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SARS-CoV-2 removal in municipal wastewater treatment plants: Focus on conventional activated sludge, membrane bioreactor and anaerobic digestion

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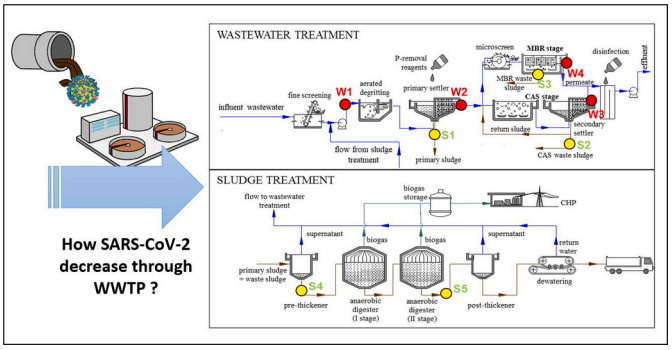
HIGHLIGHTS

- The SARS-CoV-2 concentration was not significantly reduced in primary settling.
- Log removal of SARS-CoV-2 was 1.8 ± 0.9 logs in CAS and 2.2 ± 0.7 logs in MBR systems.
- MBR improves retention effect by the ultrafiltration membranes and cake layer.
- Because high affinity with biosolids SARS-CoV-2 accumulate in primary and waste sludge.
- Negligible removal in mesophilic anaerobic digester (33 °C) due to moderate temperature.

GRAPHICAL ABSTRACT



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ABSTRACT

This work focuses on the removal of SARS-CoV-2 RNA in the various stages of a full-scale municipal WWTP characterised by two biological processes in parallel: (i) conventional activated sludge (CAS) and (ii) membrane bioreactor (MBR). The monitoring was carried out during the Omicron wave in 2022, a period characterised by a high concentration of SARS-CoV-2 in influent wastewater. The average concentration of SARS-CoV-2 in influent wastewater was 3.7×10^4 GU/L. In the primary sedimentation, the removal of SARS-CoV-2 was not appreciable. The largest log removal value of SARs-CoV-2 occurred in the biological stages, with 1.8 ± 0.9 and 2.2 ± 0.7 logs in CAS and MBR systems. The mean concentrations of SARS-CoV-2 in the CAS and MBR effluents were 6.8×10^2 GU/L and 6.4×10^2 GU/L, respectively. The MBR effluent showed more negative samples, because small particles are retained by membrane and cake layer.

The analysis of the different types of sludge confirmed the accumulation of SARS-CoV-2 in primary (5.2×10^4 GU/L) and secondary sludge (3.5×10^4 GU/L), due to the affinity of enveloped viruses towards biosolids. A SARS-CoV-2 concentration in the digested sludge equal to 4.8×10^4 GU/L denotes a negligible reduction in the mesophilic anaerobic digester at temperature of 31–33 °C.

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1. Introduction

In COVID-19 patients but also in asymptomatic individuals, the SARS-CoV-2 virus can be excreted with the stool and other bodily secretions even after respiratory symptoms have ceased, as well reviewed by Kitajima et al. (2020) and observed in patients (Xing et al., 2020). In this way, RNA fragments of SARS-CoV-2 are discharged into the sewerage system and reach the inlet of wastewater treatment plants (WWTPs) within a few hours (Foladori et al., 2022). During the period of permanence in the sewer system, which can vary widely in the order of hours depending on the length and slope (Rimoldi et al., 2020), the SARS-CoV-2 RNA undergoes a natural decay due to unfavorable conditions: presence of micropollutants, pharmaceuticals and disinfectants, or adverse physical factors such as temperature, extreme pH values or adsorbent solids (inter alia Carducci et al., 2020; Ahmed et al., 2020b). However, the time is not enough to achieve the complete inactivation and degradation of the virus. As a result, a certain amount of SARS-CoV-2 RNA can be found worldwide in untreated wastewater and exploited in the application of Wastewater-based Epidemiology (WBE) (Randazzo et al., 2020; de Llanos et al., 2022).

The increasing number of studies about the surveillance of the spread of SARS-CoV-2 using WBE has produced a large availability of data in the literature about the concentration of the virus in raw wastewater (inter alia Ahmed et al., 2020a; Saguti et al., 2021; Cutrupi et al., 2022; Monteiro et al., 2022). The sewage network operates a strong dilution of viral titers due to the large amount of water used daily per capita and the presence of industrial discharges, stormwater or infiltrations. As a results, the SARS-CoV-2 concentration in influent wastewater is relatively low and highly variable among the studies, with titers ranging from the very low concentration of 1.9 genomic unit/100 mL (19 GU/L) indicated by (Ahmed et al., 2020a) to 3×10^6 GU/L (Foladori et al., 2020). This broad range also depends on the prevalence of COVID-19 in the community and the analytical methods applied (concentration and recovery, sensitivity of assays, presence of inhibitors, etc.).

The current state of knowledge about the fate of SARS-CoV-2 in WWTPs is currently largely limited. Although many reviews have been published (Foladori et al., 2022; Serra-Compte et al., 2021), experimental studies are currently scarce both at pilot scale (Espinosa et al., 2022) and a full-scale (Kumar et al., 2021; Plaza-Garrido et al., 2023; Wang et al., 2022; Serra-Compte et al., 2021). Acquiring comprehensive data along physical, chemical and biological processes as well as sludge treatments can be difficult because viral loads progressively decrease along the plant with effluent concentrations becoming very low (near or below the LOD of the test method). Furthermore, until now, only a few studies have focused on the SARS-CoV-2 titers in waste sludge (Adelodun et al., 2022; Balboa et al., 2021) and the information is often derived from other coronaviruses (CoVs) or using spikes of surrogate viruses (Guérin-Rechdaoui et al., 2022). The reason probably lies in the difficulties in the analysis of SARS-CoV-2 in sludge matrices especially as regards the RNA extraction step (Guérin-Rechdaoui et al., 2022).

Further studies are needed to investigate SARS-CoV-2 removal in WWTPs and fate in sludge, in order to better understand the efficiency of each treatment step and the mechanisms involved. More data on the level of SARS-CoV-2 in treated effluents can help understand the spread into surface water bodies and potential concerns for wastewater reuse.

This paper reports an investigation on the removal of SARS-CoV-2 RNA in a full-scale WWTP treating municipal wastewater discharged from an urban sewershed of 86,000 inhabitants. The plant is characterised by two separated biological stages for wastewater treatment: (i) conventional activated sludge (hereinafter CAS) and (ii) membrane bioreactor (hereinafter MBR). The waste sludge was treated in a thickener and then in a mesophilic anaerobic digester. The monitoring period was from 20 January to 4 February 2022 (Fig. 1). During this period, we exploited the particularly high concentration of SARS-CoV-2 in influent wastewater that occurred during the Omicron wave, which peaked in January 2022 (Cutrupi et al., 2022). At peak, the concentration of SARS-CoV-2 RNA in wastewater was around 10^6 GU/L (Fig. 1). These high concentration is particularly advantageous for the evaluation of inactivation kinetics or decay rates, considering that surrogates are usually spiked in the reactors during laboratory experiments to increase viral concentrations. Conversely, in the cases where the concentration is low or no detectable virus can be found in the samples, a precise assessment

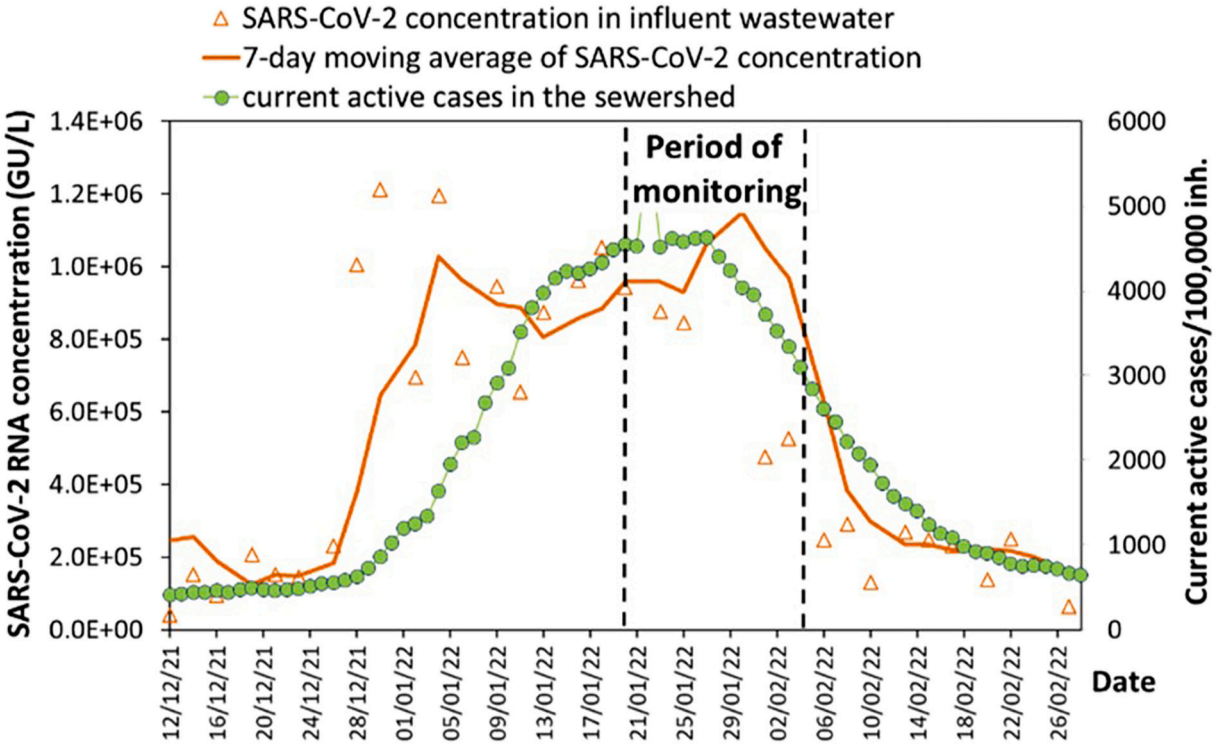

Fig. 1. SARS-CoV-2 RNA concentrations in influent wastewater compared to the current active cases during the Omicron wave in January – February 2022.

of the maximum removal efficiency is not feasible. Although the monitoring period, indicated in Fig. 1, may appear relatively short, all samples tested positive for SARS-CoV-2, which met the objective of ensuring a constantly high viral load at the entrance of the WWTP (condition similar to a tracer).

To our knowledge, the present study is one of the few studies carried out (i) in a full-scale WWTP, (ii) with a high concentration of SARS-CoV-2 in influent wastewater, (iii) comparing two biological processes, CAS and MBR, in parallel, (iv) including fluxes in sludge treatments included anaerobic digestion. In particular, papers focused on CAS and MBR are scarce at the moment (Wang et al., 2022; Serra-Compte et al., 2021). Therefore, this work provides further insights into the fate of SARS-CoV-2 along the various stages of a WWTP, contributing to increase the knowledge in this field.

2. Materials and methods

2.1. The municipal WWTP

The full-scale municipal WWTP (Trento, Italy) serves a population of 86,000 inhabitants (census data) and has a design capacity of 120,000 Population Equivalent (PE). The WWTP treats an average daily flow rate of 18,000 m^3/d and an average daily organic load of 9700 kg COD/d (COD, Chemical Oxygen Demand). The physico-chemical characterisation of influent wastewater is presented in Table 1 whereas single parameters are described in Section 2.8.

The layout of the WWTP (Fig. 2) includes the mechanical pre-treatments (fine screening and aerated degritting) followed by the primary settler (total volume 2500 m^3). The hydraulic retention time (HRT) in the primary settler is approximately 3.4 h. Afterward the pre-settled wastewater enters the biological treatment which consists of two separate stages: a conventional activated sludge process (CAS, which treats 69 % of the influent flow rate) and a membrane bioreactor (MBR, which treats 31 % of the influent flow rate).

The CAS stage is divided into 3 tanks with a total volume of 4,200 m^3 followed by secondary settlers with a total volume of 5,600 m^3. The CAS stage is operated with intermittent aeration to implement simultaneous nitrification-denitrification process. The HRT is approximately 17 h including biological tanks and secondary settlers. The average concentration of Total Suspended Solids (TSS) in the CAS stage is 6.4 g TSS/L.

The MBR stage consists of a 1,500 m^3 oxidation tank and a 312 m^3 membrane tank where the Zenon hollow-fibers for ultrafiltration (0.04 μm pore size) are installed. HRT in the MBR stage is approximately 7 h. The process requires mechanical aeration for oxidation, but also for the cleaning of the membranes. Recirculation of the aerated mixing liquor is provided from the membrane tank to the oxidation tank to uniformly distribute the sludge concentration in the reactor. The average TSS concentration in the MBR stage is 6.0 g TSS/L.

Disinfection was present but not monitored in this research. Then effluent wastewater are discharged into the receiving river.

The sludge treatment consists of a thickener, mesophilic anaerobic digester (average temperature of 33 °C) and dewatering unit.

Table 1
Physico-chemical parameters in the WWTP during the monitoring period (avg ± st.dev.).

Parameter	Influent wastewater	Pre-settled wastewater	Effluent wastewater
COD (mg/L)	578 ± 148	407 ± 66	47 ± 3
BOD_5 (mg/L)	272 ± 38	256 ± 45	5 ± 1
TSS (mg/L)	248 ± 111	109 ± 34	10.3 ± 0.6
TKN (mg/L)	75.5 ± 11.9	–	8.5 ± 1.5
NH_4^+-N (mg/L)	51.9 ± 11.3	–	7.4 ± 1.3
NO_3^--N (mg/L)	1.0 ± 0.0	–	6.8 ± 0.8
NO_2^--N (mg/L)	0.3 ± 0.0	–	3.0 ± 0.5
Total N (mg/L)	76.0 ± 11.8	–	17.7 ± 1.6
Total P (mg/L)	10.1 ± 1.5	–	1.1 ± 0.2
pH (−)	7.8 ± 0.2	7.7 ± 0.1	7.7 ± 0.1
Temperature (°C)		11.4 ± 0.3	
Flow rate (m^3/d)		17,640 ± 600	

2.2. Period of monitoring

Monitoring of WWTP was performed in the period from January 20 to February 04, 2022, a relatively short period but characterised by a peak in the spread of the Omicron wave in the population. This two-week period was consistent with the sludge retention time in the biological stages. Quantification of the concentration of SARS-CoV-2 was carried out daily in the various stages of the WWTP, as well as physico-chemical analyses of wastewater and sludge.

2.3. Sampling plan for SARS-CoV-2 monitoring

2.3.1. Sampling points

Wastewater and sludge were collected in the sampling points indicated in Fig. 2. The points along the wastewater line were as follows: (W1) influent wastewater taken in the pre-treatments after fine screening; (W2) settled wastewater taken after the primary settler; (W3) effluent from the CAS stage taken in the supernatant of secondary settler; (W4) effluent from the MBR stage taken from the permeate stream.

Sludge samples were collected in the following sampling points along the sludge line: (S1) primary sludge separated in the primary settler; (S2) waste sludge from the CAS stage; (S3) waste sludge from the MBR stage; (S4) thickened sludge, (S5) digested sludge taken at the outlet of the mesophilic anaerobic digester.

2.3.2. Sampling runs

Monitoring of SARS-CoV-2 in WWTP was performed with 8 complete sampling runs, for a total of 84 samples.

2.3.3. Sampling procedure

Influent wastewater was collected with refrigerated automatic samplers, taking aliquots of equal volume at 15 min intervals and forming 24-h composite samples in 2 L bottles.

In the other sampling points along the wastewater line, the use of automatic samples was impractical due to lack of locations, plumbing and electrical connections for the equipment. Therefore, grab samples were collected in these points. Cutrupi et al. (2021), in the same WWTP of the present research, observed that the two daily peaks of SARS-CoV-2 concentration in influent wastewater lasted about 4 h (at 9–11 in the morning and 15–17 in the afternoon), while the minimum concentration occurred during a 4-h time frame overnight. Considering 3-h and 17-h HRT in the primary and secondary stages, respectively, homogenization is promoted and thus the fluctuations in the grab samples taken were less evident.

The sampling of sludge was performed by manual collection in 100 mL Falcon tubes. All samples were immediately frozen at −20 °C until laboratory analyses.

2.4. Extraction of SARS-CoV-2 RNA from wastewater

The analysis protocol follows the direction of La Rosa et al. (2021) and is the official method of the Italian national wastewater surveillance network (SARI). This method was developed by Wu et al. (2020) and applied by the SARI network with some modifications. A volume of 45 mL was analysed for samples W1 and W2 while this volume was multiplied 12-fold for samples W3 and W4. Larger volumes are needed for effluent wastewater to increase the chances of detection, because lower titers of viral material are expected. The initial sample volume was treated with a pasteurization step (a heat bath at 56 °C for 30 min) for

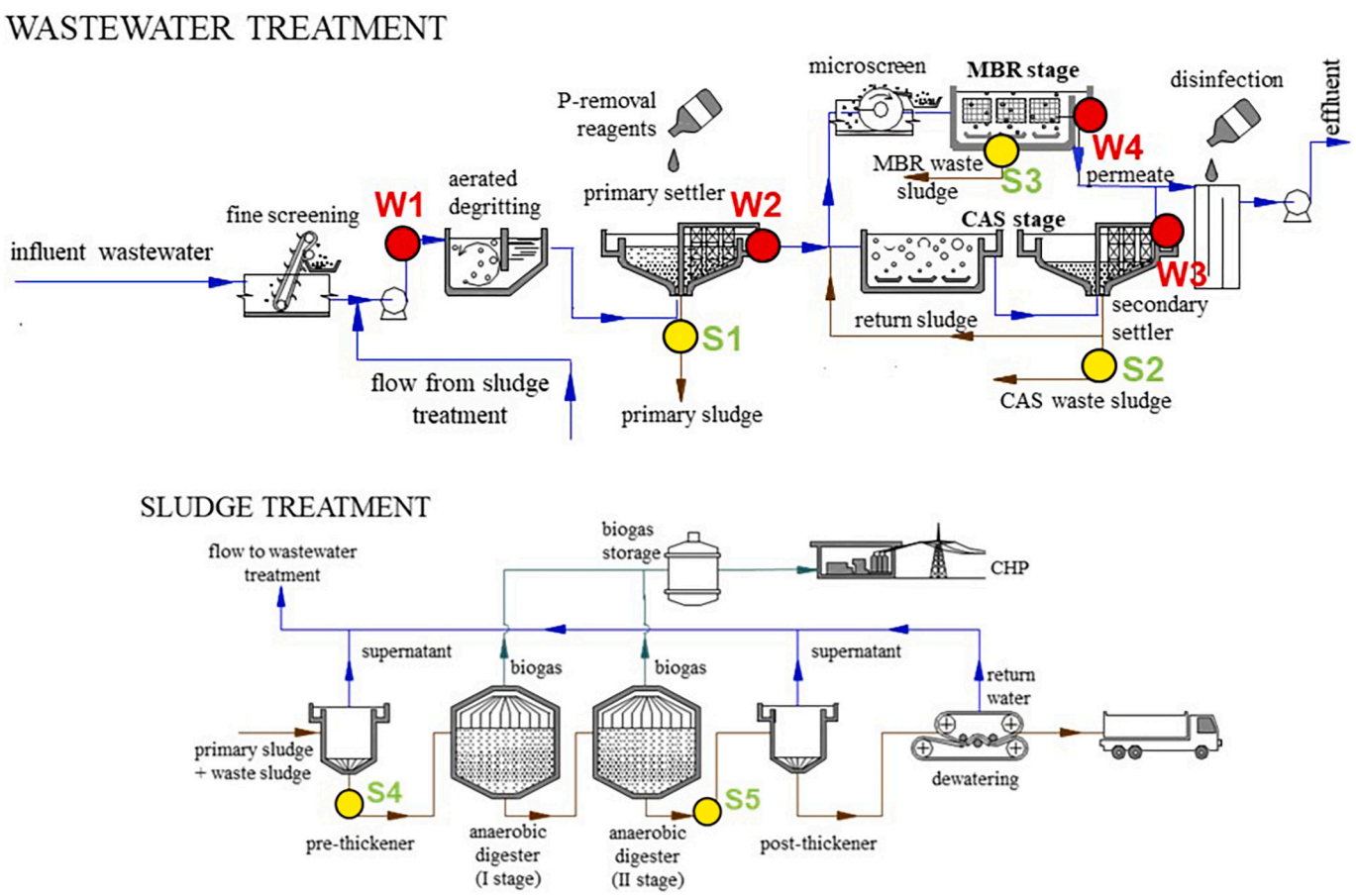

Fig. 2. Flow-sheet of the WWTP with indication of the two biological stages: CAS stage and MBR stage. Red dots indicate sampling points for wastewater. Yellow dots indicate sampling points for sludge. Letters W1-W4 and S1-S5 are described in Section 2.3.1

pathogen inactivation and then centrifuged at 4,500 ×*g* for 30 min to remove the solid fraction of the matrix. 4 g of PEG8000 and 0.9 g of NaCl were added to 40 mL of the liquid phase, mixed and centrifuged at 12,000 ×*g* for 2 h. To 480 mL of samples W3 and W4 the reagents were added in the same proportion. This step allows the precipitation of the viral material which condenses in the form of an almost invisible pellet on the walls of the tube. The newly formed liquid fraction was then discharged and the pellet was used for viral RNA extraction with a semi-automated extraction platform, eGENE-UP® (bioMérieux, Marcy l'Etoile, France) with the use of magnetic beads of silica. Nucleic acids were then eluted in 100 μL of elution buffer and PCR inhibitors, still present, were removed with the OneStep PCR Inhibitor Removal Kit (Zymo Research, CA, USA).

2.5. Extraction of SARS-CoV-2 RNA from sludge

The sludge samples were analysed as mixed liquors containing solid and liquid fractions. 8 mL of pasteurized samples were added to 32 mL of distilled water and analysed following the procedure of the Wizard® Enviro Total Nucleic Acid Kit (Promega, WI, USA). 0.5 mL of Protease Solution was added to the sample and mixed well by inversion. After a 30 min incubation at room temperature, the sample was centrifuged at 3,000 ×*g* for 10 min to remove solids. This step avoids clogging in the filtration column. 40 mL of the supernatant were decanted into clean test tubes, and specific buffers were added. After mixing, 24 mL of isopropanol was added to the tube, and the matrix was filtered into a funnel connected to the PureYield™ (Promega, WI, USA) binding column. After the passage of the sample matrix, two different wash buffers were passed through the binding column membrane. Nuclease-Free Water was pre-heated to 60 °C and used with an elution device to elute the total nucleic acids in the filtration membrane. This process was applied twice obtaining at the end 100 μL of the eluted sample. The total nucleic acids eluted were clean-up and concentrated with the use of PureYield™ Minicolumns (Promega, WI, USA) to the final volume of 40 μL.

2.6. Quantification of SARS-CoV-2 concentration in wastewater and sludge

The extracted nucleic acids were analysed using the Real-Time One-Step quantitative PCR assay harmonized with the SARI network (La Rosa et al., 2021). The designed primers and probe were used to amplify the 332 bp fragment of the SARS-CoV-2 ORF-1b gene (nsp14). Each 25 μL reaction contained 250 nM of the probe, 500 nM of forward and 900 nM of reverse primers, 1 μL of 25 × RT-PCR Enzyme, 12.5 μL of 2 × RT-PCR Buffer, and 5 μL of acid extract nucleic. The AgPath-ID One-Step RT-PCR (Life Technologies, CA, USA) was used to prepare the mix for RT-qPCR. Thermocycling conditions consisted of 30 min at 50 °C for reverse transcription, 10 min at 95 °C for DNA polymerase activation, and 45 cycles for amplification, 15 s at 95 °C and 45 s at 60 °C. The instrumental platform used was Applied Biosystems™ 7500 (ThermoFisher Scientific, MA, USA). At each cycle, a calibration curve was produced using 5 serial dilutions of material at a known concentration (from 10 to 10^5 copies/μL), obtaining slope values close to −3.32 (minimum −3.1, maximum −3.6) and $R^2 \geq 0.98$. Results were obtained from the threshold cycle (Cq) intersection with the calibration curve and then expressed as GU/L. Samples with a quantitation cycle cut-off (Cq) >40 (Cq > 40) were considered negative for the presence of the virus. In this case, the concentration of SARS-CoV-2 in the sample was calculated using a Cq value of 40 in the run in which the sample was processed.

Log removal values for SARS-CoV-2 RNA were calculated

considering influent and effluent concentrations, according in (Serra-Compte et al., 2021); when SARS-CoV-2 RNA was not detected, the log removal value was calculated using LOD. With rgards to the samples of sludge, the SARS-CoV-2 concentrations were also expressed as GU/g TSS and GU/g VSS (VSS, Volatile Suspended Solids).

2.7. LOD of SARS-CoV-2 analysis

In order to evaluate the performance characteristics of the amplification step, the assay limit of detection (ALOD) was calculated. Three different genomic concentrations were used: 1, 2 and 4 GU/μL. For each concentration, 8 replicates were performed in 3 separate PCR platforms (in total 24 replicate samples were run). The number of positive results were the followings: 24/24 for the concentration 4 GU/μL, 24/24 for the concentration of 2 GU/μL and finally 23/24 for the concentration of 1 GU/μL. The concentration of SARS-CoV-2 identifiable with 95 % probability (at least 95 % of samples containing that quantity of target are positive) is ALOD95 = 0.92 GU/μL, while ALOD50 = 0.21 GU/μL. PCR-based assays with low ALOD and a wide dynamic range are ideal for wastewater surveillance applications (Ahmed et al., 2022).

2.8. Sampling plan and analyses of physico-chemical analyses in wastewater and sludge

Samples of influent, pre-settled and effluent wastewater for physico-chemical analyses were collected with refrigerated autosamplers forming 24-h composite samples in 2 L bottles. Wastewater samples were analysed for total COD, BOD_5 (Biochemical Oxygen Demand), TSS, Total Kjeldahl Nitrogen (TKN), NH_4^+-N, NO_2^--N, NO_3^-N, Total Nitrogen (TN) and Total Phosphorus (TP), according to Standard Methods (Lipps et al., 2023) and the national Italian methods (APAT, 2003). All these analyses were performed as part of the weekly routine monitoring of the plant.

Sludge samples were collected in 100 mL Falcon tubes, together with sampling for SAR-CoV-2 (Section 2.3). The analysis performed in the sludge were TSS and VSS.

2.9. Statistics

Statistical analyses were performed using Microsoft Excel. The statistical comparison was determined by a Student's *t*-test and set at $p < 0.05$ (data analysis tool in MS-Excel, Microsoft). The paired t-test was implemented for the comparison of results between CAS and MBR effluents (paired observations in stages operated in parallel).

3. Results and discussion

3.1. Removal of physico-chemical parameters in the WWTP

The main physico-chemical parameters values in influent, pre-settled and effluent wastewater during the monitoring period are summarised in Table 1. COD concentration passed from 578 ± 148 mg COD/L in the influent to 407 ± 66 mg COD/L after the primary sedimentation (removal efficiency of 28 ± 8 %). Then the effluent COD reached 47 ± 3 mg COD/L after the biological stages.

Primary settling separated about half of the suspended solids (removal efficiency of 54 ± 14 %) and the influent concentration of 248 ± 111 mg TSS/L was reduced to 109 ± 34 mg TSS/L after sedimentation (Table 1) and to 10.3 ± 0.6 mg TSS/L in the effluent. Phosphate precipitation was performed in the primary settler using aluminium salts. Total P passed from the influent concentration of 10.1 ± 1.5 mg P/L to 1.1 ± 0.2 mg P/L in the effluent (Table 1).

Influent N consisted mainly of ammonium nitrogen and organic nitrogen, while the concentrations of NO_2^--N and NO_3^--N were negligible in the influent (Table 1). With an average influent TKN of 75.5 ± 11.9 mg N/L, the concentration in the effluent was 8.5 ± 1.5 mg TKN/L and 7.4 ± 1.3 mg NH_4^+-N/L (Table 1), demonstrating that stable nitrification occurred in the biological stages with an efficiency of 88 ± 4 %, despite the low water temperature of 11.4 ± 0.3 °C during the winter period.

Regarding the removal of total nitrogen (TN), the concentration of 76 ± 11.8 mg TN/L in the influent was reduced to 17.7 ± 1.6 mg TN/L in the effluent, with a removal efficiency of 76 ± 5 %, obtained with an improved denitrification in biological reactors by means of intermittent aeration; under this condition heterotrophic bacteria find a low-oxygen environment suitable for denitrification.

These results confirm that the monitored WWTP presents a removal efficiency of COD, TSS, TKN, total N and total P in agreement with the expected performances of low-medium loaded plants, able to meet the requirements for the discharge into surface water bodies. As this WWTP configuration is very common and widely applied, the findings on SARS-CoV-2 removal described in the following sections can be considered transferable to other similar plants.

3.2. Removal of SARS-CoV-2 in the wastewater treatments

The histograms in Fig. 3 compare the SARS-CoV-2 concentrations along the various stages of the wastewater treatment line, and in particular in raw and pre-settled wastewater and in the effluents from both CAS and MBR stages.

3.2.1. Raw wastewater and pre-settled wastewater

A percentage of 100 % of positive samples for SARS-CoV-2 RNA was found in the raw and pre-settled wastewater. The average concentration of SARS-CoV-2 in the influent wastewater was 3.7×10^4 GU/L, with a maximum value of 1.0×10^5 GU/L (Fig. 3). These values are at least an order of magnitude lower than the concentrations shown in Fig. 1 for the same period and plant. This difference was due to the different storage of the samples applied in our laboratory. In particular, freezing for a period up to 4 months was applied to the samples of Fig. 3 and not to the samples of Fig. 1; in fact, storage at −20 °C for weeks causes a decrease in viral concentration by more than half of the original concentration in fresh samples as previously demonstrated in the same WWTP in Cutrupi et al. (2021).

The average concentration of SARS-CoV-2 in pre-settled wastewater was 3.5×10^4 GU/L (Fig. 3). Although primary sedimentation removed an average of 56 % of TSS (TSS decreased from 248 to 109 mg TSS/L on average; Table 1), the average concentration of SARS-CoV-2 only decreased from 3.7×10^4 to 3.5×10^4 GU/L (small difference of 0.2×10^4 GU/L). Considering the influent flow rate of 17,640 m^3/d, the amount of SARS-CoV-2 removed daily in the primary sedimentation can be approximately estimated at 3.5×10^{10} GU/d. The amount of SARS-CoV-2 in the primary sludge can be calculated using the primary sludge flow rate of 245 m^3/d and the mean viral concentration of 5.2×10^4 GU/L (value in S1 presented in Section 3.3.1). With this calculation, the viral amount in the primary sludge was estimated to 1.3×10^{10} GU/d on average, which was different from, but in the same order of magnitude as the 3.5×10^{10} GU/d removed from raw wastewater. This is a rough comparison, while a precise "mass balance" is not allowed as extraction methods differed in wastewater, which excluded solids (see Section 2.4), and in sludge (see Section 2.5). Furthermore, SARS-CoV-2 concentrations in raw wastewater are largely variable, as indicated by the large interquartile range (IQR) in Fig. 3.

From a statistical point of view, the mean and median concentrations of SARS-CoV-2 in influent and pre-settled wastewater (Fig. 3) were statistically comparable as indicated by *t*-tests with $p > 0.05$. The viruses have a small volume and water-like density which does not favor spontaneous settling. Sedimentation can be enhanced when the virus is adsorbed on suspended solids capable of settling. As shown in Table 1, here the TSS concentration entering the primary sedimentation was relatively low, with values of 248 ± 111 mg TSS/L, corresponding to a solid content of about 0.2 % which does not facilitate the flocculation process. This result differs from the observations of Serra-Compte et al. (2021) who found a mean log removal of 0.48 ± 1.17 log of magnitude

in primary settling. For a comparison with enteric viruses, primary sedimentation contribute to a removal of these viruses of 0.1–1.0 log (Simmons and Xagoraraki, 2011; Simmons et al., 2011).

3.2.2. Secondary treatment: CAS and MBR stages

The major reduction of SARS-CoV-2 concentrations occurred in the biological stages (Fig. 3), where various removal mechanisms take part: (i) presence of large flocs which improve the adsorption and bio-flocculation of small viral particles and the subsequent separation in the secondary clarifier; (ii) natural decay due to an unfavorable environment, (iii) presence of antagonistic bacteria, which cause enzymatic breakdown and predation, contributing to the inactivation of the virus (Arslan et al., 2020; Pourakbar et al., 2022). In particular, the adsorption on solids is favored by the high hydrophobicity of SARS-CoV-2 and of CoVs in general.

In the effluents from CAS and MBR stages, the average concentration of SARS-CoV-2 was 6.8×10^2 GU/L and 6.4×10^2 GU/L, respectively (Fig. 3). The effluents from these two stages were not statistically different ($p > 0.05$). For a comparison, in the effluents from CAS, Wang et al. (2022) measured SARS-CoV-2 concentrations of 15–800 copies/L, in the same order of magnitude of our study. Instead, Wang et al. (2022) reported a range of 16–100 copies/L in the MBR effluent, slightly lower that our case. According to our experiments, also in Wang et al. (2022), the average SARS-CoV-2 concentrations after CAS and MBR were not significantly different ($p > 0.05$).

The log removal value from pre-settled wastewater to secondary treated effluents was calculated: it was 1.8 ± 0.9 and 2.2 ± 0.7 logs in CAS and MBR effluents, respectively. This removal values are slightly higher than 1.2–1.4 logarithmic units of reduction of Norovirus and Sapovirus (Taboada-Santos et al., 2020). In the study of Serra-Compte et al. (2021), activated sludge plus nutrient removal showed a SARS-CoV-2 removal of 1.37 ± 0.72 logs, while MBR treatment removed $>1.97 \pm 0.93$ logs, very similar to the value found in our monitoring. Plaza-Garrido et al. (2023) observed SARS-CoV-2 removal from 72 to 98 % in activated sludge with bio-disc.

Various studies in the literature have observed that secondary treated effluents can be negative for SARS-CoV-2 RNA (Haramoto et al., 2020; Sherchan et al., 2020). Negative sample does not mean absence of virus, but an amount of viral RNA below the LOD of the analysis. Here, negative samples were 2/9 in the CAS effluent (that means 78 % positivity), but 6/9 samples in the MBR effluent (only 34 % positivity). The MBR effluent showed more negative samples, due to the additional mechanisms of retention of small particles, such as virions, by the membrane (with cut off of 0.04 μm) and cake layer (Chaudhry et al., 2015). It is worth noting that the diameter of the coronavirus virion is approximately 60–140 nm and is covered by projections (9–12 nm), but the viral RNA fragment could be much smaller and therefore more capable of passing through the membrane. The results of MBR treatment indicate therefore that the filtration through membrane-based systems improve the efficiency of conventional activated sludge based on gravity sedimentation, in agreement with the observations of (Serra-Compte et al., 2021).

The secondary biological treatment based on CAS and MBR has an important role in the removal of the virus, but does not permit the complete elimination of SARS-CoV-2 RNA in all samples. This can be clearly seen in our case where the influent concentration of the virus was relatively high during the Omicron wave. Conversely, in the presence of lower influent concentrations, complete removal could be observed. This suggests the importance of reporting the removal of SARS-CoV-2 in secondary treatment together with the viral titer in the influent.

3.3. Removal of SARS-COV-2 in the sludge treatments

According to the flow chart in Fig. 2, the primary sludge and waste sludge (secondary sludge) extracted from the CAS and the MBR stages were conveyed to sludge treatment. Then, the mixed sludge followed the route of thickening, for the reduction of the water content, and mesophilic anaerobic digestion for the degradation of the organic matter and biogas production.

Available data on TSS concentration and VSS/TSS ratio in the sludge streams of the WWTP are synthesized in Table 2. When data were not available because the lack of measuring instrument on a certain flow, values were estimated using mass balances. TSS data were used to express the SARS-CoV-2 concentrations in sludge as GU/g TSS and GU/g VSS.

Fig. 4 shows the concentration of SARS-CoV-2 in the various sludge streams. All samples tested positive. Statistical comparison between the datasets in Fig. 4 indicates that the results were comparable in all the stages and not significantly different ($p > 0.05$ for all cases).

3.3.1. Primary and waste sludge

The average concentration of SARS-CoV-2 in primary sludge was 5.2×10^4 GU/L (Fig. 4), corresponding to 5.2×10^3 GU/g TSS and 6.2×10^3 GU/g VSS. These values are in the same order of magnitude as the values of 1.25×10^4 GU/L and 2.33×10^4 GU/L detected by Kocamemi et al. (2020) in primary sludge collected from two WWTPs in Istanbul. Primary sludge was analysed by Peccia et al. (2020) who found SARS-CoV-2 concentrations in the range from 1.7×10^6 to 4.6×10^8 GU/L. These authors proposed primary sludge as a spot for monitoring the viral concentrations in WBE applications, because the solids have an acceptable delay from the excretion (Peccia et al., 2020).

In secondary sludge (Fig. 4), the average concentration of SARS-CoV-2 was 3.5×10^4 GU/L in CAS waste sludge and 3.7×10^4 GU/L in MBR waste sludge. These values are in agreement with the results in secondary sludge reported by Kocamemi et al. (2020), who measured a range of 1.17×10^4 - 4.02×10^4 GU/L.

The concentrations of SARS-CoV-2 in primary sludge and waste sludge were therefore similar, and not statistically different. This similarity between primary and secondary sludge was also observed by Kocamemi et al. (2020). However, when comparing the viral titer in terms of GU/g TSS, the MBR sludge presents a significantly higher concentration (4.6×10^3 GU/g TSS and 5.7×10^3 GU/g VSS) than the CAS sludge (2.7×10^3 GU/g TSS and 3.4×10^3 GU/g VSS), indicating that membrane enhance the accumulation of the viral RNA in the sludge.

The viral concentrations in the CAS waste sludge and MBR waste sludge (Fig. 4) were much higher than those measured in the respective CAS and MBR effluents (Fig. 3). This confirms the important effect of accumulation of SARS-CoV-2 in secondary sludge, because the enveloped viruses have an affinity towards biosolids (Balboa et al., 2021; Kitamura et al., 2021; Mohan et al., 2021).

3.3.2. Thickened sludge

Sludge thickening was applied to reduce the moisture content of mixed sludge. The mean concentration of SARS-CoV-2 in the thickened sludge (Fig. 4) was 3.5×10^4 GU/L, very similar and not statistically different from the results in CAS and MBR waste sludge. This means that the separation of the supernatant (containing water, some suspended solids and a certain amount of virus) during thickening does not

Table 2
Characteristics of the sludge streams in the WWTP. Key: * estimated data from mass balances. ** Symbols are indicated in Fig. 2 and Section 2.3.

Points of sampling **	Type of sludge	TSS concentration (g/L)	VSS/TSS
W1	Primary sludge	10*	0.85*
W2	CAS waste sludge	12.8*	0.81 ± 0.02
W3	MBR waste sludge	8.0*	0.79 ± 0.01
W4	Thickened sludge	20*	0.80*
W5	Digested sludge	18.6 ± 2.9	0.72 ± 0.04

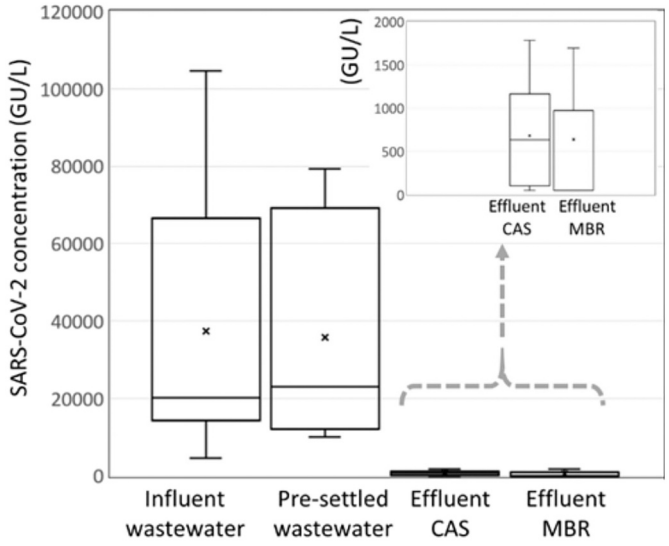

Fig. 3. Box plots of SARS-CoV-2 concentrations in influent and pre-settled wastewater and in the effluents from CAS and MBR.

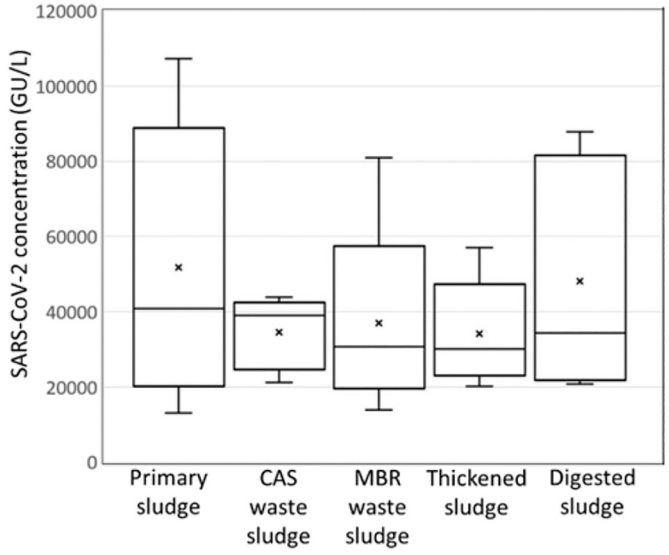

Fig. 4. Box plots of SARS-CoV-2 concentration in primary sludge and waste sludge from CAS and MBR and along the sludge treatments.

significantly change the virus concentration in the thickened sludge. In the literature, the thickener was proposed as a sampling point for sludge collection aimed at WBE application (Balboa et al., 2021). From our results, no significant differences were found in the mean and median values of viral concentrations when sampling thickened sludge or primary sludge. However, large data variability occurs in the primary sludge, as indicated by the larger IQR (Fig. 4), while the thickened sludge has a lower IQR. This means that the variability of SARS-CoV-2 concentrations in thickened sludge is smaller than in primary sludge.

3.3.3. *Digested sludge*

The presence of SARS-CoV-2 in the digested sludge from the mesophilic anaerobic digester was also monitored (Fig. 4). The mean concentration of SARS-CoV-2 in the digested sludge (Fig. 4) was $4.8 \times 10^4 \pm 3.1 \times 10^4$ GU/L. Therefore, mesophilic digestion did not significantly change the SARS-CoV-2 RNA concentration in the thickened sludge.

Studies on the effect of full-scale anaerobic digestion in mesophilic or thermophilic field on SARS-CoV-2 contained in the sludge are very scarce. Some information derives from laboratory analyses in which the pasteurization of wastewater samples was performed to reduce the health risks of operators (Rosa et al., 2020; Arora et al., 2020; Wu et al., 2020).

While temperature is known to affect the stability of coronaviruses and SARS-CoV-2 (Chan et al., 2020), mesophilic temperatures of 31–33 °C were not effective in reducing SARS-CoV-2 RNA levels. This is in agreement with other findings: SARS-CoV-2 remained stable at mesophilic temperatures of 37 °C for at least 24 h (Wang et al., 2020). In the study of Bibby and Peccia (2013) a mixture of primary and secondary sludge was treated in the mesophilic anaerobic digestion of 5 WWTPs, and CoVs were present in 80 % of samples.

Conversely, anaerobic digestion in a thermophilic range (around 55 °C) or thermal hydrolysis (150–160 °C) before anaerobic digestion, have been demonstrated in the literature to be effective in the complete inactivation of SARS-CoV-2 and are recognized as a sanitizing processes (Guérin-Rechdaoui et al., 2022; Bardi, 2021; Serra-Compte et al., 2021). In fact, high temperatures contribute to disrupt the enveloped viruses such as CoVs (Chan et al., 2011): in particular, temperature of 56 °C for 15 min inactivated the infectivity of the previous SARS CoV-1 (Chan et al., 2011), which suggests an analogous effect against SARS-CoV-2. Although the virus is effectively inactivated at thermophilic temperatures, the viral RNA remains detectable in the downstream RT-qPCR assay. For this reason, thermophilic temperatures (i.e. 56–60 °C, 30 min) are widely used to inactivate SARS-CoV-2 in clinical specimens (swabs, saliva, etc…) to make the whole workflow safer without the risk of exposure to viable SARS-CoV-2, but avoiding a significant influence on RNA copies detection (Delpuech et al., 2022; Lamy et al., 2022; Pastorino et al., 2020).

4. Conclusions

SARS-CoV-2 concentrations were monitored during the Omicron wave in the stages of a full-scale WWTP characterised by a common configuration based on primary settling and secondary biological treatments, thickening and mesophilic anaerobic digestion.

Rather than primary sedimentation, the most effective treatment for SARS-CoV-2 removal was the biological stage, such as CAS and MBR. The small volume and water-like density of the virus are not conducive to spontaneous sedimentation, while adsorption, flocculation, predation and natural decay in the biological stages led to increased viral removal. The reduction of SARS-CoV-2 concentration in the biological stages was 1.8 ± 0.9 and 2.2 ± 0.7 logs in CAS and MBR, respectively. Some effluent samples were below the LOD of the analytical method. The SARS-CoV-2 concentrations in the CAS and MBR effluents (mean of positive samples) were 6.8×10^2 GU/L and 6.4×10^2 GU/L, respectively. The MBR effluent, showed more negative samples, as small particles are retained by the ultrafiltration membrane and cake layer.

Due to the affinity of enveloped viruses towards biosolids, accumulation of SARS-CoV-2 was observed in primary sludge and secondary sludge, where the concentrations were 5.2×10^4 GU/L and 3.5×10^4 GU/L, respectively. The mesophilic anaerobic digester operated at the temperature of 31–33 °C presented a negligible reduction of the SARS-CoV-2 RNA.

CRediT authorship contribution statement

Francesca Cutrupi: Conceptualization, Investigation (RNA extraction, RT-qPCR), Writing - original draft, review &edit; Maria Cadonna: Methodology (RNA extraction, RT-qPCR), Revision of original draft; Mattia Postinghel: Methodology (RNA extraction, RT-qPCR); Paola Foladori: Conceptualization, Formal analysis, Visualization, Writing - original draft, Writing - review &edit, Funding acquisition.

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.

Data availability

Data will be made available on request.

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