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Doctoral thesis

NTNU
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Thesis for the Degree of
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Abstract

Aquaculture is one of the fastest growing food producing sectors today. Norway is the world's largest producer of farmed Atlantic salmon (*Salmo salar*), and the aquaculture industry is an important contributor to value creation and employment nationally. The land based production phase in Norwegian salmon farming has the past years been extended to include post-smolt for an increasing number of farms. This is a consequence of problems with salmon lice and diseases in open net pens, escapes, pollution, industry public relations and economic aspects of regulations and concessions. Increased production time on land in recirculating aquaculture systems (RAS) with larger fish demand more comprehensive water treatment to maintain good water quality. Higher biomass and feeding generate more intensive organic loads and particles in RAS. Furthermore, prolonging the production on land to include post-smolt may involve introduction of salt water to the systems. Organic matter and salinity will affect the water treatment significantly, in particular nitrification and the microbial water quality. Bacteria are key players in the nutrient fluxes in RAS to maintain high water quality. The motivation for this thesis was to provide more knowledge on operation and rearing regimes in RAS for salmon smolt and post-smolt production, with a special focus on microbial challenges related to organic matter and salinity.

Our first experiment evaluated the effects of enhanced particle removal with membrane filtration in RAS on concentrations of organic matter and its consequences for water quality and microbial conditions. This experiment was furthermore used to make a carbon and nitrogen mass balance. We evaluated the dynamics and fate of C and N input to RAS, and removal efficiencies of the water treatment, including a membrane, for C and N compounds. The results showed that the system with membrane filtration had higher microbial diversity, lower and shorter bacterial blooms and generally lower bacterial densities in the water than in the system without membrane filtration. The mass balance showed that membrane filtration reduced the fraction of input C and N ending up as particles in RAS. The membrane directly removed particles, reducing accumulation of C and N compounds which resulted in better water quality. The better physicochemical and microbial water quality in combination with higher temperatures led to better appetite of the fish and as a consequence, this system had less feed

waste and better fish growth than the system without membrane filtration. High organic matter loadings did not impact the nitrification efficiency negatively due to total ammonia nitrogen (TAN) limitation. This implies that as long as TAN is limiting and there is sufficient oxygen concentrations in the biofilter, increased loadings of organic matter in post-smolt production with larger fish will not suppress nitrification. Membrane filtration has shown to be a suitable technology for removal of the smallest particles and bacteria in RAS to improve water quality. However, cost-benefit analyses with membrane filtration at different life stages during Atlantic salmon production remains to be done to determine economic feasibility for the fish farmers.

Our second experiment studied how two different regimes for salinity increase in RAS affected the RAS microbiota, nitrification capacity and performance of fish. One regime was a gradual increase in salinity in a brackish water RAS with post-smolt, the other was a direct transfer of post-smolts from a low salinity brackish RAS to a high salinity/seawater RAS, both groups with subsequent transfer to sea. The results showed that salinity was a driver for bacterial succession in RAS water. This included a combination of physiological salinity adaptation processes and succession causing change in community structure and introduction of new species. We showed that it was possible to successfully increase the salinity in an operating RAS with fish without exceeding toxic concentrations of TAN and nitrite. We hypothesize this was due to the salinity history of the system and halotolerant nitrifying bacteria embedded in the biofilter biofilm. Whether one salinity adaptation strategy was better than the other in respect to the fish still remains unknown as there were no clear positive indications in either of the fish groups in the two salinity adaptation regimes both on land and at sea.

The third experiment investigated the start-up of nitrifying biofilms in freshwater and brackish water MBBR biofilters. The development of the nitrifying community assembly in the biofilm and nitrification capacity were compared in the two reactors. We observed that after 60 days of start-up, the brackish water biofilm had half the nitrification capacity of the freshwater biofilm during stress-tests, with less diverse microbial communities and lower proportion of nitrifiers. However, low ammonia and nitrite concentrations with rapidly increasing nitrate concentrations indicated that complete nitrification was established in both reactors. The results suggest that nitrification developed in comparable time in brackish and freshwater, and brackish start-up can be a strategy for bioreactors with varying salinity, like in post-smolt production.

Preface

This doctoral thesis is submitted to the Norwegian University of Science and Technology (NTNU) in Trondheim for partial fulfilment of the requirements for the degree of Philosophiae Doctor (PhD). Prof. Stein W. Østerhus at the Department of Civil and Environmental Engineering has been the main supervisor, and prof. Olav Vadstein and associate prof. Kari J.K. Attramadal at the Department of Biotechnology and Food Science have been the co-supervisors.

The work for this thesis was conducted over four years, in which 75% of the time was dedicated to research, and 25% to duty work at the Department. The duty work included assisting and lecturing in the course TVM4110 - *Water Chemistry* and co-supervision of master students.

This thesis is based on work from three experimental studies. The first study was conducted in collaboration with Sintef Ocean, and was funded by the Cooperation in Fisheries, Aquaculture and Seafood Processing (COFASP) in the European Research Area Network (ERA-NET), and the Norwegian Research Council. The second study was a collaboration with Let Sea, and was funded by Regionalt Forskningsfond Nord (RFF-Nord), Skattefunn and Let Sea AS. The third study was part of CtrlAQUA SFI, Centre for research-based innovation funded by the Research Council of Norway and the Centre partners, including Krüger Kaldnes AS. This thesis also received funding from the Department of Civil and Environmental Engineering and the Faculty of Engineering with "Strategiske omstillingsmidler (SO)" as part of the NTNU OCEANS pilot research program *Aquaculture & Environmental Interactions*.

In accordance with the guidelines of the Faculty of Engineering, the thesis contains an introduction to the research work and four scientific papers.

Acknowledgements

I would like to start thanking my awesome supervisors, Olav, Kari and Stein. It has of course been some ups and downs, but overall, I have enjoyed doing this PhD. I know a big part of that is because of you. I always felt I could come and talk to you, and that the PhD and I were in "safe hands" with you.

Olav, you always have the answer to everything. You are kind, caring, wise and you always know what to say to make me feel better and less stressed at my most pessimistic times. Kari, you are smart, funny and professional! Your profound knowledge on RAS has truly inspired me, and I identify myself with you as I think we are a bit alike. If I become anything like you, I will be happy. Stein, our "teddy bear". You are on my list of the nicest men I have met, but you are also the busiest man I have met. Being so skilled in your field, I understand that many people want your time. During our conversations, I always lost track of time and I wanted to discuss more with you. Thank you all, for everything.

A big thank you to all my PhD colleagues at Vassbygget. Chunbo, you were the best office m ate I could have asked for. And Silje, I will miss listening to all your funny stories during lunch. Ana, Blanca and Erle, I'm so glad that Vassbygget brought us together, and I now consider you all close friends. Noëmi, I think we both needed each other during corona semi-lock-down. Thank you for countless nice evenings with TV (especially Harry Potter and Babylon Berlin), cross-country skiing and being a good friend and support.

To Cynthia, for making my teaching duty a truly nice experience. Thank you for your kindness, guidance and help through difficult equations with water chemistry.

I am very grateful that I could continue to be a part of the ACMS group at Biotechnology after my master thesis. Ingrid, you are one of the reasons why I wanted to do the PhD. Thank you for everything and the nice memories (with Olav also) in Oregon. Thank you Sharada, for letting me take part in your work at Sunndalsøra, I really enjoyed our collaboration. And Stine, "flower power twins" in Berlin and good times in the "stamp" at Dønna, I will never forget.

To our amazing RAS-ORGMAT team, Kari, Trond, Deni, Anette, Gaute and Arne. You are all part of this thesis and the reason why our experiment at Sealab was a true success.

All the staff at Let Sea Bjørn, you made the time we spent at Dønna really great. You all took such good care of Anette and me, helped us during sampling and rescued us from the ferry dock.

My only family in Trøndelag, tante Elin and Thomas, I am so grateful for the hospitality you show in Meldal. For all the dinners and day-trips to Agdenes, Lensvik, Surnadalsøra, Rindal and Hoston we've had together. It has been a safe feeling to know I have family close to me

To my family, mamma, pappa, Maria, Anna and Åse. Coming home to Ersfjordbotn have been and always will be something I really look forward to. The calm atmosphere in our house, skiing, fishing and hiking together always took my mind off the PhD-work and I could just relax. Your care and support is something I value highly, and I love you all so much.

And of course, to you my Carl. Thank you for always making me believe I can accomplish everything I hope to. I am forever grateful for all your support, love and patience, which has helped me through this PhD.

Table of contents

Abbreviations

	Description
AOB	Ammonia oxidizing bacteria
DOC	Dissolved organic carbon
FTS	Flow-through system
HRT	Hydraulic retention time
MBBR	Moving bed biofilm reactor
NH_3	Ammonia
NH_4^+	Ammonium
NOB	Nitrite oxidizing bacteria
OTU	Operational taxonomic unit
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
POC	Particulate organic carbon
PON	Particulate organic nitrogen
RAS	Recirculating aquaculture system
TAN	Total ammonia nitrogen
TOC	Total organic carbon
UV	Ultraviolet
WWT	Wastewater treatment

Definitions

Smolt	Smolt is a juvenile salmon that has gone through smoltification and is adapted to a life in seawater. The size of the smolts are typically from 70 to around 120 g.
Post-smolt	Post-smolt is a term that is not clearly defined. It can refer to 1) the first stage after smoltification, 2) it can be related to size, typically when the fish are from 250 g to 1000 g, or 3) when the fish have smoltified (on land) but have not yet been transported to the sea. The latter definition for post-smolt is used in this thesis.
Salinity	Salinity is the amount of salt dissolved in a body of water. The major ions in seawater are chloride (Cl^- : 55.3%), sodium (Na^+ : 30.7%), sulphate (SO_4^{-2} : 7.6%) magnesium (Mg^{2+} : 3.7%), calcium (Ca^{2+}: 1.2%) and potassium (K^+: 1.1%). For this thesis the term is given in parts per thousand (‰ or ppt).
Freshwater	Freshwater is water with less than 1‰ of dissolved salts. In the aquaculture industry, the freshwater term may refer to water up to 3‰ salinity, as some salt is typically mixed into the production water during the freshwater juvenile stages to prevent growth of fungi.
Brackish water	Brackish water is saltwater and freshwater mixed together. By definition, it is water with a salinity between 1 - 30‰. For this thesis, brackish water refers to water with a salinity of 3‰ up to 25‰.

Seawater	Seawater is water with more than 30‰ salinity. In the ocean, the salinity is typically 33-35‰. In the aquaculture industry, seawater or marine land-based systems may refer to water with a salinity over ~25‰ and is the salinity range used in this thesis.
r/K-selection theory	An ecological theory with two generalized growth strategies that result in different community structures based on selective forces of substrate availability, growth rates and competition. The r/K- theory is not an either or, but the two opposites in a continuum.
r-strategist	r-strategists have high maximum growth rates, low substrate affinity and poor competitive ability at low substrate supply per individual. r-selection occurs when the population is below the system carrying capacity, and the population will grow with the reproductive rate r, which has given the name r-strategist. Pathogenic species are often considered opportunistic r-strategists, and this group also contain non-pathogenic species.
K-strategist	K-strategists have low maximum growth rates, high substrate affinity and high competitive ability at low substrate supply per individual. Their biomass will be close to the carrying capacity of the system, thereof the name which originated from German: Kapazitätsgrenze.
Microbially matured water	Microbially matured water is diverse, more stable to environmental perturbations and dominated by K-strategists.
Carrying capacity (CC)	The maximum population density that a system can sustain over time. The supply of degradable organic matter is typically the growth limiting factor defining CC for heterotrophic bacteria.

List of publications

Paper I — Fossmark, R.O., Vadstein, O., Rosten, T.W., Bakke, I., Košeto, D., Bugten, A.V., Helberg, G.A., Nesje, J., Jørgensen, N.O.G., Raspati, G., Azrague, K., Østerhus, S.W. & Attramadal, K.J.K. 2020. Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (*Salmo salar*). *Aquaculture*. vol. 524, 735268. DOI: 10.1016/j.aquaculture.2020.735268

Paper II — Fossmark, R.O., Vadstein, O., Attramadal, K.J.K. & Østerhus, S.W. Mass balance of carbon and nitrogen, and nitrification efficiency in recirculating aquaculture systems (RAS) with membrane filtration rearing Atlantic salmon parr (*Salmo salar*). (Manuscript in preparation)

Paper III — Fossmark, R.O., Attramadal, K.J.K., Nordøy, K., Østerhus., S.W., Vadstein, O. 2021. A comparison of two seawater adaptation strategies for Atlantic salmon post-smolt (*Salmo salar*) grown in recirculating aquaculture systems (RAS): Nitrification, water and gut microbiota, and performance of fish. *Aquaculture.* vol. 532, 735973. DOI: 10.1016/j.aquaculture.2020.735973

Paper IV — Navada, S., Sebastianpillai, M., Kolarevic, J., Fossmark, R.O., Tveten, A., Gaumet, F., Mikkelsen, Ø. & Vadstein, O. 2020. A Salty Start: Brackish water start-up as a microbial management strategy for nitrifying bioreactors with variable salinity. *Science of the Total Environment.* vol. 739, 139934. DOI: 10.1016/j.scitotenv.2020.139934

List of oral conference presentations

2019 Microbial community dynamics in recirculating aquaculture systems rearing Atlantic salmon parr (*Salmo salar*) with reduced organic loading through membrane filtration. *Aquaculture Europe, Berlin, Germany.*

2019 Increase in salinity as a driver for microbial community succession in RAS for Atlantic salmon smolt (*Salmo salar*) production. *Nordic RAS, Berlin, Germany.*

2019 Salinity as a driver for microbial community succession in the gut of Atlantic salmon (*Salmo salar*). *International fish microbiota workshop, Eugene, Oregon USA.*

Chapter 1: Introduction

This chapter gives a brief introduction to the background and motivation for the doctoral work. The knowledge gaps, scope of work and objectives are presented. An overview of the thesis structure is at the end of the chapter. A more detailed presentation of the background is given in Chapter 2.

1.1 Background and motivation for this thesis

Atlantic salmon (*Salmo salar*) production is an ongoing success story in Norway, contributed by high market prices and a continuous demand globally. In 1989, the total Norwegian salmon production was around 111 000 tons, and in 2018 it had increased to 1.28 million tons (Statistics Norway, 2019). Parallel with this over tenfold growth of salmon production the past 30 years, the environmental impacts from salmon farming are increasing. One example is the increased abundance of salmon lice, which has become a major threat to further growth of the industry (Abolofia et al., 2017). This is one out of several drivers for more land-based production of salmon. The first production stages of farmed Atlantic salmon are in land-based systems with freshwater or water with low salinity (~0-3‰) from the eggs hatch to smoltification. Traditionally, the fish are at this point moved to sea cages for the grow-out to market size adult salmon. The past years the land-based production phase has been extended to include post-smolt for an increasing number of farms. As a consequence of more land-based production, the interest in water reuse systems are challenging the traditional flow-through systems (FTS) for smolt and post-smolt cultivation (Martins et al., 2010, Dalsgaard et al., 2013). In recirculating aquaculture systems (RAS), a large share (> 90%) of the water goes through biological and physical water treatment processes and is reused, and the water consumption is therefore substantially lower than in FTS. RAS are closed systems, which allows for better control over the water quality, temperature, discharge and excluding parasites (Summerfelt et al., 2001). In

fish production in general, it is well known that optimal physicochemical water quality is crucial for fish health. The past years it has also been more recognized that the microbial water quality is an important factor in the cultivation of fish (Attramadal et al., 2012a, Pedersen et al., 2017, Rud et al., 2017, Rojas-Tirado et al., 2018, Dahle et al., 2020, Minich et al., 2020). Bacteria are necessary to maintain high water quality by conversion of waste nutrients in RAS (Blancheton et al., 2013). In addition, bacteria colonize the fish and affect them positively by e.g. improved utilization of nutrients in the gut and protection against invasion of pathogens (Nayak, 2010, Gomez et al., 2013). Optimal microbial water quality have a high abundance of beneficial bacteria that can outcompete opportunistic and potential pathogenic bacteria (Attramadal et al., 2014, Vadstein et al., 2018). Increased production time on land with larger fish demand more comprehensive water treatment to maintain good water quality. Higher biomass and feeding yield more intensive organic loads and particles in RAS. Furthermore, prolonging the production on land to include post-smolt may involve introduction of salt water to the systems. More organic matter and salinity in RAS will affect the water treatment significantly, especially nitrification and the nitrifying bacteria in the biofilter. Furthermore, organic matter is substrate for heterotrophic bacteria, and increased organic matter loads will affect the microbial water quality. More knowledge on operation and rearing regimes concerning organic matter and salinity in RAS is therefore needed to optimize the production of Atlantic salmon smolt and post-smolt, and was the motivation for this thesis.

1.2 Knowledge gaps and scope of work

A key challenge in RAS is the accumulation of organic matter as fine suspended solids and colloidal particles. Particulate organic matter (POM) originating from feed waste and faeces is typically the limiting resource determining the carrying capacity (CC) of heterotrophic bacteria in the system. A low and stable CC in the system is suggested to be a strategy for achieving an optimal microbial environment with high abundance of beneficial bacteria (Attramadal et al., 2012a, Attramadal et al., 2014). With increased post-smolt production and higher organic loads, the removal of particles will become more critical and impact the water quality negatively as today's technology does not efficiently remove the smallest particles. Membrane filtration has been proposed to supplement the conventional particle removal in RAS to

remove the smallest and colloidal particles (Viadero and Noblet, 2002, Holan et al., 2013, Holan et al., 2014a). Membrane filtration is a well-known technology, commonly used for treating drinking water and wastewater. However, the use in RAS for salmon production is not well studied, and we lack knowledge on how enhanced removal of organic matter affect the microbial water quality and fish growth. This was elaborated in **Experiment 1**.

Carbon and nitrogen are added to RAS every day through the fish feed. The parts that do not end up as fish biomass eventually become particulate or dissolved compounds which are recirculated in the system if not removed. As previously noted, the accumulation of particulate compounds is a challenge in RAS, and due to high water recirculation flows and turbulence in RAS, particles containing C and N can dissolve into forms which are more available for bacterial assimilation and energy sources (Leonard et al., 2002, McMillan et al., 2003). The carbon to nitrogen ratio (C/N) affects the competition between heterotrophic and the autotrophic nitrifying bacteria and thus the nitrification efficiency of the biofilter (Michaud et al., 2006). Although the importance of organic matter effects on nitrification is well described, there is still little knowledge on the fate of C and N input into RAS, and to what extent the different forms of these elements affect the capacity and functionality of the biofilter and general water treatment efficiency. This was also elaborated in **Experiment I**.

Salmon farmers using RAS for post-smolt production face the challenge of varying salinities during the production cycle. There are two options for the fish farmers, 1) introduce seawater into the RAS used for freshwater cultivation of juveniles or 2) move the post-smolt to a separate brackish/seawater adapted RAS. Increasing the salinity in an operating RAS changes the environmental conditions for the water treatment and the bacterial populations adapted to the freshwater system. It could be a better option as it is a gentler way of changing the environment for the fish and transportation is avoided. However, these effects on fish health have not been studied. Little is known about the bacterial dynamics and the functionality of e.g. nitrifying bacteria during the transition from pre to post-smolt conditions in RAS. How much of the bacterial community dynamics that is a physiological salinity adaption process relative to succession causing change in community structure and introduction of new species is not clear. This was elaborated in **Experiment II**.

Little is known about the time required to achieve nitrification during start-up in brackish water biofilter with virgin carriers. Some studies have shown that the start-up of nitrifying biofilms in seawater is longer than in freshwater, as initially more energy is directed towards osmoregulation than growth of the nitrifying bacteria (Nijhof and Bovendeur, 1990, Rusten et al., 2006). Therefore, the use of matured biofilm carriers with salinity-adapted bacteria have been used to accelerate the process (Kuhn et al., 2010). However, due to the high focus on biosecurity in RAS, the use of inoculum and matured biofilm carriers may not be preferred, and clean virgin carriers with synthetic wastewater during start-up must be used. More knowledge on the time required for start-up in brackish water with virgin carriers and nitrification capacity is needed, as more systems now may require salt for post-smolt production. This was elaborated in **Experiment III**.

The overall goals of this thesis were to elaborate the effects of organic matter and salinity in RAS on microbial community dynamics, nitrification functionality, general water quality and fish performance. Field studies at small and commercial scale RAS facilities with Atlantic salmon were performed to meet the research goals. To study more in detail the nitrifying community succession, nitrification kinetics and capacity of biofilters, lab-scale and semi-commercial scale reactor tests were also conducted. The tree experiments that this thesis is based on are presented in **Chapter 3.**

1.3 Research objectives and thesis structure

With the knowledge gaps described, the research objectives for this thesis are the following:

Objective 1	Obtain knowledge on enhanced particulate organic matter removal in RAS considering the effects on microbial community dynamics, general water quality and fish performance (**Paper I**).
Objective 2	Elaborate the capacity and the functionality of biofilters for handling nitrogen and carbon, and the dynamics of C and N waste in RAS in connection to relevant operational choices (**Paper II**).
Objective 3	Study how salinity increase affects bacterial succession, nitrification functionality and fish performance in RAS (**Paper III**).
Objective 4	Study and compare the nitrifying bacterial succession and capacity in biofilter biofilm during start-up of an MBBR in freshwater and brackish water (**Paper IV**).

Chapter 1 has presented general background and motivations for this thesis, the scope of work and knowledge gaps which gave the basis for the research objectives. Chapter 2 provides a more detailed background on the topics addressed in this thesis. Chapter 3 presents the experimental studies which this thesis is based on and the general research methodology. Chapter 4 presents a summary of the papers with the main results, and finally Chapter 5 discuss the main results from the papers with concluding remarks and presents some suggestions for future work. All the papers for this work are given in Appendix A. Appendix B holds the co-author statements for publishing in this thesis.

Chapter 2: Background

This chapter holds a detailed presentation of the background and knowledge status of the topics addressed in this thesis.

2.1 Atlantic salmon life cycle and commercial production

Atlantic salmon (S*almo salar*) in an anadromous fish. The wild salmon spawn and hatch in rivers, and spend the juvenile life stages as alevin, fry and parr in freshwater. Then the parr go through the seawater preparatory transformation which is called smoltification. This transformation is induced by photoperiod (McCormick et al., 1987), endocrine signals and water temperature (Specker, 1982, Björnsson et al., 2011). The transition into smolt changes the osmoregulatory management and morphological traits from a darker pigmented parr with characteristic vertical spots to a silver coloured smolt. Then the salmon smolt is ready for seawater and migrates out from the river to the sea.

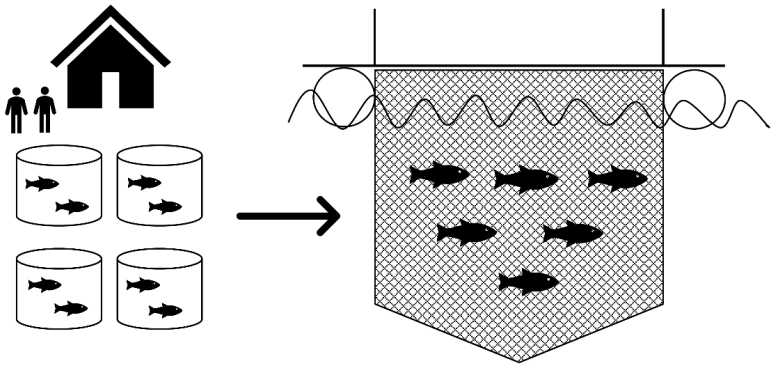

Fig. 1. Commercial production of Atlantic salmon, showing the land-based phase following grow-out in net pens in the sea.

In traditional commercial salmon production (Fig. 1), the juvenile salmon are cultivated in freshwater or water with low salinity on land in RAS or FTS until a size of around 70 to 140 g (Bergheim et al., 2009). Then the smoltification is induced, e.g. through artificial summer light regimes, higher water temperatures and/or higher salinity in the land-based systems (Imsland et al., 2014, Fjelldal et al., 2018). Afterwards the salmon are transported with well boats to sea cages for grow-out to market size of typically 4 to 6 kg. Sea cages are the main production system for the grow-out stage of salmon, and they have low investment and maintenance costs compared to land-based systems (Lekang et al., 2016). The long coastline of Norway and cold water temperatures are very suitable for salmon farming, which is the main contributor to the fact that Norway is the worldwide top commercial producer of farmed salmon (FAO, 2018).

2.2 Land-based production of smolt

The salmon success story in Norway has run into some challenges with production in sea cages open to the surrounding marine environments. As a consequence, the land-based production phase has been extended to include post-smolt for an increasing number of farms the past years. One major challenge is salmon lice, which have increased the production costs significantly, and negatively impacted fish health and public relations of the industry (Abolofia et al., 2017). Off-shore ocean farms have been designed for salmon production in recent years, and these farms are more weather exposed and outside the salmon lice areas. Cultivation in these farms need larger and more robust fish than the typical smolt, which consequently require longer production time on land. Larger post-smolt are hypothesized to be stronger than smaller smolt to handle the harsher and weather exposed conditions at sea (Lekang et al., 2016). By prolonging the land-based phase, the fish will be larger, and the time they are exposed to salmon lice, number of de-licing treatments, exposure to potential harmful algal blooms and risk for diseases at sea are reduced. Another driver for increased production time on land is higher utilization of the Maximum Allowed Biomass (MAB) at the sea localities given in the concessions for the fish farm. On land the temperatures can be better controlled and kept higher than in the sea, especially during the winter months (Kolarevic et al., 2014). Therefore, the fish will grow faster on land at higher temperatures (Barton, 1996), and as a consequence a better utilization of the production quotas may be achieved with post-smolt production. The concessions to produce salmon in open sea cages are expensive, and can cost from 150-200 000

NOK per ton MAB (Norwegian Directorate of Fisheries, 2020). In contrast, the concessions for producing salmon on land are free (Norwegian Directorate of Fisheries, 2016), making land-based production attractive for the fish farmers.

Post-smolt is not clearly defined among people working within aquaculture. For this thesis, the definition of a post-smolt is when the fish have smoltified on land but have not yet been transported to the sea. When the salmon is entering seawater, it must be able to maintain the internal osmotic balance by excreting ions (mainly Na^+ and Cl^-) and retain water (Specker, 1982). The major osmoregulatory changes that occur during smoltification are increased activity of the ion-transporting enzyme Na+, K+-ATPase (NKA) (Specker, 1982, Björnsson et al., 2011). Salmon can maintain high gill NKA activity and good hypo-osmoregulatory abilities when kept in freshwater after smoltification (Stefansson et al., 1998). This makes the seawater timing somewhat flexible in the production cycle. However, if the smolts are kept in freshwater for a long period after smoltification (this depends on temperature and salinity), the fish will partially readapt to freshwater and desmoltify (McCormick and Saunders, 1987). Changing the osmoregulation is an energy-costly process (Rivera-Ingraham and Lignot, 2017) and stressful for the fish. It causes physiological changes reducing appetite and growth (Jørgensen and Jobling, 1994) and the immune suppression (Johansson et al., 2016). Salmon have been grown to market size in RAS with only freshwater (Davidson et al., 2016), however problems with early sexual maturation was shown to be a production barrier as it reduces flesh quality and growth (McClure et al., 2007). It has been shown that salinity over 15‰ in Atlantic salmon cultivation can inhibit desmoltification (Mortensen and Damsgård, 1998), which indicates that post-smolt production should be over this salinity to achieve optimal growth.

2.3 Recirculating Aquaculture Systems

The question of sustainability in food production is frequently stressed, including land-based fish production. The traditional FTS have lower production and maintenance costs and are less complex to operate than RAS. However, as these systems have very high water consumption, they can only be built by rivers where the water flow is high, stable and not exposed to seasonal water depletion (Timmons and Ebeling, 2007). RAS on the other hand, are considered to be more environmentally friendly and economic in terms of energy for heating and water use, but

the initial investment costs are high (Tal et al., 2009). Typically, over 90% of the water is recirculated, making the hydraulic retention time (HRT) of the water long and provide a more stable rearing environment for the fish. RAS is a closed system with much better control of pollution and environmental impacts than the open marine systems (Summerfelt et al., 2001, Martins et al., 2010). A typical RAS (Fig. 2) will contain rearing tanks and a water treatment section for mechanical removal of particles, biological filtration for conversion of toxic N-compounds and organic matter, and CO_2-degassing and oxygenation (Lekang, 2013). Controlling the water quality is essential for successful farming in RAS, and aspects of economics, technology and daily manageable operations for the fish farmers are topics of discussion in aquaculture engineering. Increasing the land-based production phase to include post-smolt production impact the water treatment processes in RAS, and the systems must be dimensioned to handle more organic matter and potentially also changes in salinity. Bacteria are key players in the nutrient fluxes to maintain high water quality in RAS. They keep the concentrations of potentially toxic compounds at acceptable levels. It is therefore also important to elaborate how the microbial water quality will respond to the environmental changes in post-smolt production.

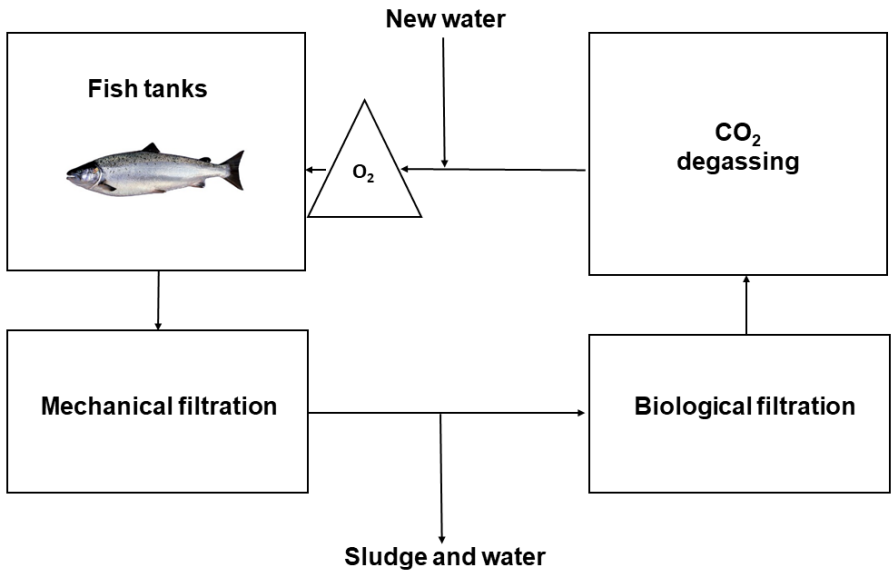

Fig. 2. RAS showing the typical water treatment processes

2.4 Bacteria in RAS and microbial management in aquaculture

RAS is a complex ecosystem with bacterial populations associated with water, biofilm and fish. There are two main groups of bacteria that participate in the nutrient fluxes in RAS: heterotrophic bacteria that degrade organic matter, and autotrophic bacteria that oxidize ammonia to nitrate, mainly in the biofilter biofilm (Blancheton et al., 2013). The heterotrophic bacteria that are in suspension in the water phase, are in close contact with the fish and can affect them both positively and negatively. The bacteria can be beneficial for the fish through metabolic and immunological relations, and by facilitating nutrient absorption in the gut, stimulating the immune system and protecting against invasion by pathogens (Gómez and Balcázar, 2008). Pathogenic and opportunistic bacteria can stress the fish and cause infections (Llewellyn et al., 2014). The water treatment and rearing environment in RAS should therefore be optimized to maintain a healthy bacterial flora, with low probability of opportunistic bacterial blooms.

Microbially matured water is dominated by beneficial/non-opportunistic stable bacterial communities that can outcompete opportunistic and potential pathogenic bacteria (Vadstein et al., 1993, Skjermo et al., 1997, Attramadal et al., 2012a). Rearing regimes selecting for matured microbial communities in the water have shown to be beneficial for cultivation of marine larvae (Skjermo et al., 1997, Attramadal et al., 2014, Attramadal et al., 2016, Vestrum et al., 2018). However, the effects are not as thoroughly studied in the production of juvenile and on-growing salmon. Vadstein et al. (1993) derived the definition of microbial matured water from the ecological theory of r/K-selection (MacArthur and Wilson, 1967, Andrews and Harris, 1986), where two generalized directions of succession occurs based on selective forces of substrate availability and competition. Opportunistic r-strategists have rapid growth rates when resources are abundant and succeed when the competition for nutrients is low (low population densities). K-strategists in contrast, have lower growth rates and thrive in environments with high competition (high population densities) as they can exploit limited resources better. Since K-strategists are not as dependent on high and balanced nutrient supplies, their biomass is close to the system carrying capacity (CC) and they are more stable and resilient to environmental fluctuations and invasion (Vadstein et al., 2004). K-selected communities are hypothesized to be more favourable for the fish health and survival, and most fish-pathogenic bacteria belong

to the category of r-strategic opportunistic microbes (Vadstein et al., 2018). Some opportunistic bacteria are commonly present in the natural aquatic microbiota, and typically these indigenous bacteria only become harmful when they are in high numbers and there are environmental conditions that impair the fish (De Schryver and Vadstein, 2014). In the RAS water treatment, a selection pressure that will promote K-strategists and reduce the possibility for opportunistic proliferation is therefore desired. This may be achieved in an environment with reduced nutrient supply per bacterium at the system CC (Attramadal et al., 2016).

2.5 Particles and organic matter in RAS

Particles in aquaculture systems are produced from decomposing food, faecal waste, and dead and living bacteria (Chen et al., 1993). Many techniques can be used to remove particles, e.g. mechanical filtration in a disk, belt or sand filter, and gravity separation (Summerfelt et al., 2001, Lekang, 2013). However, these particle removal methods only remove particles that are larger than 60 µm (Chiam and Sarbatly, 2011). The fine suspended solids, in particular the solids below 20 µm size, remains in the system and are recirculated (Chen et al., 1993, Fernandes et al., 2014). In a RAS with high flow rates and pumps that cause turbulence in the water, the larger particles can quickly disintegrate into smaller particles and dissolved fractions which are harder to remove (McMillan et al., 2003).

The fish feed is the source of carbon and nitrogen input into RAS that eventually become particles. The content of feed for salmonids contain around 50% carbon (Corner et al., 2006) and around 7.3% nitrogen (Dalsgaard and Pedersen, 2011). In salmon farming, it has been estimated that 40-48% of the carbon is lost through respiration by the fish, 14-30% is used for growth (Corner et al., 2006, Wang et al., 2012), and 15-19% are lost as faeces (Reid et al., 2009, Wang et al., 2012). If the feed conversion ratio is good (not much higher than 1), the feed waste typically constitute 3 – 5% (Reid et al., 2009). This means that 14-46% of the carbon input through feed ends up in the water as particulate or dissolved fractions and will accumulate depending on the make-up water flow and water treatment. The fate of the input nitrogen is more elaborated in **section 2.7.**

Accumulation of particles in RAS is a problem, as it lower the quality of water, induce stress on the fish and lead to decreased performance and disease resistance (Cripps and Bergheim, 2000). Particles can cause physical damage to the gills (Chapman et al., 1987), and high particle concentrations have been linked to the occurrence of bacterial gill disease and amoeba gill infestation (Bullock et al., 1994). Particles also reduce the disinfection of water by protecting the bacteria from UV-light and ozone disinfecting methods (Hess-Erga et al., 2008). Sedimentation of particles can result in formation of anaerobic zones which can cause production of toxic hydrogen sulphide (H_2S). This is even more critical with seawater addition in RAS (Letelier-Gordo et al., 2020), as seawater contain more sulphate than freshwater. Accumulation and mineralization of particles will also lead to an increase in bacterial substrate that induce heterotrophic bacterial growth, causing competition for oxygen and space in the biofilter which can reduce the nitrification efficiency (Chen et al., 2006, Michaud et al., 2006). It can also cause an increase in bacterial numbers and change the microbial community composition in the system, which may be unfavourable for the cultivated species (Holan et al., 2014a, Wold et al., 2014, Attramadal et al., 2012b). Protein skimming/foam fractionation have been used in seawater RAS to remove particles smaller than 20 µm (Brambilla et al., 2008, Barrut et al., 2013), however this technology is less efficient in freshwater. There is a need for a more advanced particle removal system for the fine suspended solids and colloidal fraction of the particles, to improve the water quality and fish performance in post-smolt production with more intensive organic loads.

2.6 Membrane filtration

Membrane filtration is a particle separation technology that efficiently remove the smaller and colloidal fractions of solids, and this technology has been proposed to supplement the conventional particle removal in aquaculture and RAS (Gemende et al., 2008, Pimentel et al., 2017). The membrane filtrations that are suitable for RAS are microfiltration and ultrafiltration. This technology remove both particulate and colloidal substances, including bacteria, and have been used for treating drinking water and wastewater (Van der Bruggen et al., 2003). However, the interest in using membrane filtration in aquaculture is still low despite the well-known problems with accumulation of small particles. The reasons for this are first of all the costs, as membrane filtration adds additional operational complexity to the RAS. The membrane also

needs frequent cleaning due to fouling (Sharrer et al., 2007, Holan et al., 2014b, Gao et al., 2019), and thorough surveillance and maintenance (Viadero and Noblet, 2002). More research is therefore needed to evaluate whether the extra cost for the membrane filtration can be balanced by potentially better water and fish quality. Membrane filtration reduce the organic load and lead to a more stable CC, with reduced nutrient supply per bacterium (Attramadal et al., 2012a, Wold et al., 2014). This in turn could select for a more microbially matured water, which it hypothesized to have been achieved in production of marine larvae (Holan et al., 2014a, Wold et al., 2014). We do, however, lack knowledge on whether microbially matured water can be obtained with membrane filtration in production of Atlantic salmon. These systems have much higher loadings of organic matter, water flows and turbulence and shorter hydraulic retention times (HRT) in the fish tanks than in the previous studies with marine larvae. Membrane filtration in RAS for salmon production should therefore be studied, to determine if it is a suitable technology for removal of the smallest particles at an acceptable cost.

2.7 Nitrification and nitrogen in RAS, and start-up of nitrifying reactors

Fish excrete ammonia as the end-product of protein catabolism, and the excretion rate is high in salmon production as its diet contain a lot of protein (Timmons and Ebeling, 2007). In aquaculture, the term total ammonia nitrogen (TAN) is used to express ammonia concentration. TAN is the sum of ammonia nitrogen (NH_3-N) and ammonium nitrogen (NH_4^+-N), which are in a pH-dependent equilibrium in the water. The toxicity increases with higher pH as the equilibrium is driven towards the un-ionized NH_3 which the fish are more permeable to (Eddy, 2005). The primary purpose of a biofilter in RAS is the biological conversion of ammonia to nitrite (NO_2^-), and then to nitrate (NO_3^-). TAN and nitrite are toxic for the fish at low concentrations. The concentrations of TAN and nitrite nitrogen (NO_2-N) should be below 2 and 0.1 mg L^{-1}, respectively, this including a safety factor for increase in pH, to avoid detrimental conditions for the fish in RAS (Reported for freshwater RAS: Norwegian Food Safety authority, 2016). For saltwater, the concentration of NO_2-N can be higher, due to protection from the Cl^- ions (Jensen, 2003).

Nitrification includes two steps; first TAN is oxidized to NO_2^- (Eq. I) by ammonia oxidizing bacteria (AOB), in the second step NO_2^- is oxidized to NO_3^- (Eq.II) by nitrite oxidizing bacteria

(NOB). Recently it has also been found nitrifying bacteria that does complete ammonia oxidation (comammox) from ammonia to nitrate (van Kessel et al., 2015). Incomplete nitrification may occur resulting in accumulation of NO_2^-. It is therefore important that both the nitrification steps take place simultaneously.

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad \text{(I)}$$

$$NO_2 + 0.5\ O_2 \rightarrow NO_3^- \quad \text{(II)}$$

The nitrifiers are autotrophic bacteria and grow on surfaces in the aerated biofilters (Schreier et al., 2010). Heterotrophic bacteria are also abundant in the biofilters where they utilize organic matter, usually DOC which is most available for them (Blancheton et al., 2013). They compete with the autotrophs for space and oxygen, and their maximum growth rate is typically higher than the nitrifiers (Zhu and Chen, 1999). Therefore, it is important that the DOC/TAN ratio in the water reaching the biofilter is low, otherwise the heterotrophs may outcompete the nitrifiers and impair nitrification efficiency (Michaud et al., 2006, Guerdat et al., 2011, Navada et al., 2020a). However, there are some contradictory results, where high C/TAN ratios have shown not to affect nitrification negatively (Bovendeur et al., 1990, Fernandes et al., 2015). Even though the importance of organic matter effects on nitrification is well studied, we still lack knowledge on the dynamics of C and N in RAS in relation to operational choices, and to what extent the different forms of these elements impact nitrification efficiency.

The content of feed for salmonid fish contain around 5.6 to 8 % nitrogen (Dalsgaard and Pedersen, 2011, Ytrestøyl et al., 2015). It has been shown that 49% of the ingested nitrogen is retained in the fish in farmed trout (Dalsgaard and Pedersen, 2011) and 38% in adult salmon (Wang et al., 2012). Of the ingested feed is 37-53% estimated to end up as TAN (Piedrahita, 2003, Drennan et al., 2006, Terjesen et al., 2013) in salmon farming in RAS. Loss of N to faeces and feed waste have been estimated to equal 8-19% (Piedrahita, 2003, Reid et al., 2009). Thus, out of the total N input from feed, 51-62% end up in the water as waste compounds in RAS.

The moving bed biofilm reactor (MBBR) is a biofilter that is suitable for nitrification in RAS. It has been successfully used for treating municipal and industrial wastewater (Ødegaard et al., 1994), and is also a common biofilter in aquaculture systems. The MBBR has small plastic

carriers on which the bacteria can grow. The biofilm carriers move freely in the water column of the reactor with the help of aeration or mechanical mixers. Ammonia conversion can be expressed with equation III. This equation assumes that no other substrate than S is limiting.

$$R = \frac{R_{max}\ S}{K_S + S} \qquad \text{(III)}$$

R: substrate removal rate (g m^{-2} d^{-1}), R_{max}: maximum substrate removal rate (g m^{-2} d^{-1}), S: substrate concentration (mg L^{-1}), K_S: half saturation constant (mg L^{-1}). At high substrate concentration, the process is a zero-order expression, and normally oxygen is the rate limiting substrate. TAN is the limiting substrate at low concentrations, and in RAS the concentration is low compared to wastewater treatment systems. Therefore, in RAS, TAN is typically the rate limiting substrate and the reaction approximates a 1st order process (Rusten et al., 2006). This impacts the nitrifying community dynamics in the biofilm, and also to what extent the C/TAN-ratio affects the nitrification kinetics (Guerdat et al., 2011, Navada et al., 2020a). We do, however, need to better understand the capacity and functionality of biofilters for handling nitrogen and carbon.

The biofilm on the carriers in e.g. MBBRs, are composed of bacteria embedded in a dynamic matrix of extracellular polymeric substances (EPS). The major components are microbial cells and cell debris, polysaccharides, water and excreted cellular products (Sutherland, 2001). The AOBs and NOBs are slow growing bacteria with limited abilities to form biofilm due to lack of EPS production (Bassin et al., 2012b). In contrast, the heterotrophic bacteria are typically fast-growing and excrete EPS which facilitates the formation of biofilm and attachment of bacteria in the matrix (Tsuneda et al., 2001). Therefore, during start-up of a nitrifying reactor, both heterotrophic and nitrifying bacteria are needed to create a biofilm matrix suitable for nitrification. It has been shown that brackish water nitrifying biofilms (20‰ salinity) have higher tolerance to increased salinity than freshwater biofilms (Gonzalez-Silva et al., 2016). This suggests that start-up of a biofilter in brackish water could be a strategy for systems with post-smolt production with variable salinity requirements. Some studies suggest that biofilm formation takes longer time in seawater compared to in fresh water, and that the nitrification capacity is lower (Nijhof and Bovendeur, 1990, Rusten et al., 2006) due to more energy use for osmoregulation. To speed up the process, the use of matured biofilm carriers with salinity-

adapted bacteria can be used to reduce the start-up time (Sudarno et al., 2010). However, due to the high focus on biosecurity in RAS, the use of inoculum and matured biofilm carriers may not be preferred, and clean virgin carriers with synthetic wastewater during start-up are used. Knowledge on the time needed for start-up in brackish water with virgin carriers and nitrification capacity is needed, to assure efficient biofilters for potential post-smolt production at higher salinity.

2.8 Salinity effects on bacteria

Organisms that live in high-salinity environments must be able to balance the cellular osmolarity with the surroundings (Oren, 2011). Osmotic stress on non-adapted bacteria will cause an outward flow of intracellular water, leading to dehydration and eventually cell death (Csonka, 1989). For the anadromous salmon that naturally adapts to higher salinity, the processes are well known during the freshwater to seawater transition. For the bacteria in RAS however, knowledge on the adaptation and/or succession of new species during environmental shift to higher salinity is still limited. Nitrification efficiency in freshwater biofilms decrease after abrupt salinity changes as the nitrifying bacteria are inhibited by osmotic stress (Gonzalez-Silva et al., 2016, Kinyage et al., 2019). Increasing the salinity in a RAS can cause accumulation of toxic concentrations of TAN and NO_2-N, and potentially lead to mortality of fish. Studies have shown that the nitrification process/efficiency can recover after or during increased salinity (Bassin et al., 2012a, Quartaroli et al., 2017, Navada et al., 2019). Seawater could therefore be introduced to the RAS used for freshwater cultivation of juveniles to avoid desmoltification and improve salmon growth. Another option is to move the fish to a separate high salinity RAS. However, this may not be preferred as it involves moving the fish, potentially causing stress and reduced growth. It is not clear how much of the bacterial community dynamics that is a physiological salinity adaptation process relative to succession causing change in community structure and introduction of new species. More knowledge is needed to understand the bacterial adaptation and the functionality of both the heterotrophic and nitrifying bacteria during the transition from freshwater to seawater in RAS.

Chapter 3: Research Methods

This chapter presents the experimental studies that were conducted to address the objectives for this thesis. The experimental set-ups are summarized, and a description of Illumina sequencing and our interpretation of sequencing data is given. The other analytical methods used are briefly presented, and more thorough descriptions of these methods are in the papers.

3.1 Experimental studies

3.1.1 Experiment 1 – Variable loading of organic matter in RAS with membrane filtration

The first experiment evaluated the effects of improved particle removal with membrane filtration in RAS on concentrations of organic matter and its consequences for water quality and microbial conditions. This experiment was furthermore used to make a carbon and nitrogen budget for RAS with and without membrane filtration. The experiment was conducted at Sealab, NTNU's Centre of Fisheries and Aquaculture in Trondheim, Norway. The experimental system with fish tanks is shown in Fig. 3.

The study was part of the Sintef Ocean project "Water treatment technology for recirculating aquaculture systems to increase efficiency by reducing the negative effects of organic matter (RAS-ORGMAT)". Two pilot-scale RAS with Atlantic salmon parr were compared, one system with membrane filtration (mRAS) and one conventional RAS (cRAS). The water exchange rate in both systems were manipulated equally to induce periods of high/increasing and low/decreasing concentrations of organic matter. The effects of variable organic matter loads on the physicochemical and microbial water quality with and without membrane filtration were studied in **Paper I**. In **Paper II**, we did a mass balance of carbon and nitrogen entering with the feed and estimated the amount of output as fish biomass and waste compounds

released to the systems. Furthermore, we studied the removal of different forms of C and N waste products (POC, DOC, PON and TAN) through the physical and biological water treatment processes. How variations in concentration of the different forms of C and N affected nitrification efficiency and the nitrifying community composition in the biofilters were also elaborated.

Fig. 3. Research facility at NTNU Sealab. This picture shows the fish hall. 12 of the tanks are connected to two (6 fish tanks each) and separate RAS water treatment loops (in a separate room) and were used in this study. Photo: Sintef Ocean

3.1.2 Experiment 2 – Salinity increase regimes in RAS

The second experiment addressed the effects of two different regimes for salinity increase in commercial-scale RAS with Atlantic salmon, following transfer to sea cages. The experiment was conducted at the Let Sea RAS facility on Dønna, in Nordland county. Salmon parr were stocked in a low salinity brackish water RAS (bRAS) at 3‰ salinity. After the fish had smoltified, half the group remained in bRAS and the other half was moved to a high salinity brackish/seawater RAS (sRAS) at 25‰. In bRAS, the salinity was increased from 3 to 26‰ over a period of 28 days, and in sRAS the salinity was increased to 28‰ and remained constant

for the same period. Afterwards, the post-smolts were transferred to two separate sea cages. We studied how the different salinity regimes affected the heterotrophic and nitrifying bacteria, the nitrification kinetics in the biofilter and the gut microbiota of the fish. Nitrification capacity tests at different salinities were performed on biofilter media from bRAS, to evaluate short term robustness of the biofilter to salinity changes (Fig.4 bottom). We documented fish growth and mortality throughout the land phase in RAS and the sea cage phase until slaughter. **Paper III** is based on work form this experiment.

Fig. 4. Anette Bugten sampling faeces from the fish for microbial analysis (top left) and Kristian Nordøy netting fish for sampling (top right). Capacity stress-tests of biofilter carriers with different salinities (bottom). Photos: R.O. Fossmark.

3.1.3 Experiment 3 –Start-up of nitrifying biofilms in brackish- and freshwater

The third experiment investigated the start-up of nitrifying biofilms in freshwater and brackish water in semi-commercial scale MBBR biofilters. The experiment was conducted at the Nofima Centre for Recirculation in Aquaculture at Sunndalsøra. The experiment was conducted in two MBBRs with freshwater (F, 0‰ salinity) and brackish water (B, 12‰ salinity). The start-up was monitored over 60 days. The reactors had virgin carriers and were fed synthetic wastewater, containing sucrose as carbon source and ammonium chloride (NH_4Cl) and sodium nitrite ($NaNO_2$) as nitrogen sources. The development of the nitrifying community assembly in the biofilm was compared in the F and B reactors. Capacity stress tests were conducted in smaller reactors with carriers from the B and F MBBRs, to determine maximum oxidation rates of ammonia (AOR_{max}) and nitrite (NOR_{max}) at the different salinity maturations. **Paper IV** is based on work from this experiment.

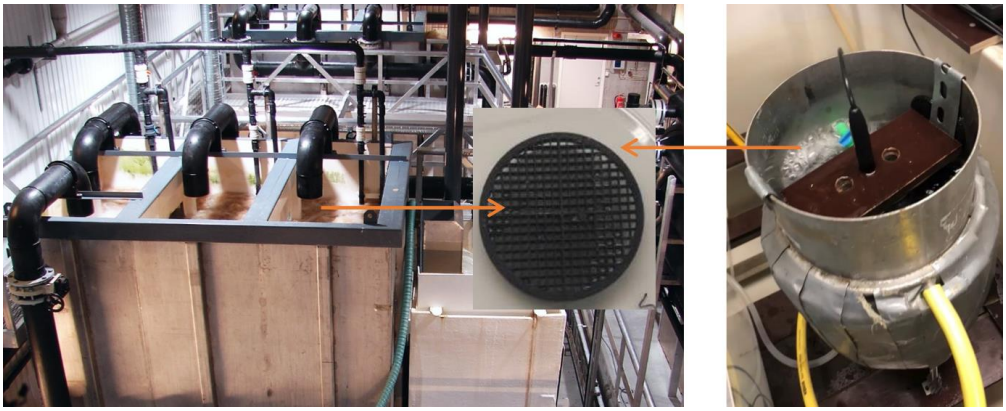

Fig. 5. Semi-commercial MBBR at Nofima Sunndalsøra (left), photo: Nofima. Small-scale reactors (right), photo: R. Fossmark. The biocarriers (centre) are AnoxK™ Chip P (Krüger Kaldnes AS, Norway).

3.2 Methodology

In all the experiments for this thesis, Illumina sequencing of a part of the bacterial 16S rRNA gene was performed on DNA-samples from RAS water, biofilter biofilm and/or fish gut content samples. Taxonomical libraries were constructed from the sequencing results, which showed the relative abundance and diversity of the bacterial communities present in the samples.

3.2.1 Illumina sequencing of the 16S rRNA gene for bacterial community composition

During the last decades, techniques for studying microbial community composition have evolved from culture-based methods to high-throughput DNA sequencing, like Illumina sequencing (Goodwin et al., 2016). This has made it possible to study microbial communities at a much higher resolution and accuracy, as culture based methods are time-consuming and biased under laboratory conditions (McCaig et al., 2001). The most common approach to investigate microbial community compositions, is by sequencing a part of the 16S rRNA gene which is amplified by PCR. The 16S rRNA gene is highly suitable to investigate microbial community compositions, diversity and phylogenetic relationships (Di Bella et al., 2013). The gene encodes the small ribosomal sub-unit and is a key element in the protein-synthesizing machinery and universally present in all bacteria. The gene is functionally preserved through evolution, and rarely subjected to horizontal gene transfer (Olsen et al., 1986). The DNA sequence of the gene contains conserved and highly variable regions. The conserved regions can be used to design universal broad-range bacterial primers to target all bacteria in the sample for PCR analysis, whereas the variable regions are used to gain taxonomic information (Clarridge, 2004). Databases of microbial rRNA gene sequences, e.g. Ribosomal Database Project, RDP (Cole et al., 2013) and Microbial database for activated sludge, MiDAS (Nierychlo et al., 2019), are used to classify the 16S rDNA sequences at various taxonomic levels. There are some drawbacks when studying bacterial communities based on the 16S rRNA-gene. Different taxa can have different copy numbers of the rrn operon (Farrelly et al., 1995), causing some bacteria to be overrepresented in 16S rRNA gene analysis. When sequencing the 16S rRNA gene, the bacteria are typically not identified at species level, as the length of the regions targeted for sequencing are short (in base pairs) and the 16S gene sequence

does not contain sufficient information to separate all sequences at species level (Fox et al., 1992). Therefore, genus is generally the highest taxonomic classification obtained. Some caution should therefore be taken when using sequencing of 16S rRNA and interpreting the results. Notwithstanding, Illumina is state of the art, and is very efficient in terms of bases sequenced per amount of time and cost at high resolution.

3.2.2 Diversity of bacterial communities

The bacterial diversity of a given environment can be quantified by the amount of variation in the bacterial communities present. Species richness and evenness are typical assessments of bacterial diversity, and high values reflect diverse bacterial communities. Species richness is the number of different species present in sample, and the species evenness describes the variations in species abundance. The alpha (α) diversity refers to the diversity of a defined unit, sample or habitat and is often represented by species richness, evenness and/or the Shannon's diversity index (Di Bella et al., 2013). The Shannon index (Shannon and Weaver, 1949) includes both species richness and their relative abundance in a sample. High values reflect communities with greater species richness and evenness, whereas lower values reflect communities with fewer species and/or low equality of abundances. The diversity between samples is beta (β) diversity, and can be described/quantified by how many shared species two samples have (e.g. presence/absence-based Sørensen-Dice similarity) and the relative abundance of the shared and not shared species (e.g. abundance-based Bray-Curtis similarity) (Chao et al., 2006).

3.2.3 Measurements of water quality

Measurements of the water quality in the experimental studies were undertaken. To quantify bacterial numbers and growth in RAS water, flow cytometry analysis and [^{3}H]-thymidine incorporation into DNA, respectively, was done. Concentrations of TOC, DOC, POC, PON, TAN, NO_2-N, NO_3-N and turbidity were measured to evaluate water quality, nitrification efficiency and to elaborate the dynamics of C and N and output of the different forms of C and N in RAS. Details on all these measurements can be found in the papers.

Chapter 4: Summary of papers

The three experimental studies resulted in 4 scientific papers, 3 of them published in international peer-reviewed journals. This chapter presents the main findings.

4.1 Paper I: *Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (Salmo salar).*

In this study two pilot-scale RAS were compared, one system with membrane filtration (mRAS) for enhanced removal of particles, and one conventional RAS (cRAS). The water exchange rate in both systems were manipulated equally to induce periods of high/increasing and low/decreasing loads of organic matter. The consequences of varying organic matter concentrations on water quality and microbial conditions were evaluated.

The results showed that in the system with membrane filtration, the concentrations of organic matter were more stable throughout the experiment for the changing organic matter loadings. As a consequence, the water in mRAS had higher bacterial diversity, lower and shorter bacterial blooms and generally lower bacterial densities than in cRAS (Fig. 6). All these variables indicate a better microbial water quality with more stable organic matter loadings through enhanced removal of particles. The physicochemical water quality was also better in mRAS in terms of lower turbidity and concentrations of POC. The average weight of the fish at the end of the experiment in mRAS was 14% and significantly higher than in cRAS, and mRAS produced more fish biomass in total. The temperature in mRAS was 1.2 °C higher than in cRAS due to membrane operation but could not explicitly explain the better growth of the fish in mRAS. The mortality was low and the same in both systems (<1%), and we did not

detect any potential pathogenic bacteria in high relative abundance. The study showed the positive effects of enhanced removal of particles and organic matter, both for the microbial and physicochemical water quality with positive consequences for fish growth.

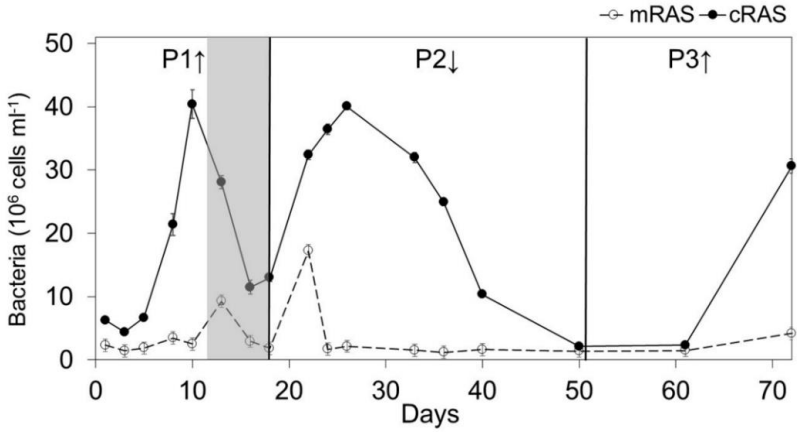

Fig 6. Bacterial densities in RAS. Data are the mean (±SD) from all samples in the same system each sampling day. Grey shading shows extra addition of water due to technical problems (**Paper I**: Fossmark et al., 2020)

4.2 Paper II: *Mass balance of carbon and nitrogen, and nitrification efficiency in recirculating aquaculture systems (RAS) with and without membrane filtration for Atlantic salmon parr (Salmo salar)*

In the same pilot-scale RAS as in **Paper I** with mRAS and cRAS, we did a mass balance of carbon and nitrogen entering with the feed and estimated the amount of output as fish biomass and waste compounds released to the systems. Furthermore, we studied the removal of different forms of C and N through the physical and biological water treatment processes, and how accumulating concentrations of different forms of C and N affected nitrification efficiency and the nitrifying community composition in the biofilters.

Our mass balance showed that out of total input C, 27-30% and 22-24% in mRAS and cRAS, respectively, were retained as fish biomass. For total input N, the fraction retained as fish biomass was 19-22% in mRAS and 16-17% in cRAS. The fish in mRAS ingested more feed and grew better (as presented in **Paper** I) and more of the input feed was therefore retained as

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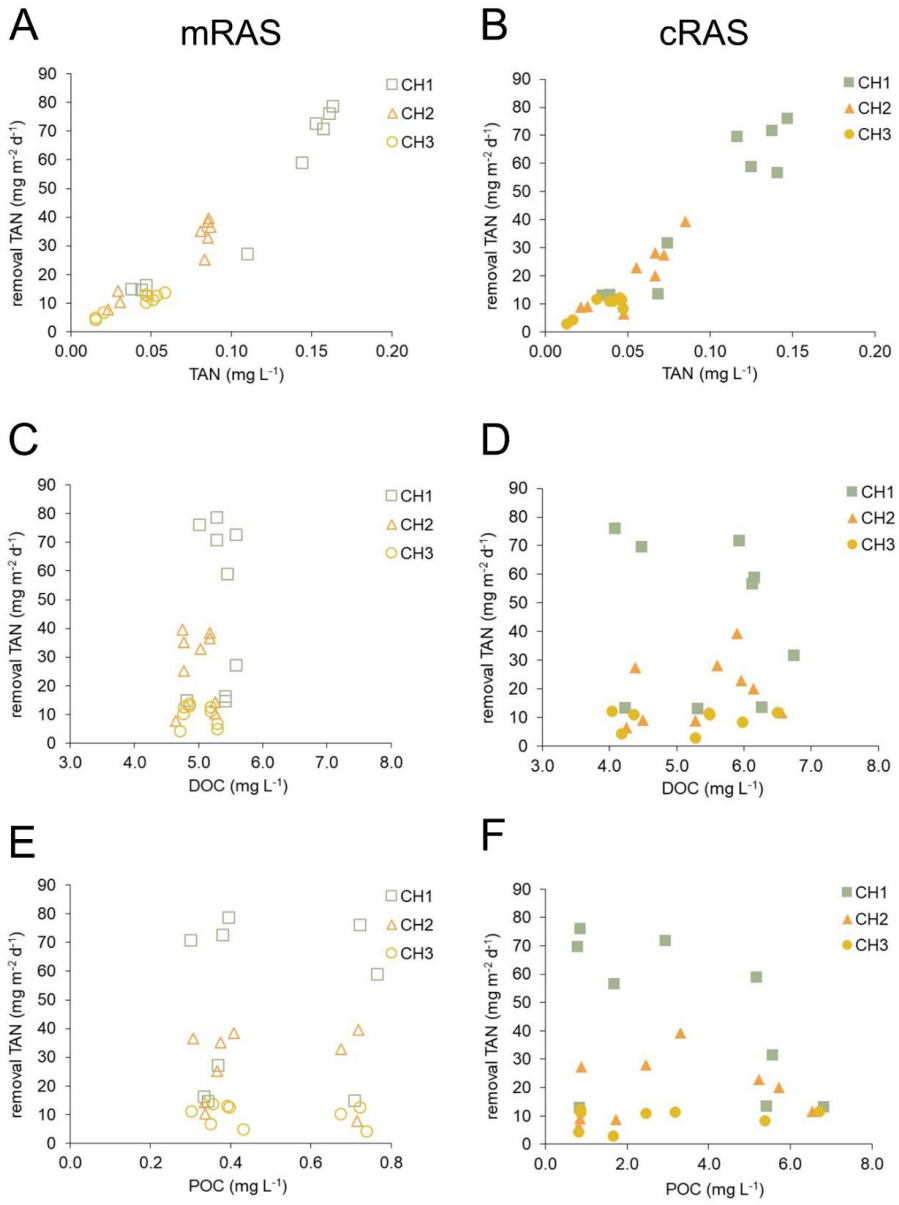

Fig. 7. TAN removal rates versus TAN, DOC and POC concentrations in each chamber (CH) of the MBBRs. Empty symbols show mRAS and filled symbols cRAS. Notice the different x-axes in E and F. (**Paper II**: Fossmark et al., *in prep*)

biomass in mRAS. The feed waste was high in both systems, but higher in cRAS due to less ingestion of feed. In mRAS the feed waste constituted 22-26%, and in cRAS 37-60% of total feed. The membrane removed 2 to 3% of total N input, and 3 to 4.5% of total C input. Thus, both the removal of C and N through membrane filtration and less feed waste resulted in less accumulation of POC, DOC and PON in mRAS, giving better water quality and fish growth.

The MBBRs removed 60 to 84% of the TAN produced by the fish, and 6 to 13% of total C input as DOC in both systems. The removal of DOC by heterotrophic bacteria in the biofilter accounted for a notable fraction of total C input. The concentrations of POC were up to 15 times higher in cRAS than in mRAS at low water exchange rate, however the nitrification efficiency was not affected negatively (Fig.7E and F). The relative abundance of nitrifying bacteria in the biofilter biofilm was 2 times higher in the mRAS MBBR than in the cRAS MBBR after the period with high concentrations of POC. In conclusion, this study showed a mass balance of the input C and N through fish feed, and that the better water quality in mRAS resulted in higher appetite of the fish than in cRAS. As a consequence, there was more particle production in cRAS due to more feed waste and less removal of particles due to no membrane filtration. Despite the fact that POC concentrations were up to 15 times higher in cRAS than in mRAS, the nitrification efficiency was not significantly different between the systems.

4.3 Paper III: *A comparison of two seawater adaptation strategies for Atlantic salmon post-smolt (Salmo salar) grown in recirculating aquaculture systems (RAS): Nitrification, water and gut microbiota, and performance of fish*

Two adaptation strategies for salinity increase in production of Atlantic salmon post-smolt in commercial-scale RAS were studied. One regime was a gradual increase in salinity from 3 to 26‰ over a period of 28 days in a brackish water RAS (bRAS) with post-smolts. The other was a direct transfer of post-smolts from a low (3‰) to a high salinity (25-28‰) brackish/seawater RAS (sRAS). Afterwards both fish groups were transferred to two separate sea cages and monitored until slaughter.

We observed a succession in the bacterial communities in the water of the system with increasing salinity (bRAS) which was driven by both salinity and fish biomass/feed load in the

system. The change in microbial community structures in bRAS was higher when increasing the salinity from 6 to 12‰, than increasing it from 12 to 26‰. Around half of the OTUs present in the water at 3‰ adapted to the higher osmotic stress during the salinity increase to 26‰ in bRAS. The same dominating nitrifying OTUs were found in the biofilters in bRAS (throughout the salinity increase) and in sRAS. In bRAS, the concentrations of TAN and NO_2-N accumulated during the salinity increase from 3 to 12‰. However, when the salinity was increased further from 12 to 26‰, the nitrification efficiency rose and there was no accumulation of TAN and NO_2-N. The capacity tests showed that when the biofilm carriers in bRAS were stressed with higher or lower salinity than the native bRAS salinity, the AOR_{max} was inhibited by 25 to 40% (Fig. 8). The degree of inhibition and lag-phases depended on the magnitude of osmotic stress in relation to the native bRAS salinity in the stress test reactors with both higher and lower salinities.

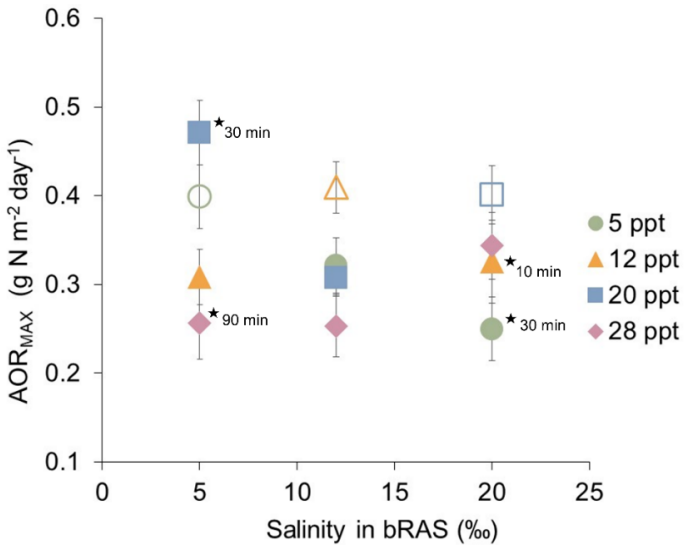

Fig. 8. Maximum ammonia oxidation capacity (AOR_{max}) ±SE of biofilter carriers from bRAS during capacity stress tests in reactors with 5, 12, 20 and 28‰ salinity (legend). Empty symbols show reactor with native bRAS salinity. Black stars show reactors with a lag-phase in the beginning of the stress test, and with indication of the length of the lag-phase. A stress test at native salinity of 28‰ was not conducted (**Paper III**: Fossmark et al., 2021).

The faecal microbiota of individual fish in bRAS evolved to become more similar to sRAS individuals as the salinity was increased. Thus, salinity was a driver of succession in the gut microbiota as well as in the RAS water. Both fish groups were diagnosed with Cardiomyopathy syndrome (CMS), causing high mortality in both groups. The fish in bRAS had a lower mortality than the fish in sRAS, but the sRAS group grew better both on land and in the sea cages. It was therefore hard to conclude whether one salinity adaptation regime was better than the other for the fish. Even though there was accumulation of TAN and NO_2-N in bRAS during the salinity increase, the average concentrations of TAN and NO_2-N in the constant high salinity sRAS were still significantly higher, but below the concentrations to avoid toxicity for the fish given by the Norwegian Food Safety Authority. Thus, the different nitrification efficiencies in the two systems probably did not impact the fish growth and mortality. We concluded in this study that salinity is a driver of succession in RAS, and that other factors such as organic loading and stochastic processes in the host also affect the microbial community compositions. We showed the possibility of increasing the salinity in an operating RAS without exceeding toxic concentrations of TAN and NO_2-N, at least if the system has been exposed to higher salinity before.

4.4 Paper IV: *A salty start: Brackish water start-up as a microbial management strategy for nitrifying bioreactors with variable salinity*

This experiment was conducted in two semi-commercial MBBRs with freshwater (F, 0‰ salinity) and brackish water (B, 12‰ salinity). The nitrifying community succession and nitrifying capacities of the reactors were compared. The start-up was monitored over 60 days.

We observed that after 60 days of MBBR start-up, the brackish water biofilm had half the nitrification capacity of the freshwater biofilm during stress-tests, with less diverse microbial communities, lower proportion of nitrifiers, and significantly different nitrifying community composition. However, low ammonia and nitrite concentrations with rapidly increasing nitrate concentrations indicated that complete nitrification was established in both reactors within 60 days (Fig. 9). Nitrite oxidation developed slightly slower in the B reactor than in the F reactor, and the diversity and relative abundance of nitrifiers in the intake freshwater was higher than in the intake seawater. The microbial community composition of the intake water sources were

more similar to the initial biofilm samples, than later in the succession. The results suggest that nitrifying biofilms developed in comparable time in brackish and freshwater. Brackish start-up can be a strategy for bioreactors with varying salinity, like in post-smolt production.

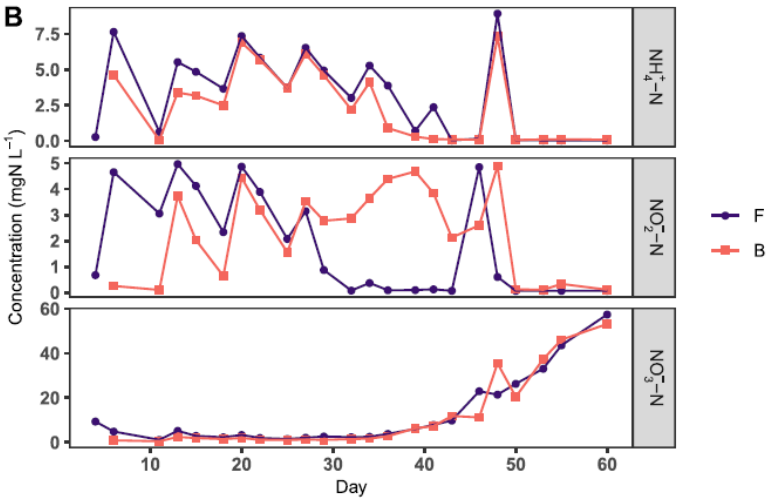

Fig. 9. Ammonia, nitrite and nitrate concentration in the freshwater (F) and brackish water (B) reactors during the study. The points have been connected to improve readability, but are not necessarily linearly related. Note the difference in the scales (**Paper IV**: Navada et al., 2020b).

Chapter 5: Discussion and conclusions

This final chapter provides a discussion of the main results and conclusive remarks. Recommendations for future work and perspectives are given at the end of the chapter

5.1 Effects of enhanced particulate organic matter removal in RAS

The first objective in this thesis was to obtain knowledge on enhanced particulate organic matter removal in RAS considering the effects on microbial community dynamics, general water quality and fish performance. In **Paper I**, we observed that the RAS with enhanced removal of particles had higher microbial diversity, lower and shorter bacterial blooms and generally lower bacterial densities in the water than in the system with only conventional particle removal. POM originating from feed waste and faeces is substrate for heterotrophic bacteria in RAS. Therefore, the concentration and degree of removal of POM is an important factor in determining the CC of the system, which in turn affects the microbial water quality. The enhanced removal of the small particles provided better conditions for K-selection, namely lower and more stable concentrations of organic matter reducing the substrate supply per bacterium (Attramadal et al., 2012a). Mature microbial communities dominated by K-strategists are predicted to have higher stability to environmental perturbations (De Schryver and Vadstein, 2014). We hypothesize that the more diverse microbial communities and lower and shorter bacterial blooms in the RAS with enhanced removal of particles, supports that microbially matured water can be achieved with membrane filtration or any removal of organic matter including small particles in RAS with juvenile salmon.

In **Paper III**, the change in organic loads in RAS affected the microbial community dynamics without any change in salinity. We observed that the increase in fish biomass (organic matter) decreased the bacterial richness, whereas a decrease in fish biomass increased the richness. Both **Paper I and III** therefore show that a lower organic loading increase the diversity of the

water microbiota. In **Paper III**, the Bray-Curtis similarities of the microbial community structures at the same salinity and change in fish biomass, was comparable to the Bray-Curtis similarity with 8.7 times salinity increase. Clearly, concentration and variation of organic matter have a large impact on the microbial community dynamics in RAS water.

In **Paper I**, the better microbial and physicochemical water quality with enhanced removal of particles did not affect the mortality of the fish. However, the fish had a 14% higher average end weight in the system with membrane filtration than the fish in the system without membrane filtration. The better fish growth in the system with better water quality could, however, also be attributed to higher temperatures in the water due to membrane operation. It is hard to conclude exactly how much of the improved growth of the fish that was attributed to the higher temperature compared to the better water quality, as it was probably a combination of both. Nevertheless, the better water quality and higher temperature led to better fish growth and a higher production of biomass at the end of the experiment. These results illustrates the potential of including membrane filtration for enhanced removal of particles in RAS to improve fish growth.

As one of the reasons why membrane filtration is rarely used in RAS are due to the high cost, the energy operating expenses (OPEX) for RAS and membrane filtration were studied through literature review. As discussed in **Paper I**, the energy OPEX of a RAS varies a lot, with studies estimating costs from 5.46 to 26 kWh kg^{-1} fish produced (Ayer and Tyedmers, 2009, d'Orbcastel et al., 2009, Summerfelt et al., 2009, Samuel-Fitwi et al., 2013, Liu et al., 2016, Song et al., 2019). It has been estimated that membrane ultrafiltration will cost 0.1-0.15 kWh m^{-3} of treated water (Pearce, 2008, Verrecht et al., 2010). Due to the high variability in estimated energy use in RAS, we could not conclude whether the cost of membrane filtration could balance the better water quality and fish health in our study. In our study, the fish were juvenile salmon parr, and previous research have shown improved growth and slightly higher survival of marine larvae in RAS when using membrane filtration (Holan et al., 2014a, Wold et al., 2014). Larvae are more fragile and susceptible to possible detrimental host-microbe interactions than larger fish. Therefore, the positive effects of better microbial water quality through membrane filtration could be more evident when the fish are more vulnerable at younger stages. The water flows will be substantially lower for hatcheries with larvae than juvenile and post-smolt salmon and affect the costs significantly. Thus, including membrane

filtration in the water treatment probably has a changing cost-benefit situation through the production cycle.

Whether membrane filtration is economically feasible in post-smolt production with higher organic loads and water flows, remains to be studied. We concluded in **Paper I** that the higher stability in physical, chemical and microbial water quality variables, indicate that the membrane filtration had a stabilizing effect and reduced the level and variation of the carrying capacity for bacteria. This is beneficial for commercial fish production in RAS which are subject to changes in organic loading due to introduction of new fish groups, weighing, vaccination, change of feed etc. through the production cycle. Enhanced particle removal will lower the change of bacterial related accidents, as the likelihood of bacterial blooms and reduced viability of the fish is lower.

5.2. Mass balance and dynamics of C and N in RAS

The second objective of this thesis was to elaborate the capacity and the functionality of biofilters for handling nitrogen and carbon, and the dynamics of C and N waste in RAS in connection to relevant operational choices. In **Paper II** we did a mass balance on the input C and N through the fish feed, to elaborate the outputs of C and N as fish biomass and waste compounds released to the systems. We showed in **Paper I** that the fish in the system with enhanced removal of particles and better water quality were significantly bigger than in the conventional system, due to better water quality and higher temperatures. The mass balance in **Paper II** that the membrane removed 2 to 3% of total N input, and 3 to 4.5% of total C input per day, reducing the fraction of input C and N ending up as particles in RAS. Thus, the membrane filtration directly removed compounds containing C and N, reducing concentrations of DOC, POC and PON which resulted in better water quality. The better water quality furthermore led to better appetite of the fish and as a consequence, this system had less feed waste and better fish growth than the system without membrane filtration. Feed is a significant cost for the salmon fish farmers, and an optimal FCR close to 1 is desired for good economic viability of the salmon production (Fry et al., 2018). The FCRs that were presented in **Paper II** were over 2 in both systems at normal feeding (2% of total biomass), thus there was more feed waste in our study than it should be at normal commercial operating conditions. The FCR

must be taken into consideration when doing cost-benefit analyses together with the OPEX of RAS as discussed in the previous section.

In **Paper II** we observed that the concentrations of DOC always were higher than POC in both systems (from 1.1 to 14 times higher) throughout the experiment. The high turbulence and water flows in the RAS caused much of the carbon to end up as DOC, which is more available for bacterial assimilation and energy source. The concentrations of POC were up to 15 times higher in the conventional system than in the system with membrane filtration at low water exchange rate, and DOC concentrations were only up to 1.2 times higher. The membrane clearly lowered the concentrations of particulate carbon, however, the effect on the dissolved fraction was less apparent as the concentrations of DOC were more similar between the systems. We saw in **Paper I** that there were significantly higher bacterial densities in the conventional system throughout the experiment, despite the little difference in DOC between the systems. Furthermore, the bacterial densities clearly correlated with the organic load in the systems. We hypothesize that the similar DOC concentrations between the systems and bacterial densities following the organic load, could be due to fast utilization of DOC by heterotrophic bacteria in the water and biofilter. In the experiment that **Paper I and II** were based on (Experiment I), we planned to measure BDOC, as this would show us how much of the carbon that was biologically degradable. Unfortunately, the quality of these measurements were not satisfactory for the manuscripts, and we could not use the results. Instead, we did a literature review to address the biodegradability of organic carbon in RAS. We found some studies which established that the dissolved organic carbon in RAS with rainbow trout (*Oncorhynchus mykiss)* is not easily biodegradable (Dalsgaard and Pedersen, 2011, Rojas-Tirado et al., 2017, Rojas-Tirado et al., 2018). This support the hypothesis that heterotrophic bacteria in the water and biofilter quickly consume the easily degradable DOC, and that the DOC we measured could have been the not easily biodegradable organic carbon. In **Paper II** we observed removal of DOC through the MBBRs, which added up to a notable fraction of total C input in the mass balance (6-13%) in both systems. We do not know where in the RAS the DOC was assimilated, and whether the DOC that was removed in the MBBRs was easily biodegradable or not easily biodegradable. If it was the latter, the mass balance probably should have had even more of the total C input removed as DOC, which was the easily biodegradable fraction quickly utilized that we did not measure.

We showed in **Paper II** that the MBBRs removed 60 to 84% of total TAN produced in both systems. Even though the concentrations of POC were up to 15 times higher in the conventional system than in the membrane system, the nitrification was not affected negatively. We assume that this was due to TAN limitation, and the linearity between ammonia oxidation and TAN concentrations (Chapter 4 Fig. 7A & B) showing typical 1st order reaction slopes that support TAN limiting conditions (Rusten et al., 2006). The relative abundance of nitrifying bacteria in the biofilter biofilm was also 2 times higher in the system with membrane filtration than in the conventional system after the period high concentrations of POC. The high concentrations of POC could have caused more heterotrophic growth in the biofilm, thus resulting in lower relative abundance of nitrifiers. In **Paper IV**, we observed that the MBBR with lower relative abundance of nitrifying bacteria (the brackish water MBBR) had half the nitrification capacity than the MBBR with higher relative abundance of nitrifying bacteria (freshwater MBBR) during stress tests. In **Paper II** the MBBR carriers were not subjected to stress-tests with non-limiting TAN conditions, however the results in **Paper IV** suggest that the MBBR in the system without membrane filtration would have had lower nitrification capacity. Maximum specific nitrification rates have shown to be proportional to fraction of nitrifiers in the biofilm community under non-limiting TAN conditions (Bassin et al., 2015). However, in commercial salmon production in RAS, the concentration of TAN must be below 2 mg L^{-1}, to avoid toxic concentrations for the fish (Norwegian Food Safety Authority, 2016). Thus, non-limiting conditions should not occur at normal operating conditions. The concentration of DOC and POC is therefore of less importance in RAS in salmon production in regard to nitrification efficiency if there is sufficient oxygen concentrations in the biofilter. However, we have shown that high concentrations of organic matter in general have a negative impact on the physicochemical and microbial water quality in RAS, affecting fish appetite and growth.

5.3 Salinity increase in RAS

The third objective of this thesis was to study how salinity increase affects bacterial succession, nitrification functionality and fish performance in RAS. Fish farmers using RAS for post-smolt production face the challenge of varying salinities in the production cycle. We have a lot of knowledge on how the salmon adapts to a life at higher salinity. However, we have less information on how the bacteria associated with the water, biofilter and fish in RAS adapt.

We observed in **Paper III** that the change in microbial community composition in the water during salinity increase in the brackish RAS was higher when increasing the salinity from 6 to 12‰ (salt concentration was increased with 6‰), than increasing it from 12 to 26‰ (salt concentration was increased with 14‰). We hypothesize that this indicate a threshold salinity for some of the bacterial populations between 6 and 12‰, and with consequences for succession. These results are in line with a previous study with hierarchical clustering of bacterial community composition similarities in the Baltic Sea (Herlemann et al., 2011). In that study, the microbial community samples clustered in three salinity ranges: 0 - 3.2‰, 4.6 - 7.7‰ and 10.5 - 30.9‰. Herlemann et al. (2011) concluded that the OTUs present at the brackish water localities were not specialised for brackish water, but adapted bacteria originating from marine and freshwater environments. Our results show similar clustering, in the two salinity ranges from 3 - 6‰, and 12 - 26‰. But we also saw that around half of the OTUs present in the water at 3‰ were able to adapt to the higher osmotic stress during the salinity increase to 26‰. This suggests that the most important threshold inducing the largest changes for the bacterial populations could be between 0 and 3‰, i. e. going from fresh to brackish water conditions. So how do the bacteria adapt to higher osmotic stress? When non-adapted bacteria are subjected to higher salt stress, the bacteria can accumulate osmolytes to protect/stabilize the proteins in the bacterial cell wall and raise the osmotic pressure in the cytoplasm to avoid plasmolysis (Arakawa and Timasheff, 1985). Osmolytes can stabilize enzyme function, and protect against salinity (Sleator and Hill, 2002). A salinity of 8.5‰ is considered isotonic, or physiological saline (He et al., 2017). Our results indicate that bacteria with better abilities of osmoregulation, potentially osmolyte accumulation, are selected for above 6‰ whereas the bacteria that do not are selected for below 12‰. Since we did not sample between 6 and 12‰, this threshold could be the isotonic salinity 8.5‰.

In **Paper III** we observed that the concentrations of TAN and NO_2-N accumulated during the salinity increase from 3 to 12‰. But afterwards, the nitrification efficiency rose, and no accumulation of TAN and NO_2-N was observed during subsequent salinity increase from 12 to 26‰. This shows indications of a threshold salinity around 12‰ for the nitrifying bacteria. However, in the biofilter biofilm it was the same dominating nitrifying OTUs throughout the salinity increase from 3 to 26‰ in the brackish water RAS and in the constant high-salinity/seawater RAS. It was therefore not a change in nitrifying community structures, but a

matter of time to adapt. When the brackish water RAS had reached 12‰ salinity, the nitrifying bacteria had adapted to a higher (than original 3‰ salinity) salinity environment, and they managed to adapt to 26‰ salinity without compromising nitrification efficiency. In commercial post-smolt production, it is no standard at which salinity to produce the fish, but many farmers typically do it from ~14 to 22‰ (Attramadal, K.J.K. 2021, personal communication). During production there could be situations when the salinity might need to be changed, e.g. due to changes in water distribution at the facility or temperature control. If a commercial RAS with post-smolt is operated at e.g. 16‰ salinity, our results showing no accumulation TAN and NO_2-N from 12 to 26‰ salinity increase, indicate that if it is necessary to use more seawater in the production giving a salinity up to 26‰, the nitrification efficiency should not decline. However, as discussed in **Paper III**, we hypothesize that the success of the salinity increase with no accumulation of TAN and NO_2-N from 12 to 26‰ was due to the salinity history in the systems. The RAS had been operated at higher salinities up to 20‰ before, and seawater priming has shown to improve salinity acclimatization (Navada et al., 2020c). The capacity tests in **Paper III** showed that when the carriers in the brackish water RAS were stressed with higher or lower salinity than the native salinity (the salinity that the system was operated with at the time of the stress-test), the AOR_{max} was in general lower than in the native salinity reactor. The inhibition of nitrification varied from 25 to 40%, depending on the stress level. This is lower than what other studies have found in freshwater (0‰) nitrifying reactors introduced to salt stress (97 to 100% inhibition: Gonzalez-Silva et al., 2016, Kinyage et al., 2019). The degrees of inhibition (25 to 40%) in our study are probably not critical in a commercial RAS, as the degrees of inhibition was during stress-tests at much higher concentrations of TAN than would be in a commercial RAS with limited TAN conditions. As the lag-phases were only 10-90 minutes, the nitrifying bacteria present must have adapted physiologically to the new salinity rather than being replaced by a nitrifier specialized for the new salinity. All of this indicates that the biofilter biofilms in the brackish water RAS in **Paper III** was embedded with nitrifying bacteria that could adapt to salinities in the ranges from 3 to 26‰, without severe loss of activity during stress-tests, although with a lag-phase.

We compared two seawater adaptation strategies and have until now discussed the effects on the bacteria in the water and the biofilter. But which strategy seemed to be better in respect to the fish? It has been hypothesized that a gradual succession in the gut microbiota is better for the fish health than sudden abrupt changes (De Schryver and Vadstein, 2014). In our study in

Paper III we hypothesized that a gradual salinity maturation of the fish gut microbiota was better than an abrupt salinity change. We observed that the faecal microbiota of fish in the brackish water RAS with increasing salinity evolved to become more similar to the fish in the seawater RAS. Thus, salinity drove the succession in the gut microbiota as well as in the RAS water. However, after transfer to the sea cages, the gut microbiota changed in both fish groups, and the similarity between the groups declined. We also observed that the water microbiota was very similar in the different fish tanks, but that this was not reflected in the faecal microbiota between individuals within a fish tank or between fish tanks within a system. This indicates that there are other factors than water microbiota involved in the assembly of the gut microbiota of Atlantic salmon smolt (Vestrum et al., 2020). We hypothesize that these factors could be selection and stochastic processes in the host, and stress caused by the transfer to the sea cages. As discussed in **Paper III,** the brackish water RAS fish group had a lower mortality than the seawater RAS fish group, however the latter fish group grew better both on land and in the sea cages. Both fish groups were also diagnosed with Cardiomyopathy syndrome (CMS), causing high mortality. It was therefore hard to conclude whether one salinity adaptation regime was better than the other for the fish. Nevertheless, we have shown that it was possible to successfully increase the salinity in an operating RAS with fish, which we hypothesize was due to the salinity history and halotolerant nitrifying bacteria embedded in the biofilm. Salinity has shown to be a driver for succession of bacterial communities in RAS, along with other factors such as organic loading in the RAS water and stochastic processes in the host. We showed indications of a threshold salinity with consequences for succession in the RAS water microbiota between 6 and 12‰, which we hypothesize is the isotonic salinity 8.5‰. The bacterial community development in the RAS water was a combination of a physiological salinity adaption process and succession causing change in community structure and introduction of new species.

5.4 Start-up of fresh and brackish water MBBRs

We have now discussed that nitrifying biofilms with some history of osmotic stress are more robust to salinity changes than biofilms that have only experienced freshwater. This implies that bacterial succession in an environment with salinity forge a halotolerant biofilm that more easily adapt to changes in salinity. We saw in **Paper III** the effects of salinity increase on

nitrification in biofilters that were mature and had been exposed to osmotic stress earlier. The fourth and last objective of this thesis was to study and compare the nitrifying bacterial succession and capacity in biofilter biofilm during start-up of an MBBR with virgin carriers in freshwater and brackish water. It is generally assumed that start-up in seawater takes much longer time than in fresh water. In **Paper IV** we found the that biofilms develop nitrification in brackish water in equivalent time as in freshwater, and considerable nitrification was obtained in both reactors within the 60 day start-up. However, the brackish water biofilm had half the nitrification capacity of the freshwater biofilm (during stress-tests) with less diverse microbial communities, lower proportion of nitrifiers, and a significantly different nitrifying community composition. In both **Paper III** and **IV**, the biofilters were colonized by bacteria in the inlet freshwater and seawater, but in different ratios to achieve their respective salinities. Thus, it was freshwater and seawater bacteria that colonized both systems/reactors, and they adapted to the salinity that they were subjected to in the RAS/MBBRs. In both **Paper III** and **IV**, the seawater was treated with UV upon entry to the facility (the freshwater was not, this is due to Norwegian regulations), thus the number of bacteria able to colonize the systems were reduced in the seawater. In **Paper IV** the microbial communities of the intake seawater was less diverse than the intake freshwater, which could be related to why the brackish water biofilm had a less diverse microbial community composition and less nitrifiers. However, the brackish MBBR was at 12‰ salinity, thus it received more freshwater (with higher diversity than seawater) than seawater. Therefore, bacteria from both water sources colonized the brackish reactor, and the freshwater reactor was only colonized by bacteria from one water source. We hypothesize that the lower diversity and relative abundance of nitrifiers in the brackish MBBR biofilm could be due to possible cell plasmolysis of the freshwater bacteria mixing with the seawater. As a consequence, less bacteria from the freshwater were able to colonize the carriers in the brackish MBBR, and to furthermore be implemented in the biofilm. In **Paper IV** the dominant AOBs and NOBs established in the brackish MBBR biofilm were also detected in both intake water sources (and they were also detected in the freshwater MBBR biofilm at low relative abundance). In **Paper III**, we found the same dominating nitrifying OTUs in the brackish and seawater RAS biofilters. In **Paper III** we did not sample the intake water of the systems, however the results in **Paper IV** indicate halotolerant nitrifying bacteria in the fresh and seawater sources.

None of the start-up MBBRs in **Paper IV** were introduced to water with higher salinity afterwards to determine whether the nitrification efficiency would have been sufficient at commercial post-smolt operating conditions. Thus, we do not know if the lower nitrification capacity and lower relative abundance of nitrifiers in the brackish MBBR would have any consequences when introduced to a commercial RAS with fish. Using a freshwater reactor would probably have a much higher inhibition of nitrification than a brackish water reactor (Gonzalez-Silva et al., 2016) when introduced to water with higher salinity. Start-up of biofilters used for post-smolt production should therefore be done in brackish water, and we have shown it is possible to do so in comparable time as in freshwater.

5.5 Future work and perspectives

Enhanced removal of particles and organic matter in RAS have shown to improve both the physicochemical and microbial water quality. We hypothesize that if the fish in the system with better particle removal had been challenged, they would have managed better than the fish in with system with only conventional particle removal, due to the better water quality and more stable rearing conditions. To determine whether the better microbial water quality would suppress opportunistic blooms, experiments with addition of known opportunistic bacteria to controlled small scale systems should be done, to see how the fish and microbial community dynamics would be affected. This could determine if the low variation in organic matter and low CC in RAS would tolerate a potential disease outbreak better and give more support to the use of water treatment technology for improved particle removal.

As more advanced water treatment for fine solids removal adds high additional cost to the RAS, the benefits must be weighed against profits for better growth of the fish and/or less mortality. The energy OPEX for membrane filtration is mostly based on the amount of water treated. With 10-15% of the recirculation flow treated as we did **Paper I**, we clearly saw positive effects both for the physicochemical and the microbial water quality. More experiments with different percentages of the recirculation flow treated should be conducted. And as we discussed in section 5.1, more experiments at different life-stages of the salmon should also be done, following cost-benefit analyses.

We have shown the possibility of increasing the salinity in an operating RAS with fish that has history of osmotic stress (**Paper III**). Future studies should elaborate whether a RAS with a history of operation only at 0-3‰ salinity (and not up to 20‰ as in our experiment) would tolerate an increase in salinity. More information of the microbial community succession during salinity increase from 0 to 3‰ should also be elaborated, as this range seems to be an important threshold for the bacterial populations. Most RAS in salmon production today are operated with some salinity (~1-3‰) also at the freshwater production stages to prevent growth of fungi and to protect against nitrite (Holan, A.B. 2021, personal communication). Thus, few RAS in salmon production today are strict freshwater systems and the range from 0 to 3‰ might not be that relevant in salmon production. Notwithstanding, it would be interesting to study the microbial community succession from strictly freshwater to brackish water conditions.

In **Paper IV** the salinity adaptation in the brackish water was at 12‰, but post-smolt production can be at higher salinities. More experiments should be done to determine if the biofilms develop in comparable time at higher salinities than 12‰. The start-up MBBRs were not introduced to water with higher salinity afterward to determine whether the nitrification efficiency would have been sufficient at commercial post-smolt operating conditions. Future studies should elaborate the robustness of the biofilters after start-up, when they are introduced to systems with fish and variations in salinity.

Our results have given us a better understanding of the microbial challenges in RAS related to variations in organic matter and salinity. This knowledge contribute towards optimizing the land-based production of Atlantic salmon smolt and post-smolt.

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Appendix A: Publications

Paper I

Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (*Salmo salar*).

Ragnhild O. Fossmark, Olav Vadstein, Trond W. Rosten, Ingrid Bakke, Deni Košeto, Anette V. Bugten, Gaute A. Helberg, Jenny Nesje, Niels O.G. Jørgensen, Gema Raspati, Kamal Azrague, Stein W. Østerhus & Kari J.K. Attramadal. 2020.

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Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (*Salmo salar*)

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ABSTRACT

A key challenge in recirculating aquaculture systems (RAS) is the accumulation of particulate organic matter, especially the fine and colloidal fraction due to low removal efficiency of today's technology. The supply of organic matter is typically the limiting resource determining the carrying capacity (CC) of heterotrophic bacteria in the system. An appropriate and stable CC is proposed as a strategy for an optimal microbial environment in RAS with less blooms of opportunistic bacteria and more stable community dynamics. In this study, we investigated the effects of including a membrane for ultrafiltration in the RAS water treatment loop (treating 10–15% of the total water flow) to reduce the amount of fine and colloidal organic matter. Atlantic salmon parr (*Salmo salar)* were reared in two pilot-scale RAS (mRAS: membrane, cRAS: conventional). To evaluate the bacterial dynamics with and without membrane filtration at different organic loadings, the water exchange rates of the systems were manipulated equally to create periods with high and low loading of organic matter. The results showed that in the mRAS water, the level of organic matter was more stable throughout the experiment for the changing organic matter loadings. As a consequence, water in mRAS had higher microbial diversity, lower and shorter bacterial blooms and generally lower bacterial densities than in cRAS. All variables indicate a better microbial environment in the water of the system with membrane filtration. Also, the physicochemical water quality was better in mRAS in terms of lower turbidity and particulate organic matter (POC), and slightly lower concentrations of total ammonia nitrogen (TAN). The composition of the microbial communities was significantly different between the two systems, and temporal variations in the community dynamics were observed in both systems during the periods with different organic loadings. At high organic loading, the genus *Mycobacterium* had high relative abundance in the cRAS water (up to 0.25) compared to mRAS (0.01–0.03). The fish in mRAS were significantly bigger (14%) than fish in cRAS at the end of the experiment, however it is hard to conclude whether the better growth in mRAS was due to higher temperatures (caused by membrane operation) or better water quality, as it was probably a combination of both. We can conclude that membrane filtration gave more stable and better physicochemical and microbial water quality, which will reduce the probability for microbially related accidents in RAS.

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1. Introduction

Optimization of water treatment to obtain and maintain a healthy bacterial flora in recirculating aquaculture systems (RAS) is gaining interest (Attramadal et al., 2012; Wold et al., 2014; Pedersen et al., 2017; Rud et al., 2017; Rojas-Tirado et al., 2018; de Jesus Gregersen et al., 2019). Bacteria are necessary for the fluxes and the conversions of nutrients in RAS to maintain high water quality (Blancheton et al., 2013). Bacteria also have direct implications for the fish, as they are highly abundant in the water and in constant contact with the mucosal surfaces of the skin, gills and gut. This close relationship can be both beneficial and detrimental for the fish. Bacteria can give positive effects through metabolic and immunological relations, such as improved utilization of nutrients in the gut and protection against invasion of pathogens (Nayak, 2010; Maynard et al., 2012; Gomez et al., 2013). On the downside, negative interactions with bacteria challenge the fish and can lead to infections (Llewellyn et al., 2014). The microbial community structures in RAS are shaped by physicochemical variables and competition for nutrients and space, and this selection has consequences for the composition of the microbial communities (De Schryver and Vadstein, 2014).

Rearing regimes selecting for mature microbial communities in the water, including RAS, have been shown to be beneficial for the cultivation of marine fish larvae (Skjermo et al., 1997; Attramadal et al., 2014; Attramadal et al., 2016; Vestrum et al., 2018; Vadstein et al., 2018a). RAS have properties that favour the development of matured communities dominated by K-strategic bacteria, which are considered to be beneficial for the fish (Attramadal et al., 2012; Attramadal et al., 2014). K-selected microbial communities can outcompete opportunistic r-strategic bacteria and lower the chance of negative bacterial interactions with the fish (Vadstein et al., 2018b). However, systems for juvenile and ongrowing salmon have not been studied in this context. There is limited knowledge of the effects of bacterial communities on salmon fish health and growth. Systems for salmon parr and smolt production have lower salinity, and substantially higher biomass, organic load and higher water exchange rate in the fish tanks than the, in this context, more studied systems for marine larvae. These differences between systems are expected to affect the microbial dynamics significantly.

The availability of nutrients is a key to the selection regime for microbially matured water. The removal of particulate organic carbon (POC) originating from feed waste and faeces in RAS is therefore essential to consider, as POC can mineralize and dissolve to become bacterial substrate. Biodegradable organic matter is the limiting substrate for heterotrophic bacteria in RAS (Blancheton et al., 2013), thus the accumulation and solubilization of degradable POC increases the bacterial carrying capacity (CC) of the system. This in turn, increases bacterial numbers and can alter the microbial community composition due to changes in the selection pressure in the RAS (Attramadal et al., 2012; Wold et al., 2014). Efficient removal of POC is also important for maintaining high physicochemical water quality (Chen et al., 1993; Cripps and Bergheim, 2000; Michaud et al., 2006; Fernandes et al., 2015). However, the large variety of particle sizes in RAS (diameters of nanometres to centimetres), challenges the choice of water treatment technology. The conventional particle removal systems used in freshwater RAS cannot efficiently remove particles smaller than 20 μm (Chen et al., 1993; Chiam and Sarbatly, 2011). Protein skimmers have shown to be efficient for fine particle removal in seawater systems (Rahman et al., 2012), however they are not as efficient in freshwater. Smaller particles may therefore accumulate and negatively affect the water quality by increasing the CC, and thus reduce the nitrification efficiency of the biofilter (Chen et al., 2006; Michaud et al., 2006; Guerdat et al., 2011), irritate the gills of the fish (Chapman et al., 1987), shield bacteria from disinfection (Hess-Erga et al., 2008) and result in formation of anaerobic zones which can yield production of toxic hydrogen sulphide (H_2S) (Tal et al., 2009).

Membrane technology with ultrafiltration (0.001–0.1 μm) or microfiltration (0.1–10 μm) has been proposed as supplement to existing particle removal technologies, especially targeting the fraction of fine and colloidal particle sizes to improve water quality in RAS (Gemende et al., 2008; Pimentel et al., 2017). By reducing the nutrient supply per bacterium, a competitive environment favouring K-selection of the bacteria in the water may be achieved (Attramadal et al., 2014). Membrane filtration have previously showed increased growth rates and reduced mortality in cultivation of marine fish larvae due to improved water quality (Holan et al., 2014; Wold et al., 2014). Our hypothesis is that RAS in general selects for microbial matured water of high stability, and that membrane filtration in RAS increases the stability even more by keeping the CC lower and more even, which will be more critical in a system with high biomass and high organic loading.

Membrane filtration in RAS with juvenile and ongrowing salmon is not well studied, and we lack knowledge on whether microbially matured water could be achieved in RAS with fresh water, high biomass and low hydraulic retention time in the rearing tanks. Membrane filtration adds additional costs and complexity to the RAS for the fish farmers, and it demands frequent cleaning due to fouling (Viadero and Noblet, 2002; Sharrer et al., 2007). More research is therefore needed to evaluate if the cost of membrane filtration for improved particle removal can be balanced by better physicochemical and microbial water quality yielding healthier salmonids. The objective of this study was to evaluate how the physicochemical and microbial water quality of RAS with and without membrane filtration were affected by high/increasing and low/decreasing loads of particles and organic matter. We hypothesized that the water in RAS with improved particle removal by membrane filtration would 1) result in lower organic loads, lower numbers of bacteria and a more stable and favourable microbially matured water, and 2) better general physicochemical water quality and nitrification efficiency in the biofilter, all of which could contribute to improved growth and survival of the fish.

2. Materials and methods

2.1. Experimental design: system configuration and rearing conditions

The experiment was conducted at Sealab, NTNU's Centre of Fisheries and Aquaculture in Trondheim, Norway. It included two separate pilot-scale RAS, the conventional RAS (cRAS) and the RAS with a membrane (mRAS), each with a total volume of 4.2 m^3 (Fig. 1). The water in the systems were from the municipal freshwater distribution system. Some seawater (pumped in from 70 *m* sea level depth in the Trondheim fjord and UV-treated) was mixed into the systems to maintain 3 ppt salinity to avoid growth of fungus. Both systems included six rearing tanks (0.40 m^3 each) and a recirculating water treatment loop containing a mechanical drum screen filter (HEX, CM Aqua Technologies, Denmark), a moving bed biofilm reactor (MBBR: Nofitech, Norway) and a water-to-air counter flow CO_2-degasser (Nofitech, Norway). The MBBR included three chambers, each filled with 0.12 m^3 of carriers with a specific surface area of 828 m^2 m^{-3} (Table 1). The membrane in mRAS was a hollow fibre, polymeric X-FLOW Compact 4.0G ultrafiltration membrane (Pentair, Netherlands). Ultrafiltration was chosen over microfiltration due to less chances of irreversible fouling (Kimura et al., 2006). The module contained two membranes in series, each with an area of 4.0 m^2 and an average pore size of 20–30 nm. The membrane treated 10–15% of the total water flow and was backwashed every 60 min (timer controlled) for 50 days, then every 15 min for the rest of the experiment (22 days). Chemical cleaning with sodium hydroxide (NaOH) and sodium hypochlorite (NaOCl) was done every 2nd week manually. The membrane was operated with constant transmembrane pressure (TMP) at 0.7–0.8 bar, with a crossflow configuration and produced 600–700 L h^{-1} permeate. The membrane maintained 98.5% recovery from feed stream on average through the experiment.

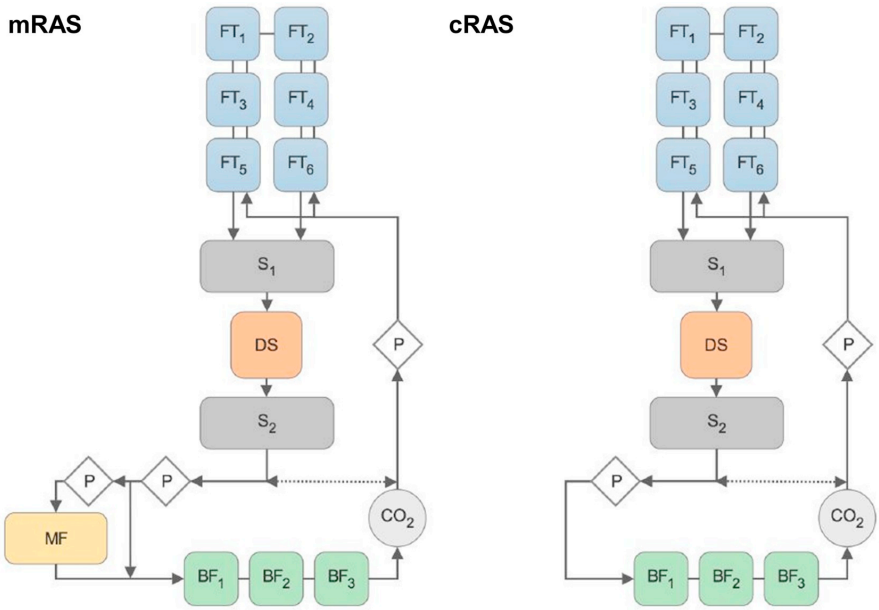

Fig. 1. Experimental set-up of the two systems, membrane RAS (mRAS) and conventional RAS (cRAS). FT = fish tank, S = sump, DS = drum screen filter, MF = membrane filtration, BF = biofilter, CO_2 = CO_2-degasser, P = pump.

Table 1
Dimensions of components in mRAS and cRAS.

Location	Parameter	Unit	
Total system	Volume	m^3	4.2
	Make-up water flow	L h^{-1}	Table 2
	HRT (volume (make-up water flow^{-1}))	days	Table 2
Culture tanks	Volume	m^3	0.40
	Flow	L min^{-1}	17
	HRT (volume (flow^{-1}))	min	23
Drum screen filter	Mesh	μm	mRAS: 20 cRAS: 60
CO_2 degasser	Area	m^2	6.85
MBBR (3 chambers)	Reactor volume empty	m^3	0.40 × 3 = 1.2
	Volume carriers	m^3	0.12 × 3 = 0.36
	Carrier specific surface	m^2 m^{-3}	828
	Biofilter carrier area	m^2	99 × 3 = 298
	Filling fraction (dry)	% of volume	30

Each fish tank was stocked with 60 individuals (6 kg m^{-3}) of Atlantic salmon parr (*Salmo salar)* with an average weight of 40 ± 4 g (± SD). After stocking, the systems were run for an acclimatization period of 70 days where the water was cross run between the systems to ensure an equal start situation. The fish came from MOWI Slørdal and had been reared in a flow-through system before arriving at Sealab. The fish were reared with an artificial winter light regime (7 L: 17D) throughout the experiment. They were fed commercial pellets (3 mm Nutra RC, Skretting, Norway) by automatic feeders (Arvo-Tec Oy, Finland) installed at each fish tank. The fish were fed every 20 min during the 7-h light period.

The experiment, excluding the 70-day acclimatization period, lasted for 72 days in total (03.04.17–13.06.17). By manipulation of the make-up water exchange rate/total hydraulic retention time (HRT) and feeding, we divided the experiment into three different periods (Table 2): Period 1 (P1) got an increasing load of organic matter (accumulating) through the period due to low water exchange rate, P2 got decreasing organic loading due to high water exchange rate (dilution), and P3 got increasing organic loading (accumulating) due to lower water exchange rate and higher feeding. At the end of P1 there was extra addition of water to both systems as one of the outlets of a fish tank in cRAS clogged, and water ran out of the tank and was lost. New water was added to both systems at day 12 to 18 to compensate for the loss in cRAS, and to assure identical conditions in both systems (not included in Table 2).

2.2. Daily management and water quality variables

The fish were inspected on a daily basis, and any mortality/abnormalities were documented. The experiment was carried out within the Norwegian animal welfare act guidelines, in accordance with the Animal Welfare Act of 20th December 1974, amended 19th June 2009, at a facility with permission to conduct experiments on fish (code 93) provided by the Norwegian Animal Research Authority (NARA) by FELASA-approved personnel. Large particles of feed waste and faeces were collected on sieves in the outlet of each fish tank and removed manually daily. Temperature and dissolved oxygen were measured with a handheld Pro2030 dissolved oxygen meter (YSI, USA) in the outlet of the tanks. The concentration of CO_2 was measured (Oxyguard, Denmark) in the pump sump after the CO_2-degasser. Total ammonia nitrogen (TAN), nitrite and nitrate were analysed in water entering the biofilter with a DR/890 Colorimeter (Hach, USA). The pH was recorded with a 3210 pH-meter (WTW™ ProfiLine™, Xylem, Germany), and alkalinity was measured through acid titration of RAS water with 0.1 M hydrochloric acid (HCl) until the titration endpoint of pH 4.5. Sodium bicarbonate ($NaHCO_3$) was added to the systems to maintain an alkalinity of 50 mg L^{-1} $CaCO_3$ throughout the experiment. All these measurements were performed either daily or every other day. The turbidity was measured in glass vials using a 2100AN turbidimeter (Hach, USA) twice a week. Samples for total and dissolved organic carbon (TOC and DOC) were collected every 2 weeks in glass vials and

Table 2
Experimental conditions in mRAS and cRAS for the three periods of different organic loading.

Day	Period	Water exchange rate (% d^{-1})	HRT_{tot} (days)	Variation in organic matter load through period	Make-up water flow ($L\ h^{-1}$)
1–18	P1	10	9.7	Increasing ↑	18
19–51	P2	60	1.6	Decreasing ↓	105
52–72	P3	30	3.5	Increasing ↑	50

conserved with acid until analysis through combustion and carbon dioxide detection (Apollo 9000 TOC-analyser, Teledyne Tekmar, USA). The water for DOC analysis was filtered through 0.45 μm nitrocellulose membrane filters (MF-Milipore™). The difference between TOC and DOC was considered to be particulate organic carbon (POC), and the percentage of the particulate fraction of TOC was calculated.

2.3. Water sampling for microbiology

Water samples for analysis of microbiology were collected at Day 1, 18, 39, 50, 66 and 72 in the outlet of fish tanks 1, 3 and 5, inside the DS filter, sump 2 and after biofiltration before CO_2-degassing (Fig. 1). The sampling was done at the same time of the day (around 10.00 AM). Water samples were filtered through a 0.22 μm sterile filter (Sterivex™) using a 60 mL syringe. The volume filtrated was approximately 200 mL for mRAS and 100–150 mL for cRAS, depending on amount of particles in the water. All the samples were stored at −20 °C until further analysis.

2.4. Sequencing of 16S rRNA gene amplicons

2.4.1. DNA extraction, PCR and Illumina sequencing preparation

DNA extraction was conducted with the QIAamp® DNA Mini Kit (Qiagen, Germany). The water filters were cut into small pieces with a sterile scalpel and put into 1.5 mL Eppendorf tubes. The manufacturers' protocol was followed with minor alterations. An extra lysis step was added to ensure lysis of Gram-positive bacteria by using an enzymatic lysis buffer (180 μL) consisting of 2 mM EDTA, 20 mM Tris-HCl (pH 8), 1.2% Triton and lysozyme (20 mg mL^{-1}), following 1 h of incubation at 37 °C. The variable region 4 (V4) of the bacterial 16S rRNA gene (Marchesi et al., 1998) was targeted for bacterial community analyses of RAS water and biofilter biofilm. The V4 region was amplified using the broad coverage primers 515F (5′-tcgtcggcagcgtcagatgtctataagagacagnnnn GTGCCAGCMGC GGTAA-3′), and 805R (5′-gtctcgtgggctcggagatgtgtataagagacagnnnnACTACNVGGGTATCTAA KCC-3′). Illumina adapter sequences are in lower case letters and were included due to subsequent amplicon sequencing. Each PCR reaction contained 0.02 U/μL Phusion Hot Start II DNA polymerase (Thermo Scientific), 0.2 mM of each dNTP (VWR), 0.3 μM of each primer (SIGMA), 2 mM $MgCl_2$ (Thermo Scientific), and reaction buffer (Thermo Scientific) in a total reaction volume of 25 μL, including 1 μL of ~1 ng/μL DNA extract as template. The PCR reactions were run with 30 cycles (T100TM Thermal Cycler, BioRad). PCR products were normalized with a SequalPrep Normalization Plate (96) kit (Invitrogen, USA), following the protocol included in the kit. Unique barcode-sequences were added to each PCR product using the Nextera XT Index kit (Illumina, USA) through an additional PCR run with 8 cycles. The barcoded PCR products were examined by agarose gel electrophoresis. The indexed amplicons were normalized again using the Normalization plate. A total of 96 samples were pooled for each illumina lane and concentrated with AmiconUltra 0.5 Centrifugal Filter (Merck Millipore, Ireland) as described by the manufacturer. The concentration and purity (A260/280 & A260/230) of the sample were measured with NanoDrop One (Thermo Scientific). The pooled amplicon libraries (96 samples in each) were sequenced on one MiSeq lane each (Illumina, USA) at the Norwegian Sequencing Centre (NSC, Oslo, Norway).

2.4.2. Processing of sequencing data

The Illumina sequencing data were processed with the USEARCH pipeline (version 9.2). Paired reads were merged, primer sequences trimmed and reads shorter than 230 base pairs were filtered out. The data went through demultiplexing and quality filtering with the Fastq_filter command with an expected error threshold of 1.0. Singletons and chimera sequences were removed, and clustering at the 97% similarity level was done, all with the UPARSE-OTU algorithm (Edgar, 2013). Finally, taxonomy assignment was performed using the Sintax script (Edgar, 2016) with the RDP reference data set (version 15) and a confidence value threshold of 0.8. The data were normalized to lowest read count (20,000) to avoid bias in diversity analyses due to variable sequencing depth. OTUs representing eukaryotic amplicons (algae) were removed before further statistical analysis. OTUs of interest were further investigated with the SeqMatch tool for type strains at the RDP website (Cole et al., 2013).

2.5. Quantification of bacterial cell densities and growth

2.5.1. Flow cytometry

Bacterial numbers were quantified by flow cytometry of water from fish tanks, sumps and the CO_2-degasser outlet three times a week. The samples were fixated with glutaraldehyde (final concentration 0.5%), snap frozen in liquid nitrogen and stored at −20 °C. Prior to analysis, the samples were diluted 1:10 with 0.2 μm filtered 0.1× TE buffer to keep the cell counts below 1000 events μl^{-1} for avoiding overload of the instrument's max read count. SYBR® Green I (Life Technologies, Thermo Fisher Scientific Inc.) DNA stain was added to the samples (final concentration 1%) to stain the bacteria. The samples were analysed with a BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, USA). The stained cells were excited with a blue laser (488 nm). The detector for green fluorescence (533 ± 15 nm) was used to detect the bacteria present in the sample. Further analysis of the results was conducted using the BD CSampler™ Software, and bacterial densities were calculated.

2.5.2. [^{3}H]-thymidine incorporation

Incorporation of [^{3}H]-thymidine into bacterial DNA was performed according to Fuhrman and Azam (1980) to estimate bacterial cell growth. Into triplicates of water samples of 1 mL, [^{3}H]-thymidine was added to a final concentration of 10 nM and total activity of 0.5 μCi (specific activity of 20 Ci $mmol^{-1}$) (PerkinElmer, USA). Incubation period was 30 min at fish tank water temperature with shaking at 200 RPM. The incubations were terminated by addition of 50% trichloroacetic acid (TCA) to a final concentration of 5%. As controls, 50% TCA was added to 0.22 μm-filtered fish tank water to a final concentration of 5% prior to [^{3}H] thymidine additions and run in parallel to measure abiotic adsorption of radioactivity. Finally, the samples were centrifuged, and the pellets were washed twice in 5% TCA. After removal of the supernatant, the pellets were suspended in 1 mL HiSafe® 3 scintillation fluid (PerkinElmer, USA). Radioactivity was counted in a PerkinElmer Tri-Carb 4910TR scintillation counter. The incorporation rate was converted to bacterial growth rate using a conversion factor of $1.1 \cdot 10^{18}$ cells per mol thymidine (Riemann et al., 1987).

2.6. Analysis and assimilation of dissolved free amino acids

Water samples of 10 mL for analysis of concentrations of dissolved free amino acids (DFAA) were filtered through 0.2 μm membrane filters and kept frozen at −20 °C until analysis by HPLC and fluorescence detection. Analysis of the amino acids followed procedure of Jørgensen and Middelboe (2006). DFAA were derivatized with *o*-phthaldialdehyde and N-isobutyryl-L-cysteine as a chiral agent (Brückner et al., 1995) and separated on a Waters XTerra RP18 3.5-μm particle column (Waters Corporation, Milford, USA) at a flow rate of 0.7 mL min^{-1}. Mobile phases were (A) aqueous solution of 5 mM $Na_2H_2PO_4$, 45 mM sodium acetate trihydrate and 7.5% methanol at pH 6.4, and (B) 100% methanol (Mopper and Furton, 1991). The derivatives were eluted with the following gradient: T_0 min (100% A, 0% B), T_{27} min (50% A, 50% B), T_{30} min (10% A, 90% B) and T_{33} min (100% A, 0% B). The HPLC equipment consisted of a Waters 2965 autosampler and pump module, and Waters 2475 fluorescence detector. For calibration, a standard mixture of 18 L amino acids was enriched with non-protein amino acids and selected D isomers of amino acids (Glu, Asp, Ser and Ala) and glucosamine (GluA, component of bacterial cells walls). Individual amino acids in the chromatograms were identified from retention times determined from the standard mixture.

Bacterial assimilation of DFAA was measured according to procedure by Jørgensen et al. (1993). A mixture of four [^{14}C] L-amino acids (Glu, Ser, Gly and Ala) in an equimolar composition at a total activity of 0.01 μCi (PerkinElmer, USA) was added to triplicate 10 mL water samples and a control with 2% formaldehyde. Addition of the tracers corresponded to about 5 nmol DFAA L^{-1}. The samples were incubated for 30 min at fish tank temperature after which the samples were fixed with formaldehyde (2% final concentration). The fixed samples were filtered through 0.2 μm membrane filters which were radio assayed by liquid scintillation counting. Respiration of the assimilated DFAA was not determined.

2.7. Measures of microbial diversity and statistical analyses

The program package PAST version 3.21 (Hammer et al., 2001) was used to calculate diversity indices and to perform statistical analyses. Alpha-diversity measures included estimated species richness Chao-1 (Chao, 1984) and evenness. Beta-diversity was calculated based on the presence/absence-based Sørensen-Dice similarity and the abundance-based Bray-Curtis similarity (Chao et al., 2006). Water quality variables were checked for normality with Shapiro-Wilk test (Shapiro and Wilk, 1965). Two sample paired *t*-tests were used on data that did follow a normal distribution, whereas Mann-Whitney-Wilcoxon-tests were used on samples that did not. Two-Sample t-tests were used to determine statistical significance between fish end-weights and estimated richness and evenness. Ordination by Principal Coordinate Analysis (PCoA) with normalized and square root transformed data based on Bray-Curtis similarities were used to visualize the similarities/dissimilarities and development of the bacterial communities in mRAS and cRAS. Similarity Percentage (SIMPER) with Bray-Curtis similarities was used for assessing which OTUs that were mostly responsible for the observed differences in community composition between groups of samples (sampling dates and RAS system). One-way permutational multivariate analysis of variance (PERMANOVA) with Bray-Curtis similarities was performed to test for statistically significant difference between different groups of samples (Anderson, 2017).

3. Results

3.1. Performance of recirculating systems and fish

There was a significant 1.2 °C higher temperature on average ($p < .0001$) in mRAS (Table 3), due to production of heat by the membrane filtration system during operation. The turbidity of cRAS was 3 times higher than in mRAS ($p < .0001$), and the water was also visually more turbid with a stronger brown colour. Concentrations of TAN were 16% higher in cRAS, and a paired t-test confirmed that the difference was statistically significant ($p = .04$). The mortality was very low in both systems (< 1%) with only 3 dead fish in each system through the experiment. The final weights of the fish in mRAS were 14% higher than in cRAS and significant ($p < .0001$) with averages (±SD) of 142.6 ± 28.4 g and 125.0 ± 23.2 g, respectively.

Table 3
Water quality variables through the experiment in mRAS and cRAS.

Variable	mRAS	cRAS
Temperature (°C)	13.7 ± 0.6[b]	12.5 ± 0.4[b]
CO_2 (mg L^{-1})	1.83 ± 0.9	1.34 ± 0.9
Salinity (ppt)	3.5 ± 0.2	3.6 ± 0.2
pH	7.75 ± 0.02	7.74 ± 0.01
Alkalinity (mg L^{-1} as $CaCO_3$)	50.1 ± 1.5	50.7 ± 0.8
TAN (mg L^{-1})[a]	0.079 ± 0.03[b]	0.092 ± 0.03[b]
NO_2 – N (mg L^{-1})[a]	0.051 ± 0.1	0.055 ± 0.1
NO_3 – N (mg L^{-1})[a]	21.2 ± 1.3	18.8 ± 1.3
Turbidity (NTU)	1.18 ± 1.03[b]	3.52 ± 2.91[b]

[a] measured in water entering the biofilter.
[b] significant difference between systems.

3.2. Concentration of organic matter

The concentration of TOC was significantly higher in cRAS for all sampling dates ($p < .01$) and correlated with the organic loading during the different periods (Fig. 2A). The difference in concentration was particularly large at the beginning of P2 (2× higher in cRAS, $p = .002$). The concentration of DOC was slightly higher in cRAS than mRAS in the beginning of P1 and the beginning of P2 (Day 27: Fig. 2B). The difference was significant ($p < .01$), but the concentration was only 0.5 mg L^{-1} higher in cRAS. No significant differences in DOC were found between the systems for the rest of P2 and in P3 ($p > .06$). In the beginning of P1, POC constituted around 25% of the total organic carbon in both systems. Through P2, the amount of particles increased in cRAS and the particulate fraction of the total organic carbon was 35 to 40% at day 27 and 36, whereas in mRAS it was 15 to 20%. In P3, the difference between the systems were even more profound, with around 7% particulate organic carbon in mRAS and 30% in cRAS. The membrane had a clear effect on the concentration of particulate organic carbon, whereas the effect was not as evident for the dissolved organic carbon.

3.3. Bacterial densities, cell production and DFAA assimilation in RAS water

The bacterial densities in the RAS water were determined from water in fish tanks, sumps and CO_2-degasser outlet and were similar in the different compartments of each RAS system (Low SD in Fig. 3). The trend in the densities of bacteria was negatively correlated to the water exchange rate and positively correlated to the organic load in both systems. The densities of bacteria were significantly higher in cRAS for all sampling dates ($p < .0001$). Three bacterial blooms were observed in cRAS, one in each period of the experiment. In mRAS, on the other hand, it was only tendencies for small blooms. During P1 with increased organic load the bloom in mRAS increased the bacterial density by a factor of 3 and in cRAS a factor of 9. This resulted in a 4 times higher maximum bacterial density in cRAS. The extra water exchange at the end of P1 (shaded grey) resulted in a quick reduction in the density of bacteria in both systems. The bloom during P2 was profound for cRAS and was 2.3 times higher than in mRAS and lasted much longer. The bacterial densities in cRAS declined a factor 20 from day 25 to the end of P2 (day 50). This reduction in bacterial numbers happened much

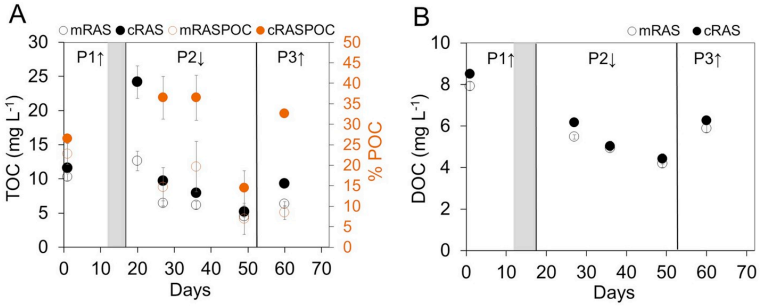

Fig. 2. A) TOC (black symbols) and %POC (brown symbols). B) DOC (black symbols) throughout the experiment. Data are the mean (±SD) from all samples in the same system (both sumps and CO_2-degasser outlet) each sampling day. Periods with different organic loading showed. Grey shading (day 12–18) shows extra addition of water due to technical problems.

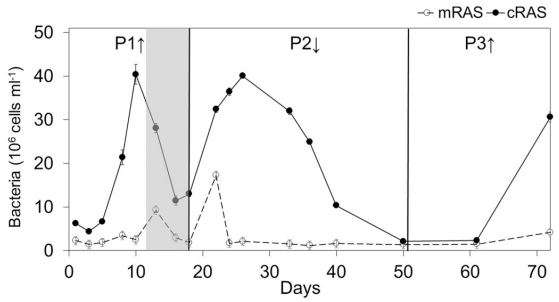

Fig. 3. Bacterial densities in RAS water through the experiment. Data are the mean (±SD) from all samples in the same system (fish tanks, sumps and CO_2-degasser outlet) each sampling day. Grey shading (day 12–18) shows extra addition of new water due to technical problems.

quicker in mRAS where it declined a factor 10 in only 2 days. During the overfeeding in P3 the bacterial densities increased by a factor 3 and 13 for mRAS and cRAS, respectively. At the end of the experiment, the density of bacteria was 7 times higher in cRAS than in mRAS. It is clear that membrane filtration reduced bacterial numbers.

The bacterial production showed minor variations in mRAS and varied significantly in cRAS during the experiment. In mRAS, the production ranged from 36 to 65 · 10^6 cells L^{-1} h^{-1}, while in cRAS the range was 27 to 915 · 10^6 cells L^{-1} h^{-1} (Fig. 4A). On three out of five sampling days (Day 1, 18 and 72) the cell production was 3.1, 16.4 and 2.2 times higher in cRAS than in mRAS and was slightly lower on the other two days. The high bacterial production at Day 18 agrees with a high increase in cell density in cRAS the following days. The bacterial assimilation of DFAA (Fig. 4B) was less variable in mRAS than in cRAS and showed the same trend as observed for the cell production. In mRAS, the assimilation ranged from 0.77 to 2.19 μg L^{-1} h^{-1}, as compared to 1.1 to 13.3 μg L^{-1} h^{-1} in cRAS. The assimilation was 2.7 to 6.1 higher in cRAS than in mRAS, except for Day 50 when the assimilation was 2× higher in mRAS. When relating assimilation of C from DFAA to C required for the measured cell production, DFAA-C was determined to sustain from 34.5 to 90.9% of the microbial C demand in mRAS and 23.5 to 80.7% in cRAS. The lowest C contribution from DFAA in cRAS occurred when the peak in cell production occurred, suggesting that DFAA were not a major C source to the cells during that period.

3.4. Microbial diversity of system water

Illumina sequencing yielded a sequencing depth of on average 80,000 reads per sample, and in 400–800 observed OTUs per sample. Before further analysis the dataset was normalized to equal sequencing depth per sample (20,000 reads), to avoid bias in the further analysis.

3.4.1. Alpha diversity of water microbiota

Estimated species richness (Chao-1) for the water samples (Fig. 5A) was significantly higher in mRAS at all samplings in P2 and P3 ($p < .001$). The difference was especially evident at Day 39, which was approximately three weeks after the systems had gone from high to low organic loading. At this sampling, estimated richness in mRAS was approximately twice that in cRAS ($p < .0001$). The evenness (Fig. 5B) of the water microbiota was higher for mRAS than cRAS during P2 and P3 (Day 39, 66 and 72; $p < .001$). The difference was especially clear at Day 39 and Day 72 when evenness was about 2× higher in mRAS than in cRAS.

3.4.2. Beta diversity of water microbiota

To examine the temporal development within and between systems of the microbial communities, we used ordination by Principal Coordinate Analysis (PCoA) based on similarity indices. Bray-Curtis and Sørensen-Dice similarities were used to quantify whether differences in microbial communities were due to differences in relative abundance or change in OTU inventory. The first two coordinates (axes) of the ordination based on Bray-Curtis similarity explained 30.1 and 16.0% of the variation in community composition between samples, respectively

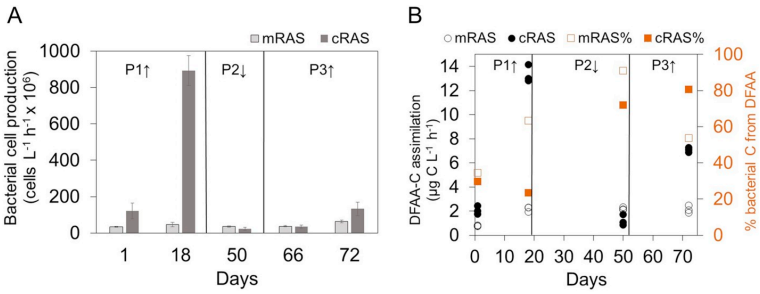

Fig. 4. A) Bacterial cell production. Data are the mean (±SD) from all samples in the same system (both sumps and CO_2-degasser outlet) each sampling day. B) Net incorporation of dissolved free amino acids (DFAA) into bacterial biomass. Contribution of DFAA-C to the bacterial C demand (%) is shown on the right (brown) y-axis in B.

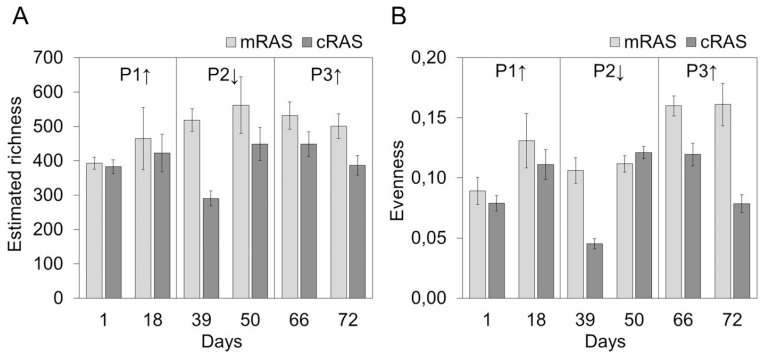

Fig. 5. A) Estimated richness (Chao-1) and B) Evenness for water samples through the experiment. Data are mean ± SD of all samples in the same system (fish tanks, sumps and CO_2-degasser outlet) at each sampling date.

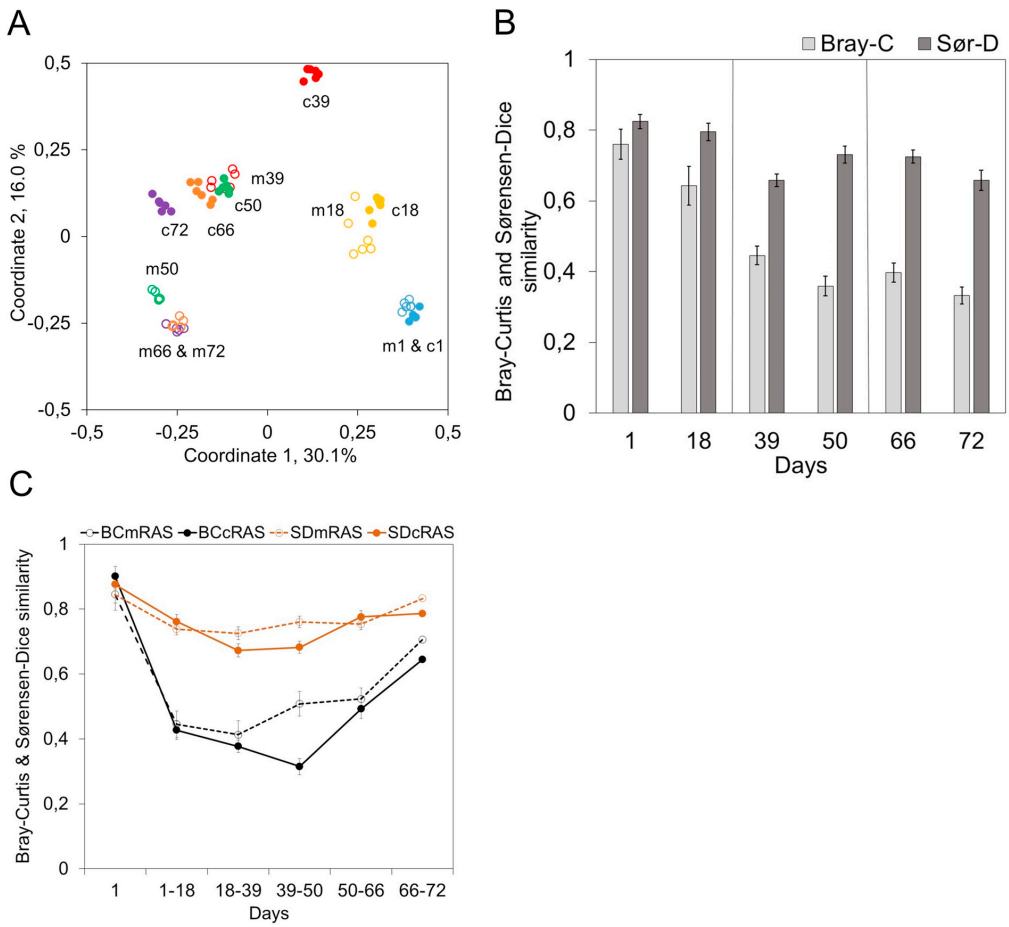

Fig. 6. A) PCoA of water samples based on Bray-Curtis similarities. m = mRAS and empty circles, c = cRAS and filled circles, following day of sampling (same colour). B) Bray-Curtis and Sørensen-Dice similarities between mRAS and cRAS. Data are mean ± SD of all values in the similarity-matrix comparing all samples within each sampling date. C) Succession of microbial communities through the experiment within each system analysed with Bray-Curtis (black symbols/lines) and Sørensen-Dice (brown symbols/lines) similarities. Data are the mean ± SD of all values in the similarity-matrix comparing water microbiota between two succeeding sampling dates within mRAS and cRAS.

(sum 46.1%: Fig. 6A). The PCoA plot shows that through P1, with increasing organic loading (Day 1 to 18), the communities of both systems developed in the same direction. The microbial community composition of the two systems were relatively similar on Day 18 following the first bacterial bloom (Fig. 3). In P2 on Day 39 (decreasing organic load), the communities of the two systems showed different succession pattern and continued to diverge until the end of the experiment. The samples from mRAS at Day 66 and 72 overlap, which is not the case for cRAS in P3. This indicates higher stability in mRAS than cRAS at the end of this period with increasing organic load. When comparing the two systems, the Sørensen-Dice similarity was higher than the Bray-Curtis similarity at all sampling dates (Fig. 6B). This indicates that variations in the relative abundances of the OTUs contributed more to the observed differences between the systems than the presence or absence of OTUs. The Bray-Curtis similarity decreased by a factor of 2 throughout the experiment, indicating that the cRAS and mRAS water microbiota developed to become increasingly dissimilar. Once the water microbiota of the systems diverged after Day 18 (Fig. 6A & B), the Bray-Curtis similarities decreased with 40% by Day 50 and stayed at the low similarity throughout the rest the experiment (Fig. 6B). Thus, when the environmental shift (change in organic loading) induced the change in the microbial communities, they never returned to become more similar to each other again. In the succession plot within each system (Fig. 6C), the Sørensen-Dice is generally higher than Bray-Curtis similarities for all sampling dates. This shows that the change in abundance of OTUs present within each RAS contributed more to the succession than contribution from new OTUs. Bray-Curtis was only notably higher in mRAS from Day 39 to 50 with values of 0.3 and 0.5 in mRAS and cRAS, respectively. From Day 66–72 the Bray-Curtis similarity was 0.05 higher in mRAS than cRAS, again supporting that mRAS could be slightly more stable at the end of the experiment with increasing organic load than cRAS. A PERMANOVA test based on with Bray-Curtis similarities confirmed that the microbial community compositions from all samplings both within and between systems were significantly different ($p < .05$). Both systems underwent succession through the experiment, and the water microbiota changed slightly more in cRAS than in mRAS (lower Bray-Curtis similarities for between-day comparisons).

3.5. Microbial community compositions in mRAS and cRAS

The taxa summary (Fig. 7) shows the development of the microbial community composition at the class level. The data are presented as relative abundances where the shown taxa are the relative share (values from 0 to 1) of reads in the sample. The most abundant bacterial classes in the water were Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Actinobacteria. During P1 with increasing organic load in the systems, the relative abundance of Betaproteobacteria increased from approximately 0.12 to 0.3 in both systems. The opposite was observed for Gammaproteobacteria, which decreased a factor 5 from 0.25–0.3 (Day 1) to 0.05–0.07 (Day 18). In cRAS there was a transitory peak in the abundance of Alphaproteobacteria from Day 18 to 39 from 0.25 to 0.4, but the abundance remained at 0.25 in mRAS. At the end of P2 (Day 50) cRAS had a relative abundance of 0.15 of Actinobacteria, whereas it was only 0.02 in mRAS.

SIMPER analysis based on Bray-Curtis similarities was performed to determine which OTUs that contributed most to the difference between systems and sampling date (samples from the same system and sampling date were grouped: Fig. 8). An OTU representing *Gemmobacter* (Alphaproteobacteria: OTU_196) was the dominating OTU in cRAS at Day 39, with a relative abundance of 0.4. This is the highest relative abundance of any OTU at one sampling date in the whole dataset, and it contributed most to the difference between systems and sampling dates (6.3%). The relative abundance in mRAS was considerably lower (0.1) at Day 39, and in both systems the relative abundance dropped to less than 0.01 by Day 50. OTU_4 (*Sphaerotilus,* Betaproteobacteria) was more relative abundant in mRAS in P2 and P3 (0.2 and 0.1) than in cRAS (0.1 and 0.05). OTU_5 (*Legionella,* Gammaproteobacteria) was highly abundant only in the beginning of the experiment at Day 1, with relative abundance of 0.27 in cRAS and 0.20 in mRAS. After Day 1 the relative abundance dropped to less than 0.01 and remained low through the rest of the experiment. The OTU table contained 57 different OTUs that belonged to the *Legionella* genus, and those OTUs were more abundant during P2 and P3 as well (Supplementary Fig. S1). An OTU representing *Mycobacterium* (Actinobacteria: OTU_7) was highly relative abundant in cRAS water towards the end of the experiment (up to around 0.25), while it had a relative abundance of <0.03 in mRAS through the whole experiment. Another group of bacteria that have been related to high particle environments in RAS which can create anaerobic zones, are sulphate reducing bacteria (SRB). In this study the relative abundance of SRBs (Supplementary Fig. S2) was higher in mRAS (0.12–0.16) than cRAS (0.01–0.06) in P2 and P3.

4. Discussion

4.1. Membrane filtration reduces the microbial CC by removal of small organic particles

The membrane treated a side stream of 10–15% of the total water flow, which significantly lowered organic carbon build-up (up to 50% lower) and consequently the density of bacteria in mRAS. This effect was especially strong when the organic loading was high. The effect of membrane filtration was evident for POC (Fig. 2A), but not for DOC (Fig. 2B). DOC is the most easily biologically degradable fraction of organic carbon, and 10–30% has typically been shown to be easily biologically degradable, depending on the water source (Søndergaard and Worm, 2001; Piech et al., 2019). In this experiment, organic matter was added to the systems every day through fish feed and production of faeces, and the reduction in POC through membrane filtration resulted in less solubilization and hydrolyzation of particles that eventually could become DOC. As more particles accumulated in cRAS, there was probably more DOC production in this system. The typically 2–6% higher DOC concentration in cRAS than in mRAS (Fig. 2B) indicate that the extra DOC produced was quickly consumed by the bacteria. In P2 the densities of bacteria decreased more rapidly after the bloom in mRAS than in cRAS (Fig. 3). In mRAS the membrane removed both bacteria and the bacterial substrate. In cRAS the bacteria and bacterial substrate were primarily removed through dilution due to water exchange. Bacteria can grow faster than the systems' HRTs (Since the HRTs were in the order of days: Table 2), causing the bacterial densities to decrease much slower in cRAS. The membrane showed a stabilizing effect on the bacterial CC during the experiment when the loading of organic matter changed. Such stabilization is thought to be beneficial for commercial fish production in RAS, which are subject to changes in organic loading due to introduction of new fish groups, weighing, vaccination, change of feed etc. through the production cycle. The level of the removal of organic matter is easy to control by deciding the size of the side stream that goes through the membrane, and one can thus avoid lowering the CC below a critical level (Holan et al., 2013; Vadstein et al., 2018b).

4.2. Membrane filtration in RAS increase the diversity of the microbial communities

The water microbiota of mRAS developed and maintained its higher diversity (i.e. richness and evenness: Fig. 5A and B), whereas cRAS microbiota developed into communities with lower diversity. It was the abundance of the different OTUs present in each RAS that changed the most, rather than the contribution from new OTUs throughout the experiment (Fig. 6B and C). Both systems were initially colonized by bacteria from the same fish and inlet water, and 70–80% of all OTUs identified were found in all samples between and within the systems. This shows that both RAS had a similar microbiome with respect to

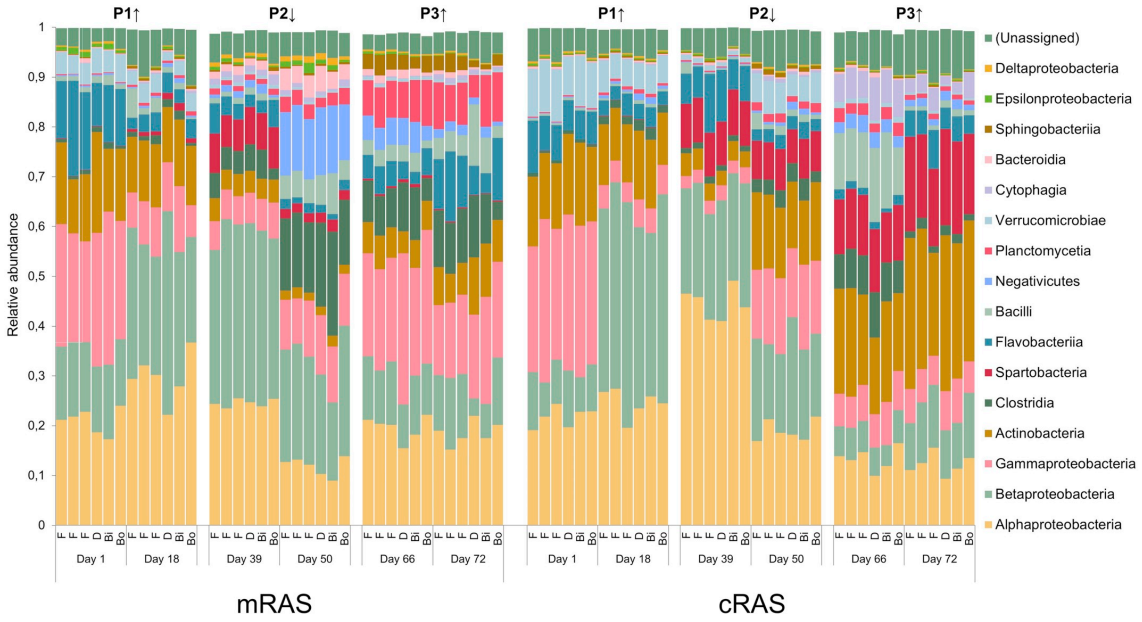

Fig. 7. Composition of water microbiota at class level showing relative abundance at the different sampling dates in mRAS and cRAS. Only taxa with >0.01 relative abundance in minimum one sample is included in this fig. F = fish tanks, D = drum screen filter, Bi = biofilter in, Bo = biofilter out.

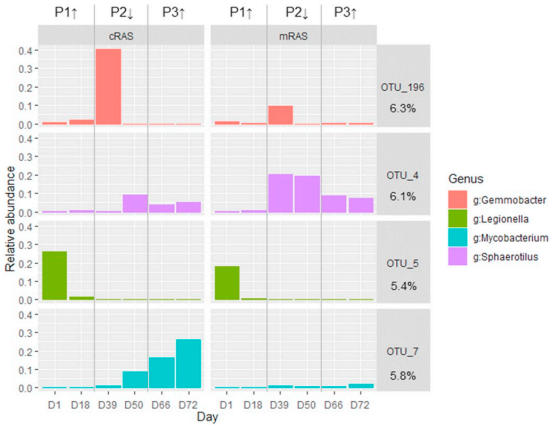

Fig. 8. Top four OTUs contributing to the difference between sampling dates and systems. All OTUs are classified for the genus (different colors) with a confidence threshold of 0.8. Relative abundance on y-axis and contribution to difference (%) from SIMPER analysis is shown below OTU IDs.

OTUs present, but that the differences in organic loading in each RAS created a selection pressure where the different bacteria succeeded to different degrees. When the organic matter loadings changed (between periods), the change in the microbial CC was not as profound in mRAS as in cRAS. This temporal destabilization in the environment caused the diversity to drop and specific bacteria became dominant, especially in cRAS, likely due to higher substrate availability. One example is OTU_196 (*Gemmobacter,* Alphaproteobacteria), which was four times more relative abundant in cRAS than mRAS on Day 39 (Fig. 8). Day 39 was approximately two weeks following the peak bacterial bloom and the organic load was decreasing. OTU_196 was not identical to any of the 16S type strains for *Gemmobacter* in the RDP database. The highest match of 97.9% was with *Gemmobacter tilapiae* (Sheu et al., 2013), a strain isolated from a freshwater pond with Tilapia fish (*Tilapia rendalli*). Sheu et al., 2013 did not report any disease of the fish and little information can be found about this genus. OTU_7 in the *Mycobacterium* genus was found in high relative abundance in cRAS. This genus includes several human pathogens (Gupta et al., 2018), and the SeqMatch tool showed that OTU_7 was not identical to any of the 16S type strains. The known species *Mycobacterium salmoniphilum* which cause mycobacteriosis in salmon farming (Aro et al., 2014) was not found to match the OTU_7 representative sequence. There were 57 different OTUs that belonged to the genus of *Legionella* (Gammaproteobacteria), and this genus was highly abundant throughout the experiment in both systems (Supplementary Fig. S1). *Legionella* is common in freshwater and soil environments, and around 50% of the species have been associated with disease in humans (Llewellyn et al., 2017). OTU_5 (Fig. 8) and the other most abundant *Legionella* OTUs (Supplementary Fig. S1) representative sequences had generally low sequence match to the different *Legionella* species in the RDP database, and *Legionella* is not known for causing losses in salmon farming. Amoeba are known to be hosts for *Legionella* as these bacteria commonly replicate intracellularly in eukaryotic hosts (Thomas et al., 2004). Thus, this high abundance of *Legionella* throughout the experiment could indicate high abundance of amoeba. Protozoan organisms were not targeted in this study, it could be interesting to focus more on the protozoa in RAS in the future. Both *Mycobacterium* and *Legionella* are commonly found in low amounts in municipal drinking water distribution systems (Waak et al., 2018; Waak et al., 2019), and the intake water for both RAS systems was municipal drinking water. The high relative abundance of *Mycobacterium* and *Legionella* in this study is probably due to the environment with high concentrations of nutrients. SRBs have received much attention recently in the commercial salmon RAS industry in Norway, as there have been reported several incidents with high salmon mortalities where production of toxic hydrogen sulphide (H_2S) is thought to have been the cause (Åtland and Stenberg, 2019). SRBs are part of the natural microbiota and are omnipresent (Vigneron et al., 2018). The relative abundance of SRBs was higher in mRAS (Supplementary Fig S2), even

though it had lower turbidity and POC than cRAS. It could be that in cRAS the higher concentrations of organic carbon caused methanogenic bacteria to compete with the SRBs in anaerobic spots (Tal et al., 2009). However, measurements to investigate this were not undertaken.

4.3. Did membrane filtration result in better water quality and performance of fish?

The membrane filtration resulted in better physicochemical water quality in terms of less particles/lower turbidity, less accumulation of organic carbon and slightly lower concentrations of TAN (Table 3). Both systems were RAS, which are considered to select for a more beneficial microbial water quality compared to traditional flow through systems (Attramadal et al., 2014; Vestrum et al., 2018). The concentrations of TOC and DOC were not noticeably higher than concentrations found in other RAS, ranging from 10 to 25 mg C L^{-1} (Krumins et al., 2001; Hambly et al., 2015). Known salmon pathogenic genera were not found in high relative abundance in either of the systems and were not studied further. However, mRAS had significantly lower bacterial densities and higher bacterial diversity. This could indicate that mRAS developed a better microbial water quality than cRAS by providing more stable conditions for K-selection, namely lower and more stable concentrations of available organic matter, supporting the hypothesis for the experiment. Mature microbial communities dominated by K-strategists are predicted to have higher stability to perturbations (De Schryver and Vadstein, 2014; Vadstein et al., 2018b), which was more seen in mRAS than in cRAS. This further supports the hypothesis that a more microbially matured water can be achieved in RAS for juvenile salmon with appropriate use of membrane filtration. Membrane filtration will reduce the probability for microbially related accidents such as blooms of pathogens and potential anaerobic conditions that can lead to production of H_2S.

The average weight of the fish at the end of the experiment in mRAS was 14% and significantly higher than in cRAS, and mRAS produced more biomass in total. The better growth in mRAS can be attributed to a combination of higher temperature and better conditions. On average the temperature was 1.2 °C higher in mRAS than in cRAS (Table 3) caused by the membrane operation. Even though the temperature range in this experiment has shown little effect on the growth of salmon (Handeland et al., 2008), it is hard to conclude exactly how much of the improved growth of the fish that can be attributed to the higher temperature compared to the better water quality. Nevertheless, both the better water quality and the higher temperature should be weighed as a positive effect of the membrane, as heating of water is considered to be a large energy cost in RAS (Badiola et al., 2017). The estimated energy operating expenses (OPEX) for salmonids in RAS varies from 5.46 to 26 kWh kg^{-1} of fish produced (Ayer and Tyedmers, 2009; d'Orbcastel et al., 2009; Summerfelt et al., 2009; Samuel-Fitwi et al., 2013; Liu et al., 2016; Song et al., 2019). The energy OPEX for membrane ultrafiltration is estimated to be 0.1–0.15 kWh m^{-3} of treated water (Pearce, 2008; Verrecht et al., 2010; Maere et al., 2011). Liu et al. (2016) reports a RAS OPEX cost of 5.4 kWh kg^{-1}, including the whole production cycle from hatching to market size salmon of 5 kg. Using the data provided by Liu et al. (2016) and adding membrane ultrafiltration (treating a side stream of 10% of the water flow) in all production stages, the energy OPEX of the membrane would equal 1.5 kWh kg^{-1} fish, which equal 27% of the total energy OPEX cost of the RAS. Song et al. (2019) reports a cost of 7.5 kWh kg^{-1} market size salmon produced. Estimating the cost of membrane filtration in that study, the membrane filtration OPEX constitutes 5.6% of the total RAS OPEX cost. Due to the high variability in estimated energy use in RAS, it is hard to conclude whether the cost of membrane filtration can be balanced by better water quality and fish growth. Moreover, inclusion of membrane filtration probably has a changing cost-benefit situation through the production cycle. It can for example, be more beneficial in stages and periods where the fish is more vulnerable to particles and the water flow is relatively low, compared to production stages with more robust fish and high water flows. More research is therefore needed with membrane filtration in RAS at the different production stages of salmon to study the cost and the benefits. What we can conclude from our study is that the higher stability in physical, chemical and microbial water quality variables, indicate that the membrane had a stabilizing effect and reduce the carrying capacity for bacteria. If the RAS and fish had been challenged, we expect that the system with membrane filtration would have managed better. The better water quality and higher temperature led to better fish growth, and illustrates the potential of including membrane filtration in RAS for salmon smolt production.

5. Conclusions

- For microbial water quality, the system with membrane filtration had smaller and shorter bacterial blooms, generally lower densities of bacteria, and more diverse microbial communities. Microbially matured water was achieved in production of Atlantic Salmon parr in RAS, and the water seemed to be more mature with membrane filtration.
- For physicochemical water quality the system with membrane filtration had lower concentrations of particles/lower turbidity, less accumulation of organic carbon and slightly lower concentrations of TAN. In general, the variability of these measurements was lower in mRAS.
- The survival of the fish was the same in both systems, and a combination of better water quality and higher temperatures resulted in better growth of fish in mRAS than in cRAS.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735268.

Declaration of Competing Interest

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

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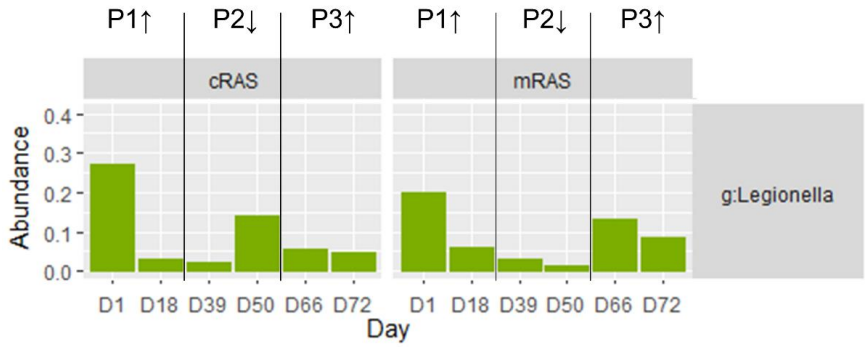
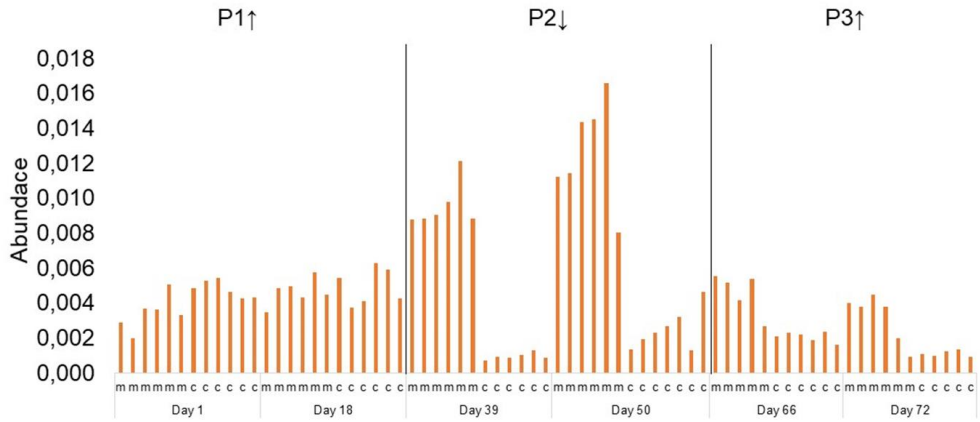
Supplementary data Paper I



Supplementary Fig.S1. Relative abundance of all *Legionella* OTUs classified over a confidence threshold of 0.8



Supplementary Fig.S2. Relative abundance of sulphate reducing bacteria. m = mRAS, c = cRAS.

Paper II

Mass balance of carbon and nitrogen, and nitrification efficiency in recirculating aquaculture systems (RAS) with and without membrane filtration for Atlantic salmon parr (*Salmo salar*)

Ragnhild O. Fossmark, Olav Vadstein, Kari J.K. Attramadal & Stein W. Østerhus.

Manuscript in preparation

This paper is awaiting publication and is therefore not included.

Paper III

A comparison of two seawater adaptation strategies for Atlantic salmon post-smolt (*Salmo salar*) grown in recirculation aquaculture systems (RAS): Nitrification, water and gut microbiota, and performance of fish

Ragnhild O. Fossmark, Kari J.K. Attramadal, Kristian Nordøy, Stein W. Østerhus & Olav Vadstein. 2021.

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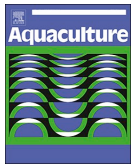

A comparison of two seawater adaptation strategies for Atlantic salmon post-smolt (*Salmo salar*) grown in recirculating aquaculture systems (RAS): Nitrification, water and gut microbiota, and performance of fish

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ABSTRACT

The land-based production phase in Norwegian Atlantic salmon farming has the past years been extended to include post-smolt for an increasing number of farmers. Post-smolt production can involve introduction of brackish/seawater to a recirculating aquaculture system (RAS) that is acclimatized to freshwater for the earlier stages of salmon production. A change from freshwater to seawater shifts the environmental conditions for fish, bacteria and water treatment processes in RAS. Two regimes for salinity increase were studied to evaluate the effects on nitrification functionality, water and gut microbiota and fish performance on land and in the sea cages. A fish group of 200,000 salmon parr were stocked in a brackish water RAS (bRAS) at 3‰ salinity. After the fish had smoltified the group was split in two, one group was kept in bRAS and the other was moved to a RAS operated at 28‰ salinity (sRAS). The bRAS was operated with a gradual increase in salinity from 3 to 26‰ over a period of 28 days, whereafter both groups were moved to two separate sea cages. Bacterial communities of water, biofilter biofilm and fish faeces were characterized by 16S rRNA amplicon sequencing. Nitrification capacity tests at different salinities were performed on biofilter media from bRAS, to evaluate short term robustness to salinity changes. Ordination based on Bray-Curtis similarities showed that in water samples in bRAS, the bacterial communities were stable from 12 to 26‰ salinity increase. The faecal microbiota of the fish showed high inter-individual variation within fish tanks, suggesting stochastic processes/drift to affect the community structures in addition to salinity increase. The same nitrifying bacteria were present in bRAS (throughout the salinity increase) and in sRAS, showing that these nitrifiers could adapt to salinities from 3 to 26‰, and 28‰. After the sea cage phase, fish from the sRAS system had in total 2.9% higher weight than the fish from bRAS, however the mortality was 15% higher in the sRAS group. Salinity was a driver for succession in RAS, and other factors such as organic load in the water and stochastic processes in the host also affected the bacterial community dynamics.

1. Introduction

Production of Atlantic salmon (*Salmo salar)* post-smolt, or large smolt, on land in recirculating aquaculture systems (RAS) is increasing in Norway. In traditional salmon farming, the fish are produced in land-based systems with freshwater or brackish water with low salinity (~0–3‰) from hatching to smoltification. Then they are moved to net pens in the sea for grow-out to market size. Handling stress during the transfer to sea, and exposure to salmon lice and delicing treatments, can reduce growth and increase mortality of the fish in the sea cages (Iversen et al., 2005; Nilsen et al., 2017). Salmon lice have become a significant challenge for salmon farmers (Abolofia et al., 2017), and is one of the drivers for extending the land-based phase to produce post-smolts to reduce the time the fish are exposed to lice in the sea cages (Dalsgaard et al., 2013). Post-smolt production will increase the fish size before transfer to sea, which is hypothesized to make the fish more robust for the exposed conditions at an open sea cage and possible sea lice attack. A second driver for increased production time on land is higher utilization of the Maximum Allowed Biomass (MAB) at the sea locality given in the concessions for the fish farm in Norway (Lekang

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et al., 2016).

From an operational perspective, the question of whether the post-smolts should be reared in freshwater or brackish/saltwater in RAS is discussed. Atlantic salmon have been grown to market size in RAS with only freshwater (Davidson et al., 2016), however problems with early sexual maturation was shown to be a production barrier as it reduces growth and flesh quality (McClure et al., 2007). Furthermore, the natural anadromous life cycle of the salmon is overlooked, and the fish may use energy to change the osmoregulation by smoltifying and desmoltifying, which will also negatively affect the growth (McCormick and Saunders, 1987; Jørgensen and Jobling, 1994; Johansson et al., 2016). It has been shown that salinity over 15‰ in Atlantic salmon cultivation can inhibit desmoltification (Mortensen and Damsgård, 1998), indicating that optimal growth of the post-smolts should be above this salinity. Higher salinity can be introduced by moving the smoltified fish from a freshwater system to a post-smolt system run with brackish or seawater (as done by e.g. Erko Settefisk AS, Stord, Norway). This may lead to stress for the fish due to handling and transport. Alternatively, seawater can be introduced into the RAS used for freshwater cultivation of juveniles. This results in a major change in the environmental conditions for the water treatment system and the functionality of the bacterial populations adapted to the freshwater system. It could, however, be a better option as it is a more gentle way of changing the environment for the fish, and because the transportation is reduced. Bacteria in RAS are vital for conversion of waste nutrients to maintain high water quality (Blancheton et al., 2013). Furthermore, bacteria have an important function for fish health, by facilitating nutrient adsorption and digestion in the gut, and for functional development including the immune system (Fraune and Bosch, 2010; Gomez et al., 2013; Llewellyn et al., 2014). However, little is known about the bacterial dynamics and the functionality of e.g. nitrifying bacteria during the transition from freshwater to seawater in RAS.

The biofilter function in RAS is especially vulnerable to salinity increase. Several studies have shown negative effects of salinity increase on nitrification efficiency and changes in bacterial community dynamics of ammonia oxidizing (AOB) and nitrite oxidizing bacteria (NOB) in wastewater treatment (WWT) (Uygur and Kargı, 2004; Aslan and Simsek, 2012; Wang et al., 2015; Cortés-Lorenzo et al., 2015). It is, however, not straightforward to transfer this knowledge to RAS for fish production as the studies have different; 1) environmental biofilm history, 2) species present in the biofilter, 3) temperatures and pH (Moussa et al., 2006), and 4) the concentrations of total ammonia nitrogen (TAN) and nitrite nitrogen (NO_2-N) in RAS with Atlantic salmon are substantially lower than in WWT. The latter can affect the nitrifying community dynamics and nitrification efficiency, as TAN usually is the rate limiting substrate in RAS, compared to oxygen in WWT (Chen et al., 2006; Rusten et al., 2006). TAN and NO_2-N in RAS with Atlantic salmon production should be below 2 mg L^{-1} and 0.1 mg L^{-1}, respectively, to avoid toxicity for the fish (reported for freshwater RAS from the Norwegian Food Safety Authority, 2016). Nitrification efficiency has shown to decrease after abrupt salinity changes as the nitrifying bacteria are inhibited by osmotic stress (Gonzalez-Silva et al., 2016; Kinyage et al., 2019). Increasing the salinity in a RAS can cause accumulation of toxic levels of TAN and nitrite, and potentially lead to mortality of the fish. Studies have shown that the nitrification process/efficiency can recover after or during increased salinity (Bassin et al., 2012; Quartaroli et al., 2017; Navada et al., 2019). Bacteria can adapt to higher salt stress (Zahran, 1997; Oren, 2011), and the increase of salinity in a RAS with fish is therefore possible.

RAS is a complex ecosystem with bacteria associated with water, fish and biofilter. Different salinities have been shown to alter the bacterial community structures in the water of RAS (Bakke et al., 2017; Rud et al., 2017), and the gut microbiota of salmon change during the transition from freshwater to seawater (Rudi et al., 2018). It is not clear how much of the bacterial community dynamics that is a physiological salinity adaptation process relative to succession causing change in community structure and introduction of new species. More knowledge is needed to understand the bacterial adaptation versus succession during such shifts in environmental conditions in RAS. The fish are exposed to a sudden and major change in environmental microbes during the transfer to sea at a vulnerable stage. Bacterial diseases documented in sea cages have been associated with the transfer of smolt to the sea (Eggset et al., 1997; Johansson et al., 2016; Hjeltnes et al., 2019). It is not known whether the bacteria associated with the smolt on land prior to transfer to sea affects the susceptibility for diseases at sea. How the changes in salinity affects the microbial water quality and nitrification efficiency in RAS, and linking it to fish performance before and after transfer to sea have not been addressed before, and was the motivation for our study.

We hypothesize that a gradual increase of salinity in RAS acclimatize both the microbiota associated with the fish and the biofilter, resulting in more robust fish at transfer to sea and a more functional biofilter on land. This hypothesis was tested experimentally in two regimes for brackish to seawater transition in production of Atlantic salmon post-smolt in RAS with subsequent transfer to sea. One regime was a gradual increase in salinity in RAS, whereas the other was a direct transfer of smolt to a RAS with high salinity. More specifically we evaluated 1) how a gradual increase in salinity in RAS affected the microbial community structure and dynamics in water and biofilter, 2) how the nitrification efficiency of the biofilter adapted to the two regimes for change in salinity, and 3) how the different salinity regimes affected fish growth and gut microbiota before and after transfer to sea cages.

2. Materials and methods

2.1. Experimental set-up and operational conditions during the study

The experiment was carried out at the LetSea RAS facility Bjørn and sea cage locality Havstein, both on/by Dønna (Nordland, Norway, 66°05′ N 12°31′ E). Two separate commercial scale RAS were used for the study. One system was a seawater/high salinity brackish water RAS (sRAS) that had previously been operated with 18–33‰ salinity for 3 years. The other system was identical, but a brackish water RAS (bRAS) operated with 3–5‰ salinity for 6 months before this experiment. Prior to that, bRAS had been operated with 20‰ salinity for 3 years. Seawater was pumped into the facility from 140 m depth, treated with a mechanical screen (200 μm) and UV. The freshwater came from the municipal freshwater distribution system. For both systems, seawater and freshwater were mixed to give their respective salinities. Each system (Fig. 1A), delivered by AKVA Group, had a total volume of 1200 m^3. They included 18 fish tanks (34 m^3) each and a water treatment loop with particle removal through a mechanical drum screen sieve (mesh 60 μm), CO_2-degasser and a split loop with 1/3 of the waterflow going through a so-called fixed fluidized biofilter (147 m^3, 50% filling, carriers with specific surface area of 600 m^2 m^{-3}, giving a total biofilm area of 44,100 m^2). Make-up water was 1% of total system volume per hour (12 m^3 h^{-1}), giving 75% water recirculation per day. The pH was controlled with automatic addition of 0.3 M sodium hydroxide (NaOH). The fish were fed commercial pellets from automatic feeders (on land: Intro Q 200, at sea: Energy X 1000 and Power Extreme 2500, Biomar).

Atlantic salmon (*Salmo salar*) parr with average weight of 37 ± 11 g (± SD), were stocked (200,000 individuals) in bRAS (06.03.2018). When the fish had smoltified and reached approximately 100 g (01.06.18), the smolt were randomized and split in two groups. One group (100,000 fish) remained in bRAS at 3‰, whereas the other group (100,000 fish) was stocked in sRAS at 25‰ (Fig. 1B). The fish were counted with an automatic fish counter (AquaScan, Norway) during stocking, to ensure the same number of individuals in each system and fish tanks. The post-smolts were kept in the two experimental RAS for 60 days to acclimatize and to resume appetite after

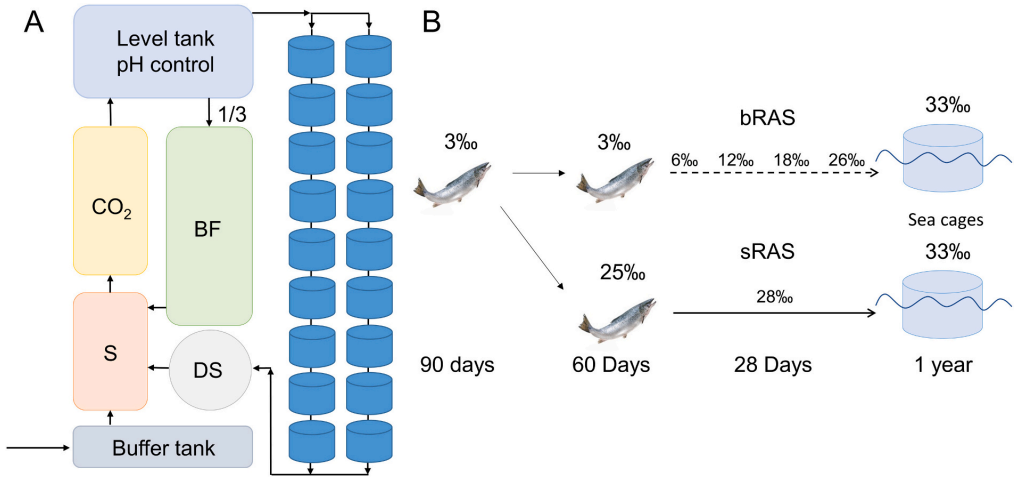

Fig. 1. A) Set-up of each RAS. 18 fish tanks (blue), DS = drum screen sieve, S = Sump, CO_2 = CO_2-degasser, BF = biofilter (1/3 of total flow), make-up water was added to the buffer tank. B) Experimental overview. All the fish were stocked in bRAS with 3‰ salinity, and after 90 days the fish group was split. Half the group remained in bRAS and the other was moved to sRAS with 25‰ salinity. Then the fish were kept in their respective RAS for 60 days, whereafter the salinity was slowly increased in bRAS over 28 days and the salinity in sRAS remained stable at 28‰. Afterwards both fish groups were transported to two separated sea cages, for grow-out to market size for 1 year.

having been moved. The salinity in sRAS was increased from 25 to 28‰ after the 60 days, and remained stable through the rest of the experiment. In bRAS the salinity was slowly increased over a period of 28 days (03.08.2018–31.08.2018). The salinity was increased with 0.5 to 1.0‰ per day. Seawater was continuously mixed into the buffer tank in bRAS, and the salinity was usually 1–2‰ higher in here than in the fish tanks as it took some time for the water to mix in the system. The experimental plan was to increase the salinity in bRAS as much as possible without exceeding a concentration of 2 mg L^{-1} TAN and 0.5 mg L^{-1} NO_2-N (Norwegian Food Safety Authority, 2016) in the 28 days. These concentrations were measured daily, and if the concentrations reached the upper threshold, the feeding in bRAS was stopped and the seawater flow into the buffer tank was reduced. After the TAN and NO_2-N concentrations declined, feeding was resumed. At day 28, a salinity of 26‰ was achieved. After the termination of the salinity experiment, both fish groups were transported separately in a well boat to two individual sea cages at Havstein. The fish grew in the sea cages for around 1 year to a size range of 4.6–4.8 kg.

2.2. Daily management, measurements of water quality and fish weighing

All fish tanks/sea cages were inspected daily, and dead fish were removed and registered. In both RAS, water quality variables were measured in the water of the level tank (Fig. 1A). Temperature and oxygen were logged automatically every 10 min in the fish tanks. Salinity was measured with a handheld salinity meter (OxyGuard, Denmark). Total ammonia nitrogen (TAN), nitrite and pH were analysed with a Photometer 7100 (Palintest, UK). Dissolved CO_2 was measured with a CO_2-analyser (OxyGuard, Denmark) once a week. All other water quality variables were measured daily. The biofilter was backwashed two times a week. At each weighing of the fish on land, 25 individual fish from each tank were weighed manually (sum: 450 fish per system) and put back into the fish tanks. After the sea cage phase, all the fish were transported to the MOWI salmon slaughterhouse on Herøy (Nordland, Norway) for weighing and slaughtering.

2.3. Sampling for microbiological analysis from water, biofilter biofilm and salmon faeces

Samples for microbiological analysis were collected in both sRAS and bRAS when the salinity of bRAS was 3, 6, 12, 18 and 26‰. Samples of the water were collected also after the fish groups were split. Water samples were collected in the outlet of three fish tanks and in water going in and out of the biofilter. The water samples (approximately 200 mL) was filtered with 60 mL syringes through sterile 0.22 μm filters (Sterivex™) to collect bacteria. To collect biocarriers form the biofilter, metal grids which held the carriers together had to be drilled open. The middle of the biofilter was most accessible for this operation, and two biofilm carriers were collected from this location. Fish for faecal sampling were netted from the fish tanks into in a smaller tank and euthanised within the guidelines for animal welfare given by the Norwegian Food Safety Authority (with an overdose of anaesthetics, Benzoak vet. 200 g L^{-1}). At each sampling, four fish were taken from the same three fish tanks as the water samples were collected. The faeces were squeezed out of the gut of the fish into a petri dish, and afterwards transferred to a sterile 2 mL Cryotube. From the sea cages, faecal samples were collected from 10 fish in each cage 3 weeks after sea transfer. All samples were stored at −20 °C until further analysis.

2.4. Nitrification capacity stress test

Nitrification capacity stress tests were performed to determine the maximum ammonia oxidation capacity (AOR_{max}). This was to evaluate short term robustness to salinity changes of the carriers in bRAS when the salinity of the system was 5, 12 and 20‰. Freshwater and seawater mixed to salinities of 5, 12, 20 and 28‰, respectively, were added to four 1 L batch reactors. Biofilter carriers from bRAS were put in the reactors (30% filling, 220 carriers giving a total surface area of 0.18 m^2 in each reactor). The water volume was 0.95 L and the carrier volume was 0.30 L in each reactor. Ammonium sulphate ($(NH_4)_2SO_4$) was added to each reactor to feed the ammonia oxidizing bacteria (AOB). Start concentration of TAN was 15 mg L^{-1} in the first stress test when tank salinity was 5‰, and 24 mg L^{-1} for the tests when tank salinity was 12 and 20‰. The nitrite oxidizing bacteria (NOB) were only fed nitrite from the oxidized ammonium. The reactors had aeration from

aquarium pumps to maintain oxygen concentration of approximately 8.2 mg L^{-1} in each reactor (measured with a Handy Polaris 2 dissolved oxygen meter, OxyGuard, Denmark). The capacity tests were run for 210 min, with samplings for TAN and NO_2-N after 10 min and then every 30 or 60 min. Approximately 20 mL of water was sampled from each reactor with a syringe and filtered through a glass microfiber filter (Whatman GF/F, GE Healthcare, UK) for particle removal. TAN, NO_2-N and pH were measured in the filtrate with Photometer 7100 (Palintest, UK). Sodium bicarbonate ($NaHCO_3$) was added to the reactors to maintain the pH at 7.5–7.9, and the temperature was 14 ± 1 °C throughout the capacity tests.

2.5. Bacterial community composition

Bacterial community composition was determined by high throughput sequencing of PCR amplicons of a ≈ 450 base pair long stretch of the 16S-rRNA gene. DNA extraction was conducted with the DNeasy 96 Blood and Tissue kit (Qiagen, Germany). The filters with water samples and biofilm carriers were cut into small pieces with a sterile scalpel and put into tubes supplied by the kit. Faecal samples were transferred directly to the tubes. The manufacturers' protocol was followed with minor alterations. An extra lysis step was added in the beginning of the protocol to ensure lysis of Gram-positive bacteria. This was done with an enzymatic lysis buffer (180 μL) consisting of 2 mM EDTA, 20 mM Tris-HCl (pH 8), 1.2% Triton and lysozyme (20 mg/mL), and 1 h of incubation at 37 °C. PCR and Illumina sequencing was done according to Fossmark et al. (2020). The exception was that the V3 and V4 region of the 16S rRNA gene was targeted for sequencing, by the use of forward primer 338F 5′- tcg tcg gca gcg tca gat gtg tat aag aga cag nnnn CCT ACG GGW GGC AGC AG-3′, and reverse primer 805R 5′- gtc tcg tgg gct cgg aga tgt gta taa gag aca g nnnn GAC TAC NVG GGT ATC TAA KCC -3′. Illumina adapter sequences are in lower case letters. The USEARCH pipeline (version 9.2) was used to process the Illumina sequencing data. All steps were done according to Fossmark et al. (2020), with the exception that reads shorter than 400 base pairs were considered co-amplified unwanted reads (e.g. mitochondrial salmon DNA), and were therefore filtered out. For water and faecal samples, the Ribosomal Database Project (RDP version 16: Cole et al., 2013) was used as reference to assign taxonomy to the OTUs. For the biofilter biofilm samples, the taxonomic assignment for nitrifying bacteria was rather poor or not classified at all with the RDP reference database. Given that nitrifying activity was observed in the experiment, this contradicted the classification given by RDP. For that reason the biofilter biofilm samples were checked with a different database, i.e. Microbial Database for Activated Sludge (MiDAS version 3: Nierychlo et al., 2019). Using MiDAS for taxonomy assignment, nitrifying OTUs were classified at high confidence threshold and the results reported for biofilter biofilm microbiota is therefore from MiDAS. The faecal samples contained many OTUs from the phylum Cyanobacteria, including chloroplasts. These OTUs were removed from the dataset before further analysis. To avoid bias due to variation in sequencing depth between samples, the water and biofilm samples were normalized to 20,000 reads (lowest read count), whereas the faecal samples were normalized to 4000 reads (lowest read count after removal of reads representing Cyanobacteria). OTUs of interest were further investigated with the SeqMatch tool for type strains at the RDP website and the BLAST search tool (Altschul et al., 1990).

2.6. Data analysis and statistics

The program package PAST version 3.21 (Hammer et al., 2001) was used to perform statistical analyses and to calculate bacterial community diversity index. Shapiro-Wilk tests (Shapiro and Wilk, 1965) were performed to check for normality in the water quality variables and fish weight data from the land phase. Two-Sample *t*-tests were used on data that were normally distributed, and Mann-Whitney-Wilcoxon-tests were used on data that did not. After the sea cage phase, the fish were automatically divided by size into weight-groups in the ranges 1–2, 2–3, 4–5, 5–6, 6–7 and over 7 kg. Afterwards all the individuals in each range were weighed, and total weight in each range was given. The average weight was based on the sum of all the fish slaughtered divided by total number of individuals. To estimate the number of individuals in each weight range, we assumed that the average weight for each of the size classes were 1.5, 2.5, 3.5, 4.5, 5.5, 6.5 and 7.5 kg. A Chi-square test (χ^2) was performed on a contingency table consisting of the estimated numbers of fish in each range in bRAS and sRAS to determine if the size frequency was significantly different between bRAS and sRAS. The Alpha-diversity index of the bacterial communities was OTU richness (total number of OTUs). Beta-diversity was calculated based on the presence/absence-based Sørensen-Dice similarity and the abundance-based Bray-Curtis similarity (Chao et al., 2006). Ordination by Principal Coordinate Analysis (PCoA) with normalized and square root transformed data based on Bray-Curtis similarities was used to visualize the similarities and development (succession) of the bacterial communities in bRAS and sRAS. One-way permutational multivariate analysis of variance (PERMANOVA) with Bray-Curtis similarities was performed to test for statistically significant difference between different groups of samples (Anderson, 2017). Maximum ammonia oxidation rates (AOR_{max}) during the capacity stress tests were determined from linear regression of TAN concentration versus time in the reactors.

3. Results

3.1. Nitrification and water quality in bRAS and sRAS

During the salinity increase, the concentrations of TAN and NO_2-N in bRAS (Fig. 2) were thoroughly monitored to prevent toxic concentrations for the fish. As the salinity increased from 3‰ to 7‰, TAN and NO_2-N increased from 0.75 to 1.8 and 0.1 to 0.3 mg N L^{-1}, respectively. Then the feeding was stopped, and the concentrations dropped to 0.4 mg L^{-1} for TAN and 0.18 mg L^{-1} for NO_2-N. The feeding was resumed, and the concentrations increased a second time to similar levels as before. The feeding was again stopped, and the concentrations declined again (0.4 mg L^{-1} TAN and 0.08 mg L^{-1} NO_2-N). A third increase in the concentrations was seen, TAN increased to 1.5 mg L^{-1} and NO_2-N to 0.17, and the feeding was stopped again. At this point, the salinity was 12‰, and thereafter TAN and NO_2-N remained below 1.1 and 0.16 mg L^{-1}, respectively. The biofilter function was relatively stable when the salinity was > 12‰.

The average temperature in bRAS was significantly higher than in sRAS (Table 1) throughout the experiment, both during the acclimatization period ($p < 0.001$, 2.6 °C higher) and during the salinity increase ($p < 0.001$, 1.0 °C higher). The temperature in the sea (which sRAS got most of the water from) was lower than the water from the freshwater distribution system (which bRAS got most of the water from), causing the temperature in bRAS to be higher. The average concentrations of TAN and nitrite were significantly higher in sRAS than in bRAS through the experiment ($p < 0.05$). Even though TAN and NO_2-N peaks were observed during the salinity increase in bRAS (Fig. 2), the concentrations in sRAS were still on average higher. This shows that the nitrification efficiency was overall lower in sRAS with stable high salinity than in bRAS with increasing salinity.

3.2. Salinity dependent nitrification capacity of biofilter carriers from bRAS during adaptation to increasing salinity

The maximum ammonia oxidation capacities (AOR_{max}) of the carriers when the salinity in bRAS was 5‰ were inhibited 25 and 37% when stressed with a salinity of 12 and 28‰, respectively (Fig. 3). The reactor stressed with 28‰ had the highest reduction and the longest lag-phase of 90 min before nitrification could be observed (Supplemental S1). When the salinity was 12‰ in bRAS, no lag-phases were

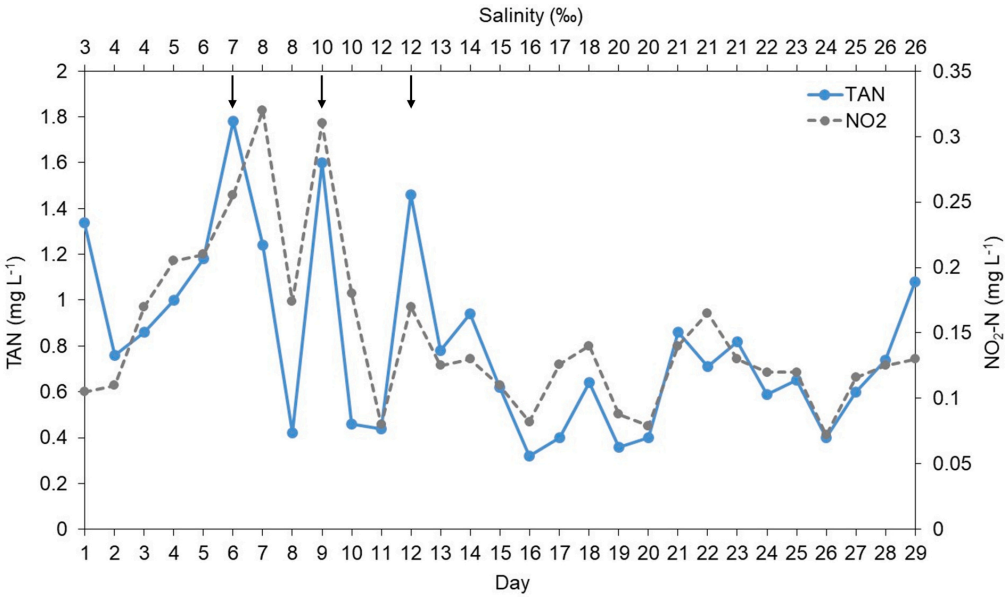

Fig. 2. Concentrations of TAN and NO_2-N in bRAS through the experiment during salinity increase. Samples collected from level tank. Arrows show stop of feeding for 1 day.

observed in any of the reactors. The AOR_{max} was inhibited by 40, 25 and 17% when stressed with 28, 20 and 5‰ salinity, respectively. When the salinity in bRAS was 20‰, the reactor stressed with 5‰ had the lowest AOR_{max} (37% reduction) and longest lag-phase. The AOR_{max} in the native salinity reactors (reactors holding the salinity that bRAS was operated with at that time) were similar in each capacity test, with around 0.40 g N m^{-2} d^{-1}. When bRAS was at 12 and 20‰ salinity, the native reactors had the highest $AOR_{max.}$ These results show that the AOBs were affected negatively by deviations from the native bRAS salinity. When bRAS was at 5‰ salinity, the reactors stressed with higher salinity (20 and 28‰) had lag-phases, and when bRAS was at 20‰ salinity, the reactors stressed with lower salinity (5 and 12‰) had lag-phases and lower AOR_{max}. More details on the regression analysis can be found in the Supplemental Table S1.

Regardless of salinity in bRAS, the concentration of NO_2-N throughout all the capacity tests was highest in the 28‰ salinity reactor and lowest in the 5‰ reactor (Fig. 4). Even though the AOR_{max} in general decreased when the carriers were subjected to higher or lower osmotic stress than their native salinity, this was not reflected in the NO_2-N concentrations. The concentrations in the 28‰ salinity reactors at the end of the capacity tests were a factor 3.4, 3.0 and 5.6 higher than in the 5‰ reactors at 5, 12 and 20‰ native bRAS salinity, respectively. These results indicate that the AOBs are negatively affected by deviations from native salinity, whereas the NOBs are negatively affected by increased salinity. Due to high oxygenation of the reactors, possible denitrification was not included in the nitrification analysis.

3.3. Bacterial community composition

The Illumina sequencing yielded a total of 4255 OTUs from the raw data. The sequencing depth was on average 40,000 reads for water and biofilter biofilm samples. For faecal samples the number of reads was variable, ranging from 4000 to 80,000 reads. However, 50 to 90% of the reads in the faecal samples were classified as *Streptophyta*, and are likely from higher plants or algae used as feed ingredients. These reads were removed from the dataset before further analysis. After normalization and removal of plant/algal OTUs, there were a total of 3064 OTUs in the water, 1602 OTUs in the biofilter biofilm and 1480 OTUs in faecal samples.

3.3.1. Nitrifying bacteria in the biofilter

In the biofilter biofilm in both systems, we observed nitrifying OTUs belonging to the following genera: *Nitrosomonas* 2 OTUs, *Nitrosospira* 1

Table 1
Water quality variables (± SD) in bRAS and sRAS.

Variable	bRAS		sRAS	
	After split 3‰	3–26‰	After split 25‰	28‰
	Day −60 - 0	Day 1–28	Day −60 - 0	Day 1–28
Temperature (°C)	16.1 ± 0.8[a]	15.2 ± 1.1[a]	13.5 ± 0.9[a]	14.2 ± 0.7[a]
Salinity (‰)	4.0 ± 1.4[a]	Fig. 2	25.7 ± 3.2[a]	28.2 ± 0.7
pH	7.0 ± 0.3	7.1 ± 0.6	7.1 ± 0.3	7.1 ± 0.2
TAN (mg L^{-1})	1.1 ± 0.7[a]	0.8 ± 0.4[a] (Fig. 2)	1.4 ± 0.9[a]	1.2 ± 0.3[a]
NO_2 – N (mg L^{-1})	0.1 ± 0.2[a]	0.1 ± 0.1[a] (Fig. 2)	0.3 ± 0.3[a]	0.2 ± 0.04[a]

[a] Statistically significant, $p < 0.05$.

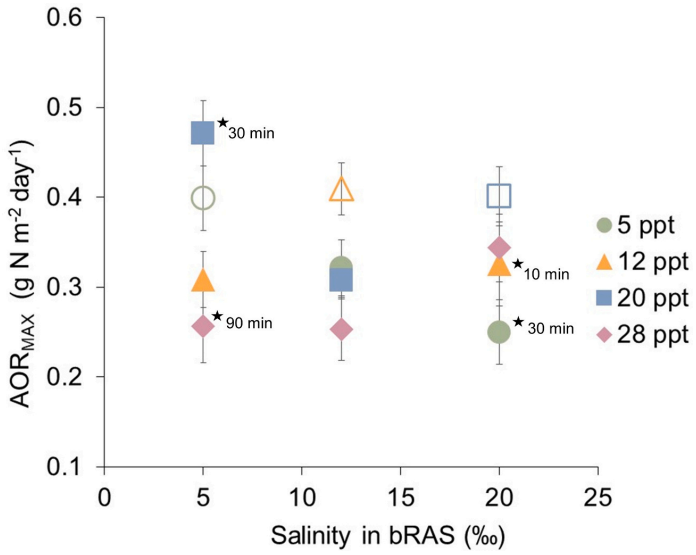

Fig. 3. Maximum ammonia oxidation capacity (AORmax) ± SE of biofilter carriers from bRAS during capacity stress tests in reactors with 5, 12, 20 and 28‰ salinity (legend). Empty symbols show reactor with native bRAS salinity. Black stars show reactors with a lag-phase in the beginning of the stress test, and with indication of the length of the lag-phase. A stress test at native salinity of 28‰ was not conducted.

OTU, *Nitrospira* 15 OTUs and *Nitrolancea* 3 OTUs. However, only 2 out of the 21 nitrifying OTUs had a relative abundance higher than 0.1%, and these were included in Fig. 5. The *Nitrosomonas* OTU_77 and *Nitrospira* OTU_45 were present in both sRAS and bRAS, thus these nitrifying OTUs can be present both at low and higher salinity. In bRAS as the salinity increased, the *Nitrospira* OTU_45 decreased in relative abundance from around 16% at Day 1 (3‰) to 10% on Day 19 (18‰), and by Day 28 (26‰) the abundance was 0.5%. The *Nitrosomonas* OTU_77 had a relative abundance of around 1% from 3 to 6‰ salinity, then it increased to 8% at 18‰ salinity. In sRAS, the relative abundance of *Nitrosomonas* OTU_77 was low throughout the experiment, around 1%, and *Nitrospira* OTU_45 had an abundance of 3–5%.

3.3.2. Bacterial community structures and succession in the water

To visualize the temporal development of the microbial communities in bRAS and sRAS water, ordination by Principal Coordinate Analysis (PCoA) based on Bray-Curtis similarities was performed. The ordination for the first two axes explained 57% of the variance in the dataset (Fig. 6A), and showed that the samples from sRAS and bRAS were fully separated on Axis 1. The samples from sRAS clustered throughout the experiment, but samples from the water going out of the biofilter were slightly separated from the water sampled from the fish tanks and water going in to the biofilter. For bRAS the samples were more spread and a succession of the microbial communities due to salinity increase can be observed through the experiment, primarily along Axis 2. Also in bRAS, the samples from the biofilter were separated from the fish tank samples. Examining the third axis of the ordination, (Fig. 6B) the samples in sRAS at the split of the systems (D-60) were clearly separated from the rest of the samples. One-way PERMANOVA confirmed that when samples were grouped by sampling day and treatment, all groups were significantly different ($p < 0.03$), including the samples of sRAS.

Bray-Curtis and Sørensen-Dice similarities were calculated to examine the succession of the microbial communities in the water between two succeeding sampling times within each system. In bRAS (Supplemental S2A), comparing the samples at the split of the systems on Day −60 and Day 1 was the most dissimilar comparison of succeeding samples, for both Sørensen-Dice and Bray-Curtis (0.5 and 0.3, respectively). The salinity was the same for this period (3‰), however this comparison had the longest interval in days and a change in fish biomass in both systems. The Bray-Curtis similarities comparing salinities 3–6‰ and 6–12‰ were around 0.4. Then after 12‰ the comparisons increased to 0.6 and were approximately at the same level as Sørensen-Dice. This indicate that the change in abundance of the OTUs present up to 12‰ contributed more to the succession of the microbial communities than introduction of new OTUs. Whereas after 12‰ and up to 26‰, the succession is equally contributed by introduction of new OTUs and change in abundance of present OTUs. This furthermore

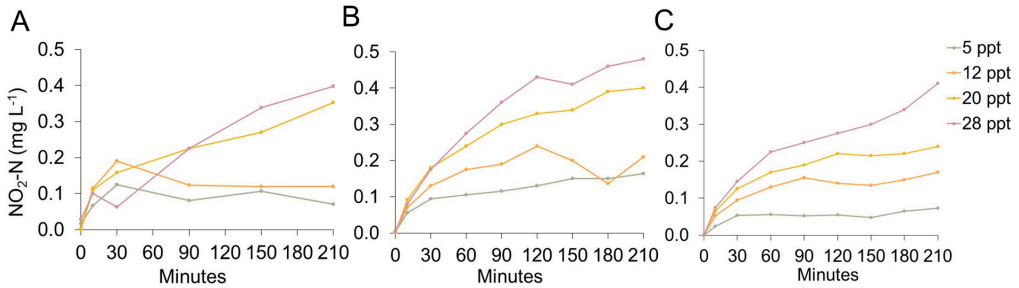

Fig. 4. Concentrations of NO_2-N in the reactors with bRAS carriers during capacity stress tests with 5, 12, 20 and 28‰ salinity (legend) when the system salinity was A) 5‰ B) 12‰ and C) 20‰.

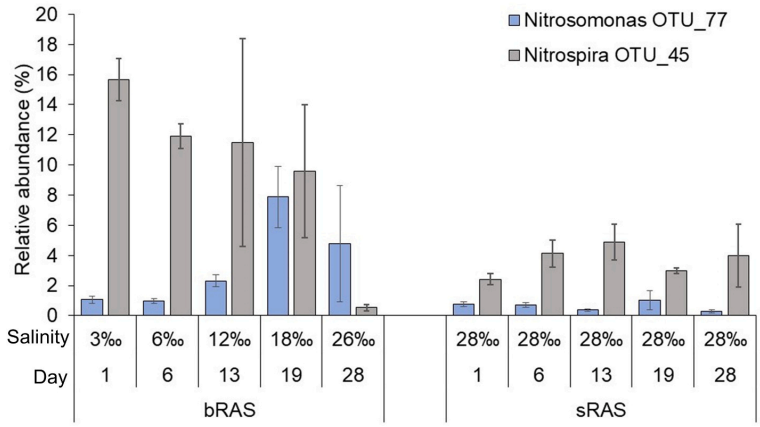

Fig. 5. Nitrifying OTUs found in the biofilter biofilm carriers in bRAS and sRAS with a relative abundance > 0.1%.

suggests that the OTUs with low abundance are replaced with new OTUs. Comparing the water samples in bRAS at Day 1 and Day 28 (3 vs 26‰) (Supplemental, Table S2), the Bray-Curtis similarity was around 0.3, and at the same value as when comparing Day −60 and Day 1. Even though the salinity was the same on Day −60 and Day 1 (3‰), the Bray-Curtis similarity is the same as when comparing the two sampling times with 8.7 times salinity increase. For sRAS (Supplemental S2B), the comparison between Day −60 and Day 1 was also the most dissimilar (Bray-Curtis: 0.37, Sørensen-Dice: 0.5). Salinity was a driver for succession, but other factors (e.g. change in fish biomass) also contributed to the change in bacterial community composition.

There were 1767 and 2171 OTUs in total found in bRAS and sRAS water, respectively. The number of OTUs that were shared in both systems was 874, constituting 50 and 40% of total OTUs in bRAS and sRAS, respectively. The most abundant OTUs in bRAS and sRAS (Fig. 7) were found in both systems. However, the general trend was that if the OTU was in high relative abundance in one system, it was low in the other. Examples of this was OTU_8, *Gemmobacter.* This OTU was highly abundant in bRAS and had very low abundance in sRAS. In bRAS OTU_8 decreased in relative abundance from 33% at Day −60 to 4% at Day 1 and then increased to 14% by Day 13 at 12‰ salinity. After 12‰ salinity, it decreased again to 5% by the end of the experiment. The relative abundance of OTU_196 (*Pseudohodobacter)* increased as the salinity increased from 3% to 22% through the experiment form Day 1 to 28. The opposite was observed for OTU_13, *Mycobacterium.* This OTU decreased in relative abundance from 19% to 3% as the salinity increased through the experiment. *Loktanella* (OTU_3) had very low abundance in bRAS at salinity 3–12‰ salinity, however at 18‰ and 26‰ salinity the abundance increased to 2 and 4%, respectively. *Loktanella* is highly abundant in sRAS (from 7 to 30% through the experiment), thus *Loktanella* succeeds with salinities higher than 12‰. Similar trends are seen for *Leucothrix* (OTU_16) as it increased from 1 to 7% in relative abundance in bRAS from 18‰ to 26‰ salinity and had a relative abundance in sRAS from 4 to 10%.

The OTU richness in bRAS at each sampling and the number of shared OTUs between sampling times are presented in Table 2. On Day 1 (3‰), the total number of OTUs were 1087, and as the salinity increased up to 26‰, there was a decrease in the number of shared OTUs from Day 1 to Day 28. Out of the total OTUs present on Day 1, 47.9% were still present on Day 28 with 26‰ salinity. From Day −60 to Day 1, the total number of OTUs increased a factor 1.73. On Day −60 half the fish group was moved to sRAS. This shows that a reduction in fish biomass and organic loading, increased the total number of OTUs. In

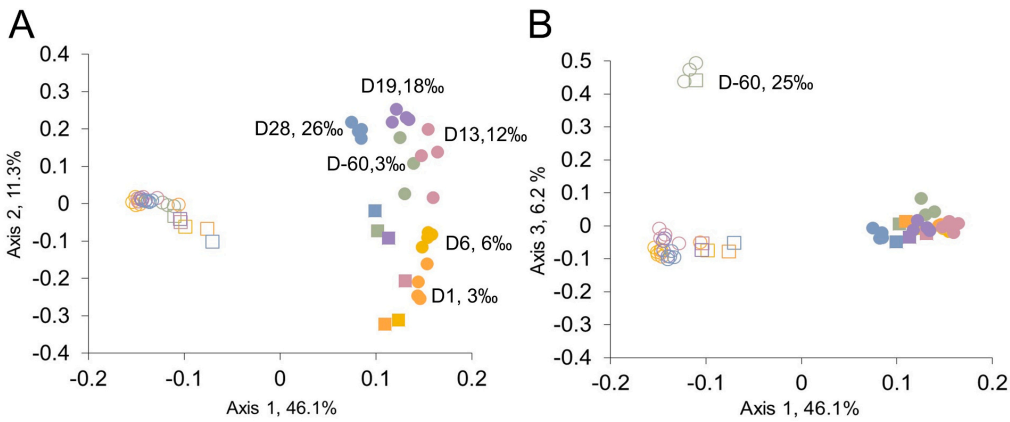

Fig. 6. Ordination by PCoA based on Bray-Curtis similarities for water samples. Filled symbols are bRAS and empty are sRAS. Circles are water going out of the fish tanks and into the biofilter, squares are water going out of the biofilter. Same colour is same sampling day, green = Day −60, orange = Day 1, yellow = Day 6, pink = Day 13, purple = Day 19, blue = Day 28.

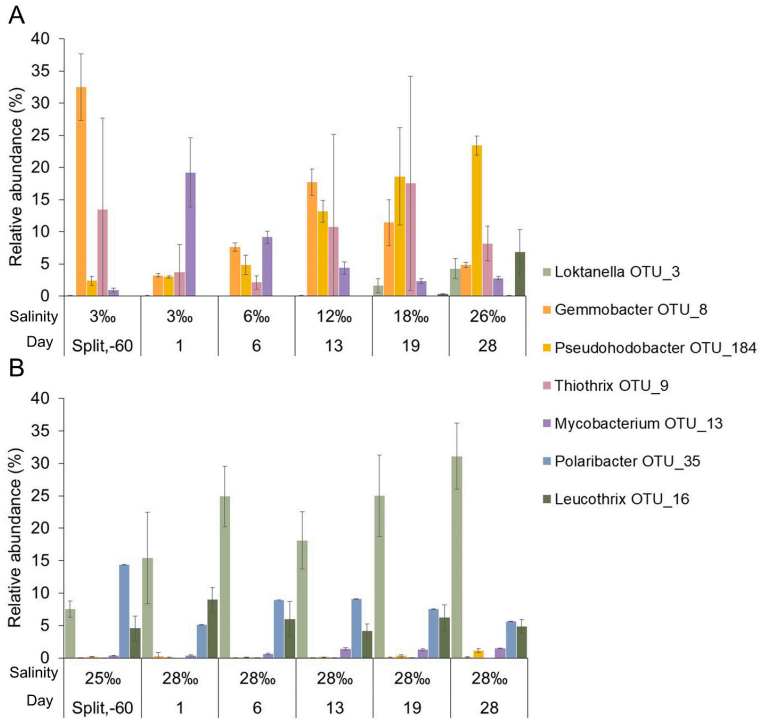

Fig. 7. Most abundant OTUs in the fish tank water of A) bRAS and B) sRAS. All OTUs are classified for the genus with a confidence threshold of 0.8.

Table 2
Matrix of the richness (total number of OTUs) at each sampling in bRAS (bold) and number of shared OTUs between the samplings. Percentage shows how many of the total OTUs at one sampling were present in the comparing sampling later in the experiment.

bRAS OTUs	Day −60, 3‰	Day 1, 3‰	Day 6, 6‰	Day 13, 12‰	Day 19, 18‰	Day 28, 26‰
Day −60, 3‰	**625**	77.4%	66.7%	66.2%	56.8%	57.4%
Day 1, 3‰	484	**1087**	54.4%	55.7%	45.5%	47.9%
Day 6, 6‰	417	591	**738**	74.5%	61.6%	60.1%
Day 13, 12‰	414	602	550	**802**	65.3%	64.8%
Day 19, 18‰	355	495	455	524	**669**	73.8%
Day 28, 26‰	359	521	444	520	494	**749**

sRAS the opposite was observed, and the total number of OTUs decreased a factor 1.4 from Day −60 (1481 OTUs) to Day 1 (1051 OTUs) as 100,000 fish were stocked in the system.

The taxa summary of bacterial orders in the water samples (Supplemental S3) showed a change in the relative abundance of the taxa in bRAS through the experiment, whereas for sRAS there was less variation. Going from Day −60 to Day 1 in bRAS, the salinity was the same (3‰), but a change in the most abundant orders Rhodobacteriales, Thiotrichales, Burkholderidales and Actinomycetales are evident. In the fish tanks, Rhodobacteriales and Thiotrichales decreased in relative abundance from 40 to 50% and 20–25% to 10% and 2–10%, respectively. Burkholderidales and Actinomycetales increased in relative abundance from 3 to 5% to 10–15%. The bacterial communities remained at these relative abundances during the salinity increase from 3 to 6‰ (Day 1–6). On Day 13 (12‰) and throughout the salinity increase to Day 28 (26‰), the relative abundance of Rhodobacteriales (40–50%), Thiotrichales (10%), Burkholderidales (5–30%) and Actinomycetales (3–5%) remained relatively stable even though the salinity more than doubled (Marked in red: Supplemental S3). The samples from the water going out of the biofilter differentiated from the fish tanks and biofilter in samples, which was evident for both systems. In sRAS, the most abundant orders were Rhodobacteriales and Flavobacteriales, and less change in the relative abundance were observed here compared to bRAS which coincides with the PCoA-plot (Fig. 6).

3.3.3. Faecal microbiota

The faecal microbiota of the individuals within the fish tanks had a Bray-Curtis similarity of around 0.5 in bRAS and 0.5–0.6 in sRAS on Day 28, when salinity was 26–28‰ (Fig. 8A). The faecal microbiota of fish in the sea cages had a lower Bray-Curtis similarity in both systems, with 0.22 and 0.30 for the bRAS and sRAS group, respectively. As the salinity increased in bRAS, the faecal microbiota of the individuals became more similar to the faecal microbiota of the sRAS individuals and increased from 0.2 at 3‰ to 0.5 at 26‰ salinity (Fig. 8B). The Bray-Curtis similarity of the individuals within a fish tank on Day 28 and the similarity between bRAS and sRAS the same day were similar and around 0.5. This means that the variation in faecal microbiota between individuals within a fish tank was comparable to the variability between the two different systems that had different water

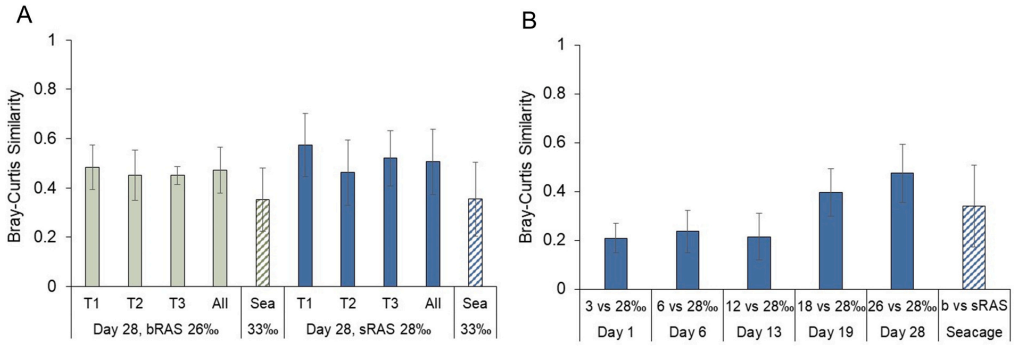

Fig. 8. A) Bray-Curtis similarities for comparing the faecal microbiota of individual fish in bRAS and sRAS within each fish tank (T1, T2 and T3), within each system (All) at Day 28, and all individuals in each sea cage (Sea). B) Bray-Curtis similarities comparing faecal microbiota in bRAS and sRAS at each sampling. Data are the mean ± SD of all values in the similarity-matrix

Table 3
Weight and mortality data of the fish in bRAS and sRAS and in the sea cages.

Period	Average weight ± SD (g)	
	bRAS	sRAS
Split of systems/acclimatization (Day −60)	91 ± 10.4[a]	108 ± 6.2[a]
Start salinity increase (Day 1)	240 ± 36.2	235 ± 11.9
End of salinity increase (Day 28)	299 ± 40.5[a]	339 ± 19.0[a]
End of sea cage phase (~1 year)	4692[a]	4817[a]
	Mortality (%)[b]	
	bRAS group	sRAS group
On land in RAS	4.3	16.7
Sea cage	23.5	26.0
Total mortality	27.8	42.7

[a] Statistically significant, $p < 0.05$.
[b] Both fish groups were diagnosed with CMS, which can explain the high mortality.

microbiota. Comparing the faecal microbiota of the fish before and after sea transfer, the Bray-Curtis similarity was 0.26 ± 0.10 and 0.30 ± 0.12 for bRAS and sRAS, respectively. A *t*-test confirmed that the difference was not significant ($p = 0.11$), thus both the fish groups changed the faecal microbiota to the same degree after sea transfer.

3.4. Performance of fish

After the split of the systems, the fish in sRAS were 19% bigger ($p < 0.001$) than the fish in bRAS (Table 3). During the acclimatization period (From Day −60 to Day 1), fish in sRAS grew a factor 2.2 and in bRAS a factor 2.6. The higher growth in bRAS can be explained by the higher temperatures and also the sRAS fish had reduced appetite after having been moved. On Day 1 when the salinity was increased, the fish in bRAS and sRAS started out with comparable weight ($p = 0.56$). At the end of the salinity increase period (Day 28) the fish in sRAS were 14% and significantly ($p < 0.001$) bigger than the fish in bRAS even though the temperature was on average 1.0 °C lower in sRAS (Table 1). From Day −60 to Day 28, the fish in bRAS and sRAS grew 208 and 231 g, respectively. The sRAS fish had lower growth in the acclimatization period after being transported to sea water, but the appetite was resumed during the salinity increase period. The opposite could be seen for bRAS. The fish grew better than in sRAS during the acclimatization period, however during the salinity increase the fish growth was reduced (despite higher temperatures). After the sea cage phase, the frequency (number of fish) in each weight range was significantly different between the bRAS and sRAS group ($p < 0.0001$). The average weight of the sRAS group was 2.6% bigger than in bRAS due to less individuals with weight < 4 kg (15 vs 10%). Both fish groups were diagnosed with the cardiac disease Cardiomyopathy syndrome (CMS), and the disease was observed both on land and at sea. The fish in both groups showed normal behaviour except for reduced appetite at the mentioned time periods. The mortality was 3.9 and 1.1 times higher in the sRAS group than the bRAS group on land and at sea, respectively. Thus, the fish in sRAS grew slightly better in total, but had a 1.5 times higher mortality in total and produced less biomass.

4. Discussion

Two regimes for transition from brackish to seawater in production of Atlantic salmon post-smolt was studied holistically, by gathering data on nitrification, composition of heterotrophic and nitrifying bacteria throughout the system and data on performance and gut microbiota of the fish.

4.1. The same nitrifying OTUs were found in both systems, and a low AOB:NOB ratio suggests presence of comammox

In bRAS, the nitrifying bacteria present in the biofilter at 12‰ were able to handle the osmotic stress of water with salinity up to 26‰, without compromising the efficiency in nitrification (Fig. 2). The capacity tests showed that when the carriers were stressed with higher or lower salinity than the native bRAS salinity, the AOR_{max} was lower than in the native salinity reactors (Fig. 3). The inhibition varied between 25 and 40%, which is lower than what have been seen in other studies with salt stress in freshwater nitrifying reactors (97–100% inhibition: Gonzalez-Silva et al., 2016; Kinyage et al., 2019). Historically, the bRAS system had always been operated with some salinity (3–5‰ salinity 6 months prior to our experiment), and also at higher salinities (20‰ for 3 years) for earlier production groups at the facility. Seawater priming have shown to improve salinity acclimatization (Navada et al., 2020), thus if bRAS had been primarily a strict freshwater (0‰) biofilter with no previous experience of osmotic stress, a higher inhibition might have been seen. As the lag-phases were only 10–90 min, depending on the stress-level, the nitrifying bacteria present must have adapted physiologically to the new salinity rather than being replaced by a nitrifier specialized for that salinity. The concentrations of NO_2-N in the reactors were always lowest in the 5‰ reactor, regardless of the native bRAS salinity. This indicate that nitrite oxidizers were more affected by salinity increase than ammonia oxidizers. However, the concentrations of nitrite were relatively low (< 0.5 mg L^{-1}) in all the reactors, and in bRAS NO_2-N did not accumulate after the salinity had

reached 12‰.

Only two OTUs identified as nitrifying bacteria were found in relative abundance over 0.1% in the biofilm. Both these OTUs were found in both systems, thus they functioned at both low and higher salinities. The abundance of *Nitrosomonas* was low, especially in sRAS and in the beginning of the experiment in bRAS. *Nitrospira* was more abundant in both systems, except for the last sampling in bRAS. *Nitrosomonas* are AOBs, and a low AOB:NOB ratio have been suggested as an indicator of presence of complete ammonia oxidation (comammox) bacteria (Fowler et al., 2018). *Nitrospira* was previously thought to be nitrite oxidizing bacteria, however recent studies have found that some *Nitrospira* species are comammox bacteria and oxidize both ammonia and nitrite (van Kessel et al., 2015; Daims et al., 2015). Comammox *Nitrospira* have been found in biofilters treating waters with low concentration of ammonia, like RAS and ground water (van Kessel et al., 2015; Gülay et al., 2016). Comammox nitrifiers could therefore be in the nitrifying consortium in the systems studied. OTU_77 had 94–96% sequence match with three *Nitrosomonas* species which have been reported to require salt for growth, *N. aestuari, N. halophila* and *N. marina* (Koops et al., 1991). Both bRAS and sRAS got the same intake water, but in different ratios to achieve the respective salinities, thus the same bacteria had been introduced to both systems and biofilter. Because both OTUs were present in bRAS (and throughout the salinity increase) and sRAS, it suggests that these nitrifying OTUs can adapt to salinity in the range from 3 to 26‰, and also 28‰.

4.2. Increasing the salinity beyond 12‰ did not change the bacterial community compositions of bRAS water

By increasing the salinity approximately 1‰ per day, a succession in the bacterial communities was observed in the water samples in bRAS (Fig. 6 and Supplemental S3). There were, however, also observed changes in the community structures when the salinity was constant in the acclimatization period. In this period, bRAS had a reduction of the organic load by moving 100,000 fish to sRAS. Whereas sRAS got a sudden and high organic load with the introduction of 100,000 fish. An increase in organic load decreased the OTU richness, whereas a relief in organic load increased the richness (Table 2). Organic matter is substrate for heterotrophic bacteria in RAS (Michaud et al., 2006), and changes in community compositions due to changes in organic matter loading have been seen in other studies (Wold et al., 2014; Fossmark et al., 2020). In bRAS, increasing the salinity from 6 to 12‰, the change in microbial community dynamics was higher than increasing it above 12‰ (Higher Bray-Curtis: Supplemental S2). It seems like there is a threshold salinity somewhere between 6 and 12‰ because increasing the salinity 2.2 times from 12 to 26‰ did not induce large changes in the community structures. The same trends have been seen in hierarchical clustering based on bacterial community composition similarities in the Baltic Sea (Herlemann et al., 2011). In that study there were clustering of samples in three salinity ranges: 0–3.2‰, 4.6–7.7‰ and 10.5–30.9‰. Herlemann et al. (2011) concluded that the OTUs present at brackish water localities were not specialized for brackish water, but adapted bacteria originating from marine and freshwater environments. In our study, half of the OTUs present in the water at 3‰ also adapted to the higher osmotic stress during the salinity increase to 26‰ in bRAS (Table 2). The OTUs with high abundance were generally the ones to adapt, whereas the OTUs with low abundance were replaced by new OTUs. This density dependent ability to adapt, suggests that low density OTUs are vulnerable to drift during the adaptation period.

The most abundant genera in sRAS were in general in low abundance in bRAS, and vice versa (Fig. 7). The OTU with highest abundance in sRAS was OTU_3 *Loktanella*. This OTU got the highest sequence match of 98.8% with the species *Loktanella acticola* (Park et al., 2017). Park et al. (2017) reports that this species grew optimally from 20 to 30‰ salinity. This corresponds well with the findings in our experiment as OTU_3 was found in higher abundance in bRAS at 18‰ to 26‰, and high relative abundance (7–30%) in sRAS. This OTU is thus selected for at higher salinities. *Loktanella acticola* is closely related to *Loktanella maritima*, which have shown to be commensal bacterium in production of lobster, and inhibit the growth of pathogenic *Vibrio parahaemolyticus* (Ranson et al., 2018). Another species in this genus, *Loktanella koreensis,* is an algicidal bacterium which have shown to be important in controlling proliferation of algae (Meyer et al., 2017). Some species of this genus thus have symbiotic/commensal interactions in different marine ecosystems. In sRAS *Leucothrix* OTU_16 was also highly abundant, and got 93.2% sequence match with the filamentous bacteria *Leucothrix mucor* (Ludwig et al., 1995). This species have been found to densely colonize cod eggs (Hansen and Olafsen, 1989) and was thought to negatively affect the embryo development due to hypoxia conditions caused by the bacteria. *Leucothrix* was also found in cultivation of lump fish in RAS, and were hypothesized to be a cause of poorer gill health of the fish (Dahle et al., 2020). *Leucothrix* could therefore have a negative effect on the bacterial water quality in RAS. The OTU with the highest abundance in the dataset was OTU_8, *Gemmobacter*, and was found in bRAS in high abundance. Little is known about this genus and for OTU_8 the highest sequence match was 98.8% with *Gemmobacter tilapiae* (Sheu et al., 2013), a strain isolated from a fresh water pond with Tilapia fish (*Tilapia rendalli*). Sheu et al., 2013 did not report any disease of the fish or other effects associated with this bacterial genus. OTU_13 *Mycobacterium* had 90.1% match with several different *Mycobacterium* species. The known salmon pathogen *Mycobacterium salmoniphilum* which causes mycobacteriosis in Atlantic salmon (Aro et al., 2014) did not match the representative sequence found in this experiment. Other genera known to be potentially pathogenic in salmon farming was not found in high abundance and were not studied further.

4.3. Salinity drove the succession of the faecal microbiota, and high inter-individual variation within the fish tanks suggests that stochastic processes also affect the succession

The faecal microbiota of individuals in bRAS evolved to become more similar to sRAS individuals as the salinity was increased (Fig. 8). Even though the water microbiota was very similar in the different fish tanks (Fig. 6 and Supplemental Table S2 Average Bray-Curtis: 0.86), this was not reflected in the faecal microbiota between individuals within a fish tank or between fish tanks within a system. This is emphasized by the fact that the similarity between individuals within a fish tank was the same as the similarity between the two RAS at Day 28 (Bray-Curtis: 0.5, Fig. 8). The same was seen when comparing the microbiota of individual fish within and between the sea cages. This shows that there are other factors than water microbiota involved in the assembly of the gut microbiota of Atlantic salmon smolt. High inter-individual differences in the gut microbiota have been seen in other studies of farmed Atlantic salmon in the same freshwater rearing systems (Dehler et al., 2017), and in farmed and wild Atlantic cod caught at the same location (Fjellheim et al., 2012; Star et al., 2013; Bakke et al., 2015). Dehler et al. (2017) and Star et al. (2013) found core OTUs that were consistently seen in all individuals, and it was discussed that the core OTUs could be important for the fish health and that they are actively selected for in the host. The OTUs who's presence differ between individuals may have redundant functions, and their presence is explained by stochasticity (Zhou and Ning, 2017). The first colonization of the gut of fish larvae could also have an impact on the gut microbiota for juvenile/adult stages, so-called priority effect (De Schryver and Vadstein, 2014). The order of species colonizing an environment is suggested to cause divergence between communities even though the environmental conditions are the same (Nemergut et al., 2013). The fish in our study were smolt and in a critical stage in their life cycle by their preparation for a life in seawater. This causes stress, which has shown to alter the intestinal lining of salmon and furthermore affect the

number of bacteria in the faeces (Olsen et al., 2002). Clearly, many factors affects the succession of the gut and faecal microbiota of salmon smolt, and salinity has shown to be one driver for the succession. It has been hypothesized that a gradual succession in the gut microbiota is better for fish health than sudden abrupt changes (De Schryver and Vadstein, 2014). After sea transfer, the faecal microbiota in both fish groups changed to the same degree (same Bray-Curtis values comparing microbiota before and after sea transfer), regardless of the salinity regime operated on land. To what extent the different salinity regimes had an effect on the faecal microbiota after transfer to sea is therefore hard to conclude on. More samples later in the sea cage phase should have been collected, to see if there were any long-term effects on the different salinity regimes.

4.4. Did the different salinity regimes affect fish growth and mortality?

Both fish groups were diagnosed with CMS, the fish from the sRAS group grew better than the bRAS group, however the sRAS fish had higher mortality. On land, the highest mortality of the sRAS fish was observed when the fish had a high growth rate. This was 4 weeks after the fish were moved to higher salinity and when the appetite was resumed. CMS affects the cardiovascular capacity (Garseth et al., 2018) and have shown to affect fish with fast growth and high condition factor (Løvoll et al., 2010). Therefore, the better growth in sRAS could in fact be the reason why more fish in this group were affected by CMS. It could also be that the sRAS fish were more susceptible to the disease due to stress caused by moving the fish from bRAS to sRAS at the spilt of the fish group, and sudden change to high salinity. It is hard to conclude whether the different salinity regimes on land affected the mortality and growth rates of the fish or not. The hypothesized positive effects of acclimatizing the microbiota associated with the water and salmon gut to higher salinity before sea transfer can therefore not be supported, as there is much uncertainty due to the CMS and what caused the RAS-groups to have different mortality and growth rates. Excluding the mortality and only including the fish weights at the end, the hypothesis is contradicted as the fish in sRAS grew better, both on land and in the sea. In the faecal microbiota, it seems like other factors such as stochastic processes and stress may undermine the effects of salinity maturation. The gradual salinity increase induced peaks in TAN and NO_2-N concentrations in bRAS, however the average concentrations in sRAS were still significantly higher during the experiment. Why TAN and NO_2-N were higher in sRAS with stable salinity could be the introduction of the 100,000 fish which increase the organic load, which have shown to negatively affect nitrification (Michaud et al., 2006; Guerdat et al., 2011). The relative abundance of nitrifiers were also lower for the sRAS biofilter, in addition to the lower temperature which could have affected the nitrification efficiency negatively. It has been shown that salmon parr exposed to TAN concentrations of up to 25 mg L^{-1} (35 μg L^{-1} NH_3-N) did not affect the growth or welfare of the fish (Kolarevic et al., 2013). Therefore, the difference in mortality and growth between the systems were probably not attributed to the differences in nitrification in the systems, as the concentrations of bRAS and sRAS were 20 times below this concentration. Nevertheless, we can conclude from this study is that increasing the salinity by 1‰ per day is possible in a RAS with salmon post-smolt without exceeding toxic concentrations of TAN and NO_2-N. However, we believe this is valid if the RAS and biofilter has experienced osmotic stress earlier and will not be true for a strict freshwater system where the inhibition of nitrification probably will be higher. The history of the biofilter must be taken into consideration for fish farmers when planning to increase the salinity in an operating RAS with fish.

5. Conclusion

Salinity has shown to be a driver of succession in RAS, and other factors such as organic load in the water and stochastic processes in the host also affected the development of bacterial communities. We found indications for a threshold salinity between 6 and 12‰, whereafter the bacterial community compositions in the water were stable to subsequent salinity increase. The biofilters in bRAS and sRAS had the same dominating nitrifying OTUs which were able to adapt to a salinity increase from 3 to 26 and 28‰. The capacity tests showed that the AOR_{max} was inhibited up to 40% when the carriers were stressed with higher or lower salinity than the native bRAS salinity. Increasing the salinity around 1‰ per day in RAS for salmon post-smolt production is possible if the biofilter have some history with osmotic stress. The fish in bRAS had a lower mortality than the fish in sRAS, however the sRAS group grew better.

Declaration of Competing Interest

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

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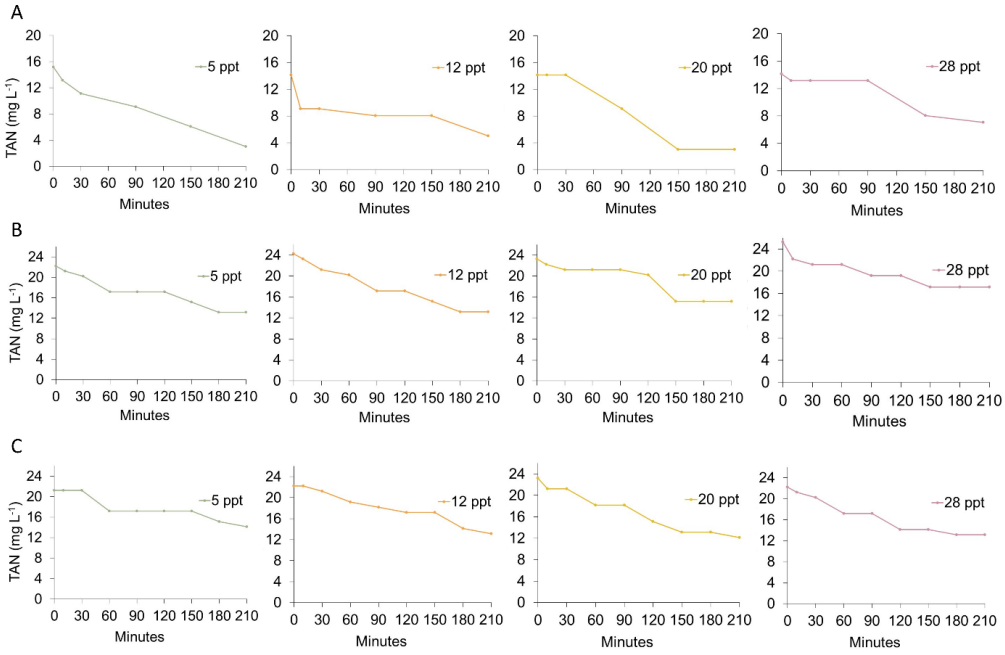
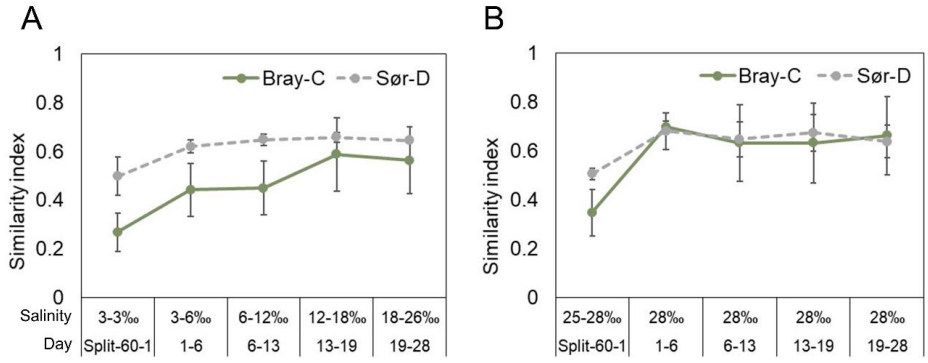
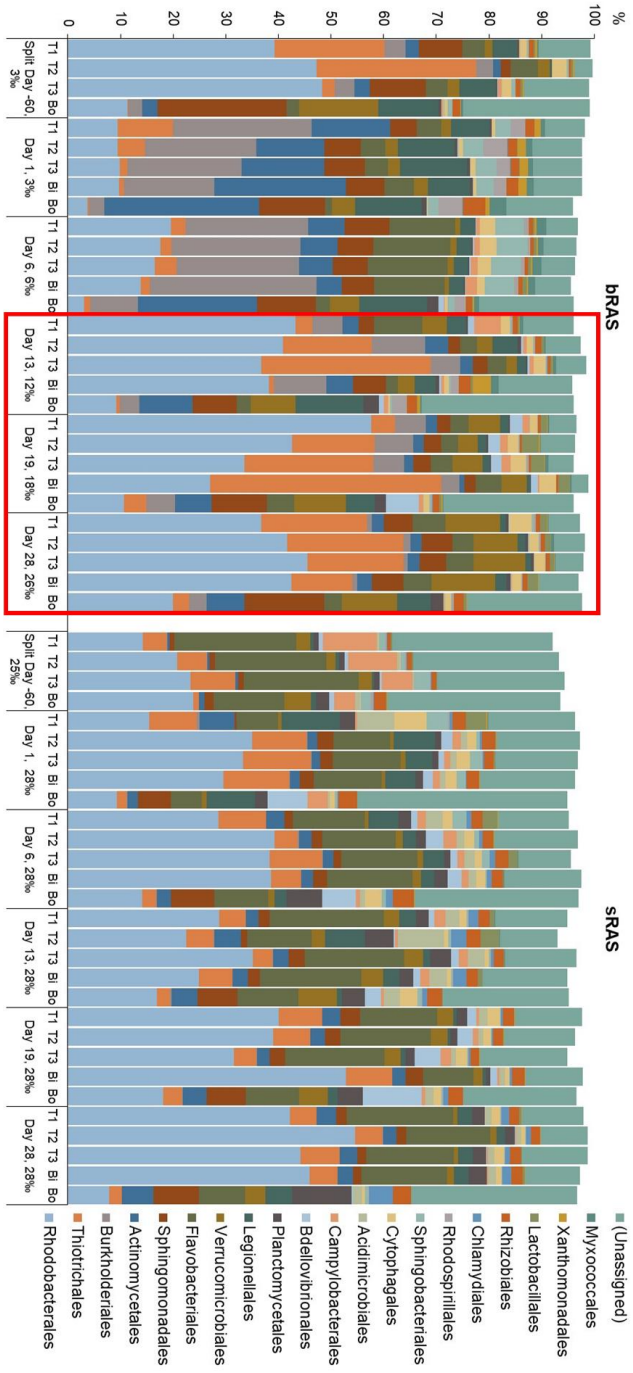
Supplementary data Paper III



Supplementary Fig. 1. Capacity tests for ammonia oxidation of bRAS carriers when the system salinity was A) 5‰ B) 12‰ and C) 20‰.



Supplementary Fig. 2. Bray-Curtis and Sørensen-Dice similarities for microbial communities in the water of A) bRAS and B) sRAS. Data are the mean ± SD (N=16) of all values in the similarity matrix comparing water microbiota samples between succeeding sampling days within each system.



Supplementary Fig. 3. Composition of water microbiota at order level showing the relative abundance in bRAS and sRAS. Only taxa with >1% relative abundance in minimum one sample is included in this figure. T = fish tank, Bi = biofilter in, Bo = biofilter out. Area marked in red showing period of salinity increase from 12 to 26‰.

Table S1. Linear regression of capacity tests for maximum ammonium oxidation rate (AOR_{max}) of biofilter carriers in bRAS at 5, 12 and 20‰ salinity.

	AOR_{max} ±SE (g N day^{-1} m^{-2})	**Lag phase (min)**	**R^2**
Stress test salinity	**5 ppt salinity in bRAS**		
5	0.40 ± 0.03	0	0.96
12	0.31 ± 0.07	0	0.90
20	0.47 ± 0.12	30	0.89
28	0.25 ± 0.04	90	0.88
	12 ppt salinity in bRAS		
5	0.34 ± 0.04	0	0.93
12	0.41 ± 0.03	0	0.97
20	0.31 ± 0.05	0	0.86
28	0.25 ± 0.04	0	0.85
	20 ppt salinity in bRAS		
5	0.25 ± 0.04	30	0.87
12	0.33 ± 0.02	10	0.97
20	0.40 ± 0.03	0	0.96
28	0.34 ± 0.04	0	0.92

Table S2. Bray-Curtis similarities for water samples. Data are the mean ± SD of all values in the similarity-matrix comparing water microbiota between fish tanks and biofilter out at the different samplings.

bRAS	D-60, 3‰	D1, 3‰	D6, 6‰	D13, 12‰	D19, 18‰	D28, 26‰	D-60, 25‰	D1, 28‰	D6, 28‰	D13, 28‰	D19, 28‰	D28, 28‰
D-60, 3‰	0.72 ± 0.07											
D1, 3‰	0.29 ± 0.05	0.82 ± 0.04										
D6, 6‰	0.29 ± 0.05	0.46 ± 0.01	0.90 ± 0.04									
D13, 12‰	0.49 ± 0.09	0.39 ± 0.06	0.47 ± 0.04	0.70 ± 0.06								
D19, 18‰	0.40 ± 0.07	0.30 ± 0.04	0.39 ± 0.04	0.62 ± 0.09	0.72 ± 0.10							
D28, 26‰	0.28 ± 0.04	0.29 ± 0.03	0.30 ± 0.02	0.46 ± 0.03	0.60 ± 0.06	0.86 ± 0.01						
sRAS												
D-60, 25‰	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.14 ± 0.02	0.76 ± 0.09					
D1, 28‰	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.11 ± 0.02	0.21 ± 0.02	0.37 ± 0.03	0.86 ± 0.03				
D6, 28‰	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.11 ± 0.01	0.18 ± 0.02	0.38 ± 0.03	0.70 ± 0.03	0.77 ± 0.07			
D13, 28‰	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.02	0.13 ± 0.02	0.20 ± 0.02	0.40 ± 0.05	0.60 ± 0.07	0.67 ± 0.07	0.72 ± 0.09		
D19, 28‰	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.14 ± 0.02	0.23 ± 0.02	0.40 ± 0.04	0.69 ± 0.02	0.71 ± 0.05	0.67 ± 0.10	0.80 ± 0.07	
D28, 28‰	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.13 ± 0.02	0.20 ± 0.02	0.35 ± 0.02	0.59 ± 0.02	0.67 ± 0.05	0.61 ± 0.06	0.70 ± 0.05	0.82 ± 0.01

Paper IV

A salty start: Brackish water start-up as a microbial management strategy for nitrifying bioreactors with variable salinity

Sharada Navada, Marianna Sebastianpillai, Jelena Kolarevic, Ragnhild O. Fossmark, Ann-Kristin Tveten, Frédéric Gaumet, Øyvind Mikkelsen & Olav Vadstein. 2020.

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A salty start: Brackish water start-up as a microbial management strategy for nitrifying bioreactors with variable salinity

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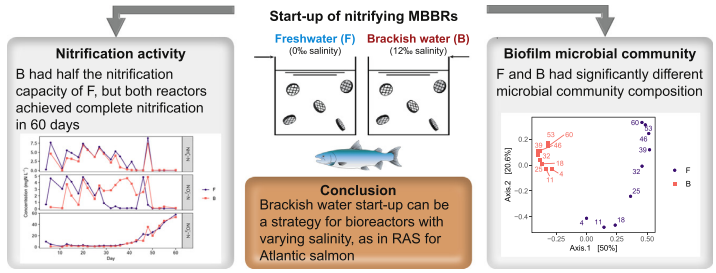
HIGHLIGHTS

- Complete nitrification was achieved in fresh- and brackish water MBBRs in 60 days
- Microbial and nitrifying community structure in the MBBRs differed significantly
- *Nitrosomonas* & *Nitrosospira* were the dominant AOB in fresh- & brackish biofilms
- *Nitrotoga* was the dominant genus of nitrite oxidizers in both treatments
- Startup in brackish water can be a strategy for bioreactors with variable salinity

GRAPHICAL ABSTRACT



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ABSTRACT

Nitrifying biofilms developed in brackish water are reported to be more robust to salinity changes than freshwater biofilms. This makes them a promising strategy for water treatment systems with variable salinity, such as recirculating aquaculture systems for Atlantic salmon. However, little is known about the time required for nitrification start-up in brackish water or the microbial community dynamics. To investigate the development of nitrifying biofilms at intermediate salinity, we compared the startup of moving bed biofilm reactors with virgin carriers in brackish- (12‰ salinity) and freshwater. After 60 days, the brackish water biofilm had half the nitrification capacity of the freshwater biofilm, with a less diverse microbial community, lower proportion of nitrifiers, and a significantly different nitrifying community composition. *Nitrosomonas* and *Nitrosospira*-like bacteria were the main ammonia oxidizers in the brackish water biofilms, whereas *Nitrosomonas* was dominant in freshwater biofilms. *Nitrotoga* was the dominant nitrite oxidizer in both treatments. Despite the lower nitrification capacity in the brackish water treatment, the low ammonia and nitrite concentration with rapidly increasing nitrate concentration indicated that complete nitrification was established in both reactors within 60 days. The results suggest that biofilms develop nitrification in brackish water in comparable time as in freshwater, and brackish start-up can be a strategy for bioreactors with varying salinity.

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1. Introduction

Variable salinity influents are often encountered in municipal and industrial water treatment systems, such as food processing, cities with seawater flushing, and land-based aquaculture (Lefebvre and Moletta, 2006; Navada et al., 2020). In the past decade, intensive land-based aquaculture has been on the rise due to the escalating global food demand, depleting oceans, and water scarcity (FAO, 2018). Recirculating aquaculture systems (RAS) are a rearing technology for producing fish in land-based facilities with the treatment and reuse of water. RAS for anadromous fish, such as Atlantic salmon (*Salmo salar*), face the unique challenge of varying salinities during the production of different life stages of the fish (Kinyage et al., 2019; Navada et al., 2020; Navada et al., 2019). From egg to the smolt phase, the fish are reared in freshwater. After smoltification, the salinity is typically increased to brackish water (12–22‰ salinity) or seawater (32‰ salinity) (Davidson et al., 2016). While the fish are physiologically adapted to tackle an increase in salinity, the microbes in the nitrifying bioreactors in RAS may be negatively impacted by salinity changes (Navada et al., 2019). In RAS, the bioreactors perform the vital task of oxidizing the ammonia produced by the fish to nitrite, and subsequently, to nitrate. As even very low concentrations of ammonia (<2 mg L^{-1} total ammonia nitrogen) and nitrite (<0.5 mgN L^{-1}) are toxic to Atlantic salmon, it is essential to maintain high and stable nitrification in RAS.

The nitrification process is typically carried out by two mutualistic microbial guilds: ammonia oxidizing bacteria (AOB) or archaea (AOA) that convert ammonia to nitrite, and nitrite oxidizing bacteria (NOB) that convert nitrite to nitrate. Recently, bacteria within the genus *Nitrospira* were shown to be capable of complete ammonia oxidation (comammox), and were also detected in RAS bioreactors (Bartelme et al., 2019; Van Kessel et al., 2015). Changes in salt concentration can disrupt the osmotic balance in the bacterial cells, leading to inhibition or plasmolysis (during salinity increase) and reducing the nitrification activity (Csonka, 1989). However, bacteria can acclimate to high salinities by maintaining osmotic balance through synthesis or uptake of compatible solutes (Oren, 2011). Several studies have explored the impact of salinity on freshwater nitrifying biofilms (Gonzalez-Silva et al., 2016; Kinyage et al., 2019; Sudarno et al., 2011). Irrespective of the method of salinity change, an initial reduction in the nitrification capacity is typically observed when the salinity is increased from 0‰ to above 10‰ (Gonzalez-Silva et al., 2016; Navada et al., 2019; Nijhof and Bovendeur, 1990). Further, complete acclimation from freshwater to higher salinities can take weeks (Bassin et al., 2012a; Navada et al., 2019). Thus, a better strategy is required for making RAS bioreactors robust to salinity changes.

A recent study showed that osmotic stress priming (prior exposure to salinity) could greatly improve salinity adaptation in freshwater nitrifying biofilms (Navada et al., 2020). This implies that the main challenge is the first salinity increase in newly matured freshwater bioreactors. One option is to have separate RAS for pre- and post-smolt operated at different salinities. This option is not always preferred, as it involves moving the fish, which can stress them and cause poor health or mortality. Moreover, separate nitrification loops for different salinities have a larger areal footprint and higher operating costs. Another option may be to initiate biofilm development at a high salinity (>10‰) and then decrease the salinity, as microbes can adapt more easily to a decrease in osmolarity than an increase (Csonka, 1989). Further, biofilms developed at high salinity will have a species inventory that is adapted (or primed) to salt, thus making them robust to future salinity increases (Navada et al., 2020). This hypothesis is supported by studies that reported brackish (10–22‰ salinity) or seawater biofilms to be more robust to salinity changes than freshwater biofilms (Gonzalez-Silva et al., 2016; Li et al., 2019; Navada et al., 2020). Thus, it appears that the bacterial succession in brackish- or seawater forges a halotolerant biofilm microbial community that can better adapt to varying salinities than freshwater biofilms.

Although start-up at a high salinity appears to be a promising strategy for RAS bioreactors, there are some constraints. At elevated salt concentration, much of the energy produced by the autotrophic activity of nitrifiers is directed towards osmoregulation, thereby reducing the energy for maintenance and growth (Oren, 2011). Indeed, studies report that nitrifying bioreactors in seawater require a much longer start-up period and have up to 60% lower nitrification rates than freshwater bioreactors (Nijhof and Bovendeur, 1990; Rusten et al., 2006). Further, due to the strong emphasis for biosecurity in aquaculture, RAS bioreactors are usually started in clean water with synthetic chemicals as nutrient sources. This makes the start-up even more time-consuming. Attempts have been made to accelerate start-up using commercial nitrifying inocula, but with mixed results (Brailo et al., 2019; Kuhn et al., 2010; Li et al., 2019; Manthe and Malone, 1987). Seeding with mature biofilm carriers or enriched halotolerant nitrifiers can improve salinity adaptation and reduce the start-up time (Sudarno et al., 2010; Zhu et al., 2016), but these are not always available and can also pose a biosecurity risk.

In our previous study, we found that brackish water biofilms were much more robust to salinity increase than freshwater biofilms, suggesting that start-up in brackish water could be a strategy to improve salinity acclimation in biofilms (Navada et al., 2020). However, the time required for start-up and the developmental phase of nitrifying biofilms in brackish water is not well researched. Although previous studies have documented the start-up of freshwater, brackish, and marine bioreactors (Bassin et al., 2012b; Jiang et al., 2019; Kumar et al., 2010; Li et al., 2019; Liu et al., 2019), no studies exist on a clean start-up in brackish water (without seeding or inoculation). This study was undertaken to compare the nitrification activity and microbial community dynamics during the start-up of semi-commercial moving bed biofilm reactors (MBBR) in freshwater (0‰ salinity) and brackish water (12‰ salinity), using virgin carriers. The goal was to determine if start-up in brackish water could be a practical strategy for industrial bioreactors with varying salinity requirements during operation, as in RAS for Atlantic salmon.

2. Materials & methods

2.1. Experimental setup and operation

The experiment was conducted in two semi-commercial RAS MBBRs at the Nofima Centre for Recirculation in Aquaculture at Sunndalsøra, Norway (Terjesen et al., 2013). The MBBRs were started up in freshwater (F, 0‰ salinity) and brackish water (B, 12‰ salinity), respectively. The system water volume was approx. 20 m^3, including the MBBR, CO_2 stripper, pump sump, and pipes. Each MBBR was filled (~40% by volume) with virgin biofilm carriers (AnoxK™ Chip P, Krüger Kaldnes AS, Norway). Both MBBRs were started up simultaneously. Due to difficulty in mixing the carriers, approximately one-third of the carriers were removed in the beginning and refilled on days 8–10. On day 2, the following chemicals were dosed: sucrose (882 g), NH_4Cl (710 g), $NaNO_2$ (572 g), $Na_2HPO_4{\cdot}12H_2O$ (207 g), KH_2PO_4 (78 g) (Zhu et al., 2016), and 200 mL of micronutrient stock solution. The micronutrient solution contained the following chemicals (mg per 2 L of deionized water): $FeCl_3{\cdot}6H_2O$ (55), $MgSO_4{\cdot}7H_2O$ (190), $CuSO_4{\cdot}5H_2O$ (5), $CoCl_2{\cdot}6H_2O$ (6), $NiCl_2{\cdot}6H_2O$ (6), $ZnSO_4{\cdot}7H_2O$ (34), $NaMoO_4{\cdot}2H_2O$ (5), and $MnCl_2{\cdot}4H_2O$ (42) (adapted from Wagner et al., 2016; Zhu et al., 2016).

The start-up was monitored over 60 days. During this period, the MBBRs were operated at 14–17 °C and pH 8, controlled by automatic dosing of sodium bicarbonate. The dissolved oxygen was maintained at 85–100% saturation. For the first 12 days, the MBBRs were operated in batch mode with internal water circulation. Due to water loss by evaporation, a continuous influent flow of 2.5 L min^{-1} was provided during the rest of the experiment (hydraulic retention time ~ 6 days). The intake water sources were pretreated as described in (Terjesen et al., 2013). Briefly, the F reactor was supplied freshwater that was

pumped from borewells, treated with silicate and degassed. For the B reactor, the freshwater (FW) and seawater (SW, filtered and UV-irradiated) intake flows were mixed to attain a salinity of 12‰. Sucrose (770–880 g) was added weekly as a carbon source to accelerate biofilm formation, as recommended by Bassin et al., 2012b. Phosphate was provided weekly as $Na_2HPO_4{\cdot}12H_2O$ (180–230 g) and KH_2PO_4 (70–90 g) to maintain the orthophosphate concentration above 0.5 mgP L^{-1}. The micronutrient solution (200 mL) was dosed weekly. Sodium nitrite (200–500 g) was supplied (approx. weekly) in the first six weeks as a substrate for the nitrite oxidizing bacteria. Ammonium chloride (610–730 g) was dosed weekly during the first five weeks. As the nitrification rate increased, this dosing was increased to 1834 g every three days during days 47–56. On days 57–59, 800 g NH_4Cl was added daily. The theoretical concentration of ammonia and nitrite in the MBBR corresponding to the dosed NH_4Cl and $NaNO_2$ is shown in Fig. 1A.

2.2. System variables

The system variables were measured daily using a handheld multimeter (Multi 3620, WTW, Germany) with sensors for temperature and pH (SenTix® 980, WTW, Germany), dissolved oxygen (Handy Polaris 2, Oxyguard, Denmark), and salinity (TetraCon® 925, WTW, Germany). Three days a week, water samples were taken from the MBBR or the MBBR effluent for the analyses of inorganic nitrogenous compounds. As the MBBRs were well aerated with the carriers in constant motion, they can be considered as continuously stirred tank reactors (CSTR) where the concentration in the effluent is equal to that in reactor. The water samples were filtered through a 0.45 μm syringe filter (Acrodisc®, VWR International) and preserved at −20 °C in 20 mL polyethylene scintillation vials (Wheaton Industries, USA). Water samples from the freshwater and seawater inlets were also collected on days 11, 39, and 61. All samples were analyzed using a flow injection autoanalyzer (Flow Solution IV, OI Analytical, USA) according to U.S. EPA Method 350.1 for ammonia and Method 353.2 for nitrite and nitrate (U.S. EPA, 1983). The orthophosphate concentration in the MBBR water was measured twice a week using a spectrophotometric kit (Method 114543, Merck, Germany). The intake water flowrates were measured using online flowmeters.

2.3. Capacity tests to measure maximum ammonia and nitrite oxidation rates

On days 56–57, capacity tests were conducted to determine the maximum oxidation rates of ammonia (AOR_{max}) and nitrite (NOR_{max}). Two stainless steel reactors (water volume ~ 7 L) were set up in a temperature-controlled water bath (13–15 °C) in batch mode. These reactors, F_{cap} and B_{cap}, were filled with freshwater and 12‰ salinity brackish water (mix of FW and SW), respectively. The reactors were well

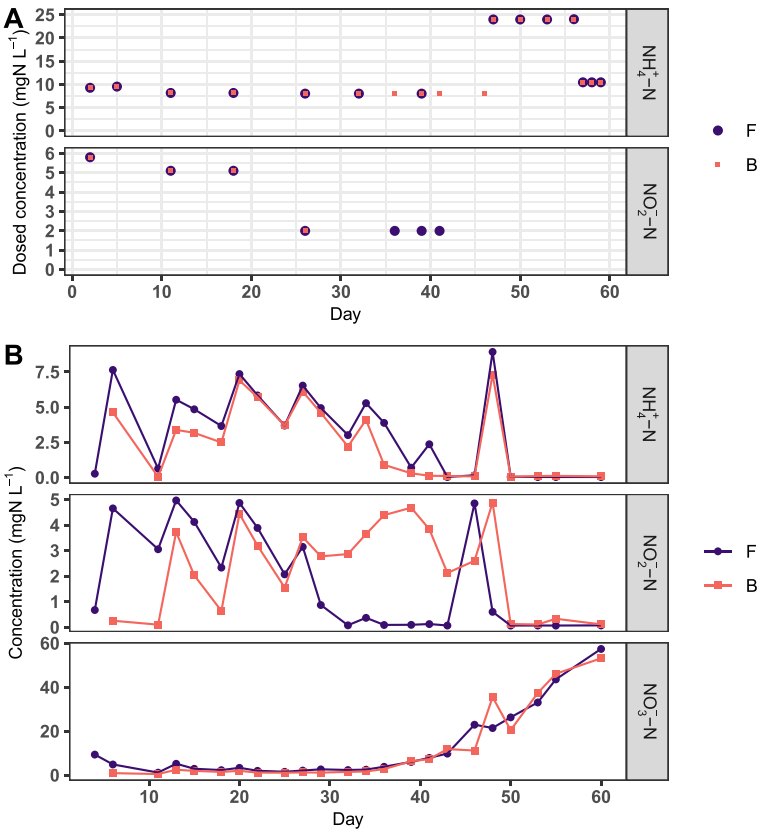

Fig. 1. A) Theoretical concentration of ammonia and nitrite in the reactors corresponding to the respective quantities of ammonium chloride and sodium nitrite dosed; B) Ammonia, nitrite, and nitrate concentration in the freshwater (F) and brackish water (B) reactors during the study. The points have been connected to improve readability, but are not necessarily linearly related. Note the difference in the scales.

aerated, and the dissolved oxygen saturation was 100–101% in all the tests. The F_{cap} and B_{cap} reactors were then filled (40% by volume) with biofilm carriers from the full-scale F and B reactors, respectively. For the ammonia capacity test, a spike solution (50 mL) was added to each reactor, resulting in an initial ammonia concentration of ~10 mgN L^{-1}. This spike solution contained 5.26 g NH_4Cl and 19.60 g $NaHCO_3$ in 1 L deionized water. In F_{cap}, the pH was adjusted by the further addition of 561 mg $NaHCO_3$ dissolved in 50 mL deionized water. The pH in the reactors was 8.1–8.3 throughout the test. Water samples were taken every 30–60 min and the ammonia concentration was measured using the phenate method with 5-10x dilution (Merck test 1.14752, Germany). The nitrite capacity test was conducted in a similar manner by adding 100 mL of a spike solution (prepared with $NaNO_2$ and deionized water) to each lab reactor, corresponding to an initial nitrite concentration of 20 mgN L^{-1}. The pH in the reactors was 7.9–8.0 during the test. Water samples (~50 mL) were collected every 10–16 min and the nitrite concentration was measured using the colorimetric method with 20x dilution (Merck test 1.14776, Germany). The photometric measurement of ammonia or nitrite was made by transferring each reacted sample to a 10 mm cuvette and subsequently analyses by a spectrophotometer (PhotoLab 6100 VIS, WTW, Germany). During each capacity test, 8–9 samples were analyzed.

2.4. 16S rRNA gene amplicon sequencing

Weekly, two biofilm carriers from each MBBR were collected and preserved at −20 °C. To study the microbial community composition of the intake water sources, samples of the freshwater and seawater were collected on days 4, 39, and 61. Each water sample (~200 mL) was filtered through a 0.22 μm filter (Sterivex™, Merck, Germany) and these filters were preserved at −20 °C.

DNA was extracted from the Sterivex™ filters and one quarter of each biofilm carrier using the DNeasy® PowerSoil Kit (Qiagen, Germany). The eluted DNA samples were stored at −20 °C. Qubit assay for dsDNA with high sensitivity (Invitrogen, Thermo Fisher Scientific) was conducted to measure the DNA concentration. For sequencing, the V3 and V4 regions of the 16S rRNA gene was targeted using broad range PCR primers with Illumina adapter sequences (338F: 5′ cgtcggcagcgtcagatgtctataaga gacagnnnnCCTACGGGWGGCAGCAG-3′ and 805R: 5′-gtctcgtgggctcggagatgtgtataagagacagnnnnn ACTA CNVGGGTATCTAAKCC-3′, Illumina adapter sequences are in lower case letters). Each PCR reaction contained 0.02 U μL^{-1} Phusion Hot Start II DNA polymerase (Thermo Scientific), 0.2 mM of each dNTP (VWR), 300 nM of each primer (SIGMA), 2 mM $MgCl_2$ (Thermo Scientific), and reaction buffer from Thermo Scientific in a total reaction volume of 25 μL, including 1 μL of ~1 ng μL^{-1} DNA extract as template. The PCR reactions were run with 30 cycles (T100TM Thermal Cycler, BioRad). PCR products were normalized with a SequalPrep Normalization Plate (96) kit (Invitrogen, USA), following the manufacturers' protocol. Unique barcode-sequences were added to each PCR product using the Nextera XT Index kit (Illumina, USA) through an additional PCR run with eight cycles. The barcoded PCR products were examined by 1% agarose gel electrophoresis. The indexed amplicons were normalized again using the normalization plate. A total of 96 samples were pooled and concentrated with Amicon Ultra-0.5 mL Centrifugal Filters (Ultracel® 3 K, Merck Millipore, Ireland) using manufacturers' protocol. The concentration and purity (A260/280 & A260/230) of the sample were measured with NanoDrop One (Thermo Scientific). The pooled amplicon libraries were sequenced on one MiSeq lane each (Illumina, USA) at the Norwegian Sequencing Centre in Oslo.

2.5. Data analyses and statistics

2.5.1. Ammonia and nitrite oxidation capacity

For each capacity test, linear regression was performed on the NH_4^+-N or NO_2^--N concentration vs time. The residuals of the linear regression model were checked for normality (Shapiro-Wilk test), homoscedasticity, and influential outliers. The maximum oxidation rates were then calculated from the slopes. The hypothesis of differences between the slopes were tested using analysis of covariance (ANCOVA) (Fox and Weisberg, 2011; Navada et al., 2019). A confidence interval of 95% was used ($\alpha = 0.05$). The data analyses were performed in R (V.3.6.1) using packages reshape and ggplot2 (Wickham, 2016, 2007).

2.5.2. Processing and analysis of microbial community data

The Illumina sequencing data were processed using the USEARCH pipeline (version 11). In the first step, pair reads were merged, primer sequences were trimmed, and all the reads shorter than 400 bp were filtered out. The next step involved quality filtering and demultiplexing using the Fastq_filter command with an expected error threshold of 1. Operational taxonomic unit (OTU) clustering was performed at 97% similarity level by implementing the UPARSE algorithm (Edgar, 2013). This also included removal of chimera sequences and singletons. Taxonomic assignment was based on the Sintax command (Edgar, 2016) with a confidence value threshold of 0.8 with Ribosomal Database Project (RDP Version 16, https://rdp.cme.msu.edu/). Nitrite oxidizing bacteria were detected at extremely low proportions using this database, which contradicted the nitratation activity in the reactors. To investigate this, DNA from the biofilm samples on days 46–60 was used to generate amplicons and sequenced on Ion Personal Genome Machine™ (Ion Torrent™, Thermo Fischer Scientific, USA) using procedures described previously (Navada et al., 2019). Briefly, the sequencing targeted seven variable regions (V2–4, V6–9) of the 16S rRNA gene and used the Curated MicroSEQ® 16S Reference Library v2013.1 combined with the Greengenes database for sequence identification. To check if the low proportion of NOB was due to differences in the classification of taxa, the Illumina sequences were also classified using the reference database Microbial Database for Activated Sludge (MiDAS3, Version 3) (Nierychlo et al., 2019). In addition to all the OTUs classified as potential nitrifying bacteria by the RDP database, the MiDAS3 database also detected the NOB genus *Nitrotoga*. This genus was found in both the MiDAS3 (Illumina sequences) and the Ion Torrent™ analyses, but not in the classification of the Illumina sequences by the RDP database. Thus, for consistency, the results reported in this study are based on Illumina sequencing classified by MiDAS3 (unless otherwise specified).

For the Illumina sequencing data, OTUs classified as archaea or unclassified at the domain level were removed. OTUs classified as cyanobacteria or plastids were also removed as they were not considered relevant. For both sequencing methods, the data was normalized to the sum of reads per sample. Further, OTUs at a maximum relative abundance of <0.1% in any sample were removed. The following data analysis was performed on the OTU table from the Illumina sequencing classified by MiDAS3 database. The α-diversity of each sample was estimated as the first-order diversity number (N_1) (Hill, 1973), richness (N_0, zero order diversity number), and evenness (N_1/N_0). Analysis of variance (ANOVA) was used to compare α-diversity indices between the two treatments based on the biofilm samples collected during days 30–60. Further, the dissimilarities in the microbial community composition of the biofilm samples were visualized using ordination by principal coordinates analysis (PCoA) based on Bray-Curtis and Sørensen-Dice distances. The succession in the microbial community was plotted as the Bray-Curtis distance between each biofilm sample and the first sample of the respective treatment. Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distances was used to test the hypothesis of equal microbial community composition between groups of samples (Anderson, 2001). Similarity percentages (SIMPER) was used to determine the main taxa contributing to the dissimilarity in the microbial communities (Clarke, 1993). Microbial data analysis was performed in R (3.6.1) using packages phyloseq and vegan (McMurdie and Holmes, 2013; Oksanen et al., 2019).

3. Results & discussion

3.1. Complete nitrification was established in both reactors within 60 days

During the start-up period, the freshwater (F) and brackish water (B) treatments showed similar trends in the ammonia and nitrate concentration (Fig. 1B). By comparison, the nitrite concentration in B was higher than in F during days 30–50, indicating a slower onset of nitrite oxidation in B compared to F. In both reactors, the nitrate concentration increased rapidly after day 40 (7 mgN m^{-2} d^{-1}), reflecting an increase in the nitrification rate. During the first 12 days, the concentration of ammonia and nitrite decreased on some days despite no dilution water flow. It is unlikely that this decrease was due to nitrification as there was no corresponding increase in nitrate. We think that the inconsistency may be due to analytical error or system fluctuations in the beginning of the experiment. Nonetheless, after the first two weeks, the ammonia and nitrite concentration were consistent with the chemical addition in both reactors. Due to the scale of this study, it was not possible to have treatment replicates. However, previous studies on the effect of salinity on medium-scale MBBRs have shown low variability among treatment replicates (Navada et al., 2020, 2019). We therefore believe that the similarities and differences in this study are due to the treatment and not due to chance and stochasticity. To the best of our knowledge, this is the first study to compare the simultaneous start-up of nitrification in freshwater and brackish water in semi-commercial RAS MBBRs. The scale of this study thus makes it extremely relevant for the design and management of bioreactors in commercial RAS.

The capacity tests at the end of the start-up period (day 60) showed that B had lower nitrification capacity than F (Table 1). As the oxidation rates were low (<100 mgN m^{-2} d^{-1}), the concentration difference between samples may have been occluded by the uncertainty in the measurements. The regression analysis could have been improved by increasing the time interval between samples and/or by taking a greater number of samples. Nonetheless, B had a consistently higher concentration of ammonia (or nitrite) than F during these tests, indicating lower nitrification rates in B (Supplementary information, Fig. A1). The F treatment had significantly higher (2×) nitrite oxidation capacity (NOR_{max}) than the B treatment, which corroborates the data from continuous operation. Further, at the end of the start-up period, the ammonia oxidation capacity (AOR_{max}) in F was 2.5× higher than in B, but the difference was only marginally significant ($p = 0.07$). In contrast, treatment B appeared to have slightly higher ammonia oxidation than F during continuous operation, especially observed during days 36–46 (Fig. 1). A previous study also reported that the nitrification capacity in brackish water biofilms is at least as high as that in freshwater biofilms (Navada et al., 2020). The nitrification rate (~0.01 gN m^{-2} d^{-1}) in both treatments was at least an order of magnitude lower than the rates reported for cold-water RAS (Rusten et al., 2006). This is likely because the concentration of ammonia and nitrite was so low (<0.5 mgN L^{-1}) during some periods that it may have limited the nitrification rate (Rusten et al., 2006). The low supply of substrate likely reduced the rate of build-up of nitrifying biomass during parts of the study, and hence the nitrification capacity. As nitrifying bacteria have a maximum doubling time of approximately one day (Keen and Prosser, 1987), we can assume that with sufficient substrate (ammonia), the nitrification capacity would double each day. Under these conditions, the nitrification capacity is projected to exceed 0.3 g m^{-2} d^{-1} within one week after day 60. Thus, with sufficient ammonia loading rate, the nitrification rate can rapidly increase to the values observed in salmonid RAS (Rusten et al., 2006). It is also possible that the oxidation rates in the capacity tests were slightly different from those in the 20 m^3 MBBRs. Planktonic bacteria could have contributed to the overall nitrification rate in the semi-commercial MBBRs, as the reactors had a retention time of around six days. These planktonic bacteria would have been excluded in the capacity tests as new water was used in the tests. It is also possible that some biomass was sloughed off the carriers when they were transferred to the lab setup. So, the batch tests may have given a slightly lower estimate of the nitrification capacity that was present in the 20 m^3 MBBRs. It should be noted that it is difficult to calculate the exact nitrification rate in the semi-commercial MBBRs due to unsteady state conditions and continuous dilution flow. However, as both reactors had similar chemical dosing and operating conditions, the nitrification rates of the two reactors can be compared relative to one another.

Despite the difference in the nitrification capacity in the batch tests, the nitrification performance in the two treatments during continuous operation was comparable. The concentration of the inorganic nitrogen compounds was similar in both reactors after day 50, with low concentration of ammonia and nitrite (<0.5 mgN L^{-1}). Moreover, in both reactors, the NOR_{max} was 3-4× higher than the AOR_{max}, indicating that complete ammonia oxidation to nitrate was achieved. This is in contrast to marine biofilm systems, which often show persistent nitrite accumulation and lower nitrite oxidation than freshwater systems during start-up (Gutierrez-Wing and Malone, 2006; Manthe and Malone, 1987; Nijhof and Bovendeur, 1990). The addition of nitrite during start-up likely facilitated the growth of NOB in our study. Previous studies have reported that without seeding or commercial inocula, nitrifying biofilms can take 100–300 days to develop in seawater (Li et al., 2019; Liu et al., 2019; Nijhof and Bovendeur, 1990). Conversely, in our study, complete nitrification was achieved in both the fresh- and brackish water bioreactors within 60 days. This strongly suggests that biofilms develop much faster in brackish water compared to seawater. As 12‰ salinity is close to isotonic conditions, the microbes likely required lesser energy to meet the osmotic requirements at this salinity than in seawater (~32‰ salinity), thus directing more energy to growth (He et al., 2017). This could explain why nitrification in the brackish water reactor started up in similar time as in the freshwater reactor. Although we did not test the salinity tolerance of the reactors in this study, previous studies provide strong evidence that brackish biofilms (10–22‰) are robust to salinity changes (Gonzalez-Silva et al., 2016; Li et al., 2019; Navada et al., 2020). Thus, start-up in brackish water can be a practical strategy for bioreactors where salinity changes are expected, such as in RAS for Atlantic salmon.

3.2. Microbial analyses

The OTU table for biofilm and water samples contained 1049 taxa, of which 394 OTUs were present in the biofilm. Ordination by PCoA based on Bray-Curtis distances showed that the biofilm microbial community composition of the two treatments was separated along the first coordinate axis (Fig. 2A). PERMANOVA analyses confirmed that the microbial

Table 1
Capacity test results for the freshwater and brackish water MBBRs. Linear regression analysis shows the maximum oxidation rate ± SE (standard error) of ammonia and nitrite (calculated from the slope), adjusted R^2, and degrees of freedom (df). Asterisks denote significant difference between the oxidation rates of the two treatments ($p < 0.05$).

	Freshwater			Brackish water			Difference
	Oxidation rate ± SE (mgN m^{-2} d^{-1})	R^2_{adj}	df	Oxidation rate ± SE (mgN m^{-2} d^{-1})	R^2_{adj}	df	*p*
Ammonia	10 ± 2	0.75	6	4 ± 2	0.16	6	0.07
Nitrite	33 ± 6	0.78	7	15 ± 4	0.61	6	**0.04***

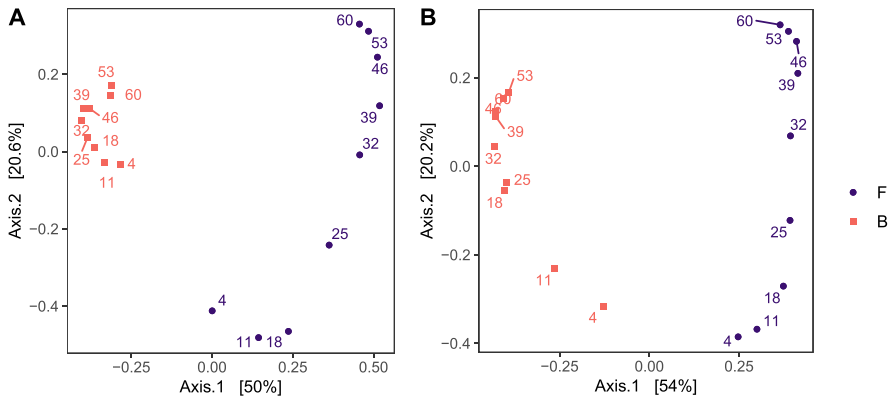

Fig. 2. Ordination by principal coordinates analysis (PCoA) based on A) Bray-Curtis (relative abundance) and B) Sørensen-Dice (presence-absence) distances between the biofilm samples. Labels indicate sampling day. Each point represents the mean data from two biofilm carriers. Square brackets show the percent variance explained by each of the coordinate axes.

community composition in the two treatments was significantly different during the study ($p < 0.001$, $R^2 = 0.44$). The Bray-Curtis dissimilarity based on relative abundance between the treatments on any given week was high (>0.85, excluding day 4 when it was 0.74). Overall, the α-diversity of the biofilm microbial community was significantly higher in F than in B, suggesting that the biofilm was further developed in F than in B (Supplementary information, Fig. A2).

3.2.1. The microbial community composition changed significantly after the first month in both biofilms

The microbial community composition of the biofilms evolved over time (Fig. 2). In both treatments, the community composition changed significantly from the first half of the study (days 0–30) to the second half (days 31–60) ($p < 0.001$, $R^2 = 0.4$–0.6). This was correlated to the nitrification activity, which increased rapidly after day 30, as inferred from the trends in the nitrite and nitrate concentration. The change in community composition after day 30 could also be observed from the proportions of different taxa (Fig. 3) and the bacterial succession in the biofilm (Supplementary information, Fig. A3–5). After day 30, the Bray-Curtis distance relative to the first biofilm sample (day 4) in B leveled off at 0.57–0.70. This contrasts with F, where the distance was much higher (0.94–0.98). Also, the relative abundance of nitrifiers increased significantly after the first month. Ordination based on Sørensen-Dice distances (presence-absence) resulted in a plot similar to that based on Bray-Curtis distances (Fig. 2B). This suggests that the compositional changes were primarily due to changes in the species inventory, and less due to changes the relative abundance of OTUs. SIMPER analysis showed that five families contributed to >50% of the difference between the first and second half of the study (Supplementary information, Tables A1–2). The proportions of *Burkholderiaceae* and *Pseudomonadaceae* decreased in the second half of the study in both treatments. In F, the proportions of *Sphingomonadaceae* and *Rhodobacteraceae* increased. The early biofilm community was likely dominated by microbes that could attach to the plastic carriers to form a biofilm. Indeed, the dominant taxa in the biofilm during days 1–30, heterotrophs within *Pseudomonadaceae* and *Burkholderiales*, can produce extracellular polymeric substances (EPS) and are reported to be initial biofilm colonizers (Winkler et al., 2018). *Psuedomonadaceae*

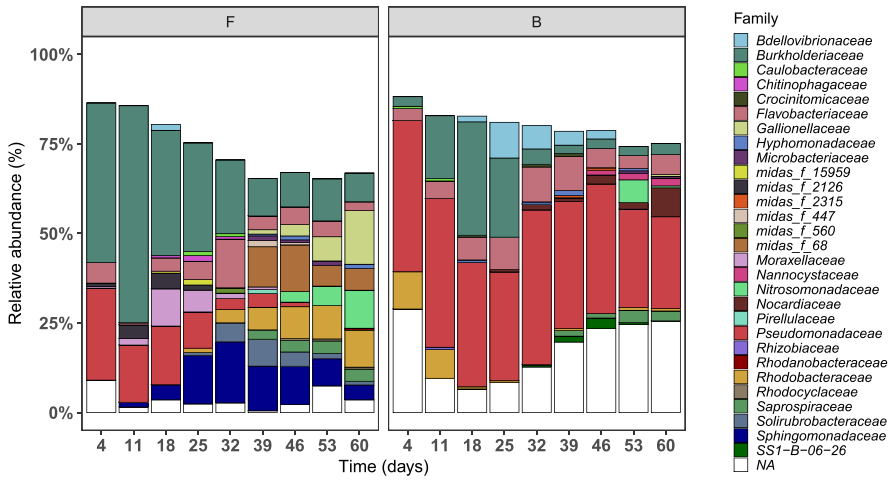

Fig. 3. Relative abundance of taxa in the freshwater (F) and brackish water (B) biofilm classified at the family level. Each data point represents the mean data from two biofilm carriers. For simplicity, only taxa present at relative abundance >1% in at least one sample are shown.

were also abundant in a previous study on marine RAS (Michaud et al., 2009). In the present study, *Sphingomonadaceae* were most abundant in the freshwater biofilm, but they have also been detected in freshwater and marine RAS MBBRs (Jiang et al., 2019; Tal et al., 2003). As the biofilm developed and grew thicker, it provided niches for bacteria with poor EPS production capability but high survivability within a biofilm matrix. Nitrifying bacteria are an example of such microbes. The alteration in the species inventory also increased the α-diversity of the biofilms during the study (Supplementary information, Fig. A2). Fewer OTUs were classified at the family level in the brackish water biofilm. It is likely that the MiDAS3 database is biased towards freshwater microbial communities, as it characterizes microbial communities in full-scale wastewater treatment plants and anaerobic digesters (Nierychlo et al., 2019), which are typically operated at zero or low salt concentrations. However, all the nitrifying OTUs classified by the RDP database were also classified by the MiDAS3 database, indicating that the characterization of the nitrifying community was not negatively affected by this bias.

3.2.2. The microbial community composition of the two biofilms was significantly different

The ordination plot showed that the microbial community composition in F evolved significantly with time, whereas it was relatively stable in B (Fig. 2A). This suggests that the biofilm development was faster in F compared to B. In the second half of the study (days 30–60), when the developing biofilm started to adapt to the environmental conditions, the Bray-Curtis dissimilarity between F and B increased to an average of 0.93. The microbial community composition of the two treatments was significantly different during this period ($p < 0.001$, $R^2 = 0.72$). SIMPER analyses showed that five families could explain >50% of the difference between treatments (Table 2). *Burkholderiaceae* and *Sphingomonadaceae* were the most abundant families in F, whereas *Pseudomonadaceae* was the most abundant in B (Fig. 3). In the second half of the study, F had greater α-diversity than B. The first-order diversity in F (40 ± 5) was twice that in B (19 ± 15). Secondly, taxa richness was significantly higher in F (99 ± 5) than in B (68 ± 28). Finally, evenness was 50% higher in F (0.41 ± 0.03) than in B (0.27 ± 0.08).

3.2.3. The nitrifying community composition in the two biofilms was significantly different

In the OTU table with biofilm and water samples, 29 OTUs were identified as likely nitrifying bacteria. Seventeen of these were detected in the biofilm samples (Supplementary information, Table A3). Ten OTUs were classified as AOB. Seven of these were classified at the family level as *Nitrosomonadaceae*, wherein six were classified at the genus level as *Nitrosomonas*. The main nitrite oxidizer in both treatments was the genus *Nitrotoga*, within the family *Gallionellaceae*. This genus was not detected by the RDP database (Fig. 4). In both reactors, the relative abundance of the nitrifying bacteria increased rapidly after day 39. During days 46–60, the nitrifying community composition of the treatments differed significantly ($p = 0.002$, $R^2 = 0.46$). The freshwater reactor had a greater proportion of nitrifiers than the brackish water reactor. On day 60, the proportion of nitrifiers in F was 28% compared to 2% in B. This may explain the higher nitrification capacity in F. Treatment F also had a greater diversity of nitrifiers than B, with 12–13 nitrifying OTUs on day 60 compared to only 2–3 OTUs in B (Fig. 5). Note that one B sample on day 53 had ~12% nitrifiers, which may be an outlier, as all the other B samples during days 46–63 contained nitrifiers at a relative abundance <3%.

We constructed a phylogenetic tree in MEGA X software to compare the AOB OTUs obtained in this study with strains of AOB in the NCBI database (Supplementary information, Fig. A6). The dominant OTU in F (OTU_37) was most similar to *N. ureae*, probably due to the low substrate concentration. The B treatments contained two main AOB OTUs. One of them (OTU_22, *Nitrosomonas*) was detected in both F and B biofilms and can be considered halotolerant. The other OTU (OTU_109, 26% likelihood *Nitrosospira*) was absent in the F samples, suggesting that it was halophilic. Although AOB belonging to the genus *Nitrosococcus* have been reported in brackish biofilms (Kumar et al., 2010), *Nitrosomonas* and *Nitrosospira* appear to be the most common AOB genera in RAS biofilms, both freshwater and marine (Liu et al., 2019; Navada et al., 2019; Tal et al., 2003). It should be noted that the microbial analysis targeted only the bacterial domain, and not archaea. Studies show that archaea may be the dominant ammonia oxidizing microorganisms in RAS (Bartelme et al., 2019; Sauder et al., 2011). However, the extent of their contribution to the nitrification functionality is uncertain (Bartelme et al., 2017; Hatzenpichler, 2012).

In this study, *Nitrotoga* was the dominant nitrite oxidizer in both treatments, with relative abundance as high as 17%. Ion Torrent sequencing was used as a complementary analysis to confirm the presence of *Candidatus Nitrotoga*. This genus was detected at a slightly higher relative abundance (~27%) by Ion Torrent than by Illumina sequencing, possibly due to differences in methodology. Although *Nitrobacter* is considered an important genus of NOB in saltwater environments (Kuhn et al., 2010; Kumar et al., 2010), it was not detected in our study. *Nitrospira* has been reported as the main NOB in RAS bioreactors at salinities ranging from freshwater to seawater (Bartelme et al., 2019; Keuter et al., 2017; Rud et al., 2017). Comammox *Nitrospira* have also been detected in freshwater RAS, with speculations that comammox thrive under the oligotrophic conditions (in terms of the substrate, ammonia) in RAS (Bartelme et al., 2019, 2017; Kits et al., 2017). We do not know if comammox *Nitrospira* were present in this study, as it is not possible to differentiate between comammox and canonical *Nitrospira* by 16S rRNA amplicon sequencing (Pjevac et al., 2017). However, *Nitrospira* was found only in a few F samples at very low relative abundance (<0.2%) (by both methods). It is reported that *Nitrotoga* can outcompete both *Nitrospira* and *Nitrobacter* at temperatures 4–10 °C (Alawi et al., 2009; Karkman et al., 2011). Therefore, we hypothesize that the dominance of *Nitrotoga* over *Nitrospira* in our study may be due to lower temperatures (14–17 °C) than in the other studies (>20 °C). As this genus has also been reported as halotolerant (Keuter et al., 2017; Navada et al., 2020, 2019), it can be an important NOB in cold-water nitrifying systems with variable salinity. Notably, the genus *Nitrotoga* was not classified by the RDP database. Future studies on cold-water nitrifying biofilms should use suitable methods to target this genus.

3.2.4. The selection pressure played a bigger role in biofilm community assembly than the initial microbial composition

The microbial community composition in the intake water was analyzed to investigate if the bacteria from these sources served as inocula for the reactors. The relative abundance of nitrifying OTUs in the FW

Table 2
SIMPER analysis showing the taxa families contributing the most to the difference between the freshwater (F) and brackish water (B) treatments in the second half of the study.

Family	Average relative abundance in F	Average relative abundance in B	Contribution	Cumulative contribution
Pseudomonadaceae	2%	34%	24%	24%
Burkholderiaceae	16%	4%	10%	34%
Sphingomonadaceae	11%	0.2%	9%	43%
midas_f_68 (Order: *Saccharimonadales*)	8%	0%	6%	49%
Gallionellaceae	5%	0.3%	4%	53%

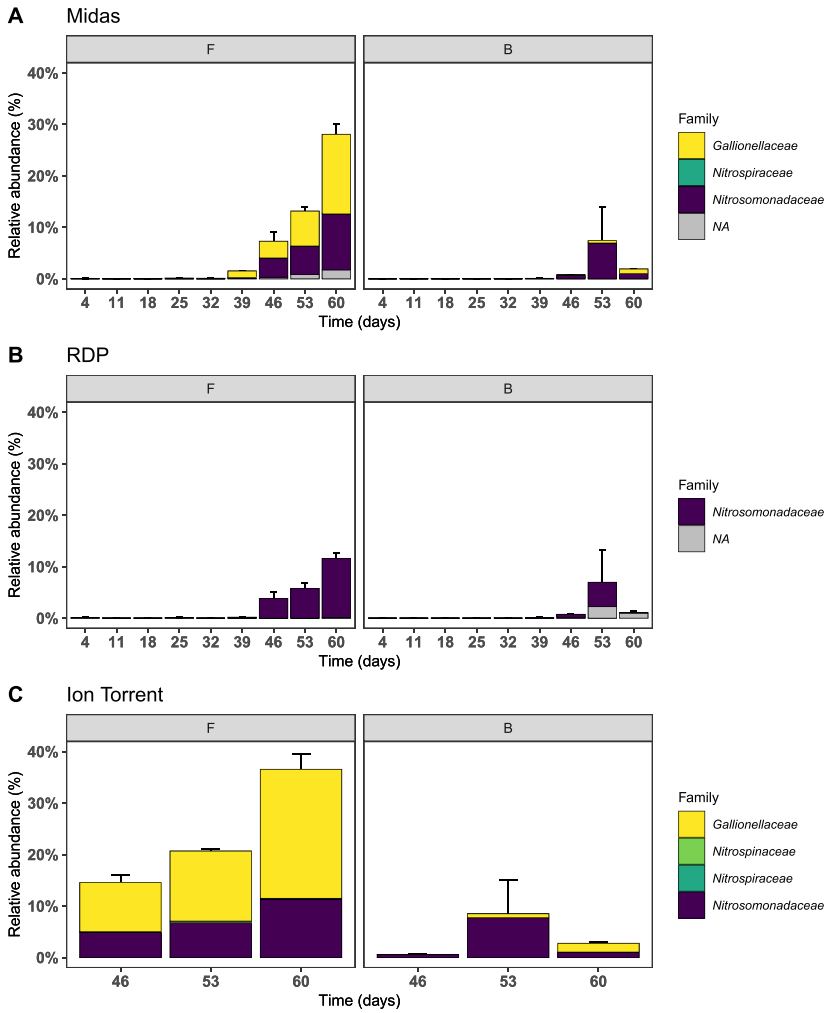

Fig. 4. Relative abundance of nitrifying bacteria in the biofilm in the freshwater (F) and brackish water (B) treatments analyzed by different methods of 16S rRNA gene amplicon sequencing. A) Illumina sequencing with classification by MiDAS3 database B) Illumina sequencing with classification by RDP database, and C) Ion Torrent™ sequencing. The OTUs are classified at the family level. Each bar shows the mean (± SD) relative abundance of total nitrifiers from two replicate biofilm carriers.

was low (<0.8%). One AOB OTU (OTU_37, *Nitrosomonas*) detected in a FW sample was also detected in the F biofilm on day 60 at ~8% relative abundance. The FW also contained a NOB OTU (OTU_33, *Nitrotoga*) that was found at 15% relative abundance in F and ~ 1% in B on day 60. Another NOB OTU (*Nitrospira)* was detected in the FW samples at 0.1–0.3% relative abundance, but it was not present in any of the biofilm samples. In the SW source, nitrifying bacteria were not detected at the set threshold. The sparseness of nitrifying bacteria in the SW was likely because of disinfection. However, two OTUs belonging to *Nitrosomonas* (OTU_22, 37) and one belonging to the genus *Nitrotoga* (OTU_33) were detected in the SW at relative abundance 0.01–0.10%. These OTUs were also detected in the F and B biofilms and in FW, suggesting that they were halotolerant. The dominant AOB (OTU_22) and NOB (OTU_33) established in the brackish biofilm were also detected in the FW and SW sources (as well as in F). This halotolerant nitrifying community may explain why salinity changes do not affect the microbial community composition in brackish water biofilms (Navada et al., 2020). However, the nitrification functionality during salinity changes is likely dependent on both the microbial community composition of the biofilm as well as the physiological response of the bacteria to osmotic stress.

After day 30, the α-diversity indices in the F biofilms were 1.5–2 times higher than in B. Given that B received bacterial inocula from both freshwater and seawater, one would have expected a higher diversity in this treatment. However, as the seawater was disinfected, the influx of bacteria (including nitrifiers) to the B reactor was lower. Further, although FW and SW had similar first-order diversity, SW had lower taxa richness and higher evenness than FW (Table 3). The lower species richness in the intake water thus narrowed the pool of bacterial species available for colonization in B. Moreover, the mixing of freshwater and seawater at the inlet of the B reactor may have caused cell plasmolysis due to the sudden change in the osmotic pressure (Csonka, 1989).

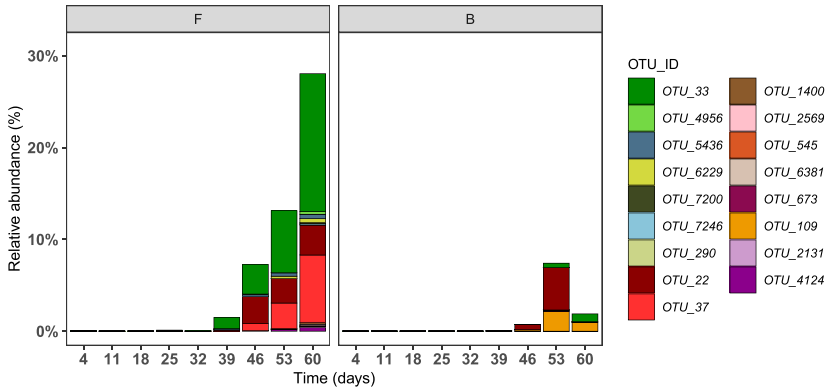

Fig. 5. Relative abundance of the nitrifying OTUs in the biofilms in the freshwater (F) and brackish water (B) treatments during the study. Each bar represents the mean of two replicate biofilm carriers. For the NOB: OTUs 33, 4956, 7246 were classified at the genus level as *Nitrotoga*; OTUs 5436, 6229, 7200 were classified as likely *Nitrotoga*; OTU_290 was classified as *Nitrospira*. For the AOB: OTUs 22, 37, 1400, 545, 6831, 673 were classified as *Nitrosomonas*; OTU_2569 was classified as likely *Nitrosomonas*; OTUs 109, 2131, 4124 were classified as likely *Nitrosospira*. See Supplementary information, Table A3 for detailed OTU classification.

Consequently, the B treatment received fewer bacteria that could adapt to the salinity in the reactor. Thus, the lower microbial diversity and nitrification performance in B may be attributed to the differences in intake water treatment in addition to the salt stress. As most fish farmers are required to disinfect the intake seawater, this study is representative of the actual industrial conditions.

The microbial community composition of the intake water sources was more similar to the initial biofilm samples. This suggests that the intake water served as a source of bacteria. However, in both treatments, the biofilm community diverged from the initial composition over time and became significantly different. In the F treatment, the Bray-Curtis distance between the biofilm and the freshwater source in the first month was 0.67, and this increased to >0.9 as the biofilm developed. In the B treatment, the biofilm composition was highly dissimilar (0.84–1.00) from the freshwater and the seawater sources throughout the study. Thus, the community assembly was more influenced by selection than dispersal (Nemergut et al., 2013), and the reactor conditions and biofilm interactions significantly influenced the bacterial succession. The opposite was observed in a study on nitrifying sludge, wherein the initial composition played a more important role than the operating conditions in the microbial community assembly (Wittebolle et al., 2009). However, biofilms are more complex than nitrifying sludge. As the bacteria in a biofilm share a common habitat, microbial interactions are crucial in determining the colonization success of a species within a biofilm. By the end of this study (days 46–60), the nitrifying community composition in the biofilm was significantly different from that in the intake water ($p = 0.002$, $R^2 = 0.32$). This suggests that a commercial nitrifying inoculum selected based on physiochemical factors alone may not necessarily succeed in colonizing the biofilm and promoting start-up. It may explain why some studies with nitrifying inocula did not succeed in accelerating start-up (Li et al., 2019; Manthe and Malone, 1987). Thus, when selecting a commercial inoculum, the survivability of the bacterial species in the biofilm and the selection pressure should be considered along with physicochemical factors. Further research is required to investigate the fitness of nitrifying species in biofilms at different salinities.

Table 3
α-diversity parameters for the freshwater and seawater intake sources. Mean (± SD) of three samples. Asterisks indicate significant difference based on a 95% confidence interval.

	Freshwater	Seawater	*p*
First-order diversity (N_1)	41.3 ± 9.9	42.3 ± 13.9	0.93
Richness (N_0)	116 ± 17	64 ± 28	0.052
Evenness (N_1/N_0)	0.35 ± 0.06	0.68 ± 0.09	**0.006***

4. Conclusions

This study investigated whether start-up in brackish water could be a strategy for nitrifying bioreactors dealing with variable salinity. The results showed that nitrification (especially nitrite oxidation) developed slightly slower in the brackish water reactor than in the freshwater reactor, possibly due to the higher salinity in the reactor and the disinfection of intake seawater. Although the intake water sources influenced the initial microbial community composition in the biofilms, the final community composition was determined by the selection pressure in each reactor. At the end of the study, the brackish water biofilm had lower diversity, and significantly different microbial and nitrifying community composition than the freshwater biofilm. Complete nitrification was established in both reactors within 60 days, indicating that start-up in brackish water can be a practical strategy to attain nitrifying biofilms robust to salinity changes. Notably, the dominant nitrite oxidizer in this study, *Nitrotoga*, was not classified by RDP database. As *Nitrotoga* are halotolerant and can be abundant (up to 20%) in cold-water RAS, future studies should use suitable methods to identify this genus.

CRediT authorship contribution statement

Sharada Navada: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Marianna Sebastianpillai:** Methodology, Investigation, Formal analysis, Writing - review & editing. **Jelena Kolarevic:** Conceptualization, Resources, Supervision, Writing - review & editing. **Ragnhild O. Fossmark:** Methodology, Writing - review & editing. **Ann-Kristin Tveten:** Methodology, Investigation, Resources. **Frédéric Gaumet:** Conceptualization, Supervision. **Øyvind Mikkelsen:** Resources, Supervision. **Olav Vadstein:** Conceptualization, Resources, Formal analysis, Supervision, Writing - review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

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Supplementary Information for

A salty start: Brackish water start-up as a microbial management strategy for nitrifying bioreactors with variable salinity

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Table A1: Families contributing the most to the differences in microbial community composition between the first and second half of the study in treatment F

Family	Relative abundance in days 1-30	Relative abundance in days 30-60	Contribution	Cumulative contribution
Burkholderiaceae	46%	16%	26%	26%
Pseudomonadaceae	18%	2%	14%	40%
Sphingomonadaceae	6%	11%	7%	47%
midas_f_68 (Order: *Saccharimonadales*)	0%	8%	7%	53%
Rhodobacteraceae	1%	8%	6%	60%

Table A2: Families contributing the most to the differences in microbial community composition between the first and second half of the study in treatment B

Family	Relative abundance in days 1-30	Relative abundance in days 30-60	Contribution	Cumulative contribution
Burkholderiaceae	19%	4%	25%	25%
Pseudomonadaceae	38%	34%	15%	40%
Rhodobacteraceae	6%	3%	7%	47%
Bdellovibrionaceae	3%	3%	6%	53%
Flavobacteriaceae	7%	8%	5%	58%

Table A3: Classification of the nitrifying OTUs in the biofilms using MiDAS3 database.

OTU_ID	AOB/ NOB	Classification by MiDAS3 database
OTU_33	NOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Gallionellaceae(0.9900),g:Nitrotoga(0.9801),s:midas_s_184(0.6763)
OTU_4956	NOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Gallionellaceae(0.9400),g:Nitrotoga(0.8836),s:midas_s_184(0.7511)
OTU_5436	NOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Gallionellaceae(0.2900),g:Nitrotoga(0.0841),s:midas_s_184(0.0151)
OTU_6229	NOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Gallionellaceae(0.2600),g:Nitrotoga(0.0676),s:midas_s_214(0.0088)
OTU_7200	NOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Gallionellaceae(0.1500),g:Nitrotoga(0.0225),s:midas_s_184(0.0032)
OTU_7246	NOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Gallionellaceae(0.9000),g:Nitrotoga(0.8010),s:midas_s_184(0.4886)
OTU_290	NOB	d:Bacteria(1.0000),p:Nitrospirae(1.0000),c:Nitrospira(1.0000),o:Nitrospirales(1.0000),f:Nitrospiraceae(1.0000),g:Nitrospira(1.0000),s:midas_s_4843(1.0000)
OTU_22	AOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Nitrosomonadaceae(1.0000),g:Nitrosomonas(0.9900),s:midas_s_654(0.4653)
OTU_37	AOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Nitrosomonadaceae(1.0000),g:Nitrosomonas(1.0000),s:midas_s_4796(0.6700)
OTU_1400	AOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Nitrosomonadaceae(1.0000),g:Nitrosomonas(1.0000),s:midas_s_4796(0.9600)
OTU_2569	AOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Nitrosomonadaceae(0.5800),g:Nitrosomonas(0.3364),s:midas_s_4796(0.1312)
OTU_545	AOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Nitrosomonadaceae(1.0000),g:Nitrosomonas(1.0000),s:midas_s_3664(0.3500)
OTU_6381	AOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Nitrosomonadaceae(0.9300),g:Nitrosomonas(0.8277),s:midas_s_654(0.3642)
OTU_673	AOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Nitrosomonadaceae(1.0000),g:Nitrosomonas(1.0000),s:midas_s_6372(0.5600)
OTU_109	AOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Nitrosomonadaceae(0.8300),g:Nitrosospira(0.2573),s:midas_s_19816(0.0798)
OTU_2131	AOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Nitrosomonadaceae(0.5500),g:Nitrosospira(0.2860),s:midas_s_19816(0.1487)
OTU_4124	AOB	d:Bacteria(1.0000),p:Proteobacteria(1.0000),c:Gammaproteobacteria(1.0000),o:Betaproteobacteriales(1.0000),f:Nitrosomonadaceae(0.7000),g:Nitrosospira(0.4620),s:midas_s_19816(0.3049)

Figure A1: Concentration of A) ammonia, and B) nitrite vs time during the capacity tests for ammonia and nitrite, respectively. The spike solution was dosed 30 and 15 min before the first sample was taken for ammonia and nitrite, respectively.

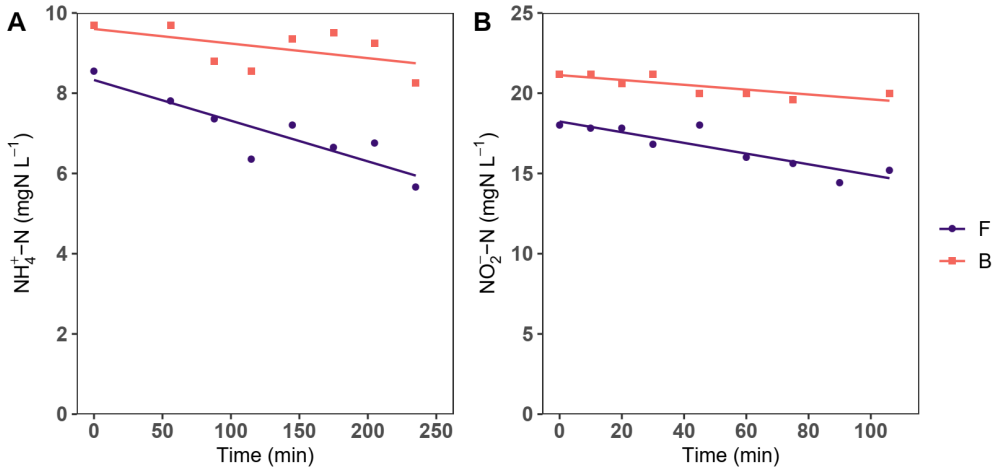


Figure A2: α-diversity in the biofilm in treatments F and B. A) Shannon diversity, B) First-order Hill number, C) Richness (zero-order Hill number), and D) Evenness. Error bars indicate standard deviation of two replicates.

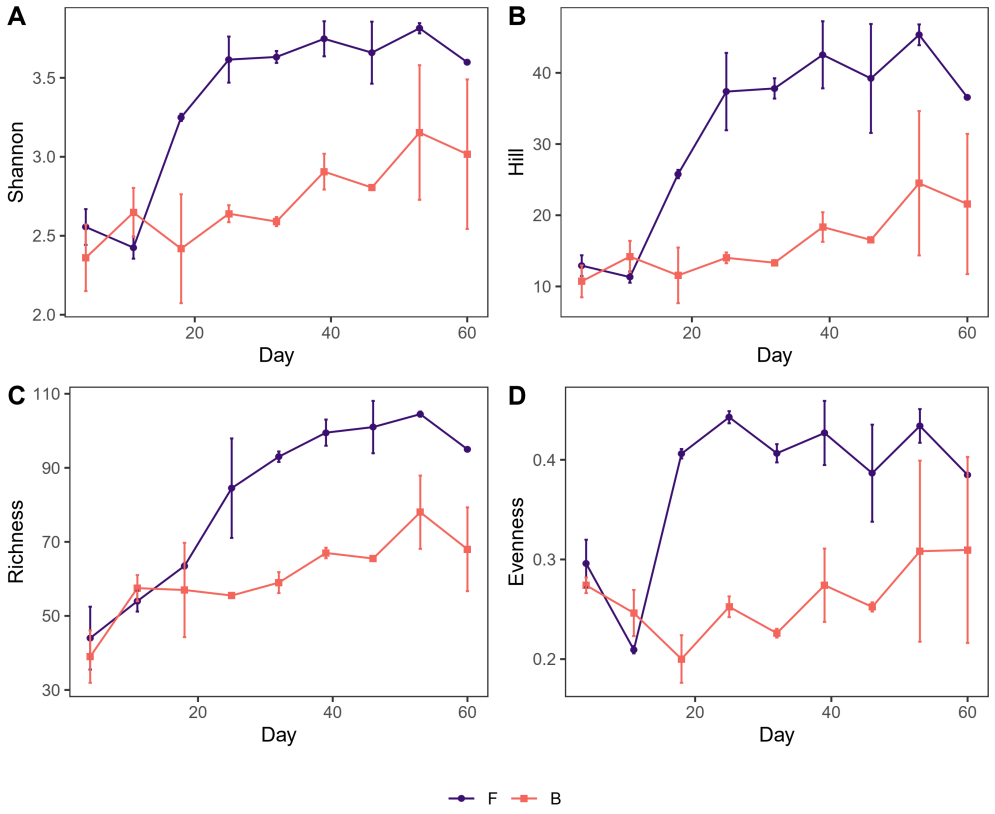


Figure A3: Bacterial succession in the freshwater (F) and brackish water (B) biofilms. For each treatment, the data points show the relative Bray-Curtis distance between the sample and A) the sample on day 4 and B) the previous sample (one week earlier). Graphs C and D show the relative Sørensen-Dice index between the sample and C) the sample on day 4 and D) the previous sample (one week earlier). Each data point is calculated from the mean distances between two replicate carriers for each sampling day.

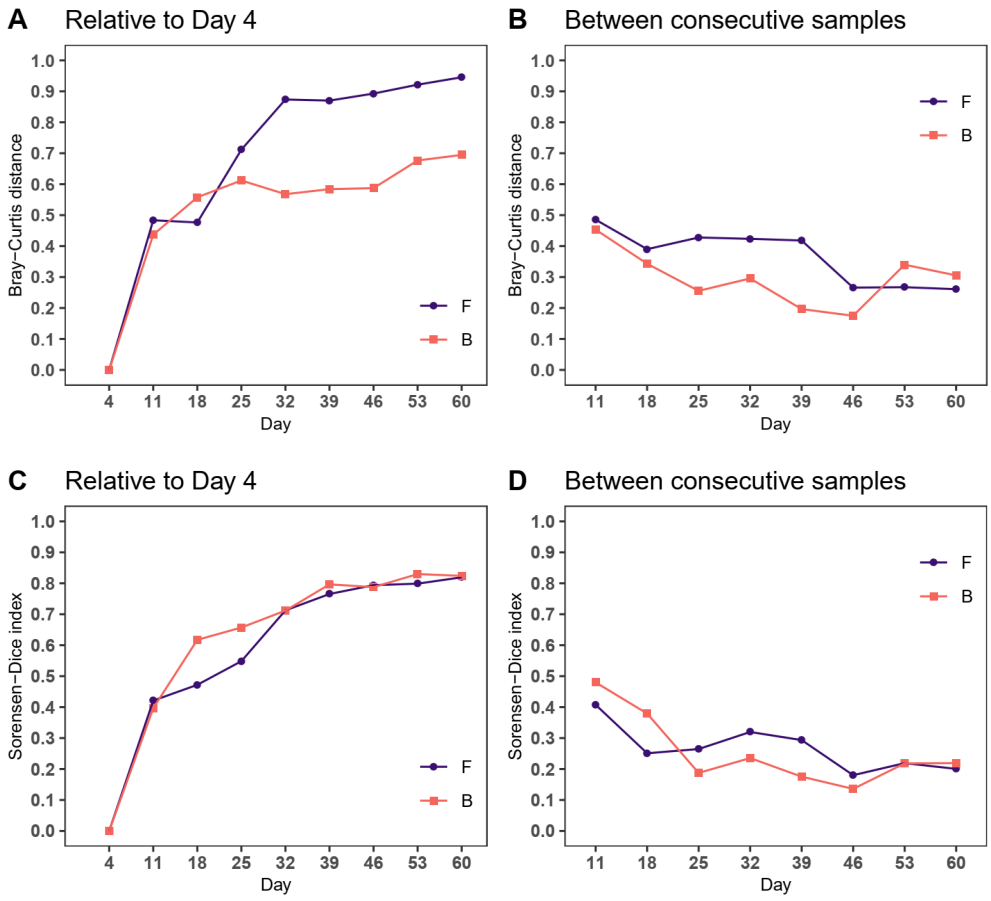


Figure A4: Relative abundance of OTUs in the biofilm classified at the phylum level using MiDAS3 database.

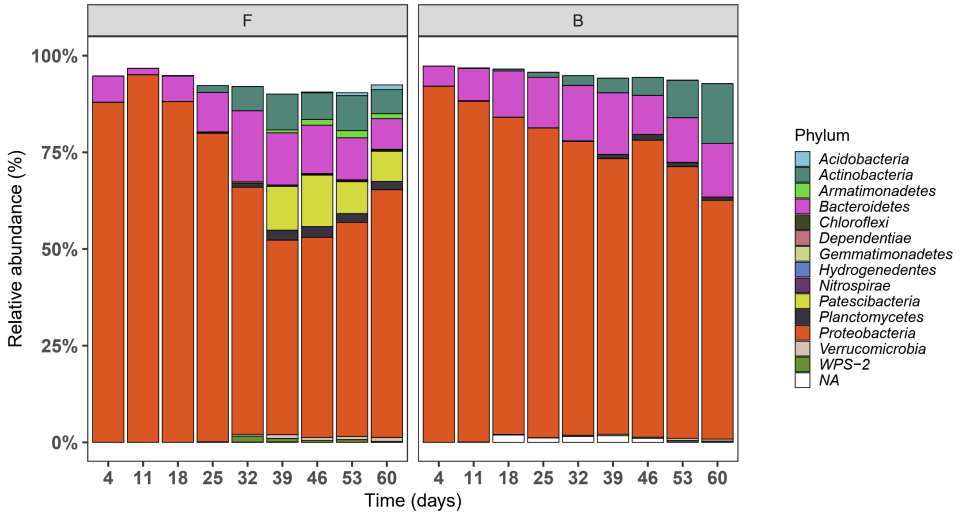


Figure A5: Relative abundance of OTUs in the biofilm classified at the class level using MiDAS3 database.

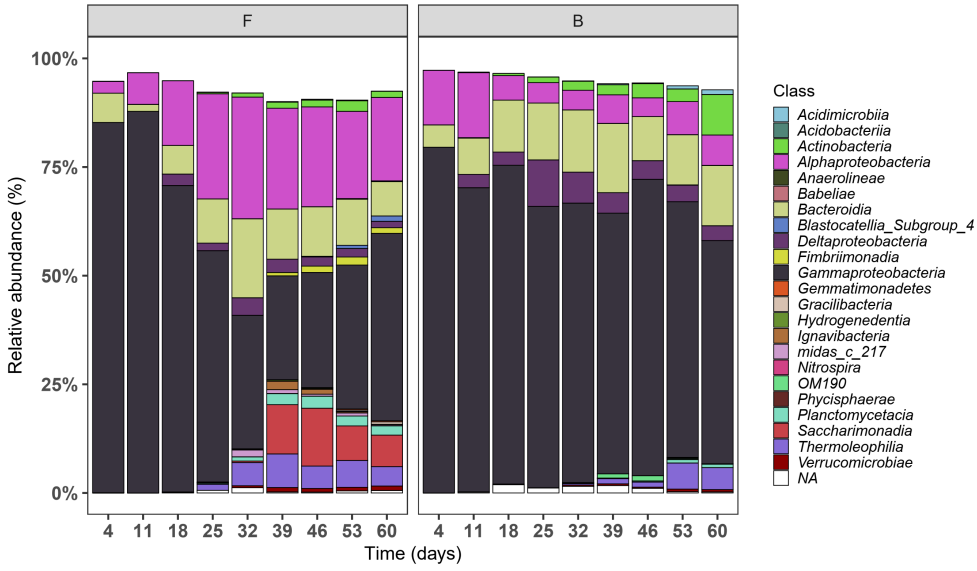
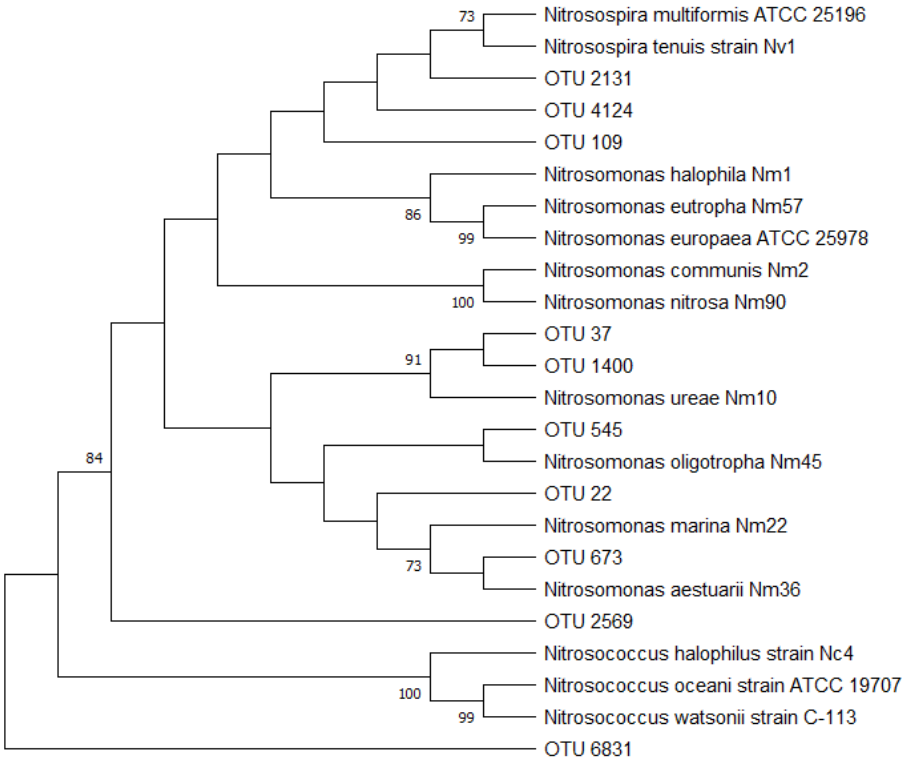


Figure A6: The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.69 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (if >70%). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated (complete deletion). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).



Strain name	NCBI Accession number
Nitrosomonas ureae strain Nm10	NR_119317.1
Nitrosomonas oligotropha strain Nm45	NR_114770.1
Nitrosomonas aestuarii strain Nm36	NR_114769.1
Nitrosomonas marina strain Nm22	NR_119316.1
Nitrosospira tenuis strain Nv1	NR_114773.1
Nitrosospira multiformis strain ATCC 25196	NR_112159.1
Nitrosomonas communis strain Nm2	NR_119314.1
Nitrosomonas nitrosa strain Nm90	NR_114772.1
Nitrosomonas halophila strain Nm1	NR_119315.1
Nitrosomonas europaea strain ATCC 25978	NR_117649.1
Nitrosomonas eutropha strain Nm 57	NR_114771.1
Nitrosococcus halophilus strain Nc4	NR_074790.1
Nitrosococcus oceani strain ATCC 19707	NR_074330.1
Nitrosococcus watsonii strain C-113	NR_074791.1

References

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X : Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol. Biol. Evol. 35, 1547–1549. doi:10.1093/molbev/msy096

Appendix B: Co-author statements

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

Microbial challenges in recirculating aquaculture systems for Atlantic salmon *(Salmo salar)* smolt and post-smolt

*) The declaration should describe the work process and division of labor, **specifically identifying the candidate's contribution**, as well as give consent to the article being included in the thesis.

*)
Declaration of co-authorship on the following article:

Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (*Salmo salar*). *Aquaculture*. vol.524. https://doi.org/10.1016/j.aquaculture.2020.735268

Ragnhild O. Fossmark contributed significantly to this experimental study, with operation of the RAS pilots and husbandry work, responsibility for the bacterial community sampling and analysis, and was the main responsible for the writing phase.

OV: significant advice on data analysis and interpretation, and guidance on structure and writing of the manuscript. **TR:** major parts of the planning, applications for funding, project management, operation of the RAS pilots and husbandry work, and data analysis. **IB:** parts of the data analysis and contribution to the writing phase. **DK:** operation of the RAS pilots, husbandry work, water quality measurements and quantification of bacteria. **AVB:** with operation of the RAS pilots, husbandry work and water quality measurements. **GAH:** operation of the RAS pilots and husbandry work, water quality measurements, and fish data analysis. **JN**: quantification of bacteria in the RAS pilots, and presentation of such results. **NOGJ:** major parts of the planning, applications for funding, data analysis and contribution to the writing phase. **GR:** water quality measurements and main responsibility for operation of the membrane. **KA:** major parts of the planning, applications for funding, project management, and responsibility for operation of the membrane. **SWØ**: parts of the data analysis and contribution to the writing phase. **KJKA:** major parts of the planning, applications for funding, project management, operation of the RAS pilots and husbandry work, advice on data analysis and contribution to the writing phase.

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark

26.11.2020
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Place, date

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Signature co-author

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I hereby declare that this article can form part of the thesis by the PhD candidate, Ragnhild Olsen Fossmark, who contributed to the article by providing guidance with the methodology for gene amplicon sequencing (Illumina), contributed to the microbial data analysis, helped with writing the associated methodology section, and co-supervised the master thesis based on this study.

Contribution of co-authors:
SN (first author) performed the detailed experimental design (including planning the sampling and analysis), the capacity tests, co-supervised the master thesis based on the study, performed data curation and analysis including statistical analysis - both physicochemical and microbiological (after generation of OTU table), data visualization, wrote the original draft and revised the subsequent versions after feedback from co-authors. **MS** analyzed the samples by 16S rRNA gene amplicon sequencing (Illumina), performed data analysis and interpretation and wrote her master thesis based on results from the physicochemical analysis and microbial data (Illumina sequencing with RDP classification). **JK** conceptualized the idea of studying the parallel startup of fresh- and brackish water bioreactors, provided resources (facility and technical help for sampling and analysis) and guidance on the manuscript. **AKT** performed gene amplicon sequencing (Ion Torrent) of biofilm (including resources, methodology) from DNA extraction until the generation of the OTU table. **FG** provided inputs to the manuscript from the industrial perspective. **ØM** provided resources (sensors, metals analysis). **OV** supervised the master thesis, provided significant guidance on data analysis and interpretation, and guided the structure and writing of the manuscript. All co-authors reviewed and provided inputs to the final manuscript.

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Trondheim, 15.01.2021
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Mass balance of carbon and nitrogen, nitrification efficiency and nitrifying community succession in recirculating aquaculture systems (RAS) with membrane filtration rearing Atlantic salmon parr *(Salmo salar).* *In prep*

Ragnhild O. Fossmark contributed significantly to this experimental study, with operation of the RAS pilots and husbandry work, major planning for sampling protocol, responsible for the physicochemical and bacterial community sampling and analysis, and was the main responsible for the writing phase.

KJKA: major parts of the planning for the RAS-pilots, applications for funding, project management, operation of the RAS pilots and husbandry work, advice on data analysis and contribution to the writing phase. **SWØ**: significant advice on the data analysis and interpretation, and contribution to the writing phase. **OV:** significant advice on data analysis and interpretation, and guidance on structure and writing of the manuscript

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark

Trondheim, 15.01.2021
……...
Place, date


…..……
Signature co-author

*)
Declaration of co-authorship on the following article:

A comparison of two seawater adaptation strategies for Atlantic salmon post-smolt (*Salmo salar*) grown in recirculating aquaculture systems (RAS): Nitrification, water and gut microbiota, and performance of fish. *Aquaculture.* vol. 532. DOI: 10.1016/j.aquaculture.2020.735973

Ragnhild O. Fossmark contributed significantly to this experimental study with planning for the sampling protocol, responsible for bacterial community sampling and analysis, and was the main responsible for the writing phase.

KJKA: Advice on data analysis and significant contribution to the writing phase. **KN:** major parts of the planning, applications for funding, project management and parts of the data analysis. **SWØ**: parts of the data analysis and contribution to the writing phase. **OV:** significant advice on data analysis and interpretation, and guidance on structure and writing of the manuscript

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark

Trondheim, 15.01.2021
……...
Place, date


…..……
Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

Microbial challenges in recirculating aquaculture systems for Atlantic salmon (Salmo salar) smolt and post-smolt

*) The declaration should describe the work process and division of labor, **specifically identifying the candidate's contribution**, as well as give consent to the article being included in the thesis.

*)
Declaration of co-authorship on the following article:

Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (*Salmo salar*). *Aquaculture*. vol.524. https://doi.org/10.1016/j.aquaculture.2020.735268

As a co-author, I have contributed to this experimental study with major parts of the planning, applications for funding, project management, operation of the RAS pilots and husbandry work, and data analysis

Ragnhild O. Fossmark contributed significantly to this experimental study, with operation of the RAS pilots and husbandry work, responsibility for the bacterial community sampling and analysis, and was the main responsible for the writing phase.

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark

..... 25/9-20
Place, date

.....
Signature co-author
Trond Rosten

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

Microbial challenges in recirculating aquaculture systems for Atlantic salmon *(Salmo salar)* smolt and post-smolt

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As a co-author, I have contributed to this experimental study with parts of the data analysis and contribution to the writing phase

Ragnhild O. Fossmark contributed significantly to this experimental study, with operation of the RAS pilots and husbandry work, responsibility for the bacterial community sampling and analysis, and was the main responsible for the writing phase.

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark

Ingrid Bakke

Ingrid Bakke

Trondheim, 25.09.2020
……...
Place, date

…..……
Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

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As a co-author, I have contributed to this experimental study with operation of the RAS pilots, husbandry work, water quality measurements and quantification of bacteria

Ragnhild O. Fossmark contributed significantly to this experimental study, with operation of the RAS pilots and husbandry work, responsibility for the bacterial community sampling and analysis, and was the main responsible for the writing phase.

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark

Deni Koseto

Trondheim, 30/9-20
Place, date

Deni Koseto
Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

Microbial challenges in recirculating aquaculture systems for Atlantic salmon *(Salmo salar)* smolt and post-smolt

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As a co-author, I have contributed to this experimental study with operation of the RAS pilots, husbandry work and water quality measurements

Ragnhild O. Fossmark contributed significantly to this experimental study, with operation of the RAS pilots and husbandry work, responsibility for the bacterial community sampling and analysis, and was the main responsible for the writing phase.

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark

Anette Bugten

TRONDHEIM., 29.09.20
Place, date

Anette Wold Bugten
Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

Microbial challenges in recirculating aquaculture systems for Atlantic salmon (Salmo salar) smolt and post-smolt

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As a co-author, I have contributed to this experimental study with operation of the RAS pilots and husbandry work, water quality measurements, and fish data analysis

Ragnhild O. Fossmark contributed significantly to this experimental study, with operation of the RAS pilots and husbandry work, responsibility for the bacterial community sampling and analysis, and was the main responsible for the writing phase.

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark

Gaute Helberg

Trondheim, 06.10.20
Place, date

Gaute Helberg
Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

Microbial challenges in recirculating aquaculture systems for Atlantic salmon (Salmo salar) smolt and post-smolt

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As a co-author, I have contributed to this experimental study with quantification of bacteria in the RAS pilots, and presentation of such results

Ragnhild O. Fossmark contributed significantly to this experimental study, with operation of the RAS pilots and husbandry work, responsibility for the bacterial community sampling and analysis, and was the main responsible for the writing phase.

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark

Jenny Nesje

Halden, 25. Sep 2020
Place, date

Jenny Nesje
Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

Microbial challenges in recirculating aquaculture systems for Atlantic salmon *(Salmo salar)* smolt and post-smolt

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As a co-author, I have contributed to this experimental study with major parts of the planning, applications for funding, data analysis and contribution to the writing

Ragnhild O. Fossmark contributed significantly to this experimental study, with operation of the RAS pilots and husbandry work, responsibility for the bacterial community sampling and analysis, and was the main responsible for the writing phase.

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark.

Copenhagen, 25-09-2020

Niels O.G. Jørgensen

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

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As a co-author, I, Gema Sakti Raspati, have contributed to this experimental study with water quality measurements and main responsibility for operation of the membrane.

Ragnhild O. Fossmark contributed significantly to this experimental study, with operation of the RAS pilots and husbandry work, responsibility for the bacterial community sampling and analysis, and was the main responsible for the writing phase.

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark.

Gema Sakti Raspati

Trondheim, 25-09-2020
……...
Place, date

…..……
Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

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As a co-author, I have contributed to this experimental study with major parts of the planning, applications for funding, project management, and responsibility for operation of the membrane.

Ragnhild O. Fossmark contributed significantly to this experimental study, with operation of the RAS pilots and husbandry work, responsibility for the bacterial community sampling and analysis, and was the main responsible for the writing phase.

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark

Dr. Kamal Azrague

Oslo, 25.09.2020

Place, date

Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

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DOI: 10.1016/j.aquaculture.2020.735973

Ragnhild O. Fossmark contributed significantly to this experimental study with planning for the sampling protocol, responsible for bacterial community sampling and analysis, and was the main responsible for the writing phase.

KJKA: Advice on data analysis and significant contribution to the writing phase. **KN:** major parts of the planning, applications for funding, project management and parts of the data analysis. **SWØ**: parts of the data analysis and contribution to the writing phase. **OV:** significant advice on data analysis and interpretation, and guidance on structure and writing of the manuscript

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark

Sandnessjøen
26.11.2020
Place, date

Kristian Nordøy
Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

Microbial challenges in recirculating aquaculture systems for Atlantic salmon *(Salmo salar)* smolt and post-smolt

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*)
Declaration of co-authorship on the following article:

A Salty Start: Brackish water start-up as a microbial management strategy for nitrifying bioreactors with variable salinity. *Science of the Total Environment*. vol. 739. https://doi.org/10.1016/j.scitotenv.2020.139934

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark, who contributed to the experimental study with bacterial community analysis, and assisted in parts of the writing phase

Sharada Navada

Trondheim, 1st Oct 2020
……...
Place, date

…..……
Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

Microbial challenges in recirculating aquaculture systems for Atlantic salmon *(Salmo salar)* smolt and post-smolt

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A salty start: Brackish water start-up as a microbial management strategy for nitrifying bioreactors with variable salinity. *Science of the Total environment.* vol. 739. https://doi.org/10.1016/j.scitotenv.2020.139934

I hereby declare that this article can form part of the thesis by the PhD candidate, Ragnhild Olsen Fossmark, who contributed to the article by providing guidance with the methodology for gene amplicon sequencing (Illumina), contributed to the microbial data analysis, helped with writing the associated methodology section, and co-supervised the master thesis based on this study.

Contribution of co-authors:
SN (first author) performed the detailed experimental design (including planning the sampling and analysis), the capacity tests, co-supervised the master thesis based on the study, performed data curation and analysis including statistical analysis - both physicochemical and microbiological (after generation of OTU table), data visualization, wrote the original draft and revised the subsequent versions after feedback from co-authors. **MS** analyzed the samples by 16S rRNA gene amplicon sequencing (Illumina), performed data analysis and interpretation and wrote her master thesis based on results from the physicochemical analysis and microbial data (Illumina sequencing with RDP classification). **JK** conceptualized the idea of studying the parallel startup of fresh- and brackish water bioreactors, provided resources (facility and technical help for sampling and analysis) and guidance on the manuscript. **AKT** performed gene amplicon sequencing (Ion Torrent) of biofilm (including resources, methodology) from DNA extraction until the generation of the OTU table. **FG** provided inputs to the manuscript from the industrial perspective. **ØM** provided resources (sensors, metals analysis). **OV** supervised the master thesis, provided significant guidance on data analysis and interpretation, and guided the structure and writing of the manuscript. All co-authors reviewed and provided inputs to the final manuscript.

Trondheim 22 10 20
……...
Place, date

Marianna Sebastianpillai
MariannaS
……
Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

Microbial challenges in recirculating aquaculture systems for Atlantic salmon *(Salmo salar)* smolt and post-smolt

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Jelena Kolarevic

Sunndalsøra, 23/10/20
Place, date

Jelena Kolarevic
Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

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*)
Declaration of co-authorship on the following article:

A salty start: Brackish water start-up as a microbial management strategy for nitrifying bioreactors with variable salinity. *Science of the Total environment.* vol. 739. https://doi.org/10.1016/j.scitotenv.2020.139934

As a co-author, I, Ann-Kristin Tveten, have contributed by performing gene amplicon sequencing (Ion Torrent) of biofilm (including resources, methodology) from DNA extraction until the generation of the OTU table.

Ragnhild O. Fossmark contributed to the article with the methodology for gene amplicon sequencing (Illumina), contributed to the microbial data analysis, helped with writing the associated methodology section and co-supervised the master thesis based on this study

I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark

Ann-Kristin Tveten

Ålesund 21/10-20
Place, date

Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

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I hereby declare that this article can form part of the named thesis by the PhD candidate Ragnhild O. Fossmark, who contributed to the experimental study with bacterial community analysis, and assisted in parts of the writing phase

Oslo, 02/10/2020
Place, date

Frederic GAUTET
Signature co-author

NTNU

DECLARATION OF CO-AUTHORSHIP

Ragnhild Olsen Fossmark applies to have the following thesis assessed:

Microbial challenges in recirculating aquaculture systems for Atlantic salmon *(Salmo salar)* smolt and post-smolt

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I hereby declare that this article can form part of the thesis by the PhD candidate, Ragnhild Olsen Fossmark, who contributed to the article by providing guidance with the methodology for gene amplicon sequencing (Illumina), contributed to the microbial data analysis, helped with writing the associated methodology section, and co-supervised the master thesis based on this study.

Contribution of co-authors:
SH (first author) performed the detailed experimental design (including planning the sampling and analysis), the capacity tests, co-supervised the master thesis based on the study, performed data curation and analysis including statistical analysis - both physicochemical and microbiological (after generation of OTU table), data visualization, wrote the original draft and revised the subsequent versions after feedback from co-authors. **MS** analyzed the samples by 16S rRNA gene amplicon sequencing (Illumina), performed data analysis and interpretation and wrote her master thesis based on results from the physicochemical analysis and microbial data (Illumina sequencing with RDP classification). **JK** conceptualized the idea of studying the parallel startup of fresh- and brackish water bioreactors, provided resources (facility and technical help for sampling and analysis) and guidance on the manuscript. **AKT** performed gene amplicon sequencing (Ion Torrent) of biofilm (including resources, methodology) from DNA extraction until the generation of the OTU table. **FG** provided inputs to the manuscript from the industrial perspective. **ØM** provided resources (sensors, metals analysis). **OV** supervised the master thesis, provided significant guidance on data analysis and interpretation, and guided the structure and writing of the manuscript. All co-authors reviewed and provided inputs to the final manuscript.

Øyvind Mikkelsen

Longyearbyen, 25/10-20
Place, date

Signature co-author

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NTNU

Norwegian University of
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