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

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**Abstract**

Community-level wastewater monitoring for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA has demonstrated useful correlation with both coronavirus disease 2019 (COVID-19) case numbers and clinical testing positivity. Wastewater monitoring on college campuses has demonstrated promising predictive capacity for the presence and absence of COVID-19 cases. However, to date, such monitoring has largely relied upon composite or grab samples and reverse transcription quantitative PCR (RT-qPCR) techniques, which limits the accessibility and scalability of wastewater monitoring. In this study, we piloted a workflow that uses tampons as passive swabs for collection and reverse transcription loop-mediated isothermal amplification (RT-LAMP) to detect SARS-CoV-2 RNA in wastewater. Results for the developed workflow were available same day, with a time to result following tampon swab collection of approximately three hours. The RT-LAMP 95% limit of detection (76 gene copies reaction$^{-1}$) was greater than RT-droplet digital PCR (ddPCR; 3.3 gene copies reaction$^{-1}$). Nonetheless, during a building-level wastewater monitoring campaign conducted in the midst of weekly clinical testing of all students, the workflow demonstrated a same-day positive predictive value (PPV) of 33% and negative predictive value (NPV) of 80% for incident COVID-19 cases. The NPV is comparable to that reported by wastewater monitoring using RT-qPCR. These observations suggest that even

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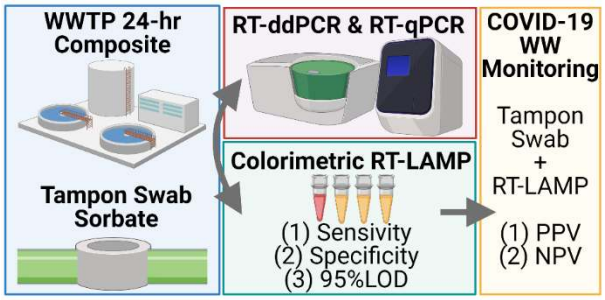
with lower analytical sensitivity the tampon swab and RT-LAMP workflow offers a cost-effective and rapid approach that could be leveraged for scalable same-day building-level wastewater monitoring for COVID-19.

Keywords: SARS-CoV-2, wastewater monitoring, environmental surveillance, RT-LAMP, building-level, near-source, passive sampling

**Highlights**

- RT-LAMP wastewater testing results available three hours after swab collection;
- Tampon swab and RT-LAMP same-day NPV of 80% and PPV of 33% for COVID-19 cases;
- Tampon swab and RT-LAMP wastewater monitoring consumables cost less than $0.25 USD per person monitored;



## 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

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grab samples reported mixed results. Detection of SARS-CoV-2 RNA lagged a three person COVID-19 outbreak by one month at one facility and preceded clinical identification of COVID-19 cases by 5 to 19 days in two other facilities (Davó et al., 2021). A recent preprint described the use of near-source tracking via twice weekly composite samples and RT-qPCR to monitor wastewater from 16 schools and showed detection frequency consistent with community COVID-19 status (Gutierrez et al., 2021).

Wastewater monitoring for SARS-CoV-2 RNA, including build-level monitoring, is being used to manage COVID-19 on university campuses throughout the United States (Harris-Lovett et al., 2021). Colleges have deployed wastewater monitoring in conjunction with other public health measures including clinical testing, contact tracing, and isolation (Travis et al., 2021) with wastewater monitoring used to guide clinical testing (Barich and Slonczewski, 2021). At the University of Arizona, wastewater surveillance with serial grab samples identified one symptomatic and two asymptomatic infections in a dorm and provided early warning of infections in a total of 13 dorms over a semester (Betancourt et al., 2021). An innovative high-throughput wastewater monitoring platform allowed for the detection of a single case of COVID-19 among 415 residents of a dorm at University of California San Diego (Karthikeyan et al., 2021). And another building-level monitoring effort leveraged composite wastewater samples and RT-qPCR performed three times weekly to identify asymptomatic COVID-19 cases on multiple occasions down to one asymptomatic infection among 150 to 200 dorm residents (Gibas et al., 2021).

Building-level wastewater monitoring could be particularly useful at universities where student behavior (Monod et al., 2021), congregate living (Reukers et al., 2021), and asymptomatic transmission (Bjorkman et al., 2021) could combine to fuel outbreaks. Complicating transmission control are asymptomatic infections, which have been observed to account for 43% (Lavezzo et al., 2020) to 50% of infections (Arons et al., 2020) among adults. Since viral loads have been

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found to be similar among asymptomatic, pre-symptomatic, and symptomatic patients (Lavezzo et al., 2020; Walsh et al., 2020) and asymptomatic and mild COVID-19 cases have been observed to shed SARS-CoV-2 RNA in stool (Park et al., 2020), wastewater monitoring offers an opportunity to screen for COVID-19 cases among building-level populations and identify cases via follow-up clinical testing (Oran and Topol, 2020).

While wastewater surveillance offers a compelling tool for building-level COVID-19 detection at universities, most of the reported monitoring efforts have depended on composite samplers to achieve representative samples over a defined time period (usually 24 hours). These samplers can be expensive and difficult to place in building service lines. Other studies have used grab samples, but such samples are “snapshots” and may not afford a reliably representative sample. A few SARS-CoV-2 wastewater monitoring efforts to date, however, have used the Moore Swab, a gauze bundle left suspended in sewers to sorb wastewater and enteric pathogens. This type of passive sampling was first used to detect *Salmonella* Paratyphi in 1948 (Barrett et al., 1980) and has also been used to detect *Vibrio cholerae* (Barrett et al., 1980) and enteric viruses (Tian et al., 2017) in wastewater. More recently, Moore swabs in combination with RT-qPCR were used to monitor wastewater at a university and were able to detect one to two COVID-19 cases in a building (Liu et al., 2020). The same study found that when used alongside grab samples, the Moore Swab allowed a greater sensitivity for SARS-CoV-2 RNA in wastewater from a hospital treating COVID-19 patients (Liu et al., 2020). Another evaluation of passive samplers (gauze, electronegative filter, and cotton buds) alongside traditional sampling techniques (flow-weighted and time-average composite, and grab samples) found that passive samplers were at least as sensitive over 24-hour deployments and a positive correlation between SARS-CoV-2 RNA concentrations in wastewater and those from passive samplers (Schang et al., 2020).

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Passive samplers, such as the Moore Swab, could make wastewater monitoring possible without the use of expensive composite samplers. However, detection and quantification of SARS-CoV-2 RNA in wastewater samples has also required the use of RT-qPCR techniques, which depend on specialized PCR equipment such as thermal cyclers. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Notomi, 2000) offers the potential to detect SARS-CoV-2 RNA in wastewater samples without the use of such equipment. RT-LAMP has been validated for rapid testing of clinical samples including serum, urine, saliva, oropharyngeal swabs, and nasopharyngeal swabs for SARS-CoV-2 RNA (Ganguli et al., 2020; Schermer et al., 2020). A colorimetric RT-LAMP kit developed by New England Biolabs using multiplexed primers targeting the N and E regions (N2 and E1) of the SARS-CoV-2 genome had accuracy greater than 90% compared to RT-qPCR and a 95% limit of detection of 59 copies per reaction when used to test heat treated saliva samples (Lalli et al., 2021). Multiplexing primers and the addition of guanidine chloride was found to increase the sensitivity five- to tenfold for colorimetric LAMP with the N2 and E1 primers yielding the best performance among seven primer sets (Zhang et al., 2020). A preprint reported the use of RT-qLAMP with primers targeting the ORF1a, E, and N genes to test wastewater samples for SARS-CoV-2 RNA without extraction in wastewater volumes up to 9.5 µL (Ongerth and Danielson, 2020).

During the current study, we piloted the application of colorimetric RT-LAMP to detect SARS-CoV-2 RNA in wastewater from tampon swabs and primary influent from WWTPs in northern Indiana and northeast Georgia using a variety of extraction and processing techniques. We assessed the sensitivity, specificity, and limit of detection of RT-LAMP for wastewater samples compared to RT-qPCR and reverse transcription droplet digital PCR (RT-ddPCR). We then used tampon swabs and RT-LAMP for rapid monitoring of building-level wastewater at the University of Notre Dame over six weeks in conjunction with ongoing public health measures to assess the positive and negative predictive value of these measures.

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## 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.

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At ND, with the assistance of utilities personnel, tampon swabs (Tampax Pearl, Super) were deployed into the wastewater collection system weekly for six weeks from approximately 8:00 am to 11:00 am at nine different locations selected to isolate individual residential halls (RH) (anonymized as RH 1 to 9). During the monitoring period, these RHs housed 1,627 students accounting for 25% of the on-campus residents. Upon retrieval from manholes, swabs were placed into sterile WhirlPak bags and stored on ice. In the lab, swabs were hand squeezed while in the WhirlPak bag to remove most of the sorbate and then aseptically placed into a 60 mL luer-lock syringe (ML60, Air-Tite Products Co, Virginia Beach, VA). The sorbate remaining in the WhirlPak bag was then poured into the syringe and pressed into a 50 mL centrifuge tube using the syringe plunger typically resulting in 25 to 35 mL of sorbate. After the first press, a volume of PBS/Tween20 solution (10 mM sodium phosphate, 0.15M NaCl, 0.05% Tween 20) was pipetted into the syringe (typically 15 to 25 mL) such that the total volume of absorbate resulting from each swab was 50 mL and pressed through the swab into the centrifuge tube. The resulting 50 mL of sorbate was then immediately concentrated or extracted as described below. For primary influent and raw sewage samples collected at UGA and a subset of samples at ND, no concentration or fractionation was performed prior to extraction. For other samples, various forms of concentration and fractionation as described below were trialed.

*2.3 Electronegative Membrane Concentration*

At ND, primary influent samples and some raw sewage composite samples were concentrated using electronegative membrane filtration as described in detail elsewhere (https://dx.doi.org/10.17504/protocols.io.bhiuj4ew). Briefly, a 100 mL aliquot of well-mixed sample was filtered through a 0.45 µm mixed-cellulose ester membrane (Pall Corporation, Port Washington, NY, USA) using a vacuum filtration assembly (Sigma-Aldrich, St. Louis, MO, USA). The membrane was then aseptically rolled into a 2 mL Garnet bead tube (Qiagen, Hilden, Germany) and frozen at -80°C until homogenization prior to extraction.

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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

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raw sewage and sorbate solids were homogenized by adding 600 uL of PM1 and 6 uL of β-mercaptoethanol (MP Biomedicals, Irvine, CA, USA) to the PowerBead tubes. These tubes were bead beat for four rounds of 20 seconds each at 4.5 M/s on a FastPrep 24 (MP Biomedicals, Irvine, CA, USA). The bead tubes were centrifuged at 13,000 x g for 1 minute and 500 uL of the resulting supernatant was transferred into a clean 2 mL microcentrifuge tube and DNA/RNA was extracted per the Qiagen protocol. Purified nucleic acids were eluted in 100 uL of RNase-free water.

*2.7 Heat Extraction & No Extraction*

A subset of 1 mL swab sorbate samples and 1 mL re-suspended solids samples were subjected to heat extraction by incubation in a heat block at 95°C for 15 minutes. After incubation, the samples, contained in 2 mL microcentrifuge tubes, were centrifuged at 13,000 x g for 2 minutes and 100 uL of supernatant was transferred to a clean 2 mL centrifuge tube for testing by RT-LAMP. A subset of primary influent samples was also tested by RT-LAMP without extraction or pre-treatment.

*2.8 RT-ddPCR*

For samples processed at ND, SARS-CoV-2 RNA was quantified using the BioRad QX200 Droplet Digital PCR (ddPCR) System and C1000 Touch Thermal Cycler (Hercules, CA, USA) as previously described in detail (Bivins et al. 2021 preprint). Reverse transcription and droplet digital PCR were performed in a single step using the One-Step RT-ddPCR Advanced Kit for Probes (BioRad, Hercules, CA, USA) using the premixed N1 assay (Liu et al. 2020). RT-ddPCR reactions were prepared in triplicate at a volume of 22 uL consisting of 4 uL sample RNA, 6.45 uL PCR-grade water, 5.25 uL 4X Supermix, 2.1 uL reverse transcriptase, 1.05 uL dithiothreitol, and 3.15 uL of premixed N1 primers and probes (resulting concentrations of 1000 nM and 250 nM, respectively) from Integrated DNA Technologies (Coralville, IA, USA). A 20 uL volume of the

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reaction mixture, prepared per the BioRad protocol, was pipette mixed and transferred into the droplet generation step. Following thermal cycling (50°C 60 minutes; 95°C 10 minutes; 40 cycles of 95°C 30 seconds and 59°C one minute; 98°C 10 minutes; 4°C hold), droplet fluorescence amplitudes were read, classified as positive or negative, and the N1 copy number calculated using manual thresholding in QuantaSoft Version 1.7.4 (BioRad, Hercules, CA, USA) such that all pertinent negative controls contained no positive droplets.

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

For samples processed at UGA, SARS-CoV-2 viral RNA was detected and quantified using a two-step reverse transcription qPCR (RT-qPCR) method. Purified RNA was converted to cDNA using an adapted protocol for Invitrogen M-MLV Reverse Transcriptase (Cat No. 28025013). For a 25 uL reaction, sample RNA (3 uL), Random Hexamer (2.5 uM, Cat No. N8080127), dNTP Mix (0.5 uM), and PCR-Grade H20 (10.25 uL) were prepared in a PCR-grade low-bind strip tube. The reaction was heated to 65°C for 5 min and then chilled at 4°C. The samples were vortexed and spun briefly, and the following reagents were added to the reaction: M-MLV 5X buffer (1X), M-MLV RT (125 U), DTT, and SUPERase•In RNase Inhibitor (10 U, Cat No. AM2694) to 25 uL. The final reaction was then incubated under the following conditions: 10 min at 25°C, 50 min at 37°C, and 70°C for 15 min.

SARS-CoV-2 cDNA copies were quantified by real-time quantitative PCR (qPCR) using TaqMan chemistry (Fast Advanced MasterMix, Cat No. 4444557). The SARS-CoV-2 N1 and N2 genes were quantified using the 2019-nCoV CDC primers and probes synthesized by IDT (Cat No. 10006713). Samples were assayed in triplicate. For each reaction, 2 uL of template cDNA was mixed with 10 uL of 2X Taq Fast Advanced MasterMix (Cat No.4444963), 1.5 uL of the IDT SARS-CoV-2 (2019-nCoV) CDC RUO Primer and Probe Kit (Cat No. 10006713), and PCR-grade water to a total volume of 20 uL. Assays were analyzed using a BioRad StepOne under the following

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reaction conditions: 95°C for 2 min; 40 Cycles x (95°C for 3 sec, 55°C for 30 sec); 4°C hold. Standard curves for the N1 and N2 assays were generated from quantification of the SARS-CoV-2 plasmid standard synthesized by IDT (4.12 kbp 2019-nCoV_N_Positive Control, Cat No. 10006625). Prior to quantification, the standard was linearized by enzymatic digestion with ScaI-HF (New England BioLabs Cat No. R3122S). A serial dilution of the linearized plasmid was assayed in triplicate.

*2.10 RT-LAMP*

SARS-CoV-2 RNA was detected by RT-LAMP using the SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit (Cat No. E2019S) from New England BioLabs (NEB) (Ipswich, MA, USA), a 30-minute 65°C colorimetric assay. The kit includes an internal control (LAMP Primer Mix targeting human RNA rActin) and a SARS-CoV-2 LAMP Primer Mix targeting the N and E genes (N2 and E1, respectively, Table S1). NEB reports positive detections observable down to 50 copies per reaction (NEB Product Specification). Each sample was assayed in triplicate RT-LAMP reactions and in parallel with an internal control for each sample, and positive controls, and negative controls for each experiment. For each reaction, template RNA (4 uL) was mixed with WarmStart Colorimetric LAMP 2X Master Mix with UDG (12.5 uL), LAMP Primer Mix (2.5 uL), guanidine hydrochloride (2.5 uL), and PCR-grade water to a final reaction volume of 25 uL. The reaction was vortexed gently and briefly spun down prior to incubation at 65°C for 30 minutes. Reactions were cooled at room temperature for 5 min before reading color change and interpreting the results per the NEB protocol. RT-LAMP results were acceptable if the internal control was successfully detected in each sample, the SARS-CoV-2 positive and negative controls (two each per experiment) were appropriately positive and negative, and the negative extraction controls were negative for both the internal control and SARS-CoV-2. When the internal control was not detected for a sample, the sample was interpreted to be inhibited.

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*2.11 COVID-19 Clinical Surveillance at ND*

During the period of wastewater monitoring at ND, COVID-19 safety protocols were in place including universal masking, physical distancing, daily health checks, and asymptomatic and symptomatic COVID-19 testing. COVID-19 testing methods included saliva-based PCR tests, primarily for asymptomatic surveillance, nasal swab PCR tests, and rapid antigen tests. All undergraduate and professional students participated in mandatory weekly surveillance testing. Students testing positive for COVID-19 and their close contacts entered isolation in residential facilities outside of their residence hall. Close contacts were tested by nasal swab PCR test on day four of isolation and rapid antigen test on day seven of isolation. If both tests were negative, close contacts departed isolation on day 7. If either test was positive, close contacts began a new 10-day period of isolation. Students testing positive for COVID-19 completed isolation per United States Centers for Disease Control and Prevention protocols with at least 10 days from symptom onset for symptomatic cases or 10 days from positive test results for asymptomatic cases. Although visitation between residence halls was restricted, the possibility of a non-resident COVID-19 case or convalescent case shedding into the wastewater system of another residence hall cannot be precluded.

Deidentified COVID-19 case data including the date of positive test, date of isolation start, and date of isolation end were acquired for the nine residence halls over the wastewater monitoring period. The research protocol was reviewed by the University of Notre Dame Institutional Review Board (21-04-6586). In addition to de-identification of the COVID-19 case data for the study, the residence halls have also been anonymized (RH1 to RH9), and the monitoring period has been anonymized by the use of elapsed days (0 to 73) rather than dates. The wastewater monitoring was performed in coordination with the ND Covid Response Unit.

*2.12 Data Analysis*

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The RT-LAMP 95% limit of detection (LOD) was estimated using N1 copy number data (N1, RT-ddPCR) and proportions of RT-LAMP reactions positive along an N1 concentration gradient. A cumulative Gaussian distribution was fit to the gradient and the 95th percentile estimated as detailed elsewhere (Bivins et al., 2021). The true negative rate (specificity) was estimated using RT-ddPCR/qPCR non-detections and paired RT-LAMP classifications. The true positive rate (sensitivity) was estimated using RT-ddPCR/qPCR detections and paired RT-LAMP classifications. The relationship between N1 copy number (RT-ddPCR/qPCR) and RT-LAMP classification was modeled using a simple logistic regression (McDonald, 2015) with statistical significance determined by likelihood ratio test (Fox, 1997) and fit assessed using Tjur's R-squared (Tjur, 2009). Comparisons between two groups (e.g. inhibition between sample types) were made using Mann-Whitney tests and between multiple groups (e.g. inhibition between extraction methods and positivity rate between sorbate fractions) using Kruskal-Wallis tests with Dunn's post test (Dunn, 1964; Kruskal and Wallis, 1952; Mann and Whitney, 1947). The positive and negative predictive values (PPV, NPV) of wastewater testing by tampon swab and RT-LAMP for COVID-19 cases was estimated for incident COVID-19 cases in the residence hall each day following wastewater monitoring out to seven days. PPV and NPV were estimated across all nine residence halls each week, among single residence halls across all weeks, and across all residence halls and all weeks (Parikh et al., 2008). In this case PPV is the probability of an incident COVID-19 case following a positive wastewater sample, and, conversely, NPV is the probability of no incident COVID-19 cases following a negative wastewater sample. All graphing and statistical analyses associated with the described experiments were performed using GraphPad Prism Version 9.0.0 (GraphPad Software, LaJolla, CA, USA).

## 3. Results

In total, 153 wastewater samples were tested via RT-LAMP. To characterize the sensitivity, specificity, and analytical sensitivity of RT-LAMP, we used 24-hour composite samples of WWTP

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influent (n = 42) and raw sewage samples collected via tampon swabs (n=7). To analyze RT-LAMP performance with various extraction and processing methods, we leveraged samples from WWTP composites (n = 43) and tampon swabs (n = 78). Lastly, during a prospective wastewater monitoring campaign at ND, we used RT-LAMP to test 59 raw sewage samples collected via tampon swabs. One tampon swab could not be recovered because it broke free while deployed in a manhole.

*3.1 Analytical sensitivity*

Using RT-LAMP positivity and RT-ddPCR N1 copy number data, we estimated the RT-LAMP 95% LOD to be 76 gene copies (GC) for a single reaction (95% CI: 67 - 87) using a fitted cumulative Gaussian distribution (Figure S1; $R^2$ = 0.997). The RT-LAMP 95% LOD is approximately 20 times our estimate of the N1 RT-ddPCR 95% LOD (Bivins et al., 2021). NEB reports “positive detection observable down to 50 copies”, which is comparable to our estimated 67% LOD (51 GC/reaction). Since the RT-LAMP kit uses N2 and E primers, our N1 LOD estimates are not directly representative of the primers in the kit; however, they do provide an estimate of the RT-LAMP LOD relative to RT-ddPCR.

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

Compared to both RT-qPCR/ddPCR non-detections (N1; n = 13), RT-LAMP demonstrated an overall true negative rate (TNR) of 46%. Interestingly, the seven false positives were all in comparison to RT-qPCR non-detections (n=9). Whereas for the four RT-ddPCR non-detections, RT-LAMP demonstrated a TNR of 100%. Sample types among the non-detections included both WWTP influent composites and swab sorbate. The experimental design does not allow us to examine whether the difference in the TNR observed between RT-qPCR (two-step) and RT-ddPCR (one-step) is attributable to differences in the analytical sensitivities of the PCR methods,

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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*

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After heat extraction, 100% of swab sorbate samples (n=5) were inhibited and remained so even after 1:10 dilution. Among the five solid fraction samples, 100% were inhibited after heat extraction, and 40% remained so even after 1:10 dilution. Given the high rate of inhibition, we abandoned heat extraction as a reliable method for detection in wastewater via RT-LAMP.

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

Lastly, we assessed the rate of RT-LAMP inhibition for samples extracted using the Viral RNA Mini Kit (UGA) and PowerViral DNA/RNA Kit (ND). For 24-hour WWTP influent composite samples (n = 9), no inhibition was observed following extraction with the Viral RNA Mini kit. But we observed a 60% inhibition rate for sorbate from swabs deployed for 24 hours extracted with the same kit (n = 5). The inhibition rate for the passive samples was significantly greater than the rate for the composite samples (Figure S3 A; p = 0.0275). Among 24-hour WWTP influent composite samples extracted with the PowerViral kit (n = 33), 18% were inhibited. While for sorbate, sorbate solid fraction, and sorbate liquid fraction samples (n = 68) from swabs deployed for four hours, the PowerViral Kit produced an inhibition rate of 4%. The inhibition rate was significantly lower for passive samplers than composite samples extracted via PowerViral (Figure S3 B; p = 0.0317). As shown in Figure S4, the difference in inhibition rates between the Viral RNA Mini Kit and PowerViral DNA/RNA kit was not statistically significant for composite samples (panel A) or for all samples (panel C). We did observe a significantly lower rate of inhibition for swab samples extracted via PowerViral compared to Viral RNA Mini (Figure S4 B; p = 0.0030). However, this difference could also be attributable to the deployment of swabs for 24 hours at UGA (Viral RNA Mini) compared to only four hours at ND (PowerViral).

*3.7 Tampon Swab Sorbate Processing*

To optimize the workflow for SARS-CoV-2 RNA detection in wastewater via RT-LAMP, we assessed the rates of inhibition and positivity between Amicon-concentrated swab sorbate, the

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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

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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%

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during the six days following wastewater testing (Figure 2A). As shown in Figure 2B, NPV was greater with a maximum of 78% on the day of wastewater testing to a day six minimum of 38%. The PPV of wastewater testing could be adversely affected by positive RT-LAMP results attributable to convalescent COVID-19 cases returning to residence halls after isolation. As shown in Figure S9, there were six instances where RT-LAMP replicates were positive despite no incident COVID-19 cases, but with returning convalescent cases in the prior seven days. In these six instances, it required four or more convalescent cases before 2 of 3 RT-LAMP replicates were positive, suggesting that a cutoff value of 67% positivity (2 of 3 replicates) could increase the PPV of the wastewater method. As shown in Figure 2A, PPV is increased to 33% when 2 of 3 positive RT-LAMP reactions are required to classify a sample as positive. This change in cutoff value leaves the NPV largely unchanged (Figure 2B). If the detection of convalescent COVID-19 cases by wastewater sampling is considered a true positive (e.g., the true detection of SARS-CoV-2 RNA shed into the wastewater system), then the PPV improves to 56% on day 0 up to 75% by day three after wastewater monitoring (Figure S11).

## 4. Discussion

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

To develop more accessible wastewater monitoring techniques, we piloted and characterized the performance of a monitoring protocol that makes use of tampon swabs and RT-LAMP to detect SARS-CoV-2 RNA in building-level wastewater. The 95% LOD for a single RT-LAMP reaction was 20 times higher than the RT-ddPCR N1 assay 95% LOD. Several studies have found that SARS-CoV-2 RNA shedding in feces can outlast nasopharyngeal shedding in up to 50% of COVID-19 patients (Elbeblaw, 2020; Jones et al., 2020; Wang et al., 2020). In such cases, the higher RT-LAMP LOD could be advantageous by allowing for convalescent cases to go undetected, while newly incident COVID-19 cases could still be detected. RT-LAMP demonstrated an overall sensitivity of 57% compared to PCR methods, and a specificity of 100%

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compared to one-step RT-ddPCR. Unfortunately, we were not able to replicate the findings of an earlier pre-print study as all of our attempts to test wastewater directly were inhibited (Ongerth and Danielson, 2020). Our attempts at heat extraction were also consistently inhibited despite the success with saliva and other clinical samples (Mahmoud et al., 2021). We found that regardless of the wastewater type (influent composite or swab sorbate) the use of an extraction kit for testing by RT-LAMP was important to produce uninhibited results.

When paired with tampon swab sorbate, the Qiagen AllPrep PowerViral DNA/RNA Kit yielded a 4% inhibition rate among all samples. Concentrating sorbate with Amicon ultrafilters proved burdensome due to clogging. Since wastewater solids have been proposed as an efficient and sensitive partition for SARS-CoV-2 RNA detection (D'Aoust et al., 2021b; Kitamura et al., 2021), we opted to abandon Amicon concentration in favor of testing the sorbate solids fraction. We found that the solids fraction yielded a comparable SARS-CoV-2 positivity and inhibition rate to ultrafilter concentrate.

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

The optimized tampon swab and RT-LAMP workflow yielded a same-day PPV of 33% and an NPV of 80% in six weeks of wastewater monitoring. Accounting for the detection of convalescent cases improves the PPV to 56%. The PPV we observed was much lower than the 82% reported during another study leveraging PEG precipitation and RT-qPCR, but the NPV we observed (80% versus 88.9%) was comparable (Betancourt et al., 2021). The specificity of the tampon swab and RT-LAMP method for COVID-19 cases was 80%, which is better than the 52% specificity reported for an ultracentrifugation and RT-qPCR method that did not distinguish new infections from convalescent (Colosi et al., 2021). Thus, the tampon swab and RT-LAMP approach may offer a specificity and NPV comparable to more sophisticated monitoring methods. Several epidemiological modeling studies have suggested that an optimal strategy for managing COVID-

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19 on college campuses should include high-frequency screening tests that are highly specific (Lopman et al., 2021; Paltiel et al., 2020). Our observations indicated that the NPV and PPV for tampon swab and RT-LAMP monitoring were maximized with wastewater monitoring daily to every three days.

*4.3 Rapidity of RT-LAMP results*

These models have also consistently emphasized rapid results reporting over sensitivity as a critical feature of effective screening. Wong et al. found that wastewater monitoring with one day to results and four days or less to follow up clinical testing could keep infection rates within 5% of those achieved by clinical testing of individuals (Wong et al., 2020). Following extraction, the RT-qPCR and RT-ddPCR workflows used in the study required 3.5 and 7 hours, respectively, to produce results. Whereas, the RT-LAMP workflow required only 1.5 hours (45 minute preparation, 30 minute incubation, 15 minutes to read results). Additional time is required for tampon swab deployment, collection, sorbate harvesting, and extraction. At ND, tampon swabs were deployed at 8:00 am, retrieve at 11:00 am, and results were transmitted to the COVID Response Unit by 3:00 pm. Though we only conducted the wastewater monitoring weekly, the workflow could easily be modified to achieve results daily by noon. For example, a tampon swab could be deployed in the sewer for 24 hours, retrieved at 8:00 am, at which time another could be deployed, and results could be reported by noon at which time clinical testing could be mobilized in response. Based on a 5-day incubation and 1.2 day medical seeking period (Lauer et al., 2020), Zhu *et al*. have suggested a 6.2-day window to efficiently interrupt transmission chains (Zhu et al., 2021). The tampon swab and RT-LAMP method described in this study is easily capable of producing monitoring results within this window. Efficient transmission control through timely wastewater results is even more important on college campuses since asymptomatic infections are more prevalent among younger populations (Bjorkman et al., 2021).

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*4.4 Wastewater Monitoring Scalability and Accessibility*

In addition to reasonable specificity, and rapid results, the tampon swab and RT-LAMP method could also afford improved accessibility to wastewater monitoring in low-resource settings. Many of the COVID-19 wastewater monitoring efforts to date, including those on college campuses, have made use of composite samplers and RT-qPCR techniques to detect and quantify SARS-CoV-2 RNA (Ahmed et al., 2020b; Harris-Lovett et al., 2021). While these techniques have proven useful for tracking COVID-19 in some communities, the expense of composite samplers and the apparatus required to perform RT-qPCR greatly limits the accessibility and scalability of wastewater monitoring for SARS-CoV-2. The World Health Organization has identified wastewater monitoring approaches for pooled testing of high-risk lower-resource settings as a critical need to expand the application of the technique (World Health Organization, 2020). While we could not avoid using a kit-based RNA extraction, the method does not require a composite sampler or thermal cycler for RT-qPCR, relying instead on tampons for sampling and basic lab equipment including centrifuges, microcentrifuges, vortexes, and single temperature incubators for swab processing and RT-LAMP testing. The per sample analytical cost was comparable between RT-ddPCR ($35) and the NEB RT-LAMP kit ($31); however, we estimate that a self-assembled RT-LAMP kit using the same primers could halve the per-sample cost once optimized. Even with the off-the-shelf kit, the per capita consumables cost for the entire workflow was approximately $0.25.

*4.5 Limitations*

There are limitations that should be considered in generalizing the findings of this study. First, our comparison of RT-LAMP and RT-qPCR/ddPCR leveraged samples from only two monitoring sites, ND and UGA. Although we made use of raw sewage and WWTP influent samples, wastewater, and therefore RT-LAMP performance, can be variable among sites. For comparison with clinical surveillance, we monitored wastewater at nine ND residence halls. We note that while

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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.

- The consumables cost of wastewater monitoring over six by tampon swab and RT-LAMP was less than $2 per person and could likely be further reduced through a self-assembled LAMP kit.

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**Data Availability**

The datasets analyzed during the current study, excluding clinical data, are available in the OSF.IO repository, https://osf.io/2jdbs/ doi: 10.17605/OSF.IO/2JDBS.

**Competing Interests**

The authors declare no competing financial or non-financial interests.

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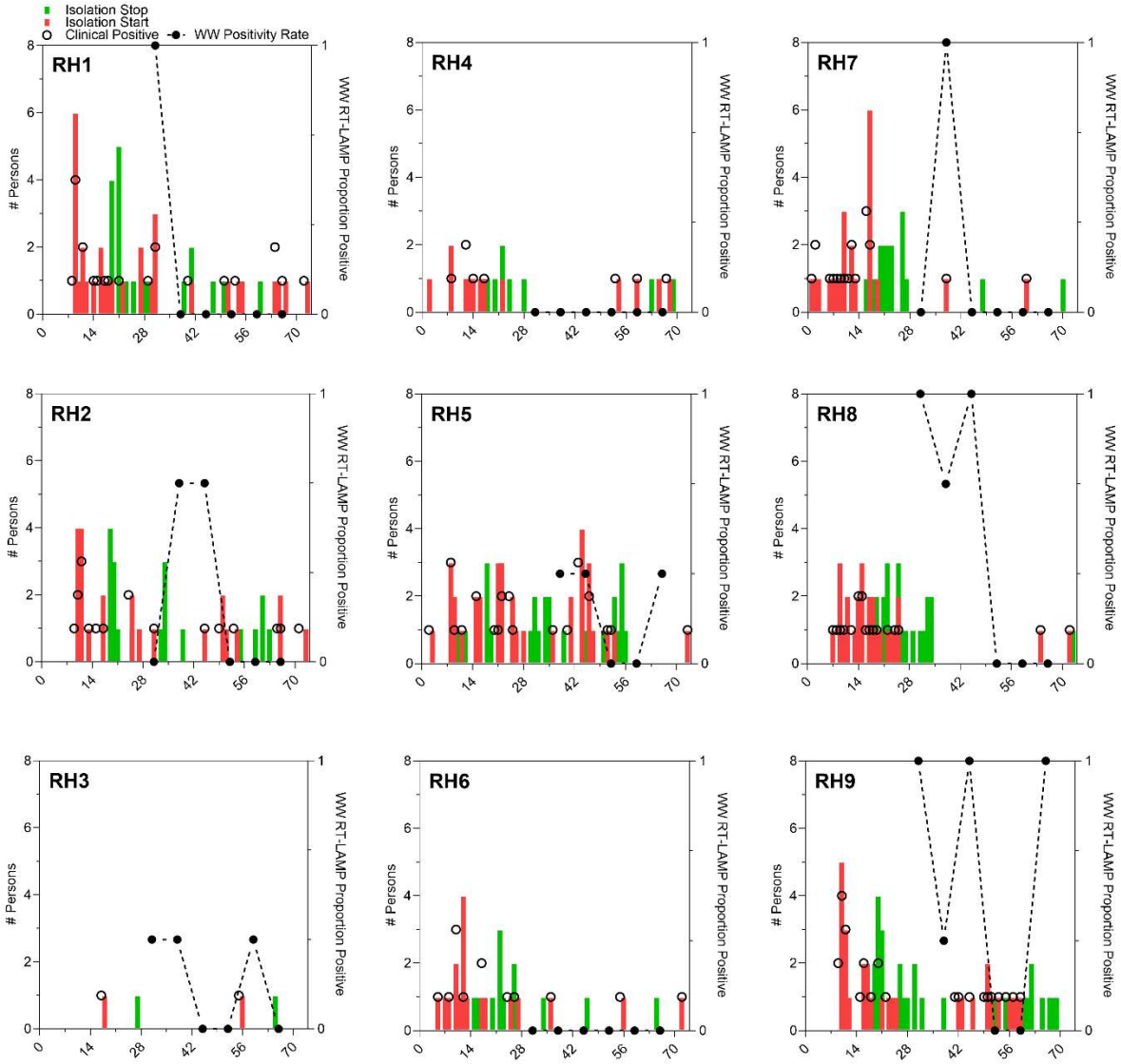


Figure 1 | Daily COVID-19 clinical positives, isolation start, and isolation stop (left y-axis), compared with the proportion of RT-LAMP reactions positive (three reactions per wastewater (WW) sample; right y-axis) for SARS-CoV-2 RNA among nine residence halls over a 73 day period (x-axis) with wastewater monitoring every seven days from day 31 to 66.

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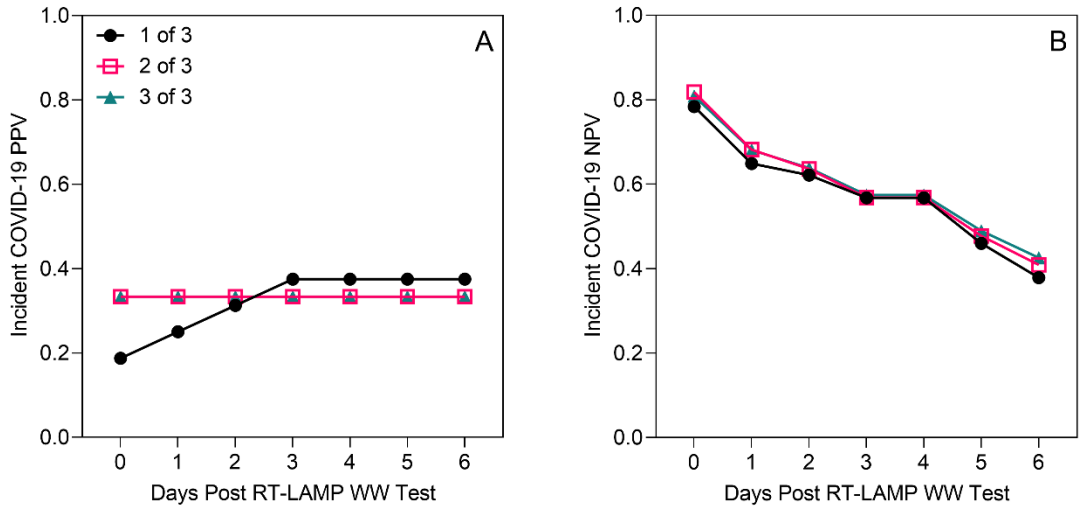


639
640
641 Figure 2 | Positive predictive value (PPV) (A) and negative predictive value (NPV) (B) in the seven
642 days following wastewater monitoring by tampon swab and RT-LAMP for three different cutoff
643 values for classification of RT-LAMP results as “positive” for SARS-CoV-2 RNA (1 of 3, 2 of 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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