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Evaluation of the suitability of a plant virus, pepper mild mottle virus, as a surrogate of human enteric viruses for assessment of the efficacy of coagulation–rapid sand filtration to remove those viruses

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ABSTRACT

Here, we evaluated the removal of three representative human enteric viruses — adenovirus (AdV) type 40, coxsackievirus (CV) B5, and hepatitis A virus (HAV) IB — and one surrogate of human caliciviruses — murine norovirus (MNV) type 1 — by coagulation–rapid sand filtration, using water samples from eight water sources for

drinking water treatment plants in Japan. The removal ratios of a plant virus (pepper mild mottle virus; PMMoV) and two bacteriophages (MS2 and φX174) were compared with the removal ratios of human enteric viruses to assess the suitability of PMMoV, MS2, and φX174 as surrogates for human enteric viruses. The removal ratios of AdV, CV, HAV, and MNV, evaluated via the real-time polymerase chain reaction (PCR) method, were 0.8–2.5-$\log_{10}$ when commercially available polyaluminum chloride (PACl, basicity 1.5) and virgin silica sand were used as the coagulant and filter medium, respectively. The type of coagulant affected the virus removal efficiency, but the age of silica sand used in the rapid sand filtration did not. Coagulation–rapid sand filtration with non-sulfated, high-basicity PACls (basicity 2.1 or 2.5) removed viruses more efficiently than the other aluminum-based coagulants. The removal ratios of MS2 were sometimes higher than those of the three human enteric viruses and MNV, whereas the removal ratios of φX174 tended to be smaller than those of the three human enteric viruses and MNV. In contrast, the removal ratios of PMMoV were similar to and strongly correlated with those of the three human enteric viruses and MNV. Thus, PMMoV appears to be a suitable surrogate for human enteric viruses for the assessment of the efficacy of coagulation–rapid sand filtration to remove viruses.

Keywords: Coagulation; Non-sulfated high-basicity PACl; Rapid sand filtration; Pepper mild mottle virus; Surface charge; Virus inactivation

1. Introduction

Human enteric viruses are one of the leading causes of nonbacterial gastrointestinal illness and can be transmitted via water. Because large numbers of human enteric viruses are excreted in the feces of patients, not only raw sewage and sewage discharges, but also drinking water sources that receive sewage discharges are often contaminated with those viruses (Albinana-Gimenez et al., 2006; Bosch, 2007). The implication is that consumption of water may result in exposure to human enteric viruses, particularly when the drinking water treatment process for virus reduction is inadequate. Assessment of the extent of virus reduction during drinking water treatment is therefore important for the prevention and control of waterborne viral diseases.

Some researchers have determined the virus removal efficiency at drinking water treatment plants (DWTPs) by using quantitative real-time polymerase chain reaction (PCR), which is a rapid, highly sensitive, highly specific means of quantifying viruses

(Albinana-Gimenez et al., 2006; Albinana-Gimenez et al., 2009; Asami et al., 2016). Because the numbers of indigenous human enteric viruses in water samples, particularly treated water, are usually below the PCR quantification limit, large-volume water samples and concentration techniques to reduce sample volume are required to estimate the concentrations of indigenous human enteric viruses (Rames et al., 2016). However, even when more than 1000 L of water are concentrated to less than several milliliters, human enteric viruses are sometimes not detected in the treated water (Albinana-Gimenez et al., 2009; Prevost et al., 2016). Accurate assessment of the efficacy of human enteric virus removal by DWTPs has therefore been hampered by the low virus concentrations in treated water.

A metagenomic analysis has revealed that a plant virus, pepper mild mottle virus (PMMoV), an RNA virus (genus *Tobamovirus*, family Virgaviridae) that infects bell, hot, and ornamental peppers, is present at concentrations up to 10^9 virus particles per gram of human feces (Zhang et al., 2006). Because human feces are the most likely source of PMMoV in surface waters and because PMMoV is more frequently detected and is present at higher concentrations and with less seasonality than human enteric viruses in surface waters, including drinking water sources (Hamza et al., 2011; Haramoto et al., 2013), PMMoV has been proposed as an indicator of fecal pollution in surface water. In

addition, the concentrations of PMMoV in drinking water sources are probably high enough to determine virus removal efficiency at DWTPs. In fact, Asami et al. (2016) successfully evaluated the virus removal efficiency of coagulation–sedimentation and rapid sand filtration at a DWTP in Bangkok, Thailand, by monitoring PMMoV concentrations during the treatment process. If the removal efficiencies of PMMoV and human enteric viruses are comparable, PMMoV could be a useful surrogate for evaluating the efficacy of drinking water treatment processes to remove human enteric viruses. Because coagulation–sedimentation followed by rapid granular filtration and in particular coagulation–rapid sand filtration are used worldwide in DWTPs to produce drinking water from surface water, whether PMMoV is an adequate surrogate for human enteric viruses in coagulation–rapid sand filtration is an important question. However, the relationship between the removal efficiencies of PMMoV and human enteric viruses in coagulation–rapid sand filtration has not yet been investigated.

In this study, we conducted laboratory-scale coagulation–rapid sand filtration experiments with water samples from eight drinking water sources across Japan to investigate the efficacy of removal of human enteric viruses via coagulation with aluminum-based coagulants, including commercially available polyaluminum chloride (PACl) and alum, followed by settling and rapid sand filtration with virgin silica sand and

in-use silica sand collected from a rapid sand filter in a DWTP. We then compared the results with PMMoV and human enteric viruses to assess the suitability of PMMoV as a surrogate for human enteric viruses. The fourth contaminant candidate list (CCL4) for drinking water, published by the U.S. Environmental Protection Agency, includes four types of human enteric viruses: adenoviruses (AdVs); enteroviruses, which include polioviruses, coxsackieviruses (CVs), and echoviruses; hepatitis A viruses (HAVs); and caliciviruses, which include noroviruses and sapoviruses (USEPA, 2016). For our study, we chose three representative CCL viruses, AdV, CV, and HAV, and a surrogate of human caliciviruses, the murine norovirus (MNV). Among AdVs and CVs, AdV type 40 and CV B5 were specifically chosen for use in this study because they are highly resistant to ultraviolet (UV) disinfection (Nwachuku et al., 2005) and free-chlorine disinfection (Cromeans et al., 2010), respectively. For comparative purposes, the removal efficiencies of bacteriophages MS2 and φX174 were also investigated because they are widely used as surrogates for human enteric viruses to evaluate virus removal via coagulation–sedimentation followed by rapid granular filtration (Nasser et al., 1995; Gerba et al., 2003; Abbaszadegan et al., 2007; Boudaud et al., 2012).

2. Materials and methods

2.1. Source water, coagulants, and filter media

The water samples used in the present study were collected from eight water sources for DWTPs in various areas of Japan. Table 1 shows the water quality data for the sources. All of the treatment plants employed coagulation with aluminum-based coagulants (PACl or alum) followed by rapid sand filtration for the production of drinking water. The source water samples were stored at 4 °C until use (up within one year after sampling) and brought to 20 °C immediately prior to use. We have confirmed that water quality parameters, i.e., turbidity, dissolved organic carbon (DOC) concentration, and UV absorbance at 260 nm (UV260, an indication of natural organic matter [NOM] concentration), of the samples were stable during sample storage period.

To investigate the effects of coagulant basicity ($[OH^-]/[Al^{3+}]$) and sulfate content on virus removal via coagulation–rapid sand filtration, we used five aluminum-based coagulants (Taki Chemical Co., Kakogawa, Japan). Specifications of the coagulants are shown in Table 2 and described in the Supplementary Information.

To investigate the effect of the age of silica sand on virus removal via coagulation–rapid sand filtration, we used virgin silica sand (effective size, 0.6 mm; uniformity coefficient, <1.3; Nihon Genryo Co., Kawasaki, Japan) and in-use silica sand (>6 years of use with hydraulic backwashing every 8 days) collected from a rapid sand filter in a

DWTP as the filter media for the rapid sand filtration. Table 3 shows the specifications of the sands.

2.2. Characterization of filter media

The effective size and uniformity coefficient of the silica sands were determined by sieve analyses.

The zeta potentials of the silica sands were determined with a Zetasizer Nano ZS (50 mW, 532-nm green laser; Malvern Instruments, Malvern, Worcestershire, UK) equipped with a surface zeta potential cell kit (ZEN1020, Malvern Instruments). The silica sand was attached to the sample holder by using double-faced adhesive tape. The sample was inserted into a disposable plastic square cuvette containing prepared Milli-Q water with a 0.01% (v/v) suspension of latex microspheres (mean diameter, 0.5 μm; 5050A, Thermo Fisher Scientific Inc., Waltham, MA, USA) as tracer particles for measurements. The alkalinity of the Milli-Q water was brought to 20 mg-$CaCO_3$/L by the addition of 0.4 mM $NaHCO_3$, and the pH was adjusted to 7 with HCl. Measurements were conducted at 25 °C and a 17° measurement angle at five different distances from the sample surface to calculate the zeta potential of the silica sand.

2.3. Human enteric viruses, MNV, bacteriophages, and PMMoV

AdV type 40 Dugan strain (ATCC VR-931), CV B5 Faulkner strain (ATCC VR-185), HAV IB HM175/18f strain (ATCC VR-1402), and MNV type 1 CW1 strain (ATCC PTA-5935) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and propagated in human lung carcinoma epithelial cells (A549 cells; ATCC CCL-185, obtained from ATCC), buffalo green monkey kidney epithelial cells (BGM cells; kindly supplied by Dr. Daisuke Sano, Hokkaido University, Sapporo, Japan), fetal rhesus monkey kidney epithelial cells (FRhK-4 cells; ATCC CRL-1688, obtained from ATCC), and murine macrophage cells (RAW264.7 cells; ATCC TIB-71, obtained from ATCC), respectively. Details of the propagation and purification of AdV, CV, HAV, and MNV have been described in previous reports (Shirasaki et al., 2016; Shirasaki et al., 2017a). The concentrations of AdV, CV, HAV, and MNV in the purified solutions were approximately $10^{5–6}$, 10^{7}, $10^{5–6}$, and 10^{6} plaque-forming units (PFU)/mL, respectively, based on the results of plaque assays (Shirasaki et al., 2016; Shirasaki et al., 2017a).

F-specific RNA bacteriophage MS2 (NBRC 102619) and somatic DNA bacteriophage φX174 (NBRC 103405) were obtained from the National Institute of

Technology and Evaluation Biological Research Center (Kisarazu, Japan), as were the *Escherichia coli* bacterial hosts in which the bacteriophages were propagated (NBRC 13965 for MS2, NBRC 13898 for φX174). Details of the propagation and purification of the bacteriophages have been described by Shirasaki et al. (2016). The concentrations of MS2 and φX174 in the purified solutions were approximately 10^{10} and $10^{7–8}$ PFU/mL, respectively, as evaluated by means of plaque assays (Shirasaki et al., 2016).

The PMMoV pepIwateHachiman1 strain (MAFF 104099) was obtained from the National Institute of Agrobiological Sciences Genebank (Tsukuba, Japan), and propagated in *Nicotiana benthamiana* (seeds kindly supplied by Dr. Kenji Nakahara, Hokkaido University). The details of propagation of PMMoV are described in Supplementary Information. The concentration of PMMoV in stock solution was approximately 10^7 lesions/mL, as evaluated by using a local lesion count assay with *Nicotiana tabacum* cv. *Xanthi-nc* (see section 2.7, seeds also kindly supplied by Dr. Kenji Nakahara).

2.4. Batch coagulation experiments

Batch coagulation experiments were conducted with 2 L of virus-spiked source water in

square plastic beakers at 20 °C. Purified solutions of human enteric viruses, MNV, and bacteriophages, and the stock solution of PMMoV, were simultaneously added to the source water at initial concentrations (C_0) of approximately 10^{2-3} PFU/mL for AdV, CV, HAV, MNV, 10^7 PFU/mL for MS2, 10^5 PFU/mL for φX174, and 10^3 lesions/mL for PMMoV. Because the purified solutions and stock solution of viruses were diluted by addition to the source water, virus addition contributed less than 0.3 mg/L of unintentional carry-over of DOC. After enough HCl or NaOH was added to the spiked water to bring the final pH to 7, a coagulant was injected into the water. The coagulant dosages added to the source-water samples were the same as the dosages used at the corresponding DWTP on the day the source water was sampled (Table 1). The water was stirred rapidly for 1 min (G = 200 s^{-1}, 196 rpm) and then slowly for 10 min (G = 20 s^{-1}, 42 rpm) with an impeller stirrer. The water was then allowed to stand for 60 min to settle the generated aluminum floc particles. Approximately 1.5 L of supernatants were then sampled from the beaker for quantification of the virus concentrations (C_s) and turbidity, and for use in the following rapid sand filtration experiments. In addition, a portion of each supernatant was filtered through a polytetrafluoroethylene membrane filter (nominal pore size 0.45 μm; Dismic-25HP, Toyo Roshi Kaisha, Tokyo, Japan) for quantification of the virus concentrations (C_{mf}), DOC, and UV260. The turbidity, DOC concentrations, and UV260-

absorbing NOM were quantified with a turbidity meter (2100Q Portable, Hach Company, Loveland, CO, USA), a total organic carbon analyzer (SIEVERS 900, GE Analytical Instruments, Boulder, CO, USA) and a UV-visible spectrophotometer (UV-1800 UV-VIS, Shimazu Corp., Kyoto, Japan), respectively.

2.5. Rapid sand filtration experiments

After the batch coagulation experiments, rapid sand filtration experiments were conducted with a plastic column (diameter, 3.6 cm; length, 50 cm) packed with silica sand. Virgin silica sand was washed with Milli-Q water and dried at 105 °C for 6 h. The washed virgin silica sand or in-use silica sand was gradually added into the column to achieve a 10-cm filter depth. Approximately 5 L of Milli-Q water was pumped upward (i.e. from the filter effluent side to the filter influent side) through the column, and then another approximately 5 L (for virgin silica sand) or 1 L (for in-use silica sand) of Milli-Q water was pumped downward through the column with a peristaltic pump to remove fines in the filter media. This upward-and-downward washing procedure was done twice when virgin silica sand was used as the filter medium. Sufficient removal of fines was confirmed by the fact that the turbidity of the filter effluent after washing was

approximately equal to that of Milli-Q water. Next, approximately 1.4 L of the supernatant from the settled sample (see section 2.4) was fed into the column at a constant flow rate (120 m/day) by the peristaltic pump. The first 300 mL of filtrate was discarded, and then the measurement of filtration time was started. Samples were taken from column filtrate after 5 and 10 min of filtration for quantification of the virus concentrations (C_{rf}) and turbidity. In addition, a portion of each filtrate was filtered through a polytetrafluoroethylene membrane filter (nominal pore size 0.45 μm; Dismic-25HP) for quantification of DOC and UV260. Unused filter medium (i.e. washed filter medium not yet used in the study) was used in each experiment to avoid viral and particle cross-contamination between experiments.

To investigate the effect of filter depth on virus removal via coagulation–rapid sand filtration, the column having a filter depth of 10 cm was connected to another same column to achieve a total filter depth of 20 cm.

2.6. Quantification of viruses by real-time PCR or real-time RT-PCR

Real-time PCR, which can detect all viruses, regardless of their infectivity or the existence of aggregates, was used to quantify viral DNA and RNA. Specifically, viral DNA of AdV

or φX174 was quantified by means of real-time PCR, and viral RNA of CV, HAV, MNV, MS2, or PMMoV was quantified by means of real-time reverse-transcription PCR (real-time RT-PCR). The details of the real-time PCR and real-time RT-PCR methods have been described by Shirasaki et al. (2017b).

2.7 Quantification of infectious PMMoV by local lesion count assay

Infectious PMMoV was quantified by a local lesion count assay with *Nicotiana tabacum* cv. *Xanthi-nc*. The seed and the seedling of *Nicotiana tabacum* cv. *Xanthi-nc* were grown and cultured via the same procedure used to grow *Nicotiana benthamiana* (Supplementary Information). After cultivation, three leaves of each plant were covered with 600 mesh carborundum, and then 100 μL of a sample serially diluted 10-fold with phosphate-buffered saline was rubbed into each leaf with a gloved finger. The virus-inoculated plant was incubated at 20 °C for 5 min, and then the inoculum mixed with carborundum was washed away by using Milli-Q water. After washing, the plant was incubated in the growth chamber at 25 °C under a long-day photoperiod (16-h light, 8-h dark) for 4–5 days. At the end of the incubation, local lesions on each leaf were counted, and the average lesion count of 1–3 plants (i.e., 3–9 leaves) prepared from a single sample

was considered as the infectious PMMoV concentration for that sample. The detection limit of the local lesion count assay was 1 lesion/300 μL, i.e. 10/3 lesions/mL, when three leaves from each plant were infected.

2.8. Electrophoretic mobility

The electrophoretic mobility of PMMoV was measured in filtered source water samples with the Zetasizer Nano ZS. A stock solution of PMMoV was suspended in the water at approximately $10^{4–5}$ lesions/mL. Details of the electrophoretic mobility measurements have been described by Shirasaki et al. (2016).

3. Results and discussion

3.1. Virus removal by coagulation–rapid sand filtration with PACl-1.5s

Figure 1a shows the removal ratios ($\log_{10}[C_0/C_s]$) of the viruses after coagulation with PACl-1.5s followed by settling under gravity in samples of eight different raw water sources. Although almost no removal (<0.3-$\log_{10}$) of viruses was observed in the absence of a coagulant at pH 7 (data not shown), coagulation with PACl-1.5s removed viruses

from the virus-spiked river water. These results indicated that the viruses stably monodispersed by electrical repulsion in the source water samples were destabilized by the addition of PACl-1.5s and became entrapped in or adsorbed to the aluminum floc particles generated during coagulation. Floc particles with the entrapped or adsorbed viruses then settled from suspension by gravity. Virus removal ratios of 0.2–2.0-$\log_{10}$ were observed under conditions when removals of turbidity, DOC, and UV260 were 76–92%, 35–52%, and 32–64%, respectively. Under the same conditions, the removal ratios of AdV, CV, HAV, and MNV were 0.9–2.0-$\log_{10}$, 0.5–1.6-$\log_{10}$, 0.8–1.1-$\log_{10}$, and 0.6–1.3-$\log_{10}$, respectively. The removal ratios depended strongly on the source water sample (Fig. S1a). For example, coagulation resulted in relatively high virus removals from source waters B, C, and E but the lowest removal ratios from source water G among the source water samples used in this study. The UV260-absorbing NOM of source water G was markedly higher than that of the other source-water samples (Table 1). The destabilization mechanisms (i.e., coordination reactions between coagulant species and carboxyl groups of the virus surface proteins or NOM) are similar for virus and NOM (Bratby, 2006). Therefore, the high NOM content of source water G probably competed with the viruses for coordination reactions and led to a reduction of virus removal via coagulation.

Figure 1b shows the removal ratios ($\log_{10}[C_0/C_{rf}]$) of the viruses after coagulation with PACl-1.5s followed by settling and rapid sand filtration with virgin silica sand. In the absence of a coagulant, settling followed by rapid sand filtration resulted in almost no removal (<0.3-$\log_{10}$) of the viruses (data not shown). Improvements of the virus removal ratios were achieved by combining coagulation and rapid sand filtration compared with coagulation alone (Fig. 1a). Therefore, the viruses entrapped in or adsorbed onto the suspended aluminum flocs were effectively removed by the subsequent rapid sand filtration. The virus removal ratios in the coagulation–rapid sand filtration with PACl-1.5s were almost independent of filtration time, and almost no differences were observed in virus removal ratios for filter depths of 10 and 20 cm (data not shown). These results indicate that the effects of filtration time and filter depth on virus removal were negligible, and most of the viruses entrapped in or adsorbed onto the suspended aluminum flocs were retained in the surface layer of the silica sand filter in the coagulation–rapid sand filtration. Accordingly, the removal ratios obtained after rapid sand filtration were expressed as the average of the values for a filter depth of 10 cm after filtration times of 5 and 10 min. The range of virus removal ratios was 0.3–2.7-$\log_{10}$ under conditions such that the turbidities of filtrates were <0.2 NTU (<0.14 kaolin turbidity degree) and the removal ratios of AdV, CV, HAV, and MNV were 1.3–2.4-$\log_{10}$, 0.8–2.5-$\log_{10}$, 1.1–2.4-$\log_{10}$, and 0.8–2.4-$\log_{10}$,

respectively. The removal ratios obtained after rapid sand filtration with source water G were the lowest among the source water samples used in the present study (Fig. S1b). This result suggests that the efficiency of virus removal during coagulation directly influences the virus removal ratios of subsequent rapid sand filtration. Improvement of coagulation efficiency is therefore essential for improvement of virus removal via coagulation–rapid sand filtration.

3.2. Effects of types of coagulant and filter medium on virus removal

As described above, the virus removal ratios obtained with source water G were markedly lower than those with other source water samples. To improve virus removal performance, we investigated the effect of coagulant type on virus removal in coagulation–rapid sand filtration using source water G. In addition, because source water B was treated with alum in its DWTP (Table 1), the virus removal performances of coagulation–rapid sand filtration with alum were investigated and then compared with virus removal with PACl-1.5s for source water B. The virus removal ratios obtained with alum were almost the same as those obtained with PACl-1.5s for source water B (Fig. 2a). A similar pattern was also observed for source water C (Fig. 2b). In contrast, virus removal ratios were

improved by using high-basicity PACls, particularly non-sulfated, high-basicity PACls (i.e., PACl-2.1ns and PACl-2.5ns), compared with PACl-1.5s for source water G (Fig. 2c). Other source water samples (i.e., low-NOM source water C; Fig. 2b, and high-NOM source water H; Fig. 2d) were also examined. Whereas the virus removal ratios obtained with non-sulfated, high-basicity PACls were almost the same as those obtained with PACl-1.5s for low-NOM source water C, approximately a 1-$\log_{10}$ improvement in virus removal ratios was achieved for high-NOM source water H, similar to the case of high-NOM source water G. These results indicated that the type of coagulant affected the virus removal performance of coagulation–rapid sand filtration, particularly when the source water contained high concentrations of NOM. The results also indicated that non-sulfated, high-basicity PACls were the most effective for removing viruses, not only with in-line coagulation–MF (Shirasaki et al., 2017b) but also with coagulation–rapid sand filtration. We have previously reported that PACl-2.1ns has a higher colloid charge density than alum, PACl-1.5s, and PACl-2.1s (Shirasaki et al., 2014). This difference is attributable to the high colloidal aluminum content and absence of sulfate in PACl-2.1ns (Shirasaki et al., 2014). In addition, PACl-2.1ns and PACl-2.5ns contain large amounts of the Al_{30} species $[Al_{30}O_8(OH)_{56}(H_2O)_{24}]^{18+}$, which are the highest-charged polycations ever characterized among PACls and confers a stronger floc formation capacity compared with

other Al species in PACls (Chen et al., 2006; Zhang et al., 2008), including alum, PACl-1.5s, and PACl-2.1s. These characteristics of PACl-2.1ns and PACl-2.5ns likely resulted in their higher virus removal performances compared to the other coagulants used in this study.

The silica sand used in DWTPs has typically been in use for several years. To investigate the effect of this use on virus removal, in-use silica sand was collected from a rapid sand filter in a DWTP, and the virus removal ratios obtained in the coagulation–rapid sand filtration with in-use silica sand were then compared with those obtained with virgin silica sand (Fig. 3). Even when in-use silica sand was used instead of virgin silica sand in the rapid sand filter, there was almost no removal (<0.3-$\log_{10}$) of viruses in the absence of a coagulant (data not shown). Although improvements of virus removal ratios were observed by combining coagulation and rapid sand filtration versus coagulation and settling alone, the virus removal ratios obtained with in-use silica sand were almost the same as those obtained with virgin silica sand for source water C (Fig. 3a). No large differences in removal ratios were observed between in-use silica sand and virgin silica sand for source water G (Fig. 3b) and source water H (Fig. 3c). We then tested the efficacy of the in-use silica sand washed in an alternative way. Approximately 1 L of source water sample that had been filtered through a polytetrafluoroethylene membrane filter (nominal

pore size 0.45 μm; Dismic-25HP) instead of Milli-Q water was pumped upward through the column. Then another approximately 1 L of filtered source water sample was pumped downward through the column via a peristaltic pump. The virus removal ratios obtained with the in-use silica sand were comparable to those obtained with virgin silica sand (data not shown). These results indicated that the age of silica sand used in the rapid sand filter did not affect the virus removal performance of coagulation–rapid sand filtration. Granular filtration including rapid sand filtration can remove particles from water by several mechanisms. When particles are larger than the void spaces in the filter, they are removed by straining. When the particles are smaller than the void spaces, they can be removed only if they contact and stick to the grains of the medium (Crittenden et al., 2012). Because the effective size and uniformity coefficient of virgin silica sand and in-use silica sand were comparable (Table 3), the straining efficiencies of the two columns packed with virgin silica sand and in-use silica sand were probably comparable. In contrast, the surface charges (i.e. zeta potentials) of the virgin silica sand and in-use silica sand were different. Virgin silica sand was more negatively charged than in-use silica sand (Table 3). However, the differences in the surface charges of the two silica sands did not affect the virus removal performance of the coagulation–rapid sand filtration because comparable removal ratios were achieved with virgin silica sand and in-use silica sand.

3.3. Relationships between removal ratios of enteric viruses, bacteriophages, PMMoV turbidity, DOC, and UV260

To investigate whether the bacteriophages MS2 and φX174, the plant virus PMMoV, and water quality parameters (turbidity, DOC, and UV260) were suitable surrogates for AdV, CV, HAV, and caliciviruses, we calculated Pearson's correlation coefficients (r) between the removal ratios of viruses, turbidity, DOC, and UV260 achieved with coagulation–rapid sand filtration (Excel Toukei BellCurve software, Social Survey Research Information Co., Tokyo, Japan; Table 4). The removal ratios obtained with AdV, CV, HAV, and MNV were strongly correlated with each other in the coagulation–rapid sand filtration experiments (r = 0.79–0.96). These viruses have similar isoelectric points (i.e. 3.6–3.8) and have shown similar electrophoretic mobilities in filtered source water samples at pH 7. For example, Shirasaki et al. (2016) and Shirasaki et al. (2017a) have reported the electrophoretic mobilities of AdV, CV, HAV, and MNV to be –1.49, –1.91, –1.69, and –1.72 (μm/s)/(V/cm), respectively, in filtered source water C (designated source water D in those previous studies). The similarities of the surface charge properties of the viruses probably resulted in the comparable removal ratios obtained for AdV, CV, HAV,

and MNV.

The removal ratios of MS2 were moderately or strongly correlated with those obtained for the three human enteric viruses and MNV (r = 0.60–0.92). However, the removal ratios of MS2 were sometimes larger than those of the three human enteric viruses and MNV (Fig. 4a). These results indicate that MS2 is probably not an appropriate surrogate for AdV, CV, HAV, and caliciviruses during coagulation–rapid sand filtration, because the removal ratios of those viruses would sometimes be overestimated if MS2 were used as a surrogate.

The removal ratios obtained for φX174 were correlated with those of the three human enteric viruses and MNV (r = 0.53–0.75), although the Pearson's correlation coefficients were lower than those in the case of MS2 (Table 4). In addition, the removal ratios of φX174 tended to be smaller than those of the three human enteric viruses and MNV (Fig. 4b). These results suggest that φX174 has the potential to be a conservative surrogate for AdV, CV, HAV, and caliciviruses during coagulation–rapid sand filtration.

The removal ratios of PMMoV were strongly correlated with those of the three human enteric viruses and MNV (r = 0.81–0.94), and the Pearson's correlation coefficients were higher than in the cases of MS2 and φX174 (Table 4). In addition, the removal ratios of PMMoV were comparable to those of the three human enteric viruses and MNV (Fig. 4c).

The isoelectric point of PMMoV is 3.2 (Shirasaki et al., 2017b), similar to those of AdV, CV, HAV, and MNV (3.6–3.8) and unlike those of MS2 and φX174, 2.2 and 1.6, respectively (Shirasaki et al., 2016; Shirasaki et al. 2017a). The electrophoretic mobility of PMMoV in filtered source water C at pH 7, –1.97 (μm/s)/(V/cm), was also similar to those of AdV, CV, HAV, and MNV, –1.91 to –1.49 (μm/s)/(V/cm), and unlike those of MS2 and φX174, –2.45 and –2.95 (μm/s)/(V/cm), respectively (Shirasaki et al., 2016; Shirasaki et al. 2017a). The similarities in the surface charge properties among PMMoV, the three human enteric viruses, and MNV probably led to the similarity of the removal ratios. These results suggest that PMMoV is a better surrogate than MS2 and φX174 and potentially a suitable surrogate for AdV, CV, HAV, and caliciviruses during coagulation–rapid sand filtration.

In contrast, there was no correlation or only a weak correlation between the removal ratios of turbidity, DOC, and UV260 versus those of the three human enteric viruses and MNV (r = –0.06 to 0.49), unlike in the cases of MS2, φX174, and PMMoV (Table 4, Fig. 4d–f). Asami et al. (2016) have reported that optimization for turbidity removal at a given operation in a DWTP employing coagulation–sedimentation and rapid sand filtration does not necessarily lead to optimization of virus removal. Our results are consistent with that assessment, because the removal ratios of turbidity were not correlated with those of the

three human enteric viruses and MNV (r = –0.06–0.12). These results indicate that it is difficult to estimate the removal ratios in a coagulation–rapid sand filtration of AdV, CV, HAV, and caliciviruses from the removal ratios of turbidity, DOC, and UV260.

3.4. Effect of virucidal activity of PACl on PMMoV removal

Because the removal ratios of PMMoV were comparable to and strongly correlated with those of the three human enteric viruses and MNV, PMMoV appeared to be a more suitable surrogate for human enteric viruses than MS2, φX174, turbidity, DOC, and UV260 for assessment of the efficacy of coagulation–rapid sand filtration in removing viruses when the virus removal ratios were determined via PCR. However, several research groups, including ours, have compared the removal ratios of infectious viruses evaluated by means of the plaque assay, which can detect only infectious viruses, and the removal ratios of all viruses evaluated via PCR after dissolution or exclusion of aggregates. Results have shown that the bacteriophages MS2 and Qβ lose their infectivity after contact with PACl (Matsushita et al., 2011; Kreissel et al., 2014). In our previous studies (Shirasaki et al., 2016; Shirasaki et al., 2017a), the removal ratios determined by the two methods were comparable for AdV, CV, MNV, and φX174, but statistically

different for HAV and MS2. It is therefore likely that the former four retain their infectivity after contact with PACl, whereas HAV and MS2 do not. These results indicate that the virucidal activity of PACl contributes to the removal of infectious HAV and MS2 during coagulation. However, the possibility that PMMoV is inactivated during coagulation has not yet been investigated. If the virus removal performances of coagulation–rapid sand filtration with PACl are evaluated by using a virus sensitive to virucidal activity (such as HAV or MS2) as a representative or a surrogate of human enteric viruses, the removal ratios of that virus, as determined via an infectivity assay, would probably overestimate the ability of coagulation–rapid sand filtration to remove viruses insensitive to virucidal activity (such as AdV and CV). We therefore compared the infectious PMMoV removal ratios obtained via the local lesion count assay and the total PMMoV removal ratios obtained via PCR after coagulation with PACl-1.5s to determine whether PMMoV was inactivated by PACl (Fig. 5). PMMoV was independently added to the source water. The removal ratios determined by the two methods did not differ for PMMoV, not only after settling but also after rapid sand filtration with virgin silica sand. These results indicate that, like AdV, CV, MNV, and φX174 but unlike HAV and MS2, PMMoV was not inactivated by contact with PACl during coagulation (i.e. PMMoV is insensitive to the virucidal activity of PACl). If the

virus removal efficacy of coagulation–rapid sand filtration is evaluated by using PMMoV, and if the removal ratios of PMMoV are evaluated by means of an infectivity assay, the results will not lead to overestimation of the ability of coagulation–rapid sand filtration to remove viruses insensitive to the virucidal activity of PACl. In addition, because the removal ratios of PMMoV determined via the local lesion count assay and PCR were almost the same, the local lesion count assay, which is inexpensive and easy to conduct without dedicated equipment, is probably a useful alternative to PCR for assessing PMMoV removal efficacy in the virus-spiking experiments. These results and those described in section 3.3, combined with the fact that the percentage of samples that are positive for PMMoV and the concentration of PMMoV in drinking water sources are much higher than those of human enteric viruses (Haramoto et al., 2012; Haramoto et al., 2013; Asami et al., 2016), suggest that PMMoV could be a useful target virus for evaluating the virus removal performances of DWTPs that employ coagulation–rapid sand filtration.

3.5. Comparison of virus removal by rapid sand filtration and membrane filtration

Disposable polymeric membrane filters with nominal pore sizes of 0.2 or 0.45 μm have

been widely used to separate dissolved substances from suspended particles and employed in many studies to simulate media filtration (Matsui et al., 2013). If the virus removal performances of media filtration and membrane filtration are comparable after coagulation, membrane filtration with disposable polymeric membrane filters could be used as a surrogate for media filtration in terms of virus removal. To determine whether membrane filtration was a useful alternative to rapid sand filtration for estimating virus removal performance in a coagulation–rapid sand filtration, we determined the relationships between virus removal ratios obtained by coagulation combined with settling and rapid sand filtration with virgin silica sand and those obtained by coagulation combined with settling and filtration through a polytetrafluoroethylene membrane with a nominal pore size of 0.45 μm (Fig. 6). Virus concentrations that were below the quantification limit of PCR (several membrane filtrate samples) were assigned the quantification limit when calculating the correlation coefficients. The virus removal ratios obtained via coagulation followed by membrane filtration were strongly correlated with those obtained in the coagulation–rapid sand filtration, regardless of the type of virus (r = 0.73–0.97). Even when the virus concentrations that were below the quantification limit of PCR were excluded from the analysis, the strong correlations between the removal ratios obtained after membrane filtration and those obtained after sand filtration were

observed (r = 0.76–0.97). However, the removal ratios obtained after membrane filtration tended to be larger than those obtained after rapid sand filtration (Fig. 6). These results indicate that filtration with a disposable polytetrafluoroethylene membrane filter with a nominal pore size of 0.45 μm does not appear to be a useful alternative to rapid sand filtration for estimating virus removal performances in a coagulation–rapid sand filtration because the virus removal ratios obtained via rapid sand filtration would sometimes be overestimated. In general, membrane properties such as pore size, hydrophobicity, and surface charge, which are related to the membrane material, have been shown to affect virus removal performance (Urase et al., 1996; van Voorthuizen et al., 2001; Shirasaki et al., 2017b). Therefore, further investigations will be necessary to identify the ideal surrogate membrane for estimating virus removal via coagulation–rapid sand filtration.

4. Conclusions

(1) Based on PCR assays, the removal ratios of AdV, CV, HAV, and MNV achieved by coagulation–rapid sand filtration with PACl-1.5s were 1.3–2.4-$\log_{10}$, 0.8–2.5-$\log_{10}$, 1.1–2.4-$\log_{10}$, and 0.8–2.4-$\log_{10}$, respectively.

(2) The type of coagulant affected the virus removal performance, but the age of silica

sand used in the rapid sand filtration did not. Coagulation–rapid sand filtration with non-sulfated, high-basicity PACls removed viruses more efficiently than did coagulation–rapid sand filtration with other aluminum-based coagulants.

(3) The removal ratios of MS2 were sometimes larger than those of the three human enteric viruses and MNV, whereas the removal ratios of φX174 tended to be smaller than those of the three human enteric viruses and MNV. In contrast, the removal ratios of PMMoV were strongly correlated with those of the three human enteric viruses and MNV and were similar to those of the three human enteric viruses and MNV. PMMoV, like AdV, CV, MNV, and φX174 but unlike HAV and MS2, was not inactivated after contact with PACl during coagulation. PMMoV, unlike MS2 and φX174, thus appears to be a suitable surrogate for human enteric viruses for assessing the efficacy of coagulation–rapid sand filtration processes to remove viruses.

(4) There was no correlation or only a weak correlation between the removal ratios of turbidity, DOC, and UV260 versus those of the three human enteric viruses and MNV. It is therefore difficult to estimate the removal ratios of AdV, CV, HAV, and caliciviruses from the removal ratios of turbidity, DOC, and UV260 via coagulation–rapid sand filtration.

(5) The virus removal ratios obtained by coagulation combined with settling and

membrane filtration with a polytetrafluoroethylene membrane filter with a nominal pore size of 0.45 μm tended to be larger than those obtained via coagulation combined with settling and rapid sand filtration. Thus, filtration with this membrane does not appear to be a useful alternative to rapid sand filtration for estimating virus removal via coagulation–rapid sand filtration.

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Table 1 – Water quality data for the source water samples, along with coagulant type and dosage.

	Sampling date	pH	Turbidity (NTU)	DOC (mg/L)	UV260 (cm^{-1})	Alkalinity (mg-$CaCO_3$/L)	Coagulant type	Coagulant dosage at sampling day (μM-Al)
A	13-Oct-15	7.7	0.4	0.6	0.012	50.0	PACl	40
B	26-Oct-15	7.6	4.6	2.0	0.030	34.0	alum	40
C	02-Sep-15	7.3	1.0	0.9	0.021	11.0	PACl	40
D	19-Oct-15	7.3	3.0	0.9	0.027	24.0	PACl	40
E	29-Sep-15	7.5	4.5	0.7	0.018	31.6	PACl	70
F	30-Sep-15	7.5	2.8	1.0	0.020	36.0	PACl	80
G	01-Oct-15	7.5	3.0	3.7	0.093	58.2	PACl	100
H	01-Oct-15	7.6	10.6	3.3	0.054	64.0	PACl	100

Table 2 – Specifications of the aluminum-based coagulants used in this study.

Coagulants	Product name	Basicity	Aluminum concentration (% [w/w] as Al_2O_3)	Sulfate concentration (% [w/w])	Relative density at 20ºC	Manufacturer
Alum	Aluminum sulfate	0.0	8.1	22.6	1.3	
PACl-1.5s	PACl 250A	1.5	10.1	2.9	1.2	
PACl-2.1s	PACl 700A	2.1	10.1	2.0	1.2	Taki Chemical Co.
PACl-2.1ns	–	2.1	10.4	0.0	1.2	
PACl-2.5ns	Takibain #1500	2.5	23.2	0.0	1.3	

Table 3 – Specifications of the sands used in this study.

Sands	Effective size (mm)	Uniformity coefficient	Zeta potential (mV)	Manufacturer
Virgin silica sand	0.61	1.27	-51.2 ± 6.6	Nihon Genryo Co.
In-use silica sand	0.65	1.24	-31.3 ± 8.7	–

Table 4 – Pearson's correlation coefficient matrix among the logarithmic removal ratios of viruses, turbidity, DOC, and UV260 achieved with coagulation–rapid sand filtration (n = 19).[a]

	AdV	CV	HAV	MNV	MS2	φX174	PMMoV	Turbidity	DOC	UV260
AdV	1.00									
CV	0.79**	1.00								
HAV	0.89**	0.93**	1.00							
MNV	0.86**	0.92**	0.96**	1.00						
MS2	0.60**	0.92**	0.79**	0.77**	1.00					
φX174	0.53*	0.75**	0.71**	0.63**	0.87**	1.00				
PMMoV	0.81**	0.94**	0.91**	0.90**	0.88**	0.76**	1.00			
Turbidity	0.12	-0.06	0.00	0.05	-0.22	-0.41	-0.08	1.00		
DOC	0.15	0.49*	0.42	0.36	0.65**	0.72**	0.47*	-0.66**	1.00	
UV260	0.10	0.48*	0.45	0.38	0.64**	0.70**	0.45	-0.56*	0.94**	1.00

[a] Asterisks indicate a statistically significant correlation (*$P < 0.05$, **$P < 0.01$).

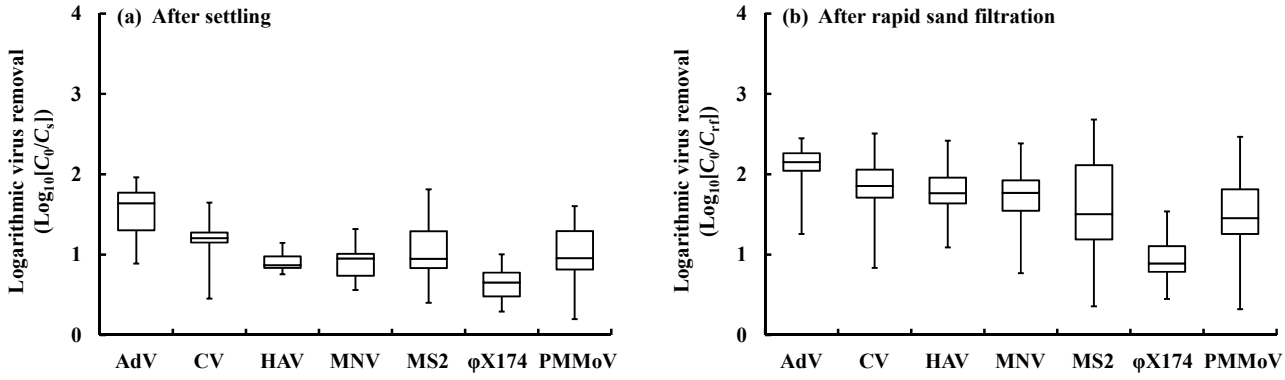


Fig. 1 – Efficacy of coagulation with PACl-1.5s followed by settling (a) or settling and rapid sand filtration with virgin silica sand (b) for the removal of virus particles from source water samples for DWTPs (8 samples collected from various locations in Japan). Virus concentrations were determined via PCR. Horizontal lines within boxes represent median values, the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively, and the upper and lower bars outside the boxes indicate the maximum and minimum values, respectively.

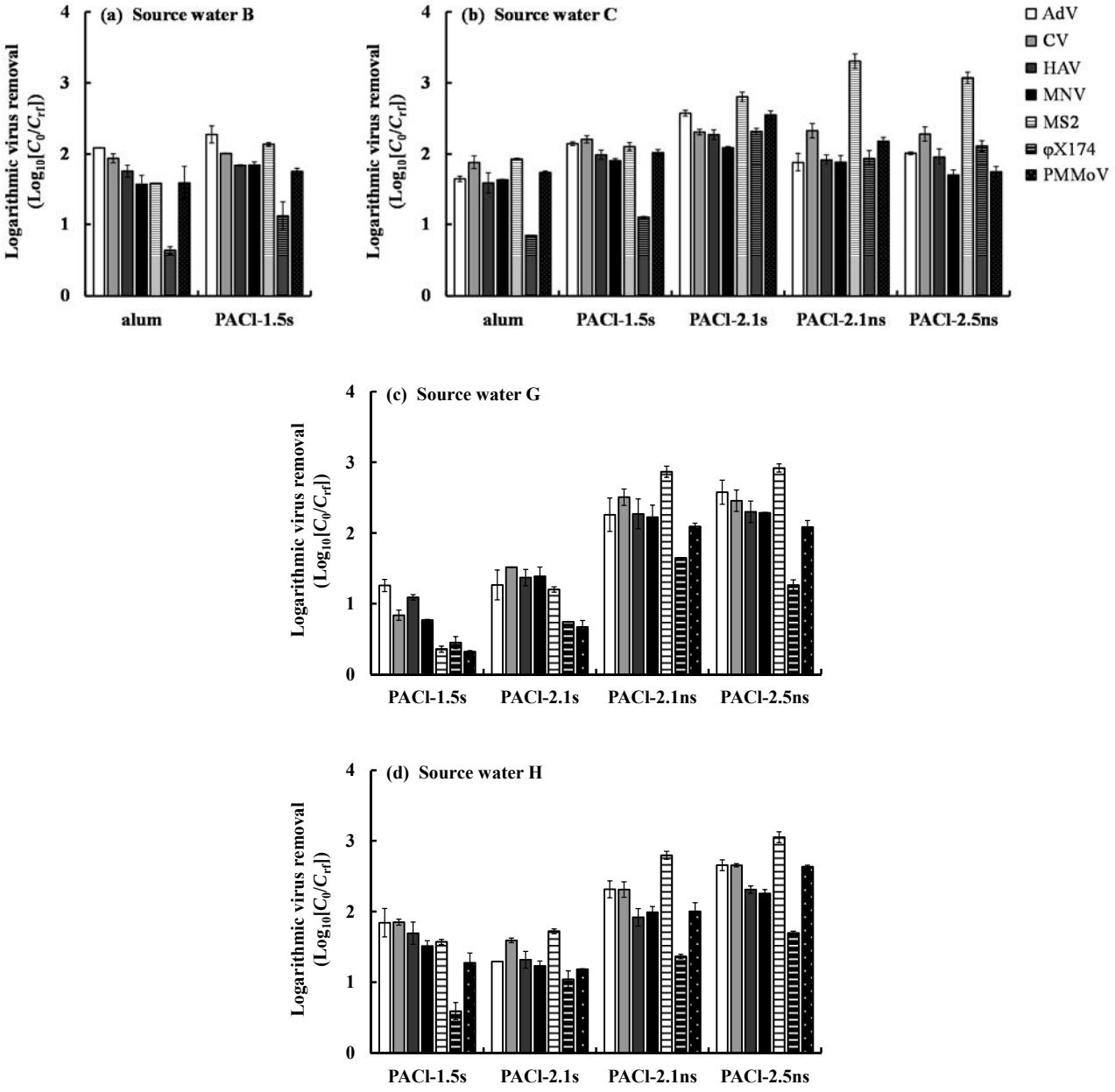


Fig. 2 – Effect of coagulant type on removal of virus particles by coagulation followed by settling and rapid sand filtration with virgin silica sand using source water B (a), source water C (b), source water G (c), or source water H (d), as evaluated by PCR. Values are the means of two or four experiments, and error bars indicate standard deviations.

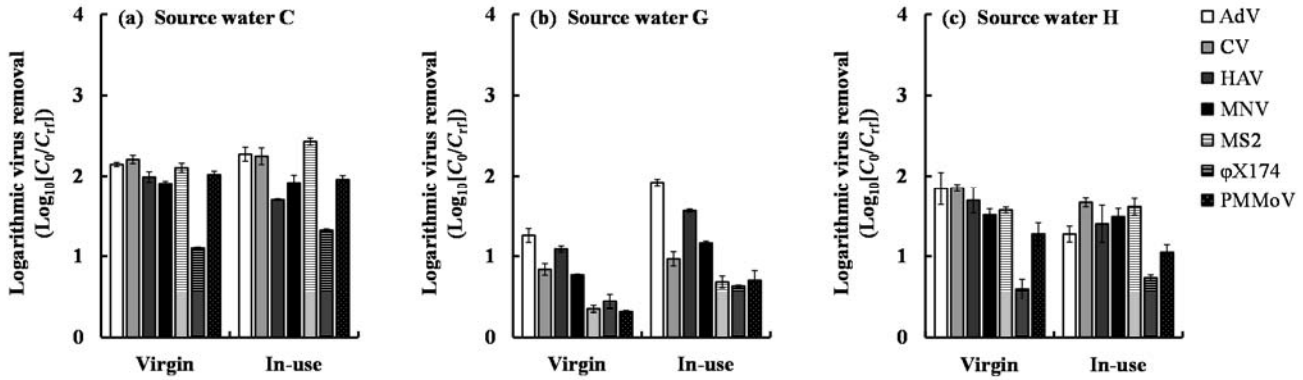


Fig. 3 – Effect of sand type on removal of virus particles by coagulation with PACl-1.5s followed by settling and rapid sand filtration using source water C (a), source water G (b), or source water H (c), as evaluated by PCR. Values are the means of duplicate experiments, and error bars indicate standard deviations. Standard deviations were calculated based on two experimental data.

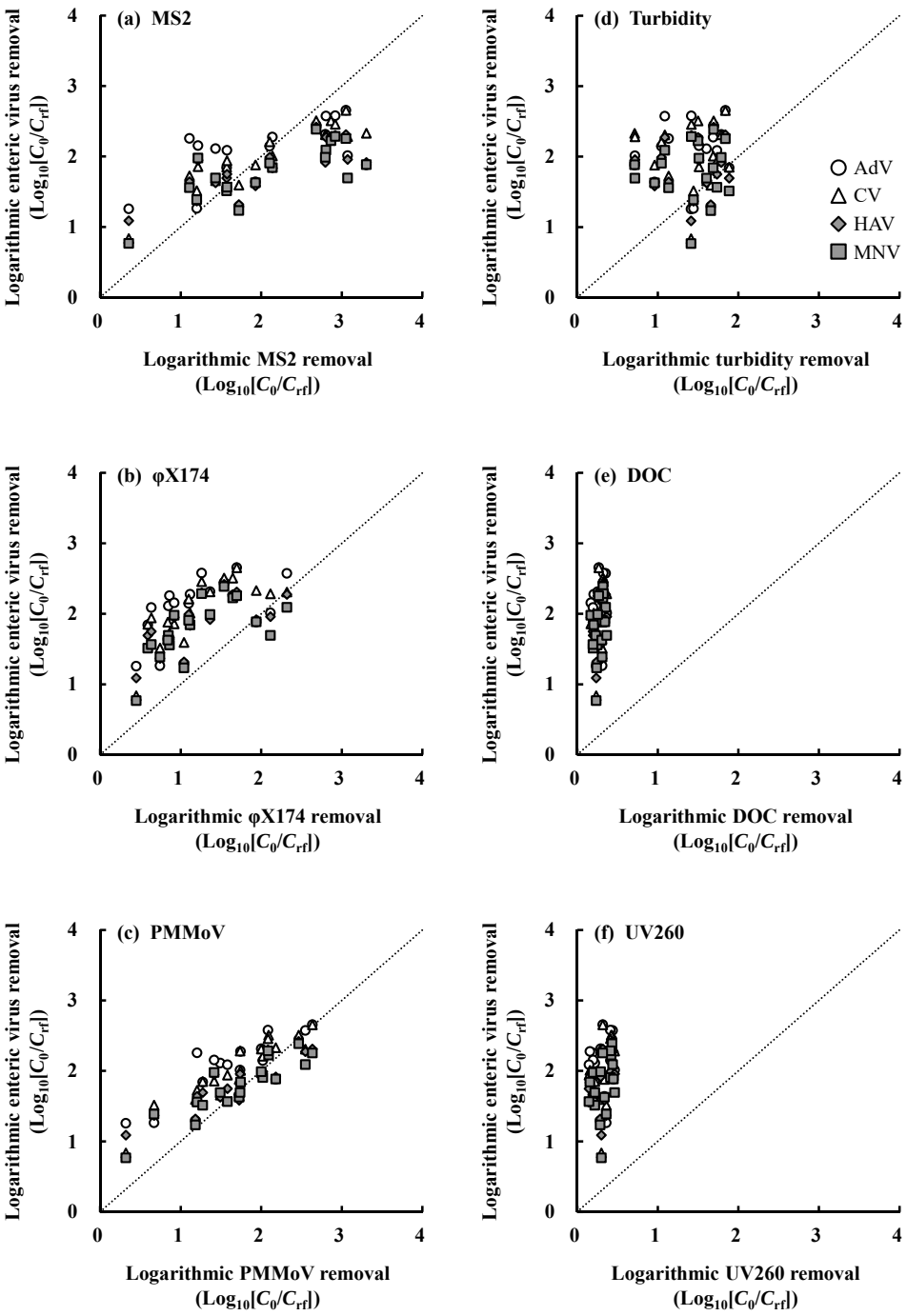


Fig. 4 – Relationships between the removal ratios of enteric viruses and those of bacteriophages MS2 (a), φX174 (b), PMMoV (c), turbidity (d), DOC (e), and UV260 (f) after coagulation followed by settling and rapid sand filtration with virgin silica sand.

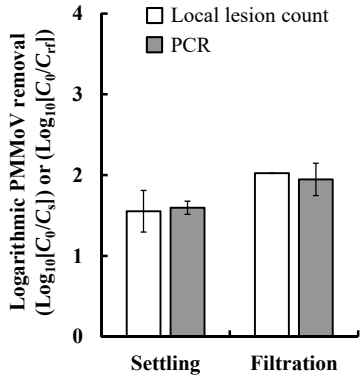


Fig. 5 – Comparison of PMMoV removal ratios evaluated by the local lesion count assay and PCR after coagulation with PACl-1.5s followed by settling or settling and rapid sand filtration with virgin silica sand. Source water, C. Filtration time of rapid sand filtration, 5 min. Values are the means of duplicate experiments, and error bars indicate standard deviations. Standard deviations were calculated based on two experimental data.

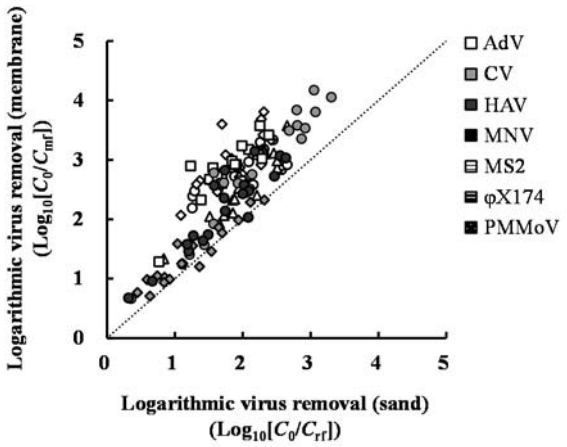


Fig. 6 – Comparison of virus removal ratios evaluated by PCR after coagulation followed by settling and rapid sand filtration with virgin silica sand, and after coagulation followed by settling and filtration through a polytetrafluoroethylene membrane with a nominal pore size of 0.45 μm.