



## Long term detection and quantification of SARS-CoV-2 RNA in wastewater in Bahrain



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### ABSTRACT

Wastewater-based epidemiology is a corroborated environmental surveillance tool in the global fight against SARS-CoV-2. The analysis of wastewater for detection of SARS-CoV-2 RNA may assist policymakers to survey a specific infectious community. Herein, we report on a long-term quantification study in Bahrain to investigate the incidence of the SARS-CoV-2 RNA in wastewater during the COVID-19 pandemic. The ~260,000 population of Muharraq Island in Bahrain is served by a discrete sewerage catchment, and all wastewater flows to a single large Sewage Treatment Plant (STP) with a capacity of 100,000 m<sup>3</sup>/day. The catchment is predominately domestic, but also serves several hospitals and Bahrain's international airport. Flow-weighted 24-h composite wastewater samples for the period February 2020 to October 2021 were analyzed for the presence of SARS-CoV-2 N1, N2 and E genes. A Spearman rank correlation demonstrated a moderate correlation between the concentration of SARS-CoV-2 N1, N2 and E genes in the wastewater samples and the number of COVID-19 cases reported on the same day of the sampling.

SARS-CoV-2 viral genes were detected in wastewater samples shortly after the first cases of COVID-19 were reported by the health authorities in Bahrain by reverse transcription-polymerase chain reaction (RT-qPCR). The viral genes were detected in 55 of 65 samples (84.62%) during the whole study period and the concentration range was found to be between 0 and 11,508 RNA copies/mL across the viral genes tested (in average N1: 518.4, N2: 366.8 and E: 649.3 copies/mL). Furthermore, wastewater samples from two COVID-19-dedicated quarantine facilities were analysed and detected higher SARS-CoV-2 gene concentrations (range 27-19,105 copies/mL; in average N1: 5044, N2: 4833 and E: 8663 copies/mL). Our results highlight the potential use of RT-qPCR for SARS-CoV-2 detection and quantification in wastewater and present the moderate correlation between concentration of SARS-CoV-2 genes with reported COVID-19 cases for a specified population. Indeed, this study identifies this technique as a mechanism for long term monitoring of SARS-CoV-2 infection levels and hence provides public health and policymakers with a useful environmental surveillance tool during and after the current pandemic.

### 1. Introduction

The recent Coronavirus Disease 2019 (COVID-19) has exposed the vulnerability of people's health across the world. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causal agent of COVID-19, is a positive single-stranded RNA virus, that causes infection and respiratory failure and has resulted in millions of deaths

(Karthika et al. 2021). SARS-CoV-2 consists of the structural proteins, encode by nucleocapsid (N), membrane (M), envelope (E) and the spike (S) genes, which are involved in binding host cell receptors and mediating membrane fusion and virus entry. Additionally, SARS-CoV-2 encodes for nine accessory proteins (open reading frame (ORFs) proteins), which play a fundamental role in virulence and pathogenesis (Mariano et al. 2020). Around 253 million confirmed cases and more

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than 5 million deaths have been reported globally as of end of November 2021 (Dong et al. 2020). The Kingdom of Bahrain recorded its first COVID-19 infection case in February 2020 and since then there have been 277,000 cases and 1,399 COVID-19-related deaths (Dong, Du and Gardner 2020, WHO, 2021a).

The SARS-CoV-2 can migrate to other tissues from airways and lungs (Zhang, Xiang, et al. 2021) and given its tolerance for extreme pH, can even pass through the stomach. The virus is thought to remain viable and replicate in intestinal epithelial cells, which, incidentally, express high levels of ACE2 (Guney and Akar 2021, Jimenez et al. 2021). Indeed, the World Health Organization (WHO) and others have reported that up to 50% of SARS-CoV-2-infected individuals present with diarrhoea, demonstrating the intestinal effects of the virus (Song et al. 2020, Wang, Hu, et al. 2020, WHO. 2020). SARS-CoV-2 can be detected in human faeces and sewage, further illustrating that the virus is not isolated to the respiratory tract (Tomasino et al. 2021b, Wurtz et al. 2021). Whilst SARS-CoV-2 has been detected in faeces of symptomatic patients, faecal shedding of the virus has also been recorded in asymptomatic, pre-symptomatic and post-symptomatic COVID-19 patients (Chen, Chen, et al. 2020); indeed, risk of faecal-oral transmission of COVID-19 can potentially increase even if individuals have no symptoms (Gu et al. 2020, Xiao et al. 2020).

The ongoing COVID-19 pandemic has demonstrated the need for reliable and quick diagnostic assays. COVID-19 diagnostic kits were quickly produced after the WHO released a diagnostic protocol on January 13, 2020, (Corman et al. 2020). The sampling for wastewater, and analytical procedures (Quantitative real-time polymerase chain reaction, RT-qPCR) have a high level of harmonization and are considered reliable, since they are based on medically validated procedures for patient testing using swab or blood samples (CDC. 2021, Medema, Been, et al. 2020).

RT-qPCR is the most extensively used molecular diagnostic (Camp et al. 2021). Monitoring viral RNA in sewage is postulated as a useful tool in extending current clinical surveillance, by providing information on the prevalence and spread of the disease in a specific population (Amereh et al. 2021). A recent survey reported that laboratories around the world employed molecular detection methods to determine the presence of the virus in wastewater, with over 90% using a RT-qPCR method (Zhou et al. 2021). Also, reverse transcription droplet digital PCR (RT-ddPCR) is accepted as a molecular detection method. The main differences can be found in the virus concentration method, where ultracentrifugation, adsorption and precipitation, polyethylene glycol (PEG) precipitation, centrifugal ultrafiltration, direct extraction or membrane filtration are the most widely used concentration tools (Cervantes-Avilés et al. 2021). Combination of all these techniques have allowed the presence of the virus to be detected in wastewater facilities before medical symptoms or positive cases were detected by the medical community (Medema, Heijnen, et al. 2020).

Although there are reports of SARS-CoV-2 monitoring in wastewater (Hata et al. 2021, Hillary et al. 2021, Tomasino et al. 2021a), there are no scientific publications reporting long-term wastewater monitoring studies in the Middle East. SARS-CoV-2 wastewater monitoring has the potential to identify infections and consequently estimate infection rates in a population that belong to a specific sewage catchment area. In many cases, the viral load measured in wastewater treatment plant samples may reflect the overall amount of virus shed by infected people (Zheng et al. 2020). Monitoring SARS-CoV-2 in wastewater maybe useful in ascertaining the status of the pandemic and steering public health decision-making (Girón-Navarro et al. 2021).

Here, we report the first long-term wastewater monitoring study in the region (focused on Muharraq Island in Bahrain), detecting SARS-CoV-2 genes by RT-qPCR, between February 2020 and October 2021. Twenty-four-hour flow-weighted composite samples of wastewater from the inlet sampling point of Muharraq Sewage Treatment Plant were collected and analyzed for the presence of the virus material and the re-

sulting concentration, expressed as gene copies/mL, determined. The resulting data were compared with the number of COVID-19 cases reported by the Bahrain health authorities for the corresponding day (Ministry of Health, Bahrain 2021). It is recognized that SARS-CoV-2 gene data were only determined within wastewater arising from the Muharraq island catchment and not the entire country. Data on the incidence of COVID-19 cases for Bahrain for individual areas were not available.

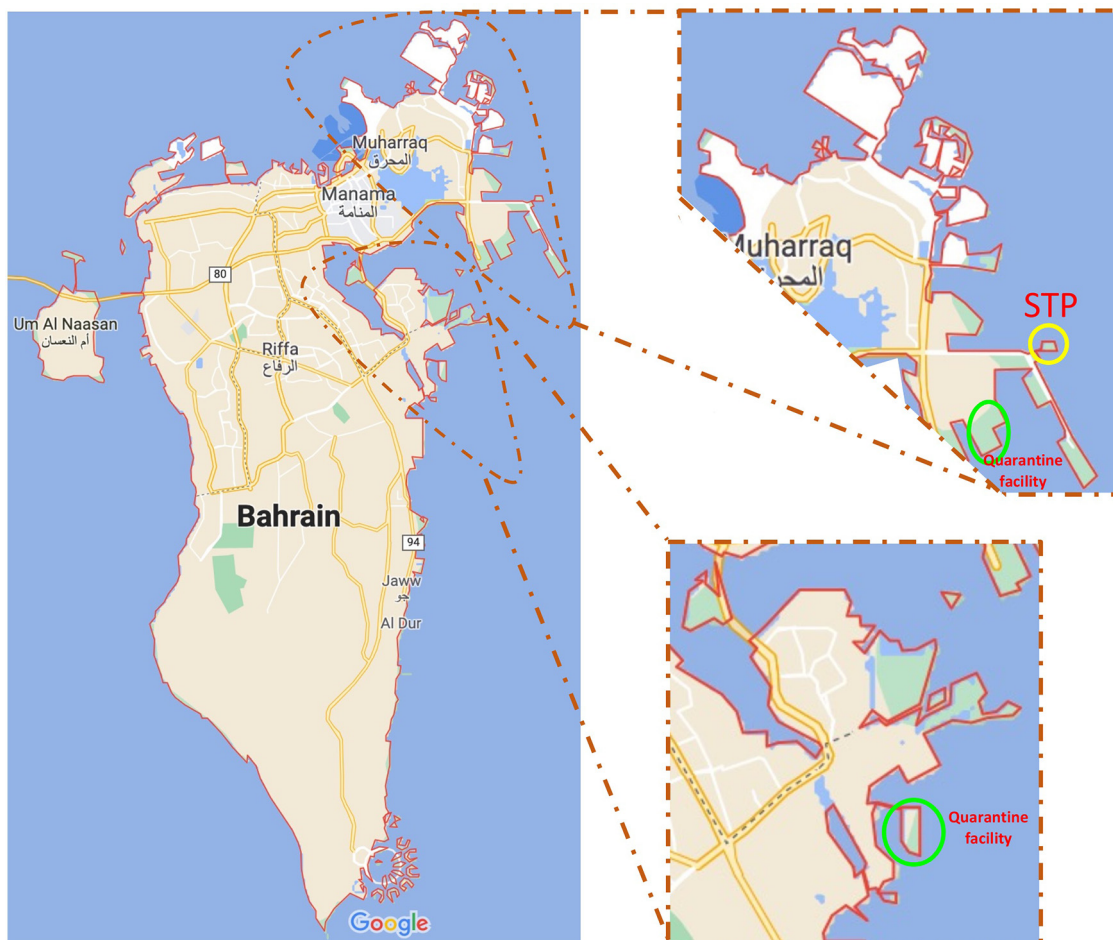
During the study, the number of reported COVID-19 cases per day varied between 0 and 3275. Peaks of positive new cases were observed on a number of occasions during the research period. A total of four epidemic peaks were registered in Bahrain during this study, during: June–July 2020, September 2020, February–April 2021, and May–June 2021, with the largest peak in May 2021. Indeed, our findings show a strong match between the quantification of SARS-CoV-2 genes (copies/mL) in wastewater from Muharraq Island and the major COVID-19 incidence peaks in Bahrain. By reporting gene detection of SARS-CoV-2 in wastewater, this study provides relevant information that can be used in further monitoring sewage-based surveillance programs in Bahrain, where perhaps other CoVs, such as MERS-CoV, should be included in the monitoring, given its high incidence in the Middle East. (Zhang, Shi, et al. 2021)

## 2. Material and methods

### 2.1. Area of study and wastewater treatment plant

The Kingdom of Bahrain is an archipelago of 33 islands. The country has a population of approximately ~1.5 million (Bahrain-Ministry-of-Information-Affairs 2021). The second largest island, Muharraq is home to a population of ~260,000 and occupies 67 km<sup>2</sup>. The majority of Muharraq is served by a discrete sewerage catchment and a single wastewater treatment works, the Muharraq Sewage Treatment Plant (STP). This plant is operated under a 29-year public private partnership (PPP) concession, between the Muharraq Sewage Company (MSC) and the Bahrain Ministries of Finance and Works. Whilst a small percentage of properties within Muharraq are serviced by septic tanks, approximately 95% of properties are served by a direct connection to the governments sewerage network (Bahrain-Ministry-of-Works 2021). This network discharges into recently constructed sewers, which, in turn, connect to a single 16.5km long, deep gravity sewer, which transports the wastewater directly to the treatment plant.

The catchment comprises primarily of low-level housing, numerous schools, a medical school, a major hospital, smaller hospitals/clinics, commercial establishments, and the Bahrain international airport. Since there is no major industry on the Muharraq island, the quality of wastewater being discharged to the sewer is typical of a domestic catchment. Due to the country's low rainfall, the flow of wastewater throughout the network does not significantly vary. Furthermore, because there are no storm overflows within the catchment area, all wastewater arising from the population served is discharged to the wastewater treatment works. Consequently, the wastewater received at the STP can be considered as a useful source of information in monitoring the incidence of SARS-CoV-2 infection in the catchment population. The Muharraq STP is a modern, conventional, sequential batch reactor activated sludge wastewater treatment plant, designed for nutrient removal with tertiary treatment, producing a final effluent compliant with WHO standards and suitable for re-use by unrestricted irrigation located in North of Bahrain as shown in Fig. 1. The plant has a design capacity of 100,000 m<sup>3</sup>/day, serving the majority of the Muharraq population. The plant comprises fine screening, grit removal, biological treatment utilizing activated sludge sequential batch reactors, incorporating nitrogen and phosphorus removal, microfiltration to 20 µm, ultra-violet disinfection (247 nm) and chlorination. The treated sewage/irrigation water is either exported for



**Fig. 1.** A map of Bahrain, annotating the location of sample collection from Muharraq island, sewage treatment plant (yellow circle) and quarantine facilities (green circles).

re-use or, when not required, discharged to sea via a dedicated long sea outfall.

## 2.2. Wastewater sample collection and storage

In this study, 65 composite sewage samples were collected at periodic intervals (at least 2 samples per month in 2020 and 3 samples per month in 2021, except for October 2021) between February 2020 and October 2021 from Muharraq STP in Bahrain. As part of the operating contract, 24-h composite flow weighted samples are collected at the inlet and outlet of the treatment works, utilizing dedicated refrigerated auto-samplers (Endress & Hauser Liquistation CSF48). The on-site laboratory is ISO17025 accredited for sampling, transportation, and the analysis of 44 chemical and microbiological parameters. Samples for the determination of SARS-CoV-2 genes were sub-sampled from the 24-h composite flow weighted samples and collected in Polytetrafluoroethylene (PTFE) 1 L bottles. These samples were either immediately frozen and stored at  $-20^{\circ}\text{C}$  or transported at  $4^{\circ}\text{C}$  to the testing laboratory for SARS-CoV-2 analysis. Six samples from the quarantine facilities in Hidd and Sitra were also collected (Fig. 1). The Sitra site specifically catered for confirmed positive cases who required medical care. Sewage tankers collect wastewater from the septic tankers located at the quarantine facilities and transported to Muharraq STP for disposal at the tanker waste discharge facility during the night, between 20:00 and 23:59. The 24-h composite sampler did not operate whilst these tankers were discharging and the sewage was pumped forward to the primary works, to avoid cross-contamination.

## 2.3. Wastewater sample processing and RNA isolation

Initial samples were analyzed according to published PEG methods (Mendema 2020). However, a direct capture method (Promega Corporation, USA) was subsequently employed and used for this study as it gave greater yields of RNA. Viral RNA extraction was carried out using Wizard® Enviro Total Nucleic Acid Kit (Promega Corporation, USA). 40 mL of wastewater was used for viral RNA isolation. The wastewater samples were treated with 0.5 mL of protease solution for 30 min at room temperature and centrifugated at 3000 g for 10 min. This step releases the nucleic acid bound to the solids into the suspension (Mondal et al. 2021). Up to 20 mL of the supernatant was mixed with 6 mL of binding buffer 1 and 0.5 mL of binding buffer 2. Isopropanol (24 mL) was mixed to the solution and passed through the PureYield™ binding column using a Vac-Man®. The PureYield™ binding column was washed twice with 5 mL and 20 mL of CWE and RWA buffers, respectively. The viral RNA was eluted using 1 mL of pre-heated ( $60^{\circ}\text{C}$ ) nuclease free water.

The viral RNA was purified using PureYield™ mini columns (Promega Corporation, USA). In total, 400  $\mu\text{L}$  of binding buffer 1, 100  $\mu\text{L}$  binding buffer 2 and 750  $\mu\text{L}$  of isopropanol was mixed with the eluted viral RNA. Solution was passed through a PureYield™ mini column by centrifugation at 10,000 rpm for 1 min. Then, the column was washed with 300  $\mu\text{L}$  and 500  $\mu\text{L}$  of CWE and RWA buffer, respectively. Finally, clean viral RNA was eluted with 40  $\mu\text{L}$  of pre-heated ( $60^{\circ}\text{C}$ ) nuclease free water. The viral RNA was stored at  $-80^{\circ}\text{C}$  for further analysis. Nuclease free water was used as a negative control in each viral RNA isolation.



## 2.4. Viral detection and quantification by RT-qPCR

The viral isolated RNA from wastewater was used to perform RT-qPCR using the detection kit for wastewater kit (Promega Corporation, USA). The nucleocapsid (N1, N2) and envelop (E) genes of SARS-CoV-2 genome were targeted by RT-qPCR (N1-Fw-GACCCCAAATCAGCGAAAT, N1-Rev-TCTGGTACTGCCAGTTGAAT-CTG, N1-P-FAM-ACCCCGCATTACGTTTGGTGGACC-/IABlack-ZEN; N2-Fw-TTACAAACATTGGCCGCAAA, N2-Rev-GCGGACATTCCGAAGAA, N2-P-FAM-ACAATTTGCCCCAGCGCTTCAG-IABlack-ZEN; E-Fw-ACAGGTACGTTAATAGTTAATAGCGT, E-Rev-ATATTGCAGCAGTACG-CACACA, E-P1-FAM-ACACTAGCCATCCTTACTGCGCTTCG-IABlack-ZEN) (Promega Corporation, USA). These primer targeted sequences were not affected by mutations in the prominent variants of concern during the time of the study (B.1.1.7 [alfa], B.1.351 [beta], B.1.617.2 [delta] and P.1 [gamma]) (Choi and Smith 2021). Sequence alignment of the primers used in this study and the variants of concern, corroborated that mutations were not located in the primer binding regions (data not shown).

To determine viral quantification, log dilutions ( $2 \times 10^1 - 2 \times 10^5$  copies/ $\mu$ L) of DNA standard were amplified across all samples to create a standard curve for each SARS-CoV-2 target genes. RT-qPCR reactions of 20  $\mu$ L were made up of 15  $\mu$ L of master mix (GOTaq® Wastewater-qPCR Master Mix (2X), GoScript® RT (50X), Prime/Probe/IAC Mix (20X), nuclease-free water) and 5  $\mu$ L of viral purified RNA. Reactions were performed on a CFX96 Real-Time Thermocycler (BIORAD) with the following cycling conditions: reverse transcription for 15 min at 45 °C, initial denaturation for 2 min at 95 °C, and 40 cycles of 15 s at 95 °C and 60 s at 62 °C. Positive and negative controls were included in all RT-qPCR runs. Concentration of viral load in wastewater was reported as RNA copies/mL.

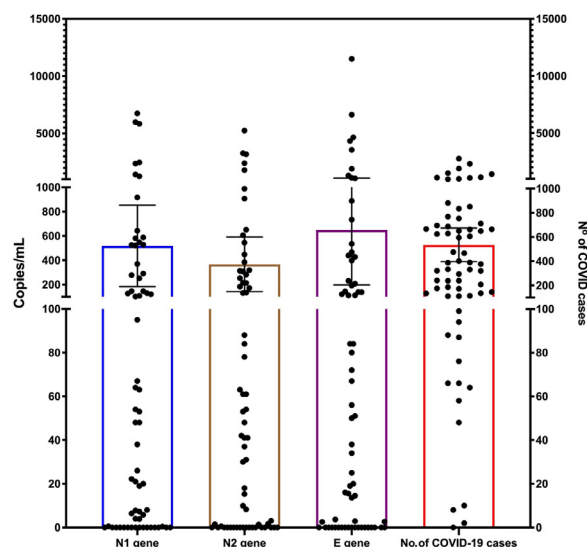
## 2.5. Statistical analysis

Descriptive statistical analysis was performed for SARS-CoV-2 gene concentration values (RNA copies/mL) and COVID-19 positive cases reported during the study. The COVID-19 daily positive cases reported by the Bahrain government were utilized as a part of this research (Ministry-of-Health-Bahrain 2021). Spearman-rank correlation was performed to assess the correlation between SARS-CoV-2 gene concentration values (copies/mL) and reported positive COVID-19 cases on the sample collection day. Level of Spearman's rank correlation coefficient ( $\rho$ ) was defined as low (<0.29), moderate (0.3–0.49), and strong (0.5–1) correlation. Mann-Whitney non-parametric test was used to compare the SARS-CoV-2 genes quantification values (copies/mL) between samples collected on Muharraq Island and the quarantine facilities. Significant  $p$  value was set up as <0.05 for all analysis. All analyses were conducted using GraphPad's Prism 9.1.1 software (La Jolla, CA).

## 3. Results

### 3.1. SARS-CoV-2 gene detection in wastewater from Muharraq Island correlated highly with reported COVID-19 case numbers in Bahrain

A total of 65 composite sewage samples from Muharraq Island were analysed for SARS-CoV-2 genes between February 2020 and October 2021 (Supplementary material 1, Table 1). Nucleoprotein N1, N2 and Envelope (E) SARS-CoV-2 mRNA levels were analysed by RT-qPCR in all samples. The presence of the three SARS-CoV-2 target genes was detected in 75%, 72% and 71% of the samples for N1, N2 and E gene, respectively, confirming the presence of SARS-CoV-2 RNA in wastewater of Bahrain's Muharraq Island. A total of 49, 47 and 46 samples were found positive and 16, 18 and 19 were negative for N1, N2 and E genes, respectively. Among the wastewater samples the maximum SARS-CoV-2 gene copies/mL values and mean values were detected for E (max value: 11,508; mean: 649.3), followed by N1 (max value: 6747; mean: 518.4)



**Fig. 2.** SARS-CoV-2 N1, N2 and E gene values (RNA Copies/mL) in wastewater samples collected from Muharraq Sewage Treatment Plant between February (2020) and October (2021), and positive COVID-19 national cases reported throughout the study per day of sample collection. Each point in graph represents the SARS-CoV-2 concentration (RNA Copies/mL) in wastewater for N1, N2 and E genes or the daily number of positive COVID-19 cases reported in Bahrain. The top of colored bar graphs represents the mean and error bars with 95% CI (Confidence Interval).

and N2 (max value: 5245; mean: 366.8) genes (Table 1 and Fig. 2). According to national COVID-19 data for Bahrain, there was only one day when zero cases were reported. The highest number of positive COVID-19 cases reported was 3274 on 29<sup>th</sup> May 2021, although this date was not sampled. The highest number of COVID-19 cases on which a sample was collected and analyzed was 2803 (May 26, 2021). 527 was the mean number of the COVID-19 cases per day for the entire study period (Table 1 and Fig. 2).

In order to evaluate the quantitative monitoring of SARS-CoV-2 of wastewater in Muharraq Island, we compared the SARS-CoV-2 gene levels (copies/mL) in wastewater with the reported positive COVID-19 cases in Bahrain over the study time. In Fig. 3, four epidemic peaks were registered in Bahrain during this study, during: 1) June–July 2020, 2) September 2020, 3) February–April 2021 and 4) May–June 2021 (Fig. 3). We found a strong match between the quantification of SARS-CoV-2 genes (copies/mL) in wastewater from Muharraq Island and the COVID-19 incidence peaks in Bahrain, especially with the highest peak that occurred in May–June in 2021 (Fig. 3). Indeed, the first peak detected for SARS-CoV-2 gene quantification went up in June 2020, which partially overlapped with the first COVID-19 peak showing that the SARS-CoV-2 was readily detectable in wastewater as soon as cases began to emerge. In September/October 2020 the second COVID-19 peak was observed with a small delay peak of SARS-CoV-2 genes at the third sampling point of September, which also happened in February 2021 for N1 and N2 genes that follow the increase of COVID-19 cases in January 2021. The subsequent peaks in 2021 (from March) were intermittent and did not follow the shape of COVID-19 peaks, only two peaks of SARS-CoV-2 N2 and E genes preceded and follow (May and June 2021) the top of the COVID-19 peak in May 2021. SARS-CoV-2 N1 gene preceded overlap and follow the COVID-19 peak in May 2021. Interestingly, SARS-CoV-2 genes tested in this study showed final peaks even when COVID-19 cases decrease from July to October 2021.

To further evaluate the correlation between SARS-CoV-2 gene quantification in wastewater and the COVID-19 cases in Bahrain, Spearman-rank correlation was performed. We found significant positive correlation between all three SARS-CoV-2 genes (copies/mL) and COVID-19

**Table 1**

Descriptive statistics of SARS-CoV-2 gene (N1, N2 and E) levels in wastewater samples collected from Muharraq Island between February (2020) and October (2021) and reported COVID-19 cases in the Kingdom of Bahrain.

	Muharraq Island, Bahrain			Bahrain
	N1 gene	N2 gene	E gene	COVID-19 Cases
Number of samples collected / days with COVID-19 cases	65	65	65	65
Number of positive sample / days with COVID-19 cases	49	47	46	64
Number of negative samples / days without COVID-19 cases	16	18	19	1
Minimum copies/mL values / COVID-19 cases	0	0	0	0
Maximum copies/mL values / COVID-19 cases	6747	5245	11508	2803
Mean - copies/mL values / COVID-19 cases	518.4	366.8	649.3	527
Std. Deviation	1351	906.6	1824	556.6
Std. Error of Mean	167.6	112.4	226.2	69.03
Lower 95% CI of mean	183.6	142.1	197.3	389
Upper 95% CI of mean	853.2	591.4	1101	664.9
Coefficient of variation	2.607	2.472	2.809	1.056

positive cases, with the E ( $\rho$ : 0.38 and  $p$  value 0.002), N1 ( $\rho$ : 0.46 and  $p$  value  $9.5e-5$ ) and N2 ( $\rho$ : 0.46 and  $p$  value  $1.4e-4$ ) gene showing a moderate correlation (Fig. 4). Overall, our results showed that measurement of SARS-CoV-2 gene concentration in wastewater from Muharraq Island correlated with COVID-19 cases in the entire Kingdom of Bahrain, highlighting it as an accurate method to determinate the level of COVID-19 in a specific population. This method may thus identify a useful scientific approach for monitoring of changes in COVID-19 prevalence in a population both during and post-pandemic.

### 3.2. Wastewater from quarantine facilities showed high SARS-CoV-2 gene concentrations, compared to wastewater from Muharraq Island

In its response to the pandemic, Bahrain set up specialist medical facilities to specifically care for and quarantine people suffering with COVID-19 who required medical intervention. We analyzed 6 composite sewage samples from quarantine facilities in Bahrain. Wastewater from the two facilities was transported to the treatment plant by dedicated sewage tankers and not via the sewerage network. (Supplementary material 1). Samples were collected from the tankers as they discharged at the dedicated tanker off-loading station on site following a specific discharge regime agreed with Bahrain health authorities. Discharge was at an allocated time of 20:00–23:59 h each day, the automatic sampler on the main treatment plant was paused to prevent any quarantine wastewater contaminating the 24-h composite sample. Samples from the tankers were collected in PTFE 1L containers and subject the same transportation, storage, and analytical procedures as the main samples. As in the samples from Muharraq island, N1, N2 and E SARS-CoV-2 genes were measured in wastewater sample for the Sitra and Hidd quarantine facilities by RT-qPCR. We did not only identified the presence of the three SARS-CoV-2 genes in all the quarantine wastewater facility samples analyzed, but found that the wastewater from these tankers had much higher concentrations of the SARS-CoV-2 genes N1 (max value: 10,700; mean: 5044), N2 (max value: 12,672; mean: 4833) and E (max value: 19,105; mean: 8663), compared with the samples collected in Muharraq STP (N1, max value: 6747; mean: 518.4. N2, max value: 5245; mean: 366.8 and E, max value: 11,508; mean: 649.3.) (Table 2).

Although, the number of samples collected from the quarantine facilities were limited to 6 samples only, we believe that this is the first-time wastewater from a dedicated medical quarantine facility, specifically dedicated to the treatment of COVID-19 patients has been sampled, analyzed, and reported, highlighting the usefulness of the RT-qPCR method to detect SARS-CoV-2 for monitoring infection of the occupants in a specific location or building/s.

We hypothesized that SARS-CoV-2 gene levels would be highest in wastewater collected from quarantine facilities, to investigate this we analyzed and compared the SARS-CoV-2 N1, N2 and E genes levels in wastewater from the Hidd and Sitra quarantine facilities and Muharraq STP. Given that only people who tested positive for COVID-19 were

residing in quarantine facilities, we were not surprised to find that the level of the three SARS-CoV-2 genes analyzed were significantly higher in the wastewater from the Hidd and Sitra quarantine facilities (Fig. 5).

## 4. Discussion

This study analyzed SARS-CoV-2 gene patterns in 24-h composite wastewater samples from Muharraq STP in Bahrain, between February 2020 and October 2021. Peaks of SARS-CoV-2 gene detection levels in wastewater from Muharraq STP correlated closely with the COVID-19 peaks reported in the whole country. Our data agrees with other studies that reported a positive correlation between SARS-CoV-2 gene levels in wastewater and confirmed COVID-19 cases (Agrawal et al. 2021, Mondal, Feirer, Brockman, Preston, Teter, Ma, Goueli, Moorji, Saul and Cali 2021, Robotto et al. 2021). However, this is the first study showing SARS-CoV-2 surveillance from wastewater in the Kingdom Bahrain. Additionally, we compared the SARS-CoV-2 gene levels from the Muharraq STP and Hidd/Sitra quarantine facilities with national COVID-19 cases published daily by the Bahrain government. Even though the STP wastewater samples were from Muharraq Island only, we found that the concentration of SARS-CoV-2 genes closely correlated with the number of confirmed COVID-19 cases in the whole country. It has been reported that the number of confirmed COVID-19 cases is likely to be underestimated (Hata, Hara-Yamamura, Meuchi, Imai and Honda 2021, Robotto, Lembo, Quaglino, Brizio, Polato, Civra, Cusato and Di Perri 2021), which may have negatively impacted our correlation analysis. Indeed, asymptomatic, or pre-symptomatic individuals could also, unknowingly, shed SARS-CoV-2 into the wastewater system and not be reported as COVID-19 positive cases. In fact, in 2020, Chen, Zhu et al., reported that the duration of shedding in the asymptomatic group is around 6 days (Chen, Zhu, et al. 2020). Additionally, some infected individuals may not have been tested, further distorting an accurate view of daily case numbers (Oran and Topol 2020). However, according to our study findings, wastewater monitoring in Muharraq Island was effective and accurate for detecting and quantifying SARS-CoV-2 and monitoring the increasing and decreasing waves of COVID-19 cases in Bahrain. Moreover, given that wastewater samples contain faeces from symptomatic and asymptomatic, pre-symptomatic and misdiagnosed individuals, this study showed how SARS-CoV-2 wastewater monitoring could be considered by Health Authorities as a complementary approach to COVID-19 surveillance and testing strategies, overcoming the issues related to underreporting COVID-19 cases (Chavarria-Miró et al. 2021). Also, systematic monitoring of SARS-CoV-2 in wastewater could be a useful tool to track COVID-19 in Bahrain when the governmental testing by qRT-PCR is no longer used post-pandemic.

Interestingly, we observed final SARS-CoV-2 gene peaks when COVID-19 cases decrease at the end of the study. According to Wu et al (2020), SARS-CoV-2 genes were detected in 55% of infected individuals for an average of 11.2 days after respiratory tract samples

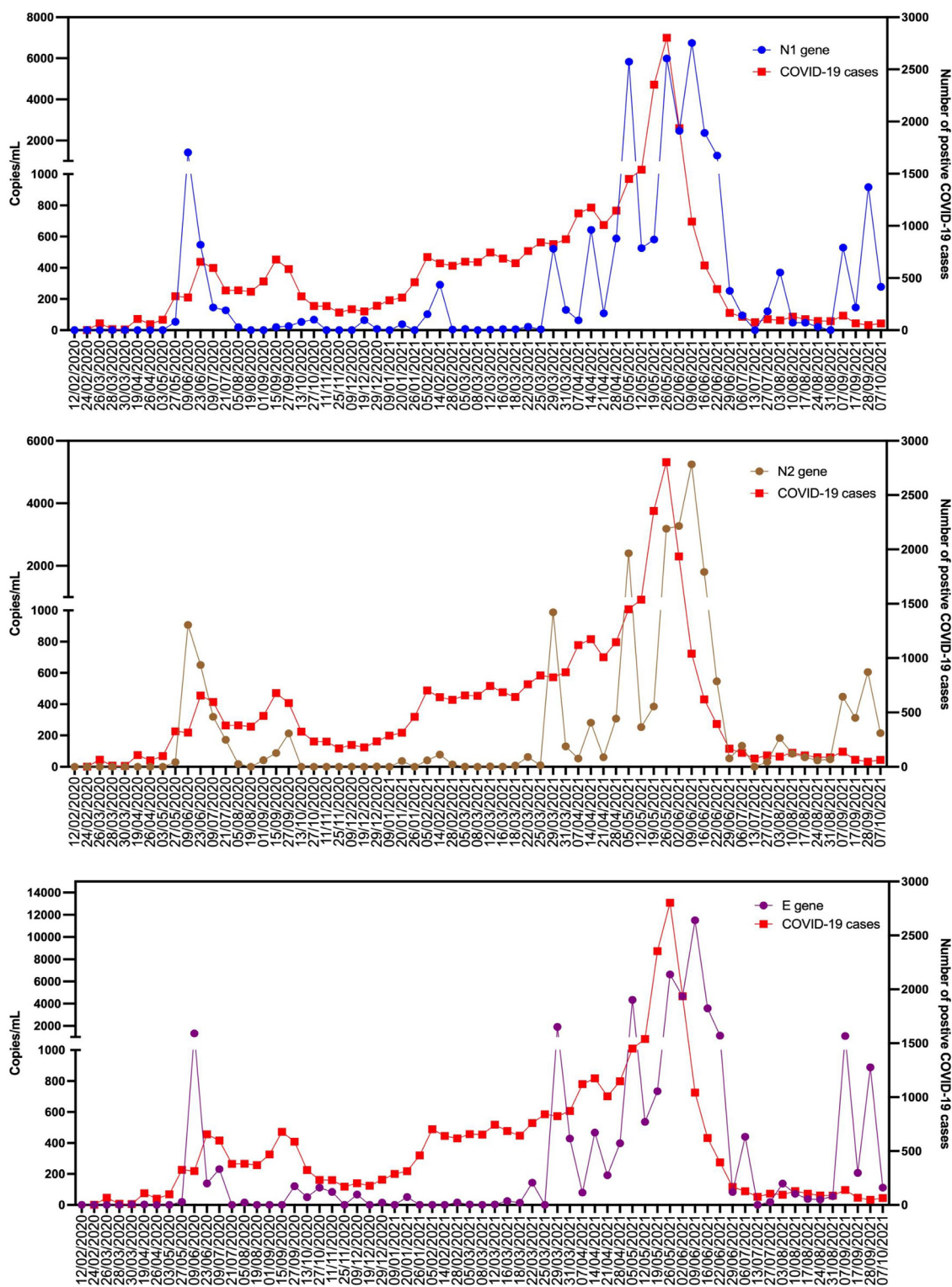


Fig. 3. Dynamics of SARS-CoV-2 genes expression (copies/mL) in wastewater from Muharraq Island and recorded positive COVID-19 cases in the Kingdom of Bahrain, between February 2020 and October 2021. Line graph showing SARS-CoV-2 genes (N1: blue, N2, brown and E: purple) expression in wastewater from Muharraq ST and positive COVID-19 cases in Bahrain (in red).

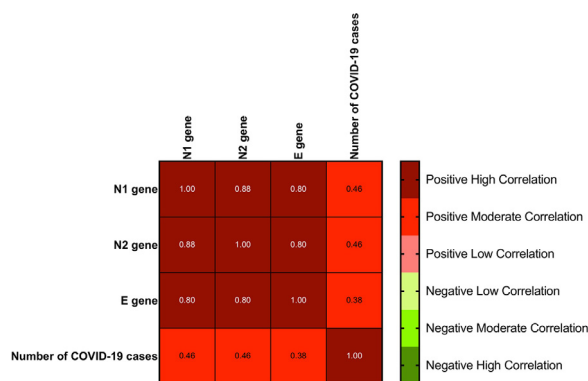
were negative, which may indicate that the virus remains replicating in the gastrointestinal system (Wu et al. 2020). Moreover, the authors hypothesized about a prolonged viral shedding in faeces up to five weeks after viral clearance in the respiratory tract, which was also supported by other studies (Santos et al. 2020, Walsh et al. 2020) which also mentioned the SARS-CoV-2 gene detection from asymptomatic cases

(Almadhi et al. 2021, Schmitz et al. 2021). Additionally, it is also possible that increases of SARS-CoV-2 in wastewater could be associated to wild animal discharges into the wastewater system. Recently, it has been shown that SARS-CoV-2 variants can infect mice, meaning their discharges should be taken into consideration when analyzing levels of the viral genes in wastewater (Pan et al. 2021, Stone et al. 2021). How-



**Table 2**  
Descriptive statistics of SARS-CoV-2 N1, N2 and E gene levels (copies/ml) in wastewater samples collected from the quarantine facilities in Hidd and Sitra, Bahrain.

	Quarantine facilities		
	N1 gene	N2 gene	E gene
Number of samples collected / days with COVID-19 cases	6	6	6
Number of positive sample / days with COVID-19 cases	6	6	6
Number of negative samples / days without COVID-19 cases	0	0	0
Minimum copies/mL values / COVID-19 cases	90	27	37
Maximum copies/mL values / COVID-19 cases	10700	12672	19105
Mean - copies/mL values / COVID-19 cases	5044	4833	8663
Std. Deviation	3388	4603	6154
Std. Error of Mean	1383	1879	2513
Lower 95% CI of mean	1489	3.086	2204
Upper 95% CI of mean	8599	9664	15122
Coefficient of variation	67.16%	95.23%	71.04%



**Fig. 4.** Spearman-rank correlation between SARS-CoV-2 gene concentration (copies/mL) in wastewater from Muharraq Island and COVID-19 cases in Bahrain, during the study period of February 2020 and October 2021. Level of Spearman's rank correlation coefficient ( $\rho$ ) was defined as low (<0.29), moderate (0.3–0.49), and strong (0.5–1) correlation. Significant  $p$  value was set at <0.05.

ever, it is worth noting that whilst this is possible, wild animals have not been observed in the Muharraq Wastewater Services Company's sewers since they were brought into service in 2014. Furthermore, given the very high concentrations of hydrogen sulphide observed in sewers in the Middle East (Al-Shammiri 2004), as a function of high temperatures, the presence of wild animals in significant numbers is unlikely; thereby reducing the potential impact on SARS-CoV-2 concentrations determined.

The Bahrain government established quarantine facilities for COVID-19 positive people that required medical intervention. We analyzed wastewater samples from the quarantine facilities in the Hidd/Sitra districts and as expected, the concentration of SARS-CoV-2 genes was higher from wastewater collected in the quarantine facilities than from STP. Up-to-date, most of the wastewater studies have focused on monitoring water treatment plants (Ahmed et al. 2020, Peccia et al. 2020, Wannigama et al. 2021), and wastewater from hospitals (Acosta et al. 2021, Gonçalves et al. 2021, Karami et al. 2021, Wang, Feng, et al. 2020). However, our work is the first to report SARS-CoV-2 genes in wastewater from quarantine facilities, that only house positive COVID-19 patients. Overall, our results from quarantine facilities demonstrated that the measurement of SARS-CoV-2 provides a reliable estimation of COVID-19 prevalence in a specific population or geographic location, as was suggested before by Galani et al 2021, who demonstrated that wastewater surveillance data can predict hospitalizations and intensive care unit admission in hospitals (Galani et al. 2021).

Although governments and scientists are now tracking the spread of SARS-CoV-2 in sewage and establishing guidelines for

surveillance programs (Agrawal, Orschler and Lackner 2021, COVID19wbec, 2021, European-Commission 2021, Kreier 2021, Sangsanont et al. 2021), it is still necessary to expand in the long term the monitoring of SARS-CoV-2 and other viruses such as MERS-CoV and newly emerging viruses. Currently, SARS-CoV-2 monitoring in wastewater is helping to track down cases, predict re-emergences, identify where to target testing, estimate numbers of infected people in specific places, and identify circulation of the new viral variants (Kreier 2021). In Barcelona (Spain), SARS-CoV-2 wastewater monitoring anticipated the original and second COVID-19 pandemic waves and levels in wastewater showed the efficacy of lockdown measures (Chavarría-Miró, Anfruns-Estrada, Martínez-Velázquez, Vázquez-Portero, Guix, Paraira, Galofré, Sánchez, Pintó and Bosch 2021). However, permanent surveillance programs were not established before the pandemic even though sewage monitoring has been used to detect polio, Hepatitis A, Norovirus outbreaks (Hellmér et al. 2014, Ivanova et al. 2019) and even illicit drug use in urban wastewater (Sulej-Suchomska et al. 2020).

In summary, SARS-CoV-2 and other virus monitoring should be included as routine testing in wastewater, given that in the past 60 years the world has seen new emergence of three novel Coronaviruses, Ebola, Avian Influenza, Zika and Dengue virus, among others (Bogler et al. 2020). Additionally, new viruses and new emergent viral strains also should be included in the routine pathogen monitoring of wastewater, as was the case for the new SARS-CoV-2 omicron variant, which was detected in wastewater in Houston and California (NPR. 2021). This was further supported by the global emergence of the Omicron variant (WHO, 2021b). It is therefore worth considering expanding work such as that reported here to further analyze the extracted RNA material for the presence of new genetic variants. As the Muharraq catchment includes Bahrain's International Airport, it is possible that new variants could be detected early from wastewater discharged at this port of entry, prior to infections being subsequently detected within the community and action taken.

## 5. Conclusions

Wastewater samples collected from Muharraq STP demonstrated that SARS-CoV-2 RNA concentration is correlated with the number of COVID-19 cases recorded in the Kingdom of Bahrain. Also, we corroborate the high SARS-CoV-2 RNA concentration levels in quarantine facilities that house COVID-19 patients. Together, our results suggest that wastewater surveillance could be a complementary approach to estimate and track the SARS-CoV-2 RNA prevalence in local communities and the monitoring should be inclusive of detection of other viruses. Even though vaccination programs are successful, SARS-CoV-2 monitoring in wastewater is indicative if the virus is still in circulation among the population.

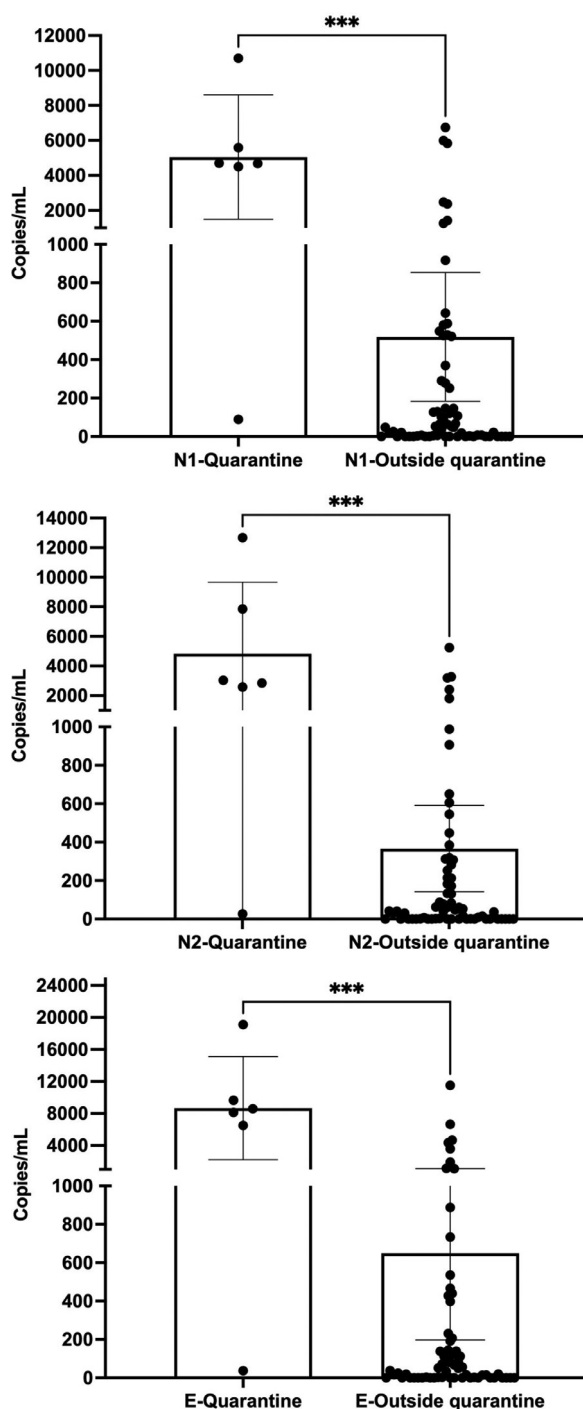


Fig. 5. Differences of SARS-CoV-2 quantitation values between samples collected in the Hidd and Sitra quarantine facilities and Muharraq Island in Bahrain (outside quarantine). Each dot represents SARS-CoV-2 gene (N1, N2 and E) levels (copies/mL) in wastewater. The top of bar graphs represents the mean and error bars show 95% CI (Confidence Interval). Data was subjected to a Mann Whitney test, and the asterisks indicate \*\*\*  $p$  value <0.001.

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**Declaration of Competing Interest**

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Peter Naylor reports financial support was provided by Muharraq Wastewater Services Co. Peter Naylor reports a relationship with Muharraq Wastewater Services Co. that includes: employment.

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**Supplementary materials**

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.hazadv.2022.100082.

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