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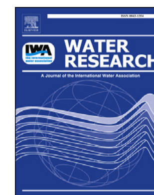

Biofilms remember: Osmotic stress priming as a microbial management strategy for improving salinity acclimation in nitrifying biofilms

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ABSTRACT

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

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1. Introduction

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

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

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

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

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

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

2. Materials & methods

2.1. Experimental setup and design

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

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

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

2.2. Nitrification performance

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

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

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

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

2.3. Microbial analyses

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

2.4. Data analysis and statistics

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

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

3. Results

3.1. Nitrification activity in the freshwater (F) treatments

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

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

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

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

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

3.3. Microbial community composition

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

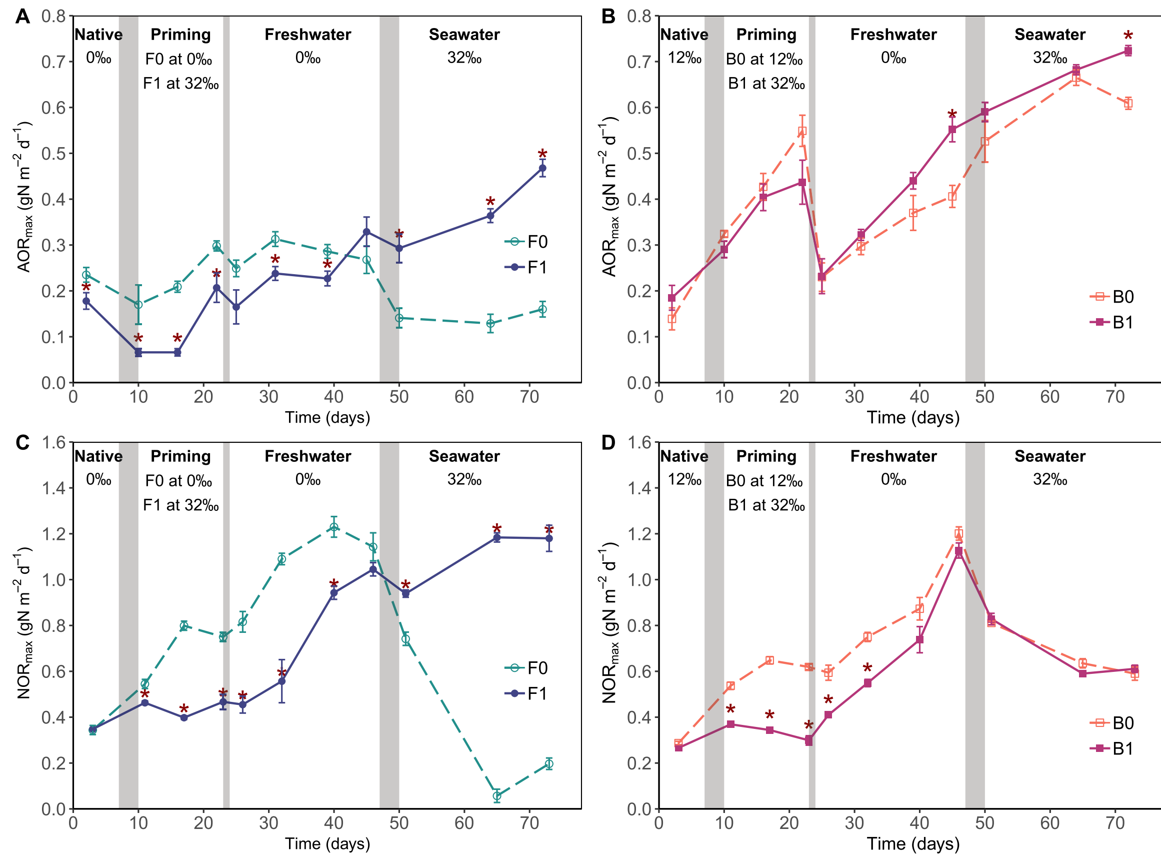

Fig. 1. Maximum ammonia and nitrite oxidation rates (AOR_{max} and NOR_{max}, respectively) during different phases of the experimental period for the freshwater and brackish water treatments. A) AOR_{max} in F0 and F1, B) AOR_{max} in B0 and B1, C) NOR_{max} in F0 and F1, and D) NOR_{max} in B0 and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

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

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

4. Discussion

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

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

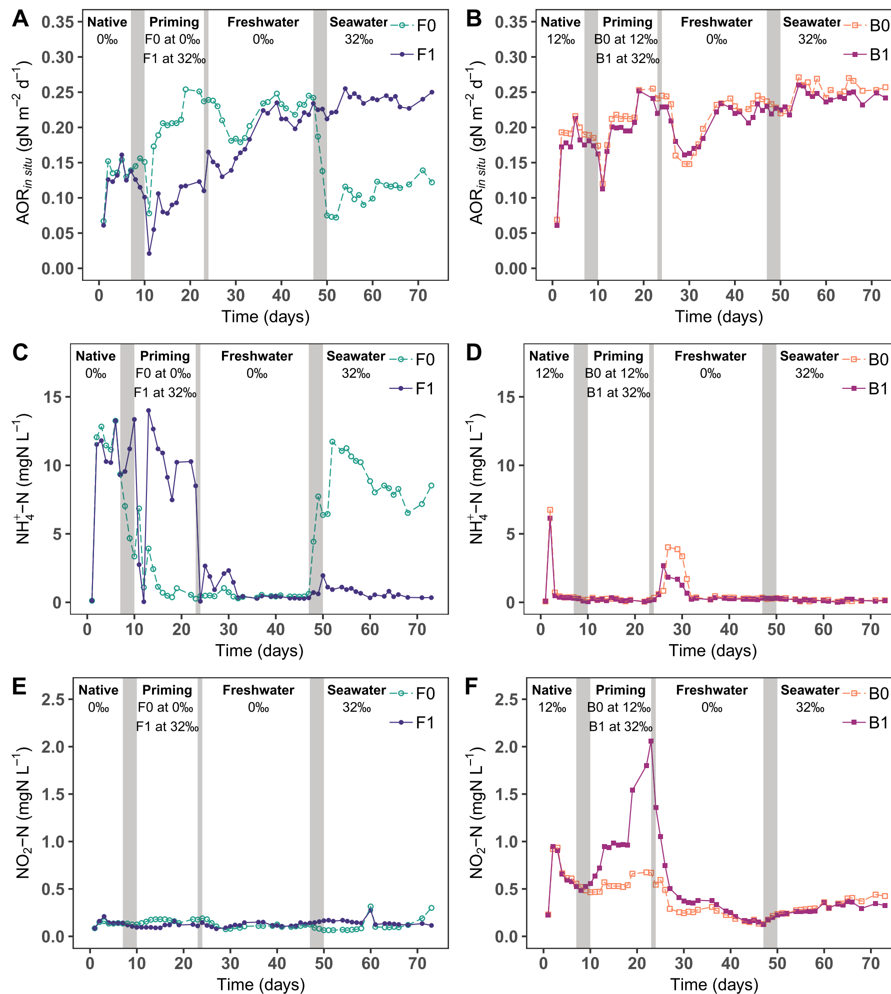

Fig. 2. Average *in situ* measurements in the MBBRs as a function of time. *In situ* specific ammonia oxidation rate ($AOR_{in\ situ}$) for treatments A) F0 and F1, and B) B0 and B1; ammonia concentration (NH_4^+-N) in the MBBR for C) F0 and F1, and D) B0 and B1; nitrite (NO_2^--N) concentration in the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

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

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

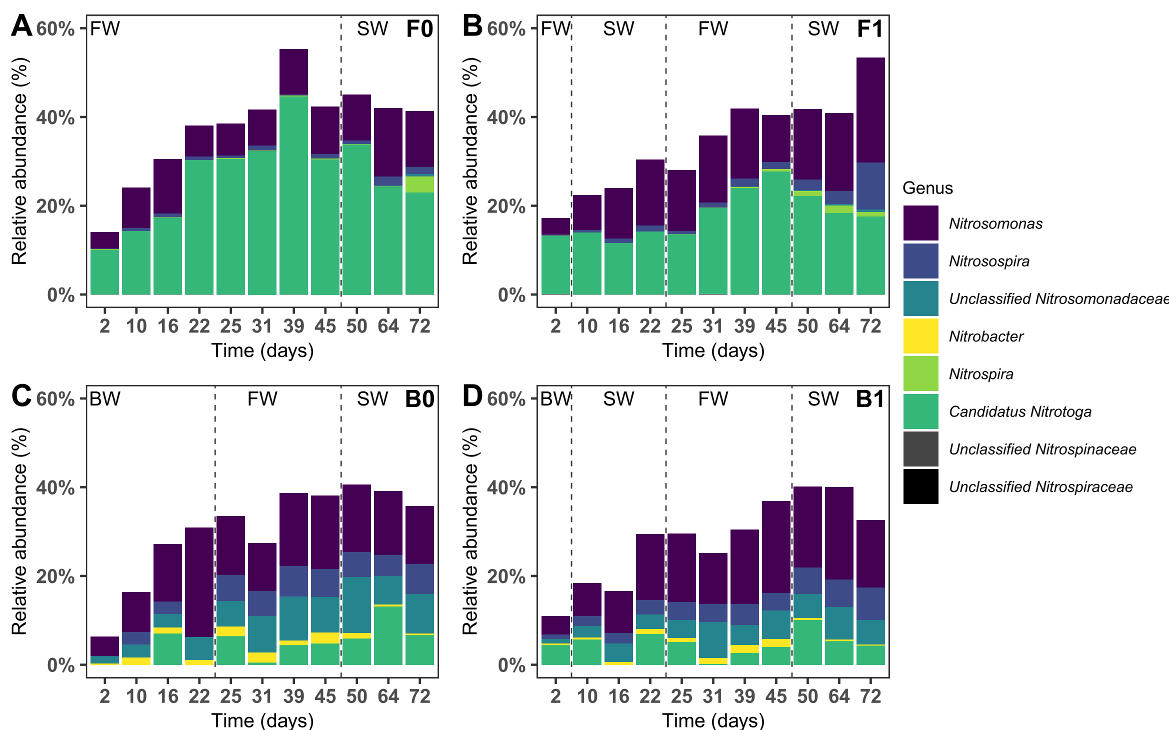

Fig. 3. Relative abundance of the nitrifying genera in treatments A) F0, B) F1, C) B0, and D) B1. Dotted lines demarcate periods in freshwater (FW), brackish-water (BW), and seawater (SW).

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

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

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

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

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

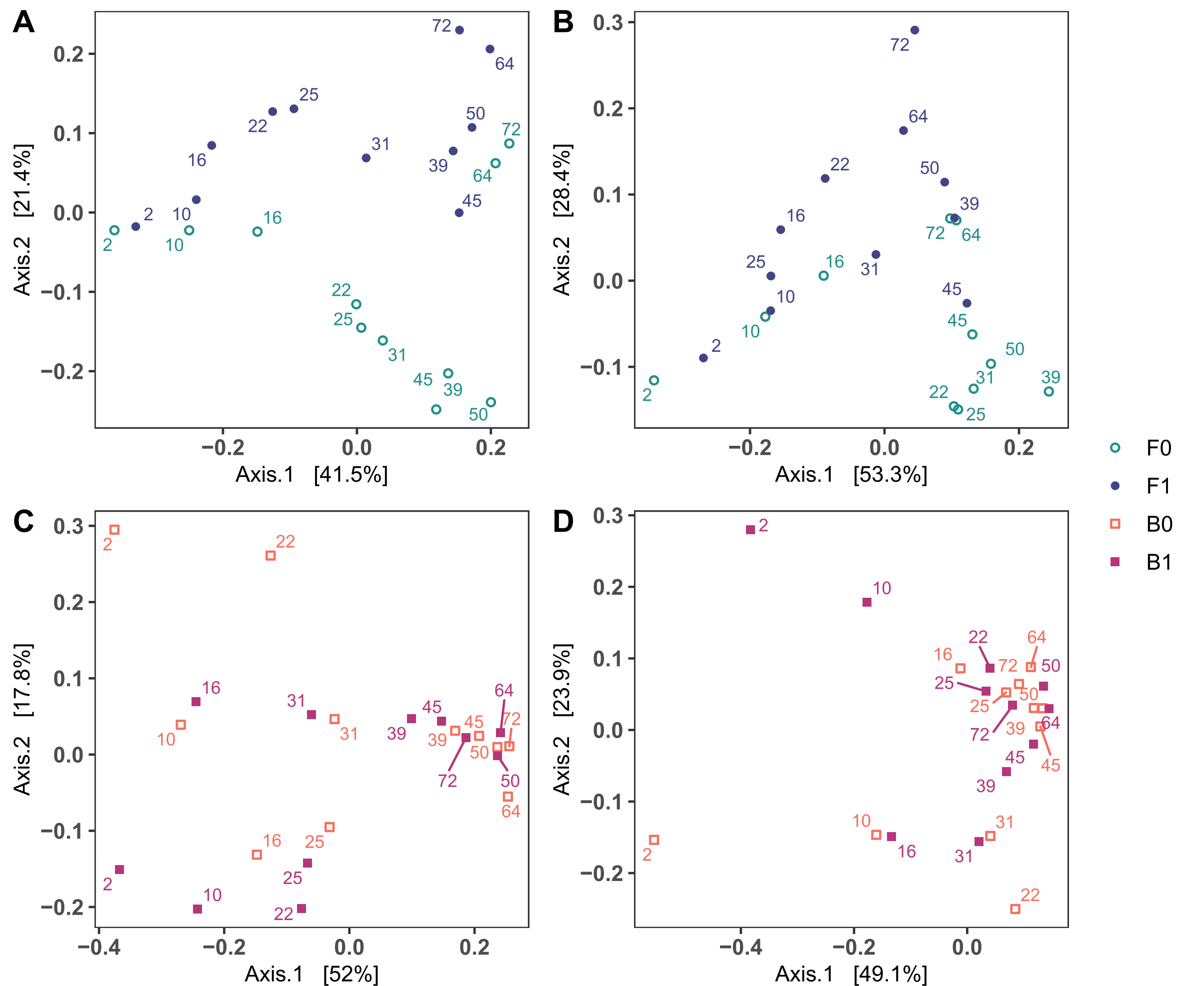

Fig. 4. Ordination by principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity between biofilm samples: A) freshwater treatments – all OTUs, B) freshwater treatments – nitrifying OTUs C) brackish water treatments – all OTUs, and D) brackish water treatments – nitrifying OTUs. Labels indicate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

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

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

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

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

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

5. Conclusions

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

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

Author contributions

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

Declaration of competing interest

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

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Appendix A. Supplementary data

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