

To the Graduate Council:

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Population (*amoA*-based) and activity (*amoA*-mRNA-based)
assessment of ammonia oxidizing bacteria (AOB)
during activated sludge wastewater treatment

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Abstract

The population size and physiological activity of ammonia-oxidizing bacteria (AOB) are two crucial rate-limiting parameters for the removal of ammonia from wastewater (Wagner et al., 1995). These two biological parameters may be affected by operational/environmental conditions (e.g., low pH, low temperature, low dissolved oxygen (DO), or short solids retention time (SRT)), and these impacts may subsequently influence ammonia removal performance in wastewater treatment systems (Okabe et al., 1996; Nogueira et al., 2002; Robinson et al., 2004; Zimmerman et al., 2004; Hallin et al., 2005). The impacts of changes in population dynamics and microbial activity of AOB on overall nitrification efficiency during wastewater treatment have not been fully addressed. The fundamental goal of this study was to assess AOB population dynamics (based on gene *amoA* measurement) and AOB physiological activity (based on *amoA* mRNA transcript measurement) as a function of changes in operational conditions (i.e., SRT, NH_3 , and, DO), and relate these molecular parameters to overall ammonia oxidation treatment performance.

This study consisted of three components to achieve the overall research goal. Component I focused on estimating long-term shifts in AOB population dynamics under different operational conditions (four SRTs, two NH_3 levels, and two DO levels) in an industrial wastewater (bench-scale activated sludge) treatment system. Results suggested that the AOB population dynamics in this treatment system were primarily influenced by SRT and NH_3 levels. Short-term changes in NH_3 concentration affected AOB activity (based on ammonia oxidation rates); however, with long-term changes in NH_3 level, the AOB population gradually shifted in short SRT systems due to ammonia induced selection of different AOB strains. Changes in DO concentration (from 3 mg/L to 0.5 mg/L) did not significantly affect AOB population dynamics. In general, overall AOB population levels did not rapidly respond to the tested operational changes. Thus, real-time fluctuations in the overall ammonia oxidation performance of the treatment system were not solely reflected by AOB population levels.

In research components II and III, *amoA* mRNA abundance was measured to determine whether this metric was related to AOB activity. In component II, an AOB culture from a laboratory enriched nitrifying system was used to examine the *amoA* mRNA response under varying NH₃ and DO levels to assess AOB physiological activity at the transcriptional level. Results indicated that *amoA* mRNA abundance quickly (few hours) responded to different ammonia levels while *amoA* DNA levels did not notably change; and, the maximum *amoA* mRNA level reflected the ammonia oxidation activity of AOB cells under each ammonia condition. However, the *amoA* mRNA level did not correspond to the significant drop in ammonia oxidation activity during DO limitation. In component III, the physiological activity of each AOB population in the industrial bench-scale system was assessed based on *amoA* mRNA measurements to determine if activity ascendancy of an AOB strain lead to its population dominance in the treatment system under two different ammonia loadings. Results indicated that the dominant AOB population had relatively higher *amoA* mRNA abundance than the other strain's allowing this group to be more active than other AOB groups.

In summary, the physiological activity measurements, based on *amoA* mRNA assessment, were responsive to some changes in operational conditions (i.e., varying NH₃ concentrations at sufficient oxygen levels). Thus, this would allow quick identification of fluxes in ammonia oxidation performance for nitrifying systems. However, shifts in ammonia oxidation rate did not strictly correlate to changes in *amoA* mRNA level suggesting that AOB oxidation activity involved additional regulatory mechanisms other than *amo* expression, possibly at the post-transcriptional level. The *amoA* mRNA assessment may reflect an AOB oxidation activity shift in some cases but cannot be solely used to evaluate ammonia oxidation performance in all nitrifying systems. Hence, traditional oxidation-rate-based estimation is still necessary for routinely monitoring ammonia oxidation performance in treatment systems. Nevertheless, the biological parameters of AOB population levels and cellular activity provide insight into the microbial processes behind nitrification thereby enhancing our understanding of the nitrogen conversion process.

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List of abbreviations

AMO:	Ammonia monooxygenase
<i>amo</i> :	gene encoding ammonia monooxygenase
<i>amoA</i> :	gene encoding subunit A of ammonia monooxygenase
ANAMMOX:	Anaerobic ammonia oxidation
AOB:	Ammonia-oxidizing bacteria
AOR:	Ammonia oxidation rate
BOD:	Biological oxygen demand
C:	(Organic) Carbon
COD:	Chemical oxygen demand
CSTR:	Continuous-flow stirred tank reactor
Ct:	Threshold cycle (in real-time PCR)
DO:	Dissolved oxygen
HRT:	Hydraulic retention time
mRNA:	messenger ribonucleic acid
N:	Nitrogen
NOB:	Nitrite-oxidizing bacteria
PCR:	Polymerase chain reaction
RT:	Reverse transcription
SRT:	Solids retention time
WWTP:	wastewater treatment plant

Chapter 1 Introduction

1.1 Background

During modern wastewater treatment, ammonia (NH_3) removal is essential to protect receiving water bodies from eutrophication and oxygen depletion (Painter, 1986; EPA, 2001). Ammonia removal from wastewater is normally achieved using engineered biological treatment processes (i.e., nitrification or anaerobic ammonia oxidation). Within these ammonia removal processes, ammonia oxidation to nitrite (NO_2^-) by ammonia-oxidizing bacteria (AOB) is the first key step in biologically transforming ammonia into stable nitrogen products (e.g., NO_3^- , N_2). Therefore, effective ammonia oxidation by AOB is prerequisite to accomplish efficient ammonia removal during biological wastewater treatment.

Ammonia oxidation performance (as well as nitrification performance) can be negatively affected by improper operational/environmental conditions (Wild et al., 1971; Sharma and Ahlert., 1977; Painter, 1986; Okabe et al., 1996; Nogueira et al., 2002; Robinson et al., 2004; Hallin et al., 2005). Rapid assessment of ammonia oxidation under various conditions is crucial to detect nitrification instability and possible system failure. Traditionally, ammonia oxidation performance has been evaluated by ammonia oxidation rate (Belser, 1979; Prosser, 1989; Daims et al., 2001 (a)). However, once retardation in ammonia oxidation is noted, this oxidation-rate-based parameter would not provide further information about the AOB microbial circumstance for understanding and solving unstable nitrification problems (Figure 1-1). Alternatively, the AOB population size and physiological activity are generally considered as two fundamental rate-limiting parameters for ammonia removal from wastewater (Wagner et al., 1995). Several studies have elucidated AOB population ecology and cell activity in activated sludge or biofilm treatment systems (Schramm et al., 1999; Gieseke et al., 2001; Dionisi et al., 2002; Harms et al., 2003), but correlations between these two parameters and overall ammonia oxidation performance have not been well understood (Kowalchuk and Stephen, 2001; Keller et al., 2002).

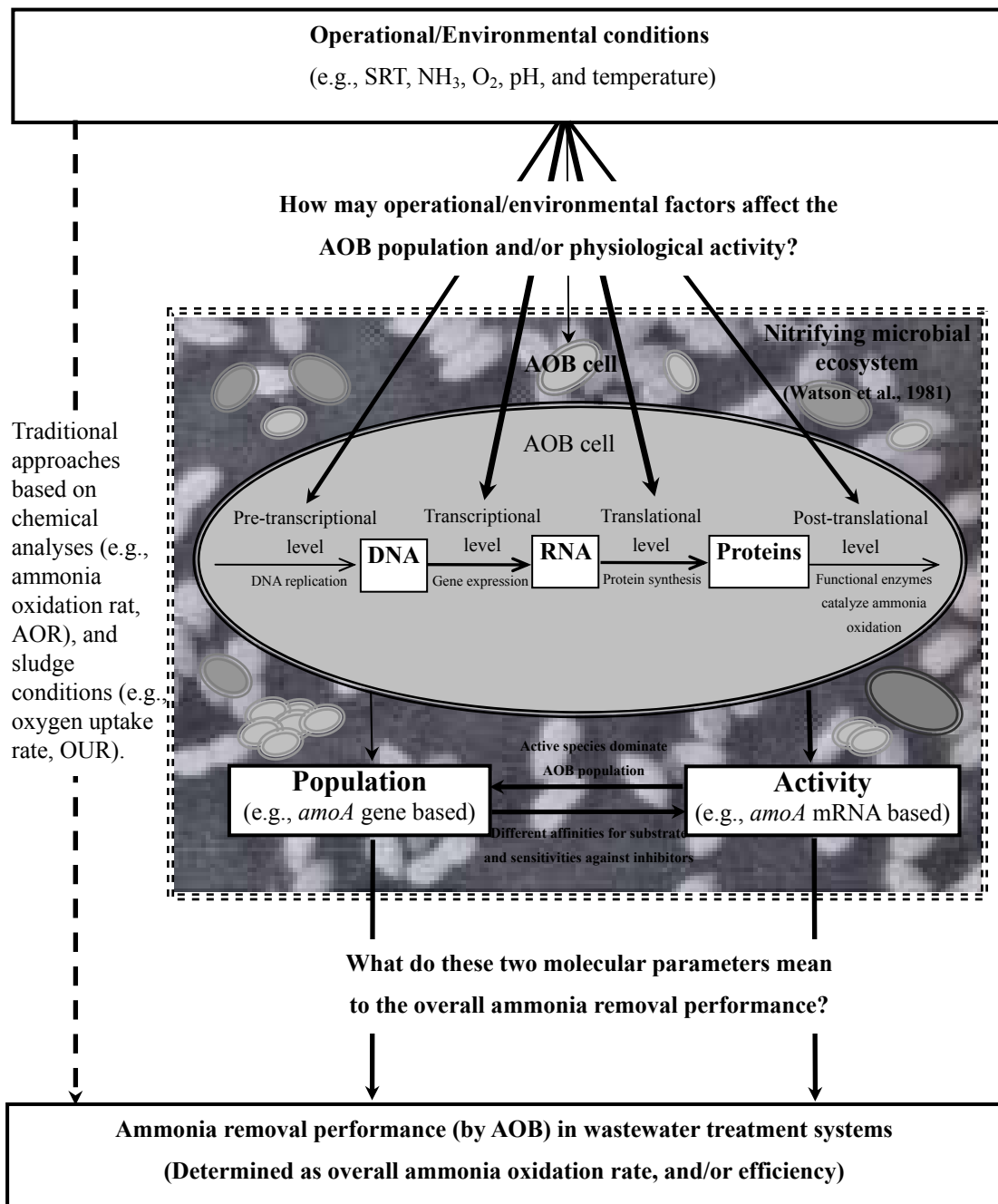


Figure 1-1. Conceptual model representing how operational/environmental factors may influence AOB population dynamics and/or cellular oxidation activity, and affect ammonia removal performance.

As suggested by the conceptual model shows in [Figure 1-1](#), the variables of concern may directly or indirectly influence the AOB activity and/or population by impacting metabolic pathways or controlling retention time of AOB cells in the treatment system ([Sayavedra-Soto et al., 1996](#); [Stein et al., 1997](#); [Stein and Arp, 1998 \(a\)](#), [Bothe et al., 2000](#); [Brandt et al., 2001](#); [Aoi et al., 2002, 2004 \(a\), \(b\)](#); [Hallin et al., 2005](#)). Consequently, variations in microbial activity and population may lead to different treatment performances in nitrification processes. This study mainly focused on examining the influences of three important operational factors (i.e., SRT, NH_3 , and DO) on AOB population size and physiological activity. The SRT directly controls biosolids concentration and microbial community structure in treatment systems by removing a fraction of the biomass under steady-state conditions. An appropriate SRT range is required to maintain slow-growing AOB in nitrifying systems ([Painter, 1986](#); [Pollice et al., 2002](#)). In addition, the NH_3 and DO concentrations are two other major factors affecting nitrification performance in wastewater treatment systems ([Hunik et al., 1992](#)) because AOB utilize NH_3 and DO as the sole energy and N-source and as a terminal electron acceptor, respectively. While short-term fluctuation in either NH_3 or DO concentrations may affect AOB activity ([Aoi et al., 2004 \(b\)](#), [Bollmann et al., 2005](#)), long-term limitation in either substrate may result in the loss of nitrification function in a treatment system due to a reduction in the number of AOB cells.

Over the past decade, it has become clear that the AOB in complex microbial communities do not belong to a single species. Based on cultivation-independent molecular approaches, diverse uncultured AOB strains, in addition to the 16 currently-defined AOB species, have been identified in various engineered systems and natural environments ([Purkhold et al., 2000](#); [Gieseke et al., 2001](#); [Purkhold et al., 2003](#); [Francis et al., 2003](#)). The *amoA* gene has been used to distinguish between closely related AOB based on the fact that it is a functional trait with higher sequence variability than 16S rDNA ([Rotthauwe et al., 1997](#); [Purkhold et al., 2000](#)). The AOB populations in several microbial ecosystems have been enumerated using the *amoA*-based quantitative real-time PCR approach ([Harms et al., 2003](#); [Okano et al., 2004](#); [Layton et al., 2005](#)). However, the manner in which the conditions affect AOB population and then impact overall ammonia

oxidation performance has not been fully addressed. Additional studies are still required to understand which AOB populations dominate in particular nitrifying systems under various operational/environmental conditions and to further reveal their physiological characteristics in order to provide critical information for optimization of nitrification.

The ammonia oxidation activity of AOB cells has been traditionally determined as average cellular oxidation rate (or nitrite production rate) by simultaneously estimating cell numbers and substrate (or product) concentrations in both batch and continuous culture systems (Belser, 1979; Prosser, 1989; Schramm et al., 1999; Harms et al., 2003). But, this parameter does not directly provide insight on the physiological activity conditions of AOB cells and has limitations in addressing specific activity variances between distinct AOB groups coexisting in mixed cultures. Recently, abundance of *amoA* mRNA transcripts has been proposed to monitor AOB physiological activity (Aoi et al., 2002). However, specific correlations between *amoA* mRNA level and ammonia oxidation performance (based on oxidation rates) have not been explicitly determined. It is necessary to clarify whether and how the *amoA* mRNA level can reflect the ammonia oxidation activity of AOB cells.

1.2 Research objectives, and hypothesis

The overall goal of this research is to assess the AOB population (based on gene *amoA* measurement), and the AOB physiology activity (based on *amoA* mRNA transcript measurement) as a function of changes in operational conditions (SRT, NH₃, and DO), and then relate these molecular parameters to overall ammonia oxidation performance.

Three major objectives of this research are:

1. To determine the effects of the operational factors SRT, NH₃ concentration, and DO level on AOB population dynamics and examine links between the AOB populations and the ammonia oxidation treatment performance,
2. To establish the *amoA* mRNA transcript level under varying NH₃ and DO levels to assess whether this physiological indicator may reflect the ammonia oxidation activity of AOB cells, and

3. To verify whether the dominant AOB has an activity supremacy and whether shifts in AOB populations are a result of changes in AOB cellular activity.

The research hypotheses are:

1. The operational factors of SRT, NH_3 , and DO will significantly affect AOB population dynamics and the ammonia oxidation treatment performance; the ammonia oxidation performance will depend upon the overall AOB population size.
2. The abundance of the *amoA* mRNA transcripts will be affected by varying NH_3 and DO levels. The *amoA* mRNA level can reflect the ammonia oxidation activity of AOB cells. And, the *amoA*-mRNA-based activity assessment will be more responsive to shifts in ammonia oxidation performance.
3. An AOB strain that produces more *amoA* mRNA will be more active and will dominate the AOB population in the treatment system.

Chapter 2 Literature reviews

2.1 Ammonia removal from wastewater

Nitrogen (N) is a major element that can be incorporated into several organic and inorganic compounds. These nitrogen-containing materials can be converted via various biological or chemical processes to other forms within the global nitrogen cycle ([Figure 2-1](#)). Some of these nitrogen-containing compounds are useful; however, some, such as ammonia/ammonium ($\text{NH}_3/\text{NH}_4^+$), are considered major pollutants in municipal, agricultural, and many industrial wastewaters. Typically, ammonia concentrations in sewage treatment systems are on the order of 1-50 mg/L as $\text{NH}_3\text{-N}$ (~60 mg/L as TKN-N); however, some industrial effluent, livestock wastewater, or landfill leachate have concentrations up to ~1,000 mg/L as $\text{NH}_3\text{-N}$ ([Prosser, 1989](#); [Hellinga et al., 1998](#); [Rittmann and McCarty, 2001](#)). Although a certain amount of ammonia in wastewaters can supply nitrogen necessary for microbial growth during wastewater bio-treatment, discharge of excess ammonia into receiving water bodies may cause eutrophication and oxygen depletion ([Painter, 1986](#)), and may also harm aquatic life ([Arthur et al., 1987](#)). Therefore, engineered processes that optimize the transformation of ammonia in wastewater to stable nitrogen compounds have been an important role in biological nutrient removal in modern wastewater treatment.

2.1.1 Ammonia conversion pathways

[Figure 2-2](#) summarizes the three primary ammonia conversion pathways. Ammonia (NH_3 with an oxidation state of -3) can be transformed to dinitrogen gas (N_2 with an oxidation state of 0) via the nitrification/denitrification process or partial-nitrification/denitrification process in which nitrate (NO_3^- with a valence of $+5$), and nitrite (NO_2^- with a valence of $+3$) are important intermediates, respectively. Alternatively, ammonium (NH_4^+) can be directly converted to dinitrogen gas via the anaerobic ammonia oxidation (ANAMMOX) process (using nitrite, previously oxidized from ammonia, as the terminal electron acceptor, [Bock et al., 1995](#); [Strous et al., 1999](#)).

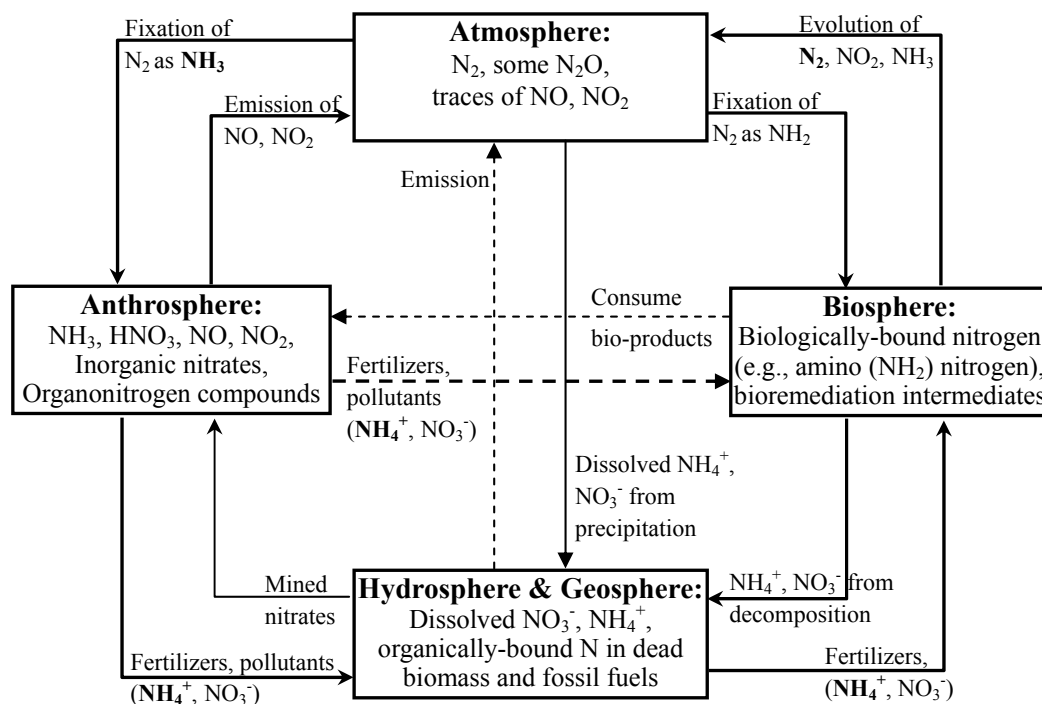


Figure 2-1. The nitrogen cycle among all spheres on the earth (Manahan, 2000).

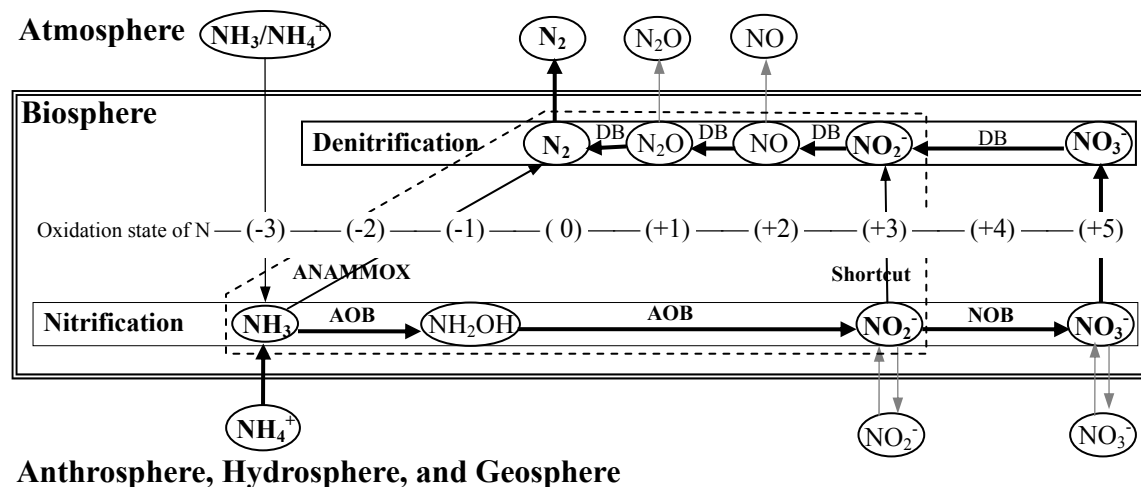
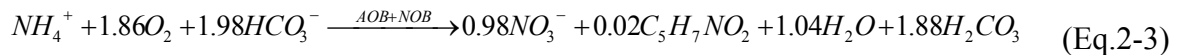
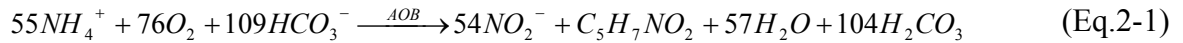


Figure 2-2. Biological transformation pathways of ammonium (AOB: ammonia-oxidizing bacteria; NOB: nitrite-oxidizing bacteria; DB: denitrifying bacteria.).

Complete ammonia removal from wastewater is most often achieved using classical nitrification/denitrification processes (Figure 2-2). Aerobic biological nitrification is a two-step oxidation process. The first step is ammonia (NH₃) oxidation to nitrite (NO₂⁻) that is mainly carried out by autotrophic ammonia-oxidizing bacteria (AOB). The second step is nitrite (NO₂⁻) oxidation to nitrate (NO₃⁻) that is carried out by autotrophic nitrite-oxidizing bacteria (NOB). The two bacterial groups work synergistically to complete the nitrification process in natural environments (e.g., ocean, lake and soil) or in engineered treatment systems (e.g., activated sludge and biofilm) usually under aerobic conditions. The ammonia oxidation (Eq.2-1), nitrite oxidation (Eq.2-2), and complete nitrification reaction (Eq.2-3) can be represented by the following equations (Brown and Caldwell, 1975; Rittmann and McCarty, 2001):



Ammonia oxidation (Eq.2-1) has been recognized as the rate-limiting step of nitrification because it proceeds more slowly than nitrite oxidation (Eq.2-2). These equations indicate that oxygen (O₂) and alkalinity (in term of bicarbonate, HCO₃⁻) are required for AOB and NOB to oxidize ammonia to nitrate via nitrite. To completely oxidize one mole of ammonia to nitrate, about 1.86 moles of oxygen and 1.98 moles of bicarbonate are theoretically required (Eq.2-3). Therefore, about 4.57 mg of oxygen is required to oxidize one mg of ammonia-N (3.43 mg oxygen to oxidize 1 mg of ammonia to nitrite and 1.14 mg oxygen to oxidize 1 mg nitrite to nitrate (Sharma and Ahlert, 1977)). Ammonia (NH₃) is believed to be the actual substrate for AOB rather than ammonium ion (NH₄⁺) (Suzuki et al., 1974; Drozd, 1976). The major N-containing product from complete nitrification is nitrate, which can be further reduced anaerobically to dinitrogen gas by a heterotrophic denitrification process. Approximate 2% of ammonia-N is theoretically used to synthesize biomass of nitrifiers (Eq.2-3).

Beside the conventional nitrification/denitrification process, an ammonia removal process combining partial nitrification (ammonia oxidation to nitrite) and denitrification

has been used to transform ammonia to dinitrogen gas via nitrite. This kind of ammonia removal process is referred to as a “shortcut” in the nitrification/denitrification process (Voets et al., 1975; Alleman, 1985; Bernet et al., 2001). This method has a dominant advantage in that less aeration energy is required for partial-nitrification compared to the conventional process. From an economic point of view, this “shortcut” nitrification/denitrification process is more appropriate when treating wastewaters with relatively high ammonia loading such as livestock (e.g., swine) wastewater and landfill leachate (Hellings et al. 1998).

2.1.2 Ammonia conversion (NH_3 oxidation to NO_2^- by AOB)

Ammonia oxidation to nitrite by AOB is the first key step (also a rate limiting step for nitrification) to transform ammonia to stable dinitrogen gas. Effective ammonia oxidation by AOB is prerequisite to achieve stable ammonia removal in wastewater treatment. However, autotrophic AOB can be out-competed by fast-growing heterotrophs because AOB have relatively low maximum growth rates, low substrate affinities, and high sensitivity to operational/environmental conditions and the presence of toxic substrates (Mobarry et al., 1996; Koops et al., 2003; Head and Oleszkiewicz, 2005). A better understanding of the microbial characteristics of AOB is necessary for optimization of nitrification (Keller et al., 2002). Researchers have carried out several studies addressing:

- (1) Bacterial diversity: What kinds of bacteria are capable of oxidizing ammonia? (Watson et al., 1981, 1989),
- (2) Taxonomy and phylogeny: What are the properties of the AOB? How can they be taxonomically classified and phylogenetically identified? (Koops et al., 1991; Head et al., 1993),
- (3) Microbial populations: How many AOB exist in the micro-ecosystems? What is the AOB percentage? (Wagner et al., 1995; Dionisi et al., 2002),
- (4) Metabolic mechanism: How do AOB oxidize ammonia to nitrite to get energy for survival? (Wood, 1986),
- (5) Enzymology: What kinds of enzymes in AOB catalyze ammonia oxidation?

- (Hooper et al., 1997),
- (6) Genetics: What genes encode the functional enzymes for nitrification? (McTavish et al., 1993; Chain et al., 2003),
- (7) Kinetics: How fast can AOB oxidize ammonia? What is the specific substrate consumption rate? (Prosser, 1989; Suwa et al., 1994), and
- (8) Activity: What are the capacities of AOB in oxidizing ammonia? (Stein et al., 1997; Aoi et al., 2002).

Among these issues, population size and physiological activity of AOB in wastewater treatment systems are generally considered the rate-limiting parameters for ammonium removal from wastewater (Wagner et al., 1995; Yuan and Blackall., 2002). However, the impact of changes in AOB population and/or activity on overall nitrification treatment performance during wastewater processing has not been fully addressed. Understanding the population size and ammonia oxidation activity of AOB cells under various operational/environmental conditions may provide fundamental knowledge for optimum design and operation of nitrifying processes. Additionally, quick and accurate methods for monitoring these two parameters may allow for a real-time determination of nitrification treatment performance and an effective response to sudden environmental impacts or operational perturbations prior to nitrification failure.

2.2 Ammonia-oxidizing bacteria (AOB)

2.2.1 General property, habitat, and optimum growth condition

Ammonia-oxidizing bacteria (AOB) represent a physiologically distinct group of nitrifying bacteria (Nitrobacteraceae family) capable of oxidizing ammonia to nitrite as a sole energy source (Watson et al., 1974; Koops and Möller, 1992). They are all gram-negative and obligate chemoautotrophic microorganisms (Koops and Möller, 1992). Most AOB grow autotrophically, assimilating carbon dioxide (CO₂) as the major carbon source (Koops and Möller, 1992). Some can also use organic compounds (e.g., acetate, pyruvate) for mixotrophic growth (Watson et al., 1989), but none of them prefer to grow heterotrophically because energy yield from heterotrophic growth is inefficient (Krümmel

and Harms, 1982). The AOB are usually obligate aerobes, but some can grow under reduced oxygen conditions (Goreau et al., 1980; Watson et al., 1989).

These chemoautotrophic ammonia oxidizers are ubiquitous in diverse environments such as soils, compost piles, fresh waters, marine, and sewage treatment systems (Watson et al., 1989; Koops and Möller, 1992). Some strains prefer eutrophic or oligotrophic environments, respectively (Koops and Möller, 1992). In nature, some AOB strains have been found in sub-optimal environments such as building sandstone (low ammonium levels), marine sediments (extremely low oxygen tensions), acid soils ($\text{pH} \leq 4$), deep oceans (temperature $\leq 5^\circ\text{C}$), deserts and hot springs (temperatures $\geq 60^\circ\text{C}$) (Watson et al., 1989). However, optimum growing conditions at temperatures around $25\text{--}30^\circ\text{C}$, and pH-value around 7.5-8.0 have been established for all currently isolated AOB species/strains (Koops and Möller, 1992).

The AOB cells may be rod, spherical (coccoid), spiral, curve rod (vibrios) or lobular shaped and many, but not all, possess extensive cytomembranes that usually form flattened vesicles or tubes within the cells and are arranged characteristically in each species (Watson et al., 1981, 1989) (Table 2-1). The marine strains have an additional cell-wall layer outside the typical gram-negative cell envelope. These observed characteristics have been used to taxonomically categorize the isolated AOB from diverse environments (Section 2.2.2).

Table 2-1. Characteristics of the shape, size of cells and the arrangement of the cytoplasmic membranes of five generally accepted genera of AOB (based on Watson et al., 1981, 1989; Koops and Möller, 1992; and Koops et al., 2003).

Genus	Cell shape	Cell size (μm)	Cytomembranes
<i>Nitrosomonas</i>	Straight rod	0.8~1.0×1.0~2.0	Peripheral, lamellar
<i>Nitrosococcus</i>	Coccoid	1.5~2.2 in diameter	Central or peripheral, lamellar
<i>Nitrospira</i>	Spiral	0.3~0.4 in diameter	Lacking
<i>Nitrosolobus</i>	Lobular	1.0~1.5 in diameter	Internal, compartmentalized
<i>Nitrovibrio</i>	Curved rod	0.3~0.4×1.1~3.0	Lacking

2.2.2 Taxonomic classification (genera, species, and strains)

Since Winogradsky first successfully isolated ammonia oxidizers in 1892, many AOB strains, isolated from either natural environments or engineered processes, have been cultured, and studied. These AOB have been traditionally classified into five generally accepted genera: *Nitrosomonas* (Winogradsky, 1892), *Nitrosococcus* (Winogradsky, 1892), *Nitrospira* (Winogradsky, 1933), *Nitrosolobus* (Watson, 1971), and *Nitrosovibrio* (Harms et al., 1976) primarily based on the shape of the cells (morphology) and the arrangement of the intracytoplasmic membranes.

The AOB strains in each of these five genus have been further characterized and taxonomically classified into several species based on their GC compositions of DNA, DNA homologies, shape and ultra-structure, salt requirements, ammonia tolerance, utilization of urea as ammonia source, and whole-cell protein patterns (Watson and Mandel, 1971; Dodson et al., 1983; Koops and Harms, 1985; Koops et al., 1991). A species is defined quite arbitrarily when strains show 70% or greater DNA homology. Sixteen defined AOB species (together with 9 undefined species) are summarized in Table 2-2 based on investigations before the early 1990s (Watson et al., 1981; Watson et al., 1989; Koops et al., 1991). Since that time, some AOB isolates have been cultured but none of them have been defined as a novel distinct species (Suwa et al., 1997). In addition, the existence of many more uncultured strains has been indicated by molecular *in situ* investigations (Koops and Pommerening-Röser, 2001; Purkhold et al., 2003).

In the genus *Nitrosomonas*, at least 13 species (10 defined, and 3 undefined) have been characterized. While *N. europaea* (Winogradsky, 1892) was known as the only recognized species in the genus *Nitrosomonas* in the early 1970s, Watson and Mandel (1971) first suggested that the 13 strains of *Nitrosomonas* they studied could be grouped into three species based on their slight differences in cell morphology and isolated sources (although their GC contents were not significantly different). This work was later confirmed by Dodson et al. (1983) based on comparison of DNA homology. In 1985, Koops and Harms indicated the presence of eight *Nitrosomonas* species (including *N. europaea*) based on analyses of GC content of DNA, DNA homologies, and genome sizes.

Table 2-2. Classification of AOB species into five genera based mainly on analyses of DNA homology (Koops and Möller, 1992).

Genus/Species (genospecies)	First report	Typical strains*	Typical origin
<i>Nitrosomonas</i>	Winogradsky, 1892		
<i>europaea</i> (12)	Winogradsky, 1892	Nm 27, 28, 35, 48, 50 ^T (C-31), 87, 89	Sewage, soil, water
<i>cryptotolerans</i> (5)	Jones et al., 1988	Nm 55 (4W30)	Sea water
<i>communis</i> (1)	Koops et al., 1991	Nm 2 ^T , 32, 40, 44	Soil
<i>ureae</i> (4)	Koops et al., 1991	Nm 4, 5, 6, 9, 10 ^T , 13, 42, 88	Soil, fresh water
<i>aestuarii</i> (6)	Koops et al., 1991	Nm 3, 11, 17, 20, 36 ^T , 62 (C-52), 64 (C-121), 69 (C-19)	Sea water
<i>marina</i> (8)	Koops et al., 1991	Nm 22 ^T , 61, 71	Sea water, salt lake
<i>nitrosa</i> (9)	Koops et al., 1991	Nm 7, 72, 90 ^T (Nc 5), 91 (Nc 6)	Sewage, pond
<i>eutropha</i> (10)	Koops et al., 1991	Nm 14, 19, 23, 24, 26, 38, 39, 53, 56, 57 ^T (C-91)	Sewage, Mud, soil
<i>oligotropha</i> (11)	Koops et al., 1991	Nm 8, 37, 43, 45 ^T , 46, 49	Sewage, soil, water
<i>halophila</i> (13)	Koops et al., 1991	Nm 1	Sea water
sp. (2)	Koops et al., 1991	Nm 34, 41	Soil
sp. (3)	Koops et al., 1991	Nm 33	Soil
sp. (7)	Koops et al., 1991	Nm 51 (C-15), 63 (C-56)	Sea water
<i>Nitrosococcus</i>	Winogradsky, 1892		
<i>nitrosus</i>	Migula, 1900	Nc 5, 6 (?) (or no longer in culture!)	?
<i>oceanus</i>	Watson, 1965, 1971	Nc 1, 9 (C-27), 10 ^T (C-107)	Sea water
<i>mobilis</i>	Koops et al., 1976	Nc 2 ^T , 3, 8	Sea water, Sewage
<i>halophilus</i>	Koops et al., 1990	Nc 4 ^T , 7	Salt lake
<i>Nitrosospira</i>	Winogradsky, 1933		
<i>briensis</i>	Winogradsky, 1933	Nsp 8, 10 ^T (C-76)	Soil
sp.	Koops et al., 1985	Nsp 1, 4 ^T (C-126, 128), 7, 16, 18, 21, 22, 25, 26	Soil, fresh water?
sp.	Koops et al., 1985	Nsp 5, 6, 12, 13, 23	Soil, fresh water?
sp.	Koops et al., 1985	Nsp 2 ^T , 15, 19	Soil, fresh water?
sp.	Koops et al., 1985	Nsp 3, 14, 17	Soil, freshwater?
<i>Nitrosolobus</i>	Watson, 1971		
<i>multiformis</i>	Watson, 1971	Nl 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13 ^T (C-71), 14, 15	Soil
sp.	Koops et al., 1985	Nl 5	Sewage
<i>Nitrosovibrio</i>	Harms et al., 1976		
<i>tenuis</i>	Harms et al., 1976	Nv 1 ^T , 2, 13	Soil
sp.	Koops et al., 1985	Nv 6, 12	Soil?

* Reports by Watson (1971), Koops and Harms (1985), Jones et al. (1988), and Koops et al. (1991); T: type strain for each species.

Three years later the second characterized species in the genus *Nitrosomonas* was defined as *N. cryotolerans* (Jones et al., 1988). More recently, eight additional species in the *Nitrosomonas* genus were defined, and the presence of at least three further species was indicated (Koops et al., 1991). Currently, ten species in the genus *Nitrosomonas* have been defined (i.e., *N. europaea*, *N. cryotolerans*, *N. communis*, *N. ureae*, *N. aestuarii*, *N. marina*, *N. nitrosa*, *N. eutropha*, *N. oligotropha*, and *N. halophila*) and three additional species in this genus belonging to genospecies 2,3 and 7 have not been defined yet. Among these 13 species in the *Nitrosomonas* genus, only four, *N. europaea*, *N. nitrosa*, *N. eutropha*, and *N. oligotropha*, are often found in sewage treatment systems. Other species originally isolated from soil, fresh water and seawater are unlikely to prefer sewage treatment systems.

The genus *Nitrosococcus* includes at least four defined species, *N. nitrosus* (Migula, 1900), *N. oceanus* (Watson, 1971), *N. mobilis* (Koops et al., 1976), and *N. halophilus* (Koops et al., 1990). Unfortunately, the species *N. nitrosus* suspected by Winogradsky (1892) and first reported by Migula (1900) was not preserved. Although Koops and Harms (1985) described two neotype strains that are similar to *N. nitrosus* in their shape and size, it was suggested that this species should be placed on the list of rejected names because its' morphology was not distinguishable from some coccoid strains in the *Nitrosomonas* genus (Koops and Möller, 1992). Three of the *Nitrosococcus* species are significantly different in arrangement of the cytomembrane. The cytomembrane in the *N. mobilis* species is similar to the *Nitrosomonas* species (arranged like flattened vesicles in the peripheral cytoplasm), while that in the *N. oceanus*, and *N. halophilus* have a more centrally located stack of flattened vesicles (Koops et al., 1976; Koops and Möller, 1992). Almost all species in the genus *Nitrosococcus* were originally isolated from marine samples, thus AOB in this genus are unlikely to be found in sewage treatment systems with the exception of *N. mobilis* (Juretschko et al., 1998).

The genus *Nitrospira* includes at least five species (Koops and Harms, 1985), but only one species, *N. briensis* (Winogradsky, 1933; Watson, 1971), has been defined. Based on analyses of GC content and DNA homology, Koops and Harms (1985) indicated the presence of four other species in this genus, but these species have not been

defined yet. It has been suggested that *Nitrosospira* spp. are widespread in the environment (Hiorns et al., 1995; Hastings et al., 1998). Although most *Nitrosospira* species were originally isolated from soil and fresh water, some isolated *Nitrosospira* strains have recently been found in sewage treatment systems (Rotthauwe et al., 1997), and in lab-scale nitrifying reactors (Schramm et al., 1998; Schramm et al., 1999).

In the genus *Nitrosolobus*, only one species, *N. multiformis* (Watson et al., 1971) has been defined. Based on DNA homology analyses (Koops and Harms, 1985), another *Nitrosolobus* species isolated from sewage systems was recognized to as different from *N. multiformis*, but it hasn't been defined yet. The genus *Nitrosovibrio* includes at least two species based on analyses of DNA homology (Koops and Harms, 1985), but only the species *N. tenuis* (Harms et al., 1976) has been defined.

2.2.3 Phylogenetic relationship of AOB

On the basis of 16S ribosomal RNA gene (rDNA) analyses, all known AOB are phylogenetically confined to two groups (beta- and gamma-subdivisions) within the Proteobacteria (the purple photosynthetic bacteria) (Woese, 1987) (Figure 2-3). Among five AOB genera, only the genus *Nitrosococcus* is not phylogenetically homogeneous. The species *Nitrosococcus oceanus*, and likely also *Nitrosococcus halophilus* belong to the gamma-subdivision of the Proteobacteria (Woese et al., 1984, 1985; Koops et al., 1990), while the species *Nitrosococcus mobilis* together with other AOB species in the genus *Nitrosomonas*, *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio* belong to the beta-subdivision of the Proteobacteria (Figures 2-3, 2-4). It has to be emphasized that two ammonia-oxidizing species of the genus *Nitrosococcus*, *N. oceanus* and *N. mobilis*, are phylogenetically not closely related although their morphologies are similar (Woese et al., 1984, 1985). Additionally, it had been suggested that *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* could be reclassified into one common genus (*Nitrosospira*) based on the homology of 16S rRNA sequences (Head et al., 1993). Therefore, it seems more appropriate to classify AOB based on genotypic 16S rRNA genes (or 16S rDNA) sequences instead of phenotypic characteristics (cell morphology, and arrangement of cytoplasmic membrane) (Koops and Möller, 1992).

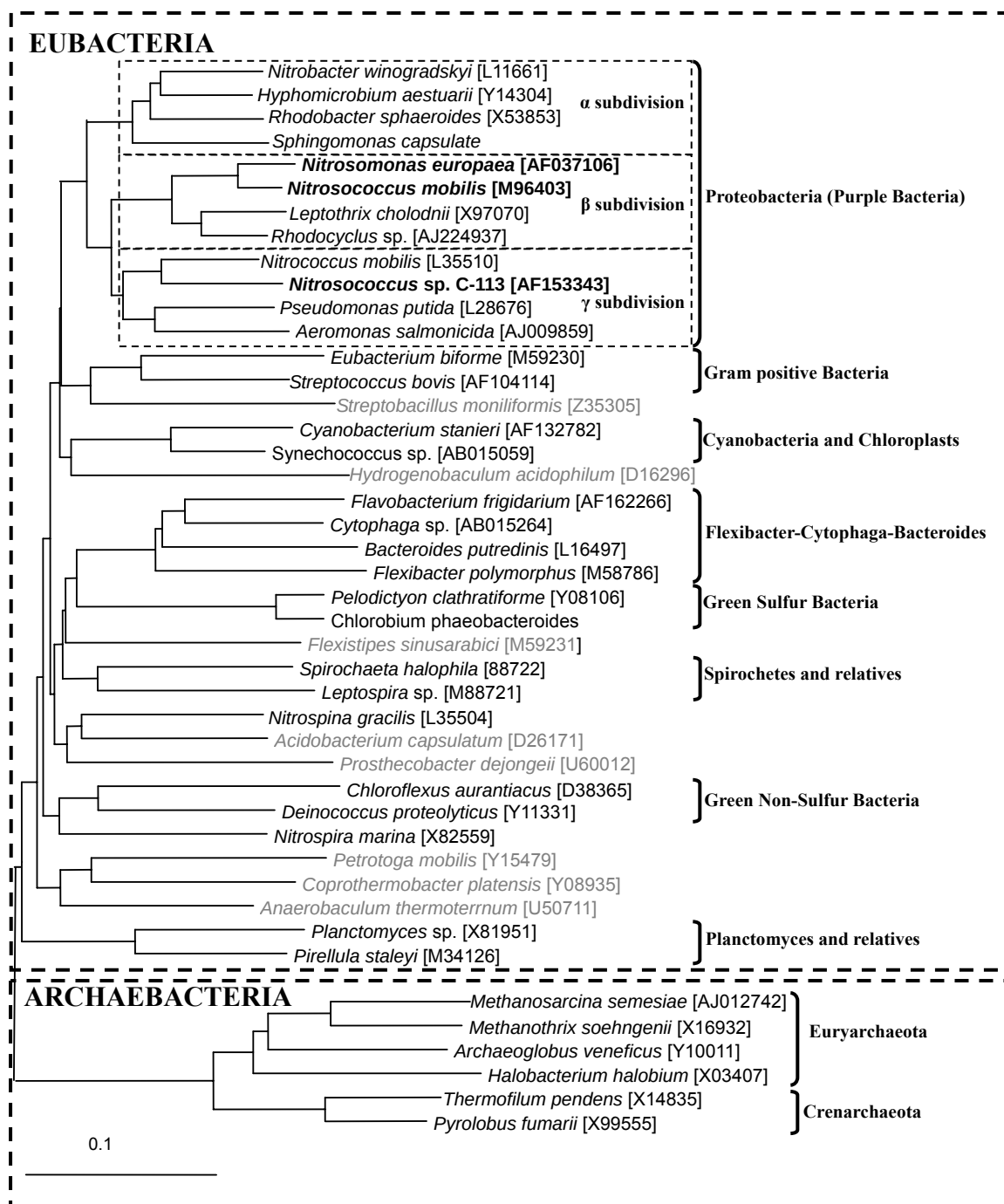


Figure 2-3. Based on comparison analysis of 16S rRNA genes sequences, all known AOB strains or isolates can be phylogenetically confined to two groups (β-, and γ-subdivisions) within the Proteobacteria of the eubacteria. (Phylogenetic tree was constructed based on 16S rDNA sequences from GenBank of the Center for Microbial Ecology, Michigan State University, and the National Center for Biotechnology Information, NCBI).

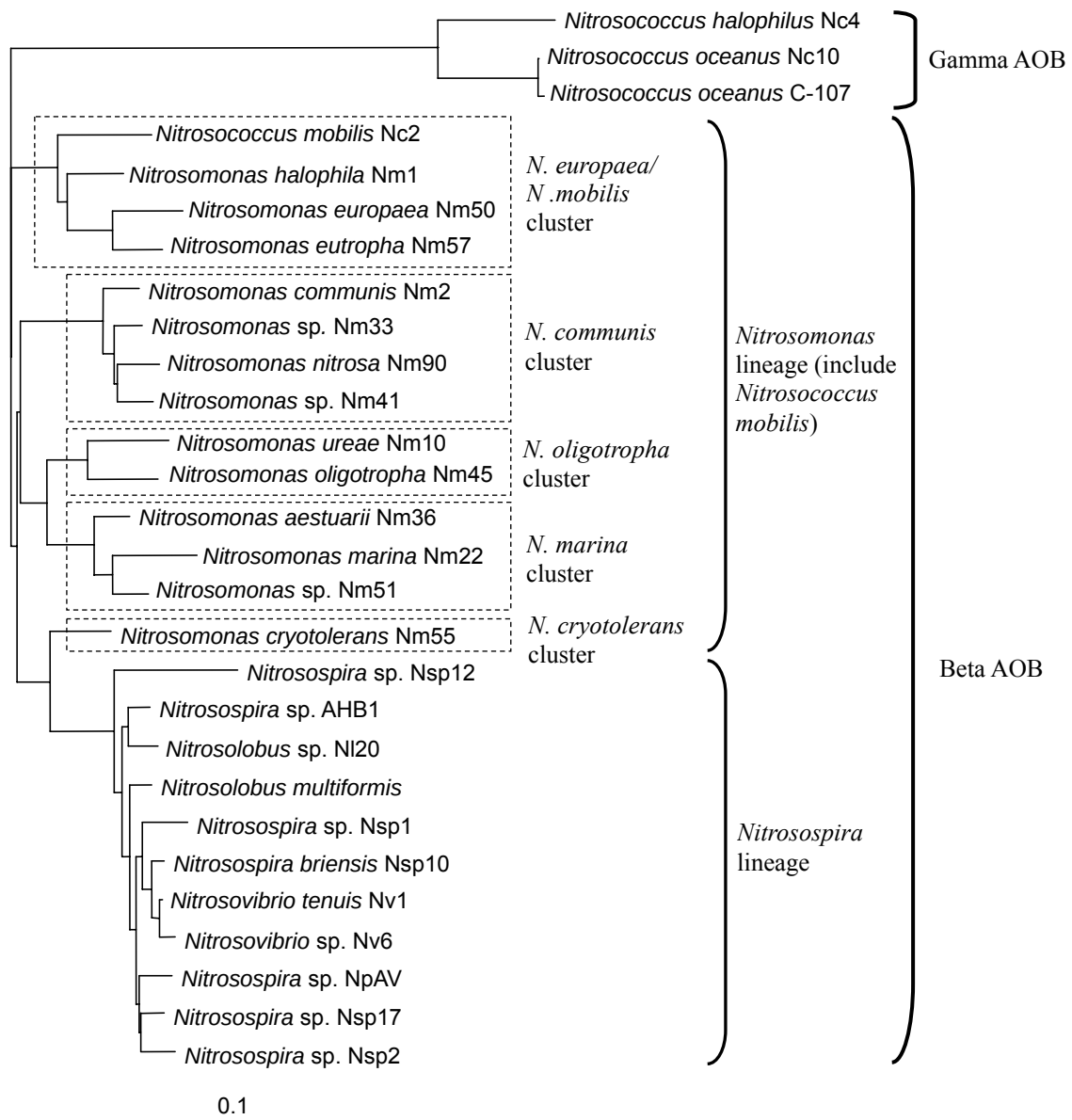


Figure 2-4. An example of the phylogenetic relationship of most defined AOB based on 16S rRNA gene (rDNA) sequences (~1300 bases) from the NCBI GenBank database (reported by Head et al., 1993; McCaig et al., 1994; Teske et al., 1994; Rotthauwe et al., 1995; Pommerening-Röser et al., 1996; Aakra et al., 2001; Takahashi et al., 2001).

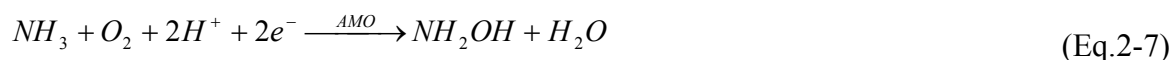
According to the 16S rDNA sequences, the beta-subdivision AOB can be separated into two major different lines of descent; *Nitrosomonas* spp. and *Nitrosococcus mobilis* can be characterized into one lineage, whereas *Nitrospira*, *Nitrosolobus* and *Nitrosovibrio* belong to another lineage (Figure 2-4). The cultured AOB in the *Nitrosomonas* cluster were usually grouped into at least five distinct lineages of descent including the *N. marina* cluster, the *N. oligotropha* cluster, the *N. europaea*/*N. Mobilis* cluster, the *N. communis* cluster, and the *N. cryotolerans* cluster (Purkhold et al., 2000; Koops and Pommerening-Röser; 2001) (Figure 2-4). Additionally, many uncultured AOB strains or isolates found in diverse environmental samples (based on phylogenetic analysis) are present in several other clusters within both the *Nitrospira* and the *Nitrosomonas* clusters (Stephen et al., 1996; Purkhold et al., 2000; Gieseke et al., 2003; Purkhold et al., 2003). A comparative analysis of genes sequences encoding the major part of the functional enzyme catalyzing ammonia oxidation in AOB (e.g., *amoA* genes encoding a subunit of a key enzyme, ammonia monooxygenase, that catalyzes the first step of ammonia oxidation, Section 2.3.3) also has been applied to refine the phylogenetic relationship between closely related AOB (Rotthauwe et al., 1995; Rotthauwe et al., 1997) (Section 2.5.2). Purkhold et al. (2000) indicated that comparative phylogenetic analyses using *amoA* DNA and 16S rDNA as marker molecules suggested similar, but not identical, evolutionary relationships of AOB.

It has been suggested that the AOB species (or strains) in each phylogenetically definable group have similar ecophysiological characteristics (such as self-flocculating, salt requirement, urease activity, and substrate affinity) that may be different from those of others phylogenetically definable groups (Koops and Pommerening-Röser; 2001). These ecophysiological differences that exist between the distinct representatives can lead to different distribution patterns of the species or groups of species, and result in different AOB population dynamics in investigating systems. For instance, the AOB in the *N. europaea*/*N. mobilis* cluster prefer eutrophic habitats like sewage treatment plants while those in the *N. oligotropha* cluster are typically found in freshwater environments (Koops and Pommerening-Röser; 2001) (Section 2.5).

2.3 Ammonia oxidation

2.3.1 Ammonia oxidation pathway and enzymes involved

The ammonia oxidation pathway and electron transport model have been studied mainly in one AOB species, *N. europaea*, and it has been assumed that the oxidation pathways are similar between AOB species (Bothe et al., 2000). In *N. europaea*, ammonia (NH₃) is first oxidized to hydroxylamine (NH₂OH) (Dua et al., 1979); and then, hydroxylamine is oxidized to nitrite (NO₂⁻). The reactions can be represented by the following equations:



It has been reported that ammonia (NH₃) rather than ammonium ion (NH₄⁺) is the substrate of the energy generating system in AOB (Suzuki et al., 1974; Drozd, 1976), but hydroxylamine (NH₂OH), the first intermediate of ammonia oxidation, is believed to be the actual energy source (Hofman and Lees, 1953). One of the two oxygen atoms in nitrite, oxidized from ammonia via hydroxylamine, is derived from dioxygen (O₂) (Dua et al., 1979; Hollocher et al., 1981), and another oxygen in nitrite is derived from water (Andersson and Hooper, 1983). Ammonia monooxygenase (AMO) is a key enzyme that catalyzes the oxidation of ammonia to hydroxylamine (Eq.2-7). Because the standard Gibbs free energy change of the direct oxidation of ammonia to hydroxylamine is positive (ca. +17kJ/mol), the enzyme AMO is required in order for this reaction to occur biologically. Hydroxylamine oxidoreductase (HAO) is the key enzyme that catalyzes the oxidation of hydroxylamine to nitrite, generating four electrons and five protons (Eq.2-8). AMO and HAO are co-dependent because AMO produces hydroxylamine as a substrate for HAO, and HAO produces electrons as the reductant for AMO except at the very beginning stage of the ammonia oxidation in which NADH probably serves as the reductant source for AMO to initiate ammonia oxidation (Bothe et al., 2000). Losing either AMO or HAO activity in AOB can cause ammonia oxidation failure in nitrification.

As shown in [Figure 2-5](#), four electrons generated from hydroxylamine oxidation pass to cytochromes C₅₅₄ and then, with four protons, are used to reduce two molecules of ubiquinone (UQ) to two molecules of ubiquinol (UQH₂) ([Wood, 1986](#)). One ubiquinol molecule is oxidized by the AMO; whereas the other ubiquinol molecule is oxidized by the cytochromes bc₁. Via these ubiquinone reduction and ubiquinol oxidation cycles, two hydroxylamine-derived electrons are transported to AMO to activate oxygen for sustaining further ammonia oxidation, while the remaining two hydroxylamine-derived electrons are diverted primarily to an electron transport chain via cytochromes C₅₅₂ and aa₃ for ATP generation or rarely to reversed electron flow for reduction of nucleotide cofactors (e.g. NAD⁺) used for CO₂ fixation and other biosynthetic reactions ([Wood, 1986](#)). Only about 0.025-0.125 % of hydroxylamine-derived electrons are used to reduce NAD⁺ for cellular growth and reproduction ([Wood, 1986](#); [Wehrfritz et al., 1993](#); [Hooper et al., 1997](#)). Because of this autotrophic mode of life, growth rates of AOB are relatively slow compared to heterotrophs. This makes it difficult to grow AOB in culture or to study AOB using cultivation-dependent methods.

2.3.2 Ammonia monooxygenase (AMO)

Information about the ammonia monooxygenase (AMO) enzyme is still insufficient at present mainly because this enzyme has not yet been purified in an active state from autotrophic AOB. Most of what is currently known about the features and structure of AMO comes from indirect evidence obtained from inhibitor and isotope studies with intact cells (mainly with *N. europaea*) or studies on a similar functional enzyme isolated from heterotrophic nitrifying bacteria ([Bothe et al., 2000](#)).

The ammonia monooxygenase (AMO) enzyme in *N. europaea*, and probably some other autotrophic AOB, is a copper-containing ([Loveless and Painter, 1968](#)), membrane-bound enzyme ([Hyman and Wood, 1985](#)). Lately, [Zahn et al. \(1996\)](#) suggested that iron is a catalytic component in the center of the AMO enzyme. This iron center is possibly a part of the oxygen activating center that is significantly separated from the substrate-binding site ([Zahn et al., 1996](#)). It is accepted that AMO consists of at least three subunits, AMO-A, AMO-B and AMO-C, with different sizes, structures and arrangements within

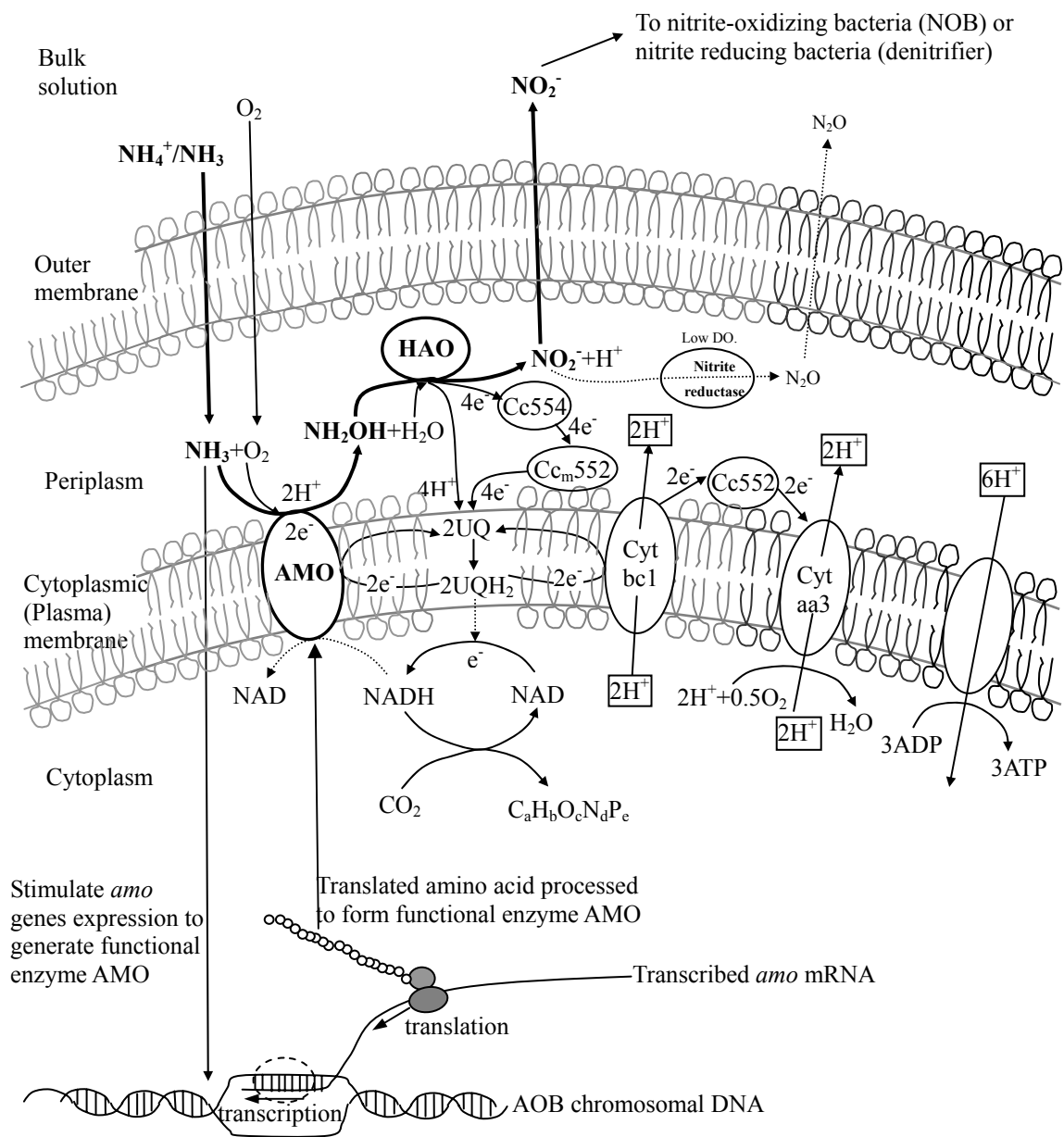


Figure 2-5. Ammonia oxidation pathways and electron transport model for ammonia-oxidizing bacteria (based on Wood, 1986; Whittaker et al., 2000).

the cytoplasmic membrane/periplasmic space of the cells (Bothe et al., 2000). In *N. europaea*, the subunit AMO-A has a molecular weight (M_r) of 27-32 kDa (Hyman and Arp, 1992), the subunit AMO-B has a M_r of 38-43 kDa (Bergmann and Hooper, 1994), and the subunit AMO-C has a M_r of 31.4 kDa (Sayavedra-Soto et al., 1998). The catalytic site of ammonia oxidation is believed to be located in AMO-A. As predicted from the amino acid sequence, the subunit AMO-A would consist of 5 transmembrane segments and a large periplasmic loop containing a disproportionate number of amino acids, while the subunit AMO-B has two transmembrane domains and two large periplasmic domains (Hooper et al., 1997). The subunit AMO-C could serve as a director that supervises AMO-A/AMO-B interactions in order to maintain AMO functionality (Klotz et al., 1997).

In addition to ammonia, AMO can catalyze the oxidation of a wide range of organic substrates such as hydrocarbons, alcohols and halocarbons (Hooper and Terry, 1973; Suzuki et al., 1976). Since these organic compounds are all non-polar, it is inferred that the active site of AMO is hydrophobic. Moreover, these organic compounds may compete with ammonia for the substrate-binding site of AMO and the available reducing power, and then inhibit the oxidation ability of AMO in AOB. Other known AMO inhibitors include U.V. light, suicide substrates (e.g., acetylene), and metal-chelating compounds such as allylthiourea and other C=S-containing compounds (Hyman and Arp, 1992) that inhibit ammonia oxidation activity of AOB at the enzyme (post-translation) level.

To date, cell-free AMO as the active enzyme has only been purified from a heterotrophic nitrifier, *Paracoccus denitrificans* (Moir et al., 1996). This purified AMO has several properties similar to the AMO enzyme family from *N. europaea*. For example, it is a quinol oxidase, is inhibited by light and a variety of chelating agents, is activated by cupric ions and consists of two subunits with molecular masses of 38 and 46 kDa (Moir et al., 1996). However, the third subunit AMO-C in the AMO from autotrophic nitrifiers was lacking in this purified enzyme.

At the post-translational level, the ammonia oxidation activity of AMO may be affected by several environmental factors such as ammonia concentration (Stein et al., 1997), dissolved oxygen concentration, pH, and temperature (Section 2.4.2). It has been

reported that the AMO activity in *N. europaea* can remain stable under short-term (24 hours) ammonia limitation (Stein and Arp, 1998 (a)) or under long-term (1~10 weeks (Bollmann et al., 2002) to 342 days (Wilhelm et al., 1998)) ammonia starvation. This may allow AOB to quickly respond to a sudden pulse of ammonia and to survive under conditions of fluctuating ammonia concentration (Bothe et al., 2000). Thus, an AMO enzyme pool within AOB in a nitrifying system may reflect the ammonia oxidation potential of the system and may serve as a novel indicator of ammonia oxidation activity (Section 2.6.5).

The AMO enzymes of AOB share many similarities with a membrane-bound particulate methane monooxygenase (pMMO) enzyme of methanotrophic bacteria despite the very different physiological role of these two enzymes in cell metabolism for both groups of bacteria (Bédard and Knowles, 1989; Holmes et al., 1995). For example, like AMO, pMMO also consists of at least two membrane-associated polypeptides of about 27 kDa and 45 kDa, has similar broad substrate and inhibitor profiles, and may contain a trinuclear copper center at the active site in the 27-kDa polypeptide (Holmes et al., 1995). Methanotrophs can oxidize ammonia and ammonia oxidizers can oxidize methane, although neither type of organism can use the energy from the reaction for growth. Additionally, both enzymes can catalyze oxidation reactions of various nongrowth substrates such as methanol (Voysey and Wood, 1987), ethylene (Hyman and Wood, 1984), cyclohexane, benzene, and phenol (Hyman et al., 1985). Moreover, amino acid sequences of these two enzymes share high similarity and conservation (75~99%) (Holmes et al., 1995). This may suggest the AMO and pMMO are evolutionarily related enzymes.

2.3.3 Genes encoding AMO (*amoCAB*)

In general, the bacterial genetic information is stored in a chromosome located in its cytoplasm. The chromosomes of most prokaryotic bacteria are constructed as a circular structure comprised of proteins and nucleic acids (double strain DNA). The DNA sequences consist of genetic codes that determine all the bacterial reproduction and metabolism. The DNA is generally replicated every generation time before bacteria

undergo cell division. Through this replication, inherited genetic information can be transferred from ancestor to new generation. The antisense strand of DNA also can be transcribed into complementary sequences of RNA including messenger RNA (mRNA), transport RNA (tRNA), and ribosomal RNA (rRNA). In a subsequent translation process, these RNA molecules work together with some proteins in a ribosome complex to produce amino acids that serve as functional enzymes (or proteins) to catalyze biological metabolism reactions or form cell structure. Therefore, it is concluded that gene expression starts with transcription of functional genes to mRNA and ends with translation of mRNA to a polypeptide through which functional enzymes are generated to catalyze substrate conversion.

The three subunits of AMO (AMO-A, AMO-B, and AMO-C) in autotrophic AOB are encoded by three genes *amoA*, *amoB* and *amoC* located on an *amoCAB* operon. The sequences of these *amo* genes have been studied in representatives of AOB from both the β -subdivision ([McTavish et al., 1993](#); [Bergmann and Hooper et al., 1994](#); [Rotthauwe et al., 1995](#); [Klotz and Norton, 1995](#); [Norton et al., 1996](#); [Klotz et al., 1997](#)), and γ -subdivision ([Alzerreca et al., 1999](#)) of Proteobacteria. Multiple copies of *amo* genes have been identified in the AOB belonging to the β -subdivision of Proteobacteria ([McTavish et al., 1993](#); [Klotz et al., 1997](#)). Currently reported *amoA* and *amoB* genes in AOB from the β -subdivision are always contiguous and preceded by the third gene, *amoC* ([Klotz et al., 1997](#)). Sayavedra-Soto et al. (1998) showed a map of *amo* locus in *N. europaea* that consisted of two copies of *amoC*, *amoA* and *amoB* genes located together on the *amoCAB* operon and an intergenic space between *amoC* and *amoA*, but not between *amoA* and *amoB* ([Figure 2-6](#)). Recently, after an entire genomic DNA sequence for *N. europaea* was studied, [Chain et al. \(2003\)](#) indicated that two copies of *amo* genes in this AOB are located separately in two *amo/hao* gene clusters with a 1,300 kb intergenic space between them. A detailed map of the *N. europaea* ATCC 19718 genome reported by [Chain et al. \(2003\)](#) is shown in [Figure 2-7](#).

The *amo* gene sequences in the genome of most AOB species have been determined and posted in several publicly accessible GenBanks. [McTavish et al. \(1993\)](#) first

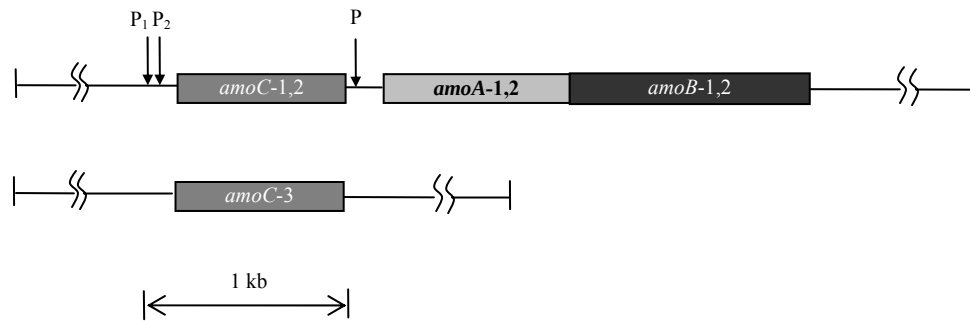


Figure 2-6. Map of the *amo* locus in *N. europaea* shows two copies of *amoA*, *amoB*, and *amoC* genes in the *amoCAB* operon, and one additional copy of *amoC* not adjacent to *amoAB*. The locations of the putative promoters for *amoCAB*, *amoC*, and *amoA* (P_1 , P_2 , and P) are indicated (based on [Sayavedra-Soto et al., 1998](#); [Hommes et al., 2001](#)).

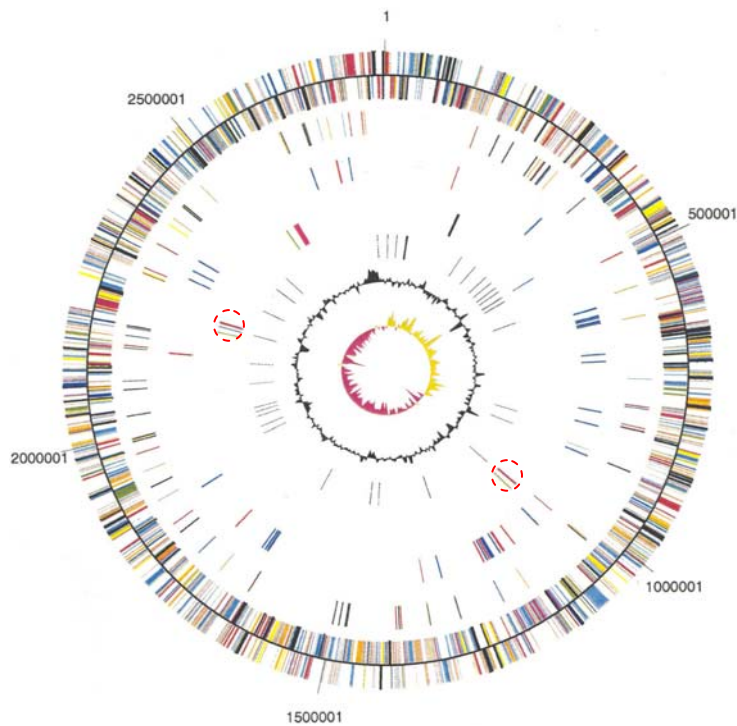


Figure 2-7. Circular representation of the 2,812,094 bp genome of *N. europaea* ATCC 19718 in which the location of *amo* gene clusters are emphasized in dash circles ([Chain et al., 2003](#)).

completed an entire *amoA* gene sequence and a partial *amoB* gene sequence of *N. europaea* genomic DNA by combining two restriction fragments (*Sau3AI* and *KpnI*). Later, Bergmann and Hooper (1994) completed the entire *amoB* gene sequence from the *N. europaea* genome, and Hommes et al. (1998) reported the *amoC* gene sequence that was later confirmed by Norton et al. (2002). After an entire genomic DNA sequence of *N. europaea* ATCC19718 was completed, it was found that two copies of the *amoCAB* gene were located at positions 1,028,378 - 1,031,451 and 2,238,597 - 2,241,670, respectively (Chain et al, 2003). The arrangement of the *amoC*, *amoA*, and *amoB* gene sequences of *N. europaea* genomic DNA is shown in Figure 2-8. The *amo* gene sequences from the genomic DNA of other AOB such as *Nitrosomonas eutropha*, *Nitrospira* sp. NpAV, *Nitrospira* sp. AHB1, *Nitrosolobus multiformis* C-71, *Nitrosococcus oceani*, and *Nitrosococcus* sp. C-113 have also been determined (Klotz and Norton, 1995; Rotthauwe et al., 1995; Norton et al., 1996; and Alzerreca et al., 1999). The *amo* gene sequences of these AOB are available at the GenBank of the National Center for Biotechnology Information (NCBI), www.ncbi.nlm.nih.gov.

The gene sizes of *amoC*, *amoA*, and *amoB* from the *amoCAB* operon of *N. europaea* genome are 816, 831, and 1263 bases, respectively (Table 2-3) (McTavish et al., 1993; Bergmann and Hooper, 1994; Hommes et al., 1998). The intergenic spacer between *amoC* and *amoA* (*C-A* I.S.) is 163 bases long (Alzerreca et al., 1999). Moreover, the sequence of non-operon *amoC* gene (*amoC*₃) of *N. europaea* also had been determined to be 825 bases (Sayavedra-Soto et al., 1998). The *amo* gene sizes of three other AOB species are also list in Table 2-3. The genes sizes are slightly different between the distinct AOB species and strains although these genes all encode similar functional enzymes (AMO) to catalyze ammonia oxidation.

Multiple copies of *amo* genes have been identified in the genome of most of the AOB from the β -subdivision of Proteobacteria such as *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrospira* sp. strain NpAV, *Nitrospira briensis*, *Nitrosolobus multiformis*, and *Nitrosovibrio tenuis* (Table 2-4). In *N. europaea*, the *amoC*, *amoA*, and *amoB* genes are present in two copies and are adjacent to each other with intergenic

```

0001 TGGTGAATAG GAGGATAAAG AGATGGCAAC TACGTTAGGA ACGAGCAGTG CCTCATCAGT CTCGTCGAGA GGCTATGACA TGTCACTGTG GTATGACTCC
                                ↳Start amoC
0101 AAATTTTACA AATTGGCAT GATTACCATG CTTCTGGTGG CAATTTTCTG GGTATGGTAT CAACGTTACT TTGCATATTC ACACGGAATG GACTCAATGG
0201 AACCCGAGTT TGATCGTGTG TGGATGGGGT TGTGGCGTGT ACATATGGCA ATCATGCCGC TGTTTGCGCT GGTACCTGG GGTGGATCC TGAACACGG
0301 TGATACGAAA GAGCAATTGG ATAATCTGGA TCCCAAACCT GAAATCAAAC GCTACTTCTA CTACATGATG TGGCTGGGTG TATACATTTT TGGTGTTTAC
0401 TGGGTGGTA GCTTCTTAC GGAGCAAGAT GCCTCCTGGC ACCAAGTGAT TATTCGTGAT ACCAGTTTCA CGCCAAGTCA CGTAGTGGTG TTTTACGGAT
0501 CATTCCCAGT GTACATCGTT TCGGTGTTG CAACCTACCT GTATGCAATG ACTCGCCTGC CATGTGTCAG CCGTGGAATT TCCTTCCCCG TGGTTATGGC
0601 GATTGCAGCG CCGTTGATGA TTCTGCCTAA CGTTGGTCTG AACGAGTGGG GTCATGCTTT CTGGTTCATG GAAGAGTTGT TCAGCGCACC ACTGCACTGG
0701 GGATTGTAG TGTGGGCTG GCGGGTCTG TTCCAGGGTG GTGTGCGAGC TCAGATCATT ACCCGTTATT CCAATCTGAC CGATGTGGTT TGAACAACCC
0801 AAAGCAAAGA AATTCTGAAT AACCGGATTG TAGCTTATTC TGGTATGTAC TCCAGGCAGA AGTTGCGCTT GGGGTACATT AAAAGAGGAA GTCTGCCCGG
                                Stop amoC→
0901 GTGCTGAAG TAGCACCTGG TCAGTCAGAA CCAGAGTGGC GTACCTTAGA GGAAAGAGG AATCGTAATG TTACTTTTAA ACTATAAGAG GGGAGGGCAG
1001 GGTGAGTATA TTTAGAACGG AAGAAATCCT GAAAGCGGCC AAGATGCCGC CGGAAGCGGT TCATATGTCA CGTCTGATTG ATGAGTTTAA TTTTCAATT
                                ↳Start amoA
1101 CTGATTATTT TGCTGGTGGG TACCTACCAC ATGCACCTTA TGCTGCTGGC AGGTGACTGG GATTTCGTGA TGGACTGGAA AGATCGTCAA TGGTGCCCGG
1201 TTGTAACGCC AATCGTGGGG ATCACCTACT GTTCGGCAAT CATGTATTAC TTGTGGGTCA ACTACGCCCA ACCGTTGGT GCAACGTTGT GTGTGTTGTG
1301 TCTGCTGATT GGTGAGTGGC TGACACGTTA CTGGGGATTC TACTGTGGTT CACACTACCC CATCAACTTC GTAACACCGG GCATTATGCT TCCGGGTGGC
                                >>amoA-1F primer>>
1401 CTGATGCTGG ACTTCACGCT GTATCTGACA CGCAACTGGC TGGTGACGGC TCTGGTTGGA GGTGGATTCT TCGGTCTGCT GTTCTATCCG GGTAACTGGC
1501 CGATTTTGG ACCAACCCAT TTGCCAATCG TTGTAGAAGG CACATGTGCT TCGATGGCTG ATTACATGGG ACATCTGTAT GTTCGTACAG GTACACCCGA
1601 GTATGTTCTG CATATTGAGC AAGGTTCACT GCGTACCTTT GGTGGTCATA CCACAGTTAT TGCAGCATTG TTCTCTGCGT TCGTATCAAT GTGTATGTTT
1701 ACCGATGGT GGTATCTTGG AAAAGTTTAC TGTACAGCCT TTTTCTACGT TAAAGGTAAA AGAGGTCGTA TCGTACATCG CAATGATGTT ACCGCATTCTG
<<amoA-2R primer<<
1801 GTGAAGAAGG CTTTCCAGAG GGGATCAAAT AAAATGGGTA TCAAAAACCT TTATAAACGG GGAGTGATGG GGCCTTACGG TGTGCTTAT GCAGTAGCCG
                                Stop amoA→ ↳Start amoB
1901 CATTAGCCAT GACAGTAACA TTGGATGTCT CGACGGTAGC AGCTCATGGG GAGCGATCCC AGGAGCCGTT CCTTCGGATG CGTACAGTTC AATGGTATGA
2001 CATCAAATGG GGTCTGAGG TAACCAAAGT TAATGAGAAC GCAAAAATCA CCGGTAAGTT TCACTTGGCT GAAGATTGGC CACGTGCAGC TGCAACACCT
2101 GATTTTCTCT TCTTTAACGT AGGAAGCCCA AGCCCCGTAT TCGTACGTTT GAGTACCAAG ATCAATGGCC ATCCATGGTT CATTTCAGGT CCTCTGCAAA
2201 TTGGCCGTGA CTATGAGTTT GAAGTCAATC TGCCTGCAGC TATTCGGGGA CGTCATCACA TGCATGCCAT GCTGAACGTC AAAGATGCAG TGCCGATTGC
2301 TGGGCCAGGT GCATGGATGA ACATTACCGG AAGCTGGGAT GATTTTACCA ACCCACTGAA ATTATTGACG GGTGAGACAA TTGATTGAGA AACCTTCAAC
2401 TTATCGAACG GTATATTCTG GCACGTTGTC TGGATGTCCA TCGGTATTTT CTGGATTGGT GTTTTACAG CACGGCCGAT GTTCTGCCA CGTAGTCGTG
2501 TATTGTTGGC CTACGGTGAT GATCTGTGTA TGGATCCGAT GGACAAAAA ATCACCTGGG TACTTGCCAT TCTGACGCTG GCGCTGGTAT GGGGTGGATA
2601 CCGTTATACG GAAATAAAC ATCCCTACAC GGTACCTATC CAGGCAGGTC AATCCAAAGT GGCAGCATTG CCAGTAGCAC CCAACCCAGT ATCGATTGTC
2701 ATTACAGATG CTAACATGA CGTACCGGGA CGTGCACTGC GTGTCACCAT GGAAGTAACC AATAATGGCG ACATACCTGT CACATTGGT GAGTTCACCA
2801 CGGCAGGCAT ACGTTTCATC AACTCTACTG GCCGTAAATA CTTGGATCCT CAGTATCCTC GTGAACATGAT CGCTGTCGGT TTGAATTCG ACGATGAAAG
2901 CGCAATTCAA CCTGGTCAGA CCAAGGAAGT GAAAATGGAA GCCAAGATG CATTGTGGGA AATCCAACGT CTGATGGCGT TGCTGGGTGA CCGGGAAGG
3001 CGTTTTGGTG GATTATTGAT GTCTTGGGAT GCAGAAGGTA ATGCCCATAT CAACAGTATT GCTGGTCCGG TGATTCCTGT CTTACACAAA CTCTGATAAA
                                Stop amoB→

```

Figure 2-8. An example of one copy of the *amo* gene sequence in the genome of *Nitrosomonas europaea* based on the NCBI GenBank database (reported by [McTavish et al., 1993](#) (bases844-2726), [Bergmann and Hooper, 1994](#) (bases2622-3200), [Hommes et al., 1998](#) (bases1-1922)). This combined sequence is >99% identified with the *amoCAB* sequences of *N. europaea* ATCC19718 reported by [Chain et al., 2003](#).

Table 2-3. Sizes* of *amo* genes, the intergenic spacer between *amoC* and *amoA* (*C-A* I.S.) and between *amoA* and *amoB* (*A-B* I.S.)

AOB	Functional genes & Intergenic space (I.S.)					Reference
	<i>amoC</i>	<i>C-A</i>	<i>amoA</i>	<i>A-B</i>	<i>amoB</i>	
		I.S.		I.S.		
<i>Nitrosomonas europaea</i>	816	163	831	0-2	1263	McTavish et al., 1993; Bergamann and Hooper, 1994; Hommes et al., 1998; Alzerreca et al., 1999
<i>Nitrosospira</i> sp. NpAV	813	223	825	0	1248	
<i>Nitrosococcus oceani</i>	786	286	744	65	1251	
<i>Nitrosococcus</i> sp. C-113	---	286	741	65	1260	
<i>Methylococcus capsulatus</i> (Bath)	783	154	744	?	1245	

*Gene sizes counted from start codon to stop codon

Table 2-4. Copy number of *amo* genes from some AOB species

AOB	Copies of <i>amo</i> genes				Reference
	Inside the			Outside the	
	<i>amoCAB</i> operon			<i>amoCAB</i> operon	
	<i>amoC</i>	<i>amoA</i>	<i>amoB</i>	<i>amoC'</i>	
<i>Nitrosomonas europaea</i>	2	2	2	1	McTavish et al., 1993
<i>Nitrosomonas eutropha</i>	2	2	2	1	Klotz et al., 1997
<i>Nitrosococcus oceani</i>	1	1	1	0	Alzerreca et al., 1999
<i>Nitrosococcus</i> C-113	1	1	1	0	Alzerreca et al., 1999
<i>Nitrosospira briensis</i>	3	3	3	1	Klotz et al., 1997
<i>Nitrosolobus multiformis</i>	3	3	3	1	Klotz et al., 1997
<i>Nitrosospira</i> NpAV	3?	3	3?	1	Norton et al., 1996
<i>Nitrosovibrio tenuis</i>	2	2	2	1	Klotz et al., 1997

spacer regions between *amoC* and *amoA* (McTavish et al., 1993; Hommes et al., 2001; Arp et al., 2002; Chain et al., 2003). The two copies of *amoCAB* genes are highly identical (>99%) (McTavish et al., 1993; Chain et al., 2003). In *Nitrosospira* sp. strain NpAV the *amoA* genes, and probably also *amoB* and *amoC*, are present in three copies with 99.6% DNA similarity among three *amoA* copies and with intergenic spacer regions between *amoC* and *amoA* (Norton et al., 1996). Moreover, these β -subdivision AOB usually have one non-operon *amoC* copy that is not adjacent to a copy of *amoAB* (Figure 2-6). On the other hand, unlike most of the β -subdivision AOB having multiple copies of *amo* genes and an individual non-operon *amoC* copy outside the *amoCAB* operon, some AOB from γ -subdivision such as *Nitrosococcus oceani* and *Nitrosococcus* sp. strain C-113 have only one copy of the *amoA*, *B*, and *C* genes and do not have the additional non-operon *amoC* genes (Tables 2-3, 2-4). These *amoC*, *amoA*, and *amoB* genes are adjacent to each other with two intergenic spacer regions between *amoC* and *amoA* and between *amoA* and *amoB* that have never been observed in the β -subdivisions AOB (Alzerreca et al., 1999).

It is believed that transcription of *amo* genes in *N. europaea*, and probably other AOB, are induced by ammonia (Sayavedra-Soto et al., 1996). The *amoA* and *amoB* genes are usually co-transcribed with *amoC* in the same messenger RNA (mRNA), but they also could be expressed independently in *Nitrosospira* sp. NpAV (Sayavedra-Soto et al., 1998). In *N. europaea*, the expression of the individual non-operon *amoC* copy outside the *amoCAB* operon was at much lower levels than the expression levels of the other two copies of *amoC* from *amoCAB* operon (Sayavedra-Soto et al., 1998). Hommes et al. (2001) identified three potential transcription start sites located at 166 and 103 bp upstream of the *amoC* start codon and 114 bp upstream of the *amoA* start codon. All these transcript sites have putative σ^{70} promoter sequences associate with them (Hommes et al., 2001). Previously, Sayavedra-Soto et al. (1996, 1998) detected three AMO transcripts in growing *N. europaea* corresponding to *amoC*, *amoCAB*, and *amoAB*, respectively. The reason for three AMO mRNAs is unknown; they may be originally derived from *amoCAB* mRNA processing or from transcription of *amoC* and *amoA* (Sayavedra-Soto et al., 1998; Arp et al., 2002). The sizes and molecular weight of polypeptides encoded from

amo genes in two AOB species are showed in [Table 2-5](#), and [Table 2-6](#), respectively. In *N. europaea*, the *amoC*, *amoA*, and *amoB* genes encode 271, 276, and 420 bases of amino acids, respectively. These polypeptides in AOB are the major elements that comprise the AMO functional enzymes.

[Hommes et al., 1998](#) used *amoA* mutants to show that both copies of *amoA* (*amoA*₁ and *amoA*₂) in *N. europaea* are expressed functionally (not silent), but could be regulated differently. Either copy of *amoA* was sufficient to support growth of *N. europaea* when the other copy was disrupted; however, the mutations in *amoA*₁ resulted in slower growth while the mutations in *amoA*₂ could be compensated and did not affect the growth rate ([Hommes et al., 1998](#)). It was suggested that the presence of two copies of the *amo* operon might allow *N. europaea* to synthesize a large amount of AMO in a short time when the ammonia substrate is abundant and to survive during low ammonia substrate condition by using only one *amo* gene copy to save vital endogenous energy ([Hommes et al., 1998](#)).

Table 2-5. Size of amino acids (bases) encoded by *amo* genes

AOB	Amino acids				Reference
	<i>AmoC</i>	<i>AmoA</i>	<i>AmoB</i>	<i>AmoC'</i> *	
<i>Nitrosomonas europaea</i>	271	276	420	274	Klotz and Norton, 1995 ; Norton et al., 1996 ; and Sayavedra-Soto et al., 1998
<i>Nitrospira</i> sp. NpAV	270	274	415	270	

**AmoC'*: the amino acid encoding by non-operon *amoC* gene that is not in *amoCAB* operon.

Table 2-6. Molecular weight, M_r, (kDa) of polypeptide encoded by *amo* genes

AOB	AMO subunits				Reference
	<i>AMO-C</i>	<i>AMO-A</i>	<i>AMO-B</i>	<i>AMO-C'</i>	
<i>Nitrosomonas europaea</i>	31.4	27~32	38~43	31.7	Klotz and Norton, 1995 ; Norton et al., 1996 ; and Sayavedra-Soto et al., 1998
<i>Nitrospira</i> sp. NpAV	31~36	30~36	---	31	

*SDS-PAGE molecular weight estimates

Ammonia oxidation activity of AOB can be affected at the *amo* gene transcriptional (Sayavedra-Soto et al., 1996), and mRNA translational (Hyman and Arp, 1995) levels by environmental factors (Section 2.4.3). It has been shown that AOB cells need more than 8 hours incubation under ammonia-free conditions to allow mRNA levels to decrease below detection limit (Sayavedra-Soto et al., 1996). This suggests that mRNA transcripts of *amo* genes are relatively more stable than other prokaryotic mRNA transcripts (on the order of a few minutes) (Stahl and Berube, 2004).

At the DNA level, the *amo* genes encoding the enzyme AMO in AOB share high sequence identity and similarities with *pmo* genes encoding pMMO in methanotrophs (Semrau et al., 1995; Holmes et al., 1995; Alzerreca et al., 1999). The *pmo* genes of γ -subdivision methanotrophs (e.g., *Methanococcus capsulatus* Bath strain) showed higher similarities with *amo* genes sequences of the γ -subdivision AOB (e.g., *Nitrosococcus oceani*) than with *amo* genes of β -subdivision AOB (Holmes et al., 1995; Alzerreca et al., 1999). The property of high similarities between the *amo* and *pmo* genes may limit detection of γ -subdivision AOB based on *amo* gene information.

2.4 Investigations of nitrification treatment performance

2.4.1 Suspended nitrifying activated sludge processes

The activated sludge process is the most widely used biological process for the treatment of municipal and industrial wastewaters (Rittmann and McCarty, 2001). Figure 2-9 shows a typical activated sludge process consisting of an aeration tank, a settling tank, a sludge recycle line, and a sludge wasting line. Activated sludge contains a microbial aggregates (or flocs) suspended in an aeration tank and is used to eliminate target pollutants (e.g., C-, N-, & P-compounds) in influent wastewaters. When the slurry of treated wastewater and microorganisms (mixed liquor) pass to the settling tank, the microbial flocs are settled from the treated wastewater. A proportion of settled biosolids is wasted to control the solids retention time (SRT) and the rest is returned to the aeration tank to maintain a stable mixed liquor suspended solids (MLSS) level for the treatment process (Rittmann and McCarty, 2001).

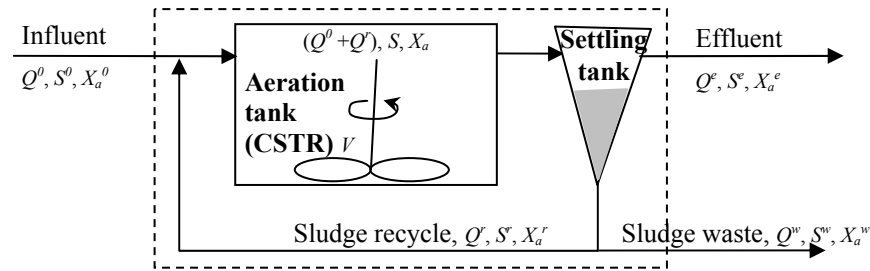


Figure 2-9. Schematic of a typical activated sludge process (Rittmann and McCarty, 2001). [Where Q = flow rate, S = substrate concentration, X_a = active sludge concentration, CSRT = continuous-flow stirred-tank reactor, V = CSTR volume, θ = influent, e = effluent, r = recycle, and w = waste.]

The activated sludge process can be employed to achieve nitrification and organic-carbon (e.g., biological oxygen demand, BOD) removal in a single aeration reactor, or two separate aeration reactors in series. In a one-sludge (or one-stage) nitrification process, heterotrophic and nitrifying bacteria coexist in a single mixed liquor and simultaneously oxidize ammonium and organic BOD (Rittmann and McCarty, 2001). The two-sludge (or two-stage) nitrification is an attempt to reduce the competition between heterotrophs and nitrifiers by oxidizing most of the organic BOD in a first stage, while the ammonia is oxidized in a second stage (Rittmann and McCarty, 2001).

Although the suspended activated sludge process has been widely applied for ammonia removal via nitrification during wastewater processing, washout of nitrifying bacteria from biological reactors can negatively affect nitrification treatment performance. Washout of nitrifying bacteria in activated sludge may be caused by several operational/environmental parameters (section 2.4.3), of which SRT is one of the important factors. An SRT greater than 10 days is generally required to maintain nitrifying bacteria (with slow growth rate and low substrate affinity) in the nitrification processes (Rittmann and McCarty, 2001).

2.4.2 Theoretical aspects for a CSTR with sludge recycling

For a continuous-flow stirred-tank reactor (CSTR) system (with sludge recycling), substrate (S) and active biomass (X_a) concentrations are theoretically dependent upon

operating SRT (θ_x) (Eq. 2-9) under steady state (as represented by the Eqs 2-10, 2-11) (Rittmann and McCarty, 2001).

$$\begin{aligned} SRT(\theta_x) &= \frac{\text{active biomass in the system}}{\text{removal rate of active biomass from system}} = \frac{X_a V}{Q^e X_a^e + Q^w X_a^w} \\ &= \frac{\text{active biomass in the system}}{\text{production rate of active biomass in system}} = \frac{X_a V}{Y(-r_{ut})V - bX_a V} \end{aligned} \quad (\text{Eq. 2-9})$$

$$S = K \frac{1 + b\theta_x}{\theta_x(Y\hat{q} - b) - 1} \quad (\text{Eq. 2-10})$$

$$X_a = \frac{\theta_x}{\theta} \frac{Y(S^0 - S)}{1 + b\theta_x} \quad (\text{Eq. 2-11})$$

[Y : true yield for cell synthesis (mg/mg); r_{ut} : substrate utilization rate; b : endogenous decay coefficient (1/day); K : concentration giving one-half the maximum rate (mg/L); $\hat{q}$: maximum specific substrate utilization rate (mg/day); S^0 : influent substrate concentration (mg/L); θ : hydraulic retention time (day).]

When an inert biomass concentration (X_i) is considered based on f_d coefficient (fraction of the biodegradable active biomass), the total volatile suspended solids (VSS) concentration (X_v) can be presented as Eq. 2-12 (Rittmann and McCarty, 2001):

$$X_v = X_a + X_i = \frac{\theta_x}{\theta} \left[X_i^o + \frac{Y(S^0 - S)(1 + (1 - f_d)b\theta_x)}{1 + b\theta_x} \right] \quad (\text{Eq. 2-12})$$

The S , X_a and X_v levels are plotted in Figure 2-10 as a function of SRT (θ_x) based on Eqs. 2-10, 2-11, and 2-12 (using the following operating and kinetic parameters: $K = 20$ mg BOD_L/L; $Y = 0.45$ gVSS_a/gBOD_L; $\hat{q} = 10$ gBOD_L/gVSS_a/day; $b = 0.21$ 1/day; HRT (θ) = 1.4 day; $S^0 = 800$ mgBOD_L/L; and $f_d = 0.8$ based on Rittmann and McCarty, 2001). The plots show that the substrate concentration (S) is inversely proportional to SRT (θ_x), whereas, the active solids concentration (X_a) and the total volatile suspended solids concentration (X_v) are directly proportional to SRT (θ_x). In other words, a CSTR system operated at a higher SRT should theoretically have lower substrate concentration and higher VSS concentration in the reactor.

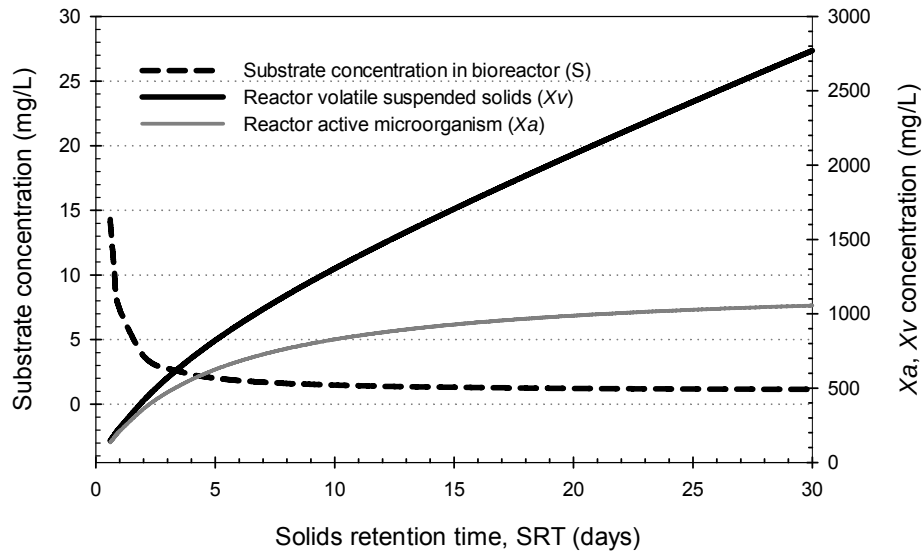


Figure 2-10. A plot showing relationships between S , X_v , X_a and θ_x .

Furthermore, a net active biomass production rate (r_{net}) can be represented as the following equation:

$$r_{net} = Q^e X_a^e + Q^w X_a^w = Y(-r_{ut})V - bX_aV = \frac{X_aV}{\theta_x} \quad (\text{Eq. 2-13})$$

Based on Eq. 2-13, the true cell synthesis ($Y(-r_{ut})V$), the endogenous decay ($-bX_aV$), and the net active biomass production (r_{net} , or X_aV/θ_x) can be plotted as a function of SRT (θ_x) (Figure 2-11). The plots show that, as SRT (θ_x) increases, the true cell synthesis remains relatively constant, the net active biomass production rate (r_{net}) significantly decreases and the endogenous decay increases. Systems operating at higher SRT theoretically have higher endogenous decay and lower net active biomass production. Since the net active biomass produced is equal to the amount of biomass wasted to maintain a steady state operating SRT, lower SRT systems require a greater amount of waste biomass than higher SRT system. Thus, lower SRT systems maintain lower levels of new biomass (or active bacterial cells) in the system. If the net production rate of slow-growing bacteria is lower than the wastage rate in lower SRT systems, population levels will decrease over time and ultimately wash out.

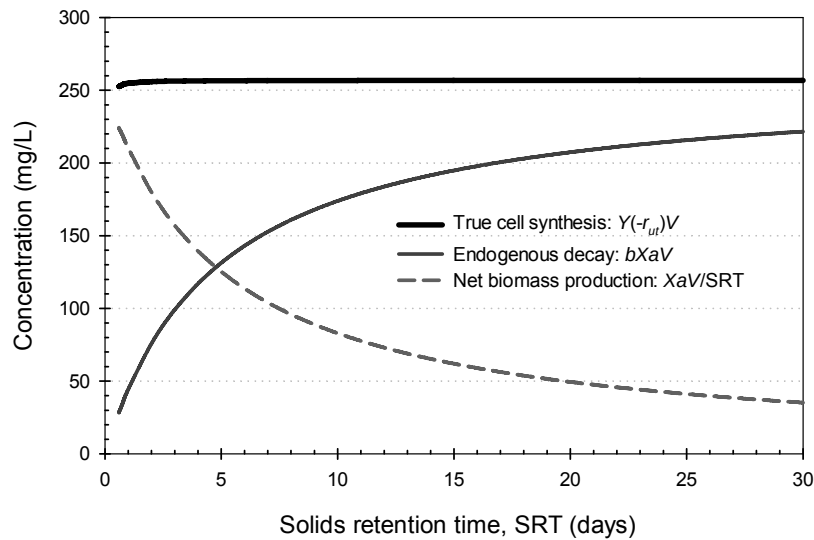


Figure 2-11. Plots show the true cell synthesis ($Y(-r_{ut})V$), the endogenous decay ($-bX_aV$), and the net active biomass production (r_{net} , or X_aV/θ_x) as a function of solids retention time (θ_x).

2.4.3 Factors affecting nitrification treatment performance

Ammonia oxidation performance (as well as nitrification performance) in either engineered systems or natural environments can be affected by several parameters (Table 2-7). These factors may affect AOB at cellular or metabolic levels by changing substrate availability (Stein and Arp, 1998 (a); Ballmann et al., 2002), prohibiting growth (Brandt et al., 2001; Risgaard-Petersen et al., 2004), controlling cell maintenance (Nogueira et al., 2002; Hallin et al., 2005), or interrupting functional gene expression or enzyme activity (Hyman and Arp, 1995; Sayavedra-Soto et al., 1996; Stein et al., 1997; Aoi et al., 2002, 2004 (a), (b); Ebie et al., 2004; Bollmann et al., 2005). Some studies have addressed changes in AOB population or activity under different operational/ environmental conditions (Ballmann and Laanbroek, 2002; Nogueira et al., 2002; Avrahami et al., 2003; Avrahami and Conrad, 2003; Park and Noguera, 2004; Hallin et al., 2005); however, the impact of changes in AOB microbial conditions on overall ammonia oxidation performance have not been explicitly addressed.

Table 2-7. Summary of operational/environmental factors that may impact ammonia oxidation in engineered systems or natural environments.

Factors	Possible impact level* (Mechanism)		Representative reference
	Cell	Metabolism	
Ammonia concentration	×	√	Tappe et al., 1999 ; Bollmann et al., 2002
Dissolved oxygen (DO)	×	√	Jayamohan et al., 1988 ; Beccari et al., 1992
Nitrite concentration	×	√	Sharma and Ahlert, 1977 ; Stein and Arp, 1998b
Solids retention time (SRT)	√	√	Sharma and Ahlert, 1977 ; Pollice et al., 2002
Hydraulic retention time (HRT)	√	√	Sharma and Ahlert, 1977 ; Nogueira et al., 2002
pH	?	√	Shammas, 1986 ; Frijlink et al., 1992
Alkalinity	×	√	Wood, 1986 ; Frijlink et al., 1992
Temperature	?	√	Randall, and Buth, 1984 ; Avrahami et al., 2003
Organic carbon (C/N ratio)	×	√	Gilmore et al., 1999 ; Nogueira et al., 2002
Inorganic carbon	×	√	Green et al., 2002 ; Wett and Rauch, 2003
Inhibitors	?	√	Beg and Atiqullah, 1983 ; Brandt et al., 2001
Light	×	√	Sharma and Ahlert, 1977 ; Prosser 1989
Micronutrients	×	√	Sharma and Ahlert, 1977
Heavy metals	×	√	Stephen et al., 1999 ; Hu et al., 2002,2003
Microbial interactions	√	√	Risgaard-Petersen et al., 2004

* The term “impact on cell level” stands for a case in which factors act directly on AOB cell maintainance; whereas the term “impact on metabolism level” refers to a case when the effects are related to ammonia oxidation pathway and AOB growth such as DNA replication, transcription (RNA production), translation (protein production), electron transportation, and energy generation. ×: not affected; √: affected; and ?: not sure.

As showed in Eq.2-3 ([Section 2.1.1](#)), ammonia, bicarbonate (alkalinity), and dissolved oxygen (DO) are three major reactants in nitrification. Thus, the nitrification rate could be affected by concentrations of these reactants in the activated sludge mixed liquor. Some researchers have studied the effect of ammonia concentration on nitrification ([Rozich and Castens, 1986](#); [Hunik et al., 1992](#); [Stein and Arp, 1998 \(a\)](#); [Tappe et al., 1999](#); [Bollmann et al., 2002](#)). In general, nitrification rates increase with increasing ammonia concentration. However, high ammonia concentration inhibition of nitrification has been reported ([Rozich and Castens, 1986](#)) and may be caused by elevated free ammonia and nitrous acid levels ([Prakasam and Loehr, 1972](#); [Anthonisen et al., 1976](#)). [Suwa et al. \(1994\)](#) suggested that there were possibly two types of AOB that predominate in the activated sludges: one was sensitive and the other was insensitive to high concentrations of ammonia. An ammonia concentration range of 35.2~70.4 (mM) (or 633.6~1267.2 mg/L) has been historically applied for the cultivation of AOB ([Engle and Alexander, 1958](#)). However, typical municipal wastewaters usually contain low levels of ammonia ([Wild et al, 1971](#); [Shieh and LaMotta, 1979 \(a\), \(b\)](#)). For treating wastewater with low ammonia concentrations, it is important to understand how AOB maintain their population and ammonia-oxidation activity under such conditions ([Tappe et al., 1999](#); [Bollmann et al., 2002, 2005](#)). Since it is believed that ammonia is the primary inducer for transcription of the *amo* gene in AOB ([Stein et al., 1997](#)), the ammonia concentration may affect the ammonia oxidation activity of AOB cells at the transcriptional level ([Sayavedra-Soto et al., 1998](#)).

The effect of the DO concentration on nitrification rate has been investigated by a number of researchers using both pure and mixed cultures ([Sharma and Ahlert, 1977](#); [Stenstrom and Poduska, 1980](#); [Laanbroek and Gerards, 1993](#); [Laanbroek et al., 1994](#)). Typically, AOB have a higher affinity for oxygen than NOB; thus, reduction of oxygen tension should repress nitrite oxidation before ammonia oxidation ([Laanbroek and Gerards, 1993](#)). Normally, the nitrification rate will be limited when the DO concentration is decreased. It has been reported that the range of DO concentration needed to reliably achieve nitrification in wastewater treatment is between 0.3 and 2.0 (mg/L) ([Stenstrom and Poduska, 1980](#)). [Beccari et al. \(1992\)](#) suggested that the effects

related to internal oxygen diffusion in flocs must be considered in evaluating overall kinetics. Both the biofloc size and DO concentration in the bulk liquid phase may affect the amount of oxygen available for nitrification (Beccari et al., 1992). Moreover, under oxygen limitation, ammonia may be converted to dinitrogen gas via shortcut nitrification/denitrification or anaerobic ammonia oxidation pathways. In this situation, using conventional kinetic analysis to estimate nitrifying activity may be biased.

Nitrification performance also depends on the amount of active AOB in the treatment system (Sharma and Ahlert, 1977). Theoretically, the nitrification rate will increase when either population or activity of AOB is increased. In a continuous flow treatment system, the AOB population is a proportion of the total bacteria (usually represented by total mixed liquor volatile suspended solids, MLVSS), which is controlled by the operational parameter SRT (Eq. 2-9). At steady-state, the amount of MLVSS wasted from the system equals the amount of MLVSS generated daily in the system and is constant when wastewater composition does not change. Thus, total MLVSS in the system is a function of SRT (Section 2.4.2).

The effects of SRT and MLVSS on ammonia-oxidation may interact with other factors. It has been reported the MLVSS concentration influences the extent of temperature and pH effects on nitrification (Shammas, 1986). To achieve nitrification at low DO concentrations (0.5-1.0 mg/L), a higher SRT is required (Stenstrom and Poduska, 1980). Additionally, treatment systems operated under varying SRT levels result in different effluent ammonia concentrations (or different ammonia oxidation performances) (Robinson et al., 2004; Zimmerman et al., 2004). In a survey of two full-scale activated sludge processes, it was extrapolated that the effect of a longer SRT on the nitrification activity was probably due to physiological changes in the AOB community rather than a change in community composition (Hallin et al., 2005).

Several investigations have assessed pH effects on nitrification (Sharma and Ahlert, 1977; Shammas, 1986). Generally, the optimum pH range for nitrification is between 8 and 9 (Brown and Caldwell, 1975). A wider range between 7 and 9 has also been suggested (Engel and Alexander, 1958) as optimal. A neutral pH range close to 7 is more

appropriate for both heterotrophs and nitrifying bacteria in the treatment systems conducting multi-function treatment for both C and N removal. Below pH 7, growth and metabolic activity of AOB decrease dramatically mainly because the $\text{NH}_3/\text{NH}_4^+$ equilibrium is shifted away from NH_3 , the true substrate for AOB (Frijlink et al., 1992).

The overall optimum temperature for the growth of nitrifying bacteria in mix culture appears to be in the range 28~36°C (Sharma and Ahlert, 1977). However, temperature is difficult to control in full-scale nitrification processes. Low temperatures in winter usually decrease the nitrification rate. Wild et al. (1971) indicated the nitrification rate at 27°C was 90% of that at 30°C, and the rate at 17°C was 50% of that at 30°C. Longer HRT and SRT are usually used to achieve complete nitrification in winter (Sharma and Ahlert, 1977).

When other parameters (temperature, pH, DO, etc.) are held constant, the degree of nitrification decreases significantly with an increase in organic loading (Prakasam and Loehr, 1972). An increase of organic loading may promote the growth of heterotrophs that compete with nitrifying bacteria for available ammonium and oxygen. Okabe et al., (1996) found that a higher influent C/N ratio retarded accumulation of nitrifying bacteria resulting in a considerably longer start-up period for complete and stable nitrification due to nitrifier competition with heterotrophs for dissolved oxygen and space in the mixed-culture biofilm.

Other factors such as inhibitors, light, micronutrients, and heavy metals may also affect the growth of nitrifying bacteria and nitrification rate (Sharma and Ahlert, 1977). Because so many factors can affect nitrification performance in wastewater treatment systems, it is necessary to operate at conditions as close as possible to the optimum to achieve effective nitrogen removal. Furthermore, it is important to carefully define and control the environmental factors when conducting investigations on nitrification and nitrifying bacteria in either batch or continuous experiments. Once it is understood how environmental factors affect nitrification performance in nitrifying systems, it becomes possible to find and solve operating problems when declining nitrification capacity is detected. This study will focus on examining the influence of three primary operational

factors (SRT, NH₃ concentration, and DO concentration) on the AOB population dynamics and cell activities.

2.4.4 Functional parameters for ammonia oxidation performance

Traditionally, overall nitrification treatment performance in a wastewater treatment system has been determined by parameters that are convenient to measure and evaluate at the system level on site (Figure 2-12). Representative performance parameters include effluent ammonia (and/or nitrate) concentrations, ammonia removal efficiency, ammonia oxidation rate, oxygen consumption rate, and/or nitrite/nitrate production rate in the treatment system. However, these parameters do not directly represent microbial conditions of nitrifying bacteria relating to nitrification function.

While the population and activity of AOB cells in wastewater treatment systems are considered as two crucial rate-limiting parameters for the removal of nitrogen from wastewater (Wagner et al., 1995; Dionisi et al., 2002), trials have been conducted to evaluate ammonia-oxidation activity at the cellular level (Figure 2-12) based on overall ammonia-oxidation rates and AOB population numbers (cell density or concentration).

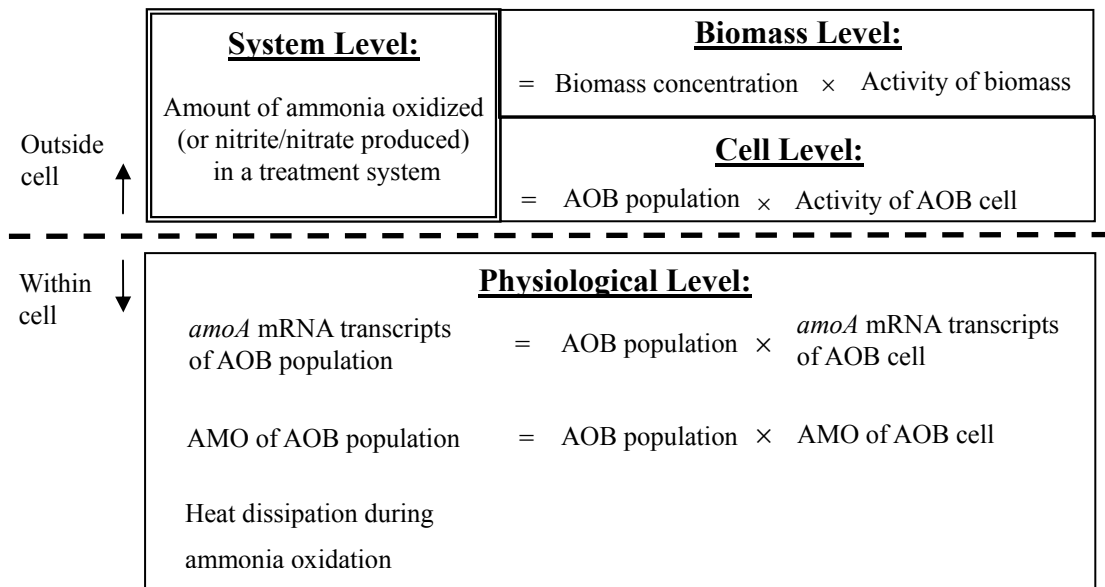


Figure 2-12. Overall ammonia-oxidation treatment performance may be represented at system, cell, and physiological level.

Additionally, the ammonia-oxidation activity has been represented at the physiological level based on calorimetric measurements related to heat dissipation during ammonia oxidation (Aulenta et al., 2002; Daverio et al., 2003), abundance of the *amoA* transcripts (mRNA) (Aoi et al., 2002, 2004 (a), (b)), and AMO activity (Stein and Arp, 1998 (a), (b)).

2.4.5 Determining overall ammonia oxidation rate

Assuming ammonia-oxidation proceeds under the equilibrium concentrations of the enzyme-substrate complex, nitrification rates can be described by the Monod equation when a non-inhibitory relationship exists between nitrification rates and substrate (ammonia-N) concentrations. The nitrification rate can be represented as the specific substrate utilization rate (mg ammonia-N oxidized per mg MLVSS per day) (Eq.2-14), the specific oxygen uptake rate (SOUR) (mg oxygen consumed per mg MLVSS per day), or the overall substrate utilization rate (mg ammonia-N oxidized per liter per day) (Eq.2-15) (Beg and Atiqullah, 1983; Shammash, 1986).

$$q = -\frac{dS}{dt} \left(\text{or } q = q_m \frac{S}{K_s + S} = \frac{r_m}{X} \frac{S}{K_s + S} = \frac{\mu_m}{Y} \frac{S}{K_s + S} \right) \quad (\text{Eq.2-14})$$

$$r = -\frac{dS}{dt} \left(\text{or } r = r_m \frac{S}{K_s + S} = q_m X \frac{S}{K_s + S} = \frac{\mu_m X}{Y} \frac{S}{K_s + S} \right) \quad (\text{Eq.2-15})$$

[Where q = specific substrate (ammonia or oxygen) utilization rate (mg-N/mg-MLVSS/day or mg-O₂/mg-MLVSS/day); r = nitrification rate (mg-N/L/day); S = substrate (ammonia-N or oxygen) concentration (mg/L); t = time (days); X = biomass (MLVSS) concentration (mg/L); q_m = maximum specific substrate utilization rate (mg-N/mg-MLVSS/day, or 1/day); K_s = affinity (half-rate) coefficient (or saturation constant) (mg/L); μ_m = maximum growth rate (mg-biomass/mg-MLVSS/day, or 1/day); Y = growth yield coefficient (mg-biomass/mg-N); r_m = maximum rate constant (mg-N/L/day).]

Suwa et al. (1994) reported different sensitivities of AOB to ammonium sulfate [(NH₄)₂SO₄] in activated sludges using the Monod and Haldane equations to describe substrate non-inhibition kinetics for (NH₄)₂SO₄-insensitive AOB strains and substrate inhibition kinetics for (NH₄)₂SO₄-sensitive AOB strains, respectively. Their results

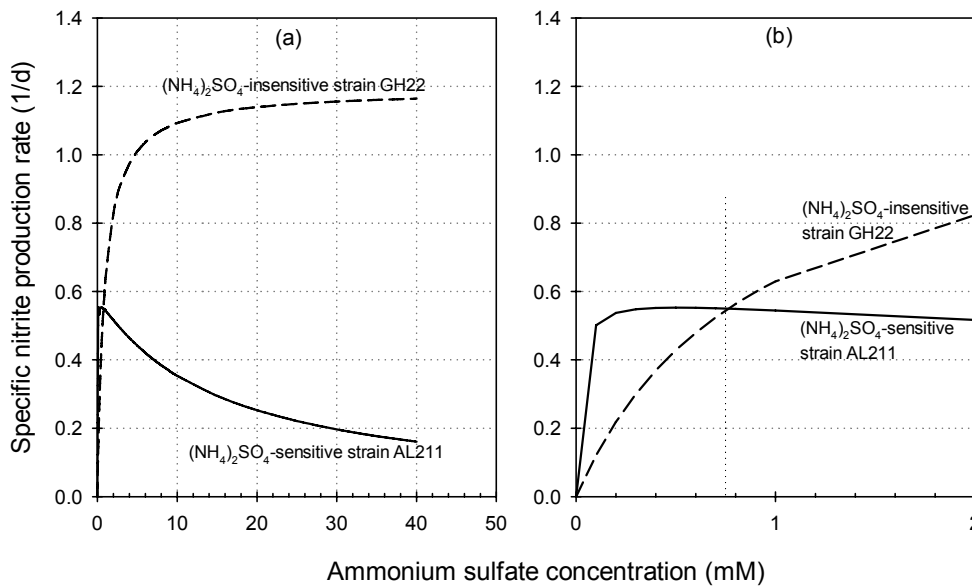


Figure 2-13. Nitrite production rates of AOB strains GH22 and AL211 as a function of $(\text{NH}_4)_2\text{SO}_4$ concentration (a); with an emphasis in a concentration range of 0~2 mM (b) (Based on kinetic parameters reported by [Suwa et al., 1994](#)).

showed that the sensitive strains had lower maximum specific nitrite production rate and lower saturation constant ([Figure 2-13 \(a\)](#)). They suggested that $(\text{NH}_4)_2\text{SO}_4$ -sensitive strains had a growth advantage in lower $(\text{NH}_4)_2\text{SO}_4$ concentrations, whereas insensitive strains had an advantage in higher $(\text{NH}_4)_2\text{SO}_4$ concentrations ([Suwa et al., 1994](#)).

Any operational factor that may change substrate (ammonia) levels may shift the predominant AOB strains and cause a change in the AOB population dynamics due to different kinetics between different AOB strains (or species). For example, an AOB strain (e.g., AL211) ([Suwa et al., 1994](#)) with a low saturation constant utilize ammonia and reproduce in low ammonium concentrations (<0.8mM) ([Figure 2-13 \(b\)](#)); thus, this strain will likely become dominant under a low ammonium concentration condition.

2.5 AOB population dynamics in micro-ecosystems

To achieve multifunctional wastewater treatment including C, N, and P removal, modern biological treatment systems usually contain various groups of bacteria, such as

heterotrophic bacteria, phosphate-accumulating organisms (PAO), nitrifying bacteria (AOB, and NOB), and denitrifying bacteria in a single reactor or in a series of reactors (Gieseke et al., 2001). Figure 2-14 presents a possible schematic model of micro-ecosystem in a stratified structure of biofloc from a multifunctional biological treatment process. It shows that the fast growing heterotrophs occupy the outer layers, where concentrates of substrates are high, while the slow growing nitrifying bacteria remain deeper inside the biofilm (Nogueira et al., 2002). Under a condition of ammonia and oxygen limitation, available ammonia and oxygen are likely utilized by heterotrophs first, resulting in less or no ammonia and oxygen for autotrophic nitrifier to utilize. In such a complex ecosystem, AOB are easily outcompeted by fast-growing heterotrophs for oxygen and ammonia because AOB have low maximum growth rates, low substrate affinities, and high sensitivity to toxic shocks or sudden pH changes (Koops and Möller, 1992). This makes it difficult to maintain stable nitrification in such engineered systems (Painter, 1986; Mobarry et al., 1996). Since AOB are key microorganisms that catalyze ammonia oxidation, it is important to develop fast and reliable methods to monitor AOB population and activity.

It has been suggested that the dominant AOB in nitrifying systems such as activated sludge or biofilm reactors are the β -subdivision members of the Proteobacteria, especially the members of *Nitrosomonas* species (Wagner et al., 1995; Mobarry et al., 1996; Schramm et al., 1996). However, *Nitrosococcus mobilis*, and *Nitrosospira* sp. also have been found as dominant AOB species in some sewage treatment systems (Wagner et al., 1998; Juretschko et al., 1998; Schramm et al., 1998, 1999). In these studies, only a single dominant AOB species has been recognized in each system. Gieseke et al. (2001) found the coexistence of two different AOB populations in a biofilm system. It was suggested that *N. oligotropha*-like AOB with a higher oxygen affinity might be active during an initial aeration period, while *N. europaea*-like AOB with a relatively lower oxygen affinity, and higher maximum ammonia conversion rate, could become more active in a late oxic period (Gieseke et al., 2001). Lately, multiple nitrifying bacterial populations including *N. mobilis*, *N. oligotropha* and *N. europaea/eutropha* related AOB were revealed in a biofilm nitrifying reactor (Gieseke et al., 2003). It was suggested that

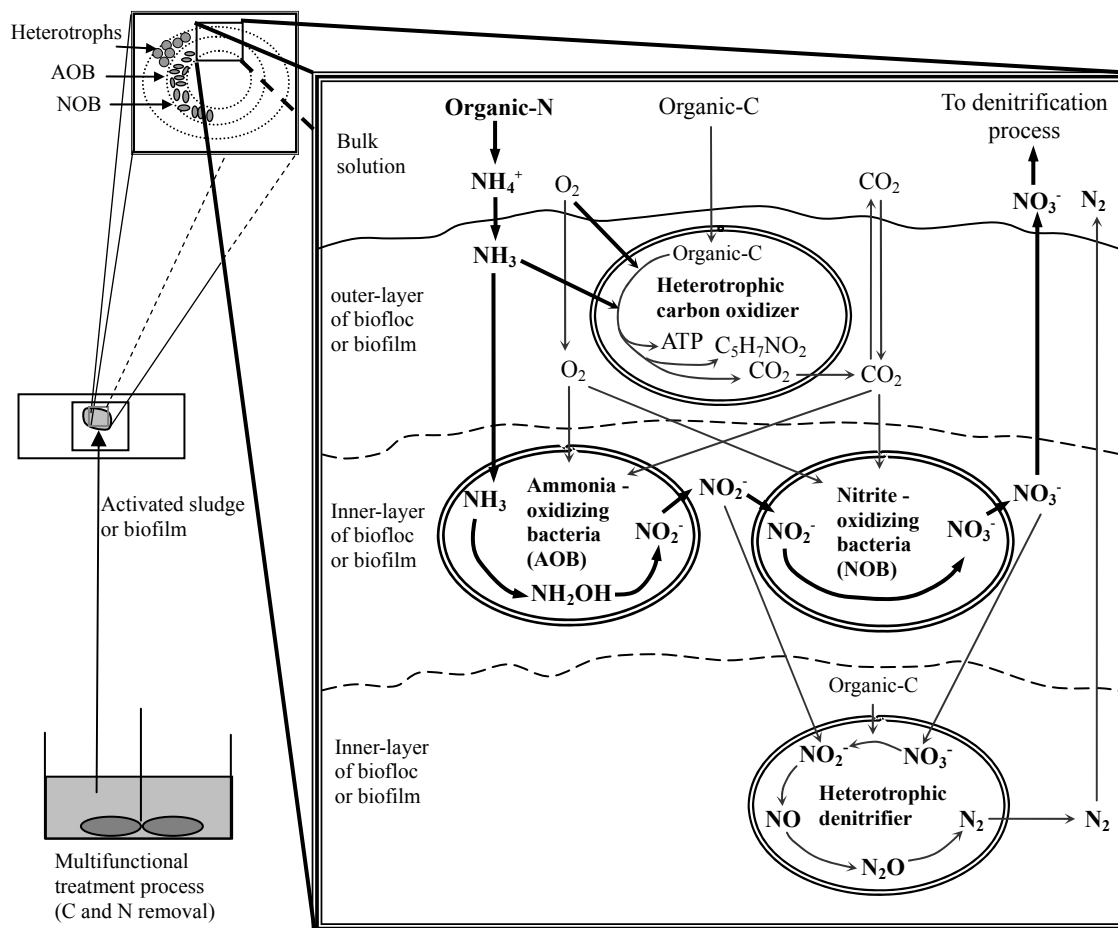


Figure 2-14. A schematic model showing diverse bacteria groups responsible for C, N removal in activated sludge flocs or biofilm from biological treatment processes.

different competitive abilities of certain AOB strains under the prevailing conditions or different sensitivities against inhibitory effects could cause particular AOB species (or strains) to be selected under specific operational/environmental conditions and become the dominant AOB population in the nitrifying system (Suwa et al., 1994, 1997; Koops and Pommerening-Röser, 2001; Bollmann and Laanbroek, 2001).

The AOB populations in nitrogen removal ecosystems have been analyzed by several conventional cultivation-dependent methods. However, due to limitations in isolating and cultivating AOB, some novel cultivation-independent methods have been promoted to investigate AOB.

2.5.1 Cultivation-independent 16S rDNA molecular approach

The 16S rRNA approach was initially applied in phylogenetical classification of AOB (Woese et al., 1984; Lane et al., 1985; Olsen et al., 1986; Head et al., 1993; Teske et al., 1994). Lately, it has been developed to identify and quantify the AOB population in various samples taken from both natural environments and engineered systems by combining other molecular tools such as polymerase chain reaction (PCR), dot blot hybridization, fluorescent-labeled oligonucleotides probe hybridization, competitive PCR and quantitative real-time PCR (McCaig et al., 1994; Hiorns et al., 1995; Voytek and Ward, 1995; Mobarry et al., 1996; Wagner et al., 1996; Ward, 1996; Stephen et al., 1996; Hovanec and DeLong, 1996; Hastings et al., 1997; Ward et al., 1997; Kowalchuk et al., 1997; Juretschko et al., 1998; Kowalchuk et al., 1998; Logemann et al., 1998; Schramm et al., 1998; Speksnijder et al., 1998; Stephen et al., 1998; Bruns et al., 1999; McCaig et al., 1999; Phillips et al., 1999; Schramm et al., 1999; Whitby et al., 1999; Phillips et al., 2000; Bano and Hollibaugh, 2000; Daims et al., 2001; Bjerrum et al., 2002; Dionisi et al., 2002; Juretschko et al., 2002; Cébron et al., 2003; Harms et al., 2003).

A combination of sequential molecular procedures based on rDNA sequences has been defined as “full-cycle rRNA analysis”, a closed-cycle and biphasic approach that consists of two phases, sequencing and probing (Figure 2-15, Amann et al., 1995). In the first phase of the “full-cycle rRNA analysis”, the genomic DNA is first extracted from sample. Target 16S rDNA gene fragment in AOB is then amplified by PCR, and cloned

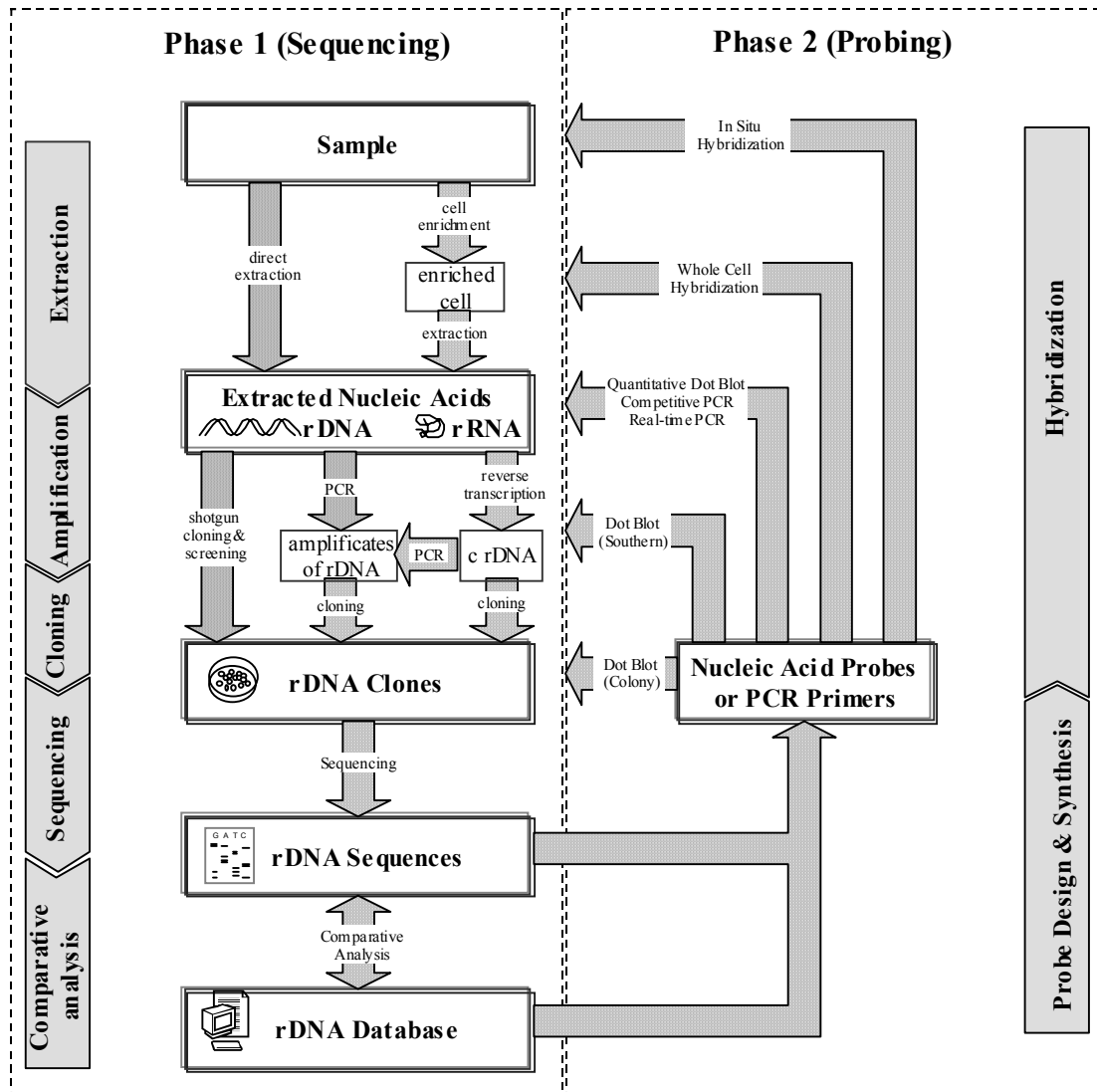


Figure 2-15. Flow chart showing the principal phases of “the full-cycle rRNA analysis” (Based on Amann et al., 1995).

into gene carrier (e.g. plasmid) to make AOB 16S rDNA gene libraries. Subsequently, the target 16S rDNA sequence is retrieved and compared with reported sequences (e.g., GenBank of NCBI) to phylogenetically identify the AOB. Sequence information also can be used to design PCR primers and fluorescent-labeled oligonucleotides probes for further investigating samples *in situ*, in culture, or in DNA extracts. In the second phase, PCR-based methods such as competitive PCR or real-time PCR using specific primer set and fluorescent-labeled probe can be used to recognize and enumerate AOB. By using these molecular analyses, it is possible to identify and quantify AOB population *in situ* without cultivation. In the biological N-removal systems, the application of 16S rDNA-based approach is a very important evolution to more quickly and precisely identify and quantify AOB, whose activity is an important rate-limiting parameter in the removal of nitrogen from sewage (Wagner, 1995).

However, application of the 16S rDNA-based approach for investigating AOB in diverse environments or engineered systems still has some limitations (Amann et al., 1995; Mobarry et al., 1996; Pommerening-Röser et al., 1996). In the initial step of nucleic acids extraction, the extraction efficiency may not be complete because not all AOB can be lysed equally well and not all genomic DNA can be equally captured in extracts. This may cause biased interpretation of the actual abundance of AOB in the analyzing ecosystems after the following quantitative analysis. Secondary, the PCR amplification step has several pitfalls. The well-known PCR problems include inhibition of PCR amplification by co-extracted contaminants (e.g., humic substances), differential amplification for different DNA molecules from different bacteria, formation of artefactual PCR products (e.g., chimeras between two different homologous molecules), and cross-reaction of primers with genes sequences from members of other phylogenetic and physiological groups (Amann et al., 1996; Suzuki and Giovannoni, 1996; Stephen et al., 1996; Wintzingerode et al., 1997; Rotthauwe et al., 1997). Third, the cloning step may also have bias mainly due to different cloning efficiencies for target gene fragments. Fourth, the comparative analysis of 16S rDNA sequences might fail to phylogenetically discriminate AOB strains because of high similarity of 16S rDNA sequences (Amann et al., 1992; Purkhold et al., 2000). Fifth, the majority of oligonucleotides used are designed

on the basis of a relatively small number of DNA sequences usually originating from pure cultures that may not represent the true diversity of bacteria in the samples investigated (Wagner et al., 1993; Utaker and Nes, 1998). Furthermore, using oligonucleotide probes designed for hybridization analysis may be biased due to difference of oligonucleotide quality, difference of cell lysis efficiency, and the presence of extracellular DNA or RNA in the samples (Utaker and Nes, 1998). Because of these limitations, applying the 16S rDNA approach to investigate AOB may bear risks in that closely-related AOB cannot be discriminated and the actual abundance of AOB in samples investigated cannot be determined.

2.5.2 The *amoA* approach

In addition to the 16S rRNA gene sequences, it is also possible to investigate AOB by using sequence information of functional genes such as *amoA*, the gene encoding the subunit of the AOB specific enzymes, ammonia monooxygenase (AMO) (Section 2.3.2, and 2.3.3). The application of the *amoA* approach for identifying and quantifying AOB is similar to the full-cycle rRNA analysis (Section 2.5.1), consisting of sequencing and probing phases. The only one significant difference is that the *amoA* gene sequences are used in the *amoA* approach instead of the 16S rRNA gene sequences.

Since McTavish et al. (1993) first sequenced a full-length *amoA* gene from genomic DNA of *Nitrosomonas europaea*, *amoA* genes sequences of other AOB have been completed (Section 2.3.3). A comparative analysis of *amoA* genes of two phylogenetically related AOB, *Nitrospira* sp. AHB1 and *Nitrosolobus multiformis* C-71 was first conducted by Rotthauwe et al. (1995). Their results showed that the identity value for the *amoA* gene sequences between these two AOB was 86.7%, while the identity of the 16S rRNA gene was at least 98.5%. In other words, *amoA* gene sequences of these two investigated AOB had a lower degree of sequence conservation (or similarity) than the 16S rRNA gene. This facilitated the application of the *amoA* gene sequences information as a molecular tool for a fine-scale differentiation of autotrophic AOB, at the species or strain level, in both environmental and cultivation studies (Rotthauwe et al., 1995).

Based on the *amoA* genes sequence information, several PCR primer pairs have been designed and applied to amplify the *amoA* genes from AOB (Table 2-8). For example, an *amoA*-based PCR primer pair, AMO-F/AMO-R, has been used to amplify the *amoA* genes of genomic DNA from pure cultures of *Nitrosomonas europaea*, *Nitrosomonas cryotolerans*, and *Nitrosococcus oceanus* and from bacteria in seawater collected offshore near the Florida Keys for estimating the distribution and relative abundance of AOB in marine (Sinigalliano et al., 1995). The primers successfully amplified 665 bases of nucleic acid from DNA of target AOB. Later, the AMO-F/AMO-R PCR primer pair was used to investigate AOB in activated sludge and biofilm systems (Juretschko et al., 1998; Daims et al., 2001 (a), (b)).

Additionally, amoA-1F/amoA-2R is a popular *amoA*-based PCR primer pair designed by Rotthauwe et al. (1997). By using this primer pair, Rotthauwe et al. (1997) successfully amplified a fragment of the *amoA* gene for AOB in environmental samples. The specific 491-bp PCR products retrieved from rice roots, activated sludge, freshwater, and an enrichment culture were used to generate *amoA* gene libraries. The comparative analysis of partial *amoA* gene sequences from randomly selected clones indicated that members of both the *Nitrosomonas* and *Nitrosospira* genera could be identified in activated sludge (Rotthauwe et al., 1997). More recently, the amoA-1F/amoA-2R primer pair has been widely applied to investigating phylogenetic relationship of AOB (Purkhold et al., 2000) and population size of AOB in biofilm and activated sludge wastewater treatment systems (Gieseke et al., 2001; Dionisi et al., 2002). Furthermore, a modified primer pair, “amoA-1F*/amoA-1R” with two bases of nucleic acid changed on the forward primer reported by Rotthauwe et al. (1997), has been used to study the AOB in soil (Stephen et al., 1999; Kowalchuk et al., 2000), and composted materials (Kowalchuk et al., 1999).

The application of the *amoA* approach for identifying and quantifying AOB also bears some of the same limitations that the 16S rDNA approach has (within sequential investigation steps such as DNA extraction (Juretschko et al., 1998), PCR amplification (Rotthauwe et al., 1997), cloning, and probing). Nevertheless, it has been suggested that the *amoA* approach represents a very powerful molecular tool for analyzing indigenous

Table 2-8. Summary of the specific PCR primer pairs based on *amo* gene sequences.

PCR primer pairs based on <i>amo</i> gene sequences	Size of amplified products (bp)	References
AMO-F/AMO-R	~665	Sinigalliano et al., 1995 ; Daims et al., 2001
AMO-F2/AMO-R2	~625	Juretschko et al., 1998
AMOF2/AMOR2R	~1719	Hastings et al., 1997 ; Hastings et al., 1998
amoA-486F/amoA-597R	~112	Mendum et al., 1999
amoA-1F/amoA-2R	~491	Rotthauwe et al., 1997 ; Purkhold et al., 2000 ; Gieseke et al., 2001 ; Dionisi et al., 2002 ;
amoA-1F*/amoA-2R	~490	Stephen et al., 1999 ; Kowalchuk et al., 2000
A189/A682	~525	Holmes et al., 1995
305F/302R	~1100	Norton et al., 2002

ammonia-oxidizing communities due to its specificity, its fine-scale resolution of closely related AOB populations, and the fact that a functional trait rather than a phylogenetic trait is detected ([Rotthauwe et al., 1997](#); [Purkhold et al., 2000](#)).

2.5.3 Identifying AOB

Identification of AOB in diverse samples can be conducted by comparative analysis of gene sequences, hybridization with designed specific probes, or amplification with specific PCR primer pairs. The AOB in investigated samples are considered the same as defined AOB or isolates when (i) the result of comparative analysis shows absolute identity (i.e., $\geq 98\%$ similarity) between nucleic acid sequence of sample and sequence of defined AOB or isolates from GenBank; (ii) the fluorescent-labeled probe specific for target AOB can hybridize with complementary oligonucleotide site within extracted DNA or genomic DNA within AOB cells; or (iii) the primer pair specific for target AOB can amplify a segment of DNA extracted from samples. According to sequence comparative analysis, the phylogenetic relationship between AOB strains in samples and defined AOB

also can be obtained. The *amo* genes sequences having high identity and less conservation (comparing with 16S rRNA gene sequences) are suggested to have more potential to identify and classify AOB ([Section 2.5.2](#))([Rotthauwe et al, 1995](#); [Rotthauwe et al, 1997](#); [Purkhold et al., 2000](#)).

2.5.4 Quantifying AOB populations

Hybridization- and quantitative PCR-based assays are two major quantitative molecular techniques that have been used to enumerate AOB populations without cultivation. The quantitative hybridization techniques are primarily based on the application of radioactively or fluorescently labeled oligonucleotide probes specific for target regions in either 16S rRNA genes or *amoA* genes from the AOB of interest. Combining fluorescent *in situ* hybridization (FISH) with confocal laser scanning microscopy (CLSM) and digital image analysis is an example of a hybridization-based quantitative tool that has been widely applied to determine AOB population size in engineered nitrifying systems ([Wagner et al., 1995](#); [Amann et al., 1995](#); [Wagner et al., 1996](#); [Schramm et al., 1998](#); [Schramm et al., 1999](#); [Okabe et al., 1999](#); [Sato et al., 2000](#); [Daims et al., 2001 \(a\), \(b\)](#); [Gieseke et al., 2001](#); [Nogueira et al., 2002](#)). This tool also can be used to analyze the spatial distribution of AOB in activated sludge flocs or biofilm (in comparison of other heterotrophic bacteria). However, due to the fact that cell numbers only can be determined from a relative small unit of sample volume under CLSM, application of this hybridization-based approach for estimating microbial population size might be biased when bacteria are not normally distributed among flocs or aggregates.

The competitive PCR (cPCR) quantitative technique is based on addition of known amounts of an internal standard to the PCR ([Diviacco et al., 1992](#)). The internal standard is a competitive template, which is constructed with a sequence as similar as possible to sequence of the template in the environmental sample and co-amplified together with the sample template. The relative amount of amplification formed from each of the templates can be quantified ([Bjerrum et al., 2002](#)). With either 16S rDNA or *amoA* genes as templates, the quantitative cPCR has been used to quantify AOB in environmental samples ([Mendum et al., 1999](#); [Stephen et al., 1999](#); [Ivanova et al., 2000](#); [Kowalchuk et](#)

al., 2000; Phillips et al., 2000; Bjerrum et al., 2002) and in engineered systems (Kowalchuk et al., 1999; Daims et al., 2001 (b); Dionisi et al., 2002). The quantitative cPCR approach has high sensitivity and accuracy, and eliminates the problems with tube-to-tube variation, contamination by PCR inhibitors, product inhibition, etc. (Piatak et al., 1993; Bjerrum et al., 2002). However, it may be difficult to achieve the same affinity of the primers for the target and competitive molecules, which may complicate quantification (Hermansson and Lindgren, 2001).

The real-time PCR quantitative technique is based on continuously monitoring accumulation of PCR products during the amplification process (Holland et al., 1991; Heid et al., 1996; Freeman et al., 1999; Dionisi et al., 2003). The PCR amplified products can be detected by using DNA-binding dyes (e.g., intercalator ethidium bromide, and SYBR[™] Green I dye), molecular beacons, hybridization probes (e.g., LightCycler[™] technology), and hydrolysis probes (e.g., TaqMan[®] probe) (Bustin, 2000). The TaqMan[®] probes are dual-labeled fluorogenic oligonucleotides that consist of two dyes, a reporter dye (e.g., 6-carboxyfluorescein, FAM) at the 5'-end and a quencher dye (e.g., Black Hole Quencher[™]) at the 3'-end. While the probe is intact, the 3'-end quencher dye quenches (or suppresses) fluorescent emission of the 5'-end reporter dye by Förster-type resonance energy transfer (FRET) through space. The fate of the probe during the extension phase of PCR is presented in Figure 2-16. After the denaturation step of PCR, two PCR primers anneal with the templates; meanwhile, the probe can anneal with the target sequence downstream from one of the primer sites if the target sequence is present. When one of the primers is extended and reaches the probe during the elongation step, the probe will be cleaved by the 5' nuclease activity of Taq DNA polymerase. This cleavage of the probe not only allows primer extension to continue to the end of the template strand obtaining a new amplicon for next cycle of PCR amplification, but also separates the 5'-end reporter dye from the 3'-end quencher dye increasing the fluorescence signal of the reporter dye. The increase in fluorescence intensity is proportional to the amount of amplicon produced. During the early exponential phase of the PCR amplification, the reaction cycles at which fluorescence exceeded a threshold limit is defined as the threshold cycle (Ct) value that is correlated to initial concentration of the template DNA. The higher the amount of initial

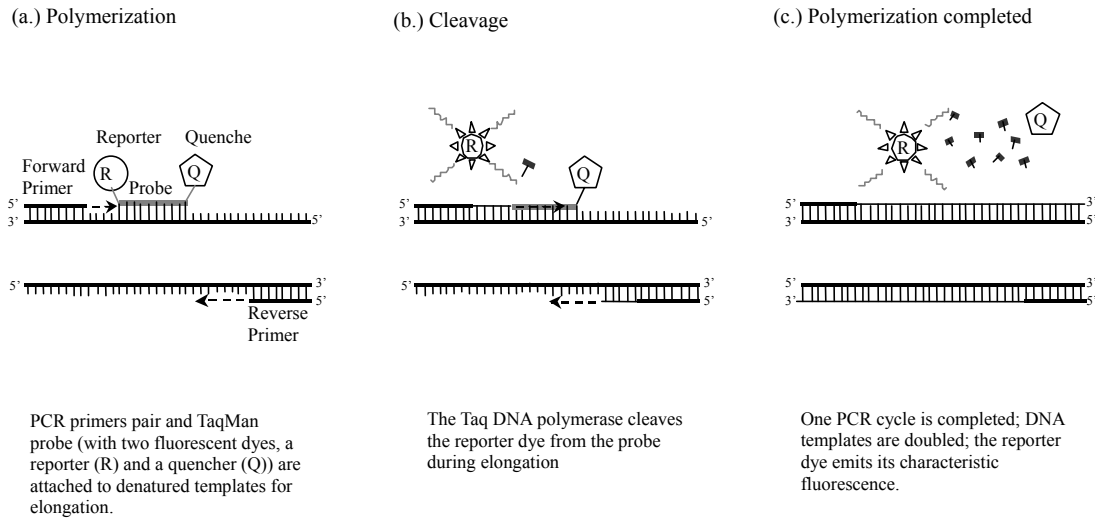


Figure 2-16. A diagram showing how reporter dye is cleaved from the probe in a TaqMan[®] probe based real-time PCR assay (Heid et al., 1996).

template DNA, the earlier the fluorescence will reach the threshold; and copy number of the initial target DNA is thereby determined by comparison to a standard curve (Grüntzig et al., 2001).

The real-time PCR quantitative technique based on utilizing the hydrolysis fluorogenic probe (e.g., The TaqMan[®] probe) has recently been promoted to investigate microbial ecology because it offers several advantages: (i) it is a highly sensitive and specific assay; (ii) the assay allows quantification to be completed during PCR reaction; (iii) it is performed in a closed-tube system that increases the sample throughput; (iv) it requires no post-PCR processing (e.g., gel electrophoresis); (v) the assay has a rapid turn-around time; and (vi) it supports the use of a normalization gene for quantitative PCR or housekeeping genes for quantitative RT-PCR controls (Heid et al., 1996).

Hermansson et al. (2001) designed a fluorogenic-probe-based real-time PCR assay to quantify the AOB population in arable soil by targeting a 16S rDNA region of the AOB. Their results suggested that increasing the amount of substrate by nitrogen fertilization increased the number of AOB in the soil. Lately, an *amoA*-based real-time PCR assay has been applied to study effects of ammonium on population size of AOB in soil (Okano et

al., 2004). Real-time PCR assays based on *amoA* gene also have been applied to investigate *Nitrosomonas oligotropha*-like and *Nitrosomonas nitrosa*-like AOB population in the full-scale municipal and industrial activated sludge nitrification system, respectively (Harms et al., 2003; Layton et al., 2005).

2.6 Ammonia-oxidation activities of AOB cells

Traditionally, the ammonia-oxidation activities of AOB have been determined by kinetic parameters such as specific ammonia oxidation rate, or specific oxygen utilization rate based on pure culture, or isolates (Belser, 1979; Prosser, 1989). The ammonia-oxidation activities of some pure-cultured AOB have been reported in a range of 4~23 (fmol/cell/hr) (Belser, 1979; Prosser, 1989). However, information is still lacking due to the limitation of cultivation-dependent methods. For instance, enumeration of AOB based on MPN technique is frequently inaccurate and consequently leads to overestimation of cell activities (Prosser, 1989). Alternatively, some molecular approaches can more accurately quantify AOB population *in situ* without cultivation and allow ammonia-oxidation activity of AOB to be estimated at cell level. Additionally, the abundance of *amoA* mRNA has been proposed to indicate AOB cellular activity at the physiological level (Aoi et al., 2002, 2004 (a), (b); Bollmann et al., 2005).

2.6.1 AOB cellular oxidation rate

The ammonia-oxidation activity of AOB cells has been represented by cellular ammonia-oxidation rate determined from the overall ammonia-oxidation rate ($\mu\text{mol/L/hr}$) and AOB population (cells/L). Selected results for municipal activated sludges are summarized in Table 2-9 in which the AOB populations were quantified using FISH (rRNA-targeted oligonucleotide probes) (Daims et al., 2001 (a)), quantitative cPCR (*amoA* gene-based) (Daims et al., 2001 (b)), or real-time PCR (*amoA* gene-based) (Harms et al., 2003). Although the AOB population and ammonia-oxidation activity may vary from system to system due to differences in wastewater composition and operating conditions, these results were in a similar range.

Table 2-9. Summary of AOB population and cellular ammonia-oxidation rate (linked with activity) in municipal activated sludges.

Reference	Quantifying Method	AOB population (cells/liter)	NH ₃ -oxidation rate (fmol/hr/cell)
Daims et al., 2001(a)	FISH (rRNA)	$9.8 \pm 1.9 \times 10^{10}$	2.3 ± 0.4
Daims et al., 2001(b)	cPCR (<i>amoA</i>)	?	16~48
Dionisi et al., 2002	cPCR (<i>amoA</i>)	$4.3 \sim 40.5 \times 10^7$	?
Harms et al., 2003	Real-time PCR (16S rRNA)	$1.2 \pm 0.9 \times 10^{10}$	7.7 ± 6.8
Harms et al., 2003	Real-time PCR (<i>amoA</i>)	$7.5 \pm 6.0 \times 10^9$	12.4 ± 7.3

The parameter of cellular ammonia-oxidation rate may represent ammonia-oxidation activity for AOB cells; however, it cannot indicate physiological condition within AOB cells which may provide further information for assessing the reasons causing shifts in ammonia oxidation treatment stability. Additionally, when multiple AOB species/strains coexist in a treatment system, an average cellular ammonia oxidation rate is determined based on an assumption that all AOB cells from distinct groups have the same cellular oxidation rate. Thus, this average cellular oxidation rate for AOB does not address specific activity variances among distinct AOB groups if their ammonia oxidation activities are not the same. Based on the current understanding that ecophysiological differences exist between the distinct AOB representatives ([Suwa et al., 1994](#), [Koops and Pommerening-Röser, 2001](#); [Bollmann and Laanbroek, 2001](#)), it is unlikely that all AOB cells from different ecophysiologicaly-distinguished groups have the same ammonia oxidation activity.

2.6.2 An *amoA* mRNA approach using Northern hybridization

Based on a concept that ammonia oxidation by chemolithotrophic AOB is completely reliant on the catalytic activity of the key enzyme, ammonia monooxygenase (AMO) ([Section 2.3.2](#)) whose active site is believed to be translated from *amoA* mRNA (transcripting from *amoA* gene), it has been assumed that the cellular ammonia oxidation

activity might be correlated with the abundance of *amoA* mRNA in AOB cells (Stein and Arp, 1998; Bothe et al., 2000; Aoi et al., 2002).

Northern hybridization has been used to examine *amoA* mRNA transcripts for *N. europaea* cell cultures incubated in a medium containing ammonia (Sayavedra-Sota et al., 1996; Stein et al., 1997; Stein and Arp, 1998 (a), (b)). Results showed that increases of relative ammonium oxidation activity (determined by NH_4^+ -dependent O_2 uptake rate) after 2~4 hours of incubation corresponded to increases in the relative amount of hybridized *amoA* (Stein and Arp, 1998 (a)) transcriptionally induced by ammonia (Sayavedra-Sota et al., 1996; Stein et al., 1997). These works were the first to mechanistically investigate the ammonia oxidation activity of *N. europaea* at the *amoA* mRNA (transcriptional), and AMO (translational, or post-translational) levels. However, abundances of the *amoA* mRNA transcripts in *N. europaea* were examined by relative label intensity of hybridized bands in Northern hybridization and were not be absolutely quantified.

2.6.3 An *amoA* mRNA approach using reverse transcription PCR (RT-PCR)

The *amoA* mRNA also can be quantified using an approach combining reverse transcription (RT) and a quantitative assay (e.g., competitive and real-time PCR). The RT approach has been used for investigating gene expression in clinical studies since the 1990s (Bustin, 2000). The first step of this analysis is generating cDNA copies from the *amoA* mRNA using reverse transcriptase and a primer complementary to the RNA sequence at the 3' region of the mRNA. In the next step, the cDNA can be further quantified by real-time PCR assay, for example, using TaqMan[®] probe. The *amoA*-mRNA-based measurement has been recently used to assess physiological activity of AOB cells (Table 2-10).

The *amoA* mRNA has been semi-quantified to investigate the response of *amoA* mRNA transcription and ammonia-oxidizing activity of AOB from a nitrifying biofilm system to the change of pH and ammonia concentration (Aoi et al., 2002) (using a sequential process involving reverse transcription (RT), PCR amplification, gel electrophoresis, and intensity measurement of isolated bands on agarose gel). In a batch

Table 2-10. Summary of ammonia oxidation activity studies based on the *amoA* mRNA approach.

Reference	Experiment mode & sample	Test factor	Approach	Test correlation*
Aoi et al., 2002	Batch (BF [†])	Ammonia,	RT-PCR & gel image	+
	Continuous (BF)	pH		-, +
Aoi et al., 2004 (a)	Batch (BF)	Ammonia,	Competitive RT-PCR	+
		Salinity,		+
		DO		+
	Continuous (BF)	pH		-, +
Aoi et al., 2004 (b)	Batch (BF)	Ammonia,	real-time RT-PCR	+
	Continuous (BF)	Salinity		-, +
Ebie et al., 2004	Batch (BF)	Process	RT-PCR & DGGE	+
	Continuous (AS [‡])	Process		+
Bollmann et al., 2005	Continuous (<i>N. briensis</i>)	Ammonia	RT-PCR & gel image	+

*: test correlation “+” stands for the change in *amoA* mRNA level corresponded to change in actual ammonia oxidation activity; while correlation “-” stands for the correspondence was not observed; †: biofilm; ‡: activated sludge.

experiment, their result showed that the relative band intensity of the RT-PCR products (represented the abundance of *amoA* mRNA transcripts) increased rapidly enough to be detected after the biofilm was transferred into an ammonia-containing solution for 2 hours. Furthermore, the relative band intensity of a sample taken after 24 hours incubation in the ammonia-containing solution was relatively stronger than that during ammonia starvation; while the band intensity representing the signals of 16S-rRNA-encoding DNA and *amoA* DNA remained almost constant under both ammonia starvation and incubation periods (Aoi et al., 2002). The increase of *amoA* mRNA was correlated with an increase of ammonia removal of about 160 (mg NH₃/L) within 24 hours. Additionally, the result of a continuous experiment showed that the relative band intensity increased about 1.5 units 2 hours after the continuous feeding system recovered from low pH (~5.5). The increase of *amoA* mRNA was correlated with an increase in ammonia removal of about 50 (mg NH₃/L) within 2 hours. These results suggested

mRNA-based detection of bacterial activity reflects the actual ammonia- oxidizing activity much more closely than other measures, such as rRNA and DNA (Aoi et al., 2002).

Recently, expression of *amoA* mRNA has been examined by competitive (Aoi et al., 2004 (a)) and real-time RT-PCR (Aoi et al., 2004 (b)) to study the relationship between actual ammonia oxidation activity and *amoA* mRNA transcript level. In short-time (5hrs) batch-mode incubations, results showed that *amoA* mRNA transcription closely corresponded to the actual ammonia oxidation activity (determined by NO_x^- -N production) under ammonia-rich, salinity, and DO-limited conditions while *amoA*-encoding DNA hardly increased (Aoi et al., 2004 (a)). During the continuous-feeding incubation, the *amoA* mRNA level did not dynamically change in response to the decrease in cell activity caused by pH reduction; nevertheless, the level significantly increased when cell activity recovered upon the increase of pH (Aoi et al., 2004 (a)).

Moreover, it was found that *amoA* mRNA levels slowly decreased (24-72 hrs) under ammonia starvation compared to a quick (1-2 hrs) increase of *amoA* mRNA level under the sudden exposure to ammonia after 72 hours of starvation (Aoi et al., 2004 (b)). In another continuously fed experiment using salinity as an inhibition factor, a marked change in *amoA* mRNA level was not observed even when ammonia oxidation activity was significantly inhibited under more than 6 wt% of NaCl, but an increase in *amoA* mRNA level corresponded to ammonia oxidation activity recovery from inhibition caused by salinity (Aoi et al., 2004 (b)).

2.6.4 Ammonia-oxidation activity of AOB under ammonium limitation

In an oligotrophic situation, AOB have to adapt to stressful conditions of ammonia limitation and starvation. Bollmann et al. (2002) carried out a growth experiment of AOB at low ammonia concentrations using *N. europaea* and the ammonia oxidizer G5-7, a close relative of *Nitrosomonas oligotropha* belonging to *Nitrosomonas* cluster 6a, enriched from a freshwater sediment. Their result showed that strain G5-7 was able to out-compete *N. europaea* at growth-limiting substrate concentrations of about 10 μM ammonia ($\approx 0.18 \text{ mg/L}$), suggesting a better growth ability of the ammonia oxidizer G5-7

at low ammonia concentration (Bollmann et al., 2002). Another result showed that *N. europaea* became almost immediately active after 1 to 10 weeks of ammonia deprivation following the addition of fresh ammonia (the added ammonia was converted within 48 to 96 h), suggesting *N. europaea* displayed a more favorable starvation response (Bollmann et al., 2002). These results indicated some AOB (like G5-7) are able to grow at lower ammonia concentrations at which point the growth of other AOB (like *N. europaea*) are halted; also, some AOB (e.g., *N. europaea*) are able to recover their physiological activity after long periods of ammonia starvation.

Bollmann et al., 2005 studied the effect of short-term ammonia starvation on *Nitrosospira briensis*, and found that a slowly-decreased activity (determined by NO_x^- production) corresponded to a decrease in *amoA* mRNA level, while no changes in 16S rRNA were observed (based on band intensities of RT-PCR products visualized in agarose gels). They also found the *amoA* mRNA was still present after a starvation period of 12 days suggesting that ammonia starvation may lead to an increase in the half-life of mRNA and result in a stabilization of the *amoA* mRNA in AOB cells (Bollmann et al., 2005).

In an ongoing project, it was hypothesized that starved AOB cells may increase the stability of the existing mRNA pool by sacrificing strict regulation of gene expression in a strategy to conserve energy (Stahl and Berube, 2004). Some batch-mode study results related to the responses of AOB to ammonia limitation and starvation suggested that ammonia oxidation activity of AOB may remain stable for a period of time (at least 10 weeks) because AOB cells and AMO enzyme in cells were not mineralized under ammonium starvation (Stein and Arp, 1998 (a); Bothe et al., 2000). Nevertheless, in a CSTR system with sludge recycling, the population of AOB cells may decline due to sludge wastage to maintain the operating SRT, if the net production rate of AOB is lower than the net wastage rate under ammonium starvation condition. It had been suggested that *N. europaea* cells were more capable of tolerating ammonia starvation in biofilms than in suspension cultures, perhaps due to the production of and response to the quorum-sensing molecule, N-acyl homoserine lactone (Stein and Arp, 1998).

2.6.5 An AMO enzyme approach

It has been well-established that ammonia oxidation by chemolithotrophic AOB is completely reliant on the catalyzing activities of the key enzyme, ammonia monooxygenase (AMO) (Section 2.3.2) whose activity could remain stable under ammonia starvation (Stein and Arp, 1998 (a); Wilhelm et al., 1998; Bollmann et al., 2002). The AMO enzyme pool within an AOB population could possibly be used to infer the maximum ammonia oxidation activity of AOB in nitrifying systems under various conditions. Since AMO has not been purified in an active state, its quantification cannot be achieved directly. Alternatively, approaches using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a PhosphorImagerTM to determine amounts of radiolabeled AMO-binding reagents (e.g., a suicide substrate, acetylene $^{14}\text{C}_2\text{H}_2$, and a metal-chelating compound, thiourea) captured by the active site of AMO, or amounts of radiolabeled inorganic carbon (e.g., $^{14}\text{CO}_2$, and $\text{Na}_2^{14}\text{CO}_3$) incorporated into AMO polypeptide have been developed to indirectly represent the quantity of AMO enzyme (Hyman and Arp, 1992,1995; Sayavedra-Soto et al., 1996; Stein et al., 1997; Stein and Arp, 1998 (a), (b)).

Based on the results from Stein and Arp (1998 (a)), increases in the relative ammonia oxidation activity (determined by NH_4^+ -dependent O_2 uptake rate) of *N. europaea* after 4~6 hours of incubation in medium containing ammonia not only corresponded to increases in the relative amount of *amoA* transcribed (Section 2.6.2), but also correlated to increases of relative density of ^{14}C -labeled AMO polypeptide (as measured by SDS-PAGE and a PhosphorImagerTM) (Stein and Arp, 1998 (a)). After 24 hours of incubation, the relative ammonia oxidation activity decreased while the relative amount of AMO polypeptide remained almost stable. It was demonstrated that the loss of ammonia oxidation activity after a 24-hour incubation period was caused by a decrease in pH and an accumulation of nitrite that post-translationally interrupted the activity of the AMO enzyme (Stein and Arp, 1998 (b)). From this work, it might be possible to determine the ammonia oxidation activity of AOB in nitrifying systems at the AMO enzyme level and investigate how operational/environmental factors affect ammonia oxidation activity of AOB at the translational and post-translational levels.

2.7 Study motivation

The AOB population and activity levels may be affected by certain operation/ environmental conditions, which may subsequently influence overall ammonia oxidation performance (Nogueira et al., 2002; Robinson et al., 2004; Hallin et al., 2005). Several studies have elucidated AOB population ecology and cellular activity in selected treatment systems (Schramm et al., 1998, 1999; Gieseke et al., 2001; Dionisi et al., 2002; Harms et al., 2003; Aoi et al., 2004; Layton et al., 2005). However, it is still not fully understood how the AOB population and activity can be affected under different conditions and how shifts in AOB population and activity may impact overall ammonia oxidation performance in nitrifying systems. This study was performed to assess the AOB population (based on *amoA* measurement) and the AOB physiology activity (based on *amoA* mRNA measurement) as a function of changes in operational conditions (i.e., SRT, NH₃, and DO), and to relate these molecular parameters to overall ammonia oxidation treatment performance (based ammonia oxidation rate).

Chapter 3 Materials and methods

3.1 Overview of experimental approach

An overview of experimental approach is shown in [Figure 3-1](#). Three research components were performed to achieve the overall goal of this study ([Section 1.2](#)). In order to investigate AOB population dynamics and activity under different operating conditions, a bench-scale activated sludge system was established to treat a low-ammonia-strength industrial wastewater under four different SRTs, two influent NH_3 levels, and two operating DO levels. Long-term (≥ 40 days) shifts in AOB population under designated operating conditions were assessed to address possible correlations between AOB population dynamics and ammonia oxidation treatment performance (Part I). It was expected that the AOB population in the treatment system would be affected by the various testing factors and that shifts in AOB population dynamics would result in changes in ammonia oxidation performance.

A second bench-scale nitrifying system was later established by introducing a synthetic high-strength ammonia substrate to enrich a nitrifying culture to study AOB cellular activity based on *amoA* mRNA measurement (using reverse transcription (RT) real-time PCR technique). Short-term changes in NH_3 and DO levels were employed to induce AOB ammonia oxidation activity shifts. This work (Part II) was conducted to achieve proposed objective 2, which focused on establishing the *amoA* mRNA transcript level in AOB cells as an indicator of ammonia oxidation activity. It was anticipated that *amoA* mRNA would respond to short-term changes in NH_3 and DO levels and that changes in ammonia oxidation rate would be reflected in *amoA* mRNA levels.

In Part III of this study, the *amoA* mRNA approach was used to examine AOB physiological activity for the industrial bench-scale system. Ascendancy of AOB cellular activity as a reason for AOB population dominance in the industrial bench-scale system was assessed. An AOB strain with a higher physiological activity was expected to dominate the AOB population in the treatment system.

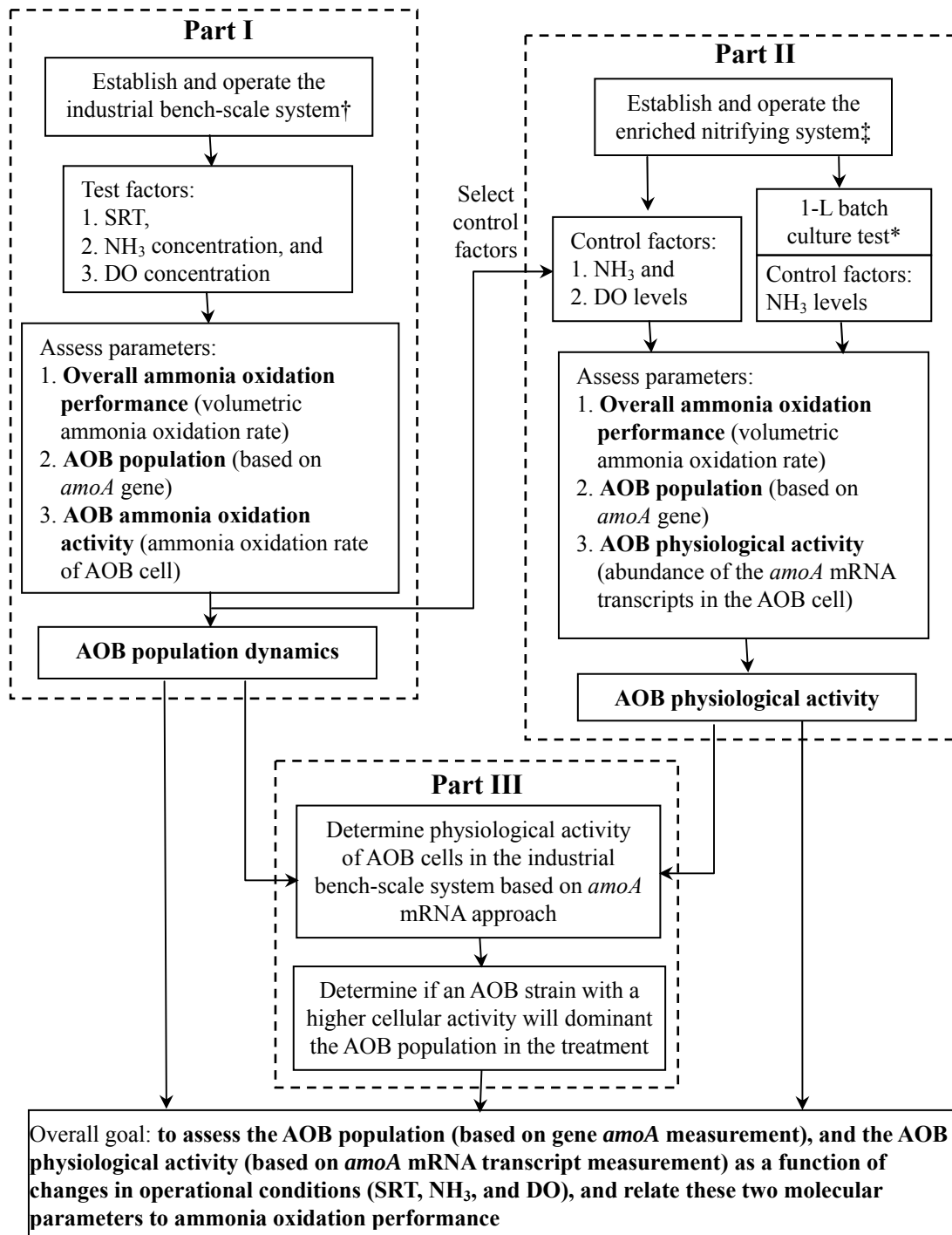


Figure 3-1. Overview of research approach used to achieve the overall goal and objectives of this study. (Note †: bench-scale activated sludge industrial wastewater treatment system; ‡: bench-scale laboratory-enriched nitrifying system; and *: continuous-feed batch culture (1 L) activity test.)

Specific tasks for each research part are described as follows. In Part I, the industrial bench-scale system (consists of four trains operated at four SRTs in parallel) was operated at three designated testing conditions (phase 1: high DO, low ammonia; phase 2: high DO, high ammonia; and, phase 3: low DO, high ammonia). Aqueous samples were collected from this treatment system and analyzed for TKN, $\text{NH}_3\text{-N}$, $\text{NO}_2^- \text{-N}$, $\text{NO}_3^- \text{-N}$ to estimate overall ammonia oxidation treatment performance (based on volumetric ammonia oxidation rate) under each operating phase. AOB *amoA* gene libraries were established to identify dominant AOB groups in the treatment system and to design specific real-time PCR primer/probe sets. Population sizes of the AOB groups found in the treatment system were then quantified (based on the *amoA* gene abundance) using newly designed *amoA*-based TaqMan[®] real-time PCR assays in order to assess the influence of SRT, NH_3 , and DO on AOB population dynamics.

In Part II, the enriched nitrifying system was operated at steady state to obtain a stable AOB culture. The *amoA* gene libraries for this system were constructed to identify dominant AOB species/strains, and to design a real-time PCR primer/probe set to quantify the dominant AOB population (based on the *amoA* copy number enumerated using a TaqMan[®] real-time PCR assay). Additionally, the physiological activity of AOB cells was determined (based on *amoA* mRNA transcripts abundance quantified using a newly-designed reverse transcription (RT) real-time PCR assay). Enriched nitrifying culture from the system was harvested to examine ammonia oxidation rates and physiological response (based on *amoA* mRNA measurement) under varying ammonia concentrations using continuous-feed batch culture (1 L) tests. In addition, the enriched nitrifying system was operated at different ammonia oxidation treatment conditions (by changing the operating NH_3 and DO levels) to assess possible correspondence between the *amoA* mRNA transcripts level and the ammonia oxidation rate (activity).

Specific tasks for Part III were to develop RT real-time PCR assays to assess the physiological activity of dominant AOB populations in the industrial bench-scale system and to examine the ascendancy in physiological activity as a reason for AOB population dominance (in the industrial bench-scale system) under two different testing ammonia loadings.

3.2 Experimental systems and samples collection

3.2.1 Industrial bench-scale system

The industrial bench-scale system consisted of four separate aeration tanks. Each tank included three 10-L baffled reactors operating in series (Figures 3-2 (a), (b); 3-3 (a), (b)). Three influent pumps (Cole-Parmer MasterFlex® L/S™, with four Easy-Load® 7518-00 pump heads) were used to pump influent wastewater from a holding drum to each of the three sections of the four reactor trains (tanks). Effluent from each train intermediate stage reactor flowed into the next stage through a baffle opening and then overflowed to a 1.9-L external secondary clarifier that provided quiescent settling and continuous recycle of activated sludge. A sludge recycle pump was used to pump the settled sludge back to the first section of each train. Each reactor was equipped with a micro-submersible pump that provided complete mixing within the tank. Aeration for all reactors was supplied as compressed air through diffuser tubing in the bottom of the reactors. A large stand was used to support the reactors and all ancillary equipment. This allowed the entire system to be placed on a bench top in a laboratory at an industrial full-scale wastewater treatment plant (WWTP).

Additionally, a DO monitor and control system (consisting of twelve Model 5500-Series DO probes, six Model D53 DO analyzers, and twelve air-blast units) was installed to monitor and maintain DO concentration at each designated level. A pH monitor and control system (consisting of four Walchem WDP300 Series pH/ORP controllers, four pH probes, and four pumps) was also installed to monitor and maintain the pH level in the first-section reactors of four trains.

At the start of the study, bioreactors of the industrial bench-scale system were seeded with activated sludge taken from the final aeration basins of the industrial full-scale WWTP using different dilution ratios (i.e., $\sim 1/8$, $\sim 3/8$, $\sim 5/8$, and $\sim 7/8$). Each of the four trains in the system was operated at an SRT of 2-, 5-, 10-, or 20-days. The operating SRT of each train was controlled by the amount of biosolids wasted daily (calculated from Eq. A-3 (Appendix A) based on daily measured MLVSS concentrations in reactors and each effluent).

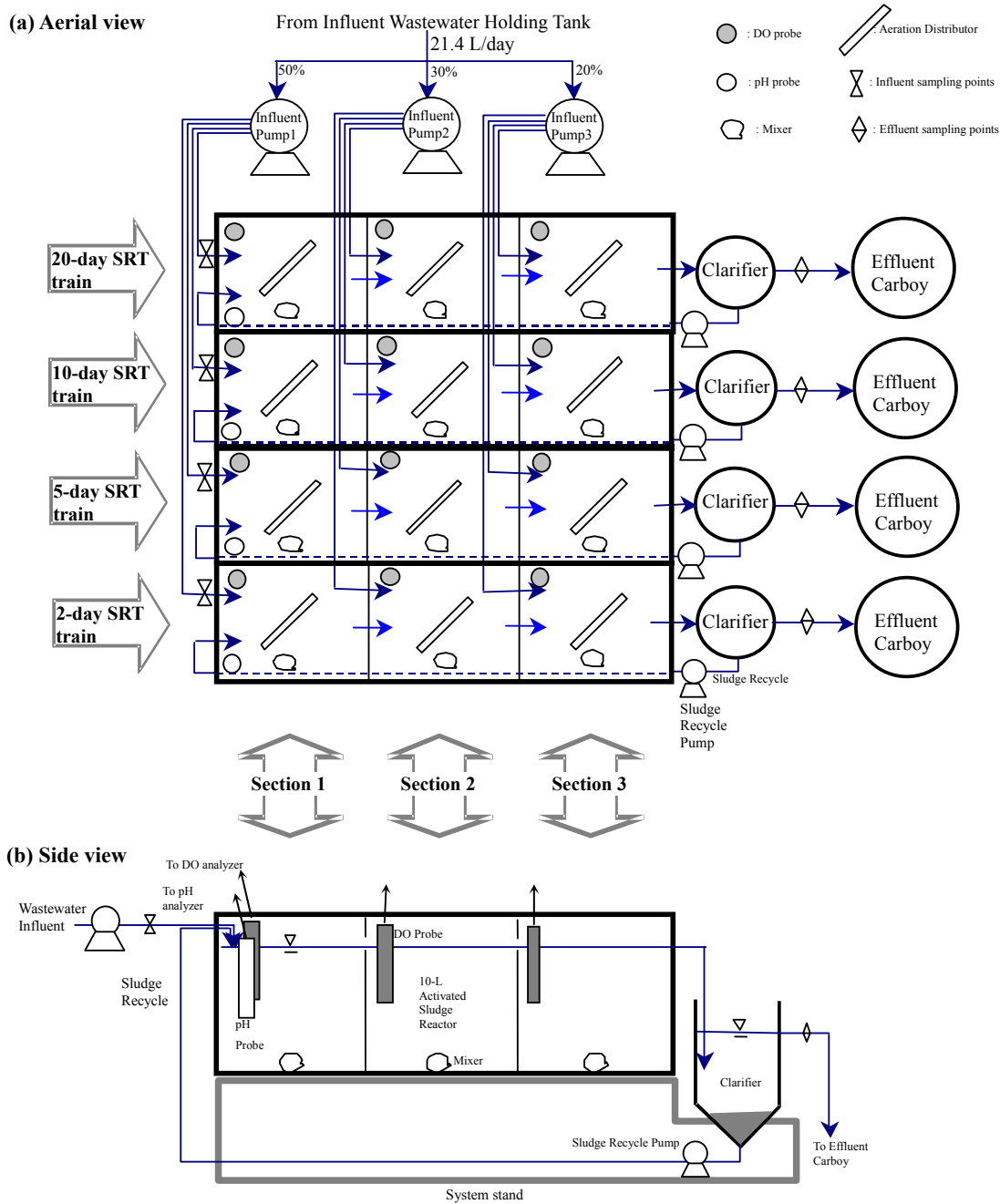


Figure 3-2. Aerial-view (a) and side-view (b) diagrams of the bench-scale activated sludge industrial wastewater treatment system.

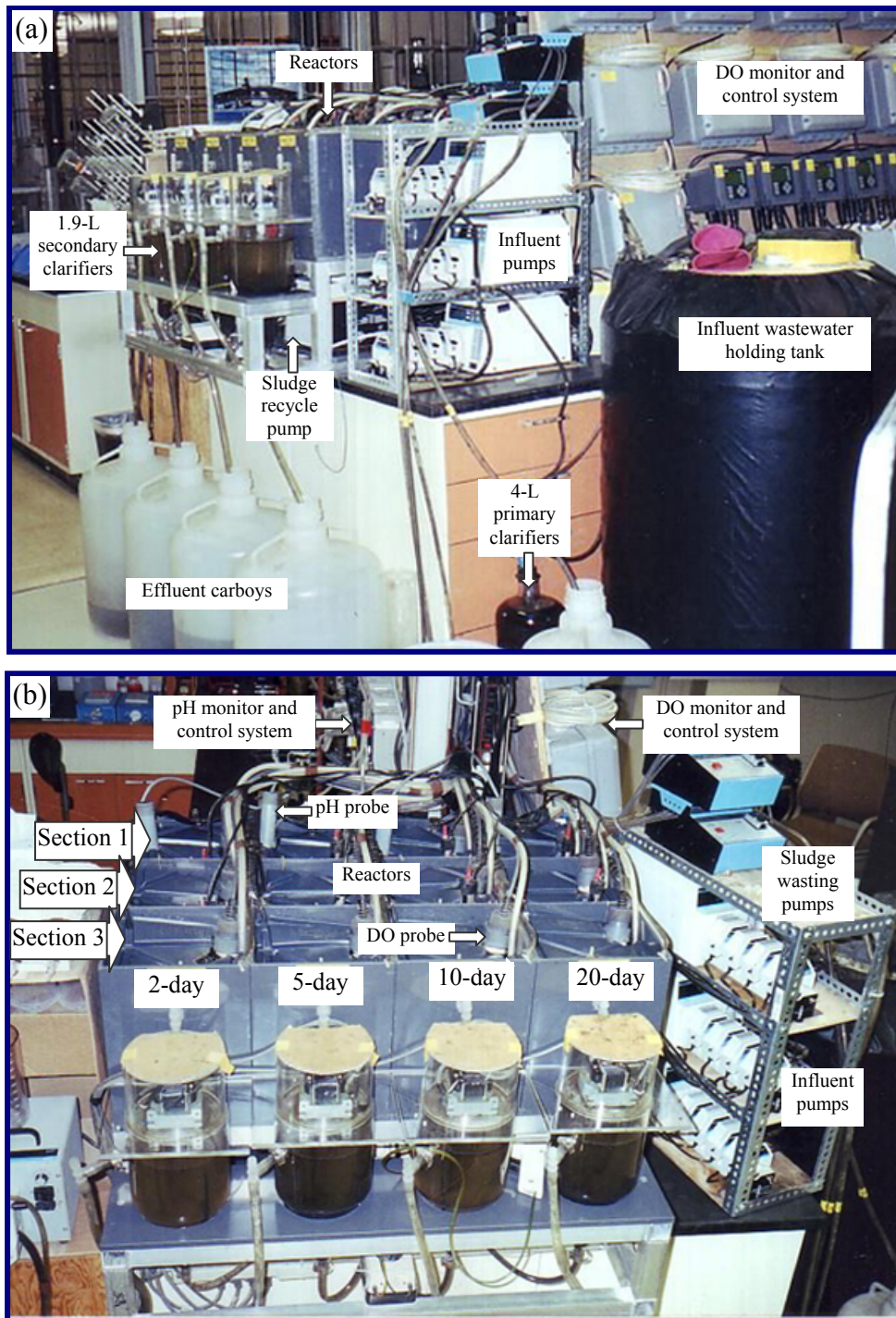


Figure 3-3. The industrial bench-scale system consisting of influent wastewater holding tank, influent pumps, sludge recycle pump, reactors (4 trans $\times$ 3 sections), clarifiers, effluent carboys, and pH and DO monitor and control systems.

The system was operated at an influent flow rate of 21.4 L/day for each SRT train (or $21.4 \text{ L/day/train} \times 4 \text{ trains} = 85.6 \text{ L/day}$ for the entire treatment system). This flow rate yielded a hydraulic retention time (HRT) of 33.6 hours (1.4 days) per train. Approximately 50% (10.7 L/day), 30% (6.4 L/day), and 20% (4.3 L/day) of the total influent flow for each SRT train (21.4 L/day) was pumped to the first-, second-, and third-section reactors, respectively. The sludge recycle rate was 43.2 L/day. Because activated sludge in each reactor was suspended and equipped with a micro-submersible pump, each reactor could be considered a CSTR system. (Criteria for mass balance in CSTR system with sludge recycling ([Section 2.4.2](#)) were implemented to study this experimental system). Under complete mixing conditions, the activated sludge in each train could be recycled approximately 2.15 times per day. Additionally, the system was operated at room temperature (range: 25-30 °C; average: 28.0 ± 0.5 °C). The pH value in the first-section reactor of each train was controlled within a neutral range (7.0-7.2) using sodium hydroxide (NaOH). The average pH value during study was 7.18 ± 0.02 .

The overall study period consisted of three operating phases ([Table 3-1](#)) in which two ammonia loadings (high and low), and two DO levels (3.0 and 0.5 mg/L) were tested in the four SRT trains. The relatively high influent ammonium loading was achieved by adding ammonium chloride (NH₄Cl) as a supplemental nitrogen source in the influent wastewater. The DO level was controlled by the DO monitor and control system. During

Table 3-1. Summary of operation days and conditions for the three operating phases in the industrial bench-scale system.

Phases	Phase 1	Phase 2	Phase 3
Operation days	1 $\Longrightarrow$ 68	68 $\Longrightarrow$ 110	110 $\Longrightarrow$ 152
NH ₄ ⁺ (mg/L)	Low (~18.9)	High* (~54.6)	High* (~52.8)
DO (mg/L)	High (3)	High (3)	Low (0.5)

* Note: The relative high influent ammonium was achieved by adding ammonium chloride (NH₄Cl) as a supplemental nitrogen source in the influent.

phases 1 and 2, the system was operated under the same DO level of 3 mg/L, but the influent ammonium concentration was approximately doubled in phase 2. During phase 3, the influent ammonium concentration was maintained at a high level while the DO level was lowered to 0.5 mg/L.

The influent wastewater for the industrial bench-scale system was taken from an equalization tank at the industrial full-scale WWTP. Fresh wastewater was transported from the sampling point to the laboratory using two 55-gallon (208.2 L) steel drums and was then pumped into a 110-gallon influent drum to feed the experimental system. Each batch of wastewater was sufficient to feed the system for approximately 3~4 days. The influent wastewater was continuously mixed in the drum. Before being pumped into the experimental system, most of solids in the influent were separated in a 4-L clarify to prevent clogging of the influent hoses.

This industrial wastewater had an average chemical oxygen demand (COD) concentration of 996.8 mg/L (information from the industrial full-scale WWTP during the time of the study), and a range of 300-1500 mg/L. It consisted of at least 25 major organic compounds. [Table 3-2](#) lists ten of the 25 major organic compounds whose COD proportions were greater than 1% of total COD in the influent. These ten major organic compounds contribute 95% of total COD in the influent ([Figure 3-4](#)). Five of the 25 major organic compounds were N-containing compounds including triethylamine $[(C_2H_5)_3N]$, N, N-dimethylformamide $[HCON(CH_3)_2]$, acetonitrile $[CH_3CN]$, dimethylamine $[(CH_3)_2NH]$, and aniline $[C_6H_5NH_2]$. These N-containing compounds would represent organic-nitrogen (proportion of TKN) components. If all of this organic-nitrogen was hydrolyzed to ammonia, an average concentration of 3.3 mg NH_3 -N/L would be produced. There was no other major ammonia source in the original wastewater, so ammonia gas was normally dissolved in the influent to the full-scale WWTP to provide a sufficient N-source for heterotrophic metabolism. After adding supplemental ammonia, the average TKN concentration was 21.5 mg/L and the average ammonia concentration was 19.1 mg/L in the influent. Thus, ammonia was the major N-source in the influent wastewater ([Figure 3-5](#)).

Table 3-2. Major organic compounds in the original industrial wastewater used to feed the industrial bench-scale system.

Compounds	Molecule	Concentration (mg/L)	COD (mg/L)	TKN (mg-N/L)
1. Acetic Acid	CH ₃ COOH	332.0	355.2	---
2. Isopropanol	(CH ₃) ₂ CHOH	61.0	146.4	---
3. Acetone	CH ₃ COCH ₃	49.0	108.3	---
4. Methanol	CH ₃ OH	55.0	82.5	---
5. Ethylene Glycol	HOCH ₂ CH ₂ OH	60.0	77.4	---
6. Triethylamine*	(C ₂ H ₅) ₃ N	13.0	45.4	1.8
7. n-Butyric Acid	CH ₃ CHCOOHCH ₃	22.0	40.0	---
8. Ethanol	C ₂ H ₅ OH	14.0	29.3	---
9. Toluene	C ₆ H ₅ CH ₃	9.2	28.8	---
10. Propionic Acid	CH ₃ CH ₂ COOH	13.0	19.6	---
Other C-containing	---	---	47.8	---
Other N-containing	---	---	16.3	1.5
Total	---	---	996.8	3.3

* TKN component.

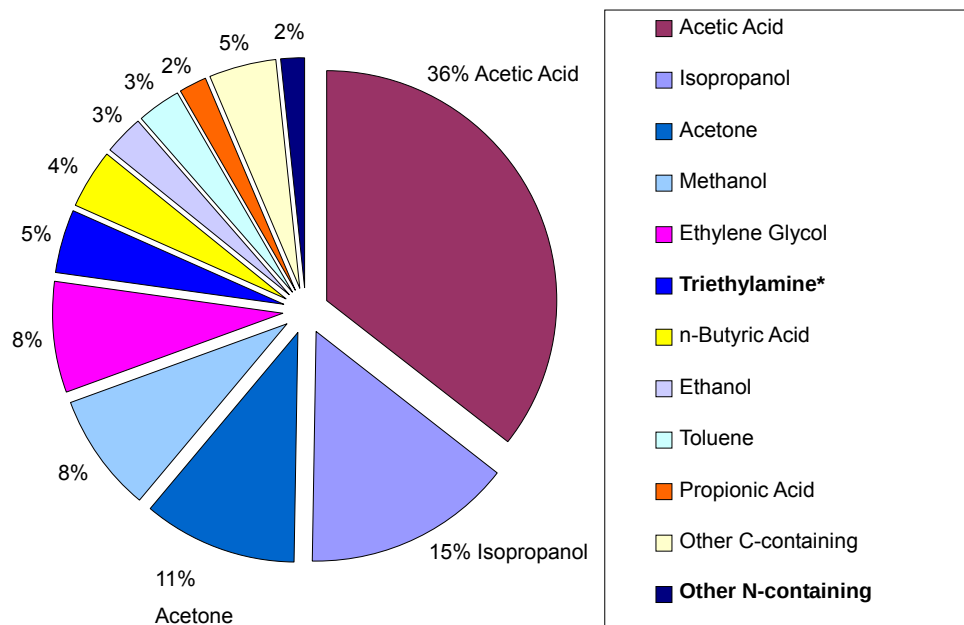


Figure 3-4. COD proportions of major organic compounds in the industrial wastewater. The N-containing compounds (*) contributed approximately 7% of total COD, and about 3.3 mg/L of organic nitrogen.

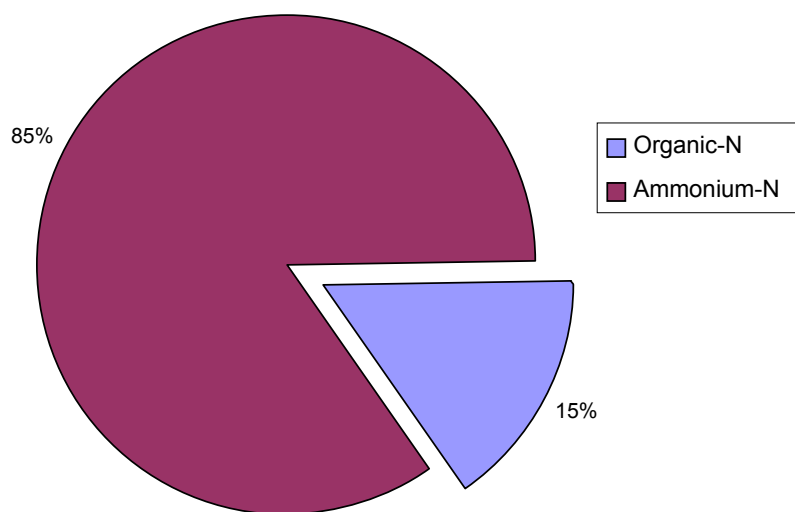


Figure 3-5. Organic-N and ammonium-N proportions of total TKN (organic-N plus ammonium-N) in the industrial wastewater. The average total TKN concentration was 21.5 mg/L of which approximately 85% was ammonium-N.

Influent and effluent samples were taken from each of four SRT trains of the experimental system five days a week and analyzed for chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), ammonia, nitrite, and nitrate ([Section 3.3](#)) as parameters of treatment performance. The influent sampling point was located before the first section of all four SRT trains, and the effluent sampling point was immediately after the clarifier of each SRT train ([Figure 3-2](#)). The influent samples for the four SRT trains were considered to be same and were combined as one influent sample every sampling day. Additionally, biosolids samples were taken from all 12 reactors five days a week to measure mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations for determining the sludge wastage volume needed to control SRT in each of four trains. Extra biosolids samples were taken from the second section of each SRT train for analyzing bacterial populations and physiological activity of AOB ([Sections 3.4, 3.5](#)). The sampling procedures were as follows:

(1) About 200 mL of sample from each of the clarifier effluent ports of the four SRT trains was collected into four 500-mL beakers. The DO, pH, and temperature values were recorded. Additionally, a 100 mL sample of 24-hour composite effluent in each effluent carboy was taken for determining effluent MLSS and MLVSS levels (Note: Effluent carboy samples were completely mixed before these samples were taken).

(2) About 200 mL of influent sample was collected into a 500-mL beaker. Meanwhile, 20 mL of mixed liquor was pipetted from each reactor in the 2- and 5-day trains and 10 mL from each reactor in the 10- and 20-day trains for determining MLSS and MLVSS in system ([Section 3.3.5](#)).

(3) About 2 mL of the effluent samples from step 1, and the influent sample from step 2 were filtered with 0.45 μm glass-fiber filters into 2-mL glass bottles with plastic caps, acidified with 4 μL of 6N sulfuric acid (H_2SO_4) (added into each glass bottle before sample filtration), and stored in a 4 °C refrigerator for later COD analysis ([Section 3.3.4](#)). The COD analyses were conducted approximately every two weeks, when sufficient samples were accumulated for running one batch of COD digestion.

(4) Five milliliters of effluent and influent samples from step 1 and step 2 were

filtered (into 15-mL plastic Falcon™ centrifuge tubes), acidified, and stored in a 4 °C refrigerator prior to TKN analysis ([Section 3.3.1](#)) at a laboratory associated with the full-scale WWTP. A total of ten TKN samples per week (5 samples: 1 influent and 4 effluent per sampling day; 2 sampling days per week) were analyzed.

(5) About 0.5 mL of each sample was filtered (into 1-mL Dionex™ Ion Chromatograph (IC) auto-sample vials), and stored in a 4 °C refrigerator for nitrite and nitrate analyses ([Section 3.3.3](#)).

(6) Ammonia (50 mL) concentration was analyzed on-site every sampling day. Concentrations of MLSS and MLVSS were also measured every sampling day in order to determine the wastage volume for each reactor of each SRT train ([Eq. 3-5](#)).

(7) While waiting for the MLSS samples to be dried, any biofilm that developed on the sidewalls of each reactor was striped off by mechanical brushing. The DO and pH probes were cleaned, and the DO and pH meters were calibrated every two weeks.

(8) Extra biosolids samples (50 mL for the 2-, and 5-day trains and 10 mL for the 10-, and 20-day trains) for genetic and transcript analysis ([Section 3.4](#) and [Section 3.5](#)) were also taken from reactors and immediately stored in a -80 °C freezer before processing.

(9) After wastage volumes were determined, a proportion of mixed liquor was removed directly from each reactor and replaced with same volume of effluent.

(10) Effluent in carboys was discarded, and new influent wastewater was added to the influent holding tank, if necessary.

It is noteworthy that the effluent samples were taken just prior to the biosolids sampling to ensure that the reactors had operated for at least 12 hours without disturbance before sampling.

3.2.2 Bench-scale enriched nitrifying system

The enriched nitrifying system included two 10-L reactors, two mixers (Thermolyne Cimarec® 3), an influent pump motor (Cole-Parmer MasterFlex® L/S™)

with two pump heads (Easy-Load[®] 7518-00), a 20-L influent substrate holding bottle, two 2-L effluent collection flasks, an aeration system and a pH monitor and control system (Figures 3-6). The two nitrifying reactors (Stable reactor and Trial reactor) were operated in parallel to enrich the nitrifying culture for subsequent activity assessment. In order to maintain a control reactor at all time, only the Trial reactor was used for testing. The Stable reactor produced the seed culture for the Trial reactor. The influent pump was used to continually inject substrate into both reactors. The effluent ports were normally closed to allow mixed liquor to accumulate for two days. Approximately 2-L of mixed liquor was withdrawn from each reactor every two days for wasting, replacing or testing purposes. When the Trial reactor was not undergoing tests, wasted mixed liquor from the Stable reactor was used to replace a same amount of mixed liquor in the Trial reactor. Each reactor was completely mixed using a mixer and a magnetic stir bar. Aeration was supplied as compressed air through diffuser tubing in the bottom of the bioreactors. A pH control system (Walchem[™] WDP300 Series) was installed to monitor and maintain pH level in the bioreactors. The entire system was set up on a bench top in a laboratory incubation room with temperature control.

The reactors contained enriched nitrifying bacteria (Hawkins, 2005) and were constantly fed with synthetic wastewater consisting of 107.1 mM NH₃-N (~1,500 mg NH₃-N/L), 1.5 mM K₂HPO₄, 1.5 mM KH₂PO₄, 0.01 mM FeSO₄, 0.016 mM EDTA, 0.5 μM CuSO₄, 0.75 mM MgSO₄, and 0.2 mM CaCl₂. A batch of 16-L fresh wastewater was made for feeding the bioreactors about every two weeks. The synthetic wastewater was autoclaved (at 121°C, 22 psi for 20 minutes) to minimize possible biofilm formation in the substrate holding bottle and heterotrophic bacteria contamination for the enriched nitrifying culture. Biosolids were not recycled in this treatment system. The influent flow rate was approximately 1 L/day. This flow rate yielded a hydraulic retention time (HRT) and a solids retention time (SRT) of approximately 10 days (biosolids were wasted as a 2-L batch every 2 days). The reactors were continually aerated with compressed air at a flow rate of 800 mL/min. The pH in the reactor was controlled at 7.20±0.01 by adding 5% of Na₂CO₃. Temperature for the incubation room was controlled at 30°C.

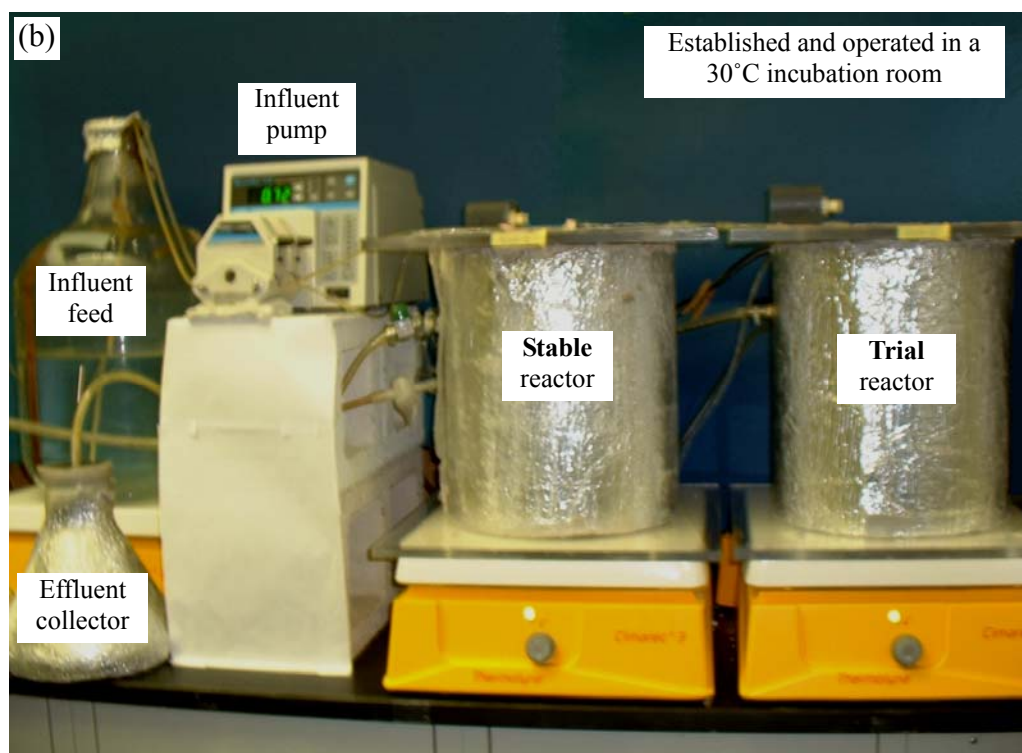
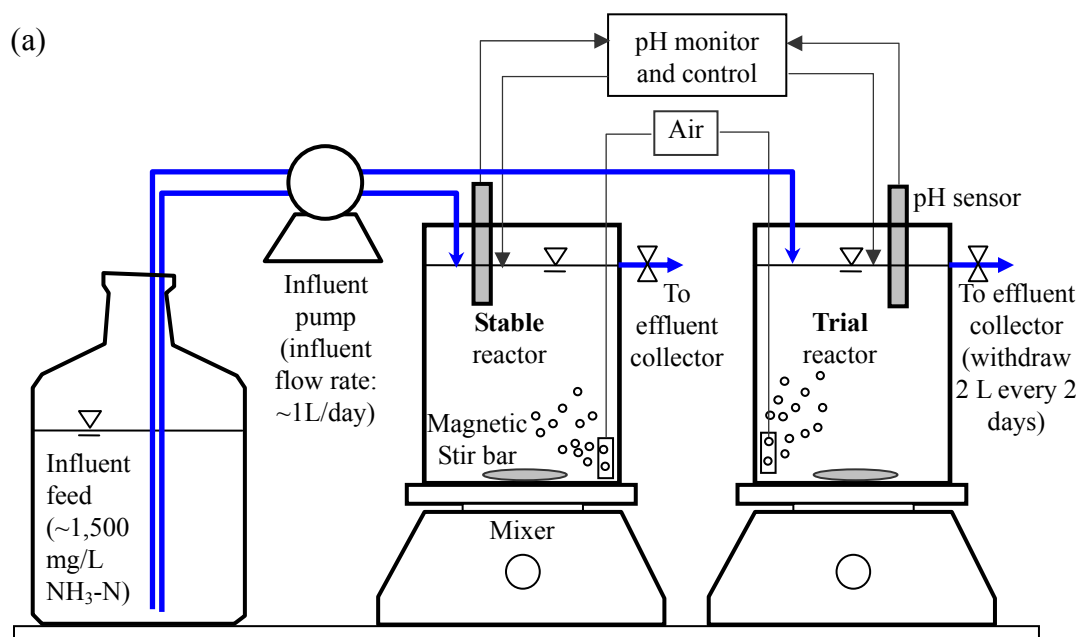
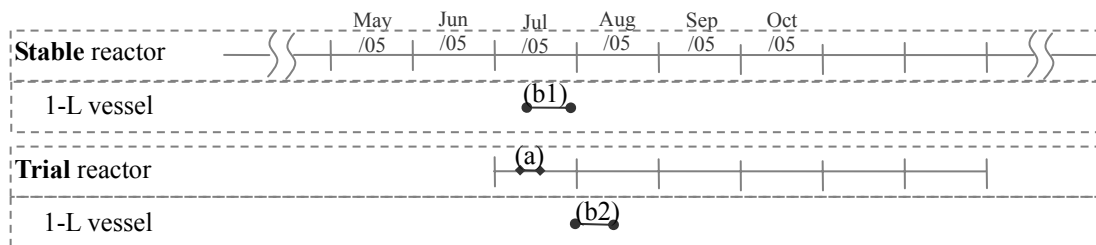


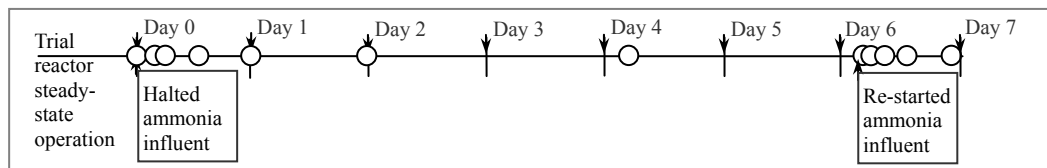
Figure 3-6. Diagram (a), and picture (b) of the enriched nitrifying system consisting of and influent holding tank, influent pump, two nitrifying reactors (Stable and Trial reactors), mixers, and effluent collectors.

Under steady-state operation, the influent and effluent samples were taken from each of two reactors approximately once a month and analyzed for ammonia, nitrite and nitrate concentrations (Section 3.3) to determine system stability with regards to ammonia oxidation. The influent samples were taken directly from the substrate holding bottle, and the effluent samples were taken directly from the reactors. Additionally, biosolids samples were also taken from each of two reactors approximately once a month to measure MLSS and MLVSS concentrations and ensure that the biosolids were maintained at a constant level. Additional biosolids samples were taken to assess the *N. europaea* population based on *amoA* DNA quantification (Section 3.4).

Designated preliminary tests for the Part II study are summarized in Figure 3-7. In order to observe significant *amoA* mRNA responses under different ammonia levels, the *amoA* mRNA-based activity study was purposely designed to start at low *amoA* mRNA levels (close to background). An *amoA* mRNA decay profile was pre-examined under ammonia starvation to devise a culture harvest strategy. The ammonia influent for the



(a) Pre-examined *amoA* mRNA decay profile under ammonia starvation using the Trial reactor



(b1) Pre-examined ammonia oxidation rate for non-starved cultures from the Stable reactor

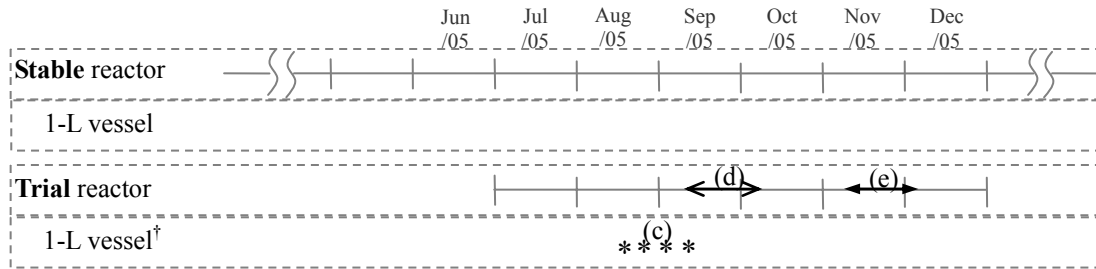
(b2) Pre-examined ammonia oxidation rate for 2-day starved cultures from the Trial reactor

Figure 3-7. Summary of preliminary examinations for *amoA* mRNA decay profile under ammonia starvation, and for ammonia oxidation rate using non-starved or 2-day starved cultures.

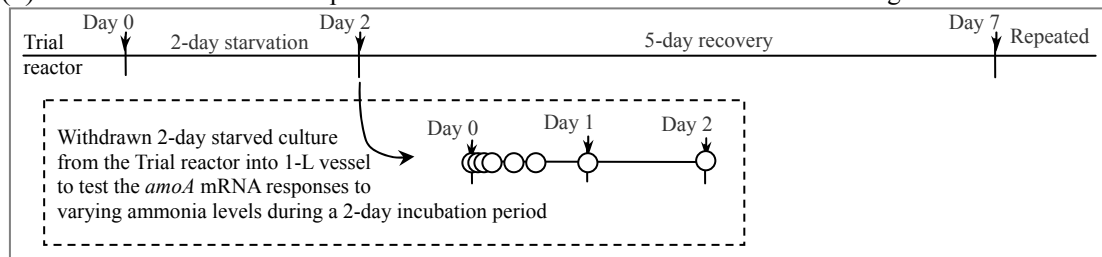
Trial reactor of the enriched nitrifying system was halted for 6.25 days to allow *amoA* mRNA to decline. During this ammonia-starving period, the pH level in the bioreactor was controlled using 1N HCl (at a level close to 7.20). Samples (2-mL) of biosolids suspension were taken at time zero, 0.21, 0.33, 0.50, 1.00, 2.00, 4.25, 6.25, 6.29, 6.38, 6.50, and 6.92 days (Figure 3-7) to analyze the abundance of *amoA* DNA and mRNA for the *N. europaea* group in order to examine the *amoA* mRNA decay profile under ammonia starvation and then to develop a strategy for harvesting low-*amoA*-mRNA-contained nitrifying cultures.

The *amoA* mRNA responses under short-term changes in ammonia loadings and DO levels were assessed using the Trial reactor of the enriched nitrifying system (Figure 3-8). The ammonia loading test was started after a 2-day ammonia starvation (based on previously-derived harvest strategy). Different ammonia loadings were achieved by adjusting influent ammonium concentrations (Figure 3-8). Approximately 1,500; 3,000; and 300 mg/L of ammonia-N were diluted from a 10,000 mg/L ammonia-N stock solution (consisting of 714.3 mM NH₃-N, 10 mM K₂HPO₄, 10 mM KH₂PO₄, 0.067 mM FeSO₄, 0.1067 mM EDTA, 3.3 μM CuSO₄, 5 mM MgSO₄, and 1.3 mM CaCl₂); and then used to feed the bioreactor at a flow rate of 1 L/day (ammonia loadings of approx. 446, 893, and 89 μmol NH₃-N/L/hr). A 5-day starvation experiment (no ammonia feeding) was conducted in between each feeding. During this test, aeration was maintained at a flow rate of ~800 mL/min (DO level around 4.1 mg/L) except for a period between days 25-30. About 4-6 samples were taken from the bioreactor during each 5-day period for chemical and molecular analyses.

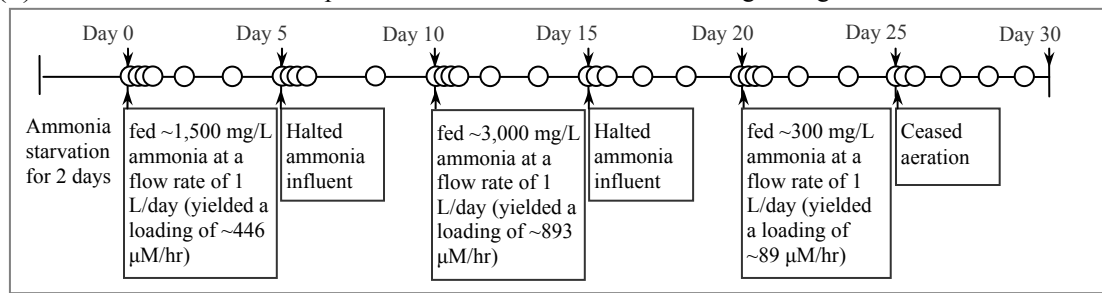
During the DO test, varying DO levels were achieved by controlling the air supply (aeration or non-aeration) while the influent ammonia concentration was held constant at ~1500 mg/L (Figure 3-8). Following normal aeration operation, a 5-day non-aeration experiment was conducted to assess the influence of DO-limitation on ammonia oxidation activity and *amoA* mRNA level in the *N. europaea* group. A total of 18 samples were taken from the bioreactor during the 25-day testing period for chemical and molecular analyses.



(c) Assessed *amoA* mRNA responses under different ammonia concentrations using 1-L vessels



(d) Assessed *amoA* mRNA responses under different ammonia loadings using the Trial reactor



(e) Assessed *amoA* mRNA responses under different DO levels using the Trial reactor

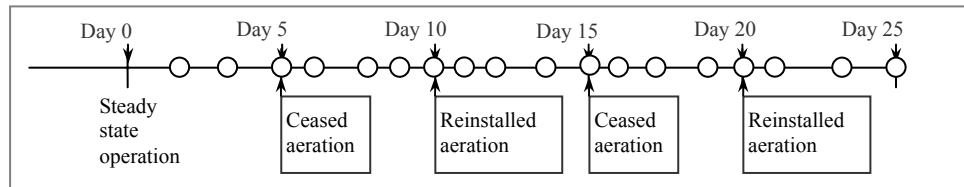


Figure 3-8. Summary of designated tests conducted for Part II study. (Note: the symbol $\dagger$ are tests conducted in 1-L vessels using starved cultures taken from the Trial reactor; and the symbol $\circ$ are sampling points).

3.2.3 Batch culture tests using 1-L vessels

Ammonia oxidation activities (based on the ammonia oxidation rate, [Eq. A-15](#), [Appendix A](#)) and the physiological response of the *N. europaea* group (based on *amoA* mRNA abundance) under varying ammonia concentrations were assessed using a 1-L batch culture taken from the enriched nitrifying system. Tests were conducted using a system consisting of a water bath (BI-TC 2002, BI Integrated recirculating water bath temperature control system for the BI-2000 Electrolytic Respirometer System), two 1-L reactor vessels (BI-RBC-1 reactor vessel for the BI-2000 Electrolytic Respirometer System), two pumps (Cole-Parmer MasterFlex[®] L/S[™], with four pump heads of Easy-Load[®] 7518-00), eight 300-mL BOD bottles (four for ammonia feeds and four for carbonate feeds), two pH monitors, and an air supply system ([Figure 3-9](#)). The water bath was controlled at a temperature of 30°C. The aeration rate was controlled at ~100 mL/min. The water bath was covered with a black box to prevent light influence on ammonia oxidation activity (light inhibits AMO enzyme activity).

In order to more precisely represent the *amoA* mRNA abundance and ammonia oxidation activity of the *N. europaea* group under each designated ammonia level, the ammonia concentration in each batch culture test was intentionally controlled at the target level. In each batch culture test, ammonia was continually fed at a rate equal to the expected ammonia oxidation rate that was pre-estimated using 1-L batch cultures with different initial ammonia concentrations. One liter of non-starved and of 2-day starved cultures were taken from the Stable and Trial reactors, respectively, and incubated for four hours in the 1-L vessels at initial ammonia concentrations of 1, 5, 10, 20, 50 and 100 mg/L (as N). Ammonia concentrations were measured at time zero, 0.5, 1.0, 1.5, 2.0, and 4.0 hours to determine ammonia oxidation rates. The ammonia oxidation rate information was used to devise feeding strategies for controlling stable ammonia and pH levels during the 1-L batch culture tests for the *amoA*-mRNA-based activity study.

The *N. europaea amoA* mRNA responses under different ammonia concentrations were assessed using one liter of 2-day starved cultures taken from the Trial reactor of the enriched nitrifying system. Tests were conducted for two days under a constant pH level

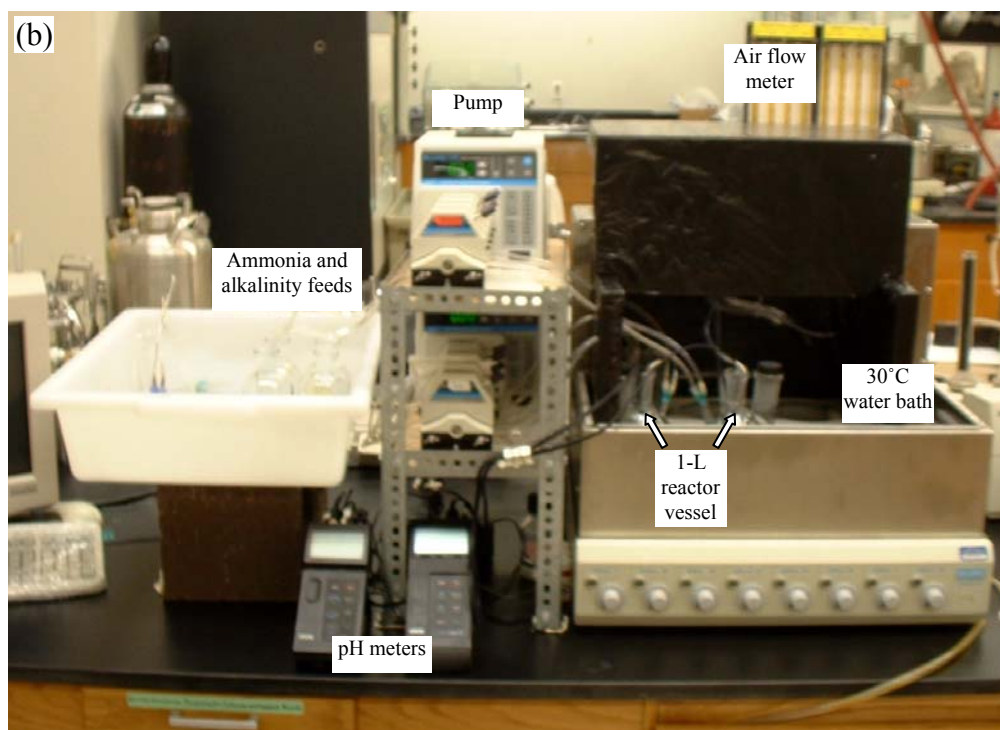
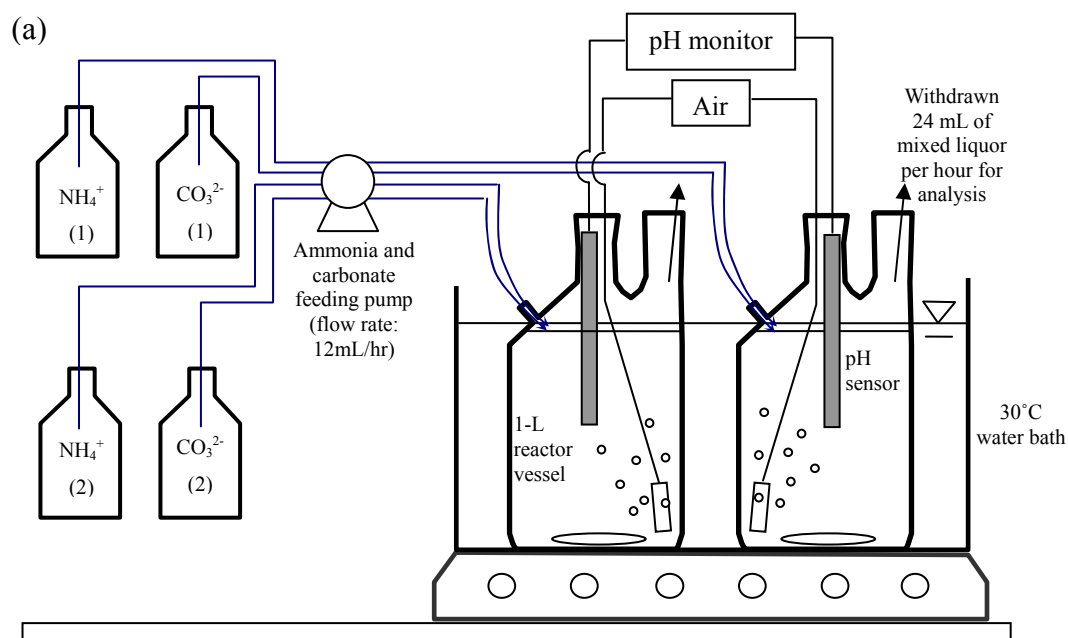


Figure 3-9. Diagram (a), and picture (b) of the system used for continuous feed 1-L batch culture tests. The system primarily consisted of a temperature control water bath, two 1-L vessels, two pH meters, and aeration system, and ammonia and carbonate feeding systems.

(ca. 7.2) and ammonia concentrations of 0 (control), 1, 5, 10, 20, 50 and 100 mg/L. Two designated ammonia levels were tested each time (Figure 3-7). Ammonia concentrations were maintained based on the feeding strategies (previously derived from the ammonia oxidation rate information) (Tables 3-3, 3-4). Constant pH level was controlled by adding carbonate at a rate just equal to the rate at which alkalinity was theoretically consumed. Because the tests were started with a 2-day starved culture (having a lower ammonia oxidation rate than the non-starved culture), feeding strategies based on the ammonia oxidation rates determined for the 2-day starved cultures (AOR_{starved}) (Table 3-3) were used for the first two hours of incubation. During 2-48 hours of incubation, feeding strategies (Table 3-4) were based on the ammonia oxidation rates determined by non-starved cultures ($AOR_{\text{non-starved}}$). The ammonia feeds were diluted from a 10,000 mg/L $\text{NH}_3\text{-N}$ stock solution (consisting of 714.3 mM $\text{NH}_3\text{-N}$, 10 mM K_2HPO_4 , 10 mM KH_2PO_4 , 0.067 mM FeSO_4 , 0.1067 mM EDTA, 3.3 μM CuSO_4 , 5 mM MgSO_4 , and 1.3 mM CaCl_2). The carbonate feeds were diluted from a 10,000 mg CO_3^{2-} /L stock solution. The feeding rate for ammonia and carbonate was set at 12 mL/hr.

Tests were started by adding 0, 0.1, 0.5, 1, 2, 5, and 10 mL of the 10,000 mg/L $\text{NH}_3\text{-N}$ stock solution into 1-L of 2-day starved cultures to yield target ammonia concentrations of 0, 1, 5, 10, 20, 50, and 100 mg $\text{NH}_3\text{-N}$ /L, respectively. Mixed liquor samples were taken directly from the vessels for both chemical and molecular analyses. Biosolids samples (4 mL) were taken from the 1-L cultures at time zero, 1, 2, 4, 8, 12, 24, and 48 hours to analyze the abundance of *N. europaea amoA* DNA and RNA. Additionally, 24 mL of mixed liquor was taken from the 1-L vessel every hour (the mixed liquor volume increased by 24 mL per hour because ammonia and carbonate were fed at the same rate of 12 mL/hr). The mixed liquor sample was centrifuged at 3500 rpm for 2 minutes using a 50-mL plastic FalconTM centrifuge tube. Twenty mL of the supernatant fraction was pipetted into a 100-mL beaker for ammonia analysis, and settled biosolids were recycled into the 1-L batch culture (when biosolids were sampled at the above-mentioned sampling times). When biosolids were not sampled, concentrated biosolids were wasted according to a factor of 0.11 mL/mg NH_3 oxidized (based on the enriched

Table 3-3. Ammonia and carbonate feeding strategies utilized to maintain constant ammonia and pH levels for the first 2-hr of incubation under different ammonia testing concentrations.

Designed ammonia concentration (mg/L) for tests	AOR _{starved} mg NH ₃ -N/L/hr (μmol/L/hr)	Ammonia (in mg/mL) addition required at a flow rate of 12 mL/hr	Carbonate (in mg/mL) addition required at a flow rate of 12 mL/hr
0	0.00 (0.0)	0.0000	0.0000
1	0.83 (59.3)	0.0692	0.2317
5	3.44 (245.7)	0.2867	0.9603
10	5.74 (410.0)	0.4783	1.6024
20	8.46 (604.3)	0.7050	2.3618
50	11.09 (792.1)	0.9242	3.0960
100	14.20 (1014.3)	1.1833	3.9642

Table 3-4. Ammonia and carbonate feeding strategies utilized to maintain constant ammonia and pH levels during incubation between 2~48 hrs under different ammonia testing concentrations.

Designed ammonia concentration (mg/L) for tests	AOR _{non-starved} mg NH ₃ -N/L/hr (μmol/L/hr)	Ammonia (in mg/mL) addition required at a flow rate of 12 mL/hr	Carbonate (in mg/mL) addition required at a flow rate of 12 mL/hr
0	0.00 (0.0)	0.0000	0.0000
1	1.03 (73.6)	0.0858	0.2875
5	4.39 (313.6)	0.3662	1.2267
10	7.41 (529.3)	0.6173	2.0681
20	11.27 (805.0)	0.9395	3.1473
50	16.41 (1172.1)	1.3678	4.5822
100	19.36 (1382.9)	1.6129	5.4033

nitrifying system operational condition that 1,000 mL biosolids were wasted when ~1,500 mg ammonia-N were oxidized every day and a ratio of 1:6 when 24 mL of mixed liquor was concentrated into a 4 mL concentrate after centrifugation and sampling for ammonia analysis). Based on real-time monitored pH values and hourly measured ammonia concentrations, fine-scale adjustments on pH and ammonia levels in the mixed liquor were achieved by adding small volumes of 10,000 mg/L CO_3^{2-} and 10,000 mg/L $\text{NH}_3\text{-N}$ stock solutions when ammonia and pH values were lower than desired levels (e.g., add 0.1 mL of 10,000 mg/L ammonia stock solution would increase ammonia concentration in the mixed liquor by 1 mg/L). In contrast, if the ammonia and pH values were higher than designated levels, the ammonia and carbonate feeds were halted briefly to allow levels drop back down. The fine-scale adjustments on ammonia concentrations were taken into account when ammonia oxidation rates were determined.

3.2.4 Biosolids samples from the industrial full-scale WWTP

The biosolids samples taken from the bench-scale industrial wastewater treatment system were stored at -80°C for approximately 3 - 4 years prior to *amoA* mRNA measurements. Target *amoA* mRNA degradation during long-term storage at -80°C was examined using fresh biosolids samples taken from the industrial full-scale WWTP. A volume of mixed liquor (500 mL) was collected from the aeration tank of the industrial full-scale WWTP on March 11, 2005 (day 0). This 500-mL sample was continually blended with a mixer while thirty 10-mL aliquots (numbered from 1 through 30) were pipetted into 15-mL plastic Falcon[™] centrifuge tubes. The prepared aliquots were immediately frozen at -80°C for about thirty minutes, and then transported on ice to another -80°C freezer located at University of Tennessee.

Six samples (#1 - #6) were analyzed for RI-27 and *N. nitrosa amoA* mRNA and DNA on day 18 as initial target levels. Afterward, 2 samples were analyzed for target mRNA and DNA on days 45, 75, 101, 129, 157, 185, 213, 228, 269, and 333, respectively. The RI-27 and *N. nitrosa amoA* mRNA and DNA concentrations were plotted over storage times to examine decay of the target nucleic acids.

3.3 Chemical analyses

The chemical analysis parameters for each sample collected from the two bench scale systems and 1-L batch culture tests are summarized in [Table 3-5](#). Parameters measured included total Kjeldahl nitrogen (TKN), ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), Chemical oxygen demand (COD), mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MLVSS).

3.3.1 Total Kjeldahl nitrogen (TKN) analysis

Analysis of influent and effluent samples for TKN concentration was performed on the industrial samples in an on-site laboratory at the full-scale WWTP according to Standard Method 4500-N_{org} D, the Macro-Kjeldahl Method ([APHA, 1998](#)).

3.3.2 Ammonia analysis

Analysis for ammonia concentration was performed according to Standard Method 4500-NH₃ D, Ammonia-Selective Electrode Method, using an Orion Model 95-12 ammonium-selective probe as suggested by the method with a minor modification in sample volume ([APHA, 1998](#)). Four 50 mL standard solutions (with concentrations of 0.1, 1, 10, and 100 mg/L ammonia-N, respectively) were prepared in 100-ml plastic beakers to calibrate the ammonia-selective probe (the calibration was started with the lowest concentration). One mL of Orion pH Adjusting Solution was added to each standard just before measurement. In order to minimize volume changes during the 1-L batch culture tests (for Part II study), the volume of sample and standard solutions was adjusted to 20 mL, and 0.4 mL of pH Adjusting Solution was added right before measurement. The beaker was then placed on a small magnetic stir plate and the probe placed into the fluid. When the meter readings (in millivolt, mV) became stable, values were recorded. When all readings for standard solutions were recorded, a standard curve was plotted. The same measuring method was employed for the samples. The samples and standards were held at the same temperature to ensure accurate measurements. After all readings for samples were recorded, the ammonia concentration of each sample was calculated using the standard curve.

Table 3-5. Chemical analysis items for samples taken from the bench-scale systems.

Analysis parameters	Samples			Method
	1. Influent	2. Biosolid	3. Effluent	
1. TKN*	I		I	4500-N _{org} D. †
2. NH ₄ ⁺	I, E, B		I,E,B	4500-NH ₃ D. †
3. NO ₂ ⁻	I, E		I,E	4110 B. †
4. NO ₃ ⁻	I, E		I,E	4110 B. †
5. COD	I		I	5220 D. †
6. MLSS		I, E	I	2540 D. †
7. MLVSS		I, E	I	2540 E. †
8. N-content*		I		EPA Method 365.4‡

NOTE: I: analysis performed on the industrial bench-scale system; E: analysis performed on enriched nitrifying system; B: analysis performed during the 1-L batch culture tests; † are analysis methods suggested in the Standard Methods for the Examination of Water and Wastewater, 20th edition (APHA, 1998); ‡ is an automated TKN analysis method suggested by EPA (Methods for the Chemical Analysis of Water and Wastes, EPA/600/4-79-020, March, 1983); and * is the analysis performed in an on-site laboratory at the industrial full-scale WWTP.

3.3.3 Nitrite, and nitrate analyses

Analyses of aqueous samples for the anions nitrite-N and nitrate-N were performed in accordance with Standard Method 4110 B, Ion Chromatography (IC) with Chemical Suppression of Eluent Conductivity using a Dionex™ DX 500 Chromatograph system with an IonPac® AS9-HC 4mm anion exchange column (APHA, 1998). For each IC run, four standards (not including a blank) and one quality control sample were prepared containing varying concentrations of both anions being analyzed in order to calibrate the IC and to ensure accurate measurements. The standard solutions consisted of both nitrite and nitrate at concentrations of 0.1, 1, 10, and 100 mg/L as N. The vials with either 0.5 mL of standard or sample were placed into the IC's auto sampler and the analysis sequence initiated. The IC system automatically plotted a calibration curve based on the standards and give concentrations for each anion in mg/L. The nitrite and nitrate concentrations were obtained from the calibration curves.

3.3.4 Chemical oxygen demand (COD) analysis

COD analysis of influent and effluent samples from the industrial bench-scale system for soluble COD was performed in accordance with Standard Method 5220 D, Closed Reflux Colorimetric Method using High-Range Hach[®] COD Test kit (Hach, Inc., Loveland, CO) (APHA, 1998). Five standards (not including a blank) were prepared for COD analysis using dried potassium hydrogen phthalate (KHP) to create a standard adsorption curve using concentrations of 200, 400, 600, 800, and 1,000 mg/L. For each test, 2 mLs of standard or sample were placed into a Hach vial (containing reagents for COD digesting: 0.1-1.0 % (w/v) chromic acid, and 80-90 % (w/v) sulfuric acid), shaken, and digested on a heating block (Hach COD reactor, Hach, Inc., Loveland, CO) for 2 hours. After cooling, the Hach vial with digested sample was analyzed using a spectrophotometer (Perkin-Elmer Lambda 3B[™] UV/VIS Spectrophotometer). A standard curve was plotted and the COD values of samples (mg/L) was then obtained.

3.3.5 MLSS and MLVSS analyses

Analysis of mixed liquor suspended solids (MLSS) was performed in accordance with Standard Method 2540 D, Total Suspended Solids dried at 103-105°C using Environmental Express 1.5µm x 47mm ProWeigh[®] glass fiber filters and a laboratory 105°C oven (APHA, 1998). All filters were pre-dried and weighed (the measured weight was defined as W_1) before use. The pre-dried and weighted filters were pre-wetted with deionized (DI) water just prior to use. For the industrial bench-scale system, four of the 24-hour composite effluent samples and twelve of the mixed liquor samples from the reactors were gently poured through the filters and washed down with DI water (one filter was used for one sample). For the enriched nitrifying system, one sample from each reactor was analyzed. The filters were then placed in aluminum dishes and dried in the 105°C oven for one hour. The filters were removed from the oven and re-weighed (this weight was defined as W_2). The values were recorded and used in the following equation to determine the MLSS and effluent suspended solids (ESS) in mg/L:

$$MLSS(or\ ESS) = \frac{(W_2 - W_1)}{V} \quad (Eq.3-1)$$

Where: W_2 = final filter weight (mg); W_1 = initial filter weight (mg); and V = sample volume (L).

Analysis of mixed liquor volatile suspended solids (MLVSS) was performed in accordance with Standard Method 2540 E, Fixed and Volatile Solids Ignited at 550°C using the dried MLSS filters and a laboratory 550°C muffle furnace (APHA, 1998). After the MLSS values were recorded, the filters were placed in aluminum dishes and ignited in the muffle furnace for no less than 15 minutes to combust the volatile portion of the sample. The filters were then removed from the oven, cooled and re-weighed (this weight was defined as W_3). These values were recorded and then used in the following equation to calculate the MLVSS and effluent volatile suspended solids (EVSS) in mg/L:

$$MLVSS(or\ EVSS) = \frac{(W_2 - W_3)}{V} \quad (\text{Eq.3-2})$$

Where: W_2 = final filter weight from MLSS analysis (mg); W_3 = final filter weight after MLVSS analysis (mg); and V = sample volume (L).

3.4 Molecular methods for DNA analysis

3.4.1 DNA extraction

Genomic DNA was extracted from biosolids samples collected from both the industrial bench-scale system and the enriched nitrifying system using a FastDNA[®] kit (Qbiogene Inc, Morgan Irvine, CA) with minor modifications. These modifications included washing the binding matrix-DNA complex twice with 80% (v/v) ethanol after the recommended salt-ethanol wash step and eluting the DNA in 200 μ L of 10 mM Tris-HCl buffer (pH 8.0) (Dionisi et al., 2002). The integrity of extracted DNA was analyzed by electrophoresis using 0.7% (w/v) agarose gel (Agarose Low EEO Electrophoresis Grade, Fisher Scientific, Pittsburgh, PA), 1X TBE, and 1X GelStar nucleic acid gel stain (FMC Corporation, Rockland, ME). The quantity of extracted DNA was measured by a fluorometer (Hoefer[™] DyNA Quant[™] 200, Amersham Biosciences, Piscataway, NJ). DNA extracts were immediately stored at -80°C for further quantification.

3.4.2 Creating *amoA* gene libraries, and analyzing sequence data

A section of the *amoA* gene in genomic DNA extracted from biosolids samples was amplified by PCR using a forward primer *amoA*-1F (GGGGTTTCTACTGGTGGT) and a reverse primer *amoA*-2R (CCCCTCKGSAAAGCCTTCTTC, K = G or T; S = G or C) (Rotthauwe et al., 1997) to target AOB genomic stretches corresponding to positions 332-349, and 802-822, respectively of the *amoA* gene sequence of *Nitrosomonas europaea* (Figure 3-10). Briefly, each PCR reaction mixture (total volume of 25 μ L) consisted of one puReTaq[™] Ready-To-Go[™] PCR bead (Amersham Biosciences, Piscataway, NJ), 23.0 μ L of nucleic acid-free HPLC grade water, 0.5 μ L of each primer (final concentrations were 400 nM), and 1.0 μ L sample template (final concentrations were approximately 2.0~2.5 ng/ μ L) (Dionisi et al., 2002). The PCR program was as follow: 5 min at 94°C, followed by 50 cycles consisting of 30 sec at 94°C, 60 sec at 60°C, and 30 sec at 72°C and a final cycle consisting of 7 min at 72°C.

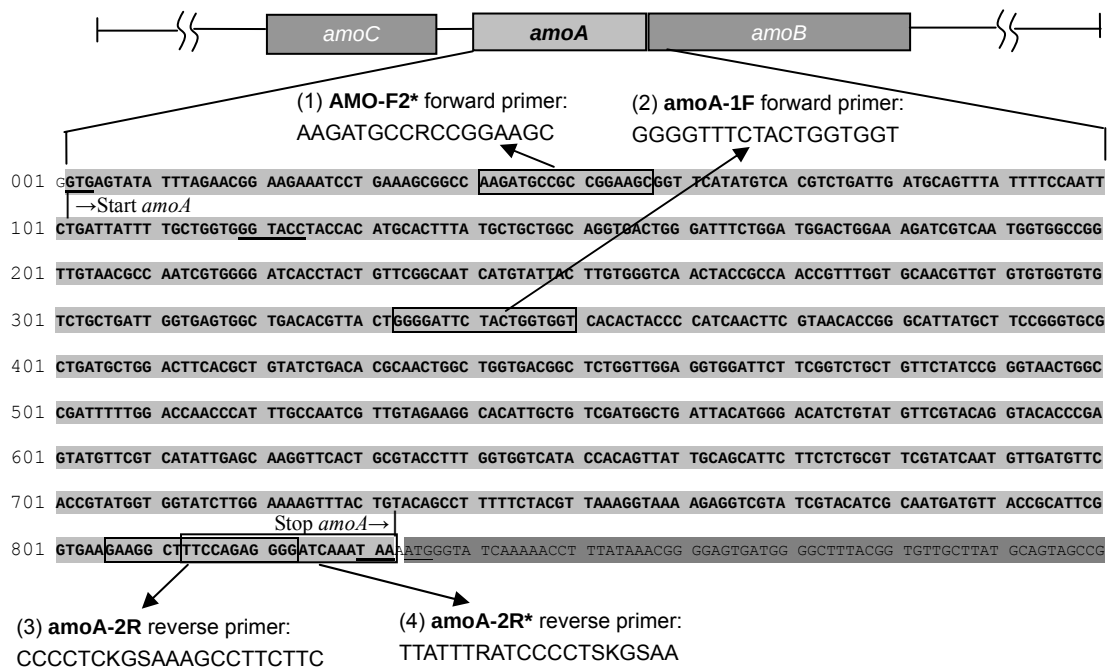


Figure 3-10. The positions of PCR primers used to amplify a segment of *amoA* gene corresponding to the positions of the *amoA* gene of *N. europaea*.

Additionally, a section of the *amoA* gene in extracted DNA (for the 2- and 5-day SRT trains of the industrial bench-scale system and the enriched nitrifying system biosolids samples) were amplified using a nested-PCR. In the first PCR amplification, a primer set AMO-F2* (AAGATGCCRCCGGAAGC)/amoA-2R* (TTATTTRATCCCCTS KGSAA, R = A or G) was first used to amplify an *amoA* segment of approximately 792 bp from extracted genomic DNA. PCR amplification was performed in 25µL (total volume) mixtures using Ready-To-Go™ PCR beads (Amersham Biosciences, Piscataway, N.J.). The PCR program was 5 min at 95°C, followed by 40 cycles consisting of 30 sec at 95°C, 45 sec at 58°C, and 30 sec at 72°C and a final cycle consisting of 10 min at 72°C. Sequentially, the previous PCR amplified products were further amplified using the primer set amoA-1F/amoA-2R* to target AOB genomic stretches corresponding to positions 332-349 and 812- 831 respectively of the *amoA* gene sequence of *Nitrosomonas europaea* (Figure 3-10). PCR amplification was also performed in 25µL (total volume) mixtures using Ready-To-Go™ PCR beads. The PCR program was as used in the first step. The PCR products were checked with gel electrophoresis using 1.5% (w/v) or 2.0% (w/v) agarose gel to make sure target gene had been amplified.

The PCR products (amplified target gene) were cloned into the plasmid vector (pCR®4-TOPO®) (Figure 3-11) by one-shot chemical transformation as described in the manufacturer instructions (TOPO TA Cloning® Kit for Sequencing, Invitrogen, Carlsbad, CA). Clones with target inserts were determined by PCR screening as described in the same instructions (Option: PCR products could further digest with restriction enzyme *ClaI* to determine possible sequence affinity). Plasmid DNA of selected clones with the target *amoA* gene was isolated with a DNA purification kit (Wizard® Plus SV Minipreps DNA Purification System, Promega, Madison, WI). The purified plasmid DNA was rechecked by restriction analysis (digesting with *EcoRI*) (Option: Digested with *EcoRI* + *ClaI* to determine possible sequence affinity). The purified plasmid DNA with the target gene of interest was sequenced at the Molecular Biology Resource Facility of the University of Tennessee (Knoxville) with an Applied Biosystems 373™ DNA sequencer (Perkin-Elmer, Foster City, CA) equipped with an ABI PRISM dye terminator cycle sequencing kit, AmpliTaq® DNA polymerase, and M13f or M13r primers.

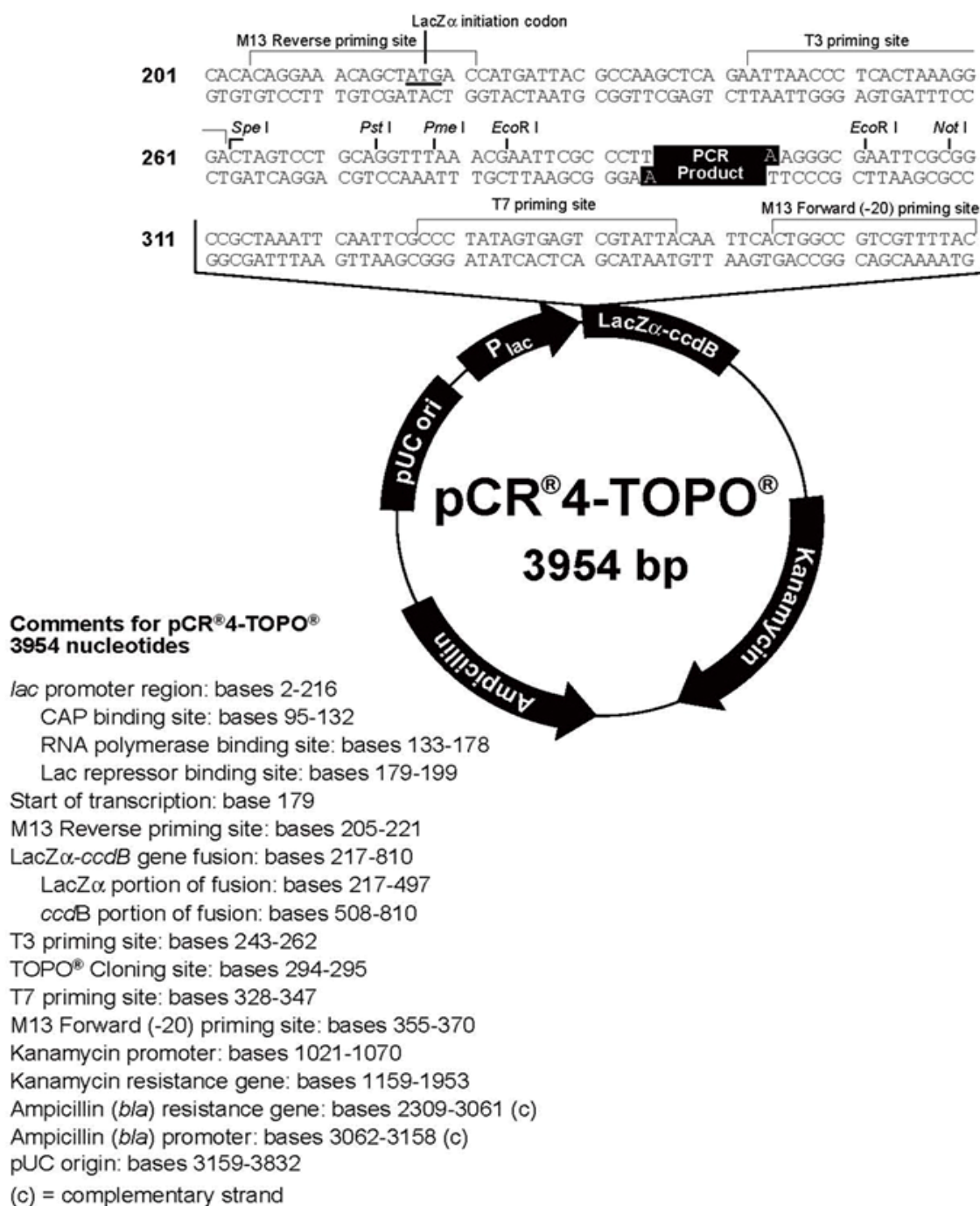


Figure 3-11. Map of pCR[®]4-TOPO[®] plasmid vector (Invitrogen, Carlsbad, CA) for carrying *amoA* gene library in this study.

The *amoA* sequences were compared with sequences in publicly accessible databases by using the program Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The CLUSTAL X (Thompson et al., 1997) software was used to align sequences from both samples and published databases. Phylogenetic trees were constructed in CLUSTAL X and visualized in TreeView® (Page, 1996).

3.4.3 Primers and probes designed

The primer pair and probe specific for total eubacterial 16S rDNA and AOB 16S rDNA were based on previously works (Dionisi et al., 2002; Harms et al., 2003) (Table 3-6). The primer set and probe for quantifying *Nitrosomonas nitrosa amoA* were based on an original design (Dionisi et al., 2002). These primers and probes were synthesized by Sigma Genosys (Sigma Genosys, The Woodlands, TX).

A forward primer amoA-[RI-27]-542f (5'>CATTGTTATCGATGGCTGACTATA>3'), a reverse primer amoA-[RI-27]-679r (5'>ACGCTGAGAAGAATGCTGCAAT>3'), and a TaqMan® probe amoA-[RI-27]-bhq-651r (5'>6-FAM-TGTATGACCACCGAACG TACGCAGTGAG-BHQ-1>3') (Table 3-6) were deduced from a fragment of *amoA* sequences aligned with the CLUSTAL X (Thompson et al., 1997), according to the guidelines provided by Applied Biosystems (<http://home.appliedbiosystems.com>; Foster City, CA). The amoA-RI-27 primer pair and probe were specific for one dominant AOB species in the industrial bench-scale system that had 99% similarity to uncultured AOB RI-27 (GenBank accession number AF532311) (Gieseke et al., 2001).

A forward primer amoA-[B2-3]-596f (5'>CGGAATACGTRAGATTGATTGA>3'), a reverse primer amoA-[B2-3]-744r (5'>GAAGAAMGCGGTGCAATAGA>3'), and a TaqMan® probe amoA-[B2-3]-bhq-713r (5'>6-FAM-TACCACCATACGCAGWACATC AGCAT-BHQ-1>3') (Table 3-6) were also deduced from a fragment of *amoA* sequences aligned with the CLUSTAL X (Thompson et al., 1997), according to the guidelines provided by Applied Biosystems (<http://home.appliedbiosystems.com>; Foster City, CA). The amoA-B2-3 primer pair and probe were specific for another dominant AOB species in the industrial bench-scale system that had 99% similarity to uncultured AOB B2-3 (GenBank accession number AF293067) (Gieseke et al., 2001).

Table 3-6. Target molecules, the associated PCR primers, and fluorescent probes used for the detection of total eubacteria 16S rDNA, AOB 16S rDNA, and AOB *amoA* DNA (specific for *N. nitrosa*, RI-27, B2-3, and *N. europaea* groups).

Target molecule	Forward and reverse primers	TaqMan [®] probes
Eubacteria	1055f:	16S-Taq1115:
16S rDNA	5'>ATGGCTGTCGTCAGCT>3'	5'>6-FAM-CAACGAGCGCAACCC-TAMRA>3'
	1392r:	
	5'>ACGGGCGGTGTGTAC>3'	
AOB	CTO 189f A/B:	TMP1:
16S rDNA	5'>GGAGRAAAGCAGGGGATCG>3'	5'>6-FAM-CAACTAGCTAATCAGRCATCRGCCGCTC-TAMRA>3'
	RT1r:	
	5'>CGTCCTCTCAGACCARCTACTG>3'	
<i>N. nitrosa</i>	amoA-Nn-548f (amonit1-f):	amoA-Nn-Taq-740r (amonit1-p):
<i>amoA</i>	5'>TTTCAATGGCAGACTACA>3'	5'>6-FAM-AATGCTGTACAGTAACTTTACCGAG-TAMRA>3'
(original)*	amoA-Nn-777r (amonit1-r):	
	5'>TTTAACAATACGGCCTCT>3'	
<i>N. nitrosa</i>	amoA-Nn-542f (nitrosa-f):	amoA-Nn-bhq-648r (amoNnTaq3r):
<i>amoA</i>	5'>TATTGCTTTCAATGGCAGACTACA>3'	5'>6-FAM-ATGGCCGCCAAAGGTACGCAGTGAA-BHQ-1>3'
(modified)†	amoA-Nn-676r (nitrosa-r):	
	5'>CCGCAAAGAACGCAGCAATC>3'	
RI-27	amoA-[RI-27]-542f:	amoA-[RI-27]-bhq-651r:
<i>amoA</i>	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:
<i>amoA</i>	5'>CGGAATACGTRAGATTGATTGA>3'	5'>6-FAM-TACCACCATACGCAGWACATCAGCAT-BHQ-1>3'
	amoA-[B2-3]-744r:	
	5'>GAAGAAMGCGGTGCAATAGA>3'	
<i>N. europaea</i>	amoA-Ne-590f:	amoA-Ne-bhq-720r:
<i>amoA</i>	5'>GTACACCTGAGTATGTWCGTC>3'	5'>6-FAM-TCCAAGATACCACCATACGTGAACATCAACA-BHQ-1>3'
	amoA-Ne-751r:	
	5'>TAACGTAGAAAAGGCTGTACAGT>3'	

* In this study, the primers and probe originally designed by [Dionisi et al., 2002](#) were used to quantify *Nitrosomonas nitrosa* population in the industrial bench-scale system; † A modified *N. nitrosa* primer/probe set ([Layton et al., 2005](#)).

Additionally, a forward primer amoA-Ne-590f (5'>GTACACCTGAGTATGTWC GTC>3'), a reverse primer amoA-Ne-751r (5'>TAACGTAGAAAAAGGCTGTACAGT >3'), and a TaqMan[®] probe amoA-Ne-bhq-720r (5'>6-FAM-TCCAAGATACCACCATA CYGTGAACATCAACA-BHQ-1>3') were deduced from a fragment of *amoA* sequences aligned with the CLUSTAL X (Thompson et al., 1997), according to the guidelines provided by Applied Biosystems (<http://home.appliedbiosystems.com>; Foster City, CA). This primer pair and probe were specific for a dominant AOB group in the enriched nitrifying system that had 93% similarity to *Nitrosomonas europaea* (GenBank accession number AF058691) or uncultured AOB isolate ENI-11 (GenBank accession number AB179055.1).

The primers and probes designed in this study were synthesized by Biosearch Technologies, Inc. (Novato, CA). Upon receipt, synthesized primers and probes were diluted to 1000 and 100 µM, respectively, using 10mM Tris-HCl buffer (pH=8.0) and then stored in -20°C freezer. Prior to processing the real-time PCR assays, or reverse transcription (RT) real-time PCR assays, the 1000-µM primers and 100-µM probes stock solutions were further diluted to 20 µM, and 10 µM working solutions using nuclease-free water.

3.4.4 Quantitative real-time PCR assays

Real-time PCR assays were developed for the quantification of total eubacterial 16S rDNA, *Nitrosomonas nitrosa*-type *amoA*, uncultured AOB RI-27 isolate *amoA* and uncultured AOB B2-3 isolate *amoA* in the genomic DNA extracted from biosolid samples of the industrial bench-scale system. Additionally, real-time PCR assays were developed to quantify total eubacterial 16S rDNA, AOB 16S rDNA and *Nitrosomonas europaea*-type *amoA* in the genomic DNA extracts for the enriched nitrifying system. The genomic DNA extracts from the 1-L batch culture tests were analyze for the *Nitrosomonas europaea* DNA.

Real-time PCR assays were run by using a DNA Engine Opticon[™] Continuous Fluorescence Detection System (MJ Research, Waltham, MA). The threshold was determined by the computer software as 5X the standard deviation of the background

fluorescence averaged over a minimum of 5 cycles at the start of the run. The threshold cycle (Ct) of each PCR reaction was automatically determined by detecting the cycle at which the fluorescence exceeds the calculated threshold. During each PCR run, the Ct values obtained from the DNA standards were used for the construction of a standard curve. The standard curve was used to calibrate target concentrations after each PCR run. All real-time PCR assays were performed (at least) in triplicate per sample, and all PCR runs included a negative control reaction (without template) and a positive control reaction (with previously amplified template). The effect of sample concentration on PCR performance was determined using dilutions of sample DNA (initial concentration of 50 ng/μL) containing 5 pg to 50 ng in nucleic-acid-free water.

Total bacterial 16S rDNA in the DNA extracts of biosolids samples from both the industrial bench-scale system and the enriched nitrifying system was quantified using primers 1055f and 1392r, and the TaqMan[®] probe 16S-Taq1115 (Dionisi et al., 2002) (Table 3-6). The PCR mix with a total volume of 25 μL contained 12.5 μL of 2X QuantiTect Probe PCR Master Mix (QuantiTect[®] Probe PCR Kit, Qiagen, Valencia, CA), 0.75 μL of 20 μM forward primer 1055f, 0.75 μL of 20 μM reverse primer 1392r, 0.625 μL of 10 μM TaqMan[®] 16S-Taq1115f probe, and 5 μL of diluted sample DNA or standard (Table 3-7). A dilution series of plasmid #931 with 16S rDNA genes (in a range of 1.0×10^2 - 1.0×10^8 copies per PCR reaction of the 16S rDNA gene) was used as standard for total 16S rDNA quantification. The quantitative real-time PCR program was as follows: 2 min at 50°C, 10 min at 95°C, and followed by 50 cycles at 95°C for 30 sec, 55°C for 45 sec, and read fluorescence (Table 3-8).

The AOB 16S rDNA in the DNA extracts of biosolids samples taken from the enriched nitrifying system was quantified using primers CTO 189fA/B and RT1r and the TaqMan[®] probe TMP1 (Harms et al., 2003). The PCR mix (total volume of 25 μL) contained 12.5 μL of 2X QuantiTect Master Mix (QuantiTect[®] Probe PCR Kit, Qiagen, Valencia, CA), 0.375 μL of 20 μM forward primer CTO 189fA/B, 0.375 μL of 20 μM reverse primer RT1r, 0.3125 μL of 10 μM TaqMan[®] TMP1 probe, and 5 μL of diluted sample DNA or standard (Table 3-7). A dilution series of plasmid #931 with 16S rDNA

Table 3-7. Components of the PCR mix for one reaction in each real-time PCR assay.

Target molecule	Ingredients	Volume/ one reaction	Final concentration
Eubacteria 16S rDNA	QuantiTect Master Mix (2 X)	12.5000 µL	1 X
	Primer 1055f (20 µM stock)	0.7500 µL	0.600 µM
	Primer 1392r (20 µM stock)	0.7500 µL	0.600 µM
	16S-Taql1115f (10 µM stock)	0.6250 µL	0.250 µM
	Nuclease-free water	5.3750 µL	---
	Diluted DNA template	5.0000 µL	~1 ng
AOB 16S rDNA	QuantiTect Master Mix (2 X)	12.5000 µL	1 X
	Primer CTO 189fA/B (20 µM stock)	0.3750 µL	0.300 µM
	Primer RT1r (20 µM stock)	0.3750 µL	0.300 µM
	TMP1 (10 µM stock)	0.3125 µL	0.125 µM
	Nuclease-free water	6.4375 µL	---
	Diluted DNA template	5.0000 µL	~1 ng
<i>N. nitrosa</i> <i>amoA</i>	QuantiTect Master Mix (2 X)	12.5000 µL	1 X
	Primer amoA-Nn-548f (20 µM)	0.7500 µL	0.600 µM
	Primer amoA-Nn-777r (20 µM)	0.7500 µL	0.600 µM
	Probe amoA-Nn-Taq-740r (10 µM)	1.2500 µL	0.500 µM
	Nuclease-free water	4.7500 µL	---
	Diluted DNA template	5.0000 µL	~5 ng
Uncultured AOB RI-27 isolate <i>amoA</i>	QuantiTect Master Mix (2 X)	12.5000 µL	1 X
	Primer amoA-[RI-27]-542f (20 µM)	0.7500 µL	0.600 µM
	Primer amoA-[RI-27]-679r (20 µM)	0.7500 µL	0.600 µM
	Probe amoA-[RI-27]-bhq-651r (10 µM)	1.2500 µL	0.500 µM
	Nuclease-free water	4.7500 µL	---
	Diluted DNA template	5.0000 µL	~5 ng
Uncultured AOB B2-3 isolate <i>amoA</i>	QuantiTect Master Mix (2 X)	12.5000 µL	1 X
	Primer amoA-[B2-3]-596f (20 µM)	0.7500 µL	0.600 µM
	Primer amoA-[B2-3]-744r (20 µM)	0.7500 µL	0.600 µM
	Probe amoA-[B2-3]-bhq-713r (10 µM)	1.2500 µL	0.500 µM
	Nuclease-free water	4.7500 µL	---
	Diluted DNA template	5.0000 µL	~5 ng
<i>N. europaea</i> <i>amoA</i>	QuantiTect Master Mix (2 X)	12.5000 µL	1 X
	Primer amoA-Ne-590f (20 µM)	0.7500 µL	0.600 µM
	Primer amoA-Ne-751r (20 µM)	0.7500 µL	0.600 µM
	Probe amoA-Ne-bhq-720r (10 µM)	0.5000 µL	0.200 µM
	Nuclease-free water	5.5000 µL	---
	Diluted DNA template	5.0000 µL	~5 ng

Table 3-8. Protocol of real-time PCR assays for quantifying eubacterial and AOB 16S rDNA (based on the information suggested in the QuantiTect® Probe PCR Handbook, Qiagen, Valencia, CA).

real-time PCR step	Quantitative real-time PCR conditions using TaqMan® probe	
	Eubacteria – 16S rDNA	AOB – 16S rDNA
1. Carryover prevention	2 min at 50°C	---
2. Initial activation	10 min at 95°C	10 min at 95°C
3. Denaturation	30 sec at 95°C	30 sec at 95°C
4. Annealing/Extension	45 sec at 55°C	45 sec at 55°C
5. Read fluorescence	read	read
6. Go to step 3 for	50 times	50 times
7. End	end	end

genes (in a range of 1.0×10^2 - 1.0×10^8 copies per PCR reaction of the 16S rDNA gene) was used as standard for total AOB 16S rDNA quantification. The quantitative real-time PCR program was as follows: 10 min at 95°C, and followed by 50 cycles at 95°C for 30 sec, 55°C for 45 sec, and read fluorescence (Table 3-8).

The *Nitrosomonas nitrosa* partial *amoA* was quantified using primers amoA-Nn-548f (or amonit1-f) and amoA-Nn-777r (or amonit1-r), and the TaqMan® probe amoA-Nn-Taq-740r (or amonit1-p). The 25-μL PCR mix contained 12.5 μL of 2X QuantiTect Master Mix® (Qiagen, Valencia, CA), 0.75 μL of 20 μM forward primer, 0.75 μL of 20 μM reverse primer, 1.25 μL of 10 μM TaqMan® probe, and 5 μL of DNA sample or standard (Table 3-7). Standards consisted of the plasmid pCR®2.1 carrying the *N. nitrosa* *amoA* gene (GenBank accession number AF420294) were adjusted to 5.0×10^1 - 5.0×10^7 copies per PCR reaction. PCR amplification conditions were as follows: 10 min at 95°C, followed by 50 cycles at 95°C for 15 sec, 54°C for 30 sec, and plate-read (Table 3-9).

The *amoA* gene fragment of uncultured AOB RI-27 isolate was quantified using previously designed primers amoA-[RI-27]-542f and amoA-[RI-27]-679r, and the

Table 3-9. Real-time PCR protocols for quantifying *N. nitrosa*, RI-27, B2-3, and *N. europaea amoA* gene (based on the suggestion in the QuantiTect® Probe PCR Handbook, Qiagen, Valencia, CA).

real-time PCR step	Quantitative real-time PCR conditions using TaqMan® probe			
	<i>N. nitrosa</i> <i>amoA</i>	RI-27 <i>amoA</i>	B2-3 <i>amoA</i>	<i>N. europaea</i> <i>amoA</i>
1. Carryover prevention	---	---	---	---
2. Initial activation	10 min at 95°C	10 min at 95°C	10 min at 95°C	10 min at 95°C
3. Denaturation	15 sec at 95°C	15 sec at 95°C	15 sec at 95°C	15 sec at 95°C
4. Annealing/Extension	30 sec at 54°C*	30 sec at 60°C*	30 sec at 56°C*	30 sec at 60°C*
5. Read fluorescence	read	read	read	read
6. Go to step 3 for	50 times	50 times	50 times	50 times
7. End	end	end	end	end

* Melting temperatures, T_m, at step 4 were determined based on Oligonucleotide Properties Calculator at <http://www.basic.nwu.edu/biotools/oligocalc.html>

TaqMan® probe amoA-[RI-27]-bhq-651r (Layton et al., 2005). The 25-μL PCR mix contained 12.5 μL of 2X QuantiTect Master Mix® (Qiagen, Valencia, CA), 0.75 μL of 20 μM forward and reverse primers, 1.25 μL of 10 μM TaqMan® probe, and 5 μL of sample DNA or standard (Table 3-7). Purified plasmid DNA carrying the RI-27 *amoA* gene (10-day *amoA* gene library clone IB-C10-26 for the industrial bench-scale system from this study) was adjusted to 1.0×10^9 (copies/μL) as standard stock solution. Standards were diluted in a range from 5.0×10^1 to 5.0×10^7 copies per PCR reaction. PCR amplification protocol was as follows: 10 min at 95°C, 50 cycles at 95°C for 15 sec, 60°C for 30 sec, and plate-read (Table 3-9).

The *amoA* gene fragment of uncultured AOB B2-3 isolate was quantified using primers amoA-[B2-3]-596f and amoA-[B2-3]-744r, and the TaqMan® probe amoA-[B2-3]-bhq-713r newly designed in this study. The 25-μL PCR mix contained 12.5 μL of 2X

QuantiTect Master Mix (QuantiTect[®] Probe PCR Kit, Qiagen, Valencia, CA), 0.75 µL of each 20 µM primer, 1.25 µL of 10 µM TaqMan[®] probe, and 5 µL of DNA sample or standard (Table 3-7). Purified plasmid DNA carrying the B2-3 *amoA* gene (2-day *amoA* gene library clone IB-C2-17 for the industrial bench-scale system from this study) was adjusted to 1.0×10^9 (copies/µL) as standard stock solution. Standards were diluted in a range from 5.0×10^1 to 5.0×10^7 copies per PCR reaction. PCR amplification protocol was as follows: 10 min at 95°C, followed by 50 cycles at 95°C for 15 sec, 56°C for 30 sec, and plate-read (Table 3-9).

The *amoA* gene of *Nitrosomonas europaea* group identified in the enriched nitrifying system was quantified using newly designed primers amoA-Ne-590f and amoA-Ne-751r, and the TaqMan[®] probe amoA-Ne-bhq-720r (Table 3-6). The 25-µL PCR mix contained 12.5 µL of 2X QuantiTect Master Mix (Qiagen, Valencia, CA), 0.75 µL of each 20 µM primer, 0.5 µL of 10 µM TaqMan[®] probe, and 5 µL of DNA sample or standard (Table 3-7). Purified plasmid DNA carrying the *amoA* gene of the *N. europaea* group (*amoA* gene library clone EN-C4 from this study) was adjusted to 1.0×10^9 (copies/µL) as standard stock solution. Standards were diluted in a range from 5.0×10^1 to 5.0×10^7 copies per PCR reaction. PCR amplification protocol was: 10 min at 95°C, followed by 50 cycles at 95°C for 15 sec, 60°C for 30 sec, and plate-read (Table 3-9).

3.5 Molecular methods for mRNA analysis

3.5.1 RNA extraction from biosolids samples

RNA was extracted from archived biosolids samples using RNeasy[®] Mini Kit (Qiagen, Valencia, CA) based on the Protocol for Isolation of Total RNA from Bacteria in RNeasy[®] Mini Handbook (Qiagen, Valencia, CA). Briefly, bacterial cells were first concentrated by centrifugation and then lysed by lysozyme (3 mg/mL in TE buffer). RNAs in lysate were isolated with RNeasy[®] mini spin column. Next, an RNase-Free DNase Set (Qiagen, Valencia, CA) was used to remove DNA from isolated RNA samples. Finally, RNA samples were washed with Buffer RPE (containing ethanol) twice and then

eluted with 100 μ L RNA storage solutions (Ambion[®], Austin, TX). Extracted RNA samples were immediately stored in -80°C freezer for further quantification.

3.5.2 Preparing the *amoA* mRNA standard

The *amoA* mRNA standards for reverse transcription (RT) real-time PCR assays were synthesized from clone libraries in which PCR products of target *amoA* gene had been cloned in a direction that anti-sense sequences were opposite to T7 priming site. (This is necessary to allow RNA polymerase to transcribe information on the anti-sense sequence.) Three *amoA* mRNA standards were prepared in this study to quantify the *amoA* mRNA level for the RI-27, B2-3, and *N. europaea* groups identified in the industrial bench-scale system and in the enriched nitrifying system, respectively.

A 100- μ L aliquot of selected clone library was used to inoculate 5 mL of LB Kan₅₀ media in a sterile 15-ml plastic Falcon[™] centrifuge tube and incubated overnight at 37°C by shaking horizontally at 200 rpm. Plasmid DNA was extracted using a DNA purification kit (Wizard[®] Plus SV Minipreps DNA Purification System, Promega, Madison, WI). Extracted plasmid DNA was further purified and concentrated via an ethanol/salt precipitation. Purified plasmid DNA was linearized using a *speI* restriction enzyme digest (Promega, Madison, WI). Each digestion reaction was performed in a 40- μ L mixture containing 29.6 μ L diethylpyrocarbonate (DEPC-treated) water, 4.0 μ L 10X Buffer B, 0.4 μ L Bovine serum albumin (BSA), 2.0 μ L *speI* restriction enzyme and 4.0 μ L concentrated plasmid. Digestion was performed at 37°C for 4 hours. Integrity of linearized plasmid DNA was evaluated on a 1 % (w/v) agarose gel.

Subsequently, a fragment of *amoA* mRNA transcript was produced using the RiboMAX[™] Large Scale RNA Production Systems (Promega, Madison, WI). In this *in vitro* transcription reaction, a T7 RNA polymerase was used to transcribe a fragment of *amoA* mRNA from the anti-sense sequence of cloned *amoA* gene library (Figure 3-12) based upon the T7 transcription protocol (Promega, Madison, WI). (A linear luciferase gene was used as positive control to produce ~1,800 bases transcripts and DEPC water was used as negative control). Briefly, each transcription reaction was conducted in a 50- μ L mixture including 10 μ L of T7 transcription buffer (5X), 5 μ L of T7 RNA polymerase,

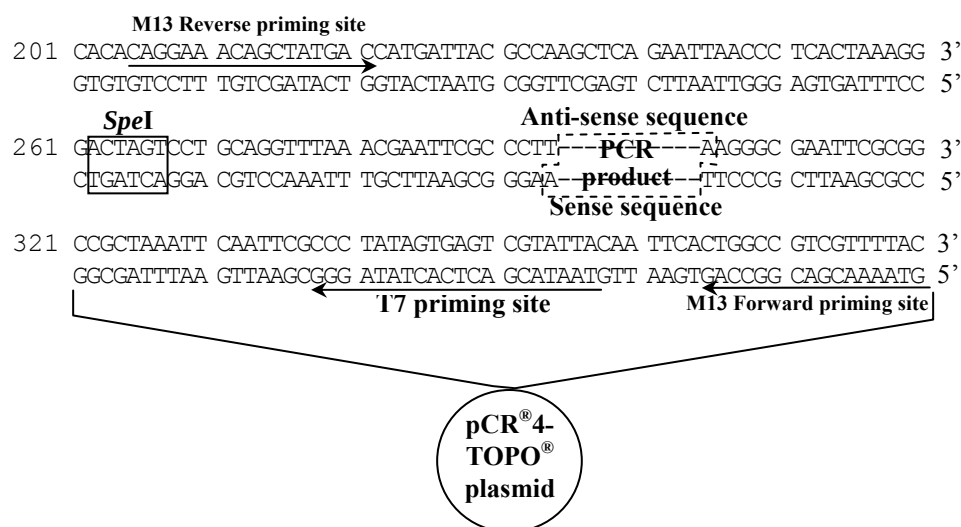


Figure 3-12. The *amoA* mRNA standard was generated from the *amoA* gene library using T7 RNA polymerase to conduct transcription using anti-sense sequence as template.

15 μ L of rNTP (25 mM), and 20 μ L of linearized plasmid DNA. The mixture was incubated at 37°C for 4 hours to allow transcription to proceed. Transcribed mRNAs were cleaned-up with RPE buffer and digested with DNA-free™ DNase (Ambion, Austin, TX) to remove residue of the transcription template (linearized plasmid DNA). After concentrating, the mRNA products were immediately stored at -80°C for further evaluation.

3.5.3 Quality and quantity evaluations for *amoA* mRNA standards

Qualities of prepared *amoA* mRNA standard stock solutions were evaluated using denaturing agarose gel (to check the integrity of mRNA), ultraviolet (UV) light absorption ratio (A_{260}/A_{280}) (to check the purity of nucleic acid or protein contaminants), and no-RT RT real-time PCR reaction (to check contamination of transcription template with linearized plasmid DNA). Quantities of prepared *amoA* mRNA standards were determined by spectrophotometric quantification and RiboGreen® RNA quantification (Molecular Probes, Eugene, OR).

The integrity of laboratory-prepared mRNA standard was checked using 1.5% (w/v) denaturing agarose gel. The agarose gel was prepared in a 50-mL mixture containing 0.75 g ultra-pure agarose (Gibco-BRL, Invitrogen, Carlsbad, CA), 36 mL DEPC water, 5 mL of 10X MOPS buffer (containing 0.2 M MOPS, 20 mM sodium acetate and 10 mM EDTA), and 9 mL of 37% (w/v) formaldehyde (used within a chemical fume hood). The solidified gel was submerged in 1X MOPS solution and pre-run for 10 minutes (with circulation of the 1X MOPS buffer between positive (+) and negative (-) electrophoresis terminal with a peristaltic pump). A 5-μL sample (i.e., mRNA standard stock solution or RNA marker) was denatured by mixing with 20 μL RNA sample buffer (consisting of 10 mL deionized formamide, 3.5 mL of 37% (w/v) formaldehyde, and 2 mL of 10X MOPS buffer) and incubating at 65°C for 15 minutes. After the pre-treated mRNA sample was cooled on ice for 2 minutes, a 2-μL aliquot of RNA loading buffer (containing 50% glycerol, 5 mM EDTA, 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol FF, and 1 mg/mL ethidium bromide (EtBr) was added right before the mRNA sample was loaded into the wells of the gel. The gel was run at 45 V (4~5 V/cm of gel) until the bromophenol blue had migrated ~ 8 cm (approx. 4 hrs). The gel was then visualized and photographed under UV illumination.

The purity of prepared mRNA standard was determined by an UV absorption ratio (A_{260}/A_{280}). The mRNA standard stock solution was diluted 1:500 (or 1:200 for RI-27, and B2-3 *amoA* mRNA standards) using 10 mM Tris-HCl (pH 7.6). The absorbance of the dilution was measured under UV wavelength of 260 nm (maximum absorption of nucleic acids, i.e., RNA or DNA) and then 280 nm (maximum absorption for proteins) using a Beckman Coulter™ (DU® 640B) spectrophotometer.

The presence of DNA contamination (residue of linearized plasmid DNA) in the prepared mRNA standard was checked by a no-RT reverse transcription (RT) real-time PCR reaction. The PCR reaction was performed in a 25-μL mix contained 12.5 μL of 2X QuantiTect Probe RT-PCR Master Mix® (Qiagen, Valencia, CA), 0.75 μL of 20 μM *amoA*-Ne-590f primer, 0.75 μL of 20 μM *amoA*-Ne-751r primer, 1.25 μL of 10 μM *amoA*-Ne-bhq-720r probe, 4.75 μL of RNase-free water, and 5 μL diluted mRNA standard solution (10^9 copies/μL of *amoA* mRNA). The QuantiTect RT mix (containing

reverse transcriptase) was not added so that only DNA target, instead of RNA, would be detected. A series of *N. europaea amoA* DNA dilutions ($10^1 \sim 10^7$ copies/ μL) was made from purified plasmid DNA of the *amoA* gene library (clone EN-C4) and served as standards to calibrate DNA in the 10^9 -copies/ μL mRNA standard solution. The PCR program was 30 min at 50°C , 15 min at 95°C , and followed by 50 cycles at 95°C for 15 sec, 60°C for 30 sec, and read fluorescence.

The quantity of the prepared mRNA standard was determined in a 1:500 (or 1:200 for RI-27, and B2-3 *amoA* mRNA standards) dilution of the stock solution in DEPC water. The absorbance of the dilution was detected under a UV wavelength of 260 nm using a spectrophotometer (Beckman Coulter™ DU® 640B). The measured absorption values were converted into concentration (in ng/ μL) using a coefficient of 40 (cf., one A_{260} unit equals approximately 40 ng/ μL of RNA when absorbance is measured in a cuvette with a 1-cm path length).

The concentration of *amoA* mRNA in the standard stock solution was confirmed by using RiboGreen® RNA Quantitation Reagent and Kit (Molecular Probes, Inc., Invitrogen, Carlsbad, CA). The measurement was based the low-range (1-50 ng/mL) experimental protocol. The sample fluorescence was read by a VersaFluor™ Fluorometer (BIO-RAD, Hercules, CA) using a final volume of 2000 μL . Additionally, the read was confirmed by a Wallac 1420 Victor2™ Multilabel Counter (PerkinElmer®, Wellesley, MA) using a final volume of 200 μL . Preparations for rRNA standard solutions were summarized in [Tables 3-10, 3-11](#). The 20X TE buffer was diluted 20-fold with nuclease-free water to produce a 1X TE working solution. The RiboGreen reagent was diluted 2000-fold with 1X TE buffer to make a 2000-fold diluted RiboGreen working solution. And, the 100,000 ng/mL standard (containing 16S and 23S rRNA from *E. coli*) was diluted 1:1000 with 1X TE buffer to generate a 100-ng/mL standard working solution. The artificial mRNA standard sample was diluted 5,000~100,000-fold using 1X TE buffer. The fluorescence of the rRNA standards and diluted samples were read at standard fluorescein wavelengths (excitation ~480 nm, emission ~520 nm). The concentration of the diluted mRNA standard sample was calibrated from the constructed rRNA standard curves.

Table 3-10. Protocol for preparing the low-range rRNA standard solutions (with a 2000- μ L final volume for each of standard) used for measurements by the VersaFluorTM Fluorometer (BIO-RAD, Hercules, CA).

Volume (μ L) of 100 ng/mL rRNA standard*	Volume (μ L) of 1X TE buffer [†]	Volume (μ L) of 2000-fold diluted RiboGreen Reagent [‡]	Final Volume (μ L)	Final Concentration (ng/mL)
1000	0	1000	2000	50
500	500	1000	2000	25
300	700	1000	2000	15
100	900	1000	2000	5
20	980	1000	2000	1
0	1000	1000	2000	0

[†]: 1X TE working solution was made by diluting 20-fold of a 20X TE buffer with nuclease-free water (DEPC-treated water).

[‡]: The RiboGreen working solution was generated by diluting concentrated RiboGreen RNA quantitation reagent 2000-fold with 1X TE buffer.

*: The 100-ng/mL standard working solution was diluted 1:1000 (from a 100,000 ng/mL standard, containing 16S and 23S rRNA from *E. coli*) with 1X TE buffer.

Table 3-11. Protocol for preparing the low-range rRNA standard solutions (with a 200- μ L final volume for each of standard) measured by the Wallac 1420 Victor2TM Multilabel Counter (PerkinElmer[®], Wellesley, MA).

Volume (μ L) of 100 ng/mL rRNA standard*	Volume (μ L) of 1X TE buffer [†]	Volume (μ L) of 2000-fold diluted RiboGreen Reagent [‡]	Final volume (μ L)	Final concentration (ng/mL)
100	0	100	200	50
50	50	100	200	25
30	70	100	200	15
10	90	100	200	5
2	98	100	200	1
0	100	100	200	0

[†]: 1X TE working solution was made by diluting 20-fold of a 20X TE buffer with nuclease-free water (DEPC-treated water).

[‡]: The RiboGreen working solution was generated by diluting concentrated RiboGreen RNA quantitation reagent 2000-fold with 1X TE buffer.

*: The 100-ng/mL standard working solution was diluted 1:1000 (from a 100,000 ng/mL standard, containing 16S and 23S rRNA from *E. coli*) with 1X TE buffer.

3.5.4 Reverse-transcription (RT) real-time PCR assays

Three RT real-time PCR assays were used to quantify the abundance of the *amoA* mRNA in the *Nitrosomonas nitrosa*, RI-27, and B2-3 AOB groups identified in the industrial bench-scale system; and, one RT real-time PCR assay was developed for quantification of the *amoA* mRNA in the *Nitrosomonas europaea* group dominant in the enriched nitrifying system. The RT real-time PCR assays were also run by using a DNA Engine Opticon™ Continuous Fluorescence Detection System (MJ Research, Waltham, MA). The RT real-time PCR assays were based on protocols suggested in QuantiTect™ Probe RT-PCR Handbook (Qiagen, Valencia, CA). In the RT real-time PCR reaction, target *amoA* mRNA was first reverse-transcribed to cDNA, and the cDNA was sequentially quantified by real-time PCR reaction. The primers and probes used for the RT real-time PCR assays were the same as those used in the real-time PCR quantifications specific for *amoA* DNA of distinct AOB groups (Table 3-6).

The *N. nitrosa amoA* mRNA concentration was quantified using the amoA-Nn-542f, amoA-Nn-676r primers and the amoA-Nn-Taq-648r probe (Table 3-6). Each reaction was performed in a 25-μL mix contained 12.5 μL of 2X QuantiTect Probe RT-PCR Master Mix (QuantiTect™ Probe RT-PCR Kit, Qiagen, Valencia, CA), 0.75 μL of 20 μM forward primer, 0.75 μL of 20 μM reverse primer, 1.25 μL of 10 μM probe, 0.25 μL of QuantiTect RT Mix, 4.5 μL of nuclease-free water, and 5 μL of sample RNA or standard (Table 3-12). Serial dilutions of the *N. nitrosa amoA* DNA ($5 \times 10^1 \sim 5 \times 10^7$ copies per PCR reaction) carried by the plasmid pCR®2.1 were used as standards to calibrate abundance of cDNA reverse-transcribed from target *N. nitrosa amoA* mRNA. The RT real-time PCR reaction program was 30 min at 50°C, 15 min at 95 °C, followed by 50 cycles at 95°C for 15 sec, 54°C for 30 sec, and read fluorescence (Table 3-13).

The *amoA* mRNA concentration of the RI-27 group was quantified using the amoA-[RI-27]-542f, amoA-[RI-27]-679r primers and the amoA-[RI-27]-bhq-651r probe. Each 25-μL reaction mix contained 12.5 μL of 2X QuantiTect Probe RT-PCR Master Mix® (Qiagen, Valencia, CA), 0.75 μL of 20 μM forward primer, 0.75 μL of 20 μM reverse primer, 1.25 μL of 10 μM probe, 0.25 μL of QuantiTect RT Mix®, 4.5 μL of nuclease-free

Table 3-12. Components for one reaction in each RT real-time PCR assay.

Target molecule	Ingredients	Volume/ one reaction	Final concentration
<i>N. nitrosa</i> <i>amoA</i> mRNA	QuantiTect RT-PCR Master Mix (2 X)	12.5000 µL	1 X
	Primer amoA-Nn-542f (20 µM)	0.7500 µL	0.600 µM
	Primer amoA-Nn-676r (20 µM)	0.7500 µL	0.600 µM
	Probe amoA-Nn-Taq-648r (10 µM)	1.2500 µL	0.500 µM
	QuantiTect RT Mix	0.2500 µL	---
	Nuclease-free water	4.5000 µL	---
	Diluted RNA template	5.0000 µL	~5 ng
Uncultured AOB RI-27 isolate <i>amoA</i> mRNA	QuantiTect RT-PCR Master Mix (2 X)	12.5000 µL	1 X
	Primer amoA-[RI-27]-542f (20 µM)	0.7500 µL	0.600 µM
	Primer amoA-[RI-27]-679r (20 µM)	0.7500 µL	0.600 µM
	Probe amoA-[RI-27]-bhq-651r (10 µM)	1.2500 µL	0.500 µM
	QuantiTect RT Mix	0.2500 µL	---
	Nuclease-free water	4.5000 µL	---
	Diluted RNA template	5.0000 µL	~5 ng
Uncultured AOB B2-3 isolate <i>amoA</i> mRNA	QuantiTect RT-PCR Master Mix (2 X)	12.5000 µL	1 X
	Primer amoA-[B2-3]-596f (20 µM)	0.7500 µL	0.600 µM
	Primer amoA-[B2-3]-744r (20 µM)	0.7500 µL	0.600 µM
	Probe amoA-[B2-3]-bhq-713r (10 µM)	1.2500 µL	0.500 µM
	QuantiTect RT Mix	0.2500 µL	---
	Nuclease-free water	4.5000 µL	---
	Diluted RNA template	5.0000 µL	~5 ng
<i>N. europaea</i> <i>amoA</i> mRNA	QuantiTect RT-PCR Master Mix (2 X)	12.5000 µL	1 X
	Primer amoA-Ne-590f (20 µM)	0.7500 µL	0.600 µM
	Primer amoA-Ne-751r (20 µM)	0.7500 µL	0.600 µM
	Probe amoA-Ne-bhq-720r (10 µM)	0.5000 µL	0.200 µM
	QuantiTect RT Mix	0.2500 µL	---
	Nuclease-free water	5.2500 µL	---
	Diluted RNA template	5.0000 µL	~5 ng

Table 3-13. Protocols of RT real-time PCR assays for quantifying *N. nitrosa*, RI-27, B2-3, and *N. europaea amoA* mRNA (modified from suggestions in QuantiTect™ Probe RT-PCR Handbook, Qiagen, Valencia, CA).

RT real-time PCR Steps	Quantitative RT real-time PCR reaction programs			
	<i>N. nitrosa</i> <i>amoA</i> mRNA	RI-27 <i>amoA</i> mRNA	B2-3 <i>amoA</i> mRNA	<i>N. europaea</i> <i>amoA</i> mRNA
1. Reverse transcription	30 min at 50°C	30 min at 50°C	30 min at 50°C	30 min at 50°C
2. Initial activation	15 min at 95°C	15 min at 95°C	15 min at 95°C	15 min at 95°C
3. Denaturation	15 sec at 95°C	15 sec at 95°C	15 sec at 95°C	15 sec at 95°C
4. Annealing/Extension	30 sec at 54°C	30 sec at 60°C	30 sec at 56°C	30 sec at 60°C
5. Read fluorescence	read	read	read	read
6. Go to step 3 for	50 times	50 times	50 times	50 times
7. End	end	end	end	end

water, and 5 µL of sample RNA or standard (Table 3-12). The artificial RI-27 *amoA* mRNA standards were diluted in a range from 10³ to 10⁹ copies per PCR reaction to calibrate the abundance of target RI-27 *amoA* mRNA. The RT real-time PCR reaction program was 30 min at 50°C, 15 min at 95 °C, and followed by 50 cycles at 95°C for 15 sec, 60°C for 30 sec, and read fluorescence (Table 3-13).

The B2-3 *amoA* mRNA concentration was quantified using the amoA-[B2-3]-596f, amoA-[B2-3]-744r primers and the amoA-[B2-3]-bhq-713r probe. Each 25-µL reaction mix contained 12.5 µL of 2X QuantiTect Probe RT-PCR Master Mix® (Qiagen, Valencia, CA), 0.75 µL of 20 µM forward primer, 0.75 µL of 20 µM reverse primer, 1.25 µL of 10 µM probe, 0.25 µL of QuantiTect RT Mix®, 4.5 µL of nuclease-free water and 5 µL of sample RNA or standard (Table 3-12). The artificial B2-3 *amoA* mRNA standards were diluted in a range from 10³ to 10⁹ copies per PCR reaction to calibrate the abundance of target B2-3 *amoA* mRNA. The RT real-time PCR reaction program was 30 min at 50°C, 15 min at 95 °C, and followed by 50 cycles at 95°C for 15 sec, 56°C for 30 sec, and read fluorescence (Table 3-13).

The *N. europaea amoA* mRNA concentration was quantified using the amoA-Ne-590f, amoA-Ne-751r primers and the amoA-Ne-bhq-720r probe. Each 25- μ L reaction mix contained 12.5 μ L of 2X QuantiTect Probe RT-PCR Master Mix[®] (Qiagen, Valencia, CA), 0.75 μ L of 20 μ M forward primer, 0.75 μ L of 20 μ M reverse primer, 0.5 μ L of 10 μ M probe, 0.25 μ L of QuantiTect RT Mix[®], 5.25 μ L of nuclease-free water and 5 μ L of sample RNA or standard (Table 3-12). The artificial *N. europaea amoA* mRNA standards were diluted in a range of 10^3 to 10^9 copies per PCR reaction to calibrate the abundance of target *N. europaea amoA* mRNA. The RT real-time PCR reaction program was 30 min at 50°C, 15 min at 95 °C, and followed by 50 cycles at 95°C for 15 sec, 60°C for 30 sec, and read fluorescence (Table 3-13).

Chapter 4 Results and discussion — Part I

(Assessments of AOB population dynamics in the industrial bench-scale system)

4.1 Industrial bench-scale system treatment performance

4.1.1 Activated sludge maintenance

The concentrations of mixed liquor volatile suspended solids (MLVSS) in each reactor and in the effluent for each treatment train of the industrial bench-scale system were measured to determine the amount of waste biomass ([Appendix A.1](#)) necessary to control the solid retention time (SRT) operating parameter for each of the four trains. During each of the three operating phases, the MLVSS concentrations were directly proportional to the SRT; longer SRTs resulted in higher MLVSS levels ([Figure 4-1, Table 4-1](#)). In each SRT train, the MLVSS concentrations of the 1st-section reactors were slightly (~ 0.2 fold) higher than that in the 2nd-, and 3rd-section reactors. This is believed to be due to the fact that recycle sludge was pumped back to the 1st-section reactors and that fast-growing heterotrophic bacteria proliferated in the 1st-section since these reactors received approximately 50% of the incoming COD, while the 2nd-, and 3rd-section only received approximately 30%, and 20% of the incoming COD, respectively.

The effluent MLVSS concentrations were notably lower than reactor MLVSS levels for each SRT treatment train ([Figure 4-1, Table 4-2](#)) suggesting that the biosolids were effectively settled in the clarifiers. During phase 2, the effluent MLVSS levels in the 20-, 10-, and 5-day SRT trains were relatively high due to inconsistent effluent flow. Although the 3/8-inch effluent channels (between the 3rd-section reactor and clarifier) were cleaned daily, scum in the reactors would sometimes block the channel, breaking through only after the water level (and pressure) increased. This break-through would occasionally cause a disturbance in the clarifiers, resulting in high MLVSS concentrations in the effluents. The effluent and waste VSS were summed to assess the total active biomass removed from the system for SRT determination. Thus, when the effluent VSS increased, the amount of wasted biomass would be decreased to maintain the appropriate SRTs.

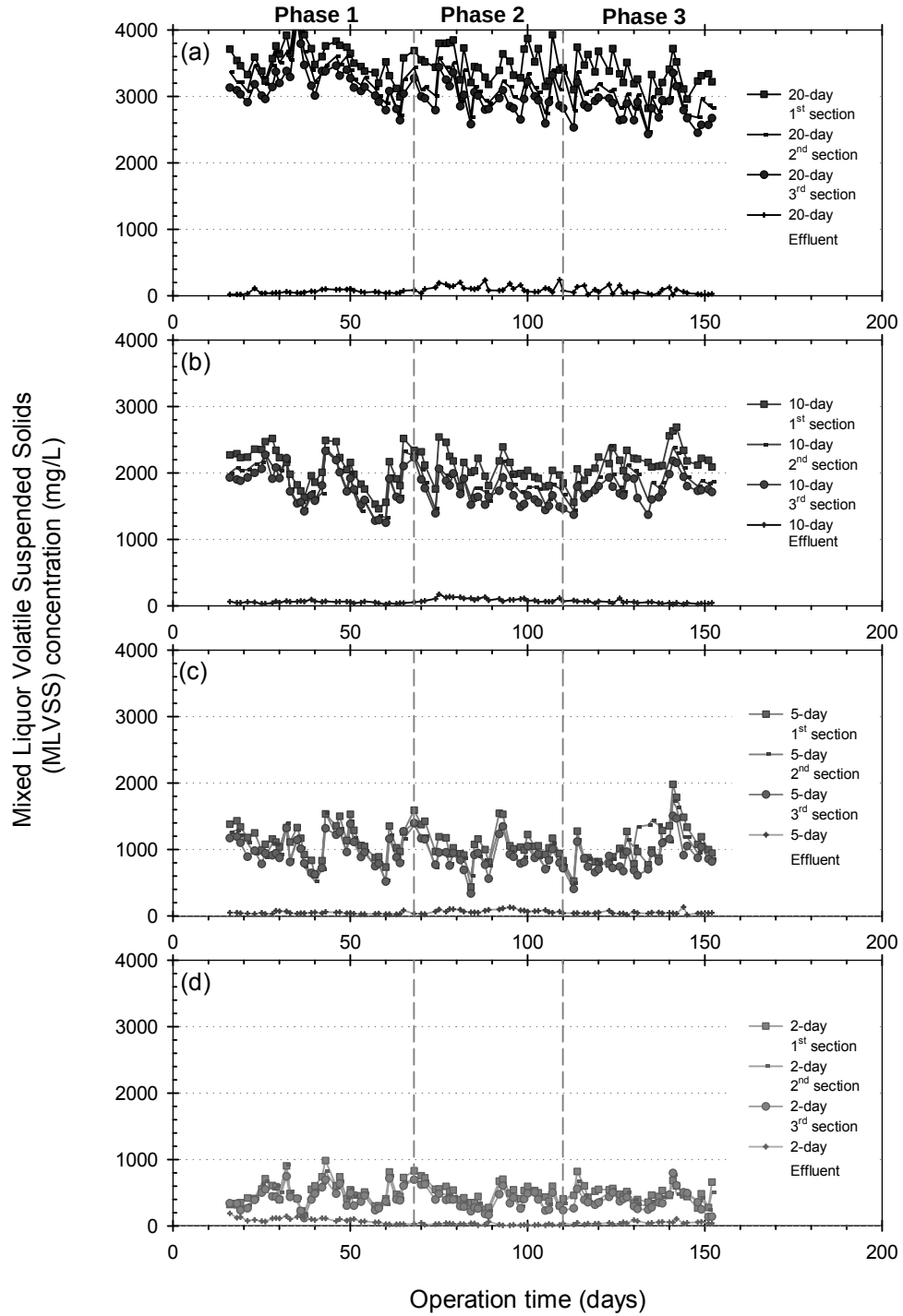


Figure 4-1. MLVSS concentrations in each of the 12 reactor units that formed the industrial bench-scale wastewater treatment system and in the effluent of each SRT train. (a) 20-day, (b) 10-day, (c) 5-day, and (d) 2-day.

Table 4-1. Average MLVSS concentrations (mg/L) with standard deviations in each reactor during the three operating phases. Coefficients of variation for the three average MLVSS concentrations were determined to assess stability of MLVSS levels over the entire operational period.

Reactors		Phase 1	Phase 2	Phase 3	Coefficients of variation* (%)
		High DO	High DO	Low DO	
		Low NH ₃	High NH ₃	High NH ₃	
20-day	1 st -section	3590(±293)	3515(±235)	3340(±239)	3.7
	2 nd -section	3357(±305)	3137(±220)	2956(±207)	6.4
	3 rd -section	3187(±283)	2968(±217)	2807(±228)	6.4
10-day	1 st -section	2080(±304)	2058(±207)	2185(±227)	3.2
	2 nd -section	1863(±315)	1802(±171)	1913(±233)	3.0
	3 rd -section	1815(±292)	1673(±181)	1762(±191)	4.1
5-day	1 st -section	1155(±242)	1064(±226)	1079(±329)	4.6
	2 nd -section	1021(±260)	920(±178)	1072(±281)	8.0
	3 rd -section	984(±230)	879(±204)	884(±249)	6.5
2-day	1 st -section	524(±195)	495(±133)	494(±122)	3.4
	2 nd -section	499(±173)	413(±129)	448(±109)	9.5
	3 rd -section	433(±154)	372(±132)	381(±145)	8.3

* Coefficient of variation for the average MLVSS concentrations during the three operating phase

Table 4-2. Average MLVSS concentrations (mg/L) with standard deviations in the effluent of each SRT train during the three operating phases.

SRT trains	Phase 1	Phase 2	Phase 3
	High DO	High DO	Low DO
	Low NH ₃	High NH ₃	High NH ₃
20-day	58 (±26)	118 (±54)	63 (±48)
10-day	54 (±15)	98 (±28)	51 (±19)
5-day	43 (±15)	75 (±26)	47 (±22)
2-day	90 (±41)	26 (±14)	50 (±19)

The MLVSS concentration is typically used to represent the abundance of active biomass in an activated sludge treatment system (Rittmann and McCarty, 2001). In order to achieve steady-state under a designated SRT for the treatment system, the active biomass must be maintained at a constant level by wasting excess bacterial cells at a rate equal to the net growth rate (Rittmann and McCarty, 2001). In this study, the average MLVSS levels in each reactor remained stable over the entire operational period (<10% variation for the average MLVSS concentrations during the three operating phases) (Table 4-1); suggesting that active biomass were maintained in each SRT train and that biomass (microbial) steady state was attained in the industrial bench-scale system.

4.1.2 Chemical oxygen demand (COD) treatment performance

The COD concentrations for the influent and effluent samples collected from the four SRT trains were measured to determine carbon oxidation performance for the heterotrophic bacteria in the industrial bench-scale system. Influent and effluent COD concentrations and COD removal efficiency for each SRT train are shown in Figure 4-2. Influent wastewater COD concentrations were highly variable during the entire study period (Figure 4-2 (a)); however, COD removal performance was fairly stable during the three test phases (Figure 4-2, Table 4-3). For most of the 1st and 2nd operational phases, effluent COD concentrations were lower than 100 mg/L, and removal efficiencies exceeded 90% (Figure 4-2). During phase 3 (DO=0.5 mg/L), average removal efficiencies were between 85~90% for each SRT which were significantly lower (p-value <0.05) than those measured during phase 2 (DO=3.0 mg/L) (Table 4-4). Thus, the lower DO level did impact COD removal performance in the industrial bench-scale system.

Average COD removal performance in the 20-day train was slightly better than that in lower SRT trains during each phase of operation (Table 4-3). Although treatment level differences between the 20-, 10-, and 5-day SRT reactors were small during each operating phase, the average COD removal efficiency of the 20-day train was significantly higher (p-value <0.05) than that of the 2-day train. These results were reasonable based on theoretical inference that the effluent substrate (e.g., COD) concentration should drop as the operational SRT increases (Section 2.4.2).

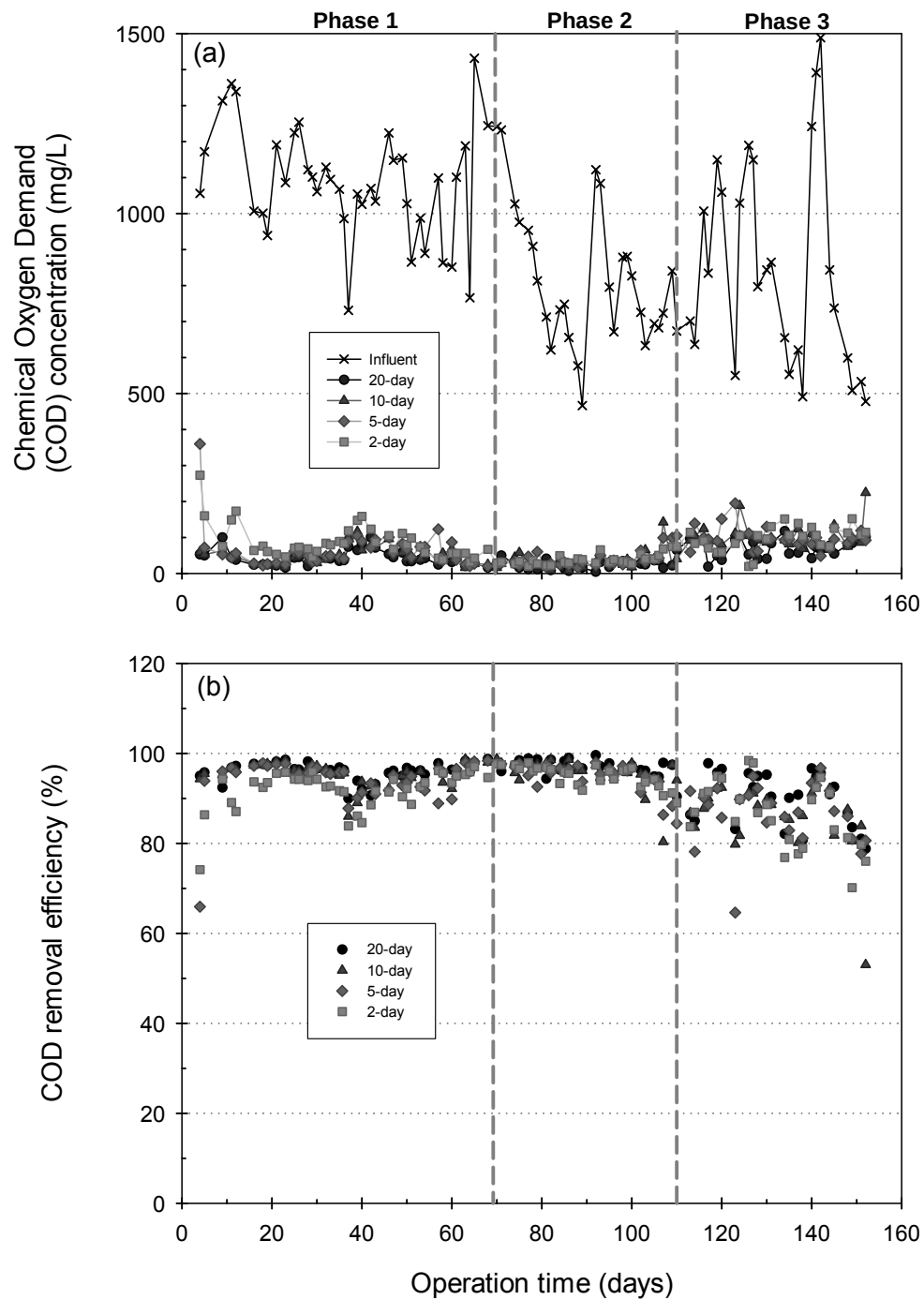


Figure 4-2. Influent and effluent COD concentrations (a), and COD removal efficiency (b) in each SRT system.

Table 4-3. Average influent and effluent COD concentrations (mg/L) with standard deviations and average removal efficiencies (%).

		Phase 1	Phase 2	Phase 3
		High DO	High DO	Low DO
		Low NH ₃	High NH ₃	High NH ₃
Influent		1084 (±157)	818 (±194)	844 (±293)
Effluent	20-day	43 (±21), 96.0%	25 (±13), 96.8%	73 (±26), 89.9%
	10-day	49 (±23), 95.3%	34 (±25), 95.6%	103 (±35), 86.1%
	5-day	63 (±56), 94.0%	40 (±24), 94.9%	98 (±31), 86.8%
	2-day	87 (±47), 91.9%	39 (±15), 94.9%	98 (±33), 86.4%

Table 4-4. Statistical t-test for differences in COD removal efficiency (%) under two unique DO levels (DO=3.0 mg/L and DO=0.5 mg/L).

	COD Removal Efficiency [†] (%)		Statistical t-test for (COD removal efficiency) _{DO=3.0} = or ≠ (COD removal efficiency) _{DO=0.5}	
	Phase 2	Phase 3	p-value [‡]	Test result
	(DO=3.0 mg/L)	(DO=0.5 mg/L)		
20-day	96.8	89.9	<0.05	(Rem.eff) _{DO=3.0} ≠ (Rem.eff) _{DO=0.5}
10-day	95.6	86.1	<0.05	(Rem.eff) _{DO=3.0} ≠ (Rem.eff) _{DO=0.5}
5-day	94.9	86.8	<0.05	(Rem.eff) _{DO=3.0} ≠ (Rem.eff) _{DO=0.5}
2-day	94.9	86.4	<0.05	(Rem.eff) _{DO=3.0} ≠ (Rem.eff) _{DO=0.5}

†: COD removal efficiency (%) = average COD removal efficiency during steady-state of two different DO operating phases (26 data points);

‡: The p-values of t-test were used to test (mean of COD removal efficiency)_{DO=3.0} = or ≠ (mean of COD removal efficiency)_{DO=0.5} using $\alpha=0.05$. When p-value < $\alpha(=0.05)$, it suggested that (mean of COD removal efficiency)_{DO=3.0} ≠ (mean of COD removal efficiency)_{DO=0.5}. Otherwise, there was no evidence to verify that two means were different.

Stable COD removal performance implied that the heterotrophic organic-carbon oxidizing bacteria grew consistently in this experimental system (as supported by the MLVSS data). Therefore, heterotrophic bacteria utilized a proportion of influent ammonium as a nitrogen nutrient source for cell metabolism and proliferation. This nitrogen would then be considered as biomass-N and removed from the system via daily sludge wasting. Therefore, the influent ammonium-N utilized by the fast-growing heterotrophic organic-carbon oxidizing bacteria would not be available to the ammonia-oxidizing bacteria (AOB) and was not included in ammonia oxidation performance estimations for the AOB in the industrial bench-scale system.

4.1.3 Ammonia removal and conversion profile

Ammonia concentrations in the influent and effluent samples were measured to determine ammonia removal efficiency ([Appendix A.2](#)) for the industrial bench-scale system. Influent ammonia concentrations were variable during each operating phase ([Figure 4-3 \(a\)](#)). During phases 2, and 3, the influent ammonia concentrations were higher than that in phase 1 due supplemental ammonia (using NH_4Cl) addition. A shock-loading period (discharge of NH_4NO_3 into the full-scale WWTP) occurred during phase 3 and resulted in a sudden increase for influent ammonia concentration (from 61.4 to 157.3 mg/L as N) for six days.

The ammonia removal efficiency in longer SRT trains (i.e., 20- and 10-day) was stable during the overall study period ([Figure 4-3 \(b\)](#)). In contrast, the ammonia removal efficiency fluctuated in the 5- and 2-day SRT trains when the NH_3 level was doubled during phase 2 and when the DO level was lowered to 0.5 mg/L during phase 3 operation. Although significant ammonia removal was observed in the treatment system during the study period, this ammonia removal was not due solely to oxidation of NH_3 by the AOB. A portion of the influent ammonia was utilized by fast-growing heterotrophs for cellular metabolism and proliferation and was not available for AOB oxidation. Therefore, ammonia conversion profiles were analyzed to determine the amount of ammonia removal that was achieved via oxidation by the AOB population during bench-scale industrial wastewater processing.

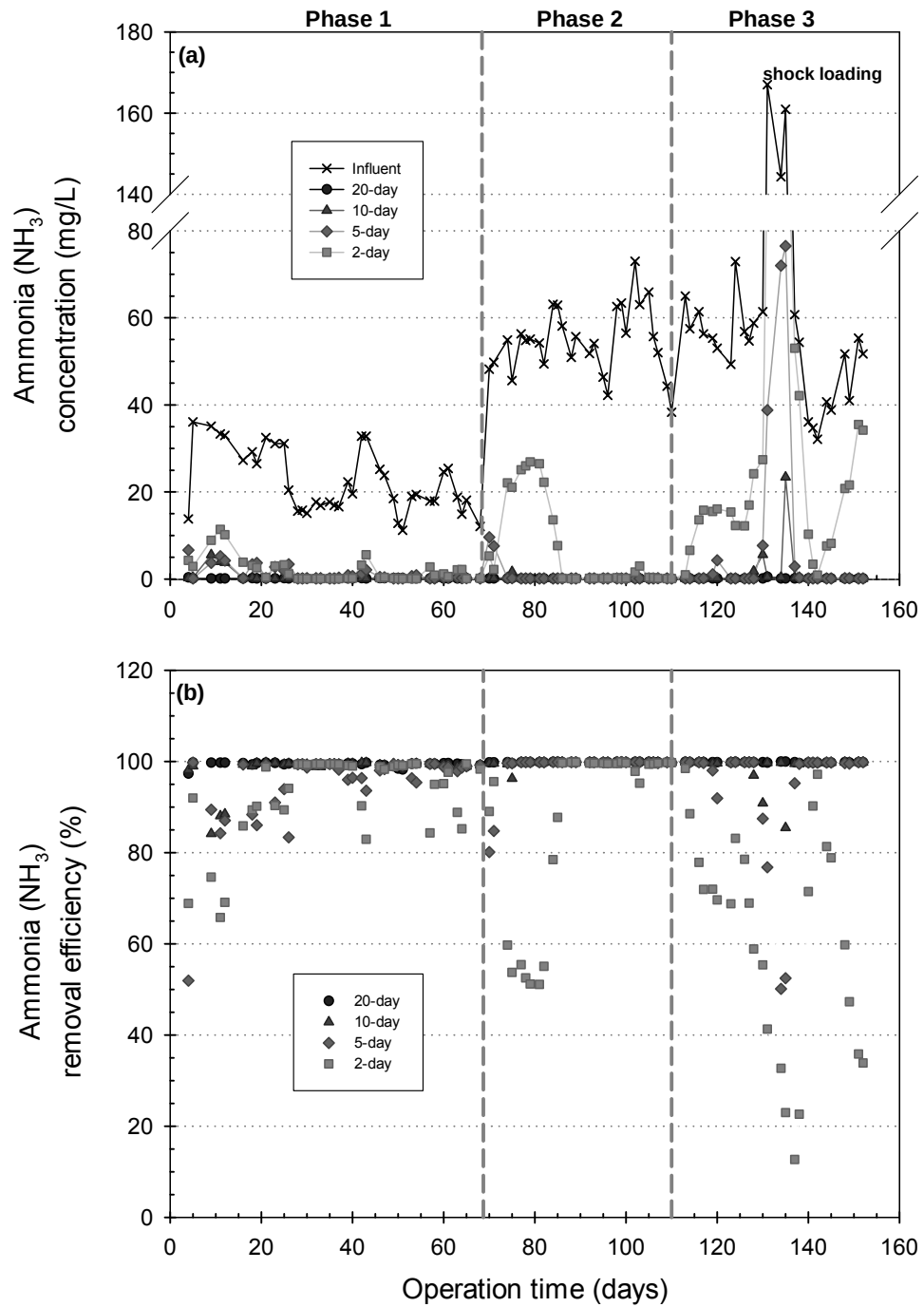


Figure 4-3. Influent and effluent ammonia concentrations (a) and ammonia removal efficiency (b) in each SRT train of the industrial bench-scale system.

Five nitrogen species including organic-N, ammonia-N, nitrite-N, nitrate-N, and biomass-N were considered to assess ammonia conversion profiles and determine AOB ammonia oxidation performance. In the influent ([Figure 4-4 \(a\)](#)), ammonia (70 ~ 94% of summed influent N) was the major nitrogen compound, while organic nitrogen, nitrite, and nitrate were relatively minor. In the effluent ([Figure 4-4 \(b ~ e\)](#)), biomass-N and nitrate were the major nitrogen products most of time, except during unstable periods in which high levels of nitrite and/or untreated ammonia was detected in the SRT effluents. In order to gain representational results for AOB ammonia oxidation performance, population sizes, and physiological activity under designated operational conditions, steady state treatment performance was attained for each operating phase. Steady state (with regard to ammonia oxidation) was defined as the period of time in which effluent ammonia concentrations remained consistently below 1 mg/L.

During phase 1 (low ammonia, and high DO operation), the average steady state influent ammonia concentration was 20.2 mg/L (as N) while average effluent ammonia concentrations were below 1.0 mg/L in all four SRT trains ([Figure 4-4, Table 4-5](#)). Most of the influent ammonia-N was converted to biomass-N (especially fast-growing heterotrophs) resulting in less ammonia available for AOB oxidation. Theoretically, the nitrogen required for heterotrophic growth could be estimated using a COD/nitrogen ratio of 30/1 (developed from operation of the industrial full-scale WWTP). In phase 1, average COD removed by heterotrophic bacteria was 1,029 mg/L, therefore nitrogen required for heterotrophic growth was ~34 mg/L, which was higher than the average ammonia-N concentration in the influent (~20.2 mg/L). This supports the conclusion that most of the influent ammonia nitrogen was utilized by heterotrophic bacteria for cell growth during phase 1. Moreover, based on mass balance estimations published by [Rittmann and McCarty \(2001\)](#) ([Section 2.4.2](#)), true bacterial growth in the four SRT trains would be theoretically similar, but not equal, based on the consistent COD and ammonia removal performances measured at each SRT ([Figures 4-2, 4-3](#)). However, the amount of net bacterial growth (equal to true growth minus endogenous decay) in the longer SRT train (e.g., 20-day) would be less than that in the shorter SRT train (e.g., 2-day) due to a higher amount of endogenous decay (i.e., endogenous-decay coefficient

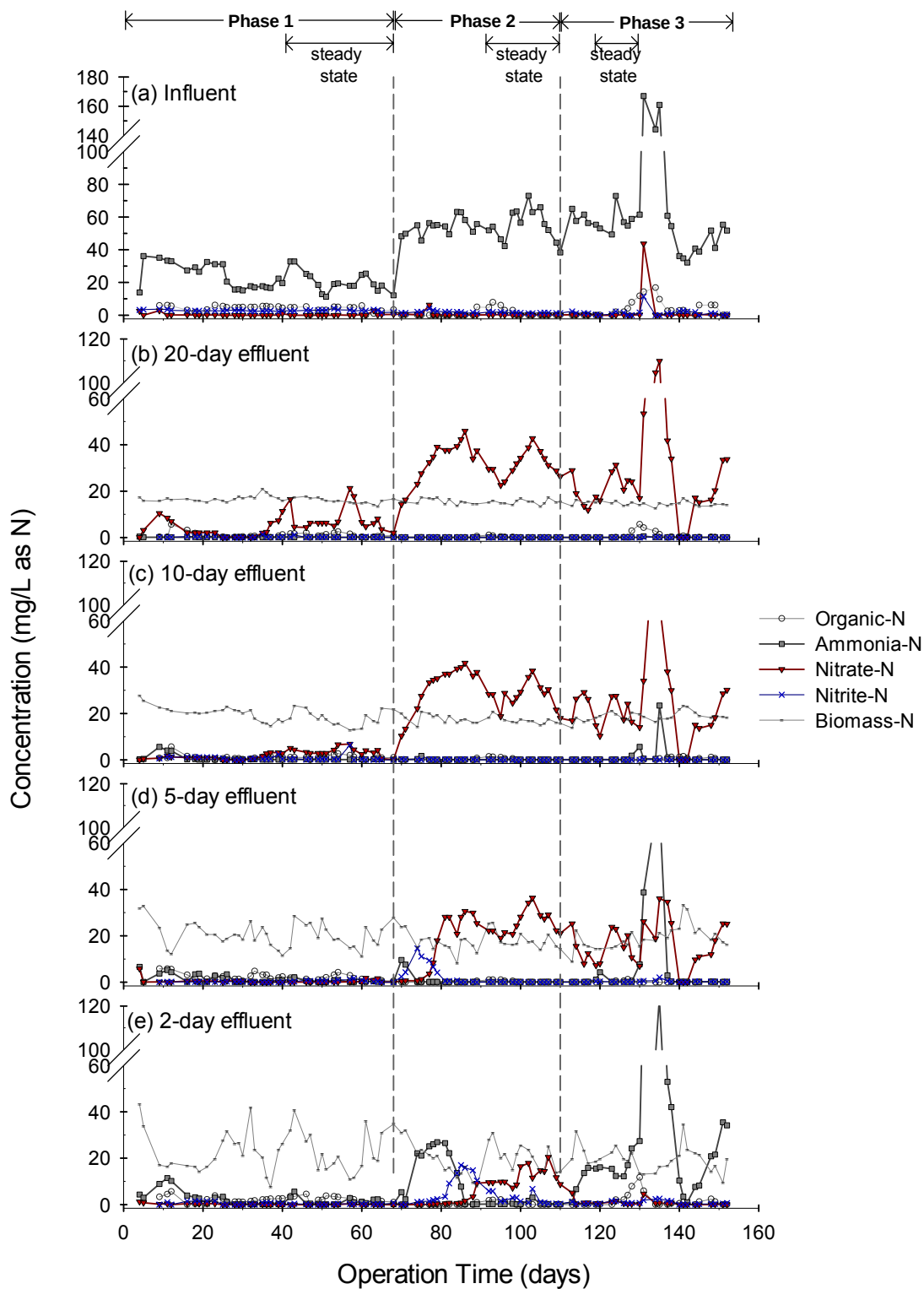


Figure 4-4. Concentrations of target nitrogen species in influent (a), and effluent (b ~ e) during three operating phases.

Table 4-5. Average influent and effluent concentrations (mg/L) of nitrogen compounds during steady-state of each test phase.

			2-day	5-day	10-day	20-day
Phase 1	Influent	Organic-N		3.3 (± 1.8)		
		NH ₄ ⁺		20.2 (± 5.9)		
		NO ₂ ⁻		2.8 (± 0.5)		
		NO ₃ ⁻		0.2 (± 0.2)		
		Total Inf. N		26.5		
	Effluent	Organic-N	2.2 (± 1.2)	1.5 (± 1.1)	1.1 (± 0.7)	0.9 (± 0.8)
		NH ₄ ⁺	0.8 (± 0.8)	0.5 (± 0.5)	<0.1	<0.1
		NO ₂ ⁻	0.2 (± 0.2)	0.4 (± 0.4)	0.6 (± 0.6)	0.2 (± 0.2)
		NO ₃ ⁻	<0.1	0.2 (± 0.2)	3.5 (± 1.6)	7.8 (± 5.0)
		Biomass N	~22.8	~21.5	~19.2	~17.0
		Total Eff. N	~26.1	~24.1	~24.5	~26.0
		Missing N	~0.4%	~2.4%	~2.0%	~0.5%
Phase 2	Influent	Organic-N		1.8 (± 1.8)		
		NH ₄ ⁺		54.9 (± 10.0)		
		NO ₂ ⁻		1.5 (± 0.3)		
		NO ₃ ⁻		0.1 (± 0.1)		
		Total Inf. N		58.3		
	Effluent	Organic-N	0.6 (± 0.6)	0.3 (± 0.3)	0.4 (± 0.4)	0.2 (± 0.2)
		NH ₄ ⁺	0.5 (± 0.5)	<0.1	<0.1	<0.1
		NO ₂ ⁻	2.3 (± 2.2)	0.0	0.0	0.0
		NO ₃ ⁻	12.1 (± 4.0)	26.2 (± 5.3)	31.6 (± 6.8)	34.2 (± 6.1)
		Biomass N	~21.0	~20.0	~18.5	~16.8
		Total Eff. N	~36.5	~46.6	~50.6	~51.3
		Missing N	~21.8	~11.7	~7.7	~7.0
Phase 3	Influent	Organic-N		3.5 (± 3.5)		
		NH ₄ ⁺		52.8 (± 4.2)		
		NO ₂ ⁻		0.8 (± 0.7)		
		NO ₃ ⁻		0.1 (± 0.1)		
		Total Inf. N		57.2		
	Effluent	biomass-N	2.5 (± 2.5)	0.3 (± 0.3)	0.2 (± 0.2)	0.7 (± 0.7)
		NH ₄ ⁺	17.1 (± 9.0)	0.9 (± 0.9)	0.6 (± 0.6)	0.1 (± 0.1)
		NO ₂ ⁻	0.5 (± 0.4)	0.2 (± 0.1)	0.0	0.0
		NO ₃ ⁻	0.7 (± 0.7)	18.7 (± 6.4)	20.6 (± 6.6)	21.6 (± 7.0)
		Biomass N	~20.1	~19.1	~18.6	~15.3
		Total Eff. N	~40.9	~39.2	~40.0	~37.7
		Missing N	~16.3	~18.0	~17.7	~19.5

Total Inf. N = Influent (Organic-N + NH₄⁺ + NO₂⁻ + NO₃⁻); Total Eff. N = Effluent (Organic-N + NH₄⁺ + NO₂⁻ + NO₃⁻ + Biomass N); Biomass N = daily wasted biomass-N; Missing N = (Total Inf. N - Total Eff. N).

multiply by active biomass concentration (high in longer SRT train) and reactor volume). This explained why the wasted biomass-N (equal to N for net growth under steady state operation) in the longer SRT train was less than that in the shorter SRT train (Figure 4-4, Table 4-5). Furthermore, high levels of endogenous decay in the longer SRT train would lead to additional ammonia available to both heterotrophs and AOB via hydrolysis of organic nitrogen from dead biomass (Rittmann et al., 1994; Kindaichi et al., 2004; Okabe et al., 2005). This would allow AOB to oxidize some ammonia in the longer SRT train even under ammonia limitation. Average nitrite plus nitrate concentrations (NO_x^-) of 8.0 mg/L and 4.1 mg/L were detected in effluent from the 20-, and 10-day trains, respectively, suggesting that ammonia oxidation occurred at low levels at these SRTs. However, significant ammonia oxidation did not occur in the 5- and 2-day SRT trains (as demonstrated by low effluent NO_x^- levels of 0.6 and 0.2 mg/L, respectively).

During phase 2 (relatively high ammonia, and high DO operation), average influent ammonia concentrations increased from 20.2 mg/L (during phase 1) to 54.9 mg/L (as N) via addition of ammonium chloride (NH_4Cl). Although a large portion of the influent ammonia-N (~ 40%) was taken up by the biomass, significant nitrification occurred in all SRT trains. Nitrifying bacteria (AOB and NOB) in the 20- and 10-day systems quickly converted the excess ammonia to nitrate via nitrite (no lag phase) (Figure 4-4 (b), (c)), while the 5-day SRT system required approximately one week to achieve complete nitrification (Figure 4-4 (d)), and the 2-day SRT system required approximately two and half weeks before nitrification was achieved (Figure 4-4 (e)). A transition period was noted in the 2-day system. For approximately the first ten days, high levels of untreated ammonia were detected in the effluent, while nitrite and nitrate concentrations in the effluent were at or below the detection limit. After about one week, nitrite levels began to rise and a corresponding drop in effluent ammonia concentration was measured. Nitrite build-up continued for about five days before the levels began to drop. Ultimately, effluent ammonia and nitrite reached low levels and nitrate was measured in the effluent. However, the nitrate production levels in the 2-day train were usually lower than that in longer SRT trains implying that the longer SRT systems had better nitrification treatment performance. Furthermore, wasted biomass-N in the 2-day train was higher than that in

longer SRT trains; this again indicates that net growth in shorter SRT systems was higher than that in longer SRT systems. When considering the nitrogen balance in each SRT train during phase 2, approximately 12% (20-day) to 37% (2-day) of nitrogen in the influent could not be recovered from the effluent. [Yang et al. \(2005\)](#) found that over 90% of nitrogen was recovered during sequencing batch reactor operation for nitrification (based on a nitrogen mass balance using ammonia, nitrite, nitrate, and biomass-N). Unbalance in nitrogen could be due to either production of other, not-measured, nitrogen forms or underestimation of biomass-N. In a biomass digestion study, [Jung et al. \(2004\)](#) showed that biomass-N levels were similar under all conditions tested (alternating aerobic/anaerobic operating periods); however, extended anaerobic treatment resulted in more missing nitrogen (~50%) than shorter periods (<10%) due to production of gaseous nitrogen. Moreover, aerobic denitrification has been found in nitrification systems ([Robertson and Kuenen, 1990](#); [Third et al., 2003](#); [Thomas et al., 2005](#)). Hence, gaseous products from denitrification (e.g., N_2 or N_2O) may account for the missing nitrogen mass noted in this study. The missing nitrogen mass was considered as a portion of ammonia-oxidation performance by AOB in this study ([Appendix A.4](#)) because influent ammonia is first oxidized to nitrite by AOB before further oxidation to nitrate and/or reduction to gaseous nitrogen compounds. Based on above-mentioned analyses, it could be surmised that ammonia oxidation by AOB occurred at a higher level in all four SRT trains during phase 2 since excess ammonia was available in the influent.

During phase 3, the operating DO level was lowered to 0.5 mg/L while ammonia supplementation continued. The average influent ammonium concentration during steady-state (not including the shock loading period) was 52.8 mg/L (as N) ([Figure 4-4](#), [Table 4-5](#)). In the 2-day SRT system, effluent nitrate levels began to drop and a corresponding rise in effluent ammonia concentration was observed ([Figure 4-4 \(e\)](#)), suggesting a drop in nitrification performance in this SRT train. In the 5-, 10-, and 20-day SRT trains, nitrate continued to be measured in the effluent while ammonia levels in the effluent remained low. However, effluent nitrate levels during this phase were lower than those measured during phase 2. This may be due to either a drop in nitrification performance or a raise in denitrification of nitrite or nitrate. It was surmised that

decreases in the effluent nitrate level for the 5-, 10-, and 20-day trains were due to enhanced denitrification during low DO operation (Third et al., 2003; Thomas et al., 2005), rather than decreased nitrification based on the fact that ammonia did not accumulate in the effluent (Figure 4-4). Thus, most of the influent ammonia available to the nitrifying bacteria in the industrial bench-scale system was oxidized to nitrite and/or nitrate, some of which was subsequently denitrified (to gaseous nitrogen compounds that were not measured in this study) under the low DO condition during phase 3. So, estimation of ammonia oxidation for AOB (Appendix A.4) included the unmeasured nitrogen mass. Based on the stated assumption and estimation, low DO operation during phase 3 significantly lowered ammonia oxidation treatment performance only in the 2-day SRT system and not in the 5-, 10-, and 20-day SRT systems.

It should be noted that a shock-loading period occurred during phase 3 operation. High levels of ammonia and nitrate were detected in the influent for the industrial bench-scale system on operational day 131 and continued for six days (Figure 4-4 (a)). The 20- and 10-day SRT systems effectively treated the shock ammonia load. Most of the effluent-N was in the nitrate form with little ammonia present in the effluent of these two longer SRT systems (Figure 4-4, Table 4-6). The 5-day SRT system only treated a portion of ammonia and additional untreated ammonia remained in the effluent. The 2-day SRT system produced very little nitrate and most of the ammonia remained untreated in its effluent. These results demonstrate that the longer SRT systems had a high capacity to receive sudden increases in ammonia loading. Researchers have noted that AOB in long SRT reactors can adjust their activity (rather than population size) to adapt to short-term changes in ammonia loading (Aoi et al., 2004 (a), (b)). Interestingly, effluent nitrate levels in the 20-, 10-, and 5-day SRT systems decreased to below detection levels after shock loading and remained there for four days (post-shock-loading period). After this period, the concentration slowly rose over time to levels measured before introduction of the shock load. These results show that ammonia shock-loading impacted nitrification performance even though the longer SRT systems quickly responded to sudden increase in ammonia loading.

Table 4-6. Average influent and effluent concentrations (mg/L) of nitrogen compounds during shock loading, and post shock loading periods.

			2-day	5-day	10-day	20-day
Shock loading	Influent	Organic-N	13.5 (±3.6)			
		NH ₄ ⁺	157.4 (±11.8)			
		NO ₂ ⁻	11.3			
		NO ₃ ⁻	43.6			
		Total Inf. N	225.8			
	Effluent	Organic-N	0.0	0.0	0.3 (±0.3)	2.0 (±1.0)
		NH ₄ ⁺	110.5 (±19.0)	74.3 (±3.2)	11.9 (±11.9)	0.1 (±0.0)
		NO ₂ ⁻	2.4 (±0.1)	1.3 (±1.0)	0.1 (±0.1)	0.1 (±0.0)
		NO ₃ ⁻	0.7 (±0.1)	27.4 (±12.3)	83.4 (±22.1)	107.0 (±3.8)
		Biomass N	~14.4	~19.4	~16.9	~13.6
		Total Eff. N	~128.0	~122.4	~112.6	~122.8
Post - shock loading	Influent	Organic-N	2.3 (±1.1)			
		NH ₄ ⁺	33.4 (±1.8)			
		NO ₂ ⁻	2.5 (±0.5)			
		NO ₃ ⁻	0.0			
		Total Inf. N	38.2			
	Effluent	Organic-N	0.7 (±0.3)	0.1 (±0.0)	1.1 (±0.5)	0.5 (±0.2)
		NH ₄ ⁺	2.2 (±1.8)	<0.1	<0.1	<0.1
		NO ₂ ⁻	0.0	0.0	0.0	0.0
		NO ₃ ⁻	0.0	0.0	0.0	0.0
		Biomass N	~28.3	~32.2	~23.0	~16.3
		Total Eff. N	~31.2	~32.4	~24.2	~16.9

Total Inf. N = Influent (Organic-N + NH₄⁺ + NO₂⁻ + NO₃⁻); Total Eff. N = Effluent (Organic-N + NH₄⁺ + NO₂⁻ + NO₃⁻ + Biomass N); Biomass N = daily wasted biomass-N.

4.1.4 Volumetric ammonia-oxidation rate

The volumetric ammonia-oxidation rate ($\mu\text{mol/L/hr}$, or $\mu\text{M/hr}$) was used to describe the ammonia-oxidation capacity for each SRT train. During phase 1 steady-state operation, average ammonia-oxidation rates were relatively low because of the small amount of ammonia available for AOB (approx. 0~11.1 $\mu\text{mol NH}_3\text{-N/L/hr}$) (Figure 4-5, Table 4-7). Longer SRT systems (20-, and 10-day) had higher rate values than the 2-day SRT system ($p\text{-value}<0.05$, Table 4-8). Low ammonia-oxidation rates confirmed the previous inference that ammonia oxidation only occurred at a low level in each of four SRT treatment trains during phase 1 (low-N operation) due to the limited ammonia available.

During the steady-state operation of phase 2 (relatively high ammonia and high DO operation), the average ammonia-oxidation rates were 58, 63, 65, and 68 $\mu\text{mol/L/hr}$ for the 2-, 5-, 10-, and 20-day SRT trains, respectively. These rate values were significantly higher than those estimated in phase 1 ($p\text{-value}<0.05$) (Table 4-9). This demonstrated that significant ammonia-oxidation occurred in all four SRT systems after addition of supplemental ammonia to the influent during phase 2. Additionally, it appeared that the higher SRT systems resulted in higher ammonia-oxidation rates (theoretically reasonable since higher SRT systems typically have better substrate (COD or ammonia) treatment performance, Section 2.4.2). However, differences between ammonia-oxidation rates for the four SRT systems were not statistically significant ($p\text{-value}>0.05$) based on results of t-test (Table 4-8).

After the operating DO level was lowered to 0.5 mg/L during phase 3, only the average ammonia-oxidation rate of the 2-day train significantly dropped (from ~58 $\mu\text{mol/L/hr}$ (phase 2, high DO) to ~27 $\mu\text{mol/L/hr}$, $p\text{-value}<0.05$, Table 4-10). The average ammonia-oxidation rates in the 20-, 10-, and 5-day SRT trains remained as high as that evaluated during phase 2 ($p\text{-value}>0.05$, Table 4-10). It was concluded that low DO operation during phase 3 significantly impacted only the ammonia oxidation performance in the 2-day train, but did not affect that in the 5-, 10-, and 20-day trains of the industrial bench-scale system.

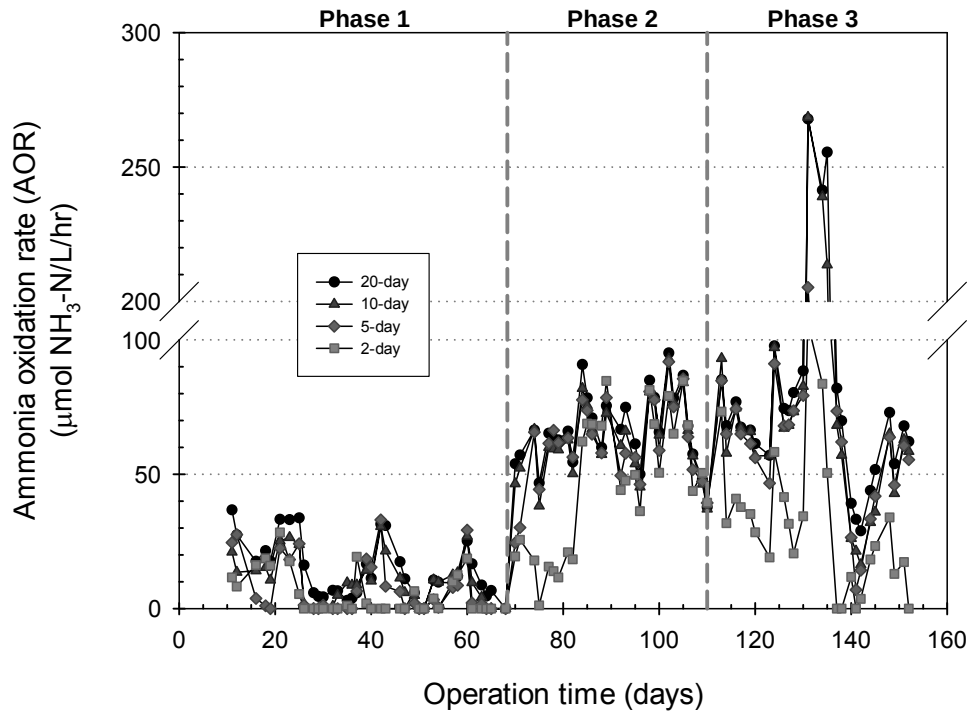


Figure 4-5. Ammonia-oxidation rate ($\mu\text{mol NH}_3\text{-N/ L/hr}$, or $\mu\text{M NH}_3\text{-N/hr}$) in each SRT train over time.

Table 4-7. Average ammonia-oxidation rate in each SRT train during steady-state operation of each test phase and during the shock-loading period (in $\mu\text{mol NH}_3\text{-N/ L/hr}$, or $\mu\text{M NH}_3\text{-N/hr}$).

		Phase 1	Phase 2	Phase 3	Shock loading
		High DO	High DO	Low DO	Low DO
		Low NH ₃	High NH ₃	High NH ₃	Shock NH ₃
NH ₃ available for AOB		0 ~ 11.1	58.8 ~ 68.3	58.8 ~ 68.3	251.7 ~ 257.1
NH ₃ oxidized by AOB	20-day	11.1 (± 9.9)	68.1 (± 16.3)	68.1 (± 15.3)	254.9 (± 13.3)
	10-day	8.1 (± 8.1)	65.0 (± 16.2)	61.8 (± 17.9)	240.4 (± 27.4)
	5-day	3.8 (± 3.8)	63.2 (± 16.4)	60.4 (± 16.2)	148.1 (± 49.7)
	2-day	0.0	58.0 (± 16.4)	26.8 (± 14.6)	80.8 (± 28.2)

Table 4-8. Results (p-value) of a statistical t-test to assess average ammonia-oxidation rates between different SRTs during three test phases.

	Phase 1			Phase 2			Phase 3		
	20-day	10-day	5-day	20-day	10-day	5-day	20-day	10-day	5-day
10-day	0.368	--	--	0.616	--	--	0.322	--	--
5-day	0.062	0.279	--	0.417	0.751	--	0.218	0.860	--
2-day	<0.05*	<0.05*	0.084	0.411	0.256	0.411	<0.05*	<0.05*	<0.05*

* statistically significant at the $\alpha=0.05$ level.

Table 4-9. Statistical t-test results comparing differences in average ammonia oxidation rate (AOR) for each SRT train during phase 1 and phase 2 operation.

	Ammonia Oxidation Rate [†]		Statistical t-test [‡] for	
	Phase 1	Phase 2	$H_0: AOR_{\text{phase1}} \geq AOR_{\text{phase2}}$	
	($\text{NH}_3 \approx 20.2 \text{ mg/L}$)	($\text{NH}_3 \approx 54.9 \text{ mg/L}$)	p-value	Test result
20-day	11.1 (± 9.9)	68.1 (± 16.3)	<0.05	$AOR_{\text{phase1}} < AOR_{\text{phase2}}$
10-day	8.1 (± 8.1)	65.0 (± 16.2)	<0.05	$AOR_{\text{phase1}} < AOR_{\text{phase2}}$
5-day	3.8 (± 3.8)	63.2 (± 16.4)	<0.05	$AOR_{\text{phase1}} < AOR_{\text{phase2}}$
2-day	0.0	58.0 (± 16.4)	<0.05	$AOR_{\text{phase1}} < AOR_{\text{phase2}}$

[†] Ammonia Oxidation Rate ($\mu\text{mol NH}_3\text{-N/L/hr}$): average ammonia oxidation rates during quasi-steady-state of two different ammonium-loading phases (14 data points during day 47-68 of phase 1; day 92-110 of phase 2).

[‡] The p-values of t-test were testing results for hypotheses of $H_0: AOR_{\text{phase1}} \geq AOR_{\text{phase2}}$; & $H_a: AOR_{\text{phase1}} < AOR_{\text{phase2}}$; using $\alpha=0.05$. When “p-value < $\alpha(=0.05)$ ”, “ $AOR_{\text{phase1}} < AOR_{\text{phase2}}$ ” was statistically acceptable; Otherwise, the analyzed data could not provide sufficient evidence to verify that “ $AOR_{\text{phase1}} \geq AOR_{\text{phase2}}$ ”.

Table 4-10. Statistical t-test results comparing differences in average ammonia oxidation rate (AOR) for each SRT train under high and low DO levels (3.0, and 0.5mg/L). Results of statistical analyses suggested that low DO operation during phase 3 did significantly (p-value<0.05) impact the ammonia oxidation rate in the 2-day SRT treatment system.

	Ammonia Oxidation Rate [†]		Statistical t-test [‡] for	
	Phase 2	Phase 3	H ₀ : AOR _{phase2} = AOR _{phase3}	
	(DO = 3.0 mg/L)	(DO = 0.5 mg/L)	p-value	Test result
20-day	68.1 (±16.3)	68.1 (±15.3)	0.695	AOR _{phase2} = AOR _{phase3}
10-day	65.0 (±16.2)	61.8 (±17.9)	0.393	AOR _{phase2} = AOR _{phase3}
5-day	63.2 (±16.4)	60.4 (±16.2)	0.478	AOR _{phase2} = AOR _{phase3}
2-day	58.0 (±16.4)	26.8 (±14.6)	<0.05	AOR _{phase2} > AOR _{phase3}

[†] Ammonia Oxidation Rate (μmole NH₃-N/ liter-hour): average ammonia oxidation rate during quasi-steady-state of two different DO operating phases (14 data points during day 92-110 of phase 2; day 120-130 and day 144-152 of phase 3).

[‡] The p-values of t-test were testing results for hypotheses of H₀: AOR_{phase2} = AOR_{phase3}; & H_a: AOR_{phase2} ≠ AOR_{phase3}; using α=0.05. When p-value < α(=0.05), it suggested that AOR_{phase2} = AOR_{phase3}. Otherwise, evidence was not sufficient to verify that AOR_{phase2} ≠ AOR_{phase3}.

It is generally considered that the ammonia-oxidation capacity in nitrifying systems may depend on AOB population size and physiological activity (Wagner et al., 1995; Mobarry et al., 1996; Dionisi et al., 2002). Based on above-mentioned results from this study, it could be expected that the population and/or activity of AOB in the industrial bench-scale system might be low (especially in the lower SRT train) during phase 1, while ammonia-oxidation reaction was relatively insignificant under ammonia limitation condition. However, the AOB population size and/or activity might have been changed as an explanation for the increases in ammonia-oxidation rate under a relatively high ammonia supply during phase 2. Moreover, the low DO operation during phase 3 might impact either AOB population or activity in the 2-day SRT train and then cause a drop in ammonia-oxidation rate. This expectation will be further examined by molecular surveys based on the quantifications for a stretch of AMO functional enzyme encoding gene (i.e., *amoA*), and transcripts (i.e., *amoA* mRNA) using real-time PCR and reverse transcription (RT) real-time PCR assays (Section 4.2, Section 6.2).

4.2 Industrial bench-scale system microbial population

Biosolids samples taken from bioreactors of the industrial bench-scale system during each operating phase were used to quantify the microbial population based on real-time PCR assays. Biosolids samples were chosen from each SRT train during steady-state of each operating phase for genetic analysis. Bacterial DNA was extracted from selected samples and used to assess target bacterial numbers. A real-time PCR assay based on previous work (Dionisi et al., 2002) was used to quantify total eubacteria by specifically targeting a section of 16S rRNA gene (16S rDNA) in bacterial genomic DNA (Section 3.4.4). Three real-time PCR assays were designed and optimized to quantify three AOB species and isolates identified in the industrial bench-scale system (including *Nitrosomonas nitrosa*, uncultured AOB RI-27, and B2-3 isolates) by specifically targeting a section of the *amoA* gene in genomic DNA of each of these AOB (Section 3.4.4). For comparison of target bacterial populations in the four SRT systems, molecular results are presented as cells per liter of mixed liquor (cells/L).

4.2.1 Population of total eubacteria

The total eubacterial numbers in each SRT system under steady state of each operating phase are plotted in Figure 4-6, and the averages are summarized in Table 4-11. Results showed that the total eubacteria numbers in the 2-day SRT train decreased somewhat after about forty days of operation, while those in the longer SRT trains remained relatively constant. However, during the steady-state of each operating period, numbers of total bacteria in each SRT train were within an order of magnitude ($1.5\text{--}12.1 \times 10^{11}$ cells/L), similar to previous results (i.e., $4.3 \pm 2.0 \times 10^{11}$ cells/L for a municipal wastewater treatment system, Harms et al., 2003) and slightly lower than $4.8 \pm 2.0 \times 10^{12}$ cells/L for an industrial full-scale WWTP (Layton et al., 2005). This demonstrated that the active bacterial population in the system was stable as a result of consistent substrate (COD and/or ammonia) utilization. Because of the relatively high influent COD levels (compared to influent ammonia levels), heterotrophic bacteria should predominate the microbial population in the industrial bench-scale system. Thus, the number of the total bacteria should be similar to the number of heterotrophic bacteria.

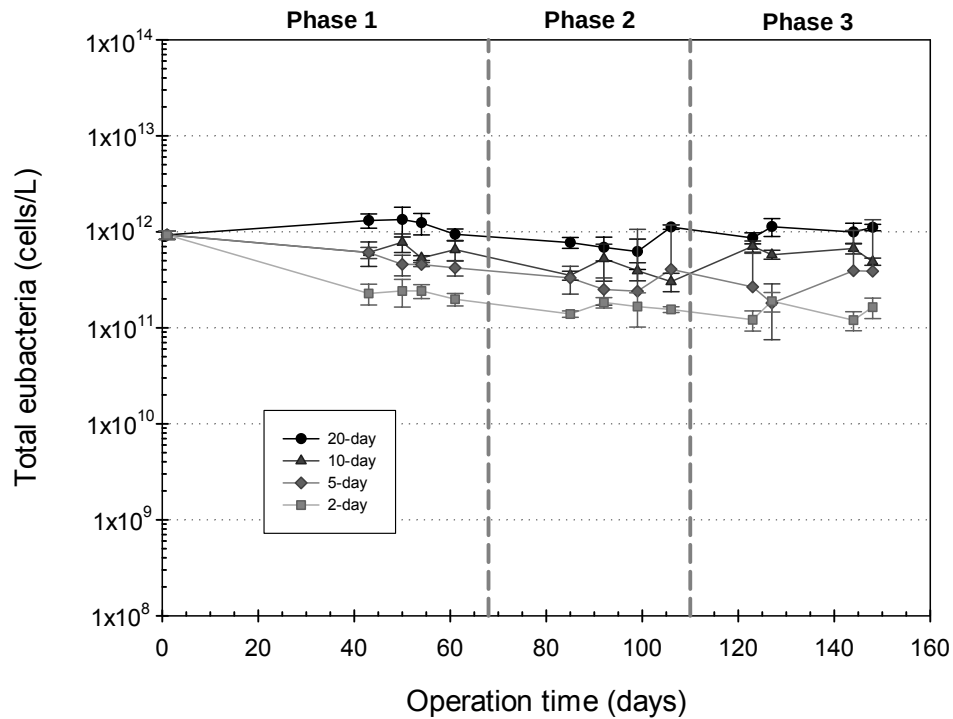


Figure 4-6. Total eubacteria concentrations in each SRT system during steady-state of the three operating phases.

Table 4-11. Average total eubacteria (cells/L) in each SRT train during steady-state of the three operating phases.

	Phase 1	Phase 2	Phase 3
	High DO	High DO	Low DO
	Low NH ₃	High NH ₃	High NH ₃
20-day	$12.10 (\pm 1.84) \times 10^{11}$	$7.99 (\pm 2.20) \times 10^{11}$	$10.20 (\pm 1.24) \times 10^{11}$
10-day	$6.42 (\pm 1.02) \times 10^{11}$	$3.92 (\pm 0.96) \times 10^{11}$	$6.10 (\pm 0.97) \times 10^{11}$
5-day	$4.85 (\pm 0.83) \times 10^{11}$	$3.07 (\pm 0.77) \times 10^{11}$	$3.07 (\pm 1.02) \times 10^{11}$
2-day	$2.27 (\pm 0.20) \times 10^{11}$	$1.61 (\pm 0.19) \times 10^{11}$	$1.48 (\pm 0.34) \times 10^{11}$

During each operating phase, the longer SRT trains had higher total bacteria numbers (in cells/L) than the shorter SRT trains (Figure 4-6, Table 4-11) which is in agreement with the higher MLVSS concentrations measured in the longer SRT trains. The linear relationship depicted in Figure 4-7 indicates that higher numbers of total bacteria were found in biosolid samples with higher concentrations of MLVSS. When the total bacteria number was normalized to mass of MLVSS, the value was essentially constant at a mean of $3.34 \pm 0.87 \times 10^8$ cells/mg MLVSS. This suggests that the SRT, DO level, influent ammonia concentration, and COD concentration did not significantly change the population of total eubacteria (predominantly heterotrophs) on a unit mass basis. However, this information was not sufficient to determine whether the populations of slow-growing autotrophic AOB in the microbial community were also stable under severe select pressures from factors of SRT, DO level, or ammonia concentration. Therefore, three *amoA*-based real-time PCR assays were developed to enumerate the AOB population and assess the AOB population dynamics under different designated operational conditions in the industrial bench-scale system (Section 4.2.4).

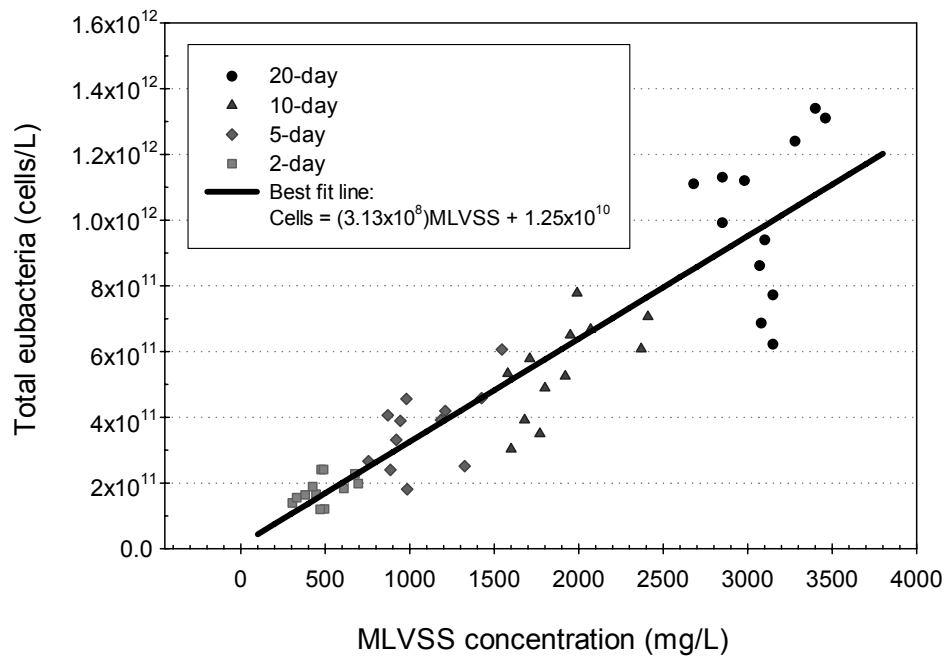


Figure 4-7. Correlation between MLVSS (mg/L) and total eubacteria (cells/L).

4.2.2 The *amoA* gene library

Target *amoA* gene in genomic DNA extracted from biosolid samples was amplified by PCR using the amoA-1F/ amoA-2R primer pair or by nested-PCR using a combination of AMO-F2*/amoA-2R* and amoA-1F/amoA-2R* primer pairs for further establishing *amoA* gene libraries for the industrial bench-scale system (Appendix B). The sizes of *amoA* genes amplified from the PCR and the nested-PCR were 491 bp (base pair) and 500 bp, respectively. The PCR products were checked on a 1.5% (w/v) agarose gel to insure that the target gene had been amplified (Figure 4-8). The PCR products were subsequently cloned into plasmid vector (pCR[®]4-TOPO[®]) by one-shot chemical transformation as described in the instructions of the manufacturer (“TOPO TA cloning[®] kit for sequencing”; Invitrogen, Carlsbad, CA).

Colonies were picked from each clone library and further analyzed by PCR screening (as described in the instructions, “TOPO TA cloning[®] kit for sequencing”; Invitrogen, Carlsbad, CA) to determine which clones carried target inserts. Figure 4-9 (a) depicts an example PCR screening result. The clones with target inserts had a PCR product of approximately 500bp after amplification with amoA-1F/amoA-2R or amoA-1F/amoA-2R* primer sets (e.g., clones A and C showed in Figure 4-9 (a)), while the clones without target inserts did not have one (e.g., clone B showed in Figure 4-9 (a)). Plasmid DNA of selected clones with target inserts was extracted and purified for sequencing. Isolated plasmid DNA was checked with restriction analysis (digested with *EcoRI*) to estimate the quantity of extract and to once again make sure it carried target gene (Figure 4-9 (b)).

Additionally, products from the PCR screening process were further digested with *ClaI* and *EcoRI* + *ClaI* to compare the digestion patterns for clones carrying an unknown target gene sequence with those for clones carrying the known target gene sequence. For instance, digestion patterns of sample S₁, and S₃ (Figure 4-10 (a)) were similar with those of R₁ (cloned gene for *N. nitrosa*) and R₂ (cloned gene of RI-27), respectively; while the digestion pattern of sample S₂ was different from that of the reference clones (R₁, R₂, and R₃). Another example showed that digestion patterns of samples S₆ and S₈ (Figure 4-10

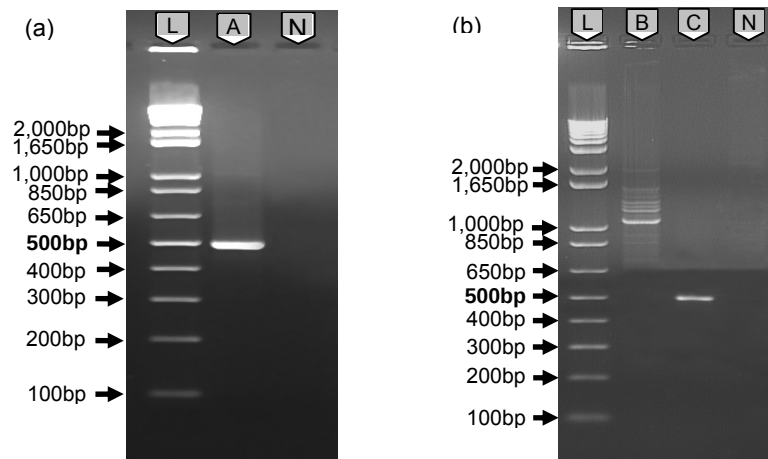


Figure 4-8. Examples of gel electrophoresis (100mV for 1~2 hours) of the *amoA* gene product in 1.5% agarose gels, (a) PCR products amplified from genomic DNA extracted from 20-day biosolid sample for day 106, and (b) nested-PCR products amplified from genomic DNA extracted from 2-day biosolid sample for day 106. (L: 1Kb Plus DNA Ladder; A: PCR product; B: nested-PCR step1 products; C: nested-PCR step2 product; N: negative control.)

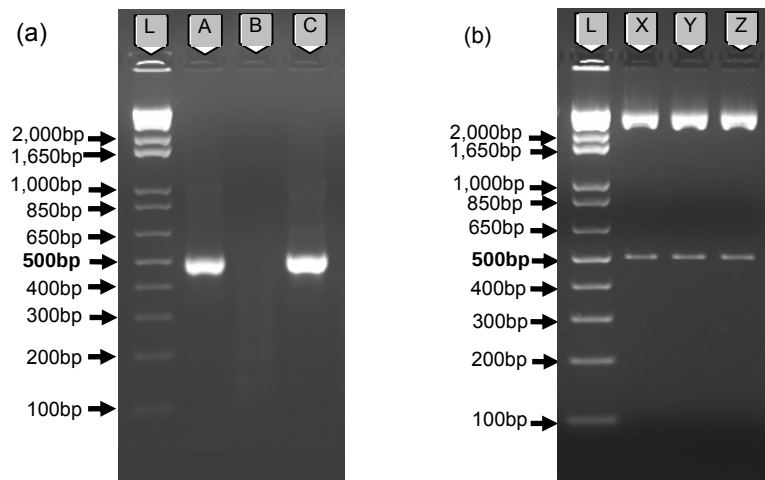


Figure 4-9. Examples of agarose gels after electrophoresis used for checking clones for target inserts by (a) PCR screening, and (b) restriction analysis (digested with *EcoRI*) after plasmid DNA purification. (L: 1Kb Plus DNA Ladder; A, B, and C: PCR products of 5-day library#1 clone41, 42, and 43, respectively; X, Y, and Z: restriction analyses of purified plasmid DNA of 5-day library #1 clone24, 27, and 45, respectively.)

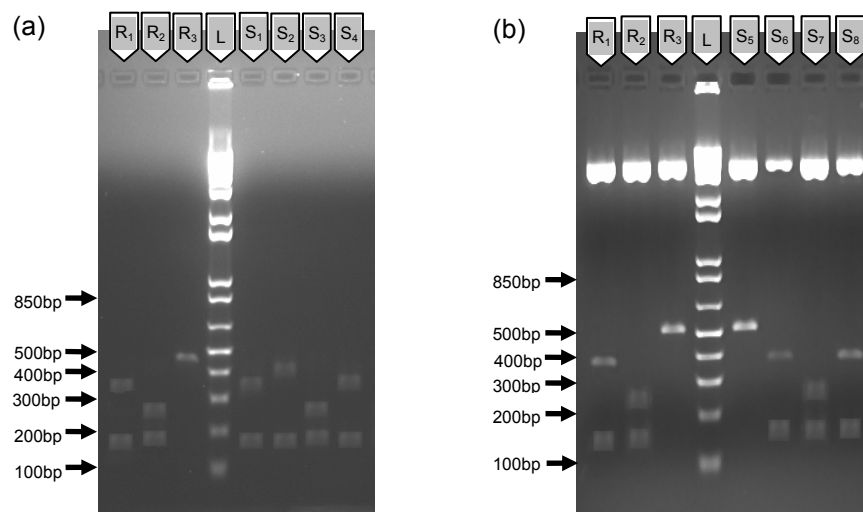


Figure 4-10. Examples of agarose gels after electrophoresis used to check clones with target inserts using (a) restriction analysis (digested with *Cla*I) followed by PCR, and (b) restriction analysis (digested with *Eco*RI + *Cla*I) after plasmid DNA purification. (L: 1Kb Plus DNA Ladder; R₁, R₂, and R₃: reference clone libraries with gene sequences of *N. nitrosa* [AJ238495], RI-27 [AF532311], and W637 [AF353251], respectively; S₁, S₂, S₃, and S₄: restriction analyses of PCR products of 2-day library #2 clone36, 39, 41, and 46, respectively; S₅, S₆, S₇, and S₈: restriction analyses of purified plasmid DNA of 2-day library #2 clone2, 38, 42, and 52, respectively.)

(b)) were similar with that of R₁ (cloned *amoA* gene for *N. nitrosa*); while the digestion pattern of sample S₅ was similar with R₃ (cloned *amoA* gene for an uncultured AOB group, W637). This additional process reduced the number of clones selected for further plasmid DNA purification and sequencing (only clones with new digestion patterns were selected). Table 4-12 summarizes the number of colonies picked from each clone library, the number of clones with target gene inserts, and the number of clones selected for sequencing. At least 30-70 clones were picked from each clone library to screen for inserts of target *amoA* gene. PCR screening results showed that 14, 9, 55, and 77 clones for the 20-, 10-, 5-, and 2-day SRT trains, respectively, had target *amoA* gene inserts (among which, 14, 9, 7, and 28 clones were subsequently selected for sequencing). Information of *amoA* sequence was used to identify AOB in the industrial bench-scale system, and to design real-time PCR primer/probe sets to quantify the found AOB groups (Section 4.2.3).

Table 4-12. Summary of (a) number of colonies picked from each clone library for further analyses, (b) number of clones with target inserts checked by PCR screening, and (c) number of clones selected for plasmid DNA purification and sequencing.

SRT	<i>amoA</i> library	Primer sets used for PCR amplification	(a) Number of colonies picked	(b) [†] Number of clones with target inserts	(c) Number of clones selected for sequencing
20-day	Library #1	amoA-1F/amoA-2R	50	14	14
10-day	Library #1	amoA-1F/amoA-2R	50	9	9
5-day	Library #1	amoA-1F/amoA-2R	50	29	3
	Library #2	AMO-F2*/amoA-2R* + amoA-1F/amoA-2R*	34	26	4
2-day	Library #1	amoA-1F/amoA-2R	50	12	12
	Library #2	AMO-F2*/amoA-2R* + amoA-1F/amoA-2R*	70	65	16

[†] The *amoA* gene library

4.2.3 Identification of AOB based on *amoA* gene

Sequences of a section of the *amoA* gene (approximately 450 bp, target minus primers sites) cloned in this study were compared with the *amoA* sequences in publicly accessible databases using the BLAST program (Altschul et al., 1990). Based on sequence comparisons of the *amoA* gene, the majority of cloned target sequences for the four SRT trains were highly affiliated ($\geq 98\%$ similarity) with *Nitrosomonas nitrosa* Nm90 (GenBank accession number AJ238495), an uncultured AOB isolate RI-27 (GenBank accession number AF532311), and another uncultured AOB isolate B2-3 (GenBank accession number AF293067). A few clones also closely matched uncultured AOB isolates W637 [AF353251], and TA921i [AF339043] (Table 4-13).

The target *amoA* sequences from both samples and published databases were aligned with the CLUSTAL X program (Thompson et al., 1997) to construct a phylogenetic tree (Figure 4-11). Results suggested that most AOB in the four SRT trains were closely related to three phylogenetically distinct groups including *N. nitrosa* Nm90 [AJ238495], an uncultured AOB isolate RI-27 [AF532311], and another uncultured AOB isolate B2-3 [AF293067]. The *N. nitrosa* and B2-3 groups were in the *N. communis* and *N. oligotropha* cluster, respectively; whereas, the RI-27 group formed a sole cluster near the *N. communis* and *N. europaea* clusters (Figure 4-11). It should be noted that the *amoA* gene sequence of clone I-212 (GenBank accession number AF420294, Dionisi et al., 2002) is from a clone library for the industrial full-scale WWTP from which this bench-scale study system was modeled. The *amoA* gene sequence of clone I-212 is similar ($\geq 98\%$ similarity) to *N. nitrosa*. In the previous work (Dionisi et al., 2002); no clones in the industrial full-scale WWTP libraries were identified as either RI-27 or B2-3. This suggested that the AOB population significantly shifted during the bench-scale system study period. Specifically, the *amoA* gene sequences of the *N. nitrosa* and RI-27 groups were revealed in the clone libraries for all four SRT trains, whereas the *amoA* gene sequence of the B2-3 group was only found in the clone libraries for the 2-, and 5-day SRT systems (Table 4-13, Figure 4-11). This suggested that different operating SRTs likely impacted AOB population dynamics within the industrial bench-scale treatment system.

Table 4-13. Identification of *amoA* clone library for the four SRT systems in the industrial bench-scale system using the published *amoA* sequences from a publicly accessible database (National Center for Biotechnology Information, NCBI) as reference.

Reference AOB	SRT	Closely matched clones	
		<i>amoA</i> Library #1	<i>amoA</i> Library #2
<i>N. nitrosa</i>	20-day	7*,12,13,31,34,40	---
	10-day	5*,11, 24	---
	5-day	2*	32
	2-day	12,15	5*,54,60
RI-27	20-day	3*,20,42,44	---
	10-day	1*, 26 [†] ,49	---
	5-day	34*,45	24
	2-day	NM	18*, 66
B2-3	20-day	NM	---
	10-day	NM	---
	5-day	NM	1*,21 [†]
	2-day	NM	16*, 17 ,21,39
W637	20-day	NM	---
	10-day	18*	---
	5-day	NM	NM
	2-day	NM	8*
TA-921i	20-day	NM	---
	10-day	NM	---
	5-day	NM	NM
	2-day	NM	2*, 40

* The typical clones selected for phylogenetic analysis; NM: no match clone; n: the clones used as standards for real-time PCR quantification; n: the clone selected to prepare real-time PCR standard; and †: the clone selected to prepare (*amoA* mRNA) RT real-time PCR standard.

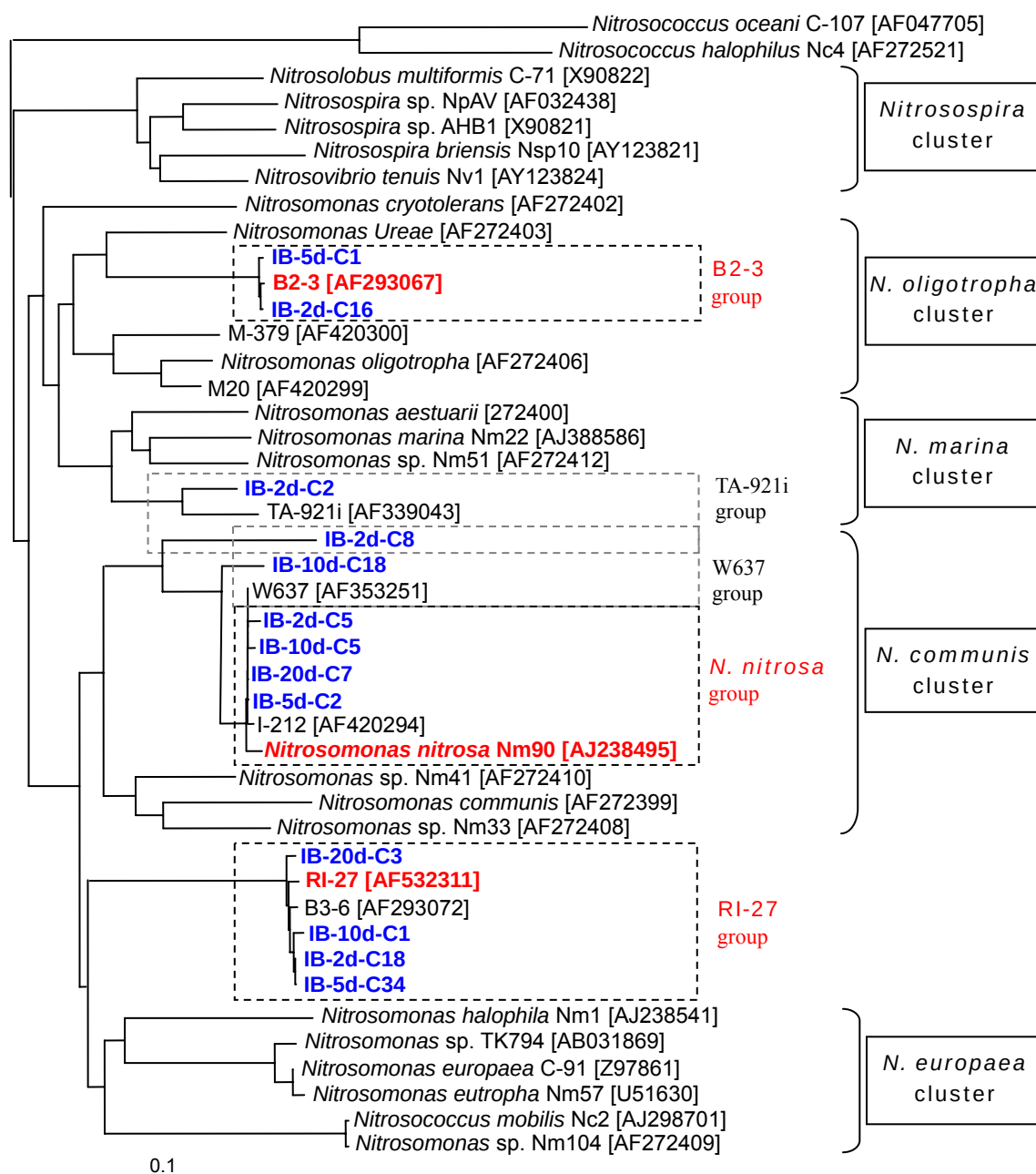


Figure 4-11. Phylogenetic relationship for cloned *amoA* gene from the industrial bench-scale system. Most of the *amoA* sequences in the clone libraries from the four SRT trains were close related ($\geq 98\%$ similarity) to *N. nitrosa* Nm90 [AJ238495], an uncultured AOB isolate RI-27[AF532311], and another uncultured AOB isolate B2-3[AF293067]. (Clone was numbered as IB-#1d-C#2, in which #1=operating SRT, and #2=clone number).

The alignment results of the target *amoA* sequences were also used to design real-time PCR primers and probes to quantify dominant AOB strains or species in the industrial system. Two real-time PCR assays using newly designed primers and probes were developed in this study to enumerate the population of RI-27 and B2-3. The result of the *amoA* sequence alignment appears in [Figure 4-12](#). The position of each real-time PCR primer and probe were highlighted in the list. When the sequences of primers and probes specific for *N. nitrosa* ([Dionisi et al., 2002](#); [Layton et al., 2005](#)) were compared with *amoA* sequences at these primer or probe sites in other AOB, more than one mismatch was found ([Table 4-14](#)). For instance, there were 4, 7, and 4 mismatches between *N. nitrosa* and RI-27 at the originally designed forward primer (amoA-Nn-548f) site, probe (amoA-Nn-Taq- 740r) site, and reverse primer (amoA-Nn-777r) site, respectively ([Figure 4-12](#), [Table 4-14](#)). Thus, the real-time PCR primer/probe set specific for *N. nitrosa* group would not quantify other AOB groups (e.g., RI-27 or B2-3).

Two new real-time PCR primer/probe sets specific for RI-27 and B2-3 were designed in this study. The sequences of these two primer/probe sets are summarized in [Table 3-6](#), and their specificities are summarized in [Table 4-15](#). The newly designed real-time PCR primer/probe set (amoA-[RI-27]-542f/amoA-[RI-27]-bhq-651r/amoA-[RI-27]-679r) was specific for the RI-27 group, and had more than one mismatch with other AOB groups ([Table 4-15](#)). There were 6, 6, and 5 mismatches between RI-27 and *N. nitrosa* at the forward primer (amoA-[RI-27]-542f) site, probe (amoA-[RI-27]-bhq-651r) site, and reverse primer (amoA-[RI-27]-679r) site, respectively. Another newly designed real-time PCR primer/probe set (amoA-[B2-3]-596f/amoA-[B2-3]-bhq-713r/amoA-[B2-3]-744r) was specific for the B2-3 and TA921i groups ([Table 4-15](#)). At these primer/probe sites, more than one mismatch in *amoA* sequence was found between B2-3 and other AOB groups. Clone IB-2d-C8 more closely matched (85% similarity) the W637 group, but the sequence was similar to the primer/probe sequences for the TA921i group ([Figure 4-11](#), [Figure 4-12](#)). Clone IB-10d-C18 aligned with the W637 group. The population of W637 group was identified with a combination set of primers/probe (amoA-Nn-542f/amoA-Nn-Taq-648r/amoA-[RI-27]-679r), and was below the detection limit ($\sim 2.3 \times 10^4$ copies/L). Thus, this group was not further considered.

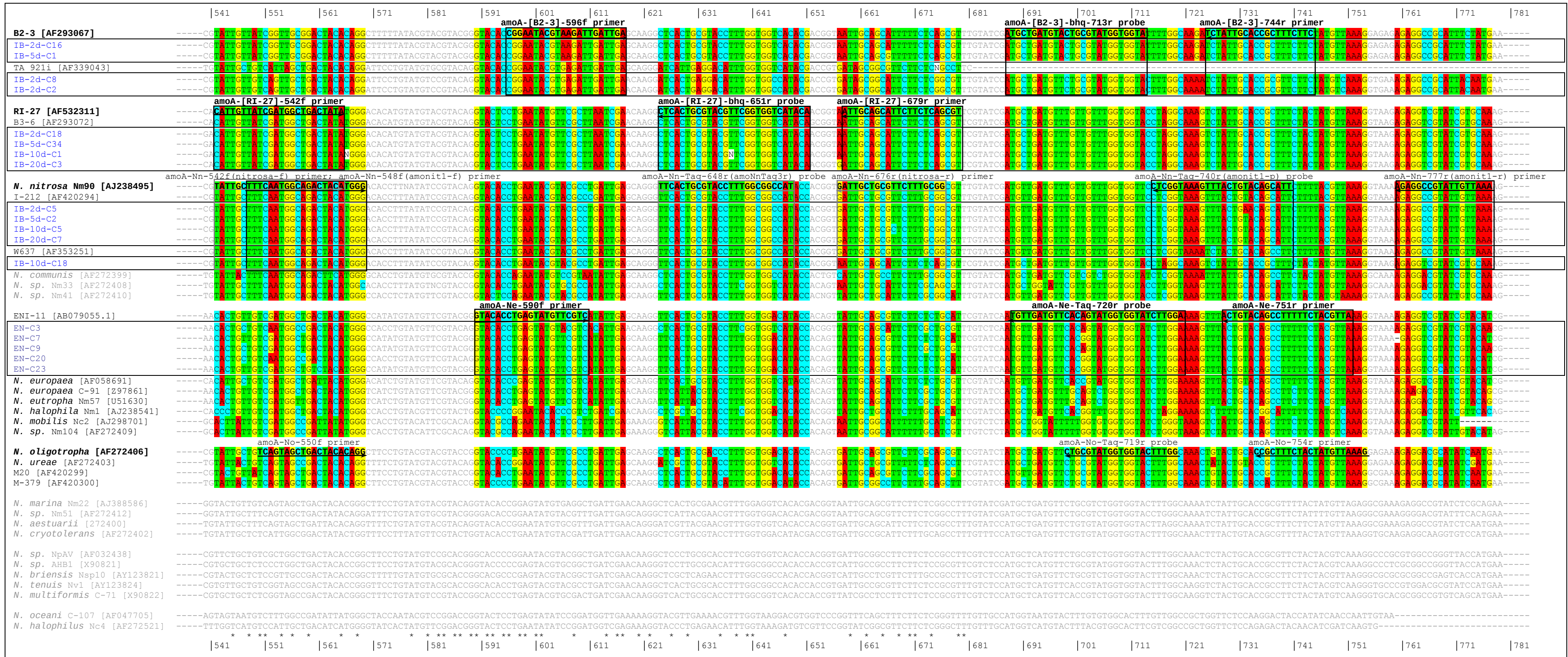


Figure 4-12. CLUSTAL X (1.64b) multiple sequence alignment. (Clone named as “IB-#1d-C#2” is *amoA* clone library for the industrial bench-scale system; clone named as “EN-C#3” is *amoA* clone library for the enriched nitrifying system for Part II study).

Table 4-14. The number of mismatches in *amoA* sequences between clones (closely related to *N. nitrosa*, W637, RI-27, B2-3, and TA921i) and sequences of real-time PCR primers and probes designed to specifically quantify *N. nitrosa*.

Clones* closely related AOB	Original [†] primers and probe specifically targeting <i>N. nitrosa</i>			Modified [‡] primers and probe specifically targeting <i>N. nitrosa</i>		
	forward primer:	TaqMan [®] probe:	reverse primer:	forward primer:	TaqMan [®] probe:	reverse primer:
	amoA-Nn- 548f	amoA-Nn- Taq-740r	amoA-Nn- 777r	amoA-Nn- 542f	amoA-Nn- bhq-648r	amoA-Nn- 676r
<i>N. nitrosa</i>	0	0~1	0	0	0	0~1
W637	0	7	4	0	0	6
RI-27	4	7	4	6	5	5~6
B2-3	7	10	5	6	4	6~7
TA921i	6	9	6	5	5	5

Note: * Typical clones closely related to *N. nitrosa* were IB-2d-C5, IB-5d-C2, IB-10d-C5, and IB-20d-C7; typical clone closely related to W637 was IB-10d-C18; typical clones closely related to RI-27 were IB-2d-C18, IB-5d-C34, IB-10d-C1, and IB-20d-C3; typical clones closely related to B2-3 were IB-2d-C16, and IB-5d-C1; and, typical clones closely related to TA921i was IB-2d-C2 (as listed in Table 4-13, Figure 4-11, and Figure 4-12); [†] an original *N. nitrosa* primer/probe set (Dionisi et al., 2002) was used in this study; [‡] a *N. nitrosa* primer/probe set designed by Layton et al., 2005.

Table 4-15. The number of mismatches in *amoA* sequences between clones (closely related to *N. nitrosa*, W637, RI-27, B2-3, and TA921i) and sequences of real-time PCR primers and probes designed in this study to specifically quantify RI-27 and B2-3.

Clone closely related AOB	Primers and probe specifically targeting RI-27			Primers and probe specifically targeting B2-3		
	forward primer:	TaqMan [®] probe:	reverse primer:	forward primer:	TaqMan [®] probe:	reverse primer:
	amoA- [RI-27]- 542f	amoA- [RI-27]- bhq-651r	amoA- [RI-27]- 679r	amoA- [B2-3]- 596f	amoA- [B2-3]- bhq-713r	amoA- [B2-3]- 744r
<i>N. nitrosa</i>	6	6	5	4~5	7~8	6
W637	6	6	0	4	5~6	1~2
RI-27	0	0	0	7	5~6	1~2
B2-3	5	4	1~2	0	0~1	0~1
TA921i	6	7	3	0	0~1	0~1

4.2.4 AOB population dynamic in industrial bench-scale system

The populations levels of *N. nitrosa*, RI-27, and B2-3 in each SRT system over time are shown in [Figure 4-13](#). The average population concentrations used to seed the industrial bench-scale system and during steady state of each operating phase are summarized in [Figure 4-14](#). These results show that *N. nitrosa* (3.35×10^9 cells/L) and RI-27 (5.25×10^9 cells/L) dominated the AOB population in the original activated sludge (from the industrial full-scale WWTP) used to seed the bench-scale system, while the B2-3 concentration (1.32×10^8 cells/L) was relatively low. After approximately six weeks of operation, the *N. nitrosa* population significantly decreased in all four SRT trains during steady-state of phase 1 ([Figure 4-13](#), [Figure 4-14](#)). This suggested that the operating conditions of phase 1 were inappropriate for *N. nitrosa*, and that RI-27 adapted to the ammonia limitation condition more effectively and became more dominant in all four SRT trains. However, both the RI-27 and B2-3 populations in the 2-, and 5-day SRT systems decreased, while these populations in the 10-, and 20-day SRT systems remained stable or increased slightly ([Figure 4-13](#), [Figure 4-14](#)) suggesting that these AOB cells were poorly maintained in the activated sludge systems operated at shorter SRTs (i.e., 2- or 5-day) under limited ammonia condition. Because ammonia-oxidation did not significantly occur in the 2-, and 5-day SRT systems during phase 1 ([Section 4.1.4](#)), the slow-growing AOB could not proliferate while fast-growing heterotrophs did by oxidizing significant amounts of carbon (e.g., COD). Since sludge wastage was based on the overall microbial net growth determined by MLVSS measurements, the AOB wastage rate was higher than the AOB net growth rate (slow under ammonia limitations) during the bench-scale transition period (operating SRT shifted from ~14-day for full-scale WWTP to 2-, or 5-day for the bench-scale system). This SRT transition resulted in and AOB population decrease in the shorter SRT systems. In contrast, the AOB populations were more stable in the 10-, and 20-day SRT systems due to the low level of ammonia-oxidation in these longer SRT trains ([Section 4.1.4](#)).

After ammonia-oxidation levels increased in all four SRT trains during phase 2, the AOB population dynamic in the 2-day SRT system was different from that in longer SRT

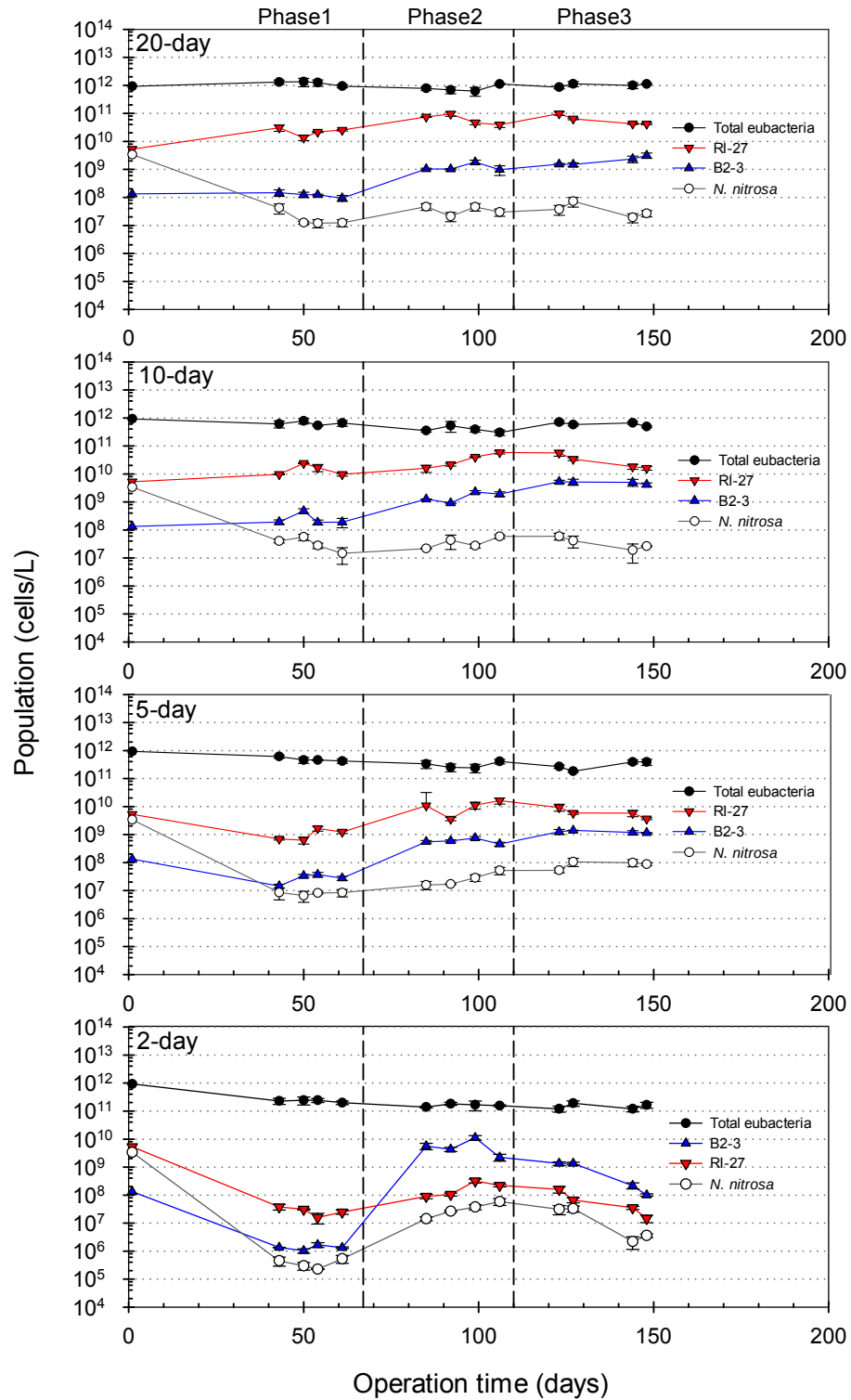


Figure 4-13. AOB population dynamics in each SRT train of the industrial bench-scale system over the three operating phases.

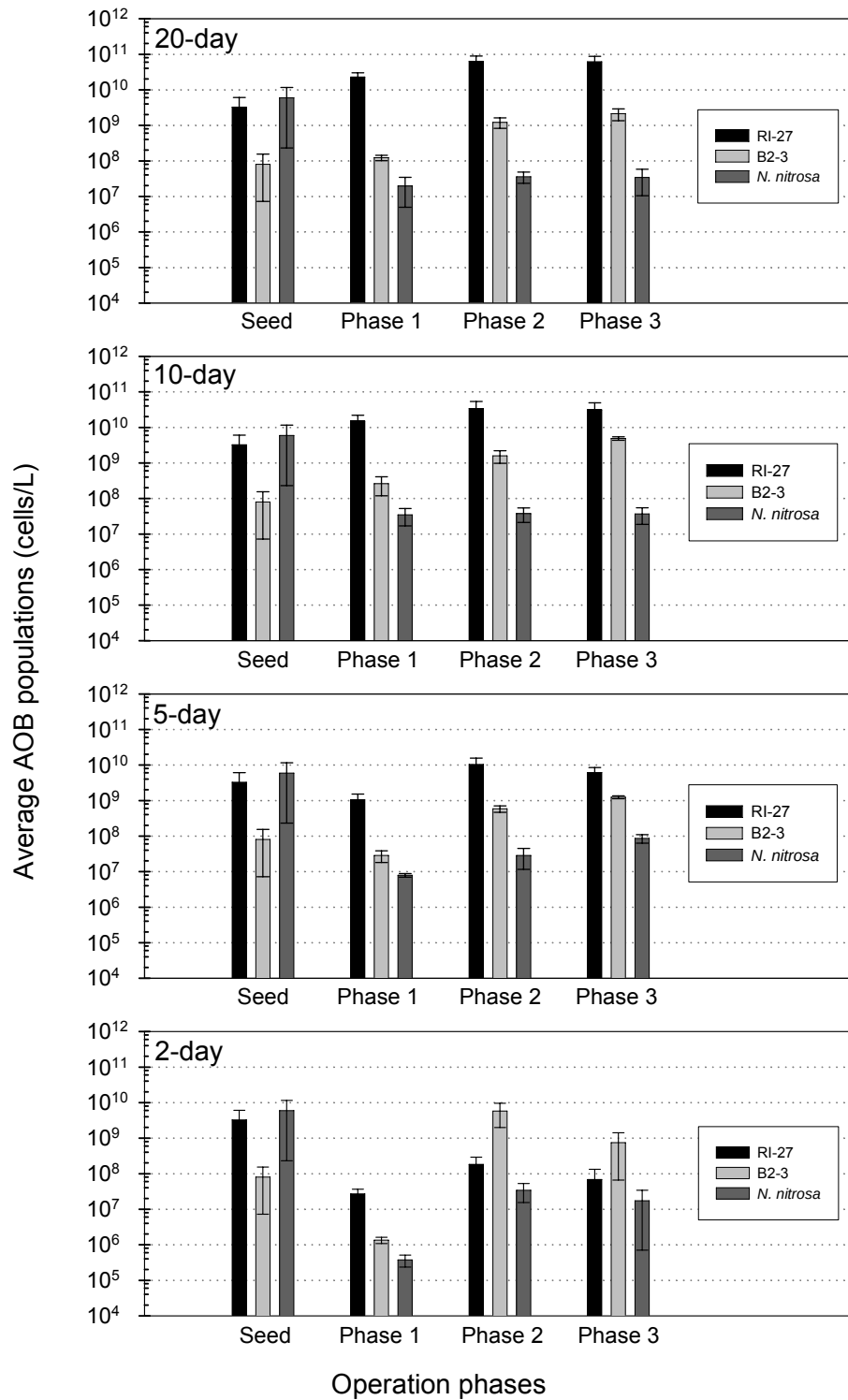


Figure 4-14. Average AOB concentrations used to seed the industrial bench-scale system and during steady-state of each operational phase.

systems. The B2-3 population increased sharply from 1.34×10^6 (cells/L) to 5.81×10^9 (cells/L) over the approximately two weeks transition period; thereby dominating the overall AOB population in the 2-day SRT system during phase 2 (Figure 4-13, Figure 4-14). Meanwhile, the B2-3 populations in the 5-, 10-, and 20-day SRT systems increased only slightly. Additionally, the *N. nitrosa* population in the 2-day SRT system also increased during phase 2, while it remained nearly stable in the 5-, 10-, and 20-day SRT systems (Figure 4-13, Figure 4-14). This suggested some operating conditions of the 2-day SRT system were more appropriate for both B2-3 and *N. nitrosa*. The RI-27 population increased somewhat in all four SRT trains during phase 2, and remained dominant in the 5-, 10-, and 20-day SRT systems. In general, populations of *N. nitrosa*, RI-27, and B2-3 all increased to different degrees during phase 2. Different AOB population dynamics between the four SRT trains suggested that variation in operating SRTs resulted in AOB population shifts because of different levels in ammonia-oxidation treatment performance between the four SRT systems after ammonia loading increased during phase 2.

After the DO level was lowered from 3.0 to 0.5 mg/L during phase 3, AOB populations of *N. nitrosa*, RI-27, and B2-3 in all four SRT systems did not significantly change based on statistics analysis (t-test) results ($p\text{-value} > 0.05$). However, the AOB populations of *N. nitrosa*, RI-27, and B2-3 in the 2-day SRT system decreased somewhat ($p\text{-value} = 0.10$) (Figure 4-13 (d), 4-14 (d)) as a result of the drop in ammonia-oxidation treatment performance ($p\text{-value} \leq 0.05$). Under low DO condition, the relatively low AOB concentration in the 2-day SRT system (compared to those in longer SRT systems) could not completely oxidize all the available ammonia from the influent and some untreated ammonia remained in the effluent as mentioned in Section 4.1.3. This decrease in the ammonia-oxidation rate resulted in a drop in AOB growth; thus, causing a decline in the AOB population in the 2-day SRT system during phase 3. In the longer SRT systems, the AOB population of *N. nitrosa*, RI-27, and B2-3 remained stable while the ammonia-oxidation rate did not significantly change ($p\text{-value} > 0.05$) in these longer SRT systems under low DO condition as indicated in Section 4.1.4.

In order to further evaluate correlations between overall ammonia oxidation performance and AOB population size, the AOB populations of *N. nitrosa*, RI-27, and B2-3 were summed to yield an overall AOB population in each SRT train during each operating phase (Table 4-16). The overall AOB concentrations were always close to the dominant AOB population levels which were likely responsible for the major ammonia-oxidation treatment in the system. The overall AOB population remained stable in 20-, 10-, and 5-day SRT systems under changing operational factors of ammonia loading and DO level. In the 2-day SRT system, the overall AOB population was more poorly maintained under severe conditions such as limited ammonia or oxygen.

The proportion of total AOB to eubacteria in each SRT system during steady-state of each operating phase was computed by dividing the summed AOB population [*N. nitrosa* + (RI-27) + (B2-3)] by the total eubacteria (Table 4-17). The percentages of AOB were similar to results from other investigations (i.e., 1.7% AOB in an activated sludge municipal wastewater treatment system, Harms et al., 2003; 5.2% AOB in an A2O system, You et al., 2003). In this study, the results showed that longer SRT systems had higher percentages of AOB over the three operating phases. Moreover, the percentages of AOB population in the four SRT systems during phase 1 (high DO, and low ammonia) were relatively lower than those for phases 2 and 3 due to limited ammonia availability. During phase 2 (high DO, and high ammonia), the percentage of AOB in the four SRT systems

Table 4-16. Total population sizes (cells/L) of the three identified AOB in the industrial system during steady-state of each operational phase.

	Phase 1	Phase 2	Phase 3
	High DO, Low NH ₃	High DO, High NH ₃	Low DO, High NH ₃
20-day	2.28×10 ¹⁰	6.47×10 ¹⁰	6.38×10 ¹⁰
10-day	1.53×10 ¹⁰	3.55×10 ¹⁰	3.59×10 ¹⁰
5-day	9.59×10 ⁸	1.10×10 ¹⁰	7.47×10 ⁹
2-day	2.89×10 ⁷	6.03×10 ⁹	8.33×10 ⁸

Table 4-17. Percentages of AOB in each SRT train of the industrial bench-scale system during steady-state of each operating phase.

		Phase 1	Phase 2	Phase 3
		High DO Low NH ₃	High DO High NH ₃	Low DO High NH ₃
20-day	Summed AOB	2.28×10^{10}	6.47×10^{10}	6.38×10^{10}
	Total eubacteria	12.10×10^{11}	7.99×10^{11}	10.20×10^{11}
	Percentage (%)	1.89	8.10	6.25
10-day	Summed AOB	1.53×10^{10}	3.55×10^{10}	3.59×10^{10}
	Total eubacteria	6.42×10^{11}	3.92×10^{11}	6.10×10^{11}
	Percentage (%)	2.38	9.06	5.89
5-day	Summed AOB	9.59×10^8	1.10×10^{10}	7.47×10^9
	Total eubacteria	4.85×10^{11}	3.07×10^{11}	3.07×10^{11}
	Percentage (%)	0.20	3.59	2.43
2-day	Summed AOB	2.89×10^7	6.03×10^9	8.33×10^8
	Total eubacteria	2.27×10^{11}	1.61×10^{11}	1.48×10^{11}
	Percentage (%)	0.01	3.74	0.56

significantly increased. The additional available ammonia provided an energy source for AOB proliferation via oxidation. During phase 3 (low DO, and high ammonia), the measured percentages of AOB population in the four SRT systems decreased (Table 4-17).

A theoretical percentage of AOB in the treatment system was calculated using the volatile suspended solids (VSS) production rates (Eq. 4-1) and the typical growth parameters (Table 4-18) for heterotrophs and AOB (Rittmann and McCarty, 2001).

$$\frac{\Delta X_v}{\Delta t} = Q(S^0 - S)Y \frac{1 + (1 - f_d)b\theta_x}{1 + b\theta_x} \quad (\text{Eq. 4-1})$$

Where $\Delta X_v/\Delta t$ = volatile suspended solids production rate (mg VSS/day); Q = hydraulic flow rate (L/day); S^0 = influent substrate concentration (mg/L); S = effluent substrate concentration (mg/L); Y = true yield (mg/mg); f_d = biodegradable fraction; b = endogenous decay coefficient; and θ_x = solids retention time (days).

The computed results were compared with measured values and are summarized in Table 4-19. In longer SRT systems (≥ 10 days), the theoretical values were comparable to the measured values; whereas, the theoretical values were higher than measured values in shorter SRT systems. This suggests that the AOB measured in this study represent most of the AOB population in the longer SRT systems. For the shorter SRTs, the discrepancy between measured and theoretical values may be due to the presence of unidentified AOB or, more likely, that the typical growth parameters utilized to determine theoretical AOB percentages may not accurately represent specific microbial conditions under varying situations (such as short SRT, and low DO). It has been suggested that significant uncertainty and variability in kinetic parameters exist among the published values (Cox, 2004). For example, heterotrophic yield coefficient values can range from 0.5 to 0.82 while autotrophic values can range from 0.26 to 0.9. The theoretical AOB percentages for the shorter SRT systems approached the measured values (Table 4-19) when the growth yields for heterotrophs and AOB were adjusted to 0.72 and 0.18, respectively. However, the uncertainty associated with these growth parameters makes comparison problematic. Nevertheless, the proportion of identified AOB provided preliminary insight to the microbial community structure in this treatment system.

Table 4-18. Typical growth parameters for heterotrophs and AOB (based on [Rittmann and McCarty, 2001](#) with modifications for a condition of 28°C).

Heterotrophs		AOB	
Y (mg VSS/mg BOD)	0.45	Y (mg VSS/mg $\text{NH}_3\text{-N}$)	0.33
b (1/days)	0.24	b (1/days)	0.20
f_d	0.8	f_d	0.8

Table 4-19. Measured and theoretical percentages of AOB in four SRT trains of the industrial bench-scale system.

	Phase 1		Phase 2		Phase 3	
	High DO		High DO		Low DO	
	Low NH_3		High NH_3		High NH_3	
	Measured	Theoretical (Modified*)	Measured	Theoretical (Modified*)	Measured	Theoretical (Modified*)
20-day	1.89 %	2.25 %	8.10 %	9.27 %	6.25 %	9.18 %
10-day	2.38 %	2.04 %	9.06 %	9.23 %	5.89 %	9.29 %
5-day	0.20 %	1.68 % (0.29 %)	3.59 %	8.99 % (3.02 %)	2.43 %	8.86 % (2.97 %)
2-day	0.01 %	1.21 % (0.04 %)	3.74 %	8.38 % (2.70 %)	0.56 %	5.38 % (1.56 %)

* Theoretical modified AOB percentages for shorter SRT systems which were determined using growth yield (Y) values of 0.72 mg VSS/mg BOD and 0.18 mg VSS/mg $\text{NH}_3\text{-N}$ for heterotrophs and AOB, respectively.

The total AOB population was correlated to the overall ammonia oxidation rate in each SRT train during each of three operating phases (Figure 4-15). (A ratio between these two parameters represented an average cellular ammonia oxidation rate for AOB cells in the industrial bench-scale treatment system, Section 4.2.5). The overall ammonia oxidation rates differed under similar AOB population levels; thus, rates did not correlate with AOB cellular concentration. Specifically, the longer SRT trains maintained a significant AOB population during phase 1 (low-N) operation but did not have the significant ammonia oxidation performance shown in phases 2 (high-N/high-DO), and 3 (high-N/low-DO). These results suggested that overall ammonia oxidation performance of the treatment system was not reflected solely by AOB population size. Variances in average cellular oxidation rates implied that AOB may adjust their cellular oxidation activities to adapt to different environmental conditions (i.e., low-N and high-N). Hence, AOB cellular activity was further assessed for the industrial bench-scale system based on a kinetic approach (Section 4.2.5) and an *amoA* mRNA approach (Section 6.2).

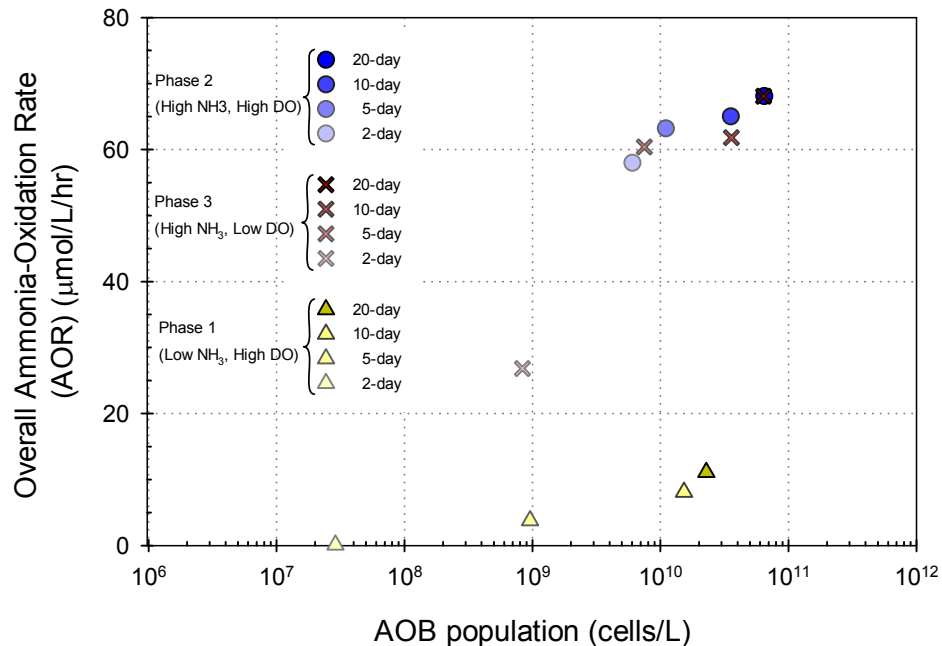


Figure 4-15. Relationships between AOB concentration (bacterial population size) and ammonia-oxidation rate in each phase of this study.

4.2.5 Competition among distinct AOB groups

It has been suggested that various AOB species (or strains) may have different affinities for substrates (ammonia and oxygen) and/or different sensitivity to inhibitors; thus particular AOB species (or strains) may dominate the AOB population under specific conditions (Suwa et al., 1994, 1997; Koops and Pommerening-Röser, 2001). The AOB population structural dynamics observed in this study suggested that the three AOB groups (*N. nitrosa*, RI-27, and B2-3) may have different kinetic features, which determine their growth rate under each operating condition. During phase 1 (low ammonia operation), the *N. nitrosa* population decreased in all four SRT trains indicating that this AOB species grew more slowly than the other two AOB groups (RI-27 and B2-3) under this condition (ammonia concentration <1 mg/L). Meanwhile, the RI-27 and B2-3 strains adapted to the limiting ammonia condition and grew more effectively. During phase 2 (high ammonia operation), both the B2-3 and *N. nitrosa* populations in the 2-day SRT train significantly increased (p-values<0.05), while those in the longer SRT trains did not. These results suggested that the relatively high ammonia level (24.3 ± 2.4 mg/L), which accumulated during the transitional period in the 2-day SRT system, was more favorable to the B2-3 and *N. nitrosa* organisms. Based on these findings, the relative growth rates for the three AOB populations appear to be: RI-27 $\geq$ B2-3 > *N. nitrosa* under low ammonia concentration (<1 mg/L), and B2-3 > *N. nitrosa* > RI-27 under higher ammonia concentration (~24 mg/L).

The growth rate of a distinct AOB group is tightly linked to both its cellular ammonia oxidation rate and growth yield (Rittmann and McCarty, 2001). Researchers have documented the cellular oxidation rate and the yield for some AOB species and strains in both pure and mixed cultures (Table 4-20); however, relative information for most other AOB strains and uncultured AOB is lacking. Some published values (Table 4-20) are diverse. It is not clear whether the yield and oxidation rate values for one AOB group differ from those for other representative AOB groups and how these two values for a representative AOB group may be changed under different operational/environmental conditions.

Table 4-20. Literature values for cellular ammonia oxidation rate, yield and maximum specific growth rate for AOB.

AOB	Oxidation rate	Reference
<i>Nitrosomonas</i> sp.	11~23 fmol NO ₂ ⁻ /cell/hr	Belser, 1979
<i>Nitrosomonas europaea</i>	1~7 fmol NO ₂ ⁻ /cell/hr	Keen and Prosser, 1987
<i>Nitrosomonas europaea</i>	1.24~23 fmol NH ₃ /cell/hr	Laanbroek and Gerards, 1993
Mixed culture	2.3±0.4 fmol NH ₃ /cell/hr	Daims et al., 2001 (a)
Mixed culture	16~43 fmol NH ₃ /cell/hr	Daims et al., 2001 (b)
<i>N. oligotropha</i> dominated	7.7±6.8 fmol NH ₃ /cell/hr	Harms et al., 2003 (16S rDNA-based)
<i>N. oligotropha</i> dominated	12.4±7.3 fmol NH ₃ /cell/hr	Harms et al., 2003 (amoA-based)
Mixed culture	0.4~15.6 fmol NH ₃ /cell/hr	Okano et al., 2004
Mixed culture	0.03~43 fmol NH ₃ /cell/hr	Coskuner et al., 2005
Mixed culture	0.5~4.0 fmol NH ₃ /cell/hr	This study* (Low-N phase)
Mixed culture	1.1~9.6 fmol NH ₃ /cell/hr	This study* (High-N phase)
Yield		
<i>Nitrosomonas</i> ATCC	4.61~6.44×10 ⁶ cells/μmol NH ₃	Belser and Schmidt, 1980
<i>Nitrosomonas europaea</i>	1.26~1.72 μg biomass/μmol NH ₃	Keen and Prosser, 1987
Not specified	5.88 μg biomass/μmol NH ₃	Prosser et al., 1989
Mixed culture	6.3 μg biomass/μmol NH ₃	Rittmann et al., 1999
Mixed culture	1.7~17.5 ×10 ⁶ cells/μmol NH ₃	Okano et al., 2004
Mixed culture	2.2~7.9×10 ⁶ cells/μmol NH ₃	This study* (Low-N phase)
Mixed culture	1.5~2.3×10 ⁶ cells/μmol NH ₃	This study* (High-N phase)
Maximum specific growth rate (μ _{max} , 1/hr)		
<i>N. europaea</i> ATCC	0.052~0.066	Belser and Schmidt, 1980
<i>N. europaea</i>	0.017~0.039	Keen and Prosser, 1987

* The yield and ammonia oxidation rate of AOB cells were determined based on the overall ammonia oxidation rate and the summed AOB population under steady-state of each operating phase assuming (1) that cell death had not occurred, and (2) that the cellular oxidation rate for each AOB cell from different groups was the same.

An average cellular ammonia oxidation rate for AOB was assessed in this study using the overall volumetric ammonia oxidation rate ($\mu\text{mol/L/hr}$) (Table 4-7), the summed AOB population (cells/L) (Table 4-16), and the assumption that all AOB cells from the three distinct groups have the same cellular oxidation rate. Results showed that most values were similar to the reported AOB ammonia oxidation rates of 2.3 ± 0.4 fmol/cell/hr (Daims et al., 2001 (a)), 7.7 ± 6.8 , and 12.4 ± 7.3 fmol/cell/hr (Harms et al., 2003) (Tables 4-20, 4-21). The relatively high oxidation rates for the 2-day SRT train during high-N operation (i.e., phases 2, and 3) may result from a fact that the dominant AOB in this shorter SRT train was under-estimated as speculated by AOB percentage (Table 4-19). Even so, the AOB cellular oxidation rates for the four SRT trains were significantly higher during high-N operation (phases 2, and 3) than during low-N operation (phase 1). These results demonstrated that the average ammonia oxidation rate of AOB cells changed under variant operational conditions (i.e., low-N/high-DO, high-N/high-DO, and high-N/low-DO).

Although the yield values obtained in this investigation were similar to those reported in other studies (Table 4-20), these mixed culture assessments may not reflect actual growth yields for the three distinct AOB groups. Additionally, if the ammonia oxidation activities among distinct AOB groups are not the same, specific activity variances cannot be addressed from determinations based on the average cellular

Table 4-21. Average ammonia oxidation rate of AOB cell (fmol/cell/hr) in the industrial bench-scale system during steady state of each operational phase.

	Phase 1	Phase 2	Phase 3
	High DO	High DO	Low DO
	Low NH_3	High NH_3	High NH_3
20-day	0.5	1.1	1.1
10-day	0.5	1.8	1.7
5-day	4.0	5.7	8.1
2-day	0.0	9.6	32.2

oxidation rate. Based on the suggestion that various AOB species (or strains) have different substrates affinities (Suwa et al., 1997; Koops and Pommerening-Röser, 2001), the three distinct AOB groups in the industrial bench-scale system more likely had variant growth yields and cellular oxidation rates under each operating condition. However, no appropriate approach is currently available to determine the AOB cellular activity in mix culture especially for uncultured AOB strains.

Two kinetically-distinct types of AOB had been found to predominate in activated sludges: one type is sensitive while the other is insensitive to substrate concentration (Suwa et al., 1994, 1997; Gieseke et al., 2001). Under low substrate levels, AOB of the substrate-sensitive group generally have higher ammonia conversion activities (due to their high substrate affinity (low K_s value)) than the substrate-insensitive group. In contrast, AOB of the substrate-insensitive group typically have a lower substrate affinity (higher K_s values), thereby becoming more active under high substrate concentrations whereas the substrate-sensitive group usually become inhibited by high substrate levels (Suwa et al., 1994; Gieseke et al., 2001). The substrate affinity (K_s value) for the *N. nitrosa* group has been reported to be 19~46 μM NH_3 (Koops and Pommerening-Röser, 2001). If the RI-27 group has a lower K_s value (higher ammonia affinity), then this organism may be more active and become dominant under low ammonia conditions (e.g., 20-day SRT train during both operational phases). In contrast, if the B2-3 group has a higher K_s value (lower ammonia affinity), it would dominate the AOB population under high ammonia conditions (e.g., the transition period in the 2-day SRT train at the beginning of phase 2).

Based on the profiles of the three AOB populations and assumptions concerning their relative growth rates, an attempt was made to predict kinetic behavior for these three AOB groups (Figure 4-16) using inhibition equations (since they have not been classically found in eutrophic (nutrient rich) environments (Gieseke et al., 2001; Koops and Pommerening-Röser, 2001). Assigned conditions (1), (2), and (3) are used to represent particular situations in which RI-27, *N. nitrosa*, and B2-3 will be the most active group, respectively. For instance, the RI-27 will be more active than *N. nitrosa* and B2-3 if ammonia concentrations range from 0 to 0.3 mM; whereas, the B2-3 will become

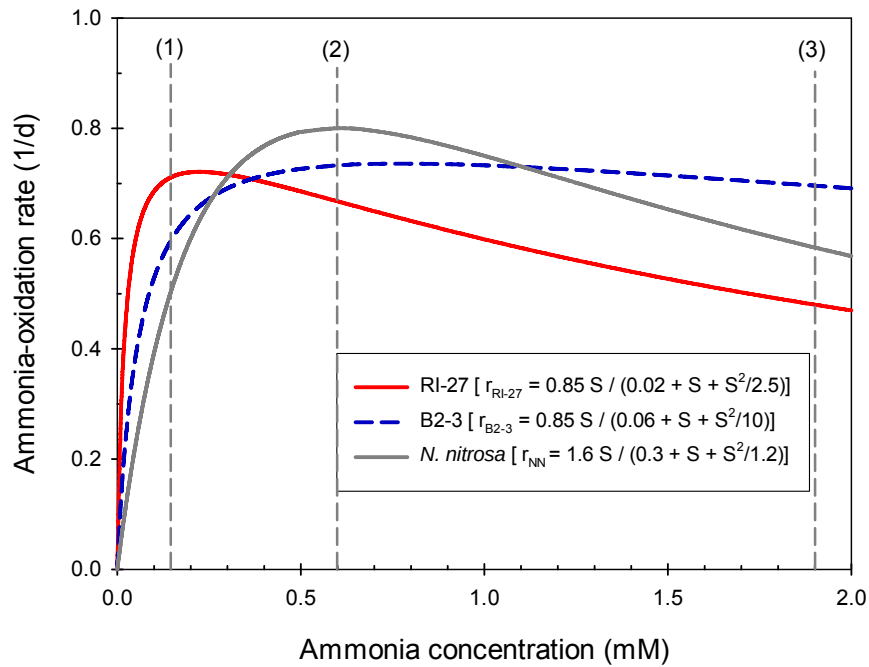


Figure 4-16. Projected kinetic behavior for the three investigated AOB based on the Haldane equation (note: not experimental data).

more active and grow faster than the other two AOB groups when the ammonia concentration is higher than 1.2 mM. Condition 2 is a situation similar to the industrial full-scale activated sludge treatment process. Under this condition, *N. nitrosa* had the highest ammonia-oxidation rate and growth rate of the three identified AOB and dominated the AOB population in the full-scale system (Layton et al., 2005). Condition 1 represents a situation in which ammonia concentrations in mixed liquor (equal to effluent concentrations in the CSTR system) are very low (similar to the operating condition of phase 1 in all four SRT systems). Under this condition, RI-27 had the highest ammonia-oxidation rate (or growth rate) among the three AOB groups and; therefore, should dominate the AOB population. Condition 3 represents a situation in which ammonium concentrations are high (as in the transition period in the 2-day system during phase 2). Under this condition, the B2-3 population had the highest ammonia-oxidation rate and growth rate; thus, should be the dominant AOB population. Based on these kinetic characteristics, the AOB population would shift under different operating conditions.

4.3 Summary for Part I

4.3.1 Effects of SRT, NH_3 , and DO on the ammonia oxidation of AOB

The long-term effect of the operational factors SRT, ammonia, and DO on the dependent parameters determined in the Part I are summarized in [Table 4-22](#). The tested dependent parameters include the overall volumetric ammonia oxidation rate ($\mu\text{mol/L/hr}$), the AOB population in (cells/L), and the average ammonia oxidation rate of AOB cells (fmol/cell/hr). Results show that these dependent parameters determined for short and long SRT systems were significantly different ($p\text{-value} < 0.05$) ([Table 4-22](#)). The longer SRT systems tend to maintain higher AOB populations and overall ammonia oxidation treatment performance than the shorter SRT systems. Moreover, changes in the influent ammonia levels impacted the overall ammonia oxidation rate and AOB population in the shorter SRT systems ([Table 4-22](#)). The AOB population sizes in the 2-, and 5-day SRT systems increased after the influent ammonia level approximately doubled during phase 2. The AOB population structure in the 2-day SRT system differed from longer SRT systems. Although the AOB populations in the longer SRT systems were relatively stable under different ammonia loadings, both the overall and the cellular ammonia oxidation rates increased in all four SRT systems during phase 2 (higher ammonia operation than phase 1) ([Table 4-22](#)). Additionally, these dependent parameters were not significantly affected by DO limitation during phase 3 except those for the 2-day SRT train. Therefore, the operational factors of SRT and ammonia affected the AOB population and the ammonia oxidation rate in the nitrifying system more significantly than DO.

4.3.2 Select control factors for studying AOB cellular activity in Part II

The operational parameter of SRT tended to directly control biosolids concentration and microbial population in the treatment system by physically removing a fraction of the biomass (net growth) under steady-state conditions. Therefore, the SRT primarily influenced the AOB population dynamics (as opposed to AOB activity at the cellular level) in the treatment systems. Differences in ammonia oxidation treatment

Table 4-22. Summary of the effects of SRT, NH₃, and DO on tested dependent parameters in the industrial bench-scale treatment system

Testing factors		Dependent parameters [†]		
		Volumetric ammonia oxidation rate	AOB population size	Ammonia oxidation rate of AOB cell
SRT	Low NH ₃ /High DO	++	++	++
Short vs. Long	High NH ₃ /High DO	++	++	++
(2-, 5-day vs. 10-, 20-day)	High NH ₃ /Low DO	++	++	++
	Summary [‡]	++	++	++
NH ₃	2-day	++	++	++
Low vs. High	5-day	++	++	+
(~18.9mg/L vs. ~53.7mg/L)	10-day	++	-	+
	20-day	++	-	+
	Summary [‡]	++	+	+
DO	2-day	++	+	+
High vs. Low	5-day	-	-	-
(3 mg/L vs. 0.5 mg/L)	10-day	-	-	-
	20-day	-	-	-
	Summary [‡]	-	-	-

†: “++”: p-value<0.05, significantly affected; “+”: 0.05<p-value<0.1, slightly affected; and “-”: 0.1<p-value, not affected.

‡: Summary = average of [(“+” number) + (“-“ number)] by counting five and higher fractions as units and disregard the rest.

performances (demonstrated by variable ammonia oxidation rates) between SRT systems (Table 4-7) resulted from variant features of diverse AOB populations responding to the condition changes (e.g., ammonia loading). Because different AOB species (or strains) dominated the AOB population in different SRT systems, comparison of the AOB cellular activities (based on the *amoA* mRNA measurement) between SRTs is difficult. Each AOB population requires different reverse-transcription real-time PCR assays for quantifying the *amoA* mRNA transcripts due to the variability in the *amoA* gene sequences. Therefore, SRT was not selected as a control factor to study subsequent AOB physiology activity in Part II of this research.

It has been reported that expression of the *amo* functional gene is induced by ammonia (Sayavedra-Soto et al., 1996; Stein and Arp, 1998 (a), (b)). In the Part I study, the influent ammonia levels (low: ~20.0mg/L and high: ~54.9mg/L) affected the overall volumetric ammonia oxidation rate and the average ammonia oxidation rate of AOB cells in all four SRT trains (Table 4-22). It is believed that short-term changes in the ammonia level could induce shifts in AOB physiological activity; and, if the changed ammonia level is maintained for a period of time, the AOB population would gradually shift due to selection of specific AOB species (or strains). The ammonia strength should directly influence the metabolic pathway of AOB cells at the *amo* gene transcriptional level (and/or the enzyme, post-translation, level) and affect the ammonia oxidation activity of AOB cells. Since short-term changes in the ammonia concentration may significantly induce short-term shifts in AOB physiology activity without changing the AOB population, influent ammonium concentration was selected as a control factor to study AOB physiology activity in Part II of this research.

Results from Part I of this study suggest that the DO level may not significantly affect AOB physiological activity since the low DO level at 0.5 mg/L did not significantly affect the dependent parameters considered in the longer SRT systems (Table 4-22). However, extremely low DO levels (<0.5 mg/L), when aeration in the treatment system is completely stopped, may impact AOB physiological activity. Since it has not been reported that DO is an inducer for *amo* functional gene expression, the DO may not directly affect AOB cellular activity at the transcriptional level. However, DO

limitation may reduce the metabolism level for oxidizing ammonia, thus the AOB cells may have to adjust genomic expression, which may change the abundance of *amo* mRNA transcripts. Therefore, the effect of DO on AOB cellular activity at the *amo* mRNA transcriptional level may be less responsive than the effect of ammonia concentration. Since the effect of DO on AOB cellular activity at the *amo* mRNA transcriptional level has not been fully addressed, DO was also selected as a control factor to study *amo* mRNA transcript changes under short-term, extremely low, DO conditions in the Part II of this study.

The ammonia oxidation performance of nitrifying systems will depend on both the AOB population size and the ammonia oxidation activity of AOB cells. Based on Part I results, it appears that the AOB population size (DNA-based quantification) does not rapidly respond to changes in operational/environmental conditions. Therefore, DNA-based techniques should not be solely used to reflect real-time fluctuations in the overall ammonia oxidation capacity of treatment systems, particularly if the physiological activity of the AOB cells has not been determined. An alternative technique that assesses the *amoA* transcript level in AOB cells (mRNA-based quantification) will likely be more responsive to changes in operational/environmental conditions and should better reflect short-term shifts in kinetic activity of the cells. AOB activity based on the *amoA* mRNA approach and the assessment of physiological activity on ammonia oxidation performance constituted the follow-up studies that were conducted in Part II.

Chapter 5 Results and discussion — Part II

(*amoA*-mRNA-based ammonia-oxidation activity in the enriched nitrifying system)

There are several reasons to use the enriched nitrifying system in Part II to study the AOB physiological activity prior to assessment of AOB activity in the industrial bench-scale system (Part III). First, the biosolid samples collected from the industrial bench-scale system may be too old to assess AOB cellular activity at the transcriptional level because target mRNA may have degraded over the long storage time (3-4 years). Second, the three AOB populations measured in the industrial system may complicate the study of AOB physiological activity if the *amoA*-mRNA based method has not been well established. Third, the enriched nitrifying system consists of primarily one nitrifying population ([Hawkins, 2005](#)) which should simplify the assessment of any short-term activity shifts in the AOB cells. And finally, the operational conditions for the enriched nitrifying system are easy to control, thereby, minimizing possible interferences in mixed microbial cultures and complex wastewater; thus, the effect of a sole designated factor on AOB physiological activity can be more accurately assessed.

5.1 Enriched nitrifying system treatment performance

5.1.1 Biomass maintenance

The Stable reactor in the enriched nitrifying system was operated under constant conditions ([Section 3.2.2](#)) to obtain a stable AOB culture to assess ammonia oxidation activity based on the *amoA* mRNA approach. In order to prevent any possible AOB population changes in the enriched nitrifying culture, designated short-term (approx. 2-5 days) experiments for *amoA*-mRNA-based activity evaluation were conducted in a second Trial reactor. This system was seeded with (wasted) enriched nitrifying culture from the Stable reactor and operated in parallel prior to experimental processing. Mixed liquor in the Trial reactor was replaced with the same volume of culture withdrawn from the Stable reactor approximately every two days to maintain a similar AOB culture for

both reactors when experimental testing was not in progress. Under normal operating conditions, both reactors were continually fed with high strength ammonia ($\sim 1,500$ mg/L as N) at a flow rate of 1 L/day. Mixed liquor was allowed to accumulate for two days; thus, about two liters of mixed liquor was withdrawn from each reactor every two days for wasting, replacing or testing. This operation yielded an SRT of about 10 days for each reactor. Although biosolids concentrations were not required to control the operating SRT in this treatment system, MLSS and MLVSS levels were occasionally measured to check the stability of the biosolids. Under typical operating conditions, the MLSS and MLVSS concentrations were 543 ± 86 and 277 ± 23 mg/L for the Stable reactor, and 490 ± 139 and 277 ± 51 mg/L for the Trial reactor (Figure 5-1). The suspended-biosolid levels in both reactors were essentially constant over the study period based on the coefficient of variation (8-28%) for analyzed data, suggesting that biomass steady state was attained in the enriched nitrifying system.

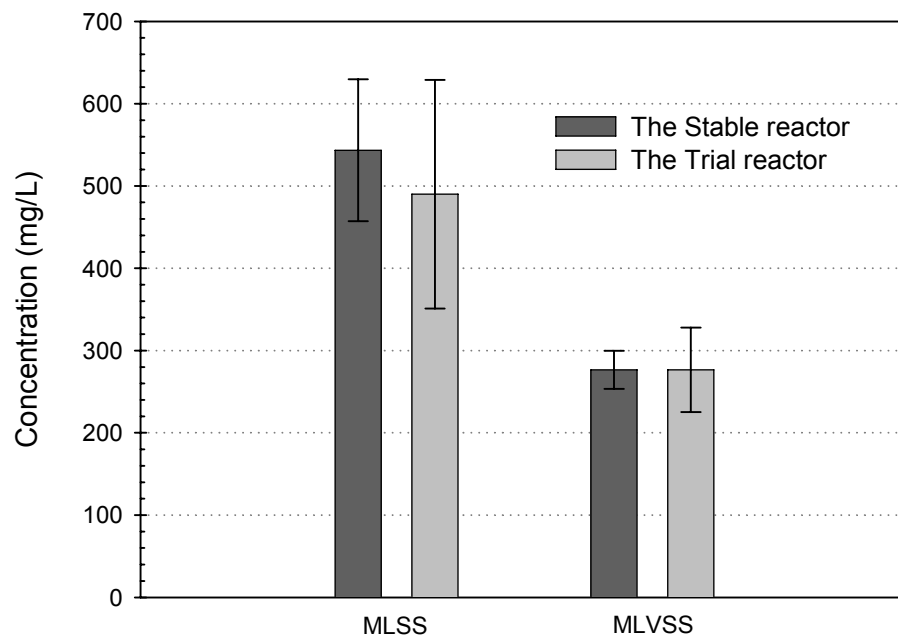


Figure 5-1. Average MLSS and MLVSS concentrations (with standard deviation) for the Stable and Trial reactors of the enriched nitrifying system.

The MLVSS concentrations detected for this system (~ 277 mg/L) were much lower than the values measured for the 10-day SRT train ($\sim 1,900$ mg/L) in the industrial bench-scale system. Because both reactors in the enriched nitrifying system were consistently fed with a carbon-free and ammonia-rich substrate to enrich the AOB culture, little organic-carbon was available for heterotrophic growth. Under this established operating condition, heterotrophic bacteria likely utilized dead biomass or metabolites (e.g., soluble microbial products, SMP) from nitrifying bacteria as suggested by [Rittmann et al. \(1994\)](#), [Jung et al. \(2004\)](#) and [Okabe et al. \(2005\)](#).

5.1.2 Ammonia conversion

The influent ammonia concentration for the enriched nitrifying system was controlled at $\sim 1,500$ mg $\text{NH}_3\text{-N/L}$ (with a measured mean of $1,487 \pm 40$ mg/L over the study period, [Figure 5-2](#)) in order to achieve a steady state for ammonia-oxidation treatment performance. When each of the reactors was fed with $\sim 1,500$ mg/L $\text{NH}_3\text{-N}$ at a flow rate of 1 L/day, the ammonia loading for each reactor was about $446 \mu\text{mol/L/hr}$.

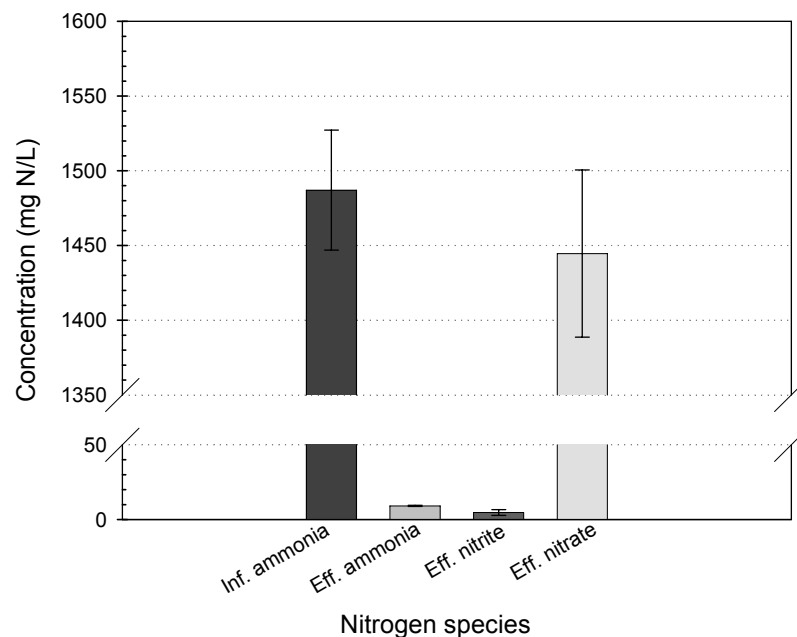


Figure 5-2. Average ammonia, nitrite and nitrate concentrations in influent (Inf.) and effluent (Eff.) for the enriched nitrifying system under steady-state operation.

Steady state for this treatment system was defined as a condition under which the effluent ammonia and NO_x^- (nitrite + nitrate) concentrations remained stable (i.e., measured values were within 10% of the mean). Under steady-state operation, the average ammonia, nitrite and nitrate levels in the reactors (equal to the concentrations in the withdrawn effluent) were 9.1 ± 0.4 , 4.7 ± 1.9 and $1,445 \pm 56$ mg/L as N, respectively (Figure 5-2). This indicated that most of the influent ammonia was oxidized to nitrite by AOB and then to nitrate by NOB with very little ammonia and nitrite remaining in the effluent. The volumetric ammonia oxidation rate determined for the enriched nitrifying system (~ 435 $\mu\text{mol/L/hr}$) was very close to the loading level of 446 $\mu\text{mol/L/hr}$. This ammonia oxidation capacity was about seven times higher than the values ($62\sim 65$ $\mu\text{mol/L/hr}$) determined for the 10-day SRT train in the industrial bench-scale system. Thus, the overall ammonia oxidation treatment performance in the enriched nitrifying system was significantly higher than the industrial system. Based on differences in the ammonia oxidation capacity, one may infer that the AOB population structure in the enriched nitrifying system would be different from that found in the industrial bench-scale system.

5.1.3 Ammonia oxidation rate

In order to derive a feeding strategy to control stable ammonia levels during a follow-up *amoA*-mRNA-based activity study (Section 5.3.3) (to obtain representative *amoA* mRNA abundance under each particular NH_3 level), the ammonia oxidation rate of the AOB culture from the enriched nitrifying system was pre-estimated under different initial ammonia concentrations (including 1, 5, 10, 20, 50, and 100 mg/L as N). Two experiments were conducted using non-starved (from the Stable reactor) and 2-day starved (from the Trial reactor) AOB cultures, respectively (Section 3.2.3). In both experiments, the ammonia levels dropped immediately after the tests were started (Figure 5-3). Ammonia was completely oxidized after 2 hours of incubation in the batch cultures when the initial ammonia concentration was lower than or equal to 10 mg/L; whereas, ammonia was not completely oxidized even after 4 hours when initial ammonia concentrations were 20, 50, and 100 mg/L. However, the ammonia levels dropped faster in the batch cultures with high initial concentrations than with low initial concentrations.

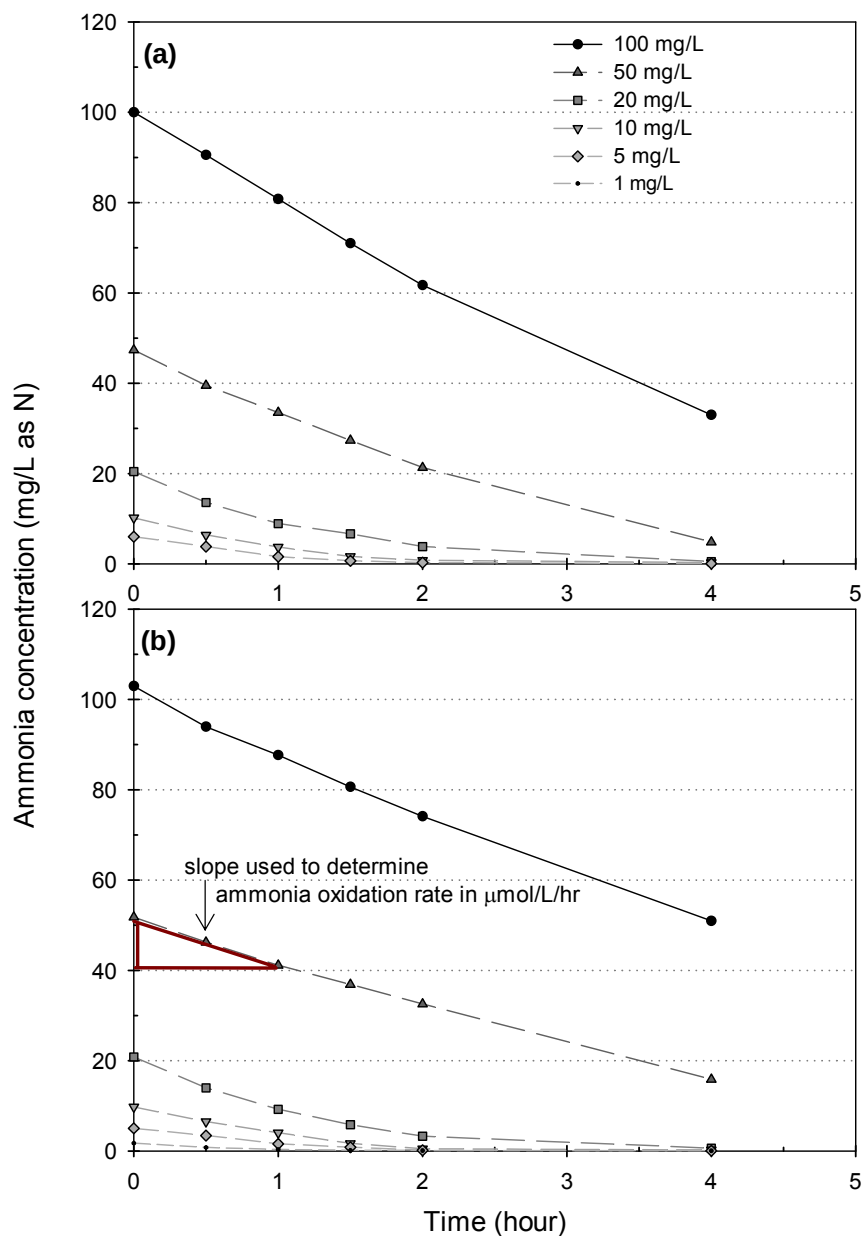


Figure 5-3. Ammonia oxidation profiles under different initial ammonia concentrations of 1, 5, 10, 20, 50 and 100 mg/L using non-starved (a), and 2-day starved (b) AOB cultures from the Stable and Trial reactors, respectively, of the enriched nitrifying system. The slope between the first three data points (from time zero to one hour) of each ammonia utilization curve was used to determine the ammonia oxidation rate (in $\mu\text{mol/L/hr}$) for the AOB culture under each particular initial ammonia concentration.

Moreover, the ammonia levels decreased faster in the batch cultures using non-starved AOB cells than with starved AOB cells, suggesting that the ammonia was oxidized more quickly in the non-starved AOB culture than in the 2-day starved AOB culture.

The slope of each ammonia utilization curve between time zero and one hour was used to determine ammonia oxidation rates (in $\mu\text{mol/L/hr}$) for each AOB culture under each particular initial ammonia concentration. Results showed that the ammonia oxidation rate increased as the ammonia concentration increased and that the ammonia oxidation rate for the non-starved AOB culture was higher than that for the starved culture (Figure 5-4).

Based on the Monod equation (Section 2.4.5), two kinetic models were developed to represent relationships between the ammonia concentrations and the ammonia oxidation rates (Figure 5-4). The maximum ammonia oxidation rates from the models were 1,667 and 1,111 $\mu\text{mol/L/hr}$ for the non-starved and starved cultures, respectively.

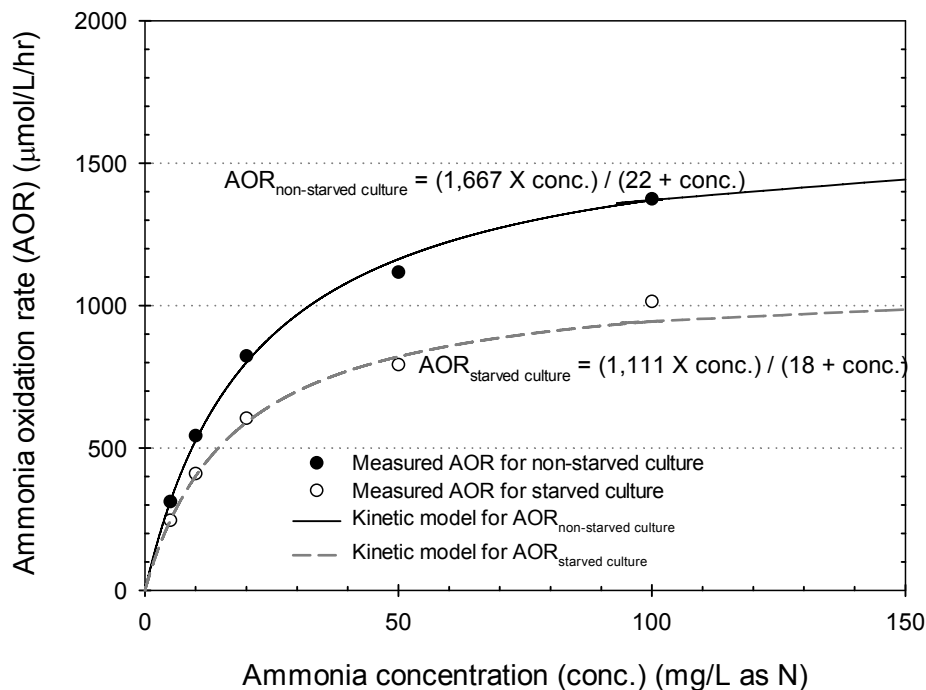


Figure 5-4. Measured and modeled ammonia oxidation rates for the AOB culture in the enriched nitrifying system under different ammonia concentrations.

Therefore, the AOB culture in the enriched nitrifying system merely performed at approximately one fourth (446 vs. 1,667 $\mu\text{mol/L/hr}$) of its maximum oxidation capacity under steady-state operation. The ammonia oxidation rate would increase if the influent ammonia loading for the enriched nitrifying system was increased. Moreover, the K_s values (substrate affinity coefficient) from the models were 22 and 18 mg/L $\text{NH}_3\text{-N}$ for the non-starved and starved cultures, respectively. These K_s values were very close to the one (~ 25 mg/L $\text{NH}_3\text{-N}$) reported for a $(\text{NH}_4)_2\text{SO}_4$ -insensitive strain GH22 (Suwa et al., 1994) suggesting that the enriched AOB in the nitrifying system may represent a group which tended to be more active under high ammonia concentration (similar to the GH22 strain).

In a follow-up study, the responses of the *amoA* mRNA transcripts to different ammonia levels were further evaluated using 1-L batch cultures (Sections 3.2.3) to assess whether or not *amoA* mRNA abundance reflects the actual ammonia oxidation rate (Section 5.3.3). In order to more precisely represent the *amoA* mRNA responses under certain ammonia concentrations, the ammonia concentration in each batch culture test was purposely controlled at a designated level based on a feeding strategy (Section 3.2.3) determined from the ammonia oxidation rate information using the kinetics models. In each batch culture test, ammonia was continually fed at a rate equal to the rate of the ammonia oxidation expected under certain ammonia level. For instance, it was expected that about 11.27 mg $\text{NH}_3\text{-N}$ would be oxidized in the one liter mixed liquor sample within a one-hour period for the batch culture containing 20 mg/L $\text{NH}_3\text{-N}$ (measured value was 11.51 mg $\text{NH}_3\text{-N}$); therefore, approximately 11.27 mg $\text{NH}_3\text{-N}$ was added to the 1-L culture every hour (continual feed of a 0.9395 mg/mL $\text{NH}_3\text{-N}$ stock solution at a flow rate of 12 mL/hr, Table 5-1) to maintain an ammonia level as close to 20 mg/L as possible. Additionally, the ammonia oxidation rate information was used to calculate theoretical alkalinity consumption rates to determine the carbonate (CO_3^{2-}) feeding strategies (Section 3.2.3) needed to maintain a stable pH level of 7.2 during the test period. In each batch culture test, carbonate was continually fed at a rate equal to the rate at which carbonate was theoretically consumed during ammonia oxidation.

Table 5-1. The ammonia oxidation rate information was used to determined ammonia and carbonate feeding strategies to maintain constant ammonia and pH levels during follow-up 1-L batch culture tests (assessing *amoA* mRNA responses under different ammonia concentrations, [Section 5.3.3](#)).

Designated ammonia levels (mg/L) for tests	Expected AOR* mg NH ₃ -N/L/hr (μmol/L/hr)	Ammonia (in mg/mL) needed to be added at a flow rate of 12 mL/hr	Carbonate (in mg/mL) needed to be added at a flow rate of 12 mL/hr
0	0.00 (0.0)	0.0000	0.0000
1	1.03 (73.6)	0.0858	0.2875
5	4.39 (313.6)	0.3662	1.2267
10	7.41 (529.3)	0.6173	2.0681
20	11.27 (805.0)	0.9395	3.1473
50	16.41 (1172.1)	1.3678	4.5822
100	19.36 (1382.9)	1.6129	5.4033

* Expected ammonia oxidation rate for non-starved culture using the best fit kinetic model.

5.2 Enriched nitrifying system microbial population

5.2.1 AOB identification based on *amoA* sequence

The *amoA* gene library for the enriched nitrifying system ([Appendix C](#)) was constructed by the same procedure used to create the *amoA* gene library for the industrial bench-scale system. Briefly, target *amoA* gene in genomic DNA extracted from enriched culture of the nitrifying system was amplified by nested-PCR using a combination of AMO-F2*/*amoA*-2R* and *amoA*-1F/*amoA*-2R* primer pairs. The PCR products were subsequently cloned into plasmid vector (pCR[®]4-TOPO[®]). Plasmid DNA of selected clones with target *amoA* gene was isolated with a DNA purification kit ([Section 3.4.2](#)). Purified plasmid DNA extracts of eight selected clones were sequenced.

Sequences of a section of the *amoA* gene were compared with the *amoA* sequences in a publicly accessible database (i.e., NCBI) using the BLAST program ([Altschul et al., 1990](#)). Based on the *amoA* sequence comparison, all eight cloned sequences for the AOB culture in the enriched nitrifying system had a ≥93% similarity to the *amoA* sequence of *Nitrosomonas europaea* (GenBank accession number AF058691) or an uncultured AOB isolate ENI-11 (GenBank accession number AB179055.1) ([Table 5-2](#)).

Table 5-2. Identification of *amoA* clone library for the enriched nitrifying system using a published database as reference.

Reference AOB	Close match clones* from the enriched nitrifying system
<i>Nitrosomonas europaea</i> [AF058691]	3, <u>4</u> , 7, 9, 20, 23, 36, 42

4: the clone selected to prepared real-time PCR standard and RT real-time PCR standard. *: clones were also affiliate with an uncultured AOB isolate ENI-11 [AB179055.1]

The target *amoA* sequences were aligned with the CLUSTAL X program (Thompson et al., 1997) in order to construct a phylogenetic tree and design real-time PCR primers and probe to quantify both *amoA* DNA and mRNA for the *N. europaea* group. The results suggested that the AOB in the enriched nitrifying system were phylogenetically related to the *N. europaea* group (Figure 5-5). No AOB from the *N. communis*, *N. marina*, *N. oligotropha*, and *N. cryotolerans* clusters were identified in this enriched nitrifying system.

Based on the *amoA* sequence alignment results there was more than one mismatch (2~9 mismatches) between the *amoA* sequences of clones for the enriched nitrifying system and those of primers and probes designed to quantify other AOB groups (e.g., *N. nitrosa*, RI-27, and B2-3) (Figure 5-6, Table 5-3). Hence, the previously-designed real-time PCR primer/probe sets would not quantify the *N. europaea* identified in the enriched nitrifying system.

Based on the *amoA* gene sequence comparisons, a new primer/probe set (amoA-Ne-590f/amoA-Ne-Taq-720r/amoA-Ne-751r) was designed to quantify the *N. europaea* *amoA* DNA and mRNA abundance. When the sequences of the newly designed primers and probe for the *N. europaea* group were compared with those of other AOB groups at corresponding positions, more than two mismatches (3-9) were found at the primers and probe sites (Figure 5-6, Table 5-4). In contrast, the clones' sequences for the enriched nitrifying system were highly identical (no mismatch, Figure 5-6, Table 5-4) to the primers and probe, suggesting the newly designed primers/probe set was suitable to quantify the *amoA* DNA and mRNA for the *N. europaea* group identified in the enriched nitrifying system.

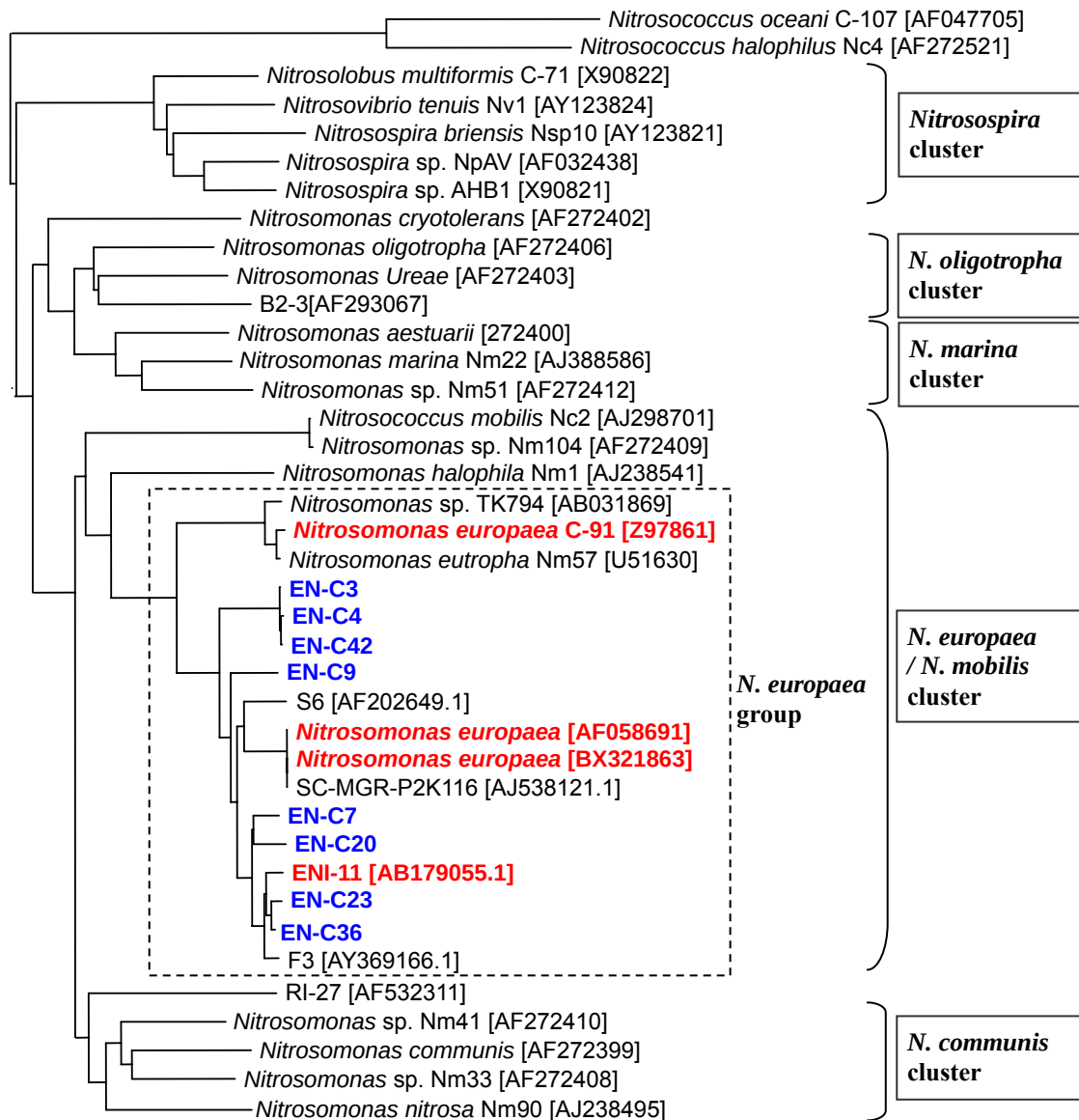


Figure 5-5. Phylogenetic relationship of AOB species/strains based on gene sequences of a section of *amoA* gene visualized in TreeView (Page, 1996). Most of the *amoA* sequences in the clone libraries from the enriched nitrifying system were similar ($\geq 93\%$) to the *amoA* sequence of *Nitrosomonas europaea* [AF058691] or an uncultured AOB isolate ENI-11 [AB179055.1]. (Clone for the enriched nitrifying system was named as EN-C#1, in which #1 is a given clone number.)

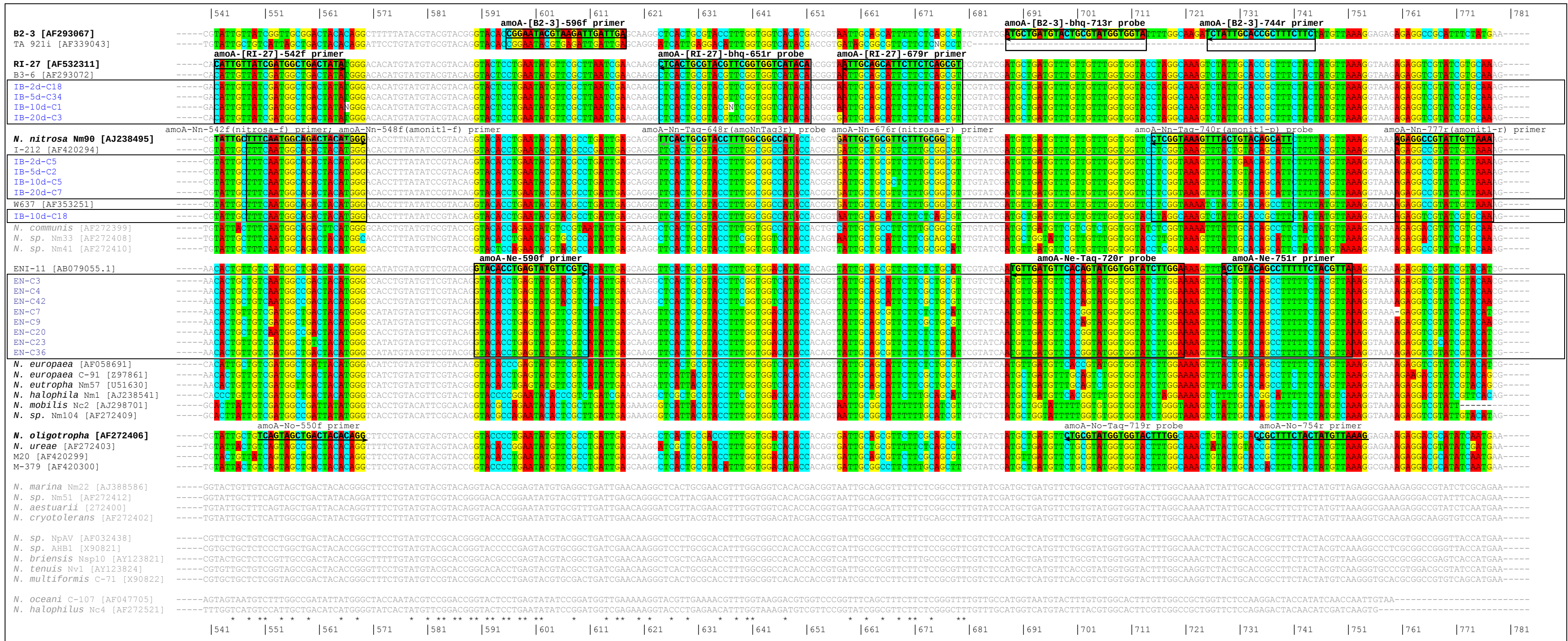


Figure 5-6. CLUSTAL X (1.64b) multiple sequence alignment for *amoA* sequences from the enriched nitrifying system *amoA* clone libraries (named as EN-C#1), the industrial bench-scale system *amoA* clone libraries (named as IB-#2d-C#3) and from published databases.

Table 5-3. The number of mismatches in *amoA* gene sequences between *amoA* clone libraries for the enriched nitrifying system and real-time PCR primers and probes designed to specifically quantify *N. europaea*, *N. nitrosa*, *N. oligotrophica* (Dionisi et al., 2002), RI-27, and B2-3.

AOB groups	Primers and probes for real-time PCR	Mismatches in <i>amoA</i> sequences at primers and probes sites
<i>N. europaea</i>	forward primer: amoA-Ne-590f	0
	probe: amoA-Ne-bhq-720r	0
	reverse primer: amoA-Ne-751r	0
<i>N. nitrosa</i>	forward primer: amoA-Nn-548f	2~4
	probe: amoA-Nn-Taq-740r	3
	reverse primer: amoA-Nn-777r	4~5
<i>N. nitrosa</i>	forward primer: amoA-Nn-542f	4~7
	probe: amoA-Nn-Taq-648r	2~4
	reverse primer: amoA-Nn-676r	4~5
<i>N. oligotrophica</i>	forward primer: amoA-No-550f	4~6
	probe: amoA-No-Taq-719r	5
	reverse primer: amoA-No-754r	5
RI-27	forward primer: amoA-[RI-27]-542f	3~6
	probe: amoA-[RI-27]-bhq-651r	2~5
	reverse primer: amoA-[RI-27]-679r	3
B2-3	forward primer: amoA-[B2-3]-596f	8~9
	probe: amoA-[B2-3]-bhq-713r	5
	reverse primer: amoA-[B2-3]-744r	6

Table 5-4. The number of mismatches in *amoA* sequences of some AOB groups when compared with sequences of the newly designed primers and probe for the *N. europaea* group.

Reference AOB	Primers and probe for specifically targeting <i>N. europaea</i> group		
	Forward primer: amoA-Ne-590f	Probe: amoA-Ne-bhq-720r	Reverse primer: amoA-Ne-751r
ENI-11 [AB179055.1]*	0	0	0
<i>N. nitrosa</i> Nm90 [AJ238495]	3	9	3
<i>N. oligotropha</i> [AF272406]	3	6	6
RI-27 [AF532311]	4	8	7
B2-3 [AF293067]	6	7	6

* An uncultured AOB isolate in *N. europaea* group whose sequences at designed primer and probe sites were highly identified (>99%) with the gene sequences from *amoA* clone libraries for the enriched nitrifying system.

5.2.2 Population structure

The number of total eubacteria in the enriched nitrifying system under steady-state conditions was quantified using a 16S rDNA-based real-time PCR assay (Dionisi et al., 2002) (Section 3.4.4). The 16S rDNA of AOB were quantified using another real-time PCR assay (Harms et al., 2003) for determining the overall AOB population. The concentration of *N. europaea* was quantified using the new primer/probe set designed in this study. Under steady-state conditions, the average *N. europaea* population ($1.77 \pm 0.45 \times 10^{11}$ cells/L) was very close to the average total eubacteria ($2.58 \pm 0.46 \times 10^{11}$ cells/L) (difference was statistically insignificant, p-value>0.01), and somewhat higher than the average total AOB ($9.03 \pm 2.61 \times 10^{10}$ cells/L). This suggests the *N. europaea* dominated the overall AOB population and was a major bacterial group (~68.6% of total eubacteria) in the enriched nitrifying system (Figure 5-7). The concentration of *N. europaea* was stable over the entire study period as demonstrated by the low standard deviation (coefficient of variation = 25.4%) for the quantified population levels. This simplified the follow-up activity studies for the enriched nitrifying system because any

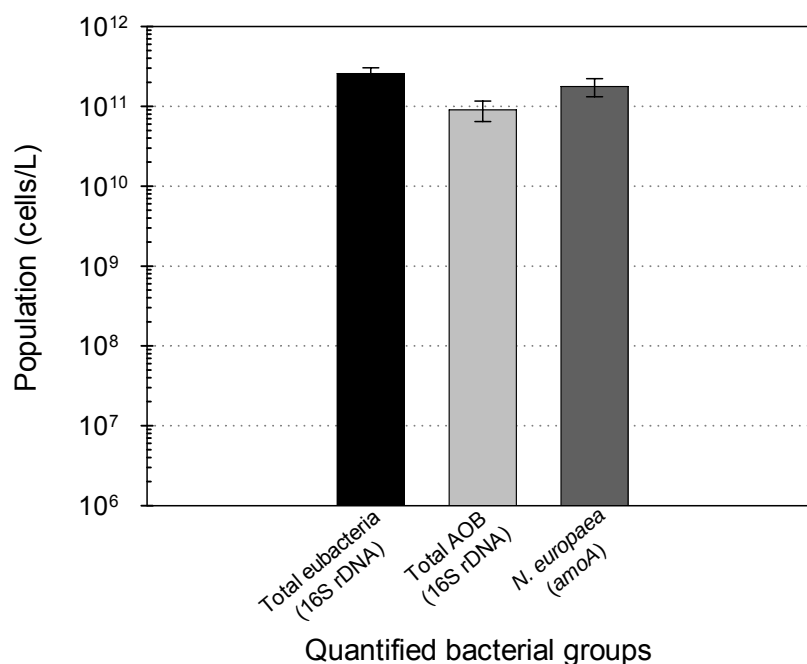


Figure 5-7. Average population sizes for total eubacteria, total AOB, and *N. europaea* groups. (Average values were based on measurements of 18 samples collected during steady operating condition over 12-month study period.)

significant change in AOB populations would complicate assessments for cellular *amoA* mRNA abundance and ammonia oxidation activity (specific primer/probe set is required to quantify *amoA* mRNA for particular AOB group). Therefore, the follow-up physiological activity study for the enriched nitrifying system was focused on the *N. europaea* group.

The proportion of the *N. europaea* population in the enriched nitrifying reactor system was approximately 68.6% (calculated by dividing the average *N. europaea* population by the average total eubacteria number). This percentage is much higher than that in the 10-day SRT train in the industrial bench-scale system (2.38~9.06%), and slightly higher than the percentage found in an autotrophic nitrifying biofilm (47.2±5.8%, using FISH) (Okabe et al., 2005). Since the enriched nitrifying system was fed with a substrate free of organic carbon, a theoretical value for the AOB percentage could not be further determined because of a lack of theoretical heterotroph abundance. Alternatively,

it has been suggested that dead biomass or the formation of soluble microbial products (SMP) from nitrifying bacteria can provide a supplementary organic substrate for heterotrophs (Rittmann et al., 1994; Kindaichi et al., 2004; Okabe et al., 2005). A theoretical AOB percentage value (61.8%) was obtained for the enriched nitrifying system using an assumption that organic-C source from dead biomass and/or metabolites of nitrifying bacteria was about 60% of the organic biomass (i.e., MLVSS). These results suggest that some heterotrophic growth occurred in the enriched nitrifying system receiving influent that was free of organic-C.

Obviously, the AOB community structure in the enriched nitrifying system (consisting predominantly of the *N. europaea* cluster) was different from that in the industrial bench-scale system (dominated by RI-27 or B2-3). It has been suggested that AOB in the *N. europaea* cluster has a high K_s value (low substrate affinity); thus, has a high activity under high ammonia concentration, and prefers eutrophic habitats (Koops and Pommerening-Röser, 2001). Under steady-state operation, both ammonia loading and concentration in the enriched nitrifying system reactor (i.e., $\sim 446 \mu\text{mol/L/hr}$ and $9.1 \pm 0.4 \text{ mg/L}$, respectively) were higher than that in the industrial bench-scale system (i.e., $59 \sim 68 \mu\text{mol/L/hr}$ and $< 1.0 \text{ mg/L}$). Therefore, the eutrophic conditions in the enriched nitrifying system allowed the *N. europaea* group to become dominant; while the RI-27 and B2-3 groups were not detected in this system.

An average cellular ammonia oxidation rate of 2.5 fmol/cell/hr was determined for the *N. europaea* cells in the enriched nitrifying system based on the overall volumetric ammonia oxidation rate (in $\mu\text{mol/L/hr}$) (Section 5.1.2), and the average *N. europaea* population (in cells/L). This calculated cellular ammonia oxidation rate was in a reasonable range when compared with previously reported results ($0.03 \sim 43 \text{ fmol/cell/hr}$) (Table 4-20). Since the *N. europaea* group was the dominant AOB population in the enriched system, it was deemed that this calculated average cellular ammonia oxidation rate represented activity conditions for the *N. europaea* group. In the next section, the effects of NH_3 and DO levels on oxidation activity and physiological conditions for *N. europaea* were assessed based on *amoA* mRNA measurement.

5.3 Activity study based on *amoA* mRNA for *N. europaea* group

It has been suggested that the abundance of *amoA* mRNA transcripts may correspond to the ammonia oxidation rate of AOB (Aoi et al., 2002, 2004). However, this correlation has not been investigated for particular AOB groups due to a lack of specific primers and probes to quantify *amoA* mRNA transcripts. In this study, a reverse transcription (RT) real-time PCR assay was developed to specifically quantify *amoA* mRNA for the *N. europaea* group (dominant AOB population in the enriched nitrifying system). An *amoA* mRNA standard stock solution was prepared for the quantitative RT real-time PCR assay to calibrate target transcripts. Response of *amoA* mRNA in the *N. europaea* to different ammonia levels was assessed using 1-L batch culture tests over a 2-day period (Section 5.3.3). In addition, *amoA* mRNA levels in *N. europaea* were examined relative to changes in ammonia loading and DO level in a 10-L reactor (the Trial reactor of the enriched nitrifying system) (Section 5.3.4 and 5.3.5). Based on results from these experiments, the correlation between *N. europaea amoA* mRNA levels and the actual ammonia oxidation activities could be assessed.

5.3.1 Preparing *amoA* mRNA standard

In order to obtain a standard to quantify *N. europaea amoA* mRNA using RT real-time PCR, the clone EN-C4 from the *amoA* library of the enriched nitrifying system was selected to make a *N. europaea amoA* mRNA standard (Section 3.5.2). This clone was grown in a sterile 15-mL centrifuge tube using 5mL of LB Kan₅₀ media. Plasmid DNA was extracted from the enriched clone library, and was linearized (by digesting with *speI* restriction enzyme) to allow a fragment of *amoA* mRNA transcripts to be synthesized in an *in vitro* transcription reaction. The integrities of purified and linearized plasmid DNA were evaluated using 1% (w/v) agarose gel (Figure 5-8). The gel image showed that the linearized plasmid DNA moved faster than non-linearized (circular) plasmid DNA and formed a single band at around the 4,000-bp marker position (actual size for the plasmid DNA with target inserted is about 4,454 bp). This result suggested that the integrity and purity of the linearized plasmid DNA was good.

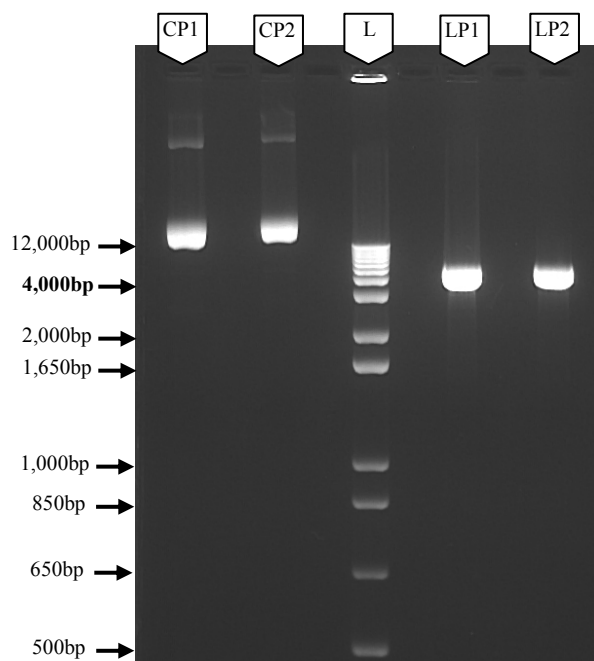


Figure 5-8. Image of gel electrophoresis (100mV for 2~3 hrs) for purified circular and linearized plasmid DNA. (L: 1Kb Plus DNA Ladder; CP1, and CP2: purified circle (non-linearized) plasmid DNA; LP1, and LP2: linearized plasmid DNA).

Subsequently, the anti-sense strand in the linearized plasmid DNA served as a template for RNA polymerase to synthesize a fragment of *amoA* mRNA transcripts using the RiboMAX™ Large Scale Transcription RNA Production System (Section 3.5.2). After the transcription product was cleaned-up with the RNeasy® Mini Kit, DNase digested, and concentrated, a stock solution of the *in-vitro* produced *amoA* mRNA standard was immediately stored at -80°C for further quality and quantity evaluations. The quality of the prepared *amoA* mRNA standard was evaluated using a denaturing agarose gel (to determine the integrity of the synthesized mRNA), an UV absorption ratio (A_{260}/A_{280}) (to determine protein contamination), and a no-RT RT real-time PCR reaction (to determine contamination of transcription template, linearized plasmid DNA) (Section 3.5.3). Additionally, the quantity of *amoA* mRNA in the prepared standard stock solution was determined by spectrophotometric quantification and RiboGreen® RNA quantitation (Section 3.5.3).

The integrity of the *amoA* mRNA standard was evaluated by formaldehyde-denaturing agarose gel (Figure 5-9). The gel image showed that the artificially-transcribed mRNA formed a single band close to the 623-bases marker position (the size of transcription product should be 585 bases, beginning with the T7 priming site to the end of the *SpeI* restriction site); while the positive control represented a single band around the 1,908-bases marker position (size should be ~1,800 bases). No band was seen in the negative control. These results demonstrated that the transcription reaction succeeded and the prepared mRNA standard had good integrity and had good quantity based on the observed band intensity (Figure 5-9).

The quality of the *amoA* mRNA preparation was further evaluated by determining the UV absorption ratio (A_{260}/A_{280}) to check the amount of protein contamination. The mRNA standard stock solution was diluted 1:500 using 10mM Tris-HCl (pH=7.6), and the absorbance of the dilution was measured under a UV wavelength of 260 nm

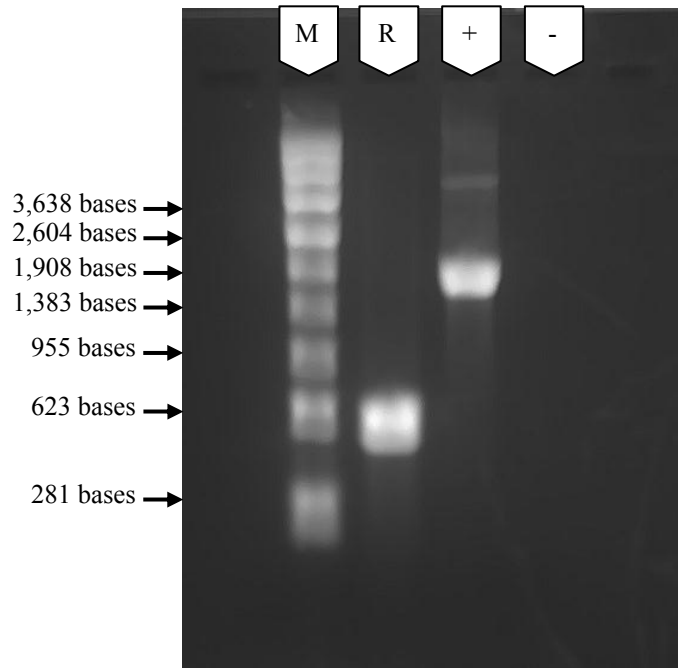


Figure 5-9. Formaldehyde-denaturing agarose gel for evaluating integrity of the *in-vitro* synthesized *amoA* mRNA (for the *N. europaea* group). (M: RNA markers; R: prepared RNA standard for calibrating *N. europaea amoA* mRNA; “+”: positive control; “-”: negative control).

(maximum absorption of nucleic acids, RNA and DNA) and then 280 nm (maximum absorption of proteins). The higher the absorption ratio is the lower proportion of protein will be in the mRNA preparation. A reference ratio of 1.8~2.0 was used to estimate nucleic acid quality (Pfaffl 2000; Sambrook and Russell, 2001). In this study, the ratio value for the buffer diluted mRNA standard was 1.97 ± 0.23 suggesting that the prepared *amoA* mRNA standard stock solution had low protein contamination and high nucleic acid quality.

Quality of the *amoA* mRNA preparation was further evaluated using a no-RT RT real-time PCR reaction to check for the presence of DNA contamination (residue of the transcription template, linearized plasmid DNA) in the mRNA standard stock solution. The mRNA standard stock solution was diluted to 10^9 copies/ μ L using DEPC H₂O based on quantification results. A series of *amoA* DNA dilutions ($10^1 \sim 10^7$ copies/ μ L) served as standards for calibrating DNA content in the 10^9 -copies/ μ L mRNA standard solution. The result indicated that the DNA concentration in the prepared dilution (10^9 copies/ μ L mRNA) was $2.70 \pm 0.75 \times 10^3$ copies/ μ L (Figure 5-10) suggesting that DNA contamination was not significant in the prepared *amoA* mRNA standard solution. Thus, the low DNA residue in the mRNA standard preparation would not bias the quantification results from the RT real-time PCR.

The quantity of the synthesized *amoA* mRNA standard for the *N. europaea* group was determined in a 1:500 dilution of the stock solution in DEPC H₂O. The absorbance of the dilution was measured under UV wavelength of 260 nm using a Beckman CoulterTM (DU[®]640B) spectrophotometer (Section 3.5.3). The measured absorption values were converted to concentration for the mRNA preparation using a coefficient of 40 (an A₂₆₀ of 1 corresponds to a 40 ng/ μ L RNA solution). The average concentration of prepared *amoA* mRNA was $1,257.0 \pm 52.5$ ng/ μ L (Table 5-5). This value was further converted to copies/ μ L using a coefficient of $\sim 3 \times 10^9$ copies/ng. This coefficient was determined as follows (using a synthesized RNA size $\approx$ 585 bases; an average RNA molecular weight $\approx$ 340 daltons/base, or g/mole/base; and, an Avogadro's number $\approx 6 \times 10^{23}$ molecules/mole, or copies/mole).

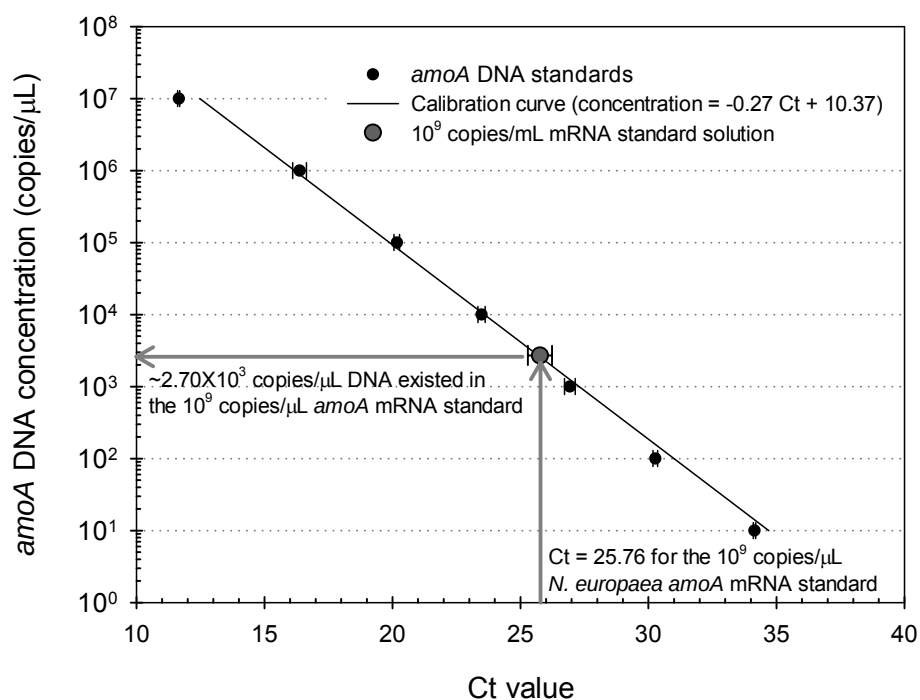


Figure 5-10. A series of *amoA* DNA standards was used to calibrate DNA content in the prepared *N. europaea amoA* mRNA standard stock solution (using 10⁹ copies/μL dilution).

Table 5-5. Concentration of the prepared *N. europaea amoA* mRNA standard measured by spectrophotometry and RiboGreen[®] assay.

Quantification methods	Concentration (ng/μL)	Concentration (copies/μL)
Spectrophotometer (Beckman Coulter [™] DU [®] 640B)	1,257±53	3.77±0.16×10 ¹²
RiboGreen [®] (measured by Wallac [†])	1,250±255	3.75±0.77×10 ¹²
RiboGreen [®] (measured by BIO-RAD [‡])	1,439±177	4.32±0.53×10 ¹²
Mean (with standard deviation)	1,301±149	3.90±0.45×10 ¹²

[†]: Wallac 1420 Victor2[™] Multilabel Counter; [‡]: BIO-RAD VersaFluor[™] Fluorometer.

$\sim 585 \text{ bases} \times \sim 340 \text{ g/mole/base} \approx 198,900 \text{ g/mole}$

$1 \text{ ng} \div \sim 198,900 \text{ g/mole} \approx 5.028 \times 10^{-15} \text{ mole}$

$(\sim 5.028 \times 10^{-15} \text{ mole}) \times (\sim 6 \times 10^{23} \text{ copies/mole}) \approx 3 \times 10^9 \text{ copies}$

Therefore, the concentration for the prepared *N. europaea amoA* mRNA was approximately $3.77 \times 10^{12} \text{ copies/}\mu\text{L}$ (Table 5-5).

The *amoA* mRNA concentration in the prepared standard stock solution was also confirmed by RiboGreen[®] RNA Quantitation assay using a 1:100,000 dilution (final volume of 200- μL measured by Wallac 1420 Victor2[™] Multilabel Counter, and final volume of 2,000- μL measured by BIO-RAD VersaFluor[™] Fluorometer) (Section 3.5.3). Concentrations determined by RiboGreen[®] RNA quantitation assay using two different detectors were $1,250 \pm 255 \text{ ng/}\mu\text{L}$, and $1,439 \pm 177 \text{ ng/}\mu\text{L}$, respectively (Table 5-5). These results were similar to the value determined by spectrophotometry. The three measured values were converted from ng/ μL to copies/ μL and with a mean of $3.90 \times 10^{12} \text{ copies/}\mu\text{L}$ (Table 5-5). This average concentration (in copies/ μL) was used to determine the dilution rate for making the 10^9 -copies/ μL standard solution from the prepared mRNA stock.

Standards were diluted from the prepared *N. europaea amoA* mRNA standard stock solution in a series from 1×10^9 to 1×10^1 copies/reaction. These standard solutions were pre-tested using a reverse transcription (RT) real-time PCR assay developed in this study. The detected fluorescence signals versus threshold cycles (Ct values) are plotted in Figure 5-11. Results indicate that the prepared mRNA did react with the newly designed primers/probe specific for the *N. europaea* group and thus was detected by the RT real-time PCR. The high standard solution had a low Ct value. An RNA extract for a biosolids sample taken from the enriched nitrifying system was also detectable (Figure 5-11) suggesting that the developed RT real-time PCR method could be used to measure the abundance of target *amoA* mRNA in the *N. europaea* group. In standard solutions lower than 1×10^3 copies/reaction, PCR reactions generated very weak signals over 51 cycles and resulted in poor Ct values. Thus, standards ranging from 1×10^3 to 1×10^9 were selected to quantify target mRNA. A good linear relationship was observed between standard concentrations and Ct values (Figure 5-12).

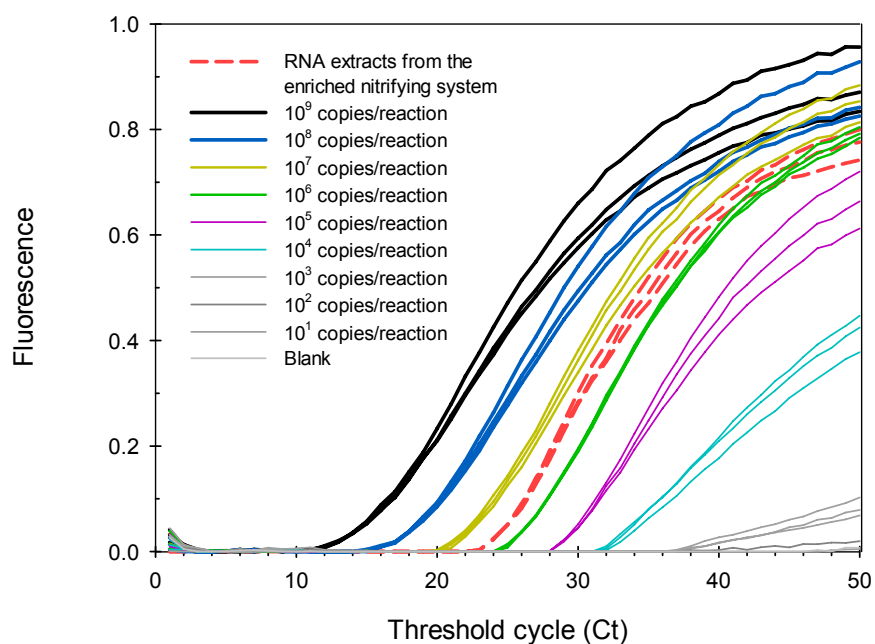


Figure 5-11. Reverse transcription (RT) real-time PCR pre-test for the prepared *N. europaea amoA* mRNA standard and RNA extracts from the enriched nitrifying system.

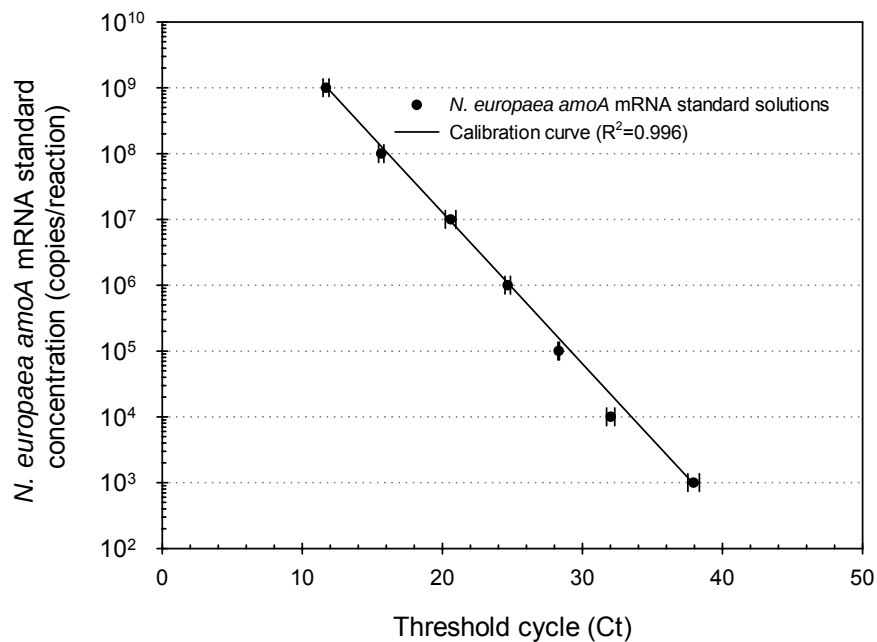


Figure 5-12. Linear relationship observed between mRNA standards and Ct values.

5.3.2 Generating a culture harvest strategy for activity studies

In order to observe significant *amoA* mRNA responses under different ammonia concentrations for the *N. europaea* group, the activity study was designed to start at low *amoA* mRNA levels (close to background). A preliminary test was conducted to assess the *N. europaea amoA* mRNA decay profile under ammonia starvation using a 10-L reactor (i.e., the Trial reactor in the enriched nitrifying system) (Section 3.2.2). The enriched culture in the Trial reactor was incubated at 30°C under ammonia-free conditions and complete aeration (at an air flow rate of about 80 mL/min/L to yield a DO level of 3~4 mg/L). Additionally, the pH in the bioreactor was controlled at a 7.20 level using 1N hydrochloric acid (HCl).

After ammonia feeding was halted, an increase in pH (from 7.20 to 7.33) was observed after 5 hours of ammonia-starvation (Figure 5-13). The pH levels were lowered to a range of 7.20-7.30 using 1N HCl. Results showed that the *N. europaea amoA* DNA

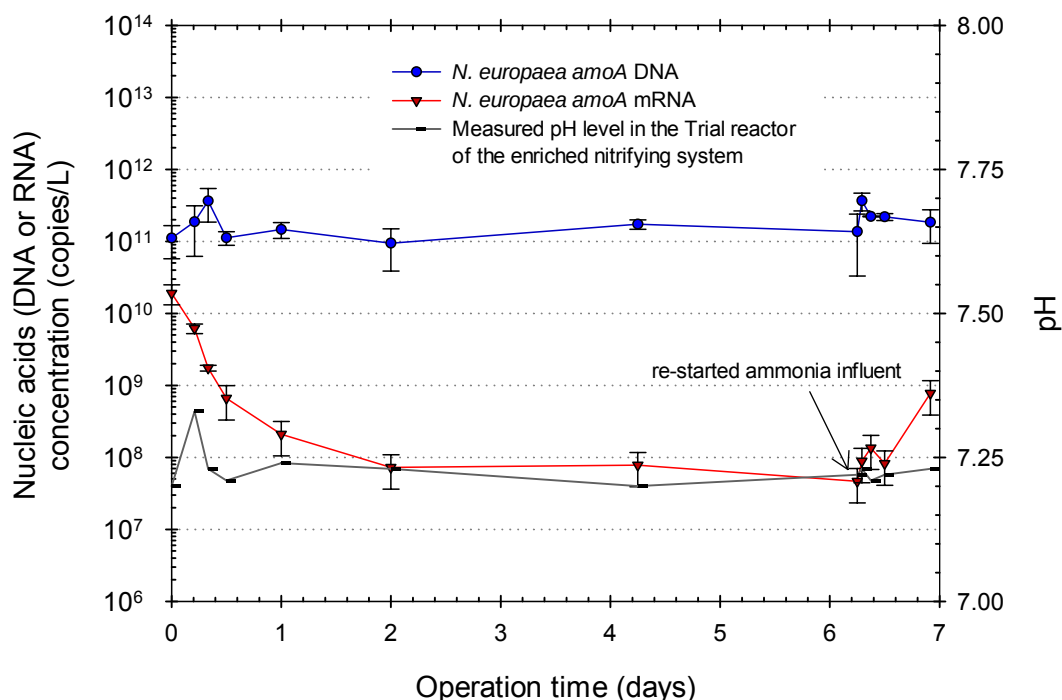


Figure 5-13. *N. europaea amoA* mRNA decay profile during ammonia starvation in the Trial reactor of the enriched nitrifying system.

levels remained stable over a 6-day ammonia starvation period; while the *amoA* mRNA abundance dropped from $1.90 \pm 0.59 \times 10^{10}$ to $7.25 \pm 3.62 \times 10^7$ copies/L during the first two days of ammonia starvation (Figure 5-13). After this decrease within the first two days, *amoA* mRNA abundance remained stable at a basal level of approximately 1×10^8 copies/L for the remainder of the incubation period under ammonia-free condition. When ammonia-feeding was re-initiated on day 6.25, a gradual increase in the *amoA* mRNA level was noted.

Similar results have been reported by Aoi et al., (2004 (b)). Those researchers found that the *amoA* mRNA level decreased 15-fold during 72 hours (3 days) of ammonia starvation with a fast decline rate in the first 24 hours. In another study, Bollmann et al. (2005) found that *Nitrosospira briensis amoA* mRNA decreased after about 6 days of ammonia starvation. The reported *amoA* mRNA declination trend was similar to what was observed in this study; however, the declination extent observed in this study (about 200-fold) was more significant. Using a Northern hybridization technique, Sayavedra-Soto et al. (1996) found that AOB cells need to be in an ammonia-free environment for more than 8 hours for AMO and HAO mRNA to decrease to undetectable levels (until bands became invisible in the Northern blot). The slow decrease in mRNA levels for AOB cells may result from the ribosome protection and 5' binding mechanisms (Carrier and Keasling, 1997), the presence of stable loop structures, or a low constitutive mRNA production fuelled by available endogenous energy (Sayavedra-Soto et al., 1996). In addition, AOB was capable of remaining in a state allowing fast recovery of ammonia oxidation activity using available endogenous energy for the preferential expression of key mRNA (e.g., *amo* and *hao*) under energy limitation (Bollmann et al., 2005).

Based on the preliminary results of *amoA* mRNA decay profiles in the *N. europaea* group under ammonia starvation condition, it was decided that an ammonia starved culture (for at least 2 days) in the 10-L reactor with pH control would be utilized to harvest cells for *amoA*-mRNA-based activity studies. In order to minimize impacts on the steady-state operation for the Stable reactor, ammonia starvation was only conducted in the Trial reactor of the enriched nitrifying system.

5.3.3 *amoA* mRNA responses under different ammonia concentrations

The response of the *N. europaea amoA* mRNA to different ammonia levels was examined in 1-L batch tests using constant ammonia concentrations (i.e., 0, 1, 5, 10, 20, 50, and 100 mg/L as N) and 2-day starved cultures from the Trial reactor of the enriched nitrifying system (Section 3.2.3). Although the ammonia level in each batch culture test was controlled by continually feeding ammonia at a rate equal to the predicted ammonia oxidation rate, small variances between the actual and designated ammonia levels would sometimes occur during the 2-day testing period. Thus, ammonia concentrations in each batch were monitored every 1-2 hours. When the measured value was more than 10% of designated level, ammonia concentration in the batch was manually adjusted (by adding additional ammonia or ceasing ammonia feeding). The measured results indicated that the ammonia concentration in each batch test was very close to the desired level (Figure 5-14, Table 5-6). In addition, the ammonia oxidation rates determined for each batch were constant after 1-2 hours incubation (Figure 5-15), and very close to the previously determined values (previously measured and modeled ammonia oxidation rates for the cultures from the enriched nitrifying system, Section 5.1.3) (Figure 5-16). These results suggest that the ammonia concentration in each batch was maintained at the designated level. Therefore, it was believed that follow-up measurements quantifying the abundance of *amoA* mRNA at each ammonia concentration represented the transcriptional response of the *N. europaea* group for that ammonia level.

Both *amoA* DNA and mRNA abundances were quantified using *N. europaea*-specific real-time PCR, and RT real-time PCR, respectively. Results showed that both the *amoA* DNA and mRNA levels in the blank batch culture (ammonia-free) did not notably change over the 2-day testing period (Figure 5-17). However, the *amoA* mRNA abundance increased, from a basal level of $1.35 \pm 0.31 \times 10^8$ copies/L to different levels ($7.09 \pm 0.51 \times 10^8$ - $3.66 \pm 0.18 \times 10^{11}$ copies/L), in the other six batches fed with various levels of ammonia while the *amoA* DNA levels remained unchanged (Figures 5-17, 5-18). The *amoA* mRNA/DNA ratio profiles basically followed the trends of the mRNA levels. After incubation started, the target mRNA levels quickly increased and reached a plateau

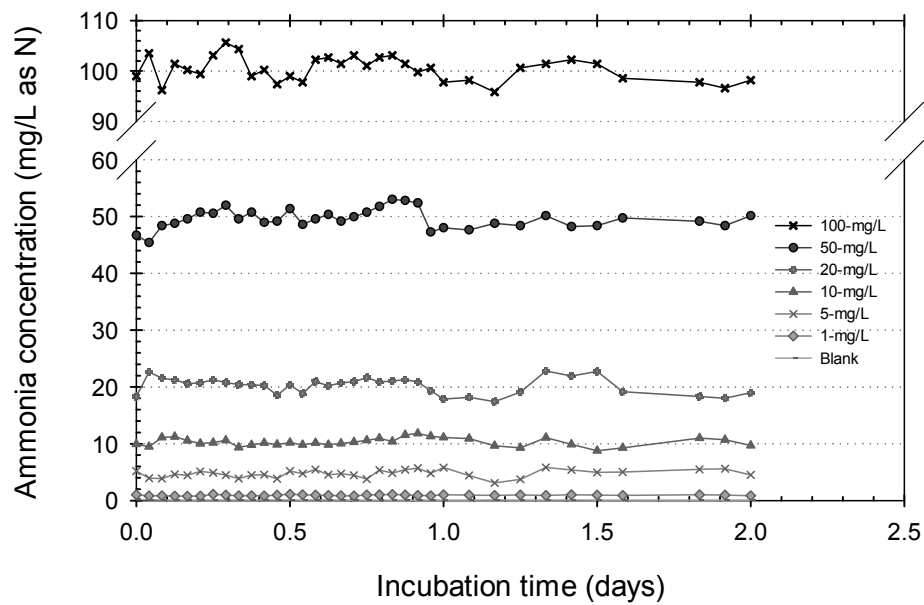


Figure 5-14. Measured ammonia concentrations in each batch culture test to study *amoA* mRNA response using different ammonia concentrations.

Table 5-6. Average ammonia concentrations and ammonia oxidation rates determined in batch culture with different ammonia concentrations during 1-L batch culture tests for *amoA*-mRNA-based activity.

Target ammonia level (mg/L as N)	Measured ammonia concentration (mg/L as N)	Calculated ammonia oxidation rate ($\mu\text{mol NH}_3\text{-N/L/hr}$)
100-mg/L	100.4 $\pm$ 2.4	1,372.4 $\pm$ 74.2
50-mg/L	49.6 $\pm$ 1.7	1,132.6 $\pm$ 41.9
20-mg/L	20.2 $\pm$ 1.5	780.2 $\pm$ 38.7
10-mg/L	10.3 $\pm$ 0.7	549.4 $\pm$ 35.9
5-mg/L	4.7 $\pm$ 0.7	380.6 $\pm$ 22.5
1-mg/L	0.9 $\pm$ 0.1	142.1 $\pm$ 14.5
Blank	<0.1 $\pm$ 0.0	0.0

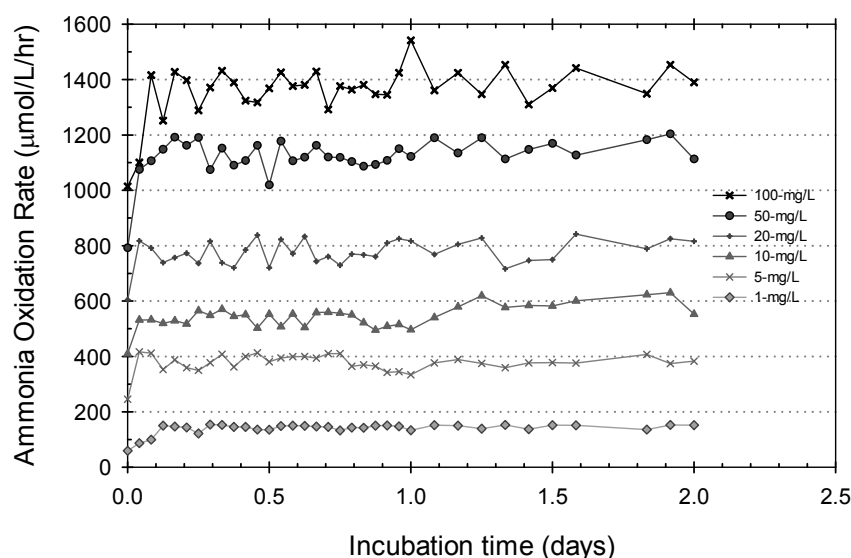


Figure 5-15. Calculated ammonia oxidation rate in each batch culture test to study *amoA* mRNA response using different ammonia concentrations.

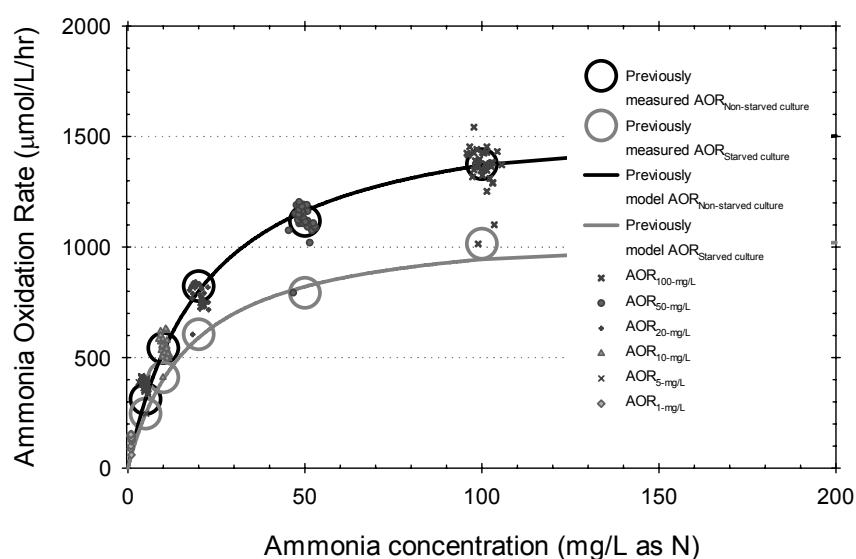


Figure 5-16. Ammonia oxidation rates determined in batch culture using different ammonia concentrations plotted together with previously measured ammonia oxidation rate (AOR_{Non-starved culture}, and AOR_{Starved culture}) and determined kinetic models for non-starved and starved cultures taken from the enriched nitrifying system.

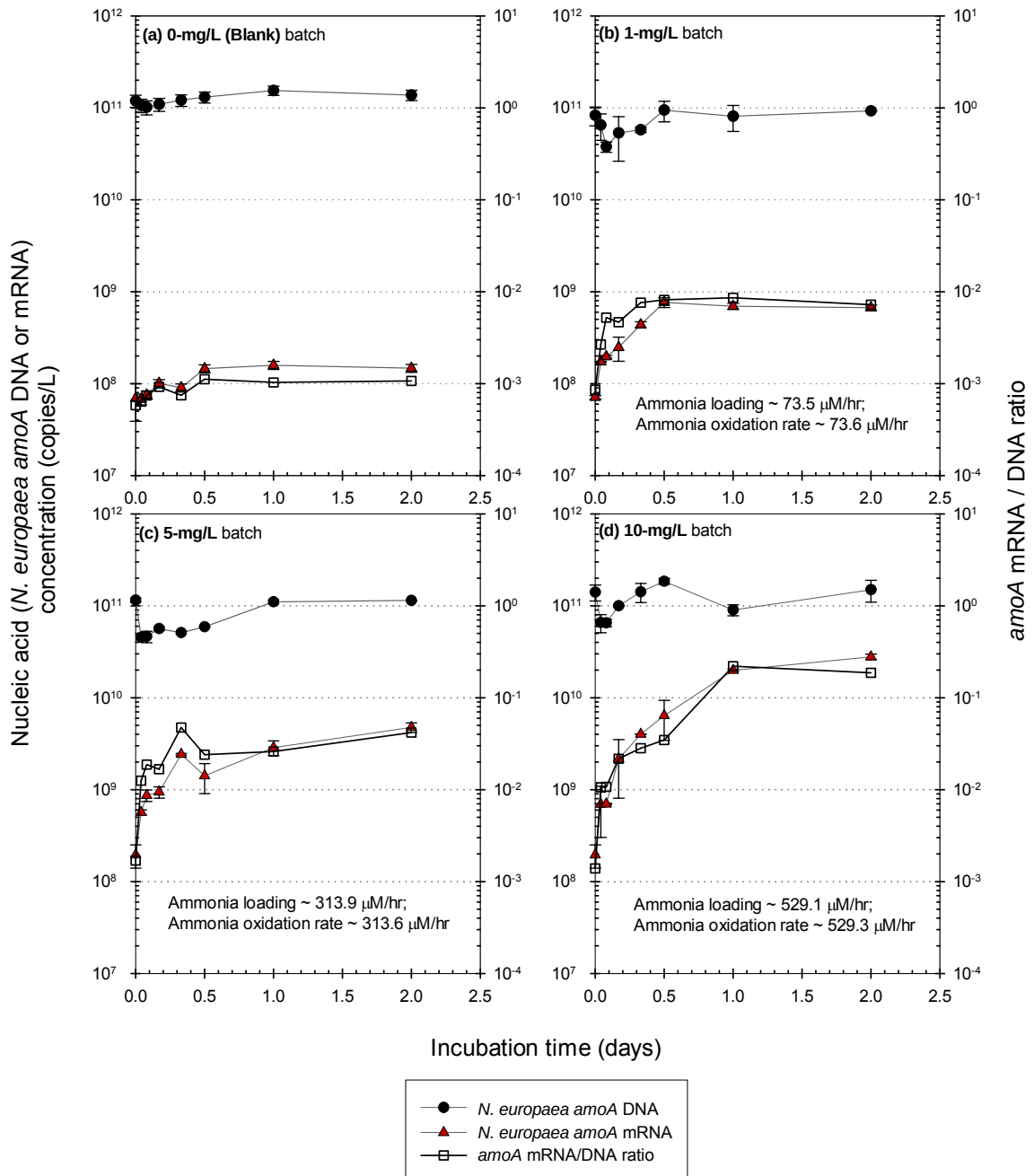


Figure 5-17. *N. europaea amoA* mRNA and DNA concentrations and mRNA/DNA ratios in batch culture tests using 0 (a), 1 (b), 5 (c) and 10 (d) mg/L of ammonia.

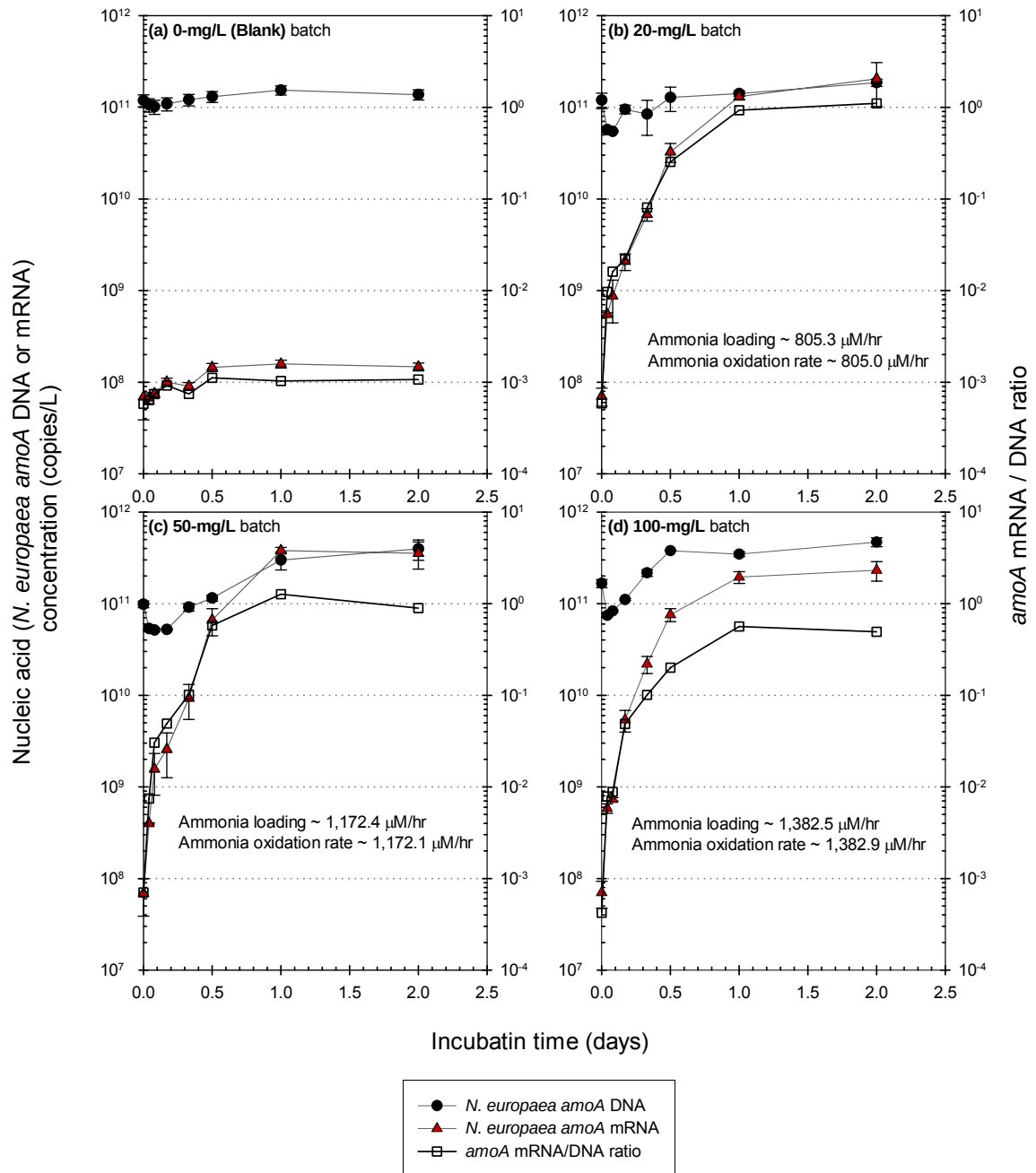


Figure 5-18. *N. europaea* *amoA* mRNA and DNA concentrations and mRNA/DAN ratios in batch culture tests using 0 (a), 20 (b), 50 (c) and 100 (d) mg/L of ammonia.

level within 0.5-1 day. Typically, the high-ammonia-concentration batch cultures had more significant shifts in *amoA* mRNA abundance than low-ammonia-concentration batch cultures. For instance, after one day incubation, the *amoA* mRNA level in the 20-mg/L batch rose to $1.31 \pm 0.11 \times 10^{11}$ copies/L while that the 1-mg/L batch only rose to $6.93 \pm 0.49 \times 10^8$ copies/L. The *amoA* mRNA plateau levels in 20-, 50-, and 100-mg/L batches were similar (p-value 0.036~0.039) (Figure 5-18, Table 5-7) suggesting that the *amoA* mRNA transcripts in the *N. europaea* become saturated when ammonia concentrations were higher than 20 mg/L. For the batch cultures with ammonia concentrations lower than or equal to 20 mg/L, the *amoA* mRNA plateau levels corresponded to the measured ammonia oxidation rates (Figure 5-19).

In a previous investigation conducted on a nitrifying biofilm system (Aoi et al., 2004 (a), (b)), *amoA* mRNA increased about 12~15 fold after a 72-hr starved nitrifying aggregate was exposed to 34.6~43.0 mM of ammonia-N for 4~6 hours. The increase in *amoA* mRNA level corresponded to enhanced ammonia oxidation activities (based on NO_x^- -N production). Thus, the researchers suggested that *amoA* mRNA can be applied as a new biomarker of ammonia oxidation activity in natural and engineered systems where many bacterial species construct complex consortia (Aoi et al., 2004 (b)). However, after *amoA* mRNA responses in the *N. europaea* group under different ammonia levels were examined in this study, it was found that increases in *amoA* mRNA abundance became retarded in the 50-, and 100-mg/L batches (Figure 5-18, Table 5-7). Thus, the regression correlation between *amoA* mRNA abundance and ammonia oxidation rate broke down for the 50-, and 100-mg/L batches (Figure 5-19). The ammonia oxidation rates determined for the 50-, and 100-mg/L batches were significantly higher than that for the 20-mg/L batch (p-value<0.05), but the *amoA* mRNA levels for these three batches were less variable. Apparently, the *amoA* mRNA level became saturated at ammonia concentrations higher than 20 mg/L. Sayavedra-Soto et al. (1996) suggested that the amount of AMO mRNA (i.e., *amo* mRNA) did not continue to increase and appeared to reach a maximum when levels above 2 mM $(\text{NH}_4)_2\text{SO}_4$ (~56 mg/L ammonia-N) were used for incubation. Based on these observations, it may be deduced that the ammonia oxidation rate did not directly relate to abundance of *amo* mRNA transcript.

Table 5-7. Average *amoA* mRNA plateau levels for different batch cultures containing various ammonia levels. A paired t-test was used to assess the difference in *amoA* mRNA plateau levels between the 20-mg/L batch and other concentration batches.

Batch concentration (mg/L NH ₃ -N)	Average <i>amoA</i> mRNA plateau levels (copies/L)	Statistical paired t-test*
100-mg/L	$2.13 \pm 0.26 \times 10^{11}$	0.0391
50-mg/L	$3.66 \pm 0.18 \times 10^{11}$	0.0360
20-mg/L	$1.68 \pm 0.52 \times 10^{11}$	---
10-mg/L	$2.38 \pm 0.57 \times 10^{10}$	0.0106
5-mg/L	$3.81 \pm 1.34 \times 10^9$	0.0087
1-mg/L	$7.09 \pm 0.51 \times 10^8$	0.0087
Blank	$1.35 \pm 0.31 \times 10^8$	0.0086

* Statistical paired t-test for testing H_0 : average of (*amoA* mRNA_{20-mg/L} - *amoA* mRNA_{other concentration batches}) = 0, a p-value < 0.05 suggests that the mean of *amoA* mRNA_{20-mg/L} $\neq$ the mean of *amoA* mRNA_{other concentration batches} is statistically acceptable.

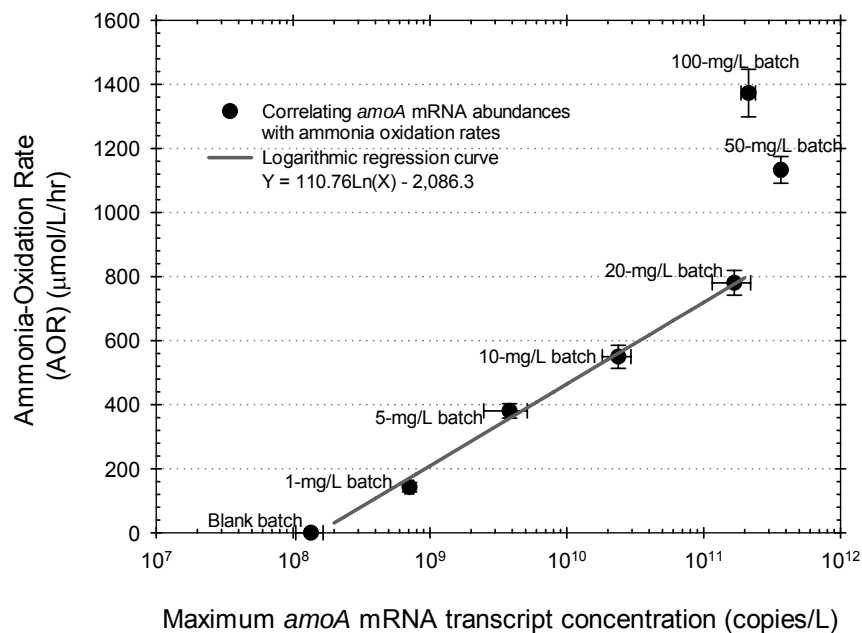
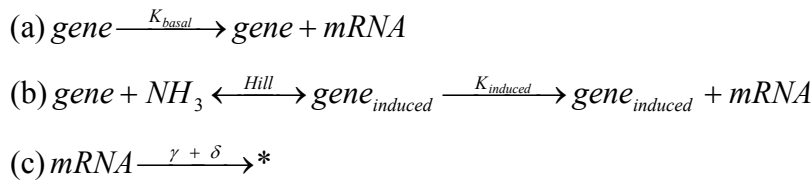


Figure 5-19. Ammonia oxidation rates correlated with maximum abundance of the *amoA* mRNA in the *N. europaea* group under various ammonia levels. The correlation broke down for the 50-, and 100-mg/L batches.

The *amoA* mRNA level for the 20-, 50-, and 100-mg/L batches increased more than 1,000-fold after 1 day of incubation (Figure 5-18). These increases were much greater than previously observed results (12~15 fold increases, Aoi et al., 2004 (a), (b)) while the ammonia test concentrations (20, 50, and 100 mg/L, or 1.43, 3.57, and 7.14 mM) were lower than those used by Aoi et al. (2004 (a), (b)) (i.e., 34.6~43.0 mM, or 484~602 mg/L ammonia-N). The *amoA* mRNA levels might have not reached a plateau level after only 4-hours of incubation in the Aoi study. At the enzyme level, it has been suggested that AMO specific activity can continue to increase if the cells are allowed to recover for longer periods after either light or acetylene (C₂H₂) inactivation (Hyman and Arp, 1992; Sayavedra-Soto et al., 1996). Thus, Sayavedra-Soto et al. (1996) believed that the amount of AMO activity (based on O₂ uptake rate or NO₂⁻ accumulation) detected at 3 hours in their investigation may represent only the amount of active AMO produced during the described incubation period, but may not indicate the maximum possible. The induction of *amoA* mRNA could follow a similar trend in responding to ammonia exposure.

In order to gain a better understanding of *amoA* mRNA abundance under varying ammonia strength, an mRNA production model was developed to describe the *amoA* mRNA response of the *N. europaea* group under different ammonia concentrations. The mRNA production and decay reactions can be illustrated by the following processes:



[Where K_{basal} is the basal mRNA production constant, $K_{induced}$ is the mRNA transcription rate constant for induced gene, γ is the mRNA decay rate constant, and the δ is the cell growth rate constant].

If the induction level (IL) for the *amo* gene is given by $[1/(1+(14/[NH_3])^2)]$ (where $[NH_3]$ = ammonia concentration in mg/L) based on the Hill equation using a Hill coefficient (h) = 2 (Weiss, 1997), then the mRNA production rate becomes Eq. 5-1.

$$\frac{d[mRNA]}{dt} = K_{basal}(1 - IL) + K_{induced}(IL) - (\gamma + \delta)[mRNA] \quad \text{(Eq. 5-1)}$$

Let $K_{basal} (1-IL) + K_{induced} (IL) = A$, and solve $[mRNA]$ at time point “ t ” in Eq. 5-1,

$$\begin{aligned}
 \frac{d[mRNA]}{dt} &= A - (\gamma + \delta)[mRNA] \rightarrow \frac{d[mRNA]}{A - (\gamma + \delta)[mRNA]} = dt \\
 \rightarrow \int_0^t \frac{d[mRNA]}{A - (\gamma + \delta)[mRNA]} &= \int_0^t dt \rightarrow \frac{\ln(A - (\gamma + \delta)[mRNA])}{-(\gamma + \delta)} \Big|_0^t = (t - 0) \\
 \rightarrow \frac{1}{-(\gamma + \delta)} \ln \frac{(A - (\gamma + \delta)[mRNA]_t)}{(A - (\gamma + \delta)[mRNA]_0)} &= t \rightarrow \frac{(A - (\gamma + \delta)[mRNA]_t)}{(A - (\gamma + \delta)[mRNA]_0)} = \exp(-t \times (\gamma + \delta)) \\
 \rightarrow (A - (\gamma + \delta)[mRNA]_t) &= (A - (\gamma + \delta)[mRNA]_0) \times \exp(-t \times (\gamma + \delta)) \\
 \rightarrow [mRNA]_t &= \frac{A - (A - (\gamma + \delta)[mRNA]_0) \times \exp(-t \times (\gamma + \delta))}{(\gamma + \delta)} \quad (\text{Eq. 5-2})
 \end{aligned}$$

Therefore, the mRNA level at time “ t ” (i.e., $[mRNA]_t$) can be modeled by Eq. 5-2 using the following parameter values: $\gamma = \ln(2)/1 = 0.69$ (1/day) (presumed *amoA* mRNA half-life was ~1 day, Aoi et al. (2004 (b)) showed that *amoA* mRNA decreased 2 fold in ~1 day under ammonia starvation); $\delta = \ln(2)/2 = 0.35$ (1/day) (corresponding to an average AOB cell doubling time of ~2 days, based on a maximum specific growth rate of 0.017-0.039 (1/hr), Keen and Prosser, 1987); $[mRNA]_0 = 10^8$ copies/L (based on measured *amoA* mRNA level after 2-day starvation in this study); $K_{basal} = 10^8 \times (\gamma + \delta) = 1.04 \times 10^8$ copies/L/day; $[mRNA]_{\text{maximum}} = 2 \times 10^{11}$ copies/L (based on maximum *amoA* mRNA level observed in this study); and, $K_{induced} = 2 \times 10^{11} \times (\gamma + \delta) = 2.08 \times 10^{11}$ copies/L/day.

Model results for *N. europaea amoA* mRNA concentrations at different ammonia levels are plotted with measured results from the 1-L batch culture tests (Figure 5-20). The modeled results do not completely fit the measured values, especially for the higher concentration batches (i.e., 20-, 50-, and 100-mg/L) during the 0.3-0.6 day incubation period. These variations between modeled and measured results might be caused by either errors that occurred during measurement of *amoA* mRNA or the existence of additional transcription mechanisms that were not considered during model development. Nevertheless, the modeled maximum *amoA* mRNA levels were very close to measured results in most tests ($\pm 3\sim 52\%$ agreement to the measured values). With the exception of

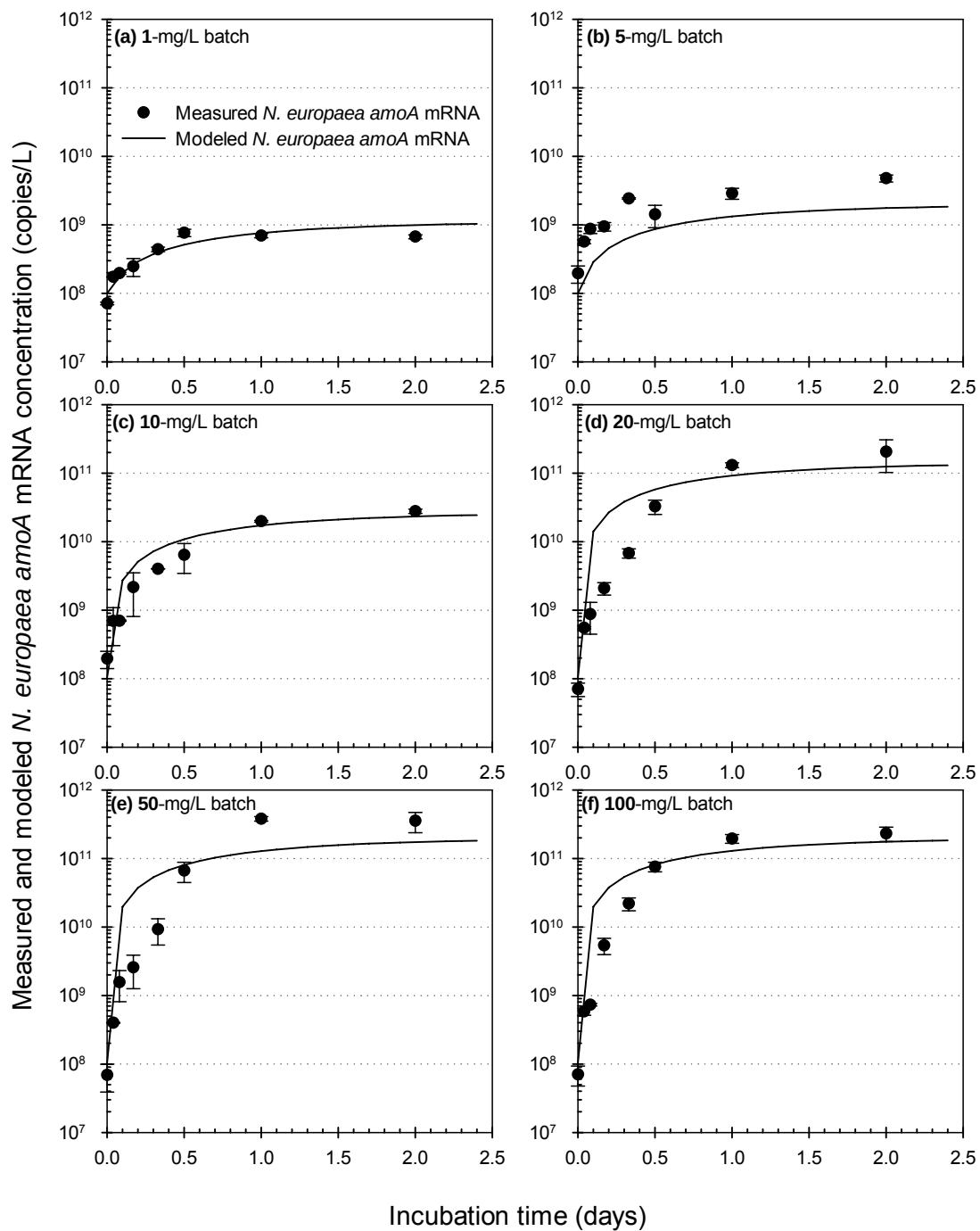


Figure 5-20. Model *amoA* mRNA production in the *N. europaea* AOB group under different ammonia concentrations (i.e., 1 (a), 5 (b), 10 (c), 20 (d), 50 (e), and 100 (f) mg/L).

the 50-mg/L batch, the maximum *amoA* mRNA levels predicted by the developed model were in agreement with measured values (Figure 5-21). A slope of 0.8265 for the best fit line suggested that the modeled maximum *amoA* mRNA levels were somewhat lower than the measured values.

Based on the presumed function for gene induction level ($IL = 1/(1+(14/[NH_3])^2)$), it appeared that *amo* gene would be quickly induced at low ammonia concentrations, and reach a 50% level at ammonia concentrations around 14 mg/L (Figure 5-22). When the ammonia concentration was higher than 14 mg/L, *amo* gene induction would become close to the saturated condition. The predicted maximum *amoA* mRNA levels after 2 days of incubation under different ammonia concentrations basically follow the same trend of the predicted induction levels (Figure 5-22). Thus, the maximum *amoA* mRNA levels would increase more rapidly under low ammonia levels, and tend to reach a saturation level at high ammonia concentrations as demonstrated in this study.

The modeled results indicated that a positive correlation for substrate might exist in the *amoA* mRNA transcription processes. Two molecules of substrate (i.e., ammonia or O₂) or energy carrier (i.g., ATP) may be involved in the initiation of *amoA* mRNA transcription as indicated by a Hill coefficient of 2. It has been suggested that *N. europaea* must use a supply of endogenous energy and intracellular metabolite pools (e.g., nucleotides and amino acids) to synthesize RNA and proteins prior to ammonia metabolism (during ammonia oxidation activity resuscitation from C₂H₂ inhibition) (Sayavedra-Soto et al., 1996).

Stein and Arp (1998 (a)) have observed some increases in *amoA* transcripts for *N. europaea* (approx. 6.5-7.5 fold raise based on relative densitometric values of the mRNA band hybridized to the *amoA* probe in Northern hybridizations) after a 4-6 hours incubation at initial ammonia concentrations of 15 and 50 mM. These researchers found that the increase for both incubations (15 and 50 mM ammonia) occurred at approximately the same rate and that the maximum increase levels for both incubates were very close (6.5 and 7.5 fold for 15- and 50-mM tests, respectively). This is similar to the saturated condition for *amoA* mRNA observed in this study.

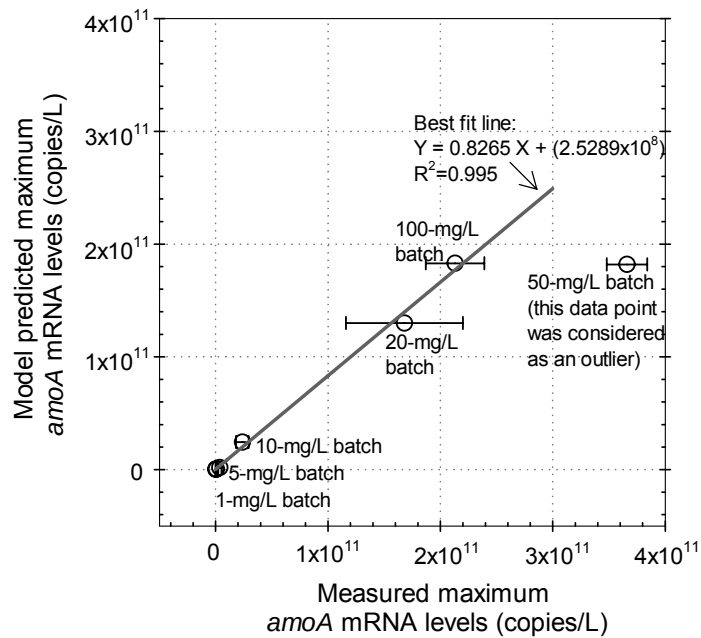


Figure 5-21. Correlation between measured and model-predicted maximum *amoA* mRNA levels under different ammonia concentrations.

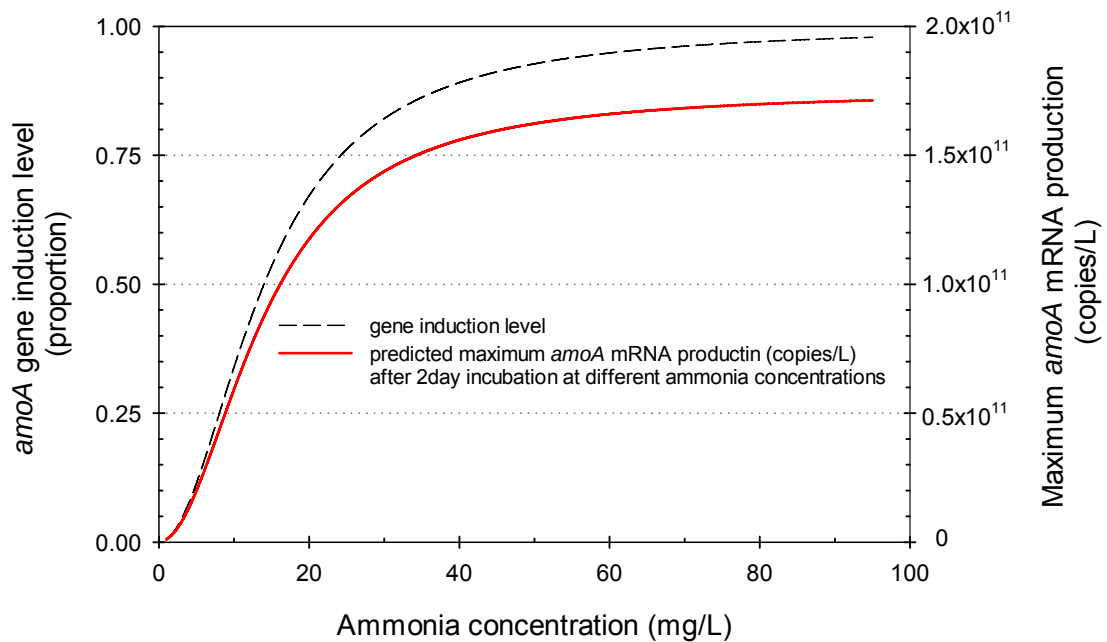


Figure 5-22. Predicted *amoA* gene induction level (IL) and maximum *amoA* mRNA production under different ammonia levels based on the developed model.

5.3.4 *amoA* mRNA responses under different ammonia loadings

Under steady-state operating conditions, the ammonia concentration in a continuous-flow nitrifying treatment system (operating at a fixed SRT) should remain stable (Eq. 2-10, Rittmann and McCarty, 2001). However, steady-state conditions may break down when ammonia loadings fluctuate (changes in either influent ammonia concentration or influent flow rate) and cause rapid ammonia level shifts in the treatment system. Therefore, the response of *N. europaea amoA* mRNA to different ammonia loadings was examined in a 10-L continuous-flow reactor (the Trial reactor of the enriched nitrifying system) using three different influent ammonia levels (i.e., ~1500, ~3000 and ~300 mg/L) and a constant influent flow rate of ~1 L/day (Section 3.2.2).

To start the experiment, influent for the Trial reactor in the enriched nitrifying system was stopped for 2 days to allow the *amoA* mRNA concentration to drop to a basal level (*ca.* 10^8 copies/L). When the bioreactor was re-fed with ammonia at an average loading of $\sim 435.1 \mu\text{mol NH}_3\text{-N/L/hr}$ (feeding 1,462 mg/L ammonia-N at a flow rate of ~ 1 L/day on day 0) (Figure 5-23 (a)), the *amoA* mRNA levels immediately responded and increased from 3.3×10^8 copies/L to an average maximum level of $6.2 \pm 0.7 \times 10^9$ copies/L within one day; whereas, the *amoA* DNA remained relatively stable (Figure 5-23 (c)). Meanwhile, the microbial culture in the bioreactor had an average ammonia oxidation rate of $415.4 \pm 44.1 \mu\text{mol/L/hr}$ (determined by NO_x production, Figure 5-23 (b)), very close to the supplied loading ($\sim 435.1 \mu\text{mol NH}_3\text{-N/L/hr}$) indicating that the ammonia oxidation efficiency was high.

Beginning on day 5, the influent feed was stopped for 5 days. The abundance of the *amoA* mRNA quickly dropped to $1.5 \pm 0.6 \times 10^8$ copies/L during the first 2-days of ammonia starvation and remained stable near the basal level for the remaining 3 days (Figure 5-23(c)). A significant decline in the ammonia oxidation rate was observed and corresponded with the drop in *amoA* mRNA level. Starting on day 10, the bioreactor was re-fed with ammonia at an average loading of $\sim 978.6 \mu\text{mol NH}_3\text{-N/L/hr}$ (feeding 3,288 mg/L ammonia-N at a flow rate of ~ 1 L/day). Again, the *amoA* mRNA levels rapidly responded to the revived ammonia-feed and increased from $8.6 \pm 0.2 \times 10^7$ to $7.8 \pm 5.6 \times 10^{10}$

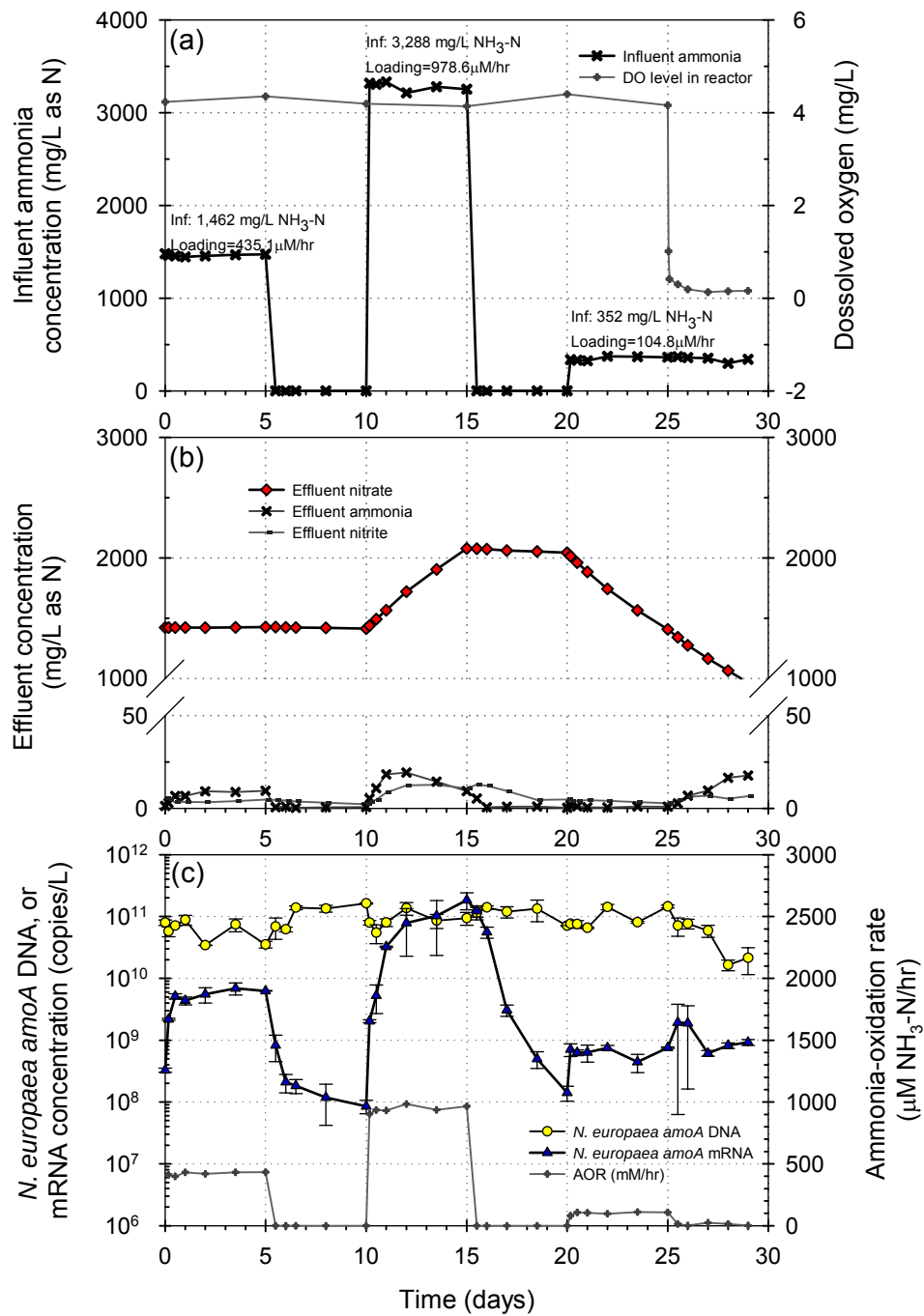


Figure 5-23. Ammonia concentrations in the influent and DO level in the bioreactor with sequential changes in ammonia loading (a), ammonia, nitrite, and nitrate concentrations in the bioreactor (same as in the effluent) (b), and ammonia oxidation rates, *N. europaea amoA* gene, and *amoA* mRNA abundance (c).

copies/L in about 2 days (Figure 5-23(c)). Meanwhile, a significant increase in the ammonia oxidation rate was noted although a small amount of ammonia (29~192 mg/L) accumulated in the reactor during this high ammonia loading operation.

After operation under high ammonia loading for 5 days (days 10-15), the bioreactor was again subjected to a 5-day ammonia starvation period (started on day 15). During this ammonia starvation period, the ammonia oxidation rate rapidly decreased to a level close to zero while the *amoA* mRNA level gradually dropped from $1.9 \pm 0.6 \times 10^{11}$ to $1.4 \pm 0.4 \times 10^8$ copies/L within the 5 days. On day 20, the bioreactor was re-fed with ammonia-N at an average loading of $\sim 104.8 \mu\text{mol NH}_3\text{-N/L/hr}$ (feeding 352 mg/L ammonia-N at a flow rate of ~ 1 L/day). Similarly, a corresponding increase in ammonia oxidation rates and *amoA* mRNA levels was observed. The increases were lower than those obtained in tests with higher ammonia loadings (i.e., ~ 435.1 , and $\sim 978.6 \mu\text{mol NH}_3\text{-N/L/hr}$). These results demonstrate that the plateau levels for both *amoA* mRNA and ammonia oxidation rate depend upon ammonia loading.

This investigation is the first to address the *amoA* mRNA response of the *N. europaea* group to three different ammonia loadings in a continuous-flow nitrifying reactor. In a similar study, the *amoA* mRNA shift for *Nitrosospira briensis* was measured during ammonia resuscitation (at an ammonia loading of $\sim 70 \mu\text{M/hr}$) in a continuous culture reactor and showed that the starved cells regained the same *amoA* mRNA level as the unstarved cells after about 4 hours of ammonia addition (Bollmann et al., 2005).

On day 25, aeration to the bioreactor was stopped to examine the impact of low dissolved oxygen (DO) level on ammonia oxidation and *amoA* mRNA transcription. After aeration ceased, residual oxygen in the bioreactor was quickly consumed and the DO level rapidly dropped from 4.16 to 0.41 mg/L in 2 hours (Figure 5-23 (a)). After this sharp declination, the DO level remained stable at 0.16 ± 0.03 mg/L. Under this low DO condition, the ammonia oxidation rate declined as reflected by a decrease in the nitrate concentration and a small ammonia accumulation in the bioreactor (i.e., 16.5-17.7 mg/L $\text{NH}_3\text{-N}$) (Figure 5-23 (b), (c)). Meanwhile, the *amoA* mRNA level remained unchanged during the 4-day low DO operation. In order to further investigate how ammonia

oxidation rates and *amoA* mRNA levels respond to different DO levels, a follow-up experiment was conducted using complete aeration (DO high at ~4 mg/L) and no aeration (DO lower than 0.2 mg/L).

5.3.5 *amoA* mRNA responses under different DO levels

The response of *N. europaea amoA* mRNA to different DO levels was examined in the same 10-L continuous-flow nitrifying reactor (the Trial reactor in the enriched nitrifying system) using two aeration strategies (i.e., complete aeration and no aeration) and a constant ammonia loading of ~441.9 $\mu\text{mol NH}_3\text{-N/L/hr}$ (feeding 1484.8 ± 41.6 mg/L ammonia-N at a flow rate of ~1 L/day) (Section 3.2.2). The bioreactor was initially aerated using compressed air (supplied at a flow rate of 800 mL/min). Measured DO levels in the bioreactor were around 4 mg/L under steady-state operation (Figure 5-24 (a)). After the aeration was halted on day 5, the DO levels quickly decreased to less than 0.5 mg/L in one hour, and then remained near 0.04 mg/L during the non-aeration period. Under this low DO condition, ammonia oxidation rates notably decreased as demonstrated by ammonia accumulation along with declines in NO_x^- ($\text{NO}_2^- + \text{NO}_3^-$) levels (Figure 5-24 (b)). However, the *amoA* mRNA concentration did not significantly decrease under this DO-limiting condition (Figure 5-24 (c)).

After aeration was re-initiated on day 10, the DO levels measured in the bioreactor rapidly increased to about 3 mg/L within a couple of hours, and then gradually reached approximately 4 mg/L in 5 days. Under this high-DO condition, the ammonia oxidation activity recovered as reflected by decreases in ammonia and increases in NO_x^- ($\text{NO}_2^- + \text{NO}_3^-$) levels (Figure 5-24 (b)). Interestingly, the *amoA* mRNA concentration rose from $9.2 \pm 0.9 \times 10^9$ to $1.6 \pm 1.0 \times 10^{11}$ copies/L within the first 2 days of high-DO operation, and then dropped back to around 1.4×10^{10} copies/L during the remaining 3 days. This increase corresponded to a revival in the ammonia oxidation rate (Figure 5-24 (c)) suggesting that the ammonia buildup, which occurred under low DO condition during days 5-10, induced oxidation activity shifts in the *N. europaea* group after aeration was re-started in the nitrifying bioreactor. A similar phenomenon was observed when these conditions were repeated during days 15-25.

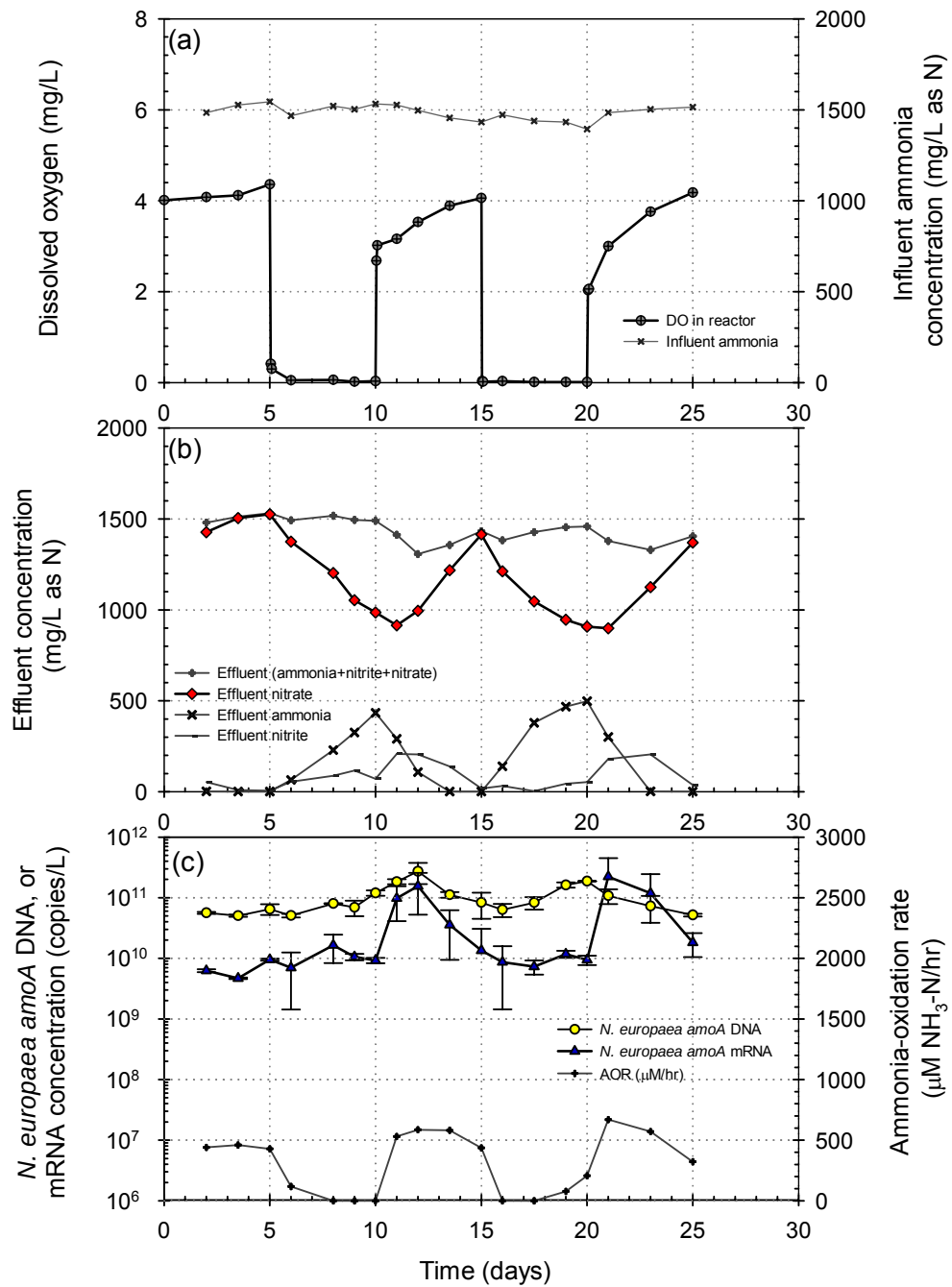


Figure 5-24. Ammonia concentration in the influent and DO levels in the bioreactor (a), ammonia, nitrite, and nitrate concentrations in the bioreactor (same as in the effluent) (b), and *N. europaea amoA* gene levels, mRNA abundance, and ammonia oxidation rates (c) under different aeration strategies (i.e., complete aeration and non-aeration).

These results were similar to those observed in a previous investigation in which the *amoA* mRNA level did not significantly change although ammonia oxidation activity (based on production of NO_x^- -N) decreased during a 6-day low-pH (approx. 5.6-6.0) operation (Aoi et al., 2004 (a)). After the pH was adjusted back to 7.5, Aoi et al. (2004 (a)) found that the *amoA* mRNA level increased about 4 fold in response to a recovery in the ammonia oxidation activity. Moreover, the same researchers also found that the *amoA* mRNA level did not notably change under saline inhibition (6-8%, w/w of NaCl) although ammonia oxidation activity dropped (Aoi et al., 2004 (b)). When the NaCl concentration was lowered to <6% (w/w), ammonia oxidation activity immediately recovered. Additionally, the *amoA* mRNA level rapidly rose then decreased because of a drop in the amount of accumulated ammonia (Aoi et al., 2004 (b)). Thus, it appears that these environmental factors (i.e., low-DO, low-pH, or high-salinity), which caused retardation in ammonia oxidation activity, do not directly impact the *amo* gene transcription to mRNA and/or the stability of *amo* mRNA during short-term shock experiment. In contrast, the AOB may sacrifice strict regulation of *amo* mRNA transcription to preserve energy when cell activity decreases as hypothesized for the AMO enzyme (Aoi et al., 2004 (a)). This characteristic may allow AOB cells to survive in nature or engineered nitrifying systems under fluctuating environmental conditions (e.g., ammonia and/or DO limitations).

Although it is believed that ammonia is the primary inducer for transcription of the *amo* mRNA in AOB (Stein et al., 1997), energy or particular molecules (e.g., O_2 , or enzymes) may also be involved in *amo* gene induction processes. Results from this study indicate that *amoA* mRNA levels remain unchanged although ammonia oxidation rates significantly dropped under DO-limiting conditions (Figure 5-24 (c)). The fact that *amoA* mRNA in *N. europaea* cells did not significantly decrease may be due to either the presence of stable mechanisms or a low constitutive mRNA production fuelled by available endogenous energy (Romeo and Zusman, 1992, Sayavedra-Soto et al., 1996; Carrier and Keasling, 1997). Additionally, *amoA* mRNA levels did not increase (along with increases in ammonia concentration) during low-DO operation (days 5-10, and 15-20, Figure 5-24 (c)) because energy was insufficient while ammonia oxidation activity

was retarded. Once the ammonia oxidation activity recovered during the subsequent aerobic operation (days 1-15, and 20-25), the *amoA* mRNA levels rapidly increased because energy became available. Likewise, no marked changes in *amoA* mRNA levels observed under either short-term low-pH or salinity inhibition in previous investigations (Aoi et al., 2004 (a)(b)) may also be due to the same mechanism that controlled mRNA levels during the DO-limitation test in this study.

5.3.6 Ammonia oxidation activity and *amoA* mRNA levels

The mean of the maximum *amoA* mRNA level from each test (i.e., varying ammonia concentrations for 1-L batch culture test, and different ammonia loadings and DO levels for continuous-flow test in the 10-L enriched nitrifying reactor) was plotted with the average ammonia oxidation rate (based on NH_3 oxidation rate or NO_x^- production rate) under each test condition (Figure 5-25). Results showed that the ammonia oxidation rate correlated with *amoA* mRNA abundance under varying ammonia concentrations and different operating ammonia loadings ($R^2 = 0.886$); higher maximum *amoA* mRNA concentration resulted in higher ammonia oxidation activity. It was deduced that the relatively higher ammonia level in the mixed liquor under high ammonia loading operation elevated the ammonia oxidation rate and also promoted *amoA* mRNA transcription; similar to what was observed in batch-mode tests conducted by Sayavedra-Soto et al. (1996) and Aoi et al. (2004 (a), (b)).

During high-DO operation (conditions similar to the ammonia loading test during days 0-5, i.e., ammonia loading of $\sim 441.9 \mu\text{mol NH}_3\text{-N/L/hr}$ and a DO level of $\sim 4.2 \text{ mg/L}$), the correlation between ammonia oxidation activity and *amoA* mRNA level also agreed with that noted under different ammonia loadings (Figure 5-25). However, under low-DO operation, the *amoA* mRNA level did not correspond to the low ammonia oxidation activity (based on oxidation rate estimation). During short-term unsuitable conditions (including low DO), both *amo* mRNA and likely AMO enzyme levels in AOB remained stable (when ammonia was not limiting); hence, AOB cells could immediately revive once the repressive condition passed. For example, ammonia oxidation rates quickly recovered after the DO level was raised (from <0.1 to $\sim 4.0 \text{ mg/L}$, Figure 5-24).

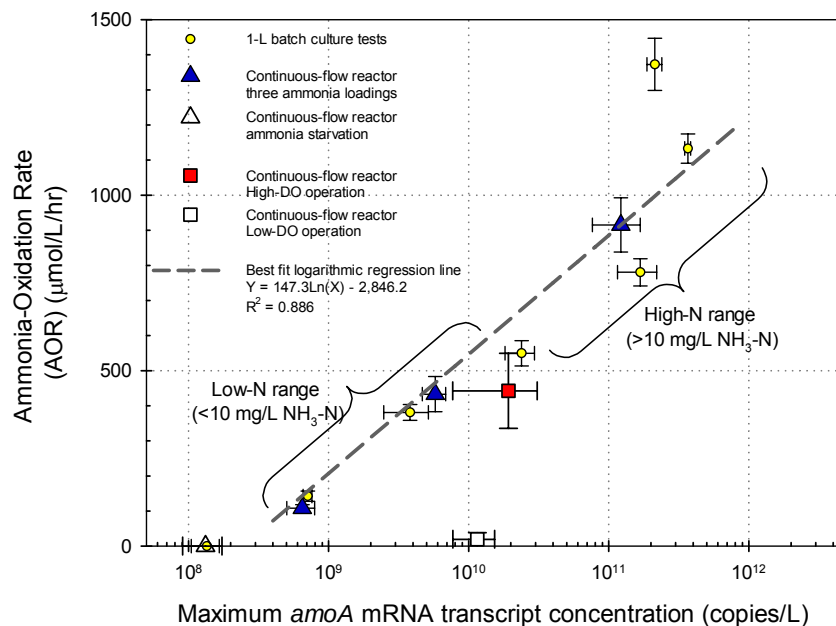


Figure 5-25. Ammonia oxidation activity corresponded to maximum *amoA* mRNA level under varying ammonia concentrations or loadings, but not under DO limitation condition.

Although the *amoA* mRNA level reflected ammonia oxidation rates under varying ammonia levels, correlation between these two parameters did not follow a strict linear relationship (i.e., 2 fold increases in the *amoA* mRNA level corresponded to ~1.2-2.4, and ~1.1-1.2 fold increases in oxidation rate under low (≤ 10 mg/L) and high (> 10 mg/L) N ranges, respectively). These results indicated that ammonia oxidation in AOB cells was not simply regulated by *amo* transcripts. Post-transcriptional regulation may, in part, control ammonia oxidation activity of AOB. Whether or not an ammonia molecule can be oxidized ultimately depends upon the catalytic function of the AMO enzyme even though the amount of AMO may not directly correlate with ammonia oxidation activity (Bock and Wagner, 2003). Any factor associated with synthesis and functionalism of the AMO enzyme may influence the overall outcome of ammonia oxidation. Therefore, *amoA* mRNA abundance may not completely illustrate the complex ammonia oxidation activity regulation that occurs in AOB cells. Nevertheless, this measurement provided typical transcriptional responses of the *amoA* gene under different operating conditions.

5.4 Summary for Part II

The short-term effects of changes in either ammonia concentration or dissolved oxygen (DO) levels on the *N. europaea* population (based on *amoA* gene quantification), the abundance of *N. europaea amoA* mRNA transcripts, and the overall ammonia oxidation rate are summarized in Table 5-8. Evaluations were based on a statistical t-test to examine differences between two means for each test condition (e.g., low vs. high ammonia concentrations, or low vs. high DO levels). When statistical testing results (i.e., p-value) were below 0.05, testing factors (i.e., NH₃ and DO) did affect the dependent parameters (i.e., oxidation rate, population, and *amoA* mRNA). Results indicated that short-term changes in either ammonia or DO level did not notably impact the *N. europaea* population (*amoA* gene based), but did significantly affect the overall ammonia oxidation rate (Table 5-8). Interestingly, *amoA* mRNA levels shifted under different ammonia loadings and concentrations, but remained unchanged when DO was limited for about 5 days. Although the *amoA* mRNA level increased during the first 2-3 days after aeration was re-initiated (i.e., days 10-13, and 20-23, Figure 5-24 (c)), concentrations

Table 5-8. Summary of the short-term effects of ammonia and DO levels on the overall volumetric ammonia oxidation rate, the *N. europaea* population, and the *amoA* mRNA abundance of the *N. europaea* group

Testing factors	Dependent parameters		
	Overall ammonia oxidation rate	<i>N. europaea</i> population	<i>N. europaea amoA</i> mRNA
Ammonia concentrations:			
Low (1, 5, 10 mg/L) vs.	++	-	++
High (20, 50, 100 mg/L)			
Ammonia Loadings:			
Low (104.8 µmol/L/hr) vs.	++	-	++
High (978.6 µmol/L/hr)			
DO Levels:			
Low (no-aeration) vs.	++	-	-
High (complete aeration)			

PS. “++”: p-value<0.05, significantly affected; “+ “: 0.05<p-value<0.1, slightly affected; and “-“:0.1<p-value, not affected.

dropped to levels measured prior to the DO-limitation. The transitional period during aeration re-initiation was not considered for statistical evaluation when low and high DO operational conditions were compared. Therefore, *amoA* mRNA abundance was not governed by DO level. Additionally, decreases in ammonia oxidation activity were not reflected in *amoA* mRNA levels under DO shifts.

The *amoA* mRNA levels corresponded to ammonia oxidation rates (based on NO_x^- production) for the *N. europaea* group under varying ammonia concentrations or loadings. In contrast, the *amoA* mRNA abundance did not change when the ammonia oxidation rate dropped under DO limiting conditions. Therefore, it was concluded that the *amoA* mRNA level may reflect a shift in ammonia-oxidizing activity under vary ammonia loadings or concentrations; however, *amo* gene transcription (e.g., *amoA* mRNA) may not correlate directly to ammonia oxidation activity of AOB under DO limitation.

However, it appeared that ammonia oxidation activity in AOB cells was not simply regulated by *amo* transcripts (as the increases in ammonia oxidation rates did not strictly correlated with *amoA* mRNA abundance, [Figure 5-25](#)). [Wei et al., \(2006\)](#) documented diverse transcriptional responses of overall genes in *N. europaea* under ammonia starvation, and found that some genes (e.g., *hao*, *cbbL*, and multicopper oxidase type 1) were more responsive in expression regulation than the *amoA* gene. Otherwise, ammonia oxidation activity of AOB may be regulated at the post-transcriptional level. Variances in availability of energy, electrons and reductant, for example, may influence the overall function of the AMO enzyme and lead to variations in ammonia oxidation performance. Thus, *amoA* mRNA abundance may reflect ammonia oxidation activity in some cases but cannot be solely used to evaluate ammonia oxidation performance in all nitrifying systems because activity variations due to possible regulation at the post-transcriptional level may exist in AOB cells.

Chapter 6 Results and discussion — Part III

(Assessing *amoA*-mRNA-based activity for AOB populations in the industrial bench-scale system)

6.1 *amoA*-mRNA-based activity study for the industrial system

In the industrial wastewater treatment system (Part I), the B2-3 group became the dominant AOB population during 2-day SRT operation after influent ammonia concentrations were approximately doubled. The RI-27 group remained dominant in the other three SRT trains (i.e., 5-, 10-, and 20-day) ([Section 4.2.4](#)). It was inferred that the relatively high ammonia level (~24.3 mg/L), which accumulated during the transitional period of high-N operation in the 2-day SRT train, was more favorable to the B2-3 organism. It would appear that the B2-3 group became more active than the RI-27 and *N. nitrosa* groups during this time. However, specific activity variances between distinct AOB groups could not be addressed from previously-applied determinations based on the average cellular ammonia oxidation rates ([Section 4.2.4](#)).

The Part II results indicated that *amoA* mRNA levels reflected shifts in ammonia-oxidizing activity under different ammonia concentrations. Therefore, an *amoA*-mRNA-based activity study was conducted on biosolids samples taken from the industrial bench-scale treatment system to assess whether the B2-3 dominance noted in the 2-day SRT train during high-N operation (phase 2) was due to an increase in B2-3 activity ([Section 6.1.3](#)). Three reverse transcription (RT) real-time PCR assays were developed to specifically quantify the abundance of *amoA* mRNA for the AOB groups (i.e., *N. nitrosa*, RI-27, and B2-3) identified in the bench-scale industrial wastewater treatment system ([Section 3.5.4](#)). Two *amoA* mRNA standard stock solutions were prepared for the quantitative RT real-time PCR assays to calibrate target transcripts of the RI-27 and B2-3 groups ([Section 6.1.1](#)). Calibration for the *N. nitrosa* transcript was based on the *amoA* DNA standard. Target *amoA* mRNA in stored AOB biosolids samples from the industrial bench-scale system was initially checked for viability using the developed RT real-time PCR assays after target mRNA standards were prepared. Additionally, because the

biosolids samples taken from the industrial bench-scale system were stored at -80°C for 3~4 years prior to *amoA* mRNA measurements, the stability of this material under -80°C storage was examined using fresh biomass samples taken from the full-scale industrial WWTP. Samples were analyzed for target *amoA* DNA and mRNA after different -80°C storage times (Section 6.1.2).

6.1.1 Preparing RI-27 and B2-3 *amoA* mRNA standards

The clones IB-10d-C26 and IB-5d-C21 from the *amoA* libraries for the industrial bench-scale system were selected to make *amoA* mRNA standards for the RI-27, and B2-3 AOB groups, respectively. The synthetic *amoA* mRNA transcripts were prepared using the same method applied to generate *N. europaea amoA* mRNA standard for the enriched nitrifying system (Section 3.5.2). Briefly, a fragment of the *amoA* mRNA transcript was produced (using the anti-sense strand of the linearized plasmid DNA as template) during an *in-vitro* reaction based on T7 transcription protocol (RiboMAX™ Large Scale RNA Production Systems, Promega, Madison, WI). After the products were cleaned up (using RNeasy mini kit), DNase digested, and concentrated, the *amoA* mRNA standard solutions were immediately stored at -80°C for further quality and quantity evaluations. The quality of the prepared RI-27 and B2-3 *amoA* mRNA standards was evaluated using a denaturing agarose gel (to check the integrity of prepared mRNA), a UV absorption ratio (A_{260}/A_{280}) (to check protein contamination), and a no-RT RT real-time PCR reaction (to check contamination of transcription template, the linearized plasmid DNA). In addition, the quantity of each prepared *amoA* mRNA standard was determined by both spectrophotometric quantification and RiboGreen® RNA quantitation.

The integrity of each prepared RI-27 and B2-3 *amoA* mRNA standard was evaluated using a formaldehyde-denaturing agarose gel. The gel image (Figure 6-1) showed that each *in-vitro* transcribed mRNA preparation formed a single band close to the 623-base marker position (the size of transcription product should have been 585 bases, beginning with the T7 priming site to the end of *SpeI* restriction site); while the positive control represented a single band around the 1,908-base marker position (size should have been ~1,800 bases). No band was observed in the negative control. These

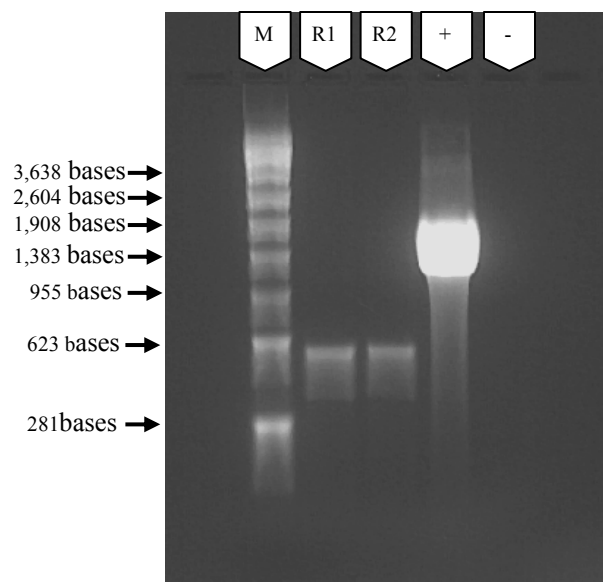


Figure 6-1. Formaldehyde-denaturing agarose gel for evaluating integrity of the artificially-transcribed *amoA* mRNA (for the RI-27 and B2-3 AOB groups). (M: RNA markers; R1: prepared RI-27 *amoA* mRNA standard; R2: prepared B2-3 *amoA* mRNA standard; “+”: positive control; “-”: negative control).

results demonstrated that the transcription reaction succeeded and that the prepared mRNA standards had good purity and integrity (as demonstrated by identical size for the products in comparison to RNA size marker).

The quality of each *amoA* mRNA preparation was further evaluated using a UV absorption ratio (A_{260}/A_{280}) to determine the amount of protein contamination. The mRNA standard stock solutions were diluted 1:200 using 10mM Tris-HCl (pH=7.6), and the absorbance of the dilutions was measured under UV wavelengths of 260 nm (maximum absorption of nucleic acids, RNA and DNA) and 280 nm (maximum absorption of proteins). The higher the absorption ratio represents a lower proportion of protein in the mRNA preparation. Ratio values for the buffer diluted RI-27 and B2-3 mRNA standards were 2.02 ± 0.13 and 1.92 ± 0.01 , respectively. This suggested that the prepared *amoA* mRNA standard stock solutions had low protein contamination and high nucleic acid quality (based on a reference ratio of 1.8~2.0 used to estimate nucleic acid quality as suggested by [Sambrook and Russell, 2001](#)).

The quality of the *amoA* mRNA preparations was further evaluated using two no-RT RT real-time PCR assays to assess the presence of DNA contamination (residue of the transcription template, i.e., linearized plasmid DNA) in each mRNA standard stock solution. The mRNA standard stock solutions were diluted to 1×10^9 copies/ μL of *amoA* mRNA using DEPC H₂O based on quantification results (Table 6-1). A series of *amoA* DNA dilutions ($1 \times 10^1 \sim 1 \times 10^7$ copies/ μL) served as standards to calibrate the DNA content in the 10^9 -copies/ μL mRNA standard solution for each AOB group (i.e., RI-27 or B2-3). Results indicated that the RI-27 and B2-3 *amoA* DNA concentrations in the prepared dilutions (10^9 copies/ μL mRNA) were $3.98 \pm 0.42 \times 10^4$ and $8.47 \pm 0.90 \times 10^3$ copies/ μL , respectively (Figure 6-2). These results demonstrated that DNA contamination was not significant in either of the *amoA* mRNA standard solutions. Thus, the low DNA residue in the mRNA standard preparations did not affect calibration results for RT real-time PCR quantification.

The quantity of the *amoA* mRNA standard for the RI-27 and B2-3 groups was determined in 1:200 dilutions of the stock solutions in DEPC H₂O. The absorbance of the dilutions was measured at a UV wavelength of 260 nm using a Beckman CoulterTM (DU[®] 640B) spectrophotometer (Section 3.5.3). The measured absorbance values were converted to concentrations for the mRNA preparations using a coefficient of 40 (an A_{260} of 1 corresponds to a 40 ng/ μL RNA solution). The average concentrations of prepared RI-27 and B2-3 *amoA* mRNA standards were 151.2 ± 7.9 and 72.4 ± 2.8 ng/ μL , respectively (Table 6-1).

Concentrations of *amoA* mRNA in the RI-27, and B2-3 stock solutions were further confirmed by RiboGreen[®] RNA quantitation assay using 5,000~10,000-fold dilutions (final volume of 200 μL) (Section 3.5.3). The fluorescence of each dilution was measured using a Wallac 1420 Victor2TM Multilabel Counter. Concentrations of RI-27 and B2-3 *amoA* mRNA determined by the RiboGreen[®] RNA assay were 137.5 ± 8.9 ng/ μL , and 89.2 ± 4.8 ng/ μL , respectively (Table 6-1). These results were similar to the values determined by spectrophotometric quantification. The two measured values were converted from ng/ μL to copies/ μL for each mRNA standard solution using a coefficient

Table 6-1. Concentrations (with standard deviations) of RI-27 and B2-3 *amoA* mRNA standards measured by spectrophotometry and RiboGreen[®] assay.

<i>amoA</i> mRNA standards	Quantification method	Concentration (ng/μL)	Concentration (copies/μL)
RI-27	Spectrophotometry	151.2±7.9	4.54×10 ¹¹
	RiboGreen [®]	137.5±8.9	4.13×10 ¹¹
	Mean	143.0±10.6	4.29±0.32×10 ¹¹
B2-3	Spectrophotometry	72.4±2.8	2.17×10 ¹¹
	RiboGreen [®]	89.2±4.8	2.68×10 ¹¹
	Mean	82.5±9.9	2.48±0.30×10 ¹¹

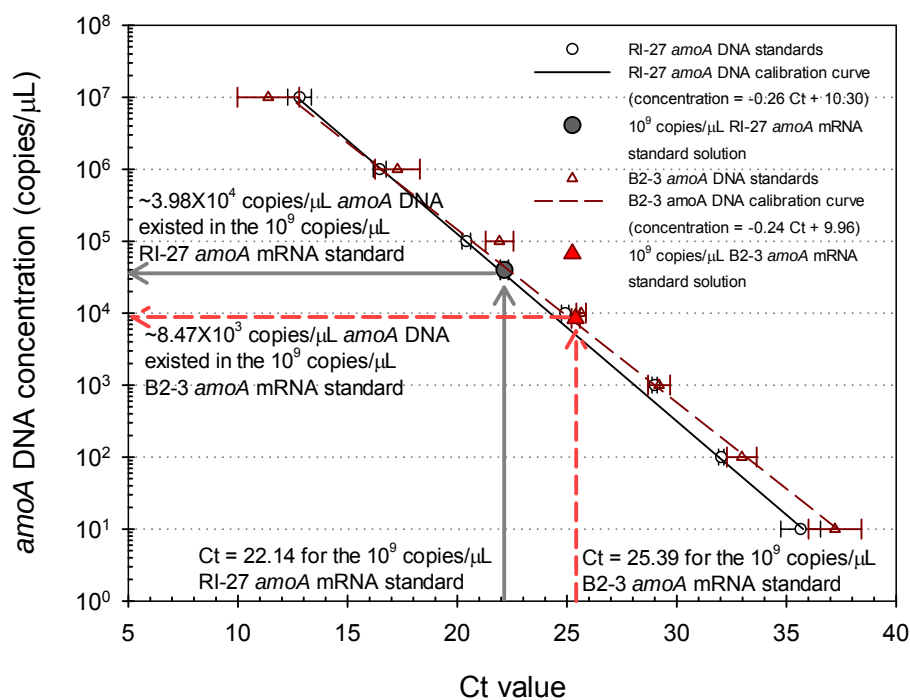


Figure 6-2. Series of *amoA* DNA standards used to calibrate DNA content in the RI-27 and B2-3 *amoA* mRNA standard stock solutions (using 10⁹ copies/μL dilution).

of $\sim 3 \times 10^9$ copies/ng (estimated as follows):

$\sim 585 \text{ bases} \times \sim 340 \text{ g/mole/base} \approx 198,900 \text{ g/mole}$

$1 \text{ ng} \div \sim 198,900 \text{ g/mole} \approx 5.028 \times 10^{-15} \text{ mole}$

$(\sim 5.028 \times 10^{-15} \text{ mole}) \times (\sim 6 \times 10^{23} \text{ copies/mole}) \approx 3 \times 10^9 \text{ copies.}$

The mean concentration values for the RI-27, and B2-3 *amoA* mRNA standards were 4.29×10^{11} and 2.48×10^{11} copies/ μL , respectively (Table 6-1). These average concentrations (in copies/ μL) were used to determine appropriate dilution rates for subsequent 10^9 -copies/ μL standard solutions.

Previous reverse transcription (RT) real-time PCR reactions for the *N. europaea* standard solutions lower than 1×10^3 copies/reaction generated very weak signals over 51 cycles, thus a series dilution from 5×10^8 to 5×10^3 copies/reaction was prepared from RI-27 and B2-3 *amoA* mRNA standard stock solutions to quantify target mRNA during RT real-time PCR quantification. These standard dilutions were preliminarily tested using two RT real-time PCR assays developed in this study prior to specifically quantify *amoA* mRNA for the RI-27 and B2-3 groups in the RNA extracts from the biosolids samples. The detected fluorescence signals versus threshold cycles (Ct values) are plotted in Figures 6-3 (a) and 6-4 (a). Results indicated that the mRNA in the prepared standard dilutions reacted with the newly designed primers/probe sets specific for the RI-27 and B2-3 groups and were efficiently detected by the RT real-time PCR reactions. The high standard solution had a low Ct value. Good linear relationships were observed between standard concentrations (ranging from 5×10^8 to 5×10^3 copies/reaction) and Ct values for both RT real-time PCR assays (Figure 6-3 (b), 6-4 (b)).

Additionally, six RNA extracts of mixed liquor suspended solids samples (three from the 2-day SRT train, and three from the 10-day SRT train) taken from the bench-scale industrial wastewater treatment system were also initially checked for viability using the RT real-time PCR reactions. The Ct values in a range of 24-32 cycles were observed from both RI-27 and B2-3 specific RT real-time PCR reactions. This suggested that target *amoA* mRNA in stored AOB MLSS samples taken from the industrial bench-scale system remained detectable after long-term (3-4 years) storage at -80°C .

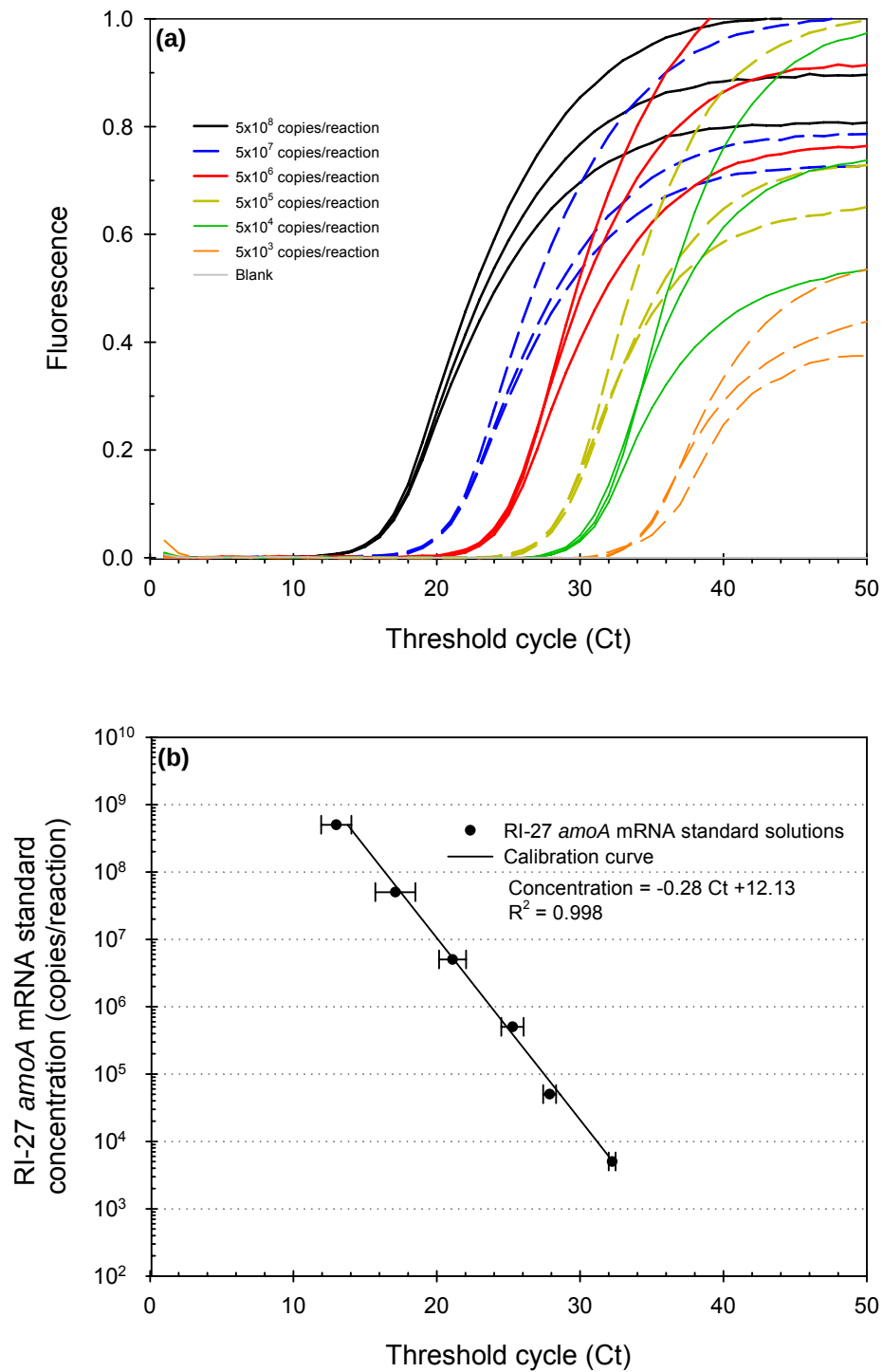


Figure 6-3. Fluorescence signal of serially diluted RI-27 *amoA* mRNA standard solutions detected during a reverse transcription (RT) real-time PCR (a) and the linear relationship between mRNA standards and Ct values (b).

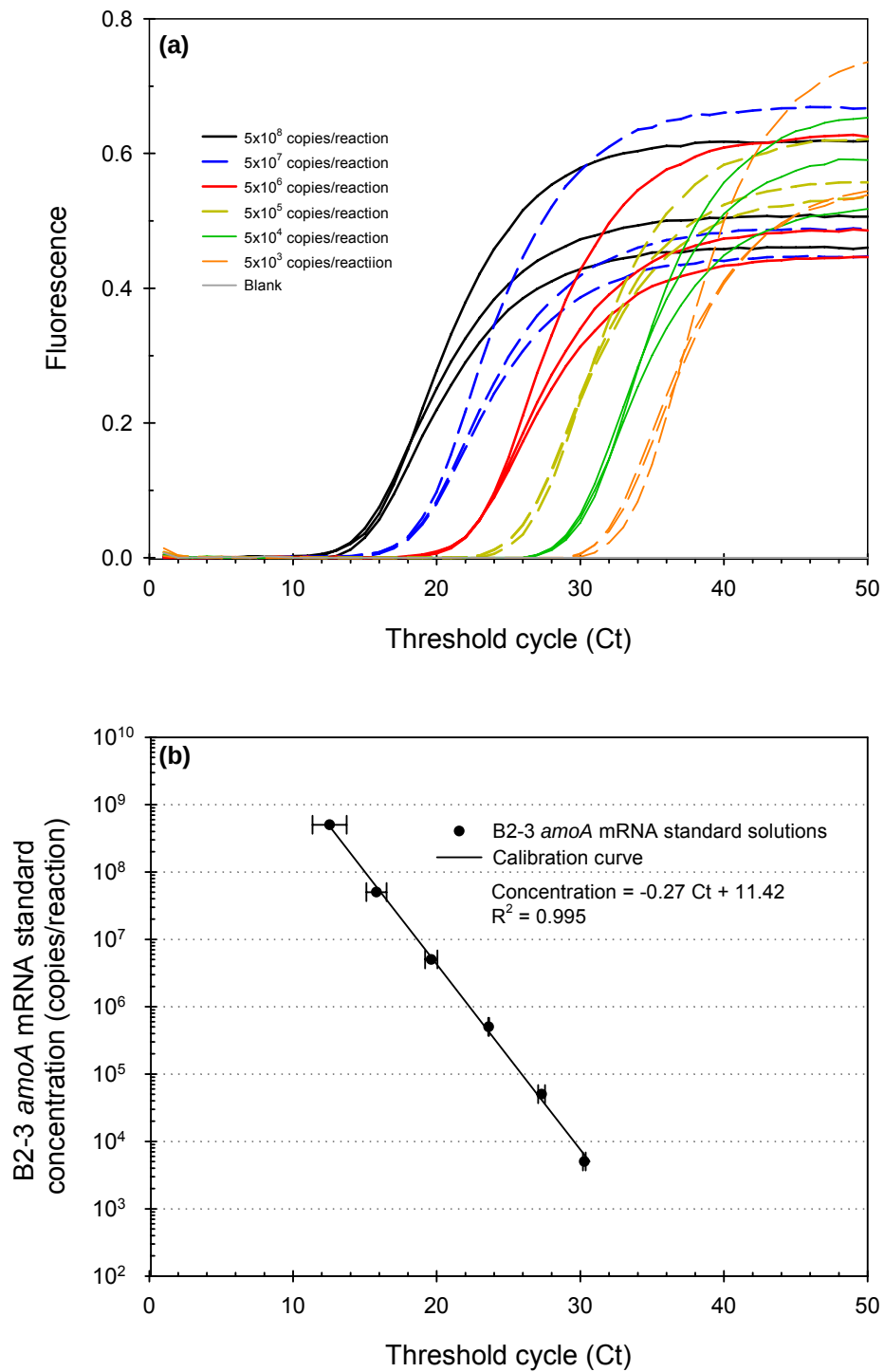


Figure 6-4. Fluorescence signal of serially diluted B2-3 *amoA* mRNA standard detected during RT real-time PCR reactions (a) and the linear relationship between mRNA standards and Ct values (b).

6.1.2 Examining *amoA* mRNA stability under -80°C storage

The decay phenomena of nucleic acids (i.e., DNA or RNA) in MLSS samples stored at a temperature of -80°C is unknown. Therefore, *amoA* DNA and mRNA stability during -80°C storage was examined using MLSS samples taken from the full-scale industrial WWTP (Section 3.2.4). Within these sampled MLSS, the RI-27 ($1.4 \pm 0.4 \times 10^9$ cell/L) and *N. nitrosa* ($6.0 \pm 0.4 \times 10^8$ cell/L) groups were identified as dominant AOB. The *amoA* DNA and *amoA* mRNA concentrations of these two dominant AOB groups in the MLSS samples, stored at -80°C, were assessed under different storage times over a 228~333 day (approximately 7-11 months) period.

The *amoA* DNA and mRNA concentrations as a function of storage time are plotted in Figure 6-5. The *amoA* mRNA decay trend was determined based on a best-fit regression line for the measured mRNA levels (excluding one outlier). Both best-fit regression functions had negative exponential power suggesting that slow deterioration of the target *amoA* mRNA occurred over the time course of this experiment (7-11 months). However, it appeared that the target mRNA abundance remained essentially at the same level when quantification variations were considered (for 6~18 repeats of each sample at different storage-times). Based on this analysis, the target mRNA in the MLSS samples from the bench-scale industrial wastewater treatment system did not significantly decay during long-term storage at -80°C. Hence, the RI-27 and *N. nitrosa amoA* mRNA levels measured after -80°C storage (Section 6.1.3) should represent mRNA levels for active biomass during treatment in the industrial bench-scale system.

6.1.3 Assessing AOB activity based on *amoA* mRNA measurements

The Part II results indicated that the *amoA* mRNA level reflected a shift in AOB ammonia-oxidizing activity under different ammonia concentrations. Therefore, an *amoA*-mRNA-based activity study was conducted for MLSS samples taken from the 2-day SRT train of the bench-scale industrial system to assess whether the B2-3 dominance noted in this SRT train during high-N operation (phase 2) was due to an increase in B2-3 activity. It was hypothesized that B2-3 activity (based on the *amoA* mRNA measurement) in the 2-day SRT train was significantly higher during high-N operation (phase 2) than

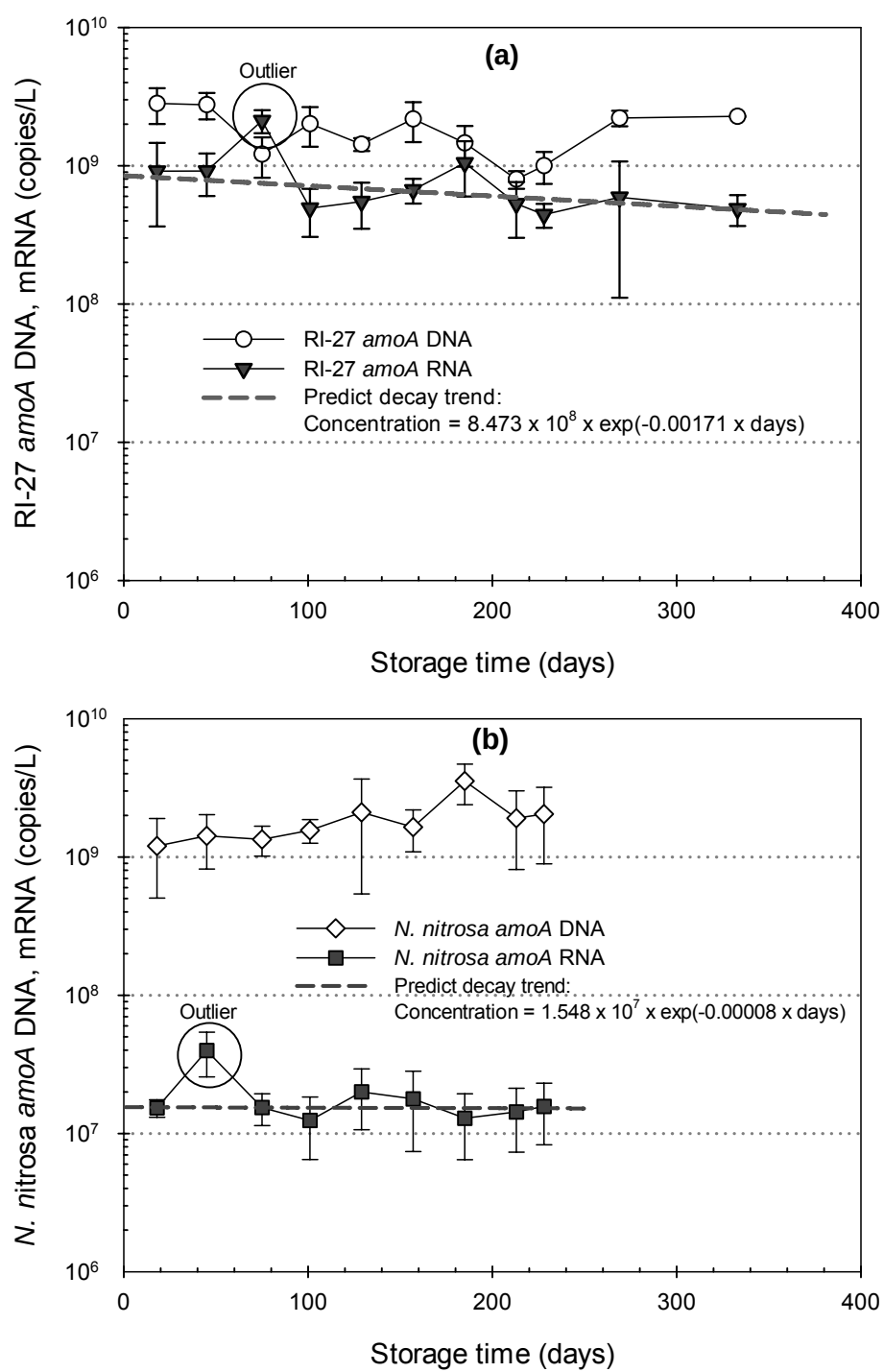


Figure 6-5. *amoA* DNA and mRNA concentrations of RI-27 (a) and *N. nitrosa* (b) measured at different -80°C storage times.

during low-N operation (phase 1). Additionally, MLSS samples from the 10-day SRT train were also analyzed for *amoA* mRNA concentrations. Results served as a reference to assess cellular activity (*amoA*-mRNA based) for the AOB groups (i.e., RI-27, B2-3, and *N. nitrosa*) identified in the bench-scale industrial system. It was anticipated that the *amoA* mRNA abundance for the dominant AOB population, i.e. RI-27, in the 10-day SRT train was higher than *amoA* mRNA for the B2-3 and *N. nitrosa* groups.

Figure 6-6 (c) shows *amoA* mRNA abundance for the RI-27, B2-3 and *N. nitrosa* groups in the 10-day SRT train (without considering any decay effect as concluded from previous *amoA* mRNA decay profile assessment at -80°C storage). Results indicated that the RI-27 *amoA* mRNA levels ($1.14 \pm 0.94 \times 10^9 \sim 1.51 \pm 0.96 \times 10^{10}$ copies/L) were much higher than the B2-3 and *N. nitrosa* *amoA* mRNA levels ($4.50 \pm 2.37 \times 10^7 \sim 1.36 \pm 0.86 \times 10^8$, and $3.28 \times 10^4 \sim 1.29 \times 10^6$ copies/L, respectively). At a cellular level, the RI-27 *amoA* transcripts were also higher than the B2-3 and *N. nitrosa* groups during both low-N and high-N phases (based on *amoA* mRNA transcribed per copy of *amoA* gene, or mRNA/DNA ratio) (Figure 6-6 (e)). These results clearly demonstrate that the RI-27 group was more active than the other two AOB groups (i.e., B2-3 and *N. nitrosa*) and likely contributed to the dominant presence of this organism in the 10-day SRT treatment system.

In the 2-day SRT train, the AOB population dynamics (Figure 6-6 (b)) was also reflected in the assessed physiology activity levels; more active AOB resulted in a higher population (based on *amoA* mRNA measurement) (Figure 6-6 (d)). In the first 10 days of high-N operation, overall *amoA* mRNA abundance for the B2-3 population sharply increased from $1.55 \pm 0.16 \times 10^6$ to $1.67 \pm 0.17 \times 10^9$ copies/L while the RI-27 and *N. nitrosa* *amoA* mRNA only increased ~2 fold (Figure 6-6 (d)). Additionally, an increase in the average *amoA* mRNA/DNA ratio was noted for the B2-3 group during high-N (phase 2) operation (Figure 6-6 (f)) suggesting an increase in B2-3 cellular activity. These results verified the hypothesis that B2-3 activity increased during high-N operation. It was surmised that the B2-3 group became more active than the RI-27 and *N. nitrosa* groups under the relatively high ammonia level that was produced during the transitional period

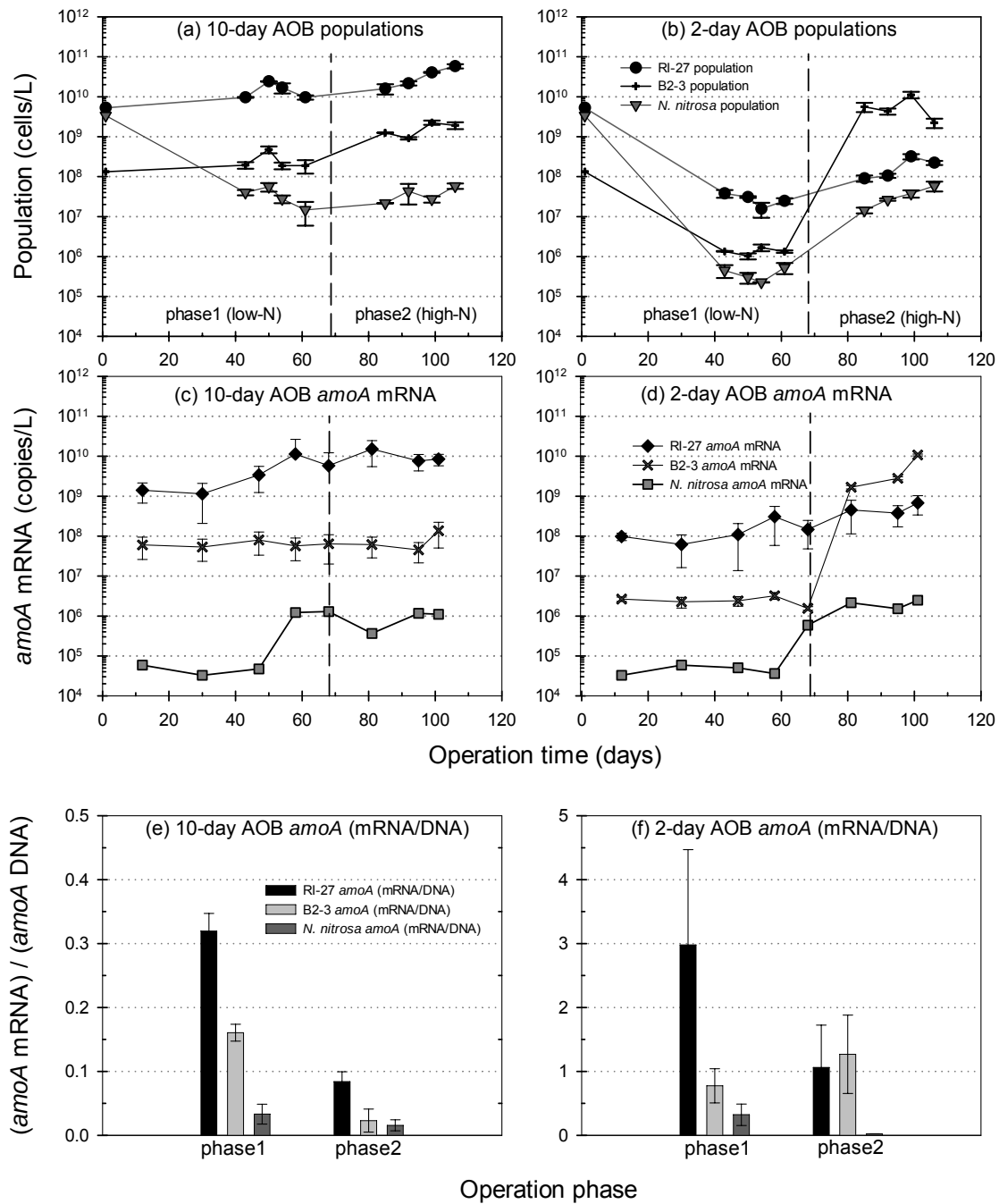


Figure 6-6. RI-27, B2-3 and *N. nitrosa* populations (*amoA* DNA based quantification) in the 10-day (a) and 2-day (b) SRT trains, cell activity (*amoA* mRNA based) in the 10-day (c) and 2-day (d) SRT trains, and average *amoA* mRNA/DNA ratios for AOB in the 10-day (e) and 2-day (f) SRT trains of the bench-scale industrial wastewater treatment system.

of the high-N operation in the 2-day SRT train. This activity increase allowed the B2-3 group to dominate the overall AOB population under such conditions (i.e., high ammonia and short SRT). Based on the *amoA* mRNA/DNA ratio appraisal, the AOB cells in the 2-day SRT train appeared to be more active (higher mRNA/DNA ratio, [Figure 6-6 \(e\), \(f\)](#)) than those in the 10-day SRT train. However, the overall AOB population in the 2-day SRT train was lower than that in the 10-day SRT train due to the short-SRT operational characteristic which dictated that large amounts of new grow AOB cells be wasted from the treatment system.

Based on these investigations, it is clear that kinetically-diverse AOB groups have different ammonia oxidation activity levels under various operational/environmental conditions. More active AOB groups tend to become dominant in the treatment system. Therefore, it is not appropriate to assume that the ammonia oxidation rates (or activities) among distinct AOB groups are the same (which was presumed in the proceeding determination of average cellular oxidation rates for the AOB groups in mixed cultures from the industrial bench-scale system, [Section 4.2.4](#)). For instance, an average cellular oxidation rate of 1.8 fmol/cell/hr (determined for the AOB population in the 10-day SRT train during high-N operation (phase 2) ([Table 4-21](#))) would not suitably represent the actual activity level for the *N. nitrosa* group which was less active than the dominant AOB group, i.e. RI-27 ([Figure 6-6 \(a\), \(c\), \(e\)](#)). In this case, the actual oxidation rate for the *N. nitrosa* group must be lower than that for the RI-27 group. The *amoA* mRNA based assessment can address specific activity variances between distinct AOB groups under different ammonia concentrations. Currently, the *amoA*-mRNA-based measurement has not been widely used to assess AOB activity because specific primers and probes to quantify *amoA* mRNA transcripts are not sufficient for most AOB groups. This study provided a method to individually examine oxidation activity for three AOB groups identified in a bench-scale industrial wastewater treatment system.

Additionally, results showed that *amoA* mRNA abundance does reflect a shift in AOB activity under different ammonia loadings. During high-N operation for the industrial bench-scale system, the overall *amoA* mRNA abundance (close to *amoA* mRNA level for the dominant AOB) was higher than that during low-N operation ([Figure](#)

6-6 (c), (e)). An increase in the RI-27 *amoA* mRNA level in the 10-day SRT train during the high-N phase (Figure 6-6 (c)) corresponded to a significant raise in the overall ammonia oxidation rate (increased from ~8 to ~65 $\mu\text{mol/L/hr}$) (Section 4.1.4). Moreover, in the 2-day SRT train, B2-3 *amoA* mRNA drastically increased during the high-N phase to a level higher than the RI-27 *amoA* mRNA concentration during low-N phase (Figure 6-6 (d)). This increase in *amoA* mRNA abundance was associated with a raise in the overall ammonia oxidation rate (increased from ~0 to ~58 $\mu\text{mol/L/hr}$).

6.2 Summary from Part III

Based on *amoA* mRNA evaluation for AOB activity in the bench-scale industrial wastewater treatment system, it was affirmed that the most active AOB group dominated the overall AOB population in the treatment system. The cellular activity of the RI-27 group (based on *amoA* mRNA /DNA ratio) was higher than those for the B2-3 and *N. nitrosa* groups resulting in RI-27 predominance in the 10-day SRT train. Additionally, the B2-3 group became the dominant AOB population in the 2-day SRT train during high-N operation due to cellular activity enhancement. These results demonstrate that cellular activity among distinct AOB groups is not the same. Specific activity variances between distinct AOB groups can be reflected by *amoA* mRNA levels. Activity ascendancy allows a particular AOB group to dominate the overall AOB population in the treatment system.

Chapter 7 Summary and conclusions

The three major objectives of this study (corresponding to each of the three research components, Part I, II, and III) were to: (1) determine the effect of the operational factors (SRT, NH_3 , and DO) on AOB population dynamics and ammonia oxidation treatment performance, (2) establish the mRNA transcript level of the *amoA* gene in AOB cells as a physiological indicator for the ammonia oxidation activity of AOB under different operational conditions (NH_3 , and DO), and (3) determine if AOB population shifts are a result of changes in AOB cellular activity (*amoA*-mRNA-based), respectively. Significant conclusions for each part of this research are summarized below.

7.1 AOB population dynamics in the industrial bench-scale system (Part I)

7.1.1 Ammonia oxidation rate (treatment performance)

- [SRT]: The overall volumetric ammonia oxidation rate ($\mu\text{mol/L/hr}$) in the industrial bench-scale system was significantly affected by SRT ($p\text{-value}<0.05$). The longer SRT system (i.e., 10-, and 20-day) had better ammonia oxidation treatment performance (higher oxidation rate) than the shorter SRT system (i.e., 2-, and 5-day).
- [NH_3]: The overall oxidation rate was also affected by ammonia loading. After the influent ammonia level approximately doubled during high-N operation (phase 2), the overall volumetric ammonia oxidation rate for each of the four SRT trains significantly increased ($p\text{-value}<0.05$).
- [DO]: Low DO operation (0.5 mg/L) did not impact the overall oxidation rate in the treatment system except for the 2-day SRT train.

7.1.2 AOB population size

- [SRT]: The AOB population size (cells/L) was significantly affected by SRT ($p\text{-value}<0.05$). Longer SRT systems (i.e., 10-, and 20-day) led to a higher AOB population than did shorter SRT systems (i.e., 2-, and 5-day).
- [NH_3]: A change in influent ammonia concentration (from low, ~ 20 mg/L, to high, $\sim$

55 mg/L) induced shifts in AOB population size for the shorter SRT trains. During high-N operation (phase 2), AOB populations in the 2-, and 5-day SRT trains significantly increased ($p\text{-value}<0.05$). Additionally, the AOB population structure in the 2-day SRT system (i.e., dominated by the B2-3 group) differed from that in the longer SRT systems (i.e., dominated by the RI-27 group).

- [DO]: Low DO level (0.5 mg/L) did not significantly impact the AOB population in the four SRT systems ($p\text{-value}>0.05$).

7.1.3 Cellular ammonia oxidation rate (cell activity)

- [SRT]: The average cellular ammonia oxidation rate (fmol/cell/hr) was significantly affected by SRT ($p\text{-value}<0.05$). AOB cells in the shorter SRT systems (i.e., 2-, and 5-day) had higher oxidation rates than those in the longer SRT systems (i.e., 10-, and 20-day).
- [NH₃]: High-N operation (phase 2) significantly changed only the oxidation rate of AOB cells in the 2-day SRT train ($p\text{-value}<0.05$).
- [DO]: Low DO operation (0.5 mg/L) did not significantly impact the cellular ammonia oxidation rate of AOB in the treatment system ($p\text{-value}>0.05$).

7.1.4 Testing result for Part I hypothesis

The AOB population and ammonia oxidation treatment performance in the bench-scale industrial wastewater treatment system were impacted to various degrees by the operational factors of SRT, NH₃, and DO. The effects of the SRT and ammonia concentration on AOB population and overall ammonia oxidation rate (for up to 42-67 days) were more significant than was low DO (0.5 mg/L).

Low AOB population levels in the 2-day SRT train after low-N operation led to a response delay in treatment performance during subsequent high-N operation. At steady-state, the overall ammonia oxidation treatment performance was not dependent upon the AOB population size. Although the longer SRT systems had relatively stable AOB populations, varied ammonia oxidation performance was noted under different ammonia loadings.

7.2 *amoA*-mRNA-based activity in the enriched nitrifying system (Part II)

Short-term (2-5 days) changes in NH_3 and DO levels were used to induce shifts in the ammonia oxidation activity of AOB cell in the enriched nitrifying system. Abundance of *amoA* mRNA for the *Nitrosomonas europaea* group was quantified to physiologically reflect ammonia oxidation activity.

7.2.1 Ammonia oxidation rate (treatment performance)

- $[\text{NH}_3]$ in 1-L batch culture tests: The ammonia oxidation rate was dependent upon ammonia concentration. A saturation relationship between ammonia level and oxidation rate was noted for the enriched nitrifying culture in batch-mode tests.
- $[\text{NH}_3]$ and $[\text{DO}]$ in the 10-L continuous-flow nitrifying reactor: Short-term changes in ammonia loading and DO level for the continuous-flow reactor resulted in different ammonia oxidation rates.

7.2.2 *N. europaea amoA* mRNA abundance

- $[\text{NH}_3]$ in 1-L batch culture tests: Response in *N. europaea amoA* mRNA to different ammonia concentrations was significant. In general, the maximum *amoA* mRNA levels increased with increasing ammonia concentration up to 20 mg/L $\text{NH}_3\text{-N}$ then became saturated at ammonia levels above this concentration. The maximum *amoA* mRNA level under different ammonia concentrations was predicted using a preliminary physiological model. Model-predicted levels were very similar to measured values. Additionally, these measured and modeled *amoA* mRNA levels correlated to ammonia oxidation rates (at $\text{NH}_3 < 20$ mg/L).
- $[\text{NH}_3]$ in the 10-L continuous-flow nitrifying reactor: The *N. europaea amoA* mRNA quickly responded to different ammonia loadings (within ~2 days), and levels corresponded to changes in ammonia oxidation rates in the trial reactor of the enriched nitrifying system.
- $[\text{DO}]$ in the 10-L continuous-flow nitrifying reactor: The *amoA* mRNA abundance did not change when ammonia oxidation rates dropped under DO-limiting conditions; thus, no correlation between *amoA* mRNA level and oxidation rate was

found during short-term low DO operation of the enriched nitrifying system.

7.2.3 Testing result for Part II hypothesis

The *amoA* mRNA abundance can serve as a physiological indicator to rapidly reflect a shift in ammonia-oxidizing activity under varying ammonia concentrations and loadings. However, it does not directly correlate with ammonia oxidation activity of AOB under DO limiting (but NH₃ sufficient) conditions. And, this metric cannot be solely used to evaluate exact ammonia oxidation performance in all nitrifying systems because activity variations due to possible regulation at the post-transcriptional level may exist.

In comparison to DNA-based quantification for AOB populations, the *amoA* mRNA abundance in AOB cells more rapidly responds to changes in operational/environmental conditions (usually on the order of hours). Thus, this physiological parameter can better reflect real-time shifts in overall ammonia oxidation performance in some cases (particularly changing ammonia concentrations).

7.3 *amoA*-mRNA-based activity for AOB in the industrial system (Part III)

An *amoA*-mRNA-based activity study was conducted on MLSS samples taken from the bench-scale industrial system to assess 1) whether the B2-3 dominance noted in the 2-day SRT train during high-N operation (phase 2) was due to an increase in B2-3 activity and 2) whether the RI-27 dominance in the 10-day SRT train was a result of activity ascendancy for this organism.

7.3.1 Comparison of *amoA* mRNA levels for distinct AOB groups

- In the 10-day SRT train, both overall *amoA* mRNA abundance (population level) and *amoA* mRNA/DNA ratio (cellular level) of the RI-27 group were higher than those for the B2-3 and *N. nitrosa* groups over the entire study period.
- In the 2-day SRT train, the B2-3 *amoA* mRNA and the mRNA/DNA ratio increased during high-N operation to levels higher than those for the RI-27 and *N. nitrosa* groups.

7.3.2 AOB dominance as a function of cellular activity

- The physiological activity of the RI-27 group was higher than that for the B2-3 and *N. nitrosa* groups resulting in RI-27 dominance in the 10-day SRT train.
- The B2-3 group became the dominant AOB population in the 2-day SRT train during high-N operation due to cellular activity enhancement.

7.3.3 Testing result for Part III hypothesis

Under particular conditions, kinetically-diverse AOB groups have different cellular activities, which are reflected in the *amoA* mRNA measurements. Activity enhancement allowed particular AOB groups to dominate the overall AOB population in the treatment system.

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Appendix A -- Data analyses

A.1 Determination of solids retention time (SRT)

The operating SRT for each train in the industrial bench-scale system was calculated using the following formula:

$$SRT = \frac{\text{Total MLVSS in train (mg)}}{[\text{Total effluent MLVSS} + \text{Total MLVSS of waste}] \text{ (mg/day)}} \quad (\text{Eq.A-1})$$

Thus, total MLVSS of waste (mg/day) equals to the following calculation.

$$\frac{\text{Total MLVSS in train (mg)}}{SRT \text{ (day)}} - \text{Total effluent MLVSS (mg/day)} \quad (\text{Eq.A-2})$$

Once the amount of wastage biomass was determined, the waste volume was then calculated by the following formula:

$$\text{Total waste volume} = \frac{\text{Total MLVSS of waste (mg)}}{\text{Average MLVSS concentration (mg/L)}} \quad (\text{Eq.A-3})$$

A.2 COD and ammonium removal performance

For estimating chemical oxygen demand (COD) treatment performance in the industrial bench-scale system, COD removal efficiency (%) was defined as:

$$COD \text{ removal efficiency (\%)} = \frac{COD_{Influent} - COD_{Effluent}}{COD_{Influent}} \times 100\% \quad (\text{Eq.A-4})$$

Ammonium removal efficiency (%) in the industrial bench-scale system was defined as the following formula:

$$NH_4^+ \text{ removal efficiency (\%)} = \frac{NH_4^+_{Influent} - NH_4^+_{Effluent}}{NH_4^+_{Influent}} \times 100\% \quad (\text{Eq.A-5})$$

A.3 Biomass-N for the industrial bench-scale system

Daily wasted biomass-N in the industrial bench-scale system was estimated using the formula below. This calculation was based on an assumption that the N-content of the

MLSS in the industrial bench-scale system at each SRT did not significantly change over time; thus a one-time-measurement of N in the MLSS was used throughout the course of the research period.

Daily wasted biomass-N (mg-N/day)

$$= \text{Total wasted MLSS (mg/day)} \times \text{average N-content (\%)} \quad (\text{Eq.A-6})$$

The N-content for biosolids sample taken from each of the four SRT trains was determined on day 93 (during phase 2) to estimate amount of nitrogen in daily-wasted biomass and to evaluate the overall fate of nitrogen in the industrial bench-scale treatment system. The biomass-N values were 7.04, 7.32, 7.71, and 7.15% (w/w) for the 20-, 10-, 5-, and 2-day SRT trains, respectively, with a mean (and standard deviation) of $7.31 \pm 0.29\%$. These results were close to the annual average of $6.88 \pm 1.13\%$ for the full-scale industrial WWTP. Because the industrial bench-scale system was fed with the same wastewater as that in the full-scale WWTP (except during high-N operation when influent ammonia levels doubled), it was presumed that the N-content in the bench-scale system biosolids did not significantly change during the overall operational period. Therefore, a mean of 7.31% was used to calculate the amount of the biomass-N wasted from each SRT train (Eq. A-6) in order to evaluate the overall fate of nitrogen in this bench-scale system.

A.4 Ammonia-oxidation performance

To evaluate ammonia-oxidation treatment performance in the industrial bench-scale system, it was assumed that any ammonia released by hydrolysis of organic-nitrogen compounds (although relative small amount) was an ammonia source for ammonia-oxidizing bacteria (AOB). Additionally, not all of the influent ammonia (including that hydrolyzed from organic-nitrogen compounds) was available to the AOB (i.e., some ammonia in the influent was used in biomass or enzyme synthesis especially by heterotrophs). Also, the industrial wastewater used as influent for the bench-scale system contained small amounts of nitrite and nitrate that could exit in the effluent with the nitrite and nitrate produced from nitrification. Moreover, other nitrogen conversion reactions (e.g., denitrification) could occur and produce other end products (especially

gaseous nitrogen compounds). Therefore, the ammonia-oxidation performance was evaluated based on the overall nitrogen balance for the treatment system (Figure A-1).

The nitrogen balance equivalent can be calculated from Eq. A-7, in which Inf = influent, Eff = effluent, org-N = organic-N and $Eff_{biomass-N}$ = biomass-N in both wasted and effluent biosolids:

$$Inf_{NH_3} + Inf_{org-N} + Inf_{NO_2^-} + Inf_{NO_3^-} = Eff_{NH_3} + Eff_{org-N} + Eff_{NO_2^-} + Eff_{NO_3^-} + Eff_{biomass-N} \quad (\text{Eq. A-7})$$

The nitrogen available for autotrophic AOB can be calculated from the following formula:

$$Inf_{NH_3} + (Inf_{org-N} - Eff_{org-N}) - Eff_{biomass-N} \quad (\text{Eq. A-8})$$

And finally, the amount of ammonia oxidized by AOB can be calculated from Eq. A-9, which should be equal to the amount of NO_x^- oxidized from ammonia (Eq. A-10):

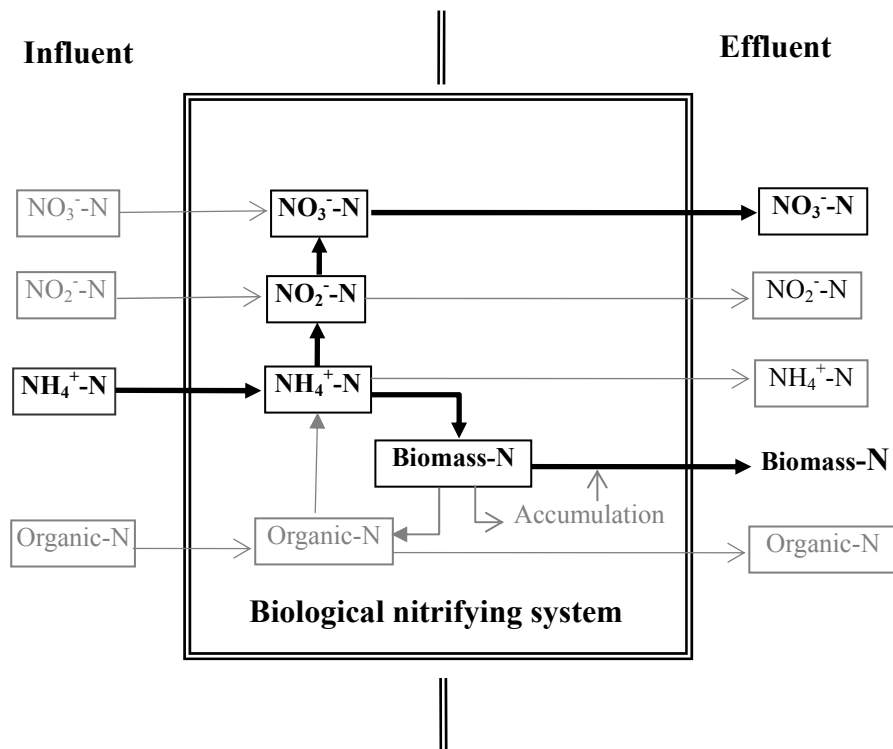


Figure A-1. Nitrogen mass balance in a CSTR biological nitrifying system under steady-state conditions.

$$(Inf_{NH_3} - Eff_{NH_3}) + (Inf_{org-N} - Eff_{org-N}) - Eff_{biomass-N} \quad (\text{Eq. A-9})$$

$$(Eff_{NO_2^-} - Inf_{NO_2^-}) + (Eff_{NO_3^-} - Inf_{NO_3^-}) \quad (\text{Eq. A-10})$$

Overall ammonia oxidation treatment performance for the industrial bench-scale system was also defined by the volumetric ammonia oxidation rate (amount of ammonia oxidized per liter per hour, i.e., $\mu\text{mol NH}_3\text{-N/L/hr}$) in each SRT train. The amount of ammonia oxidized was calculated from Eq. A-10. The quantification of overall AOB population was used to normalize the ammonia oxidation activity to the cell level (amount of ammonia oxidized per AOB cell per hour, i.e., $\text{fmol NH}_3\text{-N/cell/hr}$).

The ammonia oxidation treatment performance in the enriched nitrifying system was also defined by the volumetric ammonia oxidation rates (amount of ammonia oxidized per liter per hour, i.e., $\mu\text{mol NH}_3\text{-N/L/hr}$). (Since the influent nitrogen species for this treatment system was simply ammonia, the nitrite and nitrate levels in the influent can be ignored in Eq. A-10.) Thus, the amount of ammonia oxidized in the enriched nitrifying system was calculated as $(NO_2^- + NO_3^-)$ under steady-state operational conditions.

When designated experiments were performed in the Trial reactor of the enriched nitrifying system, the dilution factor was considered for determining ammonia oxidation rates. The amount of ammonia oxidized under non-steady state conditions (experimental period) was calculated by Eq. A-12 developed from concentration equilibrium (Eq. A-11) where $(NO_x^-)_{T1}$, $(NO_x^-)_{T2}$ are the $(NO_2^- + NO_3^-)$ concentrations at time points 1 and 2, respectively; and V_{T1} , V_{T2} are the mixed liquor volumes at time points 1 and 2, respectively; and $(NO_x^-)_{T2-T1}$ is the $(NO_2^- + NO_3^-)$ oxidized from ammonia during time points 1 and 2.

$$\frac{[(NO_x^-)_{T1} \times V_{T1}] + [(NO_x^-)_{T2-T1} \times (V_{T2} - V_{T1})]}{V_{T2}} = (NO_x^-)_{T2} \quad (\text{Eq. A-11})$$

$$(NO_x^-)_{T2-T1} \times (V_{T2} - V_{T1}) = [(NO_x^-)_{T2} \times V_{T2}] - [(NO_x^-)_{T1} \times V_{T1}] \quad (\text{Eq. A-12})$$

Therefore, the ammonia oxidation rate under non-steady state conditions ($AOR_{non-steady\ state}$)

(experimental periods) was calculated using the following formula.

$$AOR_{non-steady\ state} = \frac{[(NO_x^-)_{T2} \times V_{T2}] - [(NO_x^-)_{T1} \times V_{T1}]}{(T2 - T1)} \quad (\text{Eq. A-13})$$

The ammonia oxidation rates in the 1-L batch culture tests were determined based on ammonia concentration because the mixed liquor taken from the enriched nitrifying system had very high NO_x^- concentrations (especially NO_3^-) that resulted in insignificant shifts in the NO_x^- concentration during the 1-L batch culture tests. The amount of ammonia oxidized in a 1-L batch culture test during a time period (i.e., 1 hour) was calculated by [Eq. A-14](#).

$$NH_3 \text{ oxidized during } (T2-T1) = (NH_3)_{T1} \times V_{T1} + NH_3 \text{ added during } (T2-T1) - (NH_3)_{T2} \times V_{T2} \quad (\text{Eq. A-14})$$

Therefore, the ammonia oxidation rate in the 1-L batch culture tests was determined using the following formula.

$$\frac{(NH_3)_{T1} \times V_{T1} + NH_3 \text{ added during } (T2-T1) - (NH_3)_{T2} \times V_{T2}}{(T2 - T1)} \quad (\text{Eq. A-15})$$

A.5 Calculation for microbial population and *amoA* mRNA abundance

The molecular results of functional gene abundance were normalized from copies per liter to cells per liter (mixed liquor) using the following values: 3.6 copies of 16S rDNA per cell for total eubacteria and AOB ([Klappenbach et al., 2001](#)) and 2 copies of the *amoA* gene per cell for AOB ([McTavish et al., 1993](#)).

The *amoA* mRNA abundance was presented in copies per liter (mixed liquor). It was further normalized to *amoA* mRNA copies per gene copy (divided the *amoA* mRNA copies with *amoA* DNA copies) to assess AOB cellular activity.

A.6 Statistical test

A statistical t-test was used for data analyses at an α -value of 0.05. The test

computes whether the means of two analyzed data sets differ from each other (SAS version 8.2, SAS Institute Inc., Cary, NC). The null hypothesis was that there were no significant differences between the two means (i.e., the difference between the two means equal zero). The null hypothesis was rejected when the p-value test result was less than the target α -value (0.05). When the p-value test result was higher than the target α -value (0.05), there was insufficient evidence to verify that the means of two data sets differed from one other.

Appendix B -- *amoA* gene libraries for the industrial bench-scale system

Table B-1. The clone libraries for the industrial bench-scale system closely matched the *amoA* sequences of the *Nitrosomonas nitrosa* [AJ238495] group. (*: the typical clones selected for phylogenetic analysis).

Closely match	Partial <i>amoA</i> sequences
<i>N. nitrosa</i>	>IB-20d-C7* 1 TGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTGGTAACGGCGC 121 TGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTGGAC 181 CGACTCACTTGCCGTGCTGTTGCAGAAAGGCGTATTGCTTTCAATGGCAGACTACATGGGAC 241 ACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTGC 301 GTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGATG 361 TGATGTTTGTGTTTGGTGGTTCCCTCGGTAAAGTTTACTGTACAGCATTCTTTTACGTTA 421 AAGGTAAAAGAGGCCGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGCT 481 TTGCAGAGGGG
	>IB-20d-C12 1 TGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTGGGA 181 CCGACTCACTTGCCGTGCTGTTGCAGAAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCCTCGGTAAAGTTTACTGTACAGCATTCTTTTACGTT 421 AAAGGTAAAAGAGGCCGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTTGCCGAGGGG
	>IB-20d-C13 1 TGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTGGGA 181 CCGACTCACTTGCCGTGCTGTTGCAGAAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTGC 301 GTACCTTTGGCGGCCATACCACGGTGGTTGCTGCGTTCTTTGCGGCGTTTGTATCGTTGT 361 TGATGTTTGTGTTTGGTGGTTCCCTCGGTAAAGTTTACTGTACAGCATTCTTTTACGTTA 421 AAGGTAAAAGAGGCCGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGCT 481 TTCCCAGGGG
	>IB-20d-C31 1 TGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTGGGA 181 CCGACTCACTTGCCGTGCTGTTGCAGAAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCCTCGGTAAAGTTTACTGTACAGCATTCTTTTACGTT 421 AAAGGTAAAAGAGGCCGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTCCAGAGGGG
	>IB-20d-C34 1 TGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGTGCATTGATGATGGATATCACGTTGTACTTGACCCGTAACGGTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTGGGA 181 CCGACTCACTTGCCGTGCTGTTGCAGAAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCCTCGGTAAAGTTTACTGTACAGCATTCTTTTACGTT 421 AAAGGTAAAAGAGGCCGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTGCAGAGGGG

Table B-1. Continue.

Closely match	Partial <i>amoA</i> sequences
<i>N. nitrosa</i>	>IB-20d-C40 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTTGGA 181 CCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCTCGGTAAAGTTTACTGTACAGCATTTCTTTACGTT 421 AAAGGTAAAAGAGGCGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTTCCAGAGGGG
	>IB-10d-C5* 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTTGGA 181 CCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCTCGGTAAAGTTTACTGTACAGCATTTCTTTACGTT 421 AAAGGTAAAAGAGGCGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTTGCAGAGGGG
	>IB-10d-C11 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTTGGA 181 CCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCTCGGTAAAGTTTACTGTACAGCATTTCTTTACGTT 421 AAAGGTAAAAGAGGCGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTTCCCGAGGGG
	>IB-10d-C24 1 TGGGGTTTCTACTGGTGGTCACACTACCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTTGGA 181 CCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCTCGGTAAAGTTTACTGTACAGCATTTCTTTACGTT 421 AAAGGTAAAAGAGGCGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTTGCAGAGGGG
	>IB-5d-C2* 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTTGGA 181 CCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCTCGGTAAAGTTTACTGTACAGCATTTCTTTACGTT 421 AAAGGTAAAAGAGGCGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTTCCAGAGGGG

Table B-1. Continue.

Closely match	Partial <i>amoA</i> sequences
<i>N. nitrosa</i>	>IB-5d-C32 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTGGGA 181 CCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCTCGGTAAAGTTTACTGTACAGCATTTCTTTACGTT 421 AAAGGTAAAAGAGGCGGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTTCCAGAGGGGA
	>IB-2d-C5* 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGGTGCATTGATGTTAGGATATCACGTTGTACTTGACCCGTAACGGTTGGTAACGGCG 121 CTGACCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTGGGA 181 CCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCTCGGTAAAGTTTACTGAACAGCATTTCTTTACGTT 421 AAAGGTAAAAGAGGCGGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTTGCACAGGGGATCAAATAA
	>IB-2d-C12 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTGGGA 181 CCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCTCGGTAAAGTTTACTGTACAGCATTTCTTTACGTT 421 AAAGGTAAAAGAGGCGGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTTTCAGAGGGG
	>IB-2d-C15 1 TGGGGTTTCTACTGGTGGTCACATTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTGGGA 181 CCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGTGTTCTTTGCGGCGTTTGTATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCTCGGTAAAGTTTACTGTACAGCATTTCTTTACGTT 421 AAAGGTAAAAGAGGCGGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTTCCGAGGGG
	>IB-2d-C54 1 TNGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGAT 61 TCCGGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTTGGTAACGGC 121 GCTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTGG 181 ACCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGG 241 ACACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACT 301 GCGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGTATCGAT 361 GTTGATGTTTGTGTTTGGTGGTTCCTCGGTAAAGTTTACTGTACAGCATTTCTTTACGT 421 TAAAGGTAAAAGAGGCGGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGG 481 CTTTCACAGGGGATTAAATAA

Table B-1. Continue.

Closely match	Partial <i>amoA</i> sequences
<i>N. nitrosa</i>	>IB-2d-C60 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACGGTTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTGGGA 181 CCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCCTG 301 CGTACCTTTGGCGGCCATACCACGGTGATTGCTGCGTTCTTTGCGGCGTTTGATCGATG 361 TTGATGTTTGTGTTTGGTGGTTCCACGGTAAAGTTTACTGTACAGCATTCCTTTACGTT 421 AAAGGTAAAAGAGGCGTATTGTTAAAAGAGATGACGTTACTGCGTTTGGTGAAGAAGGC 481 TTTGCCGAGGGGATCAAATAA

Table B-2. The clone libraries for the industrial bench-scale system closely matched the *amoA* sequences of the RI-27 [AF532311] group. (*: the typical clones selected for phylogenetic analysis).

Closely match	Partial <i>amoA</i> sequences
RI-27	>IB-20d-C3* <pre> 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATTAACCTTTGTAATTCCATCAATCATGATT 61 CCAGGTGCGTTAATGTTGGATATTACTTTATATCTAACCCGTAACCTGGCTCGTGACAGCG 121 TTAATTGGTGGCGGATTCTTTGGATTATTCTTCTATCCAGGTAACCTGGGTATTTTGGGA 181 CCGACCCACTTGCCAATCGTAGTTGAAGGCACATTGTTATCGATGGCTGACTATATGGGA 241 CACATGTATGTACGTACAGGTACTCCTGAATATGTCGCTTAATCGAACAGGCTCACTG 301 CGTACGTTCCGGTGGTCATACAACGGTGATTGCAGCATTCTTCTCAGCGTTTCGTATCGATG 361 CTGATGTTTGTGTTTGGTGGTACCTAGGCAAAGTCTATTGCACCCTTTCTACTATGTT 421 AAAGGTAAGAGAGGTCGTATCGTGCAAAGAGATGACGTTACAGCATTGTTGGTGAAGAAGGC 481 TTTGCAGAGGGG </pre> >IB-20d-C20 <pre> 1 TGGGGTTTCTACTGGTGGTCACNCTATCCAATTAACCTTTGTAATTCCATCAATCANGATT 61 CCAGGTGCGTTAANGTTGGATATTACTTTATATCTAACCCGTAACCTGGCTCGTGACAGCG 121 TTAATTGGTGGCGGATTCTTTGGATTATTNTTCTATCCAGGTAACCTGGGTATTTTGGGA 181 CCGACCCCTTGCCNATCGTAGTTGAAGGCNCTTGTATCGATGGCTGACTATATGGGA 241 CACATGTATGTACGTACAGGTACTCCTGAATATGTCGCTTAATCGAACAGGCTCACTG 301 CGTACGTTCCGGTGGTCATACAACGGTAATTGCAGCATTCTTCTCAGCGTTTCGTATCGATG 361 CTGATGTTTGTGTTTGGTGGTACCTAGGCAAAGTCTATTGCACCCTTTCTACTATGTT 421 AAAGGTAAGAGAGGTCGTATCGTGCAAAGAGATGACGTTACAGCATTGTTGGTGAAGAAGGC 481 TTTCCAGGGGG </pre> >IB-20d-C42 <pre> 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATTAACCTTTGTAATTCCATCAATCATGATT 61 CCAGGTGCGTTAATGTTGGATATTACTTTATATCTAACCCGTAACCTGGCTCGTGACAGCG 121 TTAATTGGTGGCGGATTCTTTGGATTATTCTTCTATCCAGGTAACCTGGGTATTTTGGGA 181 CCGACCCACTTGCCAATCGTAGTTGAAGGCACATTGTTATCGATGGCTGACTATATGGGA 241 CACATGTATGTACGTACAGGTACTCCTGAATATGTCGCTTAATCGAACAGGCTCACTG 301 CGTACGTTCCGGTGGTCATACAACGGTAATTGCAGCATTCTTCTCAGCGTTTCGTATCGATG 361 CTGATGTTTGTGTTTGGTGGTACCTAGGCAAAGTCTATTGCACCCTTTCTACTATGTT 421 AAAGGTAAGAGAGGTCGTATCGTGCAAAGAGATGACGTTACAGCATTGTTGGTGAAGAAGGC 481 TTTGCCGAGGGG </pre> >IB-20d-C44 <pre> 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATTAACCTTTGTAATTCCATCAATCATGATT 61 CCAGGTGCGTTAATGTTGGATATTACTTTATATCTAACCCGTAACCTGGCTCGTGACAGCG 121 TTAATTGGTGGCGGATTCTTTGGATTATTCTTCTATCCAGGTAACCTGGGTATTTTGGGA 181 CCGACCCACTTGCCAATCGTAGTTGAAGGGACATTGTTATCGATGGCTGACTATATGGAC 241 ACATGTATGTACGCACAGGTACTCCTGAATATGTTTCGCTTAATCGAACAGGCTCACTGC 301 GTACGTTCCGGTGGTCATACAACGGTAATTGCAGCATTCTTCTCAGCGTTTCGTATCGATGC 361 TGATGTTTGTGTTTGGTGGTACCTAGGCAAAGTCTATTGCACCCTTTCTACTATGTTA 421 AAGGTAAGAGAGGTCGTATCGTGCAAAGAGATGACGTTACAGCATTGTTGGTGAAGAAGGCT 481 TTCCCGAGGGG </pre> >IB-10d-C1* <pre> 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATTAACCTTTGTAATTCCATCAATCATGATT 61 CCAGGTGCGTTAATGTTGGATATTACTTTATATCTAACCCGTAACCTGGCTCGTGACAGCG 121 TTAATTGGTGGCGGATTCTTTGGATTATTCTTCTATCCAGGTAACCTGGGTATTTTGGGA 181 CCGACCCACTTGCCAATCGTAGTTGAAGGGACATTGTTATCGATGGCTGACTATANGGGA 241 CACATGTATGTACGTACAGGTACTCCTGAATATGTCGCTTAATCGAACAGGCTCACTG 301 CGTACGNTCCGGTGGTCATACAACGGTAATTGCAGCATTCTTCTCAGCGTTTCGTATCGATG 361 CTGATGTTTGTGTTTGGTGGTACCTAGGCAAAGTCTATTGCACCCTTTCTACTATGTT 421 AAAGGTAAGAGAGGTCGTATCGTGCAAAGAGATGACGTTACAGCATTGTTGGTGAAGAAGGC 481 TTTCCAGAGGGG </pre>

Table B-2. Continue.

Closely match	Partial <i>amoA</i> sequences
RI-27	>IB-10d-C26 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATTAACCTTTGTAATTCATCAATCATGATT 61 CCAGGTGCGTTAATGTTGGATATTACTTTTATATCTAACCCGTAACCGGCTCGTGACAGCG 121 TTAATTGGTGGCGGATTCTTTGGATTATTCTTCTATCCAGGTAACGGGTATTTTTTGGGA 181 CCGACCCACTTGCCAATCGTAGTTGAAGGCACATTGTTATCGATGGCTGACTATATGGGA 241 CACATGTATGTACGTACAGGTACTCCTGAATATGTTTCGCTTAATCGAACAAGGCTCACTG 301 CGTACGTTTCGGTGGTCATACAACGGTAATTGCAGCATTCTTCTCAGCGTTTCGTATCGATG 361 CTGATGTTTGTGTTTGGTGGTACCTAGGCAAAGTCTATTGCACCGCTTTTCTACTACGTT 421 AAAGGTAAGAGAGGTCGTATCGTGCAAAGAGATGACGTTACAGCATTGTTGGTGAAGAAGGC 481 TTTGCAGAGGGG
	>IB-10d-C49 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATTAACCTTTGTAATTCATCAATCATGATT 61 CCAGGTGCGTTAATGTTGGATATTACTTTTATATCTAACCCGTAACGGCTCGTGACAGCG 121 TTAATTGGTGGCGGATTCTTTGGATTATTCTTCTATCCAGGTAACGGGTATTTTTTGGGA 181 CCGACCCACTTGCCAATCGTAGTTGAAGGCACATTGTTATCGATGGCTGACTATATGGGA 241 CACATGTATGTACGTACAGGTACTCCTGAATATGTTTCGCTTAATCGAACAAGGCTCACTG 301 CGTACGTTTCGGTGGTCATACAACGGTAATTGCAGCATTCTTCTCAGCGTTTCGTATCGATG 361 CTGATGTTTGTGTTTGGTGGTACCTAGGCAAAGTCTATTGCACCGCTTTTCAACTATGTT 421 AAAGGTAAGAGAGGTCGTATCGTGCAAAGAGATGACGTTACAGCATTGTTGGTGAAGAAGGC 481 TTTCCAGAGGGG
	>IB-5d-C24 1 TGGGGTTTCTACTGGTGGTCACACTATCTAATTAACCTTTGTAATTCATCAATCATGATT 61 CCAGGTGCGTTAATGTTGGATATTACTTTTATATCTAACCCGTAACGGCTCGTGACAGCG 121 TTAATTGGTGGCGGATTCTTTGGATTATTCTTCTATCCAGGTAACGGGTATTTTTTGGGA 181 CCGACCCACTTGCCAATCGTAGTTGAAGGGACATTGTTATCGATGGCTGACTATATGGGA 241 CACATGTATGTACGTACAGGTACTCCTGAATATGTTTCGCTTAATCGAACAAGGCTCACTG 301 CGTACGTTTCGGTGGTCATACAACGGTAATTGCAGCATTCTTCTCAGCGTTTCGTATCGATG 361 CTGATGCTTGTGTTTGGTGGTACCTAGGCAAAGTCTATTGCGCCGCTTTTCTACTATGTT 421 AAAGGTAAGAGAGGTCGTATCGTGCAAAGAGATGACGTTACAGCATTGTTGGTGAAGAAGGT 481 TTTCCCAGGGGATCAAATAAA
	>IB-5d-C34* 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATTAACCTTTGTAATTCGTCATCAATCATGATT 61 CCAGGTGCGTTAATGTTGGATATTACTTTTATATCTAACCCGTAACGGCTCGTGACAGCG 121 TTAATTGGTGGCGGATTCTTTGGATTATTCTTCTATCCAGGTAACGGGTATTTTTTGGGA 181 CCGACCCACTTGCCAATCGTAGTTGAAGGCACATTGTTATCGATGGCTGACTATATGGGA 241 CACATGTATGTACGTACAGGTACTCCTGAATATGTTTCGCTTAATCGAACAAGGTTCACTG 301 CGTACGTTTCGGTGGTCATACAACGGTAATTGCAGCATTCTTCTCAGCGTTTCGTATCGATG 361 CTGATGTTTGTGTTTGGTGGTACCTAGGCAAAGTCTATTGCACCGCTTTTCTACTATGTT 421 AAAGGTAAGAGAGGTCGTATCGTGCAAAGAGATGACGTTACAGCATTGTTGGTGAAGAAGGC 481 TTTCCAGAGGGGA
	>IB-5d-C45 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATTAACCTTTGTAATTCATCAATCATGATT 61 CCAGGTGCGTTAATGTTGGATATTACTTTTATATCTAACCCGTAACGGCTCGTGACAGCG 121 TTAATTGGTGGCGGATTCTTTGGATTATTCTTCTATCCAGGTAACGGGTATTTTTTGGGA 181 CCGACCCACTTGCCAATCGTAGTTGAAGGGACATTGTTATCGATGGCTGACTATATGGGA 241 CACATGTATGTACGTACAGGTACTCCTGAATATGTTTCGCTTAATCGAACAAGGCTCACTG 301 CGTACGTTTCGGTGGTCATACAACGGTAATTGCAGCATTCTTCTCAGCGTTTCGTATCGATG 361 CTGATGTTTGTGTTTGGTGGTACCTAGGCAAAGTCTATTGCACCGCTTTTCTACTATGTT 421 AAAGGTAAGAGAGGTCGTATCGTGCAAAGAGATGACGTTACAGCATTGTTGGTGAAGAAGGC 481 TTTCCCAGGGGA

Table B-2. Continue.

Closely match	Partial <i>amoA</i> sequences
RI-27	>IB-2d-C18* 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATTAACCTTTGTAATTCATCAATCATGATT 61 CCAGGTGCGTTAATGTTGGATATTACTTTATATCTAACCTGTAACCTGGCTCGTGACAGCG 121 TTAATTGGTGGCGGATTCTTTGGATTATTCTTCTATCCAGGTAACCTGGGTTATTTTGGGA 181 CCGACCCACTTGCCAATCGTAGTTGAAGGCACATTGTTATCGATGGCTGACTATATGGGA 241 CACATGTATGTACGTACAGGTACTCCTGAATATGTTTCGCTTAATCGAACAGGGCTCACTG 301 CGTACGTTTCGGTGGTCATACAACGGTAATTGCAGCATTCTTCTCAGCGTTTCGTATCGATG 361 CTGATGTTTCGTTGTTTGGTGGTACCTAGGCAAAGTCTATTGCACCGCTTTCTACTATGTT 421 AAAGGTAAGAGAGGTTCGTATCGTGCAAAGAGATGACGTTACAGCATTTGGTGAAGAAGGT 481 TTTGCACAGGGGATCAAATAA >IB-2d-C66 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATTAACCTTTGTAATTCATCAATCATGATT 61 CCAGGTGCGTTAATGTTGGATATTACTTTATATCTAACCCGTAACCTGGCTCGTGACAGCG 121 TTAATTGGTGGCGGATTCTTTGGATTATTCTTCTATCCAGGTAACCTGGGTTATTTTGGGA 181 ACGACCCACTTGCCAATCGTAGTTGAAGGCACATTGTTATCGATGGCTGACTATATGGGA 241 CACATGTATGTACGTACAGGTACTCCTGAATATGTTTCGCTTAATCGAACAGGGCTCACCG 301 CGTACGTTTCGGTGGTCATACAACGGTAATTGCAGCATTCTTCTCAGCGTTTCGTATCGATG 361 CTGATGTTTGTGTTTGGTGGTACCTAGGCAAAGTCTATTGCACCGCTTTCTACTATGTT 421 GAAGGTAAGAGAGGTTCGTATCGTGCAAAGAGATGACGTTACAGCATTTGGTGAAGAAGGT 481 TTTGCCAGGGGATCAAATAA

Table B-3. The clone libraries for the industrial bench-scale system closely matched the *amoA* sequences of the B2-3 [AF293067] group. (*: the typical clones selected for phylogenetic analysis).

Closely match	Partial <i>amoA</i> sequences
B2-3	>IB-5d-C1* <pre> 1 TGGGGTTTCTACTGGTGGTCACACTACCCGTTGAATTTTGTACTGCCATCGACAATGATA 61 CCTGGTGCATTGATGCTGGACACAATTTTATTGTTGACGGGTAACGGCTGGTTACAGCA 121 CTGTTAGGTGGTGGATTCTGGGGACTGTTTTTCTACCCCTGGTAACGGCCGATATTTGGC 181 CCAACCCACTTACCGGTCGTAGTAGAAGGCGTATTGTTATCGGTTGCGGACTACACAGGC 241 TTTTATACGTACGTACGGGTACACCGGAATACGTAAGATTGATTGAGCAAGGCTCACTG 301 CGTACCTTTGGTGGTCACACGACGGTAATTGCAGCGTTTTTCTCAGCGTTTGTATCGATG 361 CTGATGTACTGCGTATGGTGGTATTTTGGCAAGATCTATTGCACCGCTTTCTTCTATGTT 421 AAAGGAGAGAGAGGCCGCATTTCTATGAAAAATGACGTAACCGCATTTGGTGAAAAAGGC 481 TTTGCACAGGGGATTAAATAAA </pre> >IB-5d-C21 <pre> 1 TGGGGTTTCTACTGGTGGTCACACTACCCGTTGAATTTTGTACTGCCATCGACAATGATA 61 CCTGGTGCATTGATGCTGGACACAATTTTATTGTTGACGGGTAACGGCTGGTTACAGCA 121 CTGTTAGGTGGTGGATTCTGGGGACTGTTTTTCTACCCCTGGTAACGGCCGATATTTGGC 181 CCAACCCACTTACCGGTCGTAGTAGAAGGCGTATTGTTATCGGTTGCGGACTACACAGGC 241 TTTTATACGTACGTACGGGTACACCGGAATACGTAAGATTGATTGAGCAAGGCTCACTG 301 CGTACCTTTGGTGGTCACACGACGGTAATTGCAGCGTTTTTCTCAGCGTTTGTATCGATG 361 CTGATGTACTGCGTATGGTGGTATTTTGGCAAGATCTATTGCACCGCTTTCTTCTATGTT 421 AAAGGAGAGAGAGGCCGCATTTCTATGAAAAATGACGTAACCGCATTTGGTGAAAAAGGC 481 TTTGCACAGGGGATTAAATAAA </pre> >IB-2d-C16* <pre> 1 TGGGGTTTCTACTGGTGGTCACACTACCCGTTGAATTTTGTACTGCCATCGACAATGATA 61 CCTGGTGCATTGATGCTGGACACAATTTTATTGTTGACGGGTAACGGCTGGTTACAGCA 121 CTGTTAGGTGGTGGATTCTGGGGACTGTTTTTCTACCCCTGGTAACGGCCGATATTTGGC 181 CCAACCCACTTACCGGTCGTAGTAGAAGGCGTATTGTTATCGGTTGCGGACTACACAGGC 241 TTTTATACGTACGTACGGGTACACCGGAATACGTAAGATTGATTGAGCAAGGCTCACTG 301 CGTACCTTTGGTGGTCACACGACGGTAATTGCAGCATTTTTTCTCAGCGTTTGTATCGATG 361 CTGATGTACTGCGTATGGTGGTATTTTGGCAAGATCTATTGCACCGCTTTCTTCTATGTT 421 AAAGGAGAGAGAGGCCGCATTTCTATGAAAAATGACGTAACCGCATTTGGTGAAAAAGGC 481 TTTGCCAGGGGATCAAATAAA </pre> >IB-2d-C17 <pre> 1 TGGGGTTTCTACTGGTGGTCACACTACCCGTTGAATTTTGTACTGCCATCGACAATGATA 61 CCTGGTGCATTGATGCTGGACACAATTTTATTGTTGACGGGTAACGGCTGGTTACAGCA 121 CTGTTAGGTGGTGGATTCTGGGGACTGTTTTTCTACCCCTGGTAACGGCCGATATTTGGC 181 CCAACCCACTTACCGGTCGTAGTAGAAGGCGTATTGTTATCGGTTGCGGACTACACAGGC 241 TTTTATACGTACGTACGGGTACACCGGAATACGTAAGATTGATTGAGCAAGGCTCACTG 301 CGTACCTTTGGTGGTCACACGACGGTAATTGCAGCATTTTTTCTCAGCGTTTGTATCGATG 361 CTGATGTACTGCGTATGGTGGTATTTTGGCAAGATCTATTGCACCGCTTTCTTCTATGTT 421 AAAGGAGAGAGAGGCCGCATTTCTATGAAAAATGACGTAACCGCATTTGGTGAAAAAGGC 481 TTTGCCAGGGGATCAAATAAA </pre> >IB-2d-C21 <pre> 1 TGGGGTTTCTACTGGTGGTCACACTACCCGTTGAATTTTGTACTGCCATCGACAATGATA 61 CCTGGTGCATTGATGCTGGACACAATTTTATTGTTGACGGGTAACGGCTGGTTACAGCA 121 CTGTTAGGTGGTGGATTCTGGGGACTGTTTTTCTACCCCTGGTAACGGCCGATATTTGGC 181 CCAACCCACTTACCGGTCGTAGTAGAAGGCGTATTGTTATCGGTTGCGGACTACACAGGC 241 TTTTATACGTACGTACGGGTACACCGGAATACGTAAGATTGATTGAGCAAGGCTCACTG 301 CGTACCTTTGGTGGTCACACGACGGTAATTGCAGCATTTTTTCTCAGCGTTTGTATCGATG 361 CTGATGTACTGCGTATGGTGGTATTTTGGCAAGATCTATTGCACCGCTTTCTTCTATGTT 421 AAAGGAGAGAGAGGCCGCATTTCTATGAAAAATGACGTAACCGCATTTGGTGAAAAAGGC 481 TTTGCACAGGGGATCAAATAAA </pre>

Table B-3. Continue.

Closely match	Partial <i>amoA</i> sequences
B2-3	>IB-2d-C39 <pre> 1 TGGGGTTTCTACTGGTGGTCACACTACCNGTTGAATTTTGTACTGCCATCGACAGTGATA 61 CCTGGTGCATTGATGCTGGACACAATTTTATTGTTGACGGGTAACGGCTGGTTACAGCA 121 CTGTTAGGTGGTGGATTCTGGGGACTGTTTTTCTACCTGGTAACGGCCGATATTGGC 181 CCAACCCACTTACCGGTCGTAGTAGAAGGCGTATTGTTATCGGTTGCGGACTACACAGGC 241 TTTTATACGTACGTACGGGTACACCGGAATACGTAAGATTGATTGAGCAAGGCTCACTG 301 CGTACCTTTGGTGGTCACACGACGGTAATTGCAGCATTTTCTCAGCGTTTGTATCGATG 361 CTGATGTACTGCGTATGGTGGTATTTGGCAAGATCTATTGCACCGCTTCTTCTATGTT 421 AAAGGAGAGAGAGGCCGCATTTCTATGAAAAATGACGTAACCGCATTTGGTGAAAAAGGC 481 TTTGCACAGGGGATCAAATAAA </pre>

Table B-4. The clone libraries for the industrial bench-scale system closely matched the *amoA* sequences of the W637 [AF353251] group. (*: the typical clones selected for phylogenetic analysis).

Closely match	Partial <i>amoA</i> sequences
W637	>IB-10d-C18* 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATTAACCTTTGTAATTCATCAATCATGATT 61 CCGGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACCTGGTTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTGGGA 181 CCGACTCACTTGCTGTGCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGACTACATGGGA 241 CACCTTTATATCCGTACAGGTACACCTGAATACGTACGCCTGATTGAGCAGGGTTCACCTG 301 CGTACCTTTGGCGGCCATACCACGGTAATTGCAGCATTCTTCTCAGCGTTTCGTATCGATG 361 CTGATGTTTGTGTTTGGTGGTACCTAGGCAAAGTCTATTGCACCGCTTTCTACTATGTT 421 AAAGGTAAGAGAGGTCGTATCGTGCAGAAAGAGATGACGTTACAGCATTGGTGGAAGGAGGC 481 TTTGCCGAGGGG
	>IB-2d-C8* 1 TGGGGTTTCTACTGGTGGTCACACTATCCAATCAACTTTGTTACCCCATCGATCATGATT 61 CCGGGTGCATTGATGTTGGATATCACGTTGTACTTGACCCGTAACCTGGTTGGTAACGGCG 121 CTGATCGGAGGCGGATTCTTTGGTCTGTTATTCTATCCTGGCAACTGGCCGATCTTTGGT 181 CCGACCCATCTGCCGGTAGTAGTGGAAGGCGTATTGTTGTCAGTTGCTGACTACACAGGA 241 TTCCTGTATGTCCGTACAGGTACACCGGAATACGTGAGATTGATTGAACAAGGATCACTG 301 AGGACATTTGGTGGCCATACGACCGTGATAGCGGCATTCTTCTCGGCGTTTGATCCATG 361 CTGATGTTCTGCGTATGGTGGTACTTTGGCAAAATCTATTGCACCGCGTTCTTCTATGTC 421 AAAGGTGAAAGAGGCGCATTACAATGAAGAATGACGTAACGGCATTGGTGGAACAGGA 481 TTTGCCGAGGGGATCAAATAA

Table B-5. The clone libraries for the industrial bench-scale system closely matched the *amoA* sequences of the TA921i [AF339043] group. (*: the typical clones selected for phylogenetic analysis).

Closely match	Partial <i>amoA</i> sequences
TA921i	>IB-2d-C2* 1 TGGGGTTTCTACTGGTGGTCGCACTACCCGATCAACTTTGTACTGCCATCGACCATGATA 61 CCAGGCGCACTGATGCTTGACACGATCATGTTGTTGACCGGCAACTGGTTGGTTACGGCA 121 TTAAGGGTGGCGGATTCTGGGGCTTGTCTTCTATCCTGGCAACTGGCCGATCTTTGGT 181 CCGACCCATCTGCCGGTAGTAGTGGAAGGCGTATTGTTGTCAGTTGCTGACTACACAGGA 241 TTCCTGTATGTCCGTACAGGTACACCGGAATACGTGAGATTGATTGAACAAGGATCACTG 301 AGGACATTTGGTGGCCATACGACCGTGATAGCGGCATTCTTCTCGGCGTTTGATCCATG 361 CTGATGTTCTGCGTATGGTGGTACTTTGGCAAAATCTATTGCACCGCGTTCTTCTATGTC 421 AAAGGTGAAAGAGGCGCATTACAATGAAGAATGACGTAACGGCATTGGTGGAACAGGA 481 TTTCCCAGGGGATCAAATAA
	>IB-2d-C40 1 TGGGGTTTCTACTGGTGGTCGCACTACCCGATCAACTTTGTACTGCCATCGACCATGATA 61 CCAGGCGCACTGATGCTTGACACGATCATGTTGTTGACCGGCAACTGGTTGGTTACGGCA 121 TTAAGGGTGGCGGATTCTGGGGCTTGTCTTCTATCCTGGCAACTGGCCGATCTTTGGT 181 CCGACCCATCTGCCGGTAGTAGTGGAAGGCGTATTGTTGTCAGTTGCTGACTACACAGGA 241 TTCCTGTATGTCCGTACAGGTACACCGGAATACGTGAGATTGATTGAACAAGGATCACTG 301 AGGACATTTGGTGGCCATACGACCGTGATAGCGGCATTCTTCTCGGCGTTTGATCCATG 361 CTGATGTTCTGCGTATGGTGGTACTTTGGCAAAATCTATTGCACCGCGTTCTTCTATGTC 421 AAAGGTGAAAGAGGCGCATTACAATGAAGAATGACGTAACGGCATTGGTGGAACAGGA 481 TTTCCACAGGGGATCAAATAA

Appendix C -- *amoA* gene libraries for the enriched nitrifying system

Table C-1. The clone libraries for the enriched nitrifying system closely matched the *amoA* sequences of the *Nitrosomonas europaea* [AF058691] or ENI-11 [AB179055.1] group.

Closely match	Partial <i>amoA</i> sequences
<i>N. europaea</i>	>EN-C3 <pre> 1 TGGGGTTTCTACTGGTGGTCACACTACCCCATCAACTTCGTCACACCTGGCATTATGCTT 61 CCGGGTGCGCTGATGCTGGATTTCACCATGTATCTGACACGCAACTGGCTGGTAACTGCT 121 CTGGTAGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACGGCCGATCTTTGGA 181 CCGACCCATCTGCCAATCGTTGTAGAAGGAACACTGCTGTCAATGGCCGACTACATGGGC 241 CACCTGTATGTTTCGTACAGGTACACCTGAGTATGTACGTACATTGAACAAGGCTCACTG 301 CGTACCTTCGGTGGTCATACCACGGTTATTGCAGCATTCTTCGCTGCGTTTGTCTCAATG 361 TTGATGTTTACAGTATGGTGGTATCTTGAAAAAGTTACTGTACAGCCTTTTCTACGTT 421 AAAGGTAAGAGGTTCGTATCGTACAACGTAATGATGTTACTGCATTTCGGTGAAGAAGGC 481 TTTCCAGAGGGGA </pre> >EN-C4 <pre> 1 TGGGGTTTCTACTGGTGGTCACACTACCCCATCAACTTCGTCACACCTGGCATTATGCTT 61 CCGGGTGCGCTGATGCTGGATTTCACCATGTATCTGACACGCAACTGGCTGGTAACTGCT 121 CTGGTAGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACGGCCGATCTTTGGA 181 CCGACCCATCTGCCAATCGTTGTAGAAGGAACACTGCTGTCAATGGCCGACTACATGGGC 241 CACCTGTATGTTTCGTACAGGTACACCTGAGTATGTACGTACATTGAACAAGGCTCACTG 301 CGTACCTTCGGTGGTCATACCACGGTTATTGCAGCATTCTTCGCTGCGTTTGTCTCAATG 361 TTGATGTTTACAGTATGGTGGTATCTTGAAAAAGTTACTGTACAGCCTTTTCTACGTT 421 AAAGGTAAGAGGTTCGTATCGTACAACGTAATGATGTTACTGCATTTCGGTGAAGAAGGC 481 TTTCCAGAGGGGA </pre> >EN-C7 <pre> 1 TGGGGTTTCTACTGGTGGTCACACTACCCCATCAACTTCGTCACACCTGGCATTATGCTT 61 CCGGGTGCGCTGATGCTGGATTTCACCATGTATCTGACACGCAACTGGCTGGTAACTGCT 121 CTGGTAGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACGGCCGATCTTTGGT 181 CCGACACATCTGCCAATCGTTCGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGC 241 CATATGTATGTTTCGTACGGGTACACCTGAGTATGTTTCGTATATTGAGCAAGGTTCACTG 301 CGTACCTTTGGTGGACATACCACAGTTATTGCAGCGTTCTTCTCTGCATTTCGTATCAATG 361 TTGATGTTTACGGTATGGTGGTATCTTGAAAAAGTTACTGTACAGCCTTTTCTACGTT 421 AAAGGTAAGAGGTTCGTATCGTACATCGCAATGATGTTACCGCATTCGGTGAAGAAGGCT 481 TTCCAGAGGGGA </pre> >EN-C9 <pre> 1 TGGGGTTTCTACTGGTGGTCACACTATCCTATCAATTTTCGTAACACCGGGCATTATGCTT 61 CCGGGTGCGCTGATGCTGGACTTCACGCTGTATCTGACACGTAACGGCTGGTGACAGCT 121 CTGGTTGGGGGCGGATTCTTTGGTCTGCTGTTCTACCCGGGTAACGGCCGATCTTTGGT 181 CCAACGCATCTGCCAATCGTTATAGAAGGAACACTGCTGTGATGGCTGACTACATGGGC 241 CATATGTATGTTTCGTACGGGTACACCTGAGTATGTTTCGTATATTGAGCAAGGTTCACTG 301 CGTACCTTTGGTGGACATACCACAGTTATTGCAGCGTTCTTCTCTGCATTTCGTATCAATG 361 TTGATGTTTACAGTATGGTGGTATCTTGAAAAAGTTACTGTACAGCCTTTTCTACGTT 421 AAAGGTAAGAGGTTCGTATCGTACAACGTAATGATGTTACTGCATTTCGGTGAAGAAGGC 481 TTTCCGAGGGGA </pre> >EN-C20 <pre> 1 TGGGGTTTCTACTGGTGGTCACACTATCCTATCAATTTTCGTAACACCGGGTATTATGCTT 61 CCAGGTGCGCTGATGCTGGATTTCACCATGTATCTGACACGCAACTGGCTGGTAACTGCT 121 CTGGTAGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACGGCCGATCTTTGGA 181 CCGACCCATCTGCCAATCGTTGTAGAAGGAACACTGCTGTCAATGGCCGACTACATGGGC 241 CACCTGTATGTTTCGTACAGGTACACCTGAGTATGTTTCGTATATTGAGCAAGGTTCACTG 301 CGTACCTTTGGTGGACATACCACAGTTATTGCAGCGTTCTTCTCTGCATTTCGTATCAATG 361 TTGATGTTTACGGTATGGTGGTATCTTGAAAAAGTTACTGTACAGCCTTTTCTACGTT 421 AAAGGTAAGAGGTTCGTATCGTACATCGCAATGATGTTACCGCATTCGGTGAAGAAGGC 481 TTTCCAGAGGGGA </pre>

Table C-1. Continue.

Closely match	Partial <i>amoA</i> sequences
<i>N. europaea</i>	>EN-C23 1 TGGGGTTTCTACTGGTGGTCACACTATCCTATCAATTTTCGTAACACCGGGTATTATGCTT 61 CCAGGTGCGCTGATGCTGGATTTTCACGCTGTATCTGACACGCAACTGGCTGGTGACGGCT 121 CTGGTTGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACCTGGCCGATCCTTGGT 181 CCGACACATCTGCCAATCGTCGTAGAAGGAACACTGTTGTCGATGGCTGTCTACATGGGC 241 CATATGTATGTTTCGTACGGGTACACCTGAGTATGTTTCGTATATTGAGCAAGGTTCACTG 301 CGTACCTTTGGTGGACATACCACAGTTATTGCAGCGTTCTTCTCTGCATTTCGTATCAATG 361 TTGATGTTACAGGTATGGTGGTATCTTGAAAAAGTTTACTGTACAGCCTTTTTCTACGTT 421 AAAGGTAAAAGAGGTGCGATCGTACATCGCAATGATGTTACCGCATTCGGTGAAGAAGGC 481 TTTCCCGAGGGGA
	>EN-C36 1 TGGGGTTTCTACTGGTGGTCACACTATCCTATCAATTTTCGTAACACCGGGTATTATGCTT 61 CCAGGTGCGCTGATGCTGGATTTTCACGCTGTATCTGACACGCAACTGGCTGGTGACGGCT 121 CTGGTTGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACCTGGCCGATCCTTGGT 181 CCGACACATCTGCCAATCGTCGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGC 241 CATATGTATGTTTCGTACGGGTACACCTGAGTATGTTTCGTATATTGAGCAAGGTTCACTG 301 CGTACCTTTGGTGGACATACCACAGTTATTGCAGCGTTCTTCTCTGCATTTCGTATCAATG 361 TTGATGTTACAGGTATGGTGGTATCTTGAAAAAGTTTACTGTACAGCCTTTTTCTACGTT 421 AAAGGTAAAAGAGGTGCGATCGTACATCGCAACGATGTTACCGCATTCGGTGAAGAAGGC 481 TTTCCAGAGGGGA
	>EN-C42 1 TGGGGTTTCTACTGGTGGTCACACTACCCCATCAACTTCGTACACCTGGCATTATGCTT 61 CCGGGTGCGCTGATGCTGGATTTTCACCATGTATCTGACACGCAACTGGCTGGTAACTGCT 121 CTGGTAGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACCTGGCCGATCCTTGGG 181 CCGACCCATCTGCCAATCGTTGTAGAAGGAACACTGCTGTCAATGGCCGACTACATGGGC 241 CACCTGTATGTTTCGTACAGGTACACCTGAGTATGTACGTACATTGAACAAGGCTCACTG 301 CGTACCTTCGGTGGTCATACCACGGTTATTGCAGCATTCTTCGCTGCGTTTGTCTCAATG 361 TTGATGTTACAGTATGGTGGTATCTTGAAAAAGTTTACTGTACAGCCTTTTTCTACGTT 421 AAAGGTAAAAGAGGTGCGTATCGTACAACGTAATGATGTTACTGCATTTCGGTGAAGAAGGC 481 TTTCCCGAGGGGA

**Appendix D -- Real-time PCR calibration curves for the RI-27, B2-3,
and *N. europaea amoA* quantification**

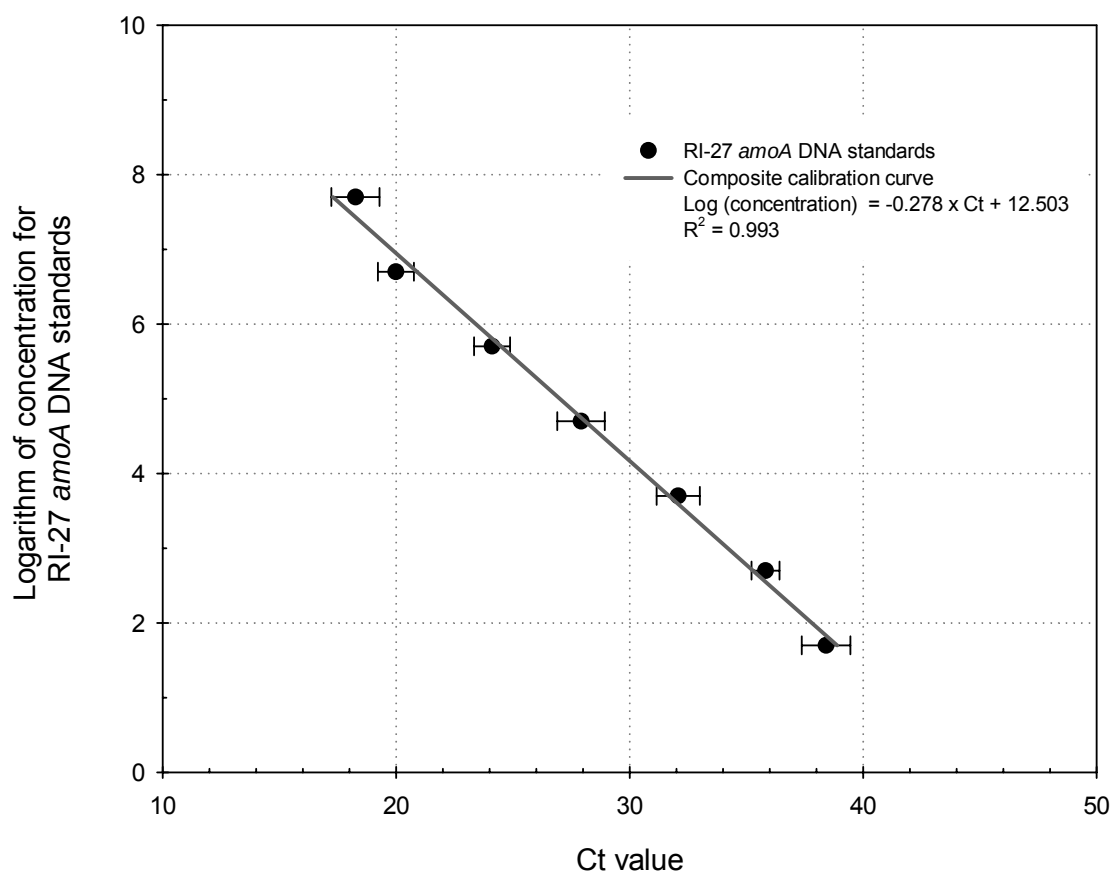


Figure D-1. Composite calibration curve for RI-27 *amoA* DNA real-time PCR quantification (generated from results of four PCR runs).

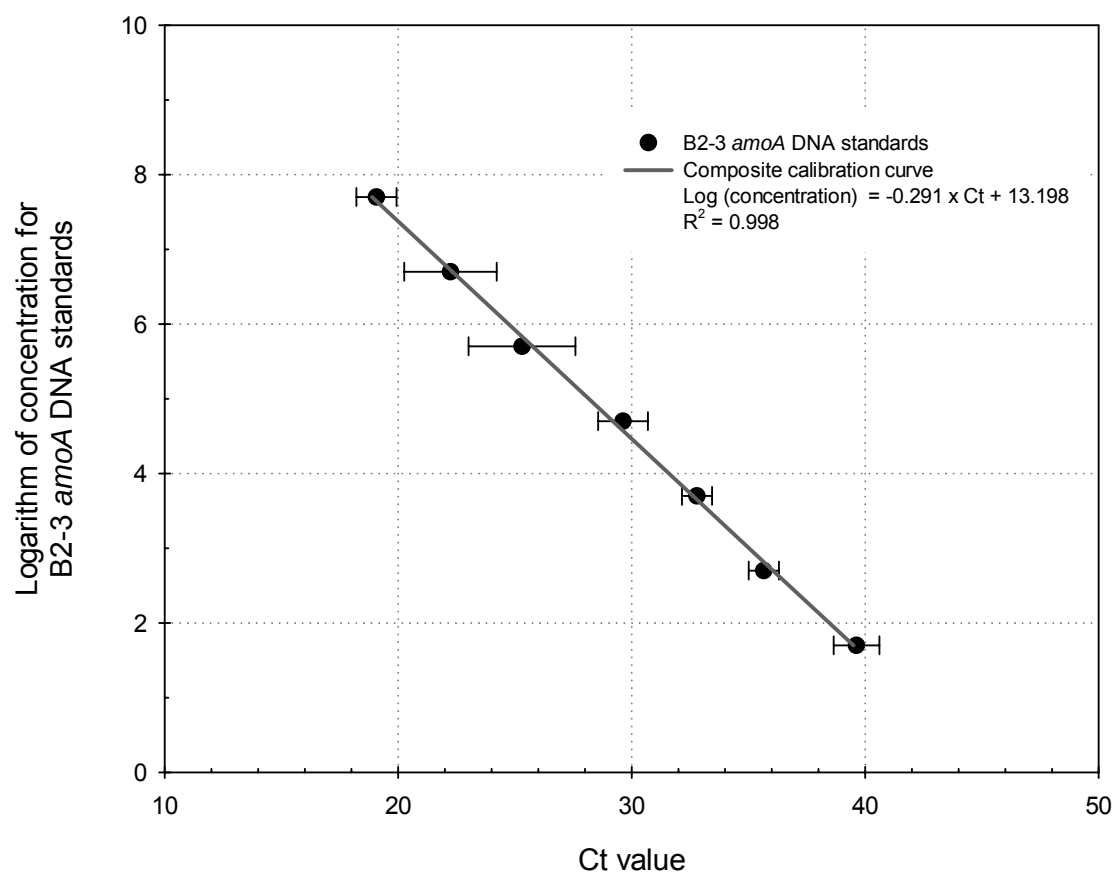


Figure D-2. Composite calibration curve for B2-3 *amoA* DNA real-time PCR quantification (generated from results of three PCR runs).

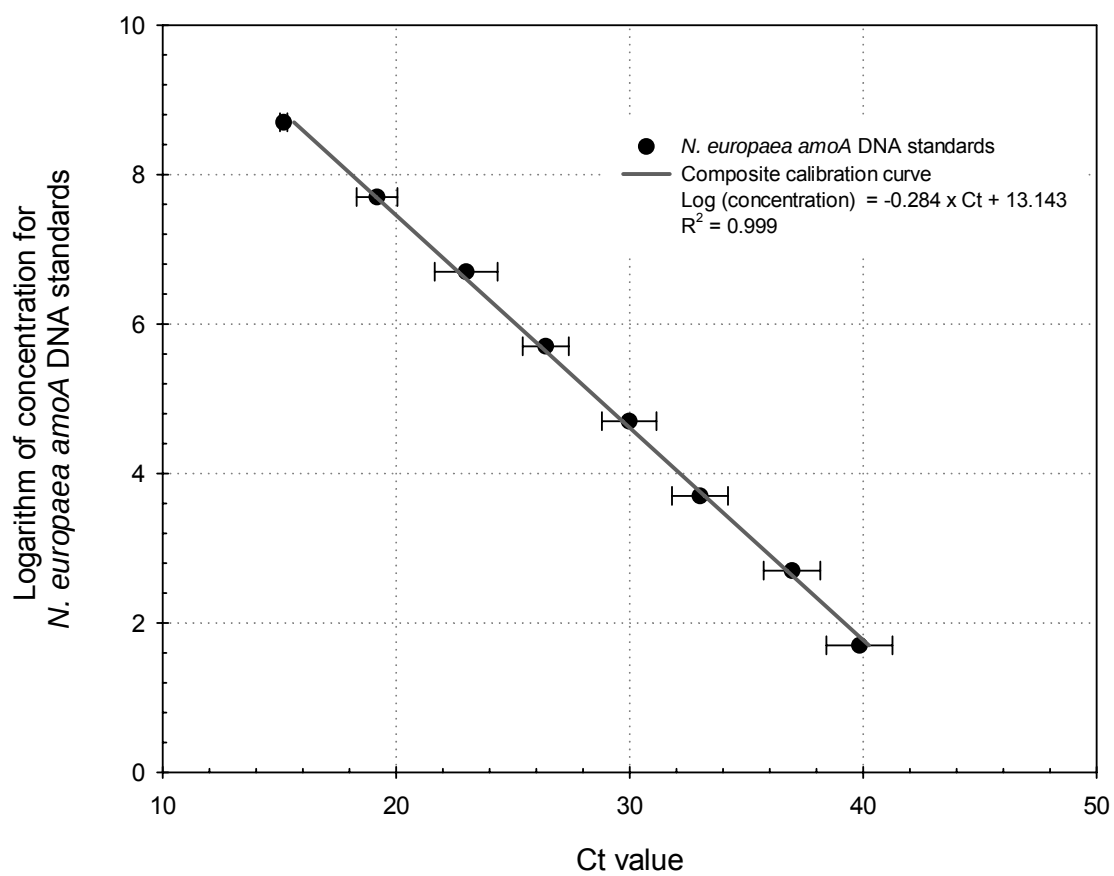


Figure D-3. Composite calibration curve for *Nitrosomonas europaea amoA* DNA real-time PCR quantification (generated from results of ten PCR runs).

Vita

Hsion-Wen, David Kuo was born in Chiayi, Taiwan on February 11th, 1971. He received a B.S. and a M.S. degree in Environmental Engineering from the National Cheng-Kung University (NCKU), Taiwan in 1994 and 1996, respectively. His master study was focused on industrial wastewater treatment using anaerobic bioreactor. After two-year compulsory military service, he worked one year as research assistant for his former advisor at NCKU prior to his graduate education in the United States.

He enrolled the graduate program at the University of Tennessee-Knoxville in January, 2001 (after studied one and a half years as non-degree student at the University of Tennessee-Martin to enforce his background in molecular biology). He completed the requirements for a Doctor of Philosophy degree in Civil Engineering in August, 2006. After graduation, he planned to serve as a post-doctoral researcher for 2-3 years before go back to Taiwan to continue his career in environmental protection.