1 **Building-level wastewater monitoring for COVID-19 using tampon**
2 **swabs and RT-LAMP for rapid SARS-CoV-2 RNA detection**
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16 **Abstract**

17 Community-level wastewater monitoring for severe acute respiratory syndrome coronavirus 2

18 (SARS-CoV-2) RNA has demonstrated useful correlation with both coronavirus disease 2019

19 (COVID-19) case numbers and clinical testing positivity. Wastewater monitoring on college

20 campuses has demonstrated promising predictive capacity for the presence and absence of

21 COVID-19 cases. However, to date, such monitoring has largely relied upon composite or grab

22 samples and reverse transcription quantitative PCR (RT-qPCR) techniques, which limits the

23 accessibility and scalability of wastewater monitoring. In this study, we piloted a workflow that

24 uses tampons as passive swabs for collection and reverse transcription loop-mediated isothermal

25 amplification (RT-LAMP) to detect SARS-CoV-2 RNA in wastewater. Results for the developed

26 workflow were available same day, with a time to result following tampon swab collection of

27 approximately three hours. The RT-LAMP 95% limit of detection (76 gene copies reaction^{-1}) was

28 greater than RT-droplet digital PCR (ddPCR; 3.3 gene copies reaction^{-1}). Nonetheless, during a

29 building-level wastewater monitoring campaign conducted in the midst of weekly clinical testing

30 of all students, the workflow demonstrated a same-day positive predictive value (PPV) of 33%

31 and negative predictive value (NPV) of 80% for incident COVID-19 cases. The NPV is comparable

32 to that reported by wastewater monitoring using RT-qPCR. These observations suggest that even

33   with lower analytical sensitivity the tampon swab and RT-LAMP workflow offers a cost-effective

34   and rapid approach that could be leveraged for scalable same-day building-level wastewater

35   monitoring for COVID-19.

36

37   Keywords: SARS-CoV-2, wastewater monitoring, environmental surveillance, RT-LAMP,

38   building-level, near-source, passive sampling

39

40   **Highlights**

41   • RT-LAMP wastewater testing results available three hours after swab collection;

42   • Tampon swab and RT-LAMP same-day NPV of 80% and PPV of 33% for COVID-19

43   cases;

44   • Tampon swab and RT-LAMP wastewater monitoring consumables cost less than $0.25

45   USD per person monitored;

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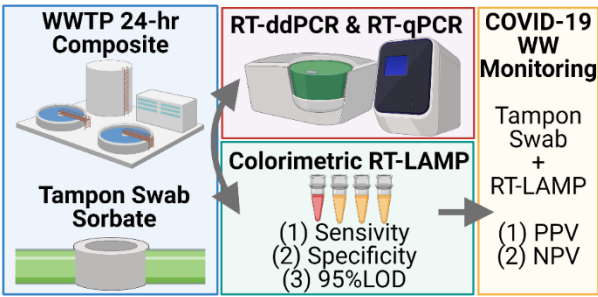
1. Introduction

Infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiologic agent of coronavirus disease 2019 (COVID-19), is accompanied by shedding of the virus, including its RNA genome, in upper and lower respiratory tract fluids and feces (Cevik et al., 2021), saliva (Wyllie et al., 2020), and urine (Kashi et al., 2020). Since these body fluids are frequently discharged to wastewater collection networks in domestic sewage, wastewater-based epidemiology (WBE; also called wastewater surveillance or wastewater monitoring) has become a useful tool for assessing community trends of COVID-19 (Bivins et al., 2020). SARS-CoV-2 RNA has been detected in untreated wastewater samples throughout the world (Ahmed et al., 2020a; Carrillo-Reyes et al., 2021; Fongaro et al., 2021; Johnson et al., 2021; Medema et al., 2020; Wu et al., 2020). Longitudinal measurements of SARS-CoV-2 RNA in wastewater influent and primary solids at wastewater treatment plants (WWTPs) have been found to correlate with COVID-19 clinical testing metrics in various communities (D'Aoust et al., 2021b; Feng et al., 2021; Gonzalez et al., 2020; Peccia et al., 2020). In many contexts increases in SARS-CoV-2 RNA in wastewater or wastewater solids have preceded increases in COVID-19 cases and hospitalizations by days to weeks (D'Aoust et al., 2021a; Nemudryi et al., 2020; Saguti et al., 2021). Thus, wastewater monitoring offers a complementary method of assessing COVID-19 trends in communities that is independent of and perhaps leads clinical testing.

While promising, monitoring SARS-CoV-2 RNA in influent at WWTPs lacks the geographic specificity to target clinical testing or other public health interventions at fine scales. Building-level monitoring, on the other hand, could inform clinical testing at specific locations on the basis of wastewater data from individual facilities, such as schools (Hassard et al., 2021) and skilled nursing facilities (Spurbeck et al., 2021). Spurbeck *et al.* used 24-hour wastewater composite samples and RT-qPCR to detect one infection among 60 skilled nursing facility residents (Spurbeck et al., 2021). Another building-level study at a skilled nursing facility using wastewater

3

75   grab samples reported mixed results. Detection of SARS-CoV-2 RNA lagged a three person

76   COVID-19 outbreak by one month at one facility and preceded clinical identification of COVID-19

77   cases by 5 to 19 days in two other facilities (Davó et al., 2021). A recent preprint described the

78   use of near-source tracking via twice weekly composite samples and RT-qPCR to monitor

79   wastewater from 16 schools and showed detection frequency consistent with community COVID-

80   19 status (Gutierrez et al., 2021).

81

82   Wastewater monitoring for SARS-CoV-2 RNA, including build-level monitoring, is being used to

83   manage COVID-19 on university campuses throughout the United States (Harris-Lovett et al.,

84   2021). Colleges have deployed wastewater monitoring in conjunction with other public health

85   measures including clinical testing, contact tracing, and isolation (Travis et al., 2021) with

86   wastewater monitoring used to guide clinical testing (Barich and Slonczewski, 2021). At the

87   University of Arizona, wastewater surveillance with serial grab samples identified one

88   symptomatic and two asymptomatic infections in a dorm and provided early warning of infections

89   in a total of 13 dorms over a semester (Betancourt et al., 2021). An innovative high-throughput

90   wastewater monitoring platform allowed for the detection of a single case of COVID-19 among

91   415 residents of a dorm at University of California San Diego (Karthikeyan et al., 2021). And

92   another building-level monitoring effort leveraged composite wastewater samples and RT-qPCR

93   performed three times weekly to identify asymptomatic COVID-19 cases on multiple occasions

94   down to one asymptomatic infection among 150 to 200 dorm residents (Gibas et al., 2021).

95

96   Building-level wastewater monitoring could be particularly useful at universities where student

97   behavior (Monod et al., 2021), congregate living (Reukers et al., 2021), and asymptomatic

98   transmission (Bjorkman et al., 2021) could combine to fuel outbreaks. Complicating transmission

99   control are asymptomatic infections, which have been observed to account for 43% (Lavezzo et

100   al., 2020) to 50% of infections (Arons et al., 2020) among adults. Since viral loads have been

101   found to be similar among asymptomatic, pre-symptomatic, and symptomatic patients (Lavezzo

102   et al., 2020; Walsh et al., 2020) and asymptomatic and mild COVID-19 cases have been observed

103   to shed SARS-CoV-2 RNA in stool (Park et al., 2020), wastewater monitoring offers an opportunity

104   to screen for COVID-19 cases among building-level populations and identify cases via follow-up

105   clinical testing (Oran and Topol, 2020).

106

107   While wastewater surveillance offers a compelling tool for building-level COVID-19 detection at

108   universities, most of the reported monitoring efforts have depended on composite samplers to

109   achieve representative samples over a defined time period (usually 24 hours). These samplers

110   can be expensive and difficult to place in building service lines. Other studies have used grab

111   samples, but such samples are "snapshots" and may not afford a reliably representative sample.

112   A few SARS-CoV-2 wastewater monitoring efforts to date, however, have used the Moore Swab,

113   a gauze bundle left suspended in sewers to sorb wastewater and enteric pathogens. This type of

114   passive sampling was first used to detect *Salmonella* Paratyphi in 1948 (Barrett et al., 1980) and

115   has also been used to detect *Vibrio cholerae* (Barrett et al., 1980) and enteric viruses (Tian et al.,

116   2017) in wastewater. More recently, Moore swabs in combination with RT-qPCR were used to

117   monitor wastewater at a university and were able to detect one to two COVID-19 cases in a

118   building (Liu et al., 2020). The same study found that when used alongside grab samples, the

119   Moore Swab allowed a greater sensitivity for SARS-CoV-2 RNA in wastewater from a hospital

120   treating COVID-19 patients (Liu et al., 2020). Another evaluation of passive samplers (gauze,

121   electronegative filter, and cotton buds) alongside traditional sampling techniques (flow-weighted

122   and time-average composite, and grab samples) found that passive samplers were at least as

123   sensitive over 24-hour deployments and a positive correlation between SARS-CoV-2 RNA

124   concentrations in wastewater and those from passive samplers (Schang et al., 2020).

125

126   Passive samplers, such as the Moore Swab, could make wastewater monitoring possible without

127   the use of expensive composite samplers. However, detection and quantification of SARS-CoV-

128   2 RNA in wastewater samples has also required the use of RT-qPCR techniques, which depend

129   on specialized PCR equipment such as thermal cyclers. Reverse transcription loop-mediated

130   isothermal amplification (RT-LAMP) (Notomi, 2000) offers the potential to detect SARS-CoV-2

131   RNA in wastewater samples without the use of such equipment. RT-LAMP has been validated for

132   rapid testing of clinical samples including serum, urine, saliva, oropharyngeal swabs, and

133   nasopharyngeal swabs for SARS-CoV-2 RNA (Ganguli et al., 2020; Schermer et al., 2020). A

134   colorimetric RT-LAMP kit developed by New England Biolabs using multiplexed primers targeting

135   the N and E regions (N2 and E1) of the SARS-CoV-2 genome had accuracy greater than 90%

136   compared to RT-qPCR and a 95% limit of detection of 59 copies per reaction when used to test

137   heat treated saliva samples (Lalli et al., 2021). Multiplexing primers and the addition of guanidine

138   chloride was found to increase the sensitivity five- to tenfold for colorimetric LAMP with the N2

139   and E1 primers yielding the best performance among seven primer sets (Zhang et al., 2020). A

140   preprint reported the use of RT-qLAMP with primers targeting the ORF1a, E, and N genes to test

141   wastewater samples for SARS-CoV-2 RNA without extraction in wastewater volumes up to 9.5

142   μL (Ongerth and Danielson, 2020).

143

144   During the current study, we piloted the application of colorimetric RT-LAMP to detect SARS-

145   CoV-2 RNA in wastewater from tampon swabs and primary influent from WWTPs in northern

146   Indiana and northeast Georgia using a variety of extraction and processing techniques. We

147   assessed the sensitivity, specificity, and limit of detection of RT-LAMP for wastewater samples

148   compared to RT-qPCR and reverse transcription droplet digital PCR (RT-ddPCR). We then used

149   tampon swabs and RT-LAMP for rapid monitoring of building-level wastewater at the University

150   of Notre Dame over six weeks in conjunction with ongoing public health measures to assess the

151   positive and negative predictive value of these measures.

152

2. Materials & Methods

2.1 Primary influent and raw sewage samples

During the experiments performed in Athens, GA, and Notre Dame, IN (USA), 24-hour time-based composite samples of primary influent were collected at eleven wastewater treatment plants (WWTPs): three located in Athens-Clarke County, GA and eight located throughout the state of Indiana. All such samples collected at WWTPs are referred to as "primary influent" throughout. In addition to primary influent, a number of wastewater samples were collected from the wastewater systems at the University of Notre Dame (ND) and neighborhoods within the Athens area, including the University of Georgia (UGA). All samples from wastewater collection systems are referred to as "raw sewage" throughout. Raw sewage samples were collected using two techniques: 24-hour time-based composite samples (for the main sewage discharge manhole at ND) and tampon swab passive samplers (detailed further below). In all cases, immediately after collection, both primary influent and raw sewage samples were stored and transported on ice or at 4°C until processed.

2.2 Tampon Swab Samplers

Tampons were used as low-cost and readily available Moore swabs for passive sampling of raw sewage in the wastewater collection system. At UGA, 100% organic cotton tampon swabs (OB Brand Organic Tampons, Super) were deployed into the wastewater collection system for 24 hours at each sampling location. After recovery, swabs were placed in sterile WhirlPak bags (Nasco, Fort Atkinson, WI) and saturated with 20 mL of sterile PBS. Saturated swabs were hand massaged for two minutes to elute viruses and then the sorbate was squeezed from the swab and collected in a sterile 50 mL centrifuge tube for immediate extraction.

176

177   At ND, with the assistance of utilities personnel, tampon swabs (Tampax Pearl, Super) were

178   deployed into the wastewater collection system weekly for six weeks from approximately 8:00 am

179   to 11:00 am at nine different locations selected to isolate individual residential halls (RH)

180   (anonymized as RH 1 to 9). During the monitoring period, these RHs housed 1,627 students

181   accounting for 25% of the on-campus residents. Upon retrieval from manholes, swabs were

182   placed into sterile WhirlPak bags and stored on ice. In the lab, swabs were hand squeezed while

183   in the WhirlPak bag to remove most of the sorbate and then aseptically placed into a 60 mL luer-

184   lock syringe (ML60, Air-Tite Products Co, Virginia Beach, VA). The sorbate remaining in the

185   WhirlPak bag was then poured into the syringe and pressed into a 50 mL centrifuge tube using

186   the syringe plunger typically resulting in 25 to 35 mL of sorbate. After the first press, a volume of

187   PBS/Tween20 solution (10 mM sodium phosphate, 0.15M NaCl, 0.05% Tween 20) was pipetted

188   into the syringe (typically 15 to 25 mL) such that the total volume of absorbate resulting from each

189   swab was 50 mL and pressed through the swab into the centrifuge tube. The resulting 50 mL of

190   sorbate was then immediately concentrated or extracted as described below. For primary influent

191   and raw sewage samples collected at UGA and a subset of samples at ND, no concentration or

192   fractionation was performed prior to extraction. For other samples, various forms of concentration

193   and fractionation as described below were trialed.

194

195   *2.3 Electronegative Membrane Concentration*

196   At ND, primary influent samples and some raw sewage composite samples were concentrated

197   using electronegative membrane filtration as described in detail elsewhere

198   (https://dx.doi.org/10.17504/protocols.io.bhiuj4ew). Briefly, a 100 mL aliquot of well-mixed sample

199   was filtered through a 0.45 µm mixed-cellulose ester membrane (Pall Corporation, Port

200   Washington, NY, USA) using a vacuum filtration assembly (Sigma-Aldrich, St. Louis, MO, USA).

201   The membrane was then aseptically rolled into a 2 mL Garnet bead tube (Qiagen, Hilden,

202   Germany) and frozen at -80°C until homogenization prior to extraction.

203

204   *2.4 Centrifugal Ultrafilter Concentration*

205   A subset of swab sorbate samples from ND were concentrated by passing 15 mL of sorbate

206   through an Amicon Ultra-15 10 kDa Centrifugal Filter Unit (MilliporeSigma, MA, USA) via a 5,000

207   x g spin for 30 minutes. The retentate was resuspended in 1 mL of PBS/Tween20 solution and

208   500 µL was transferred into a 2 mL PowerBead tube containing 0.1 mm glass beads (Qiagen,

209   Hilden, Germany) for homogenization prior to extraction. Owing to difficulty passing the entire 15

210   mL volume through the ultrafilter, this concentration method was abandoned after the first week

211   of sampling.

212

213   *2.5 Swab Sorbate Solids Fractionation*

214   Since enveloped viruses, including SARS-CoV-2, partition favorably to solids in wastewater (Li et

215   al., 2021; Ye et al., 2016), after abandoning ultrafiltration, swab sorbate samples at ND were

216   processed with emphasis on the solids fraction. Each 50 mL sorbate volume was subjected to

217   centrifugation at 10,000 x g for 10 minutes at 4°C. The supernatant was poured off and the pellet

218   was resuspended using 1 mL of PBS/Tween20 solution. A 500 µL aliquot of the resuspension

219   was transferred into a 2 mL PowerBead tube containing 0.1 mm glass beads (Qiagen, Hilden,

220   Germany) for homogenization prior to extraction. For a subset of samples, 15 mL of the resulting

221   supernatant was concentrated via Amicon as described above.

222

223   *2.6 Kit-based RNA Extractions*

224   For samples processed at UGA, RNA was extracted from 280 µL aliquots of unconcentrated

225   tampon sorbate and primary influent using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden,

226   Germany). Purified RNA was eluted in 60 uL of PCR-grade water. At ND, DNA and RNA were

227   extracted from tampon sorbate and primary influent using an AllPrep PowerViral DNA/RNA kit

228   (Qiagen, Hilden, Germany). Prior to extraction, membrane filters, Amicon ultrafilter retentate, and

229   raw sewage and sorbate solids were homogenized by adding 600 uL of PM1 and 6 uL of **β-**

230   mercaptoethanol (MP Biomedicals, Irvine, CA, USA) to the PowerBead tubes. These tubes were

231   bead beat for four rounds of 20 seconds each at 4.5 M/s on a FastPrep 24 (MP Biomedicals,

232   Irvine, CA, USA). The bead tubes were centrifuged at 13,000 x g for 1 minute and 500 uL of the

233   resulting supernatant was transferred into a clean 2 mL microcentrifuge tube and DNA/RNA was

234   extracted per the Qiagen protocol. Purified nucleic acids were eluted in 100 uL of RNase-free

235   water.

236

237   *2.7 Heat Extraction & No Extraction*

238   A subset of 1 mL swab sorbate samples and 1 mL re-suspended solids samples were subjected

239   to heat extraction by incubation in a heat block at 95°C for 15 minutes. After incubation, the

240   samples, contained in 2 mL microcentrifuge tubes, were centrifuged at 13,000 x g for 2 minutes

241   and 100 uL of supernatant was transferred to a clean 2 mL centrifuge tube for testing by RT-

242   LAMP. A subset of primary influent samples was also tested by RT-LAMP without extraction or

243   pre-treatment.

244

245   *2.8 RT-ddPCR*

246   For samples processed at ND, SARS-CoV-2 RNA was quantified using the BioRad QX200

247   Droplet Digital PCR (ddPCR) System and C1000 Touch Thermal Cycler (Hercules, CA, USA) as

248   previously described in detail (Bivins et al. 2021 preprint). Reverse transcription and droplet digital

249   PCR were performed in a single step using the One-Step RT-ddPCR Advanced Kit for Probes

250   (BioRad, Hercules, CA, USA) using the premixed N1 assay (Liu et al. 2020). RT-ddPCR reactions

251   were prepared in triplicate at a volume of 22 uL consisting of 4 uL sample RNA, 6.45 uL PCR-

252   grade water, 5.25 uL 4X Supermix, 2.1 uL reverse transcriptase, 1.05 uL dithiothreitol, and 3.15

253   uL of premixed N1 primers and probes (resulting concentrations of 1000 nM and 250 nM,

254   respectively) from Integrated DNA Technologies (Coralville, IA, USA). A 20 uL volume of the

255 reaction mixture, prepared per the BioRad protocol, was pipette mixed and transferred into the

256 droplet generation step. Following thermal cycling (50°C 60 minutes; 95°C 10 minutes; 40 cycles

257 of 95°C 30 seconds and 59°C one minute; 98°C 10 minutes; 4°C hold), droplet fluorescence

258 amplitudes were read, classified as positive or negative, and the N1 copy number calculated using

259 manual thresholding in QuantaSoft Version 1.7.4 (BioRad, Hercules, CA, USA) such that all

260 pertinent negative controls contained no positive droplets.

261

262 *2.9 Reverse Transcription - Quantitative Polymerase Chain Reaction (qPCR)*

263 For samples processed at UGA, SARS-CoV-2 viral RNA was detected and quantified using a two-

264 step reverse transcription qPCR (RT-qPCR) method. Purified RNA was converted to cDNA using

265 an adapted protocol for Invitrogen M-MLV Reverse Transcriptase (Cat No. 28025013). For a 25

266 uL reaction, sample RNA (3 uL), Random Hexamer (2.5 uM, Cat No. N8080127), dNTP Mix (0.5

267 uM), and PCR-Grade H20 (10.25 uL) were prepared in a PCR-grade low-bind strip tube. The

268 reaction was heated to 65°C for 5 min and then chilled at 4°C. The samples were vortexed and

269 spun briefly, and the following reagents were added to the reaction: M-MLV 5X buffer (1X), M-

270 MLV RT (125 U), DTT, and SUPERase•In RNase Inhibitor (10 U, Cat No. AM2694) to 25 uL. The

271 final reaction was then incubated under the following conditions: 10 min at 25°C, 50 min at 37°C,

272 and 70°C for 15 min.

273

274 SARS-CoV-2 cDNA copies were quantified by real-time quantitative PCR (qPCR) using TaqMan

275 chemistry (Fast Advanced MasterMix, Cat No. 4444557). The SARS-CoV-2 N1 and N2 genes

276 were quantified using the 2019-nCoV CDC primers and probes synthesized by IDT (Cat No.

277 10006713). Samples were assayed in triplicate. For each reaction, 2 uL of template cDNA was

278 mixed with 10 uL of 2X Taq Fast Advanced MasterMix (Cat No.4444963), 1.5 uL of the IDT SARS-

279 CoV-2 (2019-nCoV) CDC RUO Primer and Probe Kit (Cat No. 10006713), and PCR-grade water

280 to a total volume of 20 uL. Assays were analyzed using a BioRad StepOne under the following

281   reaction conditions: 95°C for 2 min; 40 Cycles x (95°C for 3 sec, 55°C for 30 sec); 4°C hold.

282   Standard curves for the N1 and N2 assays were generated from quantification of the SARS-CoV-

283   2 plasmid standard synthesized by IDT (4.12 kbp 2019-nCoV_N_Positive Control, Cat No.

284   10006625). Prior to quantification, the standard was linearized by enzymatic digestion with ScaI-

285   HF (New England BioLabs Cat No. R3122S). A serial dilution of the linearized plasmid was

286   assayed in triplicate.

287

288   *2.10 RT-LAMP*

289   SARS-CoV-2 RNA was detected by RT-LAMP using the SARS-CoV-2 Rapid Colorimetric LAMP

290   Assay Kit (Cat No. E2019S) from New England BioLabs (NEB) (Ipswich, MA, USA), a 30-minute

291   65°C colorimetric assay. The kit includes an internal control (LAMP Primer Mix targeting human

292   RNA rActin) and a SARS-CoV-2 LAMP Primer Mix targeting the N and E genes (N2 and E1,

293   respectively, Table S1). NEB reports positive detections observable down to 50 copies per

294   reaction (NEB Product Specification). Each sample was assayed in triplicate RT-LAMP reactions

295   and in parallel with an internal control for each sample, and positive controls, and negative

296   controls for each experiment. For each reaction, template RNA (4 uL) was mixed with WarmStart

297   Colorimetric LAMP 2X Master Mix with UDG (12.5 uL), LAMP Primer Mix (2.5 uL), guanidine

298   hydrochloride (2.5 uL), and PCR-grade water to a final reaction volume of 25 uL. The reaction

299   was vortexed gently and briefly spun down prior to incubation at 65°C for 30 minutes. Reactions

300   were cooled at room temperature for 5 min before reading color change and interpreting the

301   results per the NEB protocol. RT-LAMP results were acceptable if the internal control was

302   successfully detected in each sample, the SARS-CoV-2 positive and negative controls (two each

303   per experiment) were appropriately positive and negative, and the negative extraction controls

304   were negative for both the internal control and SARS-CoV-2. When the internal control was not

305   detected for a sample, the sample was interpreted to be inhibited.

306

307   *2.11 COVID-19 Clinical Surveillance at ND*

308   During the period of wastewater monitoring at ND, COVID-19 safety protocols were in place

309   including universal masking, physical distancing, daily health checks, and asymptomatic and

310   symptomatic COVID-19 testing. COVID-19 testing methods included saliva-based PCR tests,

311   primarily for asymptomatic surveillance, nasal swab PCR tests, and rapid antigen tests. All

312   undergraduate and professional students participated in mandatory weekly surveillance testing.

313   Students testing positive for COVID-19 and their close contacts entered isolation in residential

314   facilities outside of their residence hall. Close contacts were tested by nasal swab PCR test on

315   day four of isolation and rapid antigen test on day seven of isolation. If both tests were negative,

316   close contacts departed isolation on day 7. If either test was positive, close contacts began a new

317   10-day period of isolation. Students testing positive for COVID-19 completed isolation per United

318   States Centers for Disease Control and Prevention protocols with at least 10 days from symptom

319   onset for symptomatic cases or 10 days from positive test results for asymptomatic cases.

320   Although visitation between residence halls was restricted, the possibility of a non-resident

321   COVID-19 case or convalescent case shedding into the wastewater system of another residence

322   hall cannot be precluded.

323

324   Deidentified COVID-19 case data including the date of positive test, date of isolation start, and

325   date of isolation end were acquired for the nine residence halls over the wastewater monitoring

326   period. The research protocol was reviewed by the University of Notre Dame Institutional Review

327   Board (21-04-6586). In addition to de-identification of the COVID-19 case data for the study, the

328   residence halls have also been anonymized (RH1 to RH9), and the monitoring period has been

329   anonymized by the use of elapsed days (0 to 73) rather than dates. The wastewater monitoring

330   was performed in coordination with the ND Covid Response Unit.

331

332   *2.12 Data Analysis*

333   The RT-LAMP 95% limit of detection (LOD) was estimated using N1 copy number data (N1, RT-

334   ddPCR) and proportions of RT-LAMP reactions positive along an N1 concentration gradient. A

335   cumulative Gaussian distribution was fit to the gradient and the 95th percentile estimated as

336   detailed elsewhere (Bivins et al., 2021). The true negative rate (specificity) was estimated using

337   RT-ddPCR/qPCR non-detections and paired RT-LAMP classifications. The true positive rate

338   (sensitivity) was estimated using RT-ddPCR/qPCR detections and paired RT-LAMP

339   classifications. The relationship between N1 copy number (RT-ddPCR/qPCR) and RT-LAMP

340   classification was modeled using a simple logistic regression (McDonald, 2015) with statistical

341   significance determined by likelihood ratio test (Fox, 1997) and fit assessed using Tjur's R-

342   squared (Tjur, 2009). Comparisons between two groups (e.g. inhibition between sample types)

343   were made using Mann-Whitney tests and between multiple groups (e.g. inhibition between

344   extraction methods and positivity rate between sorbate fractions) using Kruskal-Wallis tests with

345   Dunn's post test (Dunn, 1964; Kruskal and Wallis, 1952; Mann and Whitney, 1947). The positive

346   and negative predictive values (PPV, NPV) of wastewater testing by tampon swab and RT-LAMP

347   for COVID-19 cases was estimated for incident COVID-19 cases in the residence hall each day

348   following wastewater monitoring out to seven days. PPV and NPV were estimated across all nine

349   residence halls each week, among single residence halls across all weeks, and across all

350   residence halls and all weeks (Parikh et al., 2008). In this case PPV is the probability of an incident

351   COVID-19 case following a positive wastewater sample, and, conversely, NPV is the probability

352   of no incident COVID-19 cases following a negative wastewater sample. All graphing and

353   statistical analyses associated with the described experiments were performed using GraphPad

354   Prism Version 9.0.0 (GraphPad Software, LaJolla, CA, USA).

355

356   **3. Results**

357   In total, 153 wastewater samples were tested via RT-LAMP. To characterize the sensitivity,

358   specificity, and analytical sensitivity of RT-LAMP, we used 24-hour composite samples of WWTP

359   influent (n = 42) and raw sewage samples collected via tampon swabs (n=7). To analyze RT-

360   LAMP performance with various extraction and processing methods, we leveraged samples from

361   WWTP composites (n = 43) and tampon swabs (n = 78). Lastly, during a prospective wastewater

362   monitoring campaign at ND, we used RT-LAMP to test 59 raw sewage samples collected via

363   tampon swabs. One tampon swab could not be recovered because it broke free while deployed

364   in a manhole.

365

366   *3.1 Analytical sensitivity*

367   Using RT-LAMP positivity and RT-ddPCR N1 copy number data, we estimated the RT-LAMP

368   95% LOD to be 76 gene copies (GC) for a single reaction (95% CI: 67 - 87) using a fitted

369   cumulative Gaussian distribution (Figure S1; R^2 = 0.997). The RT-LAMP 95% LOD is

370   approximately 20 times our estimate of the N1 RT-ddPCR 95% LOD (Bivins et al., 2021). NEB

371   reports "positive detection observable down to 50 copies", which is comparable to our estimated

372   67% LOD (51 GC/reaction). Since the RT-LAMP kit uses N2 and E primers, our N1 LOD estimates

373   are not directly representative of the primers in the kit; however, they do provide an estimate of

374   the RT-LAMP LOD relative to RT-ddPCR.

375

376   *3.2 RT-LAMP True Negative Rate (Specificity)*

377   Compared to both RT-qPCR/ddPCR non-detections (N1; n = 13), RT-LAMP demonstrated an

378   overall true negative rate (TNR) of 46%. Interestingly, the seven false positives were all in

379   comparison to RT-qPCR non-detections (n=9). Whereas for the four RT-ddPCR non-detections,

380   RT-LAMP demonstrated a TNR of 100%. Sample types among the non-detections included both

381   WWTP influent composites and swab sorbate. The experimental design does not allow us to

382   examine whether the difference in the TNR observed between RT-qPCR (two-step) and RT-

383   ddPCR (one-step) is attributable to differences in the analytical sensitivities of the PCR methods,

384   the extraction kits used (Viral RNA MiniKit vs. PowerViral DNA/RNA, respectively), or between

385   the wastewater samples collected at UGA and ND.

386

387   *3.3 RT-LAMP True Positive Rate (Sensitivity)*

388   We estimated the true positive rate (TPR) using RT-qPCR (n = 3) and RT-ddPCR (n = 27)

389   quantifications (N1 target in triplicate) compared to positivity among all RT-LAMP reactions.

390   Across all samples positive for SARS-CoV-2 RNA by RT-qPCR/ddPCR, the RT-LAMP TPR was

391   57%. A logistic regression model (Figure S2 B) fit to the data indicated that increasing N1

392   GC/reaction was associated with increasing probability of detection by RT-LAMP performed in

393   triplicate (likelihood ratio test, p = 0.0034). However, the model fit was poor (Tjur's R-squared =

394   0.24). Nonetheless, the logistic model indicates that the 50% probability of detection via RT-LAMP

395   performed in triplicate is 18 N1 GC/reaction, while the NEB-reported 50 copies yields an 83%

396   probability of detection by RT-LAMP performed in triplicate. The receiver operating characteristic

397   curve (Figure S2 C) indicates that when N1 GC/reaction are greater than 13, RT-LAMP is able to

398   achieve 80% sensitivity while minimizing false positives.

399

400   *3.4 No Extraction Inhibition Rate*

401   We attempted extraction-free RT-LAMP on five tampon swab sorbate and four 24-hour composite

402   samples of WWTP influent. The inhibition rate among the five undiluted passive samples was

403   100%. The inhibition rate for undiluted composite samples was 100% when using 7 µL or 4 µL of

404   input. After 1:10 dilution, no inhibition was observed for 7 µL of input. Given the dilution required

405   to remedy inhibition and the resulting 10x increase in the 95% LOD, we abandoned extraction-

406   free RT-LAMP as a reliable detection method.

407

408   *3.5 Heat Extraction Inhibition Rate*

409   After heat extraction, 100% of swab sorbate samples (n=5) were inhibited and remained so even

410   after 1:10 dilution. Among the five solid fraction samples, 100% were inhibited after heat

411   extraction, and 40% remained so even after 1:10 dilution. Given the high rate of inhibition, we

412   abandoned heat extraction as a reliable method for detection in wastewater via RT-LAMP.

413

414   *3.6 Viral RNA Mini versus PowerViral DNA/RNA Inhibition Rate*

415   Lastly, we assessed the rate of RT-LAMP inhibition for samples extracted using the Viral RNA

416   Mini Kit (UGA) and PowerViral DNA/RNA Kit (ND). For 24-hour WWTP influent composite

417   samples (n = 9), no inhibition was observed following extraction with the Viral RNA Mini kit. But

418   we observed a 60% inhibition rate for sorbate from swabs deployed for 24 hours extracted with

419   the same kit (n = 5). The inhibition rate for the passive samples was significantly greater than the

420   rate for the composite samples (Figure S3 A; $p = 0.0275$). Among 24-hour WWTP influent

421   composite samples extracted with the PowerViral kit (n = 33), 18% were inhibited. While for

422   sorbate, sorbate solid fraction, and sorbate liquid fraction samples (n = 68) from swabs deployed

423   for four hours, the PowerViral Kit produced an inhibition rate of 4%. The inhibition rate was

424   significantly lower for passive samplers than composite samples extracted via PowerViral (Figure

425   S3 B; $p = 0.0317$). As shown in Figure S4, the difference in inhibition rates between the Viral RNA

426   Mini Kit and PowerViral DNA/RNA kit was not statistically significant for composite samples (panel

427   A) or for all samples (panel C). We did observe a significantly lower rate of inhibition for swab

428   samples extracted via PowerViral compared to Viral RNA Mini (Figure S4 B; $p = 0.0030$).

429   However, this difference could also be attributable to the deployment of swabs for 24 hours at

430   UGA (Viral RNA Mini) compared to only four hours at ND (PowerViral).

431

432   *3.7 Tampon Swab Sorbate Processing*

433   To optimize the workflow for SARS-CoV-2 RNA detection in wastewater via RT-LAMP, we

434   assessed the rates of inhibition and positivity between Amicon-concentrated swab sorbate, the

435   solid fraction of swab sorbate, and the liquid fraction of swab sorbate during two weeks of

436   wastewater monitoring at ND. During the first week, Amicon-concentrated sorbate extracted via

437   PowerViral produced no inhibited RT-LAMP reactions and an overall SARS-CoV-2 RNA positivity

438   of 40% (11 of 27 RT-LAMP replicates) in samples collected from nine RHs. However, filtering the

439   swab sorbate through the Amicon ultrafilters required several hours of centrifugation. Given our

440   interest in a rapid testing procedure, the following week the swab sorbate was first centrifuged,

441   then the resulting supernatant was concentrated via Amicon and extracted with PowerViral. The

442   solid fraction pellet was also extracted via PowerViral. The rate of RT-LAMP inhibition among the

443   extracted supernatant samples was 38% and SARS-CoV-2 RNA was not detected in any of 24

444   RT-LAMP replicates. For the extracted solid fractions, there was no inhibition observed and the

445   SARS-CoV-2 RNA positivity was 33% among 30 RT-LAMP replicates. Both the Amicon-

446   concentrated and solids fraction samples exhibited lower rates of inhibition (Figure S5 A) and

447   higher rates of SARS-CoV-2 positivity (Figure S5 B) than the liquids fraction. Since inhibition rates

448   ($p > 0.9999$) and SARS-CoV-2 RNA positivity rates ($p > 0.9999$) were comparable between

449   Amicon-concentrate and solid fraction, we elected to continue monitoring at ND using only the

450   swab sorbate solid fraction to allow for faster processing.

451

452   *3.8 COVID-19 Clinical Data*

453   During the observation period, 143,884 COVID-19 clinical tests (symptomatic and asymptomatic)

454   were performed at ND. During the wastewater monitoring (day 31 to 66), an average of 13,748

455   clinical tests were performed each week (Figure S6). The COVID-19 positivity and case number

456   trends among the subpopulation accounted for in sewage monitoring (Figure S7) are similar to

457   the trends for the entire campus. The proportion of wastewater RT-LAMP tests that were positive

458   decreased abruptly from 30% to 0 from week 3 to week 4, and then increased slightly in the

459   following two weeks. As shown in Figure S8, this abrupt shift in wastewater positivity could not be

460   explained by a shift in domestic water use. Water use patterns in three of the RHs remained

18

461   consistent across these weeks of wastewater sampling with 19 to 23% of the daily water use

462   occurring during the 8 am to 11 am wastewater monitoring period.

463

464   *3.9 RT-LAMP PPV and NPV for COVID-19*

465   RT-LAMP wastewater testing results (proportion of positive RT-LAMP replicates), COVID-19

466   clinical positives, residents exiting the RH for isolation, and residents returning from isolation are

467   shown for each RH in Figure 1. RT-LAMP positives in wastewater were coincident with COVID-

468   19 cases on the same day on four occasions (RH1, RH2, RH7, RH9). For two residence halls

469   (RH4, RH6) RT-LAMP results were negative across the entire sampling period with one occurring

470   on the same day as a positive COVID-19 clinical test in RH4. There were also RT-LAMP positives

471   during periods without incident COVID-19 cases in RH2, RH3, RH8, and RH9.

472

473   Although the ND COVID-19 Response Unit was informed of the wastewater sampling results, the

474   clinical surveillance testing was performed independently and thus allows for an estimation of the

475   tampon swab and RT-LAMP wastewater testing PPV and NPV. PPV and NPV were calculated

476   for each day from the day of wastewater testing (day 0) out to six days after. The PPVs displayed

477   a wider range across residence halls (0 to 100%; Figure S9 A) than weeks (0 to 75%; Figure S9

478   C). In general, PPV increased from the day of wastewater monitoring to three days after as

479   incident COVID-19 cases increased in the days following. PPV could not be estimated for RH4,

480   RH6, or week 4 monitoring since there were no positive wastewater results. NPV displayed a

481   similar pattern of variation with the range observed between residence halls (0 to 100%) being

482   greater than the range between weeks of monitoring (22 to 100%). NPV decreased from the day

483   of wastewater monitoring out to three days as incident COVID-19 cases increased.

484

485   Across all residence halls and weeks, tampon swab and RT-LAMP wastewater monitoring, with

486   any replicate positive classified as a positive wastewater result, displayed a PPV of 19 to 38%

487   during the six days following wastewater testing (Figure 2A). As shown in Figure 2B, NPV was

488   greater with a maximum of 78% on the day of wastewater testing to a day six minimum of 38%.

489   The PPV of wastewater testing could be adversely affected by positive RT-LAMP results

490   attributable to convalescent COVID-19 cases returning to residence halls after isolation. As shown

491   in Figure S9, there were six instances where RT-LAMP replicates were positive despite no

492   incident COVID-19 cases, but with returning convalescent cases in the prior seven days. In these

493   six instances, it required four or more convalescent cases before 2 of 3 RT-LAMP replicates were

494   positive, suggesting that a cutoff value of 67% positivity (2 of 3 replicates) could increase the PPV

495   of the wastewater method. As shown in Figure 2A, PPV is increased to 33% when 2 of 3 positive

496   RT-LAMP reactions are required to classify a sample as positive. This change in cutoff value

497   leaves the NPV largely unchanged (Figure 2B). If the detection of convalescent COVID-19 cases

498   by wastewater sampling is considered a true positive (e.g., the true detection of SARS-CoV-2

499   RNA shed into the wastewater system), then the PPV improves to 56% on day 0 up to 75% by

500   day three after wastewater monitoring (Figure S11).

501

502   **4. Discussion**

503   *4.1 Reliable RT-LAMP Workflow and Analytical Performance*

504   To develop more accessible wastewater monitoring techniques, we piloted and characterized the

505   performance of a monitoring protocol that makes use of tampon swabs and RT-LAMP to detect

506   SARS-CoV-2 RNA in building-level wastewater. The 95% LOD for a single RT-LAMP reaction

507   was 20 times higher than the RT-ddPCR N1 assay 95% LOD. Several studies have found that

508   SARS-CoV-2 RNA shedding in feces can outlast nasopharyngeal shedding in up to 50% of

509   COVID-19 patients (Elbeblaw, 2020; Jones et al., 2020; Wang et al., 2020). In such cases, the

510   higher RT-LAMP LOD could be advantageous by allowing for convalescent cases to go

511   undetected, while newly incident COVID-19 cases could still be detected. RT-LAMP

512   demonstrated an overall sensitivity of 57% compared to PCR methods, and a specificity of 100%

513   compared to one-step RT-ddPCR. Unfortunately, we were not able to replicate the findings of an

514   earlier pre-print study as all of our attempts to test wastewater directly were inhibited (Ongerth

515   and Danielson, 2020). Our attempts at heat extraction were also consistently inhibited despite the

516   success with saliva and other clinical samples (Mahmoud et al., 2021). We found that regardless

517   of the wastewater type (influent composite or swab sorbate) the use of an extraction kit for testing

518   by RT-LAMP was important to produce uninhibited results.

519

520   When paired with tampon swab sorbate, the Qiagen AllPrep PowerViral DNA/RNA Kit yielded a

521   4% inhibition rate among all samples. Concentrating sorbate with Amicon ultrafilters proved

522   burdensome due to clogging. Since wastewater solids have been proposed as an efficient and

523   sensitive partition for SARS-CoV-2 RNA detection (D'Aoust et al., 2021b; Kitamura et al., 2021),

524   we opted to abandon Amicon concentration in favor of testing the sorbate solids fraction. We

525   found that the solids fraction yielded a comparable SARS-CoV-2 positivity and inhibition rate to

526   ultrafilter concentrate.

527

528   *4.2 RT-LAMP predictive capability compared to RT-qPCR studies*

529   The optimized tampon swab and RT-LAMP workflow yielded a same-day PPV of 33% and an

530   NPV of 80% in six weeks of wastewater monitoring. Accounting for the detection of convalescent

531   cases improves the PPV to 56%. The PPV we observed was much lower than the 82% reported

532   during another study leveraging PEG precipitation and RT-qPCR, but the NPV we observed (80%

533   versus 88.9%) was comparable (Betancourt et al., 2021). The specificity of the tampon swab and

534   RT-LAMP method for COVID-19 cases was 80%, which is better than the 52% specificity reported

535   for an ultracentrifugation and RT-qPCR method that did not distinguish new infections from

536   convalescent (Colosi et al., 2021). Thus, the tampon swab and RT-LAMP approach may offer a

537   specificity and NPV comparable to more sophisticated monitoring methods. Several

538   epidemiological modeling studies have suggested that an optimal strategy for managing COVID-

539   19 on college campuses should include high-frequency screening tests that are highly specific

540   (Lopman et al., 2021; Paltiel et al., 2020). Our observations indicated that the NPV and PPV for

541   tampon swab and RT-LAMP monitoring were maximized with wastewater monitoring daily to

542   every three days.

543

544   *4.3 Rapidity of RT-LAMP results*

545   These models have also consistently emphasized rapid results reporting over sensitivity as a

546   critical feature of effective screening. Wong et al. found that wastewater monitoring with one day

547   to results and four days or less to follow up clinical testing could keep infection rates within 5% of

548   those achieved by clinical testing of individuals (Wong et al., 2020). Following extraction, the RT-

549   qPCR and RT-ddPCR workflows used in the study required 3.5 and 7 hours, respectively, to

550   produce results. Whereas, the RT-LAMP workflow required only 1.5 hours (45 minute preparation,

551   30 minute incubation, 15 minutes to read results). Additional time is required for tampon swab

552   deployment, collection, sorbate harvesting, and extraction. At ND, tampon swabs were deployed

553   at 8:00 am, retrieve at 11:00 am, and results were transmitted to the COVID Response Unit by

554   3:00 pm. Though we only conducted the wastewater monitoring weekly, the workflow could easily

555   be modified to achieve results daily by noon. For example, a tampon swab could be deployed in

556   the sewer for 24 hours, retrieved at 8:00 am, at which time another could be deployed, and results

557   could be reported by noon at which time clinical testing could be mobilized in response. Based on

558   a 5-day incubation and 1.2 day medical seeking period (Lauer et al., 2020), Zhu *et al*. have

559   suggested a 6.2-day window to efficiently interrupt transmission chains (Zhu et al., 2021). The

560   tampon swab and RT-LAMP method described in this study is easily capable of producing

561   monitoring results within this window. Efficient transmission control through timely wastewater

562   results is even more important on college campuses since asymptomatic infections are more

563   prevalent among younger populations (Bjorkman et al., 2021).

564

565　*4.4 Wastewater Monitoring Scalability and Accessibility*

566　In addition to reasonable specificity, and rapid results, the tampon swab and RT-LAMP method

567　could also afford improved accessibility to wastewater monitoring in low-resource settings. Many

568　of the COVID-19 wastewater monitoring efforts to date, including those on college campuses,

569　have made use of composite samplers and RT-qPCR techniques to detect and quantify SARS-

570　CoV-2 RNA (Ahmed et al., 2020b; Harris-Lovett et al., 2021). While these techniques have proven

571　useful for tracking COVID-19 in some communities, the expense of composite samplers and the

572　apparatus required to perform RT-qPCR greatly limits the accessibility and scalability of

573　wastewater monitoring for SARS-CoV-2. The World Health Organization has identified

574　wastewater monitoring approaches for pooled testing of high-risk lower-resource settings as a

575　critical need to expand the application of the technique (World Health Organization, 2020). While

576　we could not avoid using a kit-based RNA extraction, the method does not require a composite

577　sampler or thermal cycler for RT-qPCR, relying instead on tampons for sampling and basic lab

578　equipment including centrifuges, microcentrifuges, vortexes, and single temperature incubators

579　for swab processing and RT-LAMP testing. The per sample analytical cost was comparable

580　between RT-ddPCR ($35) and the NEB RT-LAMP kit ($31); however, we estimate that a self-

581　assembled RT-LAMP kit using the same primers could halve the per-sample cost once optimized.

582　Even with the off-the-shelf kit, the per capita consumables cost for the entire workflow was

583　approximately $0.25.

584

585　*4.5 Limitations*

586　There are limitations that should be considered in generalizing the findings of this study. First, our

587　comparison of RT-LAMP and RT-qPCR/ddPCR leveraged samples from only two monitoring

588　sites, ND and UGA. Although we made use of raw sewage and WWTP influent samples,

589　wastewater, and therefore RT-LAMP performance, can be variable among sites. For comparison

590　with clinical surveillance, we monitored wastewater at nine ND residence halls. We note that while

591   COVID protocols during the sampling period did not allow guests into the residence halls, it is not

592   possible to completely exclude the possible shedding of SARS-CoV-2 RNA into the residence

593   hall wastewater by non-residents. The predictive performance was variable between halls and

594   weeks and the study was not designed to further investigate these differences. The tampon swabs

595   were only deployed for a three-hour interval between 8:00 am and 11:00 am. This period

596   accounted for roughly 20% of daily domestic water use, but the performance of the workflow could

597   potentially be improved with longer deployments of the tampon swabs, assuming this does not

598   lead to increased rates of inhibition. We independently monitored the wastewater from residence

599   halls during a large and robust clinical surveillance program that featured weekly testing of every

600   single student. In the midst of such a clinical surveillance effort, the predictive performance of

601   wastewater monitoring is likely to be conservative. Nonetheless, our experience suggests that

602   tampon swabs in combination with RT-LAMP could afford a specific, rapid, cost-effective, and

603   accessible screening method for building-level wastewater monitoring. As vaccination efforts

604   continue to progress, such a monitoring method may offer a scalable approach for non-intrusive

605   screening of at-risk populations.

606

607   **5. Conclusions**

608   - RT-LAMP sensitivity was 57%, specificity was 100%, 95% LOD was 76 gene copies per

609   reaction compared to SARS-CoV-2 RNA detection by RT-ddPCR.

610   - Tampon swabs combined with RT-LAMP were successfully used to detect SARS-CoV-2

611   RNA in building-level wastewater with results available the by 3 pm the same day.

612   - Over six weeks of monitoring the swab and RT-LAMP wastewater test demonstrated

613   80% negative predictive value and 33% positive predictive value compared to clinical

614   COVID-19 testing.

615   • The consumables cost of wastewater monitoring over six by tampon swab and RT-

616   LAMP was less than $2 per person and could likely be further reduced through a self-

617   assembled LAMP kit.

618

619   **Acknowledgements**

624

625   **Data Availability**

626   The datasets analyzed during the current study, excluding clinical data, are available in the

627   OSF.IO repository, https://osf.io/2jdbs/ doi: 10.17605/OSF.IO/2JDBS.

628

629   **Competing Interests**

630   The authors declare no competing financial or non-financial interests.
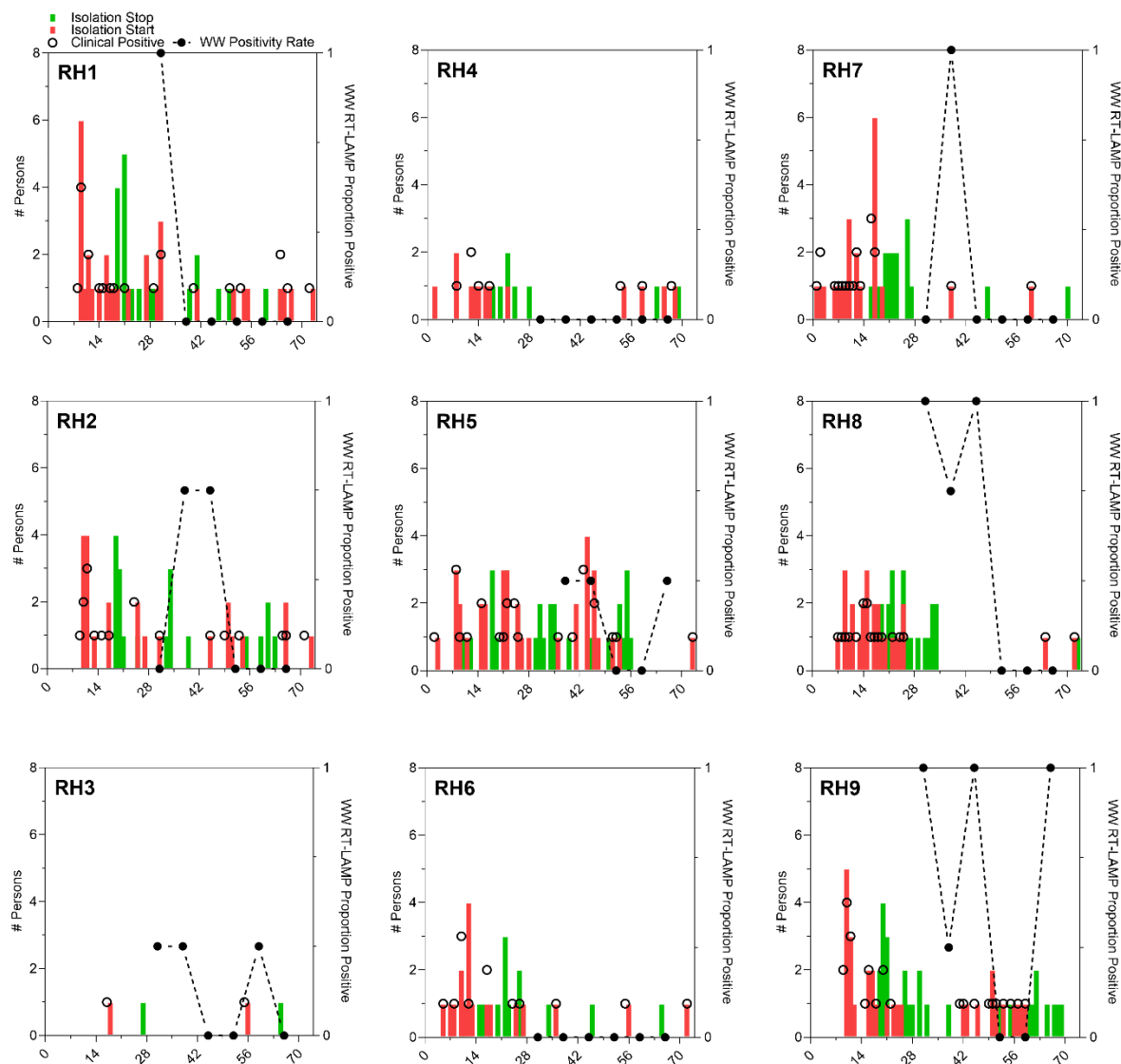
631
632
633 Figure 1 | Daily COVID-19 clinical positives, isolation start, and isolation stop (left y-axis),
634 compared with the proportion of RT-LAMP reactions positive (three reactions per wastewater
635 (WW) sample; right y-axis) for SARS-CoV-2 RNA among nine residence halls over a 73 day
636 period (x-axis) with wastewater monitoring every seven days from day 31 to 66.
637

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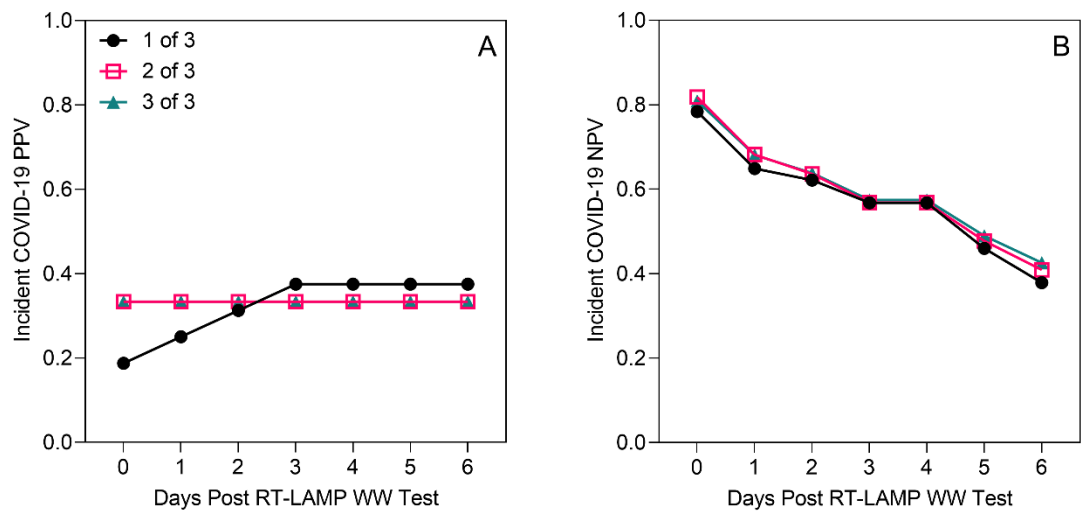
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641   Figure 2 | Positive predictive value (PPV) (A) and negative predictive value (NPV) (B) in the seven
642   days following wastewater monitoring by tampon swab and RT-LAMP for three different cutoff
643   values for classification of RT-LAMP results as "positive" for SARS-CoV-2 RNA (1 of 3, 2 of 3, or
644   3 of 3 reactions positive) as observed during monitoring of wastewater from nine residence halls
645   for six weeks.

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