than that for M-11 culture medium. The initial concentration of DOC was about 150 mg/L in CT culture medium and the concentration of DOC was constant during *P. tenue* culture. Unlike M-11 culture medium, the CT culture medium would not lead to a significant variation of AOC concentration although some variation was observed in AOC-P17 and AOC-NOX at the death phase (after 39 days). From the results, it was considered that the generation and the ingredients of EOM derived from algae could be changed by the condition of growth medium. Moreover, the AOC formation seemed to increase enormously in culture medium including organic substances with low concentration such as M-11 culture medium. The variation of AOC component derived from EOM produced in M-11 culture medium after 39 days culture was classified by molecular weight fractionation (Figure 2). The EOM was fractionated by ultra filtration membrane into four classes of molecular weight (lower than 1,000Da, 1,000–5,000Da, 5,000–10,000Da and higher than 10,000Da). The AOC produced extracellularly by *P. tenue* was mainly detected at the fraction of lower than 1,000Da of molecular weight. The ratio of AOC-P17 and AOC-NOX at the fraction of lower than 1,000Da was about 80% and 20%, respectively. The ratio of AOC-NOX decreased with increase of molecular weight and it almost disappeared in the fraction of higher than 10,000Da after all. The EOM with low molecular weight is likely to be the main organic substances contributing to the formation of AOC.

We also investigated the relationship between the chlorine dosage and residual chlorine. Figure 3 shows that the residual chlorine increases according to chlorine dosage. The chlorine was not consumed and remained in case of the fresh culture medium excluding *P. tenue*. When the chlorine dose was 30 mg/L, the residual chlorine for EOM and IOM was about 10 and 20 mg/L, respectively. Namely, the chlorine demand for the oxidization of IOM was nearly double that for the oxidization of EOM. Figure 4 shows the variation of AOC components after the chlorination. In Figure 4a, the AOC concentration shows the maximum concentration of 751 μg/L at the low chlorine dosage of 2 mg/L, revealing that AOC increased 18% compared with that before chlorination. The AOC concentration then decreased with the increase of chlorine dosage. The 5, 10 and 30 mg/L of chlorine dosage led AOC to decrease to 336 μg/L, 219 μg/L and 98 μg/L, respectively. Before chlorination, the concentration of AOC–P17 was nearly equivalent to that of AOC-NOX. The ratio of AOC–P17 to AOC, however, had rapidly decreased with chlorination. When the 30 mg/L chlorine was dosed, the AOC–NOX ingredients could account for 90% of the AOC concentration. Considering that the 2 mg/L of chlorine dosage is generally adopted for drinking water treatment, the present system may not sufficiently treat the EOM contaminated into raw water and the AOC may remain even after chlorination. Higher chlorine dosage than usual is needed for treatment of EOM not to form AOC. The AOC variation according to the chlorination of IOM is presented in Figure 4b. IOM

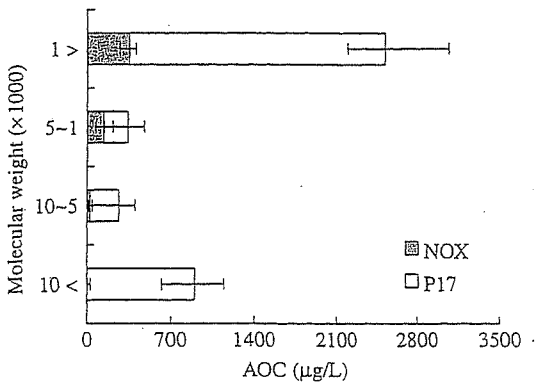

Figure 2 The molecular weight profile of AOC derived from EOM produced by *P. tenue* culture

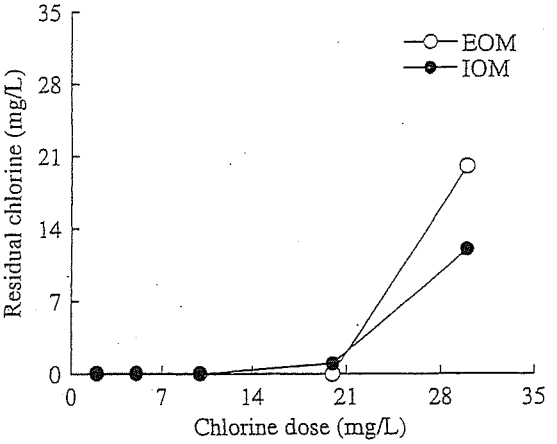

Figure 3 The relationship between chlorine dose and residual chlorine after chlorination of EOM and IOM

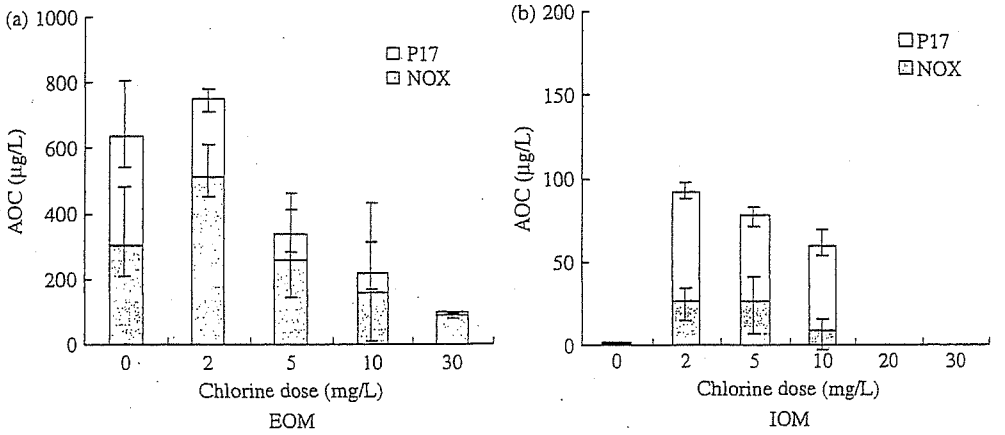

Figure 4 The variation of AOC components after chlorination of EOM and IOM: (a) EOM; and (b) IOM

was collected by centrifugation and was chlorinated using the same dosages as the experiments for EOM. Since the residual chlorine for IOM was detected after 20 mg/L of chlorine dosage as shown in Figure 3, we added 20 mg/L of chlorine dosage. The AOC concentration derived from IOM increased with increasing chlorine dosage. The AOC concentration increased to 92 and 78 μg/L after chlorination with 2 and 5 mg/L of chlorine dosage, respectively. The AOC, however, was not detected at 20 or 30 mg/L of chlorine dosage. Unlike the results for EOM, the AOC–P17 was higher than the AOC–NOX accounting for over 74% of AOC concentration. The results showed that much less AOC could be formed from IOM than from EOM by chlorination.

Conclusions

We have been interested in the variation of AOC concentration derived from algae, especially EOM. In the present study, we carried out the algal culture using different kinds of culture media and investigated the variation of AOC component in EOM fractionated by the molecular weight. Further on, we presented the fate of AOC after chlorination of EOM and IOM. The AOC could be formed enormously from EOM produced by algae grown in the culture medium including poor organic substances such as the M-11 culture medium. The concentration of AOC–P17 and AOC–NOX consisting of AOC would vary with different trends according to the type of culture medium. For the M-11 culture medium, the AOC–P17 and AOC–NOX showed a similar trend to each other and the maximum concentration was observed at the stationary phase. On the

other hand, the results for the CT culture medium including rich organic substances showed that the change of the AOC–P17 and AOC–NOX was contrary to each other. The result implied that the lower concentration of organic substances may stimulate the metabolism of algae concerning AOC component. The variation of AOC components in EOM classified by the molecular weight was presented. AOC was mainly observed at the fraction of lower than 1,000Da of the molecular weight. Namely, the EOM with low molecular weight among EOMs produced by *P. tenue* would help the formation of AOC. The behavior of AOC by the chlorination of EOM and IOM showed that the AOC could be generated more easily from EOM than IOM by chlorine oxidation. The AOC concentration after chlorination of EOM was much higher than that of IOM. The most AOC concentration depended on the AOC–NOX for EOM and the AOC–P17 for IOM. When chlorination of EOM and IOM was conducted, the concentration of AOC increased at first and then decreased. The AOC from IOM was not detected with 20 mg/L of chlorine dosage while the complete removal of AOC was not observed for EOM. The EOM could be considered to account for much of AOC formation in drinking water treatment system. Therefore we should pay more attention to the contamination of EOM in drinking water resource. Moreover, the AOC concentration reached a maximum with 2 mg/L of chlorine dosage when the chlorination of EOM and IOM was carried out. Although a chlorine dosage of 2 mg/L is generally adopted for drinking water treatment, a higher chlorine dosage is required to treat EOM not to form AOC.

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Detection System of *Cryptosporidium parvum* Oocysts by Brackish Water Benthic Shellfish (*Corbicula japonica*) as a Biological Indicator in River Water

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Abstract. The brackish water benthic shellfish, *Corbicula japonica*, was experimentally exposed to *Cryptosporidium parvum* oocysts at 1.51×10^4 oocysts/clam/day for 7 or 14 days. Oocysts were predominantly eliminated through the feces of *Corbicula japonica* in both cases by microscopic and PCR methods. The fecal excretion rates of oocysts within 4 days after the last exposure to *Corbicula japonica* were 87.6% for the 7-day exposure group and 86.0% for the 14-day exposure group. The tissue residue level of oocysts in the gastrointestinal tract 3 days after the last exposure was 2.7% of total exposed oocysts and that of 7 days was 1.1% for the 7-day exposure case and 1.6 and 0.5% for the 14-day exposure case, respectively, maintaining infectivity to cultured cells (HCT-8) *in vitro*. At the same time, field tests of *Corbicula japonica* for collecting oocysts showed that this clam could certainly collect *Cryptosporidium parvum* oocysts in the natural river and, furthermore, the gene type of *C. parvum* could be also identified proving its effectiveness as a biological indicator. The present study showed that the brackish water benthic shellfish *Corbicula japonica* may be capable of gathering and preserving *Cryptosporidium parvum* oocysts to a considerable extent under the natural ecological conditions, and further suggests the effectiveness of *Corbicula japonica* as a practical and general bioindicator for estimates of river water contamination by oocysts of *Cryptosporidium parvum*.

There are a number of reports of the detections of oocysts of the coccidian protozoan genus *Cryptosporidium parvum* (*C. parvum*) in aquatic life, especially in sea water shellfish (Fayer *et al.* 1998, 1999; Graczyk *et al.* 1998, 1999, 2001; Gomez-Bautista *et al.* 2000; Freire-Santos *et al.* 2001, 2002; Traversa *et al.* 2004; Giangaspero *et al.* 2005; Gomez-Couso *et al.* 2005). Previous studies of these shellfish have suggested that some of the shellfish offer the potential to be biological indicators of contamination of their habitat by *C. parvum* oocysts. However, quantitative time-course balance study is limited. The amount of oocysts detected in sea water shellfish is assumed to depend on the surrounding ocean currents (Gomez-Bautista *et al.* 2000), and while these sea water clams appear to be a possible hygienic biological indicator for perishables, they could not be a practical biological indicator for estimating vicinal river water contamination by *C. parvum* in its habitat. To enable a more practical and effective estimate of river water contamination by *C. parvum*, the aquatic life in fresh or brackish water must be taken into account, because it presents a more limited and stable habitat than the sea environment.

In Japan, there are a number of fresh or brackish water benthic shellfish, which feed on suspended plankton by filtration with gills. Of these, three *Corbiculidae* species are commonly consumed and considered edible: *Corbicula leana* and *Corbicula sandai*—known to be hermaphroditic, ovoviviparous, and gonochorismal, oviparous clams—live in fresh water throughout their lives, inhabiting the middle reaches of rivers and lakes, respectively. *Corbicula japonica* (*C. japonica*) is a gonochorismal, oviparous clam, which lives in brackish water and is widely bred in river-mouth brackish water areas at the estuaries of rivers. Therefore, it may offer the potential to be a biological indicator for estimates of contamination in river water by *C. parvum* oocysts, and it is available for sampling throughout the year as well as being a convenient size for experiments in the laboratory.

In a previous study on the role and the usefulness of *C. japonica* as a biological parasite indicator by single exposure of *C. parvum* oocysts to the clam, the effectiveness of *C. japonica* as a biological indicator for oocysts of *C. parvum* was suggested (Izumi *et al.* 2004). The present study investigates the role of *C. japonica* as a practical oocyst collection system in further detail by consecutive exposures of *C. parvum* oocysts to the clam.

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Materials and Methods

Keeping of the Shellfish

C. japonica with a body size of 31.2–37.4 × 27.3–32.0 × 18.2–25.0 mm and a body weight of 11.5–18.1 g were collected from the Ishikari River, Hokkaido, Japan. Ninety *C. japonica* clams were placed in a stainless steel cage so as not to overlap in a 12-L inner cistern filled with dechlorinated 10% artificial sea water (Tetra Marinsalt, Tetrawerke, Germany) of pH 7.4 and d = 1.002 equipped with an air pump (Inno β6000, Nisso, Japan) with two stick type air stones (15 × 15 × 150 mm) set under the stainless steel cage for aeration and circulation of the water, floated in an insulated outer cistern filled with fresh water maintained at 15°C by a cooler (RZ-90, REI-SEA, Japan). To remove ammonia and nitrites, an adequate amount of oxidizing bacteria (Aquarium Pharmaceuticals) was added to the water of the inner cistern. The clams were acclimatized for 6 weeks in the aquarium prior to the oocyst introduction, and they were satiated daily with proper amounts of both liquid-type chaw for invertebrates (Tetrawerke) and powdered chlorella (*Chlorella pyrenoidosa*, Sun Chlorella, Japan) suspended in water, which had a negative IFA reaction against *C. parvum* oocysts. The water of the inner cistern was periodically changed, at 9:30 a.m. every morning, with pre-adjusted dechlorinated 10% artificial sea water (15°C) to protect the shellfish from temperature shock, and the feeding was carried out about 30 min after the water change.

Administration and Recovery of C. parvum *(Balance Study)*

Oocysts of *C. parvum* (bovine type, Lot No. 8-27) were purchased from Waterborne Inc. and stored at 4°C. After 6 weeks of acclimation, 20 randomly selected control clams were removed from the aquarium, and the water had a daily dose of 1.36×10^6 oocysts of *C. parvum* (1.13×10^5 oocysts/L, 1.51×10^4 oocysts/clam) added with chaw at 10:00 a.m. For both the 7- and 14-times exposure tests, clam fecal samples and cistern water were collected daily and examined after the start of the *C. parvum* exposure, and ten randomly selected clams were removed and examined at 1, 2, 3, 4, 7, 10, and 14 days after the final addition of *C. parvum*. The treatment of the clam feces and water in the aquarium during the experiment was carried out in the same manner as the previous report with the above-mentioned time intervals.

Detection of C. parvum *Oocysts*

The details of the detection of oocysts from the clam bodies, fecal samples, and cistern water are as described previously (Izumi *et al.* 2004). Prior to the staining of the surface of the fecal oocysts by fluorescent dye, the anti-*Cryptosporidium* kit (Dynabeads GC-Combo Kit, Dynal Biotech Inc.) was used to purify the samples, followed by boiling treatment for 5 min (Inomata *et al.* 1999). The microscopic observations were carried out for the identification and determination of the *C. parvum* oocysts under a fluorescence microscope (DM LB2, Leica) with 200 times magnification.

Field Survey

A field experiment was carried out to estimate the ability of the clams to collect protozoa in the actual river. First, the shellfish was acclimatized for 6 weeks in the aquarium in the laboratory and checked to be *C. parvum* free, then groups of 60 clams were put in closed stainless-steel mesh cages and set at the three different locations (total 180 clams) in the river-mouth area of the Yanbetsu River in Hokkaido, Japan. The cages were positioned as follows: one in the center of the river-flow, and the others about 5 m from the center, to form a line across the river. The transport of the clams from the laboratory to the river was rapid in an insulated box kept at about 4°C with frozen gel packs and without water. On the 7th day after the placement in the river, the three cages were removed from the river and carried in an insulated box to the aquarium in the laboratory under the above conditions. Thereafter, fecal samples of the bivalves were collected daily for 3 days and analyzed for oocysts of *C. parvum* in the manner described previously. To estimate the relationship between the density of oocysts of *C. parvum* in the water and the actual amount trapped by the clams, the protozoa in the river water were checked twice during the survey, at the start and at the end (the 7th day) of the experiments with the clams in the river. The treatment of the water sample (10 L) was processed by a mixed cellulose ester membrane filter (A100A090C, ADVANTEC) filtration method (membrane filtration method), followed by acetone dissolution, oocyst purification by the above-mentioned anti-*Cryptosporidium* kit, and staining with fluorescent dyes. The trapping ability of *C. japonica* for oocysts of *C. parvum* in the river was made by an approximate estimate calculating the volume of water filtered by a clam per day and the density of the protozoa in the river water. Fecal samples collected in the same way at the other time from the same field were used in the following infectivity assay, DNA sequencing, and PCR-RFLP analysis.

Oocyst Infectivity Assay by Cultured Cells (HCT-8)

The *in vitro* infectivity of the oocysts recovered from the clam feces was determined by the infectivity to cultured cells (HCT-8). The basic procedure was detailed in previous reports (Upton *et al.* 1995; Slifko *et al.* 1997; Hirata *et al.* 2001; Izumi *et al.* 2004). The infection of the cultured cells was evaluated by observation of the proliferation of sporozoits stained with immunofluorescent agent in the HCT-8 cells under the fluorescence microscope with 200–1,000 times magnification.

DNA Extraction and PCR

In both tests, an aliquot of sample of excreta and the GI tract after the final exposure were checked for protozoan DNA by PCR, as reported previously, while the internal control (IC: 542 bp) was applied to only the negative control (Peng *et al.* 1997; Sulaiman *et al.* 1998).

DNA Sequencing and Analysis

The PCR product was extracted by the QIAquick Gel Extraction Kit (Qiagen) followed by purification with HPLC (TOSO 8020 DNA NPR column: 4.6 mm × 75 mm) equilibrated with 20 mM Tris-HCl/1 mM EDTA/pH 8.0. The product was eluted by a 0.200–0.275 M NaCl gradient and then ligated to a pGEM T-Easy Vector (Promega) by a Rapid DNA Ligation Kit (Roche Diagnostics), followed by transformation to the JM109 Competent Cell (Promega). The DNA sequencing of recombinant clones that held the corresponding insert of *C. parvum* was performed on an ABI 377 Automated Sequencer (Perkin Elmer) using a BigDye Primer Cycle Sequencing FS Ready Reaction Kit with a primer set of -21M13 and M13Rev (Applied Biosystems). The representative human and bovine genotypes were

deposited in GenBank with access numbers AF082521 to AF082524 showing typical human type sequence #51:G, #78:C, #100:T, #147:C and #280:T/C including the *Hae*III site (#76–79:GGCC), and typical bovine type sequence #51:A, #78:T, #100:G, #147:T and #280:C including *Bst*EII site (#76–82:GGTNACC), respectively (see Table 2).

PCR RFLP

To supplement the DNA sequencing study for differentiating the genotypes of *C. parvum*, PCR amplification of the TRAP-C2 gene was followed by RFLP (Sulaiman *et al.* 1998). Restriction enzymes, *Bst*EII and *Hae*III (Roche Diagnostics), were applied for classification between bovine and human genotypes, respectively. For the restriction fragment analysis, about 1.5 μg of amplified PCR product in a total of 20 μl of the respective restriction buffer was digested by 10 U of *Bst*EII or *Hae*III under the conditions recommended by the supplier. The digested product was fractionated on 2.0% agarose gel and visualized by ethidium bromide staining.

Measurement of the Water Filtration Rate of C. japonica

To obtain basic ecological data of *C. japonica* for the following field survey, an attempt was made to determine the water filtration rate of the clams. Ten randomly selected clams were placed in a stainless steel cage in a glass beaker with 2 L of 10% artificial seawater. The beaker was kept cooled at 15°C, and a ball-type air stone and a stirrer stone were set under the stainless steel cage to prevent chlorella powder sedimentation during the test. Two hundred milligrams of chlorella powder were suspended in water and added to the beaker. Subsequently, 10 ml of water were collected every 10 min after the addition of the chlorella; after centrifugation (3,000 rpm, 10 min) of the collected water, the chlorophyll in the precipitate was extracted with 5 ml of acetone. The extracted chlorophyll was determined as chlorophyll A and B by measurement of the wavelengths at 664, 647, and 630 nm, as *Chlorella pyrenoidosa*, a *Chlorophyceae* that contains both chlorophylls A and B (Greenberg *et al.* 1992). The calculation of the two chlorophyll concentrations was made with the following numerical formula: chlorophyll A (μg/ml) = $11.85Abs_{664} - 1.54Abs_{647} - 0.08Abs_{630}$, and chlorophyll B (μg/ml) = $- 5.43Abs_{664} + 21.03Abs_{647} - 2.66Abs_{630}$. The total chlorophyll content was calculated as the sum of chlorophyll A and B. We measured the time to the plateau level where the total chlorophyll had been removed by the clam feeding, and then estimated the filtration rate of the clams.

Analysis of Metals and Nitrogen

The basic procedures of the analysis of the metals (Al, Ba, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Pb, and Zn) in the water of the cistern were as described in the previous report (Izumi *et al.* 2004). The analysis of nitrogen for ammonia and nitrites in the water of the cistern was carried out with indophenol and colorimetric methods, respectively (Greenberg *et al.* 1992).

Results

Overall the data for maintaining the shellfish show that the conditions for this study were adequate. The basic data for maintaining the shellfish containing the metal concentration, and the nitrogen values of ammonia and nitrites in the water, which are both harmful to clams, showed almost the same values as the previous report (Izumi *et al.* 2004). None of the control clams contained *C. parvum* oocysts, confirmed both microscopically and by PCR. The aquarium water was oocyst-negative before the start of the experiment. During the experimental period of both consecutive exposure tests, the clam mortalities were below 2.0% for both the 7- and 14-day studies.

The two exposure studies reported here show that *C. parvum* oocysts were rapidly ingested in *C. japonica* and nearly all excreted in the feces irrespective of the length of the oocyst exposure period. Figure 1 shows the balance of *C. parvum* oocysts detected in the clam excreta and the gastrointestinal tracts (GI tract). In the exposure periods, the fecal excretion of oocysts was almost linear with steep slopes, showing relatively constant daily excretion rates in both tests. After the final of the 7 or 14 consecutive daily doses of *C. parvum* oocysts to *C. japonica*, the excretion patterns of oocysts were similar to that of the single administration (Izumi *et al.* 2004). Thus, the oocysts were predominantly excreted in the feces in at least three phases: a more rapid first phase for several days after cessation of the exposure, and 6 or 7 days after the last dose (the last phase), in which a small quantity of oocysts was microscopically detected, while the protozoan DNA was identified by PCR on the 7th day after the last dose in both tests. It was observed that the excretion rate in feces seemed to be slightly faster in the 7-times exposure group than in the 14-times exposure group. However, there were statistically no significant differences between the two groups (Fig. 1A–D).

Overall, the excretion of oocysts by the fecal route was predominant, and the total recovery of oocysts within the 4 days after the final dose was 87.6% in the feces and 1.5% in the water for the 7-times exposure test, and 86.0% in the feces and 1.9% in the water for the 14-times exposure test.

The oocyst residue in the tissue of the two groups after the final exposure showed that most of the oocysts in the shellfish were in the GI tract, and the amount of oocysts in the mantle and gills was negligible compared to that in the GI tract in both tests (data not shown).

The decrease rate of oocysts in both the 7- and 14-times exposure tests in the GI tract was slightly different at different time points, with the decrease in the first 3 days more rapid than later (Fig. 1E, F). The slope of the curve in the later phase was flatter and reached a plateau level 10–14 days after the final exposure. The decrease in oocysts in the GI tract appears mostly to correspond to the fecal excretion, in both magnitude and reduction pattern, like that observed in the single administration test.

The oocyst residue levels in the GI tract 1 day after the last exposure were 6.6% of the total dose for the 7-times exposure test and 4.2% for the 14-times exposure test, and 14 days later the residual levels were 0.7 and 0.2%, respectively. At 14 days after the last dose, the residue of *C. parvum* oocysts in the GI tract was microscopically and genetically detected in both dose tests, while fecal DNA was not identified at that time. This may account for some nonspecific absorbance of oocysts to the GI tract (Fig. 2A). In the exposure studies, the total amount of oocysts detected microscopically in both the excreta and water amounted to about 89.9% of the oocysts introduced during the 7-times exposure test and 88.3% during the 14-times exposure

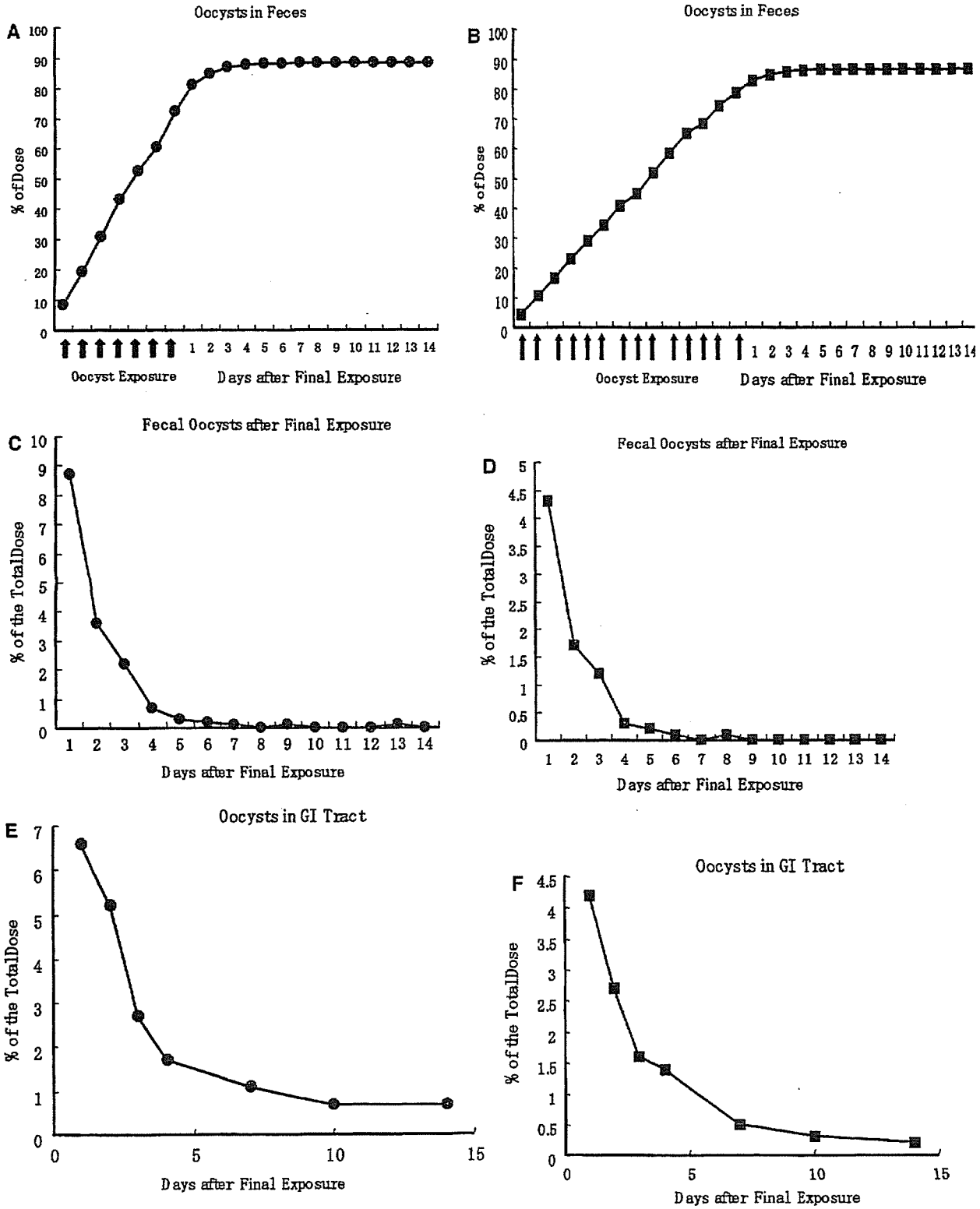

Fig. 1. The relative amounts of *Cryptosporidium parvum* oocysts detected in the excreta and GI tract of *Corbicula japonica* in the studies by consecutive oocysts exposure tests (A, C, and E: 7 doses; B, D, and F: 14 doses). **(A,B)** The cumulative percent amount of oocysts detected in the clam excreta with the exposure rate of 1.51×10^4 oocysts/clam/day of oocysts for 7 or 14 days. **(C–F)** The percent amounts of oocysts determined in clam feces and GI tract following 7 or 14 consecutive daily exposures of oocysts

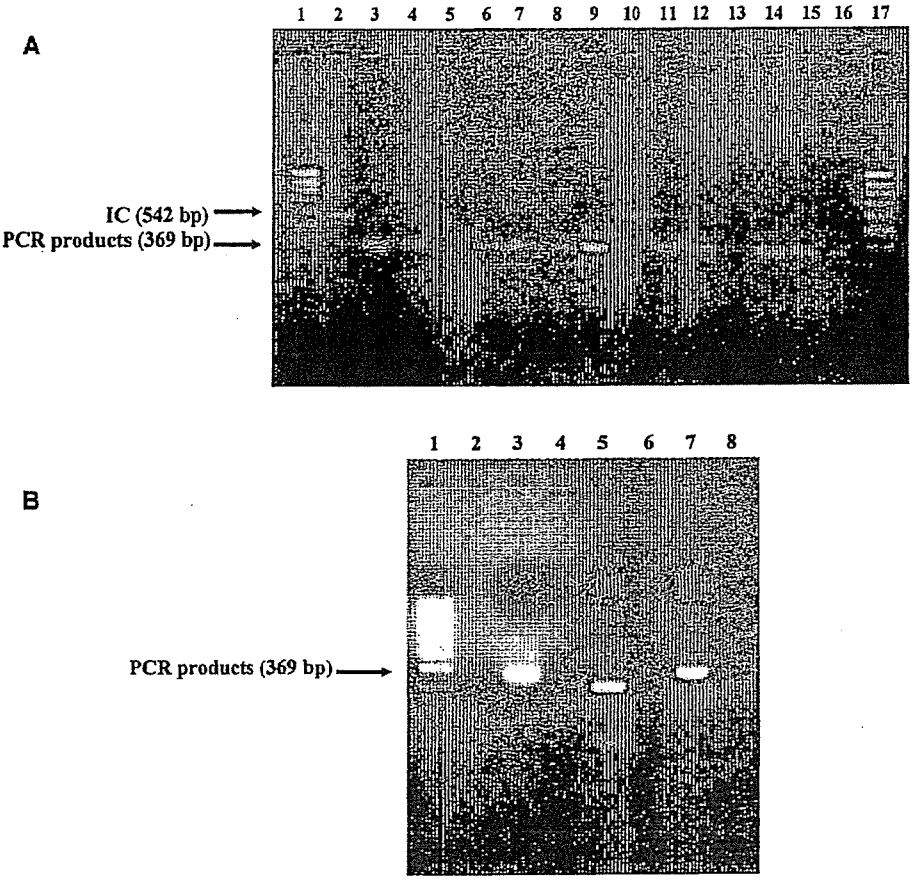

Fig. 2. Ethidium bromide–stained 2.0% agarose gel showing amplification products using TRAP-C2 primers from clam excreta and GI tract samples (A), and the results of PCR- RFLP for the fecal DNA obtained in the field survey (B). (A) Lanes 3–8; 7 exposures, lanes 11–16; 14 exposures. Lanes 1, 17: 100-bp 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

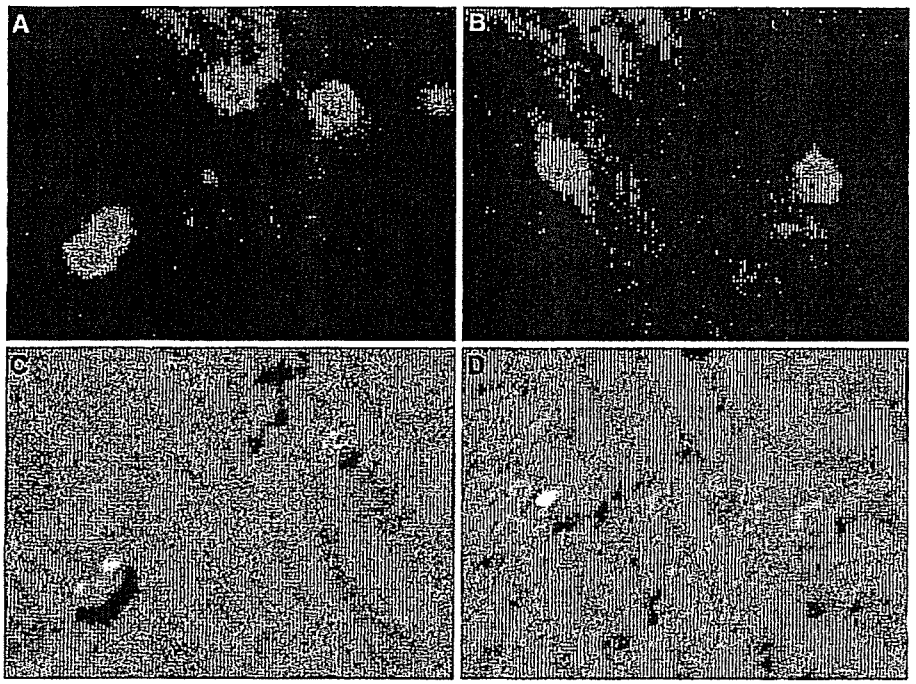

Fig. 3. Photographs of the developmental stage of *Cryptosporidium parvum* prepared from fecal samples of laboratory and field tests in HCT-8 cells 2 days after inoculation. **(A)** Fluorescence photograph of a field of foci after 2 days of inoculation using oocysts prepared from laboratory tests (14-exposure 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.

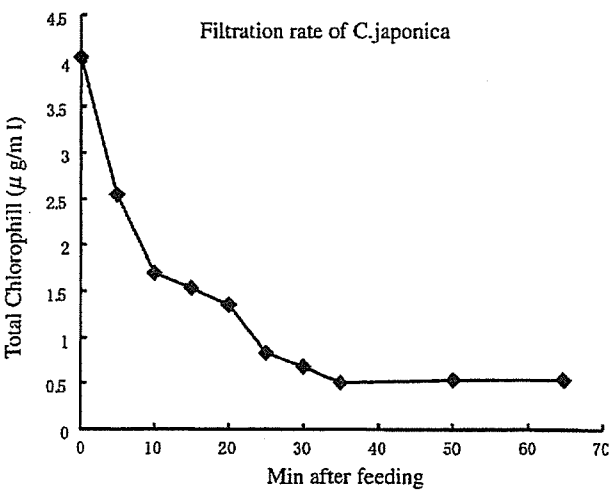

Fig. 4. Water filtration rate of *Corbicula japonica* determined by powdered chlorella. The filtration rates of the clams were calculated from the amount of extracted chlorophyll and the time till discontinuing feeding

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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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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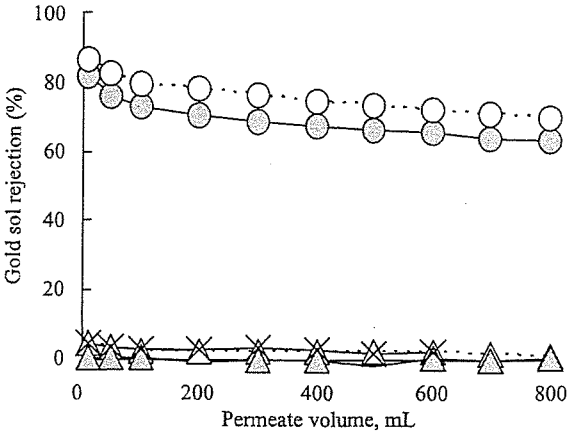

Fig. 1. Effect of membrane type on 10 nm gold sol rejection. The experiment were conducted under 50 kPa. Solid circled, Sartorius Q; open circles, Sartorius D; solid triangles, Sartorius S; open triangles, Sartorious C; x marks, GVWP.

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

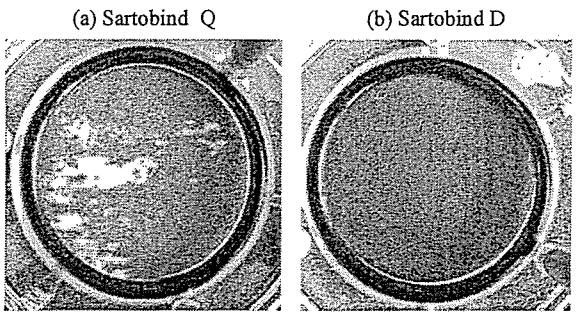

Fig. 2. Photos of membrane surface just after 100 mL of the 10 nm gold sol was filtered under 50 kPa.

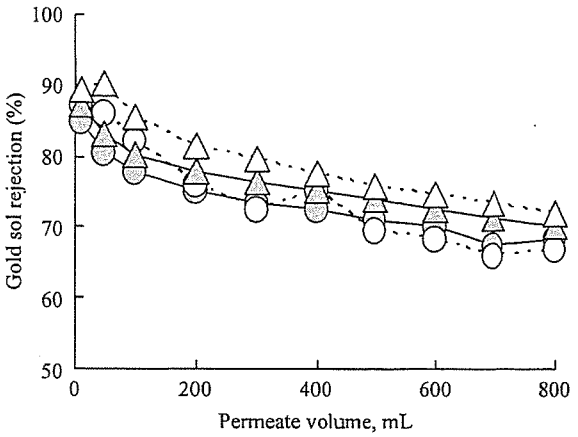

Fig. 3. Effect of size of gold sol on rejection. The experiments were conducted under 50 kPa. Solid circles, 10 nm, Q; open circles, 43 nm, Q; solid triangles, 10 nm, D; open triangles, 43 nm, D.

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.

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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

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).

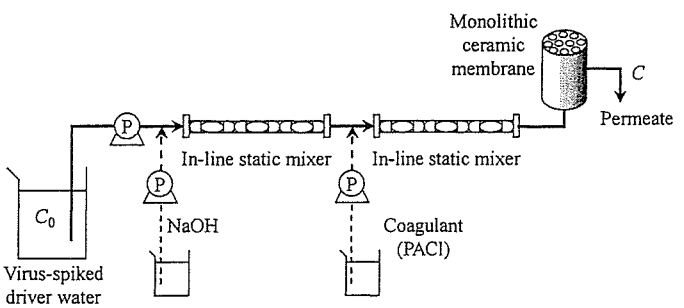

Figure 1 Diagram of experimental setup of the coagulation–MF hybrid experimental water-treatment system. C_0 and *C* mean the virus concentrations (pfu/mL) in raw-water tank and MF permeate at each sampling time, respectively

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′

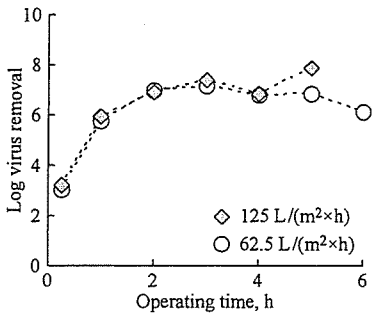

Figure 2 Effect of filtration flux on QB virus removal. The coagulant (PACl) dose was 1.08 mg/Al/L, and the coagulation time was 2.4 s

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

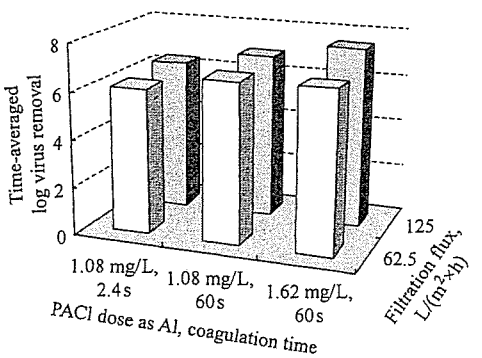

Figure 3 Overall comparison of parameters for QB virus removal by the coagulation–MF hybrid system

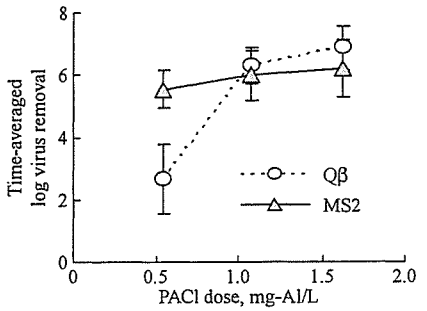

Figure 4 Comparison of virus removal between QB and MS2. The coagulation time was 2.4 s, and the flux was 62.5 L/(m^2 × h)

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

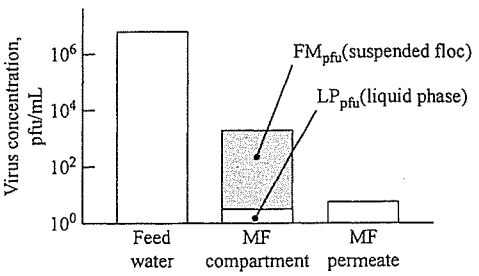

Figure 5 Infectious QB virus concentrations in the feed water, in the water in the MF compartment, and in the MF permeate after the operation of 6 h when the system was operated with a PACl dose of 1.08 mg/Al/L, a coagulation time of 2.4 and a filtration flux of 62.5 L/(m^2 × h)