Investigation of enteric adenovirus and poliovirus removal by coagulation processes and suitability of bacteriophages MS2 and φX174 as surrogates for those viruses

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Abstract

We evaluated the removal of enteric adenovirus (AdV) type 40 and poliovirus (PV) type 1 by coagulation, using water samples from 13 water sources for drinking water treatment plants in Japan. The behaviors of two widely accepted enteric virus surrogates, bacteriophages MS2 and φX174, were compared with the behaviors of AdV and PV. Coagulation with polyaluminum chloride (PACl, basicity 1.5) removed AdV and PV from virus-spiked source waters: the infectious AdV and PV removal ratios evaluated by means of a plaque-forming-unit method were 0.1–1.4-$\log_{10}$ and 0.5–2.4-$\log_{10}$, respectively. A nonsulfated high-basicity PACl (basicity 2.1)

removed infectious AdV and PV more efficiently than did other commercially available PACls (basicity 1.5–2.1), alum, and ferric chloride. The MS2 removal ratios tended to be larger than those of AdV and PV, partly because of differences in the hydrophobicities of the virus particles and the sensitivity of the virus to the virucidal activity of PACl; the differences in removal ratios were not due to differences in the surface charges of the virus particles. MS2, which was more hydrophobic than the other viruses, was inactivated during coagulation with PACl. Therefore, MS2 does not appear to be an appropriate surrogate for AdV and PV during coagulation. In contrast, because φX174, like AdV and PV, was not inactivated during coagulation, and because the hydrophobicity of φX174 was similar to or somewhat lower than the hydrophobicities of AdV and PV, the φX174 removal ratios tended to be similar to or somewhat smaller than those of the enteric viruses. Therefore, φX174 is a potential conservative surrogate for AdV and PV during coagulation. In summary, the surface hydrophobicity of virus particles and the sensitivity of the virus to the virucidal activity of the coagulant are probably important determinants of the efficiency of virus removal during coagulation.

Keywords: Bacteriophage, Coagulation, Electrophoretic mobility, Enteric virus, Hydrophobicity, Virus inactivation

1. Introduction

The presence of human enteric viruses in drinking water sources, including river water (Kishida *et al.,* 2012), lake water (Wong *et al.,* 2009), and groundwater (Abbaszadegan *et al.,* 1999), is now well recognized, mainly as a result of the development of the polymerase chain reaction (PCR) method for detecting and quantifying viruses in water. The main sources of viral contamination in drinking water sources are human waste and sewage discharges; large numbers of viruses are excreted in the stools of infected people (up to 10^{11} virus particles per gram of stool; Bosch, 2007). Whether or not the presence of viruses in drinking water sources poses a risk to human health depends on the quality of the water sources and the efficacy of drinking water treatment processes. Because increased urbanization and burgeoning human populations have resulted in increased generation of wastes and increased reuse of virus-contaminated wastewater (Bosch, 2007), understanding of virus reduction during drinking water treatment processes is becoming increasingly important for the prevention and control of waterborne diseases caused by exposure to human enteric viruses through drinking water.

In 2009, the U.S. Environmental Protection Agency published its third contaminant candidate list (CCL3) for drinking water (USEPA, 2009). The CCL3 includes 104 unregulated chemicals or chemical groups and 12 microbiological contaminants that are known or anticipated to occur in drinking water systems and may require regulation in the future. Human adenoviruses (AdVs) and the human enterovirus (EV) group, which includes polioviruses (PVs), are also listed in the CCL3.

Human AdVs (genus *Mastadenovirus,* family Adenoviridae) are non-enveloped, icosahedral viruses with diameters of 80–100 nm and a linear, double-stranded DNA genome (Jiang, 2006; Bosch, 2007). The 51 serotypes of human AdVs are divided into six subgroups (A–F), and subgroup F is composed of the enteric AdVs (types 40 and 41) that are most often associated with gastroenteritis in children (Rigotto *et al.,* 2011). These human enteric AdVs have been detected by PCR-based assays not only in human waste (Allard *et al.,* 1990), sewage (Fong *et al.,* 2010), and river water (Fong *et al.,* 2010; Kishida *et al.,* 2012) but also in treated drinking water (Lee and Kim, 2002). However, the study of the behavior of enteric AdVs during drinking water treatment processes has been hindered by the facts that these viruses are more difficult to culture than other human AdVs, that they have little or no cytopathic effect on most cell lines, and that there is no standard plaque assay for them (Jiang, 2006; Rigotto *et al.,* 2011). Therefore, their behaviors during drinking water treatment are not yet fully understood (Jiang, 2006; Mayer *et al.,* 2008). On the other hand, Cromeans *et al.* (2008) recently developed a plaque assay with human lung carcinoma epithelial cells (A549 cells) for the quantification of infectious enteric AdVs and used the assay to evaluate the efficacy of disinfection processes for these viruses (Cromeans *et al.,* 2010).

Human EVs (genus *Enterovirus,* family Picornaviridae) are icosahedral viruses with diameters of approximately 20–30 nm and a linear, positive-sense, single-stranded RNA genome. This group of viruses includes PVs, coxsackieviruses, echoviruses, and the numbered EVs (Fong and Lipp, 2005). Because PVs were the first viruses to be propagated in tissue culture, they are widely used as

representative human EVs to evaluate virus reduction during drinking water treatment processes, particularly disinfection processes (Sobsey, 1989).

Virus reduction during drinking water treatment involves two main components: (1) reduction in virus numbers by means of physical and physicochemical processes such as coagulation, media filtration, and membrane filtration and (2) reduction in virus infectivity by means of chemical and photochemical inactivation processes such as chlorination, ozonation, and ultraviolet irradiation. Multiple-barrier approaches that provide reliable physical removal along with chemical and photochemical inactivation are required for the effective control of viruses in drinking water (Shannon *et al.*, 2008), and information about the efficacy of drinking water treatment processes to remove and inactivate viruses and about virus concentrations in drinking water sources is required for quantitative microbial risk assessment (Hijnen and Medema, 2010). Data on how the efficacy of physical removal of CCL3 viruses compares to that of chemical and photochemical inactivation are limited (Gerba *et al.,* 2003; Ryu *et al.,* 2010). In particular, the ability of physical and physicochemical processes to remove enteric AdVs has not been reported. One reason for this limitation is that performing bench-scale or pilot-scale studies of human enteric virus removal is difficult owing to the labor-intensive and time-consuming procedures required for virus cultivation and for cell culture–based infectivity assays (Ryu *et al.,* 2010). Therefore, little is known about which processes and process conditions effectively remove CCL3 viruses.

In contrast to human enteric viruses, bacteriophages (i.e., viruses that infect bacteria) can be

cultivated rapidly and quantified easily and are thus often used as surrogates to evaluate the effectiveness of drinking water treatment processes (Mesquita *et al.*, 2010). Among the many types of bacteriophages, the F-specific RNA bacteriophage MS2 (genus *Levivirus*, family Leviviridae) is widely used as a surrogate for human enteric viruses to evaluate the performance of coagulation (Abbaszadegan *et al.*, 2007; Mayer *et al.*, 2008; Matsushita *et al.*, 2011; Kreissel *et al.*, 2014; Shirasaki *et al.*, 2014) and membrane filtration (Arkhangelsky and Gitis, 2008) because of the bacteriophage's morphological similarities to PVs. MS2 is an icosahedral virus approximately 20–30 nm in diameter with a linear, positive-sense, single-stranded RNA genome (Mesquita *et al.*, 2010). In addition to MS2, somatic DNA bacteriophage φX174 (genus *Microvirus*, family Microviridae) is also used as a surrogate for human enteric viruses in a wide variety of water treatment processes such as coagulation (Abbaszadegan *et al.*, 2007; Mayer *et al.*, 2008; Kreissel *et al.*, 2014) and coagulation–mixed media filtration (Abbaszadegan *et al.*, 2007). φX174 consists of a circular, single-stranded DNA genome enclosed in an icosahedral capsid with a diameter of approximately 27 nm (Mesquita *et al.*, 2010). However, the availability of data on whether these bacteriophages are actually adequate surrogates for the physical removal of CCL3 viruses is limited.

Recently, some research groups, including ours, have reported that the bacteriophages MS2 and Qβ lose their infectivity during coagulation with polyaluminum chloride (PACl) (Matsushita *et al.*, 2011; Kreissel *et al.*, 2014): specifically, during coagulation, the infectious virus concentration quantified by the plaque-forming unit (PFU) method differs substantially from the total (infectious

+ inactivated) virus concentration quantified by the real-time PCR method, indicating that some of the bacteriophages are inactivated by PACl. The detection of only infectious viruses by means of cell culture–based infectivity assays including the PFU method is necessary to assess human health risks. Nevertheless, the PCR method, which is quick, highly sensitive, and highly specific, is commonly used to evaluate virus removal efficiency in drinking water treatment plants because cell culture–based infectivity assays are labor-intensive and time-consuming (Bosch, 2007). However, if PACl exhibits virucidal activity for CCL3 viruses, as it does for bacteriophages, determination of removal ratios by PCR probably results in underestimation of the ability of coagulation to remove and inactivate CCL3 viruses because the use of PCR cannot distinguish between infectious and inactivated viruses. Therefore, to determine whether the PCR method is a satisfactory alternative to the PFU method, the PFU and PCR methods must be used in combination to determine whether CCL3 viruses are inactivated by coagulants. To our knowledge, the possibility that CCL3 viruses are inactivated during coagulation has not yet been investigated.

In this study, we conducted batch coagulation experiments with water samples from 13 drinking water sources from various regions of Japan to investigate the removal of CCL3 viruses, specifically enteric AdV and PV, by means of coagulation with PACl, including a high-basicity PACl, alum, and ferric chloride ($FeCl_3$). In addition, we experimentally compared the behaviors of MS2 and φX174 with those of enteric AdV and PV to assess the suitability of these bacteriophages as surrogates for human enteric viruses. By using a combination of PFU and PCR methods, we

quantified infectious viruses and total viruses with the goal of determining the mechanism of virus reduction (i.e., physical removal, inactivation, or both) during coagulation.

2. Materials and methods

2.1. Source water and coagulants

The water samples used in the present study were collected from 13 water sources for drinking water treatment plants in various areas of Japan (water quality data for the sources are shown in Table 1). All of these treatment plants employ coagulation with aluminum-based coagulants (PACl or alum) followed by rapid sand filtration for the production of drinking water. Five of the plants also use ozonation and biological activated-carbon filtration. The source water samples were stored at 4 °C until use and brought to 20 °C immediately prior to use.

For the batch coagulation experiments, we used three commercially available aluminum-based coagulants: PACl-1.5s, PACl-2.1s, and alum. PACl-1.5s (PACl 250A) has a basicity ($[OH^-]/[Al^{3+}]$) of 1.5, contains 10.1% (w/w) Al_2O_3 and 2.9% (w/w) sulfate, and has a relative density of 1.2 at 20 °C; the “1.5” in the name indicates the basicity, and the “s” indicates that the PACl is sulfated. PACl-2.1s (PACl 700A) is a high-basicity PACl (basicity, 2.1) containing 10.2% Al_2O_3 and 2.1% sulfate and having a relative density 1.2 at 20 °C. Alum has a basicity of 0, contains 8.2% Al_2O_3 and 22.7% sulfate, and has a relative density of 1.3 at 20 °C. PACl-1.5s, PACl-2.1s, and alum were obtained from Taki Chemical Co. (Kakogawa, Japan). These three coagulants were compared with

PACl-2.1ns, a nonsulfated (ns), high-basicity (2.1) PACl containing 10.4% Al_2O_3 and having a relative density of 1.2 at 20 °C (Taki Chemical Co.), and with an $FeCl_3$ solution prepared by dissolution of reagent-grade iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, Wako Pure Chemical Industries, Osaka, Japan) in Milli-Q water (Milli-Q Advantage, Millipore Corp., Billerica, MA, USA). Immediately prior to the coagulation experiments, the coagulants were diluted with Milli-Q water.

2.2. Human enteric viruses

Enteric AdV type 40 strain Dugan (ATCC VR-931) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The virus was propagated in human lung carcinoma epithelial cells (A549 cells, ATCC CCL-185) obtained from ATCC and maintained in 1X Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies), 100 units/mL penicillin, and 100 μg/mL streptomycin (Pen Strep, Life Technologies).

PV type 1 Sabin attenuated LSc/2ab strain was kindly provided by Dr. Hiroyuki Shimizu of the National Institute of Infectious Diseases (Tokyo, Japan). The virus was propagated in buffalo green monkey kidney epithelial cells, which were kindly supplied by Dr. Daisuke Sano of Hokkaido University (Sapporo, Japan) and maintained in 1X Eagle's minimum essential medium (with phenol red, Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine

serum, 2 mM L-glutamine (Life Technologies), 100 units/mL penicillin, 100 μg/mL streptomycin, and 1.125 g/L sodium hydrogen carbonate.

The details of the propagation and purification of AdV and PV are described in Supplementary Information.

2.3. Bacteriophages

F-specific RNA bacteriophage MS2 (NBRC 102619) and somatic DNA bacteriophage φX174 (NBRC 103405) were obtained from the NITE 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). The details of the propagation and purification of the bacteriophages are described in Supplementary Information.

2.4. Batch coagulation experiments

Batch coagulation experiments were conducted with 300 mL of virus-spiked source water in square plastic beakers at 20 °C. Purified MS2 and φX174 solutions (see Supplementary Information) were simultaneously added to the beaker at initial concentrations (C_0) of approximately 10^6 PFU/mL and $10^{4–5}$ PFU/mL, respectively; then purified AdV or PV solution (see Supplementary Information) was added to the beaker at an initial concentration (C_0) of approximately 10^2 PFU/mL or 10^3 PFU/mL. Although the initial virus concentrations in the virus-spiked source water samples differed

depending on the virus type, we confirmed that the difference in initial concentration did not affect the infectious MS2 and φX174 removal performance of coagulation with PACl-1.5s, at least in the concentration range of 10^3–10^6 PFU/mL. Because the purified virus solutions were diluted by their addition to the source water, virus addition contributed less than 0.2–0.3 mg/L of unintentional carry-over of dissolved organic carbon. After enough HCl or NaOH was added to the spiked water to bring the final pH to 7 for PACl and alum or 6 for $FeCl_3$, 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 drinking water treatment plant on the day the source water was sampled (Table 1). The water was stirred rapidly for 1 min (G = 200 s^{-1}, 94 rpm) and then slowly for 10 min (G = 20 s^{-1}, 20 rpm) with an impeller stirrer. The water was then allowed to stand for 60 min to settle the generated aluminum or iron floc particles. After the settling, approximately 100 or 120 mL of supernatants were sampled from the beaker for quantification of the AdV, PV, and bacteriophage concentrations (C_s) and turbidity. In addition, a portion of each supernatant (approximately 50 or 80 mL) was filtered through a polytetrafluoroethylene membrane filter (nominal pore size 0.45 μm; Dismic-25HP, Toyo Roshi Kaisha, Tokyo, Japan) for quantification of the AdV, PV, and bacteriophage concentrations (C_f) and the residual aluminum concentration. The turbidity was quantified with a 2100Q Portable Turbidimeter (Hach Company, Loveland, CO, USA). After the addition of 1% (v/v) nitric acid (ultrapure, Kanto Chemical Co., Inc., Tokyo, Japan) to the membrane permeate, aluminum concentration was determined by inductively coupled plasma–mass

spectrometry (Agilent 7700 series, Agilent Technologies, Inc., Santa Clara, CA, USA).

2.5. AdV and PV assay

Infectious AdV was quantified by a plaque assay recently developed by Cromeans *et al.* (2008) with some modifications. Infectious PV was quantified by means of a plaque assay described by Sano *et al.* (2001), with slight modifications. The details of the plaque assays are described in Supplementary Information.

Real-time PCR, which detects all viruses regardless of their infectivity or the existence of aggregates, was used to quantify DNA and RNA. Specifically, AdV viral DNA was quantified by real-time PCR, and PV viral RNA was quantified by real-time reverse-transcription PCR (real-time RT-PCR). The details of the real-time PCR and real-time RT-PCR methods are described in Supplementary Information including Table S1.

2.6. Bacteriophage assay

Infectious bacteriophages were quantified by determination of the number of PFUs according to the double-layer method (Adams, 1959) with *E. coli* (NBRC 13965 for MS2, NBRC 13898 for φX174) as a bacterial host. The average of the plaque counts of triplicate plates prepared from one sample was considered as the infectious bacteriophage concentration for that sample.

MS2 viral RNA was quantified by real-time RT-PCR, and φX174 viral DNA was quantified by

real-time PCR. The details of the real-time PCR and real-time RT-PCR methods are described in Supplementary Information including Table S1.

2.7. Electrophoretic mobility and hydrophobicity

The electrophoretic mobilities of AdV, PV, MS2, and φX174 were measured in prepared Milli-Q water and in filtered source water samples. The hydrophobicities of AdV, PV, MS2, and φX174 were estimated by the bacterial adherence to hydrocarbon method (Rosenberg *et al.,* 1980), with some modifications. The details of the electrophoretic mobility and hydrophobicity measurements are described in Supplementary Information including Figures S1 and S2.

3. Results and discussion

3.1. Virus removal by coagulation with PACl-1.5s

Figure 1 shows the removal ratios ($\log_{10}[C_0/C_s]$) of infectious AdV and PV, as evaluated by the PFU method, after coagulation with PACl-1.5s and subsequent settling. Because AdV and PV particles are small and the particle monodispersion is stabilized by electrical repulsion in the source water samples used in the present study (see section 3.3), no removal ($\leq$0.1-$\log_{10}$) of AdV or PV was observed in the absence of a coagulant at pH 6 or 7. In contrast, coagulation with PACl-1.5s under conditions to optimize turbidity removal (76–>99%) and minimize residual aluminum concentration ($\leq$0.05 mg-Al/L) removed AdV and PV from the virus-spiked source waters. The addition of the

coagulant destabilized the virus particle dispersion in the source waters, the particles were entrapped in or adsorbed onto the aluminum floc particles generated during coagulation, and then floc particles with the entrapped or adsorbed viruses settled from suspension under the influence of gravity during the settling process. The removal ratios of infectious AdV and PV were 0.1–1.4-$\log_{10}$ and 0.5–2.4-$\log_{10}$, respectively, and the ratios depended strongly on source water quality: for example, coagulation resulted in 0.1-$\log_{10}$ and 0.7-$\log_{10}$ removals of infectious AdV and PV from source water K but 1.4-$\log_{10}$ and 2.4-$\log_{10}$ removals from source water M.

Other researchers have also reported that aluminum-based coagulants are useful for removing human enteric viruses and that raw water quality affects virus removal. For example, Nasser *et al.* (1995) reported that the presence of humic acid in raw water negatively affects virus removal. Although the turbidity and alkalinity of raw water are well recognized as parameters that affect coagulation efficiency, Rao *et al.* (1988) found that these two parameters had no influence on virus removal. The source water samples used in the present study differed substantially with respect to their water quality parameters (which are listed in Table 1). The samples also differed with respect to the coagulant dosage used at their drinking water treatment plants (Table 1). Coagulant dosage is an important determinant of coagulation efficiency, and the optimum coagulant dosage for removing turbidity and minimizing residual aluminum concentration depends strongly on the source water quality. Additional research is necessary to elucidate the water quality parameters and process factors that have the largest impact on the efficiency of virus removal during coagulation processes.

Figure 2 shows the removal ratios ($\log_{10}[C_0/C_f]$) of infectious AdV and PV after coagulation with PACl-1.5s, settling, and subsequent membrane filtration. Because the diameters of AdV and PV particles (approximately 80–100 and 20–30 nm, respectively) are smaller than the pore size of the membrane filter (0.45 μm), no removal (<0.2-$\log_{10}$) of AdV or PV was observed in the absence of a coagulant. Compared to coagulation and settling alone (Figure 1), the combination of coagulation, settling, and membrane filtration improved AdV and PV removal ratios by approximately 2–3-$\log_{10}$: 1.9–>3.7-$\log_{10}$ and 2.4–>3.9-$\log_{10}$ removals were achieved for infectious AdV and PV, respectively. These results indicate that viruses entrapped in or adsorbed onto the aluminum floc particles that could not be excluded from suspension by gravity during settling were effectively removed by the subsequent membrane filtration.

Hijnen and Medema (2010) reviewed seven reports (published from 1975 to 2003) describing the removal of viruses, including human enteric viruses and bacteriophages, by coagulation–rapid granular filtration and estimated that the microorganism elimination credit of this process was 3.0 ± 1.4-$\log_{10}$ (range, 1.2-$\log_{10}$ to 5.3-$\log_{10}$) for viruses. Although the virus removal mechanism of media filtration differs from that of membrane filtration, the removal performances we observed for AdV and PV in the present study were similar to those reported by Hijnen and Medema.

3.2. Effects of coagulant dosage and coagulant type on virus removal

As described above, the AdV and PV removal ratios for coagulation with PACl-1.5s depended on

source water quality, and the removal ratios obtained with source water K were markedly lower than those with source water M. With the goal of improving the removal of AdV and PV, we investigated the effects of coagulant dosage and coagulant type on virus removal from source water samples B, D, and K because the water qualities of these samples were quite different from one another (Table 1), and the AdV and PV removal ratios obtained with PACl-1.5s for these samples were lower than the ratios for the other samples used in the present study (Figure 1). In addition, because source water C is treated with alum in its corresponding drinking water treatment plant (Table 1), the virus removal performances of coagulation with PACl-1.5s and with alum were compared for source water C. Infectious AdV and PV removal ratios ($\log_{10}[C_0/C_s]$) obtained by means of coagulation and settling were somewhat improved by a 2-fold increase in the dosage of PACl-1.5s (Figure 3). In addition, the virus removal ratios also depended on coagulant type: infectious AdV and PV removal ratios obtained with alum and $FeCl_3$ were almost the same as or somewhat smaller than those obtained with PACl-1.5s, whereas the ratios obtained with high-basicity PACls, particularly PACl-2.1ns, were larger than those obtained with the other coagulants used in the present study, including PACl-1.5s at a 2-fold increased dosage. We previously reported that PACl-2.1ns has higher colloid charge density than PACl-1.5s, PACl-2.1s, and alum, probably due to the high colloidal aluminum content and the absence of sulfate in PACl-2.1ns (Shirasaki *et al.,* 2014). Because high colloid charge density gives a coagulant a high capacity to neutralize negatively charged viruses during coagulation, PACl-2.1ns removed AdV and

PV more effectively than did the other coagulants.

When the process involving coagulation, settling, and filtration was used, the infectious AdV and PV removal ratios ($\log_{10}[C_0/C_f]$) were markedly increased relative to the ratios obtained with the process involving only coagulation and settling; and at least 3-$\log_{10}$ removals were achieved with all the coagulants except PACl-1.5s (Figure 4). In particular, coagulation with $FeCl_3$ resulted in more than 4-$\log_{10}$ removals of PV from all the source water samples.

3.3. Relationships between enteric virus removal and bacteriophage removal

To investigate whether the bacteriophages MS2 and φX174 were adequate surrogates for AdV and PV during coagulation, we determined the relationships between enteric virus and bacteriophage removal ratios (Figures 5 and 6). Values of the virus removal ratios that were below the detection limit were excluded from the analysis. In addition, the data obtained from experiments involving coagulation with the high-basicity PACl were also excluded because the number of samples was limited. Analysis of covariance was used to compare the differences in the relationships between enteric virus and bacteriophage removal ratios for the various coagulant types and separation processes. Excel Toukei 2012 software (Social Survey Research Information Co., Tokyo, Japan) was used to conduct the statistical analyses. Because the removal ratio data obtained by the PFU method did not satisfy several key assumptions that underlie the use of analysis of covariance, we used total virus removal ratio data determined by the PCR method (Table S2). The relationships

between total AdV removal ratio and total MS2 removal ratio did not differ significantly among the three coagulants, whether or not coagulation and settling were followed by filtration (analysis of covariance, $P > 0.63$); whereas the relationships were significantly different between after settling and after filtration regardless of the coagulant type ($P < 0.02$) (Table S2). Similar results were observed for the other comparisons shown in Table S2, except in the following two cases: comparison between alum coagulation with settling and alum coagulation with settling and filtration ($P = 0.06$), and comparison between $FeCl_3$ coagulation with settling and $FeCl_3$ coagulation with settling and filtration ($P = 0.12$). These results indicate that the relationship between enteric virus particle removal ratios and bacteriophage particle removal ratios was the same regardless of coagulant type used, whereas the relationship differed between the separation processes. Accordingly, for each separation process, the data sets for the three coagulants were combined for regression analysis.

Total MS2 removal ratios evaluated by the PCR method tended to be larger than total AdV removal ratios obtained by coagulation combined with settling and filtration (Figure 6a), and a similar trend was observed for the relationship between total PV and total MS2 removal ratios (Figure 6b). These results indicate that the behaviors of MS2 and enteric virus particles differed during coagulation and that MS2 does not appear to be an appropriate surrogate for AdV and PV, because the AdV and PV removal ratios would be overestimated if MS2 was used as a surrogate. In addition, there was no correlation or only a weak correlation between the total MS2 and total AdV

or PV removal ratios obtained by coagulation (Figure 6a,b). These relationships also support our contention that MS2 may not be an appropriate surrogate for AdV and PV.

In contrast, total φX174 removal ratios tended to be similar to or somewhat smaller than total AdV removal ratios obtained by coagulation combined with settling and filtration (Figure 6c), and a similar trend was observed for the relationship between total PV and total φX174 removal ratios (Figure 6d). These results indicate that φX174 has the potential to be a conservative surrogate for AdV and PV during coagulation. However, there was no correlation or only weak correlation between the total φX174 and total AdV or PV removal ratios obtained by coagulation (Figure 6c,d). Because the total φX174 removal ratios observed in the present study were in a limited range (approximately 0–2-$\log_{10}$) compared with those of other viruses, data limitations may have been a reason for the poor correlation. Further investigation involving additional source water samples and coagulants is necessary to determine whether φX174 can actually be used as a conservative surrogate for AdV and PV during coagulation.

The surface charge on virus particles is often invoked to explain virus removal by physicochemical water treatment processes, including coagulation (Matsushita *et al.*, 2004). To investigate why the MS2 removal ratios were larger than those of AdV and PV whereas the φX174 removal ratios were similar to or smaller than those of the enteric viruses, we compared the electrophoretic mobilities of AdV, PV, MS2, and φX174 in prepared Milli-Q water at various pH values (Figure S3) and in the filtered source water samples used in the present study at a

coagulation pH of 6 or 7 (Figure 7). In prepared Milli-Q water, the electrophoretic mobilities of the virus particles were positive at pH values less than 4 for AdV and PV and less than 2 for MS2 and φX174, whereas the electrophoretic mobilities were negative at pH values larger than 5 for AdV and PV and larger than 3 for MS2 and φX174 (Figure S3). The pH values at which the electrophoretic mobilities of AdV, PV, MS2, and φX174 were equal to zero (i.e., the isoelectric points) were 3.7, 4.1, 2.2, and 1.6, respectively. In a review of virus isoelectric point measurements, Michen and Graule (2010) reported that the isoelectric points of AdV type 5, MS2, and φX174 determined by means of laser scattering (the method used in the present study) were 4.5, 2.2–3.9, and 2.6, which are roughly in agreement with our results. In contrast, although there are no available isoelectric point data for PV measured by means of laser scattering, the isoelectric point of PV type 1 has been measured by isoelectric focusing in dense aqueous solutions and has been reported to range from 3.8 to 4.5 and from 6.8 to 8.3 (Michen and Graule, 2010); the former range is roughly in agreement with our result. Because the isoelectric points of AdV, PV, MS2, and φX174 observed in the present study were less than 4.1, these viruses are negatively charged at around neutral pH. In fact, AdV, PV, MS2, and φX174 showed negative electrophoretic mobilities in the filtered source water samples at pH 6 and 7 (Figure 7). However, the magnitude of the electrophoretic mobility depended on the virus type: under both pH conditions, φX174 tended to be the most negatively charged, followed by MS2, AdV, and PV in that order. The order of the electrophoretic mobilities of the viruses was not in accordance with the order of the removal ratios by coagulation (MS2 > PV > AdV > φX174).

Therefore, the difference in the AdV, PV, MS2, and φX174 removal ratios during coagulation did not depend solely on the difference in electrophoretic mobility between the viruses in the source water samples used in the present study. Dika *et al.* (2015) reported that in prepared de-ionized water at pH 7, MS2 is more negatively charged than φX174, in agreement with our observation in prepared Milli-Q water at the same pH (Figure S3). In contrast, φX174 tended to be more negatively charged than MS2 in the filtered source water samples, as described above, probably owing to the difference in the solvent water quality. In fact, Bellou *et al.* (2015) reported that the surface charges of MS2 and φX174 are almost the same at pH 7 when 2mM phosphate-buffered saline is used as the solvent.

We also estimated the hydrophobicities of AdV, PV, MS2, and φX174 by using the bacterial adherence to hydrocarbon method (Figure 8). AdV and φX174 remained completely in the water phase after mixing with all the tested solvents, whereas PV and MS2 were transferred to the solvent phase when *n*-octane, *p*-xylene, or toluene was used as the solvent. In addition, the percentage of MS2 remaining in the water phase after mixing with toluene was lower than that of PV. These results indicate that the viruses have different surface hydrophobicities, with MS2 being the most hydrophobic, followed by PV, AdV, and φX174 in that order. Dika *et al.* (2015) reported that MS2 is more hydrophobic than φX174, which agrees with our results. Because mechanisms that destabilize viruses and NOM during the coagulation process, that is, coordination reactions between coagulant species and carboxyl groups of the virus surface proteins or NOM, are similar for the two species

(Bratby, 2006), and because the hydrophobic fractions of NOM are removed from water more efficiently than are the hydrophilic fractions (Matilainen *et al.*, 2010), virus particles having a more hydrophobic surface can be expected to be removed during coagulation to a greater extent than viruses having a less hydrophobic surface. In fact, the order of the hydrophobicities of the viruses was in accordance with their removal ratios by coagulation (MS2 > PV > AdV > φX174). Therefore, the differences in hydrophobicity between AdV, PV, MS2, and φX174 contributed to differences in their removal ratios during coagulation.

3.4. Effect of virucidal activity of coagulant on virus removal

As described above, the behaviors of the MS2 and enteric virus particles during coagulation differed partly because of the difference in hydrophobicity between the viruses, and the removal ratios of total MS2, which has a more hydrophobic surface than the enteric viruses, tended to be larger than total enteric virus removal ratios (Figure 6a,b). In addition, the removal ratios of infectious MS2 also tended to be larger than those of infectious AdV and PV (Figure 5a,b), whereas the removal ratios of infectious φX174 were similar to or somewhat smaller than those of infectious AdV and PV (Figure 5c,d). To investigate why the removal ratios of infectious MS2, particularly those obtained by coagulation with PACl, were larger than those of infectious enteric viruses, we compared the removal ratios of infectious viruses evaluated by the PFU method and the total virus removal ratios evaluated by the PCR method for each virus (Figure 9). The removal ratios

determined by the two methods did not differ significantly for AdV and PV, no matter what type of coagulant was used (two-tailed *t*-test, $P > 0.05$; Figures 9a,b). In contrast, although no significant difference between the MS2 removal ratios determined by the two methods was observed when alum or $FeCl_3$ was used as a coagulant ($P > 0.43$), the removal ratios differed significantly when PACl was used as the coagulant ($P = 0.00$): the removal ratios of infectious MS2 were larger than those of total MS2 (Figure 9c). This difference between the two methods could be explained by the formation of aggregates consisting of several infectious MS2 particles, by inactivation of MS2 during coagulation, or by both. Some research groups, including ours, have reported that the bacteriophages MS2 and Qβ lose their infectivity after contact with PACl, whereas alum and $FeCl_3$ have no significant effect on bacteriophage infectivity (Matsushita *et al.*, 2011; Kreissel *et al.*, 2014). These results suggest that the virucidal activity of PACl contributes to the removal of infectious MS2 during coagulation, and our observation that neither alum nor $FeCl_3$ showed virucidal activity during coagulation agrees with the previous results, because the MS2 removal ratios determined by the two methods did not differ significantly, as described above.

In contrast, no significant difference between the two methods was observed for AdV or PV, even during coagulation with PACl (Figure 9a,b); in other words, these viruses were not inactivated during coagulation. In addition, Kreissel *et al.* (2014) reported that φX174 does not lose its infectivity after contact with PACl; their results agree with ours because the removal ratios of infectious φX174 were not larger than those of total φX174 no matter what type of coagulant was

used (Figure 9d). These results indicate that φX174 was also not inactivated during coagulation. Accordingly, the fact that the infectious MS2 removal ratio obtained by means of PACl coagulation was higher than the infectious PV and AdV removal ratios was due in part to the inactivation of MS2 by PACl during coagulation.

Although the mechanism of virus inactivation during coagulation with PACl remains unclear, a possible explanation for MS2 inactivation could be that its adsorption onto the host bacteria is blocked by polymeric aluminum species such as Al_{13} species $[AlO_4Al_{12}(OH)_{24}(H_2O)_{12}]^{7+}$ that are present in PACl or are formed during coagulation; monomeric aluminum species, which are the major aluminum species in alum, have been shown not to account for MS2 inactivation (Matsushita *et al.,* 2011; Kreissel *et al.,* 2014). F-specific RNA bacteriophages MS2 and Qβ, which are sensitive to the virucidal activity of PACl, have a capsid made up of 180 copies of a coat protein and one copy of a maturation protein (protein A), and the maturation protein has been shown to be responsible for the adsorption of these bacteriophages to the F-pili of the host bacteria at the initial stage of virus infection (Fauquet *et al.,* 2005). In contrast, the surface of somatic DNA bacteriophage φX174, which is not sensitive to the virucidal activity of PACl, has 12 spikes made up of a major spike protein (protein G) and a minor spike protein (protein H), both of which are thought to be involved in recognition by and binding to the receptor on the host bacteria (Olson *et al.,* 1992). AdV also has 12 vertex capsomers, which consist of a penton protein and a fiber capsid protein, and these capsomers are responsible for binding of AdV to the protein coxsackie and

adenovirus receptor on the surface of the host cell (Bosshard *et al.*, 2013). PV consists of 60 copies of surface proteins VP1, VP2, and VP3, along with the internal protein VP4; and the five-fold axis formed by five VP1s (a total of 12 sites) on the virion surface has been proposed to attach to the PV receptor on the host cell surface (Arita *et al.*, 1998). The fact that only a single protein on the MS2 surface is responsible for adsorption of the virus to the host bacteria—as opposed to 12 binding sites in the cases of AdV, PV, and φX174—might be related to the different sensitivities of MS2 and other viruses to the virucidal activity of PACl.

3.5. Relationship between AdV removal and PV removal

As described above, the differences between the removal ratios of the enteric viruses and the bacteriophages were partly due to differences in hydrophobicity and sensitivity to the virucidal activity of PACl. On the other hand, although the electrophoretic mobilities of AdV and PV were almost the same in the source water samples at pH 6 and 7 (Figure 7) and these viruses were not inactivated during coagulation, the surface of PV was more hydrophobic than that of AdV (Figure 8). These results indicate that the removal ratios of PV can be expected to be somewhat larger than those of AdV during coagulation. In fact, infectious PV was removed to a slightly greater extent than infectious AdV during coagulation (Figure 10). A similar trend was observed for the relationship between the total AdV and PV removal ratios determined by the PCR method (Figure S4). Accordingly, both the surface hydrophobicity of the virus particles and the sensitivity of the

virus to the virucidal activity of coagulant, as well as the surface charge of the virus particles, are probably important determinants of the efficiency of virus removal during coagulation.

We found that the removal ratios determined by the PFU and PCR methods showed an approximately 1:1 correlation for AdV and PV (Figure 9a,b). Therefore, the PCR method is probably a satisfactory alternative to the PFU method for evaluating removal of these viruses during coagulation.

4. Conclusions

(1) The infectious enteric AdV and PV removal ratios achieved by coagulation with PACl-1.5s ranged from 0.1 to 1.4-$\log_{10}$ and 0.5 to 2.4-$\log_{10}$, respectively, and depended strongly on source water quality.

(2) A nonsulfated high-basicity PACl (PACl-2.1ns) removed infectious AdV and PV more efficiently than did the other coagulants (PACl-1.5s, alum, and $FeCl_3$).

(3) Because the bacteriophage MS2 removal ratios tended to be larger than those of AdV and PV, owing in part to the high hydrophobicity of the MS2 virus particles and the high sensitivity of MS2 to the virucidal activity to PACl, this bacteriophage does not appear to be an appropriate surrogate for AdV and PV during coagulation.

(4) In contrast, the φX174 removal ratios tended to be similar to or somewhat smaller than those of AdV and PV because φX174, like AdV and PV, was not inactivated during coagulation, and the

hydrophobicity of φX174 was similar to or somewhat lower than the hydrophobicities of the enteric viruses. Accordingly, φX174 may have the potential to be a conservative surrogate for AdV and PV during coagulation.

(5) The surface hydrophobicity of virus particles and the sensitivity of a virus to the virucidal activity of the coagulant are probably important determinants of the efficiency of virus removal during coagulation.

Acknowledgements

We thank Dr. Hiroyuki Shimizu (National Institute of Infectious Diseases) for providing Sabin attenuated LSc/2ab strain of PV type 1. We also thank Dr. Daisuke Sano and Ms. Rie Nomachi (Hokkaido University) for providing buffalo green monkey kidney epithelial cells and teaching us the methods of cultivating and quantifying infectious PV. We thank the staff of the drinking water treatment plants for providing source water samples. This research was supported in part by a Grant-in-Aid for Young Scientists A (no. 25709044, 2013), a Grant-in-Aid for Scientific Research S (no. 24226012, 2012), and a Grant-in-Aid for Scientific Research B (no. 15H04064, 2015) from the Japan Society for the Promotion of Science; by a Health and Labour Sciences Research Grant (Research on Health Security Control) from the Ministry of Health, Labor and Welfare of Japan; and by a grant from the Kurita Water and Environment Foundation (no. 14A007, 2014).

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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	15-Jan-14	7.6	1.0	0.4	0.009	49.8	PACl	40
B	14-Jan-14	7.9	1.2	0.4	0.011	55.7	PACl	40
C	15-Aug-13	7.3	2.9	1.4	0.028	32.5	alum	40
D	15-Aug-13	7.2	1.3	0.8	0.025	14.3	PACl	40
E	07-Aug-13	7.1	13.7	0.8	0.028	21.9	PACl	50
F	14-Aug-13	7.4	6.2	1.5	0.034	39.5	PACl	50
G	19-Aug-13	8.0	1.0	0.5	0.010	47.1	PACl	50
H	20-Aug-13	6.8	2.0	0.7	0.016	37.7	PACl	60
I	14-Aug-13	7.4	1.1	1.2	0.032	46.2	PACl	70
J	06-Aug-13	6.3	17.5	1.2	0.032	40.6	PACl	80
K	23-Jul-14	8.1	4.8	2.7	0.074	103.0	PACl	110
L	06-Aug-13	7.4	9.7	1.4	0.036	49.7	PACl	120
M	07-Aug-13	7.2	27.1	3.9	0.082	73.5	PACl	200

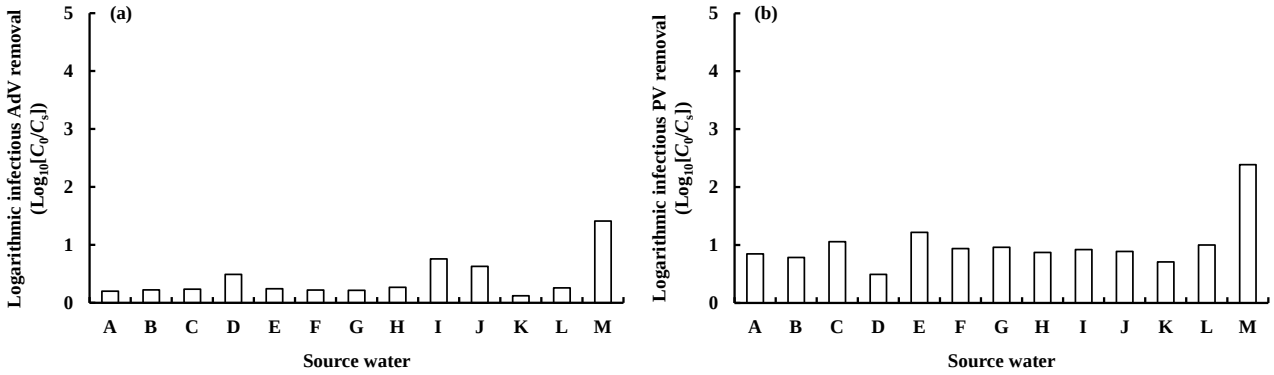

Figure 1. Effects of source water quality on removal of infectious AdV (a) and PV (b) by coagulation with PACl-1.5s and settling, as evaluated by the PFU method.

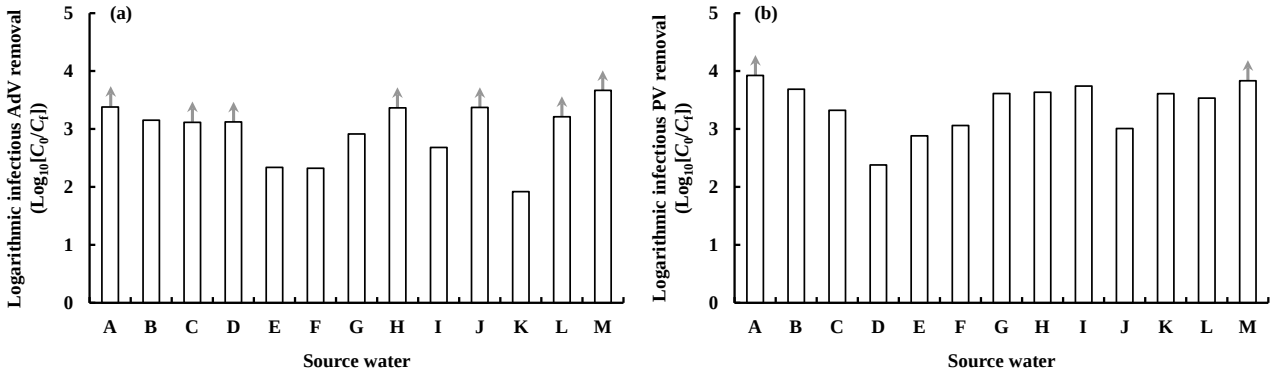

Figure 2. Effects of source water quality on removal of infectious AdV (a) and PV (b) by coagulation with PACl-1.5s, settling, and filtration, as evaluated by the PFU method. Arrows indicate that the virus concentrations were below the quantification limit (1/6 PFU/mL).

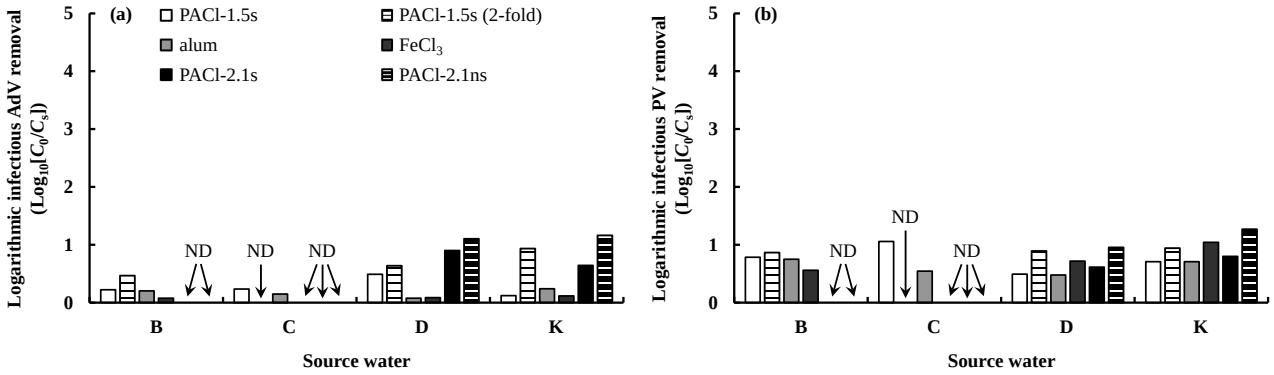

Figure 3. Effects of coagulant dosage and coagulant type on removal of infectious AdV (a) and PV (b) by coagulation and settling, as evaluated by the PFU method. ND, not determined.

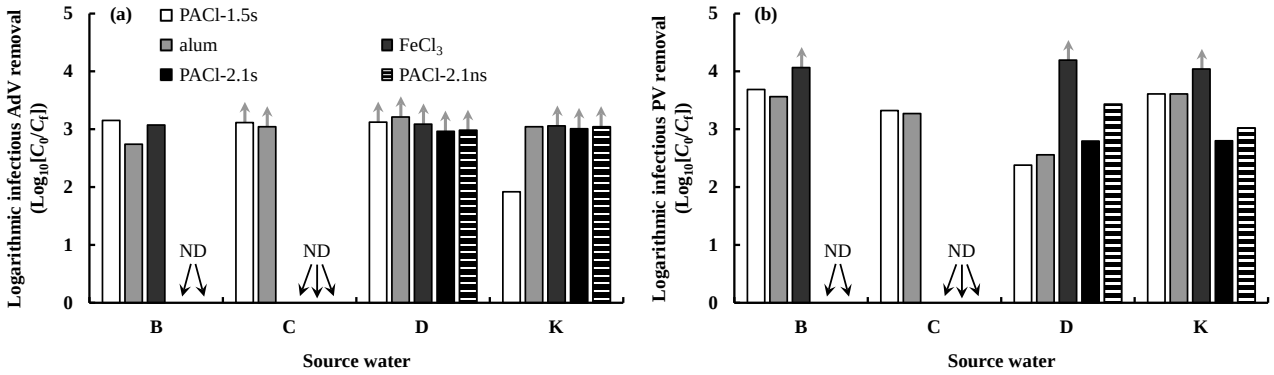

Figure 4. Effects of coagulant dosage and coagulant type on removal of infectious AdV (a) and PV (b) by coagulation, settling, and filtration, as evaluated by the PFU method. Arrows indicate that the virus concentrations were below the quantification limit (1/6 PFU/mL). ND, not determined.

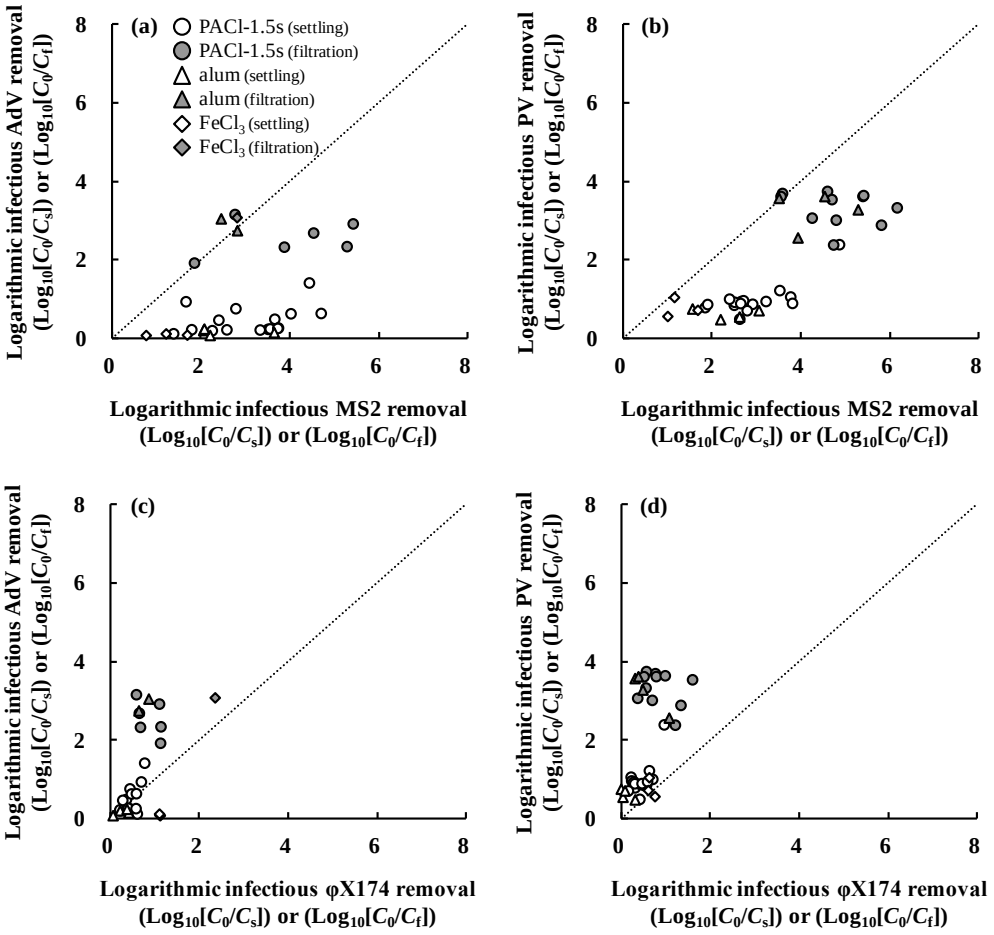

Figure 5. Relationships between infectious AdV and MS2 removal ratios (a), infectious PV and MS2 removal ratios (b), infectious AdV and φX174 removal ratios (c), and infectious PV and φX174 removal ratios (d) during coagulation.

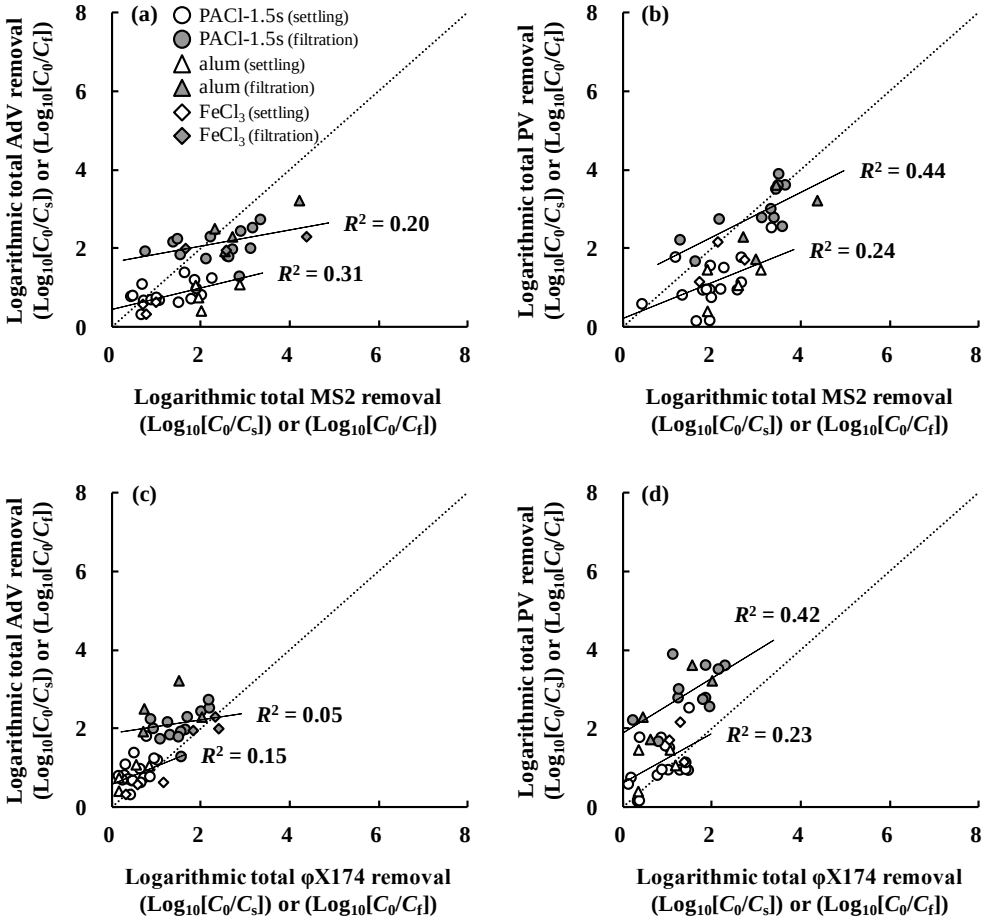

Figure 6. Relationships between total AdV and MS2 removal ratios (a), total PV and MS2 removal ratios (b), total AdV and φX174 removal ratios (c), and total PV and φX174 removal ratios (d) during coagulation.

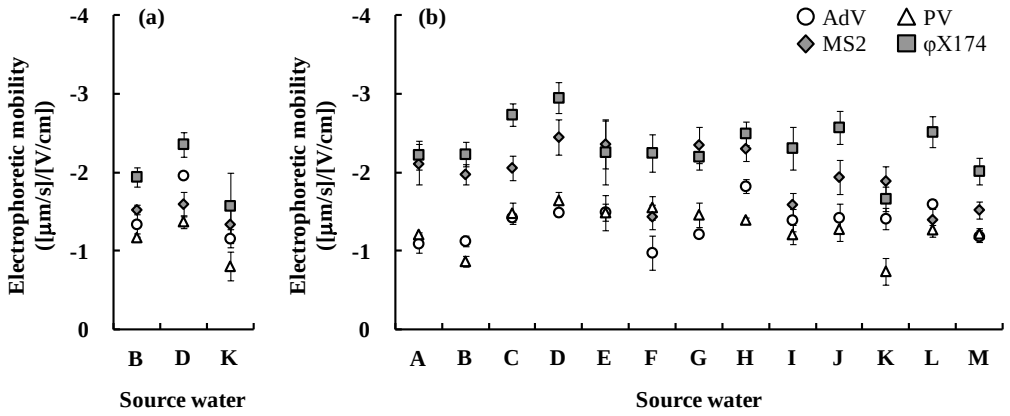

Figure 7. Electrophoretic mobilities of AdV, PV, MS2, and φX174 in filtered source water samples at pH 6 (a) and 7 (b). Values are means, and error bars indicate standard deviations (*n* = 9).

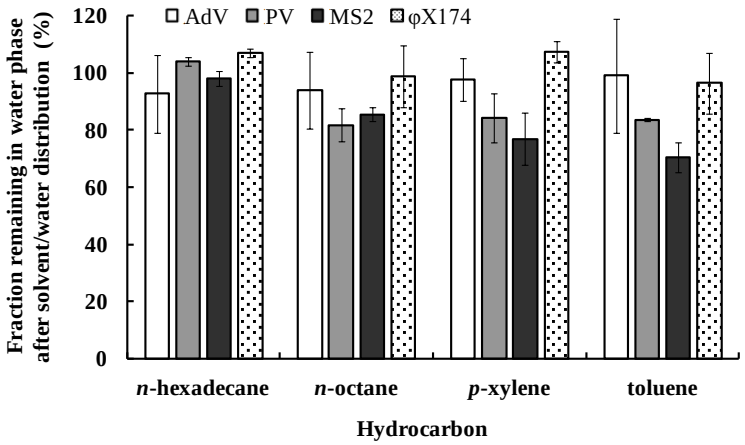

Figure 8. Hydrophobicities of AdV, PV, MS2, and φX174. Values are means, and error bars indicate standard deviations (n = 2 or 3).

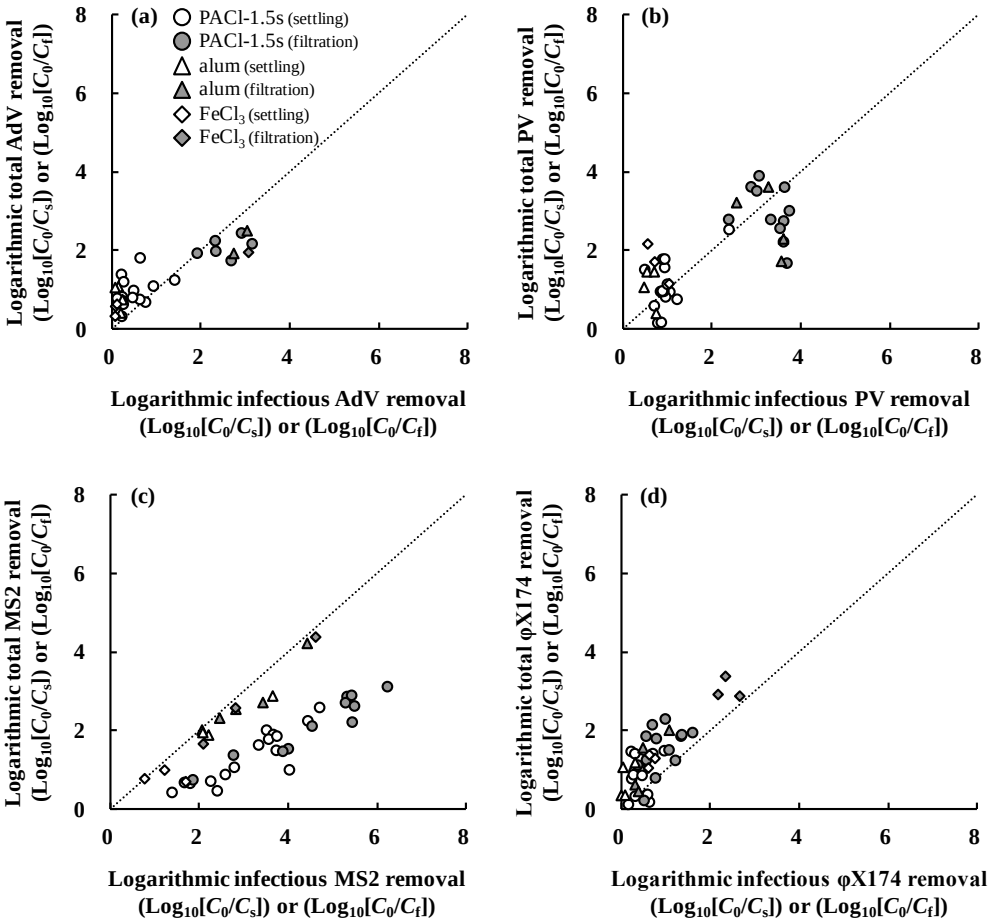

Figure 9. Comparison of infectious virus removal ratios evaluated by the PFU method and total virus removal ratios evaluated by the PCR method for AdV (a), PV (b), MS2 (c), and φX174 (d) during coagulation.

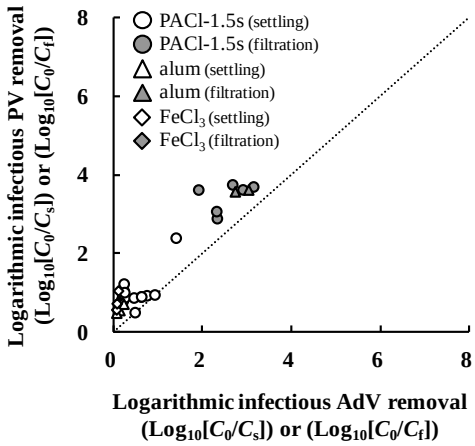

Figure 10. Relationship between infectious PV and AdV removal ratios during coagulation.