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Protocol for the assessment of viral retention capability of membranes

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ABSTRACT

A series of experiments has been carried out to determine the Log removal value (LRV) of MS2 bacteriophages suspended in various buffers (osmosed water, tap water, aqueous solutions of NaCl and phosphate buffer solution) during filtration through hollow fiber membranes made of cellulose acetate. Viral concentrations in permeate and retentate were determined using two different methods, namely plaque forming unit (PFU) counting, which reveals only infectious particles and quantitative RT-PCR which detects the total (infectious + inactivated) number of viral genomes regardless of their infectivity.

From this experimental study, we propose guidelines for preparing the challenging solutions and measuring their concentration which ensure a reliable assessment of the membrane performance.

1. Introduction

Membrane technologies used in drinking water production and waste water treatment provide an effective barrier to pathogens such as viruses as long as the membrane integrity is not compromised [1,2]. The development of a virus challenge test is a prerequisite to the assessment of the capacity of a membrane to retain viruses. Such test requires the selection of test particles, of a buffer, of quantification methods and of experimental conditions for which the data collected would be meaningful.

Although virus challenge tests are commonly performed by water authorities and companies supplying water disinfection units, no well established protocol is available today. Moreover, in the literature on this subject, the range of operating conditions is diverse and the trends reported during filtration are sometimes in contradiction with each other. For example, the solvent for suspending phages is not clearly established. Langlet et al. [3] use phosphate buffer at low concentration (typically at 0.2 mM), whereas others use distilled water [4] or milli-Q™ water [5]. Filtration time during which the feed concentration remains constant is a parameter taken into account in some works, but not always clearly specified. Acker et al. [6] recommend a filtration time shorter than 6 min without giving further details, which makes difficult the completion of membrane characterization experiments and the collection of multiple samples. Langlet et al. [3] do not specify their filtration time but explain that it depends on the permeability of the tested membranes; they recommend the filtration of 400 mL of viral suspension as a minimum, but the membrane area used is not reported.

The capacity of a membrane system to reduce the bacteria or virus content in a stream is generally quantified by the logarithmic reduction value (LRV) defined by Eq. (1):

$$\mathrm{LRV} = \mathrm{Log}_{10}\left(\frac{C_{r(t)}}{C_{p(t)}}\right) \quad (1)$$

with $C_{r(t)}$, virus concentration in retentate and $C_{p(t)}$, virus concentration in permeate at time t.

In most studies, the concentrations are the numbers of infectious viruses per millilitres determined in permeate and retentate samples, by plaque forming unit (PFU) counting. Some authors suspect this method to lead to an overestimation of the virus removal because of possible occurrence of virus aggregation in the permeate compartment [3], but aggregation may occur in the retentate as well. In addition, PFU counting is a very time-consuming method, which takes about 24 h to get a concentration in a sample.

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A promising alternative method for counting viruses, is the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), which is classically employed in molecular biology to detect the viral genome, and has been reported to be a relevant one for the evaluation of the retrovirus removal by chromatography by Lau et al. [7]. This method seems to be the most sensitive of the specific tools. On the other hand qRT-PCR is reported to detect inactivated viruses [8]. As a consequence, the detection of viruses based on qRT-PCR might lead to false positive results [9].

The virus surrogate used for the challenge tests may be an issue. Bacteriophages infecting coliform bacteria have been considered as possible indicator organisms for enteric viruses in surface and ground waters contaminated with fecal material [10,11]. As a consequence they are often used as surrogates to evaluate the pathogenic virus removal efficiency of filtration membranes used for water treatment. The bacteriophage most commonly used by scientists and industrialists to challenge membranes is MS2, a nucleic acid (single-stranded RNA) virus [e.g. 12,13]. The advantages of MS2 are many: it is one of the smallest viruses (23–30 nm in diameter) then able to reveal small defects or pores, close in size and shape (icosahedral capsid) to enteric hepatitis A virus and poliovirus, non-pathogenic and relatively inexpensive.

An analysis of the relevant literature shows, not necessarily explicitly, that virus aggregation, adsorption or inactivation interferes with the assessment of the virus removal capacity of membranes. Before the development of the PCR technique, PFU counting was the only way for checking the presence of viruses in a medium. Decreases in PFU values were interpreted in terms of "virus inactivation".

Thompson et al. [14] show that MS2 inactivation is the result of exposure to surface forces at the dynamic air–water–solid interface. Moreover, MS2 is increasingly inactivated during mixing in polypropylene tubes as the ionic strength of the suspension is raised. MS2 inactivation is minimal when the air–water interface is completely eliminated from polypropylene tubes. All batch experiments performed with glass tubes demonstrate no substantial inactivation of MS2. Theses authors conclude that viral inactivation in simple dynamic batch experiments is dependent upon (i) the presence of a dynamic air–water–solid interface (where the solid is a hydrophobic surface), (ii) the ionic strength of the suspension, (iii) the concentration of surface active compounds in the suspension, and (iv) the type of virus used.

A change in infectivity can however be the consequence of at least three mechanisms: virus inactivation, adsorption/adhesion to the walls of the equipment, aggregation (as an aggregate of several viruses produces only one "plaque").

The assessment of adsorption on any solid surface of the testing equipment or of the membrane is of major importance considering the very low concentration of viruses involved in filtration tests, in particular in the permeate. The knowledge of adsorption kinetics allows to evaluate the time to reach saturation without which an accurate evaluation of virus retention by the membrane would not be possible [15,16]. Virus adsorption is enhanced when particles and membrane charges are opposite in sign or small in magnitude. MS2 phages have an isoelectric point (IP) of 3.9 at 100 mM ionic strength [12], which suggests a significant negative charge carried by the virus at neutral pH and plays against adsorption of bacteriophages onto negatively charged membranes as those classically used in water treatment processes. Ionic strength of the fluid also plays a critical role on adsorption of viruses to surfaces as well as on their aggregation. The presence of di and trivalent cations promotes adsorption of MS2 viruses onto membranes by influencing electrostatic interactions [17].

Aggregation of MS2 particles is not observed for pH higher than the isoelectric point of the particle (pH 3.9) and ionic strengths for which interparticular repulsive electrostatic interactions are expected to be sufficiently screened (1–100 mM $NaNO_3$) [12,18]. Operating at neutral pH then allowed overcoming the aggregation process. On the other hand, Langlet et al. [18] clearly show that MS2 phages exhibit significant aggregation for pH <IP, conditions for which aggregates up to a few micrometers in size are observed. Langlet et al. [12] show that Qβ, which is another potential virus surrogate, suspended in solutions of large electrolyte concentrations aggregate over the whole range of pH from 1.5 to 7.5. This behavior is in favor of choosing MS2 as model particle for virus challenge test as compared to Qβ.

So, although the impacts of factors such as ionic strength, virus concentration and filtration time on the virus retention by membranes have often been reported, studies on the influence of these parameters have not conducted so far in a systematic way. From a technical point of view, a characterization experiment must allow time to take several permeate samples, and the concentration of the challenging solution must remain as high as possible during the test. If one accounts for the recommendation by the U.S. Environmental Protection Agency [19] regarding the control of the quality of treated surface water by membrane filtration, virus feed concentration has to be sufficiently high to allow the demonstration of up to 6.5 Log removal if the surrogate is removed to the detection limit (Eq. (1)). In addition, considering that the literature reports variations in virus concentrations by several orders of magnitude over the time of an experiment (one to a few hours), we have to set an acceptable limit of such variation. In the present project, we have therefore considered that if the virus concentration in the retentate decreases by more than 90% (one order of magnitude), then the conditions have too much changed for being considered as acceptable.

The aim of this study was then to define experimental conditions allowing a reliable determination of the virus retention capacity of a membrane used in water treatment. Specific attention has been paid to the effects of aggregation, adsorption and inactivation of viruses during filtration. Viral concentrations in permeate and retentate were determined using two different methods according to a previous study [21], namely plaque forming unit (PFU) method and qRT-PCR with RNA extraction.

We first monitor the changes in virus concentration over time in the retentate circuit, then check the role of the some selected buffers. From these experimental observations, we propose guidelines for a reliable determination of the virus retention capacity (LRV) of a membrane system.

2. Materials and methods

2.1. Bacteriophage stock preparation

All tests were performed with MS2 phage (ATCC 15597-B1) and *Escherichia coli* W 1485 (ATCC 12435) as host bacteria, obtained from Institut Pasteur (Paris). The replication method is described in a previous paper [20]. The phage stock suspension (10^{11} PFU mL^{-1}) was characterized in terms of shape and size by transmission electron microscopy (TEM) and dynamic light scattering (DLS). Despite the fairly high viral concentration, isolated and non-aggregated viruses were obtained after amplification. The reproducibility of the suspensions was checked in terms of concentration and size [21]. Dynamic light scattering revealed a single size distribution peak with a *z*-averaged hydrodynamic diameter of 26.0 nm whereas negatively stained preparations (TEM) showed a diameter of approximately 30 nm. These results are in agreement with previous studies [12], which report the diameter of the spherical MS2 to be 30 nm.

2.2. Bacteriophage assays

2.2.1. Quantification of infectious viruses by cell culture: PFU method

The plaque assay procedure used to determine the concentration in phages is as described in Furiga et al. [20]. The only difference is that in the present study, 1 mL of bacteriophage sample was collected and mixed to 9 mL of *E. coli* suspension when Furiga et al. combined 0.1 mL of bacteriophage sample with 0.9 mL of *E. coli*. As a consequence, in the present work, the detection limit of the plaque assay which corresponds to the smallest amount of phages that could be detected but not necessarily accurately quantified (results not reproducible) was 1 PFU mL^{-1} whereas the smallest amount of phages that could be quantified (reproducible results) was 30 PFU mL^{-1}. Samples were diluted when necessary using PBS (9 g L^{-1} NaCl, 0.8 g L^{-1} Na_2HPO_4, 0.1 g L^{-1} KH_2PO_4; Lonza, Verviers, Belgium) in order to decrease the concentration in bacteriophage below 300 PFU mL^{-1} which is the concentration that can easily be counted on a plate with the naked eye. PBS was preferred to other buffers in an effort to promote viral suspension stability.

2.2.2. Quantification of viral genome by qRT-PCR method

We define the concentration measured by the qRT-PCR method as the total viral RNA concentration.

The viral RNA was extracted using the QIAamp® Viral RNA Mini kit (Quiagen, Courtaboeuf, France) according to the manufacturer's instructions. Extraction was performed from 140 μL of viral suspension (standard or samples). The extracted RNA was eluted in 60 μL of buffer and immediately stored at −20 °C. qRT-PCR conditions used for MS2 detection and quantification are described in [20]. In the sampling conditions described in the previous section, the detection limit of the qRT-PCR was 10^1 equiv. PFU mL^{-1} and the quantification limit was 10^2 equiv. PFU mL^{-1} [20].

2.3. Membranes

Tests were conducted using ultrafiltration membranes prepared for this project. These are inner skinned hollow fibers (molecular weight cut-off 100 kDa – permeability 142 ± 54 L h^{-1} m^{-2} bar^{-1}) made of cellulose acetate. 15 hollow fibers (0.93 and 1.66 mm of internal and external diameter respectively) were assembled in a bench-scale module of 300 mm in length and 8 mm in internal diameter. A new module was made for each experiment. The membrane effective area per module was 91 ± 5 cm^2. The integrity of each module was tested prior to any experiment: the module was first filled with distilled water, then compressed air was injected in the retentate compartment at 1 ± 0.005 bar in a closed circuit and the transparent module shell allowed to check for bubbling. A module was considered integer when no bubble was detected at naked eye. The membrane permeability to distilled water, L_p, was determined before and after the filtration of bacteriophage suspension in order to check for membrane fouling according to the Darcy law:

$$J = \frac{Q}{A} = L_p \Delta P \quad (2)$$

where J, flux density [L h^{-1} m^{-2}], Q, filtration flow of pure solvent [L h^{-1}], A, membrane effective area [m^2], L_p, membrane hydraulic permeability [L h^{-1} m^{-2} bar^{-1}] and ΔP, trans-membrane pressure [bar].

Five values of applied pressure where systematically used for the permeability determination. The permeability is given for a temperature of 20 °C, as data were corrected when necessary for the effect of temperature on the water viscosity [22] using Eq. (3), valid for 0 °C < T < 30 °C:

$$L_p \ (20\,^{\circ}\mathrm{C}) = L_p(T)\exp(-0.0239(T-20)) \quad (3)$$

where T is the temperature [°C].

According to a French standard [23], for the characterization of ultrafiltration or microfiltration membranes, the loss in permeability by fouling during the test has to be smaller than 30% for a retention measurement to be considered as valid. For all experiments conducted in this study, we checked that the loss in L_p after bacteriophage filtration met this criterion.

In order to avoid cross contamination, a new module was used for each experiment, first rinsed with distilled water in normal filtration mode, then backwashed with a sodium hypochlorite solution at 200 ppm total free chlorine during 10 min. The module was then filled with the hypochlorite solution and after 30 min, was thoroughly rinsed with sterile water.

2.4. Ultrafiltration set-up and procedure

Experiments were performed using a laboratory cross-flow filtration apparatus. The feed tank was a 5 L glass vessel as previous studies showed that no substantial loss by adsorption of MS2 was observed when using glassware [14,21] and the pump was of positive displacement type (PCM P2MGI; Moineau, Vanves, France). The feed tank was jacketed, which allowed the temperature to be controlled (Fig. 1). The module shell was made of polyvinyl chloride and tubing of polyamide.

Experiments were conducted over 2 h in cross-flow conditions under constant transmembrane pressure (0.5 bar), at controlled temperature 20 °C ± 2 °C and at the natural pH of the water (pH 7 ± 0.5). 2 L of feed suspension was obtained by diluting the bacteriophage stock solution in various media (osmosed water, tap water microfiltered through 0.2 μm filters, distilled water containing 1 g L^{-1} NaCl, tap water containing 5 g L^{-1} NaCl, distilled water containing 9 g L^{-1} NaCl and PBS). Permeate and retentate were recycled into the feed tank. Samples (1 mL) were taken periodically and assayed using the two methods presented in Sections 2.2.1 and 2.2.2. The samples were stored in the dark at 4 °C when they were analyzed within a day (typically by PFU counting), or preserved at −80 °C until qRT-PCR analysis was performed.

In our experiments, the quantification limit of the PFU method being 30 PFU mL^{-1}, the minimum initial concentration chosen for the feed suspension was 10^8 PFU mL^{-1} in order to allow for the demonstration of up to 6.5 Log removal, according to Eq. (1) ($\mathrm{Log}_{10}[10^8/30] = 6.5$).

In addition, when no virus was detected in a permeate sample, the permeate concentration was taken equal to 30 PFU mL^{-1} in Eq. (1), the value then obtained was considered as the minimum LRV that could be claimed in the conditions of the experiment. The true LRV value was equal to or larger than this one.

Experiments were at least duplicated and the difference between two LRV was <0.5. The equipment was cleaned after each experiment by circulating a 200 ppm sodium hypochlorite solution, but used membrane modules were discarded. The system was then rinsed with distilled water until no hypochlorite was detected in permeate and retentate streams. For this, the total free chlorine concentration in the rinsing solution was assayed by adding DPD free chlorine reagent (HACH 14070-14099 Pk/100) to it and measuring the absorbance at 530 nm (HACH 2400).

2.5. Virus inactivation during filtration

We used the same model for describing the virus inactivation kinetics as Gassilloud et al. [24] (Eq. (4)) i.e. a first order reaction:

$$\mathrm{Log}_{10}\left(\frac{C_{r(t)}}{C_{r(0)}}\right) = -a_i t \quad (4)$$

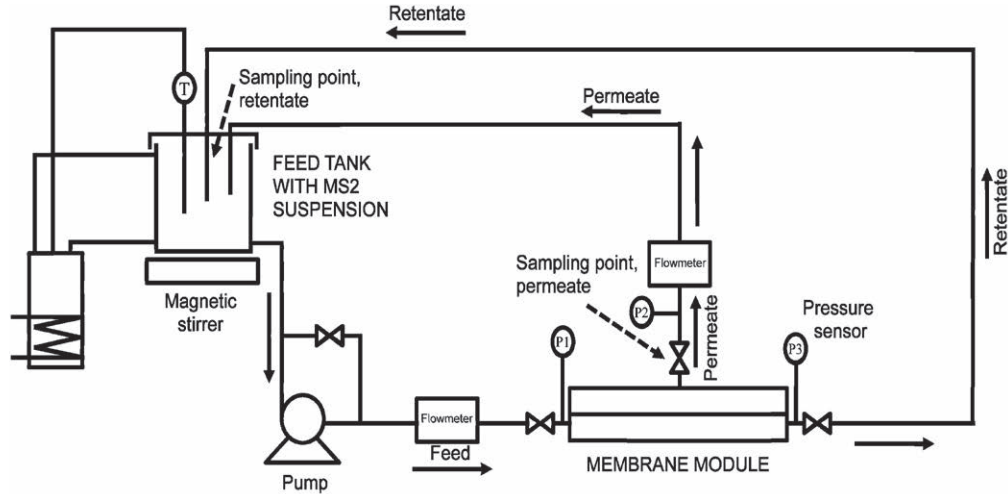

Fig. 1. Diagrammatic view of the experimental setup.

with a_i, inactivation rate constant, t, time, $C_{r(0)}$, virus concentration in retentate at time 0 and $C_{r(t)}$, virus concentration in retentate at time t.

From Eq. (4), Gassilloud et al. [24] calculate T90, the time required for the infectivity to be reduced by 90% (or 1 Log_{10}). This approach used with the PFU method characterizes inactivation but does not discriminate pure inactivation from adhesion or aggregation. This point is discussed further below.

The inactivation rate constant a_i of MS2 phages was obtained from the plot of $\text{Log}_{10}(C_{r(t)}/C_{r(0)})$ versus time. The filtration test duration ($d_{\text{filtration}}$) corresponding to the time at which the concentration in infectious particles MS2 decreased by 1 Log_{10} in the retentate (named T90 by Gassilloud et al. [24]), was then simply given by:

$$d_{\text{filtration}} = \frac{1}{a_i} \quad (5)$$

3. Results and discussion

The virus challenge filtration test requires the definition of experimental conditions which most favor the virus transmission. This means that viruses should not aggregate, adsorb on the experimental set-up (tank, membrane modules, pipes, etc.) during the test and that inactivation should be as limited as possible. In order to evaluate the extend of each phenomenon; the suspension was first circulated in an empty filtration module shell.

3.1. Preliminary experiments conducted with an empty module shell

A series of experiments was performed by circulating a MS2 suspension prepared in osmosed water in the filtration system containing an empty module shell (without membrane). The retentate was recirculated and analyzed over time by PFU and qRT-PCR. We observed in these preliminary experiments (data not shown) that the infectious virus concentration decreased from 9.2×10^7 to around 7×10^1 PFU mL^{-1} within 60 min. In the same time, the concentration in viral genomes determined by qRT-PCR decreased from 5.2×10^8 to 2×10^8 equiv. PFU mL^{-1} (and to 8.23×10^7 equiv. PFU mL^{-1} over 240 min). This decrease can be partly attributed to virus adsorption on the experimental set-up [15]. In order to compensate this virus loss by adsorption, we decided to add 2 mL of virus stock suspension at 10^{11} PFU mL^{-1} to the feed tank, 15 min after the beginning of each filtration run, according to a protocol proposed by Urase et al. [13]. This addition called "doping" in the rest of this paper, allowed to restaure the infectious virus concentration back to close to 10^8 PFU mL^{-1}, and its effect on the stabilization of infectivity is shown in Fig. 2. When viruses were detected in the permeate their concentration was almost stable after 10–15 min of filtration. We then assumed that adsorption on the walls of the permeate circuit had reached saturation.

In Fig. 2 we compare the phage concentration over time during in the filtration system without membrane. A more rapid decline in infectious MS2 particles concentration was observed in PBS (ionic strength 182 mM) than in osmosed water, leading to a concentration around 2×10^2 PFU mL^{-1} 30 min after doping. Despite the doping at 15 min, the total viral RNA decreased by 1 Log in 105 min after doping at high ionic strength (182 mM), when it was almost stable in osmosed water.

According to Gassilloud and Gantzer [15], a loss in infectious virus in an aqueous medium as measured by PFU counting can result from inactivation, adhesion to the experimental set-up and aggregation, whereas for viral genomes, the loss measured by RT-PCR can only result from adhesion. As a consequence, several hypotheses could explain our observations:

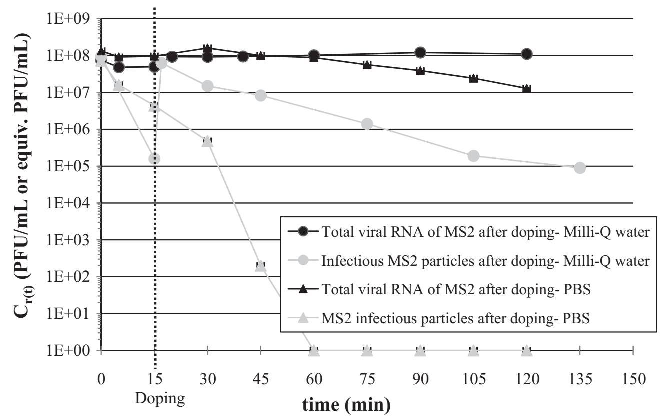

Fig. 2. Concentration in total viral RNA (in equiv. PFU mL^{-1}, qRT-PCR method) and in infectious MS2 particles (in PFU mL^{-1}, PFU method). The suspension is circulated in the filtration system without membrane (empty module shell). Phages are diluted in milli-Q™ water or PBS. Origin of time was taken at the beginning of the test when the feed concentration is ca. 1×10^8 PFU mL^{-1} ($n = 1$).

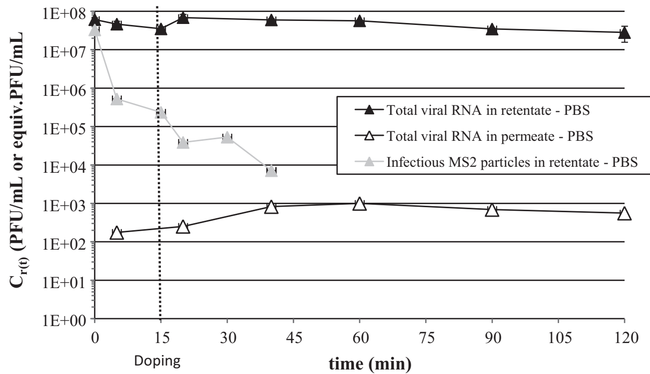

Fig. 3. Concentration in total viral RNA and in infectious MS2 particles during a 2 h filtration test with "doping" at 15 min. Solvent was PBS ($n = 2$).

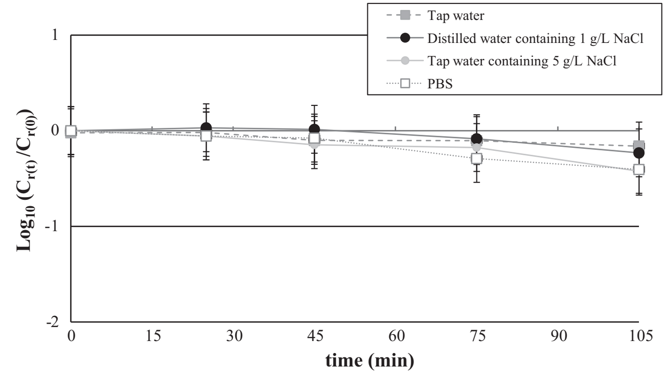

Fig. 4. Retentate concentration in total RNA viral (qRT-PCR method) during filtration test of suspensions in various media. The "doping" was taken as the origin of time, the concentration $C_r(0)$ was then equal to the total amount of viral RNA after doping ($n \geq 2$).

(i) The presence of salts might favor the damage of the virus capsid by the pump, then inactivating the viruses.
(ii) The presence of salts might favor infectious phages aggregation in the filtration system, resulting in a decrease in the number of infectious particles counted by PFU whereas in static conditions [20] the suspension prepared in PBS was stable over 2 h.
(iii) The genome reduction shown by qRT-PCR suggests a stronger adsorption on the walls of the filtration system caused by the combined effects of ionic strength and particles inactivation. Electrostatic repulsions are expected to be screened by the presence of salt in solution thus leading to an increase in virus adsorption on surfaces, both of them being generally negatively charged as mentioned in the introduction.

3.2. Aggregation, adsorption and inactivation of bacteriophages during a filtration test

Aggregation, adsorption and inactivation of MS2 particles during a filtration test were studied separately so as to distinguish which assumptions amongst those previously mentioned, is valid in order to account for these phenomena in calculating the LRV.

3.2.1. Aggregation

If the important reduction in infectious MS2 particles concentration at high ionic strength (182 mM) were due to viral aggregation, then we should expect a lower total viral RNA concentration in the permeate as a large aggregate was expected to be more rejected by the membrane than a single phage (the concentration in infectious particles in the permeate being zero, we could not make a comparison with that one). However, no decrease in total viral RNA over time was observed in the permeate by qRT-PCR (Fig. 3). We conclude that if MS2 aggregation in the retentate was significant, it would impact the virus retention by the membrane, and hence its concentration in the permeate. Therefore, one can consider that in our conditions, the membrane was challenged with a suspension of truly dispersed infectious viruses. These results are in accordance with those of Langlet et al. [3].

3.2.2. Adsorption

The influence of a buffer on adsorption during filtration can be quantified by monitoring the changes in total viral RNA determined by qRT-PCR. In Fig. 4, the $\text{Log}_{10}(C_{r(t)}/C_{r(0)})$ was plotted versus time according to Eq. (4). The origin of time and concentration was taken just after doping.

Adsorption seems to be more important in the presence of salts in the suspension. The higher the ionic strength, the steeper the total viral RNA decrease. This is consistent with a screening of repulsive electrostatic interactions by the presence of salt. However, the maximum decrease over the filtration run of 105 min which was observed with PBS (182 mM), was only 0.4 Log_{10}. The experiment conducted in the same conditions but without hollow fibers in the module shell (Section 3.1) showed a decrease by around 1 Log_{10} over the same time of 105 min after doping (symbol A in Fig. 2). The loss in total viral RNA was less important in the presence of membranes in the module probably because in this case the contact area between the suspension and tubing was reduced, the phages retained by the membrane being confined in the retentate compartment and not coming into contact with the permeate circuit and module shell.

3.2.3. Inactivation

Fig. 5 reports the changes in retentate concentration of infectious particles over time for various buffers (same as in Fig. 4). We could ascribe the decrease in PFU mL^{-1} to a decrease in concentration in isolated infectious MS2 phages in suspension as aggregation and adsorption phenomena have been dismissed. We observe a high rate of inactivation at high ionic strengths (I), starting from the nominal virus concentration, whereas at low I, we have a sharp initial loss in infectivity, then a very slow inactivation. Furiga et al. [20] report an immediate decrease in infectivity at low ionic strength by about 1 Log, whereas they do not observe such an inactivation over time when working in stirred vessels (with no pumping devices then). These results [20] shown that during filtration tests using high-ionic-strength solutions, the inactivation phenomenon was different from the osmotic stress one: the virus capsid was not totally broken, as no free RNA was detected by qRT-PCR performed without the RNA extraction step. As a consequence, the particle size was not significantly modified and the behavior of viruses during filtration was similar with regards to the sieving mechanism. This assumption was supported by the results shown in Fig. 7D and D′ as the decrease in infectious MS2 in the retentate over time (Fig. 7D) was not accompanied by an increase in total viral RNA in the permeate (Fig. 7D′).

On the other hand, according to Thompson et al. [14] the presence of salts leads to an increase of the particles attraction to the air–water–solid interface due to a decrease of the thickness of the electrostatic double layer around the particles. This greater virus sorption at the air–water-interface results in exposure of phages to inactivating forces. It is obvious that this effect is more marked when the virus is hydrophobic like MS2 but we can also expect a greater inactivation than the interface is rapidly renewed, as it was the case during our filtration tests.

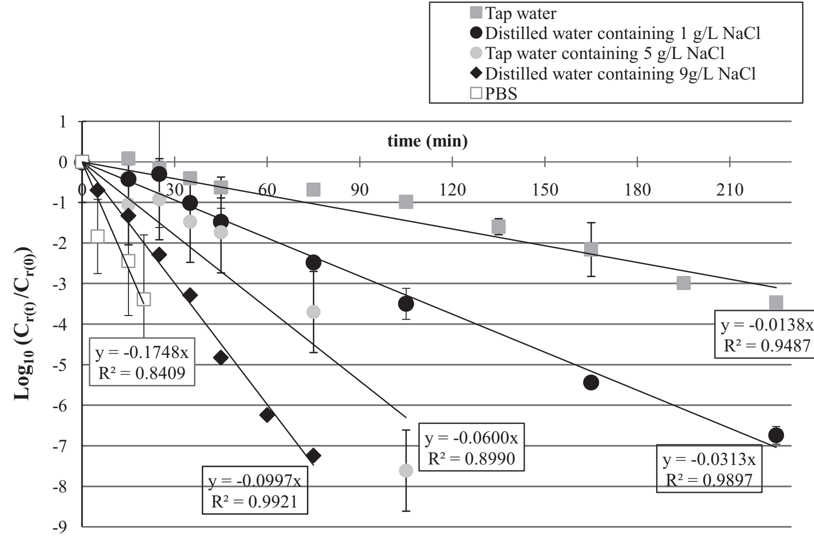

Fig. 5. Retentate concentration in infectious MS2 (PFU method) during filtration of suspensions in various media. The "doping" was taken as the origin of time, the concentration $C_r(0)$ was then equal to the total amount of viral RNA after doping ($n \geq 2$).

Table 1
Inactivation rate constant a_i (Eq. (4)) and $d_{filtration}$ (Eq. (5)), time at which the concentration of infectious MS2 has decreased by 1 Log_{10}, when phages were suspended in different media.

Quantification	Ionic strength, I (mM)	a_i (s^{-1})	a_i (min^{-1})	$d_{filtration}$ (min) (calculated)
Inactivation – tap water	3	2.30×10^{-4}	0.0138	72
Inactivation – distilled water + 1 g L^{-1} NaCl	17	5.22×10^{-4}	0.0313	32
Inactivation – tap water + 5 g L^{-1} NaCl	86	10.0×10^{-4}	0.0600	17
Inactivation – distilled water + 9 g L^{-1} NaCl	155	16.6×10^{-4}	0.0997	10
Inactivation – PBS	182	29.1×10^{-4}	0.1748	0

3.2.4. *Duration of the filtration test*

We calculated an inactivation rate coefficient a_i as the slope of the plot of $Log_{10}(C_{r(t)}/C_{r(0)})$ versus time for each buffer (Fig. 5). The filtration test duration $d_{filtration}$ (Table 1) was deduced by using Eq. (5). The higher the ionic strength, the larger the inactivation rate coefficient a_i and therefore the shorter $d_{filtration}$. In PBS, immediately after doping, over 90% of the phages initially present in the retentate were no longer infectious, therefore the evaluation of the virus retention was technically not reliable. At low ionic strengths, and typically in tap water, the filtration might be carried out for 70–80 min.

We have plotted the filtration test duration $d_{filtration}$ as a function of the ionic strength of the phage suspension in Fig. 6 and found that the empirical Eq. (6) fits the data with a correlation coefficient of 0.96, for ionic strengths between 3 mM and 180 mM:

$$d_{filtration} = -16 \ \mathrm{Ln}\, I + 85 \quad (6)$$

To sum up, when PFU counting is used to assess phage concentration in permeate and retentate, virus retention by a membrane should be determined within the characteristic time $d_{filtration}$. When the qRT-PCR method is used, the observed loss in total viral RNA in retentate is less than 1 Log_{10} even after 105 min of filtration whatever the buffer investigated (Fig. 4); therefore the duration of the filtration test can be longer in this case, but we have to keep in mind that the parameter which is measured then is not the infectivity of the permeate.

3.3. *LRV assessment*

3.3.1. *Permeate and retentate concentrations during filtration*

Fig. 7 shows MS2 concentrations in permeate and retentate collected during 2 h filtration tests, determined by PFU and by qRT-PCR methods. Data from samples collected after doping only are shown.

Infectious MS2 phage. The dashed vertical lines in Fig. 7 represent $d_{filtration}$. As discussed before, data from experiments in PBS were not valid. In tap water, conditions were acceptable for ca. 70 min,

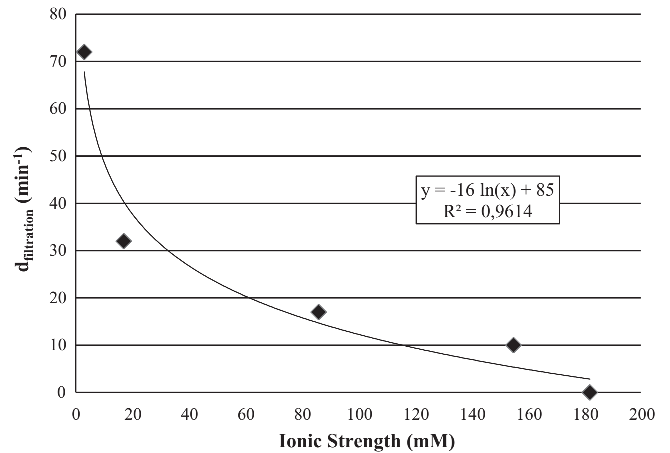

Fig. 6. Inactivation coefficient a_i (s^{-1}) as a function of ionic strength of the suspension (mM).

whereas, the time for sampling was much shorter if 5 g L^{-1} NaCl was added.

We could not detect any infectious MS2 in permeate at high salt concentrations. This can be explained by the rapid decrease in infectious MS2 concentration in the retentate. For NaCl concentrations below 5 g L^{-1}, the virus concentration in permeate was below the PFU quantification limit (30 PFU mL^{-1}) or slightly above.

Total viral RNA concentrations. The amount of total viral RNA in the retentate changed within a range considered as negligible (see Fig. 4 and comments) during a 2 h filtration run, whatever the buffer. Thus in our conditions, the experiments aimed at determining the LRV in total viral RNA could last 105 min and even more after doping. The permeate concentration in total viral RNA was below the quantification limit of qRT-PCR (10^2 equiv. PFU mL^{-1}) when the filtration was performed at low ionic strength (below 86 mM or 5 g L^{-1} NaCl), but above this limit in PBS, typically around 10^3 equiv. PFU mL^{-1} (Fig. 7D′). In these conditions, the permeate concentration was almost stable after 10–15 min of filtration. From these results we have assumed this time sufficient to reach saturation of permeate circuit (from the permeate side of the membrane to the permeate sampling point) in term of virus adsorption.

In the presence of salts, a larger amount of non-infectious viruses (because no detectable by PFU) (Fig. 7D and D′) were found in the permeate, suggesting that non-infectious viruses transfer was facilitated by high ionic strengths.

3.3.2. LRV comparison

As for an example, LRVs were finally determined for MS2 dispersed in various buffers (Fig. 8). The reported values are an average of at least two reproducible tests performed on two different modules over $d_{\text{filtration}}$ for infectious phage and over 2 h of filtration for total viral RNA. When no phage was detected in the permeate or when the concentration was below the quantification limit of the analytical method, permeate concentrations in infectious MS2 and in total viral RNA were taken equal to 30 PFU mL^{-1} and 10^2 equiv. PFU mL^{-1} respectively for the LRV calculation.

The LRVs reported in Fig. 8 are the minimum values that can be claimed in our conditions accounting for $d_{\text{filtration}}$ (for infectious MS2), retentate concentration and quantification limits of PFU and qRT-PCR methods for the permeate except for PBS (Fig. 7D′).

The minimum LRV for infectious MS2 decreased slightly for ionic strengths between 3 mM (tap water) and 155 mM (distilled water + 9 g L^{-1} NaCL).

LRV in total viral RNA was independent of the ionic strength up to 86 mM and slightly decreased for higher salt concentrations. For ionic strengths above 155 mM, the concentration in infectious

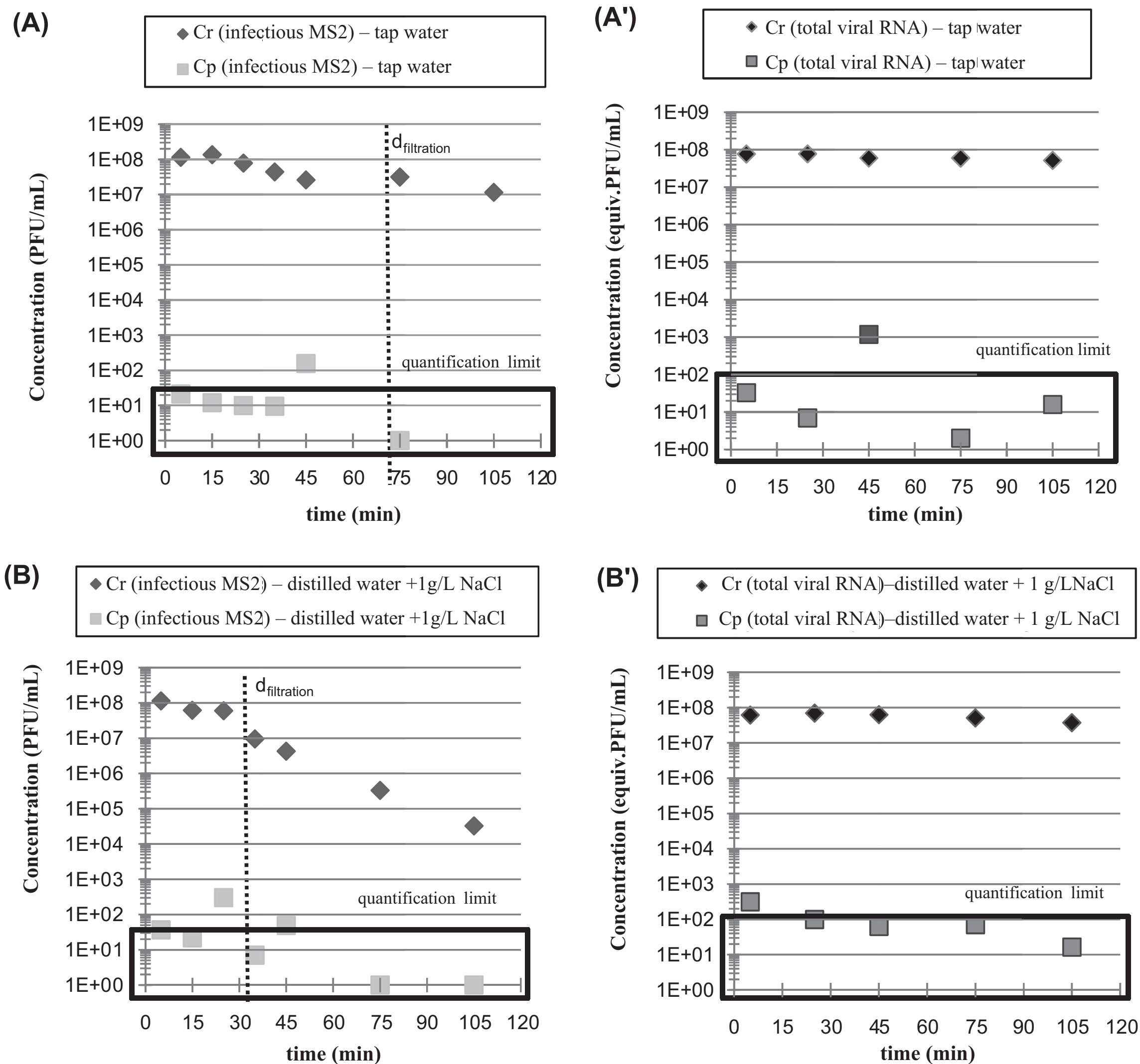

Fig. 7. Influence of the type of buffer on infectious MS2 and viral RNA concentrations in retentate and permeate during filtration. (A) Tap water, (B) distilled water containing 1 g L^{-1} NaCl, (C) filtered tap water containing 5 g L^{-1} NaCl and (D) PBS). The “doping” was taken as the origin of time ($n \geq 2$).

(C)

Cr (infectious MS2) – tap water +5g/L NaCl
Cp (infectious MS2) – tap water +5g/L NaCl

Concentration (PFU/mL)

$d_{filtration}$

quantification limit

time (min)

(C')

Cr (total viral RNA) – tap water + 5 g/L NaCl
Cp (total viral RNA) – tap water + 5 g/L NaCl

Concentration (equiv.PFU/mL)

quantification limit

time (min)

(D)

Cr (infectious MS2) – PBS
Cp (infectious MS2) – PBS

Concentration (PFU/mL)

quantification limit

time (min)

(D')

Cr (total viral RNA) –PBS
Cp (total viral RNA) –PBS

Concentration (equiv.PFU/mL)

quantification limit

time (min)

Fig. 7. (*Continued*).

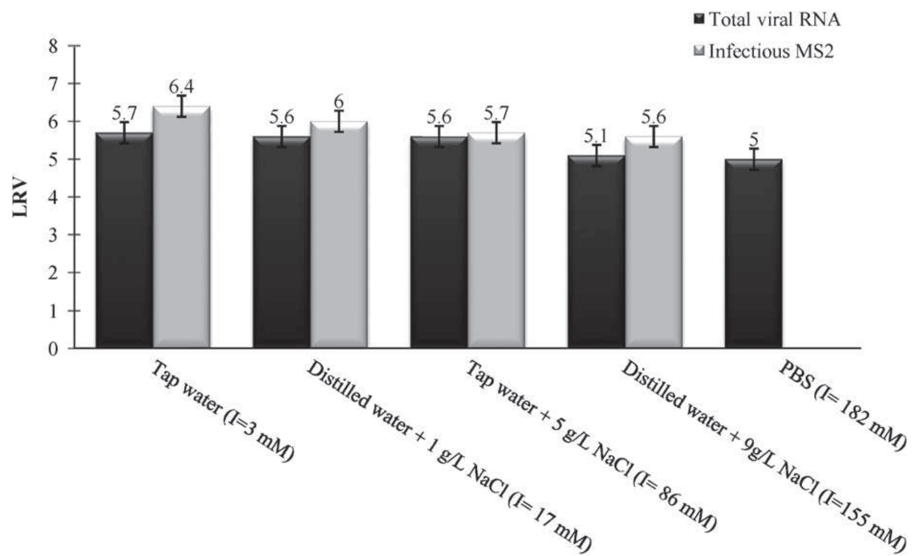

Fig. 8. Minimum LRVs in infectious MS2 and total viral RNA that can be claimed in our conditions accounting for $d_{filtration}$ (for infectious MS2), retentate concentration and quantification limits of PFU and qRT-PCR methods for the permeate (except for PBS Fig. 7D′) as a function of dilution media ($n \geq 2$).

phages decreased very rapidly, and the corresponding LRV could not be calculated. At 155 mM, $d_{filtration}$ was equal to 10 min, and the LRV in infectious phages could be calculated during this period only. Therefore, the LRVs determined by qRT-PCR over 2 h of filtration (5.1 for I = 155 mM and 5 for I = 182 mM) correspond to the removal of inactivated phages. This information is relevant with regards to public health issues, especially when the risk of virus reviviscence is concerned.

A maximum difference of 0.7 Log_{10} was observed between the LRV determined by PFU and qRT-PCR methods for ionic strengths strictly different (above or below) from 86 mM, The difference between LRV's determined by the two methods remains within the experimental uncertainty, except maybe when tap water was used, whereas Langlet et al. [3] report a difference of 1 Log_{10} in their conditions (0.2 mM PBS solution). At 86 mM which was the "minimum" ionic strength allowing to preserve the infectivity of the initial suspension [20], the difference between the LRV's was minimum. When dilution buffers have lower or higher ionic strengths, both methods should be used.

If only one quantification technique (often the traditional PFU method) is available, this study suggests the use of a low-ionic-strength solution which allows to maintain the infectivity of the initial suspension while giving enough time to run the filtration experiment. From a practical point of view, filtered tap water proved to be a very acceptable dilution medium.

4. Conclusion

The minimum LRV which can be claimed from the data collected after a membrane characterization test is limited by:

- The minimum virus concentration in the retentate during the test.
- The detection limit of the virus counting method, when applied to the permeate.

Amongst the two main methods available for virus counting, PFU counting has a lower detection limit than qRT-PCR and it enlarges the range of LRV's which can be claimed.It is well established, and we confirm here, that the virus concentration in a solution used to characterize filtration membranes may dramatically change during the test and we consider that this should be accounted for when assessing membrane LRVs, unless the test is invalidated because the conditions experienced by the membrane change too much during the test.

We suggest to collect the data used for assessing a membrane/module virus LRV within a period of time during which the change in virus concentration remains within some limits (here, 100–10%). We show that this maximum duration changes with the type of buffer used to dilute the virus stock solution, and decreases with its ionic strength (such as in Eq. (6)).

Implementing the recommendations to the assessment of a cellulose acetate membrane showed that there was no difference in our conditions between the LRV's found by PFU and qRT-PCR, and that the LRV slightly decreased when the ionic strength of the buffer increased. However, operating with low ionic strength buffers gives much more time for running the filtration tests, and filtered tap water proved in our case well adapted to this purpose.

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