Quantification of ammonia-oxidizing bacteria populations in full-scale sewage activated sludge systems and assessment of system variables affecting their performance

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T. Limpiyakorn*, F. Kurisu and O. Yagi****

*Department of Urban Engineering, Graduate School of Engineering, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan (E-mail: *miketawan@yahoo.com*)

**Research Center for Water Environment Technology, Graduate School of Engineering, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan (E-mail: *kurisu@env.t.u-tokyo.ac.jp*; *yagi@env.t.u-tokyo.ac.jp*)

Abstract This study carried out quantification of ammonia-oxidizing bacteria (AOB) populations in 12 full-scale sewage activated sludge systems that were different in ammonia removals and treatment processes during three different seasons. Experiment was divided into 3 parts: 1) analysis of AOB communities by PCR-DGGE-cloning-sequencing of 16S rRNA genes; 2) development of four real-time PCR primer sets for quantification of the particular AOB of interest; and 3) quantification of AOB populations by using the newly developed real-time PCR primer sets. The results suggested that all the primer sets gave good reproducibility and specificity for PCR amplification with the detection limits of 10^2 copies/PCR reaction. Although the 12 systems were different in several aspects, one of the identified sequence types of *Nitrosomonas oligotropha* cluster was the dominant AOB in every system and every season studied. However, the other sequence type of this cluster was not significantly involved in ammonia removals in the systems. The occurrence of *N. communis* cluster in the systems seemed to depend on the remaining oxygen concentrations in the sludge floc and thus the activity of aerobic heterotrophs in the aeration tanks. *N. europaea*–*Nitrosococcus. mobilis* solely existed in one A2O system of which the influent contained twice the chloride concentrations than those of other systems.

Keywords 16S rRNA gene; activated sludge; ammonia-oxidizing bacteria; real-time PCR; sewage treatment

Introduction

Nitrification plays a key role in the biological removal of nitrogen in activated sludge systems. The nitrification is the two-step process involving two phylogenetically unrelated groups of obligate chemolithotrophic bacteria. Ammonia is first oxidized to nitrite by ammonia-oxidizing bacteria (AOB), and nitrite is subsequently oxidized to nitrate by nitrite-oxidizing bacteria. Because of the slow growth rate of AOB, their inability to compete with heterotrophs, and their high sensitivity to many environmental factors, ammonia oxidation is the rate-limiting step of nitrogen removal in activated sludge systems. Therefore, a better understanding of the microbiology and ecology of AOB in activated sludge systems is necessary for the enhancement of system performance and control.

A number of studies have assessed the microbiology and ecology of AOB in their environments. The microbiology and ecology of AOB are suggested to differ among distinct AOB species. The distribution patterns of distinct AOB species in the environments reflect the physiological properties of the isolates observed in the laboratory (Koops and Pommerening-Roser, 2001). Among these, ammonia seems to be the most important factor in the inclusion of distinct AOB species in the environments, whereas other factors such as

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salinity are also reported to influence distinct AOB species (Koops and Pommerening-Roser, 2001, Bollmann and Laanbroek, 2002). However, beside ammonia, an understanding of how other factors in activated sludge systems manipulate distinct AOB species in the systems are still doubtful. In general, AOB found in low-ammonia systems are cited as being the same bacteria. They are often overlooked by representing their characters by the only few common members of the groups. In spite of this, their characters may differ, and they may be influenced by distinct factors in the systems.

According to the above concerns, this study carried out quantification of AOB populations in 12 full-scale sewage activated sludge systems, which were different in ammonia removals and treatment processes: anaerobic/anoxic/aerobic (A2O); anaerobic/aerobic (AO); and conventional activated sludge (AS) processes, during three different seasons: summer, autumn, and winter. The experiment was divided into three parts: 1) analysis of AOB communities by specific polymerase chain reaction (PCR) amplification followed by denaturing gel gradient electrophoresis (DGGE), cloning, and sequencing of 16S rRNA genes; 2) development of four real-time PCR primer sets for quantification of the particular AOB of interest; and 3) quantification of AOB populations by using the newly developed real-time PCR primer sets.

Methods

Sewage activated sludge samples and descriptions of sewage activated sludge systems

Samples were taken from the aeration tanks of 12 full-scale sewage activated sludge systems. The 12 systems are in eight sewage treatment plants in Tokyo that are run by the Bureau of Sewerage, Tokyo Metropolitan Government, Japan. The 12 systems were different in ammonia removals and were operated with different treatment processes: A2O; AO; and AS processes. Samples were collected from the 12 systems during three different seasons: summer (August 2001); autumn (November 2001); and winter (February 2002).

Table 1 shows treatment processes, influent characteristics, effluent characteristics, removal efficiencies, and operational parameters of the 12 systems. 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 and chloride concentrations that were twice those of other systems. BOD removal efficiencies were excellent ($>95\%$) in all systems; however, ammonia removal efficiencies differed due to the difference in system operation. Ammonia concentrations in the effluents varied among the systems in accordance with the ammonia removal efficiencies and the initial volumetric ammonia loads entering the aeration tanks. Nitrite concentrations in the effluents were less than 2 mg N/l and pH were controlled 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 seasonal variations in influent characteristics, effluent characteristics, removal efficiencies, or operational parameters were observed in all systems throughout this study.

Analysis of AOB communities in sewage activated sludge samples

Analysis of AOB communities in sewage activated sludge samples was carried out by using specific PCR amplification followed by DGGE, cloning, and sequencing of 16S rRNA genes. The details of this analysis are described elsewhere (Limpiyakorn *et al.*, 2005).

Development of four real-time PCR primer sets for quantification of the particular AOB of interest

Four new real-time PCR primer sets were developed for 16S rRNA genes of the particular AOB of interest (Figure 1 and Table 2). The particular AOB of interest are sequence type 6a-34 of *N. oligotropha* cluster, sequence type 6a-1 of *N. oligotropha* cluster,

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Table 1 Treatment processes, influent characteristics, effluent characteristics, removal efficiencies, and operational parameters of 12 sewage activated sludge systems

Parameter	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
BOD in influent	mg/l	91–139	53–141	53–141	53–141	80–133	64–132	65–100	67–90	63–111	74–103	83–94	34–73
BOD removal	%	98–99	98–99	97–99	97–99	95–96	99–100	98–99	96–99	97–98	97–99	95–97	95–97
Volumetric BOD removal	g/m^3 d	–*	–*	–*	150–350	–*	–*	–*	200–250	300–440	250–350	250–350	100–180
NH_4-N in influent	mg/l	26–30	13–16	13–16	13–16	20–24	14–18	12–18	15–18	15–18	18–19	18–21	14–17
NH_4-N in effluent	mg/l	0	0	0–1	0–2	3–12	0	1–2	0–6	0–1	0	12–19	4–16
NH_4^+-N removal	%	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	g/m^3 d	42–44	33–34	26–30	24–31	18–50	28–33	27–44	32–34	54–60	49–60	0–13	0–20
Cl^- in influent	mg/l	140–180	54–80	54–80	54–80	63–80	40–58	80–90	–*	–*	89–94	89–98	52–72
DO	mg/l	4.1	1.8	2.2	2.3	3.4	5.3	5.8	6.5	6.7	6.5	1.8	1.6

BOD, biological oxygen demand; DO, dissolved oxygen
*Data is not available

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members within *N. communis* cluster, and all members of *N. europaea–Nc. mobilis* cluster. The details of this development are described elsewhere (Limpiyakorn *et al.*, in press). Briefly, the primer sets were designed by using the ARB program (Munich, Germany) with the ssu rRNA database (Antwerp, Belgium) and they were constructed based on BODIPY®FL modified primer (Kurata *et al.*, 2001). Standard DNA, which were the pT7Blue vector possessing partial fragments of 16S rRNA genes of the target AOB, were prepared in a range of 5×10^1 to 5×10^7 copies. PCR amplification was performed in an ABI Prism SDS 7000 instrument (PE Applied Biosystems). The entire primer sets were tested for their specificities with five almost full-length 16S rRNA genes of *Ns. multiformis*, *N. europaea*, *N. cryotolerans*, *Nc. oceani*, and *Methylocystis* sp. M. and 13 partial fragments of 16S rRNA genes which were isolated from sewage activated sludge samples during the analysis of AOB communities.

Quantification of AOB populations in sewage activated sludge samples

Quantification of AOB populations in sewage activated sludge samples was performed by using the newly developed real-time PCR primer sets. 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.

Results and discussion

Analysis of AOB communities in sewage activated sludge samples was carried out by using specific PCR amplification followed by DGGE, cloning, and sequencing of 16S rRNA genes. The detailed results of this analysis are described elsewhere (Limpiyakorn *et al.*, 2005). Briefly (Figure 2), the members of the *Nitrosomonas oligotropha* cluster existed in all samples, and two of the identified sequence type of *Nitrosomonas oligotropha* cluster (sequence type 6a-1 and sequence type 6a-34) arose in most samples. Members of *N. communis* cluster occurred almost exclusively in association with A2O and AO systems (A, B1, B2, B3, C, D, and E). Members of *N. europaea–Nitrosococcus mobilis*, *N. cryotolerans*, and unknown *Nitrosomonas* clusters took place solely in system A.

Four new real-time PCR primer sets were developed to specific 16S rRNA genes of the particular AOB of interest which were recognized in the analysis of AOB communities. The detailed results of this development are described elsewhere (Limpiyakorn *et al.*, submitted). Briefly, all the primer sets performed good reproducibility and specificity of PCR amplification. The detection limits of all the primer sets were 10^2 copies/PCR reaction. Additionally, depending on the primer sets, the reliable quantified numbers were obtained when the target AOB DNA were present in more than 0.1–1% of the total AOB DNA.

Quantification of AOB populations in sewage activated sludge samples was performed by using the newly developed real-time PCR primer sets (Figure 3). Notably, the sum of the four particular AOB of interest corresponded to the total AOB numbers quantified by

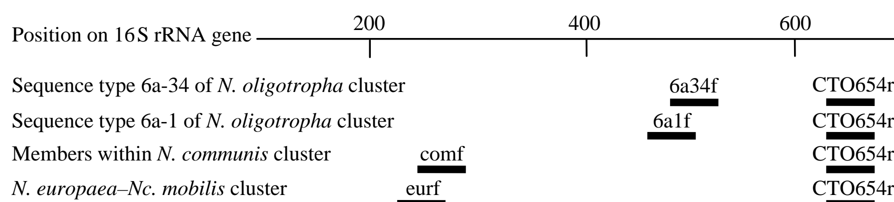

Figure 1 Positions of real-time PCR primer sets on 16S rRNA gene based on nucleotide numbers corresponding to *E. coli* numbering

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Table 2 Oligonucleotide sequences of real-time PCR primers

Primer	Sequences	Target region*
6a34f	TTCATGACGGTATCAACAGA	475–494
6a1f	CATAGCTCATGACGGTATCG	470–489
Comf	CTCGTGCTTTAAGGGTGGCC	214–233
Eurf	AAGACCTTGCGCTAAAGGAG	209–228
CTO654r	CTAGCYTTGTAGTTTCAAACGC	632–654

*Nucleotide numbers corresponding to *E. coli* numbering

the primers CTO189f and RT1r and the *Taq*Man probe TMP1 (Hermansson and Lindgren, 2001) in all samples (data not shown).

Numbers of sequence type 6a-34 of *N. oligotropha* cluster were ranging from 1.0×10^9 to 9.9×10^{10} cells/ l (Figure 4), which were close to the total AOB numbers (1.0×10^9–9.2×10^{10} cells/l). Although, the 12 systems were different in several aspects, including influent characteristics, treatment process, and system operation (Table 1), the sequence type 6a-34 were the dominant AOB in every system and every

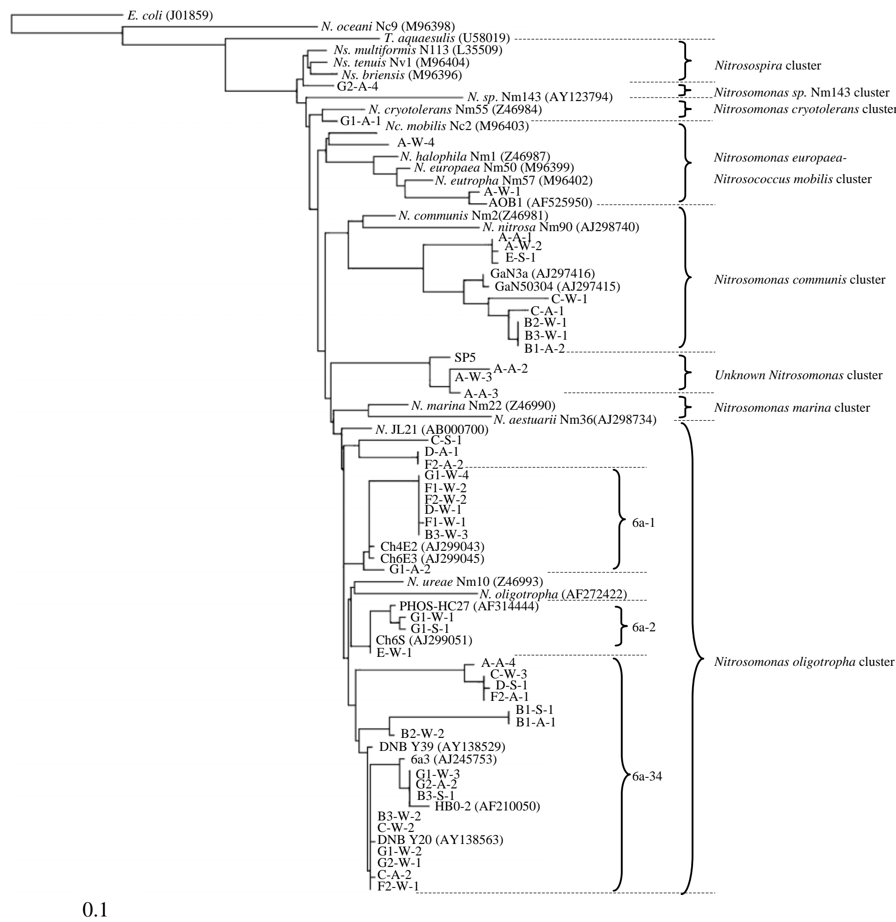

Figure 2 Phylogenetic tree constructed based on 16S rRNA genes of AOB belonging to β-Proteobacteria by adding our 397-bp sequences into the distance tree, prior constructed based on comparison of 1000-bp sequences of described AOB species (Koops *et al.*, 2003) and some non-AOB by using the ARB program. AOB genus abbreviations are *N.* for *Nitrosomonas*, *Nc.* for *Nitrosococcus*, and *Ns.* for *Nitrosospira*

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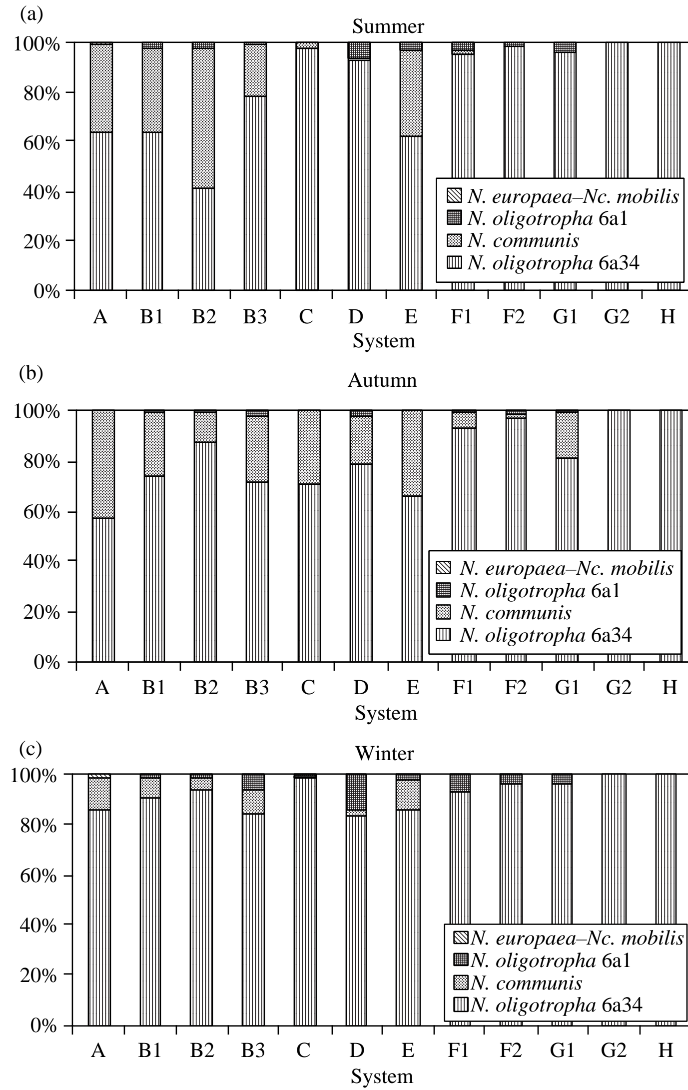

Figure 3 Compositions of AOB populations in aeration tanks of 12 sewage activated sludge systems during three different seasons

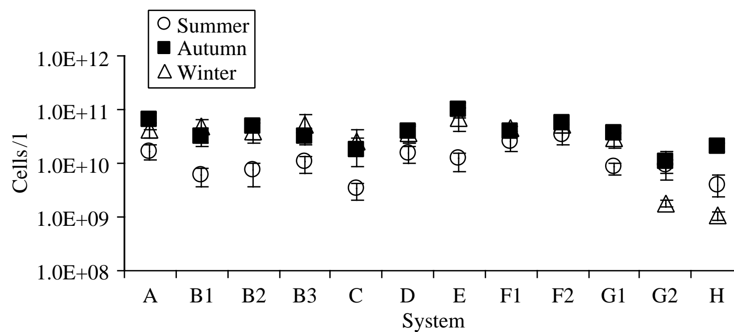

Figure 4 Numbers of sequence type 6a-34 of *N. oligotropha* cluster in aeration tanks of 12 sewage activated sludge systems during three different seasons

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studied season. This result suggested that the sequence type 6a-34 were capable of dominating in a wide range of conditions in the systems. And thus, they were the important AOB in the sewage activated sludge systems.

Numbers of sequence type 6a-1 of *N. oligotropha* cluster were ranging from 1.5×10^8 to 6.6×10^9 cells/l (Figure 5). In contrast to the sequence type 6a-34, the sequence type 6a-1 existed in small numbers in some systems. Previously, AOB associated with this sequence type (bands Ch4E2 and Ch6E3) were reported as the halosensitive AOB which were grown at growth-limiting ammonium concentration (5 μM; Bollmann and Laanbroek, 2001, Bollmann and Laanbroek, 2002). For these reasons, the sequence type 6a-1 did not arise in system A due to the chloride concentrations in the influent of this system being more than twice those of other systems (Table 1). In addition, they did not occur in systems C, G2, and H because ammonium concentrations in these systems were higher than those in other systems (approximately >5 mg N/l; Table 1). This result suggested that the sequence type 6a-1 may have been inhibited by ammonium concentration in ranges of millmolar (approximately >5 mg N/l). Hence, it can be implied that the sequence type 6a-1 existed in the sewage activated sludge systems of which ammonia was completely oxidized by other AOB. Thus, they were not significantly involved in ammonia removals in the systems.

Numbers of members within *N. communis* cluster were ranging from 8.1×10^7 to 5.2×10^{10} cells/l (Figure 6). Members of *N. communis* cluster existed in every studied season in systems A, B1, B2, B3, C, D, and E, while they were found sporadically in systems F1, F2, and G1, and none were observed in system G2 and H. Obviously (Table 1), members within the *N. communis* cluster existed in all A2O and AO systems (systems A, B1, B2, C, D, and E) and in some AS systems where the volumetric BOD removals in aeration tanks were low (systems B3, F1, and G1). Moreover, they were never observed in systems of which aeration tanks were low in DO concentrations (systems G2 and H). These results suggested that the remaining oxygen concentration in the sludge flocs in aeration tanks is possibly the significant factor for the presence of members within the *N. communis* cluster in the systems. The remaining oxygen concentration in the sludge flocs depended largely on the amount of oxygen exploited by aerobic heterotrophs. As a result, members within the *N. communis* cluster preferred A2O and AO systems to the AS system because in A2O and AO systems, aerobic heterotrophs may be less active than in the AS system due to less BOD loads entering the aeration tanks. Among AS systems, *N. communis* cluster preferred the systems whose volumetric BOD removals are low (160–400 g BOD/m^3 d). This is because in these systems, aerobic heterotrophs are grown in declining growth phase where they are less active than in the AS systems whose volumetric BOD removals are high. Previously, members of *N. communis* cluster

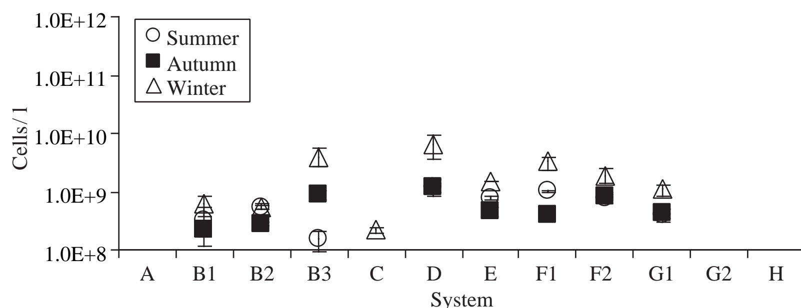

Figure 5 Numbers of sequence type 6a-1 of *N. oligotropha* cluster in aeration tanks of 12 sewage activated sludge systems during three different seasons

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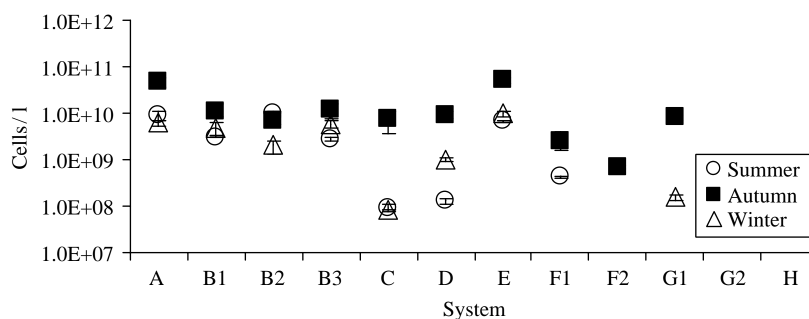

Figure 6 Numbers of *N. communis* cluster in aeration tanks of 12 sewage activated sludge systems during three different seasons

(clones GaN3a and GaN50304), which is closely related to our sequences, were reported for their lower oxygen affinity than that of the *N. oligotropha* cluster (Gieseke *et al.*, 2001). Thus, a study of the effect of oxygen in sludge floc on AOB populations would be necessary.

N. europaea–Nc. mobilis cluster arose in small numbers (3.5×10^8–6.2×10^9 cells/ l) only in system A. The outstanding physiological property of *N. europaea–Nc. mobilis* cluster is the low affinity to free ammonia ($K_s > 30\,\mu M$) and the halotolerance to moderate halophilicity (Koops and Pommerening-Roser, 2001). The higher ammonium concentrations in the influent of system A are not the primary reason for the presence of *N. europaea–Nc. mobilis* cluster, as the ammonium concentrations remained at ~2 mM, similar to those of other systems (Table 1). On the other hand, the twice higher chloride concentrations in the influent of system A than in other systems may account for the presence of the *N. europaea–Nc. mobilis* cluster.

Conclusions

In this study, *in situ* characterization of four particular AOB of interest was carried out by using the newly developed real-time PCR primer sets. Various aspects of sewage activated sludge systems, including influent characteristics, treatment processes, system operation, and seasonal variations, influenced differently distinct AOB in this low-ammonia system.

Acknowledgements

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