J Ind Microbiol Biotechnol (2010) 37:751–757
DOI 10.1007/s10295-010-0728-3

SHORT COMMUNICATION

Transcription levels (*amoA* mRNA-based) and population dominance (*amoA* gene-based) of ammonia-oxidizing bacteria

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Received: 19 February 2010 / Accepted: 16 April 2010 / Published online: 4 May 2010
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Abstract A population shift of ammonia-oxidizing bacteria (AOB) was described within a bench-scale activated sludge process treating an industrial wastewater in a previous report (Kuo et al. in Environ Eng Sci 23:507–520, 2006). In this investigation, transcriptional levels (*amoA* mRNA-based) of the three AOB groups (i.e., RI-27, B2-3, and *Nitrosomonas nitrosa*) identified in the treatment process were determined by quantitative real-time reverse transcription (RT-PCR) assays to circuitously evaluate AOB ammonia-oxidizing activity and to assess the presumed correlation between cellular activity and the dominant (greatest number) AOB population. Results demonstrated that the AOB group with higher *amoA* mRNA levels dominated the overall AOB population in the wastewater treatment process. Although AOB population dominance did not correlate well with transcripts at a normalized cellular level (*amoA* mRNA/DNA ratio), overall *amoA* mRNA levels did reflect the activity of distinct AOB groups under different N-loading conditions. Thus, an additional molecular parameter (*amoA* mRNA) was successfully utilized to assess timely shifts in AOB population structure that may impact nitrification treatment performance.

Keywords Ammonia-oxidizing bacteria (AOB) · AOB population shift · *amoA* mRNA levels · Quantitative real-time reverse transcription PCR · Industrial wastewater

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Introduction

Ammonia-oxidizing bacteria (AOB) are one of the fundamental microbial groups responsible for ammonium conversion during industrial wastewater processing. In various nitrifying systems, different AOB groups have been found to be dominant under certain prevailing conditions [7, 9, 21, 23] and shifts in AOB population structure have been reported [4, 11, 12, 18] with some affecting nitrification performance [14, 16]. It has been suggested that competitive ability and/or tolerance to inhibitors may lead to specific AOB population dominance within nitrifying systems [5, 13, 17]. Kinetic-based approaches regarding average ammonia-oxidation rate (or AOB cellular activity) have been used to explicate population dominance [8, 19, 24, 25] but kinetic parameters for many currently uncultured AOB strains remain unknown. A better understanding of population dynamics during wastewater treatment is critical to optimize nitrification performance [27, 29] especially

Table 1 Target molecules, the associated PCR primers, and fluorescent probes used for the detection of AOB *amoA* mRNA (specific for *N. nitrosa*, RI-27, and B2-3 groups) in this study

Targets	Primers	Probes
N. nitrosa	amoA-Nn-542f (nitrosa-f):	amoA-Nn-bhq-648r (amoNnTaq3r):
amoA mRNA	5′ > TATTGCTTTCAATGGCAGACTACA > 3′	5′ > 6-FAM-ATGGCCGCCAAAGGTACGCAGTGAA-BHQ-1 > 3′
	amoA-Nn-676r (nitrosa-r):	
	5′ > CCGCAAAGAACGCAGCAATC > 3′	
RI-27	amoA-[RI-27]-542f:	amoA-[RI-27]-bhq-651r:
amoA mRNA	5′ > CATTGTTATCGATGGCTGACTATA > 3′	5′ > 6-FAM-TGTATGACCACCGAACGTACGCAGTGAG-BHQ-1 > 3′
	amoA-[RI-27]-679r:	
	5′ > ACGCTGAGAAGAATGCTGCAAT > 3′	
B2-3	amoA-[B2-3]-596f:	amoA-[B2-3]-bhq-713r:
amoA mRNA	5′ > CGGAATACGTRAGATTGATTGA > 3′	5′ > 6-FAM-TACCACCATACGCAGWACATCAGCAT-BHQ-1 > 3′
	amoA-[B2-3]-744r:	
	5′ > GAAGAAMGCGGTGCAATAGA > 3′	

to identify timely shifts in AOB population structure that may adversely impact treatment processes.

Recently, AOB transcriptional responses to particular environmental conditions (e.g., ammonia supplement, ammonia deprivation, carbon deprivation, or metal stress) have been reported [2, 22, 28]. Studies have indicated that ammonia-oxidizing activity does somewhat correspond with *amoA* mRNA levels [1–3, 6]. However, it is currently unclear whether these activity and/or *amoA* mRNA changes result in any shift in AOB population. In this investigation, the transcriptional parameter *amoA* mRNA was measured to assess the shift in AOB population observed in an earlier report [14, 15]. Quantitative real-time reverse transcription (RT-PCR) assays were used to specifically measure the *amoA* mRNA abundance for three AOB groups (i.e., *Nitrosomonas nitrosa*, uncultured AOB RI-27 and B2-3) previously identified in a bench-scale activated sludge process treating an industrial wastewater [14]. Previous results showed that the dominant AOB population in a 2-day SRT (solid retention time) treatment system shifted from the RI-27 to the B2-3 group after the influent ammonium loading was changed from low-N to high-N while the RI-27 group remained dominant in longer SRT systems (i.e., 5-, 10-, and 20-day systems). The goal of this study was to utilize specific RT-PCR assays to measure *amoA* mRNA concentrations and to substantiate whether this molecular parameter correlated with AOB population dominance (greatest number) in the 2- and 10-day SRT treatment systems during bench-scale industrial wastewater treatment.

Materials and methods

The bench-scale activated sludge experimental process was described in the previous study [14]. Briefly, the process consisted of four separate treatment channels (or systems), each with three 10-l baffled reactors operating in series. The reactors were initially seeded with activated sludge collected from the aeration tanks of a full-scale industrial wastewater treatment plant (WWTP). Wastewater from the WWTP (augmented with NH_4Cl during high-N treatment) was used to continually feed the experimental process. The system was operated under different solids retention time (SRT) (i.e., 2, 5, 10, and 20 days for each of the four individual systems) and ammonia loading (i.e., low-N: 17.5 ± 5.1 mg NH_3-N/L/day and high-N: 49.3 ± 8.7 mg NH_3-N/L/day) while pH and dissolved oxygen (DO) were maintained at constant levels of 7.2 and 3 (mg/l), respectively.

Bacterial RNA of the biosolids samples taken from the industrial bench-scale process were extracted using the RNeasy® Mini Kit (Qiagen, Valencia, CA) based on the "Protocol for Isolation of Total RNA from Bacteria" described in the manufacture's handbook. The *amoA* mRNA concentrations of the three AOB groups were quantified with three, one-step quantitative RT-PCR assays using three primer pairs and three TaqMan® probes (Table 1) designed in previous studies [14, 16]. The RT-PCR assays were performed using a "DNA Engine Opticon™ Continuous Fluorescence Detection System" (Bio-Rad, Hercules, CA; former MJ Research, Waltham, MA). Each reaction of all three RT-PCR assays was performed in a 25-µl mix containing 12.5 µl of 2X QuantiTect Probe RT-PCR Master Mix® (QuantiTect™ Probe RT-PCR Kit, Qiagen, Valencia, CA), 0.75 µl of each forward primer (20 µM), 0.75 µl of each reverse primer (20 µM), 1.25 µl of each probe (10 µM), 0.25 µl of QuantiTect RT Mix, 4.5 µl of nuclease-free water, and 5 µl of RNA samples or standards. The RT-PCR reaction program was 30 min at 50°C, 15 min at 95°C, followed

by 50 cycles consisting of 15 s at 95°C, and 30 s at 54°C, 56°C, and 60°C (for the *N. nitrosa*, B2-3, and RI-27 assays, respectively).

Two *amoA* mRNA standard stock solutions were prepared for the quantitative RT-PCR assays to calibrate target transcripts of the RI-27 and B2-3 groups. Each *amoA* mRNA standard solution was prepared using an in vitro transcription reaction in which the T7 RNA polymerase was used to transcribe a fragment of *amoA* mRNA from the anti-sense sequence of a selected cloned *amoA* gene library created in the previous study [14] based on the "T7 Transcription Protocol" stated in the manufacture's technical bulletin (RiboMAX™ Large-Scale RNA Production Systems-SP6 and T7, Promega, Madison, WI). The quality of each of the prepared RI-27 and B2-3 *amoA* mRNA standards was evaluated using a formaldehyde denaturing agarose gel (to ensure the integrity of the prepared mRNA), a UV absorption ratio (A_{260}/A_{280}) (to check protein contamination), and a real-time no-RT RT-PCR reaction (real-time thermo-cycle step prior to PCR amplification was performed without adding reverse transcriptase in the reaction mix) (to test contamination of transcription template, i.e., the linearized plasmid DNA). Additionally, the quantity of each prepared *amoA* mRNA standard was determined by both spectrophotometric and RiboGreen® (Molecular Probes, Invitrogen, Eugene, OR) RNA quantification methods. The quantified *amoA* mRNA standard stock solutions were further serially diluted ten-fold to concentrations ranging from 10^8 to 10^3 copies/μl and used as standards to calibrate *amoA* mRNA concentrations for the RI-27 and B2-3 groups, respectively. Additionally, calibration for the *N. nitrosa* transcript was based on the *amoA* DNA standard. Purified plasmid DNA carrying the *N. nitrosa amoA* gene was adjusted to serial dilutions (10^7–10^1 copies/μl) and used as standards to calibrate concentration of cDNA reverse-transcribed from target *N. nitrosa amoA* mRNA. During each RT-PCR run, the *Ct* values (*X* axis) of the serially diluted standards were plotted with RNA/DNA concentrations in logarithm (*Y* axis) to construct a standard curve for calibrating the abundance of the target *amoA* transcripts. The composite calibration equations for the B2-3, RI-27, and *N. nitrosa* AOB groups were $Y_{\text{B2-3}} = -0.27\ X_{\text{B2-3}} + 11.42$ ($r^2 = 0.995$), $Y_{\text{RI-27}} = -0.28\ X_{\text{RI-27}} + 12.13$ ($r^2 = 0.998$), and $Y_{\text{Nn}} = -0.26\ X_{\text{Nn}} + 11.19$ ($r^2 = 0.997$), respectively. The quantitative results of *amoA* mRNA concentrations were converted from "copies per reaction" to "copies per liter (of mixed liquor)" and used to assess possible correlations with the population data (*amoA* DNA-based, i.e., one AOB cell has two copies of *amoA* gene) (Figs. 3a, 4a) reported in a previous study [14].

Results and discussion

The average overall *amoA* mRNA concentrations for AOBs in the 2- and 10-day SRT systems under both low- and high-N operations were correlated with the average ammonia oxidation rates (Pearson correlation coefficient $r = 0.87$) (Fig. 1). This suggested that the *amoA* mRNA measurement reflected AOB activity under different ammonia loadings. Thus, this molecular parameter was used to represent the metabolic activity of the three AOB groups and to extrapolate activity to population dominance.

A previous report indicated that the dominant AOB population in a 2-day SRT treatment system shifted from the RI-27 to the B2-3 group after the influent ammonium loading was changed from low-N to high-N; while the RI-27 group remained dominant in a 10-day SRT system [14]. We hypothesized that the relatively high ammonia-N concentration (~24.3 mg/l, Fig. 2), which accumulated during the transitional period of high-N operation in the 2-day SRT system (while ammonia-N levels in the 10-day SRT system remained below 1 mg/l), created a more favorable condition for the B2-3 organism. Thus, this B2-3 AOB group became more active than the RI-27 and *N. nitrosa* groups resulting in dominance within the AOB population in the 2-day SRT system.

During the overall study period, the RI-27 *amoA* mRNA concentrations in the 10-day SRT treatment system ranged from approximately 1.14×10^9 to 1.51×10^{10} copies/l (Fig. 3b), which were much higher than those for the B2-3 and *N. nitrosa* groups (4.50×10^7–1.36×10^8 and 3.28×10^4–1.29×10^6 copies/l, respectively). The high *amoA* mRNA levels indicate that the RI-27 group was

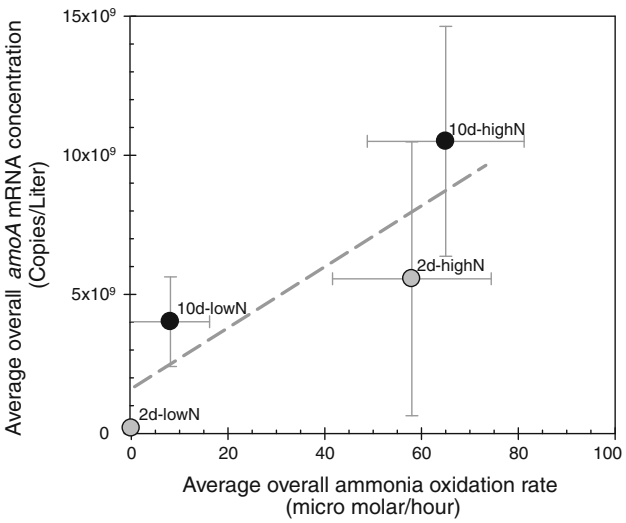

Fig. 1 Average overall *amoA* mRNA concentration correlated with the average overall ammonia oxidation rate (Pearson correlation coefficient = 0.87)

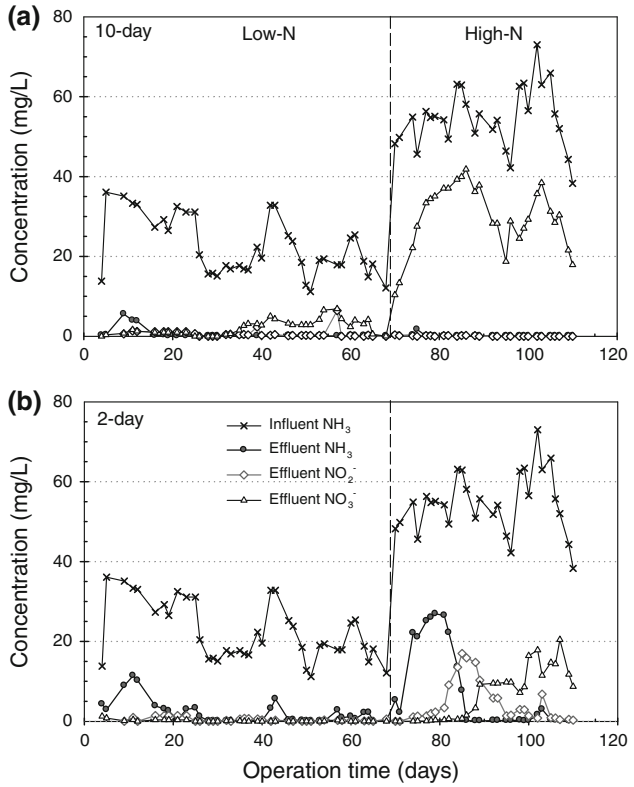

Fig. 2 Influent ammonia (NH_3) and effluent ammonia, nitrite (NO_2^-), and nitrate (NO_3^-) concentrations (mg as N/L) for the 10-day (**a**) and 2-day (**b**) SRT systems during low-N and high-N operations. The transition point from low-N to high-N on day 69 is indicated by *dashed lines*

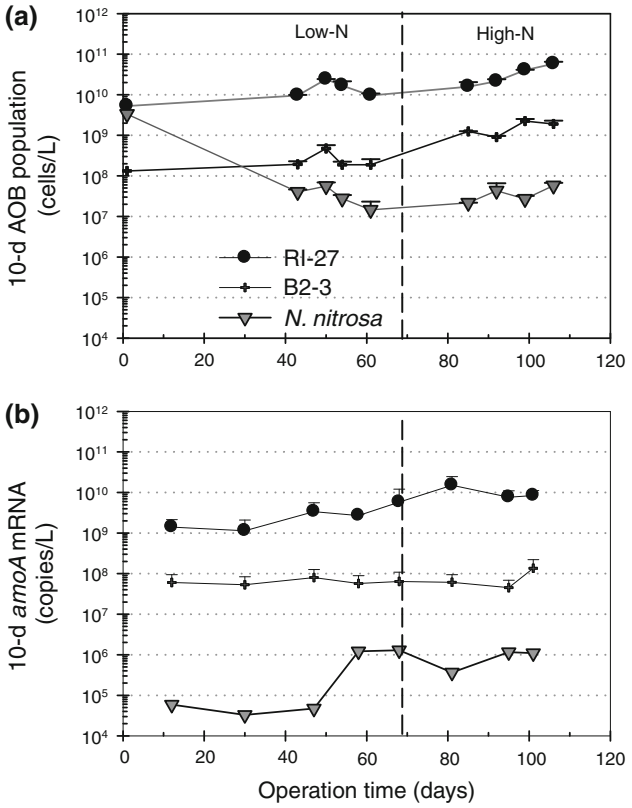

Fig. 3 RI-27, B2-3 and *N. nitrosa* populations (*amoA* DNA based quantification) (**a**) and transcription levels (*amoA* mRNA based) (**b**) in the 10-day SRT treatment system. The transition point from low-N to high-N on day 69 is indicated by *dashed lines*

more active than the other two AOB groups and this allowed the organism to dominate the overall AOB population in the 10-day SRT treatment system (Fig. 3a).

In the 2-day SRT system, the AOB population dynamics was also reflected in the assessed *amoA* mRNA levels; i.e., AOB with higher *amoA* mRNA levels dominated the overall AOB population (Fig. 4a, b). Specifically, after approximately 10 days of high-N operation, overall *amoA* mRNA abundance for the B2-3 population increased approximately 1,000-fold (from 1.55×10^6 to 1.67×10^9 copies/l) while the RI-27 *amoA* mRNA increase was approximately two-fold (Fig. 4b). These results supported the hypothesis that the B2-3 group would become more active than the RI-27 group under the relatively high ammonia level that accumulated during the transitional period between low- and high-N operation in the 2-day SRT system. This activity increase could be one major reason allowing the B2-3 group to dominate the overall AOB population under high-N and short-SRT operational conditions. The B2-3 AOB group was first reported by Gieseke et al. [9] for the AOB community structure of a biofilm. Given that the kinetic characteristics are still lacking for this organism, we surmised that the B2-3 growth rate was greater than RI-27 and *N. nitrosa* under high ammonia concentration based on the *amoA* mRNA levels observed in this study. In contrast, the growth rate of B2-3 would be lower than those of the other two AOB groups at the low ammonia concentration condition found in the 10-day SRT system making this organism a minor AOB group at the shorter SRT, as was observed.

At a normalized cellular level (based on *amoA* mRNA transcribed per copy of *amoA* gene, i.e., mRNA/DNA ratio), correlations between AOB populations and *amoA* mRNA levels (for each *amoA* gene) were not consistent. The *amoA* mRNA/DNA ratios ranged from 0.01 to 2.42 (Fig. 5). Although overall and cellular *amoA* transcripts of the RI-27 were generally higher than the B2-3 and *N. nitrosa* groups during its domination in the 10-day system under both low-N and high-N operation (Fig. 3a, b), the same phenomenon was not observed in the 2-day system (i.e., B2-3 dominated in the 2-day system during high-N operation but had a lower *amoA* mRNA/DNA ratio than RI-27). Additionally, in the 10-day system, the B2-3 *amoA* mRNA level decreased during high-N operation while its population slightly increased. Deviations for both *amoA*

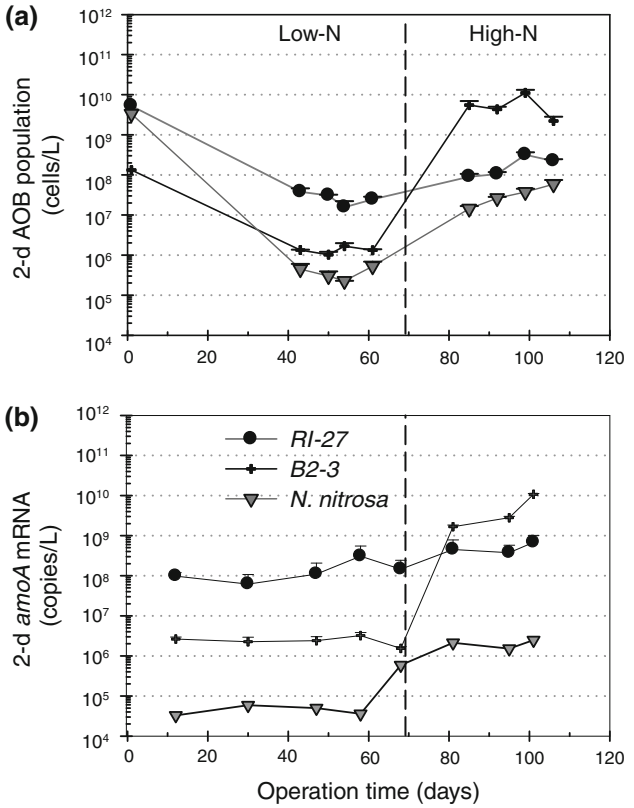

Fig. 4 RI-27, B2-3, and *N. nitrosa* populations (*amoA* DNA-based quantification) (**a**) and transcription levels (*amoA* mRNA based) (**b**) in the 2-day SRT treatment system. The transition point from low-N to high-N on day 69 is indicated by *dashed lines*

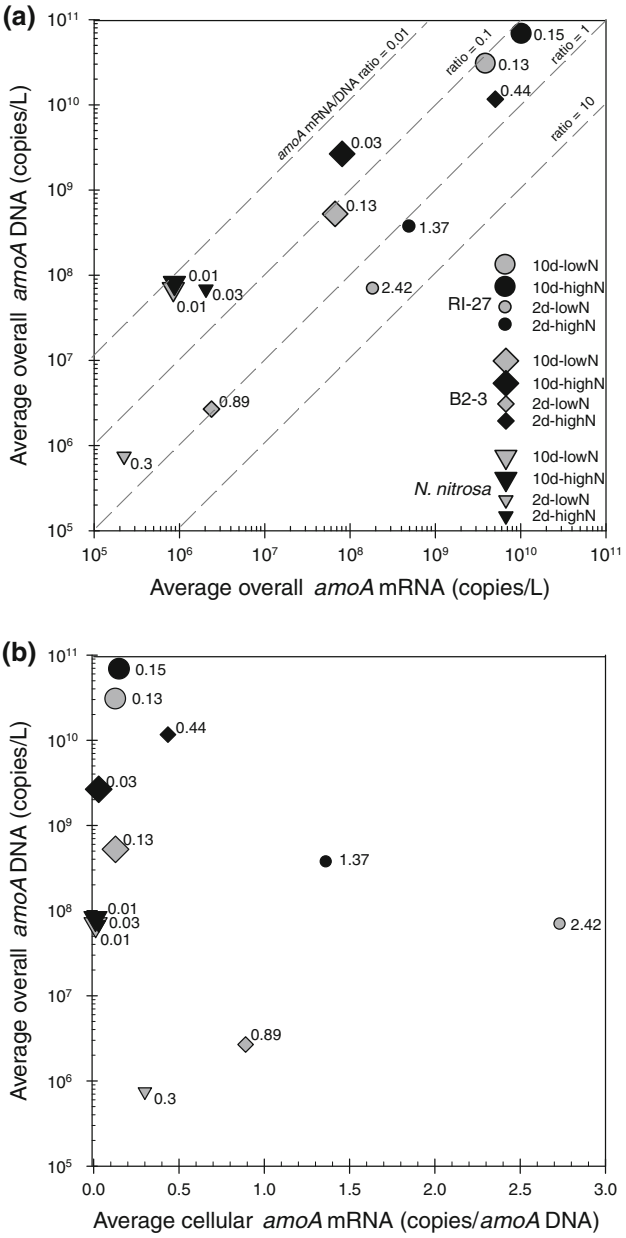

Fig. 5 Plots between average *amoA* mRNA and average *amoA* DNA showing a good correlation (Pearson correlation coefficient r was 0.95) (**a**). In contrast, diverse *amoA* mRNA/DNA ratios (from 0.01 to 2.42) did not consistently correspond to AOB population (*amoA* DNA-based) dominance (correlation coefficient $r = -0.21$) (**b**). For instance, B2-3 dominated in the 2-day system during high-N operation while its *amoA* mRNA/DNA ratio (0.44) was lower than that of RI-27 (1.37) in the same system

mRNA and DNA measurements could lead to a wide range of mRNA/DNA ratios, as was observed.

Interestingly, based on the *amoA* mRNA/DNA appraisal, the ratios for the 2-day SRT treatment system appeared to be about 10-fold higher than those in the 10-day SRT train (Fig. 5). The short-SRT operational characteristic of the 2-day treatment system dictated the daily removal of large amounts of new growth AOB cells, which led to the lower overall AOB population level at this SRT compared to the longer (10-day) SRT (Figs. 3a, 4a). While overall ammonia-oxidation performance (or oxidation rate) in the 2- and 10-day SRT systems were similar (i.e., 58 ± 16.4 and 65 ± 16.2 μM NH_3–N/h, respectively) [14], each AOB cell in the 2-day system (containing a smaller AOB population) appeared to oxidize more ammonia than that in the 10-day treatment system. Thus, the AOB cells in the 2-day system (with higher *amoA* mRNA levels) could quickly propagate under short SRT operation, thereby reducing the possible cellular washout and ultimately system failure. These observations are well supported by previous reports that indicate decreased SRT results in an increased substrate loading and oxidation rate when normalized per unit of activated sludge [10, 20].

Although multiple nitrogen species were monitored during this wastewater treatment study, approximately 13% (10-day) to 37% (2-day) of influent nitrogen could not be recovered in the effluent. The nitrogen that was unaccounted for could be due to either production of other, non-measured, nitrogen forms, or an underestimation of biomass-N. It has been suggested that aerobic denitrification

can occur in nitrification systems [26], hence, gaseous products from denitrification (e.g., N_2 or N_2O) may account for the missing nitrogen mass noted in this study. The increased denitrification activity would reduce nitrite/nitrate accumulation, which could benefit the nitrifying bacteria. Therefore, changes in denitrifier activity could affect overall AOB activity and potentially lead to an AOB population shift, particularly in shorter SRT systems with elevated denitrification activity.

Additionally, results indicate that kinetically diverse AOB groups had different *amoA* mRNA levels under various operational/environmental conditions. This study demonstrated that *amoA*-mRNA-based assessment of ammonia oxidation activity among distinct AOB groups was not the same, therefore the use of average cellular oxidation rates for multiple AOB groups in mixed cultures may not be representative of actual AOB activity and population dynamics. To date, the *amoA*-mRNA-based measurement has not been widely used to study nitrifying systems because specific primers and probes to quantify *amoA* mRNA transcripts have not been readily available for most AOB groups. This investigation has demonstrated that *amoA*-mRNA-based physiological activity for each of multiple AOB groups identified in an industrial wastewater treatment system could be examined individually to better assess nitrification population dynamics.

In summary, this work affirmed that the AOB group with higher total *amoA* mRNA levels dominated the overall AOB population during wastewater treatment although *amoA* mRNA/DNA ratios did not correlate well with the dominant AOB population. Various activities of distinct AOB groups could be reflected by overall *amoA* mRNA levels under different N-loading conditions allowing for timely assessment of the AOB population to gain a better understanding of population dynamics during wastewater treatment and to help in the optimization of nitrification performance.

Acknowledgments This work was funded by the Waste Management and Research and Education Institute of the University of Tennessee (Knoxville).

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