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Removal of pathogens by ultrafiltration from sea water

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ABSTRACT

Among water treatment processes, ultrafiltration is known to be efficient for the elimination of micro-organisms (bacteria and viruses). In this study, two pathogens were targeted, a bacterium, *Vibrio aestuarianus* and a virus, OsHV-1, with the objective to produce high quality water from seawater, in the case of shellfish productions. The retention of those microorganisms by ultrafiltration was evaluated at labscale. In the case of OsHV-1, the protection of oysters was validated by *in vivo* experiments using oysters spat and larvae, both stages being highly susceptible to the virus. The oysters raised using contaminated seawater which was then subsequently treated by ultrafiltration, had similar mortality to the negative controls. In the case of *V. aestuarianus*, ultrafiltration allowed a high retention of the bacteria in seawater with concentrations below the detection limits of the 3 analytical methods (flow cytometry, direct seeding and seeding after filtration to 0.22 μm). Thus, the quantity of *V. aestuarianus* was at least, 400 times inferior to the threshold known to induce mortalities in oysters. Industrial scale experiment on a several months period confirmed the conclusion obtained at lab scale on the *Vibrio* bacteria retention. Indeed, no bacteria from this genus, potentially harmful for oysters, was detected in permeate and this, whatever the quality of the seawater treated and the bacteria concentration upstream of the membrane. Moreover, the resistance of the process was confirmed with a stability of hydraulic performances over time for two water qualities and even facing an algal bloom. In terms of retention and resistance, ultrafiltration process was validated for the treatment of seawater towards the targeted pathogenic microorganisms, with the aim of biosecuring shellfish productions.

1. Introduction

In 2014, world oyster production reached the level of 5.2 million tonnes, corresponding to 3.3 billion euros (Buestel et al. 2009). Since shellfish production is intimately linked to the marine environment, it is therefore sensitive to the events that occur. In the case of the oyster production, the profession is indeed impacted by crises of mortalities, sometimes resulting in a decimation of the breedings as in 1920 and 1971 in France for *Ostrea edulis* and *Crassostrea angulata,* respectively. In fact, pathogenic organisms were involved in oyster diseases and massive mortalities. These recurring crises have resulted in the introduction of new and more resistant oyster species. Since the 1970s, oyster *Crassostrea gigas* has been produced almost in monoculture in France. Moreover, oyster producers are using hatchery spat, able to produce diploid and triploid selected oysters for their higher resistance to both diseases in order to supplement or even replace wild-caught spat on oyster farms (Azéma et al., 2016; Burnell and Allan, 2009; Dégremont et al., 2016a; Helm, 2004). Nevertheless, the pollution found in shellfish farms and more generally in aquaculture farms knows various origins and consequences. In hatcheries or nurseries, animals are sensitive to the water quality variations which must yet have characteristics adapted to their growth (Utting and Helm, 1985). In fact microorganisms such as parasites, bacteria, viruses and fungi can be present in feed water at harmful concentrations for aquaculture

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production (Lekang, 2013). In the case of breeding larval animals, the removal of pathogenic microorganisms is essential at this first stage of life, more vulnerable and susceptible to infections (Azéma et al., 2017; Dégremont et al., 2016a). Several virus families have been identified as pathogens in aquaculture, iridoviruses, herpesviruses, reoviruses and rhabdoviruses (Zhang and Gui, 2015). In shellfish production, herpes infections have been reported in nine different species of bivalves (Travers et al., 2015). These pathogens have been associated with significant larval mortalities in hatcheries, in France and New Zealand, and appear to be widespread worldwide. There have always been mortal episodes on *Crassostrea gigas (C. gigas)* since its introduction, however, episodes of mortality have been increased on this species since 2008, mainly affecting spat/juveniles due to a pathogenic virus OsHV-1, and affecting adults since 2012 in relation to a pathogenic bacteria *Vibrio aestuarianus* (Azéma et al., 2015; Cochennec et al., 2011; Renault, 2011; Solomieu et al., 2015). The shellfish farming profession is also confronted with different families of bacteria known to be pathogenic for animals at various steps of their development (Solomieu et al., 2015). Among the pathogenic organisms found, bacteria from the *Vibrio* group, which includes 118 species, have been associated with mortalities for many bivalve species (Travers et al., 2015). Thus, the oyster mortalities since 2008 in France are mainly linked to the OsHV-1 virus at the spat stage of life and then to specie of *Vibrio* (*aestuarianus*) mainly at the adult stage. The bacterium *Vibrio aestuarianus* was detected in 60% of the batches of hollow oysters analysed in 2014 and is therefore considered to be one of the most problematic pathogens for the French and European shellfish farming profession (Azéma et al., 2015; Travers et al., 2017). To protect oysters, purification processes can be set up in farms with the objective to deliver water with a constant quality, suitable for farming, respectful of the growth and well-being of animals. For shellfish hatcheries and nurseries, the treatment commonly applied consists of a first filtration step to remove bigger suspended particles and then a UV disinfection step to remove most of the biological organisms. The filtration is essential to avoid an excessive concentration of particles brought to animals, potentially harmful for their growth and which would favour the appearance of diseases. Processes generally used for the removal of particles are sand filtration or equivalent media, followed by a fine filtration on bags or cartridges to retain particles larger than 10 µm to 1 µm (Ford et al., 2001; Helm, 2004; Wallace et al., 2008). This process does not allow long-term protection of farms, in particular for smaller pollution (pathogenic organisms) and dissolved substances. It must therefore be combined with other processes that is why a disinfection step is needed to inactivate pathogens (Torgersen and Hastein, 1995). The most used processes are first disinfection by ultraviolet (UV) radiations or chemical oxidation processes with chlorine or ozone. In the case of UV disinfection, the dose applied depends on the targeted water quality (targeted pathogens, disinfection objectives) but also on the presence of suspended matter and on the water transmittance. This presence of suspended matter is function of the first filtration treatment efficiency. Many advantages are specific to UV, such as in situ generation, therefore no storage of toxic or dangerous products, no risk of overdose and very fast action (Summerfelt, 2003). Moreover, unlike the membrane filtration, UV radiation is not able to remove bacteria and virus, it only inactivates them. Regarding pathogenic organisms, this process has been shown to be effective for the inactivation of the OsHV-1 virus and the bacteria *Vibrio aestuarianus* by Stavrakakis et al. (2017). A dose of 50 mJ cm^{-2} is thus effective using a low-pressure UV system for both pathogen inactivation. Among the drawbacks of water treatment with UV radiation, the aging of the lamps, the revival of some microorganisms and the influence of the quality of the water (transmittance) to be treated on disinfection performances, can be mentioned (Gullian et al., 2012; Martínez et al., 2013; Qualls and Johnson, 1983). The previous step of removing suspended particles is then also necessary to guarantee the effectiveness of UV treatments that could be impacted by the presence of particles (Lekang, 2013). In addition, the generation of degradation by-products during the treatment of chemical molecules by UV was highlighted (Souissi et al., 2013). Ozone, the most powerful industrial oxidant, is used in many applications for its oxidizing and disinfecting capacities. Thus, reducing the bacterial load or improving the yield and production growth are beneficial effects of the use of ozone promoted by Powell and Scolding (2018) on the cultivation of crustaceans and molluscs. In oyster farming, the effectiveness of this process has been demonstrated for specific pathogens, the herpes virus OsHV-1 and the bacterium *Vibrio aestuarianus*. A treatment of 30 min at a concentration of 1 mg L^{-1} is effective for the protection of bivalves (Stavrakakis et al., 2017). Ozone can also be coupled with UV radiation to improve treatment. Sharrer et al. (2007) showed that this combination resulted in almost complete inactivation in coliform and heterotopic bacteria in fresh water (42.5–112.7 mJ cm^{-2}). Ozone, however, has drawbacks too: its production cost and the fact that this oxidant is toxic towards humans and animals (Lekang, 2013; Moretti et al., 1999). Powell and Scolding (2018) have highlighted a negative impact on hatching rates, growth, until mortality observations on shellfish. Finally, chemical oxidation leads to the formation of oxidation residues and disinfection by-products which can impact the species living in the natural environment, or farms in the case of a closed circuit (Delacroix et al., 2013; Kornmueller, 2007; Lazarova et al., 1999; Richardson et al., 2007).

In this paper, ultrafiltration (UF) is evaluated on its capacity to produce a high-quality water for bio-securisation. In fact, this process well used in other industrial applications, especially to treat seawater, allows a purification by a steric effect without drawbacks on the quality of the water produced (Greenlee et al., 2009; Karakulski et al., 2002; Wolf et al., 2005). For the first time, this innovative process is used to bio-secure shellfish farms against specific pathogens. The first part focuses on the specific removal of two pathogens: the herpes virus OsHV-1, and the bacterium *Vibrio aestuarianus*, known for their impact on shellfish production. In a second part, the aim is to evaluate over the long term (8 months), and at an industrial scale, the performances of the process for the abatement of the bacteria load present in the sea water feeding the process (total flora and *Vibrio* bacteria). The UF pilot used in this work was studied in terms of retention and hydraulic performance, monitored on different feed water qualities.

2. Materials and methods

2.1. Semi-industrial and lab scale ultrafiltration pilot plants

The ultrafiltration membrane used for the experiments were hollow fibres (ALTEON™ I; SUEZ environnement-Aquasource, France) in polyethersulfone with 7 channels of a 0.9 mm inside diameter and 1.2 m long. Their MWCO was 0.02 µm and initial permeability 1000 L h^{-1} m^{-2} bar^{-1} with a 8 m^2 of membrane surface (Cordier et al., 2019a,b, 2018). The semi-industrial pilot was able to treat 20 m^3 d^{-1}. The tests were all performed in dead end filtration. Hydraulic performances, Lp and TMP, respectively membrane permeability and transmembrane pressure, were registered every minute by the pilot system. Turbidity was measured and recorded every minute in the feeding tank of the UF pilot using a prob VisoTurb 700 IQ (WTW). In order to maintain a flux productivity and to sustain an efficient process, a frequent chemical cleaning (CEB) was conducted during this investigation. Chemical cleaning was a two steps procedure: first a basic solution with an addition of chlorine (pH = 9.5) in order to reach a chlorine concentration between 100 and 200 ppm in membranes depending on the treatment needed, was injected in the membranes. Then, after 30 min, the module was rinsed with permeate at 2 $m^3.h^{-1}$ and filled with an acid solution (pH = 2). After 30 min the module was finally rinsed with permeate at 2 $m^3.h^{-1}$. The limit of permeability before chemical cleaning was 250 L h^{-1} m^{-2} bar^{-1}.

For confinement constraints, a lab scale pilot was used with the same hollow fiber membranes. The membrane module, with an active

area of 0.138 m^2, was able to treat a volume of 8.5 L. These characteristics allow a volumic concentration factor (VCF) of 267, similar to the semi-industrial pilot. Hydraulic performances, Lp and TMP were collected by weighting the permeate every minute and following the pressure gauge. The transmembrane pressure applied for the ultrafiltration treatment was 0.3 bar. All the results are expressed taking into account the variation of temperature.

2.2. OsHV-1

2.2.1. Oysters and contaminated sea water

Two groups of oysters were used during the experiments: oysters selected for their higher susceptibility to OsHV-1 and *V. aestuarianus* according to Azéma et al. (2017) and unselected oysters (NSI) produced according to a standardized protocol describe by Petton et al. (2015). The selected oysters were used to prepare the contaminated sea water (*C. gigas* oysters selected for their high sensitivity to the OsHV-1 virus, age < 1 year, length = 2 cm, width = 1 cm, experimental oysters produced by Ifremer). Meanwhile, NSI which means standard oyster spat Ifremer, were used to be maintained in the ultrafiltered seawater in order to verify the virus retention. The contaminated solution was prepared by injecting a viral solution into the adductor muscle of the oyster. To perform the injection into the muscle, the oysters must be anesthetized. For this, the oysters were placed dry for 4 h before falling asleep. These stressful conditions encourage them to open more quickly and filter as soon as they are returned to water. The oysters were anesthetized (T = 20 °C) in hexahydrate $MgCl_2$ (50 g L^{-1}) for 2 h (Suquet et al., 2009). Then, they were injected with 10 µL of viral solution in the adductor muscle to allow spreading into the circulatory system. The OsHV-1 inoculum was prepared and produced according to Schikorski et al. (2011), and injections were performed 24 h before the start of the experiment. Then, oysters were placed in disinfected seawater (T = 20 °C) during 24 h. The conditions of the 3 tests, number of oysters and seawater volume used to prepare contaminated sea water, are 85/20, 200/25 and 200/25 oysters L^{-1} respectively for test 1, 2 and 3 (Morga et al., 2020). After 24 h, the sick oysters released the virus in the seawater: a contaminated seawater is obtained to be treated by ultrafiltration. The transmembrane pressure applied for the ultrafiltration treatment was 0.3 bar. Permeate weight was collected each minute in order to determinate flowrate and membrane permeability.

2.2.2. Evaluation of virus retention

Virus retention was evaluated by two methods as describe in Fig. 1: bathing and injection. For bathing, 10 × 2 spat oysters were placed into 3 × 2 pools filled with 2 L of permeate, disinfected seawater by ultrafiltration (negative control) and contaminated seawater (positive control). For the injection method, 10 × 2 spat oysters were injected with 100 µL of permeate, disinfected seawater (negative control) and contaminated seawater (positive control) containing virus. Injected oysters were then placed in 6 pools of disinfected seawater at 20 °C. Negative controls enable to verify that oysters could face experiment conditions. Meanwhile, positive controls, which were the oysters in contact with contaminated seawater, verified the pathogenicity of the OsHV-1. Each test was duplicated in order to have 2 negative controls, 2 positive controls and 2 permeates in bathing and injection evaluation. For each condition, cumulative mortality was monitored every 24 h for 7 days.

2.2.3. OsHV-1 analysis larvae mortality data analyses

For the larval experiment, mortality was analyzed by a binomial logistic regression throughout the GLIMMIX procedure (SAS® 9.4 software,Cary, NC, USA) at day 3 and day 7 post-infection according to the following model:

$$\text{logit}(\pi ij) = \mu + \text{condition}_i + \text{replicate}_{(ji)}$$

where πij is the probability of the mortality at day 3 or day 7 for oyster of the "i" condition (negative control, positive control, and permeate) (fixed factor) for the "j" replicates (6 wells)(random factor) and µ the intercept.

2.2.4. Polymerase chain reaction (QPCR) for virus analyses

Different seawater samples were collected during the filtration step: first and last millilitre of permeate, retentate and the inlet contaminated seawater (Table 1). A dead-end filtration is operated, so the permeate is divided into two identical volumes P1 and P2 to take into account the increased concentration (i.e. the membrane retention) in the hollow fibre lumen. Dead oysters were also collected to be analysed. Each sample was analysed by QPCR at Ifremer Laboratory of Genetics and Pathology of Marine Molluscs (LGPMM, La Tremblade, France). The QPCR limit detection was estimated at 10 copies of virus DNA per microliters of samples (Pepin et al., 2008).

2.3. Vibrio aestuarianus

2.3.1. Contaminated sea water

Vibrio aestuarianus GFP strain was provided in Petri dish by Ifremer LGPMM (La Tremblade, France). The *Vibrio aestuarianus* GFP is a modified strain that gets kanamycine resistant and fluorescent characteristics. The bacteria solution was prepared 24 h before the ultrafiltration tests at a 10^9 CFU L^{-1} concentration following the protocol developed by (Azéma et al., 2016). After 24 h, the bacteria solution was then seeded in a 10 L autoclaved seawater to produce a contaminated seawater at 10^6 CFU L^{-1}. 8.5 L of contaminated seawater was ultrafiltrated by the lab scale pilot. Permeate weight was collected each minute in order to determinate flowrate and membrane permeability. Performances of bacteria removal by ultrafiltration was determined by cytometry and specific bacteria analysis.

2.3.2. Cytometry and bacteria analysis

Due to its fluorescent characteristics *Vibrio aestuarianus* GFP is detectable by cytometry analysis. 800 µL of contaminated seawater, permeate, concentrate and autoclaved seawater were analysed in triplicate by flow cytometry (CyFlow-Partec-Sysmex) after 4 min with a threshold fixed on FL1 fluorescence (Aboubaker et al., 2013; Travers et al., 2017). Beside flow cytometry, two types of bacteria analysis were carried out: bacterial analysis on Petri dishes and bacteria analysis with a vacuum filtration on 0.2 µm cellulose filter before growth on Petri dishes. *Vibrio aestuarianus* analysis were carried out in salted luria broth agar medium with kanamycine which allows only *Vibrio aestuarianus* GFP growth. For the bacteria analysis, 50 µL of sample was deposed on the Petri dishes and then incubated at 20 °C during 48 h. A second bacteria analysis method was performed by filtering 500 mL of sample on the cellulose filter, then the filter was deposed on Petri dish and incubated at 20 °C during 48 h.

2.3.3. Microbiologic analyses

In shellfish culture, some species of *Vibrio* being pathogenic for oysters, their presence in the water supplying spat and larvae is also monitored. Total bacterial load and *Vibrio* were analysed in these waters by microbiologic analyses realised twice a week in the pilot feed and in permeate (both at the beginning and the end of the filtration cycle). The aim of these analyses was to verify the absence of *Vibrio* bacteria but also to estimate the total bacterial retention by UF. *Vibrio* analysis were carried out in TCBS agar medium and total bacterial load on marine agar medium (Aboubaker et al., 2013). 50 µL of each water sample was deposed on the Petri dishes and then incubated at 20 °C during 48 h. Retentions at the beginning and end of the filtration cycle are calculated from the equations below:

$$Initial\ \ retention = \frac{C_{feed} - C_{firstmL}}{C_{feed}} Final\ \ retention = \frac{C_{retentate} - C_{lastmL}}{C_{retentate}}$$

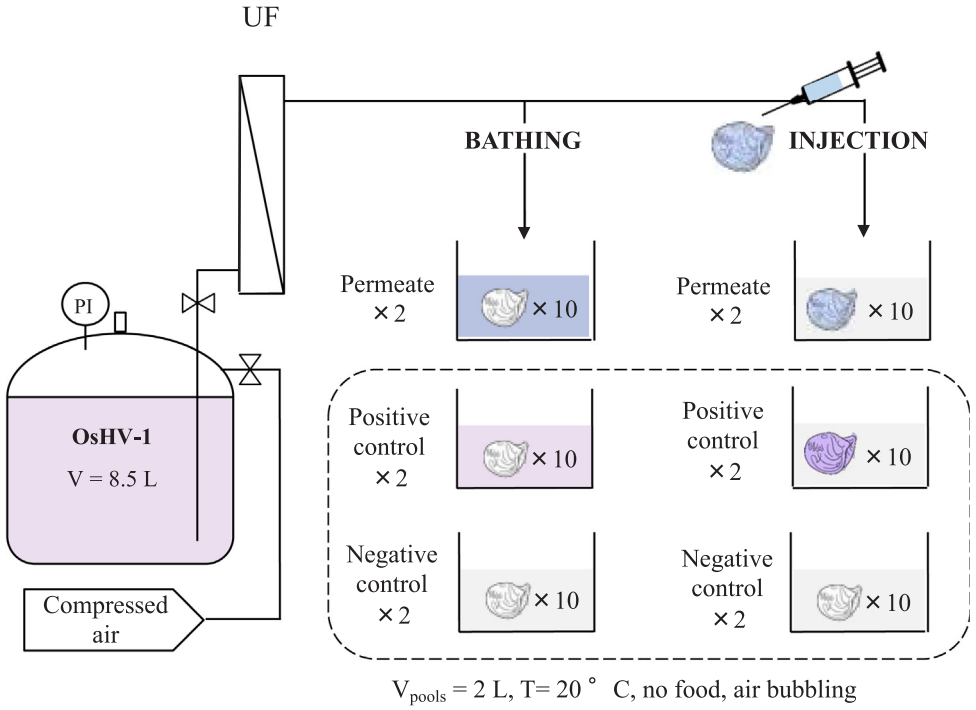


Fig. 1. Treatment of OsHV-1 – In vivo test of bathing and injection – Pink: contaminated solution with OsHV-1; blue: treated water (=permeate) and grey: disinfected seawater [TMP = 0.3 bar]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Treatment of OsHV-1 – Synthesis of analyses realized on permeates obtained.

Permeate	First mL	P1	P2	$P_{average}$ = *P1 + P2*	Last mL
Volume recovered (L)	0.05	4.2	4.2	*= 4.2 + 4.2*	0.05
In vitro analyses	×	×	×	×	×
In vivo analyses				×	
Total bacteria and *Vibrio* measurement	×			×	×

with: C_{feed}: virus concentration in the feed, $C_{retentate}$: virus concentration in the retentate, $C_{first\ mL}$: viral DNA concentration in the first mL of permeate and $C_{last\ mL}$: viral DNA concentration in the last mL.

3. Results and discussion

In this paper, ultrafiltration is evaluated on its capacity to biosecure shellfish farms and to make oyster farming more sustainable. The aim is the treatment of feed sea water for the protection of larvae and spat against pathogenic organisms. The retention and hydrodynamic performances, as well as the resistance of the process to the fouling generated on the membrane in the case of these particular pollutions, are monitored.

3.1. Specific tests for C. gigas pathogen retention: laboratory scale

The tests were carried out using a laboratory scale ultrafiltration device to meet the confinement constraints linked to working with sensitive bacteria and viruses on a production site. This device is equipped with the same membranes as the industrial-scale pilot (used for the long-time evaluation) and operates at constant pressure. The volumic concentration factor (VCF) of the industrial pilot is the same on the laboratory pilot by choosing a filtering surface of 0.13 m^2 and a volume of solution to be treated of 8.5 L, for J60 t60 conditions (i.e. Permeate flux J = 60 L h^{-1} m^{-2} and filtration time t = 60 min). In addition, the ultrafiltration tests are carried out at a transmembrane pressure (TMP) of 0.3 bar which corresponds to the maximum TMP reached during the filtration of seawater on the industrial-size pilot under similar J60 t60 conditions. Whether for OsHV-1 and *V. aestuarianus*, the general protocol followed was identical and took place in three stages: (i) preparation of a contaminated sea water, (ii) ultrafiltration of this solution with flow monitoring and (iii) verification of performance by *in vivo* and / or *in vitro* tests with positive and negative controls.

3.1.1. Virus OsHV-1 treatment: virus retention

Three experiments were carried out on the virus. The characteristics of the experiments, initial permeability measured with demineralized water, filtration time, turbidity, DNA concentration of OsHV-1 and total

Table 2
Characteristics of OsHV-1 experiments.

	Filtration		**Contaminated seawater**			
Test	Lp_0 *(L h^{-1} m^{-2} bar^{-1})*	Filtration time (min)	Turbidity *(NTU)*	*Number of oysters/Seawater Volume L*	OsHV-1 Concentration *(DNA copies μL^{-1})*	Total bacteria *(CFU mL^{-1})*
1	780	210	5.5	85 / 20	$8.7.10^2$	$4.39.10^6$
2	607	167	8.2	200 / 25	$9.1.10^1$	$8.79.10^6$
3	770	47	4.6	200 / 25	$4.2.10^2$	$1.19.10^6$

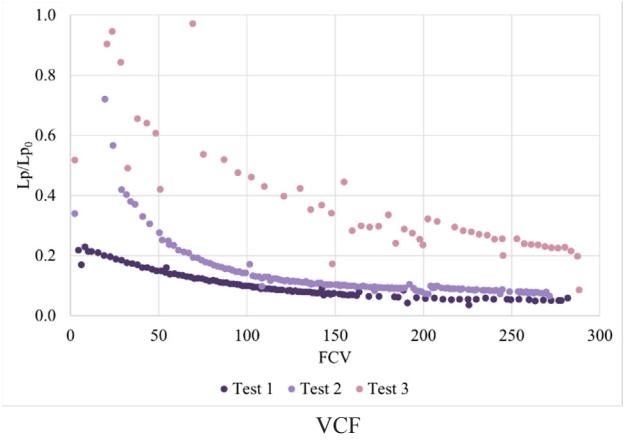


Fig. 2. Evolution of permeability vs. VCF – Retention of OsHV-1.

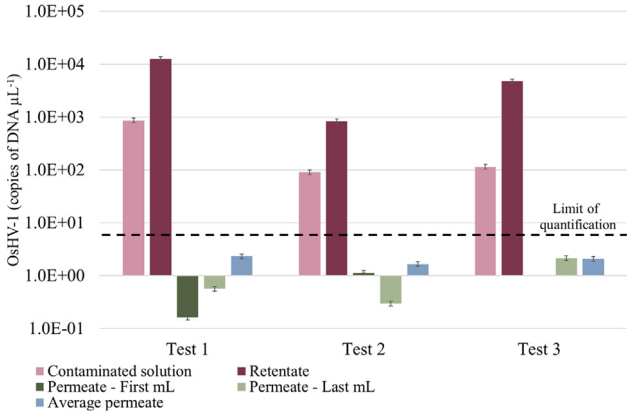


Fig. 3. DNA of OsHV-1 concentrations in samples after ultrafiltration of contaminated solution - qPCR analysis.

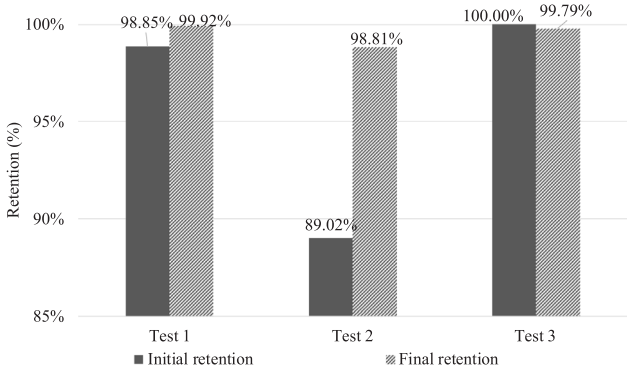


Fig. 4. OsHV-1 retentions at the beginning and end of filtration cycle for the 3 tests.

flora of the contaminated sea water are summarized in Table 2.

In the test 1, the virus concentration was judged too low. In order to work with a contaminated solution with a higher OsHV-1 DNA concentration, the number of oysters used for the preparation of the solutions for tests 2 and 3 was increased from 85 to 200 oysters (Table 2). However, contrary to what was expected, the increase of the viral concentration in the solutions was not significant. In the case of test 2, it was even lower than test 1 with less than 10^2 copies of DNA μL^{-1} in the contaminated solution. This result could be explained by a reduced susceptibility of the oysters in tests 2 and 3, because even if they were from the same batch, they were older (3 months) and larger than in test 1, as demonstrated by Dégremont (2013). 24 h post-injection, mortalities were observed among the animals used for the preparation of the contaminated seawater by OsHV-1 dedicated to the tests 1 and 2. Those dead oysters generated a viscous deposit in the solution obtained testifying to a more turbid and more loaded bacteria solutions than for test 3, especially for the test 2 as shown by the variation in filtration time. The variation of the permeability as a function of the VCF is presented in Fig. 2 for the 3 tests carried out. The differences of the number of dead oysters to prepare solution and the difference of turbidity, virus and bacterial concentration, led to different fouling generated by the filtration of the 3 solutions obtained. Moreover, the effect of these initial parameters is accentuated by the concentration factor in the membrane. Membrane fouling seems to be linked more to parameters such as suspended matter (turbidity) and the presence of bacteria than to the concentration of OsHV-1 in the seawater, it is in agreement with the pore size. In all 3 cases, the permeability loss remains reasonable with final permeate fluxes around 100 L h^{-1} m^{-2} bar^{-1} from high concentrated solutions.

The retention performance of the virus is controlled by measurements of the concentration of OsHV-1 viral DNA by QPCR analyses in the contaminated water, in the retentate and permeate. Several samples of permeate are analyzed: first mL, last mL, first 4.2 L, last 4.2 L and average permeate in order to take into account the variation of the concentration inside the membrane during the experiment. The concentrations measured on these different samples and for the 3 tests are presented Fig. 3. Whatever the viral DNA concentration in the initial solution, if virus is detected in the permeates, the measured value is always less than the QPCR quantification limit (10 copies of viral DNA μL^{-1}). The analyses revealed very low amount of virus, not quantifiable, in the permeate samples.

Retentions at the beginning and end of the filtration cycle are calculated as previously explained, for the different experiments and presented in Fig. 4. To be noted that, when the concentration measured is inferior to the quantification limit, the value used for retention calculation is this threshold, 10 DNA copies μL^{-1}.

Retention was always greater than 89% and retention of 100% was even achieved for test 3 since the virus was not detected in the first mL of permeate. The minimum value of 89% may appear low but it is important to specify that the retention values are calculated using for the limit of quantification of 10 copies of viral DNA μL^{-1}, leading to an underestimation. This underestimation is even more pronounced when the concentration in the feed is low: test 2 showed the lower retention because of a concentration in the contaminated solution of 100 copies of DNA μL^{-1}. If the virus concentrations were below the QPCR quantification limit in the 3 tests, the retention was not total since the presence of DNA from the virus is almost always detected in the permeate. Two questions arise following these results: (i) is the amount of virus still present in the permeate sufficient to induce oyster mortality? and (ii) does the DNA detected, once it has passed through the pores of the membrane (20 nm), belong to a virus able to infect oysters? Indeed, the QPCR carried out makes it possible to detect traces of viral DNA but does not specify whether this DNA is from an integrated viral particle. To answer these questions and to conclude on the performances of the UF process for the removal of this pathogenic agent, *in vivo* tests were performed. Two tests were set up to assess the infectiosity of the treated solution: bathing of oyster spat (n = 10, V = 2 L of water, in duplicate) and larvae (n = 150 larvae per 6 mL well, 6 wells per condition), and injections in spat (10 individuals injected in duplicate). To validate the results obtained, the same manipulations were each time carried out on positive and negative controls, under the same conditions. The mortalities obtained after 7 days of contact, for the immersion (or bath) and oyster injection tests on the 3 tests, are presented in Fig. 5. The dead oysters were frozen, and their flesh was then analyzed by QPCR. In all the samples analyzed, a concentration greater than or equal to 10^3 copies of viral DNA μL^{-1} was measured, which confirms that all the oysters died due to a viral infection. Regarding the

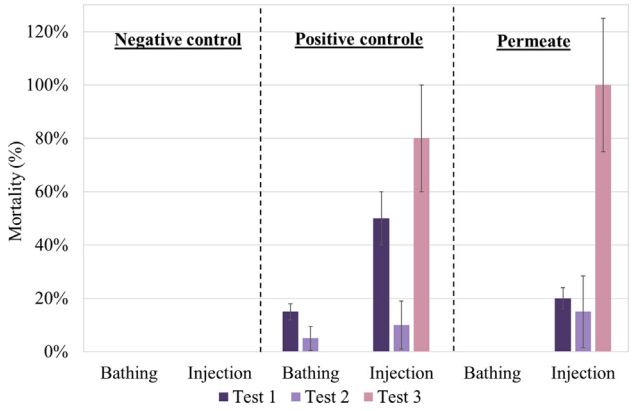
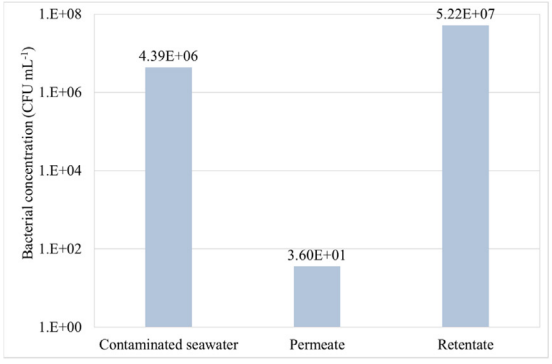


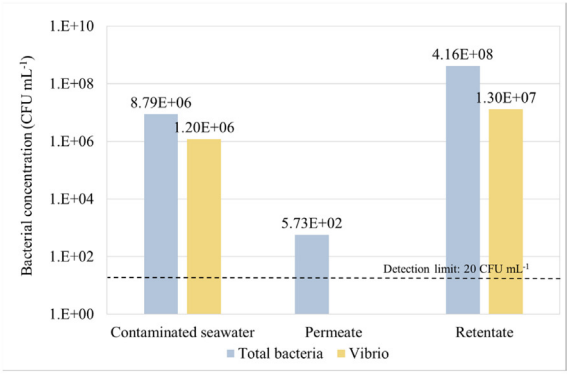
Fig. 5. In vivo tests results – Oyster spat mortalities after 7 days in contact by bathing and injection with negative, positive controls and permeate.

controls, as expected, (a) no mortality in the negative controls, either by injection or by bathing was observed. The oysters were not affected by an injection of 100 µL (Schikorski et al., 2011) and withstood the conditions of the experiment at 20 °C in ultrafiltered seawater without food during the 7 days of follow-up; (b) mortalities were observed for positive injection and bathing controls. As expected, the mortalities by injection are higher than those in bathing for each test. These results are consistent since, during the injection, the virus enters the oyster's body directly without going through the filtration and incorporation stage, as in the case where the virus is found in the bathing water (Schikorski et al., 2011). In experiment 2, low mortality was observed in the positive injection controls which could be explained by their age, close to 18 months, making the oysters more resistant to the virus. For experiment 3, the tests were therefore carried out on the selected oysters for their higher susceptibility to the virus, and the average mortality was 80% in the positive controls by injection. However, on this same experiment, no mortality was observed in bathing oysters, so no conclusion can be drawn.

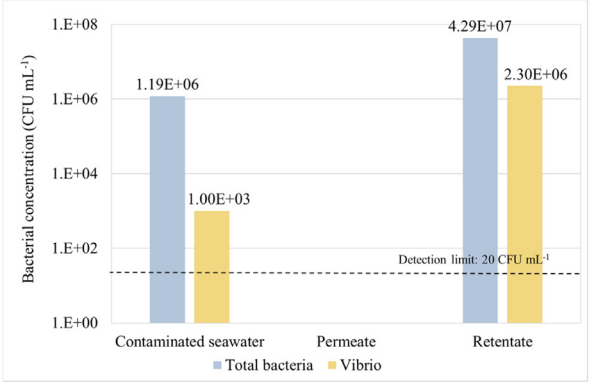
Concerning the permeate, 100% mortality was obtained on the injected oysters but none for those in bathing. These results are correlated with QPCR analyzes: the viral DNA is detected in low concentration, sufficient to kill oysters when the permeate is injected directly inside the body, but insufficient to kill oysters in bathing over 7 days. Ultrafiltration provides protection of oysters at the spat stage when the permeate is in bathing contact with the oysters, closer to real production conditions. In order to validate the effectiveness of ultrafiltration for the protection of shellfish farms, during experiment 3, the immersion or bath tests were carried out with oyster larvae 8 days old, life stage more sensitive than spat previously used (Dégremont et al., 2016b). As for spat, the larvae were brought into contact with the permeate produced and the cumulative mortalities at days 3 and 7 were compared with those obtained for bathing larvae in contaminated water and ultrafiltered seawater. At day 3, mortality was not significantly different among conditions ($P = 0.48$) with 23%, 25%, and 36% for the negative control, the permeate, and the positive control, respectively. P value lesser than 0.05 indicates strong evidence against the null hypothesis which was the absence of effect on larval mortality among conditions (ultrafiltation vs negative vs positive controls). At day 7, larvae of the positive control showed a significant higher mortality (100%) than those of the negative control (44%) and the permeate (56%) ($P < 0.01$). Although the mortality of the larvae in contact with the permeate was 12% higher than in the negative control, this



Test 1



Test 2



Test 3

Fig. 6. Total bateria and Vibrio bacteria concentrations.

difference was not significant (P = 0.23). Thus, it appears that the protection of the larvae by the ultrafiltration process was effective against OsHV-1. Mortality observed for the negative controls, as well as the permeate condition could be both related to physiological-genetic characteristics and the conditions of the 6-well plates experiment that lasted 7 days without feeding. Thus, it is common to observe some mortality for D larvae, as some of them will be unable to survive (abnormal shape, deleterious/letal genes..), as well as those near the metamorphosis (Dégremont et al., 2016b).

3.1.2. Virus OsHV-1 treatment: bacteria retention

Bacterial removal was measured on total flora and *Vibrio* bacteria, present in contaminated seawater by OsHV-1. The results put in light a reduction of at least 4 logs of the total flora in each experiment by ultrafiltration (Fig. 6). The *Vibrio* concentrations from Petri dishes in experiment 1 could not be determined because of the too large number of colonies formed on agar media (> 300 CFU). Dilutions were made for the following experiments to facilitate the enumeration. The retention of bacteria of the *Vibrio* genus obtained for experiments 2 and 3 was high since no colony was detected on the petri dishes corresponding to the permeates analyzed and this for different initial bacterial concentrations.

The initial and final retentions of total flora and bacteria (*Vibrio* genus) were determined for each experiment (Table 3), showing the high performances of the membrane to reduce the bacteria load, with removal from 4.19 to more than 6.33 log and from 2.99 to more than 7.11 log for total bacteria and *Vibrio* bacteria respectively.

As conclusion, the tests carried out with OsHV-1 virus highlight a retention of this compound by ultrafiltration membranes with values higher than 4 log which is the retention given by the membrane manufacturer for bacteriophage MS2, virus with a lower size (25 nm) than OsHV-1. However, this retention is not total because traces, not quantifiable, are detected by QPCR in the permeate. This result is confirmed by *in vivo* tests: when the permeate obtained was injected into oysters, mortalities comparable to those obtained for positive controls were noted at the spat stage. The presence of viruses in the permeate despite the pore size of the membranes could be justified by an heterogeneity of the pores of this sample of membranes or by the implementation (or manufacturing) of the micro-modules used for these tests. If the injection test validates the presence of the virus in the permeate and its retained infectiosity, it does not reflect actual hatchery conditions. The balneation tests then carried out, in real conditions, underline a protection of the oysters by the process since no bathing mortality was observed on the 3 tests after ultrafiltration. This result was confirmed with bathing larvae, the most susceptible stage of the oyster for OsHV-1. Moreover, it is important to note, that we have used harsh conditions and in reality, the feed water viral concentrations is not at this level used for these experiments. In addition, the bacteriological analyzes carried out underline the performance of the ultrafiltration for the reduction of the total flora (> 4 log) and particularly bacteria of the *Vibrio* type (> 7 log with no detection in the permeate), which represents preliminary results encouraging for specific tests on *Vibrio aestuarianus* but also for long-term follow-up tests for retention of total *Vibrio*.

Table 3
Initial and final removal of bacteria obtained during OsHV-1 experiments.

Test	Total bacteria		*Vibrio*	
	Initial removal (Log)	Final removal (Log)	Initial removal (Log)	Final removal (Log)
1	5.08	6.16	–	–
2	4.19	5.86	> 6.08	> 7.11
3	> 4.77	> 6.33	> 2.99	> 6.36

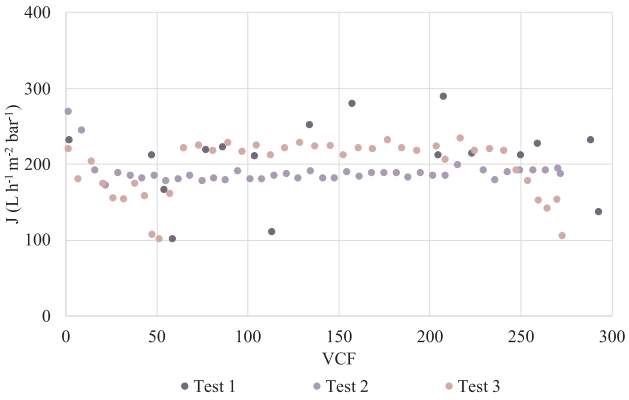


Fig. 7. Evolution of flux vs. VCF for Vibrio aestuarianus retention tests.

3.1.3. Retention of the Vibrio aestuarianus bacteria

The tests were carried out on a strain of *Vibrio aestuarianus* GFP, modified to be detectable by flow cytometry without the addition of a marker and resistant to an antibiotic, kanamycin (which makes it possible to obtain cultures specific to this bacterium). Three filtration tests, were carried out with a theorical bacterial concentration in the initial solution of 10^5-10^5 and 10^6 CFU mL^{-1}. The variation of flux as the function of the VCF is presented Fig. 7. A more moderate fouling than in the case of the treatment of viral solutions was observed. This result is explained by the quality of the matrix containing the microorganisms: in the case of the virus the solution was prepared from oysters which also brought organic matter (bacteria, faeces, rotting flesh) to the solution in addition to the virus, which is not the case for *Vibrio aestuarianus* bacteria solutions.

(i) Monitoring of retention performance by seeding on a Petri dish

Two types of sample seeding were carried out: with filtration on a cellulose filter (0.22 µm) and without filtration. Only the results of tests 2 and 3 are presented in Fig. 8a, because an external contamination appeared for the test 1. The formation of a circle concentrated in bacteria in the center of the filter was observed for the contaminated seawater which was not the case for the medium permeate and the negative control. The appearance of mold on the filters in test 3 both in permeate and negative control showed that the filtration was carried out under non-sterile conditions. However, the specific and selective medium used, LBS + kanamycin, limit the appearance of bacteria originating from handling under non-sterile conditions. The advantage of the filtration method is the concentration of bacteria in a sample containing a very small quantity leading to a detection limit of 2 CFU L^{-1} versus 20 CFU mL^{-1} in the case of direct seeding without filtration. Thus, the 500 mL of first and last 4.2 L of permeate and average permeate (which is the mix of the two permeates P1 and P2 obtained), were filtered in triplicate and no bacteria was observed on the 9 filters. The removal rate cannot be calculated with this method because the contaminated seawater is too concentrated to allow a count of the colonies formed. However, the absence of a colony in the permeate indicates high retention, the limit of detection (2 CFU L^{-1}) is therefore given for manipulations 2 and 3 as the maximum value. With the direct seeding method, no colony was observed in the permeates (first and last 4.2 L of permeates (P2) and average permeate (P1 + P2)). The initial and final retentions calculated for the 3 experiments are reported Fig. 8b. The difference between the initial and final removal is consistent with the VCF, reflecting an increase in the concentration upstream due to filtration and a zero concentration downstream from the start to the end of the experience. Ultrafiltration allowed total retention of the *Vibrio aestuarianus* bacteria with minimum abatements between 5 and 7 log.

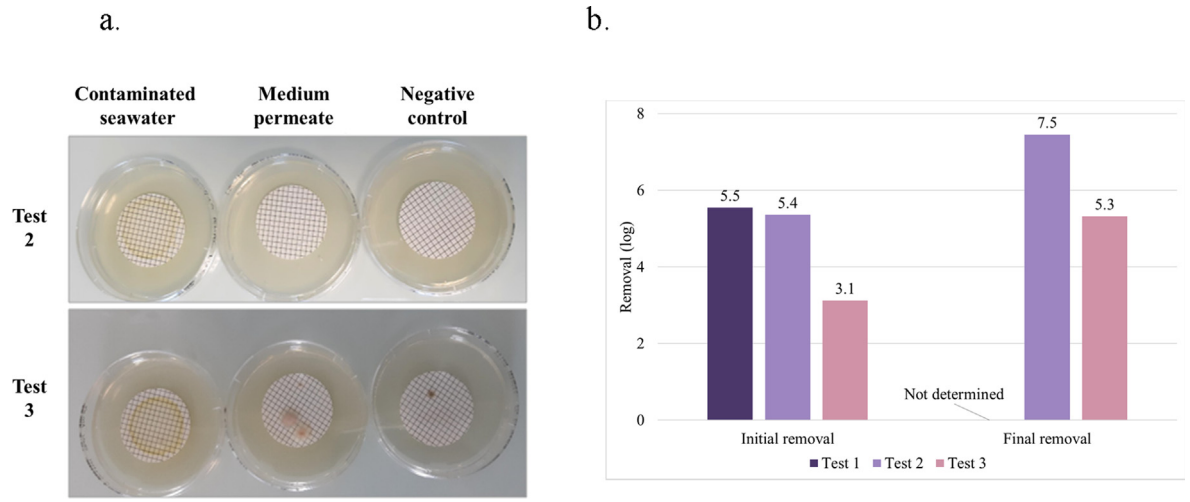


Fig. 8. Treatment of Vibrio aestuarianus - a. Pictures of filters 48 h after incubation and b. Removal of Vibrio aestuarianus calculated from direct seeding results – Seeding.

(ii) Monitoring of retention performance by flow cytometry

In order to validate the retention performance, the samples (contaminated solution, medium permeate, retentate, autoclaved seawater) were also analyzed by flow cytometry. The results are reported in Fig. 9. For all the permeates analyzed for the 3 tests, the concentration measured was below the detection limit (10^2 CFU mL^{-1}). In the case of tests 1 and 2, a second population was observed outside of zone R2 but this new population comes from ultrafiltered and autoclaved seawater used to prepare the bacterial solutions in agreement with the cytograms of the negative control.

The initial and final retentions and removal rate of the 3 experiments are given in Fig. 10. These values were calculated with the flow cytometry detection limit (i.e. 100 CFU mL^{-1}) and therefore underestimated.

The results obtained by flow cytometry are in agreement with those obtained by the method of inoculation on a Petri dish. In conclusion for *V. aestuarianus* bacteria, the three specific experiments carried out with this bacterium highlight the retention of this microorganism, allowing concentration levels in the permeate to be reached below the detection thresholds of the different methods used. Indeed, the detection threshold of the analysis by flow cytometry was 100 CFU mL^{-1}, which decreased to 20 CFU mL^{-1} in Petri dish by direct inoculation of 50 µL, versus 2 CFU L^{-1} only for the method of depositing a 0.2 µm filter

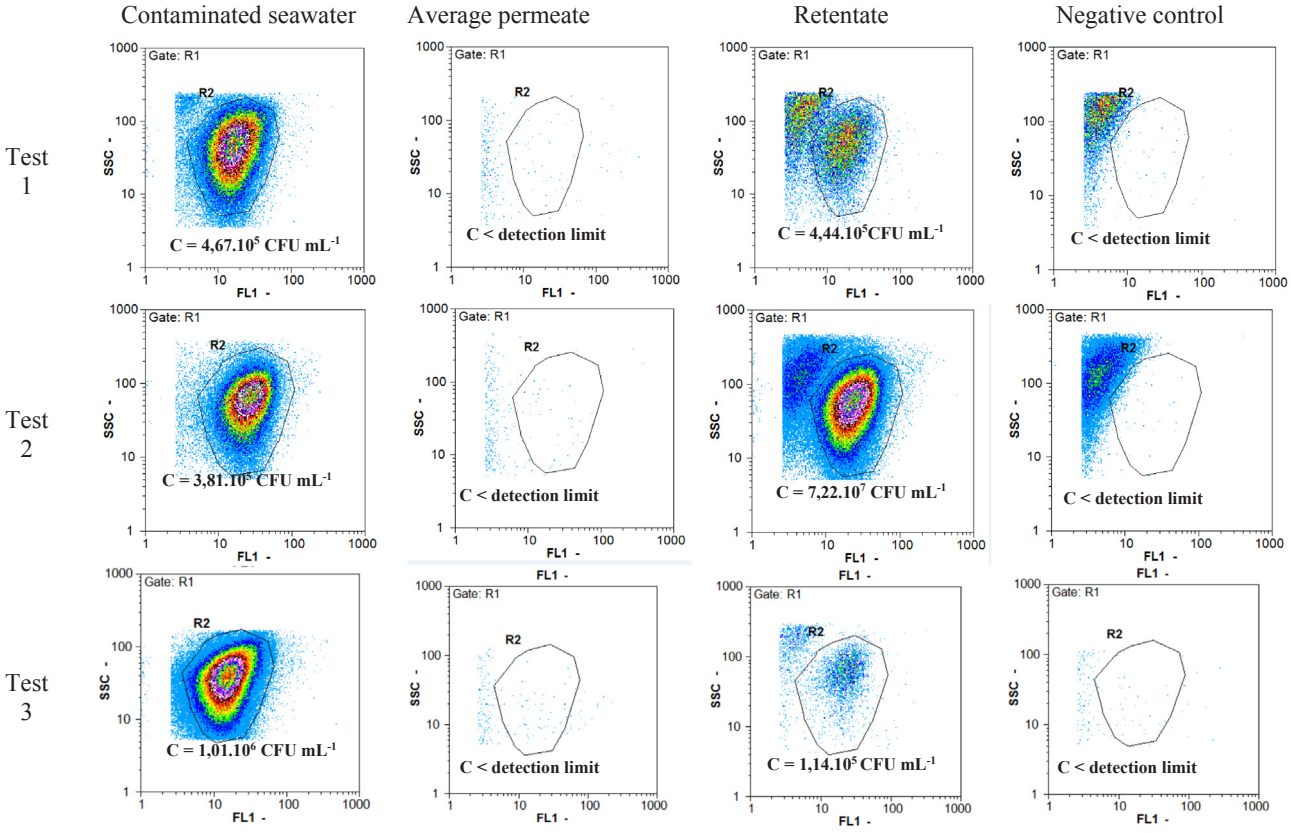


Fig. 9. Treatment of Vibrio aestuarianus – Flow cytometry analysis.

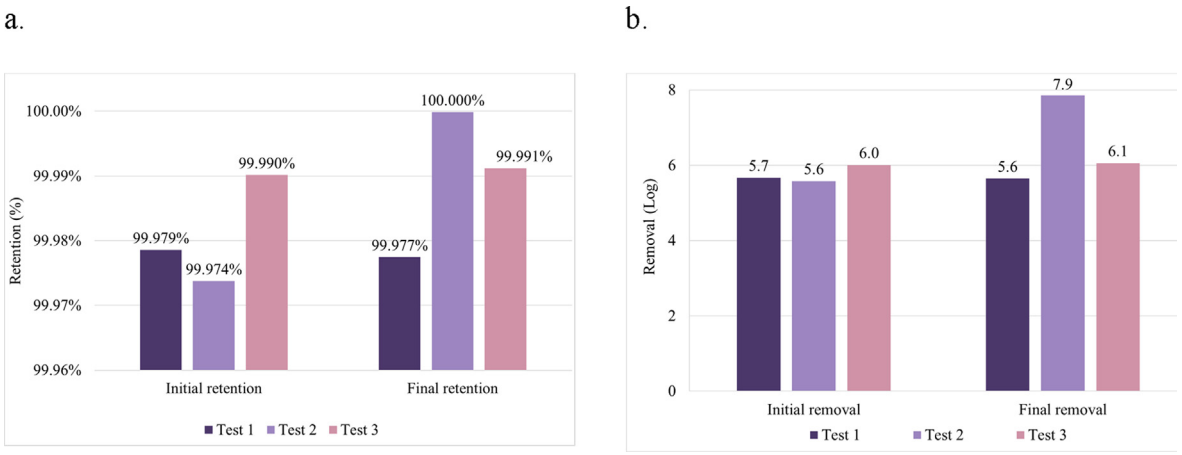


Fig. 10. Treatment of Vibrio aestuarianus - a. Initial and final retention et b. Initial and final removal – Flow cytometry analysis.

which had filtering several liters of permeate. If *in vivo* tests could not be carried out to validate the efficacy of the treatment, a study by Travers et al. (2017) estimated the minimum infective dose required to reliably induce infection in adult oysters after a 24 h immersion period in contaminated seawater, was estimated at 4×10^4 CFU mL^{-1}., i.e. 400 times more than the highest detection limit in this study. With these results, it is possible to conclude on the effectiveness of the process to protect oyster farms from this pathogenic agent.

3.2. Disinfection of water entering the hatchery / nursery on a semi-industrial scale

The objective is to validate the use of ultrafiltration for the disinfection of water entering the hatchery / nursery on a semi-industrial scale and for real conditions in feed water. The hydraulic performance of the process is therefore monitored over several months and the retention of the total flora and *Vibrio* bacteria naturally present in the water supplying the pilot are evaluated. During the period of these tests, the pilot was confronted with different qualities of seawater and even in the particular case of an algal bloom upstream of the experimental installations.

3.2.1. Bacteria removal rate

The retention performance of total bacteria is presented in Fig. 11. The measurements were carried out at the beginning (initial permeate) and at the end of the filtration cycle (final permeate). Samples from June 12 and June 17 (2019) show less than 98% of the retention, which is explained by the formation of a biofilm in the permeate lines. After cleaning the pilot with chlorine at 4 ppm on June 24, the retention of the total flora is greater than 98%, thus reflecting the effectiveness of the membrane. The concentration of total flora in the permeates is below the detection threshold of the analyze (20 CFU mL^{-1}), regardless of the bacterial concentration of the feed. This detection limit, coupled with concentrations in the feed of around 10^4 CFU mL^{-1}, explains the low removal rate of only 99%.

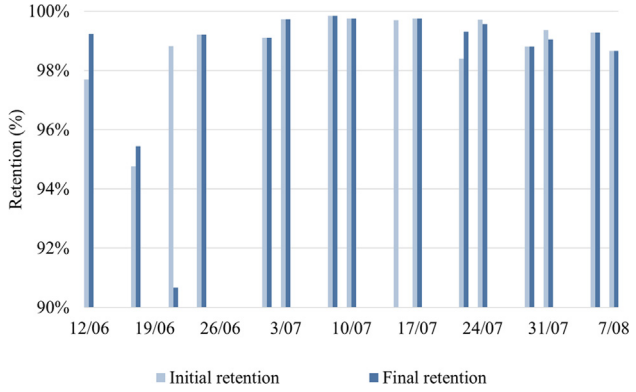


Fig. 11. Total flora retention – Semi-industrial scale.

During the two months of follow-up, no *Vibrio* bacteria was detected on the permeate samples (initial and final). The concentration fixed to calculate the retention Fig. 12, 20 CFU mL^{-1} which is the detection limit justifies the low retention obtained. The initial concentrations of bacteria of the genus *Vibrio* remained low during the follow-up (200 CFU mL^{-1} on average). Some peaks were observed in the feed water on 21 June with 5220 CFU mL^{-1} and on 24 July with 520 CFU mL^{-1}. Despite these fluctuations in bacterial concentrations, the membrane process has enabled retention since the bacteria are not quantifiable in the permeates. Ultrafiltration ensures the retention of *Vibrio* bacteria regardless of the initial concentration, the permeability of the membrane and the physico-chemical parameters of the seawater. Similar results leading to the same conclusion were obtained in winter Fig. 12b.

3.2.2. Effect on the physico-chemical parameters

The physico-chemical parameters of the feed and initial and final permeates were followed for 2 months. As expected, the pH, salinity, temperature and dissolved oxygen are not affected by the ultrafiltration treatment since the values are constant between the feed and permeates (Cordier et al., 2019b). Conversely, in Fig. 13, a reduction in the turbidity is obtained with the ultrafiltration process since it is mainly below 1 NTU in the permeates whatever the turbidity of the feed (between 1 and 9 NTU).

3.2.3. Hydrodynamic performances

The evolution of permeability and turbidity versus time is presented in Fig. 14. The peaks of turbidity recorded every 6 h correspond to the backwashing of the sand filter placed upstream of the ultrafiltration pilot plant (the peak of turbidity between 07/16 and 07/23 is not representative of the water quality- probe position error). Whatever the water quality with a turbidity from 3 to 7 NTU, the slopes relating to the membrane fouling are more significant for high turbidity. The time between two chemical cleanings is therefore shorter: before the increase of turbidity from 3 to 4 to 6–7 NTU, the time to pass from a permeability of 600 at 300 L h^{-1} m^{-2} bar^{-1} is on average 47.4 h against 24.1 h after the change in water quality.

If an increase in turbidity has an impact on the fouling, this has no effect on the recovery of initial permeability obtained after a chemical cleaning (CEB). Indeed the average initial permeability is 750 ± 50 L h^{-1} m^{-2} bar^{-1} and this remained constant over the duration of the study and on the different qualities of water. Moreover, during this

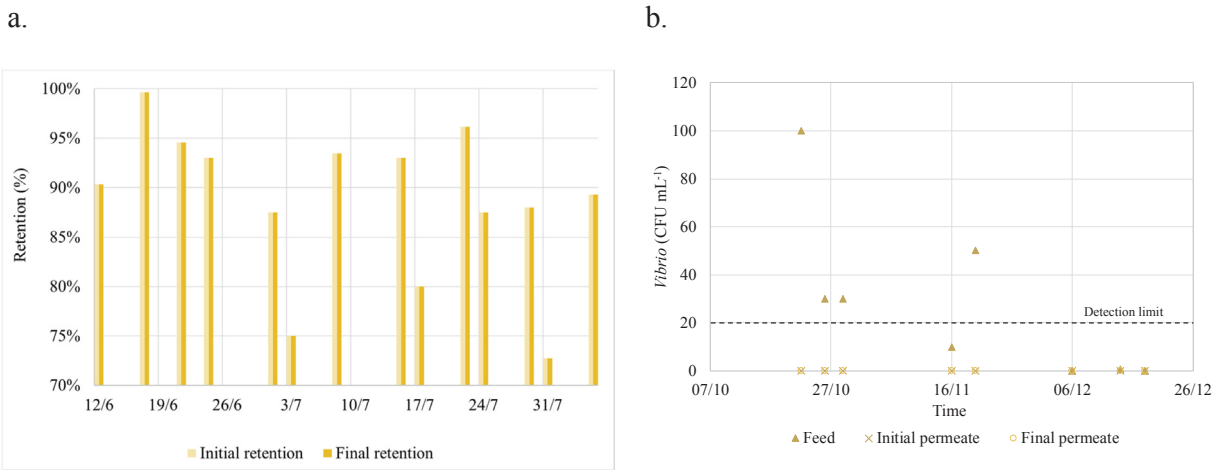


Fig. 12. a. Evolution of Vibrio retention (June - July 2019) and b. Evolution of Vibrio concentrations (October – December 2018).

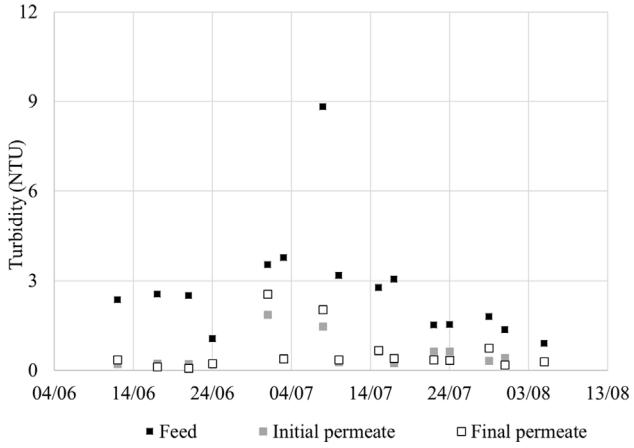


Fig. 13. Evolution of turbidity vs. time (June - August 2019).

period an algal bloom was observed. Water treatment processes commonly used in the laboratory were not able to face the pollution and the consequence was a contamination of the oyster lines maintained in the experimental hatchery/nursery. On the contrary, ultrafiltration process showed its efficiency both in terms of hydraulic performances, with a stability of the permeability and retention of pollution (bacteria, turbidity, oyster predators) with the production of a clear water free of parasites (Cordier, 2019).

4. Conclusions

Performances of the ultrafiltration process were evaluated for biosecuring farms. The first part was devoted to the validation of the process for the elimination of pathogenic organisms from the seawater flow used in oyster hatchery/nursery. Laboratory-scale trials were used to meet site security requirements, and focused on the two main pathogens affecting the French oyster production: the OsHV-1 virus and the bacteria *Vibrio aestuarianus*. It emerges from this work, that the retention of OsHV-1 was always greater than 98%, but did not reach 100%. Meanwhile, the quantity of viral DNA found for the permeate condition did not generate mortality using bathing neither on spat nor

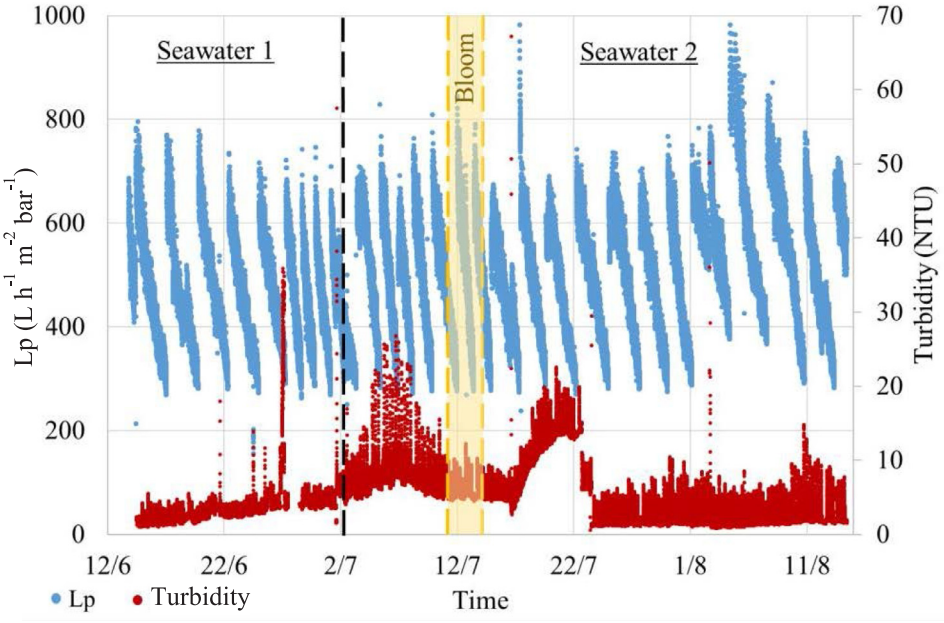


Fig. 14. Evolution of permeability of turbidity vs. time (June – August 2019) – Semi-industrial scale pilot.

larvae, the latter being very sensitive to this pathogenic agent. For the bacterium *Vibrio aestuarianus*, the retention tests of the bacterium reduced by 5–7 on a log scale for the permeate condition reaching the limits of detection regardless of the analytical techniques (seeding on the medium of specific culture of permeate samples, permeate filtration and culture of the filter on specific medium and flow cytometry). The permeate filtration tests and re-culture of the filter highlight the recovered concentrations resistant to 2 CFU L^{-1}. If these *in vitro* analyzes do not allow to conclude on the absence of virulence of the permeate on oysters, the tests carried out by Travers et al. (2017) found a concentration 400 times higher to impact the adult oysters by bathing. Thus, protection of oyster farms at the larval, spat and adult stages were obtained by the ultrafiltration process against two pathogens known worldwide for their impact on the *Crassostrea gigas* oyster production. Tests on an industrial scale, which were carried out naturally with lower but real concentrations of microorganisms, validate ultrafiltration in real conditions as a treatment for incoming seawater from shellfish structures. Bacterial abatement monitoring highlights retention with concentrations in the permeate below the detection threshold for analysis of *Vibrio* bacteria, potentially pathogenic for shellfish. This was confirmed over several months of analysis, different water qualities and bacterial loads upstream of the membranes. Regarding hydraulic and retention performances, the process was tested on two water qualities and during an algal bloom. The permeability remained constant after the chemical cleanings, and the time between these cleaning procedures did not drop below 12 h over the duration of the study, reflecting the resistance and the stability of the process.

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