Short-term Project on COVID-19 Pandemic Monitoring

unicef

Final Project Report

Weekly Surveillance of Wastewater for SARS-CoV-2 Gene Detection in Ahmedabad for Pandemic Curve Monitoring

RES/UNICEF/EH/P0237/2021/0013

Submitted by

PROF. MANISH KUMAR (PI)

Discipline of Earth Sciences, Indian Institute of Technology, Gandhinagar, Gujarat-382355

DR. MADHVI JOSHI (CO-PI)

Gujarat Biotechnology Research Centre, Gandhinagar

WEEKLY SURVEILLANCE OF WASTEWATER FOR SARS-COV-2 GENE DETECTION IN AHMEDABAD FOR PANDEMIC CURVE MONITORING

RES/UNICEF/EH/P0237/2021/0013

Submitted to:

UNITED NATIONS CHILDREN'S FUND
INDIA

Principal Investigator	:	**Dr. Manish Kumar**
	:	
Signed		
	:	
Designation		**Assistant Professor** Indian Institute of Technology Gandhinagar manish.kumar@iitgn.ac.in
Year	:	**June– 2021**

Acknowledgement

We are grateful to UNICEF India for giving attention (especially Dr. Shyamnarayan Dave and Dr. Laxmi Bhawani from Gujarat office) to our study on first proof of the capability of wastewater surveillance for COVID-19 in India and providing us a platform to carry out further research in the form of the project grant. Furthermore, the authors extend their sincere thanks to Sri. A. V. Shah from Gujarat Pollution Control Board (GPCB), for the sampling design and help during the entire study. We are grateful to the Director, Gujarat Biotechnology Research Center (GBRC), Gujarat, for his support and encouragement. The PIs highly appreciate the consistent support from the staff and the Dean, Research and Development, Indian Institute of Technology Gandhinagar. In addition to the UNICEF, the principal funder, we also like to acknowledge the associated funds from the Asia Pacific Network, Kiran C Patel Centre for Sustainable Development, and UKIERI, India, that was used for reaching this point in our WBE approach for pandemic management. In the last, we extend our sincerest appreciation to all the staff members involved directly or indirectly for accomplishing this task.

Dr. Manish Kumar

Dr. Maddhvi Joshi

(Principal Investigators**)**

Table of Contents

Project Team

Name	Degree	Position at IIT-Gn
Dr. Manish Kumar	PhD, Tokyo University, Japan	Assistant Professor
Dr. Madhvi Joshi	PhD, Saurashtra University, Gujarat	Scientist-D
Dr. Vaibhav Srivastav	PhD, Banaras Hindu University, Uttar Pradesh	Research Associate
Dr. Arbind Kumar Patel	PhD, Tezpur University, Assam	Research Associate
Ms. Shilangi Gupta	MSc, Ahmedabad University, Gujarat	Junior Research Fellow
Mr. Rajesh Rathod	ITI, Gujarat Council of Vocational Training, Gujarat	Lab–Helper

List of Figures

List of Tables

List of Annexures

Executive Summary

unicef
United Nations Children's Fund

1. Executive Summary

1.1 Background

The contagious severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for the coronavirus pandemic, has infected 11 million people in India alone by February 22nd, 2021 (WHO, 2020). A large number of asymptomatic patients exerted a never seen before challenges over the actual estimation of disease spread based on clinical surveillance (Rimoldi et al., 2020; Medema et al., 2020). Earlier studies suggested that 18-45% of patients do not have signs of infection with COVID-19 but are capable of spreading the disease and pose an adverse impact on the actual containment of the disease (Lavezzo et al., 2020; Yang et al., 2020; Mizumoto et al., 2020; Nishiura et al., 2020). As up to 67% of infected people showed SARS-CoV-2 presence in feces (Chan et al., 2020; Cheung et al., 2020; Parasa et al., 2020; Wong et al., 2020), alternative approaches such as wastewater-based epidemiology (WBE) surveillance has gained loads of recognition as a viable option that can provide early warning of the upcoming prevalence of the disease within a community (Hata et al., 2021; Kumar et al., 2021a, b,). One of the advantages of WBE is that wastewater contains feces from a huge number of people. Therefore, it may require a far fewer number samples and less labor than clinical testing to know the presence of infected persons in the area. Also, to evaluate WBE's potential as an early prediction tool for COVID-19 pandemic, it is essential to explore the correlation between the SARS-CoV-2 genetic load in wastewater and the number of cases at the district level in each country.

Overall, wastewater-based epidemiology (WBE) is a promising approach to understand the status of the disease outbreak in a certain catchment by monitoring the viral load in the wastewater, as it contains the excretion from both symptomatic and asymptomatic individuals. WBE had been an effective tool during past outbreak of other enteric viruses, such as poliovirus, hepatitis A and norovirus, it can be used as an early warning tool for the disease outbreak in a community and used to inform the efficacy of the current public health interventions. WBE data can help to estimate actual infected population due to the virus, as it covers asymptomatic and pre-symptomatic patients too, which may be underestimated by clinical surveillance.

The infectivity of SARS-CoV-2 RNA in wastewater, owing to viral shedding of infected symptomatic/asymptomatic patients, and their transmission remains under debate (Buitrago-Garcia et al., 2020). Potential community transmission associated with untreated/ treated wastewater, e.g., reuse of wastewater (inbuilt environments), aerosols of wastewater potentially exposing WWTP workers, sludge transfer activities, irrigation and recreational activities in wastewater-impacted waters, is still being debated (Barceló et al., 2020a, b; Lavezzo et al., 2020).

In the initial pandemic phase, the effluents from wastewater treatment facilities were reported mostly free from

Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2) RNA, and thus conventional wastewater treatments were generally considered effective. However, there is a lack of first-hand data on i) comparative efficacy of various treatment processes for SARS-CoV- 2 RNA removal; and ii) temporal variations in the removal efficacy of a given treatment process in the backdrop of active COVID-19 cases.

1.2 Scope and Objectives

The study intends to conduct weekly surveillance of SARS-CoV-2 to gather evidence about the COVID-19 situation at the community level. The surveillance study is to be carried out in the Ahmedabad city of Gujarat state, India and the area covered is 464 Km2, 53m elevation above the MSL, and have population of 55.7 lakhs (2011). It will cover the urban population in corporation area and urban poor in slum settlements. As the individual test of Corona patient required significant cost and time, alternatively wastewater epidemiology approach at community level for surveillance of COVID-19 would be more inclusive. In view the scale of the pandemic situation, the proposed study would develop methodology capable of predicting the worst situation rise due to COVID-19.

Following the proven concept and capabilities of detecting the RNA of Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2) in wastewater, it is imperative for the adoption of WBE on the policy level, which has been for some reason still delayed in the major parts of the globe. Under the light of above discussion, the objectives of the present study aimed at: **i)** To detect and quantify variation in the genetic material of SARS-CoV-2 in the various wastewaters of Ahmedabad to understand pandemic situation; **ii)** To have a weekly resolution of the data for three months in genetic material loadings in the various wastewater treatment plants of Ahmedabad; **iii)** To establish applicability of WBE for COVID-19 surveillance as a potential tool for public health monitoring at the community level; **iv)** To understand the pathogen diversity (viral and bacterial) from wastewater in order to establish early sign of WBE as prediction tool.

However, keeping in mind the Potential community transmission associated with untreated/ treated wastewater, e.g., reuse of wastewater (inbuilt environments), aerosols of wastewater potentially exposing WWTP workers, sludge transfer activities, irrigation and recreational activities in wastewater-impacted waters, *"a comparison and evaluation of the removal efficacy of SARS-CoV-2 RNA by conventional activated sludge (CAS) and root zone treatment (RZT) processes have been made through two months-long influent and effluent monitoring"*

Likewise, in view of the new coming/ reported variants of SARS-CoV-2, an attempt has been made for ***"Wastewater based genomic surveillance of SARS-CoV-2 (whole genome sequencing)"***

On top of that, the exponential rise in the consumption rate of certain antimicrobials during the COVID-19 pandemic in an effort to minimise the risk

of severe infections and mortality. Also, due to lack of regulations on the prescription and non-prescription use of antimicrobials and its consumption rate in India, a third additional objective aimed "***to assess the effect of imprudent consumption of ABS during the COVID-19 pandemic, comparison of the 2020 prevalence of antidrug resistance (ADR) of Escherichia coli (E. coli) with a similar survey carried out in 2018 in Ahmedabad, India using SARS-CoV-2 gene detection as a marker of ABS usage."***

1.3 Methodology

Wastewater samples were collected from nine different locations, including eight wastewater pumping stations and a single sewage treatment plant (**Fig. 1**). The samples were collected weekly for twenty-five weeks from each location during September 2020 to February 2021. A total of 224 samples were analyzed in the present study to detect SARS-CoV-2 RNA from nine different sites, comprising 199 samples from eight wastewater pumping stations and 25 samples from a single sewage treatment plant in Ahmedabad, India. All the samples were collected by grab hand sampling using 250 ml sterile bottles. Simultaneously, blanks in the same type of bottle were examined to know any contamination during the transport. The samples were kept cool in an ice-box until further process. The analysis was performed on the same day after bringing the samples to the laboratory.

Wastewater samples were centrifuged, filtered, and concentrated using polyethylene glycol (PEG) and NaCl. RNA isolation from the pellet with the concentrated virus was performed using Nucleo-Spin® RNA Virus isolation kit (Macherey-Nagel GmbH & Co. KG, Germany). The samples were spiked with MS2 phage as an internal control prior to the RNA extraction provided by TaqPathTM Covid-19 RT-PCR Kit.

Applied Biosystems 7500 Fast Dx Real-Time RT-PCR Instrument (version 2.19 software) was used for SARS-CoV-2 gene detection. In the process, the probes anneal to three specific target sequences located between three unique forward and reverse primers for the N, ORF 1ab, and S genes. The methodology is shown with Illustrative flowchart in **Fig.2**.

1.4 Key Findings and Results

1.4.1 WBE study in Ahmedabad

We detected and quantified variation in SARS-CoV-2 RNA from wastewater samples for six months (September 2020 and February 2021) to understand the pandemic situation in Ahmedabad, Gujarat, India. Among the 224 samples analyzed in the study, 212 (94.6%) were found positive, comprising at least two positive RT-PCR results targeting SARS-CoV-2 ORF1ab, S gene, and N gene assays. In addition to this, 213/224 (95.1%), 202/224 (90.2%), and 209/224 (93.3%) samples showed positive RT-PCR results for N, ORF 1b and S genes, respectively. The average Ct values for N, ORF 1ab, and S genes were 32.11, 32.74, and 33.14, respectively. The average Ct values of

internal control (MS2 bacteriophage) was 27.50, and no SARS-CoV-2 genes were detected in the negative control samples.

Monthly variation depicted a significant decline of 89.7, 63.7, and 90.1% in N, ORF-1ab, and S gene concentration (copies/L), respectively in October compared to September 2020, followed by a sharp increment in November 2020 i.e. ~25 folds in N gene, ~22 folds in ORF 1ab and ~26 folds in S gene. The PCR products for all three genes were maximum in wastewater samples of November. The descending order of monthly variation in ORF 1ab gene concentration in wastewater samples was: November> September> December> January> October> February. Likewise, decreasing order of N and S genes in wastewater samples followed a similar pattern and found in order of November> September> December> January> February> October. The genome concentration of SARS-CoV-2 RNA was maximum in the month of November (~10729 copies/ L), followed by September (~3047 copies/ L), January (1810 copies/ L), December (1802 copies/ L), February (492 copies/ L) and October (453 copies/L). The rise in genome concentration in wastewater samples collected in November was in line with a ~ 1.5-fold rise in the number of confirmed cases during the 3rd September 2020 and 26th November 2020. Trends of monthly variation in SARS-CoV-2 RNA concentration in the wastewater samples may be ascribed to a decline of 19.3% in active cases in October 2020 and a rise of 1.82% in November 2020 compared to the preceding months. A little percentage increase of 1.82% in the active cases equalled 59 cases, while the total number of active cases was 3293 in the month of November 2020. However, at the same time, a prominent rise of 17.3% (i.e., 7386 new cases) noticed in November 2020. Also, a monthly decrease of 3.73% in recovered cases was noticed in November compared to October 2020. The monthly recovery rate of patients was 16.61, 19.31, and 15.58% in September, October, and November 2020, respectively. Apart from that, people's casual and reluctant attitude during the festive season in India (mid-October to mid-Nov) might be the reason for the piercing rise in COVID-19 cases.

This finding was further supported by the relation between the percentage change in effective gene concentration level and confirmed cases, which followed a similar trend on the temporal scale with a ~1 to 2 weeks' time distance. The percentage change in the gene concentration was observed in the lead of 1-2 weeks with respect to the provisional figures of confirmed cases. SWEEP data-based city zonation was matched with the heat map of the overall COVID-19 infected population in Ahmedabad city, and month-wise effective RNA concentration variations are shown on the map. The results expound on the potential of WBE surveillance of COVID-19 as a city zonation tool that can be meaningfully interpreted, predicted, and propagated for community preparedness through advanced identification of COVID-19 hotspots within a given city.

1.4.2 Efficacy of WWTPs to remove SARS-CoV-2 RNA

This work provides a comparative account of the removal efficacy of conventional activated sludge (CAS) and root zone

treatments (RZT) based on weekly wastewater surveillance data, consisting of forty-four samples, during a two-month period. The average genome concentration was higher in the inlets of CAS-based wastewater treatment plant in the Sargasan ward (1.25×10^3 copies/ L), than that of RZT plant (7.07×10^2 copies/ L) in an academic institution campus of Gandhinagar, Gujarat, India. ORF 1ab and S genes appeared to be more sensitive to treatment i.e., significantly reduced ($p < 0.05$) than N genes ($p > 0.05$). CAS treatment exhibited better RNA removal efficacy ($p = 0.014$) than RZT ($p = 0.032$). Multivariate analyses suggested that the effective genome concentration should be calculated based on the presence/absence of multiple genes. The present study stresses that treated effluents are not always free from SARS-CoV-2 RNA, and the removal efficacy of a given WWTPs is prone to exhibit temporal variability owing to variations in active COVID-19 cases in the vicinity and genetic material accumulation over the time.

Disinfection seems less effective than the adsorption and coagulation processes for SARS-CoV-2 removal. Results stress the need for further research on mechanistic insight on SARS-CoV-2 removal through various treatment processes taking solid–liquid partitioning into account.

1.4.3 Metagenomic study of 16s RNA in wastewater samples

The results suggest no clear-cut pattern among the bacterial population and association with SARS-CoV-2 genetic load in wastewater samples. Some of the bacterial population significantly changed on monthly temporal scale but no clear-cut concluding pattern was seen. There was significant difference at the bacterial taxonomic level was observed between the untreated and treated wastewater samples. We did not have explicit raw data of the wastewater quality parameters on the sampling date, therefore cannot draw a concrete and convincing finding. The results were not promising but they indicated a possible correlation ship among the SARS-CoV-2 gene concentration and bacterial population and dynamics. Therefore, further investigation is required considering different influencing factors such as sampling timing, sewage flow rate, treatment process, and wastewater physico-chemical parameters.

1.4.4 Wastewater based genomic surveillance of the SARS-CoV-2

We have first reported, detected and identified the designated *Variant of Concern* (VoC: VOC-21APR-02; B.1.617.2) from wastewater samples using genomic surveillance approach. The key spike protein mutations that were identified in the SARS-CoV-2 genome assembly as compared to the reference Wuhan/Hu-1/2019 (EPI_ISL_402125) variant that were identified include C21618G/Thr19Arg (T19R), T22917G/Leu452Arg (L452R), C22995A/Thr478Lys (T478K), A23403G/Asp614Gly (D614G), and C23604G/Pro681Arg (P681R) from the samples collected in the month of February, 2021. The observation of the deletion at 22029 (6 bp), 28248 (6 bp) and

28271 (1 bp) were also observed and seen in the B.1.617.2 lineage. These findings point towards probably an early circulating B.1.617.2 lineage in Ahmedabad, Gujarat while clinical samples sequenced in the month of March, 2021 were detected with the cases of B.1.617.2 variant. The variants of concern (VOCs) can be more transmissible resulting in probably higher disease severity outcomes and are also known for reduced sensitivity to antibody neutralization.

1.4.5 Prevalence of antidrug resistance (ADR) in ambient water samples

To assess the effect of imprudent consumption of ABS during the COVID-19 pandemic, we compare the 2020 prevalence of antidrug resistance (ADR) of *Escherichia coli* (*E. coli*) with a similar survey carried out in 2018 in Ahmedabad, India using SARS-CoV-2 gene detection as a marker of ABS usage. We found a significant ADR increase in 2020 compared to 2018 in ambient water bodies, harbouring a higher incidence of ADR *E.coli* towards non-fluoroquinolone drugs. Effective SARS-CoV-2 genome copies were found to be associated with the ADR prevalence. The prevalence of ADR depends on the efficiency of WWTPs (Wastewater Treatment Plants) and the catchment area in its vicinity. In the year 2018 study, prevalence of ADR was discretely distributed, and the maximum ADR prevalence recorded was ~ 60%; against the current homogenous ADR increase, and up to 85% of maximum ADR among the incubated *E.coli* isolated from the river (Sabarmati) and lake (Chandola and Kankaria) samples. Furthermore, wastewater treatment plants showed less increase in comparison to the ambient waters, which eventually imply that although SARS-CoV-2 genes and faecal pollution may be diluted in the ambient waters, as indicated by low Ct-value and *E.coli* count, the danger of related aftermath like ADR increase cannot be nullified. Also, Non-fluoroquinolone drugs exhibited overall more resistance than quinolone drugs. Overall, this is probably the first-ever study that traces the COVID-19 pandemic imprints on the prevalence of antidrug resistance (ADR) through wastewater surveillance and hints at monitoring escalation of other environmental health parameters. This study will make the public and policyholders concerned about the optimum use of antibiotics during any kind of treatment.

1.5 Conclusion

1.5.1 WBE study in Ahmedabad

A temporal variation of SARS-CoV-2 RNA presence in wastewater was studied for a period of three months in Ahmedabad, India. A total 212 samples (94.6%) of the total 224 samples tested in the study were found to be positive, with at least two positive RT-PCR results targeting SARS-CoV-2 ORF1ab, S gene, and N gene assays. Monthly variation depict-ed a significant decline in all three gene targets copies in October compared to September 2020, followed by a sharp increment in November 2020. Correspondingly, the descending order of average genome

concentration was November (~10729 copies/ L) > September (~3046 copies/ L) > October (~454 copies/ L). This finding was further supported by the relation between the percentage change in genome concentration level and confirmed cases, which followed a similar trend on the temporal scale with a ~1 to 2 weeks' time distance. The results unveiled the untapped potential of WBE surveillance of COVID-19 as an early warning tool for practical use of city zonation based on SWEEP data for actual scenario and future prediction. This approach may help the authorities identify the hotspots within a city and tuning effective management interventions. Further research may be focused on quantification of correlation of SWEEP results with clinical surveillance data and development of predictive model that can translate SWEEP data for easy propagation to policy makers and common public to enhance the preparedness and management of pandemics.

1.5.2 Efficacy of WWTPs to remove SARS-CoV-2 RNA

The study concluded that influent waters present better picture in terms of SARS-CoV-2 gene monitoring; effective genome concentration should be calculated based on presence/absence of multiple genes rather the presence of one specific gene; and treatments are less effective on N-genes and the most effective for S-genes. CAS treatment exhibited better RNA removal rate (t=2.98, p=0.014) compared to the root-zone treatment (t=2.54, p=0.032). In addition, treatment plants with smaller capacity are likely to show more fluctuations in effluent water quality.

Two most critical findings from the ongoing pandemic perspectives were that the treated effluents are not always free from SARS-CoV-2 RNA, and are subject to temporal variability. We stress the need for wastewater surveillance of SARS-CoV-2 at the treatment plant scale with further investigation on the efficacy of the treatment processes on the removal of the enveloped virus such as SARS-CoV-2 as well as the genomic materials.

1.5.3 Metagenomic study of 16s RNA in wastewater samples

No clear-cut pattern among the bacterial population and association with SARS-CoV-2 genetic load in wastewater samples was observed. Some of the bacterial population significantly changed on monthly temporal scale but no clear-cut concluding pattern was seen. There was significant difference at the bacterial taxonomic level was observed between the untreated and treated wastewater samples. The results were not promising but they indicated a possible correlation ship among the SARS-CoV-2 gene concentration and bacterial population and dynamics. Therefore, further investigation is required considering different influencing factors such as sampling timing, sewage flow rate, treatment process, and wastewater physico-chemical parameters.

1.5.4 Wastewater based genomic surveillance of the SARS-CoV-2

The designated *Variant of Concern* (VoC: VOC-21APR-02; B.1.617.2) from wastewater samples has been identified using genomic surveillance approach. The variants of concern (VOCs) can be more transmissible resulting in probably higher disease severity outcomes and are also known for reduced sensitivity to antibody neutralization.

Therefore, WBE could be a useful method in early warning of the circulating novel variants and monitoring cryptic transmission of the SARS-CoV-2. Also, real time monitoring of the pandemic progression and helping the decision support system for public health interventions.

1.5.5 Prevalence of antidrug resistance (ADR) in ambient water samples

Non-fluoroquinolone drugs showed overall more resistance as compared to fluoroquinolone drugs. Tetracycline followed by norfloxacin has shown more resistance as compared to the other drugs. Despite a decrease in the prevalence of *E. coli* on the sampled river locations, the percentage resistance had been significantly increased in the year 2020 compared to year 2018. The increased consumption of antimicrobials in the pandemic period, the percentage of antidrug resistance has been increased significantly. Wastewater based epidemiology can be the key tool to monitor the antimicrobials prevalence and antidrug resistance in the pandemic situations.

1.6 Utility of knowledge

The result findings will help in providing:

a. Interactive publicly accessible genome concentrations data on web with a week lag for general public and three-day lag to public health workers, policy makers and water managers.

b. Longer time-series data to be used for various modelling and risk evaluation study.

c. An additional way to understand the efficacy of vaccine.

d. Resolution with signs indicative of temporal variation in COVID-19 patient loadings.

e. Developing advisory in the context of rapid-testing, number of testing, community clearance, hotspot identification, vaccine need identification zones as well as, to stay at home the accurate scale of the pandemic must use the environmental surveillances of SARS-CoV-2 in wastewater to supplement the individual testing and timely identification

Introduction

unicef
United Nations Children's Fund

2. Introduction

2.1 Introduction and Rationale

The global pandemic caused by severe acute respiratory syndrome 2 (SARS-CoV-2) disease has led to more than 11 million people in India alone by February 22nd, 2021 (WHO, 2020). A large number of asymptomatic patients exerted a never seen challenges over the actual estimation of disease spread based on clinical surveillance (Rimoldi et al., 2020; Medema et al., 2020). Also, the high prevalence of asymptomatic infectious persons is a matter of concern that raises doubt on the available data of active cases based on a clinical survey (Rimoldi et al., 2020; Medema et al., 2020). Therefore, alternative approaches such as wastewater-based epidemiology (WBE) are gaining recognition, and surveillance of SARS-CoV-2 transmission and real-time trend monitoring is being endorsed to trigger pandemic responses by scientific communities (Medema et al., 2020; Randazzo et al., 2020). The SARS-CoV-2 virus replicates in epithelial cells of alveoli and enterocytes of the intestinal lining in human beings due to the expression of ACE2 receptor resulting in respiratory illness and gastro-intestinal symptoms such as vomiting and diarrhoea (Ni et al., 2020; Kumar et al., 2020; Gupta et al., 2020; Zhang et al., 2020; Xiao et al., 2020). The clinical symptoms of SARS-CoV-2 infection include cough, breathing problems, diarrhoea, and fever. Different studies suggest that 48–67% of deceased persons exhibited SARS-CoV-2 RNA in the stool (Chan et al., 2020; Cheung et al., 2020; Parasa et al., 2020; Wong et al., 2020).

Due to the presence and extended excretion of SARS-CoV-2 RNA in the faecal matter of pre-symptomatic and deceased persons, WBE is gaining attention worldwide to monitor COVID-19, particularly in the developing economies with poor health infrastructure. An earlier investigation on COVID-19 patients revealed the prevalence of SARS-CoV-2 RNA in the stool of a larger population (48.1%) than patients with gastro-intestinal symptoms (17%) (Cheung et al., 2020). The latter study suggested that asymptomatic persons together with symptomatic persons, discharge viral particles in the excreta finding their way to sewage treatment plants. Interestingly, 18–45% of patients lack symptoms in the case of COVID-19 infection but are capable of transmitting the disease and can adversely affect the actual containment of COVID-19 (Lavezzo et al., 2020; Yang et al., 2020; Mizumoto et al., 2020; Nishiura et al., 2020). Haver and co-workers (2020) reported 6 to 24 times higher infection among asymptomatic and mild symptomatic individuals than confirmed cases at ten different sites in the United States based on surveillance of antibodies to SARS-CoV-2.

The wastewater encompasses SARS-CoV-2 RNA from both asymptomatic and symptomatic patients; therefore, WBE may prove its worthiness for COVID-19 surveillance to forecast the overall pandemic situation. WBE may help in identifying the hotspots and tuning the public health initiatives that will give

preparatory time to the regulatory bodies to handle the adverse situation. Further, WBE could provide an early warning of possible re-outbreaks and seasonal outbreaks in the future. The occurrence of SARS-CoV-2 RNA in wastewater has widely been reported from all corners of the world, including Spain, France, Italy, China, Netherlands, Australia, India, and Japan (Randazzo et al. 2020; Wurtzer et al., 2020; Zhang et al., 2020; Medema et al., 2020; La Rosa et al., 2020; Ahmed et al., 2020; Hata et al., 2020; Kumar et al., 2020, 2021). Although the sensitivity of WBE is comparatively less than clinical trials and largely depends on the viral load in the patient's faecal matter, earlier clues and wide acceptability of WBE suggest that this approach could be superior to clinical surveillance for the early prediction of COVID-19 status for highly populated places (Medema et al., 2020; Randazzo et al., 2020; La Rosa et al., 2020). Therefore, to evaluate WBE's potential as an early prediction tool for COVID-19 pandemic, it is essential to explore the correlation between the SARS-CoV-2 genetic load in wastewater and the number of cases at the district level in each country.

2.2 Potential transmission through wastewater

The awareness of the potential risk of SARS-CoV-2 from wastewater has increased since RNA detection of SARS-CoV-2 in wastewater reached public domains. Recently, possible transmission of COVID-19 from sewage was reported by a cohort study in Guanzhou, China (Yuhan et al., 2020). Although the occurrence of fecal-oral route transmission and potential community spread associated with untreated/treated wastewater, e.g., reuse of wastewater (inbuilt environments), aerosols of wastewater potentially exposing WWTP workers, sludge transfer activities, irrigation and recreational activities in wastewater-impacted waters, is still being debated (Barcelo et al., 2020 a,b; Lavezzo et al., 2020). However, there are growing concerns about the exposure risk of SARS-CoV-2 in natural water bodies that receive treated effluent from wastewater treatment plants (WWTPs), among citizens, administrative sectors, and policymakers. Because of the limited prior knowledge, the fate of SARS-CoV-2 from wastewater treatment to the water environment is still being scholarly speculated in a qualitative manner.

2.3 Removal of SARS-CoV-2 wastewater from wastewater treatment plants (WWTPs)

To date, we have gained knowledge on many aspects of Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2), especially on transmission, monitoring, analytical techniques, prognosis, diagnosis, models, and management aspects. However, the infectivity of SARS-CoV-2 RNA in wastewater, owing to viral shedding of infected symptomatic/asymptomatic patients, and their transmission remains under debate (Buitrago-Garcia et al., 2020). Potential community transmission associated with untreated/ treated wastewater, e.g., reuse of wastewater (inbuilt environments), aerosols of wastewater potentially exposing WWTP

workers, sludge transfer activities, irrigation and recreational activities in wastewater-impacted waters, is still being debated (Barceló et al., 2020a, b; Lavezzo et al., 2020). The two main obstacles are i) whether the viral genome load in wastewater is viable, and ii) whether wastewater treatments can completely remove SARS-CoV-2 RNA? (Balboa et al., 2021; Haramoto et al., 2020; Rimoldi et al. ,2020; La Rosa et al., 2020).

In general, wastewater surveillance of SARS-CoV-2 has focused on early-warning capability verifications [Ahmed et al., 2020; Kitajima et al., 2020; Kumar et al., 2020a, b) or protocol improvement through comparing various techniques of concentration and precipitations (Sherchan at al., 2020; Prevost et al., 2020; Lodder et al., 2020), and solid-aqueous interactions from sludge and virus interaction perspectives. However, since the beginning, subtle parallel efforts were there to check the SARS-CoV-2 RNA presence in secondary- and tertiary treated wastewater. Apart from several reports neglecting the presence of SARS-CoV-2 in treated water, Randazzo et al., 2020 confirmed 11% (2 out of 18) of secondary- and 0% (0/12) tertiary-treated water samples positive for SARS-CoV-2 RNA. Haramoto et al., (2020) detected as many as 2400 gene copies/L of SARS-CoV-2 RNA in secondary-treated wastewater, whereas raw wastewater samples were not positive with SARS-CoV-2, owing to the difference of sample amounts taken for filtration i.e. 200 mL for raw wastewater vs 5000 mL for secondary-treated wastewater. They also tested river samples, but no positive samples could be traced. Interestingly, they reported that 20% of secondary-treated wastewater samples that were found positive could not show the presence of S and ORF1a genes but the N-genes.

By 2021, more efforts started pouring, which tried to screen the treated water like Westhaus et al., (2020) reported modest SARS-CoV-2 removal from all three monitored conventional activated-sludge-based WWTP plants. They pointed out that the plant with full-scale ozonation illustrated a relatively better reduction of SARS-CoV-2 fragments in the effluent; and recommended to include membrane-based WWTP plant for future studies. On the other hand, Hasan et al., (2020) reported no positive results after monitoring 11 WWTPs effluents. They concluded that the treatment technologies used in the UAE were efficient in degrading SARS-CoV-2, and confirming the safety of treated water in the country for reuse. Similar results were reported by Balboa et al (Balboa et al., 2020) after observing WWTP in Spain for few days in both effluent and treated sludge.

We performed two months of monitoring for SARS-CoV-2 genes in untreated and treated wastewater samples, collected from two mechanically different treatment plants, viz. conventional activated sludge (CAS) process (Sargasan) and root zone treatment (RZT) (academic institution) located in Gandhinagar, India. Our main objectives were to: i) compare and evaluate the removal efficacy of SARS-CoV-2 by CAS and RZT processes through months-long influent and effluent monitoring; and ii) study temporal

variations in the removal efficacy of a given treatment process in the backdrop of active COVID-19 cases. We wish to add significant pertinent knowledge related to the actual and varying capabilities of one conventional and another zero-discharge trending root-zone treatment systems, so that infectivity can be adequately understood and appropriate information disseminated to the community. Our study is vital as transmission routes in the developing countries are many, owing to less prevalent, unproperly managed sewer systems that lead to wastewater leakages, occurrences of open defecation and common sewer overflow (CSO) situations.

2.4 Wastewater- based epidemiology (WBE) and genomic surveillance approach

Genomic signature of the SARS-CoV-2 can be deciphered through wastewater-based epidemiology (WBE) and genomic surveillance approach. The World Health organization (WHO) recognizes the environmental sewage surveillance strategies for the monitoring and detection of the viral pathogens in circulation. Even though it is challenging due to the sample heterogeneity and complex nature of the samples with fragmented nucleic acids. However, it remains a powerful tool for the detection, identification, prediction and development of an early system for the pathogen outbreaks surveillance to support the public health interventions. Pathogen in sewage and wastewater treatment sites can be helpful in development of the early warning systems. Further, it will be helpful in identification of the areas with higher prevalence of the virus in circulation among populations to aid in the non-pharmaceutical interventions (NPIs).

SARS-CoV-2 can persist in water droplets in the form of aerosols and raises several concerns on mode of transmission especially in clinical settings, hospitals and high-risk zones even though less quantifiable and definite evidence are difficult to prove otherwise. Further, investigation is required to conclusively determine the nature and extent to which it can be transmissible and cause infections.

Evidences are required to establish the hypothesis of the transmission of the SARS-CoV-2 from the wastewater sites or fecal-oral transmission routes, which remains debatable. In a study from 205 patients from China, published in early March 2020 by Wang et al. speculated the transmission of the SARS-CoV-2 via fecal route. Infection control guidelines remained silent on the airborne transmission of the SARS-CoV-2 until recently, whereas the Centre for Disease Control (CDC), USA updated the scientific briefing on 7th May, 2021 on the transmission modes of SARS-CoV-2. The virus containing large droplets remains suspended in air for minutes to hours and can be a possible source of transmission.

China sequenced and submitted a total of five environmental samples collected from the Huanan Seafood Market in Wuhan, Hubai with collection date 1 Jan, 2020 in the GISAID server. Some of the genomes were also sequenced from outer packaging of cold chain products in

Shandong province, China in September, 2020. Further, Bangladesh reported 23 genomes sequenced from the currency note swab samples in the month of August and September, 2020 collected from the local transportation, grocery shops and restaurants. While, United Arab Emirates (UAE) sequenced SARS-CoV-2 genome from the samples collected from washing table (stainless steel) swab in a bakery in December, 2020. Similarly, SARS-CoV-2 genomes from Austria were sampled from the sewage and wastewater treatment plants. Similarly, SARS-CoV-2 genomes were also sequenced from non-primate hosts and submitted to the GISAID server. The coverage and quality of these datasets is of varying degree in terms of quality of the sequenced genomes due to several factors in the environmental samples. Still, it is debatable and there is lack of direct scientific evidence to conclude whether a person can get SARS-CoV-2 infection from wastewater samples.

To the best of our knowledge no new variant of SARS-CoV-2 has been reported from the wastewater samples. Keeping in mind the early and prolonged excretion of SARS-CoV-2 virus in wastewater, it is imperative to search for a new SARS-CoV-2 variant in wastewater that would not only help in identifying new variants but also help in better understanding of the pandemic situation and tuning the public health intervention.

2.5 Antidrug resistance in ambient water samples of Ahmedabad during the COVID-19 pandemic

The exponential rise in the consumption of antimicrobials in various applications such as medical, veterinary, domestic and agricultural and their leak to aquatic ecosystems has caused the global prevalence of antidrug resistance (ADR), which is being considered a major threat to public health (Rodriguez-Mozaz et al., 2015; Chatterjee et al., 2010; Baker-Austin et al., 2006). The rate of consumption of certain antimicrobials has escalated during the COVID-19 pandemic in an effort to minimise the risk of severe infections and mortality (Miranda et al., 2020; Liu et al., 2020). Around 70% of COVID-19 patients have received antimicrobial treatment along with overuse of various antibiotics despite only 10% on average show microbial infections (Hsu, 2020). As most of the consumed drugs and their metabolites are excreted through urine and faeces, their discharge to aquatic environments depends on the removal efficiency of the WWTPs (Azuma et al., 2012; Takanami et al., 2010; Kumar et al., 2020a). If the WWTP clearing rate is low, microorganisms exposed to antimicrobials and metabolites develops mutations causing ADR (Guo et al. 2018, Kumar et al., 2020a). Thus, the increased use of antimicrobials in the current pandemic will probably pose an increased risk in terms of ADR during post COVID-19 as concerned by a number of recent studies (Kuroda et al., 2021; Lucien et al., 2021; Hsu, 2020; Kumar et al., 2020a; Asaduzzaman et al., 2020). Therefore, it is imperative to assess the effect of imprudent consumption of antimicrobial substances (ABS) during the COVID-19 pandemic.

2.6 Objectives

Following the proven concept and capabilities of detecting the RNA of Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2) in wastewater, it is imperative for the adoption of WBE on the policy level, which has been for some reason still delayed in the major parts of the globe. Under the light of above discussion, the objectives of the present study aimed at: **i)** To detect and quantify variation in the genetic material of SARS-CoV-2 in the various wastewaters of Ahmedabad to understand pandemic situation; **ii)** To have a weekly resolution of the data for three months in genetic material loadings in the various wastewater treatment plants of Ahmedabad; **iii)** To establish applicability of WBE for COVID-19 surveillance as a potential tool for public health monitoring at the community level; **iv)** To understand the pathogen diversity (viral and bacterial) from wastewater in order to establish early sign of WBE as prediction tool.

Apart from the approved objectives, we have performed further studies for better understanding and effective management of COVID-19 like pandemic/ epidemic condition. The objectives are as follows:

a.) Comparison and evaluation of the removal efficacy of SARS-CoV-2 by conventional activated sludge (CAS) and root zone treatment (RZT) processes through two months-long influent and effluent monitoring.

b.) Wastewater based genomic surveillance of SARS-CoV-2 (whole genome sequencing) to get an idea about mutants.

c.) *To assess the effect of imprudent consumption of ABS during the COVID-19 pandemic, comparison of the 2020 prevalence of antidrug resistance (ADR) of Escherichia coli (E. coli) with a similar survey carried out in 2018 in Ahmedabad, India using SARS-CoV-2 gene detection as a marker of ABS usage.*

Methodology

unicef
United Nations Children's Fund

3. Methodology

3. 1 Study area for WBE study

Ahmedabad is the seventh largest city in India and the second biggest trade centre in the western Indian region, with a population of 5.5 million (Census, 2011). It has a 1523 km sewage network assisted with forty-three sewage pumping stations. The present existing treatment capacity of the wastewater treatment plant in the city is 670 MLD in 2007 which is likely to be extended to 1075 MLD by 2021 (https://web.worldbank.org/archive/website01409/WEB/IMAGES/2010_1_1.PDF AMC Report). There are 84 urban health centres present in different ward in Ahmedabad (AMC, 2021).

3. 2 Sampling approach for WBE

In order to achieve the objective; firstly, the entire city was divided based on urban/rural as well as north and south to the Sabarmati River- the major river that dissects the city; and 29 locations had been chosen in association with Gujarat Pollution Control Board (GPCB) officials. We observed the data variations of 29 locations for the first four weeks. Thereafter, based on the significance of the variations within the data-set, we fixed thirteen locations to continue monitoring including nine different locations for the wastewater (eight wastewater pumping stations and a single sewage treatment plant) (**Fig. 1**); and four surface water locations (three lakes and one river sample). In the present study, we reported weekly data of wastewater samples collected from nine different locations for thirteen weeks during September to November 2020.

A total of 116 samples were analyzed in the present study to detect SARS-CoV-2 RNA from nine different sites, comprising 103 samples from eight wastewater pumping stations and 13 samples from a single sewage treatment plant in Ahmedabad, India. All the samples were collected by grab hand sampling using 250 ml sterile bottles (Tarsons, PP Autoclavable, Wide Mouth Bottle, Cat No. 582240, India). Simultaneously, blanks in the same type of bottle were examined to know any contamination during the transport. The samples were kept cool in an ice-box until further process. The analysis was performed on the same day after bringing the samples to the laboratory. All the analyses were performed in Gujarat Biotechnology Research Centre (GBRC), a laboratory approved by the Indian Council of Medical Research (ICMR), New Delhi.

3.3 Detection and extraction of viral RNA from wastewater samples

3.3.1 Precipitation of viral particle

30 mL samples were centrifuged at 4000×g (Model: Sorvall ST 40R, Thermo Scientific) for 40 minutes in a 50 mL falcon tube followed by filtration of supernatant using 0.22-micron syringe filter (Mixed cellulose esters syringe filter, Himedia). After filtrating 25 mL of the supernatant, 2 g of PEG 9000 and 0.437 g of NaCl (17.5 g/L) were mixed in the filtrate, and this was

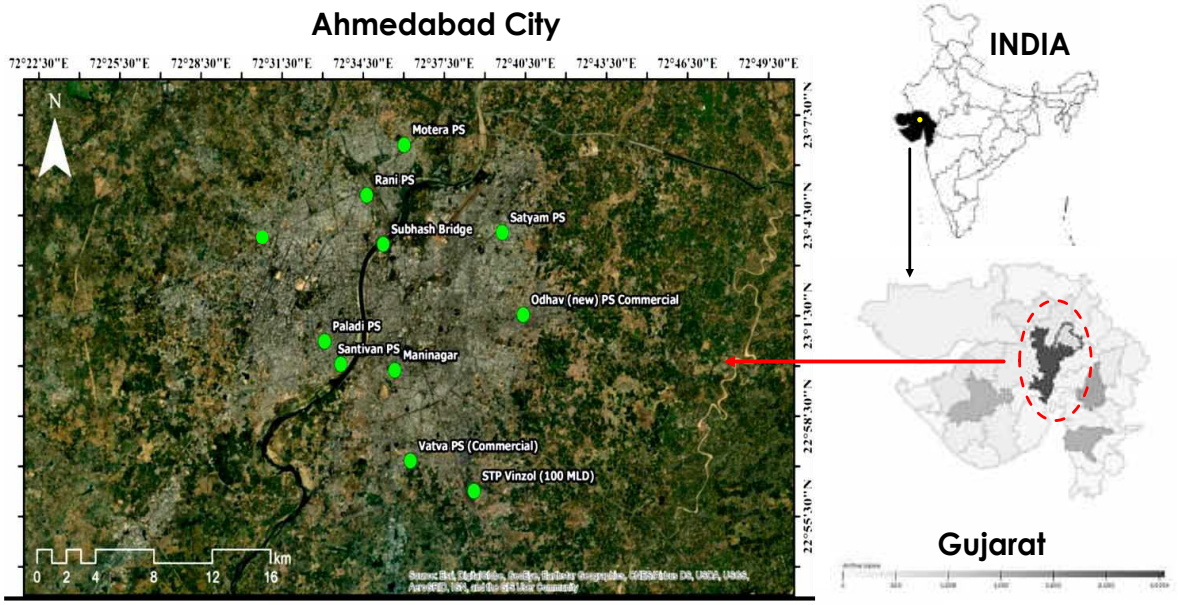


Fig. 1 Geospatial position of sampling locations in Ahmedabad city

incubated at 17°C, 100 rpm overnight (Model: Incu-Shaker™ 10LR, Benchmark). Next day, the mixture was centrifuged at 14000×g (Model: Kubota 6500, Kubota Corporation) for about 90 minutes. The supernatant was discarded after centrifugation, and the pellet was resuspended in 300µL RNase-free water. The concentrated sample was kept in 1.5ml eppendorf at -40 °C, and this was further used as a sample for RNA isolation.

3.3.2 RNA isolation, and RT-PCR

RNA isolation from the pellet with the concentrated virus was performed using NucleoSpin® RNA Virus isolation kit (Macherey-Nagel GmbH & Co. KG, Germany). The samples were spiked with MS2 phage as an internal control prior to the RNA extraction provided by TaqPathTM Covid-19 RT-PCR Kit. Some other specifics are, a) the nucleic acid was extracted by NucleoSpin® RNA Virus isolation kit and Qubit 4 Fluorometer (Invitrogen) was used for the total RNA concentrations estimation, b) MS2 phage was taken as a molecular process inhibition control for evaluating the efficiency of nucleic acid extraction and PCR inhibition. (MPC; Haramoto et al., 2018). Briefly, steps were carried out as per the guideline provided with the product manual of Macherey-Nagel GmbH & Co. KG, and RNAs were detected using reverse transcription PCR (RT-PCR).

Applied Biosystems 7500 Fast Dx RT-PCR Instrument (version 2.19 software) was used for SARS-CoV-2 gene detection. In the process, the probes anneal to three specific target sequences located between three unique forward and reverse primers for the N, ORF 1ab, and S genes. A template of 7 µl of extracted RNA was used in each reaction with TaqPath™ 1 Step Multiplex Master Mix (Thermofischer Scientific, USA). Total reaction mixture volume of 20

µL contained 10.50 µL Nuclease-free Water, 6.25 µL Master Mix, and 1.25 µL COVID-19 RT-PCR Assay Multiplex. Three controls were used, namely: positive control (TaqPath™ COVID 19 Control), one negative control (from extraction run spiked with MS2), and no template control (NTC). The RT-PCR contained 1 incubation step cycle of 25°C & 2 minutes, 1 cycle of reverse transcription 53°C & 10 minutes, 1 cycle of activation 95°C & 2 minutes, and 40 cycles of amplification, including denaturation at 95°C for 03 seconds and extension 60°C for 30 seconds. Finally, results were interpreted using Applied Biosystems Interpretive Software, and Ct values for three target genes i.e., ORF1ab, N Protein, and S Protein of SARS-CoV-2 along with MS2 used as an internal control.

3.3.3 Gene copy estimation: Quality Control and Quality Assurance

The samples were considered as positive if at least two of the three primer probe sets showed amplification. The average Ct-value of a given sample was then converted to gene copy numbers considering the equivalence of 500 copies of SARS-CoV-2 genes as 26 Ct-value (provided with the kit), and the same was extrapolated to derive approximate copies of each gene. In this semi-quantitaive method to provide the gene concentration, the calibration curve was prepared based on the well-established principle of 3.3 CT change corresponding to a 10-fold gene concentration change. The average effective gene concentration of SARS-CoV-2 present in a given sample was calculated by multiplying the RNA amount used as a template with the enrichment factor for each sample. In addition, we had calculated the gene copy numbers based on the positive control provided with kit i.e., 10^4 copies/µl and the final concentration of 25 copies per reaction. The positive control was providing the same ct values for all 3 genes, and relative to the Ct values of genes of positive controls, copy numbers have been calculated in test samples of different sources. The effective gene concentration is considered as "zero" when RT-PCR results were positive for only one gene out of three in the wastewater sample. The limit of detection has been set to 40 amplification cycle (Ct=40) in the RT-PCR analysis. The effective gene concentration was calculated by averaging the gene copies of all three genes in a particular sample.

Due to various constraints, samples were analyzed in duplicate, considering that the samples were analyzed in the batch accompanied with negative and positive controls, and each sample was spiked with known concentrations of MS2. In the event of any variations (among duplicate and controls) of more than 10%, samples were re-analyzed. It is worth noting that the primer efficiency of different genes will be slightly varied according to the primer sequence. Based on several hundreds of RTPCR run, it was found that the positive control was robust enough to provide the same Ct values for all three genes, implying no evident difference between the primer efficiency. We report both primary Ct-values and derived gene copies relative to the Ct values of positive controls for both

individual genes and effective SARS-CoV-2 gene concentration.

mapping and self-assessment on COVID–19. This application reached more than 100

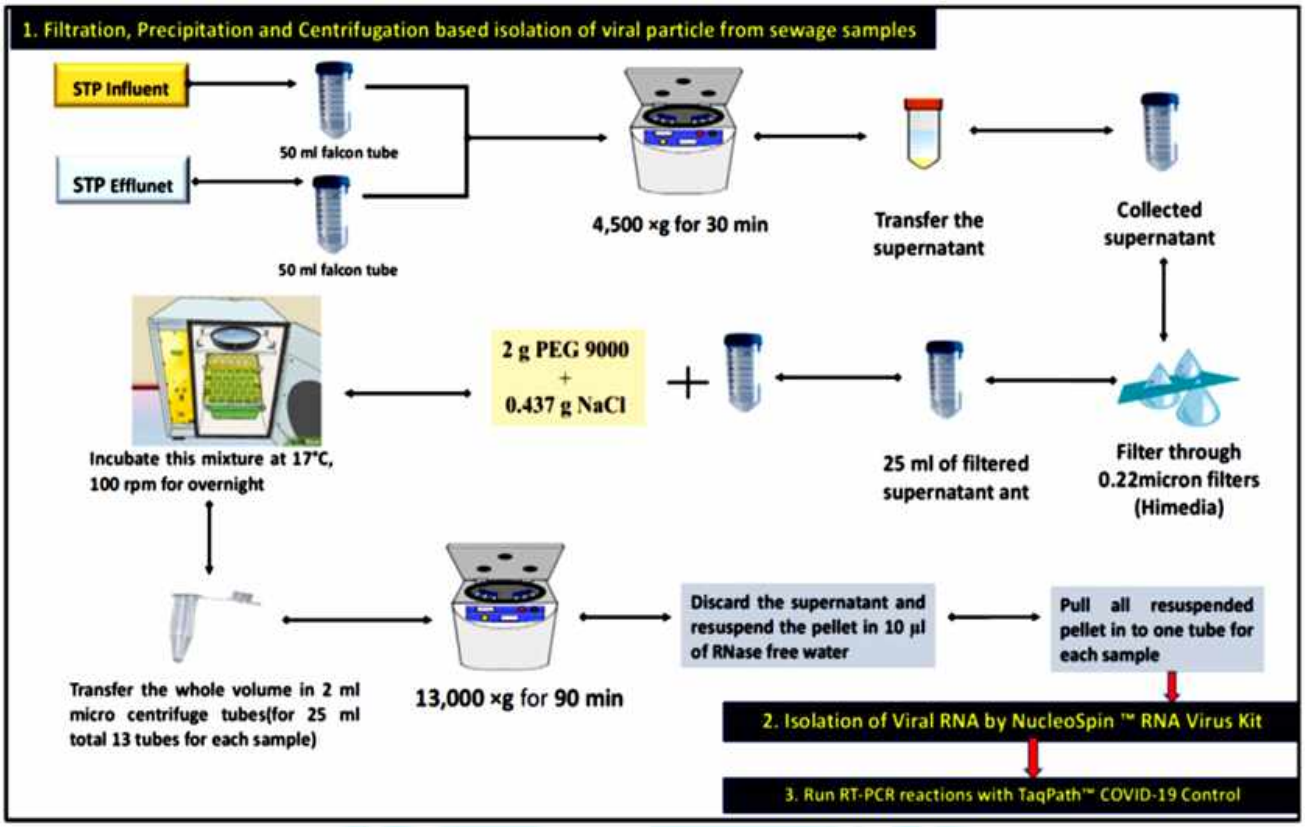


Fig. 2 Methodology of sample preparation, virus concentration, RNA extraction

3.4 Epdemiological information, data collection and interpretation

The data of affected people and their locations were obtained from the governmental mobile application 'Arogya Setu' which is published as Ahmedabad COVID-19 community vulnerability map published by SustainAbly and Accion Land Pvt. Ltd, accessible at http://google.org/crisismap/a/gmail.com/amdcovid19. 'Aarogya Setu' mobile application was launched by the Ministry of Electronics and Information Technology of the Indian government for collecting data pertaining to tracing, syndromic

million installs in 40 days (Arogya setu, Wikipedia 2021). Other information was obtained from the Ahmedabad city portal accessed using link https://ahmedabadcity.gov.in/portal/web?requestType=ApplicationRH&actionVal=loadCoronaRelatedDtls&queryType=Select&screenId=114. Several other informations can be accessed using https://ahmedabadcity.gov.in/portal/jsp/Static_pages/water_project.jsp .

Statistical Package for the Social Sciences (SPSS 21) has been used for hypothesis testing through Analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT). The OriginPro 2019b

data analysis software has been used to draw boxplots.

3.5 SARS-CoV-2 RNA removal from wastewaters

We investigated wastewater samples collected from conventional activated sludge (CAS) based treatment plant situated at the Sargasan ward of Gandhinagar (Sargasan WWTP), and from the root-zone treatment plant of an academic institution located in Gandhinagar, both located in Gujarat, India.

At the two WWTPs, influent and effluent wastewater samples were initially collected biweekly, then weekly for two months, from August to September 2020. Twenty-one grab samples, representing the treatment plant inlets and outlets of both treatment plants, were collected every Monday of the week at 10 am and placed into 250-ml sterile bottles (Tarsons, PP Autoclavable, Wide Mouth Bottle, Cat No. 582240, India). Simultaneously, blanks were included to check for contamination during travel. The samples were kept cool in an ice-box until analysis. All laboratory analyses were performed on the same day and included duplicates to ensure accuracy and precision. It is imperative to note that we evaluated the removal of SARS-CoV-2 RNA by wastewater treatment methods, including disinfection. It is therefore, final effluent was sampled after the disinfection process, which is essential in the context of risk assessment of SARS-CoV-2 in receiving water.

3.6 Metagenome analysis of the prokaryotic 16S ribosomal RNA gene

3.6.1 16S Metagenomics Kit Sequencing using Ion Torrent PGM

The 16S region was amplified with 16S Ion Metagenomics Kit ™ (Life Technologies) by 2 separate PCR reactions using primer set V3 and V4 hypervariable or V regions of the 16S rRNA. Equal volumes of V3 and V4 amplification reactions were combined. Fifty nanograms of combined amplicons were processed to make the DNA library using Ion Plus Fragment Library Kit ™ and Ion Xpress Barcodes Adapters, 1–16 ™ (Life Technologies, Grand Island, NY). Adapter-ligated and nick-repaired DNA was amplified with the following steps: 1 cycle of 95°C for 5 min; 5 cycles of 95°C for 15sec, 58°C for 15 sec, 70°C for 1 min; hold at 4°C. Each step was followed by purification using 1.4 volumes of Agencourt AMPure beads (Beckman Coulter, Inc, Atlanta, Georgia) and eluted in low Tris-EDTA buffer. Size and quantity of processed libraries were evaluated with DNA high sensitivity kit in 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA.). Each sample was adjusted to 26 picomolar concentration. Equal volumes of all samples of Ahmedabad were combined and processed with One-Touch 2 and One-Touch ES systems (Life Technologies, Grand Island, NY) according to the manufacturer' instructions. Sequencing was performed on the Ion Personal Genome Machine (PGM) using 400-bp kit and 316 v2 chip.

Base calling and run demultiplexing were performed by Torrent Suite version 4.4.2 (Life Technologies, Grand Island, NY) with default parameters. FileExporter version 4.4.0. 0 (Life Technologies, Grand Island, NY) was used to generate demultiplexed fastq files for each sample. Mean read length for both forward and reverse reads ranged between 235bp to 238 bp for all samples.

3.6.2 Data processing and preparation

The data pipeline can be described in 3 steps for this work. Pre-processing includes quality filtering and length filtering, adding read labels in order to mimic non-demultiplexed data for downstream analysis, and concatenating reads into one file. The second step involves dividing reads into 2 subsets of the 2 hypervariable regions. This step begins with aligning the reads to the Silva Database using Mothur, separating reads into forward and reverse, and binning reads based on start and stop coordinate from the Mothur alignment. The third step, Operational Taxonomic Units (OTU) clustering and taxonomic assignment, includes trimming reads and removing chimeras, clustering reads into OTUs and assigning taxonomy using Quantitative Insights into Microbial Ecology (QIIME). Finally, OTUs were compared across different V regions. Future work will be to develop a consensus OTU table, if possible, taking into account OTUs from each region.

3.7 Genomic surveillance for SARS-CoV-2 variants in wastewater

3.7.1 Library preparation, sequencing and data analysis

RNA was extracted as described in our previous studies (Kumar et al., 2020; 2021) in that, we enrich the virus particles using polyethylene glycol (PEG) method. The extracted RNA was subjected to cDNA synthesis using SuperScript-III First-Strand Synthesis System (Invitrogen/Thermo Fisher Scientific). For library preparation, we used Ion AmpliSeq Community SARS-CoV-2 panel and Ion AmpliSeq library kit Plus (Invitrogen/Thermo Fisher Scientific). Quality of the library was checked on Bioanalyzer (Agilent 2100) using DNA High Sensitivity (HS) Kit (Agilent). Sequencing was carried out on Ion S5 Plus System (Thermo Fisher Scientific) on 530 Chip and 400 bp chemistry.

3.7.2 Data filtering, trimming and genome assembly

All raw sequences were processed using the PRINSEQ-lite v.0.20.4 for quality filtering. Reads were trimmed from the right where the average quality of the 5 bp window was lower than QV25, 5 bp from the left end was trimmed. Reads with length lower than 50 bp with average quality QV25 were filtered. Quality filtered data were assembled using reference-based mapping using CLC Genomics Workbench version 12.0.3. Mapping tracks were used for variant calling and identification of the mutations. Haplotyping of the assembled genomes were carried out based on the 80% (Major allele) and 20% (Minor allele) frequency. These variants were verified and

confirmed using Integrative Genomics Viewer (IGV) after manual curation. Further, Pango-Lineages were identified using the pango-lineage classification system (https://cov-lineages.org/).

The key challenges in wastewater based genomic surveillance of the SARS-CoV-2 are: **i)** primer biases and sensitivity issues were observed and remains a plausible concern; **ii)** sample collection timing and intervals are critical parameters for optimal surveillance strategy; **iii)** assessment of the SARS-CoV-2 for the viability in cell cultures and infectivity; **iv)** effect of physicochemical wastewater treatment process on the false positive and negative detection limits

3.8 Antidrug resistance study in the Indian ambient waters

3.8.1 Sample collection and ADR analyses

The water samples were collected from 6 different locations of Ahmedabad city on 23rd June 2018, and 16th October 2020. Two locations on the stretch of Sabarmati River: Nehru Bridge (NB) and Sardar Bridge (SB); two lakes: Kankariya Lake (KL) and Chandola Lake (CL), and two WWTP locations: Chandkheda (inlet: CI and outlet: CO) and Vasna, also known as Juhapura (inlet: VI and outlet: VO), selected to assess ADR. For SARS-CoV-2 gene detection, a total of 10 locations were selected to represent various zones of the city that comprises all ADR sampling locations. We kept ADR locations low to match the number of locations tested in 2018 (Ram and Kumar, 2020). The geographical details about the selected locations are well described in our previous study by Ram and Kumar (2020). Sterile bottles (Tarson-546041) of medical grade were used to collect the samples, which were then kept in iceboxes until arrival at the laboratory. For on-site measurement of pH, EC, ORP, TDS and salinity, a multi-parameter probe, HANNA HI9828 was used. The procedure for testing the isolation of *E. coli* for ADR is likewise described in Ram and Kumar (2020). Briefly, the water samples were filtered through membranes with 0.45-µm-pore size, and *E. coli* trapped by the membranes were incubated on Chromocult® Coliform Agar ES (Merck Microbiology, Darmstadt, Germany). Each *E. coli* isolate was tested for susceptibility to six antibiotics (kanamycin, KM; tetracycline, TC; norfloxacin, NFX; ciprofloxacin, CIP; levofloxacin, LVX; and sulfamethoxazole, ST) by Kirby-Bauer method using PERLCORE® Sensitivity Test (ST) Agar (EIKEN Chemical Co., Ltd, Tokyo).

3.8.2 SARS-CoV-2 RNA detection

Same as described in **Subsection 3.3**

Results and Discussion

unicef
United Nations Children's Fund

4. Result and Discussion

We detected and quantified variation in SARS-CoV-2 RNA from wastewater samples for six months (September 2020 and February 2021) to understand the pandemic situation in Ahmedabad, Gujarat, India. Among the 224 samples analyzed in the study, 212 (94.6%) were found positive, comprising at least two positive RT-PCR results targeting SARS-CoV-2 ORF1ab, S gene, and N gene assays (**Table 1**). In addition to this, 213/224 (95.1%), 202/224 (90.2%), and 209/224 (93.3%) samples showed positive RT-PCR results for N, ORF 1b and S genes, respectively. The distribution analysis of Ct values for different genes using boxplot is represented in **Fig. 3**. The average Ct values for N, ORF 1ab, and S genes were 32.11, 32.74, and 33.14, respectively. The average Ct values of internal control (MS2 bacteriophage) was 27.50, and no SARS-CoV-2 genes were detected in the negative control samples.

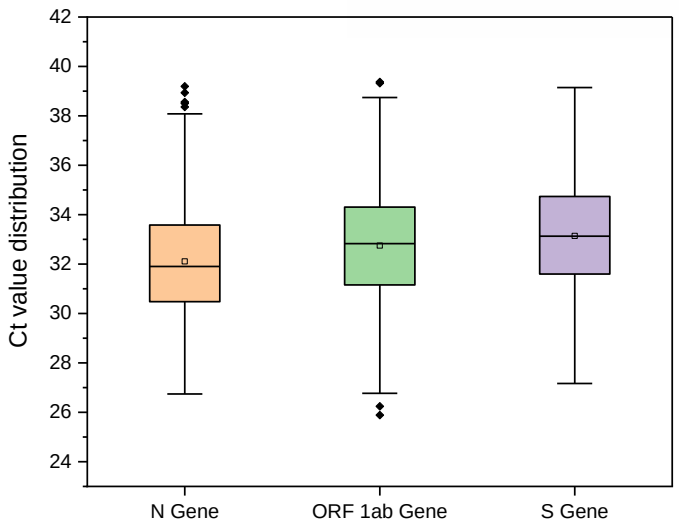


Fig. 3 Distribution of Ct values of SARS-CoV-2 genes during the study period

4.1 Monthly and Weekly Variations

Monthly variation depicted a significant decline of 89.7, 63.7, and 90.1% in N, ORF-1ab, and S gene concentration (copies/L), respectively in October compared to September 2020, followed by a sharp increment in November 2020 i.e. ~25 folds in N gene, ~22 folds in ORF 1ab and ~26 folds in S gene. The PCR products for all three genes were maximum in wastewater samples of November. The descending order of monthly variation in ORF 1ab gene concentration in wastewater samples was: November> September> December> January> October> February. Likewise, decreasing order of N and S genes in wastewater samples followed a similar pattern and found in order of November> September> December> January> February> October (**Fig. 4 a-c**). The genome concentration of SARS-CoV-2 RNA was maximum in the month of November (~10729 copies/ L), followed by September (~3047 copies/ L), January (1810 copies/ L), December (1802 copies/ L), February (492 copies/ L) and October (453 copies/L). The rise in genome concentration in wastewater samples collected in November was in line with a ~ 1.5-fold rise in the number of confirmed cases during the 3rd September 2020 and 26th November 2020 (**Fig. 4d**).

There had been a decline of 20.47% in active cases in October 2020 with respect to September, and a rise of 1.82% occurred in November 2020 compared to the preceding month i.e., October. While the increase of 1.82% in the active cases of November with respect to October is equivalent to a change of 59 cases (3,234

cases on 1st November – 3,293 on 26th November); however, the same monthly change in the total confirmed cases between October and November has been of 14.1% due to addition of 6,019 new cases to the tally of October by 26th November, 2020. Also, a monthly decrease of 4.45% in recovered cases was noticed in November compared to October 2020. The monthly recovered new cases were 16.61, 20.02, and 15.58% in September, October, and November 2020, respectively. Apart from that, people's casual and reluctant attitude during the festive season in India (mid-October to mid-Nov) might be the reason for the piercing rise in COVID-19 cases.

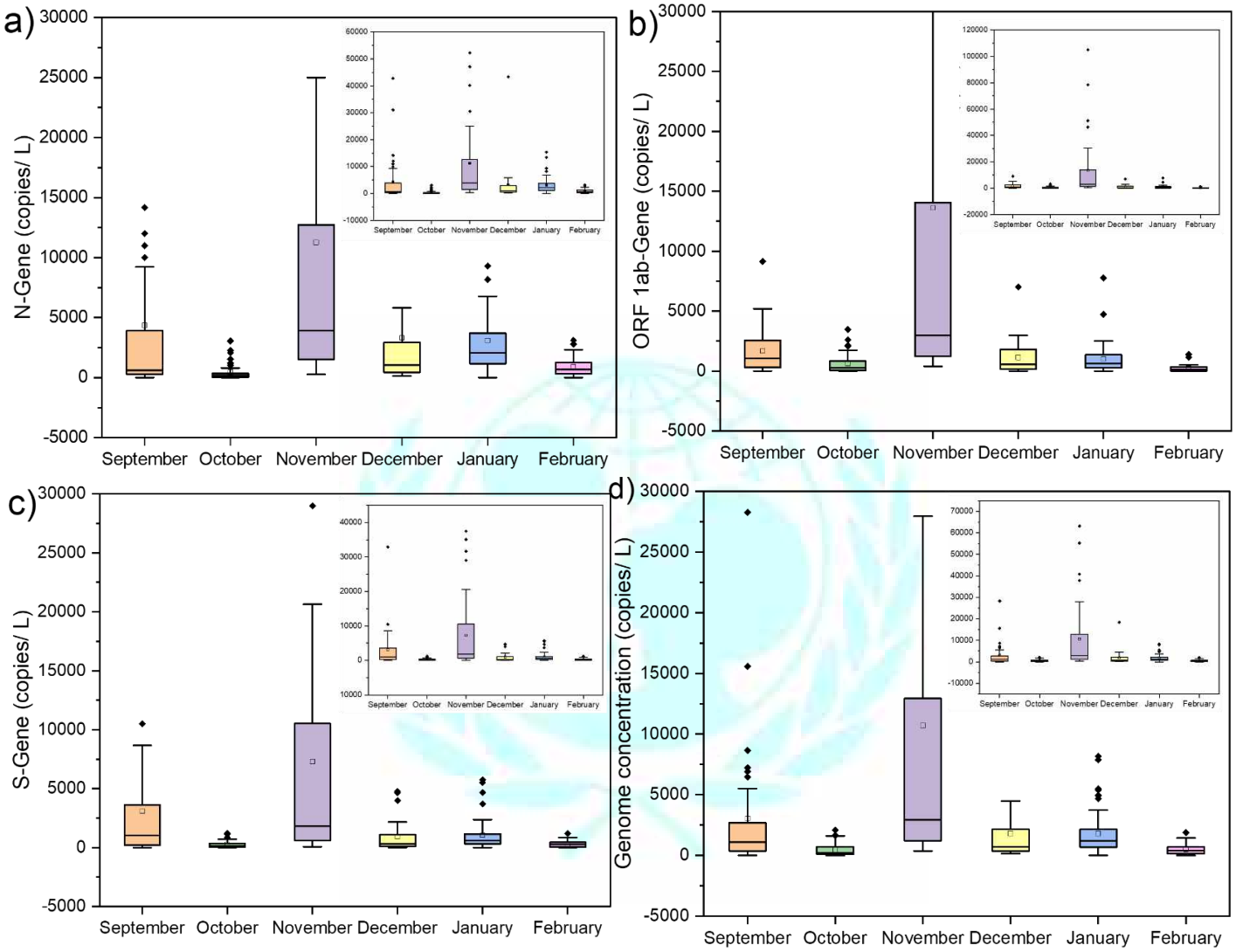


Fig. 4 Distribution of SARS-CoV-2 gene copies on a temporal scale (monthly variation)

Table 1. Temporal variation in gene copies of the SARS-CoV-2 targeted genes and genome concentration at various locations in Ahmedabad city

Station	Sampling date	September, 2020				October, 2020					November, 2020				December, 2020			January, 2021						February, 2021		
		3.09	10.09	17.09	24.09	0.1.10	8.10	15.10	22.10	29.10	5.11	12.11	19.11	26.11	14.12	21.12	28.12	4.01	8.01	11.01	15.01	22.01	29.01	8.02	15.02	22.02
	Active Cases	3671	4168	4038	4252	4122	3614	3472	3451	3372	3283	3280	3362	3293	2998	2994	2930	2894	2848	2796	2552	1991	1478	855	544	548
	SARS-CoV-2 Genes	Gene Copies (copies/ L) x 10^2																								
Motera PS	N	19.9	120	0.36	1.56	7.99	2.84	1.23	30.8	ND	28.7	70.8	522.7	57.8	7.29	5.85	50.81	34.39	8.48	31.48	11.79	11.27	19.38	6.55	5.91	15.24
	ORF	5.84	16	1.43	5.73	1.7	1.16	10	20.8	ND	3.86	104	783.2	44.4	6.06	2.31	24.44	9.75	6.59	3.69	6.51	12.91	3.12	ND	1.01	4.08
	S	4.4	71.1	0.78	4.6	1.27	1.32	3.17	11.3	0.34	3.65	63.9	350.8	18.2	7.40	2.30	8.06	22.30	5.02	4.31	3.13	6.08	3.77	0.26	3.53	7.58
	Genome	10.1	69.1	0.86	3.96	3.65	1.77	4.8	21	ND	12.1	79.5	552.2	40.1	6.92	3.49	27.77	22.15	6.69	13.16	7.14	10.09	8.76	2.27	3.48	8.97
Ranip PS	N	3.18	310.4	9.8	5.4	6.61	3.73	2.17	0.68	0.17	64.2	33.1	471	124.7	22.50	4.34	433.41	13.64	27.00	24.21	12.03	65.62	15.55	4.09	5.07	1.97
	ORF	ND	51.9	41.7	14.8	0.86	ND	13.3	3.59	0.95	29.9	30.5	463.2	101.9	28.27	0.27	70.13	5.67	17.14	15.33	ND	5.92	ND	ND	2.07	1.18
	S	0.46	105	39.2	15.2	1.67	0.51	5.91	0.18	0.78	15.8	24.8	289.9	37.04	39.97	0.42	47.54	7.49	14.81	8.92	2.94	15.83	1.74	ND	1.19	1.68
	Genome	1.22	155.8	30.2	11.8	3.05	1.41	7.14	1.48	0.63	36.6	29.4	408.1	87.9	30.25	1.68	183.69	8.93	19.65	16.15	4.99	29.13	5.76	ND	2.78	1.61
Paldi PS	N	5	40.5	3.26	ND	12.1	0.27	0.23	0.55	0.3	8.69	12.6	99.8	39.1	2.21	8.90	25.69	26.03	21.90	8.57	23.51	56.48	32.00	ND	31.03	8.41
	ORF	1.73	11.7	11.1	0.28	3.31	0.19	0.69	2.17	0.27	5.77	24.4	140.5	21.9	1.29	0.96	7.88	22.00	8.95	ND	5.10	8.85	ND	ND	13.78	3.46
	S	0.79	29.6	9.8	0.75	1.76	ND	0.66	2.78	0.69	3.52	27.2	118.9	9.93	1.74	0.58	0.84	10.72	6.32	3.97	6.44	22.38	ND	0.56	11.80	5.28
	Genome	2.51	27.2	8.07	0.34	5.74	0.15	0.53	1.83	0.42	5.99	21.4	119.7	23.6	1.75	3.48	11.47	19.58	12.39	4.18	11.68	29.24	ND	0.00	18.87	5.72
Santivan PS	N	12.4	100	3.07	1.37	2.15	2.37	0.87	0.96	ND	15.1	2.74	116.3	12	9.72	9.82	4.30	66.61	36.93	6.54	11.34	22.72	ND	9.20	11.00	23.27
	ORF	4	30.4	9.74	4.13	0.65	0.24	3.9	5.17	ND	12.2	3.89	129.6	12.9	7.36	2.42	3.41	24.28	24.30	10.91	3.25	4.70	0.72	0.82	ND	11.41
	S	3.14	86.6	10.4	4.57	1.2	ND	1.87	1.55	0.15	6.03	4.24	141.9	3.67	10.78	1.48	ND	57.41	14.26	2.74	6.03	19.12	ND	1.21	4.36	8.44
	Genome	6.51	72.3	7.74	3.36	1.33	0.87	2.21	2.56	ND	11.1	3.63	129.3	9.5	9.29	4.57	2.57	49.43	25.16	6.73	6.88	15.51	ND	3.74	5.12	14.37
Maninagar	N	5.8	48.5	6.15	0.62	15.4	3.5	2.78	ND	0.15	8.3	NA	168.6	34.5	3.49	7.01	16.26	67.67	3.51	18.52	63.21	27.96	48.70	12.60	3.26	2.00
	ORF	1.05	10.3	26.3	2.62	3.95	0.26	26.1	2.54	1.68	5.93	NA	172.7	28.3	1.38	5.68	5.13	2.18	2.54	6.17	25.12	13.69	1.30	ND	1.96	0.82
	S	1.18	20.6	35.2	2.08	2.84	0.73	12.1	ND	0.47	2.17	NA	105.2	10.4	2.96	4.73	2.21	36.99	2.75	5.44	23.56	14.31	2.46	0.48	3.17	1.31
	Genome	2.68	26.4	22.5	1.78	7.39	1.5	13.7	ND	0.77	5.47	NA	148.8	24.4	2.61	5.80	7.87	35.62	2.93	10.04	37.30	18.65	17.49	4.36	2.80	1.38
Satyam PS	N	14.1	141.7	4.91	6.48	30.4	4.88	2.23	0.21	0.28	10.2	8.21	23.2	29.5	1.89	2.54	14.25	133.93	12.03	0.43	15.70	36.81	81.68	2.16	19.05	7.06
	ORF	1.4	39.9	23	24.8	10.8	1.05	17.2	3.34	2.15	7.52	5.82	13.3	27.6	ND	1.04	3.24	47.25	6.37	1.05	11.76	19.64	ND	1.18	3.69	3.79
	S	2	78	23.3	24.8	7.01	0.36	7.09	1.69	0.51	0.68	3.03	12.1	10.3	0.92	0.81	0.74	55.06	3.21	0.89	4.80	11.25	ND	0.52	3.23	5.49
	Genome	5.82	86.5	17.1	18.7	16.1	2.1	8.85	1.75	0.98	6.15	5.69	16.2	22.5	0.93	1.46	6.08	78.75	7.20	0.79	10.75	22.56	ND	1.28	8.66	5.45
STP Vinzole	N	11	92.2	2.57	ND	20.6	ND	1.68	3.16	0.92	111.5	127.4	470.9	56.4	36.71	29.42	18.59	25.81	14.53	9.37	62.92	6.93	154.19	27.95	13.52	8.72
	ORF	3.97	22.3	34.7	ND	6.44	ND	16.8	34.7	6.8	43.9	187.1	1049	17.1	19.94	15.57	18.03	6.22	1.67	6.31	77.78	5.20	2.07	2.85	5.21	2.24
	S	6.23	51.1	37.3	ND	4.97	ND	5.98	11.6	2.52	18.5	105	374.5	11.7	12.11	4.87	17.30	4.18	0.30	4.02	23.70	8.78	5.37	3.61	2.56	4.06
	Genome	7.06	55.2	24.9	ND	10.7	ND	8.15	16.5	3.41	57.9	139.8	631.5	28.4	22.92	16.62	17.97	12.07	5.50	6.57	54.80	6.97	53.88	11.47	7.10	5.01
Odhav PS	N	38.1	427.5	5.84	1.69	22.7	0.29	2.2	ND	1.61	155.2	305.2	249.9	401.7	45.53	40.30	58.17	11.84	11.64	12.65	19.17	4.06	51.22	ND	4.02	ND
	ORF	17.9	91.4	31	3.46	9.05	ND	21.3	40.5	8.38	132.5	512.1	277.8	305.5	24.79	15.90	29.69	15.43	5.61	2.94	12.41	5.76	3.11	ND	ND	ND
	S	6.87	329.2	31.5	7.43	6.96	ND	8.57	0.15	3.36	69.3	316.5	206.4	131.7	21.61	8.32	46.51	7.44	2.45	3.71	11.55	1.95	10.33	0.30	ND	ND
	Genome	20.9	282.7	22.8	4.19	12.9	5.74	10.7	0.2	4.45	119	377.9	244.7	279.6	30.64	21.51	44.79	11.57	6.56	6.44	14.38	3.92	21.55	ND	ND	ND
Vatva PS	N	13.2	110	3.12	0.87	10.3	0.8	0.22	2.02	0.46	23.9	28.5	34.7	15.2	1.39	26.20	10.31	6.05	24.35	38.47	15.01	8.67	92.96	7.35	6.82	4.79
	ORF	7.94	35.4	15.5	0.13	4.63	0.14	7.7	17.2	2.14	6.54	23.2	31.8	10.8	1.63	5.99	2.05	ND	15.88	16.81	15.51	11.45	0.73	0.94	2.54	3.34
	S	1.33	48.7	20.2	1.01	3.01	ND	1.09	8.56	0.76	7.93	17.5	25.3	5.62	2.22	0.88	6.08	1.66	6.64	9.12	8.27	3.63	46.65	ND	5.24	3.12
	Genome	7.51	64.7	12.9	0.67	5.97	0.47	3	9.27	1.12	12.8	23	30.6	10.5	1.75	11.02	6.15	2.57	15.62	21.47	12.93	7.92	46.78	2.76	4.87	3.75

Weekly temporal variations in average SARS-CoV-2 gene copies were analyzed for SARS-CoV-2 RNA presence in samples collected from all the sampling locations in Ahmedabad and are displayed in **Fig. 4a-d**. One-way ANOVA and Duncan post hoc test ($p < 0.05$) was performed to see the significance level in gene copy variation among different sampling dates. The results showed significant differences in all three gene copies, i.e., N-gene (ANOVA, F= 7.49, $p < 0.001$), ORF-1ab genes (ANOVA, F= 5.94, $p < 0.001$), and S-gene (ANOVA, F= 8.25, $p < 0.001$) on the temporal scale (sampling dates). Similarly, differences were significant in the case of effective gene concentration (ANOVA, F= 7.12, $p < 0.001$).

The N-Gene concentration in wastewater samples collected on September 10th, 2020 was found to be significantly higher than other sampling dates, except November 26th, 2020, and lower than November 19th, 2020. The ORF 1ab gene copies/ L in wastewater samples noticed maximum on November 19th, 2020 and were significantly higher than other sampling dates. Except for November 19th, 2020, the changes in ORF 1ab gene concentration were insignificant among different sampling dates. Likewise, the highest S-Gene concentration was noticed on November 19th, 2020 ($p < 0.05$), followed by September 10th, 2020. The S gene copies/ L in wastewater samples collected on September 10th, 2020 was significantly higher than other sampling dates except for November 12th, 2020. In addition to this, the alteration in S-Gene concentration was statistically insignificant among the remaining dates. Moreover, the SARS-CoV-2 effective gene concentration was found to be maximum and significantly higher on November 19th, 2020 than others. The effective gene concentration in wastewater sampled on September 10th, 2020 was significantly higher than the samples of September 24th, 2020 and October 8th & 29th 2020. All three gene copies (i.e., N, ORF1ab, and S genes) and effective gene concentration were detected maximum on November 19th, 2020, and values were significant ($p < 0.05$) as compared to other sampling dates. The exponential rise in virus gene concentration might be due to the decline in the decreasing trend (< -0.1%, November 12th, 2020) followed by the increase in the number of active cases (i.e. 2.5% which corresponded to the 82 new cases on November 19th, 2020), compared to the earlier sampling dates.

The major implications of these temporal variations in monthly and weekly data of various genes can be explained in three ways: i) the explicit effect of variations in new confirmed cases on gene copies. In this context, it is interesting to note that change in the active cases is not showing much relationship with the WBE data; ii) there is not much difference among the individual genes and effective gene concentrations when we visualize the monthly variation; and iii) weekly variation brings out the difference among the various genes and need to normalize the data in effective gene concentrations. Weekly data explicitly confirms that N genes are much more resistant among the three and ORF-1ab seems the least sensitive gene. These two observations are clearly evident in data of 10th September

and 5th November (**Fig. 5**) when the variations/ disagreements among the various genes are explicit. The further implications of these findings are related to the required sampling event and calculations of the effective gene calculations. It is evident here is that biweekly sampling should be enough to get a trend in a given Indian city. Also, COVID-19 wastewater surveillance-based data must not be judged or evaluated based on a single particular gene of SARS-CoV-2 but its effective gene concertation based on multiple genes.

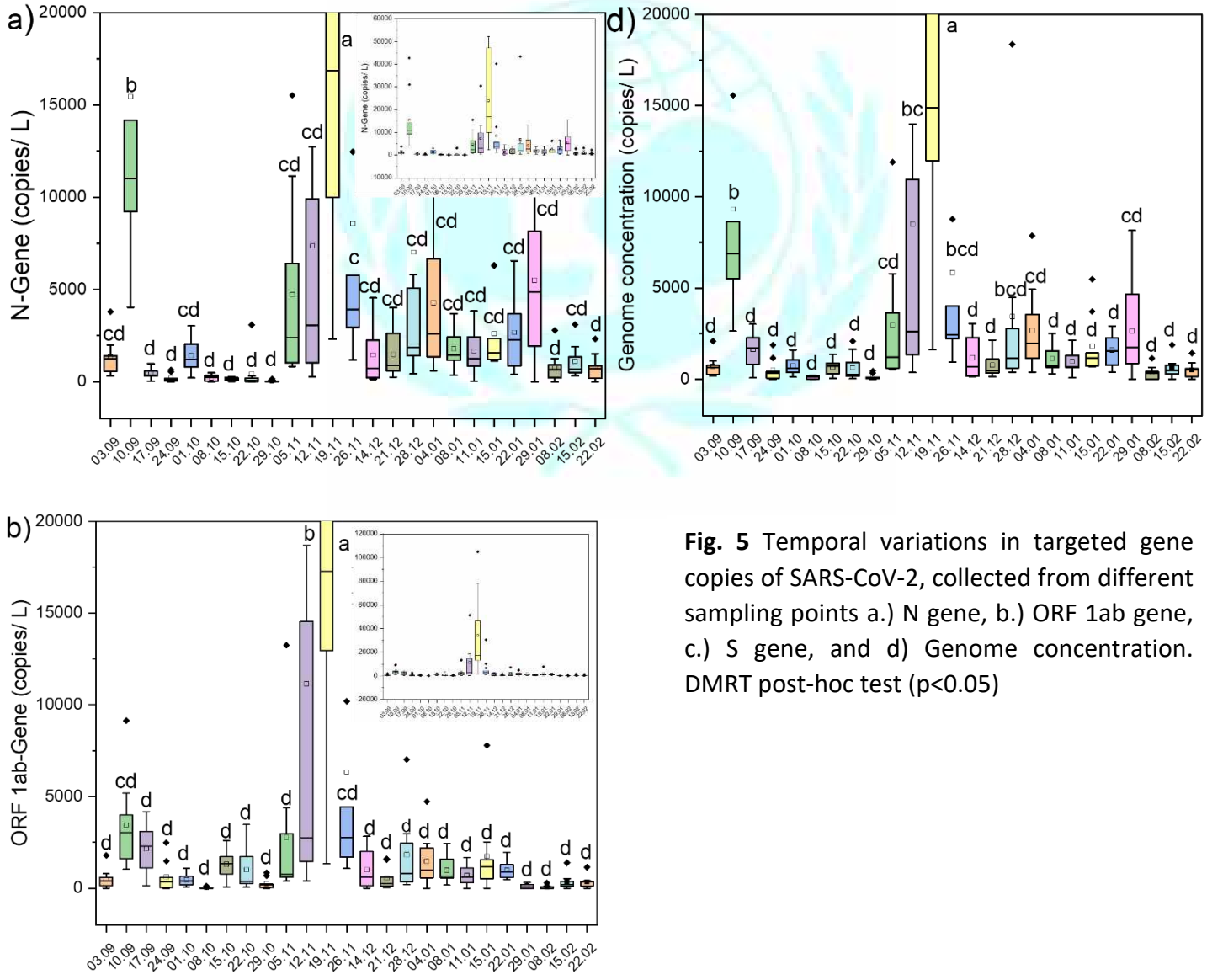


Fig. 5 Temporal variations in targeted gene copies of SARS-CoV-2, collected from different sampling points a.) N gene, b.) ORF 1ab gene, c.) S gene, and d) Genome concentration. DMRT post-hoc test ($p<0.05$)

4.2 SWEEP-based city zonation and Identification of Hot-Spots

Depending on the SARS-CoV-2 effective gene concentration in wastewater samples based on analytical results, we identified highly susceptible areas for COVD-19 infection and its transmission among the community. Although we do not have explicit epidemiological data at the ward level/ sampling locations; variations were good enough to classify a city based on SARS-CoV-2 gene concentration in wastewater samples. The north (Motera and Ranip) and east (Odhav and Satyam) zones were highly affected areas with an average effective gene concentration of ~15,574 and ~13,397 copies/L, respectively, in November (**Fig. 5a**). Likewise, in September, wastewater samples collected from the east zone showed maximum effective gene concentration (~5734 copies/ L), followed by the north zone (~3536 copies/ L). Though areas present in north and east zones showed high virus genetic load, yet a sharp rise in SARS-CoV-2 RNA was noticed in all the zones in November 2020 (**Fig. 5a**). It has also been represented in a summarised format with a comparison to the affected population in the city (**Fig.5b & c**).

It is imperative to note that 5b is a generalised status of the city as of 26th November, 2020 pertaining to the COVID-19 total confirmed cases and **Fig 5c** depicts three months change in SARS-CoV-2 effective gene concentration by bar diagram with existing positive cases of 26th November, 2020 by colour coding. Although it would have been better to provide heat maps, active case distributions and effective gene concentrations over the entire study period to understand the effectiveness of WBE surveillance; the two observations are critical i.e. i) Satyam and Vinzol locations showed opposite monthly trends of SARS-CoV-2 gene concentration. It was found to be higher in case of Vinzol for the month of November compared to Satyam, implying the capability of WBE to distinguish the parts of city based on SARS-CoV-2 gene concentration; and ii) scale of change varies among the sampling locations, therefore seems to be related to the size of the catchment and treatment plant, suggesting month-wise variation is not enough. Also, there is a need for the match between the epidemiological data and SARS-CoV-2 gene concentration in wastewater samples. Overall, despite several challenges in epidemiological and clinical data collection as well as sewage water collection and catchment delineation in India, the proper scrutiny and regular monitoring of wastewater could be useful for preparedness against adverse conditions as appeared in post-festive days in Ahmedabad.

The SWEEP technology offers a better picture of the pandemic situation at the sub-city or zone level, relying on the SARS-CoV-2 RNA concentration in wastewater samples of a particular area. SWEEP data can help to estimate the actual extent of the infection due to the SARS-CoV-2, as it covers both asymptomatic and presymptomatic patients, which may be underestimated by clinical surveillance. Therefore, SWEEP data-based zonation of

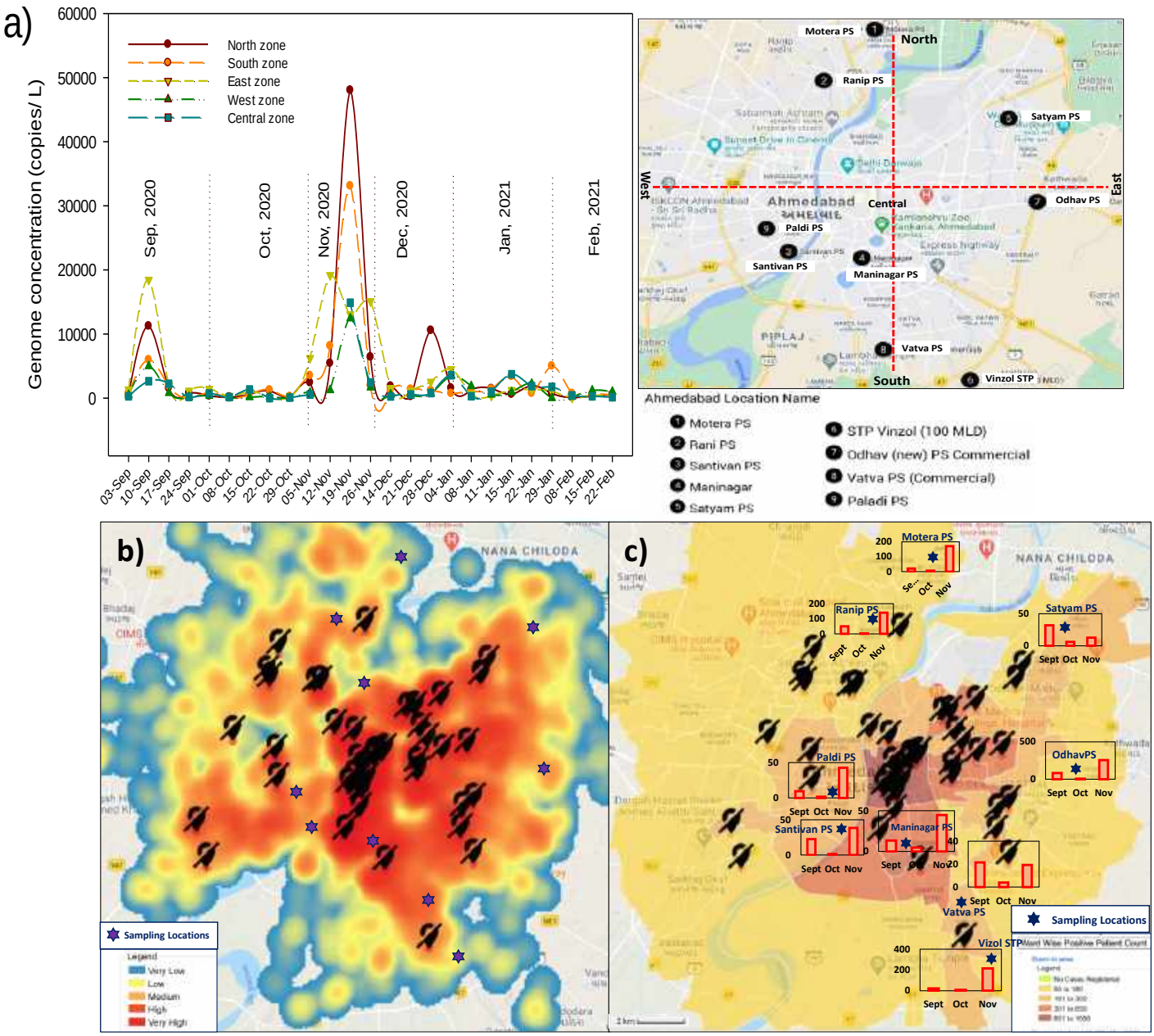


Fig. 5. a) Zone-wise Covid-19 pandemic status in Ahmedabad city; b) Heat map of the overall infected population in Ahmedabad City based on Aarogya-setu mobile application. Very low, low, medium, high and very high indicates no to up to 50, 51-180, 181-300, 301-650, and >651 registered positive covid-19 cases per ward. and c) Month-wise Effective gene concentration at the sampling locations (y-axis in bar diagrams represents SARS-CoV-2 effective gene concentration in copies x 10^2/ L wastewater samples). Note: Positive patient count has been taken on ward basis not on the population-density.

the city can help to identify hot-spots to increase the preparedness in advance. On the other hand, clinical surveillance usually fails to classify the city into distinct zones as it is more dependent on the location of test centres rather than the COVID-19 patients, and owing to asymptomatic and presymptomatic patients. This is why several study could early detect the SARS-CoV-2 RNA in wastewater, before the first

clinical report like Madema et al. (2020) in the Netherlands, La Rosa et al. (2020) in two different cities in Italy and Randazzo et al. (2020) in Spain. However, this is probably the first study where the SARS-CoV-2 RNA data has been compared with ward wise positive patient counts.

4.3 Early Warning Potential of WBE

In this view, the present research work followed our first proof concept study, where we detected SARS-CoV-2 genetic material in wastewater and proposed its wide applicability for COVID surveillance in the community (Kumar et al. 2020a). The linear regression between changes in SARS-CoV-2 effective gene concentration and the number of confirmed cases showed a positive correlation but was not statistically significant ($p = 0.135$, $R = 0.438$). There was no linear relationship between the SARS-CoV-2 gene concentration and epidemiological data. Therefore, we showed the relationship between percentage changes in effective gene concentration and confirmed cases that can be used as a pre-alarming tool, which gives a lead of ~2 weeks for the upcoming scenario (**Fig. 6**). Examining the potential of WBE for COVID-19 surveillance as a potential tool showed that the percentage change in effective gene concentration level on a particular date was in conjunction with the confirmed cases registered 1-2 weeks later on a temporal scale by the regulatory authority based on clinical tests (**Fig. 6**). For example, on October, 8th, 2020, a sharp decline of ~86% was noticed in the percentage change in the average effective gene concentration which was followed by ~0.4% decline in the percentage change in confirmed COVID cases on October, 22nd, 2020. Likewise, on November 5th, 2020, a steep hike of >22-folds in the percentage change in the average effective gene concentration was noticed compared to the earlier sampling date, which was followed by ~0.6% and 2.37% increment in the percentage change in confirmed COVID cases on November 19th and November, 26th, 2020, respectively. In the contrary, more than >1,000% and 500% increase were noticed in percentage change in SARS-CoV-2 effective gene concentration in wastewater in early September and mid-October, respectively. However, there seems no notable increase in the number of confirmed cases 1-2 weeks later. Still, this technique displayed positive prediction in most of the cases during the study period. Therefore, we can predict the severity of the pandemic situation 1-2 weeks prior to the official reports by the regulatory body based on clinical tests.

The results unravel the potential of WBE surveillance of COVID-19 as an early warning tool displayed by the adequate presence of SARS-CoV-2 genetic material in wastewater samples though limited cases were documented and based on the immediate future trends. These findings were in agreement with those of Ahmed et al. (2020b), who noticed a longitudinal decline in the presence of SARS-CoV-2 RNA with the tapering of the first epidemic wave; however, there was no concrete

relationship between virus RNA and daily cases numbers.

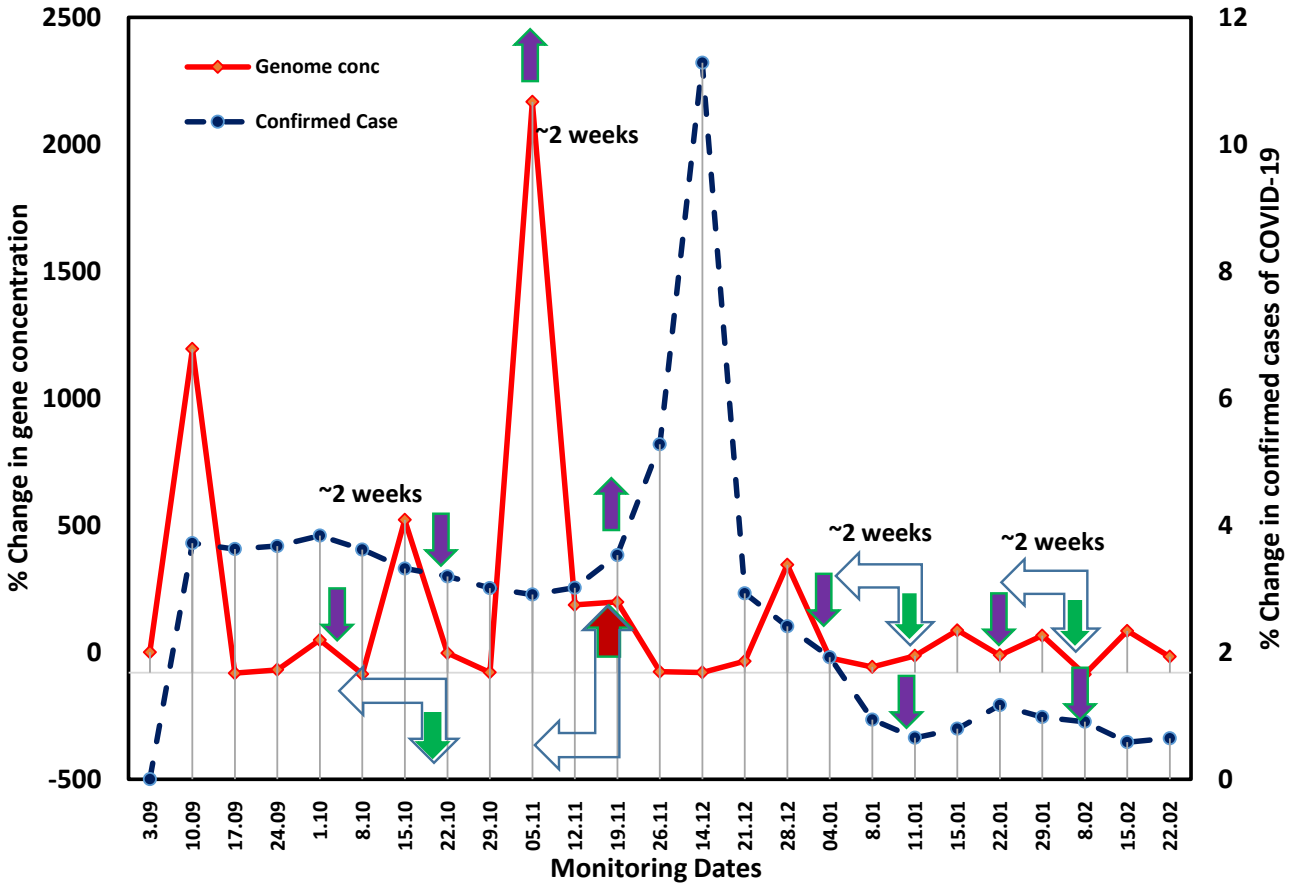


Fig. 6 Potential and evidence of wastewater based epidemiology surveillance of Covid-19 pandemic as an early warning tool

4.4 Removal efficacy of two principally different wastewater treatment plants (WWTPs) for SARS-CoV-2 removal

4.4.1 Research findings of the removal efficacy of WWTPs

We analyzed the efficacy of two treatment processes of CAS and RZT (schematic diagrams of the operating mechanism of both plants in Sargasan and academic campus are shown in **Figs. 7 a** and **b**, respectively). **Table 2** summarizes the change in the Ct-value and gene copies of SARS-CoV-2 N-genes (nucleocapsid protein), S-genes (spike glycoprotein), and ORF 1ab genes (polyprotein) before and after the treatment i.e., in the samples of influent and effluent for two months (August and September 2020) of monitoring. It also provides the date of sampling, effective genome concentration, and active COVID-cases. The Ct values of internal control (MS2 bacteriophage) ranged between 25.41 to 28.01 and 25.59 to 30.08 in the samples from Sargasan and academic institution WWTPs, respectively. No SARS-CoV-2 genes were detected in the negative control samples.

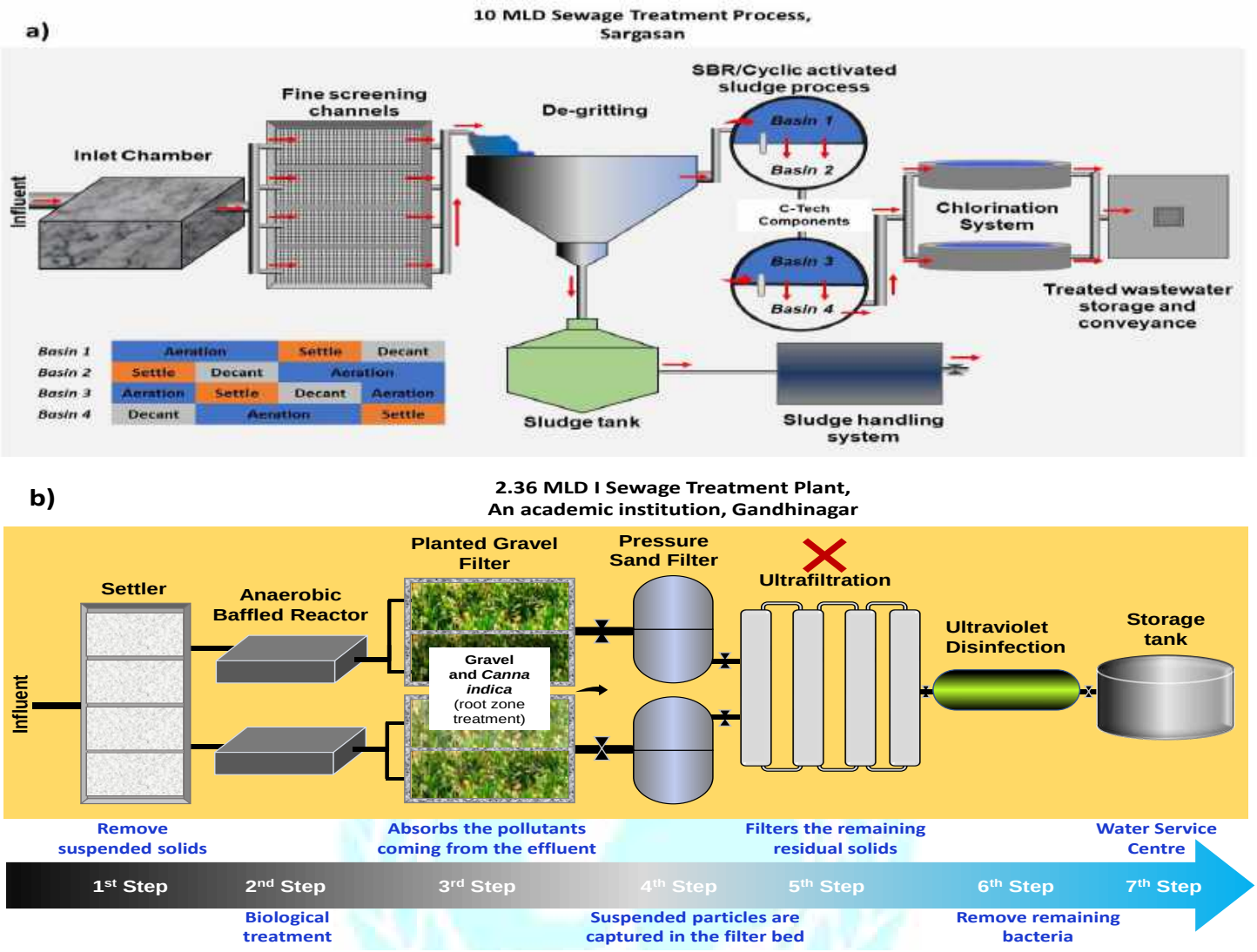


Fig.7. Simplified illustration of the layout of two wastewater treatment plants; a) Conventional Activated Sludge based WWTP in Sargasan, and b) root-zone treatment in an academic institution of Gandhinagar, India monitored during August and September, 2020

Table 2. Temporal variation in SARS-CoV-2 genetic material loading found in the influent and effluent samples collected from two different wastewater treatment plants

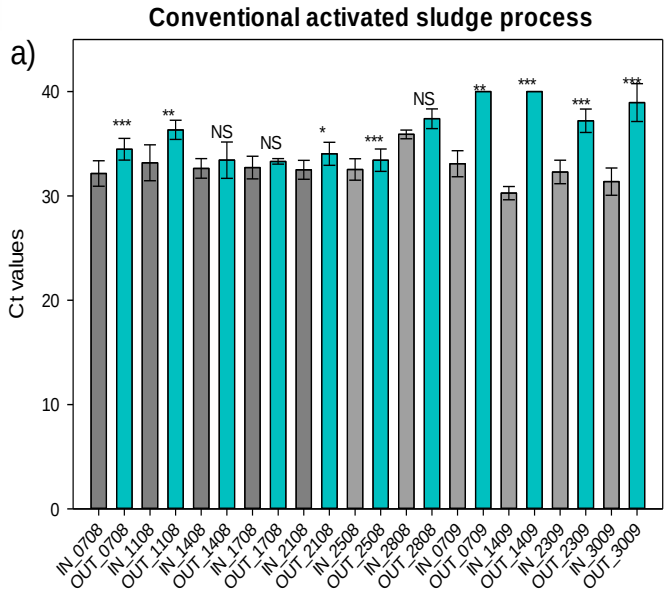
Station		Sampling date	Sampling date Vs Active/ confirmed cases Vs Gene copies										
			August 2020							September, 2020			
			07.08.20	11.08.20	14.08.20	17.08.20	21.08.20	25.08.20	28.08.20	07.09.2	14.09.20	23.09.20	30.09.20
		Active/ confirmed Cases ➡	317/1680	264/ 1793	261/ 1894	269/ 1984	271/ 2097	300/ 2208	329/2317	442/2697	496/ 2967	571/ 3337	613/ 3666
		SARS-CoV-2	Gene Copies (copies/ L) x 10^2										
Sargasan ward	Inlet	N-Gene	8.50	4.60	5.53	5.99	8.07	10.2	0.74	12.4	59.7	3.99	47.5
		ORF-Gene	5.13	1.87	48.8	3.81	4.47	3.72	1.01	5.69	24.4	13.5	13.2
		S-Gene	25.5	17.2	15.1	15.4	14.8	13.2	0.42	2.46	35.3	15.2	8.42
		SARS-CoV-2 Genome	13.0	7.89	8.50	8.41	9.10	9.05	0.98	6.85	39.8	10.9	23.0
	Outlet	N-Gene	1.80	0.60	7.86	5.07	4.94	5.99	0.67	ND	ND	0.24	0.55
		ORF-Gene	1.40	0.50	1.27	5.46	1.37	2.05	0.23	ND	ND	0.46	ND
		S-Gene	4.83	1.40	9.21	3.95	4.10	7.26	0.42	ND	ND	0.86	ND
		SARS-CoV-2 Genome	2.68	0.83	6.11	4.82	3.47	5.10	0.44	ND	ND	0.52	INC
Academic institution	Inlet	N-Gene	NA	1.83	23.6	18.3	3.50	8.62	5.05	ND	3.95	ND	1.01
		ORF-Gene	NA	0.74	14.8	11.2	0.69	8.42	8.53	ND	ND	ND	0.43
		S-Gene	NA	5.60	22.6	29.8	4.94	20.3	21.3	ND	ND	0.21	ND
		SARS-CoV-2 Genome	NA	2.72	20.3	19.8	3.04	12.5	11.6	ND	INC	INC	0.72
	Outlet	N-Gene	NA	0.72	5.25	6.07	2.90	5.46	1.67	ND	9.15	0.15	0.25
		ORF-Gene	NA	ND	4.54	2.18	0.97	3.78	0.77	0.28	ND	0.70	ND
		S-Gene	NA	1.86	4.32	13.8	1.74	7.34	2.87	ND	ND	ND	ND
		SARS-CoV-2 Genome	NA	0.86	4.70	7.35	1.87	5.52	1.77	ND	INC	0.43	INC

Low High

Paired T-tests between the inlet and outlet wastewater samples, taken on the same days, were performed to understand the significance of the SARS-CoV-2 gene removal efficacy of each treatment process, i.e., CAS process-based treatment at Sargasan (**Fig. 8a**) and RZT at an academic institution in Gandhinagar (**Fig. 8b**). We then combined the data and conducted paired T-test analyses of the significance of SARS-CoV-2 gene removal efficacy based on Ct-values obtained and various gene copies calculated for CAS (**Figs. 9a** and **c**) and RZT (**Figs. 9b** and **d**), respectively.

Overall comparison of SARS-CoV-2 genome removal efficacy of CAS and RZT is expressed through paired T-test performed on the total effective genome concentrations obtained throughout the 60 days of monitoring (**Fig. 10**). Monthly variations and their significance of SARS-CoV-2 genes removal efficacy of CAS; and RZT is presented in **Fig. 11** to understand the impact of genetic loading in the influent and its correlation with removal efficacy of the treatment processes. MVA was conducted to understand the overall impact of treatment by visualizing the PC loading in a 3-D domain for various water quality parameters and SARS-CoV- 2 gene loading of collected influent (untreated) and effluent (treated) samples during the two-month monitoring period (**Figs. 12a** and **b**).

Although there will be a considerable uncertainty, we could estimate the number of people shedding SARS-CoV-2 to wastewater. SARS-CoV-2 is contained in the human stool at 4-6 log copy/g, and assuming that the average stool weight is 500 g per day per person, that results in $5x10^6$ to $5x10^8$ copies per person per day shredded to wastewater. Assuming that our raw wastewater samples had 1000 copies/L on average, raw wastewater from Sargasan WWTP had $1x10^9$ copies per day, implying that there were 2 to 200 people shedding SARS-CoV-2 in the catchment on a day. However, there would be too many uncertainties in this calculation, due to significant decay/reduction of viral RNA during transport from toilets to WWTPs. Therefore, hereafter, only Ct-values and gene copies are compared. Further, the role of aqueous and solid-phase interactions for the quantification of SARS-CoV-2 gene concentrations has been prominently highlighted in terms of recovery of the viral RNA in the aqueous environment through solid fractions (Kitamura et al., 2020). However, we did not take sludge into account as there still needs a robust standard protocol for sludge clean up and RT-qPCR measurements to be established.



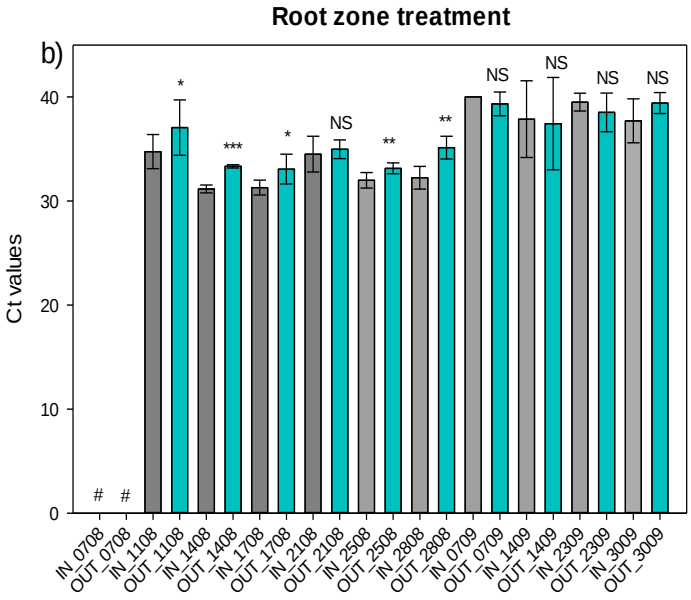


Fig. 8. Paired T-test between inlet and outlet wastewater samples taken on the same days for SARS-CoV-2 genetic load in a) Conventional activated sludge process-based treatment at Sargasan, and b) Root-zone treatment at academic institution in Gandhinagar. (where *** = p <0.01; ** = p <0.05; * = p <0.1; NS = not significant; # = data not available; and RT-PCR was run for 40 cycles)

4.4.2 Discussion on the removal efficacy of WWTPs

4.2.2.1 Significance of Treatment

Of the eleven samples collected from the inlet and outlet points of WWTPs during the study period, eight samples from Sargasan and five samples from the academic institution showed significant removal of the viral genes (**Figs. 8a** and **b**). Paired T-tests between influent and effluent wastewater show a significant reduction through CAS treatment systems except for three occasions. Reduction/removal of SARS-CoV-2 genes was highly significant (p <0.01) in nearly 50% of the samples, with non-significant removal in August only. RZT appeared effective in August but failed to show significant removal of SARS-CoV-2 RNA in September. There may be two possible explanations related to the operation of WWTPs and COVID-19 cases in the vicinity of WWTPs. The RZT was situated and precisely received waste from the campus dwellers and visitors only, and COVID-19 cases increased in September 2020. Thus, even if we assume the viral shedding contribution of visitors was non-variable, it is certain that genetic loading increased in the RZT plant during September 2020. We also suspect that operating conditions at the treatment plants were not consistent throughout the monitoring period. Nevertheless, the RZT achieved significant removal on more than 50% of the sampling dates.

Paired t-tests show that irrespective of treatment type, the N-gene is much more stable than S- and ORF-1ab genes of SARS-CoV-2 (**Figs. 9a** to **d**). Removal efficacy was highest for S genes (p <0.01) followed by ORF-1ab (p <0.05) for both treatment processes. Overall, N genes showed non-significant reduction after treatment. The ORF 1ab-gene copy numbers decreased by 84.4% (t=2.78, p=0.022) and 70.5% (t=2.30, p=0.047) in Sargasan WWTP and the academic institution WWTP, respectively (**Figs. 9c** and **d**). Likewise, S-genes were significantly removed by both treatment plants (80.5%, t=4.10, p=0.002 at Sargasan and 69.5%, t=2.84, p=0.019 at the academic institution). Conversely, the abundance of N-gene declined 83.4% at Sargasan WWTP (**Fig. 9c**) and 52.0% at the academic institution during treatment (**Fig. 9d**), but the differences in S- and N-gene removal were statistically significant (t=2.04, p=0.069 and t=1.59, p=0.147, respectively). The results showed that both the cyclic activated sludge process and root zone treatment plants of Sargasan and the academic institution

effectively removed ORF ab-genes and S-genes, but not N-genes.

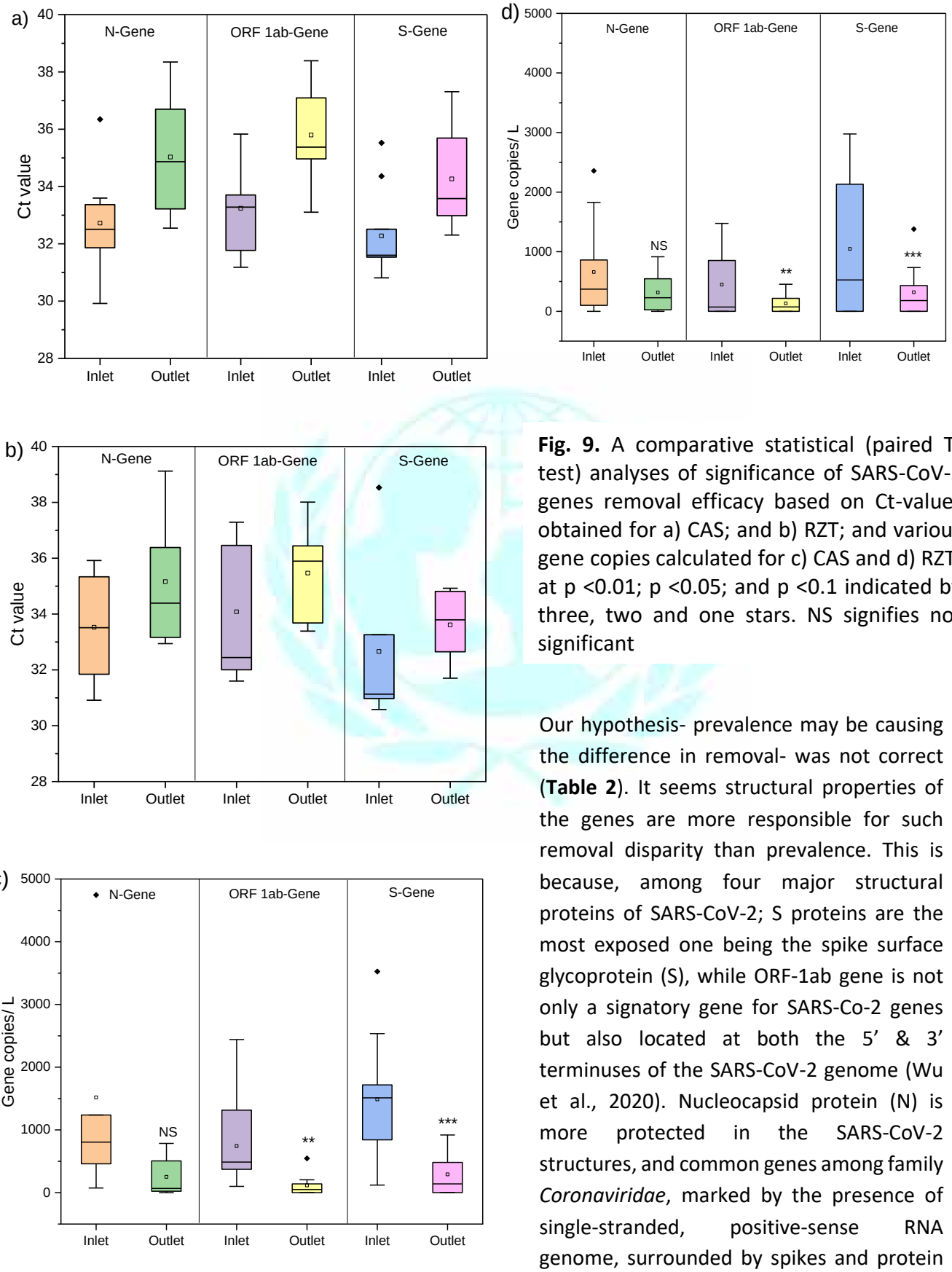


Fig. 9. A comparative statistical (paired T-test) analyses of significance of SARS-CoV-2 genes removal efficacy based on Ct-values obtained for a) CAS; and b) RZT; and various gene copies calculated for c) CAS and d) RZT; at p <0.01; p <0.05; and p <0.1 indicated by three, two and one stars. NS signifies not significant

Our hypothesis- prevalence may be causing the difference in removal- was not correct (**Table 2**). It seems structural properties of the genes are more responsible for such removal disparity than prevalence. This is because, among four major structural proteins of SARS-CoV-2; S proteins are the most exposed one being the spike surface glycoprotein (S), while ORF-1ab gene is not only a signatory gene for SARS-Co-2 genes but also located at both the 5' & 3' terminuses of the SARS-CoV-2 genome (Wu et al., 2020). Nucleocapsid protein (N) is more protected in the SARS-CoV-2 structures, and common genes among family *Coronaviridae*, marked by the presence of single-stranded, positive-sense RNA genome, surrounded by spikes and protein

envelope. Earlier studies suggested reduction of SARS-CoV-2 genetic material during wastewater treatment processes via secondary treatment such as activated sludge/ A2O/ extended aeration and tertiary treatment such as disinfection, coagulation, flocculation, sand filtration, NaClO/UV (Randazzo at al., 2020). Interestingly, none of the studies investigated the removal efficacy of a given treatment for SARS-CoV-2 RNA. In our study, both the CAS and RZT processes are found to effectively remove SARS-CoV-2 RNA. To the best of our knowledge, this is the first report assessing the effectiveness of RZT for SARS-CoV-2 RNA reduction.

4.2.2.2 Comparative efficacy of CAS and RZT processes to remove SARS-CoV-2 genes

SARS-CoV-2 RNA is substantially reduced in treated wastewater i.e. effluents of both WWTPs throughout the sampling period, as indicated by the overall comparison of SARS-CoV-2 genome removal efficacy of CAS and RZT through a paired T-test (**Fig. 10**). Although there was a significant difference in average SARS-CoV-2 genome concentration in the influents of the CAS plant at Sargasan (1.25×10^3 copies/ L) and the RZT system of an academic institution (7.07×10^2 copies/ L). Yet, both processes mostly showed effective removal at $p<0.05$. However, incomplete removal may have some environmental and health implications.

While infectivity and viability of these genomes are still being debated and researched with a general consensus of viability being less likely and thus the infectivity, there is still no study that has yet proven the chance of transmission and infectivity impossible. In such a scenario, significant removal is not enough, as such effluents will finally be received by the ambient waters. Therefore, we foresee an immediate increase in reporting of SARS-CoV-2 genes in freshwater systems like lakes, rivers, and perhaps groundwater. Several imperative hypotheses need to be tested in this regard, and the present study signifies the need of such investigations.

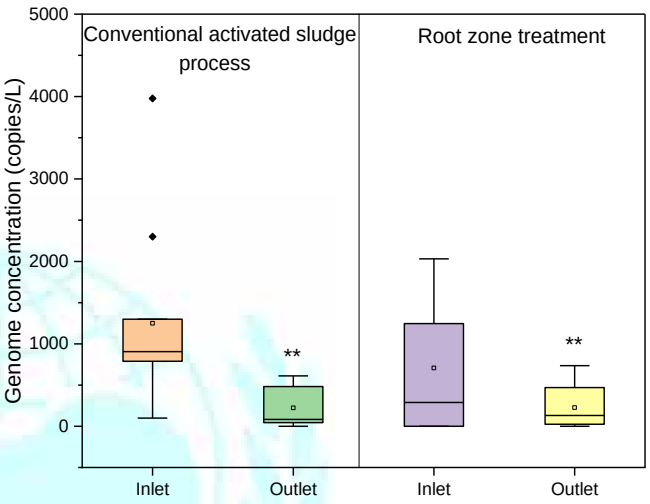


Fig. 10. Overall comparison of SARS-CoV-2 genome removal efficacy of conventional activated sludge and root-zone treatments expressed through paired T-test performed on the total effective genome concentrations obtained through out the 60 days of monitoring period. Same level of significance is used as above.

Further, we also suspect that the size of the treatment plant and operational and management consistencies, along with the quality of influent water will play a critical role in the entire research scenario of COVID-19 transmission and monitoring (Kumar et al., 2021a). As far as treatment type is concerned, the RZT will show a bit wider fluctuation than the CAS treatment process (**Fig. 10**). The low genome concentration at the academic institution WWTP is apparently

due to institutional wastewater load which was confined to the
institutional community and malfunctioning of the ultrafiltration unit of the WWTP. Conversely, the Sargasan WWTP receives municipal wastewater, resulting in the presence ofSARS-CoV-2 RNA in effluent wastewater, owing to fluctuating genetic loading in the inlet waters. We conclude that both WWTPs effectively removed viral genes, but Sargasan STP was more efficient (82.4% decrease, t=2.98, p=0.014) than the academic institution (67.9% decrease, t=2.54, p=0.032) (**Fig. 10**). It is imperative to note that we have collected samples from both treatment processes after disinfection processes and still found the genetic fragments of SARS-CoV-2 in the effluent. This observation may imply that owing to nanosized colloidal nature of genetic fragments, disinfection processes like chlorination/UV are likely to be less effective than the process of coagulation.

Overall, as PCR-based detection of RNA does not mean detection of viable SARS-CoV-2, and quantifying active (viable) SARS-CoV-2 is a difficult challenge, with so far only one lab-scale experiment reported (Bivins et al. 2020), we recommend further study for a valid discussion on implications of leftover SARS-CoV-2 RNA after the treatment. However, our data explicitly disapprove the general notion that treatment completely removes the genetic fragments of SARS-CoV-2.

4.2.2.3 Temporal variation in removal efficacy

As suspected above, we investigated the role of influent quality in terms of SARS-COV-2 genetic loading through temporal variation in the performances of both CAS and RZT systems (**Fig. 11**). For CAS plant in Sargasan ward, inlet quality in September showed higher genetic loading than that of August, which has been verified by confirmed COVID-19 cases in the city, yet removal was better in September than August 2020. When inquired with operational staff, it seems that operational inconsistencies are responsible for these results rather than the genetic material loading. While in the case of the academic institution RZT-based plant, where the operation was rather more consistent, it seems that genetic material loading in the inlet water has reflected the genome concentration left in the effluent waters. This is also very likely to be attributed to the size of plant i.e., CAS facility of Saragasan is 10,000 m3/day against 2360 m3/day of the RTZ plant of the academic institution, leading to the sensitivity of RZT plant for genetic loading in the inlet wastewater. Nevertheless, at this juncture, we take these results as indicative ones, and more convincing conclusions pertaining to the role of influent water quality, and its implication may be derived after further monitoring. Such notion has also been expressed elsewhere (Lescure et al., 2020; Hata et al., 2020b; McCarty et al., 1986).

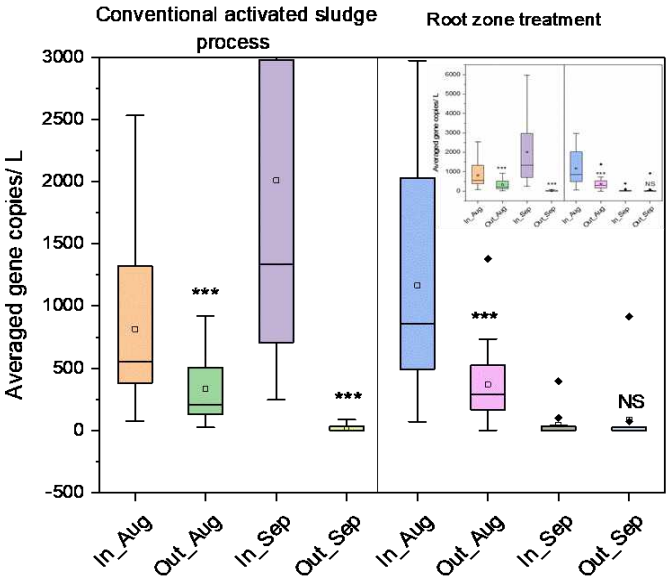
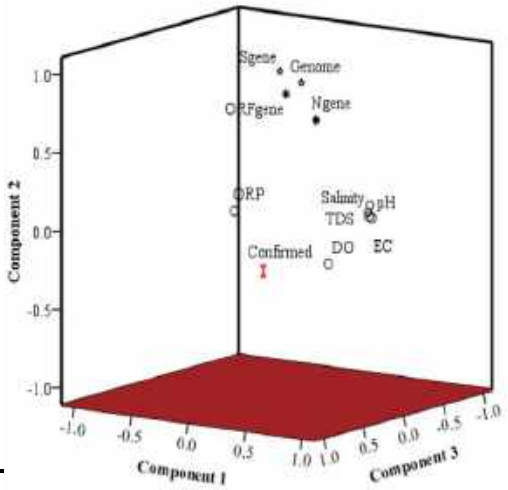


Fig. 11. A comparative statistical (paired T-test) analyses in monthly variation of significance of SARS-CoV-2 genes removal efficacy of CAS; and b) RZT; at $p < 0.01$; $p < 0.05$; and $p < 0.1$ indicated by three, two and one stars. NS signifies not significant

4.2.2.4 Treatment Impact Insight through multivariate statistical analyses

Principal component analyses show a comprehensive picture of the overall contribution and influence of treatment on SARS-COV-2 gene removal. The entire dataset obtained for influent and effluent were subjected to PCA and projected in the 3-D domain of three main PCs. Owing to more complex nature of influents, four PCs were identified after nine iterations that explain 90% of the total variance in the dataset of influent waters. The first PC explains 34% of the total variance with significant loading for in-situ water quality parameters forming a cluster (EC, TDS, Salinity, and pH) with moderate loading (0.5) for N genes. On the other hand, nearly the same (~30%) variation of data sets is explained by SARS-COV-2 genes, and genome concentrations form a cluster upper left domain with significant loadings for effective genome concentrations (0.94) followed by S-genes, ORF-1ab, and N-genes as PC2. Interestingly in influent waters, N-genes illustrated moderate to high loading as both PC1 and PC2.

After treatment, the complexion changed significantly with the overall reductions of PCs to three, explaining cumulative variations of 80% in the dataset. Another significant observation was that SARS-CoV-2 genes exhibit higher loadings than the in-situ water quality parameters in effluent waters. Order of loadings among SARS-CoV-2 genes and genome remains the same i.e., effective genome concentration > S-genes > ORF-1ab > N-genes. Confirmed COVID-19 emerged as PC3 (with moderate loading of 0.78) in influent waters, stressing the relationship of confirmed cases with SARS-CoV-2 RNA in the wastewater, but the influence was weakened in the treated water with non-significant say in the quality variations of the samples (Orive et al., 2020; Pan et al., 2020). This is the first time MVAs was used with wastewater surveillance dataset to signify the impact of treatment, which eventually proves that: i) wastewater surveillances did track COVID-19 loading of the community; ii) influent waters present a better picture in terms of SARS-CoV-2 gene monitoring; iii) effective genome concentration should be calculated based on presence/absence of multiple genes rather the presence of one specific gene; iv) N-genes are the most resistant to treatment with higher sensitivity than S and ORF-1ab genes; and v) the presence of residual SARS-CoV-2 genes after treatment is critical from the effluent quality point of view. Among the other exciting observations; the explicit grouping/clustering of SARS-CoV-2 genes and other water quality parameter; and influence of confirmed COVID-19 cases has been significant from the wastewater-based epidemiology perspectives.



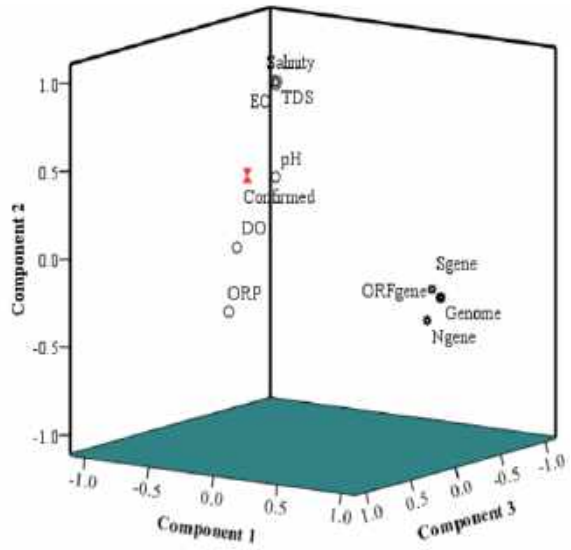
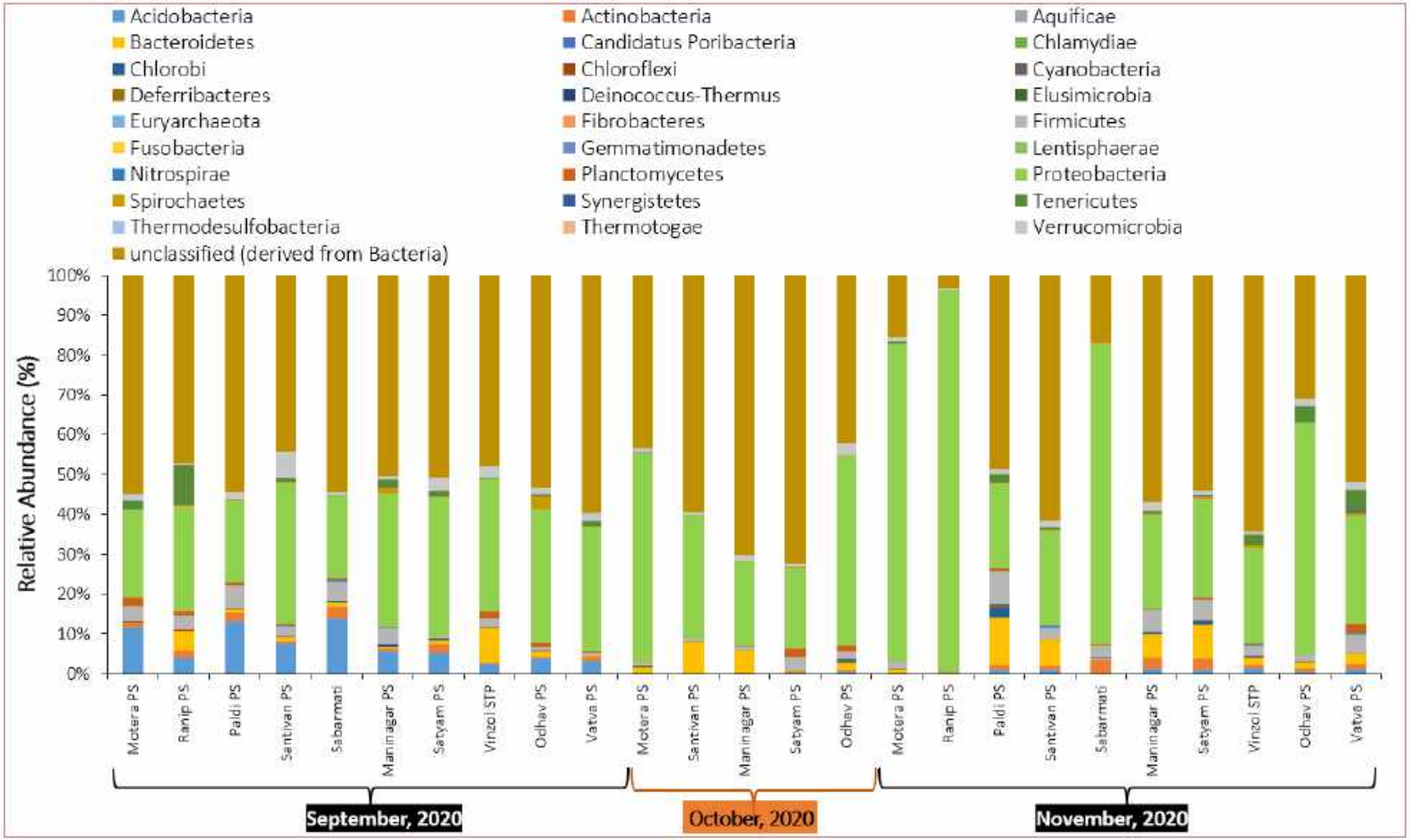


Fig. 12. Three-dimensional projection of the principal component loading for a) Influent and b) effluent; exhibiting the effect of treatment on SAR-CoV-2 genes association with other water quality parameters and confirmed cases of COVID-19

4.5 Metagenome analysis of bacterial population and their relation with SARS-CoV-2 RNA harbouring in wastewater

The results suggest no clear-cut pattern among the bacterial population and association with SARS-CoV-2 genetic load in wastewater samples. Some of the bacterial population significantly changed on monthly temporal scale but no clear-cut concluding pattern was seen. There was significant difference at the bacterial taxonomic level was observed between the untreated and treated wastewater samples. We did not have explicit raw data of the wastewater quality parameters on the sampling date, therefore cannot draw a concrete and convincing finding. The results were not promising but they indicated a possible correlation ship among the SARS-CoV-2 gene concentration and bacterial population and dynamics. Therefore, further investigation is required considering different influencing factors such as sampling timing, sewage flow rate, treatment process, and wastewater physico-chemical parameters.

a.)


Acidobacteria
Actinobacteria
Aquificae
Bacteroidetes
Candidatus Poribacteria
Chlamydiae
Chlorobi
Chloroflexi
Cyanobacteria
Deferribacteres
Deinococcus-Thermus
Elusimicrobia
Euryarchaeota
Fibrobacteres
Firmicutes
Fusobacteria
Gemmatimonadetes
Lentisphaerae
Nitrospirae
Planctomycetes
Proteobacteria
Spirochaetes
Synergistetes
Tenericutes
Thermodesulfobacteria
Thermotogae
Verrucomicrobia
unclassified (derived from Bacteria)
Relative Abundance (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Motera PS
Ranip PS
Paldi PS
Santivan PS
Sabarmati
Maninagar PS
Satyam PS
Vinzol STP
Odhav PS
Vatva PS
Motera PS
Santivan PS
Maninagar PS
Satyam PS
Odhav PS
Motera PS
Ranip PS
Paldi PS
Santivan PS
Sabarmati
Maninagar PS
Satyam PS
Vinzol STP
Odhav PS
Vatva PS
September, 2020
October, 2020
November, 2020

b.)

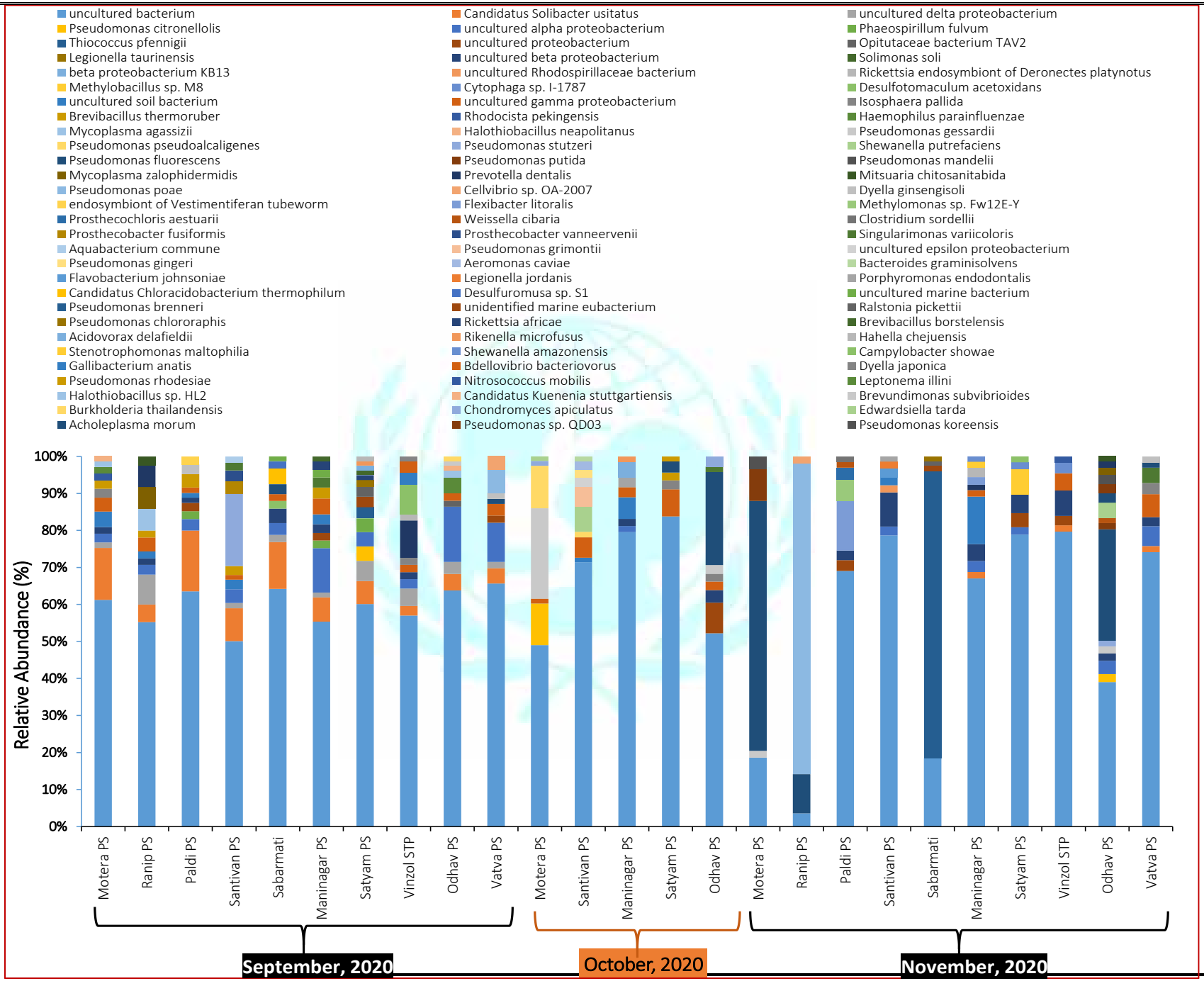


Fig. 13. Temporal variation in the bacterial population present in wastewater samples of Ahmedabad at, a) phylum level; b) species level

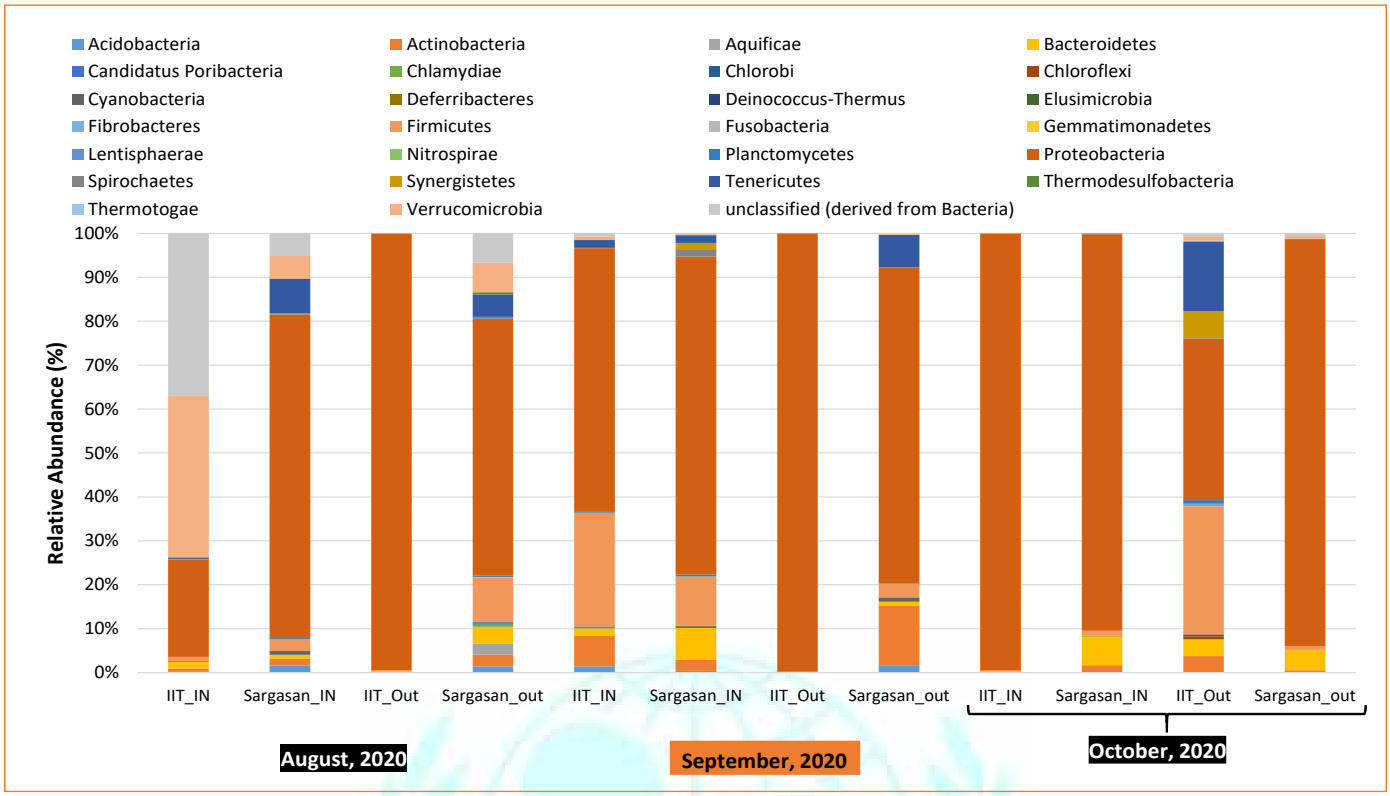
Table 3. Ecological indexes and main data comparisons

Months	Index								
	Sampling site	Taxa_S	Individuals	Dominance_D	Simpson_1-D	Shannon_H	Evenness_e^H/S	Fisher_alpha	Chao-1
10-Sep-20	Motera PS	768	82502	0.2721	0.7279	2.54	0.01651	117.1	1183
	Ranip PS	800	89031	0.2114	0.7886	2.849	0.02158	121.2	1235
	Paldi PS	602	50659	0.276	0.724	2.57	0.02171	96.01	1129
	Santivan PS	751	116172	0.2069	0.7931	2.649	0.01882	107.5	1089
	Sabarmati	756	108408	0.2862	0.7138	2.545	0.01686	109.6	1170
	Maninagar PS	746	66817	0.2325	0.7675	2.707	0.02008	117.6	1236
	Satyam PS	684	94119	0.2576	0.7424	2.646	0.02061	99.86	1093
	Vinzol STP	867	106987	0.2335	0.7665	2.716	0.01744	129	1431
	Odhav PS	795	110388	0.2965	0.7035	2.404	0.01392	115.9	1228
	Vatva PS	547	58135	0.2956	0.7044	2.411	0.02037	83.56	909.1
15-Oct-20	Motera PS	555	113036	0.2531	0.7469	2.074	0.01434	75.97	834.6
	Santivan PS	643	86313	0.3473	0.6527	2.265	0.01498	94.27	1011
	Maninagar PS	752	98503	0.4312	0.5688	2.057	0.01041	110.7	1144
	Satyam PS	522	111041	0.5258	0.4742	1.569	0.009195	70.96	964.8
	Odhav PS	571	49258	0.2239	0.7761	2.552	0.02248	90.64	1014
19-Nov-20	Motera PS	285	13149	0.3346	0.6654	2.1	0.02866	51.36	435.1
	Ranip PS	304	116147	0.6521	0.3479	0.942	0.008438	37.86	479.5
	Paldi PS	724	61460	0.2432	0.7568	2.938	0.02608	115.3	1137
	Santivan PS	894	63023	0.3633	0.6367	2.478	0.01333	147.5	1249
	Sabarmati	311	94675	0.4635	0.5365	1.662	0.01694	40.03	469.1
	Maninagar PS	878	57351	0.2351	0.7649	3.053	0.02413	147.1	1319
	Satyam PS	563	52123	0.2944	0.7056	2.823	0.02988	88.2	720.5
	Vinzol STP	738	52006	0.4141	0.5859	2.177	0.01195	121.8	1104
	Odhav PS	349	15895	0.1543	0.8457	2.996	0.05731	63.07	579.8
	Vatva PS	504	48908	0.2696	0.7304	2.88	0.03535	78.27	674.5

4.5.1 Comparison of bacterial profile between untreated and treated wastewater samples

The results suggest no clear-cut pattern among the bacterial population and association with SARS-CoV-2 genetic load in wastewater samples. Some of the bacterial population significantly changed on monthly temporal scale but no clear-cut concluding pattern was seen. There was significant difference at the bacterial taxonomic level was observed between the untreated and treated wastewater samples. We did not have explicit raw data of the wastewater quality parameters on the sampling date, therefore cannot draw a concrete and convincing finding. The results were not promising but they indicated a possible correlation ship among the SARS-CoV-2 gene concentration and bacterial population and dynamics. Therefore, further investigation is required considering different influencing factors such as sampling timing, sewage flow rate, treatment process, and wastewater physico-chemical parameters.

a.)



b.)

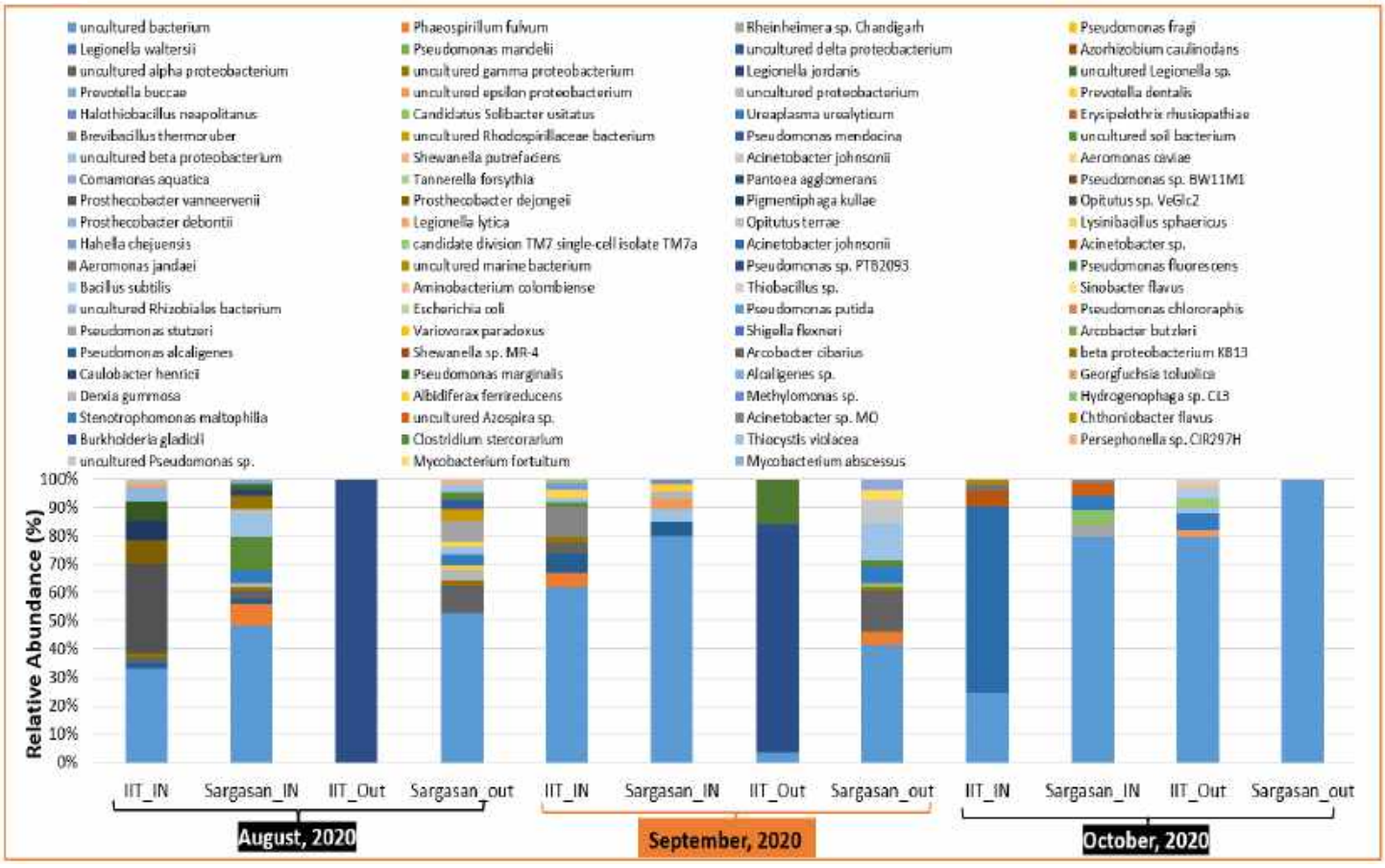


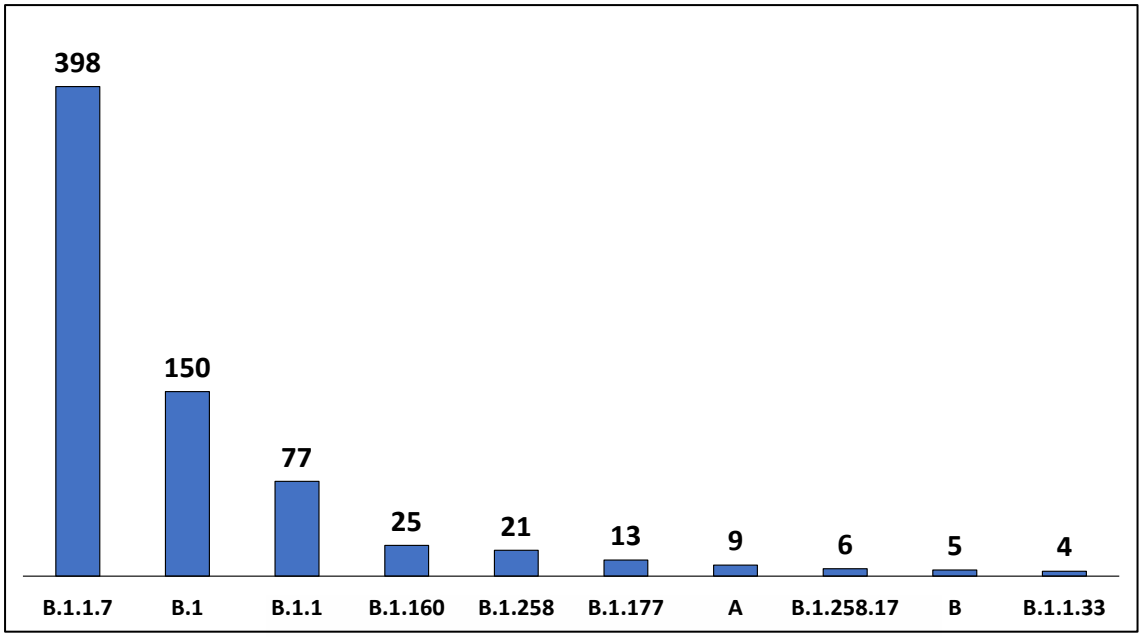
Fig. 14. Temporal variation in the bacterial population present in untreated and treated wastewater samples at, a) phylum level; b) species level

4.5 SARS-CoV-2 whole genome sequencing from the wastewater samples

Identification of the circulating variant from the wastewater provides critical information about the possible undetected cases in the populations and thus support the early warning of the coming pandemics situation in real time. Therefore, insights from our study further highlight the role of the dominant circulating variants from wastewater. Key spike protein mutations that were identified in the SARS-CoV-2 genome assembly as compared to the reference Wuhan/Hu-1/2019 (EPI_ISL_402125) variant that were identified include C21618G/Thr19Arg (T19R), T22917G/Leu452Arg (L452R), C22995A/Thr478Lys (T478K), A23403G/Asp614Gly (D614G), and C23604G/Pro681Arg (P681R) from the samples collected in the month of February, 2021. The observation of the deletion at 22029 (6 bp), 28248 (6 bp) and 28271 (1 bp) were also observed and seen in the B.1.617.2 lineage. These findings point towards probably an early circulating B.1.617.2 lineage in Ahmedabad, Gujarat while clinical samples sequenced in the month of March, 2021 were detected with the cases of B.1.617.2 variant. The variants of concern (VOCs) can be more transmissible resulting in probably higher disease severity outcomes and are also known for reduced sensitivity to antibody neutralisation (Wang et al. 2021; Davies et al. 2021). These variants often harbour multiple mutations in the spike protein and other prominent genomic regions which may result in attenuated effectiveness of SARS-CoV-2 therapeutic interventions. Therefore, it is essential to track current circulating variants and dominant mutations to identify rapidly evolving new variants to ensure an appropriate public health response and interventions. The classification of the variants of concern are defined by Public Health England (PHE), UK (https://github.com/phe-genomics/variant_definitions).

These variants are significant in terms of viral pathogenicity, virulence and transmission. E484K is located in the receptor binding ridge of the spike protein and is found in many lineages including B.1.351 (VOC-20DEC-02), P.1 (VOC-21JAN-02), A.23.1 (VUI-21FEB-01), B.1.525, B.1.1.318, P.2 (VUI-21JAN-01), B.1.324.1, a subclade of B.1.526, and P.3 (VUI-21MAR-02). This mutation reduces binding to polyclonal sera (Greaney, Loes, et al. 2021b) and escapes treatment with the antibody REGN10933 (Starr et al. 2021) which is part of the REGN-COV2 cocktail. It also results in escape from class 2 antibodies (Greaney, Starr, et al. 2021). The mutation P681H is located adjacent to the spike protein furin cleavage site and is found in B.1.1.7 (VOC-20DEC-01), B.1.1.318 and P.3 (VUI-21MAR-02), and P681R is found in A23.1 and all B.1.617 lineages. P681H has been shown to enhance cleavage of spike (Brown et al. 2021). The impact of this increased efficiency in cleavage is not clear but is one hypothesis to explain the enhanced transmissibility of B.1.1.7. Similarly, D614G is known for enhanced transmissibility of the SARS-CoV-2 (Korber et al. 2020).

a.)



b.)

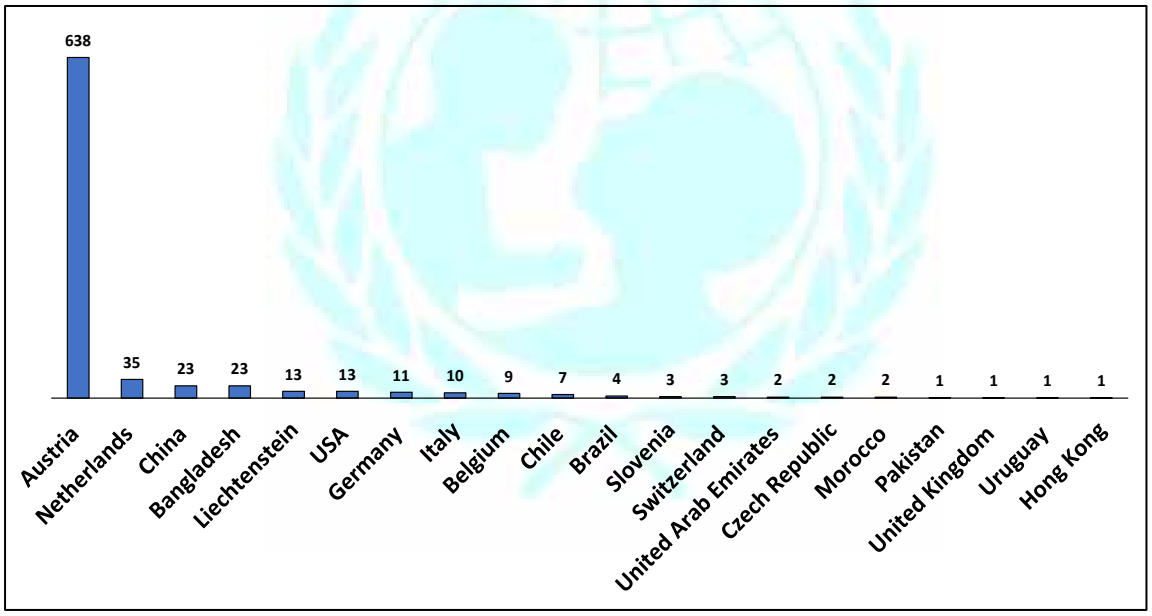


Fig. 15 a) Top 10 SARS-CoV-2 Lineages reported from Environmental Sites; **b)** Countries reported SARS-CoV-2 genomes from Environmental Sites

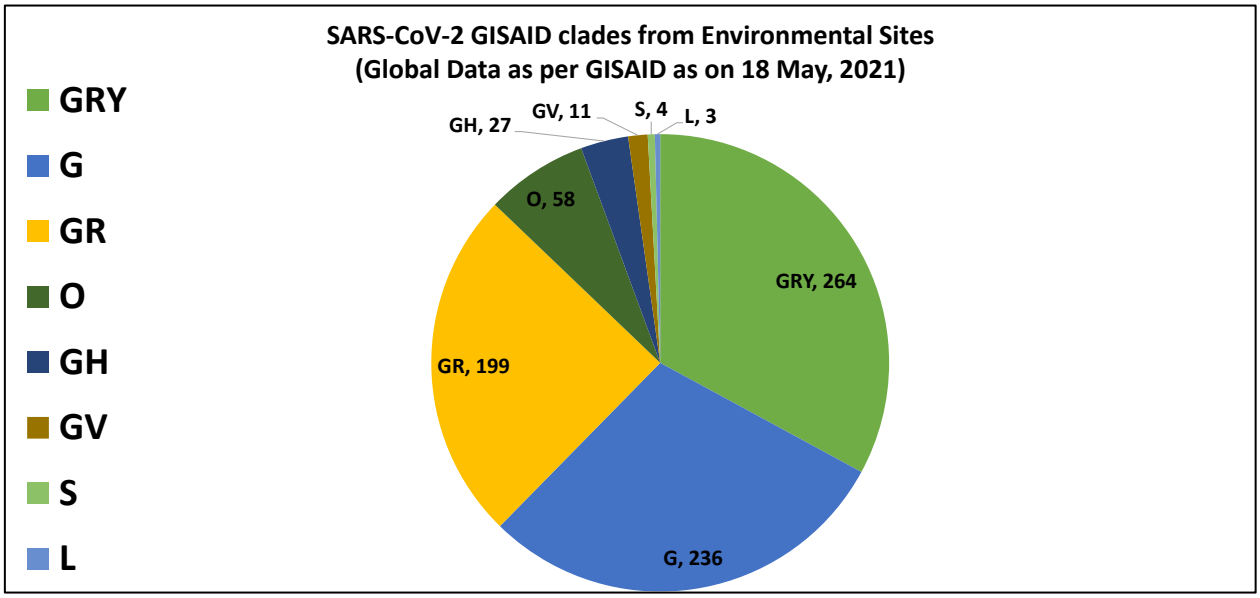


Fig. 16. SARS-CoV-2 genomes from the environmental sites. From Asian countries China (n=23), Bangladesh (n=23), United Arab Emirates (n=2), Pakistan (n=1) and Hong Kong (n=1) reported SARS-CoV-2. Globally, a total of 802 genomes were available from the samples collected from environmental sites. Austria (n=638, 79.55%) has submitted highest genomes from the sewage treatment sites. Overall, dominance of the PANGO-lineages is represented by B.1.1.7 (n=398, 49.63%), B.1 (n=150, 18.70%) and B.1.1 (n=77, 9.60%). Dominance of the GISAID clades is represented by GRY (n=264, 32.92%), G (n=236, 29.43%) and GR (n=199, 24.81%). No report of SARS-CoV-2 Whole Genome Sequence from environmental sites from India as per GISAID server as on 18 May, 2021.

Table 4a-d. Variants of Spike protein from river and waste water samples

a) Sabarmati river (Date of sampling: 22.09.2020)

Reference Position	Type	Length	Reference	Allele	Zygosity	Count	Coverage	Frequency	Forward/reverse balance	Average quality	Overlapping annotations	Coding region change	Amino acid change
21618	SNV	1	C	G	Homozygous	109	110	99.09090909	0.293577982	29.60550459	CDS: S, Gene: S	YP_009724390.1:c.56C>G	YP_009724390.1:p.Thr19Arg
21754	SNV	1	G	T	Homozygous	10	11	90.90909091	0.4	29.4	CDS: S, Gene: S	YP_009724390.1:c.192G>T	YP_009724390.1:p.Trp64Cys
21757	Insertion	1	-	C	Homozygous	9	11	81.81818182	0.444444444	27.66666667	CDS: S, Gene: S	YP_009724390.1:c.196dup	YP_009724390.1:p.His66fs
21975	SNV	1	A	C	Heterozygous	8878	17029	52.13459393	0.487497184	31.15127281	CDS: S, Gene: S	YP_009724390.1:c.413A>C	YP_009724390.1:p.Asp138Ala
22444	SNV	1	C	T	Homozygous	8314	8336	99.73608445	0.495669954	29.26798172	CDS: S, Gene: S	YP_009724390.1:c.882C>T	
23002	MNV	2	TA	GG	Homozygous	6	14	42.85714286	0.5	21.5	CDS: S, Gene: S	YP_009724390.1:c.1440_1441delinsGG	YP_009724390.1:p.Cys480_Asn481delinsTrpAsp
23164	SNV	1	T	C	Homozygous	17	18	94.44444444	0.117647059	25.17647059	CDS: S, Gene: S	YP_009724390.1:c.1602T>C	
23403	SNV	1	A	G	Homozygous	6833	6922	98.71424444	0.431582028	26.20766867	CDS: S, Gene: S	YP_009724390.1:c.1841A>G	YP_009724390.1:p.Asp614Gly
23436	SNV	1	A	G	Heterozygous	2918	6747	43.24885134	0.42152159	32.20493489	CDS: S, Gene: S	YP_009724390.1:c.1874A>G	YP_009724390.1:p.His625Arg
23784	SNV	1	A	G	Homozygous	30	30	100	0.066666667	31.16666667	CDS: S, Gene: S	YP_009724390.1:c.2222A>G	YP_009724390.1:p.Tyr741Cys
24173	SNV	1	G	T	Homozygous	8623	8684	99.29755873	0.448915691	29.17546098	CDS: S, Gene: S	YP_009724390.1:c.2611G>T	YP_009724390.1:p.Ala871Ser
24532	SNV	1	A	G	Homozygous	3475	3602	96.47418101	0.482014388	29.9657554	CDS: S, Gene: S	YP_009724390.1:c.2970A>G	

Key mutations: Thr19Arg; Asp614Gly (D614G)

b) Vinzol STP untreated wastewater (Date of sampling: 26.11.2020)

Reference Position	Type	Length	Reference	Allele	Zygosity	Count	Coverage	Frequency	Forward/reverse balance	Average quality	Overlapping annotations	Coding region change	Amino acid change
21618	SNV	1	C	G	Homozygous	2309	3546	65.11562324	0.465569511	30.30619316	CDS: S, Gene: S	YP_009724390.1:c.56C>G	YP_009724390.1:p.Thr19Arg
22917	SNV	1	T	G	Homozygous	5507	7009	78.57040947	0.491919375	30.62411476	CDS: S, Gene: S	YP_009724390.1:c.1355T>G	YP_009724390.1:p.Leu452Arg
23403	SNV	1	A	G	Homozygous	5050	5071	99.5858805	0.426336634	27.38455446	CDS: S, Gene: S	YP_009724390.1:c.1841A>G	YP_009724390.1:p.Asp614Gly
23604	SNV	1	C	G	Homozygous	11425	13530	84.44198078	0.447439825	32.26118162	CDS: S, Gene: S	YP_009724390.1:c.2042C>G	YP_009724390.1:p.Pro681Arg
23927	SNV	1	T	G	Heterozygous	10	18	55.55555556	0.1	32	CDS: S, Gene: S	YP_009724390.1:c.2365T>G	YP_009724390.1:p.Tyr789Asp
24144	SNV	1	T	G	Heterozygous	22	56	39.28571429	0	29.54545455	CDS: S, Gene: S	YP_009724390.1:c.2582T>G	YP_009724390.1:p.Leu861Trp
25101	Deletion	1	A	-	Homozygous	23	24	95.83333333	0.47826087	24.04347826	CDS: S, Gene: S	YP_009724390.1:c.3543del	YP_009724390.1:p.Glu1182fs

Key mutations: Thr19Arg; Asp614Gly (D614G)

c) Vinzol STP untreated wastewater (Date of sampling: 08.02.2021)

Reference Position	Type	Length	Reference	Allele	Zygosity	Count	Coverage	Frequency	Forward/reverse balance	Average quality	Overlapping annotations	Coding region change	Amino acid change
21618	SNV	1	C	G	Homozygous	2968	3018	98.34327369	0.403638814	30.35950135	CDS: S, Gene: S	YP_009724390.1:c.56C>G	YP_009724390.1:p.Thr19Arg
22227	SNV	1	C	T	Homozygous	8	10	80	0.375	23.5	CDS: S, Gene: S	YP_009724390.1:c.665C>T	YP_009724390.1:p.Ala222Val
22917	SNV	1	T	G	Homozygous	12103	12463	97.11144989	0.463851938	30.92084607	CDS: S, Gene: S	YP_009724390.1:c.1355T>G	YP_009724390.1:p.Leu452Arg
23403	SNV	1	A	G	Homozygous	10612	10682	99.34469201	0.437712024	27.3558236	CDS: S, Gene: S	YP_009724390.1:c.1841A>G	YP_009724390.1:p.Asp614Gly
23604	SNV	1	C	G	Homozygous	13271	13681	97.00314305	0.495591892	32.08236003	CDS: S, Gene: S	YP_009724390.1:c.2042C>G	YP_009724390.1:p.Pro681Arg

Key mutations: Thr19Arg; Asp614Gly (D614G); Leu452Arg (L452R);

Pro681Arg (P681R)

d) Vinzol STP treated wastewater (Date of sampling: 08.02.2021)

Reference Position	Type	Length	Reference	Allele	Zygosity	Count	Coverage	Frequency	Forward/reverse balance	Average quality	Overlapping annotations	Coding region change	Amino acid change
21618	SNV	1	C	G	Homozygous	2823	2897	97.44563341	0.470421537	31.10768686	CDS: S, Gene: S	YP_009724390.1:c.56C>G	YP_009724390.1:p.Thr19Arg
21987	SNV	1	G	A	Homozygous	1859	2816	66.015625	0.381387843	30.29800968	CDS: S, Gene: S	YP_009724390.1:c.425G>A	YP_009724390.1:p.Gly142Asp
22029	**Deletion**	**6**	**AGTTCA**	**-**	**Heterozygous**	**1713**	**3002**	**57.06195869**	**0.412726211**	**27.58201985**	**CDS: S, Gene: S**	**YP_009724390.1:c.467_472del**	**YP_009724390.1:p.Glu156_Arg158 delinsGly**
22227	SNV	1	C	T	Heterozygous	733	1578	46.45120406	0.416098226	24.26193724	CDS: S, Gene: S	YP_009724390.1:c.665C>T	YP_009724390.1:p.Ala222Val
22917	SNV	1	T	G	Homozygous	5410	6238	86.72651491	0.431608133	29.70887246	CDS: S, Gene: S	YP_009724390.1:c.1355T>G	YP_009724390.1:p.Leu452Arg
22995	SNV	1	C	A	Homozygous	3151	3470	90.80691643	0.437956204	32.38400508	CDS: S, Gene: S	YP_009724390.1:c.1433C>A	YP_009724390.1:p.Thr478Lys
23403	SNV	1	A	G	Homozygous	5362	5372	99.81384959	0.497575532	27.03450205	CDS: S, Gene: S	YP_009724390.1:c.1841A>G	YP_009724390.1:p.Asp614Gly
23604	SNV	1	C	G	Homozygous	7587	10306	73.6173103	0.46737841	32.08778173	CDS: S, Gene: S	YP_009724390.1:c.2042C>G	YP_009724390.1:p.Pro681Arg
24410	SNV	1	G	A	Homozygous	2427	3202	75.79637726	0.482488669	29.77173465	CDS: S, Gene: S	YP_009724390.1:c.2848G>A	YP_009724390.1:p.Asp950Asn
24775	SNV	1	A	T	Heterozygous	285	749	38.05073431	0.470175439	31.85263158	CDS: S, Gene: S	YP_009724390.1:c.3213A>T	YP_009724390.1:p.Gln1071His

Key mutations: Thr19Arg; Asp614Gly (D614G); Leu452Arg (L452R);

Pro681Arg (P681R); Thr478Lys (T478K);

22029 del

N-Gene

Asp63Gly (D63G); Arg203Met (R203M)

Asp377Tyr (D377Y); (28248 del and 28271 del)

4.7 Antidrug resistance in the ambient waters of Ahmedabad

Fig. 17 and **Fig. 18** represents the comparative sensitivity of *E.coli* towards six antibiotics including the fluoroquinolone drugs NFX (norfloxacin), CIP (ciprofloxacin), LVX (levofloxacin) as well as TCE (tetracycline drugs), KM (kanamycin monosulphate), and ST (sulfamethoxazole), at various sampling locations (CI, VI, CO, VO, NB, SB, CL, and KL) in 2018 and 2020. In 2018, the river location NB had 0% resistance for all antibiotics, whereas SB location had 40% resistance towards all antibiotics except 60% resistance for KM. SB is the central urban location. This indicates that the ADR on the urbanisation and the discharge conditions. However, in 2020, this resistance increased at both river locations for all antibiotics, except for KM at SB. For all Quinolone drugs, the antidrug resistance increased to 50% at both river locations in 2020, whereas it was varying for TCE, KM and ST. At location NB, resistance was observed to be increased for TCE, KM and ST. Whereas, at location SB, resistance increased for TCE, ST, but decreased for KM. This indicates inflow or generation of antidrug resistant *E.coli* in the river water from urbanised sources which reflect increased use of antimicrobials, due to the unavailability of COVID-19 specific drugs (Abelenda-Alonso et al., 2020; Getahun et al., 2020; Hsu, 2020). Though the prevalence of *E. coli* was highest in 2018, more antidrug resistant *E.coli* are generated in the year 2020 due to heavy usage of antimicrobials.

In 2018, no ADR was observed for any of the antibiotics at location CL and KL, except for NFX, TCE and ST at location KL. **(Fig. 17** and **Fig. 18**). However, significant resistance was observed for all antibiotics, except KM, at both lake locations with higher values at CL than KL. This indicates more urbanised discharge carrying antidrug resistant *E.coli* accumulates at the location CL. One of the major reasons for the generated resistance at CL is the occasional discharge to the CL from nearby open Pirana solid waste dumping site (Singh et al. 2008). This call for a monitoring of urban wastewater flows being discharged to the lake ecosystem. Among the sampled WWTP locations in the year 2018, at locations VI and VO, no resistance was observed for any of the antibiotics except TCE (20% in influent) (**Fig. 17** and **Fig. 18**). Whereas, at CI location resistance for NFX, LVX, TCE, KM, was observed but only found to be increasing towards CIP and KM at location CO. These results show the increase in antidrug resistance after WWTP treatment, which was consistent as reported in the studies from Sweden and Austria (Reinthaler et al., 2003; Flach et al., 2018).

Interestingly, ADR increased significantly for all antibiotics in the year 2020 at the VI and VO locations when compared to year 2018. In the year 2020, ADR was observed for all antibiotics at VI and these resistances were observed to be increasing or being constant at VO locations for all antibiotics except KM (decreased by 35%) (**Fig. 17** and **18**). Such a high increase in the resistance in treated effluent can be attributed to a long residence time.

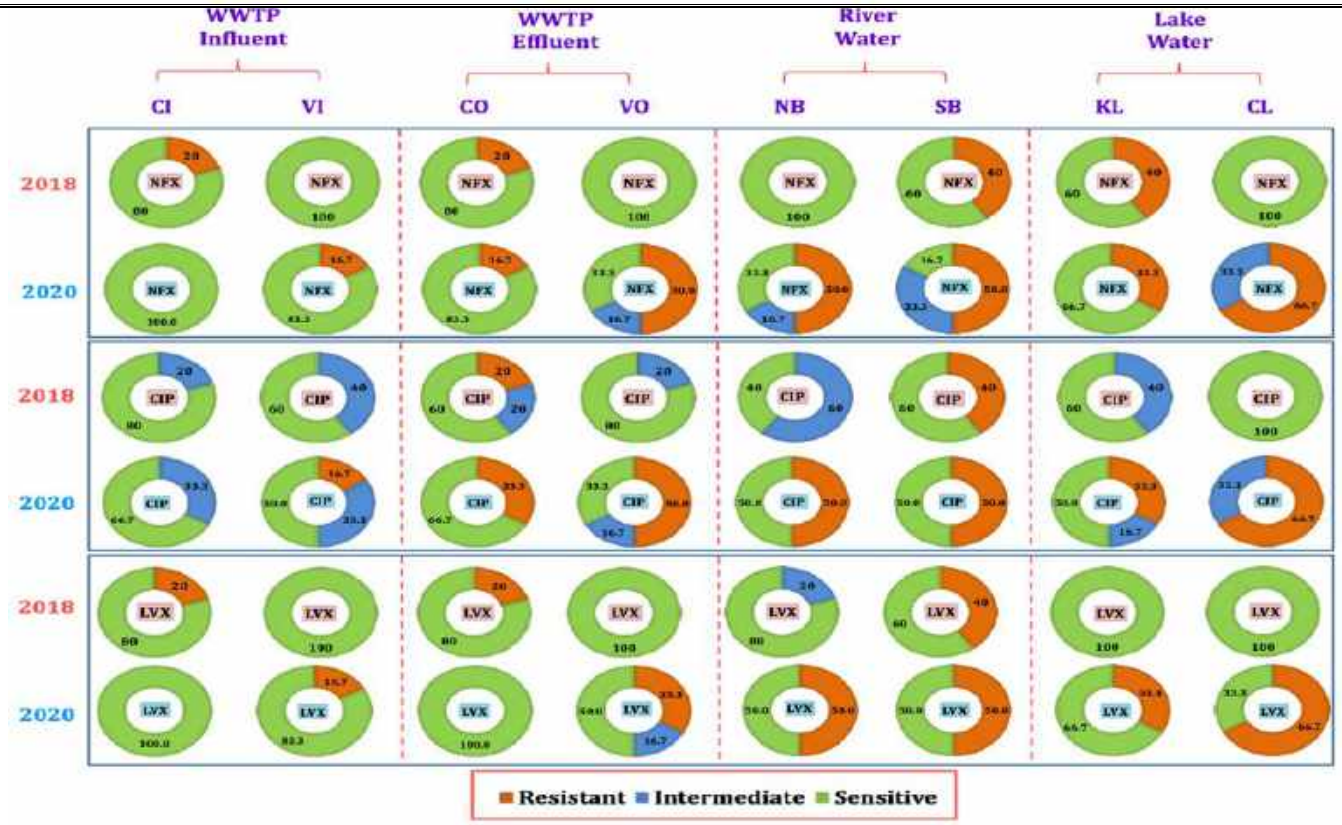


Fig. 17. Percentage of antibiotic resistance in the influents of different water compartments in years 2018 and 2020 against fluroquinolone drugs i.e. NFX (Norfloxacin), CIP (Ciprofloxacin), LVX (Levofloxacin) for locations including WWTPs CI (Chandkheda Inlet), CO (Chandkheda Outlet), VI (Vasna Inlet) and VO (Vasna Outlet); Rivers, NB (Nehru Bridge) and SB (Sardar Bridge), and Lakes, KL (Kankaria Lake) and CL (Chandola Lake).

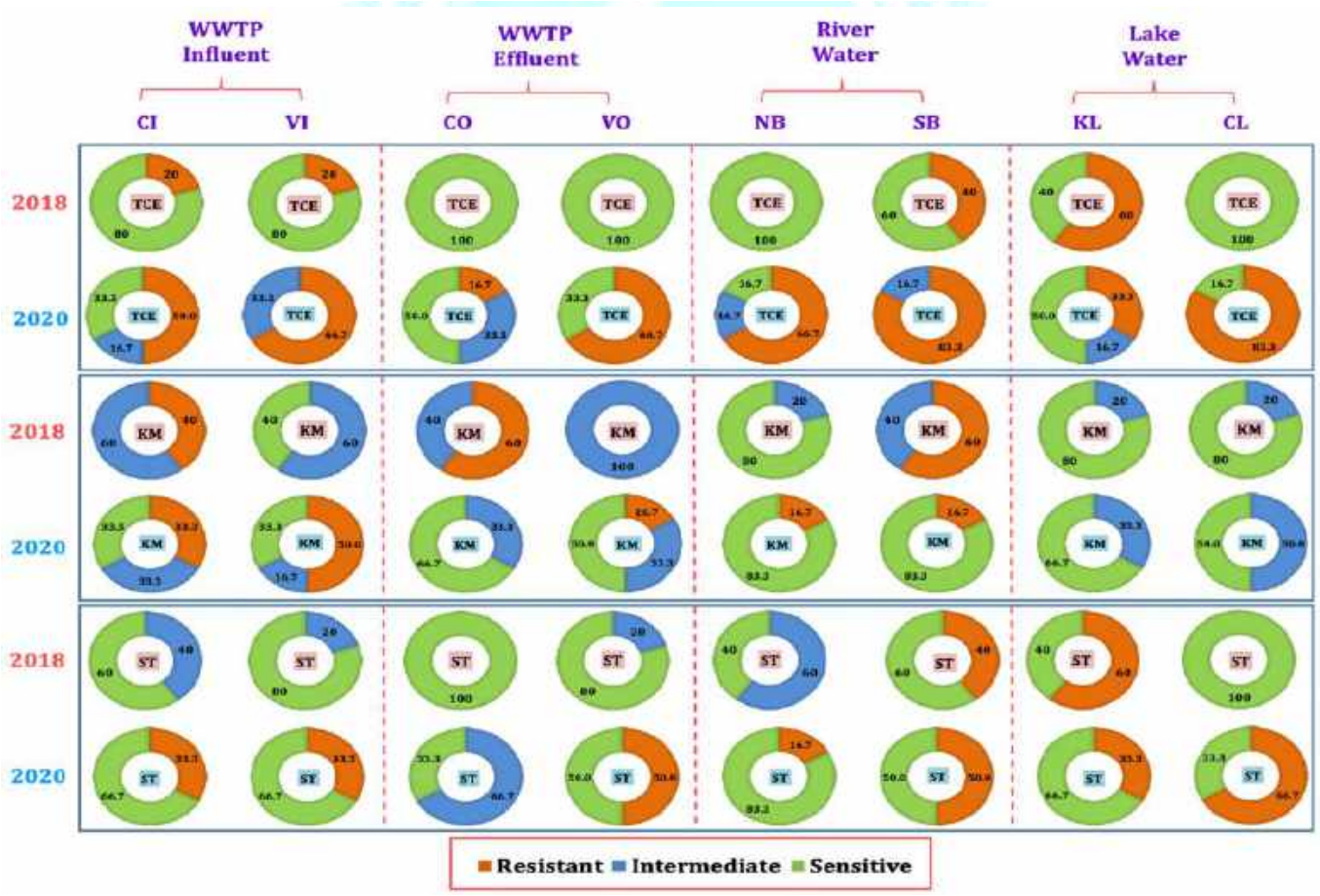


Fig. 18. Percentage of antibiotic resistance in the influents of different water compartments in years 2018 and 2020 against tetracycline drugs (TCE), aminoglycosides i.e. KM (kanamycin), and others i.e. ST (sulfamethoxazole) for locations including WWTPs CI (Chandkheda Inlet), CO (Chandkheda Outlet), VI (Vasna Inlet) and VO (Vasna Outlet); Rivers, NB (Nehru Bridge) and SB (Sardar Bridge), and Lakes KL (Kankaria Lake) and CL (Chandola Lake).

In the case of CI in the year 2020, no resistance was observed towards the quinolone drugs, whereas the observed ADR for KM, ST, and TCE, was reduced significantly at CO location. However, resistance was observed to be generated for NFX and CIP at CO in year 2020. The high resistance towards quinolone drugs is attributed to the discharge having domestic origin (Threedeach et al., 2012; Auerbach et al., 2007); because these drugs are prescribed for treatments of respiratory and urinary tract infections, their use has increased significantly during the COVID-19 pandemic (Abelenda-Alonso et al., 2020; Getahun et al., 2020; Hsu, 2020).

Fig. 19 highlights the statistical comparison of overall ADR in the year 2018 and 2020, whose causes are well described above. It is clearly seen that the mean percentage value of overall ADR was increased for the resistant strains of *E. coli* in the year 2020 than 2018, except in the case of kanamycin (remains nearly same). Whereas, the mean percentage value of overall ADR observed to be decreasing for the sensitive strains of *E. coli* in the year 2020 than 2018, except in case of kanamycin (increases). The percentage of ADR (in resistant *E.coli* strains) for almost all antibiotics: CIP, LVX, TC, KM, ST (except NFX: 89.1% change), was observed to be very significant in the year 2020 than 2018, as $p < 0.10$.

This indicates that the significant change is occurring due to increase in the mean value of percentage of ADR. Overall, the comparison of overall ADR shows a significant increase statistically in the year 2020 than 2018.

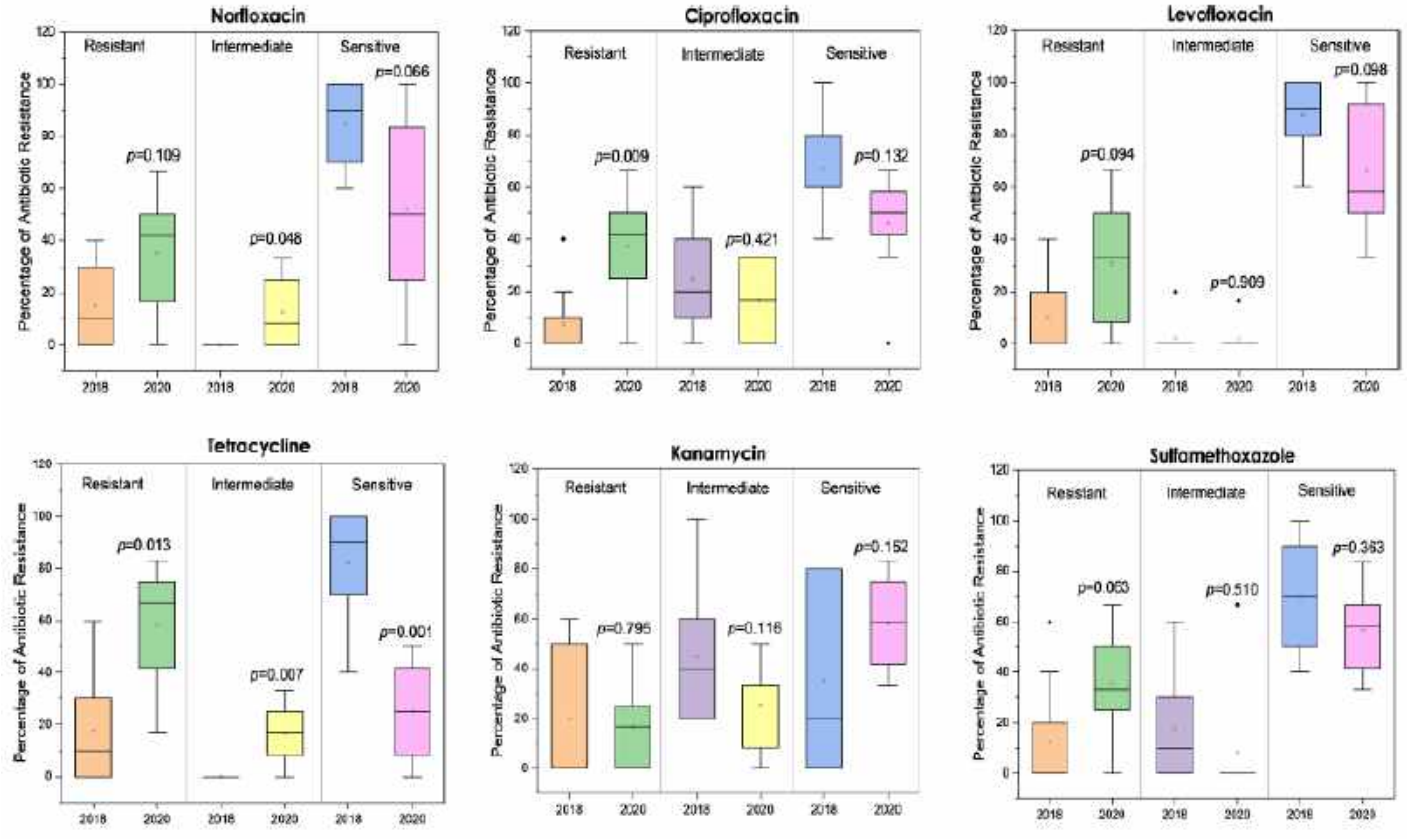


Fig. 19. Comparison of antibiotic (antidrug) resistance against various antibiotics in 2018 and 2020 with the results of a statistical T-test.

4.8. Future perspective of WBE study

we explicitly put forward an example of the effectiveness of SWEEP for the early warning of COVID-19, and emphasize the continuous long-term monitoring with the following future objectives: **i)** monitoring the COVID-19 curve in the post-vaccination period through quantifying the genetic material of SARS-CoV-2 in the wastewaters of a given city (Ahmedabad); **ii)** understanding the association of antibiotic resistance with COVID-19 prevalence; **iii)** developing an online portal with a weekly update of gene concentration with accessibility provided to the public and policymakers; **iv)** estimating the potential risk of SARS-CoV-2 in natural water bodies through various water activities using a quantitative microbial risk assessment (QMRA) framework; **v)** generating longer time-series data to further check the robustness of early warning capability of the techniques and its possible benefits; and **vi)** developing predictive modelling for connecting the missing points in SWEEP generated database, meaningful interpretations, and supporting other surveillance protocols. SWEEP can be considered for developing advisory in the context of rapid-testing, the number of testing, community clearance, hotspot identification, vaccine need identification zones, as well as making a recommendation on staying at home and implementing curfews.

In this first phase, we have explicitly shown the capability of WBE as an early warning and city zonation tool however in a country like India, where sewer systems are not complete, and treatment systems are not well-managed, it is important to have long-term monitoring for a year at the least so that precious meaningful data for the developing country can be obtained. Furthermore, a practical guide and pandemic management tools can be developed by integrating the virtues of information technology with the early warning capability of wastewater surveillance. Confidence may be generated among the commons as well as to the government agencies like Ahmedabad Municipal Corporation (AMC) for incorporating WBE into regular monitoring program for the management of the current or future COVID-like epidemic or pandemic outbreak.

The removal efficacy of the two studied WWTPs suggests that the treated effluents are not always free from SARS-CoV-2 RNA, and are subject to temporal variability. Therefore, we stress the need for wastewater surveillance of SARS-CoV-2 at the treatment plant scale with further investigation on the efficacy of the treatment processes on the removal of the enveloped virus such as SARS-CoV-2 as well as the genomic materials. The future research efforts may therefore consider the influence of genetic material loading in the influent, difference in sewage flow and treatment methods, hydraulic and sludge retention time of technology used, and serviced people.

Furthermore, ADR study in ambient water samples in Ahmedabad suggests that WBE can be the key tool to monitor the antimicrobials prevalence and antidrug resistance in the pandemic situations.

Conclusion

unicef
United Nations Children's Fund

5. Conclusions

5.1 WBE study in Ahmedabad

A temporal variation of SARS-CoV-2 RNA presence in wastewater was studied for a period of three months in Ahmedabad, India. A total 111 samples (95.7%) of the total 116 samples tested in the study were found to be positive, with at least two positive RT-PCR results targeting SARS-CoV-2 ORF1ab, S gene, and N gene assays. Monthly variation depicted a significant decline in all three gene targets in October compared to September 2020, followed by a sharp increment in November 2020. Correspondingly, the descending order of average effective gene concentration was November (~10729 copies/ L) > September (~3046 copies/ L) > October (~454 copies/ L). This finding was further supported by the relation between the percentage change in effective gene concentration level and confirmed cases, which followed a similar trend on the temporal scale with a ~1 to 2 weeks' time distance. The results unveiled the untapped potential of WBE surveillance of COVID-19 as an early warning tool for practical use of city zonation based on SWEEP data for actual scenarios and future prediction. This approach may help the authorities identify the hotspots within a city and tuning effective management interventions. Further research may be focused on quantification of correlation of SWEEP results with clinical surveillance data and development of a predictive model that can translate SWEEP data for easy propagation to policymakers and common public to enhance the preparedness and management of pandemics.

5.2 Study on the efficacy of WWTPs to remove SARS-CoV-2 RNA

Comparison of SARS-CoV-2 RNA removal efficacy of CAS and RZT, the two most used treatment systems in India, was studied through biweekly and monthly variations in their performances. We applied long-term monitoring data and performed statistical tests to understand the significance of removal and correlated it with other water quality parameters before and after deployed treatment. For the first time, MVAs used in this study along with other statistical tests highlighted the disparity in performance and statistical significance of SARS-CoV-2 RNA removal between CAS and RZT. It can be concluded that influent waters present better picture in terms of SARS-CoV-2 gene monitoring; effective genome concentration should be calculated based on presence/absence of multiple genes rather the presence of one specific gene; and treatments are less effective on N-genes and the most effective for S-genes. CAS treatment exhibited better RNA removal rate ($t=2.98$, $p=0.014$) compared to the root-zone treatment ($t=2.54$, $p=0.032$). In addition, treatment plants with smaller capacity are likely to show more fluctuations in effluent water quality.

Two most critical findings from the ongoing pandemic perspectives were that the treated effluents are not always free from SARS-CoV-2 RNA, and are subject to temporal variability. We stress the need for wastewater surveillance of SARS-CoV-2 at the treatment plant scale with further

investigation on the efficacy of the treatment processes on the removal of the enveloped virus such as SARS-CoV-2 as well as the genomic materials. The future research efforts may therefore consider the influence of genetic material loading in the influent, difference in sewage flow and treatment methods, hydraulic and sludge retention time of technology used, and serviced people. In addition, the mechanistic understanding may be generated on the SARS-CoV-2 removal using long-term step-wise sampling and monitoring of a given treatment processes. Nevertheless, our results are based on RNA fragment detection by RT-PCR, thus the abundance of viable SARS-CoV-2 in the samples can be significantly lower than the RNA-based gene copies. Therefore, research is needed for assessing infectivity through viable virus estimation, specifically for the use of reclaimed water in agriculture and drinking water supply.

5.3 Metagenomic study of 16s RNA in wastewater samples

The results suggest no clear-cut pattern among the bacterial population and association with SARS-CoV-2 genetic load in wastewater samples. Some of the bacterial population significantly changed on monthly temporal scale but no clear-cut concluding pattern was seen. There was significant difference at the bacterial taxonomic level was observed between the untreated and treated wastewater samples. We did not have explicit raw data of the wastewater quality parameters on the sampling date, therefore cannot draw a concrete and convincing finding. The results were not promising but they indicated a possible correlation ship among the SARS-CoV-2 gene concentration and bacterial population and dynamics. Therefore, further investigation is required considering different influencing factors such as sampling timing, sewage flow rate, treatment process, and wastewater physico-chemical parameters.

5.4 Wastewater based genomic surveillance of the SARS-CoV-2

We have first reported, detection and identification of designated *Variant of Concern* (VoC: VOC-21APR-02; B.1.617.2) from wastewater samples using genomic surveillance approach. The key spike protein mutations that were identified in the SARS-CoV-2 genome assembly as compared to the reference Wuhan/Hu-1/2019 (EPI_ISL_402125) variant that were identified include C21618G/Thr19Arg (T19R), T22917G/Leu452Arg (L452R), C22995A/Thr478Lys (T478K), A23403G/Asp614Gly (D614G), and C23604G/Pro681Arg (P681R) from the samples collected in the month of February, 2021. The observation of the deletion at 22029 (6 bp), 28248 (6 bp) and 28271 (1 bp) were also observed and seen in the B.1.617.2 lineage. These findings point towards probably an early circulating B.1.617.2 lineage in Ahmedabad, Gujarat while clinical samples sequenced in the month of March, 2021 were detected with the cases of B.1.617.2 variant. The variants of concern (VOCs) can be more transmissible resulting in probably higher disease severity outcomes and are also

known for reduced sensitivity to antibody neutralization.

Therefore, WBE could be a useful method in early warning of the circulating novel variants and monitoring cryptic transmission of the SARS-CoV-2. Also, real time monitoring of the pandemic progression and helping the decision support system for public health interventions.

5.5 ADR study in ambient water samples in Ahmedabad

Non-fluoroquinolone drugs showed overall more resistance as compared to fluoroquinolone drugs. Tetracycline followed by norfloxacin has shown more resistance as compared to the other drugs. Despite a decrease in the prevalence of *E. coli* on the sampled river locations, the percentage resistance had been significantly increased in the year 2020 compared to year 2018. However, the *E. coli* prevalence in STP samples was increased in the order of 102, but the pattern of antidrug resistance was not consistent. Lake locations also exhibited an increase in the antidrug resistance during the duration of pandemic. The river locations and the lake locations have shown a significant increase in the antidrug resistance, and these locations are from the highly COVID-19 infected zones of the city. The COVID-19 spread in various zones of the city has shown corresponding changes in the SARS-CoV-2 genome concentration and ADR in environmental waters. Overall, due to increased consumption of antimicrobials in the pandemic period, the percentage of antidrug resistance has been increased significantly. Wastewater based epidemiology can be the key tool to monitor the antimicrobials prevalence and antidrug resistance in the pandemic situations.

References

unicef
United Nations Children's Fund

References

A.D., Jervey, S. and Albaiu, D., 2020. Research and development on therapeutic agents and vaccines for COVID-19 and related human coronavirus diseases.

Aarogya Setu' https://en.wikipedia.org/wiki/Aarogya_Setu acessed on 20th March, 2021.

Abelenda-Alonso, G., Padull´es, A., Rombauts, A., Gudiol, C., Pujol, M., Alvarez-Pouso, C., Jodar, R., Carratal`a, J., 2020. Antibiotic prescription during the COVID-19

Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J., Tscharke, B., Verhagen, R., Smith, W.J.M., Zaugg, J., Dierens, L., Hugenholtz, P., Thomas, K. V., Mueller, J.F., 2020a. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community. Sci. Total Environ. 728.

Ahmed, W., Tscharke, B., Bertsch, P.M., Bibby, K.,Bivins, A., Choi, P., Clarke, L., Dwyer, J., Edson, J., Nguyen, T.M.H. and O'Brien, J.W., 2020. SARS-CoV-2 RNA monitoring in wastewater as an early warning system for COVID-19 transmission in the community: a temporal case study. Sci. Total Environ. p.144216.

Ahmedabad COVID-19 community vulnerability map published by SustainAbly and Accion Land Pvt. Ltd, accessible at http://google.org/crisismap/a/gmail.com/amdcovid19 accessed on 12th December, 2020.

Anderson, Elizabeth L., et al. "Consideration of the aerosol transmission for COVID-19 and public health." Risk Analysis 40.5 (2020): 902-907.

Asaduzzaman, M., Zaman, F., Rousham, E., 2020. Antibiotic consumption may be linked to exaggeration of COVID-19. Med. Hypotheses 143, 109913

Auerbach, E.A., Seyfried, E.E., McMahon, K.D., 2007. Tetracycline resistance genes in activated sludge wastewater treatment plants. Water Res. 41, 1143–1151

Azuma, T., Nakada, N., Yamashita, N. and Tanaka, H., 2012. Synchronous dynamics of observed and predicted values of anti-influenza drugs in environmental waters during a seasonal influenza outbreak. *Environmental science & technology*, *46*(23), pp.12873-12881.

Baker-Austin, C., Wright, M.S., Stepanauskas, R. and McArthur, J.V., 2006. Co-selection of antibiotic and metal resistance. Trends in microbiology, 14(4), pp.176-182.

Balboa, S., Mauricio-Iglesias, M., Rodríguez, S., Martínez-Lamas, L., Vasallo, F. J., Regueiro, B., & Lema, J. M., 2021. The fate of SARS-CoV-2 in wastewater treatment plants points out the sludge line as a suitable spot for incidence monitoring. Sci. Total Environ. 772.

Barceló, D., 2020a. Wastewater-Based Epidemiology to monitor COVID-19 outbreak: Present and future diagnostic methods to be in your radar. Case Stud. Chem. Environ. Eng. 2.

Barcelo, D., 2020b. An environmental and health perspective for COVID-19 outbreak: Meteorology and air quality influence, sewage epidemiology indicator, hospitals disinfection, drug therapies and recommendations. J. Environ. Chem. Eng.

Bivins, A., North, D., Ahmad, A., Ahmed, W., Alm, E., Been, F., Bhattacharya, P., Bijlsma, L., Boehm, A.B., Brown, J., Buttiglieri, G., Calabro, V., Carducci, A.,

Castiglioni, S., Cetecioglu Gurol, Z., Chakraborty, S., Costa, F., Curcio, S., De Los Reyes, F.L., Delgado Vela, J., Farkas, K., Fernandez-Casi, X., Gerba, C., Gerrity, D., Girones, R., Gonzalez, R., Haramoto, E., Harris, A., Holden, P.A., Islam, M.T., Jones, D.L., Kasprzyk-Hordern, B., Kitajima, M., Kotlarz, N., Kumar, M., Kuroda, K., La Rosa, G., Malpei, F., Mautus, M., McLellan, S.L., Medema, G., Meschke, J.S., Mueller, J., Newton, R.J., Nilsson, D., Noble, R.T., Van Nuijs, A., Peccia, J., Perkins, T.A., Pickering, A.J., Rose, J., Sanchez, G., Smith, A., Stadler, L., Stauber, C., Thomas, K., Van Der Voorn, T., Wigginton, K., Zhu, K., Bibby, K., 2020. Wastewater-Based Epidemiology: Global Collaborative to Maximize Contributions in the Fight against COVID-19. Environ. Sci. Technol.

Buitrago-Garcia, D., Egli-Gany, D., Counotte, M.J., Hossmann, S., Imeri, H., Ipekci, A.M., Salanti, G., Low, N., 2020. Occurrence and transmission potential of asymptomatic and presymptomatic SARS-CoV-2 infections: a living systematic review and metaanalysis. PLoS Med. 17, e1003346

Chan, P.K., Lui, G., Hachim, A., Ko, R.L., Boon, S.S., Li, T., Kavian, N., Luk, F., Chen, Z., Yau, E.M. and Chan, K.H., 2020. Serologic responses in healthy adult with SARS-CoV-2 reinfection, Hong Kong, August 2020. Emerging infectious diseases, 26(12), p.3076.

Chatterjee, S.K., Bhattacharjee, I. and Chandra, G., 2010. Water quality assessment near an industrial site of Damodar River, India. Environmental monitoring and Assessment, 161(1-4), pp.177-189.

Cheung, K.S., Hung, I.F., Chan, P.P., Lung, K.C., Tso, E., Liu, R., Ng, Y.Y., Chu, M.Y., Chung, T.W., Tam, A.R. and Yip, C.C., 2020. Gastrointestinal manifestations of SARS-CoV-2 infection and virus load in fecal samples from the Hong Kong cohort and systematic review and meta-analysis. Gastroenterology.

drug exposure of animal reservoirs. *Environmental Science & Technology*, *54*(14), pp.8503-8505.

Flach, C.F., Genheden, M., Fick, J., Joakim Larsson, D.G., 2018. A comprehensive screening of Escherichia coli isolates from Scandinavia's largest sewage treatment plant indicates no selection for antibiotic resistance. Environ. Sci. Technol. 52 (19), 11419–11428.

Getahun, H., Smith, I., Trivedi, K., Paulin, S., Balkhy, H.H., 2020. Tackling antimicrobial resistance in the COVID-19 pandemic. Bull. World Health Organ. 98 (7), 442-442.

Greenhalgh, Trisha, et al. "Ten scientific reasons in support of airborne transmission of SARS-CoV-2." The Lancet 397.10285 (2021): 1603-1605.

Guo, X., Yan, Z., Zhang, Y., Xu, W., Kong, D., Shan, Z., Wang, N., 2018. Behavior of antibiotic resistance genes under extremely high-level antibiotic selection pressures in pharmaceutical wastewater treatment plants. Sci. Total Environ. 612, 119–128.

Guo, Zhen-Dong, et al. "Aerosol and surface distribution of severe acute respiratory syndrome coronavirus 2 in hospital wards, Wuhan, China, 2020." Emerging infectious diseases 26.7 (2020): 1586.

Haramoto, E., Malla, B., Thakali, O., Kitajima, M., 2020. First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. Sci. Total Environ. 737, 140405.

Hata, A., Hara-Yamamura, H., Meuchi, Y., Imai, S. and Honda, R., 2020. Detection of SARS-

CoV-2 in wastewater in Japan during a COVID-19 outbreak. Sci. Total Environ. p.143578.

Hsu, J., 2020. How covid-19 is accelerating the threat of antimicrobial resistance. *BMJ*, *369*

https://web.worldbank.org/archive/website01409/WEB/IMAGES/2010_1_1.PDF accessed on 21st May, 2021

Jayaweera, Mahesh, et al. "Transmission of COVID-19 virus by droplets and aerosols: A critical review on the unresolved dichotomy." Environmental research (2020): 109819.

Kitajima, M., Ahmed, W., Bibby, K., Carducci, A., Gerba, C.P., Hamilton, A., Haramoto, E., Rose, J.B., 2020. SARS-CoV-2 in wastewater: state of the knowledge and research needs. Sci. Total Environ. 739, 139076.

Kitamura, K., Sadamasu, K., Muramatsu, M., & Yoshida, H., 2020. Efficient detection of SARS-CoV-2 RNA in the solid fraction of wastewater. Sci. Total Environ. 763.

Kumar, M., Alamin, M., Kuroda, K., Dhangar, K., Hata, A., Yamaguchi, H., & Honda, R. 2021a. Potential discharge, attenuation and exposure risk of SARS-CoV-2 in natural water bodies receiving treated wastewater. npj Clean Water, 4(1), 1-11.

Kumar, M., Dhangar, K., Thakur, A.K., Ram, B., Chaminda, T., Sharma, P., Kumar, A., Raval, N., Srivastava, V., Rinklebe, J. and Kuroda, K., 2021b. Antidrug Resistance in the Indian Ambient Waters of Ahmedabad during the COVID-19 Pandemic. J. Hazard. Mater. p.126125.

Kumar, M., Joshi, M., Kuroda, K., Bhattacharya, P. and Barcello, D., 2021c. First comparison of conventional activated sludge versus root-zone treatment for SARS-CoV-2 RNA removal from wastewaters: statistical and temporal significance. Chem. Eng. J.

Kumar, M., Joshi, M., Patel, A. K., & Joshi, C. G., 2020b. Unravelling the early warning capability of wastewater surveillance for COVID-19: A temporal study on SARS-CoV-2 RNA detection and need for the escalation. Environ. Res. 196. 110946

Kumar, M., Kuroda, K., Dhangar, K., Mazumder, P., Sonne, 427 C., Rinklebe, J. and Kitajima, M., 2020a. Potential emergence of antiviral-resistant pandemic viruses via environmental

Kumar, M., Kuroda, K., Patel, A.K., Patel, N., Bhattacharya, P., Joshi, M. and Joshi, C.G., 2020c. Decay of SARS-CoV-2 RNA along the wastewater treatment outfitted with Upflow Anaerobic Sludge Blanket (UASB) system evaluated through two sample concentration techniques. Sci. Total Environ. 754, p.142329.

Kumar, M., Patel, A.K., Shah, A.V., Raval, J., Rajpara, N., Joshi, M. and Joshi, C.G., 2020a. First proof of the capability of wastewater surveillance for COVID-19 in India through detection of genetic material of SARS-CoV-2. Sci. Total Environ. 746, p.141326.

Kumar, M., Thakur, A.K., Mazumder, P., Kuroda, K., Mohapatra, S., Rinklebe, J., Ramanathan, A.L., Cetecioglu, Z., Jain, S., Tyagi, V.K. and Gikas, P., 2020b. Frontier review on the propensity and repercussion of SARS-CoV-2 migration to aquatic environment. Journal of Hazardous Materials Letters, 1, p.100001.

Kuroda, K., Li, C., Dhangar, K., Kumar, M., 2021. Predicted occurrence, ecotoxicological risk and environmentally acquired resistance of antiviral drugs associated with COVID-19 in environmental waters. Sci. Total Environ. 776, 145740

La Rosa, G., Iaconelli, M., Mancini, P., Ferraro, G.B., Veneri, C., Bonadonna, L., Lucentini, L. and Suffredini, E., 2020. First detection of SARS-CoV-2 in untreated wastewaters in Italy. Sci. Total Environ. p.139652.

La Rosa, G., Iaconelli, M., Mancini, P., Ferraro, G.B., Veneri, C., Bonadonna, L., Lucentini, L., Suffredini, E., 2020. First detection of SARS-CoV-2 in untreated wastewaters in Italy. Sci. Total Environ. 736, 139652.

Lavezzo, E., Franchin, E., Ciavarella, C., Cuomo-Dannenburg, G., Barzon, L., Del Vecchio, C., Rossi, L., Manganelli, R., Loregian, A., Navarin, N. and Abate, D., 2020. Suppression of a SARS-CoV-2 outbreak in the Italian municipality of Vo'. Nature, 584(7821), pp.425-429.

Lescure, F.X., Bouadma, L., Nguyen, D., Parisey, M., Wicky, P.H., Behillil, S., Gaymard, A., Bouscambert-Duchamp, M., Donati, F., Le Hingrat, Q., Enouf, V., Houhou-Fidouh, N., Valette, M., Mailles, A., Lucet, J.C., Mentre, F., Duval, X., Descamps, D., Malvy, D., Timsit, J.F., Lina, B., van-der-Werf, S., Yazdanpanah, Y., 2020. Clinical and virological data of the first cases of COVID-19 in Europe: a case series. Lancet Infect. Dis. 20.

Liu, C., Zhou, Q., Li, Y., Garner, L.V., Watkins, S.P., Carter, L.J., Smoot, J., Gregg, A.C., Daniels,

Lodder, W., de Roda Husman, A.M., 2020. SARS-CoV-2 in wastewater: potential health risk, but also data source. Lancet Gastroenterol. Hepatol.

Lucien, M.A.B., Canarie, M.F., Kilgore, P.E., Jean-Denis, G., F´en´elon, N., Pierre, M., Cerpa, M., Joseph, G.A., Maki, G., Zervos, M.J., Dely, P., 2021. Antibiotics and antimicrobial resistance in the COVID-19 era: perspective from resource-limited settings. Int. J. Infect. Dis. 104, 250–254.

M.,Merigliano, S., De Canale, E., Vanuzzo, M.C., Besutti, V., Saluzzo, F., Onelia, F., Pacenti, M., Parisi, S.G., Carretta, G., Donato, D., Flor, L., Cocchio, S., Masi, G., Sperduti, A., Cattarino, L., Salvador, R., Nicoletti, M., Caldart, F., Castelli, G., Nieddu, E., Labella, B., Fava, L., Drigo, M., Gaythorpe, K.A.M., Brazzale, A.R., Toppo, S., Trevisan, M., Baldo, V., Donnelly, C.A., Ferguson, N.M., Dorigatti, I., Crisanti, A., 2020. Suppression of a SARS-CoV-2 outbreak in the Italian municipality of Vo. Nature 584, 425–429.

McCarty, P. L., & Smith, D. P. (1986). Anaerobic wastewater treatment. Environ. Sci. Technol. 20(12), 1200-1206

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020. Presence of SARS Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. Environ Sci Technol Lett. 7 (7), 511–516.

Miranda, C.D. and Castillo, G., 1998. Resistance to antibiotic and heavy metals of motile aeromonads from Chilean freshwater. *Science of the total environment*, *224*(1-3), pp.167-176

Mizumoto, K. and Chowell, G., 2020. Estimating Risk for Death from Coronavirus Disease, China, January–February 2020. Emerging infectious diseases, 26(6), p.1251.

Nishiura, H., Linton, N.M. and Akhmetzhanov, A.R., 2020. Serial interval of novel coronavirus (COVID-19) infections. International journal of infectious diseases.

Prevost, B., Lucas, F. S., Goncalves, A., Richard, F., Moulin, L., Wurtzer, S., 2015. Large scale survey of enteric viruses in river and waste water underlines the health status

of the local population. Environ. Int. 79, 42-50.

Randazzo,W., Truchado, P., Cuevas-Ferrando, E., Simón, P., Allende, A., Sánchez, G., 2020a. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a Low prevalence area. Water Res. 181, 115942.

Reinthaler, F.F., Posch, J., Feierl, G., Wüst, G., Haas, D., Ruckenbauer, G., Mascher, F., Marth, E., 2003. Antibiotic resistance of *E. coli* in sewage and sludge. Water Res. 37 (8), 1685–1690.

Rimoldi, S. G., Stefani, F., Gigantiello, A., Polesello, S., Comandatore, F., Mileto, D., Maresca, M., Longobardi, C., Mancon, A., Romeri, F., Pagani, C., Moja, L., Gismondo, M.R., Salerno, F.,2020. Presence and vitality of SARS-CoV-2 virus in wastewaters and rivers Sci. Total Environ. 744:140911

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D. and Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water research, 69, pp.234-242.

Sherchan, S. P., Shahin, S., Ward, L. M., Tandukar, S., Aw, T. G., Schmitz, B., ... & Kitajima, M., 2020. First detection of SARS-CoV-2 RNA in wastewater in North America: a study in Louisiana, USA. Sci. Total Environ. 743.

Singh, U.K., Kumar, M., Chauhan, R., Jha, P.K., Ramanathan, A.L., Subramanian, V., 2008. Assessment of the impact of landfill on groundwater quality: a case study of the Pirana site in western India. Environ. Monit. Assess. 141 (1), 309–321.

Takanami, R., Ozaki, H., Giri, R.R., Taniguchi, S. and Hayashi, S., 2010. Detection of antiviral drugs oseltamivir phosphate and oseltamivir carboxylate in Neya River, Osaka, Japan. *Journal of Water and Environment Technology*, *8*(4), pp.363-372.

Threedeach, S., Chiemchaisri, W., Watanabe, T., Chiemchaisri, C., Honda, R., Yamamoto, K., 2012. Antibiotic resistance of Escherichia coli in leachates from municipal solid waste landfills: comparison between semi-aerobic and anaerobic operations. Bioresour. Technol. 113, 253–258

Tiwari, S. B., Gahlot, P., Tyagi, V. K., Zhang, L., Zhou, Y., Kazmi, A. A., Kumar, M., 2021. Surveillance of Wastewater for Early Epidemic Prediction (SWEEP): Environmental and health security perspectives in the post COVID-19 Anthropocene. Environ. Res. 110831.

Tran, H. N., Le, G. T., Nguyen, D. T., Juang, R. S., Rinklebe, J., Bhatnagar, A., Lima, E.C., Iqbal, H. M. N., Sarmah, A. K., Chao, H. P., 2020. SARS-CoV-2 coronavirus in water and wastewater: A critical review about presence and concern. Environ. Res. 110265.

Wang, Wenling, et al. "Detection of SARS-CoV-2 in different types of clinical specimens." Jama 323.18 (2020): 1843-1844. Westhaus, S., Weber, F. A., Schiwy, S., Linnemann, V., Brinkmann, M., Widera, M., ... & Ciesek, S., 2020. Detection of SARS-CoV-2 in raw and treated wastewater in Germany–suitability for COVID-19 surveillance and potential transmission risks. Sci. Total Environ. 751.

Wong, M.C., Huang, J., Lai, C., Ng, R., Chan, F.K. and Chan, P.K., 2020. Detection of SARS-CoV-2 RNA in fecal specimens of patients with confirmed COVID-19: a meta-analysis. Journal of Infection.

World Health Organization Novel Coronavirus (2019-nCoV) Situation Report – 1 (2020) https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation432-reports

Wu, Y., Guo, C., Tang, L., Hong, Z., Zhou, J., Dong, X., Yin, H., Xiao, Q., Tang, Y., Qu, X., Kuang, L., Fang, X., Mishra, N., Lu, J., Shan, H., Jiang, G., Huang, X., 2020b. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. Lancet Gastroenterol. Hepatol. 5, 434–435

Xiao, F., Tang, M., Zheng, X., Liu, Y., Li, X. and Shan, H., 2020. Evidence for gastrointestinal infection of SARS-CoV-2. Gastroenterology, 158(6), pp.1831-1833.

Yang, R., Gui, X. and Xiong, Y., 2020. Comparison of clinical characteristics of patients with asymptomatic vs symptomatic coronavirus disease 2019 in Wuhan, China. JAMA Network Open, 3(5), pp.e2010182-e2010182.

Zhu, Y., Oishi, W., Maruo, C., Saito, M., Chen, R., Kitajima, M., & Sano, D., 2021. Early warning of COVID-19 via wastewater-based epidemiology: potential and bottlenecks. Sci. Total Environ. 145124.

Appendices

List of Publications

A. Published

1. Kumar, M., Joshi, M., Shah, A.V., Srivastava, V. and Dave, S., 2021. First wastewater surveillance-based city zonation for effective COVID-19 pandemic preparedness powered by early warning: A study of Ahmedabad, India. ***Science of the Total Environment**, 148367* (**Impact factor: 6.551**)

2. Kumar, M., Joshi, M., Patel, A.K. and Joshi, C.G., 2021. Unravelling the early warning capability of wastewater surveillance for COVID-19: A temporal study on SARS-CoV-2 RNA detection and need for the escalation. ***Environmental research***, *196*, p.110946. (**Impact factor: 5.715**)

3. Kumar, M., Joshi, M., Kuroda, K., Bhattacharya, P. and Barcello, D., 2021. First comparison of conventional activated sludge versus root-zone treatment for SARS-CoV-2 RNA removal from wastewaters: statistical and temporal significance. ***Chemical Engineering Journal**,130635* (**Impact factor: 10.652)**

4. Kumar, M., Dhangar, K., Thakur, A.K., Ram, B., Chaminda, T., Sharma, P., Kumar, A., Raval, N., Srivastava, V., Rinklebe, J. and Kuroda, K., 2021. Antidrug Resistance in the Indian Ambient Waters of Ahmedabad during the COVID-19 Pandemic. ***Journal of Hazardous Materials***, p.126125. (**Impact factor: 9.038**)

B. Under Review

5. Srivastava, V., Gupta, S., Patel, A., Joshi, M. and Kumar, M., 2021. Reflections of COVID-19 cases on the wastewater loading of SARS-CoV-2 RNA: A case of three major cities of Gujarat, India. (Communicated in ***Case Studies in Chemical and Environmental Engineering***) (**Impact factor: NA**)

6. Kumar, M., Mazumder, P., Deka, J. P., Srivastava, V., Mahanta, C., Rangan, L., Gupta, S., Joshi, M., Ramanathan, A L. The Spectre of 1 SARS-CoV-2 in the Ambient Urban Natural Water in Ahmedabad and Guwahati: A Tale of Two Cities (Communicated in ***Environmental Research***) (Impact factor: 5.715)

GOVERNMENT/MEDIA COVERAGE

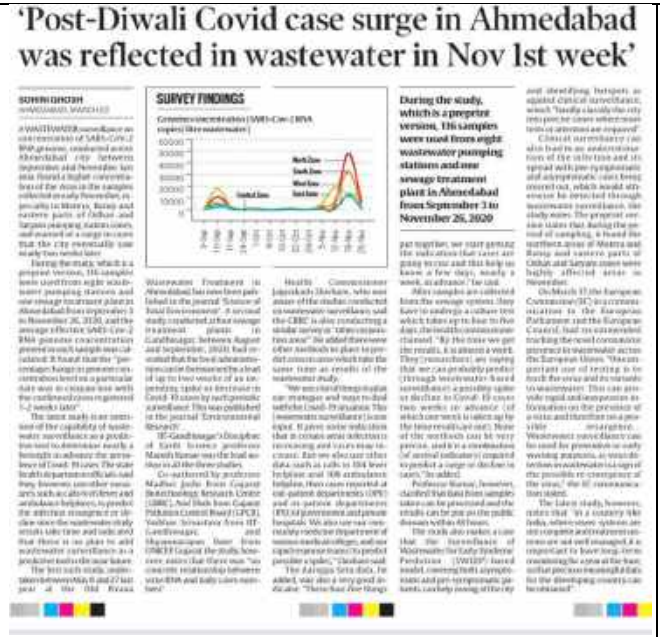
Table 5. Government and Media coverage to our research findings

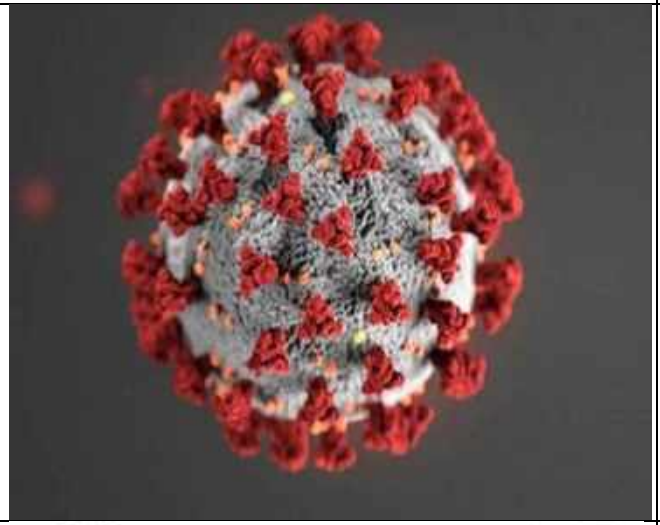
S. No.	Media news	Details and Source	Web link
1.		Title: **कोरोना वायरस के वैज्ञानिक आयाम** Source: **DD Girnara** Date: May 28, 2021	httpswww.youtube.com/watch?v=2oLK83SyQLU
2.		Source: **Ministry of Education** Date: May 20, 2021	Government Twitter handle

3.		Title: **Post pandemic environmental threats and management on world environmental day** Source: **Shobhit University, India** Date: June 5, 2021	https://bit.ly/2RLTTzt
4.		Title: **IIT-Gandhinagar wastewater study reveals resistance among microbes against antibiotics post-Covid** Source: **The Indian Express** Date: May 16, 2021	https://indianexpress.com

5.		Title: **Amid Meagre Testing, Scientists Turn to Sewage as Indicator of Coronavirus Spread in Population** Source: **News18 India** Date: MAY 01, 2020	https://www.news18.com/news/india/amid-meagre-testing-scientists-turn-to-sewage-as-indicator-of-coronavirus-spread-in-population-2600991.html
6.	 'Wastewater can help discern viral spread' Parth.Shastri@timesgroup.com Ahmedabad: The viral load in wastewater can give a fair indication of the pandemic spread and trend. That is the conclusion of a recent analysis of wastewater of Ahmedabad, Vadodara, and Gandhinagar carried out by the researchers from IIT-Gn and Gujarat Biotechnology Research Centre(GBRC). IIT-GN, GBRC ANALYSIS The paper, 'Comparative analysis of SARS-CoV-2 RNA load in wastewater from three different cities of Gujarat, India', was authored by Vaibhav Srivastava, Shilangi Gupta, Arbind Kumar Patel, and Manish Kumar of IIT-Gn. From GBRC, Madhvi Joshi was the author. The paper was recently uploaded on MedRxiv for peer review. GENOME CONCENTRATION Genome concentration in wastewater inlets: Selected areas Area and city / Genome concentration Odhav, Ahmedabad 11,900 Vinzol, Ahmedabad 5,794 Atladara, Vadodara 5,133 Kapurai, Vadodara 4,938 Ranip, Ahmedabad 3,661 Tarsali, Vadodara 3,355 Motera, Ahmedabad 1,200 Bisan, Gandhinagar 919 Paldi, Ahmedabad 590 Figures show genome copies found per 1 lakh litre of wastewater The paper said that Ahmedabad, which has the highest number of Covid-19 cases in Gujarat, had the second largest RNA copies at 2,968 per 1 lakh litre of wastewater based on the analysis of nine wastewater plants. Vadodara had the highest concentration at 3,078. Gandhinagar had 554 RNA copies per 1 lakh litre of water, added the study. The study included three genes — N, ORF1ab and S — for analysis. S and ORF were the highest in Vadodara whereas the N gene was more prominent in Ahmedabad. The samples were collected in the first week of November 2020.	Title: **Wastewater can help discern viral spread** Source: **Times of India** Date: Apr 12, 2021	https://timesofindia.indiatimes.com

		Title: **Researchers read corona outbreak signs in wastewater** Source: **Times of India** Date: May 23, 2021	https://timesofindia.indiatimes.com/
8.		**Special Issue on COVID-19** Journal: **Current Opinion in Environmental Science & Health** **Date:** March 18,2021	https://www.journals.elsevier.com/current-opinion-in-environmental-science-and-health

		Title: **Diwali Covid case surge in Ahmedabad was reflected in wastewater in Nov 1st week** Source: **Indian express** Date: March 23, 2021	https://google.com/amp/s/indianexpress.com/article/cities/ahmedabad/post-diwali-covid-case-surge-7240687/lite/…
10.		Title: **Wastewater Surveillance can warn Of Covid-19 spikes weeks in advance: IIt Gandhinagar study** Source: **Press Trust of India** Date: **Jan 07, 2021**	**https://www.firstpost.com**

11.		Title: **Gandhinagar study: Surveillance of wastewater can give up to 2-week forewarning of Covid outbreak scale** Source: **The Indian Express** Date: **January 5, 2021**	https://indianexpress.com/article/cities/ahmedabad/gandhinagar-study-covid-19-waste-water-plants-7132774/
12.		Dissemination of research from lab to ground level	

13.	 Researchers carried out sample collection for the six-month study **'CITY'S WASTEWATER SURVEILLANCE CAN HELP ISOLATE COVID HOTSPOTS'** **Studying sewage for prevalence of coronavirus can predict an outbreak 2 weeks in advance, say researchers; urge AMC to adopt the surveillance method in its Covid mgmt strategy**	Source: **Ahmedabad mirror** Date: 24 March, 2021	
14.	**India's sewage surveillance for SARS-CoV-2 going down the drain** **Not mainstreaming wastewater epidemiology is a major opportunity lost. Urgently embraced, it might still help India predict the third wave of COVID-19, and future outbreaks.** Subhra Priyadarshini doi:10.1038/nindia.2021.75 Published online 21 May 2021	Source: **Nature India** Date: 21 May, 2021	https://nindia.natureasia.com/en/nindia/article/10.1038/nindia.2021.75?fbclid=IwAR3k1ZXusbXsV1qcdCxnjRK6OJ5CbOyLRejpHk-6psczGfB5iEy4tPrG8GY

15.	 IIT-Gn study: Antibiotics resistance in wastewater microbes post-Covid	Title: **Antibiotics resistance in wastewater microbes post-Covid** Source: **The Indian Express**	https://indianexpress.com/article/cities/ahmedabad/iit-gandhinagar-wastewater-study-reveals-resistance-among-microbes-against-antibiotics-post-covid-7317656/
16.	Dear Dr Manish, I am reaching out to you on behalf of the Smart Cities Mission, Ministry of Housing and Urban Affairs. I came across your work on wastewater surveillance ("First proof of the capability of wastewater surveillance for COVID-19 in India through detection of genetic material of SARS-CoV-2") in Science of the Total Environment a few days back. We at the ministry are exploring options to deploy a large-scale health surveillance system to detect the SARS-CoV-2 virus in communities in smart cities. Wastewater surveillance is one of the most feasible options we could identify. We would like to speak with you to know more about your research work in this domain and understand the effectiveness of such a system.	Dissemination of research from lab to ground level **Cited by the Ministry** **Smart Cities Mission, Ministry of Housing and Urban Affairs**	Personal email

17.		Source: **Ministry of Education** Date: January 10, 2021	**Government Twitter handle**
18.		**Coverage of research work by National TV** Source: **Rajya Sabha TV** Date: January 07,2021	https://youtu.be/SXWZkNb5RcE

19.		Source: **The Indian Express** Date: January 5, 2021	https://indianexpress.com/article/cities/ahmedabad/gandhinagar-study-covid-19-waste-water-plants-7132774/

Photographs

unicef
United Nations Children's Fund

Motera Pumping Station (Residential)

Mega Pipeline Outlet

Nr,VN Bridge(Industrial)

Sabarmati River Nr,Subhash Bridge

Ranip Pumping Station (Commercial)

Vastrapur Lake

Paldi Pumping Station

Odhav (New Pumping Station)

Santivan Pumping Station

Chandola Lake

Maninagar Pumping Station

Kankaria Lake

Satyam Pumping Station

Odhav (New Pumping Station, Commercial)

STP Vinzol (100MLD)

Vatva Pumping Station

Vatva Pumping Station

Ahmedabad Textile Processing Association (CEPT Outlet NR,VN Bridge, Industrial)

Mega Pipeline Outlet Nr,VN Bridge(Industrial)

PUBLICATIONS

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: http://ees.elsevier.com

Wastewater surveillance-based city zonation for effective COVID-19 pandemic preparedness powered by early warning: A perspectives of temporal variations in SARS-CoV-2-RNA in Ahmedabad, India

Manish Kumar [a,*], Madhvi Joshi [b], Anil V. Shah [c], Vaibhav Srivastava [a], Shyamnarayan Dave [d]

[a] Discipline of Earth Science, Indian Institute of Technology Gandhinagar, Gujarat 382 355, India
[b] Gujarat Biotechnology Research Centre (GBRC), Sector-11, Gandhinagar, Gujarat 382 011, India
[c] Gujarat Pollution Control Board, Paryavaran Bhavan, Sector-10A, Gandhinagar, Gujarat 382010, India
[d] United Nations Children's Fund, Gujarat State Office, Gandhinagar, Sector 20, Gandhinagar, Gujarat 382021, India

ARTICLE INFO

Article history:
Received 3 January 2021
Received in revised form 6 June 2021
Accepted 7 June 2021
Available online xxx

Keywords
Wastewater based epidemiology (WBE)
COVID-19
SARS-CoV-2
Pandemic
Surveillance

ABSTRACT

Following the proven concept, capabilities, and limitations of detecting the RNA of Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2) in wastewater, it is pertinent to understand the utility of wastewater surveillance data on various scale. In the present work, we put forward the first wastewater surveillance-based city zonation for effective COVID-19 pandemic preparedness. A three-month data of Surveillance of Wastewater for Early Epidemic Prediction (SWEEP) was generated for the world heritage city of Ahmedabad, Gujarat, India. In this expedition, one hundred sixteen wastewater samples were analyzed to detect SARS-CoV-2 RNA, from September 3rd to November 26th, 2020. A total of 111 samples were detected with at least two out of three SARS-CoV-2 genes (N, ORF 1ab, and S). Monthly variation depicted a significant decline in all three gene copies in October compared to September 2020, followed by a sharp increment in November 2020. Correspondingly, the descending order of average effective gene concentration was: November (~10,729 copies/L) > September (~3047 copies/L) > October (~454 copies/L). Monthly variation of SARS-CoV-2 RNA in the wastewater samples may be ascribed to a decline of 20.48% in the total number of active cases in October 2020 and a rise of 1.82% in November 2020. Also, the monthly recovered new cases were 16.61, 20.03, and 15.58% in September, October, and November 2020, respectively. The percentage change in the gene concentration was observed in the lead of 1–2 weeks with respect to the provisional figures of confirmed cases. SWEEP data-based city zonation was matched with the heat map of the overall COVID-19 infected population in Ahmedabad city, and month-wise effective RNA concentration variations are shown on the map. The results expound on the potential of WBE surveillance of COVID-19 as a city zonation tool that can be meaningfully interpreted, predicted, and propagated for community preparedness through advanced identification of COVID-19 hotspots within a given city.

1. Introduction

The contagious severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for the coronavirus pandemic, has infected 11 million people in India alone by February 22nd, 2021 (WHO, 2020). A large number of asymptomatic patients exerted never seen challenges over the actual estimation of disease spread based on clinical surveillance (Rimoldi et al., 2020; Medema et al., 2020). Earlier studies suggested that 18–45% of patients do not have signs of infection with COVID-19 but are capable of spreading the disease and pose an adverse impact on the actual containment of the disease (Lavezzo et al., 2020; Yang et al., 2020; Mizumoto and Chowell, 2020; Nishiura et al., 2020). Cheung et al. (2020) conducted a study on a total of 4243 COVID-19 patients and detected SARS-CoV-2 RNA in feces from a higher proportion of patients (48.1%) compared to the gastro-intestinal symptoms (17%). As up to 67% of infected people showed the presence of SARS-CoV-2 RNA in feces (Chan et al., 2020; Cheung et al., 2020; Parasa et al., 2020; Wong et al., 2020), alternative approaches such as wastewater-based epidemiology (WBE) surveillance has gained loads of recognition as a viable option that can provide early warning of the upcoming prevalence of the disease within a community (Hata et al., 2021; Kumar et al., 2021a, 2021b). One of the advantages of WBE is that wastewater contains feces from a huge number of people. Therefore, it may require a far fewer number samples and less labor than clinical testing to know the presence of infected persons in the area. However, the sensitivity of WBE for SARS-CoV-2 detection is comparatively less than norovirus, presumably due to the low SARS-CoV-2 load in the patient's fecal matter and it's enveloped nature (Hata et al., 2020). Also, to evaluate WBE's potential as an early prediction tool for the COVID-19 pandemic, it is essential to explore the correlation between the SARS-CoV-2 genetic load in wastewater and the number of cases at the district level in each country.

Overall, following the proven concept and capabilities of detecting the RNA of Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2) in wastewater,

* Corresponding author at: Discipline of Earth Science, IIT Gandhinagar, India.
E-mail address: manish.kumar@iitgn.ac.in (M. Kumar)

https://doi.org/10.1016/j.scitotenv.2021.148367
0048-9697/

several limitations and bottlenecks have been put forward towards its practical applicability (Zhu et al., 2021; Tran et al., 2020). On the other hand, there is a dire need for time-series data of SARS-CoV-2 RNA concentration in the wastewater that can be matched with the actual clinical survey data to confirm the utility and predictability of wastewater surveillance. This is also imperative for the adaptation of the *Surveillance of Wastewater for Early Epidemic Prediction* (*SWEEP*) on the policy level, which has been delayed for some reason in the major parts of the globe (Tiwari et al., 2021). There has also been an active debate of varying levels of effectiveness of WBE based on the size of watersheds, catchment type, complexity of sewer systems, and population. Although the science, concepts, and knowledge pertaining to COVID-19 are still evolving and changes rapidly, it is pertinent to check how effective SWEEP can be on the urban scale, that too if cases reported from the given city have been pretty high. Under this scenario, the four major directions in the field of SWEEP may be summarised as i) substantiating the data unraveling the early warning capability of wastewater surveillance for COVID-19 through temporal studies on SARS-CoV-RNA detection; ii) need for the escalation of WBE monitoring of various parts of the globe to generate results from all the levels of COVID-19 situation; iii) developing the model that can use Ct-value obtained through SWEEP into the meaningful predictions for effective COVID-19 pandemic preparedness; and iv) collectively reach to the understanding of critical issues like removal, discharge, decay, dilution, and infectivity due to the presence of SARS-CoV-2 RNA in wastewaters (Prevost et al., 2015; Kumar et al., 2021a).

In view of this, the objective of this study was to put forward the evidence of practical applicability of SWEEP for COVID-19 pandemic management by comparing the detected concentration of SARS-CoV-2 RNA in wastewater of various parts of the city with the COVID- 19 clinical cases. The Idea is that clinical surveillance hardly classifies the city into precise zones where more tests or attention are required, while SWEEP-based information can help in zoning of the city and identifying the hotspots on a city scale. The detected concentrations of SARS-CoV-2 RNA in wastewater would reflect the true prevalence of COVID-19 infection in the sewer catchment, including clinically undiagnosed patients, while the number of clinically reported cases covers only diagnosed patients and also depends on the number of PCR diagnosis. We analyzed SARS-CoV-2 RNA in the wastewater samples (n = 116) from nine different locations, including wastewater pumping stations and sewage treatment plant (STP) and in Ahmedabad, India, from September 3rd to November 26th, 2020 (thirteen weeks), with the following objectives: a) detection and quantification of SARS-CoV-2-RNA concentration in the influent wastewater samples of Ahmedabad to understand the temporal variation in the pandemic situation over three months, b) weekly resolution of the SARS-CoV-2 RNA data for three months in wastewater samples; and c) explicating the potential of WBE for COVID-19 surveillance as a potential tool for identifying hotspots and public health monitoring at the city level.

2. Material and methods

2.1. Study area

Ahmedabad is the seventh largest city in India and the second biggest trade centre in the western Indian region, with a population of 5.5 million (Census, 2011). It has a 1523 km sewage network assisted with forty-three sewage pumping stations. The present existing treatment capacity of the wastewater treatment plant in the city is 670 MLD in 2007 which is likely to be extended to 1075 MLD by 2021 (https://web.worldbank.org/archive/website01409/WEB/IMAGES/2010_1_1.PDF AMC Report). There are 84 urban health centrespresent in different ward in Ahmedabad (AMC, 2021).

2.2. Sampling approach

In order to achieve the objective; firstly, the entire city was divided based on urban/rural as well as north and south to the Sabarmati River-the major river that dissects the city; and 29 locations had been chosen in association with Gujarat Pollution Control Board (GPCB) officials. We observed the data variations of 29 locations for the first four weeks. Thereafter, based on the significance of the variations within the data-set, we fixed thirteen locations to continue monitoring including nine different locations for the wastewater (eight wastewater pumping stations and a single sewage treatment plant) (Fig. 1); and four surface water locations (three lakes and one river sample). In the present study, we reported weekly data of wastewater samples collected from nine different locations for thirteen weeks during September to November 2020.

A total of 116 samples were analyzed in the present study to detect SARS-CoV-2 RNA from nine different sites, comprising 103 samples from eight wastewater pumping stations and 13 samples from a single sewage treatment plant in Ahmedabad, India. All the samples were collected by grab hand sampling using 250 ml sterile bottles (Tarsons, PP Autoclavable, Wide Mouth Bottle, Cat No. 582240, India). Simultaneously, blanks in the same type of bottle were examined to know any contamination during the transport. The samples were kept cool in an ice-box until further process. The analysis was performed on the same day after bringing the samples to the laboratory. All the analyses were performed in Gujarat Biotechnology Research Centre (GBRC), a laboratory approved by the Indian Council of Medical Research (ICMR), New Delhi.

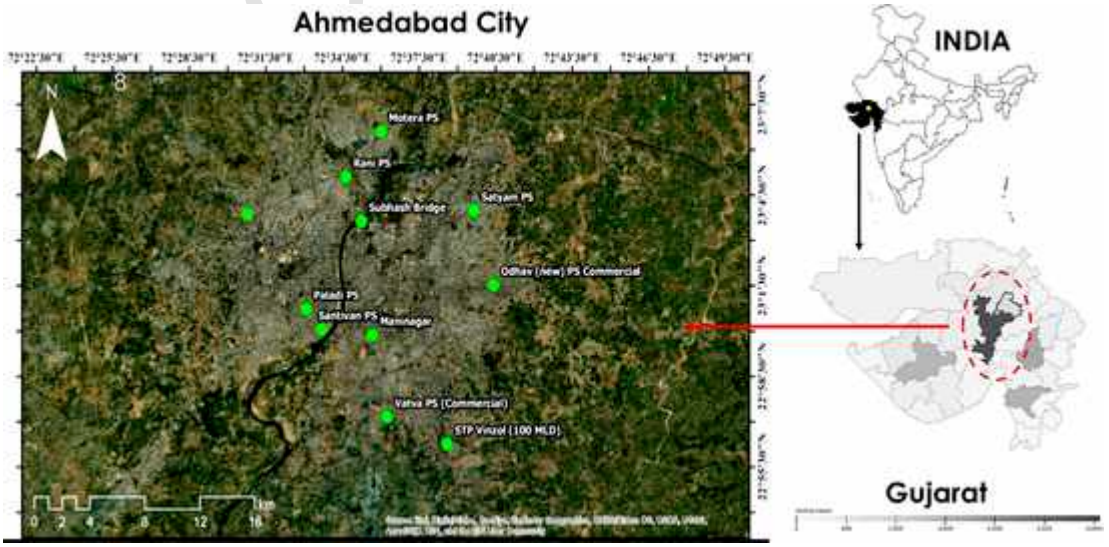


Fig. 1. Geospatial position of sampling locations in Ahmedabad city.

2.3. Detection and extraction of viral RNA from wastewater samples

2.3.1. Precipitation of viral particle

30 mL samples were centrifuged at 4000 ×*g* (Model: Sorvall ST 40R, Thermo Scientific) for 40 min in a 50 mL falcon tube followed by filtration of supernatant using 0.22-micron syringe filter (Mixed cellulose esters syringe filter, Himedia). After filtrating 25 mL of the supernatant, 2 g of PEG 9000 and 0.437 g of NaCl (17.5 g/L) were mixed in the filtrate, and this was incubated at 17 °C, 100 rpm overnight (Model: Incu-Shaker™ 10LR, Benchmark). Next day, the mixture was centrifuged at 14,000 ×*g* (Model: Kubota 6500, Kubota Corporation) for about 90 min. The supernatant was discarded after centrifugation, and the pellet was resuspended in 300 μL RNase-free water. The concentrated sample was kept in 1.5 ml eppendorf at −40 °C, and this was further used as a sample for RNA isolation.

2.3.2. RNA isolation, and RT-PCR

RNA isolation from the pellet with the concentrated virus was performed using NucleoSpin® RNA Virus isolation kit (Macherey-Nagel GmbH & Co. KG, Germany). The samples were spiked with MS2 phage as an internal control prior to the RNA extraction provided by TaqPathTM Covid-19 RT-PCR Kit. Some other specifics are, a) the nucleic acid was extracted by NucleoSpin® RNA Virus isolation kit and Qubit 4 Fluorometer (Invitrogen) was used for the total RNA concentrations estimation, b) MS2 phage was taken as a molecular process inhibition control for evaluating the efficiency of nucleic acid extraction and PCR inhibition (MPC; Haramoto et al., 2020). Briefly, steps were carried out as per the guideline provided with the product manual of Macherey-Nagel GmbH & Co. KG, and RNAs were detected using reverse transcription PCR (RT-PCR).

Applied Biosystems 7500 Fast Dx RT-PCR Instrument (version 2.19 software) was used for SARS-CoV-2 gene detection. In the process, the probes anneal to three specific target sequences located between three unique forward and reverse primers for the N, ORF 1ab, and S genes. A template of 7 μl of extracted RNA was used in each reaction with TaqPath™ 1 Step Multiplex Master Mix (Thermofischer Scientific, USA). Total reaction mixture volume of 20 μL contained 10.50 μL Nuclease-free Water, 6.25 μL Master Mix, and 1.25 μL COVID-19 RT-PCR Assay Multiplex. Three controls were used, namely: positive control (TaqPath™ COVID 19 Control), one negative control (from extraction run spiked with MS2), and no template control (NTC). The RT-PCR contained 1 incubation step cycle of 25 °C & 2 min, 1 cycle of reverse transcription 53 °C & 10 min, 1 cycle of activation 95 °C & 2 min, and 40 cycles of amplification, including denaturation at 95 °C for 03 s and extension 60 °C for 30 s. Finally, results were interpreted using Applied Biosystems Interpretive Software, and Ct values for three target genes i.e., ORF1ab, N Protein, and S Protein of SARS-CoV-2 along with MS2 used as an internal control.

2.3.3. Gene copy estimation: quality control and quality assurance

The samples were considered as positive if at least two of the three primer probe sets showed amplification. The average Ct-value of a given sample was then converted to gene copy numbers considering the equivalence of 500 copies of SARS-CoV-2 genes as 26 Ct-value (provided with the kit), and the same was extrapolated to derive approximate copies of each gene. In this semi-quantitative method to provide the gene concentration, the calibration curve was prepared based on the well-established principle of 3.3 CT change corresponding to a 10-fold gene concentration change. The average effective gene concentration of SARS-CoV-2 present in a given sample was calculated by multiplying the RNA amount used as a template with the enrichment factor for each sample. In addition, we had calculated the gene copy numbers based on the positive control provided with kit i.e., 10^4 copies/μl and the final concentration of 25 copies per reaction. The positive control was providing the same ct values for all 3 genes, and relative to the Ct values of genes of positive controls, copy numbers have been calculated in test samples of different sources. The effective gene concentration is considered as "zero" when RT-PCR results were positive for only one gene out of three in the wastewater sample. The limit of detection has been set to 40 amplification cycle (Ct = 40) in the RT-PCR analysis. The effective gene concentration was calculated by averaging the gene copies of all three genes in a particular sample.

Due to various constraints, samples were analyzed in duplicate, considering that the samples were analyzed in the batch accompanied with negative and positive controls, and each sample was spiked with known concentrations of MS2. In the event of any variations (among duplicate and controls) of more than 10%, samples were re-analyzed. It is worth noting that the primer efficiency of different genes will be slightly varied according to the primer sequence. Based on several hundreds of RTPCR run, it was found that the positive control was robust enough to provide the same Ct values for all three genes, implying no evident difference between the primer efficiency. We report both primary Ct-values and derived gene copies relative to the Ct values of positive controls for both individual genes and effective SARS-CoV-2 gene concentration.

2.4. Epdemiological information, data collection and interpretation

The data of affected people and their locations were obtained from the governmental mobile application 'Arogya Setu' which is published as Ahmedabad COVID-19 community vulnerability map published by SustainAbly and Accion Land Pvt. Ltd., accessible at http://google.org/crisismap/a/gmail.com/amdcovid19. 'Aarogya Setu' mobile application was launched by the Ministry of Electronics and Information Technology of the Indian government for collecting data pertaining to tracing, syndromic mapping and self-assessment on COVID–19. This application reached more than 100 million installs in 40 days (Arogya setu, Wikipedia 2021). Other information was obtained from the Ahmedabad city portal accessed using link https://ahmedabadcity.gov.in/portal/web?requestType=ApplicationRH&actionVal=loadCoronaRelatedDtls&queryType=Select&screenId=114. Several other informations can be accessed using https://ahmedabadcity.gov.in/portal/jsp/Static_pages/water_project.jsp.

The percentage change showed for the confirmed and active cases were calculated using the formula:

$$\%Change = \left\{ \frac{(No.\ of\ case\ in\ the\ present\ week - No.\ of\ case\ in\ the\ previ}{No.\ of\ case\ in\ the\ previous\ week} \right.$$

Statistical Package for the Social Sciences (SPSS 21) has been used for hypothesis testing through Analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT). The OriginPro 2019b data analysis software has been used to draw boxplots.

3. Results and discussions

We detected and quantified variation in SARS-CoV-2 RNA from wastewater samples for three months (September and November) to understand the pandemic situation in Ahmedabad, Gujarat, India. Among the 116 samples analyzed in the study, 111 (95.7%) were found positive, comprising at least two positive RT-PCR results targeting SARS-CoV-2 ORF1ab, S gene, and N gene assays (Table 1). In addition to this, 109/116 (93.7%) samples showed positive RT-PCR results for each N, ORF 1b, and S genes. The distribution analysis of Ct values for different genes using boxplot is represented in Fig. 2. The average Ct values for N, ORF 1ab, and S genes were 32.50, 32.36, and 33.85, respectively. The average Ct values of internal control (MS2 bacteriophage) was 28.2, and no SARS-CoV-2 genes were detected in the negative control samples.

3.1. Monthly and weekly variations

Monthly variation depicted a significant decline of 89.7, 63.7, and 90.1% in N, ORF-1ab, and S gene concentration (copies/L), respectively in October compared to September 2020, followed by a sharp increment in November 2020 i.e., ~25 folds in N gene, ~22 folds in ORF 1ab and ~26 folds in S gene. The PCR products for all three genes were maximum in wastewater samples of November, followed by September and October (Fig. 3a–c). Likewise, the effective gene concentration of SARS-CoV-2 was maximum in the month of No-

Table 1
Temporal variation in gene copies of the SARS-CoV-2 targeted genes and effective gene concentration at various locations in Ahmedabad city.
Where, ND = not detected; NA = data not available.

Station	Sampling date	September, 2020				October, 2020					November, 2020			
		3.09	10.09	17.09	24.09	0.1.10	8.10	15.10	22.10	29.10	5.11	12.11	19.11	26.11
	Active cases	3671	4168	4038	4252	4122	3614	3472	3451	3372	3283	3280	3362	3293
	Confirmed cases	32013	33204	34408	35672	37041	38381	39655	40922	42155	43381	44690	46268	48710
	SARS-CoV-2 genes	Gene copies (copies/L) × 10^3												
Motera PS	N	19.9	120	0.36	1.56	7.99	2.84	1.23	30.8	ND	28.7	70.8	522.7	57.8
	ORF	5.84	16	1.43	5.73	1.7	1.16	10	20.8	ND	3.86	104	783.2	44.4
	S	4.4	71.1	0.78	4.6	1.27	1.32	3.17	11.3	0.34	3.65	63.9	350.8	18.2
	Effective gene conc.	10.1	69.1	0.86	3.96	3.65	1.77	4.8	21	ND	12.1	79.5	552.2	40.1
Ranip PS	N	3.18	310.4	9.8	5.4	6.61	3.73	2.17	0.68	0.17	64.2	33.1	471	124.7
	ORF	ND	51.9	41.7	14.8	0.86	ND	13.3	3.59	0.95	29.9	30.5	463.2	101.9
	S	0.46	105	39.2	15.2	1.67	0.51	5.91	0.18	0.78	15.8	24.8	289.9	37.04
	Effective gene conc.	1.22	155.8	30.2	11.8	3.05	1.41	7.14	1.48	0.63	36.6	29.4	408.1	87.9
Paldi PS	N	5	40.5	3.26	ND	12.1	0.27	0.23	0.55	0.3	8.69	12.6	99.8	39.1
	ORF	1.73	11.7	11.1	0.28	3.31	0.19	0.69	2.17	0.27	5.77	24.4	140.5	21.9
	S	0.79	29.6	9.8	0.75	1.76	ND	0.66	2.78	0.69	3.52	27.2	118.9	9.93
	Effective gene conc.	2.51	27.2	8.07	0.34	5.74	0.15	0.53	1.83	0.42	5.99	21.4	119.7	23.6
Santivan PS	N	12.4	100	3.07	1.37	2.15	2.37	0.87	0.96	ND	15.1	2.74	116.3	12
	ORF	4	30.4	9.74	4.13	0.65	0.24	3.9	5.17	ND	12.2	3.89	129.6	12.9
	S	3.14	86.6	10.4	4.57	1.2	ND	1.87	1.55	0.15	6.03	4.24	141.9	3.67
	Effective gene conc.	6.51	72.3	7.74	3.36	1.33	0.87	2.21	2.56	NO	11.1	3.63	129.3	9.5
Maninagar PS	N	5.8	48.5	6.15	0.62	15.4	3.5	2.78	ND	0.15	8.3	NA	168.6	34.5
	ORF	1.05	10.3	26.3	2.62	3.95	0.26	26.1	2.54	1.68	5.93	NA	172.7	28.3
	S	1.18	20.6	35.2	2.08	2.84	0.73	12.1	ND	0.47	2.17	NA	105.2	10.4
	Effective gene conc.	2.68	26.4	22.5	1.78	7.39	1.5	13.7	ND	0.77	5.47	NA	148.8	24.4
Satyam PS	N	14.1	141.7	4.91	6.48	30.4	4.88	2.23	0.21	0.28	10.2	8.21	23.2	29.5
	ORF	1.4	39.9	23	24.8	10.8	1.05	17.2	3.34	2.15	7.52	5.82	13.3	27.6
	S	2	78	23.3	24.8	7.01	0.36	7.09	1.69	0.51	0.68	3.03	12.1	10.3
	Effective gene conc.	5.82	86.5	17.1	18.7	16.1	2.1	8.85	1.75	0.98	6.15	5.69	16.2	22.5
STP Vinzole	N	11	92.2	2.57	ND	20.6	ND	1.68	3.16	0.92	111.5	127.4	470.9	56.4
	ORF	3.97	22.3	34.7	ND	6.44	ND	16.8	34.7	6.8	43.9	187.1	1049.2	17.1
	S	6.23	51.1	37.3	ND	4.97	ND	5.98	11.6	2.52	18.5	105	374.5	11.7
	Effective gene conc.	7.06	55.2	24.9	ND	10.7	ND	8.15	16.5	3.41	57.9	139.8	631.5	28.4
Odhav PS	N	38.1	427.5	5.84	1.69	22.7	0.29	2.2	ND	1.61	155.2	305.2	249.9	401.7
	ORF	17.9	91.4	31	3.48	9.05	ND	21.3	40.5	8.38	132.5	512.1	277.8	305.5
	S	6.87	329.2	31.5	7.43	6.96	ND	8.57	0.15	3.36	69.3	316.5	206.4	131.7
	Effective gene conc.	20.9	282.7	22.8	4.19	12.9	5.74	10.7	0.2	4.45	119	377.9	244.7	279.6
Vatva PS	N	13.2	110	3.12	0.87	10.3	0.8	0.22	2.02	0.46	23.9	28.5	34.7	15.2
	ORF	7.94	35.4	15.5	0.13	4.63	0.14	7.7	17.2	2.14	6.54	23.2	31.8	10.8
	S	1.33	48.7	20.2	1.01	3.01	ND	1.09	8.56	0.76	7.93	17.5	25.3	5.62
	Effective gene conc.	7.51	64.7	12.9	0.67	5.97	0.47	3	9.27	1.12	12.8	23	30.6	10.5

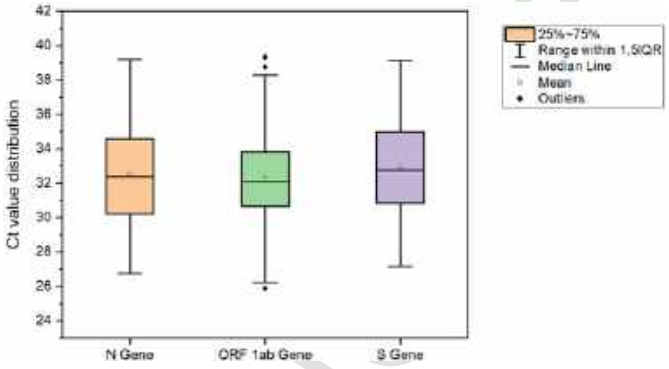


Fig. 2. Distribution of Ct values of SARS-CoV-2 genes during the study period.

vember (~10,729 copies/L), followed by September (~3047 copies/L), and October (454 copies/L) in line with a ~1.5-fold rise in the number of confirmed cases during the study period (3rd September 2020 and 26th November 2020) (Fig. 3d).

There had been a decline of 20.47% in active cases in October 2020 with respect to September, and a rise of 1.82% occurred in November 2020 compared to the preceding month i.e. October. While the increase of 1.82% in the active cases of November with respect to October is equivalent to a change of 59 cases (3234 cases on 1st November–3293 on 26th November); however, the same monthly change in the total confirmed cases between October and November has been of 14.1% due to addition of 6019 new cases to the tally of October by 26th November 2020. Also, a monthly decrease of 4.45% in recovered cases was noticed in November compared to October 2020. The monthly recovered new cases were 16.61, 20.02, and 15.58% in September, October, and November 2020, respectively. Apart from that, people's casual and reluctant attitude during the festive season in India (mid-October to mid-Nov) might be the reason for the piercing rise in COVID-19 cases.

Weekly temporal variations in average SARS-CoV-2 gene copies were analyzed for SARS-CoV-2 RNA presence in samples collected from all the sampling locations in Ahmedabad and are displayed in Fig. 4a–d. One-way ANOVA and Duncan post hoc test ($p < 0.05$) were performed to see the significance level in gene copy variation among different sampling dates. The results showed significant differences in all three gene copies, i.e. N-gene (ANOVA, $F = 7.49$, $p < 0.001$), ORF-1ab genes (ANOVA, $F = 5.94$, $p < 0.001$), and S-gene (ANOVA, $F = 8.25$, $p < 0.001$) on the temporal scale (sampling dates). Similarly, differences were significant in the case of effective gene concentration (ANOVA, $F = 7.12$, $p < 0.001$).

The N-Gene concentration in wastewater samples collected on September 10th, 2020 was found to be significantly higher than other sampling dates, except November 26th, 2020, and lower than November 19th, 2020. The ORF 1ab gene copies/L in wastewater samples noticed maximum on November 19th, 2020 and were significantly higher than other sampling dates. Except for November 19th, 2020, the changes in ORF 1ab gene concentration were insignificant among different sampling dates. Likewise, the highest S-Gene con-

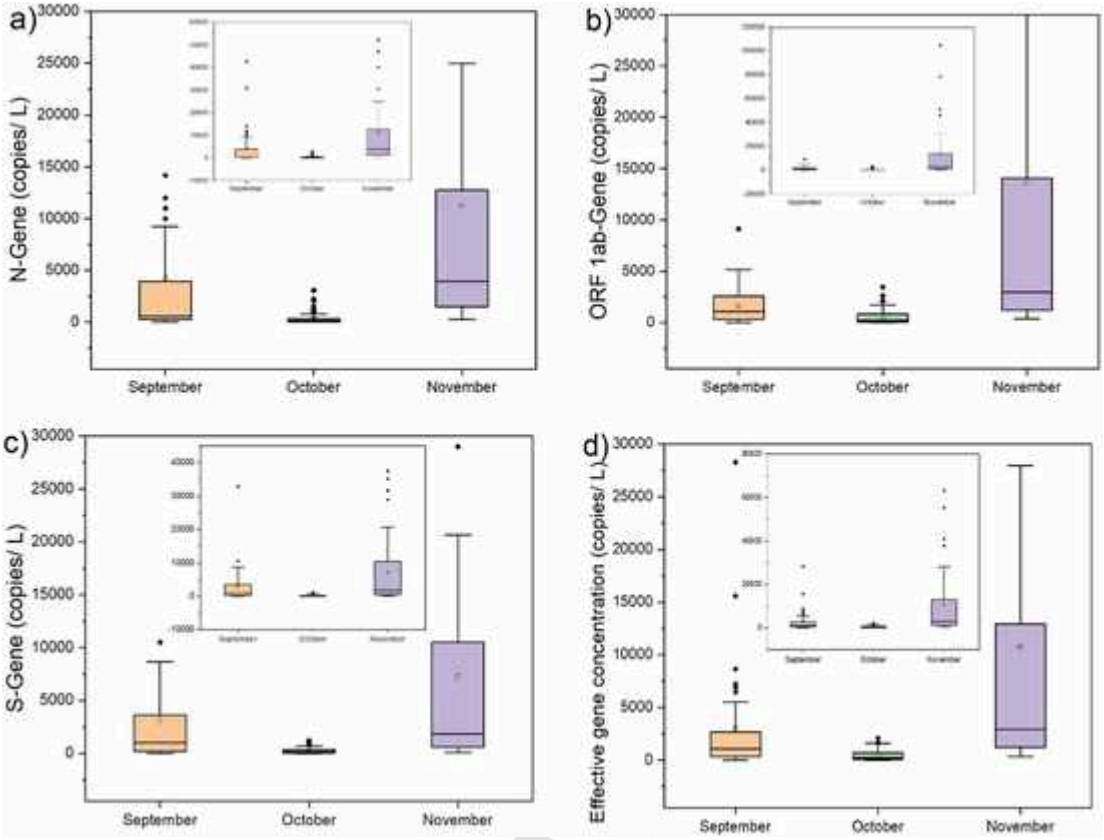


Fig. 3. Distribution of SARS-CoV-2 gene copies on a temporal scale (monthly variation).

centration was noticed on November 19th, 2020 ($p < 0.05$), followed by September 10th, 2020. The S gene copies/L in wastewater samples collected on September 10th, 2020 was significantly higher than other sampling dates except for November 12th, 2020. In addition to this, the alteration in S-Gene concentration was statistically insignificant among the remaining dates. Moreover, the SARS-CoV-2 effective gene concentration was found to be maximum and significantly higher on November 19th, 2020 than others. The effective gene concentration in wastewater sampled on September 10th, 2020 was significantly higher than the samples of September 24th, 2020 and October 8th & 29th 2020. All three gene copies (i.e. N, ORF1ab, and S genes) and effective gene concentration were detected maximum on November 19th, 2020, and values were significant ($p < 0.05$) as compared to other sampling dates. The exponential rise in virus gene concentration might be due to the decline in the decreasing trend (<−0.1%, November 12th, 2020) followed by the increase in the number of active cases (i.e. 2.5% which corresponded to the 82 new cases on November 19th, 2020), compared to the earlier sampling dates.

The major implications of these temporal variations in monthly and weekly data of various genes can be explained in three ways: i) the explicit effect of variations in new confirmed cases on gene copies. In this context, it is interesting to note that change in the active cases is not showing much relationship with the WBE data; ii) there is not much difference among the individual genes and effective gene concentrations when we visualize the monthly variation; and iii) weekly variation brings out the difference among the various genes and need to normalize the data in effective gene concentrations. Weekly data explicitly confirms that N genes are much more resistant among the three and ORF-1ab seems the least sensitive gene. These two observations are clearly evident in data of 10th September and 5th November (Fig. 4) when the varitions/disagreements among the various genes are explicit. The further implications of these findings are related to the required sampling event and calculations of the effective gene calculations. It is evident here is that biweekly sampling should be enough to get a trend in a given Indian city. Also, COVID-19 wastewater surveillance based data must not be judged or evaluated based on a single particular gene of SARS-CoV-2 but its effective gene concertation based on multiple genes.

3.2. SWEEP-based city zonation and identification of hot-spots

Depending on the SARS-CoV-2 effective gene concentration in wastewater samples based on analytical results, we identified highly susceptible areas for COVD-19 infection and its transmission among the community. Although we do not have explicit epidemiological data at the ward level/sampling locations; variations were good enough to classify a city based on SARS-CoV-2 gene concentration in wastewater samples. The north (Motera and Ranip) and east (Odhav and Satyam) zones were highly affected areas with an average effective gene concentration of ~15,574 and ~13,397 copies/L, respectively, in November (Fig. 5a). Likewise, in September, wastewater samples collected from the east zone showed maximum effective gene concentration (~5734 copies/L), followed by the north zone (~3536 copies/L). Though areas present in north and east zones showed high virus genetic load, yet a sharp rise in SARS-CoV-2 RNA was noticed in all the zones in November 2020 (Fig. 5a). It has also been represented in a summarised format with a comparison to the affected population in the city (Fig. 5b & c).

It is imperative to note that 5b is a generalised status of the city as of 26th November 2020 pertaining to the COVID-19 total confirmed cases and Fig. 5c depicts three months change in SARS-CoV-2 effective gene concentration by bar diagram with existing positive cases of 26th November 2020 by colour coding. Although it would have been better to provide heat maps, active case distributions and effective gene concentrations over the entire study period to understand the effectiveness of WBE surveillance; the two observations are critical i.e. i) Satyam and Vinzol locations showed opposite monthly trends of SARS-CoV-2 gene concentration. It was found to be higher in case of Vinzol for the month of November compared to Satyam, implying the capability of WBE to distinguish the parts of city based on SARS- CoV-2 gene concentration; and ii) scale of change varies among the sampling locations, therefore

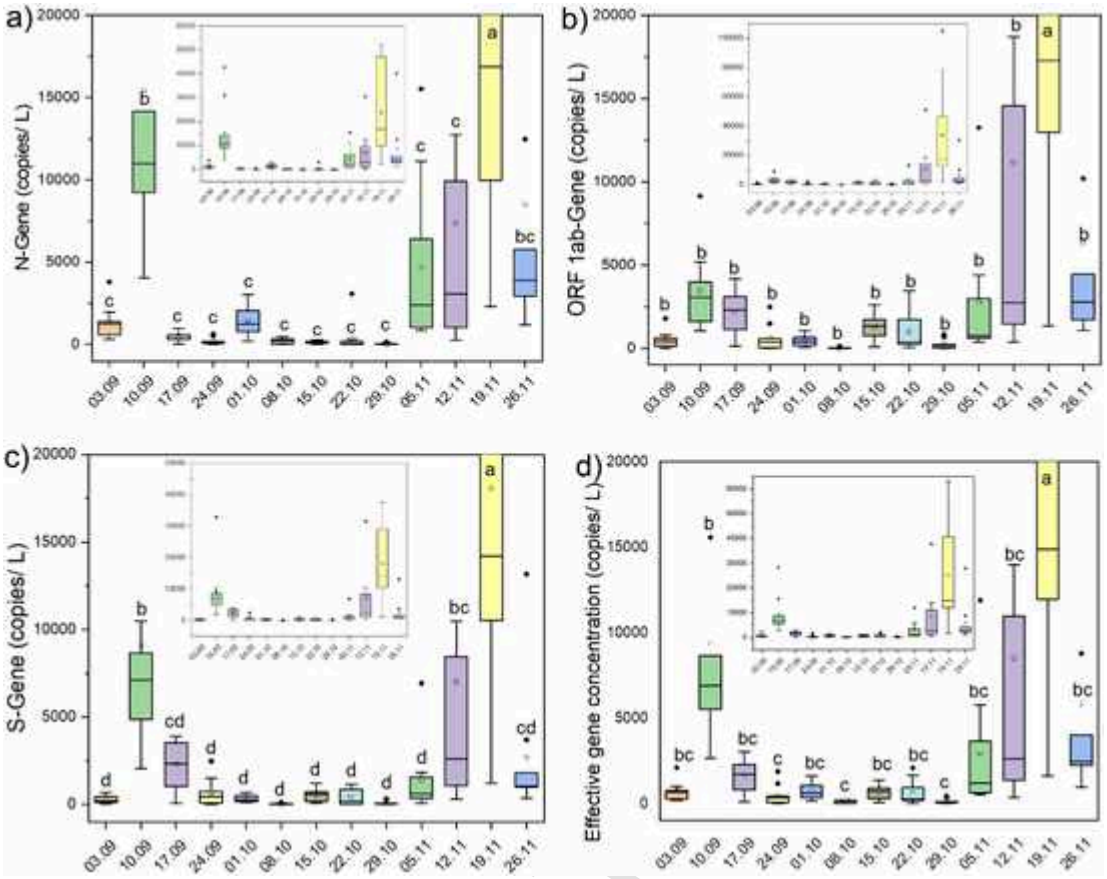


Fig. 4. Temporal variations in targeted gene copies of SARS-CoV-2, collected from different sampling points a.) N gene, b.) ORF 1ab gene, c.) S gene, and d) effective gene concentration.

seems to be related to the size of the catchment and treatment plant, suggesting month-wise variation is not enough. Also, there is a need for the match between the epidemiological data and SARS-CoV-2 gene concentration in wastewater samples. Overall, despite several challenges in epidemiological and clinical data collection as well as sewage water collection and catchment delineation in India, the proper scrutiny and regular monitoring of wastewater could be useful for preparedness against adverse conditions as appeared in post-festive days in Ahmedabad.

The SWEEP technology offers a better picture of the pandemic situation at the sub-city or zone level, relying on the SARS-CoV-2 RNA concentration in wastewater samples of a particular area. SWEEP data can help to estimate the actual extent of the infection due to the SARS-CoV-2, as it covers both asymptomatic and presymptomatic patients, which may be underestimated by clinical surveillance. Therefore, SWEEP data-based zonation of the city can help to identify hot-spots to increase the preparedness in advance. On the other hand, clinical surveillance usually fails to classify the city into distinct zones as it is more dependent on the location of test centres rather than the COVID-19 patients, and owing to asymptomatic and presymptomatic patients. This is why several study could early detect the SARS-CoV-2 RNA in wastewater, before the first clinical report like Medema et al. (2020) in the Netherlands, La Rosa et al. (2020) in two different cities in Italy and Randazzo et al. (2020) in Spain. However this is probably the first study where the SARS-CoV-2 RNA data has been compared with ward wise positive patient counts.

3.3. Early warning potential of WBE

In this view, the present research work followed our first proof concept study, where we detected SARS-CoV-2 genetic material in wastewater and proposed its wide applicability for COVID surveillance in the community (Kumar et al., 2020a). The linear regression between changes in SARS-CoV-2 effective gene concentration and the number of confirmed cases showed a positive correlation (Fig. S1) but was not statistically significant ($p = 0.135$, $R = 0.438$). There was no linear relationship between the SARS-CoV-2 gene concentration and epidemiological data. Therefore, we showed the relationship between percentage changes in effective gene concentration and confirmed cases that can be used as a pre-alarming tool, which gives a lead of ~2 weeks for the upcoming scenario (Fig. 6). Examining the potential of WBE for COVID-19 surveillance as a potential tool showed that the percentage change in effective gene concentration level on a particular date was in conjunction with the confirmed cases registered 1–2 weeks later on a temporal scale by the regulatory authority based on clinical tests (Fig. 6). For example, on October, 8th, 2020, a sharp decline of ~86% was noticed in the percentage change in the average effective gene concentration which was followed by ~0.4% decline in the percentage change in confirmed COVID cases on October, 22nd, 2020. Likewise, on November 5th, 2020, a steep hike of >22-folds in the percentage change in the average effective gene concentration was noticed compared to the earlier sampling date, which was followed by ~0.6% and 2.37% increment in the percentage change in confirmed COVID cases on November 19th and November, 26th, 2020, respectively. In the contrary, more than >1000% and 500% increase were noticed in percentage change in SARS-CoV-2 effective gene concentration in wastewater in early September and mid-October, respectively. However, there seems no notable increase in the number of confirmed cases 1–2 weeks later. Still, this technique displayed positive prediction in most of the cases during the study period. Therefore, we can predict the severity of the pandemic situation 1–2 weeks prior to the official reports by the regulatory body based on clinical tests.

The results unravel the potential of WBE surveillance of COVID-19 as an early warning tool displayed by the adequate presence of SARS-CoV-2 genetic material in wastewater samples though limited cases were documented and based on the immediate future trends. These findings were in agreement with

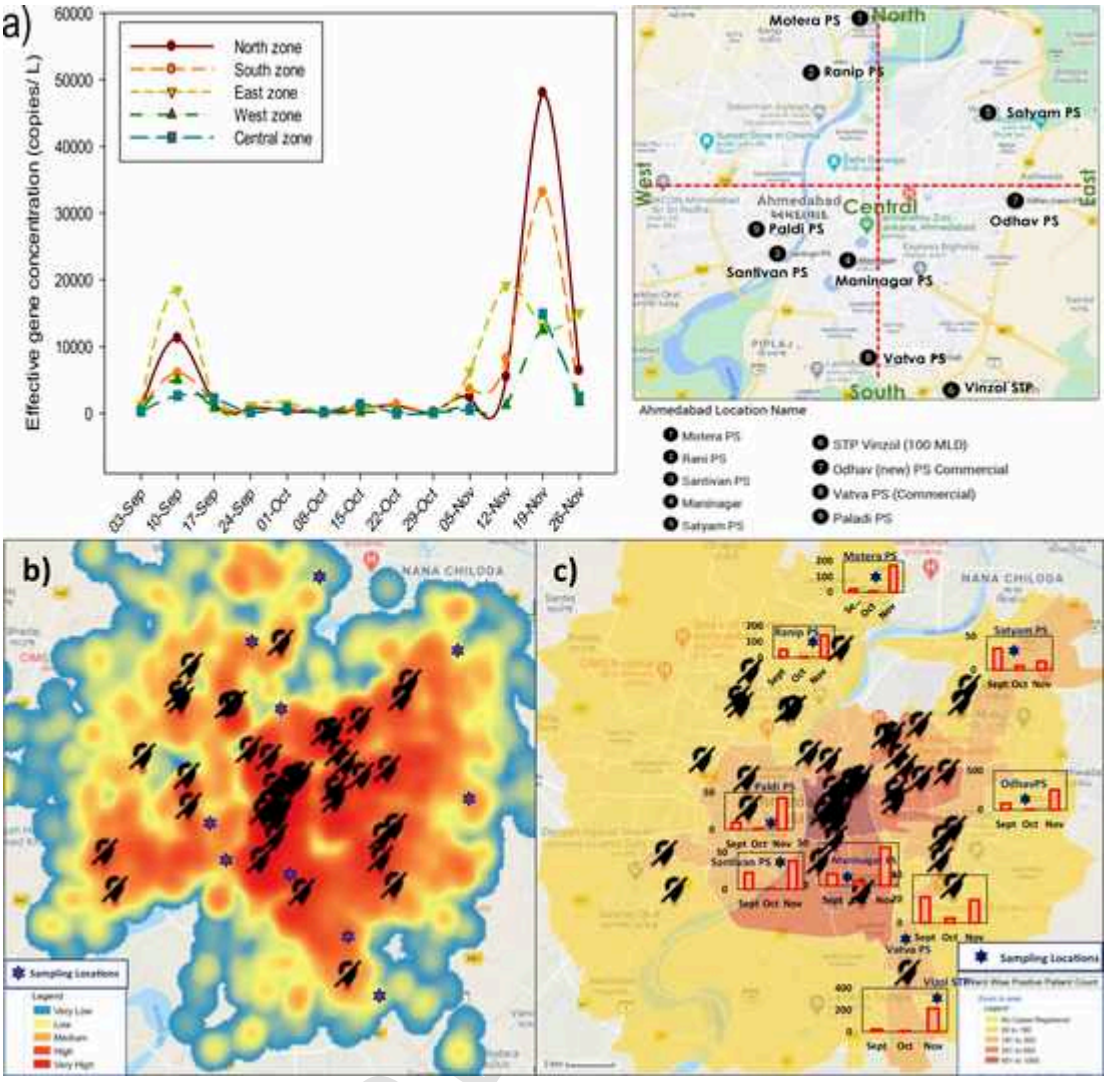


Fig. 5. a) Zone-wise Covid-19 pandemic status in Ahmedabad city; b) Heat map of the overall infected population in Ahmedabad City based on Aarogya-setu mobile application. Very low, low, medium, high and very high indicates no to up to 50, 51–180, 181–300, 301–650, and >651 registered positive covid-19 cases per ward. and c) Monthwise Effective gene concentration at the sampling locations (y-axis in bar diagrams represents SARS-CoV-2 effective gene concentration in copies $\times 10^2$/L wastewater samples). Note: Positive patient count has been taken on ward basis not on the population-density.

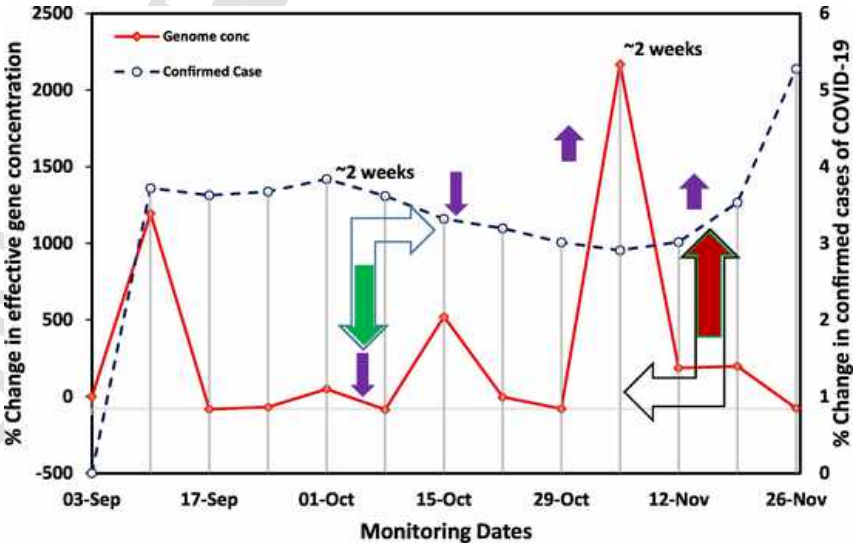


Fig. 6. Potential and evidence of wastewater based epidemiology surveillance of Covid-19 pandemic as an early warning tool.

those of Ahmed et al. (2020b), who noticed a longitudinal decline in the presence of SARS-CoV-2 RNA with the tapering of the first epidemic wave; however, there was no concrete relationship between virus RNA and daily cases numbers.

3.4. Limitations and perspectives

Epidemiological data accessible for this study has been weak as the clear catchment delineation and exact population being catered by each location is not precise. We also just matched RNA data with secondary sourced information on COVID-19 vulnerability maps. Although it may still be considered as a good beginning, yet it emphasizes the need for collaborations among the different governmental organizations. Nevertheless, we explicitly put forward an example of the effectiveness of SWEEP for the early warning of COVID-19, and emphasize the continuous long-term monitoring with the following future objectives: i) monitoring the COVID-19 curve in the post-vaccination period through quantifying the genetic material of SARS-CoV-2 in the wastewaters of a given city (Ahmedabad); ii) understanding the association of antibiotic resistance with COVID-19 prevalence (Kumar et al., 2021b); iii) developing an online portal with a weekly update of gene concentration with accessibility provided to the public and policymakers; iii) estimating the potential risk of SARS-CoV-2 in natural water bodies through various water activities using a quantitative microbial risk assessment (QMRA) framework; iv) generating longer time-series data to further check the robustness of early warning capability of the techniques and its possible benefits (Kumar et al., 2021c); and v) developing predictive modeling for connecting the missing points in SWEEP generated database, meaningful interpretations, and supporting other surveillance protocols. SWEEP can be considered for developing advisory in the context of rapid-testing, the number of testing, community clearance, hotspot identification, vaccine need identification zones, as well as making a recommendation on staying at home and implementing curfews.

In this first phase, we have explicitly shown the capability of WBE as an early warning and city zonation tool however in a country like India, where sewer systems are not complete, and treatment systems are not well-managed, it is important to have long-term monitoring for a year at the least so that precious meaningful data for the developing country can be obtained. Furthermore, a practical guide and pandemic management tools can be developed by integrating the virtues of information technology with the early warning capability of wastewater surveillance. Confidence may be generated among the commons as well as to the government agencies like Ahmedabad Municipal Corporation (AMC) for incorporating WBE into regular monitoring program for the management of the current/future COVID-like epidemic/pandemic outbreak.

4. Conclusion

A temporal variation of SARS-CoV-2 RNA presence in wastewater was studied for a period of three months in Ahmedabad, India. A total 111 samples (95.7%) of the total 116 samples tested in the study were found to be positive, with at least two positive RT-PCR results targeting SARS-CoV-2 ORF1ab, S gene, and N gene assays. Monthly variation depicted a significant decline in all three gene targets in October compared to September 2020, followed by a sharp increment in November 2020. Correspondingly, the descending order of average effective gene concentration was November (~10,729 copies/L) > September (~3046 copies/L) > October (~454 copies/L). This finding was further supported by the relation between the percentage change in effective gene concentration level and confirmed cases, which followed a similar trend on the temporal scale with a ~1 to 2 weeks' time distance. The results unveiled the untapped potential of WBE surveillance of COVID-19 as an early warning tool for practical use of city zonation based on SWEEP data for actual scenarios and future prediction. This approach may help the authorities identify the hotspots within a city and tuning effective management interventions. Further research may be focused on quantification of correlation of SWEEP results with clinical surveillance data and development of a predictive model that can translate SWEEP data for easy propagation to policymakers and common public to enhance the preparedness and management of pandemics.

Uncited references

Ahmedabad, 2020
Kumar et al., 2020b
Kumar et al., 2020c
Xiao et al., 2020

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work is funded by UNICEF, Gujarat and UKIERI. We also acknowledge the help received from Dr. Arbind K Patel, Mr. Alok Thakur, and other GBRC staffs who contributed towards sample and data analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.148367.

References

Aarogya Setuhttps://en.wikipedia.org/wiki/Aarogya_Setu2021accessed 20 March 2021

Ahmed, W., Tscharke, B., Bertsch, P.M., Bibby, K., Bivins, A., Choi, P., Clarke, L., Dwyer, J., Edson, J., Nguyen, T.M.H., O'Brien, J.W., 2020. SARS-CoV-2 RNA monitoring in wastewater as an early warning system for COVID-19 transmission in the community: a temporal case study. Sci. Total Environ. 144216. doi:10.1016/j.scitotenv.2020.144216.

AhmedabadCOVID-19 community vulnerability map published by SustainAbly and Accion Land Pvt. Ltdaccessible athttp://google.org/crisismap/a/gmail.com/amdcovid192020s 12 December 2021

Chan, P.K., Lui, G., Hachim, A., Ko, R.L., Boon, S.S., Li, T., Kavian, N., Luk, F., Chen, Z., Yau, E.M., Chan, K.H., 2020. Serologic responses in healthy adult with SARS-CoV-2 reinfection, Hong Kong, August 2020. Emerg. Infect. Dis. 26 (12), 3076. doi:10.3201/eid2612.203833.

Cheung, K.S., Hung, I.F., Chan, P.P., Lung, K.C., Tso, E., Liu, R., Ng, Y.Y., Chu, M.Y., Chung, T.W., Tam, A.R., Yip, C.C., 2020. Gastrointestinal manifestations of SARS-CoV-2 infection and virus load in fecal samples from the Hong Kong cohort and systematic review and meta-analysis. Gastroenterology. doi:10.1053/j.gastro.2020.03.065.

Haramoto, E., Malla, B., Thakali, O., Kitajima, M., 2020. First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. Sci. Total Environ. 737, 140405. doi:10.1016/j.scitotenv.2020.140405.

Hata, A., Hara-Yamamura, H., Meuchi, Y., Imai, S., Honda, R., 2020. Detection of SARS-CoV-2 in wastewater in Japan during a COVID-19 outbreak. Sci. Total Environ. 143578. doi:10.1016/j.scitotenv.2020.143578.

Kumar, M., Patel, A.K., Shah, A.V., Raval, J., Rajpara, N., Joshi, M., Joshi, C.G., 2020a. First proof of the capability of wastewater surveillance for COVID-19 in India through detection of genetic material of SARS-CoV-2. Sci. Total Environ. 746, 141326. doi:10.1016/j.scitotenv.2020.141326.

Kumar, M., Thakur, A.K., Mazumder, P., Kuroda, K., Mohapatra, S., Rinklebe, J., Ramanathan, A.L., Cetecioglu, Z., Jain, S., Tyagi, V.K., Gikas, P., 2020b. Frontier review on the propensity and repercussion of SARS-CoV-2 migration to aquatic environment. J. Hazard. Mater. Lett. 1, 100001. doi:10.1016/j.hazl.2020.100001.

Kumar, M., Kuroda, K., Patel, A.K., Patel, N., Bhattacharya, P., Joshi, M., Joshi, C.G., 2020c. Decay of SARS-CoV-2 RNA along the wastewater treatment outfitted with Upflow Anaerobic Sludge Blanket (UASB) system evaluated through two sample concentration techniques. Sci. Total Environ. 754, 142329. doi:10.1016/j.scitotenv.2020.142329.

Kumar, M., Alamin, M., Kuroda, K., Dhangar, K., Hata, A., Yamaguchi, H., Honda, R., 2021a. Potential discharge, attenuation and exposure risk of SARS-CoV-2 in natural water bodies receiving treated wastewater. npj Clean Water 4 (1), 1–11. doi:10.1038/s41545-021-00098-2.

Kumar, M., Dhangar, K., Thakur, A.K., Ram, B., Chaminda, T., Sharma, P., Kumar, A., Raval, N., Srivastava, V., Rinklebe, J., Kuroda, K., 2021b. Antidrug resistance in the Indian ambient waters of Ahmedabad during the COVID-19 pandemic. J. Hazard. Mater. 126125. doi:10.1016/j.jhazmat.2021.126125.

Kumar, M., Joshi, M., Kuroda, K., Bhattacharya, P., Barcello, D., 2021c. First comparison of conventional activated sludge versus root-zone treatment for SARS-CoV-2 RNA removal from wastewaters: statistical and temporal significance. Chem. Eng. J..

La Rosa, G., Iaconelli, M., Mancini, P., Ferraro, G.B., Veneri, C., Bonadonna, L., Lucentini, L., Suffredini, E., 2020. First detection of SARS-CoV-2 in untreated wastewaters in Italy. Sci. Total Environ. 139652. doi:10.1016/j.scitotenv.2020.139652.

Lavezzo, E., Franchin, E., Ciavarella, C., Cuomo-Dannenburg, G., Barzon, L., Del Vecchio, C., Rossi, L., Manganelli, R., Loregian, A., Navarin, N., Abate, D., 2020. Suppression of

early stage of the epidemic in the Netherlands. Enviro.n Sci. Technol. Lett. 7 (7), 511–516. doi:10.1021/acs.estlett.0c00357.
Mizumoto, K., Chowell, G., 2020. Estimating risk for death from coronavirus disease, China, January–February 2020. Emerg. Infect. Dis. 26 (6), 1251. doi:10.3201/eid2606.200233.
Nishiura, H., Linton, N.M., Akhmetzhanov, A.R., 2020. Serial interval of novel coronavirus (COVID-19) infections. Int. J. Infect. Dis.. doi:10.1016/j.ijid.2020.02.060.
Prevost, B., Lucas, F.S., Goncalves, A., Richard, F., Moulin, L., Wurtzer, S., 2015. Large scale survey of enteric viruses in river and waste water underlines the health status of the local population. Environ. Int. 79, 42–50. doi:10.1016/j.envint.2015.03.004.
Randazzo, W., Truchado, P., Cuevas-Ferrando, E., Simón, P., Allende, A., Sánchez, G., 2020. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. Water Res. 181, 115942. doi:10.1016/j.watres.2020.115942.
Rimoldi, S.G., Stefani, F., Gigantiello, A., Polesello, S., Comandatore, F., Mileto, D., Maresca, M., Longobardi, C., Mancon, A., Romeri, F., Pagani, C., Moja, L., Gismondo, M.R., Salerno, F., 2020. Presence and vitality of SARS-CoV-2 virus in wastewaters and rivers. Sci. Total Environ. 744, 140911.
Tiwari, S.B., Gahlot, P., Tyagi, V.K., Zhang, L., Zhou, Y., Kazmi, A.A., Kumar, M., 2021. Surveillance of Wastewater for Early Epidemic Prediction (SWEEP): environmental and health security perspectives in the post COVID-19 Anthropocene. Environ. Res. 110831. doi:10.1016/j.envres.2021.110831.
Tran, H.N., Le, G.T., Nguyen, D.T., Juang, R.S., Rinklebe, J., Bhatnagar, A., Lima, E.C., Iqbal, H.M.N., Sarmah, A.K., Chao, H.P., 2020. SARS-CoV-2 coronavirus in water and wastewater: a critical review about presence and concern. Environ. Res. 110265. doi:10.1016/j.envres.2020.110265. https://web.worldbank.org/archive/website01409/WEB/IMAGES/2010_1_1.PDF. (Accessed 21 May 2021).
Wong, M.C., Huang, J., Lai, C., Ng, R., Chan, F.K., Chan, P.K., 2020. Detection of SARS-CoV-2 RNA in fecal specimens of patients with confirmed COVID-19: a meta-analysis. J. Inf. Secur.. doi:10.1016/j.jinf.2020.06.012.
World Health OrganizationNovel coronavirus (2019-nCoV) situation report – 1https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation432-reports2020
Xiao, F., Tang, M., Zheng, X., Liu, Y., Li, X., Shan, H., 2020. Evidence for gastrointestinal infection of SARS-CoV-2. Gastroenterology 158 (6), 1831–1833. doi:10.1053/j.gastro.2020.02.055.
Yang, R., Gui, X., Xiong, Y., 2020. Comparison of clinical characteristics of patients with asymptomatic vs symptomatic coronavirus disease 2019 in Wuhan, China. JAMA Netw. Open 3 (5), e2010182. doi:10.1001/jamanetworkopen.2020.10182.
Zhu, Y., Oishi, W., Maruo, C., Saito, M., Chen, R., Kitajima, M., Sano, D., 2021. Early warning of COVID-19 via wastewater-based epidemiology: potential and bottlenecks. Sci. Total Environ. 145124. doi:10.1016/j.scitotenv.2021.145124.

Environmental Research 196 (2021) 110946

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Unravelling the early warning capability of wastewater surveillance for COVID-19: A temporal study on SARS-CoV-2 RNA detection and need for the escalation

Manish Kumar [a,b,*], Madhvi Joshi [c], Arbind Kumar Patel [a], Chaitanya G. Joshi [c]

[a] Discipline of Earth Science, Indian Institute of Technology Gandhinagar, Gujarat, 382 355, India
[b] Kiran C Patel Centre for Sustainable Development, Indian Institute of Technology Gandhinagar, Gujarat, India
[c] Gujarat Biotechnology Research Centre (GBRC), Sector- 11, Gandhinagar, Gujarat, 382 011, India

ARTICLE INFO

Keywords:
Coronavirus
COVID-19
SARS-CoV-2
Wastewater surveillance
Wastewater based epidemiology
Early warning system

ABSTRACT

Wastewater-based Epidemiological (WBE) surveillance offers a promising approach to assess the pandemic situation covering pre-symptomatic and asymptomatic cases in highly populated area under limited clinical tests. In the present study, we analyzed SARS-CoV-2 RNA in the influent wastewater samples (n = 43) from four wastewater treatment plants (WWTPs) in Gandhinagar, India, during August 7th to September 30th, 2020. A total of 40 samples out of 43 were found positive i.e. having at least two genes of SARS-CoV-2. The average Ct values for S, N, and ORF 1 ab genes were 32.66, 33.03, and 33.95, respectively. Monthly variation depicted a substantial rise in the average copies of N (~120%) and ORF 1 ab (~38%) genes in the month of September as compared to August, while S-gene copies declined by 58% in September 2020. The SARS-CoV-2 genome concentration was higher in the month of September (~924.5 copies/L) than August (~897.5 copies/L), corresponding to a ~2.2-fold rise in the number of confirmed cases during the study period. Further, the percentage change in genome concentration level on a particular date was found in the lead of 1–2 weeks of time with respect to the official confirmed cases registered based on clinical tests on a temporal scale. The results profoundly unravel the potential of WBE surveillance to predict the fluctuation of COVID-19 cases to provide an early warning. Our study explicitly suggests that it is the need of hour that the wastewater surveillance must be included as an integral part of COVID-19 pandemic monitoring which can not only help the water authorities to identify the hotspots within a city but can provide up to 2 weeks of time lead for better tuning the management interventions.

1. Introduction

The global pandemic caused by severe acute respiratory syndrome 2 (SARS-CoV-2) disease has led to more than 40 million confirmed cases and >1 million deaths worldwide, covering 216 countries, as of December 10th, 2020 (WHO, 2020). The high prevalence of asymptomatic infectious persons is a matter of concern that raises doubt on the available data of active cases based on a clinical survey (Rimoldi et al., 2020; Medema et al., 2020). Therefore, alternative approaches such as wastewater-based epidemiology (WBE) are gaining recognition, and surveillance of SARS-CoV-2 transmission and real-time trend monitoring is being endorsed to trigger pandemic responses by scientific communities (Medema et al., 2020; Randazzo et al., 2020). The SARS-CoV-2 virus replicates in epithelial cells of alveoli and enterocytes of the intestinal lining in human beings due to the expression of ACE2 receptor resulting in respiratory illness and gastro-intestinal symptoms such as vomiting and diarrhoea (Ni et al., 2020; Kumar et al., 2020; Gupta et al., 2020; Zhang et al., 2020; Xiao et al., 2020). The clinical symptoms of SARS-CoV-2 infection include cough, breathing problems, diarrhoea, and fever. Different studies suggest that 48–67% of deceased persons exhibited SARS-CoV-2 RNA in the stool (Chan et al., 2020; Cheung et al., 2020; Parasa et al., 2020; Wong et al., 2020).

Due to the presence and extended excretion of SARS-CoV-2 RNA in the faecal matter of pre-symptomatic and deceased persons, WBE is gaining attention worldwide to monitor COVID-19, particularly in the developing economies with poor health infrastructure. An earlier

* Corresponding author. Discipline of Earth Science, Indian Institute of Technology, Gandhinagar, Gujarat, 382 355, India.
E-mail addresses: manish.env@gmail.com, manish.kumar@iitgn.ac.in (M. Kumar).

https://doi.org/10.1016/j.envres.2021.110946
Received 23 December 2020; Received in revised form 2 February 2021; Accepted 25 February 2021
Available online 2 March 2021
0013-9351/

investigation on COVID-19 patients revealed the prevalence of SARS-CoV-2 RNA in the stool of a larger population (48.1%) than patients with gastro-intestinal symptoms (17%) (Cheung et al., 2020). The latter study suggested that asymptomatic persons together with symptomatic persons, discharge viral particles in the excreta finding their way to sewage treatment plants. Interestingly, 18–45% of patients lack symptoms in the case of COVID-19 infection but are capable of transmitting the disease and can adversely affect the actual containment of COVID-19 (Lavezzo et al., 2020; Yang et al., 2020; Mizumoto et al., 2020; Nishiura et al., 2020). Haver and co-workers (2020) reported 6 to 24 times higher infection among asymptomatic and mild symptomatic individuals than confirmed cases at ten different sites in the United States based on surveillance of antibodies to SARS-CoV-2.

The wastewater encompasses SARS-CoV-2 RNA from both asymptomatic and symptomatic patients; therefore, WBE may prove its worthiness for COVID-19 surveillance to forecast the overall pandemic situation. WBE may help in identifying the hotspots and tuning the public health initiatives that will give preparatory time to the regulatory bodies to handle the adverse situation. Further, WBE could provide an early warning of possible re-outbreaks and seasonal outbreaks in the future. The occurrence of SARS-CoV-2 RNA in wastewater has widely been reported from all corners of the world, including Spain, France, Italy, China, Netherlands, Australia, India, and Japan (Randazzo et al. 2020; Wurtzer et al., 2020; Zhang et al., 2020; Medema et al., 2020; La Rosa et al., 2020; Ahmed et al., 2020; Hata et al., 2020; Kumar et al., 2020, 2021). Although the sensitivity of WBE is comparatively less than clinical trials and largely depends on the viral load in the patient's faecal matter, earlier clues and wide acceptability of WBE suggest that this approach could be superior to clinical surveillance for the early prediction of COVID-19 status for highly populated places (Medema et al., 2020; Randazzo et al., 2020; La Rosa et al., 2020). Therefore, to evaluate WBE's potential as an early prediction tool for COVID-19 pandemic, it is essential to explore the correlation between the SARS-CoV-2 genetic load in wastewater and the number of cases at the district level in each country.

In view of this, the objective of this study was to verify the WBE approach for COVID-19 by comparing the detected concentration of SARS-CoV-2 in wastewater with the COVID- 19 cases reported by the clinical surveillance. The detected concentrations of SARS-CoV-2 RNA in wastewater would reflect the true prevalence of COVID-19 infection in the sewer catchment, including clinically undiagnosed patients, while the number of clinically reported cases covers only diagnosed patients and also depends on the number of PCR diagnosis. In the present study, we analyzed SARS-CoV-2 RNA in the influent wastewater samples (n = 43) from four wastewater treatment plants (WWTPs) in Gandhinagar, India, from August 7th to September 30th, 2020, with the following objectives: a) detection and quantification of SARS-CoV-2-RNA in the influent wastewater samples of Gandhinagar to understand the pandemic situation over 2 months b) biweekly and weekly resolution of the data for two months in genetic material loadings; and c) explicating the potential of WBE for COVID-19 surveillance as a potential tool for identifying hotspots and public health monitoring at the community level.

2. Material and methods

2.1. Sampling approach

Influent wastewater samples were collected from four different treatment plants *viz.* Basan, Jaspur, and Sargasan wards, and an academic institution present in the municipal territory of Gandhinagar (Fig. 1). The capacity of treatment plants was 2, 10, 10, and 2.36 MLD, respectively. The details of the WWTPs, including their geospatial position, capacity, treatment process, etc., are shown in Table S1. The influent wastewater samples were collected from each WWTP first biweekly, followed by weekly for two months, during August and September 2020. A total of forty-three influent wastewater samples collected from four different treatment plants were analyzed for two months. All the samples were collected by grab hand sampling using 250 mL sterile bottles (Tarsons, PP Autoclavable, Wide Mouth Bottle, Cat No. 582240, India). Simultaneously, blanks in the same type of bottle were examined to know any contamination during the transport. *In-situ* water quality parameters (pH, Electrical Conductivity, Dissolved Oxygen, Oxidation-Reduction Potential, and Total Dissolved Solids, Salinity) were examined prior to the sample collection using YSI multiparameter probe and summarized in Table S2. The samples were kept cool in an ice-box until analysis.

2.2. SARS-CoV-2 gene detection

2.2.1. Precipitation of viral particle

30 mL samples were centrifuged at 4000×g (Model: Sorvall ST 40 R, Thermo Scientific) for 40 min in a 50 mL falcon tube followed by filtration of supernatant using 0.22-μm syringe filter (Mixed cellulose esters syringe filter, Himedia). After filtrating 25 mL of the supernatant, it was treated with PEG 9000 (80 g/L), and NaCl (17.5 g/L), and this was incubated at 17 °C, 100 rpm overnight (Model: Incu-Shaker™ 10LR, Benchmark). Next day, the mixture was centrifuged at 14,000×g (Model: Kubota 6500, Kubota Corporation) for about 90 min. The supernatant was discarded after centrifugation, and the pellet was

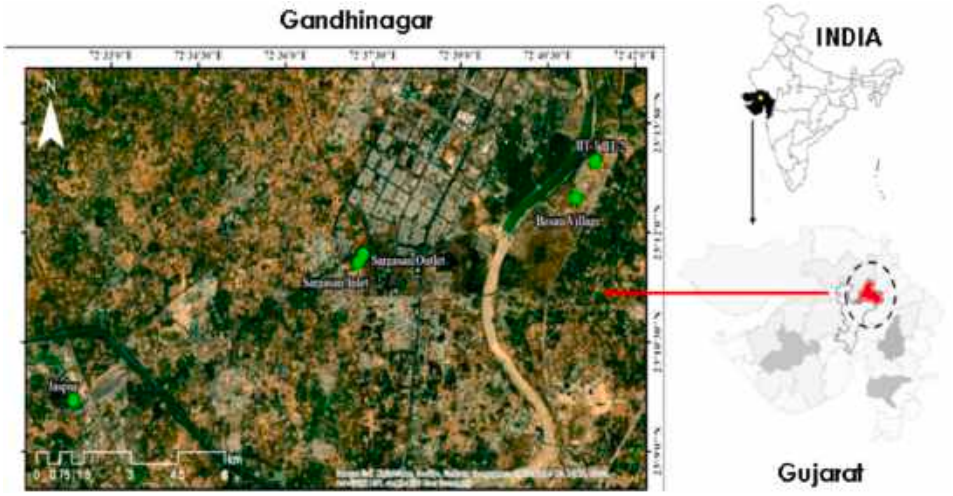


Fig. 1. Geospatial map of the sampling points in Gandhinagar, Gujarat.

resuspended in 300 μL RNase free water. The concentrated sample was kept in 1.5 mL eppendorf at −40 °C, and this was further used as a sample for RNA isolation.

2.2.2. RNA isolation, RT-PCR and gene copy estimation

RNA isolation from the pellet with the concentrated virus was performed using NucleoSpin® RNA Virus (Macherey-Nagel GmbH & Co. KG, Germany) isolation kit. MS2 phage was used as an internal control provided by TaqPathTM Covid-19 RT-PCR Kit. Some other specifics are, a) the nucleic acid was extracted by NucleoSpin@ RNA Virus isolation Kit (Applied Biosystems), and Qubit 4 Fluorometer (Invitrogen) was used for RNA concentrations estimation, b) molecular process inhibition control (MPC) was evaluated through MS2 phage for the QC/QA analyses of nucleic acid extraction and PCR inhibition (Haramoto et al., 2018). We have described methodology elsewhere (Kumar et al., 2021 and 2020a). Briefly, steps were carried out as per the guideline provided with the product manual of Macherey-Nagel GmbH & Co. KG, and RNAs were detected using real-time PCR (RT-PCR).

SARS-CoV-2 gene was detected with Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument (version 2.19 software) and for each run a template of 7 μL of extracted RNA was used with TaqPath™ 1 Step Multiplex Master Mix (Thermofischer Scientific, USA). Final reaction mixture (20 μmL) contained nuclease-free water 9 (10.50 μL), Master Mix (6.25 μL), and COVID-19 Real-Time PCR Assay Multiplex (1.25 μL). Positive control (TaqPath™ COVID 19 Control), negative control (from extraction run spiked with MS2), and no template control (NTC) were run with each batch. 40 cycles of amplification were set and results were interpreted based on the Ct values for three target genes i.e., ORF1ab, N Protein, and S Protein of SARS-CoV-2 along with that of MS2 used as an internal control.

Results were considered inconclusive if less than two genes are detected in the samples. Effective genome concentration was calculated semi-qualitatively using the equivalence of 500 copies of SARS-CoV-2 genes as 26 Ct-value (provided with the kit), and multiplying the RNA amount used as a template and the enrichment factor of waste water samples during the experimentation. The OriginPro 2019b was used for data plotting and analysis.

Test of significance and multivariate analyses (MVA) was performed with help of Statistical Package for the Social Sciences (SPSS 21) to evaluate the relatedness among various quality parameters analyzed and to delineate the principle components (PCs) governing variables in the produced data-set through orthogonal transformation that explains the captured variance in the dataset. Principle component analyses (PCA) not only reduces the dimensionality of datasets but also provides the influences of each dimensions on each other. Determining the Eigen values and Eigen vectors were the key steps in the process of PCA, following the square matrix during the formation of factor loading matrix.

3. Results and discussions

We detected and quantified variation in SARS-CoV-2 RNA from influent wastewater samples for two months (August and September) to understand the pandemic situation in Gandhinagar, Gujarat, India. Among 43 samples analyzed in the study, 40 were found positive, comprising two out of three targetted genes (Table 1). The distribution analysis of Ct values for different genes using boxplot is represented in Fig. 2a. The average Ct values for S, N, and ORF 1 ab genes were 32.66, 33.03, and 33.95, respectively. The Ct values of internal control (MS2 bacteriophage) ranged between 25.15 and 28.01. Also, no SARS-CoV-2 genes were detected in the negative control samples. The average gene copies were found to be maximum for S-gene (~1223 copies/L), followed by N-gene (~1022 copies/L) and ORF 1 ab-gene (~485 copies/L) (Fig. 2b).

3.1. The monthly variations

The monthly variation depicted a substantial rise in the average copies of N (~120%) and ORF 1 ab (~38%) genes in the month of September as compared to August, while S-gene copies declined by 58%

Table 1
Temporal variation in SARS-CoV-2 genetic material loading found in the influent and effluent samples collected from two different wastewater treatment plants.

Sampling location	SARS-CoV-2 (copies/ L)	Sampling date Vs Active cases Vs Gene copies										
		August, 2020							September, 2020			
		07.08	11.08	14.08	17.08	21.08	25.08	28.08	07.09	14.09	23.09	30.09
Active cases in Gandhinagar		367	264	261	269	271	300	329	442	496	571	613
Basan	N-Gene	484	429	1102	395	227	974	144	599	1358	22	1247
	ORF-Gene	164	239	472	319	124	362	156	105	399	34	246
	S-Gene	53	1558	1859	1779	522	1639	1320	66	70	262	116
	Genome concentration	234	742	1144	831	291	992	540	257	609	106	536
Jaspur	N-Gene	891	463	823	773	484	1024	406	494	6768	51	25
	ORF-Gene	226	382	376	577	306	665	196	105	2059	123	ND
	S-Gene	4159	1673	1662	1388	1338	1755	748	157	2967	115	ND
	Genome concentration	1759	839	954	913	709	1148	450	252	3931	97	INC
Sargasan	N-Gene	850	460	553	599	807	1020	74	1238	5966	399	4745
	ORF-Gene	513	187	488	381	447	372	101	569	2441	1352	1316
	S-Gene	2535	1719	1511	1542	1476	1324	42	246	3525	1516	842
	Genome concentration	1300	789	850	841	910	905	98	685	3977	1089	2301
Academic Institute	N-Gene	NA	183	2357	1828	350	862	505	ND	395	ND	101
	ORF-Gene	NA	74	1476	1124	69	842	853	ND	ND	ND	43
	S-Gene	NA	560	2262	2976	494	2033	2133	ND	ND	21	ND
	Genome concentration	NA	272	2032	1976	304	1246	1164	ND	INC	INC	72

#ND: Not detected; INC: Inconclusive; and NA: Not analysed.

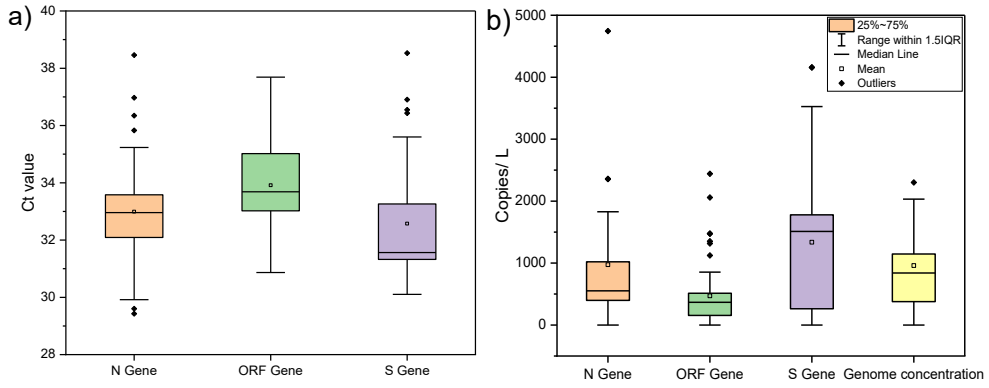


Fig. 2. Distribution of SARS-CoV-2 viral gene a) Ct values, and b) target gene copies during entire study period in Gandhinagar.

in September 2020 (Fig. 3a). The SARS-CoV-2 genome concentration was higher in the month of September (~925 copies/L) than August (~898 copies/L), corresponding to a ~2.2-fold rise in the number of confirmed cases during the study period (Fig. 3b). Temporal variations in average SARS-CoV-2 gene copies were analyzed from different WWTPs in Gandhinagar are displayed in Fig. 4a–d. One-way ANOVA and Duncan post hoc test (**$p < 0.05$**) was performed to see the significance level in gene copy variation among different sampling dates. The results showed significant differences in N-gene (ANOVA, $F = 2.68$, $p < 0.05$) and S-gene copies (ANOVA, $F = 2.20$, $p < 0.05$) on the temporal scale (sampling dates). Conversely, differences were non-significant in case of ORF-1ab genes (ANOVA, $F = 1.13$, $p > 0.05$) and genome concentration (ANOVA, $F = 1.63$, $p > 0.05$).

There are some studies available around the globe on early detection of SARS-CoV-2 RNA in wastewater, even before the first report of clinical diagnosis. For example, Madema et al. (2020) reported the presence of SARS-CoV-2 genetic material in wastewater in February, even before the official declaration of the first case in the Netherlands. Likewise, La Rosa et al. (2020) reported SARS-CoV-2 genetic material in wastewater samples before the first official documented report from two different cities in Italy. Similarly, Randazzo et al. (2020) detected SARS-CoV-2 RNA in wastewater samples from Spain. Since then, many researchers detected and reported the occurrence of SARS-CoV-2 RNA in wastewater samples and pondered its applicability for WBE surveillance (Ahmed et al., 2020, Kumar et al., 2020 a,b). However, a few studies available focused on assessing its potential on the temporal scale in relation to the changes in COVID cases.

3.2. The early warning capability

The present research work followed our first proof of the concept, where we detected SARS-CoV-2 genetic material in wastewater and proposed its wide applicability for COVID surveillance in the community (Kumar et al., 2020a). Examining the potential of WBE for COVID-19 surveillance as a potential tool showed that the percentage change in genome concentration level on a particular date was in conjunction with the confirmed cases registered 1–2 weeks later on a temporal scale by the regulatory authority based on clinical tests (Fig. 5). For example, on August 21st, 2020, a sharp decline of ~51% was noticed in the percentage change in the average genome concentration which was followed by ~0.76% decline in the percentage change in confirmed COVID cases on August 28th, 2020. Likewise, on August 25th, 2020, a steep hike of ~75% in the percentage change in the average genome concentration was noticed, which was followed by ~11% increment in the percentage change in confirmed COVID cases on September 7th, 2020. Therefore, we can predict the severity of the pandemic situation 1–2 weeks prior to the official reports by the regulatory body based on clinical tests. The results unravel the potential of WBE surveillance of COVID-19 as an early warning tool displayed by the adequate presence of SARS-CoV-2 genetic material in wastewater samples though limited cases were documented and based on the immediate future trends. These findings were in agreement with those of Ahmed et al. (2020b), who noticed a longitudinal decline in the presence of SARS-CoV-2 RNA with the tapering of the first epidemic wave; however, there was no concrete relationship between virus RNA and daily cases numbers.

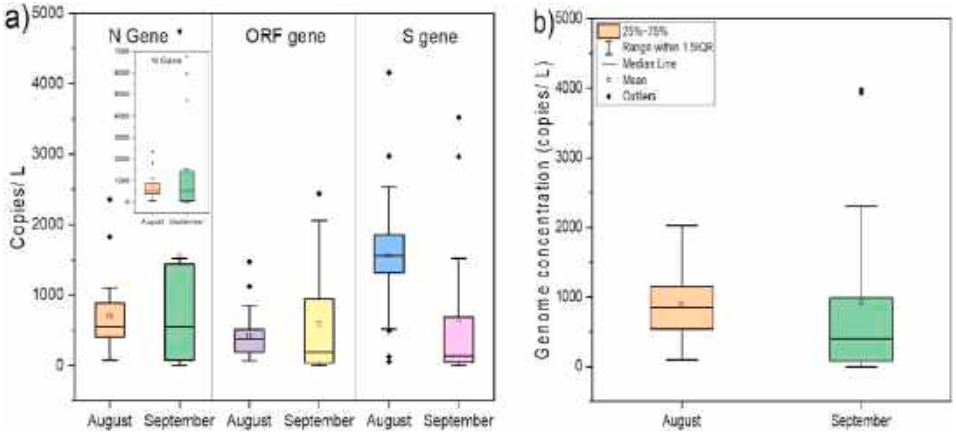


Fig. 3. Distribution of SARS-CoV-2 gene copies on a temporal scale (monthly variation), a) Target gene copies, b) Genome concentration.

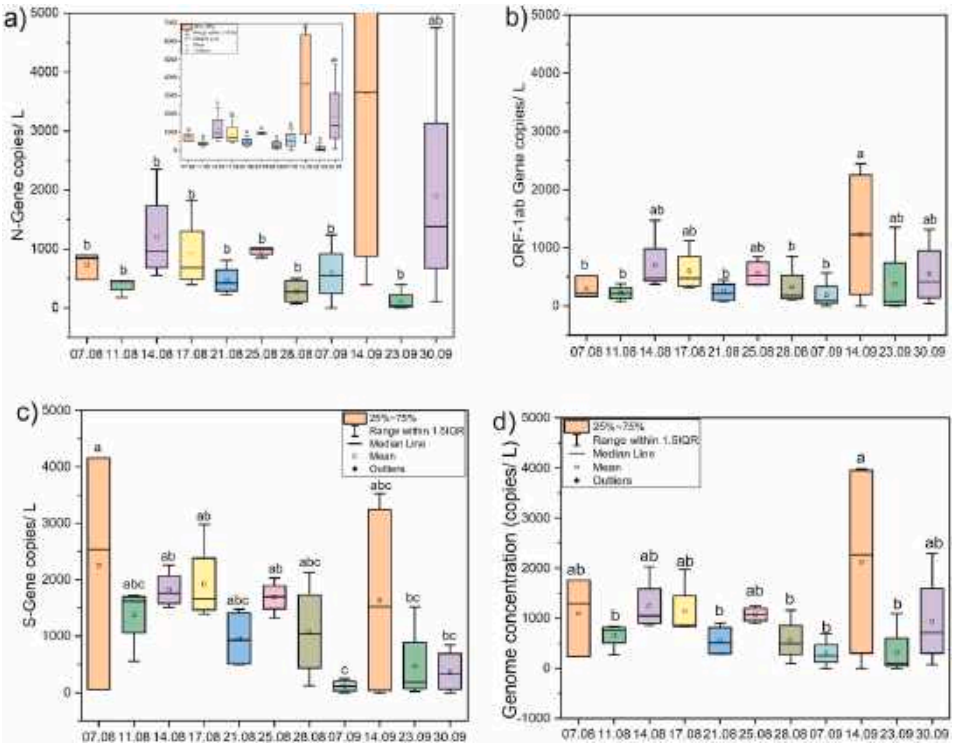


Fig. 4. Temporal variations in average SARS-CoV-2 gene copies collected from different STPs in Gandhinagar, a) N-Gene, b) ORF1 ab Gene, c) S-Gene, and d) Genome concentration. Alphabetical letters in graphs represent a statistically significant difference of $\mathbf{p < 0.05}$ by Duncan post hoc test.

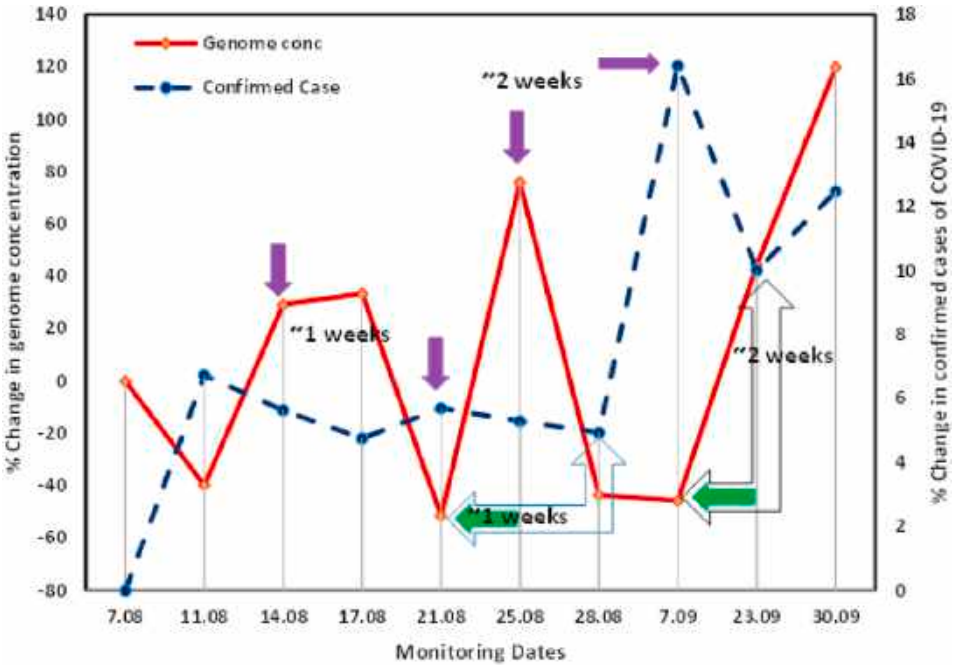


Fig. 5. Potential and evidence of wastewater-based epidemiology surveillance of Covid-19 pandemic as an early warning tool in Gandhinagar.

3.3. Relatedness with COVID-19 cases and water quality through multivariate analyses

Finally, MVA was performed to know the relation among influent wastewater physico-chemical characteristics, SARS-CoV genetic material, and pandemic status (i.e., confirmed, active, recovered, and deceased cases) through principal component analysis depicted by PCs loading in a 3-D domain during the entire two months of the monitoring period (Fig. 6a and b). A summary description of in-situ parameters (Table S3), variation explained, eigenvalue variations, and principal component loadings for influent wastewater (Table S4, Fig S1) have been provided as supplementary items.

Principal component analyses showed a comprehensive picture of the overall interaction among SARS-CoV-2 genetic material and influent wastewater characteristics. The entire dataset obtained for August and September were subjected to PCA and projected in the 3-D domain of

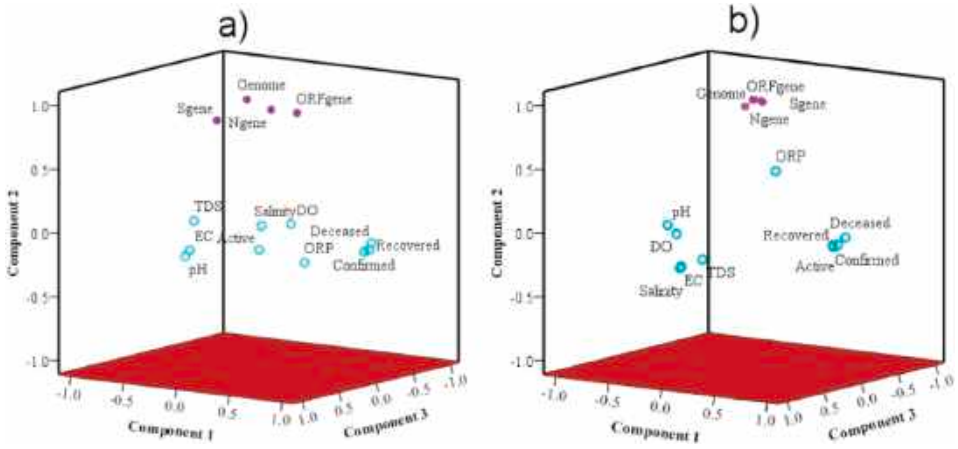


Fig. 6. Principal component analysis (PCA) to show relation/interaction among physico-chemical parameters of untreated wastewater and targeted SARS-CoV-2 gene copies, a) August; b) September 2020.

three main PCs. In the month of August, four PCs were identified that explains 76.9% of the total variance in the dataset. The first PCs explained 26.9% of the total variance with significant loading for COVID cases forming a cluster (confirmed, recovered, and active cases) with moderate loading (~0.5) of influent wastewater parameters (ORP and DO) and weak loading for ORF gene (Fig. 6a and Supplementary Table S3 and S4). On the other hand, nearly the same (~23.8%) variation of data sets is explained by SARS-CoV-2 genes, and genome concentrations form a cluster upper left domain with significant loadings for effective genome concentrations (0.98) followed by ORF-1ab, N-genes, and S-genes as PC2. Interestingly in August, the ORF 1 ab genes illustrated positive loadings in both PC1 and PC2. The PC3 and PC4 exhibited almost similar contribution (~13%) of the total variance.

In September, the complexion changed significantly with the overall reductions of PCs to three, explaining cumulative variations of 84% in the dataset. The trends were almost similar to the month of August. However, SARS-CoV-2 genes exhibited higher loadings. Order of loadings among SARS-CoV-2 genes and genome remains same i.e., effective genome concentration > ORF-1ab > N-genes > S-genes. The confirmed and active COVID cases showed a positive relationship with SARS-CoV-2 genes (ORF 1 ab, N-gene, and genome concentration), though the relationship was not strong due to weak correlation coefficients (<0.1). Though the results of MVA suggested a weak relationship between the SARS-CoV-2 genome concentration and confirmed cases, yet the percentage change in genome concentration showed a positive relation to the percentage change in the confirmed cases. This might be because of the effect of change in SARS-CoV-2 genome concentration was reflected in 1–2 weeks later data of the confirmed cases. This could also partly be ascribed to the number of confirmed cases not necessarily reflect the actual prevalence of the disease (Hata et al., 2020).

4. Conclusion

A temporal variation of SARS-CoV-2 RNA presence in influent wastewater was studied for a period of two months in Gandhinagar, India. Out of 43 samples, 40 samples were found positive, while RT-PCR showed greater sensitivity for S-gene, followed by N-gene and ORF 1 ab gene. A comparison of monthly variation demonstrated higher SARS-CoV-2 genome concentration in September (~925 copies/L) than August (~898 copies/L) in line with the ~2.2-fold rise in the number of confirmed cases during the study period. The results profoundly unravel the potential of WBE surveillance to predict the fluctuation of COVID-19 cases to provide an early warning. Our study explicitly suggests that it is the need of hour that the wastewater surveillance must be included as an integral part of COVID-19 pandemic monitoring which can not only help the authorities to identify the hotspots within a city but can provide up to 2 weeks of time lead for better tuning the management interventions. However, the capability of WBE for early warning of COVID-19 needs to be further substantiated through long-term dataset, as cross correlation of temporal patterns between SARS-CoV-2 RNA and confirmed cases is not easy to interpret with short-term data set. Second, biasness in the interpretation may arise based on the extent of effort and capacity of COVID-19 cases diagnosis.

Future outlook of WBE can have several research and application directions such as: i) continuum data on SARS-CoV-2 RNA should be obtained that can be key for future, ii) not only wastewater but treated effluents and ambient waters should also be monitored; ii) temporal variability in WBE data along with epidemiological information of the community should be made available for future comparison and predictions; iii) removal efficacy of WWTPs should not be taken for granted and virus RNA decay or accumulation perspective should be taken into account; iv) infectivity through viable virus estimation in the ambient and reclaimed waters is imperative; and iv) WBE can help to understand the efficacy of COVID-19 vaccine.

Notes

The authors declare no competing financial interest.

Credit author statement

Manish Kumar: Conceptualization, Visualization, Writing – original draft, Data curation, Interpretation, Revision & Response, Editing and Supervision, Madhvi Joshi: Data curation, Interpretation, Editing and Supervision. Arbind K Patel: Sampling, Analyses, Data curation, Chaitanya G Joshi: Guidance, and Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work is funded by Kiran C Patel Centre for Sustainable Development at IIT Gandhinagar, UNICEF, Gujarat and UK-India Education and Research Initiative (UKIERI). We acknowledge the help received from Dr. Vaibhav Srivastava who contributed towards sample and data analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.110946.

References

Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J., Tscharke, B., 2020a. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community. Science of The Total Environment, p. 138764. https://doi.org/10.1016/j.scitotenv.2020.138764.

Ahmed, W., Tscharke, B., Bertsch, P.M., Bibby, K., Bivins, A., Choi, P., Clarke, L., Dwyer, J., Edson, J., Nguyen, T.M.H., O'Brien, J.W., 2020b. SARS-CoV-2 RNA monitoring in wastewater as an early warning system for COVID-19 transmission in the community: a temporal case study. Sci. Total Environ. 144216. https://doi.org/10.1016/j.scitotenv.2020.144216.

Chan, P.K., Lui, G., Hachim, A., Ko, R.L., Boon, S.S., Li, T., Kavian, N., Luk, F., Chen, Z., Yau, E.M., Chan, K.H., 2020. Serologic responses in healthy adult with SARS-CoV-2 reinfection, Hong Kong. Emerg. Infect. Dis. 26 (12), 3076. https://doi.org/10.3201/eid2612.203833.

Cheung, K.S., Hung, I.F., Chan, P.P., Lung, K.C., Tso, E., Liu, R., Ng, Y.Y., Chu, M.Y., Chung, T.W., Tam, A.R., Yip, C.C., 2020. Gastrointestinal manifestations of SARS-CoV-2 infection and virus load in fecal samples from the Hong Kong cohort and systematic review and meta-analysis. Gastroenterology. https://doi.org/10.1053/j.gastro.2020.03.065.

Gupta, M.K., Vemula, S., Donde, R., Gouda, G., Behera, L., Vadde, R., 2020. In-silico approaches to detect inhibitors of the human severe acute respiratory syndrome coronavirus envelope protein ion channel. J. Biomol. Struct. Dyn. 1–11. https://doi.org/10.1080/07391102.2020.1751300.

Haramoto, E., Malla, B., Thakali, O., Kitajima, M., 2020. First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. Sci. Total Environ. 737, 140405. https://doi.org/10.1016/j.scitotenv.2020.140405.

Hata, A., Hara-Yamamura, H., Meuchi, Y., Imai, S., Honda, R., 2020. Detection of SARS-CoV-2 in wastewater in Japan during a COVID-19 outbreak. Science of The Total Environment, p. 143578. https://doi.org/10.1016/j.scitotenv.2020.143578.

Havers, F.P., Reed, C., Lim, T., Montgomery, J.M., Klena, J.D., Hall, A.J., Fry, A.M., Cannon, D.L., Chiang, C.F., Gibbons, A., Krapiunaya, I., 2020. Seroprevalence of antibodies to SARS-CoV-2 in 10 sites in the United States, March 23-May 12, 2020. J. Am. Med. Assoc. 180 (12), 1576–1586. https://doi.org/10.1001/jamainternmed.2020.4130.

Kumar, M., Patel, A.K., Shah, A.V., Raval, J., Rajpara, N., Joshi, M., Joshi, C.G., 2020a. First proof of the capability of wastewater surveillance for COVID-19 in India through detection of genetic material of SARS-CoV-2. Sci. Total Environ. 746, 141326. https://doi.org/10.1016/j.scitotenv.2020.141326.

Kumar, M., Thakur, A.K., Mazumder, P., Kuroda, K., Mohapatra, S., Rinklebe, J., Ramanathan, A.L., Cetecioglu, Z., Jain, S., Tyagi, V.K., Gikas, P., 2020b. Frontier review on the propensity and repercussion of SARS-CoV-2 migration to aquatic environment. J. Hazard. Mater. Lett. 1, 100001. https://doi.org/10.1016/j.hazl.2020.100001.

Kumar, M., Kuroda, K., Patel, A.K., Patel, N., Bhattacharya, P., Joshi, M., Joshi, C.G., 2020. Decay of SARS-CoV-2 RNA along the wastewater treatment outfitted with Upflow Anaerobic Sludge Blanket (UASB) system evaluated through two sample concentration techniques. Sci. Total Environ. 754, 142329. https://doi.org/10.1016/j.scitotenv.2020.142329.

La Rosa, G., Iaconelli, M., Mancini, P., Ferraro, G.B., Veneri, C., Bonadonna, L., Lucentini, L., Suffredini, E., 2020. First detection of SARS-CoV-2 in untreated wastewaters in Italy. Sci. Total Environ. 139652. https://doi.org/10.1016/j.scitotenv.2020.139652.

Lavezzo, E., Franchin, E., Ciavarella, C., Cuomo-Dannenburg, G., Barzon, L., Del Vecchio, C., Rossi, L., Manganelli, R., Loregian, A., Navarin, N., Abate, D., 2020. Suppression of a SARS-CoV-2 outbreak in the Italian municipality of Vo'. Nature 584 (7821), 425–429. https://doi.org/10.1038/s41586-020-2488-1.

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020. Presence of SARS Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in The Netherlands. Environ. Sci. Technol. Lett. 7 (7), 511–516. https://doi.org/10.1021/acs.estlett.0c00357.

Mizumoto, K., Chowell, G., 2020. Estimating risk for death from coronavirus disease, China, january–february 2020. Emerg. Infect. Dis. 26 (6), 1251. https://doi.org/10.3201/eid2606.200233.

Ni, W., Yang, X., Yang, D., Bao, J., Li, R., Xiao, Y., Hou, C., Wang, H., Liu, J., Yang, D., Xu, Y., 2020. Role of angiotensin-converting enzyme 2 (ACE2) in COVID-19. Crit. Care 24 (1), 1–10. https://doi.org/10.1186/s13054-020-03120-0.

Nishiura, H., Linton, N.M., Akhmetzhanov, A.R., 2020. Serial interval of novel coronavirus (COVID-19) infections. International Journal of Infectious Diseases. https://doi.org/10.1016/j.ijid.2020.02.060.

Parasa, S., Desai, M., Thoguluva Chandrasekar, V., Patel, H.K., Kennedy, K.F., Roesch, T., Spadaccini, M., Colombo, M., Gabbiadini, R., Artifon, E., Repici, A., Sharma, P., 2020. Prevalence of Gastrointestinal Symptoms and Fecal Viral Shedding in Patients With Coronavirus Disease 2019: A Systematic Review and Meta-analysis. JAMA network open 3 (6), e2011335. https://doi.org/10.1001/jamanetworkopen.2020.11335.

Randazzo, W., Truchado, P., Cuevas-Ferrando, E., Simón, P., Allende, A., Sánchez, G., 2020. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a Low prevalence area. Water Res. 181, 115942. https://doi.org/10.1016/j.watres.2020.115942.

Rimoldi, S.G., Stefani, F., Gigantiello, A., Polesello, S., Comandatore, F., Mileto, D., Maresca, M., Longobardi, C., Mancon, A., Romeri, F., Pagani, C., Moja, L., Gismondo, M.R., Salerno, F., 2020. Presence and vitality of SARS-CoV-2 virus in wastewaters and rivers. Sci. Total Environ. 744, 140911.

Wurtzer, S., Marechal, V., Mouchel, J.M., Maday, Y., Teyssou, R., Richard, E., Almayrac, J.L., Moulin, L., 2020. Evaluation of Lockdown Impact on SARS-CoV-2 Dynamics through Viral Genome Quantification in Paris Wastewaters. medRxiv. https://doi.org/10.1101/2020.04.12.20062679.

Wong, M.C., Huang, J., Lai, C., Ng, R., Chan, F.K., Chan, P.K., 2020. Detection of SARS-CoV-2 RNA in fecal specimens of patients with confirmed COVID-19: a meta-analysis. J. Infect. https://doi.org/10.1016/j.jinf.2020.06.012.

World Health Organization Novel Coronavirus (2019-nCoV), 2020. https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation432-reports.

Xiao, F., Tang, M., Zheng, X., Liu, Y., Li, X., Shan, H., 2020. Evidence for gastrointestinal infection of SARS-CoV-2. Gastroenterology 158 (6), pp1831–1833. https://doi.org/10.1053/j.gastro.2020.02.055.

Yang, R., Gui, X., Xiong, Y., 2020. Comparison of clinical characteristics of patients with asymptomatic vs symptomatic coronavirus disease 2019 in Wuhan, China. JAMA Network Open 3 (5). https://doi.org/10.1001/jamanetworkopen.2020.10182.

Zhang, N., Gong, Y., Meng, F., Bi, Y., Yang, P., Wang, F., 2020. Virus shedding patterns in nasopharyngeal and fecal specimens of COVID-19 patients. MedRxiv. https://doi.org/10.1101/2020.03.28.20043059.

Chemical Engineering Journal xxx (xxxx) 130635

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

First comparison of conventional activated sludge versus root-zone treatment for SARS-CoV-2 RNA removal from wastewaters: Statistical and temporal significance

Manish Kumar [a, b, *], Keisuke Kuroda [c], Madhvi Joshi [d], Prosun Bhattacharya [e], Damia Barcelo [f]

[a] Discipline of Earth Science, Indian Institute of Technology Gandhinagar, Gujarat 382 355, India
[b] Kiran C Patel Centre for Sustainable Development, Indian Institute of Technology Gandhinagar, Gujarat, India
[c] Department of Environmental and Civil Engineering, Toyama Prefectural University, Imizu 939 0398, Japan
[d] Gujarat Biotechnology Research Centre (GBRC), Sector- 11, Gandhinagar, Gujarat 382 011, India
[e] Department of Sustainable Development, Environmental Science and Engineering, KTH Royal Institute of Technology, SE-10044 Stockholm, Sweden
[f] Institute of Environmental Assessment and Water Research (IDAEA-CSIC), Barcelona, and Catalan Institute for Water Research (ICRA)-CERCA, Girona, Spain

ARTICLE INFO

Keywords:
SARS-CoV-2
COVID-19
Environmental Surveillance
Conventional activated sludge process
Root-zone treatment
Wastewater based epidemiology

ABSTRACT

In the initial pandemic phase, effluents from wastewater treatment facilities were reported mostly free from Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2) RNA, and thus conventional wastewater treatments were generally considered effective. However, there is a lack of first-hand data on i) comparative efficacy of various treatment processes for SARS-CoV-2 RNA removal; and ii) temporal variations in the removal efficacy of a given treatment process in the backdrop of active COVID-19 cases. This work provides a comparative account of the removal efficacy of conventional activated sludge (CAS) and root zone treatments (RZT) based on weekly wastewater surveillance data, consisting of forty-four samples, during a two-month period. The average genome concentration was higher in the inlets of CAS-based wastewater treatment plant in the Sargasan ward (1.25×10^3 copies/ L), than that of RZT plant (7.07×10^2 copies/ L) in an academic institution campus of Gandhinagar, Gujarat, India. ORF 1ab and S genes appeared to be more sensitive to treatment i.e., significantly reduced ($p < 0.05$) than N genes ($p > 0.05$). CAS treatment exhibited better RNA removal efficacy ($p = 0.014$) than RZT ($p = 0.032$). Multivariate analyses suggested that the effective genome concentration should be calculated based on the presence/absence of multiple genes. The present study stresses that treated effluents are not always free from SARS-CoV-2 RNA, and the removal efficacy of a given WWTPs is prone to exhibit temporal variability owing to variations in active COVID-19 cases in the vicinity and genetic material accumulation over the time. Disinfection seems less effective than the adsorption and coagulation processes for SARS-CoV-2 removal. Results stress the need for further research on mechanistic insight on SARS-CoV-2 removal through various treatment processes taking solid–liquid partitioning into account.

1. Introduction

At this juncture, when the world is facing a second winter after being threatened for the entire year with Corona Virus Disease (COVID)-19, cases are surging, with over 40 million infections and >1 million deaths [1]. To date, we have gained knowledge on many aspects of Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2), especially on transmission, monitoring, analytical techniques, prognosis, diagnosis, models, and management aspects [2–21]. However, the infectivity of SARS-CoV-2 RNA in wastewater, owing to viral shedding of infected symptomatic/asymptomatic patients, and their transmission remains under debate [22]. Potential community transmission associated with untreated/treated wastewater, e.g., reuse of wastewater (in built environments), aerosols of wastewater potentially exposing WWTP workers, sludge transfer activities, irrigation and recreational activities in wastewater-impacted waters, is still being debated [23–26] . The two main obstacles are i) whether the viral genome load in wastewater is viable; and ii) whether wastewater treatments can completely remove SARS-CoV-2 RNA? [27–38].

In general, wastewater surveillance of SARS-CoV-2 has focused on early-warning capability verifications [8,11,16,39–40]), or protocol improvement through comparing various techniques of concentration

* Corresponding author at: Discipline of Earth Science, Indian Institute of Technology Gandhinagar, Gujarat 382 355, India.
E-mail address: manish.env@gmail.com (M. Kumar).

https://doi.org/10.1016/j.cej.2021.130635
Received 19 December 2020; Received in revised form 26 April 2021; Accepted 30 May 2021
1385-8947/

and precipitations [40–43], and solid-aqueous interactions from sludge and virus interaction perspectives. However, since the beginning, subtle parallel efforts were there to check the SARS-CoV-2 RNA presence in secondary- and tertiary-treated wastewater. Apart from several reports neglecting the presence of SARS-CoV-2 in treated water, Randazzo et al., 2020 confirmed 11% (2 out of 18) of secondary- and 0% (0/12) tertiary-treated water samples positive for SARS-CoV-2 RNA. Haramoto et al., (2020) detected as many as 2400 gene copies/L of SARS-CoV-2 RNA in secondary-treated wastewater, whereas raw wastewater samples were not positive with SARS-CoV-2, owing to the difference of sample amounts taken for filtration i.e. 200 mL for raw wastewater vs 5000 mL for secondary-treated wastewater. They also tested river samples, but no positive samples could be traced. Interestingly, they reported that 20% of secondary-treated wastewater samples that were found positive could not show the presence of S and ORF1a genes but the N-genes.

By 2021, more efforts started pouring, which tried to screen the treated water like Westhaus et al., [44] reported modest SARS-CoV-2 removal from all three monitored conventional activated-sludge-based WWTP plants. They pointed out that the plant with full-scale ozonation illustrated a relatively better reduction of SARS-CoV-2 fragments in the effluent; and recommended to include membrane-based WWTP plant for future studies. On the other hand, Hasan et al., [45] reported no positive results after monitoring 11 WWTPs effluents. They concluded that the treatment technologies used in the UAE were efficient in degrading SARS-CoV-2, and confirming the safety of treated water in the country for reuse. Similar results were reported by Balboa et al [27] after observing WWTP in Spain for few days in both effluent and treated sludge.

We previously compared the decay in genetic loading of conventional and Upflow Anaerobic Sludge Blanket (UASB) treatment systems with limited data [13] and reported a gradual decay in gene copies of SARS-CoV-2 from raw influent to UASB effluent to aeration pond and to the final effluents. We then summarized that higher RNA loading translated to higher decay along with the treatment. However, data were based on two-time sampling, and a detailed investigation was recommended. It is still unclear how a varying genome loading in the influent impacts the remaining SARS-CoV-2 genome in the effluent. Therefore, it is novel to perform a comparative study, including both untreated and treated wastewater samples to assess the efficacy of treatment plants. While multivariate analysis (MVA) helps source apportionment for environmental samples, it projects unbiased relationships among parameters and their contribution to variations in the data set [39]. To date, however, reported wastewater surveillance datasets have not been large enough for MVA.

Accordingly, we performed two months of monitoring for SARS-CoV-2 genes in untreated and treated wastewater samples, collected from two mechanically different treatment plants, viz. conventional activated sludge (CAS) process (Sargasan) and root zone treatment (RZT) (academic institution) located in Gandhinagar, India. Our main objectives were to: i) compare and evaluate the removal efficacy of SARS-CoV-2 by CAS and RZT processes through months-long influent and effluent monitoring; and ii) study temporal variations in the removal efficacy of a given treatment process in the backdrop of active COVID-19 cases. We wish to add significant pertinent knowledge related to the actual and varying capabilities of one conventional and another zero-discharge trending root-zone treatment systems, so that infectivity can be adequately understood and appropriate information disseminated to the community. Our study is vital as transmission routes in the developing countries are many, owing to less prevalent, unproperly managed sewer systems that leads to wastewater leakages, occurrences of open defecation and common sewer overflow (CSO) situations.

2. Material and Methods:

2.1. Wastewater treatment plants (WWTPs)

We investigated wastewater samples collected from conventional activated sludge (CAS) based treatment plant situated at the Sargasan ward of Gandhinagar (Sargassan WWTP), and from the root-zone treatment plant of an academic institution located in Gandhinagar, both located in Gujarat, India. Schematic diagrams of the two treatment processes are shown in Fig. 1. At Sargasan WWTP (capacity: 10,000 m^3/day), the primary treatment consisted of screening by fine screening channels and grit separator tank. The secondary treatment employed was a cyclic activated sludge process operated with 3–5 h, following which the supernatant was removed from the basin and chlorinated to release as the effluent.

At the treatment plant at the academic institution (capacity: 2,360 m^3/day), the root-zone treatment (RZT) was employed as a part of an innovative Decentralized Wastewater Treatment System (DEWATS) that treats all wastewater produced by academic campus dwellers. In this plant, heavy particles and suspended solids in untreated sewage were first removed in the settler tank. Then the sewage was treated by biological treatment through the anaerobic baffled reactor, where anaerobic degradation of organic matter took place. In the third step, the sewage ran through a planted gravel filter, known as an RZT system, where the roots of the *Canna indica* absorbed organic pollutants from the sewage. In the fourth stage, sewage was passed through a pressure sand filter to reduce turbidity and BOD of the effluent. After chlorination, the final effluent was pumped to Water Service Centres in separate storage tanks. Currently the water does not go through ultrafiltration as it is pumped directly to irrigation tanks to be used for campus irrigation.

2.2. Sampling

At the two WWTPs, influent and effluent wastewater samples were initially collected biweekly, then weekly for two months, from August to September 2020. Twenty-one grab samples, representing the treatment plant inlets and outlets of both treatment plants, were collected every Monday of the week at 10 am and placed into 250-ml sterile bottles (Tarsons, PP Autoclavable, Wide Mouth Bottle, Cat No. 582240, India). Simultaneously, blanks were included to check for contamination during travel. The samples were kept cool in an ice-box until analysis. All laboratory analyses were performed on the same day and included duplicates to ensure accuracy and precision. It is imperative to note that we evaluated the removal of SARS-CoV-2 RNA by wastewater treatment methods, including disinfection. It is therefore, final effluent was sampled after the disinfection process, which is essential in the context of risk assessment of SARS-CoV-2 in receiving water [46].

2.3. Detection and extraction of viral RNA from sewage samples

2.3.1. Precipitation of virus

Thirty mL samples were centrifuged at 4000 × g for 40 min in a 50 mL falcon tube followed by filtration of supernatant using 0.22-μm syringe filter (Mixed cellulose esters syringe filter, Himedia). After filtration, 25 mL of the supernatant was treated with polyethylene glycol (PEG) and NaCl at 80 g/L and 17.5 g/L, respectively and incubated at 10 °C, 100 rpm overnight. The next day, the mixture was centrifuged for 90 min at 14000 × g and the supernatant were discarded to collect a pellet containing viruses and their fragmented genes. The pellet was resuspended in 300 μL RNase-free water and kept in 1.5 mL Eppendorf tubes at −40 °C, until further analyses.

Briefly, two mechanisms of precipitation are mediated by PEG, which is a chemically inert, nontoxic, water soluble synthetic polymer. a) PEG sterically excludes proteins from a solvent due to 'salting out ef-

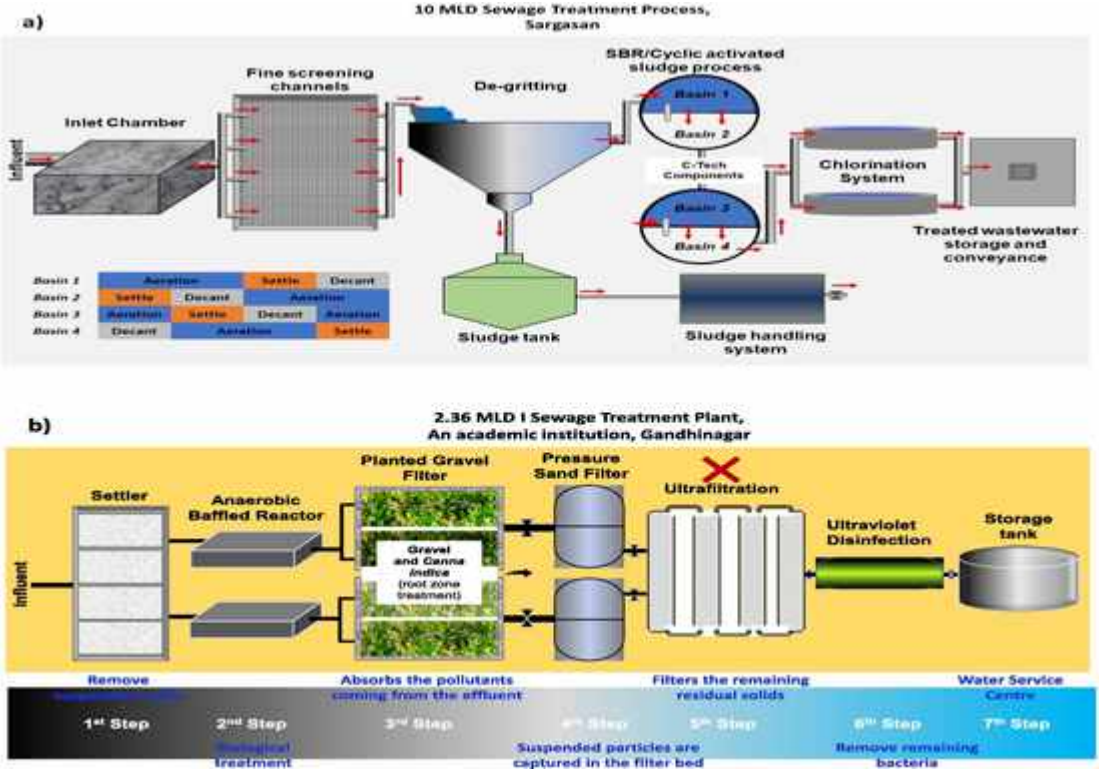


Fig. 1. Simplified illustration of the layout of two wastewater treatment plants; a) Conventional Activated Sludge based WWTP in Sargasan, and b) root-zone treatment in an academic institution of Gandhinagar, India monitored during August and September 2020.

fect' by acting as an "inert solvent sponge". And b) unfavorable thermodynamic effect on the protein surface charges by solubilized PEG, causing it to be excluded from the "protein zone", at appropriately high concentrations of polymer. The dynamics of this process is dependent on factors like protein size, their concentration and charge; pH and ionic strength of the solution; and temperature. The required amount of salt depends on the molecular weight of PEG, which counteract the "Donnan effect" and distributes viruses unequally between the phases.

2.3.2. *RNA isolation, RT-PCR and gene copy estimation*

A NucleoSpin® RNA Virus, (Macherey-Nagel GmbH & Co. KG, Germany) kit was used for RNA isolation from the pellet containing the concentrated virus. MS2 phage, provided by TaqPathTM Covid-19 RT-PCR Kit, was used as an internal control. Other specifics: a) the nucleic acid was extracted using NucleoSpin® RNA Virus Kit (Applied Biosystems), and Qubit 4 Fluorometer (Invitrogen) was used for RNA concentrations estimation; b) molecular process inhibition control was evaluated through the MS2 phage for QC/QA analyses of nucleic acid extraction and PCR inhibition [47]. We have described methodologies elsewhere [12,13]. Briefly, steps were carried out as per the guideline provided with the product manual of Macherey-Nagel GmbH & Co. KG and RNAs were detected using real-time PCR (RT-PCR).

An Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument (version 2.19 software) was used for SARS-CoV-2 gene detection. A template of 7 μL of extracted RNA was used in each reaction with TaqPath™ 1-Step Multiplex Master Mix (Thermofischer Scientific, USA). The reaction mixture volume of 20 μL contained 10.50 μL Nuclease-free Water, 6.25 μL Master Mix, and 1.25 μL COVID-19 Real Time PCR Assay Multiplex. Three controls were included: positive control (TaqPath™ COVID-19 Control); negative control (from extraction run spiked with MS2); and a no template control (NTC) [48]. The real-time PCR contained 1 incubation step cycle of 25 °C for 2 min, 1 cycle of reverse transcription 53 °C for 10 min, 1 cycle of activation 95 °C for 2 min, and 40 cycles of amplification, including denaturation at 95 °C for 3 sec and extension at 60 °C for 30 sec. Finally, results were interpreted using Applied Biosystems Interpretive Software, and Ct values for three target genes, i.e., ORF1ab, N Protein, and S Protein of SARS-CoV-2, were detected along with MS2 as an internal control.

The samples were considered as positive if at least two genes showed amplification. The average Ct-value of a given sample was then converted to gene copy numbers considering the equivalence of 500 copies of SARS-CoV-2 genes as 26 Ct-value (provided with the kit). The same was extrapolated to derive approximate copies of each gene, using the well-established principle of 3.3 CT change corresponding to a 10-fold gene concentration change. The average effective genome concentration of SARS-CoV-2 present in a given sample was calculated by multiplying the RNA amount used as a template with the enrichment factor for each sample.

It is noteworthy that the primer efficiency of different genes will be slightly varied according to the sequence of primer. However, the gene copies were numbered based on the positive control provided with kit i.e., 10^4 copies/μl and the final concentration of 25 copies per reaction. Based on several hundreds of RTPCR run, it was found that the positive control was robust enough to provide the same Ct values for all 3 genes, implying no evident difference between the primer efficiency. We report both primary Ct-values and derived gene copies relative to the Ct values of positive controls, for both individual genes and effective SARS-CoV-2 genome concentration.

Due to various constraints, samples were analyzed in duplicate, considering that the samples were analyzed in the batch accompanied with negative and positive controls, and each sample was spiked with known concentrations of MS2. In the event of any variations (among duplicate and controls) of >10%, samples were re-analyzed.

2.4. *Statistical analysis*

Box plots were prepared to explain the data variability, and one-way ANOVA was used to determine significance of the difference among the

treatment plant, various gene types and temporal variation in the SARS-CoV-2 RNA copies before and after treatment. The results obtained from ANOVA analysis were reported as ($F_{critical}$ = $F_{calculated}$, significant level P) and if $F_{calculated}$ value is greater than $F_{critical}$ value, the null hypothesis will be rejected. The Statistical Package for the Social Sciences (SPSS 21) was used for hypothesis testing and multivariate analyses (MVA) to determine the significance of removal efficacy and relatedness of various water quality parameters with SARS-CoV-2 genes through paired t-tests and principal component analyses (PCA) respectively, after Z-score data normalization [39]. A non-related principal components (PCs) was generated using orthogonal varimax rotation, and the results were projected on three-dimensional loading domain. Since the principal component analysis (PCA) are found to be useful for evidencing temporal variation caused by COVID-19 patient load and treatment, strong positive or negative correlation between a variable and a factor is indicated by a high factor loading close to 1 or −1, respectively. Three-dimensional projection of PCs is an unsupervised pattern recognition technique that groups the objects (variables) as per their similarities within a class and dissimilarities between different classes. In the present study, PCA was done using agglomeration and Ward linkage techniques.

3. Results

We analyzed the efficacy of two treatment processes of CAS and RZT (schematic diagrams of the operating mechanism of both plants in Sargasan and academic campus are shown in Fig. 1 a and b, respectively). Table 1 summarizes the change in the Ct-value and gene copies of SARS-CoV-2N-genes (nucleocapsid protein), S-genes (spike glycoprotein), and ORF 1ab genes (polyprotein) before and after the treatment i.e., in the samples of influent and effluent for two months (August and September 2020) of monitoring. It also provides the date of sampling, effective genome concentration, and active COVID-cases. The Ct values of internal control (MS2 bacteriophage) ranged between 25.41 to 28.01 and 25.59 to 30.08 in the samples from Sargasan and academic institution WWTPs, respectively. No SARS-CoV-2 genes were detected in the negative control samples.

Paired T-tests between the inlet and outlet wastewater samples, taken on the same days, were performed to understand the significance of the SARS-CoV-2 gene removal efficacy of each treatment process, i.e., CAS process-based treatment at Sargasan (Fig. 2a) and RZT at an academic institution in Gandhinagar (Fig. 2b). We then combined the data and conducted paired T-test analyses of the significance of SARS-CoV-2 gene removal efficacy based on Ct-values obtained and various gene copies calculated for CAS (Fig. 3a and c) and RZT (Fig. 3b and d), respectively.

Overall comparison of SARS-CoV-2 genome removal efficacy of CAS and RZT is expressed through paired T-test performed on the total effective genome concentrations obtained throughout the 60 days of monitoring (Fig. 4). Monthly variations and their significance of SARS-CoV-2 genes removal efficacy of CAS; and RZT is presented in Fig. 5 to understand the impact of genetic loading in the influent and its correlation with removal efficacy of the treatment processes. MVA was conducted to understand the overall impact of treatment by visualizing the PC loading in a 3-D domain for various water quality parameters and SARS-CoV-2 gene loading of collected influent (untreated) and effluent (treated) samples during the two-month monitoring period (Fig. 6a and b). A summary description of in-situ parameters (Table S1), variation explained, eigenvalue variations, and principal component loadings for influent (Table S2, Fig S1, Table S3) and effluent (Table S2, Fig S1, Table S3) are provided as supplementary material.

Although there will be a considerable uncertainty, we could estimate the number of people shedding SARS-CoV-2 to wastewater. SARS-CoV-2 is contained in the human stool at 4–6 log copy/g [49], and assuming that the average stool weight is 500 g per day per person, that results in $5x10^6$ to $5x10^8$ copies per person per day shredded to wastewater. Assuming that our raw wastewater samples had 1000 copies/L on average, raw wastewater from Sargassan WWTP had $1x10^9$ copies per day, implying that there were 2 to 200 people shedding SARS-CoV-2 in the catchment on a day. However, there would be too many uncertainties in this calculation, due to significant decay/reduction of viral RNA during transport from toilets to WWTPs. Therefore, hereafter, only Ct-values and gene copies are compared. Further, the role of aqueous and solid-phase interactions for the quantification of SARS-CoV-2 gene concentrations has been prominently highlighted in terms of recovery of the viral RNA in the aqueous environment through solid fractions [50]. However, we did not take sludge into account as there still needs a robust standard protocol for sludge clean-up and RT-qPCR measurements to be established.

Table 1

. Temporal variation in SARS-CoV-2 genetic material loading found in the influent and effluent samples collected from two different wastewater treatment plants i.e. conventional activated sludge (CAS) at Sargasan ward, and root-zone treatment (RZT) at academic institute at Gandhingar.

Station		Sampling date →	Sampling date Vs Active/ confirmed cases Vs Gene copies										
			August 2020							September, 2020			
			07.08.20	11.08.20	14.08.20	17.08.20	21.08.20	25.08.20	28.08.20	07.09.2	14.09.20	23.09.20	30.09.20
		Active/ confirmed Cases	317/1680	264/1793	261/1894	269/1984	271/2097	300/2208	329/2317	442/2697	496/2967	571/3337	613/3666
		SARS-CoV-2	Gene Copies (copies/ L) x 10^2										
Conventional Activated	Inlet	N-Gene	8.50	4.60	5.53	5.99	8.07	10.2	0.74	12.4	59.7	3.99	47.5
		ORF-Gene	5.13	1.87	48.8	3.81	4.47	3.72	1.01	5.69	24.4	13.5	13.2
		S-Gene	25.5	17.2	15.1	15.4	14.8	13.2	0.42	2.46	35.3	15.2	8.42
		SARS-CoV-2 Genome	13.0	7.89	8.50	8.41	9.10	9.05	0.98	6.85	39.8	10.9	23.0
	Outlet	N-Gene	1.80	0.60	7.86	5.07	4.94	5.99	0.67	ND	ND	0.24	0.55
		ORF-Gene	1.40	0.50	1.27	5.46	1.37	2.05	0.23	ND	ND	0.46	ND
		S-Gene	4.83	1.40	9.21	3.95	4.10	7.26	0.42	ND	ND	0.86	ND
		SARS-CoV-2 Genome	2.68	0.83	6.11	4.82	3.47	5.10	0.44	ND	ND	0.52	INC
Root-Zone Treatment (RZT)	Inlet	N-Gene	NA	1.83	23.6	18.3	3.50	8.62	5.05	ND	3.95	ND	1.01
		ORF-Gene	NA	0.74	14.8	11.2	0.69	8.42	8.53	ND	ND	ND	0.43
		S-Gene	NA	5.60	22.6	29.8	4.94	20.3	21.3	ND	ND	0.21	ND
		SARS-CoV-2 Genome	NA	2.72	20.3	19.8	3.04	12.5	11.6	ND	INC	INC	0.72
	Outlet	N-Gene	NA	0.72	5.25	6.07	2.90	5.46	1.67	ND	9.15	0.15	0.25
		ORF-Gene	NA	ND	4.54	2.18	0.97	3.78	0.77	0.28	ND	0.70	ND
		S-Gene	NA	1.86	4.32	13.8	1.74	7.34	2.87	ND	ND	ND	ND
		SARS-CoV-2 Genome	NA	0.86	4.70	7.35	1.87	5.52	1.77	ND	INC	0.43	INC

Low — High

Where, NA= data not available; ND= not detected; INC= valid but inconclusive

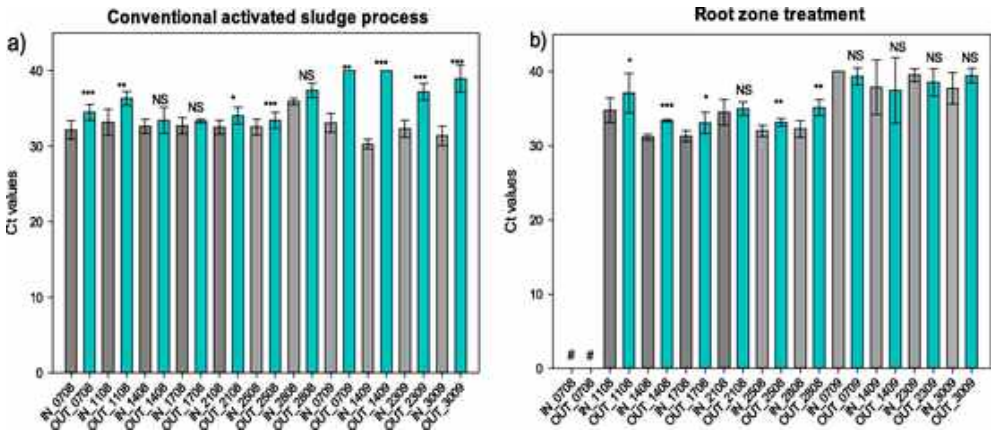


Fig. 2. Paired T-test between inlet and outlet wastewater samples taken on the same days for SARS-CoV-2 genetic load in a) Conventional activated sludge process-based treatment at Sargasan, and b) Root-zone treatment at academic institution in Gandhinagar. (where *** = $p < 0.01$; ** = $p < 0.05$; * = $p < 0.1$; NS = not significant; # = data not available; and RT-PCR was run for 40 cycles).

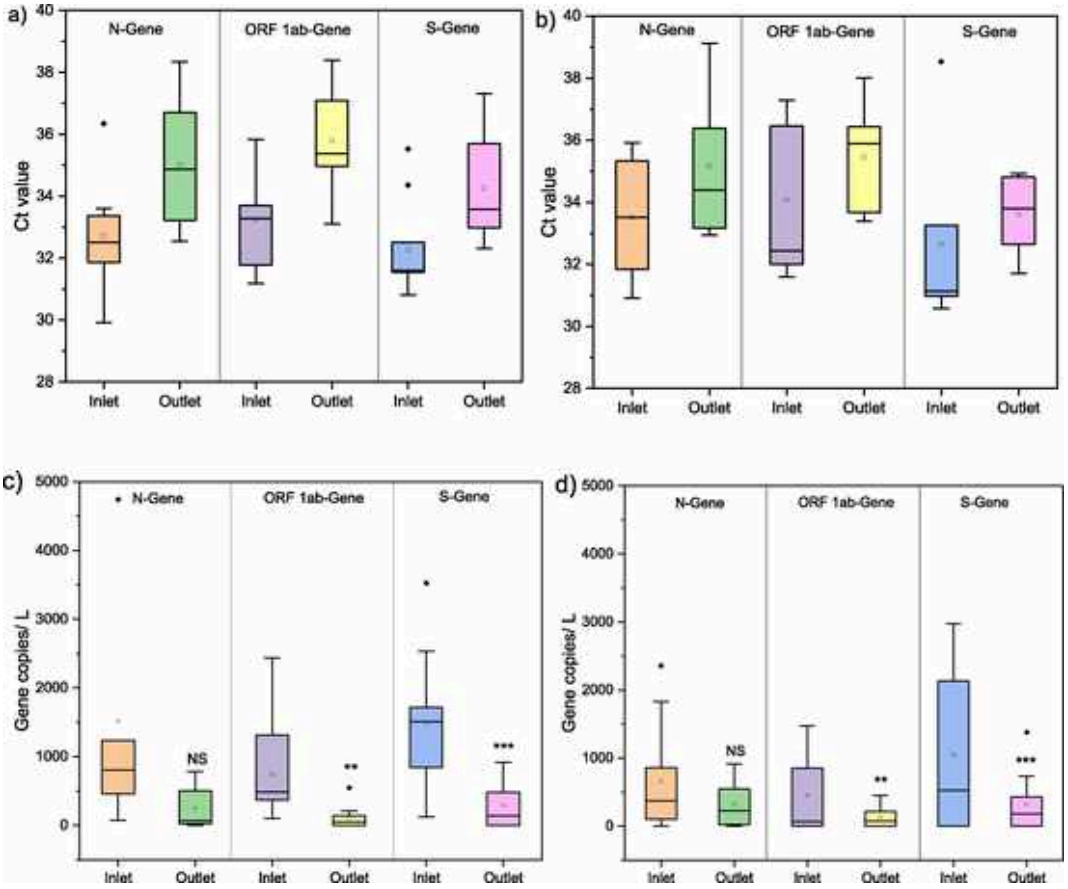


Fig. 3. A comparative statistical (paired T-test) analyses of significance of SARS-CoV-2 genes removal efficacy based on Ct-values obtained for a) CAS; and b) RZT; and various gene copies calculated for c) CAS and d) RZT; at $p < 0.01$; $p < 0.05$; and $p < 0.1$ indicated by three, two and one stars. NS signifies not significant.

4. Discussion

4.1. Significance of treatment

Of the eleven samples collected from the inlet and outlet points of WWTPs during the study period, eight samples from Sargasan and five samples from the academic institution showed significant removal of the viral genes (Fig. 2a and b). Paired T-tests between influent and effluent wastewater show a significant reduction through CAS treatment systems except for three occasions. Reduction/removal of SARS-CoV-2 genes was highly significant ($p < 0.01$) in nearly 50% of the samples, with non-significant removal in August only. RZT appeared effective in August but failed to show significant removal of SARS-CoV-2 RNA in September. There may be two possible explanations related to the operation of WWTPs and COVID-19 cases in the vicinity of WWTPs. The RZT was situated and precisely received waste from the campus dwellers and visitors only, and COVID-19 cases increased in September 2020. Thus, even if we assume the viral shedding contribution of visitors was non-variable, it is certain that genetic loading increased in the RZT plant during September 2020. We also suspect that operating condi-

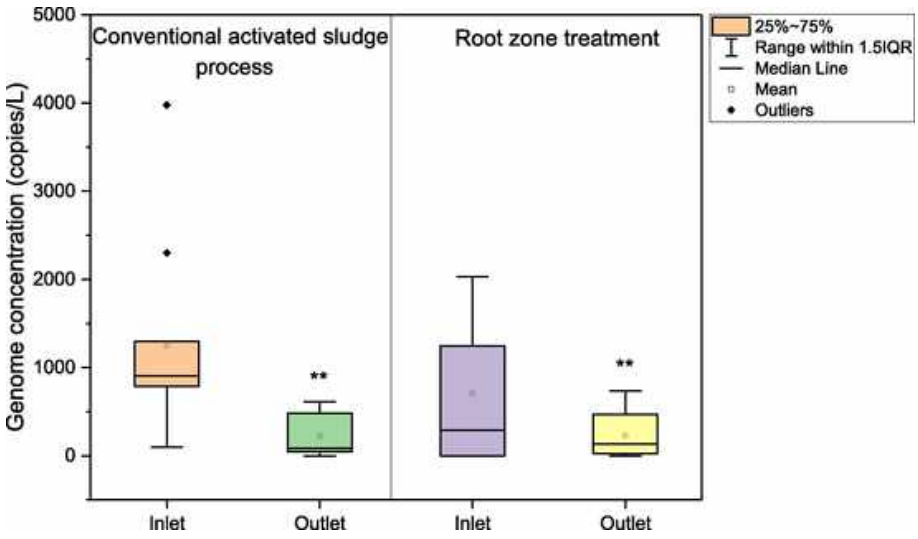


Fig. 4. Overall comparison of SARS-CoV-2 genome removal efficacy of conventional activated sludge and root-zone treatments expressed through paired T-test performed on the total effective genome concentrations obtained through out the 60 days of monitoring period. Same level of significance is used as above.

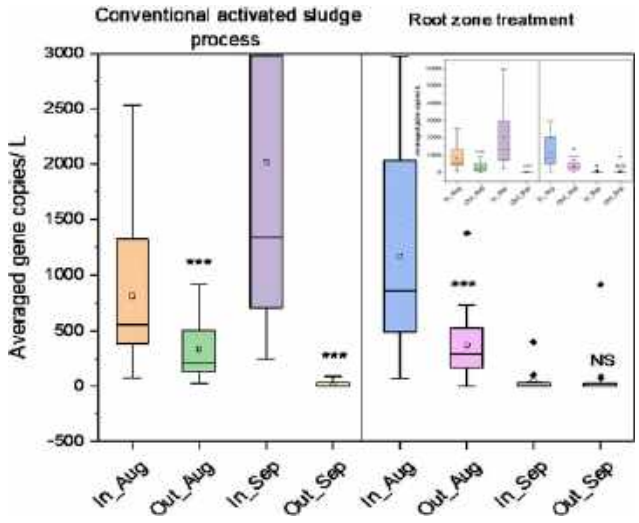


Fig. 5. A comparative statistical (paired T-test) analyses in monthly variation of significance of SARS-CoV-2 genes removal efficacy of CAS; and b) RZT; at $p < 0.01$; $p < 0.05$; and $p < 0.1$ indicated by three, two and one stars. NS signifies not significant.

tions at the treatment plants were not consistent throughout the monitoring period. Nevertheless, the RZT achieved significant removal on > 50% of the sampling dates.

Paired t-tests show that irrespective of treatment type, the N-gene is much more stable? than S- and ORF-1ab genes of SARS-CoV-2 (Fig. 3a–d). Removal efficacy was highest for S-genes ($p < 0.01$) followed by ORF-1ab ($p < 0.05$) for both treatment processes. Overall, N-genes showed non-significant reduction after treatment. The ORF 1ab-gene copy numbers decreased by 84.4% ($t = 2.78$, $p = 0.022$) and 70.5% ($t = 2.30$, $p = 0.047$) in Sargasan WWTP and the academic institution WWTP, respectively (Fig. 3c and d). Likewise, S-genes were significantly removed by both treatment plants (80.5%, $t = 4.10$, $p = 0.002$ at Sargasan and 69.5%, $t = 2.84$, $p = 0.019$ at the academic institution). Conversely, the abundance of N-gene declined 83.4% at Sargasan WWTP (Fig. 3c) and 52.0% at the academic institution during treatment (Fig. 3d), but the differences in S- and N-gene removal were statistically significant ($t = 2.04$, $p = 0.069$ and $t = 1.59$, $p = 0.147$, respectively). The results showed that both the cyclic activated sludge process and root zone treatment plants of Sargasan and the academic institution effectively removed ORF ab-genes and S-genes, but not N-genes.

Our hypothesis- prevalence may be causing the difference in removal- was not correct (Table 1). It seems structural properties of the genes are more responsible for such removal disparity than prevalence. This is because, among four major structural proteins of SARS-CoV2; S proteins are the most exposed one being the spike surface glycoprotein (S), while ORF-1ab gene is not only a signatory gene for SARS-CoV-2 genes but also located at both the 5′ & 3′-terminuses of the SARS-CoV-2 genome [37]. Nucleocapsid protein (N) is more protected in the SARS-CoV-2 structures, and common genes among family *Coronaviridae*, marked by the presence of single-stranded, positive-sense RNA genome, surrounded by spikes and protein envelope.

A comparison of the effectiveness of various wastewater treatment systems for the removal of SARS-CoV-2 genetic material is shown in Table 2. Earlier studies suggested reduction of SARS-CoV-2 genetic material during wastewater treatment processes via secondary treatment such as activated sludge/ A2O/ extended aeration and tertiary treatment such as disinfection, coagulation, flocculation, sand filtration, NaClO/UV [21]. Interestingly, none of the studies investigated the removal efficacy of a given treatment for SARS-CoV-2 RNA. In our study, both the CAS and RZT processes are found to effectively remove SARS-CoV-2 RNA. To the best of our knowledge, this is the first report assessing the effectiveness of RZT for SARS-CoV-2 RNA reduction.

4.2. Comparative efficacy of CAS and RZT processes to remove SARS-CoV-2 genes

SARS-CoV-2 RNA is substantially reduced in treated wastewater i.e. effluents of both WWTPs throughout the sampling period, as indicated by the overall comparison of SARS-CoV-2 genome removal efficacy of CAS and RZT through a paired T-test (Fig. 4.). Although there was a significant difference in average SARS-CoV-2 genome concentration in the influents of the CAS plant at Sargasan (1.25×10^3 copies/ L) and the RZT system of an academic institution (7.07×10^2 copies/ L). Yet, both processes mostly showed effective removal at $p < 0.05$. However, incomplete removal may have some environmental and health implications.

While infectivity and viability of these genomes are still being debated and researched with a general consensus of viability being less likely and thus the infectivity, there is still no study that has yet proven the chance of transmission and infectivity impossible. In such a scenario, significant removal is not enough, as such effluents will finally be

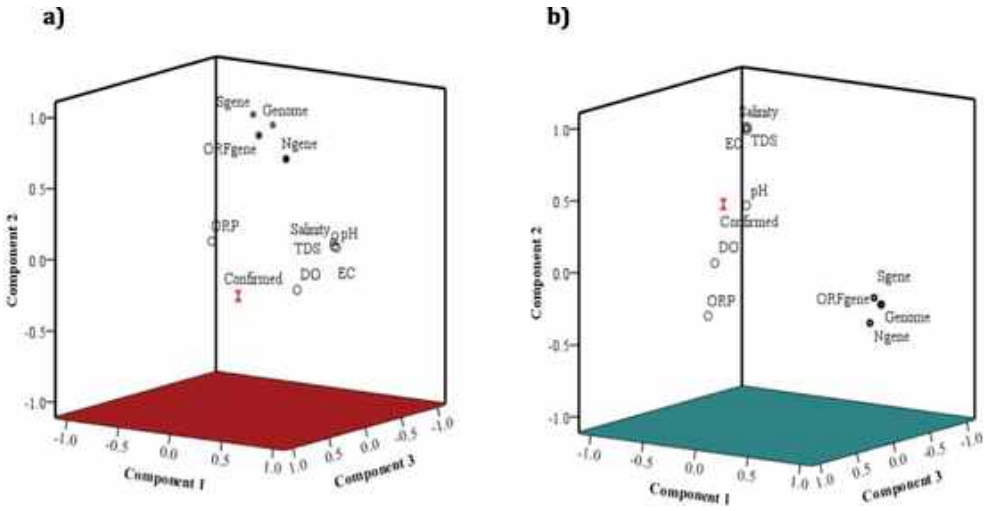


Fig. 6. Three-dimensional projection of the principal component loading for a) Influent and b) effluent; exhibiting the effect of treatment on SAR-CoV-2 genes association with other water quality parameters and confirmed cases of COVID-19.

received by the ambient waters. Therefore, we foresee an immediate increase in reporting of SARS-CoV-2 genes in freshwater systems like lakes, rivers, and perhaps groundwater. Several imperative hypotheses need to be tested in this regard, and the present study signifies the need of such investigations.

Further, we also suspect that the size of the treatment plant and operational and management consistencies, along with the quality of influent water will play a critical role in the entire research scenario of COVID-19 transmission and monitoring [13]. As far as treatment type is concerned, the RZT will show a bit wider fluctuation than the CAS treatment process (Fig. 4). The low genome concentration at the academic institution WWTP is apparently due to institutional wastewater load which was confined to the institutional community and malfunctioning of the ultrafiltration unit of the WWTP. Conversely, the Sargasan WWTP receives municipal wastewater, resulting in the presence of SARS-CoV-2 RNA in effluent wastewater, owing to fluctuating genetic loading in the inlet waters. We conclude that both WWTPs effectively removed viral genes, but Sargasan STP was more efficient (82.4% decrease, $t = 2.98$, $p = 0.014$) than the academic institution (67.9% decrease, $t = 2.54$, $p = 0.032$) (Fig. 4). It is imperative to note that we have collected samples from both treatment processes after disinfection processes and still found the genetic fragments of SARS-CoV-2 in the effluent. This observation may imply that owing to nano-sized colloidal nature of genetic fragments, disinfection processes like chlorination/UV are likely to be less effective than the process of coagulation.

Overall, as PCR-based detection of RNA does not mean detection of viable SARS-CoV-2, and quantifying active (viable) SARS-CoV-2 is a difficult challenge, with so far only one lab-scale experiment reported (Bivins et al. 2020), we recommend further study for a valid discussion on implications of leftover SARS-CoV-2 RNA after the treatment. However, our data explicitly disapprove the general notion that treatment completely removes the genetic fragments of SARS-CoV-2.

4.3. Temporal variation in removal efficacy

As suspected above, we investigated the role of influent quality in terms of SARS-COV-2 genetic loading through temporal variation in the performances of both CAS and RZT systems (Fig. 5). For CAS plant in Sargasan ward, inlet quality in September showed higher genetic loading than that of August, which has been verified by confirmed COVID-19 cases in the city, yet removal was better in September than August 2020. When inquired with operational staff, it seems that operational inconsistencies are responsible for these results rather than the genetic material loading. While in the case of the academic institution RZT-based plant, where the operation was rather more consistent, it seems that genetic material loading in the inlet water has reflected the genome concentration left in the effluent waters. This is also very likely to be attributed to the size of plant i.e., CAS facility of Saragasan is 10,000 m^3/day against 2360 m^3/day of the RTZ plant of the academic institution, leading to the sensitivity of RZT plant for genetic loading in the inlet wastewater. Nevertheless, at this juncture, we take these results as indicative ones, and more convincing conclusions pertaining to the role of influent water quality, and its implication may be derived after further monitoring. Such notion has also been expressed elsewhere [51–54].

4.4. Treatment impact insight through multivariate statistical analyses

Principal component analyses show a comprehensive picture of the overall contribution and influence of treatment on SARS-COV-2 gene removal. The entire dataset obtained for influent and effluent were subjected to PCA and projected in the 3-D domain of three main PCs. Owing to more complex nature of influents, four PCs were identified after nine iterations that explain 90% of the total variance in the dataset of influent waters. The first PC explains 34% of the total variance with significant loading for *in-situ* water quality parameters forming a cluster (EC, TDS, Salinity, and pH) with moderate loading (0.5) for N-genes (Fig. 6a and Supplementary Tables S2 and S3). On the other hand, nearly the same (~30%) variation of data sets is explained by SARS-COV-2 genes, and genome concentrations form a cluster upper left domain with significant loadings for effective genome concentrations (0.94) followed by S-genes, ORF-1ab, and N-genes as PC2. Interestingly in influent waters, N-genes illustrated moderate to high loading as both PC1 and PC2.

After treatment, the complexion changed significantly with the overall reductions of PCs to three, explaining cumulative variations of 80% in the dataset. Another significant observation was that SARS-CoV-2 genes exhibit higher loadings than the *in-situ* water quality parameters in effluent waters. Order of loadings among SARS-CoV-2 genes and genome remains the same i.e., effective genome concentration > S-genes > ORF-1ab > N-genes. Confirmed COVID-19 emerged as PC3 (with moderate loading of 0.78) in influent waters, stressing the relationship of confirmed cases with SARS-CoV-2 RNA in the wastewater, but the influence was weakened in the treated water with non-significant say in the quality variations of the samples [55–60].

This is the first time MVAs was used with wastewater surveillance dataset to signify the impact of treatment, which eventually proves that: i) wastewater surveillances did track COVID-19 loading of the

Table 2
Comparison of the effectiveness of various wastewater treatment systems for the removal of SARS-CoV-2 genetic material.

Country	City	Wastewater treatment method and types	Virus concentration method	RT-(q)PCR target region	Before treatment (gene copies /L)	After treatment (gene copies /L)	References
India	Gandhinagar	Root Zone Treatment/institutional wastewater	PEG precipitation	N gene	6.58×10^2	3.16×10^2	**Present study**
				ORF 1ab gene	4.48×10^2	1.32×10^2	
				S gene	1.05×10^3	0.32×10^3	
				Genome conc.	7.07×10^2	2.27×10^2	
		SBR/Cyclic Activated Sludge Process/chlorination Municipal wastewater		N gene	1.48×10^3	0.25×10^3	
				ORF 1ab gene	0.74×10^3	0.12×10^3	
				S gene	1.49×10^3	0.29×10^3	
				Genome conc.	1.25×10^3	0.22×10^3	
	Ahmedabad	UASB	PEG precipitation	ORF1ab, N gene S gene	3.5×10^3	<LOQ	[13]
		Aeration pond		ORF1ab	1.5×10^2 (<LOQ)	Not detected	
China		Septic tank treatment of hospital effluent	PEG precipitation	ORF1	Not detected	0.05–1.87×10^3	[20]
				N gene			
France	Paris	Municipal wastewater treatment	Ultracentrifugation	E gene	1×10^3–1×10^5	$<10 \times 10^3$	[32]
Spain	Murcia	Secondary treatment (activated sludge/A2O/extended aeration), disinfection, NaClO/UV)	Aluminium flocculation – beef extract precipitation	N gene	N1: 1.4×10^3	$<2.5 \times 10^3$	[21]
					N2: 3.4×10^3		
					N3: 3.1×10^3		
	Valencia	Municipal wastewater treatment (treatment methods not provided)	Aluminium flocculation – beef extract precipitation	N gene	N1: 1.0×10^3 – 1.0×10^4 (Averaged value)	Not detected	[21]
	Ourense	Primary settler, secondary treatment of municipal sewage	Ultrafiltration of centrifugated supernatant	N gene	7.5×10^3–1.5×10^4	Not detected	[27]
				E gene			
				RdRp gene			
Australia	Brisbane	Untreated wastewater	Adsorption-direct RNA extraction and Ultrafiltration	N Sarbeco, NIID_2019-nCOV_N	1.9×10^1 – 1.2×10^2 copies/ L	NA	[8]
USA	Southern Louisiana	Untreated wastewater, secondary treated, and final effluent	Ultrafiltration and Adsorption-elution using electronegative membrane	CDC N1, N2	3.1×10^3 – 7.5×10^3	Not detected	[40]
Netherlands	–	Untreated wastewater	Ultrafiltration	CDC N1, N2, N3, E_Sarbeco	2.6×10^3 – 2.2×10^6	NA	[61]
Italy	Milan and Rome	Untreated wastewater	PEG/dextran precipitation	RT-qPCR (RdRp), nested PCR (ORF1aband S assays)	6/12 samples found positive; gene copies were not detected	NA	[62]
Japan	Yamanashi Prefecture	Untreated influent and secondary-treated wastewater before chlorination	Electronegative membrane-vortex (EMV) method and the membrane adsorption-direct (MAD) RNA extraction method	N_sarbeco, NIID_2019-nCOV_N, CDC-N1, N-2	EMV: $<6.6 \times 10^4$ – $<8.2 \times 10^4$	EMV: $<1.4 \times 10^2$ – 2.5×10^3	[29]
					MAD: $< 4 \times 10^3$	MAD: $< 1.6 \times 10^2$	
USA	Bozeman, Montana	Untreated wastewater	Ultrafiltration	CDC N1, N2	$>3 \times 10^4$	NA	[34]
USA	Massachusetts	Untreated wastewater	PEG precipitation	CDC N1, N2, N3	$>2 \times 10^5$	NA	[63]
France	Paris	Untreated and treated wastewater	Ultracentrifugation	E_Sarbeco	$> 10^{6.5}$	$\sim 10^5$	[64]

community; ii) influent waters present a better picture in terms of SARS-CoV-2 gene monitoring; iii) effective genome concentration should be calculated based on presence/absence of multiple genes rather the presence of one specific gene; iv) N-genes are the most resistant to treatment with higher sensitivity than S and ORF-1ab genes; and v) the presence of residual SARS-CoV-2 genes after treatment is critical from the effluent quality point of view. Among the other exciting observations; the explicit grouping/clustering of SARS-CoV-2 genes and other water quality parameter; and influence of confirmed COVID-19 cases has been significant from the wastewater-based epidemiology perspectives.

5. Conclusion

A comparison of SARS-CoV-2 RNA removal efficacy of CAS and RZT, the two most used treatment systems in India, was studied through bi-weekly and monthly variations in their performances. We applied long-term monitoring data and performed statistical tests to understand the significance of removal and correlated it with other water quality parameters before and after deployed treatment. For the first time, MVAs used in this study along with other statistical tests highlighted the disparity in performance and statistical significance of SARS-CoV-2 RNA removal between CAS and RZT. It can be concluded that influent waters present better picture in terms of SARS-CoV-2 gene monitoring; effec-

tive genome concentration should be calculated based on presence/absence of multiple genes rather the presence of one specific gene; and treatments are less effective on N-genes and the most effective for S-genes. CAS treatment exhibited better RNA removal rate (t = 2.98, p = 0.014) compared to the root-zone treatment (t = 2.54, p = 0.032). In addition, treatment plants with smaller capacity are likely to show more fluctuations in effluent water quality.

Two most critical findings from the ongoing pandemic perspectives were that the treated effluents are not always free from SARS-CoV-2 RNA, and are subject to temporal variability. We stress the need for wastewater surveillance of SARS-CoV-2 at the treatment plant scale with further investigation on the efficacy of the treatment processes on the removal of the enveloped virus such as SARS-CoV-2 as well as the genomic materials. The future research efforts may therefore consider the influence of genetic material loading in the influent, difference in sewage flow and treatment methods, hydraulic and sludge retention time of technology used, and serviced people. In addition, the mechanistic understanding may be generated on the SARS-CoV-2 removal using long-term step-wise sampling and monitoring of a given treatment processes. Nevertheless, our results are based on RNA fragment detection by RT-PCR, thus the abundance of viable SARS-CoV-2 in the samples can be significantly lower than the RNA-based gene copies. Therefore, research is needed for assessing infectivity through viable virus estimation, specifically for the use of reclaimed water in agriculture and drinking water supply.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This work is funded by Kiran C Patel Centre for Sustainable Development at IIT Gandhinagar, UNICEF, Gujarat, and UKIERI. We also acknowledge the help received from Dr. Arbind K Patel and other GBRC staff who contributed towards sample and data analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2021.130635.

References

[1] World Health Organization Novel Coronavirus (2019-nCoV) Situation Report – 1 (2020) https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports.

[2] A. Bivins, D. North, A. Ahmad, W. Ahmed, E. Alm, F. Been, P. Bhattacharya, L. Bijlsma, A.B. Boehm, J. Brown, G. Buttiglieri, V. Calabro, A. Carducci, S. Castiglioni, Z. Cetecioglu Gurol, S. Chakraborty, F. Costa, S. Curcio, F.L. De Los Reyes, J. Delgado Vela, K. Farkas, X. Fernandez-Casi, C. Gerba, D. Gerrity, R. Girones, R. Gonzalez, E. Haramoto, A. Harris, P.A. Holden, M.T. Islam, D.L. Jones, B. Kasprzyk-Hordern, M. Kitajima, N. Kotlarz, M. Kumar, K. Kuroda, G. La Rosa, F. Malpei, M. Mautus, S.L. McLellan, G. Medema, J.S. Meschke, J. Mueller, R.J. Newton, D. Nilsson, R.T. Noble, A. Van Nuijs, J. Peccia, T.A. Perkins, A.J. Pickering, J. Rose, G. Sanchez, A. Smith, L. Stadler, C. Stauber, K. Thomas, T. Van Der Voorn, K. Wigginton, K. Zhu, K. Bibby, Wastewater-based epidemiology: global collaborative to maximize contributions in the fight against COVID-19, Environ. Sci. Technol. (2020), https://doi.org/10.1021/acs.est.0c02388.

[3] I. Xagoraraki, E. O'Brien, Wastewater-based epidemiology for early detection of viral outbreaks, Women in Water Qualit, Springer Nature, Switzerland, 2020, pp. 75–97.

[4] P.M. Choi B.J. Tscharke E. Donner J.W. O'Brien S.C. Grant S.L. Kaserzon ... J.F. Mueller Wastewater-based epidemiology biomarkers: past, present and future TrAC, Trends Anal. Chem. 105 2018 453 469

[5] Z. Yang, B. Kasprzyk-Hordern, C.G. Frost, P. Estrela, K.V. Thomas, Community sewage sensors for monitoring public health, Environ. Sci. Technol. 49 (2015) 5845–5846.

[6] M. Hellmér, N. Paxéus, L. Magnius, L. Enache, B. Arnholm, A. Johansson, H. Norder, Detection of pathogenic viruses in sewage provided early warnings of hepatitis A virus and norovirus outbreaks, Appl. Environ. Microbiol. 80 (21) (2014) 6771–6781.

[7] H. Asghar, O.M. Diop, G. Weldegebriel, F. Malik, S. Shetty, L. El Bassioni, C.C. Burns, Environmental surveillance for polioviruses in the Global Polio Eradication Initiative, J. Infect. Dis. 210 (suppl_1) (2014) S294–S303.

[8] W. Ahmed, N. Angel, J. Edson, K. Bibby, A. Bivins, J.W. O'Brien, P.M. Choi, M. Kitajima, S.L. Simpson, J. Li, B. Tscharke, R. Verhagen, W.J.M. Smith, J. Zaugg, L. Dierens, P. Hugenholtz, K.V. Thomas, J.F. Mueller, First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community, Sci. Total Environ. 728 (2020), https://doi.org/10.1016/j.scitotenv.2020.138764.

[9] W. Ahmed, P.M. Bertsch, K. Bibby, E. Haramoto, J. Hewitt, F. Huygens, P. Gyawali, A. Korajkic, S. Riddell, S.P. Sherchan, S.L. Simpson, K. Sirikanchana, E.M. Symonds, R. Verhagen, S.S. Vasan, M. Kitajima, A. Bivins, Decay of SARS-CoV-2 and surrogate murine hepatitis virus RNA in untreated wastewater to inform application in wastewater-based epidemiology, Environ. Res. 191 (2020) 110092.

[10] W. Ahmed, P.M. Bertsch, A. Bivins, K. Bibby, K. Farkas, A. Gathercole, E. Haramoto, P. Gyawali, A. Korajkic, B.R. McMinn, J.F. Mueller, S.L. Simpson, W.J.M. Smith, E. M. Symonds, K.V. Thomas, R. Verhagen, M. Kitajima, Comparison of virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater, Sci. Total Environ. 739 (2020), https://doi.org/10.1016/j.scitotenv.2020.139960.

[11] M. Kitajima, W. Ahmed, K. Bibby, A. Carducci, C.P. Gerba, A. Hamilton, E. Haramoto, J.B. Rose, SARS-CoV-2 in wastewater: state of the knowledge and research needs, Sci. Total Environ. 739 (2020) 139076.

[12] M. Kumar, K. Kuroda, K. Dhangar, P. Mazumder, C. Sonne, J. Rinklebe, M. Kitajima, Potential emergence of antiviral-resistant pandemic viruses via environmental drug exposure of animal reservoirs, Environ. Sci. Technol (2020), https://doi.org/10.1021/acs.est.0c03105.

[13] M. Kumar, K. Kuroda, A.K. Patel, N. Patel, P. Bhattacharya, M. Joshi, C.G. Joshi, Decay of SARS-CoV-2 RNA along the wastewater treatment outfitted with Upflow Anaerobic Sludge Blanket (UASB) system evaluated through two sample concentration techniques, Sci. Total Environ. 754 (2021), https://doi.org/10.1016/j.scitotenv.2020.142329.

[14] M. Kumar, S. Mohapatra, P. Mazumder, A. Singh, R. Honda, R. Kumari, R. Goswami, P.K. Jha, M. Vithanage, K. Kuroda, Chuxia, Making waves perspectives of modeling and monitoring of SARS-CoV-2 in aquatic environment for COVID-19 pandemic, Curr. Pollution Rep. (2020), https://doi.org/10.1007/s40726-020-00161-5.

[15] M. Kumar, S. Mohapatra, P. Mazumder, A.K. Thakur, K. Dhangar, K. Taki, S. Mukherjee, A.K. Patel, P. Bhattacharya, P. Mohapatra, J. Rinklebe, M. Kitajima, F.I. Hai, A. Khursheed, H. Furumai, C. Sonne, K. Kuroda, A chronicle of SARS-CoV-2: seasonality, environmental fate, transport, inactivation, and antiviral drug resistance, J. Hazard. Mater. (2020), https://doi.org/10.1016/j.jhazmat.2020.124043.

[16] M. Kumar, A.K. Patel, A.V. Shah, J. Raval, N. Rajpara, M. Joshi, C.G. Joshi, First proof of the capability of wastewater surveillance for COVID-19 in India through detection of genetic material of SARS-CoV-2, Sci. Total Environ. 746 (2020), https://doi.org/10.1016/j.scitotenv.2020.141326.

[17] A. Hata, R. Honda, Potential sensitivity of wastewater monitoring for SARS-CoV-2: comparison with norovirus cases, Environ. Sci. Technol. 54 (11) (2020) 6451–6452, https://doi.org/10.1021/acs.est.0c02271.

[18] A. Tang, Z. Tong, H. Wang, Y. Dai, K. Li, J. Liu, W. Wu, C. Yuan, M. Yu, P. Li, J. Yan, Detection of novel coronavirus by RT-PCR in stool specimen from asymptomatic child, China, Emerg. Infect. Dis. 26 (2020), https://doi.org/10.3201/eid2606.200301.

[19] R. Wölfel, V.M. Corman, W. Guggemos, M. Seilmaier, S. Zange, M.A. Müller, D. Niemeyer, T.C. Jones, P. Vollmar, C. Rothe, M. Hoelscher, T. Bleicker, S. Brünink, J. Schneider, R. Ehmann, K. Zwirglmaier, C. Drosten, C. Wendtner, Virological assessment of hospitalized patients with COVID-2019, Nature 581 (2020), https://doi.org/10.1038/s41586-020-2196-x.

[20] W. Zhang, R.H. Du, B. Li, X.S. Zheng, X.L. Yang, B. Hu, Y.Y. Wang, G.F. Xiao, B. Yan, Z.L. Shi, P. Zhou, Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes, Emerg. Microbes Infect. 9 (2020), https://doi.org/10.1080/22221751.2020.1729071.

[21] W. Randazzo, P. Truchado, E. Cuevas-Ferrando, P. Simón, A. Allende, G. Sánchez, SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a Low prevalence area, Water Res. 181 (2020) 115942.

[22] D. Buitrago-Garcia, D. Egli-Gany, M.J. Counotte, S. Hossmann, H. Imeri, A.M. Ipekci, G. Salanti, N. Low, Occurrence and transmission potential of asymptomatic and presymptomatic SARS-CoV-2 infections: a living systematic review and metaanalysis, PLoS Med. 17 (2020) e1003346.

[23] W. Ahmed, M. Kitajima, S. Tandukar, E. Haramoto, Recycled water safety: current status of traditional and emerging viral indicators, Curr. Opin. Environ. Sci. Health 16 (2020) 62–72.

[24] D. Barceló, Wastewater-based epidemiology to monitor COVID-19 outbreak: present and future diagnostic methods to be in your radar, Case Stud. Chem. Environ. Eng. 2 (2020), https://doi.org/10.1016/j.cscee.2020.100042.

[25] D. Barcelo, An environmental and health perspective for COVID-19 outbreak: meteorology and air quality influence, sewage epidemiology indicator, hospitals disinfection, drug therapies and recommendations, J. Environ. Chem. Eng. (2020), https://doi.org/10.1016/j.jece.2020.104006.

[26] E. Lavezzo, E. Franchin, C. Ciavarella, G. Cuomo-Dannenburg, L. Barzon, C. Del Vecchio, L. Rossi, R. Manganelli, A. Loregian, N. Navarin, D. Abate, M. Sciro, S. Merigliano, E. De Canale, M.C. Vanuzzo, V. Besutti, F. Saluzzo, F. Onelia, M. Pacenti, S.G. Parisi, G. Carretta, D. Donato, L. Flor, S. Cocchio, G. Masi, A. Sperduti, L. Cattarino, R. Salvador, M. Nicoletti, F. Caldart, G. Castelli, E. Nieddu, B. Labella,

L. Fava, M. Drigo, K.A.M. Gaythorpe, A.R. Brazzale, S. Toppo, M. Trevisan, V. Baldo, C.A. Donnelly, N.M. Ferguson, I. Dorigatti, A. Crisanti, Suppression of a SARS-CoV-2 outbreak in the Italian municipality of Vo, Nature 584 (2020) 425–429.

[27] S. Balboa, M. Mauricio-Iglesias, S. Rodríguez, L. Martínez-Lamas, F.J. Vasallo, B. Regueiro, J.M. Lema, The fate of SARS-CoV-2 in wastewater treatment plants points out the sludge line as a suitable spot for incidence monitoring, Sci. Total Environ. 772 (2021), https://doi.org/10.1016/j.scitotenv.2021.145268.

[28] M.A. Borchardt, K.R. Bradbury, M.B. Gotkowitz, J.A. Cherry, B.L. Parker, Human enteric viruses in groundwater from a confined bedrock aquifer, Environ. Sci. Technol. 41 (18) (2007) 6606–6612.

[29] E. Haramoto, B. Malla, O. Thakali, M. Kitajima, First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan, Sci. Total. Environ. 737 (2020) 140405.

[30] G. La Rosa, M. Iaconelli, P. Mancini, G.B. Ferraro, C. Veneri, L. Bonadonna, L. Lucentini, E. Suffredini, First detection of SARS-CoV-2 in untreated wastewaters in Italy, Sci. Total Environ. 736 (2020) 139652.

[31] G. Medema, L. Heijnen, G. Elsinga, R. Italiaander, A. Brouwer, Presence of SARS Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands, Environ Sci Technol Lett. 7 (7) (2020) 511–516, https://doi.org/10.1021/acs.estlett.0c00357.

[32] S. Wurtzer, V. Marechal, J.M. Mouchel, Y. Maday, R. Teyssou, E. Richard, J.L. Almayrac, L. Moulin, Evaluation of lockdown impact on SARS-CoV-2 dynamics through viral genome quantification in Paris wastewaters, medRxiv (2020), https://doi.org/10.1101/2020.04.12.20062679.

[33] S. Wurtzer, V. Marechal, J.-M. Mouchel, L. Moulin, Time course quantitative detection of SARS-CoV-2 in Parisian wastewaters correlates with COVID-19 confirmed cases, medRxiv (2020), https://doi.org/10.1101/2020.04.12.20062679.

[34] A. Nemudryi, A. Nemudraia, K. Surya, T. Wiegand, M. Buyukyoruk, R. Wilkinson, B. Wiedenheft, Temporal detection and phylogenetic assessment of SARS-CoV-2 in municipal wastewater, medRxiv (2020).

[35] S.G. Rimoldi, F. Stefani, A. Gigantiello, S. Polesello, F. Comandatore, D. Mileto, M. Maresca, C. Longobardi, A. Mancon, F. Romeri, C. Pagani, L. Moja, M.R. Gismondo, F. Salerno, Presence and vitality of SARS-CoV-2 virus in wastewaters and rivers, Sci. Total Environ. 744 (2020) 140911.

[36] M. Kumar, K. Taki, R. Gahlot, A. Sharma, K. Dhangar, A chronicle of SARS-CoV-2: part-I - epidemiology, diagnosis, prognosis, transmission and treatment, Sci. Total Environ. 734 (2020), https://doi.org/10.1016/j.scitotenv.2020.139278.

[37] Y. Wu, C. Guo, L. Tang, Z. Hong, J. Zhou, X. Dong, H. Yin, Q. Xiao, Y. Tang, X. Qu, L. Kuang, X. Fang, N. Mishra, J. Lu, H. Shan, G. Jiang, X. Huang, Prolonged presence of SARS-CoV-2 viral RNA in faecal samples, Lancet Gastroenterol. Hepatol. 5 (2020) 434–435.

[38] M. Kumar, A.K. Thakur, P. Mazumder, K. Kuroda, S. Mohapatra, J. Rinklebe, A. Ramanathan, Z. Cetecioglu, S. Jain, V.K. Tyagi, P. Gikas, S. Chakraborty, M. Tahmidul Islam, A. Ahmad, A.V. Shah, A.K. Patel, T. Watanabe, M. Vithanage, K. Bibby, M. Kitajima, P. Bhattacharya, Frontier review on the propensity and repercussion of SARS-CoV-2 migration to aquatic environment, J. Hazard. Mater. Lett. 1 (2020), https://doi.org/10.1016/j.hazl.2020.100001.

[39] M. Kumar, M. Joshi, A.K. Patel, C.G. Joshi, Unravelling the early warning capability of wastewater surveillance for COVID-19: a temporal study on SARS-CoV-2 RNA detection and need for the escalation, Environ. Res. 196 (2020) 110946.

[40] S.P. Sherchan, S. Shahin, L.M. Ward, S. Tandukar, T.G. Aw, B. Schmitz, M. Kitajima, First detection of SARS-CoV-2 RNA in wastewater in North America: a study in Louisiana, USA, Sci. Total Environ. 743 (2020), https://doi.org/10.1016/j.scitotenv.2020.140621.

[41] B. Prevost, F.S. Lucas, A. Goncalves, F. Richard, L. Moulin, S. Wurtzer, Large scale survey of enteric viruses in river and waste water underlines the health status of the local population, Environ. Int. 79 (2015), https://doi.org/10.1016/j.envint.2015.03.004.

[42] W. Lodder, A.M. de Roda Husman, SARS-CoV-2 in wastewater: potential health risk, but also data source, Lancet Gastroenterol. Hepatol. (2020), https://doi.org/10.1016/S2468-1253(20)30087-X.

[43] A.J. Prussin, J.A. Belser, W. Bischoff, S.T. Kelley, K. Lin, W.G. Lindsley, ..., L.C. Marr, Viruses in the Built Environment (VIBE) meeting report, 2020.

[44] S. Westhaus, F.A. Weber, S. Schiwy, V. Linnemann, M. Brinkmann, M. Widera, S. Ciesek, Detection of SARS-CoV-2 in raw and treated wastewater in Germany–suitability for COVID-19 surveillance and potential transmission risks, Sci. Total Environ. 751 (2020), https://doi.org/10.1016/j.scitotenv.2020.141750.

[45] S.W. Hasan, Y. Ibrahim, M. Daou, H. Kannout, N. Jan, A. Lopes, A.F. Yousef, Detection and quantification of SARS-CoV-2 RNA in wastewater and treated effluents: surveillance of COVID-19 epidemic in the United Arab Emirates, Sci. Total Environ. 764 (2020), https://doi.org/10.1016/j.scitotenv.2020.142929.

[46] M. Kumar, M. Alamin, K. Kuroda, K. Dhangar, A. Hata, H. Yamaguchi, R. Honda, Potential discharge, attenuation and exposure risk of SARS-CoV-2 in natural water bodies receiving treated wastewater, npj Clean Water 4 (1) (2021) 1–11, https://doi.org/10.1038/s41545-021-00098-2.

[47] E. Haramoto, M. Kitajima, A. Hata, J.R. Torrey, Y. Masago, D. Sano, H. Katayama, A review on recent progress in the detection methods and prevalence of human enteric viruses in water, Water Res. 135 (2018) 168–186.

[48] Y. Jung, G.S. Park, J.H. Moon, K. Ku, S.H. Beak, C.S. Lee, Kim, Seil, E.C. Park, D. Park, J.H. Lee, C.W. Byeon, J.J. Lee, J.S. Maeng, S.J. Kim, Kim, Seung Il, B.T. Kim, M.J. Lee, H.G. Kim, Comparative analysis of primer-probe sets for RT-qPCR of COVID-19 causative virus (SARS-CoV-2), ACS Infect. Dis. 6 (2020) 2513–2523.

[49] S. Zheng, J. Fan, F. Yu, B. Feng, B. Lou, Q. Zou, T. Liang, Viral load dynamics and disease severity in patients infected with SARS-CoV-2 in Zhejiang province, China, January-March 2020: retrospective cohort study, BMJ 369 (2020), https://doi.org/10.1136/bmj.m1443.

[50] K. Kitamura, K. Sadamasu, M. Muramatsu, H. Yoshida, Efficient detection of SARS-CoV-2 RNA in the solid fraction of wastewater, Sci. Total Environ. 763 (2020), https://doi.org/10.1016/j.scitotenv.2020.144587.

[51] A. Hata, H. Hara-Yamamura, Y. Meuchi, S. Imai, R. Honda, Detection of SARS-CoV-2 in wastewater in Japan during a COVID-19 outbreak, Sci. Total Environ. (2020), https://doi.org/10.1016/j.scitotenv.2020.143578.

[52] M.H. Hjelmsø, M. Hellmér, X. Fernandez-Cassi, N. Timoneda, O. Lukjancenko, M. Seidel, et al., Evaluation of methods for the concentration and extraction of viruses from sewage in the context of metagenomic sequencing, PLoS ONE 12 (1) (2017) e0170199, https://doi.org/10.1371/journal.pone.0170199.

[53] F.X. Lescure, L. Bouadma, D. Nguyen, M. Parisey, P.H. Wicky, S. Behillil, A. Gaymard, M. Bouscambert-Duchamp, F. Donati, Q. Le Hingrat, V. Enouf, N. Houhou-Fidouh, M. Valette, A. Mailles, J.C. Lucet, F. Mentre, X. Duval, D. Descamps, D. Malvy, J.F. Timsit, B. Lina, S. van-der-Werf, Y. Yazdanpanah, Clinical and virological data of the first cases of COVID-19 in Europe: a case series, Lancet Infect. Dis. 20 (2020), https://doi.org/10.1016/S1473-3099(20)30200-0.

[54] P.L. McCarty, D.P. Smith, Anaerobic wastewater treatment, Environ. Sci. Technol. 20 (12) (1986) 1200–1206.

[55] Ministry of Health & Family Welfare, Government of India. COVID-19 India update. Available from: https://www.mohfw.gov.in/.

[56] G. Orive, U. Lertxundi, D. Barceló, Do we really need to invoke heroic measures for early SARS-CoV-2 outbreak detection?, Eur. J. Epidemiol (2020), https://doi.org/10.1007/s10654-020-00654-z.

[57] Y. Pan, D. Zhang, P. Yang, L.L.M. Poon, Q. Wang, Viral load of SARS-CoV-2 in clinical samples, Lancet Infect. Dis. (2020), https://doi.org/10.1016/S1473-3099(20)30113-4.

[58] P.R. Scheuerman, S.R. Farrah, G. Bitton, Laboratory studies of virus survival during aerobic and anaerobic digestion of sewage sludge, Water Res. 25 (3) (1991) 241–245.

[59] M.E. Verbyla, J.R. Mihelcic, A review of virus removal in wastewater treatment pond systems, Water Res (2015), https://doi.org/10.1016/j.watres.2014.12.031.

[60] Y. Xu, X. Li, B. Zhu, H. Liang, C. Fang, Y. Gong, Q. Guo, X. Sun, D. Zhao, J. Shen, H. Zhang, H. Liu, H. Xia, J. Tang, K. Zhang, S. Gong, Characteristics of pediatric SARS-CoV-2 infection and potential evidence for persistent fecal viral shedding, Nat. Med. 26 (2020), https://doi.org/10.1038/s41591-020-0817-4.

[61] G. Medema, L. Heijnen, G. Elsinga, R. Italiaander, Presence of SARS-coronavirus- 2 in sewage, EST Lett. (2020), https://doi.org/10.1021/acs.estlett.0c00357.

[62] G. La Rosa, M. Iaconelli, A. Mancini, F. Bonanno, C. Veneri, L. Bonadonn, L. Lucentini, E. Suffredini, First detection of SARS-CoV-2 in untreated wastewaters in Italy, Sci. Total Environ. 736 (2020) 139652, https://doi.org/10.1016/j.scitotenv.2020.139652.

[63] F. Wu, A. Xiao, J. Zhang, X. Gu, W. Lee, K. Kauffman, W. Hanage, M. Matus, N. Ghaeli, N. Endo, C. Duvallet, K. Moniz, T. Erickson, P. Chai, J. Thompson, E. Alm, SARS-CoV-2 titers in wastewater are higher than expected from clinically confirmed cases, medRxiv (2020), https://doi.org/10.1101/2020.04.05.20051540.

[64] S. Wurtzer, V. Marechal, J. Mouchel, L. Moulin, Time course quantitative detection of SARS-CoV-2 in Parisian wastewaters correlates with COVID-19 confirmed cases, medRxiv (2020) 10–13, https://doi.org/10.1101/2020.04.12.20062679.

Journal of Hazardous Materials 416 (2021) 126125

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Antidrug resistance in the Indian ambient waters of Ahmedabad during the COVID-19 pandemic

Manish Kumar [a,*], Kiran Dhangar [a], Alok Kumar Thakur [a], Bhagwana Ram [b], Tushara Chaminda [c], Pradeep Sharma [d], Abhay Kumar [e], Nirav Raval [f], Vaibhav Srivastava [f], Jörg Rinklebe [g,h], Keisuke Kuroda [i], Christian Sonne [j], Damia Barcelo [k,l]

[a] Discipline of Earth Science, Indian Institute of Technology Gandhinagar, Gujarat 382355, India
[b] Department of Civil Engineering, Indian Institute of Technology, Gandhinagar, Gujarat, 382355, India
[c] Department of Civil and Environmental Engineering, University of Ruhuna, Galle, Sri Lanka
[d] Department of Environmental Science, Graphic Era Deemed to be University, Dehradun, Uttarakhand, India
[e] NCERT, Sri Aurobindo Marg, New Delhi- 110016, India
[f] Encore Insoltech Pvt. Ltd., Randesan, Gandhinagar, Gujarat 382007, India
[g] University of Wuppertal, School of Architecture and Civil Engineering, Institute of Foundation Engineering, Water and Waste Management, Laboratory of Soil and Groundwater Management, Pauluskirchstraße 7, 42285 Wuppertal, Germany
[h] Department of Environment, Energy and Geoinformatics, University of Sejong, Seoul, Republic of Korea
[i] Department of Environmental and Civil Engineering, Toyama Prefectural University, Imizu 939-9308, Japan
[j] Aarhus University, Department of Bioscience, Arctic Research Centre (ARC), Frederiksborgvej 399, PO Box 358, DK-4000 Roskilde, Denmark
[k] Institute of Environmental Assessment and Water Research, IDAEA-CSIC, C/ Jordi Girona 18-26, 08034 Barcelona, Spain
[l] Catalan Institute for Water Research, ICRA-CERCA, Emili Grahit 101, 17003, Girona, Spain

ARTICLE INFO

Editor: Andrew Daugulis

Keywords:
Antidrug resistance
SARS-CoV-2
COVID-19
Wastewater based epidemiology

ABSTRACT

The ongoing COVID-19 pandemic increases the consumption of antimicrobial substances (ABS) due to the unavailability of approved vaccine(s). To assess the effect of imprudent consumption of ABS during the COVID-19 pandemic, we compare the 2020 prevalence of antidrug resistance (ADR) of *Escherichia coli* (*E. coli*) with a similar survey carried out in 2018 in Ahmedabad, India using SARS-CoV-2 gene detection as a marker of ABS usage. We found a significant ADR increase in 2020 compared to 2018 in ambient water bodies, harbouring a higher incidence of ADR *E.coli* towards non-fluoroquinolone drugs. Effective SARS-CoV-2 genome copies were found to be associated with the ADR prevalence. The prevalence of ADR depends on the efficiency of WWTPs (Wastewater Treatment Plants) and the catchment area in its vicinity. In the year 2018 study, prevalence of ADR was discretely distributed, and the maximum ADR prevalence recorded was ~ 60%; against the current homogenous ADR increase, and up to 85% of maximum ADR among the incubated *E.coli* isolated from the river (Sabarmati) and lake (Chandola and Kankaria) samples. Furthermore, wastewater treatment plants showed less increase in comparison to the ambient waters, which eventually imply that although SARS-CoV-2 genes and faecal pollution may be diluted in the ambient waters, as indicated by low C_t-value and *E.coli* count, the danger of related aftermath like ADR increase cannot be nullified. Also, Non-fluoroquinolone drugs exhibited overall more resistance than quinolone drugs. Overall, this is probably the first-ever study that traces the COVID-19 pandemic imprints on the prevalence of antidrug resistance (ADR) through wastewater surveillance and hints at monitoring escalation of other environmental health parameters. This study will make the public and policyholders concerned about the optimum use of antibiotics during any kind of treatment.

1. Introduction

The exponential rise in the consumption of antimicrobials in various applications such as medical, veterinary, domestic and agricultural and their leak to aquatic ecosystems has caused the global prevalence of antidrug resistance (ADR), which is being considered a major threat to public health (Rodriguez-Mozaz et al., 2015; Chatterjee et al., 2010; Baker-Austin et al., 2006). The ADR is not only limited to the survival

* Correspondence to: Discipline of Earth Sciences, Indian Institute of Technology Gandhinagar, Room No. 336 A, Block 5, 382355 Gujarat, India.
E-mail addresses: manish.env@gmail.com, manish.kumar@iitgn.ac.in (M. Kumar).

https://doi.org/10.1016/j.jhazmat.2021.126125
Received 27 February 2021; Received in revised form 5 May 2021; Accepted 12 May 2021
Available online 24 May 2021

and infection by any particular type of microorganism, but can lead to life threatening diseases for both animals and human (Singer et al., 2008; Ferreira da Silva et al., 2007; Jiang et al., 2013). Due to lack of regulations on the prescription and non-prescription use of antimicrobials, its consumption rate in, for example, India has been increased by 105% from 2000 to 2015 while worldwide it is estimated to increase by 63% during 2010–2030 (Klein et al., 2018; Global Antibiotic Resistance Partnership GARP-India Working Group, 2011; Van Boeckel et al., 2015). On top of that, the rate of consumption of certain antimicrobials has escalated during the COVID-19 pandemic in an effort to minimise the risk of severe infections and mortality (Miranda et al., 2020; Liu et al., 2020). Around 70% of COVID-19 patients have received antimicrobial treatment along with overuse of various antibiotics despite only 10% on average show microbial infections (Hsu, 2020; Rawson et al., 2020). As most of the consumed drugs and their metabolites are excreted through urine and faeces, their discharge to aquatic environments depends on the removal efficiency of the WWTPs (Singer et al., 2008; Azuma et al., 2012; Takanami et al., 2010; Auerbach et al., 2007; Kumar et al., 2020a). If the WWTP clearing rate is low, microorganisms exposed to antimicrobials and metabolites develops mutations causing ADR (Aali et al. 2014, Alexander et al. 2020, Guo et al. 2018, Kumar et al., 2020a, 2020c) Thus, the increased use of antimicrobials in the current pandemic will probably pose an increased risk in terms of ADR during post COVID-19 as concerned by a number of recent studies (Kuroda et al., 2021; Lucien et al., 2021; Hsu, 2020; Kumar et al., 2020a; Asaduzzaman et al., 2020).

The high consumption of antimicrobials causes an increase in the prevalence of ADR in several environmental compartments including drinking, waste and groundwater, sludge, sediments and municipal solid waste leachate (Al-Judaibi, 2014; Ferreira da Silva et al., 2007; Kumar et al., 2020d; 2020e; Ram and Kumar, 2020; Zhang et al., 2015; Storteboom et al., 2010; Threedeach et al., 2012). In the case of for example *E.coli* isolates from the effluent of WWTPs have shown a higher prevalence of antidrug resistance as compared to the influent, which is probably due to poor treatment conditions, prolonged microbial activities, and chemical properties of the antimicrobial drugs (Reinthaler et al., 2003; Silva et al., 2006; Miranda and Castillo, 1998; Marcinek et al., 1998). Specifically, the conventional treatment processes at WWTPs do not completely mineralise the parent antimicrobial drugs, and generate some residues, metabolites or transformation products that may have the same biological activity as the parent drugs (Zhang et al., 2015; Kumar et al., 2020c). Thus, WWTPs are considered hotspots for the spreading ADR due to high microbial density, horizontal gene transfer (HGT), nutritional richness and the availability of antimicrobial metabolites (Zhang et al., 2015; Threedeach et al., 2012; Silva et al., 2006). Previous studies have reported a correlation between the prevalence of ADR and inefficiently treated wastewater discharge, having the abundance of *E. coli*, extravasating to river and lake waters (Na et al., 2018; Yang et al., 2017; Honda et al., 2016, 2018; Biswas et al., 2015; Akhter et al., 2014; Ram and Kumar, 2020; Kumar et al., 2020d; 2020e). Thus, a better understanding of the occurrence, distribution and frequency of antidrug resistance in the urban waters is needed to prevent or slower the rate of increase in ADR.

With the same purpose, presumptive actions are needed to study the prevalence of the ADR during wastewater treatment and the water bodies receiving the WWTP effluents. Wastewater based epidemiology (WBE) is an efficient way to trace the prevalence of ADR in highly COVID -19 infected areas, which are potentially major zones of high consumption of drugs, can be identified with the help of the WBE approach for tracing the SARS-CoV-2 genome concentration in wastewaters (Kumar et al., 2020b). Also, with the help of authorised software and apps (for example: Arogya-Setu app in India), the infected population within a certain region can be predicted. Identifying the WWTPs in such infected areas aids in correlating ADR with the elevated cases of COVID-19. Therefore, the impact of such highly contaminated zones on the prevalence of ADR in wastewaters needs to be studied well.

ADR is not included in the water quality standards and guidelines of India mostly due to the lack of proper treatment facilities in many cities where domestic wastewater is directly discharged to aquatic environments (IS10500, 2012). In this study, we select the Ahmedabad City of Gujarat Province in western India with a population of 5.6 million (2011 Census) to assess the prevalence of ADR in WWTP, lake and river locations within various zones of the city. The specific objectives of the present study are: i) to compare and discuss the prevalence of *E. coli* in the surface water and wastewater in Ahmedabad in order to have a prior knowledge of ADR pervasiveness in different compartments, ii) to analyse a comparative status of the antidrug resistance in the *E. coli* isolated from the urban waters of the city and iii) to further understand the imprints of COVID-19 situation on the status of SARS-CoV-2 genome concentration and ADR prevalence at various zones of the city.

2. Material and methods

2.1. Sample collection and ADR analyses

The water samples were collected from 6 different locations of Ahmedabad city on 23rd June 2018, and 16th October 2020 (Fig. 1). Two locations on the stretch of Sabarmati river: Nehru Bridge (NB) and Sardar Bridge (SB); two lakes: Kankariya Lake (KL) and Chandola Lake (CL), and two WWTP locations: Chandkheda (inlet: CI and outlet: CO) and Vasna, also known as Juhapura (inlet: VI and outlet: VO), selected to assess ADR. For SARS-CoV-2 gene detection, a total of 10 locations were selected to represent various zones of the city that comprises all ADR sampling locations. We kept ADR locations low to match the number of locations tested in 2018 (Ram and Kumar, 2020). The geographical details about the selected locations are well described in our previous study by Ram and Kumar (2020) (See Supplementary Information). Sterile bottles (Tarson-546041) of medical grade were used to collect the samples, which were then kept in iceboxes until arrival at the laboratory. For on-site measurement of pH, EC, ORP, TDS and salinity, a multi-parameter probe, HANNA HI9828 was used. The procedure for testing the isolation of *E. coli* for ADR is likewise described in Ram and Kumar (2020) (See Supplementary information). Briefly, the water samples were filtered through membranes with 0.45-μm-pore size, and *E. coli* trapped by the membranes were incubated on Chromocult® Coliform Agar ES (Merck Microbiology, Darmstadt, Germany). Each *E. coli* isolate was tested for susceptibility to six antibiotics (kanamycin, KM; tetracycline, TC; norfloxacin, NFX; ciprofloxacin, CIP; levofloxacin, LVX; and sulfamethoxazole, ST) by Kirby-Bauer method using PERL-CORE® Sensitivity Test (ST) Agar (EIKEN Chemical Co., Ltd, Tokyo).

2.2. SARS-CoV-2 RNA detection

The SARS-CoV-2 RNAs were isolated and detected from 30 mL wastewater samples that were centrifuged at 4000g for 40 min, followed by filtration of supernatant using 0.22-micron syringe filter (Mixed cellulose esters syringe filter, Himedia). After filtration, 25 mL of the supernatant was treated with polyethylene glycol and NaCl at 80 g/L and 17.5 g/L respectively, and incubated at 17 °C, 100 rpm overnight. The mixture was centrifuged for 90 min at 14000g and the supernatant was discarded to collect a pellet containing viruses and their fragmented genes. The pellet was re-suspended in 300 μl RNase-free water and kept in 1.5 mL vials at − 40 °C, until further analyses.

RNA isolation from the pellet with the concentrated virus was performed using NucleoSpin® RNA Virus isolation kit (Macherey-Nagel GmbH & Co. KG, Germany). The samples were spiked with MS2 phage as an internal control prior to the RNA extraction provided by TaqPathTM Covid-19 RT-PCR Kit. The nucleic acid was extracted and a Qubit 4 Fluorometer (Invitrogen) was used for RNA concentrations estimation. The molecular process inhibition control was evaluated through the MS2 phage for QA/QC analyses of nucleic acid extraction and PCR inhibition (Haramoto et al., 2018). We have described the methodologies in Kumar

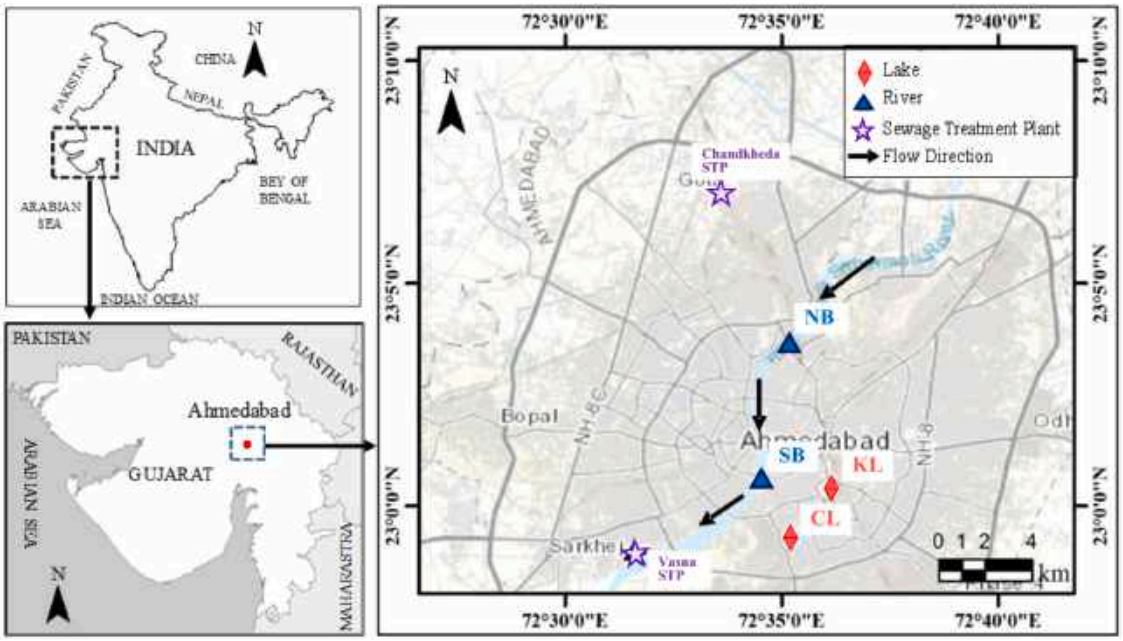


Fig. 1. Map showing the sampling locations in Ahmedabad, Gujarat (i) locations at Sabarmati River (Nehru Bridge: NB; Sardar Bridge: SB), (ii) two lakes (Kankaria Lake: KL; Chandola Lake: CL) and (iii) two different Sewage Treatment Plants (STPs) (Chandkheda STP; Vasna STP).

et al. (2021, 2020b).

Briefly, steps were carried out as per the guideline provided with the product manual of Macherey-Nagel GmbH & Co. KG and RNAs were detected using real-time PCR (RT-PCR). An Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument (version 2.19 software) was used for SARS-CoV-2 gene detection. A template of 7 µl of extracted RNA was used in each reaction with TaqPath™ 1-Step Multiplex Master Mix (Thermofischer Scientific, USA). Three controls were included: positive control (TaqPath™ COVID-19 Control); negative control (from extraction run spiked with MS2); and a no template control (NTC). Finally, results were interpreted using Applied Biosystems Interpretive Software, and Ct values for three target genes, i.e., ORF1ab, N Protein, and S Protein of SARS-CoV-2, were detected along with MS2 as an internal control.

The samples were considered as positive if at least two genes showed amplification. The average Ct-value of a given sample was then converted to gene copy numbers considering the equivalence of 500 copies of SARS-CoV-2 genes as 26 Ct-value (provided with the kit), and the same was extrapolated to derive approximate copies of each gene. The average effective genome concentration present in a given sample was calculated by multiplying the RNA amount used as a template with the enrichment factor for each sample.

2.3. Quality assurance/quality control (QA/QC) and statistical analysis

To determine the contamination occurred during transport, blanks in the same type of bottle were analysed prior to sampling. Duplicate analysis of samples was conducted to check accuracy and precision. To ensure instrument sensitivity and check cross-contamination, blanks were run for each batch of five samples. Signals were considered significant if the signal-to noise ratio was more than three. The limit of quantification (LOQ) of the overall method was defined as sample concentration equivalent to 1 copy per reaction tube, which was 1.7×10^2 copies/L. We have calculated the gene copy numbers based on the positive control provided with kit i.e., 10^4 copies/µl and the final concentration of 25 copies per reaction. Based on our experience, the same positive control is providing the same Ct values for all 3 genes analysed in this study. Hence, it is evident that primer efficiency is more or less same. Relative to the Ct values of genes of positive controls, copy numbers have been calculated in test samples of different sources.

ADR analyses were carried out in triplicate for the accuracy and precision of the data generated. Tests were repeated if the standard deviation between the triplicate was higher than 10%. Statistical analysis by Student *t*-test was done to compare the antidrug resistance caused by all six antibiotics in year 2018 and 2020, and the results were represented by Pearson's correlation coefficient (p), whose value ranges between zero to unity. The change in percentage resistance of more than 90% ($p = 0.10$) was considered significant.

3. Results and discussions

3.1. Comparison of prevalence of *E. coli*

The prevalence of *E. coli* and environmental parameters is summarised in Table 1. In 2018, the *E. coli* count was highest in river sampling locations, with maximum count of 76,600 cfu (colonies forming unit) mL^{-1}, which was the highest among the lake and WWTP locations except for the Vasana STP. This critically high prevalence is due to the river-human interactions at the riverfront, wastewater discharge or the stagnant flow conditions near the sampling locations (Pormohammad et al., 2019). This reported prevalence in the Sabarmati River was higher than the reported prevalence in rivers of tropical countries like India and Thailand (Chatterjee et al., 2010; Kumar and Sharma, 2014; Honda et al., 2016, 2018; Hamner et al., 2007; Hu et al., 2008). The higher recreational activities at KL location as compared to the CL location are the main cause of higher *E. coli* prevalence at KL (15,600 cfu mL^{-1}) than CL (3467 cfu mL^{-1}) (Kumar and Sharma, 2014; Ram and Kumar, 2020). The varying *E. coli* prevalence at STP locations (inlet and outlet) in 2018 indicates the varying amount of incoming faecal contamination and reduction ratios in the STP.

In the year 2020, the *E. coli* prevalence at STP locations was higher than in 2018 samples ranging from 950,000 to 400,000 cfu mL^{-1} at inlet locations and 19,500–32,500 cfu mL^{-1} at outlet locations. This is attributed to the increased domestic wastewater discharge from the Covid-19 lockdown which also increased the burden on municipal WWTPs resulting in less removal of *E. coli* in WWTPs. It is worth noting that, this critical *E. coli* prevalence alarms the municipal authorities to advance the disinfection processes in WWTPs, and therefore potential

Table 1
Sampling locations along with *in-situ* water quality (pH, EC, TDS, ORP and salinity) and prevalence of *E. coli* in 2018 and 2020.

Sampling Location	Year	pH	EC	TDS	ORP	Salinity	*E. coli*
Nehru Bridge (NB)	2018	8.4	1320	1090	-16	691	24,267
	2020	7.67	554	343	123.5	0.25	1400
Sardar Bridge (SB)	2018	8.00	1541	1100	2	691	76,600
	2020	7.30	533	352	115.7	0.27	5200
Kankaria Lake (KL)	2018	8.70	3015	2050	13	1350	15,333
	2020	8.58	5934	3323	30.9	2.71	13,100
Chandola Lake (CL)	2018	8.10	3240	2300	29	1510	3467
	2020	7.86	1014	590	43	0.44	ND
Chandkheda Inlet (CI)	2018	6.70	2100	1480	-274	972	4220
	2020	6.85	3745	2324	-238.6	1.87	950,000
Chandkheda Outlet (CO)	2018	7.30	1620	1400	-57	911	2893
	2020	7.52	3624	2249	118.6	1.81	32,500
Vasna Inlet (VI)	2018	6.60	1500	1060	-117	674	96,393
	2020	6.97	3254	2017	-231.7	1.61	4,000,000
Vasna Outlet (VO)	2018	6.90	1506	1070	-193	670	9467
	2020	7.34	2767	1715	90.3	1.36	19,500
ND: Not Detected	Unit	–	$\mu S\ cm^{-1}$	$mg\ L^{-1}$	mV	ppt	$cfu\ mL^{-1}$

human health effects could be reduced (Pormohammad et al., 2019). Whereas, the reduced *E. coli* prevalence in the Sabarmati river, can be attributed to the improved water quality and attenuation capacity of the river due to less human and industrial interaction. Another reason can be the dilution level of the samples collected from the river than that of the lake and WWTPs (Pormohammad et al., 2019).

3.2. Mechanism and pathways of antibiotic resistance

Though antimicrobials and antibiotics are among the essential medical interventions, increased antimicrobial resistance threatens the success of patient treatment. Antibiotic resistance has been listed as one of the three major threats to the public health in 21st century by the world health organisation (WHO) (World Health Organization, 2014). Thus, to understand and reduce the consequences of antibiotic resistance, we need to understand its mechanism. Antimicrobial resistance is expected to be the result of the environmental interactions of several organisms. As most antimicrobials consists of naturally produced compounds in nature, many of the bacteria have overcoming molecular mechanism to overcome the drugs thereby being intrinsically resistant to antimicrobials (Blair et al., 2015; Munita and Arias, 2016). However, we are here dealing with the acquired resistance by the bacteria which were originally susceptible to the particular antimicrobial.

Summarising the molecular and biochemical mechanisms of antibiotic resistance is shown in Fig. 2 (Munita and Arias, 2016). These mechanisms of antidrug are generally categorised based on genetic and mechanistic basis. In a genetic basis, antidrug resistance can be developed due to mutational resistance, horizontal and vertical gene transfer (HGT and VGT). Whereas, in a mechanistic basis, antidrug resistance can be developed due to changes in the target site, modifications of antibiotic molecule, and decreased antibiotic penetration and efflux. Fig. 2 also shows that how COVID-19 spread may impact the development of antidrug resistance. The increased pharmaceutical pollution during COVID-19 spread can increase environmental stress on bacteria or

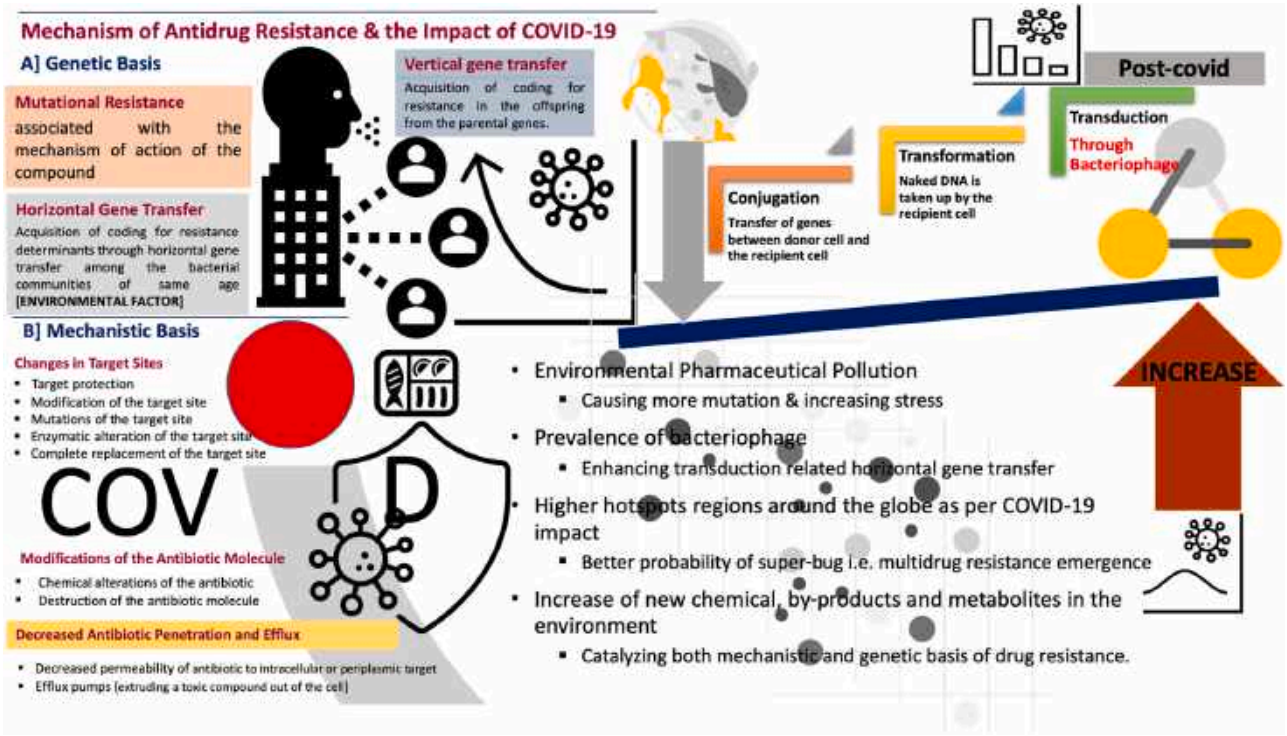


Fig. 2. Mechanism of Antidrug Resistance and the impact of COVID-19: Probable changes in molecular and biochemical triggers of an antidrug resistance.

microbes causing more mutation. This catalyses both the genetic and mechanistic basis of drug resistance. Also, the higher prevalence of bacteriophage may enhance the transduction related to HGT. Thus, the highly infected regions or hotspots of COVID-19 spread around the globe have a greater probability of the emergence of super bugs having multidrug resistance. Drugs like Remdesivir, Ivermectin, Azithromycin, Favipiravir, Chloroquine, Umiferovir, Ritonavir, Aspirin, and Hydroxycholoroquinine are going to remain under the scanner.

3.3. Comparison of occurrence of ADR

Fig. 3 and Fig. 4 represents the comparative sensitivity of *E.coli* towards six antibiotics including the fluoroquinolone drugs NFX (norfloxacin), CIP (ciprofloxacin), LVX (levofloxacin) as well as TCE (tetracycline drugs), KM (kanamycin monosulphate), and ST (sulfamethoxazole), at various sampling locations (CI, VI, CO, VO, NB, SB, CL, and KL) in 2018 and 2020. In 2018, the river location NB had 0% resistance for all antibiotics, whereas SB location had 40% resistance towards all antibiotics except 60% resistance for KM. SB is the central urban location. This indicates that the ADR on the urbanisation and the discharge conditions. However, in 2020, this resistance increased at both river locations for all antibiotics, except for KM at SB. For all Quinolone drugs, the antidrug resistance increased to 50% at both river locations in 2020, whereas it was varying for TCE, KM and ST. At location NB, resistance was observed to be increased for TCE, KM and ST. Whereas, at location SB, resistance increased for TCE, ST, but decreased for KM. This indicates inflow or generation of antidrug resistant *E.coli* in the river water from urbanised sources which reflect increased use of antimicrobials, due to the unavailability of COVID-19 specific drugs (Abelenda-Alonso et al., 2020; Getahun et al., 2020; Hsu, 2020). Though the prevalence of *E. coli* was highest in 2018, more antidrug resistant *E.coli* are generated in the year 2020 due to heavy usage of antimicrobials.

In 2018, no ADR was observed for any of the antibiotics at location CL and KL, except for NFX, TCE and ST at location KL. (Fig. 3 and Fig. 4). However, significant resistance was observed for all antibiotics, except KM, at both lake locations with higher values at CL than KL. This indicates more urbanised discharge carrying antidrug resistant *E.coli* accumulates at the location CL. One of the major reasons for the generated resistance at CL is the occasional discharge to the CL from nearby open Pirana solid waste dumping site (Singh et al. 2008). This call for a monitoring of urban wastewater flows being discharged to the lake ecosystem.

Among the sampled WWTP locations in the year 2018, at locations VI and VO, no resistance was observed for any of the antibiotics except TCE (20% in influent) (Fig. 3 and Fig. 4). Whereas, at CI location resistance for NFX, LVX, TCE, KM, was observed but only found to be increasing towards CIP and KM at location CO. These results show the increase in antidrug resistance after WWTP treatment, which was consistent as reported in the studies from Sweden and Austria (Reinthaler et al., 2003;

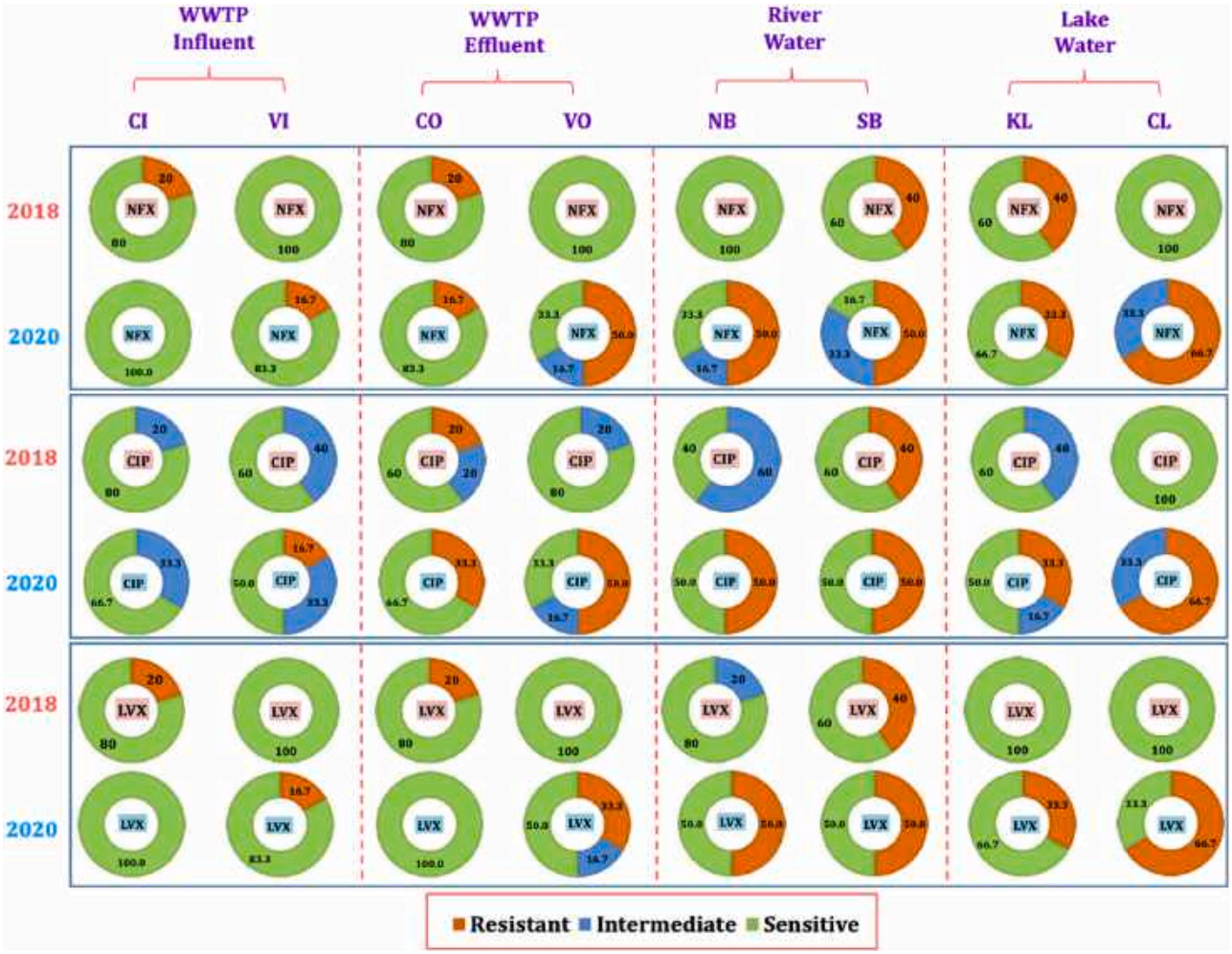


Fig. 3. Percentage of antibiotic resistance in the influents of different water compartments in years 2018 and 2020 against fluroquinolone drugs i.e. NFX (Norfloxacin), CIP (Ciprofloxacin), LVX (Levofloxacin) for locations including WWTPs CI (Chandkheda Inlet), CO (Chandkheda Outlet), VI (Vasna Inlet) and VO (Vasna Outlet); Rivers, NB (Nehru Bridge) and SB (Sardar Bridge), and Lakes, KL (Kankaria Lake) and CL (Chandola Lake).

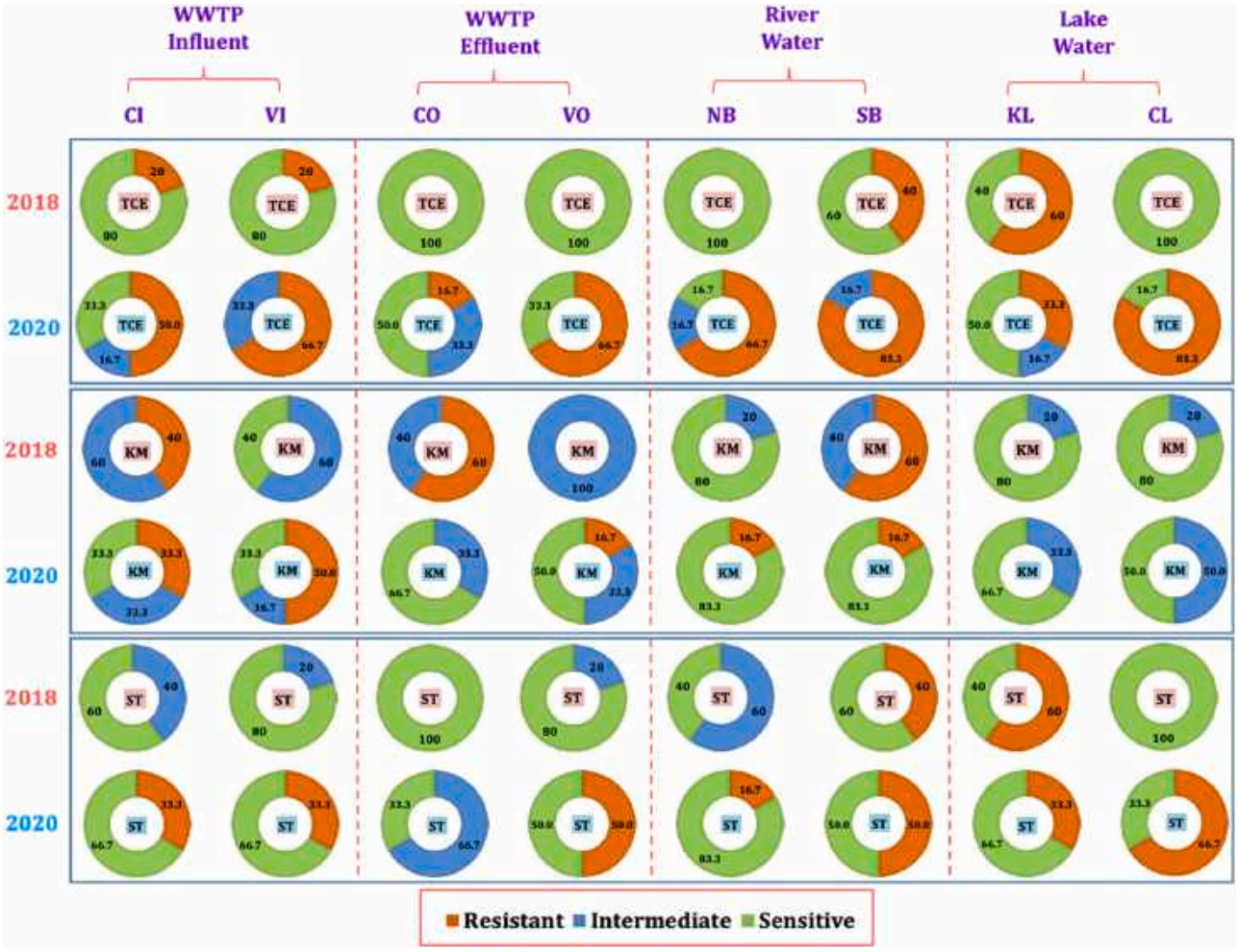


Fig. 4. Percentage of antibiotic resistance in the influents of different water compartments in years 2018 and 2020 against tetracycline drugs (TCE), aminoglycosides i.e. KM (kanamycin), and others i.e. ST (sulfamethoxazole) for locations including WWTPs CI (Chandkheda Inlet), CO (Chandkheda Outlet), VI (Vasna Inlet) and VO (Vasna Outlet); Rivers, NB (Nehru Bridge) and SB (Sardar Bridge), and Lakes KL (Kankaria Lake) and CL (Chandola Lake).

Flach et al., 2018). Interestingly, ADR increased significantly for all antibiotics in the year 2020 at the VI and VO locations when compared to year 2018. In the year 2020, ADR was observed for all antibiotics at VI and these resistances were observed to be increasing or being constant at VO locations for all antibiotics except KM (decreased by 35%) (Figs. 3 and 4). Such a high increase in the resistance in treated effluent can be attributed to a long residence time of wastewater in WWTP, where *E.coli* is in contact with the antibiotics or antibiotic residues for a long time (Honda et al., 2018). In the case of CI in the year 2020, no resistance was observed towards the quinolone drugs, whereas the observed ADR for KM, ST, and TCE, was reduced significantly at CO location. However, resistance was observed to be generated for NFX and CIP at CO in year 2020. The high resistance towards quinolone drugs is attributed to the discharge having domestic origin (Threedeach et al., 2012; Auerbach et al., 2007); because these drugs are prescribed for treatments of respiratory and urinary tract infections, their use has increased significantly during the COVID-19 pandemic (Abelenda-Alonso et al., 2020; Getahun et al., 2020; Hsu, 2020).

Overall, domestic municipal wastewater likely possesses higher concentrations of antimicrobials than any other ambient water. Aeration enhances the generation and replication of antidrug resistant *E.coli* if there is a high density and diversity of the microbial population in a given wastewater (Ram and Kumar, 2020; Kumar et al., 2020f). The advanced or hybrid wastewater treatment processes should be adopted to effectively remove the antimicrobials and their residue in order to reduce the possibility of resistance (Dhangar and Kumar, 2020). Treatment technologies such as MBR-NF/UF, MBR-UV oxidation, AS-gamma radiation was found to be very effective (removal efficiency: ~ 90–100%) for most of the antibiotics and other pharmaceuticals (Dhangar and Kumar, 2020).

The abundance in both antidrug resistance and *E. coli* count in the STPs was found to be statically related. Previously, in case of the Zenne river of Belgium, the abundance of *E. coli* and antidrug resistance increased from upstream to downstream after merging the effluent from Brussel's WWTP (Proia et al., 2018). Thus, proper and timely monitoring should be done to track such load of *E. coli* and ADR while discharging the treated effluents to the river water. From the current study, it is seen that the antidrug resistance to NFX, CIP, LVX, TCE and ST is found at most sampling locations. Such ADR generated during COVID_19 requires rigorous monitoring at local and international level through wastewater based epidemiology (Kumar et al., 2020b). However, the lack of sanitation and treatment facilities in the undeveloped and developing countries is a big challenge to monitor the spread of ADR in the environmental waters (Pormohammad et al., 2019). Perhaps the current pandemic may accelerate the upgradation of the current status of WWTP processes to tackle the pharmaceuticals and other antimicrobials successfully and to monitor ADR (Kumar et al., 2020a).

Fig. 5 highlights the statistical comparison of overall ADR in the year 2018 and 2020, whose causes are well described above. It is clearly seen that the mean percentage value of overall ADR was increased for the resistant strains of *E. coli* in the year 2020 than 2018, except in the case of kanamycin (remains nearly same). Whereas, the mean percentage

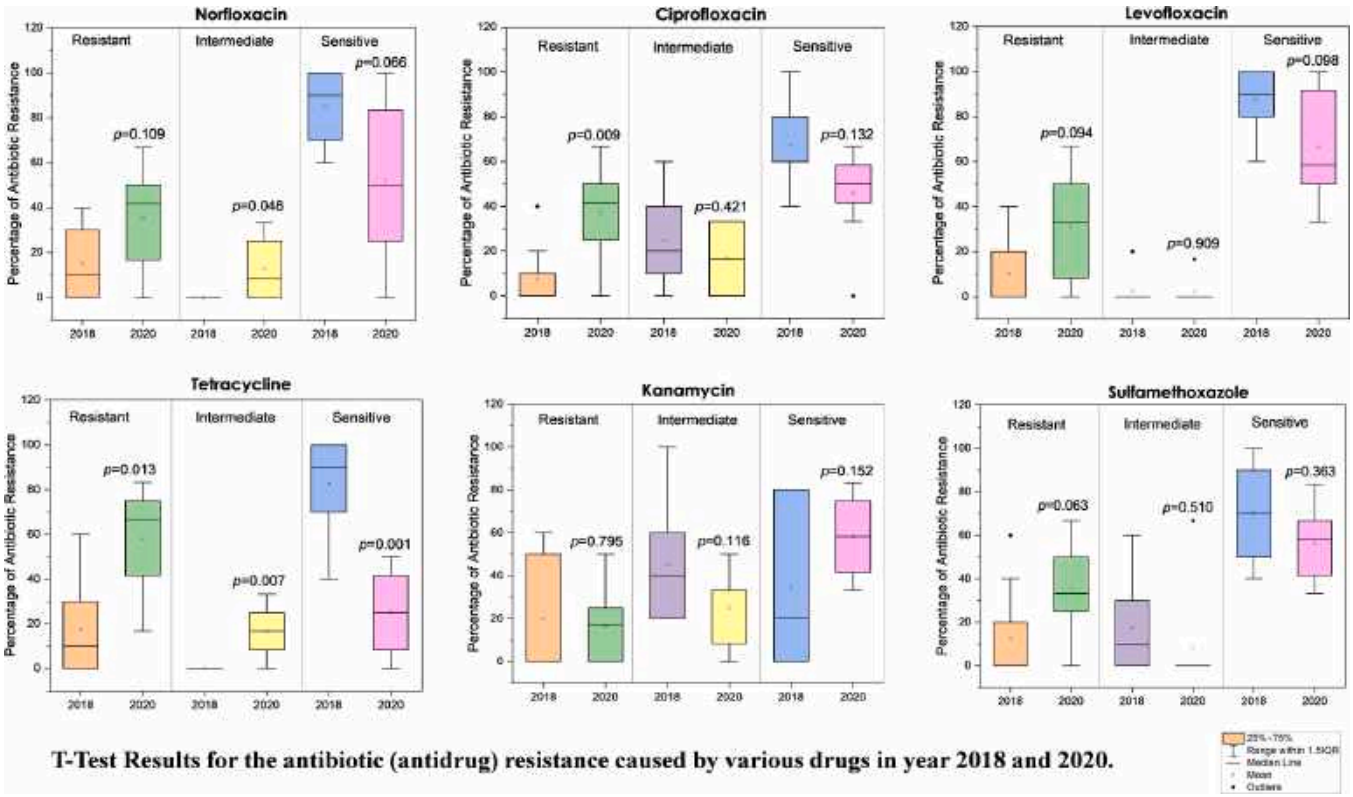


Fig. 5. Comparison of antibiotic (antidrug) resistance against various antibiotics in 2018 and 2020 with the results of a statistical T-test.

value of overall ADR observed to be decreasing for the sensitive strains of *E. coli* in the year 2020 than 2018, except in case of kanamycin (increases). The percentage of ADR (in resistant *E.coli* strains) for almost all antibiotics: CIP, LVX, TC, KM, ST (except NFX: 89.1% change), was observed to be very significant in the year 2020 than 2018, as $p < 0.10$. This indicates that the significant change is occurring due to increase in the mean value of percentage of ADR. Overall, the comparison of overall ADR shows a significant increase statistically in the year 2020 than

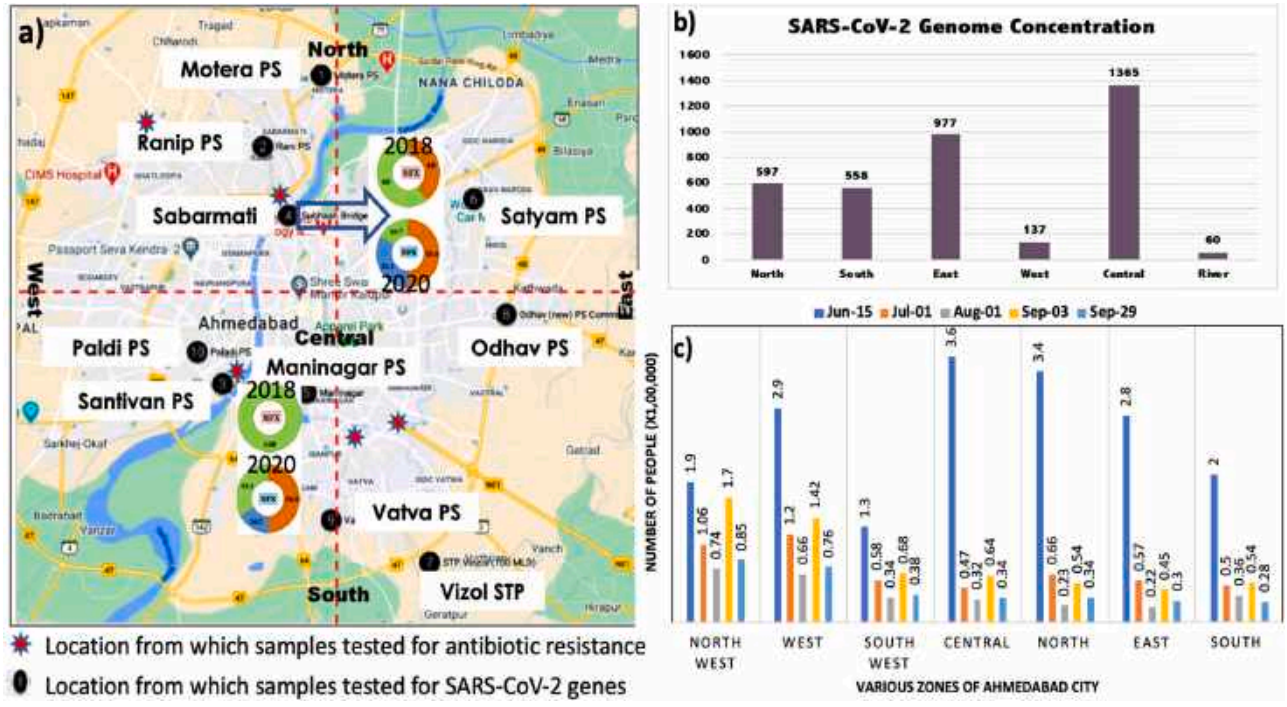


Fig. 6. Illustration depicting: a) zonation of Ahmedabad along with the sampling locations for SARS-CoV-2 RNA analyses, and antibiotic resistance bacteria (ARB) analyses. Two locations of the rivers i.e. SB and NB are shown with their increased resistance against norfloxacin between 2018 and 2020; B) Zone-wise scenario of effective SARS-CoV-2 genome concentrations (copies/L) measured in the samples and C) Number of people under threat of COVID-19 infection as predicted by Aarogya-setu application based on active cases reported and population density of a given area.

2018.

3.4. Imprints of COVID-19 spread over ADR distribution

The increased cases of COVID-19 is not surprisingly correlated to SARS-CoV-2 genes in waste and natural waters (Medema et al., 2020; Ahmed et al., 2020; Haramoto et al., 2020; La Rosa et al., 2020; Sherchan et al., 2020; Kumar et al., 2020; Nemudryi et al., 2020; Kocamemi et al., 2020). Fig. 6 represents the population under threat of COVID-19 in Ahmedabad city (as predicted by nationally authorised Arogya-Setu app), Zone-wise scenario of effective SARS-CoV-2 genome concentration (copies/L) in Ahmedabad city, sampling locations for SARS-CoV-2 RNA analyses, and ADR analyses. The population under threat of COVID-19 in various zones of the city has been predicted by Arogya-Setu app based on the confirmed cases and the population of the respective zone. Arogya-Setu is an authorised Indian COVID-19 contact tracing, syndromic mapping and self-assessment digital service provided under the Ministry of Electronics and Information Technology (MeitY), India. The sampling locations were chosen so as to cover various parts of the city. Two locations of the river i.e., SB and NB are shown with their resistance increase for Norfloxacin between 2018 and 2020. These two locations fall in the central zone of the city, which was highly affected by COVID-19, as can be seen from Fig. 6a and b. Table 2 summarises the status of the SARS-CoV-2 gene along copies with their corresponding Ct-values in the water samples collected from various parts of Ahmedabad, Gujarat on 15th October 2020. It also provides the effective genome concentration for the sampled locations. The genome concentrations were observed to be high in central, east, south and north zones of the city, which can be observed at Maninagar (1365 copies/L), Odhav PS (1070 copies/L), Satyam PS (885 copies/L), Vinzole STP (815 copies/L), and Ranip PS (714 copies/L). The sampled river location and the lake locations encompass in the same zones of the city. The high SARS-CoV-2 genome copies in these zones hint at the potential high prescription of antimicrobial drugs as a remedy to the symptoms of COVID-19. This can be the probable reason for a significant increase in ADR towards most of the drugs tested at the sampling locations in these zones. This indicates that the highly infected zones of the city, due to excessive consumption of antimicrobial drugs, have significantly impacted the antidrug resistance generated in the microorganisms. Overall, the spread of COVID-19 in the community has a prodigious correlation with the effective genome concentration of SARS-CoV-2 and with the prevalence of ADR in environmental waters.

4. Limitations

The present study compared the anti-drug resistance in *E.coli* in 2018 and 2020 with the latter prevalence of SARS-CoV-2 genes during the sampling period. Despite the correlations between increased ADR and COVID-19 spread, more future studies with rigorous sampling events are needed to conclude about the cause and effects. In addition, the concentration of pharmaceutical and personal care products (PPCPs) in the ambient environment should be monitored to quantify their increase owing to COVID-19; and then connect back to the corresponding effect on ADR for quantitative evaluation. In this study, we attempted to start a timely discussion about the likely relationships between ADR and COVID-19 spread throughout the globe. Our approach to analyse the ADR prevalence is mostly qualitative and there may be a slight possibility of both false positive and negative results. To obtain the conclusive evidence, the quantification of genetic markers for antimicrobial resistance will be helpful. In addition, one time point data may be argued -inadequate to derive a conclusion especially when samples used for ADR and SARS-CoV-2 genomes studies do not match. Hence we recommend regular monitoring along the consideration of wasetwater flow data for presenting gene flux or *E. coli* flux.

5. Conclusion

Non-fluoroquinolone drugs showed overall more resistance as compared to fluoroquinolone drugs. Tetracycline followed by norfloxacin has shown more resistance as compared to the other drugs. Despite a decrease in the prevalence of *E. coli* on the sampled river locations, the percentage resistance had been significantly increased in the year 2020 compared to year 2018. However, the *E. coli* prevalence in STP samples was increased in the order of $10^{2,}$ but the pattern of antidrug resistance was not consistent. Lake locations also exhibited an increase in the antidrug resistance during the duration of pandemic. The river locations and the lake locations have shown a significant increase in the antidrug resistance, and these locations are from the highly COVID-19 infected zones of the city. The COVID-19 spread in various zones of the city has shown corresponding changes in the SARS-CoV-2 genome concentration and ADR in environmental waters. Overall, due to increased consumption of antimicrobials in the pandemic period, the percentage of antidrug resistance has been increased significantly. Wastewater based epidemiology can be the key tool to monitor the antimicrobials prevalence and antidrug resistance in the pandemic situations.

Notes

The authors declare no competing financial interest.

CRediT authorship contribution statement

Manish Kumar: Conceptualization, Visualization, Project supervision, Writing - review & editing; **Kiran Dhangar:** Data curation, First draft, Writing - review & editing; **Alok Kumar Thakur:** Sampling and analyses in 2020, Data curation, First draft, Writing - review & editing; **Bhagwana Ram:** Sampling and analyses in 2018, Writing - review & editing; **Tushara Chaminda:** Writing - review & editing; **Pradeep Sharma:** Writing - review & editing; **Abhay Kumar:** Writing - review &

Table 2
SARS-CoV-2 Ct-values along with their corresponding gene copies in the water samples collected from various parts of Ahmedabad, Gujarat on 15th October 2020. Effective genome concentrations have also been provided in the last column.

Sampling Station	Ct values			Gene copies/ L			
	N	ORF	S	N	ORF	S	Effective gene concentration
Motera PS	35.50	32.18	33.96	123	1002	317	480
Ranip PS	34.57	31.75	32.98	217	1334	591	714
Paldi PS	38.36	36.47	36.53	23	69	66	53
Santivan PS	36.08	33.63	34.80	87	390	187	221
Sanbarmati	38.46	35.67	37.14	22	110	47	60
Maninagar	34.17	30.77	31.89	278	2605	1213	1365
Satyam PS	34.52	31.37	32.70	223	1724	709	885
Vinzole STP	34.98	31.41	32.96	168	1680	598	815
Odhav PS	34.54	31.06	32.41	220	2131	857	1070
Vatva PS	38.51	32.58	35.69	22	770	109	300

editing; **Nirav Raval:** Writing - review & editing; **Vaibhav Srivastava:** Sampling and analyses, Data curation, First draft, Writing - review & editing; **Jörg Rinklebe:** Writing - review & editing; **Keisuke Kuroda:** Writing - review & editing; **Christian Sonne:** Writing - review & editing; **Damia Barcelo:** Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work is funded by Kiran C Patel Centre for Sustainable Development at IIT Gandhinagar, UNICEF, Gujarat and UK-India Education and Research Initiative (UKIERI). We also acknowledge the help received from Dr. Shyamnarayan Dave, Dr. Madhvi Joshi, Dr. Arbind K Patel, and other GBRC and WET Lab members who contributed towards sample and data analyses.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.126125.

References

Aali, R., Nikaeen, M., Khanahmad, H., Hassanzadeh, A., 2014. Monitoring and comparison of antibiotic resistant bacteria and their resistance genes in municipal and hospital wastewaters. Int. J. Prev. Med. 5 (7), 887–894.
Abelenda-Alonso, G., Padullés, A., Rombauts, A., Gudiol, C., Pujol, M., Alvarez-Pouso, C., Jodar, R., Carratalà, J., 2020. Antibiotic prescription during the COVID-19 pandemic: a biphasic pattern. Infect. Control Hosp. Epidemiol. 41 (11), 1371–1372.
Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J., Tscharke, B., 2020. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community. Sci. Total Environ. 728, 138764.
Akhter, A., Imran, M., Akhter, F., 2014. Antimicrobial resistant coliform bacteria in the Gomti river water and determination of their tolerance level. Bioinformation 10 (4), 167–174.
Al-Judaibi, E., 2014. Infection and antibiotic resistant bacteria in developing countries: a genetic review. J. Microbiol. Res. 4 (6A), 10–17.
Alexander, J., Hembach, N., Schwartz, T., 2020. Evaluation of antibiotic resistance dissemination by wastewater treatment plant effluents with different catchment areas in Germany. Sci. Rep. 10 (1), 1–9.
Asaduzzaman, M., Zaman, F., Rousham, E., 2020. Antibiotic consumption may be linked to exaggeration of COVID-19. Med. Hypotheses 143, 109913.
Auerbach, E.A., Seyfried, E.E., McMahon, K.D., 2007. Tetracycline resistance genes in activated sludge wastewater treatment plants. Water Res. 41, 1143–1151.
Azuma, T., Nakada, N., Yamashita, N., Tanaka, H., 2012. Synchronous dynamics of observed and predicted values of anti-influenza drugs in environmental waters during a seasonal influenza outbreak. Environ. Sci. Technol. 46 (23), 12873–12881.
Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J.V., 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol. 14 (4), 176–182.
Biswas, K., Paul, D., Sinha, S.N., 2015. Prevalence of multiple antibiotic-resistant coliform bacteria in the water of river Ganga. Front. Environ. Microbiol. 1 (3), 44.
Blair, J.M., Webber, M.A., Baylay, A.J., Ogbolu, D.O., Piddock, L.J., 2015. Molecular mechanisms of antibiotic resistance. Nat. Rev. Microbiol. 13 (1), 42–51.
Chatterjee, S.K., Bhattacharjee, I., Chandra, G., 2010. Water quality assessment near an industrial site of Damodar River, India. Environ. Monit. Assess. 161 (1–4), 177–189.
Dhangar, K., Kumar, M., 2020. Tricks and tracks in removal of emerging contaminants from the wastewater through hybrid treatment systems: A review. Sci. Total Environ. 140320.
Ferreira da Silva, M., Vaz-Moreira, I., Gonzalez-Pajuelo, M., Nunes, O.C., Manaia, C.M., 2007. Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. FEMS Microbiol. Ecol. 60 (1), 166–176.
Flach, C.F., Genheden, M., Fick, J., Joakim Larsson, D.G., 2018. A comprehensive screening of Escherichia coli isolates from Scandinavia's largest sewage treatment plant indicates no selection for antibiotic resistance. Environ. Sci. Technol. 52 (19), 11419–11428.
Getahun, H., Smith, I., Trivedi, K., Paulin, S., Balkhy, H.H., 2020. Tackling antimicrobial resistance in the COVID-19 pandemic. Bull. World Health Organ. 98 (7), 442-442.
Global Antibiotic Resistance Partnership (GARP)-India Working Group, 2011. Rationalizing antibiotic use to limit antibiotic resistance in India+. Indian J. Med. Res. 134 (3), 281.
Guo, X., Yan, Z., Zhang, Y., Xu, W., Kong, D., Shan, Z., Wang, N., 2018. Behavior of antibiotic resistance genes under extremely high-level antibiotic selection pressures in pharmaceutical wastewater treatment plants. Sci. Total Environ. 612, 119–128.
Hamner, S., Broadaway, S.C., Mishra, V.B., Tripathi, A., Mishra, R.K., Pulcini, E., Pyle, B. H., Ford, T.E., 2007. Isolation of potentially pathogenic Escherichia coli O157: H7 from the Ganges River. Appl. Environ. Microbiol. 73 (7), 2369–2372.
Haramoto, E., Kitajima, M., Hata, A., Torrey, J.R., Masago, Y., Sano, D., Katayama, H., 2018. A review on recent progress in the detection methods and prevalence of human enteric viruses in water. Water Res. 135, 168–186.
Haramoto, E., Malla, B., Thakali, O., Kitajima, M., 2020. First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. Sci. Total Environ. 737, 140405.
Honda, R., Noguchi, M., Yamamoto-Ikemoto, R., Watanabe, T., 2018. Effect of sedimentation and aeration on antibiotic resistance induction in the activated sludge process. J. Water Environ. Technol. 16 (2), 94–105.
Honda, R., Watanabe, T., Sawaittayotin, V., Masago, Y., Chulasak, R., Tanong, K., Chaminda, G.T., Wongsila, K., Sienglum, C., Sunthonwatthanaphong, V., Poonnotok, A., 2016. Impacts of urbanization on the prevalence of antibiotic-resistant Escherichia coli in the Chaophraya River and its tributaries. Water Sci. Technol. 73 (2), 362–374.
Hsu, J., 2020. How covid-19 is accelerating the threat of antimicrobial resistance. BMJ 369, 1983.
Hu, J., Shi, J., Chang, H., Li, D., Yang, M., Kamagata, Y., 2008. Phenotyping and genotyping of antibiotic-resistant Escherichia coli isolated from a natural river basin. Environ. Sci. Technol. 42 (9), 3415–3420.
IS10500, B.I.S., 2012. Indian Standard Drinking Water–Specification (Second Revision). Bureau of Indian Standards (BIS),, New Delhi.
Jiang, L., Hu, X., Xu, T., Zhang, H., Sheng, D., Yin, D., 2013. Prevalence of antibiotic resistance genes and their relationship with antibiotics in the Huangpu River and the drinking water sources, Shanghai, China. Sci. Total Environ. 458, 267–272.
Klein, E.Y., Van Boeckel, T.P., Martinez, E.M., Pant, S., Gandra, S., Levin, S.A., Goossens, H., Laxminarayan, R., 2018. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc. Natl. Acad. Sci. 115 (15), E3463–E3470.
Kocamemi, B.A., Kurt, H., Hacioglu, S., Yarali, C., Saatci, A.M., Pakdemirli, B., 2020. First Data-Set on SARS-CoV-2 Detection for Istanbul Wastewaters in Turkey. medRxiv.
Kumar, A., Sharma, M.P., 2014. Application of water quality index and diversity index for pollution assessment of Kankaria Lake at Ahmedabad, India. J. Civil & Environ. Eng. 4 (3), 1.
Kuroda, K., Li, C., Dhangar, K., Kumar, M., 2021. Predicted occurrence, ecotoxicological risk and environmentally acquired resistance of antiviral drugs associated with COVID-19 in environmental waters. Sci. Total Environ. 776, 145740.
Kumar, M., Kuroda, K., Patel, A.K., Patel, N., Bhattacharya, P., Joshi, M., Joshi, C.G., 2021. Decay of SARS-CoV-2 RNA along the wastewater treatment outfitted with Upflow Anaerobic Sludge Blanket (UASB) system evaluated through two sample concentration techniques. Sci. Total Environ. 754, 142329.
Kumar, M., Kuroda, K., Dhangar, K., Mazumder, P., Sonne, C., Rinklebe, J., Kitajima, M., 2020a. Potential emergence of antiviral-resistant pandemic viruses via environmental drug exposure of animal reservoirs. Environ. Sci. Technol. 54 (14), 8503–8505.
Kumar, M., Patel, A.K., Shah, A.V., Raval, J., Rajpara, N., Joshi, M., Joshi, C.G., 2020b. First proof of the capability of wastewater surveillance for COVID-19 in India through detection of genetic material of SARS-CoV-2. Sci. Total Environ. 746, 141326.
Kumar, M., Mazumder, P., Mohapatra, S., Thakur, A.K., Dhangar, K., Taki, K., Mukherjee, S., Patel, A.K., Bhattacharya, P., Mohapatra, P., Rinklebe, J., 2021. A chronicle of SARS-CoV-2: seasonality, environmental fate, transport, inactivation, and antiviral drug resistance. J. Hazard. Mater. 405, 124043.
Kumar, M., Chaminda, T., Patel, A.K., Sewwandi, H., Mazumder, P., Joshi, M., Honda, R., 2020d. Prevalence of antibiotic resistance in the tropical rivers of Sri Lanka and India. Environ. Res. 188, 109765.
Kumar, M., Chaminda, G.T., Honda, R., 2020e. Seasonality impels the antibiotic resistance in Kelani River of the emerging economy of Sri Lanka. npj Clean Water 3 (1), 1–8.
Kumar, M., Ram, B., Sewwandi, H., Honda, R., Chaminda, T., 2020f. Treatment enhances the prevalence of antibiotic-resistant bacteria and antibiotic resistance genes in the wastewater of Sri Lanka, and India. Environ. Res. 183, 109179.
La Rosa, G., Iaconelli, M., Mancini, P., Ferraro, G.B., Veneri, C., Bonadonna, L., Lucentini, L., Suffredini, E., 2020. First detection of SARS-CoV-2 in untreated wastewaters in Italy. Sci. Total Environ. 736, 139652.
Liu, C., Zhou, Q., Li, Y., Garner, L.V., Watkins, S.P., Carter, L.J., Smoot, J., Gregg, A.C., Daniels, A.D., Jervey, S. and Albaiu, D., 2020. Research and development on therapeutic agents and vaccines for COVID-19 and related human coronavirus diseases.
Lucien, M.A.B., Canarie, M.F., Kilgore, P.E., Jean-Denis, G., Fénélon, N., Pierre, M., Cerpa, M., Joseph, G.A., Maki, G., Zervos, M.J., Dely, P., 2021. Antibiotics and antimicrobial resistance in the COVID-19 era: perspective from resource-limited settings. Int. J. Infect. Dis. 104, 250–254.
Marcinek, H., Wirth, R., Muscholl-Silberhorn, A., Gauer, M., 1998. Enterococcus faecalis gene transfer under natural conditions in municipal sewage water treatment plants. Appl. Environ. Microbiol. 64 (2), 626–632.
Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020. Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. Environ. Sci. Technol. Lett. 7, 511–516.

Miranda, C.D., Castillo, G., 1998. Resistance to antibiotic and heavy metals of motile aeromonads from Chilean freshwater. Sci. Total Environ. 224 (1–3), 167–176.
Miranda, C., Silva, V., Capita, R., Alonso-Calleja, C., Igrejas, G., Poeta, P., 2020. Implications of antibiotics use during the COVID-19 pandemic: present and future. J. Antimicrob. Chemother. 75 (12), 3413–3416.
Munita, J.M., Arias, C.A., 2016. Mechanisms of antibiotic resistance. Virulence Mech. Bact. Pathog. 481–511.
Na, G., Lu, Z., Gao, H., Zhang, L., Li, Q., Li, R., Yang, F., Huo, C., Yao, Z., 2018. The effect of environmental factors and migration dynamics on the prevalence of antibiotic-resistant Escherichia coli in estuary environments. Sci. Rep. 8 (1), 1663.
Nemudryi, A., Nemudraia, A., Wiegand, T., Surya, K., Buyukyoruk, M., Cicha, C., Vanderwood, K.K., Wilkinson, R., Wiedenheft, B., 2020. Temporal detection and phylogenetic assessment of SARS-CoV-2 in municipal wastewater. Cell Rep. Med. 1 (6), 100098.
Pormohammad, A., Nasiri, M.J., Azimi, T., 2019. Prevalence of antibiotic resistance in Escherichia coli strains simultaneously isolated from humans, animals, food, and the environment: a systematic review and meta-analysis. Infect. Drug Resist. 12, 1181–1197.
Proia, L., Anzil, A., Subirats, J., Borrego, C., Farrè, M., Llorca, M., Balcázar, J.L., Servais, P., 2018. Antibiotic resistance along an urban river impacted by treated wastewaters. Sci. Total Environ. 628, 453–466.
Ram, B., Kumar, M., 2020. Correlation appraisal of antibiotic resistance with fecal, metal and microplastic contamination in a tropical Indian river, lakes and sewage. NPJ Clean. Water 3 (1), 1–12.
Rawson, T.M., Moore, L.S., Zhu, N., Ranganathan, N., Skolimowska, K., Gilchrist, M., Satta, G., Cooke, G., Holmes, A., 2020. Bacterial and fungal coinfection in individuals with coronavirus: a rapid review to support COVID-19 antimicrobial prescribing. Clin. Infect. Dis. 71 (9), 2459–2468.
Reinthaler, F.F., Posch, J., Feierl, G., Wüst, G., Haas, D., Ruckenbauer, G., Mascher, F., Marth, E., 2003. Antibiotic resistance of *E. coli* in sewage and sludge. Water Res. 37 (8), 1685–1690.
Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242.
Sherchan, S.P., Shahin, S., Ward, L.M., Tandukar, S., Aw, T.G., Schmitz, B., Ahmed, W., Kitajima, M., 2020. First detection of SARS-CoV-2 RNA in wastewater in North America: a study in Louisiana, USA. Sci. Total Environ. 743, 140621.
Silva, J., Castillo, G., Callejas, L., López, H., Olmos, J., 2006. Frequency of transferable multiple antibiotic resistance amongst coliform bacteria isolated from a treated sewage effluent in Antofagasta, Chile. Electron. J. Biotechnol. 9 (5), 0, 0-0.
Singer, A.C., Howard, B.M., Johnson, A.C., Knowles, C.J., Jackman, S., Accinelli, C., Caracciolo, A.B., Bernard, I., Bird, S., Boucard, T., Boxall, A., 2008. Meeting report: risk assessment of Tamiflu use under pandemic conditions. Environ. Health Perspect. 116 (11), 1563–1567.
Singh, U.K., Kumar, M., Chauhan, R., Jha, P.K., Ramanathan, A.L., Subramanian, V., 2008. Assessment of the impact of landfill on groundwater quality: a case study of the Pirana site in western India. Environ. Monit. Assess. 141 (1), 309–321.
Storteboom, H., Arabi, M., Davis, J.G., Crimi, B., Pruden, A., 2010. Tracking antibiotic resistance genes in the South Platte River basin using molecular signatures of urban, agricultural, and pristine sources. Environ. Sci. Technol. 44 (19), 7397–7404.
Takanami, R., Ozaki, H., Giri, R.R., Taniguchi, S., Hayashi, S., 2010. Detection of antiviral drugs oseltamivir phosphate and oseltamivir carboxylate in Neya River, Osaka, Japan. J. Water Environ. Technol. 8 (4), 363–372.
Threedeach, S., Chiemchaisri, W., Watanabe, T., Chiemchaisri, C., Honda, R., Yamamoto, K., 2012. Antibiotic resistance of Escherichia coli in leachates from municipal solid waste landfills: comparison between semi-aerobic and anaerobic operations. Bioresour. Technol. 113, 253–258.
Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant, A., Laxminarayan, R., 2015. Global trends in antimicrobial use in food animals. Proc. Natl. Acad. Sci. 112 (18), 5649–5654.
World Health Organization, 2014. Antimicrobial Resistance: Global Report on Surveillance. World Health Organization,.
Yang, Y., Xu, C., Cao, X., Lin, H., Wang, J., 2017. Antibiotic resistance genes in surface water of eutrophic urban lakes are related to heavy metals, antibiotics, lake morphology and anthropic impact. Ecotoxicology 26 (6), 831–840.
Zhang, S., Han, B., Gu, J., Wang, C., Wang, P., Ma, Y., Cao, J., He, Z., 2015. Fate of antibiotic resistant cultivable heterotrophic bacteria and antibiotic resistance genes in wastewater treatment processes. Chemosphere 135, 138–145.

Environmental Research

The Spectre of SARS-CoV-2 in The Ambient Urban Natural Water in Ahmedabad and Guwahati: A tale of Two Cities

--Manuscript Draft--

Manuscript Number:	
Article Type:	VSI:SARS-CoV-2EnvironPoll
Section/Category:	Environmental Health & Risk Assessment
Keywords:	COVID-19; surface water; wastewater; sewage; SARS-CoV-2
Corresponding Author:	Manish Kumar, Ph.D Indian Institute of Technology Gandhinagar Gandhinagar, INDIA
First Author:	Manish Kumar
Order of Authors:	Manish Kumar
	Payal Mazumder
	Jyoti Prakash Deka
	Vaibhav Srivastava
	Chandan Mahanta
	Latha Rangan
	Shilangi Gupta
	Madhvi Joshi
	AL. Ramanathan
Abstract:	COVID-19 positive patients can egest live SARS-CoV-2 virus through faecal matter and urine, raising concerns about viral transmission through faecal-oral route and/or contaminated aerosolized water. These worries are heightened in many low and middle income nations, where raw sewage is often dumped into surface waterways and open defecation betide. In this manuscript we attempt to discern the presence of SARS-CoV-2 genetic material (ORF-1ab, N and S genes) in two urban cities of India viz., Ahmedabad, in western India with ~12 WWTPs and Guwahati, in north-east of the country with no such plants. 100% and 20% of the surface water samples had detectable SARS-CoV-2 RNA load in Ahmedabad and Gandhinagar, respectively. N-gene>S-gene>ORF-1ab-gene were readily detected in surface water of Ahmedabad, whereas, no such significant trend was found in the case of Guwahati. The high concentration of gene (ORF-1ab – 800 copies/L for Sabarmati river, Ahmedabad and S-gene – 565 copies/L for Bharalu urban river, Guwahati) found in natural waters indicates low sanitation and have various health and ecological consequences that should be investigated further.
Suggested Reviewers:	Keisuke Kuroda, PhD University of Toyama: Toyama Daigaku kuroda@pu-toyama.ac.jp
	Ryo Honda, PhD Kanazawa University: Kanazawa Daigaku rhonda@se.kanazawa-u.ac.jp
	Vinay Kumar IIT Roorkee: Indian Institute of Technology Roorkee vinayiitrp@gmail.com
	Tushara Chaminda, PhD tusharachaminda@yahoo.com
	Meththika Vithanage meththikavithanage@gmail.com

Cover Letter

Dr. Manish Kumar
Assistant Professor, Earth Sciences, 336A, Block-5
□□□□□□ □□□□□□□□□□□□□ □□□□□□□□ □□□□□□□□□
पालज, गांधीनगर, गुजरात 382 355
INDIAN INSTITUTE OF TECHNOLOGY GANDHINAGAR
PALAJ, GANDHINAGAR, GUJARAT 382 355

E-Mail : manish.kumar@iitgn.ac.in
Web : www.iitgn.ac.in/academics/es/
Tel : +91-863-814-7602 |
Office : 07923952531 | Ext: 2531(O)|
Japan Society for the Promotion of Science (JSPS) alumni
Associate Editor, Hydrological Research Letter
Associate Editor, Groundwater for Sustainable Development
https://www.researchgate.net/profile/Manish_Kumar138

IITGN

To,

Dated: 5th June, 2021

The Editor
Environmental Research

I am enclosing herewith the e-version of our manuscript entitled, "The Spectre of SARS-CoV-2 in The Ambient Urban Natural Water in Ahmedabad and Guwahati: A tale of Two Cities" for consideration for publication in your esteemed journal. We present the idea of monitoring surface waters receiving urban sewage discharge to detect viral genome load for early prediction of COVID-19 pandemic. Monitoring surface water for microbiological contaminants and their genome, along with basic water quality parameters gives insight of sanitation, wastewater management, surveillance and management of disease outbreaks.

We compared the occurrence of SARS-CoV-2 RNA in the natural waters of two urban cities of India, viz., Ahmedabad with population size of 8,059,441, having ~12 Sewage Treatment Plants (STPs) and Guwahati with population of 1,117,000 and having no STPs. Water safety begins with the preservation of natural water resources in the watershed; as a result, it is crucial to keep surface and groundwater from contamination with faeces and to prevent direct discharge of grey water into rivers, streams, lakes, wetlands, open wells, etc. The approach described in this paper can be employed in other places where sampling sewage is impossible and wastewaters are disposed into lakes, streams or rivers. The knowledge is also helpful to indicate thorough investigation of possibility of contagion in places with inadequate sanitation, where people are at risk of being exposed to polluted water or even raw sewage.

We believe that our manuscript can be given consideration for publication in your esteemed journal.

Thanking you
Best Regards

Dr. Manish Kumar

The Spectre of SARS-CoV-2 in the Ambient Urban Natural Water in Ahmedabad and Guwahati: A Tale of Two Cities

Manish Kumar[1,2*], Payal Mazumder[3], Jyoti Prakash Deka[4], Vaibhav Srivastava[1], Chandan Mahanta[5], Latha Rangan[6], Shilangi Gupta[7], Madhvi Joshi[7], AL. Ramanathan[8]

[1]Discipline of Earth Science, Indian Institute of Technology Gandhinagar, Gujarat 382 355, India

[2]Kiran C Patel Centre for Sustainable Development, Indian Institute of Technology Gandhinagar, Gujarat, India

[3]Centre for the Environment, Indian Institute of Technology Guwahati, Assam 781039, India

[4]Discipline of Environmental Sciences, Gauhati Commerce College, Guwahati, Assam 781021, India

[5]Department of Civil Engineering, Indian Institute of Technology Guwahati, Assam 781039, India

[6]Department of Bioscience and Bioengineering, Indian Institute of Technology Guwahati, Assam 781039, India

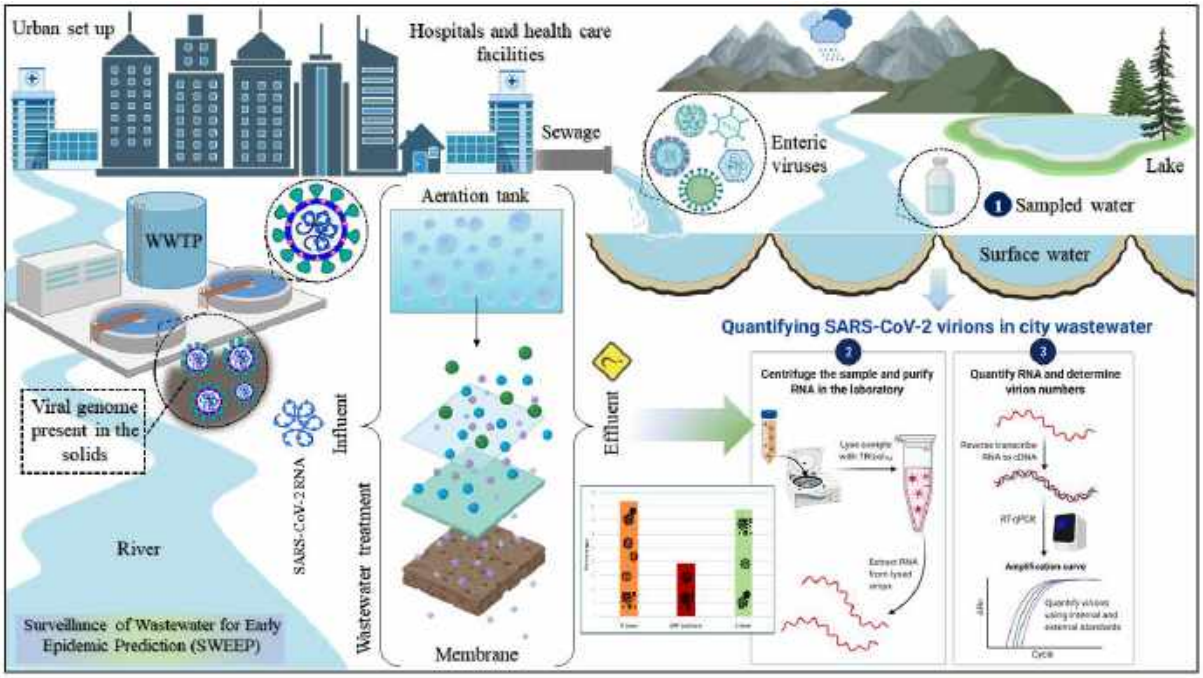
[7]Gujarat Biotechnology Research Centre (GBRC), Sector- 11, Gandhinagar, Gujarat 382 011, India

[8]School of Environmental Sciences, Jawaharlal Nehru University, New Delhi 110067, India

*Corresponding Author: manish.env@gmail.com; manish.kumar@iitgn.ac.in

Manish Kumar | Ph.D, FRSC, JSPS, WARI+91 863-814-7602 | Discipline of Earth Science | IIT Gandhinagar | India

Graphical abstract



Highlights

- Natural urban waters shows the presence of titters of SARS-CoV-2 RNA.
- Lake water receiving runoff containing SARS-CoV-2 genes reflected positive sign early.
- Viral RNA in surface water reflects inadequate sanitation and wastewater management.
- Residence time and transmission owing to viral RNA in natural waters needs further research.

Abstract

COVID-19 positive patients can egest live SARS-CoV-2 virus through faecal matter and urine, raising concerns about viral transmission through faecal-oral route and/or contaminated aerosolized water. These worries are heightened in many low and middle income nations, where raw sewage is often dumped into surface waterways and open defecation betide. In this manuscript we attempt to discern the presence of SARS-CoV-2 genetic material (ORF-1ab, N and S genes) in two urban cities of India viz., Ahmedabad, in western India with ~12 WWTPs and Guwahati, in north-east of the country with no such plants. 100% and 20% of the surface water samples had detectable SARS-CoV-2 RNA load in Ahmedabad and Gandhinagar, respectively. N-gene>S-gene>ORF-1ab-gene were readily detected in surface water of Ahmedabad, whereas, no such significant trend was found in the case of Guwahati. The high concentration of gene (ORF-1ab – 800 copies/L for Sabarmati river, Ahmedabad and S-gene – 565 copies/L for Bharalu urban river, Guwahati) found in natural waters indicates low sanitation and have various health and ecological consequences that should be investigated further.

Keywords: COVID-19, surface water, wastewater, sewage, SARS-CoV-2

Introduction

Viruses are reported to occur in the surface water and believed to impact environmental and human health (**Lu and Yu, 2018; Qu et al., 2018, Kauppinen et al., 2018; Sekwadi et al., 2018; Kuroda et al., 2015; Kumar et al., 2019; Kumar et al., 2020**). Absence of sufficient sewage collection and treatment system is likely to make the situation more severe, especially in cities of the developing countries owing to high population density, discharge of (often unregulated) domestic and industrial effluents and ineffective treatment of wastewater (**Samaraweera et al., 2019**). It is a known fact that enteric viruses can enter into the aquatic environments through several routes such as water outflows or heavy rainfall, combined sewer outflows, blockages or sanitation system failures (**Fong et al., 2010; Kumar et al., 2019**). Various enteric viruses are very similar in terms of their structure, origin and symptoms of enteric viruses. Severe Acute Respiratory Viruses (SARS) are also reported to be prevalent in wastewater and surface water despite being an enveloped virus, that rapidly degrade in the environment. The prevalence of such viruses in the aquatic environment is likely to increase considerably during the ongoing Coronavirus disease (COVID-19) pandemic situation, that pose severe health risk to humans via faecal-oral transmission or aerosolisation of water droplets containing virus (**Lodder and de Roda Husman, 2020; Naddeo and Liu, 2020**). Nonetheless, because numerous countries like India are now witnessing the largest COVID-19 peaks and a probable onset of third wave in 2021, also knowing the viable viral particles might be particularly important for Quantitative Microbiological Risk Assessment (QMRA) associated to exposure to SARS-CoV-2 contaminated water. Overall, considering the millions of infections and deaths related to COVID-19, it is highly pertinent to monitor the occurrences of SARS coronavirus 2 (SARS-CoV-2) in the freshwater and wastewater systems which is vital for human sustenance. However, faecal shedding of the virus and its detection in wastewater

might be particularly problematic in low-sanitation areas where wastewater treatment is partial or non-existent **(Kozer et al., 2021; Guerrero-Latorre et al., 2020)**.

Further, the abundance of viruses in tropical countries has not been well documented. As, the lipids of viral envelop can be easily disrupted by environmental stressors (**Pinon and Vialette, 2018**), enveloped viruses such as SARS-CoV-2 are more susceptible than non-enveloped viruses (e.g. Norovirus, Rhinovirus, etc.) under similar adverse conditions (**Gundy et al., 2009**). Although, the high temperatures and solar radiations during tropical summers can effectively lower the prevalence of viruses, COVID-19 spread in the world does not suggest such (**Carratala et al., 2013; Baker et al., 2021**). The pathway of SARS-CoV-2 reaching to the ambient waters have been plenty (**Kumar et al., 2020**), including that of short circuiting of wastewater release into the urban waters and incomplete removal of viruses during the treatment. It was found that tertiary treatment of wastewater could remove greater % of SARS-CoV-2 RNA (100%) while, that of secondary treatment (89%) (**Randazzo et al., 2020**). **de Oliveira et al, (2021)** detected SARS-CoV-2 in artificially spiked river water (filtered and unfiltered) at two different temperatures viz., 4°C and 24°C through plaque assays. On the other hand, **Haramoto et al, (2020)** reported no positive results for virus RNA in raw wastewater whereas, ~2400 gene copies/L were detected in wastewater with secondary treatment. They also sampled surface water (river) to detect the viral genome, however, there was no trace of SARS-CoV-2 RNA in river water. Surprisingly, they also observed the abundance of N genes in positive secondary treated samples but ORF-1a and S genes were not found. Although the frequency of reports on SARS-CoV-2 presence in the treated wastewater is increasing day by day (**Westhaus et al., 2021, Hasan et al., 2021**), the ambient urban waters are somehow not being monitored. Hence it is very likely that we are going to miss this opportunity to learn a lot about the pandemic situation to make our future generations capable

of understanding and manage them better. **Mancuso et al, (2021)** reviewed how SARS-CoV-2 might infiltrate the urban water cycle and subsequently spread from urban to rural water settings, posing a possible risk to crop production and, hence, human health. **Mahlknecht et al, (2021)** reported the first study on the detection of SARS-CoV-2 RNA in groundwater in Monterrey. There is currently no indication that COVID-19 may be transferred to animals or humans through polluted water (**La Rosa et al., 2020**). Despite this, the World Health Organization (**WHO, 2020**) has emphasised the need of study into the novel coronavirus persistence in environmental matrices like as surface water and wastewater.

Under the light of above discussion, we conducted SARS-CoV-2 titre monitoring in various surface waters of two Indian cities i.e. Ahmedabad in Gujarat Province and Guwahati in Assam. Cities are selected such that the former has one of the highest number of wastewater treatment plants (WWTPs) among the Indian cities i.e. Ahmedabad in western India and the latter do not have even a single treatment plant available in the city i.e., case of Guwahati in north-east India. Our main objectives were to: i) understand the frequency of positive occurrence of SARS-CoV-2 titre during weekly surveillance of the representative water bodies present in both the cites; ii) comparative assessment of the vulnerability of urban waters in a city setup among the silhouette of COVID-19 clinical cases. Our research is critical since there are several transmission pathways in underdeveloped nations due to less prevalent, poorly managed sewage systems, which result in wastewater leakages and common sewage overflow issues.

2. Materials and methods

2.1 Study area and sampling location

In the present study, three lakes i.e. Kankaria Lake, Chandola Lake, Vastrapur Lake and the Sabarmati Rivers were sampled weekly since September 3rd, 2020 to 29th December, 2020, as

a representative urban ambient water bodies in Ahmedabad **(Fig. 1a)**. In Ahmedabad, the sewage is collected through a system comprising an underground drainage network, auxiliary pumping stations (APS), Sewage Treatment Plants (STPs), and are disposed into the natural water bodies and rivers after treatment. Wastewater generated from all these development is collected by a network of underground sewers and pumping stations and is conveyed to the sewage treatment works for physical and biological treatment to meet the Gujarat Pollution Control Board (GPCB) guidelines before discharge into the nearest water body. The Ahmedabad Municipal Corporation comprises of 9 STPs, 45 Sewage Pumping Stations, and an extended Sewage Network of ~2500 km present in the city.

On the other hand, ten samples representing Dipor Bil Lake, the Brahmaputra River, the Bharalu River and the Urban Drains of the Guwahati city were taken and analysed monthly from October to December, 2020 **(Fig. 1b)**. Guwahati, known as gateway of the north-eastern India, has a concise area of 328 km^2 that exhibit rapid and unplanned urban growth with around a million of city residents as per the 2011 census. The Brahmaputra River, an international transboundary, the fifteenth longest and the ninth largest river in terms of discharge (**Pervez and Henebry, 2015**) provides one side boundary to the city. While the Bharalu River, a tributary of Brahmaputra River, flows through the dense urban region of Guwahati city and now has virtually become an urban drain. Dipor Bil Lake is a natural freshwater lake/wetland system recognised under the Ramsar Convention provides another side of the city. There is not a single STP present in Guwahati city of Assam Province. Probably the main solution of the wastewater here is the dilution owing to relatively higher rainfall (average annual precipitation of 2054 mm) with 91.9 average rainy days over a year. The perennial discharge of the Brahmaputra River is disposing the responsibility of diluting all the wastes of the city. Sampling locations in Guwahati was selected based on our previous work (**Kumar et al.,**

2019). We added two additional locations i.e. Khanapara and AIDC based on COVID-19 quarantine centre locations in the city. Overall, eight sampling locations were precisely same as described in **Kumar et al, (2019)** and two additional locations were added specific to COVID-19 pandemic. Samples were collected using composite grab sampling by mixing three samples simultaneously taken at each location.

2.2 Sample collection and preparation

The samples were collected using grab sampling technique in 500ml polyethylene sterile bottles (Tarsons, PP Autoclavable, Wide Mouth Bottle, Cat No. 582240, India) and transferred in an icebox to the laboratory at Gujarat Biotechnology Research Centre (GBRC) and refrigerated at 4^0C until further process. To take the cross-contamination during transportation into account, the sampling blanks were prepared and analysed. Samples from Guwahati was transported in a sealed ice-box by air-mail within the same day of sampling and RNA extraction was performed within 72 hrs of sampling.

Poly ethylene glycol (PEG) based precipitation method was used for concentration of the sample as explained by (**Kumar et al., 2020b**). Briefly, 30ml sample was centrifuged (Model: Sorvall ST 40R, Thermo Scientific) at 4000g for 30 minutes in a 50ml falcon tube followed by the filtration of the supernatant with a syringe filter of 0.2µ (Mixed cellulose esters syringe filter, Himedia). The filtrate was then treated with NaCl (17.5 g/L) and PEG 9000 (80 g/L) and incubated at 100 rpm overnight (Model: Incu-Shaker™ 10LR, Benchmark). The room temperature was maintained at 17 ^{0}C using air-conditioner. A protocol for the same was established before and the effect of several variables like volume of the samples, temperature, rpm speed, and amount of PEG and NaCl were already observed and standardized. To make the pellet, the solution was then subjected to ultra-centrifugation at 14000g for 90 minutes (Model: Incu-Shaker™ 10LR, Benchmark). RNase-free water was used for the resuspension

of the pellet containing viral particles, which then was stored in a 1.5ml Eppendorf tube at a temperature of -40°C until RNA isolation. The detailed work flow concept has been depicted in **Fig. 2**.

2.3 Isolation of the SARS-CoV-2 viral genome

SARS-CoV-2 RNA isolation was performed using a commercially ready-for-use kit (NucleoSpin® RNA Virus, Macherey-Nagel GmbH & Co. KG, Germany). MS2 phage (10 µL), Proteinase K (20 µL) and RAV1 buffer (600 µL) consisting of carrier RNA were mixed with 300 µL of the concentrated viral particles. MS2 phage serves as the molecular process inhibition as a test control. It was used to monitor the efficacy of RNA extraction and PCR inhibition. It should be remembered that MS2 may spontaneously exist in wastewater, so there is a risk that the retrieved MS2 may consist of both the spiked and the background viral material. As per the user manual instructions (Macherey-Nagel GmbH & Co. KG), further procedures were carried out. The last elution was done with 30 µL of kit-supplied elution buffer. Using a Qubit 4 Fluorometer (Invitrogen), RNA concentrations were checked.

The nucleic acid was analyzed to identify the S gene, N gene, and ORF1ab of SARS-CoV-2 and the internal control (MS2) with the help of RT-PCR using the TaqPath™ Covid-19 RT-PCR package (Applied Biosystems). Amplification was conducted in a reaction (25 µL) vial containing 7 µL of RNAs derived from each sample. 2 µL of the positive control (TaqPath™ COVID-19 Control) and refined 5 µL of negative control were used for the study. Nuclease-free water was applied as a template-free control in this analysis. Additional process steps were executed, as defined in the product guidebook. The RT-qPCR step consisting of 40 cycles, included UNG incubation (25 °C for 2 min), reverse transcription (53 °C for 10 min), and activation (95 °C for 2 min). The reactions were conducted and elucidated as instructed in the handbook of Applied BiosystemsTM 7500 Fast Real-Time PCR.

2.4 Data visualization

OriginPro 2019b software has been used for data analysis and to draw boxplots.

3. Results and discussion

Wastewater samples collected from surface urban waters of Ahmedabad (Sabarmati River, Kankaria, Chandola and Vastrapur lakes), Gujarat, India, revealed a considerable variation in SARS-CoV-2 genome titre. Analogy of qRT-PCR assay analysis for the determination of the virus genetic material (N, S, and ORF 1ab genes) showed 100% (4/4) positive samples. The average N-gene copies were found to be maximum in Sabarmati River (694 copies/L), followed by Kankaria (549 copies/ L) and Chandola (402 copies/L) while, Vastrapur did not show the presence of N-gene. The ORF 1ab-gene copies were found maximum in samples collected from Sabarmati River (800 copies/ L), followed by Kankaria (87 copies/L). Chandola and Vastrapur lake samples were negative for the ORF-1ab gene. Similarly, the S-gene copies climbed down from: Sabarmati River (490 copies/L)> Vastrapur (58 copies/ L)> Chandola (52 copies/ L)> Kankaria (45 copies/L). Correspondingly, a higher SARS-CoV-2 genome concentration was observed in Sabarmati River (492 copies/L), followed by Kankaria (318 copies/ L) and Chandola lake sample (75 copies/L) **(Table 1a)**. The number of active COVID-19 cases in Ahmedabad on the day of sampling matched the gene amplification and detection patterns (viral genetic load) in surface water rather well **(Fig. 3)**. The N-gene was detected in many samples even though the samples were negative for ORF-1ab gene and S-gene. This may be due to the fact that there may be sparse concentration of RNA for gene specific amplification. The box plots for Ahmedabad shows highest detection of N-gene, S-gene, ORF-1ab gene and genome concentrations in copies/L for the month of November 2020 and April 2021 **(Fig. 4)**.

The water samples collected from Guwahati (Dipor Bil lake, Brahmaputra river and WWTP at Indian Institute of Technology Guwahati (IITG) showed negative results for SARS-CoV-2 RNA. While, 1 sample near a COVID care centre and 1 sample from Bharalu urban drain tested positive for the presence of the virus genome thus showing 20% (2/10) positive results for the sampled locations. The average N-gene, ORF-1ab gene and S-gene copies were found to be maximum in the COVID care centre i.e., 9169, 4153 and 3580 copies/L than that of Bharalu urban drain. However, in the Bharalu drain the S-gene concentration was found to be the highest (565 copies/L) followed by N-gene (549 copies/L) and ORF-1ab gene (435 copies/L). Evidently, a larger genome concentration was observed in the COVID care facility (5634 copies/L) than the urban drain (516 copies/L) **(Table 1b)**. Conversely, the number of active cases rapidly decreased in the month of October, 2020 in Guwahati which, followed the trend till March, 2021 before another rise in cases from April, 2021.

The reason for negative detection of the SARS-CoV-2 gene in the Guwahati samples correspond to the decrease in clinical cases during the sampling period which, seems to be one of the lowest in the year 2020-May, 2021. The COVID care centre showed positive results as the symptomatic and asymptomatic patients were treated there. Bharalu drain, however, flows through the heat and lungs of the city and collects sewage and waste before finally joining to the Brahmaputra River. Hence, the asymptomatic cases or those who were not admitted to COVID care centres still shedding the virus would be detected in the wastewater. Guwahati city not having any WWTPs might face a lot of sanitation issues. The Bharalu river which turned into an urban drain carrying such enteric viruses might play as a hub of faecal-oral transmission. WWTPs can remove SARS-CoV-2 RNA, thus, strengthening the cities weak

health infrastructure. However, a much deeper research is still needed on the efficient removal of viral genome in WWTPs. The current results reveal the microbiological implications of sewage discharge into natural streams without prior treatment. Guwahati's urban waterways are harmed by the unmediated exude of sewage water from a population of about one million people.

India is on the verge of facing a third wave of COVID-19 among many natural calamities in 2021 e.g., several severe earthquakes in Assam and cyclone Tauktae near Gujarat coast. In such case access to safe water, health and hygiene during rehabilitation is pivotal. Therefore, all possible exposure pathways of SARS-CoV-2 is needed to be considered scientifically and point of discharge needs to identified and tested for microbial contamination along with basic water quality parameters. The findings of our study may be applied to other cities where, sewage is disposed directly into natural waterways. Particularly, the presence of SARS-CoV-2, along with other waterborne pathogens released in open surface waters, may provide a risk of infection to anyone who come into contact with such water downstream. It is crucial to note, however, that in the current study, only SARS-CoV-2 genetic material has been identified in waterways, and the virus's survival in contaminated waterways is unknown. Furthermore, because zoonotic spill over episodes are common in the Coronaviridae family, viral propagation into the environment has an undisclosed influence on domestic animals and wildlife health (**Franklin & Bevins, 2020**). Eventually, if diagnostic equipment's are restricted, the abundance of the viral genome can be employed as a surveillance criteria for a prompt warning system monitoring main sewage discharges across the city, assisting in the containment of the pandemic (**Bivins et al., 2020**).

Conclusion

The persistence of the SARS-CoV-2 virus and the viral RNA in various water matrixes is a current research subject. In the context of intermittent lockdown and progressive rise in COVID cases in India, we attempted to investigate the occurrence of SARS-CoV-2 genetic signature in two metropolitan cities of India viz., Ahmedabad (Western zone) and Guwahati (North-Eastern zone). The sustenance of the viral RNA load in urban surface waters in both the cities were congruent to the trends in active clinical COVID-19 cases. Lack of wastewater treatment coverage might be a contributing reason to the elevated probability of a COVID-19 pandemic. Water safety begins with the preservation of natural water resources in the watershed; as a result, it is crucial to keep surface and groundwater from contamination with faeces and to prevent direct discharge of grey water into rivers, streams, lakes, wetlands, open wells, etc. Surface waters receiving direct sewage or effluent discharge can be targeted for surveillance of SARS-CoV-2 genome and thus, can provide a lot of insights on rise in transmissions, sanitation, future risks and management. The approach described in this paper can be employed in other places where sampling sewage is impossible and wastewaters are disposed into lakes, streams or rivers. The knowledge is also helpful to indicate thorough investigation of possibility of contagion in places with inadequate sanitation, where people are at risk of being exposed to polluted water or even raw sewage.

Acknowledgement:

This work is funded by UNICEF, Gujarat and UKIERI. We also acknowledge the help received from Mr. Alok Thakur, and other GBRC staffs who contributed towards sample and data analyses.

References

Bivins, A., North, D., Ahmad, A., Ahmed, W., Alm, E., Been, F., Bhattacharya, P., Bijlsma, L., Boehm, A. B., Brown, J., Buttiglieri, G., Calabro, V., Carducci, A., Castiglioni, S., Cetecioglu Gurol, Z., Chakraborty, S., Costa, F., Curcio, S., De Los Reyes, F. L., … Bibby, K. (2020). Wastewater-Based Epidemiology: Global Collaborative to Maximize Contributions in the Fight against COVID-19. *Environmental Science and Technology*, *54*(13), 7754–7757. https://doi.org/10.1021/acs.est.0c02388

de Oliveira, L. C., Torres-Franco, A. F., Lopes, B. C., Santos, B. S. Á. da S., Costa, E. A., Costa, M. S., Reis, M. T. P., Melo, M. C., Polizzi, R. B., Teixeira, M. M., & Mota, C. R. (2021). Viability of SARS-CoV-2 in river water and wastewater at different temperatures and solids content. *Water Research*, *195*, 117002. https://doi.org/10.1016/j.watres.2021.117002

Estimating mortality from COVID-19: Scientific brief, 4 August 2020. (n.d.). Retrieved June 1, 2021, from https://www.who.int/publications/i/item/WHO-2019-nCoV-sci-brief-environmentalSampling-2020-1

Franklin, A. B., & Bevins, S. N. (2020). Spillover of SARS-CoV-2 into novel wild hosts in North America: A conceptual model for perpetuation of the pathogen. *Science of the Total Environment*, *733*, 139358. https://doi.org/10.1016/j.scitotenv.2020.139358

Guerrero-Latorre, L., Ballesteros, I., Villacrés-Granda, I., Granda, M. G., Freire-Paspuel, B., & Ríos-Touma, B. (2020). SARS-CoV-2 in river water: Implications in low sanitation countries. *Science of the Total Environment*, *743*(February), 0–4. https://doi.org/10.1016/j.scitotenv.2020.140832

Kozer, E., Rinott, E., Kozer, G., Bar-Haim, A., Benveniste-Levkovitz, P., Klainer, H., Perl, S., & Youngster, I. (2021). Presence of SARS-CoV-2 RNA on playground surfaces and water fountains. *Epidemiology and Infection*, *149*. https://doi.org/10.1017/S0950268821000546

Kumar, M., Patel, A. K., Shah, A. V., Raval, J., Rajpara, N., Joshi, M., & Joshi, C. G. (2020). First proof of the capability of wastewater surveillance for COVID-19 in India through detection of genetic material of SARS-CoV-2. *Science of the Total Environment*, *746*, 141326. https://doi.org/10.1016/j.scitotenv.2020.141326

Kumar, M., Ram, B., Honda, R., Poopipattana, C., Canh, V. D., Chaminda, T., & Furumai, H. (2019). Concurrence of antibiotic resistant bacteria (ARB), viruses, pharmaceuticals and personal care products (PPCPs) in ambient waters of Guwahati, India: Urban vulnerability and resilience perspective. *Science of the Total Environment*, *693*, 133640. https://doi.org/10.1016/j.scitotenv.2019.133640

Kumar, M., Thakur, A. K., Mazumder, P., Kuroda, K., Mohapatra, S., Rinklebe, J., Ramanathan, A., Cetecioglu, Z., Jain, S., Tyagi, V. K., Gikas, P., Chakraborty, S., Tahmidul Islam, M., Ahmad, A., Shah, A. V., Patel, A. K., Watanabe, T., Vithanage, M., Bibby, K., … Bhattacharya, P. (2020). Frontier review on the propensity and repercussion of SARS-CoV-2 migration to aquatic environment. *Journal of Hazardous Materials Letters*, *1*, 100001. https://doi.org/10.1016/j.hazl.2020.100001

Kuroda, K., Nakada, N., Hanamoto, S., Inaba, M., Katayama, H., Do, A. T., Nga, T. T. V., Oguma, K., Hayashi, T., & Takizawa, S. (2015). Pepper mild mottle virus as an indicator and a tracer of fecal pollution in water environments: Comparative evaluation with wastewater-tracer pharmaceuticals in Hanoi, Vietnam. *Science of the Total Environment*, *506–507*, 287–298. https://doi.org/10.1016/j.scitotenv.2014.11.021

La Rosa, G., Bonadonna, L., Lucentini, L., Kenmoe, S., & Suffredini, E. (2020). Coronavirus in water environments: Occurrence, persistence and concentration methods - A scoping review. In *Water Research* (Vol. 179). https://doi.org/10.1016/j.watres.2020.115899

Mahlknecht, J., Padilla Reyes, D. A., Ramos, E., Reyes, L. M., & Álvarez, M. M. (2021). The presence of SARS-CoV-2 RNA in different freshwater environments in urban settings determined by RT-qPCR: Implications for water safety. *Science of the Total Environment*, *784*, 147183. https://doi.org/10.1016/j.scitotenv.2021.147183

Mancuso, G., Perulli, G. D., Lavrnic, S., Morandi, B., & Toscano, A. (2021). Sars-cov-2 from urban to rural water environment: Occurrence, persistence, fate, and influence on agriculture irrigation. A review. *Water (Switzerland)*, *13*(6). https://doi.org/10.3390/w13060764

Naddeo Vincenzo and Liu Haizhou. (2020). Enhanced Reader.pdf. In *Environmental Science Water Research and Technology* (Vol. 6, p. 1213). https://doi.org/https://doi.org/10.1039/D0EW90015J

Figures

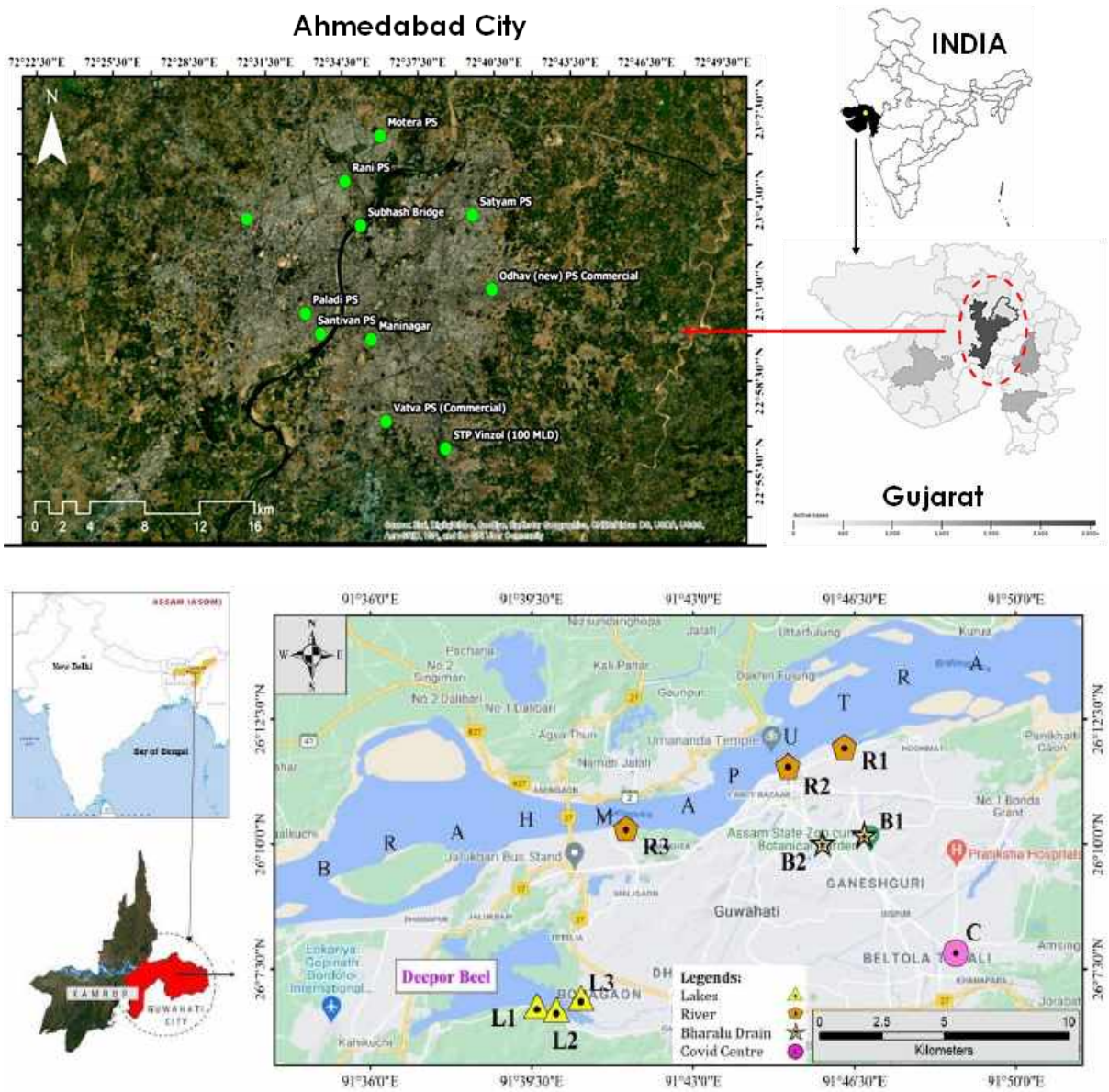


Fig.1. Map depicting the sampling sites in (a) Ahmedabad, Gujarat and (b) Guwahati, Assam

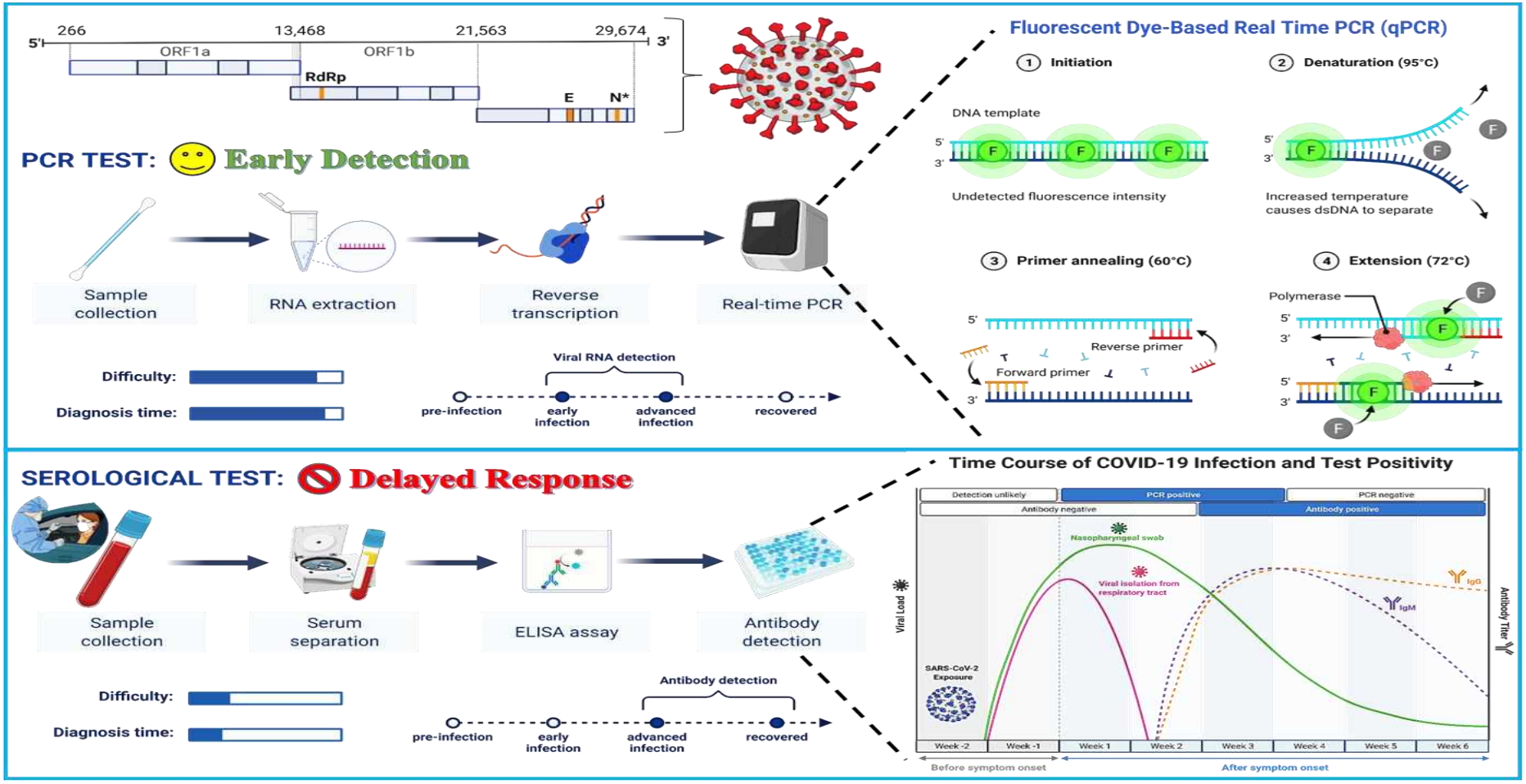


Fig.2. Advantage of qRT-PCR based detection of SARS-CoV-2 RNA over clinical tests for early detection, prediction and management of COVID-19 pandemic .

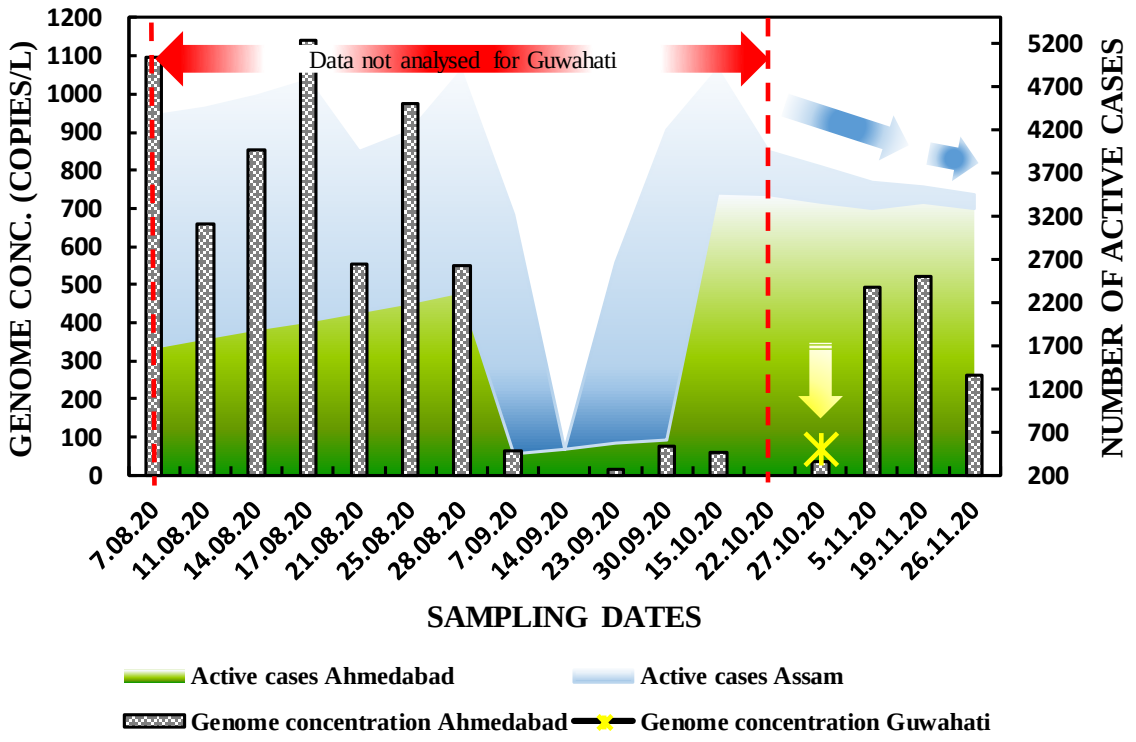


Fig.3. SARS-CoV-2 genome concentration as compared to clinical positive active cases in Ahmedabad and Guwahati, India

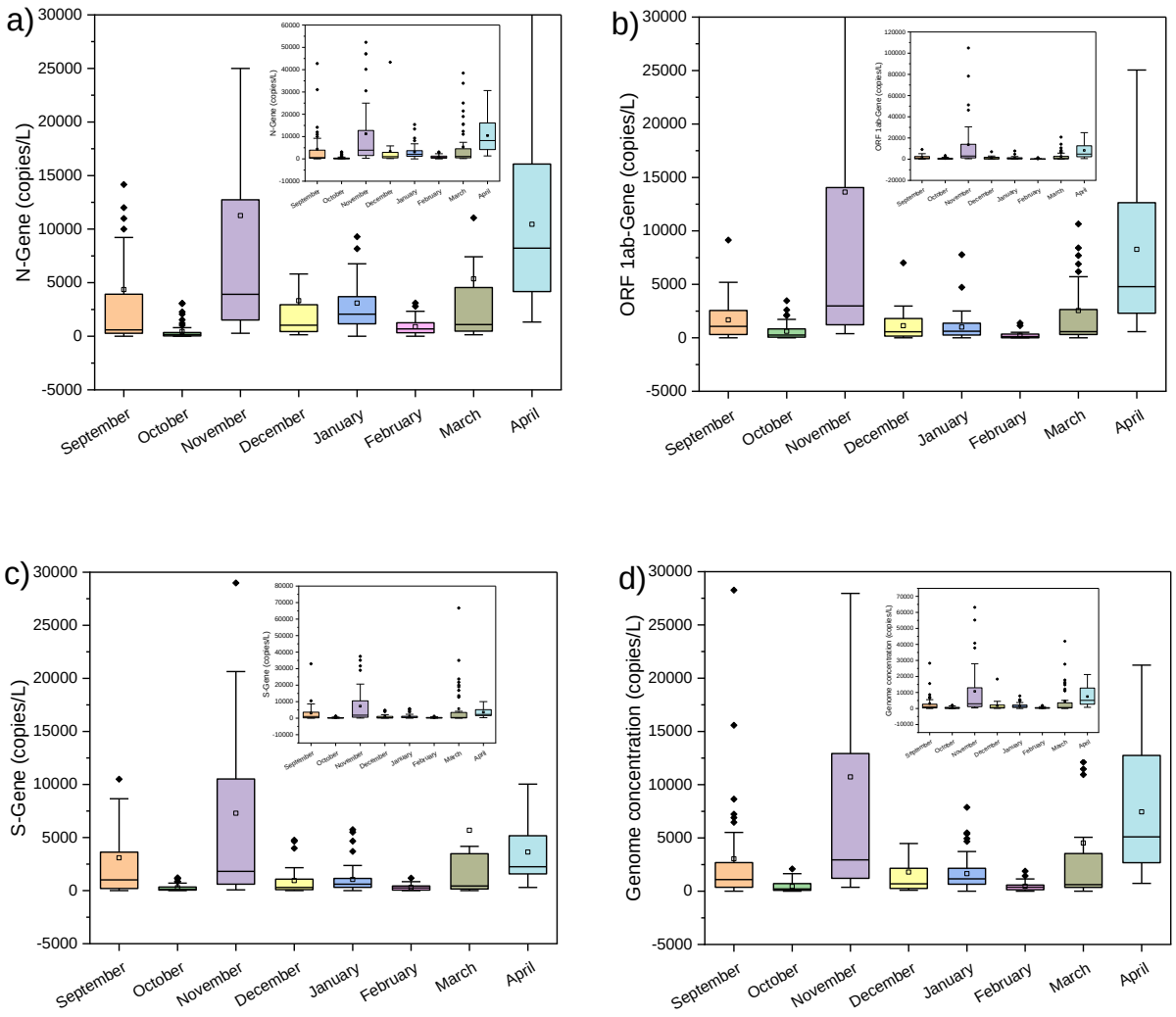


Fig. 4. Box and whiskers plots of SARS-CoV-2 (a) N gene, (b) ORF 1ab gene, (c) S gene, and (d) genome concentration in Ahmedabad, Gujarat

Tables

Table 1a: Occurrence of SARS-CoV-2 RNA traces in the freshwater samples collected from different locations in Ahmedabad.

Sampling date	Location	Ct value			Gene copies (copies/ L)			
		N	ORF	S	N	ORF	S	Genome
03.09.20	*Kankaria Lake*	36.01	ND	ND	90	0	0	INC
	Chandola Lake	ND	ND	36.94	0	0	52	INC
	Vastrapur Lake	ND	ND	36.75	0	0	58	INC
	Sabarmati River	ND	ND	38.34	0	0	24	INC
10.09.20	*Kankaria Lake*	33.10	36.08	ND	549	87	0	318
	Chandola Lake	ND	ND	ND	0	0	0	0
	Vastrapur Lake	ND	ND	ND	0	0	0	0
	Sabarmati River	ND	ND	ND	0	0	0	0
17.09.20	*Kankaria Lake*	37.87	ND	ND	31	0	0	INC
	Chandola Lake	ND	ND	ND	0	0	0	0
	Vastrapur Lake	ND	ND	ND	0	0	0	0
	Sabarmati River	ND	ND	ND	0	0	0	0
24.09.20	*Kankaria Lake*	ND	ND	37.21	0	0	45	INC
	Chandola Lake	37.33	ND	ND	402	0	0	INC
	Vastrapur Lake	ND	ND	ND	0	0	0	0
	Sabarmati River	39.24	ND	38.80	14	0	18	16
01.10.20	*Kankaria Lake*	35.67	ND	ND	111	0	0	0
	Chandola Lake	35.31	ND	39.64	137	0	12	75
	Vastrapur Lake	ND	ND	ND	0	0	0	0
	Sabarmati River	35.51	ND	ND	122	0	0	INC
08.10.20	*Sabarmati River*	37.70	35.78	36.86	34	104	55	64
15.10.20		38.46	35.67	37.14	22	110	47	60
22.10.20		ND	ND	ND	0	0	0	0
29.10.20		33.07	32.52	35.57	559	800	118	492
05.11.20		ND	ND	ND	0	0	0	0
12.11.20		ND	ND	ND	0	0	0	0
19.11.20		ND	ND	36.96	0	0	52	INC
26.11.20		ND	ND	ND	0	0	0	0
14.12.20		34.70	35.42	33.27	199	129	490	273
21.12.20		ND	ND	ND	0	0	0	0
28.12.20		32.73	33.80	39.96	694	350	10	351

Low High

Where; ND= Not detected, and INC= Detected but data inconclusive

Table 1b: SARS-CoV-2 gene concentration in wastewater samples collected from Guwahati.

Sampling date	Location	Ct Value			Gene copies (copies/ L)			
		N-Gene	ORF-Gene	S-Gene	N-Gene	ORF-Gene	S-Gene	Genome concentration
27.10.20	Dipor Bil (Boragaon)	ND	ND	ND	ND	ND	ND	ND
	Khanapara	29	30.1	30.3	9169	4153	3580	5634
	AIDC	33.1	33.5	33.1	549	435	565	516
	Uzan Bazar	ND	ND	ND	ND	ND	ND	ND
	Dipor Bil-1	ND	ND	ND	ND	ND	ND	ND
	Bhangaghar	ND	ND	ND	ND	ND	ND	ND
	Kharguli	ND	ND	ND	ND	ND	ND	ND
	Pandu	ND	ND	ND	ND	ND	ND	ND
	Dipor Bil-2	ND	ND	ND	ND	ND	ND	ND
	WW/IITG	ND	ND	ND	ND	ND	ND	ND

High Low

Conflict of Interest and Authorship Conformation Form

Please check the following as appropriate:

- All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

- This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

- The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

- The following authors have affiliations with organizations with direct or indirect financial interest in the subject matter discussed in the manuscript:

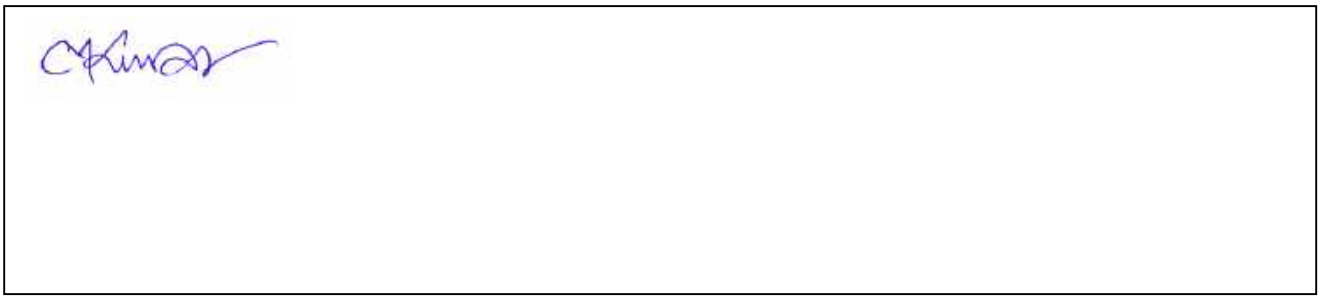
Author's name Affiliation

Manish Kumar[1,2*], [1]Discipline of Earth Science, Indian Institute of Technology Gandhinagar, Gujarat 382 355, India
[2]Kiran C Patel Centre for Sustainable Development, Indian Institute of Technology Gandhinagar, Gujarat, India
Payal Mazumder[3], [3]Centre for the Environment, Indian Institute of Technology Guwahati, Assam 781039, India
Jyoti Prakash Deka[4], [4]Discipline of Environmental Sciences, Gauhati Commerce College, Guwahati, Assam 781021, India
Vaibhav Srivastava[1], [1]Discipline of Earth Science, Indian Institute of Technology Gandhinagar, Gujarat 382 355, India
Chandan Mahanta[5], [5]Department of Civil Engineering, Indian Institute of Technology Guwahati, Assam 781039, India
Latha Rangan[6], [6]Department of Bioscience and Bioengineering, Indian Institute of Technology Guwahati, Assam 781039, India
Shilangi Gupta[7], [7]Gujarat Biotechnology Research Centre (GBRC), Sector- 11, Gandhinagar, Gujarat 382 011, India
Madhvi Joshi[7], [7]Gujarat Biotechnology Research Centre (GBRC), Sector- 11, Gandhinagar, Gujarat 382 011, India
AL. Ramanathan[8], [8]School of Environmental Sciences, Jawaharlal Nehru University, New Delhi 110067, India

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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



Comparative analysis of SARS-CoV-2 RNA load in wastewater from three different cities of Gujarat, India

Vaibhav Srivastava[1], Shilangi Gupta[1], Arbind Kumar Patel[1], Madhvi Joshi[3], Manish Kumar[1,2*]

[1]Discipline of Earth Science, Indian Institute of Technology Gandhinagar, Gujarat 382 355, India

[2]Kiran C Patel Centre for Sustainable Development, Indian Institute of Technology Gandhinagar, Gujarat, India

[3]Gujarat Biotechnology Research Centre (GBRC), Sector- 11, Gandhinagar, Gujarat 382 011, India

*Corresponding Author: manish.env@gmail.com; manish.kumar@iitgn.ac.in

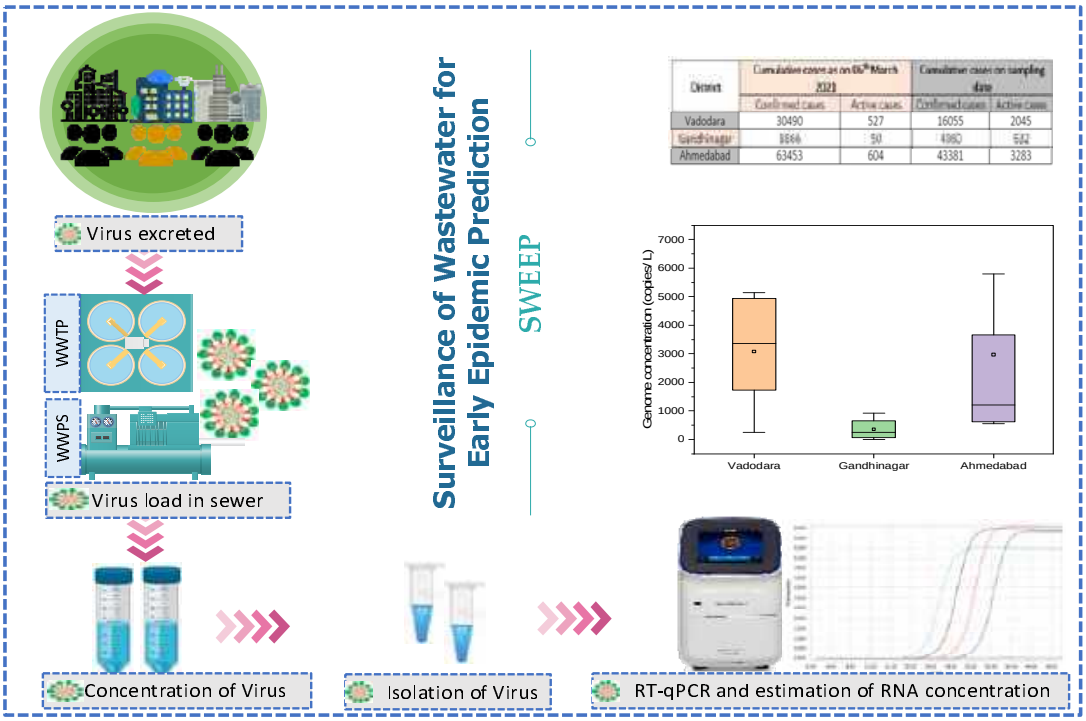
[1]Discipline of Earth Science, Indian Institute of Technology Gandhinagar, Gujarat 382 355, India

[2]Kiran C Patel Centre for Sustainable Development, Indian Institute of Technology Gandhinagar, Gujarat, India

[3]Gujarat Biotechnology Research Centre (GBRC), Sector- 11, Gandhinagar, Gujarat 382 011, India

Manish Kumar | Ph.D, FRSC, JSPS, WARI+91 863-814-7602 | Discipline of Earth Science | IIT Gandhinagar | India
Affiliate | Kiran C Patel Centre for Sustainable Development, IITGN
Member | Global Collaboration on WBE of COVID-19
Member | National Executive Committee, IWA, India
South Asia Representative | METREL Specialist Group, IWA
Editorial Board | Nature Partner Journal (NPJ) Clean Water
Editorial Board | Science of the Total Environment
Editorial Board | Case Studies in Chemical and Environmental Engineering
Editor | Hydrological Research Letter & Groundwater for Sustainable Development
Managing Guest Editor | Journal of Hazardous Material, Elsevier
Managing Guest Editor | Journal of Environmental Management, Elsevier
Google Scholar | *Research Gate* | *ORCID* | *Twitter* | *SCOPUS* | *Impactio*
Alt. email: manish.env@gmail.com ; kumarken2016@gmail.com

Graphical abstract



Abstract

The scientific community has widely supported wastewater monitoring of SARS-CoV-2 due to the early and prolonged excretion of coronavirus in the faecal matter. In the present study, eighteen influent wastewater samples from different wastewater treatment plants and pumping stations (5 samples from Vadodara city, 4 from Gandhinagar, and nine from Ahmedabad city) were collected and analyzed for the occurrence of SARS-CoV-2 RNA in Gujarat province, India. The results showed the highest SARS-CoV-2 genome concentration in Vadodara (3078 copies/ L), followed by Ahmedabad (2968 copies/ L) and Gandhinagar (354 copies/ L). The comparison of genome concentration more or less corresponded to the number of confirmed and active cases in all three cities. The study confirms the potential of the Surveillance of Wastewater for Early Epidemic Prediction (SWEEP) that can be used at a large scale around the globe for better dealing with the pandemic situation.

Keywords: COVID-19; SARS-CoV-2; Wastewater based epidemiology (WBE); Pandemic; Management

1. Introduction

Identifying the emergence and dissemination of the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) biohazard, which remains a global threat after even a year now into the 2019 coronavirus disease (COVID-19) pandemic. While some nations are now attempting to test every person (e.g., Korea and Iceland) to collect population-wide data, this method is inefficient, sluggish, and cost-prohibitive for most nations around the globe [1]. Based on a clinical study, the high pervasiveness of asymptomatic contagious individuals raises doubts about the available data on active cases [2,3]. Wastewater-based epidemiology (WBE) is drawing worldwide attention to COVID-19 surveillance due to the prevalence and protracted exudation of SARS-CoV-2 RNA in the feces of pre-symptomatic and deceased individuals, especially in developed economies with weak health infrastructure.

In India, the first case of COVID-19 was detected when a student returned from Wuhan, China, on 30th January 2020 [4]. Thenceforth, the number of infections has seen a steady spike. India has effectuated international travel bans and a stringent lockdown and curfew in the country to control the spread. Nonetheless, tropical countries like India are at higher risk due to relatively large and dense population demography, inadequate infrastructure, and healthcare services to meet very high demands. Gujarat, India, has recorded 272811 cumulative cases of COVID-19 (active cases: 3025), as of 06th March 2021. The details of the pandemic situation in Vadodara (VABO), Gandhinagar (GN), and Ahmedabad (AMD) have been shown in **Table 1** [5,6,7].

Table 1. Comparison of SARS-CoV-2 pandemic in three different cities of Gujarat, India

District	Cumulative cases as on 06th March 2021		Cumulative cases on sampling date	
	Confirmed cases	Active cases	Confirmed cases	Active cases
Vadodara	30490	527	16055	2045
Gandhinagar	8866	50	4980	632
Ahmedabad	63453	604	43381	3283

To better understand the skill and possible implementation of WBE surveillance of the novel coronavirus, the wastewater analysis for the occurrence of SARS-CoV-2 RNA was performed in three different cities (VABO, GN, and AMD) Gujarat, India, and comparisons were made with the clinical survey-based data. We also studied the temporal variance in the viral RNA concentration in STPs during post lockdown time in GN and AMD cities of Gujarat, India [8,9]. The prime goal of the present study was to substantiate the Surveillance of Wastewater for Early Epidemic Prediction (SWEEP) potential to know the extent of COVID infection by comparing the SWEEP data with clinical survey-based secondary data. Also, it will persuade the authorities and policymakers to incorporate WBE surveillance into the regular monitoring program and policy framework to manage current or future COVID-19 like pandemic situations efficiently.

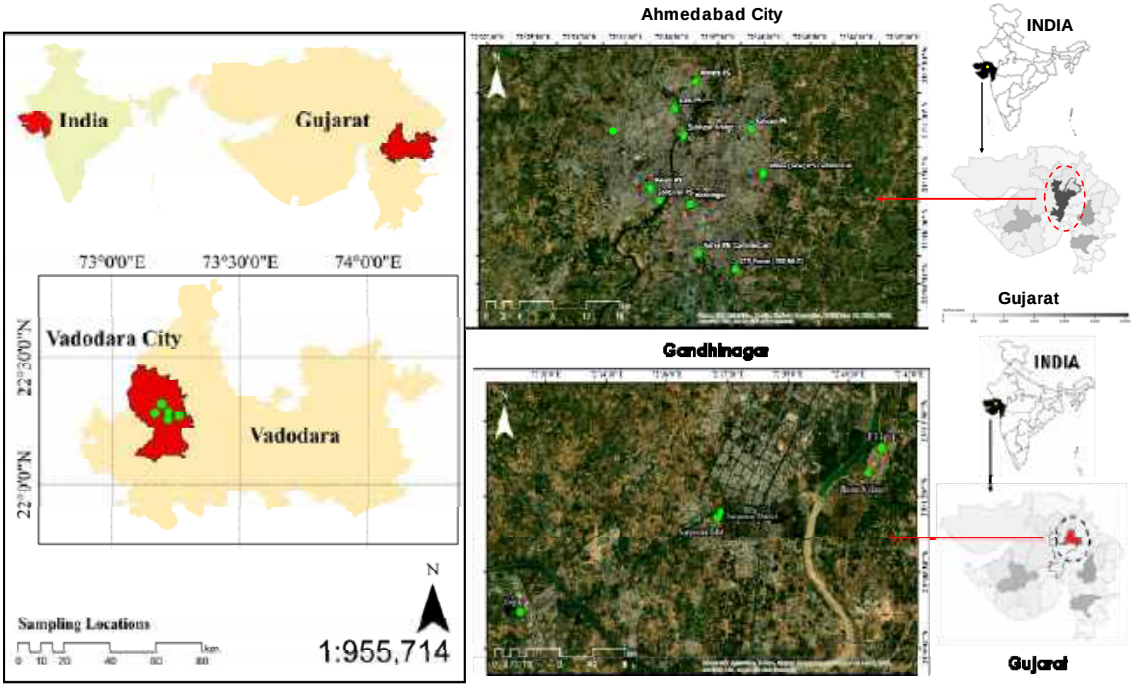


Fig. 1 Geospatial position of sampling locations in three different cities of Gujarat, India

2. Materials and methods

2.1 Sampling location

In the present study, eighteen influent wastewater samples (5 samples from Vadodara city, 4 from Gandhinagar, and nine from Ahmedabad city) were collected and analyzed for the presence of SARS-CoV-2 genetic material **(Fig. 1)**. In all three cities, the sewage is collected through a system comprising an underground drainage network, auxiliary pumping stations (APS), Sewage Treatment Plant, and disposal into the natural water bodies and rivers after treatment. Wastewater generated from all this development is collected by a network of underground sewers and pumping stations and is conveyed to the sewage treatment works for physical and biological treatment to meet the Gujarat Pollution Control Board (GPCB) guidelines before discharge into the nearest water body.

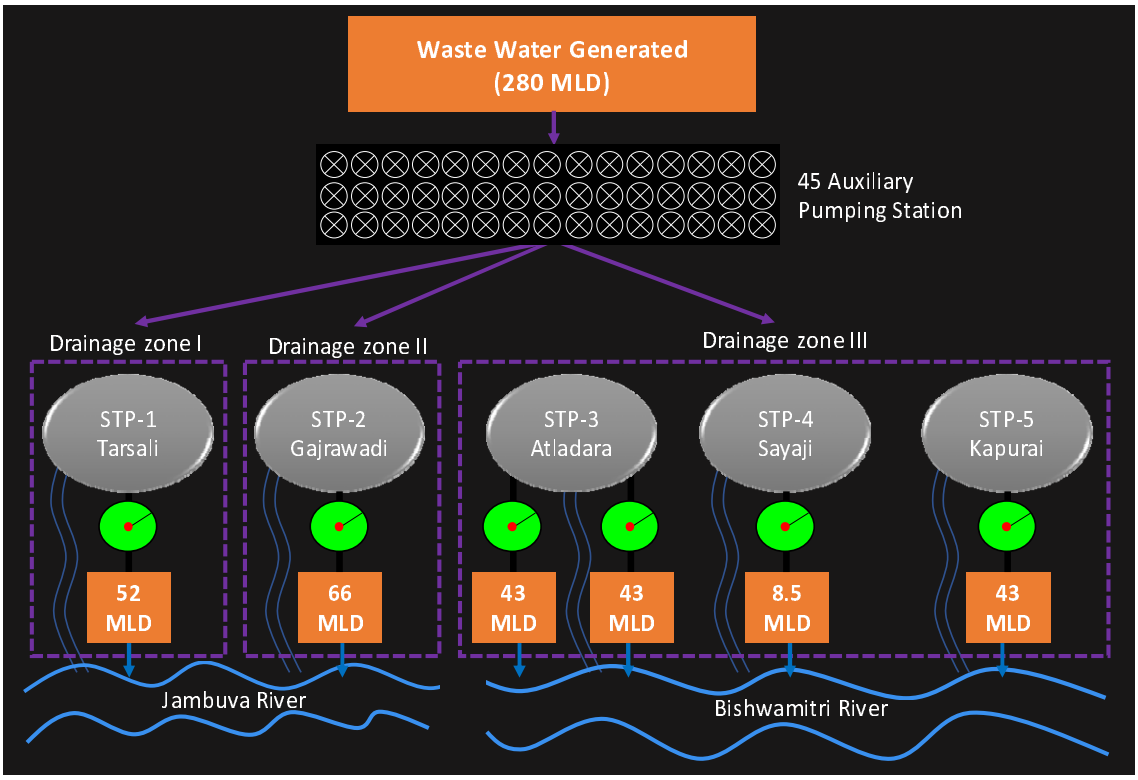


Fig.2. Sampling points and layout of the wastewater treatment in Vadodara, Gujarat, India

Vadodara Municipal Corporation has three drainage zones for the sewerage system based on the city's natural topography. Each of the drainage zones has a sewage treatment plant (STP). The sewage from drainage zones-I and II is disposed into the Jambuva River which, ultimately joins the River Vishwamitri. The sewage from drainage zone-III is disposed into the River Vishwamitri. The schematic layout of the wastewater treatment in Vadodara is shown in **Fig. 2.** Sewage Disposal Works Department of Vadodara includes 6 STPs & 49 Auxiliary/Main Pumping stations (APS/MPS). In the APS, the wastewater (sewage) from various parts of the city is collected in the wet well of the APS and then pumped to the Main Pumping Station and ultimately to the STP for treatment. Based on the natural topography of the Vadodara city sewerage system is divided into three drainage zones.

Likewise, the Ahmedabad Municipal Corporation comprises 9 STPs, 45 Sewage Pumping Stations, and an extended Sewage Network of ~2500 km present in the city. In Gandhinagar, the entire city's wastewater is first collected in the Sargasan Drainage Pumping Station via the underground pipe network. Thereafter, it is pumped and transferred mainly to the Jaspur and Sargasan STPs, where treatment processes occur. Details of the sampling locations, such as geospatial positions, capacity of the treatment plant, and wastewater source, are given in **Table 2**.

2.2 Sample collection and preparation

The untreated wastewater was collected from different locations in three cities, i.e., Vadodara (VABO), Gandhinagar (GN), and Ahmedabad (AMD) of Gujarat province, India. A total of 5 influent samples were collected from five STPs of VABO, 4 samples from STPs in GN, and 9 samples (8 from pumping stations and one from STP) from AMD in the first week of November 2020. The grab sampling method was used for the sample collection in 500ml polyethylene sterile bottles (Tarsons, PP Autoclavable, Wide Mouth Bottle, Cat No. 582240, India). Collected samples were transferred in an icebox to the laboratory and refrigerated at 4^0C until further process. A sampling blank was also prepared to examine the cross-contamination during transportation. The experiments were performed at Gujarat

Biotechnology Research Centre (GBRC), an approved laboratory by the Indian Council of Medical Research (ICMR), New Delhi.

Table 2. Details of Sampling locations

Sl No.	Sampling Location	Lat	Long	Wastewater source	Capacity (MLD)
Vadodara (VBO)					
1.	Tarsali	22°15'30.5"N	73°13'10.7"E	Residential & commercial	52
2.	Gajarawadi	22°17'12.3"N	73°13'13.8"E	Residential & commercial	66
3.	Kapurai	73°15'42.3"E	73°10'04.9"E	Residential & commercial	43
4.	Atladra	22°19'02.9"N	73°11'37.3"E	Residential & commercial	43+43
5.	Sayaji Garden	22°16'15.4"N	73°15'42.3"E	Residential	8.5
Gandhinagar (GN)					
6.	Basan inlet	23.12.28.4"N	72.40.56.3"E	Residential	2
7.	Sargasan inlet	23.11.42.4"N	72.37.18.1"E	Residential & commercial	10
8.	Jaspur inlet	23.09.40.7"N	72.32.20.3"E	Residential & commercial	76
9.	Academic institution	23°12'58.6"N	72°41'18.6"E	Institutional and residential	2.36
Ahmedabad (AMD)					
10.	Motera pumping station	23°06'36.9"N	72°36'0.9"E	Residential	NA
11.	Ranip pumping station	23°25'06.3"N	72°34'37.7" E	Commercial	NA
12.	Paldi pumping station	23°00'44.2"N	72°33'4.1" E	Commercial	NA
13.	Santivan pumping station	23°00'03.5"N	72°33'40.1" E	Residential	NA
14.	Maninagar pumping station	22°59'52.5"N	72°35'39.8" E	Residential	NA
15.	Satyam pumping station	23°03'59.6"N	72°39'38.5" E	Residential	NA
16.	STP vinzole	22°56'16.3"N	72°38'36.8" E	Residential & commercial	100
17.	Odhav pumping station	23°01'31.9"N	72°40'25.5" E	Commercial	NA
18.	Vatva pumping station	22°57'11.1"N	72°36'15.8" E	Commercial	NA

2.3 Concentration methods

The concentration method consisted of a PEG 9000 (80 g/L) and NaCl (17.5 g/L) precipitation protocol previously described by Kumar et al., 2020 [10] for wastewater samples. 30ml sample was centrifuged (Model: Sorvall ST 40R, Thermo Scientific) at 4000g for 30 minutes in a 50ml falcon tube followed by the filtration of the supernatant with a syringe filter of 0.2µ (Mixed cellulose esters syringe filter, Himedia). The 25ml sample filtrated was then treated with the NaCl (17.5 g/L) and PEG 9000 (80 g/L) and incubated at 17°C, 100 rpm

overnight (Model: Incu-Shaker™ 10LR, Benchmark). The sample was then transferred in an oak ridge tube for further centrifugation (Model: Incu-Shaker™ 10LR, Benchmark) at 14000g for 90 minutes, ultimately forming the pellets. RNase-free water was used for the resuspension of the viral particles after discarding the supernatant. The sample was then stored in a 1.5ml Eppendorf tube at a temperature of -40°C for RNA isolation.

2.4 Isolation of the SARS-CoV-2 viral genome

Using a commercially ready-for-use kit (NucleoSpin® RNA Virus, Macherey-Nagel GmbH & Co. KG, Germany), SARS-CoV-2 RNA isolation was performed. MS2 phage (10 µL), Proteinase K (20 µL) and RAV1 buffer (600 µL) consisting of carrier RNA were mixed with 300 µL of the concentrated viral particles. MS2 phage serves as the molecular process inhibition as a test control [11]. It was used to monitor the efficacy of RNA extraction and PCR inhibition. It should be remembered that MS2 may spontaneously exist in wastewater, so there is a risk that the retrieved MS2 may consist of both the spiked and the background viral material. As per the user manual instructions (Macherey-Nagel GmbH & Co. KG), further procedures were carried out. The last elution was done with 30 µL of kit-supplied elution buffer. Using a Qubit 4 Fluorometer (Invitrogen), RNA concentrations were checked.

The nucleic acid was analyzed to identify the S gene, N gene, and ORF1ab of SARS-CoV-2 and the internal control (MS2) with the help of RT-PCR using the TaqPath^{TM} Covid-19 RT-PCR package (Applied Biosystems). Amplification was conducted in a reaction (25 µL) vial containing 7 µL of RNAs derived from each sample. 2 µL of the positive control (TaqPath™ COVID-19 Control) and refined 5 µL of negative control were used for the study. Nuclease-free water was applied as a template-free control in this analysis. Additional process steps were executed, as defined in the product guidebook. The RT-qPCR step consisting of 40 cycles, included UNG incubation (25 °C for 2 min), reverse transcription (53 °C for 10 min), and activation (95 °C for 2 min). The reactions were conducted and elucidated as instructed in the handbook of Applied BiosystemsTM 7500 Fast Real-Time PCR.

2.5 Data visualization

OriginPro 2019b software has been used for data analysis and to draw boxplots.

3. Results and discussion

Wastewater samples collected from three cities (Vadodara, Gandhinagar, and Ahmedabad) of Gujarat, India, showed a great variation in SARS-CoV-2 RNA load. Comparison of RT-PCR assay findings for the detection of SARS-CoV-2 RNA (N, ORF 1ab, and S genes) among three cities showed 100% positive samples in Vadodara (5/5), 75% in Gandhinagar (3/4), and 100% in Ahmedabad (9/9). The average N-gene copies were found to be maximum in AMD (4731 copies/L), followed by VABO (3179 copies/ L) and GN (243 copies/ L). The ORF 1ab-gene copies were found maximum in wastewater samples collected from VABO (3730 copies/ L), followed by AMD (2756 copies/ L) and GN (611 copies/ L). Similarly, the descending order of S-gene copies was: VABO (2325 copies/ L)> AMD (1417 copies/ L)> GN (207 copies/ L). Conclusively, a greater genome concentration was noticed in VABO (3078 copies/ L), trailed by AMD (2968 copies/ L) and GN (354 copies/ L). The distribution of SARS-CoV-2 gene copies in wastewater samples collected from three cities is depicted in **Fig. 3.** Also, the variation in gene copies of the SARS-CoV-2 targeted genes and genome concentration in wastewater samples is shown in **Table 3**.

The trends of virus genetic load were more or less in line with the number of confirmed and active cases, which were highest in VABO, followed by AMD and GN (**Table 1**). A very nominal difference in the SARS-CoV-2 genome concentration in wastewater samples of VBO and AMD was noticed despite a difference of more than two-folds in the cumulative number of confirmed cases and above 1000 active cases in AMD compared to VBO on sampling date **(Table 1).** This trend can be ascribed to the fact that samples were collected from STPs (5) in VABO; while in the case of AMD, samples were mainly collected from pumping stations (8). Therefore, the concentration of SARS-CoV-2 RNA might be higher in STPs as compared to the sewage pumping station that was reflected in the analysis in VABO. However, some other factors such as population density, city development plan, sewerage system, health amenities, and management strategies may influence the SARS-CoV-2 genetic load in wastewater samples.

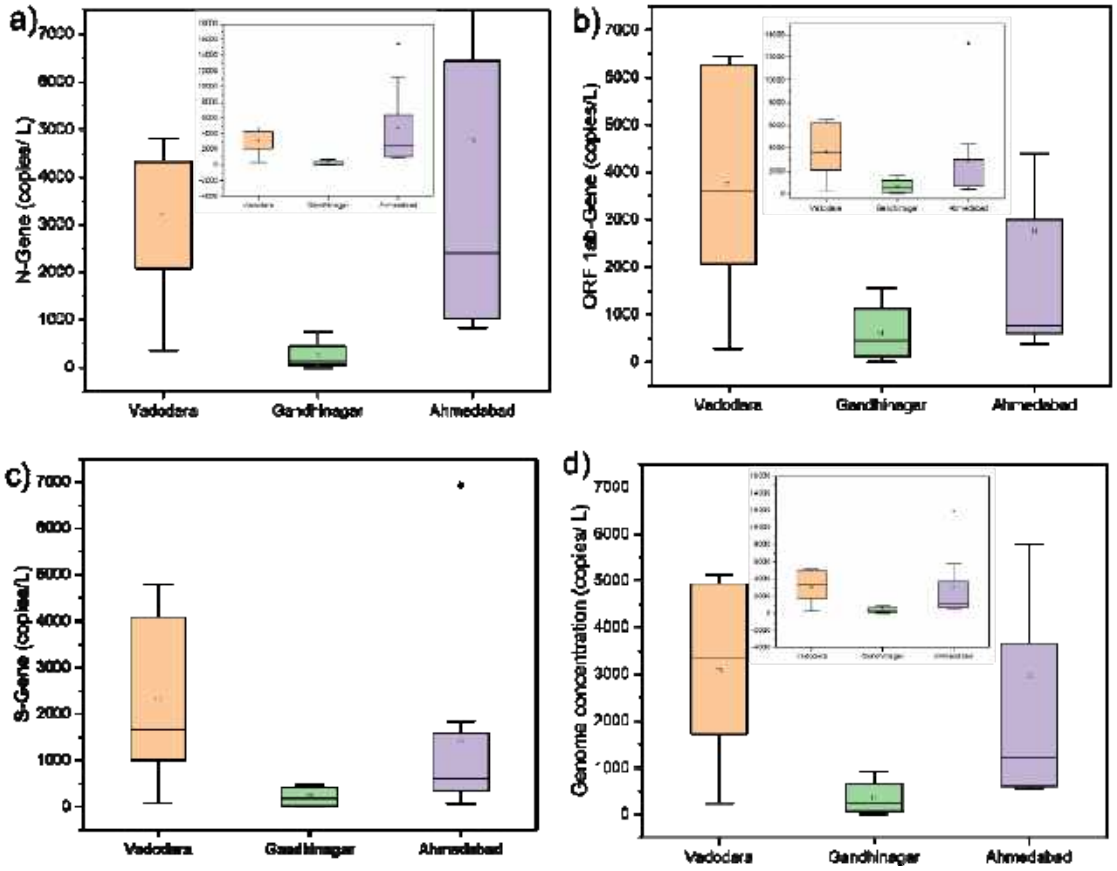


Fig.3. Distribution of SARS-CoV-2 gene copies, collected from three different cities of Gujarat, India; a.) N gene, b.) ORF 1ab gene, c.) S gene, and d) Genome concentration

The results were in agreement with Kumar et al. [8], who studied weekly temporal variation in SARS-CoV-2 genetic material concentration in wastewater samples targeting N, ORF 1ab, and S genes in a two-month study in Gandhinagar. The results suggested a positive correlation between SARS-CoV-2 genome concentration in wastewater and the number of confirmed cases, which was found higher in the month of September compared to August 2020, corresponded to ~2.2 folds increase in confirmed cases during the study period. Likewise, in another three-month (September to November 2020) weekly analysis of wastewater samples from 9 different locations in Ahmedabad city showed similar trends, and the maximum SARS-CoV-2 genome concentration was noticed in November (~10729 copies/ L), trailed by September (~3047 copies/ L), and October (454 copies/ L) in line with a

~ 1.5-fold rise in the confirmed cases during the study period. The decrease in SARS-CoV-2 concentration in October subjected to a decline of 20.5% in active cases (~844 cases), while a significant rise in virus RNA in November 2020 was due to a rise of 1.82% in active cases (~59 cases). Though the rise in active cases was nominal in November, but at the same time, a sharp rise of >7000 new cases (17.3%) was reported in November 2020 [9]. There are many other studies in the public domain from different parts of the world, such as the Netherlands [3], Spain [12], the USA [13,14], Paris [15], China [16], India [8,10], Australia [17], etc. which support WBE surveillance of COVID-19.

Table 3. Variation in gene copies of the SARS-CoV-2 targeted genes and genome concentration in wastewater samples, collected from three different cities of Gujarat, India

Sampling date	Station	Ct value			Gene copies (copies/ L)			
		N	ORF	S	N	ORF	S	Genome concentration
02.11.2020	**Vadodara**							
	STP-1 Tarsali	29.9	30.31	31.43	4814	3594	1656	3355
	STP-2 Gajrawad	31.09	31.1	32.16	2086	2069	1012	1722
	STP-3 Atladara	30.04	29.53	29.91	4346	6261	4792	5133
	STP-4 Sayaji	33.76	34.18	35.96	360	275	93	243
	STP-5 Kapurai	30.06	29.49	30.13	4292	6449	4072	4938
02.11.2020	**Gandhinagar**							
	Basan Inlet	32.65	31.53	33.32	733	1551	474	919
	Jaspur Inlet	36.00	34.53	36.91	91	222	53	122
	IIT inlet	ND	ND	ND	0	0	0	0
	Sargasan inlet	35.20	32.78	34.04	147	672	301	373
05.11.2020	**Ahmedabad**							
	Motera PS	30.63	33.65	33.73	2873	386	365	1208
	Ranip PS	29.50	30.58	31.50	6415	2986	1580	3661
	Paldi PS	32.39	33.02	33.79	869	577	352	599
	Santivan PS	31.57	31.88	32.95	1506	1220	603	1110
	Maninagar PS	32.46	32.98	34.57	830	593	217	547
	Satyam PS	32.14	32.61	36.50	1024	752	68	615
	STP Vinzole	28.75	30.03	31.27	11148	4386	1848	5794
	Odhav PS	28.31	28.52	29.39	15523	13247	6930	11900
	Vatva PS	30.90	32.83	32.53	2390	654	793	1279

One of the main advantages of WBE is that it includes both asymptomatic and symptomatic individuals, therefore can give a better picture of the pandemic situation as compared to clinical-based secondary data, which includes only symptomatic patients and rely on the number and efficiency of clinical tests. Therefore, under certain circumstances, it is possible

that despite an increase in SARS-CoV-2 RNA load in wastewater, no significant change in COVID cases may observe. Consequently, SWEEP technology can provide the actual extent of the infection at sub-city or zone levels and help in identifying the hot spots within a city.

4. Conclusion

A comparison of SARS-CoV-2 RNA presence in wastewater samples from three cities of Gujarat unveiled the highest load in VBO, followed by AMD and GN. The virus genetic material showed a positive correlation with the number of confirmed and active cases in all three cities. Also, the genome concentration more or less corresponded to the number of confirmed and active cases in the present study. The study concludes that regular monitoring of wastewater samples could be used to know the pandemic situation in a particular area and help in tuning the management interventions efficiently. Though WBE has immense potential that must be exploited and included in the policy framework around the globe; however long-scale time-series data along with epidemiological information is required to substantiate the robustness of this technology. Also, future emphasis should be paid to developing a predictive model using WBE and clinical survey data for a better understanding of the situation to the policymakers and enhancing the preparedness and management of epidemic/ pandemic situations.

Notes:

The authors declare no competing financial interest.

Acknowledgement:

This work is funded by UNICEF, Gujarat and UKIERI. We also acknowledge the help received from Mr. Alok Thakur, and other GBRC staffs who contributed towards sample and data analyses.

References

[1] Hart, O, E., Halden, R. U. 2020. Computational analysis of SARS-CoV-2/COVID-19 surveillance by wastewater-based epidemiology locally and globally: Feasibility, economy,

opportunities and challenges. Sci. Total Environ. 730, 138875. https://doi.org/10.1016/j.scitotenv.2020.138875

[2] Rimoldi, S. G., Stefani, F., Gigantiello, A., Polesello, S., Comandatore, F., Mileto, D., Maresca, M., Longobardi, C., Mancon, A., Romeri, F., Pagani, C., Moja, L., Gismondo, M.R., Salerno, F.,2020. Presence and vitality of SARS-CoV-2 virus in wastewaters and rivers. Sci. Total Environ. 744:140911

[3] Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020. Presence of SARS Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. Environ Sci Technol Lett. 7 (7), 511–516. https://doi.org/10.1021/acs.estlett.0c00357

[4] WHO. Coronavirus disease (COVID-19), India situation report. WHO India 2020. https://www.who.int/india/emergencies/india-situation-report

[5] Amdavad Municipal Corporation, Gujarat, India. 2021. https://ahmedabadcity.gov.in/portal/web?requestType=ApplicationRH&actionVal=loadCoronaRelatedDtls&queryType=Select&screenId=114

[6] Gandhinagar Municipal Corporation, Gujarat, India. 2021. http://gandhinagarmunicipal.com/corona_dashboard/

[7] COVID India, Gujarat District Wise COVID Data, Gujarat, India. 2021. https://www.covid19india.org/state/GJ

[8] Kumar, M., Joshi, M., Patel, A. K., & Joshi, C. G., 2021a. Unravelling the early warning capability of wastewater surveillance for COVID-19: A temporal study on SARS-CoV-2 RNA detection and need for the escalation. Environ. Res. 110946. https://doi.org/10.1016/j.envres.2021.110946

[9] Kumar, M., Joshi, M., Shah, A. V., Srivastava, V., & Dave, S., 2021b. First wastewater surveillance-based city zonation for effective COVID-19 pandemic preparedness powered by early warning: A study of Ahmedabad, India. *medRxiv*. https://doi.org/10.1101/2021.03.18.21253898

[10] Kumar, M., Patel, A.K., Shah, A.V., Raval, J., Rajpara, N., Joshi, M. and Joshi, C.G., 2020. First proof of the capability of wastewater surveillance for COVID-19 in India through detection of genetic material of SARS-CoV-2. Sci. Total Environ. *746*, p.141326. https://doi.org/10.1016/j.sci378totenv.2020.141326

[11] Haramoto, E., Kitajima,M., Hata, A., Torrey, J.R., Masago, Y., Sano, D., Katayama, H., 2018. A review on recent progress in the detection methods and prevalence of human enteric viruses in water. Water Res. 135, 168–186. https://doi.org/10.1016/j.watres.2018.02.004

[12] Randazzo, W., Truchado, P., Cuevas-Ferrando, E., Simón, P., Allende, A., Sánchez, G., 2020. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. Water Res. 526 (1), 135–140. https://doi.org/10.1016/j.bbrc.2020.03.044181, 115942.

[13] Wu, Y., Guo, C., Tang, L., Hong, Z., et al., 2020a. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. Lancet Gastroenterol. Hepatol. 5, 434–435. https://doi.org/10.1016/S2468-1253(20)30083-2.

[14] Nemudryi, A., Nemudraia, A., Surya, K., Wiegand, T., Buyukyoruk, M., Wilkinson, R., Wiedenheft, B., 2020. Temporal detection and phylogenetic assessment of SARSCoV- 2 in municipal wastewater. medRxiv https://doi.org/10.1101/2020.04.15.20066746 2020.03.22.20041079.

[15] Wurtzer, S., Marechal, V., Mouchel, J.M., Moulin, L., 2020. Time course quantitative detection of SARS-CoV-2 in Parisian wastewaters correlates with COVID-19 confirmed cases. medRxiv https://doi.org/10.1101/2020.04.12.20062679 2020.04.12.20062679

[16] Zhang, W., Du, R.H., Li, B., Zheng, X.S., Yang, X. Lou, Hu, B., Wang, Y.Y., Xiao, G.F., Yan, B., Shi, Z.L., Zhou, P., 2020. Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes. Emerg. Microbes Infect. 9 (1), 386–389. https://doi.org/10.1080/22221751.2020.1729071.

[17] Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J. W., ... & Mueller, J. F., 2020. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community. Sci. Total Environ. *728*, 138764. https://doi.org/10.1016/j.scitotenv.2020.138764