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SPECIALTY SECTION
This article was submitted to
Marine Fisheries, Aquaculture and
Living Resources,
a section of the journal
Frontiers in Marine Science

RECEIVED 05 September 2022
ACCEPTED 23 November 2022
PUBLISHED 08 December 2022

CITATION
Mota VC, Brenne H, Kojen M,
Marhaug KR and Jakobsen ME (2022)
Evaluation of an ultrafiltration
membrane for the removal of fish
viruses and bacteria in
aquaculture water.
Front. Mar. Sci. 9:1037017.
doi: 10.3389/fmars.2022.1037017

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Evaluation of an ultrafiltration membrane for the removal of fish viruses and bacteria in aquaculture water

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Ultrafiltration (UF) membranes are used to successfully remove waterborne virus and bacteria from wastewater and drinking water. However, UF membrane application in aquaculture water treatment is limited. In this study we evaluate the performance of a capillary polyethersulfone UF membrane to remove two benchmark waterborne fish pathogens: i) the infectious pancreatic necrosis virus - IPNV, which is an unenveloped icosahedral virus, and ii) the bacterium *Aeromonas salmonicida*, which is a Gram-negative, facultative anaerobic bacilli. Moreover, the UF membrane bench-scale unit was tested at two temperatures according to salmonid aquaculture: low (4 - 7°C) and high (16 - 19°C). Sterilised natural seawater was spiked with laboratory cultured pathogens, the water was filtrated, and the membrane permeate collected. Both pathogen solution and permeate were evaluated using a cell culture method to estimate the colony-forming units (CFU/ml) for bacteria presence, a median tissue culture infectious dose (TCID₅₀/ml) assay for virus presence, and real-time quantitative polymerase chain reaction (RT-qPCR) for both bacteria and virus presence. The membrane permeate was negative for both virus and bacteria for all analysis and for both low and high temperatures. The results from this bench-scale study are encouraging for the application of UF membrane technology in aquaculture water treatment to prevent virus and bacteria outbreaks. Further studies should validate this UF membrane technology results in commercial aquaculture conditions.

KEYWORDS

Aeromonas salmonicida, infectious pancreatic necrosis virus - IPNV, Atlantic salmon pathogens, polyether sulfone membrane, ultrafiltration (UF), aquaculture water treatment

Introduction

Membrane filtration is typically a pressure-driven process to force water particles through a membrane and retain them. The water that passes through the membrane is known as filtrate or permeate. Microfiltration, ultrafiltration, nanofiltration and reverse osmosis are the main membrane processes differentiated by the pore size. Ultrafiltration (UF) membrane is an emerging technology being applied in a wide range of industries from drinking water production to wastewater treatment to remove fine solids and microorganisms (Goswami and Pugazhenth, 2020). However, UF membrane technology to treat aquaculture water is limited (Chiam and Sarbaty, 2011).

Currently UF membrane in aquaculture applications is used as support of ultraviolet (UV) irradiation and it was shown to eliminate waterborne microorganisms, small particles, and humic acids (M. Jakobsen personal communication, July 2022). A promising use of UF membrane technology in aquaculture is a stand-alone unit to treat production facilities intake water by removing waterborne viruses and bacteria. Despite the high biosecurity features in aquaculture facilities (Mota et al., 2022), pathogen outbreaks occurred account for several mass mortality events (Murray et al., 2014; Wiik-Nielsen et al., 2017). UF filters pore sizes range from 20 to 100 nm and are composed of non-cellulosic synthetics (such as polyvinylidene fluoride, polyacrylonitrile, or polyethersulfone) that are resistant to heat and chemical attacks, possess a low protein-binding characteristic, and are reusable (Winona et al., 2001; Olszewski et al., 2005). A recent review on membrane filtration usage to remove bacteria and virus from water showed the promising use of this technology for water purification (Goswami and Pugazhenth, 2020). A particular UF membrane technology, a capillary fiber membrane made of polyethersulfone, was reported to be able to process large water volumes with extreme durability both in a drinking water treatment plant (Ming Chew et al., 2015) and in a textile wastewater treatment plant (Sahinkaya et al., 2019). The few applications of UF membrane technology in aquaculture are combined with ultraviolet (UV) irradiation. A stand-alone application of UF membrane technology can also be relevant for aquaculture operations. However, before an application of this disinfection barrier in aquaculture is suitable, it is necessary to evaluate its removal efficiency of relevant fish viruses and bacteria in aquaculture water quality.

In this study we evaluated the performance of a capillary polyethersulfone UF membrane to remove two benchmark waterborne salmonids pathogens: i) the infectious pancreatic necrosis virus (IPNV), which is an unenveloped icosahedral virus, and ii) the bacterium *Aeromonas salmonicida*, which is a Gram-negative, facultative anaerobic bacilli. Moreover, the removal efficiency was evaluated at low (4 - 7°C) and high (16 - 19°C) water temperatures for salmonids.

Material and methods

Experimental design

Two pathogens (IPNV and *A. salmonicida*) at two water temperatures (low: 4 - 7 °C and high: 16 - 19 °C) were used to test the removal performance of an UF membrane. Each condition was replicated three times in a total of 12 tests. Each test consisted in spiking sterile seawater (10 L) with a laboratory grown pathogen (virus or bacteria) and pumping the water through an UF membrane bench-scale unit. Water samples were collected from the seawater pathogen solution and from the membrane permeate to assess the presence and quantity of pathogens.

Water matrices and chemical analysis

The water used in this study was collected from surface seawater (0 - 1 m depth) at Håjafjorden 69°80'42.8"N 19° 01'58.8"E (Tromsø, Norway) during May and June 2022. After collection, the water was sterilised using a high-pressure steam steriliser (SX-700E, TOMY Digital Biology, Japan) and stored refrigerated until testing; at 4°C for low temperature test and 16°C for high temperature test. The sterilised seawater was analysed for the presence of IPNV and *A. salmonicida*, and it was negative for both microorganisms. Further, the water was characterised for salinity and pH (Tetra con 925 and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany), turbidity (ORION AQ4500, Thermo Scientific®, Thermo Fisher Scientific, USA), and ultraviolet transmittance (UVT %), an indirect measure of turbidity (Table 1). The water absorbance at $\lambda=254$ nm (uniSPEC 2 Spectrophotometer, Lab logistics group, Germany) was measured and the UVT was calculated as follows:

$$\text{Equation 1: } UVT = 10^{-a} \times 100$$

where:

UVT = ultraviolet transmittance (%)

a = absorbance at 254 nm wavelength

Ultrafiltration membrane bench-scale unit

The UF membrane was installed as a bench-scale unit (FiiZK Aqua, Trondheim, Norway) and the experimental trials were carried out in two refrigerated rooms at Nofima research facilities (Tromsø, Norway). The UF membrane bench-scale unit is illustrated in Figure 1A. Briefly, it consisted of a cylindrical tank number 1 to add the pathogen solution, a magnet gear pump (MDG-M4S6B220, Iwaki CO, Japan), a water flow meter, a temperature sensor, a water pressure sensor, a UF membrane

TABLE 1 Water quality and operation conditions during ultrafiltration membrane test.

Parameters ¹	<i>A. salmonicida</i>	IPNV
Water quality		
Salinity (ppt)	34.0 ± 0.5	33.2 ± 2.0
pH	8.3 ± 0.0	8.7 ± 0.1
Turbidity (ntu)	2.1 ± 0.3	0.4 ± 0.1
UVT (%)	45 ± 5	95 ± 1
Operation conditions		
Pressure (bar)	0.36 ± 0.07	0.40 ± 0.10
Water flow (L/min.)	0.43 ± 0.02	0.41 ± 0.04
Contact time (min.)	24 ± 1	26 ± 3
Membrane flux (L/m ² /h)	51.5 ± 2.2	49.6 ± 5.2

¹Values measured before ultrafiltration membrane and shown as mean ± standard deviation.

(Figure 1B), and a cylindrical tank number. 2 to collect the membrane permeate. The capillary membrane consisted of 63 fibres, each fibre had 7 capillaries with an inner diameter of 0.9 mm (dizzer[®] modules with Multibore[®] 0.9 membrane, Inge GmbH, Germany) and was 50 cm in length measuring a total area of 0.5 m². The membrane material was polyethersulfone and the pore size was approx. 20 nm. The membrane was operated as inside-out filtration, i.e., pressured water and particles were forced into the membrane fibres capillaries where water and particles smaller than 20 nm escape forming the membrane permeate which flowed to a cylindrical tank number 2. Standard commercial operation settings were used in the tests (Table 1), such as pressure < 1 bar and flux ~50 L/h/m².

consisted in filtering 10 L of ultrapure water (18.2 MΩ/cm, PURELAB Chorus, ELGA Lab water, Veolia, USA) mixed with a sodium hypochlorite solution of 5% active chlorine (Thermo scientific, Belgium) at a concentration 5 ml/L (v/v), followed by rinsing the unit with 10 L of ultrapure water to remove any traces of the disinfection solution. The disinfection efficiency was assessed twice, one for IPNV and one for *A. salmonicida*, by collecting water samples for microorganism detection analysis and was negative both times. At the end of each test, the membrane permeate was treated with the above sodium hypochlorite solution at a concentration 20 ml/L (v/v) for 48h - 72h before the water was disposed into the domestic wastewater system.

Disinfection of bench-scale unit

The bench-scale unit was disinfected between each test using a sodium hypochlorite solution. The disinfection protocol

Virus and bacterium preparation

IPN virus was isolated from the pylorus of an infected Atlantic salmon (*Salmo salar*) and preserved in a minimum

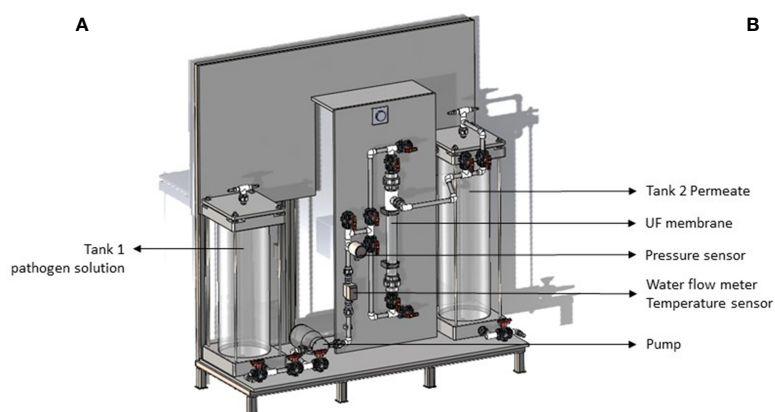


FIGURE 1 Sketch of the ultrafiltration membrane bench-scale unit (A) and picture of the membrane tested (B).

essential media (MEM; Ref. 21090-022, Gibco, USA) with 2% fetal bovine serum (FBS, product number F7524, Sigma Aldrich) at -80 °C. The virus titration was estimated $1 \times 10^{4.1}$ TCID₅₀/ml and $1 \times 10^{7.8}$ TCID₅₀/ml. At the day of each trial IPNV serum was defrosted and 18.4 ml of virus stock solution was added to the 10 L refrigerated sterile seawater.

Aeromonas salmonicida subsp. *salmonicida* bacteria was isolated from the headkidney of Atlantic salmon and preserved in a glycerol and liquid heart infusion (BHI) solution at -80 °C. Prior to each test, bacteria isolates were defrosted and grown on blood agar standard Petri plates with 2% NaCl for 5 days. After, 1 colony forming unit (CFU) was transferred to 20 ml of BHI broth 4 days prior to testing for further bacteria growth. Two days before testing, 20 ml BHI culture was transferred to 400 ml of BHI liquid culture. This bacteria stock solution was 10^5 CFU/ml and 100 ml was added to the 10 L refrigerated sterile seawater.

Water sampling and filtration

IPNV water samples from both unit tanks were collected in triplicate (100–200 ml) per test and filtered under vacuum through an electropositive charge filter disc (Zeta Plus 1MDS, Lenntech, The Netherlands) using a filtration pump (Milliflex Oasis, Millipore, Molsheim, France). The filter was stored in 100 µl nuclease-free water (Life technologies, USA) at -80 after filtration. For virus RNA extraction the whole filter was placed in a lysis buffer (RNAAdvance Tissue kit, A32646, Beckman Coulter) and incubated for 30 min. at 37 °C. Thereafter, the solution (300 µL) was transferred to tubes for further extraction in a Biomek15 automated workstation (Beckman Coulter). Extracted RNA (9 µL) was used to make cDNA, using High-Capacity RNA-to-cDNA™ Kit (4387406, Applied Biosystems).

A. salmonicida water samples from both tanks were collected in triplicate (100 ml) per test and filtered under vacuum through a cellulose ester MCE 0.45 µm sterilised filter (Millipore, Molsheim, France) using a filtration pump (Milliflex Oasis, Millipore, Molsheim, France). Filters were transferred to 2 ml tubes containing 200 µl of MilliQ water and heated for 10 min at 95°C to disrupt the bacteria cells. Bacteria lysate was used as a template in the real time quantitative polymerase chain reaction (RT-qPCR) assay.

Real-time quantitative polymerase chain reaction assay

QuantStudio™ Real-Time PCR System (Applied Biosystems, United States) was used to perform RT-qPCR. The RT-qPCR assays were performed in a final volume of 20 µl reaction mixture containing 2 µL of DNA template, 5.6 µL of nuclease-free water, 10 µl of 2X PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, USA) and 1.2 µl of primer. (Table 2). All samples were run in duplicate. The thermocycling parameters were as follows: 20 s of preincubation at 95°C, amplification with 40 cycles at 95°C for 1 s and 60°C for 20 s. A dissociation stage for 1 s followed at 95°C, 20 s at 60°C, and 1 s at 95°C. A five-step standard curve of 2-fold dilution series was prepared from pooled cDNA to determine the amplification efficiencies.

Median tissue culture infectious dose (TCID₅₀) assay

Prior to virus quantification, chinook salmon embryo cells (CHSE-214, LOT 00/F/031, ECACC, England) were grown in culture flasks with 9 mL of MEM and 8% FBS for one week at 20°C with 5% CO₂. Two days prior to virus quantification CHSE culture was moved to the 96-well microtiter plates (200 µL/well).

To quantify the virus, samples were added in 200 µl volumes to six wells per dilution (10-fold serial dilution, 2% FBS and MEM) of 96-well microtiter plates containing CHSE. The plates were incubated at 20°C with 5% CO₂ for 7 days. After the incubation period, the 96-well microtiter plates were examined for infection by visible cytopathic effect (CPE) using an inverted phase contrast microscope (TMS-F, Nikon, Japan). TCID₅₀ was calculated according to the method of Kärber (1931).

Bacteria plating and counting of colony-forming units

A. salmonicida water samples from both tanks were collected in duplicate (100 ml) per test and filtered under vacuum through a cellulose ester MCE filter as described above. Filters were immediately placed in blood agar 2% NaCl standard Petri plates and incubated at 12°C for 3–5 days. After the incubation period, bacteria colonies were counted, and the results calculated as

TABLE 2 List of primers used for the detection of the reference genes by RT-qPCR.

Gene	Orientation	Primer sequence (5'-3')	Amplicon size (bp)	Reference
AerSal	Forward	CGG AAC GTA ATC TGA ATT GTT CTT TTC	112	(Hiney et al., 1992; Balcazar et al., 2007)
	Reverse	ATT GCT TAT CGA GGC AGC CAA C		
IPNV specific	Forward	CCT GAC CTA CAA CAG CCT GAT GT	114	(Julin et al., 2009)
	Reverse	TCG AAC CCT GTT GGT AGA TTC A		

colony forming units per ml of sample (CFU/mL). Only plates with CFU between 25 and 250 were considered.

Results and discussion

Two benchmark waterborne salmonid pathogens were selected to represent a typical pathogen in these fish species' aquaculture operations. The infectious pancreatic necrosis virus (IPNV), which is an unenveloped icosahedral virus with an average size of around 55 – 70 nm is responsible for abnormal erratic corkscrew swimming and anorexia in fish (Dobos, 1995; Dopazo, 2020). The *A. salmonicida* is a Gram-negative bacterium with an average size 500 – 2000 nm, and is the causative agent of furunculosis, a bacterial septicaemia of salmonid fish (Pickup et al., 1996; Dallaire-Dufresne et al., 2014). The removal efficiency of water microorganisms was previously shown to be membrane and water quality specific (Goswami and Pugazhenthii, 2020). Microfiltration and ultrafiltration are the main membrane sizes used for water disinfection and their pore size range from 10 – 5000 nm (Jacangelo et al., 1997). For example, a suspension of poliovirus, one of the smallest known viruses that ranges between 28 - 30 nm, was found to pass through a microfiltration membrane but to be retained in a UF membrane (Madaeni et al., 1995). The UF membrane used in the current study has an approximate pore size of 20 nm (according to the manufacturer). The removal efficiency of IPNV and *A. salmonicida* by the UF membrane used in this study was 100%, i.e., the membrane permeate was found to be virus and bacteria free in all the replicated tests. Through consideration of the sizes of the target organisms and the membrane pore size, it was apparent that removal of these organisms was due to the membrane acting as a physical barrier that retained the microorganisms' particles.

UF membrane pore size was previously reported to be influenced by temperature (8.9 - 28.5 °C), where the pore size reduces at lower temperatures (Ma et al., 2017). In the current study, the UF performance was evaluated at a low (4 - 7°C) and high (16 - 19°C) temperature ranges for salmonid aquaculture, and both resulted in a 100% virus and bacteria removal. However, the pressure (bar) before the membrane was 11% higher at low temperature when compared to higher temperature, which may be a result on the effect of temperature on pore size as described by Ma et al. (2017).

Bacteria and virus removal were evaluated according to two different methods: TCID₅₀/CFU plating and RT-qPCR. Table 3 shows RT-qPCR threshold cycle (Ct) values, estimated titres as TCID₅₀/ml and colony forming units as CFU/ml for both pathogen solution and membrane permeate. Virus titres and bacteria plating returned similar results as RT-qPCR. However, further membrane validation in commercial conditions should consider RT-qPCR method, as it generates faster results, and the primers can be designed specifically for the microorganism of interest.

Conclusion

The filtration of IPNV and *A. salmonicida* suspensions using a 20 nm capillary polyethersulfone membrane completely removed the microorganism. Water temperature did not affect membrane removal efficiency in the tested range (4 - 19 °C), though lower temperatures resulted in higher membrane water pressure. Moreover, both classical microorganism detection techniques, i.e., virus titres and bacteria plating, and the advanced detection technique RT-qPCR returned the same findings. Further studies should validate this UF membrane technology in commercial aquaculture conditions.

TABLE 3 Water temperature, RT-qPCR cycle threshold (Ct) values, estimated virus titres (TCID₅₀/ml) and bacteria colony forming units (CFU/ml).

Test	Temperature (°C)	RT-qPCR (Ct)		Virus titres (TCID ₅₀ /ml)		Bacteria counting (CFU/ml)	
		Pathogen Solution	Membrane permeate	Pathogen Solution	Membrane permeate	Pathogen solution	Membrane permeate
<i>A. salmonicida</i>							
Low temperature	6.5 ± 1.7	24.3 ± 0.8	N*	-	-	2.5 x 10 ³ ± 7.1 x 10 ²	N
High temperature	18.7 ± 2.1	26.7 ± 0.9	N	-	-	≤ 1 x 10 ^{4#}	N
IPNV							
Low temperature	3.8 ± 0.2	24.7 ± 0.8	N	1 x 10 ^{3.7} ± 1 x 10 ^{1.4}	N	-	-
High temperature	16.4 ± 0.4	31.5 ± 0.4	N	1 x 10 ^{4.8} ± 1 x 10 ^{0.5}	N	-	-

*Negative (N) shows Ct values ≥ 35, no CFU plate growth or no infected cells observed.

#CFU counting < 25, only the counted dilution plated is shown.

Values are shown as mean ± standard deviation.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

Author contributions

VM: Conceptualization, Project administration, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. HB: Conceptualization, Methodology, Formal analysis, Writing – review & editing. MK: Conceptualization Methodology, Resources. KM: Conceptualization. MJ: Conceptualization, Visualization, Writing – review & editing. All authors contributed to the article and approved the submitted version.

Funding

This project is part of CtrlAQUA SFI, Center for research-based innovation, and funded by the Research Council of Norway (grant number 237856/O30) and the partners of the center.

Acknowledgments

The authors are thankful for Are Nylund and Heidrun Nylund (University of Bergen, Norway) for kindly providing the electropositive charge filter discs and Lill-Heidi Johansen

(Nofima, Norway) for helping with IPNV protocols. The authors would also like to acknowledge the dedication to construction of the UF unit by the technical staff in FiiZK AkvaFresh AS.

Conflict of interest

The authors VM and HB 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 MK, KM and MJ declare the following financial interests or personal relationships which may be considered as potential competing interests: professional relationship with a commercial company selling the ultrafiltration membrane tested.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.1037017/full#supplementary-material>

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