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Norwegian Institute of Food, Fisheries and Aquaculture Research

Nanofiltration of intake water in RAS: Effect on fish performance, health and welfare

Illustration: Nofima

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Nanofiltration of intake water in RAS: Effect on fish performance, health and welfare

Tittel:
Nanofiltrering av inntaksvann i RAS: Effekter på fiskens ytelse, helse og velferd

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Summary/Recommendation:
The experiment tested the effect of nanomembrane filtration in RAS on the salmon's physiology, welfare and growth. A specially designed membrane was used to remove sulphate from seawater and to adjust salinity from 32 to 12 ‰. A RAS where salinity was adjusted to 12 ‰ by mixing raw seawater and freshwater was used as control. The fish were kept in the two systems for 10 weeks before being transferred to tanks with seawater flow through for 4 weeks. Histology and measurements of gene expression in the gills, intestine, and skin were done and blood samples were taken to measure the content of cortisol and blood plasma ions after transfer to seawater. Post smolt in RAS with nanofiltered water grew slightly better compared to traditional RAS and the same tendency was maintained after transfer to seawater. There were no differences between systems in welfare indicators or skin morphology. In RAS with nanofiltration, some individuals showed an increase in the area of the gill lamella covered by mucus-producing cells and a lower number of chloride cells on the secondary lamellae in the gills. The effect of nanofiltration on gene expression in skin and gills was small, with only two genes that were differentially expressed in skin and 28 genes in gills.

Sammendrag på norsk:
Forsøket testet effekten av nanomembranfiltrering i RAS på laksens fysiologi, velferd og vekst. Ved nanofiltrering ble sulfatinnholdet i sjøvann redusert og saltholdigheten endret fra 32 til 12 ‰. Et RAS hvor saltholdigheten ble justert til 12 ‰ ved å blande sjøvann og ferskvann ble brukt som kontroll. Fisken gikk i de to systemene i 10 uker før den ble overført til kar med gjennomstrømning av sjøvann i 4 uker. Det ble gjort histologi og målinger av genuttrykk i gjeller, tarm og skinn og blodprøver ble tatt for å måle innholdet av kortisol og ioner i blodplasma etter overføring til sjøvann. Resultatene viste at postsmolt i RAS med nanofiltrert vann vokste litt bedre enn i tradisjonell RAS også etter overføring til sjøvann. Det var ingen forskjeller i velferdsindikatorer eller skinn-morfologi hos laksen i de to produksjonssystemene. I gjellene var det noen effekter av nanofiltrering, den viktigste var en økning i arealet av gjellelamellene som var dekket av slimproduserende celler, og et lavere antall kloridceller på de sekundære lamellene i gjellene. Effekten av nanofiltrering på genuttrykk i skinn og gjeller var liten, med kun to gener med ulikt uttrykk i skinn og 28 gener i gjeller.

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1 Introduction

Intensive systems with recirculation of water (RAS) have become the dominating production facility for Atlantic salmon smolts and post smolt during the last decade. These intensive production systems with high stocking density, high temperature and high feed loads makes managing water quality increasingly important to ensure fish welfare and performance. Keeping the fish on land for a prolonged period has also led to the use of brackish water in RAS to ensure optimal growth (Ytrestøyl et al., 2020), but also to induce seawater tolerance and prevent desmoltification in a larger fish. However, the use of seawater in RAS may potentially generate problems with formation of H_2S which is extremely toxic to fish. Seawater has a high concentration of sulphate (2700 mg/l) compared to freshwater (2 mg/l), and during anaerobic conditions in sludge sulphate-reducing bacteria may form H_2S which can reach concentrations that can be toxic (Letelier-Gordo et al., 2020), and cause sublethal effects and mortalities (Ortiz et al., 1993, Kiemer et al., 1995). Several incidences of mass mortalities have occurred in RAS facilities where H_2S is suspected to be the cause (Dalsgård 2019). Detection and monitoring of H_2S concentration is not straight forward, so another solution is to remove the sulphate from seawater by using nanofiltration. Nanofiltration with pore sizes < 0.01 µm can remove sulphate and other ions like Mg^{2+} and Ca^{2+} from seawater producing brackish water with preferred salinity. The sulphate concentration can be reduced to < 15 mg/l which may significantly reduce the risk of H_2S formation. A salinity of 12 ppt has been shown to improve growth of fish in RAS compared to freshwater and seawater (Ytrestøyl et al., 2021, 2022). Membrane filtration can also remove pathogens and particles and thus improve the biosecurity in RAS facilities and potentially improve fish growth and welfare. However, new applications need to be tested to make sure there are no unintended side effects of the obtained water quality on fish health and welfare, both in the RAS phase and after transfer to seawater. The transfer to seawater is a critical period for the salmon, with increased mortality and reduced growth performance. The fish must switch its osmoregulatory systems from taking up ions from the water in freshwater to drink seawater and actively secrete ions to maintain osmotic balance in seawater. High occurrence of infectious diseases during this period can be associated with increased pathogens pressure and systemic immune suppression during smoltification indicated by down-regulation of multiple genes (Johansson et al., 2016), which persists for several months after seawater transfer (Karlsen et al., 2018). The skin and gills are important barriers for fish robustness and disease resistance, so gill and skin condition were the primary targets for health evaluation in this study.

To test the effect of specially designed nanofiltration membrane for sulphate removal from seawater, two identical semi-commercial RAS at Nofima Centre for Recirculation Aquaculture (NCRA) at Sunndalsøra were used. In one RAS a membrane delivered by Akvafresh for nanofiltration of seawater was installed with the purpose of removing sulphate and desalinating the seawater to a salinity of 12 ppt. The other RAS was also run on 12 ppt, but the water was a mix of ground freshwater and seawater pumped from a depth of 40 m. Both systems were stocked with post smolt Atlantic salmon for a period of 12 weeks before both systems were switched to flow through full strength seawater for a period of 4 weeks before the study was ended. The aim of the final part of the experiment was to mimic transfer of post-smolts into seawater cages and to follow fish performance, health and welfare during this period. The experiment was conducted in accordance with guidelines provided in Norwegian and European legislations related to animal research, and a formal approval of the experimental protocol was given by the Norwegian Food Safety Authority.

The main hypothesis to be tested was:

“There is no difference between membrane filtered RAS and conventional RAS in terms of fish health, welfare and performance”.

2 Methods

2.1 Animal husbandry and experimental design

The experimental design and timeline are presented in Figure 1. Atlantic salmon smolts (start weight 140 g) were produced at Nofima research station in Sunndalsøra using photoperiod manipulation (6 weeks with short day (12 h light, 12 h darkness) followed by six weeks of 24 h light). After smoltification fish were moved from the flow through system with fresh water into 8 x 3.3 m^3 octagonal tanks supplied with RAS water and Nofima Centre for recirculating Aquaculture (NCRA).

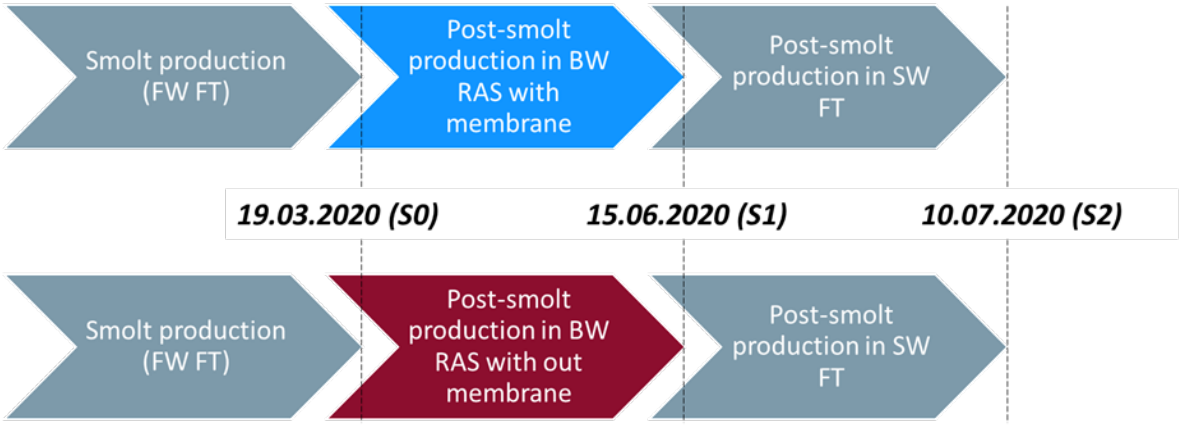

Figure 1 Experimental design, the dates indicate start of different experimental phases. FW=Freshwater, BW= Brackish water, SW = seawater, FT= Flow through, RAS=recirculation aquaculture system. Dotted lines indicate the time when samples were taken, both dates and the sampling codes (S0-S2).

Four tanks were connected to RAS in which make-up seawater was desalinated using nanofiltration membrane producing brackish water with salinity of 12 ppt (MembRAS). Prior to the start of the experiment MembRAS was emptied for water and the whole system volume was exchanged with water produced with nanofiltration membrane. This was done to secure that fish entering MembRAS were never in contact with non-filtered seawater and potential opportunistic pathogens. The remaining four tanks with post-smolts received water from control brackish (12 ppt) RAS where water was not filtered. The 12 ppt salinity of the control RAS was obtained by mixing approximately 1/3 of make-up flow from seawater taken from a depth of 40 m, and the remainder of the make-up flow from freshwater groundwater wells. Seawater was filtered and UV-treated before it was pumped into the RAS while the groundwater was treated with silicate to prevent potential copper toxicity historically observed at the research station.

The design of the RAS used in this trial is described in detail in Terjesen et al., 2013. In short, water from fish tank was treated using microscreen belt filter, followed by three chamber moving bed bioreactor (MBBR), degassing column and oxygen addition before the water was returned to the tanks (Figure 2). In the present experiment, we used a cloth with larger mesh size for the microscreen belt filter (120 µm) compared to the published description (Terjesen et al., 2013).

Temperature (PT100, Hyptech, Drammen, Norway) was controlled at 12 ^{o}C and measured continuously in each RAS during the experiment. Salinity was measured daily in both systems using a portable Multi 3410 meter and TetraCon ® 925-3 conductivity probe (WTW GmbH, Weilheim, Germany). All tanks were equipped with individual oxygenation down-flow bubble contactors and oxygen saturation was kept > 85 % in all tanks during the experiment. The water velocity in the tanks was adjusted during the trial

to 1.0 body length per second (bl s-1). The set pH point of 7.5 was maintained in both systems using pH online probe connected to Walchem WDP 320 (Holliston, MA, U.S.) control system that was controlling addition of bicarbonate using a dosing system (IWAKI EW, Tokyo, Japan).

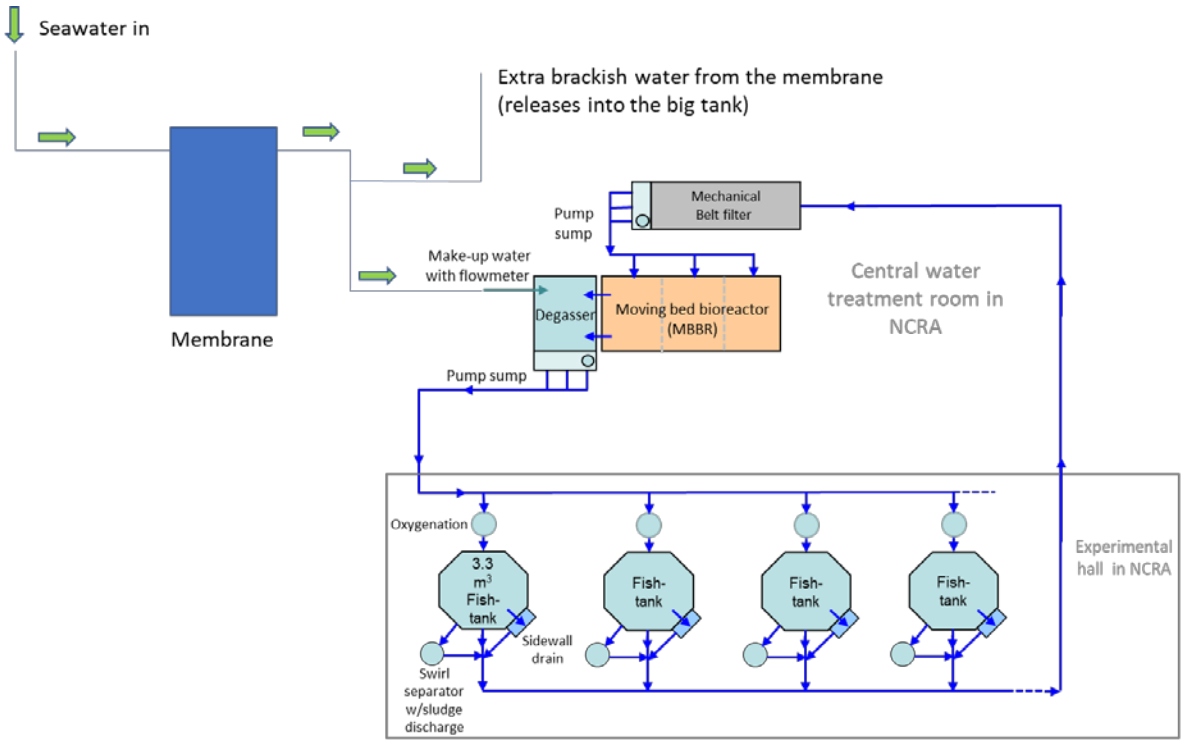

Figure 2 MembRAS overview with fish tanks and water treatment process. Membrane used in the experiment was oversized and excess of brackish water that was produced was redistributed as shown in the figure. Control RAS had the same set-up apart from the lack of membrane treatment of intake water.

After 10 weeks of production in the RAS, water quality in the rearing tanks was switched to full strength sea water and flow through system for a period of 4 weeks. The tanks were equipped with a system for collection of uneaten feed, so that feed intake could be measured. This was done during the seawater phase of the experiment to access effects on fish appetite. The feed intake was determined according to Helland, Grisdale-Helland and Nerland (1996).

2.2 Water quality and biological samplings

Detailed water quality analysis was done by NTNU. In addition, water quality was documented at the tank outlet level (n=4 for each RAS) on four occasions: at the start of the experiment (18.03.2020), end of RAS phase (10.06.2020), start of seawater phase (16.06.2020) and end of experiment (07.07.2020).

Samples of fish were taken before the experiment started (S0, 18.03), at the end of the RAS phase (S1, 15.06), and at the end of the 4 weeks in seawater (S2, 10.07) (Figure 1).

2.3 Scoring of operational welfare indicators (OWI)

OWI's were scored on two occasions, at the end of RAS production phase (09.06.2020) and at the end of the experiment (08.07.2020) according to Noble et al., (2018). In total, 30 individuals from each tank were examined on each occasion for eye and snout damage, jaw deformities, emaciation, skin damages and dorsal, caudal, pectoral and pelvic fin damage.

Fish were euthanised prior to scoring using overdose of tricaine methane sulphonate, MS 222 (Argent Chemical Laboratories, Redmond, WA, USA). At the same time individual weight was recorded (0.1 g accuracy) as well as individual length (0.1 cm accuracy).

2.4 Bactiquant analysis

Quantification of total bacteria in water, from the tank surface and from the MBBR biomedia surface of both RAS and MembRAS was done using Bactiquant® Water test-kit and Bactiquant® Surface test-kit (Mycometer, Copenhagen, Denmark) respectively. On 10.06.2020 water was collected from two tanks receiving water from MembRAS and from two tanks with control RAS water and swabs from the tank walls were collected for analyses. In addition, five biomedia from each MBBR chamber (n=3) and each RAS were sampled. Sampled water, tank surface swabs and biomedia were analyzed using above mentioned kits and according to the manufacturer's guidelines.

2.5 Histology

2.5.1 AI-model

Histology samples of skin and gills were collected at S0, S1 and S2 (19.03, 15.06 and 10.07 respectively) and analysed for gill histology (Table 1). AI-analysis of skin were conducted according to (Sveen et al., 2021), and gill AI-analysis according to our newly developed gill model on the same platform (Figure 3).

Table 1 Number of samples analyzed by the AI-model

	S0 (19.03)	S1 (15.06)	S2 (10.07)
AI - histology	Skin (N = 20) Gill (N = 19)	Skin (N = 12 MembRAS, N = 14 RAS) Gill (N = 15 MembRAS, N = 16 RAS)	Skin (N = 19 MembRAS, N = 17 RAS) Gill (N = 17 MembRAS, N = 16 RAS)

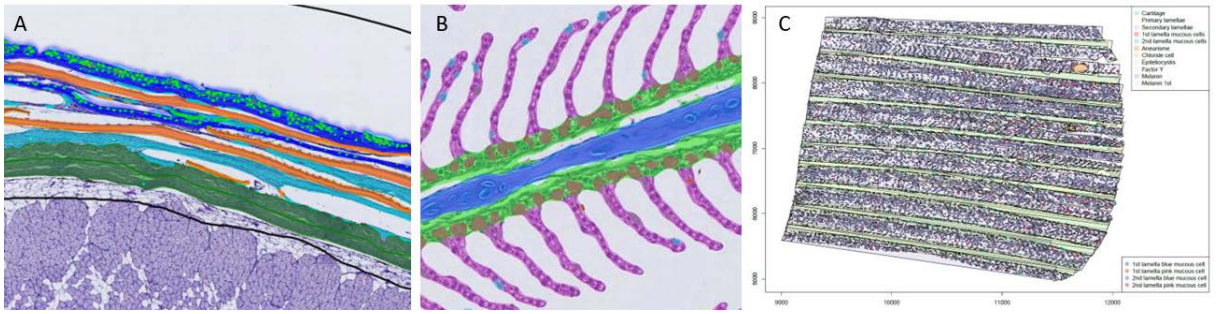

Figure 3 AI model of skin and gills. A) Tissue section of skin (AB/PAS stained), with artificial colour overlay as presented by the AI-model. Dark green (DCT), light blue (LCT), orange (Scale), ark blue (epidermis) with green circles (mucous cells). B) Section of gill pink (2nd lamellae), light blue (mucous cell), green (1st lamellae), red (chloride cells), blue (cartilage). C) Reconstruction gill based on data generated in the AI-model. Identified features as indicated in the plot.

2.5.2 Manual histopathological scoring of gills

In addition to AI-analysis, a subsample of gills was scored manually. A total of 45 gills were scored: five gills from S0, five gills from each tank 204 (RAS), 205 (MembRAS), 211 (RAS) and 212 (MembRAS) from time-point S1 after the end of RAS phase (20 gills), and the corresponding number of gills from the corresponding tanks on time-point S2, after seawater phase (20 gills). The gill score made up of a total of 19 categories based on the different tissue responses that can potentially be recorded in gill tissue, and these categories are further divided into two main groups based on whether the changes are

regarded as readily reversible or whether they are regarded as less reversible (Table 2). This is further reflected in the score, where less reversible changes are weighted more than readily reversible changes. In general, each category is scored in the way that tissue lesions seen in up to 10 % of the tissue is given score 1, distribution of tissue lesion in 10-50 % of the tissue is given score 2, and distribution of tissue lesion in above 50 % of the gill section is given score 3. For mucous cell metaplasia, an average occurrence of up to three mucous cell pr lamella is given score 1, occurrence of 3 to 6 mucous cells is given score 2, and occurrence of 7 or more mucous cells in average is given score 3. Score on the less reversible categories is multiplied by a factor of 2, and the total gill-score for each gill is presented as the sum of each single score. All available gill tissue on each section was evaluated.

Table 2 Manual scoring of tissue responses in the gills (independent of causative agent), into categories (readily reversible changes represented on yellow background, and less reversible changes presented on red background); each category is scored on a scale of 0 – 3, and less reversible changes are further multiplied by two.

<table>
<tr><td>Mucous cell metaplasia</td><td rowspan="10">Readily reversible tissue changes/assumed less impact on gill function: each category given as score 0-3</td><td>Lamellar epithelial hyperplasia</td><td rowspan="10">Less reversible tissue changes/assumed higher impact on the gill function: each category given as score 0-3, and each score multiplied by 2</td></tr>
<tr><td>Thickening of the outer margin of the lamellae (“clubbing”)</td><td>Fresh closed lamellar bleedings (acute aneurisms)</td></tr>
<tr><td>Lamellar oedema (“lifting”)</td><td>Organized closed lamellar bleedings (scare tissue of aneurisms)</td></tr>
<tr><td>Lamellar or interlamellar epithelial hypertrophy</td><td>Lamellar bleeding</td></tr>
<tr><td>Congestions thrombosis lamellae</td><td>Lamellar fusion</td></tr>
<tr><td>Congestion oedema filament</td><td>Thickening filament - inflammation</td></tr>
<tr><td>Cartilage deformity</td><td>Thickening filament – epithelial hyperplasia</td></tr>
<tr><td>Thickening distal filament (epithelial proliferation)</td><td>Inflammation single lamellae</td></tr>
<tr><td></td><td>Degeneration/necrosis pillar cells</td></tr>
<tr><td></td><td>Necrosis lamellae/filament</td></tr>
</table>

2.6 Microarray

Microarray analyses were performed on gill and skin. Samples were homogenized in FastPrep 96 (MP Biomedicals, Eschwege, Germany) and RNA was extracted on Biomek 4000 robot using Agencourt RNAdvance Tissue kit (Qiagen, Oslo, Norway). RNA concentration was measured with NanoDrop™ One (Thermo Fisher Scientific, Waltham, MA USA) and quality was assessed with Agilent Bioanalyzer 2100. Nofima’s 15 k Atlantic salmon DNA oligonucleotide microarrays SIQ-6 were manufactured by Agilent Technologies (Santa Clara, CA USA), and the reagents were purchased from the same provider. RNA amplification and labelling were performed with a One-Color Quick Amp Labelling Kit and a Gene Expression Hybridization kit was used for fragmentation of the labelled RNA. After overnight hybridization in an oven (17 h, 65 °C, rotation speed 0.01 g), arrays were washed with Gene Expression Wash Buffers 1 and 2 and scanned with an Agilent scanner. Nofima’s Bioinformatic package STARS was used for data analyses. Global normalization was performed by equalizing the mean intensities of all microarrays. The individual values for each feature were divided by the mean value of all samples producing expression ratios (ER). The log2-ER values were calculated and normalized with locally weighted nonlinear regression (Lowess) and differential expression was assessed by criteria: > 1.75-fold and $p < 0.05$ (t test).

2.7 Statistical analysis and calculations

Specific growth rate (% day-1) between two sampling points was calculated as:

SGR = (ln BW2 – ln BW1) x 100/d (BW= bodyweight (g), d = number of days)

The thermal growth coefficient, TGC, was calculated as:

TGC =1000*(BW2 1/3- BW11/3) x (number of day degrees)-1

The feed intake per tank was calculated by taking the difference between the amount of feed fed to each tank and the amount of uneaten pellet collected. Individual daily and cumulative feed intake were calculated by dividing the feed intake per tank with the number of fish in the pen.

Statistical analysis on growth, and blood ion and cortisol concentrations were performed in SAS Jmp. A one-way ANOVA with RAS system as the fixed factor was performed for each trial period or sampling point. P -values < 0.05 were considered significant.

3 Results

3.1 Water quality

At the start of the RAS phase of the experiment (18.03.2020) significantly higher temperature, pH, salinity, alkalinity and turbidity were documented in the MembRAS system compared to the Control RAS (Table 3). At the end of the RAS phase pH, conductivity and alkalinity were significantly higher in MembRAS tanks compared to tanks in the Control system (Table 3). In the seawater flow through phase the only significant difference was in alkalinity directly after transfer (16.06.2020) that was higher in the tanks originating from the control RAS compared to MembRAS (Table 3).

*Table 3 Water quality recorded at the tank outlet level in each RAS (n=4, per RAS) during the experiment; at the start (18.03.2020) and end (10.06.2020) of RAS phase and start (16.06.2020) and end of seawater phase (07.07.2020). Given values are averages ± STDEV; * indicates significant (p < 0,05) differences between two RAS at each time point.; NM- not measured.*

Experimental phase	RAS phase				Flow through phase			
Date	18.03.202		10.06.2020		16.06.2020		07.07.2020	
Water quality parameter (unit)/system	RAS	MembRAS	RAS	MembRAS	RAS	MembRAS	RAS	MembRAS
Oxygen (%)	98.5 ± 0.3	98.6 ± 0.7	92.8 ± 1.5	93.8 ± 1.7	90.5 ± 3.5	89.0 ± 1.0	90.5 ± 3.3	89.0 ± 1.5
Temp (°C)	11.8 ± 0.0	12.0 ± 0.0*	12.6 ± 0.0	12.8 ± 0.0	8.4 ± 0.0	8.4 ± 0.0	8.6 ± 0.0	8.6 ± 0.1
pH	7.8 ± 0.0	7.9 ± 0.0*	7.6 ± 0.0	7.7 ± 0.0*	7.8 ± 0.0	7.8 ± 0.0	7.7 ± 0.0	7.7 ± 0.0
Coductivity (ms/cm)	NM		19.5 ± 0.0	20.6 ± 0.0*	53.2 ± 0.0	53.2 ± 0.0	53.1 ± 0.1	53.1 ± 0.1
Salinity (ppt)	11.4 ± 0.1	11.9 ± 0.0*	11.4 ± 0.0	12.1 ± 0.0	34.1 ± 0.1	34.1 ± 0.0	31.5 ± 5.0	33.9 ± 0.0
TSS (mg/L)	4.6 ± 3.0	5.2 ± 0.8	3.8 ± 1.0	3.0 ± 0.8	5.0 ± 2.1	3.9 ± 2.7	9.1 ± 1.9	8.6 ± 1.1
Alkalinity (mg/L)	43.7 ± 1.1	51.2 ± 1.3*	40.2 ± 0.3	58.1 ± 1.2*	83.0 ± 3.5	77.0 ± 2.6*	96.3 ± 4.2	97.5 ± 1.0
CO_2 (mg/L)	1.6 ± 0.1	1.7 ± 0.0	2.6 ± 0.1	2.6 ± 0.1	4.4 ± 0.1	4.5 ± 0.2	5.5 ± 0.1	5.6 ± 0.2
TAN (mg/L)	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1
NO_2-N (mg/L)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
NO_3-N (mg/L)	8.1 ± 3.2	10.0 ± 4.0	42.1 ± 10.5	34.9 ± 9.5	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.8 ± 0.0
Turbidity (NTU)	3.9 ± 0.2	5.1 ± 0.2*	3.8 ± 1.0	3.0 ± 0.8	1.1 ± 0.7	0.6 ± 0.1	0.3 ± 0.0	0.2 ± 0.0

3.2 Fish growth parameters

At the start of the experiment the fish weighed 140 g. At the end of the RAS phase (S1) there was a close to significant difference between treatments (402 g MembRAS, vs. 366 g RAS, p = 0.06, Figure 4A). Both SGR and TGC were significantly higher in the MembRAS compared to the control RAS (Figure 4B). At the end of seawater phase (S2), fish from the MembRAS were on average 556 g and fish from control RAS were 491 g (p = 0.0009). Fish from the MembRAS also had a higher feed intake during the 4 weeks in seawater (p < 0.02, Figure 4C, D). The temperature variation in the RAS period was 12.4 ± 0.6 °C for MembRAS and 12.2 ± 0.6 °C for RAS (Figure 4E).

Figure 4 A) Weight development during the experiment (g). B) SGR and TGC values for the whole experiment. C) Feed intake after seawater transfer D) Cumulative feed intake in seawater E) Temperature in the RAS phase. N=4 tanks per treatment. MembRAS indicates RAS with nanofiltration membrane and RAS is the control system without nanofiltration.

3.3 External morphological indicators

The severity of external damage was low for all parameters and there were no significant differences between the systems (Table 4). Over time a small increase in eye and skin damage was observed while fin status remained similar throughout the experiment. No opercular shortages or vertebral deformities were observed during the experiment. The prevalence of damages is given in (Table 5).

Table 4 The average score for main morphological welfare indicators at the end of the RAS and SW phase for individuals from RAS and MembRAS

Sampling point	Treatment	Eye damage	Skin damage	Dorsal fin	Caudal fin	Pectoral fin	Pelvic fin
End of RAS phase (S1)	RAS	1.3	1.8	1.5	1.3	1.7	1.6
	MembRAS	1.4	1.9	1.3	1.4	1.4	1.3
End of SW phase (S2)	RAS	1.8	2.0	1.5	1.4	1.4	1.6
	MembRAS	1.6	2.0	1.3	1.3	1.3	1.4

Table 5 The prevalence (% individuals) of individuals showing changes in scored morphological indicators (score > 0) at the end of the RAS and SW phase for RAS and MembRAS

Sampling point	Treatment	Eye damage	Snout damage	Jaw deformitie	Emaciatio	Skin damage	Dorsal fin	Caudal fin	Pectoral fin	Pelvic fin
End of RAS phase (S1)	RAS	62	1	0	7	100	71	66	59	47
	MembRAS	73	2	0	4	100	64	83	60	48
End of SW phase (S2)	RAS	85	0	1	3	100	63	87	58	46
	MembRAS	82	3	0	1	100	66	93	59	53

3.4 Cortisol and Blood ions

High cortisol levels were found before start of the experiment (S0, Figure 5A). At the end of the RAS phase (S1) cortisol levels were lower, and not significantly different between MembRAS and control. At the end of experiment after 4 weeks in flow through seawater (S2), significantly higher cortisol concentrations were found in the MembRAS treatment (p=0.02). There were no significant differences between treatments in the RAS phase in serum Cl and Na concentrations. At the end of the RAS phase there was a tendency for a higher Mg concentration in MembRAS (p=0.07), but after 4 weeks in seawater there were no differences between treatments.

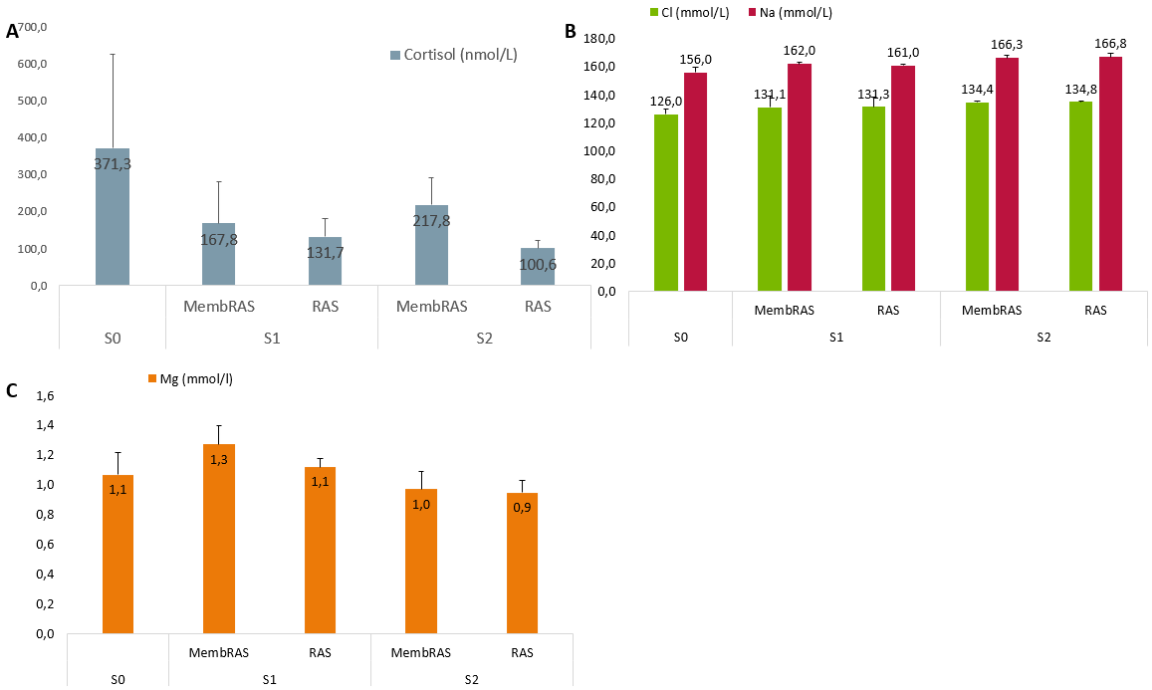

Figure 5 Serum cortisol and Cl, Na and Mg in MembRAS and RAS. S0 is before start of the experiment, S1 = end of RAS phase, S2 = end of seawater phase. A) Cortisol, B) Na and Cl, C) Mg, all values are means + SEM, (N = 4).

3.5 Bactiquant

Significantly lower BQV were measured in the MembRAS water and on the surface of the tanks in this system. Higher average BQV were measured from the biomedia collected in MembRAS but they were not significantly different compared to Control RAS (Figure 6).

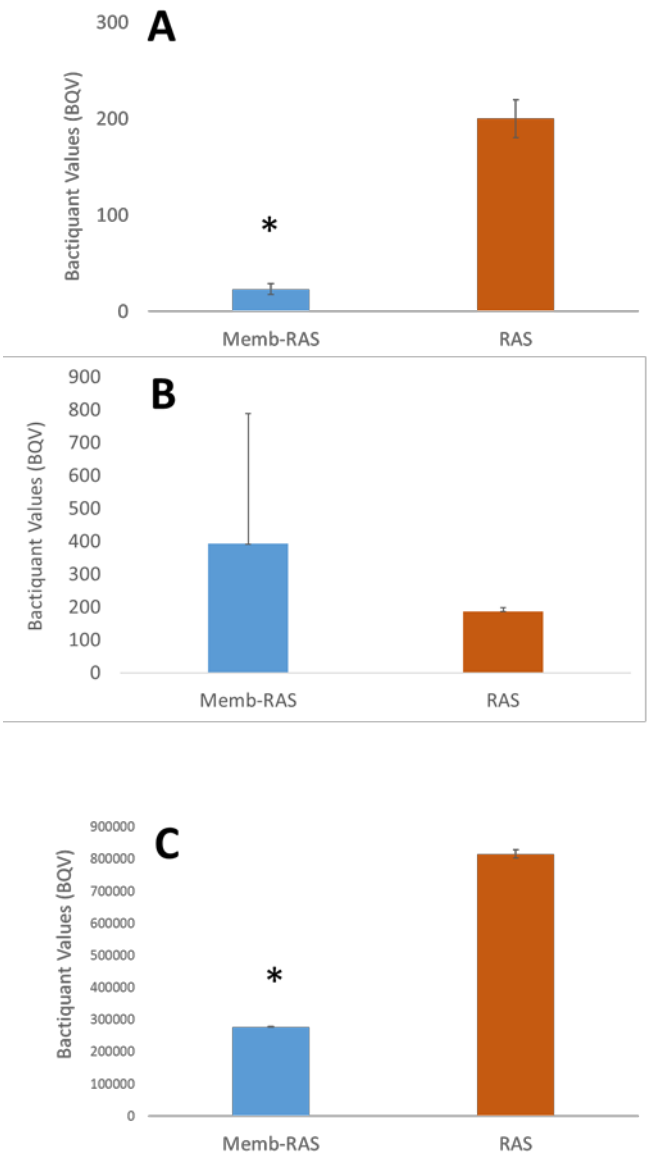

*Figure 6 Bactiquant values (BQV) for RAS with filtered water (MembRAS) and control RAS (RAS): A) tank surface B) biomedia surface; C) tank water. Values represented by columns are average values (n=2 for tank samples and n=3 for biomedia surface) ± STDEV; * indicates significant differences (p<0,05) between Control RAS and MembRAS.*

3.6 Microarray

Effect of membrane filtration on gene expression was minor. At the end of water treatment (S1), the numbers of DEG in gill and skin were respectively 25 and two (Figure 7). At S2 difference between the study groups further decreased in gill and slightly enhanced in skin (five and eleven DEG). Of note was higher expression of cytochrome P450 1A1 (*cyp1a1*) in gills at S1. This enzyme plays a key part in biodegradation of organic contaminants. Difference at both time points was shown by only one gene: *angiogenin-1 precursor / rnase zf3,* which was 6 to 7-fold stimulated in membRAS. The encoded protein can be involved in vascularization and defence against bacteria. *Inducible NO synthase* 2 (downregulated in membRAS) is also known as an immune effector and regulator of various processes including osmoregulation in smolt gills. Downregulated *mucin-5b* is a major mucus component. Only eight immune genes were differentially expressed in gill at S1, four and four genes showed higher levels in membRAS and RAS. One and five immune genes were respectively up and downregulated in

membRAS at S2 in skin. Based on our experience in Atlantic salmon transcriptomics, only downregulation of *cyp1a1* in membRAS might indicate functional changes: reduced exposure to contaminants in membrane filter water. This emblematic marker of responses to xenobiotics suggested contamination, which was reduced with the membrane. At the second time-point expression of cyp1a1 further increased leveled out in both groups. However, difference between membRAS and RAS was relatively small. Other DEG belong to many functional groups. Expression changes in only one or two genes per functional group or pathway is unlikely to affect the condition and performance of fish.

Gene	MembRAS RAS fold, S1	MembRAS RAS fold, S2
Gill		
Cytochrome P450 1A1	***-1.97***	1.08
Angiogenin-1 precursor / RNase ZF3	***6.82***	***5.98***
Nitric oxide synthase 2, inducible	***-2.94***	-1.77
Mucin-5B	***-1.75***	-2.01
Alpha-tropomyosin	***2.17***	-1.15
Anaphase-promoting complex subunit 7	***1.96***	-1.52
Annexin A2a	***1.75***	-1.18
Antigen peptide transporter	***-3.42***	2.36
Breast cancer 2, early onset	***1.80***	-1.18
C1Q and TNF related protein 3	***5.53***	1.51
C-C motif chemokine 19 precursor-1	***-2.14***	1.14
CQ067 protein	***-1.87***	-1.20
Defensin beta 4	***1.76***	-1.32
Deoxyribonuclease gamma-like	***-1.82***	-1.23
Dystonin	1.53	***1.81***
Enolase 3-1	***3.09***	-1.02
Four and a half LIM domains 1b	***1.99***	1.00
GTPase IMAP family member 4-like	***-1.81***	1.05
Heat shock protein 70	1.01	***3.46***
Histidine ammonia-lyase	***-1.79***	-1.18
Keratin, type 1, gene 19d	***1.78***	-1.37
MAM domain-containing protein 2	***2.27***	-1.13
Microfibrillar-associated protein 5-like	***1.90***	-1.40
Nephronectin	2.00	***1.75***
Potassium channel tetramerisation domain 5	***2.03***	1.24
Rhamnose binding lectin STL2	***1.97***	-1.09
TCR-like	***-2.15***	-1.38
Troponin T2d, cardiac	***11.80***	1.03
Viperin	1.35	***2.02***

Gene	MembRAS RAS fold, S1	MembRAS RAS fold, S2
Skin		
Antigen peptide transporter	***-0.43***	1.58
C-C motif chemokine 20-like	0.96	***-0.13***
CD209 antigen-like protein E (c209e)	0.21	***-0.83***
CD4-like	0.45	***-0.85***
Chemokine (C-C motif) ligand 34b, duplicate 5	-0.09	***-1.36***
enolase 3-1	0.13	***-1.02***
GMP Giant mucus protein	0.09	***-0.83***
myosin light chain 3	***-0.02***	***-2.46***
Neuroendocrine convertase 2	0.84	0.11
Proepiregulin	0.20	***-0.98***
Pyruvate kinase, muscle, b	***-0.07***	***-1.52***
Serum amyloid A5	0.21	***-1.07***
uncharacterized LOC106605369	0.32	***-1.17***

Figure 7 Differentially expressed genes (DEG), membRAS to RAS folds. Significant differences are highlighted with bold italics

3.7 Histology of skin and gills

3.7.1 Skin

For skin, there was no effect of the treatment, and the effects of time were as expected. An increase in abundance of the connective tissue compartment (dermis) together with a thickening of the dense connective tissue (DCT) was observed as a response of time. The correlation between mucous cell area and the epidermal area was also high (R > 0.7) for all parameters (Table 6), which may indicate a normal state of the epithelial tissue. The microarray results showed large differences in numbers of DEGs between production systems (FT and RAS/MembRAS, S0/S1) and between RAS/MembRAS and seawater (S1/S2). However, these results were not reflected in observable changes in skin morphology. A summary of the skin responses is presented in Figure 8 and 9.

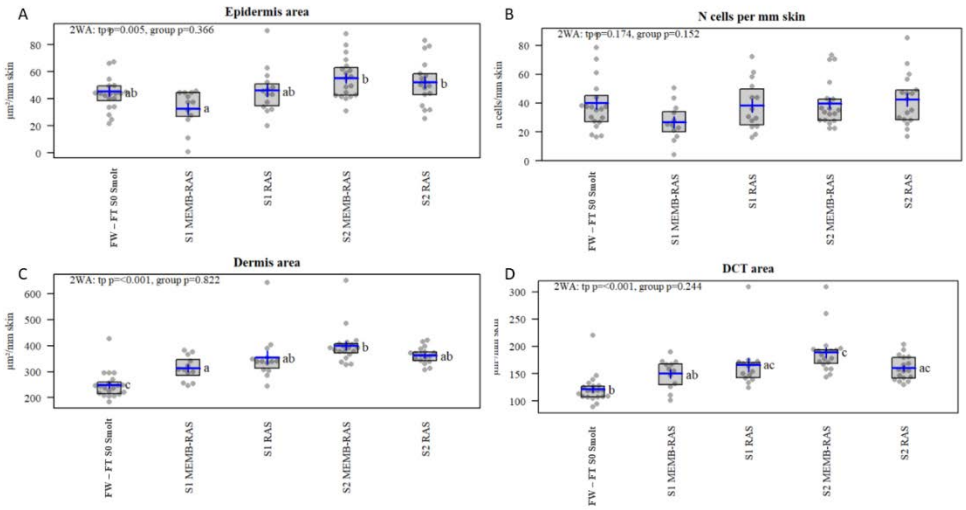

Figure 8 Plots show the areas of skin components. A) Epidermis; B) Number (N) of mucous cells per mm of skin; C) Dermis area and D) Dense connective tissue (DCT). The grey boxes indicate the 2nd and 3rd quartiles (central 50 % of the values). The blue horizontal lines indicate the mean values and blue vertical lines the +/- SEM. Two-way ANOVA p-values are indicated at the top of each plot. In case of a significant results (any $p < 0.05$), a Tukey post-hoc test was calculated and lowercase letters next to the mean indicate statistical differences. Groups which do not share a letter were significantly different from each other ($p < 0.05$). S0-S2 are sampling points (see Figure 1 for explanation).

Table 6 Correlation between epidermis and mucus cell area. The table shows the correlation estimates (Pearson), R2, p-values and number of samples in the respective group.

	Group	R	R2	P	n
## 1	S0 FW-FT	0.80581	0.64932	2e-05	20
## 2	S1 MembRAS	0.8311	0.69072	0.00081	12
## 3	S1 RAS	0.77128	0.59487	0.00124	14
## 4	S2 MembRAS	0.70608	0.49855	0.00073	19
## 5	S2 RAS	0.76896	0.59129	0.00031	17

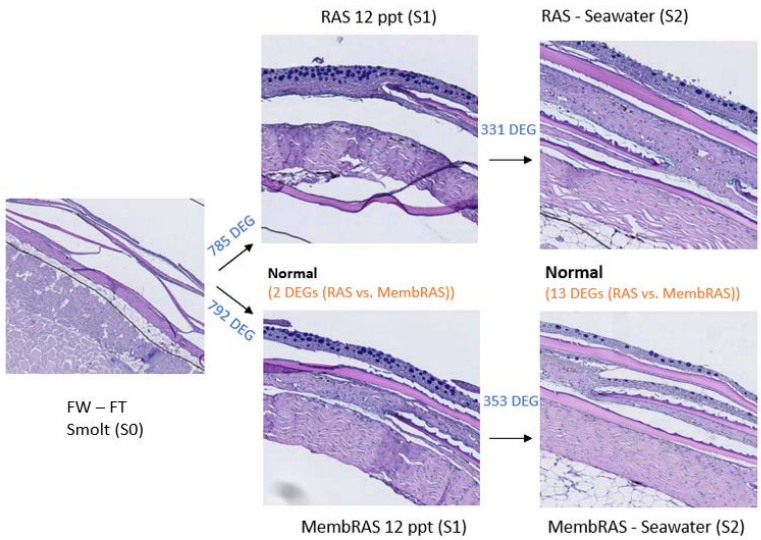

Figure 9 Summary of results, skin – The different production systems did not have an impact on the general morphology of the skin. There were very few differences in DEG at S1, and S2 between MembRAS and RAS (orange text) treated fish. The transcriptional response from fresh water to RAS systems, and RAS to seawater is indicated in the figure (blue text).

3.7.2 Gill

AI-model

Changes related to both time and treatment were identified for gill morphology. The ratio of secondary lamellae area to primary lamellae area increased with time, but no effect of treatment (Figure 10A). The main identified effect of MembRAS at S1, was an increase in area (%) of mucous cells on the secondary lamella, with a higher number of purple mucous cells per area of secondary lamella in the MembRAS treatment. The second effect of the treatment was an overall higher % of chloride cells on the secondary lamellae in the Control RAS group (P = 0.037) (Figure 10E). For the primary lamella, chloride cell % of total lamellae area increased with time, with no effect of treatment.

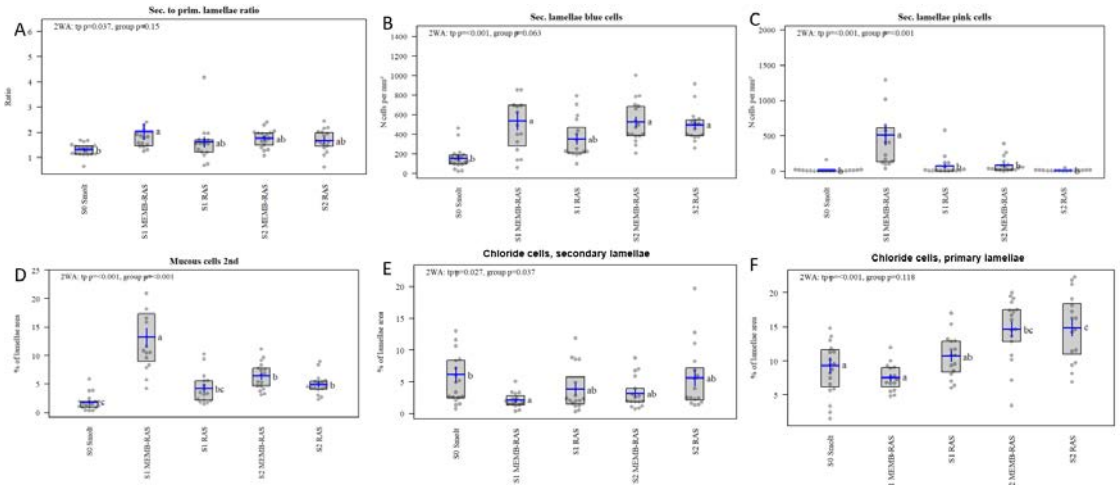

Figure 10 Plot show area of gill components, and mucous cell number. A) secondary to primary lamella ratio; B) Total number of mucous cells per mm2 of secondary lamellae; C) Number of pink mucous cells per mm2 of secondary lamellae. D) % of mucous cell area of total secondary lamella area E % of chloride cells of total secondary lamella area F % of chloride cells of total primary lamella area. Statistics as previous plot. S0-S2 are sampling points (see Figure 1 for explanation)

Histopathological changes in the gill

Manual scoring resulted in the following figures:

- Timepoint S0 (control): All gills were evaluated as having mild changes, with an average gill score of 6,5 (STDEV.s: 2,17)
- Timepoint S1 MembRAS: One gill was evaluated as having mild changes, while nine gills were evaluated as having moderate changes. Average gill score was 14,1 (STDEV.s: 3,28)
- Timepoint S1 RAS: Five gills were evaluated as having mild changes, while five gills were evaluated as having moderate changes. Average gill score was 10,1 (STDEV.s: 2,38)
- Timepoint S2 MembRAS: Nine gills were evaluated as having mild changes, while one gill was evaluated as having moderate changes. Average gill score was 8,7 (STDEV.s: 1,49)
- Timepoint S2 RAS: Eight gills were evaluated as having mild changes, while two gills were evaluated as having moderate changes. Average gill score was 8,1 (STDEV.s: 2,38)

All observed histopathological changes were unspecific and could not be related to any specific cause. No signs of infectious agents or characteristic lesions of such were observed in the investigated material. Hence, all observed changes are assumed to steam from environmental influences. Gill score at timepoint S1 MembRAS (Figure 11) was found to be significantly higher than the other groups (one-way ANOVA followed by Tukey multiple comparisons of means with 99 % family-wise confidence level). However, there was a trend of higher gill score for all gills at the end of the RAS phase (timepoint S1) compared with the other samplings, but this was not found to be statistically significant for timepoint S1 RAS. After four weeks in FT seawater (S2), the gills in both treatments normalized and few moderate changes were observed.

To further characterize the reversible histopathological processes in the gills, samples from 2 days post seawater transfer, one fish per replicate tank, was checked qualitatively for histopathological changes. All samples showed typical morphological features of gills newly transferred to seawater, with rough or "spiky" appearance of the respiratory epithelial tissue (Figure 12). In addition, similar histopathological findings as identified in S1 and were present in the gills, such as mucous cell metaplasia, and lifting of respiratory epithelial tissue (Figure 12). The summary of all gill histology data is provided in Figure 13.

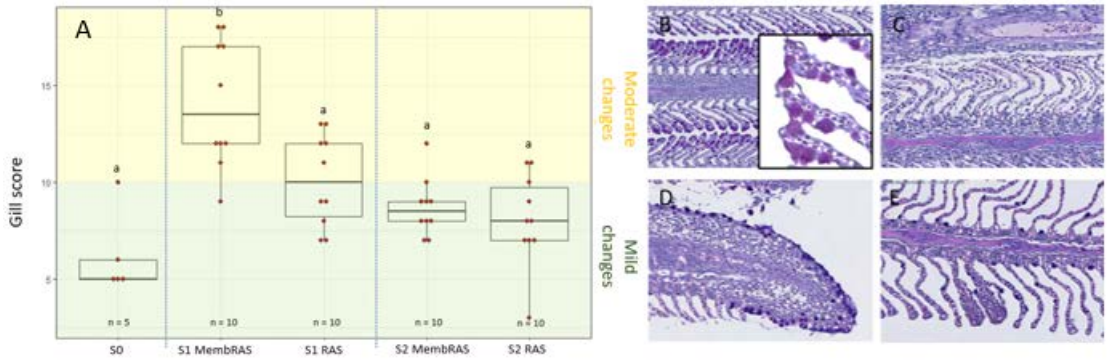

Figure 11 A) Graphical representation of histopathological gill score. Different letters indicated statistical difference. B – E) Typical histopathological findings in histological material from S1. B) Mucous cell metaplasia (with dominance of pink mucous cells), C) Necrotic respiratory epithelial tissue, D) Oedema in epithelial tissue of the primary lamellae, E) Infiltration of inflammatory cells in secondary lamellae.

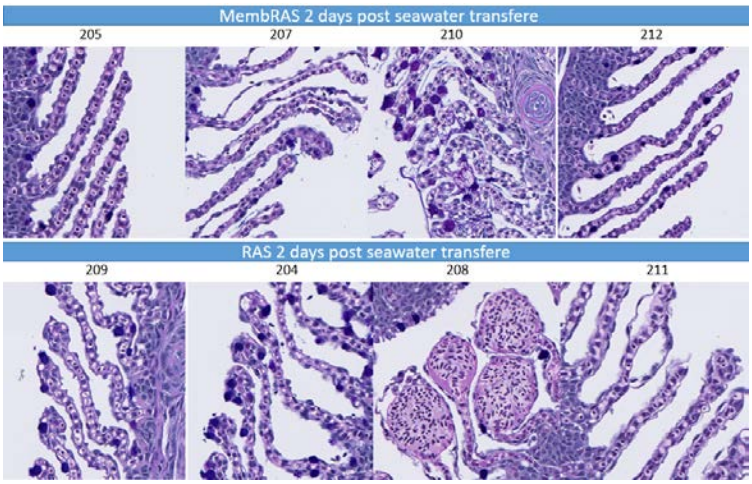

Figure 12 Typical histopathological findings two days post seawater transfer. Tank and treatment as indicated.

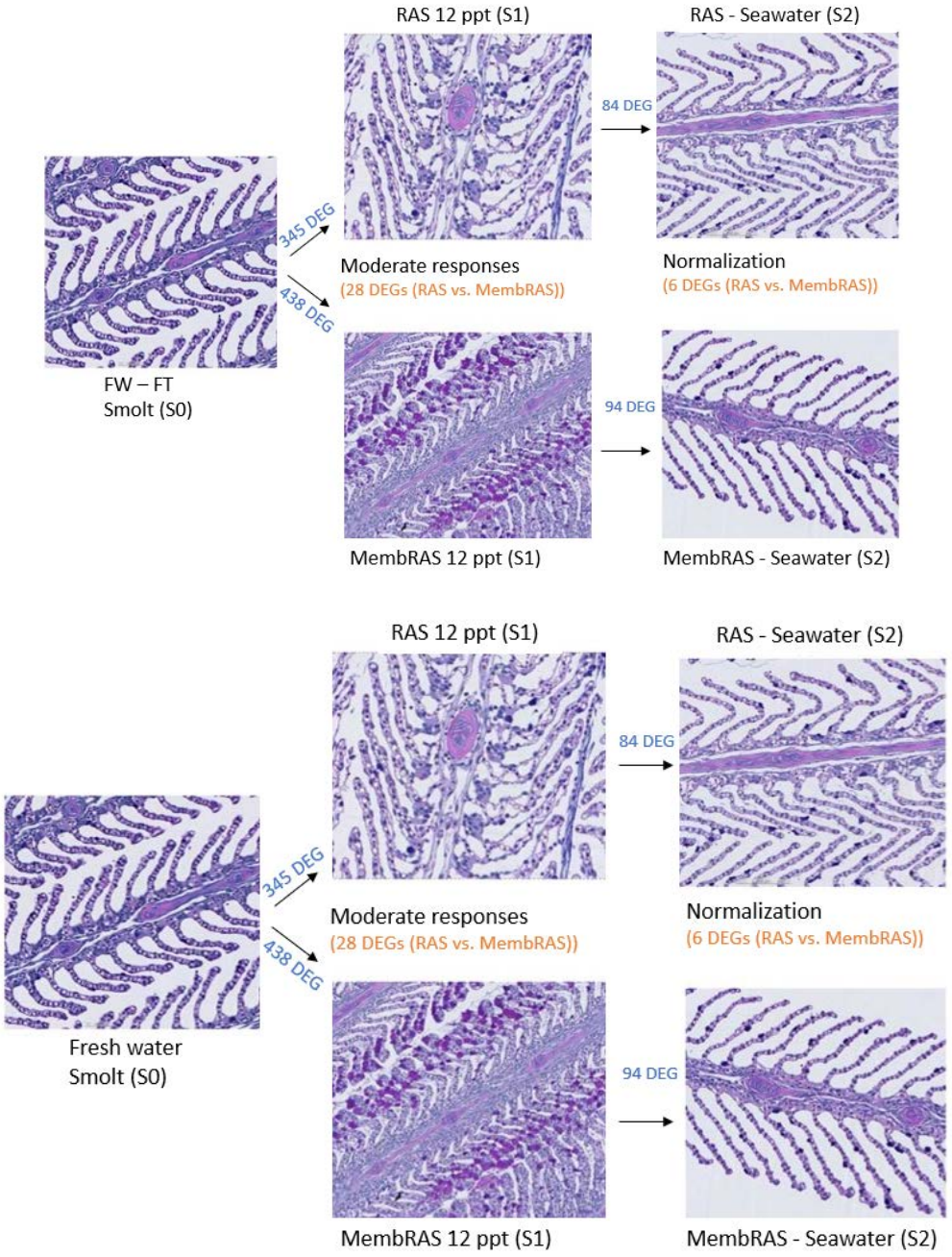

Figure 13 Summary of results, gill – moderate histopathological changes are observed in the gills after transition from freshwater flow through system to brackish RAS systems. Metaplasia and shift from blue to purple mucous cells is the most consistent finding in the MembRAS treatment. There were few differences in DEG at S1 and S2 between MembRAS and RAS (orange text) treated fish. The transcriptional response from freshwater flow through system to brackish RAS systems, and brackish RAS to seawater flow through system is indicated in the figure (blue text).

4 Discussion and conclusion

Post smolt in RAS with nanofiltered water grew slightly better compared to traditional RAS and the same tendency was maintained after transfer to seawater (final average weights were 556 and 491 g, respectively). The mean temperature was slightly higher in the MembRAS due to nanofilter operation compared to the control (0.2 °C), and higher temperature promote growth. However, this was accounted for by calculating the TGC (thermal growth coefficient) for both treatments which account for the day-degree sum (DGR) during the trial. The higher growth in the MembRAS group cannot be explained, but it is known that in seawater sulphate and other ions are abundant and are actively secreted to the surrounding water by the gills and kidney. This process require energy, which then cannot be used for growth. If nanofiltration removes ions from water, the energy requirements for maintaining osmotic homeostasis may be reduced.

Apart from difference in temperature during RAS phase we documented higher pH, salinity, alkalinity, and conductivity in MembRAS compared to the control. This can be contributed to the change in the chemical composition of water that was consequence of nano-filtration and the two different ways we achieved desired salinity in the two systems; in control RAS unfiltered seawater was mixed with ground fresh-water while in MembRAS full strength seawater was nano-filtrated to decrease salinity and remove sulphate. A separate report from NTNU explains in more depth chemical composition of water in both systems. The remaining of measured water quality parameters with known negative effect on fish (CO_2, NO_2-N, TAN) were within recommended values for salmon.

Bactiquant measurements indicated expected effect of nanofiltration as the overall bacteria presence was significantly lower in MembRAS water and on the tank walls in tanks. The same effect was not observed on the bacteria value from the biofilter media. It is known that bacterial communities in biofilters are more stable and diverse compared to water and tank surfaces (Dahle et al., 2020). This can be the reason why nanofiltration did not have as pronounced effect on biofilter as it did on bacterial communities in other RAS locations. Prolonged effect of nano-filtration on biofilter communities should be further investigated.

There were no differences between the treatments in operative welfare indicators or any morphological differences in the skin of the salmon between the two production systems. For skin, there was no effect of the treatment, and the effects of sampling time were as expected. The correlation between mucous cell area and the epidermal area was also high for all parameters, which may indicate a normal state of the epithelial tissue.

For gill morphology, changes related to both time and treatment were identified. The most important effects of nanofiltration were an increase in the area of the gill lamella which was covered by mucous-producing cells and a lower number of chloride cells on the secondary lamellae in the gills. Manual scoring of gill histopathology showed that moving fish from freshwater flowthrough system to brackish RAS with nanofiltration increased the gill score. However, there was a trend of higher gill score for all gills at the end of the RAS phase compared with the other samplings, but this was not found to be statistically significant. It is important to emphasise that most of the changes were referred as minor or moderate and we speculate that they were related to the environmental causes. After four weeks in FT seawater, the gills in both treatments normalized and few moderate changes were observed. Thus, the negative effects of brackish RAS on the gills were reversible in this study. Both RAS in this experiment had belt mechanical filters with rather large mash size of 120 µm due to the producer limitations (eg. screens with smaller mash sizes are not available on the market). In commercial RAS it is common to remove particles that are > 40-60 mm using mechanical filtration. It is possible that the presence of larger particles in both systems were the cause for some of the changes observed on the gills in both RAS. This is further strengthened by the fact that in seawater with lower turbidity no negative effect on

gill histology were observed. Additional analysis of the chemical composition of water over time might shed more light on the causes for gill change observed in two RAS.

The effect of nanofiltration on gene expression in skin and gills was small, with only two genes with different expression in skin and 25 genes in gills after fish were moved from fresh-water flow through system to brackish RAS phase, and even fewer differences after the seawater phase. One DEG deserves attention. Strong up-regulation of cypa1a in control RAS suggested the possible presence of chemical hazard.

5 Literature

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