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Candidatus Scalindua, a Biological Solution to Treat Saline Recirculating Aquaculture System Wastewater

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Highlights:

- The anammox process is a promising technique to treat nitrogen-rich marine RAS WW.
- The anammox strain *Ca.* Scalindua was successfully acclimated to high salinity marine RAS wastewater.
- Despite a slight decrease in population over the time, *Ca.* Scalindua remained the major species within the granules and was able to maintain a high nitrogen removal rate while exposed to RAS WW in the absence of TE supplementation.

Abstract: Recirculating aquaculture systems (RAS) are promising candidates for the sustainable development of the aquaculture industry. A current limitation of RAS is the production and potential accumulation of nitrogenous wastes, ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-), which could affect fish health and welfare. In a previous experiment, we have demonstrated that the marine anammox bacteria *Candidatus* Scalindua was a promising candidate to treat the wastewater (WW) of marine, cold-water RAS. However, the activity of the bacteria was negatively impacted after a direct exposure to RAS WW. In the current study, we have further investigated the potential of *Ca.* Scalindua to treat marine RAS WW in a three-phase experiment. In the first phase (control, 83 days), *Ca.* Scalindua was fed a synthetic feed, enriched in NH_4^+, NO_2^- and trace element (TE) mix. Removal rates of 98.9% and 99.6% for NH_4^+ and NO_2^-, respectively, were achieved. In the second phase (116 days), we gradually increased the exposure of *Ca.* Scalindua to nitrogen-enriched RAS WW over a period of about 80 days. In the last phase (79 days), we investigated the needs of TE supplementation for the *Ca.* Scalindua after they were fully acclimated to 100% RAS WW. Our results show that the gradual exposure of *Ca.* Scalindua resulted in a successful acclimation to 100% RAS WW, with maintained high removal rates of both NH_4^+ and NO_2^- throughout the experiment. Despite a slight decrease in relative abundance (from 21.4% to 16.7%), *Ca.* Scalindua remained the dominant species in the granules throughout the whole experiment. We conclude that *Ca.* Scalindua can be successfully used to treat marine RAS WW, without the addition of TE, once given enough time to acclimate to its new substrate. Future studies need to determine the specific needs for optimal RAS WW treatment by *Ca.* Scalindua at pilot scale.

Keywords: "*Candidatus* Scalindua"; anaerobic ammonium oxidation (anammox); recirculating aquaculture system (RAS); wastewater treatment; trace elements

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1. Introduction

The world population has reached 8 billion at the end of 2022, and is predicted to reach 9.7 billion by 2050 [1]. There is an urgent need to increase healthy, nutritious and sustainable food production to feed this growing world population [2]. Fish products are rich in high quality protein and are generally considered to be healthy (i.e., rich in long-chain omega-3 fatty acids, vitamins and trace elements) [3]. With the stagnation of capture fisheries, there is a global consensus about the importance of the aquaculture sector as an essential source of food [2,3]. The intensification of this sector is, however, accompanied by the raising of environmental concerns, especially regarding the leakage of nutrients to the environment [4]. It is, therefore, important to develop novel techniques for treatment of wastewater (WW) from fish farms, including chemical, mechanical and biological filtrations, in order to further develop this sector in a sustainable way [5–8].

Land-based closed and semi-closed fish farming systems, such as recirculating aquaculture systems (RAS) have been proposed as potential candidates to improve the sustainability of the aquaculture sector. These systems allow for a high degree of water reuse and provide a more stable farming environment and reduced environmental impact compared to traditional open-cage systems [9–12].

In RAS, nitrifying bacteria convert the ammonium (NH_4^+) produced by the fish into nitrate (NO_3^-) via nitrite (NO_2^-) in the presence of oxygen (O_2) (Figure 1A). As a result, NO_3^- can slowly accumulate over time and reach concentrations which could affect fish health and welfare [10,13,14]. High NO_3^- can be managed through biological conversion of NO_3^- to nitrogen gas (N_2) in anaerobic denitrifying biofilters, or by regular water exchanges [15,16]. However, denitrification compartments are not always present in RAS, as this process can lead to the formation and accumulation of intermediate, extremely toxic substances (i.e., NO_2^-, NO and N_2O) [17,18]. As a result, part of the water has to be exchanged regularly in systems without a denitrification compartment, which constitute the vast majority of RAS today.

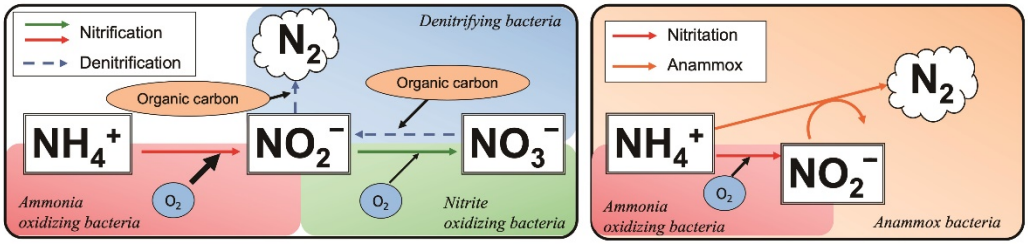

Figure 1. The conventional nitrogen removal process in RAS, with nitrification and denitrification compartments (**A**); the nitritation (partial nitrification)/anammox process (**B**).

The anammox (anaerobic ammonia oxidation) process is a cost-effective and environmentally-friendly way to remove nitrogen compounds from WW [19]. It is a chemoautotrophic biological process, where 1 mol of NH_4^+ is transformed into 1.02 mol of N_2 gas using NO_2^- (1.32 mol) as the electron acceptor and, therefore, needing a partial nitrification (nitritation, Figure 1B) [20–22]. The additional advantages of this process are its low requirements for both O_2 and organic carbon sources, as well as a low production of global warming gases [23–26]. Therefore, the anammox process has attracted more and more attention throughout the world. The anammox-related nitrogen removal process presented here provides a new perspective for future RAS WW treatment.

In 2013, Borin and colleagues [27] detected the presence of the marine strain of anammox, "*Candidatus* Scalindua" (hereafter *Ca.* Scalindua) in deep marine hypersaline gradient systems in the Eastern Mediterranean Sea. The anammox granules were found at 204‰ salinity (204 g L^{-1}). Furthermore, a high nitrogen removal rate (10.7 kg N m^{-3} day^{-1}) was obtained in an anaerobic sludge blanket reactor [28]. Moreover, these marine anammox strains could maintain stable nitrogen removal at a salinity of 50 ‰ [29]. These results sug-

gest that the anammox process has good potential to treat saline (>30‰) WW from marine aquaculture systems [30–32]. Despite these promising results, few studies have focused on the use of anammox bacteria for treatment of marine WW in aquaculture to date.

In our previous study, we have demonstrated that *Ca.* Scalindua remained the dominant species found in the granules of our anaerobic anammox rector throughout the different experimental phases, including after exposure to RAS WW [33]. In this experiment, the anammox was either fed a standard synthetic anammox feed enriched with NH_4^+ and NO_2^- and supplemented with a mix of trace elements (TE) or they were abruptly exposed to 100% RAS WW enriched with NH_4^+ and NO_2^- for short periods, in the absence or presence of TE. This TE mix, composed of 9 TE (Table 1)) was developed when the first freshwater anammox species was isolated and is currently used as a standard in anammox cultures worldwide [34]. The nitrogen removal capacity of the anammox was negatively affected by the abrupt changes to 100% RAS WW without TE, while a subsequent addition of TE led to a slight recovery [33]. We concluded that the reduction in activity could be caused by the abrupt change of medium (from synthetic feed to RAS WW), either because of high levels of unknown compounds (i.e., NO_3^-) present in the RAS WW, and/or the lack/or inappropriate concentration of certain key TE in this new medium.

Table 1. Composition of the TE mix (as reported by van de Graaf and colleagues [34]).

TE	B	Co	Cu	Fe	Mn	Mo	Ni	Se	Zn
Concentration (μg L^{-1})	2.4	28.3	63.6	1840	274.8	96.4	46.9	47.9	97.8

In the current study, we aim to investigate in greater detail, the growth mechanisms and medium requirements of the marine anammox bacteria *Ca.* Scalindua for use in marine RAS. The specific objectives were to investigate: (1) if a slow adaptation to RAS WW could lead to a better functioning of the *Ca.* Scalindua, and (2) if the TE supplementation is necessary for the normal functioning of this marine strain of anammox bacteria. As all the 9 TE elements used in the standard feed [34] are already present in seawater [33], there might not be a need for a supplementation.

2. Materials and Methods

2.1. RAS WW Collection and Characteristics

RAS WW (Table 2) was collected from the aquarium facilities of the University of Gothenburg (Gothenburg, Sweden), hosting a pilot-scale research and development facility for development of land-based marine RAS at low temperatures (ca. 10 °C). The fish species in the marine RAS were rainbow trout (*Oncorhynchus mykiss*) and Atlantic wolffish (*Anarhichas lupus*). The water samples were collected 3–8 h after the feeding of the animals to capture a peak in NH_4^+ [35–37] and immediately used to prepare the feed for the anammox reactors.

Table 2. Physicochemical characteristics of the RAS WW. Values show the average and standard deviation during the period.

Parameter	Salinity (‰)	NH_4^+ (mg-N L^{-1})	NO_2^- (mg-N L^{-1})	NO_3^- (mg-N L^{-1})	pH	TSS [1] (mg L^{-1})	COD [2] (mg-C L^{-1})	Total P [3] (mg-P L^{-1})
Value	29 ± 1	6.22 ± 2.34	2.24 ± 0.69	1.81 ± 0.88	7.3 ± 0.3	58.8 ± 5.6	39.5 ± 5.3	2.6 ± 0.7

[1] TSS, Total dissolved solids; [2] COD, Chemical oxygen demand; [3] Total P, Total phosphorous.

2.2. Reactors Operation

Ca. Scalindua granules were harvested from an up-flow column anammox stock culture that has been operating with a continuous supply of inorganic nutrient media containing NH_4^+ (28 mg-N L^{-1}) and NO_2^- (34 mg-N L^{-1}) [34] at the University of Hiroshima (Higashihiroshima, Japan) for more than 10 years [38–40]. A biomass (ca. 5 g wet weight) was brought to the University in Gothenburg in August 2019 and was used as inoculum into

a glass column reactor (Ø 50 mm; volume, 325 cm^3, KF-50, AS ONE, Tokyo, Japan, Figure 2) with a nonwoven fabric sheet (Japan Vilene, Tokyo, Japan) as biofilm carrier material. The reactor was fed with synthetic marine WW (Aquaforest, Brzesko, Poland) supplemented with nitrogen (NH_4^+ and NO_2^-) and TE, as described earlier [33,34] (Table 1). The reactor temperature was maintained at 28 °C throughout the experimental period, and the initial hydraulic retention time (HRT) was 4.6 h (Table 3). The reactor reached a steady state (removal rates of NH_4^+ and NO_2^- over 95% and it was kept under the same conditions until the start of the experiment (ca. 700 days after the initial inoculation). The reactor used in this study was operated for 280 days in 3 experimental phases.

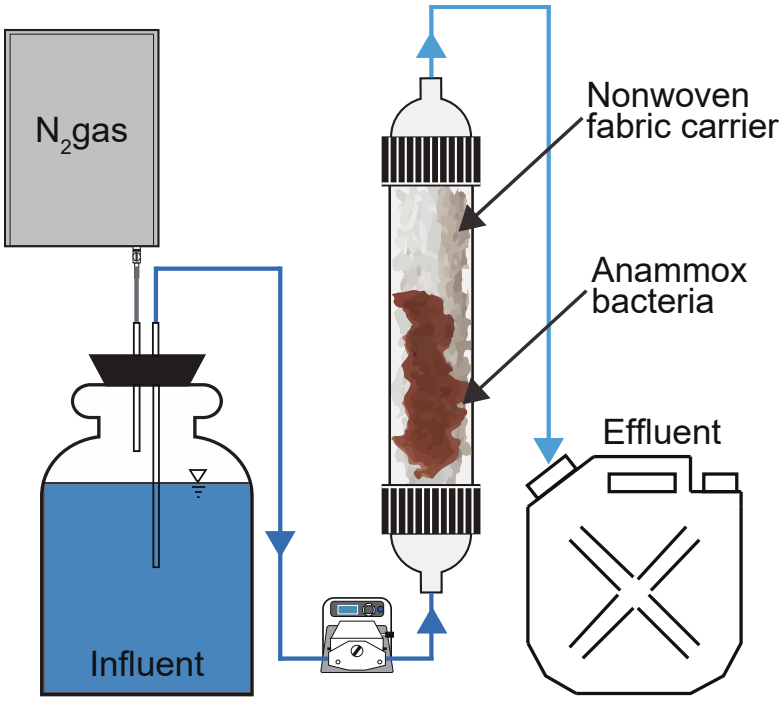

Figure 2. Schematic drawing of the up-flow column reactor.

Table 3. Operational conditions of the column rector.

Phase	Period (d)	AS/RAS [1]	TE [2]	HRT (h)	Nitrogen Loading Rate (g-TN L^{-1} day^{-1}) [3]
1	0–83	AS	+	4.6	0.38 ± 0.03
2	84–200	RAS	+	4.4	0.32 ± 0.06
3	201–280	RAS	−	4.8	0.35 ± 0.04

[1] AS, Artificial seawater; RAS, recirculating aquaculture system WW; [2] TE: Trace element mix (+, presence; −, absence), reported by van de Graaf and colleagues [34]; [3] Values show the average and standard deviation during the period.

During Phase 1 (days 0–83), the reactor was fed with the same synthetic marine WW supplemented with nitrogen (NH_4^+ and NO_2^-) and TE as was arranged earlier [33,34]. During Phase 2 (days 84–200), the reactor was exposed to gradually increasing concentrations of RAS WW. The substitution of the synthetic feed to 100% RAS WW was gradual; 10–30% of RAS WW was substituted every 10–20 days. The RAS WW characteristics were adjusted to approximately match the standard medium for *Ca*. Scalindua regarding salinity (29‰), NH_4^+ (28 mg-N L^{-1}), NO_2^- (30 mg-N L^{-1}) and inorganic carbon ($KHCO_3$, 1000 mg L^{-1}) [38,39]. During Phases 1 and 2, the feed was supplemented with a mix of TE. In the final phase, Phase 3 (days 201–280), the reactor was fed with 100% RAS WW, still supplemented with nitrogen, but in the absence of TE. During Phases 2 and 3, the reactor was slowly adapted to RAS WW with fluctuating concentrations of NH_4^+, NO_2^- and NO_3^- (6.22 ± 2.34 mg-N L^{-1}, 2.24 ± 0.69 mg-N L^{-1} and 1.81 ± 0.88 mg-N L^{-1} on average,

respectively, Table 2). The NH_4^+ and NO_2^- supplementation was adapted accordingly to ensure concentrations of these compounds, similar to Phase 1 (Figure 3). However, there was some slight fluctuation in terms of concentration regarding these nitrogen compounds, mimicking what can occur in a full-scale RAS. Although the inlet concentrations varied, the system maintained a high removal efficiency of the compounds (Figure 4).

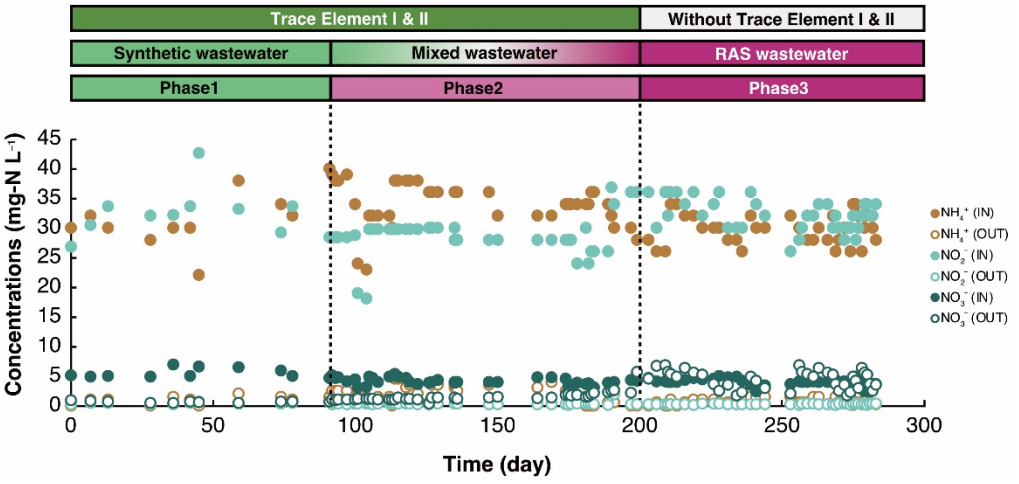

Figure 3. Concentration of NH_4^+, NO_2^- and NO_3^- in the influent (filled circles) and effluent (open circles) throughout the experiment.

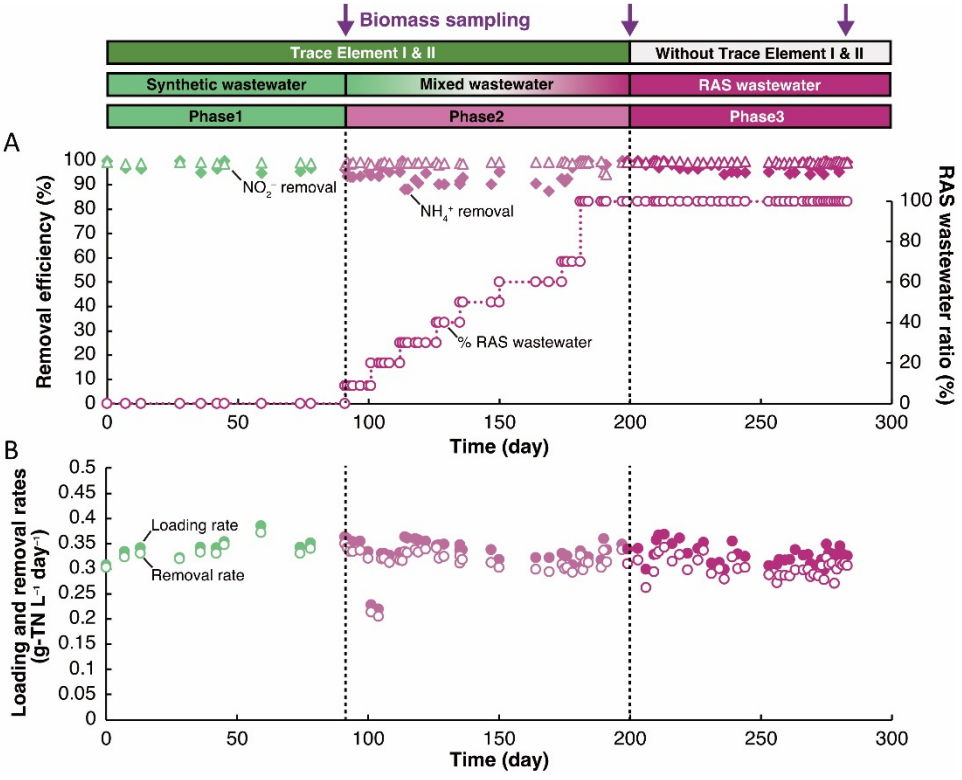

Figure 4. Anammox performance in the reactor. (**A**) NH_4^+ (closed diamonds) and NO_2^- (open triangles) removal efficiencies. (**B**) Nitrogen loading and removal rates (filled and open circles, respectively). Dotted lines and open circle indicate changes in operational phase (artificial seawater or RAS WW, the presence of TE). RAS, recirculating aquaculture system WW; TE, trace element solutions. Purple arrows indicate biomass sampling on days 83, 200, and 280.

Each WW feed was flushed with N_2 gas for at least 30 min before adding the different chemicals to achieve a concentration of dissolved O_2 below 0.5 mg L^{-1}, and the pH was adjusted to ca. 7.0 with a solution of 1 mol L^{-1} H_2SO_4 [34]. The influents were continuously introduced into the reactor using a peristaltic pump (Masterflex L/S Economy Drive, Cole-Parmer Instruments, Vernon Hills, IL, USA).

2.3. Analytical Methods

The total nitrogen (TN) loading and removal rates were calculated based on the concentrations of the nitrogenous compounds (NX; NH_4^+, NO_2^-, and NO_3^-) as well as the HRT (Figures 3 and 4, Table 3), according to the following:

$$\text{Removal efficiency (\%)} = [[\text{Influent NX-N (mg-N L}^{-1})] - [\text{Effluent NX-N (mg-N L}^{-1})]]/[\text{Effluent NX-N (mg-NL}^{-1})] \times 100$$

$$\text{Removal rate (g-TN L}^{-1}\text{ day}^{-1}) = [[\text{Influent NX-N (mg-N L}^{-1})] - [\text{Effluent NX-N (mg-N L}^{-1})]] \times [\text{Influent volumetric flow rate (L day}^{-1})]/[\text{Volume of the tank (L)}]$$

$$\text{HRT (h)} = \text{Volume of the reactor (L)}/\text{Influent volumetric flow rate (L h}^{-1})$$

Salinity, O_2 and temperature was determined using a conductivity meter portable Multimeter pHenomenal MU 6100 H (VWR international, Radnor, PA, USA). Analyses of total suspended solids (TSS) were carried out in accordance with the Standard Methods [41]. COD and Total P were measured with a DR-2800 UV-visible spectrophotometer (Hach-Lange, Dusseldorf, Germany) using the LCK1814 and the powder pillow PhosVer 3 (ascorbic acid method, 8048) methods, respectively (Hach-Lange, Dusseldorf, Germany).

The NH_4^+ and NO_2^- concentrations were determined using the powder pillow methods (salicylate method, 8155, and diazotization method, 8507, respectively, Hach-Lange, Dusseldorf, Germany) and the DR-2800. The concentrations of NO_3^- were determined using ion-exchange chromatography (HPLC 20A; Shimadzu, Kyoto, Japan) with a Shodex Asahipak NH2P-50 4D anion column (Showa Denko, Tokyo, Japan) and UV–Vis detector (SPD-20AV, Shimadzu) after filtration of samples through 0.2-µm pore-size PTFE membranes (Advantec, Tokyo, Japan) [42].

2.4. Microbial Community Analysis

To determine the potential changes in the microbial community composition during the different phases, biomass samples for the amplicon sequencing were collected from the reactor at the end of each experimental phase (days 83, 200 and 280). DNA was extracted using a FastDNA SPIN kit for soil (MP Biomedicals, Santa Ana, SC, USA). PCR amplification of the bacterial 16S rRNA gene was performed with a primer set for amplification of the V3-V4 region as follows: 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GGACTACHVGGGTATCTAATCC-3′). The details of PCR amplification were as described previously [43]. PCR products were purified using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA) according to manufacturer instructions. Purified DNA was sequenced using a MiSeq platform with a MiSeq reagent kit (v.3; Illumina, San Diego, CA, USA).

Obtained sequences were trimmed and assembled as described previously [44]. Sequence data were analyzed using QIIME 2 Core 2020.2 distribution [45]. Operational taxonomic units (OTUs) were assigned with the SILVA 132 database [46]. OTUs that accounted for >0.5% of the total reads were used for bar-plots representation. The sequence data in the present study was deposited in the DNA Data Bank of Japan (DDBJ) database under the DDBJ/EMBL/GenBank accession number DRA015143.

2.5. Fluorescence In Situ Hybridization (FISH)

Biomass samples were collected from the up-flow column reactor at the end of each experimental phase (days 83, 200, and 280). Sample fixation and the following FISH procedure were described previously [47]. The probes used in this study were as follows: a mixture of EUB338, II, III, and IV probes labelled with Alexa Fluor 647 specific for all bacteria [48,49] and Sca1129b probes labelled with Alexa Fluor 555 specific for *Ca*. Scalindua [39]. Hybridized samples were observed with an Axiomager M1 epifluorescence microscope with a 100 W HBO lamp. Images were obtained using an AxioCam MRm version 3 FireWiremonocrome camera and AxioVision software, version 4.5 (Carl Zeiss, Oberkochen, Germany).

3. Results

3.1. Reactor Performance

During Phase 1 (synthetic WW and TE), the TN removal rate showed high efficiency while maintaining constant TN loading rates (Figure 4). Once the reactor continued to maintain a stable state (from day 0 to 83), the average TN loading and removal rates were 0.32 ± 0.05 and 0.30 ± 0.03 g-TN L^{-1} day^{-1}, respectively (HRT 4.6 h). At the end of Phase 1 (day 83), the removal efficiency had reached 94% for NH_4^+ and 99% for NO_2^-, showing a successful establishment of the anammox process in the reactor [38].

During Phase 2 (days 84–200), the use of RAS WW instead of artificial seawater was implemented with a gradual substitution within about a 3-month period (10–30% every 10–20 days, Figure 4). The average TN loading rate during this phase was kept constant at 0.32 ± 0.06 g-TN L^{-1} day^{-1}. During this phase, the removal efficiencies of NH_4^+ and NO_2^- were 94,3% and 98,8%, respectively (HRT 4.4 h) and the TN removal rate was 0.31 g-TN L^{-1} day^{-1}. These results clearly indicated that the method of acclimation lead to the removal of nitrogenous compounds by the anammox microorganisms at a high level of efficiency.

During Phase 3, RAS WW was used without the addition of TE and the HRT was slightly increased to 4.8 h (i.e., a slight decrease of TN loading rate). The NH_4^+ and NO_2^- removal efficiencies showed high efficiency in removal even during this phase.

3.2. Microbial Community Analysis and FISH

A total of 25 603, 30 130 and 28 109 non-chimeric reads and 139, 172 and 206 operational taxonomic units (OTUs) were obtained from days 83, 200 and 280, respectively. In this study, OTUs that accounted for >0.5% of the total reads were used for the analysis, and OTUs accounting for <0.5% of the reads were grouped as "Others" (Figure 5). *Ca.* Scalindua was identified as the most abundant species in the reactor throughout the experiment. Interestingly, the relative abundances of *Ca.* Scalindua did not drastically change over time. Despite a slight decrease (from 21.4% at the end of Phase 1 and 16.7% at the end of the experiment), it remained the most dominant species in the anammox granule, even during the phases with 100% RAS WW. The stability of the population of *Ca*. Scalindua, throughout all the different experimental phases, was also supported by FISH observations (Figure 6).

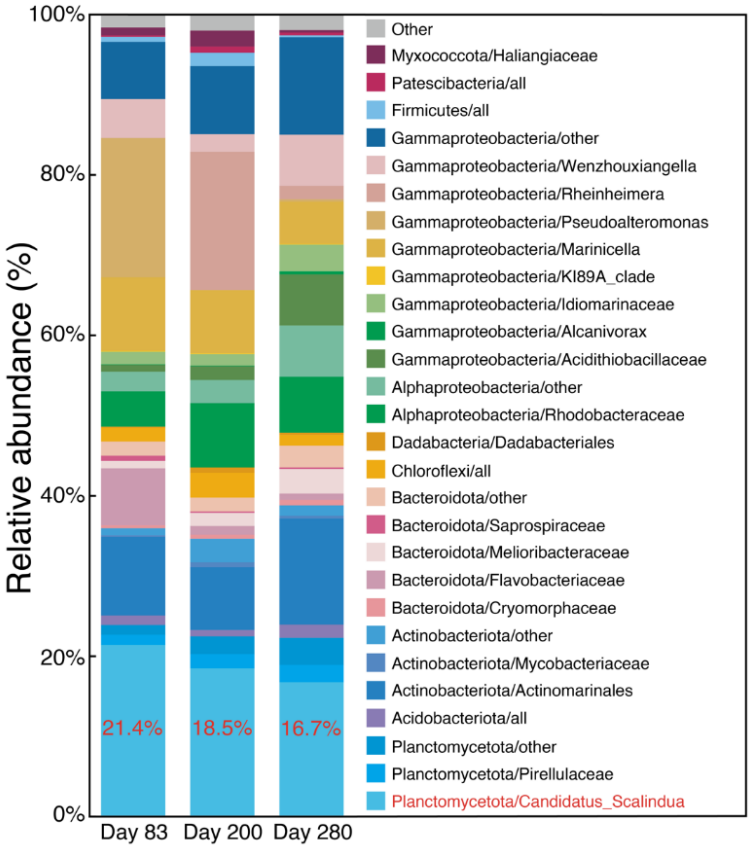

Figure 5. Microbial community composition at the end of each experimental phase (days 83, 200 and 280), based on 16S rRNA gene amplicon sequencing. Red percentages correspond to the relative abundance of the marine anammox *Ca.* Scalindua.

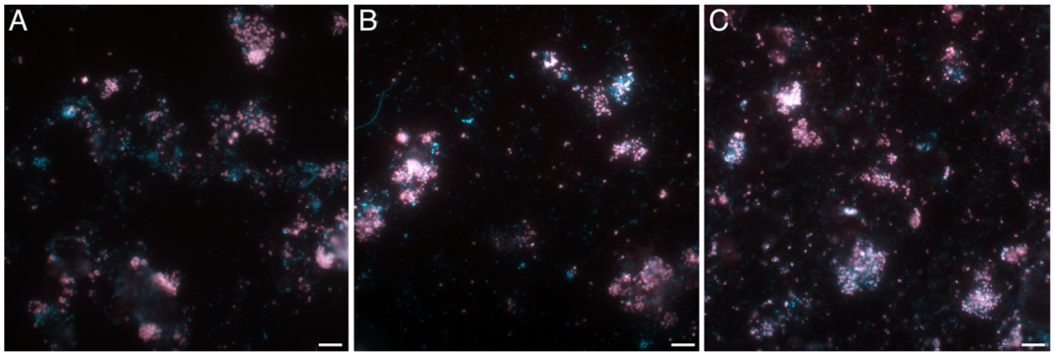

Figure 6. FISH micrographs of biomass collected from the column reactor at days 83 (**A**), 200 (**B**) and 280 (**C**). FISH was performed with Alexa Fluor 647-labelled EUB338mix probe (cyan) for all bacteria, and an Alexa Fluor 555-labelled Sca1129b probes (red) for *Ca.* Scalindua. *Ca.* Scalindua appears magenta and other bacteria appear blue. Scale bars represent 10 µm.

4. Discussion

4.1. Reactor Performance

The marine anammox species *Ca.* Scalindua has been isolated from coastal sediments from Hiroshima bay in 2007 [38]. Since then, inocula from the original samples have been successfully cultivated in Japan and Sweden, using the standard protocol developed by van de Graaf and colleagues [34]. During the first 83 days of our experiment, we continued to feed the anammox reactor with standard feed in order to maintain a steady state of high removal efficiencies with the utilization of synthetic WW as feed for the microorganisms. The efficiencies in terms of removal rate were extremely high and stable, with 98.3%

and 99.5% on average for NH_4^+ and NO_2^-, respectively. These removal rates remained stable when the synthetic feed was gradually replaced by RAS WW during the second phase (days 84–200), with average removal rates of 95.9% and 99.2% for NH_4^+ and NO_2^-, respectively. These removal rates were comparable to the removal rates obtained with the original sediment from Hiroshima bay (95% and 99%, respectively) [38]. These results confirm both the successful establishment and upkeep of the anammox process and the successful acclimation to RAS WW. The gradual increase of RAS WW, slowly replacing the standard feed, in the anammox feed method helped the microorganism to acclimate to this new substrate, as seen with the high and stable removal rates observed during this phase. Unlike what we observed in our previous experiment, the nitrogen removal efficiency was not compromised when allowed to slowly acclimate to RAS WW [33].

In this experiment, we further hypothesized that the reduction in removal rate observed could be due to the absence of certain key TE missing from the RAS WW [33]. Therefore, the last phase of our experiment was designed to verify this hypothesis, if once adapted to 100% marine RAS WW, supplemented with TE, the biomass would be able to maintain the removal capacity over time using pure marine RAS WW without TE supplementation. During this last phase, we continued to observe relatively high and stable removal rates for both NH_4^+ and NO_2^- (92.1% and 98.7%, respectively). Therefore, we can conclude that the required TE must have been present in appropriate quantities in the marine RAS WW to allow for the normal functioning of the anammox granules.

Today, the vast majority of anammox reactors are cultivated using a feed supplemented in 9 TE, which was originally designed for the first freshwater anammox bacteria isolated [34]. TE are essential for the normal functioning, growth and proliferation of the anammox bacteria [50–52]. It is essential to identify the specific TE requirements for each anammox species, as insufficient concentrations can limit normal cell functioning, division and proliferation within the granule, while too high concentrations can negatively impact the anammox process and potentially be lethal for the bacteria [51–54]. It is reasonable to hypothesize that different species of anammox bacteria may have different TE requirements. Marine strains of anammox bacteria, such as *Ca.* Scalindua, may have requirements that differ to those of the freshwater anammox most frequently studied [33,51,55]. To date, knowledge of the specific requirements regarding each individual TE is still scarce [51]. We have previously shown that all those 9 TE were present in the sea salt (Sealife, Marinetech, Tokyo, Japan) used to make the artificial marine water for the RAS from which RAS WW was used [33]. Finally, we have maintained the optimal culture condition for *Ca.* Scalindua throughout the different experimental phases. We have maintained strict anaerobic conditions through the degassing of the feed with N_2 gas prior connection to the reactor. *Ca.* Scalindua was further fed with RAS WW containing low NO_3^- levels and supplemented with NH_4^+ and NO_2^- [34]. As those optimal culture conditions might not be met in commercial-scale RAS, future studies need to investigate the performance of *Ca.* Scalindua under these real-life conditions.

4.2. Microbial Community

FISH and microbiological analysis have reflected a relatively stable population of *Ca.* Scalindua during all the phases of the experiment. Despite a slight decrease over time, *Ca.* Scalindua remained the main bacterial phylum present in the granule across the three experimental phases.

Overall, the relative abundance of bacterial phyla in the biomass samples also remained relatively stable throughout the different phases of the experiment. The small changes observed in the bacterial communities could be attributed to the high protein concentrations and/or the presence of other organic and inorganic residues (including NO_3^-) from waste, fish feces and uneaten feed in the RAS WW during Phases 2 and 3. Indeed, we observed an increase in non-chimeric reads and number of OTUs after the addition of RAS WW to the anammox feed. This additional load of bacteria from the RAS WW could have led to an artefact resulting in a slight reduction of *Ca.* Scalindua in

the granule. This is supported by the fact that most of the changes observed concerning bacterial groups belonged to the *Gammaproteobacteria* class. These bacteria, mostly aerobic, are found in freshwater and marine environments including RAS, where they are often the predominant species [56–59]. Slight changes in the bacterial communities over time in the RAS, from which we obtain the WW, could therefore be the reason for the transient, differential colonization of anammox granules over the course of the experiment. Further studies specifically designed to follow the evolution and quantification of the biomass could help detail this.

At the taxonomic level, the main differences observed concern some changes in relative abundance within the class of *Gammaproteobacteria*. Here, we witnessed the total disappearance of *Pseudoalteromonas* after Phase 1 that were replaced by *Rheinheimera* in Phase 2, which are then almost totally replaced by *Wenzhouxiangella* and *Acidithiobacillaceae* during Phase 3 (Figure 5).

Pseudoalteromonas is a genus of gram-negative rods belonging to the family *Pseudoalteromonadaceae* [60]. Members of this family have very versatile metabolic capacities and are thought to play important ecological roles in marine environments [60]. These organisms accounted for a third of the species found in the denitrifying compartment of the denitrification bioreactor of an experimental, warm-water marine RAS [61]. This disappearance could be caused by the change of environment and less favorable conditions for these organisms driven by the addition of RAS WW.

Rheinheimera is a genus of gram-negative bacteria from the family of *Chromatiaceae*. These chemoheterotrophic organisms are widely found in freshwater and seawater environments, including fishponds, but prefer aerobic or microaerobic environments to grow [62–66]. These organisms may have originated from the RAS WW, being aerobic and probably bringing microorganisms from the system, during the slow introduction period during Phase 2. However, after the next phase, these bacteria were exposed to more than 200 days of anaerobic conditions, which most probably have impacted their growth and their relative abundance within the granules [62].

The *Wenzhouxiangella* genus belongs to the gram-negative *Wenzhouxiangellacea* family, they are found in marine, hypersaline, and soda lake environments [67]. Although some species are obligately aerobic and chemoheterotrophic [68], some species have shown a successful adaptation and steady growth under anaerobic conditions in the presence of NO_3^-, which corresponds well to the environment in the anammox reactor [67].

5. Conclusions and Perspectives

This study demonstrates that *Ca.* Scalindua can be used as an alternative denitrifying component in RAS. We have shown that a slow and gradual exposure of *Ca.* Scalindua resulted in a successful acclimation to full marine RAS WW, with maintained high removal rates (over 92%) of both NH_4^+ and NO_2^- throughout the different experimental phases.

The activity of *Ca*. Scalindua was not negatively affected by the organic compounds present in the marine RAS WW nor by the absence/imbalance of certain TE, as hypothesized earlier. The current study, thus, suggests that anammox reactors containing marine strains such as *Ca.* Scalindua could be directly applied to treat RAS WW, without TE supplementation. This represents a clear advantage of using marine anammox strains in marine RAS, as seawater already contains all the essential TE for the normal growth and functioning of these bacteria.

In this study, we maintained optimal culture condition for *Ca.* scalindua throughout the experimental phases. We maintained strict anaerobic conditions and *Ca.* Scalindua was fed with RAS WW containing high concentrations of NH_4^+ and NO_2^- and low concentration NO_3^-. Future studies need to investigate the performance of *Ca.* Scalindua under typical up-scaled RAS conditions regarding nitrogen compounds and O_2, in order to validate the use of marine anammox bacteria as nitrogen treatment technology in commercial scale RAS.

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