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

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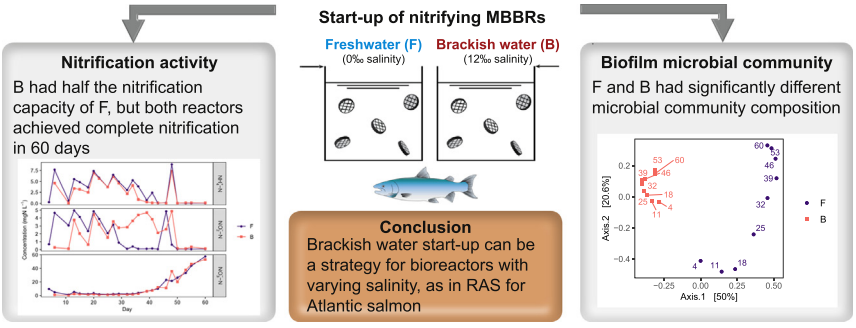
HIGHLIGHTS

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

GRAPHICAL ABSTRACT



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ABSTRACT

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

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1. Introduction

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

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

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

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

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

2. Materials & methods

2.1. Experimental setup and operation

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

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

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

2.2. System variables

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

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

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

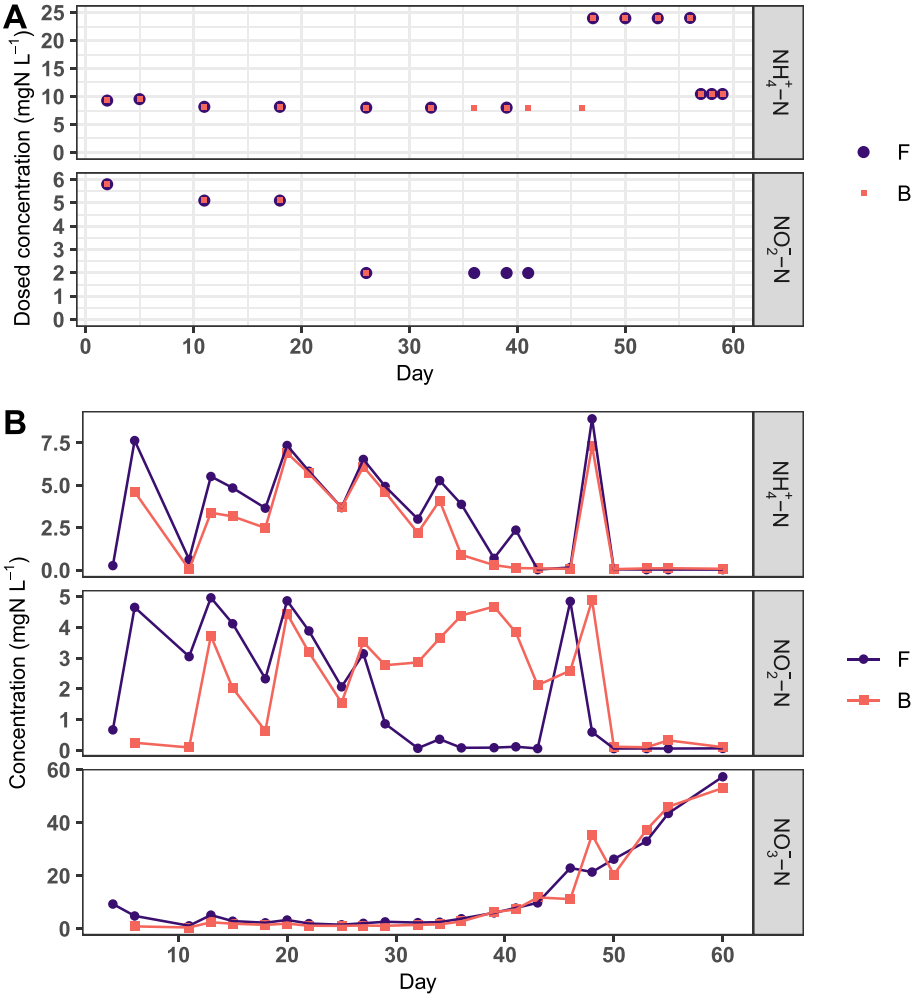

Fig. 1. A) Theoretical concentration of ammonia and nitrite in the reactors corresponding to the respective quantities of ammonium chloride and sodium nitrite dosed; B) Ammonia, nitrite, and nitrate concentration in the freshwater (F) and brackish water (B) reactors during the study. The points have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

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

2.4. 16S rRNA gene amplicon sequencing

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

DNA was extracted from the Sterivex™ filters and one quarter of each biofilm carrier using the DNeasy® PowerSoil Kit (Qiagen, Germany). The eluted DNA samples were stored at −20 °C. Qubit assay for dsDNA with high sensitivity (Invitrogen, Thermo Fisher Scientific) was conducted to measure the DNA concentration. For sequencing, the V3 and V4 regions of the 16S rRNA gene was targeted using broad range PCR primers with Illumina adapter sequences (338F: 5′ cgtcggcagcgtcagatgtctataaga gacagnnnnCCTACGGGWGGCAGCAG-3′ and 805R: 5′-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***

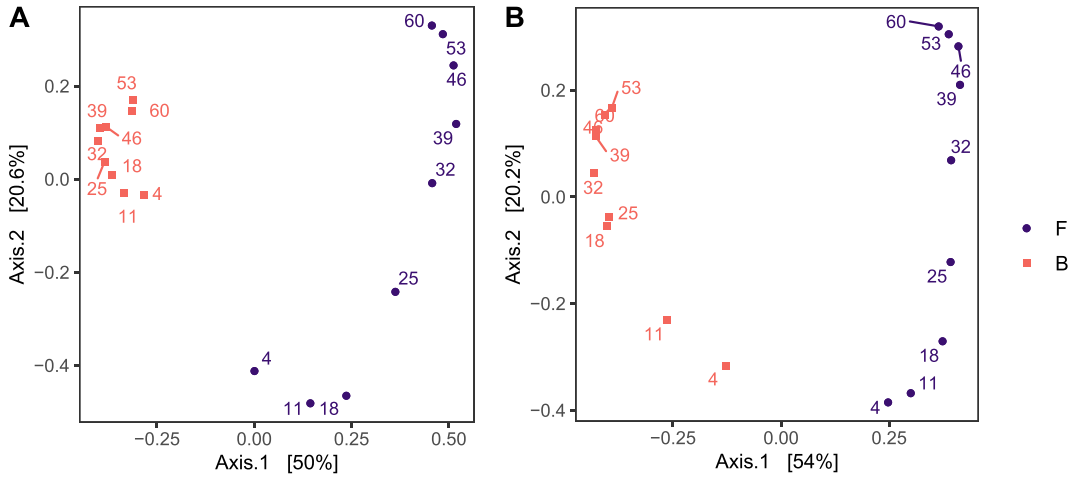

Fig. 2. Ordination by principal coordinates analysis (PCoA) based on A) Bray-Curtis (relative abundance) and B) Sørensen-Dice (presence-absence) distances between the biofilm samples. Labels indicate sampling day. Each point represents the mean data from two biofilm carriers. Square brackets show the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

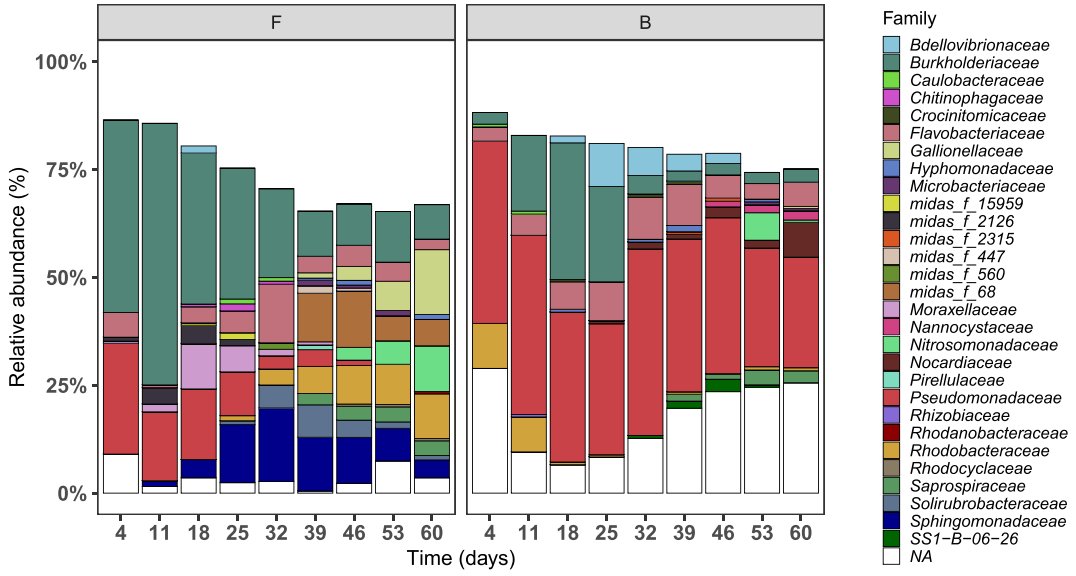

Fig. 3. Relative abundance of taxa in the freshwater (F) and brackish water (B) biofilm classified at the family level. Each data point represents the mean data from two biofilm carriers. For simplicity, only taxa present at relative abundance >1% in at least one sample are shown.

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%

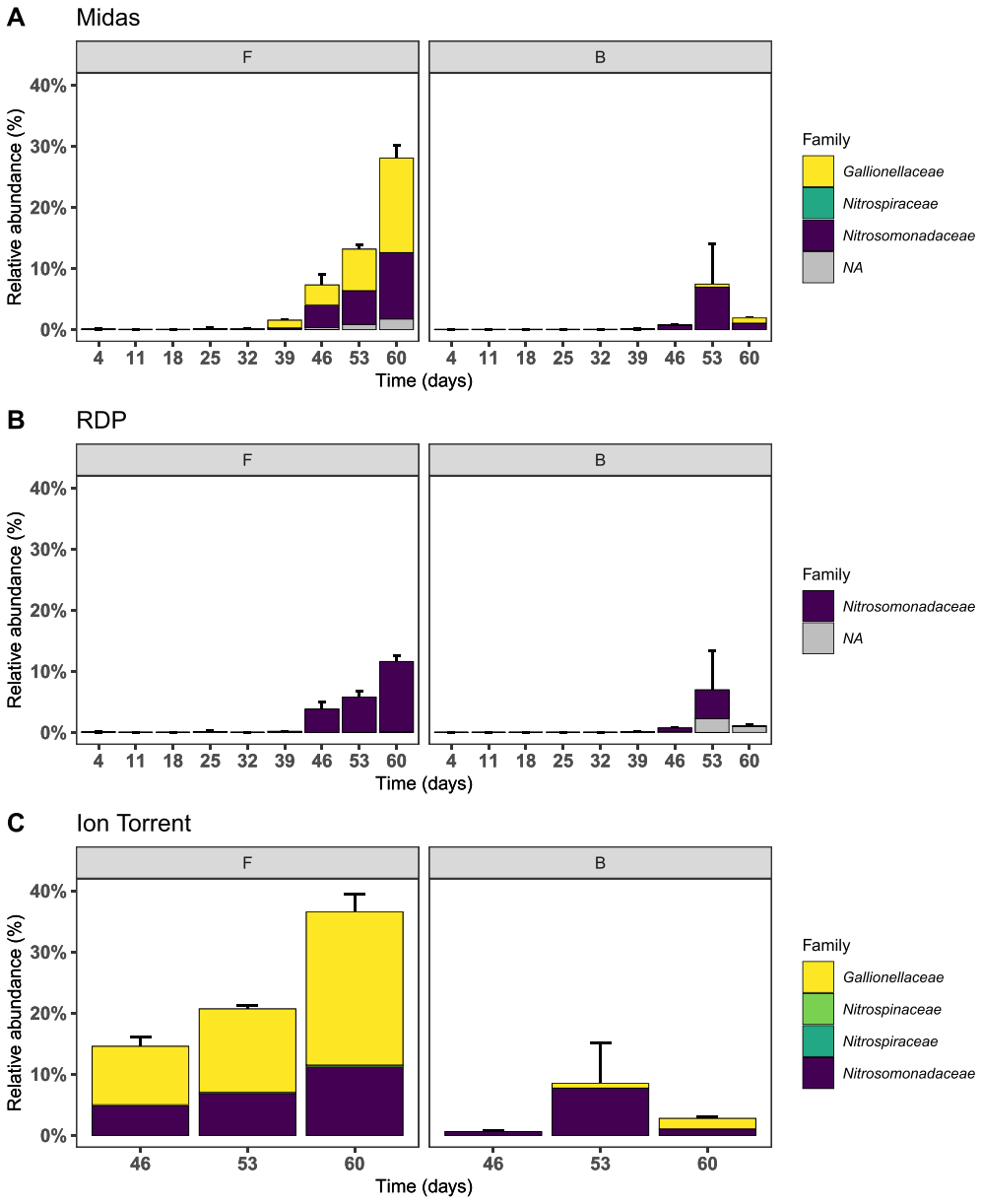

Fig. 4. Relative abundance of nitrifying bacteria in the biofilm in the freshwater (F) and brackish water (B) treatments analyzed by different methods of 16S rRNA gene amplicon sequencing. A) Illumina sequencing with classification by MiDAS3 database B) Illumina sequencing with classification by RDP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

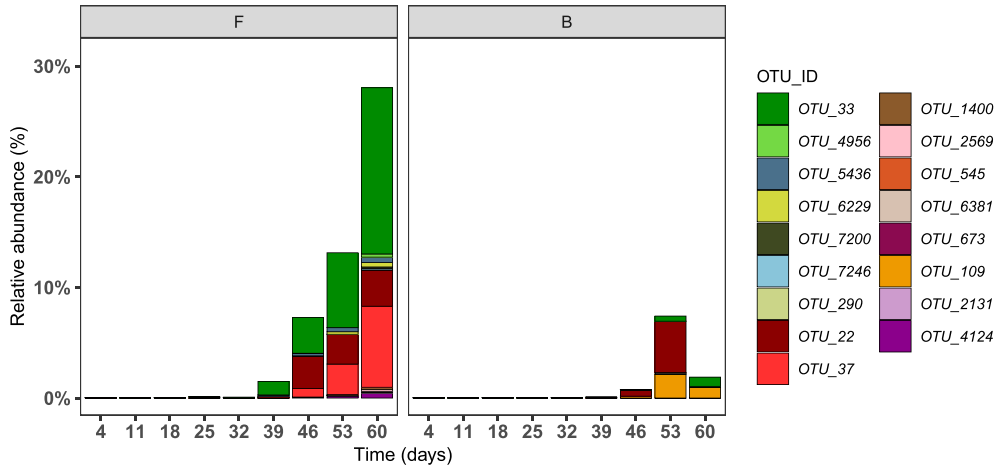

Fig. 5. Relative abundance of the nitrifying OTUs in the biofilms in the freshwater (F) and brackish water (B) treatments during the study. Each bar represents the mean of two replicate biofilm carriers. For the NOB: OTUs 33, 4956, 7246 were classified at the genus level as *Nitrotoga*; OTUs 5436, 6229,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

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Appendix A. Supplementary data

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