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Abstract: Anaerobic ammonium oxidation (anammox) involves the microbiological oxidation of ammonium with nitrite as the electron acceptor and dinitrogen gas as the main product. The Scalindua species, an anammox genus that dominates natural habitats, plays an important role in catalyzing the loss of nitrogen from marine environments. Until now, a few Scalindua species have been reported to be enriched from sea sediments. The objective of this study is to enrich marine anammox bacteria with coastal sediments in Hiroshima Bay as the inoculums. The enrichment was achieved using a continuous upflow column reactor with synthetic sea water containing ammonium and nitrite. After 48 day incubation, a simultaneous decrease in ammonium and nitrite was observed. A total nitrogen removal rate of 1.16 kg-N m-3 day-1 was attained after 306 day incubation when the nitrogen loading rate was 1.32 kg-N m-3 day-1. Phylogenetic analysis revealed that the sequence similarity between the marine anammox-like bacteria in this reactor and the unidentified Candidatus Scalindua sp. was 96-98%. We successfully enriched marine anammox bacteria in the sediments of Hiroshima Bay by using synthetic sea water. Further studies are needed to investigate the characteristics of marine anammox bacteria, including optimal pH, temperature, and nitrogen loading rate.

# Enrichment of Marine Anammox Bacteria in Hiroshima Bay Sediments

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**Abstract**
Anaerobic ammonium oxidation (anammox) involves the microbiological oxidation of ammonium with nitrite as the electron acceptor and dinitrogen gas as the main product. The *Scalindua* species, an anammox genus that dominates natural habitats, plays an important role in catalyzing the loss of nitrogen from marine environments. Until now, a few *Scalindua* species have been reported to be enriched from sea sediments. The objective of this study is to enrich marine anammox bacteria with coastal sediments in Hiroshima Bay as the inoculums. The enrichment was achieved using a continuous upflow column reactor with synthetic sea water containing ammonium and nitrite. After 48 day incubation, a simultaneous decrease in ammonium and nitrite was observed. A total nitrogen removal rate of 1.16 kg-N $m^{-3}$ $day^{-1}$ was attained after 306 day incubation when the nitrogen loading rate was 1.32 kg-N $m^{-3}$ $day^{-1}$. Phylogenetic analysis revealed that the sequence similarity between the marine anammox-like bacteria in this reactor and the unidentified Candidatus *Scalindua* sp. was 96-98%. We successfully enriched marine anammox bacteria in the sediments of Hiroshima Bay by using synthetic sea water. Further studies are needed to investigate the characteristics of marine anammox bacteria, including optimal pH, temperature, and nitrogen loading rate.

**Keywords**
Anaerobic ammonium oxidation (anammox); enrichment; marine anammox bacteria

## INTRODUCTION

Anaerobic ammonium oxidation (anammox) entails the microbiological oxidation of ammonium with nitrite as the electron acceptor and dinitrogen gas as the main product, and is mediated by a group of deep-branching *Planctomycete*-like bacteria (Strous *et al*., 1999). Anammox bacteria have been detected in different wastewater treatment facilities and natural environments in the world (Schmid *et al*., 2005). Currently, five genera of anammox bacteria have been reported and named: *Brocadia*, *Kuenenia*, *Scalindua*, *Jettenia*, and *Anammoxoglobus* (Kuenen, 2008). The *Scalindua* group is 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). In addition, studies involving $^{15}N$ tracers have also detected the anammox activity in estuary sediments in Japan (Amano *et al*., 2007; Nakajima *et al*., 2008). It is currently estimated that anammox bacteria may be responsible for at least 50% of nitrogen removal from marine ecosystems (Devol, 2003), although this value is not undisputed (Ward *et al*., 2009). A better understanding of the ecophysiology (i.e., microbial community structures and *in situ* activities) of marine anammox bacteria is important regarding the global nitrogen cycle in marine ecosystems.

Until now, marine anammox bacteria as well as wastewater anammox bacteria have not yet been isolated in pure culture. Recently, only a few *Scalindua* species have been reported to be enriched from sea sediments (Nakajima *et al*., 2008; van de Vossenberg *et al*., 2008; Kawagoshi *et al*., 2009). van de Vossenberg *et al*. have reported that no anammox bacteria were detected when artificial seawater was used for the enrichment, while two species of marine anammox bacteria were successfully enriched when a medium-based Red Sea salt was used (van de Vossenberg *et al*.,

2008). Nakajima *et al*. have also established an enrichment culture of marine anammox bacteria using deep sea water (Nakajima *et al*., 2008). It is, however, unknown what factors in media contribute to the enrichment of marine anammox bacteria.

We hypothesized that the short HRT might prevent substrate limitation and accumulation of inhibitory substances, resulting in the establishment of the enrichment with feeding low concentrations of media. The objectives of this study were, therefore, to enrich the marine anammox bacteria using a column reactor with coastal sediments in Hiroshima Bay as the inoculum, and to investigate the community structure in the biofilm using a phylogenetic analysis based on 16S RNA genes and fluorescence in situ hybridization (FISH).

## MATERIALS AND METHODS

### Sediment samples

Surface sediment samples were collected using a plastic core sampler from Hiroshima Bay; in the Seto Inland Sea during July 2007 (Location I) and July 2008 (Location II). The location and characteristics of sampling point are listed in Table 1.

### Column reactors

Two glass column reactors (reactor I and II) were operated in parallel with a nonwoven fabric sheet (Japan Vilene Co., Ltd., Tokyo, Japan) as the biofilm carrier materials (Fig. 1). Surface sediment samples (upper 2 cm) weighing 1 g (wet weight) collected during 2007 and 2008 were inoculated in reactor I and reactor II, respectively, fed with same composition of synthetic marine nutrient medium. The reactor volume was 56 $cm^3$ and the surface area of the biofilm carrier was 54.6 $cm^2$. The temperature was maintained at 20˚C. The initial hydraulic retention time (HRT) of the reactor I and II were 2.0 and 0.9 h, respectively. A synthetic marine nutrient medium was used, containing 35 g/L of an artificial sea salt (SEALIFE, Marine Tech. Co., Ltd., Address: 10-6 Nihonbashi-Odenmacho, Chuou-ku, Tokyo, Japan 103-0011) supplemented with 0.3 to 1.4 mM $(NH_4)_2SO_4$, 0.3 to 1.4 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. The total nitrogen loading and removal rates were calculated based on the concentrations of $NH_4^+$, $NO_2^-$, and $NO_3^-$, and the HRT.

**Table 1** Overview of sampling points in this study

| | Location I | Location II |
|---|---|---|
| Date | July 2007 | July 2008 |
| Location | 34˚17.5'N, 132˚23.2'E | 34˚21.4'N, 132˚30.7'E |
| Depth (m) | 25.5 | 7.4 |
| Temperature (˚C) | 19.9 | 19.4 |
| pH | 8.0 | 8.0 |
| Salinity (psu) | 32.79 | 32.05 |
| $NH_4^+$ (mg-N/L) | 0.90 | 0.25 |
| $NO_2^-$ (mg-N/L) | 0.44 | 0.03 |
| $NO_3^-$ (mg-N/L) | 1.56 | 0.08 |

**Analytical method**

The seawater quality (see above) was determined using a multi-parameter water quality meter (AAQ1183, JFE ALEC Co., Ltd., Kobe, Japan). The concentration of $NH_4^+$ was determined by using a UV-visible spectrophotometer (DR-4000, Hach Co., Loveland, CO, USA). The concentrations of $NO_2^-$ and $NO_3^-$ were determined, using ion-exchange chromatography (HPLC 10Avp; SHIMADZU Co., Kyoto, Japan) with an IC-C1 cation column and an IC-A3 anion column after filtration with 0.2 μm pore-size membranes (Advantec Co., Ltd., Tokyo, Japan).

**DNA extraction and PCR amplification**

Total DNA was extracted from the anammox biomass in the column reactor II after 162 days of operation, using a Fast DNA spin kit (Bio 101; Qbiogene Inc., Carlsbad, CA, USA) as described in the manufacturer's instructions. 16S rRNA gene fragments from the isolated total DNA were amplified, using a ONE Shot LA PCR MIX kit (TaKaRa Bio Inc., Ohtsu, Japan) and a Planctomycetales-specific primer set with pla46f (Neef *et al*., 1998) and univ1390r (Zheng *et al*., 1996). The PCR condition targeted for the anammox bacteria was as follows: 4 min of initial denaturation at 94°C, followed by 30 cycles of 45 s at 94°C, 50 s at 58°C, and 3 min at 72°C. Final extension was carried out for 10 min at 72°C. The PCR reaction was performed with a total volume of 50 μl, and 1 μg of DNA was added as template DNA. The PCR products were electrophoresed in a 1% (wt/vol) agarose gel.

**Cloning and phylogenetic analysis**

PCR products were ligated into a pCR-XL-TOPO vector and transformed into One Shot Escherichia coli cells following the manufacturer's instructions (TOPO XL PCR cloning kit; Invitrogen, Carlsbad, CA, USA), and then clone libraries were constructed. All DNA sequencing was performed by Dragon Genomics Center, TaKaRa Bio Inc. (Yokkaichi, Japan). The sequences were compared with similar sequences of the reference organisms, using a BLAST search (Altschul *et al*., 1990). Sequences with 97% or greater similarity were grouped into operational taxonomic units (OTUs), using the Similarity Matrix program from the Ribosomal Database Project (Maidak *et al*., 1997). Phylogenetic tree was constructed by ARB software with the neighbor-joining method. Bootstrap resampling analysis for 1,000 replicates was performed to estimate the confidence of the tree topologies.

**FISH analysis**

In situ hybridization was performed according to the procedure described by Okabe *et al*. (1999). A model Axioimager.M1 epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) was used

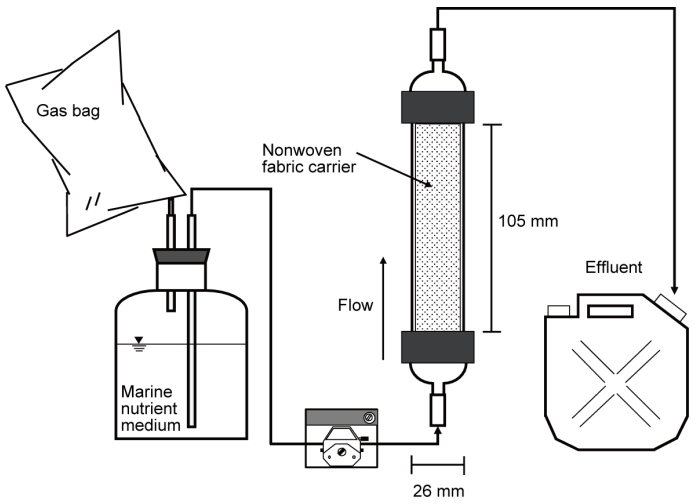


**Figure 1** Schematic drawing of an up-flow fixed-bed column reactor

for the observation. The 16S rRNA-targeted oligonucleotide probes used in this study were EUB338 (Amann *et al*., 1990), EUB338-II (Daims *et al*., 1999) and EUB338-III (Daims *et al*., 1999) for most bacteria, and Sca1309 (Schmid *et al*., 2003) for marine anammox bacteria. To detect all bacteria, the probes (EUB338mix) were used in the equimolar mixture together with probes EUB338, EUB338II, and EUB338III. The probes were labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine 5-isothiocyanate (TRITC) at the 5' end.

## RESULTS AND DISCUSSION

### Enrichment of marine anammox bacteria using column reactors

In reactor I, no simultaneous oxidation of $NH_4^+$ and $NO_2^-$ was observed during 200 day incubation (data not shown). On the other hand, in the reactor II, the simultaneous oxidation of $NH_4^+$ and $NO_2^-$ was observed after 48 day incubation (Fig. 2). In this period, the $NH_4^+$ and $NO_2^-$ removal efficiencies were 25% and 34%, respectively. A 97% $NO_2^-$ removal efficiency was observed on day 114. These results clearly suggest that anammox reaction proceeded in the reactor II. After the 97% removal efficiency was attained in the reactor II, the nitrogen loading rate was gradually increased by increasing the $NH_4^+$ and $NO_2^-$ concentrations and/or reducing the HRT up to 0.7 h. After 300 days of reactor operation, the $NH_4^+$ and $NO_2^-$ removal efficiencies increased to 95% and 99%, respectively. In addition, a total nitrogen removal rate of 1.16 kg-N $m^{-3}$ $day^{-1}$ was attained on day 306 when the nitrogen loading rate was 1.32 kg-N $m^{-3}$ $day^{-1}$ (HRT, 0.63 h). This value has never been reported before (Table 2). The nitrogen stoichiometric ratio on day 306 was 1:1.21:0.15 for conversion of $NH_4^+$ and $NO_2^-$ to the production of $NO_3^-$. This stoichiometric ratio was a little different from the previously reported ratio of 1:1.32:0.26 (Strous *et al*., 1998). The possible reason for no anammox reaction in the reactor I is due to the lower initial population of anammox bacteria. In this study, the shorter HRT resulted in the rapid detection of the anammox reaction within 2 months, compared with the 3 months by van de Vossenberg *et al*. (2008) and 8 months by Nakajima *et al*. (2008). In addition, the lower initial concentrations of $NH_4^+$ and $NO_2^-$ in the influent may also be important because higher concentrations of $NH_4^+$ and $NO_2^-$ dissociated from the actual concentrations in the sediments, resulting in an inhibitory effect for the marine anammox bacteria. Since the concentrations of adding salts were constant except ammonia and nitrite, it is not clear what kind of component in the medium influence the growth of marine anammox bacteria. Further studies incubating with single-salt based medium are needed to clarify the influence of medium composition on the growth of *Scalindua* species.

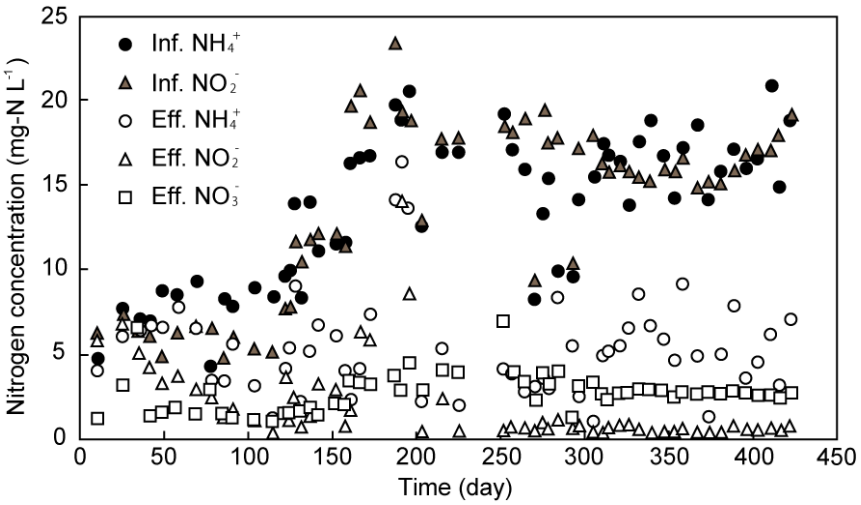


**Figure 2** Changes in ammonia, nitrite, and nitrate concentrations

**Table 2** Comparison of different enrichments

| | Nakajima *et al*., 2008 | van de Vossenberg *et al*., 2008 | In this study (reactor II) |
|---|---|---|---|
| Loading rate (g-N $L^{-1}$ $day^{-1}$) | 0.08 | 0.420 | 1.32 |
| Conversion rate (g-N $L^{-1}$ $day^{-1}$) | 0.07 | 0.345 | 1.16 |
| Reactor type | Column reactor | Sequencing batch reactor | Column reactor |
| Stoichiometry | Not available | 1:1.22:0.22 | 1:1.21:0.15 |
| HRT (h) | 24 | 72 | 0.63 to 0.9 |
| Closest species, Sequence similarity (%) | Uncultured plancmycete 3-8b6, 99% Candidatus "*Scalindua wagneri*", 97% | Gullmar Fjord sediment clone, 98% Candidatus "*Scalindua brodae*", 99.5% | Scalindua sp., 97% Candidatus "*Scalindua wagneri*", 96% |

**Phylogenetic analysis**

We analyzed the 93 clones with 1,385 bp related to the *Scalindua* group within the anammox bacteria (Fig. 3). The sequence of the OTU clone SUPa6 was closely related to the Candidatus *Scalindua* sp. enrichment culture clone 15L (EU142947), with 97% sequence similarity. Furthermore, the sequence similarities of the OTU SUPa6 to other proposed anammox bacteria, namely, "Candidatus *Scalindua brodae*" (AY254883), "Candidatus *Scalindua sorokinii*" (AY257181), and "Candidatus *Scalindua marina*" (EF602039) were 97.1, 97.0, and 97.5%, respectively. On the other hand, the sequence of the OTU clone SUPb6 was closely related to the "Candidatus *Scalindua wagneri*" (AY254882), with 96% sequence similarity. The frequency of OTU SUPa6 plus SUPb6 was 94%, suggesting that these two OTU were dominant group in this reactor II. These results clearly indicate that at least two different *Scalindua* species were present in this enrichment culture. The sequence similarities of the OTU SUPg2 and SUPe11 to the closest *Scalindua* sp. (EU142947) were 95 and 92%, respectively.

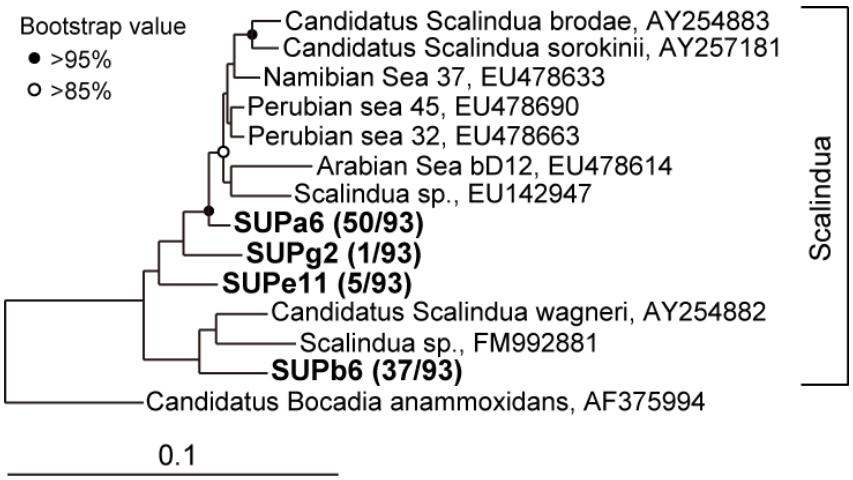


**Figure 3** Phylogenetic tree of enriched marine anammox bacteria

**FISH analysis**

To confirm the presence of marine anammox in this column reactor, we performed FISH analysis with the probe Sca1309 specific to the *Scalindua* group. The cells hybridized with the probe Sca1309 (Fig. 4) accounted for more than 90% of the total bacteria hybridized with the probe EUB338mix. The species of marine *Scalindua* enriched in this study are typical anammox bacteria, showing the ring-shape cells with a FISH as reported in another study (Schmid *et al*., 2003). It is noted that other coexisting bacteria are phylogenetically unknown at present. As shown in the phylogenetic analysis, two *Scalindua* species were enriched, and other bacterial groups also exist in this column reactor. Other probes, therefore, should be applied in the future to specifically identify the dominant *Scalindua* species and the coexisting bacterial groups to investigate their ecophysiological roles.

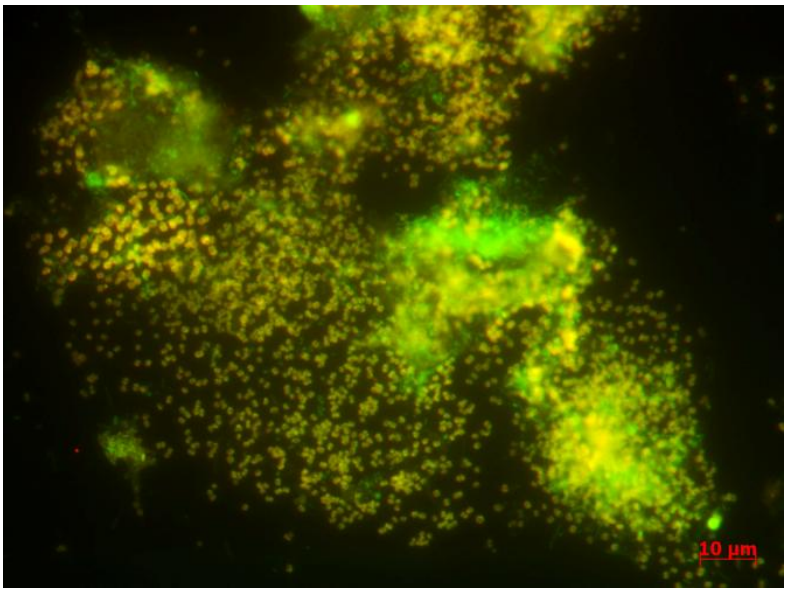


**Figure 4** A FISH image of anammox bacteria in the column reactor with FITC-labeled probe EUB338mix and TRITC-labeled probe Sca1309

## CONCLUSIONS

Within 2 months incubation, we successfully enriched marine anammox bacteria inoculated from the sediment of Hiroshima Bay with a synthetic medium containing sea salt and the standard anammox medium. A total nitrogen removal rate of 1.16 kg-N $m^{-3}$ $day^{-1}$ was attained with 0.63 h of HRT. Phylogenetic analysis clearly demonstrated that anammox bacteria, which are affiliated with at least two different *Scalindua* groups, are present. Further studies are needed to investigate the characteristics of marine anammox bacteria, such as the optimal pH, temperature, and optimal nitrogen loading rates.

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