Influence of temperature and salinity on microbial structure of marine anammox bacteria

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Abstract
Anaerobic ammonium oxidation (anammox) is a type of biological oxidation mediated by a group of *Planctomycete*-like bacteria. Members of the genus "*Candidatus* Scalindua" are mainly found in marine environments, but not exclusively. This group is cultured using different inoculums and conditions; however, its optimal growth conditions are not clear. Additionally, little is known about the factors that influence on the activity and the selection of population of marine anammox bacteria. This study was conducted to investigate the influence of temperature and salinity on the marine anammox community. To accomplish this, an up-flow fixed-bed column reactor was operated, and quantitative FISH with probes specific to dominant marine anammox bacteria was conducted. Anammox activity was observed at 20 and 30°C, but not at 10°C. A nitrogen removal rate of 0.32 kg TN m^{-3} day^{-1} was obtained at 20°C. These results suggest that temperature affects the activity (nitrogen removal rate) of anammox bacteria, while salinity does not affect the activity in the marine anammox biofilm.

Keywords: Marine anammox bacteria; salinity; temperature; community structure

INTRODUCTION

Anaerobic ammonium oxidation (anammox), which is the biological oxidation of ammonium with nitrite as the electron acceptor and dinitrogen gas as the main product, is mediated by a group of *Planctomycete*-like bacteria (Strous et al., 1999). Anammox bacteria have been detected in different wastewater treatment facilities and in natural environments worldwide (Schmid et al., 2005). To date, five genera of anammox bacteria have been reported: "*Candidatus* Kuenenia", "*Candidatus* Brocadia", "*Candidatus* Scalindua", "*Candidatus* Jettenia", and "*Candidatus* Anammoxoglobus" (Kuenen, 2008). Members of the genus "*Candidatus* Scalindua" are mainly found in marine environments, such as the Black Sea (Kuypers et al., 2003) and the Arabian Sea, as well as along the coasts of Namibia, Chile, and Peru (Woebken et al., 2008), but not exclusively (Schmid et al., 2003). In addition, studies involving ^{15}N tracers have also detected anammox activity in the sediments of estuaries in Japan (Amano et al., 2007; Nakajima et al., 2008).

To date, marine anammox bacteria and wastewater anammox bacteria have not been isolated in pure culture. Recently, a few "*Candidatus* Scalindua" species were enriched from sea sediments (Nakajima et al., 2008; van de Vossenberg et al., 2008; Kawagoshi et al., 2009; Kindaichi et al., 2011a). Table 1 shows the information of ranges for temperature and salinity with respect to anammox bacteria. Van de Vossenberg et al. (2008) reported that two species of "*Candidatus* Scalindua" were enriched at 15 and 23°C. Nakajima et al. (2008) reported that two species of "*Candidatus* Scalindua" were enriched at 25°C. Kindaichi et al. (2011a) successfully enriched two marine anammox species at 20°C in previous study. Additionally, Li et al. (2011) reported that the anammox bacterial community structure and abundance in estuary sediments showed strong seasonal dynamics. Furthermore, they found that abundance of anammox bacteria could be influenced by environmental factors such as temperature, ammonium concentration, and salinity. Amano et al. (2011) reported that "*Candidatus* Scalindua" and "*Candidatus* Kuenenia"-like species coexisted in brackish sediment, as well as in estuarine sediments, in subtropical

Table 1. Marine anammox species detected or cultured under various temperature and salinity.

Species	Temperature (°C)	Salinity (%)	Reference
"*Ca.* Scalindua brodae"	15	3.3	van de Vossenberg et al. (2008)
"*Ca.* Scalindua marina"	23	3.3	van de Vossenberg et al. (2008)
"*Ca.* Scalindua wagneri"	25	-	Nakajima et al. (2008)
"*Ca.* Scalindua" spp. "*Ca.* Kuenenia" spp.	22.1-23.5	1.9-2.2	Amano et al. (2011)
"*Ca.* Scalindua wagneri" "Ca. Scalindua marina"	20	3.5	Kindaichi et al. (2011a)

mangrove-aquaculture ecosystems in which the salinity was 1.9-2.2%. Although members of the genus "*Candidatus* Scalindua" has successfully been enriched at different temperatures and salinity levels, the optimal temperature and salinity for the enrichment of marine anammox bacteria are poorly understood.

The objective of this study was to clarify the influence of temperature and salinity on the nitrogen removal rate and microbial community structure of marine anammox bacteria. To accomplish the objective, we operated a column reactor in five phases with the enrichment culture, which were previously enriched at 20°C and 3.5% salinity (Kindaichi et al., 2011a), as the inoculum. We measured the nitrogen removal activity per unit of reactor volume as the primary criterion to evaluate the anammox activity in each phase. At first, we operated the reactor at 30 and 10°C to investigate whether anammox bacteria have the activity without adaptation to the lower temperature. Subsequently, we operated the reactor at 1.75 and 0.875% salinity to investigate the anammox activity at each salt concentration after the reactor was operated at 20°C, which was the same condition as the inoculum, to recover the anammox activity. We also monitored the fraction of anammox bacteria in each phase, because two species of anammox bacteria were present in the inoculum. Quantitative fluorescence *in situ* hybridization (FISH) was then conducted to analyze the population dynamics of the marine anammox bacteria.

MATERIALS AND METHODS

Column reactor

An up-flow fixed-bed glass column reactor was operated with a nonwoven fabric sheet (Japan Vilene Co., Tokyo, Japan) as the biomass carrier as previously described (Tsushima et al., 2007; Kindaichi et al., 2011b). A biomass weighing 1 g (wet weight) was collected from another reactor

Table 2. Operational conditions.

Phase	Time (day)	Temperature (°C)	Salinity (%)
1	0-92	30	3.5
2	92-156	10	3.5
3	156-231	20	3.5
4	231-236	20	1.75
5	236-260	20	0.875

after 341 days of operation in which the nitrogen removal activity per unit of reactor volume was 0.96 kgTN m^{-3} day^{-1} (Kindaichi et al., 2011a) and was used as the inoculum. The reactor volume was 206.8 cm^3, and the surface area of the biofilm carrier was 117 cm^2. The temperatures in Phase 1, 2, and 3 to 5 were 30, 10, and 20°C, respectively (Table 2). The hydraulic retention time (HRT) calculated based on the empty reactor volume was maintained for 1.9 h. The total nitrogen loading and removal rates were calculated based on the concentrations of NH_4^+, NO_2^-, and NO_3^-, and the HRT.

Synthetic wastewater

A synthetic marine nutrient medium was used. Specifically, this medium contained 3.5% of an artificial sea salt (Sealife Marine Tech. Co., Tokyo, Japan) in Phases 1-3, 1.75% in Phase 4, and 0.875% in Phase 5 (Table 2). The medium was also supplemented with 1.3 mM $(NH_4)_2SO_4$, 1.3 mM $NaNO_2$, 1.0 mM $KHCO_3$, 0.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM $CaCl_2 \cdot 2H_2O$, and 1 ml of trace element solutions I and II, as described by Van de Graaf et al. (1996). The medium was flushed with N_2 gas for at least 1 h before adding the nutrients to achieve a concentration of dissolved oxygen (DO) below 0.5 mg/L.

Analytical method

The NH_4^+ concentration was determined using Nessler's method with a UV-visible spectrophotometer (DR-2800, Hach Co., Loveland, USA). The concentration of NO_2^- and NO_3^- were determined using ion-exchange chromatography (HPLC 10Avp, Shimadzu Co., Kyoto, Japan) with a Shodex Asahipak NH2P-50 4D anion column (Showa Denko, Tokyo, Japan) and a UV-VIS detector (SPD-10A, Shimadzu Co., Kyoto, Japan) after filtration of the samples through 0.2-μm pore-size membranes which were made of cellulose acetate (Advantec Co., Tokyo, Japan).

FISH analysis

In situ hybridization was conducted according to the procedure described by Okabe et al. (1999), and a model LSM5 PASCAL confocal laser-scanning microscope equipped with an Ar ion laser (488 nm) and a HeNe laser (543 nm) (Carl Zeiss, Oberkochen, Germany) was used to observe the samples. The 16S rRNA-targeted oligonucleotide probes used in this study were EUBmix (composed of EUB338, EUB338 II, and EUB338 III) for most bacteria, Amx368 for all anammox bacteria, and Sca1309, Sca1129a, and Sca1129b for marine anammox bacteria as shown in Table 3. The probes were labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine

Table 3. Probes used in this study.

Probes	Sequence (5' to 3')	Specificity	Reference
EUBmix			
EUB338	GCT GCC TCC CGT AGG AGT	Most bacteria	Amann et al. (1990)
EUB338II	GCA GCC ACC CGT AGG TGT	*Planctomycetales*	Daims et al. (1999)
EUB338III	GCT GCC ACC CGT AGG TGT	*Verrucomicrobiales*	Daims et al. (1999)
Amx368	CCT TTC GGG CAT TGC GAA	All anammox	Schmid et al. (2003)
Sca1309	TGG AGG CGA ATT TCA GCC TCC	genus *"Ca.* Scalindua"	Schmid et al. (2003)
Sca1129a	TAC CCG GCA CAA CCC GCT	husup-a2	Kindaichi et al. (2011a)
Sca1129b	TAC TCG GCA TTA CCC GAT	husup-a7	Kindaichi et al. (2011a)

5-isothiocyanate (TRITC) at the 5' end. The average fraction was determined from at least 10 representative laser scanning microscopy projection images obtained from each biofilm sample using the standard software package with the LSM5 PASCAL provided by Carl Zeiss (Kindaichi et al., 2004).

RESULTS AND DISCUSSION

Effects of temperature and salinity

We operated the up-flow fixed-bed glass column reactor under different conditions to clarify the influence of temperature and salinity on the marine anammox community. Figure 1 shows the results of time courses of the nitrogen loading rate and removal rate. The nitrogen loading rate remained at 0.4-0.5 kg TN m^{-3} day^{-1} in which percentages of NH_4^+-N and NO_2^--N were 50 and 50%, respectively, throughout the operation of the reactor (Days 0-260), and the HRT was fixed at 1.9 h. When another reactor was operated at 20°C and 3.5% salinity with the same biomass (1 g wet weight), the initial nitrogen removal activity per unit of reactor volume was 0.06 kg TN m^{-3} day^{-1} (data not shown). During Phase 1 (Days 0-92), nitrogen removal gradually increased until Day 49, at which point the NO_2^- removal efficiency was 91%. After that, the reactor performance remained stable. In Phase 2 (Days 92-156) the nitrogen removal rate decreased remarkably. Additionally, the anammox activity in Phase 2 was four times lower than that of Phase 1, and this activity did not recover throughout the phase. Taken together, these results suggest that low temperatures influence the activity of marine anammox bacteria. Dosta et al. (2008) also reported that the activity of other freshwater anammox bacteria was lost at 15°C, while the activity was observed at 18°C. In Phase 3 (Days 156-231), the nitrogen removal rate was recovered to the levels observed in Phase 1, and this removal stabilized after Day 203. The maximum nitrogen removal rate (0.32 kg TN m^{-3} day^{-1}) when the ammonium and nitrite removal efficiencies were 61.4 and 89.7%, respectively. The nitrogen stoichiometric ratio on Day 203 was 1: 1.2: 0.13 for consumption of ammonium and nitrite and for the production of nitrate. These results clearly demonstrate that temperature has a strong effect on

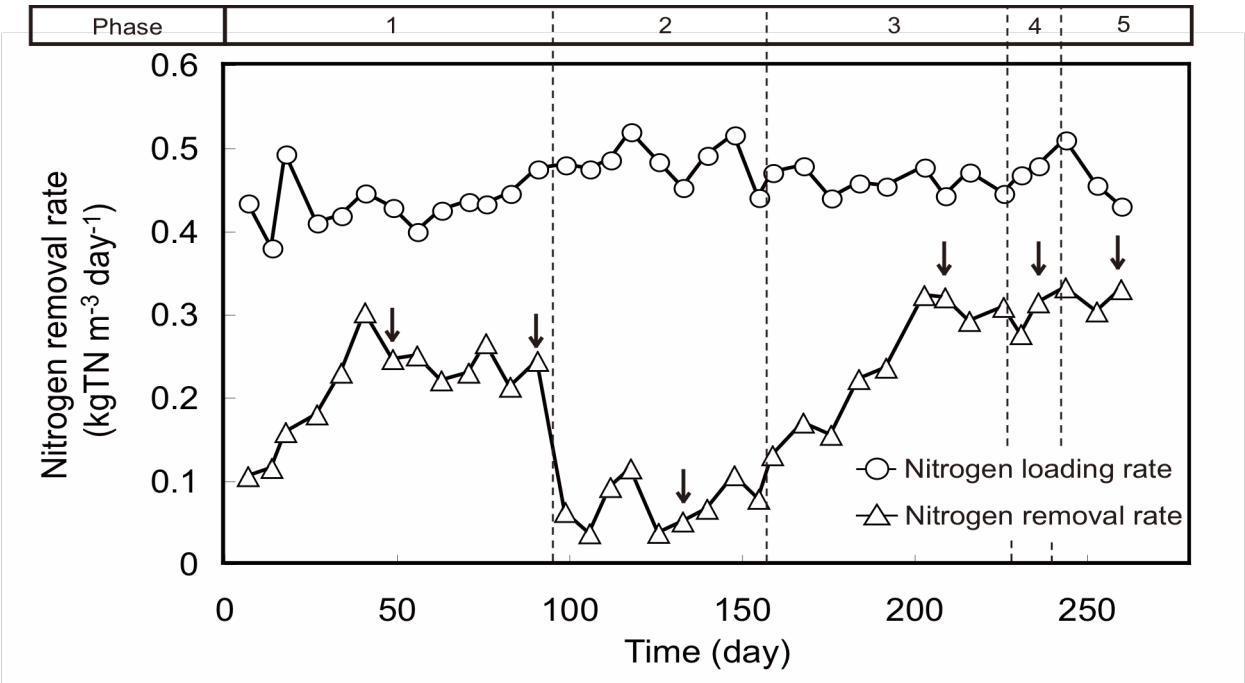

Figure 1. Time course of the nitrogen loading rate and nitrogen removal rate of the anammox reactor. Arrows show the sampling for FISH analysis.

marine anammox activities, and these tendencies are comparable to those observed in a study conducted by van de Vossenberg et al. (2008). According to the Arrhenius-type relationship, it would be expected that the nitrogen removal activity in Phase 1 (operated at 30°C) is higher than in Phase 3 (operated at 20°C). However, our data showed that the nitrogen removal activity per unit of reactor volume at 20°C is similar to that at 30°C. It is possible that the higher activity in Phase 3 is due to the growth of anammox bacteria in the reactor compared with the Phase 1 (at 30°C).

In Phases 1-3, the salinity in the medium was 3.5%; however, this level was reduced in the remaining phases. The salinity in the medium was reduced by 1.75% in Phase 4 (Days 231-236). The nitrogen removal activity per unit of reactor volume did not changed as well as the case of temperature changed. Therefore, salinity was further decreased to 0.875%, even though the phase time was not longer period. In Phase 5 (Days 236-260), the nitrogen removal rate remained stable, even though the salinity was further reduced to 0.875%. These results suggest that salinity has little effect on the activities of marine anammox bacteria.

FISH analysis

FISH analysis was conducted to determine if the two marine anammox species were present in the inoculum. As shown in Figure 2, FISH using probes Sca1129a and Sca1129b clearly revealed that two different species of "*Candidatus* Scalindua" were present (Fig. 2A, 2B). The bacterial cells that hybridized with Sca1129a (targeting husup-a2-like organisms) and Sca1129b (targeting husup-a7-like organisms) probes were doughnut-shaped with a diameter of approximately 1-μm as reported by Kindaichi et al. (2011a). It is noted that the husup-a7-like organisms and the husup-a2-like organisms closely related to "*Candidatus* Scalindua wagneri" and "*Candidatus* Scalindua marina", respectively (Kindaichi et al., 2011a).

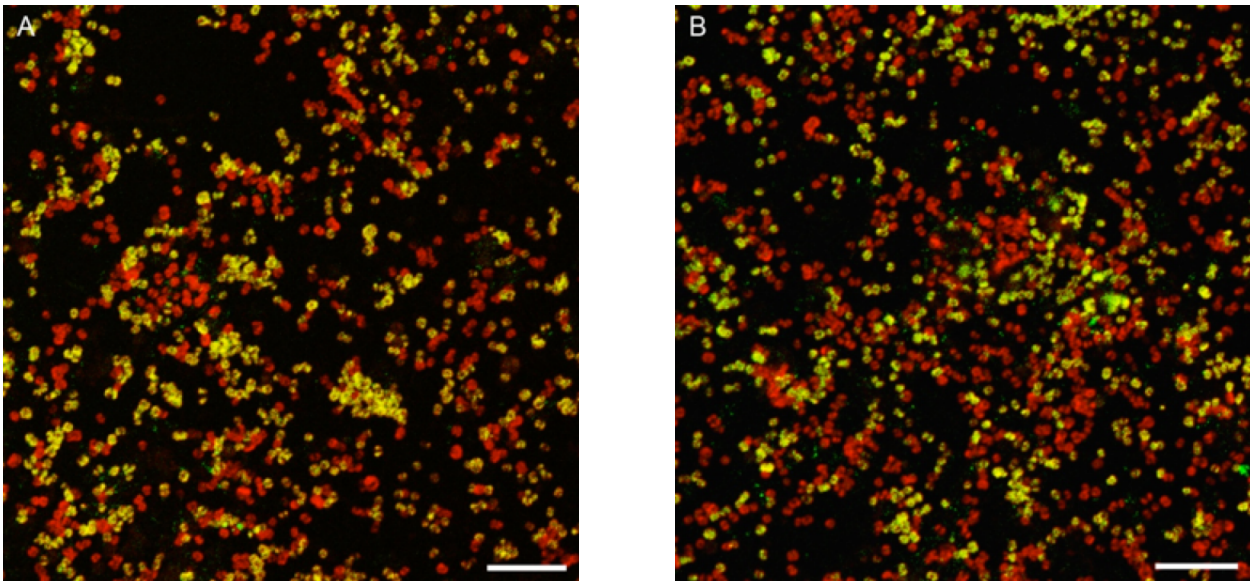

Figure 2. Confocal laser scanning microscopy images of marine anammox biofilm after homogenization. The biomass sample was obtained from the reactor on Day 234. (a) Green signals show FISH conducted using the FITC-labeled probe Sca1129a. Red signals show FISH conducted using the TRITC-labeled probe Amx368. (b) Green signals show FISH conducted using the FITC-labeled probe Sca1129b. Red signals show FISH conducted using the TRITC-labeled probe Amx368. The bars indicate 10 μm.

Population dynamics of marine anammox bacteria

Figure 3 shows the population dynamics of the two types of anammox bacteria. The changes in the population of two marine anammox species (husup-a2-like organisms and husup-a7-like organisms) were analyzed using quantitative FISH analysis, because these marine anammox species coexisted in the inoculum. The fractions of Amx368/EUBmix were similar to the sum of the fraction of the husup-a2-like organisms in each phase. This result indicates that the most anammox bacteria were constituted by the husup-a2-like organisms and husup-7-like organisms through the operation. The fraction of the husup-a7-like organisms was more than two times higher than that of the husup-a2-like organisms in the inoculum. In Phase 1, only the husup-a7-like organisms were found to be present. This result showed that only the husup-a7-like organisms contributed to nitrogen removal at 30°C. Conversely, the fraction of marine anammox bacteria (Sca1129b/EUBmix in Fig. 3) increased by up to 60% in Phase 2. Although the husup-a7-like organisms existed at 10°C, the nitrogen removal rate decreased, which showed that the activity of the husu-a7-like organisms might be very low. In Phase 3, both the husup-a7-like organisms and the husup-a2-like organisms were detected at 20°C. One possible explanation is that the operational condition at 20°C is suitable for the growth of husup-a2-like organisms. The fraction of anammox bacteria (Amx368/EUBmix) in Phase 3 was lower than that of the inoculum, which is similar to the results of a study conducted by Kindaichi et al. (2011a) who reported that two species of marine anammox bacteria were enriched from sediment at this temperature. This result indicated that coexisting bacteria have the higher growth rate, and thus they out-competed anammox bacteria for an abrupt increase of temperature. These results suggest that, among marine anammox species, the husup-a7-like organisms have a wide optimum range of temperature and husup-a2-like organisms have very narrow temperature growth range.

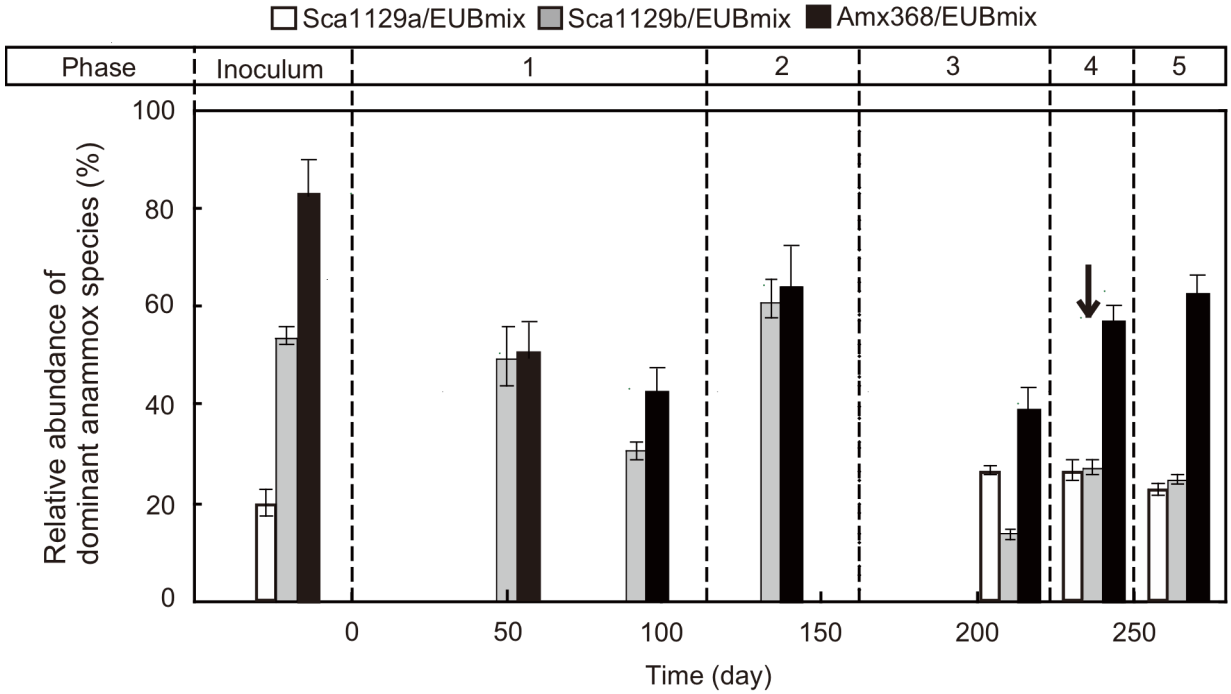

Figure 3. Relative abundance of the dominant species husup-a2-like organisms (white bars) and husup-a7-like organisms (grey bars), and all anammox bacteria detected with probe Amx368 (black bars). Relative abundance is shown as the percentage of each specific probe signal in a microscopic field to the EUBmix probe signal. The error bars indicate standard deviations. The arrow (Day 234) shows the sampling time for FISH images as shown in Fig. 2.

During Phase 4, husup-a2-like and husup-a7-like organisms were also detected, even though the salinity changed rapidly. The composition of the husup-a7-like organisms increased, although the composition of the husup-a2-like organisms did not. During Phase 5, two species of marine anammox bacteria were found to be present even though the salinity had been further reduced to 0.875%. Furthermore, the fraction of the husup-a7-like organisms and husup-a2-like organisms did not change with decreasing amounts of salinity, while the fraction of total anammox bacteria (Amx368/EUBmix) slightly increased. It is possible that the population of the coexisting bacteria decreased in Phase 4 and 5. These results support that the nitrogen removal activity per unit of reactor volume did not changed in Phase 4 and 5 even though the salinity changed (Fig. 1). Although Li et al. (2011) reported that there was negative correlation between the abundance of anammox bacteria and salinity, our data did not show such a negative correlation. Additionally, the change in salinity did not influence the fraction of the husup-a2-like organisms or husup-a7-like organisms. However, the doubling time of members of the genus "*Candidatus* Scalindua" (especially husup-a2-like organisms) might be very high; therefore, additional studies with longer phases should be conducted.

CONCLUSIONS

We investigated the influence of temperature and salinity on the activity and community structure of marine anammox bacteria with continuous operation of the reactor under different conditions. We used biomass that included two groups of organisms within the genus "*Candidatus* Scalindua" and related to clones husup-a2 and husup-a7. Anammox bacteria activity was observed at 20 or 30°C and at 1.75 or 0.875% salinity. The husup-a7-like organisms were detected using FISH analysis of samples collected throughout the operation. Conversely, the husup-a2-like organisms were only detected at 20°C. These results suggest that temperature influences the activity (nitrogen removal rate) of anammox bacteria, while salinity does not affect the microbial community structure in the marine anammox biofilm.

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