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Communities of ammonia-oxidizing bacteria in activated sludge of various sewage treatment plants in Tokyo

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Abstract

We investigated ammonia-oxidizing bacteria in activated sludge collected from 12 sewage treatment systems, whose ammonia removal and treatment processes differed, during three different seasons. We used real-time PCR quantification to reveal total bacterial numbers and total ammonia oxidizer numbers, and used specific PCR followed by denaturing gel gradient electrophoresis, cloning, and sequencing of 16S rRNA genes to analyze ammonia-oxidizing bacterial communities. Total bacterial numbers and total ammonia oxidizer numbers were in the range of 1.6×10^{12}–2.4×10^{13} and 1.0×10^{9}–9.2×10^{10} cells l^{-1}, respectively. Seasonal variation was observed in the total ammonia oxidizer numbers, but not in the ammonia-oxidizing bacterial communities. Members of the *Nitrosomonas oligotropha* cluster were found in all samples, and most sequences within this cluster grouped within two of the four sequence types identified. Members of the clusters of *Nitrosomonas europaea–Nitrosococcus mobilis*, *Nitrosomonas cryotolerans*, and unknown *Nitrosomonas*, occurred solely in one anaerobic/anoxic/aerobic (A2O) system. Members of the *Nitrosomonas communis* cluster occurred almost exclusively in association with A2O and anaerobic/aerobic systems. Solid residence time mainly influenced the total numbers of ammonia-oxidizing bacteria, whereas dissolved oxygen concentration primarily affected the ammonia-oxidizing activity per ammonia oxidizer cell.

Keywords: Activated sludge; Ammonia-oxidizing bacteria; Real-time PCR; Sewage treatment; 16S rRNA gene sequences

1. Introduction

Nitrification, the two-step process by which ammonia is oxidized to nitrate via nitrite, plays a key role in the biological removal of nitrogen in wastewater treatment systems. The process involves two phylogenetically unrelated groups of obligately chemolithotrophic bacteria. Ammonia-oxidizing bacteria (AOB) first oxidize ammonia to nitrite, and subsequently nitrite-oxidizing bacteria oxidize nitrite to nitrate by. Because of the slow growth rate of AOB, their high sensitivity to many environmental factors, and their inability to outcompete heterotrophs, ammonia oxidation is a rate-limiting step of nitrogen removal in wastewater treatment systems [1]. For this reason, a better understanding of the ecology and microbiology of ammonia-oxidizing bacteria in wastewater treatment systems is necessary to enhance treatment performance and control.

Recently developed molecular tools include sequence analysis of the 16S rRNA and *amoA* genes to reveal AOB populations and communities in various environments. In combination with clone libraries or denaturing

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gradient gel electrophoresis (DGGE), the application of specific PCR amplification [2–4] provides clarification of the ammonia oxidizing community in detail. Implementation of fluorescence in situ hybridization (FISH) [1,5–9] makes it possible to analyze complex communities of ammonia-oxidizing bacteria and estimate their numbers. More recently, PCR-based quantification techniques allow precise enumeration of AOB populations in the environments [10–12].

The distribution patterns of distinct AOB species in the environments reflect the physiological properties of AOB isolates observed in the laboratory [13]. Among these, ammonia seems to be the most important factor for the inclusion of distinct AOB species, while other factors such as salinity are also reported to influence their appearance in the environments [6,13–15]. In general, members of the *Nitrosospira* spp. or/and the *Nitrosomonas oligotropha* clusters are the dominant ammonia-oxidizing bacteria in the environments low in ammonia, whereas members of the *Nitrosomonas europaea–Nitrosococcus mobilis* cluster comprise the majority of AOB in the environments that are rich in ammonia.

Although a number of studies have assessed the ecology and microbiology of ammonia-oxidizing bacteria in wastewater treatment systems [1,5–9,11,12,15–18], our understanding of those in the sewage treatment systems is still uncertain. The effects of other factors than ammonia on individual ammonia oxidizer species in sewage treatment systems are poorly understood. AOB found in these low-ammonia systems are often cited as being the same bacteria. Some individual species are overlooked by representing their characters by those of the only few common members of the groups. However, their characteristics may differ, and they may be influenced by distinct factors in the systems.

Because of these concerns, we investigated ammonia-oxidizing bacteria in activated sludge collected from 12 sewage treatment systems. We used real-time PCR quantification to reveal total bacterial numbers and total AOB numbers and we used specific PCR amplification followed by DGGE, cloning, and sequencing of 16S rRNA genes to identify members of AOB communities. We focused on the effects of influent characteristics, treatment processes, system operation, and seasonal variation on the total AOB numbers and AOB communities.

2. Materials and methods

2.1. Samples of sewage activated sludge and description of sewage treatment systems

Activated sludge samples were collected from the aeration tanks of 12 sewage treatment systems. These systems are in use in eight sewage treatment plants in Tokyo, which are run by the Bureau of Sewerage, Tokyo Metropolitan Government, Japan. The 12 systems differed in ammonia removal and were operated with different treatment processes: anaerobic/anoxic/aerobic (A2O); anaerobic/aerobic (AO); and conventional activated sludge (AS) processes. Samples were collected from the 12 systems during three different seasons: summer (August 2001); autumn (November 2001); and winter (February 2002). Mixed-liquor suspended solids (MLSS) concentrations were determined on the day of sampling. The sludge from approximately 2 mg of MLSS 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 °C until analysis.

Details of the treatment processes, influent and effluent characteristics, removal efficiencies, and operational parameters of the 12 systems are listed in Table 1. Systems B1, B2, and B3, systems F1 and F2, and systems G1 and G2 were located in plants B, F, and G, respectively. Plant B received sewage from a single sewer line, and the sewage was split among systems B1, B2, and B3 for treatment. In contrast, multiple sewer lines entered plants F and G; as a result, the various systems in both plants received different sewage. However, the characteristics of the influents were expected to be similar because the areas from which the sewages were collected were near each other. The treatment processes of the 12 systems varied: systems A and B1 are A2O processes; systems B2, C, D, and E are AO processes; and systems B3, F1, F2, G1, G2, and H are AS processes.

Biological oxygen demand (BOD) in the influents ranged from 34 to 141 mg l^{-1}, while ammonium concentrations were between 12 and 30 mg N l^{-1}. The characteristics of the influents did not vary notably among the systems, except for system A. This system was associated with influent ammonia concentrations of 26–30 mg N l^{-1} and chloride concentrations that were double those of other systems. These differences arose because system A received sewage mostly from commercial areas without rainwater, whereas the other systems served household areas and received combined sewage. In addition, the location of system A, an artificial island in the sea, might tend to increase the chloride concentration in the influent of this system.

BOD removal efficiencies were excellent ($\geqslant$95%) in all systems; however, ammonia removal efficiencies differed among them. Completed ammonia removal was achieved in systems A, B1, B2, D, F2, and G1. Ammonia removal was poor in systems G2 and H, possibly because of insufficient oxygen. Ammonia concentrations in the effluents varied according to the difference of ammonia removal among the systems. Nitrite concentrations in the effluents were less than 2 mg N l^{-1} and pH were maintained between 6.2 and 7.4 in all systems.

Temperature in the 12 systems ranged from 14 to 22 °C in winter to 27 to 31 °C in summer. No marked

Table 1
Treatment processes, influent- and effluent characteristics, removal efficiencies, and operational parameters of 12 sewage treatment systems; information provided by Tokyo Metropolitan Government

Parameters	Unit	System											
		A	B1	B2	B3	C	D	E	F1	F2	G1	G2	H
Treatment process		A2O	A2O	AO	AS	AO	AO	AO	AS	AS	AS	AS	AS
Aeration tank volume	m^3	6400	7450	11,175	44,700	10,230	11,760	31,050	15,520	37,840	97,200	268,800	202,320
Volumetric flow to aeration tank	$m^3\ m^{-3}\ d^{-1}$	1.8–2.1	2.8–3.4	2.3–2.8	2.4–3.0	2.2–4.6	2.2–2.5	3.3	2.6–3.0	4.1–4.9	3.5–4.1	3.0–3.8	2.6–2.9
BOD in influent[a]	$mg\ l^{-1}$	91–139	53–141	53–141	53–141	80–133	64–132	65–100	67–90	63–111	74–103	83–94	34–73
NH_4-N in influent[a]	$mg\ l^{-1}$	26–30	13–16	13–16	13–16	20–24	14–18	12–18	15–18	15–18	18–19	18–21	14–17
Cl^-in influent[a]	$mg\ l^{-1}$	140–180	54–80	54–80	54–80	63–80	40–58	80–90	–[c]	–[c]	89–94	89–98	52–72
NH_4-N in effluent[a]	$mg\ l^{-1}$	0	0	0–1	0–2	3–12	0	1–2	0–6	0–1	0	12–19	4–16
NO_2-N in effluent[a]	$mg\ l^{-1}$	0	0	0	0–1	1	0	0	0–1	0	0	0–2	0–1
BOD removal[a]	%	98–99	98–99	97–99	97–99	95–96	99–100	98–99	96–99	97–98	97–99	95–97	95–97
NH_4^+-N removal[a]	%	100	98–100	96–99	87–97	40–90	100	85–97	69–98	97–100	100	0–40	7–89
Volumetric NH_4^+-N removal[a]	$g\ m^{-3}\ d^{-1}$	42–44	33–34	26–30	24–31	18–50	28–33	27–44	32–34	54–60	49–60	0–13	0–20
HRT[b]	h	24.9	16.1	12.7	9.2	7.3	14.0	9.8	8.5	5.4	6.3	7.3	9.0
SRT[b]	Day	16.5	14.2	8.6	7.2	4.6	12.6	7.9	6.3	11.7	5.7	4.5	7.1
MLSS[b]	$mg\ l^{-1}$	2122	1629	1422	1256	1085	1249	1844	1339	1698	1159	1009	1088
MLDO[b]	$mg\ l^{-1}$	4.1	1.8	2.2	2.3	3.4	5.3	5.8	6.5	6.7	6.5	1.8	1.6

HRT, hydraulic retention time; SRT, solid residence time; MLSS, mixed liquor suspended solid; MLDO, mixed liquor dissolved oxygen.

[a] All influent characteristic, effluent characteristic, and removal efficiency values were analyzed from one-day grab samples collected on the day close to the day of sludge collection.

[b] All operational parameters were the averages of the three months in which sludge was collected.

[c] Data not available.

seasonal variations in influent characteristics, removal efficiencies, or operational parameters were observed in all systems throughout this study. However, solid residence time (SRT) and dissolved oxygen (DO) concentration in system H varied during the studied seasons, and these variations will be discussed later.

2.2. DNA extraction

DNA was extracted directly from samples using FastDNA SPIN kits for soil (Bio 101, Vista, CA, USA) with a small modification at the initial step: 1 ml of sodium phosphate buffer solution was added to and mixed with the sample, and then the tube was sonicated for 30 s on ice. The remaining steps followed the manufacturer's instructions. The product from DNA extraction was verified by electrophoresis in 1% agarose (TaKaRa LO3, Tokyo, Japan).

To minimize the variation in DNA extraction, the templates used for real-time PCR quantification were prepared from the mixture of DNA, which was extracted in triplicate for a sample.

2.3. Real-time PCR quantification of total bacteria and total AOB

Real-time PCR quantification of 16S rRNA genes of total bacteria was performed using the primers 1055f and 1392r and the *Taq* Man probe 16Staq1115 as previously described [12]. The oligonucleotide sequences of the primers and the *Taq* Man probe are shown in Table 2. The PCR mixture was prepared in a total volume of 25 μl using the *Taq* Man Universal PCR Master Mix kit (PE Applied Biosystems), 15 pmol of forward primer 1055f, 15 pmol of reverse primer 1392r, 6.25 pmol of *Taq* Man probe 16Staq1115, and standard DNA or extracted DNA from samples. The standard DNA, which was the pT7Blue T-Vector (Novagen, Darmstadt, Germany) possessing 16S rRNA gene of *N. europaea*, was prepared ranging from 5×10^1 to 5×10^7 copies. The extracted DNA from a sample was prepared for three different 10-fold dilutions, and each of the dilutions was real-time PCR quantified in duplicate. PCR amplification was performed in an ABI Prism SDS 7000 instrument (PE Applied Biosystems) under conditions of 3 min at 50 °C and 10 min at 95 °C followed by 45 cycles of 30 s at 95 °C, 1 min at 50 °C, and 20 s at 72 °C. To calculate the cell numbers of bacteria from the quantified numbers of 16S rRNA gene, it is assumed that the average 16S rRNA gene copies per genome of bacterial cell is 3.6 based on the average 16S rRNA gene copies found in cultured bacteria [19].

Real-time PCR quantification of 16S rRNA genes of total AOB was performed using the primers CTO 189f and RT1r and the *Taq* Man probe TMP1 as described earlier [10]. The oligonucleotide sequences of the primers and the *Taq* Man probe are shown in Table 2. The PCR mixture was prepared in a total volume of 25 μl using the *Taq* Man Universal PCR Master Mix kit, 7.5 pmol of a 2:1 ratio of forward primers CTO 189fA/B and CTO 189fC, 7.5 pmol of reverse primer RT1r, 3.125 pmol of *Taq* Man probe TMP1, and standard DNA or extracted DNA from samples. The standard DNA, which was the pT7Blue T-Vector possessing 16S rRNA gene of *N. europaea*, was prepared in a range of 4×10^1 to 4×10^7 copies. The extracted DNA from a sample was prepared for three different 10-fold dilutions, and each of the dilutions was real-time PCR quantified in duplicate. PCR amplification was performed in an ABI Prism SDS 7000 instrument under conditions of 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C and 1 min at 60 °C. To calculate the cell numbers of ammonia-oxidizing bacteria from the quantified numbers of 16S rRNA genes, it is assumed that AOB possess one copy of 16S rRNA gene per genome [20].

2.4. PCR, DGGE, cloning, sequencing, and phylogenetic analysis

We used the primers CTO189f and CTO654r [2] to amplify 465-bp fragments of 16S rRNA genes of AOB

Table 2
Primers and probes used in this study

Primer or probe	Nucleotide sequence (5′–3′)	Reference
Primers		
1055f	ATGGCTGTCGTCAGCT	[12]
1392r	ACGGGCGGTGTGTAC	[12]
CTO 189A/Bf	GGAGRAAAGCAGGGGATCG	[2]
CTO 189Cf	GGAGGAAAGTAGGGGATCG	[2]
RT1r	CGTCCTCTCAGACCARCTACTG	[10]
CTO 654r	CTAGCYTTGTAGTTTCAAACGC	[2]
Probes[a]		
16STaq1115 (5′-FAM and 3′-TAMRA)	CAACGAGCGCAACCC	[12]
TMP1 (5′-FAM and 3′-TAMRA)	CAACTAGCTAATCAGRCATCRGCCGCTC	[10]

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine.

belonging to Betaproteobacteria. The oligonucleotide sequences of the primers are shown in Table 2. The extracted DNA was PCR-amplified using the primer set (the forward primer had a GC clamp) for 35 cycles in a 50-μl reaction volume. DNA eluted from bands excised from DGGE gels and colonies picked directly after cloning were amplified for 20–25 cycles using the primer set lacking the GC clamp in a 50-μl reaction volume. The PCR mixture was prepared using Ampli*Taq* Gold DNA polymerase (PE Applied Biosystems, CA, USA) following the manufacturer's instructions with 1 pmol of each primer. PCR amplification was performed in a T3 thermocycler (Biometra, Gottingen, Germany) under the conditions of 5 min at 95 °C followed by 35 cycles of 30 s at 92 °C, 30 s at 57 °C, and 45 s at 72 °C (+1 s each cycle), followed by 5-min final extension at 72 °C.

DGGE was performed according to the modification of a described method [21]. We used 8% polyacrylamide gels, and the urea–formamide denaturant gradient was 35–50%. Gels were run on the D Code system (Bio-Rad Laboratories, Hercules, CA, USA) for 16 h at 60 °C and 75 V. After electrophoresis, the gels were stained with Vistra Green (Amersham Pharmacia Biotech, Tokyo, Japan) and visualized with a fluorescent image scanner (Fluorimager 595, Molecular Dynamics, Sunnyvale, CA, USA). Prominent bands were excised and dissolved in 30 μl sterilized water. DNA was recovered from the gel by freeze–thawing three times.

Each target fragment of DNA recovered from the DGGE gel was purified by cloning with pT7Blue T-Vector and DH5-α competent cells (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. Positive clone colonies were amplified directly using the primer set with the additional GC-clamp, and the products again were subjected to DGGE to check their migration. The target DNA fragments were then excised and reamplified. Before sequencing, small DNA fragments and excess primers were removed from the PCR products by Microcon spin columns (Millipore, Tokyo, Japan). The purification procedure followed the manufacturer's instructions.

Sequencing reactions were run according to the manufacturer's instructions with 20 ng of the PCR product, the ABI Big Dye Terminator kit version 3.1 (PE Applied Biosystems), and the CTO primer set. After excess primers and dye terminators from the products of sequencing reaction were removed using Centri-sep spin columns (PE Applied Biosystems), the products were analyzed in an ABI 310 DNA sequencer (PE Applied Biosystems).

The completed 397-bp from 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]). Phylogenetic tree was constructed using the ARB program package. We added our 397-bp sequences into the distance tree, which was previously constructed based on comparison of 1000-bp sequences of all AOB, which are available in the SSU rRNA database, and some related non-AOB, which were used as outgroup sequences. Additionally, our 397-bp sequences and 397-bp sequences of described AOB species [22] and some related non-AOB were calculated based on 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).

2.5. Accession numbers for nucleotide sequences

The partial sequences of 16S rRNA genes obtained in this study were submitted to the DDBJ database under accession numbers AB176856–AB176884.

3. Results

3.1. Total bacterial numbers and total AOB numbers

We used real-time PCR quantification to reveal total bacterial numbers and total AOB numbers in samples of sewage activated sludge. Fig. 1 shows total bacterial numbers in the aeration tanks of the 12 systems. Total bacterial numbers were in a range of 1.6×10^{12}–2.4×10^{13} cells l^{-1}. Fig. 2 shows total AOB numbers, percentage of total AOB in total bacterial populations, and ammonia-oxidizing activities per cell of AOB in the aeration tanks of the 12 systems. Total AOB numbers were between 1.0×10^{9} and 9.2×10^{10} cells l^{-1}, and can be accounted for 0.01–2.8% of total bacterial populations. Ammonia-oxidizing activities per cell were calculated from the total AOB numbers and volumetric ammonia removal in the aeration tanks. The ammonia-oxidizing activities per cell were ranging from 0 to 49.6 fmol $cell^{-1}$ h^{-1}.

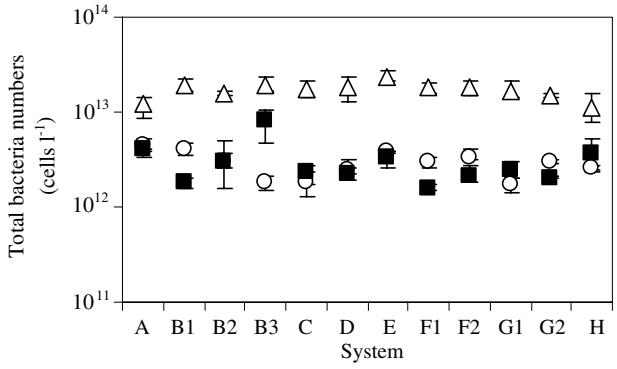

Fig. 1. Total bacterial numbers in aeration tanks of 12 sewage treatment systems: ○, summer; ■, autumn; △, winter.

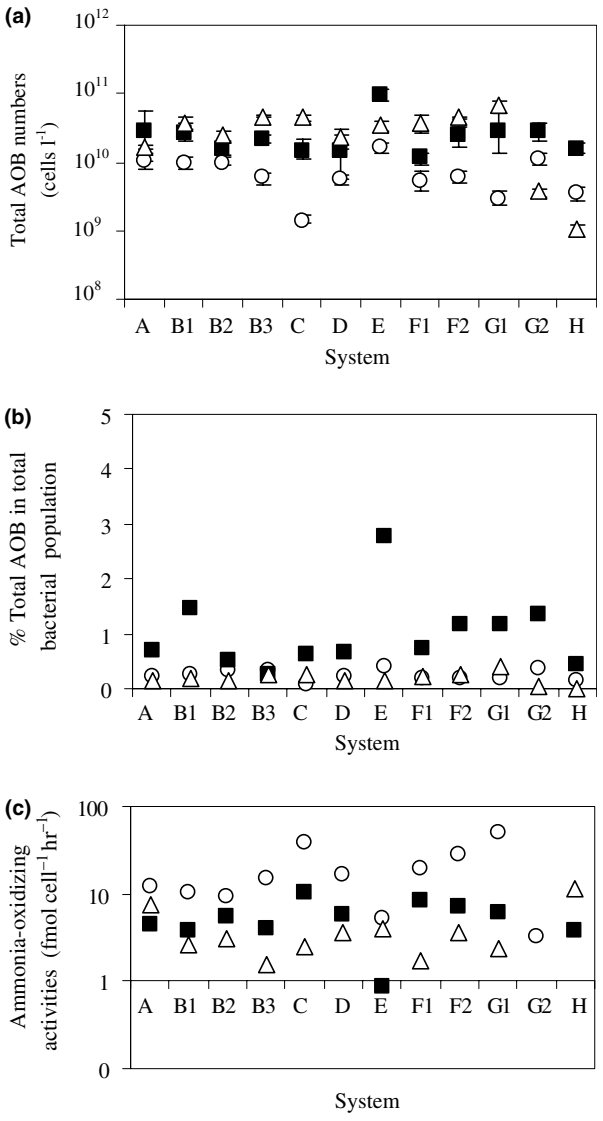

Fig. 2. Total AOB numbers (a), proportion of total AOB within total bacterial populations (b), and ammonia-oxidizing activities per AOB cell (c) in aeration tanks of 12 sewage treatment systems: ○, summer; ■, autumn; △, winter.

3.2. Analysis of samples of sewage activated sludge by specific PCR amplification followed by DGGE, cloning, and sequencing of 16S rRNA genes

We used specific PCR amplification followed by DGGE, cloning, and sequencing of 16S rRNA genes to reveal the structure of AOB communities in the samples of sewage activated sludge. Analysis of duplicate samples by DGGE showed that the band patterns obtained were reproducible (data not shown). Fig. 3 shows the image of a DGGE gel of the PCR-amplified products of samples taken from the 12 systems in autumn (November 2001). On the right side of the figure, the locations of the bands excised are labelled with the names of the AOB clusters identified. Because the locations of the bands excised differed for system A, which led to different sequences and thus identification of different AOB clusters, the names of the AOB clusters are labelled separately on the left side of the figure. After the PCR-amplified products of all samples had been electrophoresed in DGGE gels, a total of 81 representative bands were selected for sequencing. To prevent the confusion with the bands selected directly from the original gel images, all bands were excised from the gels, reamplified, and analyzed on the new gels to clarify an individual band position before selection for sequencing.

We constructed phylogenetic trees using several treeing methods. Fig. 4 shows a phylogenetic tree based on 16S rRNA genes of AOB belonging to Betaproteobacteria. All the tree methods resulted in the same grouping of AOB sequences analyzed. In addition, the grouping of AOB clusters in the trees remained the same except for the "unknown-*Nitrosomonas*" cluster, the positions of which varied with the method used. The unknown-*Nitrosomonas* cluster can be included into *N. oligotropha* cluster or can be recognized as another independent cluster within *Nitrosomonas* spp. The sequences of many of the bands analyzed reveal their relation to the *N. oligotropha* cluster (*Nitrosomonas* cluster 6a). Because many

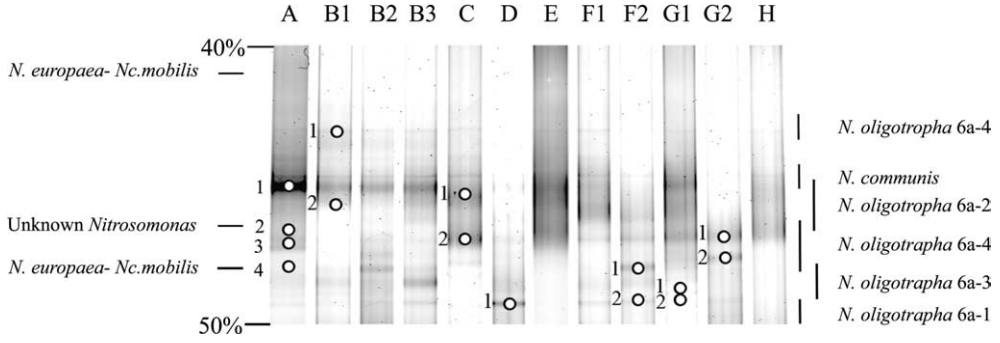

Fig. 3. DGGE images of PCR-amplified products of samples taken from 12 sewage treatment systems in autumn (November 2001). Marks and numbers indicate excised bands from which sequences were determined. AOB genus abbreviations are *N.* for *Nitrosomonas* and *Nc.* for *Nitrosococcus*.

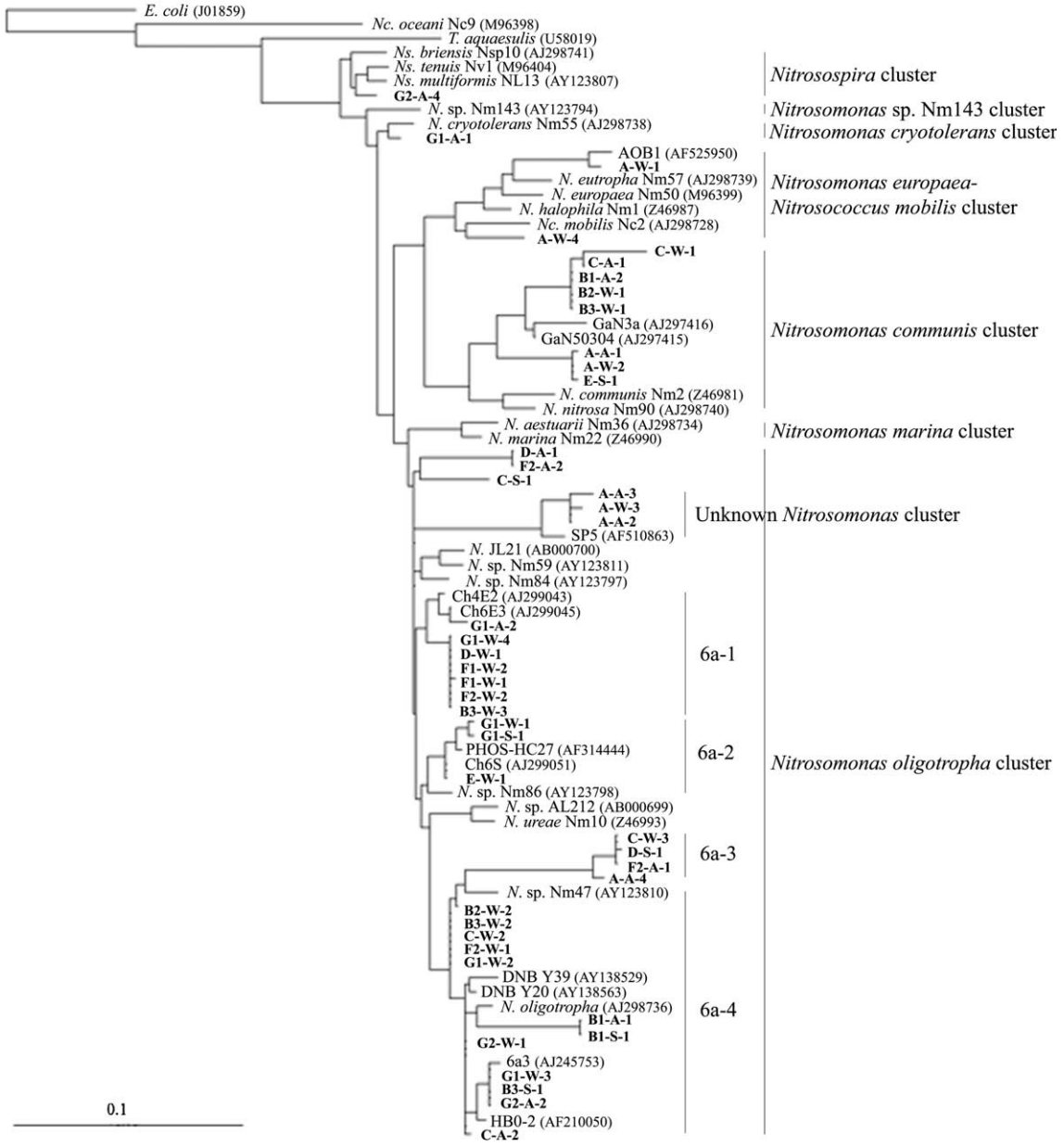

Fig. 4. Phylogenetic tree showing 16S rRNA genes of AOB belonging to Betaproteobacteria with addition of 397-bp sequences from our study into the distance tree that was previously based on comparison of 1000-bp sequences of described AOB [22] using the ARB program package. AOB genus abbreviations are *N.* for *Nitrosomonas*, *Nc.* for *Nitrosococcus*, and *Ns.* for *Nitrosospira*. Codes of the DGGE bands of this study are shown in bold; the first character indicates the sample name, the second character denotes the season (S, summer, A, autumn, W, winter, respectively), followed by the band number. AOB clusters are depicted on the right side of the tree.

sequences that are affiliated to this cluster are distributed in various environments and their properties somewhat differ, for the purpose of further discussion, the sequences identified as associated with this cluster were allocated into four sequence types based on the grouping of the AOB sequences with several treeing methods. In the previous study [14], members of the *N. oligotropha* cluster that originated from enriched brackish and freshwater were separated into three sequence types. Because the sequences found in this study differed from those of the previous one with respect to their source, the classification system of the previous study is not applicable for this one. In our classification scheme, *N. ureae* (another representative of cluster 6a) are not associated with a particular sequence type because they were not closely related to any other sequence found in this study. The ability of the primers CTO189f and CTO654r to amplify all clusters of AOB belonging to Betaproteobacteria was confirmed when members of all AOB clusters, except those of *Nitrosomonas marina* and the *N.* sp. Nm143,

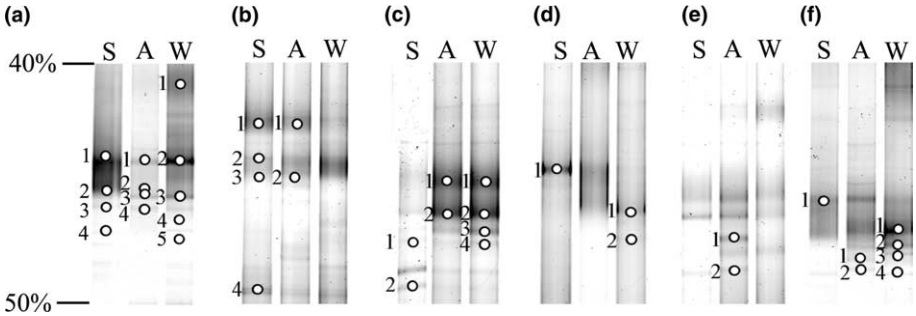

Fig. 5. DGGE images of the PCR-amplified products of samples taken from sewage treatment systems during three seasons to illustrate the seasonal variation in the AOB communities: (a) system A, (b) system B1, (c) system C, (d) system E, (e) system F2, and (f) system G1. The character above each lane (S, A, or W) stands for season (summer, autumn, or winter respectively). Marks and numbers indicate the excised bands from which sequences were determined.

were recovered from the samples. Most of the AOB sequences analyzed showed 96–99% identity to the AOB sequences available in the database. However, a few sequences, which were closely related to *N. communis*, all sequences of the unknown-*Nitrosomonas* cluster, and some type 6a-3 sequences of the *N. oligotropha* cluster, showed slightly lower homology (95%).

3.3. Effect of seasonal variation on AOB communities

PCR-amplified products of samples taken during different seasons were analyzed on the same denaturing gel to highlight the effects of seasonal changes on the communities of ammonia-oxidizing bacteria (Fig. 5). Most systems (except B3, E, F2, and G2) exhibited similar band patterns for the three seasons. Moreover, the sequence analysis showed that the AOB communities of most systems were nearly identical regardless of the season (Table 3); there were no seasonal variations in the common AOB, and only particular AOB exhibited the seasonal variation in some systems.

3.4. Distribution of AOB in sewage treatment systems

Table 3 lists and summarizes the related AOB sequences found in each sample. Most of the bands analyzed were related to *Nitrosomonas* spp. rather than *Nitrosospira* spp. The highest number of bands analyzed grouped within the *N. oligotropha* cluster. Members of this cluster were found in all samples. Within this cluster, sequence types 6a-1 and 6a-4 were found in most samples. Only system A contained members of the *N. europaea–Nc. mobilis* cluster *and* the unknown-*Nitrosomonas* cluster. Members of the *N. cryotolerans* cluster were present almost solely in system A and appeared only during some seasons in systems D, F2, and G1. Further, systems A, B1, B2, B3, C, D, and E contained members of the *N. communis* cluster; interestingly, all of these systems (except B3) were operated as either A2O or AO processes. It is not possible to discern a trend in the relationship between the sewage activated sludge system and the presence of the *Nitrosospira* cluster, which was recovered from only one sample.

4. Discussion

The total bacterial numbers in a range of 1.6×10^{12}–2.4×10^{13} cells l^{-1} in the 12 systems (Fig. 1) fairly corresponded to the numbers mentioned earlier in a municipal wastewater treatment system: 4.55×10^{12}–1.04×10^{13} cells l^{-1} by dot blot hybridization [11]; $4.3 \pm 2.0 \times 10^{11}$ cells l^{-1} by real-time PCR quantification based on 16S rRNA genes of total bacteria (the primers 1055f and 1392r and the *Taq* Man probe 16STaq1115) [12].

The total AOB numbers between 1.0×10^{9} and 9.2×10^{10} cells l^{-1} in the 12 systems can be accounted for 0.01–2.8% of total bacterial populations (Fig. 2(a) and (b)). Checking the primer and probe matches shows that the reverse primer RT1r and the *Taq* Man probe TMP1 completely target most AOB sequences analyzed. This confirms that most AOB sequences analyzed were included in the real-time PCR quantification using this primer set. The total numbers of ammonia-oxidizing bacteria in the 12 systems were compatible to the numbers reported previously in a municipal wastewater treatment system: $1.2 \pm 0.9 \times 10^{10}$ cells l^{-1} (2.9% of total bacterial populations) by real-time PCR quantification based on 16S rRNA genes of total AOB (the primers CTO 189f and RT1r and the *Taq* Man probe TMP1) and $7.5 \pm 6.0 \times 10^{9}$ cells l^{-1} (1.7% of total bacterial populations) by real-time PCR quantification based on *amoA* genes of the *N. oligotropha* [12]. The total numbers of ammonia-oxidizing bacteria varied among the seasons studied; the largest population size occurred during winter, followed by autumn and summer (Fig. 2(a)). However, a proportion of total AOB within the total bacterial populations increased (0.5–3%) during autumn, whereas in summer and winter it remained the same (0.01–1.1%) (Fig. 2(b)). The increased

Table 3
Summary of related sequences of ammonia-oxidizing bacteria in samples of 12 sewage treatment systems

AOB cluster	System																							
	A			B1			B2			B3			C			D			E			F1			F2			G1			G2			H		
	S	A	W	S	A	W	S	A	W	S	A	W	S	A	W	S	A	W	S	A	W	S	A	W	S	A	W	S	A	W	S	A	W	S	A	W
Nitrosospira sp.																														+						
Nitrosomonas communis	+		+		+			+			+			+				+			+														
Nitrosomonas europaea–Nitrosococus mobilis	+	+	+															+			+														
Nitrosomonas cryotolerans	+	+														+							+			+									
Unknown *Nitrosomonas*	+	+																																		
Nitrosomonas oligotropha, sequence type 6a-1				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+	+	+	+	+						
Nitrosomonas oligotropha, sequence type 6a-2																			+	+	+										+	+	+			
Nitrosomonas oligotropha, sequence type 6a-3	+	+	+							+		+	+	+	+	+	+	+						+												
Nitrosomonas oligotropha, sequence type 6a-4				+	+	+	+	+	+	+	+	+	+	+	+										+	+	+	+	+	+	+	+	+	+	+	+

S, summer; A, autumn; W, winter.

abundance during the autumn was possibly caused by the decrease in the ratio between BOD and NH_4-N concentrations in the influents during this season (data not shown).

The ammonia-oxidizing activities per AOB cell ranging from 0 to 49.6 fmol cell^{-1} h^{-1} in the 12 systems (Fig. 2(c)) were consistent with the numbers reported before: 7.7 and 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 (primers CTO 189f and RT1r and the *Taq* Man probe TMP1) and *amoA* genes of *N. oligotropha*, respectively [12]; and 9–23 fmol cell^{-1} h^{-1} for AOB isolates [23]. The ammonia-oxidizing activities per cell varied during the seasons studied. The highest ammonia-oxidizing activities per cell occurred during summer, followed by those during autumn and winter. Moreover, the total AOB numbers and the ammonia-oxidizing activities per AOB cell depended largely on temperature variation and altered with every temperature level: 27–31 °C during summer, 19–26 °C during autumn, and 14–22 °C during winter.

Although the total numbers of ammonia-oxidizing bacteria fluctuated over seasons, no marked seasonal variation in the composition of AOB communities was observed. The AOB communities were fairly stable in all systems during the six months of study. No seasonal variations in the common ammonia-oxidizing bacteria were observed. Only particular ammonia oxidizers exhibited seasonal variation in some systems. We therefore state that AOB communities change little over temperatures ranging from 14 to 31 °C.

Sequence analysis of 16S rRNA genes revealed that most of the electrophoretic bands were closely related to *Nitrosomonas* spp., not to *Nitrosospira* spp. This finding corresponds to several previous studies [5–9,15,16,24], but is in contrast to few studies in which mainly *Nitrosospira* spp. were detected [25,26]. Nonetheless, based on the *amoA* approach, it is notable that *Nitrosospira* species are not important ammonia-oxidizing bacteria in full-scale wastewater treatment systems analyzed so far [27].

We recovered members of the *N. oligotropha* cluster from all samples, and they comprised the majority of the bands analyzed. Although the forward primer CTO189f has a single mismatch with some members of the *N. oligotropha* cluster, the sequences closely related to this cluster could easily be recovered from our samples. Sequences of the *N. oligotropha* cluster are often recovered from oligotrophic environments, including freshwater sediment [28], wastewater treatment systems receiving low-ammonia influents [9,11,12,16], and chloraminated drinking water distribution systems [29,30]. The study of 36 isolates of the *N. oligotropha* cluster, most of which were from oligotrophic freshwater, revealed very low K_s values, ranging from 1.9 to

4.2 μM free ammonia [13]. Ammonium concentrations in the influents of every system in this study were <2 mM (28 mg N l^{-1}) (Table 1), and in the systems where nitrification was achieved, ammonium concentrations in the aeration tanks were under the detection limit. Thus, volumetric ammonia removals could be estimated as less than 60 g m^{-3} d^{-1} in all systems. This low ammonia level may be the key factor promoting the predominance of the *N. oligotropha* cluster in most systems in this study.

Sequence types 6a-1 and 6a-4 from the *N. oligotropha* cluster were distributed in most systems in most seasons studied. Sequence group 6a-1 is comprised of the sequences from continuous culture enrichment at growth-limiting ammonia concentration (5 μM; bands Ch4E2 and Ch6E3) [28], whereas sequence group 6a-4 is comprised of the sequences recovered from an aeration tank of a laboratory-scale single sludge nitrification–denitrification systems receiving a 0.8-mM ammonia in the influent (clones DNB_Y20 and DNB_Y39) [16], fresh water sediment (clone 6a3), and drinking water reservoirs (clone HBO-2). In the past, ecological and physiological differences among ammonia-oxidizing bacteria within the *N. oligotropha* cluster have been overlooked because of representing their characteristics with only one or two common members of the group. Recently, the characteristics of AOB within this cluster were suggested to somewhat differ depending on sequence type [14]. As our study shows, only two sequence types within this cluster were widely and abundantly distributed in most systems. Factors such as ammonia affinity, oxygen affinity, sensitivity to salt and/or nitrite may differ among sequence types. For example, salinity is known to affect the AOB communities in oligotrophic environments [14]. The effect of each environmental factor on AOB within this cluster needs further study to be able to better understand the implications of the presence of various sequence types.

We recovered members of the *N. europaea–Nc. mobilis* cluster only from system A. The outstanding physiological property of the *N. europaea–Nc. mobilis* cluster is the low affinity to free ammonia ($K_s > 30$ μM) [13], a characteristic consistent with this cluster's preference for eutrophic environments. Although system A contained higher ammonium concentrations in the influent than other systems (Table 1), these ammonia concentrations were not significantly different from those of other systems (~2 mM). In addition, ammonium concentrations in the aeration tank of system A were all the time below the detection limit, and volumetric ammonium removals of this system were in the same range as those of other systems. Therefore, ammonia is not responsible for the appearance of the *N. europaea–Nc. mobilis* cluster in system A. The study on 27 isolates of *N. europaea* and seven isolates of *Nc. mobilis* revealed halotolerant to moderately halophilic characteristic of this cluster [13]. Earlier molecular-level investigations supported this by showing the predominance of *N. europaea* cluster in alkaline, high-salinity environments [31] and in an industrial wastewater treatment systems treating saline wastewater [6]. The double chloride concentration in the influent of system A compared to other systems may thus explain the presence of the *N. europaea–Nc. mobilis* cluster in this system.

Besides those of the *N. europaea–Nc. mobilis* cluster, also the members of the *N. cryotolerans* cluster and of the unknown-*Nitrosomonas* cluster were present almost solely in system A. Assuming the difference in halotolerance or halosensitivity among distinct AOB species, the high chloride concentration in the influent of this system may have caused its AOB community differ from those in other systems. *N. cryotolerans* has been reported as obligately halophilic bacterium [13]. However, the salt tolerance of the unknown-*Nitrosomonas* cluster is unknown because ammonia-oxidizing bacteria affiliated to this cluster have not been isolated and characterized yet. The most closely related AOB to the unknown-*Nitrosomonas* cluster is N. sp. JL21 (accession number AB000700) with only 95% similarity in the partial 16S rRNA gene sequence. Due to the short sequence length, it is impossible at this moment to clarify whether the unknown-*Nitrosomonas* group belongs to *N. oligotropha* cluster or represents the putative novel AOB cluster.

Members of the *N. communis* cluster were found in all A2O and AO systems; but they were mostly absent from AS systems. Thus far, only two 16S rRNA gene sequences affiliated with this cluster (clones GaN50304 and GaN3a) [9] have been detected in the environments. This may have been caused by the fact that many of the commonly used primers contain mismatches to members of the *N. communis* cluster [9]. The reverse primer CTO654r does not target all members of the *N. communis* cluster recognized so far. However, using this primer set, many members of the *N. communis* cluster could be recovered from our samples. This difference is possibly because the sequences of our *N. communis* at the positions corresponding to the primer region match to the reverse primer and thus are different from those of all recognized AOB. The members of the *N. communis* cluster obtained from our samples were closely related to those recovered earlier (clones GaN50304 and GaN3a) from the top 100 μm of the biofilm surface of a sequencing batch reactor, which operated with different phases of aeration and non-aeration for the purposes of nitrogen and phosphate removal. Similarly as this earlier study, we found that the members of the *N. communis* cluster were present almost exclusively in A2O and AO systems, which were operated with different phases of aeration and non-aeration. These AOB may thus have an advantage

in environments with fluctuating oxygen levels or during the absence of oxygen. These results reflect the distinct ability of ammonia-oxidizing bacteria to survive or even prosper under special conditions. Along this line, oscillation of oxygen concentrations may be another factor supporting growth or survival of particular AOB.

Total AOB numbers and ammonia-oxidizing activities per AOB cell are discussed to clarify the reasons underlying the reduced ammonia removal in some systems (G1, G2, and H). Various aspects of system operation were found to affect the total AOB numbers and the ammonia-oxidizing activities per cell of AOB.

G1 and G2 are AS systems that received similar-strength wastewater with the same volumetric flows (Table 1). Both systems were operated with the similar SRT, but the amounts of airflow to wastewater were different (data not shown). The airflow to wastewater was sufficient in system G1, but not in system G2. As a result, DO concentrations in the aeration tank of system G1 were in a range of 5–8 mg l^{-1}, whereas those of system G2 were around 2 mg l^{-1} (Table 4). Thus, only system G1, but not system G2, achieved ammonia removal. Although ammonia removal in both systems was different, this value did not reflect the total AOB numbers; in contrast, it did reflect the ammonia-oxidizing activities. This result suggested that oxygen supply and DO concentrations did not influence the total numbers of ammonia-oxidizing bacteria. On the other hand, they did affect the ammonia-oxidizing activities per cell.

In system H, ammonia removal efficiencies and volumetric ammonia removal fluctuated during the seasons studied (Table 5). Nitrification was achieved only during autumn, not during summer or winter. Although no change in the influent characteristics was noted, operation of the system varied during the season studied. Proper operation was provided only during the autumn, whereas insufficient oxygen was supplied during the summer, and SRT was too short during the winter. The proper operation led to the highest total numbers of ammonia-oxidizing bacteria during the autumn. Longer SRT in summer seemingly supported higher total AOB numbers during this season. However, neither SRT nor total AOB numbers related to the volumetric ammonia removal and the ammonia-oxidizing activities per cell. The ammonia-oxidizing activities were dependent on DO concentrations. We can conclude that SRT mainly influenced the total AOB numbers, whereas the DO concentration primarily affected the ammonia-oxidizing activity per cell. Indeed, other studies support our observation about the effect of SRT on the total AOB numbers [32] and the effect of oxygen concentrations on the ammonia-oxidizing activity [14,33].

Table 4
Ammonia removal, total AOB numbers, ammonia-oxidizing activities per AOB cell, and DO concentrations in aeration tanks of systems G1 and G2

Parameters	Unit	G1			G2		
		Summer	Autumn	Winter	Summer	Autumn	Winter
NH_4^+-N removal[a]	%	100	100	100	40	0	11
Volumetric NH_4^+-N removal[a]	g m^{-3} d^{-1}	49	60	53	13	0	0
Total AOB number	cells l^{-1}	2.9×10^{9}	2.9×10^{10}	6.7×10^{10}	1.1×10^{10}	2.8×10^{10}	3.7×10^{9}
NH_4^+-N oxidizing activity	fmol $cell^{-1}$ h^{-1}	50	6	2	3	0	0
MLDO[b]	mg l^{-1}	5.2	6.6	7.7	1.6	1.0	2.0

MLSS, mixed liquor suspended solid; MLDO, mixed liquor dissolved oxygen.
[a] All removal efficiency values were analyzed from one-day grab samples collected on the day close to the day of sludge collection.
[b] All operational parameters were the averages of the month in which sludge was collected.

Table 5
Ammonia removal, total AOB numbers, ammonia-oxidizing activities per AOB cell, and operational parameters of system H

Parameter	Unit	Season		
		Summer	Autumn	Winter
NH_4^+-N removal[a]	%	7	89	30
Volumetric NH_4^+-N removal[a]	g m^{-3} d^{-1}	0	20	4
Total AOB number	cells l^{-1}	3.4×10^{9}	1.2×10^{10}	1.0×10^{9}
MLSS[b]	mg l^{-1}	1270	1125	870
SRT[b]	Day	8.2	6.0	3.8
NH_4^+-N oxidizing activity	fmol $cell^{-1}$ h^{-1}	0	4	12
MLDO[b]	mg l^{-1}	0.6	1.1	3.2

MLSS, mixed liquor suspended solid; SRT, solid residence time; MLDO, mixed-liquor dissolved oxygen.
[a] All removal efficiency values were analyzed from one-day grab samples collected on the day close to the day of sludge collection.
[b] All operational parameters were the averages of the month in which sludge was collected.

5. Conclusions

In this study, we mainly discussed the ammonia-oxidizing bacteria found in activated sludge of sewage treatment systems. We noted the effects of influent characteristics, treatment processes, system operation, and seasonal variation on the total AOB numbers and the AOB communities. We showed that the ammonia-oxidizing bacteria observed in these low-ammonia systems differ and are influenced by distinct environmental factors. However, the discussion primarily reflected qualitative results because accurate numbers of particular AOB were unavailable. Further studies are necessary to develop quantitative techniques for particular AOB, especially for those within the *N. oligotropha* cluster and the *N. communis* cluster, and to better clarify the roles of these ammonia oxidizers in low-ammonia environments.

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