DTU Aqua
National Institute of Aquatic Resources

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DTU Aqua Report no. 476-2025

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Preface

This report presents the results from the project “New measures to improve water quality with protein skimming, biofiltration and ozone (ProBiOzon)”, journal no. 33111-I-21-076. The European Maritime and Fisheries Fund (EMFF) and the Danish Fisheries Agency funded the project. The project leader was Lars-Flemming Pedersen, and the project period was from March 2021 to October 2023.

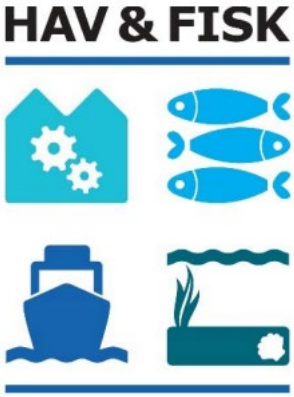
The ProBiOzon project aimed to test, document and develop water treatment technologies to reduce bacteria, organic matter and microparticles in water from recirculating aquaculture systems (RAS). The work included collaboration with industrial partners, commercial RASs, equipment suppliers, the Danish Aquaculture Producer Organization, and DTU Aqua.
We investigated the potential of different technical approaches, including protein skimming, biofiltration, and ozone associated with fish health investigations.

The project included four experimental work packages:
Protein skimming (DTU Aqua Hirtshals; chapter 4), *Effects of ozone* (Oxyguard and DTU Aqua Hirtshals and Lyngby; chapter 5), *Fish Health and pathogens* (DTU Aqua Lyngby; chapter 6) *Biological filtration* (DTU Aqua Hirtshals; chapter 7).

The close collaboration with a commercial freshwater model trout farm and a land-based seawater RAS with Atlantic salmon production allowed long-term experiments with practical and applicable purposes. We appreciate the project's financial support and acknowledge Nr. Vium Dambrug (Videbæk) and Danish Salmon (Hirtshals) for the collaboration and facility access.

DTU Aqua, Hirtshals, February 2025.

Lars-Flemming Pedersen
Senior researcher



Content

1. Extended summary

This report describes the main results from the EMFAF project “New measures to improve water quality with protein skimming, biofiltration and ozone (ProBiOzon)”. The project was led by DTU Aqua and included public research organizations (DTU Aqua Hirtshals & Lyngby), private technology suppliers (OxyGuard Int. A/S), commercial fish farm (Danish Salmon and Nr. Vium model trout farm), and the Danish Aquaculture Organization.

The project focused on applied research with test and development in collaboration with the aquaculture sector, with experimental activities from spring 2022 to fall 2023. The research activities included three types of water treatment approaches: protein skimming, ozone treatment, and biofiltration. The activities combined treatment and removal efficiency on several water quality parameters with veterinarian investigations of fish used in the experiment. The experiments were done under controlled conditions and at two commercial RAS and included several prolonged experiments from 2 weeks up to 40 weeks. Main findings of the project work packages are listed below.

Protein skimming
Prolonged experiments with protein skimmers were conducted at two different commercial aquaculture facilities to assess removal efficiency and improve operation. At the saltwater RAS Danish Salmon, two full-scale protein skimmer models were tested and compared under commercial operation, with controlled ozone dosages and hydraulic retention time. Both models showed similar removal efficiencies based on single-pass water analysis. The hydraulic retention time did not affect removal efficiency, while increasing the ozone dosage significantly improved the removal efficiency of microbes and microparticles, with up to 60% removal of microbial activity in a single pass. An ozone dose of between 7 and 10 g of O_3 per kg of feed was determined to be ideal for this specific salmon RAS.

Another study focused on testing and developing a new concept: a sustainable passive protein skimmer (PPS) prototype designed for model trout farms. This prototype uses existing airlifts to generate foam. Three PPS prototypes were constructed and tested in different raceways at freshwater model trout farm, Nr. Vium Dambrug. Testing and sampling campaigns were conducted during winter and spring and demonstrated a large removal capacity of organic matter, microparticles, and microorganisms.

Extrapolation based on prototypes covering the area of one airlift showed that up to 70% of the available particulate organic matter (measured as BOD_{5part}) could be removed within a day. The removal efficiencies of microbial activity and microparticles were also substantial, reducing their abundance by more than half in the raceway. The current hurdle to implementing advanced filtration technologies is the cost. However, the novel PPS prototype uses the existing airlifts, eliminating additional operational expenses and allowing for low-cost construction. This together with the very high removal efficiency makes PPS a potential key technology to address some of the current challenges in aquaculture.

Ozone treatment
To evaluate the impact of ozone treatment, a continuous 4-week field trial was conducted at a type 3 model trout farm. Ozonated water was compared with untreated water from the same fish

farm. The trial aimed to document the effects of ozone treatment on aquaculture water and simultaneously test and verify its influence on several water quality parameters. Despite fluctuations in the water matrix over the 30-day experimental period, ozonation led to the overall improvements in water quality, without negatively affecting fish welfare. Ozone production was measurable, and a distinct ozone gradient was observed within the system; however, no ozone was detected in the fish tanks. Ozone had a significant effect on several key water quality parameters. It improved UVT and reduced turbidity, resulting in more transparent water. A substantial reduction of microbial activity and biomass was observed with a single pass in ozone-treated systems., Although TAN and nitrite concentrations were high and fluctuating during the trial, ozone effectively oxidized nitrite to the non-toxic nitrate. Due to the short duration of the treatment, the effects of ozone on COD and BOD_5 remained inconclusive.

Fish health and pathogens

The effect of foam fractionation (FF) treatment and ozone treatment on fish health was evaluated through a series of trials. First, a brief pilot study was set up by exposing 10 groups of 40 naïve rainbow trout fry to increasing concentration of organic waste material (foam fraction) for four days, followed by a four-day recovery period. Differences in feeding behaviour were observed in the two highest concentrations (1:6 and 1:3 dilution ratios). Importantly, the pathogen *Flavobacterium psychrophilum* was detected in the organic waste introduced to the fish. Following this pilot trial, a more comprehensive experimental trial was set up to couple organic waste exposure with FF devices, to analyse the changes in microbial water quality and fish health upon treatment. In parallel, a known bacterial pathogen (*Yersinia ruckeri*) was introduced to assess the ability of the FF treatment to modify transmission dynamics and concentrate the pathogen within the foam fraction. The trial was conducted in eight semi-closed RAS systems (30 L tanks; four conditions set in duplicates) over a 11-week period. The survival probability assessed using (Kaplan-Meier estimates was slightly improved in the treated tanks, both in the absence ($p=0.055$) and presence ($p=0.066$) of pathogen infection. *Y. ruckeri* was detected in higher numbers among co-habitants in untreated tanks compared to the those treated with FF. A similar trend was observed regarding *F. psychrophilum*, a pathogen that was introduced with organic waste equally in all tanks. Molecular analysis of water indicated that Y. ruckeri concentrations were approximately five times higher in untreated tanks compared to FF-treated ones, suggesting the ability of FF to lower the circulating pathogen loads. Furthermore, when comparing water and foam fraction from the same system, *Y. ruckeri* concentration were consistently higher in the foam fraction at the most time points, reinforcing FF's potential to lower the amount of circulating pathogen in the systems.

The effect of water ozonation was assessed in the previously described experimental set-up (Oxyguard). Groups of rainbow trout (average weight 20 gr) were placed in four experimental tanks at a stocking density of 14 kg/m^3 of water. Conditions were set in duplicates to compare ozonation treatment to the untreated control systems. Over a one-month period, the observed survival was close to 100% in both conditions, with an average mortality of 1.2% in ozonated tanks and 2% in untreated tanks, and no statistically significant difference being observed ($p=0.16$, Kaplan-Meier survival probability estimation). Fish were sampled (N=10-14 per condition) at the start, midpoint and endpoint of the experiment. Growth was assessed by measuring weight and length, with no significant differences observed between the ozonated and untreated groups at any time point. Internal organs were examined for bacterial infections and compared with rainbow trout from the farm raceways connected to the experimental tanks. In the race-

ways, three known bacterial pathogens (*F. psychrophilum, A. salmonicida, Y. ruckeri*) were detected. In the experimental tanks, *F. psychrophilum* was found both in ozonated and untreated tanks; however, by the final sampling point, no pathogens were detected in fish from the ozonated tanks. Further analyses are ongoing to study the changes in the total microbial communities both in the water and on the host.

Biofiltration

A new biofiltration concept utilized compressible bioelements made of polyurethane, referred to as “French press biofilters,” was developed and tested. These biofilters were installed in a 20 m^3 RAS with 250 kg trout and operated over an 18-week period. The biofilters were tested at two different surface loading rates (high and low hydraulic retention time) and removal efficiencies of dissolved N (TAN and nitrite), organic matter, bacterial load, UVT, and turbidity were assessed. The biofilters demonstrated high treatment efficiency, ease of use, and immediate applicability. Single-pass TAN removal efficiency reached up to 80%, averaging 70% under high flow conditions and 40% at low flow conditions. Substantial reductions of organic matter were consistently observed. When the biofilter elements were squeezed/compressed, significant amounts of organic matter could be easily and safely retrieved.

Following compression and drainage, the biofilter removal performance was either unaffected or slightly reduced, emphasizing that frequent backwashing can be implemented without compromising efficiency. The concept was validated under controlled conditions and is expected to have several practical applications use, e.g. during the start-up of RAS or as a new tool/treatment method to mitigate TAN and nitrite accumulation in RAS.

Nitrification performance during start-up in saltwater was tested in a comprehensive experimental setup. The study tested four different types of biofilter elements -PR Plast PP, PR Plast PE, Mutag PE bioelements, and Levapor polyurethane foam- installed in six biofilters connected to a commercial seawater salmon RAS. Regular spiking experiments were conducted to quantify TAN and nitrite removal under controlled conditions. Volumetric TAN and nitrite removal rates were investigated at both low and high substrate concentrations (1° and 0° order conditions) over a 40-week period. PP and PE bioelements were colonized very slowly during the first two months after start-up. However, their volumetric TAN removal rates (VTR) increased linearly thereafter, stabilizing at 300-350 g TAN/m^3/d by the end of the 40-week trial. In contrast, foam bioelements showed substantial removal rates as early as one week into the trial, peaking after 12 weeks with TAN removal rates reaching up to 575 g/m^3/day at 13°C. However, performance declined over time, and by the end of the trial, VTR for foam bioelements fell below those of the other materials tested. The study provides new information to the aquaculture industry and identify new research question to achieve and maintain high and stable biofilter performance.

2. Dansk resumé

Denne rapport beskriver hovedresultaterne fra EHFAF-projektet "ProBiOzon" Forbedret vandkvalitet med proteinskimming, biofiltrering og ozon. Projektet blev ledet af DTU Aqua og bestod af forskningsorganisationer (DTU Aqua Hirtshals & Lyngby), private teknologileverandører (Oxyguard), kommercielle anlæg (Danish Salmon og Nr. Vium dambrug) og Dansk Akvakultur Organisation.

Projektet fokuserede på anvendt forskning med test og udvikling i samarbejde med akvakultursektoren, og havde diverse forsøgsaktiviteter i perioden fra foråret 2022 til efteråret 2023. Forskningsaktiviteterne omfattede tre typer vandbehandlingsmetoder: proteinskimning, ozonbehandling og biofiltrering. Aktiviteterne kombinerede målinger af rensningseffektivitet på en række vandkvalitetsparametre, sammenholdt med veterinære undersøgelser af fisk anvendt i forsøgene. Forsøgene blev udført under kontrollerede betingelser ved DTU Aqua og ved to kommercielle RAS og omfattede en række langvarige eksperimenter fra 2 uger op til 40 uger. De vigtigste resultater af de enkelte arbejdspakker er angivet nedenfor.

Protein skimning
Længerevarende forsøg med proteinskimmere blev udført på to forskellige kommercielle akvakulturanlæg for at udvikle og dokumentere fjernelseseffektivitet og forbedre driften.
Ved Danish Salmon blev to forskellige proteinskimmere testet og sammenlignet i fuld skala under kommerciel drift, ved tre kontrollerede ozondoseringsniveauer og to hydrauliske retentionstider. Begge typer af proteinskimmere viste enslignende fjernelseseffektivitet baseret på målinger af vandanalyse ved enkeltpassage. De testede hydrauliske retentionstider påvirkede ikke fjernelseseffektiviteten, hvorimod en forøget af ozondosering resulterede i store forbedringer i fjernelseseffektiviteten af bakterier og mikropartikel antal, med op til 60% fjernelse af bakteriel aktivitet i en enkelt passage. En dosis på mellem 7 og 10 g O_3 pr. kg foder blev bestemt til at være ideel til det pågældende landbaserede saltvands RAS.

En anden undersøgelse omhandlede test og udvikling af et nyt rensekoncept, et såkaldt bæredygtigt passivt proteinskimmer (PPS) til brug på model dambrug. Prototypen gør brug af de eksisterende airlifte til at danne og opsamle skum. Tre PPS-prototyper blev konstrueret og testet i forskellige opdrætsenheder på Nr. Vium Dambrug. Test- og prøvetagningskampagner blev gennemført vinter og forår, hvor PPS viste en stor fjernelseskapacitet af organisk materiale, mikropartikler, og mikrober. Såfremt prototypen blev udvidet til at dække en hel airlift, vil op imod 70 % af det tilgængelige partikulære organiske materiale (som BI_{5part}) kunne fjernes på én dag.

Reduktion af mikrobiel aktivitet og mikropartikler var også betydelig, idet der ud fra en dækket airlift blev estimeret en samlet fjernelse svarende til halvdelen af mængden i raceway systemet. Udgift til anskaffelse og omkostning til drift er en vigtig faktor og potential hindring for at implementere avancerede filtreringsteknologier er omkostningerne. Idet den nye PPS-prototype bruger de allerede installerede belufterbrønde/airlifte er der ingen yderligere driftsomkostninger og PPS kan bygges relativt billigt. Dette sammen med den meget høje fjernelseseffektivitet gør PPS til en potentiel fremtidig teknologi der med fordel kan implementeres og løse nogle af akvakulturens udfordringer med at sikre god vandkvalitet.

Ozon behandling

For at klarlægge effekten af ozon behandling, blev et 4-ugers kontinuerligt forsøg udført i felten, ved at RAS vand fra et model 3 dambrug blev ozoneret og sammenlignet med ikke-ozoneret RAS vand fra det samme dambrug. Undersøgelsen havde til formål at dokumentere effekten af ozon-behandling på akvakulturs-vand og samtidigt teste and verificere den potentielle effekt af ozonbehandling på flere vandkvalitetsparametre. Selvom vandmatrixen svingede over den 30 dages forsøgsperiode, blev vandet fra model 3 dambruget overordnet forbedret i kvalitet ved ozonering, uden at det havde en negativ effekt på fiskenes velfærd. Produktionen af ozon var målbar, og en klar gradient af restozon blev observeret efter ozon behandling, uden at der var en skadelig mængde ozon i fisketankene. Ozon havde en tydelig effekt på flere vigtige vandparametre. Ozon forbedrede vandets UVT samt turbiditet, hvorved vandet blev mere klart. En tydelig reduktion i bakterier blev observeret ved en enkelt passering gennem ozonerings behandlingen. Antallet af døde bakterier steg derfor også efter ozonering. Under forsøgsperioden svingede koncentrationen på TAN og nitrit. Ikke desto-mindre blev der påvist en reduktion af det toksiske nitrit til det ikke-toksiske nitrat. På grund af forsøgets pilot-skala, var effekten af ozon på COD og BOD_5 uklar.

Fiskehelse og patogener

Effekten af vandbehandling (proteinskimning eller ozon-behandling) på fiskehelse og fiskepatogener blev undersøgt. Først vha. et piloteksperiment, hvor 10 grupper, hver af 40 naive regnbueørredyngel blev udsat for forskellige koncentrationer af organisk affaldsmateriale (skum fraktion) over en 4 dages periode, efterfulgt af en 4 dages rekonvalescensperiode. Ændring i adfærd hos fiskene i forbindelse med fodring blev observeret blandt fisk der gik i vand med de to højeste koncentrationer af skum (1:6, 1:3). En vigtig detalje var, at patogenet *Flavobacterium psychrophilum* blev fundet i det tilsatte skum/foamate. Som opfølgning på piloteksperiment blev der opstillet et infektionsforsøg hvor tilsætning af skum blev kombineret med akvarier med/uden proteinskimmere, for at analysere ændringerne i den mikrobielle vandkvalitet og fiskehelse i forbindelse med proteinskimning. Parallelt hermed blev et kendt bakterielt fiskepatogen (*Yersinia ruckeri*) tilsat for at undersøge, om proteinskimning (foamate fraktionering) kunne ændre på patogen transmissionsdynamikken samt koncentrere patogenet i skum-fraktionen/foamate fraktionen. Eksperimentet løb over 11 uger og bestod af semi-recirkulerede RA systemer, 8 individuelle 30 L tanke (4 forskellige opsætninger i duplikater. Sandsynligheden for overlevelse (Kaplan-Meier test.) var lidt højere i de behandlede tanke (= +proteinskimning), både uden (p=0.055) og med (p=0.066) patogeninfektion. *Yersinia ruckeri* var til stede i flere af co-habitanterne i ikke-behandlede tanke sammenlignet med i co-habitanter i behandlede tanke. Samme trend sås for *F. psychrophilum*, patogenet som blev introduceret med skummet i alle tanke. Den molekylære kvantificering af vandet, hvor tanke med *Y. ruckeri* infection blev sammenlignet, viste, at den højeste koncentration af *Y. ruckeri* var ca. 5 gange højere i ikke-behandlede tanke i forhold til behandlede tanke, hvilket viser at proteinskimning/foamate fraktionering kan nedsætte mængden af cirkulerende patogen. Yderligere blev det vist, at *Y. ruckeri* koncentrationen var højere i skumfraktionen end i vandet fra det samme system ved de fleste undersøgelsestidspunkter, hvilket tyder på, at proteinskimning/foamate fraktionering kan nedsætte mængden af cirkulerende patogener i systemerne.

Effekten af ozon-behandling af vand blev undersøgt i et eksperimentelt set-up beskrevet tidligere (Oxyguard). Regnbueørreder (gns. vægt 20 gram) blev overført til 4 tanke med tætheden 14 kg/m^3 vand. Forsøget blev opstillet i duplikat, hvor to tanke modtog ozon-behandlet vand og to tanke ubehandlet vand og forløb over 28 dage. Fiskeoverlevelsen under begge forhold var

tæt på 100%, med en gennemsnitlig dødelighed på hhv. 1.2% i behandlede og 2% i ubehandlede tanke, og ingen statistisk signifikant forskel blev fundet (p=0.16, Kaplan-Meier survival probability estimation). Prøveindsamling af fisk (N=10-14/behandling) blev foretaget i starten, undervejs samt ved afslutningen af eksperimentet. Væksten blev undersøgt ved at måle vægt og længde på de individuelle fisk – der sås ingen forskel mellem grupperne. Der blev taget prøver fra indre organer hos fisk til bakteriologisk undersøgelse som blev sammenlignet med de tilsvarende prøver fra fisk der gik i raceway, der var forbundet med forsøgstankene. I fisk fra raceways blev der fundet tre kendte bakterielle patogener (*F. psychrophilum, A. salmonicida, Y. ruckeri*); i forsøgstankene blev der fundet *F. psychrophilum* i både behandlede og ubehandlede tanke. Dog blev der ved sidste prøvetagning ikke fundet patogener i fisk fra de ozon-behandlede tank. Yderligere analyser vil undersøge og belyse ændringer i de mikrobielle samfund i vand og på fisken.

Biofiltrering

Et nyt koncept baseret på anvendelse af komprimerbare bioelementer lavet af skumgummi blev udviklet og testet. Disse ”stempelkande" biofiltre blev installeret i et 20 m^3 RAS anlæg med 250 kg ørred og undersøgt over en periode på 18 uger. Biofiltrene blev testet ved to forskellige overfladebelastning (høj og lav hydraulisk retentionstid), og renseeffektivitet overfor opløst N (TAN og nitrit), organisk stof, bakteriel belastning, UVT og turbiditet, blev undersøgt. Stempelkande filtrene var meget effektive og behandlingskapaciteten viste sig at være stabil høj, nem at bruge og direkte anvendelig. Fjernelse ved enkelt-passage viste, at TAN-fjernelse effektivitet var op til 80%, i gennemsnit 70% og 40% ved højt og lavt flow. Der blev fundet betydelig reduktion af organisk stof (resultater)med en jævn, og ensartet fjernelse over tid. Når biofilterelementerne blev presset sammen, kunne betydelige mængder af organisk stof udvindes på en nem og sikker måde.

Efter sammenpresningen og dræning var stempelfiltrenes ydeevne påvirket eller svagt nedsat, hvilket understreger, at en høj returskylningsfrekvens kan implementeres og sikre effektiv og innovativ procedure. Konceptet blev verificeret under kontrollerede forhold og det forventes, at have en række anvendte anvendelser, eks. under opstart af RAS eller som en ny (mobil) renseenhed til at reducere TAN- eller nitrit akkumulering i RAS.

Nitrifikationsprocesserne (omsætningen af ammonium on nitrite) under opstart i saltvand blev testet i et stort og omfattende forsøg. Undersøgelsen omfattede fire forskellige typer biofilterelementer (PR Plast, PP og PE; Mutag PE bioelementer og Levapor polyurethanskum), som blev installeret i seks biofiltre koblet til et kommercielt saltvands lakse-RAS. Undersøgelsen bestod af regelmæssige spikeforsøg, hvor fjernelseshastigheden af TAN og nitrit blev kvantificeret under kontrollerede forhold. De volumetriske TAN- og nitritfjernelseshastigheder blev undersøgt ved lave og høje substratkoncentrationer (1° og 0° ordensbetingelser) over en periode på 40 uger. PP- og PE-bioelementer blev koloniseret meget langsomt i løbet af de første to måneder efter opstart, men efterfølgende steg den volumetriske TAN-fjernelse (VTR) lineært og nåede stabile niveauer (300-350 g TAN/m^3/d) efter 40 uger. Derimod viste bioelementer af skum betydelige fjernelsesrater efter første uge, og toppede efter 12 uger med TAN-fjomsætningsrater på op til 575 g/m^3/d ved 13°C. Ydeevnen faldt dog over tid, og ved forsøgets var VTR lavere sammenlignet de andre testede bioelementer. Undersøgelsen giver ny information til akvakulturindustrien og peger på nye løsninger til at opnå og opretholde høj og stabil biofilterperformance.

3. Introduction

Water quality remains a critical focus in all aquaculture facilities (Lindholm-Lehto, 2023). This applies across a range of systems from flow through ponds and low-tech farms with low/restricted water reuse (type 1 model trout farm) to recirculation aquacultures systems relying moderate water exchange and biofilters (type 3 model trout farms using 1-5 m^3 water pr. kg feed), as well as FREA systems and land-based RAS (< 1 m^3 water pr. kg feed). The need and requirements for water treatment – mechanical, biological, or chemical – varies between systems and is under constant surveillance. Ongoing research and development efforts aim to improve rearing practices to make fish production more sustainable. Currently, some Danish model trout farms have production conditions that call for further improvement, as they may face some of the circumstances listed below:

- High fish mortality rates
- High operational costs, such as
  - Electricity, depuration, insufficient or suboptimal treatment routines
  - Delays and reduced utilization of production units (i.e., during startup of biofilter or in periods with depressed feeding associated with impaired water quality)
- Excessive use of chemical disinfectants
- Foam formation and related problems (equipment and fish).

Addressing these issues is essential to promote the implementation and acceleration of more efficient, sustainable fish production methods. The goal is to reduce environmental impacts (lower CO_2 footprint and improved effluent quality) and decrease the use of chemical disinfectants.

3.1 Background

Production of fish at high intensities can be achieved by provision of sufficient make-up water or by reusing treated water. Over the past two decades, flow through fish farms have been replaced or substituted by model trout farms, which allows reduced water consumption, stable conditions, improved water treatment, and reduced discharge (Jokumsen & Svendsen, 2010; Heldbo et al, 2013). When water is recirculated and treated biologically and mechanically, like in type 3 model trout farms, its quality and composition changes. The retention time increases, leading to higher concentrations of dissolved and particulate organic matter, which in turn will increase microbial biomass. A common method for controlling pathogens and reducing microbial loads involves the use of chemical disinfectants such as formalin, hydrogen peroxide, peracetic acid, or sodium chloride (Pedersen et al, 2013). Ozone and UV are existing alternative or supplements to the chemical disinfectants; however, being mainly applied in closed, intensive RAS (de Jesus Gregersen et al, 2022; Kovacs et al, 2023).

Direct ozonation have not yet been applied in model trout farms, due to the high investment and running costs (Janning et al, 2012). However, since ozone generation has become more efficient, there is potential to test whether water disinfection with ozone can replace the suboptimal use of chemical disinfectants by providing improved water quality and enhanced fish performance.

3.2 Water treatment and water quality

Stable high oxygen concentration, reduced CO_2 levels, acceptable pH, and minimal dissolved iron concentrations can be achieved by aeration, degassing, base addition, and prefiltration. Excreted ammonium/ammonia may accumulate in systems with high feed loading but can be avoided with a well-designed biofilter compartment. While the accumulation of particulate organic matter can be prevented by using mechanical treatment units such as sludge cones and drum filters, managing suspended biosolids, microbes, and microparticles is more difficult (Table 3-1). Fixed bed biofilters serve as a primary treatment unit, entrapping particulate matter and facilitating its degradation by microorganisms. They require regular maintenance or cleaning through a process called backwashing. Recently, protein skimming (or foam fractionation) has shown promising potential for removing waterborne microbes or microparticles. Although it has been previously only applied in saltwater systems, the foam can also get produced and removed in freshwater RAS.

Table 3-1. Effects of RAS cleaning devices and disinfection methods on system microbiota (modified after Vadstein et al., 2018).

Water treatment	Type/principle	Mode of action on bacteria in the water phase		
		Reducing	Inactivating	Removing
Sludge cones	Solids removal	+	-	-
Swirl separator	Solids removal	+	-	-
Drum filter	Solids removal	+	-	+
Pool vacuum cleaner	Solids removal	+	-	+
Contact/particle filter	Solids entrapment/degradation	+	-	+
Membrane	Solids removal	+	-	+
Fixed bed biofilter	**Solids entrapment, biofilm, and reduction of dissolved C**	**+**	**-**	**+**
Moving bed biofilter	Biofilm formation/release reduction of dissolved C	(+)	-	-
Protein skimmer	**Removal of microparticles, bacteria and dissolved C**	**+**	**+**	**+**
Clay	Flocculation/sedimentation	+	-	-
Ozone	**Disinfection**	**+**	**+**	**+**
UV	Disinfection	-	+	-
Formaldehyde/		+	+	-
Peracetic acid/H_2O_2	Chemical disinfection	-	+	-

3.3 Project structure

The project has been carried out by DTU Aqua in collaboration with Oxyguard, Aquacircle, Nr. Vium Dambrug, Danish Salmon, and the Danish Aquaculture Producer Organisation.

The project was comprised of six work packages (WP), of which two included project management, administration meetings, and dissemination at seminars (including teaching and reporting). Four WPs included practical investigations, with experiments and applied research with aquaculture water subjected to different types of water purification and water treatment (Figure 3-1).

The experiments were carried out at DTU Aqua's controlled experimental facilities at the Section for Aquaculture located in Hirtshals and at DTU Aqua's experimental facility in Lyngby. In addition, trials, and documentation of the cleaning technologies (protein skimming and ozone) were

conducted at Danish Salmon, Hirtshals (commercial land-based saltwater salmon RAS) and at Nr. Vium Dambrug (a commercial model farm producing trout in freshwater.

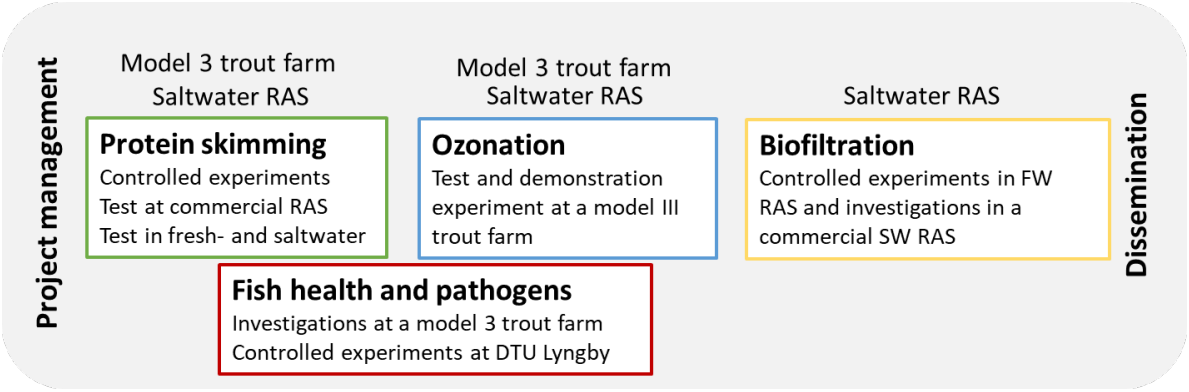


Figure 3-1. Description of project structure. Protein skimming (WP2), Ozonation (WP3), Biofiltration (WP4) and Fish health (WP5).

3.4 Aim of the project

The aims of project ProBiOzon were to test, document and develop new cleaning technologies on freshwater and saltwater farms to reduce the environmental impact and create better rearing conditions. The investigated cleaning technologies included protein skimming, ozone treatment and biofiltration. In combination with the treatment technologies, fish welfare were evaluated.

Details of each of the work packages are described in the subsequent chapters: 4) Protein skimming, 5) Ozone treatment, 6) Fish health and pathogens, and 7) Biofiltration.

4. Protein skimming

4.1 Background

The accumulation of organic matter and microparticles in RAS is a multifaceted problem stemming from several sources: uneaten feed, faecal matter, excretion of metabolic waste, biofilms, and the continuous influx of particulate matter from external sources. When coupled to low water change rate, these lead to accumulation of both dissolved and particulate organic matter. This will compromise water quality by increasing turbidity, increasing oxygen consumption, elevating concentrations of nitrogenous compounds, and creating conditions conducive to the proliferation of bacteria. Maintaining high water quality is imperative for maximizing the growth rates, survival rates, and overall well-being of the aquatic organisms being cultivated. Furthermore, the environmental sustainability of RAS practices depends on minimizing nutrient discharges and their potential to harm surrounding ecosystems. To address these issues, RAS operators are continuously seeking innovative, efficient, and sustainable methods to control accumulation of organic matter and microparticles.

Typical organic matter removal methods in aquaculture are settling of organic matter or removal by filtration (drum filters). While both methods are extensively used, they are limited on the size of particles they can affect, as only particles above 50 µm can be easily removed, leaving the remaining particulate and dissolved matter behind. One promising technology for organic matter removal is foam fractionation. Foam fractionation (or protein skimming) is a separation process that uses of the adsorption properties of surfactants to concentrate and remove both dissolved and particulate compounds from the water (Aalto et al., 2022a; Brambilla et al., 2008; Chen et al., 1994, 1993a, 1993b; de Jesus Gregersen et al., 2021; Figueiras Guilherme et al., 2020). Foam fractionators remove organic matter by mixing air bubbles and water and creating foam. Surfactants are the core of foam fractionation (Figure 4-1).

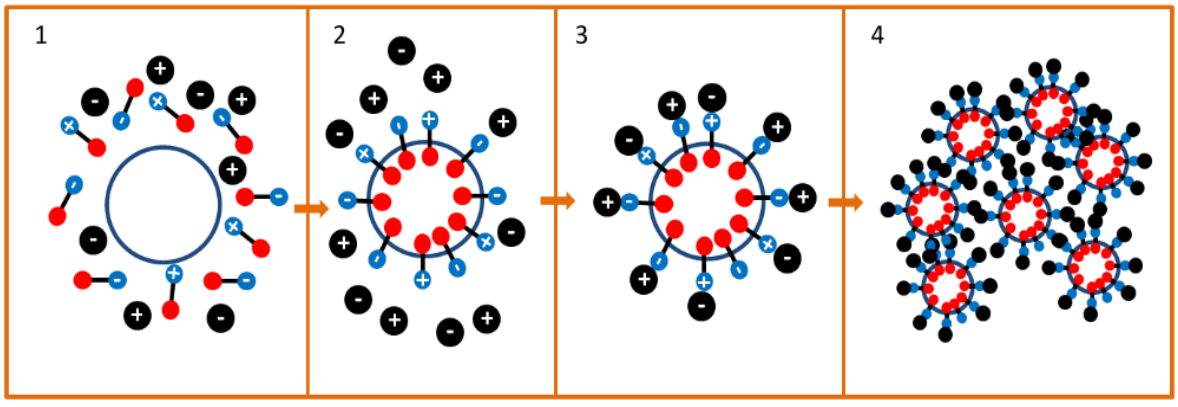


Figure 4-1. 1) Surfactants (blue and red molecules) are the drivers behind protein skimmers foam formation. 2) The hydrophobic end (red circle) of the surfactant pokes into an air bubble, while the hydrophilic end (blue circle) stays in the water. 3) The hydrophilic end is generally charged (positively or negatively) and attracts molecules with opposite charges. 4) Air bubbles with their attached surfactants and additional molecules join to form foam (de Jesus Gregersen, 2020).

Surfactants are molecules that possess both a hydrophobic end, which tends to repel water, and a hydrophilic end, which has an affinity for water. Consequently, the hydrophobic end seeks to escape the water, while the hydrophilic end prefers to remain immersed in it. This behaviour

leads to the hydrophilic end inserting itself into an air bubble, with the hydrophobic end staying within the water. Most surfactants carry an electrical charge, either positive or negative, which attracts oppositely charged molecules, ultimately resulting in the formation of foam (Brambilla et al., 2008; Timmons and Ebeling, 2010).

While foam fractionation has been successfully in indoor saltwater facilities over the last decade, it's potential within the specific and challenging conditions of RAS, both in saltwater and freshwater environments, remains a topic of active research, with a clear lack of concrete information on the use of foam fractionation in commercial conditions. Recent research has shown that foam fraction can be applied in fresh water with very high removal of organic matter (de Jesus Gregersen et al., 2021; Jafari et al., 2021), which until recently was not considered to be possible. However, while the practical application in pilot scale was demonstrated, its applicability to commercial systems remains to be tested.

Aim

In the work package 2, the applicability of foam fractionation in commercial freshwater and saltwater RAS was tested in two experiments. In the first one, the commercial operation of foam fractionation in saltwater RAS was tested under different operational conditions. In the second set of trials, a freshwater foam fractionator was developed and tested in a commercial model trout farm (MTF).

4.2 Comparison of large-scale skimmers in a commercial saltwater RAS

A full detailed description of this section can be found in the publish article titled "Evaluating protein skimmer performance in a commercial seawater recirculating aquaculture system (RAS)" attached to this report (Chapter 10.1).

4.2.1 Introduction

Foam fractionation in saltwater systems has been applied in large commercial facilities over the last decade. By removing micro particles and dissolved organic matter, foam fractionation leads to a reduction on the amount of organic matter in systems, which in turn reduced the available "food" for bacteria and leads to a reduction the systems carrying capacity, resulting in the lower microbial activity and improved system performance. Foam fractionation is typically paired with the use of ozone (O_3). Ozone is a very strong oxidant, with strong oxidative properties (Aalto et al., 2022a; Hess-Erga et al., 2008; Powell and Scolding, 2018; Summerfelt and Hochheimer, 1997), which also enhances solids removal (Davidson et al., 2011; de Jesus Gregersen et al., 2021; Rueter and Johnson, 1995). This combination tends to lead to improved performance and higher organic matter removal rates than foam fractionation alone. However, due to the toxicity of ozone, excess doses of ozone can have detrimental effects on the production, requiring tight control over the doses applied. Contact time is a key factor on the operation of foam fractionation and disinfection capabilities of ozone.

This study aimed to better understand the optimal operational conditions (contact time and ozone dose) of two commercial-scale foam fractionators and to quantify the effects of foam fractionation in a commercial setting (single pass).

4.2.2 Experimental design and set up

The trials were conducted at Danish Salmon A/S, Hirtshals, Denmark. Two commercial foam fractionators (Ratz 2500 Hi, CM Aqua Technologies Aps, Denmark and (Helgoland 2500 ×4500

LE315, Erwin Sander Elektroapparatebau GmbH, Germany, Figure 4-2) were installed on the same 7000 m^3 full strength saltwater grow-out facility (full description of the system can be found in the attached publication (Kovács et al., 2023) (see 10.1). To test relevant parameters, a 2×2×3 multifactorial experiment was conducted, testing 2 foam fractionators, two hydraulic retention times (HRT; low HRT - 1.8min and high HRT - 2.2 min) , and 3 dose levels of ozone (0, 7 and 14g O_3 per kg of feed) (Table 4-1). Each test was repeated 3 times. Samples were collected before and after the foam fractionator to measure changes in different parameters.

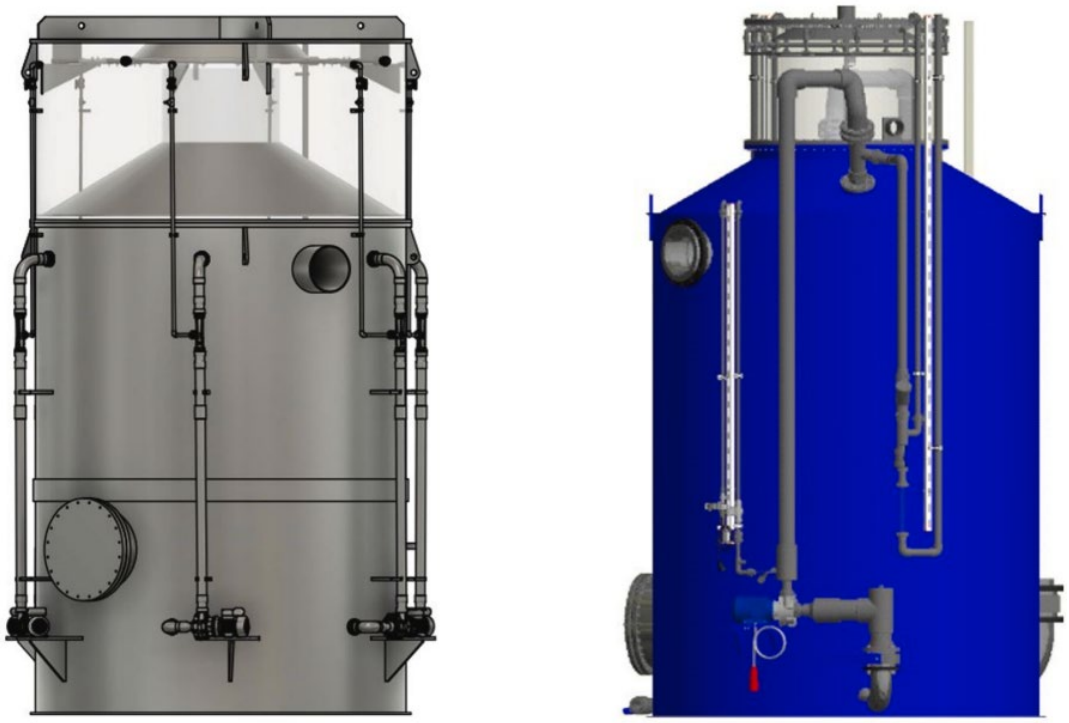


Figure 4-2. The two types of protein skimmer models tested: S1 (Ratz 2500 Hi, CM Aqua Technologies Aps, Denmark) on the left and S2 (Helgoland 2500 x 4500 LE-315, Erwin Sander Elektroapparatebau GmbH, Germany) on the right.

Table 4-1. Specifications and primary operational parameters of the two protein skimmers during the experiment.

Parameters	**Ratz 2500 Hi**	**Helgoland 2500 x 4500 LE-315**
Water volume (m^3)	17.2	16.7
Water flow (m^3 h^{-1})		
Low HRT (1.8 min)	573±5	556±5
High HRT (2.2 min)	469±5	455±5
O_3 dose (g h^{-1}) (doses varied based on feeding)		
T0	0	0
T7	390-520	390-520
T14	780-1040	780-1040
Air flow rate (m^3 h^{-1})	23±2	22±2

4.2.3 Results

The operation of both skimmers allowed for one of the first assessment of foam fractionation under full scale commercial operation, as well as the comparison of similar sized commercial units. The operational condition with the largest impact was the ozone dose. The application of foam fractionation without ozone resulted in low removal efficiency of microbial activity; only a 4.1± 9.3%. A significant removal efficiency (microbial inactivation) was achieved by higher ozone doses. ($p < 0.001$): being was 28.9±7.6% in T7 and 60.3±8.2% in T14 during a single pass (Figure 4-3).

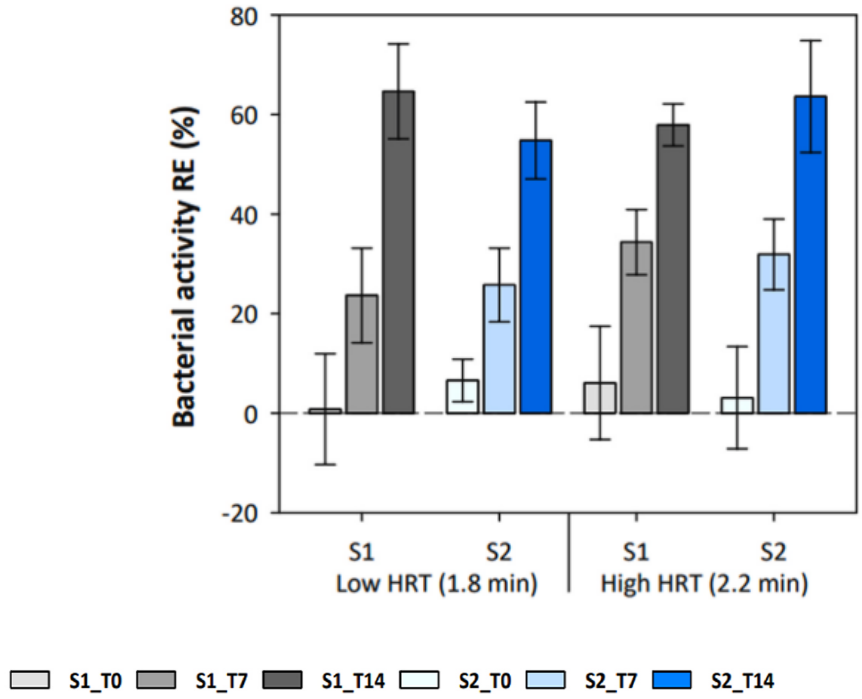


Figure 4-3. Mean ± sd (n = 3) one-pass removal efficiencies (RE) of microbial activity. The treatment combinations included two types of protein skimmers (S1 and S2), two levels of hydraulic retention times (HRT) (1.8 and 2.2 min as Low and High) and three ozone doses (0, 7, and 14 g O_3/kg feed as T0, T7, and T14).

Ozone affected the removal efficiency of particles similarly to microbial activity, the efficiency increasing with increasing ozone dose ($p<0.001$). Removal efficiency of the number of particles increased from 2.6% in the T0 treatment, to 37.7 and 51.8% in T7 and T14 respectively (Figure 4-4).

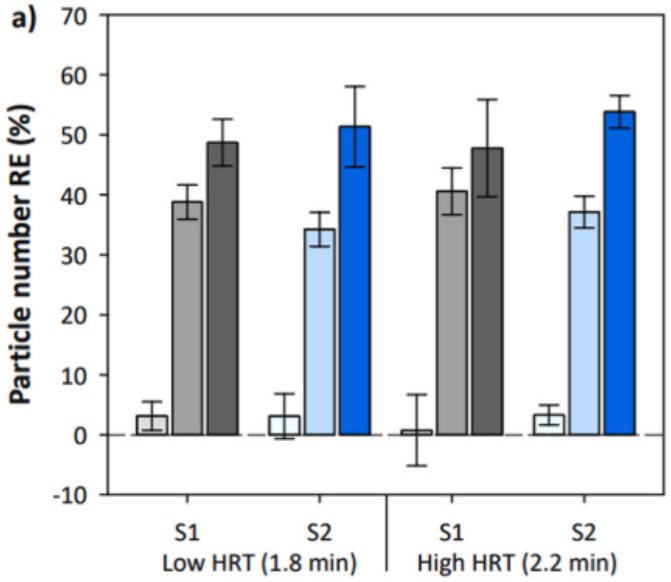


Figure 4-4. Mean ± sd (n = 3) one-pass removal efficiencies (RE) of total number of particles. The treatment combinations included two types of protein skimmers (S1 and S2), two levels of hydraulic retention times (HRT) (1.8 and 2.2 min as Low and High) and three ozone doses (0, 7, and 14 g O_3/kg feed as T0, T7, and T14).

Strong correlations were found between multiple factors. Notably, there was a correlation of over 0.87 between microbial activity and particle numbers, and the associations between microbial activity and both ORP and TRO were similarly strong (0.89 and 0.91, respectively). In general, both foam fractionators performed similarly, with the only significant difference found in the redox potential (ORP), where S1 achieved higher levels of ORP (Figure 4-5). Increasing HRT had also limited impacts, only affecting the ORP and total residual oxidants (TRO) present in the system (an increase in both cases, with increase in FRT).

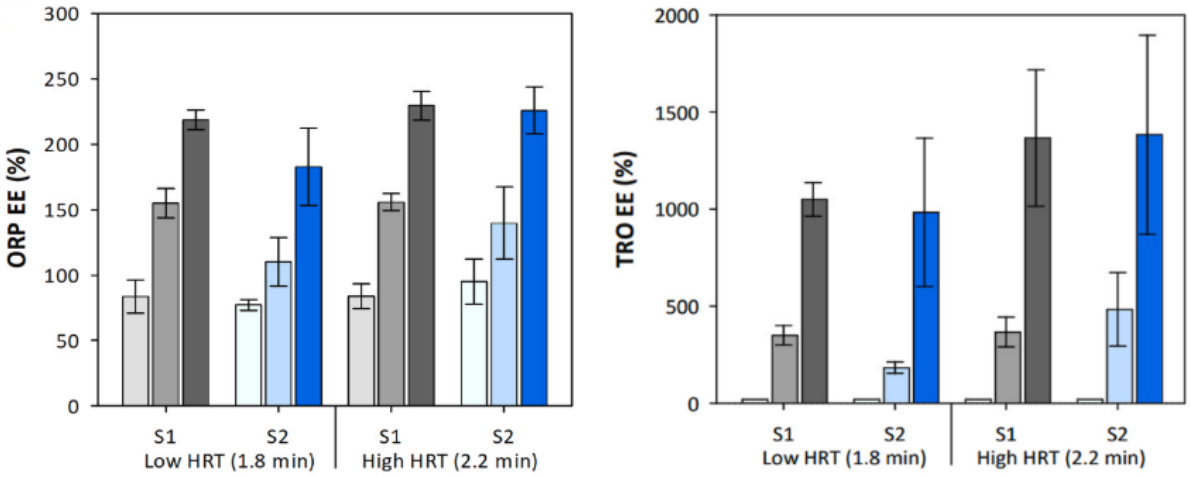


Figure 4-5. Mean ± sd (n = 3) ORP and TRO enhancement efficiencies (EE) based on different HRT and ozone doses. The treatment combinations included two types of protein skimmers (S1 and S2), two levels of hydraulic retention times (HRT) (1.8 and 2.2 min as Low and High) and three ozone doses (0, 7, and 14 g O_3/kg feed as T0, T7, and T14).

4.2.4 Discussion

Impact of Protein Skimmer Design

The performance of the two types of protein skimmers was found to be identical across several water quality parameters. This is likely due to their similar setups in flow pattern and dimensions. The oxidation-reduction potential (ORP) was the only parameter influenced by the design, being higher in S1 compared to S2. As ORP reflects the water's "oxidation power," this result indicates a higher ozone concentration and the production of oxidative radicals within the protein skimmer (Gonçalves and Gagnon, 2011). The difference in ORP between S1 and S2 can be attributed to S1's gas recycling system, which recirculates a portion of the degassed air and ozone back to the reaction chamber, unlike S2, which only receives fresh gases. While the utilization of ozone is economically significant, the higher ORP with S1 did not result in significant improvements in other water quality measures, indicating that the protein skimmer performance in both S1 and S2 can be considered equally effective under the given conditions.

Influence of Hydraulic Retention Time (HRT)

HRT is a critical operational factor in protein skimming. Prolonged HRT enhances the contact time between gas and water, thereby increasing surfactant extraction (Buckley et al., 2022; Wheaton et al., 1979). However, some studies have reported negligible or even negative long-term effects of extended HRTs on solid removal (Peng and Jo, 2003; Weeks et al., 1992). Typical HRT in commercial settings is around 2 min. In this trial, the minimum and maximum achievable HRT values (constrained by the pumps employed) were chosen. Given the low improvements observed, the slight benefits of prolonged HRT may be counteracted by the higher daily water turnover when using a shorter HRT.

A longer HRT substantially increased the ORP in S2, while the levels of total residual oxidants (TRO) increased in both protein skimmers. This is because extended contact time exposes the same water to a larger ozone volume, leading to an increase in oxidation potential and by-product formation (Summerfelt, 2003). Our study did not examine the potential formation of trihalomethanes (THM) or halogenated bromates, which can pose a risk when ozone is overdosed in

saltwater (Legube, 2011). Additionally, a significant interaction effect between the protein skimmer and HRT revealed that changes in ORP were more pronounced with S2 than S1, where ORP had already plateaued at low HRT. This suggests that within the current system, the actual dissolved ozone concentration reached saturation at the highest average ORP level of 500 mV (Summerfelt and Hochheimer, 1997).

Impact of Ozone Dose

Without ozone, microparticles and microbial activity were not consistently reduced during a single passage through the protein skimmers, but ozone addition significantly improved the removal of microbial activity, which was nearly doubled with the increased ozone dose. Previous research suggests that ozone destroys cell membranes and nucleic acids, making it effective in removing hydrophilic, free-living bacteria that are challenging to eliminate with protein skimming alone (Sharrer and Summerfelt, 2007; Summerfelt, 2003). Our observations corroborate the findings of Figueiras Guilherme et al. (2020), who found that the integration of O_3 resulted in enhanced microalgal removal, and de Jesus Gregersen et al. (2020), who demonstrated the removal of a significant portion of living microorganisms in RAS through disinfection.

Ozone, a potent oxidizing agent, can enhance the decomposition of organic compounds and the breakdown of complex molecules when integrated with protein skimming (Lekang, 2007). Although ozone decreased turbidity and increased UV transmittance (UVT), the outcomes were inconsistent. This was likely due to limitations in measurement accuracy, minor changes during a single pass and the consistently clean RAS water maintained by continuous ozonation and protein skimming before and during sampling. The particle size distribution (PSD) analysis indicated that, without ozone, the protein skimmer had a limited efficiency in reducing particle number, volume, and surface area. It was more efficient at removing larger (6–12 µm) particles than the smaller ones. Conversely, when O_3 was introduced, the total particle removal increased significantly, with the highest removal shifting from larger to smaller particles (1–2 µm).

Water Quality relations

Microbial activity exhibited a significant correlation with PSD metrics, implying that most of the removed particles in the 1–12 µm size range were microorganisms. This explains the observed low ozone demand. Microorganisms in RAS can exist as free-living, associated with microparticles, or embedded in biofilm or bioflocs. The PSD analysis suggested that most particles were primarily free-living microbes. With ozone addition, microbial removal increased significantly due to cell destruction, while overall particle removal showed only a slight increase.

Turbidity correlated moderately with all metrics except UVT, indicating that the water cloudiness was primarily due to suspended solids, including microorganisms (Schumann and Brinker, 2020). When particles and microbes are removed, turbidity improves in a similar manner. All PSD metrics were significantly correlated with the rise in ORP and TRO, reflecting the influence of ozone introduced into the water and the system's ozone demand dynamics.

The differential impact of ozone on microbial activity and particle removal highlights the presence of a significant number of biofilm-bound bacteria, which potentially exceed the 1–12 µm size range. Investigating a broader size range (e.g., 1–80 µm) would be essential to draw further conclusions on the PSD and microbial activity relations. Nonetheless, in an intensive commercial-scale RAS, microparticle concentrations are assumed to be primarily driven by microorganisms. Inhibition of microbial activity was closely associated with the TRO concentration, as ozone, while being selective in its reactions, can produce by-products with a wide reactivity range and strong oxidizing capacity. Given the presence of microbes in the 1–12 µm range and

the relatively low ozone demand, the system had substantial by-product formation and subsequent TRO concentration increase.

4.2.5 Conclusions

Overall, the findings suggest that the combination of protein skimming and moderate ozone doses improves overall water quality and regulates critical water quality factors, especially trough the inactivation of microorganisms, resulting in a reduction of the microbial activity and microparticles, in a commercial saltwater RAS. Further investigations focusing on foamate-based mass balances will yield additional insights into the protein skimmer's functioning.

The study identified the ozone dose as the main factor influencing several water quality indicators. However, higher doses nearing the system's ozone demand fostered TRO formation. Therefore, a 7 g O_3/kg feed dose appeared to optimize performance without compromising production. Incrementing the HRT affected only particle volume removal and ozone-related measurements, implying that the benefits of the longer 2.2 min HRT might be offset by the increased daily water turnover achieved with the shorter 1.8 min HRT. Furthermore, the design of the protein skimmer impacted only ORP levels, without significant enhancements in other water quality metrics. Consequently, both S1 and S2 designs were considered equally effective.

4.3 Test and development of passive foam fractionators

4.3.1 Introduction

A full detailed description of this study is available in the published article “Testing of a passive foam fractionator prototype in a commercial recirculating trout farm” attached to this report (Chapter 10.3)

Foam fractionation in freshwater has, until recently, been considered difficult and relatively inefficient. This is due to the lower surface tension of freshwater vs. saltwater. Foam fractionation relies on the production of foam by mixing air and water. Smaller bubbles produce higher surface areas of contact between the water and the bubbles, which in turn improves foam fractionation efficiency. This is easier to achieve in saltwater which has higher surface tensions than freshwater. However, in a previous study by de Jesus Gregersen et al., (2021) foam fractionation in freshwater were found to have very high organic matter removal capability and substantially improve overall system performance, in line with saltwater foam fractionation. However, commercial application is still debated due to the high cost involved in acquiring the equipment and the achievable efficiencies in commercial settings.

This study aimed at developing a low-cost foam fractionation prototype to be used in model 3 trout farms and test its efficiency under commercial conditions (Figure 4-6).

Figure 4-6. Model trout farm.

4.3.2 Experimental design and set up

All tests were conducted at Nr. Vium Dambrug, a model 3 trout farm located near Videbæk, in Midwest Jutland, Denmark (Figure 4-7). The farm consisted of 4 main production units (raceways). To account for system variability and check for process efficiency, 3 units were sampled at each visit. To test the efficiency of the prototype freshwater foam fractionator, multiple sampling events were made to the farm at different times of the year to account for production variability, as well as environmental changes (e.g., temperature).

Figure 4-7. Pictures of Nr. Vium Dambrug, with a fish section on the first picture and the fixed bed biofilter section on the second

Prototype of passive foam fractionator

Model trout farms use airlifts to aerate and move the water around the facilities (Figure 4-8a). These airlifts consist of deep sections where air is injected at the bottom. The rise of the air bubbles to the surface due to the lower density of the injected air in the water creates movement of

the water. An unintended consequence of this is the formation of foam in the farms (Figure 4-8b).

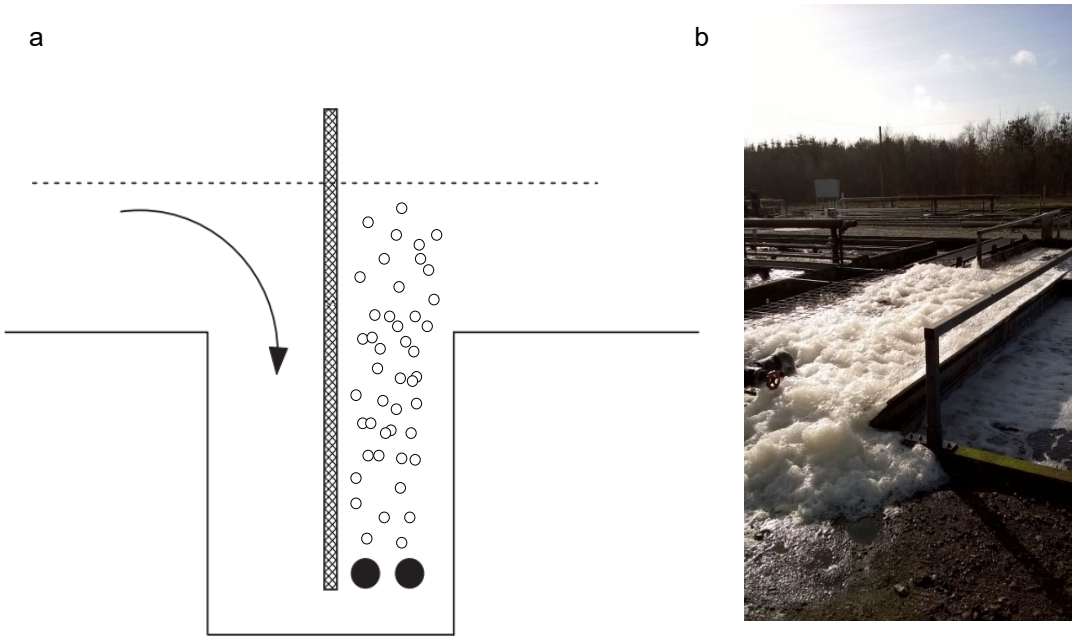


Figure 4-8. a) Schematic of an airlift, with injectors (black circles) at the bottom of a deep shaft injecting air bubbles into the water (modified from (Jokumsen and Svendsen, 2010).
b) Foam formation over an airlift at a model trout farm.

Each of the four rearing systems have 6 airlifts per raceway. The air lift tested measured 1.9 m by 5 m, with a surface area of 9.5 m^2. In simple terms, the prototype consists of an inverted rectangular funnel, measuring 100 cm in length, 50 cm in width and 50 cm in height, occupying a surface area of 0.5 m^2 (Figure 4-9). The funnel walls are place at a 45^o angle. The prototype was made of polyethylene. It was held over the airlifts on a set of 4 stainless steel arms with height adjustment, so fine tuning of removal can be achieved. Foam overflows through the middle, into two channels on either side of the opening. Collection ports at the end of the channels allow for sample collection. To compare efficiencies, three identical prototypes were made and tested on three different production units.

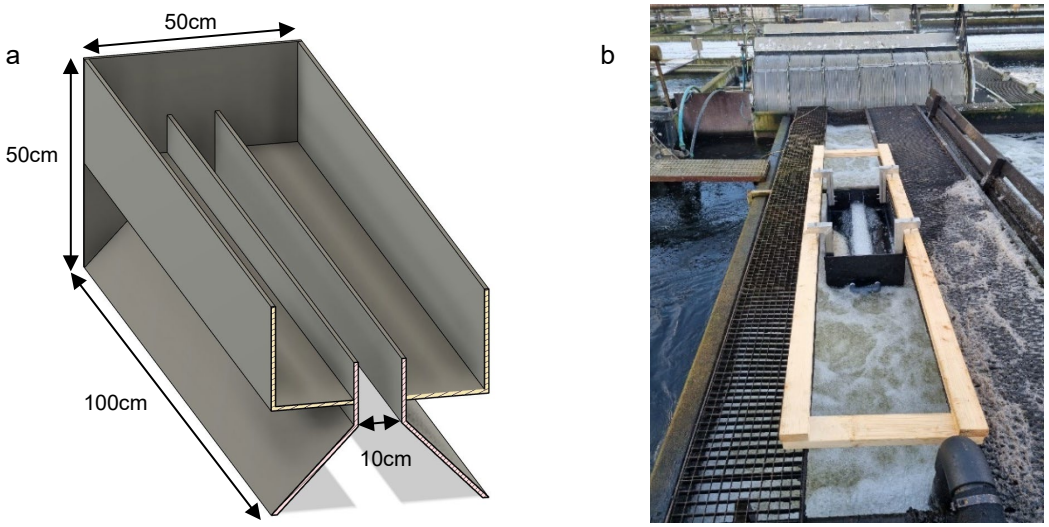


Figure 4-9. a) Schematic view of the prototype foam fractionator. b) Foam fractionator installed in a commercial facility, with an operational area of 0.5m^2.

Sampling

During sampling days, the prototypes where randomly installed in each of the 3 raceways and adjusted as needed. Once everything was set up, the prototypes where emptied and then foam was collected for 1 minute. The total amount of foam collected was transferred to a 10 L container and the exact volume of sample collected noted down.

During the first sample collection on each of the systems, a 5 L water sample from the production unit was also collected.

Each unit was sampled multiple times (Figure 4-10). The number of samples collected varied depending on the sampling day, but a total of 48 samples were collected, 24 during the late fall/winter (17/11-22, 07/12-22, and 18/01-23), and 24 during spring (23/05-23 and 30/05-23).

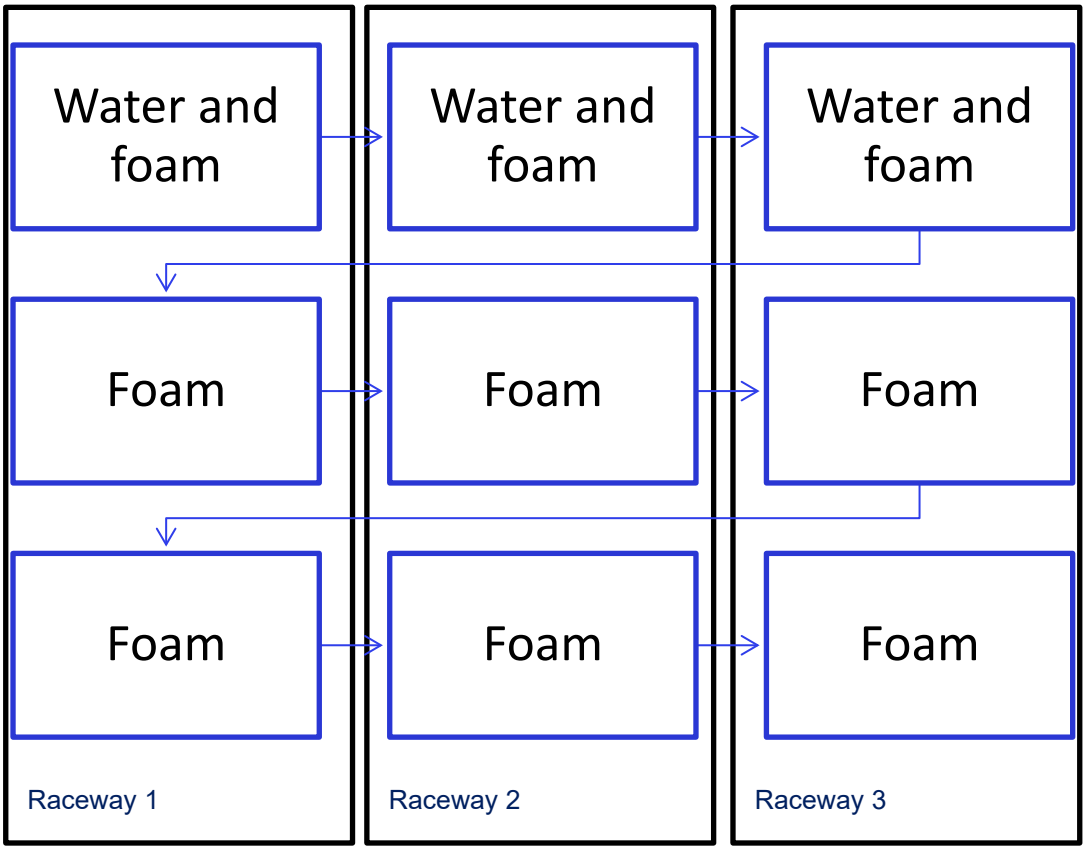


Figure 4-10. Sampling order used in all sampling dates.

Sample analysis

The samples collected (both water and foam) were all treated in the same way. A subsample of 2 L was placed in a beaker over a magnetic stirrer, with a stirring bar inside, to ensure proper mixing of the samples. The samples where then split in individual samples for analysis.

Microbial activity was measured using BactiQuant (Mycometer A/S, Denmark), expressing microbial activity as BQ values. Organic matter was measured has both biological oxygen demand after 5 days (BOD_5) and chemical oxygen demand (COD). In the case of BOD_5, total (unfiltered samples) and dissolved (samples filtered on a 0.45 µm, Advantec® membrane filter, Toyo Roshi Kaisha Ltd, Japan) fractions where measured. The particulate fraction ($BOD_{5\text{-PART}}$) was calculated as the difference between the total and the dissolved fractions.

BOD_5 was measured following ISO 5818 (1989) modified by the addition of allylthiourea (ATU). COD was measured following ISO 6060 (1989). Microparticles between 10 µm and 200 µm were measured using an AccuSizer 780 SIS (Particle Sizing Systems, Santa Barbara, CA, USA). Total P concentration was measured following ISO6878 (2004), while NO_3 was measured according to ISO7890-1 (1986). NO_3 was the only measurement performed exclusively on the water samples.

Calculations

To estimate the potential removal capacity under commercial conditions, the calculations were made assuming a full airlift was fitted with the prototypes and was operated constantly over 24 hours. The airlift measured 1.9 m by 5 m (approximately 9.5 m^2), about 19 times the size of the used prototype. The results were calculated in relation to the amount present in the water of the

production unit. Therefore, the results are presented as relative removal efficiency (% of the total present in the raceway)

First, the total amount of each metric in the production raceway was calculated as:

$$total\ in\ the\ production\ unit\ = Volume\ of\ production\ unit * concentration$$

For each of the prototype, the total amount of each metric removed was calculated for each individual sample as:

$$total\ removed\ (sample\ 1) = Volume\ of\ foam\ (sample\ 1) * concentration(sample\ 1)$$

To calculate the potential maximum removal efficiency, the following equation was used:

$$Potential\ maximum\ removal\ efficiency = \frac{\frac{(total\ remove\ sample\ 1 + \cdots + total\ remove\ sample\ n)}{n} * 1440 * 19.5}{Total\ in\ the\ production\ unit}$$

Where n is the total number of samples collected in each day for each prototype, 1440 minutes in a day, and 19.5 is the number of prototypes that would fit in one airlift.

4.3.3 Results

Foam production occurred in all sampling days and in all raceways, irrespective of the amount of natural occurring foam in the production units (Figure 4-11). Overall, there was a large variation on the amounts removed, across different sampling days, and within the same sampling day.

In general, the removal of particulate matter was more efficient compares to the fraction of dissolved biodegradable organic matter (Figure 4-12.).

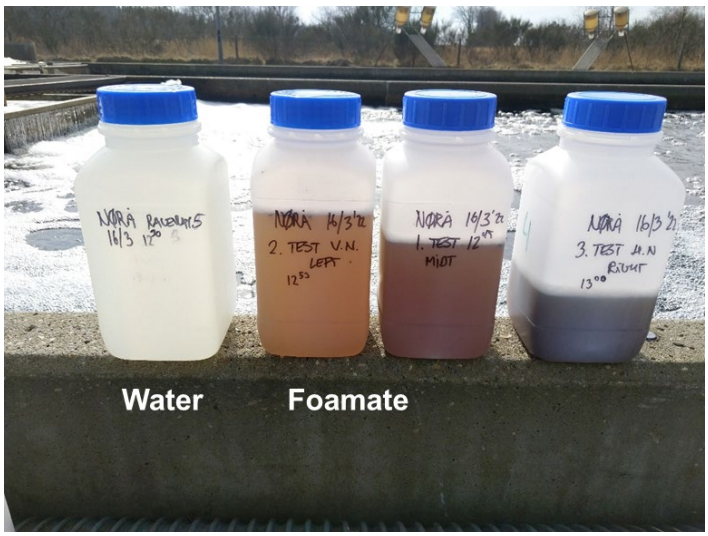


Figure 4-11. Foamate samples collected in a trout farm using foam fractionation. The left sample is with water from the raceway, while the other three jars contain passively collected foamate with different concentration.

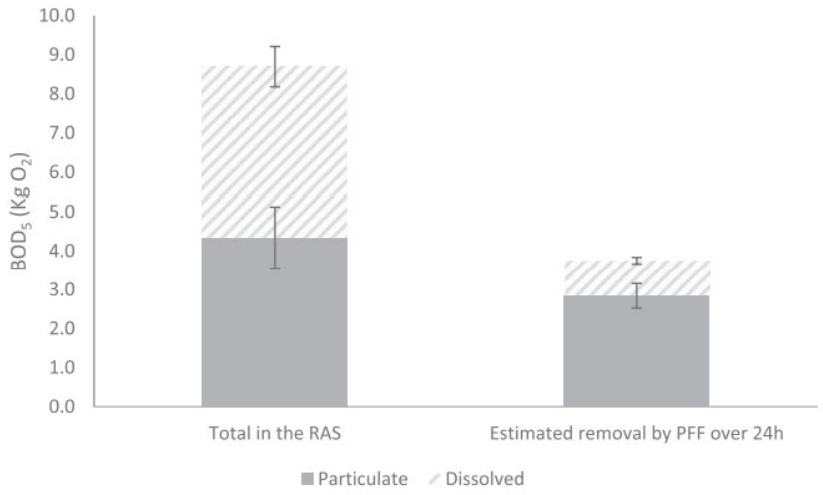


Figure 4-12. The amount of BOD_5 (kg O_2) present in the production unit (particulate and dissolved) and estimated removal capacity. Total in RAS was calculated based on the concentration in the water multiply by the total volume of the production unit. Estimated volume remove was calculated based on the concentration removed, multiplied by the estimated volume remove over 24h if a full airlift was used.

The calculated removal efficiency of organic matter varied between 29 and 172% of all organic matter present in the water (Table 4-2), with and average removal efficiency of 75% measured as BOD_5. Two of the raceways had similar removal efficiencies in both winter and spring, being 105% in winter and 86% in the spring in the raceway 3 and 76% in the winter and 70% in the spring in the raceway 4. On the contrary, in raceway 2, the removal efficiencies were improved from winter to spring, going from 52% to 99%. The removal efficiency of organic matter (measured as COD) showed a similar trend with an average removal capacity of 68% over 24h. Microbial activity was highly affected by the foam fractionation, the average removal efficiency being 233%.

Table 4-2: Water quality parameters from the commercial freshwater recirculating trout farm. The values represent mean± std.dev. based on sampling in three similar raceway systems on three sampling events in winter (N=9) and two sampling events in the spring (N=6).

Water quality parameter	Winter		Spring		Average	
	Average	St dev	Average	St dev	Average	St dev
Particle Number (# ml^{-1})	1056	554	1067	335	1060	479
BOD_{5TOT} (mg O_2 l^{-1})	6.6	2.3	3.9	1.2	5.5	2.4
BOD_{5DISS} (mg O_2 l^{-1})	3.6	1.0	1.5	0.2	2.8	1.3
BOD_{5PART} (mg O_2 l^{-1})	3.0	2.2	2.3	1.2	2.8	1.9
COD_{TOT} (mg O_2 l^{-1})	17.7	6.5	20.8	3.6	18.9	5.7
Bactiquant (BQV)	29486	26410	14337	10790	23449	22812
Total P (mg P l^{-1})	0.18	0.10	0.15	0.08	0.17	0.10
NO_3-N (mg N l^{-1})	9.4	2.3	13.8	1.7	11.2	3.0
Temperature (°C)	6.3	0.8	13.9	0.5	8.2	3.3

The foam had between 9 to 29 times more microbial abundance and activity than the water. This up-concentration factor was calculated by dividing the concentration in the foam (Table 4-3) with the concentration in the water. Particles in the water were also efficiently removed by the passive foam fractionator prototype, with a calculated average of 178% removal. The removal efficiency of volume of particles increased significantly from winter (158%) to spring (382%). Total P removal efficiencies were also substantial and calculated to be 41% over 24h.

Table 4-3. Average foam volume and concentration of selected water quality variables measured in the foam collected by the PFF over a period of one minute. Values presented as mean± standard error of the mean (SEM), based on three replicated sampling events in winter (N=24) and two replicated sampling events in the spring (N=24).

Foam samples	Volume of foam (l^{-1})	Particle Number (# ml^{-1})	BOD_{TOT} (mg O_2 l^{-1})	BOD_{DISS} (mg O_2 l^{-1})	BOD_{Part} (mg O_2 l^{-1})	COD_{TOT} (mg O_2 l^{-1})	Bactiquant (BQV)	Total P (mg P l^{-1})
Winter								
Average	5.1	1.41E+04	40.5	10.7	29.7	111	4.73E+05	1.05
SEM	0.3	2.19E+03	4.9	1.8	3.7	17.8	1.85E+05	0.25
Spring								
Average	7.5	2.22E+04	19.1	6.2	12.8	71.1	1.78E+05	0.67
SEM	0.3	3.43E+03	1.2	0.6	0.9	5.9	2.17E+04	0.06
Combined								
Average	6.3	1.82E+04	28.2	8.2	20.1	91.0	2.96E+05	0.82
SEM	0.3	2.12E+03	2.8	0.9	2.1	9.8	7.86E+04	0.11

4.3.4 Discussion

Foam fractionation in fresh water has been shown to be efficient and have the capability to significantly improve water quality (Chen et al., 1993a; de Jesus Gregersen et al., 2021; Weeks et al., 1992). However, its applicability in commercial settings is still rare. The prototype developed and tested in this trial showed good potential removal efficiencies across various metrics tested.

In a previous study by de Jesus Gregersen et al. (2021), it was shown that foam fractionation could not completely remove all organic matter, even when using an over-dimensioned skimmer. This is due to the lack of affinity of some types of organic matter to form foam. The process relies on the presence of surfactants and surface charged (Timmons and Ebeling, 2010). Once they are removed, system reaches a new steady state, and, as there is less organic matter to generate foam , the overall efficiency of foam fractionation tends to decline.

This study indicates that using a single airlift compartment equipped with the protein skimmer prototypes can achieve a substantial removal capacity, and at the same time, requires relatively low construction and operational costs, as modifying and retrofitting the existing airlift will be minimal.

Natural foam formation in MTFs is not homogenous, as it includes diurnal and seasonal fluctuations. Fish farmers report much lower production of foam during the cold months compared to the other seasons, which is partly due to the feeding patterns and temperature. While clear visual differences in the amount of foam were observed at the farm during this trial, they were not related to a single factor. There was some variation in the results obtained, but overall, the results showed similar effects in both winter and summer months. This suggests that while natural foam occurrence is reduced during the colder periods, the prototypes are still capable of removing large amounts of organic matter, particles, and microbes during this period.

Feeding (period) is a major factor determining foam formation at the fish farm. Foam levels fluctuate throughout the day in response to feeding activities. The oil present in the feed reduces the surface tension, thereby inhibiting foam formation and greatly reducing natural foam occurrence (Timmons et al., 1995). Typically, natural foam starts to occur and build up in MTFs in the late afternoon, once feeding has stopped, and continues building up until feeding resumes the following morning. In this field trial, samples were collected during the feeding period (due to practical matters related to sample processing). This may have affected the observed removal efficiencies, since foam production is expected to be lowest during that period. If the efficiency of the prototype follows the natural production of foam in the raceway higher removal efficiencies could likely be achieved post-feeding. However, this was not included in the current experiment and remains to be tested.

The prototype primarily targeted the particulate organic matter fractions. This greater elimination of particulate matter is commonly observed during foam fractionation (Brambilla et al., 2008; Chen et al., 1993a; Timmons et al., 1995). The significant reduction of particulate matter is likely responsible for the observed decrease in total phosphorus (P) levels, as a substantial portion of total P is particle-bound (Dalsgaard and Pedersen, 2011). This suggests that foam fractionation could be an effective tool for managing P discharge from aquaculture, as a higher concentration of P on the foam would facilitate end of pipe treatment.

Despite being most efficient at removing particulate matter, the prototype also showed potential for extracting up to 55% of all dissolved organic matter. This could be a game changer, as there are currently almost no technologies available that can deal with dissolved organic matter in RAS. The particle removal efficiency might be somewhat overestimated, The foaming process aggregates molecules together, meaning that small particles are clustered into larger particles. The measurement equipment used in this study can only quantify microparticles larger than 10 µm, and it is possible that many particles became detectable in the foam fractionation process. In RAS, most particles are below 20 µm (de Jesus Gregersen et al., 2020, 2019; Fernandes et al., 2014).

Previous studies have shown that foam fractionation itself is ineffective at removing bacteria from water (Figueiras Guilherme et al., 2020; Kovács et al., 2023). This is primarily due to hydrophilic nature and low adhesion properties of suspended single bacteria limiting affinity for the foaming process (Zita and Hermansson, 1997). In a commercial testing of full-scale saltwater foam fractionation (Section 4.2.; Kovács et al., 2023), a low removal of microbial activity over a single pass on two distinct commercial skimmers was found. This was, however, significantly improved once ozone was added. Likewise, in a control trial exploring technics to remove algae from the water (Figueiras Guilherme et al., 2020), microalgae removal only occurred when disinfectants were added and cell walls were destroyed. The current trial demonstrated one of the highest potential microbial removal rates alongside particulate matter. This is likely due to the nature of the bacteria in the water. In model trout farms, most bacteria live attached to particles (de Jesus Gregersen et al., 2019; Pedersen et al., 2017). Therefore, as particles are removed from the water, the associated surface attached bacteria are also removed.

The removal of both particulate and dissolved organic matter from the water will lower the microbial presence and activity, due to an overall reduction of the systems carrying capacity (Aalto et al., 2022a; Fossmark et al., 2020). During the trial, it was estimated that the foam fractionator would produce a volume of foam at 6-8 m^3/hour which represent approx.10 % of the system vol-

ume. While this is a large volume of discharge water, it is in general, at least 10 times more concentrated than the normal exchanged water, which could potentially help with end of pipe treatment, where higher concentrations and lower volumes are beneficial. Further modification of the passive foam fractionator could include designs to up concentrate and further enhance the removal efficiency and reducing the foamate volume.

The results of the field sampling are both interesting and promising. Accumulation of organic matter has various negative impacts on fish production, with increased turbidity, increased O_2 consumption and CO_2 production from microbes consuming organic matter, increased microbial activity due to an increase in the systems carrying capacity, reduction in biofilter performance, among others (de Jesus Gregersen et al., 2019; Fossmark et al., 2020; Ling and Chen, 2005; Michaud et al., 2006; Pedersen et al., 2017; Schumann and Brinker, 2020). Even the lowest levels of removal obtained during the field trials led to significant improvements on water quality. A constant challenge in model trout farms is the control of the microbial water quality and the need to use expensive chemicals to keep acceptable conditions. By removing bacteria directly and reducing the amount of food available for bacteria, it is likely that a large reduction in the use of chemicals could be achieved, which would ultimately result in a reduction of cost.

Installation and operational cost are important when considering the implementation of foam fractionation as an additional water treatment process in a RAS. The estimated cost of building and installing the prototypes over an existing full air lift would be < 100.000 DKK. Moreover, as the prototypes make use of the airlifts already installed and in operation at the farms, there are no expect operational cost, apart from some cleaning and adjustments. As model trout farms have more than one airlift, so the removal capacity could be increased or decreased based on the number of airlifts used for foam fractionation.

4.3.5 Conclusions

Effective methods of removing organic matter are essential to modern aquaculture. However, reliable, and affordable ways to deal with organic matter, especially microparticles and dissolved organic matter, are still few. The prototype tested in the current trial shows a great potential, both due to the very high potential removal rates, but also by its low cost and easiness of construction at the farms. While the results still need to be confirmed in larger scale, even the lowest removal rates obtained could have significant benefits for production. However, further testing should be conducted in larger scale to access is full potential applicability.

5. Effects of ozone on water quality – measures from a field trial

5.1 Background

Ozone (O_3) is a highly reactive and oxidizing gas reacting fast and in low concentrations, first with the easily degradable dissolved organic matter (DOM) and inorganic pollutants, and then with the decreasingly reductive pollutants (von Sonntag and von Gunten, 2012). Ozonation is a well-known disinfection method that has several benefits for water quality (Bullock et al., 1997; Powell and Scolding, 2018), when utilized with correct design.

Ozone is antimicrobial, meaning that it can replace chemical disinfectants, and the ozone dosage can be adjusted according to the need for disinfection (continuous/periodic). It improves water quality by oxidizing natural organic matter (NOM) and reducing the COD, while it removes color and suspended solids improving the visibility of the water (Davidson et al., 2011; Summerfelt et al., 2009; Summerfelt and Hochheimer, 1997). Ozone has been used for algae elimination for years. It accelerates protein degradation and improves coagulation and filtration processes (Antoniou and Andersen, 2012). The oxidation-reduction reaction (redox) level is increased and the oxygen concentration in the water is stabilized. It contributes to odorant reduction (geosmin and 2-methylisoborneol; MIB), improving the taste of fish (Gonçalves and Gagnon, 2011). Ozone inhibits infectious viruses (Owsley, 1991), bacteria (Colberg and Lingg, 1978; Liltved et al., 1995; Summerfelt et al., 2009; Tango and Gagnon, 2003), and protozoa (Tipping, 1988) in several aquaculture systems resulting in improved growth (Good et al., 2011). Therefore, ozone can remarkably improve the water quality in RAS and the welfare of the farmed fish.

There are also some disadvantages; if overdosed, ozone can form toxic by-products (Antoniou and Andersen, 2012), pronounced in saltwater in reaction with chlorine, bromine, and iodine, in order to take advantage of ozone's oxidative effect, it should be produced as close to the place where it is to be used. Ozone vapors are harmful to health, for which specific measures are required for safe indoor use. Safe ozone formation and correct transfer to the water phase as well as adjusting the ozone dose so that it corresponds to the water matrix, reduce these risks, and by directing the ozonated water to the inlet of the facility's biofilters there is no risk of the fish being exposed to excess ozone. Alternatively, ozonated water can pass through a UV source to ensure the breakdown of any ozone residuals.

On certain types of facilities (Model 3 fish farms), the average fish mortality is >10%, which has hampered economic profitability over a number of years. The operating loss and the time spent handling dead fish are considerable, to which is added the expected reduced feed conversion and large expenses for water treatment with auxiliary substances. Therefore, the microbial pressure in the production water in recirculated facilities should be reduced. Previously it was considered that ozone treatment was not economically justifiable in outdoor trout facilities with moderate recirculation (Janning et al., 2012). The potential application and long-term effects have recently been described by (Aalto et al., 2022a; de Jesus Gregersen et al., 2021).

De Jesus Gregersen et al. (2022) and Aalto et al. (2022a), who monitored water quality parameters in some indoor, pilot-scale freshwater RAS. Few studies have tested and verified ozone treatment under commercial aquaculture conditions (Davidson et al., 2021; Kovács et al., 2023;

Schroeder et al., 2015), but there is a lack of empirical experience and knowledge on how ozone works when applied in outdoor RAS.

This investigation aimed to document the effect of ozone treatment on aquaculture water and simultaneously test and verify the potential effects of ozone on several water quality parameters. This was done during a continuous 4-week field trial where RAS water from a model 3 fish farm was ozonated and compared with untreated RAS from the same fish farm.

5.2 Operation during the experiment - sensor recordings

5.2.1 Materials & Methods

Experimental setup

Testing and documentation of ozone under commercial conditions took place at Nr. Vium Dambrug (Model 3 fish farm). Experimental treatments were performed in conjunction with the facility, through a side stream. The setup included four tanks of 700 L volume, each stocked with 10 kg juvenile (ca. 10 g each) rainbow trout (*Oncorhynchus mykiss*) - two ozone-treated tanks (Ozone A & B) and two untreated control tanks (Tank C & D). Two sets of serial trickling filters (TF 1 & 2) which were connected to a pump sump, providing degassed water to the tanks (Fig. 5-1A). The water was flowing through the fixed bed biofilters, where “IN” reflects the position of water intake to the oxygen cone, which was used as a reaction chamber for dissolving ozone in water, and the control tanks, and “R” is raceway water upstream the sections of fixed biofilters (Fig. 5-1B). The water exchange was three times per hour in all fish tanks. The fish were fed 200 g of 1½ mm feed pellets per day per tank. Dead fish, if any, were collected and registered daily and stored at -20°C.

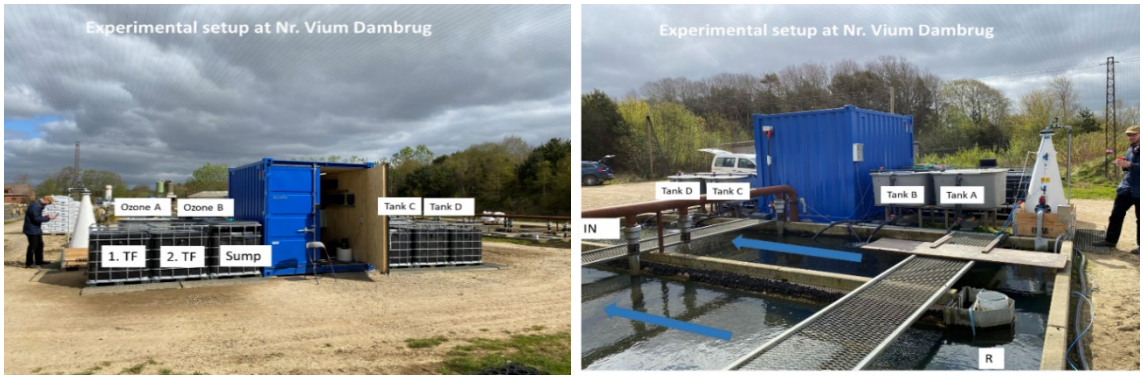


Figure 5-1. A) Front view of the setup at Nr. Vium Dambrug, with two ozone-treated fish tanks (Ozone A & B) and two untreated controls (Tank C & D). The setup also included two serial trickling filters (TF 1 & 2) and a pump sump to both sets of tanks. B) The blue arrows show the direction of water through the fixed bed biofilters, where “IN” reflects the position of water intake to the oxygen cone and the control tanks, and “R” is raceway water upstream the sections of fixed biofilters.

A 10-feet insulated container supplied with pump-frequency converters, Gaia ozone generator (Water ApS), compressor, cooling unit, OxyGuard Pacific control system and emergency oxygen valves, was installed between the two experimental setups (Fig. 5-1A). Data from dissolved oxygen (DO), CO_2, pH, redox, and ozone probes were logged in the Pacific system. The probes were cleaned regularly to avoid biofouling. Data was also stored in real-time online in a cloud-based management system (Cobália) and could be accessed remotely.

Figure 5-2. Gaia ozone generator.

The water-cooled Gaia ozone generator (Figure 5-2) was connected to the facility's supply of pure oxygen and to a compressor regularly supplying necessary nitrogen from atmospheric air. Under these conditions, the ozone generator produced 20 -25 g O_3/hour.

The water in the ozone treatment passed through an oxygen cone at a flow of 10 m^3/h, where water was mixed with ozone and oxygen at high pressure, allowing an elevated amount of ozone to be dissolved, with constant concentration of ~2 mg ozone per liter water during the experiment (Figure 5-3).

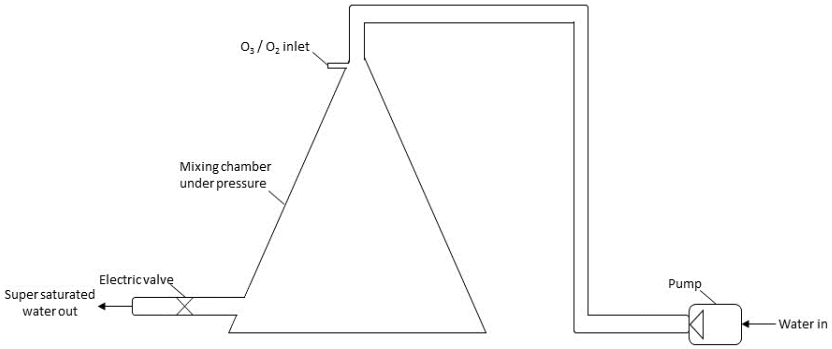


Figure 5-3. Schematic representation of an oxygen cone. Untreated water is pumped on the top of the cone, high concentration of ozone gas is also injected in the cone, and it is dissolved into water. The ozone treated water exits the cone and enters the pump sump.

The ozone generator delivered gas, consisting of 10% ozone and 90% oxygen. The supersaturated water was passed through trickling filters, to bring down the oxygen saturation to 100%, before entering the pump sump where DO, CO_2, pH, redox, and ozone sensors were installed. The water from the pump sump was delivered to the 2 fish tanks (tank A and B) (Figure 5-4)

with a constant flow, allowing the ozonated water to be replaced 3 times/hour. The ozonated water consisted of 0.05% of the total volume of the farm. The ozonated water after the fish tanks ended up back to the farm. Therefore, no real recirculation occurred; every time water samples were collected were results of a single pass from the ozone treatment. DO probes were also installed in both of the fish tanks together with oxygen diffusers.

Figure 5-4. Ozonation set-up on a model 3 pond farm. The container, the tanks, the biofilters, feeding pump, the Gaia ozone generator, and the OxyGuard Pacific control systems that were used in the experimental trial can be seen.

Both the ozonated water and the untreated water passed through the trickling filters for degassing – and then ended up in pump sumps, where DO, CO_2, pH, and redox sensor were installed before entering the 4 fish tanks at the same water flow. Oxygen diffusers were installed as well.

5.2.2 Results & Discussion

Operation during the experiment - sensor recordings

The experimental trail started on the 4/7 to 2/8 -23. During the experiment, O_2 (DO), CO_2, temperature, pH, redox, O_3, and pressure in the oxygen cone were continuously monitored by OxyGuard sensors and were logged on Cobália, via the OxyGuard Pacific system. All sensors were installed prior to experimental trial, except the ozone sensor, which was installed on 17/7 -23, due to unexpected redox and DPD measurements. However, the system depended on the water intake - which always was from the production raceways. Every alteration to the water in this system was reflected in the research tanks as well e.g., temperature fluctuations, pH adjustment, adding salt, adding formaldehyde and any other “treatment” to the overall system. No remarkable changes were observed between the treatments, in terms of pH, CO_2, and redox, and therefore, these results are not presented in this report.

Oxygen

The oxygen was decreasing in all four tanks until the 13/6 -23, where oxygen boost in each tank was deployed, through a normally open (NO) safety valve. The water temperature rose to approx. 18°C (summer), and the fish grew well - thus the water holds less O_2, and the growing fish need more O_2. The DO during the experiments was fluctuating among the tanks. After the safety valve was used for oxygenation within the tanks, the oxygen saturation level did not go below 80%. The ozone treatment (Tank A: 8.13±1.66 mg/L; Tank B: 8.17±1.87 mg/L) had lower average oxygen concentration compared to the control treatment (Tank C: 10.75±2.63 mg/L; Tank D: 8.55±1.03 mg/L; Figure 5-5). Oxygen dosing proved to be harder than expected, with unequal oxygen consumption/delivery in each tank, with Tank C having the largest difference. Having four individual oxygen supplies and normally closed (NC) valves with independent control might be a solution, should similar trials be carried out in the future.

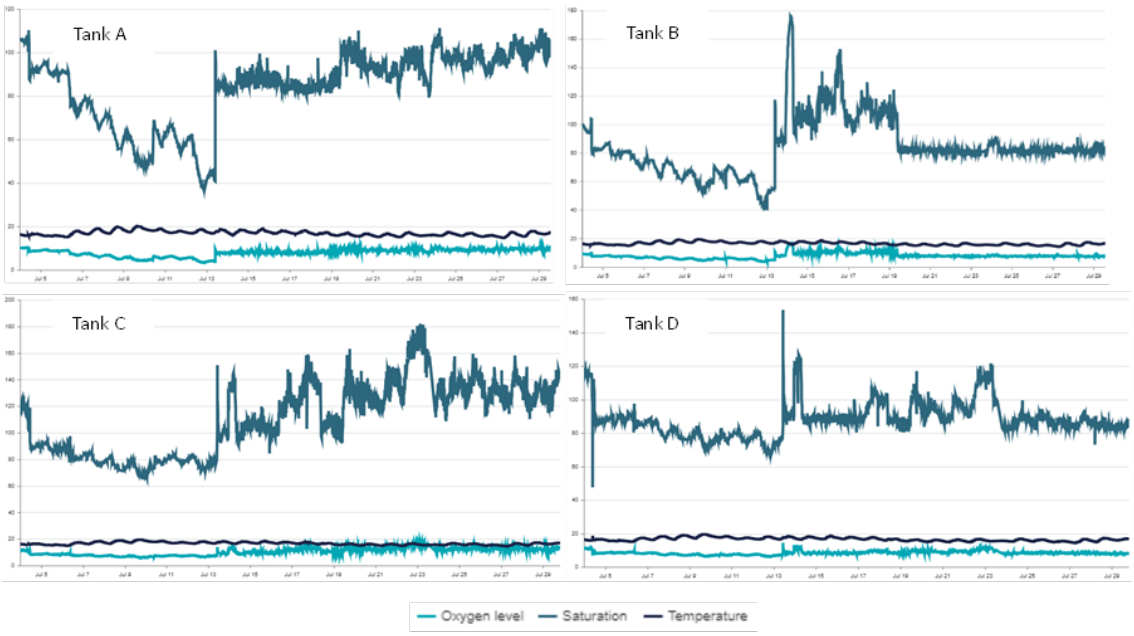


Figure 5-5. Dissolved oxygen concentration (Oxygen level, saturation, and temperature in ozone treated tanks (A and B) and control tanks (C and D) during the experiment.

Ozone and pressure

The data from the 16/6 and 17/6 -023 were from measurements in the first trickling filter after the ozone treatment. A high concentration of ~83 µg O_3/L was measured. When the sensor was moved into the pump sump after both trickling filters showed a significantly lower ozone concentration. The O_3 concentration in the ozone pump sump had a mean value of 17.88 µg/L ± 9.82 (Figure 5-6a). The pressure in the oxygen cone was relatively stable through the whole period (1.26±0.08 bar; Figure 5-6b).

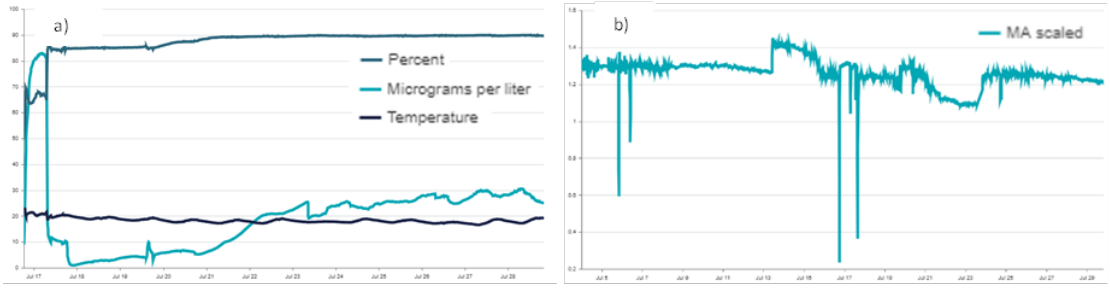


Figure 5-6. a) Ozone in ozone pump sump (ozone concentration in µg/L), percent (% saturation), and temperature (°C), and b) Pressure in oxygen cone, mA scaled to pressure (bar).

5.3 Effects of ozone on water quality – measures from the field

5.3.1 Materials & Methods

Experimental setup

Experimental setup is described under section 5.2.1.

Sampling scheme and procedures

Water samples were collected from 4/6-2/8 -23, handled and stored according to the standard operational procedures for each of the upcoming analyses. The water samples were subjected to various water quality analyses (Table 5-1, Table 5-2) e.g., ozone, UVT, turbidity, microbial water quality (BactiQuant assay, Hydrogen peroxide assay, live/dead), COD, BOD_5, TAN, nitrite, and nitrate. Several sampling positions were selected to monitor ozone concentrations and quantify water quality parameters (Fig. 1b). Samples were collected from nine sampling points:

- Common inlet to the setup (after the biofilters)
- Outlets from tank A & B with ozone-treated water
- Outlets from tank C & D with un-ozonated water
- Inlet to trickling filter 1&2 and sump
- Raceway (untreated control; before the biofilters).

Table 5-1. Overview of sampling dates and types of samples and analysis.

Experimental period of ozone treatment (2023)			Ozone	UVT	Turbidity	BactiQuant	H_2O_2 decay	Live/ dead	COD	BOD_5	TAN	Nitrite	Nitrate
Date	Day	Week											
04-07	0	start	X										
05-07	1	start	X	X	X	X	X	X	X	X	X	X	X
12-07	8	1	-	X	X	X	X	X	X	X	X	X	X
18-07	14	2	X										
19-07	15	2	X	X	X	X	X	X	X	X	X	X	X
02-08	29	4	-	X	X	X	X	X	X	X	X	X	X

Ozone measurements were made immediately after sampling. Approx. 100 mL water was collected individually from the selected sampling positions and was analyzed indoors, within one minute after sampling.

Microbial activity (and inhibition hereof) was measured on three occasions (8 a.m., 9 a.m., and 10 a.m.) per sampling day (N=27). This was done by filtering a 10.0 mL water sample through a

sterile filter and keeping the filter at 5°C until analysis at arrival at DTU Aqua in Hirtshals. The methods used to determine microbial activity were Bactiquant® (Reeslev et al., 2011) and hydrogen peroxide degradation assay (Pedersen et al., 2019). The total number as well as the proportion of dead microbial cells was quantified by flow cytometry in the afternoon upon arrival to the laboratory (Aalto et al., 2022b). The remaining water quality parameters were measured from a water sample of 5 L kept cold during transportation and at 4 °C until analysis. For BOD_5, samples were processed the following day, COD samples were conserved with 1% v/v of 4 M H_2SO_4, and dissolved N was measured on sterile filtered samples in 15 mL vials. Turbidity and UVT were measured on untreated samples.

Analyses of selected water quality parameters

Table 5-2. Overview of water quality parameters measured in the study.

Measuring parameters	Notes and references
Ammonium, nitrite, and nitrate concentration	Spectrophotometric analysis based on Danish Standard 223,224
UVT and turbidity Water clarity i.e., UV transmission (254 nm) and the water's content of particles/turbidity based on the measurement of light scattering.	UV Spectrophotometric Analysis and Turbidity Using Hach Handheld Meters and Probes.
COD (Chemical oxygen demand) Measurement of total dissolved and particulate organic matter in the form of "chemical" oxygen consumption.	Hach cuvette test in different measurement ranges (i.e. 5-60 g/L O_2 and 15-150 mg/L O_2) - filtration of raw samples with 0.45 µm filter.
BOD_5 (Biological oxygen demand over 5 days) Measurement of dissolved and particulate organic material that can be used by bacteria and implies a "biological" oxygen consumption.	Measurement of oxygen consumption at 20.0°C according to the Danish standard. Filtration of raw samples with 0.45 µm filter.
Microbial activity and abundance	Measurement of the total microbial activity in water, using both the H_2O_2 method, BactiQuant and dual staining flow cytometry. Microbial activity measured as turnover of H_2O_2 at a constant temperature of 22°C or by filtration of known volume and temp. and quantification with BactiQuant or by flow cytometry technique.
Ozone concentration	In water, samples measured as a colour reaction and determined spectrophotometrically by Hach cuvettes test as chlorine equivalents (Total residual oxidants).

5.3.2 Results and discussion

Residual ozone

Ozone was recorded in the different degassing compartments and from the outlet of the tanks connected (Figure 5-7). The highest concentrations were registered in the first trickling filter with concentrations up to 0.20 mg Cl_2 eq./L which is equal to 0.88 mg O_3/L. A clear ozone gradient was found within the test unit, and ozone detected from the outlet had diminished to 0.02-0.04 mg Cl_2 eq./L which is equal to 0.013-0.027 mg O_3/L (Figure 5-7). Wedemayer et al., (1979) has reported that an ozone residual of 0.002 mg/l would be a safe level of ozone when culturing rainbow trout. Based on the literature, the exact level of ozone that damages gills or kills rainbow trout is between 0.008-0.06 mg/L (Roselund, 1975; Wedemayer et al., 1979). According to (Bullock et al., 1997), ozone levels as high as 0.08 mg/l were measured during fish mortalities. Therefore, the concentrations that were detected in the outlet, even though were higher than 0.002 mg/L, no effect was observed on fish upon visual inspection. Consistent levels of ozone in the compartments was observed in all measurement occasions, with a tendency that maximum ozone decreased when measured in the morning (5/7 and 19/7 vs. 4/7 and 18/7-23). No ozone was measured in inlet water samples (after the biofilters), and the single registration of ozone in tank C (< 0.01 mg Cl_2 eq./L) reflects a value close to the lower detection level of the measurement.

On 12/7-23, no ozone was detected with the DPD, most probably due to a potential leak. That is also reflected in the following results from this date. As a measure of action, an installation of ozone probes, to have complete and remote overview of the ozonation process was decided.

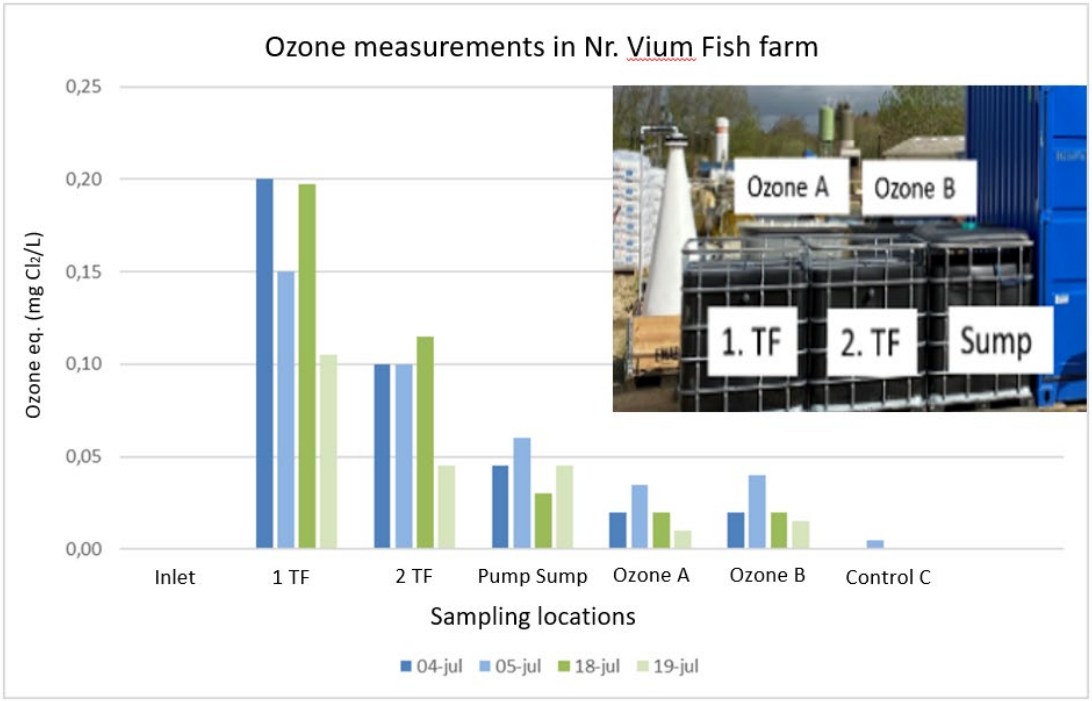


Figure 5-7. Measurement of ozone at the start of the experiment (4-5/7 -23) and after 2 weeks of operation (18-19/7 -23). The samples are taken at 18:20-19:30 (4/7 and 18/7 -23) and the following mornings (08:15-09:15).

Water clarity

Water transparency is vital for aquaculture systems management. The fish can see the feed, resulting in increased growth, improved FCR and limited feed waste. Water transparency is a good indicator of water quality and is described by the transmission of UV_{254}. UVT represents the amount of light which is absorbed by particles and dissolved substances within a water sample. Turbidity on the other hand is a measurement of how cloudy the water is, due to e.g., algae, particles, coloring because of dissolved metals ions, humic substances, and microorganisms. Among the expected outcomes of the ozonation there were the enhancement of UVT (Figure 5-8) and the turbidity (Figure 5-9) which led to the increased transparency of the water. On 12/7, even with the reduced ozone dosage, there was an improvement in the water clarity to a lower extent as it is observed on the other days.

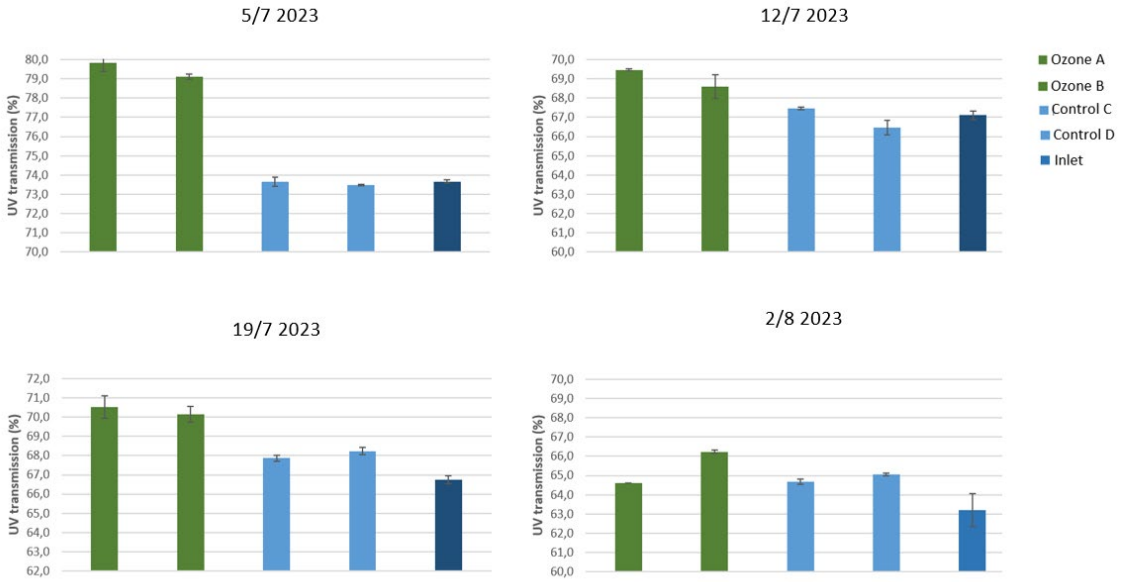


Figure 5-8. The UV transmission in % during single passage over fish tanks with and without ozone measured at different sampling points. The bars show the average ± sd of samples collected on 5/7, 12/7, 19/7, and 2/8 -23.

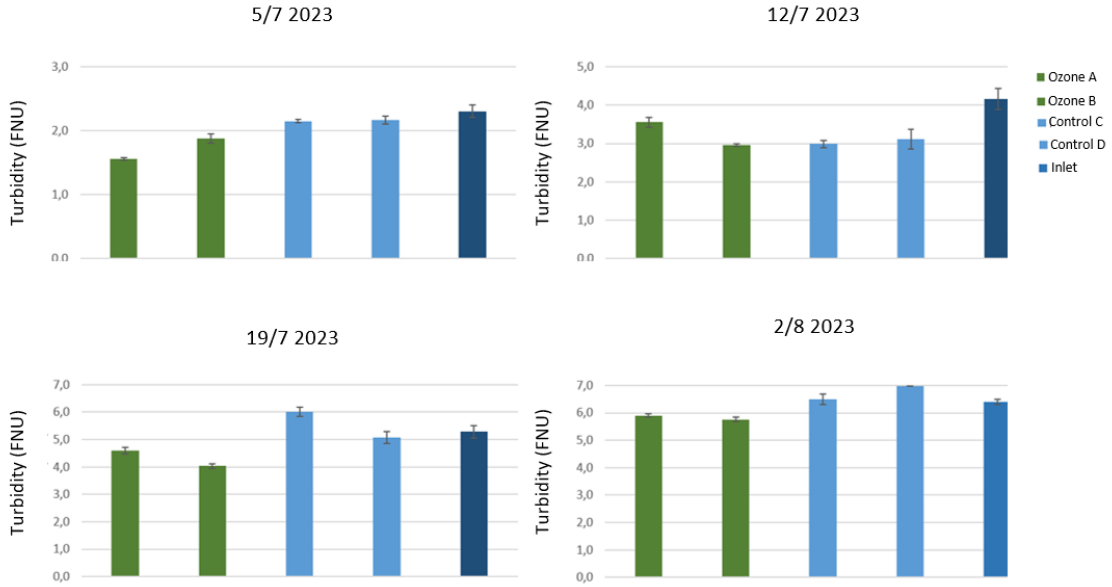


Figure 5-9. The turbidity during single passage over fish tanks with and without ozone measured at different sampling points. The bars show the average ± sd of samples collected on 5/7, 12/7, 19/7, and 2/8 -23.

Organic matter

The most common methods to assess organic matter in aquatic environments are gross indicators such as the biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), or turbidity. Both total BOD_5 and COD (BOD_{TOT} and COD_{TOT}) comprise the total content of organic matter but can be subdivided into a particulate and a dissolved fraction (COD_{PART} and COD_{DISS}). COD will always be higher than the BOD since some compounds will not be biologically degraded within 5 days, and some can only be oxidized chemically. This is confirmed also by the results of this study, where COD ranged between 18 and 40 mg O_2/L (Figure 5-10), whereas the BOD_5 ranged between 4 and 14 mg O_2/L (Figure 5-11). There was variability in terms of COD in the control samples over time. The dissolved fraction was the dominant one (Figure 5-10). Due to short-term treatment, the ozone effect on COD was not clear. A similar trend than in COD was observed with $BOD_{5\text{-}TOT}$: there was variability in the control samples over time and the par-

ticulate fraction was the dominant one (Figure 5-11). Due to short-term treatment, the ozone effect on BOD_5 was not clear, but higher $BOD_{5\text{-}TOT}$ concentrations were observed in the ozone treated samples.

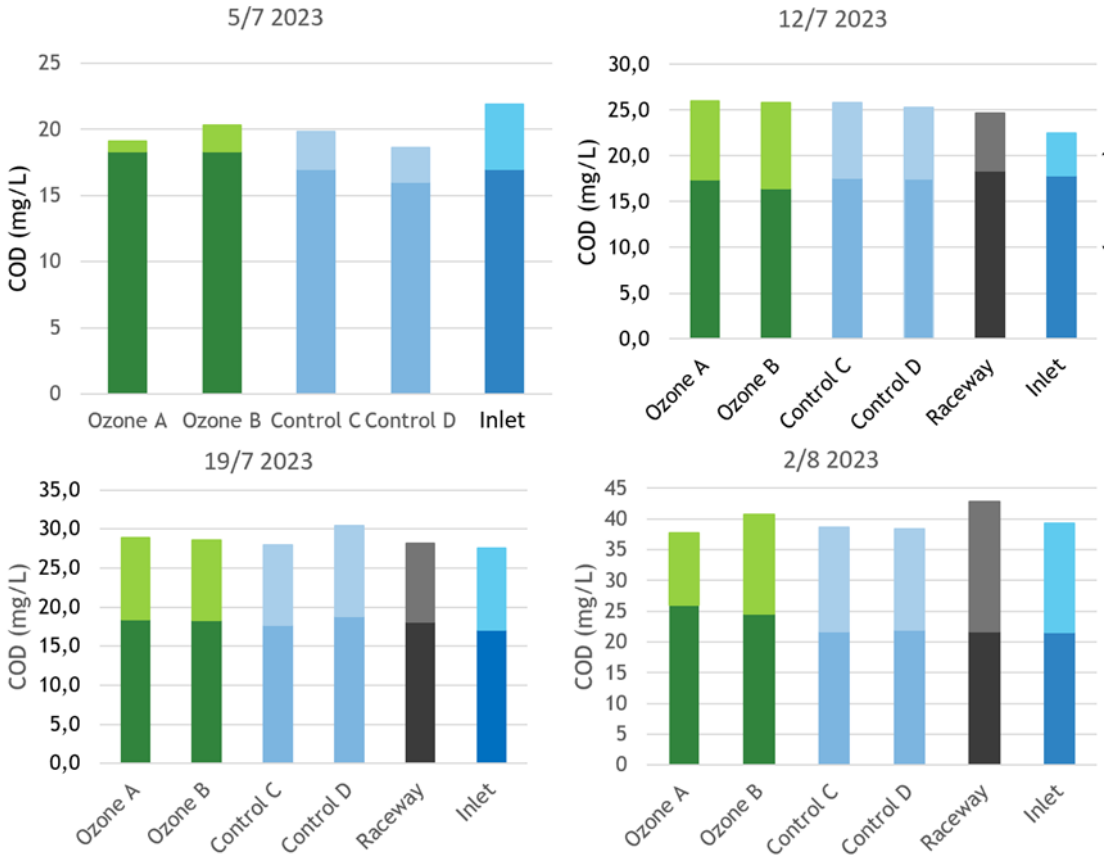


Figure 5-10. Concentrations of the total biological oxygen demand (COD_{TOT}) over fish tanks with and without ozone measured at different sampling points; the lower part of the bars represents the dissolved (COD_{DISS}) and the upper part the particulate (COD_{PART}) COD concentrations during the trial.

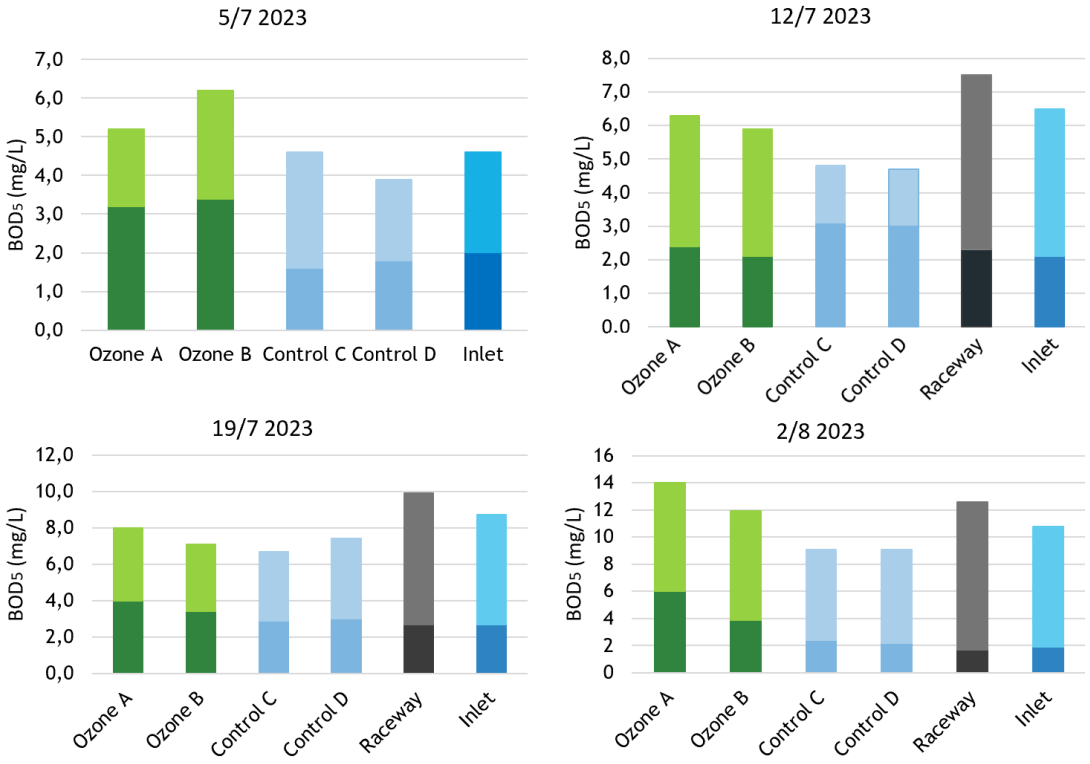


Figure 5-11. Concentrations of the total biological oxygen demand ($BOD_{5\text{-}TOT}$) over fish tanks with and without ozone measured at different sampling points; the lower part of the bars represents the dissolved ($BOD_{5\text{-}DISS}$) and the upper part the particulate ($BOD_{5\text{-}PART}$) BOD_5 concentrations during the trial.

When dissolved organic matter is oxidized by ozone, biodegradable organic matter is formed. Low ozone dosages could not break down the molecules, but it might have enlarged the molecules by breaking double bonds and adding oxygen atoms in the parental compound and forming a secondary molecule with higher molecular weight (Von Sonntag and Von Gunten, 2012). These larger compounds could have been removed by filtration and sedimentation or be degraded by bacteria, since the newly formed hydroxyl groups are easier to break down.

To obtain more information in relation to organic matter characterization in RAS water, the biodegradability index i.e., the ratio between $BOD_{5\text{-}TOT}$ and COD_{TOT} was calculated. If $BOD_{5\text{-}TOT}/COD_{TOT}$ is >0.6 then the organic matter is easily biodegradable, if $BOD_{5\text{-}TOT}/COD_{TOT}$ is between 0.3 and 0.6 the organic matter is average biodegradable, and if $BOD_{5\text{-}TOT}/COD_{TOT}$ is <0.3 the organic matter is not easily biodegradable (Srinivas, 2008). The mean biodegradability index for $BOD_{5\text{-}TOT}/COD_{TOT}$ of all samples was 0.3, indicating that the organic waste in the RAS water was average or not easily biodegradable and that the soluble fraction of the organic matter in the water was composed of high amounts of hard to degrade and/or non-biodegradable substances.

In the present study, the water was ozonated and was passing through two trickling filters (TF, Fig. 5-1), a pump sump before entering the fish tanks and then back to the raceway. The experimental setup only allowed measures from a single pass treatment, and the effects of ozone are therefore less pronounced than if the setup had reflected a RAS with repeated/continuous ozonation.

Microbial activity

The microbial activity, measured with the BactiQuant assay, showed large differences over time, as the values in inlet water ranged between 12000 and 340000 BQV (Figure 5-12). However, the ozone treatment caused a significant microbial inactivation, when was measured both with BactiQuant (Figure 5-12, Figure 5-13) and the Hydrogen peroxide assay (Figure 5-14). Even with a single pass, the ozone reduced the microbial activity by 60-70% (Figure 5-12). The results of flow cytometry corroborate these results, as the number of living cells decreased (Figure 5-15) and consequently the proportion of dead cells of all cells increased (Figure 5-16) in ozone treated tanks. On 12/7-23, there was no statistical difference in the microbial activity or abundance, independently of the determination method that was used, between the ozone- treated and control water samples. This was a result of a technical defect, where a pressure drop in the oxygen cone caused insufficient ozone concentration to be transferred. This was confirmed by measures of absence of ozone in the trickling filters. To deal with this, ozone sensors were installed to have a complete overview of the ozonation process, and a leak was rectified.

Figure 5-12. Microbial activity based on Bactiquant measurements at five different sampling points and four sampling times. The bars show the average ± sd of samples collected and fixed at 08:30, 09:30 and 10:30.

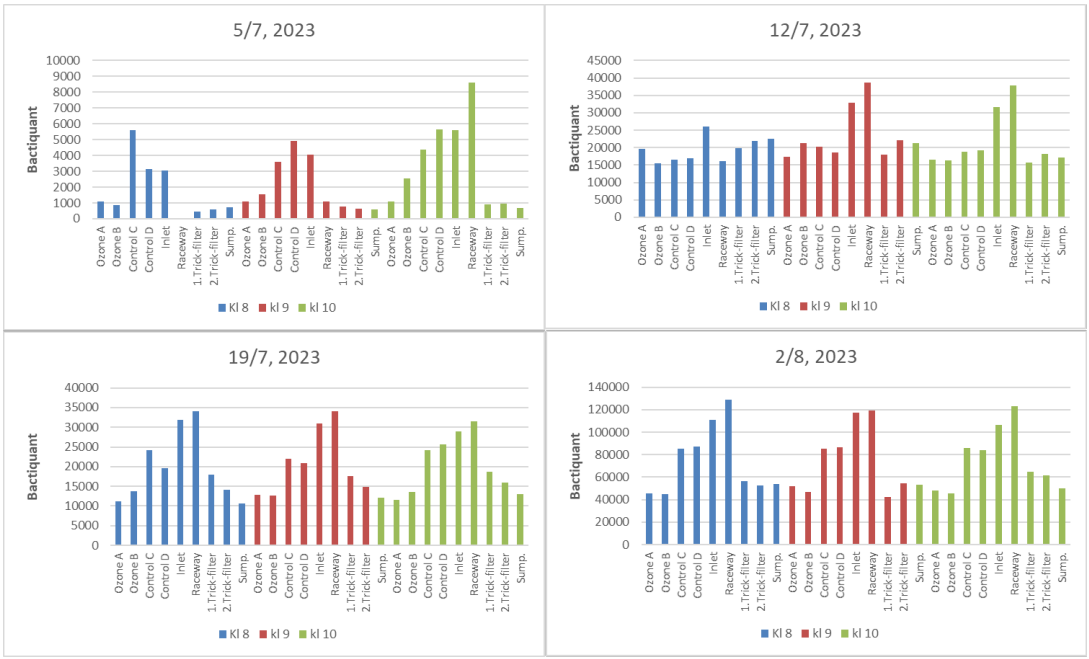


Figure 5-13. Microbial activity based on Bactiquant measurements at 9 sampling locations at 08:30, 09:30, and 10:30 on 5/7, 12/7, 19/7, and 2/8, 2023.

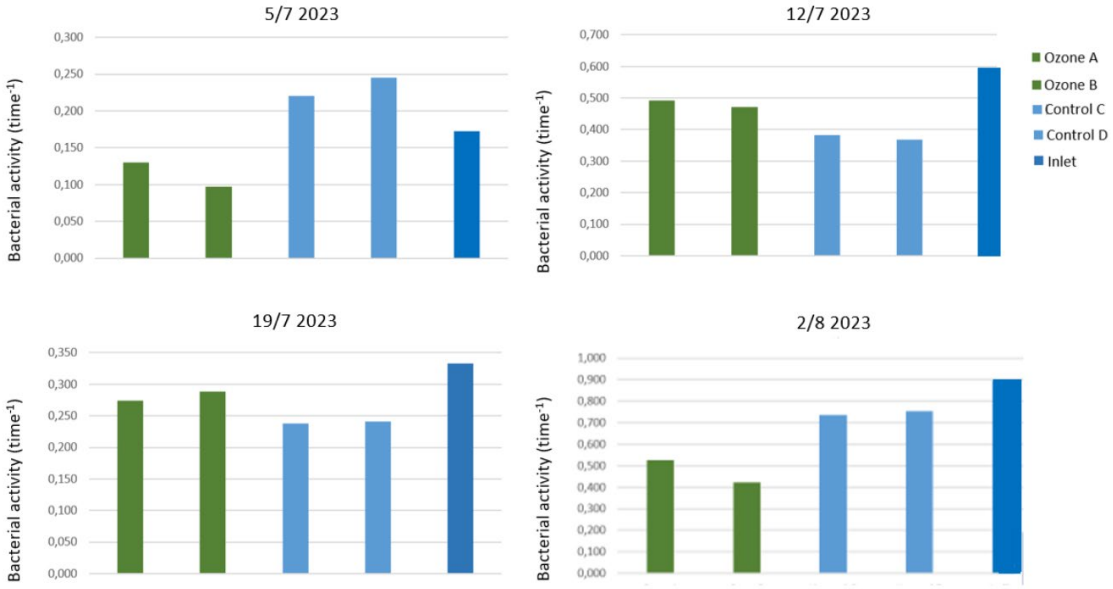


Figure 5-14. Microbial activity based on the hydrogen peroxide degradation method at five different sampling points and four sampling times. The bars show the average ± sd of samples collected between 11:00 and 12:00 on 5/7, 12/7, 19/7, and 2/8 -23, were stored in a refrigerator and analysed in Hirtshals.

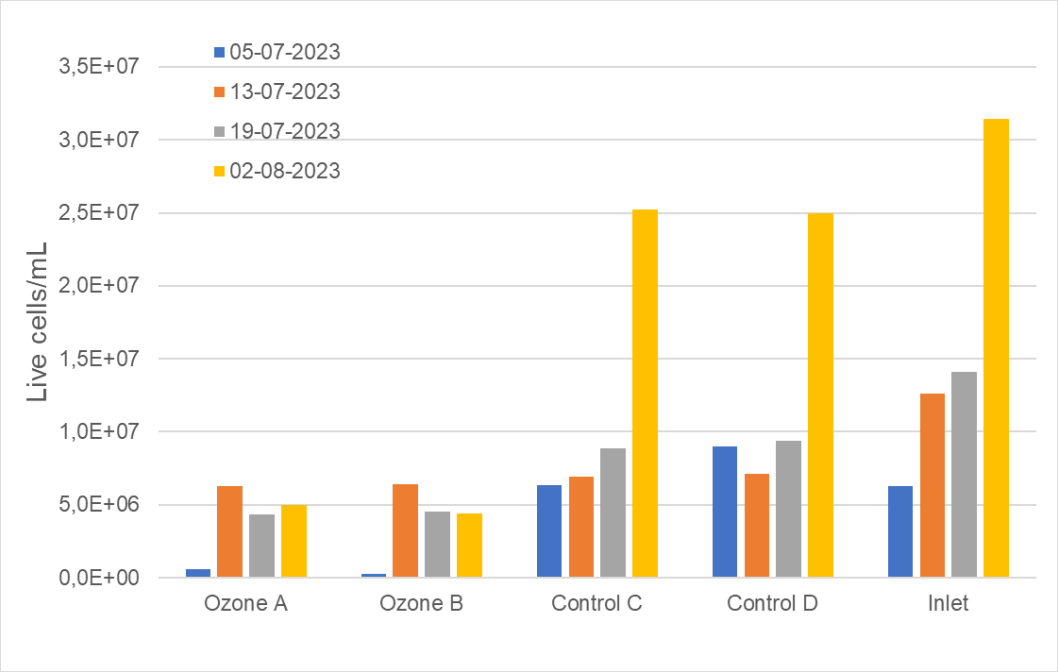


Figure 5-15. Number of living cell per mL measured with flow cytometry at five different sampling locations and four different sampling times.

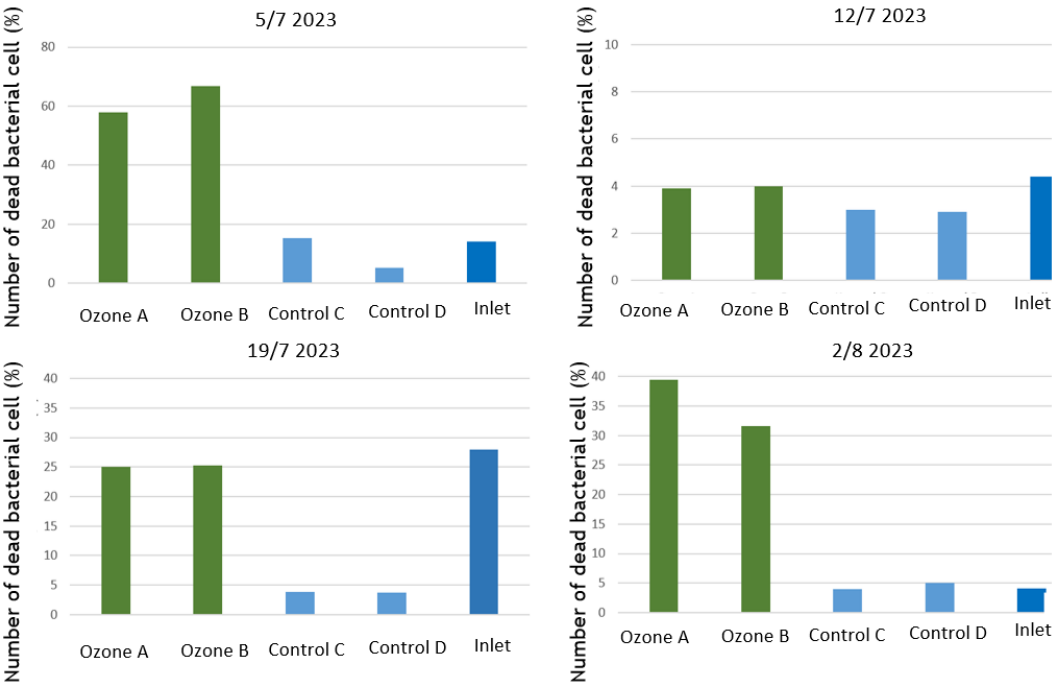


Figure 5-16. The proportion of dead microbial cells of all cells during single passage at five different sampling points and four sampling times.

Total ammonia nitrogen and nitrite

TAN and nitrite concentrations in periods were relatively high and their concentrations in inlet water samples (after biofilters), varied greatly over time. Ozone did not have any effect in TAN (Figure 5-17). Ammonia is not readily oxidized by ozone (Timmons and Ebeling, 2010), unless pH levels are 9 or above (Rice et al., 1981). However, a significant nitrite decrease was seen upon ozonation (87% on 5/7, 66% on 19/7, and 53% on 2/8; Figure 5-18).

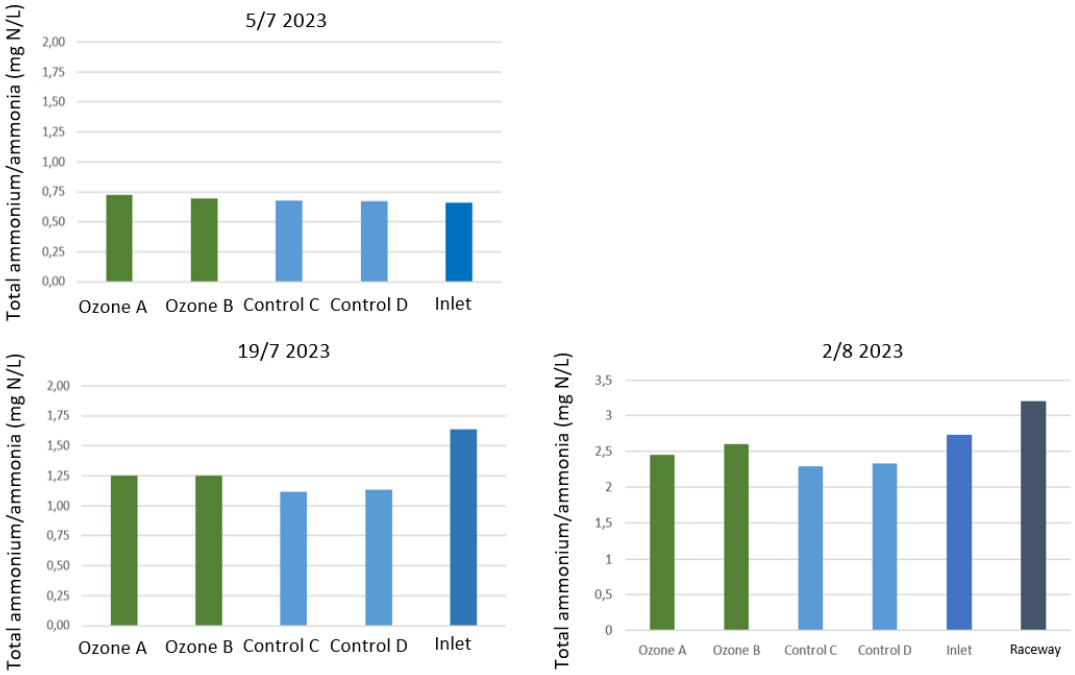


Figure 5-17. The Total ammonia nitrogen (TAN) concentration during single passage at six different sampling locations. Samples were collected on 5/7, 19/7, and 2/8 -23.

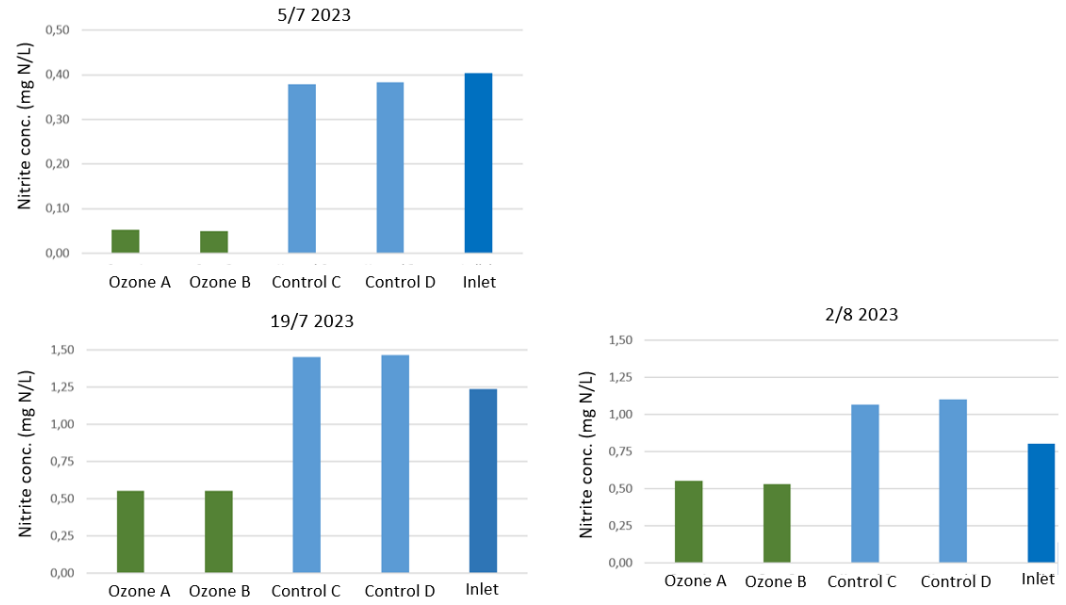


Figure 5-18. The nitrite concentration during single passage at six different sampling locations. Samples were collected on 5/7, 19/7, and 2/8 -23.

5.3.3 Conclusions

Although the water matrix fluctuated over time (30 days), the overall water quality of the model trout 3 farm was improved upon ozonation, and the fish welfare was not negatively affected by the treatment. The ozone production was measurable, and a clear ozone gradient was observed within the system, while no ozone was detected within the fish tanks. Ozone had a clear effect on several key water quality parameters. Ozone contributed to improvement of UVT and turbidity resulting in more transparent water. Substantial reduction of bacteria was found with a single pass when was measured both with BactiQuant and H_2O_2 assay and the number of dead cells increased in ozone-treated systems. During the experimental trial, high and fluctuating TAN and nitrite concentrations were measured. Nevertheless, ozone oxidized nitrite to the not toxic nitrate. When comparing day 1 to day 30, there was not a linear trend of improvement in none of the measured parameters, which was expected since only 0.05% of the total volume was ozonated and was returning to the fish farm again. It would be interesting for future projects to upscale this set up in full-scale trials.

5.4 Effects of ozone on fish performance and pathogen abundance

5.4.1 Background

Ozonation treatment is a tool/technology implemented in aquaculture facilities as part of the biosecurity plan. This tool is applied to achieve disinfection of water and inactivate fish pathogens by virtue of its anti-microbial activity. Such inactivation of pathogens occurs through production of reactive oxygen species (ROS) (Gardoni et al., 2012), which can damage cellular components such as cell walls, membranes, viral envelopes, and DNA (Burleson et al., 1975).

Ozonation is known to have an impact on the overall microbial communities of the water in aquaculture (Aalto et al., 2022a) , but it is not so clear whether changes in the microbial communities caused by ozonation will have an effect on fish health. In this chapter, we intend to increase the understanding of how ozonation will impact the overall pathogen load in the water and the effect of ozone treatment on fish health.

5.4.2 Materials & Methods

Experimental setup

Experimental setup is described under section 5.2.1.

Sampling

The fish were kept in the four tanks for 28 days, where samples of fish and water were collected at three time points: the day after the fish had been added to the tanks, after 2 weeks and after 4 weeks at the day of termination of the experiment. Details regarding the sampling timepoints, sample types and water sampling sites can be seen in Figure 5-19 and Figure 5-20.

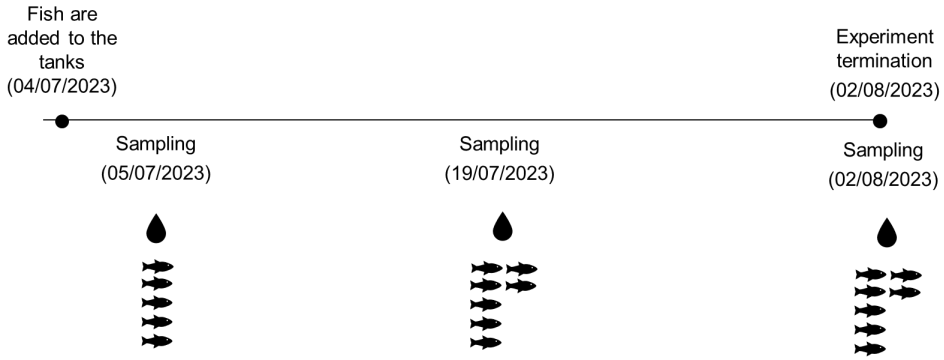


Figure 5-19. Timeline of fish experiment.

Fish sample collection:

- For bacteriological examination
- For qPCR (molecular quantification)
- For microbiome analysis.

Fish sampling consisted of samples for bacteriology, for qPCR analysis and for microbiome analysis. Mucus and gill sampling were done on the farm immediately after the fish had been taken up from the water and euthanized, whereas further sampling was done at the laboratory later on the same day. The fish were kept cold during transportation to the lab.

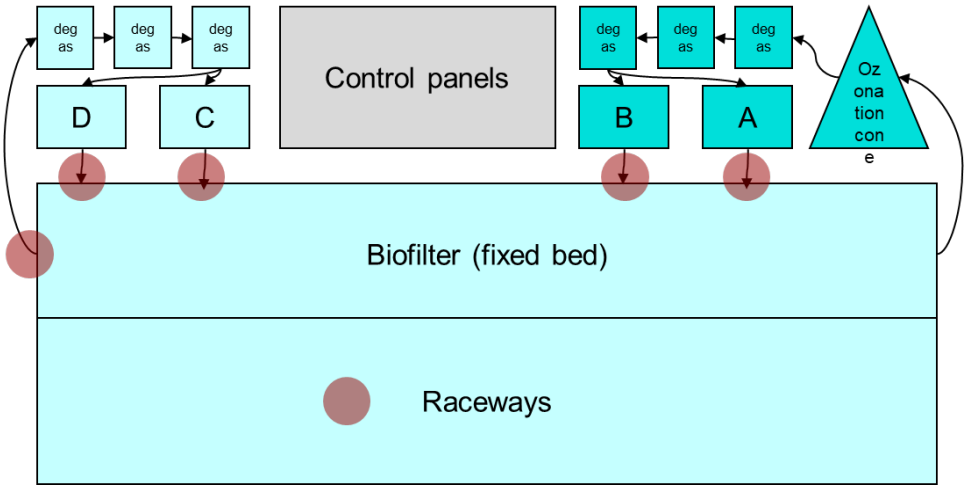


Figure 5-20. Schematic presentation of water sampling sites.

Water samples were collected at the 3 timepoints (Figure 5-19) from the sites described in 5.2.1 (Figure 5-20). The water samples were kept cold during transportation to the lab, where they were filtered.

Water filtration

Water samples were collected in sterile 2 L glass bottles (Figure 5-21) and processed as previously described (Zarantonello and Cuenca, 2024). Filtration was performed in replicates for each sample, until membrane clogging. Filters were then treated with propidium MonoAzide

(PMA) dye. Filters were aseptically sectioned and placed in 2 mL sterile Eppendorf tubes, then snapped frozen on dry ice. For each filter, an un-treated control was also prepared.

Figure 5-21. Water samples were collected at the experiment site for molecular analysis. The water samples were transported, filtered, and treated on the same day in the laboratories of DTU Aqua, Lyngby.

5.4.3. Results

Survival analysis

During the one-month experiment, the mortalities in the four tanks were very low (Figure 5-22, Figure 5-23).

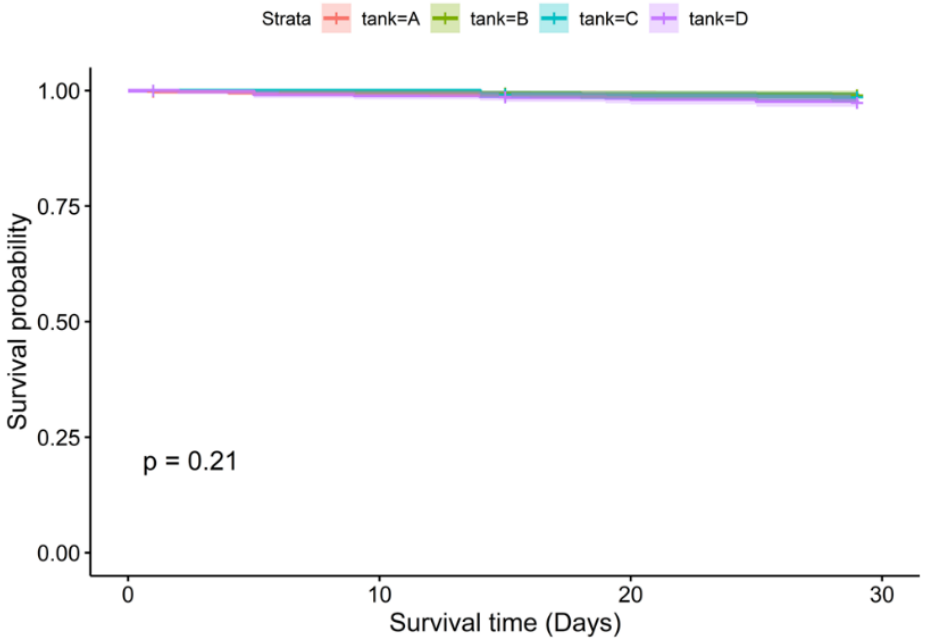


Figure 5-22. Survival analysis between all experimental tanks.

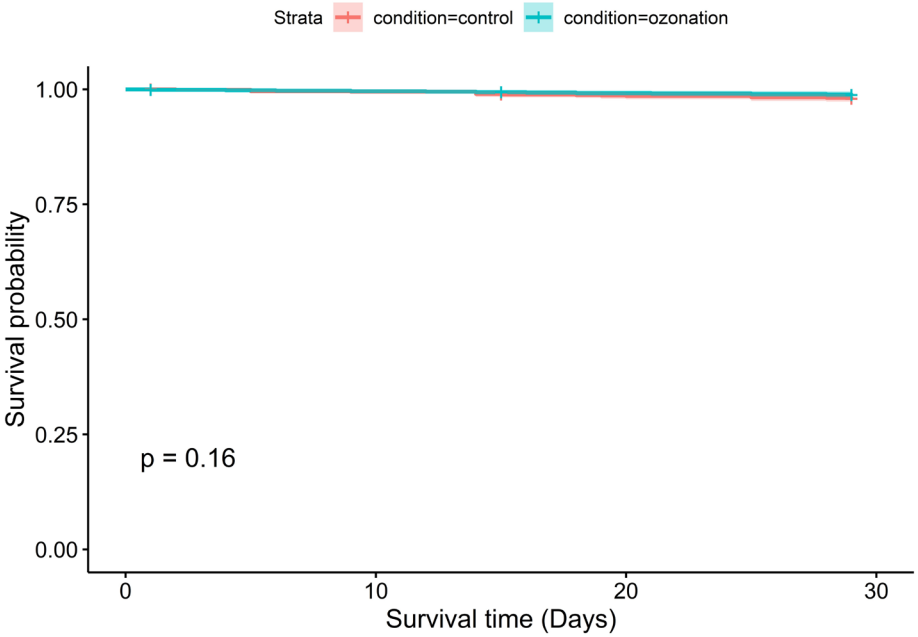


Figure 5-23. Survival analysis ozone treated (A, B) and control (C, D) tanks.

The mortalities during the observed period were overall low and no statistical difference was observed between conditions (Kaplan-Meier survival probability estimation p=0.16).

Fish growth performance

The fish sampled at the three sampling points (N=10-14 fish/treatment) were measured individually, and the average of length and weight in the individual tanks for each time point are shown in Figure 5-24 and Figure 5-25.

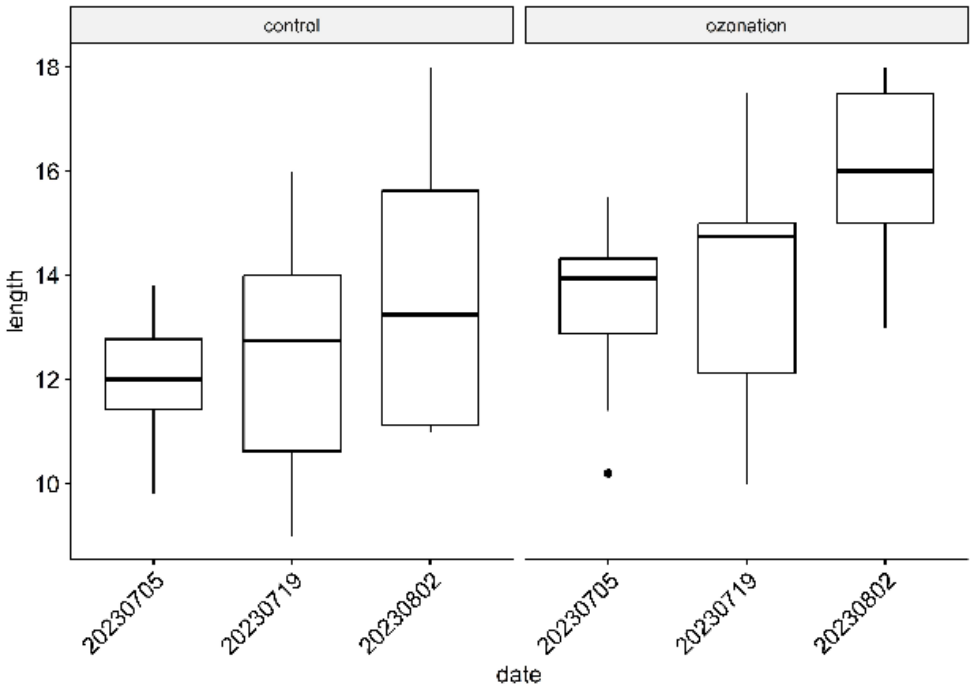


Figure 5-24. Average fish length (cm) over timepoints (N=10-14)

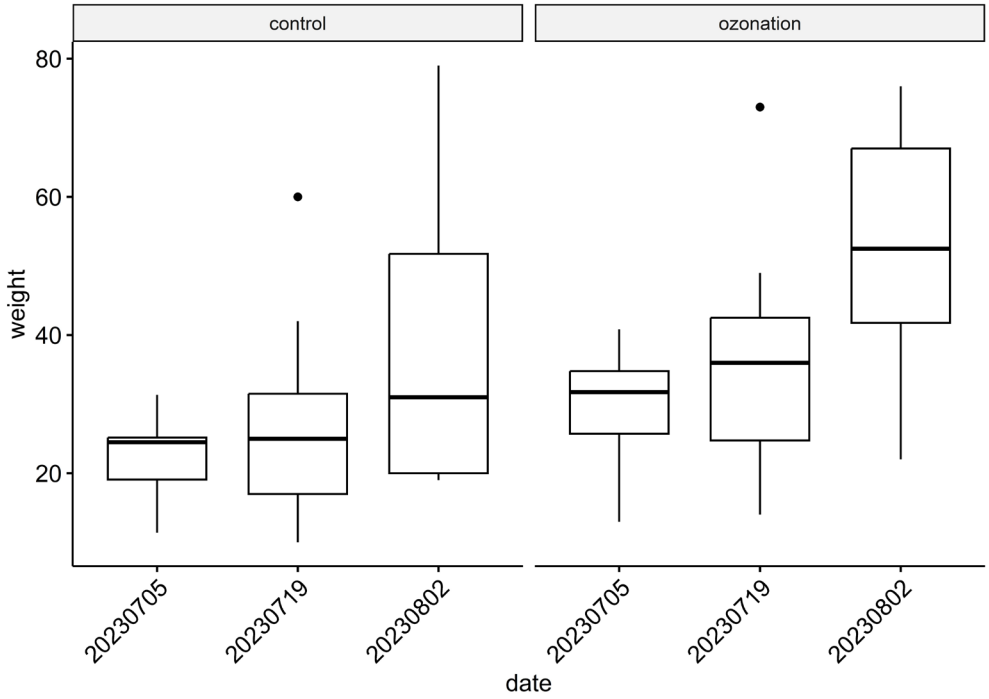


Figure 5-25. Average weight (gr) over timepoints (N=10-14)

Fish size in the two tanks with ozonation was slightly higher when compared with fish sampled from control tanks at the final sampling point.

Furthermore, the bulk weight was measured for each tank at the termination of the experimental trial. Bulk measurement results are the following:

- Tank A: 17.1 kg
- Tank B: 16.5 kg
- Tank C: 16.7 kg
- Tank D: 16.2 kg.

Bacteriological examination

The bacteriological examination of fish showed that fish picked randomly from the raceway batch that were transferred to the four experimental tanks did not harbor any fish pathogens, whereas the fish pathogens *Flavobacterium psychrophilum*, *Aeromonas salmonicida,* and *Yersinia ruckeri* could be isolated from sick fish sampled from the same batch (Table 5-3). From the fish sampled from the four tanks during the experiment (76 fish in total), *F. psychrophilum* could be isolated from the inner organs of 6 fish.

Table 5-3. Fish individuals collected and euthanized on sampling days (N=5-7).

	TANK A	TANK B	TANK C	TANK D	RACE-WAYS (random)	RACE-WAYS (sick)
SAMPLING DAY 1 (05/07/2023)	No pathogens detected	1 fish: *F. psychrophilum*	No pathogens detected	1 fish: *F. psychrophilum*	No pathogens detected	3 fish: *F. psychrophilum* 1 fish: *A. salmonicida*
SAMPLING DAY 14 (19/07/2023)	No pathogens detected	2 fish: *F. psychrophilum*	1 fish: *F. psychrophilum*	No pathogens detected		4 fish: *F. psychrophilum* 2 fish: *A. salmonicida*
SAMPLING DAY 28 (02/08/2023)	No pathogens detected	No pathogens detected	No pathogens detected	1 fish: *F. psychrophilum*		4 fish: *F. psychrophilum* 4 fish: *A. salmonicida* 1 fish: *Y. ruckeri*

The fish from the four tanks that died during the trial were collected and frozen. At the end of the trial, the internal organs of all dead fish were sampled for bacteriology and PCR to determine the potential presence of known pathogens at the time of death. In Table 5-4, it is possible to see the results of the bacteriological investigations. The same results were plotted over the time of death and grouped by condition in Figure 5-26.

Table 5-4. The proportions of dead individuals harboring the known pathogens over the total number of dead individuals in each tank. Individuals reaching humane endpoint were collected and frozen over the experimental trial duration and were subjected to bacteriological analysis at the time of experiment termination.

	TANK A	TANK B	TANK C	TANK D
F. psychrophilum	1/7	0/5	1/7	0/13
A. salmonicida	1/7	4/5	2/7	4/13
Y. ruckeri	1/7	0/5	0/7	0/13

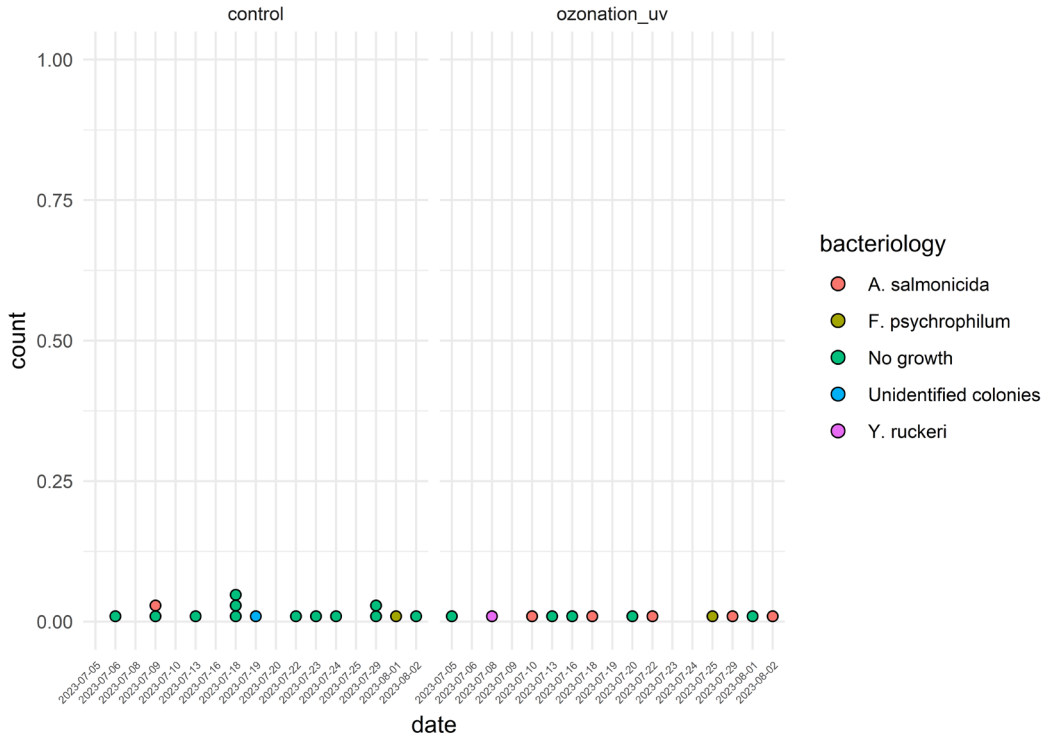


Figure 5-26. The same data as in Table 5-3 and Table 5-4, showed on a timeline (date of death) and grouped by condition (treated A+B; untreated C+D).

5.4.3 Discussion

The fish used for this field trial were not naïve and originated from the batch of fish that were already in the raceway, whereto the four experimental tanks were connected. Therefore, the fish already had been exposed to potential pathogens that were in the system before the onset of the trial.

The sampling of sick fish from the raceway at the beginning of as well as during the trial showed that the fish pathogens *F. psychrophilum*, *Y. ruckeri,* and *A. salmonicida* were in system. These pathogens might have been moved over to the four tanks, either together with the fish or in the water, as the water for both raceway and tanks were part of the same water recirculation system. This means that what this trial is elucidating is what ozone treatment of water does to transmission of pathogens to the tanks via the water as well as if it over time will lower the infection level in the specific tanks. The pathogens will not be completely removed, as they might have already been transferred to the tanks by the fish, but the infection pressure might be lowered, as the water originating from the same system where there are several pathogens have been ozone treated. After one month, no differences in mortalities between tanks treated with ozone and untreated tanks were observed. This could be caused by the short duration (4 weeks) of this experiment. Further investigation of the water samples taken will also elucidate if there were lower levels of pathogens in water from tanks that had been ozone treated. Additional examinations will also be performed on the water samples to determine the total composition of microbial communities and to investigate possible microbial taxa preferentially eliminated by ozonation treatment.

At a macroscopical level, the water from untreated tanks was visibly more turbid and showed a greener pigmentation (Figure 5-21), which might confirm previous studies stating that oxidants

are able to also prevent potential algal blooms (Chen et al., 2009). Therefore, algae quantification and comparison between conditions might also be worth investigating in future experiments.

The fact that the dead fish during the trial were frozen on the day of death and first all examined by the end of the trial are likely biasing the result, meaning that more of the fish in reality harbored pathogenic bacteria. This might be further elucidated when samples from dead fish are screened by specific qPCR methods targeting individual pathogens. For sure it can be concluded by the bacteriological examinations of the dead fish that the pathogens can be found among fish in the four experimental tanks.

The fact that the fish in the tanks might have lived under better conditions than the fish in the raceway, when it comes to e.g., fish density, access to feed, might lead to that the beneficial effect of ozonation will not be as visible, as if the fish density had been higher in the tanks. A comparison of fish densities in tanks versus raceway shows that the fish density in the tanks were 10 kg biomass in 0.7 m^3 water at the onset of the trial, whereas it was approx. 50 kg biomass per m^3 water in the raceway.

6. Fish health and pathogens

Fish health and welfare can be seriously impacted by different pathogens, among them pathogens of bacterial origin. The level of bacteria in recirculated aquaculture systems (RAS farms) can be high and is further increased by the presence of organic waste particles. Protein skimming and ozonation are all features affecting the microbial level in RAS, meaning they can also influence the level of fish pathogenic bacteria. Chapter 6 will explore how these two systems can influence fish pathogenic bacteria and thereby also the health and welfare of fish in RAS.

6.1 Effects of foamate on fish performance and pathogen abundance

6.1.1 Background

Organic waste residues can impact microbial growth as well as fish welfare and performance in aquaculture. In this chapter, we investigate how the exposure to organic waste can influence the welfare of fish.

In our first pilot experiment, we exposed rainbow trout fry to increasing concentrations of foamate (foamate in the subsequent chapter refers to the material removed by protein skimmer in RAS, also referred to as foam fractionation treatment) to assess its impact on:

- Fish survival
- Behavior (feeding, swimming)
- Growth performance.

Additionally, foamate microbial composition was examined to assess:

- Possible pathogenic bacteria content
- Differences in composition between batches as well as overtime (to test bias in the experiment).

The foamate used in this experiment originated from a RAS system at DTU Aqua Hirtshals.

6.1.2 Materials and methods

Experimental setup

Rainbow trout eyed eggs from a Danish commercial fish farm officially registered free of IPNV, IHNV, VHSV, and bacterial kidney disease (BKD) were disinfected by an iodofor, solution, and were then hatched and grown in a dedicated Specific Pathogen Free (SPF) area in the facilities of the Section for Fish and Shellfish Diseases (EURL, DTU Aqua, Kgs. Lyngby, DK). Once fish reached an average weight of 4 g, 10 groups of 40 fish were transferred to 10 independent tanks (8 L each) in the high containment infection facility of the fish facility. Fish were fed commercial feed pellets twice per day, in the morning and in the afternoon just after exposure to the foamate solutions. The tanks were independently oxygenated, and the flow-through systems ensured a water exchange of 2 L/hour. Foamate from a protein skimming system was repeatedly sent from DTU Aqua, Hirtshals for the duration of the trial to perform the exposure experiment. Starting from day 0, tanks were subjected to increasing concentrations of organic waste in water in duplicate, for a total of 5 experimental conditions: 1:3, 1:6, 1:12, and 1:24 foamate in water, and control (no foamate exposure) (Figure 6-1).

Figure 6-1. Experimental tanks exposed to organic waste material (foam fraction). From left to right: control tank, 1:3, 1:6, 1:12, 1:24 foam to water ratio exposed tanks.

The daily exposure consisted of stopping the water exchange flow, lowering the level to 6 L in each tank, 2 hours of exposure to foamate, whereafter the water flow was re-opened. The exposures were repeated once every day for 4 days. On exposure day 4, 5 fish in each tank were randomly collected and euthanized for sampling (Figure 6-2). Fish were then let to recover for a period of 4 days, and on day 9, 5 fish were again randomly collected and euthanized for sampling. The experiment was thereafter terminated.

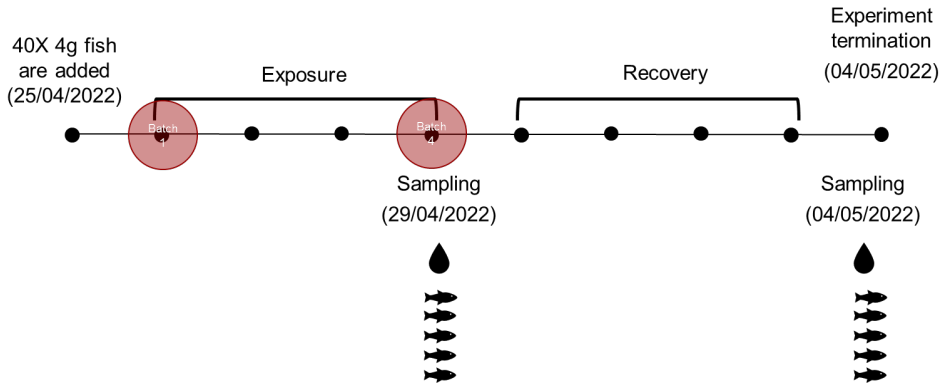


Figure 6-2. Sampling timeline.

Sampling

On day 4 and day 9, five fish were randomly collected from each tank and euthanized with a lethal dosage of benzocaine 4% in water. Weight and length of each fish was measured and recorded. Fish were aseptically dissected, and internal organs were sampled for bacteriological examination (spleen, kidney, brain). Each sample was plated on both blood agar (BA) and tryptone yeast extract salts agar (TYES-A) plates.

Additionally, water and foamate samples were collected in sterile glass bottles and immediately processed as follows. Sequential filtrations were performed first on a fiberglass 100 um-pore membrane and later on a 0.22 µm PVDF membrane, until membrane clogging. Filters were aseptically sectioned and placed in 2 mL sterile Eppendorf tubes, then snapped frozen on dry ice. The foamate batches used on day 1 and 4 of exposure were analyzed on the day of exposure and after 7 weeks of storage at 4°C. Additionally, a dilution of 1:6 foamate in water, and water from the experimental facilities were also analyzed.

Foamate batches used on day 1 and 4 of exposure were additionally diluted 1:10 and plated on TYES agar plates to compare the abundance of Colony Forming Units (CFUs) between foamate batches.

Nucleic acid extraction

For nucleic acid extraction, MagMAX Microbiome Ultra Nucleic Acid Isolation Kit (Applied Biosystems) was used in conjunction with the automated extraction system KingFisher Flex (Thermo Fisher Scientific) with modifications as previously described (Zarantonello and Cuenca, 2024). Eluted nucleic acid was stored at -20°C until subsequent processing.

16S rRNA gene library preparation and sequencing

DNA was quantified on a spectrophotometer (Nanodrop 2000). 10 ng of each sample were used as a template for library preparation using the SQK-16S024 kit (Oxford Nanopore Technologies) following the manufacturer's recommendations. Amplicons were purified using Ampure beads and quantified using Qubit HS DNA kit. Libraries were pooled in equimolar ratios and loaded on a FLO-001 flow cell for sequencing following manufacturer's guidelines.

Data analysis

Statistical analysis of fish weight and length was carried out using R Statistical Software (R Core Team, 2022), using pairwise comparisons with Wilcoxon rank sum test between experimental groups. Raw sequencing data were basecalled with high accuracy (quality threshold phred=9), de-multiplexed, and primers, adapters, barcodes, and chimeras were removed using Guppy (ONT). Resulting reads were filtered by length, discarding reads <1000 bp and > 2000 bp. Quality control was performed using fastqc. Pre-processed reads were classified with Emu (v3.3.1; (Curry et al., 2022) taxonomic classifier using RDP with NCBI taxonomy database. Diversity metrics were computed using package *phyloseq* and data was plotted package *ggplot2*.

6.1.3 Results

Reduced survival and behavioural changes

No reduced survival was observed in any of the experimental conditions upon foamate treatment for the duration of the experimental trial. However, rainbow trout groups exposed to the two highest concentrations of foamate (1:3, 1:6) showed changes in their feeding behavior after exposure. As opposed to normal chasing of feed pellets observed in the other conditions, fish were indifferent to the feed. Such a change in behavior could suggest a higher degree of stress, lower appetite, or even impaired capacity to sense the presence of feed pellets in their proximity.

Growth performance

Fish collected for sampling at the end of the exposure period were measured in weight and length. Similarly, all individuals remaining at the termination of the experiment were also measured (N=60/group). Despite the observed changes in feeding behavior after exposure in the highest concentrations of foamate, no significant difference was observed between conditions at termination point (Figure 6-3).

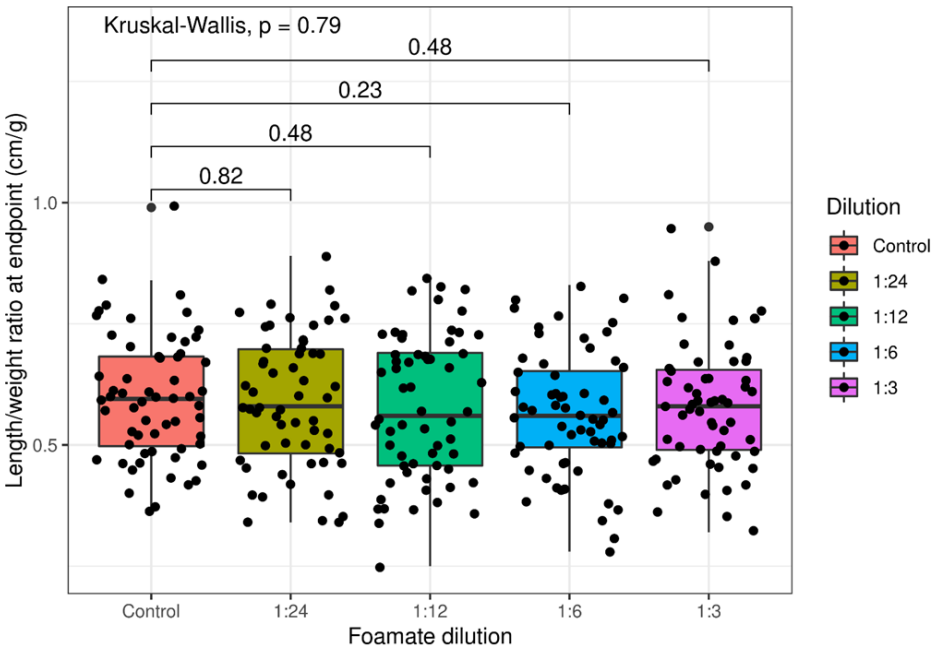


Figure 6-3. Comparison of length/weight ratio (expressed in cm/gr) between the experimental groups (control group and four foamate dilution groups).

Bacteriological examination

Foam fractionation is expected to concentrate organic particles from the water and eliminate them. Therefore, it was hypothesized that bacteria associated with organic particles are removed together with the foam. Hence, not only fish were exposed to an increased concentration of organic waste, but also to a higher concentration of bacteria, with an unknown composition. In order to investigate if foamate exposure subjected the fish to a higher chance of bacterial infection, rainbow trout were randomly collected at the end of the exposure period (day 4) and at the end of the recovery period (day 9) and sampled (from internal organs) for bacteriological examination. No bacterial growth was observed, apart from isolation of *Flavobacterium psychrophilum* from one fish sampled 4 days after exposure in the group exposed to 1:6 dilution of foamate.

Foamate bacteriological examination

Dilutions of foamate (10-fold) were plated on TYES agar plates to determine the CFU counts for two different batches (used on day 1 and day 4 of exposure). The two batches showed similar abundance of CFU/mL.

First day exposure: 1.4×10^7 CFU/mL

Fourth day exposure: 6.7×10^6 CFU/mL

Microbial diversity of foamate by batch, filter type, storage time

The microbial content of the foam fraction was unknown. Because it was our intention to use the foam exposure in combination with a bacterial infection in the next phase of the experiment, we needed to know 1) if the foam bacterial content was uniform across batches; 2) if filter type used for water filtration impacted the detectable taxa; 3) if storage time impacted the microbial composition of the foam fraction. To answer these questions, two batches of foamate used for exposure (namely day 1 and day 4) were filtered on the day of exposure, 7 weeks later, and with 2 different filter types. The bacteria obtained on the filters were subsequently analyzed through

16S rRNA gene metabarcoding. The resulting bacterial compositions were uniform across the 2 batches. Unsurprisingly, the main driver of changes in composition seemed to be the filter type used for filtration. Figure 6-4 shows the relative abundances of bacteria across batches and control water at the class level.

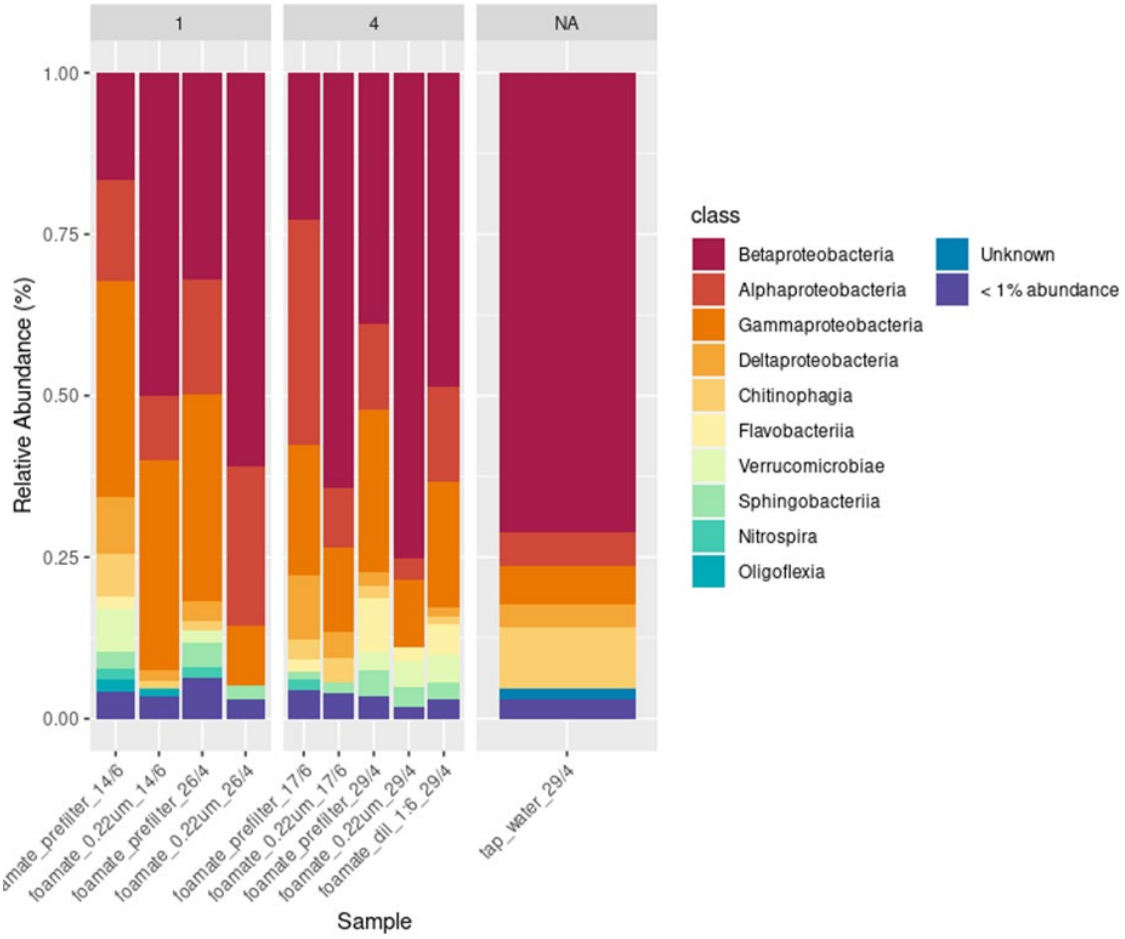


Figure 6-4. The relative abundance of bacterial classes (16S rRNA gene metabarcoding) of two batches (1 and 4) of foamate on week 1 (26/4 and 29/4) and week 7 (14/6 and 17/6), filtered through 0.22 µm or 100 µm filters. Additionally, the composition of tap water (shown on the right) and the composition of one of the dilutions (1:6) was analyzed.

Surprisingly, no dramatic changes in bacterial composition were observed after 8 weeks of storage at 4°C. As shown in Figure 6-5, the species richness (calculated as Chao1 alpha diversity index) between week 1 and week 7 was not significantly different between storage times.

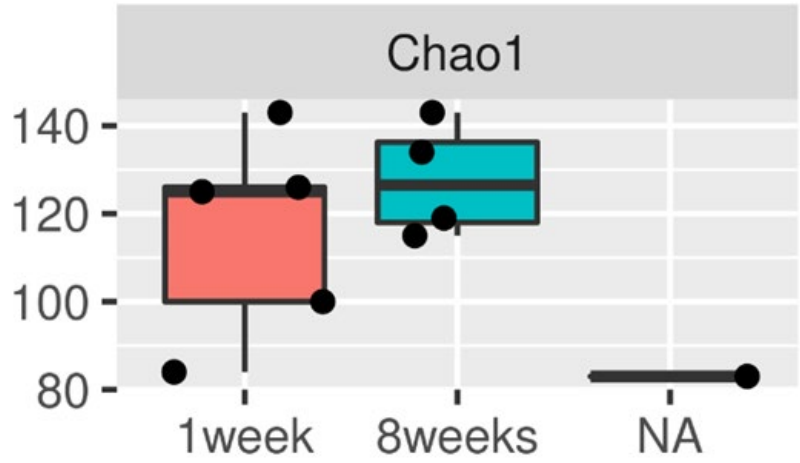


Figure 6-5. Species richness of foamate sample stored either 1 week or 7 weeks at 4 degrees.

6.1.4 Discussion

No reduced survival was observed during the experiment. Changes in feeding behavior were noticeable at least in the two highest foamate concentrations. However, this change in behavior did not cause growth changes in the different groups, maybe because the fish were only subjected to foamate during a short period of 4 days. The short trial period can also very well be the reason that no mortalities were seen in any of the groups. Therefore, no strong conclusions on growth performance or mortality rate in connection with foamate exposure can be drawn based on this experiment.

Surprisingly, a known bacterial pathogen for rainbow trout, *F. psychrophilum* was found in otherwise clean fish originating from a batch of SPF fish. This leads to the conclusion that the used foamate contained *F. psychrophilum* in such concentration that it could infect the fish and thereby be present in internal organs after four days exposure.

As expected, the specific filter type will change the bacterial taxa that are detected. Because most studies of bacterial composition of water use 0.22 µm filters only, we chose to carry on the subsequent part of the study with this filter type. Without pre-filtration, the volume of water that can be filtered for each sample is significantly lower, but still sufficient to proceed with the analyses. Species richness does not change over storage time; however, it only represents the genomic content of the sample, but does not provide information on the bacterial activity changes over time.

Alive bacteria in the different batches of received foamate was estimated by 10-fold dilutions plating, which showed that a similar amount of CFUs/mL were present. However, the plating was not repeated after 8 weeks to estimate the number of alive bacteria after storage time, since our main purpose in this case was to confirm that the CFU number per volume unit in each batch of foamate was comparable.

6.2 Protein skimming and impaired water quality on fish performance

6.2.1 Background

From our pilot study on organic waste (foamate) exposure (6.1), we know that:

- The foamate did not induce visible effects (in terms of behaviour, weight, mortality) in fry of rainbow trout during our experimental trial, regardless of the foamate concentration.
- Foamate hosted *F. psychrophilum*.
- Foamate preserved at 4°C did not significantly change in bacterial composition over time (for at least 7 weeks).

Foam fractionation is a water treatment that is supposed to separate organic waste residues from the water. As such, it is hypothesized that also the bacteria associated with organic particles should be eliminated from the water.

In this part of the project, we investigated this hypothesis with the objective of testing the potential of the protein skimming treatment to:

- Modify the health, growth and performance of fish exposed to organic waste material (foamate).
- Eliminate pathogenic bacteria during active cohabitation with infected fish, and as such.
- Alter microbial disease dynamics and transmission in the fish.
- Modify the microbial composition on the fish and in the water.

6.2.2 Materials and methods

Experimental setup

Rainbow trout eyed eggs from a Danish commercial fish farm officially registered free of IPNV, IHNV, VHSV and bacterial kidney disease (BKD) were disinfected by an iodoform solution, then hatched and grown in a dedicated Specific Pathogen Free (SPF) area in the facilities of the Section for Fish and Shellfish Diseases (EURL, DTU Aqua, Kgs. Lyngby, DK) using recirculated tap water disinfected by UV light. Once fish reached an average weight of 1.5 g, 8 groups of approximately 100 fish were transferred to the high containment infection facility in 8 independent tanks (30 L each) at a constant temperature of 12±1°C. Fish were fed commercial feed pellets (2%) twice per day, in the morning and in the afternoon just after exposure. The tanks were independently aerated, and partially recirculating systems were ensured by individual biofilters.

Figure 6-6. Experimental set-up in the high containment infection facilities in DTU Aqua, Lyngby. Each 30 L tank was individually recirculated (biofilters can be seen on the floor for each tank). On the foreground, one of the tanks treated with foam fractionation is shown: the water from the biofilter is directed towards the protein skimmer device, where foam is created and overflowed to a separate reservoir (white tank on the floor). The water fraction from the protein skimmer is instead redirected to the main tank.

Each tank (Figure 6-6) had an overflow system as well as addition of water (1.5 L/h) regularly, ensuring that the water in the tanks would not run low, as foamate (and thereby water) was removed from some of the tanks. Once a day the tanks were cleaned with the removing of roughly

1/3 of the water in the tanks. Foamate from a foam fractionation system was repeatedly sent from DTU Aqua, Hirtshals for the duration of the trial in order to perform the exposure experiment. Starting from day 0, all biofilters were primed with foamate. Figure 6-7 shows the timeline, including sampling of fish and water, of the experiment. From the moment of biofilter priming, newly received batches of foamate were added repeatedly twice per week until the end of the experiment. Water was sampled from each tank on day 13 after priming the filters. Individual protein skimmers were installed in 4 of the tanks and activated on day 15. On day 22, both water and foam were sampled, together with 5 fish from each tank. On day 32, 20 randomly selected fish in each of the 8 tanks were injected with 0.05 ml of either mock sterile media or $3.8x10^4$ CFU/fish of *Y. ruckeri* 100415-1/1. A liquid culture (KB) with the isolate (originating from and kept at -80 degrees in a 15-20% glycerol stock) had been incubated at 20ºC for 48 h. The bacterial culture was thereafter diluted in the same media. The infections were performed in order to obtain four experimental conditions, each in duplicate, as depicted in Table 6.1.

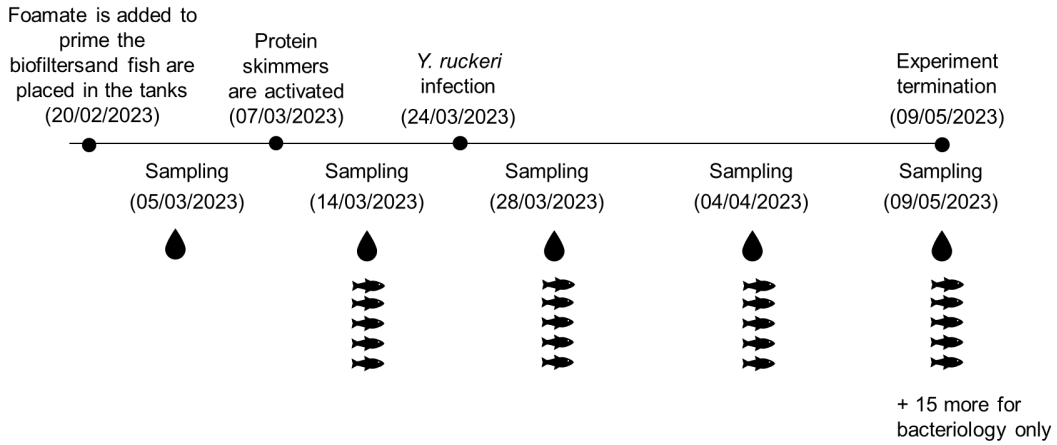


Figure 6-7. Timeline for the experiment.

Table 6-1. The division of tanks in experimental conditions.

	Foam fractionation	No treatment
Y. ruckeri infection	Tanks 5,6	Tanks 3,4
Mock infection	Tanks 1,2	Tanks 7,8

After infection, water, foam and 5 fish from each tank were sampled on day 36, 43, and 78 (day of termination of the experimental trial). Survival was recorded daily, and individuals reaching humane endpoint were euthanized with a lethal dosage of benzocaine 4% in water.

Sampling

On day 22, 36, 43, and 78, 5 fish were randomly collected from each tank and euthanized with a lethal dosage of benzocaine 4% in water (on day 90, 15 additional fish were sampled for bacteriological analysis; see Figure 6-7). Weight and length of each fish was measured and recorded. Fish were aseptically dissected, and internal organs were sampled for bacteriological examina-

tion (spleen, kidney, brain). Each sample was plated on both BA and TYES agar plates. Similarly, all dead individuals along the trial were collected and sampled for bacteriological analysis. Kidney samples were also collected and snap frozen in sterile 2 mL Eppendorf tubes. Water and foamate samples were collected in the morning just before cleaning and placed in sterile glass bottles, then immediately filtered until the membrane clogging. The total filtered volume was recorded for every sample. Filters were aseptically sectioned and placed in 2 mL sterile Eppendorf tubes, then snap frozen on dry ice. Representative foamate batches were also filtered in the same way.

Nucleic acid extraction

For nucleic acid extraction from filter membranes, MagMAX Microbiome Ultra Nucleic Acid Isolation Kit (Applied Biosystems) was used in conjunction with the automated extraction system KingFisher Flex (Thermo Fisher Scientific), with modifications as previously described (Zarantonello and Cuenca, 2024).

For kidney material extraction, NucleoMag VET kit was used with the automated extraction system Kingfisher Flex (Thermo Fisher Scientific). Eluted nucleic acid was stored at -20°C until subsequent processing.

Positive controls (*Y. ruckeri* isolate used for infection) and negative controls (filters only, reagents only) were extracted along samples in every plate.

***Y. ruckeri* quantification by qPCR analysis**

To quantify *Y. ruckeri* in both internal organs (kidney) and environmental (water and foam) samples, a previously designed and validated probe-based qPCR assay was used (Miller et al., 2017), targeting the glutamine synthetase gene (*gln*A).

Primer sequences:

FW: 5’-TCCAGCACCAAATACGAAGG-3’

RV: 5’-ACATGGCAGAACGCAGAT-3’

Probe: 5’-AAGGCGGTTACTTCCCGGTTCCC-3’An internal control assay targeting the ELF1alpha rainbow trout gene was used in conjunction to the *Y. ruckeri* assay to assess baseline presence of the host genome. Artificial DNA control (GBlock, IDT) for *Y. ruckeri* has been used as a standard for the assay. Sequence from *Yersinia ruckeri* in the gBlock:

5’-ATCGATGATATCGAAGGCGCATGGAACTCCAGCACCAAATACGAAGGTGG-
TAACAAAGGCCATCGTCCCGCAGTAAAAGGCGGTTACTTCCCGGTTCCCCCAGTTGATTCC
GCGCAAGATCTGCGTTCTGCCATGTGTTTAACCATGGAAGATATGGGTCTGGTTG-3’

Luna Universal Probe Master Mix was used according to the manufacturer’s indications to perform qPCR. Data was processed on CFX Manager Software (BioRad).

Data analysis

Statistical analysis and data representation were performed on R Statistical Software (R Core Team, 2022). Survival analysis was performed using the Kaplan-Meier estimator with R packages *survival* and *survminer*.

6.2.3 Results

Survival analysis

Mortality was recorded along the experimental trial to perform survival probability analysis across experimental conditions (Table 6-2).

Table 6-2. Mortalities in the different tanks during the experiment.

Tank	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Before 24/3	15	3	12	20	7	12	23	18
After injection	6	3	21	9	4	22	5	3
Left at termination	44	59	26	35	52	29	36	48
Total sampled	35	35	35	35	35	35	35	35
Total (at the beginning)	**100**	**100**	**94**	**99**	**98**	**98**	**99**	**104**

Kaplan-Meier survival probability estimation was performed and compared across conditions, including injected fish and co-habitants together. When comparing between all four experimental conditions (Figure 6-8), the survival probability in the non-infected tanks was much higher ($p < 0.0001$), indicating that the infection and co-habitation trial with *Y. ruckeri* was effective in lowering the survival probability both in treated and untreated tanks. Interestingly, for each pair of experimental conditions, a slightly higher survival probability was estimated in the tanks where protein skimmers were installed.

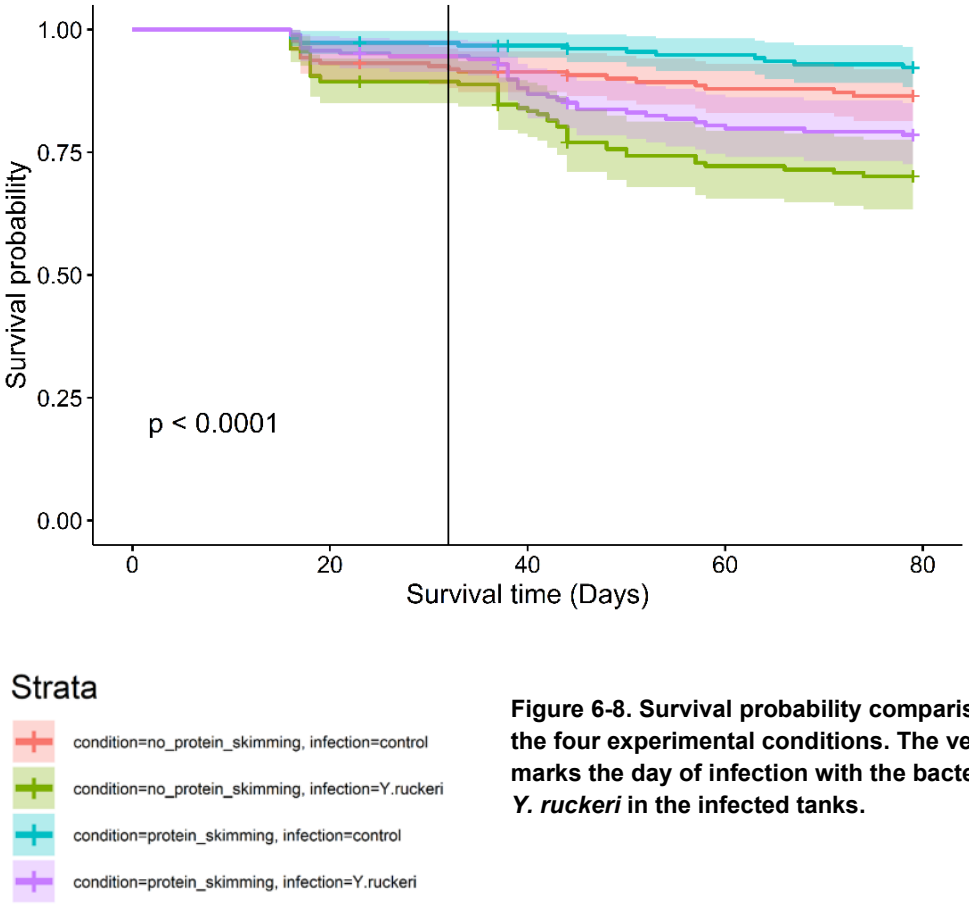


Figure 6-8. Survival probability comparison between the four experimental conditions. The vertical line marks the day of infection with the bacterial pathogen *Y. ruckeri* in the infected tanks.

Survival probability was then compared between infected tanks only, to assess the effect of water treatments (foam fractionation, no treatment) on fish survival in the presence of a pathogen. Although not statistically significant, the survival probability is slightly increased in the tanks where protein skimmers were present (p=0.055; Figure 6-9).

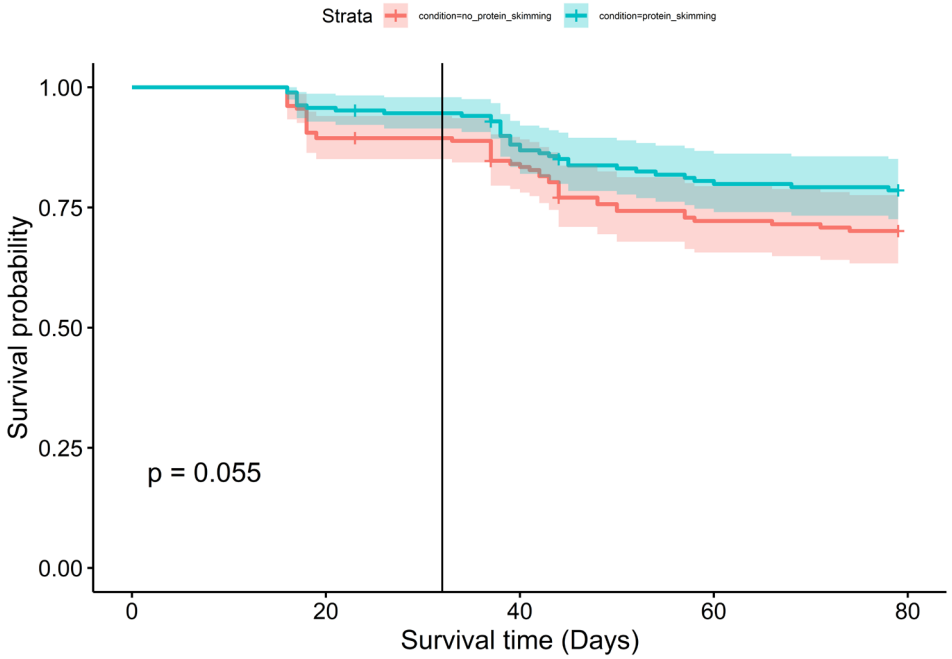


Figure 6-9. Survival probability comparison between infected tanks. The vertical line marks the day of infection with the bacterial pathogen *Y. ruckeri* in the infected tanks.

Similarly, the test was finally performed also in non-infected tanks, to assess the effect of protein skimming on fish survival probability. Again, as in the infected tanks, the survival probability is slightly increased in the presence of protein skimmers, although the difference is not statistically significant (p=0.066; Figure 6-10).

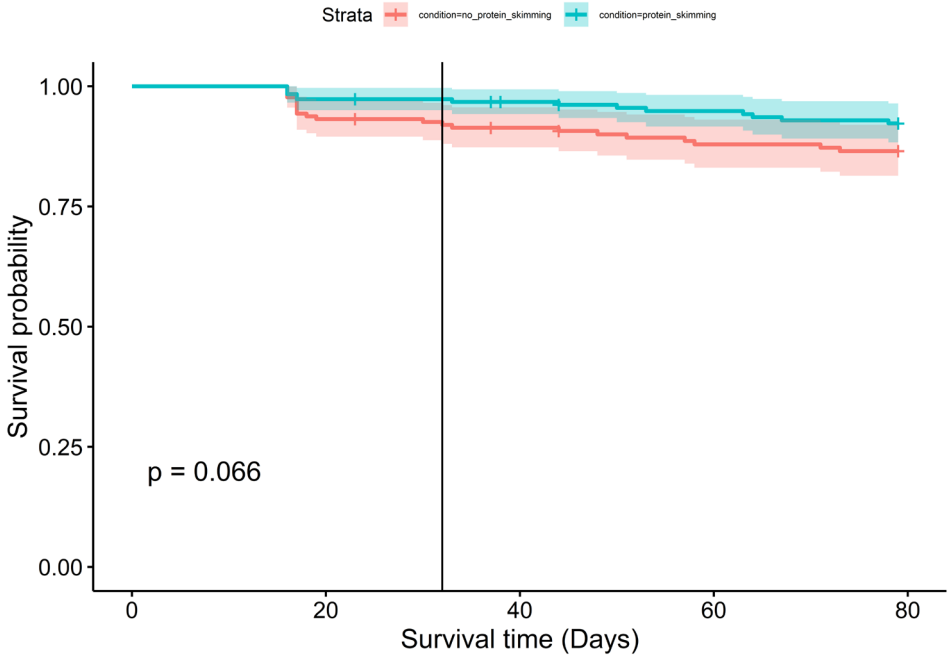


Figure 6-10. Survival probability comparison between non-infected tanks. The vertical line marks the day of infection with the bacterial pathogen *Y. ruckeri* in the infected tanks.

Bacteriological examination of co-habitants reaching humane endpoint

Internal organs (brain, kidney, and spleen) from co-habitants reaching humane endpoint were plated on BA (Figure 6-11) and TYES agar (Figure 6-12) to assess if morbidity occurred due to an infection with a known pathogen, and specifically, if *Y. ruckeri* was being transmitted effectively in infected tanks. *Yersinia ruckeri* was found in almost equal number of dead co-habitants when comparing tanks with protein skimming or without (4-5 positive individuals). Surprisingly, one cohabitant from a control tank with no protein skimming was also found positive for *Y. ruckeri*. Additionally, an infection with another known rainbow trout pathogen, *F. psychrophilum*, was found to be present in all tanks. This was expected, as was known from previous analyses, that *F. psychrophilum* could be found in the foamate from the DTU Aqua Hirtshals facility in all tanks.

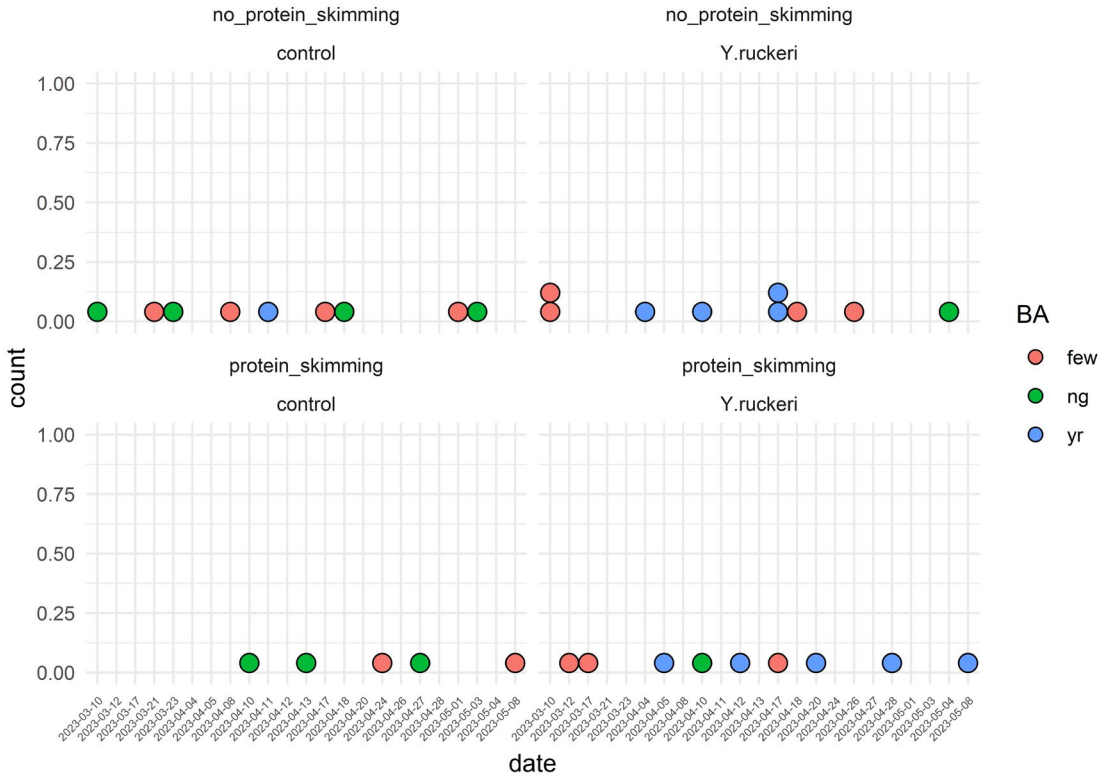


Figure 6-11. Results from the bacteriological examination on BA of dead co-habitants. The figure legend refers to either no bacterial growth (ng), few unidentified colonies (few), or *Y. ruckeri* presence (yr).

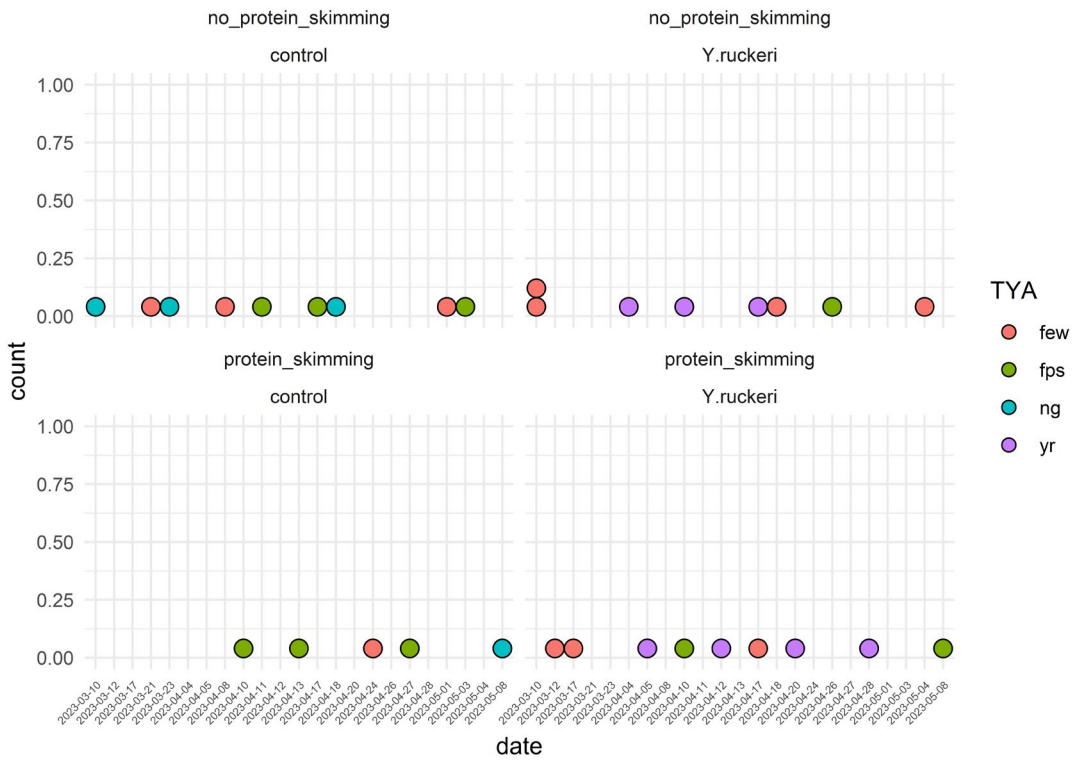


Figure 6-12. Results from the bacteriological examination on TYES agar of dead co-habitants. The figure legend refers to either no bacterial growth (ng), few unidentified colonies (few), *Y. ruckeri* presence (yr), or *F. psychrophilum* presence (fps).

Bacteriological examination of co-habitants sacrificed at sampling points

Similarly, internal organs (brain, kidney, spleen) were plated on BA (Figure 6-13) and TYES agar (Figure 6-14) to assess the presence of pathogens from the sampled individuals (N=10/group) (40/group on the termination day).

Although the sample size was too low to draw straightforward conclusions, the results showed that *Y. ruckeri* was present in more co-habitants in the un-treated tanks, compared to the ones treated with protein skimming, at least in the sampled individuals. Tanks with no infection showed no *Y. ruckeri* growth at all.

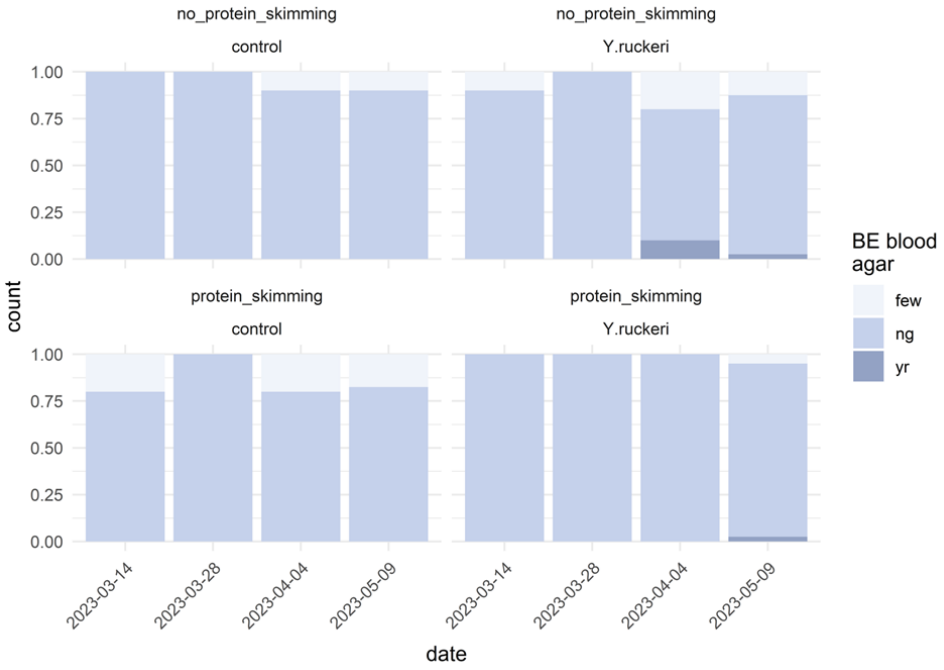


Figure 6-13. Results from the bacteriological examination on BA of sacrificed co-habitants at specific sampling points. The figure legend refers to either no bacterial growth (ng), few unidentified colonies (few), or *Y. ruckeri* presence (yr).

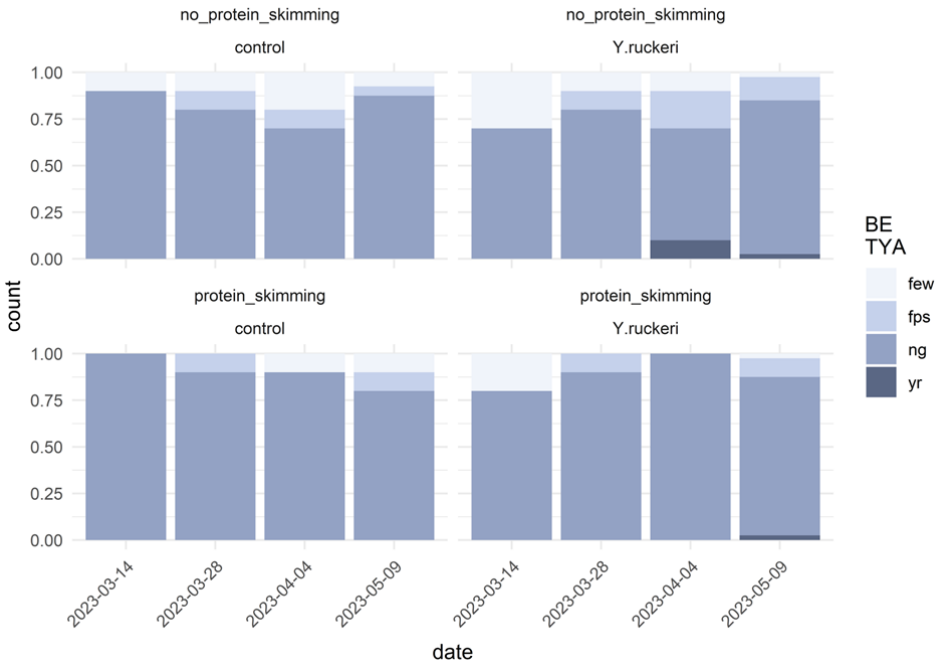


Figure 6-14. Results from the bacteriological examination on TYES agar of sacrificed co-habitants at specific sampling points. The figure legend refers to either no bacterial growth (ng), few unidentified colonies (few), *Y. ruckeri* presence (yr), or *F. psychrophilum* presence (fps).

Molecular quantification of *Y. ruckeri* in sampled co-habitants

Yersinia ruckeri was quantified in kidney samples of co-habitants at different time points (N=10/group). Molecular quantification was performed by qPCR. The assay confirmed the results of bacterial examination: no *Y. ruckeri* was found in the kidneys of cohabitants, except for 1 fish in a tank with no protein skimming and infected with *Y. ruckeri*.

Molecular quantification of Y. ruckeri in water and fractioned foamate

The pathogen was also quantified in the water from the tanks, and in the foam that was excluded by protein skimmers. Infection-negative tanks were completely clear of *Y. ruckeri* at all time points, as expected. On the other hand, *Y. ruckeri* was found in varying concentrations in all infected tanks. The highest concentration was found to be on day 43 (13 days after infection) in one of the untreated tanks, hinting at the ability of foam fractionation to lower the pathogen quantity in the recirculating systems (Figure 6-15).

To further investigate this hypothesis, *Y. ruckeri* copy number/mL was also compared between the water coming from the tank and the foam corresponding to the same tank (Figure 6-16). In both tanks analyzed, the concentration of *Y. ruckeri* was higher in the foamate at almost all time points, with an exception in tank 6 in the middle of infection (2023-03-28).

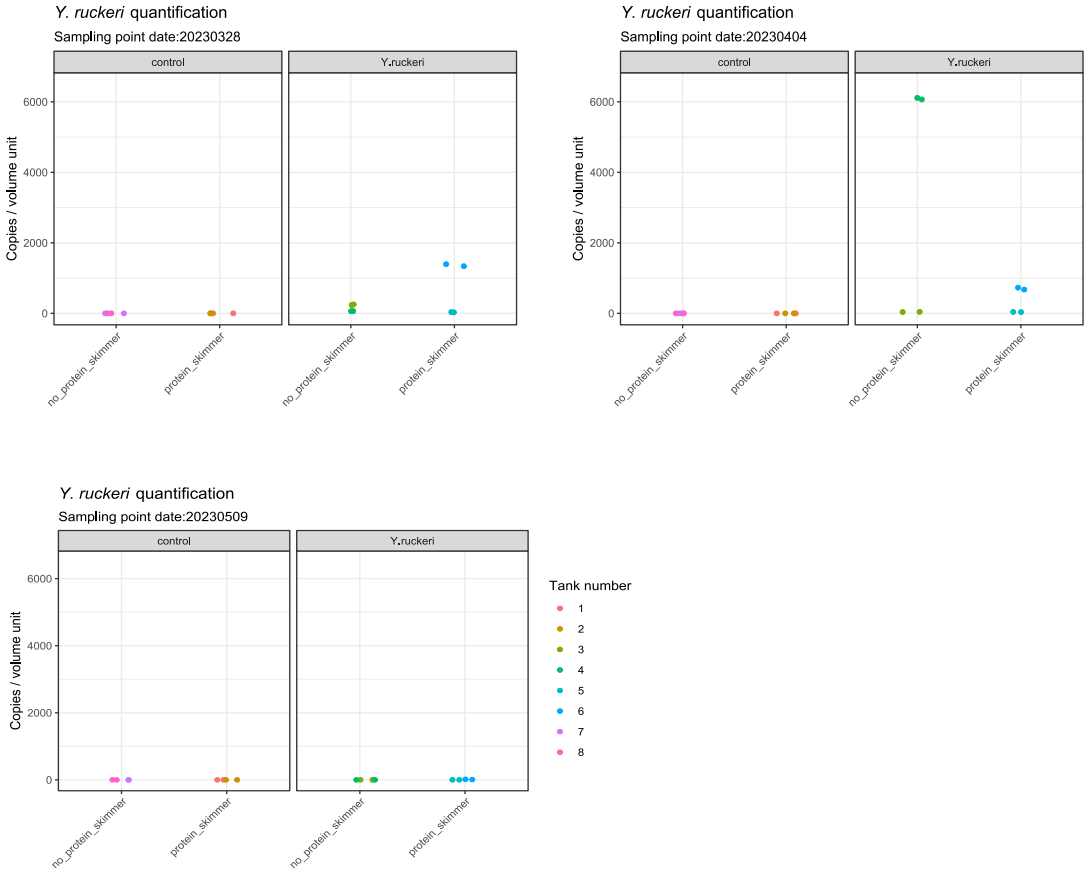


Figure 6-15. *Y. ruckeri* copy nr/volume unit (100 mL) in the water at 3 time points: start (2023-03-28), middle (2023-04-04) and end (2023-05-09) of infection, grouped by experimental condition. The dot plot shows qPCR technical replicates for each sampling point and tank. The tank of origin of each sample is color coded as described in the figure legend.

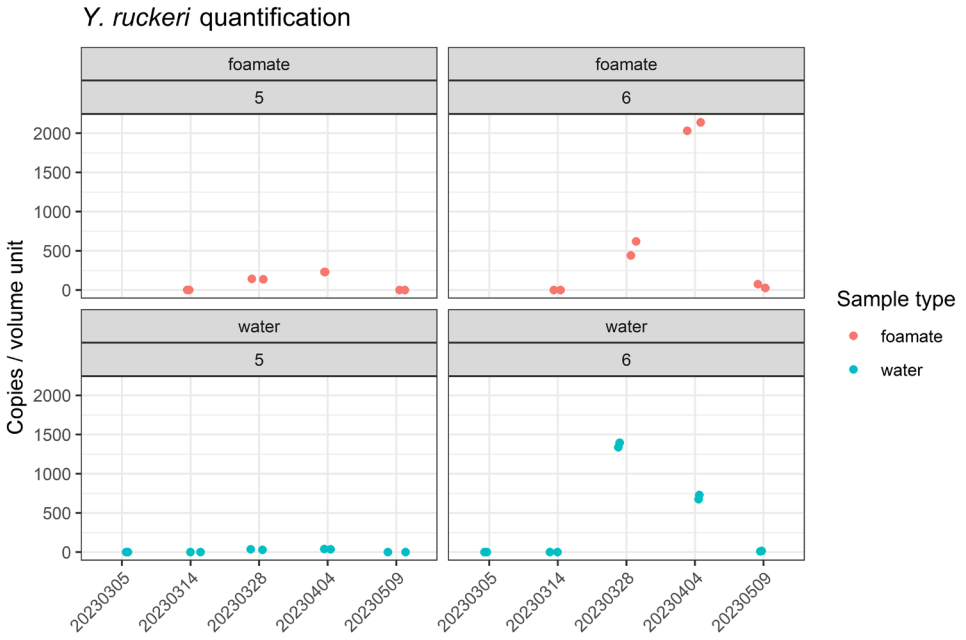


Figure 6-16. *Yersinia ruckeri* copy number/volume unit (100 mL) in foamate and water from the same tanks (tanks with fish infected with *Y. ruckeri* and with protein skimmers, i.e., tanks 5 and 6). The dot plot shows qPCR technical replicates for each sampling point in foamate or water of tanks 5 (left) or 6 (right).

Growth performance evaluation across experimental conditions

Figures: Figure 6-17 and Figure 6-18 show the growth performance of the fish across experimental conditions, Figure 6-17 focusing on length (cm) and Figure 6-18 weight (g).

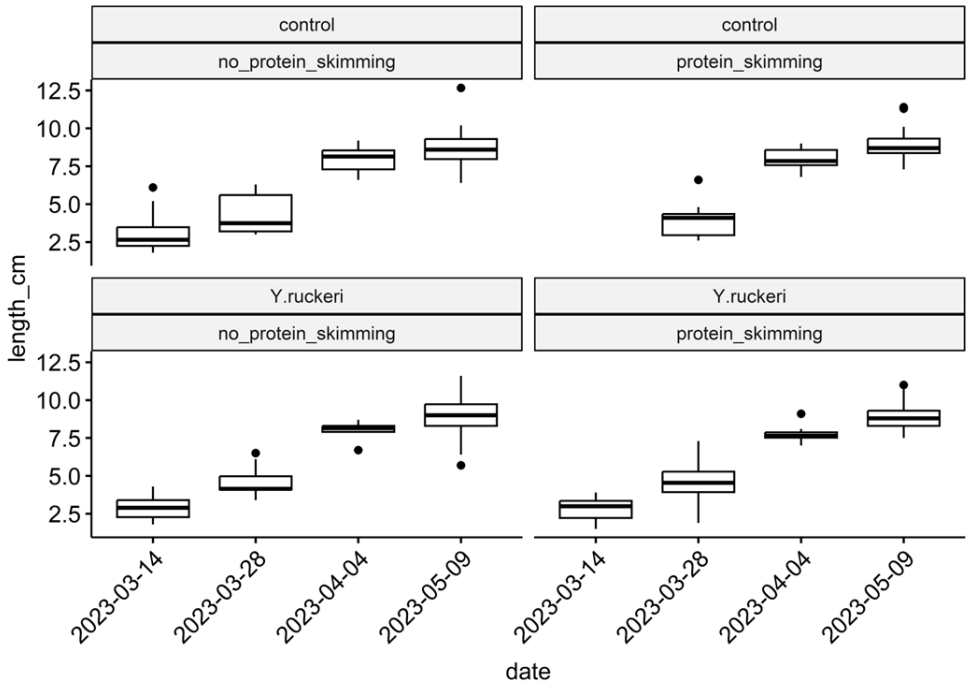


Figure 6-17. The length (cm) of fish in the four experimental condition groups over experimental time-points (N=10/group; N=40/group on the last time-point).

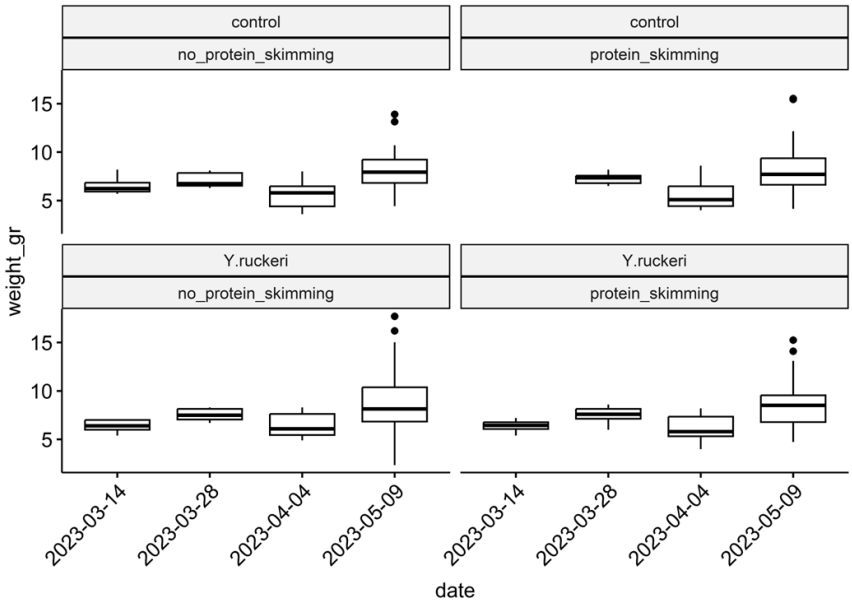


Figure 6-18. The weight (g) of fish in the four experimental condition groups over experimental time-points (N=10/group; N=40/group on the last time-point).

6.2.4 Discussion

Even if not statistically significant, survival probabilities were higher in the treated tanks, both in the presence of a known pathogen or in absence of it. This may point to the ability of foam fractionation to reduce stress on the farmed animals, therefore improving their performance.

Bacteriological examination of sampled fish at different time points showed a higher number of individuals affected by pathogenic bacteria in untreated tanks with respect to tanks treated with foam fractionation. *Yersinia ruckeri* levels in the water reached a way higher concentration in the untreated tanks than in the treated ones. As a possible explanation, we hypothesized that the pathogen was being concentrated in the foam fraction. Molecular analysis of the foam fraction revealed that indeed the pathogen was generally more concentrated in the foam than in the water in the tanks. Overall, we can conclude that the use of protein skimmers may be useful to reduce the bacterial load in the water, and that this also applies to pathogens, which are also being removed from the system and concentrated in the foam fraction.

However, the experimental trial set up had limitations:

- The experimental set up had many sources of variability, among them the most important is the daily manual calibration of the protein skimmers. The organic particle removal rate was not stable over time, and this causes variability between replicate tanks but also among time points for the same tank.
- The bacteriological examination of rainbow trout internal organs at the different time points highlights the presence of *F. psychrophilum* in all conditions, which was expected given that it was harbored by the foamate itself. This is another source of variability, given that the survival probability is also affected by the presence of this pathogen. In our case, since we are looking at the ability of protein skimmers to reduce all sorts of bacteria, and not only *Y. ruckeri*, the presence of *F. psychrophilum* actually added further data to state that fish in the treated tanks were less prone to infection.

7. Biological filtration

Biofiltration includes the important microbial nitrification process where ammonium is oxidized via nitrite to nitrate. Biofiltration is a central treatment process in all aquaculture facilities that re-uses water, as it ensures low TAN and nitrite levels. The two-step nitrification process includes

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2H^+$$

$$NO_2^- + ½\ O_2 \rightarrow NO_3^-$$

The equation below includes inorganic carbon and biomass formed of the combined processes

$$NH_4^+ + 1.86\ O_2 + 1.98\ HCO_3^- \rightarrow 0.020C_5H_7NO_2 + 0.98NO_3^- + 1.88CO_2 + 1.04H_2O$$

Biofiltration also includes the removal of dissolved and particular organic matter, which positively affects the water quality. The microbial processes involve both slow autotrophic nitrification and much faster heterotrophic processes (C oxidation and N assimilation in the presence of oxygen and denitrification at anoxic conditions). The microbial conversions of dissolved N and C lead to biomass yields which is seen as biofilm growth. The biofilm will be released from moving bed bioelements due to the shear forces of mixing or accumulate in fixed bed biofilters.

The type of biofilter (fixed vs. moving bed), size, dimension, N-loading rate, temperature, salinity, filling ratio, carrier material, acclimatization, and hydraulic retention time all influence the biofilter performance (Chen et al., 2006; Malone and Pfeiffer, 2006; Pedersen et al., 2015; Suhr and Pedersen, 2010). Organic matter also affects the biofilter, typically by overgrowing surface areas and potentially outcompeting the nitrifying bacteria for space and available oxygen. Organic matter gets trapped either as particulate organic matter in fixed bed biofilter and/or as biofilm due to excessive microbial growth. In such cases, an uneven flow distribution (preferential flow) may occur due to channel formation, and mechanical backwashing is required. The protocols for this are well developed, but it is time- and energy-consuming. Substantial amounts of water are often required, and the removed biofilm is difficult to drain effectively. Here, we tested how to improve effective and gentle backwashing and to capture and remove organic matter by using a new approach with foam bioelements (Section 7.1.).

The startup and colonization of biofilters is an area with substantial economic perspectives. The initial colonization and maturation of nitrification in biofilters typically take 4-6 weeks in freshwater. In cold seawater, this can easily last 4-6 months before stable and sufficient nitrifying bacteria are established. The main obstacles relate to the slow growth of nitrifying bacteria and the requirement of a suitable surface for them to colonize. In section 7.2, we describe a study comparing four different types of commercially available bioelements during startup and colonization in seawater at a commercial salmon RAS.

The purpose of this work package was: i) *to reduce the microbial pressure in the water phase*, improve water quality, and reduce the need for chemical additives by *optimizing organic matter removal in biological filters* and ii) to *test colonization and performance of nitrifying bacteria during startup in salt water*. Based on the studies, we aimed to get new knowledge about microbial growth and retention in biofilters, to test and develop new methods to remove bacteria and particulate matter in biofilters, and document colonization and nitrogen removal capacity in saltwater biofilters.

7.1 Test of fixed bed biofilters with Levapor foam

7.1.1 Background

Recirculating aquaculture systems (RAS) with a high feed loading and low water exchange can experience severe accumulation of microparticles, such as suspended bacteria and particulate organic matter, which deteriorates water quality and can harm fish health (Pedersen et al., 2012, Pedersen et al., 2017; Schumann and Brinker, 2020). Protein skimming can remove microparticles (de Jesus Gregersen et al., 2021; de Jesus Gregersen and Pedersen, 2022; Vadstein et al., 2018) or the particles can be trapped in fixed bed biofilters (Fernandes et al., 2017). While fixed bed biofilters are effective for particle removal, they can become clogged over time, losing efficiency and increasing the risk of hydrogen sulfide (H_2S) formation if not properly maintained. Biofilter backwashing helps to mitigate these issues by reducing biofilms and unwanted organic matter, but is time-consuming and often associated with suboptimal cleaning.

This study aimed to develop a new efficient water treatment unit to remove bacteria and organic matter from RAS. This was done using Levapor® polyurethane foam (https://levapor.com/levapor-an-ideal-mbbr-ifas-carrier/), which has a promising composition and can be squeezed (Figure 7-3). Biofilter media is used in moving bed biofilters and has a high volumetric nitrogen (N) removal rate due to the structure and surface area (Plesner et al, 2021; Qi et al, 2022). The study included pilot-scale testing of tailor-made fixed-bed biofilters, focusing on their cleaning and removal performance with and without backwashing (BW). Controlled batch removal analysis and mass balances of relevant water quality parameters were performed to determine treatment efficiency at various hydraulic retention times (HRT).

7.1.2 Materials and methods

Experimental setup

The studies were conducted at DTU Aqua, Hirtshals, in a 20 m^3 freshwater RAS facility with 200-250 kg rainbow trout distributed in two tanks (Figure 7-1A). The facility was in stable operation for several months before the start of the experiment, where the fish received 2.0 kg of feed per day and a daily water change of 2.8 m^3. The specific experimental setup tested consisted of four 16 L reactors, each with 10 liters of foam rubber (Levapor® bioelement; Figure 7-1B). The reactors were made of transparent PVC pipes (Ø 160 mm) and supplied with water from the top (fixed bed down flow bioreactor). Each reactor was fitted with valves and water flow meters to control constant flow. Each reactor also had a piston to compress the medium and a drain at the bottom to collect the sludge to facilitate cleaning and rinsing of the foam rubber medium. The four reactors were supplied with water from a manifold connected to the pump sump.

The experiment was performed in duplicate, with two identical reactors [F1 and F2] with low hydraulic retention time (HRT) of 2.4 min. (400 L/h) and two reactors [F3 and F4] with high HRT of 16 min (flow rate of 60 L/h). The experiment lasted 18 weeks, from mid-July to the end of November 2022. The reactors were tested during the first six weeks, after which they were turned off, drained, and disconnected for four weeks. After week 10, the medium (4 x 10 liters of foam rubber) was filled back into the four reactors, which were tested for another 4 weeks. Finally, the water quality in the RAS plant was monitored for another month to assess the reactors' possible impact on the system level (Figure 7-2).

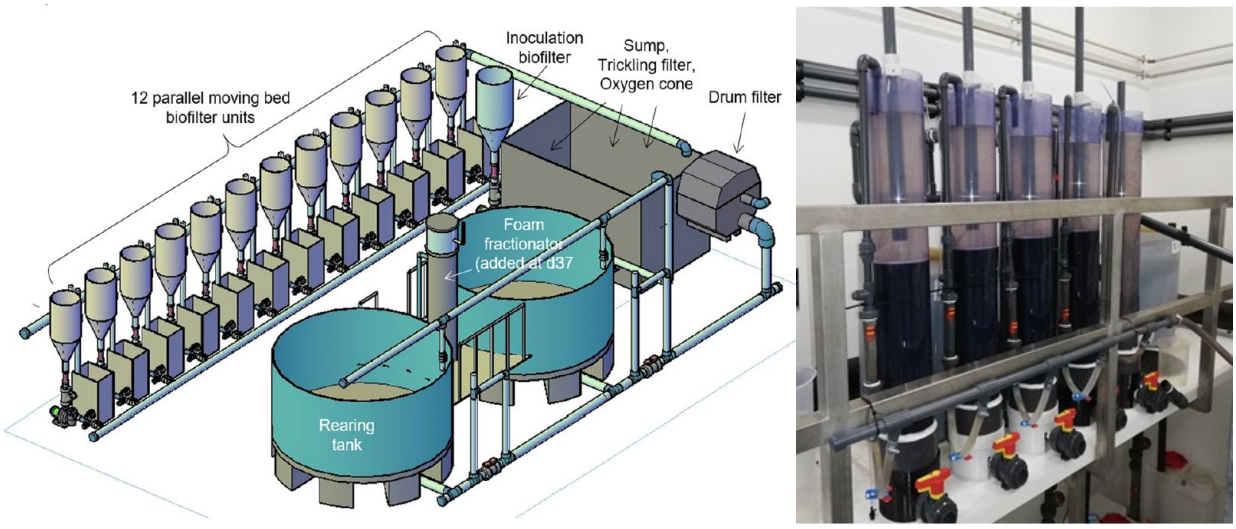


Figure 7-1. Left: RAS setup consisting of two 5 m^3 fish tanks with a total of approx. 250 kg rainbow trout, oxygen cone, cooling coil, Hydrotech drum filter (60 µm mesh), trickling filters with Exponet 200 and 12 identical, 80 L parallel biofilters. Feeding, water exchange, and cleaning measures reflect water quality similar to the production water in a model 3 trout farming. The four reactors with 10 L foam rubber (right) were connected in parallel to this RAS.

Test with Levapor (2*2 HRT)	Pause (drained)	Re-test with Levapor (2*2 HRT)	Pause
Week 1-6	Week 7-10	Week 11-14	Week 15-18

Figure 7-2. Experimental design with 2 periods (weeks 1-6 and weeks 11-14) of active operation of fixed bed biofilters with Levapor.

Sampling and analysis

Single pass efficiency – measurement of inlet and outlet concentration

Two sets of 1 L pooled water samples were collected from the common inlet and each reactor outlet to determine the single-pass efficiency (Table 7-1). The two sets of samples were taken 5 minutes apart at the same time of day.

Table 7-1. Sampling locations, frequency, and analysis

Sampling locations	Frequency	Analysis
Inlet to and outlet from the reactor with Levapor (before backwash)	Weekly (9 a.m.)	Microbial activity H_2O_2 assay, BactiQuant, Ammonium, Nitrite, turbidity and UVT
Sludge collected from squeezing/backwash	Weekly (9 a.m.)	Microbial activity H_2O_2 assay, BactiQuant, dry matter, dissolved and particulate COD
Inlet to and outlet from the reactor with Levapor (after backwash)	Monthly (10:15)	Microbial activity H_2O_2 assay, BactiQuant, Ammonium, Nitrite, turbidity and UVT
System water from the fish tank	Twice a week (8.30 a.m.)	Microbial activity H_2O_2 assay, BactiQuant, Ammonium, Nitrite, turbidity and UVT Alkalinity, pH, temp. dissolved and particulate COD

Sludge

When cleaning/draining the reactors to collect accumulated sludge, the water supply was stopped and approx. Three liters of water was drained from each reactor to avoid overflow during the backwash process. The Levapor foam media was then flushed back by squeezing it five times until the PVC plate reached a depth of approx. 20 cm from the bottom of the reactors (Figure 7-3). The resulting sludge was drained. The remaining particulate organic material was removed by filling the reactors with 10 L of system water, and the medium was gently mixed before the water was drained again. The total amount of drainage from the first and second rinsing (approx. 20-23 L) was measured, and homogeneous sub-samples of this were used for analysis and mass balance of the sludge sample. Initially (weeks 1 and 2), the protocol was tested and modified, of which not all samplings were used for analysis.

Figure 7-3. Picture of Levapor bioelements, and a sketch of the principle of standardized backwashing/squeezing the bioelements to extract collected organic matter.

Measurement of inlet and outlet after backwashing (single passage)

At week 4 and week 12 (15/8 -22 and 24/10 -22), supplementary sampling of the inlet and outlet was carried out to examine the possible effect of backwashing. The samples were taken as above in duplicates 5 min apart, one hour after a backwash procedure.

Basic monitoring of the RAS system

Throughout the 18-week trial period, two weekly water samples (grab samples) were collected from the pump sump to document and monitor the associated water quality and any effect of the filters at system level.

Calculations

Based on single pass ([inlet]-[outlet]), the reactors' removal efficiency (RE) was calculated as:

$$RE = \frac{(V_{in} - V_{out})}{V_{in}} \times 100$$

where: V_{in}: inlet value (FNU, BQV, mg/L, %, k); V_{out}: outlet value (FNU, BQV, mg/L, %, k).

Based on the sludge fraction, the relative substance retention was calculated for dry matter and total and dissolved COD for measurements over a week:

$$Relative\ substrate\ retention = V_s \times S_v$$

where: V_s: sludge concentration (mg/L); S_v: Sludge volume (L) per week.

The sludge's microbial activity (BactiQuant and hydrogen peroxide assay) was measured on a sub-sample. The values reflect an average of the values of the replicates within the same group. The "Enhancement factor" reflects the degree of up concentration in the foam compared to the water.

7.1.3 Results

During the 18-week-long experimental period, no fish mortality was recorded. The foam reactors significantly removed dissolved substances and particulate matter and reduced the microbial abundance from the first sampling set and throughout the trial.

Single pass removal efficiency

Ammonium

Ammonium was significantly reduced during a single pass from the start until the end of the experiment (Figure 7-4A). The foam reactors with high retention time were most stable and had a significant highest TAN removal efficiency. The TAN removal efficiency of >80% was measured after one week, with an overall mean removal efficiency of 71±8% over the test period. The TAN concentration was between 0.08 and 0.22 mg TAN/L during the 18 weeks, with an average of 0.17 mg TAN/L. The foam reactors with low HRT also removed TAN from week 1, but at a relatively lower RE of 20 to 60%, the average TAN removal efficiency being 41±12% per passage. TAN removal efficiency was not affected by a 4-week long break or by backwashing events.

Based on the flow (0.40 and 0.06 m^3/h), average TAN removal efficiency (%), and TAN concentration at the inlet (0.17 mg TAN/L), *the estimated TAN removal rates* were:

Low HRT: (0.17 mg TAN/L × 10^3 × 41% × 0.40 m^3/h) ÷ 0.01 m^3 × 24 h d^{-1} = **67 g TAN/m^3/d**
High HRT: (0.17 mg TAN/L × 10^3 × 71% × 0.06 m^3/h) ÷ 0.01 m^3 ×24 h d^{-1}= **17 g TAN/m^3/d**

Nitrite

Nitrite was also reduced during a single pass from the start until week 6 (Phase 1; Figure 7-4B). The nitrite removal efficiency in low HRT biofilter increased from 20% at week 1 to approx. 70% after 6 weeks (mean 54%). The REs were much lower and more uniform in foam filters with low HRT, averaging 13% in weeks 1- 6.

Based on the flow (0.06 and 0.40 m^3/h), average NO_2- removal efficiency (%), and nitrite concentration (0.11 mg nitrite-N/L), *the estimated apparent nitrite removal rate* was:

Low HRT: (0.11 mg NO_2-N/L × 10^3 × 13% × 0.40 m^3/h) ÷ 0.01 m^3 × 24 h d^{-1} = **14 g NO_2-N/m^3/d**
High HRT: (0.11 mg NO_2-N/L × 10^3 × 54% × 0.06 m^3/h) ÷ 0.01 m^3 × 24 h d^{-1} = **8.6 g NO_2-N/m^3/d**

The total nitrite removal (TAN removal and apparent nitrite removal) was estimated to be **81 g NO_2-N/m^3/d** and **26 g NO_2-N/m^3/d** for low HRT and high HRT, respectively.

In phase 2 (week 11-14), after the intended break, the interruption substantially impaired both sets of foam reactors (Figure 7-4). At weeks 10 and 11, nitrite accumulated during single passes (negative removal efficiency). The most extreme inhibition was observed at week 11,

where nitrite-N increased from 0.13 mg NO_2-N/L in the inlet to 0.19 mg NO_2-N/L in the outlet of the low HRT (-43% removal) and to 0.33 mg NO_2-N/L in the outlet from the high HRT reactors (-149% removal). The formed nitrite concentration was slightly higher than the calculated TAN removal. The nitrite removal was recovered in weeks 13 and 14, however at a lower level than prior to the break.

Microbial activity

Microbial activity in the water averaged 55.900±6.900 (SE), ranging from 19.600 to 115.500 BQV over the period of 18 weeks. The microbial activities were significantly reduced in both types of foam filters. Foam filters with high HRT removed 19-65% from microbial activity measured as BactiQuant, averaging 37% with removal measured after the first week. The low HRT foam filters removed from 0-30% (average 15%) with a single event without any reduction. Over time, the removal efficiency increased in the high HRT and the interruption after week 6 did not have any clear impact on this.

Similar patterns were identified using hydrogen decay as an index of microbial activity. Here, a single pass in high HRT resulted in an approximate 27% reduction (range 5-48%), with variation from week to week. The reduction in the low HRT groups averaged 8 % (range -3-28%) and was unaffected by the break after week 6 (Figure 7-4C, D).

UVT and turbidity

The UVT and turbidity improved linearly in foam filters with high HRT, starting at 8% at week 1 and > 40% at week 6 (Figure 7-4E, F). The low HRT foam reactors showed a similar improvement at a lower level, from 2 to 16%. The 4-week break from week 6 caused only a minor relative reduction in RE for both types of foam filters. UVT was improved by 2% to 10%, being most pronounced in HRT foam filters and increasing over time.

Figure 7-4. Results of removal efficiency of fixed bed bioreactors with Levapor foam. The removal efficiencies (RE) are calculated as ([Inlet]-[Outlet]) ×100/[Inlet] and reflect average ± sd (N=4). The reactors were operated with 400 l/h, ~ 2.4 min hydraulic retention time [Low HRT] or 60 L/h ~ 14 min HRT [High HRT]. A) Total ammonium N, B) Nitrite-N, C) BactiQuant, D) H_2O_2 decay assay, E) Turbidity, and F) UVT.

Effects of backwashing on removal efficiency

Ammonium and nitrite

Ammonium removal was unaffected by backwashing when measurements before backwash and 1 hour later were compared. Both sets of biofilters were found to have a slightly higher removal efficiency after backwash when compared to RE measures just before this (Figure 7-5). Similarly, removal efficiencies of nitrite were also improved just after backwashing events (week 4 and week 14), with > 10% points for the high HRT biofilters. These findings are important from an applied and practical point of view. They indicate that any handling/squeezing can be made without jeopardizing the performance of the nitrifying bacteria. Future experiments should look into optimizing the frequency of backwashing/squeezing the foam, to ensure optimal solids removal and extraction and maintain dissolved N-removal.

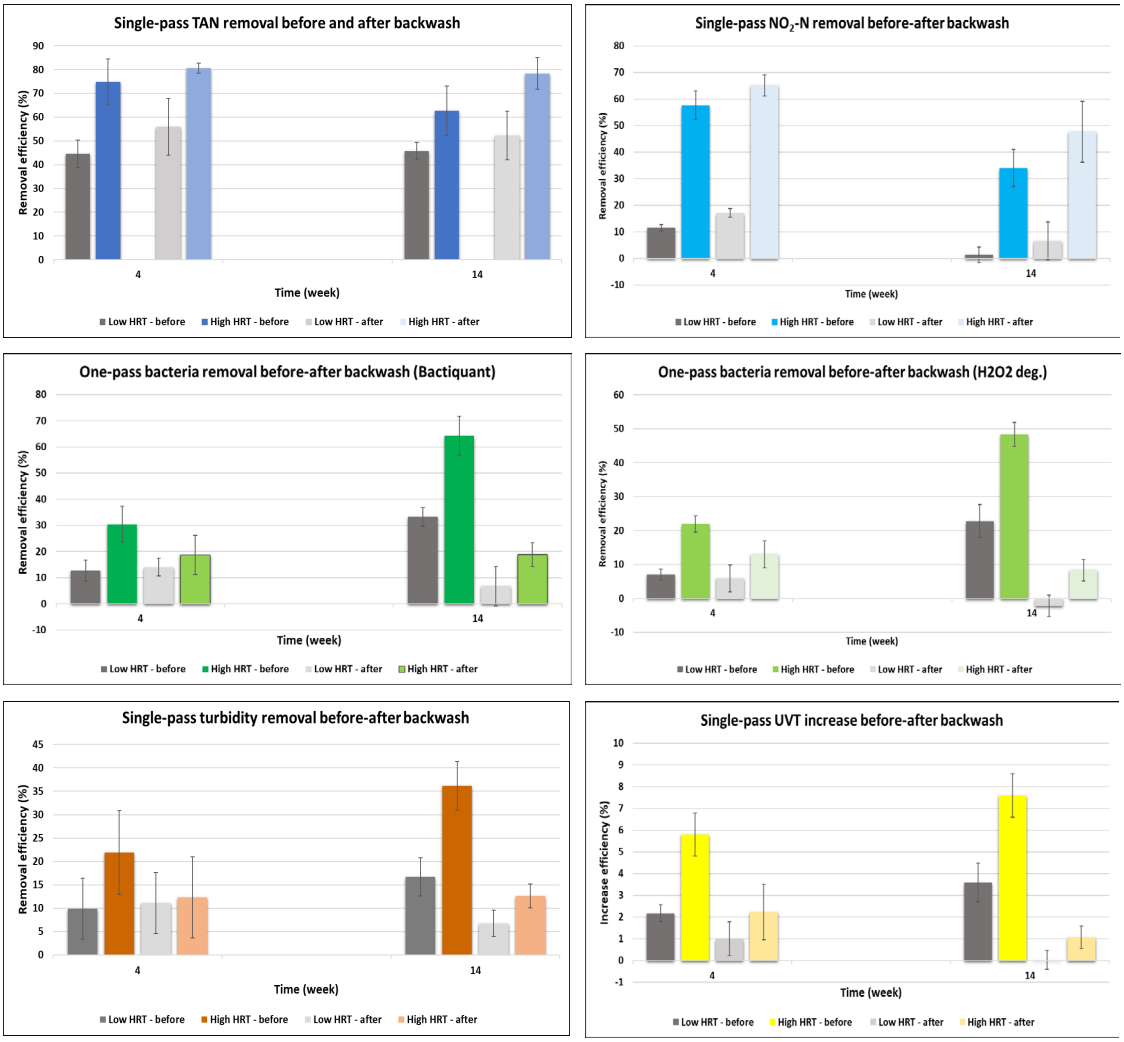


Figure 7-5. Removal efficiencies of fixed bed bioreactors with Levapor foam before and 1 hour after backwashing/squeezing the foam. The removal efficiencies (RE) are calculated as ([Inlet]-[Outlet]) ×100/[Inlet] and reflect average ± sd (N=4). The reactors were operated with 400 l/h, ~ 2.4 min hydraulic retention time [Low HRT] or 60 L/h ~ 14 min HRT [High HRT]. A) Total ammonium-N, B) Nitrite-N, C) BactiQuant, D) H_2O_2 decay assay, E) Turbidity, and F) UVT.

Microbial activity

The single pass removal of microorganisms (measured as microbial activities) was affected by backwashing. The removal efficiency of microbial activity in the high HRT biofilters dropped by 13% at week 4 and 40% at week 14, when measured as changes in BactiQuant and rates of H_2O_2 decay. In the low HRT foam filters, no change in BQV or H_2O_2 was observed in week 4, but a highly significant reduction in microbial activity (both BQV and H_2O_2) was observed after backwashing in week 14.

UVT and turbidity

Backwashing event reduced the removal efficiency of turbidity and UVT.

In all tests (except for low HRT filters measured at week 4), REs were reduced by 10-25%. Despite this temporarily reduced efficiency as compared to undisturbed conditions, both filters had a significant positive effect on water quality also after backwashing. It is expected that the

squeezing of the foam material will lead to reduced biofilm and increased porosity, thereby ensuring a more homogeneous distribution of water to biofilm.

Retention of organic matter

There was a substantial buildup of organic matter in the foam filters during the experiment. Based on the results above, there were no indications of any net release from the filters: water leaving the filters was of better quality that the water entering the filters.

The amount of organic matter trapped in foam showed a clear and consistent pattern over time. The filters operated with high flow (low HRT) were able to trap more organic matter than the ones with high HRT (lower flow), see. Fig. 7-6.).

Figure 7-6. Representative subsamples of reject water (20-23 L) based on squeezing organic loaded from foam biofilters with low HRT (left) and high HRT (right). Photo by Bence Dániel Kovács.

In the low HRT biofilter, 35-41 g (38±1 g) dry matter was collected once weekly during the 14 weeks. The high HRT biofilters held back from 22-28 g (25±0.7 g) dry matter, also very stable and not affected by a period of disconnection (Figure 7-7). The amount of organic matter removed corresponds to 2.5-3.8 kg dry matter/m^3 foam per circle (1 week). The removal capacity was constant over the period, and we assume that the duration between squeezing events was too long. With more frequent drainage, it is expected that substantially more of the organic matter can be withdrawn. This will increase the efficiency of the water treatment and based on the results above, this can take place without affecting nitrification.

The results from the COD analysis show a similar pattern, with a slight increase at the end of the trial. Here stable and high concentrations were found in the low HRT biofilters ranging from 18-31 g O_2 in 10-liter foam elements (26±1.2 g O_2), and approximately 60% less in high HRT (7±0.7 g O_2).

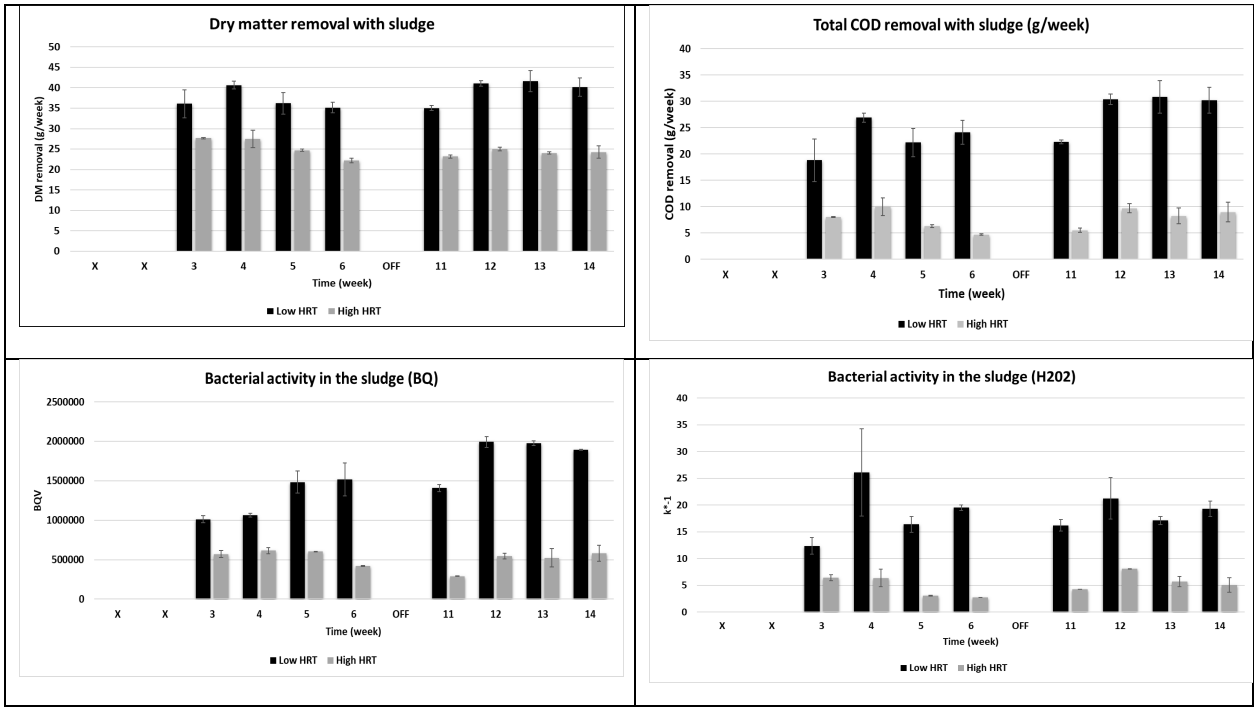


Figure 7-7. Results of reject water composition based on backwashing/squeezing of Levapor foam in two types of foam biofilter reactors. Low HRT filter was operated with 400 l/h, ~ 2.4 min hydraulic retention time: High HRT with 60 L/h ~ 14 min HRT. A) Dry matter B) Total COD, C) BactiQuant, and D) H_2O_2 decay assay. The values are calculated using the concentration and drainage volume, based on a duplicate test with average ± sd.

The microbial activity, measured as BactiQuant or H_2O_2 degradation rates, also revealed substantial removal capacity by the foam filters. The foam filters with low HRT exceeded the filter with less flow, leading to reject water with $1x10^6$ – $2x10^6$ BQ values/mL. Considering the average water content at $56x10^3$ BQ values/mL, the filters managed to up-concentrate with a factor of 20-38 times (the total volume of the reject water not considered).

The filters operated a high HRT and had less bacteria entrapped in the foam. BQV ranged from $2.90 \cdot 10^5$ to $6.10 \cdot 10^5$ ($5.2 \cdot 10^5 \pm 4.3 \cdot 10^4$). Again, future studies should look into optimizing the squeezing frequency, most likely to backwash more frequently and/or adjust the flow.

RAS water quality during the experiment

The RAS water quality, based on biweekly sampling and analysis is depicted in Figure 7-8. Overall, the RAS is considered very stable with constant conditions, which is reflected by the water analysis. The original hypothesis was that foam filters would lead to improved water quality due to enhanced water treatment. This is not fully supported by the results below. It has to be taken into account that the RAS relied on additional water treatment units, such as 12 biofilters, a drum filter, and a foam fractionator. The biofilters and drum filter were performed constantly throughout the 18 weeks and ensured a certain level of dissolved N and organic matter, respectively. The foam fractionator was operated fulltime but adjusted *ad hoc*, and since it was the only treatment unit capable of removing biosolids and microparticles, we cannot rule out the uncontrolled operation may have blurred the picture by the noise associated with inconsistent removal. So, the overall evaluation is that TAN and nitrite are kept low and stable, and elevated nitrate proves and confirms the feed loading and the stability of the RAS operation.

The microbial activity tended to be slightly elevated in the periods without the foam filters in operation (week 7-10 & 15-18), being, however, not consistent (week 1 and 2 deviates).

7.1.4 Perspectives

The results from the controlled 18-week study provide different new information. By applying the foam as a fixed sandwich filter, a number of significant improvements in water quality were documented. High and stable water treatment efficiency was documented in terms of dissolved N reduction, entrapment, and reduction of bioavailable organic matter as well as purification effect. Furthermore, the results show that the filters were effective already after one week from start and potentially earlier, as this was the first measurement.

The potential benefits include:

- Highly and efficient TAN and NO_2-N removal from the start of operation
- Very effective reduction of organic matter and microbial load in a single passage
- Improved backwashing management and efficient removal of reject water
- More than 70% TAN removal in a single passage.
  A very low TAN levels (< 0,20 mg TAN/L), this accounted to 70 g TAN/m^3/d which can be ≥ 5 times increased under TAN unlimited conditions (TAN > 1 mg TAN/L)
- Use relatively small foam filters as a plug-in unit, i.e., during the start-up of RAS.
- Maintenance and backwashing of the biofilter does not affect TAN removal, and most other water quality parameters are not or only slightly reduced.
- High HRT (low flow) benefits single pass removal efficiency (N, bacteria, UVT, turbidity).
- Low HRT (high flow) benefits removal with sludge (dry matter, COD).

It remains to be evaluated how flow, design of reactors and maintenance frequency can optimize the removal of dissolved N, microparticles and bioavailable organic matter. With the tested flow rates, size of biofilters and cleaning frequency, the filters showed removal on several parameters. Release of organic matter from the foam reactors or clogging in the foam reactors was not observed on any occasions, suggesting the loading rates to be increased. Future studies will look into scaling up the foam reactors and further optimizing the operational parameters (e.g., HRT, loading and frequency of backwashing). They can also investigate the effect of foam filters on geosmin levels in the RAS, how much and how efficiently biosolids can be trapped and valorized and to which extend the treatment can be applied in saltwater RAS, without forming anaerobic conditions and leak of H_2S.

7.2 Nitrification performance of different bioelements during startup in a commercial seawater RAS

7.2.1 Background

This study aimed to evaluate the cold start-up of four types of bio elements in a commercial saltwater recirculating aquaculture system. The selected biofilter elements were distributed into identical biofilters connected to a smolt unit (weeks 1-15) and a grow-out production unit (weeks 15-40).

The nitrification performances for each type of biofilter elements were assessed by transferring and testing subsamples in reactors under controlled conditions and spiked with TAN or nitrite.

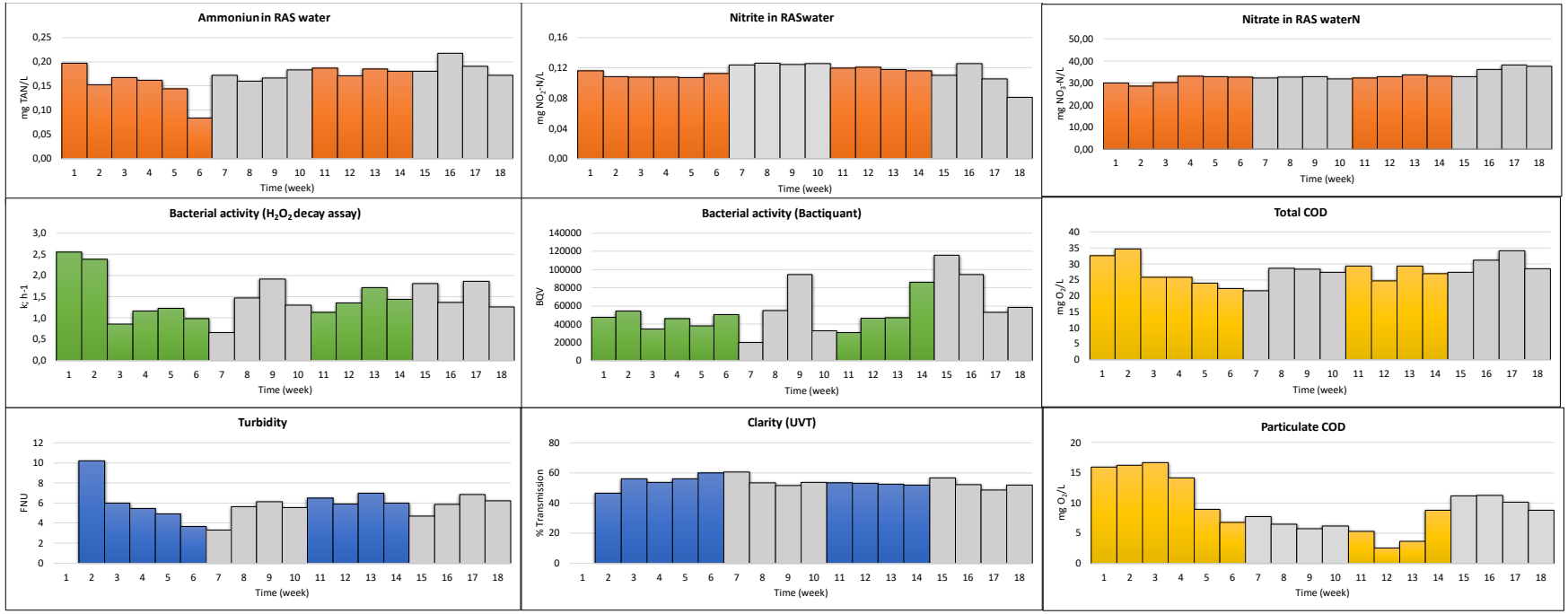


Figure 7-8. Water quality parameters from the experimental RAS during the 18-week trial. Each bar represents an average of two weekly grab samples. Experimental conditions in the 20 m^3 freshwater RAS (15-16 °C, pH 7.0-7.2) included 250 kg large rainbow trout with 2 kg feed/day, and a daily exchange of 2.8 m^3 water.

7.2.2 Material & Methods

Experimental design with bio elements and reactors

The experiment was conducted at Danish Salmon A/S, Hirtshals, Denmark, lasting 40 weeks (from summer 2022 to spring 2023). The experimental setup included four types of bio elements and two types of biofilters (Figure 8-9). The four types of bioelements included: **1) RK BioElement PP** (750 m^2/m^3), 2) **RK BioElement PE** (750 m^2/m^3), 3) **Levapor** (2500 m^2/m^3), and 4) **Mutag Biochip** (5500 m^2/m^3). The biofilters were operated as moving bed bioreactors, in cylindrical units (R1-R2-R3-R4) or in tray reactors (T1 & T2). All four types of bioelements were tested in the cylindrical reactors; Levapor and Mutag were additionally also tested in tray reactors (Figure 7-9, Figure 7-10).

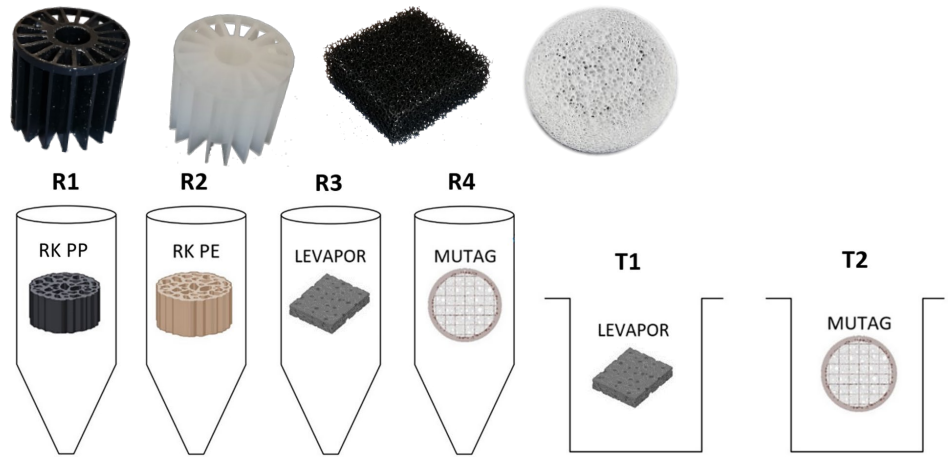


Figure 7-9. Experimental set-up with different bio-elements and reactors.
R: 90 l cylindrical reactors; T= 120 l tray reactors.

Table 7-2. Operational conditions of the tested bio-elements and reactors.

	Cylinder	Cylinder	Cylinder	Cylinder	Tray	Tray
	RK Plast PP (Black)	**RK Plast PE (White)**	**Levapor**	**Mutag**	**Levapor**	**Mutag**
Media vol. (L)	36	36	22.5	36	30	48
Approx. adj. water vol. (L)	71	71	78	73	117	112
Approx. water flow (L/h)	1065	1065	1170	1095	1755	1680
Air flow rate (L/min)*	4-5	4-5	4-6	5-6	20	20

***** adjusted from time to time depending on the media movement.

The operational conditions are described in Tables 7-2 and 7-3. The two types of reactors differed in shape, flow pattern and volume. The cylindrical reactors (90 L) received water from the bottom (up-flow), and the tray reactors (120 L) received water in a horizontal manifold with a vertical tangential flow pattern. The reactor filling ratios were 40% for R1, R2, R4, and T2, while only 25% for Levapor in R3 and T1. The hydraulic retention time (HRT) was approximately 4 min.

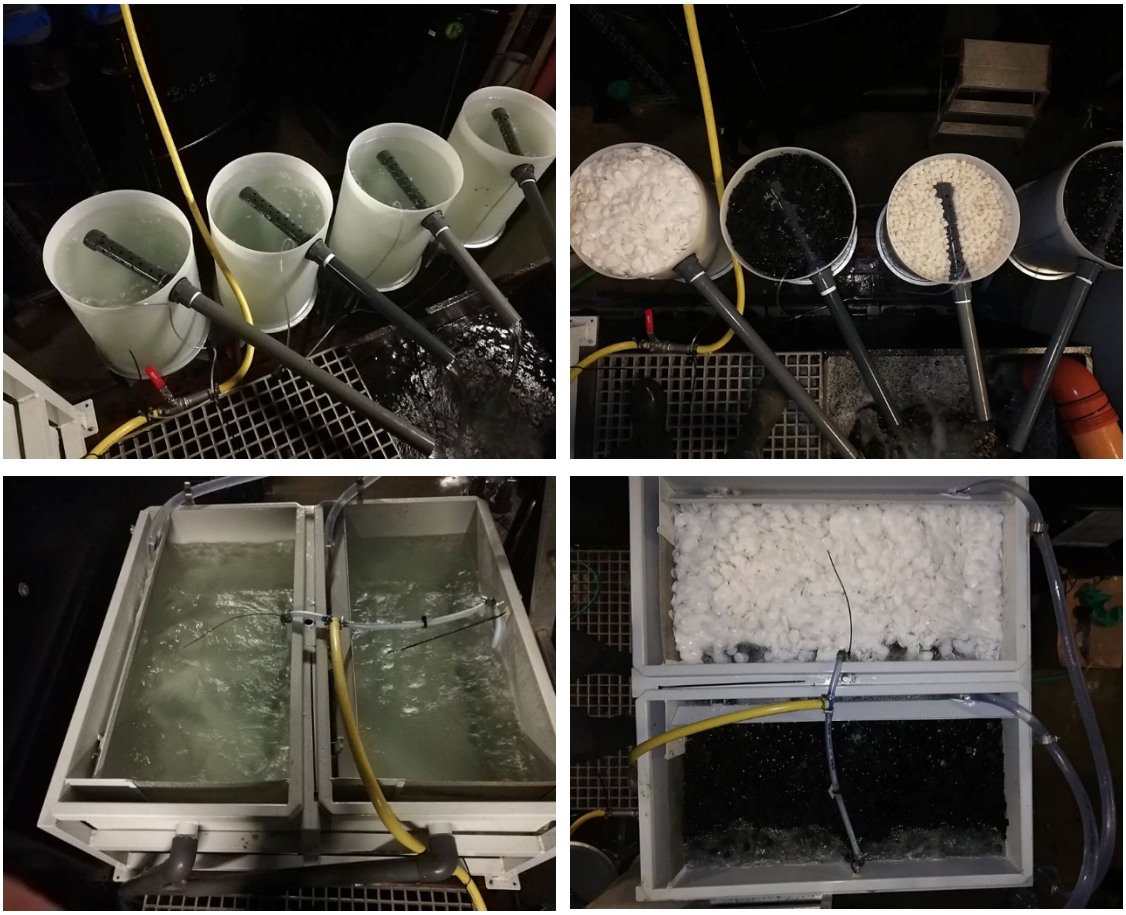


Figure 7-10. Pictures of the experimental setup at Danish Salmon. The top pictures show the 4 cylindrical reactors prior to and immediate after transfer of bioelements. The bottom picture shows the two-tray reactor prior to and immediate after transfer of white RK PE bioelements and black Levapor bioelements. Photo by Bence Dániel Kovács.

Commercial test RAS

Initially, the reactors received RAS water from the water treatment system of a full-strength saline (34 ppt) smolt facility (Figure 7-11). Out of experimental control, but due to an industry-driven decision to change the future operation of the smolt facility, including changes in salinity, the six reactors were timely and carefully transferred to a full-strength saline grow-out facility in week 15 (11/10/2022) (Figure 7-11). The average biomass, feeding and water quality parameters of the two systems are shown in Table 7-3.

Figure 7-11. Pictures of the setup in the smolt facility (Top) and the grow-out production unit (Bottom) Photo by Bence Dániel Kovács.

In both systems, the reactors were supplied with water through a submersible pump (Terada S-500, TERADA PUMP MFG. CO., LTD, Japan). The water was then split through a manifold to supply each reactor. Each reactor was equipped with valves and water flow meters to ensure precise water flow measurement and monitoring. Moreover, two separate air pumps (Mistral II 400, AB Aqua Medic Gmbh, Germany) were used to supply air to the cylindrical and tray reactors.

Table 7-3. Average biomass, feeding and water quality parameters of the two systems.

Experimental conditions	Smolt	Grow-out
Testing period (Weeks)	(1-14)	(15-40)
Standing biomass (t)	8±4.5	191±21
Feeding (~1% of BW/d) (kg)	81±55	1429±220
TAN (mg N/L)	0.8±0.3	0.7±0.2
NO_2-N (mg N/L)	0.7±0.5	0.7±0.4
NO_3-N (mg N/L)	145±44	114±28
COD tot. (mg O_2/L)	29±5.1	31±3.6
COD diss. (mg O_2/L)	31±3.5	31±3.9
pH	7.4±0.2	7.3±0.1
Alkalinity (mmol/L)	2.1±0.6	3.8±1.1
Temperature (°C)	13±0.9	11.2±0.2
Salinity (‰)	33-34	33-34

Assessment of biofilter performance and nitrification activity

Both ammonium oxidation (TAN removal) and nitrite oxidation (nitrite removal) were measured at high and low substrate concentrations. TAN and NO_2-N spike tests were conducted in the biofilters or in separate reactors to assess the nitrification activity of the different bioelements (Table 7-4). Maximum TAN and nitrite removal (under the given conditions) were calculated from spiking trials with 4 mg N/L (substrate-unlimited conditions; 0° order kinetics) and spiking trials with 1 mg N/L (substrate-limited conditions; 1° order kinetics), as described by Kinyage & Pedersen, (2016) and Aalto et al, (2022b).

In the first two months of the experiment, weekly tests were conducted with three tests performed on the culture reactors and one in duplicate on the lab-scale reactors per month. From the second to the sixth month, two tests were conducted monthly, with one test in the culture reactors and one in duplicate on the lab-scale reactors. For the remaining period, monthly tests were conducted in duplicate on the lab-scale reactors (Figure 7-12).

Table 7-4. Overview of the experiment to separately quantify ammonium and nitrite removal in biofilters installed at Danish Salmon. Each event included 4 sets of spiking (NH_4^+ or NO_2^- at low and high concentrations) for each type of biofilter elements/reactor. Each individual spiking test consisted of 8 samples collected within 1½ hours.

Experimental period (weeks)	Spike in biofilter reactors with all bioelements	Spike in small lab.-scale reactors with transferred subsamples of bio elements
Week 1-8	3 tests/months in all biofilters	1 test/month of all bioelements
Week 9-24	1 biweekly test in all biofilters	1 biweekly test of all bioelements
Week 24-40		1 test pr. month of all bioelements

During the tests, both the culture and the lab-scale reactors were filled with clean seawater. In the culture reactors, media volume, water volume and airflow rates were applied as outlined in Table 7-2.

In the lab-scale reactors, 4 L of water and 1 L of media were used with airflow rates of 1-2 L/min. After spiking, 10 ml water samples were collected (0.2 um sterile filtered) at 5, 15, 30, 45, 60, 75, and 90 minutes during the 1.5-hour tests. Temperature and pH were measured at the beginning and end of each test.

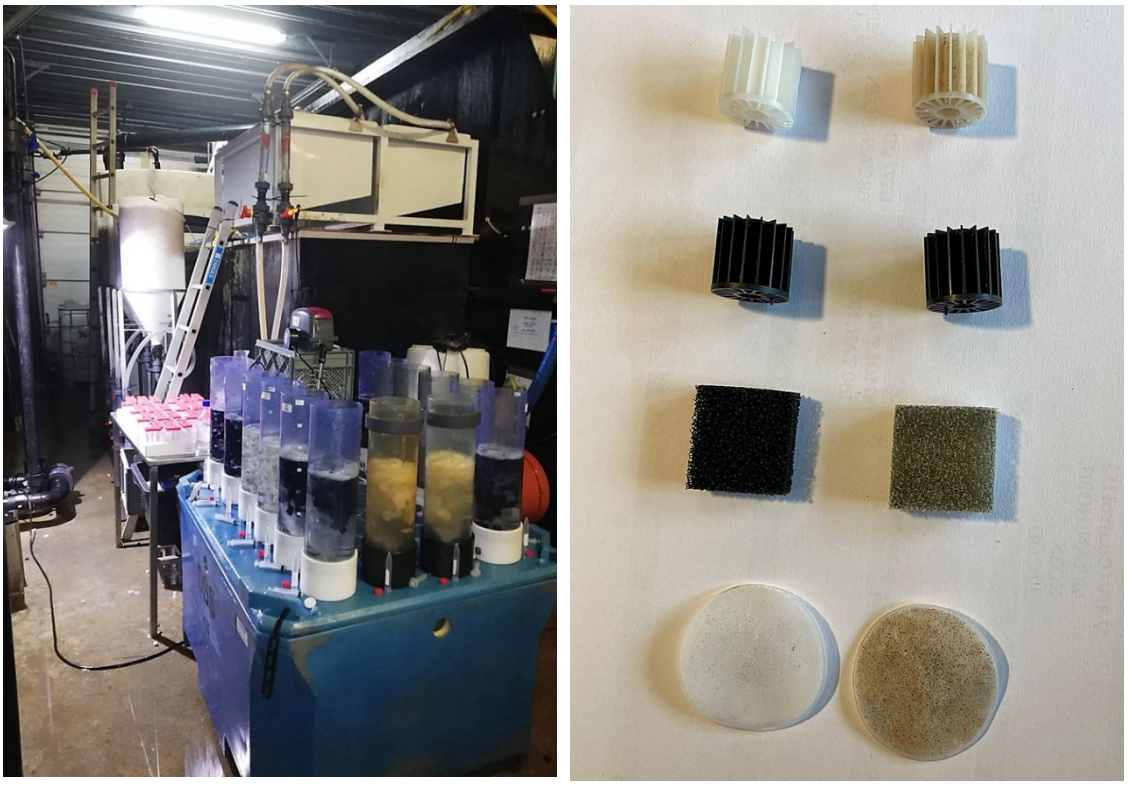


Figure 7-12. Left: Example of nitrification capacity tests performed with bioelements transferred from biofilters to 12 reactors and spiked with either TAN or nitrite. Right: example of bioelements before test (Day 0) and after 6 months. Photo by Bence Dániel Kovács.

Data analysis

The conserved water samples were analyzed according to National standard methods for TAN and NO_2-N. Volumetric removal rates (VRR) were calculated from the time-related reduction of TAN or nitrite in each trial.

Maximum, substrate-unlimited removal rates (0° order) were calculated based on substrate concentrations above 1 mg N/L using least square linear regression. The slope of the regression line, the total volume of the bio elements, and the total water volume of the reactor were then used to calculate the 0° order volumetric removal rate of TAN or NO_2-N, VTR_{0a} and VNR_{0a} ($g/m^3/d$).

Substrate-dependent (limited) removal rates (1° order) were calculated based on substrate concentrations below 1 mg N/L and linear regression on log-transformed substrate concentra-

tions. The slope of the log-transformed data's regression line was multiplied by the reactor's total water volume and divided by the total volume of bio elements. This calculation provided the volumetric 1° order rate for TAN and nitrite as VTR_{1a} and VTR_{1a} (d).

VRRs are expressed as absolute values for trials conducted on the culture reactors and as average ± standard deviation for trials conducted on duplicate lab-scale reactors.

7.2.3 Results and Discussion

The trial was conducted and completed during 40 weeks of operation in a full-strength seawater RAS. In week 15, the experimental setup had to be moved from the smolt facility to the grow-out system due to an unforeseen coming change. The two production systems differed in size and biomass (Figure 7-11AB; however, the RAS water composition was similar. From weeks 4-7, the feed was severely reduced in the smolt facility due to the movement of smolts (Figure 7-13). This is reflected by a reduction in the TAN and nitrite concentration (Figure 7-14). An advantage of the current experiment was that all six biofilters received the same amount of water and faced the same drop and fluctuations in loading rates. Aside from that initial period, TAN levels ranged from 0.6 to 0.8 mg TAN/L, which is in the 1 order area limiting the nitrification performance. The nitrite concentrations were slightly higher and ranged wider (from 0.5 to 1.4 mg NO_2-N/L).

The nitrate concentrations demonstrate a high feed loading (Pedersen et al, 2012) and relatively stable conditions, which can also be seen in the amount of organic matter measured as chemical oxygen demand (Figure 7-15).

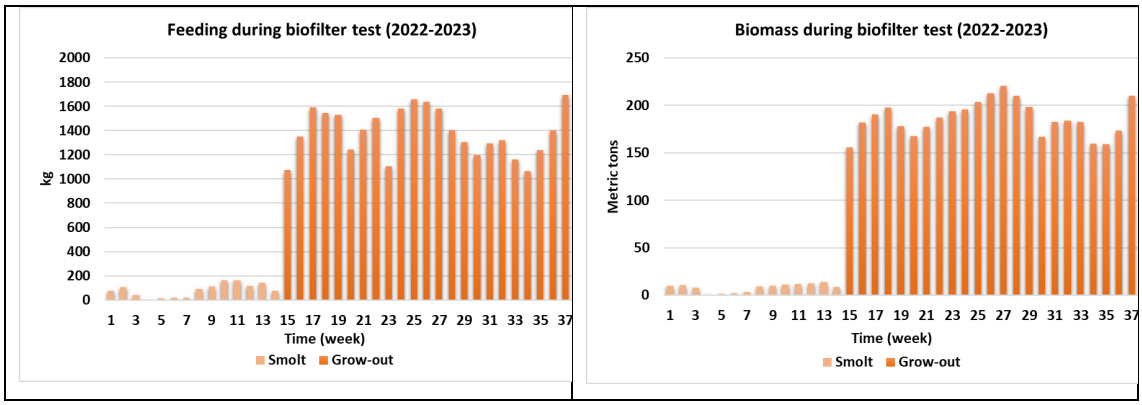


Figure 7-13. Daily records of feeding in the two seawater RAS tested and the corresponding standing biomass. The biofilter setup was moved from the smolt to the grow-out facility at week 15.

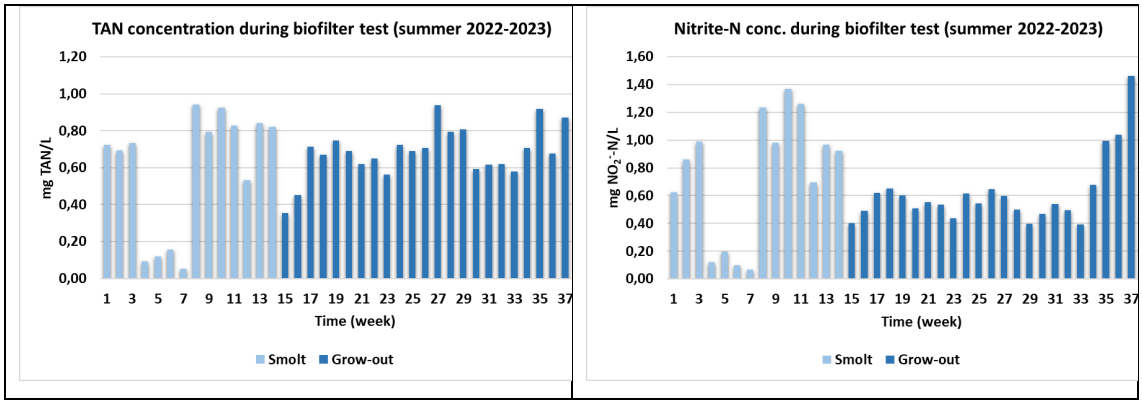


Figure 7-14. Average daily concentrations of TAN and nitrite in the two seawater RAS tested.

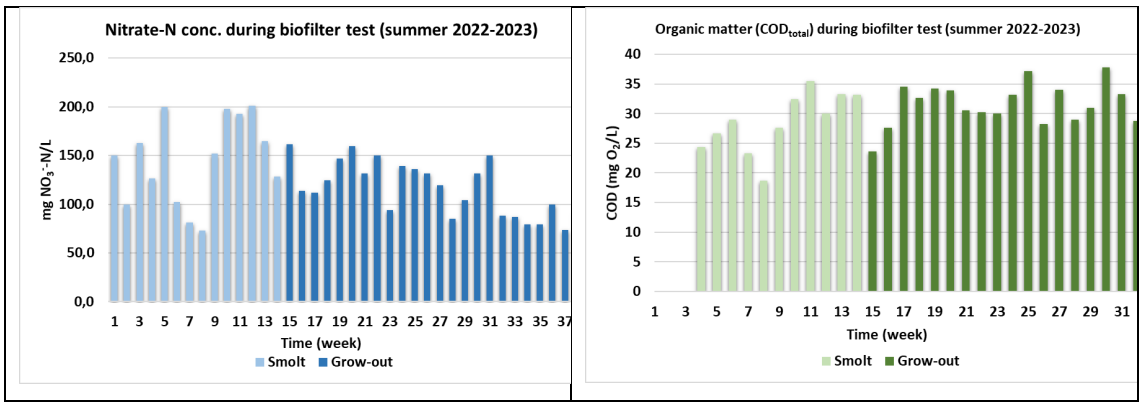


Figure 7-15. Average daily nitrate concentrations and organic matter content (measured on unfiltered water samples as COD_{TOT}) in the two seawater RAS tested.

Ammonium removal during startup and colonization in saltwater RAS.

Below, the results of the nitrification performance of each type of bioelement are presented, covering maximum TAN and nitrite removal (0°order kinetics) as well as [TAN] and [NO_2-N] dependent removal (substrate limiting 1° order kinetics).

RK Plast

RK Plast biomedia, both the white polyethylen (PE) and the black polypropylen (PP), had a similar very low colonization of ammonium oxidizing bacteria for the first 8 eight weeks (Figure 7-16 A,B). Here both 0° and 1° order removal rates were ≤ 10 g TAN//m^3/d. From week 10, a gradual linear increase from 40 g TAN/m^3/d to 250 g TAN/m^3/d was observed (0° removal). Likewise, the 1° order removal rate constant increased from 40 to approx. 400/d (week 10 – week 36-40).

The nitrite oxidation activity was lower than the TAN oxidation, and the initial colonization period lasted 12-14 weeks where the VNR ≤ 5 g TAN//m^3/d. The VNR increased slowly from week 14-week 24 (5 to 15 g NO_2-N/m^3/d), to increase much faster up to 160 NO_2-N/m^3/d at week 40. The concentration-dependent 1°order nitrite removal followed the same pattern, with very low activity for the 24 weeks, for then to increase substantially (Figure 7-16 C,D). The PP and PE bioelements had very similar dynamic and performance ranges throughout the experimental period. Neither TAN nor nitrite removal activity seemed to reach a stable maximum level within the experimental period, except for the 1° TAN removal which plateaued around 400 d^{-1}.

Facts

RK Plast: TAN and nitrite removal rates increased throughout the trial and did not reach steady state or a max. plateau. Highest 1 °order TAN removal rates at the end of the experiment.

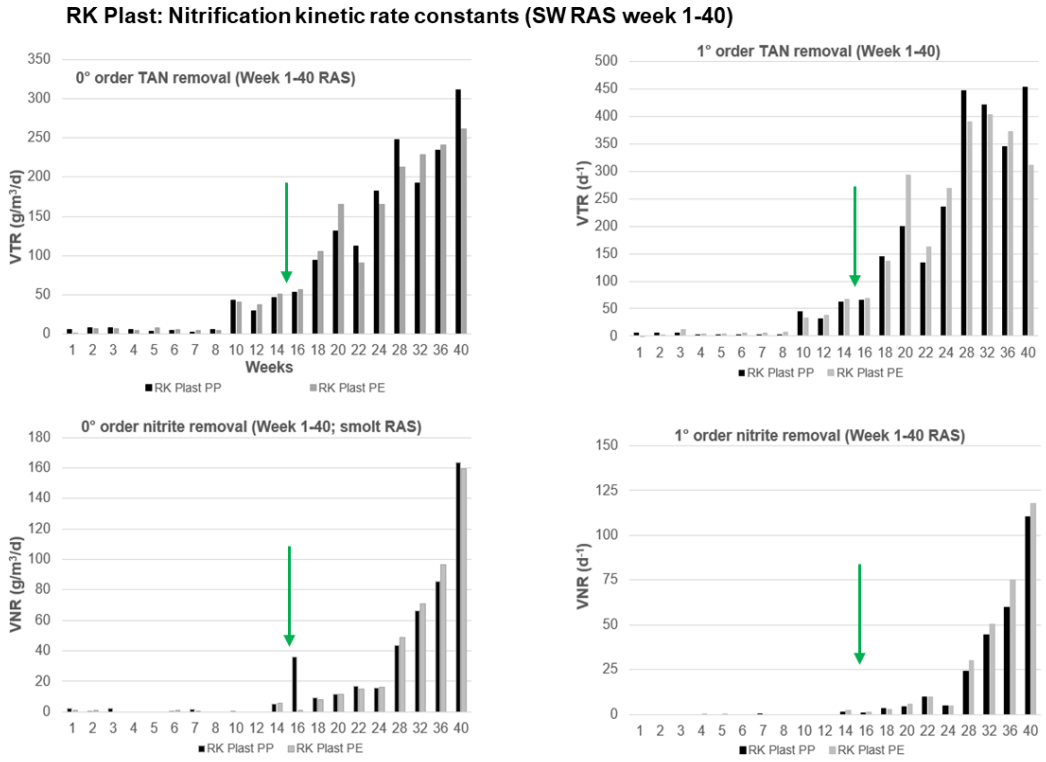


Figure 7-16. Temporal development nitrifying microbial activity measured on RK Plast bioelements 40 weeks after startup in seawater. Top left: Maximum TAN removal rates (0° Order kinetics), top right: concentration-dependent TAN removal rates (1° Order kinetics); bottom left: Maximum NO_2-N removal rates, and bottom right: concentration-dependent NO_2-N removal rates (1° Order kinetics). The green arrow indicates (week 15) when biofilters were moved from smolt to grow-out facilities. The bioelements were tested in parallel cylindrical biofilters, RK Plast PP = polypropylene (Black), and RK Plast PE= polyethylene (white).

Mutag Bioelements

The Mutag bioelements showed a similar pattern as RK Plast with faster colonization of AOB than NOB. The volumetric ammonium removal ranged from 10-35 g TAN/m^3/d during the first 8 weeks, followed by an increase up to approx. 300 g TAN/m^3/d occuring after 24 weeks. This level was maintained for the remaining 16 weeks of testing (Figure 7-17A). The [TAN]-dependent removal showed a more distinct pattern, with low, but detectable removal during the first 8 weeks. From week 10 to 20, the 1° VTR was approx. 200 d^{-1}; these values increase to 350 d^{-1} in the last part of the study (week 20-40).

The nitrite removal development differed from the TAN removal, with low VNR (≤ 15 g NO_2^- N/m^3/d) during the first 8 weeks. A sharp increase in VRN appeared after 12 weeks, rising from 25 NO_2^-N/m^3/d to 300 NO_2^-N/m^3/d at week 40. The first-order nitrite removal rates were identical to the zero-order VNR, though reaching slightly lower max values after 40 weeks (Figure 7-17D). The study showed consistent results for Mutag's performance regardless of whether it was operated in a cylindric biofilter or a tray biofilter. Both TAN and nitrite removal rates followed the same temporal development (at low and high concentrations) and the few individual differences observed were not linked to the type of biofilter (Figure 7-17).

Facts
Mutag: TAN removal rates increased throughout the trial and reach steady state at approx. 300. The nitrite removal rates increased throughout the trial reaching levels of approx. 300. Highest 0°order TAN removal rates, as well as highest nitrite (0° and 1 ° order) at the end of the experiment.

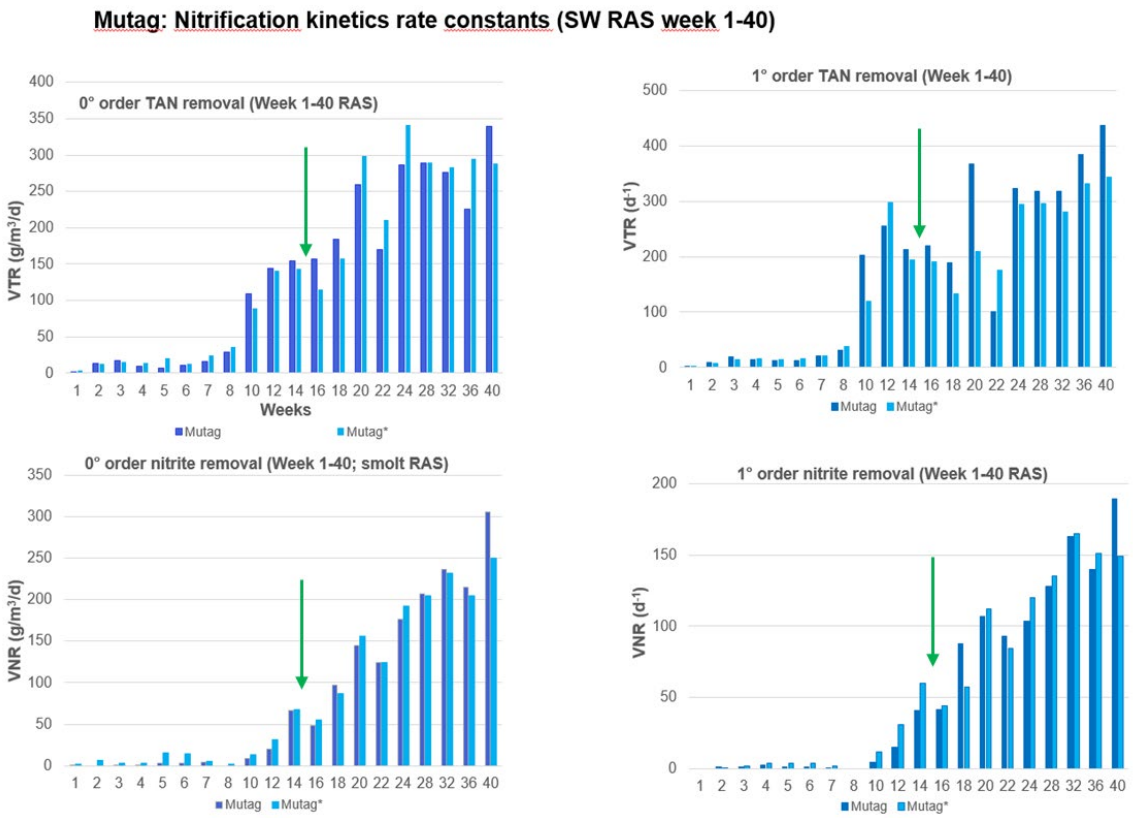


Figure 7-17. Temporal development nitrifying microbial activity measured on Mutag bioelements 40 weeks after startup in seawater. Top left: Maximum TAN removal rates (0°Order kinetics), top right: concentration dependent TAN removal rates (1° Order kinetics); bottom left: Maximum NO_2-N removal rates, and bottom right: concentration dependent NO_2-N removal rates (1° Order kinetics). The green arrow indicates (week 15) when biofilters were moved from smolt to grow-out facilities. The Mutag bioelements were tested in two parallel biofilters, a cylinder biofilter (Dark blue bars) and a horizontal tray (Light Blue bars*).

Levapor

Levapor showed a unique and different temporal pattern of AOB and NOB activity during the 40 weeks. Immediately after the start, Levapor had significantly high VRT ≥ 100 g TAN/m^3/d from weeks 2 – 7. The VTR then increased and peaked at 575 g TAN/m^3/d after 12 weeks, whereafter, the removal rate dropped exponentially and remained at 100-200 g TAN/m^3/d from week 20 to week 40 (Figure 7-18). A similar pattern was observed for the [TAN] dependent VTR, with very high values (2-350 d^{-1}) from weeks 2-7. After a peak lasting from weeks 8-12, VTR decreased and remained low until the end of the trial. During the last half of the study, Levapor bioelements from the tray biofilter had generally higher VTRs than the Levapor

bioelements from the cylindric biofilter (Figure 7-18), though both followed the same temporal pattern.

Similar to TAN removal by Levapor, nitrite was immediate removed at rates around 50 g NO_2-N/m^3/d during week 3-8 Figure 7-18C. Peak removal occurred in weeks 10, 12 and 14, followed by a severe drop from week 16 and onwards. The Levapor samples collected from the tray biofilter had higher VNRs than the Levapor from the cylindridal biobiofilter (approx. 150 vs. 75 g NO_2-N/m^3/d during weeks 18-28). The same temporal pattern (fast initiation of nitrite removal, peak removal during week 10-14, and reduced nitrite removal with better performance in Levapor from the tray biofilter compared to the cylinderic biofilter) was also observed for the nitrite-limited conditions (1º Order; Figure 18-8D). The results, based on the described conditions during the prolonged study, are somehow unexpected. The sudden drop in nitrification performance of Levapor (in both types of biofilters) after approximately three months of operations were not observed in any of the other four biofilter tested. All six biofilters were carefully moved from the smolt to the grow-out RAS in a short time, with similar, stable conditions. The reduced performance during the second half of the study, was more pronounced in Levapor elements from the cylindrical biofilter compared to the tray biofilter.
A previous aquaculture study with Levapor in a freshwater RAS showed VTR of 5-600 g TAN/m^3/d after five to eight weeks in a similar type of biofilter (Plesner et al., 2021). The study lasted two months during which no indications of reduced performance was observed.
A potential explanation of the observed reduction of VTR and VNR after three months in the present study may be related to a physical impairment of the media under the conditions tested. Here the porous media could be affected by clogging due to microbial growth and insufficient mixing in high density seawater. It remains to be tested, if this is the case and whether increased, vigorous aeration can maintain sufficient mixing and aeration of the Levapor elements, thereby maintaining a high ammonium and nitrite removal and preventing a time-related reduction.

Facts
Levapor: Fastest colonization and removal during week 0-12. Highest TAN removal rates up to 575 g TAN/m^3/d and highest nitrite removal up to 350 NO_2-N/m^3/d. After week 12, drops were recorded at maximum TAN and nitrite removal rates (0° order) as well as under sub-strate limiting conditions.

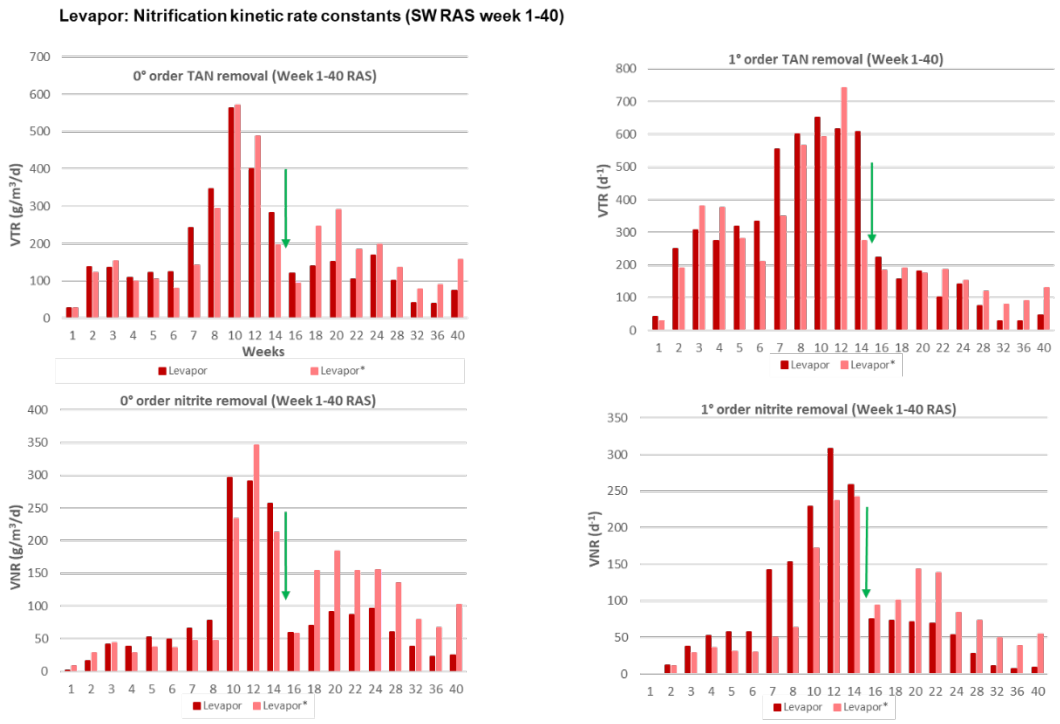


Figure 7-18. Temporal development nitrifying microbial activity measured on Levapor bioelements 40 weeks after startup. Top left: Maximum TAN removal rates (0° Order kinetics), top right: concentration-dependent TAN removal rates (1° Order kinetics); bottom left: Maximum NO_2-N removal rates, and bottom right: concentration-dependent NO_2-N removal rates (1° Order kinetics). The green arrow indicates (week 15) when biofilters were moved from smolt to grow-out facilities. The Levapor bioelements were tested in two parallel biofilters, in a cylinder biofilter (red bars) and in a horizontal tray (Light red bars).

Nitrification removal characteristics of the three types of bioelements

The combined temporal nitrification performance of all four types of bioelements are shown in Figure 3-1. It is important to emphasize that the nitrification performance was measured and evaluated under a given set of equal conditions at a commercial saltwater RAS, which does not nescessarily encompase ideal operating conditions in terms of filling ratio, airflow and hydralic retention time.

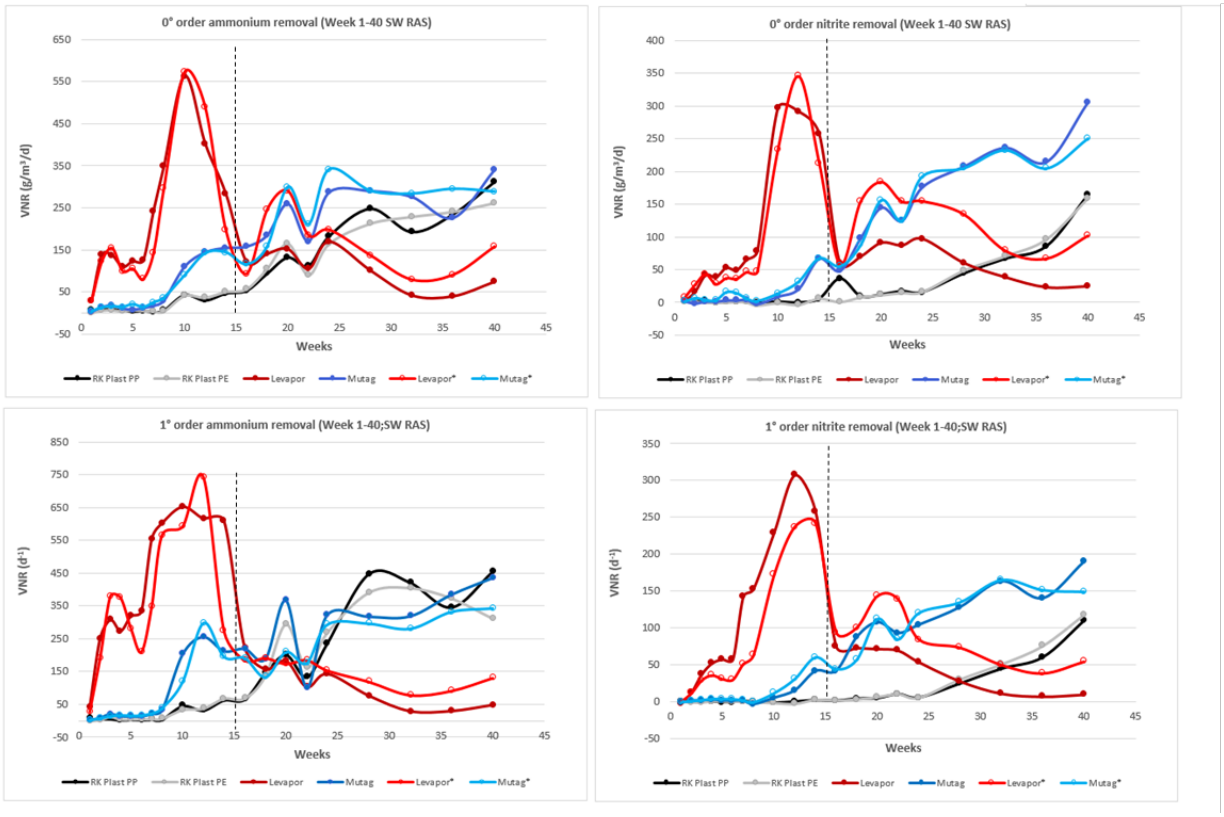


Figure 7-19. Nitrification performance of four types of biofilter elements in six biofilters during start-up in a commercial saltwater RAS: RKplast black PP (black) RK Plast white PE (grey) both in cylindrical biofilters; Mutag in a cylindrical biofilter (dark blue) and in a horizontal tray (light blue), and Levarpor a in cylindical biofilter (dark red) and in a horizontal tray (light red). 0° TAN and nitrite removal rates measured at high TAN or nitrite concentration; 1° TAN and nitrite removal rates measured at low TAN or nitrite concentration.

Mutag

Reactors filled with Mutag demonstrated a gradual increase in performance over time, leading to the highest overall performance after 40 weeks amongst all media tested (Figure 8-19). This was documented using Mutag bioelements from both a cylindrical biofilter and from a horizintal tray, which revealed similar VTR and VNR. The physical mixing and integration of media into the water phase was generally slow, lasting 6-8 weeks, which was potentially attributed to the low density of the media. Improved mixing conditions may have increased VTR and VNRs further than was recorded. Notably, by the end of the trial, the average NO_2-N removal rates were the highest of all media tested, while TAN removal rates were similar to those of RK.

RK Plast

Similarly to Mutag, RK Plast gradually increased performance over time. This media type was found to have the best and movement and fastest mixing/ integration into the water phase within 2-3 weeks. By the end of the trial, the average TAN removal rates were similar to Mutag; the NO_2-N removal rates were higher than Levapor but lower than Mutag (Figure 7-19).

Levapor

Initailly, the reactors with Levapor showed a several fold significant higher AOB and NOB activity compared to the other two types of bioelements tested (Figure 7-19). The highest VTR (575 g/m^3/d) and VNR (350 g N/m^3/d) were measured at weeks 10 and 12. After that, both AOB and NOB performance dropped substantially in both biofilters in the smolt facility.

The structure and porosity of the Levapor media favoured colonization or entrapment of nitrifying bacteria from the beginning. This may have caused the elevated high AOB and NOB activity, but it may also partly explain why the performance dropped after three months. Provided that the media were overgrown by biofilm or trapped particulate organic matter, this may have impaired proper hydraulic mixing and decreased the mass transfer from the water into the biofilm (Prehn et al., 2012).

It remains to be tested whether the active carbon layer in Levapor plays a role in the initial elevated TAN removal. The results from the french press study (Section 7.1) showed that maintenance of fixed Levapor elements was associated with significant amounts of trapped organic matter/biofilm which could be removed by washing/squeezing, without affecting the AOB and NOB activity.

The movement of the experimental setup from the smolt to the grow-out facility in week 15 was associated with only minor changes in water quality (Table 7-3). *Though the transfer of bioelements was gentle, some of the Levapor elements were floating or maintained passively near the surface*. The mixing of the Levapor bioelements ceased, and despite increased aeration, Levapor bioelements were not in constant motion or fully mixed as opposed to the other types of bioelements. The study tested Mutag and Levapor in two different types of biofilter compartments. Mutag remained unaffected in terms of mixing and nitrification performance (regardless of the movement and the types of biofilters), whereas Levapor showed reduced performance which was most pronounced in the cylindric biofilter due to suboptimal mixing conditions. Studies remain to test whether full-strength salinity (with a higher density) negatively affects the mixing of Levapor or if increased, vigerous aeration or squeezing /backwashing of the elemenst may have a positive effect.

7.2.4 Conclusions and perspectives

The study provides the first detailed and prolonged set of AOB and NOB activities obtained systematically from a commercial saltwater RAS. It included 40 weeks of continuous operation and allowed us to compare different types of bioelements that received the same water. Furthermore, two types of biofilters were used.

In summary, the study showed:

1) During the first eight weeks, there was very slow colonization (<10 g TAN/m^3/d) in the traditional PP and PE bioelements. Only slightly higher TAN removal (10-20 g TAN/m^3/d) in PE Mutag chips in the same period, whereas polymeric foam exceeded 100 g TAN/m^3/d after the second startup week.

2) A linear increase in TAN removal rates (at high and low TAN levels) from week 10 for RK Plast up to 300 g TAN/m^3/d. Maximum nitrite removal rates of RK Plast were below 20 g NO_2-N/m^3/d for 24 weeks, then increasing up to 160 NO_2-N/m^3/d. There were no differences in VTR and VNR between white PE and black PP bioelements.
3) Mutag PE bioelements showed a step increase in TAN removal rates (at high and low TAN levels) from week 10 and stabilized at 300 g TAN/m^3/d from week 24. Nitrite removal rates of Mutag bioelements increased linearly from week 10, reaching appr. 250 g NO_2-N/m^3/d at the end. There were only small differences in VTR and VNR between Mutag bioelements operated in cylinders vs. horizontal trays.
4) The highest TAN removal rates (575 g TAN/m^3/d) were recorded with Levapor at week 12, with similarly high nitrite removal of 350 g NO_2-N/m^3/d. Hereafter, both VTR and VNR dropped to approx. 150 g TAN/m^3/d and 100 g NO_2-N/m^3/d. The removal rates measured on elements in the horizontal trays were generally higher than rates recorded in the cylindric biofilters.
5) For all three types of bioelements tested, there was a strong correlation or similarity between the max TAN removal rates measured under satiated conditions (0°) and TAN removal rates measured under low TAN concentrations (substrate limited conditions)

The findings obtained under the given conditions revealed that colonization of nitrifying bacteria is slow and requires patience. Active and stable RAS water is often not an option during biofilter maturation. Therefore, the values obtained in the present study are based on "*ideal conditions*" in terms of TAN, nitrite, biodegradable material, phosphate, nutrients and trace elements – and bacteria. The VTR and VNR values measured are likely higher than those found in experimental setups with synthetic water with ammonium chloride, sodium nitrite, and inoculum.

The rates obtained can be used to calculated VTR and VNR at other temperatures. Assuming the temperature effects are similar in fresh and saltwater, VTR and VNR increases by 7% and 5% pr. °C, respectively (Kinyage and Pedersen, 2016).

The rapid and high TAN and nitrite removal capacity of Levapor also opens up new ways of combining or operating biofilters. A contingency plan could involve a plug-in bioreactor with Levapor during unintended TAN or nitrite accumulation. The potential of using biofilters with combined bioelements could also become an option to be tested. Future studies should also look into depending factors and corresponding actions to ensure stable and high nitrification performance in polymeric foam.

8. Perspectives

8.1 Outcome of the project

The project mainly aimed to address water quality challenges and develop sustainable solutions. We identified and demonstrated two new water treatment techniques to improve RAS's microbial water quality.

The **passive airlift protein skimmer** has great potential in model trout farms, where simple technology can substantially improve the water quality.

The passive skimmer suddenly presents a realistic solution to control unwanted foam, which removes a significant fraction of the bacteria and particulate matter in the rearing water. This is a new step forward towards improved water treatment, and the proposed technique fills a void in the current selection of treatment solutions.

The French-press solution with fixed squeezable **foam bioelements** also represents a novel technique to entrap organic matter and reduce bacteria and microparticles in RAS. The treatment concept was documented during a period of 18 weeks and showed consistent removal efficiency of both dissolved N and bacteria as well as efficient cleaning and backwashing. The potential of this new treatment device is large, as it can be used for different purposes in RAS. This could be used periodically to enhance ammonium and nitrite removal or it could be a permanent solution to remove bacteria and microparticles from the water.

Both the passive airlift skimmer and the French-press biofilter were tested on a pilot scale, and future work will include testing and developing larger, commercial-scale units (see below). The new treatment solutions can benefit several model trout farms and fully recirculated aquaculture systems.

Demonstrating **ozone treatment** of model trout farm water gave practical experience over 28 days and showed different effects on water quality. Ozone was generated and controlled by Gaia and Cobalia (Oxyguard) and successfully delivered to two tanks with juvenile rainbow trout. Ozone significantly reduced microbial activity by up to 70% compared to untreated tanks in a single pass and improved water clarity, decreased turbidity and reduced nitrite. The study is one of the first to deliver ozone via an oxygen cone on an outdoor fish farm, and the findings provide guidelines for further large-scale ozonation.

The project also documented that two different types of full-scale protein skimmers performed quite similarly when compared. The importance of ozone dosing at different levels was described. This information is important to finetune and optimize protein skimming under commercial conditions.

The long-term study of the colonization of nitrifying bacteria in full-strength seawater also revealed relevant documentation. It took two-three months before any significant ammonium or nitrite was removed by traditional bioelements, whereas foam bioelements showed high removal performance almost immediately. The latter dropped after three months, while the PE and PP bioelements gained momentum and showed high and stable performance over time. Future studies should look into how to ensure high-performance TAN and nitrite removal of foam bioelements in seawater, and, likewise, measures to speed up colonization in PE and PP bioelements will have a substantial economic effect.

8.2 Future perspectives

Water quality control remains one of the most important aspects of successful RAS operation.

The project has identified different technologies and concepts, among which some can become cost-effective solutions. The transfer from pilot scale to full scale relies on industrial partners, and we assume that some of the ideas can be implemented. Among these, DTU Aqua plans to continue researching these topics, ideally with new research projects in collaboration with industrial partners, which actively contributed with input and feedback at a aquaculture seminar (Fig 8-1).

Future research activities can include:

- Development, test and implementation of a full-scale passive protein skimmer
- Construction and test of a large-scale French presser
- Enrichment, priming or manipulating bioelements to speed up colonization in seawater
- New methods to reduce and/or remove bacteria from RAS water.

Figure 8-1. The results from the project were presented at Vingsted Centret to an audience representing the aquaculture industry by fish farmers, systems designers, consultants, manufacturers (i.e., protein skimmers, sensors, loggers, bioelements), feed companies and Danish Aquaculture.

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10. Appendix. Supplementary study results

10.1 Scientific manuscript about protein skimmers in SW RAS

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Evaluating protein skimmer performance in a commercial seawater recirculating aquaculture system (RAS)

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10.2 Scientific manuscript about passive foam fractionator in a model trout farm

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Testing of a passive foam fractionator prototype in a commercial recirculating trout farm

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ABSTRACT

Foam fractionation has emerged as a technical solution to reduce the build-up of microparticles and dissolved organic matter in recirculating aquaculture systems (RAS). However, commercial application in freshwater RAS is challenging and expensive. In the present study, a simple, low-cost passive foam fractionation (PFF) prototype was developed and tested under commercial conditions. The prototype was tested in a Model Trout Farm (MTF) in three different production raceways during winter and spring to assess the operation and removal potential. A number of different water quality parameters, including organic matter, particles, bacterial activity, and phosphorus were examined in the system water and in the removed foamate. Overall, the PFF prototype removed particles as well as particulate and dissolved organic matter, reduced the amount of bacteria and total phosphorus in the water, regardless of sampling time and place. By utilizing the existing airlifts in the MTF, the associated cost of construction and operation was kept low. Overall, the results demonstrate that the passive foam fractionation has the potential to help address some of aquaculture's pressing issues in a cost effective manner.

1. Introduction

Accumulation of organic matter and microparticles in recirculating aquaculture systems (RAS) have been highlighted as major concerns (Badiola et al., 2012) as available technologies applied for high-flow water filtration have a very low capability for removal of these components (Ji et al., 2020b). The accumulation of organic matter can cause different problems, i.e. impairment of biofilters (Chen et al., 2006; Michaud et al., 2006; Zhang et al., 1994), increase oxygen consumption and elevated CO_2 levels due to bacterial presence and degradation of organic matter (Leonard et al., 2002), as well as increase the turbidity and decrease the ultra-violate transmission (UVT) of the water (Holan et al., 2014; Spiliotopoulou et al., 2018). An additional practical problem at some fish farms can also include the unintended, periodic formation of foam, which is a nuisance and difficult to control and remove.

Foam fractionation is one of the very few technologies that can remove microparticles and dissolved organic matter from RAS (Timmons and Ebeling, 2010). The principle relies on the water-to-air interaction of protein-like substances in the water. The foam fractionator design facilitates transfer of small surface-charged, hydrophilic molecules in the water to air bubbles, allowing them to aggregate in large structures which can then be released and removed in the foamate (Burghoff, 2012; Lekang, 2007; Zhang and Zhang, 2019). Foam fractionation has been shown to improve water quality, by removal of microparticles (Chen et al., 1993a; de Jesus Gregersen et al., 2021; Ji et al., 2020a; Pfeiffer et al., 2024), reduction of the bacterial loads (Aalto et al., 2021; Brambilla et al., 2008), reduction of organic matter (Chen et al., 1993a; Peng and Jo, 2003; Weeks et al., 1992), and improvements in turbidity and UVT (de Jesus Gregersen et al., 2021). Previous studies have shown that foam fractionation can be as effective at removing particles from the water as drum filters with mesh sizes of 120, 90 and 60 μm (Ji et al., 2020b).

The foam formation and thereby the potential removal efficiency can be affected by several different factors (Lekang, 2007). The surface tension of the water is crucial for the foam removal process, and the presence of hydrophobic substances, for example, lipids in the water during feeding, suppresses the foam formation. Foam fractionation has mainly been considered an effective removal process in salt water, due to increased surface tension (Jafari et al., 2022; Lekang, 2007; Timmons and Ebeling, 2010). However, (de Jesus Gregersen et al., 2021) showed under replicated conditions, that foam fractionation could reduce 60 % of the organic matter in pilot scale RAS, as well as 61 % of bacterial

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10.3 List of abbreviations

- AOB – Ammonium oxidizing bacteria
- $BOD_{5\text{-}TOT}$ – Total Biological oxygen demand (5-days) from untreated samples
  $BOD_{5\text{-}DISS}$ – Dissolved Biological oxygen demand (5-days) from 0.22 µm filtered samples
- $BOD_{5\text{-}PART}$ – Particulate Biological oxygen demand (5-days) – as BOD5_Total - BOD5_Diss
- BQV - BactiQuant value
- COD_{TOT} Total Chemical oxygen demand from untreated sample
- COD_{DISS} Dissolved Chemical oxygen demand from 0.2 µm filtered samples
- COD_{PART} Particulate Chemical oxygen demand as COD_{TOT} - COD_{DISS}
- FF - Foam fractionation
- *K* - H_2O_2 decay rate (h^{-1})
- NOB – nitrite oxidizing bacteria
- NTU - nephelometric turbidity unit
- OPO – ozone-produced oxidants typically recorded as mg Cl_2/L
- ORP – oxygen redox potential in mV
- PE – Polyethylene
- PP - polypropylene
- PPM – part per million
- PN - Total particle number
- PS – Protein skimmer
- PSA - total particle surface area
- PV - total particle volume
- RAS - Recirculating aquaculture system
- SNR - surface-specific nitrite removal (g $N/m^2/d$)
- SSA - Specific Surface Area
- STR – surface-specific TAN removal (g $N/m^2/d$)
- TAN – Total ammonium Nitrogen
- TRO – Total residual oxidants typically recorded as mg Cl_2/L
- TSS – Total suspended solids
- UVT – Ultraviolet transmission (% transmission at 254 nm)
- VNR - volumetric nitrite removal (g $N/m^2/d$)
- VTR – volumetric TAN removal (g $N/m^2/d$)

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