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# Tolerance of the Marine Anammox *Candidatus* Scalindua to High Nitrate Concentrations: Implications for Recirculating Aquaculture Systems

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**Highlights:**

- The anammox process has significant potential to treat nitrogen-rich marine RAS WW.
- The marine anammox species *Ca.* Scalindua demonstrated effective treatment of synthetic WW with high $NO_3^-$ levels typically encountered in RAS, at a laboratory scale.
- Despite a relative decline in the population over time, *Ca.* Scalindua remained a key species within the anammox granules and sustained a high nitrogen removal rate over a period of 262 days of exposure to elevated $NO_3^-$ levels.

**Abstract:** Recirculating aquaculture systems (RAS) hold significant potential for sustainable aquaculture by providing a stable, controlled environment that supports optimal fish growth and welfare. In RAS, ammonium ($NH_4^+$) is biologically converted into nitrate ($NO_3^-$) via nitrite ($NO_2^-$) by nitrifying bacteria. As a result, $NO_3^-$ usually accumulates in RAS and must subsequently be removed through denitrification in full RAS, or by regular water exchanges in partial RAS. The marine anammox bacteria *Candidatus* Scalindua can directly convert toxic $NH_4^+$ and $NO_2^-$ into harmless nitrogen gas ($N_2$) and has previously been identified as a promising alternative to the complex denitrification process or unsustainable frequent water exchanges in marine RAS. In this study, we evaluated the impact of high $NO_3^-$ levels typically encountered in RAS on the performance and abundance of *Ca.* Scalindua in a laboratory-scale bioreactor. The bacterial composition of the granules, including the relative abundance of key nitrogen-cycling taxa, was analyzed along with the functional profile (i.e., $NH_4^+$ and $NO_2^-$ removal efficiencies). For this purpose, a bioreactor was inoculated and fed a synthetic feed, enriched in $NH_4^+$, $NO_2^-$, minerals and trace elements until stabilization (Phase 1, 52 days). $NO_3^-$ concentrations were then gradually increased to 400 mg·$L^{-1}$ $NO_3^-$-N (Phase 2, 52 days), after which the reactor was followed for another 262 days (Phase 3). The reactor maintained high removal efficiencies; 88.0 ± 8.6% for $NH_4^+$ and 97.4 ± 1.7% for $NO_2^-$ in Phase 2, and 95.0 ± 6.5% for $NH_4^+$ and 98.6 ± 2.7% for $NO_2^-$ in Phase 3. The relative abundance of *Ca.* Scalindua decreased from 22.7% to 10.2% by the end of Phase 3. This was likely due to slower growth of *Ca.* Scalindua compared to heterotrophic bacteria present in the granule, which could use $NO_3^-$ as a nitrogen source. Fluorescence in situ hybridization confirmed the presence of a stable population of *Ca.* Scalindua, which maintained high and stable $NH_4^+$ and $NO_2^-$ removal efficiencies. These findings support the potential of *Ca.* Scalindua as an alternative filtering technology in marine RAS. Future studies should investigate pilot-scale applications under real-world conditions.

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**Citation:** Roques, J.A.C.; Unegbu, E.; Fujii, N.; Marqué, A.; Micolucci, F.; Sundell, K.S.; Kindaichi, T. Tolerance of the Marine Anammox *Candidatus* Scalindua to High Nitrate Concentrations: Implications for Recirculating Aquaculture Systems. *Water* **2024**, *16*, 3705. https://doi.org/10.3390/w16243705

Academic Editor: Anastasios Zouboulis

Received: 30 October 2024
Revised: 19 December 2024
Accepted: 20 December 2024
Published: 22 December 2024

**Keywords:** *Candidatus* Scalindua; anaerobic ammonium oxidation (anammox); nitrate tolerance; recirculating aquaculture system (RAS); wastewater treatment

## 1. Introduction

The global population is predicted to reach 9.7 billion by 2050 [1]. Fish products, which are rich in high-quality protein and nutrients, are ideal food for the growing population [2]. For the past three decades, the aquaculture sector has been widely recognized as an essential food provider [2,3]. However, its intensification raises environmental concerns, including eutrophication from the discharge of nutrient-rich wastewater (WW) [4]. Developing innovative WW treatment techniques is therefore essential to the sustainable growth of this sector [5–8].

Recirculating aquaculture systems (RAS) are a sustainable alternative to traditional open-cage farming. These land-based closed systems allow for high water reuse and stable conditions for fish farming [9–12]. In RAS, the ammonium ($NH_4^+$) excreted by the fish is converted into nitrite ($NO_2^-$) and then nitrate ($NO_3^-$), in the presence of oxygen ($O_2$). As a result, $NO_3^-$ can slowly accumulate over time and can reach concentrations that could affect the health and welfare of the fish (100–1000 mg·$L^{-1}$ $NO_3^-$-N) [10,13–15].

Although generally considered the least toxic among the three nitrogenous wastes, high levels of $NO_3^-$ are toxic to fish and need to be dealt with [13,15,16]. Therefore, it is important for the farmers not to exceed certain levels of $NO_3^-$ to avoid health and welfare issues. These levels are highly species- and stage-specific. For instance, the health and performance of juvenile rainbow trout (*Oncorhynchus mykiss*) but of not post-smolt Atlantic salmon (*Salmo salar*) were affected by chronic levels of up to 100 mg·$L^{-1}$ $NO_3^-$-N [14,15]. The feed intake of African catfish (*Clarias gariepinus*) was impacted by chronic exposure to 379 ± 33 mg·$L^{-1}$ $NO_3^-$-N, while juvenile pikeperch (*Sander lucioperca*) were unaffected when exposed to similar levels [17,18]. Too-high $NO_3^-$ can be managed through denitrification, the biological conversion of $NO_3^-$ into nitrogen gas ($N_2$) by denitrifying bacteria in anaerobic conditions, or by regular water exchanges [19,20]. However, denitrification compartments are not always included in RAS, as this process is quite complex. Improper placement in the system may result in the formation and accumulation of intermediate highly toxic compounds, including $NO_2^-$, nitric oxide (NO) and nitrous oxide ($N_2O$) [21,22]. As a result, a portion of the water must be exchanged regularly in systems lacking denitrification filters, which constitutes the majority of RAS today. In the meantime, alternative WW cleaning pathways are being investigated. Current topics include electrochemical oxidation [23], biochar [24] and anammox [25,26].

The anammox (anaerobic ammonia oxidation) process is a cost-effective and environmentally friendly method of removing nitrogen wastes ($NH_4^+$ and $NO_2^-$) from WW [27]. It is carried out by bacteria belonging to the phylum *Planctomycetes* and the order *Candidatus* Brocadiales. Within this order, five genera from two families are currently known to perform the anammox process. Among these, *Candidatus* Scalindua (hereafter referred to as *Ca.* Scalindua) is currently the only known marine anammox bacterium [28]. The anammox process is a chemoautotrophic process, where 1 mol of $NH_4^+$, the electron donor, is transformed into 1.02 mol of $N_2$ gas using 1.32 mol of $NO_2^-$ as the electron acceptor [29–31]. Anammox requires minimal external carbon and emits fewer greenhouse gases; thus, it has become a globally recognized process over the past three decades [32–35].

Previous research highlights that the anammox process may already occur naturally in both freshwater and marine RAS, contributing to nitrogen removal in ways that have not yet been fully quantified or understood [36–38]. The presence of anammox bacteria in RAS biofilters could potentially explain gaps in nitrogen budgets that cannot be accounted for by nitrification and denitrification alone [39]. However, while anammox reactors have recently shown promising results in pilot-scale freshwater RAS, demonstrating effective nitrogen removal [40], their application in marine RAS remains largely unexplored. Developing

such targeted reactors would enable us to harness this process more effectively, providing a sustainable solution to nitrogen management in aquaculture WW.

We have previously demonstrated, at the laboratory scale, the potential of *Ca.* Scalindua to remove $NH_4^+$ and $NO_2^-$ in marine RAS WW, achieving removal efficiencies over 92% after acclimation [26]. However, we have also shown that the nitrogen removal performance of *Ca.* Scalindua could be altered by compounds present in the RAS WW, such as $NO_3^-$ [25]. Therefore, the specific requirements for optimal WW treatment in real RAS conditions using *Ca.* Scalindua must be identified. The nitrification process in RAS will result in a temporal accumulation of $NO_3^-$, and while the physiological effects of high $NO_3^-$ in fish are well documented, the possible impact on the anammox bacteria performance and the bacterial community profile is less clear, especially in marine environments. In freshwater granules dominated by *Broccadia* spp., 53 mg·$L^{-1}$ $NO_3^-$-N can (temporarily) mitigate the toxicity of high $NO_2^-$ (327 mg·$L^{-1}$) in the absence of $NH_4^+$ as an electron donor [41]. Furthermore, the presence of high $NO_3^-$ in the media could promote the development of competitive denitrifying bacteria. This could potentially affect the anammox activity and bacterial community in the long term [42]. In a low-strength WW treatment with slightly lower $NH_4^+$ (21.7 mg·$L^{-1}$) and $NO_2^-$ (25 mg·$L^{-1}$) loading rates than in our previous experiments, 10 weeks of $NO_3^-$ accumulation significantly affected the anammox process and bacterial community in freshwater *Candidatus* Kuenenia stuttgartiensis granules [43]. The same species was also able to degrade close to 50 mg·$L^{-1}$ $NO_3^-$-N in batch experiments without an external electron donor, through an internal $NO_3^-$ reduction process [44]. Thus, the effects of $NO_3^-$ in freshwater anammox species are complex and not yet fully understood.

When it comes to the marine species *Ca.* Scalindua, research on this topic is, to the best of our knowledge, currently non-existent. Therefore, it is essential to investigate the impact of high $NO_3^-$ levels, which are found in real RAS, on *Ca.* Scalindua activity and bacterial community to validate this process in RAS under real-world conditions.

While previous studies on microbial diversity in RAS have focused on community composition under standard conditions, our study is unique, as it examines the acclimation of *Ca.* Scalindua to high $NO_3^-$ levels, a factor which is often overlooked. Using synthetic WW, we isolate the effects of $NO_3^-$ accumulation on microbial dynamics, providing controlled insights into anammox resilience and adaptability. This research bridges the gap between controlled experimental conditions and the complexities of real RAS, where fluctuating organic matter and feed contaminants present additional challenges.

In the current study, we aimed to fill this knowledge gap by chronically exposing granules containing the marine anammox bacteria *Ca.* Scalindua to high, realistic concentrations of $NO_3^-$, mimicking RAS conditions at the laboratory scale. These concentrations, which can reach up to 400 mg·$L^{-1}$ $NO_3^-$-N, are known to impact the health and welfare of fish [17,18]. We investigated the effect of these high $NO_3^-$ levels on the nitrogen removal rate and bacterial community structure of the anammox granule.

## 2. Materials and Methods

### *2.1. Reactor 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 $NH_4^+$ and $NO_2^-$ [45] at the University of Hiroshima (Higashihiroshima, Japan) for over 13 years [46,47]. A biomass sample of approximately 5 g (wet weight) was delivered to the University of Gothenburg (Sweden) in August 2019 and used as inoculum to initiate a new culture in a glass column reactor (Ø 50 mm; volume, 325 $cm^3$, KF-30, AS ONE, Tokyo, Japan, Figure 1) with a non-woven fabric sheet as biofilm carrier material (Japan Vilene, Tokyo, Japan). The reactor was maintained at a constant temperature of 28 °C in an incubator (INCU-Line IL 112 Prime incubator, VWR international, Radnor, PA, USA) and fed with synthetic marine WW (salinity, 29‰, Aquaforest, Brzesko, Poland) supplemented with nitrogen (28 mg·$L^{-1}$ $NH_4^+$-N and 34 mg·$L^{-1}$ $NO_2^-$-N), inorganic carbon ($KHCO_3$,

1000 mg·L$^{-1}$), minerals ($CaCL_2$, $KH_2PO_4$, $MgSO_4$) and nine trace elements (TE) with EDTA, as described earlier [25,45]. After all the different chemicals were added, the WW feed was flushed with pure $N_2$ gas for at least 30 min to obtain a dissolved $O_2$ concentration below 0.5 mg·L$^{-1}$. The pH was adjusted to 7.0–7.5 with a solution of glacial sulfuric acid ($H_2SO_4$) [45]. The influents were continuously introduced into the reactor using a peristaltic pump (Masterflex L/S Economy Drive, Cole-Parmer Instruments, Vernon Hills, IL, USA).

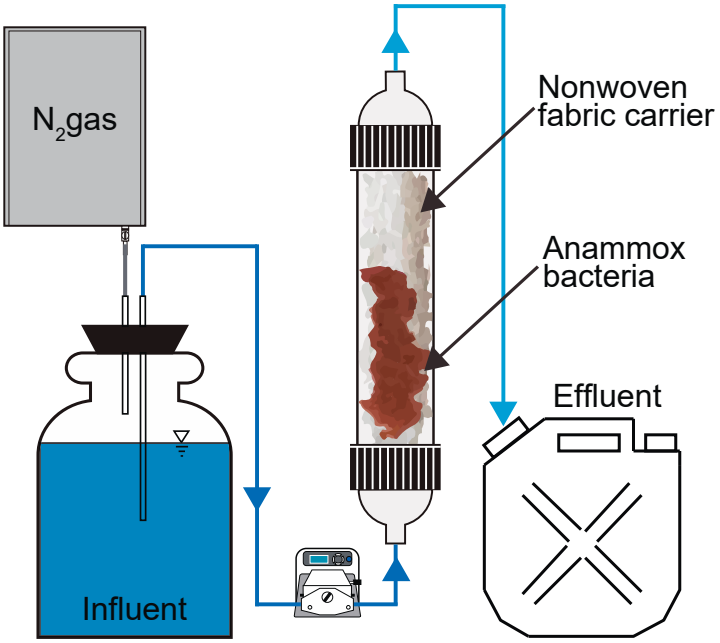


**Figure 1.** Schematic drawing of the up-flow column reactor.

### *2.2. Experimental Protocol*

On 10 January 2023 (approximately 1230 days after the initial inoculation), 6.5 g of biomass from the stock reactor was inoculated into a new reactor of the same volume as the original inoculum (325 cm$^3$) using a new non-woven fabric sheet of the same type (Japan Vilene, Tokyo, Japan). The reactor was followed for 368 days in three experimental phases.

During Phase 1 (stabilization, days 0–52), the reactor was operated with the same synthetic WW feed supplemented with $NH_4^+$, $NO_2^-$, $KHCO_3$, minerals and TE as was used in previous experiments [25,45]. In Phase 2 (days 53–105), the reactor was subjected to gradually increasing concentrations of $NO_3^-$, starting from 25 mg·L$^{-1}$ $NO_3^-$-N and reaching 400 mg·L$^{-1}$ $NO_3^-$-N on day 105. $NO_3^-$ concentrations were doubled every 9–20 days over this 52-day period. During Phase 3 (days 106–368), the $NO_3^-$ was kept at 400 mg·L$^{-1}$ $NO_3^-$-N without any additional changes. Apart from changes in $NO_3^-$ (and, consequently, in the nitrogen loading rate), all other environmental parameters—temperature (28 °C), salinity (29‰), $O_2$ (below 0.5 mg·L$^{-1}$), pH and HRT—remained consistent (Table 1, Figure 2).

**Table 1.** Operational conditions of the column rector in the three experimental phases.

| Phase | Period (d) | [$NO_3^-$-N] (mg·L$^{-1}$) | HRT (h) | pH Influent | pH Effluent | Nitrogen Loading Rate (g-TN·L$^{-1}$·day$^{-1}$) | Nitrogen Removal Rate (g-TN·L$^{-1}$·day$^{-1}$) |
|---|---|---|---|---|---|---|---|
| 1 | 0–52 | 0 | 5.4 ± 0.4 | 7.16 ± 0.18 | 7.51 ± 0.21 | 0.28 ± 0.03 | 0.21 ± 0.07 |
| 2 | 53–105 | 25 → 400 | 5.8 ± 0.4 | 7.24 ± 0.31 | 7.54 ± 0.34 | 0.81 ± 0.44 | 0.21 ± 0.02 |
| 3 | 106–368 | 400 | 6.2 ± 0.8 | 7.45 ± 0.27 | 7.64 ± 0.54 | 1.85 ± 0.30 | 0.29 ± 0.08 |

Note: The values represent the average ± standard deviation for the selected period.

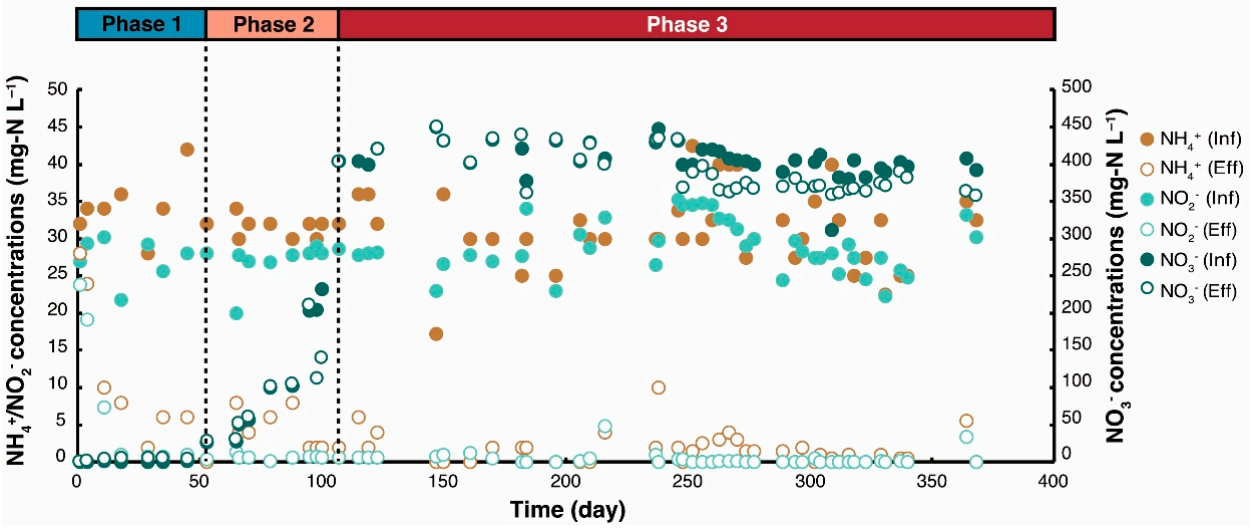


**Figure 2.** Concentration of $NH_4^+$, $NO_2^-$ and $NO_3^-$ in the influent ('Inf', filled circles) and effluent ('Eff', open circles) throughout the experiment.

### 2.3. Analytical Methods

The individual removal efficiencies of all the nitrogen compounds, the total nitrogen (TN) loading and removal rates, as well as the HRT (Table 1, Figure 3) were calculated as follows:

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

where Nx is the desired nitrogen compound among $NH_4^+$, $NO_2^-$, or $NO_3^-$.

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

where NX is the sum of $NH_4^+$, $NO_2^-$, and $NO_3^-$.

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

Salinity, $O_2$ and temperature were determined using a conductivity meter portable Multimeter pHenomenal MU 6100 H (VWR international, Radnor, PA, USA). Concentrations of $NH_4^+$ were determined using the powder pillow methods (salicylate method, 8155, Hach-Lange, Dusseldorf, Germany), and concentrations of $NO_2^-$ were determined using the LCK341 kit (Hach-Lange, Dusseldorf, Germany), both using the DR-2800 spectrophotometer (Hach-Lange, Dusseldorf, Germany). $NO_3^-$ concentrations below 200 mg·$L^{-1}$ $NO_3^-$-N 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, Kyoto, Japan) after filtration of samples through 0.2 µm pore-size PTFE membranes (Advantec, Tokyo, Japan) [48]. Concentrations above 200 mg·$L^{-1}$ $NO_3^-$-N were measured using the LCK339 kit and the DR-2800 spectrophotometer (Hach-Lange, Dusseldorf, Germany), after being diluted 200 times to prevent the salinity from interfering with the kit.

### 2.4. Microbial Community Analysis

Biomass samples for amplicon sequencing were collected at the end of Phase 1, when the reactor was stabilized (day 38), as well as one and two months into Phase 3 (days 139 and 167) and in the final month of the experiment (day 331). DNA was extracted using a FastDNA SPIN kit for soil (MP Biomedicals, Santa Ana, CA, USA). PCR amplification of

the bacterial 16S rRNA gene was performed targeting the V3-V4 region using the primer set 341F and 805R, as described earlier [49]. PCR products were purified using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA). 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 [50]. Sequence data were analyzed using QIIME 2 Core 2023.2 distribution [51]. Amplicon Sequence Variants (ASVs) were assigned via the SILVA 138.1 database [52]. ASVs that accounted for less than 1% of the total reads were used for bar-plots representation (Figure 4). The sequence data in the present study were deposited in the DNA Data Bank of Japan (DDBJ) database under the DDBJ/EMBL/GenBank under Bioproject ID PRJDB17738 and accession number DRA018222.

### *2.5. Fluorescence In Situ Hybridization (FISH)*

Biomass samples were collected from the up-flow column reactor at the same timepoints as for the microbial community analysis (days 38, 139, 167 and 331). Sample fixation and the following FISH procedure were performed as previously described [53]. The probes used for FISH included a mixture of EUB338, II, III, and IV probes labelled with Alexa Fluor 647, which target all bacteria [54,55], and Sca1129b probes labelled with Alexa Fluor 555, which are specific to *Ca.* Scalindua [47]. Hybridized samples were observed with an AxioImager Z2 epifluorescence microscope with a 100 W HBO lamp (Carl Zeiss, Oberkochen, Germany). Images were obtained using an AxioCam 712 mono camera and AxioVision software, version 4.5 (Carl Zeiss, Oberkochen, Germany).

## 3. Results

### *3.1. Reactor Performance*

During Phase 1 (stabilization), $NH_4^+$ and $NO_2^-$ removal efficiencies reached around 80–100% after 18 days (Figure 3A), while $NO_3^-$ exhibited a high negative removal efficiency (i.e., it was produced) of $-442.6 \pm 412.6\%$. These values indicate the successful establishment of the anammox process in the new experimental reactor [46]. Once the reactor was stable (from day 19 onwards), the TN loading and removal rates were $0.27 \pm 0.04$ and $0.22 \pm 0.04$ g-TN·$L^{-1}$·$day^{-1}$ respectively, and the HRT was 5.4 h (Table 1, Figure 3B).

During Phase 2 (days 53–105), $NO_3^-$ was slowly introduced to the reactor. The TN loading rate slowly increased with increasing $NO_3^-$ concentrations. High $NH_4^+$ and $NO_2^-$ removal efficiencies were maintained at $88.0 \pm 8.6\%$ and $97.4 \pm 1.7\%$, respectively (Figure 3A). The production rate of $NO_3^-$ was still negative but decreased to $-3.5 \pm 4.2\%$. The TN loading rate gradually increased from 0.34 to 1.65 g-TN·$L^{-1}$·$day^{-1}$ and the TN removal rate was $0.21 \pm 0.02$ g-TN·$L^{-1}$·$day^{-1}$ on average, with an HRT of 5.8 h (Table 1, Figure 3B).

During Phase 3 (days 106–368), $NO_3^-$ concentrations were kept constant in the influent. $NH_4^+$ and $NO_2^-$ removal efficiencies continued to be high during this phase as well: $95.0 \pm 6.5\%$ and $98.6 \pm 2.7\%$, respectively (Figure 3A). The $NO_3^-$ removal efficiency was positive during this phase ($5.2 \pm 6.6\%$). The TN loading rate was $1.85 \pm 0.30$ g-TN·$L^{-1}$·$day^{-1}$, and the TN removal rate was $0.29 \pm 0.08$ g-TN·$L^{-1}$·$day^{-1}$ (Table 1, Figure 3B). The HRT was slightly increased to 6.2 h (i.e., a slight increase in TN loading rate due to the high $NO_3^-$ concentrations, as shown in Table 1).

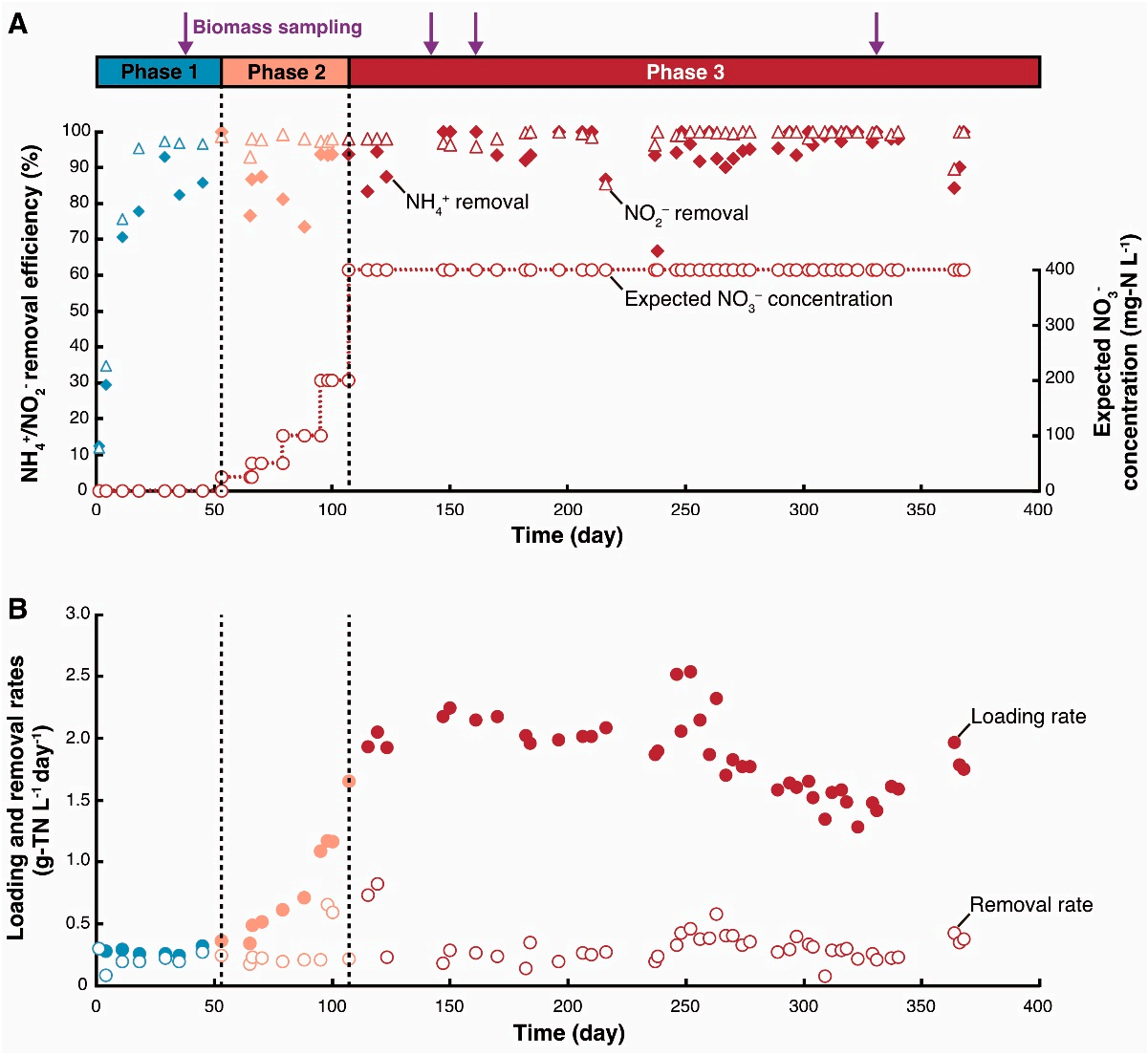


**Figure 3.** Anammox performance in the reactor. (**A**) $NH_4^+$ (closed diamonds) and $NO_2^-$ (open triangles) removal efficiencies (%) and expected $NO_3^-$ concentrations (dotted lines and open circles). (**B**) Nitrogen loading and removal rates (filled and open circles, respectively). Purple arrows indicate biomass sampling on days 38, 139, 167 and 331.

### *3.2. Microbial Community Analysis and FISH*

A total of 52,097, 56,823, 54,763 and 65,120 non-chimeric reads, along with 169, 289, 235 and 248 ASVs, were obtained from days 38, 139, 167 and 331, respectively. In this study, ASVs that represented more than 1% of the total reads were used for the analysis; ASVs accounting for less than 1% of the total reads were grouped as "Others" (Figure 4). *Ca.* Scalindua was among the most abundant species in the reactor throughout the different phases (Figure 4). The relative abundances of *Ca.* Scalindua did not change between the two first time-points. The relative abundance was 22.7% at the end of the stabilization period (day 38) and 22.1% after approximately one month at 400 mg·$L^{-1}$ $NO_3^-$-N (day 139). However, the relative abundance of *Ca.* Scalindua dropped to 13.3% and 10.2% after two and nine months of exposure to high $NO_3^-$. The population abundance of *Ca.* Scalindua during all experimental phases was further corroborated by FISH observations (Figure 5).

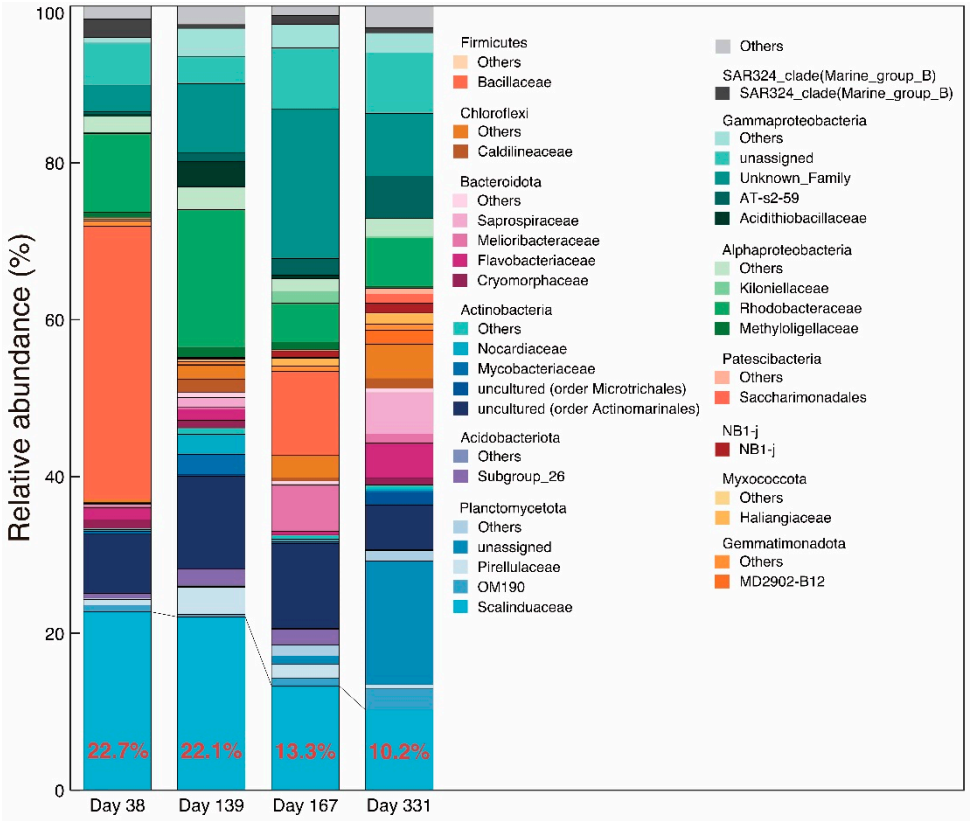


**Figure 4.** Microbial community composition at the end of Phase 1 (day 38), and after one, two and nine months of exposure to 400 mg·L$^{-1}$ $NO_3^-$-N (days 139, 167 and 331, respectively). Analysis based on 16S rRNA gene amplicon sequencing. Red percentages correspond to the relative abundance of the marine anammox *Ca.* Scalindua.

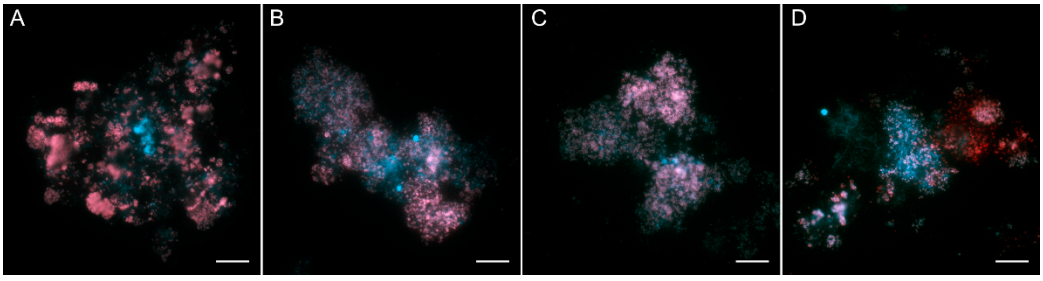


**Figure 5.** FISH micrographs of biomass collected from the reactor on days 38 (**A**), 139 (**B**), 167 (**C**) and 331 (**D**). FISH analysis utilized the Alexa Fluor 647-labelled EUB338mix probe (cyan) targeting all bacteria, while the Alexa Fluor 555-labelled Sca1129b probe (red) was used specifically for *Ca.* Scalindua. *Ca.* Scalindua appears magenta and other bacteria appear blue. Scale bars indicate 10 µm.

## 4. Discussion

### *4.1. Reactor Performance*

The marine anammox species used in this study, *Ca.* Scalindua, was isolated from coastal sediments in Hiroshima bay over 17 years ago [46]. Inoculum from the original samples have been further successfully cultivated in Japan (since 2007) and in Sweden (since 2019), using the protocol developed by van de Graaf and colleagues almost 30 years ago [45].

During the first 52 days of our experiment, we fed the newly inoculated anammox reactor with standard feed in order to maintain the anammox process and achieve a steady state of high removal efficiencies [46,47,53]. Once the removal efficiencies for both $NH_4^+$ and $NO_2^-$ were high and stable (over 80%), we gradually introduced $NO_3^-$ to the system.

We started with 25 mg·L$^{-1}$ $NO_3^-$-N and reached 400 mg·L$^{-1}$ $NO_3^-$-N after 52 days by doubling the dose every 9–20 days. During this second phase, we maintained high removal efficiencies for both $NH_4^+$ and $NO_2^-$, with averages of 88.0% and 97.4%, respectively. In the third phase, where the $NO_3^-$ was kept constant at 400 mg·L$^{-1}$ $NO_3^-$-N for almost nine months, we continued to observe high removal efficiencies of 95.0% and 98.6% for $NH_4^+$ and $NO_2^-$ respectively. These high values were comparable to those of the original sediment from Hiroshima bay (95% and 99%, respectively) [46]. These results confirm both the successful establishment and maintenance of the anammox process, as well as the effective acclimation of *Ca.* Scalindua to high $NO_3^-$ levels typically found in RAS [15,56].

Too-high levels of $NH_4^+$ and $NO_2^-$ are both known to potentially inhibit the anammox process, with a general consensus that the inhibition threshold for $NO_2^-$ (between 100 and 280 mg·L$^{-1}$ $NO_2^-$-N) is lower than the one of $NH_4^+$ (over 770 mg·L$^{-1}$ $NH_4^+$-N) [57]. High concentrations of $NO_3^-$ could also impact the anammox process, as $NO_3^-$ can also act as alternative electron acceptor [44]. $NO_2^-$ is more favourable and is preferable to $NO_3^-$ as electron acceptor under normal conditions, since it yields more energy and its reduction requires fewer enzymatic steps than for $NO_3^-$ [58]. However, if the concentrations of $NO_3^-$ are high, the anammox process could prioritize $NO_3^-$ reduction over $NO_2^-$ reduction, leading to less efficient nitrogen removal [44,58]. This was not observed in this experiment, where the concentrations of $NO_3^-$ were approximately twelve times higher than those of $NO_2^-$ (400 mg·L$^{-1}$ $NO_3^-$-N and 34 mg·L$^{-1}$ $NO_2^-$-N respectively). Under real RAS conditions, where $NO_2^-$ is not supplemented and typically remains below 1 mg·L$^{-1}$ [59], while $NO_3^-$ levels typically range from 10 to 400 mg·L$^{-1}$ [10,14,17], the process dynamics may differ and will warrant further investigations. In our experiment, we used synthetic WW enriched with $NO_2^-$ (34 mg·L$^{-1}$ $NO_2^-$-N) to maintain reactor stability, while we tested $NO_3^-$ concentrations comparable to real RAS conditions. In real RAS, $NO_2^-$ levels should ideally not exceed 0.1–0.5 mg·L$^{-1}$ $NO_2^-$-N [60], resulting in a higher $NO_3^-$:$NO_2^-$ ratio. This could influence electron acceptor preference, potentially shifting towards $NO_3^-$ reduction. It is also important to note that, while our results demonstrate successful acclimation of *Ca.* Scalindua to high $NO_3^-$ concentrations, the study was conducted using synthetic WW, which differs from the complex real RAS WW. Real RAS conditions, including fluctuations in organic matter and other nitrogenous compounds, as well as potential contaminants from fish feed (lipids, proteins, and trace metals), may influence reactor performance and microbial community dynamics.

### *4.2. Microbial Community*

Both FISH and microbiological analysis indicated the presence of *Ca.* Scalindua populations throughout the experiment. Despite a (relative) decrease over time, *Ca.* Scalindua remained one of the key bacterial phyla in the granules across all three phases.

None of current lineage of anammox bacteria exists as pure culture, but instead cohabit within granules with heterotrophic bacteria [61,62]. Some these heterotrophic bacteria phyla that comprise major component of the granules in this study, including the *Planctomycetes*, *Firmicutes*, *Proteobacteria*, *Bacteroidota*, *Chloroflexi* and *Acidobacteria*, are also generally important components of the microbial community in anammox bioreactors [61–63]. Organisms from these phyla can also be major fermentative acidogens under anaerobic and microaerobic environmental conditions in granules [64]. High $NO_3^-$ concentrations are known to impact bacterial communities in biofilms, resulting in potential biodiversity loss and tendencies towards community simplification [65]. These changes could influence community resilience and metabolic interactions, potentially altering the overall efficiency and stability of the system. One of the notable changes in bacterial community is the initial fluctuation of *Firmicutes* (*Bacillaceae*) in the first few months of the experiment, followed by their quasi-disappearance at the end of the experiment. *Firmicutes* was the dominant phylum at the beginning of the experiment (34.9%). Its disappearance could be attributed to a disturbance by $NO_3^-$. This was also seen in a previous study where the addition of 25–100 mg·L$^{-1}$ $NO_3^-$-N to groundwater led to a decrease in the relative *Firmicutes*

abundance from 87.6% to 54.2%–60.4% [66]. On the other hand, the proportion of *Proteobacteria*, *Bacteroidota* (including *Flavobacteriaceae*), *Chloroflexi* and *Planctomycetes* (excluding *Ca.* Scalindua) gradually increased in the granules over time. Some bacteria from these phyla are capable of using $NO_3^-$ as an electron acceptor, either through denitrification or $NO_3^-$ reduction, both in freshwater and marine environments, including RAS [67–69]. In addition, the anaerobic heterotrophic *Proteobacteria* and *Bacteroidota* such as *Flavobacteriaceae* could have benefited from the higher $NO_3^-$ sensitivity of the *Firmicutes* to outcompete this phylum [69], while some photosynthetic *Chloroflexi* could have scavenged organic matter from dead *Firmicutes* or other bacteria to flourish [70].

The exact role of these heterotrophic bacteria in the anammox granule has yet to be uncovered. One study showed that most organisms present in the granules were capable of $NO_3^-$ respiration, leading to the formation of $NO_2^-$ [71]. However, the high removal efficiencies (over 97%) of $NO_2^-$ in our study suggest that this was not the case, as the secondary production of $NO_2^-$ would lead to $NO_2^-$ accumulation over time.

Although we noticed a diminution of the relative abundance of *Ca.* Scalindua in the granule (from around 22% at the beginning of the experiment and one month after reaching high $NO_3^-$ levels, to 10.2% after almost nine months exposure), the relative proportion of all *Planctomycetes* (including *Ca.* Scalindua) increased from 24.5% to 30.6%. The anammox process is known to be driven by bacteria from the phylum *Planctomycetes,* to which *Ca.* Scalindua belongs [63,72]. However, the other *Planctomycetes* identified in this study (*Pirellulaceae*, OM190) are not known to be able to perform the anammox process, which is specific to the order Brocadiales [73].

We observed a 25% increase in non-chimeric reads and a 47% increase in ASVs between days 38 and 331. The apparent decrease in the relative abundance of *Ca.* Scalindua over time may simply be due to its slower growth rate compared to other heterotrophic bacteria present in the granules (14.4 days, [53]). These coexisting bacteria could thrive on excess $NO_3^-$ as their nitrogen source and use metabolic compounds produced by *Ca.* Scalindua as their carbon source [74]. This hypothesis is supported by the positive removal efficiency of $NO_3^-$ during this phase and the changes in microbial community with an increase in phylum capable of $NO_3^-$ removal. FISH results further suggest a stable population of *Ca.* Scalindua throughout the three experimental phases. Thus, we conclude that the *Ca.* Scalindua population in the column reactor was sufficient to maintain high nitrogen removal rates, despite its slight reduction in relative abundance within the anammox granule, confirming the granule's potential for treating RAS WW.

Our analysis provides insights into community shifts but does not resolve the exact metabolic roles of heterotrophic bacteria within the granule. Further studies employing metagenomic or transcriptomic approaches could elucidate the functional contributions of these bacteria, particularly their interactions with anammox organisms under high $NO_3^-$ conditions. Additionally, quantifying the biomass of anammox organisms and their associated granules would provide valuable information for assessing reactor performance and microbial growth dynamics.

### *4.3. Future Directions: Scaling Up Ca. Scalindua Applications in RAS*

Building on our previous study [26], our work demonstrates the potential of *Ca.* Scalindua to treat marine RAS WW, at the laboratory scale, highlighting its ability to operate without the need for TE supplementation and its tolerance to high $NO_3^-$ concentrations. For successful scaling up, further studies must validate the robustness of *Ca.* Scalindua under real RAS conditions, including transient changes in nitrogen loading rates and operational disturbances typical of commercial systems. Pilot-scale investigations will be crucial to identify key operational parameters, such as resilience to variable influent quality, hydraulic retention times, optimal reactor positioning.

Similarly to traditional denitrification loops in full RAS, an anammox reactor may need to be separated from the rest of the system to ensure optimal anaerobic conditions for *Ca.* Scalindua [19]. For instance, a two-step SHARON (single-reactor high-activity

ammonium removal over nitrite)-anammox process could be employed, where $NO_2^-$, the dominant final product from the SHARON reactor, is fed to the anaerobic anammox reactor [75]. Alternatively, recent studies have demonstrated the successful enrichment of anammox bacteria and an effective nitrogen removal efficiency (89.4% for TN, 93.1% for $NH_4^+$ and 93.5% for $NO_2^-$) using a pilot-scale up-flow anaerobic sludge blanket (UASB) reactor in freshwater RAS [40]. This UASB reactor, coupling anammox and denitrification, enables stable anammox activity with high nitrogen removal potential could be tested in marine RAS. Finaly, a one-step process in a submerged bed reactor could be used, allowing ammonium-oxidizing bacteria and anammox bacteria to coexist in a low-oxygen environment. In this setup, oxidation of $NH_4^+$ to $NO_2^-$ by ammonium-oxidizing bacteria would maintain low $O_2$ conditions in the reactor, while *Ca.* Scalindua converts the remaining $NH_4^+$ and the newly formed $NO_2^-$ into $N_2$ [76]. These steps will provide the necessary foundation for designing full-scale systems, but further research must also evaluate their effects on both reactor performance and microbial community composition. Given the slow growth of *Ca.* Scalindua, ensuring the stability of the granules is critical to prevent them from being outcompeted by faster-growing bacteria in RAS biofilters.

## 5. Conclusions and Perspectives

This study demonstrated that the anammox activity of *Ca.* Scalindua granules was not impacted after chronic exposure to high $NO_3^-$ concentrations that could be observed in RAS under controlled laboratory conditions. Despite the reduction in relative abundance of *Ca.* Scalindua in the granules, its removal efficiencies for both $NH_4^+$ and $NO_2^-$ remained high and constant throughout the different experimental phases. The relative decrease in abundance is most likely due to the slow growth of this bacteria compared to other heterotrophic bacteria present in the granules. FISH results also suggested a relatively stable presence of *Ca.* Scalindua throughout the experiment. Therefore, we concluded that the gradual exposure of *Ca.* Scalindua to high $NO_3^-$ levels resulted in successful acclimation of the bacteria.

Our study thus suggests that anammox granules containing *Ca.* Scalindua could be directly applied to treat marine RAS WW as an alternative to the traditional nitrification–denitrification pathway commonly employed in RAS.

However, scaling up to real-case systems could present challenges and is in need of further investigation. Throughout the different experimental phases of this study, optimal culture conditions for *Ca.* scalindua were maintained, including strict anaerobic conditions and the use of a synthetic WW feed enriched with $NH_4^+$ and $NO_2^-$. Future studies should explore the positioning and the performance of *Ca.* Scalindua under typical RAS conditions concerning $NH_4^+$, $NO_2^-$ and $O_2$ concentrations. In addition, future research should explore the combined effects of $NO_3^-$, organic matter and feed by-products on microbial communities and water quality to develop comprehensive WW strategies for RAS. These next steps are essential to validate the application of this bacterium as an alternative treatment technology at a commercial scale in marine RAS.

**Author Contributions:** Conceptualization: J.A.C.R., F.M. and T.K.; Data curation: J.A.C.R., N.F. and T.K.; Formal analysis: J.A.C.R., N.F., A.M. and T.K.; Funding acquisition: J.A.C.R., K.S.S. and T.K.; Investigation: J.A.C.R., E.U., N.F. and T.K.; Methodology: J.A.C.R., E.U., N.F., A.M. and T.K.; Project administration: J.A.C.R.; Resources: J.A.C.R., K.S.S. and T.K.; Software: N.F. and T.K.; Supervision: J.A.C.R., K.S.S. and T.K.; Validation: J.A.C.R. and T.K.; Visualization: J.A.C.R. and T.K.; Writing—original draft: J.A.C.R.; Writing—review and editing: J.A.C.R., E.U., N.F., A.M., F.M., K.S.S. and T.K. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was conducted within the frame of the MIRAI project and was supported by FORMAS (2020-00867), The Birgit och Birger Wåhlströms Minnesfond för den bohuslänska havs-och insjömiljön, Stockholm, Sweden, STINT, Stockholm, Sweden (mobility grant for internationalization, MG2019-8483), JSPS KAKENHI Grant Numbers JP20KK0244, JP21K19866, Japan, and JSPS Bilateral Program, Grant number JPJSBP120239926, Japan.

**Data Availability Statement:** Publicly available datasets were analyzed in this study. The sequence data in the present study were deposited in the DNA Data Bank of Japan (DDBJ) database under the DDBJ/EMBL/GenBank under the DDBJ/EMBL/GenBank under the Bioproject ID PRJDB17738, accession number DRA018222.

**Acknowledgments:** The authors are grateful to Linda Frank Hasselberg for her technical assistance and Miyuki Roques for the language editing.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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