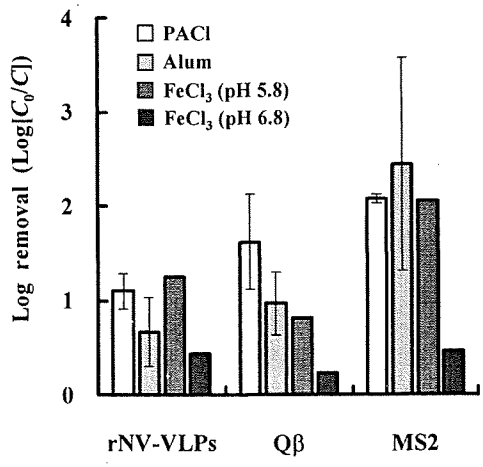

図-6. 各凝集剤におけるrNV-VLPs, Qβ, MS2の除去率比較: Coagulant dose 40 μM

後）のrNV-VLPs, Qβ, MS2の除去率を図-6に示す. 比較のため, pH 6.8における$FeCl_3$を用いた実験結果についても示す. 図より, rNV-VLPsにおいては, 凝集剤としてPAClおよび$FeCl_3$（pH 5.8）を用いた場合に1 log以上の除去率が得られた. 一方, Alum, $FeCl_3$（pH 6.8）を用いた場合の除去率はそれぞれ0.7, 0.4 logであった. Qβにおいては, PAClを用いた場合の除去率が1.6 logと最も高く, また, MS2においてはPACl, Alum, $FeCl_3$（pH 5.8）のいずれにおいても2 log程度の除去率が得られた. この結果から, PAClが最も効果的にrNV-VLPs, Qβ, MS2を除去できることが分かった.

rNV-VLPs, Qβ, MS2の除去率を比較すると, $FeCl_3$（pH 6.8）を用いた場合を除き, rNV-VLPsとQβは全体として約1 log程度の除去率であったのに対し, MS2は約2 log程度の除去率となった. 欧米諸国においては, MS2が病原性ウイルスの代替指標として幅広く用いられているが, 本研究で行った凝集沈澱処理においては, rNV-VLPsの除去率はMS2の除去率よりも低く, Qβの除去率と同程度であったため, MS2に比べてQβがヒトノロウイルスの指標と成り得る可能性が考えられた.

我が国の水道水質基準には, 微生物に関する項目として一般細菌, 大腸菌が規定されているが, ウイルスに関する基準は現在のところない. 一方, U.S.EPAは, 浄水処理（ろ過および消毒）によって表流水, 地下水の腸管系ウイルスを4 log除去あるいは不活化することを規定している[27]. 本研究で行った凝集沈澱処理においては, rNV-VLPsの除去率は最大で1.7 log（$FeCl_3$, 60 μM-Fe, pH 5.8）であったことから, ヒトノロウイルスを4 log除去あるいは不活化するためには, 後段にろ過処理, 消毒処理等を組み合わせる必要があると考えられる.

4. 結論

本研究では, 細胞培養系が確立されていないヒトノロウイルスの凝集沈澱処理性を, 粒子形状が保証されたヒトノロウイルスの外套タンパク粒子（rNV-VLPs）を用いることによって評価した. また, 病原性ウイルスの代替指標として広く用いられている大腸菌ファージQβ, MS2との処理性比較を行った.

本研究で得られた知見を以下にまとめる.

(1) rNV-VLPsを用いることにより, ヒトノロウイルスの凝集沈澱処理性を評価することができた.

(2) 凝集沈澱処理におけるrNV-VLPsの除去率は, 凝集剤添加濃度40 μMにおいて0.4-1.2 logであり, PACl, $FeCl_3$（pH 5.8）を用いた場合に1 log以上の除去率が得られた.

(3) 凝集沈澱処理におけるrNV-VLPsの除去率は, MS2の除去率よりも低く, また, Qβの除去率と同程度であった. 従って, MS2に比べてQβがヒトノロウイルスの指標と成り得る可能性が考えられた.

謝辞：本研究は, 科学研究費補助金　若手研究(B)「ウイルス外套タンパク粒子を用いたノロウイルスの浄水処理性の検討」（平成19-20年度, 課題番号 19760368, 代表　松下拓）ならびに厚生労働科学研究費補助金「飲料水の水質リスク管理に関する統合的研究」（平成19-21年度, 課題番号 19310801, 主任研究者　松井佳彦）により行われた. 記して感謝の意を表す.

参考文献

1) Havelaar A.H., Olphen M.V. and Drost Y.C.: F-specific RNA bacteriophage are adequate model organisms for enteric viruses in fresh water, *Applied and Environmental Microbiology*, Vol. 59, No. 9, pp. 2956-2962, 1993.

2) U.S. Environmental Protection Agency: Drinking Water Contaminant Candidate List and Regulatory Determinations, Contaminant Candidate List 3 (CCL 3), U.S. Environmental Protection Agency, Washington DC., 2008.

3) Grabow W.O., Gauss-Müller V., Prozesky O.W. and Deinhardt F.: Inactivation of hepatitis A virus and indicator organisms in water by free chlorine residuals, *Applied and Environmental Microbiology*, Vol. 46, No. 3, pp. 619-624, 1983.

4) Herbold, K., Flehmig B. and Botzenhart K.,: Comparison of ozone inactivation, in flowing water, of hepatitis A virus, poliovirus 1, and indicator organisms, *Applied and Environmental Microbiology*, Vol. 55, No. 11, pp. 2949-2953, 1989.

5) Nasser A., Weinberg D., Dinoor N., Fattal B. and Adin A.: Removal of hepatitis A virus (HAV), poliovirus and MS2 coliphage by coagulation and high rate filtration, *Water Science and Technology*, Vol. 31, No. 5-6, pp. 63-68, 1995.

6) Zheng D.P., Ando T., Fankhauser R.L., Beard R.S., Glass R.I. and Monroe S.S.: Norovirus classification and proposed strain nomenclature, *Virology*, Vol. 346, No. 2, pp. 312-323, 2006.

7) Park G.W., Boston D.M., Kase J.A., Sampson M.N. and Sobsey M.D.: Evaluation of liquid- and fog-based application of sterilox hypochlorous acid solution for surface inactivation of human norovirus, *Applied and Environmental Microbiology*, Vol. 73, No. 14, pp. 4463-4468, 2007.

8) 北島正章，遠矢幸伸，松原康一，原本英司，宇田川悦子，片山浩之，大垣眞一郎：新たな代替指標としてマウス分離株を用いた水道水中のノロウイルスの塩素耐性の解明，環境工学研究論文集，Vol. 45, pp. 361-370, 2008.

9) Thurston-Enriquez J.A., Haas C.N., Jacangelo J., Riley K. and Gerba C.P.: Inactivation of feline calicivirus and adenovirus type 40 by UV radiation, *Applied and Environmental Microbiology*, Vol. 69, No. 1, pp. 577-582, 2003.

10) Thurston-Enriquez J.A., Haas C.N., Jacangelo J. and Gerba C.P.: Chlorine Inactivation of Adenovirus Type 40 and Feline Calicivirus, *Applied and Environmental Microbiology*, Vol. 69, No. 7, pp. 3979-3985, 2003.

11) Duizer E., Bijkerk P., Rockx B., Groot A., Twisk F. and Koopmans M.: Inactivation of Caliciviruses, *Applied and Environmental Microbiology*, Vol. 70, No. 8, pp. 4538-4543, 2004.

12) Thurston-Enriquez J.A., Haas C.N., Jacangelo J. and Gerba C.P.: Inactivation of enteric adenovirus and feline calicivirus by ozone, *Water Research*, Vol. 39, No. 15, pp. 3650-3656, 2005.

13) Jiang X., Wang M., Graham D.Y. and Estes M.K.: Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein, *Journal of Virology*, Vol. 66, No. 11, pp. 6527-6532, 1992.

14) White L.J., Ball J.M., Hardy M.E., Tanaka T.N., Kitamoto N. and Estes M.K.: Attachment and entry of recombinant Norwalk virus capsids to cultured human and animal cell lines, *Journal of Virology*, Vol. 70, No. 10, pp. 6589-6597, 1996.

15) Parker T.D., Kitamoto N., Tanaka T., Huston A.M. and Estes M.K.: Identification of genogroup I and genogroup II broadly reactive epitopes on the norovirus capsid, *Journal of Virology*, Vol. 79, No. 12, pp. 7402-7409, 2005.

16) Redman J.A., Grant S.B. and Olson T.M.: Filtration of recombinant Norwalk virus particles and bacteriophage MS2 in quartz sand: importance of electrostatic interactions, *Environmental Science and Technology*, Vol. 31, No. 12, pp. 3378-3383, 1997.

17) Someya, Y., Takeda N. and Miyamura T.: Complete nucleotide sequence of the chiba virus genome and functional expression of the 3C-like protease in *Escherichia coli*, *Virology*, Vol. 278, No. 2, pp. 490-500, 2000.

18) 植竹久雄: ウイルス学（第4版）, pp. 34, 理工学社, 1995.

19) Langlet J., Gaboriaud F., Duval J.F.L. and Gantzer C.: Aggregation and surface properties of F-specific RNA phages: implication for membrane filtration processes, *Water Research*, Vol. 42, No. 10-11, pp. 2769-2777, 2008.

20) Adams M.H.: *Bacteriophages*, Interscience Publishers, New York, NY, USA, pp. 450-454, 1959.

21) 片山浩之，嶋崎明寛，大垣眞一郎：陰電荷膜を用いた酸洗浄・アルカリ誘出によるウイルス濃縮法の開発，水環境学会誌，Vol. 25, No. 8, pp. 469-475, 2002.

22) O'Connell K.P., Bucher J.R., Anderson P.E., Cao C. ., Khan A.S., Gostomski M.V. and Valdes J.J.: Real time fluorogenic reverse transcription-PCR assays for detection of bacteriophage MS2, *Applied and Environmental Microbiology*, Vol. 72, No. 1, pp. 478-483, 2006.

23) Utagawa E.T., Takeda N., Inouye S., Kasuga K. and Yamazaki S.: 3'-Terminal sequence of a small round structured virus (SRSV) in Japan, *Archive of Virology*, Vol. 135, No. 1-2, pp. 185-192, 1994.

24) Katayama K., Hansman G.S., Oka T., Ogawa S. and Takeda N.: Investigation of norovirus replication in a human cell line, *Archive of Virology*, Vol. 151, No. 7, pp. 1291-1308, 2006.

25) American Water Works Association, in: F.W. Pontius (Ed.), Water Quality and Treatment, A Handbook of Community Water Supplies, 4th ed., McGraw-Hill, New York, pp. 300-305, 1990.

26) Abbaszadegan M., Mayer B.K., Ryu H. and Nwachuku N.: Efficacy of removal of CCL viruses under enhanced coagulation conditions, *Environmental Science and Technology*, Vol. 41, No. 3, pp. 971-977, 2007.

27) U.S. Environmental Protection Agency: National Primary Drinking Water Standards, EPA816-F-01-007, Office of Water, U.S. Environmental Protection Agency, Washington, DC, 2001.

(2009. 5. 22 受付)

Evaluation of Removal Performance of Human Norovirus during Drinking Water Treatment Process by using Recombinant Norovirus VLPs

Nobutaka SHIRASAKI[1], Taku MATSUSHITA[1], Yoshihiko MATSUI[1], Atsushi OSHIBA[1], Toshifumi URASAKI[1] and Koichi OHNO[1]

[1]Division of Built Environment, Graduate School of Engineering, Hokkaido University

Norovirus (NV) is the prototype strains of a group of human caliciviruses responsible for epidemic outbreaks of acute gastroenteritis in the worldwide. As a result of the lack of a mammalian cell culture model for this virus, the studies on drinking water treatment such as separation and disinfection processes are still hampered. In the present study, we tried to investigate the removal performance of NV as particles during the coagulation–sedimentation process by using recombinant NV virus-like particles (rNV-VLPs) that are morphologically and antigenically similar to native NV. In addition, the behaviors of bacteriophage Qβ and MS2 were also experimentally investigated to be compared with that of rNV-VLPs, and then to discuss the suitability of these bacteriophages as surrogates for NV. Approximately 1-log removal was observed for rNV-VLPs with 40 μM-Al of polyaluminum chloride dosage in the coagulation–sedimentation process. In addition, the removal ratios of MS2 were approximately 1-log larger than that of rNV-VLPs and Qβ. Accordingly, Qβ is more suitable surrogates for native NV compared to the MS2 in the coagulation–sedimentation process.

WATER RESEARCH 43 (2009) 605–612

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/watres

Comparison of behaviors of two surrogates for pathogenic waterborne viruses, bacteriophages Qβ and MS2, during the aluminum coagulation process

N. Shirasaki, T. Matsushita, Y. Matsui, T. Urasaki, K. Ohno*

Division of Built Environment, Graduate School of Engineering, Hokkaido University, N13W8, Sapporo 060-8628, Japan

ARTICLE INFO

Article history:
Received 9 May 2008
Received in revised form
2 October 2008
Accepted 6 November 2008
Published online 19 November 2008

Keywords:
Bacteriophages
Coagulation
Electrophoretic mobility
Particle size distribution
Real-time RT-PCR
Virus inactivation

ABSTRACT

Differences in the behaviors of two surrogates for pathogenic waterborne viruses, F-specific RNA bacteriophages Qβ and MS2, were investigated during the coagulation process by using river water spiked with these bacteriophages. The particle size and electrophoretic mobility of Qβ and MS2 were similar, but the removal performances of infectious Qβ and MS2, as measured by a plaque forming unit (PFU) method, differed markedly during the coagulation process. The removal ratio of the infectious Qβ concentration was approximately 2 log higher than that of the infectious MS2 concentration at all coagulant doses tested. The total Qβ and MS2 bacteriophage concentrations, which were measured by a real-time reverse transcription-polymerase chain reaction (RT-PCR) method and represented the total number of bacteriophages regardless of their infectivity, were similar after the coagulation process, suggesting that the behaviors of Qβ and MS2 as particles were similar during the coagulation process. The difference between total concentration and infectious concentration indicated that some of the bacteriophages were probably inactivated during the coagulation process. This difference was larger for Qβ than MS2, meaning that Qβ was more sensitive to the virucidal activity of the aluminum coagulant. Analysis of the PFU and real-time RT-PCR findings together suggested that the difference in removal performances of Qβ and MS2 during the coagulation process was probably caused by differences not in the extent of bacteriophage entrapment in the aluminum floc particles but in the sensitivity to virucidal activity of the aluminum coagulant.

1. Introduction

During drinking water treatment, coagulation is an essential process for combining small particles into larger aggregates. Small particles in the drinking water source, such as viruses, that will not settle from suspension by gravity are destabilized and combined into larger aggregates during the coagulation process; this allows the small particles to be effectively removed by subsequent sedimentation and filtration processes. Several studies have reported the usefulness of the coagulation process for the removal of enteric viruses and bacteriophages, which are viruses that infect bacteria (Guy et al., 1977; Havelaar et al., 1995; Nasser et al., 1995).

Some bacteriophages have been evaluated as possible indicators for enteric viruses. For instance, F-specific RNA bacteriophage concentrations are highly correlated with those of enteric viruses in a wide range of water environments and water treatment processes (Havelaar et al., 1993). Because of their morphological similarity to hepatitis A viruses and polioviruses, F-specific RNA bacteriophage MS2 is widely used

* *Corresponding author.* Tel.: +81 11 706 7281; fax: +81 11 706 7279.
E-mail address: nobutaka@eng.hokudai.ac.jp (N. Shirasaki).
0043-1354/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.watres.2008.11.002

as a surrogate for pathogenic waterborne viruses in Europe and the United States (Jacangelo et al., 1995; Meng and Gerba, 1996; Redman et al., 1997; Shin and Sobsey, 1998, 2003; Sobsey et al., 1998; Meschke and Sobsey, 2003; Thurston-Enriquez et al., 2003; Zhu et al., 2005; Fiksdal and Leiknes, 2006), whereas the F-specific RNA bacteriophage Qβ tends to be used as a surrogate in Japan (Kamiko and Ohgaki, 1989; Urase et al., 1996; Otaki et al., 1998).

Previous studies have compared the behaviors of Qβ and MS2 in the physicochemical treatment process. Their behaviors are similar in the process of adsorption onto solid surfaces (such as cellulose, kaolin, carbon black and river sediment; Sakoda et al., 1997), the sandy aquifer treatment process (Dowd et al., 1998), and the direct microfiltration treatment process (Herath et al., 2000), whereas the sensitivities of Qβ and MS2 to ultraviolet radiation are significantly different (Blatchley et al., 2008). The behaviors of these two bacteriophages in the coagulation process may be different as well, although these differences have not been investigated except for previous studies of our research group (Matsui et al., 2003; Matsushita et al., 2004). If significant differences do exist, then Qβ and MS2 cannot be used as equivalent surrogates to evaluate the effectiveness of treatment processes for the removal of pathogenic waterborne viruses.

Virucidal activity during the water treatment process has been widely investigated. Ultraviolet radiation (Kamiko and Ohgaki, 1989; Meng and Gerba, 1996; Sobsey et al., 1998; Thurston-Enriquez et al., 2003) as well as disinfectants such as free chlorine, chlorine dioxide (Sobsey et al., 1998), chloramines (Shin and Sobsey, 1998) and ozone (Shin and Sobsey, 2003) are well known to inactivate viruses. Our research group has reported the virucidal activity of aluminum coagulant (Matsui et al., 2003; Matsushita et al., 2004). In our previous studies, we used the plaque forming unit (PFU) method in an attempt to determine the mechanisms and kinetics of the virucidal activity of aluminum coagulant. During coagulation of the virus suspension, although the majority of viruses were entrapped in the aluminum floc particles, some of the viruses remained suspended in the liquid phase. Not all the suspended viruses were infectious; some were likely inactivated by the virucidal activity of aluminum coagulant. Thus, virus removal during the coagulation process includes two mechanisms: entrapment in floc particles and inactivation. The PFU method, however, can detect neither the viruses entrapped in the floc particles nor the inactivated viruses judging from its measurement principle. Therefore, even though the virus concentration measured by the PFU method decreases in the liquid phase during the coagulation process, it remains unclear whether the decrease results from the entrapment in floc particles, inactivation, or both.

The reverse transcription-polymerase chain reaction (RT-PCR) method is capable of amplifying small regions of viral nucleic acid. Thus, RT-PCR can detect infectious as well as inactivated viruses, unless the target nucleic acid is lost, but not all viruses entrapped in the floc particles can be detected by this method. Calculation of the concentration of entrapped virus particles simply entails subtracting the concentration of total viruses including infectious and inactivated viruses in the liquid phase, as determined by RT-PCR, from the total virus concentration as measured in the raw water. Likewise, subtracting the concentration of infectious viruses, as determined by the PFU method, from the total virus concentration, as determined by RT-PCR, gives the number of inactivated viruses. Therefore, combining the PFU and RT-PCR methods allows entrapped viruses and inactivated viruses in the coagulation process to be clearly distinguished.

Our objectives were to investigate and compare the behaviors of two bacteriophages, Qβ and MS2, during the coagulation process by using both PFU and real-time RT-PCR methods and to elucidate what caused the differences.

2. Materials and methods

2.1. Source water and coagulant

River water was sampled from the Toyohira River (Sapporo, Japan; water quality shown in Table 1) on 12 October 2007. Polyaluminum chloride (PACl) (250A; 10.5% Al_2O_3, relative density 1.2 at 20 °C; Taki Chemical Co., Ltd., Hyogo, Japan) was used for coagulation experiments.

2.2. Bacteriophages

The bacteriophages Qβ (NBRC 20012) and MS2 (NBRC 102619) were obtained from the NITE Biological Research Center (NBRC, Chiba, Japan). Qβ is the prototype member of the genus *Allolevivirus* in the virus family *Leviviridae*, and MS2 is the prototype member of the genus *Levivirus* in the *Leviviridae*. The genomes of these two bacteriophages 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 (The Universal Virus Database of the International Committee on Taxonomy of Viruses). 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 (2000×*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; Centriplus-100, Millipore Corp., Billerica, MA, USA) to prepare the bacteriophage stock solution. The concentration of each bacteriophage stock solution was approximately 10^{12} PFU/mL.

2.3. Batch coagulation experiments

Batch coagulation experiments were conducted with 200 mL of bacteriophage-spiked river water in glass beakers at 20 °C. The bacteriophage stock solution was added to the beaker at

Table 1 – Water quality of the Toyohira River.

pH	7.5
DOC (mg/L)	0.90
OD260 (cm^{-1})	0.027
Turbidity (NTU)	0.50
Alkalinity (mg-$CaCO_3$/L)	19.1

approximately 10^6 or 10^8 PFU/mL and mixed with an impeller stirrer. PACl was injected into the water as a coagulant at dosages of 0.54, 1.08 or 1.62 mg-Al/L. The pH of the water was immediately adjusted to, and maintained at, 6.8 using HCl. The water was stirred rapidly for 2 min ($G = 200\ s^{-1}$, 61 rpm) and then slowly for 28 min ($G = 20\ s^{-1}$, 13 rpm). The water was then left at rest for 20 min to settle the aluminum floc particles generated. Samples were taken from the beaker before coagulant dosing and after rapid mixing, slow mixing, and settling for quantification of the bacteriophage concentrations.

To quantify the bacteriophage concentration in the liquid phase of the floc mixture, the suspended floc particles were separated from the mixture by centrifugation (2000×*g*, 10 min), and the bacteriophage concentration in the supernatant was measured by the PFU and the real-time RT-PCR methods (see Section 2.4).

In addition, to quantify the bacteriophage concentration in the floc particles, the particles were dissolved by raising the pH of the water to 9.5 with NaOH in 12% beef extract (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) solution and the water was vortexed for 5 h at 4 °C by using direct mixer (DM-301, As One Corp., Osaka, Japan) at 2000 rpm. Beef extract was used in an effort to prevent the inactivation of bacteriophages during floc dissolution (Matsui et al., 2003). Samples of raw water and rapid mixing were taken from the beaker directly for the floc dissolution, because these samples were considered to be mixed completely during the sampling. In contrast, the floc particles settled during the coagulation process were resuspended after the settling process by strong mixing with a magnetic stirrer at 1400 rpm for subsequent sampling and floc dissolution (floc dissolution procedure was described above). After floc dissolution, the bacteriophage concentration in the sample was measured by the PFU and real-time RT-PCR methods.

Because substances such as natural organic matter (Abbaszadegan et al., 1993; Kreader, 1996) and beef extract (Abbaszadegan et al., 1993; Sano et al., 2003) are known to inhibit the amplification of the viral genome by PCR, each sample was diluted 10-fold with Milli-Q water (Milli-Q Advantage, Millipore Corp.) before the real-time RT-PCR quantification.

2.4. *Bacteriophage assays*

2.4.1. *PFU method*

The infectious bacteriophages were enumerated according to the double-layer method (Adams, 1959) by using the bacterial host *E. coli* (NBRC 13965). The average of plaque counts of triplicate plates prepared from one sample was considered as the infectious bacteriophage concentration.

2.4.2. *Real-time RT-PCR method*

Viral RNA of bacteriophages was quantified by the real-time RT-PCR method, which detects viruses regardless of their infectivity. We defined concentration measured by the real-time RT-PCR method as total bacteriophage concentration. For quantification of bacteriophages in the raw water and liquid phase of the floc mixture, viral RNA was extracted from 140 μL of sample with a QIAamp Viral RNA Mini Kit (Qiagen K.K., Tokyo, Japan) to obtain a final volume of 60 μL. For quantification of bacteriophages in the floc dissolution sample containing beef extract, a 100-μL sample was heated at 90 °C for 10 min and then cooled to 4 °C for 1 min in a thermal cycler (Thermal Cycler Dice Model TP600, Takara Bio Inc., Shiga, Japan) to extract viral RNA by destroying the capsid. 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. 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 50 cycles of 95 °C for 15 s and 60 °C for 1 min in an ABI Prism 7000 Sequence Detection 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 PFU method and the number of cycles for amplification in the PCR process, which is based on the assumption that the freshly prepared stock solution did not contain any inactivated bacteriophages.

2.5. *Particle size distribution*

Particle size distribution of bacteriophages was measured in prepared Milli-Q water and filtered river water. To bring the alkalinity to 20 mg-$CaCO_3$/L, 0.4 mM $NaHCO_3$ was added to the Milli-Q water, and the pH of was adjusted to 6.8 with HCl. River water was filtered through a stirred ultrafiltration cell (Model 8400, Millipore Corp.) with ultrafiltration membrane (molecular weight cutoff 100,000, regenerated cellulose; Ultrafiltration Disks, YM-100, Millipore Corp.) to exclude the large particles, and the pH was adjusted to 6.8 with HCl. The Milli-Q and river water samples were kept for 1 day at 20 °C to stabilize the pH. Just before the measurement of particle size distribution, each bacteriophage was suspended at approximately 10^{10} PFU/mL in the prepared Milli-Q water or filtered river water using the bacteriophage stock solution. The particle size distribution of the bacteriophages was measured with a fiber-optic dynamic light-scattering spectrophotometer (FDLS-3000, Otsuka Electronics Co., Ltd., Osaka, Japan) 200 or 400 times for each sample at 25 °C and at a 90° measurement angle.

2.6. *Electrophoretic mobility*

In Milli-Q water and river water prepared as described in Section 2.5, an electrophoretic light-scattering spectrophotometer (ELS-6000, Otsuka Electronics Co., Ltd.) was used to measure electrophoretic mobility. Just before the measurement, each bacteriophage was suspended at approximately 10^{10} PFU/mL in the prepared Milli-Q water or filtered river water using the bacteriophage stock solution. The electrophoretic mobility of the bacteriophages was measured 25 times for each sample at 25 °C and at a 15° measurement angle.

Table 2 – Oligonucleotide sequences of the primers and 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′-GGG CAC GTG TTT TGA TCG A-3′	690–708	
	TaqMan probe	5′-AGG CGC TCC GCT ACC TTG CCC T-3′	650–671	

3. Results and discussion

3.1. *Particle size distribution*

Fig. 1 shows the particle size distributions of Qβ and MS2 in the prepared Milli-Q water and filtered river water at pH 6.8. In both the Milli-Q and river water, the particle sizes were distributed over the range of 20–30 nm. These values correspond with the particle diameters previously reported for Qβ and MS2 (The Universal Virus Database of the International Committee on Taxonomy of Viruses). Thus, we can assume that no virus–virus aggregate was generated and these two bacteriophages were stably monodispersed in the raw water used (without coagulant dosing).

Langlet et al. (2008) reported that Qβ had a tendency to aggregate in solutions with high ionic strength: Qβ aggregated in deionized water with 100 mM $NaNO_3$ at neutral pH (ionic strength, approximately 1×10^{-1}), whereas it did not aggregate in deionized water with 1 mM $NaNO_3$ (ionic strength, approximately 1×10^{-3}). The ionic strength of both our prepared Milli-Q water and the filtered river water was approximately 1×10^{-3}.

3.2. *Effect of coagulant dose on infectious bacteriophage removal*

Fig. 2 shows the effect of coagulant dose on infectious bacteriophage removal, as measured by the PFU method after settling without centrifugal separation. Bacteriophage removal was not observed without PACl dosing, and the removal ratio of infectious bacteriophages ($\log[C_0/C]$) increased with coagulant dose. At a PACl dose of more than 1.08 mg-Al/L, more than 2-log removal of infectious bacteriophage was achieved for both Qβ and MS2. Therefore, the coagulation process is effective for the removal of infectious bacteriophages. Other studies have reported the usefulness of the coagulation process for removing infectious enteric viruses and infectious bacteriophages (Guy et al., 1977; Havelaar et al., 1995; Nasser et al., 1995).

In our study, however, the removal performances of Qβ and MS2 were quite different. The removal ratio of infectious Qβ was approximately 2 log higher than that of infectious MS2 at each of the PACl doses tested. This difference is detailed in the following section.

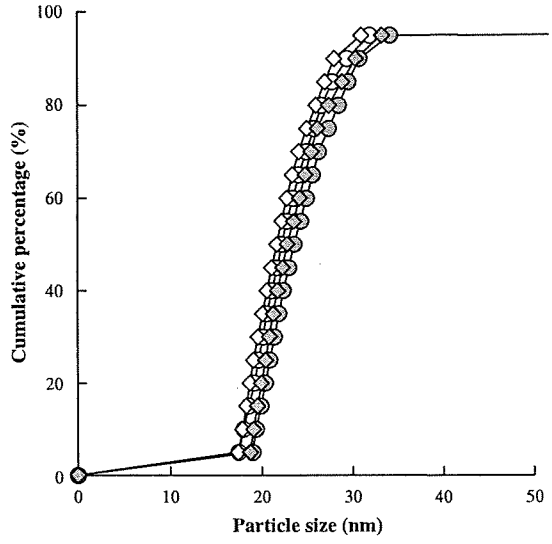

Fig. 1 – Particle size distribution of Qβ (white) and MS2 (gray) in the prepared Milli-Q water (circles) and filtered river water (diamonds), based on the number of particles. Values are the means of 200 or 400 measurements. Bacteriophage concentration in each sample was approximately 10^{10} PFU/mL.

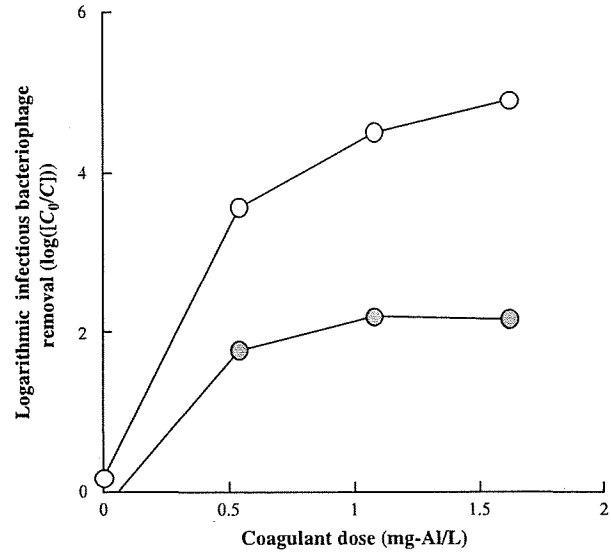

Fig. 2 – Effect of coagulant dose (0.54, 1.08 or 1.62 mg-Al/L) on infectious bacteriophage removal after settling without centrifugal separation. White and gray symbols represent Qβ and MS2, respectively. Initial bacteriophage concentrations in raw water were approximately 10^6 PFU/mL.

3.3. *Changes in the bacteriophage concentrations in the liquid phase of the floc mixture*

To investigate why the removal performances of Qβ and MS2 were different in the coagulation process, the changes in bacteriophage concentrations in the liquid phase of the floc mixture were measured during the coagulation process (Fig. 3). Although the DOC concentration of bacteriophage-spiked river water increased with the bacteriophage feed concentration, owing to the unavoidable uptake of the residual component of the culture medium, the removal ratios did not differ among initial concentrations of 10^6, 10^7 and 10^8 PFU/mL for both bacteriophages (data not shown). This finding suggests that the DOC component from the bacteriophage culture solution did not affect bacteriophage removal during the coagulation process.

The total bacteriophage concentrations of Qβ and MS2 decreased as coagulation progressed (Fig. 3a). These reductions from the initial concentrations in raw water were probably due to the entrapment of bacteriophages in the aluminum floc particles, because the floc particles entrapping the bacteriophages were excluded from the liquid phase by the centrifugal separation. At each stage of the coagulation process, the reduction in Qβ concentration was similar to that of MS2, with 2-log reductions achieved for both bacteriophages by the end of the process (Fig. 3a). This result suggests that the particle behaviors of Qβ and MS2 were the same during the coagulation process; this possibility is supported by the particles' electrophoretic mobility, an important factor in the coagulation process. The electrophoretic mobilities of Qβ and MS2 were compared using the *t*-test (two-tail) based on 0.01 level of significance. No significant ($P > 0.01$) difference in the electrophoretic mobility was observed between the two bacteriophages in the filtered river water at pH 6.8, whereas a significant ($P < 0.01$) difference was observed in the prepared Milli-Q water (Fig. 4). Therefore, these two bacteriophages behaved in a similar manner as particles in the river water.

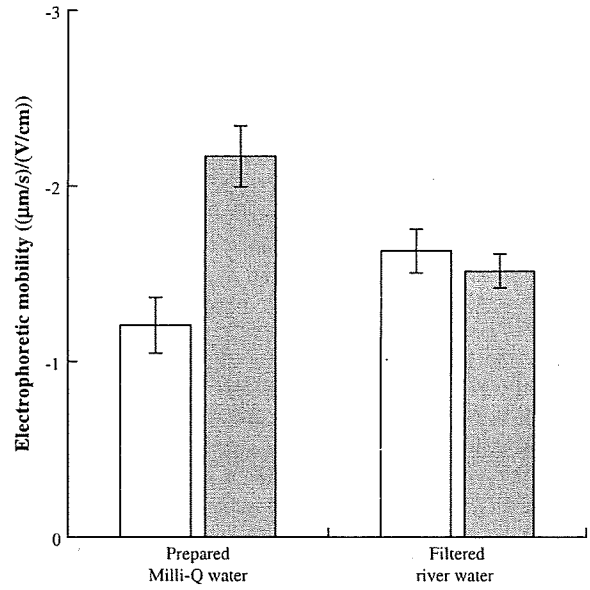

Fig. 4 – Electrophoretic mobility of Qβ (white) and MS2 (gray) in the prepared Milli-Q water and filtered river water. Values are the means and standard deviation of 25 measurements. Bacteriophage concentration of each sample was approximately 10^{10} PFU/mL.

Differences were observed between total bacteriophage concentration (Fig. 3a) and infectious bacteriophage concentration (Fig. 3b). The same phenomenon was

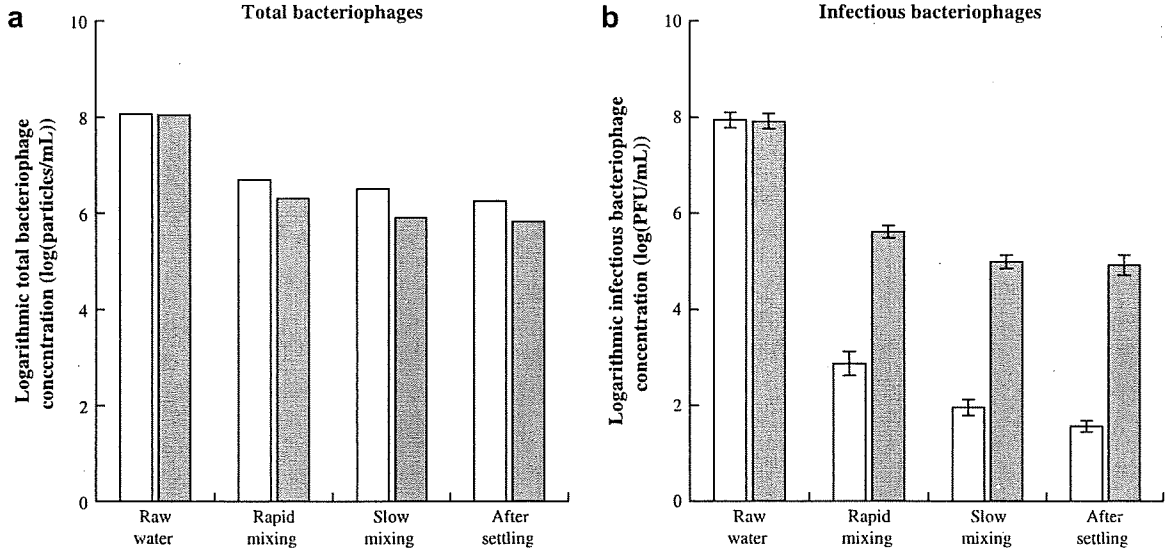

Fig. 3 – Changes in the concentrations of total bacteriophages (a) and infectious bacteriophages (b) in the liquid phase of the floc mixture during the coagulation process. The concentrations of all samples were measured after centrifugal separation. White and gray columns represent Qβ and MS2, respectively. Values of infectious bacteriophage concentrations are means and standard deviation of three replicates for Qβ and five replicates for MS2. Initial bacteriophage concentrations of raw water were approximately 10^8 PFU/mL. PACl dosage was 1.08 mg-Al/L.

observed during the coagulation–microfiltration (MF) process with 1.08 mg-Al/L of PACl (Shirasaki et al., in press): total Qβ concentration was larger than infectious Qβ concentration in the MF permeate. Because the pore size of the MF membrane used in the coagulation–MF process was 0.1 μm, the maximal size of microflocs which passed through the membrane was 0.1 μm. If the microfloc is assumed to be an aggregate of Qβ (50% cumulative percentage of particle size distribution of Qβ was 0.022 μm as shown in Fig. 1) having fractal dimension of 3.0, the 0.1-μm aggregate consists of 94 Qβ particles $((0.1\ \mu m/0.022\ \mu m)^{3.0} = 94)$. In other words, aggregates consisting more than 94 Qβ particles cannot pass through the MF membrane. If the difference between the total and infectious Qβ concentrations in the MF permeate is caused by aggregation alone, the difference is theoretically calculated at 2.0 log (log(94) = 2.0). Tambo and Watanabe (1979) reported that the fractal dimension of aluminum floc particles produced by PACl addition is in the range of 1.5–2.0. Then, the required difference which are theoretically calculated from the reported fractal dimension is in the range of 1.0 log $(\log(0.1\ \mu m/0.022\ \mu m)^{1.5})$–1.3 log $(\log(0.1\ \mu m/0.022\ \mu m)^{2.0})$. Actually, the differences between total and infectious Qβ concentrations which were observed in the coagulation–MF process were 1.1, 1.7, 2.3 and 2.4 logs at operation time of 1, 2, 3 and 4 h, respectively. These differences are more than the theoretically required differences. This means that the difference in the total and infectious bacteriophage concentrations cannot be explained by aggregation alone. A part of the bacteriophages must have been inactivated during the coagulation process. Therefore, in the present study, we believe that some the bacteriophages were inactivated during the coagulation process. Other researcher has also introduced the combination of the PFU and RT-PCR methods to discuss the virus inactivation during soil column filtration process, and suggest that the difference in the results between these two methods express the virus inactivation (Meschke and Sobsey, 2003).

The total and infectious bacteriophage concentrations differed markedly between Qβ and MS2: a 4-log difference was observed for Qβ, whereas less than a 1-log difference was observed for MS2. Thus, Qβ was more sensitive to virucidal activity of PACl than was MS2. Our research group previously reported on the virucidal activity of PACl (Matsui et al., 2003; Matsushita et al., 2004) and demonstrated that Qβ was the most sensitive to inactivation among four types of bacteriophages tested (Qβ, MS2, P1 and T4). The difference between Qβ and MS2 in their sensitivity to PACl may have resulted in the difference in the reduction of infectious bacteriophage concentrations: after settling, a 6-log reduction was achieved for Qβ, whereas a 3-log reduction was observed for MS2 (Fig. 3b). The virucidal activity of PACl for Qβ probably occurred in the liquid phase during the coagulation process.

The mechanism underlying the virucidal activity of the aluminum coagulant is not understood clearly. Matsui et al. (2003) reported that the infectivity of a bacteriophage did not decrease when the bacteriophage was exposed to a solution containing "preformed" aluminum hydroxide floc prepared by preneutralizing a solution of reagent-grade aluminum chloride with 1 M sodium carbonate at pH 7.5 for 3–19 days. This finding suggests that factors other than the presence of amorphous aluminum hydroxide rendered the bacteriophage inactive. PACl, which contains polymeric aluminum species, has been reported to induce much greater virucidal activity than monomeric aluminum coagulants, including reagent-grade aluminum chloride and aluminum sulfate, and alum used in actual treatment plants (Matsui et al., 2003; Matsushita et al., 2004). Thus, the polymeric aluminum species might play an important role in the virucidal activity. We

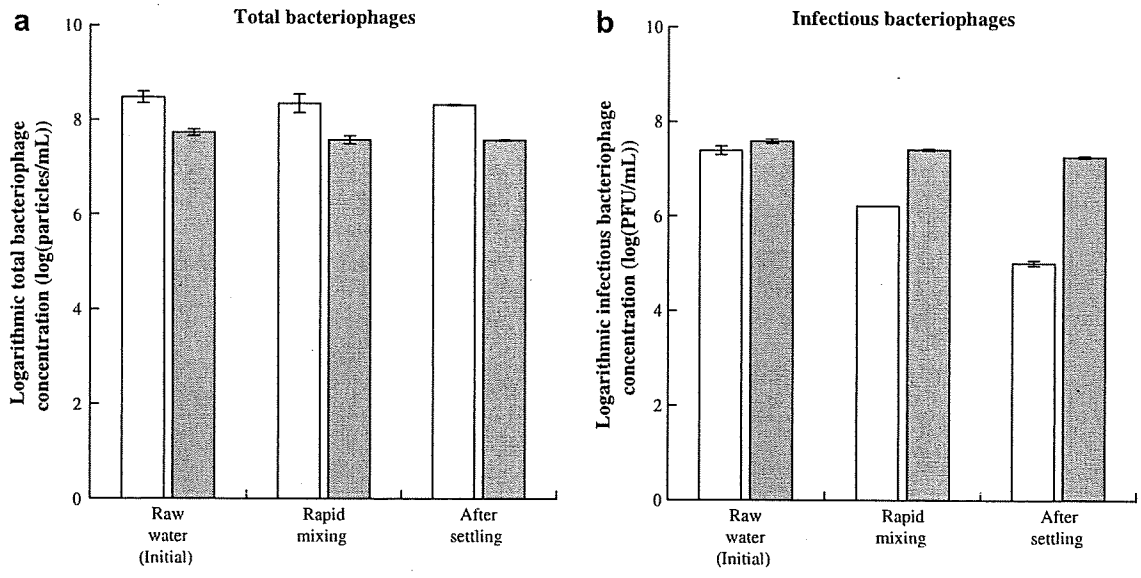

Fig. 5 – Changes in the concentrations of total bacteriophages (a) and infectious bacteriophages (b) of the floc mixture during the coagulation process. The concentrations of all samples were measured after floc dissolution. White and gray columns represent Qβ and MS2, respectively. Values are means and standard deviation of two replicates. Initial bacteriophage concentrations in raw water were approximately 10^8 PFU/mL. PACl dosage was 1.08 mg-Al/L.

imagine that some polymers formed during the hydrolysis of aluminum coagulant might sorb strongly to bacteriophages, either rendering them inactive or preventing infectivity, although further study is needed.

Our results revealed significant differences in the behaviors of Qβ and MS2 during the coagulation process. When the performance of a treatment plant employing the coagulation process is evaluated by using Qβ or MS2 as a surrogate for pathogenic waterborne viruses, the results should be judged carefully while considering the differences in behaviors described in this paper.

3.4. *Changes in the bacteriophage concentrations of the floc mixture after floc dissolution*

Once bacteriophages are entrapped in the aluminum floc particles during the coagulation process, they cannot be enumerated directly by the PFU method or real-time RT-PCR method. To quantify the bacteriophages in the floc particles, these particles were dissolved and the total bacteriophage concentration was measured. This value was then compared with the total bacteriophage concentration in the raw water (initial value) to confirm whether the floc particles formed during the coagulation process were dissolved completely. For both Qβ and MS2, the total bacteriophage concentrations recovered to their initial values at each stage of the coagulation process (Fig. 5a), indicating the complete dissolution of the floc particles.

The infectious MS2 concentrations in samples taken after rapid mixing and settling almost completely recovered to the initial value upon floc dissolution (Fig. 5b), meaning that no significant virus inactivation was observed for MS2 in the floc particles. This finding is in accord with those of Zhu et al. (2005), who suggested that MS2 was not inactivated by aluminum coagulant according to jar tests with 1–5 mg-Al/L: the infectious MS2 concentrations before and after coagulation, settling, and resuspension of the coagulated sludge were not statistically different. In contrast, infectious Qβ concentrations in the sample after rapid mixing and settling were still 1–2 log lower than the initial value after floc dissolution, indicating that the infectious Qβ concentration did not recover to its initial value (Fig. 5b). Therefore, Qβ was inactivated not only in the liquid phase but also in the floc particles during the coagulation process.

When the raw water in a drinking water treatment plant is polluted with a virus, the infectious virus is expected to be concentrated in the treatment sludge produced during the coagulation process; this would potentially increase the risk of infection when exposed to this sludge. In contrast, if the virucidal activity of the aluminum coagulant were consistent with that demonstrated for Qβ, most of the virus in the sludge would be inactivated. Therefore, the risk associated with exposure to the sludge could be decreased by using aluminum coagulant.

4. Conclusions

(1) Although their particle size and electrophoretic mobility were similar, the removal performances of infectious Qβ and MS2 during the coagulation process differed markedly: the removal ratio of infectious Qβ was approximately 2 log higher than that of infectious MS2 at all the PACl doses tested.
(2) Use of a combination of PFU and real-time RT-PCR methods revealed that the difference in behaviors between Qβ and MS2 during the coagulation process was probably not due to the difference in the extent of entrapment in the aluminum floc particles but to the difference in sensitivity to the virucidal activity of PACl. Significant inactivation of Qβ was observed during the coagulation process, whereas little or no inactivation of MS2 was observed.
(3) When the performance of a treatment plant employing the coagulation process is evaluated by using either Qβ or MS2, the results should be carefully judged considering the differences in their behaviors.

Acknowledgement

This research was supported in part by a Grant-in-Aid for the Encouragement of Young Scientists (no. 19760368, 2007) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, a Grant-in-Aid (no. 19310801, 2007) from the Ministry of Health, Labor, and Welfare of Japan, and the Kajima Foundation's Research Grant (2007).

REFERENCES

Abbaszadegan, M., Huber, M.S., Gerba, C.P., Pepper, I.L., 1993. Detection of enteroviruses in groundwater with the polymerase chain reaction. Applied and Environmental Microbiology 59 (5), 1318–1324.

Adams, M.H., 1959. Bacteriophages. Interscience Publishers, New York, pp. 450–454.

Blatchley III, E.R., Shen, C., Scheible, O.K., Robinson, J.P., Ragheb, K., Bergstrom, D.E., Rokjer, D., 2008. Validation of large-scale, monochromatic UV disinfection systems for drinking water using dyed microspheres. Water Research 42 (3), 677–688.

Dowd, S.E., Pillai, S.D., Wang, S., Corapcioglu, M.Y., 1998. Delineating the specific influence of virus isoelectric point and size on virus adsorption and transport through sandy soils. Applied and Environmental Microbiology 64 (2), 405–410.

Fiksdal, L., Leiknes, T.O., 2006. The effect of coagulation with MF/UF membrane filtration for the removal of virus in drinking water. Journal of Membrane Science 279 (1–2), 364–371.

Guy, M.D., McIver, J.D., Lewis, M.J., 1977. The removal of virus by a pilot treatment plant. Water Research 11 (5), 421–428.

Havelaar, A.H., Olphen, M.V., Drost, Y.C., 1993. F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. Applied and Environmental Microbiology 59 (9), 2956–2962.

Havelaar, A.H., Olphen, M.V., Schijven, J.F., 1995. Removal and inactivation of viruses by drinking water treatment processes under full-scale conditions. Water Science and Technology 31 (5–6), 55–62.

Herath, G., Yamamoto, K., Urase, T., 2000. The effect of suction velocity on concentration polarization in microfiltration membranes under turbulent flow conditions. Journal of Membrane Science 169 (2), 175–183.

Jacangelo, J.G., Adham, S.S., Laîné, J.M., 1995. Mechanism of *Cryptosporidium*, *Giardia*, and MS2 virus removal by MF and UF. Journal of the American Water Works Association 87 (9), 107–121.

Kamiko, N., Ohgaki, S., 1989. RNA coliphage QBeta as a bioindicator of the ultraviolet disinfection efficiency. Water Science and Technology 21 (3), 227–231.

Katayama, H., Shimazaki, A., Ohgaki, S., 2002. Development of virus concentration method using negatively charged membrane by alkaline elution after acid rinse. Journal of Japan Society on Water Environment 25 (8), 469–475 (in Japanese).

Kreader, C.A., 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Applied and Environmental Microbiology 62 (3), 1102–1106.

Langlet, J., Gaboriaud, F., Duval, J.F.L., Gantzer, C., 1996. Aggregation and surface properties of F-specific RNA phages: implication for membrane filtration processes. Water Research 42 (10-11), 2769–2777.

Matsui, Y., Matsushita, T., Sakuma, S., Gojo, T., Mamiya, T., Suzuoki, H., Inoue, T., 2003. Virus inactivation in aluminum and polyaluminum coagulant. Environmental Science and Technology 37 (22), 5175–5180.

Matsushita, T., Matsui, Y., Inoue, T., 2004. Irreversible and reversible adhesions between virus particles and hydrolyzing-precipitating aluminum: a function of coagulation. Water Science and Technology 50 (12), 201–206.

Meng, Q.S., Gerba, C.P., 1996. Comparative inactivation of enteric adenoviruses, poliovirus and coliphages by ultraviolet irradiation. Water Research 30 (11), 2665–2668.

Meschke, J.S., Sobsey, M.D., 2003. Comparative reduction of Norwalk virus, poliovirus type 1, F+ RNA coliphage MS2 and *Escherichia coli* in miniature soil columns. Water Science and Technology 47 (3), 85–90.

Nasser, A., Weinberg, D., Dinoor, N., Fattal, B., Adin, A., 1995. Removal of hepatitis A virus (HAV), poliovirus and MS2 coliphage by coagulation and high rate filtration. Water Science and Technology 31 (5–6), 63–68.

O'Connell, K.P., Bucher, J.R., Anderson, P.E., Cao, C.J., Khan, A.S., Gostomski, M.V., Valdes, J.J., 2006. Real time fluorogenic reverse transcription-PCR assays for detection of bacteriophage MS2. Applied and Environmental Microbiology 72 (1), 478–483.

Otaki, M., Yano, K., Ohgaki, S., 1998. Virus removal in a membrane separation process. Water Science and Technology 37 (10), 107–116.

Redman, J.A., Grant, S.B., Olson, T.M., 1997. Filtration of recombinant Norwalk virus particles and bacteriophage MS2 in quartz sand: importance of electrostatic interactions. Environmental Science and Technology 31 (12), 3378–3383.

Sakoda, A., Sakai, Y., Hayakawa, K., Suzuki, M., 1997. Adsorption of viruses in water environment onto solid surfaces. Water Science and Technology 35 (7), 107–114.

Sano, D., Fukushi, K., Yoshida, Y., Omura, T., 2003. Detection of enteric viruses in municipal sewage sludge by a combination of the enzymatic virus elution method and RT-PCR. Water Research 37 (14), 3490–3498.

Shin, G.A., Sobsey, M.D., 1998. Reduction of Norwalk virus, poliovirus 1 and coliphage MS2 by monochloramine disinfection of water. Water Science and Technology 38 (12), 151–154.

Shin, G.A., Sobsey, M.D., 2003. Reduction of Norwalk virus, poliovirus 1, and bacteriophage MS2 by ozone disinfection of water. Applied and Environmental Microbiology 69 (7), 3975–3978.

Shirasaki, N., Matsushita, T., Matsui, Y., Kobuke, M., Ohno, K. Comparison of removal performance of two surrogates for pathogenic waterborne viruses, bacteriophage Qβ and MS2, in a coagulation–ceramic microfiltration system. Journal of Membrane Science, in press, doi:10.1016/j.memsci.2008.10.037.

Sobsey, M.D., Battigelli, D.A., Shin, G.A., Newland, S.S., 1998. RT-PCR amplification detects inactivated viruses in water and wastewater. Water Science and Technology 38 (12), 91–94.

Tambo, N., Watanabe, Y., 1979. Physical characteristics of flocs-I. The floc density function and aluminum floc. Water Research 13 (5), 409–419.

Thurston-Enriquez, J.A., Haas, C.N., Jacangelo, J., Riley, K., Gerba, C.P., 2003. Inactivation of feline calicivirus and adenovirus type 40 by UV radiation. Applied and Environmental Microbiology 69 (1), 577–582.

The Universal Virus Database of the International Committee on Taxonomy of Viruses. <http://phene.cpmc.columbia.edu/index.htm> (accessed 17.04.08).

Urase, T., Yamamoto, K., Ohgaki, S., 1996. Effect of pore structure of membranes and module configuration on virus retention. Journal of Membrane Science 115 (1), 21–29.

Zhu, B., Clifford, D.A., Chellam, S., 2005. Virus removal by iron coagulation–microfiltration. Water Research 39 (20), 5153–5161.

Journal of Membrane Science 326 (2009) 564–571

Contents lists available at ScienceDirect

Journal of Membrane Science

journal homepage: www.elsevier.com/locate/memsci

Comparison of removal performance of two surrogates for pathogenic waterborne viruses, bacteriophage Qβ and MS2, in a coagulation–ceramic microfiltration system

N. Shirasaki*, T. Matsushita, Y. Matsui, M. Kobuke, K. Ohno

Division of Built Environment, Graduate School of Engineering, Hokkaido University, N13W8, Sapporo 060-8628, Japan

ARTICLE INFO

Article history:
Received 8 September 2008
Received in revised form 22 October 2008
Accepted 24 October 2008
Available online 5 November 2008

Keywords:
Bacteriophages
Coagulation
Ceramic microfiltration
Virus inactivation
Virus removal

ABSTRACT

The removal performance of two surrogates for pathogenic waterborne viruses, F-specific RNA bacteriophages Qβ and MS2, was evaluated during the coagulation–ceramic microfiltration process. River water spiked with these bacteriophages was used to investigate differences in their behaviors. Infectious and total (infectious + inactivated) bacteriophage concentrations were measured by plaque forming unit and real-time reverse transcription–polymerase chain reaction methods, respectively. Removal of infectious Qβ and MS2 was similar under each coagulation condition. Approximately 6-log reduction was achieved for both bacteriophages at 1.08 mg-Al/L of coagulant dose and 5-min coagulation time. At least 4-log reduction occurred even when coagulant dose and coagulation time were reduced to 0.54 mg-Al/L and 1.8 s, respectively. In contrast, removal of total Qβ and MS2 differed markedly. Removal of total MS2 was approximately 2-log larger than that of total Qβ, possibly owing to selective interaction with the cake layer, although the particle diameters and electrophoretic mobilities of Qβ and MS2 were similar. The total number of bacteriophages retained in the microfiltration compartment after 4-h filtration was similar for Qβ and MS2, but there were approximately 3 log fewer infectious Qβ than infectious MS2, probably owing to the difference in sensitivity to the virucidal activity of aluminum coagulant.

1. Introduction

Low-pressure membrane (microfiltration and ultrafiltration membranes) technologies provide important alternatives to separation processes such as sedimentation and rapid sand filtration. Microfiltration (MF) is effective for the reduction of turbidity and removal of bacteria, algae, and protozoa. Among MF membranes, ceramic 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 and other inorganic membranes [1]. These properties of ceramic membranes allow the use of strong acids and bases in chemical cleaning, application of high pressure for hydraulic backwashing, and operation at a high filtration flux. However, MF membranes alone cannot be expected to efficiently remove pathogenic waterborne viruses such as hepatitis A viruses, polioviruses, and noroviruses, because membrane pore sizes are not small enough. Some studies have reported insufficient virus removal by MF membrane alone [2–5], although the U.S. Environmental Protection Agency requires 4-log removal/inactivation of viruses [6].

Using a coagulation process as pretreatment for the MF process may mitigate membrane fouling and improve permeate water quality [7–9]. In addition, a high ratio of virus removal was achieved by the combination of coagulation and MF processes. Zhu et al. [10] reported a >4-log removal of viruses at pH 6.3 with 10 mg-Fe/L of ferric chloride and 0.22-μm pore size of polyvinylidene fluoride MF membrane, whereas MF alone achieved a <0.5-log removal. Fiksdal and Leiknes [11] reported that no virus removal was obtained without a coagulation/flocculation pretreatment process, whereas the combination of coagulation/flocculation and MF (0.2-μm pore size of polyethersulfone) processes with 3–5 mg-Al/L of commercial aluminum-based coagulant provided from 6.7- to >7.5-log removal of viruses. Our research group also reported the effectiveness of a coagulation–ceramic MF system [12,13]: a >6-log removal of viruses was achieved by two types of 0.1-μm pore size ceramic MF systems (positive pressure-driven monolith type and negative pressure-driven immersed type) with 0.5–1.0 mg-Al/L of polyaluminum chloride.

To evaluate the virus removal performance of the membrane filtration process, bacteriophages (i.e., viruses that infect

* Corresponding author. Tel.: +81 11 706 7281; fax: +81 11 706 7279.
E-mail address: nobutaka@eng.hokudai.ac.jp (N. Shirasaki).

0376-7388/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.memsci.2008.10.037

bacteria) have been used as possible indicators for enteric viruses. Because of their morphological similarity to hepatitis A viruses and polioviruses, F-specific RNA bacteriophage MS2 is widely used as a surrogate for pathogenic waterborne viruses in Europe and the United States [2,10,11,14–16] and the F-specific RNA bacteriophage Qβ tends to be used as a surrogate in Japan [4,5,12,13,17,18]. Previous studies have compared the behaviors of Qβ and MS2 in the coagulation process and direct MF process. The removal of these bacteriophages in the coagulation process with polyaluminum chloride differed markedly [19,20], whereas their removal was similar in the direct MF process with a 0.1-μm pore size ceramic membrane [21]. Therefore, the removal of Qβ and MS2 in the coagulation–MF process may differ as well, although these differences have not been investigated fully. If marked differences do exist, then Qβ and MS2 cannot be used as equivalent surrogates to evaluate the effectiveness of coagulation–MF processes for the removal of pathogenic waterborne viruses.

The effectiveness of membrane filtration processes, including the coagulation–MF process, for virus removal is generally evaluated based on the results of the plaque forming unit (PFU) method, which can detect only infectious viruses. However, a decrease in the infectious virus concentration is not only due to physical removal (e.g., sieving, adsorption) during the membrane separation process, but also to virucidal activity of aluminum coagulant [19,20] during the coagulation pretreatment. The mechanisms of virucidal activity of aluminum coagulant remain unclear. Inactivated viruses in the MF permeate might recover their infectivity in the water distribution system. However, the PFU method cannot detect the inactivated viruses that leak into MF permeate, which might underestimate the risk of infection. In other words, the removal performance of virus particles during the coagulation–MF process may be overestimated. The PFU method cannot distinguish whether a decrease results from physical removal, virus inactivation during the treatment process, or both. In contrast, the polymerase chain reaction (PCR) method can detect infectious viruses as well as inactivated viruses, unless the target nucleic acid is lost. Accordingly, a combination of the PFU and PCR methods allows physical removal and virus inactivation in the treatment processes to be clearly distinguished. To date, only our research group has investigated the virus removal performance and mechanisms of both infectious and inactivated viruses in the coagulation–MF process [17,18].

Our objectives in the present study were to investigate the behavioral differences of Qβ and MS2 during the coagulation–ceramic MF system using both PFU and real-time reverse transcription–polymerase chain reaction (RT-PCR) methods and to elucidate what caused those differences.

2. Materials and methods

2.1. Source water, coagulant, and MF membranes

River water was sampled on 12 October 2007 from the Toyohira River (Sapporo, Japan; water quality shown in Table 1). Polyaluminum chloride (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 (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), which was installed in a stainless-steel casing.

Table 1
Water quality measures of the Toyohira River.

pH	7.5
DOC (mg/L)	0.90
OD260 (cm^{-1})	0.027
Turbidity (NTU)	0.50
Alkalinity (mg-$CaCO_3$/L)	19.1

2.2. Bacteriophages

The bacteriophages Qβ (NBRC 20012) and MS2 (NBRC 102619) were obtained from the NITE Biological Research Center (NBRC, Chiba, Japan). Qβ is the prototype member of the genus *Allolevivirus* in the Leviviridae, and MS2 is the prototype member of the genus *Levivirus* in the Leviviridae. The genomes of these two bacteriophages 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 [22]. 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 (2000 × 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 with a centrifugal filter device (molecular weight cutoff 100,000, regenerated cellulose; Centriplus-100, Millipore Corp., Billerica, MA, USA) to prepare the bacteriophage stock solution.

2.3. Coagulation–ceramic MF experiments

The experimental setup is shown in Fig. 1. The river water was spiked with either Qβ or MS2 in the raw water tank at approximately 10^6 or 10^8 PFU/mL. The raw water tank was constantly mixed with an impeller stirrer during the experiments. The raw water was fed into the system at a constant flow rate (83.3 L/m^2 h) by a peristaltic pump. Hydrochloric acid was added before the first in-line static mixer (hydraulic retention time 1.8 s; 1/4-N40-172-0, Noritake Co., Ltd., Nagoya, Japan) to maintain the MF permeate at pH 6.8. 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 or 1.08 mg-Al/L). To obtain the two coagulation times, the in-line static mixer (hydraulic retention time 1.8 s) and a combination of the in-line static mixer and a subsequent tygon tube reactor (total hydraulic retention time 5 min) were used as the second in-line static mixer. 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 1 h.

2.4. Extraction of bacteriophage from ceramic MF compartment

After the filtration experiments, the water (floc mixture) in the MF compartment was withdrawn by gravity. To quantify the bacteriophage concentration in the liquid phase of the floc mixture, the suspended floc particles were separated from the mixture by centrifugation (2000 × g, 10 min), and then the bacteriophage concentration in the supernatant was measured by the PFU and real-time RT-PCR methods (see Section 2.5): the PFU method measured the concentration of infectious bacteriophages, and the real-time RT-PCR method measured the concentration of total bacteriophages regardless of their infectivity. To quantify the bacteriophage concentrations in the suspended aluminum floc particles, the floc particles were dissolved by raising the pH of the water to 9.5 with aqueous sodium hydroxide in 12% beef extract (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) solution and vortexing the water intensely for 5 h at 4 C. Beef extract was used in an effort to prevent the inactivation of virus during floc dissolution [19]. The bacteriophage concentrations in the floc mixture were

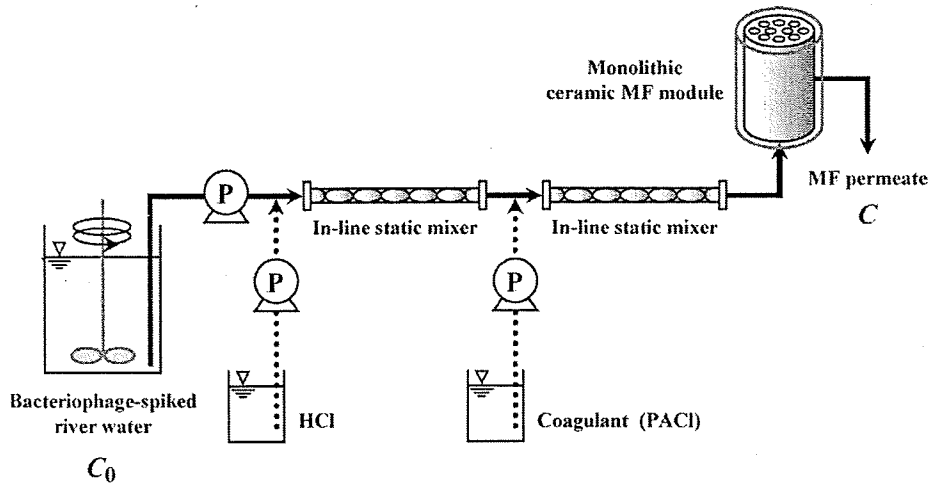

Fig. 1. Schematic diagram of the experimental coagulation–ceramic MF system. C_0 and C are the bacteriophage concentrations (PFU or particles/mL) in the raw water tank and the MF permeate, respectively, at each sampling time.

then measured by the PFU and real-time RT-PCR methods. In addition, to elute floc particles retained on the membrane surface after the floc mixture had been withdrawn by gravity, hydraulic backwashing (pressure 0.5 MPa) with 200 mL of Milli-Q water (Milli-Q Advantage, Millipore Corp.) was conducted using a nitrate gas cylinder. The floc particles in the backwash eluent were dissolved by the method described above. The bacteriophage concentrations in the backwash eluent were measured by the PFU and real-time RT-PCR methods. Finally, the quantities of bacteriophages in the solid phase in the MF compartment were calculated from the bacteriophage concentrations in the floc mixture and the backwash eluent.

Because beef extract is known to inhibit the amplification of the viral genome by PCR [23,24], each floc dissolution sample containing beef extract was diluted 10-fold with Milli-Q water before the real-time RT-PCR quantification.

2.5. Bacteriophage assays

2.5.1. PFU method

The infectious bacteriophages were enumerated according to the double-layer method [25] using the bacterial host *E. coli*. The average of plaque counts of triplicate plates prepared from one sample was considered as the infectious bacteriophage concentration.

2.5.2. Real-time RT-PCR method

Viral RNA of bacteriophages was quantified by the real-time RT-PCR method, which detects viruses regardless of their infectivity. We defined concentration measured by the real-time RT-PCR method as total bacteriophage concentration. For quantification of bacteriophages in the raw water, MF permeate, and liquid phase of the floc mixture, 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. For quantification of bacteriophages in the floc dissolution sample containing beef extract, a 100-μL sample was heated at 90 °C for 10 min and then cooled to 4 °C for 1 min in a thermal cycler (Thermal Cycler Dice Model TP600, Takara Bio Inc., Shiga, Japan) to extract viral RNA by destroying the capsid. 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. The cDNA solution was then amplified by 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 50 cycles of 95 °C for 15 s and 60 °C for 1 min in an ABI Prism 7000 Sequence Detection 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 PFU method and the number of cycles for amplification in the PCR process, which is based on the assumption that the freshly prepared stock solution did not contain any inactivated bacteriophages.

2.6. Electron micrography

Qβ and MS2 were observed with an electron microscope. Ten microliters of each bacteriophage stock solution (see Section 2.2) was placed on a 400-mesh copper grid with collodion membrane (Nissin EM Corp., Tokyo, Japan) and adsorbed to the grid for 1 min. Excess solution on the grid was drained with filter paper, and

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	[26]
	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	[27]
	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	

bacteriophages were negatively stained with 10 μL of 2% phosphotungstic acid (pH 7.0) for 45 s. After the excess strain was drained off, each grid was examined with a transmission electron microscope (TEM, JEM-1210, Jeol Ltd., Tokyo, Japan). Particle diameter of each bacteriophage was expressed as the mean and standard deviation of 20 randomly chosen particles on the electron micrograph.

2.7. Electrophoretic mobility

Electrophoretic mobility of bacteriophages was measured in prepared Milli-Q water and filtered river water. To bring the alkalinity to 20 mg-$CaCO_3$/L, 0.4 mM $NaHCO_3$ was added to the Milli-Q water, and the pH was adjusted to 6.8 with HCl. River water was filtered through a stirred ultrafiltration cell (Model 8400, Millipore Corp.) with ultrafiltration membrane (molecular weight cutoff 100,000, regenerated cellulose; Ultrafiltration Disks, YM-100, Millipore Corp.) to exclude the large particles, and the pH was adjusted to 6.8 with HCl. The Milli-Q and river water samples were kept for 1 day at 20 °C to stabilize the pH. Just before the measurement of electrophoretic mobility, each bacteriophage was suspended at approximately 10^{10} PFU/mL in the prepared Milli-Q water or filtered river water using the bacteriophage stock solution. The electrophoretic mobility of the bacteriophages was measured with an electrophoretic light-scattering spectrophotometer (ELS-6000, Otsuka Electronics Co., Ltd., Osaka, Japan) 25 times for each sample at 25 °C and at a 15° measurement angle.

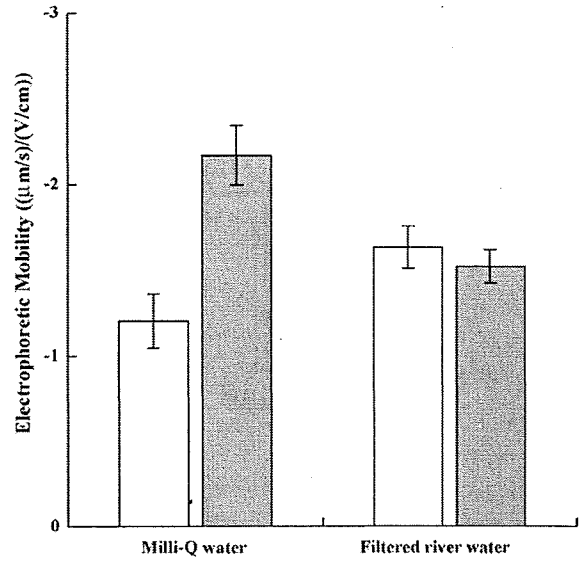

Fig. 3. Electrophoretic mobility of Qβ (white) and MS2 (gray) in the prepared Milli-Q water and filtered river water. Values represent the mean and standard deviation of 25 measurements. Bacteriophage concentration of each sample was approximately 10^{10} PFU/mL.

3. Results and discussion

3.1. Particle diameter and electrophoretic mobility

Fig. 2 shows the electron micrographs of Qβ and MS2. Nearly the same particle diameters were observed: 23.5 ± 0.8 nm for Qβ and 22.5 ± 1.0 nm for MS2. These values correspond with the particle diameters previously reported for Qβ and MS2 [22].

To investigate the electrokinetic properties of bacteriophages, the electrophoretic mobility (i.e., surface charge) was measured in the prepared Milli-Q water and filtered river water (Fig. 3). The surface charge on virus particles is often invoked to discuss the virus removal by physicochemical water treatment processes such as coagulation [20], media filtration [28–30], and membrane filtration [4,14]. The electrophoretic mobility of Qβ and MS2 were compared using the *t*-test (two tail) based on 0.01 level of significance. In the present study, the electrophoretic mobility of Qβ in the prepared Milli-Q water was significantly ($P < 0.01$) less than that of MS2. Thus, the capsid proteins of these two bacteriophages consist of different types of amino acid, so that the surface charges of the bacteriophages differed according to the difference in the dissociation of functional groups in constitutive amino acids. In the prepared Milli-Q water, the behaviors of these bacteriophages might be different during the coagulation–ceramic MF process. In contrast, no significant ($P > 0.01$) difference in electrophoretic mobility was observed in the filtered river water, possibly because the ionic strength of the river water is large enough to compress the effective thickness of the diffuse layer around the bacteriophage particles, which would allow the attractive van der Waals interaction to dominate [31] and reduce the difference in the electrophoretic mobility accordingly. Alternatively, multivalent ions present in the river water influenced the electrophoretic mobility. In this river water, Qβ and MS2 are expected to behave in a similar manner as would particles during the coagulation–ceramic MF process. Moreover, the surface charges of these two bacteriophages were negative at this pH condition (Fig. 3). This result is supported by previous studies [30,32] in which the isoelectric point (p*I*) of Qβ and MS2 were reported at around 5.3 and 3.9, respectively. The negative surface charge of Qβ and MS2 indicate that virus–virus aggregates were unlikely to be generated

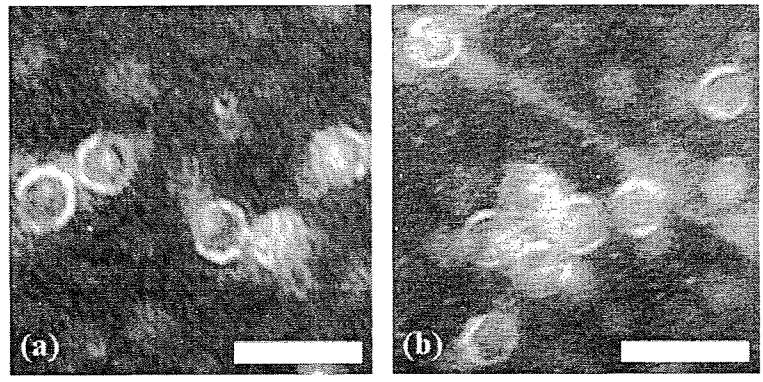

Fig. 2. Negative-strain electron micrographs of (a) Qβ and (b) MS2. The scale bar represents 50 nm.

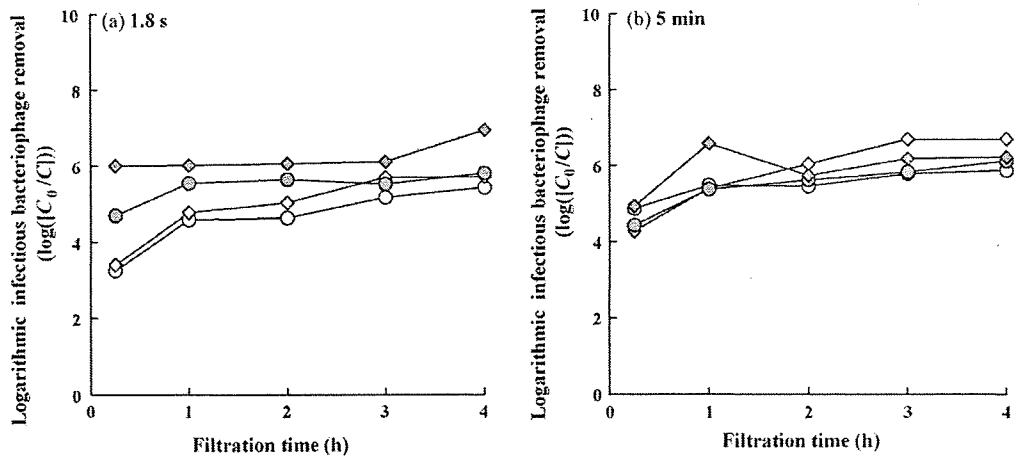

Fig. 4. Effect of coagulant dose and coagulation time on infectious bacteriophage removal. Open and solid symbols represent Qβ and MS2, respectively. Circles and diamonds represent coagulant dosage of 0.54 and 1.08 mg-Al/L, respectively. Coagulation times were 1.8 s (a) and 5 min (b). Initial bacteriophage concentrations of raw water were approximately 10^6 PFU/mL.

and these two bacteriophages were stably monodispersed in the raw water used (without coagulant dosing).

3.2. *Effect of coagulant dose and coagulation time on infectious bacteriophage removal*

Fig. 4 shows the effect of coagulant dose and coagulation time on the infectious bacteriophage removal obtained by the PFU method in the coagulation–ceramic MF system (initial concentration of each bacteriophage was approximately 10^6 PFU/mL). Because the diameters of both bacteriophages, as confirmed by electron microscopy, are smaller than the nominal pore size of MF membrane (0.1 μm), nonaggregated viruses are expected to pass through the MF membrane. Although other researchers have reported adsorptive interactions between virus and membrane surface, such as electrostatic and hydrophobic interactions, as some of the important factors for virus removal in the MF process [4,14], no removal of Qβ and MS2 was observed during the 4-h filtration without coagulation pretreatment (data not shown). This result suggests that the effect of electrostatic and hydrophobic interactions were negligible in this ceramic MF process. In contrast, addition of the coagulation pretreatment was effective in removing infectious bacteriophages under all coagulation conditions (Fig. 4). The removal ratio (log[C_0/C]) of both infectious bacteriophages gradually increased with filtration time, and a >5-log removal ratio was achieved for each infectious bacteriophage after 4 h of filtration. In a study using Qβ, our research group previously reported the factors that contribute to the time-course increase in the virus removal ratio [18]: growth of a cake layer that accumulated on the membrane surface and accumulation of foulants on the internal structure of the membrane pores probably account for the time-course increase in the virus removal ratio in the coagulation–ceramic MF system.

The effect of coagulant dose (0.54 mg-Al/L vs. 1.08 mg-Al/L) on infectious bacteriophage removal was observed with the 1.8-s coagulation time (Fig. 4a). The removal ratio of each bacteriophage increased with coagulant dose, but the effect was not large, perhaps because the infectious bacteriophage concentrations in the MF permeate were close to its detection limit (10^0 PFU/mL, approximately 6-log removal) with the 1.08 mg-Al/L coagulant dose. In contrast, Matsushita et al. [13] reported that the coagulant dose strongly affected the virus removal by the coagulation–ceramic MF system: the time-averaged virus removal was only 2.8 log at a 0.54 mg-Al/L coagulant dose, whereas 1.08 mg-Al/L achieved a 6.4-log time-averaged virus removal of infectious Qβ. This result does not correspond with the present findings, even though the same coagulation–ceramic MF system was used in both studies. One reason for the discrepancy is the difference in source water. The previous study used Toyokawa River (Aichi, Japan) water (DOC 1.1 mg/L, OD260 0.037 cm^{-1}) as the source [13]. DOC and OD260 of Toyokawa River water were higher than those of Toyohira River water (Table 1). These water qualities possibly affected the virus removal performance in the coagulation process. Accordingly, the removal performances of infectious bacteriophages between the previous and the present study were different at 0.54 mg-Al/L coagulant dose. The coagulation time also affected the infectious bacteriophage removal: extending the coagulation time from 1.8 s to 5 min somewhat increased the removal ratio of infectious bacteriophages. Thus, coagulant dose and coagulation time affected the infectious bacteriophage removal in this coagulation–ceramic MF system.

Fig. 5 summarizes the time-averaged infectious bacteriophage removal in the coagulation–ceramic MF system. According to the U.S. Environmental Protection Agency National Primary Drinking

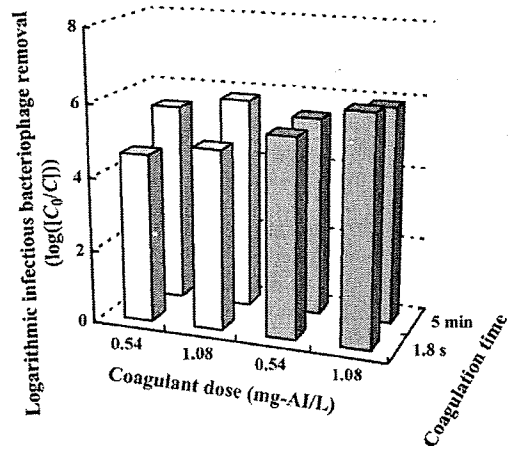

Fig. 5. Filtration time-averaged infectious bacteriophage removal. White and gray columns represent Qβ and MS2, respectively. Initial bacteriophage concentrations of raw water were approximately 10^6 PFU/mL.

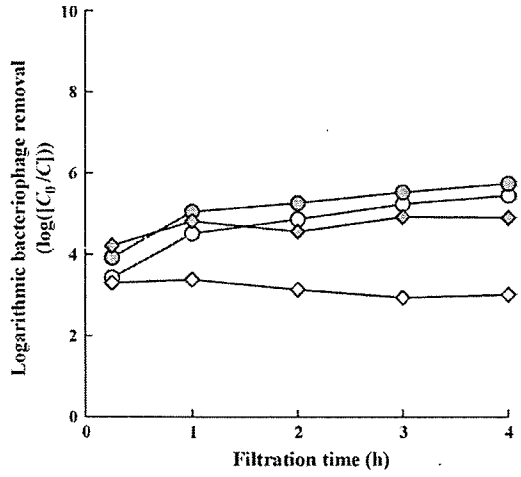

Fig. 6. Infectious bacteriophage and total bacteriophage removals in the coagulation–ceramic MF system. Open and solid symbols represent Qβ and MS2, respectively. Circles and diamonds represent infectious bacteriophages and total bacteriophages, respectively. Initial bacteriophage concentrations of raw water were approximately 10^8 PFU/mL. Coagulant dosage was 1.08 mg-Al/L, and coagulation time was 1.8 s.

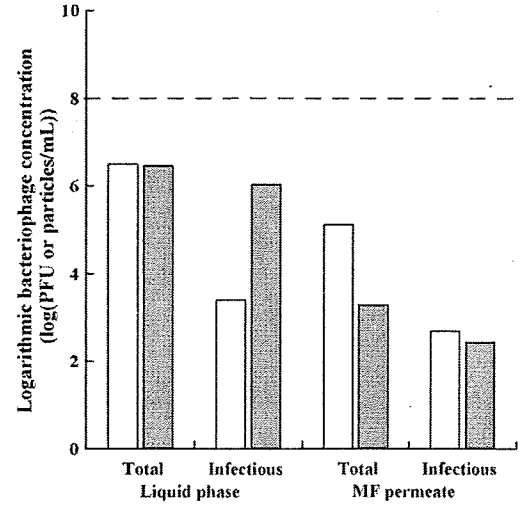

Fig. 7. Bacteriophage concentrations in the liquid phase in the MF compartment and in the MF permeate. White and gray columns represent Qβ and MS2, respectively. Initial bacteriophage concentrations of raw water were approximately 10^8 PFU/mL (indicated by dotted line). Coagulant dosage was 1.08 mg-Al/L, and coagulation time was 1.8 s.

Water Standards, enteric viruses must be removed or inactivated by 4 log from source water by filtration, disinfection, or a combination of these technologies. This 4-log removal ratio was achieved for infectious Qβ and MS2 under each coagulation condition. Even when the system was operated with a coagulant dose of 0.54 mg-Al/L and a coagulation time of 1.8 s, a >4-log of removal ratio for infectious bacteriophages was achieved. Thus, the coagulation–ceramic MF system can effectively remove infectious viruses. Further virus protection is expected by the combination with additional disinfection processes, such as chlorination, ozonation, and UV radiation.

No large difference was observed between the removal ratio of infectious Qβ and MS2 in the coagulation–ceramic MF system, although Qβ was removed slightly less effectively than MS2 at the 1.8-s coagulation time.

3.3. Removal performances of infectious bacteriophages and total bacteriophages

As described above, no large difference was observed between the removal ratio of infectious Qβ and MS2 at approximately 10^6 PFU/mL of initial bacteriophage concentration. To elucidate whether the removal ratios of infectious bacteriophages are actually different or not, the initial bacteriophage concentration of raw water was increased from 10^6 to 10^8 PFU/mL under the same operating conditions of the coagulation–ceramic MF system. In this case as well, high removal ratios were achieved for both infectious bacteriophages (Fig. 6; cf. Fig. 4a for 10^6 PFU/mL). Thus, the initial bacteriophage concentration did not seem to affect the removal performance of infectious bacteriophage, at least in the range of 10^6–10^8 PFU/mL. In addition, the removal ratios of the two infectious bacteriophages were almost the same at an initial concentration of 10^8 PFU/mL, indicating that the behaviors of infectious Qβ and MS2 were similar during the coagulation–ceramic MF process.

In contrast, the removal ratios of total bacteriophages for Qβ and MS2 obtained by the real-time RT-PCR method differed markedly (Fig. 6). The removal ratio of total Qβ was approximately 3 log and was almost constant during 4 h of filtration, whereas the removal ratio of total MS2 was more than 4 log and gradually increased with filtration time. A marked difference in the particle behaviors of Qβ and MS2 was observed in this coagulation–ceramic MF system, although similar morphological and electrokinetic properties were confirmed (Fig. 3). This difference is detailed in the following section.

3.4. Effect of enmeshment in aluminum floc particles and cake layer on bacteriophage removal

To investigate what caused the difference in particle behaviors of Qβ and MS2, the concentrations of infectious and total bacteriophage were measured in the liquid phase in the MF compartment and MF permeate at the end of the 4-h filtration (Fig. 7). Decreases in the total concentrations of Qβ and MS2 in the liquid phase compared to their initial concentrations in raw water (approximately 10^8 PFU/mL) were probably due to entrapment in or adsorption to the aluminum floc particles. The entrapped or adsorbed bacteriophages would be excluded from the liquid phase by the centrifugal separation just before the measurement of bacteriophage concentration. For both Qβ and MS2, >1.6-log reductions were observed; that is, 97% of bacteriophages were entrapped in or adsorbed to the aluminum floc particles before the ceramic MF process. Thus, the particle behaviors of Qβ and MS2 were the same during the coagulation pretreatment, as expected based on the particle diameter and electrophoretic mobility of these two bacteriophages.

A difference between total and infectious bacteriophage concentrations was observed in the liquid phase in the MF compartment. Therefore, some of the bacteriophages were inactivated during the coagulation process. The difference between two concentrations for Qβ was markedly larger than that of MS2. Thus, Qβ was more sensitive to virucidal activity of PACl than was MS2. Our group previously reported on the virucidal activity of PACl [19,20].

The total bacteriophage concentrations of Qβ and MS2 in the MF permeate were lower than that in the liquid phase in the MF compartment, suggesting that additional removal occurred when the bacteriophages passed through the ceramic MF membrane. Because the pore size of the ceramic MF membrane used in the present study is larger than the diameters of both bacteriophages, virgin membrane (i.e., devoid of cake layer) is not expected to remove nonaggregated bacteriophages by physical sieving. Alu-

minum floc particles generated in the coagulation pretreatment gradually covered the membrane surface over the filtration time and formed a cake layer, which would act as a barrier to nonaggregated bacteriophages. The cake layer, consisting of various materials such as kaolinite [2], *E. coli* [3], and powdered activated carbon [33], has been reported to increase virus removal performance in the MF process. The cake layer consisted mainly of aluminum floc in the present study, and possibly helped to remove the nonaggregated bacteriophages by reducing the effective pore size of the MF membrane. In addition, aluminum floc (i.e., amorphous aluminum hydroxide, $Al[OH]_3$) tends to be positively charged at pH 6.8, because its isoelectric point is in the pH range of 7.0–9.0 [31]. Therefore, the cake layer in the present study was positively charged and would adsorb negatively charged bacteriophages, which would further contribute to the removal of nonaggregated bacteriophages.

The removal ratio of total MS2 by the cake layer was approximately 2 log higher than that of total Qβ (Fig. 7), although their particle diameters and electrophoretic mobilities were similar. One possible explanation is that the hydrophobicity of MS2 and Qβ might be different. Hydrophobic force contributes to the adsorption of protein to aluminum-phosphate adjuvant [34]; therefore, a difference in the bacteriophages' hydrophobicity might cause the difference in the interaction between viral surface protein and the aluminum floc particles retained on the membrane surface. Another possible explanation is that the surface charge of an inactivated bacteriophage might differ from that of an infectious one. The mechanism of virucidal activity of aluminum coagulant remains unclear. However, some polymers formed during the hydrolysis of aluminum coagulant might sorb to the bacteriophage adsorption protein, and the adsorption protein might be morphologically altered. This modification might render the adsorption protein nonfunctional, which may induce the inactivation. Jones et al. [35] reported that the structures of the proteins lysozyme, ovalbumin, and bovine serum albumin were altered to less thermally stable forms by their adsorption to the surface of aluminum salt, which supports our hypothesis. This modification—forming a complex with aluminum species followed by inactivation—might change the surface charge of the bacteriophages. A difference between Qβ and MS2 in the surface charge after the modification might alter the electrostatic interaction between the inactivated bacteriophages and the cake layer, resulting in the selective removal of total bacteriophages: inactivated MS2 might be retained preferably by the cake layer as compared to inactivated Qβ.

3.5. Mass balance of bacteriophages in the coagulation–ceramic MF system

After a 4-h filtration, we determined the quantity of bacteriophages in the MF compartment using a combination of PFU and real-time RT-PCR methods (Fig. 8). Although the total number of Qβ was similar to total MS2 both in the liquid and solid phases, the number of infectious Qβ was approximately 3 log smaller than infectious MS2 in both phases. These differences were probably due to different sensitivities of the bacteriophages to the virucidal activity of PACl.

The total number of bacteriophages was at least 3 log greater in the solid phase than that in the liquid phase for Qβ and MS2. This result suggests that most of the bacteriophages (>99.9%) in the MF compartment were entrapped in or adsorbed to the aluminum floc particles and then included within the solid phase.

In the present study, the total number of bacteriophages in the MF compartment (liquid phase + solid phase) was less than that calculated theoretically by the mass balance of bacteriophages in the MF compartment (i.e., total number of bacteriophages in the raw water minus that in the MF permeate). The recovery ratios of Qβ and MS2 from the MF compartment were 35% and 65%, respectively. Thus, the mass balance of these two bacteriophages was not completely accounted for in the coagulation–ceramic MF system, probably because of the accumulation of bacteriophages on the internal structure of the membrane pores. This result is in accord with our previous findings for Qβ [18]. Thus, accumulation of bacteriophages in the internal structure of the membrane pores also contributed to the high removal performance for Qβ and MS2 in the coagulation–ceramic MF system.

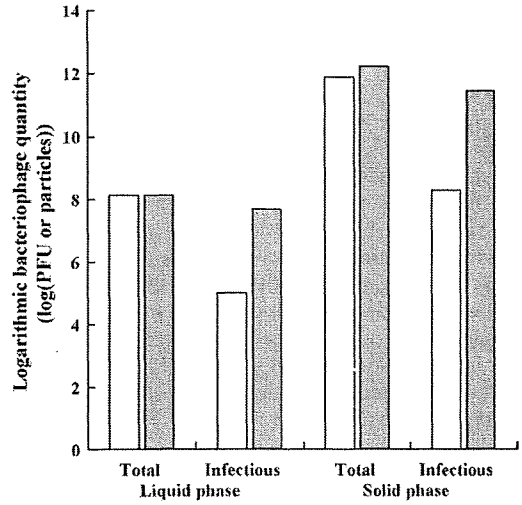

Fig. 8. Quantity of bacteriophages in the MF compartment. White and gray columns represent Qβ and MS2, respectively. Initial bacteriophage concentrations of raw water were approximately 10^8 PFU/mL. Coagulant dosage was 1.08 mg-Al/L, and coagulation time was 1.8 s.

The mechanisms of virucidal activity of PACl have yet to be elucidated. Inactivated viruses in the MF permeate might recover their infectivity in the water distribution system. Therefore, in addition to the infectious virus concentration, it is very important to evaluate the total number of viruses to ensure the safety of treated water. Our results revealed marked difference in the behaviors of Qβ and MS2 as particles during the coagulation–ceramic MF process. When the performance of a treatment plant employing the coagulation–MF process is evaluated using Qβ, MS2, or another virus as a surrogate for pathogenic waterborne viruses, the behavior of infectious virus as well as inactivated virus must be considered. The results should be judged carefully according to their different behaviors.

4. Conclusions

(1) The coagulation–ceramic MF system effectively removed infectious Qβ and MS2, and their removal performances were similar under each coagulation condition. Approximately 6-log reduction was achieved for both bacteriophages when the system was operated at coagulant dose of 1.08 mg-Al/L with 5-min coagulation time. At least 4-log reduction occurred even when the coagulant dose and the coagulation time were reduced to 0.54 mg-Al/L and 1.8 s, respectively.

(2) The removal performances of total Qβ and MS2 during the coagulation–ceramic MF process were quite different, although their particle diameters and electrophoretic mobilities were similar. The removal ratio of total MS2 was approximately 2 log higher than that of total Qβ at the end of the 4-h filtration because of selective interaction with the cake layer.

(3) The total number of Qβ was similar to that of total MS2 both in the liquid and solid phases in the MF compartment after 4-h filtration, whereas the number of infectious Qβ was approximately 3 log smaller than that of infectious MS2 in both phases. These differences are due to the sensitivity of the bacteriophages to the virucidal activity of PACl.
(4) Our results revealed marked differences in the particle behaviors of Qβ and MS2 during the coagulation–ceramic MF process. When the performance of a treatment plant employing the coagulation–MF process is evaluated using Qβ, MS2, or another virus as a surrogate for pathogenic waterborne viruses, the behavior of infectious viruses as well as inactivated ones must be considered, because inactivated viruses in the MF permeate might recover their infectivity in the water distribution system. The results should be judged carefully according to their different behaviors.

Acknowledgements

We thank Norikazu Isoda (Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University), who instructed us in the method of electron microscopic analysis. This research was supported in part by a Grant-in-Aid for the Encouragement of Young Scientists (no. 19760368, 2007) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-in-Aid (no. 19310801, 2007) from the Ministry of Health, Labour and Welfare of Japan, and the Kajima Foundation's Research Grant (2007).

References

[1] R. Sondhi, R. Bhave, G. Jung, Applications and benefits of ceramic membranes, Membr. Technol. 11 (2003) 5.
[2] J.G. Jacangelo, S.S. Adham, J.M. Laîné, Mechanism of *Cryptosporidium*, *Giardia*, and MS2 virus removal by MF and UF, J. AWWA 87 (1995) 107.
[3] S.S. Madaeni, A.G. Fane, G.S. Grohmann, Virus removal from water and wastewater using membranes, J. Membr. Sci. 102 (1995) 65.
[4] T. Urase, K. Yamamoto, S. Ohgaki, Effect of pore structure of membranes and module configuration on virus retention, J. Membr. Sci. 115 (1996) 21.
[5] M. Otaki, K. Yano, S. Ohgaki, Virus removal in a membrane separation process, Water Sci. Technol. 37 (1998) 107.
[6] National Primary Drinking Water Standards, Office of Water, U.S. Environmental Protection Agency, Washington, DC, EPA816-F-01-007, 2001.
[7] J.D. Lee, S.H. Lee, M.H. Jo, P.K. Park, C.H. Lee, J.W. Kwak, Effect of coagulation conditions on membrane filtration characteristics in coagulation-microfiltration process for water treatment, Environ. Sci. Technol. 34 (2000) 3780.
[8] T. Carroll, S. King, S.R. Gray, B.A. Bolto, N.A. Booker, The fouling of microfiltration membranes by NOM after coagulation treatment, Water Res. 34 (2000) 2861.
[9] S.J. Judd, P. Hillis, Optimisation of combined coagulation and microfiltration for water treatment, Water Res. 35 (2001) 2895.
[10] B. Zhu, D.A. Clifford, S. Chellam, Virus removal by iron coagulation-microfiltration, Water Res. 39 (2005) 5153.
[11] L. Fiksdal, T.O. Leiknes, The effect of coagulation with MF/UF membrane filtration for the removal of virus in drinking water, J. Membr. Sci. 279 (2006) 364.
[12] Y. Matsui, T. Matsushita, T. Inoue, M. Yamamoto, H. Hayashi, H. Yonekawa, Y. Tsutsumi, Virus removal by ceramic membrane microfiltration with coagulation pretreatment, Water Sci. Technol.: Water Supply 3 (2003) 93.
[13] T. Matsushita, Y. Matsui, N. Shirasaki, Y. Kato, Effect of membrane pore size, coagulation time, and coagulant dose on virus removal by a coagulation-ceramic microfiltration hybrid system, Desalination 178 (2005) 21.
[14] E.M. Voorthuizen, N.J. Ashbolt, A.I. Schäfer, Role of hydrophobic and electrostatic interactions for initial enteric virus retention by MF membranes, J. Membr. Sci. 194 (2001) 69.
[15] B. Zhu, D.A. Clifford, S. Chellam, Comparison of electrocoagulation and chemical coagulation pretreatment for enhanced virus removal using microfiltration membranes, Water Res. 39 (2005) 3098.
[16] B.S. Oh, H.Y. Jang, Y.J. Jung, J.W. Kang, Microfiltration of MS2 bacteriophage: effect of ozone on membrane fouling, J. Membr. Sci. 306 (2007) 244.
[17] T. Matsushita, Y. Matsui, N. Shirasaki, Analysing mass balance of viruses in a coagulation-ceramic microfiltration hybrid system by a combination of the polymerase chain reaction (PCR) method and the plaque forming units (PFU) method, Water Sci. Technol. 53 (2006) 199.
[18] N. Shirasaki, T. Matsushita, Y. Matsui, K. Ohno, M. Kobuke, Virus removal in a hybrid coagulation-microfiltration system: investigating mechanisms of virus removal by a combination of PCR and PFU methods, Water Sci. Technol.: Water Supply 7 (2007) 1.
[19] Y. Matsui, T. Matsushita, S. Sakuma, T. Gojo, T. Mamiya, H. Suzuoki, T. Inoue, Virus inactivation in aluminum and polyaluminum coagulant, Environ. Sci. Technol. 37 (2003) 5175.
[20] T. Matsushita, Y. Matsui, T. Inoue, Irreversible and reversible adhesions between virus particles and hydrolyzing-precipitating aluminum: a function of coagulation, Water Sci. Technol. 50 (2004) 201.
[21] G. Herath, K. Yamamoto, T. Urase, The effect of suction velocity on concentration polarization in microfiltration membranes under turbulent flow conditions, J. Membr. Sci. 169 (2000) 175.
[22] The Universal Virus Database of the International Committee on Taxonomy of Viruses (ICTV). http://phene.cpmc.columbia.edu/index.htm (accessed 17 April 2008).
[23] M. Abbaszadegan, M.S. Huber, C.P. Gerba, I.L. Pepper, Detection of enteroviruses in groundwater with the polymerase chain reaction, Appl. Environ. Microbiol. 59 (1993) 1318.
[24] D. Sano, K. Fukushi, Y. Yoshida, T. Omura, Detection of enteric viruses in municipal sewage sludge by a combination of the enzymatic virus elution method and RT-PCR, Water Res. 37 (2003) 3490.
[25] M.H. Adams, Bacteriophages, Interscience, New York, 1959, pp. 450–454.
[26] H. Katayama, A. Shimazaki, S. Ohgaki, Development of virus concentration method using negatively charged membrane by alkaline elution after acid rinse, J. Japan Soc. Water Environ. 25 (2002) 469 (in Japanese).
[27] K.P. O'Connell, J.R. Bucher, P.E. Anderson, C.J. Cao, A.S. Khan, M.V. Gostomski, J.J. Valdes, Real time fluorogenic reverse transcription-PCR assays for detection of bacteriophage MS2, Appl. Environ. Microbiol. 72 (2006) 478.
[28] D.H. Taylor, R.S. Moore, L.S. Sturman, Influence of pH and electrolyte composition on adsorption of poliovirus by soils and minerals, Appl. Environ. Microbiol. 42 (1981) 976.
[29] J.A. Redman, S.B. Grant, T.M. Olson, Filtration of recombinant Norwalk virus particles and bacteriophage MS2 in quartz sand: importance of electrostatic interactions, Environ. Sci. Technol. 31 (1997) 3378.
[30] S.E. Dowd, S.D. Pillai, S. Wang, M.Y. Corapcioglu, Delineating the specific influence of virus isoelectric point and size on virus adsorption and transport through sandy soils, Appl. Environ. Microbiol. 64 (1998) 405.
[31] American Water Works Association, in: F.W. Pontius (Ed.), Water Quality and Treatment: A Handbook of Community Water Supplies, 4th ed., McGraw-Hill, New York, 1990.
[32] A. Sakoda, Y. Sakai, K. Hayakawa, M. Suzuki, Adsorption of viruses in water environment onto solid surfaces, Water Sci. Technol. 35 (1997) 107.
[33] H.K. Oh, S. Takizawa, S. Ohgaki, H. Katayama, K. Oguma, M.J. Yu, Removal of organics and viruses using hybrid ceramic MF system without draining PAC, Desalination 202 (2007) 191.
[34] R.H. Al-Shakhshir, F.E. Regnier, J.L. White, S.L. Hem, Contribution of electrostatic and hydrophobic interactions to the adsorption of proteins by aluminum-containing adjuvants, Vaccine 13 (1995) 41.
[35] L.S. Jones, L.J. Peek, J. Power, A. Markham, B. Yazzie, C.R. Middaugh, Effects of adsorption to aluminum salt adjuvants on the structure and stability of model protein antigens, J. Biol. Chem. 280 (2005) 13406.

Development and evaluation of a reverse transcription-loop-mediated isothermal amplification assay for rapid and high-sensitive detection of *Cryptosporidium* in water samples

A. Inomata, N. Kishida, T. Momoda, M. Akiba, S. Izumiyama, K. Yagita and T. Endo

ABSTRACT

We describe a novel assay for simple, rapid and high-sensitive detection of *Cryptosporidium* oocysts in water samples using a reverse transcription-loop-mediated isothermal amplification (RT-LAMP). The assay is based on the detection of 18S rRNA specific for *Cryptosporidium* oocysts. The detection limit of the developed RT-LAMP assay was as low as 6×10^{-3} oocysts/test tube, which theoretically enables us to detect a *Cryptosporidium* oocyst and perform duplicated tests even if water samples contain only one oocyst. The developed RT-LAMP assay could more sensitively detect *Cryptosporidium* oocysts in real water samples than the conventional assay based on microscopic observation.

Key words | Cryptosporidiosis, *Cryptosporidium*, LAMP, oocysts, RT-LAMP

A. Inomata
Department of Environmental Health and Toxicology,
Tokyo Metropolitan Institute of Public Health,
3-24-1, Hyakunin-cho,
Shinjuku, Tokyo 169-0073, Japan
E-mail: *Akiko_Inomata@member.metro.tokyo.jp*

N. Kishida
M. Akiba
Department of Water Supply Engineering,
National Institute of Public Health,
2-3-6 Minami, Wako, Saitama 351-0197,
Japan
E-mail: *kishida@niph.go.jp*;
akiba@niph.go.jp

T. Momoda
Biochemical Research Laboratory,
Eiken Chemical CO., LTD,
Nogi 143, Nogi-machi, Shimotsugagun,
Tochigi 329-0114, Japan
E-mail: *takayoshi_momoda@eiken.co.jp*

S. Izumiyama (corresponding author)
K. Yagita
T. Endo
Department of Parasitology,
National Institute of Infectious Diseases,
Toyama 1-23-1, Shinjuku,
Tokyo 162-8640,
Japan
E-mail: *izmym@nih.go.jp*;
kyagi@nih.go.jp;
tendo@nih.go.jp

INTRODUCTION

Members of the genus *Cryptosporidium* are protozoan parasites that can cause the gastrointestinal disease cryptosporidiosis (O'Donoghue 1995). Cryptosporidiosis remains a public health concern, as demonstrated by continued outbreaks of this disease (Nichols 2008). Waterborne cryptosporidiosis is particularly important because *Cryptosporidium* oocysts are resistant to disinfectants (such as chlorine) commonly used for water treatment (Peeters *et al.* 1989; Carpenter *et al.* 1999). Waterborne outbreaks have been reported in not only developing countries but also developed countries. The most notorious outbreak occurred in Milwaukee, Wisconsin in 1993 where more than 400,000 suspected and 5,000 confirmed cases of clinical cryptosporidiosis (MacKenzie *et al.* 1995; Cicirello *et al.* 1997; Corso *et al.* 2003).

doi: 10.2166/wst.2009.599