CHAPTER II

LITERATURE REVIEW

2.1 Microbial nitrification

Nitrification is central to the global nitrogen cycle and involves the oxidation of ammonia to nitrate (through nitrite) by two physiologically distinct groups of organisms: autotrophic ammonia- and nitrite-oxidizers. Ammonia-oxidizing organisms convert ammonia to nitrite through hydroxylamine using ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). Autotrophic nitrite oxidizers subsequently use the enzyme nitrite oxidoreductase (NOR) to convert nitrite to nitrate, which can be assimilated or subjected to denitrification processes. In anaerobic environments, ammonia can be converted to molecular nitrogen by the anammox process by several enzymatic steps (represented by dashed arrows).

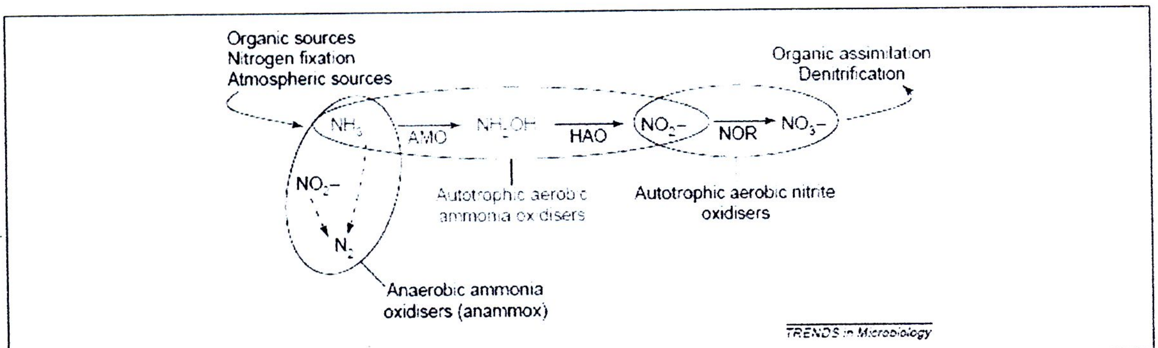

Figure 2.1 Autotrophic ammonia oxidation during nitrification (Nicol *et al.*, 2006).

The ammonia oxidation is the rate-limiting step of nitrification. In the soil environment, it can lead to substantial amounts of net nitrogen loss through subsequent denitrification or leaching of nitrate. In the marine environment, ammonia oxidation is an important component of nitrogen mineralization from organic sources and the removal of anthropogenic nitrogen inputs in coastal waters. Ammonia oxidation is also a key step in the removal of nitrogen during wastewater treatment (Prosser *et al.*, 2002 and Kowalchuk *et al.*, 2001).

Ammonia oxidation is considered to be carried out largely by autotrophic ammonia-oxidising bacteria (AOB) that form two distinct monophyletic groups within the α- and β- proteobacteria. To date, most cultured strains belong to the β-subgroup (Kowalchuk *et al.*, 2001) and β-proteobacterial AOB are believed to be the dominant ammonia oxidizers in most environments. The same as AOB, Cultivation-independent molecular methods show that members of the kingdom Crenarchaeota within the domain Archaea represent a substantial component of microbial communities in aquatic and terrestrial environments. In 2004, metagenomic studies have revealed that such Crenarchaeota contain ammonia monooxygenases genes which related to those of bacterial. Furthermore, the first marine chemolithoautotrophic strain was isolated that uses ammonia as a sole energy source (Konneke *et al.*, 2005).

It is questionable from these recent discoveries that who is the important organisms playing the key role in nitrification process. In most environments, autotrophic AOB are considered to be the most important organisms that responsible for ammonia oxidation. However, in most mesophilic environments, Crenarchaeota are more abundant than AOB populations. Wuchter *et al.* (2006) and Leininger *et al.* (2006) have found that most mesophilic Crenarchaeota are AOA, and that these organisms are the numerically dominant ammonia oxidizers in the ocean and in soils respectively.

2.2 Ammonia-oxidizing bacteria (AOB)

The lithoautotrophic ammonia-oxidizing bacteria (AOB) are well defined by their fundamental metabolism. Ammonia serves as the sole energy source and carbon dioxide is used as the carbon need. Together with the lithotrophic nitrite-oxidizing bacteria (NOB), the AOB catalyze the nitrification process ($NH_3 \rightarrow NO_2 \rightarrow NO_3$), which has a key step in natural nitrogen cycling.

2.2.1 Phylogeny of ammonia-oxidizing bacteria

2.2.1.1 16S rRNA-based phylogenetic tree

The first phylogenetic analyses of AOB were carried out by Woese and co-workers in the 1980s (Woese *et al.*, 1984; Woese *et al.*, 1985) and demonstrated that two phylogenetically distinct groups of AOB exist. The major group, containing the genera *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus*, belongs to the class Betaproteobacteria, while the second group of AOB, represented by two species of the genus *Nitrosococcus*, is affiliated with the class Gammaproteobacteria.

The genera *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* are closely related, and 16S rRNA phylogeny provides no convincing support for subdivision of this lineage into three genera, although morphological and ecophysiological differences exist between the different genera. It has therefore been suggested these genera be lumped into the single genus *Nitrosospira* (Head *et al.*, 1993). In contrast, the cultured nitrosomonads can be subdivided into six lineages which are consistently retrieved using different treeing methods and which have parsimony bootstrap support of above 90%. This phylogenetic substructure is also retrieved if all betaproteobacterial AOB isolates (56 nitrosomonads and 48 nitrosospiras), for which a 16S rRNA sequence longer than 1000 bases has been determined, are included in the treeing analysis (Figure 2.2 and 2.3).

2.2.1.2 AmoA-based phylogenetic tree

Recently, the *amoA* gene coding for the active site polypeptide of the ammonia monooxygenase has been used as an additional phylogenetic marker molecule for AOB (McTavish *et al.*, 1993; Klotz and Norton, 1995; Rotthauwe *et al.*, 1995; Suwa *et al.*, 1997; Hommes *et al.*, 1998; Alzerreca *et al.*, 1999; Yamagata *et al.*, 1999; Horz *et al.*, 2000; Purkhold *et al.*, 2000; Aakra *et al.*, 2001a; Casciotti and Ward, 2001; Purkhold *et al.*, 2003). PCR primers that allow amplification of a 453-bp fragment of this gene are generally used in these studies (Rotthauwe *et al.*, 1997; modified by Stephen *et al.*, 1999). Phylogeny inference based on the deduced amino acid sequence of the *amoA* gene fragment is overall consistent with the 16S rRNA phylogeny of AOB. Members of the genera *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* form a tight monophyletic grouping with no obvious substructure. Within the nitrosomonads the *N. europaea/Nc. mobilis* lineage and the *N. marina* lineage are also found with all treeing methods, while the *N. communis* and the *N. oligotropha* lineage are not always monophyletic (Figure 2.4). If *amoA* nucleic acid sequences are used for phylogenetic analysis, basically the same picture emerges with the exception that the *N. europaea/eutropha* lineage is no longer monophyletic.

If compared to the 16S rRNA-based phylogeny of AOB, AmoA analysis does provide less resolution, reflecting that a relatively short (151 positions) and highly conserved (93 positions have an identical amino acid in at least 98% of the betaproteobacterial AOB) amino acid sequence stretch is used as marker. The information content of AmoA sequences could be significantly extended in future studies by the development of primers that allow the amplification of more complete *amoA* gene fragments. First attempts in this direction were recently published by Norton *et al.*, 2002.

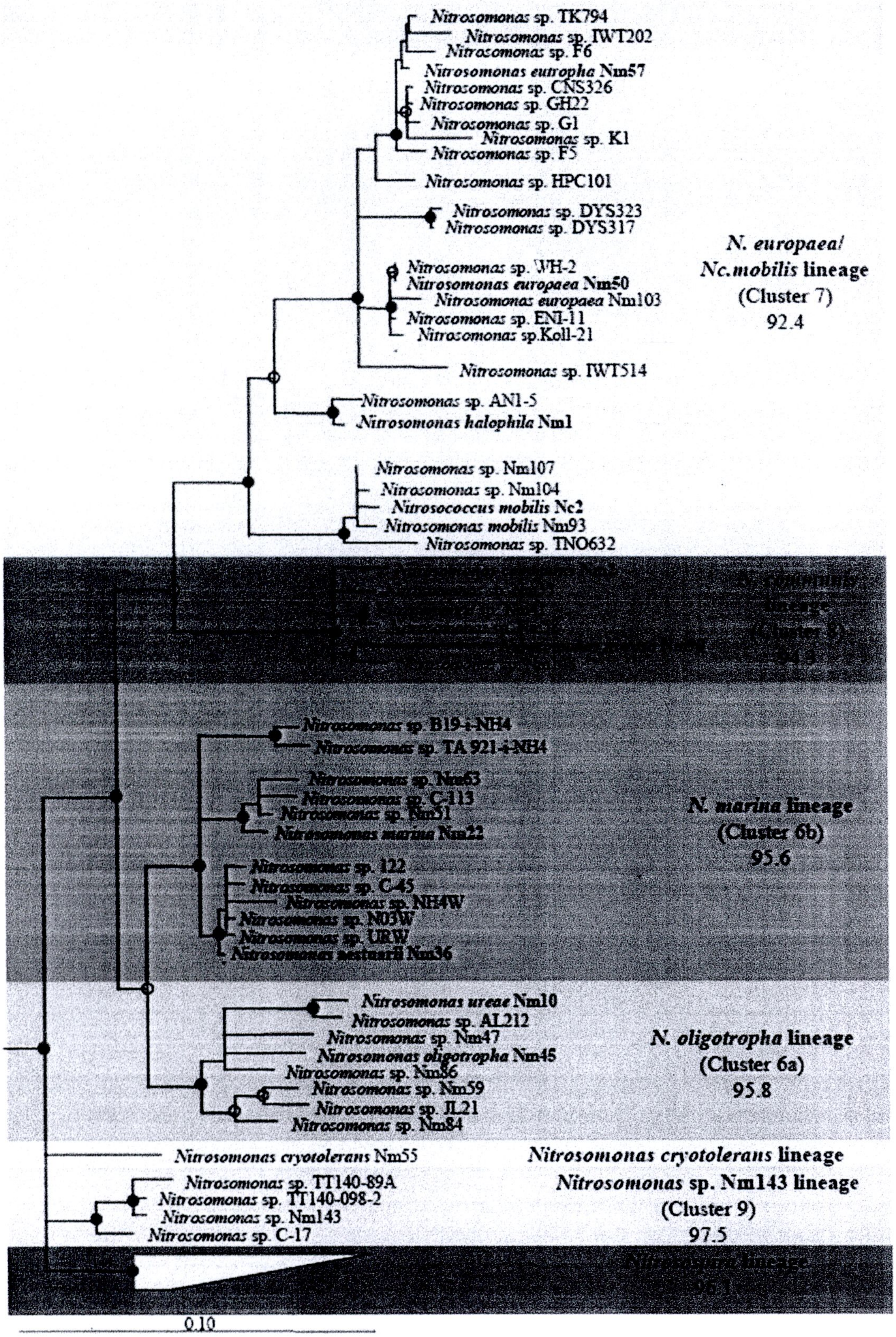

Figure 2.2 16S rRNA based phylogenetic tree of the nitrosomonads. (Koops *et al.*, 2003)

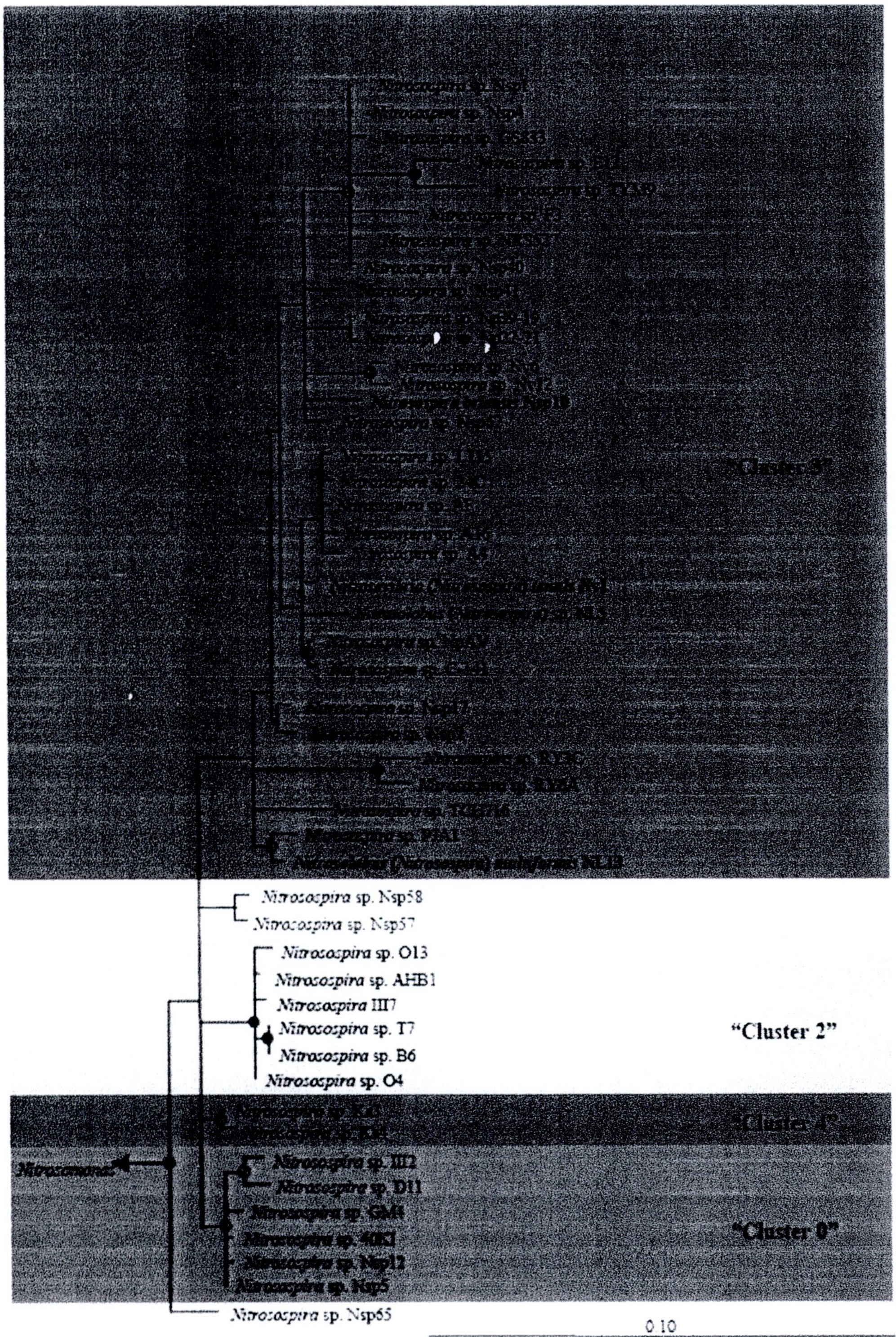

Figure 2.3 16S rRNA-based phylogenetic tree of the highly related genera *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio*. (Koops *et al.,* 2003)

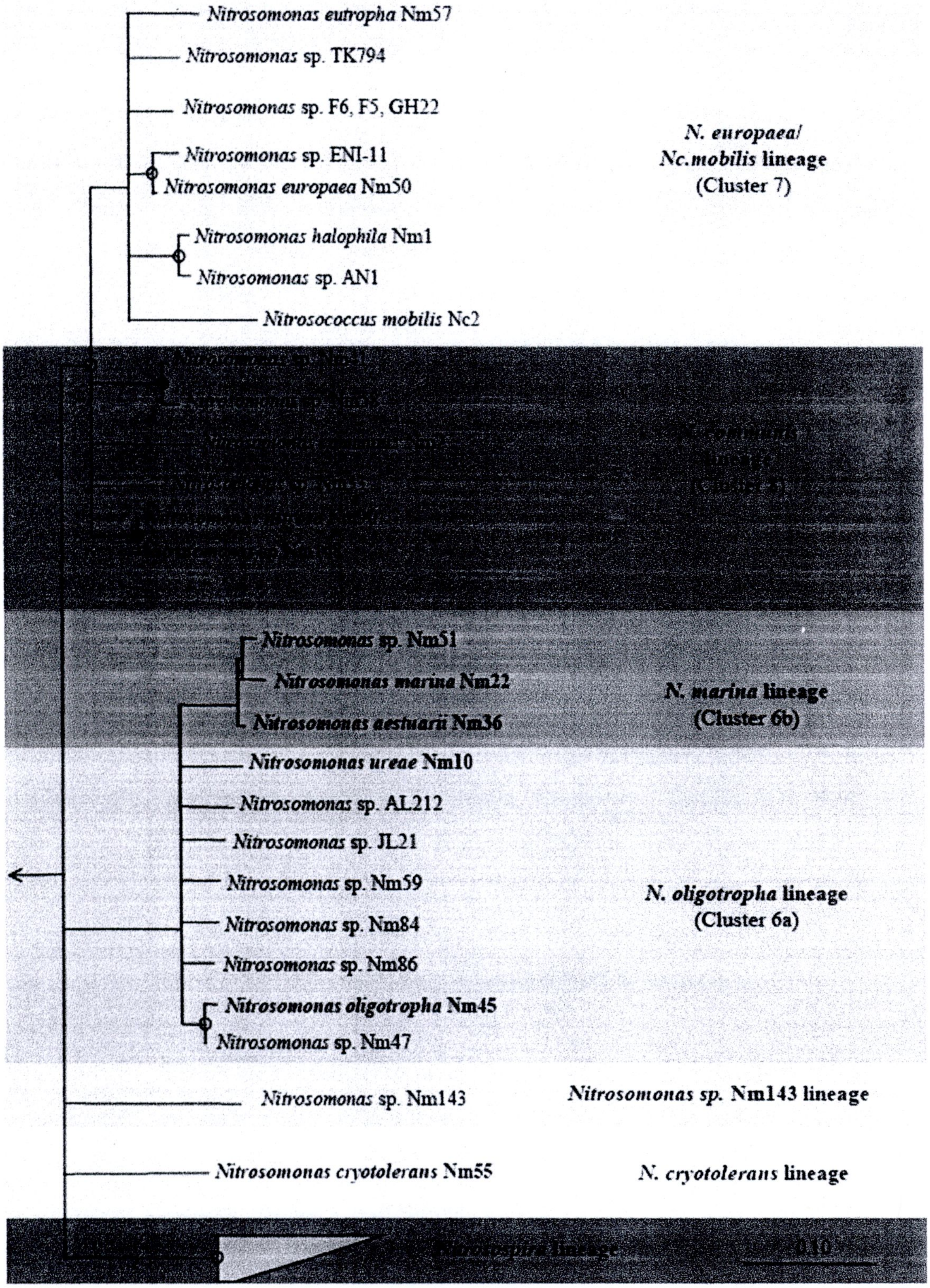

Figure 2.4 AmoA-based phylogenetic tree of the betaproteobacterial AOB. (Koops *et al.,* 2003)

2.2.2 Factors influencing communities of ammonia-oxidizing bacteria

2.2.2.1 Ammonia concentration

The distribution pattern of AOB in nature is coupled to geological, biological and anthropogenic sources of reduced nitrogen because ammonia is the essential energy source for these organisms. Consequently, they have adapted to a broad range of different ammonia concentrations in the diverse environments, reflected by different affinity constants for ammonia. This is one of the most important factors influencing the distribution patterns of AOB in nature (Suwa *et al.*, 1994; Suwa *et al.*, 1997; Stehr *et al.*, 1995a; Koops and Pommerening-Roser, 2001).

2.2.2.2 The tolerance of increasing of ammonia concentrations

Due to ammonia is a toxic compound. The tolerance of increasing ammonia concentrations (Table 2.1) is another aspect affecting the distribution patterns of AOB (Bollmann and Laanbroek, 2001).

2.2.2.3 Urease activity

The presence or absence of urease activity was observed to be of ecophysiological relevance for AOB. This property is of special importance in acidic environments, where free ammonia is missing as substrate because it is nearly quantitatively ionized to ammonium. Under such conditions, only those AOB species that can use urea as an alternative ammonia source can survive (De Boer and Laanbroek, 1989; De Boer *et al.*, 1991; Jiang and Bakken, 1999; Burton and Prosser, 2001). Possession of urease seems also to be an essential property for AOB that successfully colonize oligotrophic soils or aquatic environments.

2.2.2.4 Different salt requirements, salt tolerances and salt sensitivities

Distribution of AOB in nature also is affected by different salt requirements, salt tolerances and salt sensitivities of the distinct species (Table 2.1). This is of special importance for their distribution patterns in aquatic systems (such as rivers, lakes, estuaries, marine environments and salt lakes) that significantly differ in salinity (Koops *et al.*, 1990; Koops *et al.*, 1991).

Table 2.1 Characteristics and preferred habitats of described species of the ammonia-oxidizing bacteria (Koops *et al.*, 2003).

Species	G+C (mol%)	Carboxy-somes	Urease activity	Substrate (NH_3) affinity (K_s in μM)	Maximum ammonia tolerance NH_4Cl (in mM; pH 8.0)	Salt requirement	Maximum salt tolerance (in mM)	Preferred habitats
Nitrosomonas europaea	50.6–51.4	–	–	30–61	400	–	400	Sewage disposal plants, eutrophic freshwater and brackish water
Nitrosomonas eutropha	47.9–48.5	+	–		600	–	400	
Nitrosomonas halophila	53.8	+	–		400	–	900	
Nitrosococcus mobilis	49.3	–	–		250	–	500	
Nitrosomonas communis	45.6–46.0	–	–	14–43	250	–	250	Soils (not acid) and eutrophic freshwater
Nitrosomonas nitrosa	47.9	+	–	19–46	100	–	300	
Nitrosomonas ureae	45.6–46.0	–	–	1.9–4.2	200	–	200	Oligotrophic freshwater and natural soils
Nitrosomonas oligotropha	49.4–50.0	–	+		50	–	150	
Nitrosomonas marina	47.4–48.0	–	–	50–52	200	–	800	Marine environments
Nitrosomonas aestuarii	45.7–46.3	–	–		400	–	600	
Nitrosomonas cryotolerans	45.5–46.1	–	–	42–59	400	–	550	Marine environments
Nitrosolobus multiformis	53.5	ND	– –	ND	50	–	200	Soils (not acid)
Nitrosovibrio tenuis	53.9	ND	– –	ND	100	–	100	Soils, rocks and freshwater
Nitrosospira briensis	54	ND	– –	ND	200	–	250	Soils, rocks and freshwater
Nitrosococcus oceani	50–51	ND	–	ND	1000	–	1100	Marine environments
Nitrosococcus halophilus	50–51	ND	–	ND	500	–	1800	Marine environments and salt lakes

Symbols and Abbreviations: +, present; –, not present; – –, present in some strains; and ND, no data.

2.2.2.5 Different oxygen concentration

The AOB are aerobes. Although their oxygen affinity constants are relatively high (Painter, 1986), AOB can also survive at extremely low oxygen concentrations (Goreau *et al.*, 1980). Even ammonia oxidation under anaerobic conditions is being discussed (Schmidt and Bock, 1997; Schmidt and Bock, 1998; Zart *et al.*, 2000). However, specific selection of distinct AOB species by different oxygen concentrations has not yet been reported in the literature.

2.2.2.6 Temperature

Temperature also may be of importance for distribution patterns of AOB in nature. This has been revealed in some publications (Golovacheva *et al.*, 1976; Jones *et al.*, 1988; Jiang and Bakken, 1999). Beside environments characterized by constant high or low temperatures, such as hot springs or permafrost soils, environments showing pronounced temperature changes, such as rock surfaces, might harbor interesting AOB. However, it is not enough information for now to allow general conclusions on their AOB diversity.

2.2.3 Dominant populations of ammonia-oxidizing bacteria in wastewater treatment plant

Urea and ammonia are the most frequently found nitrogen compounds in sewage. In wastewater treatment plants, AOB oxidize ammonia to nitrite, which is subsequently converted to nitrate by the NOB. Nitrate is then removed from the sewage by denitrifying bacteria via anaerobic respiration. The slow growth rate of AOB and their susceptibility to pH and temperature swings as well as to several sewage compounds is responsible for frequent failure of the nitrification in municipal and industrial WWTPs.

2.2.3.1 Municipal wastewater treatment plant

Isolation techniques indicate that in standard municipal WWTPs, *N. eutropha* seems to be the dominant representative (Watson and Mandel, 1971b; Koops and Harms, 1985), but *N. europaea* and *Nc. mobilis* have also been repeatedly cultivated (Juretschko *et al.*, 1998; Koops and Pommerening- Roser, 2001). All these species belong to the same *Nitrosomonas* lineage (Figure 2.2)

2.2.3.2 Industrial wastewater treatment plant

In industrial WWTPs, the cultivation of representatives of members of the *N. oligotropha* lineage as well as *N. nitrosa* has been regularly reported (Koops and Harms, 1985; Suwa *et al.*, 1997). In laboratory experiments, a remarkable high tolerance of members of the *N. oligotropha* lineage to heavy metals was observed, and the production of significant amounts of exopolymeric materials by these species was suggested to be the major reason for this tolerance (Stehr *et al.*, 1995b). This resistance to heavy metals may be responsible for the presence of members of this lineage in special WWTPs.

2.2.4 Co-metabolism of organic compounds by ammonia-oxidizing bacteria

AOB, which is obligate chemolithotrophic aerobe using ammonia as a sole energy source, is widely for the oxidation of hydrocarbon substrates through the action of ammonia monooxgenase (AMO) (Arciero *et* al., 1989). During oxidation of ammonia to nitrite, AMO catalyzes the oxidation of ammonia to hydroxylamine. Subsequently, hydroxylamine is oxidized to nitrite by hydroxylamine oxidoreductase

(HAO). During the last process four electrons are released. Two of four electrons transfer to AMO in order to activate oxygen and maintain steady-state rate of ammonia oxidation. The rest two electrons are used in another oxidation reaction which is called co-metabolism (Arciero *et al.*, 1989; William and Daniel, 1993). Currently, many hydrocarbons and halogenated hydrocarbons which are able to be degraded by co-metabolism of AOB such as in Figure 2.5 show ethylene is degraded by co-metabolism of AOB.

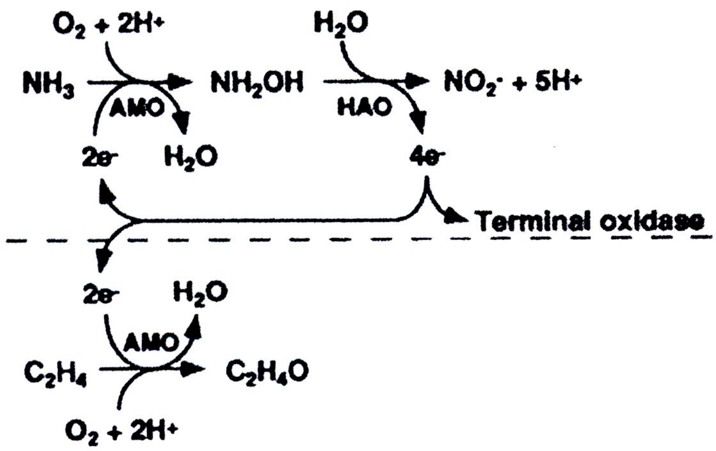

Figure 2.5 Co-metabolism of ethylene by AOB
(William and Daniel, 1993)

In mixed culture, in batch experiments with nitrifying activated sludge (NAS), 0.050 mg/l of EE2 was degraded completely within 6 days by oxidizing ammonium at rate of 50 mg NH_4^+/gDW/ hr and degrading EE2 at maximum rate of 1 µg/gDW/hr (Vader *et al.*, 2000). Furthermore, in initial concentration of 1 mgL^{-1} of estrogen were degraded with NAS by the degradation rate of 0.056 hr^{-1} for E1, 1.3 hr^{-1} for E2, 0.030 hr^{-1} for E3, and 0.035 hr^{-1} for EE2. By using inhibitor for ammonia monooxygenase, the key enzyme for ammonia oxidation by AOB confirmed that NAS significantly degrade E1, E2, E3 and EE2. In NAS, E1, E2 and E3 were degraded by heterotrophic bacteria whereas EE2 was degraded by AOB (Shi *et al.*, 2004). As for pure culture, ammonia-oxidizing bacteria (AOB), *Nitrosomonas europaea*, degraded 0.4 mg/l estrogens with constant biodegradation rates of 0.0022 mg/l/hr for E1, 0.0020 mg/l/hr for E2, 0.0016 mg/l/hr for E3 and 0.0019 mg/l/hr for EE2. Corresponding ammonia consumption rates were 1.5 $mgNH4^{+}$-N/l/hr for E1, 1.45 $mgNH4^{+}$-N/l/hr for E2, 1.35 $mgNH4^{+}$-N/l/hr for E3 and 1.55 $mgNH4^{+}$-N/l/hr for EE2 (Shi *et al.*, 2004).

A variety of biological treatment technologies for the destruction of chlorinated hydrocarbons, such as TCE, are now under development. Microorganisms that can grow on TCE as a sole carbon or energy source have not yet been isolated. However, several physiologically diverse types of bacteria can cometabolically dechlorinate and then partially or fully degrade TCE. For instance, Vogel and McCarty (1985) found that TCE could be cometabolically transformed when used as a nongrowth-supporting electron acceptor by methanogens under anaerobic conditions, while Wilson and Wilson (1985) found that methanotrophic bacteria could cometabolize TCE aerobically by means of the enzyme methane monooxygenase (MMO) when methane was supplied as a primary substrate. Recently, several studies have indicated that chlorinated aliphatic compounds, including TCE, can be cometabolized by nitrifying bacteria (*Nitrosomonas europaea*) (Ely *et al.*, 1995; Arciero *et al.*, 1989; Hyman *et al.*, 1995). It was found that the enzyme ammonia monooxygenase (AMO) wasinvolved in these reactions. Usually, aerobic cometabolic processes are preferred overanaerobic ones because TCE can be mineralized to CO_2 , H_2 O and Cl^- without the accumulation of stable and carcinogenic intermediates such as vinyl chloride (Vogel *et al.*, 1985). However, due to the low solubility of methane gas, it is difficult to handle this primary substrate for methanotrophs in cometabolism of TCE. Thus, using the more soluble of ammoniarammonium instead of methane as a primary substrate for nitrifying bacteria may increase the operation efficiencies in in situ bioremediation of TCE.

2.3 Ammonia-oxidizing archaea (AOA)

Cultivation-independent molecular surveys show that members of the kingdom Crenarchaeota within the domain Archaea represent a substantial component of microbial communities in aquatic and terrestrial environments. In 2004, metagenomic study of the Sargasso Sea by shotgun DNA sequencing, Venter *et al.* found the presence of an ammonia monooxygenase gene (*amo*A–like gene) on an archaeal-associated scaffold and indicated the potential role of archaea in nitrification processes of the ocean. After that a search for homologues of the *amoA* and *amo*B-like genes of the soil clone revealed several highly similar genes in the Sargasso Sea dataset, which enabled these marine genes to be assigned also to Crenarchaeota. This is the first link between *amo*-like genes and mesophilic Crenarchaeota. This evidence also led to the hypothesis that non-thermophilic Crenarchaeota of soil could be ammonia oxidizers (Schleper *et al.*, 2005). Furthermore, Treusch *et al.* (2005) demonstrated that incubation of soil in the presence of ammonia resulted in a significant increase of crenarchaeal amoA expression compared with controls incubated without ammonia. These experiments suggested that the identified genes encoded an ammonia monooxygenase. The ultimate confirmation of AOA activity was achieved by cultivation of a mesophilic crenarchaeote from a marine aquarium in Seattle, USA (Konneke *et al.*, 2005). *Nitrosopumilus maritimus* is phylogenetically placed within the 'marine' group 1.1a lineage (Figure 2.6). It grows chemolithoautotrophically, using ammonia as a sole energy source, and seems to grow at similar rates and densities as cultured AOB with near-stoichiometric conversion of ammonia to nitrite (Konneke *et al.*, 2005). With primers designed from the *amo* gene sequences obtained in the metagenomic studies of Treusch *et al.* (2005) and Venter *et al.* (2004), the authors amplified highly similar *amo*ABC-like genes from *N. maritimus*, which again suggested that these genes encode the key metabolic enzyme of AOA. The first molecular evidence demonstrate the archaeal *amo*A gene to be pervasive in areas of the ocean that are critical for the global nitrogen cycle – including the base of the euphotic zone, suboxic water columns and coastal/estuarine sediments (Francis *et al.*, 2005). They use specific PCR primers specifically targeting the archael *amo*A to investigate the distribution and diversity of AOA in water columns and sediments of the ocean. For the first time, these data indicated that many marine Crenarchaeota might be capable of ammonia oxidation. The archaeal amoA sequences revealed diverse and distinct AOA communities associated with different habitats and sampling sites, with little overlap between water columns and sediments. There are two evidences, showing that AOA are more abundant than AOB in marine and soil environments. In the North Sea, the archaeal amoA abundance was 1–2 orders of magnitude higher than those of bacterial nitrifiers; and , in the North Atlantic, crenarchaeotal amoA copy numbers are also 1–3 orders of magnitude higher than those of bacterial amoA (Wuchter *et al.*, 2006). Leininger *et al.* (2006)

demonstrated that *amo*A gene copies of Crenarchaeota were up to 3,000-fold more abundant than bacterial *amo*A genes. High amounts of crenarchaeota-specific lipids, including crenarchaeol, correlated with the abundance of archaeal *amo*A gene copies. Reverse transcription quantitative PCR studies and complementary DNA analysis using novel cloning-independent pyrosequencing technology demonstrated the activity of the archaea in situ and supported the numerical dominance of archaeal over bacterial ammonia oxidizers. They assume that Crenarchaeota may be the most abundant ammonia-oxidizing organisms in soil ecosystems on earth. Moreover, AOA have also recently been detected in nitrifying wastewater treatment bioreactors used to remove ammonia from wastewater by using PCR primers targeting archaeal *amo*A gene (Park *et al.*, 2006). All of these sequences showed similarity to sequences previously found in soil and sediments. AOA were distributed primarily in four major phylogenetic clusters (A, B, C, and D) This study clearly demonstrates the presence of molecular markers for AOA, including an archaeal amoA cluster (cluster D) that may be widespread in activated sludge bioreactors.

2.3.1 Phylogeny of AOA

Phylogenetic tree showed major non-thermophilic and cultivated (hyper)thermophilic lineages within the kingdom Crenarchaeota. Lineage descriptions follow those of Schleper *et al.* (Schleper *et al.*, 2005). Two lineages with representatives that have crenarchaeal AMO genes are highlighted from marine and soil environments. Pairwise distances (with LogDet–Paralinear correction) of unambiguously aligned positions were calculated using variable sites only (estimated from a maximum-likelihood model). Bootstrap support was calculated using maximum likelihood, distance and parsimony methods (100, 1000 and 1000 replicates, respectively) with values at major nodes representing the most conservative value from all three methods (expressed as a percentage). Multifurcation indicates where the relative branching order of major lineages could not be determined in the majority of bootstrap replicates with all methods. The scale bar represents an estimated 0.05 changes per nucleotide position (Nicol *et al.*, 2006).

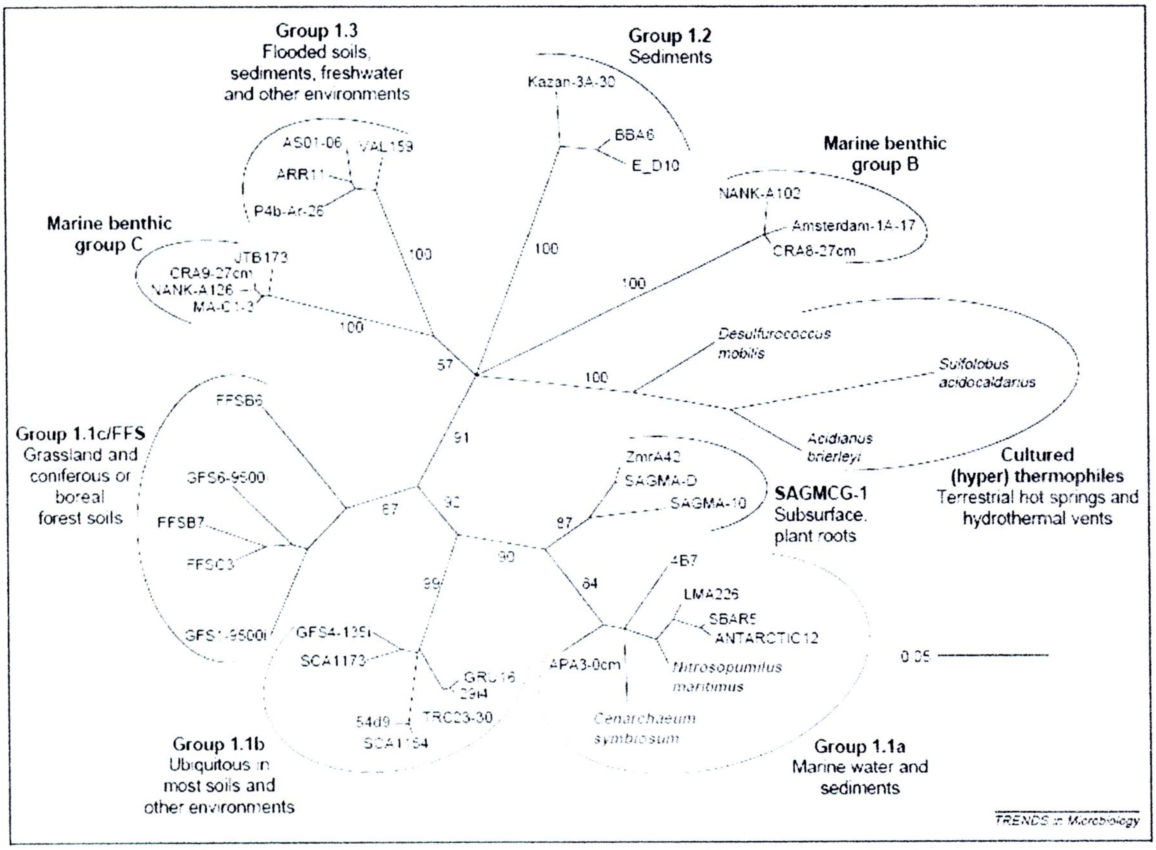

Figure 2.6 Phylogenetic tree of AOA (Nicol *et al.*, 2006)

2.3.2 Factors influencing communities of ammonia-oxidizing archaea

2.3.2.1 Ammonium concentration

A pure culture of Crenarchaeota grew to a maximal density of 1.4 x10^7 cells/ml at 28 °C in defined medium containing 500 μM ammonium, with a minimum generation time of 21 h. This cell density is approximately three orders of magnitude greater than that observed for marine Crenarchaeota in natural bacterioplankton samples. Ammonium typically reaches concentrations of < 0.03–1μM in the open ocean and < 0.03–100 μM in coastal waters (Konneke *et al.*, 2005). The maximum growth rate of Crenarchaeota in culture (0.78 d^{-1}) was higher than the range of rates estimated for natural bacterioplankton communities, which vary between 0.05 and 0.3 d^{-1}. Moreover, Francis *et al.* found that NH_4^+ might also be expected to influence AOA community structure. Coastal permeable sediments from Huntington Beach are characterized by consistently elevated NH_4^+ concentrations caused by hydrologic connection with groundwater, which is highly enriched in NH_4^+ (groundwater [NH_4^+] >150 μM). The archaeal *amoA* library from this site was the most diverse in his study.

2.3.2.2 Organic material

The addition of organic compounds, even in very low concentrations, appeared to inhibit the growth of Crenarchaeota in culture (Konneke *et al.*, 2005). Thus, organic material excreted by other organisms (for example, phototrophic primary producers) and a low concentration of ammonium may limit the abundance of marine Crenarchaeota in the environment.

2.3.2.3 Habitat/geographic location

Using PCR primers designed to specifically target archaeal *amoA*, Francis *et al.* find AOA to be pervasive in areas of the ocean that are critical for the global nitrogen cycle, including the base of the euphotic zone, suboxic water columns, and estuarine and coastal sediments. Diverse and distinct AOA communities are associated with each of these habitats, with little overlap between water columns and sediments. Within marine sediments, most AOA sequences are unique to individual sampling locations, whereas a small number of sequences are evidently cosmopolitan in distribution (Francis *et al.*, 2005).

2.3.2.4 Salinity

Differences in AOA community composition in San Francisco Bay, the largest estuary on the west coast of the United States, are likely associated with salinity (Francis *et al.*, 2005). Sequences from the 30.5-practical salinity units (psu) Central San Francisco Bay site were distributed throughout a number of different regions of the tree. While all of the low-salinity (0.5 psu) North San Francisco Bay sequences fell exclusively into one distinct phylogenetic cluster. From these result, they assumed that more salinity were responsible for more diversity of AOA.

2.3.2.5 Season

PCR amplification of archaeal 16S rRNA gene by using a general archaeal primer followed by phylogenetic analysis of sequenced denaturing gradient gel electrophoresis (DGGE) fragments revealed that Crenarchaeota dominated the archaeal community from late fall to early spring (Wuchter *et al.*, 2006). In December, abundances of Crenarchaeota decreased considerably, for reasons presently unclear, whereas ammonium levels remained relatively constant at ~9μM.

While in early January, crenarchaeotal abundance again increased substantially by 1 order of magnitude, coinciding with a decrease in ammonia levels from 10 to 3 μM.

2.3.2.6 Depth of soil

To analyse the distribution of AOA and AOB at different depths, Leininger *et al.* investigated an agricultural soil, which has been treated with different amounts and qualities of fertilizers for more than 100 years, and a natural, pristine calcareous grassland soil site. Bacterial amoA genes in the former declined significantly with depth in the unfertilized and inorganically fertilized sites while archaeal amoA genes stayed high, resulting in a maximal AOA to AOB ratio of 3,000 (Leininger *et al.*, 2006).

2.3.2.7 Bioavailability of nitrogen and carbon

Both archaeal and bacterial amoA copy numbers varied little with depth at the site treated additionally with manure and these higher levels of archaeal and bacterial amoA were associated with the highest bioavailability of nitrogen and carbon (Leininger *et al.*, 2006).

2.3.2.8 Operational system and retention time of wastewater treatment system

All of the PCR-positive samples were collected from WWTPs operating with aerated-anoxic processes (i.e., the Orbal and VLRprocesses), in which extremely low DO concentrations are maintained, enabling simultaneous nitrification and denitrification. Additionally, AOA-positive samples were collected from WWTPs operating with long retention times (>15 days of solids retention time, >24 h of hydraulic retention time). Thus, it is possible that either or both of these features (low DO levels and long retention times) facilitate the growth of AOA (Park *et al.*, 2006)

2.3.2.9 Oxygen level

Most of the marine Crenarchaeota located within the suboxic layer are putative nitrifiers and live at oxygen levels ≤1 mM (Coolen *et al.*, 2007). Moreover, the presence of crenarchaeol in the oxygen minimum zone of the Arabian Sea, where oxygen levels were less than 5 μM, provided indirect evidence that marine Crenarchaeota are capable of thriving at low oxygen levels (Sinninghe Damsté *et al.*, 2002b).

2.3.2.10 Sulfide

Different phylotypes of marine Crenarchaeota (both 16S rDNA and *amoA*) were found in the top of the sulfidic zone, and those phylotypes were not detected in the suboxic zone. Living marine Crenarchaeota were present even in the sulfidic waters with up to a few tens of mM sulfide (Coolen *et al.*, 2007).

2.4 Molecular technique

Developed molecular tools, incorporating sequence analysis of the 16S rRNA and *amoA* genes, allow investigating ammonia oxidizer in the environments without concerning to culture-dependent techniques. In combination with clone libraries or denaturing gradient gel electrophoresis (DGGE), the application of specific polymerase chain reaction (PCR) amplification (Kowalchuk *et al.*, 1997; Rotthauwe *et al.*, 1997; Nicolaisen and Ramsing, 2002) provides clarification of the ammonia oxidizer community at the species level. The implementation of fluorescence in situ hybridization (FISH) (Wagner *et al.*, 1995; Mobarry *et al.*, 1996; Juretschko *et al.*, 1998; Wagner *et al.*, 1998; Okabe *et al.*, 1999; Gieseke *et al.*, 2001) makes it possible to analyze in situ complex community structure of AOB and estimate their numbers. The use of PCR-based quantification techniques, such as competitive PCR and real-time PCR, supports enumeration of AOB populations in the environments (Hermansson and Lindren, 2000; Dionisi *et al.*, 2002; Harms *et al.*, 2003). Recently, the gene encoding for the active site of ammonia monooxygenase (*amoA*) has been established as molecular marker for AOB diversity research in natural ecosystems and in engineered systems (Gieseke *et al.*, 2001; Horz *et al.*, 2000; Rotthauwe *et al.*, 1997). In addition, a putative *amoA* gene has been detected in an autotrophic marine ammonia-oxidizing archaea (Konneke *et al.*, 2005), but the amino acid sequence are of low similarity to bacterial AMO-encoding genes. Consequently, we can use specific primer (Table 2.2) targeting both 16S rRNA and *amoA* gene of AOB and AOA

Table 2.2 Specific primer targeting 16S rRNA and *amoA* gene of AOB and AOA

	Targeting gene	Primer	Nucleotide sequence ($5'–3'$)	Reference
AOB	16S rRNA	CTO189A/Bf-GC	CGCCCGCCGCGCGGCGGGCGGGG CGGGGGCACGGGGGGAGRAAAG CAGGGGATCG	Kowalchuck *et al.*, 1997)
		CTO 189Cf-GC	CGCCCGCCGCGCGGCGGGCGGGG CGGGGGCACGGGGGGAGGAAAG TAGGGGATCG	Kowalchuck *et al.*, 1997)
		CTO 654r	CTAGCYTTGTAGTTTCAAACGC	Kowalchuck *et al.*, 1997)
	amoA gene	amoA 1F	GGGGTTTCTACTGGTGGT	Rotthauwe *et al.*, 1997
		amoA 1F-GC	CGCCGCGCGGCGGGCGGGGCGG GGGCGGGGTTTCTACTGGTGGT	Rotthauwe *et al.*, 1997
		amoA 2R	CCCCTCTGCAAAGCCTTCTTC	Rotthauwe *et al.*, 1997
AOA	16S rRNA	A21F	TTCCGGTTGATCC[CT]GCCGGA	Delong, 1992
		A958R	[C/T]CCGGCGTTGA[A/C]TCCAATT	Delong, 1992
	amoA gene	Arch-amoAF	STAATGGTCTGGCTTAGACG	Francis *et al.*, 2005
		Arch-amoAR	GCGGCCATCCATCTGTATGT	Francis *et al.*, 2005