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Detection of SARS-CoV-2 RNA in wastewater from dormitory buildings in a university campus: comparison with individual testing results

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ABSTRACT

Wastewater-based epidemiology (WBE) for monitoring COVID-19 has been largely used to detect the spread of the disease at the community level. From February to December 2022, we collected 24-h composite sewage samples from dormitory buildings in George Mason University (Fairfax, Virginia, USA) housing approximately 5,200 resident students. SARS-CoV-2 RNA extraction was achieved using an automated system based on magnetic nanoparticles. Analysis of SARS-CoV-2 RNA was performed using reverse transcription quantitative PCR based on the Centers for Disease Control and Prevention (CDC) N1 and N2 assays. From the 362 samples collected, 86% showed positive detection of SARS-CoV-2 RNA. Wastewater monitoring was able to detect SARS-CoV-2 RNA in 96% of the samples from buildings housing students with COVID-19. Over the period of study, we observed significant correlations between the SARS-CoV-2 concentration (copy number mL^{-1}) in wastewater and the number of positive cases on campus based on individual saliva testing. Although several reports have been published on the wastewater monitoring of COVID-19 in university campuses, our study is one of the very few that provides results that were obtained during the last phase of the pandemic (roughly the year 2022), when the large majority of students were vaccinated and back on campus.

Key words: clinical saliva testing, COVID-19, polymerase chain reaction (PCR), SARS-CoV-2, university campuses, wastewater-based epidemiology (WBE)

HIGHLIGHTS

- Monitoring the spread of COVID-19 in a university campus.
- WBE is proven to be a reliable method to detect pathogens in communities.
- 310 of 362 samples (85.6%) showed positive detection of SARS-CoV-2 RNA.
- We observed significant correlations between wastewater and individual testing results in the campus.
- Although papers have been published in this work, our study is one of the few that covers the end phase of the pandemic.

GRAPHICAL ABSTRACT

INTRODUCTION

The infectious disease, COVID-19, is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Andersen *et al.* 2020). COVID-19 is traditionally tested in individuals via collection of nasopharynx or throat swabs, or saliva samples, and detection of SARS-CoV-2 through reverse transcription quantitative PCR (RT-qPCR) or antibody and antigen assays (Gonzalez *et al.* 2022). However, individual testing has been shown to be sometimes difficult to implement due to a variety of factors, including the cost of analyses, the availability of clinical tests, the difficulty to reach certain layers of the population, and the willingness of individuals to be tested (Keshaviah *et al.* 2021; Ahmed *et al.* 2022). These difficulties have led to the development of alternate methods, such as monitoring SARS-CoV-2 RNA in wastewater to complement individual testing. Unlike individual testing, wastewater-based epidemiology (WBE) is based on the analysis of sewage, which allows researchers to monitor a large number of individuals from the analysis of a few samples. The approach can therefore capture the health status of a community as a whole, reaching all layers of the population, regardless of the individual's ability or willingness to be tested. WBE uses the analysis of a limited number of samples compared to individual testing, making this method cost-effective and less sensitive to supply shortages.

Over the recent years, WBE has been recognized for its potential to provide insights into community health and environmental exposure, including illicit drugs consumption, pharmaceuticals use/abuse, water pollution, and the occurrence of pathogens and antimicrobial resistance (Randazzo *et al.* 2020; Sims & Kasprzyk-Hordern 2020; Picó & Barceló 2021). While the nation is moving to a post-pandemic phase of COVID-19, the need for monitoring for potential new outbreaks, including new variants of SARS-CoV-2, remains critical. Since the emergence of the COVID-19 disease, WBE has gained increasing interest for monitoring the spread of SARS-CoV-2 at the community level. WBE has allowed monitoring the prevalence of SARS-CoV-2 in populations and detecting variants of concern with minimal inconvenience to the resident community (Sherchan *et al.* 2020; Sims & Kasprzyk-Hordern 2020). The recognition of the potential of WBE to guide the COVID-19 response has led the Centers for Disease Control and Prevention (CDC) to launch in 2020 the National Wastewater Surveillance System (NWSS) (Kirby 2021).

WBE is based on the aggregated community signal and can therefore inform on community-level trends of a disease that may be undetectable via individual testing until later stage of an epidemic. In addition, WBE is a useful pre-screening tool to target the clinical testing needs within a community. RNA from SARS-CoV-2 is shed in feces of 50–68% of infected individuals, both asymptomatic and pre-symptomatic, and is detectable for up to 30 days after the initial infection (Wu *et al.* 2020). Because only a portion of infected individuals are believed to shed the virus in sewage, WBE only applies to the analyses of sewage systems serving a rather large number of individuals. Current technology potentially allows detection of a single infected individual within a population of 10,000 (Karthikeyan *et al.* 2021). Although this level of sensitivity would make it possible to detect the virus in a specific residential area, or even in a single building, it may not be effective during low infection rates. Recent studies have shown that monitoring wastewater systems has the potential to predict COVID-19 outbreaks before they may spread and monitor the evolution of the disease at the community level without the need for individual testing (Harris-Lovett *et al.* 2021). Along with other PCR-based methods, WBE offers the possibility to quickly develop an assay to target new pathogens or variants. With the development of next-generation sequencing (NGS) technologies, it might be possible to use WBE for the detection of a range of pathogens in wastewater, even without prior knowledge of its presence (i.e., untargeted analysis).

Since the onset of the COVID-19 pandemic in early 2020, multiple reports have been published about WBE monitoring of SARS-CoV-2. Wastewater surveillance of SARS-CoV-2 has ranged from individual university buildings to large municipalities (Randazzo *et al.* 2020; Sherchan *et al.* 2020; Wu *et al.* 2020; Betancourt *et al.* 2021; Harris-Lovett *et al.* 2021). Most published reports indicated successful detection of SARS-CoV-2 RNA in some of the sewage samples tested. Moreover, several publications reported significant correlations between wastewater analysis and regional public health epidemiological data (Peccia *et al.* 2020; Randazzo *et al.* 2020; D'Aoust *et al.* 2021). WBE studies for SARS-CoV-2 monitoring have focused on multiple types of samples, including untreated wastewater (sewage), treated wastewater at different treatment stages, and surface water (WWTPs) (Haramoto *et al.* 2020; Peccia *et al.* 2020; Randazzo *et al.* 2020; Zhao *et al.* 2022). Although the overall workflow of the analysis of SARS-CoV-2 RNA in wastewater is rather similar in most studies (sewage sample collection, RNA concentration/extraction, RNA detection, and virus quantification), a large variety of protocols have been published (Harris-Lovett *et al.* 2021; Pecson *et al.* 2021). Collection of both grab samples and composite samples was reported (Randazzo *et al.* 2020; Wurtz *et al.* 2021). Concentration of the virus was performed using a range of methods, including electronegative

membrane (ENM) filtration, polyethylene glycol (PEG)-NaCl precipitation, ultrafiltration, and magnetic nanoparticle concentration (e.g., Nanotrap®) (D'Aoust *et al.* 2021; Gibas *et al.* 2021; Karthikeyan *et al.* 2021; Nagarkar *et al.* 2022). Most studies used the CDC published assays based on the PCR detection of the nucleocapsid genes N1 and N2 (Wu *et al.* 2020; Scott *et al.* 2021), although other markers, such as the World Health Organization (WHO) E-Sarbeco (Gonzalez *et al.* 2022), have been utilized. Various quality control tests have been proposed, such as the use of fecal markers for normalization of the results (e.g., pepper mild mottle virus (PMMoV)), matrix recovery spikes (e.g., MS2 bacteriophage, bovine coronavirus), as well as positive and negative controls (Wu *et al.* 2020; D'Aoust *et al.* 2021; Scott *et al.* 2021; Nagarkar *et al.* 2022). Detection of SARS-CoV-2 RNA and supplemental markers has been performed using RT-qPCR (Wu *et al.* 2020) or reverse-transcriptase droplet digital PCR (RT-ddPCR) (Nagarkar *et al.* 2022); it is noteworthy that the two methods are characterized by different sensitivities and may lead to different results. Results were typically reported as virus or gene copy number per volume of water (e.g., sewage, wastewater) or mass (e.g., biosolids) of sample. Although the lack of standardized methods may impair the comparability of the results, an interlaboratory assessment including 36 standard operating procedures (SOPs) showed good reproducibility across the different methods, even though the reproducibility within a single SOP was higher (Pecson *et al.* 2021). If the comparison included different protocols for most steps of the analytical workflow, it did not assess the effect of the sampling method (i.e., grab vs. combined sampling) and the molecular markers used (all SOPs used the CDC N1 and N2 markers), which are procedures that can significantly affect the results.

The main objective of this study was to determine the prevalence of SARS-CoV-2 RNA in untreated wastewater in the Main Campus of George Mason University (GMU, Fairfax, Virginia, USA). A secondary objective was to compare the SARS-CoV-2 RNA concentration in wastewater with the number of positive cases among GMU resident students when detected by individual testing. During the year 2022, a series of variants were observed in the USA, with Omicron B.1.1, BA.2, BA.2.12.1, BA.5, BQ.1, and BQ.1.1 being the most prevalent (CDC, Genomic Surveillance for SARS-CoV-2 Variants). Although several publications have reported successful detection of SARS-CoV-2 in sewage from small communities, such as university campuses, most of the prior studies were conducted in 2020 and 2021 (Harris-Lovett *et al.* 2021). Our study provides results obtained over the last phase of the pandemic (roughly the year 2022), when most students were back on campus and vaccinated, and when the rate of individual student testing was largely reduced.

EXPERIMENTAL SECTION

Sample collection

Untreated wastewater samples were collected from manholes of the sewage system flowing from residence halls on the GMU Main Campus (Fairfax, Virginia, USA). The monitoring focused on collector lines serving major dormitory buildings in an attempt to capture the health status of the largest number of resident students with the resources available. 24-h combined samples were collected weekly from February to December 2022. Six sites serving dorm buildings housing approximately 2,900 students (representing 56% of the Main Campus resident population) were sampled from February to August 2022, and 12 sites serving dorm buildings housing approximately 5,200 students (representing 98% of the main campus resident population) were sampled from August to December 2022 (Figure 1). On some rare instances, no significant volume was collected by the autosampler due to a low sewage flow, in which case a grab sample was obtained at the collection time instead of a 24-h combined sample. Also, two sites were not sampled in the first weeks of the study because of technical difficulties with installation of the autosamplers. Samples were collected using refrigerated autosamplers installed in selected manholes (ISCO GLS Compact Samplers). The autosamplers were deployed for a 24-h period from Monday night. The autosamplers were programed to collect 180 mL of raw wastewater every 30 min for 24 h, producing a combined sample of approximately 8.65 L. After collection, samples were kept on water ice in a cooler (temperature 4–10 °C), transported to the laboratory within an hour, and immediately processed.

RNA concentration and extraction

Wastewater samples were processed in biosafety level 2 (BSL-2) labs. The raw wastewater was first subjected to pasteurization for pathogen inactivation by incubation 20 min in a water bath at 65 °C (Wu *et al.* 2020). Two replicate aliquots of 10 mL were collected for each sample. About 50% of the samples were spiked with *Escherichia coli* MS2 bacteriophage (*Emesvirus zinderi*) as a matrix recovery control (Zeptometrix, Buffalo, New York, USA) to the final concentration of 4,000 copies/μL

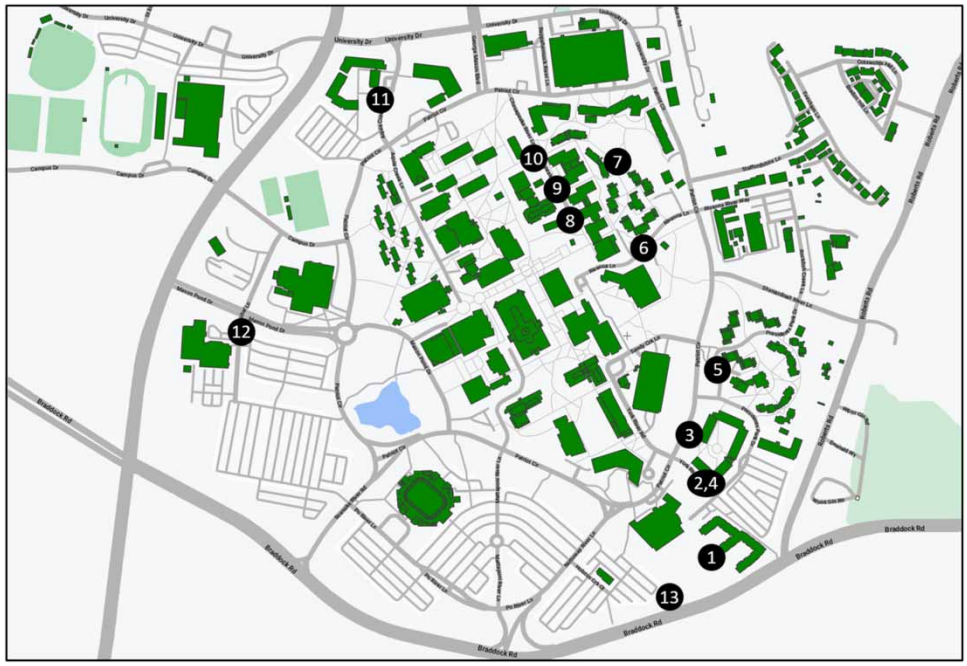

Figure 1 | Sites and dormitory buildings in George Mason University Main Campus used for untreated wastewater collection: 1: Potomac Heights, 2: Liberty Square 1, 3: Liberty Square 2, 4: Liberty Square 3, 5: Presidents' Park, 6: Essex-Carroll, 7: Hampton Roads-Eastern Shore, 8: Blue Ridge, 9: Sandbridge, 10: Northern Neck, 11: Whitetop-Rogers, 12: Ángel Cabrera Global Center, and 13: East Campus.

(Agrawal *et al.* 2021). Samples were then used for RNA concentration and extraction according to Karthikeyan *et al.* (2021). In brief, 10 mL of the sample was mixed with Nanotrap® Magnetic Virus Particles (Ceres Nanosciences, Manassas, Virginia, USA) and concentrated using an automated KingFisher™ Flex robot (Thermo Fisher, Waltham, Massachusetts, USA). 450 µL of the RNA concentrate were collected and extracted using the MagMAX™ Total Nucleic Acid Isolation Kit (Thermo Fisher) following the manufacturer's recommendations (Karthikeyan *et al.* 2021).

RT-qPCR analysis

RNA detection and quantification were performed by RT-qPCR using a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher) with the TaqPath™ 1-Step RT-qPCR Master Mix. For RT-qPCR detection of SARS-CoV-2, the N1 and N2 primers and probes (targeting two SARS-CoV-2 nucleocapsid (N) genes) were obtained from the Promega (Madison, Wisconsin, USA) (Center for Disease Control & Prevention 2020). Each sample was also analyzed for the PMMoV, a sewage fecal marker (Sherchan *et al.* 2020; Graham *et al.* 2021), and the spiked bacteriophage MS2. For all markers, the cycling conditions were as follows: RT for 15 min at 50 °C, initial denaturation for 2 min at 95 °C, and 45 cycles including denaturation for 30 s at 95 °C and annealing/extension for 30 s at 60 °C (reaction volume 20 µL). Each plate contained the samples, as well as positive and negative (no template) controls. Positive controls for SARS-CoV-2 and bacteriophage MS2 were obtained from Twist Bioscience (San Francisco, California, USA) and ZeptoMetrix (Buffalo, New York, USA), respectively. The RNA copy numbers were calculated using standard curves obtained with commercial standard RNA for N1, N2, and MS2, and RNA extracts from hot chili pepper sauce (Tabasco, Avery Island, Louisiana, USA) for PMMoV (Colson *et al.* 2010). Based on the linearity of the standard curves, the limit of quantification (LOQ) of SARS-CoV-2 RNA was lower than 2,000 copy numbers mL^{-1} for both N1 and N2 assays (the LOQ is defined as the lowest concentration of the calibration standards within the linear range of the standard curve). The LOQ of PMMoV and MS2 bacteriophage RNA was lower than 2,000 and 90,000 copy number mL^{-1}, respectively. A positive detection of the SARS-CoV-2 RNA in a sample was recorded if a PCR signal was detected with at least one nucleocapsid assay (either N1 or N2), for at least one of the two replicates, and a cycle threshold below 40. The list of primers and probes used in this study is given in Supplementary material, Table S1.

Individual saliva testing performed at GMU

Saliva-based COVID-19 testing was conducted at GMU using the Advanta™ Dx SARS-CoV-2 RT-PCR Assay (Fluidigm Corporation, South San Francisco, California, USA). The saliva-based assay is a non-invasive alternative to the more conventional nasopharyngeal swab-based assay. Individuals scheduled for testing were instructed not to eat, drink (except water), vape, smoke, chew gum, or use nasal sprays, throat lozenges/sprays, mouthwash or breath fresheners 30 min prior to saliva collection. Unstimulated whole saliva samples (min 1 mL) were collected in 5-mL sterile DNase- and RNase-free tubes by the passive drooling method. The assay was based on an automated, real-time PCR system (Biomark HD device) using the microfluidic technology, which allowed a lower sample volume and a higher testing capacity. The assay was authorized under FDA Emergency Use Authorization (EUA) and was CE-IVD marked under the In Vitro Diagnostics Directive (IVDD 98/79/EC). In short, the assay workflow involved the following steps: (1) specimen preparation (dilution of saliva in PBS and RNA Secure), (2) heat inactivation, and (3) one-step RT and pre-amplification reactions in a 96-well thermal cycler (Applied Biosystems, Beverly, Massachusetts, USA), (4) preparation of the final assay mixes, final pre-amplified samples, and final control mixes for real-time PCR, (5) preparation of the Advanta Dx IFC (integrated fluidic circuit) by injecting control line fluid and pipetting the assay and sample mix into the IFC, (6) loading the IFC on the IFC Controller RX, (7) perform thermal-cycling and collect data on the Biomark HD, and (8) analyze the data using the Real-Time PCR Analysis software and export the results using the Advanta Dx SARS-CoV-2 RT-PCR Assay interpretive software (Balaska *et al.* 2021). The N1, N2, and RNase P primers and probes (CDC) were used for the three reaction steps (RT, pre-amplification and qPCR). A no template control, a negative extraction control, and a positive control were loaded on each 96-well sample plate. The assay has been shown to provide an accuracy comparable to that of nasopharyngeal-based testing, with a sensitivity of 88.5% and specificity of 98.1% in diagnostic samples (Balaska *et al.* 2021; Butler-Laporte *et al.* 2021). The limit of detection (LOD) of the Advanta Dx SARS-CoV-2 RT-PCR was determined to be seven copies per reaction (Balaska *et al.* 2021). The saliva analysis was performed at GMU (Institute for Advanced Biomedical Research (IABR) building, Manassas, Virginia, USA), with a processing capability of about 10,000 tests per week.

Data analysis

For comparison between SARS-CoV-2 in sewage and individual testing on the whole Main Campus, we used several metrics: we first averaged the N1 and N2 copy numbers mL^{-1} of individual buildings and building groups (*average copy number* mL^{-1}). The average N1 and N2 copy numbers mL^{-1} were then normalized by the PMMoV copy numbers, reflecting the amount of fecal material in the sewage (*normalized average copy number* mL^{-1}). To account for the different numbers of students in different buildings, we also computed the average of the N1 and N2 copy numbers mL^{-1} weighted by the student population housed in these buildings or building groups (*weighted average copy number* mL^{-1}).

Saliva testing results were adjusted to match the buildings in which students resided with the groups of buildings from which the wastewater was tested. Since wastewater was analyzed once a week (24-h composite sample), we compare wastewater results with the weekly numbers of the new COVID-19-positive cases recorded.

RNA copy numbers (including the average and normalized average numbers) and standard curves (including R^2 and amplification efficiency) were computed using Excel (Microsoft Office 2021). Spearman's rank correlation coefficients (ρ) and the associated p-values were calculated with SPSS version 26.0 (IBM, Chicago, Illinois, USA) using the function 'Correlate'. We used the Spearman's rank correlation because most of our data were not normally distributed (based on Kolmogorov–Smirnov and Shapiro-Wilk tests). The interpretation of the correlations was as follows: 0.9–1.0: very high, 0.7–0.9: high, 0.5–0.7: moderate, 0.3–0.5: low, and 0.0–0.3: negligible) (Mukaka 2012).

RESULTS AND DISCUSSION

Detection of target and control RNAs in wastewater

The concentration of SARS-CoV-2 RNA in wastewater ranged from not detected to 3.71×10^6 copy numbers mL^{-1} based on N1 assay and 6.24×10^6 copy numbers mL^{-1} based on N2 assay. The average and median concentration of PMMoV marker in wastewater samples were 4.39×10^8 and 3.15×10^8 copy numbers mL^{-1}, respectively. The standard curves for N1, N2, PMMoV, and MS2 showed an amplification efficiency of 99.4, 98.1, 103.9, and 100.8 respectively, with $R^2 \geq 0.98$ in all cases. The percent recovery for the extraction method (ratio of the concentration of MS2 quantified by RT-qPCR and the theoretical MS2 concentration based on the initial spike) ranged from 0.003 to 107.5%, with an average equal to 5.3%

and a median equal to 1.4%. The percentage of samples with a recovery rate above 0.01% was 98.4%. No results were omitted for the statistical analysis because a recovery rate > 0.00% was observed for all samples. However, the recovery rate was not used further in the statistical analysis because of the issues traditionally associated with the spike recovery controls (Kantor *et al.* 2021; Zambrana *et al.* 2022). In addition to the absence of a standardized approach for integrating the recovery control results, many factors may affect differentially the recovery efficiency of the spiked control (and the SARS-CoV-2 RNA) (reviewed in Kantor *et al.* 2021). The most commonly cited factors include the variability of the RNA concentration and the efficiency of RNA extraction and PCR amplification, which are differentially affected by the wastewater characteristics (e.g., inhibitors, particulate matter, temperature) and the nature of the recovery control (e.g., size, encapsulation, degradability, viral aggregation). Other difficulties may include the variability of the ratio of intact-to-nonintact viruses, which may affect the RNA recovery and detection. Consequently, the error propagation that originates from the correction of the results using the recovery rate may lead to a larger variability within the original data (Kantor *et al.* 2021).

All samples showed positive detection of the PMMoV RNA, even though some variability was present. The PMMoV marker can serve as a natural internal control for the PCR reaction, therefore ensuring that the absence of positive detection of SARS-CoV-2 (based on the N1 and N2 makers) in some samples was not caused by inhibition of the PCR reaction or degradation of the virus in wastewater (Zambrana *et al.* 2022).

Positive and negative controls were as expected, i.e., the positive controls produced a positive signal, and the negative (no template) controls did not show amplification.

Detection of SARS-CoV-2 RNA in sewage in GMU main campus

From February to December 2022, untreated wastewater samples were collected weekly from sewage flowing from selected dorm buildings in GMU Fairfax Main Campus. Weekly samples were collected at six sites from February to August covering (approximately 56% of the resident population) and from 12 sites from August to December covering (approximately 98% of the resident population). Samples were analyzed for quantification of SARS-CoV-2 RNA (copy numbers mL^{-1}) using RT-qPCR. Wastewater results were then compared with results from individual testing (saliva testing) conducted on residential students during the same period. Although clinical testing was performed routinely on students in residential halls, the testing frequency and rules changed several times during the study period, making it difficult to define systematic testing rules. By the fall of 2022, individual testing dropped sharply, which was likely related to the relaxation of the university's testing rules, the increase of home testing, and personal fatigue or unwillingness to test post-vaccination because of a perceived reduced risk (Alvarez *et al.* 2023).

From February 1 to December 13, 2022, we collected a total of 362 samples, of which 310 (=85.6%) showed positive detection of SARS-CoV-2 RNA (positive detection indicates a positive signal in at least one assay (N1 or N2), for at least one replicate, and with a cycle threshold below 40). Out of the 164 samples associated with positive cases from clinical testing, 157 samples (=95.7%) showed positive detection of SARS-CoV-2 by RT-qPCR – this is remarkable considering that, except for one dormitory building (Global Center, which was used to isolate students who tested positive), only few positive cases were generally reported in a single building. This level of sensitivity has been reported by several other authors (Gibas *et al.* 2021; Zambrana *et al.* 2022; Sharaby *et al.* 2023). For instance, Sharaby *et al.* (2023) reported positive detection of approximately one infected individual over 1,000.

For 159 samples (=43.9% of the total), a positive RT-qPCR signal was detected although no positive individuals were reported in the building at the time of sample collection. A similar observation was reported on WBE studies conducted in other decentralized closed systems, such as hospitals, nursing homes, prisons, and cruise liners (Harris-Lovett *et al.* 2021; Scott *et al.* 2021; Wong *et al.* 2021; Sharaby *et al.* 2023). This observation can easily be explained by the fact that not all individuals in a given building were tested during the sampling time window – asymptomatic individuals for instance tend not to seek testing (it is generally accepted that at least 20% of individuals infected with SARS-CoV-2 remain asymptomatic). Also, the increase of tests conducted at home likely contributed to reducing the number of positive individuals recorded by the university. Finally, during the wastewater collection time, toilets in the building may have been used by visitors or staff personnel who do not reside in the dorm.

The percentages of positive detection in sewage reported in comparable studies focusing on small populations varied widely, from approximately 3% (Zambrana *et al.* 2022) to 82% (Betancourt *et al.* 2021), but were generally lower than the percentage of positive samples observed in our study (approximately 86%) (Supplementary material, Table S2). For instance, Gibas *et al.* (2021) (University of North Carolina, Charlotte, North Carolina, USA) reported that over the sampling period

from September 28 to November 23, 2020, only 16.6% of the samples (55 out of 332) showed a positive signal in at least one of the two qPCR replicates. Similarly, based on an 8-month monitoring campaign from October 2020 to June 2021, Sharaby *et al.* (2023) (Technion University, Haifa, Israel) reported that only 11.5% of the 523 processed samples were positive for the detection of SARS-CoV-2. This can be explained by the sampling period showing different national/regional COVID-19 trends. However, many of the prior studies were conducted over a period of time with high prevalence of COVID-19 based on the national weekly new cases (Supplementary material, Figure S1). The higher percentage of positive wastewater samples detected in our study, during which the weekly national new cases was generally lower than in the previous phases, is intriguing and can be explained by a higher number of asymptomatic, mild, and non-reported or non-tested COVID-19 cases during the end of the pandemic phase. Comparison with studies conducted in earlier phases of the pandemic must be undertaken with caution due several factors, including the increased vaccination rates and potential changes in testing behavior (Reese *et al.* 2021).

Comparison between sewage and individual testing: whole GMU Main Campus

For comparison between SARS-CoV-2 in sewage and individual testing on the whole Main Campus, we used several metrics, including the *average copy number* mL^{-1} (average values across all sites), the *normalized average copy number* mL^{-1} (PMMoV-normalized average values), and the *weighted average copy number* mL^{-1} (average values weighted by the building occupancy).

Over the year 2022, the SARS-CoV-2 copy numbers mL^{-1} determined using N1 and N2 assay showed very high correlations: Spearman rank coefficient (ρ) = 0.98 ($p < 0.001$) for the average copy numbers mL^{-1}, $\rho = 0.96$ ($p < 0.001$) for the PMMoV-normalized copy numbers mL^{-1}, and $\rho = 0.95$ ($p < 0.001$) for the weighted average copy numbers mL^{-1} (interpretation of the correlations were as follows: 0.9–1.0: very high, 0.7–0.9: high, 0.5–0.7: moderate, 0.3–0.5: low, and 0.0–0.3: negligible) (Mukaka 2012). The highly correlated N1 and N2 copy numbers mL^{-1} allowed us to average the N1 and N2 values (referred to as *N1/N2 copy number* mL^{-1}) as an estimation of the SARS-CoV-2 RNA concentration in wastewater. As indicated in other reports, the copy numbers mL^{-1} associated with N1 and N2 are typically consistent, even though N1 is considered more sensitive than N2 at low level of detection (Feng *et al.* 2021; Scott *et al.* 2021). Also, a very high correlation was observed between the simple average and weighted average N1/N2 copy numbers mL^{-1}: $\rho = 0.99$ ($p < 0.001$). On the other hand, a lower correlation was observed between the non-normalized and PMMoV-normalized N1/N2 copy numbers mL^{-1}: $\rho = 0.76$, $p < 0.001$.

Several metrics were available as indicator of the total number of resident students on Main Campus tested positive for COVID-19 based on saliva testing. These metrics included the weekly new positive cases, the weekly positivity rate (number of positive cases divided by the number of individual tested), and the number of students in isolation in the Global Center. The saliva testing assay has been shown to provide an accuracy comparable to that of nasopharyngeal-based testing, with a sensitivity of 88.5% and specificity of 98.1% in diagnostic samples (Balaska *et al.* 2021; Butler-Laporte *et al.* 2021).

Table 1 shows the Spearman's rank correlation coefficients (ρ) and associated p-values for the relationships between wastewater SARS-CoV-2 concentrations and individual testing metrics, as well as the Fairfax County, Virginia, and US COVID-19 numbers (CDC database) for the entire period of the study (February to December 2022). A low, but significant correlation was observed between the average N1/N2 copy numbers mL^{-1} and the weekly new positive cases ($\rho = 0.48$, $p < 0.001$). A similar low correlation was detected between the average N1/N2 copy numbers mL^{-1} and the positivity rate ($\rho = 0.46$, $p < 0.001$). Correlations with the number of students in isolation in the Global Center, as well as correlations with the Fairfax County, Virginia, and US COVID-19 data, were non-significant. The correlation between the PMMoV-normalized N1/N2 copy numbers (instead of N1/N2 copy number) and the weekly new positive cases was lower, although significant ($\rho = 0.41$, $p < 0.001$). The use of the weighted N1/N2 copy numbers (instead of N1/N2 copy number) did not affect much the correlation with the total weekly average ($\rho = 0.45$, $p < 0.001$). These results are also displayed graphically in Figure 2 and Supplementary material, Figure S2.

Although higher correlations are expected when the population considered is higher (e.g., wastewater treatment plants serving large communities), many reports focusing on rather small populations (e.g., university campuses) reported similarly significant correlations between wastewater SARS-CoV-2 concentrations and individual testing results (Supplementary material, Table S2). Near-source surveillance has several benefits, such as reduced dilution of the sample, lower RNA degradation, and fewer inhibitors. It also includes some potential disadvantages such as the oversized effect of other wastewater

Table 1 | Correlation matrix between selected wastewater copy numbers mL^{-1} and saliva testing metrics for the period of study (February to December 2022) (The metrics used for calculation of the correlation coefficients were described in the text and the footnote)

		Weekly positives[a]	Weekly positivity rates (%)	Weekly isolation at GC	Fairfax County weekly cases[b]	VA State weekly cases[b]	USA nationwide cases[c]
Average N1/N2	Spearman's correlation	0.484**	0.456**	0.243	0.240	0.118	0.094
	Sig. (2-tailed)	0.001	0.002	0.108	0.113	0.439	0.537
Average normalized N1/ N2	Spearman's correlation	0.414**	0.297*	0.247	0.296*	0.200	0.147
	Sig. (2-tailed)	0.005	0.048	0.102	0.048	0.189	0.335
Weighted average N1/N2	Spearman's correlation	0.448**	0.448**	0.212	0.242	0.129	0.100
	Sig. (2-tailed)	0.002	0.002	0.162	0.110	0.398	0.512

**Correlation significant at the 0.01 level.
*Correlation significant at the 0.05 level.
[a]Number of observations, $n = 44$.
[b]Data obtained from the Virginia Department of Health (USA), COVID-19 Dashboards.
[c]Data obtained from the Centers for Disease Control and Prevention (USA), COVID Data Tracker.

inputs. Nevertheless, comparison between our study and other smaller-scale studies is difficult due to the different correlation metrics used. For instance, Wright *et al.* (2022) reported a correlation coefficient $r = 0.71$ ($p < 0.01$) between wastewater and individual testing number over the fall semester 2020 in a large southwestern university. However, significant correlations between wastewater and individual testing were not always observed. Studying the prevalence of COVID-19 in Tulane University (New Orleans, Louisiana, USA) during the fall semester 2020, Scott *et al.* (2021) observed correlations ranging from non-significant ($r = 0.064$, $p = 0.835$) to highly significant depending on the group of buildings considered ($r = 0.692$, $p = 0.006$). Low or absence of correlation between wastewater SARS-CoV-2 concentration and individual testing

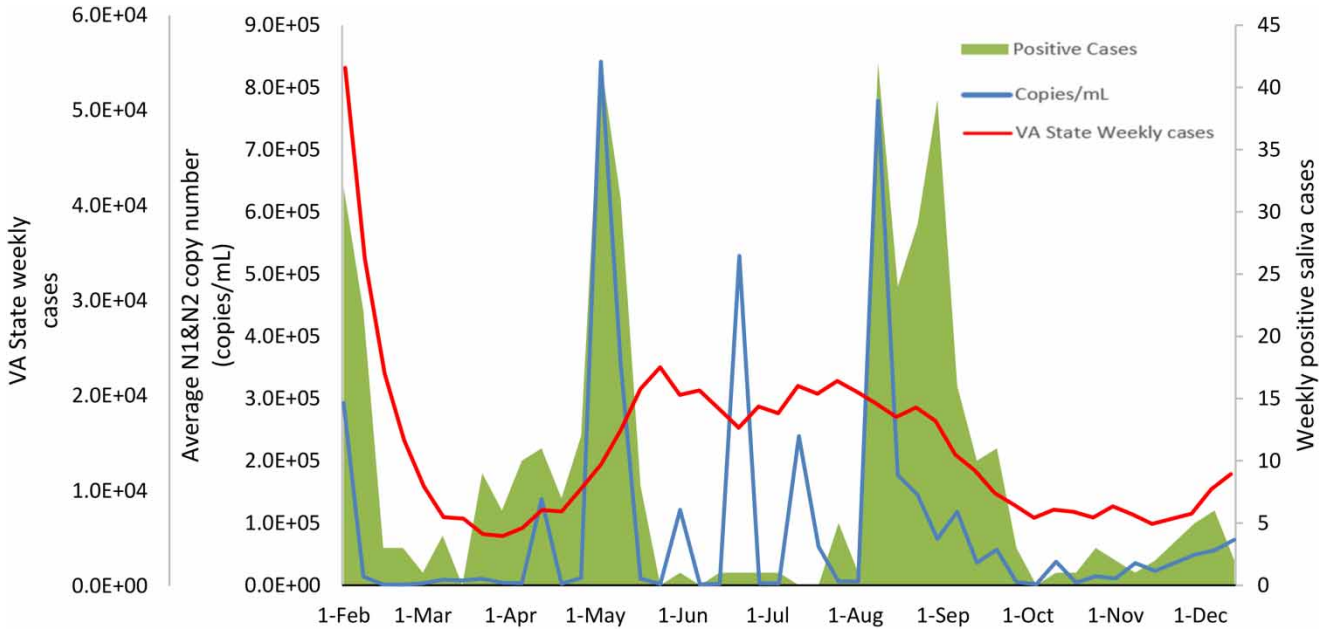

Figure 2 | Trends of SARS-CoV-2 concentration in wastewater, number of weekly positive cases among resident students in GMU Main Campus, and weekly news cases in the state of Virginia during the period of study (February to December 2022). The results are based on the analysis of 362 wastewater samples.

data can be explained by multiple factors affecting either wastewater or individual testing metrics. Biases affecting wastewater SARS-CoV-2 concentrations may include variable shedding of the SARS-CoV-2 virus in wastewater, the water usage per person, the stability of the virus, and the RNA extraction method used (Kotay *et al.* 2022; Zambrana *et al.* 2022). Although studies have provided evidence of SARS-CoV-2 fecal shedding in symptomatic, asymptomatic, and vaccinated individuals, these observations have not been quantitatively examined (Arts *et al.* 2023). To the best of our knowledge, differential shedding of SARS-CoV-2 variants has not been investigated. Biases affecting individual testing data may include the percentage of students tested and the temporary presence of guests or staff employees in the building (see above).

WBE data normalization is a common data measurement correction for any variations resulting from dilution of wastewater, the abundance of fecal matter, and other various liquids that may enter the sewage network (e.g., dishwashers, laundry machines, showers, infiltration from surface runoff, etc.) (Maal-Bared *et al.* 2023). Several reports included normalization of the wastewater copy numbers mL^{-1} using the PMMoV copy numbers mL^{-1}. However, no general agreement seems to exist that normalization increased the correlations between wastewater qPCR signals and reported COVID-19 cases. Often, as in our study, normalization using a fecal marker was not shown to positively affect correlations between wastewater concentrations and positive cases (Feng *et al.* 2021; Kantor *et al.* 2021; Zambrana *et al.* 2022). In some cases, however, fecal normalization was shown to improve correlations between wastewater and individual testing data (D'Aoust *et al.* 2021; Scott *et al.* 2021). Some authors have suggested that fecal normalization was more useful when comparing results obtained with different methods or in different studies (Simpson *et al.* 2021; Zambrana *et al.* 2022).

The low correlations between the N1/N2 copy numbers and the positivity rate determined by saliva testing may be unexpected. However, the positivity rate depended on the number of students tested which was variable depending on the academic semesters and the changing policies for on-campus testing. During the fall 2022, there was a drop of the number of individuals tested on campus (the weekly average number of students tested dropped from 890 in August to 25 in December), likely because of prevalence of at-home testing and the unwillingness of people to be tested (Alvarez *et al.* 2023).

Table 2 shows the Spearman's rank correlation coefficients (ρ) and p-values between the SARS-CoV-2 copy numbers in wastewater and the individual testing metrics, as well as the Fairfax County, Virginia, and US COVID-19 numbers (CDC database) for the second part of the study, when all the 12 sites were sampled (August to December 2022). Generally speaking, higher correlations were observed between wastewater and individual testing data, as well as with the county, state, and national COVID-19 numbers. For instance, the positive correlation between the average N1/N2 copy numbers and the weekly new positive cases was high when considered over these four months only ($\rho = 0.78$ vs. 0.48, $p < 0.001$). A remarkable

Table 2 | Correlation matrix between selected wastewater copy numbers mL^{-1} and saliva testing metrics for the period involving 12 sampling sites (August to December 2022) (The metrics used for calculation of the correlation coefficients were described in the text and the footnote)

		Weekly positives[a]	Weekly positivity rates (%)	Weekly isolation at GC	Fairfax County weekly cases[b]	VA State weekly cases[b]	USA nationwide cases[c]
Average N1/N2	Spearman's Correlation	0.779**	0.588*	0.321	0.779**	0.792**	0.789**
	Sig. (2-tailed)	0.000	0.013	0.209	0.000	0.000	0.000
Average normalized N1/N2	Spearman's Correlation	0.681**	0.444	0.293	0.363	0.615**	0.588*
	Sig. (2-tailed)	0.003	0.074	0.254	0.152	0.009	0.013
Weighted average N1/N2	Spearman's Correlation	0.635**	0.404	0.284	0.637**	0.674**	0.625**
	Sig. (2-tailed)	0.006	0.107	0.269	0.006	0.003	0.007

**Correlation significant at the 0.01 level.

*Correlation significant at the 0.05 level.

[a]Number of observations, $n = 17$.

[b]Data obtained from the Virginia Department of Health (USA), COVID-19 Dashboards.

[c]Data obtained from the Centers for Disease Control and Prevention (USA), COVID Data Tracker.

observation is that our wastewater numbers, which showed low or no significant correlations with regional or national reported COVID-19 cases when considered over the year 2022, revealed high correlations when considered over the period from August to December 2022 (e.g., $\rho = 0.79$, $p < 0.001$ for the correlation between the average N1/N2 copy numbers mL^{-1} and US COVID-19 numbers mL^{-1}). As observed before, normalization of the SARS-CoV-2 copy numbers mL^{-1} by the PMMoV copy numbers mL^{-1} did not improve the correlation between wastewater numbers and individual testing data.

These higher correlations between wastewater analyses and individual testing at the university, county, state, and national level can be explained by the larger number of individuals sampled (the 12 sites covered about 98% of the Main Campus resident students), as well as by the period considered which coincided with the fall semester, during which the student population was likely to be more stable than over the entire year 2022 (i.e., the average number of 'resident' students living in GMU Fairfax Campus was approximately 5,200 in the spring and fall and 1,300 in the summer). Indeed, increasing the number of individuals involved in the study has been shown to result in stronger correlations between wastewater and individual testing results (see the following) (Scott *et al.* 2021; Kotay *et al.* 2022).

Comparison between sewage and individual testing: individual buildings

The 12 sites sampled over the period from August to December 2022 were chosen based on the high number of students in the buildings served by the collector sampled, except for two sites: East Campus which was chosen because it collected wastewater from all dorms as well as other buildings of Fairfax Campus (with the exclusion of athletic facilities located on the west of campus), and the Global Center which was chosen because it housed resident students in isolation after they were tested positive for COVID-19.

Significant correlations (low, moderate or high) between the N1/N2 average copy numbers and the weekly positive cases were observed in a majority of buildings: Global Center ($\rho = 0.39$, $p = 0.008$), Sandbridge ($\rho = 0.44$, $p = 0.020$), Hampton Road ($\rho = 0.52$, $p < 0.001$), Potomac Heights ($\rho = 0.34$, $p = 0.025$), Northern Neck ($\rho = 0.53$, $p = 0.045$), President Park ($\rho = 0.61$, $p = 0.016$), Liberty Square 3 ($\rho = 0.58$, $p = 0.020$), and East Campus ($\rho = 0.78$, $p < 0.001$). The absence of significant correlations for a number of buildings is likely explained by the generally small number of students tested positive in a given building during a given week (in about 20% of the wastewater samples tested positive for SARS-CoV-2, only one positive case was identified in the building based on individual testing). Other authors have similarly reported higher correlations between wastewater copy numbers mL^{-1} and COVID-19 cases when considering a larger number of students. For instance, Zambrana *et al.* (2022) recorded significant correlations at the whole-campus level (Level 1), but non-significant correlations at the building level (Level 3) (considering non-normalized data). More specifically, the authors observed that the sites with the highest number of students showed the highest correlation between antigen testing and wastewater results: e.g., sites A with 3,060 students showed a correlation coefficient (ρ) = 0.75 ($p < 0.05$), site G with a population of 2,505 showed $\rho = 0.62$ ($p < 0.05$), and sites B, C, and D with populations of 485, 650, and 960, respectively, showed no statistically significant correlations. These findings underscore the pivotal role of the sample size in determining significant correlations between wastewater and individual testing results.

Limitations and challenges

Although wastewater detection of SARS-CoV-2 has been increasingly recognized as an alternative way to monitor the spread of COVID-19 at the community level, the methodology is associated with several limitations making the comparison between wastewater results and individual testing data challenging. Even though wastewater monitoring can easily be operated consistently and on a regular basis, individual testing policies and willingness to be tested make the percentage of occupants in a building variable and sometimes unpredictable, leading to discrepancies between wastewater virus concentrations and percentage of positive cases. Another difficulty comes from the shedding of SARS-CoV-2 virus in wastewater with can vary greatly from individual to individual and may continue for several weeks after positively-tested individuals have returned to their building after isolation (Kotay *et al.* 2022; Zambrana *et al.* 2022). This study used the N1 and N2 genes to detect SARS-CoV-2 RNA. However, it is noteworthy that other genes, with potential different sensitivities (e.g., Envelop gene – E gene, open reading frame 1 – abORF1ab, RNA-dependent RNA polymerase – RdRp), have been used for detection of SARS-CoV-2 in wastewater. Another limitation arises from the presence in a building of potentially infected individuals who are not registered as residents (Zambrana *et al.* 2022). Also, the increase of tests conducted at home likely contributed to reducing the number of positive individuals recorded by the university.

Our technical protocol, although shared between several studies, also included limitations. Our sampling schedule, despite spanning 24 h, was conducted weekly and may miss wastewater variability happening over the entire week. Pasteurization of the wastewater samples, which was recommended for lab safety, is known to reduce the concentration of SARS-CoV-2 RNA in the samples (Islam *et al.* 2022). Finally, the matrix recovery controls (i.e., MS2), even though not used to adjust the SARS-CoV-2 copy numbers, were only run for a subset of samples, which may not account for the full range of recovery. Because of the resources available, six sites were monitored during the first phase of the study (covering approximately 2,900 student or 56% of the GMU Fairfax Main Campus population) and 12 sites during the second phase of the study (covering approximately 5,200 students or 98% of the GMU Fairfax Main Campus population). However, the buildings sampled during the first phase of the study were randomly dispersed across campus, and we expect them to be representative of the GMU resident population at that time. Several problems are related to small-scale of the study. For instance, it has been sometimes difficult to find sampling point allowing to link a specific building to a sewage effluent (e.g., some manholes were not found or not accessible, some sewage lined received water from multiple building or only a part of a building), making difficult to relate wastewater copy numbers to positive individual cases. Small numbers of students in buildings during holidays have led to low sewage flows causing sample collection issues (Gibas *et al.* 2021; Kotay *et al.* 2022; Wartell *et al.* 2022).

CONCLUSION

This report presents results of a wastewater monitoring study of SARS-CoV-2 RNA in a university campus during the last phase of the pandemic, when vaccination rates were higher and individual testing rates were decreasing. Our data showed that wastewater monitoring could successfully detect SARS-CoV-2 RNA in sewage from buildings housing even few COVID-19 cases. Significant correlations were observed between wastewater results and individual testing data at the campus level (approximately 5,200 resident students), although correlations at the building level (approximately 200–700 resident students) were only observed in half the buildings sampled, likely because of the smaller number of individuals involved. Correlations between wastewater results and individual testing data were not affected (and even increased) during the fall 2022, even though the numbers of individuals tested on campus decreased dramatically. Together with other similar reports, our results indicate that wastewater-based monitoring is a valuable tool to determine the trend of COVID-19 and complement individual testing data in a campus student population.

Although WBE studies started in the 1980s with the Polio Global Eradication Initiative, the field has gained increasing attention with the COVID-19 pandemic. Soon after the onset of the pandemic, wastewater-based monitoring was recognized as an approach that could complement individual testing. Consequently, a number of reports describing such studies have been published, which, for the most part, focused on sewage serving large communities (e.g., main sewage collector, wastewater treatment plant), therefore allowing more reliable correlations between wastewater results and COVID-19 cases in the community. Several studies have been conducted on smaller communities, including university campuses, hospitals, schools, etc.). Despite the smaller scale, results from these studies (including ours) similarly showed significant correlations between wastewater copy numbers and individual testing cases, which is remarkable because of the differences between the two methodologies. Nevertheless, wastewater-based detection of viral markers still contains gaps in knowledge, as illustrated by the large variability of the recovery rate obtained from spiked surrogate viruses for example. Another difficulty originates from the lack of knowledge in regards to the number of viral particles shed by infected individuals, and the variation in this quantity over the course of the disease. Other uncertainties arise from the unclear relationship between the number of individuals and water usage in a building, as well as the effect of the wastewater characteristics on the viral RNA stability or detectability. Increased knowledge about these factors, which would help with the implementation of wastewater-based monitoring in the case of a new pandemic, will require a multidisciplinary approach involving biochemists, engineers, and epidemiologists.

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DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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