measured by the PFU method has been observed during the coagulation process (Shirasaki et al., 2009), indicating that some of the bacteriophages were probably inactivated by PACl. Because rNV-VLPs lack RNA, which is necessary for infection and replication in host cells, we cannot discuss the fate of infectivity of NV in the treatment process. If PACl exhibits virucidal activity for native NV as well as for bacteriophages, >4-$\log_{10}$ removal (including inactivation) of native NV might be achieved during coagulation–rapid sand filtration.

4. Conclusions

(1) Application of rNV-VLPs in laboratory-scale experiments enabled us to estimate the removal performance of the coagulation–rapid sand filtration process for NV as particles.
(2) The coagulation–rapid sand filtration process with PACl and $FeCl_3$ (pH 5.8) at a coagulant dose of 40 μM-Al or -Fe achieved approximately 3-$\log_{10}$ removals of rNV-VLPs—larger than those achieved with alum or $FeCl_3$ (pH 6.8).
(3) The removal performance for MS2 was somewhat larger than that for rNV-VLPs in the coagulation–rapid sand filtration process. Accordingly, MS2 is not recommended as an appropriate surrogate for native NV. By comparison, the removal performance for Qβ was similar to, or smaller than, that for rNV-VLPs. However, the removal performances for rNV-VLPs and Qβ differed between the coagulation process and the following rapid sand filtration process. Therefore, Qβ also is not recommended as an appropriate surrogate for native NV.

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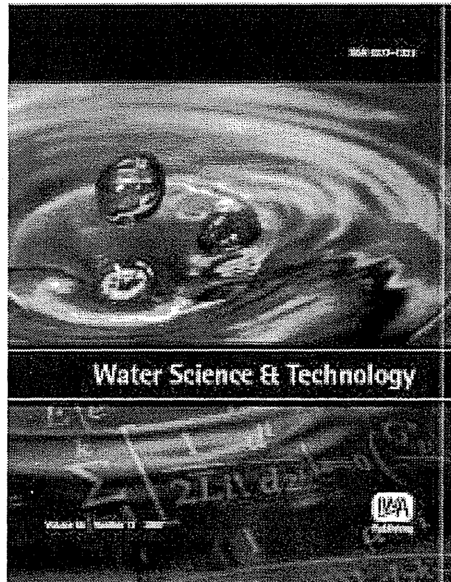

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Evaluation of norovirus removal performance in a coagulation–ceramic microfiltration process by using recombinant norovirus virus-like particles

N. Shirasaki, T. Matsushita, Y. Matsui, T. Urasaki, A. Oshiba and K. Ohno

ABSTRACT

Norovirus (NV) is a prototype strain of a group of human caliciviruses responsible for epidemic outbreaks of acute gastroenteritis worldwide. Because of the lack of a cell culture system or an animal model for this virus, studies on drinking water treatment such as separation and disinfection processes are still hampered. In the present study, we investigated NV removal performance as particles during a coagulation–ceramic microfiltration (MF) process by using recombinant NV virus-like particles (rNV-VLPs), which are morphologically and antigenically similar to native NV. We also experimentally investigated the behaviors of two widely accepted surrogates for pathogenic waterborne viruses, bacteriophages Qβ and MS2, for comparison with the behavior of rNV-VLPs. More than 4-log removal was observed for rNV-VLPs with a 1.08 mg-Al/L dose of polyaluminium chloride in the coagulation–ceramic MF process. This high removal ratio of rNV-VLPs satisfies the U.S. Environmental Protection Agency requirement of 4-log removal or inactivation. In addition, the removal ratios of Qβ and MS2 were approximately 2-log and 1-log, smaller than the ratio of rNV-VLPs. Accordingly, both bacteriophages have the potential to become appropriate surrogates for native NV in the coagulation–ceramic MF process, and, of the two, Qβ is the more conservative surrogate.

Key words | coagulation–ceramic microfiltration, ELISA, norovirus, real-time RT-PCR, virus-like particles

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INTRODUCTION

Ceramic microfiltration (MF) membranes have attracted attention in the field of drinking water treatment because they can withstand extreme acidity and alkalinity and higher operating pressures than polymeric or other inorganic membranes. These properties of ceramic membranes allow the use of strong acids and bases for chemical cleaning, application of high pressure for hydraulic backwashing, and operation at a high filtration flux. However, ceramic MF membranes alone cannot remove pathogenic waterborne viruses efficiently, because the membrane pore sizes are not small enough to remove viruses. To compensate for this disadvantage, a coagulation process has sometimes been used as a pretreatment before ceramic MF. In fact, coagulation–ceramic MF systems have already been applied to the treatment of drinking water in Japan, and our research group has showed these systems to be useful for virus removal by using the bacteriophages Qβ and MS2 as surrogates for pathogenic waterborne viruses (Matsui *et al.* 2003; Matsushita *et al.* 2005; Shirasaki *et al.* 2009*a*,*b*).

Among the pathogenic waterborne viruses, norovirus (NV; formerly known as small round structural virus and Norwalk-like virus) is one of most important human pathogens, causing epidemic and acute gastroenteritis worldwide. Although it has been 30 years since NV was first identified, studies of this virus, including experiments in the field of drinking water treatment, are still hampered by

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the lack of a cell culture system or an animal model (Zheng *et al.* 2006). Accordingly, the removal performance of NV in the drinking water treatment process has not been investigated fully. In recent years, expression of the NV genome in a baculovirus expression system has resulted in the production of recombinant NV virus-like particles (rNV-VLPs) (Jiang *et al.* 1992) that are morphologically and antigenically similar to native NV (White *et al.* 1996). In the present study, we used rNV-VLPs to investigate the removal performance of NV in a coagulation–ceramic MF process. We also experimentally investigated the behaviors of the bacteriophages Qβ and MS2 for comparison with the behavior of rNV-VLPs, and evaluated their suitability as surrogates for NV. This study represents the first attempt to apply rNV-VLPs to the evaluation of the removal performance of NV in a drinking water treatment process.

MATERIALS AND METHODS

Source water, coagulant, and MF membranes

Water was sampled from the Toyohira River (Sapporo, Japan; water quality shown in Table 1) on 12 June 2008. Two commercial aluminium coagulants, polyaluminium chloride (PACl 250A; 10.5% Al_2O_3, relative density 1.2 at 20°C; Taki Chemical Co., Ltd., Hyogo, Japan) and alum (8.1% Al_2O_3, relative density 1.3 at 20°C; Taki Chemical Co., Ltd.), were used for the coagulation process. A flat type of ceramic MF membrane (nominal pore size 0.1 μm, effective filtration area 0.0007 m^2; NGK Insulators, Ltd., Nagoya, Japan), which was installed in an acrylic-resin casing, was used.

Preparation of rNV-VLPs

Subgenomic cDNA fragments of NV (AB042808, GI/4, Chiba407/1987/JP) genome were artificially synthesized.

Table 1 | Water quality of the Toyohira River

pH	7.2
DOC (mg/L)	0.76
OD260 (cm)	0.019
Turbidity (NTU)	0.63
Alkalinity (mg-$CaCO_3$/L)	17.2

The fragments contained the entire second and third open reading frames of the NV genome. The cDNA was subcloned into a baculovirus transfer vector, and then the transfer vector was transfected into silkworm cells. The expressed rNV-VLPs were separated from the cell lysate by centrifugation and dialysis.

Quantification of rNV-VLPs

rNV-VLPs were detected by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (NV-AD (II), Denka Seiken Co., Ltd., Tokyo, Japan). The assay was performed according to the manufacturer's instructions. Optical densities at 450 nm and 630 nm in a 96-well microplate were measured with a microplate reader (MTP-300, Corona Electric Co., Ltd., Ibaraki, Japan). The rNV-VLP concentration detection limit with the ELISA kit was approximately 10^8 VLPs/mL.

Preparation of bacteriophages

F-specific RNA bacteriophages Qβ (NBRC 20012) and MS2 (NBRC 102619) were obtained from the NITE Biological Research Center (NBRC, Chiba, Japan). The bacteriophages Qβ and MS2 are widely used as surrogates for pathogenic waterborne viruses because of their morphological similarities to hepatitis A viruses and polioviruses, which are important viruses to remove during drinking water treatment. The genomes of these two bacteriophages each contain a single molecule of linear, positive-sense, single-stranded RNA, which is encapsulated in an icosahedral protein capsid with a diameter of 24–26 nm. Each bacteriophage was propagated for 22–24 h at 37°C in *Escherichia coli* (NBRC 13965) obtained from NBRC. The bacteriophage culture solution was centrifuged (2,000 × g, 10 min) and then passed through a membrane filter (pore size 0.45 μm, hydrophilic cellulose acetate; Dismic-25cs, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate was purified by using a centrifugal filter device (molecular weight cutoff 100,000, regenerated cellulose; Amicon Ultra-15, Millipore Corp., Billerica, MA, USA) to prepare the bacteriophage stock solution.

Quantification of bacteriophages

Viral RNA of the bacteriophages was quantified by real-time RT-PCR. Viral RNA was extracted from 200 μL of sample with a QIAamp MinElute Virus Spin Kit (Qiagen K. K., Tokyo, Japan) to obtain a final volume of 20 μL. The extracted RNA solution was added to a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems Japan Ltd., Tokyo, Japan) for the RT reaction, which was conducted at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 s, followed by cooling to 4°C in the thermal cycler (Thermal Cycler Dice Model TP600, Takara Bio Inc., Shiga, Japan). The cDNA solution was then amplified by a TaqMan Universal PCR Master Mix with UNG (Applied Biosystems Japan Ltd.), 400 nM of each primer (HQ-SEQ grade, Takara Bio Inc.), and 250 nM of TaqMan probe (Applied Biosystems Japan Ltd.). The oligonucleotide sequences of the primers and the probes are shown in Table 2. Amplification was conducted at 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min in an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems Japan Ltd.).

The standard curve for the real-time RT-PCR method was based on the relationship between the infectious bacteriophage concentration of a freshly prepared stock solution measured by the plaque forming unit (PFU) method (Adams 1959) and the number of cycles of PCR amplification.

Coagulation–ceramic MF process

Batch coagulation experiments were conducted with 400 mL of river water in glass beakers at 20°C. rNV-VLPs, Qβ, and MS2 were simultaneously added to the beakers at approximately 10^{11} VLPs/mL and 10^8 PFU/mL, respectively, and mixed with an impeller stirrer. PACl or alum was injected into the water as the coagulant at a dose of 0.54 or 1.08 mg-Al/L. The pH of the water was immediately adjusted to, and maintained at, 6.8 using HCl or NaOH. The water was stirred rapidly for 2 min ($G = 200\,s^{-1}$) and then slowly for 28 min ($G = 20\,s^{-1}$). The water was then left at rest for 20 min to allow the generated aluminium floc particles to settle. The supernatant was fed through a ceramic MF membrane by a peristaltic pump at 83 L/(m^2·h). The raw water (C_0; before coagulant dosing) and the MF permeate (C; after 15, 30, 60, 90, and 120 min of filtration) was collected for quantification of the rNV-VLPs, Qβ, and MS2.

Electron microscopy

rNV-VLPs were observed with an electron microscope. Ten microlitres of rNV-VLP stock solution was put on a 400-mesh copper grid with a collodion membrane (Nissin EM Corp., Tokyo, Japan) and adsorbed to the grid for 1 min. Excess solution on the grid was drained from the side of the grid with filter paper, and rNV-VLPs were negatively stained with 10 μL of 2% phosphotungstic acid (pH 5.5) for 45 s. After the excess stain was drained off, the grid was examined with a transmission electron microscope (TEM, H-7650, Hitachi High-Technologies Corp., Tokyo, Japan). Particle diameter of rNV-VLPs was expressed as the mean and standard deviation of any 10 particles on the electron micrograph.

RESULTS AND DISCUSSION

Characteristics of the rNV-VLPs produced

Figure 1 shows an electron micrograph of rNV-VLPs. The presence of particles was confirmed on the electron

Table 2 | Oligonucleotide sequences of the primers and the probes used in real time RT-PCR quantification of Qβ and MS2

Viruses		Oligonucleotide sequences	Positions	References
Qβ	Forward primer	5′-TCA AGC CGT GAT AGT CGT TCC TC-3′	49–71	Katayama *et al.* (2002)
	Reverse primer	5′-AAT CGT TGG CAA TGG AAA GTG C-3′	187–208	
	TaqMan probe	5′-CGA GCC GCG AAC ACA AGA ATT GA-3′	147–169	
MS2	Forward primer	5′-GTC GCG GTA ATT GGC GC-3′	632–648	O'Connell *et al.* (2006)
	Reverse primer	5′-GGC CAC GTG TTT TGA TCG A-3′	690–708	
	TaqMan probe	5′-AGG CGC TCC GCT ACC TTG CCC T-3′	650–671	

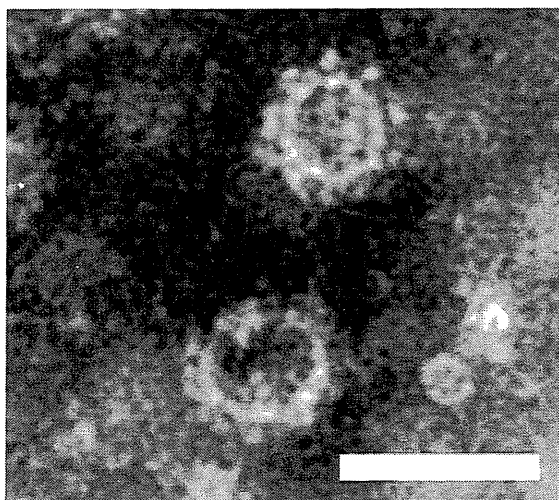

Figure 1 | Negatively stained electron micrograph of rNV-VLPs. Scale bar, 50 nm.

micrograph, and rNV capsid proteins spontaneously self-assembled into VLPs during expression. The rNV-VLP particle diameter was 35.7 ± 3.2 nm, measured by randomly choosing 10 particles on the electron micrograph. This value roughly corresponds to the particle diameter (approximately 38 nm) previously reported for native NV (Someya *et al.* 2000). This result suggests that rNV-VLPs were successfully produced by the baculovirus-silkworm expression system, and were morphologically similar to native NV.

Removal performance in the coagulation–ceramic MF process with PACl

Figure 2 shows the change in the removal ratio ($\log[C_0/C]$) of rNV-VLPs, Qβ, and MS2 with filtration time in the coagulation–ceramic MF process with PACl. Because the diameters of rNV-VLPs (35.7 ± 3.2 nm), Qβ, and MS2 (24–26 nm) are smaller than the nominal pore size of the ceramic MF membrane (0.1 μm), no removal (<0.2-log) of rNV-VLPs or either bacteriophage was observed without coagulation pretreatment (data not shown). In contrast, the coagulation–ceramic MF process effectively removed rNV-VLPs, Qβ, and MS2 at PACl doses of 0.54 (Figure 2a) and 1.08 mg-Al/L (Figure 2b). In addition, an effect of the coagulant dose (0.54 mg-Al/L vs. 1.08 mg-Al/L) on the removal of rNV-VLPs and both bacteriophages was observed: the filtration time-averaged removals of rNV-VLPs, Qβ, and MS2 were only 1.6-log, 0.5-log, and 0.9-log, respectively, at the coagulant dose of 0.54 mg-Al/L, whereas with the dose of 1.08 mg-Al/L, the removals were > 2.9-log, 2.4-log, and 3.3-log for rNV-VLPs, Qβ, and MS2, respectively. This dose effect was most likely due to an increase in floc size with the increased coagulant dose. Increasing the coagulant dose from 0.54 to 1.08 mg-Al/L increased the size of the aluminium floc particles with adsorbed/entrapped rNV-VLPs or bacteriophages, and subsequently increased the amount of aluminium floc particles that settled out from the suspension during the

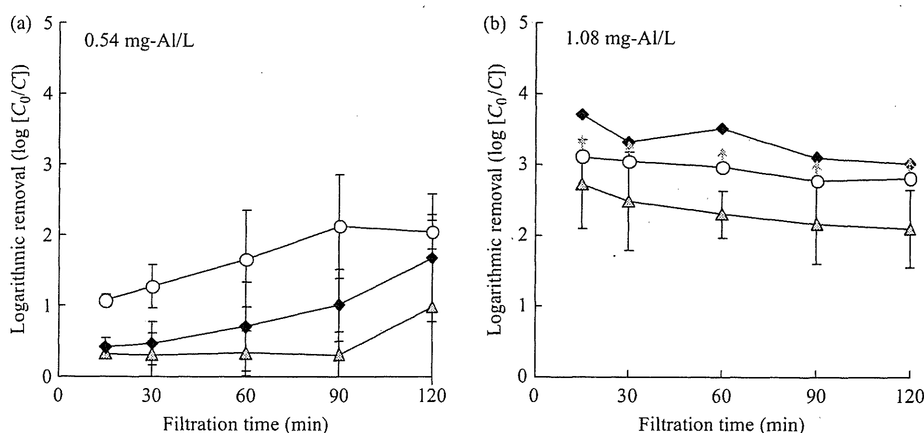

Figure 2 | rNV-VLPs, Qβ and MS2 removal by a coagulation–ceramic MF process with PACl coagulant at doses of (a) 0.54 mg-Al/L and (b) 1.08 mg-Al/L. Circles, triangles, and diamonds represent rNV-VLPs, Qβ and MS2, respectively. The arrows indicate values greater than those that could be estimated accurately by ELISA.

settling process. Consequently, the amount of aluminium floc particles that passed through a ceramic MF membrane was decreased, leading to a difference in the removal ratios of the rNV-VLPs and the two bacteriophages between PACl doses of 0.54 and 1.08 mg-Al/L.

The removal ratios of rNV-VLPs, Qβ, and MS2 gradually increased with filtration time at the PACl dose of 0.54 mg-Al/L. Ultimately, 2.0-log, 1.0-log, and 1.7-log removals were obtained for rNV-VLPs, Qβ, and MS2, respectively, at the end of the 2-h filtration. As described above, because the size and amount of aluminium floc particles that settled out from suspension were probably smaller with a PACl dose of 0.54 mg-Al/L than with one of 1.08 mg-Al/L, many of the aluminium floc particles were fed into the ceramic MF membrane and accumulated on the membrane surface as a cake layer during the MF process. This cake layer would act as a barrier to rNV-VLPs and both bacteriophages, causing the removal ratios of rNV-VLPs, Qβ, and MS2 to gradually increase with the growth of the cake layer at the PACl dose of 0.54 mg-Al/L.

In contrast, the removal ratios of rNV-VLPs, Qβ, and MS2 gradually decreased with filtration time at the PACl dose of 1.08 mg-Al/L. Our research group has demonstrated that the number of virus particles in the MF compartment increases with filtration time in the coagulation–ceramic MF system (Shirasaki *et al.* 2007). Although the experimental procedures of the present study and our previous study (Shirasaki *et al.* 2007) differed in terms of the coagulation process (batch coagulation vs. in-line coagulation), rNV-VLPs and both bacteriophages also probably accumulated in the MF compartment with filtration time in the present study. Accordingly, the concentrations of rNV-VLPs, Qβ, and MS2 in the MF permeate increased with the accumulation of rNV-VLPs and both bacteriophages in the MF compartment. In addition, because many of the aluminium floc particles had settled out from suspension during the settling process prior to the MF process with the 1.08 mg-Al/L PACl dose, the effect of the cake layer was probably smaller than with the 0.54 mg-Al/L PACl dose. The combination of these two phenomena caused the leakage of the rNV-VLPs and the two bacteriophages into the MF permeate, decreasing their removal ratios with filtration time.

The removal performance for rNV-VLPs, Qβ, and MS2 with the 1.08 mg-Al/L PACl dose at the end of the filtration (2 h) was still higher than that with the 0.54 mg-Al/L PACl dose, although the removal ratios decreased with filtration time as described above. Therefore, a PACl dose of 1.08 mg-Al/L was more effective for the removal of rNV-VLPs and both bacteriophages than the dose of 0.54 mg-Al/L in the coagulation–ceramic MF process.

Removal performance in the coagulation–ceramic MF process with alum

Figure 3 shows the change in the removal ratio of rNV-VLPs, Qβ, and MS2 with filtration time in the coagulation–ceramic MF process with alum. The coagulation–ceramic MF process effectively removed rNV-VLPs, Qβ, and MS2 at alum doses of 0.54 (Figure 3a) and 1.08 mg-Al/L (Figure 3b). In addition, an effect of the coagulant dose (0.54 mg-Al/L vs. 1.08 mg-Al/L) on the removal of rNV-VLPs and of both bacteriophages was also observed with alum: the filtration time-averaged removals of rNV-VLPs, Qβ, and MS2 were only 1.9-log, 0.9-log, and 2.3-log, respectively, at the 0.54 mg-Al/L coagulant dose, whereas the 1.08 mg-Al/L dose achieved time-averaged removals of $>$ 3.1-log, 1.3-log, and 3.1-log for rNV-VLPs, Qβ, and MS2, respectively.

The removal ratios of rNV-VLPs, Qβ, and MS2 gradually increased with filtration time at the 0.54 mg-Al/L alum dose, probably owing to the aluminium floc particles accumulating on the membrane surface as a cake layer during the MF process. Ultimately, removals of 2.3-log, 1.0-log, and 2.7-log were obtained for rNV-VLPs, Qβ, and MS2, respectively, at the end of the 2-h filtration. In contrast, the removal ratios of rNV-VLPs, Qβ, and MS2 gradually decreased with filtration time at the 1.08 mg-Al/L alum dose, for the same reason as in the PACl experiment, described above.

The removal performance for rNV-VLPs, Qβ, and MS2 with the 1.08 mg-Al/L alum dose at the end of the filtration (2 h) was also higher than that with the 0.54 mg-Al/L alum dose, as in the PACl experiments. Accordingly, the alum dose of 1.08 mg-Al/L was more effective for removal of rNV-VLPs and both bacteriophages than the 0.54 mg-Al/L alum dose in the coagulation–ceramic MF process.

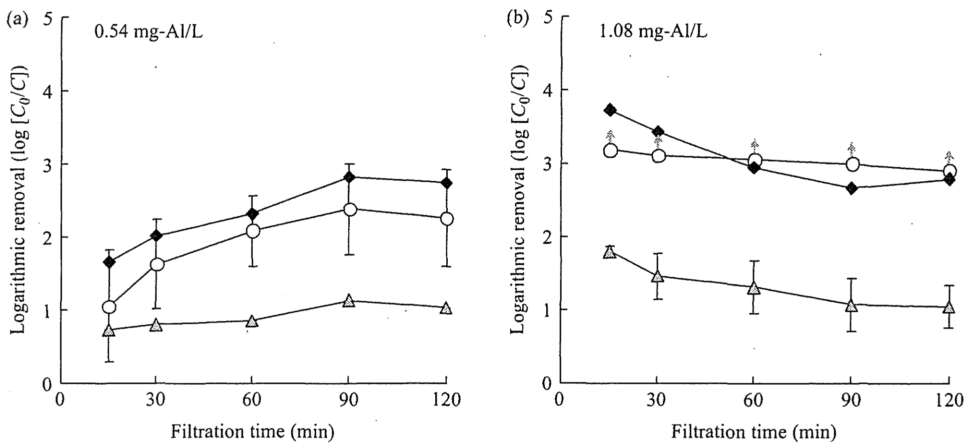

Figure 3 | rNV-VLPs, Qβ and MS2 removal by a coagulation–ceramic MF process with alum coagulant at doses of (a) 0.54 mg-Al/L and (b) 1.08 mg-Al/L. Circles, triangles, and diamonds represent rNV-VLPs, Qβ and MS2, respectively. The arrows indicate values greater than those that could be estimated accurately by ELISA.

Comparison of removal performances between PACl and alum

Because the concentration of rNV-VLPs in the MF permeate with the 1.08 mg-Al/L coagulant dose (Figures 2b and 3b) was less than the detection limit of the ELISA kit (approximately 10^8 VLPs/mL), further concentration using a centrifugal filter device (molecular weight cutoff 30,000, regenerated cellulose; Amicon Ultra-15, Millipore Corp.) was performed in the present study to evaluate 4-log removal, as regulated by the U.S. Environmental Protection Agency (USEPA) National Primary Drinking Water Standards (U.S. Environmental Protection Agency 2001). An approximately 10-fold concentration was obtained by the concentration method for rNV-VLPs in the MF permeate.

Figure 4 shows the effect of coagulant type (PACl vs. alum) on the removals of rNV-VLPs, Qβ, and MS2 in the coagulation–ceramic MF process with a 1.08 mg-Al/L coagulant dose. The experiment with PACl achieved >4-log removal of rNV-VLPs regardless of the filtration time, whereas with alum 3.3-log to >3.9-log removals were observed. This means that the experiment with PACl more effectively removed rNV-VLPs than that with alum, and that it satisfied the USEPA requirement of 4-log removal/inactivation. The difference in removal performance between PACl and alum was possibly due to differences in the characteristics of the aluminium floc particles generated during the coagulation process. Gregory & Dupont (2001) reported that aluminium floc particles formed with PACl are larger, stronger, and more readily separated by the settling process than those formed with alum. In the present study, because the rNV-VLPs were adsorbed to or entrapped in the aluminium floc particles, and settled out from suspension during the settling process or were separated during the MF process, the difference in the characteristics of the aluminium floc particles between PACl and alum may account for the difference in the removal performance for rNV-VLPs of the coagulation–ceramic MF process. Further investigation is needed.

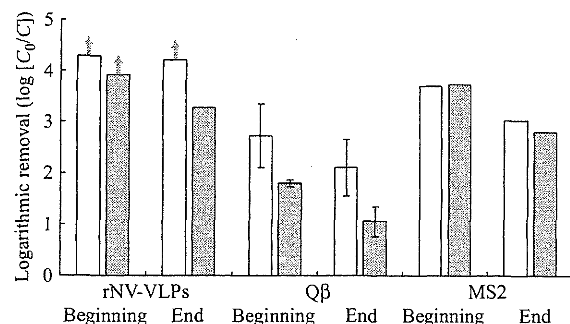

Figure 4 | Effect of coagulant type on the removal of rNV-VLPs Qβ and MS2 at the beginning and end of filtration in a coagulation–ceramic MF process. White and gray columns represent PACl and alum, respectively. The coagulant dose was 1.08 mg-Al/L. The arrows indicate values greater than those that could be estimated accurately by ELISA.

The removal ratios of Qβ and MS2 were smaller than those of rNV-VLPs in the coagulation–ceramic MF process with both aluminium coagulants: the ratio of Qβ was approximately 2-log smaller than that of rNV-VLPs, and the MS2 ratio was approximately 1-log smaller. Accordingly, both bacteriophages have the potential to become appropriate surrogates for native NV in the coagulation–ceramic MF process, with Qβ being the more conservative surrogate of the two. We imagine that the characteristics of rNV-VLPs, Qβ, and MS2 such as surface charge and hydrophobicity affect the removal performance in the coagulation–MF process. Further investigation of differences in surface properties between rNV-VLPs and the two bacteriophages is needed.

Our research group has shown that PACl exhibits virucidal activity during the coagulation process (Shirasaki *et al.* 2009*a*,*b*): an approximately 4-log difference between the total (infectious + inactivated) concentration measured by real-time RT-PCR and the infectious concentration measured by the PFU method was observed during the coagulation process, indicating that some of the bacteriophages were probably inactivated by PACl. Because rNV-VLPs lack RNA, which is necessary for infection and replication in host cells, we cannot discuss the fate of infectivity of NV in the treatment process. If PACl exhibits virucidal activity for native NV as well as for bacteriophages, >4-log removal (including inactivation) of native NV might be easily achieved during the coagulation–ceramic MF process.

CONCLUSIONS

- The removal performance of NV as particles was evaluated by using rNV-VLPs in a coagulation–ceramic MF process.
- Both coagulation type and coagulant dose affected the removal performance of rNV-VLPs: the experiment with a 1.08 mg-Al/L PACl dose achieved high ratios of rNV-VLP removal, >4-log, which satisfies the USEPA requirement of 4-log removal/inactivation.
- The removal ratios of rNV-VLPs were higher than those of Qβ and MS2 in the experiments with the 1.08 mg-Al/L coagulant dose: the Qβ ratio was approximately 2-log smaller than that of rNV-VLPs, and the MS2 ratio was approximately 1-log smaller. This means that both bacteriophages have the potential to become appropriate surrogates for native NV in the coagulation–ceramic MF process, with Qβ being the more conservative of the two.

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Feasibility of in-line coagulation as a pretreatment for ceramic microfiltration to remove viruses

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ABSTRACT

The feasibility of in-line coagulation as a pretreatment for ceramic microfiltration (MF) was verified by comparing its efficiency in the removal of viruses with that of the traditional mechanical mixing approach for coagulation, and by examining the effect of coagulant dose and coagulation time on virus removal. The in-line coagulation–ceramic MF system efficiently removed bacteriophage Qβ and MS2: removal ratios were >8.2 log for infectious viruses and >5.4 log for total (infectious + inactivated) virus particles. These values were similar to those of the mechanical coagulation–ceramic MF system. The in-line coagulation system has potential as a useful pretreatment for the removal of viruses as an alternative to the mechanical mixing system, because the former efficiently removes viruses and has a smaller footprint in treatment plants. For the in-line coagulation–ceramic MF system, a coagulant dose of 1.08 mg-Al/L and a coagulation time of 1 min were required to achieve a high level of virus removal. Infectious Qβ and MS2 were removed to similar levels by the two precoagulation methods tested, but the removal of total MS2 particles was higher than that of Qβ particles, possibly because of the selective interaction with the cake layer.

Key words | bacteriophages, ceramic microfiltration, in-line coagulation, virus inactivation, virus removal

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INTRODUCTION

Membrane filtration processes using microfiltration (MF) and ultrafiltration (UF) membranes have been widely used to produce drinking water. One of the primary reasons for the increasing use of membrane filtration is that it can be used to remove pathogenic microorganisms such as *Cryptosporidium* and *Giardia*. Complete removal of *Cryptosporidium parvum* oocysts and *Giardia muris* cysts was achieved by direct MF/UF membranes (Jacangelo *et al.* 1995). By comparison, because viruses are the smallest pathogenic microorganisms among the causative agents of waterborne disease, varying levels of virus removal have been reported for direct MF (<0.5 to >6 log)/UF (0.4 to >6 log) processes (Jacangelo *et al.* 1995; Urase *et al.* 1996; Otaki *et al.* 1998; Hu *et al.* 2003; Arkhangelsky & Gitis 2008; Langlet *et al.* 2009). The 4-log virus removal required by the US Environmental Protection Agency (USEPA 2001) is regularly not satisfied by MF/UF processes alone, particularly direct MF processes.

Recently, coagulation as a pretreatment for membrane filtration has become attractive because it is expected to improve removal of organic compounds (Yuasa 1998; Kim *et al.* 2006; Choi *et al.* 2008; Kimura *et al.* 2008) and to mitigate membrane fouling (improve flux decline) (Kunikane *et al.* 1995; Judd & Hillis 2001; Oh & Lee 2005; Cho *et al.* 2006; Kim *et al.* 2006; Choi *et al.* 2008; Kimura *et al.* 2008). The combination of coagulation and MF was shown to achieve higher levels of virus removal than that by MF alone (Zhu *et al.* 2005; Fiksdal & Leiknes 2006).

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Our group has also reported the usefulness of a coagulation–ceramic MF system for virus removal (Matsui *et al.* 2003*a*; Matsushita *et al.* 2005): a >6-log removal of viruses was achieved by this system with an aluminium coagulant.

In the coagulation–MF processes, coagulation conditions, including coagulant dose and coagulation time, affect membrane performance. Although many studies have focused mostly on the influence of membrane fouling (e.g. Lee *et al.* 2000; Judd & Hillis 2001; Oh & Lee 2005; Cho *et al.* 2006; Kimura *et al.* 2008), few researchers have reported the effect of coagulant dose (Matsui *et al.* 2003*a*; Matsushita *et al.* 2005; Zhu *et al.* 2005; Fiksdal & Leiknes 2006) and coagulation time (Matsushita *et al.* 2005) on virus removal. Zhu *et al.* (2005) combined polyvinylidene fluoride MF with jar coagulation (without settling) and reported a dramatic increase in virus removal with an increase in the dose of ferric chloride. Matsushita *et al.* (2005) also reported that coagulant dose strongly affects virus removal: only 2.8-log removal was achieved with 0.54 mg-Al/L polyaluminium chloride (PACl) in the in-line coagulation–ceramic MF process, whereas 6.4-log and 7.4-log removal was achieved with 1.08 and 1.62 mg-Al/L PACl, respectively. Matsushita *et al.* (2005) also revealed that a longer coagulation time provides a greater reduction in virus level.

Coagulation as a pretreatment for membrane filtration can be introduced by mechanical mixing or in-line mixing. Mechanical mixing is widely employed in the traditional treatment of drinking water consisting of coagulation, flocculation, sedimentation and rapid sand filtration processes. For these processes, rapid mixing and slow mixing tanks are installed in the system to allow colloids in water, following addition of the coagulant, to coagulate and flocculate. The MF process can be an alternative to the rapid sand filtration process as a physical barrier. Three applications are available for mechanical mixing in coagulation pretreatment. Raw water can be treated with the coagulant in the rapid mixing tank and then subjected to MF without slow mixing and sedimentation. Alternatively, raw water can be treated in the rapid mixing tank and then passed through a slow mixing tank only or through a slow mixing tank and then a sedimentation tank before MF. Treatment of the raw water with coagulant in the rapid mixing tank only is often applied to the ceramic MF process (Yonekawa *et al.* 2004), whereas the application of a slow mixing tank and a sedimentation tank is often used together with organic membranes (Bakersfield Water Treatment Plant, California, http://www.water-technology.net/projects/bakersfield/, accessed 4 March 2009; Columbia Heights Filtration Plant, Minneapolis, http://www.water-technology.net/projects/columbia/, accessed 4 March 2009).

As an alternative procedure, in-line coagulation as a pretreatment for MF has been tested in bench-scale and pilot-scale experiments (Judd & Hillis 2001; Matsui *et al.* 2003*a*; Matsushita *et al.* 2005; Oh & Lee 2005; Cho *et al.* 2006; Meyn *et al.* 2008) because of its advantages over mechanical mixing, including reduction of coagulant dose, coagulation time and energy consumption (Oh & Lee 2005; Meyn *et al.* 2008). Comparisons of the operation performance of these types of mechanical mixing are limited. The efficiency of dissolved organic carbon (DOC) removal has been compared among three different types of mechanical mixing for coagulation with 6 mg-Fe/L iron chloride dosing including in-line mixing (hydraulic retention time (HRT), 45 s), one-stage mechanical rapid mixing (HRT, 6.8 min), and two-stage flocculation with mechanical rapid mixing and slow mixing (total HRT, 20 min) for the ceramic MF process, and the efficiency was found to be almost the same, regardless of the mixing type (Meyn *et al.* 2008). The effect on virus removal has not been investigated, except in a previous study by our group (Matsui *et al.* 2003*a*).

The virus removal performance of membrane filtration processes, including the coagulation–MF process, is frequently evaluated by using bacteriophages (i.e. viruses that infect bacteria) as indicators of enteric viruses (Jacangelo *et al.* 1995; Urase *et al.* 1996; Otaki *et al.* 1998; Hu *et al.* 2003; Matsui *et al.* 2003*a*; Matsushita *et al.* 2005; Zhu *et al.* 2005; Fiksdal & Leiknes 2006; Arkhangelsky & Gitis 2008; Langlet *et al.* 2009; Shirasaki *et al.* 2009*a*). The plaque-forming unit (PFU) method is commonly used for quantification of bacteriophages, because it measures the concentration of infectious viruses. However, the removal of infectious viruses by the coagulation–MF process is due not only to physical removal during the membrane separation process but also to the virucidal activity of the aluminium coagulant (Matsui *et al.* 2003*b*; Matsushita *et al.* 2004; Shirasaki *et al.* 2009*b*) during the coagulation pretreatment.

In a previous study, our group applied the PFU method together with the polymerase chain reaction (PCR) method

to measure the concentration of infectious viruses as well as inactivated viruses, to evaluate the performance of the coagulation-MF process in removing infectious viruses and inactivated viruses (Shirasaki *et al.* 2009*a*). We found a difference between total (infectious + inactivated) and infectious virus concentrations just before MF, indicating inactivation of viruses during coagulation. Although the mechanisms underlying the virucidal activity of the aluminium coagulant remain unclear, inactivated viruses in the MF permeate might recover their infectivity in the water distribution system. Virus removal performances evaluated by the PFU method might underestimate the risk of infection because this method cannot count the inactivated viruses that pass into the MF permeate.

The effects of coagulation conditions on the performance of infectious and inactivated virus removal have not been investigated. By comparison, the effects of the different mixing methods (in-line mixing vs. mechanical mixing) (Matsui *et al.* 2003*a*), coagulant dose (Matsui *et al.* 2003*a*; Matsushita *et al.* 2005; Zhu *et al.* 2005; Fiksdal & Leiknes 2006) and coagulation time (Matsushita *et al.* 2005) on the removal of infectious viruses have been widely investigated by using the PFU method alone.

Our objective in the present study was to verify the feasibility of in-line coagulation as a pretreatment for ceramic MF by comparing its efficiency for virus removal with that of the traditional mechanical mixing approach for coagulation, as well as by examining the effect of coagulant dose and coagulation time on virus removal. The efficiency of virus removal in these experiments was assessed by using the PFU method, to measure the concentration of infectious bacteriophage, and real-time reverse transcription-polymerase chain reaction (RT-PCR) method, to measure the concentration of total bacteriophage.

MATERIALS AND METHODS

Source water, coagulant and MF membranes

River water was sampled from the Toyohira River (Sapporo, Japan) on 12 June 2008 and was subjected to water quality analyses (Table 1). PACl (250A; 10.5% Al_2O_3, relative density 1.2 at 20°C; Taki Chemical Co., Ltd, Hyogo, Japan) was used for the coagulation process. The membrane used was a monolithic ceramic MF module installed in a stainless-steel casing (61-channel tubular; nominal pore size 0.1 μm, effective filtration area 0.048 m^2, membrane diameter 0.03 m, membrane length 0.1 m; NGK Insulators, Ltd, Nagoya, Japan).

Table 1 | Water quality of the Toyohira River

pH	7.2
DOC (mg/L)	0.76
OD260 (cm^{-1})	0.019
Turbidity (NTU)	0.63
Alkalinity (mg-$CaCO_3$/L)	17.2

Bacteriophages

The F-specific RNA bacteriophages Qβ (NBRC 20012) and MS2 (NBRC 102619) were obtained from the NITE Biological Research Center (NBRC, Chiba, Japan). The bacteriophages Qβ (Urase *et al.* 1996; Otaki *et al.* 1998; Matsui *et al.* 2003*a*; Matsushita *et al.* 2005; Langlet *et al.* 2009; Shirasaki *et al.* 2009*a*) and MS2 (Jacangelo *et al.* 1995; Hu *et al.* 2003; Zhu *et al.* 2005; Fiksdal & Leiknes 2006; Arkhangelsky & Gitis 2008; Langlet *et al.* 2009; Shirasaki *et al.* 2009*a*) are widely used as surrogates for pathogenic waterborne viruses because of their morphological similarities to hepatitis A viruses and polioviruses, which need to be removed by the treatment of drinking water.

Qβ is the prototype member of the genus *Allolevivirus* in the family Leviviridae, and MS2 is the prototype member of the genus *Levivirus* in the family Leviviridae. The Qβ and MS2 genomes comprise a single molecule of linear positive-sense, single-stranded RNA encapsulated in an icosahedral protein capsid with a diameter of 24-26 nm (The Universal Virus Database of the International Committee on Taxonomy of Viruses, http://phene.cpmc.columbia.edu/index.htm, accessed 17 April 2008). Each bacteriophage was propagated for 22-24 h at 37°C in *Escherichia coli* (NBRC 13965) obtained from NBRC. The bacteriophage culture solution was centrifuged (2,000 × *g*, 10 min) and then passed through a membrane filter (pore size 0.45 μm, hydrophilic cellulose acetate; Dismic-25cs, Toyo Roshi Kaisha, Ltd, Tokyo, Japan).

To prepare the bacteriophage stock solution, the filtrate was purified by using a centrifugal filter device comprising a regenerated cellulose membrane with a molecular weight cut off of 100,000 (Amicon Ultra-15, Millipore Corp., Billerica, Massachusetts).

In-line coagulation–ceramic MF experiments

The setup for the in-line coagulation–ceramic MF experiments is schematically depicted in Figure 1. The river water, placed in the raw water tank, was spiked with either Qβ or MS2 at approximately 10^8 PFU/mL. Throughout the experiments, the raw water was mixed constantly with an impeller stirrer. The raw water was fed into the system at a constant flow rate ($83.3\,L/(m^2 h) = 2.0\,m/d$) by a peristaltic pump. To maintain the MF permeate at pH 6.8, hydrochloric acid or sodium hydroxide was added to the water before it reached the first in-line static mixer (HRT 1.8 s; 1/4-N40-172-0, Noritake Co., Ltd, Nagoya, Japan).

PACl was injected after the first in-line static mixer and before the second in-line static mixer at a constant dose rate (0.54, 1.08 or 1.62 mg-Al/L). To obtain the three different coagulation times, the in-line static mixer (G value 260/s, HRT 1.8 s), and a combination of the in-line static mixer and a subsequent Tygon® tube reactor (total HRT 1 or 5 min), were used as the second in-line static mixer. The total HRT was controlled by the length of the Tygon® tube reactor. After the PACl had been mixed in, the water was fed into the ceramic MF module in dead-end mode. Filtration was performed for 4 h without any backwashing. Bacteriophage concentrations in the raw water tank and in the MF permeate were measured every hour.

Mechanical coagulation–ceramic MF experiments

The setup for mechanical coagulation–ceramic MF experiments is schematically depicted in Figure 2. The river water, placed in the raw water tank, was spiked with either Qβ or MS2 at approximately 10^8 PFU/mL. Throughout the experiments, the raw water was mixed constantly with an impeller stirrer. The raw water was fed into the system at a constant flow rate (100 mL/min) by a peristaltic pump. To maintain the MF permeate at pH 6.8, hydrochloric acid or sodium hydroxide was added to the water before it reached the first in-line static mixer (HRT 1.2 s). pH-adjusted raw water was introduced and PACl was injected into the rapid mixing chamber with an impeller stirrer (G value 200/s, 109 rpm; hydraulic retention time 5 min) at a constant dose rate (1.08 mg-Al/L).

After the PACl had been mixed in, the water was passed through five chambers for slow mixing (G value 20/s, 23 rpm; HRT 5 min × 5 chambers) and then through a rectangular settler for settling of the aluminium floc particles (HRT 20 min). At the end of the rapid mixing or settling, the water was fed into the ceramic MF module in dead-end mode at a constant flow rate 67 mL/min, which

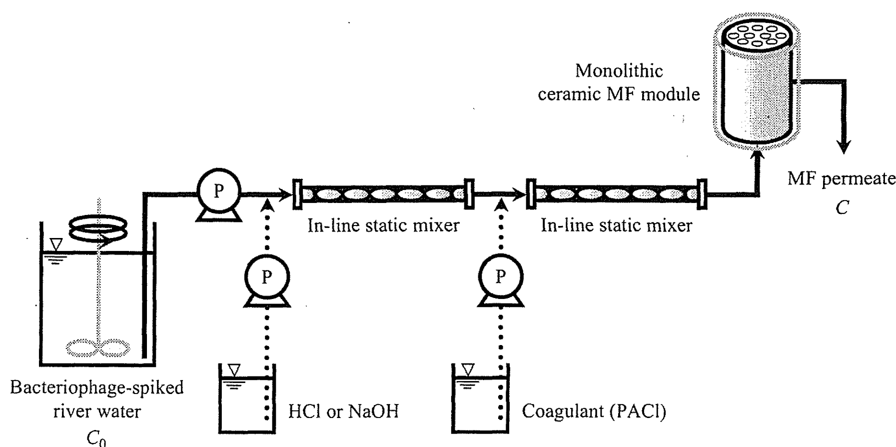

Figure 1 | In-line coagulation–ceramic MF system. C_0 and C are the bacteriophage concentrations (PFU or particles/mL) in the raw water tank and the MF permeate, respectively, at each sampling time.

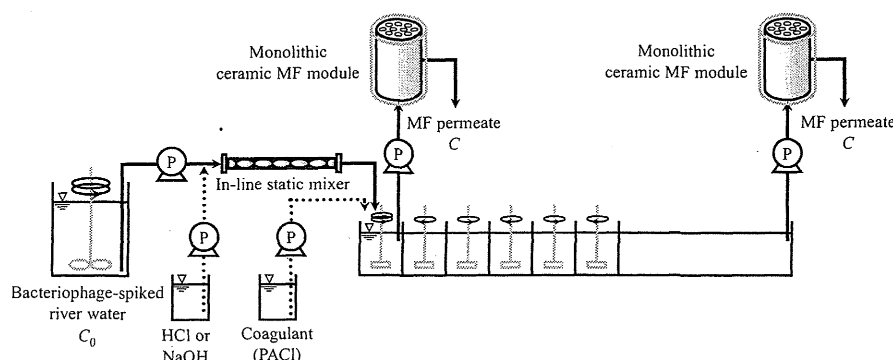

Figure 2 | Mechanical coagulation–ceramic MF system. C_0 and C are the bacteriophage concentrations (PFU or particles/mL) in the raw water tank and the MF permeate, respectively, at each sampling time.

was equivalent to 83.3 L/(m^2 h). Filtration was performed for 4 h without any backwashing. Bacteriophage concentrations in the raw water tank and in the MF permeate were measured every hour.

Bacteriophage assay

PFU method

The infectious bacteriophages were enumerated according to the double-layer method (Adams 1959) by using the bacterial host *E. coli* (NBRC 13965).

Serially diluted raw water or MF permeate (1 mL) was poured onto a solid bottom agar plate followed by 0.3 mL of host *E. coli* culture mixed with 3 mL of molten top agar. The plates were incubated for 16–24 h at 37°C. To measure the concentration of infectious bacteriophage in the water samples, we calculated the average plaque counts of triplicate plates prepared from one sample on plates with 30 to 300 PFU, which we considered a countable number of plaques, and determined the PFU/mL.

For quantification of low infectious bacteriophage concentrations (i.e. <30 PFU/mL) in the MF permeate, 50 mL of MF permeate was mixed with 5 mL of bacterial host *E. coli* culture and 50 mL molten agar, and the mixture was then poured into 10 plates (without bottom agar). The plates were incubated for 16–24 h at 37°C. We calculated the PFU/mL by dividing the total plaque counts for the 10 plates by the sample volume (50 mL).

Real-time RT-PCR method

Viral RNA of bacteriophages was quantified by real-time RT-PCR method. Real-time RT-PCR method detects the virus genome and therefore detects viruses regardless of their infectivity. We defined the concentration measured by real-time RT-PCR method as the total bacteriophage concentration. For quantification of bacteriophages in the raw water and MF permeate, viral RNA was extracted from 200 μL samples with a QIAamp MinElute Virus Spin Kit (Qiagen K.K., Tokyo, Japan) to obtain a final volume of 20 μL. The viral RNA was then subjected to RT reaction by using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems Japan Ltd, Tokyo, Japan). RT reaction was conducted at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 s, followed by cooling to 4°C in a thermal cycler (Thermal Cycler Dice Model TP600, Takara Bio Inc., Shiga, Japan). The resultant cDNA was then amplified by using a TaqMan Universal PCR Master Mix with UNG (Applied Biosystems Japan Ltd), 400 nM of each primer (HQ-SEQ grade, Takara Bio Inc.), and 250 nM of TaqMan probe (Applied Biosystems Japan Ltd). The oligonucleotide sequences of the primers and the probes are listed in Table 2. Amplification was conducted at 50°C for 2 min, 95°C for 10 min, and then for 40 cycles at 95°C for 15 s and 60°C for 1 min in an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems Japan Ltd).

We constructed a standard curve for real-time RT-PCR method based on the relationship between the infectious

Table 2 | Oligonucleotide sequences of the primers and the probes used in real-time RT-PCR quantification of Qβ and MS2

Viruses		Oligonucleotide sequences	Positions	References
Qβ	Forward primer	5′-TCA AGC CGT GAT AGT CGT TCC TC-3′	49–71	Katayama *et al.* (2002)
	Reverse primer	5′-AAT CGT TGG CAA TGG AAA GTG C-3′	187–208	
	TaqMan probe	5′-CGA GCC GCG AAC ACA AGA ATT GA-3′	147–169	
MS2	Forward primer	5′-GTC GCG GTA ATT GGC GC-3′	632–648	O'Connell *et al.* (2006)
	Reverse primer	5′-GGC CAC GTG TTT TGA TCG A-3′	690–708	
	TaqMan probe	5′-AGG CGC TCC GCT ACC TTG CCC T-3′	650–671	

bacteriophage concentration (PFU/mL) of freshly prepared bacteriophage stock solution, assumed not to contain inactivated bacteriophages, and the number of PCR amplification cycles.

RESULTS AND DISCUSSION

Infectious bacteriophage removal by the in-line coagulation–ceramic MF system

Figure 3 shows the effect of coagulant dose and coagulation time on the removal of infectious bacteriophage, assessed by the PFU method, by the in-line coagulation–ceramic MF system. Because the diameters of Qβ and MS2 were smaller than the nominal pore size of the ceramic MF membrane (0.1 μm), there was no removal of these infectious bacteriophages in the absence of coagulation pretreatment (data not shown). Other researchers also have reported an insufficient removal ratio for infectious Qβ and MS2 by MF in the absence of coagulation pretreatment: 1–2 log for Qβ (Urase *et al.* 1996) and <1 log for MS2 (Jacangelo *et al.* 1995).

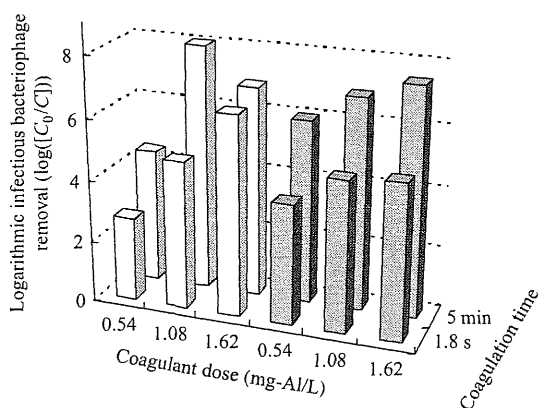

Figure 3 | Effect of coagulant dose and coagulation time on the filtration time-averaged infectious bacteriophage removal in the in-line coagulation–ceramic MF system. White and shaded columns represent Qβ and MS2, respectively.

By comparison, in-line coagulation pretreatment improved the removal ratios ($\log[C_0/C]$) for infectious Qβ and MS2 by MF (Figure 3) because these phages were negatively charged at pH 6.8 (Shirasaki *et al.* 2009*a*) and became adsorbed to/entrapped by the positively charged aluminium floc particles (amorphous aluminium hydroxide, $Al[OH]_3$) generated during coagulation pretreatment. The aluminium floc particles exceeded the pore size of the ceramic MF membrane and were removed during the filtration process. More than 4-log removal was achieved for both bacteriophages, except at a coagulant dose of 0.54 mg-Al/L and a coagulation time of 1.8 s.

According to the USEPA National Primary Drinking Water Standards, 4-log removal or inactivation of enteric viruses from source water is required by filtration, disinfection or a combination of these technologies (USEPA 2001). Our hybrid system successfully met this requirement with a sufficient coagulant dose (≥1.08 mg-Al/L). Some researchers have also reported the usefulness of precoagulation to improve the removal of viruses by MF (Matsui *et al.* 2003*a*; Matsushita *et al.* 2005; Zhu *et al.* 2005; Fiksdal & Leiknes 2006). MF alone does not remove viruses, but the coagulation–MF system is effective in removing infectious viruses: 6–7-log for Qβ (Matsui *et al.* 2003*a*) and 6.7 to >7.5-log for MS2 (Fiksdal & Leiknes 2006).

An increase in the coagulant dose reduced the number of infectious bacteriophages (Figure 3). The time-averaged reduction in infectious Qβ and MS2 increased from 2.7 to 6.5-log and from 3.9 to 5.0-log, respectively, with an increase in the coagulant dose (0.54 to 1.62 mg-Al/L)

at a coagulation time of 1.8 s. The same positive effect of coagulation has been reported in other studies (Matsui *et al.* 2003*a*; Matsushita *et al.* 2005). This effect was most likely due to an increase in floc size with increasing coagulant dose (Judd & Hillis 2001). Increasing the coagulant dose from 0.54 to 1.08 mg-Al/L increased the size of aluminium floc particles with adsorbed/entrapped bacteriophages, and subsequently reduced the number of aluminium floc particles that passed through a ceramic MF membrane.

An increase in coagulation time also reduced the number of infectious Qβ and MS2. An extension of the coagulation time from 1.8 s to 5 min increased the removal ratios for Qβ and MS2 at any coagulant dose, most likely because the floc size during the coagulation process increased with time. Our results indicated that a coagulation time of 1.8 s was insufficient to achieve a high removal ratio for infectious phages.

Overall, there was no difference in the removal ratio between infectious Qβ and MS2 when the system was operated with a sufficient coagulant dose of ≥1.08 mg-Al/L and at a coagulation time of 5 min. This result was consistent with the findings of our previous study (Shirasaki *et al.* 2009*a*). Under these conditions, a removal ratio of more than 6.9-log, based on time-averaged reduction, was achieved for Qβ and MS2 by the in-line coagulation–ceramic MF system. This removal ratio was almost the same as or higher than the removal ratios obtained with direct UF (0.4 to >6 log) (Jacangelo *et al.* 1995; Urase *et al.* 1996; Otaki *et al.* 1998; Hu *et al.* 2003; Arkhangelsky & Gitis 2008) and direct nanofiltration (NF) (1.9 to >6 log) (Urase *et al.* 1996; Otaki *et al.* 1998; Hu *et al.* 2003) processes. Thus, the in-line coagulation–ceramic MF system is a potential alternative to UF or NF processes for the removal of infectious viruses. Our results also demonstrated that a high removal ratio could be achieved with effective control of coagulant dose and coagulation time. We propose that a PACl dose of 1.08 mg-Al/L is required to achieve a high removal ratio of infectious viruses in the present in-line coagulation–ceramic MF system.

Total bacteriophage removal by the in-line coagulation–ceramic MF system

Figure 4 shows the effect of coagulant dose and coagulation time on total bacteriophage removal, assessed by real-time RT-PCR method, by the in-line coagulation–ceramic MF system. A low removal ratio (<1.0-log) was observed for total Qβ with a coagulant dose of 0.54 mg-Al/L, but a high removal ratio of more than 4-log was achieved by increasing the coagulant dose from 0.54 to 1.08 mg-Al/L. In addition, extension of the coagulation time from 1.8 s to 5 min increased the removal ratio for total Qβ from 4.0-log to 4.4-log at a coagulant dose of 1.08 mg-Al/L. Thus, both coagulant dose and coagulation time were important factors dominating the removal performance not only of infectious Qβ but also of total Qβ in the in-line coagulation–ceramic MF system.

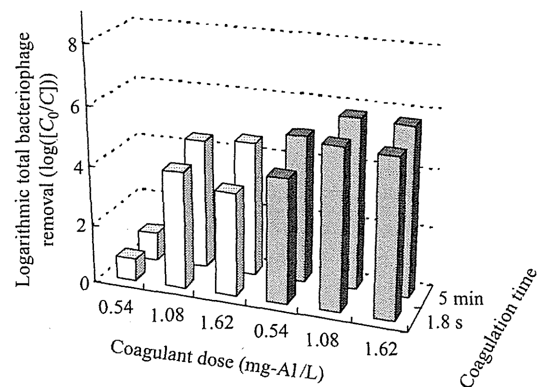

Figure 4 | Effect of coagulant dose and coagulation time on the filtration time-averaged total bacteriophage removal in the in-line coagulation–ceramic MF system. White and shaded columns represent Qβ and MS2, respectively.

By comparison, our in-line coagulation–ceramic MF system achieved a high removal ratio of >4-log for total MS2 at all coagulation conditions. Although particle diameters and electrophoretic mobilities were almost the same for Qβ and MS2 (Shirasaki *et al.* 2009*a*), a marked difference in removal ratio was observed between total Qβ and MS2. The removal ratio for total Qβ was lower than that of total MS2 under all coagulation conditions. This difference was most likely the result of differences between the interactions of Qβ and MS2 with the aluminium floc particles accumulated on the membrane surface as a cake layer. It is possible that MS2 has a higher affinity for the cake layer than does Qβ (Shirasaki *et al.* 2009*a*).

Abbaszadegan *et al.* (2007) and Mayer *et al.* (2008) reported that the removal ratio for MS2 was lower than that of adenoviruses, feline caliciviruses, coxsackieviruses, echoviruses and polioviruses by an enhanced coagulation

process using ferric chloride, and these researchers concluded that MS2 was an appropriate surrogate for enteric viruses. In contrast, our results suggest that Qβ may be a more appropriate surrogate than MS2, because we found that Qβ was more difficult to remove than MS2 by our in-line coagulation–ceramic MF system. Langlet *et al.* (2009) evaluated the efficiency of virus removal by direct MF and UF processes, also by using real-time RT-PCR method, and, consistent with our findings, their results demonstrated that the removal ratio for Qβ was lower than that of MS2; they concluded that Qβ was a better candidate than MS2 for characterizing membrane virus removal. We believe that Qβ, rather than MS2, has the potential to become a conservative surrogate for the evaluation of virus removal performance by drinking water treatment processes, including coagulation–MF processes.

The removal ratios of infectious Qβ and MS2 (Figure 3) were higher than those for total Qβ and MS2 (Figure 4). This result indicates that a large proportion of the bacteriophage population was inactivated by the coagulation–MF process. Our group previously reported that Qβ and MS2 were inactivated during the coagulation process and that Qβ was more sensitive to the virucidal activity of PACl than MS2 was (Matsui *et al.* 2003*b*; Matsushita *et al.* 2004; Shirasaki *et al.* 2009*b*). The removal efficiency of total Qβ is greater than that of MS2, but because of differences between these two bacteriophages in their sensitivity to the virucidal activity of PACl, the removal ratio of infectious Qβ and MS2 becomes almost the same.

In summary, the time-averaged removal ratios for total Qβ and MS2 were 4.4-log and 5.8-log, respectively, at a coagulant dose of 1.08 mg-Al/L and at a coagulation time of 5 min. These values were almost the same as or higher than the removal ratios by the direct UF process (1.5 to >6 log) (Langlet *et al.* 2009). Thus, the in-line coagulation–ceramic MF system is a potential alternative to the UF process for the removal of total viruses as well. From our observations, we propose that a PACl dose of 1.08 mg-Al/L is required to achieve a high removal ratio not only of infectious viruses but also of total viruses in the present in-line coagulation–ceramic MF system.

Comparison of removal performance between the in-line and mechanical coagulation processes

As described above, a PACl dose of 1.08 mg-Al/L was required to achieve high removal ratios for both infectious and total bacteriophages in the present in-line coagulation–ceramic MF system. To verify the feasibility of in-line coagulation as a pretreatment for the ceramic MF process, we compared the removal performances of Qβ and MS2 by in-line mixing and the traditional mechanical mixing approach at the same PACl dose (1.08 mg-Al/L).

Figure 5 shows a comparison of virus removal performance between the in-line and the mechanical coagulation–ceramic MF systems. Mechanical rapid mixing followed by MF achieved high removal ratios of >8.2-log for infectious and >4.3-log for total phage for both Qβ and MS2.

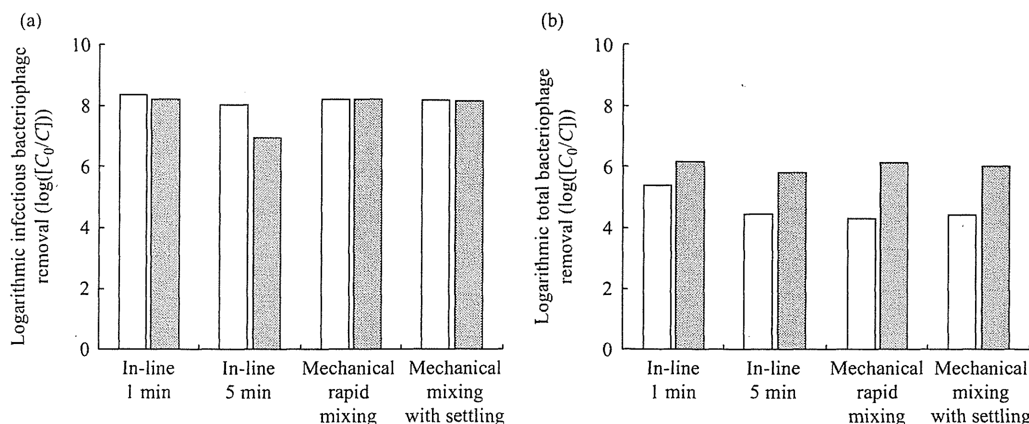

Figure 5 | Effect of mixing type of coagulation pretreatment on the removal of infectious bacteriophage (a) and total bacteriophage (b). White and shaded columns represent Qβ and MS2, respectively. PACl dosage was 1.08 mg-Al/L.

Our result for infectious Qβ removal agreed with that from the previous study (approximately 7.5-log) (Matsui *et al.* 2003*a*). Removal ratios did not improve even when slow mixing and settling processes were incorporated between the rapid mixing and MF processes. This result indicates that 5 min of rapid mixing was sufficient to achieve high removal ratios for infectious and total bacteriophage in the coagulation–ceramic MF system with mechanical mixing. Additional slow mixing and settling processes are not necessary for virus removal, enabling the actual treatment plant to be compact.

The high removal ratio for Qβ and MS2 achieved with the mechanical mixing system was similar to that achieved with equivalent coagulation time of 5 min in the in-line mixing system, demonstrating that in-line coagulation has potential as a pretreatment for MF giving high virus removal as an alternative to the mechanical mixing system. In addition, even when the coagulation time in the in-line mixing system was reduced to one-fifth (i.e. 1 min), high virus removal ratios of >8.2-log for infectious and >5.4-log for total bacteriophage, were still achieved (Figure 5). This means that 1 min of coagulation time is enough to achieve a high level of virus removal in the present in-line coagulation–ceramic MF system, leading to a reduction in the footprint required for the HRT in the actual treatment plant.

The removal performances for the two bacteriophages were similar for all tested mixing procedures, although the characteristics of aluminium floc particles (i.e. size, fractal dimension, and so on) fed into the ceramic MF module most likely differed depending on the mixing type. One possible explanation is that, during rapid mixing, regardless of mixing type, most of the bacteriophage population was adsorbed to/entrapped by aluminium floc particles that were larger than the pore size of the MF membrane and would thus be removed by the MF process. However, the small proportion of the bacteriophage population adsorbed to/entrapped by aluminium floc particles smaller than the MF pore size would pass through the system. It is possible that some of the aluminium floc particles would not be larger than the MF pore size, even after slow mixing and settling processes, and would pass through the MF membrane. Note that although a fraction of the bacteriophage population was not removed by the MF process, the removal ratio was very high. Enlargement of the floc size by traditional treatment with coagulation and sedimentation processes is not necessary for the filtration with a ceramic MF (Lerch *et al.* 2005). In-line coagulation for 1 min was enough to meet the requirement for bacteriophage removal prior to the MF process.

Another possibility is that the contribution to virus removal by the cake layer or foulant may be the same in the three mixing types tested. Formation of the cake layer on the surface of the membrane and the deposition of foulant on the internal wall of the pore of the membrane over time plays an important role in the performance of virus removal not only during the direct MF process (Jacangelo *et al.* 1995; Madaeni *et al.* 1995) but also during the coagulation–MF process (Shirasaki *et al.* 2008). The amount of aluminium floc particles introduced into the MF module for both the in-line mixing system and the rapid mechanical mixing system was approximately three times as high as that for the mechanical mixing system followed by settling, because approximately 75% of the total amount of aluminium floc particles had settled before the water was fed into the MF module. However, the cake layer and foulant do not always contribute to virus removal (Shirasaki *et al.* 2008). It is possible that the extent of cake layer formation and foulant deposition contributing to virus removal is similar in all three types of mixing system.

Here, we propose that a PACl dose of 1.08 mg-Al/L and a coagulation time of 1 min are required to achieve high removal ratios of infectious and total viruses in the present in-line coagulation–ceramic MF system. Virus removal by the in-line coagulation–ceramic MF system is similar to that by the coagulation–ceramic MF system incorporating mechanical mixing. However, virus removal may be affected by the quality of the source water. To elucidate whether the coagulation conditions employed in the present study are sufficient for all water sources, further investigations using a wide variety of water sources are needed.

CONCLUSIONS

1. The performance of the in-line coagulation–ceramic MF system in removing the bacteriophages Qβ and MS was efficient at a coagulant dose of 1.08 mg-Al/L, and its