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Unveiling the viral escape: Quantification of microfloc-bound viruses in precoagulation and membrane filtration

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ABSTRACT

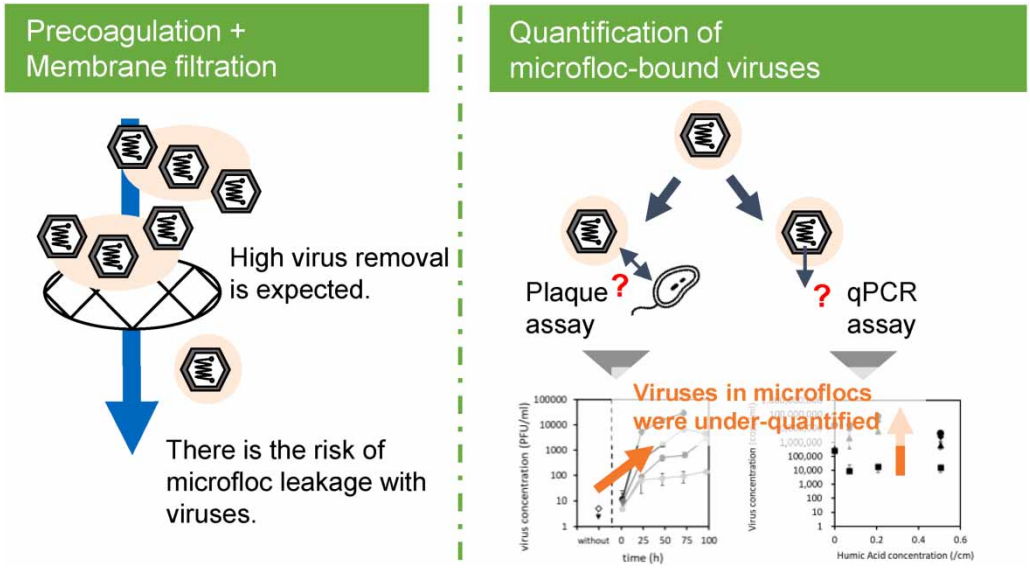
The implementation of precoagulation before the physical removal process is expected to achieve a high virus removal rate. However, viruses may form small flocs and subsequently escape into the effluent during physical removal processes. This study evaluated how viruses in the microflocs could be quantified using conventional virus quantification methods (plaque assay and quantitative polymerase chain reaction (qPCR)) to reveal the risk of underestimating virus concentration. In this study, the microfloc dissolution phenomenon in phosphate buffer solution was employed as a floc dissolution test. Viruses in microflocs formed under the experimental conditions. assuming water treatments, were quantified before and after floc dissolution. The findings revealed that virus concentrations increased by 1.0–3.9 log plaque-forming units/mL according to the plaque assay and by 1.7–4.0 log copies/mL according to the qPCR. This increase occurred after the dissolution of microflocs that were prepared in the humic acid test water. In the case of treated wastewater, virus concentrations increased in all samples according to the plaque assay and in seven of eight samples according to the qPCR. Our results indicate the necessity of careful consideration of virus quantification after precoagulation and physical removal systems.

Key words: membrane filtration, precoagulation, virus removal, water reuse

HIGHLIGHTS

- Microfloc-bound viruses were quantified using plaque and qPCR assays.
- Microflocs were dissolved in phosphate buffer.
- Virus concentration increased after floc dissolution, as quantified using the two methods.
- Microfloc leakage can cause viral risk underestimation.

GRAPHICAL ABSTRACT



INTRODUCTION

Climate change is causing extreme weather events, including droughts, making water scarcity a serious problem in several regions worldwide. In many cases, the potable water reuse of reclaimed water is now a practical option, where the capability to remove pathogenic microorganisms by water treatment trains, instead of solely relying on water quality standards, is essential for ensuring water safety. The World Health Organization (WHO) Guidelines for Drinking Water Quality have set a risk reduction target, recommending disability-adjusted life years of 10^{-6} as an acceptable annual health risk per capita (WHO 2011).

Enteric viruses are one of the most challenging pathogenic microorganisms to remove because of their nanosize and high persistence against various water treatments (WHO 2011). Enteric viruses can cause infections in humans even with a few virions (Kirby *et al.* 2014). To achieve the acceptable health risk targets of the WHO, reducing rotavirus concentrations in drinking water to levels below 1.1×10^{-5} plaque-forming unit (PFU)/L is necessary (WHO 2011). However, the quantification of such a small number of viruses in water necessitates the concentration of huge water volumes, which is impractical for routine application. As an alternative, the log reduction value (LRV) of the entire water treatment process has been recommended to ensure a safe virus level. To ensure virus removal capability in the entire process, evaluating the virus removal value for each process in the treatment train is essential.

Viruses can be eliminated through two primary methods: disinfection and physical removal. In water treatment, particularly in water reclamation treatment, specific LRVs are assigned to selected treatment processes. According to the WHO guidelines for water reuse, the validated viral LRV for microfiltration (MF) and ultrafiltration (UF) is zero, which is significantly lower than six for ultraviolet treatment or chlorination (WHO 2017). Furthermore, in California, USA, where water reclamation is being promoted, no viral LRV was credited to MF and UF membrane filtration (Olivieri *et al.* 2016).

The virus LRV during the treatment process can be calculated by quantifying the virus concentration before and after treatment. Although various methods are available for virus quantification, quantification by counting PFUs is considered the gold standard because it exclusively measures infectious viruses. However, the plaque assay may underestimate the virion concentration in treated water because of the difficulty in detecting inactivated and aggregated viruses (Gerba & Betancourt 2017). Physical removal evaluation necessitates the consideration of the total number of remaining viruses rather than solely focusing on the number of infectious ones. Therefore, using a method capable of quantifying the total number of viruses, including inactivated ones, is allowed, such as quantitative polymerase chain reaction (qPCR) that amplifies the viral gene and detects the fluorescence of the probe, regardless of virus infectivity. The qPCR assay is widely used for detecting and quantifying enteric viruses in water because of its high specificity and shorter measurement time than the cell culture assay (Lipp *et al.* 2001; Fong & Lipp 2005).

To enhance the efficiency of physical removal processes, pretreatment strategies, such as coagulation–sedimentation and coagulation alone, are occasionally used (Viegas *et al.* 2015; Asami *et al.* 2016; Yasui *et al.* 2016; Lee *et al.* 2017; Kato *et al.* 2018). For example, many water treatment facilities have employed a combination of coagulation–sedimentation and sand filtration (Asami *et al.* 2016; Kato *et al.* 2018). In fact, coagulation and rapid sand filtration result in higher LRVs than processes without coagulation (Shirasaki *et al.* 2010). In water reclamation facilities, coagulation with membrane filtration is also practiced (Viegas *et al.* 2015; Yasui *et al.* 2016; Lee *et al.* 2017). The coagulation process is recognized for its effectiveness in enhancing the removal rate of challenging substances, such as viruses and dissolved organic matter, which are difficult to eliminate through conventional membrane filtration, such as MF and UF (Huang *et al.* 2009; Heffron & Mayer 2016). In fact, higher viral LRVs have been reported for the bench-scale combination of membrane filtration and coagulation processes compared with filtration alone (Matsui *et al.* 2003a; Zhu *et al.* 2005; Fiksdal & Leiknes 2006; Shirasaki *et al.* 2009; Guo & Hu 2011; Meyn *et al.* 2012; Matsushita *et al.* 2013; Heffron & Mayer 2016). Yasui *et al.* (2023) reported virus removal exceeding 6-log through a coagulation and ceramic MF system that assumed a water reclamation system. This is attributed to the ability of the coagulation process to facilitate virus agglomeration with other substances in water, thereby enhancing the physical removal of viruses. However, acknowledging the possibility of viruses leaking into treated water in the form of small flocs is important because studies have reported residual infectivity of viruses even in floc states (Matsui *et al.* 2003b; Matsushita *et al.* 2004; Shirasaki *et al.* 2009).

The detection of viruses in microflocs may be challenging using conventional virus quantification methods (e.g., plaque and qPCR assays). Virus aggregates may lead to an underestimation of virus concentrations in plaque assays (Gerba & Betancourt 2017). When the plaque assay is performed on viruses in microflocs, only one plaque is expected to form from each floc, even if it contains multiple viruses, potentially resulting in an underestimation of virus concentration. Furthermore, successful detection of viruses within flocs was achieved by performing a plaque assay after floc dissolution using beef extract (Matsushita *et al.* 2004), indicating that viruses in flocs retain their infectivity. Conversely, the plaque assay without floc dissolution did not detect viruses. Similar considerations arise when quantifying viruses in microflocs that can pass through membranes. Furthermore, the quantification of viruses in microflocs using the qPCR is also questionable. The extraction of viral genes from microflocs may pose challenges during the gene extraction step before the qPCR assay.

Undetected microfloc-bound viruses lead to the underestimation of virus risk in filtrates of coagulation and membrane filtration processes. This study aims to evaluate the quantification of viruses in microflocs using plaque and qPCR assays, and virus quantifications were conducted after the dissolution of microflocs. Microflocs were formed in humic acid test water or treated wastewater, assuming water reclamation systems, and dissolved in phosphate buffer (PB). PB dilution is widely used for microbiological samples, and our study also evaluated the effects of microfloc contact time with PB on microfloc-bound virus quantification.

METHODS

Cultivation of the bacteriophage MS2 and measurement by the plaque assay

Bacteriophage MS2 (ATCC 15597-B1) was used in the experiments. MS2 has been widely used as an indicator virus to evaluate enteric virus removal performance in water treatment processes (WHO 2011; CCR 2015). MS2 has an icosahedral shape and negative charge under neutral conditions, similar to most enteric viruses (Michen & Graule 2010). To prepare the MS2 stock solution, MS2 was cultured with the host bacteria *Escherichia coli* K12 F^+ A/λ in Luria Bertani (LB) solution overnight at 38 °C with shaking. The MS2 culture solution was centrifuged and filtered through a 0.2-μm membrane (cellulose acetate, syringe filter, ADVANTEC) to separate the host bacteria. A stock solution with a concentration of approximately 10^{11} PFU/mL was obtained and stored at 4 °C until the experiment.

The concentration of MS2 in the samples was determined using the monolayer plaque assay on an LB agar medium. The host bacterium, as mentioned earlier, was previously incubated at 38 °C for 4 h with shaking. Sample aliquots were serially diluted and poured into plates containing the host bacteria and LB agar in duplicate. The plates were then incubated overnight at 38 °C, and virus plaques were subsequently counted.

Preparation of humic acid test water and water quality control of treated wastewater

Humic acid test water was used to create microflocs with viruses and assumed organic matter in the source water (referred to as the challenge test water by WHO 2014). To prepare the humic acid stock solution, 100 mg of humic acid (Sigma Aldrich,

Tokyo, Japan) was added to 100 mL of 1 M sodium hydroxide solution. The mixture was stirred for 2 h for dissolution. The solution was neutralized with 5 M HCl, and the remaining undissolved humic acid was removed by centrifugation and filtration through a hydrophilic polytetrafluoroethylene membrane (pore size = 0.45 μm, syringe filter, ADVANTEC, Tokyo, Japan). The humic acid stock was diluted with MilliQ water to prepare the test water for the experiment. The test water without humic acid was prepared by diluting the humic acid-free solution without the addition of humic acid using the same process. In total, four types of test water were prepared (254-nm absorbance: 0, 0.071, 0.207, and 0.505 cm^{-1}). All test water types were adjusted to have similar pH (average, 7.5 ± 0.37) and electrical conductivity (average, 1,035 ± 23 μS/m) (Table 1) and tested thrice, except for the 0.505 cm^{-1} test water, which was tested twice, excluding a sample where the plaque assay did not provide quantification data.

In addition to the humic acid test water, treated wastewater was collected from two actual wastewater treatment plants (WWTPs) located in the greater Tokyo area, Japan, in 2020 from June to November. These treatment plants employed conventional activated sludge processes as part of their regular operation. Before the experiment, the free chlorine concentration was checked (≤0.04 mg/L) to confirm that it was sufficiently low and would not inactivate viruses. In the experiment, two sample types were used: grab samples, which were collected once, and composite samples, which were collected over several days. The use of composite samples aimed to minimize variations in the water quality of treated wastewater from day to day and homogenize the water quality. The pH of the samples stored in the freezer, which were used as the composite sample, was higher than that of the grab samples. The specific water quality is shown in Table 2. All samples were tested thrice, except for the A-1 sample, which was tested twice, excluding a sample where the plaque assay did not provide quantification data.

Microfloc formation test

In total, 200 μL of the MS2 stock solution was added to 2 L of the water sample, resulting in an initial concentration of approximately 10^7 PFU and copies/mL. For the flocculant, high-basicity polyaluminum chloride (PACl, PAX-XL 19, Kemira) was used as the coagulant. The multivalent cations in PACl neutralize particles' negative charges and enhance agglomeration via their bridging effect. The PACl solution was prepared by diluting it with MilliQ water to achieve a PACl

Table 1 | Water quality of each humic acid test water ($n = 1$)

Humic acid concentration (cm)	pH before coagulation	Conductivity (μS)	pH after coagulation	Humic acid concentration after filtration (cm)	Humic acid removal (%)
0.000	7.9	1,058	7.4	–	–
0.071	7.5	1,016	7.6	0.063	11.3
0.207	7.0	1,051	7.3	0.13	37.2
0.505	7.6	1,013	7.5	0.014	97.2

Table 2 | Water quality of the treated wastewater

No.	Month	Type	Stored temperature (°C)	pH (measure acidity)	EC (μS/m)	Turbidity (NTU)	E254 abs (cm)
A-1	Jul.	Composite	−20	8.5	538	0.70	0.110
A-2	Jul. & Aug.	Composite	−20	8.1	530	0.63	0.107
A-3	Oct.	Grab sample	4	7.0	700	1.04	0.138
A-4	Oct.	Grab sample	4	7.1	636	0.90	0.124
A-5	Oct.	Composite	4	7.1	677	1.02	0.131
B-1	Jun. & Jul.	Composite	−20	8.2	386	1.30	0.086
B-2	Oct.	Grab sample	−20	7.2	495	1.23	0.115
B-3	Oct.	Grab sample	4	7.1	425	0.87	0.086
B-4	Oct.	Composite	4	7.2	482	0.95	0.099
B-5	Oct. & Nov.	Composite	4	7.2	485	0.85	0.107

concentration of 25 mg/L at the time of mixing. High-basicity PACl is known for its strong ability to enhance virus coagulation (Shirasaki *et al.* 2016) and was used in a study involving water reclamation treatments (Yasui *et al.* 2023). Raw water, along with the PACl solution, was introduced into the system and passed through a static mixer (1/2-N40-172-0, Noritake Co., Ltd, Japan) at a flow rate of 500 mL/min for rapid mixing (*G*-value: approximately 5,500 s^{-1}; the calculation formula is shown in Supplementary Information). Immediately after the mixing step, 10 mL of flocculated water was filtered through a cellulose acetate syringe filter (pore size, 0.2 μm; diameter, 25 mm; ADVANTEC). This filtration step removed flocs larger than 0.2 μm in size from the water sample. These microflocs (<0.2 μm) are smaller than some types of MF membrane pores and may have been passed through coagulation and filtration systems in previous studies.

Microfloc dissolution test

In this study, the samples were diluted with PB after the coagulation and filtration steps, and the increase in virus concentration indicated the presence of microflocs in the filtrate. For dilution, 1/15 M (67 mM) PB (pH 7.2, Wako, Tokyo, Japan) was prepared. This PB concentration is commercially available and is commonly used for diluting samples in microbiological experiments, particularly for plaque assays. To justify the PB concentration, 20, 40, and 67 mM PBs were prepared.

The flocculation and membrane filtrates were diluted with PB 10 times and mixed using a vortex mixer. The diluted solution was then maintained at a constant temperature of 25 °C, and the virus concentration was quantified using a plaque assay every other day for 5 days. In cases where 20 and 40 mM PB were used for dilution, 67 mM PB was further diluted with MilliQ water. PB was autoclaved before the experiment.

RNA extraction, reverse transcription and real-time qPCR

Before RNA extraction, the samples not subjected to microfloc dissolution were diluted with MilliQ water 10 times to adjust the concentration. From each diluted sample, a 140-μL aliquot was taken, and a 60-μL RNA extract was obtained using the QIAampViral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This extraction kit can be used when viruses are quantified in the challenge tests of water treatments. The extracted viral RNA was then subjected to a reverse transcription (RT) step conducted using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and 15 μL of cDNA was obtained. RNA extraction was also performed on virus-free MilliQ water, which was also used as a negative control for the data collection policy. RNA extraction and RT steps were performed 5 days after the microfloc formation and dissolution tests.

Quantification using the qPCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystem) with TaqMan Gene Expression Master Mix (Applied Biosystem). The MS2 primers and probes were similar to those used by Wolf *et al.* (2010). The data were considered valid when the Ct value of the negative control was >37, and the value of Ct = 37 was used as the detection limit.

RESULTS AND DISCUSSION

Verification of PB concentration during microfloc dissolution

To justify the effects of PB concentration on floc dissolution, three PB solutions (20, 40, and 67 mM) were prepared. Microflocs in this section were formed in humic acid test water and were not dissolved in MilliQ water (shown in the Supplementary Information). Figure 1 shows the changes in bacteriophage MS2 concentration after sample dilution by PB quantified using the plaque assay. The increase in the observed MS2 concentration following the floc dissolving test suggests that the presence of microflocs leads to an underestimation of the actual virus concentration. As shown in Figure 1, MS2 concentrations gradually increased in 40 and 67 mM PB, whereas floc dissolution was not observed in 20 mM PB using the plaque assay. MS2 concentrations in 67 mM PB were higher than those in 40 mM PB. PB concentration was a significant factor that dissolved microflocs. PB dilution is commonly used before the plaque assay to determine virus concentrations. A selected PB concentration (67 mM, commercial concentration) was used for serial dilution before the plaque assay. However, the duration of PB dilution was not standardized. Our results suggest that the virus concentration after the coagulation steps varies depending on the timing of the plaque assay and the concentration of PB used for dilution.

Figure 2 shows the changes in MS2 concentration before and after the floc dissolution test quantified using the qPCR. All qPCR assays were performed 4 days (96 h) after the microfloc formation and dissolution tests. As the PB concentration increased, the gaps in the MS2 concentration after floc dissolution increased. MS2 and PB concentrations exhibited positive

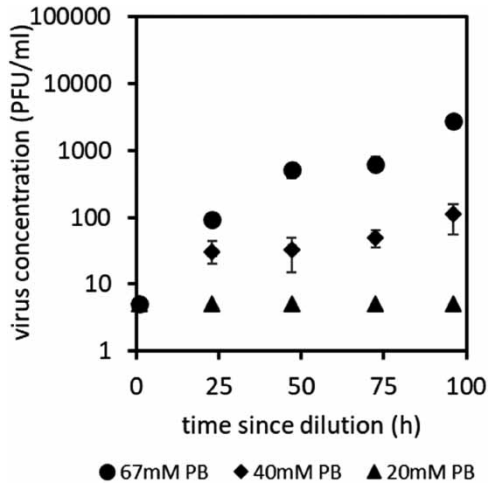


Figure 1 | MS2 concentration changes after floc dissolution for each PB concentration quantified using the plaque assay at a humic acid concentration of 0.207 cm^{-1}. Circles, diamonds, and triangles indicate 20, 40, and 67 mM PB concentrations for floc dissolution, respectively.

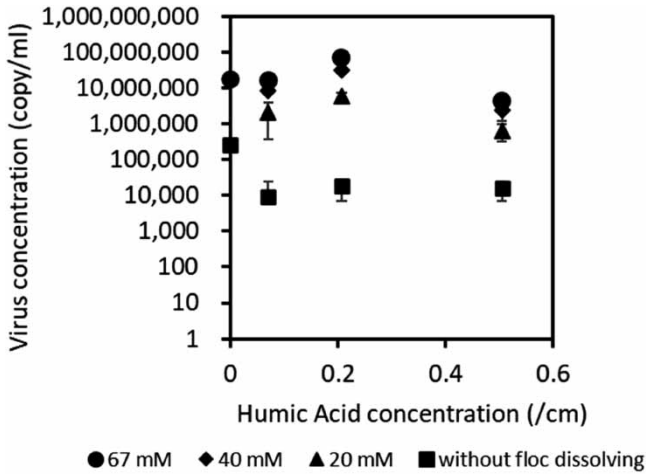


Figure 2 | MS2 concentration changes for each PB concentration quantified using the qPCR after floc dissolution. Circles, diamonds, and triangles indicate 20, 40, and 67 mM PB concentrations, respectively. The squares show concentration before floc dissolution. The humic acid concentration of 0.505 cm^{-1} is shown as the average of $n = 2$ and the others are the median of $n = 3$. Error bars indicate the range between the maximum and minimum concentrations. RNA extraction and RT were performed 4 days (96 h) after the microfloc formation and dissolution tests.

correlations ($R^2 \geq 0.99$, details are shown in the Supplementary Information). As shown in Figure 2, the MS2 concentration, as quantified using the qPCR assay after floc dissolution, also increased with a higher PB concentration. In the case of 67 mM PB, the MS2 concentration after floc dissolution was nearly equivalent to that of raw water (10^7 copies/mL, by the qPCR assay). This indicates that the removal of MS2s by the membrane was limited, and the microflocs were sufficiently dissolved in 67 mM PB. Conversely, in the case of 20 mM PB, the MS2 concentrations quantified using the qPCR increased after floc dissolution, whereas those quantified using the plaque assay did not exhibit a similar increase. This discrepancy may be attributed to the ability of the qPCR method to detect the virus in smaller microflocs not detected by the plaque assay. Possible explanations for the mechanism of floc dissolution are as follows: (1) phosphate reacted with the polymer of aluminum and (2) the positive charge of aluminum was neutralized or weakened by the negative charge of phosphate. Based on these results, 67 mM PB was selected to dissolve the microflocs in the next experiments.

Microfloc formation and dissolution in humic acid test water

Figure 3 shows the changes in MS2 concentration quantified using the plaque assay after floc dissolution with humic acid test water as the feedwater. In cases where floc dissolution was not performed, no viruses were detected in any filtrate sample, and the virus concentrations were <5 PFU/mL. As shown in Figure 3, the MS2 concentration in the filtrate increased over time after floc dissolution. Although MS2 reduction may occur during the 4 days of the floc dissolving test to some extent, these results imply the presence of small flocs that passed through the membrane during this experiment and contained infectious viruses. The magnitude of MS2 concentration escalation after floc dissolution varied depending on the humic acid concentration (Figure 3). A minimal increase was observed at the highest humic acid concentration. Augmented humic acid levels might facilitate floc enlargement and enhance virus entrapment on the membrane. Because of the difficulty in measuring floc sizes formed with humic acid using dynamic light scattering methods, floc sizes were not confirmed.

The results quantified using the qPCR assay are shown in Figure 2 (in 67 mM and without dissolving). The results presented in Figure 2 indicate that the virus concentration quantified using the qPCR assay also increased after floc dissolution. In particular, in the test water with humic acid, a notable concentration difference of 2.4–3.6 log was observed, indicating the challenges associated with quantifying microfloc-bound viruses using the qPCR. RNA extraction from viruses that reside within microflocs may be inhibited. When precoagulation was performed in the absence of humic acid, the difference in MS2 concentration before and after floc dissolution was 1.9 log, which was 0.5–1.7 log lower than that with humic acid. These microflocs primarily comprised coagulants, MS2 particles, host cell debris, and medium components in the MS2 stock. In such microflocs, the extraction of viral RNA was likely to be more successful than that in microflocs formed with humic acid. Even in cases where the floc comprises virus particles and coagulants, the detection of viruses using the qPCR assay remains challenging. The effects of microflocs on the qPCR assay should be considered even when dealing with feedwater characterized by low turbidity or dissolved organic matter.

The MS2 concentration in the filtrate after the floc dissolution tests was approximately 10^7 copies/mL (Figure 2), indicating that the use of PB for floc dissolution resulted in nearly the same MS2 concentration as the initial value. The highest MS2 LRV following floc dissolution was obtained in the test water with the highest humic acid concentration of 0.505 cm^{-1}, which was 1.46 (96.5%). Note that the removal rate of humic acid under the same conditions was 97.2%. The water quality under each experimental condition is shown in Table 1. Elevated initial humic acid concentrations corresponded to higher humic acid removal rates. In raw water without turbidity, such as the test water used in this study, the formation of flocs larger than the membrane pore size (0.2 μm) solely through the rapid mixing process is considered challenging. In the case of the high humic acid test water, the accumulation of humic acid on the membrane surface was visually observed during filtration, potentially enhancing virus removal efficiency. The possibility of enhancing virus removal by the cake layer has been reported

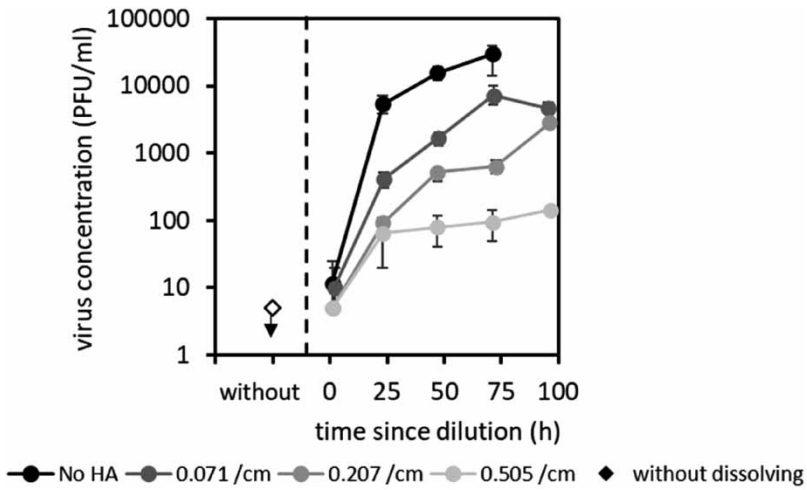


Figure 3 | Virus concentration in the filtrate before and after floc dissolution quantified using the plaque assay. Diamonds and circles represent the concentration before and after floc dissolution, respectively. The *x*-axis shows the time elapsed since floc dissolution started. The humic acid concentration of 0.505 cm^{-1} is shown as the average of $n = 2$ and the others are the median of $n = 3$. Error bars indicate the range between the maximum and minimum concentrations. No virus was detected in any sample before floc dissolution. These samples are shown in open plots with arrows.

in several studies (Shirasaki *et al.* 2009; Kreißel *et al.* 2012; Yin *et al.* 2015). However, microfloc leakage still occurred even in the presence of cake layers. This study highlighted the risk of overlooking the presence of a significant number of viruses.

Microfloc formation and dissolution in treated wastewater

In this section, treated wastewater was used as feedwater to form microflocs, assuming coagulation and MF treatments in water reclamation systems. Figure 4(a) and 4(b) shows the results of floc dissolution after flocculation and membrane filtration using actual treated wastewater from WWTP A and B as raw water in the greater Tokyo area in Japan. Although some variations were observed, an increase in the MS2 concentration after floc dissolution was observed. In treated wastewater B, the virus concentration after floc dissolution was consistently higher than that before dissolution (Figure 4(b)). In A-1 and A-2, a similar trend was observed (Figure 4(a)). In the case of A-3, A-4, and A-5, the concentration increases were slightly compared with the others (Figure 4(a)). Figure 5 illustrates the filtrate MS2 concentration before and after

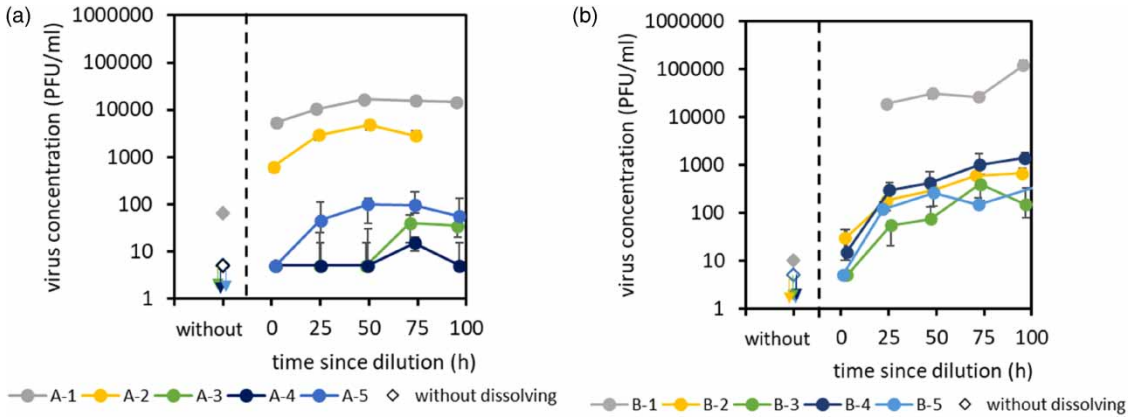


Figure 4 | Changes in the virus concentration after floc dissolution quantified using the plaque assay. (a) Microflocs formed in wastewater from WWTP A and (b) microflocs formed in wastewater from WWTP B. Plot colors are as follows: A/B-1: gray, A/B-2: yellow, A/B-3: right green, A/B-4: dark blue, and A/B-5: right blue. The diamonds and circles represent concentrations before and after floc dissolution, respectively. The *x*-axis shows the time elapsed since floc dissolution started. Sample IDs are denoted by A-1 to -5 and B-1 to -5. The corresponding water quality details are available in Table 2. For A-1, $n = 2$ and for the others, $n = 3$ median values are shown. Error bars indicate the max/min concentrations.

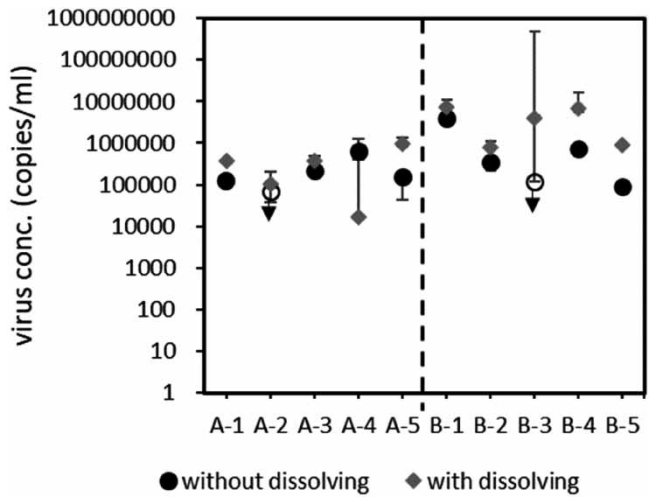


Figure 5 | Results of floc dissolution in treated wastewater quantified using the qPCR. The diamonds and circles represent concentrations before and after floc dissolution, respectively. Open plots with arrows indicate including undetected samples. Sample IDs are denoted by A-1 to -5 and B-1 to -5. The corresponding water quality details are available in Table 2. A-1 to -5 are microflocs formed in wastewater samples from WWTP A, and B-1 to -5 are microflocs in wastewater from WWTP B. For A-1, $n = 2$ and for the others, $n = 3$ median values are shown and error bars indicate the maximum/minimum concentrations.

floc dissolution, as quantified using the qPCR. Significant differences in MS2 concentrations were observed between A-5, B-4, and B-5 (one-sided *t*-test using log-concentration, A-5: $p < 0.05$, B-4: $p < 0.05$, B-5: $p < 0.05$). Although the possibility of MS2 reduction during 4 days of the floc dissolving test should be noted, this suggests that microflocs (<0.2 μm) were partially formed in the treated wastewater. Some studies have used the mixing method using a static mixer (Judd & Hillis 2001; Matsui *et al.* 2003a; Shirasaki *et al.* 2009; Matsushita *et al.* 2013), which is also anticipated to be employed in water treatment. Our findings indicate the possibility of forming small flocs with a size of <0.2 μm under certain conditions, which could lead to their leakage into the filtrate after MF. The effect of water quality on microfloc formation will be discussed later.

As shown in Figure 4, quantified using the plaque assay, the MS2 concentration after floc dissolution was 1.4–3.0 log higher in samples A-1, A-2, and B-1, which had higher pH levels (approximately 8) than the other samples. Although MS2 was not detected before floc dissolution in most cases, it was detected from the filtrates in these samples, indicating that the viruses were not perfectly coagulated. The stability of MS2 at pH 8 was reported by Feng *et al.* (2003); therefore, viruses may not be inactivated in these samples. Note that these alkaline samples were stored in a freezer, which may have resulted in a higher pH because of the release of carbon dioxide during the freezing process. In contrast, viruses were not detected in neutral pH samples (A-3, A-4, A-5, B-2, B-3, B-4, and B-5) before floc dissolution. The low pH of feedwater can improve coagulation between small particles and viruses (Guo & Hu 2011; Ding *et al.* 2016; Lee *et al.* 2017). Furthermore, the bacteriophage MS2 has an isoelectric point of 3.1–3.9 (Michen & Graule 2010), indicating that it tends to aggregate in more acidic water. Therefore, virus aggregation may have been hindered under alkaline pH 8 conditions, resulting in the detection of MS2s even before floc dissolution. The higher virus concentration in alkaline samples after floc dissolution indicates that some viruses formed microflocs and subsequently leaked into the filtrate. The alkaline pH may have inhibited the growth of flocs, contributing to this phenomenon.

In the subsequent analysis, the results for A-3, A-4, and A-5 and those for B-3, B-4, and B-5 were used to discuss the difference between treated wastewater A and B. The increase in the MS2 concentration quantified using the plaque assay in A was 0.48–1.3 log, whereas that in B was 1.5–2.3 log (Figure 4). Significant differences in MS2 concentration quantified using the qPCR assay were observed in A-5, B-4, and B-5 (Figure 5, one-sided *t*-test using log-concentration; A-5: $p < 0.05$, B-4: $p < 0.05$, B-5: $p < 0.05$). These results suggest that the microflocs in B were more effectively dissolved or that the number of microflocs formed in A was less. The difference in the concentration before and after floc dissolution quantified using the qPCR assay was larger in B than in A (Figure 5). In some cases, no difference in the concentration was observed in A (Figure 5). This indicates that microfloc formation was not as significant in treated wastewater A as it was in treated wastewater B. As shown in Table 2, the concentration of organic matter (E254 absorbance) in treated wastewater A was relatively higher than that in treated wastewater B. The electrical conductivity in A was also relatively higher than that in B, indicating a higher presence of ions in A. These differences in water quality may have promoted flocculation, leading to the entrapment of viruses in larger flocs. The effect of water quality on the size of formed flocs could be evaluated in future studies.

Table 3 shows the increase of MS2 concentration after floc dissolution. In the MS2 removal test using treated wastewater from WWTP A and B, both samples exhibited similar virus LRVs under neutral conditions when floc dissolution was not performed (the initial concentration in raw water averaged 7.3 log copies/mL). However, after 3 days of floc dissolution, treated wastewater B showed an increase in the virus concentration of 1.5–2.3 log PFU/mL, as quantified using the plaque assay (Table 3). The overall MS2 concentration in the filtrate was higher than that in treated wastewater A, indicating that the LRVs obtained using wastewater B were lower than those obtained using wastewater A. However, it should be noted that the MS2 concentration of wastewater A and B, without floc dissolution, was below the detection limit as quantified by the plaque assay (Figure 4). Similar results were obtained using humic acid test water (Figures 2 and 3). These findings indicate that the virus concentration before floc dissolution led to an overestimation of LRVs by membrane filtration. In the treated wastewater, the difference in the LRV was approximately 1.5–2.3. However, in the humic acid test water, the MS2 concentration after floc dissolution increased as the humic acid concentration decreased, with a maximum increase of 4.6 log (Figure 3). Therefore, depending on the water quality, the presence of microflocs may further contribute to the overestimation of LRVs. In the plaque assay, MS2 was not detected in most filtrate samples before the floc dissolution tests, indicating the potential presence of viruses after coagulation and filtration, even if undetected. Lower MS2 concentrations were quantified before the floc dissolution tests using the qPCR. Both quantification methods – the plaque and qPCR assays – pose a risk of overestimating virus removal values using coagulation and filtration systems. Although coagulation and membrane filtration, as well as coagulation and sand filtration, are widely employed in water treatment plants, it is necessary to verify whether the correct evaluation of viral LRVs is being achieved. Moreover, precoagulation systems capable of forming sufficiently large flocs

Table 3 | Median MS2 log-concentration increases after floc dissolving tests

Water type	Plaque assay[a]	qPCR assay
Humic acid – 0 cm^{-1}	>3.9	1.7
Humic acid – 0.071 cm^{-1}	>3.1	3.2
Humic acid – 0.207 cm^{-1}	>2.1	3.6
Humic acid – 0.505 cm^{-1}[b]	>1.2	2.5
Wastewater A-1[b]	2.4	0.47
Wastewater A-2	2.7	>0.18
Wastewater A-3	>0.90	0.25
Wastewater A-4	>0.48	−1.5
Wastewater A-5	>1.3	0.83
Wastewater B-1	3.2	0.30
Wastewater B-2	2.1	0.37
Wastewater B-3	>1.9	>1.5
Wastewater B-4	>2.3	0.99
Wastewater B-5	>1.5	1.0

[a]MS2 concentration after the floc dissolution test for 3 days was used as the concentration after floc dissolution.
[b]Average values were shown because of a limited sample number ($n = 2$).

are required. The experimental setup (e.g., mixing intensity) and water chemistry might affect the floc size distribution; however, this point remains unclear. A future study is needed to elucidate the conditions under which microflocs are formed.

CONCLUSION

This study evaluated the quantification of viruses in microflocs using the plaque and qPCR assays, exploiting the dissolution phenomena in PB solution. Experimental conditions assumed for water reclamation were selected, and virus concentrations before and after microfloc dissolution were quantified. After floc dissolution, the virus concentrations quantified using the plaque and qPCR assays increased, indicating that microfloc-bound viruses were undetected or underquantified using these conventional quantification methods. This trend was observed in microflocs formed in humic acid test water and treated wastewater. In particular, in the case of plaque assay quantification, viruses were not detected in most filtrates before floc dissolution, and the PB concentration used for floc dissolution made the difference between quantified concentrations. Our findings elucidated a novel concern – the possibility of microfloc-bound virus leakage in some water types, leading to the underestimation of viral risk after precoagulation and physical removal treatments.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

REFERENCES

Asami, T., Katayama, H., Torrey, J. R., Visvanathan, C. & Furumai, H. 2016 Evaluation of virus removal efficiency of coagulation-sedimentation and rapid sand filtration processes in a drinking water treatment plant in Bangkok, Thailand. *Water Research* **101**, 84–94. https://doi.org/10.1016/j.watres.2016.05.012.

Ding, Q., Yamamura, H., Murata, N., Aoki, N., Yonekawa, H., Hafuka, A. & Watanabe, Y. 2016 Characteristics of meso-particles formed in coagulation process causing irreversible membrane fouling in the coagulation-microfiltration water treatment. *Water Research* **101**, 127–136. https://doi.org/10.1016/j.watres.2016.05.076.

Feng, Y. Y., Ong, S. L., Hu, J. Y., Tan, X. L. & Ng, W. J. 2003 Effects of pH and temperature on the survival of coliphages MS2 and Q? *Journal of Industrial Microbiology and Biotechnology* **30** (9), 549–552. https://doi.org/10.1007/s10295-003-0080-y.

Fiksdal, L. & Leiknes, T. 2006 The effect of coagulation with MF/UF membrane filtration for the removal of virus in drinking water. *Journal of Membrane Science* **279** (1–2), 364–371. https://doi.org/10.1016/j.memsci.2005.12.023.

Fong, T.-T. & Lipp, E. K. 2005 Enteric viruses of humans and animals in aquatic environments: Health risks, detection, and potential water quality assessment tools. *Microbiology and Molecular Biology Reviews* **69** (2), 357–371. https://doi.org/10.1128/mmbr.69.2.357-371.2005.

Gerba, C. P. & Betancourt, W. Q. 2017 Viral aggregation: impact on virus behavior in the environment. *Environmental Science and Technology* **51** (13), 7318–7325. https://doi.org/10.1021/acs.est.6b05835.

Guo, H. & Hu, J. Y. 2011 Optimization study of a hybrid alum coagulation-membrane filtration system for virus removal. *Water Science and Technology* **64** (9), 1843–1850. https://doi.org/10.2166/wst.2011.147.

Heffron, J. & Mayer, B. K. 2016 Emerging investigators series: Virus mitigation by coagulation: Recent discover and future directions. *Environmental Science Water Research and Technology* **2** (3). https://doi.org/10.1039/c6ew00060f.

Huang, H., Schwab, K. & Jacangelo, J. G. 2009 Pretreatment for low pressure membranes in water treatment : A review. *Environmental Science and Technology* **43** (9), 3011–3019.

Judd, S. J. & Hillis, P. 2001 Optimisation of combined coagulation and microfiltration for water treatment. *Water Research* **35** (12), 2895–2904. https://doi.org/10.1016/S0043-1354(00)00586-8.

Kato, R., Asami, T., Utagawa, E., Furumai, H. & Katayama, H. 2018 Pepper mild mottle virus as a process indicator at drinking water treatment plants employing coagulation-sedimentation, rapid sand filtration, ozonation, and biological activated carbon treatments in Japan. *Water Research* **132**, 61–70. https://doi.org/10.1016/j.watres.2017.12.068.

Kirby, A. E., Shi, J., Montes, J., Lichtenstein, M. & Moe, C. L. 2014 Disease course and viral shedding in experimental Norwalk virus and Snow Mountain virus infection. *Journal of Medical Virology* **86** (12), 2055–2064. https://doi.org/10.1002/jmv.23905.

Kreißel, K., Bösl, M., Lipp, P., Franzreb, M. & Hambsch, B. 2012 Study on the removal efficiency of UF membranes using bacteriophages in bench-scale and semi-technical scale. *Water Science and Technology* **66** (6), 1195–1202. https://doi.org/10.2166/wst.2012.299.

Lee, S., Hata, A., Yamashita, N. & Tanaka, H. 2017 Evaluation of virus reduction by ultrafiltration with coagulation–sedimentation in water reclamation. *Food and Environmental Virology* **9** (4), 453–463. https://doi.org/10.1007/s12560-017-9301-9.

Lipp, E. K., Lukasik, J. & Rose, J. B. 2001 Human enteric viruses and parasites in the marine environment. *Methods in Microbiology* **30**, 559–588. https://doi.org/10.1016/s0580-9517(01)30063-6.

Matsui, Y., Matsushita, T., Inoue, T., Yamamoto, M., Hayashi, Y., Yonekawa, H. & Tsutsumi, Y. 2003a Virus removal by ceramic membrane microfiltration with coagulation pretreatment. *Water Science and Technology: Water Supply* **3** (5–6), 93–99. https://doi.org/10.2166/ws.2003.0154.

Matsui, Y., Matsushita, T., Sakuma, S., Gojo, T., Mamiya, T., Suzuoki, H. & Inoue, T. 2003b Virus inactivation in aluminum and polyaluminum coagulation. *Environmental Science and Technology* **37**, 5175–5180. https://doi.org/10.1021/es0343003.

Matsushita, T., Matsui, Y. & Inoue, T. 2004 Irreversible and reversible adhesion between virus particles and hydrolyzing-precipitating aluminium: A function of coagulation. *Water Science and Technology* **50** (12), 201–206. https://doi.org/10.2166/wst.2004.0714.

Matsushita, T., Shirasaki, N., Tatsuki, Y. & Matsui, Y. 2013 Investigating norovirus removal by microfiltration, ultrafiltration, and precoagulation-microfiltration processes using recombinant norovirus virus-like particles and real-time immuno-PCR. *Water Research* **47** (15), 5819–5827. https://doi.org/10.1016/j.watres.2013.07.004.

Meyn, T., Leiknes, T. O. & König, A. 2012 MS2 removal from high NOM content surface water by coagulation – ceramic microfiltration, for potable water production. *AIChE Journal* **58** (7), 2270–2281. https://doi.org/10.1002/aic.12731.

Michen, B. & Graule, T. 2010 Isoelectric points of viruses. *Journal of Applied Microbiology* **109** (2), 388–397. https://doi.org/10.1111/j.1365-2672.2010.04663.x.

Olivieri, A., Crook, J., Anderson, M., Bull, R., Drewes, J., Haas, C., Jakubowski, W., Mccarty, P., Nelson, K., Rose, J., Sedlak, D., Wade, T., Simhon, A., Pileggi, V., Flemming, C. A., Bicudo, J. R., Lai, G. & Manoharan, M. 2016 *Expert Panel Final Report: Evaluation of the Feasibility of Developing Uniform Water Recycling Criteria for Direct Potable Reuse*, Vol. 17, Issue 5. Available from: www.nwri-usa.org.

Shirasaki, N., Matsushita, T., Matsui, Y., Kobuke, M. & Ohno, K. 2009 Comparison of removal performance of two surrogates for pathogenic waterborne viruses, bacteriophage Qβ and MS2, in a coagulation-ceramic microfiltration system. *Journal of Membrane Science* **326** (2), 564–571. https://doi.org/10.1016/j.memsci.2008.10.037.

Shirasaki, N., Matsushita, T., Matsui, Y., Oshiba, A. & Ohno, K. 2010 Estimation of norovirus removal performance in a coagulation-rapid sand filtration process by using recombinant norovirus VLPs. *Water Research* **44** (5), 1307–1316. https://doi.org/10.1016/j.watres.2009.10.038.

Shirasaki, N., Matsushita, T., Matsui, Y. & Marubayashi, T. 2016 Effect of aluminum hydrolyte species on human enterovirus removal from water during the coagulation process. *Chemical Engineering Journal* **284**, 786–793. https://doi.org/10.1016/j.cej.2015.09.045.

CCR 2015 *State Water Resources Control Board Regulations Related to Recycled Water*, pp. 1–81. Available from: http://www.waterboards.ca.gov/drinking_water/certlic/drinkingwater/Lawbook.shtml.

Viegas, R. M. C., Mesquita, E., Campinas, M., Inocêncio, P., Teixeira, A. P., Martins, J. & Rosa, M. J. 2015 Water reclamation with hybrid coagulation-ceramic microfiltration: First part of a long-term pilot study in Portugal. *Journal of Water Reuse and Desalination* **5** (4), 550–556. https://doi.org/10.2166/wrd.2015.122.

Wolf, S., Hewitt, J. & Greening, G. E. 2010 Viral multiplex quantitative PCR assays for tracking sources of fecal contamination. *Applied and Environmental Microbiology* **76** (5), 1388–1394. https://doi.org/10.1128/AEM.02249-09.

World Health Organization 2011 *Guidelines for Drinking-Water Quality*, 4th edn. WHO (World Health Organization), Geneva, p. 340. https://doi.org/10.1016/S1462-0758(00)00006-6.

World Health Organization 2014 *WHO International Scheme to Evaluate Household Water Treatment Technologies Harmonized Testing Protocol: Technology Non-Specific. February*, 1–5.

World Health Organization 2017 *Potable Reuse Guidance for Producing Safe Drinking-Water*. Available from: https://www.who.int/water_sanitation_health/publications/potable-reuse-guidelines/en/.

Yasui, N., Suwa, M., Sakurai, K., Suzuki, Y., Tsumori, J., Kobayashi, K., Takabatake, H., Lee, S. T., Yamashita, N. & Tanaka, H. 2016 Removal characteristics and fluctuation of norovirus in a pilot-plant by an ultrafiltration membrane for the reclamation of treated sewage. *Environmental Technology (United Kingdom)* **37** (21), 2793–2801. https://doi.org/10.1080/09593330.2016.1164760.

Yasui, M., Ikner, L., Yonetani, T., Liu, M. & Katayama, H. 2023 Effects of surface hydrophobicity on the removal of F-specific RNA phages from reclaimed water by coagulation and ceramic membrane microfiltration. *Water Science & Technology* **87** (9), 2304–2314. https://doi.org/10.2166/wst.2023.133.

Yin, Z., Tarabara, V. V. & Xagoraraki, I. 2015 Human adenovirus removal by hollow fiber membranes: Effect of membrane fouling by suspended and dissolved matter. *Journal of Membrane Science* **482**, 120–127. https://doi.org/10.1016/j.memsci.2015.02.028.

Zhu, B., Clifford, D. A. & Chellam, S. 2005 Virus removal by iron coagulation-microfiltration. *Water Research* **39**, 5153–5161. https://doi.org/10.1016/j.watres.2005.09.035.

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