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Whole campus wastewater surveillance of SARS-CoV-2 for COVID-19 outbreak management

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ABSTRACT

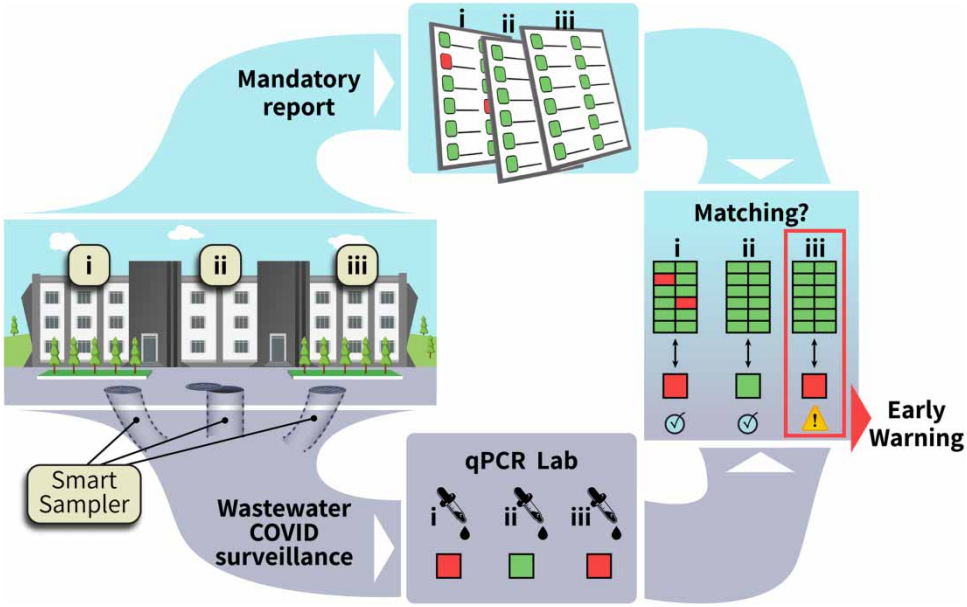
In this long-term study (eight months), a wastewater-based epidemiology program was initiated as a decision support tool for the detection and containment of COVID-19 spread in the Technion campus. The on-campus students' accommodations (~3,300 residents) were divided into housing clusters and monitored through wastewater SARS-CoV-2 surveillance in 10 manholes. Results were used to create a 'traffic-light' scheme allowing the Technion's COVID-19 task force to track COVID-19 spatiotemporal spread on the campus, and consequently, contain it before high morbidity levels develop. Of the 523 sewage samples analysed, 87.4% were negative for SARS-CoV-2 while 11.5% were positive, corroborating morbidity information the COVID-19 task force had. For 7.6% of the SARS-CoV-2 positive samples, the task force had no information about positive resident/s. In these events, new cases were identified after the relevant residents were clinically surge tested for COVID-19. Hence, in these instances, wastewater surveillance provided early warning helping to secure the health of the campus residents by minimising COVID-19 spread. The inflammation biomarker ferritin levels in SARS-CoV-2 positive sewage samples were significantly higher than in negative ones. This may indicate that in the future, ferritin (and other biomarkers) concentrations in wastewater could serve as indicators of infectious and inflammatory disease outbreaks.

Key words: COVID-19, disease management, in-sewer surveillance, metabolic marker, SARS-CoV-2, wastewater-based epidemiology

HIGHLIGHTS

- SARS-CoV-2 in-sewer monitoring was used as a decision support tool for minimising COVID-19 spread in the Technion.
- Of the positive sewage samples, 91% corroborated diagnosed cases and 7.6% served as an early warning since no positive cases were known.
- No false-positive/negative results were obtained.
- Ferritin, an inflammation biomarker, levels were significantly higher in positive sewage samples.
- Sewer monitoring may make surge testing partially redundant

GRAPHICAL ABSTRACT



INTRODUCTION

The ongoing COVID-19 pandemic caused by the novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) poses a significant threat to global health security and stability. It has a daunting effect on healthcare systems, economies, and societies. Monitoring the spread of the COVID-19 pandemic is crucial for its containment. Nevertheless, the ability to clinically test large populations routinely depends on the voluntary response of the populace and entails substantial financial costs and logistical complexity. Thus, a complementary method for clinical testing should be used in order to routinely monitor the virus prevalence and spread.

Wastewater-based epidemiology (WBE) is an epidemiological tool used for monitoring poliovirus and for tracking infectious aetiological agents that are shed in human faeces and urine (La Rosa *et al.* 2020a). Recent studies have shown that SARS-CoV-2 is excreted through human stool (Cheung *et al.* 2020; Holshue *et al.* 2020) and its RNA can be successfully detected in wastewater (Betancourt *et al.* 2021; La Rosa *et al.* 2020b; Medema *et al.* 2020a; Thompson *et al.* 2020; Westhaus *et al.* 2021; Yaniv *et al.* 2021; Bar-Or *et al.* 2022; Islam *et al.* 2022). People infected by the virus tend to excrete it before being diagnosed or start showing clinical symptoms. Moreover, asymptomatic COVID-19-positive individuals do excrete the virus in their faeces. Medema *et al.* (2020b) reported that SARS-CoV-2 was detected in wastewater treatment plants 6 days before the first cases were reported (through clinical testing) in The Netherlands. In the same line, Yaniv *et al.* (2021) reported in-sewer detection of SARS-CoV-2 about a week before a morbidity outbreak was detected in an Israeli city. COVID-19 WBE has been applied in several countries at various locations such as wastewater treatment plants (e.g. Aguiar-Oliveira *et al.* 2020; Ahmed *et al.* 2020a; La Rosa *et al.* 2020b; Medema *et al.* 2020b; Polo *et al.* 2020; Trottier *et al.* 2020; Wu *et al.* 2020; Sangsanont *et al.* 2022a, 2022b; Grube *et al.* 2023) and in-sewer monitoring (e.g. Wilder *et al.* 2021; Yaniv *et al.* 2021). These studies exhibit the advantage of WBE in assessing the morbidity status of the population in a given catchment area and also its potential to act as a morbidity outbreak early warning system. Non-intrusive in-sewer surveillance for monitoring of a residential building for COVID-19 cases (Colosi *et al.* 2021; Wong *et al.* 2021), or in airline and cruise ship sanitation systems (Ahmed *et al.* 2020b) and universities (Betancourt *et al.* 2021; Gibas *et al.* 2021; Scott *et al.* 2021; Kotay *et al.* 2022; Sumpaico-Tanchanco *et al.* 2022) not only can serve as an early warning of new cases but also may lead to targeted clinical testing and identification of infected individuals, hence preventing potential disease transmission and consequential outbreak. WBE can also be utilised as a complementary technique by providing information on inflammatory markers, where the increase in their concentration may signal the presence of disease in a community (Sims & Kasprzyk-Hordern 2020).

Ferritin, which plays an important role in the storage of intracellular iron, is widely used in clinical diagnostics and for monitoring a wide range of infectious diseases, as many of them are associated with iron overload or deficiency (Wang *et al.* 2010). During infection, ferritin protects the host by limiting iron availability to pathogens (Letendre & Holbein 1984; Wooldridge & Williams 1993; Pieracci & Barie 2005). Several studies reported high blood-serum levels of ferritin in severe COVID-19 patients (Mehta *et al.* 2020; Zhou *et al.* 2020; Ozgür *et al.* 2021). However, to the best of our knowledge, its fate in sewage was not yet investigated.

The goal of this study was to develop and implement a near-source wastewater surveillance (WWS) program in order to protect the health of students residing on campus, detect and contain COVID-19 outbreaks in their early stages, minimise their spread on the campus, and thus to allow the Technion university campus (Haifa, Israel) to remain open.

The research and surveillance program commenced on October 2020 and terminated on June 2021, covering the second and third COVID-19 waves in Israel. It was used as a front-line defence to restrain COVID-19 spread within the campus. In-sewer SARS-CoV-2 detection led to clinical testing of all individuals residing in the sub-catchment contributing to the specific manhole, and intervention by the university COVID-19 task force. In parallel, ferritin (inflammatory marker protein, see above) concentrations were monitored in order to reveal possible association with SARS-CoV-2 levels in the wastewater.

METHODS

The core of SARS-CoV-2 WBE is in-sewer detection. The virus is excreted by humans through faeces. Previous works have shown that people preferentially defaecate at home rather than at work/public toilets (e.g. Friedler *et al.* 1996; Shteynberg 2015). Hence, the WWS program on the campus mainly targeted students' accommodation and not offices, classes, etc.

Study site

The study acted as the first layer of the 'safe and open campus' initiative of the Technion, designed to contain and minimise the spread of COVID-19 on campus. The team was in close contact with the Technion's COVID-19 task force and the results of each sampling day were immediately reported to the task force. To avoid bias, the personnel who performed the analyses were informed of clinically detected COVID-19-positive (CD-COVID-P) persons in the contributing houses clusters only after the results were reported to the task force.

Figure 1 depicts the 11 monitored manholes, the contributing building clusters to each manhole, and the related number of people (ranging from ~180 to ~1,060 persons/manhole). Overall, 103 buildings were monitored, housing 3,310 residents (students and their family members). These included most on-campus housing as well as several office buildings and two kindergartens. Samples were collected from manholes prior to mixing with other sewer lines, thus samples were specific to the defined building cluster. Some manholes included flows from upstream ones (e.g. manhole 2 discharges to manhole 1, 5 to 6, 7 to 11 and both (7 and 11) to 9).

As part of the tenancy agreement, residents were required to report COVID-19-related information, such as clinical test result and date, and quarantine start and end dates. Moreover, as part of national policy, all people entering the campus had to fill a declaration stating that they were not COVID-19 positive and/or are not obliged to go on isolation. This information helped the Technion task force to manage the epidemic on campus, and as a by-product, enabled the validation of the accuracy and detection limits of the WWS campaign.

Wastewater sampling

Smart automatic continuous sewage samplers (Kando Ltd Israel) were installed in the 11 manholes (Figure 1). Each sampler consisted of a peristaltic pump, an 8 L plastic collection tank, and a remote-control application via cellular communication. Once samplers were installed within the manholes they were covered with the original lids, thus disruption to passers-by and transportation was minimal. WWS started on 22 October 2020, a few weeks before the beginning of the semester, and shortly after the beginning of the second COVID-19 wave in Israel. By November 10th all samplers were deployed. The smart samplers were programmed to sample twice a week, on Mondays and Wednesdays, when most dormitories inhabitants are usually on campus. Two hundred and fifty mL of raw sewage samples were pumped at 30 min intervals from 7:00 to 23:00, totalling 7.5 L on each sampling day at each monitored manhole. The next morning (Tuesday and Thursday) composite samples were collected from each manhole. Samples were mixed vigorously, then 50 mL were taken for RNA extraction, and 1 L was taken for chemical analyses. All samples were transported to the laboratory in a cooler (4 °C) and analysed immediately.

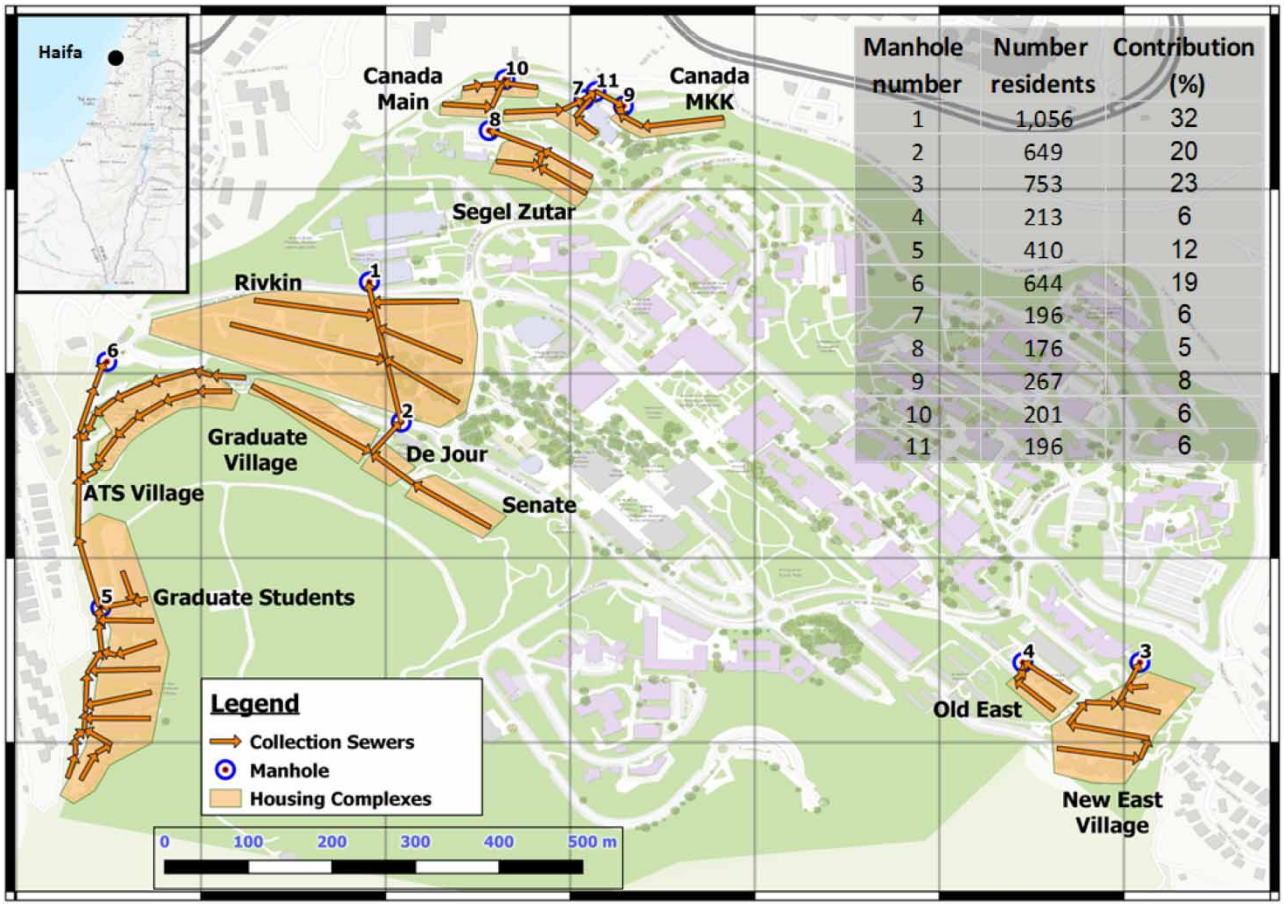


Figure 1 | Layout of the SARS-CoV-2 wastewater surveillance scheme in Technion campus (Haifa, Israel). Inset top-left – location of the Technion in Israel. Inset top right – Number of residents contributing to each manhole. Monitored manholes are indicated by numbers (1–11). Orange areas – monitored building clusters. Arrows show the sewage flow direction.

Sewage samples pretreatment

Samples were either processed without pretreatment (raw) or after pretreatment using Amicon centrifugal ultrafilters. Samples pretreatment and concentration procedures comprised of several steps based on Medema *et al.* (2020a). Briefly, 50 mL of each raw sewage sample was transferred into 50 mL sterile tubes and centrifuged (Biofuge Primo Heraeus Centrifuge; Kendro Laboratory Products, Germany) for 10 min, 4,000 *g*. Then, Amicon centrifugal ultrafilters with a molecular weight cut-off of 10 kDa (Merck Millipore, Tullagreen, Ireland) were used to further concentrate the samples. The Amicon ultrafilters were pre-centrifuged for 10 min, 4,000 *g* with 2 mL of NaOH (1 M), to optimise pore membrane activity, and washed thoroughly with distilled water. Afterward, 15 mL of each sample were transferred into the Amicon tubes and centrifuged (Heraeus Megafuge 1.0R Centrifuge; Thermo-Scientific, MA, US) for 10 min, 4,000 *g* at 4 °C (concentrated sample volumes were up to 1.5 mL (approximately 15 times more concentrated).

RNA extraction

Wastewater often contains diverse inhibitors, such as fats, proteins, and other substances, which adversely affect PCR reaction efficiency (Gibas *et al.* 2021). Therefore, a set targeting the SARS-CoV-2 Envelope small membrane protein (E-gene) was used for the qPCR. It has been previously demonstrated that E-gene detection has very high sensitivity (Corman *et al.* 2020), and that the minimum detection limit for this gene is far below the estimated viral load for COVID-19-positive persons (Chan *et al.* 2020). Therefore, using these primers allowed reliable detection and quantification of the viral concentrations in the domestic sewage of the campus. Another advantage is that on one hand, these primers are highly specific for SARS-CoV-2

(no cross-reactivity with other SARS-CoV viruses) while on the other, they allow the detection of different SARS-CoV-2 variants.

Pre-treated raw and concentrated sewage samples were transferred into pre-sterilised 1.5 mL Eppendorf tubes and kept on ice. RNA extraction was then performed on ice using the NucleoSpin RNA extraction kit (Macherey-Nagel GmbH, Duren, Germany) according to the manufacturer's instructions. RNA was extracted from each sample in duplicate using two methods as described above (totalling four replicates for each sample). The processing volume for each repetition was 200 μL with 60 μL elution volume. After nucleic acid extraction, all eluates were directly analysed by reverse transcriptase polymerase chain reaction (RT-qPCR) for the detection of SARS-CoV-2 RNA. Eluates were kept for further analyses at −80 °C.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Positive samples were detected by the accumulation of fluorescent signal in a modified RT-qPCR assay based on the Charité/Berlin Protocol according to the WHO recommendations designed to target and quantify the presence of SARS-CoV-2 gene encoding the E-gene (Vogels *et al.* 2020). The primers used were E-Sarbeco-F (ACAGGTACGTTAATAGTTAATAGCGT) and E-Sarbeco-R (ATATTGCAGCAGTACGCACACA). These primers targeted the E-gene at the 26,141–26,253 genome positions (according to SARS-CoV-2, GenBank NC_004718) as described by Corman *et al.* (2020). For detection and quantification of the E-gene qPCR products a TaqMan probe with a dye label (FAM) on the 5′ end and a minor groove binder and non-fluorescent quencher (NFQ) on the 3′ end (FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1) was used. RT-qPCR reactions were performed in 25 μL reaction volumes containing 5 μL of the eluted sample RNA, 0.8 μL of each primer, 0.4 μL TaqMan probe, 12.5 μL 1× One Step PrimeScript III RT-PCR mastermix (Takara Bio, Shiga, Japan), and 5.5 μL of ultrapure DNase/RNase-free Distilled Water (Takara Bio, Shiga, Japan). Samples were analysed by RT-qPCR using a CFX Opus 96 (Bio-Rad Ltd) and thermal cycling was performed at 50 °C for 10 min (reverse transcription), then 95 °C for 1 min (denaturation) followed by 45 cycles of 95 °C for 10 s, 55 °C for 30 s with plate read and fluorescence acquisition at the end of each cycle. Results were obtained on the same day, 7–10 h after sample collection. The cycle threshold (Ct) was defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds the background level). Ct values were determined using a manual threshold method with the threshold set mid-point through the exponential phase of amplification, as per standard practice in clinical PCR assays. A positive result was defined as amplification detected above the threshold within 42 cycles. Ct values between 42 and 45 were considered as not detected. Images of the amplification curves were taken directly from the analysis software (supplementary Fig. S1).

Synthetic single-stranded RNA of 420 base-pairs length was used as both positive control for the PCR reaction and for the creation of calibration curves for each run. For the calibration curves, a series of six four-fold dilutions were used in each PCR run to ensure proper and accurate conversion of the detected sample Ct values into RNA quantities of each eluate. A duplicate of non-template controls containing sterile ultrapure RNase-free water instead of a sample eluate at each run was used, to omit any DNA or RNA template from the reaction. This served as a general control for extraneous nucleic acid contamination of the assay. Additionally, a no reverse transcriptase control using synthetic single-stranded RNA in the absence of the reverse transcriptase was used. This control was used to reveal whether DNA contamination in an RNA preparation occurred. All fluorescence curve data analysis was done using CFX Maestro Software (Bio-Rad Ltd).

Viral concentrations (Gene Copies per L, GC/L) were calculated using Equations (1) and (2) for raw wastewater (RWW) and concentrated samples (CWW), respectively:

$$\mathrm{GC}\ (\#\ /\mathrm{L})_{\mathrm{RWW}} = \frac{10^{\text{calibration equation}} * \text{elution vol. } (\mu\mathrm{L})}{\text{Reaction vol. } (\mu\mathrm{L}) * \text{RWW vol. used for RNA extraction (L)}} \quad (1)$$

$$\mathrm{GC}\ (\#\ /\mathrm{L})_{\mathrm{CWW}} = \frac{10^{\text{calibration equation}} * \text{elution vol. } (\mu\mathrm{L}) * \text{initial volume}}{\text{Reaction vol.}(\mu\mathrm{L}) * \text{Final vol. } (\mu\mathrm{L}) * \text{CWW vol. used for RNA extraction (L)}} \quad (2)$$

One of the aims of the study was to decrease the limit of detection (LOD) to allow high-resolution identification of infected individuals from sewage samples. To achieve this objective, the RNA extraction procedure and qPCR assay were modified. First, samples were pretreated using Amicon centrifugal ultrafilters to concentrate the nucleic acid contents. Pre-concentration using ultrafiltration may lower LOD, as shown by Yaniv *et al.* (2021). Secondly, the E-gene was targeted as the viral gene marker, since it exhibits high analytical sensitivity, and significantly lower detection thresholds compared to other SARS-CoV-2 structural genes, routinely used in clinical settings (e.g. RdRP and N), as reported by Corman *et al.*

(2020). In addition, results were defined as positive for amplification detected above the threshold within 42 cycles, and not 40 cycles, as per clinical practice standard operation. This is a major difference because extending the criteria to 42 cycles substantially lowered the assay's LOD compared to other reported assays. More negative controls (no amplification and no template controls) were added to avoid false-positive results and thus, we were able to substantially decrease the LOD to 1.7×10^1 GC/L in sewage samples.

Normalised viral load: SARS-CoV-2 RNA copy number relative contribution

Normalised SARS-CoV-2 viral load (NVL, load/(person·day)) was obtained by Equation (3), using the measured SARS-CoV-2 viral content (GC/L), total nitrogen (TN) concentration in the wastewater samples and typical *per capita* nitrogen load in domestic wastewater (11.6 g N per person per day; Friedler *et al.* 2013). This method is in line with Yaniv *et al.* (2021) who have shown a good correlation between NVL based on TN and NVL based on flow and contributing population size:

$$\mathrm{NVL}\left(\frac{\mathrm{GC}}{\mathrm{person}\cdot\mathrm{day}}\right) = \frac{\text{RNA copy number}\left(\frac{\mathrm{GC}}{l}\right)\cdot 11.6\left(\frac{g-N}{\mathrm{person}\cdot\mathrm{day}}\right)}{\mathrm{TN}\left(\frac{g-N}{l}\right)} \tag{3}$$

Chemical analyses

Sewage samples (1 L) were analysed for TOC, TN, and TSS. To minimise health risk, samples were disinfected prior to the analyses by UV_{254}-irradiation (7 mW/cm^2, VL_230.G, Lamp part T-30.C, Cole Parmer Co., US), for 2 h while being continuously stirred. Disinfected samples were filtered through glass fibre filters 1.2 μm (GFA, Whatman, UK). Filters were used for measuring TSS, and filtrate was analysed for TOC and TN using a TOC-TN analyser (TOC-V CPH, equipped with TN measuring unit, TNM-1; Shimadzu, Japan). Analyses were conducted according to APHA (2017).

Ferritin analysis protocol followed Davidov *et al.* (2020). Filtered samples were concentrated by automatic solid phase extraction (SPE; Dionex Autotrace 280, Thermo-Scientific, MA, US). SPE was conducted using Strata XL 500 mg/6 mL cartridges (phenomenex®, USA) that were preconditioned with 6 mL methanol, 6 mL acetone, and 6 mL deionised water at pH 2. Approximately 900 mL filtered samples were loaded on cartridges at a rate of 8 mL/min. Cartridges were then washed with 6 mL of 5% methanol in deionised water and air-dried for 30 min. Ferritin was extracted from the cartridges with 15 mL methanol and 15 mL acetone. The eluent was evaporated to complete dryness. Dry vessels were washed with 1 mL methanol yielding an overall concentration factor of 900 for ferritin. Samples were analysed by High Performance-Liquid Chromatography (HPLC) with Photo-Diode Array (PDA) detector (LC-20AD prominence Liquid Chromatograph, Sil-20AC HT prominence Auto-sampler, SPD-M20A prominence Diode Array detector and CTO-20AC prominence Column oven, by SHIMADZO, Japan). Samples (50 μL) were injected onto a PS-C18 column kept at 20 °C (Luna® Omega, 4.6 × 150 mm, 3 μm particles, phenomenex®, CA USA). A bi-solvent system (A and B) was used at 1 mL/min flow rate. The initial solvent mixture was 95% A (deionised water + 0.1% trifluoracetic acid) and 5% B (acetonitrile + 0.1% trifluoracetic acid), then varied to 20% A: 80% B within 16 min, and kept for 2 min, and back to 95% to 5% for additional 2 min. Light absorbance was measured between 200 and 400 nm by the PDA detector, and quantification was done using the light absorbance at 210 nm. Ferritin concentration was compared to standard ferritin solution provided by Meyron-Holtz E (see acknowledgements)

As aforementioned, to ensure workers' health wastewater samples were disinfected by UV irradiation prior to the chemical analyses. Thus, experiments were conducted to evaluate UV irradiation effects of ferritin concentration. Ninety-five mL ferritin was added to 4 L tap water, the solution was continuously mixed, and concentrations were measured before and after UV irradiation.

Data and statistical analyses were performed using JMP Statistical Discovery LLC software from SAS Institute Inc (NC, USA).

RESULTS AND DISCUSSION

SARS-CoV-2 WWS

The in-sewer monitoring campaign spanned from 27 October 2020 to 30 June 2021 (eight months). During this period, 52 positive COVID-19 cases were reported by campus residents. Overall, 523 sewage samples were collected and analysed. The results were used to create a 'traffic-light' scheme which aided the Technion's COVID-19 task force to track COVID-19 temporal and spatial spread on campus (Figure 2). Eighty-seven percent of the samples were COVID-19 negative (458 samples), while 13% were positive. The latter indicated that at least one 'excreta contributor' to the manhole was COVID-19 positive. The overall mean Ct value of positive samples was 36.43 ± 2.01 (range from 30.96 to 39.83 cycles) with a corresponding viral concentration of $1.09 \times 10^5 \pm 6.27 \times 10^4$ SARS-CoV-2 GC/L (ranging from 1.7×10^1 to 3.67×10^6 GC/L).

The WWS succeeded to trace one clinically confirmed infected person out of 1,056 contributing individuals (SARS-CoV-2 and was detected twice in manhole #1; Figures 1 and 2). It should be noted that manhole #1 had the largest contributing population, thus the normalised LOD (one infected person/population size) may have not been reached. The current normalised detection (one infected person/ ~ 1,000 persons) is about five times higher than the figure reported by Gibas *et al.* (2021) who detected a single asymptomatic individual out of 150–200 people.

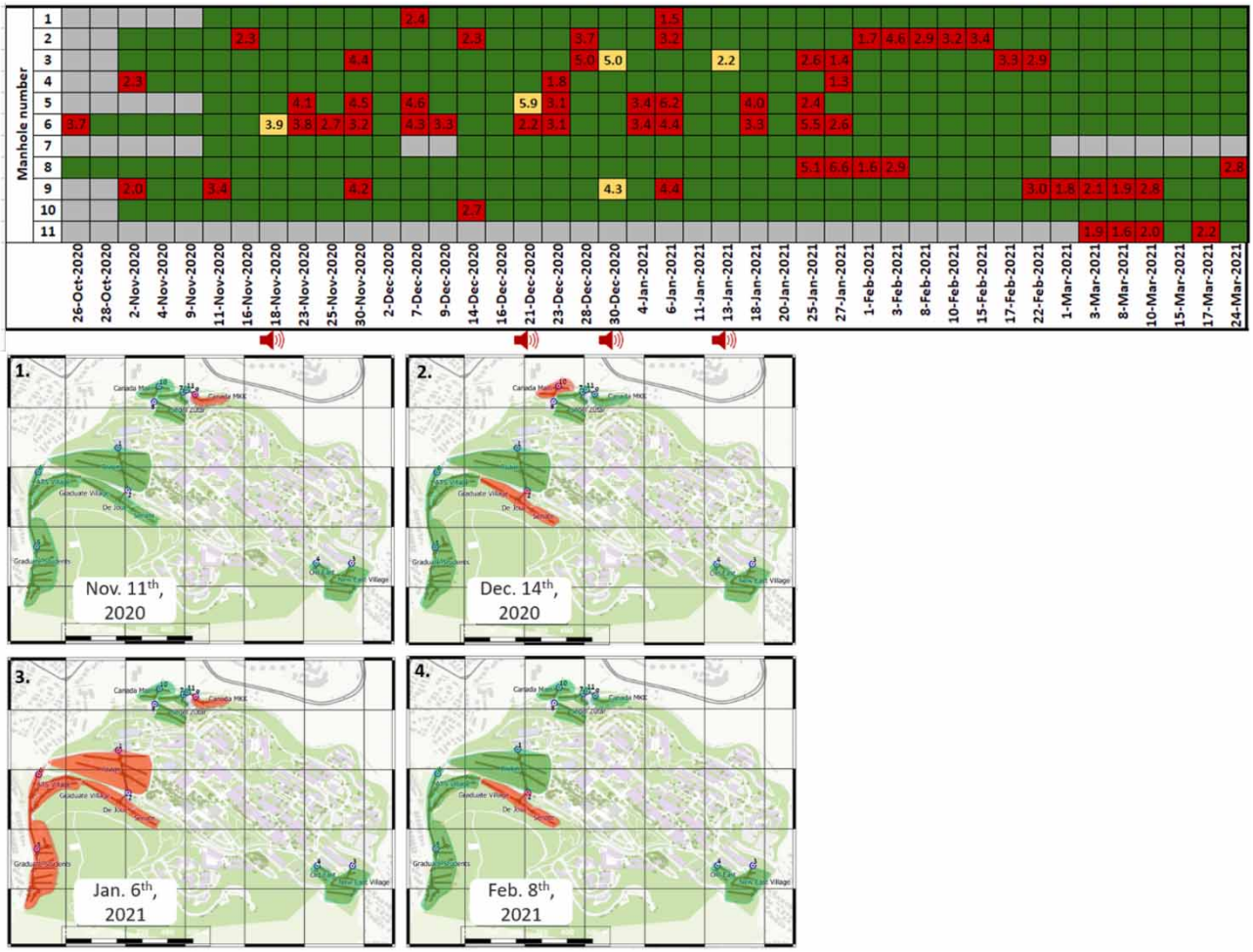


Figure 2 | 'Traffic-light' scheme of the SARS-CoV-2 surveillance in the Technion campus. Top – overall dashboard: Green rectangles – SARS-CoV-2 not detected, Yellow – early warning (detected in WW but not known to the Technion COVID-19 task force), Red – positive (detected in WW and known), Grey – not sampled/analysed). Numbers – mean Log GC/L (of all four repetitions) Horn symbol – early warning result. From 29.3.21 to 30.6.21 (end of the program) SARS-CoV-2 was not detected at all, hence data not shown. Bottom – example of four sampling days: Monitored sewer lines and Students' residences marked by dark green or orange lines/areas. Dark Green – SARS-CoV-2 'free' areas; Orange – SARS-CoV-2 detected.

During the first month of the study, few manholes were detected positive (only one positive manhole on each sampling day). However, as the semester progressed, positive detection events became more frequent, and more than one manhole was detected positive on each sampling day. The increase in detection frequency continued until 6 January, when SARS-CoV-2 was detected at five manholes simultaneously, which constituted half of the examined sewage manholes.

The WWS results were compared each sampling day with the CD-COVID-P cases known to the Technion's COVID-19 task force (reported by campus residents and employees). Based on the data of the task force, reported CD-COVID-P cases were then attributed to each manhole. A total of 98.9% of the WWS results matched the data the task force had, with 87.4% not detected in the sewage and no reported cases in the contributing population, and 11.5% were detected in the sewage and verified CD-COVID-P cases (Figure 3). Six WWS results (1.15% of the total number of samples and 9% of the total number of SARS-CoV-2 positive samples) did not match the CD-COVID-P cases known at that time to the Technion's task force.

Of the six positive WWS results mentioned above, five detections were the most important. In these, SARS-CoV-2 RNA was detected in the sewage before the COVID-19 task force knew of any CD-COVID-P individual in the respective housing clusters. In these events, all residents of the relevant housing clusters (contributing to the SARS-CoV-2 positive manhole) were asked to undergo COVID-19 PCR test immediately. In all five events, at least one new CD-COVID-P case was identified and was asked to quarantine. This emphasises the importance of WWS, as an early warning tool for COVID-19 (or other) epidemic management.

In one incident the task force data indicated one CD-COVID-P student living in the houses cluster contributing to a specific manhole, but no SARS-CoV-2 RNA was detected in the sewage of this manhole. Following this discrepancy, an investigation performed by the task force revealed that the CD-COVID-19-P student left the campus (without notice) a day before the sampling day, and therefore, the sample was negative.

The results obtained emphasise the efficiency of using near-source WWS as early warning and for tracking the current trend and spatial distribution of the epidemic. Similar importance is reported by recent studies also conducted at near-source WWS (e.g. Colosi *et al.* 2021; Wong *et al.* 2021). In addition, our results also revealed the capacity of long-term WWS for the continuous monitoring of SARS-CoV-2 in the community that can pinpoint temporal and spatial trends of COVID-19 morbidity at their onset and thus prevent and minimise the spread of the disease. Further, long-term continuous monitoring also provides the ability to distinguish between new cases and recovered ones. The same is anticipated to hold true for other diseases (known or not yet known), the vectors of which are excreted by humans.

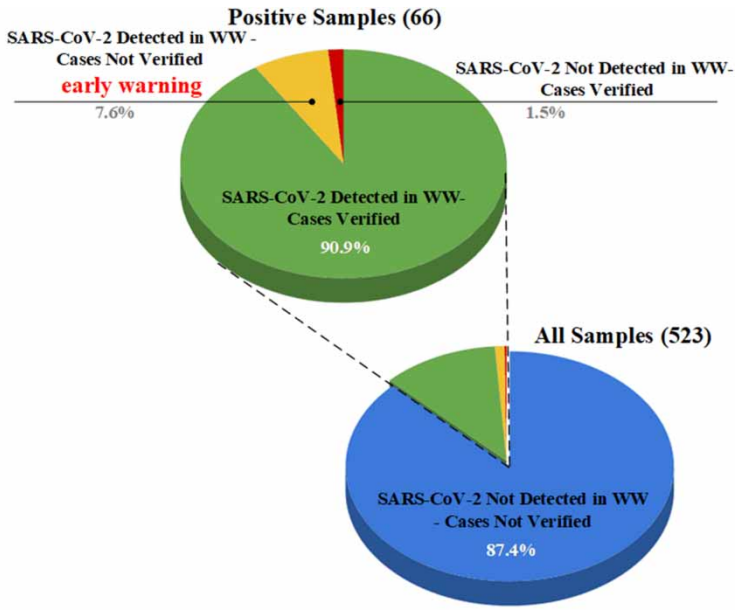


Figure 3 | SARS-CoV-2 wastewater surveillance detection compared to clinically detected COVID-19 cases known to the COVID-19 task force.

SARS-CoV-2 WWS detection rates increased from November 2020 to January 2021 and decreased from the end of January 2021 to the end of March 2021, since then SARS-CoV-2 were not detected through the WWS, and new CD-COVID-P cases were not reported in the campus until the end of the study. This pattern preceded the prevalence and spread of COVID-19 in Israel (data obtained from the Israel Ministry of Health COVID-19 dashboard) by about 3 weeks. Kolmogorov–Smirnov test showed a statistically significant fit and correlation ($p < 0.05$) between the two curve trends (Figure 4). The exponential phase of the third COVID-19 outbreak in Israel started between November–December 2020. Through the WWS a considerable increase in NVL load was detected ~2–3 weeks earlier, at the beginning of November (Figure 4). The peak number of cases in Israel was observed in mid-January 2021, whereas the on-campus NVL peaked approximately 2 weeks earlier, during the first week of January. It should be noted that although the campus may be considered as an 'isolated community' many residents leave campus during weekends to visit their hometown, family, and friends across the country, and thus, the campus population actually represents different areas of the state and not only the campus itself. Both curves were significantly affected by the national vaccination campaign, which first started only for persons older than 60 years, and then expanded to younger people. At the beginning of February 2021 SARS-CoV-2 vaccine was available to all people older than 16 years, which led to a decline in morbidity, which was evident both in the campus sewage and in the whole state in general.

Comparison between pretreatments

The mean Ct of positive samples after raw sewage extraction was 36.6 ± 2.2 with a corresponding mean viral concentration of 1.52×10^4 GC/L. The Amicon ultrafilters pretreatment of the same samples yielded similar results with a mean Ct of 36.4 ± 2.4 (viral concentration of 12.4×10^4 GC/L). Paired samples *t*-tests showed no statistically significant differences between the Ct values and viral RNA concentrations obtained by the two extraction methods. A similar phenomenon was reported by Bar-Or *et al.* (2022). In addition, significant correlations were found between Ct values and subsequent viral concentrations obtained by the two methods ($r_s = 0.64$ and 0.68, $p < 0.001$, respectively), indicating that the results were consistent and reproducible when comparing these two extraction methods.

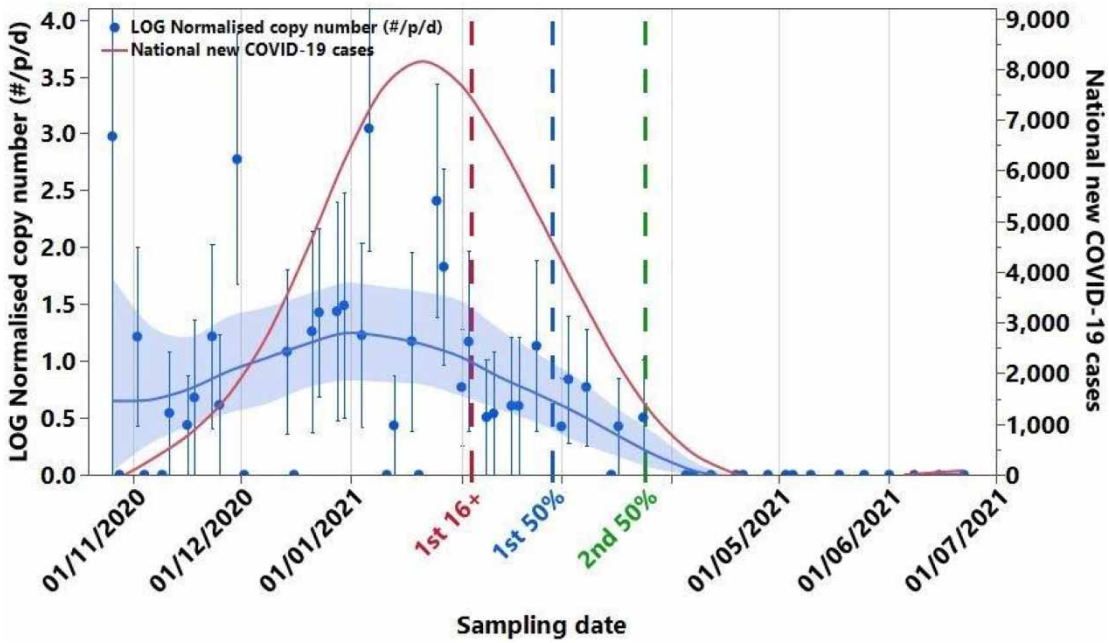


Figure 4 | Normalised viral load of SARS-CoV-2 detected by wastewater surveillance on campus (blue) and number of new COVID-19 cases in Israel (data from Israel Ministry of Health) from November 2021 to July 2021. Vertical dotted lines: red – beginning vaccination to 60 + years old persons; blue – 50% of the population (16 + years old) vaccinated once; green – 50% of the population (16 + years old) vaccinated twice. Blue dots – Log normalised copy number of all manholes mean; Error bars – standard error of Log normalised copy number of all manholes; Blue line – Spline trend line (lambda – 0.8) of Log normalised copy number of all manholes; Shaded blue – confidence of fit of the spline trend.

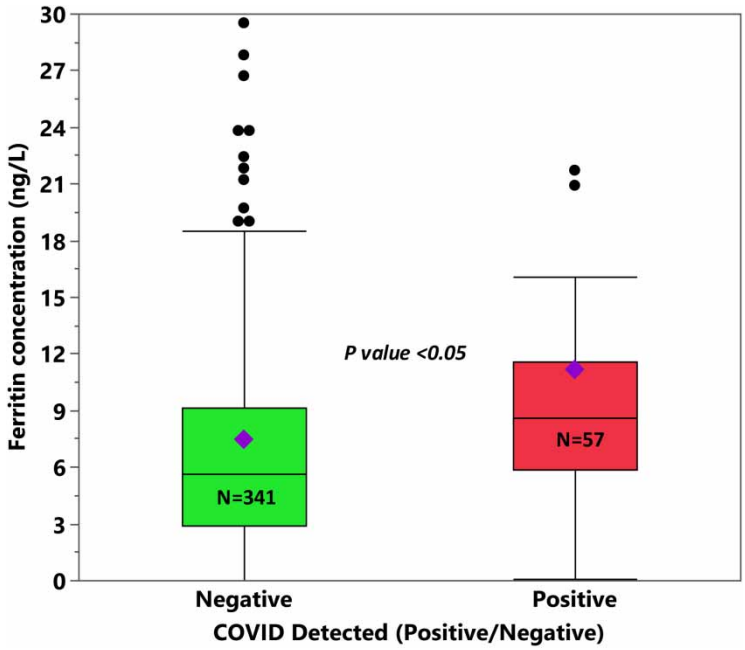


Figure 5 | Ferritin concentration in negative vs. positive SARS-CoV-2 wastewater samples. Diamonds – mean values, Horizontal black line – median values.

Discrepancies between the methods occurred in seven 'suspected-positive' samples (11% of the positive samples): six were found positive only after pretreatment with Amicon ultrafilters, while only one was found positive after raw sewage extraction but negative after Amicon ultrafilters pretreatment. This hints that pre-concentration potentially lowers the LOD, and thus may be more suitable for areas of low morbidity. This is corroborated by Yaniv *et al.* (2021) who has shown that pre-concentration lowers the detection limit.

Ferritin and its interrelation with SARS-CoV-2 in wastewater

TOC, TN, and TSS mean concentrations were 83.6 ± 1.9 (range 16.5–783), 82.4 ± 1.17 (range 10.2–248), and 294.7 ± 62.3 (43–4,708) mg/L respectively. These wide ranges are typical in near-source measurements (e.g. Friedler & Butler 1996).

Thirty percent degradation of ferritin was observed during disinfection experiments of tap water samples spiked with ferritin (not shown). These were carried out with tap water that exhibits high UV_{254} radiation transparency. Thus, UV_{254} disinfection employed on RWW that contains high pollutants concentrations and thus exhibits much lower UV_{254} transparency) most probably resulted in much lower ferritin degradation, and the ferritin values measured in this study were assumed to provide a reliable estimation of the values present wastewater.

Ferritin was detected and measured in 393 out of 401 samples analysed (98%), while in four samples it was below the detection limit. Its concentrations ranged between 0.19 and 1,079 ng/L, with mean and median concentrations of 8.2 ± 0.5 and 6.2 ng/L, respectively. As expected, its concentrations were significantly and positively correlated with TOC ($r = 0.51$, $p < 0.001$) and TN ($r = 0.25$, $p < 0.001$) which corroborates its human excreta origin.

Ferritin concentrations did not directly correlate with the concentrations of SARS-CoV-2 on a one-to-one basis. However, a significant difference in ferritin concentrations was found between SARS-CoV-2 negative and positive samples (one-way ANOVA: $p < 0.01$). Both mean and median ferritin concentrations were higher in SARS-CoV-2 positive samples (11.2 and 8.6 ng/L, respectively) compared to SARS-CoV-2 negative samples (7.45 and 5.6 ng/L, respectively; Figure 5). This difference corresponds to a mean increase of 33.5% in ferritin concentration in SARS-CoV-2 positive samples. Additionally, a two-way contingency analysis showed that ferritin was detected with varying concentrations in all 58 SARS-CoV-2 positive samples where ferritin was also measured. On the other hand, ferritin was also detected in 335 out of the 343 samples that were SARS-CoV-2 negative, albeit with significantly lower concentrations, as mentioned previously.

It should be noted that studies that monitored ferritin levels in blood-serum samples of COVID-19 patients reported significant differences in ferritin levels between severe and mild patients (Ozgür *et al.* 2021) and one order of magnitude higher concentration in non-survivors and survivors (Mehta *et al.* 2020; Zhou *et al.* 2020). In the current study, all COVID-19 patients developed only mild symptoms if at all (no cases of hospitalisation due to illness), hence, their excreted ferritin levels were supposed to be rather in the low range according to the literature.

CONCLUSIONS

Understanding the dynamics of SARS-CoV-2 by WWS can lead to efficient monitoring and control of this pandemic in urban areas. The results of the current study demonstrate that WWS can serve also as an early warning detection system prior to detection through clinical testing of the population. Spatially distributed sampling points at manholes with defined contributing catchment areas will allow predicting SARS-CoV-2 (and other disease outbreaks) imminent resurgences through the assessment of spread in a given area and the proportion of areas with virus activity in a city. The methodology was found effective in identifying spatially based hotspots of COVID-19, aiding in taking measures to contain eruptions. In a small community such as the university campus, the method may allow unknown cases of infected persons to be identified. These observations are important, as they demonstrate that WWS in relatively small yet representative populations might allow the prediction of resurgence onset on a much larger scale.

Ferritin levels (a biomarker of inflammatory diseases excreted by humans) in SARS-CoV-2 positive wastewater samples were higher (statistically significant) than in negative samples. Therefore, ferritin (and other biomarkers) presence should be examined with a broader perspective and could be used in the future in order to assess the general burden of disease within monitored populations. To that end, further research is needed to establish a baseline ferritin level in wastewater and human excreta.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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