Doctoral theses at NTNU, 2021:33

Sharada Navada

Salinity acclimation strategies for nitrifying bioreactors in recirculating aquaculture systems

Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
Philosophiae Doctor
Faculty of Natural Sciences
Department of Chemistry

NTNU
Norwegian University of
Science and Technology

Sharada Navada

Salinity acclimation strategies for nitrifying bioreactors in recirculating aquaculture systems

Thesis for the Degree of Philosophiae Doctor

Trondheim, February 2021

Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Chemistry

NTNU
Norwegian University of
Science and Technology

NTNU
Norwegian University of Science and Technology

Thesis for the Degree of Philosophiae Doctor

Faculty of Natural Sciences
Department of Chemistry

ISBN 978-82-471-4995-9 (printed ver.)
ISBN 978-82-471-4978-2 (electronic ver.)
ISSN 1503-8181 (printed ver.)
ISSN 2703-8084 (online ver.)

Doctoral theses at NTNU, 2021:33

Printed by NTNU Grafisk senter

An ode to microbes

Everywhere, yet invisible
Tiny, yet tough
Both benign and terrifying
Of it we can't speak enough.

When Leeuwenhoek spotted it
Little did he know
The strength of this ancient creature
A friend or a foe?

What we began by avoiding,
We now gladly embrace,
Creating infinite possibilities
For science and the human race.

—Sharada Navada

Abstract

With the global population poised to exceed 9 billion by 2050, food production is going to be a huge challenge. Recirculating aquaculture systems (RAS) can be a sustainable solution for producing seafood in land-based systems with water treatment and reuse. As the most valuable seafood commodity, salmonids are a commonly farmed species in RAS. Anadromous fish such as Atlantic salmon (*Salmo salar*) are typically reared in freshwater RAS until they are smoltified (seawater-adapted), and then transferred to sea cages for further growth and harvest. Due to several factors, the past decade has seen a burgeoning interest in growing salmon to a larger size in RAS, both in coastal and land-locked locations. However, the varying salinity requirements pose a challenge to the water treatment processes in RAS, particularly the nitrifying bioreactor that removes the ammonia produced by the fish. As both ammonia and nitrite are extremely toxic to fish, maintaining the nitrification efficiency during salinity changes is vital. The aim of this PhD was to investigate strategies to acclimate nitrifying bioreactors to salinity variations, so that salinity changes may be conducted in a safe manner for the fish.

The first study showed that the rate of salinity increase did not influence the nitrification activity in moving bed biofilm reactors (MBBR) during a transfer from fresh- to seawater. Ammonia oxidation capacity was mainly dependent on the salinity ($\sim$2.7% decrease per ‰) and acclimatization time ($\sim$2.1% recovery per day), with nearly complete recovery after 39 days in seawater. Salinity increments as low as 1‰ day^{-1} led to a 50-90% reduction in activity upon seawater transfer, suggesting that continuous daily salinity increments are impractical in a freshwater RAS with fish. However, if an initial decrease in nitrification activity is acceptable, large salinity increments (for e.g., 6-15‰ day^{-1}) with a long acclimatization period appear to be the most practical strategy. To increase the salinity tolerance of nitrifying bioreactors, we tested the following microbial management strategies.

The second study showed osmotic stress priming (prior exposure to seawater) to be an effective strategy for improving the nitrification activity in freshwater bioreactors. Upon seawater transfer, ammonia oxidation capacity in the primed biofilms decreased by <10% compared to 55% in the unprimed biofilms. In contrast, brackish water (12‰ salinity) biofilms were inherently robust to salinity increase, with no decrease in ammonia oxidation upon seawater transfer. As seawater priming is a time-intensive strategy, start-up in brackish water was considered as an alternative strategy. Thus, a third study was conducted to compare the parallel start-up of nitrification in two semi-commercial RAS MBBRs in fresh- and brackish water. Complete nitrification was established in both reactors within 60 days. However, the bacterial succession was slower in the brackish biofilm and the nitrification capacity was approximately half that in the freshwater biofilm. To accelerate start-up while conferring salinity resistance, the fourth study compared the salinity tolerance of newly started freshwater MBBRs seeded with biofilm carriers acclimated to fresh- or brackish water. The reactors seeded with brackish biofilm had only a 20% reduction in ammonia oxidation capacity upon seawater transfer, compared to ∼70% in the reactors seeded with freshwater biofilms. Seeding with brackish biofilm can thus be applied as a start-up strategy in RAS bioreactors. Nitrite concentration should be closely monitored after salinity changes, as nitrite oxidation can be compromised at higher salinities.

In all the studies, *Nitrosomonas* and *Nitrotoga* were the dominant genera of ammonia and nitrite oxidizers, respectively. This suggests that species within these genera are halotolerant and play an important role in cold-water RAS. A fifth study was performed to investigate if the addition of an osmolyte cocktail (containing 1mM each of trehalose, sucrose, glycine betaine, proline, carnitine, and ectoine) could aid salinity acclimation in biofilms. Osmolytes did not improve nitrification activity after a salinity increase from freshwater to seawater, likely because they were taken up by heterotrophic bacteria for osmoregulation or as substrate.

Further research should involve methods such as metaproteomics to characterize the species composition at different salinity regimes in RAS bioreactors, and to identify the biological pathways involved in salinity acclimation. Future studies should also investigate the optimum salinity for post-smolt RAS. This may be around 12‰, as it is close to isotonic conditions and thus energetically favorable both for the fish and the nitrifiers. While the salinity acclimation strategies were motivated by the challenges in RAS, they are also applicable for the treatment of high or variable salinity effluents produced by other industries. These strategies will improve the design and management of nitrifying bioreactors, especially in RAS, thus bringing us one step closer towards sustainable food production for the future.

Preface

This Industry PhD study was conducted as part of the CtrlAQUA SFI - Centre for research-based innovations in closed-containment aquaculture, the broad objective of which is: "To develop technological and biological innovations to make closed-containment aquaculture systems (CCS) a reliable and economically viable technology, for use in strategic parts of the Atlantic salmon production cycle, thus contributing significantly to solving the challenges limiting the envisioned growth in aquaculture." The PhD was established as a collaboration between Nofima, Krüger Kaldnes AS, and NTNU, who are also partners in the CtrlAQUA consortia. The goal was to investigate ways of changing the salinity in RAS bioreactors in a safe manner for the fish. As pioneers in the moving bed biofilm reactor (MBBR) technology as well as one of the leading suppliers of RAS, Krüger Kaldnes AS was a natural choice for industry collaboration on this PhD.

The PhD consisted of four main studies, three of which have been published in international journals, while the fourth manuscript is in progress. A fifth study was also performed during a four-month exchange at the Environmental Biotechnology department at TU Delft, Netherlands. Unfortunately, the experimental plan for this study had to be changed considerably due to the COVID-19 pandemic. As a result, the experimental work is still in progress and only preliminary results are presented here. The results from the PhD have been disseminated through various media, such as scientific conference presentations, posters, and videos. The findings from these studies have resulted not only in the development of strategies that can be practically applied in commercial RAS, but also provided a deeper insight into the response of the microbial community composition in biofilms to salinity changes. Thus, I hope that this thesis will satisfy both the readers interested in the engineering applications, as well as those curious about the underlying microbes that may explain the observations.

Acknowledgments

I had been warned that having more than one PhD supervisor could be a nightmare. Despite having more than seven supervisors during the PhD, I have been extremely fortunate. Although I have never been with all my four main supervisors at the same place and time (yet), they have always been there whenever I needed guidance. I would like to thank my university supervisor, Prof. Øyvind Mikkelsen, for his ever-cheerful demeanor and for procuring resources to aid my PhD. I am deeply indebted to my company supervisor, Dr. Frédéric Gaumet, for convincing me to do the PhD that led me to find my husband, and for his Saggitarian wisdom and empathy throughout these four years (and more!). I would like to thank my kind and supportive supervisor, Dr. Jelena Kolarevic, for stabilizing my PhD after all the changes in supervisors and for encouraging me to always aim high. Although I met my last supervisor Prof. Olav Vadstein only halfway through the PhD, I feel like I have known him for a long time. I am amazed by his sharp intellect, his kind consideration to people, and his dedication to giving a thorough review of everything that crosses his desk. I also thank my manager at Krüger Kaldnes, Christian Rønning, for organizing my responsibilities such that I could manage both the company work and the PhD without burning out.

Next, I would like to thank my plethora of previous supervisors and mentors - Dr. Bendik F. Terjesen and Marius Hægh for conceiving the PhD, Dr. Andries Kamstra for rescuing me in the first study when I had no direction, Dr. Vasco Mota for the immense help on the first manuscript, and the team at AnoxKaldnes (Thomas, Sofia, Maria and Maria) for guiding me through my first MBBR study. I want to thank Prof. Mark van Loosdrecht at TU Delft for the cool attitude that gave me the confidence to perform a study even during the tricky COVID situation - those nice coffee conversations were my only human contact in the two months of lockdown. I am also inspired by his sense of adventure and curiosity to get out of his comfort

zone. I thank my post-doc supervisor, Dr. Michele Laureni (TU Delft), for finding humor in mundane situations and for his frank admission to loving his cave life during the pandemic.

I would like to thank my co-workers and students who helped me. I wish to thank Dr. Ann-Kristin Tveten for conducting the microbiological lab analysis - the brusque exterior hides a wonderfully kind heart. Chris - the funniest, most opti-Chris-tic, extroverted person I have ever met and the best housemate. You really kept the mood up during the first experiment when I was struggling to find solid ground. Claudia (queen of the spectrophotometer) – you are the most cheerful and agreeable coworker one could wish for, who never complains about anything (except the turtles). Marianna - I could not have asked for a better masters student during the third experiment; self-driven, hardworking, and a critical thinker. Ragnhild – I am glad that I got to know you through our parallel PhDs, and thank you for introducing me to Olav. Vishwesh – learning R would have been tough without you, thanks! Thank you, Zejia, for the short but happy time we had together biking in Delft. I also thank the wonderful staff at Nofima, notably Britt-Kristin, Frode, Yuriy, Dag, Jascha, May-Britt, and Bernhard for their creative ideas and support during the experiments.

I would like to thank Krüger Kaldnes and the Research Council of Norway (RCN) for funding this Industry PhD (# 270888). I am fortunate to be a part of the CtrlAQUA SFI center for research-based innovation, funded by the RCN and the Center partners (# 237856). Through the Center, I got to know a great group of cutting-edge researchers. I am also grateful to Dr. David Weissbrodt and Dr. Sanni Aalto in the PhD assessment committee, who provided valuable feedback for improving the quality of this thesis.

Lastly, I can't thank my family enough. My sister, Numrata, for making me feel like I am the top researcher in the whole wide world. My husband aka home supervisor, Jeevith, for bearing with more than two years of long-distance relationship, for making me laugh through tense times, and for the incessant support throughout the PhD. My mother, for encouraging me to push my boundaries harder and to give my very best to everything I do. I am deeply inspired by her extraordinary vision, intense courage, and tranquillity in the harshest of situations. Finally, I am eagerly awaiting a new addition to our family – the one who really motivated me to finish the PhD earlier than planned!

List of articles

1. **Article I** - Navada, S., Vadstein, O., Tveten, A.-K., Verstege, G.C., Terjesen, B.F., Mota, V.C., Venkataraman, V., Gaumet, F., Mikkelsen, Ø., Kamstra, A., 2019. *Influence of rate of salinity increase on nitrifying biofilms*. J. Clean. Prod. 238.

2. **Article II** - Navada, S., Vadstein, O., Gaumet, F., Tveten, A.-K., Spanu, C., Mikkelsen, Ø., Kolarevic, J., 2020. *Biofilms remember: Osmotic stress priming as a microbial management strategy for improving salinity acclimation in nitrifying biofilms*. Water Res. 115732.

3. **Article III** - Navada, S., Sebastianpillai, M., Kolarevic, J., Fossmark, R.O., Tveten, A.K., 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*. Sci. Total Environ. 739.

4. **Article IV** - Navada, S., Gaumet, F., Tveten, A.-K., Kolarevic, J., Vadstein, O. *Seeding with brackish water biofilm as a start-up strategy for salinity acclimation in freshwater nitrifying bioreactors*. Manuscript in progress.

5. **Article V** - Navada, S., Laureni, M., Vadstein, O., van Loosdrecht, M.C.M.. *Investigating the exogenous addition of osmolytes as a salinity acclimation strategy in nitrifying biofilms*. Manuscript in progress.

List of published articles not included in this thesis:

1. Navada, S., Knutsen, M.F., Bakke, I., Vadstein, O., 2020. *Nitrifying biofilms deprived of organic carbon show higher functional resilience to increases in carbon supply*. Sci. Rep. 10, 7121.

2. Aslam, S.N., Navada, S., Bye, G.R., Mota, V.C., Terjesen, B.F., Mikkelsen, Ø., 2019. *Effect of CO_2 on elemental concentrations in recirculating aquaculture system tanks*. Aquaculture 511.

List of conference proceedings:

1. Navada, S.; Gaumet, F.; Tveten, A-K; Kolarevic, J.; Vadstein, O. (2020) *Seeding as a salinity acclimation strategy in nitrifying bioreactors*. Oral presentation at Smolt production in the future - 6th Conference on Recirculating Aquaculture. Nofima; Sunndalsøra (Virtual conference).

2. Navada, S.; Gaumet, F.; Vadstein, O.; Spanu, C.; Mikkelsen, Ø.; Kolarevic, J. (2019) *Improving salinity adaptation in nitrifying bioreactors by seawater priming*. Oral presentation at AE2019. European Aquaculture Society; Berlin.

3. Navada, S.; Sebastianpillai, M.; Gaumet, F.; Vadstein, O.; Fossmark, R.O.; Mikkelsen, Ø; Kolarevic, J. (2019) *Salinity change strategies for biofilters in RAS for Atlantic salmon (Salmo salar)*. Oral presentation at NordicRAS; Berlin.

4. Navada, S.; Vadstein, O.; Gaumet, F.; Tveten, A-K.; Spanu, C.; Mikkelsen, Ø.; Kolarevic, J. (2019) *Improving salinity adaptation of nitrifying biofilms with seawater priming*. Poster at 8th IWA Microbial Ecology & Water Engineering Specialist Conference (MEWE) ; Hiroshima.

5. Navada, S.; Vadstein, O; Tveten, A-K.; Terjesen, B. F.; Mota, V. C.; Gaumet, F.; Mikkelsen, Ø.; Kamstra, A. (2019) *Influence of rate of salinity increase on nitrifying biofilms*. Oral presentation at 8th IWA Microbial Ecology & Water Engineering Specialist Conference (MEWE); Hiroshima.

6. Sebastianpillai, M.; Navada, S.; Fossmark, R. O.; Vadstein, O. (2019) *Nitrifying capacity and microbial community structure during start-up of freshwater and brackish water moving bed biofilm reactors in recirculating aquaculture systems*. Poster at NBS Contact Meeting. Norske Kjemiske Selskap; Røros.

7. Navada, S. (2018) *Effect of change in water salinity on nitrification in moving bed biofilm reactors (MBBR)*. Oral presentation at Smolt production in the future – 5th Conference on Recirculating Aquaculture. Nofima AS; Sunndalsøra.

8. Navada, S.; Verstege, G. C.; Terjesen, B. F.; Mikkelsen, Ø.; Gaumet, F.; Mota, V. C.; Kamstra, A. (2018) *Biofilter salinity change strategies for Atlantic salmon post-smolt production in RAS*. Oral presentation at AQUA 2018. World Aquaculture Society, European Aquaculture Society; Montpellier.

9. Navada, S. (2017) *Improving salmon production with better water quality - The Eco-Way*. Oral presentation at Oslo Innovation Week ; Oslo.

10. Navada, S. (2017) *Lower energy need with better biofilter*. Oral presentation at AQUA NOR ; Trondheim.

11. Navada, S. (2017) *Secrets of a biofilter - Why microscopic things matter*. Oral presentation at AQUA NOR ; Trondheim.

Other dissemination:

1. Article (based on **Article III**) in Aquacultural Engineering Society (AES) newsletter in August 2020 issue

2. Multimedia (videos)

    - How fast can one switch from freshwater to seawater?
    - Improving salmon production with better water quality – the eco-way | Sharada Navada | Oslo Innovation Week 2017

Contents

To my darling mother, sister, and husband

Part I

Overview of thesis

Chapter 1

Introduction

"The best way to predict your future is to create it"
—Abraham Lincoln

1.1 Background

The 21st century has seen an unprecedented growth in human population. Feeding this burgeoning population will be one of the biggest challenges faced in the coming decades. But food production and distribution is also responsible for ∼25% of the global anthropogenic greenhouse gas emissions (GHG), and nearly half of this is through the production of animal protein (Parker et al., 2018; Smith et al., 2014). Seafood may be a sustainable solution to ensure a stable food future, as it produces 10x lower GHG than the production of red meat (Nijdam et al., 2012; Searchinger et al., 2018). There also appears to be a trend towards increased seafood consumption. From 1961 to 2017, the global fish consumption increased at the rate of 3.1% annually, while the consumption of other animal protein increased by only 2.1% annually (FAO, 2020). But over-exploitation has depleted the oceans, with over 34% of stocks fished at biologically unsustainable levels (FAO, 2020). Parallelly, the share of aquaculture in the global fish production has leaped from 26% in 2000 to 46% in 2016-2018 (FAO, 2020). Moreover, aquaculture has a much lower carbon footprint, water usage, and feed conversion ratio than the production of other animal protein (Marine Harvest, 2018; Nijdam et al., 2012). Thus, aquaculture could hold the key to sustainable production of animal protein to feed our planet.

1.1.1 Salmonid aquaculture

The production of salmonids, particularly Atlantic salmon, is reported to be one of the most profitable and technologically advanced fish production industries worldwide (FAO, 2020). Atlantic salmon (*Salmo salar*) is an anadromous fish that grows from the eggs to parr phase in freshwater. After the parr phase (30-70g), it undergoes a physiological and morphological transition to acquire seawater tolerance. This process is called smoltification and a salmon that has just completed this transition is defined as a smolt (Fig. 1.1). During the growth phase after smoltification, the fish is referred to as a post-smolt. More specifically, a post-smolt is a salmon that is sea-water adapted (smoltified) and weighs up to 1000g ("CtrlAQUA definitions", 2015) (Fig. 1.2). Traditionally, Atlantic salmon are reared in land-based freshwater systems until the smolt stage and thereafter, transferred to net-pens in the sea for further growth and harvest. Currently, about 73% of the world's salmon is farmed (Marine Harvest, 2018).

Figure 1.1: Atlantic salmon smolt (Source: Nofima)

In the past decade, the salmon industry has faced several major challenges, such as sea lice, fish escape and high mortality of the fish upon transfer from land-based rearing units to the sea (Bergheim et al., 2009; Lekang et al., 2016; Summerfelt et al., 2016; Terjesen et al., 2013a; Terjesen et al., 2013b). As a result, an increasing number of fish farmers are interested in cultivating salmon to larger sizes (250-2000g) in land-based systems or in closed-containment systems in the sea (Davidson et al., 2016; Ey, 2019). The growth of salmon to a larger size before transfer to open net-pens has several advantages due to improved control

Figure 1.2: Atlantic salmon post-smolt (Source: Nofima)

of the production environment and fish health. Larger fish are reported to be more robust and perform better when transferred to the sea (Ey, 2019; Holan and Kolarevic, 2015; Ytrestøyl et al., 2020). Moreover, fewer changes in fish nets are required during the production cycle, which implies fewer fish handling situations and consequently, less stress on the fish and fewer opportunities to escape (Lekang et al., 2016). Further, the reduced production time in the sea leads to a more efficient utilization of sea cage licenses while reducing the risk from environmental parasites, such as sea lice. From an environmental perspective, shifting a greater part of the production to land-based systems increases the potential for the treatment and recycling of sludge, that would otherwise be discharged into the oceans (Ey, 2019).

1.1.2 Recirculating aquaculture systems (RAS)

Land-based fish farms can be operated either as flow-through water systems or as recirculating aquaculture systems (RAS), where most of the water is treated and reused. For the production of larger fish, flow-through systems are seldom feasible as they require a continuous supply of enormous quantities of clean water. RAS have several advantages over the traditional flow-through systems, such as decreased water usage and better waste management (Badiola et al., 2012; Piedrahita, 2003). Thus, in the past decade, there has been a shift towards intensive RAS, where >98% of the system volume is treated and recycled per hour (Bregnballe, 2015). The RAS technology has also been used to produce fish in regions where aquaculture is not possible in the sea due to geographical or legal restrictions, such as Europe, China, and the USA. With increasing environmental restrictions on net-pen farming and changing ocean temperatures disrupting the ideal water conditions for fish farming, the future of net-pen farming appears bleak. The RAS technology has the potential to provide fresh seafood in the

unpredictable future. In particular, RAS can be advantageous in urban areas where space and water are limiting, and in countries with stringent environmental regulations (Bartelme et al., 2017). Despite the many advantages, RAS typically require high investment costs. Thus, high-value species are the most attractive for RAS investors, and interest in this sector is growing rapidly. Salmonids are the main species of choice for RAS as they have been the most valuable traded seafood commodity since 2013, accounting for 19% of internationally traded fish products (FAO, 2020). Worldwide, the annual production of Atlantic salmon in RAS is projected to double from ∼1000 kilo tonnes in 2019 to ∼2000 kilo tonnes by 2021 (Ey, 2019).

A conventional RAS consists of fish tanks and water treatment systems to treat and recirculate the water (Fig. 1.3). A typical fish farm can contain several RAS departments, each containing a set of fish tanks connected to a common water treatment unit. The departments are designed to increase biosecurity and to accommodate the requirements of the fish at different life stages. In the tanks, the fish retain only about 20-50% of the feed nutrients (mainly carbon, nitrogen and phosphorus), while the remaining nutrients are released into the water in the particulate or dissolved form (Wang et al., 2013). The recirculating water typically goes through the following treatment steps: mechanical filtration, biological treatment, degassing, and oxygenation. The particulate waste from the fish tanks includes uneaten feed, suspended solids and particulate organic matter. These are removed through mechanical filtration. The fish excrete nitrogen, mainly as dissolved ammonia, which is converted to nitrate in the biological treatment process. The concentration of nitrate is diluted to acceptable levels by a continuous supply of new disinfected water. After the biological treatment, the water is sent to a degasser to strip off carbon dioxide (CO_2) (a by-product of feed combustion). The water is then oxygenated and returned to the fish tanks. Optionally, a portion of the recirculating water flow may be treated with ozone or disinfected to increase clarity of the water and reduce the risk of pathogens in the RAS. The growth and welfare of the fish is highly dependent on the water quality environment (Colt, 2006; Terjesen et al., 2013a; Thorarensen and Farrell, 2011). The water quality in a RAS may be affected by system changes, such as overfeeding, equipment malfunctioning etc. Fluctuations in the water quality can, in turn, affect the water treatment systems and consequently, the fish. Therefore, it is essential to ensure good water quality in a RAS.

1.1.3 Ammonia production and toxicity

Nitrogen is an essential nutrient for all organisms. Fish feed contains nitrogen as amino acids in protein. Salmon retain 30-50% of the nitrogen in the ingested feed, while the rest is excreted through gill diffusion, gill cation exchange,

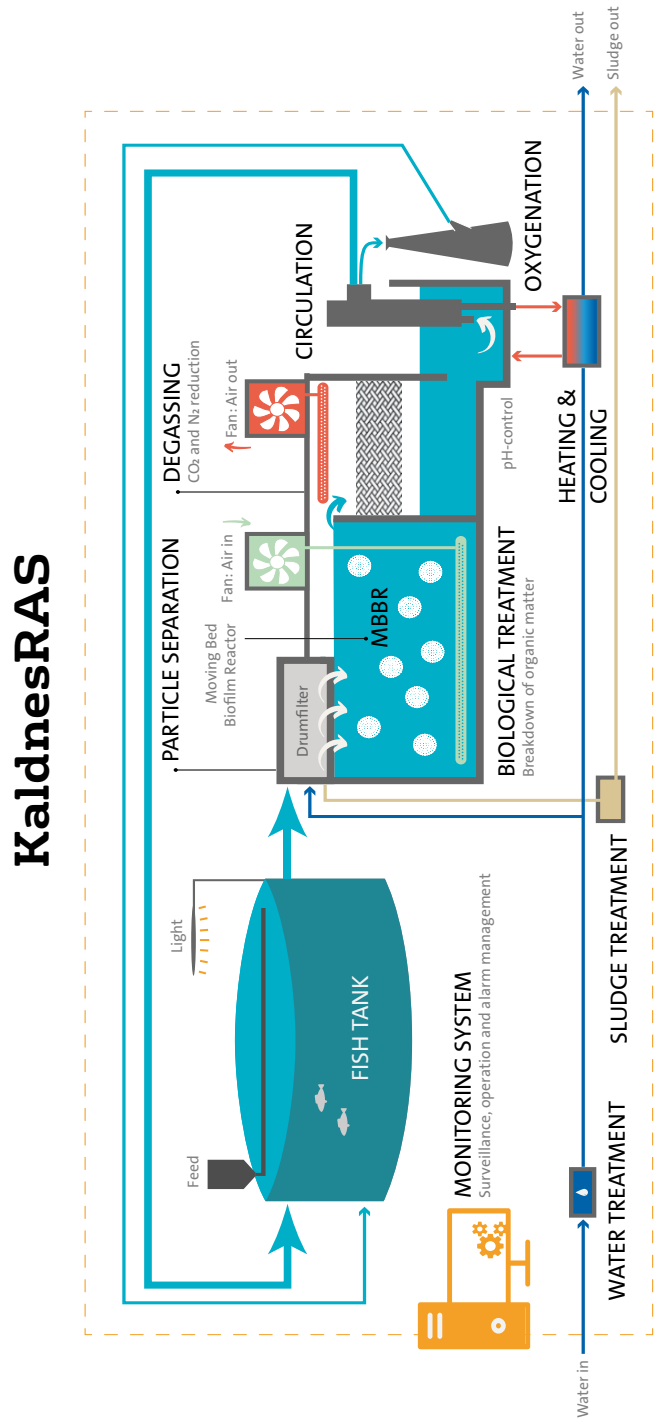


Figure 1.3: Schematic of a typical recirculating aquaculture system (RAS) (Source: Krüger Kaldnes AS)

urine and feces (Hagopian and Riley, 1998; Wang et al., 2013; Wang et al., 2012). Although the fish can excrete nitrogenous waste as amino acids or protein, the main nitrogenous metabolite produced after protein catabolism is ammonia (Thorarensen and Farrell, 2011). About 60-90% of the total nitrogen excreted by salmon is present as un-ionized ammonia (NH_3) and ammonium ion (NH_4^+), which together comprise total ammonia nitrogen (hereafter referred to as "ammonia" for simplicity) (Hagopian and Riley, 1998). In addition, accumulated feces and uneaten feed may also be proteolyzed and aminated to form ammonia (Hagopian and Riley, 1998). Of the two forms of ammonia, un-ionized ammonia is the most toxic, with the recommended safe levels for salmon being 0.012-0.025 mg L^{-1} NH_3 (Hjeltnes et al., 2012; Thorarensen and Farrell, 2011).

The percentage of total ammonia in the form of NH_3 depends on the pH, salinity, alkalinity, and temperature (Anthonisen et al., 1976; Thorarensen and Farrell, 2011). pH is the most influential factor, with higher pH resulting in a higher fraction of NH_3 (Thorarensen and Farrell, 2011). Salinity may also influence the toxicity of ammonia through the increased permeability of the biological membrane to NH_4^+ (Eddy, 2005; Noble et al., 2018). However, while some studies suggest that ammonia is more toxic to salmonids at intermediate salinity than in fresh- or seawater, other studies report the opposite (Alabaster et al., 1979; Eddy, 2005; Fivelstad, 1988). Other factors such as the dissolved oxygen concentration, feeding regime, and exercise can also impact toxicity (Eddy, 2005). While the exact tolerance of salmonids to ammonia depends on various factors, the recommended maximum concentration for chronic exposure is as low as 2 mgN L^{-1} (for farmed salmon at 12°C, pH 6.8) (Noble et al., 2018). Thus, it is essential to continuously remove ammonia from the system to maintain its concentration at the levels deemed safe for the fish.

"Microorganisms will give you anything you want if you know how to ask them"
—Kinichiro Sakaguchi

1.2 Biological nitrogen conversion

1.2.1 Biofilm reactors for biological water treatment

In a typical RAS, ammonia accumulation is prevented by the biological treatment of the recirculating water in biofilm reactors. Biofilms are "aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface" (Flemming et al., 2016). Greater than 90% of the biofilm is comprised of an extracellular matrix (Flemming and Wingender, 2010). The matrix serves a

multitude of functions such as providing stability and protection against various factors like desiccation, radiation, and metal toxicity (Flemming and Wingender, 2010; Hall-Stoodley et al., 2004). Biofilm processes are particularly desirable in RAS, as they allow higher biomass retention times and prevent the washout of beneficial microbes, specifically the nitrifying microorganisms. Several types of biofilm reactors have been implemented in RAS for achieving nitrification, such as fixed bed biofilters, trickling filters, fluidized bed biofilters, and the moving bed biofilm reactor (MBBR) (Timmons and Ebeling, 2010). The MBBR is a widely used technology in RAS due to its compactness, even load distribution, low head-loss and no requirement for backwashing (Ødegaard et al., 1994; Rusten et al., 2006) (Fig. 1.4). The MBBR consists of a tank filled with specially designed plastic carriers on which the biomass is grown (Fig. 1.5). The carriers move freely in the reactor by aeration or mechanical mixing. The frequent collisions due to mixing slough off the excess biofilm from the carriers, eliminating the need for back-washing. As such, the MBBR is an attractive self-cleaning technology for achieving ammonia removal in RAS.

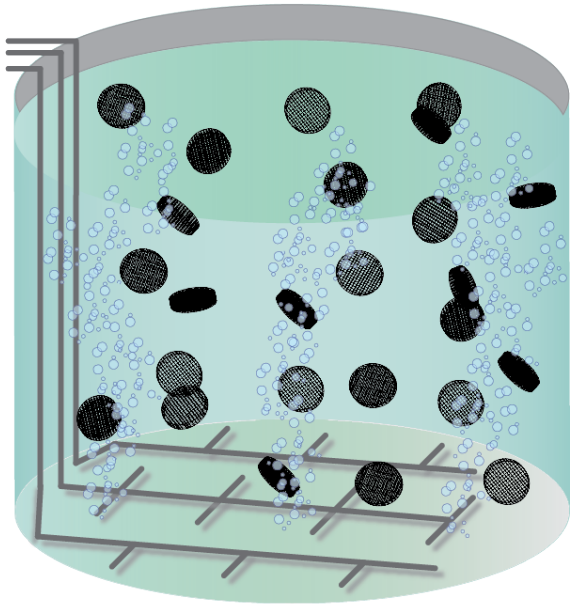


Figure 1.4: Schematic of a moving bed biofilm reactor (MBBR)

The biofilm matrix enables certain microbial behaviors that would otherwise be impossible in planktonic cells. This is because the close proximity of cells within the matrix leads to complex microbial interactions, including the formation of synergistic micro-consortia (Flemming and Wingender, 2010). Moreover,

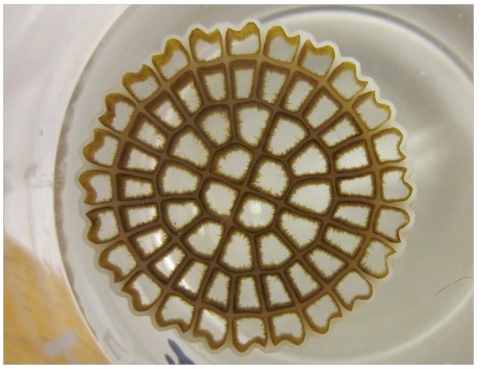


Figure 1.5: Biofilm growth on an AnoxK^{TM} K5 biofilm carrier. (Source: AnoxKaldnes)

the stratified nature of the biofilm creates a diffusion gradient of nutrients and resources across the biofilm, favoring different microorganisms in the niches within the strata (Flemming et al., 2016). Thus, biofilm reactors can accomplish various functions, depending on the type of microbes in the biofilm, availability of resources, and the interactions within the biofilm. In RAS, the biofilms contain nitrifying microbes that perform ammonia conversion. In addition, degradable organic matter produced by the fish promotes the growth of heterotrophic bacteria on the biofilms. These bacteria can compete with the nitrifiers for resources (such as oxygen and space) and may inhibit nitrification activity, especially when the organic loading is high or varying (Navada et al., 2020; Rittmann and McCarty, 2001). However, heterotrophs can be beneficial under stable conditions, as they help maintain a low concentration of dissolved organic matter in the fish tanks, and thereby control the growth of opportunistic bacteria (potential pathogens). Thus, it is important to maintain a stable microbial community in the bioreactor as it provides a microbially beneficial environment to the fish (Vadstein et al., 2018). Although the degradation of organic matter is important, ammonia conversion through nitrification is the most crucial function performed by RAS bioreactors.

1.2.2 Nitrification

Typically, nitrification is a two-step microbiological process where ammonia is first oxidized to nitrite and subsequently, to nitrate (Equations 1.1, 1.2) (Ekama et al., 2020). The two steps are performed by two microbial guilds: ammonia oxidizing microorganisms (AOM) and nitrite oxidizing bacteria (NOB), respectively. Bacteria capable of complete ammonia oxidation to nitrate (comammox) also exist within the genus *Nitrospira*, and have been detected in RAS bioreactors (Bartelme et al., 2019; Daims et al., 2015). The ammonia oxidizing bacteria (AOB) and NOB tend to exist in close proximity within a biofilm

and form a synergistic micro-consortium (Okabe et al., 1995; Schramm et al., 2000). The nitrite produced by ammonia oxidation acts as a substrate for the NOB, while the AOB are benefited by the removal of the potentially inhibitory product, nitrite, by the NOB. The nitrifying microbes are obligate aerobes and are very sensitive to low-oxygen concentrations (Rittmann and McCarty, 2001). This is seldom a problem in RAS, where the dissolved oxygen is always maintained high for the fish (>80% saturation). Nitrifiers are also autotrophic, using inorganic carbon instead of organic carbon as a carbon source (Rittmann and McCarty, 2001). Finally, they are chemolithotrophs, as they use a reduced form of inorganic nitrogen as an electron donor. This provides lesser energy than organic electron donors, and nitrifiers therefore have a slower growth rate than heterotrophs (Rittmann and McCarty, 2001). Nitrification processes usually require a suitable base to maintain the pH, as approximately two strong-acid equivalents are produced per mole of NH_4^+ oxidized (Ekama et al., 2020; Rittmann and McCarty, 2001).

$$NH_4^+ + 1.5O_2 \longrightarrow NO_2^- + H_2O + 2H^+ \tag{1.1}$$

$$NO_2^- + 0.5O_2 \longrightarrow NO_3^- \tag{1.2}$$

Conventionally, bacteria were thought to be the main nitrifying domain in RAS, and nitrification kinetics were mainly based on the genera *Nitrosomonas* and *Nitrobacter* (Timmons and Ebeling, 2010). These genera were studied the most as they were common in wastewater treatment plants and easily cultivable (Ekama et al., 2020). But recent studies show that non-canonical nitrifiers such as ammonia oxidizing archaea (AOA), and species belonging to the genera *Nitrospira* and *Nitrotoga* can also be abundant in RAS bioreactors (Bartelme et al., 2017; Hüpeden et al., 2016; Kruse et al., 2013). This is mainly due to massive technological leaps in microbiological methods such as 16S rRNA gene amplicon sequencing, that enable a detailed characterization of the microbial community at a relatively low cost.

Several factors can affect the nitrification rate, such as the temperature, pH, dissolved oxygen saturation, organics, substrate concentration, and salinity (Chen et al., 2006). In an MBBR, oxygen or ammonia is the limiting substrate and the nitrification rate is highly dependent on these variables (Rusten et al., 2006). The concentration of these variables within the biofilm depends on the thickness of the biofilm and the diffusion gradient. In a RAS, the dissolved oxygen saturation is usually high (∼80-100%), whereas the ammonia concentration is maintained

low (< 2 mgN L^{-1}). Thus, ammonia is typically the limiting substrate in a RAS MBBR. Unlike conventional wastewater treatment that allows a certain degree of control in the operating conditions, most of the operational parameters in a RAS are constrained by the requirements of the fish. During normal production of salmon, most of the operational parameters are held relatively stable. However, a special case is when the fish undergoes the physiological transition from parr to smolt. After this transition, the salinity is typically increased from freshwater to brackish- or seawater, usually within a few days. Under this perturbation in water quality, it is essential to ensure the proper functioning of all the water treatment processes to avoid undue stress to the fish.

"The cure for anything is salt water: sweat, tears or the sea"
—Isak Dinesen

1.3 Impact of salinity change on nitrifying bioreactors in RAS

1.3.1 Salinity change in RAS

In the past, land-based RAS were mainly used to rear salmon during the early life phase, typically up to 80-100 g smolt. Therefore, the water treatment systems for this life stage were usually designed for freshwater. However, for post-smolt, it is desirable to operate the RAS in brackish- or seawater. The saltwater aids osmoregulation in the fish, enabling it to adapt to the higher salinity before transfer to the sea. Although salmon can also be grown to 4-5kg in freshwater (Davidson et al., 2016), salinity can provide distinct growth and welfare advantages over freshwater. Besides being the natural environment for a smoltified fish, saline water provides several advantages, such as stress counteraction, prevention and control of diseases, and a general improvement in the condition and survival of the fish. Moreover, rearing post-smolt in a saline environment ($\geq$12‰) can prevent the loss of hypo-osmoregulatory capacity (desmoltification) (Mortensen and Damsgard, 1998; Ytrestøyl et al., 2020). Post-smolts also tend to show a preference for salinities >12‰ (Noble et al., 2018).

Therefore, it is common practice to increase the salinity after the salmon are smoltified, and to rear the post-smolt at a higher salinity. RAS designed for rearing parr, smolt, and post-smolt must be capable of operating under different salinities, ranging from freshwater ($\sim$0‰ salinity) to brackish water ($\sim$12-22‰) to full-strength seawater (32-35‰). Although the fish could also be produced in separate RAS departments in freshwater and saline water, it is more expensive.

Moreover, it requires more frequent fish handling, which is stressful for the fish. Thus, instead of having separate RAS, it is preferable to increase salinity in the RAS during the smoltification stage. After the fish are harvested, the salinity is decreased to prepare the system for the next batch of parr. Salinity increases are more crucial than salinity decreases as they must be performed while the fish are in the system. While a smoltified fish can adapt easily to an increase in salinity, the water treatment systems in the RAS can be severely impacted. For example, the addition of salt can disrupt the biological treatment process and reduce the efficiency of the carbon dioxide degasser (Chen et al., 2006; Moran, 2010a, 2010b).

Particularly, the nitrification performance can be severely affected by salinity changes, as the microbes responsible for the nitrification process can be sensitive to salinity (Csonka, 1989; Madigan et al., 2018). For salmon, not just ammonia, but also nitrite, can be highly debilitating and can cause mortality. The recommended concentration of nitrite is as low as 0.1 mgN L^{-1} for NO_2^- in soft freshwater (Noble et al., 2018; Thorarensen and Farrell, 2011). However, fish can tolerate higher nitrite concentrations in saline water due to the presence of chlorides (Gutiérrez et al., 2019; Kroupova et al., 2005). The final product of the nitrification reaction, nitrate, is relatively much less toxic to the fish with the maximum recommended concentration for post-smolt being up to 100 mgN L^{-1} (Davidson et al., 2017). A malfunctioning in the nitrification system can lead to the rapid accumulation of ammonia or nitrite in a RAS, thereby impairing fish health or causing mortality in extreme cases.

Apart from the direct impact of a reduction in nitrification efficiency, a salinity increase can also lead to other risks. As seawater contains a much higher concentration of sulfate than freshwater, the risk of hydrogen sulfide (H_2S) formation is greater in saline RAS. Hydrogen sulfide is highly toxic to salmon at concentrations even below 1 mg L^{-1} and has been suspected to cause mass mortality in several fish farms (Letelier-Gordo et al., 2020). Nitrate is the preferred electron acceptor for bacteria under anoxic conditions, and can play an important role in the control of H_2S (Torun et al., 2020). During a salinity increase, the reduction in nitrification efficiency can decrease the concentration of nitrate, leading to an increased risk of H_2S, especially in the presence of degradable organic matter. Given the combined risks associated with ammonia, nitrite and H_2S, it is extremely important to ensure proper functioning of the nitrification process during salinity changes in a RAS.

1.3.2 Salinity adaptation mechanisms in microorganisms

Perhaps the most versatile of all life forms, prokaryotes have evolved to sense and adapt to changes in their environment, including the osmolarity of the extracellular medium. In order to perform cell processes such as growth and division, the intracellular osmotic pressure must be greater than that of the growth medium (Oren, 1999; Sleator and Hill, 2001). A salinity change in the environment of a microbial cell can disrupt the osmotic balance between the interior and exterior of the cell. This osmotic pressure difference causes an instantaneous efflux or influx of water (and/or solutes), depending on whether the salinity shock is hyper- (salinity increase) or hypoosmotic (salinity decrease) (Csonka, 1989; Sleator and Hill, 2001). Of the two, hypoosmotic shock is less severe as the rigid bacterial cell walls may withstand the increase in pressure due to the water influx. Hyperosmotic shock, on the contrary, causes the cell to shrink by reducing the cytoplasmic volume – a process called plasmolysis (Csonka, 1989). Sudden plasmolysis can inhibit several physiological processes, including nutrient uptake and cell activity (Csonka, 1989; Madigan et al., 2018). If the hyperosmotic shock is not too severe, the cells may recover from plasmolysis and adapt to the environment (Csonka, 1989).

A higher extracellular salinity (hyperosmotic environment) implies a higher external osmolarity, and the cell must increase its internal osmolarity accordingly to maintain the osmotic balance. To accomplish this, the cells can resort to one of the two main osmoadaptation strategies: (i) the salt-in cytoplasm strategy and (ii) the organic osmolyte (or compatible solute) strategy (Csonka, 1989; Oren, 1999; Sleator and Hill, 2001). The salt-in strategy requires a high salt (KCl) concentration in the cytoplasm. Extensive structural adaptations are required to adapt to the high ionic strength inside the cell, and therefore, this strategy is only adopted by obligate halophiles (Oren, 2011; Sleator and Hill, 2001). The second strategy involves a bi-phasic response, where the first phase is an increase in K^+, followed by a considerable increase in the cytoplasmic concentration of osmolytes (by synthesis and/or uptake) (Sleator and Hill, 2001). Osmolytes can be accumulated at high concentration in the cell as they carry no charge at physiological pH. All halotolerant microorganisms use this strategy to adapt to salinity fluctuations as it offers a high degree of flexibility (Oren, 2011; Sleator and Hill, 2001). The ability of a microorganism to survive at a high salinity depends on the energy generated during dissimilatory metabolism and the mode of osmotic adaptation (Oren, 2011).

1.3.3 Salinity tolerance of nitrifying microorganisms

Within the bacterial domain, the ammonia oxidizers belong to three main lineages. Of these, two lineages represent the genera *Nitrosomonas* and *Nitrosospira* within the class β-proteobacteria (Prosser et al., 2014). The third lineage is the genus *Nitrosococcus* within the γ-proteobacteria class (Prosser et al., 2014). The salt tolerance of the species within the genus *Nitrosomonas* varies greatly. While *N. oligotropha* is has a maximum salt tolerance of about 100 mM ($\sim$6‰), *N. europaea* is moderately salt tolerant (up to 400 mM or 23‰) (Koops et al., 2006). The genus also contains obligately halophilic species such as *N. marina*, *N. aestuarii*, and *N. cryotolerans* (Koops et al., 2006). Species within the genus *Nitrosospira* are known to have a low salt tolerance (up to 250 mM or 15‰) (Koops et al., 2006). In contrast, the *Nitrosococcus* lineage has only been found in marine environments and is reported to be obligately halophilic (Koops et al., 2006). Within the archaeal domain, members of the phylum *Thaumarchaeota* can perform ammonia oxidization. AOA have been found to dominate AOB in a vast variety of environments, suggesting that they play a major role in the environmental nitrogen cycle (Stieglmeier et al., 2014). Recent studies have shown that AOA can be abundant in RAS bioreactors under a wide range of salinities (Bartelme et al., 2017; Bartelme et al., 2019; Sauder et al., 2011). RAS could be an ideal environment for the proliferation of AOA, as AOA are adapted to relatively low ammonia concentrations compared to AOB (Sauder et al., 2011; Stieglmeier et al., 2014). However, despite the high relative abundance in environmental systems, the contribution of AOA towards the overall nitrification activity is not yet well established (Bernhard and Bollmann, 2010; Hatzenpichler, 2012).

The known nitrite oxidizers belong to the genera *Nitrobacter, Nitrotoga, Nitrococcus, Nitrospina, Nitrolancea*, and *Candidatus Nitromaritima* (Daims et al., 2016). Except for *Nitrolancea*, all the known genera of NOB have been detected in marine systems (Daims et al., 2016), suggesting that these genera contain some species that are at least halotolerant, if not halophilic. Members of the *Nitrospinae* family (including *Nitrospina* and *Candidatus Nitromaritima*) have only been detected in marine systems, suggesting an obligately halophilic lifestyle (Daims et al., 2016).

1.3.4 Impact of salinity change on nitrification activity

Several studies have researched the impact of salinity on the nitrification process across a wide variety of systems – such as activated sludge (Bassin et al., 2012; He et al., 2017; Moussa et al., 2006), aerobic granular sludge (Bassin et al., 2011; Wang et al., 2017), fixed bed biofilters (Cortes-Lorenzo et al., 2015; Nijhof and Bovendeur, 1990; Sudarno, 2011), and MBBR (Gonzalez-Silva, 2016). Although

the quantitative effect of salinity on nitrification differs across these studies, there is consensus that an increase in salinity generally inhibits the nitrification process. However, a salinity increase from 0 to ∼10‰ appears to have a slight positive or no impact on the ammonia oxidation rate (Aslan and Simsek, 2012; Bassin et al., 2012; Cortes-Lorenzo et al., 2015; Sudarno, 2011), although not without exceptions (Kinyage et al., 2019; Sanchez et al., 2004). This is likely because these salinities are close to the isotonic point (∼9‰), which may reduce the energy required for osmoregulation and hence, make more energy dispensable for growth and metabolism (He et al., 2017). In this thesis, adaptation to salinity refers to adaptation to salinities > 10‰, unless specified otherwise.

In a nitrifying bioreactor, salinity changes can impact the AOM and NOB to different extents. There are conflicting views on which of the two is more impacted by a salinity increase. Some studies found that AOM are more influenced than the NOB (Hunik et al., 1993; Moussa et al., 2006; Sharrer et al., 2007), whereas others reported the opposite (Aslan and Simsek, 2012; Bassin et al., 2011; Nijhof and Bovendeur, 1990; Sudarno et al., 2011). However, as the studies used vastly different set-ups and microbial cultures, the discrepancy may be due to the differences in experimental conditions, the initial microbial community composition or salinity change methods. Nonetheless, several studies have reported nitrite accumulation after an increase in salinity, suggesting that nitrite oxidation may be compromised under saline conditions (Bassin et al., 2011; Cortes-Lorenzo et al., 2015; Gonzalez-Silva, 2016; Nijhof and Bovendeur, 1990). The relatively slower growth rate and lower energy yield of NOB compared to AOB may make them more susceptible, as nitrite oxidation may not generate sufficient energy for osmoregulation at elevated salt concentrations (Oren, 2011).

Studies show that nitrifying bioreactors can adapt to a prolonged exposure to salinity (Bassin et al., 2012; Sharrer et al., 2007). Salinity changes are also usually accompanied by a shift in the microbial community composition (Bassin et al., 2012; Cortes-Lorenzo et al., 2015; Gonzalez-Silva, 2016; Luo et al., 2016; Sudarno, 2011). This community shift likely eliminates microorganisms that cannot survive at higher salinities, and selects for halotolerant or halophilic microorganisms instead. Shifts in the community composition of nitrifiers are also reported, with the appearance or disappearance of certain taxa at higher salt concentrations (Bassin et al., 2011; Cortes-Lorenzo et al., 2015; Moussa et al., 2006). For e.g., increasing the salinity from freshwater to ∼33‰ resulted in a loss of *Nitrosomonas oligotropha* (Moussa et al., 2006). In the same study, *Nitrosomonas europaea* was detected at salinities as high as 66‰. Similarly, the nitrite oxidizer *Nitrospira* was reported to disappear at salinities above 33‰, and was correlated to high nitrite accumulation in the system (Bassin et al., 2011).

Other studies also report the depletion of *Nitrospira* at salinities above 16‰ (Moussa et al., 2006; Rud et al., 2017). Some studies reported a shift in the AOB population upon salinity increase (Cortes-Lorenzo et al., 2015; Gonzalez-Silva, 2016; Sudarno et al., 2010). In contrast, Bassin et al., 2011 reported no shift in the AOB population and another study continued to detect *Nitrospira* in a biofilter after an increase from freshwater to seawater (Sudarno et al., 2010). In yet another study, > 96% of AOB and > 40% of NOB were common to freshwater, brackish water (22‰ salinity) and seawater biofilms (Gonzalez-Silva et al., 2016). Moreover, nitrifiers have also been detected in estuarine systems with salinities varying from freshwater to seawater (Santos et al., 2018; Ward et al., 2007). This suggests that several nitrifiers may be capable of adapting to varying salinities.

1.3.5 Need for adaptation strategies to salinity change

Salinity changes have a more complex effect on biofilms than on an individual cell. In biofilms, the EPS produced by the bacteria can retain water and decrease the salt stress, thus protecting the cells in the biofilm against desiccation (Flemming et al., 2016). Thus, a salinity increase can induce the formation of EPS as a defense mechanism (Corsino et al., 2017; Wang et al., 2015). Salt can also strengthen the biofilm structure due to better settling characteristics (Goode and Allen, 2011). Further, biofilms can respond to prolonged salinity changes by the physiological adaptation of the existing microbes, and/or by shifting the microbial community composition towards microbes that are more suited to the salinity change regimes. The choice of the adaptation strategy adopted may depend on the intensity and duration of the salinity change (Shade et al., 2012).

Salinity change may be performed in a variety of ways. Not only the magnitude of salinity, but the method in which the salinity is changed – gradually, step change or a shock change – may influence the nitrification performance (Moussa et al., 2006). Although the bioreactor performance can recover after a few days, a shock change in salinity is reported to have a drastic reduction in nitrification rate during the initial days (Gonzalez-Silva, 2016; Nijhof and Bovendeur, 1990). An alternative strategy is to increase the salinity gradually. With this strategy, the salinity can be changed with almost no reduction in nitrification rate (Bassin et al., 2012; Bassin et al., 2011; Sharrer et al., 2007). However, it is a time-intensive strategy and can take several days or months. In a RAS, the salinity must be changed within a few days, so none of these strategies are suitable. Inoculation with salt-acclimated biomass or commercial nitrifying consortia has also been shown to improve salinity adaptation (Cui et al., 2016; Panswad and Anan, 1999; Shi et al., 2012; Sudarno et al., 2010). However, in a RAS, inoculation material can be expensive, difficult to procure, and can pose a biosecurity risk. Moreover, inoculation with salt-adapted microorganisms may not be effective during sudden

increases in salinity (Vyrides, 2015). Finally, the starting inoculum may not be suitable for the given environmental conditions. In that case, microbes in the inoculum may be outcompeted by the local flora and the selection pressure will determine the final microbial community. To the best of our knowledge, there exist no established strategies for increasing the salinity acclimation in non-inoculated nitrifying biofilms.

Chapter 2

Aims & Scope

"Begin at the beginning and go on till you come to the end; then stop"
—Lewis Caroll, Alice in Wonderland

2.1 Research aims and objectives

The overall objective of this PhD was to develop strategies for changing the salinity in RAS in a safe manner for the fish. Specifically, the goal was to find strategies to minimize the negative impact of salinity increase on the nitrification performance and improve salinity acclimation in nitrifying bioreactors. With this objective, the PhD was structured with the following aims:

- To identify the optimum rate of salinity increase
  - By comparing the impact of different rates of salinity increase on the nitrification activity in bioreactors (**Article I**)
- To investigate microbial management strategies to increase the salinity tolerance of nitrifying bioreactors
  - By studying the impact of seawater priming on the salinity tolerance of fresh- and brackish water biofilms (**Article II**)
  - By comparing the start-up of nitrification at different salinities (**Article III**)
  - By comparing salinity tolerance in bioreactors inoculated (seeded) with biofilm acclimated to different salinities (**Article IV**)
  - By investigating if the exogenous addition of osmolytes could improve salinity acclimation (**Article V**)
- In each of the above-mentioned scenarios, to gain a comprehensive understanding of the underlying microbial dynamics by characterizing the microbial community composition in the biofilm at different salinity regimes

2.2 Experimental approach and scope of the PhD

All the experiments were conducted on MBBRs supplied with synthetic medium containing ammonia and nutrients. Fish were not used in any of the studies due to ethical concerns. The experimental set-up used in **Articles I, II, and IV** was specially designed and constructed for this PhD by the candidate, with help from the supervisors and technicians at Nofima (Appendix, Fig. A.1). As the scale of an MBBR can influence the performance (Kamstra et al., 2017), the reactors were designed with a relatively large volume (∼37 L) compared to standard lab reactors (∼1 L). This made the results more reliably translatable to commercial RAS. Due to the scale of the reactors, only two replicates were used per treatment. The salinity in the reactors was changed gradually overnight by adjusting the salinity of the dilution water to the reactor. This is more representative of the salinity change method applied in an commercial RAS than a shock change in salinity. Tests were performed to confirm that the observed reduction in ammonia was primarily due to nitrification in the biofilm, and not due to bacteria in the water phase or other nitrogen removal processes (Appendix, Section A.2). In **Article III**, MBBRs from a semi-commercial RAS were used (Appendix, Fig. A.2). In addition to the main setup, glass reactors were used in **Article IV** to perform capacity tests (Appendix, Fig. A.3). In **Article V**, aerated lab beakers were operated as MBBRs (Appendix, Fig A.4). In all the studies, we performed capacity tests to estimate the total nitrifying potential. During each test, the reactors were operated in batch mode and initially dosed with a known concentration of ammonia and/or nitrite. Water samples were then analyzed for ammonia and/or nitrite at regular intervals to measure the rate of decrease. These were used to determine the zero-order reaction rate, and then normalized to the protected surface area of the biofilm carriers to calculate the maximum specific oxidation rates of ammonia (AOR_{max}) or nitrite (NOR_{max}). Capacity tests are a better indication of the nitrification activity than *in situ* rates or removal efficiencies, as the measured rates are independent of the substrate (ammonia or nitrite). In **Articles I-IV**, the analytical methods used to measure the nitrogenous compounds were compatible with seawater (Appendix, Table A.1), whereas in **Article V**, correction factors were used to adjust for salinity.

The microbial community composition was characterized by 16S rRNA gene amplicon sequencing. The microbial lab analysis (from DNA extraction to the operational taxonomic unit (OTU) table) was conducted by co-authors or outsourced to a laboratory, and is therefore not included in the scope of this PhD. Although ammonia oxidizing archaea have also been detected in RAS bioreactors, only the bacterial domain was targeted in this study due to resource limitations. Biofilm structure and EPS can also play an important role in salinity tolerance of biofilms but were beyond the scope of the present study. Besides prokaryotes,

eukaryotes such as protozoa or rotifers can also influence the microbial community and function by predating on bacteria. The study of eukaryotic organisms was not investigated in this PhD. Besides impacting the nitrification process, salinity changes can introduce other associated risks in RAS, such as H_2S formation. This is an important topic but was beyond the scope of this PhD. In the next section, we discuss the results of the studies within the above-mentioned experimental framework and scope.

Chapter 3

Discussion of results

"The solution often turns out more beautiful than the puzzle"
—Richard Dawkins

3.1 How fast should the salinity be increased? (Article I)

The objective of the first study (**Article I**) was to find the optimum rate of salinity increase that could be sustained while maintaining acceptable nitrification activity in the bioreactor. It was hypothesized that a smaller salinity increment would result in a lower negative impact on the nitrification performance, as the microbes were expected to adapt better to small changes than large changes. The results showed that irrespective of the rate of salinity change, the ammonia oxidation capacity (AOR_{max}) decreased by 50-90% upon increasing the salinity from freshwater to seawater (Fig. 3.1). Thus, it appears difficult to increase the salinity in a freshwater RAS without the risk of ammonia accumulation, especially if the bioreactor has never been exposed high salinity. Moreover, because the RAS is usually operated at the peak capacity during the salinity increase, there is little room for accommodation to the reduction in nitrification rate. It should be noted that there were no fish tanks in our studies and the hydraulic retention time (HRT) of the system was only 6-12h (**Articles I, II, IV**). In comparison, a commercial RAS has a much higher HRT ($\sim$6-10 days) with a total system volume that is typically 6-12x the MBBR volume. Thus, it is difficult to predict the ammonia concentration in a RAS based on the results from our experimental setup. However, the percent change in AOR_{max} and nitrite oxidation capacity (NOR_{max}) can be considered representative, and the fish feeding rate may be correspondingly reduced to prevent ammonia/nitrite accumulation. For e.g., during a salinity increase from freshwater

to seawater, the feeding rate may be reduced by 50-90% to compensate for the loss of nitrification activity. However, reducing the feeding rate can be detrimental to fish welfare as discussed in Section 3.2.1.

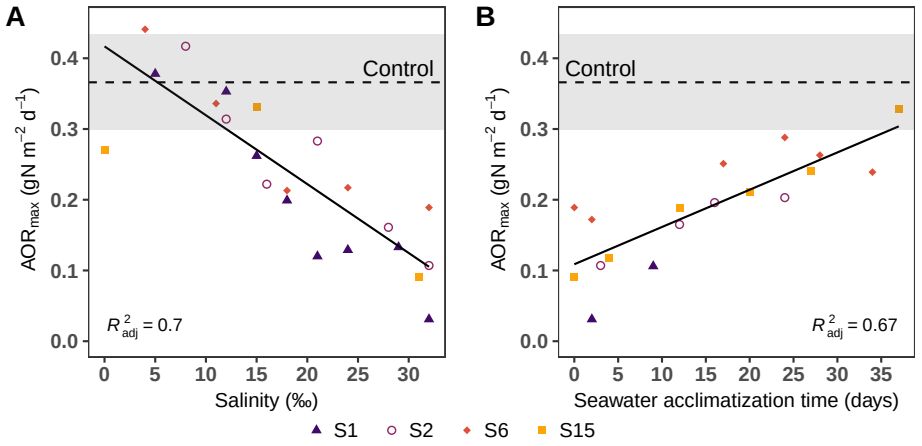


Figure 3.1: Linear regression analyses showing the correlation between A) AOR_{max} and salinity and B) AOR_{max} and seawater acclimatization time. The salinity was increased from freshwater (0‰ salinity) to seawater (32‰ salinity) at different rates of daily salinity increment: 0 (C, control), 1 (S1), 2 (S2), 6 (S6), and 15‰ day^{-1} (S15), respectively. The dashed line and the shaded region represent the average control AOR_{max} and its standard deviation, respectively. (Fig. 4 in **Article I**)

Contrary to our hypothesis, the treatment with the smallest salinity increment, S1 (1‰ day^{-1}), had the maximum reduction (∼90%) in AOR_{max} upon seawater transfer. This suggests that small daily salinity increments do not offer any advantage over large salinity increments. Further, the AOR_{max} was statistically independent of the rate of salinity change, and depended only on the salinity (∼2.7% decrease per ‰) and acclimatization time in seawater (∼2.1% recovery per day). Consequently, the treatment with the largest salinity increment, S15 (15‰ day^{-1}), had the highest AOR_{max} at the end of the 41-day study. Thus, if there are no fish in the RAS, large salinity increments appear to be the most practical strategy to increase the salinity. This strategy can be applied, for e.g., during start-up or before the introduction of a new batch of fish. We applied this strategy in **Articles II** and **IV** by performing the salinity changes over 1-3 days to optimize the duration of the experiments.

Notably, the AOR_{max} in S15 was comparable to that in the control after 39 days in seawater (**Article I**). Further, in **Articles II** and **IV**, the AOR_{max} in bioreactors acclimated to seawater was comparable or higher than that in freshwater (although

this may also have been due to the maturation of the biofilm). These findings oppose the traditional view that seawater bioreactors have a lower nitrification rate than freshwater bioreactors (Chen et al., 2006; Nijhof and Bovendeur, 1990; Rusten et al., 2006). Our studies suggest that if sufficient acclimation time is provided, biofilms can develop similar nitrification rates in seawater as in freshwater.

3.2 Strategies to improve salinity acclimation in nitrifying bioreactors

3.2.1 Osmotic stress priming (prior exposure to seawater) (Article II)

The results from **Article I** and other studies (Bassin et al., 2012; Gonzalez-Silva, 2016) showed that although freshwater bioreactors can adapt to high salinity, it is difficult to prevent the initial loss of activity during the period immediately following the salinity increase. In such a scenario, the fish feeding rate must be decreased to prevent the accumulation of toxic ammonia. However, this implies several days of reduced feeding, leading to compromised fish growth and stress. Further, starved fish may be more susceptible to ammonia toxicity than actively feeding fish (Hjeltnes et al., 2012). Thus, we needed a strategy to make bioreactors more robust to salinity changes, so that the salinity may be increased with the least possible reduction in nitrification performance.

Priming is a phenomenon where organisms exposed to a mild environmental stress show an improved response to a more severe stress in the future (Rillig et al., 2015). In microorganisms, priming is usually achieved through phenotypical modifications, such as changes in gene expression or metabolism (Hilker et al., 2016; Mitchell et al., 2009; Rillig et al., 2015). However, in a biofilm, priming can have a more complex effect. Perturbations (especially in the initial growth phase) can not only change the phenotypical response of the microorganisms, but also alter the microbial community composition and biofilm morphology, making them more resilient against similar perturbations in the future (Cabrol et al., 2016; Ohashi et al., 1995; Rillig et al., 2015; Saur et al., 2016). Although the effect of osmotic stress priming has been observed in microbial cultures (Andrade-Linares et al., 2016; Jenkins et al., 1990), its effect on biofilms was not well researched.

Thus, the second study was designed to study the effect of osmotic stress priming on newly developed fresh- and brackish water biofilms (**Article II**). Brackish water at 12‰ salinity was chosen, as smoltified fish are reported to perform better in RAS at this salinity than at higher salinities (Ytrestøyl et al., 2020). Further, previous studies showed that the nitrification performance decreases rapidly when the salinity is increased from freshwater to 10-15‰, suggesting this salinity range

to be a "break-point" for the change in microbial activity (**Article I**, Kinyage et al., 2019). We hypothesized that the primed treatments would be more robust to salinity increase than the unprimed treatments.

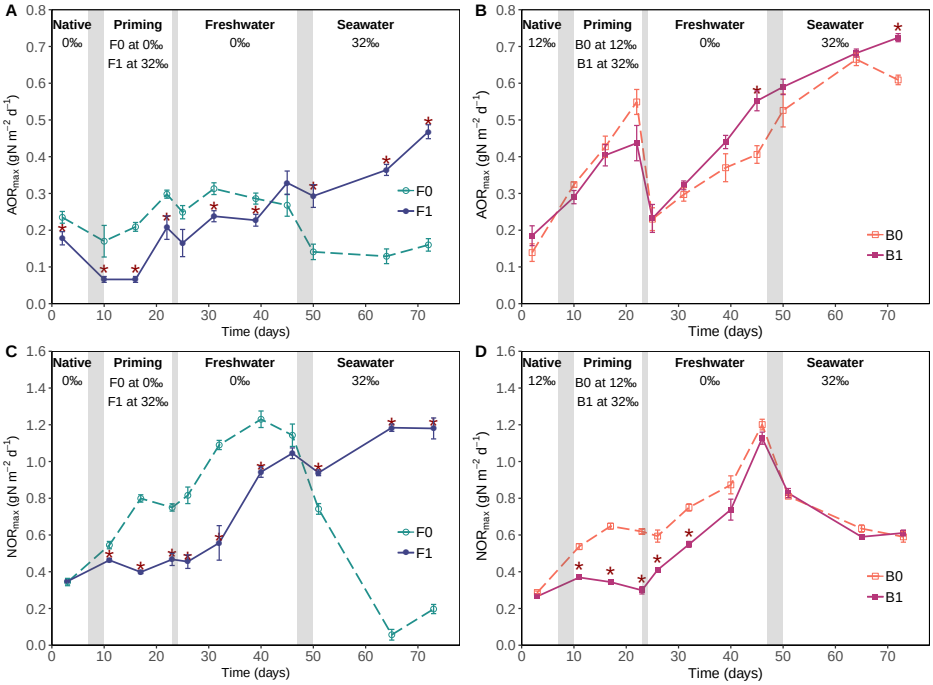


Figure 3.2: Maximum ammonia and nitrite oxidation rates (AOR_{max} and NOR_{max}, respectively) during the different experimental phases for the freshwater and brackish water treatments. A) AOR_{max} in F0 and F1, B) AOR_{max} in B0 and B1, C) NOR_{max} in F0 and F1, and D) NOR_{max} in B0 and B1. Salinities during the different phases are shown in ‰ (parts per thousand). Gray shaded regions indicate days of salinity change. In each graph, asterisks above the data points indicate that the primed treatment was significantly different from the unprimed treatment ($p < 0.05$). Note the differences in the y-axes scales. (Fig. 1 in **Article II**)

The primed freshwater treatment (F1) had a significantly higher nitrification activity than the unprimed treatment (F0) upon salinity increase from fresh- to seawater (Fig. 3.2). In fact, after a small initial decrease (< 10%) after seawater transfer, the nitrification rate in F1 increased rapidly (which could also be partly due to the developing biofilm). In comparison, F0 had a 55% decrease in AOR_{max}. This strongly suggests that seawater priming improved salinity acclimation. In contrast to the freshwater biofilms, priming did not have any significant effect on nitrification activity in the brackish water biofilms, especially after the freshwater phase. Moreover, the brackish treatments did not undergo any reduction in

AOR_{max} upon seawater transfer, and instead, showed an increase in the seawater phase. This indicates that biofilms developed in brackish water are already "primed" and inherently robust to salinity increase. However, in contrast to the freshwater treatments, slight nitrite accumulation was observed in the brackish treatments in the seawater phases. This is discussed further in Section 3.2.3.

The results show that bioreactors that have previously been exposed to high salinity, adapt better to salinity increases in the future. This was also demonstrated in a recent study where the salinity was increased at ~0.5-1‰ d^{-1} in salinity-primed RAS bioreactors, while keeping ammonia accumulation within acceptable levels for the fish (with reduced feeding on only a few days) (Fossmark et al., 2021). Thus, newly started bioreactors are the most susceptible to drastic drops in nitrification due to salinity increase. To address this issue, suitable start-up strategies are required to make the bioreactor salinity tolerant before introducing the fish to the RAS. A feasible strategy is the seawater priming of freshwater bioreactors during the start-up phase. An alternative strategy could be a direct start-up in brackish water followed by a subsequent reduction in salinity to freshwater before the introduction of the parr.

3.2.2 Start-up in brackish water (Article III)

As the priming strategy required two salinity changes and at least two weeks for seawater priming, we wanted to explore if brackish water start-up could be a more practical strategy in RAS. Further, brackish water biofilms appeared to have a relatively higher AOR_{max}, although this may also have been due to the different histories of the biofilms (**Article II**). However, little is known about the start-up time of brackish water biofilms. Seawater bioreactors are known to require much longer to start up than freshwater bioreactors (Chen et al., 2006; Nijhof and Bovendeur, 1990). Thus, for saline RAS, a typical "time-saving" strategy is to start a bioreactor in freshwater and gradually adapt it to the desired salinity (Chen et al., 2006; Nijhof and Bovendeur, 1990). However, adaptation to salinity is also time-consuming and can take several weeks or months (**Article I**, Bassin et al., 2012; Gonzalez-Silva, 2016). Thus, we conducted the third study to investigate whether a direct start-up in brackish water could be a practical strategy in RAS (**Article III**). In this study, two semi-commercial RAS MBBRs were started up simultaneously in fresh- and brackish water, respectively.

At the end of the study, the brackish water biofilm had approximately half the AOR_{max} and NOR_{max} of the freshwater biofilm. The microbial analysis also showed that bacterial succession was slower in the brackish biofilm than in the freshwater biofilm. Nonetheless, complete nitrification was achieved in both reactors after 60 days (Fig. 3.3), in contrast to 100-300 days required for the

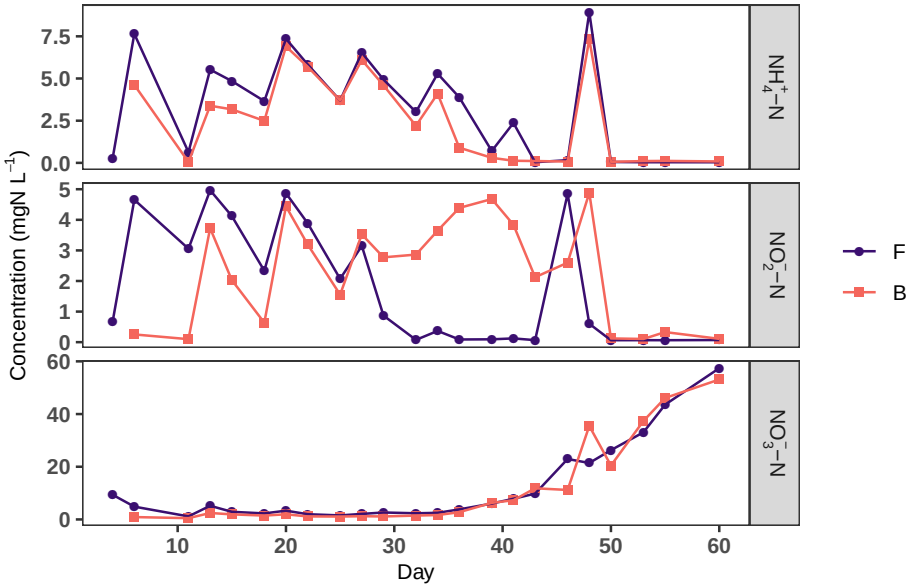


Figure 3.3: Ammonia, nitrite, and nitrate concentration in the freshwater (F) and brackish water (B) reactors during the study. The points have been connected to improve readability, but are not necessarily linearly related. Note the difference in the scales. (Fig. 1B in **Article III**)

start-up of nitrifying biofilms in seawater (Li et al., 2019; Liu et al., 2019; Nijhof and Bovendeur, 1990). Thus, a brackish start-up appears to be a feasible strategy in salmonid RAS. Before the introduction of parr to the RAS, the salinity must be reduced to accommodate the fish. However, it should be noted that a salinity decrease can also impact the nitrification performance. In **Article II**, the brackish water biofilms showed a ∼50% drop in AOR_{max} when the salinity was reduced from 12 or 32‰ to freshwater. The same study also showed a slight accumulation of ammonia after freshwater transfer, suggesting that around 1-2 weeks are required for the AOR_{max} to fully recover. Further, as nitrite accumulated during the saline phase, the nitrite concentration was high after freshwater transfer and took a few days to subside. Thus, it is recommended to acclimate the bioreactor for at least two weeks after a salinity reduction before introducing the fish to the RAS.

3.2.3 Seeding with brackish-water biofilm during freshwater start-up (Article IV)

In **Article III**, we found that a brackish start-up was a feasible strategy for RAS. However, the biofilm developed slower in brackish water than in freshwater. Moreover, a two-week period was recommended to adapt the bioreactor to freshwater before introducing the fish. To investigate if the start-up period could be shortened while conferring salinity tolerance to the bioreactor, we undertook the fourth study (**Article IV**). This study consisted of three treatments started with virgin carriers and seeded (10%) with biofilm carriers acclimated to freshwater (F), brackish water (B) and a 1:1 mix of both (FB). All reactors were started in freshwater, and the salinity was increased to seawater after 47 days of start-up. The results showed that the AOR_{max} reduced only by ∼20% in the brackish treatment, whereas it reduced by 65-75% in the other two treatments (Fig. 3.4). Further, the AOR_{max} recovered the fastest in the B treatment (∼5 days), followed by FB (∼10 days) and finally F (> 1 month). The results show that seeding with brackish water biofilm was the most effective in improving salinity acclimation in the bioreactors.

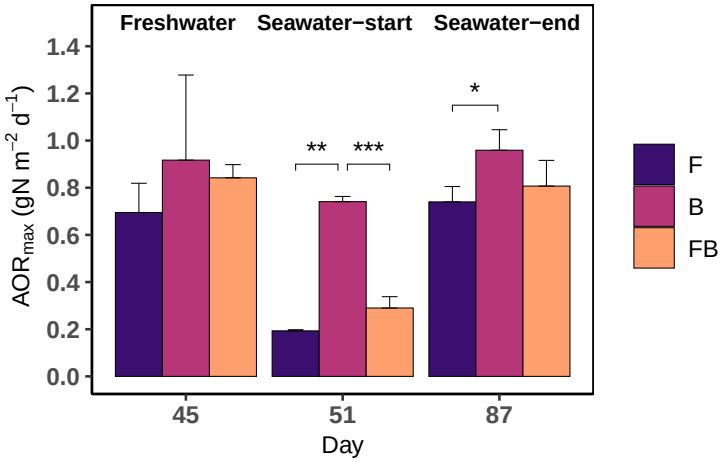


Figure 3.4: Capacity test data showing the maximum ammonia oxidation rate (AOR_{max}) in the main reactors. The treatments were seeded with freshwater-acclimated carriers (F), brackish water acclimated carriers, (B) and a 1:1 mix of fresh- and brackish water acclimated carriers (FB), respectively. The tests were conducted before (day 45, freshwater) and after (day 51, seawater-start) salinity increase to seawater, and 37 days after complete transfer to seawater (day 87, seawater-end). Salinity was changed from freshwater to seawater during days 47-50 in daily increments (∼10‰ d^{-1}). Significant differences between treatments on each day are marked by asterisks (where * denotes $0.01 < p < 0.05$, ** denotes $0.001 < p < 0.01$, and *** denotes $p < 0.001$). Note the difference in y-axes scales. (Fig. 3A in **Article IV**)

Not only was the B treatment robust to salinity increase, it achieved a high nitrification rate ($\sim$0.9 gN m^{-2} d^{-1}) within 45 days of start-up in freshwater. The rate is two orders of magnitude higher than that observed in unseeded MBBRs after 60 days of start-up (**Article III**). This could be due to the combined effect of seeding as well as the supply of substrate at non-limiting conditions. Notably, despite containing some brackish seed carriers, the performance of the FB treatment was more similar to that of the F treatment, especially immediately after the salinity increase. This is likely because the nitrifying community composition of the F and FB treatments were more similar to each other compared to that in B. This suggests that the initial period in freshwater provided a competitive advantage to the freshwater species in FB over the halotolerant brackish water species, thus making the newly developed biofilm less robust to salinity changes.

Nitrite did not accumulate in any of the treatments immediately after seawater transfer. However, the nitrite concentration increased in treatment B after one week in seawater and subsided after two weeks. Similar nitrite accumulation occurred in the F and FB treatments, but after about a month in seawater. In the last week of the study, nitrite concentration was < 5 mgN L^{-1} in the B and FB treatments, but 12-30 mgN L^{-1} in treatment F. The capacity tests at the end of the study also showed significant nitrite accumulation in all the treatments, with the lowest accumulation in treatment B. This indicates that although the AOR_{max} recovered to the original levels, nitrite oxidation did not recover completely after more than a month in seawater. Although some studies report that AOB are more affected than NOB (Moussa et al., 2006; Sharrer et al., 2007), our findings support the view that nitrite oxidation is generally lower than ammonia oxidation in saline water (**Articles I-IV**). One notable exception is that no nitrite accumulation was observed in the freshwater treatments in **Article II**. However, nitrite accumulation at high salinities has also been reported by several previous studies (Gonzalez-Silva et al., 2016; Nijhof and Bovendeur, 1990; Sudarno et al., 2011). This suggests that despite the lower toxicity of nitrite in saline water, care should be taken after a salinity increase to ensure that the nitrite levels are safe for the fish.

3.2.4 Can the exogenous addition of osmolytes improve salinity acclimation? (Article V)

In our studies, several nitrifying taxa were detected across different salinities (**Articles I-IV**). This suggests that many nitrifiers in the biofilm are halotolerant and can adapt to varying salinities. Halotolerant microorganisms typically accumulate osmolytes to adapt to an increase in the extracellular salinity (Sleator and Hill, 2001). However, the *de novo* synthesis of osmolytes is energetically expensive (Oren, 2011; Sleator and Hill, 2001). Alternatively, microorganisms

can take up osmolytes from the growth medium, and this strategy has been shown to be effective over a range of microorganisms, from methanogens to anammox bacteria (Vyrides and Stuckey, 2017). For nitrifiers, the net energy generated by autotrophic oxidation is low and therefore, surviving at high salinities is especially challenging (Oren, 2011). As the uptake of osmolytes from the medium is energetically more favorable than synthesis (Sleator and Hill, 2001; Vyrides and Stuckey, 2017), it could be a promising strategy for salinity adaptation in nitrifying microorganisms. Thus, in the fifth study, we investigated the effect of exogenous addition of an osmolyte cocktail on salinity acclimation in nitrifying biofilms (**Article V**). The osmolyte cocktail contained 1mM each of trehalose, sucrose, gylcine betaine, proline, carnitine, and ectoine.

The exogenous addition of the osmolyte cocktail did not improve salinity acclimation in MBBRs immediately after a salinity increase from freshwater to seawater. Further, two days after the salinity increase, there was a near complete inhibition in the nitrification activity in the treatment with osmolytes. This was accompanied by the growth of heterotrophic bacteria in the medium. It is likely that the heterotrophic growth (both in the biofilm and planktonic) was facilitated by osmolyte uptake by the heterotrophs for osmoregulation or as substrate. This would have increased the competition between the heterotrophs and nitrifiers (Rittmann and McCarty, 2001), and can explain the inhibition in the nitrification activity. Thus, in this study, not only did the osmolyte addition not improve salinity adaptation, but in fact, prolonged exposure to the osmolytes severely reduced the nitrification activity. Future studies should investigate the effect of individual osmolytes at different concentrations, along with metaproteomic analyses to explore the potential for osmolyte uptake as a salinity acclimation strategy in nitrifying biofilms.

"Everything is everywhere, but the environment selects"
—L.G.M. Bass Becking and M.W. Beijerinck

3.3 How does the microbial community composition in the biofilm respond to salinity changes?

3.3.1 Total microbial community composition

Apart from structural changes, salinity changes can influence a biofilm in two fundamentally different ways: through physiological adaptation of the existing microbes, or by a shift in the community composition towards microbes more suited to the salinity regime (Bassin et al., 2012; Bassin et al., 2011; Gonzalez-Silva, 2016). **Article I** showed that the although the rate of salinity change did not impact the nitrification activity, it did influence the microbial community composition in the biofilm. The microbes in the treatment with the largest salinity increment (S15, 15‰ day^{-1}) appear to have adapted to the salinity change, whereas in the other treatments, the community composition evolved to select for microorganisms more suited to the frequent salinity changes. It should be noted that the salinity was increased gradually in daily increments, in contrast to other studies where the salinity was increased in steps or shock changes (Bassin et al., 2012; Gonzalez-Silva, 2016; Kinyage et al., 2019; Moussa et al., 2006). This may have given the bacteria time to adapt to the increasing salinity by K^+ uptake or by the synthesis of compatible solutes. Shock changes in salinity may impact the nitrification more severely. Previous studies on unprimed biofilms reported > 95% inhibition in ammonia oxidation rate after a shock transfer from fresh- to seawater (Gonzalez-Silva, 2016; Kinyage et al., 2019), in contrast to our studies (55-75% reduction after a salinity increase over 1-3 days) (**Articles I, II, IV**). Other salinity change regimes, such as step changes with longer intervals between salinity changes may produce different responses in the biofilm and should be further investigated. Nonetheless, a gradual change in salinity by adjusting the salinity of the influent (as in **Articles I, II, IV**) is the most practical scenario in a commercial RAS.

The community composition and species inventory of biofilms developed in freshwater and brackish water (12‰ salinity) differed significantly (**Article III**). Further, while seawater priming caused a significant shift in the community composition in freshwater biofilms (mainly in the heterotrophic community), it did not affect the community composition in brackish water biofilms (**Article II**). This suggests that the microbes in brackish water biofilms are capable of adapting to seawater. Moreover, although a salinity increase from 0 to 8‰ did not change the AOR_{max} (**Article I**), a further increase to 12‰ resulted in a 14-26% reduction

(**Article I, II**). Thus, ∼12‰ salinity may be a critical point for salinity adaptation in microorganisms. This is corroborated by previous studies where the nitrification activity dropped significantly when salinity was increased beyond 8-15‰ (Bassin et al., 2012; Bassin et al., 2011; Fossmark et al., 2021; Gonzalez-Silva et al., 2016; Kinyage et al., 2019). Several studies also report that a salinity increase from 0 up to 10‰ appears to either not affect or to increase the nitrification rate (Aslan and Simsek, 2012; Bassin et al., 2012; Cortes-Lorenzo et al., 2015; Sudarno, 2011; Vendramel et al., 2011). This could likely be attributed to lower energy requirements for osmoregulation at isotonic conditions (∼9‰ salinity) (He et al., 2017). When salinity is increased beyond the isotonic level, energy and nutrients will be directed towards osmoregulation, making less energy available for cell growth and leading to a reduction in activity (Oren, 2011). Likely due to similar reasons, teleost species such as Atlantic salmon also seem to perform better in RAS at intermediate salinities (12-22‰) than in seawater (Ytrestøyl et al., 2020). Thus, ∼12‰ may be the optimum salinity for the operation of salmonid RAS. The small decrease in nitrification rate from 0 to 12‰ salinity can be compensated by a temporary reduction in the fish feeding rate. Operating at salinities lower than seawater can also offer other advantages, such as higher CO_2 degassing efficiency and lower risk of equipment corrosion.

Early perturbations in young biofilms are influential in shaping the biofilm structure and the eventual microbial community composition (**Article II**; Cabrol et al., 2016; Saur et al., 2016). This implies that strategies adopted during the start-up phase of nitrifying bioreactors can be highly effective in molding the initial community composition and the consequent microbial activity. Besides avoiding the risk of harming the fish, this is a strong motivation for implementing salinity acclimation strategies in the start-up phase of RAS bioreactors. However, the microbial community composition in a RAS is not static. Except **Article I**, all the studies contained newly developed biofilms. The eventual activity and microbial community composition of mature biofilms can be different from those in developing biofilms. The original community composition may be modified through community assembly processes such as dispersal (through the introduction of microorganisms in influent water or fish feed), drift (stochastic changes) and selection (competition between taxa, especially during system fluctuations) (Nemergut et al., 2013). Compared to the synthetic medium used in this PhD, RAS water contains a higher concentration of complex organic matter. It also contains a high concentration of suspended microbes that can interact with the microbes on the biofilm, thereby influencing the community composition in the biofilm. Thus, when subjected to the same salinity regimes, the microbial community composition in a RAS bioreactor may evolve differently than in this PhD. As the bacterial density is very high in biofilms, the microbial community composition in

a bioreactor can play an vital role in selecting favorable bacteria (K-selection) and preventing pathogen entry in a RAS (Schryver and Vadstein, 2014; Vadstein et al., 2018). Thus, it is important to ensure a favorable and stable microbial community in RAS bioreactors.

3.3.2 Composition of the nitrifying community

The nitrifying community composition in freshwater biofilms was influenced by salinity changes (**Articles I, II**). However, the same nitrifying taxa were present before and after the salinity changes, and the differences in the nitrifying community composition were mainly due to changes in the relative abundance of the different taxa. This suggests that physiological adaptation rather than a community shift is the preferred adaptation mechanism for nitrifiers in biofilms (**Article I, II**). It also suggests that several nitrifying microorganisms are halotolerant and can adapt to a wide range of salinities. This hypothesis is supported by a previous study where the dominant nitrifying OTUs in fresh-, brackish- (22‰ salinity) and seawater biofilms were detected in all three systems (Gonzalez-Silva et al., 2016). Another study showed that the same nitrifying taxa were present in RAS bioreactors throughout a salinity increase from 3 to 28‰, showing that they adapted to the higher salinity (Fossmark et al., 2021). Nitrifiers have also been detected in estuarine systems, indicating that they are capable of adapting to frequent salinity fluctuations (Bernhard and Bollmann, 2010; Santos et al., 2018; Ward et al., 2007). However, microbes in the biofilm may also be protected against osmotic stress through changes in the biofilm morphology, such as the production of EPS (Corsino et al., 2017; Flemming et al., 2016; Wang et al., 2015). Future studies should investigate strategies to increase the salinity tolerance of biofilms by studying the combined effect of salinity changes on the biofilm morphology and the microbial community.

Freshwater biofilms generally had higher proportions of NOB compared to AOB (**Articles I-IV**). The community composition corresponded well with the nitrification activity, as NOR_{max} was generally higher than AOR_{max} in freshwater (**Article II, III**). In general, the proportion of AOB increased with salinity, while the proportion of NOB decreased (**Article I-IV**). In samples with prolonged exposure to seawater, the AOB increased to a greater relative abundance than the NOB (**Article I, II**). Consequently, biofilms grown in brackish water had AOB:NOB>1 (**Article II, III**). However, this was not observed in **Article IV**. The changes in the AOB:NOB ratio across salinities was reflected in the nitrification activity. In **Articles II** and **IV**, although the AOR_{max} recovered during the seawater phase, the NOR_{max} decreased further a few days or weeks after seawater transfer (except in F1 in **Article II**). We do not know the reason for this delayed response of the NOB. Nitrite accumulation at higher salinities has also been reported by

other studies (Bassin et al., 2011; Cui et al., 2009; Gonzalez-Silva, 2016; Nijhof and Bovendeur, 1990). As nitrite oxidation generates lower energy than ammonia oxidation, it is possible that this energy is insufficient to sustain osmoregulation and growth over a prolonged period at elevated salinities (Oren, 2011; Rittmann and McCarty, 2001). Further investigation using advanced methods such as functional metagenomics may provide an insight into the metabolic response of AOB and NOB under acute and chronic exposure to salinity. This would help determine the dominant species at different salinities and the pathways involved in their salinity adaptation. Further, understanding the transporter genes present in the selected species would facilitate the identification of osmolytes that could potentially aid salinity acclimation.

In general, biofilms at intermediate salinity had a higher α-diversity of nitrifiers (**Article I, II, IV**). This was not observed in **Article III**, likely because the brackish water biofilm had not developed to the same extent as the freshwater biofilm. We speculate that intermediate salinities favor a greater functional redundancy compared to freshwater, as they can accommodate both halotolerant and halophilic microorganisms, whereas freshwater environments can only harbor non-halophilic (or at most, halotolerant) microorganisms. The AOB detected in our studies were β-proteobacteria belonging to the genera *Nitrosomonas* and *Nitrosospira*. Although the genus *Nitrosococcus* is reported to be obligately halophilic and abundant in marine systems (Koops et al., 2006), it was not detected in any of our studies. *Nitrosomonas* was almost exclusively the only AOB genus in the freshwater biofilms (**Articles I, II, III**). In contrast, biofilms at intermediate salinity or in seawater contained the genus *Nitrosospira* in addition to *Nitrosomonas* (**Articles I-IV**). The main nitrite oxidizing genera in our studies belonged to *Nitrotoga* (also known as *Candidatus Nitrotoga*), *Nitrospira*, *Nitrobacter* and *Nitrospina* (very low abundance). *Nitrotoga* was the dominant NOB in nearly all the biofilm samples (**Article I-IV**). However, the biofilms under saline conditions also contained small proportions of *Nitrospira* and *Nitrobacter* (**Article I, II**), suggesting that these genera may be superior competitors than *Nitrotoga* under fluctuating or intermediate salinities. These genera have been detected in marine systems and therefore, contain at least some species that are halotolerant or halophilic (Daims et al., 2016). Complete ammonia oxidation by comammox *Nitrospira* may also play a role in RAS nitrifying bioreactors, although they appear to be mostly present in fluidized sand filters operated in freshwater (Bartelme et al., 2019). As comammox *Nitrospira* cannot be differentiated by 16S rRNA sequencing (Pjevac et al., 2017), it was not investigated in this PhD. The microbial community was mainly classified at the family or genus level due to the limitations of 16S rRNA sequencing method. However, different salinity regimes may also lead to major differences at the species level. This should be investigated

in further studies using advanced microbiological techniques to identify the species selected under different salinity regimes.

Most aquaculturists have traditionally focused on *Nitrobacter* as the representative NOB in RAS bioreactors (Timmons and Ebeling, 2010). However, *Nitrotoga* was the dominant nitrite oxidizer in our studies, with relative abundance as high as ∼40% (**Articles I-IV**). Temperature appears to be a determining factor as *Nitrotoga* is reported to have a competitive advantage over *Nitrobacter* and *Nitrospira* at lower temperatures (5-10°C) (Alawi et al., 2009; Karkman et al., 2011). *Nitrotoga* has also been detected in wastewater treatment plants and RAS at low temperatures (7-16°C) (Hüpeden et al., 2016; Lücker et al., 2015). Thus, this genus can be the dominant NOB in cold-water RAS and should be considered when evaluating the nitrification kinetics. However, *Nitrotoga* has also been detected in a warm marine RAS, suggesting that this genus may be present over a wider range of temperatures than previously believed (Keuter et al., 2017). Moreover, the dominance of *Nitrotoga* in all our studies throughout the entire spectrum of salinities– from freshwater to seawater – strongly suggests that this NOB genus has a high adaptability towards salinity changes. In our studies, *Nitrotoga* was detected in the fresh- and seawater intake water sources, which may partly explain its presence in the biofilm (**Article III, IV**). However, other NOB such as *Nitrospira* were also detected in the intake water. The dominance of *Nitrotoga* despite the presence of other NOB strongly suggests that environmental factors selected for this particular genus. Notably, this genus was not classified by the Ribosomal Database Project (RDP) (**Article III**). Thus, suitable methods should be used for the detection of this genus.

In addition to the strategies explored in this PhD, salinity tolerance may also be increased by adding inoculum adapted to salt (Cui et al., 2016; Panswad and Anan, 1999; Shi et al., 2012; Sudarno et al., 2010). However, it involves costs and biosecurity risks associated with the introduction of external biological matter to a RAS. That said, certified pathogen-free commercial inoculum may be a potential strategy for increasing the salinity tolerance through initial colonization of the biofilm. However, as previously mentioned, the ultimate microbial community composition established will depend on the dispersal and selection pressures in the system. For instance, only some of nitrifying taxa in the biofilms were detected in the source water (**Article III**), suggesting that selection pressure played a greater role in establishing the community composition than the starting inoculum. This in contrast to some studies that suggest the opposite (Keuter et al., 2017; Wittebolle et al., 2009). However, seeding with biofilm carriers did influence the nitrifying community composition in newly developed freshwater biofilms in **Article IV**, although it did not influence the final composition after seawater acclimation. This

suggests that the species in seeded biofilm carriers may be more successful than commercial inocula in colonizing virgin carriers, as they are already adapted to survive in a biofilm. Indeed, this was the case in a recent study where seed carriers were more effective in accelerating the start-up period than commercial inoculum (Roalkvam et al., 2020). The same study also showed that the biofilm contained some nitrifying taxa that were not detected in the inoculum, indicating that the local taxa were preferentially selected, and could out-compete certain taxa in the commercial inoculum. Thus, local salinity acclimation strategies appear more beneficial than the addition of commercial inocula in RAS bioreactors, and should be further investigated.

"Knowing is not enough, we must apply. Willing is not enough, we must do."
—Bruce Lee

3.4 Industrial application - Bioreactor start-up strategies

A summary of the salinity acclimation strategies investigated in this PhD is presented in Fig. 3.5. Herein, we discuss the application of these strategies in the industrial context. In a RAS for Atlantic salmon parr, the bioreactor is usually started up before the introduction of the fish, with the supply of partially disinfected water, chemicals and fish feed. After nitrification is established, parr are introduced to the fish tanks and reared in freshwater. At this stage, a slight salinity increase (up to 5‰) may sometimes be desirable to prevent fungal diseases or to mitigate the toxic effects of nitrite. This may be safely done in a RAS with fish, as a salinity increase from 0‰ up to ∼8‰ has only a small impact on the nitrification activity (**Article I**). After the fish have smoltified, the salinity is typically increased from ∼0‰ (freshwater) to 12-35‰ (brackish- or seawater) to accommodate the requirements of smolt (or post-smolt). However, irrespective of the rate of salinity change, a salinity increase beyond 10-12‰ in unprimed freshwater biofilms can result in a significant drop in nitrification activity (**Article I**). This implies that it is difficult to increase the salinity beyond 10-12‰ in a freshwater RAS without compromising fish welfare. Thus, we investigated start-up strategies to make the bioreactor tolerant to salinity increase before the introduction of the fish. Broadly, three bioreactor start-up strategies are suggested (Fig. 3.6). Although the strategies have not been tested in a RAS with fish, the findings of this PhD strongly support their feasibility. These strategies may be practically implemented in an industrial RAS where it is desired to increase the salinity from freshwater to brackish- or seawater during the fish production cycle.

3.4.1 Strategy A - Osmotic stress priming

Seawater priming could be a strategy for increasing salinity tolerance in the start-up phase of RAS bioreactors (**Article II**). The bioreactor should be started up in freshwater (∼8 weeks, **Article III**), after which the salinity is increased to seawater. This salinity increase may be conducted gradually in large increments over a few days (for e.g. 1-3 days) as the rate of salinity change does not influence the nitrification activity (**Article I**). After two weeks of seawater priming, the salinity should be decreased to freshwater in the same manner. The nitrification activity is unlikely to be affected by the salinity decrease. However, the bioreactor should be monitored for a short period (∼1 week) at this salinity to ensure the nitrification performance is satisfactory before the parr are introduced. Overall, this strategy requires two salinity changes and ∼11-12 weeks.

SALINITY ACCLIMATION STRATEGIES FOR NITRIFYING BIOREACTORS

Rate of salinity increase?	Osmotic stress priming	Start-up in brackish water	Seeding with brackish biofilm	Addition of osmolytes?
Article I	**Article II**	**Article III**	**Article IV**	**Article V**
Result: Rate of salinity increase did not affect nitrification activity. *Strategy*: Large but gradual salinity increments followed by a long acclimatization period	*Result*: Osmotic stress priming improved salinity acclimation in freshwater biofilms. Brackish biofilms were inherently robust to salinity increase. *Strategy*: Prior exposure to high salinity (for freshwater biofilms)	*Result*: Biofilms achieved complete nitrification in brackish water in comparable time as in freshwater. *Strategy*: Direct start-up in brackish water (Note: Microbial succession and nitrification activity may be slower than in freshwater)	*Result*: Seeding with biofilm carriers acclimated to brackish water improved salinity acclimation in freshwater bioreactors. *Strategy*: Addition of brackish biofilm carriers during the start-up of freshwater bioreactors (Note: Temporary nitrite accumulation can occur at high salinity)	*Result*: Exogenous addition of osmolytes did not aid salinity acclimation in nitrifying biofilms. *Strategy*: Not applicable; further research required

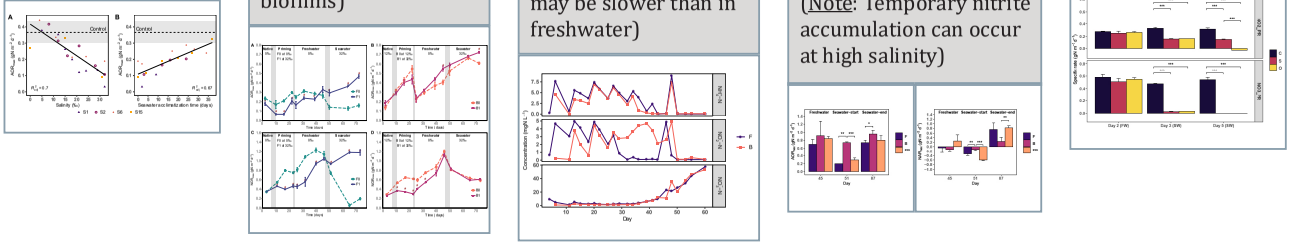


Figure 3.5: Summary of the salinity acclimation strategies investigated in this PhD

3.4.2 Strategy B - Start-up in brackish water

As biofilms can develop complete nitrification in brackish water (12‰ salinity) in comparable time as in freshwater (**Article III**), start-up in brackish water could be a strategy for RAS bioreactors. In this strategy, the bioreactor is started up at 12‰ salinity. During the start-up, chemical dosing should be increased according to the nitrification rate, so that the concentration of the substrate (ammonia/nitrite) does not limit the growth of the nitrifiers. As the nitrite oxidation rate is often low at higher salinities (**Articles II, III, IV**), nitrite may accumulate initially. When the desired nitrification rate is reached ($\sim$8 weeks), the salinity can be decreased to freshwater to prepare the system for the incoming parr. This may decrease the ammonia oxidation rate (**Article II**). Nitrite oxidation rate will likely increase in freshwater (**Article II**), and this can be helpful in decreasing the nitrite concentration if it is initially high. It is thus recommended to operate the bioreactor in freshwater for 2-4 weeks, until the ammonia oxidation rate and nitrite concentration have reached the desired levels. This strategy requires only one salinity change and $\sim$10-12 weeks.

3.4.3 Strategy C - Freshwater start-up with brackish biofilm seeding

A third strategy for improving the salinity tolerance of RAS bioreactors can be a freshwater start-up with the seeding of brackish water (12‰ salinity) biofilm carriers (**Article IV**). In general, fish farmers are reluctant to introduce external biological matter to a RAS, as it can pose a biosecurity risk. Thus, a prerequisite for this strategy is the availability of brackish water biofilm carriers on-site. If the fish farm contains another RAS department with a mature brackish water MBBR, carriers can be extracted from this MBBR for seeding. Note that this will slightly increase the biosecurity risk, as biological matter is exchanged between departments. Another alternative would be to design a small isolated MBBR that is started up and matured before the construction of the RAS MBBRs. Carriers from this MBBR can then be used for seeding the RAS MBBRs. The microbial inoculum provided by the seeding leads to rapid colonization of the carriers and high nitrification rates can be achieved within a short period. Moreover, as no salinity changes are required in this strategy, the start-up period is relatively short ($\sim$7 weeks).

3.4.4 Overview and application of the strategies

If brackish biofilm carriers are available, strategy C is recommended as it requires the least amount of time. Else, strategy A or B may be applied. After the bioreactor has been started up, parr are introduced to the system. The fish are reared in freshwater for a few weeks or months (depending on the production plan) until

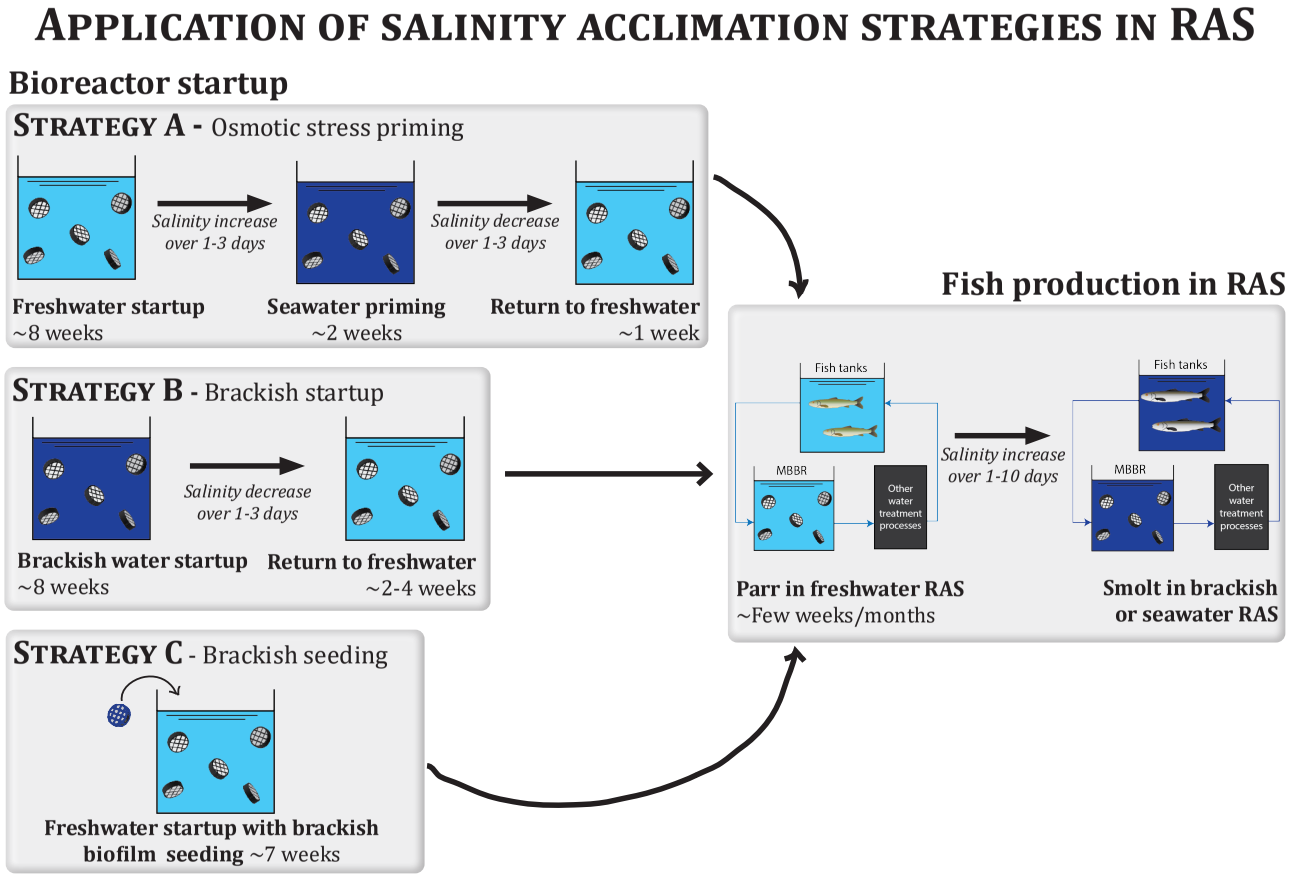


Figure 3.6: Industrial application of the salinity acclimation strategies developed in this PhD. The infographic describes three start-up strategies that can make nitrifying bioreactors more tolerant to a salinity increase, thus improving the bioreactor performance during the fish production cycle in a RAS.

they are smoltified. Thereafter, the salinity can be rapidly increased to brackish- or seawater by adjusting the salinity of the dilution water (**Article I**). In a typical RAS, this can be done within 1-10 days, depending on the flow and salinity of the incoming water. Nitrite concentration should be carefully monitored after the salinity increase to ensure that it is within the recommended levels. The post-smolt are harvested when they grow to the desired size. After emptying and cleaning the fish tanks, the system can be refilled with freshwater to prepare for the next batch of parr. After the first batch of fish, the subsequent salinity changes in the RAS will likely have a smaller impact on the nitrification activity due to the priming effect (**Article II**).

Some fish farms can also have dedicated RAS departments for post-smolt that are operated at a constant salinity level (typically brackish- or seawater). In such RAS, the bioreactors can be started directly in brackish water, as this can be faster than to start in freshwater and later increase the salinity (based on **Articles I and III**). To accelerate the start-up, the bioreactor can be seeded with biofilm carriers acclimated to brackish water. However, having separate RAS for parr and post-smolt is expensive, and also increases the challenges associated with fish handling and transfer. Thus, it may be preferable to rear parr and post-smolt in the same RAS, with the salinity adjusted as required. The above-mentioned strategies fill an important knowledge gap by addressing the challenge of salinity changes in the commercial production of Atlantic salmon in RAS. The application of these strategies can improve the bioreactor performance, thereby enhancing the production and welfare of the fish.

Chapter 4

Conclusions and future perspectives

"The truth is, most of us discover where we are headed when we arrive"
—Bill Watterson

This PhD resulted in the development of several strategies for improving the salinity acclimation of nitrifying bioreactors. While the PhD was motivated by the challenges faced in salmonid RAS, the findings can also be applied to other water treatment systems dealing with high or variable salinity, such as tanning or food-processing industries. Our results suggest that irrespective of the rate of salinity change, it is difficult to safely increase salinity in a freshwater RAS without a severe reduction in the ammonia oxidation rate. However, in a RAS without fish, large salinity increments with a long acclimatization period can be a practical strategy for increasing salinity.

As the first salinity increase is the most challenging, bioreactors should be made salinity tolerant before the first batch of fish are introduced. Osmotic stress priming (prior exposure to high salinity) can be an effective strategy to improve salinity acclimation in freshwater biofilms. However, future studies should research the long-term impacts of seawater priming to investigate whether seawater tolerance is sustained over periods longer than a few weeks. Start-up in brackish water (12‰ salinity) can also be a practical strategy, as brackish biofilms were inherently robust (or 'primed') to salinity increase and complete nitrification was established in comparable time as in freshwater. However, biofilms developed in brackish water had a slower bacterial succession and about half the nitrification capacity of freshwater biofilms. To reduce the start-up time while conferring salinity tolerance, seeding with brackish water biofilm carriers is recommended during the start-up

of freshwater bioreactors. Careful monitoring during and after salinity changes is vital, as temporary nitrite accumulation can occur even several days after a salinity increase.

Physiological adaptation rather than an alteration in the nitrifying taxa appeared to be the dominant mechanism for salinity adaptation in biofilms, suggesting that several nitrifiers are halotolerant. Across all our studies, *Nitrosomonas* was the dominant genus of AOB. *Nitrotoga* was the dominant nitrite oxidizer at all salinities, indicating that this genus is halotolerant and can play an important role in cold-water RAS. This contradicts the traditional notion of *Nitrobacter* and *Nitrospira* as the main NOB in RAS. At different salinity regimes, major differences may arise not only at the genus level, but also at the species level. Future researchers should investigate this using advanced microbiological techniques to gain a better understanding of the specific selection mechanisms under different salinity regimes. Non-canonical nitrifiers, such as AOA and comammox *Nitrospira* have also been detected in RAS bioreactors, but were not investigated in this study due to resource limitations. This may have led to some loss in scientific understanding of the process ecology. Further, as RAS water is more complex than synthetic medium, the impact of different salinity regimes on the biofilm community composition in a RAS could be different from that in this PhD.

In addition to the microbial community composition, salinity acclimation may also be influenced by the biofilm morphology, such as EPS. Future studies should investigate the role of EPS and the possibility to manipulate it to improve salinity adaptation in biofilms. Contrary to our hypothesis, the exogenous addition of osmolytes did not improve salinity adaptation in nitrifying biofilms, likely due to the uptake of osmolytes by the heterotrophs instead of the nitrifiers. Future studies should test individual osmolytes at different concentrations, accompanied by metaproteomic analyses to investigate the salinity adaptation mechanisms in nitrifying biofilms. This may help identify osmolytes that are preferentially taken up by nitrifiers. The exogenous addition of such osmolytes could be a strategy for salinity acclimation in nitrifying biofilms.

Based on our studies and existing literature, 12‰ could be the optimum salinity for operating post-smolt RAS. The temporary reduction in nitrification activity upon an increase from 0‰ to 12‰ salinity may be compensated by reduced fish feeding. A salinity decrease from 12‰ to 0‰ is also less acute than a decrease from seawater. Further, a lower salinity can alleviate other challenges experienced in seawater RAS, such as corrosion and lower CO_2 degassing efficiency. As 12‰ is close to isotonic, both the fish and the bacteria are expected to expend lesser energy for osmoregulation, making more energy available for growth. Finally,

this salinity level can prevent desmoltification and may be more favorable for fish growth and welfare than higher salinities, at least in RAS. However, as post-smolts can prefer salinities >12‰, the optimum salinity may be slightly higher than 12‰. Future studies should investigate the optimum salinity for salmonid RAS from a holistic perspective.

The salinity acclimation strategies developed in this PhD have already begun to be implemented in industrial RAS bioreactors. These strategies have the potential to enhance RAS performance during salinity changes and thus improve fish health and welfare. The ability to operate safely during varying salinities is a vital development that can aid the shift of salmonid production from the oceans to RAS, thereby bringing us one step closer to a sustainable food future.

Bibliography

Alabaster, J. S., Shurben, D. G., & Knowles, G. (1979). The effect of dissolved oxygen and salinity on the toxicity of ammonia to smolts of salmon, Salmo salar L. *J. Fish Biol*, *15*, 12.

Alawi, M., Off, S., Kaya, M., & Spieck, E. (2009). Temperature influences the population structure of nitrite-oxidizing bacteria in activated sludge. *Environmental Microbiology Reports*, *1*(3), 184–190.

Andrade-Linares, D. R., Lehmann, A., & Rillig, M. C. (2016). Microbial stress priming: A meta-analysis. *Environmental Microbiology*, *18*(4), 1277–1288.

Anthonisen, A. C., Loehr, R. C., Prakasam, T. B. S., & Srinath, E. G. (1976). Inhibition of nitrification by ammonia and nitrous acid. *Journal (Water Pollution Control Federation)*, *48*(5), 835–852.

Aslan, S., & Simsek, E. (2012). Influence of salinity on partial nitrification in a submerged biofilter. *Bioresource Technology*, *118*, 24–29.

Badiola, M., Mendiola, D., & Bostock, J. (2012). Recirculating Aquaculture Systems (RAS) analysis: Main issues on management and future challenges. *Aquacultural Engineering*, *51*, 26–35.

Bartelme, R. P., McLellan, S. L., & Newton, R. J. (2017). Freshwater recirculating aquaculture system operations drive biofilter bacterial community shifts around a stable nitrifying consortium of ammonia-oxidizing archaea and comammox Nitrospira. *Frontiers in Microbiology*, *8*.

Bartelme, R. P., Smith, M. C., & Sepulveda-villet, O. J. (2019). Component Microenvironments and System Biogeography Structure Microorganism Distributions in Recirculating Aquaculture and Aquaponic Systems. *4*(4), 1–15.

Bassin, J. P., Kleerebezem, R., Muyzer, G., Rosado, A., Van Loosdrecht, M., & Dezotti, M. (2012). Effect of different salt adaptation strategies on the microbial diversity, activity, and settling of nitrifying sludge in sequencing batch reactors. *Applied Microbiology and Biotechnology*, *93*(3), 1281–1294.

Bassin, J. P., Pronk, M., Muyzer, G., Kleerebezem, R., Dezotti, M., & van Loosdrecht, M. C. M. (2011). Effect of elevated salt concentrations on the aerobic granular sludge process: Linking microbial activity with microbial community structure. *Applied and Environmental Microbiology*, *77*(22), 7942–7953.

Bergheim, A., Drengstig, A., Ulgenes, Y., & Fivelstad, S. (2009). Production of Atlantic salmon smolts in Europe-Current characteristics and future trends. *Aquacultural Engineering*, *41*(2), 46–52.

Bernhard, A. E., & Bollmann, A. (2010). Estuarine nitrifiers: New players, patterns and processes. *Estuarine, Coastal and Shelf Science*, *88*(1), 1–11.

Bregnballe, J. (2015). *A guide to recirculation aquaculture : an introduction to the new environmentally friendly and highly productive closed fish farming systems* (tech. rep.). Food; Agriculture Organization of the United Nations.

Cabrol, L., Poly, F., Malhautier, L., Pommier, T., Lerondelle, C., Verstraete, W., Lepeuple, A. S., Fanlo, J. L., & Roux, X. L. (2016). Management of Microbial Communities through Transient Disturbances Enhances the Functional Resilience of Nitrifying Gas-Biofilters to Future Disturbances. *Environmental Science and Technology*, *50*(1), 338–348.

Chen, S., Ling, J., & Blancheton, J. P. (2006). Nitrification kinetics of biofilm as affected by water quality factors. *Aquacultural Engineering*, *34*(3), 179–197.

Colt, J. (2006). Water quality requirements for reuse systems. *Aquacultural Engineering*, *34*(3), 143–156.

Corsino, S. F., Capodici, M., Torregrossa, M., & Viviani, G. (2017). Physical properties and Extracellular Polymeric Substances pattern of aerobic granular sludge treating hypersaline wastewater. *Bioresource Technology*, *229*, 152–159.

Cortes-Lorenzo, C., Rodriguez-Diaz, M., Sipkema, D., Juarez-Jimenez, B., Rodelas, B., Smidt, H., Gonzalez-Lopez, J., Cortés-Lorenzo, C., Rodríguez-Díaz, M., Sipkema, D., Juárez-Jiménez, B., Rodelas, B., Smidt, H., & González-López, J. (2015). Effect of salinity on nitrification efficiency and structure of ammonia-oxidizing bacterial communities in a submerged fixed bed bioreactor. *Chemical Engineering Journal*, *266*, 233–240.

Csonka, L. N. (1989). Physiological and genetic responses of bacteria to osmotic stress. *Microbiological reviews*, *53*(1), 121–47.

CtrlAQUA definitions. (2015). Retrieved July 15, 2020, from https://ctrlaqua.no/about/definitions/

Cui, Y. W., Zhang, H. Y., Ding, J. R., & Peng, Y. Z. (2016). The effects of salinity on nitrification using halophilic nitrifiers in a Sequencing Batch Reactor treating hypersaline wastewater. *Scientific Reports*, *6*, 1–11.

Cui, Y., Peng, C., Peng, Y., & Ye, L. (2009). Effects of salt on microbial populations and treatment performance in purifying saline sewage using the MUCT process. *Clean - Soil, Air, Water*, *37*(8), 649–656.

Daims, H., Lebedeva, E. V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R. H., Von Bergen, M., Rattei, T., Bendinger, B., Nielsen, P. H., & Wagner, M. (2015). Complete nitrification by Nitrospira bacteria. *Nature*, *528*(7583), 504–509.

Daims, H., Lücker, S., & Wagner, M. (2016). A New Perspective on Microbes Formerly Known as Nitrite-Oxidizing Bacteria. *Trends in Microbiology*, *24*(9), 699–712.

Davidson, J., Good, C., Williams, C., & Summerfelt, S. T. (2017). Evaluating the chronic effects of nitrate on the health and performance of post-smolt Atlantic salmon Salmo salar in freshwater recirculation aquaculture systems. *Aquacultural Engineering*, *79*, 1–8.

Davidson, J., May, T., Good, C., Waldrop, T., Kenney, B., Terjesen, B. F., & Summerfelt, S. (2016). Production of market-size North American strain Atlantic salmon Salmo salar in a land-based recirculation aquaculture system using freshwater. *Aquacultural Engineering*, *74*, 1–16.

Eddy, F. B. (2005). Ammonia in estuaries and effects on fish. *Journal of Fish Biology*, *67*(6), 1495–1513.

Ekama, G., Wentzel, M., & van Loosdrecht, M. (2020). Nitrogen Removal. In G. Chen, M. van Loosdrecht, G. A. Ekama, & D. Brdjanovic (Eds.), *Biological wastewater treatment - principles, modeling and design* (2nd). IWA Publishing.

Ey. (2019). *The Norwegian Aquaculture Analysis 2019* (tech. rep.).

FAO. (2020). *The State of World Fisheries & Aquaculture 2020. Sustainability in action* (tech. rep.).

Fivelstad, S. (1988). Waterflow Requirements for Salmonids in Single-pass and Semi-closed Land-based Seawater and Freshwater Systems. *Aquacultural Engineering*, *7*(10), 183–185.

Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*, *8*(9), 623–633.

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: An emergent form of bacterial life. *Nature Reviews Microbiology*, *14*(9), 563–575.

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*, *532*(July 2020), 735973.

Gonzalez-Silva, B. M., Jonassen, K. R., Bakke, I., Østgaard, K., & Vadstein, O. (2016). Nitrification at different salinities: Biofilm community composition and physiological plasticity. *Water Research*, *95*, 48–58.

Gonzalez-Silva, B. M. (2016). *Salinity as a driver for microbial community structure in reactors for nitrification and anammox* (Doctoral dissertation). Norwegian University of Science & Technology.

Goode, C., & Allen, D. G. (2011). Effect of Calcium on Moving-Bed Biofilm Reactor Biofilms. *Water Environment Research*, *83*(3), 220–232.

Gutiérrez, X. A., Kolarevic, J., Takle, H., Baeverfjord, G., Ytteborg, E., & Fyhn Terjesen, B. (2019). Effects of chronic sub-lethal nitrite exposure at high water chloride concentration on Atlantic salmon (Salmo salar, Linnaeus 1758) parr. *Aquaculture Research*, *50*(9), 2687–2697.

Hagopian, D. S., & Riley, J. G. (1998). A closer look at the bacteriology of nitrification. *Aquacultural Engineering*, *18*(4), 223–244.

Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004). Bacterial biofilms: From the natural environment to infectious diseases.

Hatzenpichler, R. (2012). Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. *Applied and Environmental Microbiology*, *78*(21), 7501–7510.

He, H., Chen, Y., Li, X., Cheng, Y., Yang, C., & Zeng, G. (2017). Influence of salinity on microorganisms in activated sludge processes: A review. *International Biodeterioration and Biodegradation*, *119*, 520–527.

Hilker, M., Schwachtje, J., Baier, M., Balazadeh, S., Bäurle, I., Geiselhardt, S., Hincha, D. K., Kunze, R., Mueller-Roeber, B., Rillig, M. C., Rolff, J., Romeis, T., Schmülling, T., Steppuhn, A., van Dongen, J., Whitcomb, S. J., Wurst, S., Zuther, E., & Kopka, J. (2016). Priming and memory of stress responses in organisms lacking a nervous system. *Biological Reviews*, *91*(4), 1118–1133.

Hjeltnes, B., Baeverfjord, G., Erikson, U., Mortensen, S., Rosten, T., & Østergård, P. (2012). *Risk Assessment of Recirculation Systems in Salmonid Hatcheries* (tech. rep.). Vitenskapskomiteen for mattrygghet (VKM).

Holan, A. B., & Kolarevic, J. (2015). *Postsmoltproduksjon i resirkulert sjøvann på land* (tech. rep.). Nofima.

Hunik, J., Meijer, H., & Tramper, J. (1993). Kinetics of Nitrobacter agilis at extreme substrate , product and salt concentrations. *Applied and environmental microbiology*, *40*, 442–448.

Hüpeden, J., Wegen, S., Off, S., Lücker, S., Bedarf, Y., Daims, H., Kühn, C., & Spieck, E. (2016). Relative Abundance of Nitrotoga spp. in a Biofilter of a Cold-Freshwater Aquaculture Plant Appears To Be Stimulated by Slightly Acidic pH. *Applied and Environmental Microbiology*, *82*(6), 1838–1845.

Jenkins, D. E., Chaisson, S. A., & Matin, A. (1990). Starvation-induced cross-protection against osmotic challenge in Escherichia coli. *J. Bacteriol.*, *172*(5), 2779–2781.

Kamstra, A., Blom, E., & Terjesen, B. F. (2017). Mixing and scale affect moving bed biofilm reactor (MBBR) performance. *Aquacultural Engineering*, *78*, 9–17.

Karkman, A., Mattila, K., Tamminen, M., & Virta, M. (2011). Cold temperature decreases bacterial species richness in nitrogen-removing bioreactors treating inorganic mine waters. *Biotechnology and Bioengineering*, *108*(12), 2876–2883.

Keuter, S., Beth, S., Quantz, G., Schulz, C., & Eva, S. (2017). Longterm monitoring of nitrification and nitrifying communities during biofilter activation of two marine recirculation aquaculture systems (RAS). *International Journal of Aquaculture and Fishery Sciences*, *3*, 051–061.

Kinyage, J. P. H., Pedersen, P. B., & Pedersen, L. F. (2019). Effects of abrupt salinity increase on nitrification processes in a freshwater moving bed biofilter. *Aquacultural Engineering*, *84*, 91–98.

Koops, H.-P., Purkhold, U., Pommerening-Röser, A., Timmermann, G., & Wagner, M. (2006). The Lithoautotrophic Ammonia-Oxidizing Bacteria. *The prokaryotes* (pp. 778–811).

Kroupova, H., Machova, J., & Svobodova, Z. (2005). Nitrite influence on fish: A review. *Veterinarni Medicina*, *50*(11), 461–471.

Kruse, M., Keuter, S., Bakker, E., Spieck, E., Eggers, T., & Lipski, A. (2013). Relevance and Diversity of Nitrospira Populations in Biofilters of Brackish RAS. *PLoS ONE*, *8*(5), 1–9.

Lekang, O. I., Salas-Bringas, C., & Bostock, J. C. (2016). Challenges and emerging technical solutions in on-growing salmon farming. *Aquaculture International*, *24*(3), 757–766.

Letelier-Gordo, C. O., Aalto, S. L., Suurnäkki, S., & Bovbjerg, P. (2020). Increased sulfate availability in saline water promotes hydrogen sulfide production in fish organic waste. *Aquacultural Engineering*, *89*, 102062.

Li, C., Liang, J., Lin, X., Xu, H., Tadda, M. A., Lan, L., & Liu, D. (2019). Fast start-up strategies of MBBR for mariculture wastewater treatment. *Journal of Environmental Management*, *248*, 109267.

Liu, D., Li, C., Guo, H., Kong, X., Lan, L., Xu, H., Zhu, S., & Ye, Z. (2019). Start-up evaluations and biocarriers transfer from a trickling filter to a moving bed bioreactor for synthetic mariculture wastewater treatment. *Chemosphere*, *218*, 696–704.

Lücker, S., Schwarz, J., Gruber-Dorninger, C., Spieck, E., Wagner, M., & Daims, H. (2015). Nitrotoga-like bacteria are previously unrecognized key nitrite oxidizers in full-scale wastewater treatment plants. *ISME Journal*, *9*(3), 708–720.

Luo, W., Phan, H. V., Hai, F. I., Price, W. E., Guo, W., Ngo, H. H., Yamamoto, K., & Nghiem, L. D. (2016). Effects of salinity build-up on the performance and bacterial community structure of a membrane bioreactor. *Bioresource Technology*, *200*, 305–310.

Madigan, M. T., Bender, K. S., Buckley, D. H., Sattley, W. M., & Stahl, D. A. (2018). *Brock Biology of Microorganisms* (15th ed.). Pearson Education Limited.

Marine Harvest. (2018). *Salmon Farming Industry Handbook* (tech. rep.).

Mitchell, A., Romano, G. H., Groisman, B., Yona, A., Dekel, E., Kupiec, M., Dahan, O., & Pilpel, Y. (2009). Adaptive prediction of environmental changes by microorganisms. *Nature*, *460*(7252), 220–224.

Moran, D. (2010a). Carbon dioxide degassing in fresh and saline water. II: Degassing performance of an air-lift. *Aquacultural Engineering*, *43*(3), 120–127.

Moran, D. (2010b). Carbon dioxide degassing in fresh and saline water. II: Degassing performance of an air-lift. *Aquacultural Engineering*, *43*(3), 120–127.

Mortensen, A., & Damsgard, B. (1998). The effect of salinity on desmoltification in Atlantic salmon. *Aquaculture*, *168*, 407–411.

Moussa, M. S., Sumanasekera, D. U., Ibrahim, S. H., Lubberding, H. J., Hooijmans, C. M., Gijzen, H. J., & Van Loosdrecht, M. C. (2006). Long term effects of salt on activity, population structure and floc characteristics in enriched bacterial cultures of nitrifiers. *Water Research*, *40*(7), 1377–1388.

Navada, S., Knutsen, M. F., Bakke, I., & Vadstein, O. (2020). Nitrifying biofilms deprived of organic carbon show higher functional resilience to increases in carbon supply. *Scientific Reports*, *10*(1), 7121.

Nemergut, D. R., O'Neill, S. P., Bilinski, T. M., Stanish, L. F., Knelman, J. E., Wickey, P., Schmidt, S. K., Darcy, J. L., Lynch, R. C., Ferrenberg, S.,

& Fukami, T. (2013). Patterns and processes of microbial community assembly. *Microbiology and Molecular Biology Reviews*, *77*(3), 342–356.

Nijdam, D., Rood, T., & Westhoek, H. (2012). The price of protein: Review of land use and carbon footprints from life cycle assessments of animal food products and their substitutes. *Food Policy*, *37*(6), 760–770.

Nijhof, M., & Bovendeur, J. (1990). Fixed film nitrification characteristics in sea-water recirculation fish culture systems. *Aquaculture*, *87*(2), 133–143.

Noble, C., Gismervik, K., Iversen, M. H., Kolarevic, J., Nilsson, J., Stien, L. H., & Turnbull, J. F. (2018). *Welfare Indicators for farmed Atlantic salmon: tools for assessing fish welfare* (C. Noble, K. Gismervik, M. H. Iversen, J. Kolarevic, J. Nilsson, L. H. Stien, & J. F. Turnbull, Eds.).

Ødegaard, H., Rusten, B., & Westrum, T. (1994). A new moving bed biofilm reactor - applications and results. *Water Science and Technology*, *29*(10-11), 157–165.

Ohashi, A., Viraj de Silva, D. G., Mobarry, B., Manem, J. A., Stahl, D. A., & Rittmann, B. E. (1995). Influence of substrate C/N ratio on the structure of multi-species biofilms consisting of nitrifiers and heterotrophs. *Water Science and Technology*, *32*(8), 75–84.

Okabe, S., Hirata, K., & Watanabe, Y. (1995). Dynamic changes in spatial microbial distribution in mixed-population biofilms: Experimental results and model simulation. *Water Science and Technology*, *32*(8), 67–74.

Oren, A. (1999). Bioenergetic Aspects of Halophilism. *Microbiology and molecular biology reviews*, *63*(2), 334–348.

Oren, A. (2011). Thermodynamic limits to microbial life at high salt concentrations. *Environmental Microbiology*, *13*(8), 1908–1923.

Panswad, T., & Anan, C. (1999). Impact of high chloride wastewater on an anaerobic/anoxic/aerobic process with and without inoculation of chloride acclimated seeds. *Water Research*, *33*(5), 1165–1172.

Parker, R. W., Blanchard, J. L., Gardner, C., Green, B. S., Hartmann, K., Tyedmers, P. H., & Watson, R. A. (2018). Fuel use and greenhouse gas emissions of world fisheries. *Nature Climate Change*, *8*(4), 333–337.

Piedrahita, R. H. (2003). Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. *Aquaculture*, *226*(1-4), 35–44.

Pjevac, P., Schauberger, C., Poghosyan, L., Herbold, C. W., van Kessel, M. A., Daebeler, A., Steinberger, M., Jetten, M. S., Lücker, S., Wagner, M., & Daims, H. (2017). AmoA-targeted polymerase chain reaction primers for the specific detection and quantification of comammox Nitrospira in the environment. *Frontiers in Microbiology*, *8*, 1–11.

Prosser, J., Head, I., & Stein, L. (2014). The Family Nitrosomononadaceae. In E. Rosenberg, E. F. Delong, S. Lory, E. Stackebrandt, & F. Thompson (Eds.), *The prokaryotes* (4th, pp. 901–918). Springer.

Rillig, M. C., Rolff, J., Tietjen, B., Wehner, J., & Andrade-Linares, D. R. (2015). Community priming-Effects of sequential stressors on microbial assemblages. *FEMS Microbiology Ecology*, *91*(5), 1–7.

Rittmann, B. E., & McCarty, P. L. (2001). *Environmental biotechnology: Principles and Applications*. Tata McGraw Hill Education.

Roalkvam, I., Drønen, K., Dahle, H., & Inger, H. (2020). Comparison of active biofilm carriers and commercially available inoculum for activation of biofilters in marine recirculating aquaculture systems (RAS). *Aquaculture*, *514*, 734480.

Rud, I., Kolarevic, J., Holan, A. B., Berget, I., Calabrese, S., & Terjesen, B. F. (2017). Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. *Aquacultural Engineering*, *78*, 50–62.

Rusten, B., Eikebrokk, B., Ulgenes, Y., & Lygren, E. (2006). Design and operations of the Kaldnes moving bed biofilm reactors. *Aquacultural Engineering*, *34*(3), 322–331.

Sanchez, O., Aspe, E., Marti, M., & Roeckel, M. (2004). The effect of sodium chloride on the two-step kinetics of the nitrifying process. *Water Environment Research*, *76*(1), 73.

Santos, J. P., Mendes, D., Monteiro, M., Ribeiro, H., Baptista, M. S., Borges, M. T., & Magalhães, C. (2018). Salinity impact on ammonia oxidizers activity and amoA expression in estuarine sediments. *Estuarine, Coastal and Shelf Science*, *211*, 177–187.

Sauder, L. A., Engel, K., Stearns, J. C., Masella, A. P., Pawliszyn, R., & Neufeld, J. D. (2011). Aquarium nitrification revisited: Thaumarchaeota are the dominant ammonia oxidizers in freshwater aquarium biofilters. *PLoS ONE*, *6*(8).

Saur, T., Escudié, R., Santa-Catalina, G., Bernet, N., & Milferstedt, K. (2016). Conservation of acquired morphology and community structure in aged biofilms after facing environmental stress. *Water Research*, *88*, 164–172.

Schramm, A., De Beer, D., Gieseke, A., & Amann, R. (2000). Microenvironments and distribution of nitrifying bacteria in a membrane-bound biofilm. *Environmental Microbiology*, *2*(6), 680–686.

Schryver, P. D., & Vadstein, O. (2014). Ecological theory as a foundation to control pathogenic invasion in aquaculture. *The ISME Journal*, *8*, 2360–2368.

Searchinger, T., Waite, R., Beringer, T., Forslund, A., Guyomard, H., Le Mouël, C., Manceron, S., & Marajo-Petitzon, E. (2018). *Creating a sustainable food future* (tech. rep.). World Resources Institute. World Resources Institute.

Shade, A., Peter, H., Allison, S. D., Baho, D. L., Berga, M., Bürgmann, H., Huber, D. H., Langenheder, S., Lennon, J. T., Martiny, J. B., Matulich, K. L., Schmidt, T. M., & Handelsman, J. (2012). Fundamentals of microbial community resistance and resilience. *Frontiers in Microbiology*, *3*, 1–19.

Sharrer, M. J., Tal, Y., Ferrier, D., Hankins, J. A., & Summerfelt, S. T. (2007). Membrane biological reactor treatment of a saline backwash flow from a recirculating aquaculture system. *Aquacultural Engineering*, *36*(2), 159–176.

Shi, K., Zhou, W., Zhao, H., & Zhang, Y. (2012). Performance of halophilic marine bacteria inocula on nutrient removal from hypersaline wastewater in an intermittently aerated biological filter. *Bioresource Technology*, *113*, 280–287.

Sleator, R. D., & Hill, C. (2001). Bacterial osmoadaptation : the role of osmolytes in bacterial stress and virulence. *FEMS Microbiology Letters*, *26*, 49–71.

Smith, P., Bustamante, M., Ahammad, H., Clark, H., Dong, H., Elsiddig, E. A., Haberl, H., Harper, H., House, J., Jafari, M., Masera, O., Mbow, C., Ravindranath, N. H., Rice, C. W., Robledo Abad, C., Romanovskaya, A., Sperling, F., & Tubiello, F. (2014). Agriculture, Forestry and Other Land Use (AFOLU) Coordinating Lead Authors: Lead Authors. In O. Edenhofer, R. Pichs-Madruga, Y. Sokona, J. C. Minx, E. Farahani, S. Kadner, K. Seyboth, A. Adler, I. Baum, S. Brunner, P. Eickmeier, B. Kriemann, J. Savolainen, S. Schlomer, C. von Stechow, & T. Zwickel (Eds.), *Climate change 2014: Mitigation of climate change. contribution of working group iii to the fifth assessment report of the intergovernmental panel on climate change*. Cambridge University Press.

Stieglmeier, M., Alves, R., & Schleper, C. (2014). The Phylum Thaumarchaeota. In E. Rosenberg, E. F. Delong, S. Lory, E. Stackebrandt, & F. Thompson (Eds.), *The prokaryotes* (4th ed., pp. 348–362). Springer.

Sudarno, U., Winter, J., & Gallert, C. (2011). Effect of varying salinity, temperature, ammonia and nitrous acid concentrations on nitrification of saline wastewater in fixed-bed reactors. *Bioresource Technology*, *102*(10), 5665–5673.

Sudarno, U. (2011). *Nitrification in Fixed Bed Reactors Treating Saline Wastewater* (Doctoral dissertation). Karlsruhe Institute of Technology.

Sudarno, U., Bathe, S., Winter, J., & Gallert, C. (2010). Nitrification in fixed-bed reactors treating saline wastewater. *Applied Microbiology and Biotechnology*, *85*, 2017–2030.

Summerfelt, S. T., Mathisen, F., Holan, A. B., & Terjesen, B. F. (2016). Survey of large circular and octagonal tanks operated at Norwegian commercial smolt and post-smolt sites. *Aquacultural Engineering*, *74*, 105–110.

Terjesen, B. F., Rosten, T. W., & Ulgenes, Y. (2013a). *Betydning av vannmiljøet ved produksjon av laksefisk i lukkede systemer i sjø* (tech. rep.).

Terjesen, B. F., Ytrestøyl, T., Kolarevic, J., & Calabrese, S. (2013b). Effects of water salinity and exercise on Atlantic salmon performance as postsmolts in land-based closed- containment systems. *Aquaculture Innovation Workshop No. 6, Vancouver, British Columbia October 27-28, 2014*.

Thorarensen, H., & Farrell, A. P. (2011). The biological requirements for post-smolt Atlantic salmon in closed-containment systems. *Aquaculture*, *312*(1-4), 1–14.

Timmons, M., & Ebeling, J. (2010). *Recirculating Aquaculture* (3rd). Ithaca Publishing Company LLC.

Torun, F., Hostins, B., Teske, J., De Schryver, P., Boon, N., & De Vrieze, J. (2020). Nitrate amendment to control sulphide accumulation in shrimp ponds. *Aquaculture*, *521*.

Vadstein, O., Attramadal, K. J., Bakke, I., & Olsen, Y. (2018). K-selection as microbial community management strategy: A method for improved viability of larvae in aquaculture. *Frontiers in Microbiology*, *9*, 1–17.

Vendramel, S., Dezotti, M., & Sant'Anna, G. L. (2011). Nitrification of an industrial wastewater in a moving-bed biofilm reactor: Effect of salt concentration. *Environmental Technology*, *32*(8), 837–846.

Vyrides, I. (2015). Anaerobic treatment of organic saline waste/wastewater: Overcome salinity inhibition by addition of compatible solutes. In L. Behari, S. Nilotpala, P. Sandeep, P. Barada, & K. Mishra (Eds.), *Environmental microbial biotechnology*.

Vyrides, I., & Stuckey, D. C. (2017). Compatible solute addition to biological systems treating waste/wastewater to counteract osmotic and other environmental stresses: a review. *Critical Reviews in Biotechnology*, *37*(7), 865–879.

Wang, X., Andresen, K., Handa, A., Jensen, B., Reitan, K., & Olsen, Y. (2013). Chemical Composition and release rate of waste discharge from atlantic salmon farm. *Aquaculture Environment Interactions*, *4*, 147–162.

Wang, X., Olsen, L., Reitan, K., & Olsen, Y. (2012). Discharge of nutrient wastes from salmon farms: environmental effects, and potential for integrated multi-trophic aquaculture. *Aquaculture Environment Interactions*, *2*(3), 267–283.

Wang, Z., Loosdrecht, M. C. M. V., & Saikaly, P. E. (2017). Gradual adaptation to salt and dissolved oxygen : Strategies to minimize adverse effect of salinity on aerobic granular sludge. *Water Research*, *124*, 702–712.

Wang, Z., Gao, M., She, Z., Wang, S., Jin, C., Zhao, Y., Yang, S., & Guo, L. (2015). Effects of salinity on performance, extracellular polymeric substances and microbial community of an aerobic granular sequencing batch reactor. *Separation and Purification Technology*, *144*, 223–231.

Ward, B. B., Eveillard, D., Kirshtein, J. D., Nelson, J. D., Voytek, M. A., & Jackson, G. A. (2007). Ammonia-oxidizing bacterial community composition in estuarine and oceanic environments assessed using a functional gene microarray. *Environmental Microbiology*, *9*(10), 2522–2538.

Wittebolle, L., Verstraete, W., & Boon, N. (2009). The inoculum effect on the ammonia-oxidizing bacterial communities in parallel sequential batch reactors. *Water Research*, *43*(17), 4149–4158.

Ytrestøyl, T., Takle, H., Kolarevic, J., Calabrese, S., Timmerhaus, G., Rosseland, B. O., Teien, H. C., Nilsen, T. O., Handeland, S. O., Stefansson, S. O., Ebbesson, L. O., & Terjesen, B. F. (2020). Performance and welfare of Atlantic salmon, Salmo salar L. post-smolts in recirculating aquaculture systems: Importance of salinity and water velocity. *Journal of the World Aquaculture Society*, *51*(2), 373–392.

Part II

Compilation of Articles

Article I

Journal of Cleaner Production 238 (2019) 117835

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

Influence of rate of salinity increase on nitrifying biofilms

Sharada Navada [a, b, *], Olav Vadstein [c], Ann-Kristin Tveten [d], Gerhardus C. Verstege [e], Bendik Fyhn Terjesen [f, 1], Vasco C. Mota [f], Vishwesh Venkataraman [a], Frédéric Gaumet [b], Øyvind Mikkelsen [a], Andries Kamstra [g]

[a] Department of Chemistry, NTNU - Norwegian University of Science and Technology, N-7491, Trondheim, Norway
[b] Krüger Kaldnes AS (Veolia Water Technologies), N-3241, Sandefjord, Norway
[c] Department of Biotechnology and Food Science, NTNU - Norwegian University of Science and Technology, N-7491, Trondheim, Norway
[d] Department of Biological Sciences, NTNU - Norwegian University of Science and Technology, N-6009, Ålesund, Norway
[e] Aquaculture and Fisheries Group, Wageningen University, 6700, AH Wageningen, the Netherlands
[f] Nofima AS, N-9291, Tromsø, Norway
[g] Kamstra Consult, 2064, WH Spaarndam, the Netherlands

ARTICLE INFO

Article history:
Received 29 May 2019
Received in revised form
18 July 2019
Accepted 29 July 2019
Available online 29 July 2019

Handling Editor: Panos Seferlis

Keywords:
Salt acclimatization
Osmotic stress adaptation
Marine recirculating aquaculture systems (RAS) biofilter
Microbial community resistance and resilience
Atlantic salmon post-smolt
Candidatus nitrotoga

ABSTRACT

A strategy for rapid increase in salinity with minimal impact on nitrification is important for ammonia removal from saline effluents, especially in recirculating aquaculture systems with high water reuse. To study the influence of the rate of salinity increase on nitrification, continuously operated moving bed biofilm reactors were transferred from freshwater (0‰ salinity) to seawater (32‰ salinity) at five different rates of salinity change: 0 (control), 1, 2, 6, and 15‰ day^{-1}. Each daily change was conducted gradually overnight. The results showed that at salinities higher than 4–8‰, the ammonia oxidation capacity decreased linearly with salinity and reduced by 50–90% upon complete seawater transfer, with the greatest reduction in the 1‰ day^{-1} treatment. Thereafter, it increased linearly with time, with little difference between treatments. Overall, the biofilm microbial communities in the control and the 15‰ day^{-1} treatment were highly similar, while those in the other treatments shifted significantly with time and had greater species diversity, richness, and evenness of nitrifiers. *Candidatus Nitrotoga* was the dominant nitrite oxidizing bacteria in all treatments throughout the study, indicating that this recently discovered group may tolerate salinities up to 32‰. The results suggest that although the rate of salinity increase influences the microbial community composition, it only weakly influences ammonia oxidation capacity, which mainly depends on salinity and seawater acclimatization time. Therefore, for rapid seawater acclimatization of freshwater nitrifying biofilms, increasing the salinity continuously in two days may be a better strategy than increasing the salinity over a month, provided an initial decrease in ammonia oxidation is acceptable. The findings can aid in the shift from net-pen fish farming to recirculating aquaculture systems, thereby lowering the ecological impacts of seafood production.

1. Introduction

The biological process of nitrification is commonly used for ammonia removal in a wide variety of applications, including industrial, municipal, and agricultural wastewater treatment. Nitrification can be negatively impacted by salinity variations (Lay et al., 2010; Moussa et al., 2006; Wang et al., 2017). This is of special concern in recirculating aquaculture systems (RAS); land-based fish production systems that include nitrification units for the removal of ammonia produced by the fish. Intensive RAS are viewed as a sustainable solution to the rising global seafood demand, as they use much lesser water than flow-through production systems and can have a lower ecological impact than marine fisheries, where 10% of the catch is discarded (Zeller et al., 2018). Anadromous fish such as Atlantic salmon (*Salmo salar*) are typically grown in freshwater (~0‰ salinity) during the young life stages of the fish (parr), and in the later growth stages (post-smolt), in brackish water or seawater (10–22‰ and 32‰ salinities, respectively) (Davidson et al., 2016). The latter phase is typically carried out in net-pens

* Corresponding author. Department of Chemistry, NTNU - Norwegian University of Science and Technology, N-7491, Trondheim, Norway.
E-mail address: sharada.navada@ntnu.no (S. Navada).
[1] Present Address: Cermaq Group AS, N-0102, Oslo, Norway

https://doi.org/10.1016/j.jclepro.2019.117835

that discharge nutrient and toxic waste directly into the sea (Ayer and Tyedmers, 2009), and also increase the sea lice parasitic pressures, thereby harming migrating wild salmon smolts and the marine ecosystem. The shift to post-smolt production from net-pens to RAS is challenged by the requirement for increasing the salinity, which can reduce nitrification efficiency, leading to toxic ammonia and nitrite accumulation (Kinyage et al., 2019; Nijhof and Bovendeur, 1990). Besides RAS, salinity increase strategies may also be required for industrial bioreactors when only freshwater inoculum is available. Additionally, as seawater bioreactors require a longer startup period than freshwater bioreactors, nitrifying bioreactors are typically started in freshwater and later acclimatized to increasing salinity (Chen et al., 2006; Nijhof and Bovendeur, 1990). Therefore, it is important to develop an optimal procedure for increasing the salinity in nitrifying freshwater bioreactors, with the least possible impact on the nitrification activity.

In aerobic nitrifying processes, two distinct microbial guilds are known to co-exist: ammonia oxidizing bacteria (AOB) or archaea, which convert ammonia to nitrite; and nitrite oxidizing bacteria (NOB), which convert nitrite to nitrate. Recently, microorganisms capable of complete ammonia oxidation to nitrate have also been discovered (Daims et al., 2015). The negative impact of salinity increase on nitrification is usually attributed to the dehydration or plasmolysis of microbes, or a reduction in cell activity (Csonka, 1989; Madigan et al., 2018; Uygur and Kargi, 2004). If the hyperosmotic shock is not too severe, the bacteria may be temporarily inhibited but thereafter, adapt to the increased external osmotic pressure by producing compatible solutes (Csonka, 1989; Moussa et al., 2006). Alternatively, the microbial community composition may alter due to changed selection pressure and consequential succession, and thereby adapt to the new environmental conditions. The response of bacteria under disturbances may depend on the intensity and duration of the disturbance (Shade et al., 2012); in this case, the magnitude and rate of salinity change.

Nitrification may be influenced by the manner in which the salinity is changed – as a shock dose or gradual change (Moussa et al., 2006). Freshwater bioreactors subjected to a shock change to seawater show a drastic reduction in nitrification, although they start recovering after a few days (Gonzalez-Silva, 2016; Nijhof and Bovendeur, 1990). Conversely, although adaption to a gradual increase in salinity is possible with almost no decrease in nitrification, it can take several days or months (Bassin et al., 2012, 2011; Sharrer et al., 2007). Inoculation with saltwater acclimated seeds may speed up adaptation to salinity (Cui et al., 2016; Panswad and Anan, 1999; Shi et al., 2012; Sudarno et al., 2010), but is not always easily available, and can pose a biosecurity risk to the fish in RAS. As far as we know, no protocol exists for increasing the salinity in non-inoculated freshwater bioreactors within a short time-span, while maintaining an acceptable nitrification efficiency throughout.

Although several studies have reported the impact of salinity changes on nitrification (Bassin et al., 2011; Cortes-Lorenzo et al., 2015; Cui et al., 2016; Gonzalez-Silva et al., 2016; Kinyage et al., 2019; Sudarno, 2011), none have compared different rates of salinity change. Bassin et al. (2012) demonstrated that small increments in salinity had a lower negative impact on nitrification than a large one-step increase, but both the strategies tested had the same overall rate of salinity change (0‰ to 20‰ salinity in 108 days). To the best of our knowledge, the effect of different salinity increase rates on nitrification is not well studied, including whether the adaptation process is a physiological adaptation or a succession with changes in the species inventory of nitrifying microbes. Therefore, it is not clear which salinity change strategy can achieve better nitrification within the same time: small salinity increments over a long period, or large salinity increments followed by an acclimatization period. The objective of this study was to compare the impact of salinity increase rate on nitrification and microbial communities in moving bed biofilm reactors (MBBRs) transferred from freshwater to seawater. We hypothesized that 1) the nitrification activity would be better maintained under smaller salinity increments and; 2) microbial community composition would be influenced by the rate of salinity change.

2. Materials and methods

2.1. Experimental setup

The study was conducted at the Nofima Centre for Recirculation in Aquaculture (NCRA) in Sunndalsøra, Norway. The experimental setup consisted of ten continuously operated plastic MBBRs, with 37 L water volume each (45 cm × 35 cm x 40 cm). Five treatments were run in duplicate: C (control), S1, S2, S6, and S15 with salinity increase rates of 0, 1, 2, 6, and 15‰ day^{-1}, respectively (Fig. 1). Salinity change was started at the end of day 0. The experiment was conducted at 12.2 ± 0.3 °C and pH 7.9 ± 0.1 for 41 days. Two weeks prior to the start of the experiment, the reactors were filled with freshwater and mature biofilm carriers (AnoxK™ Chip P, Krüger Kaldnes AS, Norway) with a specific surface area of 900 $m^2 m^{-3}$ (~35% by volume). To minimize reactor bias, the biomedia were intermixed and redistributed to the reactors five days before commencing the experiment. The biomedia were sourced from the third MBBR chamber of NCRA's freshwater Atlantic salmon smolt RAS, Grow-out Hall 1 (Terjesen et al., 2013). This RAS MBBR had been operated in freshwater at 12 °C and pH 7.2 for several months prior to the experiment and had never been exposed to seawater before.

The experimental MBBRs were randomly distributed into two temperature-controlled water baths, with one control treatment reactor in each (Fig. 2). The temperature in each water bath was controlled using a thermostat (TRD, Schego, Germany), a heater (Titanium tube 600 W, Schego, Germany), and continuous cold freshwater flow. Each MBBR was aerated with an air blower (MSB-2-355/102-220T, Ventur Tekniska, Sweden) via an air diffuser. The air flow rate was 51 ± 5 NL min^{-1}, which ensured uniform mixing of the carriers and provided oxygen for nitrification (dissolved oxygen saturation > 70%). The freshwater and seawater water sources to the facility were pre-treated (Terjesen et al., 2013). Briefly, the freshwater was pumped from bore wells, treated with silicate and degassed, and the seawater was filtered and UV-irradiated. The two

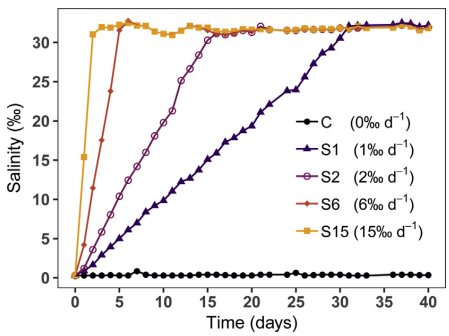


Fig. 1. Experimental design with salinity for the different treatments in ‰ (parts per thousand). The control treatment (C) was always operated in freshwater. Treatments S1, S2, S6, and S15 were transferred from freshwater (0‰) to seawater (32‰) at salinity increase rates of 1, 2, 6, and 15‰ d^{-1}, respectively.

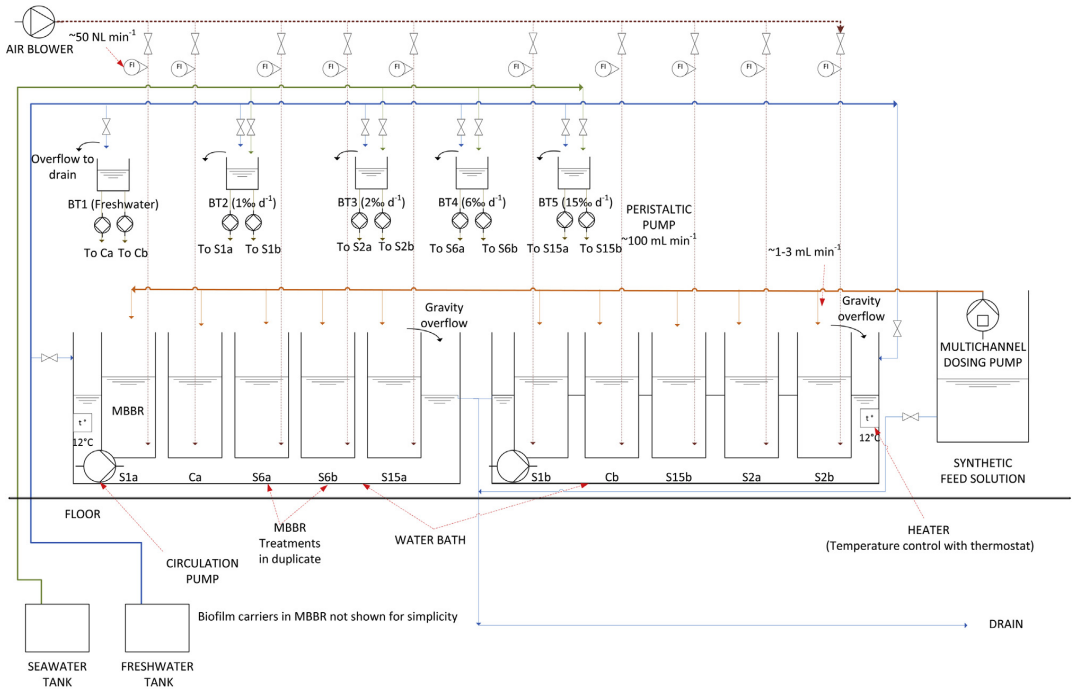


Fig. 2. Schematic diagram of the experimental setup. Continuously operated MBBRs with five treatments in duplicate, placed in temperature-controlled water baths. Treatments S1, S2, S6, and S15 were transferred from freshwater to seawater at salinity increase rates of 1, 2, 6, and 15‰ d^{-1}, respectively (duplicates denoted by suffixes 'a' and 'b'). The control treatment (C) was always operated in freshwater (0‰ salinity). The salinity in each treatment was changed by controlling the salinity in the respective buffer tank (BT) by adjusting the freshwater and seawater flows.

water sources were continuously mixed at the desired ratio in five 2 L buffer tanks, and this makeup water was supplied to the duplicate reactors of each treatment using peristaltic pumps (WPX1-P1/8 L2, Welco, Japan). The treatment salinity was changed by adjusting the flows of freshwater and seawater to these buffer tanks. The MBBR makeup flow rate was 101 ± 5 mL min^{-1}, corresponding to a hydraulic retention time of 6 h. The sampling and analyses were conducted every morning. Salinity changes in the buffer tanks were performed at the end of the day, thereby increasing the MBBR salinity gradually overnight before the next sampling.

A synthetic feed solution was prepared in a 250 L tank with freshwater and was supplied to each MBBR using a multichannel pump (520Du Pump/505CA pump head, Watson-Marlow, England). This solution had an ammonia concentration of 736 ± 85 mgN L^{-1} as $(NH_4)_2SO_4$ and contained the following nutrients per mgN L^{-1} of ammonia: 11.4 mg L^{-1} $CaCO_3$ as $NaHCO_3$, 0.1 mg L^{-1} Mg as $MgSO_4$, 0.1 mg L^{-1} orthophosphate-P as $Na_2HPO_4.12H_2O$, and 0.003 mg L^{-1} Fe as $FeCl_3.6H_2O$ (adapted from (Zhu et al., 2016)). The initial ammonia loading rate to each reactor was 0.23 gN m^{-2} d^{-1}, which is in the design range for RAS (Rusten et al., 2006; Terjesen et al., 2013). In certain periods, the feed flow rate was reduced by ~30% in some treatments to maintain the effluent ammonia concentration in the relevant range for RAS (Table 1).

For each reactor, the system parameters were measured daily in the reactors using a handheld multimeter (Multi 3630, WTW, Germany) with sensors for pH and temperature (SenTix® 940–3, WTW, Germany), dissolved oxygen (FDO® 925–3, WTW, Germany), and salinity (TetraCon® 925–3, WTW, Germany). Air flow rate was measured with rotameters (VA A-8RR, Kytola®, Finland).

2.2. Nitrification performance

The nitrification performance was gauged by the *in situ* ammonia oxidation rate ($AOR_{in\ situ}$), the ammonia oxidation capacity or maximum ammonia oxidation rate (AOR_{max}), and the effluent nitrite concentration. $AOR_{in\ situ}$ was calculated for each MBBR as the difference of the influent and the effluent ammonia mass flow rates, normalized to the total protected surface area of the biofilm carriers. The water quality in the MBBR was taken to be the same as that of the MBBR effluent, as the reactors were completely mixed. Pseudo-steady state over 24 h was assumed. $AOR_{in\ situ}$ was expected to depend on the ammonia concentration (first-order reaction), as the MBBRs were operated at low effluent ammonia concentrations typical in RAS. Water samples of the MBBR effluent and the feed solution were collected daily in 20 mL scintillation vials (PE, Wheaton Industries, USA) and preserved at −20 °C. The ammonia concentration in the thawed samples was analyzed using a flow injection Autoanalyzer (Flow Solution IV, OI Analytical, College Station, TX, USA) using the salicylate method, as per U.S. EPA method 350.1 (U.S. EPA, 1983). The method detection limit was 0.05 mgN L^{-1}. Different calibration standards were used for each salinity range: 0, 5, 10, 15, 20, 25, 28, and 32‰.

To determine the maximum ammonia oxidation rate (AOR_{max}, zero-order reaction), capacity tests were conducted. These tests

Table 1
Periods of normal and low ammonia loading rates for the different treatments, along with the corresponding effluent ammonia concentration (minimum − maximum) during those periods.

Ammonia loading rate (gN m^{-2} d^{-1})	Normal (0.21 ± 0.05)		Low (0.08 ± 0.04)	
Treatment	Experimental days	NH_4^+-N (mgN L^{-1})	Experimental days	NH_4^+-N (mgN L^{-1})
Control	0–40	0.10–0.54	NA	NA
S1	0–27	0.01–9.79	28–40	0.57–2.73
S2	0–40	0.10–6.09	NA	NA
S6	0–5, 15–40	0.10–6.41	6–14	0.20–1.34
S15	0, 20–40	0.18–5.93	1–19	0.24–1.30

NA: Not applicable.

were performed at salinity increases of 3–7‰ for S1 and S2, at all different salinities for S6 and S15, and every 7–10 days for the control and the treatments after seawater transfer. For each capacity test, the MBBR was run in batch mode by removing the reactor inlets, and 0–220 mL of synthetic feed solution was added to the reactor to achieve an initial ammonia concentration of 4–5 mgN L^{-1} in the MBBR. Water samples were collected from the reactor every 5–20 min for about 1–4 h. These samples were also frozen to −20 °C and later analyzed in the Autoanalyzer to determine the ammonia concentration.

The nitrite concentration in the MBBRs was measured using powder pillows (method HI 93707) and a photometer (C203 2008, Hanna Instruments, Canada) for the first ten days. For the remainder of the study, nitrite was measured using a test kit (APHA, 1992) and a spectrophotometer (PhotoLab 6100 VIS, WTW, Germany). This method was less time-consuming, and more samples could be analyzed concurrently. The method detection limit was 0.02 mgN L^{-1}.

2.3. Microbial community analyses

Before each capacity test, three biofilm carriers were collected from each MBBR and preserved at −80 °C until analyses. In the lab, 10 × 20 mm pieces were cut out from the thawed carriers and placed into 1.5 mL tubes containing ATL buffer (Qiagen®, Netherlands). Biofilm was detached in a Qiagen® Tissuelyser II (30hz s^{-1}, 10 min) and DNA was extracted using Qiagen® DNeasy blood and tissue kit. The biofilm samples were centrifuged at 2500 rpm for 10 min, and Proteinase K was added before overnight incubation. After lysis, spin-column DNA purification was conducted, followed by two-step elution with 80 and 40 μL AE buffer. For quality control and to optimize PCR amplification, DNA yield in the eluate was determined by Qubit™ 3.0 (Invitrogen, Thermo Fisher Scientific, USA) using Qubit™ dsDNA BR assay kit.

PCR amplification and purification of amplified products was performed with Ion 16S™ Metagenomics Kit (Life Technologies, Thermo Fisher Scientific, USA) using 6 μL template. The amplification products were purified by Mag-Bind® TotalPure NGS (Omega Bio-Tek, USA). Gel electrophoresis was performed as a quality control step to ensure the presence of DNA amplification products. For quality control, DNA amplicon concentration was measured by Qubit™ 3.0 and Qubit™ dsDNA HS assay kit. Samples were diluted to obtain 50 ng in 79 μL for library preparation. Libraries were prepared using Ion Plus Fragment Library kit (Ion Torrent™, Thermo Fischer Scientific, USA) and Ion Xpress™ Barcode Adapters 1–44. Barcoded libraries were controlled with Bioanalyzer (Agilent Technologies, USA) and Agilent High Sensitivity DNA Kit, before being diluted to a concentration of 100 pM and amplified onto ion sphere particles (ISP) by emulsion PCR. Enriched ISPs were sequenced on Ion PGM™ using Ion PGM™ Hi-Q™ View Sequencing Kit according to manufacturer's protocol.

2.4. Data analysis and statistics

2.4.1. Physicochemical parameters

AOR_{max} on a given day was calculated by performing linear regression on the combined ammonia concentration vs time data from the capacity tests of each treatment (both duplicates). The points used for linear regression had an ammonia concentration greater than 0.5 mgN L^{-1} and at least a 2% difference from the following sample. The Autoanalyzer malfunctioned during the analyses of capacity tests S15-day 11, S6-day 13, and S1-day 28 (duplicate B) and therefore, these data were excluded from the analyses. For each capacity test, the Shapiro-Wilk test and q-q plots were used to check for normality of the residuals ($\alpha = 0.05$) and potential outliers, and measurement errors outside the plausible range were removed ($[NH_4^+\text{-N}] > 7.5$ mgN L^{-1}, 5 data points). A minimum of eight data points was used for each regression. Linear regression was also performed on: a) AOR_{max} vs salinity (during transfer from freshwater to seawater) and, b) AOR_{max} vs days after complete seawater transfer. The slopes of the regression lines were compared in R (V3.5.2) using analysis of covariance (ANCOVA), wherein differences were considered significant at $p < 0.05$ (Fox and Weisberg, 2011). For comparisons with the control, the treatment AOR_{max} on a given day was compared with the two nearest controls. All physicochemical parameters are reported as mean ± standard deviation; while calculated variables (such as AOR_{max}) are reported as mean ± standard error.

2.4.2. Microbial analysis

Raw sequencing data were analysed in Ion Reporter™ software using the Metagenomics 16s w1.1 workflow (Thermo Fisher Scientific, USA) with QIIME as an integrated software. The software uses the Curated MicroSEQ® 16S Reference Library v2013.1 combined with the Greengenes database for sequence identification. Workflow parameters: detecting primers at both ends, read length filters of 120 bp after trimming primers, 2 unique reads to be valid, 90% minimum alignment coverage, genus cut-off 97%. Ion Reporter™ assembles amplicon fragments to a consensus strain covering all 1500bp of the 16S rRNA gene. Results were obtained as individual amplicons from each of the seven variable regions (V2-4, V6-9) or as consensus strain with assigned operational taxonomic units (OTU) on family, genus and species level, which were subsequently aligned to generate an OTU table. The OTU table was filtered to remove cyanobacteria and normalized to the sum of sample reads. OTUs with a maximum of less than 0.1% in any sample were filtered out. The resulting data was analysed by calculating the α-diversity (first order Hill number (Hill, 1973)), richness, evenness, and relative abundance of nitrifying OTUs in individual samples. Ordination was performed using principal coordinates analysis (PCoA) to compare samples based on Bray-Curtis similarities (β-diversity). Data analysis was performed in R (V3.5.2) using packages phyloseq and vegan (McMurdie and Holmes, 2013; Oksanen et al., 2019).

3. Results

3.1. AOR_{max} during transfer from freshwater to seawater

The ammonia oxidation capacity (AOR_{max}) in the freshwater control varied during the study, especially, on days 0 and 40, when the AOR_{max} was approximately 25% lower compared to the rest of the experimental period (Fig. 3). Overall, the control had an average AOR_{max} of 0.37 ± 0.07 gN m^{-2} d^{-1} and the percent changes in AOR_{max} are reported relative to this value. During the transfer from freshwater to seawater (32‰ salinity), AOR_{max} showed a negative linear correlation with salinity for S1, S2, and S6 (Table 2). Moreover, the slope of AOR_{max} vs salinity did not differ significantly between treatments ($p = 0.24$) and had a weighted mean value of 9.7 ± 1.4 mgN m^{-2} d^{-1} $‰^{-1}$ (Table 2, Fig. 4A). At salinities up to 12‰, AOR_{max} in the treatments was not significantly lower than in the control. AOR_{max} reduced significantly when each treatment reached seawater salinity (Fig. 4A). Treatment S1 had the lowest AOR_{max} among all the treatments at 0.03 ± 0.02 gN m^{-2} d^{-1} (~90% reduction). In comparison, AOR_{max} in both S2 and S15 was 25–30% of the control average, whereas S6 had the highest AOR_{max} among all treatments at 0.18 ± 0.03 gN m^{-2} d^{-1} (~50% reduction).

3.2. AOR_{max} after complete seawater transfer

After complete seawater transfer, the AOR_{max} showed a marginally significant linear increase with acclimatization time in seawater. The recovery rate was quantified as the slope of the regression line between AOR_{max} and days after seawater transfer. The recovery rates of the treatments were not significantly different, except between S6 and S15. Therefore, regression was performed on the combined data from all treatments, showing that AOR_{max} increased with the acclimatization time at a rate of 5.3 ± 0.9 mgN m^{-2} d^{-2} (Table 3, Fig. 4B). At the end of the 41-day study, AOR_{max} in S6 and S15 was not significantly different from that in the control. Further, AOR_{max} in S15 was the highest among all treatments (0.33 ± 0.01 gN m^{-2} d^{-1} ~ 90% of the control average), while S1 had the lowest (0.11 ± 0.01 gN m^{-2} d^{-1} ~ 30% of the control average) (Fig. 3).

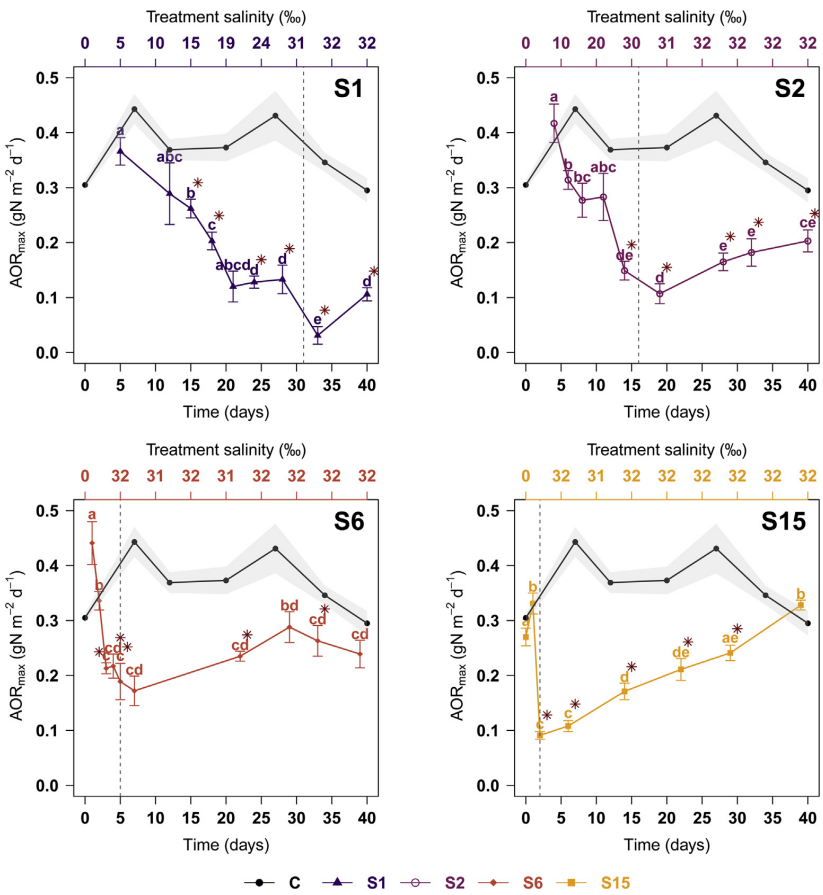


Fig. 3. Maximum ammonia oxidation rate (AOR_{max}) for treatments S1 (1‰ d^{-1}), S2 (2‰ d^{-1}), S6 (6‰ d^{-1}), and S15 (15‰ d^{-1}), compared to the freshwater control C (0‰ d^{-1}). Error bars and grey shaded region indicate standard errors for the treatment and the control, respectively. Data with an asterisk (*) are significantly different from the two nearest control data points ($p < 0.05$). Within each treatment, data with no letters in common are significantly different. The dotted line on each graph indicates the day on which the treatment was completely transferred to seawater.

Table 2
Linear regression on AOR_{max} vs salinity during salinity increase from freshwater to seawater, for each individual treatment and for all treatments. Note that for S1, S2 and S6, AOR_{max} was first measured at salinities 5, 8, and 4‰, respectively, and not at 0‰. Correlations were considered significant at $p < 0.05$ and are denoted by an asterisk (*).

Treatment	Decrease in AOR_{max} with salinity ± SE (mgN m^{-2} d^{-1} ‰$^{-1}$)	df	*p*	Adjusted R^2
S1	12.9 ± 1.8	6	0.0004*	0.88
S2	11.1 ± 2.3	4	0.008*	0.82
S6	9.0 ± 2.2	3	0.03*	0.80
S15	5.9 ± 5.5	1	0.5	0.07
All	**9.7 ± 1.4**	**20**	**0.000001***	**0.70**

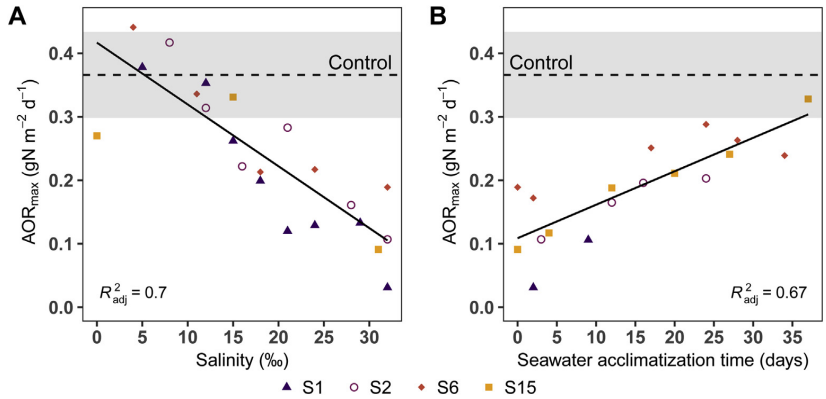


Fig. 4. Linear regression analyses on AOR_{max} from all treatments showing the correlation between A) AOR_{max} and salinity and B) AOR_{max} and seawater acclimatization time. The dashed line and the shaded region represent the average control AOR_{max} and its standard deviation, respectively.

Table 3
Linear regression on AOR_{max} vs days after seawater transfer for treatments S2, S6, S15, and all treatments (treatment S1 not shown as it had only two data points). The recovery rate after complete seawater transfer is measured as the slope of the regression line. Correlations were considered significant at $p < 0.05$ and are denoted by an asterisk (*).

Treatment	AOR_{max} recovery rate ± SE (mgN m^{-2} d^{-2})	df	*p*	Adjusted R^2
S2	4.7 ± 1.2	2	0.055	0.84
S6	2.6 ± 1.0	4	0.057	0.54
S15	6.0 ± 0.5	4	0.0002*	0.97
All	**5.3 ± 0.9**	**16**	**0.00002***	**0.67**

3.3. In situ ammonia oxidation rate and nitrite concentration

In S1 and S2, $AOR_{in\ situ}$ remained at the control level until approximately 20‰ salinity, after which it declined as the salinity increased further (Fig. 5A). $AOR_{in\ situ}$ in each treatment decreased significantly when the treatment reached seawater. Throughout the study, the freshwater control had a steady $AOR_{in\ situ}$ of 0.23 ± 0.01 gN m^{-2} d^{-1}, which was nearly equal to the ammonia loading rate to the MBBR. After a few days in seawater, $AOR_{in\ situ}$ in all treatments (except S1, which had low ammonia loading) increased, reaching 80–90% of the control $AOR_{in\ situ}$ in the final week.

Overall, the nitrite concentration in S15 was the highest, followed by S6, S2, and S1 (Fig. 5B). In S2, S6, and S15, nitrite was relatively high in the last week of the study (0.5–1.4 mgN L^{-1}) compared to the control (0.12–0.34 mgN L^{-1}), even though AOR_{max} had significantly recovered. The nitrite concentration in S1 was low and relatively stable throughout the study (0.07–0.38 mgN L^{-1}).

3.4. Microbial community analyses

Out of the 1371 OTUs sequenced, 29 were identified as nitrifying bacteria. Of these, 20 OTUs were present at relative abundance greater than 0.1%. The ammonia oxidizing bacteria (AOB) detected at the genus (species) level were *Nitrosomonas (N. cryotolerans, N. eutropha, N. marina, N. oligotropha, N.* sp., *N. ureae)*, *Nitrosospira (N. multiformis, N.* sp.*)*, and *Nitrosovibrio (N. tenuis)*; and the nitrite oxidizing bacteria (NOB) were *Candidatus Nitrotoga (nitrotoga)*, *Nitrospira (N. marina, N. moscoviensis, N. nitrospira, N.* sp.*)*, and *Nitrobacter (N. hamburgensis, N. vulgaris)*. Overall, the nitrifying OTUs constituted less than 51% of the community in all samples, while the rest were likely heterotrophic bacteria (Fig. 6). In the control, the proportion of nitrifiers increased over time. The α-diversity (first order Hill number) of the nitrifiers was significantly higher in S1-6 (9.3 ± 0.3) than in the control and S15 (5.7 ± 0.4). Evenness of the nitrifiers was also significantly higher in S1-6 (0.58 ± 0.01) than in the control and S15 (0.48 ± 0.03). The same trend was observed in richness. *Nitrosomonas* was the dominant AOB in the control and S15, while in S1-6, *Nitrosospira* was more abundant than *Nitrosomonas* during salinity increase. *Candidatus Nitrotoga* was the dominant NOB in all treatments. Ordination by PCoA based on Bray-Curtis similarities showed that the total microbial communities of the control and S15 were similar (Fig. 7A). The control on day 0 was highly dissimilar from the other control samples. Compared to S15, S1-6 were much more different from the control, especially along the first coordinate. Similar trends were observed for the nitrifying OTUs (Fig. 7B).

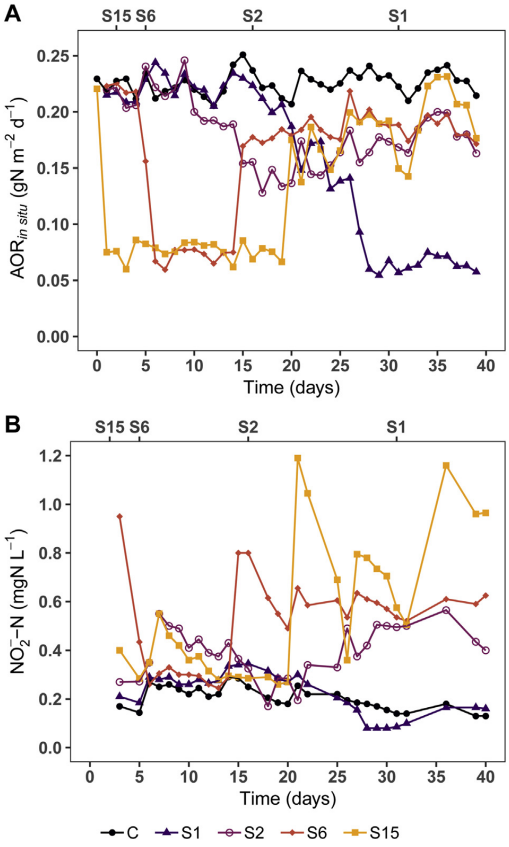


Fig. 5. For the different treatments A) *in situ* ammonia oxidation rate ($AOR_{in\ situ}$), and B) nitrite concentration in the MBBR. Labels above the graphs indicate point of complete transfer to seawater for each treatment. $AOR_{in\ situ}$ was calculated by the ammonia mass balance for each MBBR. S1, S6, and S15 had low ammonia loading rates (0.08 ± 0.04 gN m^{-2} d^{-1}) on days 28–40, 6–14, and 1–19, respectively.

4. Discussion

On complete transfer to seawater, the smallest salinity increment treatment, S1 (1‰ day^{-1}), had the lowest AOR_{max} among all treatments, contrary to what was hypothesized. Overall, AOR_{max} depended mainly on salinity and seawater acclimatization time, and was only slightly influenced by salinity change rate. In contrast, the microbial communities did appear to be influenced by the salinity increase rate and shifted differently depending on the treatment.

4.1. AOR_{max} decreased linearly with salinity

In each treatment (except S15), the AOR_{max} decreased linearly with salinity during the transfer from freshwater to seawater (Table 2). Further, statistical results showed that the decrease in the AOR_{max} was only dependent on the salinity, and independent of the salinity change rate (Fig. 4A). However, the AOR_{max} at 32‰ salinity (seawater) differed significantly between treatments, indicating that the rate of salinity change may have had an influence on the AOR_{max}. As far as we know, this is the first time that the relationship of AOR_{max} with salinity has been modeled for MBBRs under salinity change. An apparent linear decrease in the ammonia oxidation rate with salinity was also observed in other studies (Bassin et al., 2011; Gonzalez-Silva et al., 2016; Moussa et al., 2006; Uygur and Kargi, 2004). In contrast, in a recent MBBR study, AOR_{max} inhibition appeared sigmoidal with salinity (Kinyage et al., 2019). These differences may be attributed to different experimental setups and environmental variables in the studies. In this study, the control AOR_{max} varied but did not appear to follow any trend. The control on day 0 had the lowest AOR_{max}, likely because of biofilm sloughing during the redistribution of biomedia. The other control variations were probably random but should be kept in mind when evaluating the performance of the other treatments.

4.2. AOR_{max} was only slightly impacted at salinities up to 10–15‰

The AOR_{max} was slightly high compared to the control average for the first capacity tests of S1, S2, and S6 (4–8‰ salinity), suggesting that salinity increase had a positive effect on the value (Fig. 4A). Alternatively, this relative increase may be attributed to variations in the control. These findings are consistent with studies that reported salt concentration up to 10‰ either increased (Aslan and Simsek, 2012; Bassin et al., 2012) or had little negative impact on the ammonia oxidation rate (Cortes-Lorenzo et al., 2015; Sudarno, 2011; Vendramel et al., 2011). This is likely because isotonic conditions favor microbial metabolism (He et al., 2017). Further, at salinities of 10–15‰, AOR_{max} reduced only by 5–15%, contradicting studies that report 50–95% decrease in AOR_{max} in this salinity range (Gonzalez-Silva et al., 2016; Moussa et al., 2006; Vendramel et al., 2011; Wang et al., 2017). Conversely, AOR_{max} in fixed-bed biofilters were not negatively impacted at salinities of 14–20‰ (Karkman et al., 2011; Nijhof and Bovendeur, 1990; Sudarno et al., 2010). This apparent discord may be due to the differences in environmental factors or the type of nitrifying systems, for e.g. sludge vs biofilms. Biofilms may be more resistant to salinity changes than sludge, as the extrapolymeric matrix in biofilms may act as a protection against osmotic stress for the residing microorganisms (Baho et al., 2012).

4.3. Small salinity increments decreased AOR_{max} more than large salinity increments

While S1 had the maximum reduction in AOR_{max} amongst all treatments immediately after seawater transfer (~90% reduction), S6 had the lowest (~50% reduction) (Fig. 4A). To the best of our knowledge, this is the first study where ammonia oxidation was more reduced by a small salinity increment than a large salinity increment. Most related studies have performed shock or step changes in salinity (Bassin et al., 2012; Gonzalez-Silva, 2016; Moussa et al., 2006) whereas, in the present study, each daily salinity increment was gradually performed by controlling the salinity in the makeup flow to the reactors. The gradual salinity increment in this study may have given the microbes time to produce the compatible solutes required to adapt to the external osmotic pressure, thus preventing plasmolysis and successfully surviving the salinity increments. This hypothesis is supported by the similarity in microbial community composition between S15 and the control. Increasing the salinity by adjusting the makeup flow composition is likely more practical in full-scale MBBRs than a sudden increment in salinity, and should, therefore, be further researched.

4.4. AOR_{max} increased linearly with seawater acclimatization time

In seawater, AOR_{max} of all treatments showed a positive linear

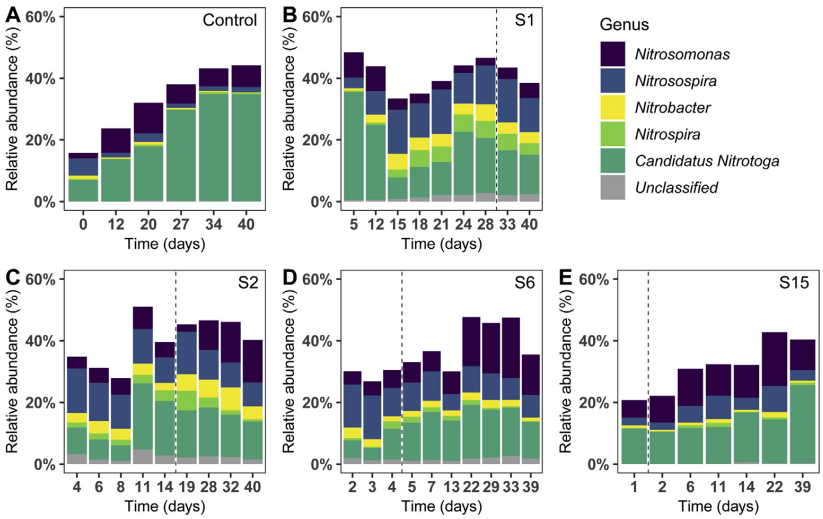


Fig. 6. Relative abundance of nitrifying genera in the biofilm for treatments A) Control, B) S1, C) S2, D) S6, and E) S15. Samples to the right of the dotted line are after complete seawater transfer.

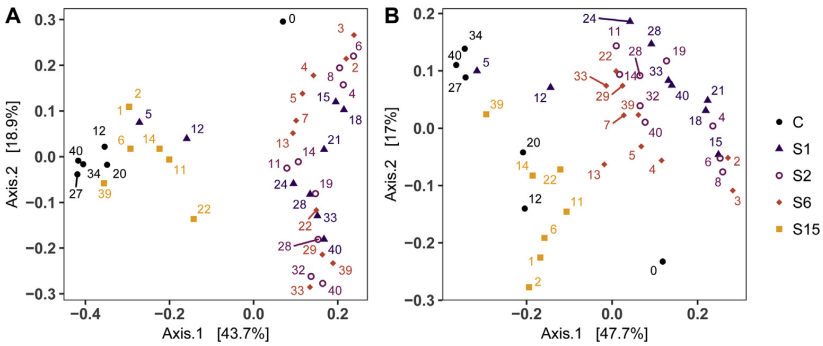


Fig. 7. Ordination by principal coordinates analysis (PCoA) based on Bray-Curtis similarities with A) all OTUs and B) nitrifying OTUs. Labels indicate sampling day. Square brackets show percentage variance explained by each coordinate axis. Treatments S1, S2, S6, and S15 were completely transferred to seawater on days 31, 16, 5, and 2, respectively.

correlation with time after seawater transfer (Fig. 4B). For each treatment, the weak correlation between the AOR_{max} and acclimatization time was likely because of the low number of observations. However, in less than 41 days in seawater, S6 and S15 had recovered to 65–90% of the AOR_{max} in freshwater, with 15–70% higher AOR_{max} than S1 and S2. This indicates that large salinity increments may be more practical than small salinity increments for commercial MBBRs. Specifically, for a RAS, in periods when the ammonia loading rate is low, the salinity may be changed in 2–5 days and the MBBR may be allowed to recover before increasing the loading rate. Moreover, this finding may be used to reduce the long startup time for seawater bioreactors (Chen et al., 2006; Nijhof and Bovendeur, 1990), by starting in freshwater and transferring to seawater within a few days, with allowance for a subsequent recovery period for seawater acclimatization. This strategy may also be applied when it is not possible to inoculate with saltwater acclimated seeds due to biosecurity constraints or unavailability of appropriate seeding material.

4.5. *In situ* nitrification performance

As capacity tests are intensive, $AOR_{in\ situ}$ was used as a proxy when the capacity tests could not be performed. In general, $AOR_{in\ situ}$ results were in accord with AOR_{max}. However, some periods of low $AOR_{in\ situ}$ were likely because of low loading and/or low nitrification. At low ammonia loading rates, as in RAS or in tertiary nitrifying bioreactors, nitrification is often limited by the ammonia concentration and $AOR_{in\ situ}$ may be lower than AOR_{max} (Rusten et al., 2006). Therefore, maximum ammonia oxidation rates are better indicators of nitrification than *in situ* ammonia oxidation rates or removal efficiencies, as also advised by (Moussa et al., 2006).

There are opposing views as to which process is more inhibited by salinity changes – ammonia oxidation (Moussa et al., 2006; Wang et al., 2017) or nitrite oxidation (Aslan and Simsek, 2012; Bassin et al., 2011; Sudarno, 2011). In this study, nitrite accumulation in S2, S6, and S15 indicates that nitrite oxidation was more impacted than ammonia oxidation. However, the relatively low

concentration of nitrite in seawater in this study (<1.5 mgN L^{-1}) suggests that nitrite oxidation rate was close to $AOR_{in\ situ}$, and not as severely inhibited as in other studies (Cortes-Lorenzo et al., 2015; Gonzalez-Silva, 2016). During some periods, nitrite oxidation may have been limited by the substrate production rate due to different ammonia loading and oxidation rates. Thus, to better compare the impact of salinity change rates on nitrite oxidation, nitrite capacity tests should be conducted.

4.6. Microbial communities were influenced by salinity increase rate

The microbial community composition in S15 was very different compared to the other treatments (Fig. 7). The similarity between S15 and the control suggests that the bacteria were only temporarily inhibited by the salinity increase and regained activity by adapting to the altered environmental conditions. Conversely, in S1-6, the microbial community composition shifted with time, as a response to salinity change and adaptation. This difference underlines that the responses of microorganisms to disturbances are dependent on the intensity and duration of the disturbance (Shade et al., 2012), and on the recovery time.

Higher species diversity, richness, and evenness of nitrifiers in S1-6 suggests that these treatments had greater functional redundancy. The continual salinity increases in S1-6 may have opened niches for populations which were either more capable of tolerating frequent salinity variations or preferred intermediate salinities. This hypothesis is supported by the shift in the dominant AOB from *Nitrosospira* during salinity increase, to *Nitrosomonas* after seawater acclimatization (Fig. 6). Similarly, *Nitrospira* and *Nitrobacter* were more abundant in S1-6 than in S15, and the abundance of *Nitrospira* decreased after seawater transfer in all treatments. Other studies have also reported that *Nitrospira* could tolerate brackish water but disappeared at salinities above 22‰ (Bassin et al., 2011; Rud et al., 2016).

The dominant NOB in this study, *Candidatus Nitrotoga*, is reported to be a K-strategist with a moderate affinity for substrate (Nowka et al., 2015; Wegen et al., 2019). Moreover, it prefers lower temperatures compared to *Nitrobacter* and *Nitrospira* and can outcompete them at 5–10 °C (Alawi et al., 2009; Karkman et al., 2011). These factors explain its dominance in biofilms in RAS for salmonids (this study; (Hüpeden et al., 2016)), which are operated at cool temperatures and low nitrite concentrations (<1 mgN L^{-1}). Although *Candidatus Nitrotoga* in pure cultures could only tolerate salinities up to 5–10‰ (Ishii et al., 2017; Wegen et al., 2019), they have been detected in marine RAS at 29–37‰ salinity (Keuter et al., 2017). Its continued presence throughout this study indicates that this NOB can adapt to salt concentrations up to 32‰, highlighting that salt tolerance in complex microbial environments may differ from those in pure cultures due to interactions between microorganisms (Ilgrande et al., 2018).

The increase in the proportion of nitrifiers in the control was likely due to the maturation of the biofilm. The other treatments were also possibly influenced by this maturation effect, as S1-6 had a higher proportion of nitrifiers than the control and S15, despite having a lower AOR_{max}. In these treatments, the nitrifiers were either inhibited or the heterotrophic bacteria were reduced by the salinity increase. Alternatively, some dead cells may have been included in the analysis, as all PCR-quality DNA are quantified in amplicon sequencing. However, the shifts in the proportions of different nitrifying genera, especially in S1-6, indicate that the changes in microbial communities were dynamic. In this study, both freshwater and halotolerant/halophilic strains of nitrifying genera were detected. Moreover, the presence of obligate halophiles, such as *N. marina* (Koops et al., 2006), suggests that the salinity increase opened new niches for marine bacteria.

Although the microbial communities differed between treatments, the AOR_{max} was only weakly influenced by the salinity change rate. Other studies have also reported that nitrifying microbial communities with different species inventory may exhibit the same nitrification activity (Bassin et al., 2012; Moussa et al., 2006). This phenomenon is likely due to high functional redundancy among taxa (Berga et al., 2017). Understanding the responses of microbes to salinity is important, as it can aid in improving bioreactor design and management, and in selecting suitable inoculum for saline bioreactors.

5. Conclusions

The aim of this study was to investigate if small daily salinity increments could be a better strategy than large daily salinity increments to adapt freshwater nitrifying MBBRs to seawater. In conclusion:

- The ammonia oxidation capacity of the MBBRs was only weakly influenced by the salinity increase rate, but decreased linearly with salinity (~2.7% decrease per ‰) and increased linearly with seawater acclimatization time (~2.1% recovery per day). This finding suggests that there is no advantage of a small salinity increment over a large salinity increment. Therefore, it appears practical to increase salinity continuously in a couple of days and allow more time for acclimatization to full salinity instead of increasing the salinity in smaller increments over a month.
- Microbial communities may tolerate large gradual increments in salinity with little change in composition. In comparison, continual changes in salinity over a long period may induce a shift in communities to increase diversity and functional redundancy of nitrifying bacteria to adapt to the constant perturbations.
- These results can aid in the shift from net-pen fish production to lower ecological impact RAS. This study may also help manage nitrifying bioreactors for saline industrial or municipal effluents, especially when salt-acclimated inoculum is unavailable. As this study showed that the salinity could not be increased within a month without a decrease in nitrification, other seawater adaptation strategies should be investigated to increase the salinity resistance of nitrifying biofilms.

Author contributions

By CRediT taxonomy: Conceptualization and experiment design: SN, BFT, AK, FG. Methodology/Resources: AK, ØM, AKT, SN. Investigation: SN, GCV, AKT. Formal analysis: SN, OV, AK, VCM. Visualization: SN, VV. Supervision: AK, OV, BFT, VCM, ØM, FG. Writing original draft: SN, VCM, OV, AKT. Critical review of manuscript: All.

Acknowledgements

This project is a part of CtrlAQUA SFI, Center for research-based innovation funded by the Research Council of Norway and the Center partners, including Krüger Kaldnes AS (#237856/O30, #270888/O30). The authors would like to deeply thank Frode Nerland and Britt Kristin Megård Reiten for assisting with the construction of the experimental setup, and the R&D team at AnoxKaldnes for scientific guidance.

References

Alawi, M., Off, S., Kaya, M., Spieck, E., 2009. Temperature influences the population structure of nitrite-oxidizing bacteria in activated sludge. Environ. Microbiol. Rep. 1, 184–190.

APHA, 1992. Standard Methods for the Examination of Water and Wastewater, Method 4500-NO2 B. American Public Health Association, Washington D.C., U.S.A.

Aslan, S., Simsek, E., 2012. Influence of salinity on partial nitrification in a submerged biofilter. Bioresour. Technol. 118, 24–29.

Ayer, N.W., Tyedmers, P.H., 2009. Assessing alternative aquaculture technologies: life cycle assessment of salmonid culture systems in Canada. J. Clean. Prod. 17, 362–373.

Baho, D.L., Peter, H., Tranvik, L.J., 2012. Resistance and resilience of microbial communities - temporal and spatial insurance against perturbations. Environ. Microbiol. 14, 2283–2292.

Bassin, J.P., Kleerebezem, R., Muyzer, G., Rosado, A.S., Van Loosdrecht, M.C.M., Dezotti, M., 2012. Effect of different salt adaptation strategies on the microbial diversity, activity, and settling of nitrifying sludge in sequencing batch reactors. Appl. Microbiol. Biotechnol. 93, 1281–1294.

Bassin, J.P., Pronk, M., Muyzer, G., Kleerebezem, R., Dezotti, M., van Loosdrecht, M.C.M., 2011. Effect of elevated salt concentrations on the aerobic granular sludge process: linking microbial activity with microbial community structure. Appl. Environ. Microbiol. 77, 7942–7953.

Berga, M., Zha, Y., Székely, A.J., Langenheder, S., 2017. Functional and compositional stability of bacterial metacommunities in response to salinity changes. Front. Microbiol. 8, 1–11.

Chen, S., Ling, J., Blancheton, J.P., 2006. Nitrification kinetics of biofilm as affected by water quality factors. Aquacult. Eng. 34, 179–197.

Cortes-Lorenzo, C., Rodriguez-Diaz, M., Sipkema, D., Juarez-Jimenez, B., Rodelas, B., Smidt, H., Gonzalez-Lopez, J., 2015. Effect of salinity on nitrification efficiency and structure of ammonia-oxidizing bacterial communities in a submerged fixed bed bioreactor. Chem. Eng. J. 266, 233–240.

Csonka, L.N., 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. 53, 121–147.

Cui, Y.W., Zhang, H.Y., Ding, J.R., Peng, Y.Z., 2016. The effects of salinity on nitrification using halophilic nitrifiers in a Sequencing Batch Reactor treating hypersaline wastewater. Sci. Rep. 6, 1–11.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R.H., Von Bergen, M., Rattei, T., Bendinger, B., Nielsen, P.H., Wagner, M., 2015. Complete nitrification by Nitrospira bacteria. Nature 528, 504–509.

Davidson, J., May, T., Good, C., Waldrop, T., Kenney, B., Terjesen, B.F., Summerfelt, S., 2016. Production of market-size North American strain Atlantic salmon Salmo salar in a land-based recirculation aquaculture system using freshwater. Aquacult. Eng. 74, 1–16.

Fox, J., Weisberg, S., 2011. An {R} Companion to Applied Regression, second ed. (Thousand Oaks CA).

Gonzalez-Silva, B.M., 2016. Salinity as a Driver for Microbial Community Structure in Reactors for Nitrification and Anammox. Norwegian University of Science and Technology.

Gonzalez-Silva, B.M., Jonassen, K.R., Bakke, I., Østgaard, K., Vadstein, O., 2016. Nitrification at different salinities: biofilm community composition and physiological plasticity. Water Res. 95, 48–58.

He, H., Chen, Y., Li, X., Cheng, Y., Yang, C., Zeng, G., 2017. Influence of salinity on microorganisms in activated sludge processes: a review. Int. Biodeterior. Biodegrad. 119, 520–527.

Hill, M.O., 1973. Diversity and evenness: a unifying notation and its consequences. Ecology 54, 427–432.

Hüpeden, J., Wegen, S., Off, S., Lücker, S., Bedarf, Y., Daims, H., Kühn, C., Spieck, E., 2016. Relative abundance of nitrotoga spp. in a biofilter of a cold-freshwater aquaculture plant appears to Be stimulated by slightly acidic pH. Appl. Environ. Microbiol. 82, 1838–1845.

Ilgrande, C., Leroy, B., Wattiez, R., Vlaeminck, S.E., Boon, N., Clauwaert, P., 2018. Metabolic and proteomic responses to salinity in synthetic nitrifying communities of Nitrosomonas spp. and Nitrobacter spp. Front. Microbiol. 9, 1–12.

Ishii, K., Fujitani, H., Soh, K., Nakagawa, T., Takahashi, R., Tsuneda, S., 2017. Enrichment and physiological characterization of a cold-adapted nitrite-oxidizing nitrotoga sp. from an eelgrass sediment. Appl. Environ. Microbiol. 83, 1–14.

Karkman, A., Mattila, K., Tamminen, M., Virta, M., 2011. Cold temperature decreases bacterial species richness in nitrogen-removing bioreactors treating inorganic mine waters. Biotechnol. Bioeng. 108, 2876–2883.

Keuter, S., Beth, S., Quantz, G., Spieck, E., S, K., 2017. Longterm monitoring of nitrification and nitrifying communities during biofilter activation of two marine recirculation aquaculture systems (RAS). Int. J. Aquac. Fish. Sci. 3, 051–061.

Kinyage, J.P.H., Pedersen, P.B., Pedersen, L.F., 2019. Effects of abrupt salinity increase on nitrification processes in a freshwater moving bed biofilter. Aquacult. Eng. 84, 91–98.

Koops, H.-P., Purkhold, U., Pommerening-Röser, A., Timmermann, G., Wagner, M., 2006. The Lithoautotrophic Ammonia-Oxidizing Bacteria. The Prokaryotes.

Lay, W.C.L., Liu, Y., Fane, A.G., 2010. Impacts of salinity on the performance of high retention membrane bioreactors for water reclamation: a review. Water Res. 44, 21–40.

Madigan, M.T., Bender, K.S., Buckley, D.H., Sattley, W.M., Stahl, D.A., 2018. Brock Biology of Microorganisms, fifteenth ed. Pearson Education Limited.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8.

Moussa, M.S., Sumanasekera, D.U., Ibrahim, S.H., Lubberding, H.J., Hooijmans, C.M., Gijzen, H.J., Van Loosdrecht, M.C.M., 2006. Long term effects of salt on activity, population structure and floc characteristics in enriched bacterial cultures of nitrifiers. Water Res. 40, 1377–1388.

Nijhof, M., Bovendeur, J., 1990. Fixed film nitrification characteristics in sea-water recirculation fish culture systems. Aquaculture 87, 133–143.

Nowka, B., Daims, H., Spieck, E., 2015. Comparison of oxidation kinetics of nitrite-oxidizing bacteria: nitrite availability as a key factor in niche differentiation. Appl. Environ. Microbiol. 81, 745–753.

Oksanen, J., Guillaume Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, Gavin L., Solymos, P., Stevens, M.H.H., Eduard, S., Wagner, H., 2019. Vegan: Community Ecology Package. R Package Version 2.5-4.

Panswad, T., Anan, C., 1999. Impact of high chloride wastewater on an anaerobic/anoxic/aerobic process with and without inoculation of chloride acclimated seeds. Water Res. 33, 1165–1172.

Rud, I., Kolarevic, J., Holan, A.B., Berget, I., Calabrese, S., Terjesen, B.F., 2016. Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. Aquacult. Eng.

Rusten, B., Eikebrokk, B., Ulgenes, Y., Lygren, E., 2006. Design and operations of the Kaldnes moving bed biofilm reactors. Aquacult. Eng. 34, 322–331.

Shade, A., Peter, H., Allison, S.D., Baho, D.L., Berga, M., Bürgmann, H., Huber, D.H., Langenheder, S., Lennon, J.T., Martiny, J.B.H., Matulich, K.L., Schmidt, T.M., Handelsman, J., 2012. Fundamentals of microbial community resistance and resilience. Front. Microbiol. 3, 1–19.

Sharrer, M.J., Tal, Y., Ferrier, D., Hankins, J.A., Summerfelt, S.T., 2007. Membrane biological reactor treatment of a saline backwash flow from a recirculating aquaculture system. Aquacult. Eng. 36, 159–176.

Shi, K., Zhou, W., Zhao, H., Zhang, Y., 2012. Performance of halophilic marine bacteria inocula on nutrient removal from hypersaline wastewater in an intermittently aerated biological filter. Bioresour. Technol. 113, 280–287.

Sudarno, U., 2011. Nitrification in Fixed Bed Reactors Treating Saline Wastewater. Karlsruher Institute for Technology.

Sudarno, U., Bathe, S., Winter, J., Gallert, C., 2010. Nitrification in fixed-bed reactors treating saline wastewater. Appl. Microbiol. Biotechnol. 85, 2017–2030.

Terjesen, B.F., Summerfelt, S.T., Nerland, S., Ulgenes, Y., Fjæra, S.O., Megård Reiten, B.K., Selset, R., Kolarevic, J., Brunsvik, P., Bæverfjord, G., Takle, H., Kittelsen, A.H., Åsgård, T., 2013. Design, dimensioning, and performance of a research facility for studies on the requirements of fish in RAS environments. Aquacult. Eng. 54, 49–63.

U.S. EPA, 1983. Methods for Chemical Analysis of Water and Wastes. U.S. Environmental Protection Agency, Cinciannati, OH, USA.

Uygur, A., Kargi, F., 2004. Salt inhibition on biological nutrient removal from saline wastewater in a sequencing batch reactor. Enzym. Microb. Technol. 34, 313–318.

Vendramel, S., Dezotti, M., Sant'Anna, G.L., 2011. Nitrification of an industrial wastewater in a moving-bed biofilm reactor: effect of salt concentration. Environ. Technol. 32, 837–846.

Wang, Z., van Loosdrecht, M.C.M., Saikaly, P.E., 2017. Gradual adaptation to salt and dissolved oxygen: strategies to minimize adverse effect of salinity on aerobic granular sludge. Water Res. 124, 702–712.

Wegen, S., Nowka, B., Spieck, E., 2019. Low temperature and neutral pH define Candidatus Nitrotoga sp. as a competitive nitrite oxidizer in co-culture with Nitrospira defluvii. Appl. Environ. Microbiol. AEM 02569, 18.

Zeller, D., Cashion, T., Palomares, M., Pauly, D., 2018. Global marine fisheries discards: a synthesis of reconstructed data. Fish Fish. 19, 30–39.

Zhu, S., Shen, J., Ruan, Y., Guo, X., Ye, Z., Deng, Y., Shi, M., 2016. The effects of different seeding ratios on nitrification performance and biofilm formation in marine recirculating aquaculture system biofilter. Environ. Sci. Pollut. Res. 23, 14540–14548.

Article II

Water Research 176 (2020) 115732

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Biofilms remember: Osmotic stress priming as a microbial management strategy for improving salinity acclimation in nitrifying biofilms

Sharada Navada [a, b, *], Olav Vadstein [c], Frédéric Gaumet [b], Ann-Kristin Tveten [d], Claudia Spanu [e, 1], Øyvind Mikkelsen [a], Jelena Kolarevic [f]

[a] Department of Chemistry, NTNU - Norwegian University of Science and Technology, N-7491, Trondheim, Norway
[b] Krüger Kaldnes AS (Veolia Water Technologies), N-3241, Sandefjord, Norway
[c] Department of Biotechnology and Food Science, NTNU - Norwegian University of Science and Technology, N-7491, Trondheim, Norway
[d] Department of Biological Sciences, NTNU - Norwegian University of Science and Technology, N-6009, Ålesund, Norway
[e] Department of Toxicology, University of Cagliari, 09124, Cagliari, Italy
[f] Nofima AS, N-6600, Sunndalsøra, Norway

ARTICLE INFO

Article history:
Received 23 December 2019
Received in revised form
10 March 2020
Accepted 16 March 2020
Available online 20 March 2020

Keywords:
Nitrification
Biofilter salt acclimatization
Acquired stress resistance
Predictive response strategy
Seawater conditioning
Marine recirculating aquaculture systems (RAS)

ABSTRACT

With increasing freshwater scarcity and greater use of seawater, fluctuating salinities are becoming common in water treatment systems. This can be challenging for salinity-sensitive processes like nitrification, especially in recirculating aquaculture systems (RAS), where maintaining nitrification efficiency is crucial for fish health. This study was undertaken to determine if prior exposure to seawater (priming) could improve nitrification in moving bed biofilm reactors (MBBR) under salinity increase from freshwater to seawater. The results showed that seawater-primed freshwater MBBRs had less than 10% reduction in nitrification activity and twice the ammonia oxidation capacity of the unprimed bioreactors after seawater transfer. The primed biofilms had different microbial community composition but the same nitrifying taxa, suggesting that priming promoted physiological adaptation of the nitrifiers. Priming may also have strengthened the extrapolymeric matrix protecting the nitrifiers. In MBBRs started up in brackish water (12‰ salinity), seawater priming had no significant impact on the nitrification activity and the microbial community composition. These bioreactors were inherently robust to salinity increase, likely because they were already primed to osmotic stress by virtue of their native salinity of 12‰. The results show that osmotic stress priming is an effective strategy for improving salinity acclimation in nitrifying biofilms and can be applied to water treatment systems where salinity variations are expected.

1. Introduction

Nitrification is a widely used process for ammonia removal in wastewater treatment. However, this biological process is sensitive to variations in salinity, as the nitrifying microorganisms can be inhibited or lysed by changes in the osmotic pressure (Csonka, 1989; Madigan et al., 2018). Several industrial and municipal effluents have fluctuating salt concentrations, such as those from tanneries, food processing, or cities with seawater flushing (Cui et al., 2009; Lefebvre and Moletta, 2006; Yu et al., 2002). Variable salinity is also common in recirculating aquaculture systems (RAS). RAS are land-based fish production systems with water treatment processes, including nitrifying bioreactors for biological ammonia removal. In RAS for Atlantic salmon (*Salmo salar*), the salinity is typically increased from freshwater to brackish water or seawater after smoltification (Kinyage et al., 2019; Navada et al., 2019). Especially in RAS and in effluents discharged to water bodies with aquatic life, maintaining nitrification efficiency during salinity variations is essential, as both ammonia and nitrite (an intermediate in the nitrification process) can be extremely toxic at concentrations as low as 1 mgN L^{-1} (Timmons and Ebeling, 2010).

Studies have shown that the nitrification activity begins to decrease significantly at salinities higher than ~10‰ (Bassin et al.,

* Corresponding author. Department of Chemistry, NTNU - Norwegian University of Science and Technology, N-7491, Trondheim, Norway.
E-mail address: sharada.navada@ntnu.no (S. Navada).
[1] Present address: University of Milan, 20122, Milan, Italy.

https://doi.org/10.1016/j.watres.2020.115732

2012a; Navada et al., 2019). Thus, in this study, salinity acclimation will refer to adaptation to salinities above 10‰. Although nitrifying bioreactors can be acclimated to higher salinities, it is difficult to avoid a loss in nitrification rate in the initial period after salinity increase (Bassin et al., 2012a; Gonzalez-Silva et al., 2016; Navada et al., 2019). Moreover, the acclimatization process is slow and can take weeks or even months (Bassin et al., 2012a, 2011; Sharrer et al., 2007). Salt-adapted inocula can reduce, but not necessarily eliminate the negative impact of salinity change on nitrification (Panswad and Anan, 1999; Shi et al., 2012). Further, in RAS, inoculation can pose a biosecurity risk to the fish. It is, therefore, necessary to develop a strategy to increase the salinity resistance of nitrifying biofilms, so that salinity changes may be performed without hindering the bioreactor performance.

Studies have shown that the performance of bioreactors may be influenced by their operational history (Cabrol et al., 2016; Saur et al., 2016). Bioreactors are often more functionally stable to environmental disturbances, such as toxic substances or high nutrient loading, when previously exposed to a smaller dose of the same disturbance (Cabrol et al., 2016 and references within). Perturbations of biofilms, especially in the initial growth phase, may influence the microbial community succession and the structure of the finally established community (Cabrol et al., 2016; Ohashi et al., 1995; Saur et al., 2016). Alternatively, the microorganisms in the biofilm may respond physiologically to the perturbations and become more tolerant to future stresses.

Priming, also called predictive response strategy or acquired stress tolerance, is a phenomenon where microorganisms exposed to a mild external stress show an improved response to a more severe stress in the future, usually through phenotypical modifications, such as changes in gene expression or metabolism (Hilker et al., 2016; Mitchell et al., 2009; Rillig et al., 2015). Priming as a physiological strategy has been observed in bacterial cultures subjected to osmotic stress (Andrade-Linares et al., 2016; Jenkins et al., 1990). However, the response of microbial communities to priming may differ greatly from that of pure cultures. Priming of microbial communities can not only modify the physiological phenotype of the microorganisms, but also alter the microbial community composition due to differences in the priming capabilities of the community members and complex microbial interactions (Rillig et al., 2015). Studies on the response of nitrifying microbial communities to osmotic stress priming are limited. A study on nitrifying sludge showed that adaptation to 10 g Cl^- L^{-1} (~16‰ salinity) did not improve the nitrification performance at higher salinities (Moussa et al., 2006). To the best of our knowledge, the effect of osmotic stress priming in nitrifying microbial communities, especially in biofilms, has not been well researched.

The objective of this study was to determine if seawater priming could improve salinity acclimation in nitrifying moving bed biofilm reactors (MBBR). It was hypothesized that the primed treatment would undergo lesser reduction in nitrification performance than the unprimed treatment when the salinity was increased from freshwater to seawater. With this objective, we investigated the effect of seawater priming on MBBRs initially started up in fresh- and brackish water, respectively.

2. Materials & methods

2.1. Experimental setup and design

The experiment was performed on continuously operated MBBRs with four treatments in duplicate. The setup was similar to that described in Navada et al. (2019). Two treatments (F0, F1) were started in freshwater (FW), whereas the other two (B0, B1) were started in brackish water (BW) at 12‰ salinity (Table 1). Thereafter, F1 and B1 were transferred to seawater (SW, 32‰ salinity) by increasing the salinity in gradual daily increments (~10-11‰ day^{-1}) over three and two days, respectively. These treatments were operated in seawater for two weeks, while F0 and B0 were operated at their native salinities. Thereafter, all reactors were transferred to FW (in approx. one day) and operated for 24 days. Finally, all reactors were transferred to seawater (salinity increased in the same manner as in the priming stage) and operated for 31 days.

The biofilm carriers (AnoxK™ Chip P, Krüger Kaldnes AS, Norway) were started in a FW and BW RAS MBBR with fish feed, NH_4Cl, $NaNO_2$, and $NaHCO_3$, with no fish in the system. These carriers had been used previously in the RAS, so they were disinfected with acid and base prior to start-up. The experimental reactors were filled with biofilm carriers (~35% by volume) from the FW and BW RAS MBBRs after two and four weeks of start-up, respectively. To acclimatize the carriers to the experimental system, the reactors were operated for one month on synthetic medium. The synthetic medium had an ammonia concentration of 700–1130 mgN L^{-1} with the following nutrients per mg of NH_4^+-N: 7.14 mg $CaCO_3$ (supplied by $NaHCO_3$), 0.1 mg P as $Na_2HPO_4 \cdot 12H_2O$, 0.1 mg P as KH_2PO_4, 0.1 mg Mg as $MgSO_4$, and 0.003 mg Fe as $FeCl_3$ (Zhu et al., 2016). Sucrose (2-4 g) was added daily in the last 19 days of the acclimatization period (with few exceptions) to boost biofilm growth by heterotrophic bacteria (Bassin et al., 2012b). To minimize reactor bias, the biomedia were intermixed and redistributed to the reactors ten days before starting the experiment.

The experiment was started after the acclimatization period. The MBBRs were operated at 12.4 ± 0.5 °C, pH 7.5 ± 0.3 and aerated with an air flow of 40 NL min^{-1} (dissolved oxygen saturation 80-100%). Each reactor was provided synthetic medium at a flow rate of 2–3 mL min^{-1}, corresponding to an average ammonia loading rate of 0.22 ± 0.04 gN m^{-2} d^{-1}. In addition, dilution water was provided to each reactor from a buffer tank (one per treatment) via a multichannel peristaltic pump (Ismatec ISM404 MCP, Cole-Parmer, USA). Salinity changes for each treatment were performed by controlling the salinity in buffer tanks by adjusting the freshwater and seawater flow rates, thus changing the reactor salinity gradually (Navada et al., 2019). In the freshwater and seawater phases, the hydraulic retention time (HRT) was approximately 5 h (dilution flow 119 ± 7 mL min^{-1}). During the native and priming phases, the reactors had a higher HRT of ~12 h (dilution flow 50 ± 3 mL min^{-1}), as this period was designed to simulate the start-up phase of MBBRs. Temperature, pH, dissolved oxygen, salinity, and flow rates of synthetic medium, dilution water and air were measured daily (with few exceptions) using the methods described in Navada et al. (2019). Ammonia (in the synthetic medium and in each reactor) and nitrite concentration in each reactor were measured using the phenate method and colorimetric method, respectively (APHA, 2017).

2.2. Nitrification performance

The *in situ* ammonia oxidation rate ($AOR_{in\ situ}$) in each reactor was calculated by ammonia mass balance, assuming pseudo-steady

Table 1
The salinities of the four treatments during each operational phase. The F and B treatments were started in fresh- and brackish water, respectively. The treatments with suffix '1' were seawater primed.

Operational phase	Treatment salinity (‰)				Experimental days	Duration (days)
	F0	F1	B0	B1		
Native	0	0	12	12	1–7	7
Priming	0	32	12	32	10–23	14
Freshwater	0	0	0	0	24–47	24
Seawater	32	32	32	32	50–92	43

state over 24 h (Navada et al., 2019). For each MBBR, the maximum ammonia oxidation rate (AOR_{max}) and the maximum nitrite oxidation rate (NOR_{max}) were determined by performing capacity tests. Each test was conducted by running the MBBR in a batch mode by closing the inlets and outlets. The reactor was spiked with 170–350 mL of either synthetic medium or a spike solution to obtain an initial ammonia concentration of 4–18 mgN L^{-1} in the MBBR. The ammonia spike solution had the same proportions of nutrients per NH_4^+-N as the synthetic medium (except iron) made up in deionized water. No spike was added if the *in situ* ammonia concentration was already high (>4 mgN L^{-1}). Ammonia concentration was measured every 5-31 min during the capacity test. The nitrite capacity test was performed similarly. Each reactor was spiked with 8–20 mg NO_2^--N L^{-1} by adding 200–250 mL of a spike solution made with $NaNO_2$ and deionized water. Water samples were analyzed every 5–15 min to determine the NO_2^--N concentration.

2.3. Microbial analyses

Before each ammonia capacity test, three biofilm carriers were sampled from each reactor and preserved at −80 °C. For each test, one biofilm sample from each treatment was analyzed by 16S rRNA gene amplicon sequencing on Ion Personal Genome Machine™ using the procedures for analysis and data processing described in Navada et al. (2019). Briefly, 10 × 20 mm pieces were cut out from the thawed carriers and placed into 1.5 mL tubes containing ATL buffer (Qiagen®, Netherlands). Biofilm was detached in a Qiagen® Tissuelyser II (30hz s^{-1}, 10 min). PCR amplification was performed with Ion 16S™ Metagenomics Kit (Cat no: A26216, ThermoFisher). The kit includes two sets of primer pools targeting variable regions V2,4,8 and V3,6,7,9, respectively. Sequences are deposited in Genbank with accession number PRJNA614452.

2.4. Data analysis and statistics

AOR_{max} (or NOR_{max}) was calculated from the slope of regression lines of the NH_4^+-N (or NO_2^--N) concentration vs time. As there was little difference between duplicate reactors, the combined data from both reactors were used to fit a regression line for each treatment, with a minimum of 14 samples per test (Supplementary Information B). Analysis of covariance (ANCOVA) was used to detect significant differences between the slopes of the regression lines of the primed and unprimed treatments (Fox and Weisberg, 2011; Navada et al., 2019). On days 74–75, nitrification activity in F0 ceased suddenly due to suspected metals deficiency (Supplementary Information, Section A.1). Therefore, all analyses are reported for days 1–73, unless otherwise specified.

For the microbial analysis, the operational taxonomic unit (OTU) table was normalized to the sum of sample reads. OTUs with a maximum of less than 0.1% in any sample were filtered out. The α-diversity of each sample was calculated using the first-order diversity number ($N_1 = e^H$, where H refers to the Shannon diversity index), richness (count of OTUs, N_0), and evenness (N_1/N_0) (Hill, 1973). Ordination was performed using principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity between the biofilm samples. Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis and Sørensen-Dice indices was used to test the hypothesis of equal community composition between groups of biofilm samples (Anderson, 2001). Differences were considered statistically significant at a confidence interval of 95% ($p < 0.05$). Physicochemical variables are reported as mean ± standard deviation (SD), whereas calculated variables (such as AOR_{max}) are reported as mean ± standard error (SE). Data analyses and visualization were performed in R (V3.4.0) using packages vegan, phyloseq, and ggplot2 (McMurdie and Holmes, 2013; Oksanen et al., 2019; Wickham, 2016).

3. Results

3.1. Nitrification activity in the freshwater (F) treatments

The primed treatment (F1) had significantly higher ammonia oxidation capacity (AOR_{max}) than F0 in the seawater phase (Fig. 1A). Until day 45, the AOR_{max} in F0 (operated in FW) was relatively stable at 0.26 ± 0.05 gN m^{-2} d^{-1}. In this period, the AOR_{max} in F1 was lower than in F0, likely due to a reduction during the priming phase. However, after the second transfer to seawater (day 50), F1 showed no significant reduction in AOR_{max}, which, in fact, increased by 80% after three weeks in seawater. In contrast, AOR_{max} in F0 decreased by 55% upon transfer to seawater. The $AOR_{in\ situ}$ and ammonia concentration in the MBBRs were in alignment with the AOR_{max} (Fig. 2A, C).

The nitrite oxidation capacity (NOR_{max}) was also higher in F1 than in F0 in the seawater phase (Fig. 1C). Until the seawater phase, the NOR_{max} in F1 was 9–50% lower than that in F0. After transfer to the SW phase, NOR_{max} in F1 initially reduced by ~10%, but increased to ~35% higher than that in the FW phase after two weeks in seawater. In contrast, NOR_{max} in F0 initially decreased by 35% on transfer to seawater, and decreased further by 90% after 15 days. Throughout the study, NOR_{max} was higher than AOR_{max} in both treatments (except in F0 on day 64), indicating complete ammonia oxidation to nitrate. Nitrite concentration in the MBBRs during normal operation was consistently below 0.4 mgN L^{-1} (days 1–73) (Fig. 2E).

3.2. Nitrification activity in the brackish water (B) treatments

In general, the AOR_{max} in B0 and B1 did not differ significantly during the study (Fig. 1B). Further, the AOR_{max} in both treatments was not impacted by seawater transfer (both in the priming and SW phases). Upon transfer to freshwater, the treatments suffered a 45-60% reduction in AOR_{max}. However, within three weeks in FW, AOR_{max} in B1 recovered completely, whereas B0 recovered to 80% of the original AOR_{max}. In the seawater phase, the AOR_{max} did not decrease; rather it increased by 30–50% after three weeks in seawater. The $AOR_{in\ situ}$ in both treatments was limited by the ammonia substrate during most of the study (Fig. 2B, D).

In contrast to AOR_{max}, the NOR_{max} was negatively impacted by salinity increase. During the priming phase, B1 (at 32‰ salinity) had significantly lower NOR_{max} than B0 (12‰ salinity) (Fig. 1D). After day 40, the NOR_{max} in the two treatments did not differ significantly. In the SW phase, the NOR_{max} reduced to half the capacity in freshwater. In the priming and seawater phases, NOR_{max} was lower than AOR_{max} (Fig. 1B, D). During the priming phase, the nitrite concentration in B1 increased to a maximum of 2 mgN L^{-1} (Fig. 2F). But after transfer to freshwater, the nitrite concentration in both treatments decreased to less than 0.2 mgN L^{-1} in three weeks. In the SW phase, although the nitrite concentration increased slightly, it was still below 0.5 mgN L^{-1}.

3.3. Microbial community composition

Of the 1434 OTUs detected in the biofilm, 25 were identified as nitrifying bacteria. After filtering out the rare OTUs, 452 OTUs remained, including 15 nitrifying OTUs (Supplementary Information, Table A.1). The nitrifying bacteria constituted up to 55% of the total reads; the rest were likely heterotrophic bacteria (Fig. 3, Supplementary Information Fig. A.6). The AOB OTUs that could be classified at the genus (species) level were *Nitrosomonas* (*N.* sp.)

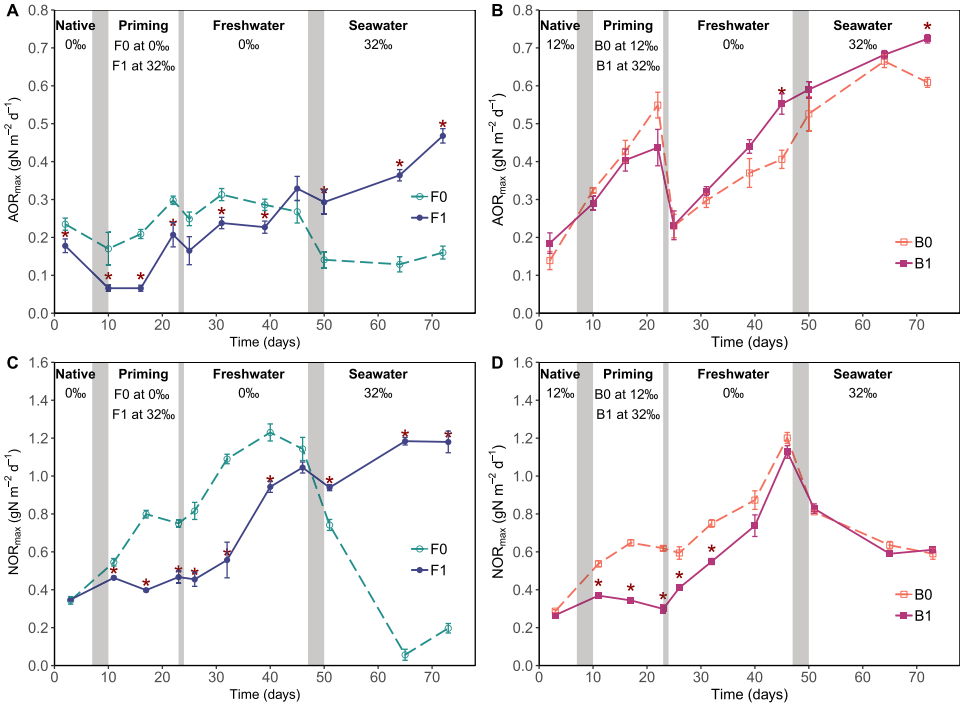


Fig. 1. Maximum ammonia and nitrite oxidation rates (AOR_{max} and NOR_{max}, respectively) during different phases of the experimental period for the freshwater and brackish water treatments. A) AOR_{max} in F0 and F1, B) AOR_{max} in B0 and B1, C) NOR_{max} in F0 and F1, and D) NOR_{max} in B0 and B1. Salinities during the different phases are shown in ‰ (parts per thousand). Gray shaded regions indicate days of salinity change. In each graph, asterisks above the data points indicate that the primed treatment was significantly different from the unprimed treatment ($p < 0.05$). Note that the y-axis scales in the top and bottom graphs are different.

and *Nitrosospira* (*N. multiformis*). The NOB were: *Candidatus Nitrotoga* (*nitrotoga*), *Nitrospira* (*N. marina*, *N.* sp.), and *Nitrobacter* (*N. vulgaris*). Four OTUs were classified only to the family level as *Nitrosomonadaceae* (2 OTUs), *Nitrospiraceae* (1 OTU), and *Nitrospinaceae* (1 OTU). *Nitrosomonas* was the dominant AOB genus in all the treatments. Among the NOB, *Candidatus Nitrotoga* was the dominant genus, although a few samples in the B treatments had *Nitrobacter* as the most abundant. *Nitrospira* was detected exclusively in the F treatments, whereas *Nitrobacter* was detected only in the B treatments. For both the F and B treatments, the α-diversity in the primed and unprimed treatment did not differ significantly, both based on all the OTUs and the nitrifying OTUs, except for evenness of the nitrifying community in B0 and B1 ($p = 0.04$) (Table 2).

For the F and B treatments separately, ordination by PCoA showed that time was the primary factor influencing both the total and nitrifying microbial community composition (Fig. 4). Further, on any given day, the Bray-Curtis similarity between the nitrifying communities of the primed and unprimed treatments was 64–90% (F treatments) and 56-92% (B treatments) (Supplementary Information, Fig. A.5). The PERMANOVA results showed that the community composition of F0 and F1 on days 31–50 were significantly different, both based on the Bray-Curtis index ($R^2 = 0.48$, $p = 0.03$) and the Sørensen-Dice index ($R^2 = 0.45$, $p = 0.03$). However, the nitrifying community composition of F0 and F1 was significantly different only based on the Bray-Curtis index ($R^2 = 0.52$, $p = 0.03$), but not on the Sørensen-Dice index ($R^2 = 0.13$, $p = 0.32$). For the B treatments, both the overall and nitrifying community composition of B0 and B1 were not significantly different throughout the experiment, based on both the distance metrics ($p > 0.2$).

4. Discussion

Growth history can influence biofilm behavior (Ohashi et al., 1995; Saur et al., 2016). In addition to having different native salinities, the biofilm carriers in F and B treatments had slightly different histories before being transferred to the experimental reactors. The F carriers had been disinfected and stored dry before start-up in the RAS MBBR, whereas the B carriers had been disinfected in the BW RAS MBBR and immediately started up again. The B carriers were also cultured two weeks longer in the RAS MBBRs than F before being transferred to the experimental system. Nonetheless, the nitrification activity in all treatments was similar on day 1, indicating that all treatments had similar nitrifying capacity in the beginning of the study (Fig. 1). Thus, the differences in salinity acclimation of the F and B treatments are primarily attributed to the difference in native salinity.

The primed treatment F1 had twice the AOR_{max} and 20% higher NOR_{max} than F0 upon transfer to the SW phase (Fig. 1A, C). This provides strong evidence that seawater priming increased salinity acclimation in the biofilm. Salinity acclimation in nitrifying biofilms may be achieved by physiological adaptation of the existing nitrifiers (Bassin et al., 2011; Navada et al., 2019) or by a shift in the microbial community composition to favor more halotolerant bacteria (Bassin et al., 2012a; Gonzalez-Silva, 2016). The acclimation strategy may be influenced by the manner of salinity change

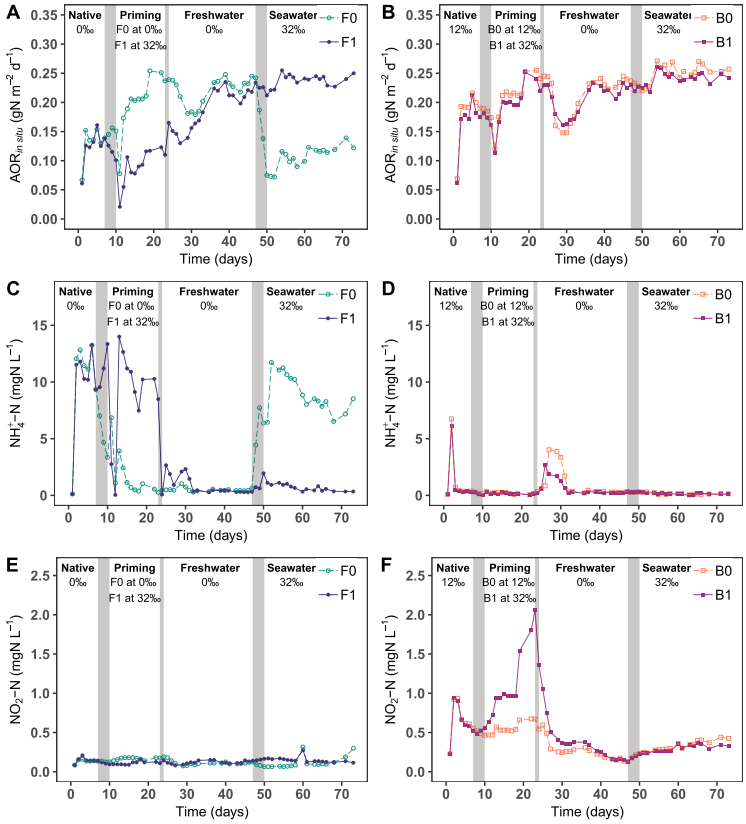


Fig. 2. Average *in situ* measurements in the MBBRs as a function of time. *In situ* specific ammonia oxidation rate ($AOR_{in\ situ}$) for treatments A) F0 and F1, and B) B0 and B1; ammonia concentration (NH_4^+-N) in the MBBR for C) F0 and F1, and D) B0 and B1; nitrite (NO_2^--N) concentration in the MBBR for E) F0 and F1, and F) B0 and B1. $AOR_{in\ situ}$ was calculated by the daily ammonia mass balance for each MBBR, assuming pseudo steady-state over 24 h. Salinities during the different phases are shown in ‰ (parts per thousand). Gray shaded regions indicate days of salinity change. Differences between duplicates were low and are not shown for simplicity.

(Bassin et al., 2012a; Navada et al., 2019). In this study, the microbial community composition in F1 changed after the priming phase (days 31–50) and became significantly different from that in F0. This indicates that the species inventory in the biofilm was influenced by priming. The compositional change was mainly due to changes in the heterotrophic community rather than in the nitrifying community. As the heterotrophic group has higher functional redundancy than nitrifiers, a larger range of microorganisms within this group can perform the same function in different salinity regimes. Despite the change in the overall community composition, the α-diversity did not change, likely because of trade-offs between priming ability and competitiveness (Rillig et al., 2015). Also, the nitrifying community composition in F0 and F1 remained highly similar (up to 90% similarity) and the same nitrifying taxa were present in both treatments. This suggests that the higher nitrification activity in F1 was due to a physiological adaptation to salinity through the production of compatible solutes rather than a compositional change in the nitrifying bacteria. The dominant AOB in this study, the genus *Nitrosomonas*, is reported to be able to adapt to seawater (Bassin et al., 2011; Foesel et al., 2008). The dominant NOB, *Candidatus Nitrotoga*, can also survive salinity increase from freshwater to seawater (Navada et al., 2019) and has been detected in marine RAS biofilms (Keuter et al., 2017). Spearman rank correlation between the nitrifying OTUs in the F and B treatments separately showed that most of the significant correlations were positive (Supplementary Information, Fig A.7). This suggests that the growth of all the nitrifying bacterial species in the various salinity regimes was similar, and that the competition between the species was not very strong. The high osmotic tolerance of nitrifying bacteria underscores the immense versatility of this bacterial group to survive in different salinities, despite the existence of exclusive freshwater and marine species. A previous study supports this observation by reporting that although freshwater, brackish water (22‰ salinity), and seawater biofilms contained several unique nitrifying OTUs, the dominant OTUs at each salinity were detected in all the three treatments (Gonzalez-Silva et al., 2016).

The nitrifying taxa were present at different relative abundances in the two F treatments, indicating that priming affected the taxa to different extents. In general, the proportion of AOB was greater in F1, whereas NOB were present at a greater relative abundance in F0. Consequently, the average ratio of AOB to NOB in F1 was double that in F0. Although F1 had the same proportion of AOB as F0 on day 45 (~12%, ~90% of which was *Nitrosomonas*), F1 had more than double the relative abundance of AOB in F0 at the end of the SW

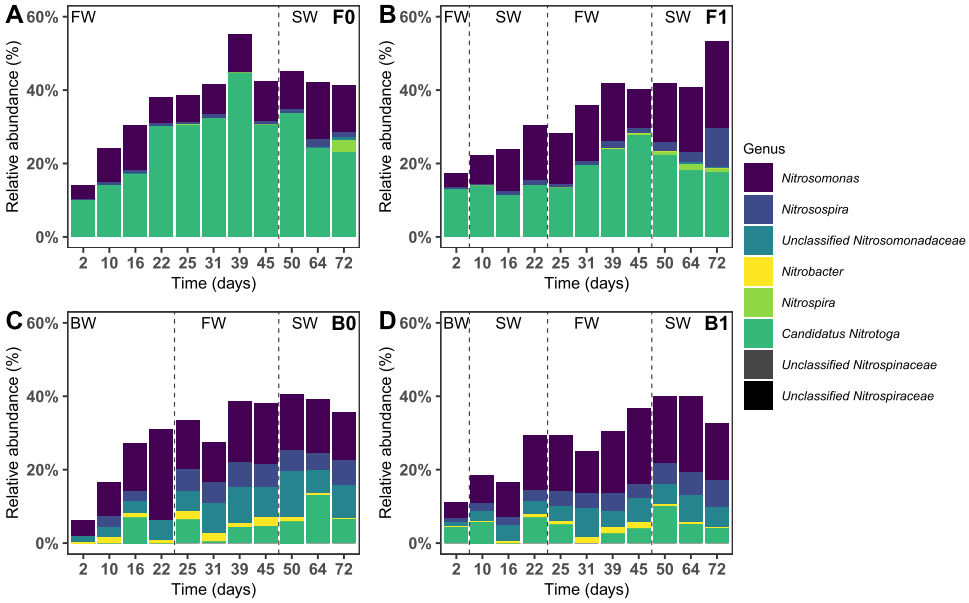


Fig. 3. Relative abundance of the nitrifying genera in treatments A) F0, B) F1, C) B0, and D) B1. Dotted lines demarcate periods in freshwater (FW), brackish-water (BW), and seawater (SW).

Table 2
The α-diversity is shown as the average (±SE) first-order diversity number, richness, and evenness during days 1–73, calculated separately for all OTUs and the nitrifying OTUs. For both the F and B treatments, the primed and unprimed treatments were not significantly different based on any of these measures ($p > 0.05$), except evenness of the nitrifying community in B0 and B1 ($p = 0.04$).

ALL OTUs	**F0**	**F1**	**B0**	**B1**
First-order diversity (N_1)	34.2 ± 3.1	41.8 ± 2.9	53.1 ± 2.8	61.6 ± 4.7
Richness (N_0)	93.5 ± 4.4	100.5 ± 2.1	114.2 ± 3.1	127.9 ± 4.7
Evenness (N_1/N_0)	0.36 ± 0.02	0.41 ± 0.02	0.46 ± 0.01	0.47 ± 0.02
NITRIFIERS	**F0**	**F1**	**B0**	**B1**
First-order diversity (N_1)	4.0 ± 0.2	4.5 ± 0.2	6.5 ± 0.4	6.0 ± 0.2
Richness (N_0)	7.3 ± 0.4	7.6 ± 0.5	9.8 ± 0.6	10.5 ± 0.2
Evenness (N_1/N_0)	0.56 ± 0.03	0.60 ± 0.03	0.66 ± 0.02	0.57 ± 0.01

phase. On day 72, the proportion of *Nitrosomonas* was twice as high in F1 (24%) than in F0 (12%), and the proportion of *Nitrosospira multiformis* was 10x higher in F1 (10%) than in F0 (1%). This suggests that priming increased the salinity acclimation of these AOB. It also indicates that AOB were more competitive than NOB in seawater, as also observed in other biofilm studies (Aslan and Simsek, 2012; Bassin et al., 2011). As ammonia oxidation is considered the rate-limiting step in the nitrification process, the increase in the proportion of AOB in F1 after seawater transfer could explain why F1 had higher nitrification capacity than F0 despite having similar nitrifying communities. The lower capacity in F0 could also have been due to inhibition of nitrifying bacteria or lower biomass of nitrifiers in F0.

The increased salt during priming may also have strengthened the biofilm structure through better settling characteristics (Goode and Allen, 2006; Moussa et al., 2006) or by shifting the overall microbial community composition towards bacteria that were efficient at producing extracellular polymeric substances (EPS). Thus, the nitrifying bacteria could have been protected against osmotic stress by the hydrated microenvironment created by the surrounding EPS (Baho et al., 2012; Flemming and Wingender, 2010). This hypothesis is plausible as nitrifiers are often found in the deeper layers of the biofilm (Okabe et al., 1996), likely because nitrifiers have low EPS production ability and slower growth rates than heterotrophs (Tsuneda et al., 2001). The protective nature of the extrapolymeric matrix may also explain why salt acclimation did not improve the salinity adaptation of nitrifying sludge in a previous study (Moussa et al., 2006). Future studies should include quantification of EPS to test this hypothesis.

In contrast to the F treatments, the nitrification activities of B0 and B1 were similar after SW transfer, indicating that seawater priming had no influence on the salinity acclimation (Fig. 1B, D). Moreover, salinity increase did not negatively impact the ammonia oxidation capacity in both B treatments. Thus, it appears that brackish water biofilms are inherently robust to salinity increase. From another perspective, it may be stated that the B treatments were already "primed" due to their native salinity of 12‰. This finding is partly in accordance with another brackish water MBBR study (22‰ salinity) where AOR_{max} reduced only by 15% after SW transfer (Gonzalez-Silva et al., 2016). However, our study contradicts other studies on brackish water adapted sludge (~11-16‰ salinity) where the reduction was 50–90% (Bassin et al., 2011; Moussa et al., 2006). This difference suggests that young brackish water biofilms may be more resilient to salinity increase than nitrifying sludge or mature biofilms. Alternatively, the distinct responses to salinity changes may have been due to different initial nitrifying communities selected by the different operating conditions (temperature, pH, ammonia loading rate/concentration etc.) in these studies. In both B treatments, the microbial community composition (both total and nitrifying) was similar throughout the experiment, indicating that brackish water biofilms contain bacteria that can physiologically adapt to varying salinities. As the B

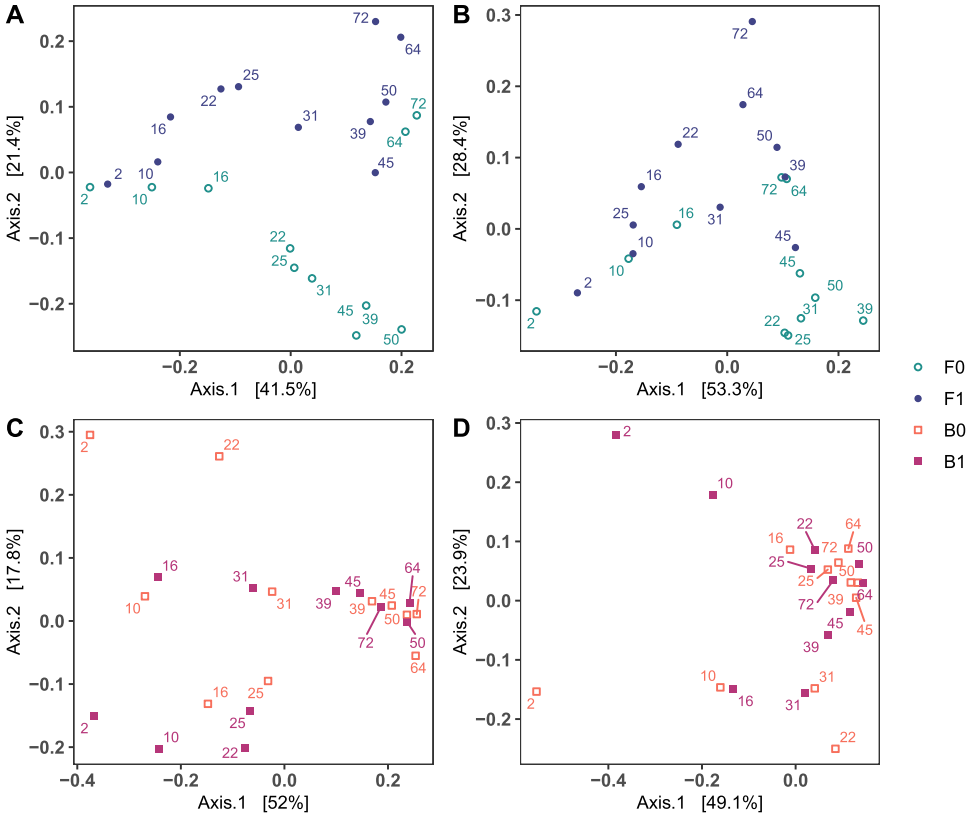


Fig. 4. Ordination by principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity between biofilm samples: A) freshwater treatments – all OTUs, B) freshwater treatments – nitrifying OTUs C) brackish water treatments – all OTUs, and D) brackish water treatments – nitrifying OTUs. Labels indicate sampling day. Square brackets show percent variance explained by each coordinate axis.

treatments had received bacteria from both FW and SW, their biofilms had a greater α-diversity than the F treatments. Further, although *Nitrosomonas* and *Candidatus Nitrotoga* were the dominant AOB and NOB in both the F and B treatments, the B treatments had a higher proportion of *Nitrosospira* and *Nitrobacter*. The greater α-diversity of nitrifiers in the B treatments may have provided functional redundancy at different salinities.

Notably, AOR_{max} in both the B treatments decreased after freshwater transfer without any change in the nitrifying community. The salinity decrease may have temporarily inhibited the nitrifying bacteria, especially obligately halophilic strains present in the biofilm. This observation contradicts a study where the ammonia oxidation capacity increased by 30% when the salinity was reduced from 20‰ (native salinity) to 0‰ (Gonzalez-Silva et al., 2016), but corroborates other studies where a decrease from the native salinity slightly reduced ammonia oxidation (Bassin et al., 2011; Sudarno et al., 2011). Bacteria are generally more resistant to a salinity decrease than a salinity increase. This is because a hypoosmotic shock usually only increases the cell volume slightly, whereas a hyperosmotic shock can cause plasmolysis (Csonka, 1989). Interestingly, AOR_{max} in B1 recovered faster than in B0 in the FW phase, suggesting that priming may have increased the capability of the biofilm to adapt to different salinities, perhaps through cross-protection.

Although all treatments had similar AOR_{max} and NOR_{max} in the beginning of the experiment, AOR_{max} increased more rapidly in the B treatments than in F. During most of the study, AOR_{max} in B was significantly greater than in F, suggesting that ammonia oxidation may be higher in BW biofilms than in FW biofilms, perhaps due to isotonic conditions (He et al., 2017) or greater α-diversity of nitrifiers. In general, the nitrite oxidation capacity increased during the FW phase, but was lower in the priming and SW phases (except in F1). Although this finding contradicts some studies (Moussa et al., 2006; Sharrer et al., 2007), it is in agreement with several other studies where NOB were more negatively affected by salinity increase than AOB (Aslan and Simsek, 2012; Bassin et al., 2011; Dinçer and Kargi, 1999; Jeong et al., 2018; Nijhof and Bovendeur, 1990). Moreover, in all treatments, the NOR_{max} was greater than the AOR_{max} immediately after SW transfer, and no nitrite accumulation was observed. But after two weeks in seawater, NOR_{max} was lower than AOR_{max} in all treatments except F1, indicating that the nitrite oxidizers did not acclimatize to the salinity, unlike the ammonia oxidizers (Fig. 1). The delayed response of the NOB highlights the importance of monitoring nitrification activity during the acclimatization period after seawater transfer. At high salt concentrations, nitrite oxidation may not generate enough energy to make

osmoregulation thermodynamically favorable (Oren, 2011). So it is especially remarkable that F1 had no decrease in nitrite oxidation, unlike that reported in many nitrification studies at high salinities (Bassin et al., 2011; Jeong et al., 2018; Sudarno et al., 2011). The differences in AOR_{max} and NOR_{max} between the F and B treatments can be related to the microbial community composition, as the B treatments had a greater proportion of AOB than NOB, while the opposite was true for F.

This study showed that osmotic stress priming, which has strong evidence in pure cultures (Andrade-Linares et al., 2016; Berga et al., 2017), can be applied to nitrifying biofilms. Thus, in bioreactors treating variable salinity effluents, the biofilms can be exposed to high salinity during the early stages of maturation to increase robustness to salinity fluctuations in the future. Specifically, in RAS for Atlantic salmon, the bioreactor can be primed before the introduction of fish into freshwater to avoid the adverse effects of salinity changes during fish production. However, many questions remain to be answered, such as, what is the minimum intensity and duration for stress priming and for how long can biofilms retain this "memory"? Further studies are required to optimize the salinity level and duration of osmotic stress priming. Also, young biofilms may be more easily influenced by environmental conditions than mature biofilms (Saur et al., 2016). In the present study, the changes in the nitrifying community until day 50 appeared to be primarily due to biofilm maturation, as seen by the overall increase in the proportion of the nitrifiers with time (Fig. 3). Further, the biofilm in this study retained the memory for at least three weeks. Other studies have proposed a time scale of two weeks as the characteristic time for microbial community development in biofilms and the conservation of biofilm memory (Saur et al., 2016). If that is the case, when the freshwater phase extends to more than a few weeks, the salinity adaptation capability may decrease. Further research is required to investigate this hypothesis. Another topic for future research is whether biofilms possess cross protection capability, for example, increased resistance to osmotic stress after prior exposure to other stressors, such as temperature or pH.

5. Conclusions

This study was undertaken to find a strategy for maintaining nitrification efficiency during salinity changes in MBBRs, especially in RAS. The results of this study showed that.

- Seawater priming changed the microbial community composition of freshwater biofilms and greatly improved nitrification during the next salinity increase. However, the nitrifying taxa did not change, suggesting that priming improved salinity acclimation through physiological adaptation of the existing nitrifiers and also perhaps by strengthening the biofilm structure.
- In contrast to freshwater biofilms, nitrification in brackish water biofilms was not influenced by priming. Also, salinity increase did not negatively affect the nitrification, nor did it change the microbial community composition. This indicates that brackish water biofilms are inherently robust to salinity increase and contain bacteria that can adapt to varying salinities.
- In conclusion, osmotic stress priming can be used as an effective microbial management strategy for improving salinity acclimation in nitrifying biofilms. Hence, prior exposure to high salinity can help biofilms adapt to salinity increases in the future. Future studies should investigate the optimal duration and intensity of osmotic stress required for priming, as well as the extent of time these biofilm "memories" can last.

Author contributions

By CRediT taxonomy: Conceptualization and experiment design: SN, FG, JK. Methodology/Resources: JK, ØM, AKT, SN. Investigation: SN, AKT, CS. Formal analysis: SN, OV. Supervision: JK, OV, FG. Writing original draft: SN, OV. Critical review of manuscript: All.

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.

Acknowledgements

This project is a part of CtrlAQUA SFI, Center for research-based innovation funded by the Research Council of Norway and the Center partners, including Krüger Kaldnes AS (#237856, #270888). The authors would like to thank the staff at Nofima for technical support and Syverin Lierhagen at NTNU for the metal analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2020.115732.

References

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26, 32–46.
Andrade-Linares, D.R., Lehmann, A., Rillig, M.C., 2016. Microbial stress priming: a meta-analysis. Environ. Microbiol. 18, 1277–1288.
APHA, 2017. In: Standard Methods for the Examination of Water and Wastewater. American Public Health Association.
Aslan, S., Simsek, E., 2012. Influence of salinity on partial nitrification in a submerged biofilter. Bioresour. Technol. 118, 24–29.
Baho, D.L., Peter, H., Tranvik, L.J., 2012. Resistance and resilience of microbial communities - temporal and spatial insurance against perturbations. Environ. Microbiol. 14, 2283–2292.
Bassin, J.P., Kleerebezem, R., Muyzer, G., Rosado, A.S., Van Loosdrecht, M.C.M., Dezotti, M., 2012a. Effect of different salt adaptation strategies on the microbial diversity, activity, and settling of nitrifying sludge in sequencing batch reactors. Appl. Microbiol. Biotechnol. 93, 1281–1294.
Bassin, J.P., Kleerebezem, R., Rosado, A.S., Van Loosdrecht, M.C.M., Dezotti, M., 2012b. Effect of different operational conditions on biofilm development, nitrification, and nitrifying microbial population in moving-bed biofilm reactors. Environ. Sci. Technol. 46, 1546–1555.
Bassin, J.P., Pronk, M., Muyzer, G., Kleerebezem, R., Dezotti, M., van Loosdrecht, M.C.M., 2011. Effect of elevated salt concentrations on the aerobic granular sludge process: linking microbial activity with microbial community structure. Appl. Environ. Microbiol. 77, 7942–7953.
Berga, M., Zha, Y., Székely, A.J., Langenheder, S., 2017. Functional and compositional stability of bacterial metacommunities in response to salinity changes. Front. Microbiol. 8, 1–11.
Cabrol, L., Poly, F., Malhautier, L., Pommier, T., Lerondelle, C., Verstraete, W., Lepeuple, A.S., Fanlo, J.L., Roux, X. Le, 2016. Management of microbial communities through transient disturbances enhances the functional resilience of nitrifying gas-biofilters to future disturbances. Environ. Sci. Technol. 50, 338–348.
Csonka, L.N., 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. 53, 121–147.
Cui, Y., Peng, C., Peng, Y., Ye, L., 2009. Effects of salt on microbial populations and treatment performance in purifying saline sewage using the MUCT process. Clean 37, 649–656.
Dinçer, A.R., Kargi, F., 1999. Salt inhibition of nitrification and denitrification in saline wastewater. Environ. Technol. 20, 1147–1153.
Flemming, H.C., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8, 623–633.
Foesel, B.U., Gieseke, A., Schwermer, C., Stief, P., Koch, L., Cytryn, E., De La Torré, J.R., Van Rijn, J., Minz, D., Drake, H.L., Schramm, A., 2008. Nitrosomonas Nm143-like ammonia oxidizers and Nitrospira marina-like nitrite oxidizers dominate the nitrifier community in a marine aquaculture biofilm. FEMS Microbiol. Ecol. 63, 192–204.
Fox, J., Weisberg, S., 2011. An R Companion to Applied Regression, Second. Ed. Thousand Oaks CA.

Gonzalez-Silva, B.M., 2016. Salinity as a Driver for Microbial Community Structure in Reactors for Nitrification and Anammox. Norwegian University of Science and Technology.
Gonzalez-Silva, B.M., Jonassen, K.R., Bakke, I., Østgaard, K., Vadstein, O., 2016. Nitrification at different salinities: biofilm community composition and physiological plasticity. Water Res. 95, 48–58.
Goode, C., Allen, D.G., 2006. Effect of calcium on moving-bed biofilm reactor biofilms. Water Environ. Res. 83, 220–232.
He, H., Chen, Y., Li, X., Cheng, Y., Yang, C., Zeng, G., 2017. Influence of salinity on microorganisms in activated sludge processes: a review. Int. Biodeterior. Biodegrad. 119, 520–527.
Hilker, M., Schwachtje, J., Baier, M., Balazadeh, S., Bäurle, I., Geiselhardt, S., Hincha, D.K., Kunze, R., Mueller-Roeber, B., Rillig, M.C., Rolff, J., Romeis, T., Schmülling, T., Steppuhn, A., van Dongen, J., Whitcomb, S.J., Wurst, S., Zuther, E., Kopka, J., 2016. Priming and memory of stress responses in organisms lacking a nervous system. Biol. Rev. 91, 1118–1133.
Hill, M.O., 1973. Diversity and evenness: a unifying notation and its consequences. Ecology 54, 427–432.
Jenkins, D.E., Chaisson, S.A., Matin, A., 1990. Starvation-induced cross-protection against osmotic challenge in *Escherichia coli*. J. Bacteriol. 172, 2779–2781.
Jeong, D., Cho, K., Lee, C.H., Lee, S., Bae, H., 2018. Effects of salinity on nitrification efficiency and bacterial community structure in a nitrifying osmotic membrane bioreactor. Process Biochem. 73, 132–141.
Keuter, S., Beth, S., Quantz, G., Spieck, E., S, K., 2017. Longterm monitoring of nitrification and nitrifying communities during biofilter activation of two marine recirculation aquaculture systems (RAS). Int. J. Aquac. Fish. Sci. 3, 051–061.
Kinyage, J.P.H., Pedersen, P.B., Pedersen, L.F., 2019. Effects of abrupt salinity increase on nitrification processes in a freshwater moving bed biofilter. Aquacult. Eng. 84, 91–98.
Lefebvre, O., Moletta, R., 2006. Treatment of organic pollution in industrial saline wastewater: a literature review. Water Res. 40, 3671–3682.
Madigan, M.T., Bender, K.S., Buckley, D.H., Sattley, W.M., Stahl, D.A., 2018. Brock Biology of Microorganisms, fifteenth ed. Pearson Education Limited.
McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS One 8.
Mitchell, A., Romano, G.H., Groisman, B., Yona, A., Dekel, E., Kupiec, M., Dahan, O., Pilpel, Y., 2009. Adaptive prediction of environmental changes by microorganisms. Nature 460, 220–224.
Moussa, M.S., Sumanasekera, D.U., Ibrahim, S.H., Lubberding, H.J., Hooijmans, C.M., Gijzen, H.J., Van Loosdrecht, M.C.M., 2006. Long term effects of salt on activity, population structure and floc characteristics in enriched bacterial cultures of nitrifiers. Water Res. 40, 1377–1388.
Navada, S., Vadstein, O., Tveten, A.-K., Verstege, G.C., Terjesen, B.F., Mota, V.C., Venkataraman, V., Gaumet, F., Mikkelsen, Ø., Kamstra, A., 2019. Influence of rate of salinity increase on nitrifying biofilms. J. Clean. Prod. 238.
Nijhof, M., Bovendeur, J., 1990. Fixed film nitrification characteristics in sea-water recirculation fish culture systems. Aquaculture 87, 133–143.
Ohashi, A., Viraj de Silva, D.G., Mobarry, B., Manem, J.A., Stahl, D.A., Rittmann, B.E., 1995. Influence of substrate C/N ratio on the structure of multi-species biofilms consisting of nitrifiers and heterotrophs. Water Sci. Technol. 32 (8), 75–84.
Okabe, S., Hiratia, K., Ozawa, Y., Watanabe, Y., 1996. Spatial microbial distributions of nitrifiers and heterotrophs in mixed-population biofilms. Biotechnol. Bioeng. 50, 24–35.
Oksanen, J., Guillaume Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, Gavin L., Solymos, P., Stevens, M.H.H., Eduard, S., Wagner, H., 2019. Vegan: Community Ecology Package. R Package Version 2.5-4.
Oren, A., 2011. Thermodynamic limits to microbial life at high salt concentrations. Environ. Microbiol. 13, 1908–1923.
Panswad, T., Anan, C., 1999. Impact of high chloride wastewater on an anaerobic/anoxic/aerobic process with and without inoculation of chloride acclimated seeds. Water Res. 33, 1165–1172.
Rillig, M.C., Rolff, J., Tietjen, B., Wehner, J., Andrade-Linares, D.R., 2015. Community priming-Effects of sequential stressors on microbial assemblages. FEMS Microbiol. Ecol. 91, 1–7.
Saur, T., Escudié, R., Santa-Catalina, G., Bernet, N., Milferstedt, K., 2016. Conservation of acquired morphology and community structure in aged biofilms after facing environmental stress. Water Res. 88, 164–172.
Sharrer, M.J., Tal, Y., Ferrier, D., Hankins, J.A., Summerfelt, S.T., 2007. Membrane biological reactor treatment of a saline backwash flow from a recirculating aquaculture system. Aquacult. Eng. 36, 159–176.
Shi, K., Zhou, W., Zhao, H., Zhang, Y., 2012. Performance of halophilic marine bacteria inocula on nutrient removal from hypersaline wastewater in an intermittently aerated biological filter. Bioresour. Technol. 113, 280–287.
Sudarno, U., Winter, J., Gallert, C., 2011. Effect of varying salinity, temperature, ammonia and nitrous acid concentrations on nitrification of saline wastewater in fixed-bed reactors. Bioresour. Technol. 102, 5665–5673.
Timmons, M.B., Ebeling, J.M., 2010. Recirculating Aquaculture, third ed. Aquaculture. Ithaca Publishing Company LLC, Ithaca, NY, U.S.A.
Tsuneda, S., Park, S., Hayashi, H., Jung, J., Hirata, A., 2001. Enhancement of nitrifying biofilm formation using selected EPS produced by heterotrophic bacteria. Water Sci. Technol. 43, 197–204.
Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.
Yu, S.M., Leung, W.Y., Ho, K.M., Greenfield, P.F., Eckenfelder, W.W., 2002. The impact of sea water flusing on biological nitrification-denitrification activated sludge sewage treatment process. Water Sci. Technol. 46, 209–216.
Zhu, S., Shen, J., Ruan, Y., Guo, X., Ye, Z., Deng, Y., Shi, M., 2016. The effects of different seeding ratios on nitrification performance and biofilm formation in marine recirculating aquaculture system biofilter. Environ. Sci. Pollut. Res. 23, 14540–14548.

Article III

Science of the Total Environment 739 (2020) 139934

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

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

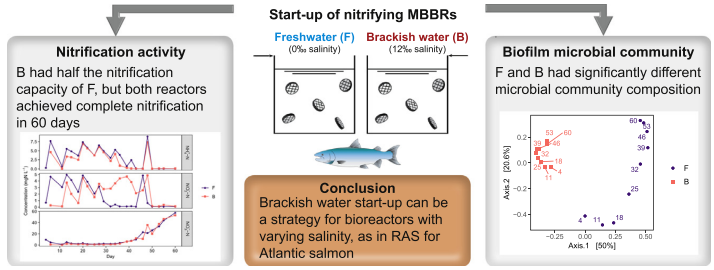
Sharada Navada [a,b,*], Marianna Sebastianpillai [c,1], Jelena Kolarevic [d], Ragnhild O. Fossmark [e], Ann-Kristin Tveten [f], Frédéric Gaumet [b], Øyvind Mikkelsen [a], Olav Vadstein [c]

[a] Department of Chemistry, NTNU Norwegian University of Science and Technology, N-7491 Trondheim, Norway
[b] Krüger Kaldnes AS (Veolia Water Technologies), N-3241 Sandefjord, Norway
[c] Department of Biotechnology and Food Science, NTNU Norwegian University of Science and Technology, N-7491 Trondheim, Norway
[d] Nofima AS, N-6600 Sunndalsøra, Norway
[e] Department of Environmental and Civil Engineering, NTNU Norwegian University of Science and Technology, N-7031 Trondheim, Norway
[f] Department of Biological Sciences, NTNU Norwegian University of Science and Technology, N-6009 Ålesund, Norway

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



ARTICLE INFO

Article history:
Received 4 April 2020
Received in revised form 1 June 2020
Accepted 1 June 2020
Available online 05 June 2020

Editor: Frederic Coulon

Keywords:
Osmotic stress
Saline RAS
Biofilter salt acclimatization
Marine MBBR
Biofilm microbial community composition
Nitrotoga

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.

* Corresponding author at: Department of Chemistry, NTNU Norwegian University of Science and Technology, N-7491 Trondheim, Norway.
E-mail address: sharada.navada@ntnu.no (S. Navada).
[1]Present address: Mowi Sandøra, N-9180 Skjervøy, Norway.

https://doi.org/10.1016/j.scitotenv.2020.139934

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

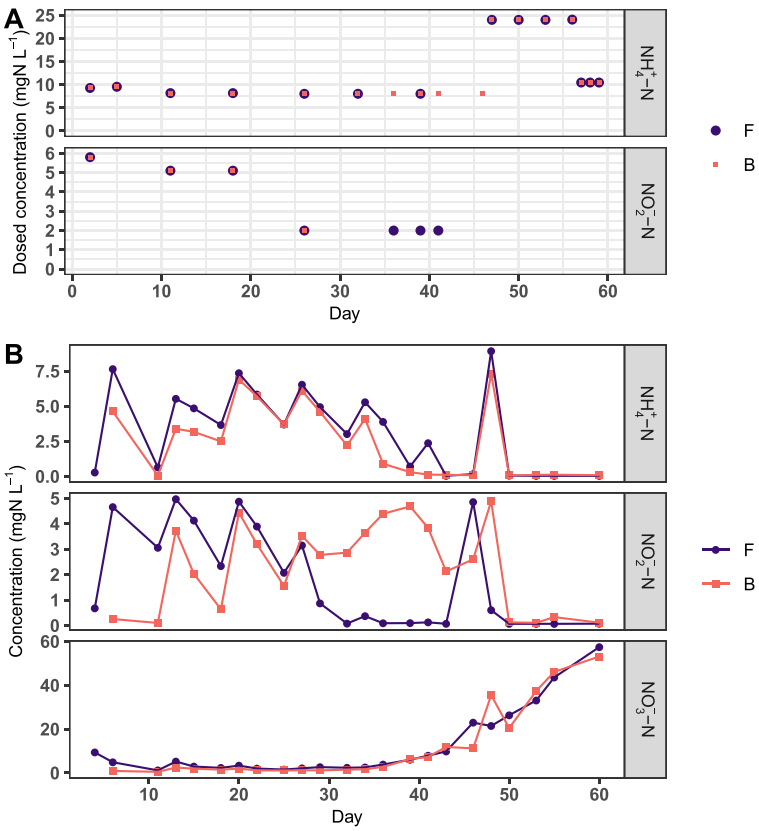


Fig. 1. A) Theoretical concentration of ammonia and nitrite in the reactors corresponding to the respective quantities of ammonium chloride and sodium nitrite dosed; B) Ammonia, nitrite, and nitrate concentration in the freshwater (F) and brackish water (B) reactors during the study. The points have 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′-gtctcgtgggctcggagatgtgtataagagacagnnnn 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***

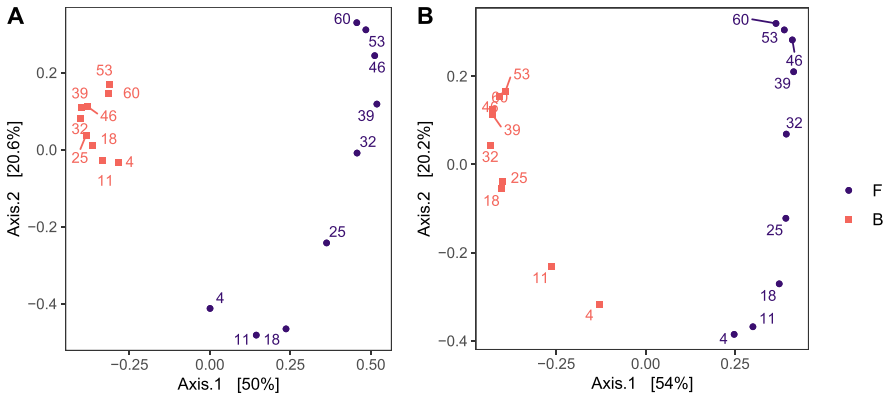


Fig. 2. Ordination by principal coordinates analysis (PCoA) based on A) Bray-Curtis (relative abundance) and B) Sørensen-Dice (presence-absence) distances between the biofilm samples. Labels indicate sampling day. Each point represents the mean data from two biofilm carriers. Square brackets show the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

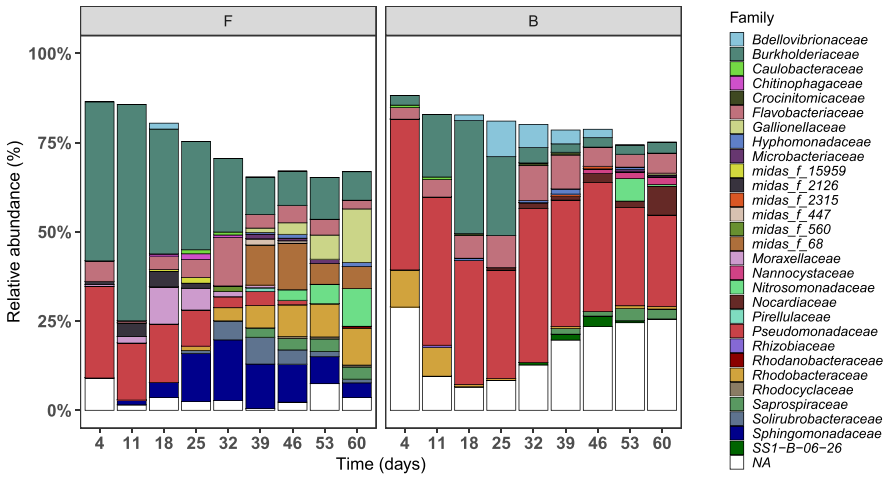


Fig. 3. Relative abundance of taxa in the freshwater (F) and brackish water (B) biofilm classified at the family level. Each data point represents the mean data from two biofilm carriers. For simplicity, only taxa present at relative abundance >1% in at least one sample are shown.

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%

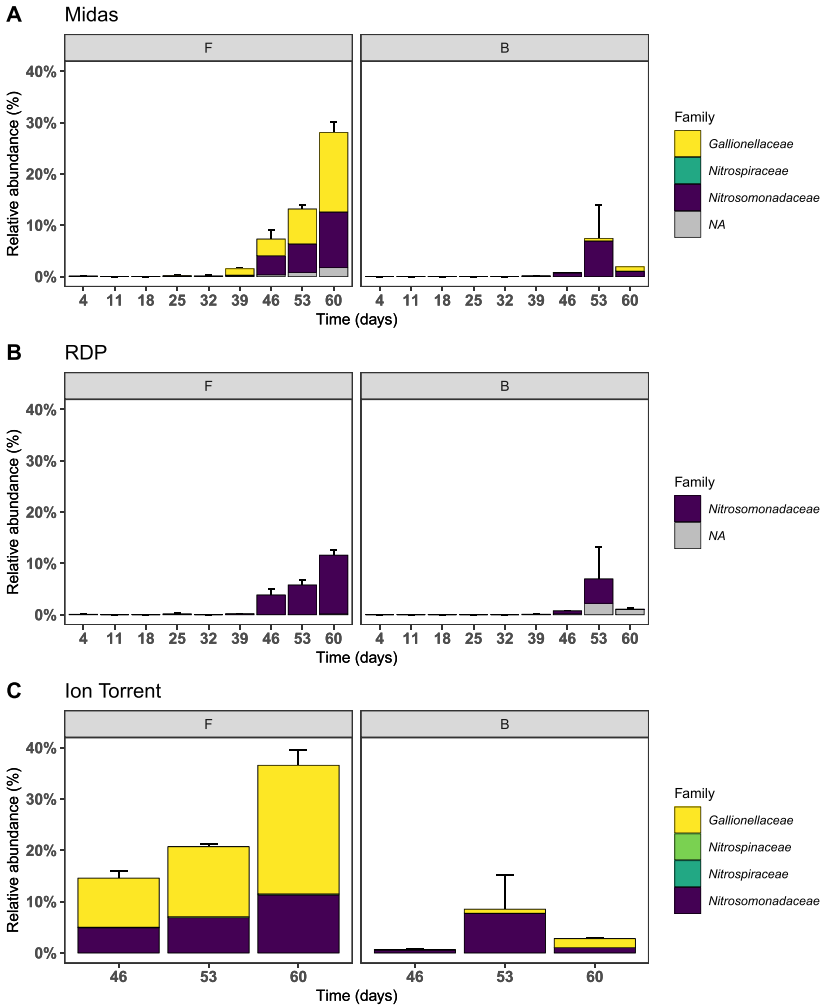


Fig. 4. Relative abundance of nitrifying bacteria in the biofilm in the freshwater (F) and brackish water (B) treatments analyzed by different methods of 16S rRNA gene amplicon sequencing. A) Illumina sequencing with classification by MiDAS3 database B) Illumina sequencing with classification by RDP 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).

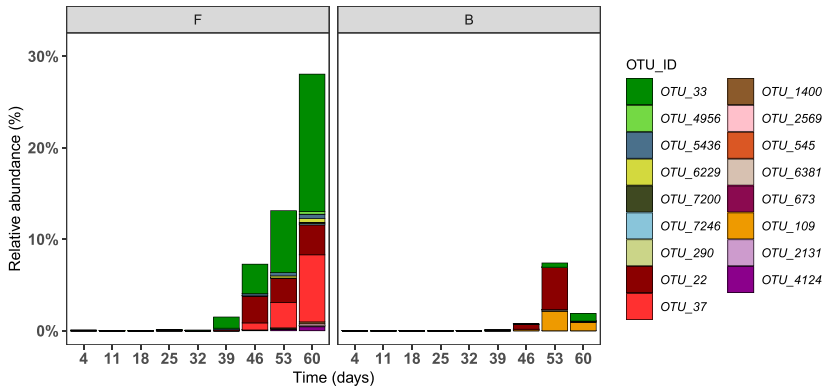


Fig. 5. Relative abundance of the nitrifying OTUs in the biofilms in the freshwater (F) and brackish water (B) treatments during the study. Each bar represents the mean of two replicate biofilm carriers. For the NOB: OTUs 33, 4956, 7246 were classified at the genus level as *Nitrotoga*; OTUs 5436, 6229, 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.

Acknowledgements

This project is a part of CtrlAQUA SFI, Center for research-based innovation funded by the Research Council of Norway (#237856, #270888) and the Center partners, including Krüger Kaldnes AS. We are grateful to Prof. Ingrid Bakke (NTNU) for helpful insights on the microbial analysis. We thank the Nofima staff for sampling and technical support, and Dag Egil Bundgaard for the physicochemical lab analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.139934.

References

Alawi, M., Off, S., Kaya, M., Spieck, E., 2009. Temperature influences the population structure of nitrite-oxidizing bacteria in activated sludge. Environ. Microbiol. Rep. 1, 184–190.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol 26, 32–46.

Bartelme, R.P., McLellan, S.L., Newton, R.J., 2017. Freshwater recirculating aquaculture system operations drive biofilter bacterial community shifts around a stable nitrifying consortium of ammonia-oxidizing archaea and comammox Nitrospira. Front. Microbiol. 8.

Bartelme, R.P., Smith, M.C., Sepulveda-Villet, O.J., Newton, R.J., 2019. Component microenvironments and system biogeography structure microorganism distributions in recirculating aquaculture and aquaponic systems. mSphere 4, 1–15.

Bassin, J.P., Kleerebezem, R., Muyzer, G., Rosado, A.S., Van Loosdrecht, M.C.M., Dezotti, M., 2012a. Effect of different salt adaptation strategies on the microbial diversity, activity, and settling of nitrifying sludge in sequencing batch reactors. Appl. Microbiol. Biotechnol. 93, 1281–1294.

Bassin, J.P., Kleerebezem, R., Rosado, A.S., Van Loosdrecht, M.C.M., Dezotti, M., 2012b. Effect of different operational conditions on biofilm development, nitrification, and nitrifying microbial population in moving-bed biofilm reactors. Environ. Sci. Technol. 46, 1546–1555.

Brailo, M., Schreier, H.J., McDonald, R., Maršić-Lučić, J., Gavrilović, A., Pećarević, M., Jug-Dujaković, J., 2019. Bacterial community analysis of marine recirculating aquaculture system bioreactors for complete nitrogen removal established from a commercial inoculum. Aquaculture 503, 198–206.

Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18, 117–143.

Csonka, L.N., 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. 53, 121–147.

Davidson, J., May, T., Good, C., Waldrop, T., Kenney, B., Terjesen, B.F., Summerfelt, S., 2016. Production of market-size North American strain Atlantic salmon Salmo salar in a land-based recirculation aquaculture system using freshwater. Aquac. Eng. 74, 1–16.

Edgar, R., 2016. SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. bioRxiv 074161.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996–998.

FAO, 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the Sustainable Development Goals. Food and Agriculture Organization of the United Nations, Rome.

Fox, J., Weisberg, S., 2011. An R Companion to Applied Regression. Second. ed. (Thousand Oaks CA).

Gonzalez-Silva, B.M., Jonassen, K.R., Bakke, I., Østgaard, K., Vadstein, O., 2016. Nitrification at different salinities: biofilm community composition and physiological plasticity. Water Res. 95, 48–58.

Gutierrez-Wing, M.T., Malone, R.F., 2006. Biological filters in aquaculture: trends and research directions for freshwater and marine applications. Aquac. Eng. 34, 163–171.

Hatzenpichler, R., 2012. Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. Appl. Environ. Microbiol. 78, 7501–7510.

He, H., Chen, Y., Li, X., Cheng, Y., Yang, C., Zeng, G., 2017. Influence of salinity on microorganisms in activated sludge processes: a review. Int. Biodeterior. Biodegrad. 119, 520–527.

Hill, M.O., 1973. Diversity and evenness: a unifying notation and its consequences. Ecology 54, 427–432.

Jiang, W., Tian, X., Li, L., Dong, S., Zhao, K., Li, H., 2019. Temporal bacterial community succession during the start-up process of biofilters in a cold-freshwater recirculating aquaculture system. Bioresour. Technol. 287.

Karkman, A., Mattila, K., Tamminen, M., Virta, M., 2011. Cold temperature decreases bacterial species richness in nitrogen-removing bioreactors treating inorganic mine waters. Biotechnol. Bioeng. 108, 2876–2883.

Keen, G.A., Prosser, J.I., 1987. Steady state and transient growth of autotrophic nitrifying bacteria. Arch. Microbiol. 147, 73–79.

Keuter, S., Beth, S., Quantz, G., Spieck, E., S, K., 2017. Longterm monitoring of nitrification and nitrifying communities during biofilter activation of two marine recirculation aquaculture systems (RAS). Int. J. Aquac. Fish. Sci. 3, 051–061.

Kinyage, J.P.H., Pedersen, P.B., Pedersen, L.F., 2019. Effects of abrupt salinity increase on nitrification processes in a freshwater moving bed biofilter. Aquac. Eng. 84, 91–98.

Kits, K.D., Sedlacek, C.J., Lebedeva, E.V., Han, P., Bulaev, A., Pjevac, P., Daebeler, A., Romano, S., Albertsen, M., Stein, L.Y., Daims, H., Wagner, M., 2017. Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. Nature 549, 269–272.

Kuhn, D.D., Drahos, D.D., Marsh, L., Flick, G.J., 2010. Evaluation of nitrifying bacteria product to improve nitrification efficacy in recirculating aquaculture systems. Aquac. Eng. 43, 78–82.

Kumar, V.J.R., Joseph, V., Philip, R., Singh, I.S.B., 2010. Nitrification in brackish water recirculating aquaculture system integrated with activated packed bed bioreactor. Water Sci. Technol. 61, 797–805.

Lefebvre, O., Moletta, R., 2006. Treatment of organic pollution in industrial saline wastewater: a literature review. Water Res. 40, 3671–3682.

Li, C., Liang, J., Lin, X., Xu, H., Tadda, M.A., Lan, L., Liu, D., 2019. Fast start-up strategies of MBBR for mariculture wastewater treatment. J. Environ. Manag. 248, 109267.

Liu, D., Li, C., Guo, H., Kong, X., Lan, L., Xu, H., Zhu, S., Ye, Z., 2019. Start-up evaluations and biocarriers transfer from a trickling filter to a moving bed bioreactor for synthetic mariculture wastewater treatment. Chemosphere 218, 696–704.

Manthe, D.P., Malone, R.F., 1987. Chemical addition for accelerated biological filter acclimation in closed blue crab shedding systems. Aquac. Eng. 6, 227–236.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8.

Michaud, L., Lo Giudice, A., Troussellier, M., Smedile, F., Bruni, V., Blancheton, J.P., 2009. Phylogenetic characterization of the heterotrophic bacterial communities inhabiting a marine recirculating aquaculture system. J. Appl. Microbiol. 107, 1935–1946.

Navada, S., Vadstein, O., Tveten, A.-K., Verstege, G.C., Terjesen, B.F., Mota, V.C., Venkataraman, V., Gaumet, F., Mikkelsen, Ø., Kamstra, A., 2019. Influence of rate of salinity increase on nitrifying biofilms. J. Clean. Prod. 238.

Navada, S., Vadstein, O., Gaumet, F., Tveten, A.-K., Spanu, C., Mikkelsen, Ø., Kolarevic, J., 2020. Biofilms remember: osmotic stress priming as a microbial management strategy for improving salinity acclimation in nitrifying biofilms. Water Res. 176, 115732. https://doi.org/10.1016/j.watres.2020.115732.

Nemergut, D.R., O'Neill, S.P., Bilinski, T.M., Stanish, L.F., Knelman, J.E., Wickey, P., Schmidt, S.K., Darcy, J.L., Lynch, R.C., Ferrenberg, S., Fukami, T., 2013. Patterns and processes of microbial community assembly. Microbiol. Mol. Biol. Rev. 77, 342–356.

Nierychlo, M., Andersen, K.S., Xu, Y., Green, N., Albertsen, M., Dueholm, M.S., Nielsen, P.H., 2019. Species-level Microbiome Composition of Activated Sludge - Introducing the MiDAS3 Ecosystem-specific Reference Database and Taxonomy. bioRxiv.

Nijhof, M., Bovendeur, J., 1990. Fixed film nitrification characteristics in sea-water recirculation fish culture systems. Aquaculture 87, 133–143.

Oksanen, J., Guillaume Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, Gavin, L., Solymos, P., Stevens, M.H.H., Eduard, S., Wagner, H., 2019. Vegan: Community Ecology Package. R Package Version 2.5–4.

Oren, A., 2011. Thermodynamic limits to microbial life at high salt concentrations. Environ. Microbiol. 13, 1908–1923.

Pjevac, P., Schauberger, C., Poghosyan, L., Herbold, C.W., van Kessel, M.A.H.J., Daebeler, A., Steinberger, M., Jetten, M.S.M., Lücker, S., Wagner, M., Daims, H., 2017. AmoA-targeted polymerase chain reaction primers for the specific detection and quantification of comammox Nitrospira in the environment. Front. Microbiol. 8, 1–11.

Rud, I., Kolarevic, J., Holan, A.B., Berget, I., Calabrese, S., Terjesen, B.F., 2017. Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. Aquac. Eng. 78, 50–62.

Rusten, B., Eikebrokk, B., Ulgenes, Y., Lygren, E., 2006. Design and operations of the Kaldnes moving bed biofilm reactors. Aquac. Eng. 34, 322–331.

Sauder, L.A., Engel, K., Stearns, J.C., Masella, A.P., Pawliszyn, R., Neufeld, J.D., 2011. Aquarium nitrification revisited: Thaumarchaeota are the dominant ammonia oxidizers in freshwater aquarium biofilters. PLoS One 6.

Sudarno, U., Bathe, S., Winter, J., Gallert, C., 2010. Nitrification in fixed-bed reactors treating saline wastewater. Appl. Microbiol. Biotechnol. 85, 2017–2030.

Sudarno, U., Winter, J., Gallert, C., 2011. Effect of varying salinity, temperature, ammonia and nitrous acid concentrations on nitrification of saline wastewater in fixed-bed reactors. Bioresour. Technol. 102, 5665–5673.

Tal, Y., Watts, J.E.M., Schreier, S.B., Sowers, K.R., Schreier, H.J., 2003. Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in a closed recirculated mariculture system. Aquaculture 215, 187–202.

Terjesen, B.F., Summerfelt, S.T., Nerland, S., Ulgenes, Y., Fjæra, S.O., Megård Reiten, B.K., Selset, R., Kolarevic, J., Brunsvik, P., Bæverfjord, G., Takle, H., Kittelsen, A.H., Åsgård, T., 2013. Design, dimensioning, and performance of a research facility for studies on the requirements of fish in RAS environments. Aquac. Eng. 54, 49–63.

U.S. EPA, 1983. Methods for Chemical Analysis of Water and Wastes. U.S. Environmental Protection Agency, Cincinnati, OH, USA.

Van Kessel, M.A.H.J., Speth, D.R., Albertsen, M., Nielsen, P.H., Op Den Camp, H.J.M., Kartal, B., Jetten, M.S.M., Lücker, S., 2015. Complete nitrification by a single microorganism. Nature 528, 555–559.

Wagner, F.B., Nielsen, P.B., Boe-Hansen, R., Albrechtsen, H.J., 2016. Copper deficiency can limit nitrification in biological rapid sand filters for drinking water production. Water Res. 95, 280–288.

Wickham, H., 2007. Reshaping data with the reshape package. J. Stat. Softw. 21, 1–20.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.

Winkler, M.K.H., Meunier, C., Henriet, O., Mahillon, J., Suárez-Ojeda, M.E., Del Moro, G., De Sanctis, M., Di Iaconi, C., Weissbrodt, D.G., 2018. An integrative review of granular

sludge for the biological removal of nutrients and recalcitrant organic matter from wastewater. Chem. Eng. J. 336, 489–502.

Wittebolle, L., Verstraete, W., Boon, N., 2009. The inoculum effect on the ammonia-oxidizing bacterial communities in parallel sequential batch reactors. Water Res. 43, 4149–4158.

Zhu, S., Shen, J., Ruan, Y., Guo, X., Ye, Z., Deng, Y., Shi, M., 2016. The effects of different seeding ratios on nitrification performance and biofilm formation in marine recirculating aquaculture system biofilter. Environ. Sci. Pollut. Res. 23, 14540–14548.

Article IV

Seeding with brackish-water biofilm as a startup strategy for salinity acclimation in freshwater nitrifying bioreactors

Sharada Navada[a,b,*], Frédéric Gaumet[b], Ann-Kristin Tveten[c], Jelena Kolarevic[d], Olav Vadstein[e]

[a] Department of Chemistry, NTNU - Norwegian University of Science and Technology, N-7491 Trondheim, Norway

[b] Krüger Kaldnes AS (Veolia Water Technologies), N-3241 Sandefjord, Norway

[c] Department of Biological Sciences, NTNU Norwegian University of Science and Technology, N-6009 Ålesund, Norway

[d] Nofima AS, N-6600 Sunndalsøra, Norway

[e] Department of Biotechnology and Food Science, NTNU - Norwegian University of Science and Technology, N-7491 Trondheim, Norway

Abstract

High or variable salinity effluents can be challenging for the biological nitrification process in water treatment systems. A special case is recirculating aquaculture systems (RAS) for salmonids where the salinity is typically increased during the production cycle. As a disruption in the nitrification process can be detrimental to the fish, it is vital to maintain nitrification performance in RAS during salinity changes. This study investigated whether seeding with salinity-acclimated carriers during freshwater start-up could improve salinity adaptation in nitrifying bioreactors. Moving bed biofilm reactors (MBBR) were started with virgin carriers and seeded with mature biofilm carriers acclimated to freshwater (F), brackish water (B, 12‰ salinity) and a 1:1 mix of both (FB). All reactors were started up in freshwater and the salinity was increased to seawater after ∼7 weeks. While the F and FB treatments had a 65-75% decrease in ammonia oxidation capacity immediately after seawater transfer, B had only a ∼20% reduction. After 40 days in seawater, ammonia oxidation recovered completely and became similar in all treatments. However, nitrite accumulation was observed in all treatments several days after the salinity increase, with the least accumulation in B. The type of seeding influenced the composition of the nitrifying community in the new biofilms (developed in freshwater), but did not influence the final composition after ∼6 weeks of seawater acclimation. The findings indicate that seeding with brackish water biofilm is a viable strategy for accelerating start-up and improving salinity acclimation in freshwater nitrifying bioreactors. However, as nitrite can be toxic to fish, it is important to closely monitor the nitrite concentration for a prolonged period (several days or weeks) after salinity increase.

Keywords

salt acclimatization; osmotic stress; ammonia nitrogen oxidation; marine recirculating aquaculture systems (RAS); biofilter inoculation

1 Introduction

Recirculating aquaculture systems (RAS) are a technology for producing fish in land-based systems with water treatment and reuse. RAS for growing anadromous fish such as Atlantic salmon (*Salmo salar L.*) face the special challenge of salinity increase after smoltification i.e., when the fish has undergone a physiological transition that allows it to adapt from freshwater to seawater. Thus, RAS for salmonids may be operated on variable salinities ranging from fresh-(0-3‰ salinity) to brackish- (12-22‰ salinity) to seawater (32-35‰ salinity) during different production periods. Salinity changes can disrupt the performance of the water treatment processes in the RAS, especially the nitrification process (Chen et al., 2006). Nitrification is a biological process where the toxic ammonia produced by the fish is successively converted to nitrite and nitrate. Typically, nitrification is a two-step process performed by two distinct microbial guilds: ammonia oxidizing microorganisms (AOM, includes ammonia oxidizing bacteria (AOB) and archaea (AOA)) that perform the first step of oxidizing ammonia to nitrite; and nitrite oxidizing bacteria (NOB) that convert nitrite to nitrate. Some species within the genus *Nitrospira* are capable of complete ammonia oxidation to nitrate (Daims et al., 2015). The microbes performing the nitrification process can be sensitive to salinity changes (Madigan et al., 2018). Thus, freshwater bioreactors can undergo a severe reduction in nitrification rate when the salinity is increased (Bassin et al., 2011; Gonzalez-Silva, 2016; Moussa et al., 2006; Navada et al., 2020b; Navada et al., 2019). As a reduction in the nitrification efficiency can quickly lead to ammonia/nitrite accumulation and a consequent risk to the fish, it is necessary to develop strategies to increase the salinity tolerance of RAS bioreactors.

A previous study showed that the salinity tolerance of nitrifying freshwater biofilms can be increased by seawater priming (Navada et al., 2020b). This implies that the first salinity increase is the most disruptive, whereas subsequent salinity changes have a lower impact on the nitrification rate. Thus, it is important to make the bioreactor robust to salinity before the fish are introduced into the system. Although seawater priming is a promising strategy, it is time-intensive, as salinity acclimation during the priming stage can require two weeks (Navada et al., 2020b). Brackish water (12-22‰ salinity) biofilms are more robust to salinity increases compared to freshwater biofilms, likely due to inherent "priming" (Gonzalez-Silva et al., 2016; Li et al., 2019; Navada et al., 2020b). Further, microbes can adapt more easily to a reduction in salinity than an increase (Csonka, 1989). This suggests that a feasible strategy is to start in brackish- or seawater, followed by a reduction in salinity before the fish are introduced to the RAS. However, nitrifying bioreactors usually take longer to start up at higher salinities, especially

in seawater (Chen et al., 2006; Nijhof and Bovendeur, 1990). Our recent study showed that complete nitrification can commence in brackish water (12‰ salinity) bioreactors in similar time as in freshwater (Navada et al., 2020a). However, the nitrification capacity in the brackish water biofilm was only half that in freshwater and the microbial community composition was still evolving, suggesting that the brackish biofilm did not develop to the extent as the freshwater biofilm. Further, a salinity decrease in brackish water biofilms can also lead to an initial reduction in the ammonia and nitrite oxidation capacity (Navada et al., 2020b). This implies that an acclimation period of ∼2-3 weeks may be necessary to adapt the reactor to freshwater before introducing the fish to the system. Thus, we wanted to investigate if the bioreactor start-up time could be reduced while conferring salinity tolerance simultaneously.

In industrial and municipal water treatment systems, seed carriers or commercial inocula are commonly added to reduce the startup time by providing the initial bacterial culture (Nogueira et al., 2002). Inoculation has been shown to accelerate the start-up of nitrifying bioreactors in aquaculture (Carmignani and Bennett, 1977; Perfettini and Bianchi, 1990). Moreover, the addition of halophilic bacteria can improve salinity adaptation in nitrifying bioreactors (Panswad and Anan, 1999; Shi et al., 2012; Sudarno et al., 2010). However, commercial salinity-adapted inocula may be expensive, difficult to procure, and can pose a biosecurity risk in RAS. Further, commercial inoculum may eventually be outcompeted by the local microbial community. Thus, adding biofilm carriers matured at the same RAS facility appears to be a better strategy than the addition of commercial inocula. For instance, a previous study showed that startup time for seawater bioreactors reduced with the addition of seed media, but not with commercial nitrifying inoculum or with freshwater seed media (Bower and Turner, 1981). Further, if the salinity in the bioreactor is different from the original salinity of the seed carriers for a prolonged period, the microbial community composition may shift. In one study, bioreactors that were started in freshwater with a commercial nitrifying inoculum adapted to 11‰ salinity had higher diversity than a seawater bioreactor with the same inocula (Grommen et al., 2005). In the same study, the ammonia-oxidizing community composition became more diverse after approximately one month in freshwater, which could indicate the selection of freshwater bacteria. Thus, although salt-acclimated inoculum can improve the salinity tolerance, changes in the water salinity may lead to competition between the inoculated and the local microbes, selecting for those that are most suited to the salinity regime. We know of no studies that investigate the salinity tolerance of nitrifying bioreactors that have been seeded with salinity-acclimated biofilm during the freshwater startup phase.

The objective of this study was to compare the salinity acclimation in nitrifying moving bed biofilm reactors (MBBR) started up in freshwater with seed carriers acclimated to freshwater, brackish water, and a 1:1 mix of both. We hypothesized that nitrification in the reactors seeded with salinity-acclimated carriers would be less impacted by a salinity increase than those with non-acclimated carriers. Further, we investigated the nitrification activity after the salinity increase to study the salinity acclimation in the bioreactors.

2 Materials & Methods

2.1 Experimental design and setup

The experimental setup was similar to that described previously (Navada et al., 2019). The experiment was performed on continuously operated MBBRs with three treatments in duplicate. All the reactors were started in freshwater with white virgin carriers and seeded with black mature biofilm carriers acclimated to different salinities. Treatments F and B were seeded with freshwater (0‰ salinity) and brackish water (12‰ salinity) acclimated biofilm carriers respectively, whereas treatment FB was seeded with a 1:1 mix of fresh- and brackish water acclimated biofilm carriers (Fig. 1). The seed carriers constituted 10% of the total carriers in each MBBR. After 47 days of start-up in freshwater, salinity in all the reactors was increased to 32% (seawater) over three days (salinities $\sim$0‰ $\rightarrow$10‰ $\rightarrow$20‰ $\rightarrow$32‰) as described in Navada et al., 2020b. Thereafter, the reactors were monitored for 40 days to observe the recovery after seawater transfer.

The biofilm carriers used for seeding were taken from a fresh- and brackish water RAS MBBR at the Nofima Center for Recirculating Aquaculture, Sunndalsøra, Norway. Prior to the experimental period, these carriers were transferred into two experimental MBBRs (37 L water volume each) in fresh- and brackish water, respectively. These experimental reactors were continuously operated at 13-14°C and pH 8.1 under similar ammonia loading rates for eight weeks (loading rate $\sim$1 gN m^{-2} d^{-1} at the end of 8 weeks). This was to ensure that the fresh- and brackish water carriers had similar operating conditions before the experiment. On day 0, six experimental MBBRs were filled ($\sim$35% by volume) with white virgin carriers and seeded with black mature biofilm carriers ($\sim$10% of total carriers). The virgin and seed carriers were the same size and shape (AnoxK^{TM} Chip P, Krüger Kaldnes, Norway). In treatment FB, the brackish water seed carriers were marked with a cable tie to distinguish them from the freshwater seed carriers (Supplementary Information, Figure S1).

The MBBRs were operated at 15°C, pH 7.6 $\pm$ 0.3. The reactors were aerated with an average airflow of 50 L min^{-1} during the experiment (dissolved oxygen

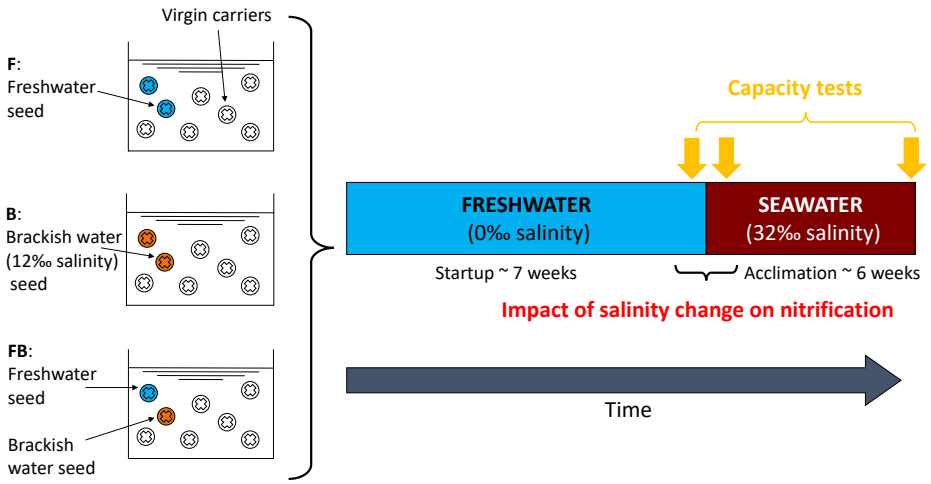


Figure 1: Experimental design. White virgin biofilm carriers were started up in MBBRs with different seeding strategies. Treatments F and B were seeded with black mature biofilm carriers acclimated to fresh- and brackish water, respectively. Treatment FB was seeded with a 1:1 mix of fresh- and brackish water acclimated carriers. The seed carriers constituted 10% of the total carriers in each reactor. The MBBRs were started up in freshwater and thereafter transferred to seawater to compare the impact of salinity change on nitrification performance.

saturation 50-100%). During the first 20 days of start-up, 8.3 g of D+ saccharose was added daily to each reactor, to boost biofilm formation by heterotrophic bacteria (Bassin et al., 2012). The reactors were operated on synthetic medium (flow rate ~0.5-8.5 mL min^{-1}) with an ammonia concentration of 670 – 2260 mgN L^{-1} and macronutrients (Navada et al., 2020b). In addition, extra $NaHCO_3$ was added to the synthetic medium (1.2-3.5 g $NaHCO_3$ L^{-1}) to ensure residual alkalinity and maintain the pH. Per 250 L of synthetic medium, 100 mL of a micronutrient solution was added. The micronutrient solution contained trace elements in the following concentrations (g L^{-1}): 1.828 $CuSO_4 \cdot 5H_2O$, 1.875 $CoCl_2 \cdot 6H_2O$, 1.883 $NiCl_2 \cdot 6H_2O$, 11.262 $ZnSO_4 \cdot 7H_2O$, 1.768 $NaMoO_4 \cdot 2H_2O$, and 13.943 $MnCl_2 \cdot 4H_2O$ (adapted from Wagner et al., 2016). During the freshwater start-up phase, the same ammonia loading rate was provided to all treatments, and this was increased to adjust to the increasing nitrification rate (by increasing the flowrate or the concentration of ammonia in the medium). After seawater transfer, we adjusted the ammonia loading rate to different treatments so that the ammonia concentration in the reactor was high enough to not be the limiting substrate (>1 mgN L^{-1}). In addition to the synthetic medium, dilution

water (flow rate 96 ± 6 mL min^{-1}) was provided to each reactor via a common buffer tank. The salinity in the reactors was controlled by adjusting the salinity of this buffer tank by blending freshwater and seawater in the desired ratio (Navada et al., 2019).

Temperature, pH, dissolved oxygen, salinity, feed flowrate, makeup flow rate, and air flow were measured using methods described previously (Navada et al., 2019). Ammonia and nitrite concentration were measured using the respective Merck test kits (Navada et al., 2020b). To measure the nitrate concentration, water samples were filtered with Acrodisc® and frozen to -20°C. The nitrate concentration in the thawed samples was measured using a flow injection autoanalyzer (Flow Solution IV, OI Analytical, USA) using Method 353.2 (U.S. EPA, 1983).

2.2 Nitrification performance

On days 45, 51, and 87, capacity tests were conducted to determine the maximum ammonia oxidation rate (AOR_{max}) and maximum nitrite accumulation rate (NAR_{max}). Each capacity test was performed by operating the MBBR in a batch mode (Navada et al., 2020b). Each MBBR was dosed a spike solution (200-220 mL) containing $(NH_4)_2SO_4$, $NaHCO_3$ (7.14 g as $CaCO_3$ per g NH_4^+-N), and $NaNO_2$ prepared in deionized water. The concentration of the spike solution was adjusted as per the *in situ* ammonia and nitrite concentration to obtain a starting concentration of 15-33 mgN L^{-1} (ammonia) and 6-20 mgN L^{-1} (nitrite) in the MBBR. After adding the spike, water samples were taken every 7-20 minutes, filtered with 0.45µm Acrodisc® syringe filters, and frozen at -20°C. During each test, eight samples were taken per reactor. The samples were analyzed using the flow injection autoanalyzer mentioned previously, according to U.S. EPA Method 350.1 for ammonia and Method 353.2 for nitrite and nitrate (U.S. EPA, 1983).

On days 46, 52, and 88, separate capacity tests were conducted on the white virgin carriers in jacketed glass MBBRs (effective volume: 1 L). The goal was to separately measure the nitrification capacity in the newly developed biofilm. The glass reactors were filled with either fresh- or seawater, according to the salinity in the main MBBRs. From each main MBBR, 51 white carriers were extracted, rinsed gently, and transferred to the glass reactors. Each glass MBBR was dosed with 5-10 mL of spike solution to achieve a starting concentration of 24-35 mgN L^{-1} (ammonia) and 5-20 mgN L^{-1} (nitrite) in the MBBR. Water samples were taken and analyzed using the same procedures as the main capacity tests. The operating conditions in the glass reactors were similar to those in the main reactors. After the tests, the carriers were returned to the main MBBRs.

2.3 Analysis of nitrifying community composition

On day 0, four black carriers were sampled from each of the fresh- and brackish water MBBRs (used for seeding). On days 45 and 88, two virgin (white) and two seed (black) biofilm carriers were sampled from each reactor and preserved at -80°C. From the FB reactors, the fresh- and brackish water seed carriers were collected separately (two each). To investigate the community composition of the intake water, two samples each were taken from the freshwater (days 27 and 45) and seawater (days 51 and 88) sources. Each water sample (∼200 mL) was filtered through a 0.22 µm filter (Sterivex^{TM}, Merck, Germany) and these filters were preserved at -20°C. The samples were analyzed by 16S rRNA gene amplicon sequencing on Ion Personal Genome Machine^{TM} using the methods described previously (Navada et al., 2020b; Navada et al., 2019). Sequences will be deposited in Genbank with accession numbers.

2.4 Data analysis

The specific *in situ* ammonia oxidation rate (AOR) in each reactor was calculated by the ammonia mass balance (assuming pseudo steady state between sampling) normalized to the total protected surface area of the biofilm carriers. The specific *in situ* nitrite oxidation rate (NOR) was calculated similarly from the difference between the mass of ammonia oxidized and the nitrite in the MBBR effluent. The ammonia oxidation efficiency (AOX) was calculated based on the influent and effluent ammonia concentration. The nitrite oxidation efficiency (NOX) was calculated by subtracting the amount of nitrite in the effluent from the amount of ammonia oxidized, normalized to the latter. For the capacity tests, the slope of the ammonia (or nitrite) concentration vs time was calculated by robust regression. Linear least squares regression is influenced by outliers and extreme data points. To reduce the sensitivity to unusual data, we chose to perform robust regression instead of removing outliers. Robust regression corrects for potential outliers by downweighing data points with higher residuals (Fox and Weisberg, 2018). To measure the zero-order kinetics, only the data points where the concentration of ammonia was >0.5 mgN L^{-1} were considered for analysis. Normality of the residuals were checked using Shapiro-Wilk tests. The AOR_{max} and NAR_{max} were calculated from the slopes of the ammonia and nitrite vs time, respectively. When necessary, the maximum nitrite oxidation rate (NOR_{max}) was calculated from the sum of the slopes of ammonia and nitrite vs time. The hypotheses of similarity of slopes between treatments was tested using analysis of covariance (ANCOVA) (Fox and Weisberg, 2011; Navada et al., 2019).

The operational taxonomic unit (OTU) table from the microbial analysis was normalized to the sum of sample reads. OTUs with a maximum of less than 0.1%

in any sample were removed. The α-diversity of each sample was estimated by calculating three indicators: richness (count of OTUs, N_0), first-order diversity number ($N_1 = e^H$, where H refers to the Shannon diversity index), and evenness (N_1/N_0) (Hill, 1973). Principal coordinates analysis (PCoA) was used as an ordination method to visualize the Bray-Curtis and Sørensen-Dice distances between samples. Subsequently, we performed permutational multivariate analysis of variance (PERMANOVA) to test the hypotheses of equal community composition between groups of samples (9999 permutations) (Anderson, 2001). The 'betadisper' function (package: vegan) was used to test the assumption of multivariate homogeneity of dispersions (variances between replicates). We used the function 'pairwise.adonis' (9999 permutations) to compare the pairwise differences between the β-diversity of the treatments (Martinez Arbizu, 2020). Analysis of variance (ANOVA) followed by Tukey posthoc test was used to test the hypothesis of equal distances between the nitrifying community composition of treatment pairs on days 45 and 88. A confidence interval of 95% was used for all statistical analyses. The data analysis and statistics were conducted in R software (Version 4.0) with packages MASS (for robust regression using function 'rlm' with psi = bisquare), vegan, phyloseq, and ggplot2 (H. Wickham, 2007; McMurdie and Holmes, 2013; Oksanen et al., 2019).

3 Results

3.1 Nitrification activity (*in situ*)

The nitrification rate in the freshwater phase increased rapidly after the first month, as seen by the increase in the nitrate concentration (Fig. 2A), AOR, and NOR (Fig. 2B). There was a slight difference in the development of nitrification activity between treatments: nitrification developed the fastest in treatment FB, next in F, and slowest in B. The AOX and NOX in F and FB treatments were consistently >95% after day 30 until the salinity change (Fig. 2C). However, the brackish water treatment achieved this level of efficiency after day 39, indicating a slower onset of nitrification than in the other two treatments. During the freshwater phase, the ammonia loading rate was adjusted to adapt to the increasing ammonia oxidation rate. The ammonia concentration varied between 0.03 – 30 mgN L^{-1} (except treatment F on day 45, where the ammonia concentration was 62 mgN L^{-1}). With one exception (day 45, F), nitrite was consistently below 10 mgN L^{-1} throughout the freshwater phase.

Upon salinity increase from freshwater to seawater, nitrification in the B treatment was the least impacted. Immediately after seawater transfer (day 50), AOR and NOR in both F and FB reduced by 60-65%, whereas it reduced only by ∼20%

in B. However, the reduction in B was not statistically significant and the AOR recovered within ∼5 days. After about 10 days in seawater, the nitrification rate (AOR and NOR) in FB recovered to similar levels as before the salinity change. In comparison, the AOR in treatment F took about a month to recover, while the NOR had not recovered completely by the end of the study. In all the treatments, the ammonia concentration was >1mgN L^{-1} after seawater transfer (with few exceptions), suggesting that the AOR was not limited by the substrate.

Nitrite concentration continued to be <10 mgN L^{-1} in the first five days after salinity change. However, after day 58, the nitrite concentration increased to 22 mgN L^{-1} in one of the B treatments, but subsided after day 62. A similar nitrite peak occurred in the other B replicate on day 69 (18 mgN L^{-1}). After two weeks in seawater (day 74), the nitrite concentration in the B treatment was mostly <5 mgN L^{-1}. Similar peaks were observed in the other treatments, with nitrite values as high as 48 mgN L^{-1}. The peaks occurred at different times in the treatment replicates, indicating a component of stochasticity in the events.

3.2 Nitrification capacity tests

At the end of the freshwater phase (days 45-46), there was no significant difference between the AOR_{max} in the treatments, both in the main and the glass capacity tests (Fig. 3A, B). On day 45, the average AOR_{max} in the main reactors was 0.82 gN m^{-2} d^{-1}. Nitrite did not accumulate in any of the tests except in one of the FB replicates where nitrite accumulated during the main test (Fig. 3C, D). In this reactor, the AOR_{max} (0.6 gN m^{-2} d^{-1}) and NOR_{max} (∼0.06 gN m^{-2} d^{-1}) during the main capacity test were much lower than the AOR and NOR observed during continuous operation (0.8-1.0 gN m^{-2} d^{-1}). Thus, the reason for this discrepancy is puzzling. During all the other tests, the nitrite concentration decreased, indicating that nitrite oxidation was faster than ammonia oxidation.

Immediately after seawater transfer (days 51-52), AOR_{max} in the F, B, and FB treatments reduced by 72, 19, and 66%, respectively. The B treatment had the highest nitrification rate, with 3.8x and 2.6x higher AOR_{max} than F and FB, respectively. In the glass reactors, the B treatment had 8x and 5.6x higher AOR_{max} than F and FB, respectively. This suggests that the newly developed biofilm contributed most to the difference between treatments, rather than the seed biofilm. Nitrite did not accumulate in any of the main or glass capacity tests, suggesting that nitrite oxidation was equally or less severely impacted by the salinity increase than ammonia oxidation. The NAR_{max} showed a similar trend in the main and glass reactors. Nitrite accumulation was the least (NAR_{max} most negative) in the FB treatment, followed by F and then B. It should be noted that the nitrite accumulation rate depends on the nitrite oxidation rate as well as the ammonia

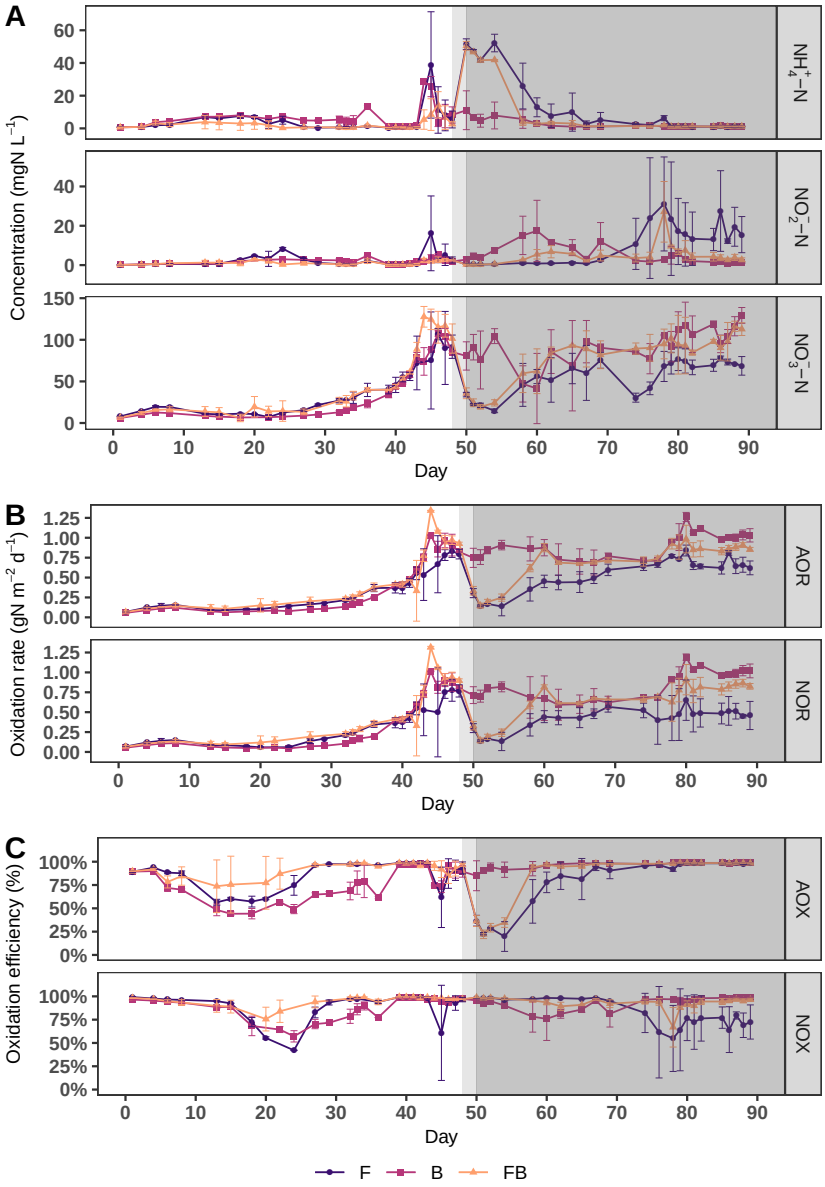


Figure 2: Nitrification performance in the treatments seeded with carriers acclimated to freshwater (F), brackish water (B) and a 1:1 mix of freshwater and brackish water acclimated carriers (FB) during the study. The graphs show as a function of time, A) Concentration of ammonia, nitrite, and nitrate; B) Oxidation rates of ammonia (AOR) and nitrite (NOR); and C) Oxidation efficiency of ammonia (AOX) and nitrite (NOX). Salinity was increased from freshwater to seawater over days 47-50. Each data point represents the mean (±SD) of two reactors. Note the difference in y-axes scales in graph A.

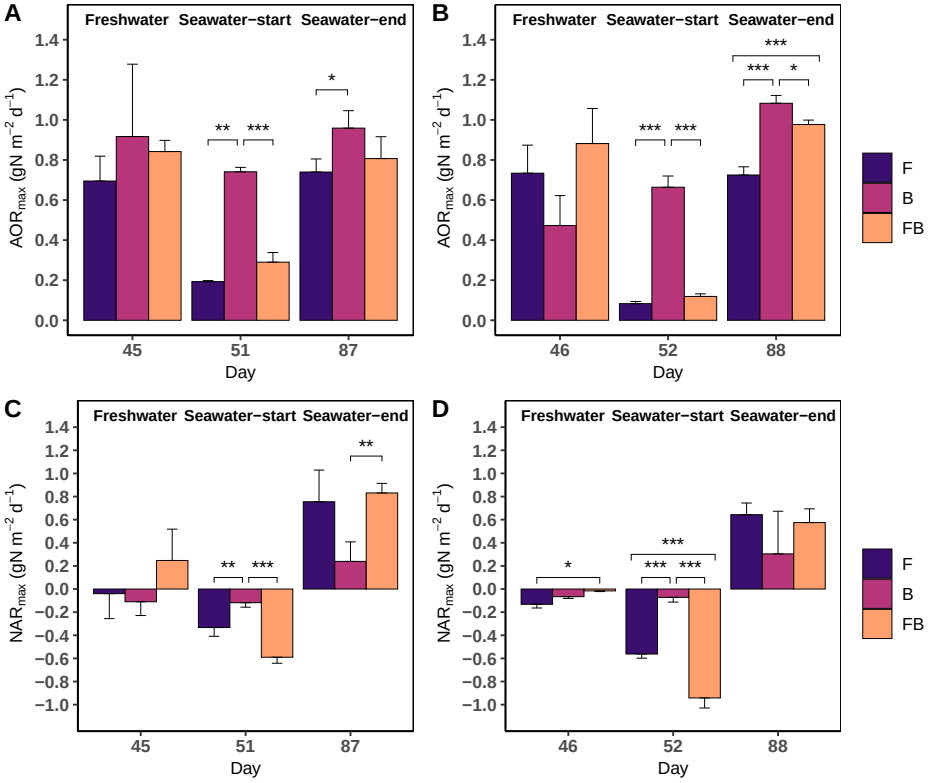


Figure 3: Capacity test data showing the maximum ammonia oxidation rate (AOR_{max}) and nitrite accumulation rate (NAR_{max}) in the A) main reactors (37 L) and B) glass reactors (1 L) with white virgin carriers only. The treatments were seeded with freshwater-acclimated carriers (F), brackish water acclimated carriers, (B) and a 1:1 mix of fresh- and brackish water acclimated carriers (FB), respectively. The tests were conducted before (days 45-46, freshwater) and after (days 51-52, seawater-start) salinity increase to seawater, and 37 days after complete transfer to seawater (days 87-88, seawater-end). Salinity was changed from freshwater to seawater during days 47-50 in daily increments (∼10‰ day^{-1}). Significant differences between treatments on each day are marked by asterisks (where * denotes $0.01 < p < 0.05$, ** denotes $0.001 < p < 0.01$, and *** denotes $p < 0.001$). Note the difference in y-axes scales.

oxidation rate. On day 51, the estimated NOR_{max} (main) was similar in B and FB treatments (∼0.87 gN m^{-2} d^{-1}), whereas F had a ∼40% lower NOR_{max} (∼0.53 gN m^{-2} d^{-1}).

At the end of the seawater phase (days 87-88), the AOR_{max} increased in all the treatments. The B treatment had the highest nitrification rate, with 30 and 20%

higher AOR_{max} than the F and FB treatments, respectively. A similar trend was observed in the glass capacity tests, where B had 50 and 10% higher AOR_{max} than the F and FB treatments, respectively. However, in all three treatments, considerable nitrite accumulation was observed. In the B treatment, the NOR_{max} did not change significantly during the seawater phase. However, in the F and FB treatments, nitrite oxidation decreased significantly in the main reactors (NOR_{max} $\sim$0.02 gN m^{-2} d^{-1}). This may have been due to inhibition by the accumulating nitrite ($\sim$30-40 mgN L^{-1} at the end of the test in these treatments). In the glass reactors, the nitrite oxidation was not as impacted, likely due to the slightly lower nitrite concentrations, compared to the main tests.

3.3 Community composition of the nitrifying bacteria

The sequencing effort resulted in a total of 1093 OTUs, with 18 of them identified as nitrifying bacteria. After applying the threshold of 0.1%, 518 OTUs remained and no nitrifying OTUs were lost in this process.

3.3.1 Differences between virgin carriers of the treatments

On day 45, the white virgin carriers in the FB treatment had the highest first order diversity and richness, whereas the B treatment had the lowest (Supplementary Information, Fig. S2). However, the α-diversity indices of the treatments became more similar on day 88. The PCoA ordination plot suggested that the nitrifying community composition in the virgin carriers of the three treatments were different on day 45 and became more similar on day 88 (Fig. 4). This was confirmed by the PERMANOVA analysis based on Bray-Curtis indices that showed a significant difference between treatments on day 45 ($p < 0.001$, $R^2 = 0.74$). Pairwise PERMANOVA comparisons of the treatments on day 45 showed a significant difference between all three pairs based on both distance indices ($p = 0.03$). On day 88 (seawater phase), the difference between treatments was also significant ($p < 0.001$), but a lower proportion of the variance was explained by the grouping ($R^2 = 0.50$) than on day 45. This suggests that the treatments became more similar over time. The test for homogeneity of variances within groups (based on Bray-Curtis distances) failed on day 45 ($p \sim 0.02$). However, PERMANOVA is relatively robust to heterogeneity in multivariate dispersions (Anderson and Walsh, 2013), and the ordination plots indicate that the differences between treatments were mainly due to the location effects rather than dispersion effects (Fig. 4). All the statistical analysis based on the Sørensen-Dice distances showed similar trends as for the Bray-Curtis, suggesting that the differences between treatments were partly due to differences in taxa composition.

On day 45, the Bray-Curtis distances between B and the other two treatments were

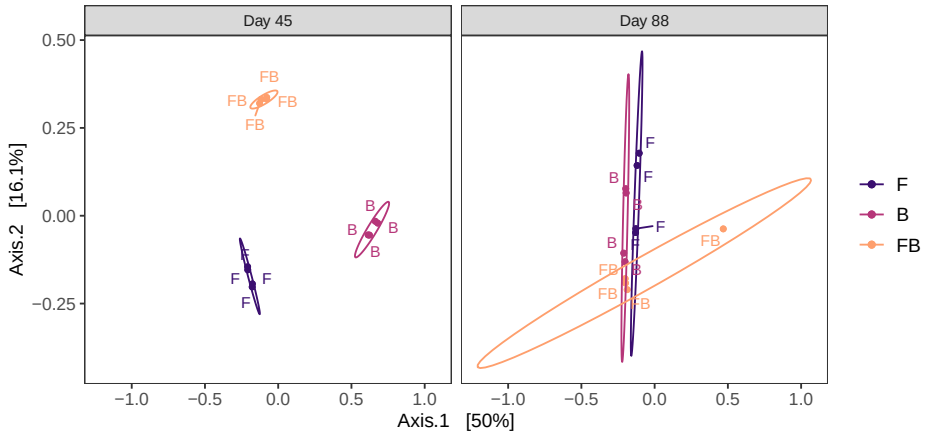


Figure 4: Ordination plot using principle coordinates analysis (PCoA) based on Bray-Curtis distances between the nitrifying OTUs in the virgin carriers on days 45 and 88. The ordination was performed on all samples simultaneously, and the graphs are faceted by day to increase clarity. Labels indicate treatment. Square brackets show the percent variance explained by each of the coordinate axes.

significantly greater than that between F and FB (Fig. 5A). This indicates that the community composition of the B treatment was the most dissimilar from that of F and FB (Bray-Curtis distance ∼0.95). As F and FB were relatively more similar (Bray-Curtis distance ∼0.57), this may explain the similar extent of reduction in nitrification activity upon seawater transfer. This was also true based on the Sørensen-Dices distances (Fig. 5B), as F and FB had more common nitrifying taxa than B (see Section 3.3.3). However, there was no significant difference in the distances between treatments on day 88; a further indication that the community composition became more similar over time (Fig. 5B, D).

3.3.2 Comparison with seed carriers

The ordination plots based on Bray-Curtis (Fig. 6) and Sørensen-Dice indices (Supplementary Information, Fig. S3) suggested that there was a significant difference between the nitrifying community composition of the fresh- and brackish water seed carriers on day 0. This was confirmed by PERMANOVA analysis based on both Bray-Curtis and Sørensen-Dice indices (p ∼0.03, R^2 = 0.80-0.96). The ordination plot also showed that the nitrifying community composition in the virgin and the seed carriers evolved over time. The PERMANOVA analysis (based on both distance indices) confirmed that the nitrifying community composition in the virgin carriers in each of the treatments evolved significantly from day 45 to 88 (p ∼0.03, R^2 = 0.43-0.93). The virgin

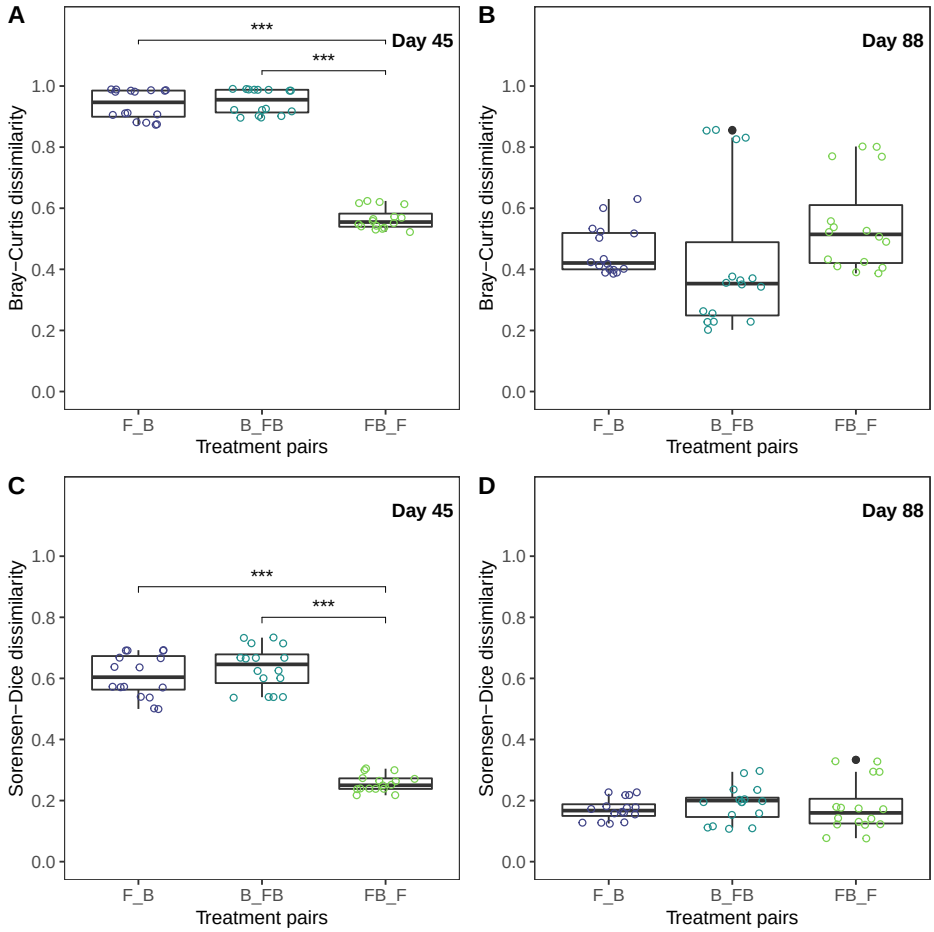


Figure 5: Bray-Curtis (A, B) and Sørensen-Dice (C, D) dissimilarities between the nitrifying community composition of the virgin biofilm carriers of the treatments on days 45 (A, C) and 88 (B, D). Significant differences between treatment pairs on each day are marked by asterisks (where *** denotes $p < 0.001$). Treatment pairs without asterisks were not significantly different ($p > 0.05$).

carriers on day 45 in each treatment were also compared with the respective seed carriers on day 0 and on day 45 based on both distance indices (Supplementary Information, Fig. S4, S5). Both the distance indices showed similar trends. The virgin carrier in the B treatment was the most dissimilar from its seed carrier (both on day 0 and day 45; Bray-Curtis dissimilarity ∼0.95), while F was the most similar (Bray-Curtis dissimilarity ∼0.24) . The virgin carriers in B also had a much lower richness than the seed carriers (Supplementary Information, Fig. S2,

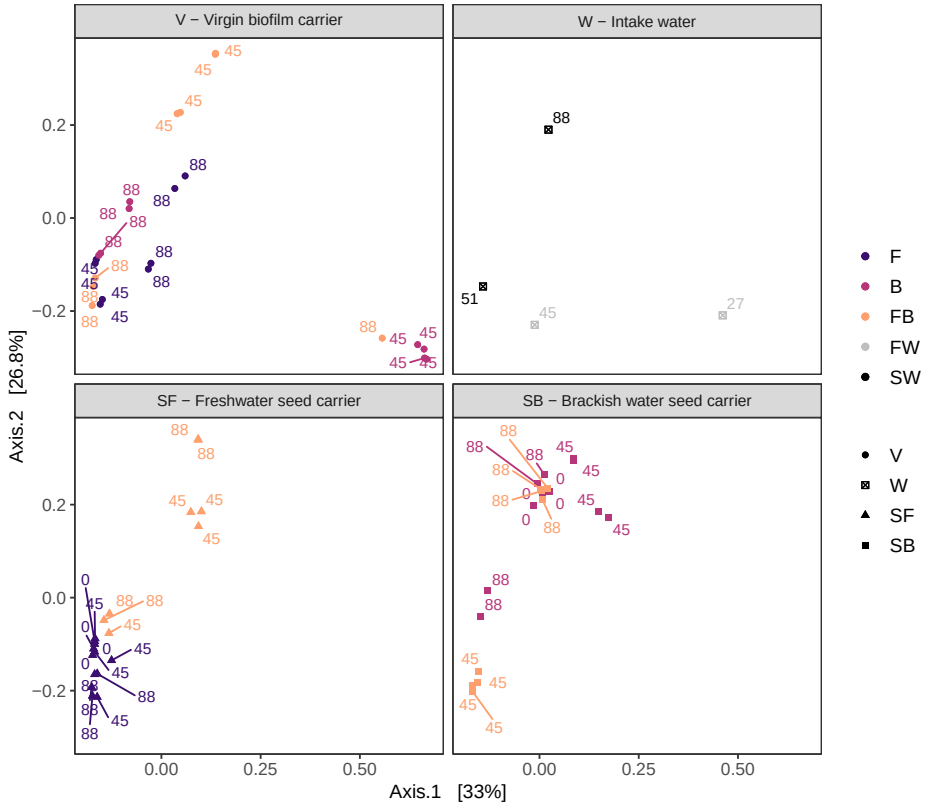


Figure 6: Ordination plot using principle coordinates analysis (PCoA) based on Bray-Curtis distances between the nitrifying OTUs of the microbial samples. The ordination was performed on all samples simultaneously, and the graphs are faceted by sample type to increase clarity. Sample types: Virgin biofilm carrier in the three treatments (V), intake water (W, where FW and SW refer to fresh- and seawater), freshwater seed carriers (SF, present in the F and FB treatments), and brackish water seed carriers (SB, present in the B and FB treatments). Labels indicate sampling day. Square brackets show the percent variance explained by each of the coordinate axes.

S6). Surprisingly, the composition of the virgin carrier in the FB treatment on day 45 was more similar to the brackish water seed than to the freshwater seed on day 0. However, it was equally dissimilar from the fresh- and brackish water seed carriers on day 45.

3.3.3 Relative abundance of nitrifying bacteria

Across the study, *Nitrosomonas* and *Candidatus Nitrotoga* were the main genera of AOB and NOB, respectively (Fig. 7). *Nitrosospira* and *Nitrospira* were also

detected, but at relatively lower abundances. The B treatment on day 45 had an extremely low relative abundance of nitrifiers (∼1%) compared to F (37-50%) and FB (45-60%). There were some differences between replicates, especially in the virgin carriers in FB treatment (Supplementary Information, Fig. S7), whereas the seed biofilm carriers had a more uniform composition across replicates (Supplementary Information, Fig. S8).

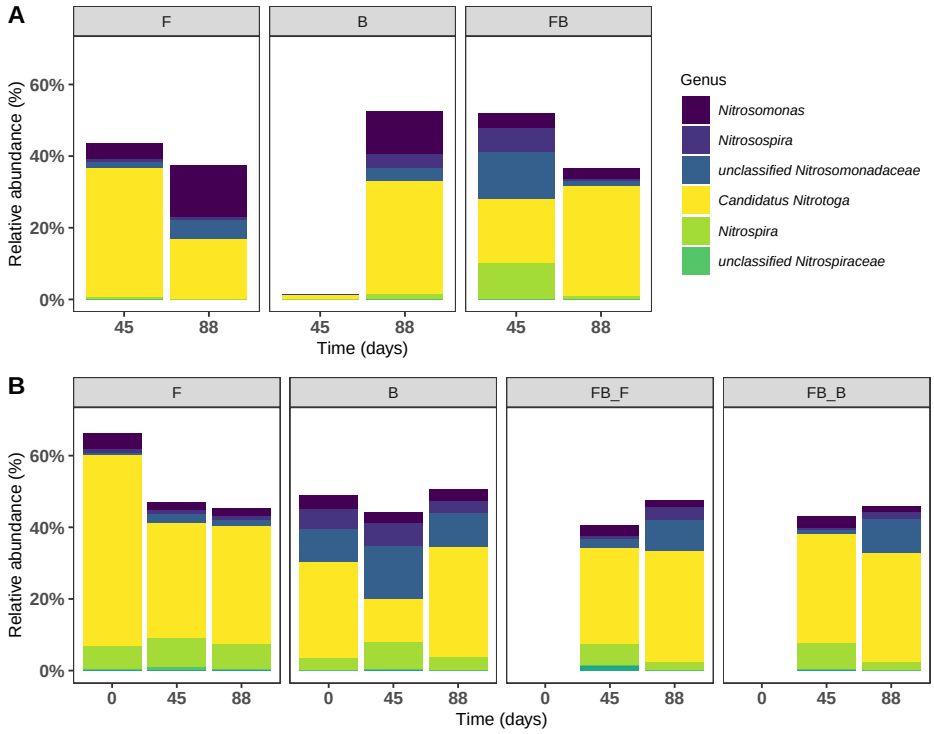


Figure 7: Relative abundance of the different genera of nitrifying bacteria in A) virgin biofilm carriers and B) seed biofilm carriers of the three treatments on days 0 (fresh- or brackish water), 45 (freshwater) and 88 (seawater). In plot B, FB_F and FB_B refer to the fresh- and brackish water seed carriers, respectively, in treatment FB. Each bar represents the average of four replicate biofilm carriers.

4 Discussion

This study was undertaken to investigate whether seeding with salinity-acclimated biofilm could improve salinity adaptation in freshwater nitrifying bioreactors. The results of the study showed that upon a salinity increase from freshwater to seawater, MBBRs seeded with carriers acclimated to brackish water (treatment B)

had 2-3x higher ammonia oxidation capacity (AOR_{max}) than those seeded with freshwater acclimated carriers (F) or a combination of fresh- and brackish water acclimated carriers (FB). This shows that seeding with brackish water acclimated carriers improved salinity acclimation, and can be a viable start-up strategy for RAS bioreactors with variable salinity requirements. Although previous studies have shown that the addition of salinity-acclimated culture can improve nitrification performance in nitrifying sludge or saline nitrifying bioreactors (Panswad and Anan, 1999; Roalkvam et al., 2020; Shi et al., 2012; Sudarno et al., 2010), this is the first study to show the efficacy of this strategy in freshwater biofilm reactors. In comparison to F and FB, the B treatment had very little reduction in nitrification capacity ($\sim$20%) and recovered quickly. In a RAS, this temporary decrease in nitrification capacity may be compensated through reduced fish feeding for a few days.

Notably, the nitrite concentration was low immediately after seawater transfer, but significant nitrite peaks were observed after a few days in seawater. Although there is divided opinion on whether AOB or NOB are more affected by a salinity increase, nitrite accumulation at elevated salinities has been reported by several studies (Bassin et al., 2011; Jeong et al., 2018; Nijhof and Bovendeur, 1990). A delayed reduction in nitrite oxidation and consequent nitrite accumulation after a salinity increase was also observed in our previous studies (Navada et al., 2020b; Navada et al., 2019). This is important because nitrite can be severely toxic to the fish at concentrations as low as 0.1 mgN L^{-1} in soft freshwater (Timmons and Ebeling, 2010). However, the toxicity of nitrite to fish is considerably reduced in the presence of chlorides (Gutiérrez et al., 2019; Kroupova et al., 2005). Thus, some nitrite accumulation may be acceptable in saline RAS, provided the salinity is high enough to mitigate the nitrite toxicity. Nonetheless, it is important to monitor the nitrite concentration for several days after a salinity increase, so that suitable measures can be taken to prevent nitrite toxicity to the fish (for e.g., reduced feeding or the addition of salt to increase the chloride concentration).

The nitrification capacity in the glass reactors with white virgin carriers was similar to that in the main reactors. This suggests that the overall nitrification activity in the main MBBRs can be attributed mainly to the newly developed biofilm on the virgin carriers rather than to the seed carriers alone. Moreover, the different responses of the treatments to salinity increase was likely due to the different nitrifying community composition in the virgin biofilms before the salinity increase (day 45). The difference in community composition also indicates that the type of seeding influenced the eventual community composition in freshwater. Interestingly, despite the presence of brackish water seed carriers, FB showed a similar response in AOR_{max} as F upon salinity increase. This was likely because the F and FB

treatments were more similar in nitrifying community composition compared to B. As the FB treatment was operated in freshwater during start-up, this could have favored the freshwater species over the brackish water species. The reduction in the nitrification rate observed in F and FB upon seawater transfer is similar to that observed in freshwater bioreactors in our previous studies (∼65-75%) (Navada et al., 2020b; Navada et al., 2019). However, it is lower than other studies that reported a >90% inhibition (Gonzalez-Silva et al., 2016; Kinyage et al., 2019). This is possibly because the salinity increase in our studies was gradual (by changing the salinity of the intake), whereas salinity was changed in a shock manner in the other studies.

In this study, nitrification rates of ∼0.8 gN m^{-2} d^{-1} were attained within 45 days of start-up at 15°C. In contrast, the freshwater start-up of an un-inoculated biofilter at 24°C took up to 150 days to attain similar rates (Nijhof and Bovendeur, 1990). In our previous study, we observed rates <0.1 gN m^{-2} d^{-1} after 60 days of start-up of unseeded semi-commercial RAS MBBRs (Navada et al., 2020a). However, the MBBRs in that study were substrate limited (ammonia < 0.5 mgN L^{-1}) during several periods. Hence, the higher rates in our study are likely a combined effect of seeding and the availability of substrate at non-limiting concentrations. However, it should be noted that due to the low ammonia tolerance of the fish, nitrification rate in an operational RAS is typically substrate-limited and will depend on the ammonia concentration (Chen et al., 2006; Rusten et al., 2006). In the freshwater phase, FB had a slightly higher AOR than the other two treatments, likely due to the higher diversity in the nitrifying community composition. But despite starting with only brackish seed carriers, the overall nitrification rate in B was comparable to the F and FB treatments after 45 days in freshwater. This indicates that the substrate loading rate plays a greater role in determining the nitrification rate than the salinity of the seeded carriers.

Notably, the replicate reactors in this study showed a greater variation in the activity and community composition compared to our previous studies using the same setup (Navada et al., 2020b; Navada et al., 2019). Even under similar environmental conditions, the order of community assembly can influence the community structure and function, and result in divergence of communities (Nemergut et al., 2013). Thus, the larger variation between replicates may have been due to the higher uncertainty in the order of species colonization in new biofilms, thus involving a greater component of stochasticity in community assembly compared to mature biofilm carriers. Future studies on bioreactor start-ups should include sufficient replicate reactors to ensure the statistical strength of the studies.

The initial community composition can play a more important role than the

operating conditions in microbial community assembly (Wittebolle et al., 2009). The same was also observed in the nitrite oxidizing community in a marine bioreactor, but not in the ammonia oxidizing community (Keuter et al., 2017). In our study, the community composition in the newly developed freshwater biofilms was influenced by the initial community composition due to the seeding. However, the final composition in seawater was independent of the seeding, suggesting that the environmental conditions selected for the final community in this case. The survival of a certain species will depend on the combined effect of the initial community composition, environmental selection, and dispersal (Nemergut et al., 2013). Thus, the initial species may be out-competed by the other microbes if selection (due to the environmental conditions for e.g. salinity) and dispersal (due to the intake water) dominate the community assembly. This may also explain why some studies with commercial inocula showed a reduction in the start-up time (Bower and Turner, 1984; Kuhn et al., 2010), whereas others did not (Bower and Turner, 1981, 1984; Li et al., 2019; Manthe and Malone, 1987). In a biofilm, the interaction between microbes (such as competition or mutualism) can play an important role in the selection process. Because the biofilm carriers contain taxa that are already selected for life in a biofilm, the addition of seed carriers can be a more effective strategy than adding commercial inocula, as also shown by a recent study (Roalkvam et al., 2020).

The nitrifying community composition of the virgin carrier in the B treatment was highly dissimilar from seed carriers on days 0 and 45 (Bray-Curtis $\sim$0.95). This is also evident from the low proportion and diversity of nitrifying bacteria in the virgin carrier on day 45. This suggests that the better salinity acclimation in B was not due to similar community composition as in the brackish water seed. However, the B treatment likely selected for nitrifying bacteria that were halotolerant, as it had the best salinity acclimation despite the lowest diversity and richness. In contrast, despite having the highest taxa diversity, FB underwent a severe reduction in nitrification upon salinity increase. Although diversity and stability are generally thought to be positively correlated (Ives and Carpenter, 2007), the effect of diversity on salinity acclimation in nitrifying biofilms is not very evident (Gonzalez-Silva et al., 2016; Navada et al., 2020b). Factors other than the community composition may also influence the salinity acclimation in biofilms. For instance, extracellular polymeric substances (EPS) produced by the bacteria can protect against salinity stress (Flemming et al., 2016). Further, although the B treatment had extremely low proportion of nitrifiers (<2%), the similar nitrification activity of all treatments suggests that the nitrifying biomass was similar across treatments. Hence, the total biomass in B was likely much higher than in the others. As heterotrophs can preferentially occupy the upper layers of the biofilm (Matsumoto et al., 2007; Okabe et al., 2002), the higher

abundance of heterotrophs in B may have protected the nitrifying bacteria in the deeper layers from osmotic stress. Future studies should investigate the effect of EPS and other factors on the salinity acclimation in nitrifying biofilms.

5 Conclusions

This study showed that seeding can be a microbial management strategy to control the community composition of nitrifiers and functionality in newly developed biofilms. However, a common selection pressure may even out the differences within six weeks, as was observed in the seawater phase. Seeding with biofilm carriers acclimated to brackish water significantly improved salinity acclimation, and should be added during the start-up of nitrifying bioreactors requiring variable salinity during operation (such as in RAS). Nitrite oxidizers may require a longer period to acclimatize to seawater than ammonia oxidizers, and nitrite concentration should, therefore, be closely monitored for several days after a salinity increase. The nitrifying community composition of the brackish water biofilm was highly dissimilar from that in the seeded biofilm, suggesting that factors other than community composition may influence the functionality, and should be investigated in further studies.

Author contributions

By CRediT taxonomy: **SN**: Conceptualization, Experiment design, Methodology, Investigation, Formal analysis, Visualization, Writing – Original Draft. **FG**: Experiment design, Supervision. **AKT**: Methodology, Investigation, Resources. **JK**: Resources, Supervision. **OV**: Experiment design, Formal analysis, Supervision. All co-authors reviewed and approved the final manuscript.

Acknowledgements

This project is a part of CtrlAQUA SFI, center for research-based innovation funded by the Research Council of Norway (# 237856, # 270888) and the partners of the Center, including Krüger Kaldnes AS. The authors would like to thank Dag Egil Bundgaard (Nofima) for performing the flow injection analyses. We would like to thank Yuriy Marchenko, May-Britt Mørkedal, and the other staff at Nofima for technical help. We also thank Prof. Øyvind Mikkelsen (NTNU) for his support.

Supplementary Information

Supplementary information

References

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, *26*, 32–46.

Anderson, M. J., & Walsh, D. C. I. (2013). PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? *Ecological Monographs*, *83*(4), 557–574.

Bassin, J. P., Kleerebezem, R., Rosado, A. S., Van Loosdrecht, M. C. M., & Dezotti, M. (2012). Effect of different operational conditions on biofilm development, nitrification, and nitrifying microbial population in moving-bed biofilm reactors. *Environmental Science and Technology*, *46*(3), 1546–1555.

Bassin, J. P., Pronk, M., Muyzer, G., Kleerebezem, R., Dezotti, M., & van Loosdrecht, M. C. M. (2011). Effect of elevated salt concentrations on the aerobic granular sludge process: Linking microbial activity with microbial community structure. *Applied and Environmental Microbiology*, *77*(22), 7942–7953.

Bower, C. E., & Turner, D. T. (1981). Accelerated nitrification in new seawater culture systems: Effectiveness of commercial additives and seed media from established systems. *Aquaculture*, *24*, 1–9.

Bower, C. E., & Turner, D. T. (1984). Evaluation of two commercial nitrification accelerators in closed seawater culture systems. *Aquaculture*, *41*, 155–159.

Carmignani, G. M., & Bennett, J. P. (1977). Rapid start-up of a biological filter in a close aquaculture system. *Aquaculture*, *11*, 85–88.

Chen, S., Ling, J., & Blancheton, J. P. (2006). Nitrification kinetics of biofilm as affected by water quality factors. *Aquacultural Engineering*, *34*(3), 179–197.

Csonka, L. N. (1989). Physiological and genetic responses of bacteria to osmotic stress. *Microbiological reviews*, *53*(1), 121–47.

Daims, H., Lebedeva, E. V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R. H., Von Bergen, M., Rattei, T., Bendinger, B., Nielsen, P. H., & Wagner, M. (2015). Complete nitrification by Nitrospira bacteria. *Nature*, *528*(7583), 504–509.

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: An emergent form of bacterial life. *Nature Reviews Microbiology*, *14*(9), 563–575.

Fox, J., & Weisberg, S. (2011). *An R Companion to Applied Regression* (Second). Thousand Oaks CA.

Fox, J., & Weisberg, S. (2018). *Robust Regression in R* (tech. rep.).

Gonzalez-Silva, B. M., Jonassen, K. R., Bakke, I., Østgaard, K., & Vadstein, O. (2016). Nitrification at different salinities: Biofilm community composition and physiological plasticity. *Water Research*, *95*, 48–58.

Gonzalez-Silva, B. M. (2016). *Salinity as a driver for microbial community structure in reactors for nitrification and anammox* (Doctoral dissertation). Norwegian University of Science & Technology.

Grommen, R., Dauw, L., & Verstraete, W. (2005). Elevated salinity selects for a less diverse ammonia-oxidizing population in aquarium biofilters. *FEMS Microbiology Ecology*, *52*(1), 1–11.

Gutiérrez, X. A., Kolarevic, J., Takle, H., Baeverfjord, G., Ytteborg, E., & Fyhn Terjesen, B. (2019). Effects of chronic sub-lethal nitrite exposure at high water chloride concentration on Atlantic salmon (Salmo salar, Linnaeus 1758) parr. *Aquaculture Research*, *50*(9), 2687–2697.

H. Wickham. (2007). Reshaping data with the reshape package. *Journal of Statistical Software*, *21*(12), 1–25.

Hill, M. O. (1973). Diversity and Evenness: A Unifying Notation and Its Consequences. *Ecology*, *54*(2), 427–432.

Ives, A. R., & Carpenter, S. R. (2007). Stability and diversity of ecosystems. *Science*, *317*(5834), 58–62.

Jeong, D., Cho, K., Lee, C. H., Lee, S., & Bae, H. (2018). Effects of salinity on nitrification efficiency and bacterial community structure in a nitrifying osmotic membrane bioreactor. *Process Biochemistry*, *73*, 132–141.

Keuter, S., Beth, S., Quantz, G., Schulz, C., & Eva, S. (2017). Longterm monitoring of nitrification and nitrifying communities during biofilter activation of two marine recirculation aquaculture systems (RAS). *International Journal of Aquaculture and Fishery Sciences*, *3*, 051–061.

Kinyage, J. P. H., Pedersen, P. B., & Pedersen, L. F. (2019). Effects of abrupt salinity increase on nitrification processes in a freshwater moving bed biofilter. *Aquacultural Engineering*, *84*, 91–98.

Kroupova, H., Machova, J., & Svobodova, Z. (2005). Nitrite influence on fish: A review. *Veterinarni Medicina*, *50*(11), 461–471.

Kuhn, D. D., Drahos, D. D., Marsh, L., & Flick, G. J. (2010). Evaluation of nitrifying bacteria product to improve nitrification efficacy in recirculating aquaculture systems. *Aquacultural Engineering*, *43*(2), 78–82.

Li, C., Liang, J., Lin, X., Xu, H., Tadda, M. A., Lan, L., & Liu, D. (2019). Fast start-up strategies of MBBR for mariculture wastewater treatment. *Journal of Environmental Management*, *248*, 109267.

Madigan, M. T., Bender, K. S., Buckley, D. H., Sattley, W. M., & Stahl, D. A. (2018). *Brock Biology of Microorganisms* (15th ed.). Pearson Education Limited.

Manthe, D. P., & Malone, R. F. (1987). Chemical addition for accelerated biological filter acclimation in closed blue crab shedding systems. *Aquacultural Engineering*, *6*, 227–236.

Martinez Arbizu, P. (2020). pairwiseAdonis: Pairwise multilevel comparison using adonis. R package version 0.4.

Matsumoto, S., Terada, A., Aoi, Y., Tsuneda, S., Alpkvist, E., Picioreanu, C., & van Loosdrecht, M. C. (2007). Experimental and simulation analysis of community structure of nitrifying bacteria in a membrane-aerated biofilm. *Water Science and Technology*, *55*(8-9), 283–290.

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE*, *8*(4).

Moussa, M. S., Sumanasekera, D. U., Ibrahim, S. H., Lubberding, H. J., Hooijmans, C. M., Gijzen, H. J., & Van Loosdrecht, M. C. (2006). Long term effects of salt on activity, population structure and floc characteristics in enriched bacterial cultures of nitrifiers. *Water Research*, *40*(7), 1377–1388.

Navada, S., Sebastianpillai, M., Kolarevic, J., Fossmark, R. O., Tveten, A. K., Gaumet, F., Mikkelsen, Ø., & Vadstein, O. (2020a). A salty start: Brackish water start-up as a microbial management strategy for nitrifying bioreactors with variable salinity. *Science of the Total Environment*, *739*.

Navada, S., Vadstein, O., Gaumet, F., Tveten, A. K., Spanu, C., Mikkelsen, Ø., & Kolarevic, J. (2020b). Biofilms remember: Osmotic stress priming as a microbial management strategy for improving salinity acclimation in nitrifying biofilms. *Water Research*, *176*.

Navada, S., Vadstein, O., Tveten, A.-K., Verstege, G. C., Terjesen, B. F., Mota, V. C., Venkataraman, V., Gaumet, F., Mikkelsen, Ø., & Kamstra, A. (2019). Influence of rate of salinity increase on nitrifying biofilms. *Journal of Cleaner Production*, *238*.

Nemergut, D. R., O'Neill, S. P., Bilinski, T. M., Stanish, L. F., Knelman, J. E., Wickey, P., Schmidt, S. K., Darcy, J. L., Lynch, R. C., Ferrenberg, S., & Fukami, T. (2013). Patterns and processes of microbial community assembly. *Microbiology and Molecular Biology Reviews*, *77*(3), 342–356.

Nijhof, M., & Bovendeur, J. (1990). Fixed film nitrification characteristics in sea-water recirculation fish culture systems. *Aquaculture*, *87*(2), 133–143.

Nogueira, R., ıs Melo, ı. F., Purkhold, U., Wuertz, S., & Wagner, M. (2002). Nitrifying and heterotrophic population dynamics in biofilm reactors: effects of hydraulic retention time and the presence of organic carbon. *Water Research*, *36*, 469–481.

Okabe, S., Naitoh, H., Satoh, H., & Watanabe, Y. (2002). Structure and function of nitrifying biofilms as determined by molecular techniques and the use of microelectrodes. *Water Science and Technology*, *46*(1-2), 233–241.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., & Wagner, H. (2019). *Vegan: Community ecology package* [R package version 2.5-6].

Panswad, T., & Anan, C. (1999). Impact of high chloride wastewater on an anaerobic/anoxic/aerobic process with and without inoculation of chloride acclimated seeds. *Water Research*, *33*(5), 1165–1172.

Perfettini, J., & Bianchi, M. (1990). The comparison of two simple protocols designed to initiate and stimulate ammonia oxidation in closed aquaculture systems. *Aquaculture*, *88*, 179–188.

Roalkvam, I., Drønen, K., Dahle, H., & Inger, H. (2020). Comparison of active biofilm carriers and commercially available inoculum for activation of biofilters in marine recirculating aquaculture systems (RAS). *Aquaculture*, *514*, 734480.

Rusten, B., Eikebrokk, B., Ulgenes, Y., & Lygren, E. (2006). Design and operations of the Kaldnes moving bed biofilm reactors. *Aquacultural Engineering*, *34*(3), 322–331.

Shi, K., Zhou, W., Zhao, H., & Zhang, Y. (2012). Performance of halophilic marine bacteria inocula on nutrient removal from hypersaline wastewater in an intermittently aerated biological filter. *Bioresource Technology*, *113*, 280–287.

Sudarno, U., Bathe, S., Winter, J., & Gallert, C. (2010). Nitrification in fixed-bed reactors treating saline wastewater. *Applied Microbiology and Biotechnology*, *85*, 2017–2030.

Timmons, M., & Ebeling, J. (2010). *Recirculating Aquaculture* (3rd). Ithaca Publishing Company LLC.

U.S. EPA. (1983). *Methods for chemical analysis of water and wastes*. U.S. Environmental Protection Agency.

Wagner, F. B., Nielsen, P. B., Boe-Hansen, R., & Albrechtsen, H. J. (2016). Copper deficiency can limit nitrification in biological rapid sand filters for drinking water production. *Water Research*, *95*, 280–288.

Wittebolle, L., Verstraete, W., & Boon, N. (2009). The inoculum effect on the ammonia-oxidizing bacterial communities in parallel sequential batch reactors. *Water Research*, *43*(17), 4149–4158.

Supplementary Information

Seeding with brackish-water biofilm as a startup strategy for salinity acclimation in freshwater nitrifying bioreactors

Sharada Navada[a,b,*], Frédéric Gaumet[b], Ann-Kristin Tveten[c], Jelena Kolarevic[d], Olav Vadstein[e]

[a] Department of Chemistry, NTNU - Norwegian University of Science and Technology, N-7491 Trondheim, Norway

[b] Krüger Kaldnes AS (Veolia Water Technologies), N-3241 Sandefjord, Norway

[c] Department of Biological Sciences, NTNU Norwegian University of Science and Technology, N-6009 Ålesund, Norway

[d] Nofima AS, N-6600 Sunndalsøra, Norway

[e] Department of Biotechnology and Food Science, NTNU - Norwegian University of Science and Technology, N-7491 Trondheim, Norway

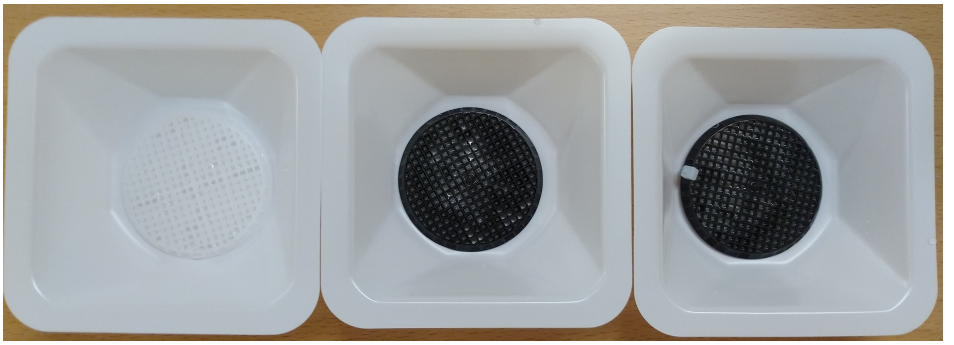


Figure S1: Pictures (left to right) of white virgin carrier, black mature biofilm carrier used for seeding, and brackish-water acclimated seed carrier in treatment FB marked with a cable tie to distinguish it from the freshwater seed carrier.

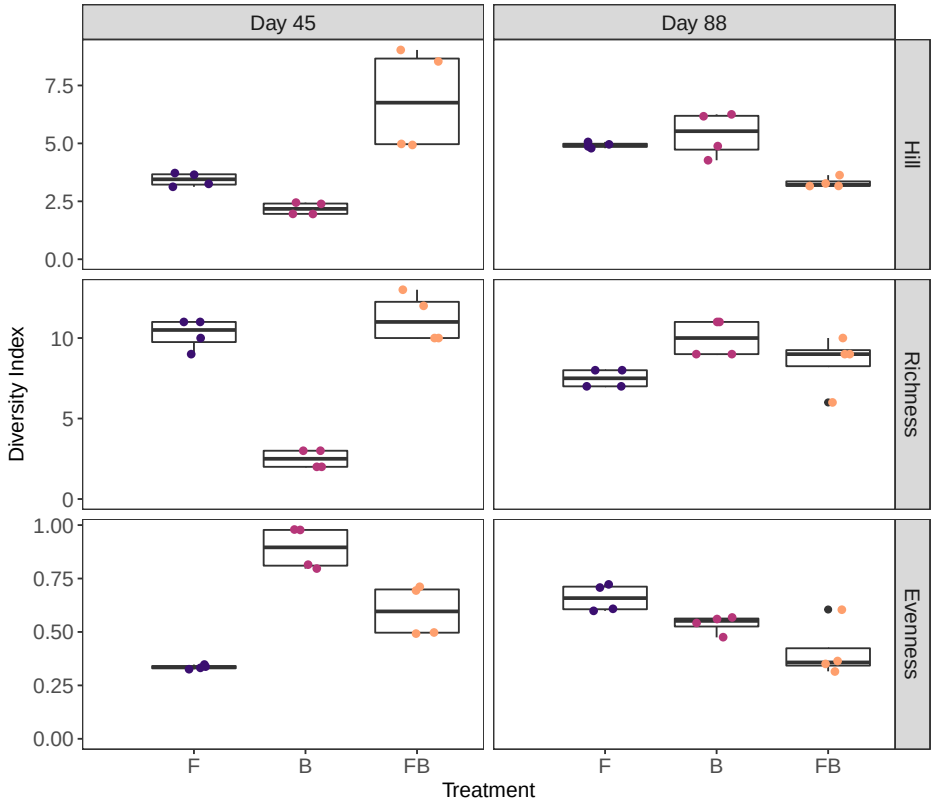


Figure S2: α-diversity indices – first order diversity (Hill number), richness, and evenness in the virgin biofilm carriers on days 45 and 88. Note the differences in y-axes scales.

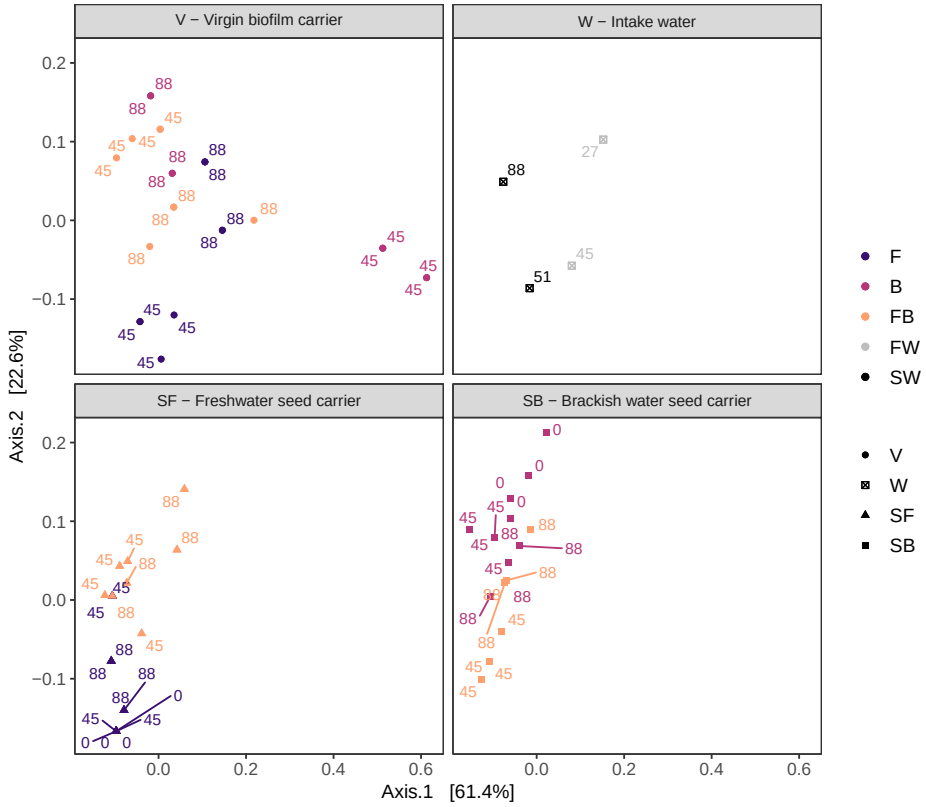


Figure S3: Ordination plot using Principle coordinates analysis (PCoA) based on Sørensen-Dice (presence/absence) distances between the nitrifying OTUs of the microbial samples. The ordination was performed on all samples simultaneously, and the graphs are faceted by sample type to increase clarity. Labels indicate sampling day. Square brackets show the percent variance explained by each of the coordinate axes.

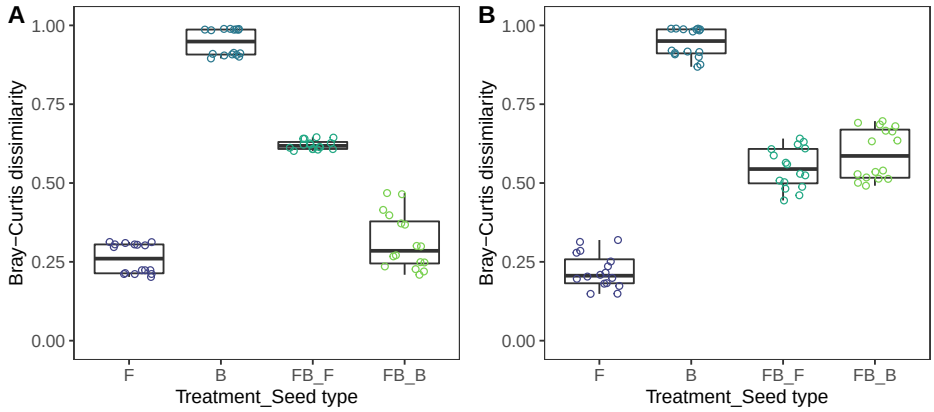


Figure S4: Bray-Curtis dissimilarity between the virgin carriers of each treatment on day 45 and A) fresh- and brackish water seed carriers on day 0; B) the respective seed carriers in each treatment on day 45.

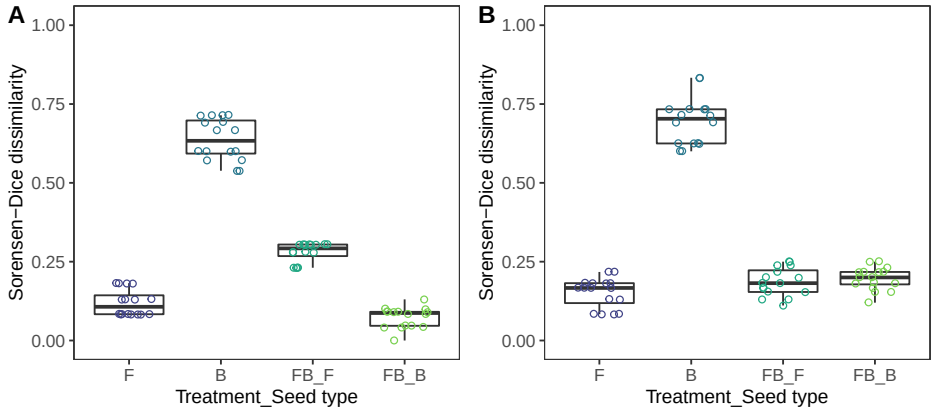


Figure S5: Sørensen-Dice dissimilarity between the virgin carriers of each treatment on day 45 and A) fresh- and brackish water seed carriers on day 0; B) the respective seed carriers in each treatment on day 45.

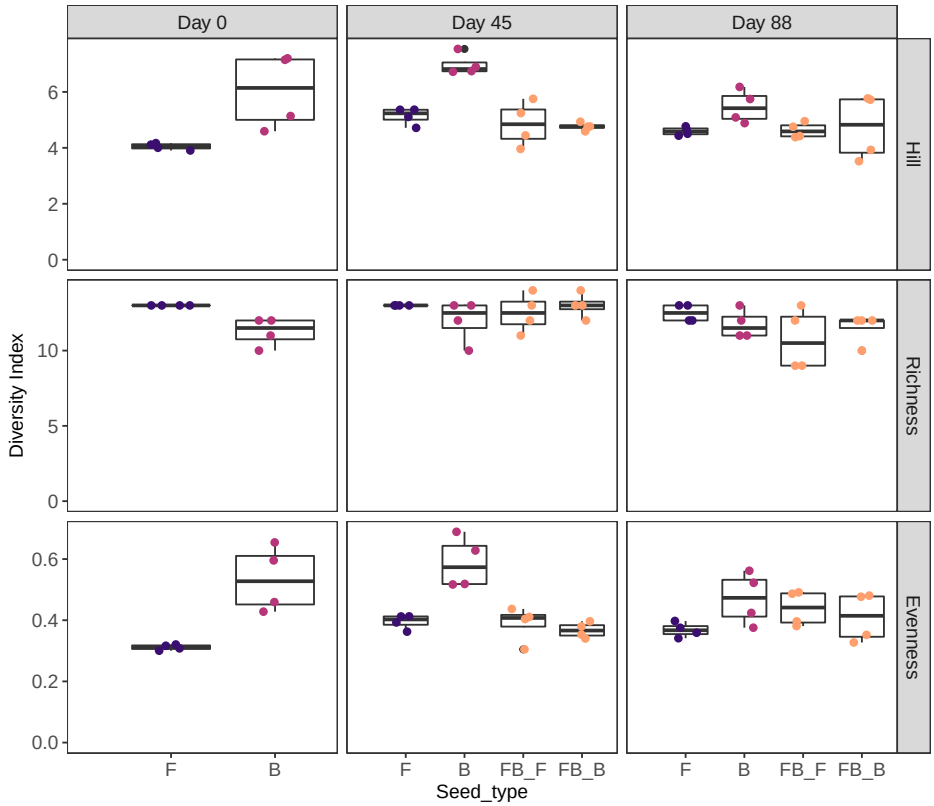


Figure S6: α-diversity indices – first order diversity (Hill number), richness, and evenness in the seeded biofilm carriers on days 0, 45, and 88. FB_F and FB_B refer to the fresh- and brackish water seed carriers, respectively, in treatment FB. Note the differences in y-axes scales.

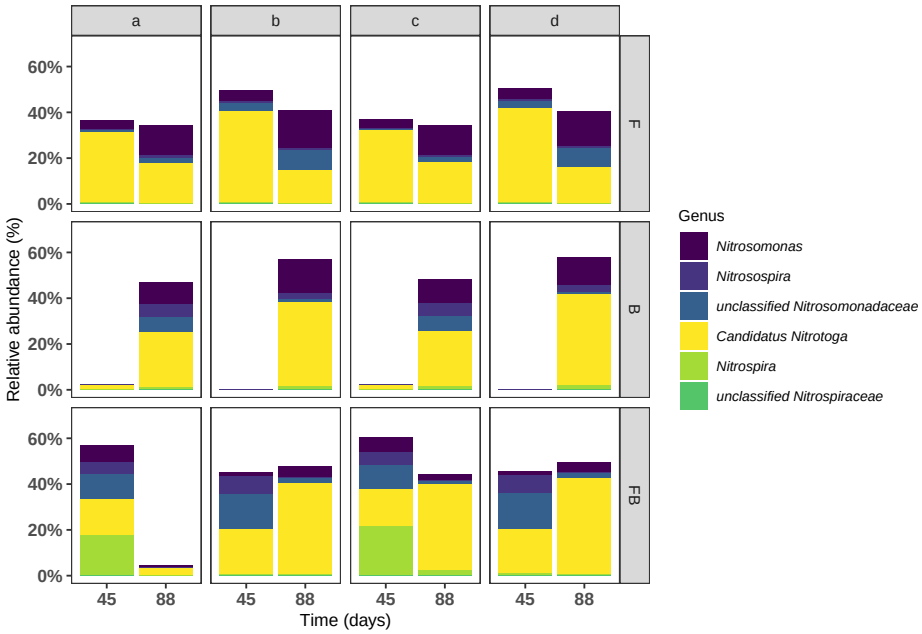


Figure S7: Relative abundance of the different genera of nitrifying bacteria in the virgin biofilm carriers of treatments F, B, and FB on days 45 (freshwater) and 88 (seawater). The plot shows the composition of in the biofilm replicates (a,b,c,d) in each treatment.

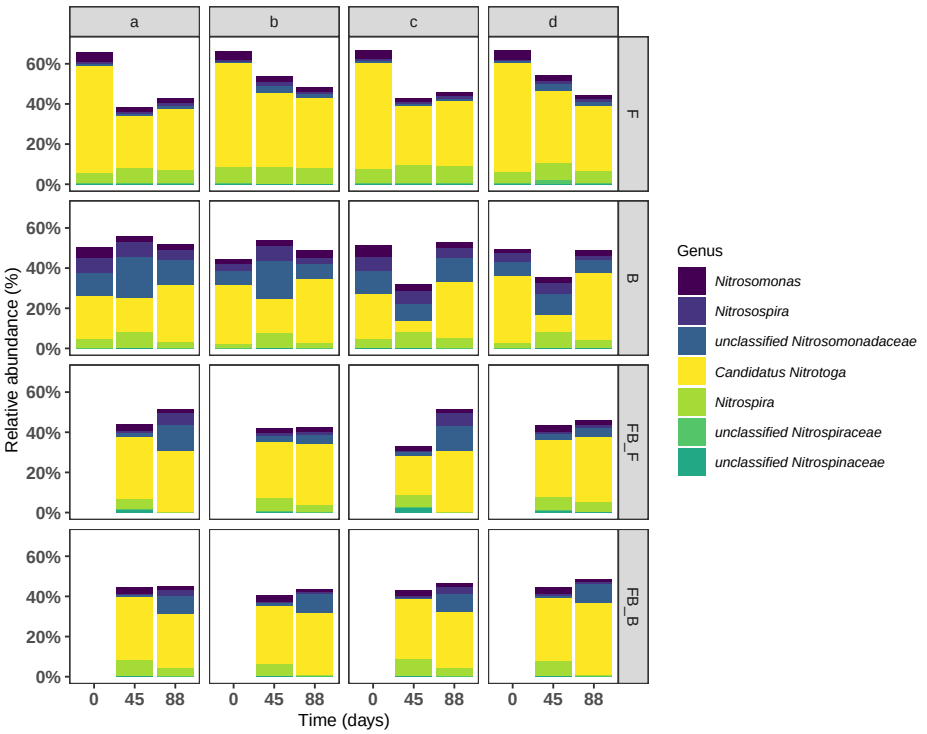


Figure S8: Relative abundance of the different genera of nitrifying bacteria in the seed biofilm carriers of treatments F, B, and FB on days 0 (fresh- or brackish water), 45 (freshwater) and 88 (seawater). The plot shows the composition of in the biofilm replicates (a,b,c,d) in each treatment.

Article V

Investigating the exogenous addition of osmolytes as a salinity acclimation strategy in nitrifying biofilms

Sharada Navada[a,b,*], Michele Laureni[c], Olav Vadstein[d], Mark van Loosdrecht[c]

[a] Department of Chemistry, NTNU - Norwegian University of Science and Technology, N-7491 Trondheim, Norway

[b] Krüger Kaldnes AS (Veolia Water Technologies), N-3241 Sandefjord, Norway

[c] Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, Van der Maasweg 9, Delft 2629 HZ, the Netherlands

[d] Department of Biotechnology and Food Science, NTNU - Norwegian University of Science and Technology, N-7491 Trondheim, Norway

Note: *Due to the COVID-19 pandemic, there were significant changes and delays in this study. Thus, here we present only the preliminary results from the study.*

Abstract

Several industries produce high or variable salinity effluents. This can be challenging for the microorganisms involved in the biological water treatment of these effluents. The uptake of osmolytes from the environment is a salinity acclimation strategy that has been shown to be effective over a range of microorganisms. In this study, we investigated the impact of the addition of an osmolyte cocktail (1mM each of trehalose, sucrose, glycine betaine, proline, carnitine, and ectoine) on nitrifying biofilms undergoing a salinity increase from freshwater to seawater. The experiment was conducted on moving bed biofilm reactors (MBBR) operated in a sequencing batch mode. The addition of osmolytes did not improve the nitrification activity on the first day after seawater transfer. Moreover, after two days in seawater, the treatment with osmolytes showed a severe reduction in the nitrification activity. This was accompanied by the putative growth of heterotrophic microorganisms in the medium facilitated by the uptake of osmolytes as substrate. Thus, the reduction in nitrification activity was likely due to the competition between the heterotrophs and nitrifiers for resources (such as oxygen) and/or osmolytes. Future studies should investigate the impact of individual osmolytes at different concentrations, as their potential as growth substrate and osmoregulators may vary. Such studies should be accompanied by metaproteomic or metabolomic analyses to determine the salinity adaptation mechanisms in nitrifying biofilms.

Keywords

salt acclimation; osmoprotectant; compatible solute; osmotic stress; osmoregulation; halotolerance

1 Introduction

About 5% of the total annual wastewater produced worldwide is saline (Vyrides, 2015). Several industries produce high or variable salinity effluents, such as oil refineries, aquaculture, seafood processing and tanneries (Lefebvre and Moletta, 2006; Vyrides, 2015). Variable salinity is challenging for biological water treatment processes because it can affect the metabolism and activity of the microorganisms by causing osmotic stress (Madigan et al., 2018; Sleator and Hill, 2001). In particular, hyperosmotic (salinity increase) changes are more detrimental than hypoosmotic (salinity decrease) changes (Csonka, 1989). The biological systems can eventually adapt to the new salinity, but the adaptation period can be very long (weeks to months) (Bassin et al., 2012; Gonzalez-Silva et al., 2016; Vyrides, 2015). Moreover, in many systems, it is common to have great variations due to process fluctuations rather than a gradual increase in salinity (Lefebvre and Moletta, 2006) and salinity may be different during different periods (Vyrides, 2015). Inoculation with salt-adapted microorganisms or with salt-adapted sludge has been shown to improve salinity acclimation (Cui et al., 2016; Sudarno et al., 2010; Vyrides and Stuckey, 2017). However, this strategy may not work during sudden increases in salinity and suitable inocula can be expensive or difficult to procure (Vyrides, 2015). Therefore, a more effective strategy is required for rapid salinity adaptation in microorganisms.

In principle, microbial cells must maintain an intracellular osmotic pressure greater than that of the environment to enable growth and cell division (Sleator and Hill, 2001). Microorganisms use two main strategies to adapt to salinity increase – i) the "salt-in" strategy and ii) the compatible solute strategy (Csonka, 1989; Sleator and Hill, 2001). In the "salt-in" strategy, the microbes increase their intracellular ion concentration to balance the external osmolarity (Vyrides and Stuckey, 2017). Owing to the high intracellular ionic strength, extensive structural adaptions are required, thus making this strategy exclusive to strictly halophilic bacteria (Sleator and Hill, 2001). The second strategy offers a greater degree of flexibility and is commonly used by halotolerant bacteria. Halotolerant microorganisms have a bi-phasic response to salinity increase. The primary response is an increase in the concentration of K^+ (and its counter-ion glutamate), followed by a secondary response of an increase in the cytoplasmic concentration of compatible solutes through synthesis or uptake (Sleator and Hill, 2001). Compatible solutes, also known as osmolytes, are highly soluble molecules that do not interact with proteins, thus enabling them to accumulate at high intracellular concentrations without interfering with the cell function (Sleator and Hill, 2001). Not just one, but several osmolytes may be associated with salinity adaption, depending on the magnitude of salinity change and exposure time (Saum and Müller, 2007;

Vyrides and Stuckey, 2017). The synthesis of osmolytes depends not only on the salinity but also on the nutrient supply, as a significant amount of nutrients are consumed during osmolyte production (Schimel et al., 2007). Moreover, the synthesis of osmolytes is energetically expensive, and the uptake of osmolytes from the environment is an energetically cheaper option (Oren, 2011). As opposed to osmolytes that accumulate inside the cells, osmoprotectants are compounds that stimulate bacterial growth at high osmolality when provided in the growth medium (Wood, 2007). Among the commonly used osmolytes and osmoprotectants are sugars (sucrose, trehalose etc.) and amino acids (glycine betaine, carnitine, proline, ectoine, choline etc.) (Oren, 1999; Sleator and Hill, 2001; Vyrides and Stuckey, 2017). Several studies have shown that the exogenous addition of osmolytes can alleviate salinity stress across a wide variety of microorganisms (Vyrides and Stuckey, 2017 and references within). Adding the osmolyte before changes in osmotic stress (rather than during) is suggested to prevent the negative effects of salinity change (Vyrides and Stuckey, 2017). Although the external osmolyte concentration in previous studies ranges from 0.1 to 150 mM, most of the studies used concentrations of the order of 1 mM (Vyrides and Stuckey, 2017). Moreover, higher concentrations (order of 100 mM or above) may also inhibit the uptake of osmolytes by microbes (Vyrides and Stuckey, 2017).

While several microbial communities such as methanogens, denitrifiers and anammox have been found capable of osmoprotectant uptake, little is known about the salinity acclimation mechanisms in nitrifying microorganisms (Vyrides and Stuckey, 2017). Nitrification is a two-step process consisting of ammonia oxidation by ammonia oxidizing bacteria (AOB) and archaea (AOA), followed by nitrite oxidation by nitrite oxidizing bacteria (NOB). Within the genus *Nitrospira*, some species can perform both the nitrification steps (comammox) (Daims et al., 2015). Compared to heterotrophs, relatively less energy is produced during the autotrophic oxidation by nitrifiers, and the energy produced may not be sufficient to sustain growth at very high salt concentrations (Oren, 2011). However, nitrifiers are found at high abundance in estuaries that frequently encounter salinity fluctuations, suggesting that several genera of nitrifying microorganisms are halotolerant (Bernhard and Bollmann, 2010; Santos et al., 2018; Ward et al., 2007). This is also supported by studies on nitrifying bioreactors where the same taxa were present across different salinities (Fossmark et al., 2021; Gonzalez-Silva, 2016; Navada et al., 2020). Thus, we hypothesize that the uptake of osmolytes may especially alleviate salinity stress in nitrifying microorganisms, as it is energetically more favorable than *de novo* synthesis. We are aware of only three studies on the effect of osmolytes on nitrifiers, and with mixed results. In one study, the addition of a cocktail of osmolytes (betaine, trehalose, proline, 3-[N-morpholino]propanesulfonic acid, taurine, and γ-amino-n-butyric

acid) did not enhance microcolony formation of *Nitrosomonas europaea* at salt concentration >0.1 M NaCl (∼6‰ salinity) (Wood and Sörensen, 1998). However, the study did not use real seawater, and was thus lacking K^+ ions that play an important role in osmoregulation (Sleator and Hill, 2001; Vyrides and Stuckey, 2017). In another study, *Nitrobacter* was capable of trehalose production and uptake of glycine betaine and sucrose from the medium (Vyrides and Stuckey, 2017). In a third study, the concentration of glutamine and proline increased in aerobic nitrifying granules, but no other osmolytes were analyzed (Wan et al., 2014).

As far as we know, there exist no studies on the exogenous addition of osmolytes on nitrification in aerobic microbial communities with both nitrifying and heterotrophic bacteria. The goal of this study was to investigate the effect of the exogenous addition of osmolytes on nitrifying biofilms undergoing salinity increase from freshwater to seawater. We hypothesized that the exogenous addition of osmolytes would alleviate salinity stress and lead to a lower reduction in nitrification rate upon salinity increase.

2 Materials & Methods

2.1 Experimental design and setup

Aerated lab beakers (0.5 L water volume) were used as moving bed biofilm reactors (MBBR). These were operated in a sequencing batch mode with synthetic medium exchanged every day (∼24h hydraulic retention time (HRT)). Three treatments were operated in triplicate: control with no salinity change (C), salinity change to seawater without the addition of osmolytes (S), and salinity change to seawater with the addition of an osmolyte cocktail (O). All reactors were started in freshwater medium with mature biofilm carriers (AnoxK™ Chip P, Krüger Kaldnes AS, Norway). After two days in freshwater media (days 1-2), the biofilm carriers in treatments S and O were subjected to hyperosmotic shock by transfer to synthetic seawater media. In treatment O, we added an osmolyte cocktail to the seawater medium. This osmolyte cocktail provided 1 mM each of trehalose, sucrose, glycine betaine (betaine hydrochloride), proline, carnitine, and ectoine. Treatments S and O were operated for three days (days 3-5) in the saline media. Treatment C was operated in freshwater throughout the study.

The biofilm carriers were obtained from a nitrifying MBBR in a commercial freshwater recirculating aquaculture system (RAS) for Atlantic salmon smolt. The MBBR had been operated at 12°C, pH 7.4, and 1‰ salinity at the time of collection, and had never been exposed to salinities higher than 5‰. Due to restrictions caused by the COVID-19 pandemic, these carriers had to be stored

for two months at 4°C. To revive the carriers before the experiment, they were transferred (∼25% fill by volume) into a large aerated reactor (4.5 L water volume). After five days, the nitrification rate reached a plateau, and these carriers were transferred to the experimental beakers. These beakers were maintained at room temperature (17-18°C). As there was no automatic pH control, the pH was 8.3-8.8 during the study, which is slightly above the pH optimum of 7.0-8.5 for nitrification (Ekama et al., 2020). To minimize evaporation, the air was humidified by bubbling through water. The dissolved oxygen saturation in the reactors was 80-90% during the study. Each reactor was filled with 15 biofilm carriers (∼20% fill by volume). The synthetic medium was modified from Bassin et al., 2011 to have an ammonia concentration of 100 mgN L^{-1}. The medium comprised of NH_4Cl (7.14 mM), $MgSO_4 \cdot 7H_2O$ (0.72 mM), KCl (0.94 mM), K_2HPO_4 (0.84 mM), and KH_2PO_4 (0.42 mM), and a trace element solution (1 mL L^{-1}) as described in (Vishniac and Santer, 1957). A stoichiometric quantity of alkalinity required for nitrification was added to the synthetic medium as $NaHCO_3$ (14.28 mM). The seawater medium had the same composition as the freshwater medium, with 35 g L^{-1} Instant Ocean® sea salt in addition to the other chemicals (∼35‰ salinity). To maintain similar alkalinity and pH in the freshwater medium as in the seawater medium, extra $NaHCO_3$ (2.40 mM) was added to the freshwater medium.

2.2 Sampling and analysis

Nitrification capacity tests were conducted on days 2, 3, and 5 to calculate the maximum specific ammonia oxidation rate (AOR), independent of the substrate concentration (zero-order rate). During each 24h test, 4-7 water samples were collected in 2 mL Eppendorf tubes. The samples were either filtered through a 0.45 µm syringe filter (Millex-HV PVDF, Sigma Aldrich, Netherlands) or centrifuged to extract the supernatant (13000 rpm, 5 min, 4°C). The samples from day 2 were analyzed immediately using ammonia test kits (LCK 303, Hach Lange, Germany) and thereafter frozen at -20°C. All the samples from days 3 and 5 were frozen. To measure the nitrite and nitrate concentration, the samples were later thawed and analyzed using Thermo Scientific™ Gallery™ Discrete Analyzer (Thermo Fisher Scientific™, Waltham, USA). Seawater samples with known concentration were used for calibration. From these, correction factors were calculated and applied to the seawater samples to adjust for the salinity interference. Temperature, pH, and dissolved oxygen were measured using handheld sensors (AppliSens®, Netherland) with a controller (Applikon®, Netherlands). Conductivity was measured using a sensor with a multiparameter analyzer (Consort C3010, Belgium).

2.3 Data analysis and statistics

The ammonia oxidation rate on day 2 was calculated from the regression line for the ammonia concentration vs time (Supplementary Information, Fig. S1). The rate was normalized to the total protected surface area of biofilm carriers to calculate the specific rate of ammonia oxidation (AOR). The specific production rates of nitrite (NO2_PR) and nitrate (NO3_PR) were calculated similarly from the regression lines of nitrite or nitrate vs time (Supplementary Information, Fig. S1, S2, S3). Analysis of covariance (ANCOVA) was used to test the hypothesis of differences between treatments replicates and between treatments (Fox and Weisberg, 2011; Navada et al., 2019). As there was no significant difference between treatment replicates in most of the tests ($p < 0.05$), the combined data from all three replicates was used for the regression analysis on each day. The data analyses and visualization were performed in R (version 4.0) using packages car, reshape, and ggplot2.

3 Results & Discussion

In the freshwater phase (day 2), there was no significant difference in any of the nitrification performance indicators (AOR, NO2_PR and NO3_PR) between treatments (Fig. 1). The average ammonia oxidation rate was 0.85 $\pm$ 0.05 gN m^{-2} d^{-1} and a significant nitrite accumulation was observed (NO2_PR = 0.26 $\pm$ 0.04 gN m^{-2} d^{-1}). This is likely because the previously cooled carriers did not get sufficient time to revive and adjust to the rapid increase in ammonia loading rate. On day 3 (the first day after seawater transfer), the seawater treatments (S and O) had a $\sim$50 and 94% reduction in the NO2_PR and NO3_PR, respectively. This indicates that the exogenous addition of the osmolyte cocktail did not significantly improve salinity acclimation in the nitrifying biofilms.

Two days after the salinity increase (day 5), the nitrate concentration in the seawater treatments was negligible during the first 8h after media exchange. This indicates a complete loss of nitrite oxidation activity. Further, on day 5, the NO2_PR in the O treatment was -0.03 gN m^{-2} d^{-1}, in contrast to the positive NO2_PR (0.18 gN m^{-2} d^{-1}) in the S treatment. The residual nitrite in O was consumed within the first 8h (from $\sim$1 to < 0.5 mgN L^{-1}). This indicates that both the ammonia and nitrite oxidation were almost completely inhibited in the O treatment on day 5. Further, the medium in the O treatment was turbid with an optical density (OD600) that was an order of magnitude higher than that of the other two treatments (0.05 compared to 0.007). This suggests a higher growth of free-living/pelagic bacteria in O compared to the other treatments. This was confirmed by light microscopy that showed a significantly higher abundance of bacterial cells (putative heterotrophs) in O. As the osmolytes

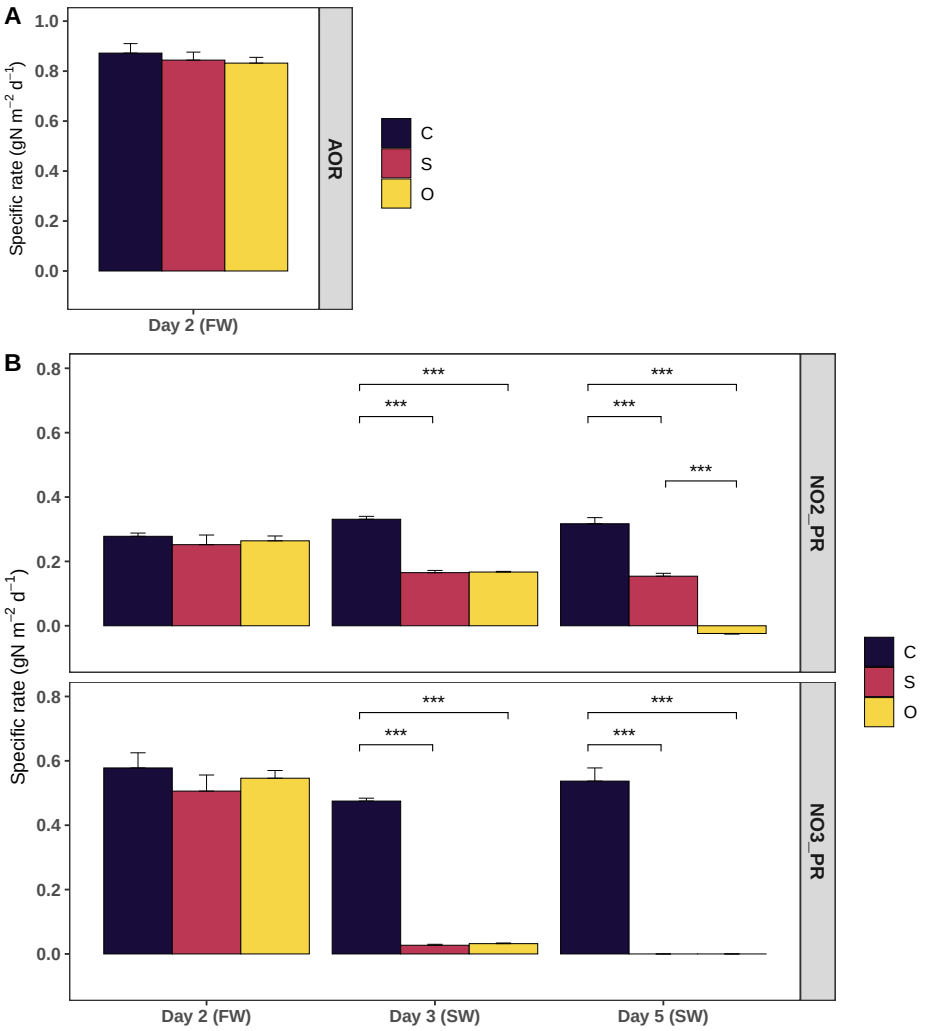


Figure 1: A) Specific ammonia oxidation rate (AOR) on day 2 (freshwater), and B) specific production rates of nitrite (NO2_PR), and nitrate (NO3_PR) on days 2 (freshwater), 3 and 5 (treatments S and O in seawater). For each day, asterisks indicate significant differences between pair-wise treatments (where *** denotes $p < 0.001$). Treatments without asterisks are not significantly different ($p > 0.05$).

are easily degradable organic compounds, they could have been consumed as substrates by the heterotrophs. The ratio of heterotrophs to nitrifiers can be as high as 4:1 even in nitrifying biofilms grown without an organic carbon source (Navada et al., 2020; Navada et al., 2019). Thus, the osmolytes could have been taken up by both the planktonic and biofilm-attached heterotrophs. Although Vyrides and Stuckey, 2017 argue that the biodegradation of compatible solutes is less pronounced at higher salinities, we observed a significant growth of planktonic heterotrophs in our study that can only be attributed to the uptake of osmolytes as substrate. As a majority of osmolyte studies have been on model heterotrophic bacteria like *E. Coli* (Rojas et al., 2014; Wood, 2015), the heterotrophs could also have been superior competitors relative to the nitrifiers for the uptake of osmolytes for osmoregulation. Both scenarios would have promoted the growth and activity of heterotrophs and thereby suppressed nitrification through microbial competition for resources, such as oxygen (Rittmann and McCarty, 2001). Thus, the osmoregulatory impact of the osmolytes on nitrification activity was confounded with the effect of the increased competition between the heterotrophs and nitrifiers.

As an osmolyte cocktail was used, we cannot determine which of the osmolytes contributed the most to the suppression in nitrification activity. The response of the microbial community also depends on the type and concentration of osmolyte. It is possible that the growth of the heterotrophs could have been avoided by reducing the concentration of the highly degradable osmolytes (such as trehalose and sucrose) or by providing only the amine-based osmolytes. Further, some studies suggest that glycine betaine may be inhibited by other osmolytes (Feeney et al., 2014; Mendum and Smith, 2002). Thus, future studies should investigate different osmolytes separately under different ranges of concentrations. Investigating the genomes of nitrifying species may also provide an insight into the potential pathways for salinity acclimation in these microorganisms. For e.g., transporter genes (treT) for trehalose (an osmolyte) are present in the genomes of some nitrifying bacteria such as *Nitrosomonas (N. aestuarii Nm36, N. halophila Nm1, N. marina Nm71)* and *Nitrospira (N. sp. QS2205, N. moscoviensis NSP M-1, N. defluvii)*, suggesting that these species may be capable of trehalose uptake from the medium. Future studies should investigate if there exist unique osmoprotectants that can selectively aid osmoregulation in nitrifiers, while not promoting the growth of heterotrophs.

4 Conclusions

In this study, the exogenous addition of osmolytes did not improve salinity acclimation in nitrifying biofilms transferred from freshwater to seawater. In fact, after 48h of exposure to salt, the addition of osmolytes significantly decreased

the nitrification activity. This was likely due to the uptake of osmolytes by the heterotrophs (as a substrate or for osmoregulation), which consequently led to a reduction of nitrification activity due to the competition between the heterotrophs and nitrifiers for oxygen and/or osmolytes. We plan to perform further research on the metagenomic and metaproteomic level to investigate the physiological response of the dominant microorganisms to the salinity increase. Future studies should investigate the impact of individual osmolytes at different concentrations to identify osmolytes that are preferentially taken up by nitrifying microorganisms for salinity acclimation.

Author contributions

By CRediT taxonomy: **SN**: Conceptualization, Experiment design, Methodology, Investigation, Formal analysis, Visualization, Writing – Original Draft. **ML**: Experiment design, Formal analysis, Supervision. **OV**: Formal analysis, Supervision. **MCML**: Experiment design, Resources, Supervision. All co-authors reviewed and approved the final manuscript.

Acknowledgements

This project was funded by TU Delft, Krüger Kaldnes AS and the Research Council of Norway (# 270888). ML was supported by a VENI grant from the Dutch Research Council (NWO) (# VI.Veni.192.252). The authors thank Sirious Ebrahimi (TU Delft) for help with setting up the experiment, and Roel van de Wijgaart and Francesc Corbera Rubio for the physicochemical analysis at TU Delft. We also thank Øyvind Mikkelsen (NTNU), Frederic Gaumet (Krüger Kaldnes AS), and Jelena Kolarevic (Nofima) for their support.

Supplementary Information

Supplementary Information

References

Bassin, J. P., Kleerebezem, R., Muyzer, G., Rosado, A., Van Loosdrecht, M., & Dezotti, M. (2012). Effect of different salt adaptation strategies on the microbial diversity, activity, and settling of nitrifying sludge in sequencing batch reactors. *Applied Microbiology and Biotechnology*, *93*(3), 1281–1294.

Bassin, J. P., Pronk, M., Muyzer, G., Kleerebezem, R., Dezotti, M., & van Loosdrecht, M. C. M. (2011). Effect of elevated salt concentrations on the aerobic granular sludge process: Linking microbial activity with microbial community structure. *Applied and Environmental Microbiology*, *77*(22), 7942–7953.

Bernhard, A. E., & Bollmann, A. (2010). Estuarine nitrifiers: New players, patterns and processes. *Estuarine, Coastal and Shelf Science*, *88*(1), 1–11.

Csonka, L. N. (1989). Physiological and genetic responses of bacteria to osmotic stress. *Microbiological reviews*, *53*(1), 121–47.

Cui, Y. W., Zhang, H. Y., Ding, J. R., & Peng, Y. Z. (2016). The effects of salinity on nitrification using halophilic nitrifiers in a Sequencing Batch Reactor treating hypersaline wastewater. *Scientific Reports*, *6*, 1–11.

Daims, H., Lebedeva, E. V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R. H., Von Bergen, M., Rattei, T., Bendinger, B., Nielsen, P. H., & Wagner, M. (2015). Complete nitrification by Nitrospira bacteria. *Nature*, *528*(7583), 504–509.

Ekama, G., Wentzel, M., & van Loosdrecht, M. (2020). Nitrogen Removal. In G. Chen, M. van Loosdrecht, G. A. Ekama, & D. Brdjanovic (Eds.), *Biological wastewater treatment - principles, modeling and design* (2nd). IWA Publishing.

Feeney, A., Johnston, C., Govender, R., O'Mahony, J., Coffey, A., & Sleator, R. D. (2014). Analysis of the role of the Cronobacter sakazakii ProP homologues in osmotolerance. *Gut Pathogens*, *6*(15).

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*, *532*(July 2020), 735973.

Fox, J., & Weisberg, S. (2011). *An R Companion to Applied Regression* (Second). Thousand Oaks CA.

Gonzalez-Silva, B. M., Jonassen, K. R., Bakke, I., Østgaard, K., & Vadstein, O. (2016). Nitrification at different salinities: Biofilm community composition and physiological plasticity. *Water Research*, *95*, 48–58.

Gonzalez-Silva, B. M. (2016). *Salinity as a driver for microbial community structure in reactors for nitrification and anammox* (Doctoral dissertation). Norwegian University of Science & Technology.

Lefebvre, O., & Moletta, R. (2006). Treatment of organic pollution in industrial saline wastewater: A literature review. *Water Research*, *40*(20), 3671–3682.

Madigan, M. T., Bender, K. S., Buckley, D. H., Sattley, W. M., & Stahl, D. A. (2018). *Brock Biology of Microorganisms* (15th ed.). Pearson Education Limited.

Mendum, M. L., & Smith, L. T. (2002). Characterization of glycine betaine porter I from Listeria monocytogenes and its roles in salt and chill tolerance. *Applied and Environmental Microbiology*, *68*(2), 813–819.

Navada, S., Vadstein, O., Gaumet, F., Tveten, A. K., Spanu, C., Mikkelsen, Ø., & Kolarevic, J. (2020). Biofilms remember: Osmotic stress priming as a microbial management strategy for improving salinity acclimation in nitrifying biofilms. *Water Research*, *176*.

Navada, S., Vadstein, O., Tveten, A.-K., Verstege, G. C., Terjesen, B. F., Mota, V. C., Venkataraman, V., Gaumet, F., Mikkelsen, Ø., & Kamstra, A. (2019). Influence of rate of salinity increase on nitrifying biofilms. *Journal of Cleaner Production*, *238*.

Oren, A. (1999). Bioenergetic Aspects of Halophilism. *Microbiology and molecular biology reviews*, *63*(2), 334–348.

Oren, A. (2011). Thermodynamic limits to microbial life at high salt concentrations. *Environmental Microbiology*, *13*(8), 1908–1923.

Rittmann, B. E., & McCarty, P. L. (2001). *Environmental biotechnology: Principles and Applications*. Tata McGraw Hill Education.

Rojas, E., Theriot, J. A., & Huang, K. C. (2014). Response of Escherichia coli growth rate to osmotic shock. *Proceedings of the National Academy of Sciences*, *111*(21), 7807–7812.

Santos, J. P., Mendes, D., Monteiro, M., Ribeiro, H., Baptista, M. S., Borges, M. T., & Magalhães, C. (2018). Salinity impact on ammonia oxidizers activity and amoA expression in estuarine sediments. *Estuarine, Coastal and Shelf Science*, *211*, 177–187.

Saum, S. H., & Müller, V. (2007). Salinity-dependent switching of osmolyte strategies in a moderately halophilic bacterium: Glutamate induces proline biosynthesis in Halobacillus halophilus. *Journal of Bacteriology*, *189*(19), 6968–6975.

Schimel, J., Balser, T. C., & Wallenstein, M. (2007). Microbial stress-response physiology and its implications for ecosystem function. *Ecology*, *88*(6), 1386–1394.

Sleator, R. D., & Hill, C. (2001). Bacterial osmoadaptation : the role of osmolytes in bacterial stress and virulence. *FEMS Microbiology Letters*, *26*, 49–71.

Sudarno, U., Bathe, S., Winter, J., & Gallert, C. (2010). Nitrification in fixed-bed reactors treating saline wastewater. *Applied Microbiology and Biotechnology*, *85*, 2017–2030.

Vishniac, W., & Santer, M. (1957). The Thiobacilli. *Microbiology and molecular biology reviews*, *21*, 195–213.

Vyrides, I. (2015). Anaerobic treatment of organic saline waste/wastewater: Overcome salinity inhibition by addition of compatible solutes. In L. Behari, S. Nilotpala, P. Sandeep, P. Barada, & K. Mishra (Eds.), *Environmental microbial biotechnology*.

Vyrides, I., & Stuckey, D. C. (2017). Compatible solute addition to biological systems treating waste/wastewater to counteract osmotic and other environmental stresses: a review. *Critical Reviews in Biotechnology*, *37*(7), 865–879.

Wan, C., Yang, X., Lee, D. J., Liu, X., Sun, S., & Chen, C. (2014). Partial nitrification of wastewaters with high NaCl concentrations by aerobic granules in continuous-flow reactor. *Bioresource Technology*, *152*, 1–6.

Ward, B. B., Eveillard, D., Kirshtein, J. D., Nelson, J. D., Voytek, M. A., & Jackson, G. A. (2007). Ammonia-oxidizing bacterial community composition in estuarine and oceanic environments assessed using a functional gene microarray. *Environmental Microbiology*, *9*(10), 2522–2538.

Wood, J. M. (2007). Bacterial Osmosensing Transporters. *Methods in enzymology: Osmosensing and osmosignaling* (pp. 77–107). Elsevier Masson SAS.

Wood, J. M. (2015). Bacterial responses to osmotic challenges. *Journal of general physiology*, 1–8.

Wood, N. J., & Sörensen, J. (1998). Osmotic stimulation of microcolony development by Nitrosomonas europaea. *FEMS Microbiology Ecology*, *27*, 175–183.

Supplementary Information

Investigating the exogenous addition of osmolytes as a salinity acclimation strategy in nitrifying biofilms

Sharada Navada[a,b,*], Michele Laureni[c], Olav Vadstein[d], Mark van Loosdrecht[c]

[a] Department of Chemistry, NTNU - Norwegian University of Science and Technology, N-7491 Trondheim, Norway

[b] Krüger Kaldnes AS (Veolia Water Technologies), N-3241 Sandefjord, Norway

[c] Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, Van der Maasweg 9, Delft 2629 HZ, the Netherlands

[d] Department of Biotechnology and Food Science, NTNU - Norwegian University of Science and Technology, N-7491 Trondheim, Norway

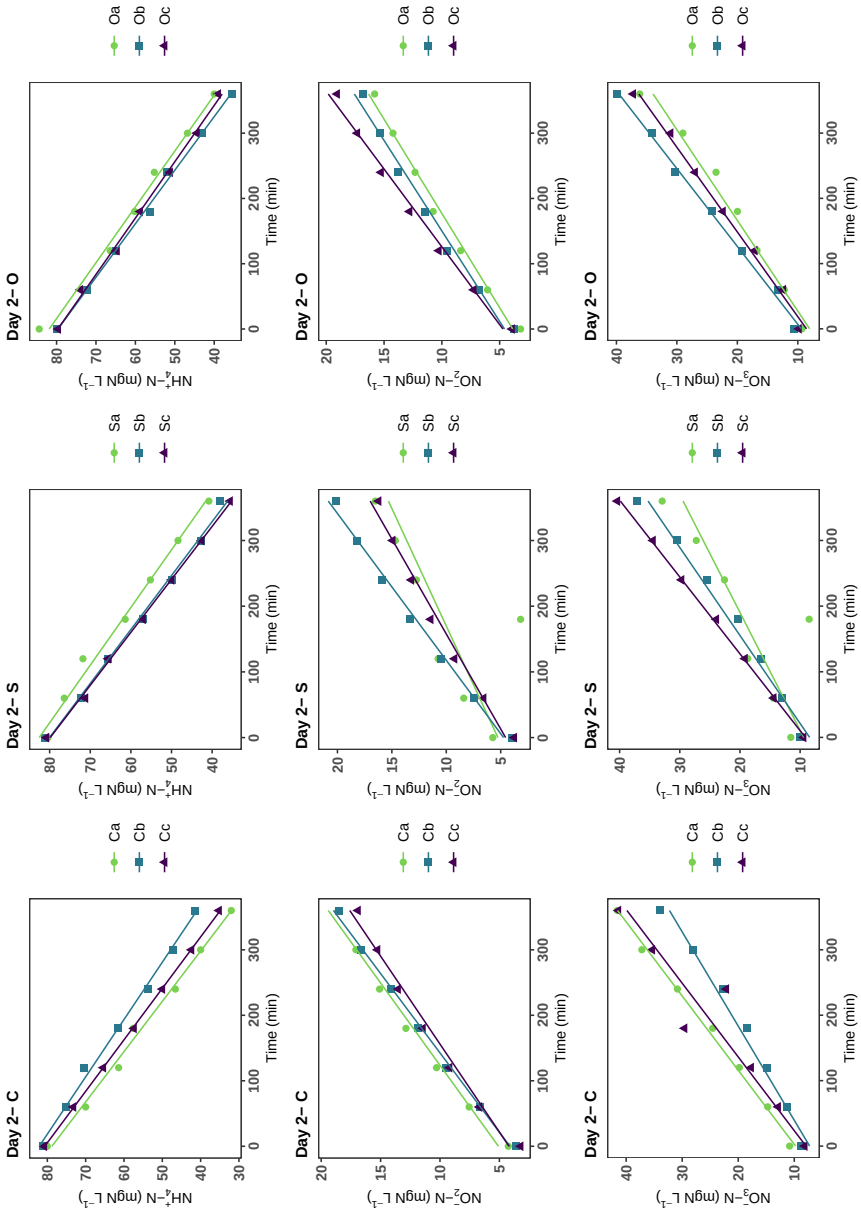


Figure S1: Day 2 - Concentration of ammonia, nitrite and nitrate vs time in the replicates of each treatment

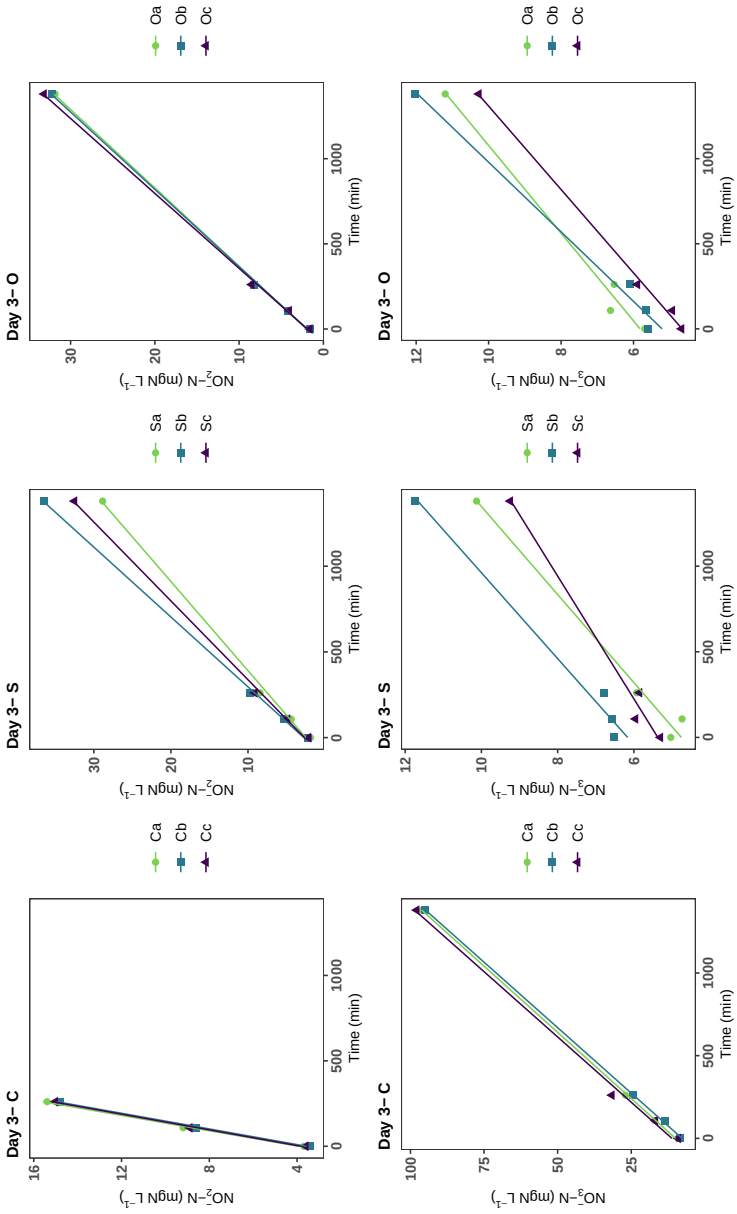


Figure S2: Day 3 - Concentration of nitrite and nitrate vs time in the replicates of each treatment. Only the first three nitrite concentration points were considered for the control treatment, as the nitrite was completely reduced within 24h. The graphs show read values adjusted for salinity factor.

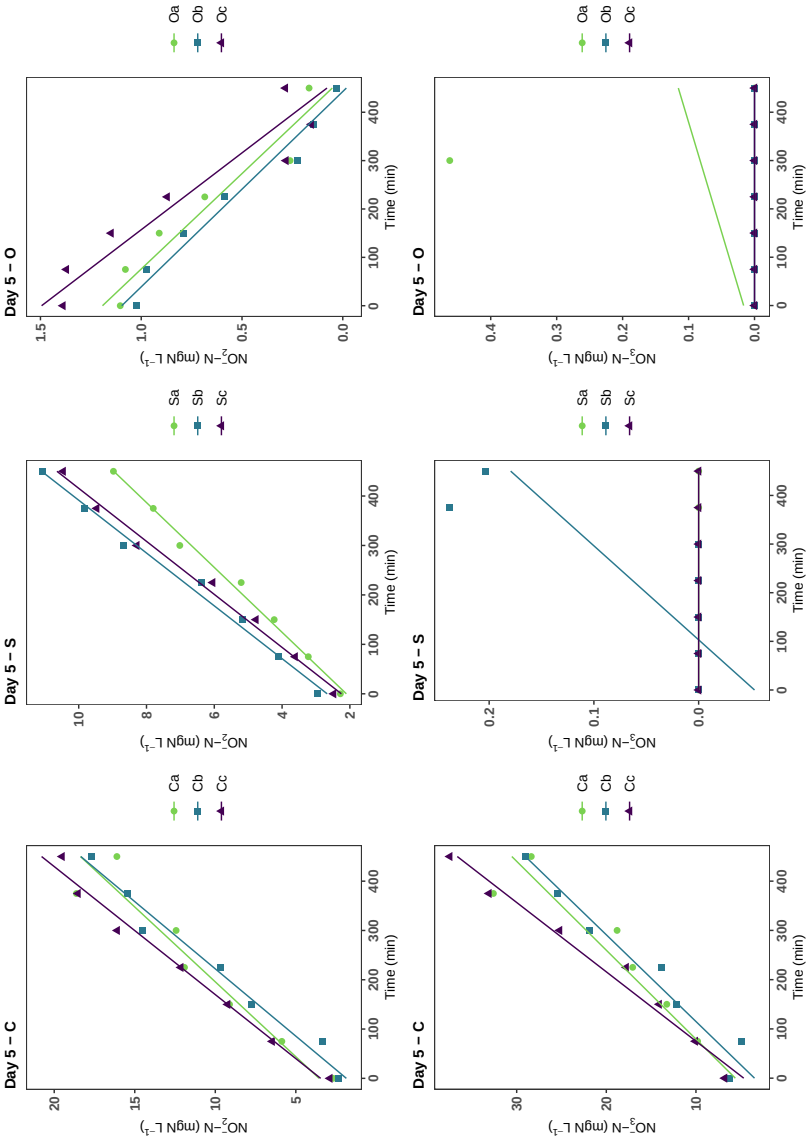


Figure S3: Day 5 - Concentration of nitrite and nitrate vs time in the replicates of each treatment. The graphs show read values adjusted for salinity factor. Most of the nitrate read values in S and O treatments were negative and were considered as zero for the analysis.

Appendix

Appendix A

Appendix

A.1 Photos of the experimental setups

Figure A.1: Experimental setup in **Articles I, II, and IV**. The setup was modified slightly based on each study.

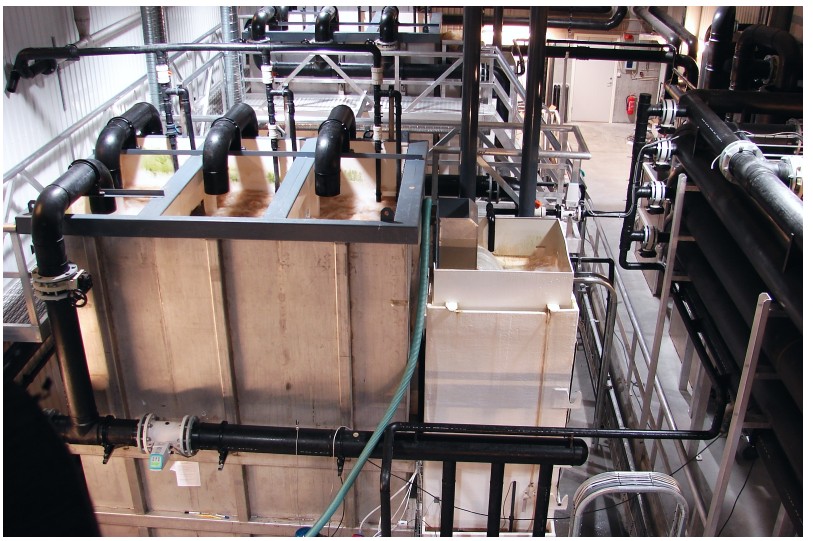


Figure A.2: Semi-commercial MBBR at Nofima (**Article III**)

Figure A.3: Glass MBBRs used for capacity tests in **Article IV**

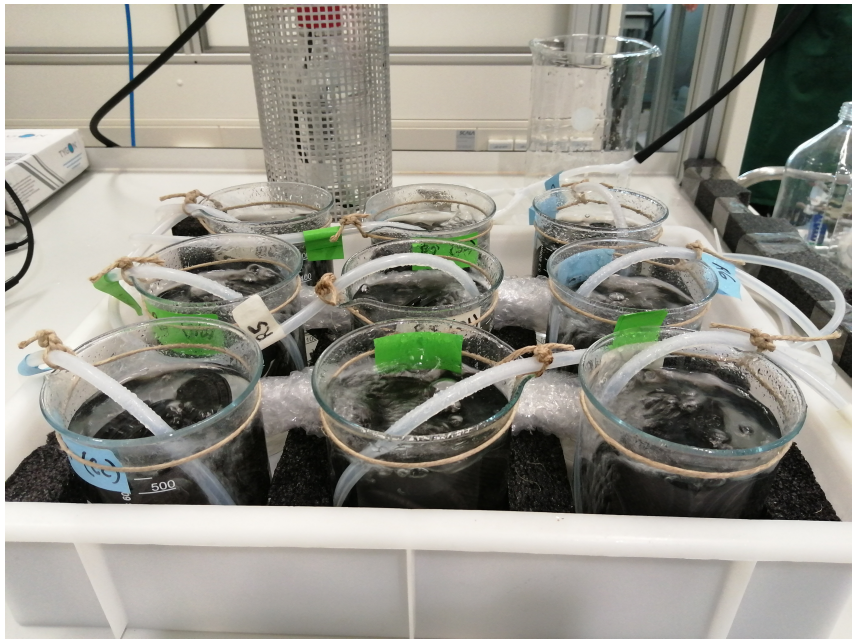


Figure A.4: Experimental setup at TU Delft (**Article V**)

A.2 Tests for ammonia removal in the water phase and by other processes

In **Article I**, additional tests were performed to measure the extent of ammonia removal attributed to nitrifying bacteria on the biofilm carriers, compared to the bacteria in the water phase of the MBBR and other ammonia-consuming processes. Three capacity tests were conducted simultaneously in aerated 5 L buckets: i) Control (C'): carriers (∼40% by volume) and water from a control MBBR; ii) Water (W): only water from the same control MBBR and no carriers; iii) Inhibitor (I): same as test C', with Allyl-thiourea (ATU) (NTH600, WTW, Germany) added to inhibit nitrification (5 mg ATU per liter of sample). Dissolved oxygen was also measured during these tests. The ammonia and oxygen consumption in the control (C') reactor were significantly higher than in the water phase (W) and nitrification inhibition (I) reactors (Fig. A.5). The control reactor showed a linear decrease in the ammonia concentration with time (slope = -0.07 mgN L^{-1} min^{-1}, p = 0.0001, $R^2 \sim 1.00$), whereas reactors W and I showed no significant correlation between the ammonia concentration and time (p = 0.74 and 0.76, respectively). The results indicate that the ammonia and oxygen consumption in the water phase or due to other processes, such as heterotrophic activity, was negligible. Hence, the measured ammonia consumption may be predominantly attributed to the nitrification process in the biofilm carriers.

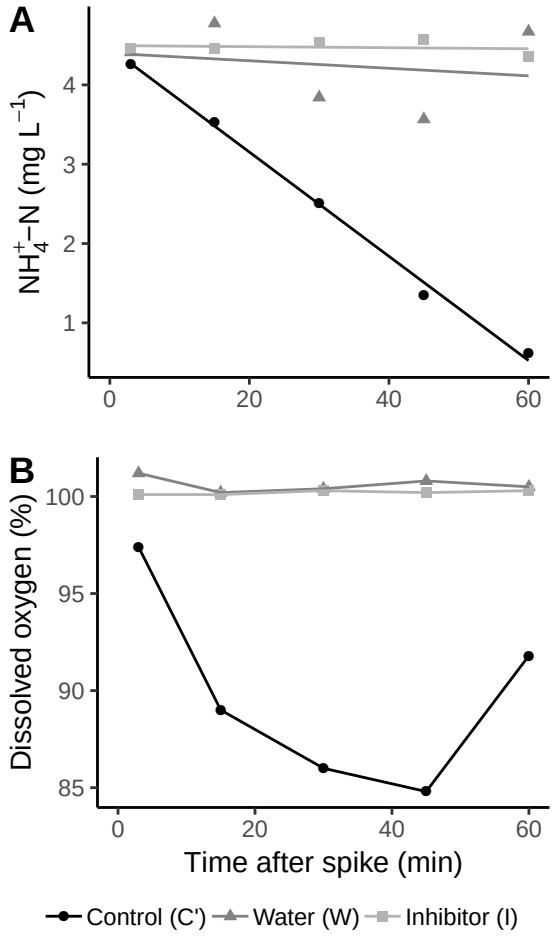


Figure A.5: Ammonia removal in the water phase and by processes other than nitrification. Simultaneous comparison of the ammonia and oxygen consumption in three 5 L reactors with i) biofilm carriers and water from a control MBBR (Control, C'), ii) water phase without carriers (Water, W), and iii) due to processes other than nitrification, same setup as C' but with the addition of a nitrification inhibitor (Inhibitor, I). The graphs show A) the ammonia concentration, and B) the dissolved oxygen in the reactors (as an indicator of oxygen consumption) during the capacity test.

A.3 Analytical methods for nitrogenous compounds

Compound	Method	Standard Method	Measuring range	Confidence interval	Seawater compatibility	Dilution range
Ammonia	Merck test kit 1.14752	Analogous to US EPA method 350.1 and APHA 4500-NH3 F	0.05 – 3.00	± 0.06	Suitable for seawater with the addition of 0.1 mL of 5M NaOH	1 – 20
	Merck test kit 1.00683	Analogous to US EPA method 350.1 and APHA 4500-NH3 F	5 – 150	± 2	Suitable for seawater	10 – 20
	Autoanalyzer	US EPA method 350.1	0.1 – 10	NA	Suitable for seawater with seawater calibration standards	1 – 10
Nitrite	Merck test kit 1.14776	Analogous to US EPA 354.1 and APHA 4500-NO2 B	0.02 – 1.00	± 0.02	Suitable for seawater	1 – 100
	Autoanalyzer	US EPA method 353.2	0.005 – 10	NA	Suitable for seawater with seawater calibration standards	1 – 20
Nitrate	Autoanalyzer	US EPA method 353.2	0.005 – 30	NA	Suitable for seawater with seawater calibration standards	1 – 5

Table A.1: Description of analytical methods used to measure ammonia, nitrite and nitrate, along with seawater compatibility

ISBN 978-82-471-4995-9 (printed ver.)
ISBN 978-82-471-4978-2 (electronic ver.)
ISSN 1503-8181 (printed ver.)
ISSN 2703-8084 (online ver.)

NTNU
Norwegian University of
Science and Technology