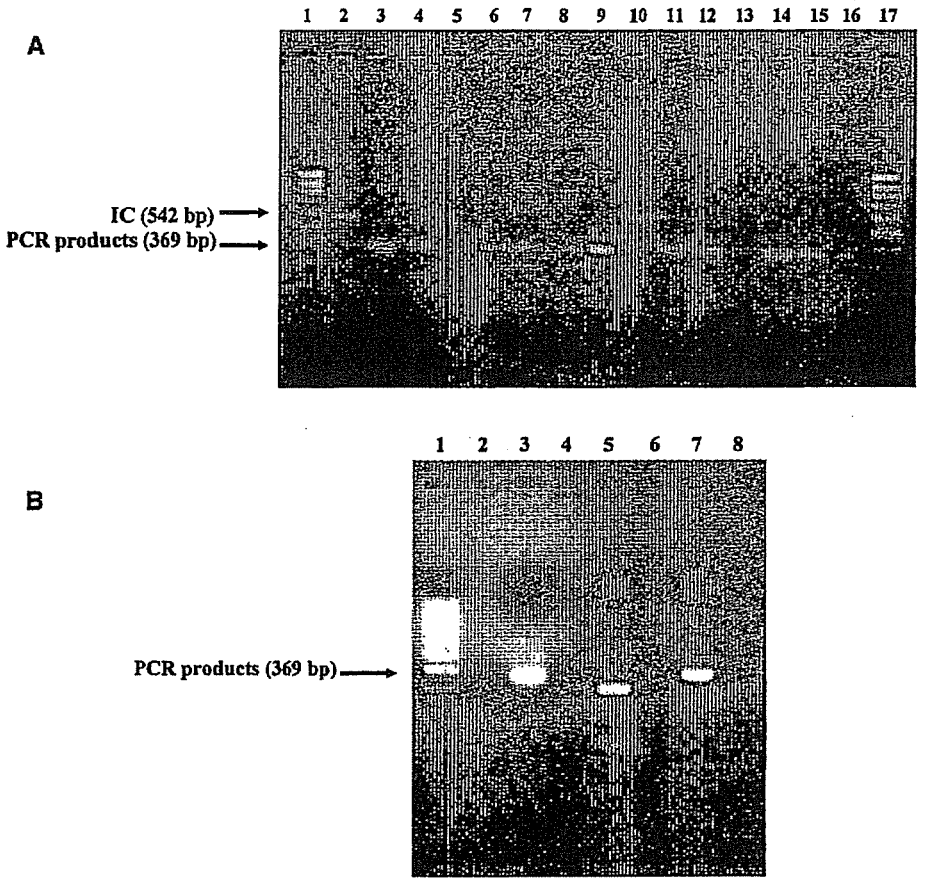

Fig. 2. Ethidium bromide–stained 2.0% agarose gel showing amplification products using TRAP-C2 primers from clam excreta and GI tract samples (A), and the results of PCR- RFLP for the fecal DNA obtained in the field survey (B). (A) Lanes 3–8; 7 exposures, lanes 11–16; 14 exposures. Lanes 1, 17: 100-bp molecular marker; lanes 2, 10: negative control containing internal control (542 bp); lanes 3, 11: fecal sample 1 day after cessation of doses; lanes 4, 12: fecal sample 3 days after cessation of doses; lanes 5, 13: fecal sample 14 days after cessation of doses; lanes 6, 14: GI tract sample 1 day after cessation of doses; lanes 7, 15: GI tract sample 3 days after cessation of doses; lanes 8, 16: GI tract sample 14 days after cessation of doses; lane 9: positive control (369 bp). (B) The TRAP-C2 PCR-amplified product after digestion with *Bst*EII (lane 5, bovine type specific marker) and *Hae*III (lane 7, human type specific marker) restriction enzymes and agarose gel electrophoresis. Lane 1: 100-bp molecular marker, lane 3: positive control (369 bp)

test. Furthermore, the tendencies of balance studies here may be similar, in both final magnitude and excretion pattern, to the previously reported single dose test with *C. japonica* (Izumi *et al.* 2004), while the present excretion rates are somewhat faster, presumably owing to the lower daily exposure amounts of oocysts per clam (about 1/4) than in the previous single exposure test.

The rates of excystation of oocysts in feces to HCT-8 cells were about 65–70% in both tests, and sporozoits were detected in HCT-8 cells 2 days after inoculation, proving the infectivity of oocysts in the feces of *C. japonica* (Fig. 3A,C).

In the field survey, 23 *C. parvum* oocysts were detected in a 10-L river water sample at the first collection, and 14 oocysts were observed in the river water at the second collection (at the end of the survey). With respect to the protozoa in the clam excreta, there was an average of 0.8 *C. parvum* oocysts per clam during the first three days after the withdrawal of the clams to the aquarium in the laboratory (Table 1). Accordingly, protozoan DNA extraction from fecal matter was attempted, and it was applied to both DNA sequencing and PCR-RFLP analysis. As shown in Table 2, the sequence analysis showed that the fecal isolate of the clam in the field survey conserved typical bovine type sequences including the *Bst*EII site when compared with representative human and bovine genotypes deposited in the GenBank (Sulaiman *et al.* 1998).

A PCR-RFLP analysis was made to visually discriminate between human and bovine-type isolates. After digestion by restriction enzymes and gel electrophoresis, the resulting band pattern was consistent with the size of the bovine genotype predicted by the mapping analysis, which was not in disagreement with the DNA sequencing data (Table 2). Thus, it was further shown by PCR-RFLP that the DNA extracted from clam excreta in the field survey contained the *Bst*EII site distinctive of the bovine type. However, it had no *Hae*III site distinctive of the human type (Fig. 2B). On the other hand, the infectious activity of oocysts to HCT-8 cells in the field survey was ascertained at a rate of about 80% of excystation, a rate that was slightly higher than the value in laboratory tests (Fig. 3B,D).

The filtration rate of *C. japonica* measured under the above-mentioned conditions was calculated to be about 300–400 ml/h/clam (Fig. 4). Assuming that the clam feeding time is about half a day, the total filtration water volume per day can be estimated as about 3.6–4.8 L/clam under the present conditions.

Discussion

According to the results in the balance studies and oocyst infectivity assay by HCT-8 cells, *C. parvum* oocysts were proved to be rapidly taken up by *C. japonica*. However, they were not really digested as a foodstuff for the clams, and they were predominantly excreted in the feces with almost intact

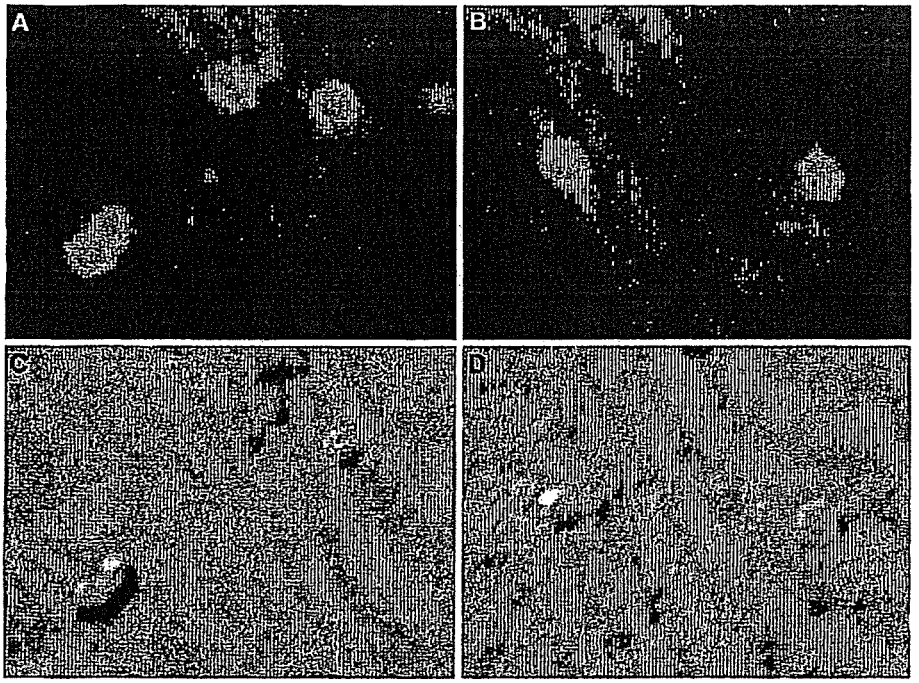

Fig. 3. Photographs of the developmental stage of *Cryptosporidium parvum* prepared from fecal samples of laboratory and field tests in HCT-8 cells 2 days after inoculation. **(A)** Fluorescence photograph of a field of foci after 2 days of inoculation using oocysts prepared from laboratory tests (14-exposure test). **(B)** Same as A, using oocysts prepared from the field survey. **(C)** Normarski interference-contrast photomicrograph of the field in A. **(D)** Same as C, the photograph corresponding to B

Table 1. Recovery of oocyts from *Corbicula japonica* applied to the field survey

	Day after withdrawal			
Group	1st	2nd	3rd	Total
A	102	46	23	171
B	79	20	14	113
C	84	26	11	121
Total	265	92	48	405

Group B was set in the center of the river, at a 0.7-m depth, and the others were set in a line at intervals of 5 m from the center, by side across the river.

Table 2. Difference between human and bovine genotypes of *Cryptosporidium parvum* based on multiple alignment deposited in the GenBank with accession no. AF082524 to AF082524

Position (no.)	Human genotype	Bovine genotype	Genotype (field survey)
51	G	A	A
78	C	T	T
100	T	G	G
147	C	T	T
280	T/C	C	C
76–79	*Hae*III site[a]	—	—
76–82	—	*Bst*EII site[b]	*Bst*EII site[b]

↓: resection enzyme cutting position.
[a] GG ↓ CC.
[b] G ↓ GTNACC.

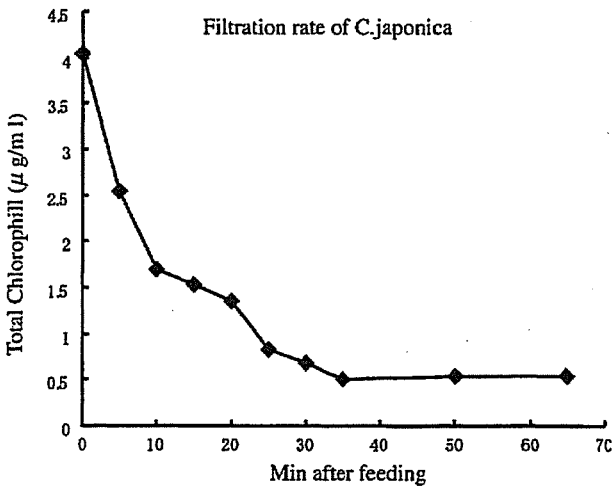

Fig. 4. Water filtration rate of *Corbicula japonica* determined by powdered chlorella. The filtration rates of the clams were calculated from the amount of extracted chlorophyll and the time till discontinuing feeding

infectivity under the present conditions as in the single exposure study (Izumi *et al.* 2004). The difficulty of digesting oocysts by *C. japonica* may be a reason why there were only small differences in the excretion and retention pattern for oocysts in the balance studies when compared with the single exposure test, suggesting that *C. parvum* oocysts may pass through the GI tract of the clam at a nearly constant rate and that they may be substantially excreted without digestion. It is also suggested that this estuarine shellfish, *C. japonica*, could be useful and important in practical attempts to recover *C. parvum* oocysts from water samples by fecal sedimentation. The feces of *C. japonica* are liable to precipitate for its high density and low viscosity in comparison with those of *Unionidae* clams, which may suggest the usefulness of

C. japonica as a biological indicator for collecting oocysts under natural environmental conditions.

A field survey was undertaken to check the potential of *C. japonica* as a biological indicator for oocysts in the natural river. We have frequently succeeded in collecting oocysts from the feces of *C. japonica* set in a river. However, especially the detection efficiency of the oocysts in the river water by the membrane filtration method tends to vary according to the natural parameters, i.e., turbidity, etc. Hence, we will continue to make efforts hereafter to collect much more useful data. This time, we tried to inspect a field test recently carried out to estimate the ability of *C. japonica* for collecting oocysts under a natural environment. The Yanbetsu River (the river width: 30 m; water depth: 0.7 m) where the test took place is in the eastern part of Hokkaido, Japan, and *C. parvum* oocysts have been detected there by our laboratory. However, this river was not used for the source of water supply, and it was decided to set clams in this river. Considering that the average number of oocysts in the river water during the field test was calculated to be about 18.5/10 L, the arithmetical average between the oocyst counts on the first and the last day, and with an estimated filtration volume of clams of about 3.6–4.8 L/clam/day, the number of oocysts ingested by *C. japonica* may be estimated as 6.7–8.9 clam/day. Therefore, the total intake of oocysts by a clam during the 7 days of the field test was presumed as 46.9–62.3/clam/week, and for a group of 60 clams, it would be 2,814–3,738 oocysts/week. After the withdrawal of the clams from the river, the fecal oocysts of the clam of each of three groups were determined and the results are shown in Table 1. The number of oocysts detected was as follows: 79–102 for the 1st day, 20–46 for the 2nd day, and 11–32 for the 3rd day, so the total number of oocysts determined for three days was 113–171 corresponding to 3.0–6.1% of the total estimated intake. Comparing these oocyst capture rates in the field test with those of the 7-times exposure test in Figure 1, the estimated oocyst recovery in the field survey was 20–40% of the corresponding laboratory test. The reasons for the low oocyst recovery in the field test may due to the following natural conditions: first, the water flow rate of the river depends on the surrounding natural circumstances closely linked with the weather factors; second, there may be the daily fluctuations in the oocyst density in the water; third, all clams may not always be in the feeding state, which would tend to vary with the various water conditions including temperature and suspended plankton density in the habitat of the clam. The average oocyst detection amount was assumed to be 2.1–2.7/clam under the present conditions in the field test, so if the oocyst amount in the water is reduced from 18.5/10 L to 1.0/10 L, the detection amount would be estimated to be reduced to 0.11–0.15/clam. With this, 6.6–9.0 oocysts should theoretically be detected by a group of 60 clams. These values may be practical allowing for checking of the river water contamination by *C. parvum* oocysts.

An infectivity assay and PCR-RFLP analysis in the field survey was also carried out on another day, and showed the existence of oocyst infectivity to host cells with a higher excystation rate (about 80%) than the laboratory values, which may depend on the freshness of the oocysts, and the bovine-type gene in the collected fecal oocysts, which may be accounted for by the latest contamination from a cattle farm up stream.

These results suggest that to carry out the qualitative investigation on *C. parvum* oocysts in a river, the practical detection limit of oocysts in the water by *C. japonica* may be around 0.5–1.0/10 L. To ensure catching *C. parvum* oocysts, the number of clams/group set in the river should be more than 100/group. If possible, clams should be set at several points near the center flow of the river. The practical detection limit of oocyst by the membrane filtration method is supposed to be about 2–3/10 L under the clear water condition, as the efficiency of the detection considerably depends on the amount of suspended substances in the water especially at the time of the melting snow. On the other hand, it was shown in our laboratory that the collection ability of *C. japonica* was not liable to vary by the turbidity of the water, perhaps owing to its natural muddy habitat. So, it was supposed that the practical oocysts detection limit of *C. japonica* might be slightly lower than that of the membrane filtration method.

As evaluated by the above results, the brackish water shellfish, *Corbicula japonica*, may be employed as a practical and effective biological indicator or collection system for *C. parvum* oocysts in river water. Further, *C. japonica* is adequate in size for the treatment and easy to obtain, as it is a ubiquitous edible clam in Japan, and not so difficult to handle in practical field use. There are, however, some points to consider when keeping the clam under artificial conditions for long periods of time.

For the immediate future, it may be necessary to employ a biological indicator to detect *C. parvum* oocysts for at least two reasons. First is to realize a stable and precise checking system for protozoa in the river water to complement conventional membrane filtration methods generally carried out in Japan, and second is to introduce a food hygiene parameter utilizing edible aquatic organisms such as oysters and cockles. To achieve this, the surrounding aquatic organisms should be investigated for suitability as biological indicators for detecting *C. parvum* oocysts, and the indicator found the most suitable to a particular river ecology should be used, while the preservation of the river ecosystem must first be given careful consideration (Miller *et al.* 2005).

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References

Fayer R, Graczyk TK, Lewis EJ, Trout JM, Farley CA (1998) Survival of infectious *Cryptosporidium parvum* oocysts in seawater and eastern oysters (*Crassostrea virginica*) in the Chesapeake Bay. Appl Environ Microbiol 64:1070–1074

Fayer R, Lewis EJ, Trout JM, Graczyk TK, Jenkins MC, Higgins J, Xiao L, Lal AA (1999) *Cryptosporidium parvum* in oysters from commercial harvesting sites in the Chesapeake Bay. Emerg Infect Dis 5:706–710

Freire-Santos F, Oteiza-Lopez AM, Castro-Hermida JA, Garcia-Martin O, Ares-Mazas ME (2001) Viability and infectivity of oocysts recovered from clams, *Ruditapes philippinarum*,

experimentally contaminated with *Cryptosporidium parvum*. Parasitol Res 87:428–430

Freire-Santos F, Gomez-Couso H, Ortega-Inarrea MR, Castro-Hermida JA, Oteiza-Lopez AM, Garcia-Martin O, Ares-Mazas ME (2002) Survival of *Cryptosporidium parvum* oocysts recovered from experimentally contaminated oysters (*Ostrea edulis*) and clams (*Tapes decussatus*). Parasitol Res 88:130–133

Giangaspero A, Molini U, Iorio R, Traversa D, Paoletti B, Giansante C (2005) *Cryptosporidium parvum* oocysts in seawater clams (*Chameleagallina*) in Italy. Prev Vet Med 69:203–212

Gomez-Bautista M, Ortega-Mora LM, Tabares E, Lopez-Rodas V, Costas E (2000) Detection of infectious *Cryptosporidium parvum* oocysts in mussels (*Mytilus galloprovincialis*) and cockles (*Cerastoderma edule*). Appl Environ Microbiol 66:1866–1870

Gomez-Couso H, Freire-Santos F, Hernandez-Cordova GA, Ares-Mazas ME (2005) A historical study of the transit of *Cryptosporidium parvum* oocysts through clams (Tapes decussatus). Int J Food Microbiol 102:57–62

Graczyk TK, Farley CA, Fayer R, Lewis EJ, Trout JM (1998) Detection of *Cryptosporidium* oocysts and *Giardia* cysts in the tissues of eastern oysters (*Crassostea virginica*) carrying principal oyster infectious diseases. J Parasitol 84:1039–1042

Graczyk TK, Fayer R, Lewis EJ, Trout JM, Farley CA (1999) *Cryptosporidium* oocysts in Bent mussels (*Ischadium recurvum*) in the Chesapeake Bay. Parasitol Res 85:518–521

Graczyk TK, Marcogliese DJ, de Lafontaine Y, Da Silva AJ, Mhangami-Ruwende B, Ppieniazek NJ (2001) *Cryptosporidium parvum* oocysts in zebra mussels (*Dreissena polymorpha*) : evidence from the St. Lawrence River. Parasitol Res 87:231–234

Greenberg AE, Clesceri LS, Eaton AD (1992) Standard methods for the examination of water and wastewaster, 18th ed. American Public Health Association, Washington, D.C. American Water Works Association, Denver. Water Environment Federation, Alexandria

Hirata T, Shimura A, Morita S, Suzuki M, Motoyama N, Hoshikawa H, Moniwa T, Kaneko M (2001) The effect of temperature on the efficacy of ozonation for inactivating *Cryptosporidium parvum* oocysts. Water Sci Technol 43:163–166

Inomata A, Oshimi A, Tanaka S (1999) Investigation of DAPI staining method of *Cryptospordium parvum* oocysts. J Jpn Water Work Assoc 68:32–36

Izumi T, Itoh Y, Yagita K, Endo T, Ohyama T (2004) Brackish water benthic shellfish (*Corbicula japonica*) as a biological indicator for *Cryptosporidium parvum* oocysts in river water. Bull Environ Contam Toxicol 72:29–37

Miller WA, Atwill ER, Gardner IA, Miller MA, Fritz HM, Hedrick RP, Melli AC, Barnes NM, Conrad PA (2005) Clams (*Corbicula fluminea*) as bioindicators of fecal contamination with *Cryptosporidium* and *Giardia* spp. in freshwater ecosystems in California. Int J Parasitol 35:673–684

Peng MP, Xiao L, Freeman AR, Arrowood MJ, Escalate A, Weltman AC (1997) Genetic polymorphism among *Cryptosporidium parvum* isolates supporting two distinct transmission cycles. Emerg Infect Dis 3:567–573

Slifko TR, Friedman D, Rose JB, Jakubowski W (1997) An *in vitro* method for detecting infectious *Cryptosporidium* oocysts with cell culture. Appl Environ Microbiol 63:3669–3675

Sulaiman IM, Xiao L, Yang C, Escalante L, Moore A, Beard CB, Arrowood MJ, Lal AA (1998) Differentiating human from animal isolates of *Cryptosporidium parvum*. Emerg Infect Dis 4:681–685

Traversa D, Giangospero A, Molini U, Iorio R, Paoletti B, Otranto D, Giansante C (2004) Genotyping of *Cryptosporidium* isolates from *Chamelea galena* clams in Italy. Appl Environ Microbiol 70:4367–4370

Upton SJ, Tilley M, Brillhart DB (1995) Effect of select medium supplements on *in vitro* development of *Cryptosporidium parvum* in HCT-8 cells. J Clin Microbiol 33:371–375

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Behavior of gold colloid as model viruses during filtration through adsorptive ion exchange membranes

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1. Introduction

Mainly because of the more stringent regulation and improvement in detection apparatus, virus removal from drinking water treatment remains an important research activity. Viruses of concern, such as hepatitis A and poliovirus, are usually too small to be removed by conventional drinking water treatment processes. Membrane separation processes such as reverse osmosis and nanofiltration can effectively produce potable water free of virus. However, operating costs required for these membrane systems are very high due to the elevated pressure required to maintain filtration. Larger pore membranes (i.e. lower energy demand), such as standard ultrafiltration and microfiltration, cannot always guarantee total virus rejection. Adsorptive ion exchange membranes have attracted attention in the field of desalination, food and pharmaceutical productions, and industrial effluents treatment; they are powerful tools for separation or concentration of ionic species [1]. Because of their ionic nature, viruses can be adsorbed by these membranes. Some researchers already tested ion exchange membranes for the production of virus vectors for gene therapy and virus vaccines [2]. However, these membranes are challenging to characterize for virus removal in drinking water treatment due to the difficulties in culturing and measuring viruses. The pore size of these membranes is usually large, so high fluxes are expected. The objective of the present study was to investigate the potential for gold colloids to characterize particle capture by ion exchange membranes for virus removal applications.

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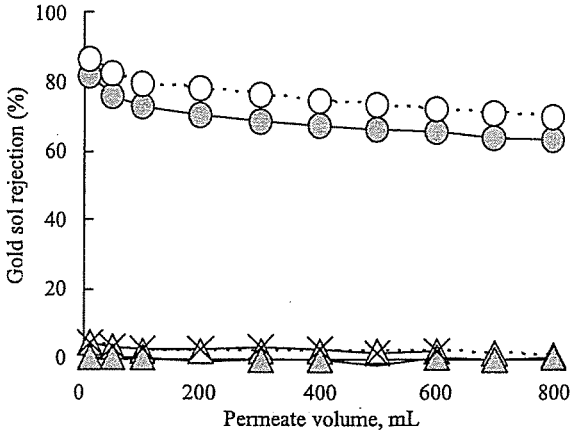

Fig. 1. Effect of membrane type on 10 nm gold sol rejection. The experiment were conducted under 50 kPa. Solid circled, Sartorius Q; open circles, Sartorius D; solid triangles, Sartorius S; open triangles, Sartorious C; x marks, GVWP.

2. Materials and methods

Four types of ion exchange membranes (pore diameter >3 μm) were used: Sartobind Q (strong anion exchanger), D (weak anion exchanger), C (strong cation exchanger) and S (weak cation exchanger), along with a typical size exclusion membrane (0.2 μm, Millipore GVWP). Two different sizes of gold colloids (10 and 43 nm) were prepared by diluting gold (III) chloride solution with citrate ion according to the literature [3] with minor modification as follows: 95 mL of 2.7×10^{-4} M of potassium tetrachloroaurate

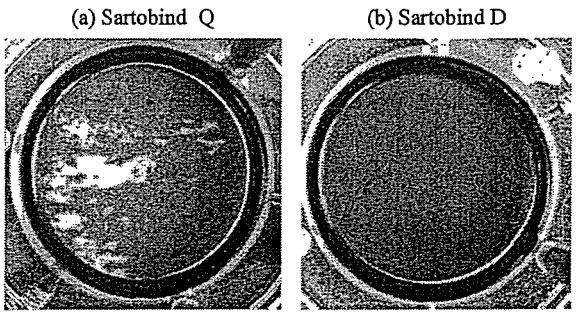

Fig. 2. Photos of membrane surface just after 100 mL of the 10 nm gold sol was filtered under 50 kPa.

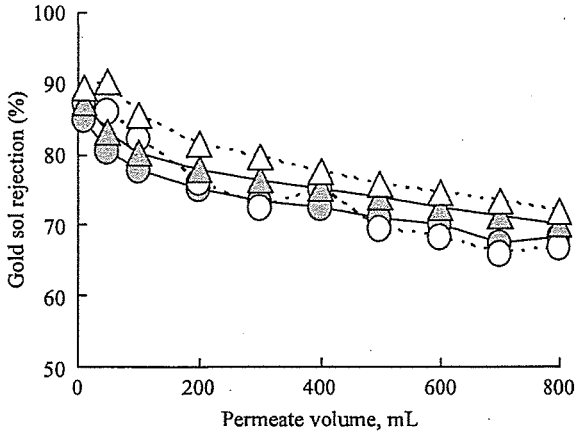

Fig. 3. Effect of size of gold sol on rejection. The experiments were conducted under 50 kPa. Solid circles, 10 nm, Q; open circles, 43 nm, Q; solid triangles, 10 nm, D; open triangles, 43 nm, D.

in 2.7×10^{-4} M of hydrochloric acid was boiled with intense stirring, and 1.5 mL (for 43 nm colloids) or 5 mL (for 10 nm colloids) of 1.7×10^{-4} mM of trisodium citrate was added. After 10 min of boiling, the mixture was cooled to room temperature, and then diluted 10 times with Milli-Q water before use. The concentrations for the 10 and 43 nm colloids are expected to on the order of 10^{15} and 10^{13} per liter respectively [3]. All experiments were conducted in a batch cell of 110 mL capacity equipped with membranes. The gold solution was continuously fed into the cell under constant pressure by using nitrogen gas (50 or 100 kPa), and filtered by the membrane in dead-end mode. The flux was measured with a Sartorius balance by continuous scaling the permeate weight. Concentration of gold sol was measured with Varian UV-visible spectrophotometer at 520 nm.

3. Results and discussion

3.1. Effect of membrane type on gold sol rejection

Flux obtained for the four ion exchange membranes were similar, and more than three

times higher that of GVWP membrane under 50 kPa pressure (data not shown). Gold sol (10 nm) was rejected by anion exchangers (Q and D), but was not rejected by cation exchangers (C and S) and GVWP membrane (Fig. 1). Because the pH of the gold sol solution was 5.2–5.6, charges of membranes Q and D were positive whereas those of membranes C and S were negative [2]. At this pH, the gold sol was negative, and therefore it was rejected by membranes Q and D. Although membrane Q is indicated as a strong ion exchanger and probably has a larger negative charge than membrane D, its removal ratio was smaller than that of membrane D. This might be due to the existence of unutilized area on the membrane (white patches in Fig. 2) possibly caused by maldistribution of pore size and/or ion exchange group; however further study is needed.

3.2. *Effect of pressure on gold sol rejection*

When filtration pressure was increased, rejection decreased. As the increase in pressure raised flux rate, the contact time of gold sol with the ion exchange group of the membrane was reduced, limiting adsorption and rejection rate.

3.3. *Effect of size of gold sol on its rejection*

Rejection of 43 nm sol was higher than for 10 nm colloids for both Q and D membranes, even though the zeta potential of 43 nm sol (–23 mV, measured with Brookhaven zeta potential analyzer) was less negative than that of 10 nm sol (–45 mV) (Fig. 3). As a result, rejection of 10 nm colloids was expected to be higher. This discrepancy in rejection was probably due to the particle concentration of 10 nm sol, which was more than ten times higher that of 43 nm sol.

4. Conclusions

The anion exchange membranes had high rejection of the gold sols with flux three times greater than those of GVWP, whereas the cation exchange membranes and GVWP showed little capture as expected. The increase in pressure decreased the rejection rate of the gold sols indicating that residence time and binding kinetics may be a factor in the capture efficiency. Although 43 nm sol was less negative than 10 nm sol, its rejection was larger than that of 10 nm sol possibly because of the difference in the particle concentrations.

References

[1] T. Xu, Ion exchange membranes: state of their development and perspective, J. Membr. Sci., 263 (2005) 1–29.

[2] R. Specht, B.B. Han, J.O. Carlson, P. Czermak, A. Wolf, O-W. Reif and S.R. Wickramasinghe, Densonucleosis virus purification by ion exchange membranes, Biotechnol. Bioeng., 88(4) (2004) 465–473.

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

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Abstract Virus removal experiments using river water spiked with bacteriophages were conducted by an in-line coagulation–ceramic microfiltration hybrid system to investigate the effects of filtration flux (62.5 and 125 L/(m^2 × h)) and type of virus (Qβ and MS2) on virus removal. In addition, the mass balance of viruses through the hybrid system was analysed by quantifying the infectious and inactive viruses by a combination of the polymerase chain reaction (PCR) method and the plaque forming units (PFU) method. Even when the system was operated at high filtration flux (125 L/(m^2 × h)), high virus removal (>6 log) with short coagulation time (2.4 s) was successfully achieved by dosing polyaluminium chloride (PACl) at more than 1.08 mg-Al/L. Removal performances were different between Qβ and MS2, although their diameters are almost the same: greater virus removal was achieved for MS2 at PACl dosing of 0.54 mg-Al/L, and for Qβ at PACl dosing of more than 1.08 mg-Al/L. The combination of the PCR and PFU methods revealed that two phenomena, adsorption to/entrapment in aluminium floc and virucidal activity of PACl, partially account for the high virus removal in the coagulation–MF hybrid system.

Keywords Coagulation; microfiltration (MF); PCR; virus

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Introduction

Ceramic membranes have attracted attention in the field of drinking water treatment in Japan because they are less breakable than membranes made of other materials. However, in general, ceramic membranes are microfiltration (MF) devices, so their pore sizes are not small enough to exclude particles with diameters less than tens of nanometres. Included among such small particles are some of the pathogenic waterborne viruses that draw attention in drinking water treatment, such as hepatitis A virus and poliovirus, both of which are approximately 20 nm in diameter. Therefore, these viruses cannot be excluded by ceramic membranes alone. In order to compensate for this disadvantage, it was proposed that coagulation, which is usually employed to destabilise and aggregate small particles and then to remove them under gravity, be used in combination with ceramic microfiltration. Wickramasinghe *et al.* (2004) reported that the addition of a cationic flocculant to the microfiltration process resulted in a 4-log reduction of minute virus of mice from CHO cell suspensions. Our research group has also reported the usefulness of the coagulation–ceramic microfiltration hybrid system for virus removal (Matsushita *et al.*, 2005): a greater than 6-log reduction of viruses was achieved by the hybrid system with sufficient dosing (1.08 mg-Al/L) of polyaluminium chloride coagulant (PACl). Moreover, a coagulation time of only 2.4 s was required to achieve such a high rate of

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virus removal, even though the system was operated without a sedimentation tank (Matsushita *et al.*, 2005), because the coagulation rate was very fast and the aggregates rapidly reached sizes larger than the membrane's pore size after coagulant had been added. All these experiments were conducted at a filtration flux of 62.5 L/(m^2 × h), but the rapid coagulation rate would allow higher-filtration flux operations, enabling the footprint of the treatment plant to be smaller.

In the hybrid system, virus removal increased with operating time (Matsushita *et al.*, 2005). One possible explanation for the increasing rate of virus removal is the accumulation of a cake layer on the surface of the membrane during the MF process. Matsui *et al.* (2003a) reported that a cake layer accumulated on the membrane surface enhanced the virus removal in a coagulation–immersed ceramic microfiltration system, the result was obtained from the comparison in virus concentrations between in the MF module and in the MF permeate. In addition, our research group suggested that the virucidal activity of the aluminium coagulant (Matsui *et al.*, 2003b) possibly enhances the virus removal and also partially accounts for the increasing rate of virus removal in the coagulation–microfiltration hybrid system (Matsushita *et al.*, 2005). However, neither the mechanisms of the increasing rate of virus removal nor any direct evidence for virucidal activity in the hybrid system have been previously investigated.

The above-mentioned results were obtained by using a bacteriophage Qβ as a model virus (Matsui *et al.*, 2003a; Matsushita *et al.*, 2005). Some researches dealing with the membrane process were conducted by using bacteriophage MS2 as a model virus (Jacangelo *et al.*, 1991, 1995; Lovins *et al.*, 2002, Hu *et al.*, 2003); MS2 is currently the most studied F-RNA coliphage (Havelaar *et al.*, 1993).

The objectives of the present study were: (1) to investigate the possibility of operating the coagulation–microfiltration hybrid system at a higher filtration flux (125 L/(m^2 × h)); (2) to compare the removal performance between two types of virus, Qβ and MS2, and (3) to analyse the mass balance of viruses throughout the hybrid system – quantifying the infectious and inactive viruses in the feed water, the membrane compartment and the filtrate by a combination of the polymerase chain reaction (PCR) method and the plaque forming units (PFU) method – in order to evaluate the contributions of cake layer formation and the virucidal activity of the coagulant to virus removal in the hybrid system.

Materials and methods

Source water, MF membranes and coagulant

Water samples from Toyokawa River (Aichi, Japan) were collected on 10 November 2004, transported in polyethylene tanks and stored at 4 °C until use. Total organic carbon and ultraviolet absorbance at 260 nm were 1.1 mg/L and 0.043 cm^{-1}, respectively. The membrane used in the present study was a monolithic ceramic module (multichannel tubular, nominal pore sizes 0.1 μm, NGK Insulators, Ltd., Nagoya, Japan), which was installed in a stainless steel casing. The coagulant, polyaluminium chloride (PACl; 10% Al_2O_3, 62.5% basicity, Sumitomo Chemical Co. Ltd., Tokyo, Japan), was diluted before use so that a fixed quantity could be added to each water sample.

Virus used

We used two bacteriophages, Qβ (NBRC 20012) and MS2 (NBRC 20015), obtained from the NITE Biological Resource Center (NBRC, Chiba, Japan), as model viruses. The genomes of Qβ and MS2 each consist of a single-stranded RNA molecule encapsulated in an icosahedral protein shell (capsid) approximately 23 nm in diameter without an envelope. Qβ and MS2 are widely used as surrogates for waterborne viral pathogens because of their morphological similarities to hepatitis A virus and poliovirus, which are

important to remove during treatment of drinking water. Qβ and MS2 were propagated for 22–24 h at 37 °C in *Escherichia coli* F^+ (NBRC 13965) obtained from NBRC. The respective Qβ and MS2 cultures were centrifuged (3000 × *g*, 10 min) and then filtered through a 0.45-μm pore-size membrane filter (cellulose acetate; DISMIC-25cs; Toyo Roshi Kaisya, Ltd., Tokyo, Japan). The filtrate was purified by using a centrifugal filter device (molecular weight cutoff: 100,000; Centriplus-100; Millipore Corp., Billerica, MA, USA) to prepare the virus stock solution, with which we reduced the TOC increase by spiking the river water with the stock solution to less than 0.1 mg/L.

Experimental setup

The experimental setup is shown in Figure 1. The river water was spiked with virus in a raw-water tank at 2–20 × 10^6 pfu/mL. The river water was fed into the system at a constant flow rate (62.5, and 125 L/(m^2 × h)) by a peristaltic pump. Aqueous sodium hydroxide was added before the first in-line static mixer (N40-172, Noritake Co., Ltd., Nagoya, Japan, hydraulic retention time; 2.4 s), with the dose being regulated so as to maintain the pH of the MF permeate at 6.8. PACl was injected after the first in-line static mixer and before the second at various dosing rates (1.08 and 1.62 mg Al/L). Two types of mixing units were used as the second in-line static mixer: an in-line mixer whose hydraulic retention time was 2.4 s, and a tube reactor with extended coagulation time of 57.6 s, which was installed after the second in-line static mixer (total retention time, 60 s). After mixing with PACl, the water was fed into the MF module in dead-end mode. The filtration lasted for 6 h without any backwash. Virus concentrations in the raw water tank and in the MF permeate were measured every hour.

Quantification of virus in MF compartment

At the end of 6-h of operation, water (floc mixture) in the MF compartment was withdrawn by gravity after demounting the MF membrane unit from its casing. To quantify the virus concentration in the liquid phase of the floc mixture, the mixture was centrifuged (3,000 × *g*, 10 min) and then the virus concentration in the supernatant was measured by the PFU method described below, which expresses the concentration of infectious viruses in the liquid phase of the floc mixture (LP_{pfu}). Next, to quantify the viruses in the suspended aluminium floc, as well as in the liquid phase of the floc mixture, the floc was dissolved by raising the pH of the water to 9.5 with NaOH in 6% beef extract (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) solution and vortexing it intensely for 5 h. Beef extract was used in an effort to prevent the floc-dissolution conditions themselves from inactivating the virus (Matsui *et al.*, 2001).

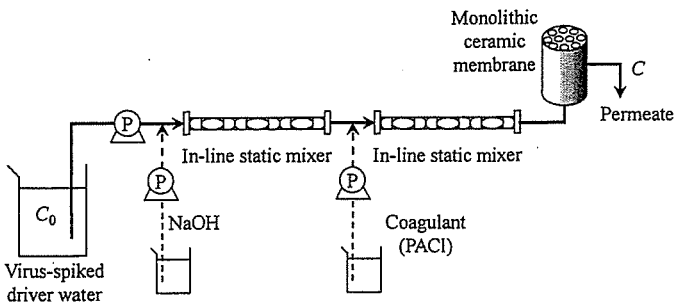

Figure 1 Diagram of experimental setup of the coagulation–MF hybrid experimental water-treatment system. C_0 and *C* mean the virus concentrations (pfu/mL) in raw-water tank and MF permeate at each sampling time, respectively

The virus concentrations in the floc mixture were measured by two methods, as described below; the PFU method measured the concentrations of infectious viruses (FM_{pfu}), and the PCR method measured the concentrations of virus particles regardless of their infectivity (FM_{pcr}). On the other hand, in order to elute floc particles that might have been retained on the ceramic membrane surface despite the force of the water's withdrawal, a backwash with 200 mL of sulphuric acid (pH 2) was employed to the MF compartment at a rate of 187.5 L/(m^2 × h). Ten milliliters of the backwash eluent was vortexed for 5 h to dissolve any floc particles therein. The virus particle concentration in the resulting solution was measured by the PCR method, which expresses the virus particle concentration in the retained floc on the membrane surface (RF_{pcr}).

Viral assay

In order to measure the concentration of infectious virus, the PFU method was employed according to the agar overlay method (Adams, 1959) using the bacterial host *E. coli* F^+. Average plaque counts of triplicate plates prepared from one sample gave the virus concentration.

For the quantification of Qβ virus particles regardless of their infectivity, the real-time PCR method was employed with a reverse transcription (RT) reaction. One hundred microliters of sample were heated at 90 °C for 10 min in a thermal cycler (Prism 7000 Sequence Detection System, Applied Biosystems Japan, Tokyo, Japan) to extract viral RNA by destroying a capsid. The RNA solution was supplemented by a one-step TaqMan RT-PCR reagent (Applied Biosystems Japan), a 50 nM concentration of each primer and a 250 nM concentration of TaqMan probe. The oligonucleotide sequences of the primers and the probe are indicated in Table 1. The RT-PCR was basically performed according to the protocol prepared by Applied Biosystems. The incubation was conducted as follows: 48 °C for 30 min, followed by 95 °C for 10 min, 50 cycles of 95 °C for 15 s, and 60 °C for 60 s.

Results and discussion

Effect of flux on virus removal

Figure 2 shows the effects of filtration flux on Qβ virus removal when the system was operated with 1.08 mg-Al/L of PACl (coagulation time 2.4 s). Because the diameter of the virus was smaller than the pore size of the MF membrane used in the present study, non-flocculated virus is expected to pass through the MF membranes; indeed, no reduction in virus levels was observed in an experiment run without PACl (data not shown). However, virus reduction was observed in experimental runs with a PACl dosage of 1.08 mg-Al/L, indicating that the coagulation pretreatment effectively flocculated the viruses so that the size of the floc exceeded the pore size of the MF membrane. The performance at a filtration flux of 125 L/(m^2 × h) was almost the same as that at 62.5 L/(m^2 × h), ensuring a high virus removal (>6 log) even when the system was operated at a high filtration flux.

Figure 3 shows an overall comparison of operational parameters, PACl dose, coagulation time and filtration flux for Qβ virus removal by the coagulation–MF hybrid system. Extending the coagulation time from 2.4 to 60 s increased virus removal, but the

Table 1 Oligonucleotide sequences of the primers and the probe used in the PCR quantification for QB

Primer QB +	5′–TCA AGC CGT GAT AGT CGT TCC TC–3′
Primer QB –	5′–AAT CGT TGG CAA TGG AAA GTG C–3′
TaqMan probe	5′–CGA GCC GCG AAC ACA AGA ATT GA–3′

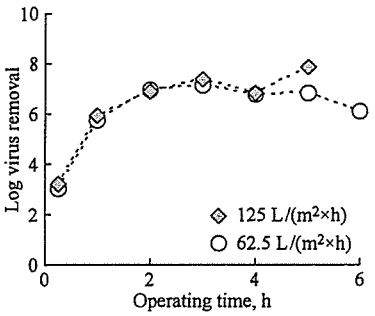

Figure 2 Effect of filtration flux on QB virus removal. The coagulant (PACl) dose was 1.08 mg/Al/L, and the coagulation time was 2.4 s

increase was not remarkably large. Increasing the PACl dose from 1.08 to 1.62 mg-Al/L also increased virus removal, but that increase was not remarkably large either. Our research group has already reported that 0.54 mg-Al/L of PACl dosing was insufficient for effective virus removal at a filtration flux of 62.5 L/(m^2 × h) even when the system was operated with a relatively long coagulation time of 60 s (Matsushita *et al.*, 2005). Thus, the PACl dose could be predicted to be insufficient when the system was operated at higher filtration flux (125 L/(m^2 × h)) with a coagulation time of less than 60 s. Likewise, coagulation pretreatment with 1.08 mg-Al/L of PACl dosing would be necessary for high virus removal (>6 log) at a filtration flux of 125 L/(m^2 × h). In other words, high virus removal with a short coagulation time of only 2.4 s could be achieved even at the high-filtration flux operation when PACl was dosed to the system at more than 1.08 mg-Al/L.

Comparison of removal between two viruses tested

Figure 4 shows a comparison of virus removals between Qβ and MS2. Although the diameters of Qβ and MS2 are almost the same, their removals by the coagulation–MF hybrid system were different: at 0.54 mg-Al/L of PACl dosing, MS2 was removed much more effectively than Qβ was. In contrast, at more than 1.08 mg-Al/L dosing, the removal of Qβ was slightly more effective than that of MS2. In water treatment, the major mechanisms with inorganic coagulants including PACl are charge neutralisation and sweep coagulation, and the sweep coagulation predominates with higher dosages of aluminium salts (Amirtharajah and Tambo, 1991). In the present study, charge neutralisation may predominate with PACl dosing at 0.54 mg-Al/L, whereas sweep coagulation may

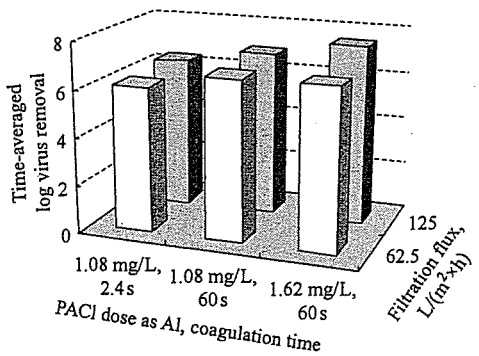

Figure 3 Overall comparison of parameters for QB virus removal by the coagulation–MF hybrid system

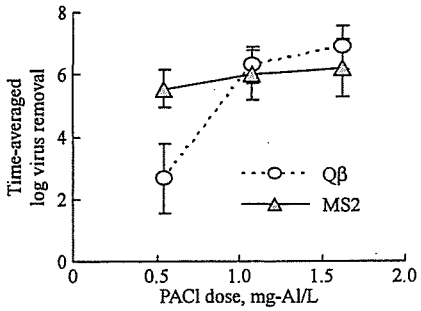

Figure 4 Comparison of virus removal between QB and MS2. The coagulation time was 2.4 s, and the flux was 62.5 L/(m^2 × h)

predominate with PACl dosing at more than 1.08 mg-Al/L. The effectiveness of the charge neutralisation and sweep coagulation for virus removal by the inorganic coagulant may vary between virus types, possibly depending on their surface charges. MS2 might tend to be more easily removed by the charge neutralisation than Qβ is. In contrast, Qβ might be more easily removed by the sweep coagulation than MS2 is. This tendency may be attributable to the difference in performance of virus removal observed in the present study; further study is needed.

Mass balance of virus

Infectious Qβ virus concentration was measured in the water sample withdrawn from the MF compartment at the end of the 6-h operation. Virus concentration in the MF compartment was measured both after centrifugation (LP_{pfu}) and after floc dissolution (FM_{pfu}). Figure 5 shows a comparison of infectious virus concentrations in the feed water, in the water in the MF compartment and in the MF permeate. The infectious virus concentration in the water in the MF compartment was much smaller than that in the feed water. This indicates that the virus was not simply concentrated in the MF compartment, although the infectious virus concentration in the MF permeate was much smaller than that in the feed water; the reason for the imbalance is discussed below.

In the MF compartment, the infectious virus concentration in the floc mixture (FM_{pfu}) was more than 3 log greater than that in the liquid phase of the floc mixture (LP_{pfu}). This indicates that most of the infectious viruses (>99.9%) had been adsorbed to, or entrapped in, the floc particles so that they were retained in the MF compartment, this retention plays an important role in the high virus removal by the membrane. The infectious virus concentration in the MF permeate was almost the same as the LP_{pfu}, suggesting that a

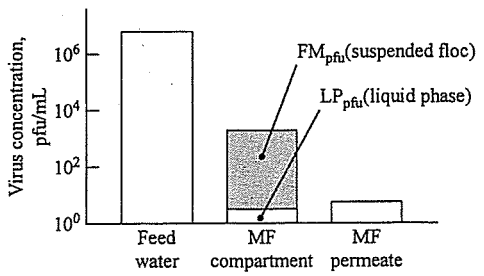

Figure 5 Infectious QB virus concentrations in the feed water, in the water in the MF compartment, and in the MF permeate after the operation of 6 h when the system was operated with a PACl dose of 1.08 mg/Al/L, a coagulation time of 2.4 and a filtration flux of 62.5 L/(m^2 × h)

cake layer possibly accumulated on the membrane surface would not be a barrier for the virus. Jacangelo *et al.* (1995) reported that a cake layer that was accumulated on the membrane surface by loading 8 and 16 g/sq of kaolinite prior to feeding virus increased virus removal by the membrane, indicating that the formation of a cake layer has the potential to enhance the removal of viruses by membrane filtration. Other researchers also reported that cake layer accumulated on the membrane surface improved virus removal by the membrane (Madaeni *et al.*, 1995; Farahbakhsh and Smith, 2004). Our research group conducted a virus removal study by a coagulation–immersed ceramic MF hybrid system, and reported that virus filtration by the floc retained on the membrane surface, as well as virus inactivation by the coagulant and virus adsorption onto suspended floc particles, were the mechanisms of virus removal by the coagulation–immersed MF hybrid system (Matsui *et al.*, 2003a). In our previous study, water samples for viral counting were withdrawn from the MF compartment when the system was in operation. In the present study, water samples were withdrawn after the operation was over and then the water feeding was stopped. Therefore, in the present study, viruses that had been in the liquid phase when the system was operational may be capable of being adsorbed to the suspended/retained floc in the short interval before the feeding is stopped. Thus, this post-adsorption might decrease virus concentration in the liquid phase down to that in the MF permeate. Accordingly, the cake layer might not serve to remove the virus in the present study; further investigation is needed.

In order to determine the reason for the imbalance in infectious virus concentration described above and to analyse the mass balance of virus in the MF compartment, the PCR technique was employed to the floc mixture withdrawn from the MF compartment (FM_{pcr}) and the backwash eluent (RF_{pcr}). Figure 6 shows the virus amounts measured by the various methods at the end of 6 h of operation. In the floc mixture withdrawn from the MF compartment, the concentration of virus particles (FM_{pcr}) was 3.4 log greater than that of the infectious virus (FM_{pfu}), indicating that most of the viruses in the floc mixture (>99.9%) were non-infectious. Thus, the virucidal activity of the aluminium coagulant (Matsui *et al.*, 2003b) plays an important role in the high virus removal by the hybrid system. The concentration of virus particles in the backwash eluent (RF_{pcr}) was 1.7 log greater than that in the floc mixture (FM_{pcr}). This means that the floc particles retained on the membrane surface, which was eluted by the backwash, contained a high amount of virus particles.

Although the backwash with sulphuric acid of pH 2 could extract a high amount of the virus from the membrane, the concentration of virus particles (RF_{pcr}) was still 0.8 log less than the theoretical value, which was calculated from the infectious virus concentrations in

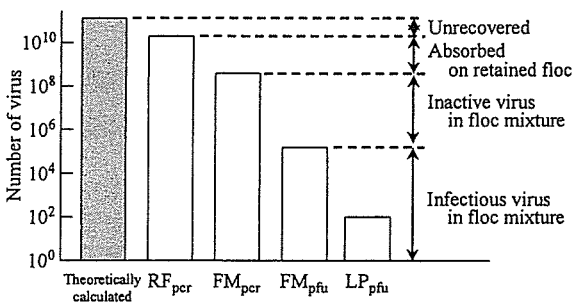

Figure 6 Mass balance of QB virus in the MF compartment at the end of the operation of 6 h when the system was operated with a PACl dose of 1.08 mg-Al/L, a coagulation time of 24 and filtration flux of 62.5 L(m^2 × h)

the feed water and the MF permeate: RF_{pcr} was 13% of the theoretically calculated value. This means that the technique we employed could not elute all viruses from the membrane unit, possibly owing to the strong adsorption of viruses on the internal structure of the membrane. Otherwise, virus particle concentration might be underestimated in the PCR method because the sulphuric acid used in the backwash process might break the viral RNA as well as the viral capsid. Alternatively, the concentration of virus particles (both infectious and inactive virus) in the MF permeate could not be measured because it was lower than the detection limit of the PCR method employed in the present study. Therefore, inactive viruses might leak from the system, contributing to overestimation of the theoretical value; further study is needed.

Overall, two phenomena, adsorption to/entrapment in aluminium floc and virucidal activity of PACl, partially account for the high virus removal in the coagulation–MF hybrid system.

Conclusions

1. A high virus removal (>6 log) with a short coagulation time of only 2.4 s could be achieved even at high-flux operation (125 L/($m^2 \times h$)) when PACl was dosed to the system at more than 1.08 mg-Al/L.
2. Although the diameters of Qβ and MS2 are almost the same, their removals were different: greater virus removal was achieved for MS2 at PACl dosing of 0.54 mg-Al/L, and for Qβ at PACl dosing of more than 1.08 mg-Al/L.
3. The combination of the PCR and PFU methods revealed that two phenomena, adsorption to/entrapment in aluminium floc and virucidal activity of PACl, partially account for the high virus removal in the coagulation–MF hybrid system.

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References

Adams, M.H. (1959). *Bacteriophages*, Interscience, New York, NY, USA, pp. 450–454.

Amirtharajah, A. and Tambo, N. (1991). Mixing in water treatment. In *Mixing in coagulation and flocculation*, Amirtharajah, A., Clark, M.M. and Trusell, R.R. (eds), AWWA Research Foundation, Denver, CO, USA, pp. 3–34.

Farahbakhsh, K. and Smith, D.W. (2004). Removal of coliphages in secondary effluent by microfiltration: mechanisms of removal and impact of operating parameters. *Wat. Res.*, **38**, 585–592.

Havelaar, A.H., Van Olphen, M. and Drost, M.Y. (1993). F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. *Appl. Environ. Microbiol.*, **59**, 2956–2962.

Hu, J.Y., Ong, S.L., Song, L.F., Feng, Y.Y., Liu, W.T., Tan, T.W., Lee, L.Y. and Ng, W.J. (2003). Removal of MS2 bacteriophage using membrane technologies. *Wat. Sci. Technol.*, **47**(12), 163–168.

Jacangelo, J.G., Laîné, J.M., Carns, K.E., Cummings, E.W. and Mallevialle, J. (1991). Low-pressure membrane filtration for removing *Giardia* and microbial indicators. *AWWA*, **83**(9), 97–106.

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

Lovins, W.A., III, Taylor, J.S. and Hong, S.K. (2002). Micro-organism rejection by membrane systems. *Environ. Eng. Sci.*, **19**(6), 453–465.

Madaeni, S.S., Fane, A.G. and Grohmann, G.S. (1995). Virus removal from water and wastewater using membranes. *J. Membrane Sci.*, **102**, 65–75.

Matsui, Y., Matsushita, T., Imai, Y., Sakuma, S. and Inoue, T. (2001). Virucidal activity of coagulant PACl and alum. *Proceedings of IWA 2nd World Water Congress in CD-ROM*, Berlin, Germany.

Matsui, Y., Matsushita, T., Inoue, T., Yamamoto, M., Hayashi, Y., Yonekawa, H. and Tsutsumi, Y. (2003a). Virus removal by ceramic membrane microfiltration with coagulation pretreatment. *Wat. Sci. Technol.: Wat. Supply*, 3(5), 93–99.

Matsui, Y., Matsushita, T., Sakuma, S., Gojo, T., Mamiya, T., Suzuoki, H. and Inoue, T. (2003b). Virus inactivation in aluminium and polyaluminium coagulation. *Environ. Sci. Technol.*, **37**(22). 5175–5180.

Matsushita, T., Matsui, Y., Shirasaki, N. and Kato, Y. (2005). Effect of membrane pore size, coagulation time, and coagulant dose on virus removal by a coagulation-ceramic microfiltration hybrid system. *Desalination*, **178**(1–3), 21–26.

Wickramasinghe, S.R., Han, B., Carlson, J.O. and Powers, S.M. (2004). Clearance of minute virus of mice by flocculation and microfiltration. *Biotechnol. Bioeng.*, **86**(6), 612–621.

Bull. Environ. Contam. Toxicol. (2004) 72:29–37

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Environmental Contamination and Toxicology

Brackish Water Benthic Shellfish (*Corbicula japonica*) as a Biological Indicator for *Cryptosporidium parvum* Oocysts in River Water

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The coccidian protozoan genus *Cryptosporidium* belonging to *Apicomplexa* is capable of infecting the gastrointestinal or respiratory tracts of a wide range of vertebrates (Spano *et al.* 1998). Six *Cryptosporidium* species are so far distinguished on the basis of differences in oocyst morphology, site of infection and host specificity (Morgan *et al.* 1995). *Cryptosporidium parvum* (*C. parvum*) is particularly considered a significant human and livestock pathogen causing cryptosporidiosis, severe diarrhea, and wasting which is not currently treatable with antimicrobial drugs, as well as no effective vaccine is available (Shianna *et al.* 1998).

Contamination of drinking water with *C. parvum* oocysts from human and animal feces occasionally causes massive outbreaks of cryptosporidiosis (Johnson *et al.* 1995). In present water purification systems, the removal of fine particles such as *C. parvum* oocysts is mainly based on the rapid filtration process utilized widely in Japan. However, the removal of oocysts by filtration is not necessarily complete, and *C. parvum* oocysts are resistant to chlorination (Carraway *et al.* 1996). If the amounts of oocysts in raw water are high and they cross through the filtration barriers, oocysts could become present in the finished water. Reported evidence supporting water-borne transmission of the parasite as an important mode of spreading is present for some documented epidemics (Awad-el-Kariem *et al.* 1994). There are several different methods available for detecting *C. parvum* oocysts in environmental water samples. Most commonly, direct and indirect microscopic visualization of oocysts is carried out using fluorescent dyes or fluorescein-conjugated antibodies (Johnson *et al.* 1995). The immunofluorescence assay (IFA) is a widely used method to detect oocysts in water, however the efficiency of the detection depends on the amount of suspended substances in the water, and this varies greatly according to the natural environmental conditions.

Benthic shellfishes, *Corbicula japonica* (*C. japonica*) live in restricted downstream brackish water areas where the terminal points of the rivers, and feed on suspended plankton by filtration with gills. Hence, *C. japonica* may be a possible biological indicator for net estimates of contamination levels in river water by *C. parvum* oocysts.
This study investigated and evaluated the role and the usefulness of *C. japonica* as a biological indicator or collection system for *C. parvum* oocysts, useful to explore effective, and stable oocysts gathering methods regardless of the surrounding natural conditions.

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MATERIALS AND METHODS

C. japonica with body size 33.3–43.1 × 29.6–37.1 × 18.0–24.1 mm and body weight 15.5–16.5 g were collected from the Ishikari River, Hokkaido, Japan. One hundred and fifty individual *C. japonica* clams were placed in a stainless steel cage in a 12 L aquarium which was filled with dechlorinated 10% artificial sea water (Tetra Marinsalt, Tetrawerke, Germany) of pH 7.4 and d = 1.002. The aquarium was kept at 15°C with a cooler (RZ-90, REI-SEA, Japan) and an air pump (Inno · β6000, Nisso, Japan) with two stick type air stones set under the stainless steel cage for aeration and circulation of the water. To remove ammonia and nitrites, an adequate amount of oxidizing bacteria (Nisso, Japan) was added to the water. The clams were acclimated for 6 weeks in the aquarium prior to the oocyst introduction, and they were fed daily with liquid-type chaw for invertebrates (Tetrawerke) and a proper amount of chlorella (*Chlorella pyrenoidosa*, Sun Chlorella, Japan) which had a negative IFA reaction against *C. parvum* oocysts. The water of the aquarium was changed daily.

Oocysts of *C. parvum* (bovine type, Lot No.01-6) suspended in storage solution (PBS containing penicillin, streptomycin and gentamicin) from Waterborne Inc. (USA) were stored at 4°C. After 6 weeks acclimation, twenty randomly selected control clams were removed from the aquarium, and the water had 8.75×10^6 oocysts of *C. parvum* (7.29×10^5 oocysts/L, 6.73×10^4 oocysts/clam) added. Ten randomly selected clams of the 130 remaining were examined at 2, 4, 6, 8, and 16 hrs after start of the exposure, and also 1, 2, 3, 4, 7, 10, and 14 days after the *C. parvum* addition. Clam feces at the bottom of the aquarium and 12 L water samples were collected at the time points detailed above.

The shells of the ten removed clams were opened by cutting the anterior and posterior adductor muscles with a scalpel, and the gills, gastrointestinal tract (GI tract) and mantle were excised in centrifuge tubes with PBS (GIBCO BRL, USA), followed by grinding with a mixer (IKA Labor Technik, Switzerland). According to the amount of contaminants, the ground fluids were diluted 100 to 1,000 times with a dilution buffer (B100-20, Waterborne, Inc.). For the DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Sigma, USA) assay, samples were boiled for 5 min (Inomata *et al.* 1999), followed by staining with a direct immunofluorescent assay kit (Aqua-Glo G/C Direct, Waterborne, Inc.) and DAPI treatment in a shaking water bath incubator at 37°C. The treated samples were applied on four well microscope slides (C. A. Hendley Ltd, U.K.), dried in an incubator at 37°C, and DABCO (1,4-diazabicyclo-2,2,2-octane, Sigma)-glycerine was added dropwise. The identification and determination of *C. parvum* oocysts were carried out under a fluorescence microscope (ECLIPSE E800, Nikon, Japan) with 200 times magnification. The wavelength for detection of *C. parvum* oocysts were 450–490 nm (B excitation) for FITC (fluorescein isothiocyanate), 365 nm for DAPI, and 510–560 nm (G excitation) for chlorophyll in algaes.

The staining treatment of *C. parvum* oocysts in the collected clam feces sediment was conducted in the same manner as described above, while the water sample was processed by a mixed cellulose ester membrane filter (A100A090C, ADVANTEC, Japan) dissolution method, followed by acetone dissolution, the removal of contaminants by the Percoll (Pharmacia Biotech, USA)-sucrose density gradient method and the staining with

fluorescence dyes in a manner similar to that above. Recovery efficiency of the *C. parvum* oocysts in water samples was determined by adding 1.0×10^3 *C. parvum* oocysts to 6 L of water.

The *in vitro* qualitative detection of infectious *C. parvum* oocysts with cultured cells (HCT-8) was carried out according to the procedure reported previously (Hirata *et al.* 2001; Slifko *et al.* 1997; Upton *et al.* 1995). Thus, feces on day 3 pooled in PBS was prepared for purification of *C. parvum* oocysts by the density gradient method, thereafter the purified oocysts were processed with acid and a succeeding 30 min trypsin (DIFCO, USA) treatment, before subjection to the *in vitro* cultured cell method for the detection of infectious *C. parvum* oocysts. The sporozoits in HCT-8 cells were stained with immonofluorescent agents (Sporo-Glo, Waterborne, Inc., USA) and observed under the fluorescence microscope with 200 times magnification.

An aliquot of the fecal sample at 14 days was analyzed for *Cryptosporidium* DNA by PCR, using TRAP-C2-F (CAT ATT CCC TGT CCC TTG AG) and TRAP-C2-R (TGG ACA ACC CAA ATG CAG AC) primer pair (Science Tanaka, Japan) corresponding to positions 848-867 on the coding strand and 1180-1199 on the negative strand of GenBank sequence X77586, respectively. Purified oocysts were rinsed twice by repeated suspension in 100 μL sterile distilled water, and the aliquot (10 μL) was subsequently subjected to DNA extraction. The oocysts were ruptured by 1.0% SDS (GIBCO BRL) in TE buffer (Nippon Gene, Japan) at 100°C for 30 min and the supernatent was subjected to phenol /chloroform /isoamyl alcohol (24/24/1, GIBCO BRL) extraction, precipitated with absolute ethanol (Wako Pure Chemical Industries, Japan), washed with 70% ethanol, and resuspended in TE buffer. The PCR reaction in a total volume of 30 μL consisted of 1×PCR buffer (Clontech, USA), 0.2 mM each dNTP (Clontech), 0.2 μM of each specific primer, 2.5 U of Taq polymerase (Promega, USA) with antiTaq (Clontech), and 50 copies of internal control. After an initial hot start with both 5 min at 80°C and 5 min at 94°C, DNA amplification was carried out for 30 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extention at 72°C for 1 min in a thermal cycler (GeneAmp PCR System 9700, Perkin-Elmer, USA). An additional cycle of 7 min at 72°C was added for strand completion. The PCR products (369 bp) were analyzed by 3.0% agarose gel electrophoresis and ethidium bromide staining. For the internal control (IC:542 bp), it was prepared from the PCR product (369 bp) and the fragment (173 bp) of the *Bss*HII digested and Klenow blunted multiple cloning site from pBluescript KS$^-$ by the rapid DNA Ligation Kit (Roche, Switzerland).

The measurement of metals (Al, Ba, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Pb, and Zn) in the water sample was performed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, OPTIMA 3300DV, Perkin-Elmer) (Greenberk *et al.* 1992). The sample water was pretreated with 1% HNO_3 and 0.1 mg/L yttrium as the internal standard, then subjected to ICP-AES under recommended conditions. The determination of nitrogen derived from ammonia and nitrites in the aquarium was carried out according to the indophenol and colorimetric methods (Greenberk *et al.* 1992), respectively.

RESULTS AND DISCUSSION

It is not simple to keep benthic freshwater or brackish water shellfishes for appropriately long periods in an artificial environment. Therefore, several daily basic check points for shellfish keeping were observed, removal of ammonia and nitrites, the temperature and pH of the aquarium water, aeration and stirring of the water, the choice of clam chow, and the amount of daily chow consumption by clam *etc.* The clam mortality was below 2% for more than 4 months in this laboratory aquarium. In this study, clam mortality was zero during both the acclimation and experimental periods.

The metal concentrations, which might influence on the water organisms, in the aquarium water were maintained during the experimental period and were as follows: Al, 0.034 ± 0.005; Ba, 0.009 ± 0.001; Ca, 80.5 ± 0.550; Cd, <0.001; Cr, <0.001; Cu, 0.015 ± 0.003; Fe, 0.021 ± 0.001; Mg, 144 ± 5.00; Mn, <0.001; Pb, <0.001; and Zn, 0.010 ± 0.002 mg/L. The nitrogen values of ammonia and nitrites, which were both harmful to clam, were below the detection limit (0.05 and 0.005 mg/L, respectively) during the experiments. The filtration method could not be adopted for trapping of ammonia and nitrites to avoid the absorption of oocysts by filter fiber, while application of oxidizing bacteria might be considered to be adequate judging from the almost perfect removal of ammonia and nitrites in the aquarium during the experimental period.

Feeding to clam was conducted daily 30 min after water exchange. The chow was freshly prepared everyday by suspending powdered chlorella and liquid-type chow in water, while the appropriate daily amount of chow was presumed to be 1.5–2.0 mg/clam for powdered chlorella, and 1.0–1.5 μL/clam for liquid-type chaw, respectively, estimated by the chow consumption and residue in the aquarium.

Recovery efficiency of *C. parvum* oocysts in the water samples by the mixed cellulose ester membrane filter dissolution method was ranged from 71 to 78% (mean ± SD = 74.1 ± 5.8%, CV = 8.0%).

None of the control clams contained *C. parvum* oocysts, confirmed by both microscopic and PCR methods. The aquarium water was oocyst-negative before the start of the experiment.

The study showed that a single exposure of *C. parvum* oocysts (6.67×10^4 oocysts/clam) to *C. japonica* resulted in a relatively rapid intake and excretion of oocysts. Fig. 1 shows the results of the balance study on the percentage of *C. parvum* oocysts detected in clam bodies and feces. The oocyst intake to the clam body was rapid, and practically no oocysts were detected in the water 2 hrs after dose. The oocysts in the clams were almost all in the GI tract, and here reached the maximum at 2 hrs after dose, thereafter they gradually reduced (Fig. 1-A, and -B). The oocyst distribution in the shellfishs 2 hrs after exposure showed that about 90% of the dose was present in the GI tract, 5.0% in the mantle (Fig. 1-B, and -D) and 0.1% in the gills (data not shown). The excretion of oocysts into feces was the predominant route and about 85% (83.4 and 1.2% in the feces and water, respectively) of the oocysts were recovered 4 days after exposure (Fig. 1-E). The total recovery of oocysts was 90% 14 days after exposure. As shown in Fig. 1-F, the oocyst excretion in feces can be presumed to start more than 4 hrs after exposure, and reaches the maximum excretion level at 8 hrs after exposure. The biological half-life ($T_{1/2}$) for the *C. parvum* oocysts in the clams was estimated to be about 24 hrs. The oocysts were excreted into feces in several phases, more rapid in the first phase, and 6 days after exposure (end of the last phase), little of the oocysts