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# Removal of pepper mild mottle virus by full-scale microfiltration and slow sand filtration plants

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It is important to evaluate the removal of enteric viruses by drinking water treatment processes so that viral infection risk can be assessed and managed. However, evaluating the removal of enteric viruses by full-scale treatment processes can be challenging due to the low numbers of viruses and the presence of substances in the water samples that inhibit detection. In this study, we evaluated the removal of pepper mild mottle virus (PMMoV) by microfiltration (MF) and slow sand filtration (SSF) at two full-scale drinking water treatment plants in Japan, quantifying virus concentrations with real-time polymerase chain reaction (qPCR). The removal of PMMoV by MF ranged from 0.0 to >0.9 $\log_{10}$, although concentrations were below the detection limit for half of the treated water samples. SSF removed PMMoV by up to 2.8 $\log_{10}$; however, the removal efficiency decreased to 0.0–1.0 $\log_{10}$ under cold water temperatures. Process control showed that nucleic acid extraction and qPCR efficiency were inhibited in nearly 40% of water samples. Dilution, DAX-8, and ferrihydrite treatments for purification were effective in mitigating these inhibitory effects.

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## INTRODUCTION

Human enteric viruses, which are excreted in high number in the feces of infected people, have frequently been found in water sources such as surface water and groundwater.[1–4] These viruses are relatively resistant to water treatment processes and have occasionally been detected in treated drinking water and tap water, including in Brazil, China, Japan, Korea, and Ghana.[5–9] This is a public health concern because only a few viral particles can result in disease.[10] Several outbreaks in the USA, Australia, and Finland[11–13] were caused by enteric viruses in contaminated drinking water, which affected a large population.

To ensure drinking water is safe from viral contamination, ideally the presence of pathogenic viruses should be monitored in finished drinking water. However, this is not practical or feasible because determining the safety level of drinking water requires large water samples ($10^4$–$10^5$ L).[14] In addition, there are methodological limitations to discriminate between infectious and noninfectious viruses in water samples.[15,16] Thus, the assessment of viral infection risk in drinking water supplies is essential for protecting public health.

The World Health Organization[14,17] has recommended a Water Safety Plan to promote a safe drinking water supply. This plan proposes a multi-barrier approach to ensure the safety of drinking water that covers all the treatment steps from source to tap; this implies that each unit process in a drinking water treatment plant (DWTP) is a critical point for controlling the risks. Quantitative microbial risk assessment has been widely used to support implementation of the Water Safety Plan. In quantitative microbial risk management, data about the virus concentration in the water source together with a given safety target (e.g., no more than one in 10,000 people infected per year[18]) are used to determine virus reduction necessary in the DWTPs.[19,20] Thus, assessing the effectiveness of the removal of viruses by water treatment processes is a key component of the assessment of the risk of viral infection. However, the data about virus removal needed for the quantitative microbial risk assessment often rely on laboratory or pilot-scale studies, which may not adequately reflect the full-scale performance of the process being assessed.

Viruses can be reduced effectively by disinfection treatment; nevertheless, it is essential to evaluate the performance of other physical treatment processes to ensure robust multi-barrier treatment. There have been studies of virus removal by conventional water treatment processes, such as coagulation, sedimentation, and rapid sand filtration,[21,22] but there are only limited data on virus removal by microfiltration (MF) and slow sand filtration (SSF) processes in full-scale DWTPs.[23,24] Rapid sand filtration is combined with pretreatment by coagulation–sedimentation and can therefore be influenced by the remaining coagulant; MF and SSF processes are not combined with any pretreatment, which can affect the efficiency of virus removal. Unlike other countries (e.g., US) where regulation for turbidity only sets for tap water (must be less than 5 NTU), Japan strictly controls the turbidity level not only for water at the tap (<2° or approximately 2.8 NTU) but also for water upon treatment. This requires water treatment plants to be equipped with a system capable of maintaining turbidity at <0.1° (approximately 0.14 NTU), in accordance with the Interim Guideline for *Cryptosporidium* Treatment in the Water Supply.[21] The efficiency of virus removal by water treatment systems in Japan may therefore be unique.

Quantitative real-time polymerase chain reaction analysis (qPCR) is widely used to evaluate virus removal at DWTPs because of its rapidity, specificity, and high sensitivity. However, owing to the low concentration of viruses present in water, particularly treated water, it is necessary to enrich their number prior to qPCR detection, which is achieved by concentrating them from a large

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volume of water. During such virus concentration processes, it has been observed that some substances (e.g., humic acids) co-concentrated with the target viruses inhibited the subsequent qPCR detection process.[25,26] Several procedures have been developed to remove these inhibiting substances or to mitigate their effect on the environmental water sample assessments, but none has been fully effective because their efficiencies are highly dependent on the type of environmental matrix involved.[27] There is therefore a need for an effective strategy to mitigate this inhibition of qPCR by drinking water matrices.

The aim of the present study was to evaluate the virus removal efficiency of MF and SSF processes at two full-scale DWTPs in Japan, both subject to the turbidity regulations. The study focused on the pepper mild mottle virus (PMMoV), a plant virus belonging to the genus *Tobamovirus* in the family *Virgoviridae*. PMMoV has been proposed as a virus indicator for fecal pollution in water sources and as a useful process indicator that is readily detectable in water treatment systems.[21,22,28–30] Water samples (before and after MF or SSF processing) were collected over a period of one year and levels of the virus were determined by qPCR. In addition, the extent of qPCR inhibition in the water samples was investigated by spiking with cucumber green mottle mosaic virus (CGMMV) as a molecular process control. Finally, sample dilution and purification methods were tested for their ability to mitigate the effect of qPCR inhibition.

## RESULTS AND DISCUSSION

### Physicochemical parameters and indicator bacteria

The profile of physicochemical parameters and indicator bacteria for raw water samples and water treated with MF (Plant A) or SSF (Plant B) during the sampling period are shown in Table 1. At Plant A, the levels of pH (6.9 ± 0.3), turbidity (<1.0 NTU), electrical conductivity (102 ± 26 µs/cm), temperature (18.4 ± 1.9 °C), *E. coli* (0.0 ± 0.0 cfu/100 mL), and total coliforms (1.6 ± 1.9 cfu/100 mL) in raw water (from a groundwater source) were all relatively stable over the sampling period. None of these parameters showed a large change after MF treatment. At Plant B, the levels of the physicochemical and bacterial parameters in the raw water (from a lake source) were as follows: pH, 7.7 ± 0.4; turbidity, 2.1 ± 1.0 NTU; electrical conductivity, 288 ± 34 µs/cm; temperature, 14.3 ± 5.6 °C; *E. coli*, 0.4 ± 0.8 cfu/100 mL; and total coliforms, 145.8 ± 175.6 cfu/100 mL. The standard deviations show that these parameters remained relatively constant, with the exception of water temperature, which could be greatly influenced by weather conditions (Fig. 1). SSF treatment greatly reduced turbidity and total coliform level, but there were no large changes in the other parameters (Table 1).

### Application of mitigation processes

A total of 29 out of the 48 samples exhibited the molecular control efficiency (*E*) greater than 10% to obtain valid PMMoV concentrations. The remaining 19 samples (*E* <10%) were subjected to dilution and mitigation processes (Table S1). Ten-fold dilution improved the *E* value to ≥10% in 11 out of the 19 samples to give the valid PMMoV concentration. Further 10-fold (total 100-fold) dilution to the remaining eight samples improved the *E* values ≥10% to give valid PMMoV concentrations only to four samples, while the other four samples contained PMMoV below the limit of detection. These remaining four unquantified samples were further subjected to the DAX-8 and the Fh treatments, where the *E* values of these samples (3–5%) were <10%. Therefore, the PMMoV concentrations for these samples were considered to range from the quantified results here up to the limit of detection with 100-fold dilution.

Overall, the results indicated that the use of the molecular control to evaluate the efficiency of RNA extraction and RT-qPCR was necessary for determining accurate concentrations of the target virus in the water samples. The sample dilution mitigated the inhibition effectively, but this approach could result in false-negative results if the sample was diluted below the limit of detection. DAX-8 and Fh treatments were also effective for

**Table 1.** Physicochemical and index bacteria parameters of raw water samples and samples treated by microfiltration (MF) or slow sand filtration (SSF) during the sampling period from May 2017 to April 2018

| | | pH | Turbidity (NTU) | EC (µs/cm) | Temperature (°C) | *E. coli* (cfu/100 mL) | Total coliform (cfu/100 mL) |
|---|---|---|---|---|---|---|---|
| MF (Plant A) | Raw water | 6.9 ± 0.3 | <1.0 | 102 ± 26 | 18.4 ± 1.9 | 0.0 ± 0.0 | 1.6 ± 1.9 |
| | Treated water | 7.0 ± 0.5 | <1.0 | 104 ± 21 | 18.7 ± 1.8 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| SSF (Plant B) | Raw water | 7.7 ± 0.4 | 2.1 ± 1.0 | 288 ± 34 | 14.3 ± 5.6 | 0.4 ± 0.8 | 145.8 ± 175.6 |
| | Treated water | 7.8 ± 0.5 | <1.0 | 292 ± 31 | 15.7 ± 6.2 | 0.0 ± 0.0 | 6.2 ± 1.7 |

Results are presented as mean ± standard deviation ($n = 12$)
*EC* electrical conductivity

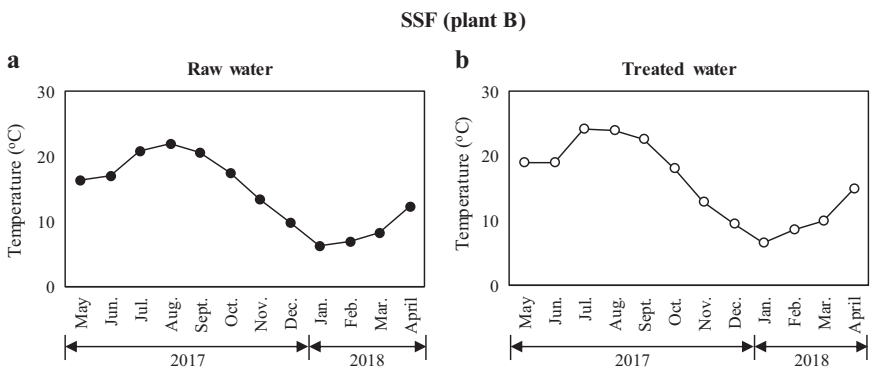


**Fig. 1** Monthly temperatures at Plant B of raw water **a** and water after treatment with slow sand filtration **b**

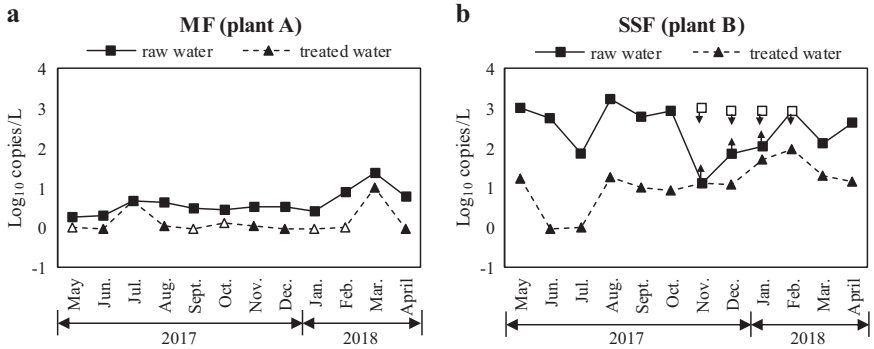


**Fig. 2** Profiles of pepper mild mottle virus concentration in raw (squares) and treated water (triangles). **a** Treatment by microfiltration at Plant A. **b** Treatment by slow sand filtration at Plant B. The white triangles indicate the limit of detection (0.9–1.6 copies/L) for the negative samples. The solid squares with arrows indicate positive results with the presence of inhibition ($E = 3$–5%). The unfilled squares with arrows indicate the possible highest concentration (886–977 copies/L) based on the negative results by 100-fold dilution ($E \geq 10\%$)

reducing the inhibitory substances but their efficiencies differed between water samples. Together, these results suggested that more than one purification method may be needed for the desired mitigation.

### PMMoV removal by MF

The performance of the MF process at Plant A at removing indigenous PMMoV is shown in Fig. 2a for the period May 2017 to April 2018. The PMMoV concentration in the raw water (from a groundwater source) remained stable at a low level ($0.6 \pm 0.3$ $\log_{10}$ copies/L (mean ± SD, $n = 12$)). In the treated water, PMMoV was always <1.0 $\log_{10}$ copies/L ($0.2 \pm 0.4$ $\log_{10}$ copies/L (mean ± SD, $n = 12$)), with half of the samples under the limit of detection, indicating that the MF membrane was able to remove PMMoV only from 0.0 to >0.9 $\log_{10}$. PMMoV is a rigid rod-shaped virus with diameter of 18 nm and a length of 300–310 nm. Thus, it could possibly penetrate through the 0.1 µm nominal pore size of the MF membrane, resulting in the low efficiency of virus removal. This result was consistent with those of previous studies that indicated that MF membranes (with pore sizes 0.1–0.22 µm) were not effective for removing enteric viruses (including adenovirus, coxsackievirus, hepatitis A virus, and murine norovirus) or bacteriophages (MS2 and Qβ) by size exclusion.[24,31] PMMoV and most enteric viruses carry a negative charge at neutral pH because their isoelectronic points are mostly <7.0.[32] Hydrophilic MF membranes based on PVDF are also negatively charged at neutral pH. Electrostatic interactions between the viruses and membrane surface did not appear to take place, allowing the viruses to pass easily through the MF membrane. This is consistent with previous studies in which limited virus removal (<0.5 $\log_{10}$) was achieved by using hydrophilic and negatively charged MF membranes (made from PVDF, polytetrafluoroethylene, or polycarbonate).[24]

### PMMoV removal by SSF

The profiles of PMMoV concentration before and after the SSF process at Plant B are shown in Fig. 2b for the period May 2017 to April 2018. The concentrations of PMMoV in raw water (from a lake) ranged from 1.1 to 3.3 $\log_{10}$ copies/L ($2.4 \pm 0.6$ $\log_{10}$ copies/L (mean ± SD, $n = 12$). During the period from November 2017 to January 2018, the level of PMMoV in the raw water was estimated to range from 1.1 to 1.8 $\log_{10}$ copies/L to <3.0 $\log_{10}$ copies/L. In these estimates, the lower value was the positive PMMoV result while inhibitors were still present ($E = 3$–5%), whereas the upper value of the undetermined sample was the detection limit obtained after mitigating for the inhibition by 100-fold sample dilution ($E > 10\%$), as described earlier. The level of PMMoV in treated water was in the range −0.02–2.0 $\log_{10}$ copies/L ($1.1 \pm 0.6$ $\log_{10}$ copies/L (mean ± SD, $n = 12$)). This showed that PMMoV removal remained steady at approximately 2.0 $\log_{10}$ between May and October 2017, decreased greatly to 0.0–1.0 $\log_{10}$ ($E = 3$–5%) or <1.8 $\log_{10}$ ($E > 10\%$) between November and March 2017, and then increased to 1.5 $\log_{10}$ in April 2018. These results clearly demonstrated that PMMoV removal by SSF was greatly reduced from November 2017 to March 2018. During this period, most of the physicochemical parameters remained relatively stable (pH, 7.08–8.41; turbidity, 1.0–4.46 NTU; and electrical conductivity, 242–331 µs/cm), although the water temperature varied (13.4–6.2 °C), with temperatures considerably colder than at the other times of the year (Table 1 and Fig. 1). This suggests that the low water temperatures may have influenced the efficiency of PMMoV removal. The viruses could be removed by the upper layers of the sand bed through adsorption onto the sticky biofilm known as Schmutzdecke[23,33], which contains many adsorption sites such as extracellular polymeric substances, proteins, polysaccharides, and lipids.[34] In addition, the removal or inactivation of viruses might occur due to biological activity such as predation and antagonism by microorganisms.[35,36] Extracellular enzymes and protease produced by microorganisms could also degrade viruses.[37,38] During low water temperatures, biological activity and the metabolism of bacteria and other microorganisms in the biofilm could be affected, thus resulting in lower virus removal.[35,39] Besides, the efficiency of virus removal by SSF was possibly reduced by desorption of viruses from biofilms over extended periods of operation.[35]

At times without the impact of the low water temperatures (<13.4 °C between November 2017 and March 2018), the SSF removed the PMMoV in a range from 1.8 $\log_{10}$ to 2.8 $\log_{10}$. The efficiency of virus removal efficiency in this study was similar to that reported in some previous studies of SSF,[40,41] and lower than the efficiency reported in some other studies[23,42]; all used the same filtration rates. However, the sand bed depth in the previous studies with the highest removal efficiency (>4.0 $\log_{10}$) was twice that used in the current study.[23] Greater bed depth could provide better virus removal in SSF due to the longer contact time. It is also possible that the different efficiencies of virus removal resulted from different virus types and differences in the quality of the water sources.[23,43]

To assess the risks of viral infection, the Guidelines for Canadian Drinking Water Quality[44] gives credits the SSF process with 2.0 $\log_{10}$ virus removal. This credit was determined based on numerous pilot-scale studies and a limited number of full-scale studies where SSF with proper design and operation can effectively achieve an average 2.0 log removal for enteric viruses. However, the results of the present study indicated that <1.8 $\log_{10}$ was achieved during the period of cold water temperatures. It is therefore possible that the risk assessment for drinking water treated by the SSF process during the winter season could be

**Table 2.** PMMoV removal efficiency by different filtration systems

| Filtration systems | Scales | Target waters | Removal efficiency ($Log_{10}$) | References |
|---|---|---|---|---|
| SSF | Full-scale | Drinking water | >0.0–2.8 | Current study |
| RSF | Full-scale | Drinking water | −0.24–0.39 | Kato et al.[21] |
| | | | 0.78–1.26 | Asami et al.[22] |
| MF | Full-scale | Drinking water | 0.0−>0.9 | Current study |
| | Lab-scale | Drinking water | <1.0[a,b] or 2.0−>4.9[b,c] | Shirasaki et al.[24] |
| UF | Pilot-scale | Reclaimed water | 2.8 | Lee et al.[29] |
| | Lab-scale | Drinking water | 3.5–>4.0[c] | Shirasaki et al.[24] |

*SSF* slow sand filtration, *RSF* rapid sand filtration, *MF* microfiltration, *UF* ultrafiltration
[a]Hydrophilic and negatively charged membrane
[b]Data was obtained from graphs
[c]Hydrophobic and positively charged membrane

overestimated. In addition, because processing at Plant B consisted of only SSF and chlorination, the provision of drinking water safe from viruses may rely solely on the performance of the chlorination.

### Removal of PMMoV by different filtration systems

Removal of PMMoV by different filtration systems (including SSF, rapid sand filtration (RSF), MF, and ultrafiltration (UF)) is compared in Table 2. SSF was able to remove PMMoV up to 2.8 $log_{10}$ (current study), which was slightly better than RSF with removals ranging from −0.24 to 1.26 $log_{10}$.[21,22] In MF system, the hydrophilic and negatively charged membrane removed PMMoV from <1.0 $log_{10}$ (previous study[24]) to >0.9 $log_{10}$ (current study), which was less effective than the hydrophobic and positively charged membrane (from 2.0 $log_{10}$ to higher than 4.9 $log_{10}$).[24] UF system also effectively removed PMMoV since the nominal pore size of the UF membrane (1–100 kDa) was greatly smaller than the size of viruses; the removal ratio of PMMoV was from 2.8 $log_{10}$ to higher than 4.0 $log_{10}$.[24,29]

In summary, the removal of indigenous PMMoV by the MF process at Plant A ranged from 0.0 to >0.9 $log_{10}$. The removal of indigenous PMMoV by the SSF process at Plant B was >0.0–2.8 $log_{10}$. The performance of the SSF process at removing viruses was stable at warm water temperatures but greatly reduced at cold water temperatures. Based on calculations using CGMMV as the molecular control, nearly 40% (19/48) of all the water samples greatly inhibited the process of RNA extraction and RT-qPCR detection ($E < 10\%$). This inhibition was mitigated effectively by sample dilution and purification by DAX-8 and Fh treatments, although the application of more than one purification method may be required to achieve the desired mitigation.

## METHODS

### Sample collection and virus concentration method

Water samples were collected monthly between May 2017 and April 2018 from the two DWTPs (Plants A and B) in Japan. The treatment process flows for the two plants are shown in Fig. 3. Plant A used a groundwater source, producing an average of 29,400 $m^3$ of drinking water per day. The treatment processes of Plant A comprised MF and chlorination. Table 3 summarizes the characteristics of the MF membrane used. Plant B used lake water source, producing an average 41,600 $m^3$ of drinking water per day. The treatment processes of Plant B comprised SSF and chlorination. The characteristics of the SSF are summarized in Table 4.

Water samples were collected monthly from the raw water intakes and after processing by MF (at Plant A) or SSF (at Plant B). The volumes of the water samples are shown in Fig. 3. The virus concentration processes for the raw and treated water samples ($n = 48$) were employed onsite using a negatively charged membrane, as previously described.[45] In brief, $MgCl_2$ was continuously added to the water samples by gravity flow to obtain a

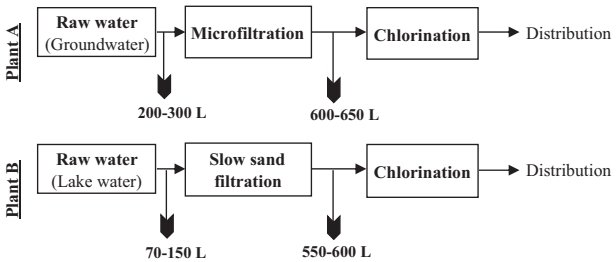


**Fig. 3** Process flow and sampling points for the two drinking water treatment plants

**Table 3.** Characteristics of microfiltration at plant A

| Characteristic | MF (Plant A) |
|---|---|
| Membrane material | Hydrophilic polyvinylidene fluoride |
| Active surface area | 75 $m^2$ (220 mm diameter × 2300 mm length) |
| Nominal pore size | 0.1 μm |
| Transmembrane pressure | 0.07 MPa |
| Configuration | Hollow fiber |
| Operation mode | Dead end |
| Number of membrane units | 6 |
| Number of membrane modules in each unit | 22 |
| Average permeate flux (total) | 4.7 $m^3/m^2$ day |

**Table 4.** Characteristics of slow sand filtration at Plant B

| Characteristic | SSF (Plant B) |
|---|---|
| Bed depth | 0.70–0.84 m |
| Effective media size | 0.30–0.45 mm |
| Uniformity coefficient | 1.89 |
| Maximum diameter | 1.69 mm |
| Minimum diameter | 0.27 mm |
| Filtration rate | 0.17 m/h |

Uniformity coefficient is the mesh diameter of a sieve which retains 60% by weight of the material under test, divided by its effective media size[35]

final concentration of 25 mM, and the mixture was then passed through a cartridge-type filter (Opticap XL2, 0.1 $m^2$ area, 0.5 pore size; Merck Millipore, MA, USA). Next, magnesium ions were washed out by passing $H_2SO_4$ solution (2 L, 0.5 mM, pH 3.0) through the filters. After this, the viruses were eluted with 200 mL of NaOH solution (1.0 mM, pH 10.8) and the eluates were immediately mixed with 1 mL of 100 mM $H_2SO_4$ and 2 mL of 100 × Tris-EDTA buffer for neutralization. Finally, the eluates were concentrated using a Centricon plus-70 filter unit (Merck Millipore), according to the manufacturer's instruction, to obtain a final volume of ~700 µL. The concentrated samples were stored at −20 °C until analysis.

## Physicochemical water quality parameters

Physicochemical water quality parameters were measured onsite immediately after collecting the samples. Turbidity was measured using a 2100Q portable turbidimeter (HACH, Tokyo, Japan). Electrical conductivity, pH, and water temperature were measured using a HI 98129 water tester (HANNA, Tokyo, Japan).

## Quantification of indicator bacteria and PMMoV

*Escherichia coli* and total coliforms were quantified for all samples using a filter unit (37 mm monitor unit, 77 mm diameter, 0.45 µm; ADVANTEC, Tokyo, Japan) and m-coliBlue24® Broth (HACH) within 6 h of sample collection.

The concentrated samples (140 µL) were extracted using QIAamp Viral RNA Mini Kits (Qiagen, Tokyo, Japan), according to the manufacturer's protocol. The extracted viral RNA was subjected to reverse transcription (RT) using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Tokyo, Japan). Real-time quantitative PCR (qPCR) was conducted using the StepOnePlus Real-Time PCR System (Applied Biosystems) and TaqMan Gene Expression Master Mix (Applied Biosystems). The sequences of primers and probe were as described in a previous study on the detection of PMMoV.[46] The detection limit of qPCR runs was 1 copy per reaction. The detection limits were 1.8–3.4 copies/L and 0.9–1.6 copies/L for raw and treated water samples from plant A (MF), respectively, and were 4.6–13.4 copies/L and 0.8–1.3 copies/L for raw and treated water samples from plant B (SSF), respectively. The titer of viral genomes was determined from a calibration curve using plasmid DNA that contained the target sequence (with 10-fold serial dilutions, $1.0 \times 10^0$ to $1.0 \times 10^4$) for each amplification. The concentration of the plasmid was predetermined by the digital PCR system (Applied Biosystems). The result of calibration curves running in triplicates showed qPCR efficiency ranging from 81 to 88% and $R^2$ values ranging from 0.97 to 0.99.

## Evaluation of viral RNA extraction and RT-qPCR efficiency

CGMMV was used as a molecular control to evaluate the efficiency of RNA extraction and RT-qPCR because of its phylogenetic and morphological similarities to the PMMoV target virus.[21,22,47] In brief, 1.4 µL of CGMMV stock (around $10^8$ copies/mL), which was provided by Japan's National Institute of Agrobiological Sciences, was spiked into all the concentrated water samples and Milli-Q water (as a control). The efficiency of CGMMV recovery (for both RNA extraction and RT-qPCR) was determined by comparing the copy number between the concentrated water sample and control. It was calculated from the equation $E = C/C_0 \times 100$, where $E$ is the efficiency of molecular control for RNA extraction and RT-qPCR, $C_0$ is the CGMMV copy number obtained from the control, and $C$ is the CGMMV copy number obtained from the target concentrated sample.

The detection of the target virus (PMMoV) in the concentrated water samples was considered valid when $E$ was ≥10%. This level was assumed to have the good efficiency of nucleic acid extraction and RT-qPCR detection.[21,48] The samples with $E < 10\%$ were judged to be highly inhibited and so were subjected to the purification method described in the following section.

## Sample dilution and purification methods for mitigating RT-qPCR inhibitory effects

To mitigate the inhibitory effects on viral RT-qPCR detection of the inhibited samples (i.e., those with $E < 10\%$), the samples were diluted and purified as shown in the flowchart in Fig. 4. First, dilutions by factors of 10 and 100 were performed because this has been reported to mitigate RT-qPCR inhibition effectively.[49] If the $E$ value of the sample was ≥10% after the dilutions, the PMMoV concentration in the sample was used for the analysis. If the $E$ value remained <10%, the sample was further purified by treatments with DAX-8 and ferrihydrite (Fh), as described below. Finally, if the $E$ value remained <10%, the highest PMMoV concentration was used in further analysis.

DAX-8 treatment was originally developed by Schriewer et al.[50] and modified by Dr. Akihiko Hata (private communication) to remove humic acid and other hydrophobic organic matter. A hydrophobic resin (DAX-8)

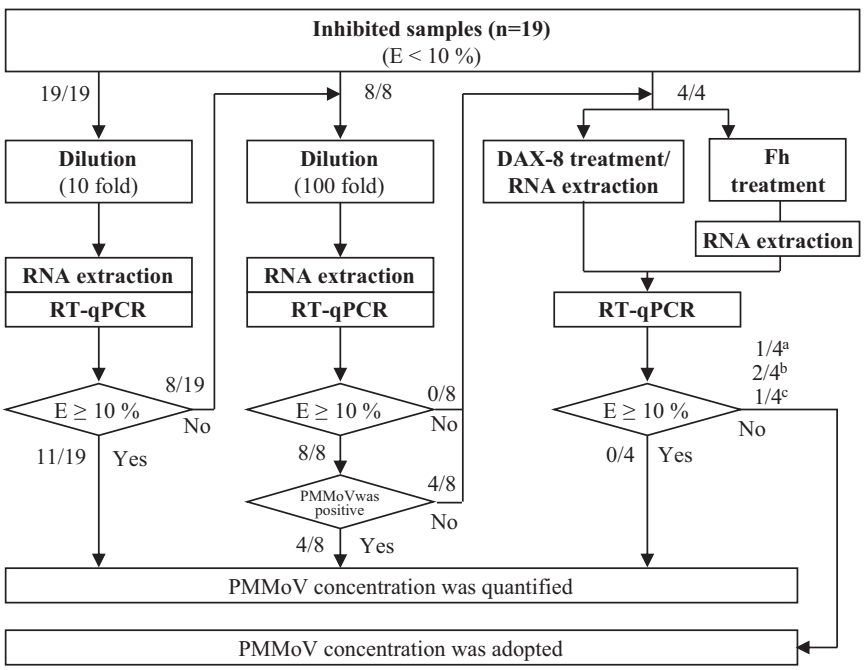


**Fig. 4** Flowchart for the sample dilution and purification methods used for the inhibited samples. $E$ represents the efficiency of molecular control calculated using cucumber green mottle mosaic virus. [a]After DAX-8 treatment, $E$ value <10%, but PMMoV concentration was positively quantified. [b]After Fh treatment, $E$ value <10%, but PMMoV concentration was positively quantified. [c]After DAX-8 or Fh treatments, $E$ value <10%, and PMMoV was non-detected

was purified and stored in an equal volume of 99.5% ethanol, as described previously.[51] The DAX-8 treatment was conducted during the RNA extraction process, as described in previous studies.[21,22] In brief, the DAX-8 solution (140 µL) was mixed well with 140 µL of the concentrated sample (1:1 ratio). The DAX-8/sample mixture was then filtered using a 0.45-µm polyvinylidene fluoride (PVDF) centrifugal filter unit (Ultrafree-MC-HV; Merck Millipore, Tokyo, Japan) with centrifugation at 8000 rpm for 1 min, which removed the DAX-8 with the absorbed inhibitors. The primary filtrate was then combined with 560 µL of the lysis buffer (AVL, Qiagen) provided with the RNA extraction kit. In addition, 560 µL of the lysis buffer was mixed with the DAX-8 resin retained in the filter unit, incubated for 10 min at room temperature, and centrifuged again at 8000 rpm for 1 min. The secondary filtrate was then combined with the primary filtrate and subjected to RNA extraction, as described earlier. Finally, the extracted RNA was further purified by gel filtration with an Illustra Microspin S-300 HR column (GE Healthcare, Tokyo, Japan), according to the manufacturer's instructions, to remove low molecular weight organic substances. The purified filtrate was then analyzed with RT-qPCR, as described earlier.

Fh treatment was developed by Canh et al.[52] to remove humic acid and inhibitory substances from environmental water samples. In brief, Fh solution was prepared following the procedures described by Leone et al.[53]. The $UV_{254}$ absorbance of the water samples was adjusted to ~1.5 $cm^{-1}$ by diluting with Milli-Q water (nearly 5-fold dilution). The Fh solution (40 µL) was then added into the water sample (140 µL) to obtain final concentration of 1000 mgFe/L, which was considered optimum for mitigating the inhibitory effects for the samples with $UV_{254}$ absorbance at ~1.5 $cm^{-1}$.[52] The Fh/sample mixtures were vortexed for 5 min and then filtered using a 0.45-µm PVDF centrifugal filter unit (Ultrafree-MC-HV; Merck Millipore) with centrifugation at 12,000 rpm for 5 min to remove the Fh particles with the absorbed inhibitors. The filtrates (140 µL) were subjected to RNA extraction, as described earlier.

### Statistical analyses

The R statistical software was utilized for statistical analyses. Kaplan–Meier (KM) method with NADA package in R was used to calculate the mean and standard deviation for the data of virus concentration containing non-detects.

## DATA AVAILABILITY

The data that support the findings of this study are available in the published open access on the figshare repository [https://doi.org/10.6084/m9.figshare.8231105.v2].

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## AUTHOR CONTRIBUTIONS

V.D.C. conducted the experiments, analysed the data, and wrote the manuscript. H.F. revised the manuscript. H.K. developed the idea, supervised the work, and revised the manuscript.

## ADDITIONAL INFORMATION

**Supplementary Information** accompanies the paper on the *npj Clean Water* website (https://doi.org/10.1038/s41545-019-0042-1).

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