Effects of ammonium and nitrite on communities and populations of ammonia-oxidizing bacteria in laboratory-scale continuous-flow reactors

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Abstract

This study investigated the effects of ammonium and nitrite on ammonia-oxidizing bacteria (AOB) from an activated sludge process in laboratory-scale continuous-flow reactors. AOB communities were analyzed using specific PCR followed by denaturing gel gradient electrophoresis, cloning and sequencing of the 16S rRNA gene, and AOB populations were quantified using real-time PCR. To study the effect of ammonium, activated sludge from a sewage treatment system was enriched in four reactors receiving inorganic medium containing four different ammonium concentrations (2, 5, 10 and 30 mM NH_4^+-N). One of several sequence types of the *Nitrosomonas oligotropha* cluster predominated in the reactors with lower ammonium loads (2, 5 and 10 mM NH_4^+-N), whereas *Nitrosomonas europaea* was the dominant AOB in the reactor with the highest ammonium load (30 mM NH_4^+-N). The effect of nitrite was studied by enriching the enriched culture possessing both *N. oligotropha* and *N. europaea* in four reactors receiving 10-mM-ammonium inorganic medium containing four different nitrite concentrations (0, 2, 12 and 22 mM NO_2^--N). *Nitrosomonas oligotropha* comprised the majority of AOB populations in the reactors without nitrite accumulation (0 and 2 mM NO_2^--N), whereas *N. europaea* was in the majority in the 12- and 22-mM NO_2^--N reactors, in which nitrite concentrations were 2.1–5.7 mM (30–80 mg N L^{-1}).

Introduction

Nitrification, the two-step process by which ammonia is oxidized to become nitrite and subsequently to nitrate, plays a key role in biological nitrogen removal in wastewater treatment systems. The process involves two phylogenetically unrelated groups of obligate chemolithotrophic bacteria. Ammonia is first oxidized to nitrite by ammonia-oxidizing bacteria (AOB), and subsequently nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB). Because AOB have slow growth rates and are sensitive to many environmental factors, the rate-limiting step of nitrification is the ammonia oxidation. Therefore, enhancement of the performance of wastewater treatment systems requires a better understanding of the microbiology and ecology of AOB.

The distribution patterns of distinct AOB species in the environments reflect the physiological properties of AOB isolates observed in the laboratory (Koops & Pommerening-Roser, 2001). Among several factors, ammonia and nitrite – the sole energy source and the product of ammonia oxidation – are two of the most important factors affecting the occurrence of distinct AOB species in the environments.

A study of AOB isolates suggested differences in ammonia affinities among distinct AOB species (Koops & Pommerening-Roser, 2001). Indeed, many observations in wastewater treatment systems showed that the occurrence of distinct AOB species in the system was related to the ammonia affinity of the species. In general, members of the *Nitrosomonas oligotropha* cluster or/and the *Nitrosospira* cluster are the most common AOB found in systems with low ammonium loads (Ballinger *et al.*, 1998; Schramm *et al.*, 1998; Dionisi *et al.*, 2002; Harms *et al.*, 2003; You *et al.*, 2003; Limpiyakorn *et al.*, 2005, 2006b), whereas members of the *Nitrosomonas europaea–Nitrosococcus mobilis* cluster are found in systems with high ammonium loads (Juretschko

et al., 1998; Wagner *et al.*, 1998; Pynaert *et al.*, 2003). However, most of the evidence so far tends to be indirect or circumstantial. Very few studies provided the direct evidence showing how differences in AOB community structure relate to ammonium load (Princic *et al.*, 1998). As a result, the threshold of ammonium load that shifts the community structure of AOB in wastewater treatment systems is not clearly understood.

To date, nitrite is known to inhibit the ammonia-oxidizing activity of AOB (Muller *et al.*, 1995; Philips & Verstraete, 2001); however, data on the inhibitory effect of nitrite on distinct AOB species is very limited. The only study undertaken on this revealed that a very high nitrite concentration of 21.4 mM NO_2^--N inhibited the activity of AOB closely related to *N. oligotropha* but not *Nitrosomonas eutropha* (Suwa *et al.*, 1994). In practice, the accumulation of nitrite occurs in certain environments and this may result in the selection of AOB species. One example is in culturing medium during isolation of AOB when NOB vanished. Another example may be in novel nitrogen removal processes, such as SHARON (Hellinga *et al.*, 1998), where partial nitrification is promoted to limit the oxidation of ammonia to nitrite.

The objective of this study is to investigate the effects of ammonium and nitrite on AOB from an activated sludge process in laboratory-scale continuous-flow reactors. The effect of ammonium was studied by enriching activated sludge taken from a sewage treatment system in four reactors receiving inorganic medium containing four different ammonium concentrations (2, 5, 10 and 30 mM NH_4^+-N). To study the effect of nitrite, the enriched culture taken from the 10-mM NH_4^+-N reactor, which possessed both *N. oligotropha* and *N. europaea*, was enriched in four reactors receiving 10-mM-ammonium inorganic medium containing four different additional nitrite concentrations (0, 2, 12 and 22 mM NO_2^--N). To identify AOB communities, specific PCR amplification followed by denaturing gel gradient electrophoresis (DGGE), cloning and sequencing of the 16S rRNA gene were used, and then AOB populations were quantified by real-time PCR.

Materials and methods

Seed

Seed used for the ammonium study was activated sludge taken from an aeration tank of a full-scale sewage treatment system in August 2001 and February 2002. This system is run by the Bureau of Sewerage, Tokyo Metropolitan Government, Japan. From August 2001 to February 2002, biological oxygen demand (BOD) in the influent of this system ranged from 63 to 111 mg L^{-1}, whereas ammonium concentrations were between 1.1 and 1.3 mM (15 and 18 mg N L^{-1}). BOD and ammonium removal efficiencies of this system were excellent (97–98% and 97–100%, respectively). Nitrite concentrations in the aeration tank were < 0.07 mM (1 mg N L^{-1}), and pH was controlled between 7 and 8. The seed used for the nitrite study was inoculated from the reactor enriched with 10-mM-ammonium inorganic medium for 48 days in the ammonium study.

Reactors

Seed was enriched in continuous-flow completely mixed reactors without sludge recycling. The total volume of the reactor was 2 L, with an effective volume of 1.5 L. To obtain the optimum condition for AOB growth, the temperature was kept at 25 °C, the dissolved oxygen concentration was controlled at around 2 mg L^{-1}, pH was maintained in a rage of 7.4–7.8 and mixing was provided at a rotating speed of 300 r.p.m. Inorganic medium was introduced into all reactors at a fixed dilution rate of 0.01 h^{-1}. All equipment and media were sterilized before use. The reactors and inorganic medium were autoclaved before use, and the air for aeration was filtered with a 0.45-μm membrane filter.

Inorganic medium

Ammonium and nitrite concentrations in inorganic medium were varied to provide the different loads of ammonium and nitrite for each reactor without changing the dilution rate. The medium contained NH_4Cl or $NaNO_2$, 40 mg of $MgSO_4 \cdot 7H_2O$, 40 mg of $CaCl_2$ $2H_2O$, 200 mg of KH_2PO_4, 1 mg of $FeSO_4 \cdot 7H_2O$, 0.1 mg of Na_2-$Mo_4O_4 \cdot 2H_2O$, 0.2 mg of $MnCl_2 \cdot 4H_2O$, 0.02 mg of Cu-$SO_4 \cdot 5H_2O$, 0.1 mg of $ZnSO_4 \cdot 7H_2O$, and 0.002 mg of $CoCl_2 \cdot 6H_2O$ per liter. To study the effect of ammonium, the activated sludge taken from the sewage treatment system was enriched in four reactors receiving inorganic medium containing four different ammonium concentrations: 2, 5, 10 and 30 mM NH_4^+-N (28, 70, 140 and 420 mg N L^{-1} or volumetric ammonium loads of 0.5, 1.2, 2.4 and 7.1 mM day^{-1}, respectively). To study the effect of nitrite, the enriched culture taken from the 10-mM NH_4^+-N reactor was enriched in four reactors receiving 10-mM-ammonium inorganic medium containing four different additional nitrite concentrations: 0, 2, 12 and 22 mM NO_2^--N (0, 28, 168, 308 mg N L^{-1} or volumetric nitrite load of 0, 0.5, 2.9 and 5.3 mM day^{-1}, respectively).

Ammonium, nitrite, and nitrate concentrations

Ammonium, nitrite and nitrate concentrations in bulk were measured using the Cuvette Test, LCK 305, LCK 342 and LCK 339, respectively (DR Lange, Dusseldorf, Germany) according to the manufacturer's instructions.

Sampling and DNA extraction

Enriched cultures were taken from the reactors periodically. Mixed liquor suspended solid (MLSS) concentrations were determined on the day of sampling. Approximately 2 mg MLSS of the culture was transferred into a 1.7-mL Eppendorf tube and centrifuged at 14 000 ***g*** for 10 min. The supernatant was removed, and the pellet was kept at $-20\,^{\circ}C$ until analysis.

DNA was extracted from samples using FastDNA SPIN kits for soil (Bio 101, Vista, CA), with a minor modification as described previously (Limpiyakorn *et al.*, 2005). To minimize the variation in DNA extraction, templates used for real-time PCR quantification were prepared by mixing the DNA that was extracted in triplicate for a sample.

AOB communities

AOB communities in samples were analyzed using specific PCR amplification followed by DGGE, cloning and sequencing of the 16S rRNA gene, as described elsewhere (Limpiyakorn *et al.*, 2005). Briefly, DNA extracted from a sample was amplified with PCR using the primer set CTO189f-CTO654r (the forward primer had an additional GC clamp; Table 1) (Kowalchuck *et al.*, 1997) with Ampli*Taq* Gold DNA polymerase (PE Applied Biosystems, CA) in a T3 thermocycler (Biometric, Gottingen, Germany). DGGE was performed using 8% polyacrylamide gels, and the urea–formamide denaturing gradient was 35–50%. Each target fragment of DNA recovered from the DGGE gel was purified by cloning using the pT7Blue T-Vector and DH5-α competent cells (Toyobo, Tokyo, Japan). Before sequencing, small DNA fragments and excess primers were removed from the PCR products by Microcon spin columns (Millipore, Tokyo, Japan). Sequencing reactions were run with the ABI Big Dye Terminator kit version 3.1 (PE Applied Biosystems) using the primer set CTO189f-CTO654r. After excess primers and dye terminators from the products of the sequencing reaction had been removed using Centri-sep spin columns (PE Applied Biosystems), the products were analyzed in an ABI 310 DNA sequencer (PE Applied Biosystems).

In total 442 bp from the 465-bp analyzed sequences were aligned with sequences from the ssu rRNA database (Antwerm, Belgium) using the ARB program package (Department of Microbiology, Technische Universitat Munchen, Munich, Germany; http://www.arb-home.de). A phylogenetic tree was constructed using the ARB program package by adding analyzed 442-bp sequences into the distance tree, which was prior constructed on the basis of a comparison of the 1000-bp sequences of all AOB (available in the ssu rRNA database) and some related non-AOB used as outgroup sequences. In addition, the analyzed 442-bp sequences and the 442-bp sequences of described AOB species (Koops *et al.*, 2003) and some related non-AOB were calculated on the basis of maximum parsimony, maximum likelihood and distance analyses, using the external software provided in the ARB program package (PHYLIP DNAPARS, AxML, and Phylip Distance Method, respectively).

AOB populations

AOB populations in samples were quantified using real-time PCR. DNA extracted from a sample was quantified via real-time PCR in an ABI Prism SDS 7000 (PE Applied Biosystems). The extracted DNA was prepared for three different 10-fold dilutions, and each of the dilutions was quantified via real-time PCR in duplicate.

Table 1. Primers and probes used in this study

Primer or probe	Nucleotide sequence (5′–3′)	Sequence position*	Reference
Primers†			
CTO189A/Bf	GGAGRAAAGCAGGGGATCG	189–207	Kowalchuck *et al*. (1997)
CTO189Cf	GGAGGAAAGTAGGGGATCG	189–207	Kowalchuck *et al*. (1997)
eurf	AAGACCTTGCGCTAAAGGAG	209–228	Limpiyakorn *et al*. (2006a)
comf	CTCGTGCTTTAAGGGTGGCC	214–233	Limpiyakorn *et al*. (2006a)
RT1r	CGTCCTCTCAGACCARCTACTG	283–304	Hermansson & Lindgren (2001)
6a1f	CATAGCTCATGACGGTATCG	470–489	Limpiyakorn *et al*. (2006a)
6a34f	TTCATGACGGTATCAACAGA	475–494	Limpiyakorn *et al*. (2006a)
CTO654r	CTAGCYTTGTAGTTTCAAACGC	633–654	Kowalchuck *et al*. (1997)
CTO 654r (5′-BODIPY®FL)	CTAGCYTTGTAGTTTCAAACGC	633–654	Kowalchuck *et al*. (1997)
1055f	ATGGCTGTCGTCAGCT	1055–1070	Harms *et al*. (2003)
1392r	ACGGGCGGTGTGTAC	1378–1392	Harms *et al*. (2003)
Probes‡			
TMP1 (5′-FAM and 3′-TAMRA)	CAACTAGCTAATCAGRCATCRGCCGCTC	226–256	Hermansson & Lindgren (2001)
16STaq1115 (5′-FAM and 3′-TAMRA)	CAACGAGCGCAACCC	1100–1115	Harms *et al*. (2003)

**Escherichia coli* numbering.

†BODIPY®FL, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid.

‡FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine.

Quantification of the 16S rRNA gene of total bacteria was performed using the primers 1055f and 1392r and the *Taq*Man probe 16Staq1115 (Table 1) (Harms *et al.*, 2003). PCR mixture and conditions were as described previously (Limpiyakorn *et al.*, 2005). Standard DNA was the pT7Blue T-Vector (Novagen, Darmstadt, Germany) possessing the 16S rRNA gene of *N. europaea* prepared in a range of from 5×10^1 to 5×10^7 copies. To calculate the cell numbers of total bacteria from the quantified numbers of 16S rRNA genes, it is assumed that the average number of 16S rRNA gene copies per genome of bacterial cells was 3.6; this was based on the average number of 16S rRNA gene copies found in cultured bacteria (Klappenbach *et al.*, 2001).

Quantification of the 16S rRNA gene of total AOB was performed using the primers CTO189f and RT1r and the *Taq*Man probe TMP1 (Table 1) (Hermansson & Lindgren, 2001). The PCR mixture and conditions were as described previously (Limpiyakorn *et al.*, 2005). The standard DNA was the pT7Blue T-Vector possessing the 16S rRNA gene of *N. europaea*, prepared in a range of 4×10^1 to 4×10^7 copies. To calculate the cell numbers of total AOB from the quantified numbers of 16S rRNA genes, it is assumed that AOB possess one copy of 16S rRNA gene per genome, on the basis of the number of 16S rRNA gene copies found in AOB (Aakra *et al.*, 1999).

Quantification of the 16S rRNA genes of specific AOB was performed using four recently developed primer sets (Table 1) (Limpiyakorn *et al.*, 2006a). The primer sets targeted 16S rRNA genes of sequence type 6a-1 of the *N. oligotropha* cluster (primer set 6a1f-CTO654r), sequence types 6a-34 of the *N. oligotropha* cluster (primer set 6a34f-CTO654r), members within the *Nitrosomonas communis* cluster (primer set comf-CTO654r), and the *N. europaea–Nitrosococcus mobilis* cluster (primer set eurf-CTO654r). The standard DNAs for the primer sets were pT7Blue T-Vectors (Novagen) possessing 16S rRNA gene fragments of bands F2-W-2, B3-W-2, B3-W-1 and 30NH_4-4 (Limpiyakorn *et al.*, 2006a), respectively, which were prepared as 10-fold dilution series in ranges of from 3×10^1 to 3×10^8, 5×10^1 to 5×10^8, 3×10^1 to 3×10^8 and 6×10^1 to 6×10^8 copies per reaction, respectively. The DNA copy numbers were converted to cell numbers in the same manner as that used for total AOB.

Accession numbers for nucleotide sequences

The obtained partial sequences of 16S rRNA genes were submitted to the DDBJ/EMBL/GenBank databases under accession numbers AB222789–AB222820.

Results

Ammonium, nitrite and nitrate concentrations

In the study on the effect of ammonium (Fig. 1), although inorganic medium with various ammonium concentrations was introduced continuously, ammonium concentrations in all reactors reached steady-state conditions at almost the detection limit (0.07 mM) after certain periods of operation (in 2-, 5- and 10-mM NH_4^+-N reactors after 4 days of operation and in the 30-mM NH_4^+-N reactor after 16 days

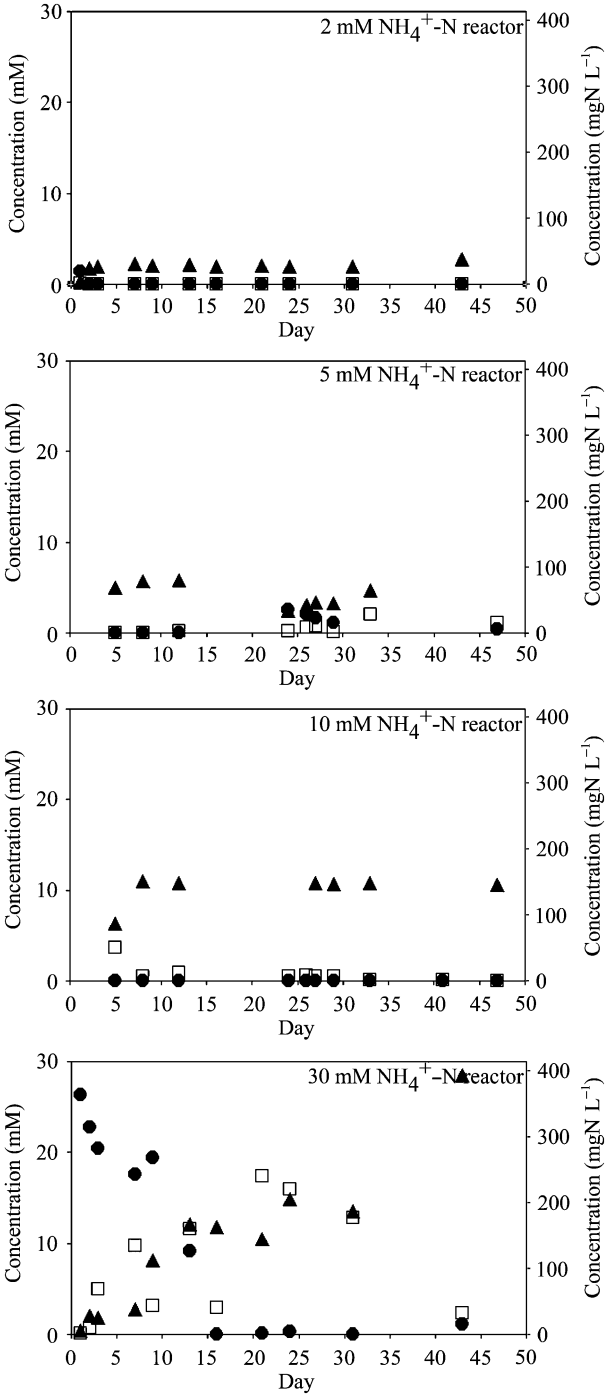

Fig. 1. Concentrations of ammonium (•), nitrite (□) and nitrate (▲) in the reactors receiving inorganic medium containing different ammonium concentrations.

of operation). However, there were small peaks of ammonium that occurred in the 5-mM NH_4^+-N reactor during day 24 and 47 of operation (0.4–2.5 mM, 5–35 mg N L^{-1}) and in the 30-mM NH_4^+-N reactor on day 43 (1.1 mM, 16 mg N L^{-1}). Nitrite concentrations in 2-, 5- and 10-mM NH_4^+-N reactors were almost always less than the detection limit (0.04 mM) after 7 days of operation, whereas in the 30-mM NH_4^+-N reactor it was in the range of 2.1–7.1 mM (30–100 mg N L^{-1}) after 40 days (data after 48 days of operation not shown).

In the study on the effect of nitrite (Fig. 2), although 10-mM-ammonium inorganic medium with various additional nitrite concentrations was provided continuously, ammonium in all reactors was completely oxidized. No nitrite was found in the control and 2-mM NO_2^--N reactors; however, in the 12- and 22-mM NO_2^--N reactors nitrite was found in the range of between 7.1 and 28.6 mM (100–400 mg N L^{-1}) during the first 20 days of operation and thereafter decreased to 2.1–5.7 mM (30–80 mg N L^{-1}).

AOB communities

AOB communities in samples were analyzed using specific PCR amplification, followed by DGGE, cloning and sequencing of the 16S rRNA gene. Analysis of duplicate samples showed that the DGGE band patterns obtained were reproducible (data not shown). To prevent confusion when bands were selected directly from the original gel images, all bands were excised from the gels, reamplified and run on new gels to clarify individual band positions before selection for sequencing. All phylogenetic methods provided the same grouping of AOB sequences analyzed, and the grouping of each AOB cluster on the phylogenetic trees remained the same. All bands analyzed were related closely to sequence types 6a-1 or 6a-34 of the *N. oligotropha* cluster or to the *N. europaea* cluster (Tables 2 and 3; Fig. 3).

Effect of ammonium on AOB communities and populations

To study the effect of ammonium, activated sludge from a sewage treatment system was enriched in four reactors receiving inorganic medium containing 2, 5, 10 or 30 mM NH_4^+-N. Although the seed sludge used for all reactors was collected from the same system, AOB populations in the seed sludge varied slightly depending on the times they were taken (i.e. 5- and 10-mM NH_4^+-N reactors collected in August 2001; 2- and 30-mM NH_4^+-N reactors collected in February 2002). In both batches of seed sludge, sequence type 6a-34 of the *N. oligotropha* cluster was the dominant AOB, whereas the *N. europaea–Nc. mobilis* cluster and members within the *N. communis* cluster were not detected. A difference between the batches was found in sequence type 6a-1 of the *N. oligotropha* cluster; this AOB was not detected

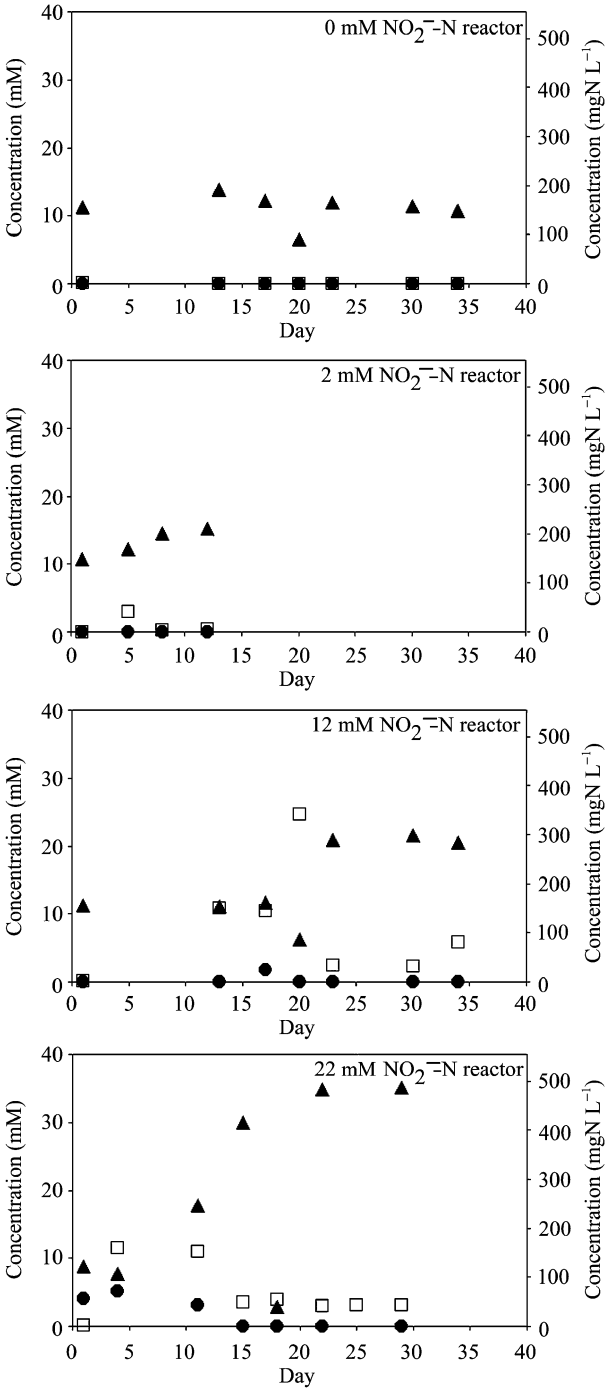

Fig. 2. Concentrations of ammonium (•), nitrite (□) and nitrate (▲) in the reactors receiving 10-mM-ammonium inorganic medium containing different additional nitrite concentrations.

in the seed sludge taken for the 2- and 30-mM NH_4^+-N reactors, but it was found in small numbers in the seed sludge taken for the 5- and 10-mM NH_4^+-N reactors (Table 2).

Analysis of AOB communities (Table 2 and Fig. 3) showed that all bands analyzed for the 2-mM NH_4^+-N

Table 2. Summary of related sequences of AOB in the reactors receiving inorganic medium containing different ammonium concentrations

	Reactor											
	2 mM NH_4^+			5 mM NH_4^+			10 mM NH_4^+			30 mM NH_4^+		
AOB cluster	0	15	40	0	15	40	0	15	40	0	15	40
Nitrosomonas europaea–Nitrosococus mobilis								+	+		+	+
Nitrosomonas oligotropha, sequence type 6a-1				+	+	+	+	+				
Nitrosomonas oligotropha, sequence type 6a-34	+	+	+	+	+	+	+	+	+	+		

0, 15 and 40; day 0, 15 and 40.

Table 3. Summary of related sequences of AOB in the reactors receiving 10-mM-ammonium inorganic medium containing different additional nitrite concentrations

	Reactor											
	0 mM NO_2^-			2 mM NO_2^-			12 mM NO_2^-			22 mM NO_2^-		
AOB cluster	0	15	30	0	15	30	0	15	30	0	15	30
Nitrosomonas europaea–Nitrosococus mobilis	+	+	+	+	+	+	+	+	+	+	+	+
Nitrosomonas oligotropha, sequence type 6a-1												
Nitrosomonas oligotropha, sequence type 6a-34	+	+	+	+	+	+	+	+	+	+	+	+

0, 15 and 30; day 0, 15 and 30

reactor related closely only to sequence type 6a-34 of the *N. oligotropha* cluster, whereas those for the 5-mM NH_4^+-N reactor were associated with sequence types 6a-1 and 6a-34 of the *N. oligotropha* cluster. In the 10-mM NH_4^+-N reactor, bands related closely to sequence types 6a-1 appeared only during the early operation period. After 24 days of operation, bands identified as *N. europaea* arose in this reactor and existed until the end of operation. In contrast to the other reactors, no band associated with *N. oligotropha* was recovered from the 30-mM NH_4^+-N reactor. Only bands related closely to *N. europaea* were detected in this reactor.

Results from the analysis of AOB populations (Fig. 4) revealed the numbers of each AOB species in the reactors. In the 2-mM NH_4^+-N reactor, sequence type 6a-34 of the *N. oligotropha* cluster was the predominant AOB during the whole operation. Numbers of sequence type 6a-1 of the *N. oligotropha* cluster, the *N. europaea–Nc. mobilis* cluster and members within the *N. communis* cluster were under the detection limits in this reactor. In the 5-mM NH_4^+-N reactor, sequence type 6a-34 comprised the majority of AOB populations during the whole operation. Sequence type 6a-1 existed in the seed sludge taken for the 5-mM NH_4^+-N reactor, and this sequence type was found in small numbers during the whole operation in this reactor. Numbers of the *N. europaea–Nc. mobilis* cluster and members within the *N. communis* cluster were under the detection limits in this reactor. The 10-mM NH_4^+-N reactor possessed a greater diversity of AOB species than any other reactors in the ammonium study. The AOB population in this reactor consisted of sequence type 6a-1, sequence type 6a-34 and *N. europaea*. Among these, sequence type 6a-34 comprised the majority of AOB populations during the whole operation. Sequence type 6a-1 existed in the seed sludge taken for the 10-mM NH_4^+-N reactor and continued in the early operation period, but this sequence type disappeared after 24 days of operation. Although numbers of *N. europaea* were under the detection limits in the seed sludge, this species' numbers rose significantly after 9 days of operation and persisted until the end of operation. Numbers of AOB within the *N. communis* cluster were under the detection limits in this reactor. In the 30-mM NH_4^+-N reactor, *N. europaea* comprised the majority of AOB populations during the whole operation. Sequence type 6a-34, the dominant AOB in the seed sludge, disappeared from this reactor after 24 days of operation. Numbers of sequence type 6a-1 and members within the *N. communis* cluster were under the detection limits in this reactor.

In most reactors in the ammonium study, the numbers of the dominant AOB quantified using the four primer sets for the particular AOB (sequence type 6a-34 of the *N. oligotropha* cluster in the 2-and 5-mM NH_4^+-N reactors and *N. europaea* in the 30-mM NH_4^+-N reactor) corresponded to the total AOB numbers quantified using the primers CTO189f and RT1r and the *Taq*Man probe TMP1. However, a discrepancy between the numbers of the dominant AOB (sequence type 6a-34 of the *N. oligotropha* cluster) and the total AOB numbers arose in the 10-mM NH_4^+-N reactor. The numbers of sequence type 6a-34 corresponded to total AOB numbers only during the early operation period. Between 9 and 24 days of operation, the numbers of this sequence type rose by one order of magnitude over the total

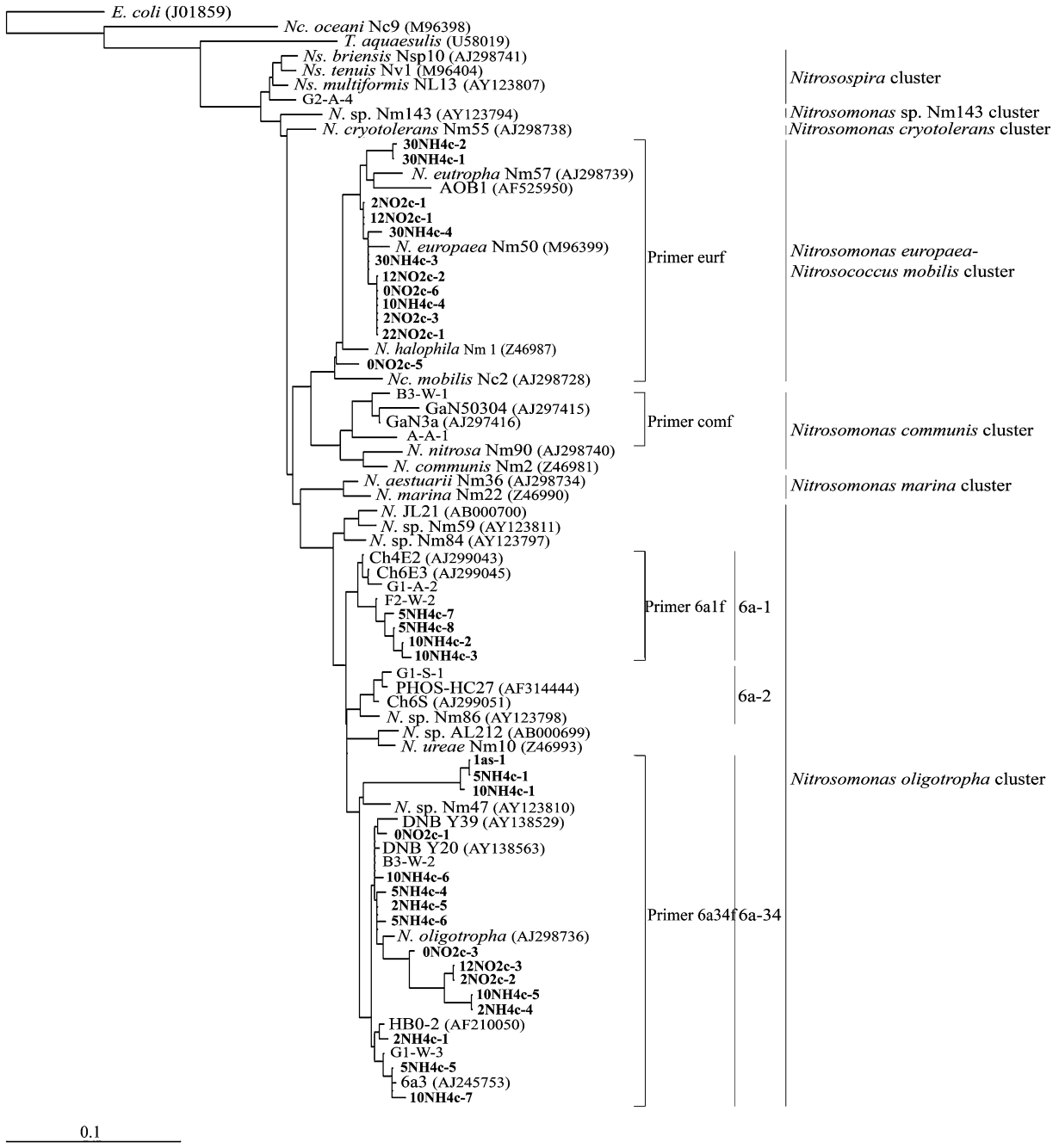

Fig. 3. Phylogenetic tree constructed on the basis of the 16S rRNA gene of AOB belonging to *Betaproteobacteria* by adding analyzed 442-bp sequences into the distance tree, prior constructed from comparison of 1000-bp sequences of described AOB species (Koops *et al.*, 2003) and some related non-AOB using the ARB program package. AOB genus abbreviations are *N.* for *Nitrosomonas*, *Nc.* for *Nitrosococcus* and *Ns.* for *Nitrosospira*. DGGE bands of this study are shown in bold, with the first characters indicating reactor name followed by band number (e.g. 30NH4c-2: band 2 of the 30-mM NH_4^+-N reactor).

AOB numbers. By checking the sequence match, it was found that sequence type 6a-34 in the 10-mM NH_4^+-N reactor (band $10NH_4$c-6 in Fig. 3) contained two mismatches at the 3′ end of the reverse primer RT1r. Therefore, the underestimated numbers of the total AOB may have arisen when the primers CTO189f and RT1r and the *Taq*-Man probe TMP1 were applied to the enriched culture from this reactor. In contrast, the sequence match showed that the sequence of band $10NH_4$c-6 completely matched the primer set for sequence types 6a-34 of the *N. oligotropha* cluster.

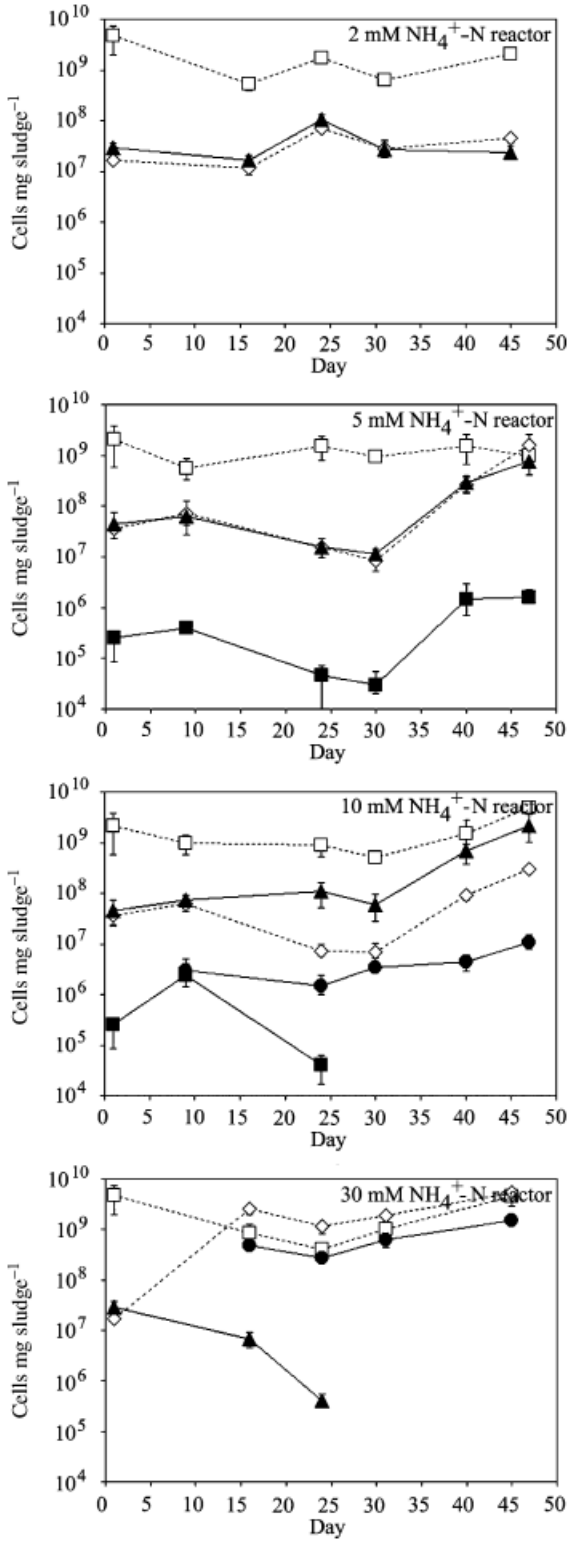

Fig. 4. Numbers of total bacteria (□), total AOB (◇) and particular AOB (■, sequence type 6a-1 of the *N. oligotropha* cluster; ▲, sequence types 6a-34 of the *N. oligotropha* cluster; •, *N. europaea–Nc. mobilis* cluster) in samples taken from the reactors receiving inorganic medium containing different ammonium concentrations.

This confirms that band 10NH_4c-6 is included in the quantification with this primer set.

Effect of nitrite on AOB communities and populations

To study the effect of nitrite, enriched culture from the 10-mM NH_4^+-N reactor was enriched in four reactors receiving 10-mM-ammonium inorganic medium containing 0, 2, 12 or 22 mM NO_2^--N. This study examined whether nitrite inhibited AOB with high ammonia affinity (e.g. *N. oligotropha* cluster) differently compared with those with low ammonia affinity (e.g. *N. europaea–Nc. mobilis* cluster). Because the ammonium study showed that both *N. oligotropha* and *N. europaea* coexisted in the 10-mM NH_4^+-N reactor, the enriched culture from this reactor was used as the seed for all reactors in the nitrite study. In addition, 10-mM-ammonium inorganic medium was added to all reactors in this study to provide the conditions under which both kinds of AOB can coexist.

Analysis of AOB communities (Table 3 and Fig. 3) showed that sequence type 6a-34 of the *N. oligotropha* cluster and *N. europaea* coexisted in all reactors in this study.

Although *N. oligotropha* and *N. europaea* coexisted in all reactors, analysis of AOB populations (Fig. 5) suggested that the numbers of each AOB species varied among the reactors. In the control and 2-mM NO_2^--N reactors, sequence type 6a-34 of the *N. oligotropha* cluster was the dominant AOB, whereas numbers of the *N. europaea–Nc. mobilis* cluster were one order of magnitude lower than sequence types 6a-34. In contrast, in the 12 and 22-mM NO_2^--N reactors, *N. europaea* became the dominant AOB population within 10 days of operation. In these two reactors, the numbers of *N. europaea* were one to two orders of magnitude higher than sequence type 6a-34. The numbers of sequence type 6a-1 of the *N. oligotropha* cluster and members within the *N. communis* cluster were under the detection limits in all reactors in this study.

Discussion

The ammonium study suggested that *N. oligotropha* was the predominant AOB in 2, 5, and 10-mM NH_4^+-N reactors, whereas *N. europaea* comprised the majority of AOB populations in 30-mM NH_4^+-N reactor (Fig. 6). The reason for the divergence of AOB species in different reactors in the ammonium study may be explained by the distinction of ammonia affinity among AOB species. Studies from isolated and mixed cultures revealed that *N. oligotropha* has lower *K* values (0.030–0.075 mM NH_4^+ plus NH_3) than other AOB (Stehr *et al.*, 1995), whereas *N. europaea* has higher *K* values (0.88–1.96 mM and 0.4–7.0 mM NH_4^+) (Prosser, 1989; Laanbroek *et al.*, 1994). This makes the *N. oligotropha* cluster the most common AOB found in oligotrophic environments,

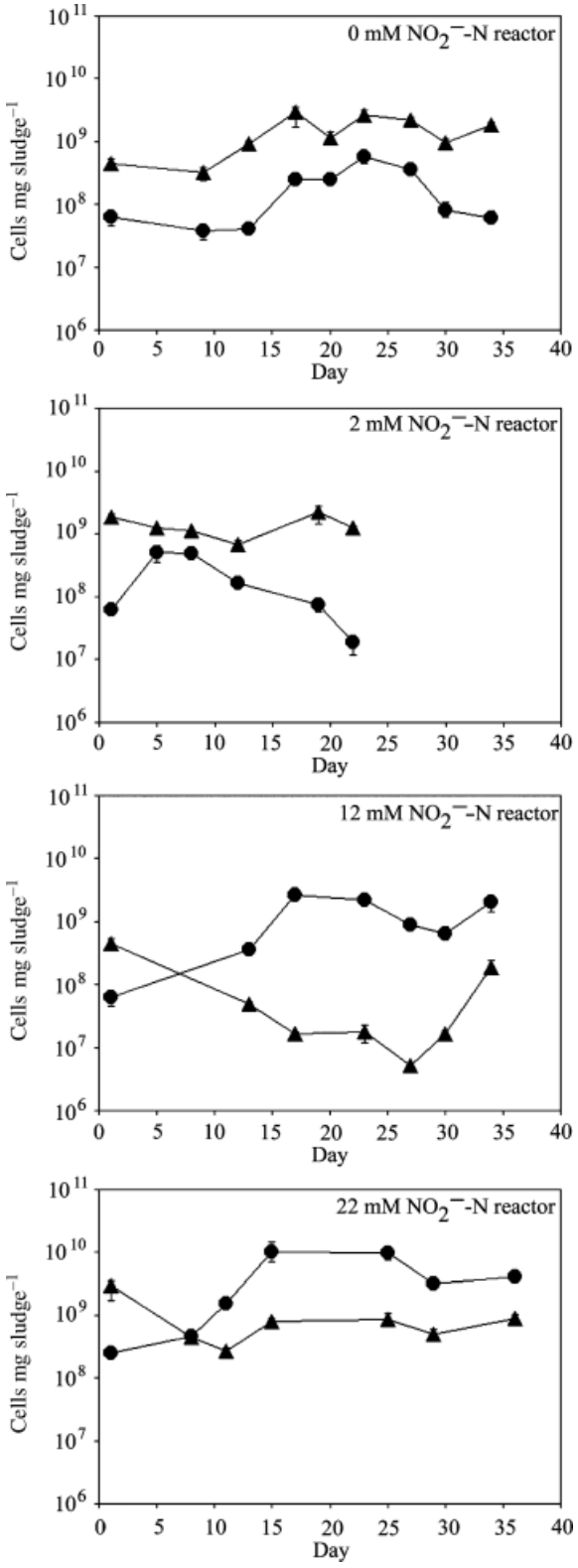

Fig. 5. Numbers of particular AOB (▲, sequence types 6a-34 of the *N. oligotropha* cluster; •, *N. europaea–Nc. mobilis* cluster) in samples taken from the reactors receiving 10-mM-ammonium inorganic medium containing different additional nitrite concentrations.

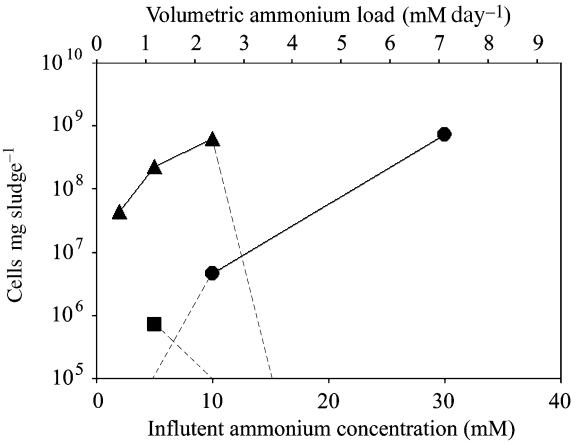

Fig. 6. Numbers of particular AOB (■, sequence type 6a-1 of the *N. oligotropha* cluster; ▲, sequence type 6a-34 of the *N. oligotropha* cluster; •, *N. europaea–Nc. mobilis* cluster) at the steady state in the reactors receiving inorganic medium containing different ammonium concentrations.

including wastewater treatment systems with low ammonium load (Ballinger *et al.*, 1998; Gieseke *et al.*, 2001; Dionisi *et al.*, 2002; Harms *et al.*, 2003; Limpiyakorn *et al.*, 2005, 2006b), freshwater sediment (Bollmann & Laanbroek, 2001) and chloraminated drinking water systems (Regan *et al.*, 2002, 2003), and *N. europaea* the most common AOB found in eutrophic environments. From this explanation, it is likely that ammonium concentration is the most important factor in the inclusion of AOB species in the reactors. However, in fact, the results from 5- and 30-mM NH_4^+-N reactors were in contradiction of the above statement. In the 5-mM NH_4^+-N reactor, although the ammonium concentrations at the steady-state condition were between 0.4 and 2.5 mM, nearly the level of *K* values of *N. europaea*, instead of the *N. europaea* cluster, the *N. oligotropha* cluster was the dominant AOB in this reactor throughout the experiment. In contrast, although those in the 30-mM NH_4^+-N reactor were almost always lower than the detection limit (0.07 mM), which were much lower that the *K* values of *N. europaea*, the *N. europaea* cluster predominated in this reactor. Therefore, the question arises of whether not only ammonium concentration but also volumetric ammonium load are the factors influencing the inclusion of AOB species in the reactors. As a result, it seemed that *N. oligotropha* was the predominant AOB when volumetric ammonium load was lower than 2.4 mM day^{-1} (33 mg N L^{-1} day^{-1}; 2-, 5- and 10-mM NH_4^+-N reactors), whereas *N. europaea* comprised the majority of AOB populations in the reactor with a volumetric ammonium load of 7.1 mM day^{-1} (100 mg N L^{-1} day^{-1}; 30-mM NH_4^+-N reactor). Although this study did not examine volumetric ammonium loads between 2.4 and 7.1 mM day^{-1} (33 and 100 mg N L^{-1} day^{-1}), *N. europaea* is likely the dominant AOB within this loading range, as they

began to appear in the reactor with a volumetric ammonium load of 2.4 mM day^{-1} (33 mg N L^{-1} day^{-1}; 10-mM $NH4^+$ reactor). In addition to ammonium, nitrite concentration may also result in the occurrence of distinct AOB species in the reactors. Only in the 30-mM NH_4^+-N reactor did nitrite accumulate (2.1–17.4 mM, 33–244 mg N L^{-1}); no accumulated nitrite was found in the 2-, 5- and 10-mM NH_4^+-N reactors. This level of nitrite may be responsible for the selection of the *N. europaea* cluster in the 30-mM NH_4^+-N reactor. Detailed effects of nitrite will be discussed further in the nitrite study.

The previous studies estimated the amounts of ammonium used for biomass synthesis to be 10% and 20% of those removed in full-scale municipal wastewater treatment systems (Daims *et al.*, 2001; Harms *et al.*, 2003). If the value of 10% was used for this study, ammonia-oxidizing activities per cell calculated based on the numbers of the dominant AOB (sequence types 6a-34 of the *N. oligotropha* cluster for the 2-, 5- and 10-mM NH_4^+-N reactors and *N. europaea* for the 30-mM NH_4^+-N reactor) will be 5.3, 18.7, 8.2 and 3.1 fmol cell^{-1} h^{-1}, respectively. All values are in the ranges of those reported previously: 9–23 fmol cell^{-1} h^{-1} for AOB isolates (Belser & Schmidt, 1980); 0.9–10.5 fmol cell^{-1} h^{-1} in 12 sewage treatment systems (solid residence time ranging from 3.9 to 16.3 days) during autumn (19–26 °C), estimated from real-time PCR quantification of 16S rRNA genes of total AOB (the primers CTO 189f and RT1r and the *Taq*Man probe TMP1) (Limpiyakorn *et al.*, 2005); and 7.7–12.4 fmol cell^{-1} h^{-1} in a municipal wastewater treatment system, estimated from real-time PCR quantification of 16S rRNA genes of total AOB (the primers CTO 189f and RT1r and the *Taq*Man probe TMP1) and *amoA* genes of *N. oligotropha*, respectively (Harms *et al.*, 2003). Surprisingly, in this study, although the growth rate of *N. europaea* seemed to be higher than that of *N. oligotropha*, one cell of *N. oligotropha* seemed to consume more ammonium than that of the *N. europaea* when the ammonia-oxidizing activities among the reactors were compared. This result may suggest the differences in the growth yield and perhaps the maintenance energy among distinct AOB species. If this proved to be the case it would have very important implications for how AOB and nitrification are modeled in the reactors with different levels of ammonium – currently a single value for these parameters is typically assumed, but if they change as community composition changes, there could be important consequences for the performance of wastewater treatment systems. Further study may need to clarify the detailed relationship between the ammonium level (ammonium concentration and volumetric ammonium load) and kinetic parameters (ammonia affinity, specific growth rate, and growth yield) including the ammonia-oxidizing activity per cell for different AOB species in wastewater treatment systems.

Also, this study suggested the effect of ammonium on AOB within the *N. oligotropha* cluster (sequence types 6a-1 and 6a-34; Fig. 4). By considering 5- and 10-mM NH_4^+-N reactors, sequence type 6a-34 survived and became the dominant AOB in a wider range of volumetric ammonium loads, whereas sequence type 6a-1 only existed in the minority in the reactor with the lower volumetric ammonium load (the 5-mM NH_4^+-N reactor). Previous studies showed that sequences of 6a-34 type have been recovered from a wide variety of environments, for example, in the aeration tank of a laboratory-scale single sludge nitrification-denitrification system with a 0.8-mM ammonium influent (clones DNB_Y20 and DNB_Y39) (Ballinger *et al.*, 1998), in freshwater sediment (clone 6a3) and in drinking water reservoirs (clone HBO-2). Recently, their numbers were revealed for the first time in activated sludge taken from 12 full-scale sewage treatment systems (Limpiyakorn *et al.*, 2006b). This study suggested that although the 12 systems differed in several aspects, including influent characteristics, treatment processes and system operation, the sequences of 6a-34 type were able to become dominant in all 12 systems. Sequence type 6a-1 was reported previously in freshwater sediment (Speksnijder *et al.*, 1998) and during continuous culture enrichment at the growth-limiting ammonium concentration of 0.005 mM (bands Ch4E2 and Ch6E3) (Bollmann & Laanbroek, 2001). Their numbers in activated sludge taken from 12 full-scale sewage treatment systems (Limpiyakorn *et al.*, 2006b) suggested that this AOB was sensitive to ammonium concentrations in the millimolar range.

Although no external organic carbon source was supplied, the majority of bacterial populations in 2-, 5- and 10-mM NH_4^+-N reactors were not AOB (Fig. 4). The coexistence of a number of heterotrophic bacteria with nitrifying bacteria was found in autotrophic environment without an external organic carbon source (Okabe *et al.*, 1999). This may be due to the fact that in this environment heterotrophic bacteria can use soluble microbial product produced by AOB and NOB for their growth (Kindaichi *et al.*, 2004).

The nitrite study showed that, although *N. oligotropha* and *N. europaea* occurred in all reactors, the numbers of each AOB species varied among the reactors (Fig. 5). Sequence type 6a-34 of the *N. oligotropha* cluster was the dominant AOB in the control and 2-mM NO_2^--N reactors, in which no nitrite was observed. In contrast, in the 12- and 22-mM NO_2^--N reactors, in which nitrite was over a range of from 2.1–5.4 mM (30–80 mg N L^{-1}), *N. europaea* was the dominant AOB. These results suggest that the growth and activity of sequence type 6a-34 may have been depressed by nitrite concentrations within the range of 2.1–5.4 mM (30–80 mg N L^{-1}), whereas this nitrite concentration inhibits *N. europaea* to a lesser extent. Thus, *N. europaea* was able to take over dominance from *N. oligotropha* and

complete the ammonia oxidation in the presence of nitrite, in spite of the fact that the ammonium load provided was more favorable for *N. oligotropha* than for *N. europaea*. Previous studies have shown that nitrite is capable of causing deterioration in ammonia oxidation by AOB (Muller *et al.*, 1995; Philips & Verstraete, 2001). At nitrite concentrations of 3–5 mM (42–70 mg N L^{-1}), ammonia oxidation rates were halved as compared with 0–2 mM (0–14 mg N L^{-1}) (Muller *et al.*, 1995). Most AOB populations in this previous study may have been *N. oligotropha*, as the seed sludge used was taken from sewage treatment systems and the medium used contained only 5 mM NH_4^+-N. Although a few studies have suggested the inhibitory effect of nitrite on the ammonia oxidation of AOB, thus far only one study has reported on this for each distinct AOB species (Suwa *et al.*, 1994). That study showed that the very high nitrite concentration of 21.4 mM NO_2^--N inhibited the ammonia-oxidizing activities of AOB isolates closely related to *N. oligotropha* but not *N. eutropha*.

In certain environments, nitrite accumulation occurs, and this may influence the occurrence of distinct AOB species. The most common circumstance may be in the culturing medium during isolation of AOB, in which nitrite accumulates as the condition provided encouragement to the growth of AOB over other microorganisms including NOB. When AOB with moderate to high affinity to ammonia, such as members within *N. oligotropha* cluster, are targets for isolation, the accumulated nitrite in the culturing medium may inhibit their growth and make isolation difficult. As a result, special attention should be paid to avoid nitrite accumulation during isolation of this AOB. Another example is in the novel nitrogen removal processes, such as SHARON (Hellinga *et al.*, 1998). In these processes, partial nitrification is promoted to oxidize ammonia over nitrite, causing high nitrite accumulation in the processes. Evidence from molecular analysis suggested that in the SHARON process, *Nitrosomonas eutropha*, a member of *N. europaea* cluster, predominated (Logemann *et al.*, 1998). However, as the application of this process is limited to only wastewater that is high in ammonium, the explanation for the occurrence of the *N. eutropha* in this process should be attributed to the high ammonium load rather than the accumulated nitrite.

In summary, this study clarified detailed effects of ammonium and nitrite on AOB from an activated sludge process in laboratory-scale continuous-flow reactors. The study on the effect of ammonium demonstrated that *N. oligotropha* predominated in the reactors with lower ammonium load, whereas *N. europaea* was the dominant AOB in the reactor with the highest ammonium load. The study on the effect of nitrite suggested that accumulated nitrite concentrations of 2.1–5.4 mM (30–80 mg N L^{-1}) more significantly inhibited the growth and activity of *N. oligotropha* than those of *N. europaea*.

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