ARTICLE

Wastewater surveillance of SARS-CoV-2 RNA and detection of emerging viral variants from wastewater in the city of Manila, Philippines

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ABSTRACT

Wastewater-based epidemiology (WBE) of SARS-CoV-2 has been explored in many countries during the past years of the COVID-19 pandemic (2021-2022) along with cost-efficient large-scale monitoring of infection. In this study, samples (n=88) were collected for three consecutive months at four different sites (A - hospital, B - immediate hospital-government offices, C - residential, D - residential-commercial). RT-qPCR was used to quantify SARS-CoV-2 RNA, concentrations, specifically the N1 and N2 genes, in samples collected from a sewer network from a hospital to a community in the City of Manila, Philippines. Monte Carlo Simulation software was then used to compute the viral RNA prevalence in the wastewater matrix. Whole genome sequencing was then conducted to determine the emerging SARS-CoV-2 viral variants in wastewater. Results showed a higher detection rate of N1 genes compared to N2 genes. The highest concentration of viral RNA was obtained from Site A, while the lowest concentration was obtained from Site C. All 13 sequenced samples were representatives of Omicron variants, five of which were detected prior to their first detection in clinical samples. Notably, three Omicron subvariants were detected in both wastewater and clinical samples within the same sampling period. These show that SARS-CoV-2 RNA has potential as a biomarker for WBE in Manila, Philippines. However, further studies are needed to optimize the viral RNA recovery protocols and plans to recommend policies for efficient sewer line management should go hand in hand for the successful integration of this system in the City of Manila.

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INTRODUCTION

The COVID-19 pandemic has made a huge impact on the health and economy of many nations, including the Philippines. During

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the pandemic, many countries have resorted to non-pharmaceutical interventions, such as lockdowns, social distancing, and community quarantines. However, the effectiveness of such interventions remains largely unproven (Hartley and Perencevich 2020; Peak et al. 2020). More importantly, in countries such as the Philippines, with crowded housing, large households, and a mostly poor population, such measures have been largely unpopular and would have been probably unsustainable if prolonged (Corburn et al. 2020).

Predicting pandemic outbreaks can be achieved by using appropriate tools and parameters that are closely related to the affected population, like environmental monitoring (Scientific Advisory Group for Emergencies 2020). Manifestations of gastrointestinal tract infections, including diarrhea, were frequently observed among COVID-19 patients (Villapol 2020). Moreover, SARS-CoV-2 was detected in fecal samples from both symptomatic and asymptomatic COVID-19 patients (Chen et al. 2020; Wang et al. 2020; Park et al. 2021). Studies have shown that the SARS-CoV-2 virus is shed in wastewater, making WBE an important tool for epidemiological studies of COVID-19 outbreaks (Kitajima et al. 2020; Larsen and Wigginton 2020; Michael-Kordatou et al. 2020; da Silva et al., 2025; Yang, W. et al., 2025; Phan, T. et al., 2025).

Numerous countries conducted wastewater surveillance for the presence of SARS-CoV-2 as an early detection measure of COVID-19 outbreaks. The Wisconsin Department of Health Services and Scottish Environment Protection Agency have each demonstrated in their respective locales that wastewater surveillance could monitor the environmental concentration of the virus to supplement their clinical case monitoring programs (Wisconsin Department of Health Services, 2024; Scottish Environment Protection Agency, 2020). These wastewater surveillance efforts were paired with various SARS-CoV-2 detection methods, similar to those used in clinical detection techniques, to measure viral concentrations in wastewater.

Detection of SARS-CoV-2 RNA in wastewater and COVID-19 case monitoring may reveal the presence of asymptomatic virus carriers (Chan et al. 2023; Koza and Li 2024). This may complement national vaccination efforts by monitoring the effects on disease rates and on disease severity, in addition to obtaining a more reliable prevalence of SARS-CoV-2 infection in a locale (Anneser et al. 2022; Chan et al. 2023). This study aims to demonstrate that wastewater-based epidemiology (WBE) is applicable to the Philippine context, specifically in Manila. This study also demonstrates that COVID-19 variants can be detected through sequencing of the RNA samples extracted from wastewater.

MATERIALS AND METHODS

Historical Analysis of SARS-CoV-2 Infection

Historical analysis of SARS-CoV-2 infection progression in the City of Manila was conducted through gathering documents and mapping trends from local health officers, and following the wastewater discharge route from the Philippine General Hospital (PGH) towards a community wastewater pumping plant. The sewer system in Manila has not yet been modernized and remains affected by various external factors. Although wastewater follows a set path from the general hospital to the wastewater pumping station, the sampling points also receive wastewater that flows from other interconnected sewer lines that converge at the same station. Additionally, the system is also affected by rainfall and by seawater intrusion, which vary with changes in tide levels. As all these factors affecting the dilution of the samples, the study improved the yield by increasing the volume of the wastewater samples for the initial filtration from 100ml to 200ml during the filtration step before the extraction of DNA and RNA.

Data considered for this study included clinical case reports of COVID-19, 14 days before and after the scheduled wastewater sampling, as well as the total number of residents, water consumption rates, and barangay boundaries. These were collected to acquire a better understanding of the temporal and spatial changes of COVID-19 viral concentration in wastewater, characteristics of the susceptible population, and duration, infection and recovery rates of COVID-19.

Wastewater sampling and preprocessing

Planning and acquisition of permits for wastewater sampling activities were conducted in coordination with the hospital engineering office and the specific water concessionaire in the City of Manila. In this study, 1.0-L of wastewater grab samples were aseptically collected in triplicate (n=88) from four different sites (A - PGH, B - Padre Faura St., C - Intramuros, D - Binondo) (Figure 1). The frequency of sampling was twice a week for three months (05 July 2022 to 15 September 2022), following diurnal sampling per day based on the data detailing the daily periods (between 7:00–9:00 AM and 11:00AM–1:00PM) of highest water consumption of the target population. To determine the appropriate sewer manholes for the sampling, various factors were considered, foremost of which was the hospital discharge route that was expected to be the source point for COVID-19 in wastewater. Other factors included the collection of environmental and engineering data onsite, specifically the in-situ wastewater temperature, pH, total dissolved solids, and the respective coordinates for each sampling site. The members of the study team ensured the use of proper personal protective equipment during sample collection. No collection of clinical samples was conducted.

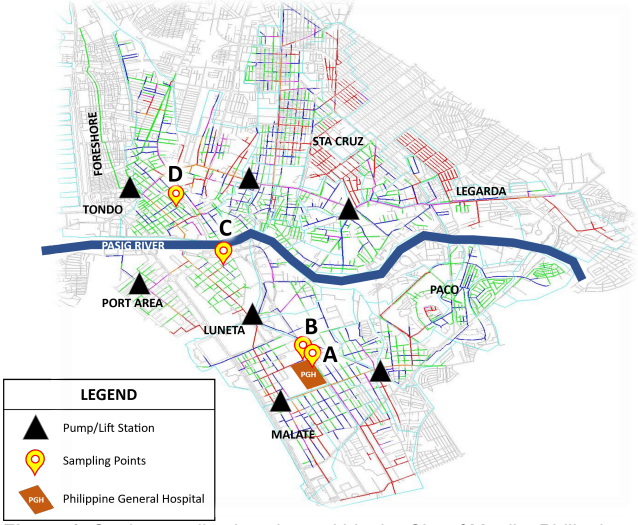

Figure 1: Study sampling locations within the City of Manila, Philippines.

Chlorine neutralization of all wastewater samples was then done using 1% sodium hypochlorite 30 minutes after sample collection. All samples were then stored at 4°C in portable, and properly sealed hand-held coolers, and transported to the BSL-2 laboratory in the University of the Philippines Manila for processing. The wastewater samples per site were combined into one composite sample, and aliquots were prepared for RNA extraction. Samples were stored at -20°C until further analyses. Sample and data processing were subsequently conducted, as illustrated in Figure 2. During the sampling period of the study, the samples were temporarily preserved by freezing. Samples were then immediately processed, by batch, as scheduled by the team after the sampling period was finished. Short-term storage (12, 14, or 16 months) had no significant impact on the viral gene abundance of the wastewater sample (Williams et al. 2024).

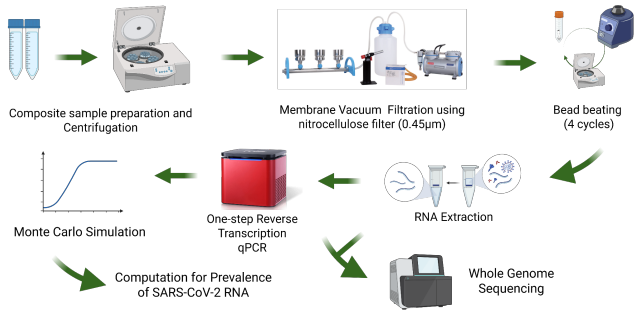

Figure 2: Schematic diagram for wastewater surveillance of COVID-19.

Total Viral RNA Extraction

Before total viral RNA extraction, four (4) grams of polyethylene glycol (8% w/v) and 0.9 g sodium chloride were added to a 40 mL raw wastewater sample. The resulting mixture was centrifuged at 3,500X g for 30 minutes at 4° C to separate the large debris, facilitating faster processing in the succeeding steps. Afterwards, the resulting mixture was filtered through a 0.45 µm mixed nitrocellulose membrane. To maximize the yield and collect the RNA that were attached to the large debris, the pellet formed after centrifugation was also resuspended and filtered through the same filter. The filtrate was discarded, and the residue and filter membrane were further processed by transferring to new microcentrifuge tubes and put through bead-beating steps (4 cycles of centrifugation at 5,000X g and 1 min vortexing). The resulting mixture was centrifuged at 13,000X g for 1 min, and the formed pellet was discarded. Total viral RNA from the supernatant was extracted using Qiagen RNA Viral Mini (Qiagen) according to the manufacturer's protocol. At least duplicate extraction was conducted per sampling site.

RT-qPCR of SARS-CoV-2 RNA in Wastewater

Quantification of SARS-CoV-2 RNA N1 and N2 genes from wastewater was conducted using Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR). Samples with cycle of quantification (Cq) values of 40 and below were considered positive for the gene being quantified (Jafferali et al 2021; Zhang et al 2022). RT-qPCR runs were conducted using the QuantiNova Probe RT-PCR Kit (Qiagen) following the manufacturer's instructions. SARS-CoV-2 N1 and N2 primers from the SARS-CoV-2 N1+N2 Assay Kit (Qiagen) were used. In addition, 2019-nCoV_N_Positive Control (Integrated DNA Technologies) and nuclease-free water were used as positive and negative controls, respectively. A standard curve was generated for every RT-qPCR run by preparing a 10-fold dilution of the positive control.

The RT-qPCR assay results were stated as concentrations, i.e., *gene copies/L wastewater*. These values were used to calculate the prevalence and persistence of SARS-CoV-2 RNA. This was followed by the development of models for the use of wastewater surveillance in monitoring the circulating COVID-19 in the community.

Whole genome sequencing of RNA

To determine the emerging SARS-CoV-2 viral variants in wastewater, 13 extracted RNA samples (with Cq values of less than or equal to 35) were sent to the Philippine Genome Center (PGC) for whole genome sequencing. Illumina COVIDSeq (Illumina, Inc.) was used during the library preparation of the RNA samples, following the manufacturer's instructions. An automated SARS-COV-2 Variant Typing online platform was used to process the raw RNA sequences via Exatype by Hyrax BioSciences (https://exatype.com/), with reference to the SARS-CoV-2 Wuhan-Hu-1 strain NC_045512.2 (https://www.ncbi.nlm.nih.gov/nuccore/1798174254/).

Degradation of biomarkers

The following equations, i.e., Equation 1, Equation 2, and Equation 3, as well as the accompanying explanations of their variables, were based from the study of Hart and Halden (2020). The degradation of SARS-CoV-2 RNA gene biomarkers (N1 and N2) in wastewater can be computed based on the exponential decay formula in Equation 1 (Hart& Halden, 2020).

Equation 1:

$$N(t) = N_0 \left(\frac{1}{2}\right)^{\frac{t}{t_{\frac{1}{2}}}}$$

where:

N(t) – concentration of undecayed biomarker (gene copies) after wastewater sampling;
N_o – initial concentration of the biomarker from the time of excretion and release into the wastewater system;
$t_{1/2}$– known half-life of the biomarker; and
t – time interval from initial time (time = 0h) to the actual time of measurement (time = t).

Scenario of wastewater collection and conveyance

This study has a different context from the McMahan scenario, where samples were obtained at influent sewer point(s) relative to the wastewater treatment plant (McMahan et al., 2021). It can be assumed that an outflow location further from the source would promote the mixing and dissolution of SARS-CoV-2 RNA from stool, further breaking up solid waste particles as they move towards the treatment plant. This may give a different value to SARS-CoV-2 RNA when samples are taken very near the source (i.e., nearer to the building or point of discharge). SARS-CoV-2 RNA may remain within the stool until the solid parts disintegrate and eventually get mixed with the flow. This may mean increased amounts of RNA are collected along the wastewater sample.

Other scenarios that could modify the amount of SARS-CoV-2 RNA found in collected samples were a) the presence of a septic tank before the collection point; b) the intermittent flow from the building (sanitary) sewer, which may be insufficient for the solid components to degrade and RNA mixed with the wastewater; and c) the temporary storage effect of manholes on the wastewater by detaining solid components and resulting to lower SARS-CoV-2 RNA collected along the flow. One finds that manhole locations provide breaks (i.e., open to the atmosphere) to sewer pipes, as opposed to closed connections.

Site A (Outflow of Philippine General Hospital)

An equivalent daily value (liters/day) drawn from total daily flows without capturing an hourly pattern can give an overestimate or underestimate of this average daily flow value. At outfall locations, or at entry points to WWTPs, the flows may always be present because of a larger catchment, unlike building sewers where flows are more intermittent.

The source of wastewater from these areas comes from two buildings comprising four (4) wards, each with water closets and sinks for plumbing fixtures. At the time of study, neither building was metered for water use. The estimate of water use per hospital bed is assumed to be between 0.5 m^3 to 1 m^3/day per staffed bed. The total number of beds from all wards was estimated to be around 65. This corresponds to about 33-65 m^3/ day at Site A. A uniform distribution in this range of flow was assigned to the Monte Carlo simulations to give daily outflows.

Site B (Sewer at Padre Faura St.)

This block covers nine (9) sewered areas under the institutional use category, comprising mainly school buildings, government buildings, and a hospital. The sampling point is a third sewer manhole about 170 meters from the first manhole, which covers

outflow from the nine sewered areas. The total sewer flow (for 4 months) was 33,717 m^3 corresponding to 1,116.5 m^3/day. Figure 3 shows the arrangements of buildings and the collection point SM1. Within this area, the residential water use consumption was 48.5 cubic meters per day based on metered readings values of a 30-day monthly total. The range of flows used for the simulation was between 47.5 to 49.5 cubic meters per day. A uniform distribution applied to this range was used to estimate daily water use in calculations. Figure 3 shows the arrangements of buildings and the collection point SM1.

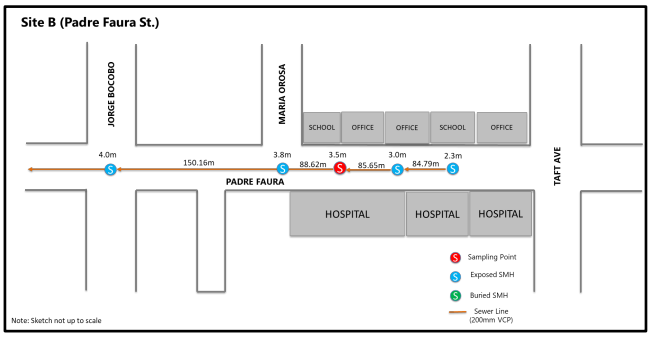

Figure 3: Site B collection point (in red) serving the block along Padre Faura between Taft Ave. and Maria Orosa St.

In terms of wastewater volumes from the sewer shed leading to the manhole location, the estimate is drawn from the water metered connections of buildings and 100% converted to wastewater. Similarly, in the absence of readily available information on daily metered sewer flow, the calculations can have uncertainties, as the volume of water provided were monthly totals from each area and converted to daily flows.

Site C (Sewer at Intramuros)

This sampling point located at the intersection of Anda St. and Legaspi St. covers the blocks that are north and east of Legaspi St. within Intramuros. About five (5) blocks cover seventeen (17) establishments mainly under the commercial use category, comprising mainly dormitories, eateries, and residential housing. The sampling point joins several pipes and manholes. The average daily sewer flow (for 4 months) was 65 m^3 per day, which corresponds to a total of 9,810 m^3. A range between 58 to 72 m^3 per day was used for the simulations using uniform distribution in calculating daily flow. Figure 4 shows the arrangements of buildings and the collection point SMH2.

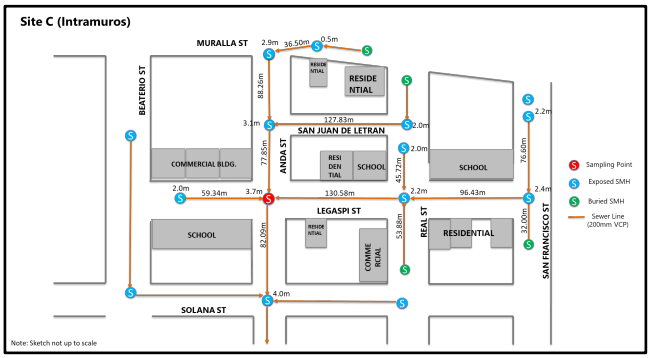

Figure 4: Collection point (in red) serving the blocks in Site C (Intramuros).

Site D (Sewer at Binondo)

This sampling point is located at the intersection of San Nicholas St. and Ilang-Ilang St. and covers the blocks that are east of San Nicolas St.

About three (3) blocks cover seventeen (17) establishments mainly under the commercial use category, comprising mainly dormitories, eateries, and residential housing. The sampling point joins two main pipes and two manholes. The average daily sewer flow (for 4 months) was 105.6 m^3 per day, which corresponds to a total of 15,940 m^3. A range between 98.5 to 103 m^3 per day was used for the simulations using uniform distribution in calculating daily flow. Figure 5 shows the arrangements of buildings and the collection point SMH2.

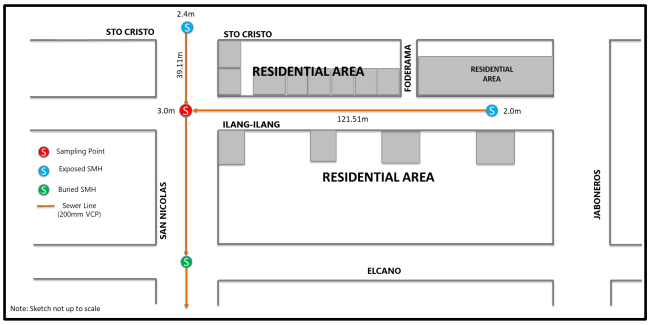

Figure 5: Collection point (in red) serving the blocks in Site D (Binondo).

The adjusted half-lives of SARS-CoV-2 RNA N1 and N2 genes as biomarkers were determined using the obtained wastewater temperature of the samples, published biomarker half-lives at ambient temperatures, and the Arrhenius Equation, similar to the methodology of Hart & Halden, (2020) as illustrated in Equation 2.

Equation 2:

$$R_2 = R_1 \times Q_{10}^{\left(\frac{T_2 - T_1}{20°C}\right)}$$

where:

R_1 represents the initial decay rate, calculated as the negative natural logarithm of two divided by the known biomarker half-life at ambient temperature (Laidler, 1984)

Solving for the half-life, Equation 3 is obtained (Hart & Halden, 2020).

Equation 3:

$$t_{\frac{1}{2},2} = t_{\frac{1}{2},1} \times \frac{ln\,(2)}{ln\,(2) \times Q_{10}^{\left(\frac{T_2 - T_1}{20°C}\right)}}$$

where:

$t_{\frac{1}{2},1}$ – reported half-life of the biomarker at 20°C ambient temperature;

$t_{\frac{1}{2},2}$ – computed half-life, adjusted to the temperature of the wastewater sample;

T_1 – temperature at which $t_{\frac{1}{2},1}$ was derived from;

T_2 –temperature at which $t_{\frac{1}{2},2}$ was derived from (20°C); and

Q_{10} –temperature-dependent rate change factor, considering 10°C increase in wastewater temperature within ambient temperature (20°C)

The variable Q_{10} was assigned a value of 3 for this study, as recommended in Hart and Halden (2020). Provided that seasonal temperature changes are not a factor due to the brief duration of the research (3 months), detection of the biomarker from the wastewater sampling point is possible, given that it is released into the wastewater system at a constant rate and would undergo degradation at a rate proportional to its residence time within the system (Hart & Halden, 2020).

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Equation 4:

$$Persons\ infected = \frac{\left(\frac{RNA\ copies}{liter}\right) * \left(\frac{X\ liters}{day}\right)}{\left(\frac{Y\ g\ feces}{person\ per\ day}\right) * \left(\frac{RNA\ copies}{g\ feces}\right)}$$

where:

Numerator – mass load concentrations of SARS-CoV-2 RNA copies per liter of wastewater generated in a study site in the City of Manila;
Denominator – estimated amount of SARS-CoV-2 RNA copies per (infected) person per day.

Note that *X liters/day* in the numerator was calculated based on the estimated water consumption rate of the contributing population to the specific wastewater sampling site. In consultation with the water concessionaire, the actual average daily water consumption of the establishments connected to the wastewater path towards the specific sampling site was used. This was due to the installation issues of the wastewater flow rate meter on the sewer manhole itself.

Monte Carlo simulation was based on the assumptions of uniform distribution values for the daily water flow, the RNA copies per liter, grams of feces per day and RNA copies per feces (g) contributing to the wastewater sample. The daily water use was based on a monthly value converted to a daily value. Only a few samples showed traceable RNA copies per liter. The estimates in the amount of feces per person and its fecal weight were drawn from the literature. Variables were allowed to vary, such as the RNA copies/liter and X liters per day. The RNA copies/liter was based on the actual value obtained from RT-qPCR results, while the X liters per day was based on the actual average water consumption of contributing households and establishments to the particular wastewater sampling site.

RESULTS AND DISCUSSION

Prevalence of SARS-CoV-2 RNA from wastewater

The RT-qPCR data was used to compute the estimated number of persons infected with COVID-19 contributing to the sampling location. The prevalence of SARS-CoV-2 RNA in wastewater was determined by calculating the ratio of infected persons to the total population contributing wastewater to the sampling point (Ahmed et al. 2020), using Monte Carlo Simulation Software (Figure 6).

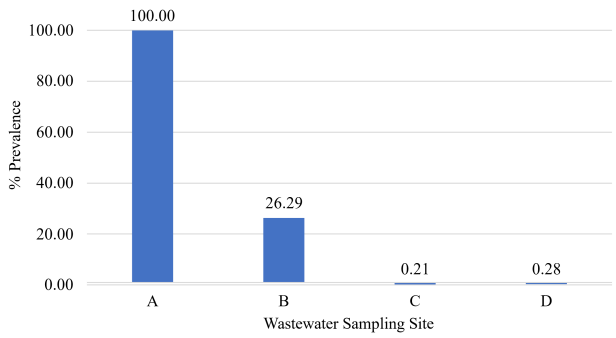


Figure 6: Prevalence of SARS-CoV-2 RNA in wastewater (Mean Persons Infected/Total Population) (n=88).

For Site A, since this sampling point is directly connected to the COVID-19 wards that houses the hospital-admitted COVID-19 patients, the study assumed that the total population contributing to this specific part of the hospital wastewater system corresponds to the actual number of COVID-19 cases that were clinically reported in the site, the computed prevalence for this site was 110.22% (Computed mean = 65.10; Total Population = 63). Thus, the study was able to detect 100% of the reported COVID-19 cases in Site A using Monte Carlo Simulation.

The computed high prevalence of SARS-CoV-2 RNA in Site A correlates with the high number of clinical case reports similar to Cavany et al. (2022). This high viral RNA prevalence is mainly because Site A is a large hospital that also provides COVID-19 testing and treatments. Hospitals are the main treatment facilities for active COVID-19 infections, which could result in more concentrated viral RNA shedding in hospital wastewater compared to that in community sewage systems (Koza and Li 2024). Moreover, confined patients typically exhibit severe COVID-19 symptoms, which can be associated with higher viral loads and longer shedding durations (Cavany et al. 2022; Koza and Li 2024).

The prevalence of SARS-CoV-2 from sampling site B was 26.29% (Computed mean = 1645.19; Total Population = 6,443). The computed prevalence was significantly higher than the average reported number of COVID-19 clinical cases (Mean Site B clinical cases = 1.5). A higher computed prevalence was expected from sampling site B since more establishments (i.e., government offices, schools, and hospitals) were contributing to the sampling sewer manhole. The actual population is unknown because the input for this sampling point is non-residential. The input reflected toilet users from unknown residences, thus, barangay census data may not be applicable. The sewer shed is comprised mainly of government offices, schools, and commercial establishments. This may be attributed to the possible viral shedding of infected workers and visitors from these non-residential establishments using restrooms, both symptomatic and asymptomatic (Cavany et al. 2022). Having no connected residential establishments, the data for the actual total population contributing to Site B was unknown. Thus, infected individuals contributing to Site B may have been from either the hospital (Site A) and/or from non-residential areas connected to Site B.

For sampling sites C and D, the computed prevalence of SARS-CoV-2 was less than 1%. Site C had a prevalence of 0.21% (Computed mean = 12.41; Total Population = 5,902), while Site D had a prevalence of 0.28% (Computed mean = 15.54; Total Population = 5,173). Interestingly, the computed low prevalence of SARS-CoV-2 RNA corresponded to the low number of reports of clinical cases in the residential areas contributing to these sites (Mean clinical cases: Site C=0.136; Site D=0.091). The less than 1% clinical cases also correspond to the low (0 or 1) reported COVID-19 clinical cases among the residential areas contributing to the sampling location. These results were similar to the correlation trends observed in other studies, where the concentration of viral RNA used to compute the prevalence of SARS-CoV-2 in wastewater was correlated to the number of infected individuals contributing to the sampling site (Medema et al. 2020; Westhaus et al. 2021; Street et al. 2021).

Several variables in Eq. (4) were based on values reported in the literature that were missing in this study. One of these was the actual value of initial undecayed RNA copies per gram of feces present in each sample. This value was based on the literature for SARS-CoV-2 RNA shedding in feces (Wölfel et al. 2020; F. Wu et al. 2020). Monte Carlo Simulation software was used to simulate 1000 scenarios based on the range from the RT-qPCR data, which includes the standard curve in each RT-qPCR run for quantification of gene copies per microliter of extracted sample. This corresponds

to the number of estimated persons infected in each sampling site, considering a range of RNA copies per gram of feces reported in the literature. Variable recovery of viral RNA from wastewater samples could be attributed to similarly variable fecal load, which was unknown in the present study (Rafiee et al. 2021; Kmush et al. 2022). Several studies have reported estimates of COVID-19 fecal shedding, ranging from 10^2 to 10^4 RNA copies per gram of feces (Wölfel et al. 2020; F. Wu et al. 2020). However, the amount of viral RNA fecal shedding varies depending on the severity of the COVID-19 infection (Zhang et al. 2020; Wölfel et al. 2020).

For an accurate computation of the number of persons infected using WBE data, the exact time of COVID-19 clinical case reporting must be noted (Reese et al. 2021). Unfortunately, during the sampling activities of this study, the COVID-19 clinical case data was made available on a per week basis by the hospital and the local government health department. Weekly case reporting does not accurately reflect daily case number fluctuations. This may make it inappropriate for estimating viral infection rates for periods shorter than a week (Reese et al. 2021; Street et al. 2021).

The uncertainty in time and frequency of fecal release of COVID-19-positive patients was another factor which could have led to inconsistent viral RNA concentration recovery (Reese et al. 2021). Samples can only contain viral RNA from patients who defecated prior to sample collection. With this in mind, the investigators acquired information on the periods of increased estimated city water usage which was hypothesized to correspond to increased usage of water in restrooms. Consequently, the timing and duration of viral shedding related to COVID-19 infection could also affect the accuracy of WBE, as some infected individuals may have high viral shedding during the first week of infection, while others may experience delayed peak shedding (Alvarez et al. 2023).

Important viral indicators suitable for long-term monitoring of outbreaks

To identify important viral indicators suitable for long-term monitoring of outbreaks, the mean initial SARS-CoV-2 RNA was computed per gene primer used, considering the variable data obtained from the RT-qPCR results (Table 1). Results indicated higher SARS-CoV-2 RNA N1 gene mean concentration (N_o=1,999.88 gene copies/L wastewater), compared to N2 gene (N_o=9.87 gene copies/L wastewater).

Table 1: Mean degradation of SARS-CoV-2 RNA N1 and N2 genes quantified from wastewater samples.

Parameters	N1 Gene	N2 Gene
N_0 – initial RNA gene quantity excreted and discharged in the wastewater sampling location (gene copies/L wastewater)	1,999.88	9.87
N(t) - undecayed RNA gene quantity after sampling time (gene copies/L wastewater)	1,197.06	6.51
(t)1/2,2 - half-life, at seasonally and spatially adjusted wastewater temperature calculated	7.19 hrs.	7.76 hrs.

Consequently, based on the RT-qPCR experiments, the SARS-CoV-2 N1 gene was detected in 43% of the total wastewater samples, while only 5% of the samples tested positive for the SARS-CoV-2 N2 gene (Figure 7). The SARS-CoV-2 N1 gene was detected from all sampling sites throughout the sampling period, while the SARS-CoV-2 N2 gene was detected in Sites A and B only. The resulting viral RNA copies per liter of wastewater were then compared to the reported COVID-19 clinical cases during the same sampling period, from July 5, 2022 to September 15, 2022 (Figure 8).

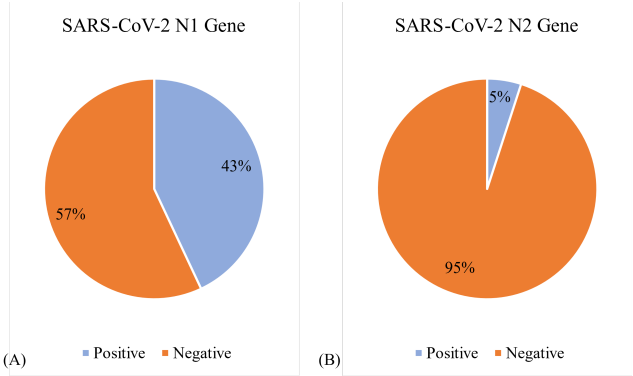


Figure 7: Percent detection of SARS-CoV-2 RNA (A) N1 and (B) N2 genes from wastewater samples (n=88).

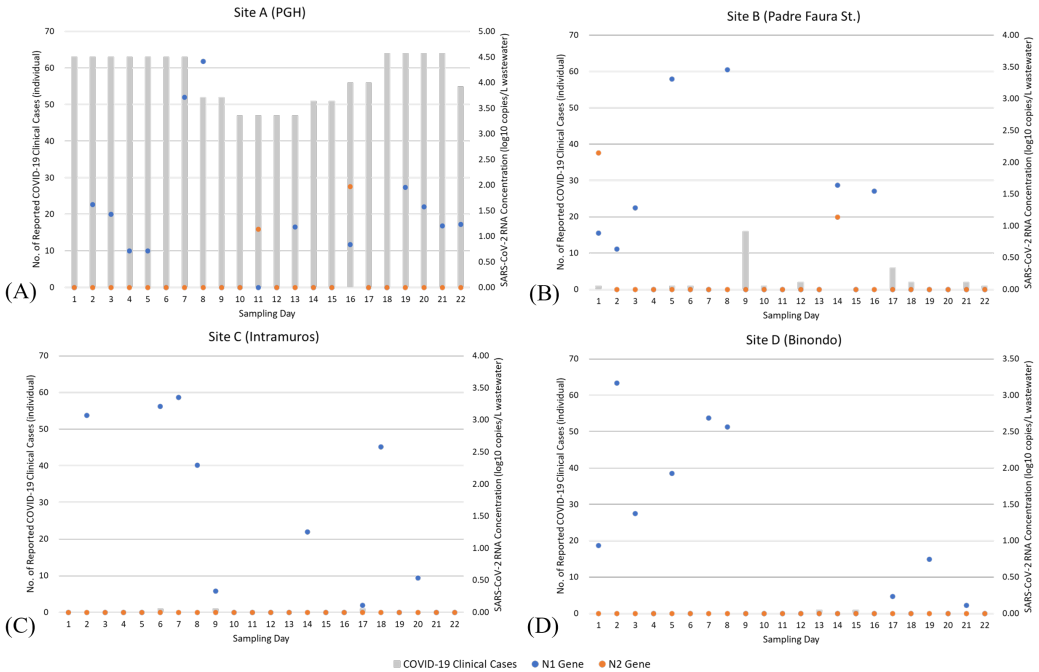


Figure 8: SARS-CoV-2 RNA viral load in wastewater and reported COVID-19 clinical cases from July 5, 2022 to September 15, 2022 for Sites A to D (n=88).

Among the study sampling sites, Site A had the most quantified SARS-CoV-2 RNA N1 gene from wastewater and had the most frequent detection of both SARS-CoV-2 genes from wastewater samples throughout the sampling period (Site A=14; Site B: 9; Site C= 8; Site D=9). Other studies have made similar observations where N1 was also detected more frequently in wastewater samples compared to N2 (Hong et al. 2021; Grube et al. 2023). N1 gene was more sensitive than N2 gene, which may be due to its resilience and lower susceptibility to degradation e.g., to total organic carbon and pH changes (Hong et al. 2021). Vogels et al. (2020) had also reported that the same N1 primer-probe set used in their study exhibited more sensitivity than that of the N2 primer-probe set. However, in their study, the primer sensitivity tests were conducted on clinical samples from COVID-19 patients.

On the other hand, other sites (Sites B, C, and D) had much fewer reported cases (0 to 1 case), in coherence with the lower detection rate from wastewater samples. This may be due to the slightly lower detectability of N2 compared to N1, consistent with several existing wastewater studies for COVID-19 in different countries (Hong et al. 2021; Feng et al. 2021). The present study in congruence with other studies has shown that the N1 gene is a more suitable viral indicator for long-term COVID-19 outbreak monitoring via wastewater surveillance. While either gene can be detected, the N1 gene may be prioritized due to its higher frequency of detection, particularly if resources permit only one gene to be targeted.

However, the reduced detectability of N2 relative to N1 is not universally expressed in all COVID-19 WBE studies. The N1 gene can also initially show higher detectability in the initial part of wastewater collection and can shift to higher N2 gene detection for the succeeding weeks of their study (Hinz et al. 2022). Further elucidation of the mechanisms of these genes in wastewater may be needed to explain this kind of detection rate shifting while considering other factors.

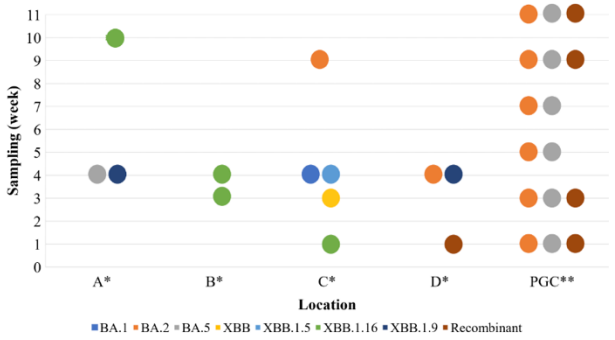
Persistence of SARS-CoV-2 RNA from Wastewater

The persistence of SARS-CoV-2 RNA from wastewater was analyzed based on the survival of the viral RNA (half-life), the time elapsed from the estimated shedding of COVID-19 patients, and wastewater sampling time. Other factors affecting gene half-life include several environmental factors, such as temperature, suspended solids, loads of organic matter, matrix dilution, and the presence of chemicals that could possibly eliminate the SARS-CoV-2 RNA from the wastewater (Tran et al. 2021; Mogili et al. 2024). Based on the results, the SARS-CoV-2 RNA N1 gene mean half-life during the project's sampling was 7.19 hours, while the N2 gene's mean half-life was 7.76 hours (both at 20.00°C). The computed half-lives of these genes were shorter than the reported 0.64 day, or 15.36 hours half-life (Bivins et al. 2020). Shorter viral RNA half-lives could negatively affect the accuracy and efficiency of WBE for COVID-19 monitoring because this indicates faster degradation of the viral RNA in the matrix. This greatly affects the appropriateness of timing and frequency of sampling (Bivins et al. 2020; Reese et al. 2021). Additionally, environmental factors like temperature could influence the recovery of RNA from the wastewater matrix. This is due to the decreased stability of RNA in elevated environmental temperatures (Paul et al. 2021; Aboubakr et al. 2021; Tiwari et al. 2022; Li et al. 2023). This was evident in the current study, in which the obtained mean wastewater temperature (31.11°C) was higher than that reported in other studies, a factor that could have caused the lower viral RNA concentration recovered from the wastewater samples.

Emerging SARS-CoV-2 viral variants in wastewater

Thirteen RNA extracts with 35 or fewer Cq values were sent to the Philippine Genome Center (PGC) for whole genome sequencing. These 13 out of 88 wastewater samples met the criteria of PGC for COVID-19 RNA sequencing of having Cq ≤35 to ensure successful sequencing, while the remaining wastewater samples with Cq values > 35 were not included. Low viral load was generally quantified from wastewater samples in this study, which may be attributed to the lower number of COVID-19 cases that were reported during the 3-month sampling period. Also, the present study's sample size was relatively smaller compared to other studies. Most COVID-19 wastewater sequencing studies conducted sampling for longer periods (8 to 24 months) during the peak of the pandemic with higher reported community infection and sampled mostly from wastewater treatment plants (Khan et al. 2023; Yousif et al. 2023; Hasing et al. 2023). The detected SARS-CoV-2 viral variants in wastewater were compared to the sequences submitted to the Global Initiative on Sharing All Influenza Data (https://gisaid.org/hcov19-variants/).

Analysis showed that all 13 wastewater samples were representative of the SARS-CoV-2 Omicron variant (Figure 9), which were in coherence with the circulating Omicron subvariants (BA.2, BA.5, and recombinant) from Philippine clinical samples from July to September 2022, as reported by PGC. Interestingly, wastewater samples collected by the study during the same period detected additional 5 new Omicron subvariants (BA.1, XBB, XBB.1.5, XBB.1.6, XBB.1.9), based on the Global Initiative on Sharing All Influenza Data (https://gisaid.org/hcov19-variants/).



Nb.: * Wastewater samples
** Philippine clinical samples sequenced by Philippine Genome Center (PGC).

Figure 9: SARS-CoV-2 Omicron variants from wastewater samples (July-September 2022) (n=13).

Interestingly, the Omicron subvariant BA.1 was detected in wastewater from Site C on 25 July 2022 (Week 4), five months after it was last detected among Philippine clinical samples in April 2022. Additionally, several Omicron subvariants were detected from wastewater a few months before they were detected in Philippine clinical samples. One was the Omicron subvariant XBB which was detected in wastewater from Site C on 21 July 2022 (Week 3). Two months after its wastewater detection, XBB was then detected for the first time in Philippine clinical samples in September 2022. Other notable Omicron subvariants were XBB.1.5 and XBB.1.9, which were both detected in wastewater samples in Week 4, on 26 July 2022, and 28 July 2022, respectively. These two Omicron subvariants were then reported in clinical samples for the first time, 6 months after wastewater detection (https://gisaid.org/hcov19-variants/). Another notable event was the frequent detection of Omicron subvariant XBB.1.16 in wastewater samples from sampling weeks 1, 3, 4, and 10. In terms of clinical surveillance of PGC, XBB.1.16 was first detected in January 2023. Similar findings were reported in other studies, in which certain COVID-19 variants were detected in wastewater several days before their first clinical detection (Iwamoto et al. 2023; Shempela et al. 2024). Literature also showed evidence on co-detection of several variants from both wastewater and clinical samples, such as BA.2.86, BA.5 (Shempela et al. 2024).

Interestingly, they also detected variants in wastewater that were absent in sequenced clinical samples, similar to the current study.

Regarding the variety of subvariant detection per sampling location, Site C showed the most Omicron subvariants (BA.1, BA.2, XBB, XBB.1.5, and XBB.1.16). On the other hand, Site B only detected XBB.1.16 for 2 consecutive weeks (Weeks 3 and 4).

These results support the promising utility of WBE in monitoring circulating COVID-19 variants in wastewater from contributing individuals in the community. More importantly, WBE can detect variants, regardless of whether individuals are symptomatic (Medema et al. 2020; Karthikeyan et al. 2022). WBE likewise has the benefit of data collection independent of patient consent. Moreover, when incorporated with genome sequencing, WBE can be used to provide real-time monitoring of viral mutations, which may aid in predicting the emergence or re-emergence of variants of concern (Trigo-Tasende et al. 2023).

Model development for COVID-19 WBE

Water consumption data from the water concessionaire was used to estimate the wastewater load ((X liters)/day). This was used to compute the number of persons infected contributing to the different sampling points or manholes.

Figures 10 to 13 show the model for the estimated number of persons infected based on the quantified SARS-CoV-2 RNA from wastewater, daily water meter consumption, and viral RNA shedding in feces simulated using Monte Carlo software (Oracle). Results showed that in all sampling sites, the predicted total number of the population contributing to the sampling site (bins) and persons infected are directly proportional. Consequently, the cumulative frequency is expected to reach a plateau as the number of bins increases. For Figures 10-13, the trend of the predicted frequency of the persons infected with SARS-CoV-2 (blue) decreases as the total number of population increases. This means that a higher number of total population decreases the chance of detecting the infected individual in the same sampling site. The number of clinical cases in this section refers to the predicted value based on the Monte Carlo stimulation. This means that the predicted or computed mean of persons infected for each sampling site was based on the current total population per site. Interestingly, the computed mean of persons infected for sampling sites A, C, and D is slightly higher than the mean reported clinical COVID-19 cases in each area (A= 57; C=2; D=1).

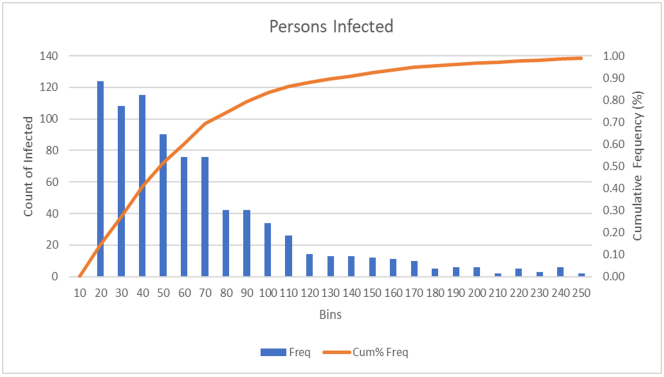


Figure 10: Estimated persons infected by COVID-19 using Monte Carlo Simulation of 1000 cases (Site A) (μ = 65.10; SD = 75.36).

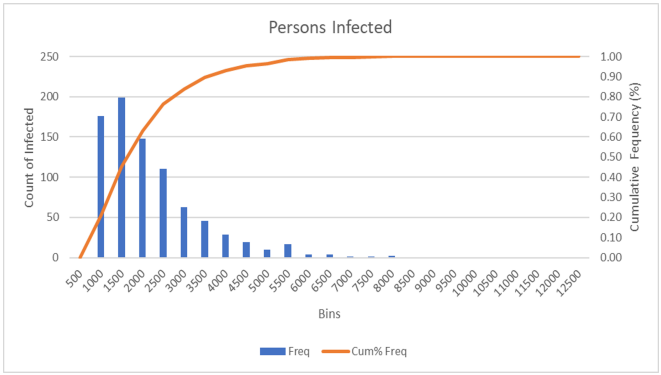


Figure 11: Estimated persons infected by COVID-19 using Monte Carlo Simulation of 1000 cases (Site B) (μ = 1645.19; SD = 1267.03).

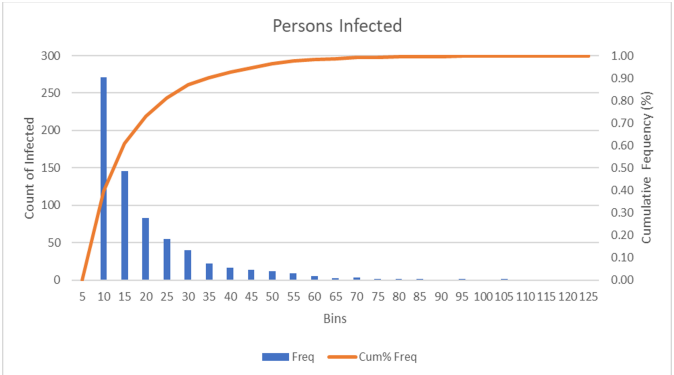


Figure 12: Estimated persons infected by COVID-19 using Monte Carlo Simulation of 1000 cases (Site C) (μ = 12.41; SD = 13.60).

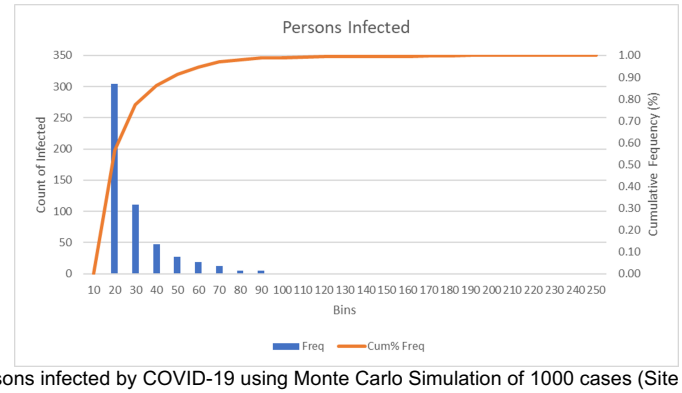


Figure 13: Estimated persons infected by COVID-19 using Monte Carlo Simulation of 1000 cases (Site D) (μ = 15.54; SD = 17.17).

However, sampling site B (Figure 11) showed a significantly higher mean number of persons infected (μ = 1645.19) than the mean reported clinical COVID-19 cases in the barangays contributing to site B ($\bar{x}$ = 2). These results can be explained by the types of establishments contributing to the sewer manhole in site B. Most of the establishments in the area were hospitals and government offices, contributing to a greater population and water consumption. Though low clinical cases were reported in this site, the temporary movement of infected individuals (asymptomatic or symptomatic) was not monitored.

The numbers of reported clinical cases in previous sections were the actual number of COVID-19 infections recorded by the local government units within the sampling duration of this study. This study resulted in higher predicted or computed mean of persons infected when compared to the actual number of officially reported infections, providing evidence that higher infection frequency may be present in the community, which could include both reported and unreported symptomatic and asymptomatic COVID-19 cases.

The model makes many assumptions when used in the Manila context, particularly a) the nearness of the source to the location of the sample and actual daily flows to a collection point, b) the estimate of RNA copies/gram feces, and c) the estimate of the half-life. Providing the values to the model results in a practical range of values for infected persons; however, in most cases, these differ from the number of clinical cases reported during sampling.

The strongest evidence of possible SARS-CoV-2 infection was taken from wastewater samples from sewer discharges from Site A. Yet the model still requires further experiments to help build a better understanding of SARS-CoV-2 RNA copies/gram-feces, sewer flow, and conveyance as a function of proximity to the source of wastewater.

This study used reported values of SARS-CoV-2 RNA copies/gram-feces because determination from individual samples was out of the study scope and was impossible during the study period in Metro Manila in 2022. The model predicts that when Cq is < 30, RNA copies/gram-feces at scales of $1x10^2$ to $1x10^4$ can suggest the presence of community infections (Wölfel et al. 2020; F. Wu et al. 2020). This was observed in the few samples taken from Site A.

Association of the amount of SARS-CoV-2 RNA in wastewater with community case data, and environmental persistence

Spearman correlation was used to analyze the association of the amount of SARS-CoV-2 RNA in wastewater with community case data (reported clinical cases) and environmental persistence (computed half-life). Interestingly, SARS-CoV-2 N1 gene half-life was moderately correlated to the clinical case data and was statistically significant (p-value <0.05), as compared with N2 gene. This might be due to its more frequent detection in wastewater samples in this study. Gene half-life can also be influenced by the nature of the wastewater matrix itself, such as possible dilution, temperature, pH, suspended solids and organic matter loads in wastewater, and the presence and concentration of disinfectants and other chemicals to possibly eliminate the virus (Tran et al. 2021; Mogili et al. 2024).

Wastewater SARS-CoV-2 RNA gene quantity was very weakly correlated with both SARS-CoV-2 RNA gene half-life and the reported number of COVID-19 clinical cases (Table 2 (A)). The N2 gene half-life was likewise very weakly correlated to clinical data (Table 2 (B)). Similar weak correlation results were also reported in other literature, which can be associated with viral shedding load, duration, and patient percentage with gastrointestinal stress (Hong et al. 2021). Moreover, this weak correlation may have been influenced by the sampling method used in the current study. Like Hong et al. in 2021, the study used grab sampling. Though literature has reported successful recovery of SARS-CoV-2 RNA via wastewater grab sampling, there is an uncertainty regarding the timely capture of fecal release from COVID-19-positive patients (Ahmed et al. 2020; Randazzo et al. 2020; Hong et al. 2021).

Table 2: Total correlation (with R values) of (A) quantified COVID-19 RNA genes in wastewater, with the computed RNA gene half-lives, and reported clinical cases of COVID-19 in the City of Manila, and (B) SARS-CoV-2 RNA gene half-lives with the reported COVID-19 clinical cases, (P < 0.05).

(A)

SARS-CoV-2 RNA Gene in Wastewater	SARS-CoV-2 RNA Gene Half-life ($t_{1/2,2}$)	Number of COVID-19 Clinical Cases
N1	Very Weak (0.1493)	Very Weak (0.1001)
N2	Very Weak (0.1221)	Very Weak (0.1201)

(B)

SARS-CoV-2 RNA Gene Half-life ($t_{1/2,2}$)	Number of COVID-19 Clinical Cases
N1	Moderate* (0.4664)
N2	Very Weak (0.0870)

Nb.: Correlation coefficient values: 0.00-0.19 "very weak"; 0.20-0.39 "weak"; 0.40-0.59 "moderate"; 0.60-0.79 "strong"; 0.80-1.0 "very strong"
*statistically significant (p-value < 0.05)

CONCLUSION

This study showed evidence of detecting SARS-CoV-2 RNA and its Omicron subvariants from urban wastewater samples in the City of Manila, from a hospital to a community sewer route. SARS-CoV-2 N1 gene detection rate was higher as compared to N2 gene in all wastewater samples tested, which was in accordance to previously reported results in other literature. The highest viral RNA prevalence was detected in Site A, a hospital facility, where most clinical cases of COVID-19 were also reported during the sampling duration. Detection of new Omicron subvariants from wastewater samples ahead of clinical sample detection during the same sampling period in the Philippines supports the possible utility of wastewater surveillance of circulating SARS-CoV-2 RNA.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Conceptualization – LMMD, RCA, RMT; methodology – LMMD, RCA, RMT; Formal analysis – RCA, RMT, LMMD; Investigation – RCA, RMT, NQC; writing – original draft preparation: RCA, RMT; writing—review and editing – RCA, LMMD, RMT, AHO, ABB, MABP, MASS, NQC; supervision – LMMD; funding acquisition – LMMD, RCA. All authors have read and agreed to the published version of the manuscript.

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