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아나목스 농후배양에서 암모니아 산화균의 자생 특성

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Presence and Growth of Ammonia-oxidizing Bacteria in Anaerobic Ammonium Oxidation Enrichment

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Abstract

Anaerobic ammonium oxidation (AMX) is a cost-efficient biological nitrogen removal process. The coexistence of ammonia-oxidizing bacteria (AOB) in an AMX reactor is an interesting research topic as a nitrogen-related bacterial consortium. In this study, a sequencing batch reactor for AMX (AMX-SBR) was operated with a conventional activated sludge. The AOB in an AMX bioreactor were identified and quantified using terminal restriction fragment length polymorphism (T-RFLP) and real-time qPCR. A T-RFLP assay based on the ammonia monooxygenase subunit A (amoA) gene sequences showed the presence of *Nitrosomonas europaea*-like AOB in the AMX-SBR. A phylogenetic tree based on the sequenced amoA gene showed that AOB were affiliated with the *Nitrosomonas europaea/mobilis* cluster. Throughout the enrichment period, the AOB population was stable with predominant *Nitrosomonas europaea*-like AOB. Two OTUs of amoA_SBR_JJY_20 (FJ577843) and amoA_SBR_JJY_9 (FJ577849) are similar to the clones from AMX-related environments. Real-time qPCR was used to quantify AOB populations over time. Interestingly, the exponential growth of AOB populations was observed during the substrate inhibition of the AMX bacteria. The specific growth rate of AOB under anaerobic conditions was only 0.111 d^{-1}. The growth property of *Nitrosomonas europaea*-like AOB may provide fundamental information about the metabolic relationship between the AMX bacteria and AOB.

Key words : Ammonia-oxidizing bacteria, Anaerobic ammonium oxidation, Terminal restriction fragment length polymorphism, Sequencing, Real-time qPCR

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1. Introduction

Complete removal of nitrogen is extremely important in wastewater treatment because this nitrogen contributes to the eutrophication of receiving waters. Moreover, the toxicity of NH_4^+ and NO_2^- has a direct threat to aquatic life. The conventional nitrogen removal process consists of two main processes: nitrification and denitrification. Nitrification is carried out sequentially by aerobic chemolithoautotrophic ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Previously, oxidation of NH_4^+ and NO_2^- by chemolithoautotrophic nitrifiers was thought to be restricted to oxic environments. The discovery of a novel pathway for anaerobic ammonium oxidation (AMX) by Planctomyces provided the opportunity to develop AMX into a novel nitrogen-removal process. AMX is an anoxic microbiological process in which NH_4^+ and NO_2^- are converted to dinitrogen gas by reaction (1) (Strous et al., 1998).

$$1.0NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

In the early 1990s, the first direct evidence of the anaerobic oxidation of NH_4^+ has been discovered at Gist-Brocades (Delft, The Netherlands) and it was noted that NH_4^+ disappeared from the reactor effluent by the production of nitrate with a concurrent increase in dinitrogen gas production on a denitrifying pilot plant (Jetten et al., 1998). The first discovered AMX bacterium was named *Candidatus Brocadia anammoxidans,* which belongs to the *Planctomyces*. Many scientists also discovered other AMX bacteria from wastewater treatment plants and marine environments and to date three genera have been described: Brocadia, Kuenenia and Scalindua (Dalsgaard et al., 2005). In AMX bioreactors, the AMX reaction must be supplied with NO_2^- which could be provided by oxidation of NH_4^+ to NO_2^- by AOB. AOB are responsible for the first step in nitrification and most of them are the species of β-subdivision of the class *Proteobacteria.*

Most of the physiological and kinetic data available for AOB have been based on a small group of cultured isolates. However, AOB are slow-growing and difficult to isolate in pure culture. In addition, cultured isolates may not represent the dominant AOB in the environment (Purkhold et al., 2000; Rowan et al., 2003). These limitations can be remedied using molecular techniques, which allow a more complete understanding of the diversity and distribution of AOB in natural environments than is offered by cultivation-based methods alone. Especially, real-time qPCR with sets of group-specific primers and probes provides a powerful approach to study the population dynamics of AOB. The quantitative analysis of AOB provides valuable knowledge on the relationship between the AOB and coexisting bacteria which share common substrates. For example, real-time qPCR revealed that the complex microbial communities of AOB, NOB and archaea for biological wastewater treatment are dynamically shifted according to the environmental conditions (Abzazou et al., 2018).

The diverse interaction of AOB with coexisting bacteria has been reported for sea and soil (Li et al., 2017; Nitahara et al., 2017). Under the oxygen-limited conditions, clusters of AMX bacteria are often found with clusters of AOB, which convert NH_4^+ into NO_2^- and consume the inhibitory oxygen (Sliekers et al., 2002; Third et al., 2001). AOB can also coexist with AMX bacteria under anaerobic conditions (Qiao et al., 2009; Quan et al., 2008). However, the relationship between AMX activity and the quantity of AOB is not known. In this research, the objectives were to identify and quantify AOB in an AMX enrichment bioreactor during the start-up and substrate inhibition periods using molecular biological techniques. The qualitative and quantitative approaches revealed the close relationship between AOB and AMX bacteria in the dynamic conditions of the common substrate, i.e., NH_4^+, in an AMX reactor.

2. Materials and Methods

2.1 Enrichment of AMX bacteria

A sequencing batch reactor for anaerobic ammonium oxidation (AMX-SBR) with a working volume of 2.5 L was operated for approximately five months (i.e., 159 days). The SBR was seeded with activated sludge; the initial biomass concentration was 1.13 g VSS/L. Temperature was maintained at 35 °C and the agitation speed was 75 rpm. To remove the dissolved oxygen completely the medium was purged with argon gas before and after being added to the SBR. The reaction was carried for 7 days for each cycle (Anjali and Sabumon, 2017). The ratio of NH_4^+- to NO_2^--nitrogen was approximately 1:1. The compositions of the enrichment medium are described in the previous study (Bae et al., 2010). The substrate nitrogen (SN) concentration, which is the sum of NH_4^+- and NO_2^--nitrogen, was controlled to reveal the maximum substrate loading which is tolerable for the AMX-SBR.

2.2 T-RFLP and sequencing

Nine samples were collected for Days 28, 56, 77, 101, 108, 115, 124, 127 and 153. To investigate the microbial community structure of AOB in the AMX-SBR, real-time qPCR, T-RFLP, and sequencing were conducted. To isolate

DNA, samples of homogenous activated-sludge were obtained from the SBR. A Power Soil™ DNA kit (Mo Bio Laboratories, US) was used to isolate the DNA following the manufacturer's protocol. For the amplification of the bacterial ammonia monooxygenase subunit A (*amoA)* gene for cloning and T-RFLP, primers *amoA*-1F (5'-GGGGTTTCTACTGGTGGT-3') and *amoA*-2R (5'-CCCCTCKGSAAAG CCTTCTTC-3') were used. For T-RFLP, the forward primer *amoA*-1F and reverse primer *amoA*-2R were labeled with fluorophores, FAM and HEX, respectively (Park and Noguera, 2004). The PCR mixture consisted of 15 μl of 2× PCR pre-Mix (SolGent, Korea), 1 μL of each primer (10 μM), 1 μL of DNA template, and 12 μL of deionized water. The PCR cycles were as follows: 1 cycle of 2 min at 95℃, 30 cycles of 20 s at 95℃, 40 s at 57℃, 40 s at 72℃ then 1 cycle of 5 min at 72℃. Cycles were performed using a MyCycler™ Thermal Cycler (BIO-RAD, USA). The amplified *amoA* gene was cloned in a pGEM®-T Easy Vector System (Promega, USA). The ligation mixture was transformed into HIT™ competent cells (Real Biotech Corp., Taiwan). Plasmids containing *amoA* gene inserts were sequenced by SolGent Co. (Korea). Forty-seven *amoA* partial sequences obtained and Phylogenetic analysis was used to determine relationships among the sequences. Sequences were aligned using the ClustalX 1.81 program. Clones with more than 97% sequence similarity were grouped into the same operational taxonomic unit (OTU). The phylogenetic tree was constructed using the neighbor-joining (NJ) method. The reliability of internal branches was assessed using 1,000 bootstrap replicates. The affiliation of the amoA gene was searched by BLAST (blast.ncbi.nlm.nih.gov). For T-RFLP, the PCR product was purified using the gel extraction and purification kit (Qiagen, USA). Purified PCR product was digested with endonuclease *Taq*I (10U) (Takara, Japan) at 65℃ for 3 h. Fragments were run on an ABI 371X sequencer (Perkin-Elmer Corp., USA) and analyzed using GeneScan 3.7 software (Applied Biosystems, USA).

2.3 Real-time qPCR

Real-time qPCR assays were performed to quantify 16S rDNA of β-subclass AOB. Two forward primers CTO 189fA/B (5'-GGAGRAAAGCAGGGGATCG-3') and CTO 189fC (5'-GGAGGAAAGTAGGGGATCG-3'), one reverse primer RT1r (5'-CGTCCTCTCAGACCARCTACTG-3'), and the TaqMan probe TMP1 (5'-CAACTAGCTAATCAGR CAT CRGCCGCTC-3') were used for AOB quantification (Hermansson and Lindgren, 2001). Primers CTO 189fA/B and CTO 189fC were used at a 2:1 ratio (Kowalchuk et al., 1997). The real-time qPCR assays were performed in duplicate with a total volume of 25μl reaction mixture, consisting of 15μl of 2× PCR pre-mix of ABI (Applied Biosystems, USA), 0.5μl of each primer (15 μM), 0.25μl (12.5 μM) of TaqMan probe, 10.75 μL of deionized water and 0.5 μL of template DNA. All manipulations were performed in laminar airflow and in low light to prevent light-activated degradation of the fluorescently labeled oligonucleotide probes. The PCR cycling condition was as follows: 1 cycle of 2 min at 50℃, 1 cycle of 10 min at 95℃, 30 cycles of 15 sec at 95℃ and 60 s at 60℃. All tubes were maintained on ice and in the dark during transport to the spectrofluorimetric thermal cycler, Prism 7300 sequence detection system (Applied Biosystems, USA). All PCR runs included control reactions without template DNA to test for possible non-specific amplification. The standard curves for AOB were constructed using a series of DNA concentrations prepared from the plasmid vector carrying the 16S rDNA gene of a *Nitrosomonas europaea*-like AOB related clone, which was obtained from a clone library constructed during this study.

3. Results and Discussion

3.1 Nitrogen removal of anaerobic ammonium oxidation

The AMX enrichment was conducted with the AMX-SBR for 159 days (Fig. 1). The nitrogen loading rate ranged from 0.003 and 0.098 kg-N/m^3-d (Fig. 2). At the start-up of the enrichment, NH_4^+ and NO_2^- concentrations were 18.6±6.7 and 13.8±7.1 mg-N/L, respectively. TN removal efficiency was maintained as lower than 70% until Day 42. The NO_2^- removal efficiency of 62.6±33.7% was higher than that of the NH_4^+ removal efficiency of 40.7±57.7%. It was expected that the heterotrophic respiration using NO_2^- as an electron acceptor resulted in the active removal of NO_2^- by denitrification. The extra-organic carbon was not supplied for the denitrification, but heterotrophic denitrifying bacteria can rely on organic carbon caused by in the degradation of bacteria which were incapable of metabolizing added NH_4^+, NO_2^- and HCO_3^-. The SN concentrations were gradually increased from 31.3 to 248.5 mg-N/L until Day 84. In this period, an average TN removal efficiency was 99.8±0.3%. At the intensive substrate loading (i.e., 631.2, 685.1 and 496.0 mg/L of SN) on Days 95, 101 and 108, the AMX-SBR showed significantly lowered TN removal efficiencies from 60.7 to 7.3%. The AMX activity was recovered by reducing the SN concentration to NH_4^+ of 52.8～177.8 mg-N/L and NO_2^- of 54.0～207.0 mg/L (Days 115-139). The TN removal was stabilized at 88.9±2.0%. The stabilized nitrogen removal rate was 0.066±0.003 kg-N/m^3-d for the last four batch operations (Fig. 2). It was known that NH_4^+ and NO_2^- are toxic to AMX bacteria (Lackner et al., 2014). In this study,

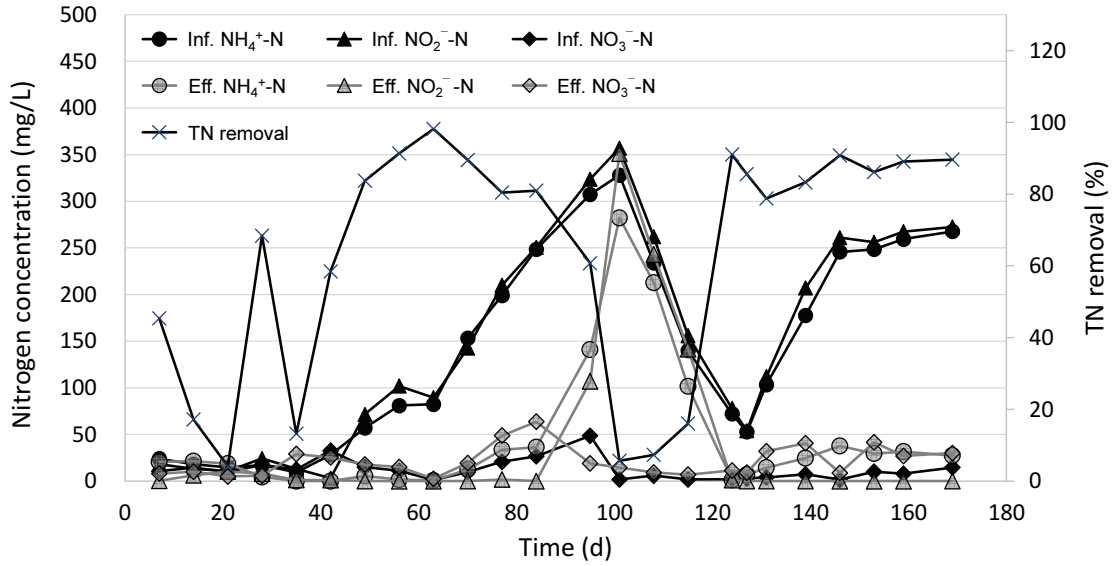

Fig. 1. The profile of nitrogen and TN removal efficiency of the anaerobic ammonium oxidation in the sequencing batch reactor.

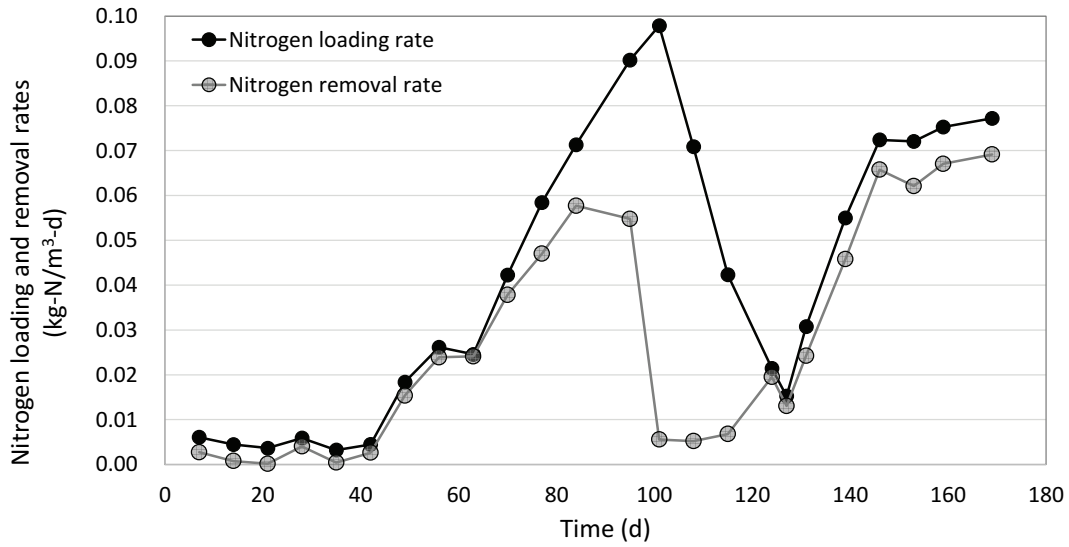

Fig. 2. Nitrogen loading and removal rates of the anaerobic ammonium oxidation in a sequencing batch reactor.

AMX bacteria are tolerant to high-level NH_4^+ and NO_2^- of around 250 mg-N/L. The high substrate tolerance of enriched AMX bacteria can be attributed to the long residence time of 7 days for the AMX-SBR reactor. It was speculated that the longer contact time with high SN concentration resulted in better tolerance to substrate toxicity. However, in the previous study, the SBR showed a high tolerance to hydraulic shock rather than an up-flow continuous reactor (Jin et al., 2008). Thus, the adaptation mechanism of AMX bacteria to high SN should be investigated in depth. For example, the metabolism change with low- and high-substrate loadings can be monitored by transcriptomic technology. Also, there is great interest in the excellent resistance of immobilized AMX bacteria to inhibitory factors for practical applications (Lotti et al., 2012).

3.2 Taxonomic information of the predominant AOB

During the AMX enrichment, the genomic DNA was extracted to identify the taxonomic affiliation and growth dynamics of AOB. The change in the microbial community structure of AOB during the operation was monitored using T-RFLP fingerprinting. Double labeled T-RFLP together with the *Taq*I restriction enzyme offered the phylogenetic information of AOB for the nine samples of Days 28, 56, 77, 101, 108, 115, 124, 127 and 153. As shown in Fig. 3, two predominant terminal restriction fragments (T-RFs) for forward and reverse at 219 and 270 bp, respectively, indicate that *N. europaea* were major AOB (Bae et al., 2011). In addition, relatively low peaks at 491 bp for forward and reverse T-RFs suggest possible coexistence of diverse AOB including *Nitrosomonas oligotropha/communis/europaea/*

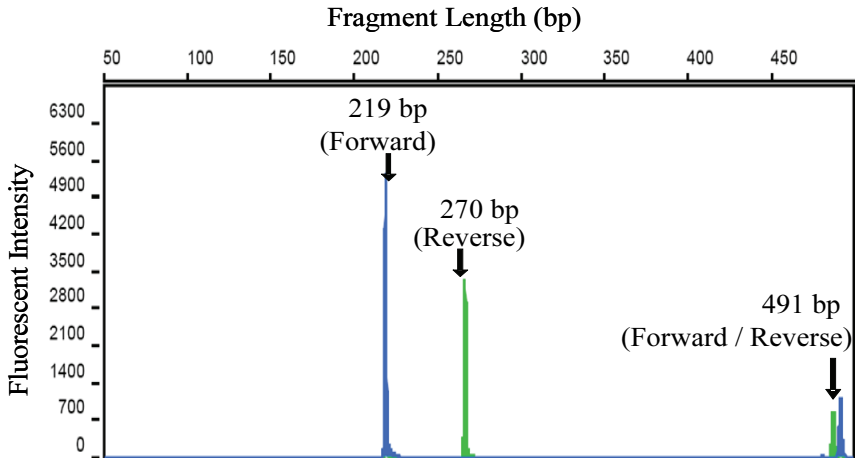

Fig. 3. The T-RFLP pattern for AOB, digested by TaqI using amoA-1F and amoA-2R primer set.

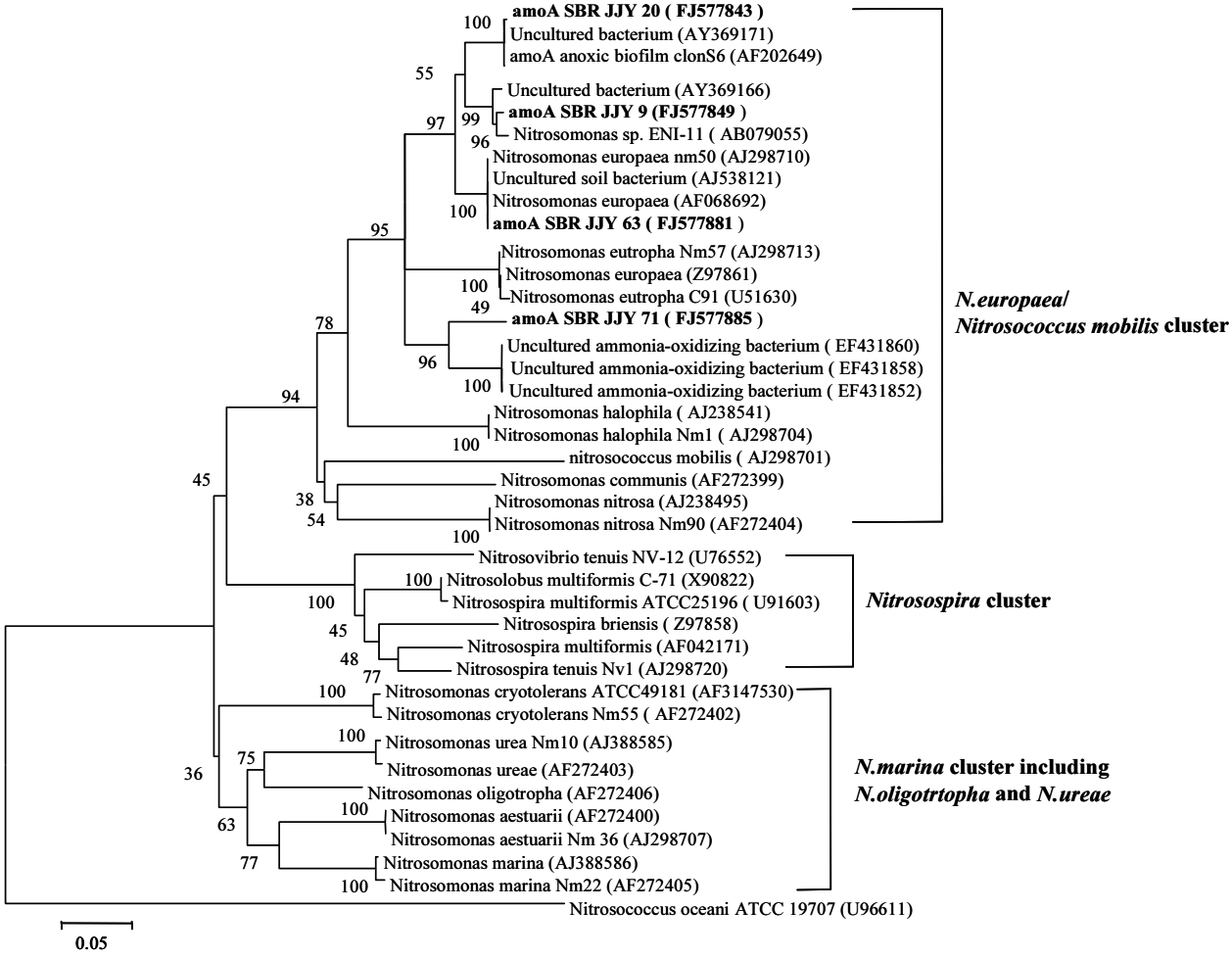

Fig. 4. Neighbor-joining phylogenetic tree based on *amoA* gene of β-proteobacterial AOB. Sequences in this study were depicted in bold. Scale bar indicates 5% sequence difference. The root represents the *amoA* gene sequence of γ-subclass AOB, *Nitrosococcus oceani* ATCC 19707 (U96611).

cryotolerans lineage (Bae et al., 2011). The predominant *Nitrosomonas europaea*-like AOB are the most commonly isolated and well-known AOB because they out-compete other AOB in environments that are rich in NH_4^+ (Hagopian et al., 1998; Limpiyakorn et al., 2005; Schramm et al. 1998; Sedlacek et al., 2020). In addition, the *N. europaea*-like AOB are less sensitive to high NH_4^+ salt concentration (i.e., 8400 mg-N/L) (Lim et al., 2008). In addition, *Nitrosomonas*

sp. is capable of anaerobic denitrification (Abeliovich and Vonshak, 1992). Nine samples in a time series showed no significant change. Therefore, it was considered that increase in SN concentration of the AMX-SBR had an insignificant effect on the composition of AOB at the genus level.

The predominant AOB were confirmed by cloning and sequencing based on the *amoA* gene. Four OTUs of amoA_SBR_JJY_20 (FJ577843), amoA_SBR_JJY_9 (FJ577849), amoA_SBR_JJY_63 (FJ577881) and amoA_SBR_JJY_71 (FJ577885) were defined among 47 clones at the cutoff of 3%. OTUs were closely related to the *Nitrosomonas europaea*/*mobilis* cluster (Fig. 4). The relative abundance of the OTUs were 2.1, 59.6, 21.3 and 17.0% for amoA_SBR_JJY_9, 20, 63 and 71, respectively. The representative OTUs were compared to clones of *N. europaea*-like AOB which have been collected from the anoxic bioreactor in previous studies (Table 1). Interestingly, the predominant amoA_SBR_JJY_20 (FJ577843) is similar to the clones from AMX-related environments and a partial nitrification reactor. The most similar clone of amoA_SBR_JJY_20 was AF202649 which was found from an anoxic biofilm which conducts the AMX reaction (Schmid et al., 2000). This implies that *Nitrosomonas europaea*/*mobilis* cluster is strongly associated with AMX bacteria. Also, the OTU with

Table 1. Affiliation of the *amoA* gene clones analyzed in this study

No.	OTU name (Accession number)	Number of sequences	% Similarity	Accession number of reference sequence (Organism)	Source of reference sequence (Nation)	Title
1	amoA_SBR_JJY_20 (FJ577843)	28	99.4	AF202649 (*amoA* anoxic biofilm clone S6)	Anoxic biofilm (Germany)	Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation.
			91.6	AB291772 (uncultured bacterium)	Sludge from partial nitrification reactor (Japan)	Microbial community that catalyzes partial nitrification at low oxygen atmosphere as revealed by 16S rRNA and *amoA* genes.
			92.1	AF532304 (uncultured ammonia-oxidizing bacterium)	Biofilm reactor (Portugal)	Nitrifying and heterotrophic population dynamics in biofilm reactors: effects of hydraulic retention time and the presence of organic carbon.
2	amoA_SBR_JJY_63 (FJ577881)	10	99.4	AL954747 (*Nitrosomonas europaea* ATCC 19718)	Isolated strain	Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea.*
			99.6	EF222047 (uncultured bacterium)	10m depth in water column (Germany)	Comparative analysis of ammonia monooxygenase (*amoA*) genes in the water column and sediment-water interface of two lakes and the Baltic sea.
			99.6	EF222034 (uncultured ammonia-oxidizing β-proteobacterium)	2m depth in water column (Germany)	
3	amoA_SBR_JJY_71 (FJ577885)	8	85.5	EF431860, EF431858 (uncultured ammonia-oxidizing bacterium)	Swine waste water treatment plants (Taiwan)	Nitrification performance and microbial ecology of nitrifying bacteria in different wastewater treatment plants.
			89.2	EU221324 (uncultured *Nitrosomonas* sp.)	Ammonium oxidizing enrichment culture (Netherlands)	Physiological and phylogenetic study of an ammonium-oxidizing culture at high nitrite concentrations.
4	amoA_SBR_JJY_9 (FJ577849)	1	98.4	AB079054, AB079055 (*Nitrosomonas* sp. ENI-11)	Isolated strain	Physical map location of the multicopy genes coding for ammonia monooxygenase and hydroxylamine oxidoreductase in the ammonia-oxidizing bacterium *Nitrosomonas* sp. strain ENI-11.
			98.6	AY369166 (uncultured bacterium)	Activated sludge (China)	Analysis of the microbial community composition and transition in the activated sludge of a lab-scale deammonification reactor by molecular methods.

the lowest abundance (amoA_SBR_JJY_9, FJ577849) is also affiliated to the AOB from a lab-scale AMX reactor (AY369166 in China). These two groups are expected to have a metabolic advantage to survive in an anaerobic and nitrogen-rich environment such as AMX reactors. The second dominant sequence of amoA_SBR_JJY_63 (FJ577881) is close to AOB in water environments. The amoA_SBR_JJY_71 (FJ577885) is related to the AOB from a swine wastewater treatment plant and an AOB culture which is tolerable to high NH_4^+ and NO_2^- concentration. This group of AOB might grow in the toxic environment of high loading of NO_2^- in the AMX-SBR.

3.3 Growth characteristics of AOB

The population dynamics of AOB responded to the AMX activity (Fig. 5). The average copies of 16S rDNA were low (i.e., 1.53×10^6 copies/mL) and remained steady during the AMX start-up period. However, the population of AOB increased quickly. Based on the four concentration values on Days 101, 108, 115 and 124, the non-linear least squares method for the exponential growth function showed a specific growth rate of 0.111 d^{-1} during the period of substrate inhibition of AMX bacteria. Thereafter, the concentration of AOB decreased sharply as soon as the AMX activity was recovered. The reason for the sharp growth of AOB is speculated that the catalyzing metabolism of AOB is stimulated by the high concentration of SN in the AMX-SBR during the period of substrate inhibition.

The syntrophy of aerobic and anaerobic ammonia oxidizers was expected to be beneficial to nitrogen removal by the production of NO_2^- by AOB. AOB possess superior growth kinetics than AMX bacteria by order of magnitude with favorable conditions of oxygen and NH_4^+ (Wett et al., 2010). However, there has been no clear evaluation of the cooperation or competition between AMX bacteria and AOB. In this study, quantitative analysis using real-time qPCR revealed a negative relationship between AMX activity and the growth rate of AOB. Under anaerobic conditions, they might compete for common substrates because *Nitrosomonas* sp. can oxidize NH_4^+ anaerobically using NO_2 (Schmidt et al., 2002). When the substrate inhibition lowered the affinity of AMX bacteria towards substrates, AOB have a chance to utilize the substrates without competition with AMX bacteria. Under aerobic conditions, the maximum specific growth rate of AOB ranges up to 2.71 d^{-1} (Mannucci et al., 2020; Park and Noguera, 2004). In this study, the specific growth rate of AOB under anaerobic conditions was only 0.111 d^{-1}. Thus, the growth rate is not comparable to the optimal growth of AOB in aerobic conditions. However, this exponential growth rate is similar to the specific growth rate of AMX bacteria, 0.114 d^{-1} of the AMX-SBR (data not shown). The similar specific growth rates well explain the immediate and comparable growth for each of these groups of ammonium oxidizers at different concentrations of SN concentrations.

4. Conclusions

In this study, An AMX-SBR with a conventional activated sludge was operated to reveal the relationship of AOB growth and AMX activity during the AMX enrichment for 159 days. The AMX-SBR showed efficient TN removal

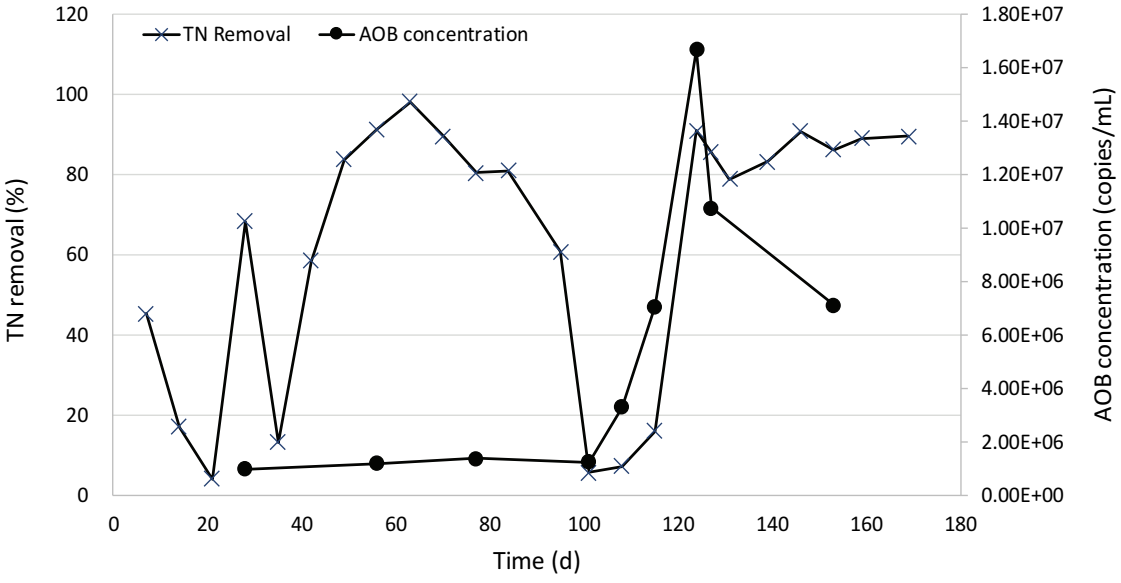

Fig. 5. The growth of AOB during the enrichment of anaerobic ammonium oxidation bacteria.

efficiency of 99.8±0.3% by enduring the intensive substrate loading. However, the high SN concentrations of 631.2, 685.1 and 496.0 mg/L resulted in the serious inhibition of AMX activity. Overall the enrichment period, T-RFLP and sequencing based on the amoA gene revealed that the AOB population was stable with predominant *Nitrosomonas europaea*-like AOB. In particular, the OTUs of amoA_SBR_JJY_20 (FJ577843) and amoA_SBR_JJY_9 (FJ577849) are similar to the clones from AMX-related environments. This implies that the coexistence of AOB and AMX bacteria is a common phenomenon for the AMX process. The specific growth rate of AOB (0.111 d^{-1}) measured by real-time qPCR was similar to that of AMX bacteria. The similar specific growth rates suggest that the growth of comparable metabolic activities of AOB and AMX bacteria under different conditions of substrate loading in the AMX bioreactor.

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