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Influence of rate of salinity increase on nitrifying biofilms

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ABSTRACT

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

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1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Experimental setup

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

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

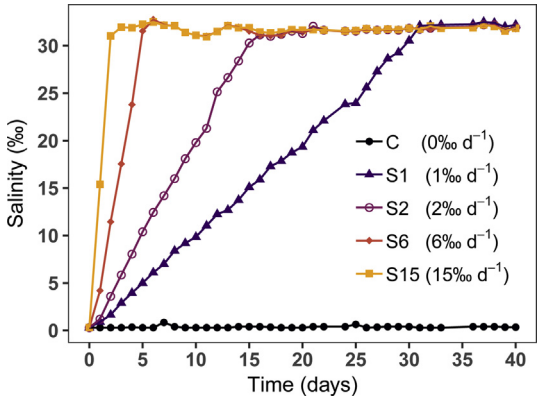

Fig. 1. Experimental design with salinity for the different treatments in ‰ (parts per thousand). The control treatment (C) was always operated in freshwater. Treatments S1, S2, S6, and S15 were transferred from freshwater (0‰) to seawater (32‰) at salinity increase rates of 1, 2, 6, and 15‰ d^{-1},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

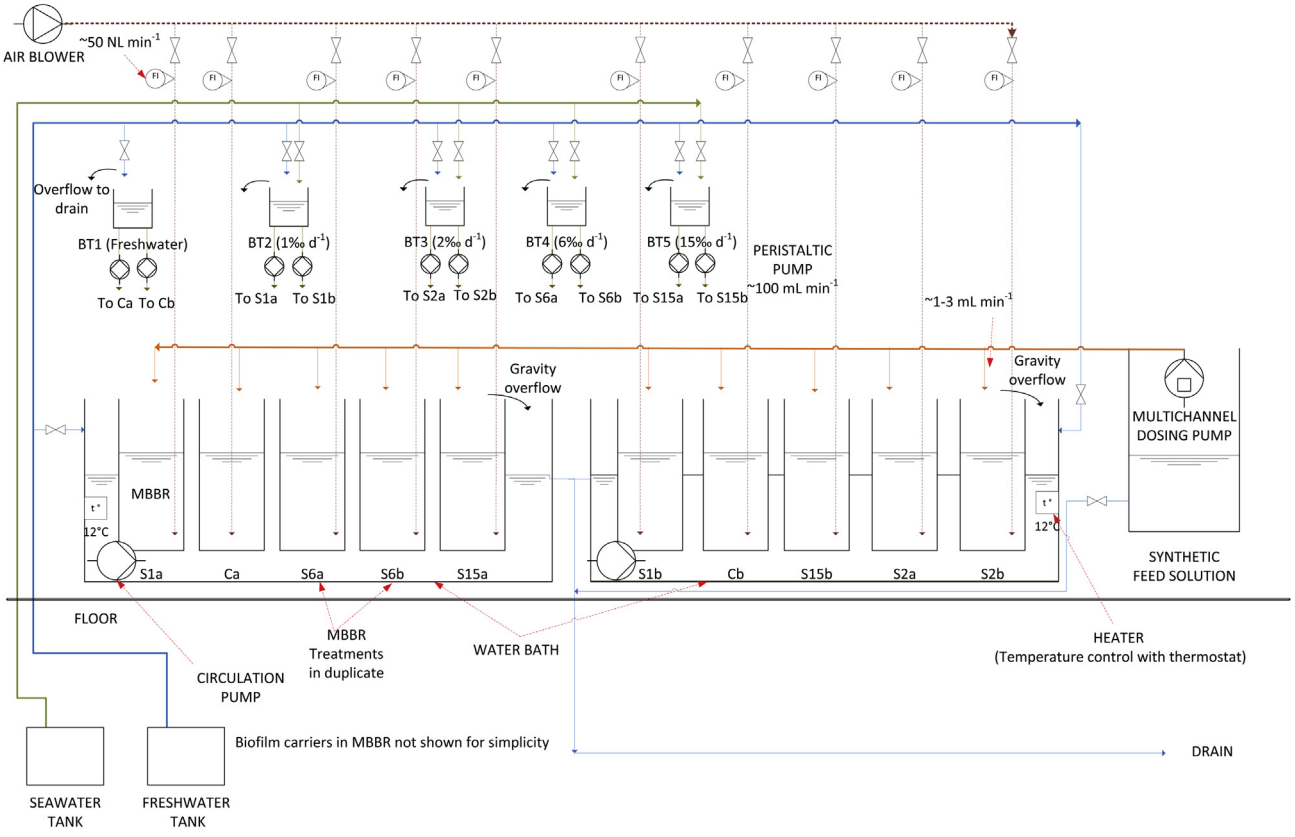

Fig. 2. Schematic diagram of the experimental setup. Continuously operated MBBRs with five treatments in duplicate, placed in temperature-controlled water baths. Treatments S1, S2, S6, and S15 were transferred from freshwater to seawater at salinity increase rates of 1, 2, 6, and 15‰ d^{-1}, respectively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

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

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

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

2.2. Nitrification performance

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

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

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

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

NA: Not applicable.

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

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

2.3. Microbial community analyses

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

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

2.4. Data analysis and statistics

2.4.1. Physicochemical parameters

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

2.4.2. Microbial analysis

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

3. Results

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

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

3.2. AOR_{max} after complete seawater transfer

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

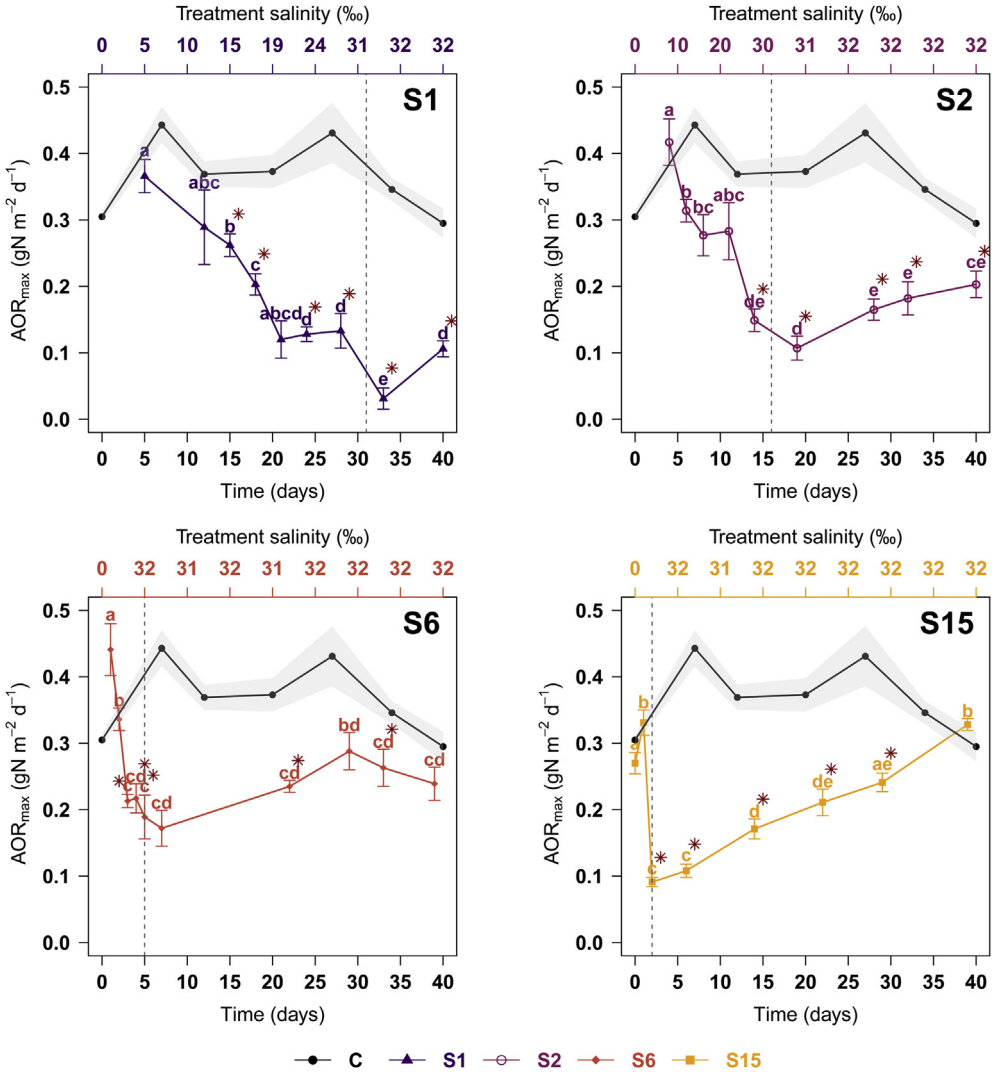

Fig. 3. Maximum ammonia oxidation rate (AOR_{max}) for treatments S1 (1‰ d^{-1}), S2 (2‰ d^{-1}), S6 (6‰ d^{-1}), and S15 (15‰ d^{-1}), compared to the freshwater control C (0‰ d^{-1}). Error bars and grey shaded region indicate standard errors for the treatment and the control, respectively. Data with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

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

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

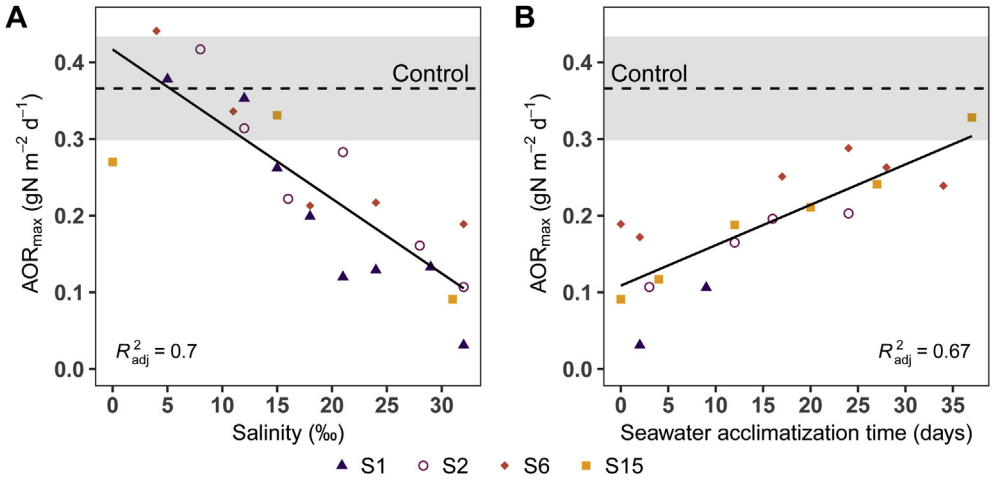

Fig. 4. Linear regression analyses on AOR_{max} from all treatments showing the correlation between A) AOR_{max} and salinity and B) AOR_{max} and seawater acclimatization time. The dashed line and the shaded region represent the average control AOR_{max} and its standard deviation, respectively.

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

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

3.3. *In situ* ammonia oxidation rate and nitrite concentration

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

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

3.4. Microbial community analyses

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

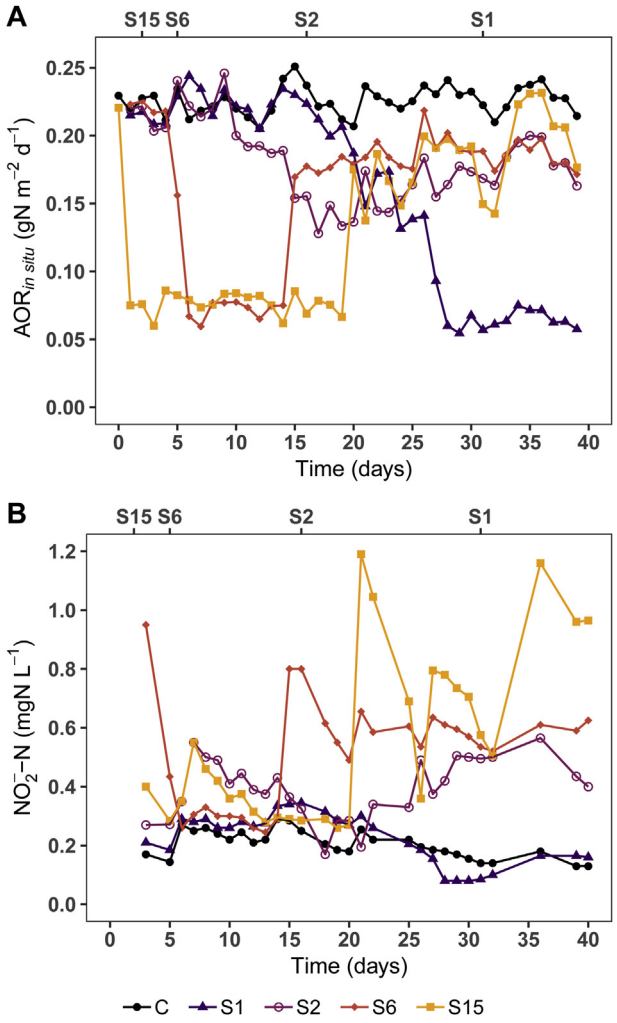

Fig. 5. For the different treatments A) *in situ* ammonia oxidation rate ($AOR_{in\ situ}$), and B) nitrite concentration in the MBBR. Labels above the graphs indicate point of complete transfer to seawater for each treatment. $AOR_{in\ situ}$ was calculated by the ammonia mass balance for each MBBR.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

4. Discussion

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

4.1. AOR_{max} decreased linearly with salinity

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

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

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

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

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

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

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

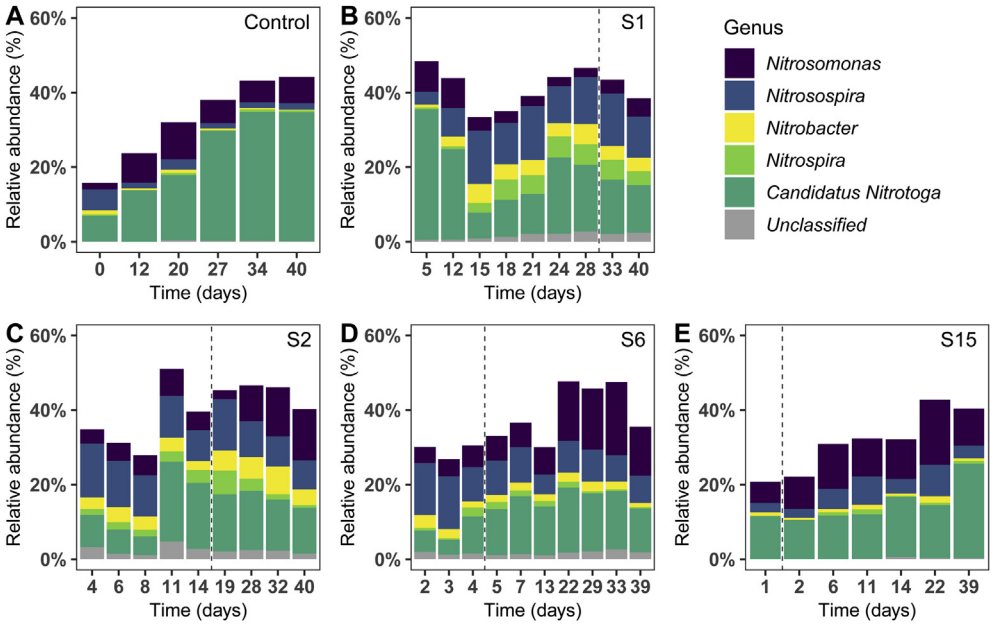

Fig. 6. Relative abundance of nitrifying genera in the biofilm for treatments A) Control, B) S1, C) S2, D) S6, and E) S15. Samples to the right of the dotted line are after complete seawater transfer.

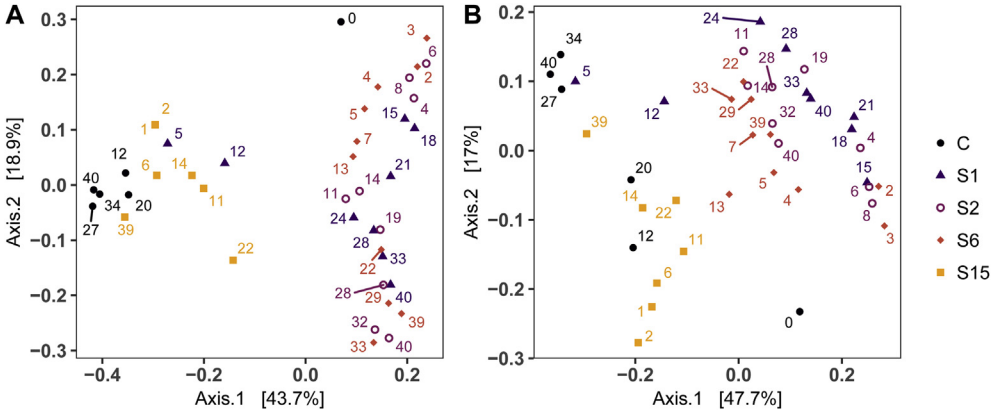

Fig. 7. Ordination by principal coordinates analysis (PCoA) based on Bray-Curtis similarities with A) all OTUs and B) nitrifying OTUs. Labels indicate sampling day. Square brackets show percentage variance explained by each coordinate axis. Treatments S1, S2, S6, and S15 were completely transferred to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

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

4.5. *In situ* nitrification performance

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

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

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

4.6. Microbial communities were influenced by salinity increase rate

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

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

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

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

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

5. Conclusions

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

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

Author contributions

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

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