Enrichment of marine anammox bacteria from seawater-related samples and bacterial community study
Y. Kawagoshi, Y. Nakamura, H. Kawashima, K. Fujisaki, K. Furukawa and A. Fujimoto

ABSTRACT
Anaerobic ammonium oxidation (anammox) is a novel nitrogen pathway catalyzed by anammox bacteria which are obligate anaerobic chemoautotrophs. In this study, enrichment culture of marine anammox bacteria (MAAOB) from the samples related to seawater was conducted. Simultaneous removal of ammonium and nitrite was confirmed in continuous culture inoculated with sediment of a sea-based waste disposal site within 50 days. However, no simultaneous nitrogen removal was observed in cultures inoculated with seawater-acclimated denitrifying sludge or with muddy sediment of tideland even during 200 days. Nitrogen removal rate of 0.13 kg/m³/day was achieved at nitrogen loading rate of 0.16 kg/m³/day after 320th days in the culture inoculated with the sediment of waste disposal site. The nitrogen removal ratio between ammonium nitrogen and nitrite nitrogen was 1:1.07. Denaturing gradient gel electrophoresis (DGGE) analysis indicated that an abundance of the bacteria close to MAAOB and coexistence of ammonium oxidizing bacteria and denitrifying bacteria in the culture.

Key words | anammox, bacterial community, enrichment culture, marine anammox bacteria, nitrogen removal

INTRODUCTION
Anaerobic ammonium oxidation (anammox) is a novel and unique nitrogen metabolic pathway first reported in 1995 (Mulder et al. 1995). In anammox reaction, ammonium is anaerobically oxidized by nitrite and nitrogen gas (N₂) is produced via hydrazine (Strous et al. 1998). Anammox bacteria are obligatory anaerobic chemoautotroph which can grow using free energy obtained from oxidation-reduction reactions of nitrite and ammonium. The properties of anammox bacteria are not only unique but also valuable for ammonium removal process. In conventional ammonium removal process using nitrification-denitrification reaction, enough amount of energy is needed for complete oxidation of ammonium to nitrate or nitrite in nitrification reaction and organic carbon source is required in denitrification reaction. In contrast to this, only half amount of ammonium is oxidized to nitrite and no organic carbon source is required in “nitritation-anammox process”, therefore it is possible to reduce the requirements for aeration energy and carbon source. In addition, since nitrogen removal rate in anammox reactor is higher than that of denitrification (Ahn 2006), scale-down of treatment facilities is expected. On the other hand, anammox bacteria have still not been isolated and their growth rate was known to be quite low (Strous et al. 1997), thus the establishment of dense and stable enrichment culture is important for the application of anammox bacteria to practical nitrogen removal process.

Anammox bacteria in various aqueous environment have been reported (Dalsgaard et al. 2003; Kuypers et al. 2005; Schmid et al. 2005; Tal et al. 2006; Kartal et al. 2007) and several full-scale treatment plants utilizing anammox
bacteria have been started up (van der Star et al. 2007). Although the presence of marine anammox bacteria (MAAOB) has also been detected in anoxic seawater environment using gene analyses (Kuypers et al. 2003; Amano et al. 2007), there have been only a few reports related to cultivation of MAAOB (Nakajima et al. 2008; van de Vossenberg et al. 2008). In these studies, seeding sludge was taken from sediment of seawater in common harbor area, and MAAOB were enriched using the medium prepared with natural seawater (Nakajima et al. 2008) or with the salt made from natural seawater (van de Vossenberg et al. 2008). In this study, enrichment culture of MAAOB from three types of the samples related to seawater environment, which were expected as good inoculum sources, was studied.

MATERIALS AND METHODS

Seawater-related samples as inoculum sources for MAAOB enrichment culture

Three types of seawater-related samples were used as the inoculum sources for enrichment culture of MAAOB as follows: 1) denitrifying sludge acclimated with seawater, 2) muddy sediment in tideland, 3) sediment in a sea-based waste disposal site. The denitrifying sludge was collected from the denitrification reactor utilising seawater as dilution water in a wastewater treatment facility of brewing company. The muddy sediment was collected at tideland in Kumamoto port. The area around sampling point is covered with seawater (1–4 m depth) in full tide and becomes tideland in low tide. The waste disposal site sediment was collected from the sea-based waste disposal site in the Seto Inland Sea of Japan (Kawagoshi et al. 2003). The floating sediment of the sea bottom was collected at a depth of 9 m.

Culture medium for MAAOB enrichment

MAAOB-medium (30 g NaCl, 6 g MgSO4·7H2O, 5 g MgCl2·6H2O, 1 g CaCl2·2H2O, 0.5 g KHCO3, 20 mg SrCl2·6H2O, 700 mg KCl, 100 mg NaBr, 20 mg H3BO3, 2 mg NaF, 0.08 mg KI, 54 mg K2HPO4, 40 mg-N (NH4)2SO4, 40 mg-N NaNO2, and 1 ml of trace element solution-I and solution-II (Imajo et al. 2004a,b) in 1,000 ml sterilized pure water, pH 7.8) including nitrite and ammonium was used for enrichment culture of MAAOB. The concentrations of nitrite nitrogen (NO2−-N) and ammonium nitrogen (NH4+-N) were both adjusted 50 mg/l or 100 mg/l for batch cultures, and changed in the range from 40 mg/l to 80 mg/l for continuous cultures. The medium was deoxidized by Argon gas purge in order to make anaerobic condition before use.

Reactor configuration and operation

Schematic of the reactor for enrichment culture is shown in Figure 1. A 500 ml of cylindrical column reactor including a non-woven fabric (Japan Vilene Co. Ltd., Japan) of 260 cm2 as a biomass carrier was used. The each inoculum sample weighing 3 g (wet weight) was put through a 0.5 mm-mesh strainer and applied to the non-woven fabric. The MAAOB-medium of 500 ml and the fabric were put into the reactor. When the reactor was started up, the medium was circulated between the reactor and a 300 ml of glass bottle containing the fresh MAAOB-medium with a peristaltic pump, and sequential batch cultivation was carried out. The culture temperature was maintained at 30°C through all experiments. When confirming the decrease of both NH4+-N and NO2−-N concentrations, the medium was continuously fed into and drawn from the reactor (continuous culture). Continuous culture was started at HRT of 48 h and nitrogen load was gradually increased. The reactor was covered to prevent growth of phototrophic bacteria.
PCR-DGGE

DNA was extracted from the biomass sample in each reactor. The 16S rRNA was amplified by polymerase chain reaction (PCR) with “touchdown” method (Don et al. 1991). The DNA fragments containing the regions v.3–v.5 were amplified with two kinds of primer-sets of UKf1 with GC-clamp and UKr1, and UKf2 with GC-clamp and UKr1 (Kawagoshi et al. 2009) for the detection of anammox bacteria. The UKf1 was used for broad range of anammox bacteria and the UKf2 was specially designed for the detection of Candidatus Scalindua sorokini (Kuypers et al. 2003) and Candidatus Scalindua brodae (Schmid et al. 2003). In addition, bacterial universal primer-set of GM5f with GC-clamp and DS907r (Teske et al. 1996) was used in order to investigate the bacteria coexisting with anammox bacteria. DNA extraction and amplification were performed according to a previously reported method (Nerenberg et al. 2008). Denaturing gradient gel electrophoresis (DGGE) was performed using a denaturing gradient gel ranged from 20% to 80% denaturant. Electrophoresis was run at 200 V and 60°C for 5h. DNA bands on the DGGE gel were excised and re-amplified by PCR as described above for direct DNA sequence. DNA sequence was performed using CEQ8000 (Beckman Coulter, Fullerton, USA) in accordance with manufacturer’s protocol. The sequences of DNA bands were compared with FASTA DNA database (Pearson & Lipman 1988). The sequence alignment and phylogenetic analysis were conducted using MEGA software version 4 (Tamura et al. 2007).

RESULTS AND DISCUSSION

Sequential batch cultivation for acclimation of MAAOB

In general, it has been known that anammox bacteria have quite low growth rate (Strous et al. 1997) and their cultivation is difficult by batch culture using suspended biomass (Imajo et al. 2004a,b). Anammox bacteria are obligate anaerobes (Strous et al. 1997) and have suitable temperature for growth depending on the type of species (Isaka et al. 2008). They have still not been isolated. Imajo et al. compared four types of reactors for enrichment of anammox bacteria and demonstrated that continuous culture using biomass carrier or bacterial granules were effective to maintain enough length of sludge retention time (SRT) for enrichment of anammox bacteria (Imajo et al. 2005). Therefore, non-woven fabric was used as a biomass carrier in this study. At the beginning, sequencing-batch cultivation was conducted to promote an acclimation of anammox bacteria.

Figure 2 shows the change of NO$_2^-$-N and NH$_4^+$-N concentrations in batch culture inoculated with the denitrifying sludge (a), muddy sediment in tideland (b), sediment of the sea-based waste disposal site (c). Decrease of NO$_2^-$-N was observed from the first batch culture in all reactors and only a little decrease of NH$_4^+$-N was confirmed in the reactors (b) and (c). No nitrate production was observed in all reactors. In anammox reaction, the molar-reaction ratio of NH$_4^+$-N, NO$_2^-$-N and NO$_3^-$-N is 1.0: 1.32: 0.26 (Strous et al. 1998), which was quite different from these results. In addition, no PCR product was amplified from any biomass.
samples in all reactors by using the 16S rRNA primers for the detection of anammox bacteria (data not shown). From these results, it was presumed that the $\text{NO}_2^-$ decrease in these reactors was due to endogenous denitrification or sulphur denitrification using reductive sulphur contained in the inoculum sources. Although no evidence of anammox reaction was confirmed in all cultures at this time, it was considered that the bacteria in the inoculum source adhered to the non-woven fabric. Therefore, the reactors were switched to continuous culture operation in order to promote the enrichment of MAAOB.

**Continuous culture for MAAOB enrichment**

Figure 3 shows the time course of $\text{NO}_2^-$ and $\text{NH}_4^+$ concentrations in influent and in effluent with HRT. In the reactor inoculated with the denitrifying sludge (Figure 3(a)), continuous culture was started at 50 mg/l of influent $\text{NO}_2^-$ and $\text{NH}_4^+$ and 48 h of HRT. The effluent $\text{NO}_2^-$ decreased to less than 25 mg/l during 20 days suggesting denitrifying activity, but the effluent $\text{NH}_4^+$ was still 35 mg/l. When influent $\text{NO}_2^-$ was adjusted at 70 mg/l on the 27th day, effluent $\text{NO}_2^-$ rapidly increased to almost the same level as the influent concentration (50 mg/l) suggesting the inhibition to denitrifying activity by the high concentration of nitrite (Strous et al. 1999). Therefore, influent $\text{NO}_2^-$ was reduced to 30 mg/l. The effluent $\text{NO}_2^-$ concentration gradually decreased to less than 2.0 mg/l within 55 days afterwards, however, $\text{NH}_4^+$ decreased by only 10 mg/l. Even after that, no decrease of $\text{NH}_4^+$ was observed although $\text{NO}_2^-$ was consumed for about 120 days. From these results, it was considered that the enrichment of MAAOB was difficult in this reactor.

In the muddy sediment-inoculated reactor (Figure 3(b)), 50 mg/l $\text{NO}_2^-$ was completely consumed from the beginning, however, $\text{NH}_4^+$ was remained at 30 mg/l in effluent suggesting endogenous denitrification in this reactor as well as the reactor inoculated with denitrifying sludge. On the 60th day, influent $\text{NO}_2^-$ concentration was adjusted at 65 mg/l to make the ratio between the concentrations of $\text{NO}_2^-$ and $\text{NH}_4^+$ to 1:1.3, which is stoichiometric reaction ratio in anammox reaction. Although a slight consumption of $\text{NH}_4^+$ was temporally observed, both $\text{NO}_2^-$ and $\text{NH}_4^+$ concentrations increased. Therefore, influent $\text{NO}_2^-$ and $\text{NH}_4^+$ were both reduced to 40 mg/l, however, 10–20 mg/l $\text{NO}_2^-$ and 20–30 mg/l $\text{NH}_4^+$ were still remained, which suggests no anammox reaction in this reactor.

![Figure 3](https://iwaponline.com/wst/article-pdf/61/1/119/447821/119.pdf)

Figure 3 | Time course of $\text{NO}_2^-$ and $\text{NH}_4^+$ concentrations in continuous culture inoculated with (a) denitrifying sludge derived from seawater, (b) muddy sediment in tideland, (c) sediment in sea-based waste disposal site.
In the reactor inoculated with the sediment of the sea-based waste disposal site, only NO$_2^-$-N consumption was observed in the beginning of the culture (Figure 3c) as well as in other reactors, however, NH$_4^+$-N concentration decreased on the 35th day. Furthermore, simultaneous removal of NO$_2^-$-N and NH$_4^+$-N of 40 mg/l was observed after 120 days. As anammox reaction was presumed at this point, HRT was gradually reduced from 48 h to 24 h without changing nitrogen concentration, and then the nitrogen concentration was raised again. Finally, the total nitrogen removal efficiency of more than 80% was achieved at nitrogen loading rate (NLR) of 0.16 kg/m$^3$/day on the 330th day. The reaction-ratio of NH$_4^+$-N and NO$_2^-$-N for about 200 days after the 120th day was 1:1.07, which was close to the stoichiometric value of anammox reaction (1:1.32), therefore the enrichment of MAAOB and nitrogen removal by anammox reaction were presumed in this reactor although almost no nitrate was produced. No nitrate production may indicate a concurrent occurrence of denitrification reaction in the reactor. From these results, it was suggested that the choice of appropriate inoculums is important to establish the MAAOB enrichment culture.

Enrichment culture of MAAOB has been succeeded in the medium using natural seawater (Nakajima et al. 2008) or the salts made from natural seawater (van de Vossenberg et al. 2008) so far. No anammox activity or cells could be detected in the medium including artificial seawater inoculated with the sediment of the Gullmar Fjord (van de Vossenberg et al. 2008). In contrast to these reports, we could obtain the enrichment culture of MAAOB in the artificial seawater medium (MAAOB-medium) suggesting the difference between the type of bacteria in our culture and others. The culture temperature in this study was also different from other studies. We considered that 30°C is appropriate for cultivation of MAAOB as because that the anammox biomass derived from freshwater environment were well cultured at more than 30°C even though natural fresh water environment temperature would be less than 30°C. On the other hand, MAAOB cultures were carried out at less than 25°C so far (Nakajima et al. 2008; van de Vossenberg et al. 2008). No serious problem caused by high temperature (50°C) was observed through the experimental period in this study. However, further study should be needed for temperature condition because that temperature might affect on MAAOB growth and anammox activity (van de Vossenberg et al. 2008).

**Bacterial community in the MAAOB-enriched biomass**

PCR was performed using the specific primer of MAAOB detection and bacterial universal primer for the biomass attached to the non-woven fabric in the reactors. The target DNA fragments were amplified from the samples in the reactors inoculated with the waste disposal site sediment, but not in other reactors. DGGE results are shown in Figure 4 with dominant bands identified with a number. Two and 7 dense DNA bands were detected on the DGGE gel applied the PCR product amplified by UKf1-UKr1 and by GM5f-DS907r, respectively. Identity of DNA bands on the DGGE gel was listed in Table 1. The main band (no. 1) was similar to marine anammox planctomycete UKU-1 (accession number: AB433172), which was identified as a marine anammox bacteria in our previous report (Kawagoshi et al. 2009), and most similar to *Candidatus Scalindua wagneri* (Schmid et al. 2003) and. The other DNA band (no. 2) was most similar to Planctomycete GMD15D02, which was identified as a non-anammox bacteria although similarity match was only 78%. Phylogenetic tree constructed with the DNA band of no. 1 and related anammox bacteria is shown in Figure 5. The band no. 2 formed with MAAOB such as JMK-2 and separately from other anammox bacteria, thus it was considered that the band no. 1 bacteria was enriched and contributed to anammox reaction in the reactor.

---

**Figure 4** | DGGE picture conducted using the biomass in the continuous culture inoculated with sea-based waste disposal site sediment.
Among 7 DNA bands on the DGGE gel amplified by bacterial universal primer, four DNA bands (no. 5, 7, 8, 10) showed more than 96% similarity to previously reported bacteria (Table 1). The band no. 5 was identified as the bacteria close to an ammonium oxidizing bacteria, no. 7 was close to denitrifying photosynthetic bacteria, and no. 8 was close to nitrogen fixing bacteria. The reactor scale used in this study was small and dissolved oxygen (DO) concentration was not controlled strictly, thus ammonium oxidizing bacteria may consume a slight DO and make anoxic condition suitable for anammox bacterial growth. Coexistence of denitrifying bacteria is consistent with the results of higher removal ability of nitrate and nitrite than that in theoretical anammox reaction, which may suggest that anammox is coupled with denitrification reaction in the reactor.

**Table 1** List of bacteria presumed as an aligned species by homology comparison of DNA bands in DGGE

<table>
<thead>
<tr>
<th>Primer</th>
<th>Band no.</th>
<th>Closest candidate by homology search</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>UKf1 &amp; UKr1</td>
<td>1</td>
<td><em>Candidatus&quot;Scalindua wagnerii&quot;</em></td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Anammox planctomycete JMK-2</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Planctomycete GMD15D02</td>
<td>78</td>
</tr>
<tr>
<td>GM5f &amp; DS907r</td>
<td>3</td>
<td><em>Flavobacteriaceae</em> bacterium 'BSD RB 42'</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td><em>Lachnospira pectinoschiza</em></td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td><em>Nitrosomonas europaea</em> ATCC 19718</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td><em>Maricaulis maris</em> MCS10</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td><em>Roseobacter denitrificans</em> OCh 114</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td><em>Mesorhizobium</em> sp. BNC1</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Mixed culture isolate koll13</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td><em>Azoarcus</em> sp. EbN1</td>
<td>97</td>
</tr>
</tbody>
</table>

**Figure 5** Phylogenetic tree constructed from partial 16S rRNA sequences of the DNA band-1 and known relatives of anammox bacteria (accession number).
CONCLUSIONS

Establishment of enrichment culture of MAAOB from seawater-related samples was conducted and the following results were obtained.

(1) Simultaneous removal of ammonium and nitrite at the reaction ratio that $\text{NH}_4^+:\text{NO}_2^{-} = 1:1.07$ in the culture inoculated with the sediment of sea-based waste disposal site after 320 days.

(2) Nitrogen removal rate of 0.15 kg/m$^3$/day was achieved at the nitrogen loading rate of 0.16 kg/m$^3$/day, and increasing of MAAOB abundance in the culture was indicated.

(3) The MAAOB close to anammox planctomycete JMK2 and Candidatus Scalindua wagneri were identified in the biomass of the culture and coexistence of ammonium oxidizing bacteria and denitrifying bacteria was suggested by DGGE analysis.

(4) No symptom of MAAOB enrichment was observed in the culture inoculated with denitrifying sludge derived from seawater and muddy tideland sediment, indicating that how to choose the appropriate inoculums is quite important for establishment of MAAOB enrichment culture.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Grand-in-Aid for Scientific Research (no. 20560508) from the Japan Society for the Promotion of Science for managing this study.

REFERENCES


