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Retention of the Tulane virus, a norovirus surrogate, by ultrafiltration in seawater and production systems

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Abstract :

Shellfish as a foodstuff must meet sanitary quality objectives for the protection of consumers and this quality is closely linked to the water. The oyster industry considered this challenge related to contaminations and currently, the major risk of disease is due to the presence of norovirus (NoV) since all oyster-consuming countries report outbreaks of gastroenteritis linked to the presence of this microorganism. Ultrafiltration has already demonstrated to be efficient for viral protection of oyster farms in previous studies. In this work, retention by ultrafiltration of Tulane virus, a NoV surrogate, was evaluated. The effect of virus concentration in the feed on the ultrafiltration efficiency has been assessed. Low retentions of about 1 log were observed at the lowest viral concentrations. At higher concentrations, an increase of retention up to 5 log was obtained. These results highlight the potential overestimation of UF efficiency during laboratory experiments realized at high concentrations, compared to low concentrations found in environmental resources. In agreement with other studies, higher retentions at high concentrations could be explained by formation of viral aggregates, which could facilitate the steric exclusion but also modify the electrostatic and hydrophobic interactions between isolated viruses/aggregates and membrane. Virus retentions with a fresh mineral water (Evian water) and seawater were compared. Seawater achieved higher retention rates for Tulane virus due to the membrane fouling.

Highlights

► Removal of the Tulane virus, a norovirus surrogate, by ultrafiltration. ► Effect of the virus concentration and type of water on the retention. ► Ultrafiltration to protect shellfish and production systems. ► A solution to depurate contaminated oysters.

Keywords : Norovirus ; Ultrafiltration ; Oyster ; Concentration effect ; Retention rate

1. Introduction

Shellfish production area are classified based on a fecal bacterial indicator (*Escherichia coli*). Shellfish that does not comply with this criterion need to be depurated before marketing. This regulation decreased the number of outbreaks linked to bacteria among shellfish consumers, however viral outbreaks still occurred (Savini et al. 2021). Among all pathogens that bivalve molluscs can accumulate, notifications from the Rapid Alert System for Food and Feed (RASFF) of the European Commission over the last 20 years showed that norovirus (NoV) contamination was reported in 34 notifications out of 63 (European Commission, 2021). NoVs are the main cause of acute viral gastro-enteritis in all age groups of humans (Atmar et al., 2018). Each winter there is a peak of gastroenteritis in the population, leading to a huge amount of viral particles in sewage (Schaeffer et al., 2018). Sewage treatment plants applied diverse technologies, with varying efficiencies on NoV removal (Sano et al., 2015). The membrane bioreactor process has been demonstrated to be one of the most efficient for the elimination of small particules such as human enteric viruses and we previously demonstrated that it increases shellfish quality (Miura et al., 2015; Schaeffer et al., 2018). However other events such as direct discharges of untreated sewage, overflow after flooding, waste input from boats also contribute to coast water contaminations. A baseline surveys conducted in Europe over two years showed that one-in-three EU oyster production area was contaminated with NoV (European Food Safety Authority and European Centre for Disease Prevention and Control, 2019).

Standard post harvesting depuration process (oysters placed in tanks continuously supplied with clean seawater (UV treatment generally used) for 24 to 96 h), initially developed to eliminate bacteria, is not efficient toward viruses that can be resistant and remain for several weeks in bivalve tissues *via* specific ligands (McLeod et al., 2017). When contamination by NoV in oysters is detected, depending on the country, the production areas may be closed and products withdrawn from the market leading to a potential huge economic impact for this sector. However, two other options are possible: (i) the use of new treatment processes in depuration systems to provide treated seawater free of NoV potentially leading to an increase of efficiency and speed of decontamination; (ii) the use of new treatment processes in closed shellfish production systems with treated seawater free of NoV. Ultrafiltration has been used more and more for the drinking water production (Ferrer et al., 2015; Gentile et al., 2018). Ultrafiltration is a membrane process in which the driving force is a pressure difference with a molecular weight cut off between 2 and 300 kDa According to the pore size (for example 10 nm), UF process can be effective to remove pathogenic microorganisms such as bacteria and

parasites, but also viruses, smaller in size than other pathogens and without addition of chemical product in water (Ferrer et al., 2015). Membrane suppliers generally reported a virus reduction in the order of 4 log (99.99% retained viruses) for UF modules, but some research studies have observed different retentions. Indeed, Jacangelo, (1995) observed a viral reduction greater than 6 log with a molecular weight cut-off (MWCO) of 100 kDa, while Urase et al. (1994) obtained only 2.5 log abatement with larger viruses and membranes with a lower MWCO (30 kDa). Despite the membrane MWCO lower than the sizes and molecular weights of viruses, UF is not always able to fully retain viruses. To better understand the retention of viruses by UF membranes, the understanding of the different separation mechanisms and the impact of different filtration factors must be studied. The UF retention mechanisms are governed not only by the steric exclusion of compounds by the membrane pores, but also by electrostatic and hydrophobic interactions. Regarding the ultrafiltration of pathogenic viruses and microorganisms, several studies have observed the importance of steric retention. For several membranes with similar characteristics (charge, composition, hydrophobicity) with different pore sizes, the retention of the same virus increases when the pore size decreases (Jacangelo et al., 1995; Langlet et al., 2009; Shirasaki et al., 2017). Likewise, the retention of different viruses on the same membrane respects the order of virus size and larger viruses are better retained (Arkhangelsky and Gitis, 2008; Urase et al., 1996). However, some authors have explained the passage through the permeate of viruses larger than the pore size by the presence of abnormal pores not included in the pore size distribution of the membrane (Lu et al., 2017; Urase et al., 1996, 1994) and that other mechanisms could influence viral retention. Van Voorthuizen et al. (2001) considered that the adsorption of a virus with an overall neutral charge on a negatively charged membrane was possible and promoted retention, while the retention of negatively charged viruses (on the same membrane) was disadvantaged because adsorption was impossible. Even though a virus has an overall zero charge, it actually has a positive and negative charge distribution. Depending on the distribution of these charges, especially on the virus capsid, the adsorption of the virus on the negative membrane can therefore be increased. This observation was confirmed by ElHadidy et al. (2013), while conversely Gentile et al. (2018) observed that with a negatively charged virus on a neutral membrane, the adsorption was impossible and poor retention was observed. Thus, the adsorption of viruses onto membranes is promoted by attraction of opposite charges. Several authors have observed that virus retention can be caused by repelling similar strong charges, limiting the approach of viruses to the membrane surface (Arkhangelsky and Gitis, 2008; ElHadidy et al., 2013; Gentile et al., 2018; Lu et al., 2017).

Overall, most of enteric viruses and membranes used are negatively charged in the environmental whose pH is in the range (6-8) (Langlet et al., 2009; Lu et al., 2017; Shirasaki et al., 2017). If the zeta potentials of membranes and viruses are sufficiently strong (greater than approximately –30 mV), strong electrostatic repulsions can therefore increase retention. The load distribution and the shape of the capsid can then play an important role (Jiang et al., 2014; Langlet et al., 2009). In addition to electrostatic interactions, interactions related to the hydrophobic / hydrophilic properties of viruses and membranes can also lead to adsorption and thus influence retention. Thus, on a hydrophobic membrane, the retention of viruses with a hydrophobic tendency is greater than that of more hydrophilic viruses thanks to the adsorption generated by hydrophobic interactions (Arkhangelsky and Gitis, 2008; Duek et al., 2012). These hydrophobic viruses are also retained more effectively by hydrophobic membranes than on hydrophilic membranes because they cannot adsorb to hydrophilic membranes, which reduces viral retention (Langlet et al., 2009; Lu et al., 2017; Pontius et al., 2009). As with the electrostatic interaction, it is difficult to predict the hydrophobic interactions of viruses with the membrane due to the complex structures of the viruses. The virus is composed of an amphiphilic assembly and although the overall structure of the virus tends to be more or less hydrophobic. The hydrophobicity of viruses is difficult to quantify and more often the hydrophobic tendencies of several viruses are compared against each other. The three types of mechanisms of steric exclusion as well as hydrophobic and electrostatic interactions should be considered when studying virus retention by UF membranes. Size exclusion is the predominant mechanism when the size of the virus to be retained is much larger than the pore size of the membranes. When the order of magnitude of the size of the virus is the same as the one of the pores of UF membranes (from 1 to 100 nm), steric exclusion does not ensure the total retention of the virus (Jacangelo et al., 1995; Lu et al., 2017). The membrane fouling (inside and/or on the membrane) is also reported as a cause of modification of virus retention. This fouling is influenced by the composition of the water, but also by the choice of filtration mode (dead-end or tangential). The composition of the water can influence the retention by various mechanisms such as the virus adsorption to particles, retention by cake formation or by irreversible fouling (Jacangelo et al., 1995). The cake formation can allow increased retention by creating a second filtration barrier.

In the context of closed shellfish depuration or production systems, the aim of this work is to fully assess the performances of the ultrafiltration (UF) process to retain the Tulane virus, as a surrogate of NoV, in seawater. The objectives are to understand the potential transfer of the virus through the membranes as a function of the water quality (salinity, concentration in

organic matters) and the initial virus concentration. This study is agreement with the FAO (Food and Agriculture Organisation) virtual workshop on innovations on aquatic health management towards reduction of antimicrobial use and mitigation of antimicrobial resistance organized the 26 of October 2021 during which 3 of the 16 projects concerned ultrafiltration in the field of aquaculture and the pathogen removal.

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2. Material and methods

2.1.Viruses and cell culture preparation

Tulane virus (TV) strain M033 (provided by T. Farkas, Cincinnati children's hospital, Cincinnati, USA) was propagated in confluent monolayers of LLC-MK2 cells (ATCC® CCL-7™, Manassas, VA) as previously described (Farkas et al., 2008). Mengovirus (MgV) strain pMC0 (provided by A. Bosch, University of Barcelona) was propagated in HeLa cells as previously described (Martin et al., 1996). When cytopathic effects (CPE) were complete, cultures were frozen and thawed (-20°C) three times, and cell debris was removed by centrifugation at 1,000 × g for 30 min. The supernatant, which contained viral particles, was stored at –80 °C in aliquots.

2.2. Membranes

Membranes used were polymeric multichannel hollow fibers (ALTEONTM I, SUEZ Aquasource®, France), made with hydrophilic polyether sulfone (PES) and a porogenic hydrophilic polymer (polyvinylpyrrolidone, PVP). Their external diameter was 4 mm and they were composed of 7 channels with an inner diameter of 0.9 mm. The active length of fiber was 25 cm which provided a specific surface of 4.95×10^{-3} m² and an internal volume of 1.11 mL. The MWCO was between 150 and 200 kDa due to a membrane pore size distribution centered around 20 nm. The average initial membrane permeability with ultrapure water was 720 ± 100 $L.h^{-1}.m^{-2}.bar^{-1}$ (Figure 1). This membrane is used to produce drinking water in France (Nancy, Orléans, l'Haÿ-les-Roses), Croatia (Dubrovnik), Switzerland (Lutry), Italia (Castiglione de Fiorantino), etc. and recently for different aquaculture applications (Cordier et al., 2018, 2019ab, 2020abcd, 2021).

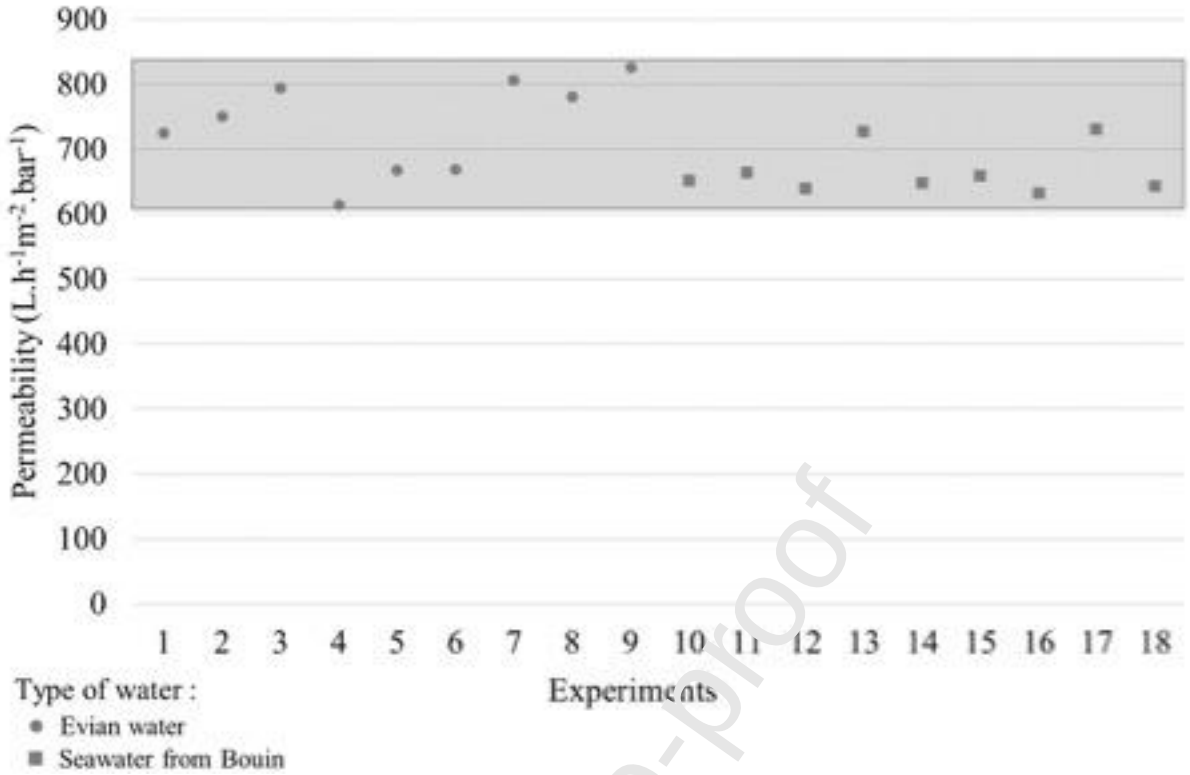


Figure 1: Initial membrane permeabilities (with ultrapure water at 20°C) for all the membrane modules used

2.3.Virus and water type

Two types of water were used: Evian mineral water and seawater from the Bourneuf Bay in Bouin (Vendée (85), France). This natural seawater was filtrated (10 µm and 1 µm) and disinfected with UV. The influences of virus concentration and water type were tested respectively with three different virus concentrations in the feed between 0.36 and 10^6 RNA copies.mL^{-1}. For each concentration and for each type of water, three replicates are used to improve the precision of the results. The characteristics of seawater and Evian water are given in Table 1.

Table 1: Evian water and seawater average characteristics

	Conducivity (µS. cm^{-1})	Turbidity (FNU)	Total Organic Carbon (TOC) (mgC.L^{-1})	pH	Total dry residue (mg.L^{-1})	Suspended solids (mg.L^{-1})	Dissolved Oxygen (mg.L^{-1})	Ammoniacal nitrogen (mgN.L^{-1})	Anions: Cl^- ; SO_4^{2-} (mg.L^{-1})	Cations: Na^+ ; Mg^{2+} (mg.L^{-1})
Evian Water	590	0.05	< 0.2	7.2	309				10 ; 14	6.5 ; 26
Seawater	50,000	1.27	2.3	8.1	37,000	2	103.1	0.019	20,800 ; 2,767	1,200 ; 9,700

2.4. Filtration procedure

Membrane modules were made of one multichannel hollow fiber membrane into a PVC external shell with an epoxy plug on each side of the module. After the module potting, membranes were rinsed with ultrapure water under different transmembrane pressures (TMP) with a maximum at 1.0 ± 0.1 bar to remove the preservative agent (glycerin). Water permeability (Lp_0) was then measured with ultrapure water (Figure 2). Experiments were performed in dead-end filtration mode with constant TMP: approximately 0.3-0.4 bar. To avoid any contamination between experiments, a new membrane module was used for each. The feed solution was a suspension of viruses in 480 mL of the tested water. 80 mL of this feed solution was sampled to measure the feed concentration. The volume of 80 mL is the minimum value to determine the virus concentration even in the feed and the permeate. 400 mL were introduced in the feed tank and passed through the membrane: pure pressurized air was connected to the feed tank containing the virus solution, which was connected to the membrane module. During the filtration process, permeate is collected over time. Almost every 5 to 10 s, its weight is recorded by an electronic balance ($\Delta m = \pm 0.01$ g; Mark Bell, Berlin, Germany) to calculate the permeate flux (J in $L.h^{-1}.m^{-2}$) from the permeate flow (Q in $L.h^{-1}$) and the membrane surface (S in m^2). The temperature is used to correct the measured flux at 20 °C in agreement with the variation of water viscosity. The permeate flux values allowed monitoring the fouling during filtration. The first 80 mL of the collected permeate represented the initial permeate sample and the last 80 mL the final permeate sample. Between those two samples, 240 mL were collected then stirred and only 80 mL out of 240 mL were kept (medium permeate sample) for analysis. Thus, four samples were taken during each filtration (feed solution, initial, medium and final permeate) and further analyzed for virus detection.

2.5. Sample analysis

(i) Water samples were ultracentrifugated for 1 hour at 100,000 x g at 4°C under a volume of 11 mL or 70 mL, depending on the expected concentration. MgV was added to all samples at a final concentration of 2×10^6 RNA copies. After supernatant elimination, pellets were resuspended in 500 µL of Phosphate-Buffered Saline (PBS) and let for 5 min. Nucleic acids (NA) was subsequently extracted by using the NucliSENS kit and the NucliSens miniMAG purification system (bioMérieux, Lyon, France) following the manufacturer's instructions, with 2 mL lysis buffer, 50 µL magnetic silica and eluted in 100 µL of the elution buffer. Nucleic acids from cultured virus were extracted using the same kit on a subsample of the cell culture supernatant.

(ii) Primers, probes and real-time reverse-transcription-PCR (rRT-PCR). For mengovirus and Tulane virus, *r*RT-PCR was conducted as previously described (Drouaz et al., 2015; ISO 15216-1, 2017). The rRT-PCR was carried out using the UltraSense One-Step quantitative RT-PCR system (Life Technologies, France), with 5 µL of undiluted extracted NA per well (25 µL reaction) under triplicate. Amplifications were performed in an Mx3000P quantitative PCR (qPCR) system (Agilent Technologies, France).

(iii) rRT-PCR controls and quantification. Filtered tips and dedicated rooms were used to prevent sample contamination. One negative-amplification control (sterile, RNase-free water) was included in each amplification series. (a) Extraction efficiency: MgV was used to evaluate the extraction efficiency. The C_T value of the undiluted samples (seeded with $2x10^6$ RNA copies of MgV) was compared to the C_T value of the positive control used in the extraction series, and to a standard curve made by end point dilution of this positive control. This standard curve allows the calculation of the slope and the difference in C_T between the controls and samples (ΔC_T) was used to determine the recovery efficiency using the equation $100 \times e^{-0.6978 \times \Delta C_T}$ and was expressed as a % for each sample. Only samples with extraction efficiencies above 10% were considered for quantification. (b) Quantification: C_T values of the triplicate amplifications were compared. If a variation >1 C_T unit was observed, the amplification was repeated, and all three C_T values were averaged. In case of one negative well, a substituted value of 41 was applied. After these verification steps, the number of RNA copies in each positive sample was estimated by comparing the C_T value of the sample to standard curves derived from plasmid containing nucleotides 3300-4299 of the Tulane virus M33 (GenBank accession no. EU391643-1). The final concentration in the sample was then back-calculated based on the volume of NA and expressed per mL of water.

2.6. Treatment of the virus retention as a function of the virus concentration

Data of Logarithmic Reduction Value (LRV) as a function of virus concentration in the feed suspensions were analyzed with the software GraphPad Prism 9.0. Best fit models were determined for each set of data, considering each Y replicate as individual value. Curves were modelized with a 95 % confidence interval.

3. Results

As NoV cannot be propagated in large quantities in cell culture the TV was used as a surrogate. We previously demonstrated the potential of this virus, member of the recovirus genus (ReCV) of the *Caliciviridae* family, to mimic NoV behavior in oyster tissues (Drouaz

et al., 2015; Polo et al., 2018). Importantly for this study the shape and the size (30 nm) of the TV is similar to NoV (Farkas et al., 2008).

3.1. Permeate flux and additional resistance

Figure 2 shows the variation of dimensionless membrane permeability as a function of the volumetric concentration factor (VCF) for different types of water. It should be recalled that the experiments were carried out in dead-end mode at constant TMP.

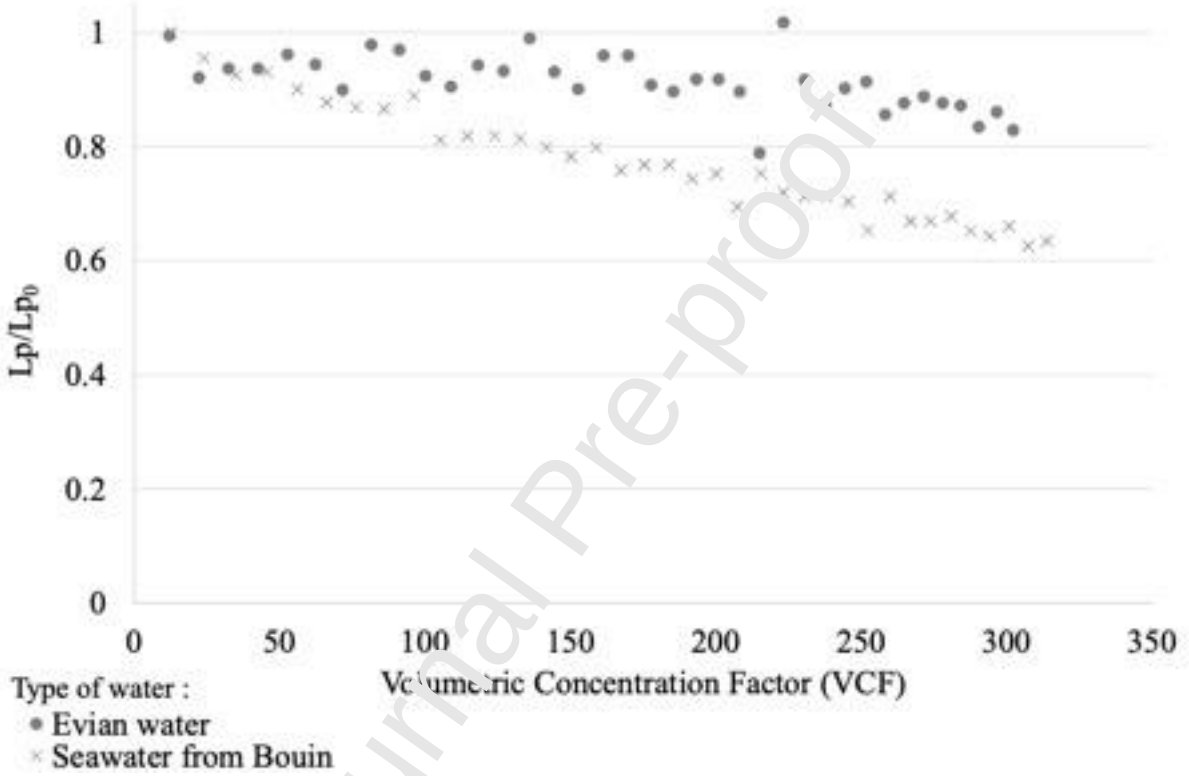


Figure 2. Variation of the dimensionless membrane permeability as the function of VCF for different types of water [TMP = 0.3 bar, Feed concentration =2.5×10^2 RNA copies.mL^{-1}]

Figure 2 highlights a slight decrease of permeability as a function of VCF for Evian water. This decrease is explained by the virus accumulation in the lumen of the membrane and an increasingly fouling without a significant impact on permeate flux and permeability. For filtration with seawater, a greater decrease is observed due to the accumulation of viruses but also of other compounds such as organic matters present in seawater. To obtain a global view of the impact of the virus concentration on the permeate flow, the permeability variation as a function of the virus concentration for each initial concentration, considering the VCF, was plotted for the three replicates. However, the initial permeability of the membrane is not strictly the same for each experiment. To consider the initial water permeability of each module individually it is the variation of the fouling resistance which is plotted. Figure 3

shows the variation of fouling resistance as a function of the theoretical concentration upstream of the membrane for Evian water, assuming a 100 % virus retention.

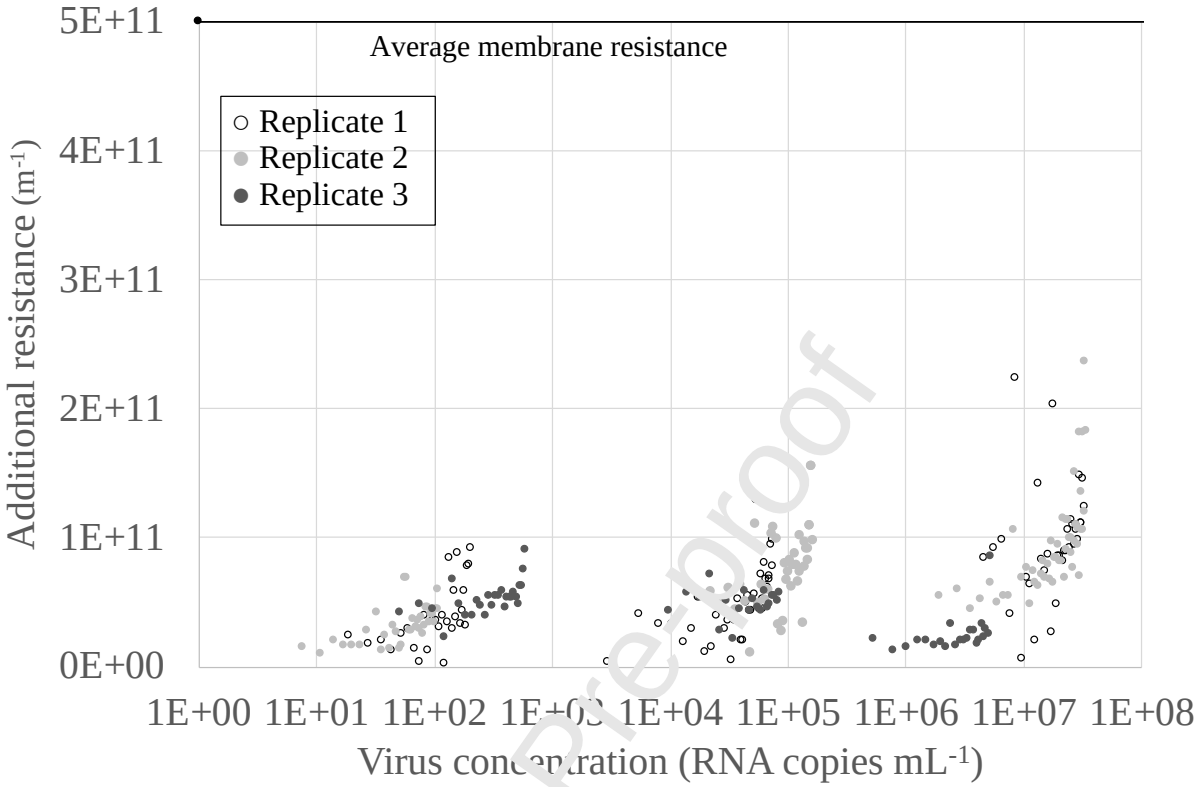


Figure 3. Variation of the additional resistance as the function of the upstream virus concentration [Retention rate = 100 %, Evian]

In Figure 3 (a) all the results of the 9 experiments are represented in the same figure with an similar variation of the additional resistance for each group of replicates. This implies a good reproducibility of the experiments for each concentration but also for different concentrations. However, for this highest feed concentration, replicate #3 tends to have a behaviour closer to the one of lower concentrations and this will be discussed in paragraph 3.2. (b) The fouling resistance (additional resistance) is very low compared to the membrane resistance, thus testifying to the purity of the filtered virus samples but also explaining the scatter plot for low concentrations (experimental error). (c) As expected, the fouling resistance increases as a function of the concentration for each initial concentration but also over the concentration range from 0.36 to 3.36 $\times 10^7$ RNA copies.mL^{-1} representing respectively the lowest concentration in the membrane lumen and the maximum final concentration obtained upstream of the membrane.

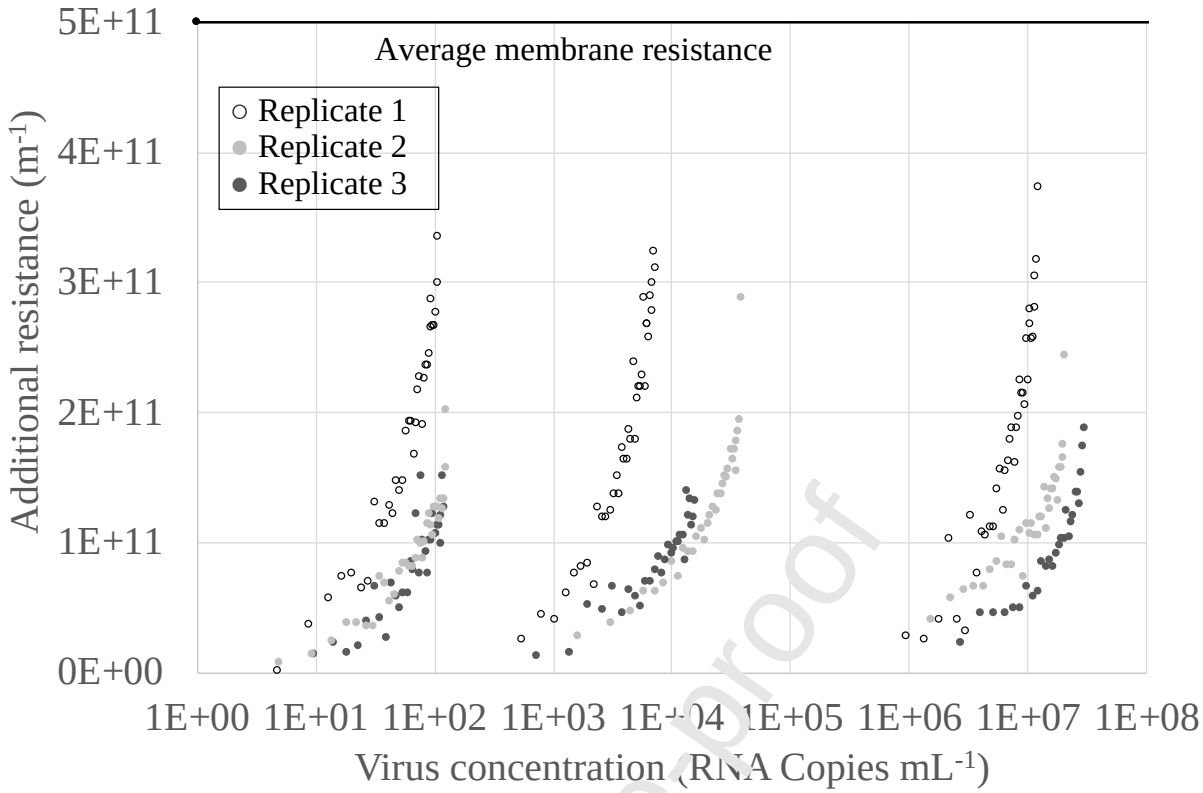


Figure 4. Variation of the additional resistance as the function of the upstream virus concentration [Retention rate = 100 %, seawater]

Figure 4 shows the evolution of the additional resistance as a function of the theoretical concentration upstream of the membrane for seawater.

In Figure 4 (a) identically, all the results of the 9 experiments are represented in the same figure with very close variations for each group of replicates. This implies good reproducibility of the experiments by concentration but also for different concentrations. (b) The fouling resistance varies more strongly than Evian water, indicating the seawater is more loaded with suspended and/or organic matter than Evian water. In addition, as the fouling due to viruses is negligible or very low (Figure 3), the variations are similar regardless the initial concentration.

3.2. Virus retention: case of Evian water

The retention rate is determined using three retention rate calculations because only average permeates (3 in number: initial, medium and final permeates), although representative of all permeates, are obtained for each experiment. First, the overall retention is calculated relatively to the initial and average concentrations of the permeates obtained by analyses. This retention rate does not take into account the concentration upstream of the membrane which increases with time, but it is the retention rate calculated by drinking water producers for the same reasons of non-accessibility to the real concentration in the lumen or the variation of

permeate concentration with time. Secondly, average retention rate is calculated considering the three permeate concentrations obtained by analyses and the three median concentrations of retentate corresponding to these 3 collection times (i.e. for these 3 permeates). This retention rate is closer to reality because it considers the increase in concentration in the retentate calculated by mass balance. However, it assumes that the permeate concentration remains constant for the duration of each permeate sample. The third retention rate is calculated by considering that the concentration in the permeate varies with time and that the concentration of the 3 analyzed permeates is the concentration of the permeate for a median volume. In this case, using the calculated concentration of the retentate for this median volume, three median retention rates are obtained for each experiment. These retention rates are calculated from the results obtained for each filtration. In the case of the highest virus concentration, the feed, the first, median and final permeate concentrations are respectively 1.1×10^5, 1.05×10^3, 8.37×10^2 and 2.68×10^1 RNA copies.mL^{-1}. Regarding the quality of the treated water, Tulane virus is detected in permeates thus UF does not lead to a total retention of this microorganism. The same conclusion was observed in the case of OsHV-1: virus was measured in permeate but, with retention rates higher than 98 %, the concentrations obtained in treated water were too low to have an impact on oysters (Cordier et al., 2020).

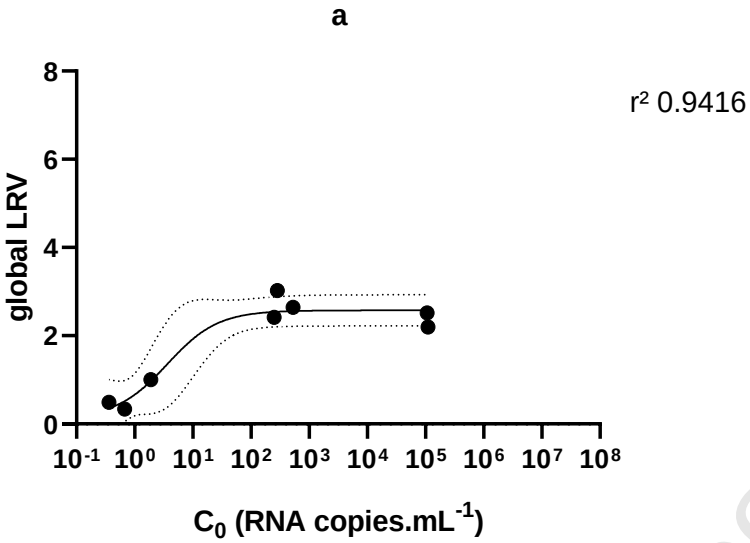
The initial measured virus concentration is 1.1×10^5 RNA copies.mL^{-1} for a theoretical value of 2.08×10^5 RNA copies.mL^{-1}. This difference can be explained by the difficulty to prepare calibrated virus solutions and RT-PCR quantification accuracy (Polo et al., 2018). The overall retention rate (used by drinking water producers) is 99.36 %, i.e. a LRV of 2.2 for an initial concentration of 1.1×10^5 RNA copies.mL^{-1}. Jacquet et al. (2021) found similar results with an enteric virus CV-B5 (of same size: 30 nm) in Evian water: LRV was equal to 2.5. Table 3 shows the virus concentrations in the lumen of the membrane considering the increase of this concentration according to the other two calculation methods and the corresponding LRVs for two replicates (#1 and #2), still in the case of the highest concentrations in virus.

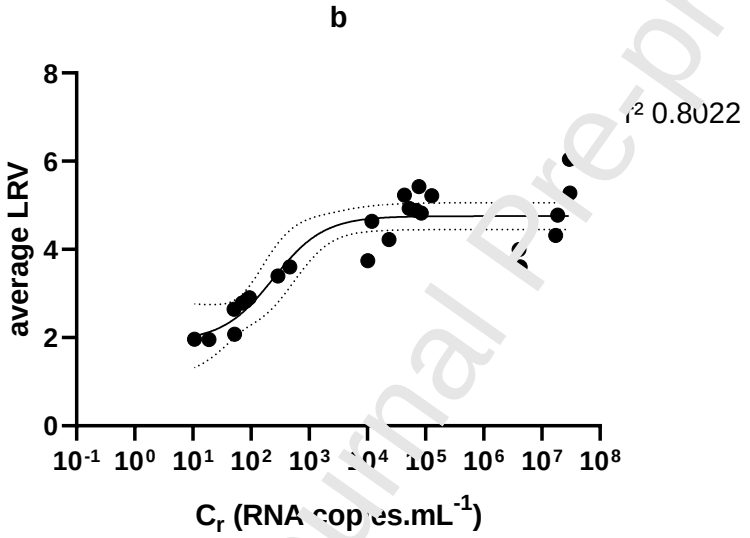
Table 3 leads to several conclusions. The LRV values, calculated from the median value or an average upstream concentration, are consistent. The upstream concentration goes from a value of 1.14×10^5 to 2.97×10^7 RNA copies.mL^{-1}, i.e. an increase of 260 whereas if the retention was complete, this increase would be 347 (= feed volume / fiber volume). Since the retention is not complete, these values are consistent. It can be seen that the higher the virus feed concentration, the greater the reduction, which can reach values of 6 at the end of the experiment. In Table 3, the bold values of replicate 1 are in perfect agreement with that of

replicate 2. For replicate #3, the concentration of the feed solution is very low compared to the other 2 replicates (84 % difference) and will not be considered in the results. This highlights the interest of replicates and the link with hydrodynamics (Figure 3) where this replicate was not strictly in the range of results.

Table 3: Median and average concentrations (RNA copies.mL^{-1}) and median and average LRV [Highest feed concentration, replicate # 1 **// replicate # 2**, Evian water]

Calculated median concentration in retentate	Median LRV	Calculated average concentration in retentate	Average LRV
3.48×10^{6} // $\mathbf{3.47\times10^{6}}$	3.52 // **3.93**	4.24×10^{6} // $\mathbf{4.07\times10^{6}}$	3.61 // **4.00**
1.64×10^{7} // $\mathbf{1.82\times10^{7}}$	4.29 // **4.77**	1.74×10^{7} // $\mathbf{1.88\times10^{7}}$	4.32 // **4.78**
2.92×10^{7} // $\mathbf{2.95\times10^{7}}$	6.04 // **5.26**	2.97×10^{7} // $\mathbf{3.05\times10^{7}}$	6.04 // **5.28**





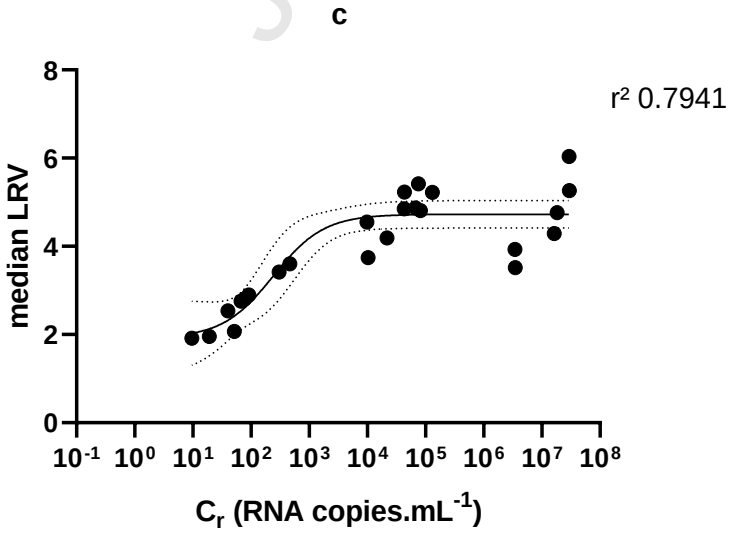


Figure 5. Varation of global, average, median LRV as the function of the virus concentration inside the membrane [Evian]

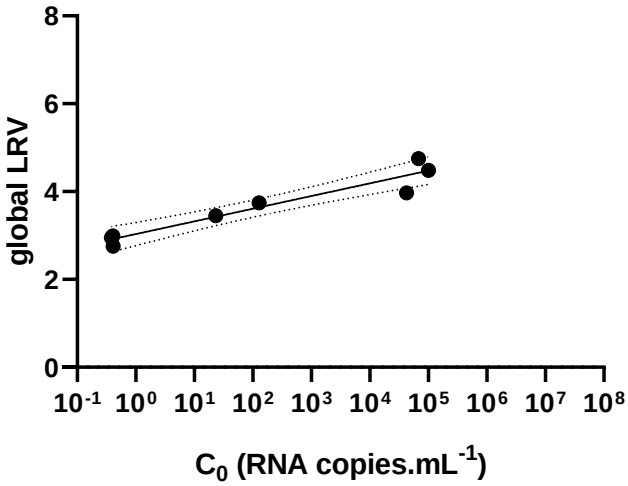
The impact of feed concentration (Cf) (i.e. concentration inside the membrane) on the retention of viruses was assessed (Figure 5) for the 3 calculations of LRV. It must be noted that viruses were detected and quantified in all the feed and in almost all permeate samples allowing to estimate a LRV in each experimental condition. If the quantification in the permeate is lower than the detection limit, the virus concentration is taken equal to these detection limits i.e. 1.82 and 0.286 RNA copies.mL^{-1} for highest and lowest ranges of feed concentration respectively. The best fit model for the "Evian" was determined as the model Pade (1,1) approximant. This model corresponds to the equation type: Y = (A0 + A1*X)/(1 + B1*X). No constraint has been given on the A0, A1 and B1 parameters. For global LRV (Figure 5-a), results showed an increase in virus retention with feed concentration. This increase goes from a LRV of less than 1 for the lowest concentrations to a stabilized LRV at around 2.5-3. This variation and the range of LRV are in agreement with the results of Jacquet et al. (2021) and puts in light that the retention of viruses increases due to their potential agglomeration when the concentration increases.

Figure 5 also gives the average LRV (Figure 5-b) and the median LRV (Figure 5-c) as a function of the upstream virus concentration (C_r). When the variation of concentration in the membrane lumen is now considered, the figures show that the two calculation modes give similar values and the LRV variations are identical to those of the global LRV. Here, median and average retentions increase and seem to stabilize for very high concentrations at around 5 log. This value is consistent with what is reported by the membrane manufacturers and with the results of Jacquet et al. (2021) in terms of variation. The retention analysis by Jacquet et al. (2021) on the agglomeration of viruses when the concentration increases to explain the increased retention of viruses therefore remains valid.

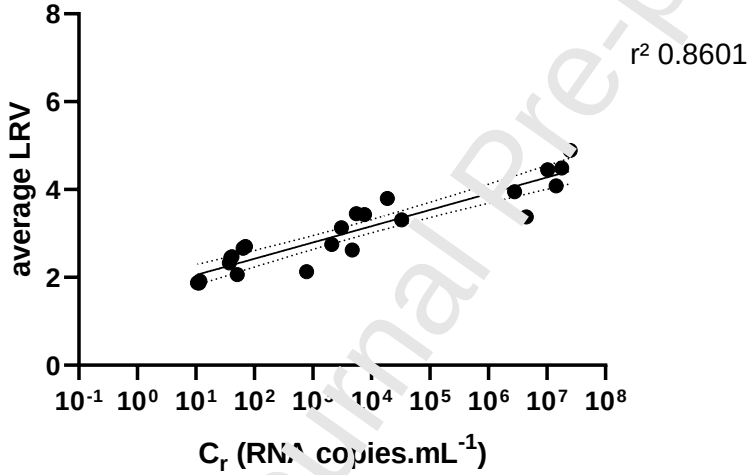
3.3. Virus retention: case of seawater

In the case of seawater, results are plotted together in Figure 6 which shows different values and variations of retention. For the seawater data, the best fit model was the Semilog line (X is log – Y is linear), corresponding to the equation type : Y=Yintercept + Slope*log(X). No constraint has been given on the parameters.

a



b



c

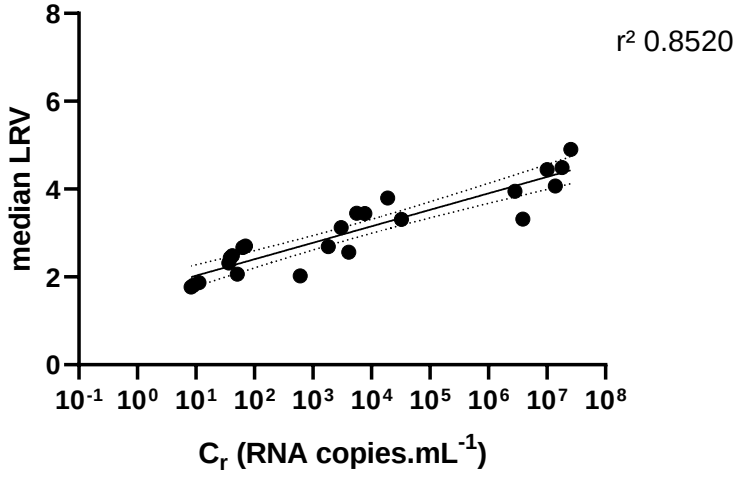


Figure 6 : Varation of global, average, median LRV as the function of the virus concentration inside the membrane [Seawater]

The presence of salts in the water to be filtered is an important factor that can influence viral retention by UF membranes, but this phenomenon remains complex. The overall ionic strength of the solution, but also the nature of the salts can affect the different retention mechanisms (Antony et al., 2012). In general, virus retention is improved for low ionic strengths (Dishari et al., 2015). The influence of ionic strength on the retention of phage PP7 was studied for different salts in solution by Gentile et al. (2018). It was observed that the presence of salts led a decrease of the phages' zeta potential. For retention by a negatively charged membrane (which is the case with membranes studied with a zeta potential between -5 mV and -15 mV for pH 7-8) electrostatic repulsions are disadvantaged by the presence of salt at high concentration. As for the overall charge of viruses, the nature of the salts present in solution can also influence the charge of the membrane surface (Gentile et al., 2018): for a constant ionic strength of 10 mM, the zeta potential of a membrane has been measured close to 0 mV in the presence of divalent salts, while it was strongly negatively charged (- 45 mV) in the presence of monovalent salts, thus influencing the electrostatic interactions of adsorption or repulsion. This study highlighted that the cations, and particularly the divalent cations Ca^{2+} and Mg^{2+} in contact with the membrane, caused a decrease of electrostatic repulsions by their positive charge. Moreover, they also prevented the adsorption of phages due to their strong hydrodynamic radius, resulting in decreased retention of viruses (Huang et al., 2012; Wu et al., 2017). On the contrary, the presence of monovalent cations promotes negative charges and electrostatic repulsions. The presence of salts can also influence hydrophobic interactions in addition to electrostatic interactions. On a hydrophobic membrane, the retention of MS2 is favored by the presence of different salts ($CaCl_2$ and NaCl), which promote hydrophobic adsorption. On the other hand, on a hydrophilic membrane, the presence of the $CaCl_2$ salt increases the retention of the MS2 phages, whereas the presence of the NaCl salt does not influence the retention: the monovalent ions of the NaCl salt only modify the hydrophobic interactions and not the electrostatic interactions. Thus, this generates a difference in phage retention on hydrophobic membranes but not on hydrophilic membranes where hydrophobic adhesion is not possible. In the case of the PSF membranes studied, the polysulfone is hydrophobic but hydrophilic agents are grafted to make the hydrophilic membrane from a hydrophobic material. However, whatever the grafted hydrophilic agents, the membranes studied are more hydrophobic than acetate membranes since they are much more sensitive to variations of the UV index or the level of organic

matter. In addition, Jacquet (2021) has shown that increasing salinity decreases the zeta potential of the membrane but the membrane fouling increases it whatever the salinity.

The modification of the retention is therefore complex with seawater. This type of water generates an increase in retention due to the presence of monovalent cations and the increase of the membrane zeta potential due to fouling, but at the opposite, the increase in salinity, in general, means that virus retention is disadvantaged (Dishari et al., 2015).

The shape of the curves obtained in this study (Figure 6), however, provides the beginnings of an explanation. Due to the pore size of ultrafiltration membrane, the salt concentration was the same whatever the virus concentration and the virus retention increases with the virus concentration upstream. Different additional resistance curves with seawater are observed (Figure 4); they would also follow a semi-log variation due to the fouling of the membranes. The use of natural water would cause fouling, thus increasing the retention of viruses. For the global LRV, this phenomenon is very marked because it does not take into account the increase in the upstream concentration. In the state of art, studies have shown similar results. In a study carried out on the influence of the composition of water, it was shown that for water without natural organic matter (NOM), the effect of electrostatic repulsions, ahead of size and hydrophobicity effects, was modified by the NOM addition which could both impact the aggregation of the NOM-viruses and the membrane fouling of the membrane with pore blockage, making size exclusion predominate over other mechanisms (Cruz et al., 2017). About the retention by a cake formation or by irreversible fouling (Jacangelo et al., 1995), the formation of a cake can allow increased retention by creating a second filtration barrier. For example, the formation of a kaolinite cake on the membrane surface thus led to an increase of the MS2 phage retention by a UF membrane from 1.2 to 3.7 log and the decrease of the permeate flux (Jacangelo et al., 1995) as observed in Figure 2. Fouling, whether reversible or not, is often reported as a cause of increased retention (Carvajal et al., 2017; Czemak et al., 2008). The explanation is often the decrease of the size and the number of pores per fouling which promotes steric exclusion (Reeve et al., 2016; Wickramasinghe et al., 2010; Yin et al., 2015).

Table 4 gives the values of global LRV as the function of the feed concentration and the type of water (i.e. fouling with seawater in comparison to Evian water). It is important to note that this natural seawater was filtrated (10 µm and 1 µm) and disinfected with UV, so the fouling is reduced in comparison to untreated water and it can be expected that the use of natural seawater would lead to higher fouling and even better retention.

Table 4: Range of global LRV as the function of the feed concentration as the function of the type of water [3 replicates for each condition]

	Global LRV – Seawater	Global LRV – Evian water
Low feed concentration [0.36-1.89 RNA copies.mL^{-1}]	[2.76-2.95]	[0.34-1]
Middle feed concentration [23-525 RNA copies.mL^{-1}]	[2.46-3.74]	[2.41-3.02]
High feed concentration [42,000-109,000 RNA copies.mL^{-1}]	[3.97-4.75]	[2.19-2.52]

For the other two LRV calculations, the concentration factor being considered, the differences are less marked.

4. Conclusion

When process has to be validated for NoV, TuV has been proposed as a surrogate. We previously demonstrated the potential of this virus, member of the recovirus genus (ReCV) of the *Caliciviridae* family, to mimic NoV behavior in oyster tissues (Drouaz et al., 2015; Polo et al., 2018). Importantly for this study the shape and the size (30 nm) of the TV is similar to NoV (Farkas et al., 2008). UF for small virus retention has been assessed regarding various experimental conditions in terms of virus concentration and type of water. Three virus concentrations were used between 0.36 and 1.5×10^{5} RNA copies.mL^{-1} to reflect real virus concentration of virus found in natural waters and the one used in laboratory studies. The latter mainly focus on feed with high virus concentrations, whereas seawater or more broadly natural waters are largely less concentrated. Moreover, two types of water, Evian water and seawater, were used to mimic viral contamination in seawater and rivers. Three retention rates are calculated to consider (i) the global retention rate of the membrane as for drinking water industry but in this case the variation of virus concentration in the lumen of the membrane is not taken into account during the filtration step (ii) the average retention rate which considers that during the permeate sampling, its concentration is not affected by the upstream concentration and (iii) the median retention rate which considers the variation of the permeate concentration during the sampling. In the case of Evian water, study about the effect of feed concentration showed a better retention efficiency for highest feed concentrations. For global LRV and for the lowest feed concentrations used, LRVs of less than 1 were obtained. With the feed concentration, an increase of global LRV was observed and the global LRV stabilizes

at around 2.5-3. This variation and the range of LRV are in agreement with the results of Jacquet et al. (2021) and show that the retention of viruses increases due to their agglomeration when the concentration increases. If we consider the variation of upstream concentration during the filtration step, results put in light that the two calculated retentions rates (median and average) give similar values and the LRV variations are identical to that of the global LRV. Here, median and average retentions increase and seem to stabilize for very high concentrations at around 5 log. This value is consistent with what is reported by the membrane manufacturers and with the results of Jacquet et al. (2021) in terms of variation. The retention analysis by Jacquet et al. (2021) on the agglomeration of viruses when the concentration increases to explain the retention of viruses therefore remains valid. The impact of the type of water on virus retention has been evaluated, resulting in a non-similar variation and a better retention efficiency with seawater (Bouin, France) compared to Evian water. The presence of salts in the seawater to be filtered is an important factor that can influence viral retention by UF membranes. If this phenomenon remains complex, the shape of the curves of additional resistance and retention as the function of the virus concentration provides the beginnings of an explanation. The salt concentration was constant whatever the virus concentration because of the pore size in ultrafiltration and the retention increases with the virus concentration upstream. The use of natural water would cause even more fouling, thus increasing the retention of viruses.

To conclude, ultrafiltration led to high retention of Tulane virus, used here as a surrogate of NoV, virus most frequently implicated in oyster related outbreaks worldwide. Even if the retention is yet not complete, the concentration in permeates is presumably enough to improve seawater quality and thus to prevent oyster contamination for example in the case of closed production systems, Tests with natural seawater and whatever the feed concentration validate ultrafiltration in real conditions with high retention rates. Tests in industrial scale and/or with real NoV are in progress and will be the subject of a forthcoming paper.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Removal of the Tulane virus, a norovirus surrogate, by ultrafiltration

Effect of the virus concentration and type of water on the retention

Ultrafiltration to protect shellfish and production systems

A solution to depurate contaminated oysters