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Quantification of anaerobic ammonium-oxidizing bacteria in enrichment cultures by real-time PCR

Running title: Quantification of ANAMMOX bacteria by real-time PCR

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Abstract

The anaerobic ammonium-oxidizing (ANAMMOX) bacteria were enriched from a rotating disk reactor (RDR) biofilm in semi batch cultures. Based on fluorescence *in situ* hybridization (FISH) analysis, this enrichment led to a relative population size of 36% ANAMMOX bacteria. Phylogenetic analysis revealed that all the detected clones were related to the previously reported ANAMMOX bacteria, Candidatus *Brocadia anammoxidans* (AF375994), with 92% sequence similarity. Furthermore, we successfully developed a real-time polymerase chain reaction (PCR) assay to quantify populations of ANAMMOX bacteria in the enrichment cultures. For this real-time PCR assay, PCR primer sets targeting 16S ribosomal RNA genes of ANAMMOX bacteria were designed and used. The quantification range of this assay was 6 orders of magnitude, from 8.9×10^1 to 8.9×10^6 copies per PCR, corresponding to the detection limit of 3.6×10^3 target copies mL^{-1}. A significant correlation was found between the increase in copy numbers of 16S rRNA gene of ANAMMOX bacteria and the increase in nitrogen removal rates in the enrichment cultures. Quantifying ANAMMOX bacterial populations in the enrichment culture made it possible to estimate the doubling time of the enriched ANAMMOX bacteria to be 3.6 to 5.4 days. The real-time PCR assay gave comparable population sizes in the enrichment cultures with the FISH results. These results suggest that the real-time PCR assay developed in this study is useful and reliable for quantifying the populations of ANAMMOX bacteria in environmental and engineering samples.

Keywords; Anaerobic ammonium oxidation (ANAMMOX), 16S rRNA approach, real-time PCR.

1. Introduction

The anaerobic ammonium oxidation (ANAMMOX) process, in which ammonium and nitrite are directly oxidized to nitrogen gas under anoxic conditions, is emerging as one of the efficient and cost-effective alternative to conventional nitrogen removal processes from ammonia rich wastewater (Mulder *et al*., 1995; Jetten *et al*., 1999). The advantages of the anammox process over the conventional combination of nitrification and denitrification processes are lower oxygen demand and no requirement for external carbon sources. However, the ANAMMOX process has been recognized as being difficult to apply for practical wastewater treatments (Abma et al., 2005). A major obstacle common to application of the ANAMMOX process is the requirement of a long start-up period due to mainly slow growth rates of ANAMMOX bacteria (the doubling time was reported to beapproximately 11 days) (Strous *et al*., 1998). Additionally, since ANAMMOX bacteria are strictly anaerobes and autotrophs, they are difficult to be cultured. Thus, they have not been isolated in pure culture yet. Therefore, a better understanding of the physiology and kinetics of ANAMMOX bacteria is obvious of paramount importance in implementing the ANAMMOX process as a manageable wastewater treatment technology.

Quantification of ANAMMOX bacteria has been attempted by using the fluorescence *in situ* hybridization (FISH) method (Isaka *et al*., 2006). FISH is, however, time consuming and sometimes difficult to use in complex microbial samples due to the formation of dense microbial clusters (Third *et al*., 2001) and low contents of rRNA molecules per cell. Real-time polymerase chain reaction (PCR) has recently been applied and optimized to quantify nitrifying bacteria that usually form dense clusters in biofilms (Kindaichi *et al*., 2006) and river sediments (Nakamura *et al*., 2006). The real-time PCR is based on continuous monitoring of fluorescence intensity throughout

the PCR reaction. Therefore, this assay is a fast, reliable, sensitive and convenient method to enumerate uncultured, slow-growing and cluster-forming ANAMMOX bacteria. However, quantification of ANAMMOX bacteria by real-time PCR assay has not been reported to date. Reliable quantification method is essential to determine kinetic parameters (e.g., growth rate) of slow-growing ANAMMOX bacteria.

The primarily goal of this study is therefore to develop a real-time PCR assay to quantify the copy numbers of 16S rRNA gene of ANAMMOX bacteria. To achieve this goal, we first enriched ANAMMOX bacteria from biomass in a fully submerged rotating disk reactor (RDR) by semi batch cultures and designed new specific real-time PCR primer sets for hitherto-reported ANAMMOX bacteria including clones retrieved from the enrichment culture. Thereafter, we applied the real-time PCR assay to evaluate its feasibility and to determine populations of the enriched ANAMMOX bacteria.

2. Material and Methods

2.1. Semi batch enrichment cultures

Enrichment cultures were carried out in 500-mL Erlenmeyer flasks containing a synthetic nutrient medium for ANAMMOX bacteria (see below) in the dark at 37˚C under anoxic condition. Twenty-five mL of the seeding sludge (approximately 50 mg [dry weight] per L) was taken from a lab-scale fully submerged rotating disc reactor (RDR) that exhibited ca. 25% of total nitrogen loss for over 1 year and inoculated into the batch cultures. The synthetic nutrient medium consisted of $(NH_4)_2SO_4$ (1.0-4.6 mM), $NaNO_2$ (1.0-4.6), $KHCO_3$ (5.0), KH_2PO_4 (0.2), $MgSO_4 \cdot 7H_2O$ (1.2), $CaCl_2 \cdot 2H_2O$ (1.4), and 1 mL of trace element solution I and II (van de Graaf *et al*., 1996). Na_2SO_3 (0.01) was used to remove a trace amount of dissolved oxygen from the medium. Anaerobic condition was obtained by vacuum evacuation and backfilling with high purity argon

gas for several times. The pressure of the headspace was maintained slightly above atmospheric pressure. The Erlenmeyer flasks were then sealed with butyl rubber stopper with sampling ports. The pH was set at 7.5, measured and adjusted with sulfuric acid regularly. Variable amounts of ammonium and nitrite were added into the cultures with syringes, whenever substrates were almost exhausted. The medium was exchanged with the fresh one when ANAMMOX activity dropped.

2.2. Trace study with ^{15}N nitrite

To confirm occurrence of ANAMMOX reaction in the batch cultures, the trace studies with [^{15}N] $NaNO_2$ were carried out in 100-mL serum bottles, which were sealed with butyl rubber stopper and aluminum seal to prevent O_2 contamination. The biomass taken from the RDR were inoculated to the serum bottles containing the same medium and incubated as mentioned above. Gas samples of 0.1 mL were taken from the headspace by a hypodermic syringe after vigorous shaking. Different N_2 gases ($^{14-14}N_2$, $^{14-15}N_2$ and $^{15-15}N_2$) were analyzed with a gas chromatograph and mass spectrometer (5972A, Hewlett-Packard, CA., USA) equipped with a thermal conductivity detector (TCD).

2.3. Analytical procedure

The concentrations of NH_4^+, NO_2^- and NO_3^- were determined by ion-exchange chromatography using a DX-100 (DIONEX, CA., USA) with an IonPac CS3 cation column and IonPac AS9 anion column after filtration with 0.2-μm pore size membranes (ADVANTEC, Tokyo, Japan).

2.4. DNA extraction and PCR amplification

Total DNA was extracted from enrichment culture samples (approximately 0.2 mL) with Fast DNA spin kit (Bio 101, Qbiogene Inc., CA., USA), as described in the manufacturer's instructions. 16S rRNA gene fragments from the extracted total DNA were amplified with *Taq* DNA polymerase (TaKaRa Bio Inc., Ohtsu, Japan) with the *Planctomycetales* specific primer set Pla46-1387R (Schmid *et al*., 2001) and *Eubacteria* targeted primer set Bac11F-1387R (Weisberg *et al*. 1991) as shown in **Table 1**. The PCR products were electrophoresed on a 1% (w/v) agarose gel.

2.5. Cloning and sequencing of the 16S rRNA gene 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; Invitrogen, CA., USA). Almost full sequencing of the 16S rRNA gene inserts (about 1,350 bp) was performed by an automatic sequencer (ABI Prism 3100 Avant Genetic Analyzer; Applied Biosystems, CA., USA) with a BigDye terminator Ready Reaction kit (Applied Biosystems). All sequences were checked for chimeric artifacts by the CHECK_CHIMERA program in the Ribosomal Database Project (Maidak *et al*., 1997) and compared with similar sequences of the reference organisms by a BLAST search (Altschul *et al*., 1990). Sequence data were aligned with the CLUSTAL W package (Thompson *et al*., 1994). Clones with more than 97% sequence similarity were grouped into the same operational taxonomic unit (OTU), and their representative sequences were used for phylogenetic analysis. Phylogenetic trees were constructed with the neighbor-joining method (Saito and Nei 1987). Tree topology was also tested using the maximum-parsimony method. Bootstrap resampling analysis for 100 replicates was performed to estimate the confidence of the tree topologies.

2.6. Sample fixation and fluorescence in situ hybridization

Biomass samples taken from the enrichment cultures were fixed in 4% paraformaldehyde solution, resuspended in a 1:1 mixture of phosphate-buffered saline (PBS) and absolute ethanol, and stored at -20˚C as previously described by Okabe *et al*. (1999). *In situ* hybridization was carried out as described previously (Amann *et al*., 1990). The 16S rRNA-targeted oligonucleotide probes used in this study are listed in **Table 2**. The oligonucleotide probes were labeled with either tetramethylrhodamine-5-isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) at the 5' end (TaKaRa, Ohtsu, Japan). Samples hybridized with probes were mounted with the Slow Fade Light antifading kit (Molecular Probes, OR., USA). All images were recorded with an LSM 510 confocal laser scanning microscope (Carl Zeiss, Inc., Germany) equipped with an Ar laser (488 nm) and a HeNe laser (543 nm). All images were combined, processed and analyzed with the standard software package provided with the LSM510. The numbers of probe AMX820-hybridized ANAMMOX bacterial cells (cells cm^{-2}) were determined by multiplying the total 4'6'-dimidino-2-phenylindole (DAPI)-stained cells and the ratios of total probe-hybridized cells to total DAPI-stained cells as described previously (Savichtcheva *et al.*, 2005). This measurement was performed in duplicate and then the average cell numbers of the probe AMX820-hybridized ANAMMOX bacteria were reported in this study. Total DAPI-stained cells were enumerated by the direct-counting method of Hobbie *et al*. (1977) after staining DAPI. The ratios of total probe AMX820-hybridized ANAMMOX cells to total DAPI-stained cells were determined by direct counting at least 20 randomly chosen microscopic fields for each sample, which corresponded to 600 to 900 DAPI-stained cells.

2.7. Primer design for real-time PCR

Two new specific primer sets of AMX809F-AMX1066R and AMX818F-AMX1066R for 16S rRNA genes of hitherto-reported ANAMMOX bacteria including the clones obtained from the semi batch enrichment cultures were designed in this study using the ARB software package (Ludwig *et al*., 2004.) and the Primer Express software package provided by Applied Biosystems (Foster city, CA., USA). The specificity of the primers was checked and confirmed using PROBE_MATCH of the ARB program. In addition, to test the specificity of the primer sets, real-time PCR amplifications with the newly designed primers were performed with plasmid DNAs that carry target and closely related but non-target clone sequences, which were obtained during the cloning analysis of the RDR biomass (**Table 3**). The optimal conditions were experimentally adjusted.

2.8. Real-time PCR

Copy numbers of 16S rRNA gene of ANAMMOX bacteria in the semi batch cultures were quantified with the real-time PCR assay. SYBR Green real-time PCR assay was conducted using the specific real-time PCR primer sets designed in this study. Each PCR mixture (25 μL) was composed of 12.5 μL of 1× SYBR Green PCR master mix (Applied Biosystems), 300 nM of each forward and reverse primers, 100 μg mL^{-1} of BSA (Sigma, Germany) and either 2.5 μL of template DNA or 10^1 to 10^8 copies per well of the standard vector plasmid carrying ca. 1,350 bp of 16S rRNA gene of the clone related to Candidatus *Brocadia anammoxidans* (AF375994). PCR amplification and detection were performed in MicroAmp Optical 96-well reaction plates with optical cap (Applied Biosystems). The template DNA in the reaction mixtures was amplified and monitored with an ABI prism 7000 Sequence Detection System (Applied Biosystems). The PCR temperature program was initiated with 2 min at 50˚C and 10 min at 94˚C,

followed by 40 cycles of 15 sec at 94˚C and 1 min at 60˚C. A melting curve analysis for SYBR Green assay was done after amplification to distinguish the targeted PCR product from the non-targeted PCR product.

The mass per copy of plasmid DNA was estimated to be 2.8×10^{-19} g-DNA per copy, according to the calculation previously reported (Labrenz *et al*., 2004) and the assumption that average molecular weight of one base pair is 330 Da.

2.9. Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the BG OTU1 used for the phylogenetic tree analysis is AB264123.

3. Results and Discussion

3.1. Enrichment of ANAMMOX bacteria

The concentrations of nitrogen compounds were monitored in the enrichment batch cultures. A typical result is shown in **Fig. 1**. From the beginning of the cultivation, ANAMMOX reaction (simultaneous decreases in NH_4^+ and NO_2^- with slightly increase in NO_3^-) was observed and the nitrogen removal rate was 3.0 μmol L^{-1} h^{-1} on the day 19 (data not shown). The medium was exchanged with the fresh one a few times per month when ANAMMOX activity dropped. The nitrogen removal rate gradually increased and finally attained 292 μmol L^{-1} h^{-1} on the day 410 as shown in **Fig. 1**. The nitrogen stoichiometric ratio was 1:1.38:0.29 for conversion of NH_4^+ and NO_2^- to the production of NO_3^- between days 384 and 410. This stoichiometric ratio was similar to the previously reported ratio of 1:1.32:0.26 (Strous *et al*., 1998), suggesting occurrence of ANAMMOX reaction in the culture. The color of the biomass gradually changed from dark brown to bright pink during cultivation.

In this study, the efficient enrichment of ANAMMOX bacteria could be successfully achieved by inoculating the biomass taken from the lab-scale fully submerged RDR that exhibited a moderate total nitrogen loss (ca. 25%) and exchanging the medium regularly. We often observed that the activity of ANAMMOX bacteria dropped and the concentration of total organic carbon (TOC) increased when the medium was not exchanged for 2 months (data not shown). In addition, it is important to inoculate small amounts of appropriate seed sludge to batch cultures for successful enrichment of ANAMMOX bacteria as described in other studies (Egli *et al*., 2001; Toh *et al*., 2002). This is probably because introduction of organic or toxic compounds contained in the initial seed sludge and accumulation of by-products during cultivation inhibits the activity of ANAMMOX bacteria (van de Graaf *et al*., 1996; Jetten *et al*., 1999).

3.2. Trace study with ^{15}N nitrite

Before $^{15}NO_2^-$ addition, the ratios of $^{14\text{-}14}N_2$ (*m/z* 28), $^{14\text{-}15}N_2$ (*m/z* 29), and $^{15\text{-}15}N_2$ (*m/z* 30) in the headspace of 100-mL serum bottles were measured to be 98.6, 1.2 and 0.2%, respectively. This ratio changed to 42.8, 54.4, and 2.8%, respectively, after 150-day incubation with $^{15}NO_2^-$ (data not shown). A significant increase of $^{14\text{-}15}N_2$ (*m/z* 29) indicated that nitrogen losses observed in the batch cultures were mainly due to ANAMMOX reaction since $^{14\text{-}15}N_2$ was produced only via ANAMMOX reaction. Furthermore, only a small amount (2.8%) of $^{15\text{-}15}N_2$ (*m/z* 30) was produced, which suggested that ordinary denitrification hardly occurred.

3.3. Phylogenetic analysis

After the repetitive ANAMMOX reaction was observed in the semi batch enrichment culture, the 16S rRNA gene clone library was constructed from the biomass taken at day

390 with the primer set of Pla46 and 1387R (**Fig. 2**). All sixteen clones were grouped into only one OTU (BG OTU 1), on the basis of having more than 97% sequence similarity within an OTU. The BG OTU 1 was related to Candidatus *Brocadia anammoxidans* (AF375994) with 92% sequence similarity and Planctomycete KSU-1 gene (AB057453) that was obtained from a continuous up-flow column reactor filled with nonwoven carriers (Fujii *et al*., 2002) with 95% sequence similarity. The low sequence similarity between these clones and previously reported ANAMMOX bacteria indicated that our ANAMMOX bacteria represented by the clones were most likely classified novel species of ANAMMOX bacteria.

To analyze whole microbial community in the enrichment culture, the other clone library was also constructed with the primer set Bac11F and 1387R. In total, 44 clones were analyzed. No clones that are belonging to *Planctomycetales* were detected. Twelve clones out of forty-four (the detection frequency of 27%) were affiliated with *Betaproteobacteria* such as *Acidovorax* sp. and *Dechloromonas* sp. (data not shown). This result indicated that ANAMMOX bacteria and other heterotrophic bacteria coexisted in this enrichment culture. The detail ecophysiological roles and functions of these coexisting heterotrophic bacteria in the ANAMMOX cultures are not clear at present.

3.4. Fluorescence in situ hybridization

Probe Pla46- and AMX820-hybridized ANAMMOX bacteria were below the detection limit of FISH analysis (less than 10^5 cell mL^{-1}, Amann *et al*., 1995) until the day 350, even thought ANAMMOX reaction was observed. The ANAMMOX bacteria were first detected with PLA46 and AMX820 probes and enumerated on day 380. All bacterial cells hybridized with AMX820 probe were approximately 1-μm diameter

doughnut-shaped cell as reported by Schmid *et al*. (2001) (**Fig. 3**). The abundances of ANAMMOX bacteria were 1.2 (± 0.4) × 10^6 and 3.3 (± 0.6) ×10^7 (±SD) cell mL^{-1} on days 380 and 410, accounting for 2% and 36% of total DAPI-stained cells, respectively. The nitrogen removal rates on days 380 and 410 were approximately 0.4 and 7.0 mol-N m^{-3} day^{-1}, respectively. No aerobic ammonium-oxidizing bacteria belonging to *Betaproteobacteria* and nitrite-oxidizing bacteria (e.g., the genus *Nitrobacter* and *Nitrospira*) were detected with the probe NSO190, NIT3 and Ntspa662 in this batch culture.

Isaka *et al*. (2006) have recently reported that the cell concentration of ANAMMOX bacteria was 4.3×10^7 cells mL^{-1} when the nitrogen removal rate was 66 mol-N m^{-3} day^{-1} in a continuous anaerobic biological filtered (ABF) reactor. Thus, the specific nitrogen removal rate was calculated to be 1.5×10^{-12} mol-N $cell^{-1}$ day^{-1}. In this study, the specific nitrogen removal rates were calculated to be 1.2×10^{-13} and 8.6×10^{-14} mol-N $cell^{-1}$ day^{-1}, which were about 6 - 18 times lower than that of the value reported by Isaka *et al*. (2006). This is probably because they used the continuous reactor in which the nitrogen loading rate was maintained high, whereas we used the semi batch culture in which substrate exhaustion and accumulation of unknown by-products occurred during the cultivation, which obviously reduces the ANAMMOX activity.

3.5. Quantification of ANAMMOX bacteria by real-time PCR

Based on 16S rRNA gene sequences obtained from the enrichment culture, real-time PCR primer sets (AMX809F-AMX1066R and AMX818F-AMX1066R) were designed for Candidatus *Brocadia anammoxidans*, Candidatus *Kuenenia stuttgartiensi*s and the BG OTU 1 (**Table 1**). The specificity of the primers was checked against the ARB database. The AMX809F, AMX818F, and AMX1066R had no mismatches to 9.6%,

8.0%, and 9.0% of 188 16S rRNA gene sequences presented in the order *Planctomycetales* and no mismatches to <0.1%, <0.1% and <0.1% of 24,123 16S rRNA gene sequences belonging to the order other than *Planctomycetales*, respectively. These results indicate that the primers had a high specificity to 16S rRNA gene sequences belonging to previously reported ANAMMOX bacteria groups.

The specificities of the primer combination of AMX809F-AMX1066R and AMX818F-AMX1066R were experimentally evaluated by real-time PCR using plasmid DNAs of clones previously obtained from the RDR biomass (**Table 3**). Real-time PCR analysis for target clones (no mismatch with both primer sets) resulted in detection of ca. 9.0×10^6 target copies per PCR. The real-time PCR detected 1 to 2 orders of magnitude lower copy numbers ($10^4 – 10^5$ target copies per PCR) when the clones contained 1 or 2 mismatches. With more than 2 mismatches, no significant amplification was observed for all clones (less than 10^3 copies per PCR).

The specificity of the primer sets was also checked by clone sequence analysis after PCR amplification of 16S rRNA genes with these primer sets. All clone sequences obtained from PCR amplification with these primer sets were closely related to BG OTU 1 with more than 98% sequence similarity, and clone sequences belonging to other than ANAMMOX bacteria were not detected at all with these primer sets (data not shown).

The standard curves for ANAMMOX bacteria were constructed from a series of 10-fold dilutions of a plasmid DNA carrying a 16S rRNA gene of the BG OTU 1 ranging from 2.5×10^{-8} to 2.5×10^{-3} ng of DNA per well for each primer set (**Fig. 4**). The consistency of the real-time PCR assay with these primers was confirmed by the strong linear inverse relationship between the threshold cycle numbers and the copy numbers of 16S rRNA gene of ANAMMOX bacteria for both primer sets ($R^2 = 0.99$). The

amplification efficiencies were more than 96% (the slopes were -3.42 and -3.39, respectively) (**Fig. 4**). The linear range of quantification for real-time PCR assays for both primer sets was 6 orders of magnitude, from 8.9×10^1 to 8.9×10^6 copies per PCR, and thus the detection limit for this assay was 8.9×10^1 target copies (corresponding to 3.6×10^3 target copies mL^{-1}) (**Fig. 4**). Melting curve analysis consistently showed only one observable peak at a melting temperature (T_m=83.4). No detectable peaks that were associated with primer-dimer artifacts or other non-specific PCR amplification products were observed.

The copy numbers of 16S rRNA gene of ANAMMOX bacteria in the enrichment batch cultures were quantified and plotted against the nitrogen removal rates (**Fig. 5**). There was a liner relationship between them (R^2 = 0.81). Based on this result, nitrogen removal rate per copy was calculated to be 7.2×10^{-14} mol-N $copy^{-1}$ day^{-1}. This value is in the same range (2.4×10^{-13} to 8.6×10^{-14} mol-N $cell^{-1}$ day^{-1}) that was determined based on the FISH counts. These results imply that the quantification of copy numbers of ANAMMOX bacterial 16S rRNA gene by the real-time PCR was reliable.

The copy numbers of 16S rRNA gene of ANAMMOX bacteria in the enrichment batch culture were 5.5 (± 1.1) $\times 10^3$, 1.7 (± 0.1) $\times 10^6$ and 8.2 (± 0.2) $\times 10^7$ copies mL^{-1} on the days 350, 380, and 410, respectively (**Fig. 6**). This indicated that ANAMMOX bacteria increased about 300 times from the day 350 to day 380 and 50 times from the day 380 to day 410, respectively. Based on these results, the doubling time of ANAMMOX bacteria was estimated to be 3.6 days for the former 30 days and 5.4 days for the latter 30 days (average doubling time was 4.3 day). The slower growth in the late stage was probably due to accumulation of by-products or frequent exhaustions of the substrates since the ANAMMOX activity became high. The doubling time estimated in this study was between the values reported previously. Strous *et al*. (1998) reported that

the doubling time was 11 days, which was calculated from biomass yield and nitrogen removal rate. In contrast, Isaka *et al*. (2006) recently reported the much shorter doubling time of 1.8 days for ANAMMOX bacteria growing in a continuous bioreactor by using FISH direct counting method. However, they did not characterize the phylogenetic identification of the ANAMMOX bacteria. It is likely that the doubling time of ANAMMOX bacteria differs from species to species and under different environments; further research is required to understand more details of growth kinetics of ANAMMOX bacteria in complex microbial communities.

4. Conclusions

We obtained the enrichment culture of ANAMMOX bacteria from a fully submerged RDR biofilm and confirmed the repetitive ANAMMOX reaction. 16S rRNA clone analysis revealed that all clones obtained in the enrichment culture were related to Candidatus *Brocadia anammoxidans* with 92% sequence similarity, indicating that our enriched ANAMMOX bacteria were most likely novel ANAMMOX species. In addition, we successfully developed the real-time PCR assay for quantification of 16S rRNA gene of ANAMMOX bacteria with the newly designed primer sets. Using this real-time PCR assay, the doubling time of the ANAMMOX bacteria enriched in this study was estimated to be 3.6 to 5.4 days. The real-time PCR assay is a useful tool to quantify very slow-growing ANAMMOX bacteria in various environmental and engineering samples. This assay will be used to screen appropriate seed sludges with high abundance of ANAMMOX bacteria for rapid and efficient start-up of ANAMMOX bioreactors in the future.

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Table 1. A list of 16S rRNA targeted-oligonucleotide primers used in this study.

	Specificity	Sequence (5'-3')	Target site[a]	References
AMX809F	Anammox bacteria[b]	GCCGTAAACGATGGGCACT	809-826	This study
AMX818F	Anammox bacteria[b]	ATGGGCACTMRGTAGAGGGGTTT	818-839	This study
AMX1066R	Anammox bacteria[b]	AACGTCTCACGACACGAGCTG	1047-1066	This study
Pla46	*Planctomycetales*	GACTTGCATGCCTAATCC	46-63	Neef *et al*. 1998
Bac11F	Bacteria	GTTTGATCCTGGCTCAG	11-37	Weisberg *et al*. 1991
1387R	Bacteria	GGGCGGWGTGTACAAGGC	1387-1403	Weisberg *et al*. 1991

[a]16S rRNA position according to *Escherichia coli* numbering

[b]Candidatus *Brocadia anammoxidans*, Candidatus *Kuenenia stuttgartiensis* and the clones (BG OUT 1) obtained in this study.

Table 2. A list of 16S rRNA targeted-oligonucleotide probes used in this study.

Probe	Specificity	Probe sequence (5' to 3')	Target site[a]	FA (%)[b]	Reference
EUB338	Most Bacteria	GCTGCCTCCCGTAGGAGT	338 - 355	–[c]	Amann *et al*. 1990
EUB338 III	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	338 - 355	–[c]	Daims *et al*. 1999
PLA46	*Planctomycetales*	GACTTGCATGCCTAATCC	46 - 63	30	Neef *et al*. 1998
AMX820	ANAMMOX bacteria[d]	AAAACCCCTCTACTTAGT	820 - 841	40	Schmid *et al*. 2001
NSO190	Betaproteobacterial ammonia-oxidizing bacteria	CGATCCCCTGCTTTTCTC C	189 - 207	55	Mobarry *et al*. 1996
NIT3	*Nitrobacter* spp.	CCTGTGCTCCATGCTCCG	1035 - 1052	55	Wagner *et al*. 1996
CNIT3	Competitor for NIT3	CCTGTGCTCCAGGCTCCG			Wagner *et al*. 1996
Ntspa662	genus *Nitrospira*	GGAATTCCGCGCTCCTCT	662 - 679	53	Daims *et al*. 2001
CNtspa662	Competitor for Ntspa662	GGAATTCCGCTCTCCTCT			Daims *et al*. 2001

[a] 16S rRNA position occording to *Escherichia coli* numbering.

[b] Formamide concentration in the washing buffer.

[c] Usable at any formamide concentrations.

[d] Candidatus *Brocadia anammoxidans, Kuenenia stuttgartiensis* and the clones (BG OUT 1) obtained in this study.

Table 3. Specificity test for AMX809F, AMX818F and AMX1066R primers by real-time PCR.

Plasmid DNA	the closest strain	Simirality (%)	NM[a]/AMX809F	NM[b]/AMX818F	NM[c]/1066R	Target copies/ AMX809F-1066R	Target copies/ AMX818F-1066R
BG OTU 1	Candidatus *Brocadia anammoxidans*	92	0	0	0	9.0E+06	8.9E+06
RDR1[e]	Candidatus *Brocadia anammoxidans*	94	1	3	1	2.1E+05	2.0E+04
RDR15	Candidatus *Brocadia anammoxidans*	90	2	1	1	2.4E+04	1.5E+05
RDR16	Candidatus *Brocadia anammoxidans*	90	2	1	2	3.3E+04	9.6E+04
RDR18	Candidatus *Brocadia anammoxidans*	95	3	1	2	9.0E+03	2.0E+05
RDR20	Candidatus *Brocadia anammoxidans*	94	3	3	5	ND[d]	ND
Negative control 1 (AY538107)	*Bacteroides fragilis*	90	9	7	10	ND	ND
Negative control 2	*Bacteroides fragilis* (X8393)	100	6	9	12	ND	ND

[a] NM: Numbers of mismatch with sequences of primer, AMX809F

[b] NM: Numbers of mismatch with sequences of primer, AMX818F

[c] NM: Numbers of mismatch with sequences of primer, AMX1066R

[d] ND: Not detected

[e]RDR 1, 15, 16, 18, and 20: these clones were obtained from the RDR biofilm previously.

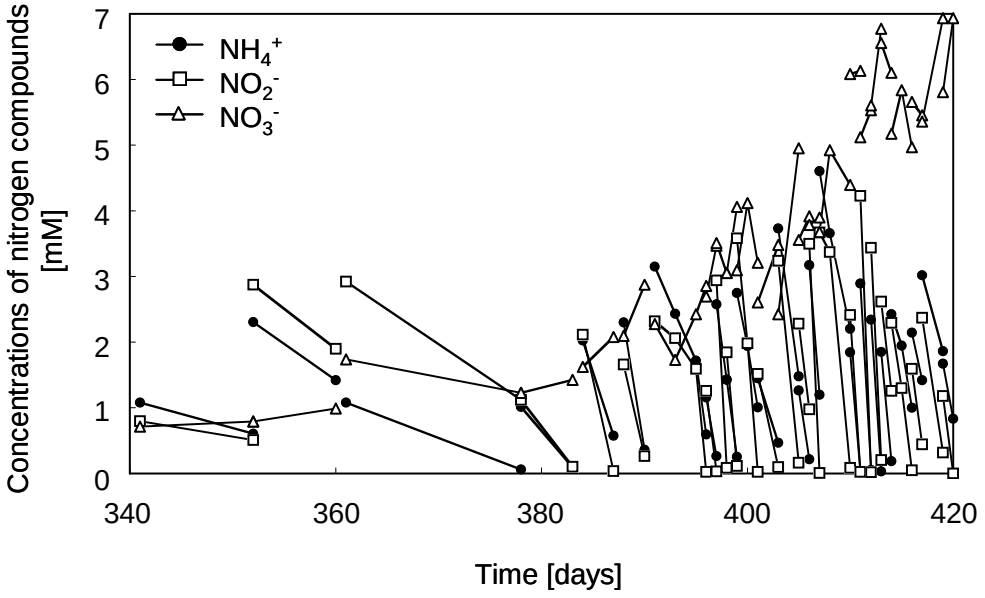

Figure 1. Time course of soluble nitrogen compounds of ammonium, nitrite, and nitrate in the semi batch enrichment culture between the days 340 and 420.

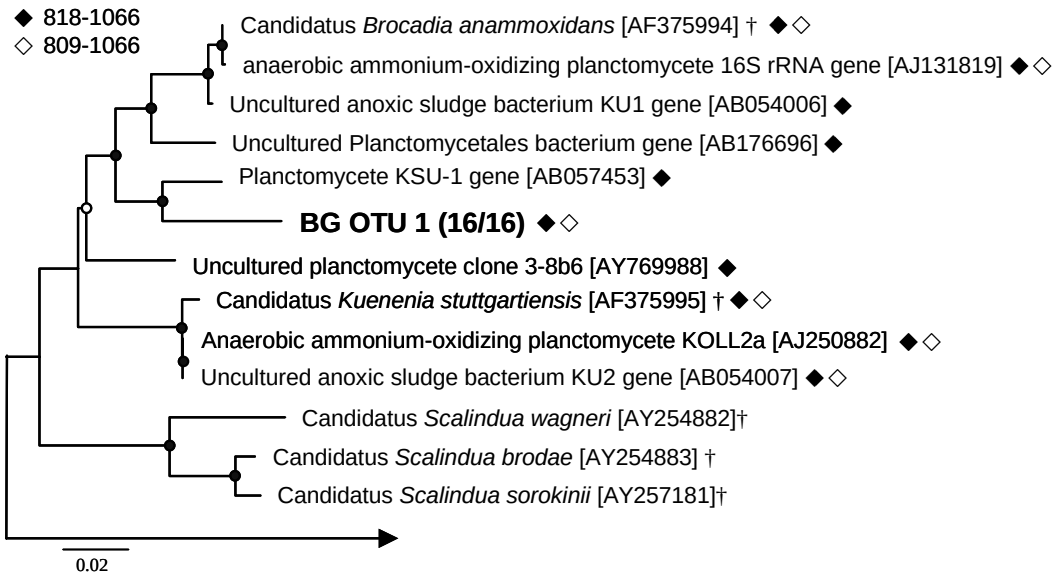

Figure 2. Phylogenetic tree of the clones obtained from the semi batch enrichment culture, based on neighbor-joining analysis for almost full length of 16S rRNA gene sequences. PCR amplification of 16S rRNA genes was conducted with the primer set of Pla46 and 1387R. Bootstrap values > 95 are indicated • and the values < 95 are indicated ○ at the nodes, respectively. The numbers in parentheses indicate the detection frequencies of identical clones in total clones analyzed. The bar represents 2% estimated sequence divergence. BG OTU 1 represents the clones retrieved from the biomass in the semi batch enrichment culture experiment on the day 390. † indicates the previously reported ANAMMOX bacteria. ◇ and ◆ indicate the clone sequences that are completely matched with those of the real-time PCR primer sets of AMX809F-AMX1066R (◇) and AMX818F-AMX1066R (◆), respectively.

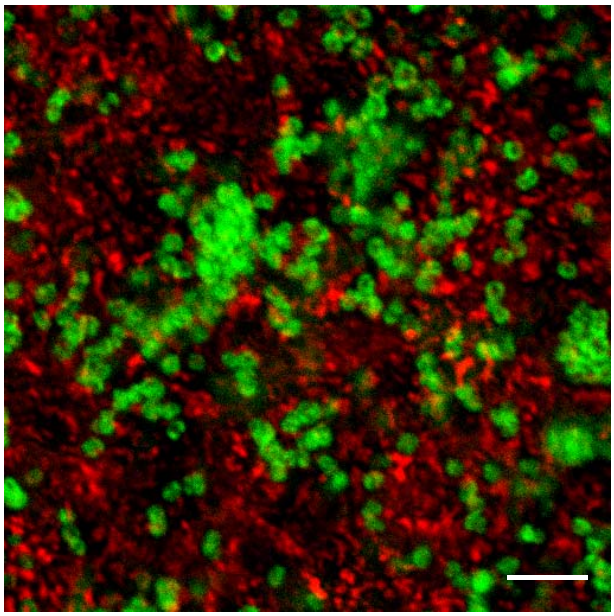

Figure 3. CSLM projection image after simultaneous *in situ* hybridization of the enriched biomass with TRITC-labeled AMX820 and FITC-labeled EUB338mix (EUB338 + EUB338III). The bar represents 5 µm.

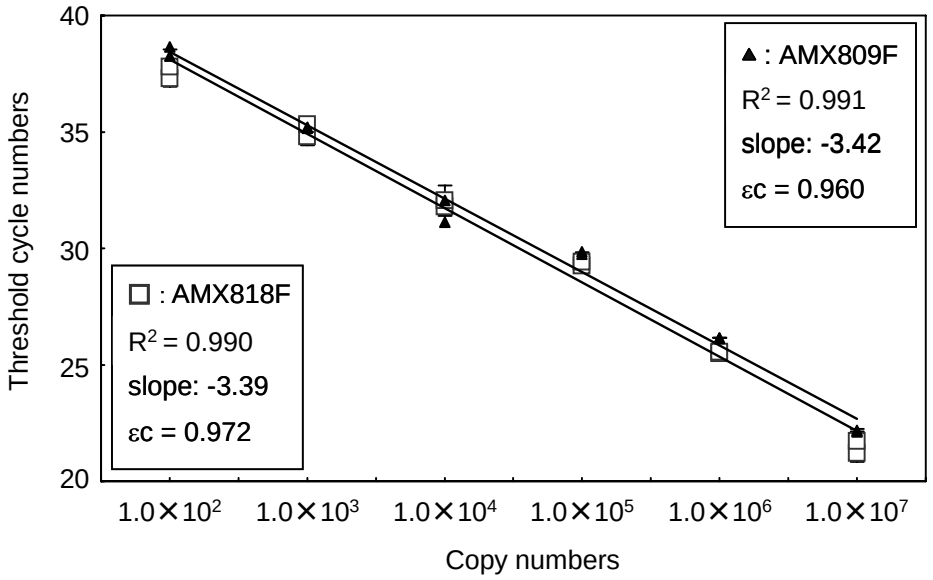

Figure 4. The standard curves were generated from serial 10-fold dilutions (from 0.9×10^7 copies to 0.9×10^2 copies) of plasmid DNA carrying 16S rRNA gene of BG OUT 1 for the primer sets of AMX809F-AMX1066R and AMX818F-AMX1066R, respectively. The curves were obtained from duplicate reactions. Error bars indicate the standard deviation of duplicate reactions. s indicates the slope. Amplification efficiency was calculated from the equation, $\varepsilon c = 10^{(1/s)} - 1$.

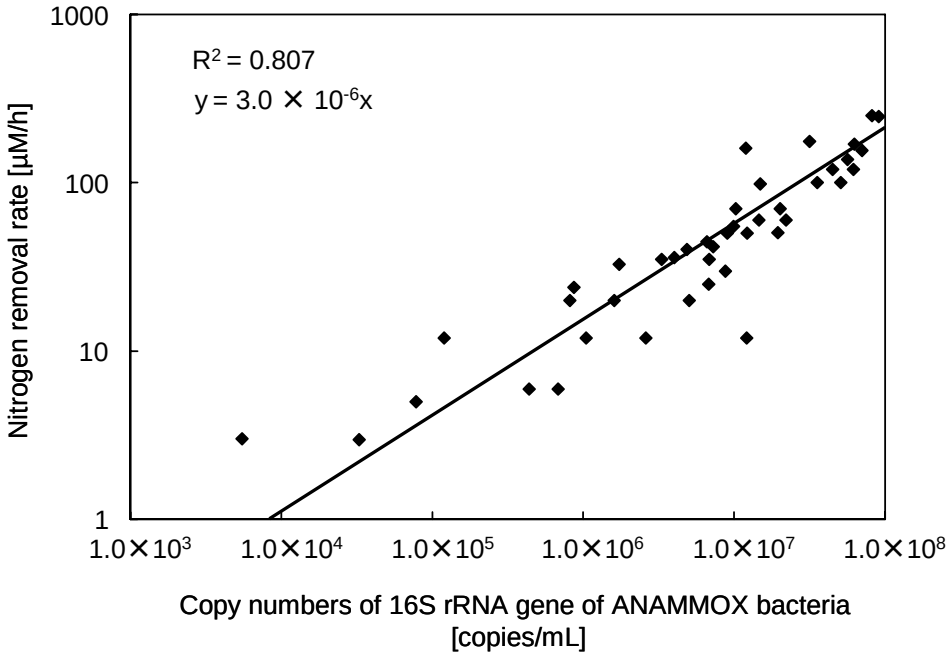

Figure 5. A relationship between nitrogen removal rates and copy numbers of 16S rRNA gene of ANAMMOX bacteria quantified by real-time PCR assay in the enrichment batch cultures.

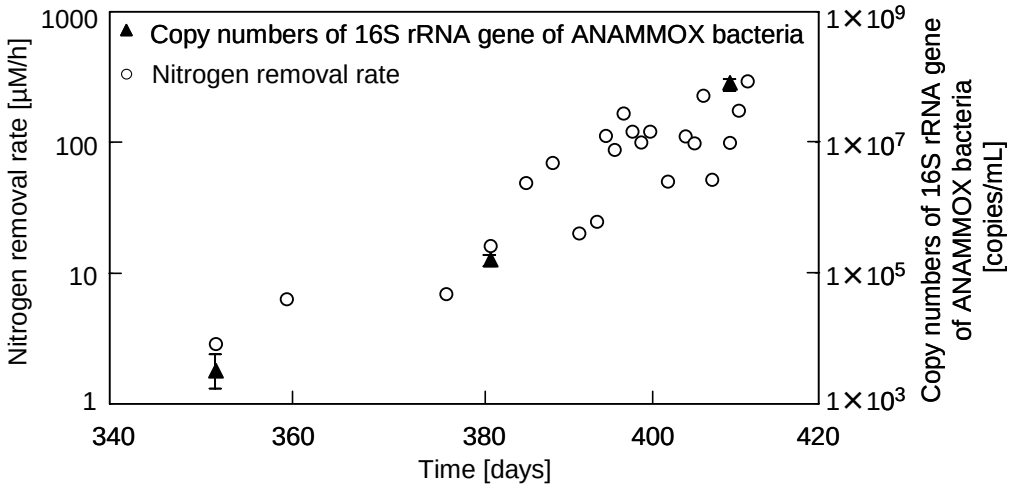

Figure 6. Time course of nitrogen removal rates (○) and the copy numbers of 16S rRNA gene of ANAMMOX bacteria (▲) in the enrichment batch culture determined by real-time PCR.