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Seeding as a start-up strategy for improving the acclimation of freshwater nitrifying bioreactors to salinity stress

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ABSTRACT

Recirculating aquaculture systems (RAS) for anadromous fish such as salmonids often require salinity changes during the production cycle. However, high or variable salinity can disrupt the biological nitrification process, which can be detrimental to the fish due to the accumulation of toxic ammonia or nitrite. Thus, it is vital to maintain sufficient nitrification capacity in RAS during salinity changes. This study investigated whether seeding with salinity-acclimated carriers in the freshwater start-up phase could increase the salinity tolerance of 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), or a 1:1 mix of both (FB). All duplicate reactors were started up in freshwater and the salinity was increased to seawater (~32‰ salinity) after ~7 weeks. While F and FB 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 the treatments several days after the salinity increase, with the least accumulation in B and the highest in F. The type of seeding influenced the composition of the nitrifying microbial community in the new biofilms (in the freshwater phase). However, the composition in the treatments became similar after ~6 weeks in seawater. The findings indicate that seeding with brackish water biofilm carriers is a potential strategy for accelerating start-up and improving the acclimation of freshwater nitrifying bioreactors to salinity stress. However, nitrite oxidizing bacteria may require a longer period for salinity adaptation. Thus, it is important to closely monitor the nitrite concentration for a prolonged period (several days or weeks) after a salinity increase.

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: 1) ammonia oxidizing microorganisms (AOM, includes ammonia oxidizing bacteria (AOB) and archaea (AOA)) that perform the first step of oxidizing ammonia to nitrite; and 2) nitrite oxidizing bacteria (NOB) that convert nitrite to nitrate (Madigan et al., 2018). Some species within the genus *Nitrospira* are capable of complete ammonia oxidation to nitrate (van Kessel et al., 2015). The microbes performing the nitrification process

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can be sensitive to salinity changes (Madigan et al., 2018). Especially, freshwater bioreactors can undergo a severe reduction in nitrification rate when the salinity is increased (Bassin et al., 2011; Gonzalez-Silva et al., 2016; Kinyage et al., 2019; Moussa et al., 2006; 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.

Our previous study showed that the salinity tolerance of nitrifying freshwater biofilms can be increased by seawater priming (Navada et al., 2020b). The study found that biofilms that have been primed to osmotic stress, i.e. previously exposed to salinities >12‰, undergo a lower decrease in nitrification rates when the salinity is increased. This implies that in a freshwater bioreactor, the first salinity increase to seawater is the most disruptive, whereas subsequent salinity increases have a lower impact on the nitrification performance. Thus, it is important to make the bioreactor salinity tolerant before the fish are introduced into the system. Although seawater priming is a promising strategy, it is time-intensive and the bioreactor may take up to two weeks to recover nitrification activity after transfer to seawater (Navada et al., 2020b). In contrast, 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). This suggests that a feasible strategy is to start in brackish- or seawater, followed by a reduction in salinity before the parr 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 uninoculated 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, salinity decrease in brackish water biofilms can also lead to an initial decrease 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 adding the fish to the system. We wanted to investigate if we could reduce the start-up time of the bioreactor while conferring salinity tolerance simultaneously.

In industrial and municipal water treatment systems, seed carriers or commercial inocula are commonly added to reduce the start-up time by providing the initial bacterial culture (Nogueira et al., 2002). Inoculation has been shown to accelerate 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 may pose a biosecurity risk in RAS. Further, commercial inoculum may 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 start-up time for seawater bioreactors reduced with the addition of seed media, but not with commercial nitrifying bacteria inoculum or with freshwater seed media (Bower and Turner, 1981). Another study showed that biofilm carriers were more effective than commercial inocula in initiating nitrification under marine conditions (Roalkvam et al., 2020). However, we are aware of no studies that have investigated the salinity tolerance of nitrifying bioreactors seeded with salinity-acclimated biofilm during the freshwater start-up 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, or 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 stress than those with non-acclimated freshwater carriers. Further, we operated the system for several weeks after the salinity increase to study the acclimation in the bioreactors.

2. Materials and 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 (water volume ~37 L each). All the reactors were started in freshwater with white virgin carriers, and seeded with black mature biofilm carriers acclimated to freshwater (treatment F), 12‰ salinity brackish water (treatment B), or a 1:1 mix of the two (treatment FB) (Fig. 1). All the treatments were run in duplicate. The seed carriers constituted 10% of the total carriers in each MBBR. After 47 days of start-up in freshwater, the salinity in all the reactors was increased to 32% (seawater). This salinity increase was performed over three days (salinities ~0‰ ➔ 10‰ ➔ 20‰ ➔ 32‰) by adjusting the salinity of the intake water, as described in Navada et al., 2020b. Thereafter, the reactors were monitored for 40 days to observe the recovery of nitrification capacity and the microbial community composition after seawater transfer.

The biofilm carriers used for seeding were taken from a fresh- and brackish water RAS MBBR with Atlantic salmon at the Nofima Center for Recirculating Aquaculture, Sunndalsøra, Norway. Prior to the experimental period, these carriers were transferred into two experimental MBBRs 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 ~ 1 gN m^{-2} d^{-1} at the end of 8 weeks). This was done to ensure that the fresh- and brackish water carriers had similar operating conditions before the experiment. On day 0, six experimental MBBRs were filled (~35% by volume) with white virgin carriers and seeded with black mature biofilm carriers (~10% of total carriers). The virgin and seed carriers were the same size and shape (AnoxK™ Chip P, Krüger Kaldnes, Norway) with a specific surface area of 900 m^2 m^{-3}. In treatment FB, the brackish water seed carriers were marked with a cable tie to distinguish them from the freshwater seed carriers (Supplementary Information, Fig. A.1).

The MBBRs were operated at 15 ± 0.7 °C, pH 7.6 ± 0.3. The reactors were aerated with an average airflow of 50 L min^{-1} during the experiment (dissolved oxygen 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 a macronutrient composition as described in 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 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). All the chemicals were procured from Merck, VWR International. During the freshwater start-up phase, the same ammonia loading rate was provided to all treatments, and this was increased (by increasing the flowrate or the concentration of ammonia in the medium) to adjust to the increasing nitrification rate. 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).

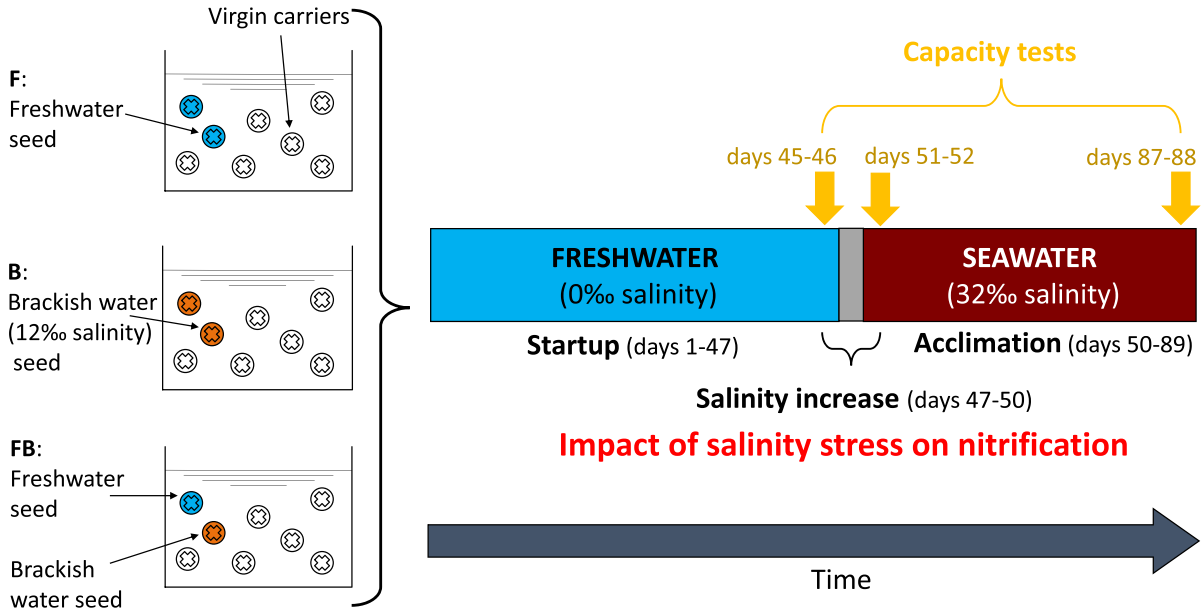

Fig. 1. Experimental design. Virgin biofilm carriers were started up in MBBRs with different seeding strategies. Treatments F and B were seeded with mature biofilm carriers acclimated to fresh- and brackish water, respectively. Treatment FB was seeded with a 1:1 mix of fresh- and brackish water acclimated carriers. Two replicate reactors were used per treatment. 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 stress on nitrification performance.

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 daily (with few exceptions) using the respective Merck test kits (Navada et al., 2020a). To measure the nitrate concentration, water samples were filtered through 0.45 μm syringe filters (Acrodisc®, VWR International) 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. Capacity tests to determine nitrification performance

Capacity tests were conducted on days 45, 51, and 87 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 to obtain an *in situ* ammonia and nitrite concentration of 15–33 mgN L^{-1} and 6–20 mgN L^{-1}, respectively, in the MBBR. After adding the spike, water samples were taken every 7–20 min, filtered with 0.45 μm 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).

In addition to the tests on the main reactors, separate capacity tests were conducted on the white virgin carriers in jacketed glass MBBRs (effective volume: 1 L) on days 46, 52, and 88. The goal was to measure the capacity in the white carriers only, to obtain an estimate of the nitrification rate in the newly developed biofilm. The glass reactors were filled with either freshwater or seawater, according to the salinity in the main MBBRs. From each main MBBR, 51 white carriers were collected, rinsed gently (to remove residual ammonia, nitrite or nitrate), and transferred to the glass reactors. Each glass MBBR was dosed with 5–10 mL of spike solution to achieve a starting ammonia and nitrite concentration of 24–35 mgN L^{-1} and 5–20 mgN L^{-1}, respectively, in the MBBR. Water samples were taken and analyzed using the same procedures as in 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 community composition of the nitrifying bacteria

On day 0, four black biofilm carriers each were sampled from the fresh- and brackish water MBBRs used for seeding. At the end of the freshwater and seawater phases (days 45 and 88, respectively), two virgin (white) and two seed (black) biofilm carriers were sampled from each reactor of treatments F and B. For treatment FB, two virgin carriers, two freshwater seed carriers, and two brackish water seed carriers were sampled from each reactor. All the carriers were preserved at −80 °C. 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™, 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™ using the methods described previously (Navada et al., 2020b; Navada et al., 2019). Sequences are deposited in Genbank with accession number PRJNA716159.

2.4. Data analysis

The surface specific *in situ* ammonia oxidation rate (AOR) in each reactor was calculated by the ammonia mass balance normalized to the total protected surface area of the biofilm carriers (Eq. (1)). The surface 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 (Eq. (2)). The ammonia oxidation efficiency (AOX)

was calculated based on the influent and effluent ammonia concentration (Eq. (3)). 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 (Eq. (4)). Pseudo steady state was assumed between sampling times (>24 h). *t*-tests were used to test the hypothesis of similar rates or efficiencies between treatments or time-points.

$$AOR = \frac{\left(C_{a,i}Q_i - C_{a,o}Q_o\right)}{SA} \quad (1)$$

$$NOR = \frac{\left(C_{a,i}Q_i - C_{a,o}Q_o\right) - C_{n,o}Q_o}{SA} \quad (2)$$

$$AOX = 1 - \frac{C_{a,o}Q_o}{C_{a,i}Q_i} \quad (3)$$

$$NOX = 1 - \frac{C_{n,o}Q_o}{C_{a,i}Q_i - C_{a,o}Q_o} \quad (4)$$

where $C_{a,i}$ is the concentration of ammonia in the synthetic medium,

$C_{a,o}$ is the concentration of ammonia in the MBBR,

$C_{n,o}$ is the concentration of nitrite in the MBBR,

Q_i is the flowrate of the synthetic medium to the MBBR,

Q_o is the flowrate out of the MBBR ($= Q_i +$ flowrate of the dilution water)

SA is the total protected surface area of the biofilm carriers in the MBBR

For the capacity tests, the slope of the ammonia (or nitrite) concentration vs time was calculated by robust regression. We chose to perform robust regression instead of removing outliers, as robust regression corrects for potential outliers by downweighing data points with higher residuals (Fox and Weisberg, 2012). To calculate the zero-order kinetics, only the data points where the concentration of ammonia (or nitrite) was >0.5 mgN L^{-1} were used 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 concentration vs time, respectively (Supplementary Information B). When necessary, the maximum nitrite oxidation rate (NOR_{max}) was calculated from the sum of the slopes of ammonia and nitrite concentration 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 dissimilarity 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 by PERMANOVA (Martinez Arbizu, 2020). Analysis of variance (ANOVA) followed by Tukey posthoc test was used to test the hypothesis of equal dissimilarities 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 (McMurdie and Holmes, 2013; Oksanen et al., 2019; Wickham, 2016).

3. Results

3.1. In situ nitrification activity

The nitrification rate increased rapidly after the first month in freshwater, as seen by the increase in nitrate concentration (Fig. 2A), AOR and NOR (Fig. 2B). After day 30, the AOX and NOX in the F and FB treatments were consistently >95% until the salinity change (Fig. 2C). However, the brackish water treatment (B) achieved this efficiency after day 39, indicating a slightly slower onset of nitrification. 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 ($p > 0.2$) and the average 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. These results indicate that seeding with brackish biofilm significantly improved the tolerance and acclimation to salinity stress.

During most of the study, the AOR was not substrate-limited as the ammonia concentration was >0.5 mgN L^{-1}. Nitrite concentration was <10 mgN L^{-1} in the freshwater phase and the first five days after the salinity increase. However, between days 58–69, the nitrite concentration increased to 18–22 mgN L^{-1} in the B reactors. The peaks declined after two weeks in seawater (day 74), and thereafter, the nitrite concentration was mostly <5 mgN L^{-1}. Similar peaks were observed in the other treatments, with nitrite concentration 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. Towards the end of the study, the NOR was 30, 1, and 5% lower than the AOR for the F, B, and FB treatments, respectively. Thus, NOB appear to require a longer acclimatization period at higher salinity than AOB.

3.2. Nitrification capacity tests

At the end of the freshwater phase (days 45–46), there was no significant difference in the AOR_{max} between 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 ± 0.11 gN m^{-2} d^{-1}. Nitrite concentration did not increase 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}) in 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}). 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 3.8× and 2.6× higher AOR_{max} than F and FB, respectively. In the glass reactors, the B treatment had 8× and 5.6× higher AOR_{max} than F and FB, respectively. This suggests that the virgin carriers, and not the seed carriers, contributed most to the difference between treatments. Nitrite concentration decreased during the main and 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 lowest (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 both the nitrite

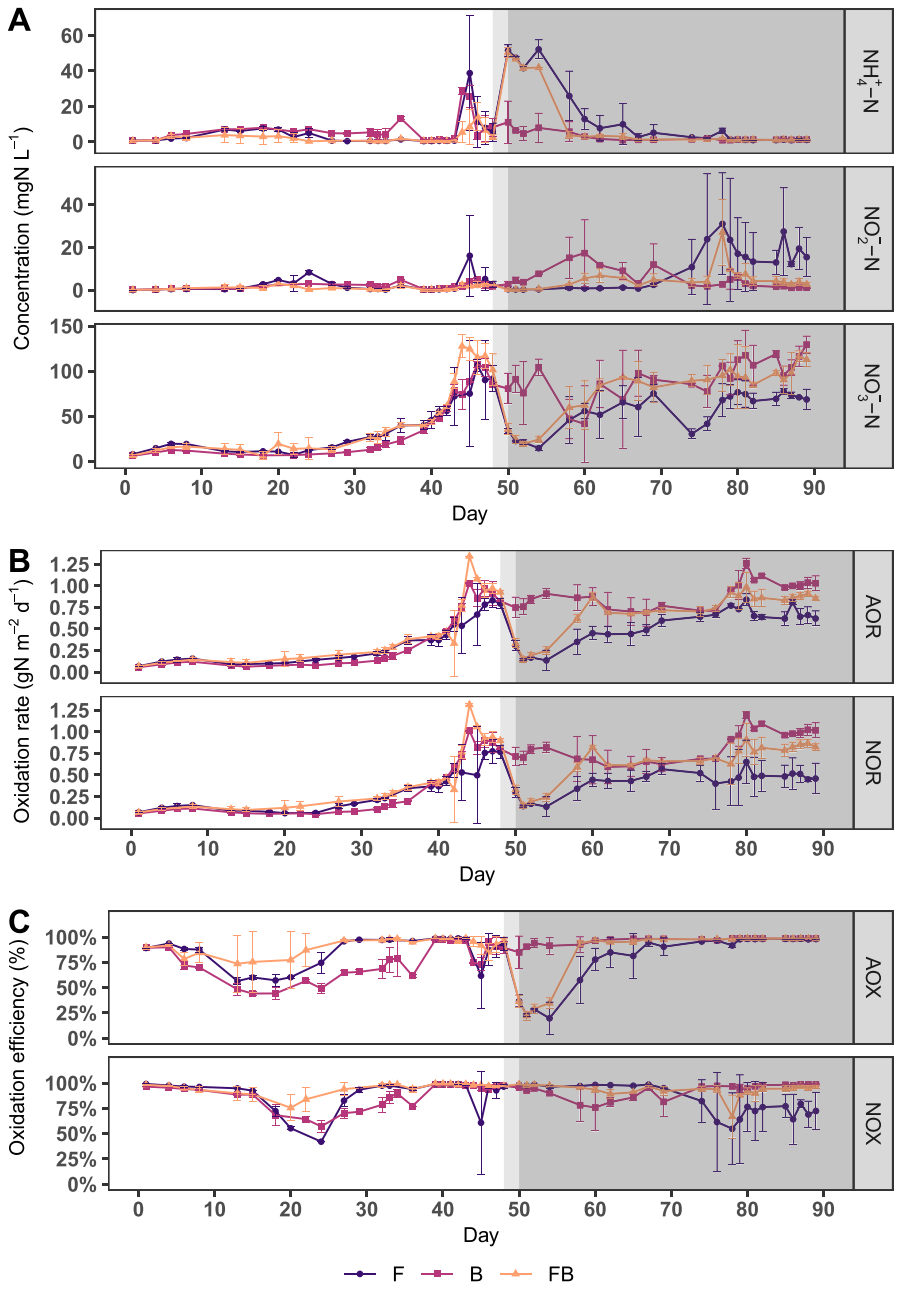

Fig. 2. Nitrification performance in the treatments seeded with carriers acclimated to freshwater (F), brackish water (B) and a 1:1 mix of freshwater and brackish water acclimated carriers (FB) during the study. The graphs show as a function of time, A) Concentration of ammonium, nitrite, and nitrate;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

oxidation rate and the ammonia 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 an approx. 40% lower NOR_{max} (~0.53 gN m^{-2} d^{-1}).

After nearly 6 weeks in seawater (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 the three treatments, considerable nitrite accumulation was observed. In the B treatment, 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} ~ 0.02 gN m^{-2} d^{-1}). This may have been due to inhibition by the accumulating nitrite (~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, due to the slightly lower nitrite concentrations, compared to the main tests.

3.3. Composition of the nitrifying community composition

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, but no nitrifying OTUs were lost in this process.

3.3.1. Differences between virgin carriers of the treatments

On day 45, the virgin carriers in the FB treatment had the highest first order diversity (8.1 ± 2.1) and richness (12.3 ± 1.5), whereas the B treatment had the lowest (diversity 2.5 ± 0.8; richness 2.8 ± 1.0) (Supplementary Information, Fig. A.2). However, the differences in the α-diversity indices between the treatments decreased from day 45 to 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

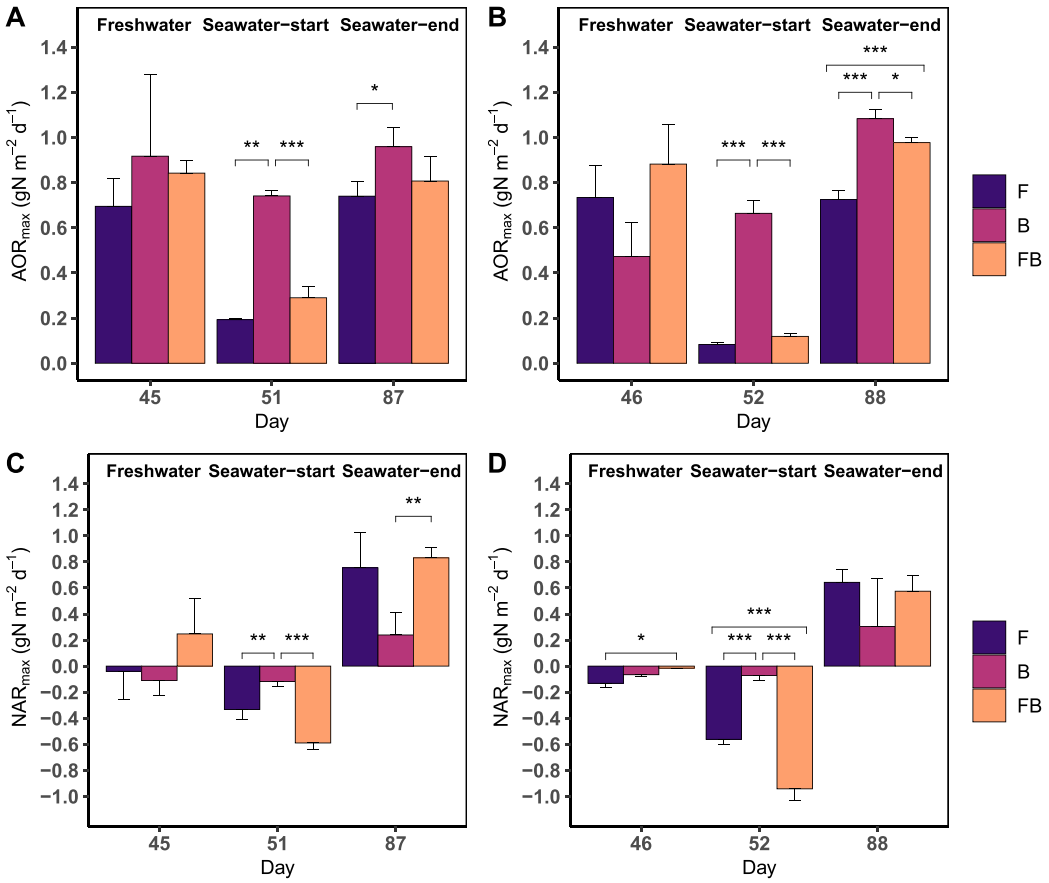

Fig. 3. Capacity test data showing the maximum ammonia oxidation rate (AOR_{max}) and nitrite accumulation rate (NAR_{max}) in the A) main reactors (37 L) and B) glass reactors (1 L) with white carriers only. The treatments were seeded with freshwater-acclimated carriers (F), brackish water acclimated 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 increased from freshwater to seawater during days 47–50 in daily increments (~10‰). Each bar represents the mean (±SE) of two reactors. 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.

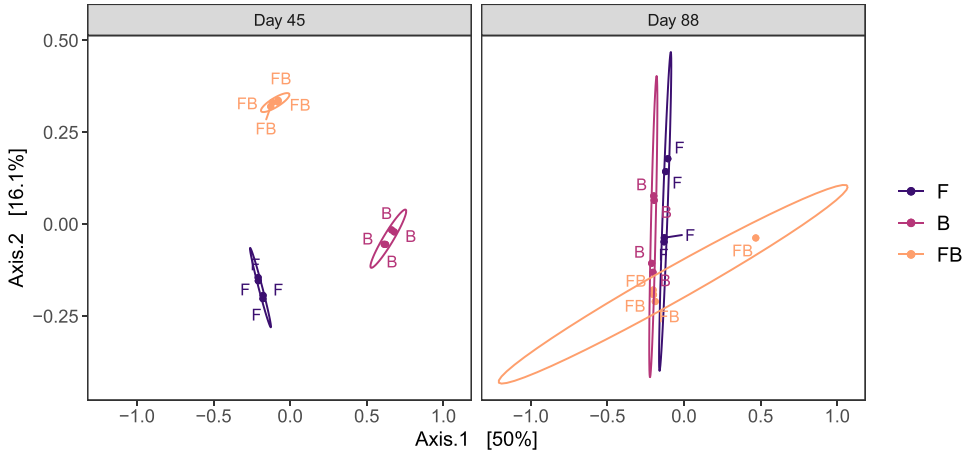

Fig. 4. Ordination plot using principle coordinates analysis (PCoA) based on Bray-Curtis dissimilarity between the nitrifying OTUs in the virgin biofilm samples on days 45 and 88. The ordination was performed on all samples simultaneously, and the graphs are faceted by day to increase clarity. Labels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

dissimilarity ($p < 0.001$, $R^2 = 0.74$). Pairwise PERMANOVA comparisons on day 45 showed a significant difference between all three treatment pairs based on both the Bray-Curtis and Sørensen-Dice dissimilarity 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 dissimilarity) 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 location effects rather than dispersion effects (Fig. 4). All the statistical analysis based on the Sørensen-Dice dissimilarity 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 dissimilarities between B and the other two treatments were 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 dissimilarity ~0.95). As F and FB were relatively more similar (Bray-Curtis dissimilarity ~0.57), this may explain the similar extent of reduction in nitrification activity upon seawater transfer. This was also true based on the Sørensen-Dice dissimilarity (Fig. 5C), as F and FB had more common nitrifying taxa than B (see Section 3.3.3). However, there was no significant difference in the dissimilarity indices between treatments on day 88; a further indication that the community composition became more similar after the salinity increase (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. A.3) suggested 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 ($p \sim 0.03$, $R^2 = 0.80–0.96$). The ordination plot also suggested that the nitrifying community composition in the virgin carriers and the seed carriers evolved over time. The PERMANOVA analysis (based on both dissimilarity indices) confirmed that the nitrifying community composition in the virgin carriers in each of the treatments evolved significantly from day 45 to 88 ($p \sim 0.03$, $R^2 = 0.43–0.93$). The virgin carriers on day 45 in each treatment were also compared with the respective seed carriers on day 0 and on day 45 (two comparisons for treatment FB) based on both dissimilarity indices (Supplementary Information, Fig. A.4, 5). Both the dissimilarity indices showed similar trends. The virgin carrier in the B treatment was the most dissimilar to its seed carrier (both on day 0 and day 45; Bray-Curtis dissimilarity ~0.95). The virgin carriers in B also had a much lower richness than the seed carriers (Supplementary Information, Fig. A.2, A.6). In contrast, the Bray-Curtis dissimilarity between the virgin and seed carriers of F was only ~0.24. 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 to the fresh- and brackish water seed carriers on day 45.

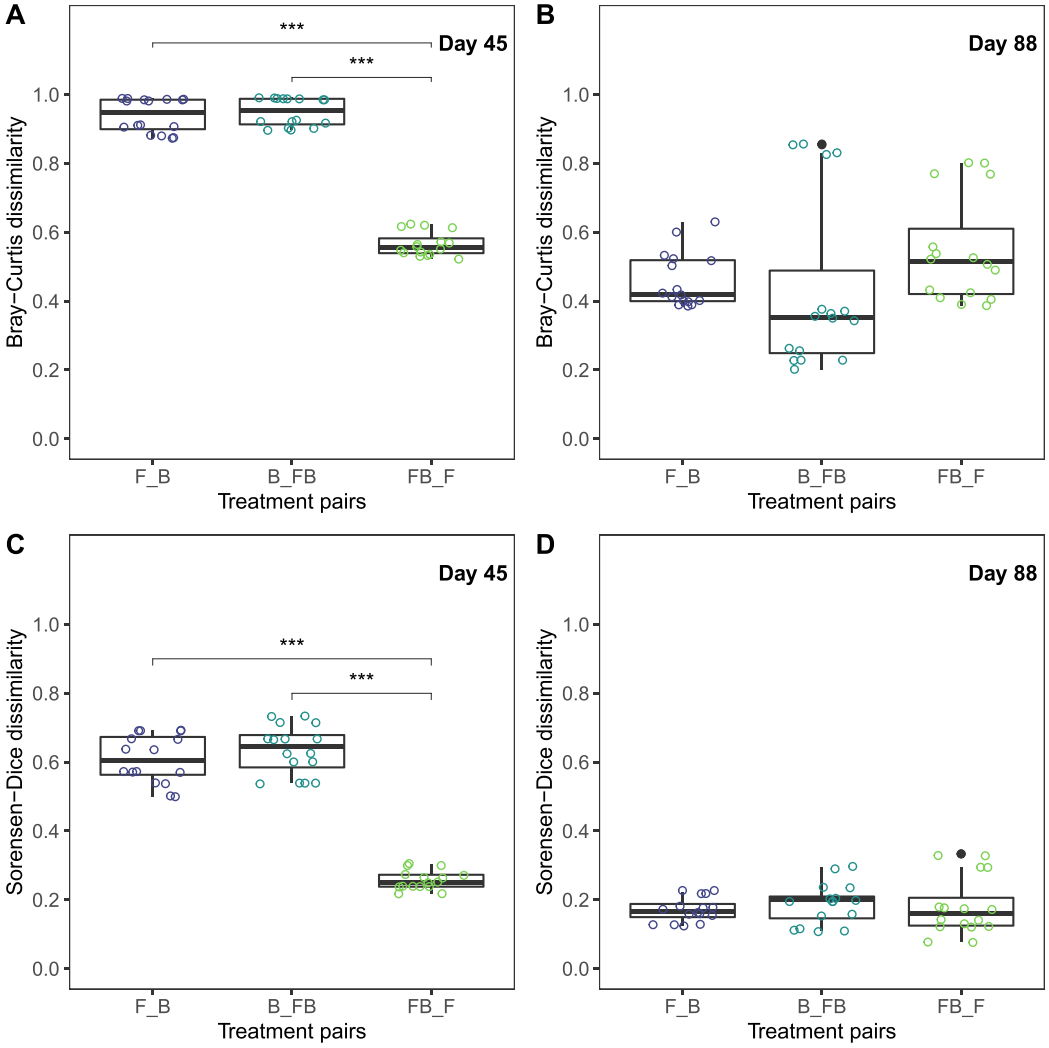

Fig. 5. Box plots of the Bray-Curtis (A, B) and Sørensen-Dice (C, D) dissimilarities between the nitrifying community composition of the virgin biofilm carriers of the treatments on days 45 (A, C) and 88 (B, D). Significant differences between treatment pairs on each day are marked by asterisks (where *** denotes $p < 0.001$, $n = 16$). Treatment pairs without asterisks were not significantly different ($p > 0.05$).

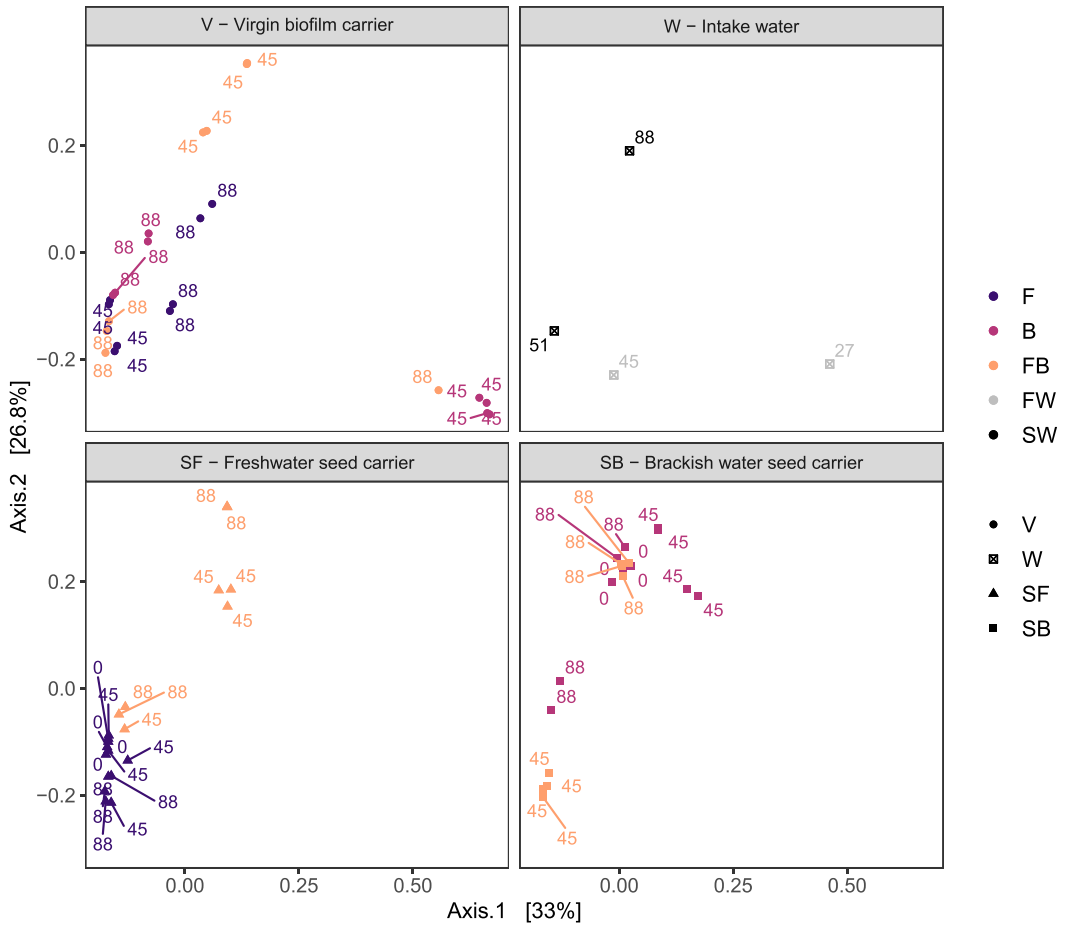

Fig. 6. Ordination plot using principle coordinates analysis (PCoA) based on Bray-Curtis dissimilarity between the nitrifying OTUs of the microbial samples. The ordination was performed on all samples simultaneously, and the graphs are faceted by sample type to increase clarity. Sample types: Virgin 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 on the axes' titles show the percent variance explained by each of the coordinate axes.

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 extremely low relative abundance of nitrifiers (~1%) compared to F (37–50%) and FB (45–60%). There were some differences in the nitrifying community composition between replicates, especially in the virgin biofilms in FB treatment (Supplementary Information, Fig. A.7), whereas the seed carriers had a more uniform composition across replicates (Supplementary Information, Fig. A.8).

4. Discussion

The results of this study show that seeding with brackish water acclimated biofilm can be a potential start-up strategy for RAS bioreactors with variable salinity requirements. Upon a salinity increase from freshwater to seawater, MBBRs seeded with carriers acclimated to brackish water (treatment B) had 2-3× 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). 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). Ours is the first study to show that this strategy can improve the tolerance and acclimation of freshwater biofilm reactors subjected to a salinity stress. The reduction in the nitrification rate observed in F and FB upon seawater transfer (~65–75%) was similar to that observed in unprimed freshwater bioreactors in our previous studies (Navada et al., 2020b; Navada et al., 2019). In contrast, the B treatment had only a small reduction in ammonia oxidation capacity (~20%) and recovered quickly. In a RAS, this temporary decrease in nitrification capacity can be compensated through reduced fish feeding for a few days.

Notably, the nitrite concentration was low immediately after seawater transfer in all treatments, but significant nitrite peaks were observed after a few days in seawater. A delayed drop in the nitrite oxidation rate and consequent nitrite accumulation after a salinity increase was also observed in our previous studies (Navada et al., 2020b, Navada et al., 2019). It is possible that the growth of the nitrite oxidizers was limited by the substrate due to the low nitrite concentration during the freshwater phase. 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). 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 chloride (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

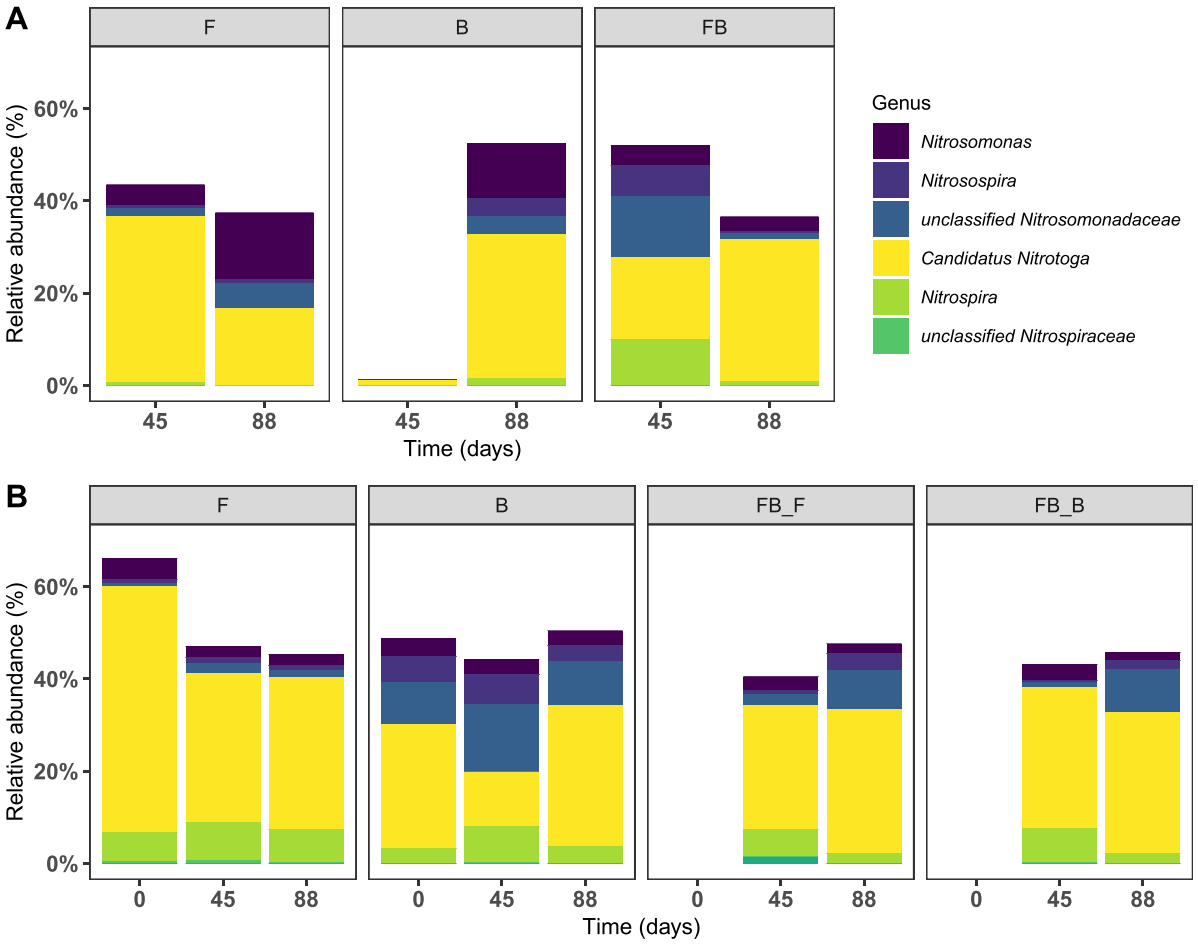

Fig. 7. Relative abundance of the different genera of nitrifying bacteria in A) virgin biofilm carriers and B) seed biofilm carriers of the three treatments on days 0 (fresh- or brackish water), 45 (freshwater) and 88 (seawater). In plot B, FB_F and FB_B refer to the fresh- and brackish water seed carriers,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

nitrite toxicity to the fish (for e.g., reduced feeding or the addition of salt to increase the chloride concentration).

The glass capacity tests indicated 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. Moreover, the different responses of the treatments to salinity stress was likely due to the differences in the 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 community composition established in freshwater. Interestingly, despite the presence of brackish water seed carriers, the AOR_{max} in FB showed a similar response as in F immediately after the salinity increase. This was likely because the nitrifying community composition in these treatments was more similar compared to that in B. As the FB treatment was operated in freshwater during start-up, this could have favored the freshwater species over the brackish water species. However, the overall recovery in the seawater phase was faster in the FB treatment than in F, especially the nitrite oxidation. Thus, despite no improvement in the salinity tolerance (immediately after the salinity stress), the brackish water seeding in FB did improve salinity acclimatization to some extent in the seawater phase.

The nitrifying community composition in the virgin carriers of the B treatment was highly dissimilar from its seed carriers on days 0 and 45 (Bray-Curtis ~0.95). 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 stress. Although diversity and stability are assumed 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). The salinity acclimation in biofilms may also be influenced by factors other than the community composition, such as the structure of the biofilm. 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; Okabe et al., 1996), the higher abundance of heterotrophs in B may have protected the nitrifying bacteria in the deeper layers from osmotic stress. Heterotrophic bacteria can also enhance the production of extracellular polymeric substances (EPS) in nitrifying biofilms (Tsuneda et al., 2001), which can protect against salinity stress (Flemming et al., 2016). It should be noted that the community composition in a RAS bioreactor could differ significantly from that in this study, as RAS water is more complex and contains a higher concentration of organic matter than the synthetic medium used in our study. The relative abundance of heterotrophs would likely be higher in RAS biofilms, which may increase the salinity tolerance of the nitrifying bacteria by virtue of a thicker biofilm or the secretion of EPS. Future studies should

investigate the effect of EPS and other factors on salinity acclimation in nitrifying biofilms in RAS bioreactors.

In this study, nitrification rates of ~0.8 gN m^{-2} d^{-1} were attained within 45 days of freshwater start-up at 15 °C. In contrast, the freshwater start-up of an un-inoculated fixed bed 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 (NH_4^+-$N < 0.5$ 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 during most periods. 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). 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 variability in the order of species colonization in new biofilms, thereby involving a greater component of stochasticity in community assembly compared to mature biofilm carriers. Future studies on bioreactor start-ups should include sufficient replicates to ensure the statistical power 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 selection due to the environmental conditions was most important for the final community composition in this case. The changes in cell density of a species depends on the combined effect of the initial community composition, selection, drift, and dispersal (Nemergut et al., 2013). Thus, the initial species may be outcompeted 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 succeeded in accelerating the start-up with commercial inocula (Bower and Turner, 1984; Kuhn et al., 2010), whereas others did not (Bower and Turner, 1984; Bower and Turner, 1981; Li et al., 2019; Manthe and Malone, 1987). Commercial inocula may also pose a biosecurity risk in RAS. Further, in a biofilm, the interaction between microbes (such as competition and 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 more effective than adding commercial inocula, as also shown by a recent study (Roalkvam et al., 2020). Thus, seeding with biofilm from a pathogen-free bioreactor appears to be a more biosecure and effective strategy than the addition of commercial nitrifying inoculum to a RAS.

5. Conclusions

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

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.736663.

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