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Effects of Ionic Strength on Bacteriophage MS2 Behavior and Their Implications for the Assessment of Virus Retention by Ultrafiltration Membranes[∇]

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Bacteriophage MS2 is widely used as a surrogate to estimate pathogenic virus elimination by membrane filtration processes used in water treatment. Given that this water technology may be conducted with different types of waters, we focused on investigating the effects of ionic strength on MS2 behavior. For this, MS2 was analyzed while suspended in solutions of various ionic strengths, first in a batch experiment and second during membrane ultrafiltration, and quantified using (i) quantitative reverse transcriptase PCR (qRT-PCR), which detects the total number of viral genomes, (ii) qRT-PCR without the RNA extraction step, which reflects only particles with a broken capsid (free RNA), and (iii) the PFU method, which detects only infectious viruses. At the beginning of the batch experiments using solutions containing small amounts of salts, losses of MS2 infectivity (90%) and broken particles (20%) were observed; these proportions did not change during filtration. In contrast, in high-ionic-strength solutions, bacteriophage kept its biological activity under static conditions, but it quickly lost its infectivity during the filtration process. Increasing the ionic strength decreased both the inactivation and the capsid breakup in the feed suspension and increased the loss of infectivity in the filtration retentate, while the numbers of MS2 genomes were identical in both experiments. In conclusion, the effects of ionic strength on MS2 behavior may significantly distort the results of membrane filtration processes, and therefore, the combination of classical and molecular methods used here is useful for an effective validation of the retention efficiency of ultrafiltration membranes.

A large range of human enteric pathogenic viruses (e.g., enteroviruses, noroviruses, rotaviruses, and hepatitis A and E viruses) may be present in natural water and may, even at low concentrations, be responsible for many serious human diseases (12). To predict the presence of fecal pollution in water and to assess the performance of disinfection systems, viral contamination can be estimated either by specifically detecting pathogenic viruses or by evaluating the level of fecal contamination using indicators that are supposed to be representative of pathogenic microorganisms of enteric origin. Bacterial indicators like *Escherichia coli* have proved to be inadequate for the assessment of viral contamination (16). Many studies have considered *E. coli* bacteriophages attractive candidates for predicting virus survival in water (3, 14, 26, 34), and among them, F-specific RNA phages have been recommended as a surrogate to evaluate pathogenic virus removal by membrane filtration used in water treatment (18, 20).

The technique of water disinfection using ultrafiltration membranes is of growing interest (20, 29). This method appears as an alternative or supplement to conventional techniques of chemical (chlorination or ozonation) or physical (UV radiation) disinfection, which presents a significant number of drawbacks, such as production of toxic by-products and the fact that, in most cases, microorganisms are only partially inactivated and remain present in the treated water (8, 25).

F-specific RNA phages (family *Leviviridae*) are viruses that primarily infect Gram-negative bacteria, which possess a plasmid coding for an F or sex pilus. Among them, bacteriophage MS2 (genus *Levivirus*, genogroup I) is a nonenveloped virus with a 27-nm protein icosahedral capsid surrounding a single-stranded RNA genome of 3,569 nucleotides (21). Because its size and structural properties are similar to those of many human pathogenic enteric viruses and because it can be quantified more easily and faster, the bacteriophage MS2 has been widely used as an indicator to quantify virus retention by a selected membrane and this technique has proved to be useful (18, 20, 29). However, many methodological problems may appear, notably because of the physical and chemical properties of this RNA phage.

Many environmental factors can influence the behavior of viruses in water, such as temperature (9), pH (9), high hydrostatic pressure (15), the presence of surface active compounds (37), and the ionic strength of the solution (37). These factors may induce viral inactivation, adhesion to support, and/or aggregation (11, 13). Inactivation consists of a loss of viral infectivity and is the consequence of a modification/deterioration of the capsid or genome. This phenomenon is the only one that can be taken into account to measure the real reduction of infectious viruses in water. Most published studies have used

only cell culture to quantify infectious phages and do not take into account all the factors that can result in loss of infectious virus particles, such as aggregation of the phages or adhesion of the phages to surfaces. However, these factors may lead to an overestimation of removal processes such as membrane filtration. Therefore, quantification methods that take these three phenomena (capsid modification, aggregation, and adhesion to support) into account and that determine the involvement of each phenomenon in the process are useful for a true assessment of the effectiveness of membrane treatment.

Recently, methods based on direct detection of bacteriophage genomes have been described, such as quantitative reverse transcriptase PCR (qRT-PCR) (7), RT-PCR with reverse line blot hybridization (40), monoplex real-time RT-PCR (27, 28), and multiplex real-time RT-PCR (22, 42). It is now clearly recognized that the viral genome persists longer than the infectivity of the virus concerned (8). Furthermore, in theory, quantification of the viral genome by PCR methods is not affected by the aggregation process.

In order to obtain real information on the virus' behavior and to estimate the true effectiveness of processes for removing viruses from water, it seems essential to take both the influence of environmental factors and the methods used to quantify the virus into consideration. Many studies using bacteriophage MS2 as an indicator to detect fecal contamination or to assess the performance of membranes have been conducted using different types of waters of various ionic strengths, such as seawater (4, 6, 22, 33), tap water (17), river water (6, 42), or storm water (6). However, it has been shown that raising the ionic strength of the solution causes increased electrostatic attractions between colloid-sized particles (e.g., bacteria and viruses) and the air-water interface (AWI) (38, 41). Therefore, we focused our study on the investigation of the effects of ionic strength on bacteriophage MS2 behavior. For this, phage MS2 was analyzed while suspended in aqueous solutions of various ionic strengths, first in a batch experiment and then during a 2-h ultrafiltration using a hollow fiber membrane. Moreover, in order to take all behaviors of MS2 (inactivation, adhesion, and aggregation) into account, we combined three methods of quantification: qRT-PCR, which detects the total number of viral genomes; qRT-PCR without the RNA extraction step, which reflects only particles with a broken capsid (free RNA); and the PFU method, to detect only infectious viruses.

MATERIALS AND METHODS

Bacteriophage MS2 stocks. Bacteriophage MS2 (ATCC 15597-B1) was replicated using *Escherichia coli* W1485 (ATCC 12435) according to the Pasteur Institute (Paris, France) procedure with slight modifications. Briefly, 1 ml of MS2 stock was mixed with 1 ml of *E. coli* suspension in 10 ml tryptone-yeast extract medium containing 14 g/liter tryptone (Oxoid, Ltd., Basingstoke, Hampshire, United Kingdom), 7 g/liter yeast extract (AES Chemunex, Bruz, France), 7 g/liter NaCl (Sigma-Aldrich Co., St. Louis, MO) and 2.5 g/liter magnesium sulfate heptahydrate (Sigma-Aldrich Co., St. Louis, MO). Then, 0.8 ml of this preparation was distributed into inclined tubes containing tryptic soy agar (bioMérieux, Craponne, France) and incubated for 22 h at 37°C. After replication, the inclined agar was washed with 5 ml of phosphate-buffered saline (PBS) (9 g/liter NaCl, 0.8 g/liter Na_2HPO_4, 0.1 g/liter KH_2PO_4; Lonza, Verviers, Belgium) on a rotator agitator (S150 orbital incubator; Stuart Scientific, Lille, France) for 10 min at room temperature and under gentle stirring. Viral suspension was then recovered and centrifuged at 3,000 × *g* for 20 min at 10°C (Megafuge 1.0R; Heraeus Sepatech, Harz, Germany) and the supernatant filtered through a 0.2-μm filter

(Minisart NML; Sartorius Stedim Biotech, Goettingen, Germany) to remove any remaining bacterial residual. The final viral concentration quantified by the double agar layer procedure (19) was 10^{11} PFU/ml. The viral suspension was then stored at −80°C.

Quantification of infectious phage by cell culture: the PFU method. Bacteriophage MS2 was enumerated according to the double-agar-layer procedure (19) with the bacterial host mentioned above. When necessary, logarithmic dilutions of MS2 samples were performed using PBS in order to decrease the numbers of plaques to between 30 and 300 per plate. Briefly, 0.1 ml of bacteriophage sample and 0.9 ml of *E. coli* suspension, prepared in PBS at approximately 1×10^8 CFU/ml (optical density at 640 nm [OD_{640}] of 0.1), were combined in a glass tube for 10 min. Then, 3 ml of molten tryptic soy agar containing 10 g/liter tryptone (Oxoid, Ltd., Basingstoke, Hampshire, United Kingdom), 5 g/liter yeast extract (AES Chemunex, Bruz, France), 5 g/liter NaCl (Sigma-Aldrich Co., St. Louis, MO), 2.5 g/liter magnesium sulfate heptahydrate (Sigma-Aldrich Co., St. Louis, MO), and 9 g/liter agar (AES Chemunex, Bruz, France), precooled to 45°C, were dispensed into each glass tube, and after mixing, the content was poured onto petri dishes containing tryptic soy agar and allowed to harden for 10 min. After overnight incubation at 37°C, plates were examined for plaque formation and the number of bacteriophage MS2 (PFU) per ml of sample was calculated. Following this protocol, the detection and quantification limits were fixed to 10^1 PFU/ml and 3×10^2 PFU/ml, respectively.

Quantification of viral genome by qRT-PCR. Viral RNA was extracted using a QIAamp viral RNA minikit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Extraction was performed on 140 μl of viral suspension (standard or samples). Extracted RNA was eluted in 60 μl of elution buffer and immediately stored at −20°C.

The primers used for MS2 detection and quantification have previously been described (28). qRT-PCR was carried out using a SuperScript III Platinum SYBR green one-step qRT-PCR kit (Invitrogen, Cergy Pontoise, France) with a 25-μl reaction mixture containing 0.5 μl of Superscript III RT/Platinium *Taq* mix, 12.5 μl of 2× SYBR green reaction mixture with a final $MgSO_4$ concentration of 3 mM, and 0.2 mM each deoxynucleoside triphosphate (dNTP), and 0.2 μM each forward and reverse MS2 primer, made up to 20 μl with RNase-free water. Five microliters of extracted RNA was added. qRT-PCR was performed using an iCycler IQ5 real-time PCR detection system (Bio-Rad, Marnes-la-Coquette, France) under the following conditions: incubation at 50°C for 10 min (RT reaction) followed by an activation step at 95°C for 5 min and then 45 cycles of amplification with denaturation at 95°C for 10 s and annealing/extension at 61°C for 30 s. Depending on the melting temperature (T_m) of a specific amplicon, the melt curve identifies a characteristic T_m that can distinguish between amplicons that differ by only a single base. For our study, a melt curve analysis was performed immediately following amplification with 95°C for 1 min and 55°C for 1 min, followed by 81 repeats of heating for 10 s, starting at 55°C with 0.5°C increments. Negative controls (RNase-free water) were added in each assay.

In order to construct standard curves, 10-fold dilutions of phage MS2 stocks (10^{11} to 10^1 PFU/ml) were made with PBS. The dilutions were analyzed by the double agar layer procedure to define concentrations in PFU/ml, and then viral RNA was extracted from each dilution. The threshold cycle (C_T) values of each dilution amplified in triplicate were plotted as a function of the logarithm of the starting quantity of phage MS2 (PFU/ml). Standard curves were then used to determine the MS2 starting quantity of samples in equivalents of PFU/ml. The slopes (*s*) of the standard curve were used to determine the PCR efficiency (*E*) in conformity with $E = 10^{(-1/s)} - 1$ (23). Thus, a standard curve with a slope of −3.33 corresponded to a reaction with an efficiency value of 100%.

The breakup of the bacteriophage capsid was analyzed by qRT-PCR performed without the RNA extraction step. In this case, 5 μl of samples was added to the 20-μl reaction mixture before the qRT-PCR was performed. The results were then corrected by the extraction dilution factor (140:60) to obtain the MS2 starting quantity of samples. For samples suspended in high concentrations of salts, the lack or decrease of free MS2 RNA (broken capsid) could be attributed to an inhibition of the PCR amplification by salts. To eliminate this phenomenon, samples tested without the RNA extraction step were also quantified by adding only 0.5 μl to the wells. Under these experimental conditions, we checked that the amplification and quantification of MS2 genome by qRT-PCR were not disturbed.

Batch experiments using solutions of various ionic strengths. Batch experiments were performed using sterilized 250-ml glass bottles (Dominique Dutscher, Brumath, France). All solutions used (pH 7 ± 0.5) were tested for their lack of RNase activity against the MS2 genome for 4 h, and their ionic strength (conductivity), expressed in μS/cm, was measured at 20°C with a conductivity meter (Cond 330i/SET; WTW, Weilheim, Germany).

MS2 stock solution (kept in PBS) was diluted in 100 ml of autoclaved ultrapure
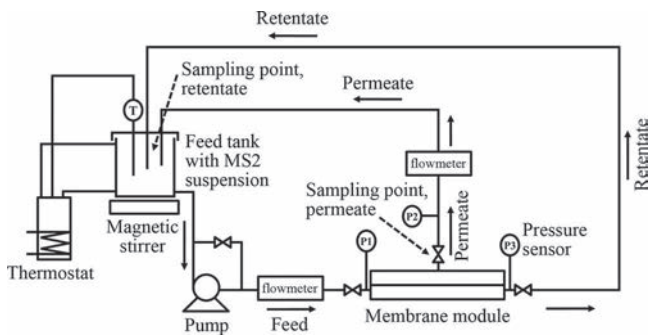
FIG. 1. Schematic representation of the laboratory cross-flow mode filtration apparatus.

water (conductivity of 1 ± 0.2 μS/cm), tap water filtered through a 0.2-μm filter (conductivity of 300 ± 5 μS/cm), or sterile PBS (conductivity of $16{,}500 \pm 500$ μS/cm) to obtain an experimental suspension with a final concentration of 1×10^8 PFU/ml and homogenized for 30 s at room temperature. Samples were collected then (time zero) and again after 30 min, 1 h, 2 h, 3 h, and 4 h, and MS2 concentration was determined by cell culture, and qRT-PCR with and without the RNA extraction step, as previously described. Each quantification of MS2 samples was performed in duplicate, and each condition was tested in triplicate. For each condition, data were expressed as means $\pm$ standard deviations of $\log_{10} C/C_0$, where C was the mean of the MS2 quantity determined by one of the three quantification methods at time x and C_0 the mean of the initial MS2 quantity determined by qRT-PCR with RNA extraction in the experimental suspension (1×10^8 PFU/ml) after homogenization (time zero).

To determine the effects of ionic strength on bacteriophage, MS2 stock solution was suspended at the concentration of 1×10^8 PFU/ml in 100 ml of filtered tap water containing from 0 to 9 g/liter of NaCl added, with the last concentration corresponding to those present in PBS. After 30 s of homogenization (time zero), MS2 concentration in each solution was determined by cell culture, and qRT-PCR with and without the RNA extraction step, as previously described.

Membrane filtration experiments. Experiments were conducted with 15 ultrafiltration hollow fiber membranes assembled in a 30-cm bench scale module in a laboratory cross-flow mode filtration apparatus. The apparatus (Fig. 1) was composed of a 5-liter glass vessel (Bioblock, Illkirch, France) and a pump (PCM P2MGI; Moineau, Vanves, France), which fed the feed suspension to the membrane module at the desired flow rates. The selected ultrafiltration hollow fiber membrane (LIFEA; Degrémont Technologies-Aquasource, Toulouse, France) was made of cellulose triacetate with internal/external configuration and a molecular mass cutoff of 100 kDa. A new membrane was used for each experiment. Each bench scale module was tested prior to the experiment to check its integrity. A bench scale module was considered intact when no loss of pressure was detected.

Filtration experiments were performed over 2 h, with the module under cross-flow filtration conditions under a pressure of 50,000 Pa (constant transmembrane pressure) and at room temperature. MS2 stock solution was suspended in 2 liters of tap water filtered through a 0.2-μm filter, PBS, or filtered tap water containing 5 g/liter of NaCl (conductivity of $9{,}500 \pm 500$ μS/cm) to obtain an MS2 feed suspension with a final concentration of 1×10^8 PFU/ml. After 30 s of homogenization at room temperature (time zero), MS2 feed suspension was circulated in the system (60 liters/h; Reynolds number [Re] = 1,800) with recycling of the retentate and permeate in the feed tank. Fifteen minutes after the start of filtration, the suspension was "doped" by adding 2 ml of phage stock suspension at 1×10^{11} PFU/ml to the feed suspension in order to saturate the MS2 adsorption in the filtration apparatus and thus to maintain a phage concentration of 1×10^8 PFU/ml in the system as previously described (30, 39).

Samples of viral suspensions were collected at different times at sampling points (Fig. 1) in the feed tank (retentate) and just after filtration (permeate), and the MS2 concentration in each sample was determined by cell culture and qRT-PCR with and without the RNA extraction step, as previously described. Experiments were carried out in duplicate. For each condition, data were expressed as means $\pm$ standard deviations of $\log_{10} C/C_0$, where C was the mean of the MS2 quantity determined by one of the three quantification methods at time x and C_0 the mean of the initial MS2 quantity determined by qRT-PCR with RNA extraction in the feed suspension (1×10^8 PFU/ml) after homogenization (time zero).
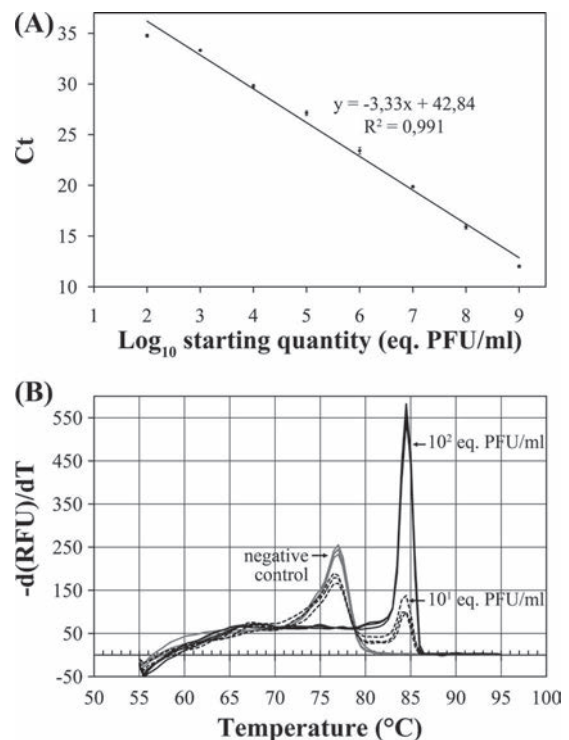

FIG. 2. Standard (A) and melting (B) curves of bacteriophage MS2 obtained using quantitative RT-PCR. (A) Results are expressed as means $\pm$ standard deviations of results from triplicate experiments. (B) The peak at 84.5°C represents MS2-specific amplification, while the peak at 77°C represents nonspecific amplicons that are observed in negative controls and in samples analyzed by qRT-PCR without the RNA extraction step. The quantification and detection limits of the qRT-PCR correspond to 10^2 and 10^1 equivalents of PFU/ml, respectively. Experiments were performed in triplicate. dT, deoxyribosylthymine.

RESULTS

Efficiency and sensitivity of qRT-PCR assays. qRT-PCR assays were run in triplicate with RNA extracted from a 10-fold serial dilution of phage MS2 in PBS (10^9 to 10^0 PFU/ml). No C_T value was obtained for negative controls (RNase-free water). A linear relationship was observed from 10^9 to 10^2 eq PFU/ml between the C_T values and the $\log_{10}$ of the starting quantity of phage MS2, with a slope of -3.33, a squared correlation coefficient of 0.991, and efficiency estimated to be 99.7% (Fig. 2A).

From 10^9 to 10^2 eq PFU/ml of phage MS2, a specific amplification curve was obtained for each of triplicate wells and the melting temperature of the amplified fragment was 84.5°C (Fig. 2B). A nonspecific amplification curve at 77°C was obtained for negative controls (Fig. 2B). For samples at 10^1 eq PFU/ml, a specific amplification curve was obtained at 84.5°C but also a nonspecific amplification curve at 77°C; at this concentration, MS2 was detected but could not be quantified accurately. Thus, the quantification and detection limits of qRT-PCR were 10^2 eq PFU/ml and less than or equal to 10^1 eq PFU/ml, respectively. When phage from stock was added to wells without the RNA extraction step, no specific amplification curve was observed at 84.5°C but a nonspecific amplifica-
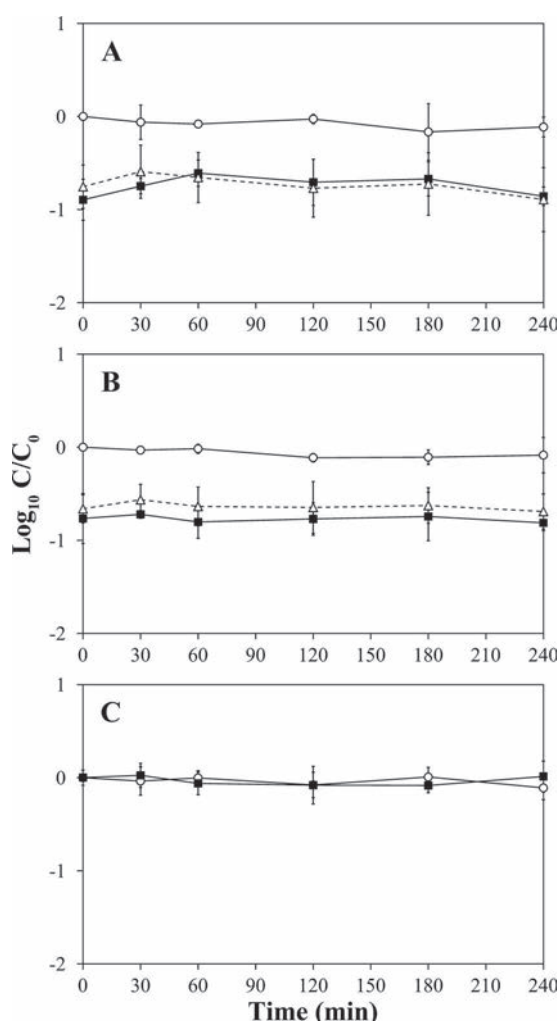
FIG. 3. Batch experiments of bacteriophage MS2 suspended in ultrapure water (A), tap water (B), and PBS (C). C/C_0 is the ratio of the quantity of total viral genomes (circles), broken particles (triangles), or infectious phage (squares) at time x over the initial quantity of total viral genomes in experimental suspension (1×10^8 PFU/ml) after homogenization (time zero). Results are expressed as means ± standard deviations of results from triplicate experiments.

tion curve was obtained at 77°C (Fig. 2B). If free RNA was present in the sample analyzed without RNA extraction (breakup of the MS2 capsid), the specific amplification curve at 84.5°C was observed (data not shown).

Effects of ionic strength on bacteriophage MS2: batch experiments. The quantities of infectious phage (PFU method), total viral genomes (qRT-PCR with RNA extraction), and broken particles (qRT-PCR without RNA extraction) in autoclaved ultrapure water (conductivity of 1 ± 0.2 μS/cm), filtered tap water (conductivity of 300 ± 5 μS/cm), and sterile PBS (conductivity of $16,500 \pm 500$ μS/cm) containing 1×10^8 PFU/ml of phage MS2 were followed for 4 h (Fig. 3). In all these three solutions, the number of total MS2 genomes remained stable over the time, as did the numbers of infectious and broken particles, although differences were observed.

At the beginning of the experiment, the infectivities of phage MS2 suspended in ultrapure water (Fig. 3A) and in tap water

containing small amounts of salts (Fig. 3B) decreased significantly (Student's t test; $P < 0.05$) by 0.9 ± 0.2 $\log_{10}$ and 0.8 ± 0.3 $\log_{10}$, respectively. In addition, at the same time, a large quantity of particles with a broken capsid (free RNA) was detected in these solutions, with significant (Student's t test; $P < 0.05$) differences of 0.8 ± 0.2 $\log_{10}$ in ultrapure water and 0.7 ± 0.2 $\log_{10}$ in tap water relative to the total quantity of MS2 genomes. In other words, dilution of MS2 in ultrapure water resulted in a $67.1\% \pm 9.6\%$ loss of capsid structural integrity and an additional $19.2\% \pm 9.2\%$ reduction of infectivity by breakup of the capsid (total reduction in infectivity of $86.3\% \pm 5.8\%$). In contrast, in PBS (Fig. 3C), all the bacteriophage kept their biological activity and no particle with capsid breakage was detected (under the detection limit).

Effects of ionic strength on bacteriophage MS2: filtration experiments. The membrane filtration experiments were performed for 2 h using tap water or using PBS from a feed suspension of phage MS2 of 1×10^8 PFU/ml. The numbers of viruses present in the retentate and permeate were quantified by cell culture (infectious phage), qRT-PCR (total viral genomes), and qRT-PCR without the RNA extraction step (broken particles). In order to focus our study on phage behavior in the retentate, the selected membrane had a very high retention capacity. This was confirmed by the fact that in the permeate, in all the experiments done, and whatever the solution used for filtration, the quantities of infectious phage, total MS2 genomes, and broken particles were under the quantification limit (data not shown).

At the beginning of the experiment, the infectivity of phage suspended in tap water (Fig. 4A) decreased by 0.6 ± 0.1 $\log_{10}$ and a breakup of the capsid was observed (-0.5 ± 0.0 $\log_{10}$). The concentrations of total MS2 genome and broken virus (free RNA) remained constant throughout the filtration, whereas the number of infectious phage decreased slightly. The same results were obtained in ultrapure water (data not shown).

In contrast, in PBS (Fig. 4B), bacteriophage was very sensitive to sheer stress, and during the filtration process, it quickly lost its infectivity, with a decrease in the retentate of 2.2 ± 0.5 $\log_{10}$ in 5 min and no infectious phage detected after only 40 min. On the other side, the concentration of total viral genomes remained constant over time in the retentate and no free RNA was detected.

Effects of various amounts of NaCl on MS2 behavior. The behavior of MS2 suspended at the concentration of 1×10^8 PFU/ml in tap water containing various amounts of NaCl (0 to 9 g/liter) was evaluated at time zero in batch experiments. The results obtained with tap water containing from 7 to 9 g/liter of NaCl were equivalent to those obtained with 6 g/liter of NaCl added (data not shown).

With increasing NaCl amounts added, the level of infectious particles increased by 0.7 ± 0.2 $\log_{10}$ between 0 and 5 g/liter of NaCl, whereas the quantity of viruses with a broken capsid significantly decreased, with a dramatic reduction at 4 to 5 g/liter (Fig. 5). No significant decrease was observed in genome quantity in any of the solutions. In tap water containing 5 g/liter (conductivity of $9,500 \pm 500$ μS/cm), the concentration of infectious particles was equivalent to those of total MS2 genomes and no particle with a breakup of the capsid could be
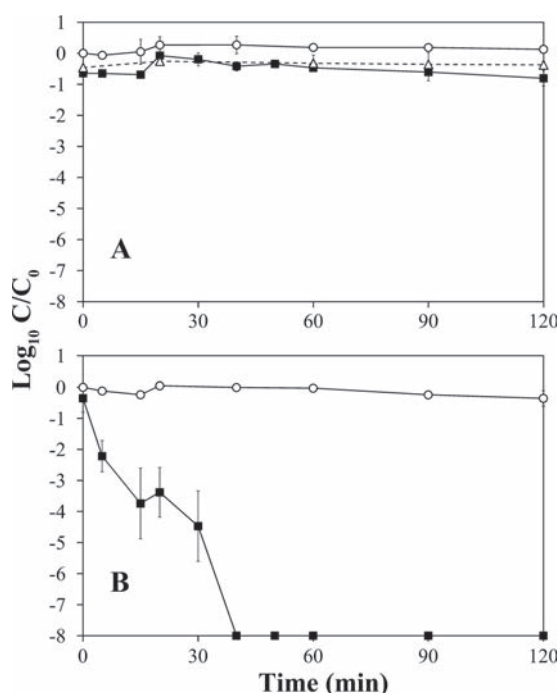
FIG. 4. Retentates of membrane filtration experiments of bacterio-phage MS2 suspended in tap water (A) and PBS (B). C/C_0 is the ratio of the quantity of total viral genomes (circles), broken particles (triangles), or infectious phage (squares) at time x over the initial quantity of total viral genomes in feed suspension (1×10^8 PFU/ml) after homogenization (time zero). Results are expressed as means ± standard deviations of results from duplicate experiments. The detection limit of the PFU method corresponds to -8 on the y axis.

FIG. 6. Batch experiments (A) and retentates of membrane filtration experiments (B) with bacteriophage MS2 suspended in tap water containing 5 g/liter of NaCl. C/C_0 is the ratio of the quantity of total viral genomes (circles), broken particles (triangles), or infectious phage (squares) at time x over the initial quantity of total viral genomes in the experimental suspension (1×10^8 PFU/ml) after homogenization (time zero). Results are expressed as means ± standard deviations of results from triplicate experiments. The detection limit of the PFU method corresponds to -8 on the y axis.

quantified (Fig. 5). These observations were stable over 4 h (Fig. 5 and 6A).

As for batch experiments, no broken particle (free RNA) was quantified during the filtration process when the phage was
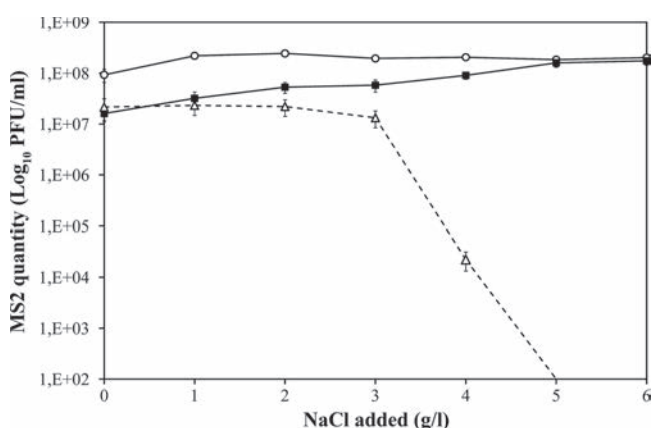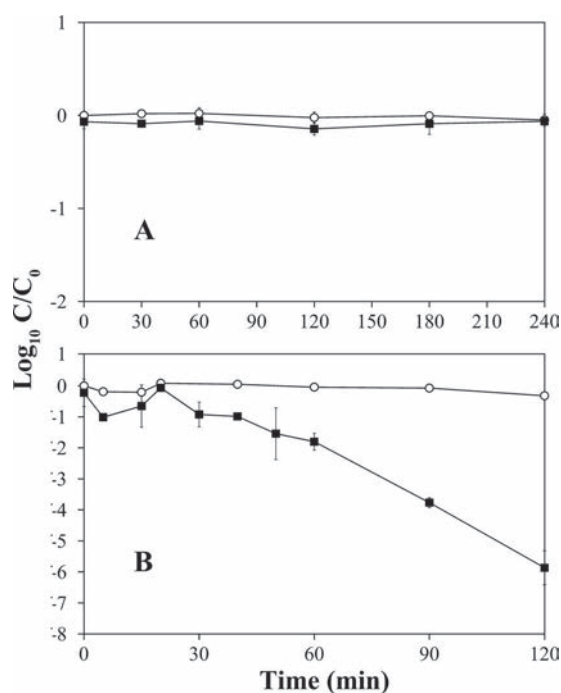

FIG. 5. Batch experiments with bacteriophage MS2 suspended in tap water in the presence of increasing concentrations of NaCl added (0 to 6 g/liter). The quantity of total viral genomes (circles), broken particles (triangles), or infectious phage (squares) in each solution was quantified at the beginning of the experiment, after homogenization (time zero). Results are expressed as means ± standard deviations of results from triplicate experiments. The quantification limit of the qRT-PCR is 10^2 eq PFU/ml.

suspended in tap water containing 5 g/liter of NaCl (Fig. 6B). Although MS2 infectivity was maintained for a longer time during the filtration process, a significant decrease of the number of infectious particles in the retentate of 5.9 ± 0.5 $\log_{10}$ after 120 min was observed. The total quantity of viruses quantified by qRT-PCR in the retentate remained constant throughout the experiment.

DISCUSSION

In this study, the ionic strength of the solution showed significant and opposite effects on the bacteriophage MS2 behavior, depending on the experimental conditions. It thus seems necessary to consider these effects in the retention capacity analysis of a selected membrane by using MS2 as indicators.

In batch experiments, the transition from the high-ionic-strength stock solution (PBS) to ultrapure water or tap water caused a loss of up to 90% of phage MS2 infectivity. Among these noninfectious phage, a small proportion was detected by the original technique of qRT-PCR performed without the RNA extraction step, which detects the free viral RNA and can therefore quantify particles with a broken capsid. Thus, in this case, a part of the loss of biological activity observed may be directly attributed to the breakup of the capsid, which results in RNA release.

The dramatic loss of infectious phage observed in batch experiments could also be explained by aggregation, adsorp-

tion to surfaces, and/or damage to the capsid. Langlet et al. (24) have recently demonstrated that aggregation of phage MS2 results in a strong decrease of PFU counts. Under our batch experimental conditions, aggregation can be excluded, as the pH of the solution was higher than the pI (pI 3.9) of MS2 (24) and, in the stock solution, phage was not aggregated at all as defined in a previous work by transmission electron microscopy and dynamic light scattering (Nanosizer) (30). The adsorption of viruses to the recipient surfaces can also be considered negligible because the number of total genomic MS2 particles detected by qRT-PCR remained constant throughout the batch experiment. Regarding the capsid, it fulfills some crucial functions, such as RNase protection and attachment to host cell receptors on the membranes of susceptible bacteria, necessary for the infection of host cells (10). Because conformational changes of the proteins in the capsid might weaken viral stability or affect attachment to the cell receptor, the infectivity of viruses requires the functional integrity of the capsid. It has been shown that osmotic effects could be responsible for the breakdown of the capsid structure (2, 5). Thus, the loss of infectivity of phage MS2 observed in batch experiments with low-ionic-strength solutions could only be due to effects, ranging from damage to breakup, caused by osmotic shock to the capsid. Batch experiments with bacteriophage MS2 suspended in tap water containing various amounts of NaCl confirmed this hypothesis; the greater the ionic-strength difference between the MS2 stock solution (PBS) and the experimental solution, the higher the osmotic shock and thus the level of damage to the capsid.

At the beginning of the filtration of phage suspended in tap water, a decrease of infectious phage and a breakup of the capsid were observed in the retentate. As for batch experiments, these observations were stable over time and could be attributed to the osmotic shock. In contrast, during filtration experiments performed using high-ionic-strength solutions, a rapid loss of MS2 infectivity was observed, which cannot be directly attributed to the osmotic effects, as these results were not observed in batch experiments. These observations suggest that ionic forces have other deleterious effects on phage MS2 during filtration experiments. This hypothesis was confirmed by the observation that filtration experiments under intermediate conditions (tap water containing 5 g/liter NaCl) induced intermediate effects on MS2 biological activity.

First, under our filtration experiment conditions, adhesion of MS2 on the membrane and membrane toxicity can be excluded as influences on MS2 biological activity because similar observations were obtained during the same experiments carried out with an empty membrane module (shell without membrane) and with various tested membranes made of polysulfone, polyethersulfone, or polyvinylidene fluoride and with internal/external or external/internal configurations (data not shown). Second, as the number of total genomic MS2 particles detected by qRT-PCR remained constant throughout the filtration processes, the total disappearance of infectious viruses may not result from their adsorption on system surfaces. The latter observation also eliminates aggregation as the main phenomenon responsible for the decrease of infectious phage observed, considering the dramatic loss of infectivity of up to 10^8 $\log_{10}$. Regarding damage to the capsid, during filtration experiments using high-ionic-strength solutions, the capsid was not totally broken, as no free RNA was detected by qRT-PCR performed without the RNA extraction step.

The exposure of phage to inactivating interfaces has been put forward to explain this inactivation. Two inactivating interfaces have been reported: (i) an air-water interface (AWI) (1, 38) and (ii) a dynamic triple-phase boundary (TPB), an interface at which the gas (air), liquid (water), and solid (surfaces) phases intersected (37). It has been reported that exposing MS2 suspensions to these interfaces produces the greatest damage to the specific viral components that are related to infection and that this phenomenon is significantly increased in a dynamic system and when the ionic strength of the solution is increased (36, 37). However, there was no air (or gas, except for the gas dissolved in solution, of course) in any part of our filtration system apart from the feed tank. Thus, under our experimental conditions, AWI and TPB interfaces could occur only in this reservoir, a 5-liter glass vessel in which the conditions were similar to those used in batch experiments. Moreover, as described by Thompson and Yates (37), MS2 inactivation by interaction with these interfaces cannot occur in the presence of glass surfaces but only with hydrophobic surfaces. As loss of infectious particles in high-ionic-strength solutions was observed only during filtration experiments, it seems that this phenomenon was linked to other parts of our filtration system. It is also important to note that in our filtration process, some pipes were made of polyvinyl chloride (PVC) and polyamide, hydrophobic surfaces known to induce MS2 inactivation in the presence of the TPB interface in high-ionic-strength solutions (37). These observations led us to foresee that a surface-water interface (SWI) between system components and solutions could induce phage inactivation and that this phenomenon increased with the ionic strength of the solution.

On the other hand, it is possible that the sheer stresses provided by the combination of the pump and a high-ionic-strength solution caused an alteration of the surface components of the capsids, which prevented them from exercising their role on bacteria cells. Interestingly, work done on phage concentration by ultrafiltration on a large sample of storm water uncovered no relationship between conductivity and phage inactivation (32). Such differences could be explained by various experimental factors. However, the nature of the pump used could be predominant. Indeed, most of work on phage concentration was performed with a peristaltic pump (17, 31, 32). The pump used in this study was a screw pump in which water comes into contact with the internal mechanism of the pump, which generates sheer waves and turbulences, which is not the case of a peristaltic pump. Nevertheless, further work will be undertaken to demonstrate this hypothesis.

The use, in most reported studies (9, 15, 38, 41), of only one quantification technique (generally the traditional PFU method) could lead to misestimated results. Indeed, the PFU method does not clearly distinguish between the real inactivation of phage (infectivity loss) and other phenomena, like aggregation, adsorption, and capsid damage. For example, filtration experiments carried out with high-ionic-strength solutions (PBS) induced the rapid disappearance of infectious particles, while the total number of genomes remained high and constant. In this study, the combination of the PFU method and molecular tools allowed us to clearly point out that the dra-

matic reduction in the phage number quantified by the PFU method in the retentate observed during the filtration experiment performed using PBS was due to a loss of infectivity caused by damage to the capsids and not to a real disappearance/destruction of particles, as the total number of phage detected by qRT-PCR was constant, and no free RNA was detected.

Under our experimental conditions and for the selected membrane, whatever the ionic strength of the solution and whatever the phage physiological state, the technique of qRT-PCR allowed an assessment of the membrane retention capacity without misestimation, which was not the case for the PFU method. Under all the conditions tested, the MS2 titers determined by qRT-PCR were the same in both the feed solution and the retentate. However, unlike the PFU method, this molecular technique does not distinguish between infectious and noninfectious viruses, like damaged and broken particles (free RNA). Thus, whether for bacteriophages or enteric viruses, the presence of the viral genome of these markers in an environmental sample is not proof of the presence of infectious viruses, and therefore, this technique alone cannot provide a relevant evaluation of membrane retention efficiency or, for that matter, public health infection risk.

On the other hand, an advantage of qRT-PCR is the possibility of using inactive particles as a surrogate. For this application, detection of free RNA by qRT-PCR performed without the RNA extraction step might be of interest to ensure that the MS2 genomes detected by qRT-PCR correspond to those of intact inactivated phage. However, the results of our filtration experiments showed that this verification was not always necessary, because under the conditions described for the filtration experiments carried out using tap water, even though free RNA was present in large quantities in the retentate, free RNA in the permeate could not be quantified, given that it did not pass through the tested membrane. This observation corroborated studies of the secondary structure of MS2 RNA, which showed that the RNA structure was not linear but rather complex, with 60 to 80% of the RNA in the form of base-paired loops and associated proteins (35). Not necessarily required for studying membranes, this technique of qRT-PCR performed without the RNA extraction step mainly enabled us to highlight some effects, such as osmotic shock, to quantify some of the noninfectious particles, and to check our assays.

In conclusion, through the use of three quantification methods that allow the determination of the functional, biological, and structural (intact, damaged, or broken capsid) states of bacteriophage MS2, this study clearly demonstrates that the ionic strength of the solution in which the phage is suspended induces significant effects on MS2 behavior and that these effects may significantly affect the results of membrane filtration experiments. Moreover, it seems that these observations are the same for other F-specific RNA phages since preliminary experiments conducted in our laboratory have already shown similar results for the Qβ bacteriophage, also commonly considered for the study of membranes (39). Thus, for membrane validation experiments, it seems important to note that the type of water to be treated has to be taken into account in order to monitor the real concentration of the MS2 suspension during the whole challenge. Moreover, given the great diversity of the types of treated water, the quantification of these indicators by the PFU method currently used alone could cause significant misestimation of membrane filtration effectiveness. Therefore, the combination of classical and molecular methods as in this work should be systematically used for the development of a membrane filtration process for each new type of equipment and for each water type in order to select an appropriate detection procedure and to correctly validate the "in-use" efficiency of ultrafiltration membranes.

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