

Quantification of *amoA* gene abundance and their *amoA* mRNA levels in activated sludge by real-time PCR

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Abstract The transcription level of *amoA* mRNA encoding a subunit of ammonia monoxygenase (AMO) in ammonia-oxidizing bacteria (AOB) was quantified by reverse transcription-polymerase chain reaction (RT-PCR) methods in combination with real-time PCR technology. The effects of ammonia concentration and dissolved oxygen (DO) on the transcription levels of *amoA* mRNA and 16S rRNA in AOB were evaluated in batch experiments with nitrifying sludge taken from a lab-scale reactor treating artificial wastewater. A batch incubation without ammonia resulted in a rapid decrease, within four hours, in the transcription level of *amoA* mRNA to as low as 1/10 of that at the beginning of the experiment, while the 16S rRNA level in AOB was almost constant. After subsequent incubation with 3 mM ammonia for eight hours, a small increase in the transcription level of *amoA* mRNA occurred, but ammonia oxidation proceeded in the interim. Copy numbers of *amoA* mRNA showed an almost fixed value for over eight hours in the absence of dissolved oxygen.

Keywords Ammonia monoxygenase; ammonia-oxidizing bacteria; *amoA*; real-time PCR; reverse transcription

Introduction

Nitrification and denitrification are common biological processes for the removal of nitrogen from municipal sewage and industrial wastewater. Nitrification is a key step in this task because of the slow growth rates of nitrifying bacteria. Therefore, an abundance of nitrifying bacteria and their activities are essential to maintain superior wastewater processing performance and to develop operational strategies for nitrogen removal. The recent development of molecular biological tools such as fluorescent in situ hybridization (FISH) and PCR-DGGE based on 16S rDNA and rRNA provides sensitive and rapid detection for nitrifying bacteria (Wagner *et al.*, 1995; Kowalchuk *et al.*, 1997). However, these techniques are not capable of analyzing cell activities or of indicating a direct response to a change in the environmental conditions of the bioreactor.

Analysis of mRNAs as indicators of gene expression should significantly enhance our understanding of active functional groups such as nitrifying/denitrifying bacteria in the environment (Oldenburg *et al.*, 2003). Ammonia-oxidizing bacteria (AOB) such as *Nitrosomonas europaea* convert ammonia to nitrite through the mediation of two enzymes: ammonia monoxygenase (AMO) and hydroxylamine oxidoreductase (HAO) (Wood, 1986). The AMO is coded by the genes *amoC*, *amoA*, and *amoB*, which are cotranscribed as 3.5 kbp mRNA (Sayavedra-Sato *et al.*, 1998). It has been reported that *amoA* performs as a typical function gene of AOB for the analysis of the nitrifying community because the gene encodes the subunit that carries the active site of the enzyme (Rottehaue *et al.*, 1997).

A real-time PCR, recently developed, offers all the advantages of the conventional PCR such as high sensitivity and specificity, and quantification of the PCR product (Bassler *et al.*, 1995). A number of studies have utilized real-time reverse transcription (RT)-PCR to investigate the expression of a function gene, such as *amoA*, in a variety of environments (Goerke *et al.*, 2001). This technique has the potential of real-time monitoring of specific functional activity in a complex community.

In general, the nitrification/denitrification processes in domestic wastewater treatment were carried out by repetition of aerobic/anaerobic conditions or intermittent aeration conditions. This repetition could place AOB in stressful conditions by changes in ammonia concentration and dissolved oxygen (DO) for 2 or 3 hours. In most of the previous studies, pure cultures of AOB were utilized to focus on the transcriptional induction of *amoA* genes (Stein *et al.*, 1997, 1998; Hommes *et al.*, 2001). However, only a few studies have reported on the transcription levels of *amoA* in AOB grown in a complex community such as activated sludge (Ebie *et al.*, 2002). Moreover, the impact of DO on AOB has not been reported at all yet. In the present study, the effect of changes in DO and ammonia concentration on the *amoA* mRNA transcription level was investigated using nitrifying sludge subjected to transient aerobic-anaerobic periods. Real-time PCR technology was employed for the quantification of 16S rDNA and *amoA* gene in AOB and the quick monitoring of *amoA* mRNA in nitrifying sludge.

Materials and methods

Pure culture and nitrifying sludge

Nitrifying sludge was developed in a lab-scale reactor that contained sponge material (0.7 cm cubes) as a biomass attachment site. Table 1 shows the composition of the synthetic wastewater fed to the reactor. The ammonia oxidation efficiency of the reactor achieved a level of over 90% at a loading rate of 200 mg-N/L/day. The temperature was maintained at 20°C. A pure culture of *N. europaea* (IFO 14298) was prepared for calibration of gene quantification and DNA standards for real-time PCR.

Batch experiment

An experiment with controlled ammonia concentrations (controlled-ammonia experiment) was conducted using three flasks (1.0 L) containing 0 mM ammonia, 3 mM ammonia, and a mixture of 3 mM ammonia and 3 mM nitrite medium. Thirty pieces of sponge cube holding nitrifying sludge were transferred immediately from the reactor to a flask containing 0 mM ammonia, and incubated for 8 h at 20°C. The sponge cubes in the 0 mM ammonia medium were then transferred to the 3 mM ammonia medium, and subsequently to the mixed medium. The total experimental duration was 24 h. DO was kept over 5 mg/L throughout the experimental period. An experiment with controlled DO concentrations (controlled-DO experiment) was performed in a similar manner, while ammonia was kept at 3 mM in

Table 1 Composition of synthetic wastewater fed to lab-scale reactor

Composition	(g/100 L)	
NH ₄ Cl	15.28	
NaHPO ₄ · 12H ₂ O	2.31	
NaCl	1.01	
KCl	0.47	
CaCl ₂ · 2H ₂ O	0.47	
MgSO ₄ · 7H ₂ O	1.67	
NaHCO ₃	93.72	
Fe-EDTA	0.03	
CH ₃ COONa	5.47	10.94

the medium. Nitrifying sludge samples for quantification of *amoA* mRNA were prepared by removing from the flask and squeezing two of the sponge cubes at appropriate intervals throughout both experiments.

DNA and RNA extraction

DNA and total RNA were extracted and purified from nitrifying sludge sampled from the test flask. The sludge was collected by centrifugation at $12\text{ k} \times g$ for 5 min at 4°C , and the resulting pellet was washed twice with 1 mL of buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.1) to remove the ammonia completely. DNA extraction and purification were performed as described by Miller *et al.* (1999) by using a lysis buffer containing sodium dodecyl sulfate and beads beating. For RNA extraction, the sludge pellets had been stored in RNA Later (Ambion) at 4°C to maintain the yield in the same conditions and to maintain the quality of the extracted RNA. The pellet and 1 mL of Isogen (Nippon Gene, Japan) were added to a 2 mL screw-cap tube containing 0.5 g of glass beads (0.2 mm in diameter). Lysis of cells was done twice by repetition of beads beating at 5,000 rpm for 2 min (Mini-BeadBeater, Biospec Products, Inc.), and then incubated at 60°C for 20 min. After the addition of 0.2 mL of chloroform, the tube was vortexed for 15 sec and centrifuged at $12\text{ k} \times g$ for 15 min. The aqueous phase was transferred into a 1.5 mL tube, 0.5 mL of isopropyl alcohol was added, and the mixture was incubated at 20°C for 15 min. After ethanol precipitation, the purified RNA was resuspended in 0.2 mL of TE (10 mM Tris-HCl, 1 mM EDTA). Fifty units of DNase I (TaKaRa, Japan) were added to the RNA extraction to eliminate contaminating genomic DNA. The DNA and total RNA were quantified by UV spectrophotometry with a Gene Quant Pro (Amersham Pharmacia Biotech).

PCR primer set

Table 2 shows PCR primers used in this study for amplification of the genes in AOB. The primer set of CTO189f/RT1r was selected to amplify a 116 bp DNA fragment in the V2 region of 16S rDNA of AOB. The primer set of *amoA*-1F and *amoA*-2R target stretches corresponding to positions 332 to 349 and 802 to 822, respectively, of the open reading frame for the *amoA* gene sequence of *N. europaea*.

RT reaction

Reverse transcription was performed using a RNA PCR kit (AMV) Ver. 2.1 (TaKaRa, Japan) and random primer (5'-NNNNNNNNN-3'). The RT reaction was carried out with a thermal cycler (i-cycler, Bio Rad) at 30°C for 10 min, 55°C for 30 min, 99°C for 5 min and 5°C for 5 min (on ice).

Real-time PCR

Real-time PCR was performed on a smart cycler (TaKaRa Biomedical). In the PCR assay, the TaKaRa Ex taq R-PCR version (TaKaRa Biomedical) was used as recommended by the manufacturer. SYBR Green I was applied as a double-stranded DNA binding fluorescent

Table 2 PCR primers used in this study for quantification of 16S rDNA and *amoA* gene in AOB

Primer	Nucleotide sequence (5'-3')	Sequence position	Target	Reference
CTO189fA/B*	GGAGRAAAGCAGGGGATCG	189-207	BAOB	Kowalchuk <i>et al.</i> (1997)
CTO189fC*	GGAGGAAAGTAGGGGATCG	189-207	BAOB	Kowalchuk <i>et al.</i> (1997)
RT1r	CGTCTCTCAGACCARCTACTG	283-304	BAOB	Hermansson <i>et al.</i> (2001)
<i>amoA</i> - 1F	GGGGTTTCTACTGTTGGT	332-349	BAOB	Rottehouwe <i>et al.</i> (1997)
<i>amoA</i> - 2R	CCCCTCKGSAAGCCTTCTTC	802-822	BAOB	Rottehouwe <i>et al.</i> (1997)

* A mixture of CTO189fA/B and CTO189fC at the weight ratio of 2:1 was used as the forward primer

dye. The PCR amplification of 16S rDNA, *amoA* gene, and 16S rcDNA, *amoA* cDNA derived from reverse transcription of total RNA, was performed at 95°C for 120 sec and then for 40 cycles of four steps consisting of 10 sec at 95°C, 15 sec at annealing temperature, 10 sec at 72°C and 5 sec at 88°C. The annealing temperatures were 61°C and 59°C for the primer set of CTO189f/RT1r and *amoA*-1F/*amoA*-2R, respectively. In all experiments, appropriate negative controls containing no template DNA or RNA were subjected to the same procedure to exclude or detect any possible contamination or carryover. Melting curves were also routinely checked to confirm quantification of the desired products. The standard curve ranging from 10^1 – 10^6 copies/tube was constructed by using a serial dilution of real-time PCR products from the genome of *N. europaea* with each primer set. In real-time PCR analysis, quantification is based on the threshold cycle, which is inversely proportional to the logarithm of the initial copy number. The threshold cycle values obtained for each sample were compared with a standard curve to determine the initial copy number of the target gene.

FISH analysis

For in-situ hybridization with fluorescence-labeled oligonucleotide probe targeting 16S rRNA, Nmn657 (5'TGGAATTCCACTCCCCTCTG3', *Escherichia coli* position: 657–667) (Araki et al., 1999) specific for genus *Nitrosomonas* was used in this study. The probe was 5' labeled with tetramethylrhodamin-isothiocyanate (TRITC). The protocol described by Amann (1995) was used, the hybridization conditions being a temperature of 46°C and a formamide concentration of 15%.

Results and discussion

Gene quantification by real-time PCR technology

According to theoretical calculations, *N. europaea* has 3.15×10^5 genomes/ng of DNA. In detail, this genome copy is calculated from a genome size of 2.81 Mbp in *N. europaea* (Chain et al., 2003) and an average molecular weight of 326 for the four nucleotides. Table 3 shows the number of genes in the DNA extracted from a pure culture of *N. europaea* and the nitrifying sludge sampled from the lab-scale reactor when the batch experiment had just started. The copy number of 16S rDNA in the DNA extracted from the pure culture was determined to be 1.9×10^5 copies/ng, a figure similar to that obtained in the theoretical calculation. On *amoA* gene (*amoA*-1F/*amoA*-2R), the expression volume was found to be 3.3×10^5 copies/ng. On the other hand, 16S rDNA gene and *amoA* gene numbered 3.0×10^4 copies/ng and 1.8×10^5 copies/ng, respectively, in the extracted DNA from the nitrifying sludge. If the genome size of the extracted DNA from the nitrifying sludge is formed at 4.6 Mbp, which is the same as for *E. coli*, the equivalent DNA has 2.2×10^5 genomes/ng. In the samples, 16S rDNA gene of AOB includes 3.0×10^4 copies/ng. If the 16S rDNA gene is assumed to have one copy per genome, it is estimated that the population of AOB in nitrifying sludge represents 13.6% of the total number of cells.

Figure 1 shows FISH analysis with an Nmn657 probe specific for genus *Nitrosomonas* of β -subclass *proteobacteria* in nitrifying sludge samples. Genus *Nitrosomonas* organisms formed a tight cluster in sludge flocs, as shown in the picture. For the microscopic counting of cells, the ratio of the total number of FISH-detected *Nitrosomonas* to that of DAPI-stained cells in any 10 fields of view was $5.2\% \pm 4.4\%$. This value is smaller than that obtained from the gene quantification of the proportion of AOB cells, which was 13.6%. There are two possible reasons: either the *Nitrosomonas* cells overlapped each other when the direct cell count was made; or more than one genome was present in one cell. The latter possibility results from preferable conditions such as a C/N ratio of 0.4 in the artificial substrate being maintained for higher activation of AOB.

Table 3 Gene numbers of 16S rDNA and *amoA* gene of AOB in equivalent DNA from *Nitrosomonas europaea* culture and nitrifying sludge sample by real-time PCR methods

Sample	<i>Nitrosomonas europaea</i>	DNA	Nitrifying sludge	DNA
Primer set	CTO189f/RT1r	<i>amoA</i> – 1F/2R	CTO189f/RT1r	<i>amoA</i> – 1F/2R
Target	16S rDNA	<i>amoA</i> – gene	16S rDNA	<i>amoA</i> – gene
Copies/ng	1.9×10^5	3.3×10^5	3.0×10^4	1.8×10^5

Impact of changes in ammonia concentration on *amoA* mRNA levels

The nitrifying sludge absorbed by each sponge cube was cultured in a vial containing 0 mM, 0.1 mM, 1 mM, 10 mM or 50 mM ammonia for 4 days (96 hr). Figure 2 shows the number of *amoA* mRNA (cDNA) copies in total-RNA extracted from the nitrifying sludge cultivated in different ammonia concentrations. The transcription level of *amoA* mRNA was absolutely dependent on the change in ammonia concentration; and a higher

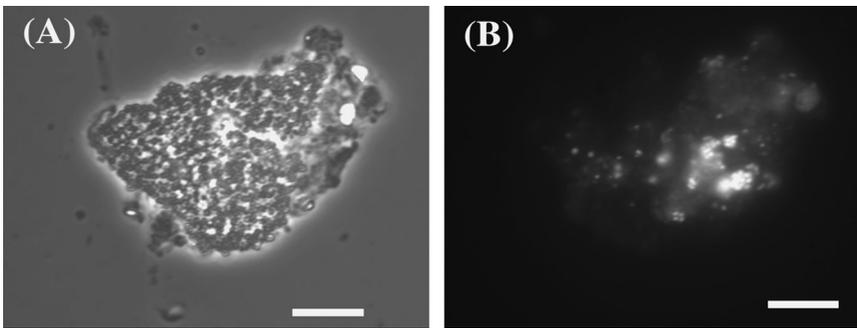


Figure 1 In-situ hybridization with fluorescent oligonucleotide probe (Nm657) specific for the genus of *Nitrosomonas*. Identical microscopic fields were viewed by phase contrast (A) and by G-excitation (B). Scale bars represent 10 μ m

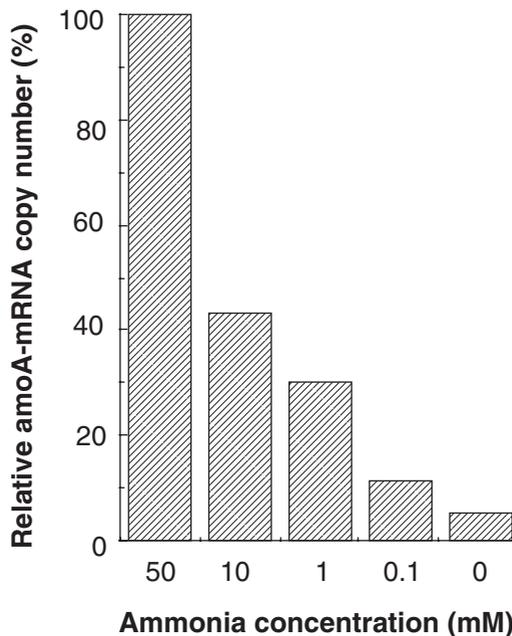


Figure 2 Copy numbers of *amoA* mRNA in the nitrifying sludge incubated with different ammonia concentration for 96 hours by real-time RT-PCR

concentration of ammonia induced an increase in the transcription. Therefore, the methodology for quantification of *amoA* mRNA applied in this study could be applicable for determining those transcription levels.

Figure 3 shows the impact of the ammonia concentration on the number of *amoA* mRNA copies in the equivalent amount of total-RNA extracted from samples in the controlled-ammonia experiment. The experiment was conducted at various concentrations of ammonia and nitrite, but at constant pH, water temperature and DO concentration. In the ammonia starvation period of Run 1, *amoA* mRNA decreased rapidly to as low as 1/10 at the beginning of the experiment. After an increase in ammonia concentration (3 mM) in Run 2, the expression of *amoA* mRNA did not recover to the same level as at the start of Run 1. In Run 3, where there was a mixture of nitrite (3 mM) and ammonia (3 mM), the expression volume of *amoA* mRNA decreased slightly. However, the expression increased at the end of the run. The RT product (cDNA) from 16S rRNA of genus *Nitrosomonas* in nitrifying sludge was also quantified with the CTO189f/RT1r primer set. The value of cDNA was almost constant at $4.8 \pm 2.2 \times 10^6$ copies/ng throughout the experimental period from Run 1 to Run 3.

Induction of *amoA* mRNA occurs at ammonia concentrations as low as 0.013 mM, according to Sayavedra-Soto's report (Sayavedra-Soto *et al.*, 1996). In a complex microbial community, an ammonia concentration sufficient to induce *amoA* would be generated by cell decomposition. For this reason, *amoA* mRNA was maintained at the 10^4 copies/ng level after 8 h of starvation (Run 1), and also after 96 h under the same starvation conditions (Figure 2) as were previously mentioned. The transcription of *amoA* mRNA was not activated as much as at the beginning of the experiment under conditions of 3 mM of ammonia, while accumulation of nitrite in the medium was found in Run 2. Therefore, ammonia oxidation may have been promoted by the residue of AMO enzyme in cells formed before Run 2. It appears that the *amoA* mRNA transcription level and the ammonia oxidizing activity cannot be linked directly.

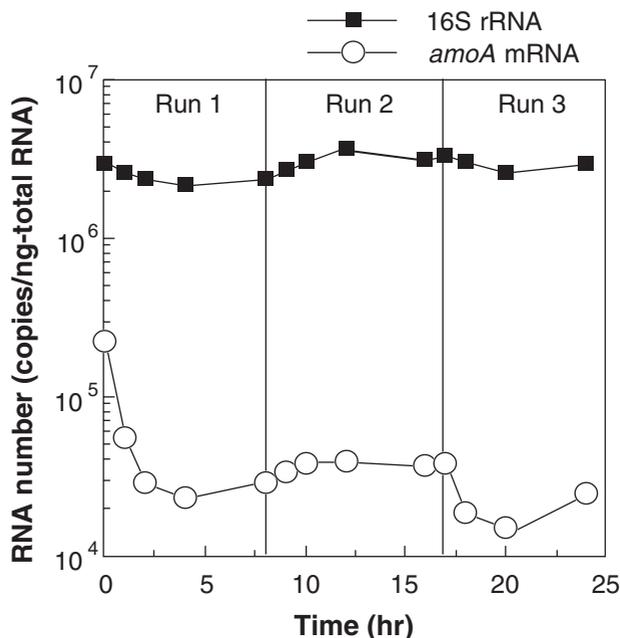


Figure 3 Changes in copy number of *amoA* mRNA and 16S rRNA in nitrifying sludge during the controlled-ammonia experiment

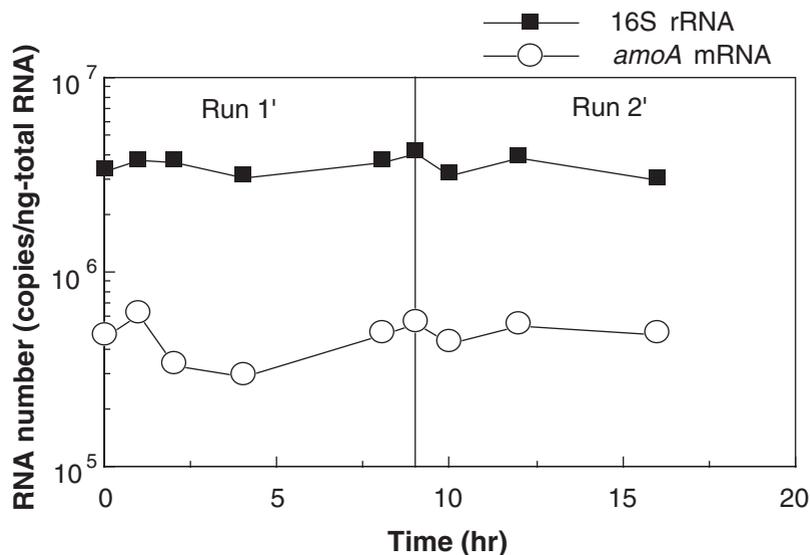


Figure 4 Changes in copy number of *amoA* mRNA and 16S rRNA in nitrifying sludge during the controlled-DO experiment

Impact of changes in DO concentration on *amoA* mRNA levels

Figure 4 shows the change in the copy number of *amoA* mRNA in the controlled-DO experiment. In Run 1, *amoA* mRNA did not show a significant change in DO at 0 mg/L. Then DO was set again at 5 mg/L after 8 hr in Run 2'. However, *amoA* mRNA showed no significant change. In Run 1', anoxic conditions were maintained for 35 h. The expression of *amoA* mRNA fell as low as 44% of that at the beginning.

The transcription of *amoA* mRNA was continued while ammonia oxidation was stopped by 8 h of anoxic conditions. In this experiment, the concentration of the AMO enzyme was not measured. Therefore, it is not clear: 1) whether the electron acceptor was present during enzyme production in anoxic conditions, or 2) whether the translation or post-translation step was inhibited by some agent other than transcription.

Conclusions

The findings of this experiment are summarized as follows.

1. Utilizing the (RT)-PCR method, it was possible to trace the impact on the expression of *amoA* mRNA of ammonia-oxidizing bacteria in nitrifying sludge by varying the concentration of ammonia or nitrite at intervals of a few hours.
2. After 8 h of ammonia starvation, the expression of *amoA* mRNA decreased to 1/10. After the ammonia concentration again began to increase at 3 mM, the expression of *amoA* mRNA recovered slightly but ammonia oxidation still continued.
3. Under ammonia starvation, the expression level of *amoA* mRNA was kept at a level of 10⁴ copies/ng per total RNA extracted.
4. The expression of *amoA* mRNA of ammonia oxidizing bacteria did not have a significant effect during the 8 h in the absence of DO.

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