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Article

Molecular Monitoring of SARS-CoV-2 in Different Sewage Plants in Venice and the Implications for Genetic Surveillance

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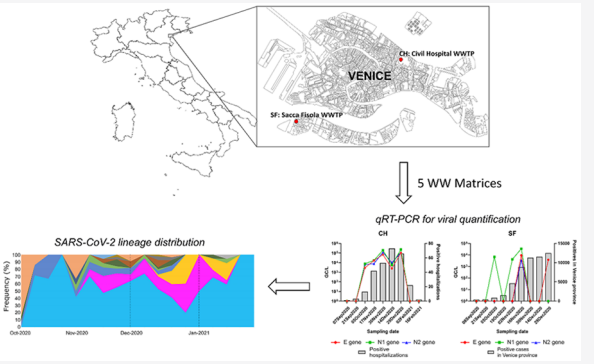
ABSTRACT: Wastewater-based epidemiology is now widely used as an indirect tool to monitor the spread of SARS-CoV-2. In this study, five different sample matrices representing diverse phases of the wastewater treatment process were collected during the second wave of SARS-CoV-2 from two wastewater treatment plants (WWTPs) serving the Civil Hospital and Sacca Fisola island in Venice, Italy. Positive SARS-CoV-2 detections occurred at both WWTPs, and data on viral genome detection rate and quantification suggest that the pellet (i.e., the particulate resulting from the influent) is a sensitive matrix that permits reliable assessment of infection prevalence while reducing time to results. On the contrary, analysis of post-treatment matrices provides evidence of the decontamination efficacy of both WWTPs. Finally, direct sequencing of wastewater samples enabled us to identify B.1.177 and B.1.160 as the prevalent SARS-CoV-2 lineages circulating in Venice at the time of sampling. This study confirmed the suitability of wastewater testing for studying SARS-CoV-2 circulation and established a simplified workflow for the prompt detection and characterization of the virus.



KEYWORDS: *SARS-CoV-2, sewage, wastewater, wastewater treatment plants, wastewater-based epidemiology, genetic surveillance*

INTRODUCTION

After the appearance and rapid global spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a huge effort was devoted to identifying the best and most cost-effective strategies for virus surveillance and tracing. Among the available solutions, wastewater-based epidemiology (WBE) has attracted attention within the scientific community. Currently, this approach is being extensively applied to monitor the presence and emergence of other known viruses (ref 1 and references therein) such as norovirus, hepatitis A virus, and poliovirus[2,3] and can provide a detailed methodological background for the development of analytical methods for the detection of SARS-CoV-2 in sewage. Indeed, although SARS-CoV-2 is primarily a respiratory pathogen, the rationale for monitoring wastewater is driven by the fact that the virus can colonize the gastrointestinal tract and eventually be shed through feces of positive patients, thus entering the sewage system.[4] Previous studies have confirmed that viral shedding occurs in 48−67% of infected individuals[5,6] with a persistence in stool from 2 to 47 days after the onset of symptoms.[5] Because fecal shedding occurs in both symptomatic and asymptomatic patients, the wastewater surveillance of SARS-CoV-2 may represent a quick and indirect tool for monitoring infection trends in a community, complementing information from direct clinical testing.

Initial data on the molecular detection of viral RNA in sewage in The Netherlands and Italy identified positive samples collected within a few days of the first recorded clinical cases.[7,8] Consequently, a growing number of WBE studies reported the positive detection of SARS-CoV-2 in wastewater as an indicator of the true incidence of the virus in the population (Australia,[9] Japan,[10] United States,[11] and India[12]), proving the utility of sewage for surveillance and early warning.[13,14] However, despite the doubtless benefits and prospects of SARS-CoV-2 WBE, several open questions remain. First, additional information is required to define the best technical

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approach and sample type to provide robust analytical data about the presence of SARS-COV-2 in specific areas in a timely and cost-effective manner. To date, WBE studies have focused on analyzing the influent, i.e., untreated water.[15] However, taking into consideration the affinity of enveloped viruses for biosolid particles,[16,17] one can assume that some information might be lost along the treatment line, raising questions about the possibility of using other solid phases to track COVID-19 prevalence in a community.[10−12,16,18−23] In addition, analyses of secondary and tertiary treated waters may provide key information about the fate of the virus after the completion of decontamination treatments. However, studies aiming to assess and compare the efficacy of different wastewater treatment plants (WWTPs) in removing viral traces from different catchment areas are still limited.[11,23] Numerous disinfection processes for treating wastewater are now available, and new technologies are constantly being developed to guarantee the inactivation of potentially harmful microorganisms. Although the infectivity of the virus at the end of the treatment line is negligible and hence does not pose a risk to humans,[24] obtaining new information about the efficacy of different treatment processes in catchment areas of different population sizes and densities is still of interest.

There is also an important need to assess the sensitivity of sewage monitoring in detecting COVID-19 outbreaks at their onset and accurately predicting the number of positive individuals in the local population. Modeling studies have estimated the minimum number of shedders per catchment required to detect traces of viral RNA, ranging from as low as 0.005% (100 shedders per 2 000 000) to 0.1% (1 shedder in 1000).[19,25] However, test sensitivity appears to be highly dependent on the decay of the virus in wastewater, which in turn is affected by the effect of temperature and by the transit time of stool in the sewage system.[26] Accordingly, to routinely apply wastewater surveillance for the estimation of SARS-CoV-2 infection prevalence, it is essential to acquire new data (i) to define the minimum number of shedders in a population required for the identification of new SARS-CoV-2 outbreaks and (ii) to validate the accuracy of recently proposed methods that estimate the number of infected individuals from sewage data with a high degree of confidence.[27,28]

Another key aspect of WBE is the genetic surveillance of SARS-CoV-2 in sewage. Leveraging current next-generation sequencing (NGS) technologies offers an unprecedented opportunity to characterize the viral genetic diversity in different communities and promptly detect recent introductions of variants of concern or new variants of SARS-CoV-2 at a population level. This approach has been successfully applied in several countries, including France,[29,30] the United States,[31−33] Israel,[34] The Netherlands, and Belgium.[35] Importantly, if coupled with targeted sequencing from clinical cases, WW sequencing has the potential to increase the coverage of information and provide critical data for the genetic surveillance and monitoring of SARS-CoV-2.

The case study of Venice is of major interest because of its uniqueness, with more than 7000 septic tanks and 200 localized biological WWTPs that treat wastewater before releasing it into the city's canals using a parceled, noncentralized sewage system. The peculiarity of Venice also resides in its hydrological features, which intimately link the city with the surrounding lagoon environment, as well as its relevance as a tourism destination that attracts thousands of visitors from abroad. Focusing on this complex scenario, our study aims to provide new data on sewage monitoring through the investigation of five different wastewater matrices collected during the second wave of SARS-CoV-2 (late 2020 to early 2021) along the treatment line of two WWTPs with different operating practices. A selection of positive samples were sequenced to assess the predominant SARS-CoV-2 lineage circulating within the local community at the time of sampling. Overall, our study allows us (i) to propose a new analytical approach based on the testing of the solid component of untreated wastewater for the detection and sequencing of SARS-CoV-2 RNA, (ii) to provide new information about the sensitivity and accuracy of WBE to predict the number of shedders, (iii) to compare the efficacy of two WWTPs in removing SARS-CoV-2 RNA, and (iv) to obtain new information about the suitability of different matrices for the genetic characterization of SARS-CoV-2 at a population level.

MATERIALS AND METHODS

Wastewater Treatment Plants and Sampling Scheme. Wastewater samples were collected from the WWTP (i) at the Civil Hospital (CH) in the historic center of Venice, which collects the waste from all of the hospital wards and the services with a capacity of 4000 equivalent inhabitants and a flow discharge of 500 m^3/day, and (ii) at Sacca Fisola (SF), serving a small urban community of ∼750 equivalent inhabitants located on the Giudecca island, with a flow discharge of 144 m^3/day (Figure S1). The CH and SF plants are both membrane bioreactor (MBR) plants that couple activated sludge technology with the separation of sludge by filtering membranes, replacing the original sedimentation with gravity. The CH plant effluent is treated with an ultraviolet (UV) disinfection step before being discharged into the lagoon in the Fondamenta Nove area. In contrast, the discharge from the WWTP of SF flows into the Canale della Giudecca without any additional disinfection treatment.

Grab samples were collected in the morning (10:00 a.m. to 12:00 p.m.) approximately every 2 weeks, from September 2020 to February 2021 for the CH and from September 2020 to December 2020 for SF. Five hundred milliliters of material was collected in plastic containers and stored at −80 °C for a minimum period of 1 month to a maximum of 6 months, until processed. Five different matrices for each WWTP and date of sampling were gathered and analyzed for the presence of SARS-CoV-2 RNA: (i) the influent matrix (IN), (ii) the pellet resulting from the clarification of influent samples prior to concentration (PT), (iii) the sludge (SL), (iv) the effluent after all treatments still in the plants (OUT), and (v) the effluent (EF) once it enters Venice's canals, grabbed at the discharge point of the WWTPs.

Viral Quantification in Field Samples. Prior to the analysis of field samples, procedures for the concentration of aqueous samples, isolation of RNA, and quantification of the viral genome by qRT-PCR were optimized. The detailed optimization workflow is provided in the Supporting Information. Samples were thawed under refrigeration and incubated at 56 °C for 30 min to inactivate potentially harmful pathogens and guarantee operator safety.[7,36−38] Fifty milliliters of IN, OUT, and EF samples were spiked with a known amount of infectious bronchitis virus (IBV; 5 μL of virus with a hemoagglutination titer of 1:256) serving as the process internal control for the concentration step and centrifuged at 3214*g* for 15 min at 4 °C to remove debris and large particulates. In this phase, suspended solids from all influent wastewater samples formed a visible pellet (PT), which was stored and subsequently analyzed. Forty-five milliliters of the clarified supernatant was concentrated using

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Table 1. Samples Subject to NGS Analysis and Sequencing Results

WWTP	date	matrix	Ct values (E gene)	GC/L (E gene)	% genome	lineage
CH	October 5, 2020	SL	29.19	1.89×10^7	0.09	–
CH	November 30, 2020	IN	31.81	6.88×10^4	63.81	B.1.177[a]
CH	November 30, 2020	PT	31.62	2.84×10^6	96.21	B.1.177[b]
CH	December 29, 2020	IN	31.34	1.04×10^5	99.76	B.1.160[c]
CH	December 29, 2020	PT	31.93	1.78×10^6	95.15	B.1.160[d]
SF	November 2, 2020	SL	32.10	2.51×10^6	54.05	–
SF	November 16, 2020	IN	34.58	9.35×10^3	54.80	–
SF	November 16, 2020	PT	37.09	3.67×10^4	23.30	–

[a]Sequences were submitted to GISAID[60] with an identification name of VEN-IZSVe-21RS1495_VE_CH-IN. [b]Sequences were submitted to GISAID[60] with an identification name of VEN-IZSVe-21RS2156−3_VE_CH_PT. [c]Sequences were submitted to GISAID[60] with an identification name of VEN-IZSVe-21RS1496_VE_CH-IN. [d]Sequences were submitted to GISAID[60] with an identification name of VEN-IZSVe-21RS1498_VE_CH-PT.

the Amicon Ultra-15 with a NMWL of 30 kDa (Millipore) through three consecutive centrifugation rounds of 15 mL each (3214*g* for 10 min at 4 °C), to obtain approximately 900 μL of the concentrated sample. In addition, a washing-out step was performed by reconstituting the concentrate with 15 mL of nuclease-free water followed by a centrifugation round to yield a concentrate of ∼200 μL. Subsequently, a final step of column filter washing with 100 μL of nuclease-free water was carried out. Washing water was pooled with the concentrate sample to yield a final volume of approximately 300 μL of concentrate for subsequent RNA extraction.

The IN, PT, and OUT samples were subjected to RNA purification using the Quick Viral RNA kit (Zymo research) (starting volume of 200 μL, elution volume of 50 μL), while the QIAamp Viral RNA kit (QIAGEN) was adopted for EF samples (starting volume of 140 μL, elution volume of 60 μL). The intype IC-RNA (Indical Bioscience) was added to each sample during the lysis phase in a quantity equal to $^1/_{10}$ of the sample elution volume. SL samples (starting volume of 3 mL) were processed using the RNAeasy Powersoil total RNA kit (QIAGEN) according to the manufacturer's protocol.

qPCR reactions for the E, N1, and N2, targeting viral genomic regions within the envelope and nucleocapsid protein genes,[39,40] were assembled and performed as described in the Supporting Information and Table S1. Three technical replicates were predisposed for each specimen. The conformity of IN, PT, OUT, and EF samples was assessed by co-amplifying the IC-RNA (expected Cq of 28 ± 3),[41] while for SL samples, the process was verified through the amplification of the human RNase P gene[42] in a separate reaction. When necessary, to further confirm the negativity of doubtful samples, a qRT-PCR targeting IBV was also run (procedure modified from ref 43). Conforming specimens were considered positive for SARS-CoV-2 when they (i) tested positive with a Cq of <40 in at least two technical replicates within one single viral target or (ii) yielded one positive replicate with a Cq of <40 for at least two different viral targets.

For absolute quantification, standard curves were generated for all of the viral genes analyzed using 10-fold dilutions of target-specific plasmids, from 10^4 to -10^0 GC/μL. Calibration curves were included in each session and were run in triplicate. For each specimen, quantitative results for E, N1, and N2 obtained by interpolation and expressed as GC/L (mean of technical replicates) were plotted in time course graphs together with the number of positive cases recorded, using GraphPad Prism 8. Spearman's correlation coefficient was calculated for each viral target, to assess the correlation between analytical data and COVID-19 records.

Estimates of Infected Individuals Based on Average Daily Wastewater Flow. To assess the minimum number of shedders necessary to detect with sufficient sensitivity SARS-CoV-2 RNA in sewage, and to identify the most reliable method for inferring the number of estimated infected cases (EIC) based on viral quantification in WWTP samples, two different formulas (McMahan et al.,[27] termed "J" hereafter, and Saththasivam et al.,[28] termed "N" hereafter) were tested. To validate these prediction models, estimates were based on data of the influent samples from the CH, because the exact number of infected people was available from hospital daily bulletins. While the two approaches entail the same factors, the absolute values used for fecal load (α) and fecal shedding (β) are different. The formula proposed by Saththasivam and collaborators[28] was modified as follows:

$$N = (\mathrm{CRNA} \times F)/\alpha\beta$$

where CRNA is the measured SARS-CoV-2 RNA concentration at the inlet of the WWTP (sum of RNA copies per liter of the IN and PT fractions), F is the volumetric flow rate of the WWTP (liters per day, equal to a 499.2 m^3 average daily flow rate), α is the fecal load (grams per day per person), and β is fecal shedding (RNA copies per gram).

In contrast to Saththasivam et al.,[28] factors related to losses in the sewer were not taken into account because the WWTP of the CH is a closed plant. The results for the E, N1, and N2 genes obtained from the influent samples were plotted on a graph as the EIC on the different sampling days using GraphPad Prism 8.[44]

For each tested formula, the mean Δ(EIC) value, which indicates the mean value of the difference between the real number of cases and the mathematical estimate calculated herein, was used to compare the accuracy of the two formulas.

Next-Generation Sequencing and Bioinformatics Analysis. Eight positive samples for SARS-CoV-2 RNA from the CH and SF that were representative of the different sample matrices were subject to Illumina NGS sequencing. Candidate samples were selected as follows: (i) GC/L ≥ 1.78×10^6 in at least one of the three target genes or (ii) samples positive for more than one sample matrix on the same date, independent of the viral load recorded. This latter requirement had the purpose of comparing possible differences in sequencing quality as well as verifying the presence of the same variants in the different matrices compared [e.g., IN vs PT for November 30, 2020, at the CH (see Table 1)].

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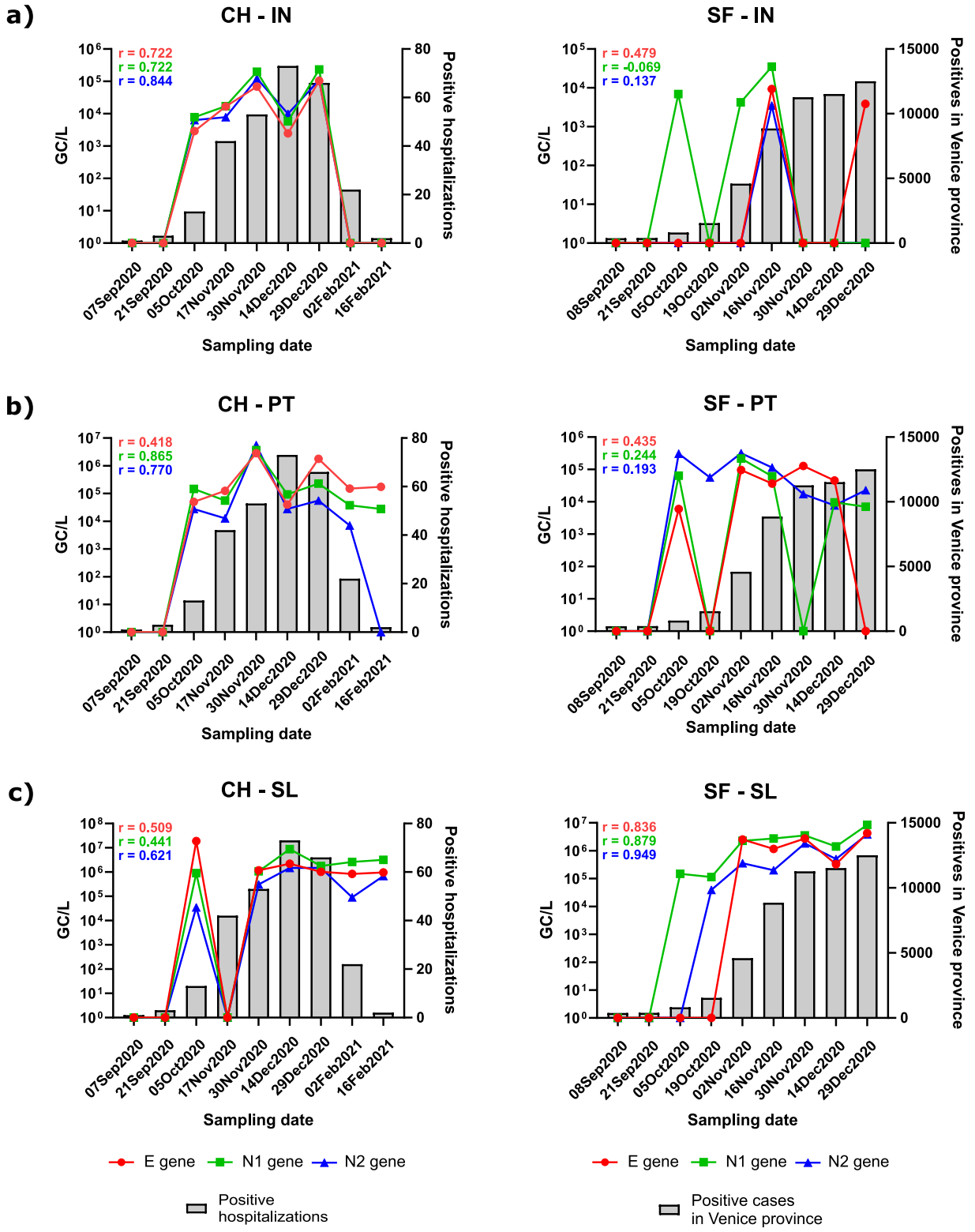

Figure 1. Detection of SARS-CoV-2 RNA in the (a) IN, (b) PT, and (c) SL matrices from the CH and SF WWTPs. GC/L values (lines, left *y*-axis) are plotted against the real number of COVID-19 positive cases registered for each sampling date (histograms, right *y*-axis). For the Civil Hospital, the real number of positive hospitalizations was used as a reference. For Sacca Fisola, the overall number of positives in the province of Venice was used. The *x*-axis reports the sampling date. GC/L values were obtained from the amplification of E, N1, and N2 targets that are color-coded as follows: red circles for E, green squares for N1, and blue triangles for N2. For each target, the Spearman's correlation coefficient *r* with respect to the number of COVID-19 cases is reported.

Wastewater samples were analyzed for the presence of SARS-CoV-2 RNA using the ARTIC amplification protocol (ARTIC nCoV-2019 V3 Panel, Integrated DNA Technologies). RNA from each sample was reverse transcribed using the LunaScript RT SuperMix (New England Biolabs), and 5 μL of cDNA was amplified in duplicate with the ARTIC primer set (Integrated

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DNA Technologies) and the Q5 Hot Start High Fidelity DNA Polymerase (New England Biolabs). The following conditions were utilized: 98 °C for 30 s, 95 °C for 15 s, and 63 °C for 5 min (35−40 cycles), with a 4 °C holding step. Amplicons were purified using 0.8× AMPure XP beads (Beckman Coulter) and quantified using the Qubit dsDNA HS Assay kit (ThermoFisher Scientific). Libraries were prepared with the Illumina DNA Prep kit (Illumina) according to the manufacturer's instructions, and quality was checked using the Bioanalyzer High Sensitivity DNA kit (Agilent Technologies). Libraries were pooled at equimolar concentrations and sequenced on the MiSeq platform (Illumina) with Miseq Reagent kit version 2 in 150 PE mode.

Read quality was assessed using FastQC.[45] Cutadapt[46] was employed to perform quality filtering and adapter trimming. Reads were aligned against the Wuhan SARS-CoV-2 genome (NCBI Reference Sequence NC_045512.2) using BWA-MEM.[47] GATK (ref 48 and references therein) and Picard Tools (http://broadinstitute.github.io/picard/) were used to correct potential errors, realign reads around insertions and deletions (indels) with respect to the reference genome, and recalibrate base quality. Variants call was performed with LoFreq*.[49] Consensus sequences were produced with a script developed in house. Single-nucleotide polymorphisms (SNPs) and indels with a frequency ≥50% were reported in the consensus, while positions with a coverage <10 were masked with the N IUPAC symbol. The minimum genome coverage considered for reliable lineage assignment was 60%. Lineage assignment was performed using the Pangolin tool[50] and confirmed with Nextclade Web[51] (https://clades.nextstrain.org).

RESULTS AND DISCUSSION

Detection of SARS-CoV-2 RNA in Different Matrices and WWTPs of Venice. The mean recovery efficiency of SARS-CoV-2 assessed during the optimization phase was 79 ± 25% for all of the matrices and molecular targets investigated. The analytical procedure optimized in here appeared to have a slightly better yield compared to those of previous studies.[7,9,13,52] Detailed information about the performance of analytical protocols is provided in Tables S2 and S3.

SARS-CoV-2 RNA was quantified at each time point in the IN, OUT, EF, and SL samples, as well as in PT, resulting from influent sample clarification prior to concentration. For all of the samples (n = 45 for the CH; n = 45 for SF), the viral load was expressed as the mean of positive technical replicates for each target. Results are summarized in Figure 1 and Table S4. Standard deviations for each gene and sample are reported in Table S5. SARS-CoV-2 RNA was detected in 41 samples (45.6%), with positive detections mostly occurring in pretreatment matrices (IN, PT, and SL), showing 36 positives from 54 samples investigated (66.6%). Conversely, in post-treatment matrices (OUT and EF), SARS-CoV-2 RNA was detected in 5 out of 36 samples analyzed (13.9%).

At the CH, the only sampling time with no viral detection for any of the five matrices analyzed was the oldest collection date (September 7, 2020) corresponding to the beginning of the second wave of SARS-CoV-2 in Italy when only one positive local hospitalization was reported. Similarly, in SF, the first positive signal was detected on October 5, 2020, consistent with the low number of COVID-19 cases reported in the province during the early phase of the monitoring campaign. From early October until the end of the monitoring period (December 2021 for SF and February 2021 for the CH), all samples from both sites tested positive for at least two target genes.

The viral load estimated for the pretreatment water samples (IN+PT) contained most of the genetic material and reflected the observed trend of SARS-CoV-2 circulation in the population. To assess whether viral quantification of IN+PT correlated with infection prevalence in the population, the RNA quantifications obtained for these matrices were compared with the number of cases reported in the regional daily bulletins (Figure 1). For the CH, reference was made to the number of hospitalized patients with COVID-19, while for SF, whose facility serves a small catchment area, the number of positives reported refers to the entire province of Venice [~850000 people (Figure 1)]. At the CH, for all sampling times but one (December 14, 2020), quantitative data obtained for IN and PT matrices followed the same trend of the number of hospitalized patients reported in the bulletins, with consistent results among the three target genes (mean IN GC/L = 6.09×10^4; mean PT GC/L = 7.55×10^5). Correlation analysis comparing positive cases against the CH wastewater's SARS-CoV-2 RNA concentrations returned a highly positive correlation index for all targets in IN and PT matrices (Figure 1). The decreased viral load observed in both IN and PT matrices at the time of the maximum number of hospitalizations at the CH on December 14, 2020, could be hypothetically explained by high tides occurring between December 13 and 16 in the Venice lagoon. However, decreases in viral load caused by the effect of wastewater dilution remain just speculation, and the impact of tides in the detection and quantification of microorganisms needs to be studied further. In contrast to the CH, several discrepancies were observed in SF with respect to the trend of the contagion curve for the entire province of Venice, with greater variation in the GC estimates among the three genes analyzed. These results were expected given the limited number of Giudecca inhabitants (750) afferent to the SF WWTP. Accordingly, SARS-CoV-2 RNA detection at SF (mean IN GC/L = 1.05×10^4; mean PT GC/L = 8.41×10^4) reflects the presence of outbreaks in this small portion of the Venetian population, thereby leading to possible mismatches with the number of positive cases reported for the entire population of the province of Venice by regional bulletin, and a consequent weaker Spearman's correlation index for IN and PT (Figure 1).

Special consideration is needed for SL and for the particulate derived from the clarification on the inlet samples prior to concentration, here named PT. Overall, SL showed the highest detection rate and SARS-CoV-2 RNA concentration levels (Figure 1c; mean GC/L for the CH = 2.64×10^6; mean GC/L for SF = 2.02×10^6) compared to those of aqueous matrices from both sites. Such a high viral load may be the result of intrinsic muddy/solid waste material accumulation over time in the treatment plants. In turn, quantitative data from SL most likely reflect cumulative data on SARS-CoV-2 circulation over time, rather than an assessment at a specific time point, as suggested by the tail-shaped trend for the E, N1, and N2 targets shown in Figure 1. Accordingly, high correlation indices for SL are most likely biased by the effect of material stagnation, and thus artifactual. Thus, although sludge (primary settling solid) may represent a more sensitive matrix for SARS-CoV-2 RNA detection compared to other inlet matrices,[16] particular attention should be paid if information about viral circulation at a single data point is required. In contrast, promising results were obtained by the analyses of the influent-derived PT matrix, showing higher positive SARS-CoV-2 RNA detections and

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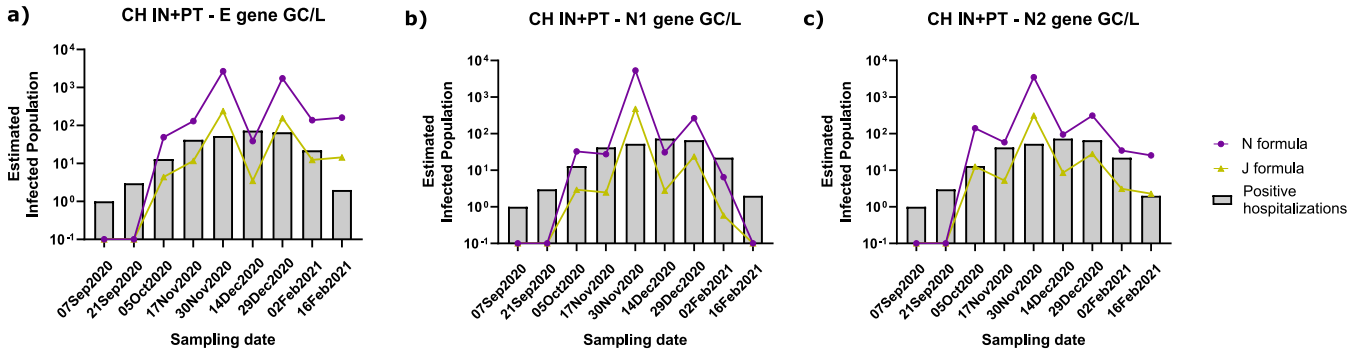

Figure 2. Modeling of SARS-CoV-2 prevalence in the Civil Hospital (CH) based on wastewater analysis sample data (IN+PT matrices) by using the N formula (purple line) and the J formula (yellow line). The accuracy of the two formulas was tested and compared by plotting the estimated infected cases (lines) against the real cases number (histograms) for the (a) E gene, (b) N1 gene, and (c) N2 targets (*y*-axis) at each time point (*x*-axis).

quantification for all of the viral targets (Figure 1 and Table S4). While the IN matrix is commonly employed for environmental monitoring studies, the use of its derived solid component has been rarely assessed. Only one paper has reported the importance of this matrix,[23] while in other studies, it is considered as a sample fraction to be tested jointly with the IN concentrate.[7,19,53] Notably, Jorgensen et al.[19] reported more frequent SARS-CoV-2 RNA detection in the supernatant than in the suspended solid particulate. Conversely, our results demonstrate that PT analyses led to the detection of a higher number of positive samples (14 and 9 in the PT and IN matrices, respectively) (Table S4), which better reflect the number of positive cases reported in daily bulletins (Figure 1). In addition, RNA extracted from PT was qualitatively good for sequencing purposes (see Identification of Prevalent SARS-CoV-2 Lineages).

Inference of the Number of Positive Cases Based on SARS-CoV-2 RNA Quantification. While qRT-PCR is highly informative and widely used for environmental detection of viruses, there are downsides due to the difficulty in correlating viral load with the real number of infected individuals in the community served by the treatment plant.[27,54,55] In our study, the quantification of SARS-CoV-2 RNA in influent sewage from the CH having a known number of hospitalized patients provides an opportunity to define the minimum number of positives required to successfully detect SARS-CoV-2 in WW samples and to evaluate the accuracy of existing models in the estimation of the number of positives starting from genome quantification data from sewage.

The analysis of hospital plants for WBE monitoring was first reported by Jorgensen et al.,[19] who used this information to assess the detection limit below which it is not possible to obtain an estimated number of shedders. After monitoring a WWTP serving a hospital facility during a major COVID-19 outbreak (February to April 2020), Jorgensen and colleagues[19] estimated a sensitivity of the method between 1 virus shedder per 1000 people and 2 virus shedders per 10 000 people (in the range of 0.02−0.1% of the infected community). In our study, the first SARS-CoV-2 RNA detection in IN, PT, and SL occurred with 13 hospitalized (Table S4), reflecting a population size equivalent to ∼4000 persons (i.e., ∼0.3% positives). Unfortunately, it was not possible to assess the sensitivity of the method with a lower infection prevalence due to the lack of samples.

Two different formulas for defining SARS-CoV-2 prevalence starting from WWTP monitoring, corresponding to those provided by McMahan et al.[27] ("J") and Saththasivam et al.[28] ("N"), were also tested (Figure 2 and Table S6). While both approaches adequately reflected the trend of hospitalization with consistent results among the three targets, they showed significant differences in the absolute value of estimated infected cases. These discrepancies are likely due to the different values of fecal load and fecal shedding considered within each formula. Overall, the N formula tends to overestimate the number of infected cases with respect to the real number of positives, as reported also by other groups.[28,54,55] Conversely, the J formula provided values of estimated positive individuals closer to the real number of hospitalized people reported in the daily bulletins, suggesting that this calculation method is more accurate. However, it must be noted that discrepancies between the number of positives as estimated from sewage testing and the official records based on clinical confirmation and contact tracing are widely expected due to several factors that make these calculations extremely complex,[27,54,55] namely, the effective number of people served by the plant, the presence of asymptomatic individuals, and environmental and climate variables (e.g., high tide in the case of the Venice WWTP).[55]

Comparisons between the CH and SF WWTPs and Decontamination Efficacy. Venice is characterized by a widespread network of wastewater treatment plants serving the municipal territory. To date, approximately 7000 individual treatment systems have been built in the historic center, most of which (6800) consist of septic tanks and a small number (200) of electromechanical biological systems, such as sequencing batch reactors (SBRs) and membrane bioreactors (MBRs). SBRs and MBRs represent the next-generation sewage treatment plants and were implemented to serve catchment areas where the population is greater than an equivalent of 100 inhabitants. Given the heterogeneity and spread of WWTPs in the area, Venice represents an interesting case study for testing the effectiveness of different technologies adopted in the treatment plants to decontaminate samples that contain pathogens. Both WWTPs included in this study exploit the MBR technology, with the exception of a final disinfection step of UV treatment used in the CH purification plant.

Laboratory results highlight the presence of SARS-CoV-2 RNA traces in posttreatment waters, a finding similar to those of Ahmed et al.[9] and Rimoldi et al.[24] Although there is little diversity between the disinfection plants, there is variation in the number of positive samples obtained from postpurification waters (OUT and EF) at the two sites. Specifically, a higher number of OUT and EF samples obtained from the SF-WWTP tested positive for SARS-CoV-2 RNA (n = 4 of 9), while one

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positive record was obtained for CH WWTP samples (Table S4). Despite additional samples being required to confirm this observation, our data suggest that there may be differences in the effectiveness of the two plants in removing viral traces. This variability is most likely related to the final step of UV treatment implemented in the CH WWTP that is known to degrade RNA[56] by causing photochemical modification, cross-linking, and oxidative damage. In fact, while MBRs alone are not always successful in completely removing viral contaminants (such as enteroviruses, noroviruses, and rotaviruses),[57,58] the association with a final UV disinfection step could increase the efficiency of the treatment process. However, it is noteworthy that when postpurification waters are considered, a higher number of positives from SF were detected in EF samples. Hence, the possibility that such genomic signals might derive from the pouring of WW into the lagoon directly from septic tanks or urban runoff from domestic wastewater cannot be excluded. It also should be noted that there is a lack of direct correspondence between pretreatment (IN and PT) and posttreatment (OUT and EF) matrices sampled on the same day. This difference can be easily explained by the stagnation time of wastewater in the system during the different disinfection steps, which in turn vary between the two plants.

Although the detectability of SARS-CoV-2 RNA in posttreatment samples may be cause of concern, it is important to stress that it is not necessarily a sign of infectivity and does not represent a risk to humans. Indeed, to date no viable virions have been isolated from posttreatment wastewater samples testing positive for SARS-CoV-2 by molecular assays,[24] and most studies have confirmed the complete removal of viral RNA traces at the end of an MBR disinfection line.[11,20,59]

Identification of Prevalent SARS-CoV-2 Lineages. SARS-CoV-2 reads were obtained for all of the sequenced samples, confirming positive qRT-PCR detections of viral genetic material in the pretreatment matrices (IN and PT) and in SL. Genome coverage ranged between 0.09% and 99.76% (Figure S2). Overall, IN and PT samples with higher GC/L values ($>6.88 \times 10^4$) showed the highest coverage values that allowed lineage assignment, although it was not possible to assess the minimal viral load required for the Pangolin lineage identification because of the limited number of samples subject to NGS (Table 1). In contrast, SL samples showing a high viral content provided unsatisfactory coverage. This has been observed in other studies,[32,35] and it is likely due to the RNA degradation that may occur during sludge stagnation in the WWTP or to the decontamination treatment itself.

Pangolin lineages were assigned on the basis of the consensus sequences obtained and could be clearly identified for the three samples with >95% coverage. The lineage identified for the IN sample collected on November 30, 2020, was not assigned with Pangolin due the high number of ambiguous bases (N) (>33%). However, this sample presents all mutations typical of lineage B.1.177 with the exception of ORF1b:P314L as it is located in a portion of the genome not covered by reads. For the remaining samples with lower coverage, the SARS-CoV-2 lineage could not be established by either Pangolin or manually.

Notably, the CH sample collected on November 30, 2020, yielded highly similar sequences assigned to lineage B.1.177 in both IN and PT matrices. Similarly, IN and PT samples collected at the CH in December 2020 yielded identical consensus sequences belonging to lineage B.1.160. Importantly, these results are consistent with data from the genetic surveillance activities that report B.1.177 and B.1.160 as the most common lineages in the Veneto region at the time of sampling, with frequencies of 53% for B.1.177 and 17% for B.1.160 in November and December 2020 (Figure S3; https://www.izsvenezie.it/caratteristiche-genetiche-sars-cov-2-veneto-3/). In addition, the agreement between the sequence data obtained from IN and PT matrices, without a substantial loss of mapping quality and/or resolution for variant detection, confirmed the reliability of influent particulate samples for molecular detection and characterization of SARS-CoV-2.

Implications and Perspective of Molecular Monitoring of SARS-CoV-2 at WWTPs. SARS-CoV-2 surveillance based solely on clinical testing and contact tracing leaves a number of unanswered questions about the dynamics of virus spread. As a consequence, additional tools are needed to strengthen our ability to predict outbreaks in a timely manner and obtain biological samples that contain the greatest amount of information to assist decision making.[61] Genetic surveillance for SARS-CoV-2 in WWTP samples is considered an appealing approach for covering these gaps for its cost effectiveness and because it provides data about a pool of individuals at a single time point. Overall, this study confirms the suitability of WBE as a low-cost solution for tracking the presence of SARS-CoV-2 and for identifying the predominant viral lineages as a supplementary tool, although its use for surveillance as an independent variant is currently under validation.[62]

The ability to detect SARS-CoV-2 RNA at the CH WWTP at the beginning of the second wave with only a few patients hospitalized with COVID-19 suggests that testing a small number of wastewater samples represents a far more cost-effective way to obtain population-wide information compared to targeted individual testing. The positivity trends observed for both the CH and SF over the monitoring period reflect well the number of reported positive cases in the same time slot, further corroborating the usefulness of WBE in monitoring the progress of an infection, identifying new outbreaks, and estimating prevalence. In addition, testing influent untreated solid particles obtained just after a clarification step, thereby avoiding time-consuming laboratory procedures, provides a reliable, fast, and inexpensive alternative for improving and accelerating long-term environmental monitoring. Conversely, due to the possible accumulation of material, sludge appears to be unsuitable for following viral spreading in real time, despite its high sensitivity.

This study represents the first attempt to detect, quantify, and sequence the SARS-CoV-2 genome in different Venice WWTPs and in treated waters discharged into the canals, paving the way for future studies on this city, unique for the impressive amount and types of WWTPs and for the number of people in transit. Future investigations on larger numbers and types of plants, potentially representative of different social contexts, such as tourist accommodation, schools, hospitals, etc., will allow the monitoring of new and existing outbreaks and shed light on the spread, introduction, establishment, and extinction of SARS-CoV-2 variants.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.2c00013.

Locations of the wastewater treatment plants sampled in this study (Figure S1); additional details about analytical methods optimization and yield; real-time RT-PCR oligonucleotides used in this study (Table S1); recovery

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and concentration efficiency of SARS-CoV-2 RNA in artificially spiked IN, OUT, and EF (Table S2); recovery and concentration efficiency of IBV in artificially spiked IN, OUT, and EF (Table S3); raw data on time course detection of SARS-CoV-2 RNA in different WW sample matrices from the CH and SF treatment plants (Table S4); standard deviations (SD GC/L) calculated (a) for the CH (b) for SF and (c) for IN+PT matrices of the CH for comparison with the number of hospitalized cases (Table S5); inference of the number of infected cases based on viral load (Table S6); genome coverage for WW samples subject to NGS (Figure S2); SARS-CoV-2 lineage distribution in the Veneto region (Figure S3) (PDF)

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Author Contributions

I.B. and A.M. contributed equally to this work, and M.M. and V.P. contributed equally to this work. C.Z., M.M., V.P., I.M., A.F., C.T., G.F., C.C., T.P., F.S., and L.B. conceived and designed the study. C.C., A.M., I.B., A. Berton, and C.B. collected WW samples. G.T., C.C., and G.F. retrieved information about WWTPs. F.B. and A. Bortolami provided SARS-CoV-2 and IBV strains for assay optimization. I.B., V.P., G.D.R., A.M., and F.B. optimized laboratory procedures. I.B. and A.M. concentrated the samples, extracted the RNA, and performed qRT-PCR. E.P., E.G., E.G.Q., and A.F. performed NGS and bioinformatics analysis. A.M. and I.B. statistically analyzed data. I.B., A.M., V.P., M.M., E.G., and A.F. interpreted results and wrote the manuscript. E.C.H. critically reviewed the manuscript. All of the authors approved the manuscript.

Notes

The authors declare no competing financial interest.

Sequences obtained from samples VEN-IZSVe-21RS1495_VE_CH-IN, VEN-IZSVe-21RS2156−3_VE_CH_PT, VEN-IZSVe-21RS1496_VE_CH-IN, and VEN-IZSVe-21RS1498_VE_CH-PT are available on GISAID as entries EPI_ISL_7283383, EPI_ISL_7283619, EPI_ISL_7283552, and EPI_ISL_7283609, respectively.

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