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Removal performance of SARS-CoV-2 in wastewater treatment by membrane bioreactor, anaerobic-anoxic-oxic, and conventional activated sludge processes

Rongxuan Wang a, Md. Alamin a, Shohei Tsujib, Hiroe Hara-Yamamurab, Akihiko Hatac, Bo Zhao d,e, Masaru Ihara e,f, Ryo Honda b,e,⁎

a Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Japan
b Faculty of Geosciences and Civil Engineering, Kanazawa University, Kanazawa, Japan
c Department of Environmental and Civil Engineering, Toyama Prefectural University, Imizu, Japan
d Key Laboratory of Integrated Regulation and Resource Development of Shallow Lakes of Ministry of Education, College of Environment, Hohai University, Nanjing, PR China
e Research Center for Environmental Quality Management, Graduate School of Engineering, Kyoto University, Otsu, Japan
f Faculty of Agriculture and Marine Science, Kochi University, Nankoku, Japan

HIGHLIGHTS
• SARS-CoV-2 RNA in influent wastewater was detected at 3.3–6.0 log10 copies/L.
• The total LRV by the target WWTPs was at least 2.5 logs or higher.
• The MBR had more stable reduction of SARS-CoV-2 RNA than CAS and A2O.
• SARS-CoV-2 RNA in final effluent was all below 20 copies/L after chlorination.
• PMMoV is a good indicator to ensure the reduction of SARS-CoV-2 in WWTP.

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ABSTRACT
The potential risk of SARS-CoV-2 in treated effluent from a wastewater treatment plant (WWTP) is concerned since SARS-CoV-2 is contained in wastewater during the COVID-19 outbreak. However, the removal of SARS-CoV-2 in WWTP has not been well investigated. The objectives of this study were (i) to clarify the removal performance of SARS-CoV-2 during wastewater treatment, (ii) to compare the removal performance of different secondary treatment processes, and (iii) to evaluate applicability of pepper mild mottle of virus (PMMoV) as a performance indicator for the reduction of SARS-CoV-2 RNA in wastewater treatment. Influent wastewater, secondary-treatment effluent (before chlorination), and final effluent (after chlorination) samples were collected from a WWTP from May 28 to September 24, 2020, during the COVID-19 outbreak in Japan. The target WWTP had three parallel treatment systems employing conventional activated sludge (CAS), anaerobic-anoxic-oxic (A2O), and membrane bioreactor (MBR) processes. SARS-CoV-2 in both the liquid and solid fractions of the influent wastewater was concentrated and quantified using RT-qPCR. SARS-CoV-2 in treated effluent was concentrated from 10 L samples to achieve a detection limit as low as 10 copies/L. The log reduction value (LRV) of SARS-CoV-2 was 2.7 ± 0.86 log10 in CAS, 1.6 ± 0.50 log10 in A2O, and 3.6 ± 0.62 log10 in MBR. The lowest LRV observed during the sampling period was 2.8 log10 in MBR, 1.2 log10 in CAS, and 1.0 log10 in A2O process, indicating that the MBR had the most stable reduction performance. PMMoV was found to be a good indicator virus to evaluate reduction performance of SARS-CoV-2 independent of the process configuration because the LRV of PMMoV was significantly lower than that of SARS-CoV-2 in the CAS, A2O and MBR processes.
1. Introduction

The coronavirus infectious disease 2019 (COVID-19) pandemic caused over 220 millions of confirmed infections and over 4 million deaths worldwide (World Health Organization, 2021). Because patients infected with SARS-CoV-2 shed the virus in feces (Miura et al., 2021), the detection of SARS-CoV-2 RNA in influent wastewater at wastewater treatment plants (WWTPs) has been reported during the COVID-19 outbreak in various countries (Tanimoto et al., 2022; Hata et al., 2021; La Rosa et al., 2020; Medema et al., 2020; Peccia et al., 2020; Sherrchun et al., 2020). SARS-CoV-2 RNA has also been detected in the treated effluent of WWTPs at low concentrations (Haramoto et al., 2020; Tandukar et al., 2022; Westhaus et al., 2021). Nevertheless, infectious viral SARS-CoV-2 particles in wastewater have not been detected to date (Solosey, 2022). Infectious SARS-CoV-2 reportedly decays faster in wastewater than does viral RNA (Bivins et al., 2020). Thus, it has been speculated that the potential risk of COVID-19 transmission via a wastewater route is quite low or still controversial (Ahmed et al., 2021a; Ahmed et al., 2021b; Albert et al., 2021; Kumar et al., 2021a; Zaneti et al., 2021). However, as long as SARS-CoV-2 RNA is detected, the lack of detected infectious viral particles does not mean its absence but also its possible presence under the detection limit. Indeed, a cohort study reported possible transmission via a wastewater route in conjunction with poor sanitation (Yuan et al., 2021). Although the potential infection risk of SARS-CoV-2 in wastewater is unknown, it is important to investigate the reduction of SARS-CoV-2 in wastewater treatment plants to securely control and minimize its potential risk.

A membrane bioreactor (MBR) process has better virus removal performance than a conventional activated sludge (CAS) process (Kitajima et al., 2014; Kuo et al., 2010; Li et al., 2021; Simmons et al., 2011), and is promising for the removal of emerging viruses (Simmons et al., 2011). Most past studies have targeted non-enveloped enteric viruses, which are composed of protein capsids. While, SARS-CoV-2 is an enveloped virus, which have wrapping with phospholipid bilayer membrane. Reduction of enveloped virus in wastewater treatment process was reportedly more efficient than non-enveloped viruses because the enveloped virus is more vulnerable (Gundy et al., 2009; Ye et al., 2016). To date, there has only been a limited number of studies on the reduction of SARS-CoV-2 RNA in WWTPs. In CAS, the reduction of SARS-CoV-2 RNA in WWTPs has been reported to be 1.0–4.1 log_{10} (Abu Ali et al., 2020; Saguti et al., 2021; Serra-Compte et al., 2021), while only one study has reported the reduction of SARS-CoV-2 in an MBR (Serra-Compte et al., 2021). In the study, the reduction was reported to be 1.0–2.9 log_{10}, although the SARS-CoV-2 in the influent wastewater was quantified only for the liquid fraction (Serra-Compte et al., 2021). Actually, SARS-CoV-2 was reportedly abundant in both liquid and solid fractions (Alamin et al., 2022; Forés et al., 2021; Juel et al., 2021; Kitamura et al., 2021). Therefore, the reported LRV of SARS-CoV-2 in MBR has possibly been underestimated in previous studies.

The objective of this study is to evaluate reduction of SARS-CoV-2 in WWTPs by MBR and other process configurations. To this end, reduction of SARS-CoV-2 was investigated in a WWTP that have parallel treatment processes of CAS, A2O and MBR. Total reduction of SARS-CoV-2 in wastewater was investigated by quantifying SARS-CoV-2 in both the solid and liquid fractions of the wastewater. Moreover, applicability of PMMoV as a performance indicator for SARS-CoV-2 reduction in wastewater treatment was assessed. The outcomes of this study are useful for ensuring the reduction of SARS-CoV-2 RNA in wastewater, thus reducing the possible risk of infection in water environments.

2. Materials and methods

2.1. Wastewater treatment plant and sampling

Influent wastewater and treated effluents were collected from a municipal WWTP in Japan twice a month from May 28 to September 24, 2020. The targeted WWTP received municipal wastewater from 270,000 of population via the two influent channels #1 and #2, which covered 32% of the total population in the entire city. The wastewater was treated in parallel using the three treatment systems of CAS, MBR, and anaerobic-anoxic-oxic (A2O) processes (Fig. 1). The wastewater from the influent channel #1 was distributed to the CAS and MBR processes, while all of the wastewater from the influent channel #2 was treated by the A2O process. The secondary-treatment effluents of the channels #1 and #2 were chlorinated, and then merged before final discharge. The target WWTP satisfied the local regulations for effluent quality during the study period (Table S6). The flow rate for the wastewater distributed into each system was summarized in Table S1. The collected samples and volume are: 250 mL of wastewater from influent channels #1 and #2, 10 L of secondary-treatment effluent from the CAS, MBR and A2O processes, and 10 L of the final effluent after the chlorinated effluents from both channels merged. All samples were collected in plastic containers sterilized using gamma-ray irradiation, and immediately transported to the laboratory on ice. The influent wastewater samples were stored at ~ 80 °C until analysis. Immediately after sampling the final effluent, sodium thiosulfate was added to the final concentration at 50 mg/L to quench the residue chlorine. Effluent samples were processed to measure the virus concentration within 24 h after sampling.

![Fig. 1. Treatment flow of the target wastewater treatment plant (WWTP).](image-url)
2.2. Virus concentration and RNA extraction

Polyethylene glycol (PEG) precipitation is a conventional method used to determine the concentration of viruses in wastewater samples. Recently, PEG precipitation has been reported to effectively concentrate SARS-CoV-2 RNA in wastewater (Alamin et al., 2022; Hata et al., 2021; Sapula et al., 2021; Torri et al., 2021). In this study, the viral RNA in the influent wastewater samples was concentrated following Alamin et al. (Alamin et al., 2022). First, the solid fraction of the wastewater was collected by centrifuging 40 mL of each sample at 3000 xg at 4 °C for 30 min, then resuspended in 500 μL of phosphate buffer solution. The viral RNA in the secondary effluents and final effluents were first concentrated using a hollow-fiber ultrafiltration membrane unit (APS25SA, Asahi Kasei) within 24 h after sampling. The membrane unit was conditioned by circulating 200 mL of 5 % FBS solution prior to sample filtration. After 10 L of the effluent sample had been filtered, the virus concentration retained in the membrane unit was recovered using an elution buffer containing 0.1 % (v/v) Tween 80, 100 mg/L sodium polyphosphate, and 10 ppm (v/v) antifoam A. The recovered concentrate (approximately 100 – 200 mL) was subsequently concentrated further using PEG precipitation as described above, but with the solid fraction included as well. The concentrate samples were stored at −80 °C until RNA extraction. Viral RNA was extracted from 140 μL of the concentrate using a QIAamp Viral RNA Mini Kit (Qiagen, USA).

2.3. Quantification of SARS-CoV-2

The extracted SARS-CoV-2 RNA was quantified using two-step RT-qPCR. After RNA extraction, 5 μL of the RNA extract was subjected to reverse transcription (RT) with PrimeScript RT Master Mix (Takara Bio, Japan) to obtain 25 μL of cDNA. SARS-CoV-2 RNA was quantified using qPCR with Premix Ex Taq (Probe qPCR), ROX Plus (Takara Bio, Japan), and the CDC 2019-nCoV_N1 (CDCN1) primer and probe was used for quantification of SARS-CoV-2 RNA (Table S2). SARS-CoV-2 RNA was detected using a real-time PCR system (Quanstudio 5, Thermo, USA). The thermal cycling conditions for the SARS-CoV-2 assay was as follows: initial denaturation at 95 °C for 20 s, followed by 45 cycles of denaturation at 95 °C for 5 s, and primer annealing and extension reaction at 60 °C for 30 s. The limit of detection (LOD) and limit of quantitation (LOQ) in the influent wastewater were approximately 3 log_{10} copies/L and 4 log_{10} copies/L, respectively (Table S3). The LOD and LOQ in the secondary-treatment effluent and final effluent were approximately 10 copies/L and 100 copies/L, respectively (Table S3).

2.4. Quantification of process control (PMMoV)

F-specific bacteriophage (F-phage) and pepper mild mottle virus (PMMoV) were used as process controls, according to Hata et al. (Hata et al., 2021). F-phage was used as a process control to evaluate the efficiency of the virus concentration step, while PMMoV was used to evaluate the detection efficiency for the entire process, i.e., concentration, RNA extraction and RT-qPCR assay. The F-phage concentration in each sample was quantified before and after the concentration step using conventional plaque assay using Salmonella typhimurium WG49 as the host strain. The recovery of F-phage was determined based on the concentrations before and after the concentration process using Eq. (1).

$$R_{F-phage} = \frac{C_f}{C_0} = \frac{V_e}{V_i}$$

where, $R_{F-phage}$: concentration efficiency of F-phage, $C_f$ (PFU/mL): the F-phage concentration in the virus concentrate, $V_e$ (μL): total volume of the concentrate, $V_i$ (μL): volume of the raw wastewater sample subjected to the concentration process, $C_0$ (PFU/μL): the F-phage concentration in the raw wastewater sample.

The PMMoV for each sample before and after the concentration step was quantified using two-step RT-qPCR. Extraction and quantification of the viral RNA were performed in the same manner as for SARS-CoV-2. The influent wastewater samples were directly subjected to RNA extraction. The treated effluent wastewater samples were subjected to RNA extraction after primary virus concentration using the hollow-fiber ultrafiltration unit, as described above before RNA extraction. To evaluate the detection efficiency of PMMoV, PMMoV in the samples that had been concentrated using PEG precipitation were also quantified in the same manner. Recovery efficiency of PMMoV was calculated from the proportion of the detected concentration after the virus concentration to the reference concentration without the virus concentration step. The primer and probe used for quantification of PMMoV were shown in Table S2. The thermal cycling conditions for the PMMoV assay were as follows: initial denaturation at 95 °C for 20 s, followed by 40 cycles of denaturation at 95 °C for 15 s, and primer annealing and extension at 60 °C for 60 s.

The recovery efficiency of F-phage was 5.6–85 % in the liquid fraction of the influent wastewater, 1.2–48 % in the solid fraction, and 1.0–3.4 % in the treated effluents (Tables S4, S5). The detection efficiency of PMMoV ranged 1.3–219 % in the liquid fraction of influent wastewater and 4.8–183 % in the solid fraction, and 5.7–328 % in the treated effluent. The detection efficiencies of F-phage and PMMoV were consistent with the reported values in previous study (Alamin et al., 2022).

2.5. Determination of the log reduction value (LRV)

Removal performance of SARS-CoV-2 and PMMoV was evaluated using the log reduction value (LRV), which was calculated based on the total loading of the virus in the influent wastewater and treated effluent for each process, using Eqs. (2)–(6). For LRV in CAS and MBR, the virus concentration in the influent wastewater from channel #1 was used in the calculation of the LRV. For the calculation of LRV in the entire WWTP and the A2O process, the virus concentration in the influent wastewater was calculated as the flow-rate weighted average of the channels #1 and #2 because the target WWTP and A2O process received wastewater from both influent channels. For calculation of LRV by chlorination, the virus concentration before chlorination was calculated as the flow-rate weighted average of the secondary-treatment effluent from the CAS, A2O and MBR processes.

$$R_{WWTP} = - \log_{10} \left( \frac{X_e}{X_i} \right) = - \log_{10} \left( \frac{C_{WWTP} \cdot (Q_{CAS} + Q_{MBR} + Q_{A2O})}{C_{Gi} \cdot Q_i + C_{G2} \cdot Q_2} \right)$$

(2)

$$R_{CAS} = - \log_{10} \left( \frac{C_{CAS} \cdot Q_{CAS}}{C_{Gi} \cdot Q_{CAS}} \right)$$

(3)

$$R_{MBR} = - \log_{10} \left( \frac{C_{MBR} \cdot Q_{MBR}}{C_{Gi} \cdot Q_{MBR}} \right)$$

(4)

$$R_{A2O} = - \log_{10} \left( \frac{C_{A2O} \cdot Q_{A2O}}{C_{Gi} \cdot Q_{A2O} + C_{G2} \cdot Q_2} \right)$$

(5)

$$R_{CI} = - \log_{10} \left( \frac{C_{WWTP} \cdot (Q_{CAS} + Q_{MBR} + Q_{A2O})}{C_{WWTP} \cdot (Q_{CAS} + C_{CAS} + C_{MBR} + C_{A2O} + C_{A2O} \cdot Q_{A2O})} \right)$$

(6)

where $R_{WWTP}$: LRV by the entire process of the target WWTP; $R_{CAS}$, $R_{MBR}$, $R_{A2O}$: LRV by CAS, MBR, and A2O, respectively; $R_{CI}$: LRV by chlorination; $X_i$: the total virus loading in the influent; $X_e$: the total virus loading in the effluent.

$C_{WWTP}$: the virus concentration in the final effluent of the target WWTP (copies/L); $C_{CAS}$, $C_{MBR}$, $C_{A2O}$: the virus concentration in treated effluent of CAS, MBR, and A2O, respectively (copies/L); $C_{Gi}$, $C_{G2}$: the virus concentration in wastewater of influent channels #1 and #2, respectively (copies/L).
The total concentration of SARS-CoV-2 RNA, which was calculated as the sum of the concentration in the liquid and solid fractions, was $3.7 - 6.0 \log_{10}$ copies/L in the influent channel #1 and $3.3 - 4.4 \log_{10}$ copies/L in the influent channel #2 (Fig. 2a). The concentration of SARS-CoV-2 in the channel #1 was significantly higher than that in the channel #2 ($p = 0.01 < 0.05$). All of the influent samples were detected above limit of detection (LOD). The range of the detected concentration was not significantly different between the reported range of $4.1 - 4.5 \log_{10}$ copies/L during the first wave of the COVID-19 outbreak in Japan (Hata et al., 2021). High concentrations of SARS-CoV-2 were detected from May 28 to June 23 although there were no clinically confirmed cases. This indicated that there were undiagnosed infected people because of the limited capacity of clinical testing in Japan. Significant correlations were not observed between SARS-CoV-2 concentrations in wastewater and confirmed cases of COVID-19 in the target city (Fig. 2a). One possible reason for the low correlation between SARS-CoV-2 RNA levels in wastewater and the number of confirmed cases is that the clinical PCR testing was limited during the early stages of the outbreak in Japan due to the low testing capacity. Hata et al. (Hata et al., 2021) also reported the detection of SARS-CoV-2 RNA in wastewater in late May 2020 despite the number of newly confirmed cases being zero or below 1 per 100,000 population. Another possible reason for the low correlation is that the sampling interval was too long to capture any change in the infection trend. More frequent sampling at least once a week is suggested to obtain the correlated data (Ahmed et al., 2021a; Ahmed et al., 2021b; Giraud-Billoud et al., 2021; Kumar et al., 2021b).

SARS-CoV-2 RNA was detected both in liquid and solid fractions of the influent wastewater. In influent wastewater, SARS-CoV-2 RNA was mostly detected at higher concentration in the solid fraction (Fig. 2b; Table S3). The detected SARS-CoV-2 concentrations in each fraction of wastewater were $3.7 \pm 0.59$ log$_{10}$ copies/L in liquid fraction and $4.1 \pm 0.83$ log$_{10}$ copies/L in solid fraction. Out of all 18 influent samples, five samples resulted in negative for SARS-CoV-2 RNA in the liquid fraction, while only one sample resulted in negative in the solid fraction. Abundance of SARS-CoV-2 RNA in the solid fraction have also been reported in past studies (Tanimoto et al., 2021; Graham et al., 2021; Kitamura et al., 2021; Li et al., 2021). Kitamura et al. (Kitamura et al., 2021) reported that the SARS-CoV-2 RNA concentration in the liquid and solid fractions was $3.0 - 3.6$ log$_{10}$ copies/L and $2.2 - 4.1$ log$_{10}$ copies/L, respectively, while Li et al. (Li et al., 2021) reported $1.4 - 6.2$ log$_{10}$ copies/L and $1.2 - 5.1$ log$_{10}$ copies/L, respectively. Alamin et al. (Alamin et al., 2022) reported that SARS-CoV-2 spiked in wastewater was partitioned in both liquid and solid fractions. Hence, the sum of the SARS-CoV-2 RNA concentration in liquid and solid fractions were used as the total SARS-CoV-2 concentration in the influent wastewater for the calculation of log$_{10}$ reduction values in the following discussion.

### 3.2. Removal of SARS-CoV-2 by CAS, A2O and MBR processes

The range of the detected SARS-CoV-2 RNA concentration secondary-treatment effluent (before chlorination) was $1.2 - 2.9$ log$_{10}$ copies/L in CAS, $1.8 - 3.1$ log$_{10}$ copies/L in A2O and $1.2 - 2.0$ log$_{10}$ copies/L in MBR processes (Fig. 3). Of the nine effluent samples taken for each process, two samples from the CAS, four samples from the A2O process, and three samples from the MBR were below the LOD. Though the average of the detected SARS-CoV-2 concentrations after secondary treatment were not significantly different among CAS, A2O and MBR ($p > 0.05$). However, the highest SARS-CoV-2 concentration in the MBR effluent was $2.0$ log$_{10}$ copies/L, 1 log$_{10}$ lower than that observed in the CAS and A2O processes. This indicates that MBR effluent had stably lower SARS-CoV-2 than CAS and A2O effluents. The SARS-CoV-2 RNA concentration after secondary treatment found in the present study was lower than previously reported values (Table 1). (Haramoto et al., 2020) reported RNA concentration was detected as $3.4$ log$_{10}$ copies/L at highest in treated effluent in CAS process, while the LOD of the effluent ranged from 2.1 to $3.4$ log$_{10}$ copies/L. The lower detected concentration in effluent in this study than the previous studies was probably caused not by better reduction performance at the
target WWTP, but by the lower LOD of effluent (0.79–1.4 log). Importantly, the effluent concentration after secondary treatment was independent of the influent concentration for the CAS, A2O, and MBR processes during the study period.

LRV by secondary treatment ranged 0.69–5.3 or higher among CAS, A2O and MBR. Average and standard deviations of LRV were 2.7 ± 0.86 in the CAS, 1.6 ± 0.50 in the A2O, and 3.6 ± 0.62 in the MBR on those days when both influent and secondary treated effluent was detected above the LOD (Fig. 4). The LRVs in A2O nor MBR were not significantly different from LRV in CAS (p > 0.05). The lowest LRV in MBR was 2.8, which was higher by >1.5-log compared to the lowest LRVs in CAS and A2O (1.2 log and 1.0 log, respectively). Hence, the MBR achieved a more stable reduction of SARS-CoV-2 RNA than CAS and A2O. The highest LRVs were around 5 log or higher in all processes, having no remarkable difference among the different processes. The reduction of SARS-CoV-2 by CAS observed in this study was consistent with previous studies, which have reported LRVs of 1.0–4.1 log (Abu Ali et al., 2020; Saguti et al., 2021; Serra-Compte et al., 2021). However, the reduction of SARS-CoV-2 by MBR observed in this study was remarkably higher than the reported values by Serra-Compte et al. (Serra-Compte et al., 2021), which was 1.0–2.9 log. In Serra-Compte et al. (Serra-Compte et al., 2021), only liquid fractions of influent wastewater were targeted for SARS-CoV-2 quantification in MBR. SARS-CoV-2 RNA is more often abundant in the solid fraction of wastewater (Forés et al., 2021; Juel et al., 2021; Kitamura et al., 2021). Also in this study, SARS-CoV-2 was often more abundant in the solid fraction of influent wastewater than in the liquid fraction (Fig. 2b). Hence, LRV by MBR reported by Serra-Compte et al. (Serra-Compte et al., 2021) was possibly underestimated. The major mechanism for virus reduction in wastewater treatment is the adsorption of viral particles on sludge flocs and removal of the sludge where the viruses are adsorbed (Zhu et al., 2021). Membrane filtration in MBR has better reduction of sludge particles than gravity sedimentation in CAS. Therefore, the actual LRV of SARS-CoV-2 for an MBR is expected to be equal to or higher than that for CAS process, as observed in the present study.

The LRV of SARS-CoV-2 from the CAS and A2O processes observed in the present study was higher than those of enteric viruses reported in past studies, whereas LRV of SARS-CoV-2 from the MBR was not remarkably different from those of enteric viruses in previous studies. LRV of norovirus has been reported to be 1.3–2.9 in CAS, 1–2 in A2O and 4.6–5.7 in MBR (Chaudhry et al., 2015; Kitajima et al., 2014; Miura et al., 2015). The reported LRV of enterovirus was 2–3 in CAS, 0.5–1.0 in A2O and 3.4–5.1 in MBR (Francy et al., 2012; Miura et al., 2015; Simmons et al., 2011; Tandukar et al., 2020). LRVs of adenovirus have been reported 2.0–3.0 in CAS, 0.4–1.6 in A2O and 3.7–5.6 in MBR (Chaudhry et al., 2015; Francy et al., 2012; Kuo et al., 2011; Tandukar et al., 2020). These studies show that the lowest LRV of MBR was higher than that by CAS and A2O processes for some enteric viruses. A higher reduction of SARS-CoV-2 than enteric viruses in wastewater treatment was predicted by Kumar et al. (Kumar et al., 2021a). SARS-CoV-2 is expectedly more adhesive to sludge flocs than non-enveloped enteric viruses because SARS-CoV-2 is an enveloped virus with a relatively hydrophobic lipid bilayer membrane. Ye et al. (Ye et al., 2016) reported that the enveloped viruses of murine hepatitis virus (MHV) and p6 in wastewater exhibited four times greater adsorption onto solid particles than the non-enveloped viruses of MS2 and T3. The greater reduction of SARS-CoV-2 in CAS and A2O observed in the present study indicates that adsorption onto sludge flocs is one of the major mechanisms underlying the removal of SARS-CoV-2 reduction in these processes. In contrast, the reduction of SARS-CoV-2 in MBR was not remarkably different from that of non-enveloped enteric viruses. This suggests that other mechanisms also play important role in reduction of SARS-CoV-2 by MBR, as discussed in detail below.

SARS-CoV-2 RNA in the chlorinated final effluent was all under detection limit, which was approximately 10 copies/L. The entire reduction of SARS-CoV-2 RNA by the WWTP was expected at least 3.5 log or higher. The minimum LRV in the target WWTP was at least 2.5. In past studies, SARS-CoV-2 RNA in effluent after chlorination was also reported as below detection limit of 2 log10 copies/L (Serra-Compte et al., 2021) or 2.6 log10 copies/L (Randazzo et al., 2020). This illustrates that SARS-CoV-2 RNA remained in the final effluent of the WWTP at very low concentrations, as predicted by Kumar et al. (Kumar et al., 2021a). The LRV of SARS-CoV-2 by the entire process observed in this study was comparable to or higher than typical LRV of norovirus and enterovirus, which ranged 1.5–3.9 log10 copies/L (Jumat et al., 2017; Sano et al., 2016). The reduction of SARS-CoV-2 RNA by chlorination process was at least 1.0 log or higher. The LRV of SARS-CoV-2 by chlorination was higher than the reported LRV of other enteric viruses. The reduction of enterovirus was <0.5 log by chlorination process (Jumat et al., 2017). The limited LRV by chlorination was probably because some viral RNA remain even after the virus is inactivated. Hence, reduction of SARS-CoV-2 infectivity is expected to be higher than viral RNA. Bivins et al. (Bivins et al., 2020) reported that SARS-CoV-2 RNA was more persistent than infectious SARS-CoV-2. Therefore, concentration of infectious SARS-CoV-2 in treated effluent is expected to be far less than the concentration present in wastewater.

### Table 1: Reduction of SARS-CoV-2 at various wastewater treatment processes.

| Country | Treatment system | Concentration before treatment (log10 copies/L) | Concentration after treatment (log10 copies/L) | Log removal value | Reference |
|---------|------------------|-----------------------------------------------|-----------------------------------------------|------------------|----------|
| Japan   | CAS              | 3.7–6.0                                       | 0.8–2.9                                       | 2.8 ± 0.86       | This study |
|         | MBR              | 3.7–6.0                                       | 1.2–2.0                                       | 3.6 ± 0.62       |          |
|         | A2O              | 3.3–4.4                                       | 0.86–3.0                                      | 1.6 ± 0.50       |          |
|         | Chlorination     | 1.2–2.9                                       | <1.1–1.8                                      | >1.0 ± 0.39      |          |
| Spain   | Activated sludge | 3.3 ± 0.7                                     | 2.3 ± 0.4                                     | 1.0 ± 0.54       | (Serra-Compte et al., 2021) |
| France  | Activated sludge + nutrient removal followed by clarification | 3.7 ± 0.7 | 2.3 ± 0.7 | 1.4 ± 0.72 | (Serra-Compte et al., 2021) |
|         | MBR              | 3.9 ± 0.9                                     | 2.1 ± 0.35                                    | 2.0 ± 0.93       | (Kumar et al., 2021b) |
| India   | SBR + chlorination | 3.2                                           | 2.4                                           | 0.77             |          |
|         | Constructed wetland | 2.8                                           | 2.5                                           | 0.32             |          |
| Spain   | Various WWTPs    | 5.0–6.0                                       | ND*–5.0                                       | –                | (Randazzo et al., 2020) |

ND*: not detected (detection limit: approximately 5 log copy/L), CAS: conventional activated sludge process, MBR: membrane bioreactor process, A2O: anaerobic-anoxic-oxid process, SBR: sequencing batch reactor process.
SARS-CoV-2 RNA detected in this study. Westhaus et al. (Westhaus et al., 2021) also reported no infectious potential for treated wastewater following the exposure of human Caco-2 cells to a treated wastewater sample that contained 22 copies/mL of SARS-CoV-2 RNA. In the present study, SARS-CoV-2 RNA was found at much lower level than in Westhaus et al. (Westhaus et al., 2021), thus infectious SARS-CoV-2 is unlikely to be present at significant levels in the WWTP effluent in the present study. Moreover, Kumar et al. (Kumar et al., 2021a; Kumar et al., 2021c) estimated that the potential risk of SARS-CoV-2 via WWTP effluent was quite low, with the LRV of SARS-CoV-2 in WWTPs assumed to be much higher than for other enteric viruses. The results of the present study support their assumption regarding the high reduction of SARS-CoV-2 in WWTPs. Therefore, the potential risk of SARS-CoV-2 infection via exposure to treated effluent from the target WWTP in this study is likely to be quite limited.

There was no clear correlation found between the LRV and operating parameters, such as the mixed-liquor suspended solids (MLSS), sludge retention time (SRT), hydraulic retention time (HRT), aerobic HRT (A-HRT), dissolved oxygen (DO), temperature, pH, and conductivity (Fig. S1). MBR has been reported to have better reduction of enteric viruses than CAS. However, the average reduction of SARS-CoV-2 RNA was not significantly different between MBR and CAS in the present study. The major mechanism for viruses reduction in WWTP are (i) adsorption on sludge particles and (ii) separation of sludge where viral particles are adsorbed (Hirani et al., 2014; Miura et al., 2018). Moreover, the longer SRT allows better biological degradation of viral particles as well as to maintain MLSS (Hirani et al., 2014). It is known that SARS-CoV-2 is sensitive to high temperature. However, no clear relationship between these parameters and the LRV of SARS-CoV-2 RNA has not been found so far, probably because many factors simultaneously affect the reduction of SARS-CoV-2 in WWTPs.

In this study, the MBR achieved an LRV of at least 2.8-log for SARS-CoV-2 RNA, while that for the CAS and A2O processes was sometimes as low as 1.0–2.0. This indicates that the MBR has the advantage of stable reduction for both non-enveloped enteric viruses and enveloped viruses. However, the LRV of viruses in an MBR also depends on the properties of the membrane, including its pore size and membrane material. The MBR in the present study was equipped with an MF membrane, whose nominal pore size was larger than the size of SARS-CoV-2 viral particles, and it is possible that an MBR with an ultrafiltration (UF) membrane would achieve the greater removal of SARS-CoV-2. Further research is thus required on the removal of enveloped viruses such as SARS-CoV-2 by MBR with different membrane settings. In this study, the SARS-CoV-2 RNA concentrations in the treated effluent fluctuated independently from those in the influent wastewater. However, the determinative factors were not clarified, probably because many factors were simultaneously involved. Further investigation is necessary to reveal the impact of operating parameters on reduction of SARS-CoV-2 in WWTPs.

3.3. Potential of PMMoV as a performance indicator for SARS-CoV-2 removal

The requirements for a performance indicator for virus reduction in wastewater treatment are that: (i) the indicator virus is constantly abundant in wastewater independent of the outbreak situation in the sewershed, (ii) the indicator virus has a high enough concentration to be detected even after treatment, (iii) LRV of the indicator virus is comparable to or lower than the LRV of the target virus in order to secure the reduction of the target virus by monitoring of the indicator virus (Farkas et al., 2020). In the present study, the observed concentration of PMMoV in influent wastewater was 7.6 ± 0.33 log_{10} copies/L and 6.5 ± 0.43 log_{10} copies/L in the channels #1 and #2, respectively. Hence, PMMoV was constantly abundant in wastewater independent of the COVID-19 outbreak situation in the sewersheds. PMMoV was also consistently detected in treated effluent at sufficiently higher concentration than LOD. In secondary treatment effluents before chlorination, PMMoV concentrations were 6.1 ± 0.50 log_{10} copies/L in CAS process, 6.0 ± 0.34 log_{10} copies/L in A2O, and 5.3 ± 0.057 log_{10} copies/L in MBR. The detected concentrations in the final treated effluent were 4.6 ± 0.17 log_{10} copies/L, while the LOD of PMMoV was 1.5 ± 0.14 log copies/L (Table S4, S5). Hence, PMMoV was present in the detectable range even after treatment, independent of the process configuration. The LRV of PMMoV from the influent to the final effluent after chlorination was 2.3 ± 0.39 log, which was comparable to or lower than the LRV of SARS-CoV-2 (Fig. 5d). In secondary treatment before chlorination, LRV of PMMoV were 1.7 ± 0.45 in CAS, 1.1 ± 0.31 in A2O process and 2.4 ± 0.30 in MBR (Fig. S5). The obtained results were consistent with the range of previous studies, which was reportedly 1.7–3.7 log in CAS process and 0.9–1.9 log in MBR process (Papp et al., 2020; Wilhelm et al., 2010). The LRV of PMMoV observed in each secondary treatment was mostly lower than the LRV of SARS-CoV-2 RNA in the CAS, A2O, and MBR processes (Fig. 5). Lower LRV of PMMoV than norovirus was also reported in the past study, indicating that PMMoV is relatively persistent in wastewater treatment processes (Chen et al., 2021). These results indicate that the
LRV of PMMoV was consistently lower than the LRV of SARS-CoV-2 RNA independent of the process configuration. Thus, it is possible to ensure the reduction of SARS-CoV-2 by monitoring the LRV of PMMoV in WWTPs. Thus, PMMoV has good potential for use as a performance indicator for SARS-CoV-2 reduction in wastewater treatment processes.

4. Conclusions

In the target WWTP, SARS-CoV-2 RNA in influent wastewater was detected at concentration of 3.3 × 10^3 to 6.0 log_{10} copies/L. SARS-CoV-2 RNA in the final effluent after chlorination was all below the LOD at approximately 10 copies/L. The estimated total LRV after disinfection was at least 2.5 or higher. Importantly, the SARS-CoV-2 RNA concentration in the effluent was independent of the influent concentration in all of the secondary treatment processes. In secondary treatment, the observed LRVs of SARS-CoV-2 was 2.7 ± 0.86 in CAS, 1.6 ± 0.50 log in A2O, and 3.6 ± 0.62 log in MBR. The LRVs of SARS-CoV-2 in CAS and A2O were higher than the typical LRV of non-enveloped enteric viruses. The MBR had the most stable reduction performance of the three processes because the lowest LRV with the MBR was 2.8, which was higher by >1.5-log compared to the lowest LRVs in CAS and A2O (1.2 log and 1.0 log, respectively). In this study, the operating parameters (e.g., the DO, HRT, MLSS, SRT, Temperature, etc) had no significant relationship with reduction of SARS-CoV-2. PMMoV is a potentially good performance indicator for reduction of SARS-CoV-2 in various treatment processes, because (i) PMMoV was constantly abundant in influent wastewater independent of COVID-19 epidemic situation in the sewershed, (ii) PMMoV was present at detectable concentration even after treatment, and (iii) LRV of PMMoV was mostly lower than that of SARS-CoV-2 RNA with the CAS, A2O, and MBR processes. PMMoV would be a useful performance indicator to secure reduction of SARS-CoV-2 in wastewater treatment.

CRediT authorship contribution statement

Rongxuan Wang: Methodology, Investigation, Formal analysis, Writing - original draft, Visualization. Md. Alamin: Investigation. Shohei Tsuji: Investigation. Hiroe Hara-Yamamura: Investigation, Writing-review & editing. Akihiko Hata: Methodology, Writing-review & editing. Bo Zhao: Investigation. Masaru Ihara: Resources, Supervision. Ryo Honda: Conceptualization, Methodology, Writing - review & editing, Funding acquisition, Project administration.

Data availability

Data will be made available on request.

Declaration of competing interest

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

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Appendix A. Supplementary data

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