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Phylogenetic and functional marker genes to study ammonia-oxidizing microorganisms (AOM) in the environment

Pilar Junier · Verónica Molina · Cristina Dorador · Ora Hadas · Ok-Sun Kim · Thomas Junier · Karl-Paul Witzel · Johannes F. Imhoff

Abstract The oxidation of ammonia plays a significant role in the transformation of fixed nitrogen in the global nitrogen cycle. Autotrophic ammonia oxidation is known in three groups of microorganisms. Aerobic ammonia-oxidizing bacteria and archaea convert ammonia into nitrite during nitrification. Anaerobic ammonia-oxidizing bacteria (anammox) oxidize ammonia using nitrite as electron acceptor and producing atmospheric dinitrogen. The isolation and cultivation of all three groups in the laboratory are quite problematic due to their slow growth rates, poor growth yields, unpredictable lag phases, and sensitivity to certain organic compounds. Culture-independent approaches have contributed importantly to our understanding of the diversity and distribution of these microorganisms in the environment. In this review, we present an overview of approaches that have been used for the molecular study of ammonia oxidizers and discuss their application in different environments.

Keywords AOB · AOA · Anammox · Functional marker · Phylogenetic marker

P. Junier
Laboratory of Microbial Ecology, University of Neuchatel,
2009 Neuchatel, Switzerland

P. Junier (✉)
Environmental Microbiology Laboratory,
École Polytechnique Fédérale de Lausanne,
CE 1 644 (Centre Est), Station 6,
1015 Lausanne, Switzerland
e-mail: Pilar.Junier@epfl.ch

V. Molina
Departamento de Oceanografía & Centro de Investigación Oceanográfica en el Pacífico Sudoriental,
Universidad de Concepción,
Casilla 160-C,
Concepción, Chile

C. Dorador
Departamento de Acuicultura, Facultad de Recursos del Mar,
Universidad de Antofagasta,
Antofagasta, Chile

O. Hadas
Israel Oceanographic and Limnological Research,
The Yigal Allon Kinneret Limnological Laboratory,
P.O. Box 447, Migdal 14950, Israel

O.-S. Kim
School of Biological Sciences and Institute of Microbiology,
Seoul National University,
56-1 Shillim-dong, Kwanak-gu,
Seoul 151-742, Republic of Korea

T. Junier
Computational Evolutionary Genomics Group,
University of Geneva,
1211 Geneva, Switzerland

K.-P. Witzel
Max-Planck-Institute for Evolutionary Biology,
24306 Plön, Germany

J. F. Imhoff
Leibniz-Institute of Marine Sciences IFM-GEOMAR,
Duesternbrooker Weg 20,
24105 Kiel, Germany

Introduction

Ammonia oxidation is generally regarded as the rate-limiting step on autotrophic nitrification in a wide range of environments (Kowalchuk and Stephen 2001). Until recently, autotrophic ammonia oxidation was thought to be restricted to two groups of aerobic bacteria called ammonia-oxidizing bacteria (AOB), responsible for the oxidation of ammonia to nitrite. In the past few years, a series of new discoveries have challenged this view. First, the existence of anaerobic ammonia oxidation (anammox), which had been hypothesized based on thermodynamic calculations (Broda 1977), was confirmed in a pilot denitrifying wastewater treatment plant (Mulder et al. 1995). Further studies demonstrated that the anammox process is carried out by bacteria (van de Graaf et al. 1995) that were identified as members of the *Planctomycetes* (Strous et al. 1999). Second, analysis of metagenomic libraries from seawater (Venter et al. 2004) and soil (Treusch et al. 2005) revealed the existence of putative genes involved in ammonia oxidation in genomic fragments derived from uncultivated Crenarchaeota. The confirmation of archaeal ammonia oxidation was obtained by the cultivation of an ammonia-oxidizing archaeon (AOA) named Candidatus *Nitrosopumilus maritimus* (Könneke et al. 2005). Recently, the cultivation of two other thermophilic AOA, Candidatus *Nitrosocaldus yellowstonii* (de la Torre et al. 2008) and Candidatus *Nitrososphaera gargensis* (Hatzenpichler et al. 2008), has been reported.

Ammonia oxidation is central to the global nitrogen cycle. Nitrate, the product of aerobic ammonia and nitrite oxidation, is a fixed inorganic nitrogen source available for primary production in aquatic environments (Capone and Carpenter 1982). However, in terrestrial ecosystems, leaching of nitrate (e.g., from fertilized soil), other NO_x compounds (through incomplete denitrification by ammonia oxidizers; Conrad 1996) and N_2 (only in the case of anammox; Kartal et al. 2007), can lead to a net loss of nitrogen. Anaerobic ammonia oxidation maybe an important sink for fixed inorganic nitrogen in aquatic environments (Thamdrup and Dalsgaard 2002; Dalsgaard et al. 2003; Kuypers et al. 2003, 2005), and it is used for the removal of nitrogen from wastewater in sewage treatment plants (Jetten et al. 2001; Schmidt et al. 2003). Additionally, the cometabolic activity of enzymes involved in ammonia oxidation (i.e., ammonia monooxygenase, AMO) may be used as a mechanism for initiating the removal of certain pollutants (e.g., chlorinated aliphatics, alkanes, alkenes, or aromatics) from soils, waters, and sediments (Arp et al. 2001).

Ammonia-oxidizing microorganisms (AOM) are a diverse microbial group (Fig. 1) found in most environments where ammonia is available: soils, freshwater, and marine habitats. AOM are slowly growing bacteria with low maximum growth yields in cultures. Without the use of multiple complimentary approaches, we would have a very restricted vision of their abundance, function, diversity, and distribution in nature. This review will focus on phylogenetic and functional molecular marker genes used so far to study AOM and in addition will discuss the application of these markers for ecological studies.

The 16S rRNA gene

The most traditionally used phylogenetic marker for studying microbial communities is the 16S rRNA gene. AOB represent one of the bacterial groups for which the amplification and analysis of the 16S rRNA gene has been successfully used (Bothe et al. 2000; Kowalchuk and Stephen 2001; Juretschko et al. 1998; McCaig et al. 1994; Prosser and Embley 2002; Voytek and Ward 1995). AOB are divided into two monophyletic lineages based on their 16S rRNA gene sequences (Head et al. 1993; Purkhold et al. 2000, 2003; Teske et al. 1994). The first lineage belongs to the betaproteobacteria (beta-AOB) and comprises *Nitrosomonas* (including *Nitrosococcus mobilis*) and *Nitrosospira* (including *Nitrosolobus* and *Nitrosovibrio*) species. The second lineage, affiliated with the gammaproteobacteria (gamma-AOB), contains *Nitrosococcus oceani* and *Nitrosococcus halophilus*. The phylogenetic coherence of AOB has prompted the design of several specific polymerase chain reaction (PCR) primers and fluorescent in situ hybridization (FISH) probes (Hastings et al. 1997; Hiorns et al. 1995; Kowalchuk et al. 1997; McCaig et al. 1994; Mobarry et al. 1996; Pommerening-Roeser et al. 1996; Stephen et al. 1996; Utaker and Nes 1998; Voytek and Ward 1995; Wagner et al. 1995; Ward et al. 1997). However, the 16S rRNA gene sequence similarity is very high (even more than 99% within the genus *Nitrosospira*). Sequencing of the intergenic spacer between the 16S and the 23S rRNA genes has been suggested as an alternative to differentiate closely related species. Differences in size (ranging from ca. 400 to 700 bp) and lower sequence similarity (43–96%) suggested that this is a valuable complementary tool to the 16S rRNA-based approaches (Aakra et al. 1999). However, it has not been widely used for environmental studies.

So far, the study of anammox bacteria in natural assemblages has been usually based on 16S rRNA phylogenetic markers. Like AOB, 16S rRNA gene sequence analyses of anammox bacteria show that they form a monophyletic clade within the phylum *Planctomycetes*. However, a higher 16S rRNA gene sequence divergence has been observed among anammox bacteria (<87.1% identity; Schmid et al. 2005; Woebken et al. 2008) compared

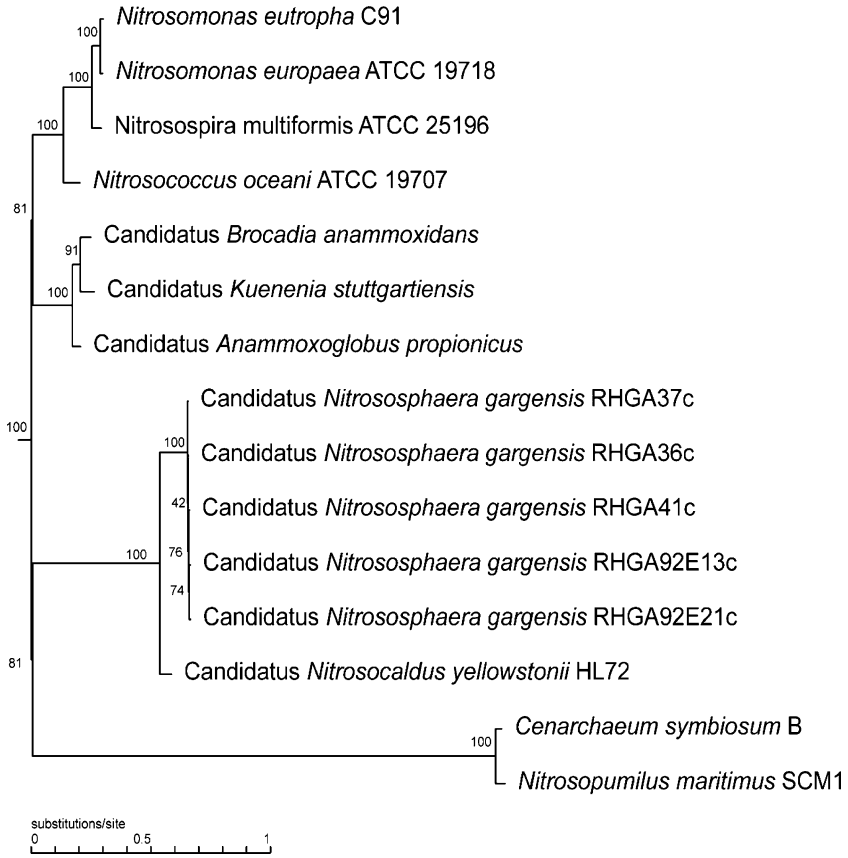

Fig. 1 Phylogenetic tree based on 16S rRNA gene sequences for different ammonia-oxidizing microorganisms. The sequences were aligned with Muscle (Edgar 2004) using a maximum of 24 iterations. A maximum-likelihood tree was built from the resulting alignment with PhyML (Guindon and Gascuel 2003) using the JTT model, 16 substitution rate classes, and maximum-likelihood estimates of parameters. Bootstrap values (%) from 1,000 bootstraps are indicated

to AOB (<89.5% similarity; Purkhold et al. 2000). Although amplification with nonspecific 16S rRNA gene primers has been used to characterize environmental communities with high abundance of anammox bacteria (e.g., anammox bioreactors), in samples with low representation of anammox, nonspecific 16S rRNA clone libraries are not a good choice. Therefore, some specific primers, mainly derived from FISH probes, have been suggested as an alternative (Schmid et al. 2005). Unlike in other *Planctomycetes*, in anammox bacteria, the 16S rRNA, the 23S rRNA, and an intergenic sequence between them are cotranscribed together as an operon. This intergenic sequence has been used for designing FISH probes that can be used for the specific detection of anammox (Schmid et al. 2001). FISH has been particularly useful for the study of these bacteria because of their characteristic doughnut shape generated by the presence of the "anammoxosome", a special compartment of the cell where the anammox process takes place (Schmid et al. 2005).

Up to now, AOA appear not to form a monophyletic clade but rather to belong to different lineages within the Crenarchaeota. The worldwide distribution and numerical importance of Crenarchaeota has been demonstrated in several 16S rRNA gene surveys. Phylogenetic analysis revealed distinct lineages that in general reflect a certain level of ecological differentiation based on the environment (Nicol and Schleper 2006). Genes that are possibly involved in nitrification have been found among some of these lineages from both soil and aquatic environments. A comparison of diversity studies based on the 16S rRNA gene and archaeal *amoA* indicates a substantial congruence in the phylogeny of crenarchaeal ribosomal and *amo* genes (Prosser and Nicol 2008). However, the 16S rRNA-defined diversity is significantly greater than the diversity associated with ammonia-oxidizing activity (Nicol and Schleper 2006; Prosser and Nicol 2008). Since it remains unknown whether AOA represent a monophyletic clade, as it is the case for AOB and anammox, the use of specific 16S rRNA gene primers for studying AOA is not a promising approach.

Functional markers

The main pitfall of the 16S rRNA gene as a molecular marker is that it is not necessarily related to the physiology of the target organisms (Calvo and Garcia-Gil 2004; Kowalchuk and Stephen 2001), and a slight lack of specificity may shift the spectrum of sequences amplified by PCR toward phylogenetically related but physiologically and ecologically different organisms (Bothe et al. 2000; Junier et al. 2008a). This is clearly

the case for AOB (Junier et al. 2008a) and most likely also for AOA and anammox. Another reason for using functional gene markers is that certain microbial guilds (i.e., microorganisms performing a common function) are not numerically dominant, as it is the case for AOM, and are hardly detectable using a 16S rRNA gene-based approach. Therefore, functional markers such as the genes encoding key enzymes involved in a specific metabolic pathway, e.g., ammonia oxidation, have been considered as an alternative for ecological studies (Rotthauwe et al. 1997).

In aerobic AOB and AOA, ammonium is first oxidized by the membrane-bound enzyme ammonia monooxygenase (Arp et al. 2002; Könneke et al. 2005). In the case of AOB, the second and energy-producing step is the oxidation of hydroxylamine to nitrite by hydroxylamine oxidoreductase (HAO; Arp et al. 2002). In AOA, an alternative mechanism for channeling electrons has been proposed, since available genome information does not support the existence of genes homologous to *hao* and other essential proteins found in AOB (Hallam et al. 2006a). Like aerobic ammonia oxidizers, anammox bacteria use ammonia oxidation as energy-generating mechanism for autotrophic growth. However, they use nitrite (instead of oxygen) as an electron acceptor. In anammox, a different metabolic pathway combines ammonia oxidation and denitrification (specifically nitrite reduction and N_2 production) in a single process (Kartal et al. 2007). Thus, only part of the ammonia oxidation pathway and consequently the potential functional markers derived from it is common to both aerobic and anaerobic AOM. Table 1 gives a summary of the functional molecular markers available for studying AOM in environmental samples.

Ammonia monooxygenase genes

In all known AOB, the genes encoding the enzyme AMO belong to an operon with the structure *amoCAB* (Chain et al. 2003; Klotz et al. 2006; Norton et al. 2002, 2008; Stein et al. 2007). Multiple copies of the operon are present in the genomes of beta-AOB (Chain et al. 2003; Norton et al. 2008; Stein et al. 2007), whereas a single copy has been reported for gamma-AOB (Klotz et al. 2006). Despite the potential of using the whole *amoCAB* operon for molecular studies, only a portion of the gene *amoA* has been generally used as a molecular marker to study the diversity of AOB (Rotthauwe et al. 1997). It has been argued that this region, which is relatively short (around 450 bp) and highly conserved, provides less resolution than the 16S rRNA gene (Koops et al. 2003; Purkhold et al. 2003). For environmental studies, the advantages of the higher specificity of the *amoA* approach may outweigh this disadvantage.

Since the publication of the first *amoA* sequence of *Nitrosomonas europaea* (McTavish et al. 1993), the number of partial and full-length sequences available in public databases has increased significantly (Fig. 2). Several PCR primers to amplify *amoA* have been published (Holmes et al. 1995; Hoshino et al. 2001; Juretschko et al. 1998; Nicolaisen and Ramsing 2002; Nold et al. 2000; Norton et al. 2002; Okano et al. 2004; Purkhold et al. 2000; Rotthauwe et al. 1997; Stephen et al. 1999; Sinigalliano et al. 1995; Webster et al. 2002), and a recent comparison of these primers has indicated that there are considerable differences in their performance and specificity (Junier et al. 2008b). The analysis of AMO-encoding genes has been extended to *amoC* and *amoB* (Calvo and Garcia-Gil 2004; Norton et al. 2002; Purkhold et al. 2000). A nested PCR approach to amplify environmental *amoCAB* sequences has recently been established, providing an increase of the sensitivity in detecting *amo* genes in samples with low abundances of AOB (Junier et al. 2009).

Interestingly, the genome projects of *N. europaea* (Chain et al. 2003), *Nitrosomonas eutropha* (Stein et al. 2007), and *Nitrosospira multiformis* (Norton et al. 2008) have revealed the existence of two additional conserved genes, *orf4* and *orf5*, immediately following the *amoCAB* operon (Fig. 3). The genomes of *N. europaea* and *N. multiformis* also encode singletons of *amoC* and *orf4*/*orf5*. It has been suggested that these unclustered copies may extend the flexibility for expression of the ammonia catabolic inventory under fluctuating ammonia concentrations (Norton et al. 2008). In the gamma-AOB *N. oceani*, two additional genes, *orf1* and *orf5*, have been identified as belonging to the *amoCAB* operon. While *orf5* is homologous to the *orf5* in beta-AOB, *orf1* has no equivalent in beta-AOB. Both genes, recently designated *amoR* and *amoD*, respectively, are cotranscribed with *amoCAB*, forming the specific gamma-AOB *amoRCABD* operon (El Sheikh et al. 2008), which is specific for gamma-AOB. So far, none of these genes has been used for environmental studies.

As mentioned above, homologs to the *amoA* gene were initially detected in genomic community studies and cultured AOA (Könneke et al. 2005; Treusch et al. 2005; Venter et al. 2004). Homologs to *amoC* and *amoB* also have been found, although the structure of an *amoCAB* operon has not been observed in AOA (Nicol and Schleper 2006). Several primer sets have been developed to amplify archaeal *amoA* (Francis et al. 2005; Könneke et al. 2005; Park et al. 2008; Tourna et al. 2008; Treusch et al. 2005; Urakawa et al. 2008), allowing the identification and quantification of AOA (Beman et al. 2008; Mincer et al. 2007). Currently, archaeal *amoA* sequences are submitted to the GenBank at a faster pace than bacterial *amoA* sequences (Fig. 2).

Table 1 Summary of functional molecular markers used to study ammonia-oxidizing microorganisms in environmental samples

Enzyme	Gene	Group	Primer	Sequence (5′–3′)	Reference
AMO	*amoA*	Bacteria	amoA21f(AMO-F2)	AGA AAT CCT GAA AGC GGC	Sinigalliano et al. (1995)
			amoA34f	GCG GCR AAA ATG CCG CCG GAA GCG	Molina et al. (2007)
			amoA4Of (AMO-F2)	AAG ATG CCG CCG GAA GC	Juretschko et al. (1998)
			amoA49f	GAG GAA GCT GCT AAA GTC	Junier et al. (2008b)
			amoA60r (304R)	TAY CGC TTC CGG CGG CAT TTT CGC CGC	Norton et al. (2002)
			amoA121f (amoA-3F)	ACC TAC CAC ATG CAC TT	Webster et al. (2002)
			amoA151f (A189)	GGN GAC TGG GAC TTCTGG	Holmes et al. (1995)
			amoA154f (301F)	GAC TGG GAC TTC TGG CTG GAC TGG AA	Norton et al. (2002)
			AmoA154fs	GAC TGG GAC TTC TGG	Junier et al. (2008b)
			AmoA187f (amoA-1FF)	CAA TGG TGG CCG GTT GT	Hoshino et al. (2001)
			amoA310f (amoA-3F)	CGT GAG TGG GYT AAC MG	Purkhold et al. (2000)
			amoA332f (amoA-1F)	GGG GTT TCT ACT GGT GGT	Rotthauwe et al. (1997)
			amoA332fHY (amoA1F mod)	GGG GHT TYT ACT GGT GGT	Stephen et al. (1999)
			amoA337p (A337)	TTC TAC TGG TGG TCR CAC TAC CCC ATC AAC T	Okano et al. (2004)
			amoA349r	ACC ACC AGT AGA AAC CCC	Junier et al. (2009)
			amoA359rC (amoA-4R)	GGG TAG TGC GAC CAC CAG TA	Webster et al. (2002)
			amoA627r	CGT ACC TTT TTC AAC CAT CC	Junier et al. (2008b)
			amoA664f	GCS TTC TTC TCN GCS TTTC	Junier et al. (2009)
			amoA665r (AMO-R2)	GCT GCA ATA ACT GTG GTA	Juretschko et al. (1998)
			amoA680r (A682 mod)	AAV GCV GAG AAG AAW GC	Nold et al. (2000)
			amoA681r (A682)	GAA SGC NGA GAA GAA SGC	Holmes et al. (1995)
			amoA686r (AMO-R)	GAT ACG AAC GCA GAG AAG	Sinigalliano et al. (1995)
			amoA802f	GAA GAA GGC TTT SCM GAG GGG	Junier et al. (2009)
			amoA820r (Amoa-2R′)	CCT CKG SAA AGC CTT CTT C	Okano et al. (2004)
			amoA822r (amoA-2R)	CCC CTC KGS AAA GCC TTC TTC	Rotthauwe et al. (1997)
			amoA822rTC (amoA-2R-TC)	CCC CTC TGC AAA GCC TTC TTC	Nicolaisen and Ramsing (2002)
			amoA822rTG (amoA-2R-TG)	CCC CTC TGG AAA GCC TTC TTC	Okano et al. (2004)
			amoA828r (302R)	TTT GAT CCC CTC TGG AAA GCC TTC TTC	Norton et al. (2002)
		Archaea	Arch-amoAF	STA ATG GTC TGG CTT AGA CG	Francis et al. (2005)
			Arch-amoAR	GCG GCC ATC CAT CTG TAT GT	Francis et al. (2005)
			amo196F	GGW GTK CCR GGR ACW GCM	Treusch et al. (2005)
			amo227R	CRA TGA AGT CRT AHG GRT ADC C	Treusch et al. (2005)
			amo247	CCA ACC AWG CWC CYT TKG CDA CCC	Treusch et al. (2005)
			CrenAmo1F	AAT GGT CTG GCT WAG ACG C	Könneke et al. (2005)
			CrenAmo1R	GAC CAR GCG GCC ATC CA	Könneke et al. (2005)
			crenAMO_F	ATG GTC TGG CTA AGA CGM TGT A	Hallam et al. (2006b)
			cren AMO_F	CCC ACT TTG ACC AAG CGG CCA T	Hallam et al. (2006b)
			Arch-amoA26F	GAC TAC ATM TTC TAY ACW GAY TGG GC	Park et al. (2008)
			Arch-amoA417R	GGK GTC ATR TAT GGW GGY AAY GTT GG	Park et al. (2008)
			CG I.1b-amoAF	ATA GTT GTA GTT GCT GTA AAT AG	Park et al. (2008)
			CG I.1b-amAR	CTC TAG AGG GTC TCT GAC CAG	Park et al. (2008)
			Arch-amoAF	GCT CTA AAT ATG ACA GTA TAC	Park et al. (2008)

Table 1 (continued)

Enzyme	Gene	Group	Primer	Sequence (5′–3′)	Reference
			Arch-amoAR	AYC ATG TTG AAY AAT GGT AAT GAC	Park et al. (2008)
			CG I.1b-amoAF	GTA CAT TAT TGA CAA TCA ACG C	Park et al. (2008)
			CG I.1b-amoAR	ATC CTA RYG CAA ACC AAG CTC	Park et al. (2008)
			CrenamoA616r	GCC ATC CAT CTG TAT GTC CA	Tourna et al. (2008)
			Arch-amoA-79F	ATT AAY GCA GGW GAY TAY A	Urakawa et al. (2008)
			Arch-amoA-479R	TAT GGT GGY AAY GTD GGT C	Urakawa et al. (2008)
			Arch-amoA-479R	AAT GGT CTG GST TAG AMG	de la Torre et al. (2008)
	amoB	Bacteria	amoB44r (amoB-4R)	GCT AGC CAC TTT CTG G	Purkhold et al. (2000)
			amoB160 (amoBMf)	TGG TAY GAC ATK AWA TGG	Calvo and Garcia-Gil (2004)
			amoB506r (308R)	TCC CAG CTK CCG GTR ATG TTC ATC C	Norton et al. (2002)
			amoB660r (amoBMr)	RCG SGG CAR GAA CAT SGG	Calvo and Garcia-Gil (2004)
			amoB1179r	CCA AAR CGR CTT TCC GG	Junier et al. (2008b)
			amoB1179rGam	GCA AAG CGG CTG TCT GG	Junier et al. (2008b)
		Archaea	CrenAmo2.1F	CAC GGT GTM CAA GCA CA	Könneke et al. (2005)
			CrenAmo2.2R	RAT TAC YTG CCA VGG TC	Könneke et al. (2005)
	amoC	Bacteria	amoC58f	CTA YGA CAT GTC RTC GTG G	Junier et al. (2008a, b)
			amoC763f (305F)	GTG GTT TGG AAC RGI CAR AGC AAA	Norton et al. (2002)
		Archaea	CrenAmo3.1F	ATG GCA CAR ATG CCS GC	Könneke et al. (2005)
			CrenAmo3R	GGT ATW GAT CTG TAC AA	Könneke et al. (2005)
HAO	*hao*	Bacteria	haof1	TGC GTG GAR TGY CAC	Schmid et al. (2008)
			haoR3	AGR TAR GAK YSG GCA AA	Schmid et al. (2008)
			haoF4	AAY CTK CGC TCR ATG GG	Schmid et al. (2008)
			haoR2	GGT TGG TYT TCT GKC CGG	Schmid et al. (2008)
HZO	*hzo*	Anammox	hzocl1F1	TGY AAG ACY TGY CAY TGG	Schmid et al. (2008)
			hzocl1R2	ACT CCA GAT RTG CTG ACC	Schmid et al. (2008)
			hzo1F1l	TGY AAG ACY TGY CAY TGG G	Schmid et al. (2008)
			hzocl1R2	ACT CCA GAT RTG CTG ACC	Schmid et al. (2008)
			hzocl2aF1	GGT TGY CAC ACA AGG C	Schmid et al.(2008)
			hzocl2aR1	TYW ACC TGG AAC ATA CCC	Schmid et al. (2008)
			hzocl2aF1	GGT TGY CAC ACA AGG C	Schmid et al. (2008)
			hzocl2aR2	ATA TTC ACC ATG YTT CCA G	Schmid et al. (2008)
			hzocl2aF2	GTT GTG MTG MWT GTC ATG G	Schmid et al. (2008)
			hzocl2aR1	TYW ACC TGG AAC ATA CCC	Schmid et al. (2008)
Nitrite reductase	*nirK*	Bacteria	Cunir3	CGT CTA YCA YTC CGC VCC	Casciotti and Ward (2001)
			Cunir4	GCC TCG ATC AGR TTR TGG	Casciotti and Ward (2001)
	nirS	Anammox	Scnir372F	TGT AGC CAG CAT TGT AGC GT	Lam et al. (2009)
			Scnir845R	TCA AGC CAG AC CAT TTG CT	Lam et al. (2009)
NorB	*norB*	Bacteria	norB1	CGN GAR TTY CTS GAR CAR CC	Casciotti and Ward (2005)
			norB2	GAC AAR HWY TAY TGG TGG T	Casciotti and Ward (2005)
			norB3	CCY TCV ACC CAG ASA TGC AC	Casciotti and Ward (2005)
			norB6	TGC AKS ARR CCC CAB ACB CC	Casciotti and Ward (2005)
			norB7	CCR TGG STR WAR WAR TTS AC	Casciotti and Ward (2005)
			norB8	CRT ADG CVC CRW AGA AVG C	Casciotti and Ward (2005)
Accase	*accB*	Archaea	ACAC254f	GCT GAT GCT ATA CAT CCW GGW TAY	Auguet et al. (2008)
			ACAC720r	GCT GGA GAT GGA GCY TCY TCW ATT	Auguet et al. (2008)

AMO ammonia monooxygenase, *HAO* hydroxylamine oxidoreductase, *HZO* hydrazine oxidoreductase, *NorB* nitric oxide reductase, *ACCase* biotin carboxylase

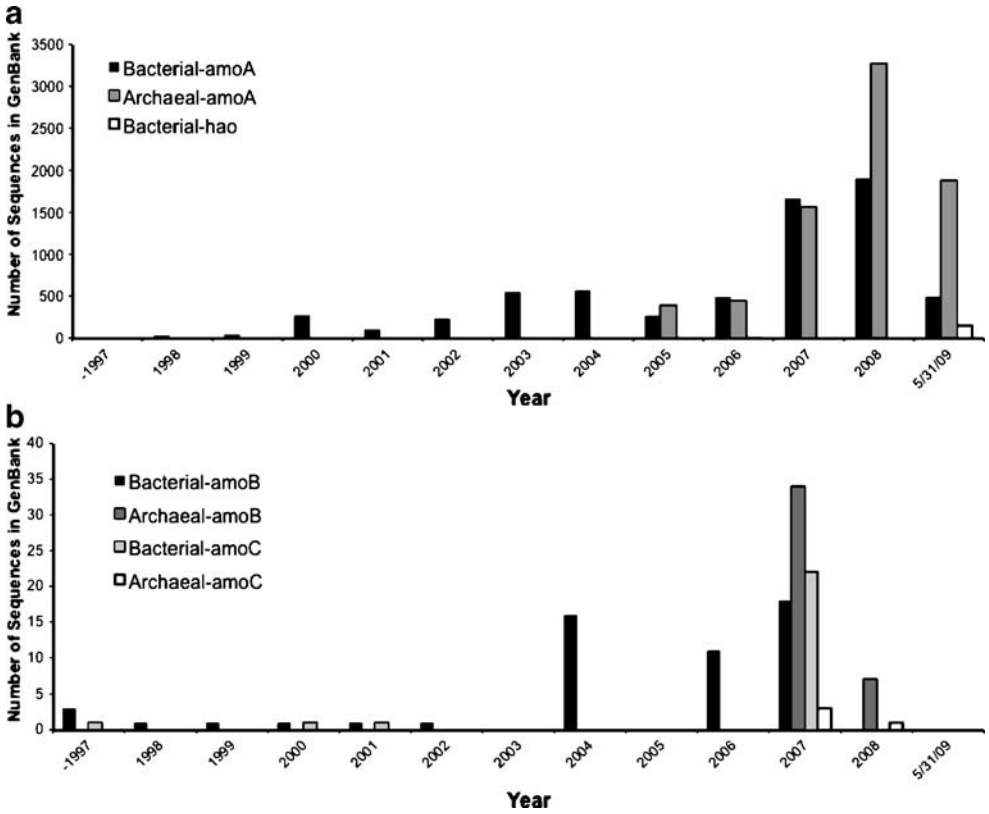

Fig. 2 Cumulative number of *amoCAB/hao* sequences submitted to the GenBank database. **a** *amoA* and *hao/hzo* genes. **b** *amoB* and *amoC*

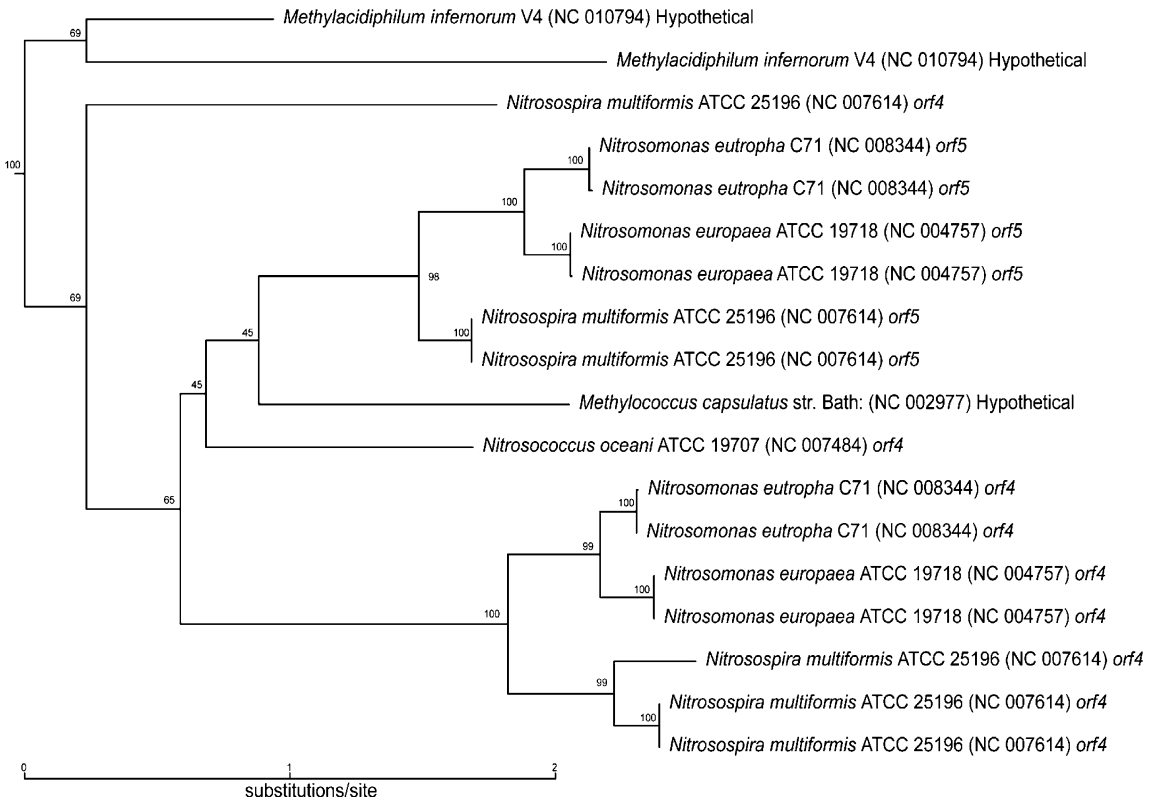

Fig. 3 Phylogenetic tree based on sequences homologs to *orf4* and *orf5* found in the genome of ammonia-oxidizing bacteria and methane-oxidizing bacteria. For methods, see Fig. 1

Hydroxylamine/hydrazine oxidoreductase genes in aerobic and anaerobic ammonia-oxidizing bacteria

One of the few potentially common functional markers for aerobic and anaerobic ammonia-oxidizing bacteria is the gene encoding the octahaem cytochrome c (OCC) proteins, hydroxylamine oxidoreductase in AOB, and hydrazine oxidoreductase (HZO) in anammox. HAO is responsible for the dehydrogenation of hydroxylamine to nitrite (the second step of ammonia oxidation) and is proposed as the protein relaying the electrons to the ubiquinone pool via two interacting cytochromes, *c*554 and *cM*552 (Hooper et al. 2005). Due to its soluble nature, HAO is the best-studied functional component of aerobic ammonia oxidation (Igarashi et al. 1997). In anammox, HZO converts hydrazine to N_2 (Strous et al. 2006), generating the proton motive force for energy production. HAO and HZO can oxidize the alternate substrate (hydroxylamine and hydrazine, respectively; Hooper et al. 2005), and functional and sequence similarities between both enzymes have been described (Klotz et al. 2008; Shimamura et al. 2007; Strous et al. 2006).

Multiple copies of the *hao* gene have been revealed in the genome sequencing projects of beta-AOB (Arp et al. 2007; Chain et al. 2003; Norton et al. 2008; Stein et al. 2007), as part of a gene cluster including three additional genes, *orf2* and *cycAB* (although *cycB* is absent in one of the copies in *N. europaea* and *N. eutropha* (Norton et al. 2008)). The genome analysis of Candidatus *Kuenenia stuttgartiensis* revealed eight highly divergent octahaem protein regions as possible candidates for HZO (Strous et al. 2006). The genes encoding the HAO/HZO proteins appear to be suitable as functional and phylogenetic biomarkers because of their highly conserved sequence across different species (Klotz et al. 2008), and recently, several sets of degenerate primers were designed based on the available *hao*/*hzo* sequences (Schmid et al. 2008).

Phylogenetic analyses of the *hao*/HAO sequences obtained with a collection of cultured representatives of AOB were congruent with those reported for the 16S rRNA gene and *amoA*/AmoA (Schmid et al. 2008), suggesting that the *hao* gene may be an alternative molecular marker for AOB. However, the *hao* gene is also present in nonammonia oxidizers, including methane-oxidizing bacteria (MOB) and other microorganisms (Ward et al. 2004, Bergmann et al. 2005). The in silico analysis of the degenerate primers designed to amplify *hao* (Schmid et al. 2008) suggested that they are specific for AOB, although stringent conditions are required to avoid cross-amplification with non-AOB also containing *hao* copies (e.g., MOB and the sulfur oxidizer *Silicibacter pomeroyi*; Moran et al. 2004).

Analysis of the *hzo* genes from anammox proved to be more complex than in the case of *hao* for AOB since in the genome of Candidatus *K. stuttgartiensis*, several possible OCC protein-encoding genes were identified (Strous et al. 2006). The phylogeny of *hzo*/HZO revealed the existence of three clusters of sequences. At least one of these, cluster 1, is in agreement with the previously published rRNA phylogeny of anammox bacteria. The analyses of the primers with cultures, man-made, and environmental samples produced positive results opening the promise of the use of *hzo* as a phylogenetic and functional marker in the future (Schmid et al. 2008).

Other potential candidates for functional markers

Genes involved in nitrogen metabolism

Other genes encoding enzymes involved in the nitrogen metabolism have been found in AOB (Arp et al. 2007). One of these genes, the copper-containing dissimilatory nitrite reductase gene (*nirK*), was detected in several isolates of beta-AOB by PCR with nonspecific primers (Casciotti and Ward 2001). The topology of the *nirK* tree corresponds to those of 16S rRNA and *amoA*. These results suggest that *nirK* sequences retrieved from the environment may include sequences from ammonia-oxidizing bacteria, although specific *nirK*-AOB primers have not been designed yet. Another gene that has been shown to be present in some AOB is the one encoding for the large subunit of nitric oxide reductase (*norB*). However, amplification was not successful in all *Nitrosospira* strains tested (Casciotti and Ward 2005), and therefore, it is not clear if it is useful as a general functional marker of AOB.

A putative nitrite reductase (*nirS*) gene coding for cytochrome *cd-1* was also found in anammox. NirS is postulated to participate in the oxidation of nitrite to nitric oxide, which forms hydrazine together with ammonium in a process catalyzed by the hydrazine hydrolase (Strous et al. 2006). This putative *nirS* has been used as molecular marker to study the presence and potential activity of anammox in the environment by reverse transcriptase PCR (Lam et al. 2009).

Autotrophic growth

Although autotrophy is one of the features common to AOM and most AOB, heterotrophic growth has been reported for several AOB (Arp et al. 2007). In the case of AOB, the genes coding the ribulose-biphosphate carboxylase/oxygenase (RuBisCO, *cbbL* and *cbbS*) could be considered as a molecular marker. However, evidence from the genome projects of AOB indicate that the *cbb* operon has been subjected to horizontal gene transfer (Norton et al. 2008). The genes for RuBisCO do not appear to be present in AOA and anammox.

In AOA, genes corresponding to the 3-hydroxypropionate and citric acid cycles were identified (Hallam et al. 2006a). One of the enzymes responsible for the fixation of CO_2 in this metabolic pathway is the acetyl CoA carboxylase (ACCase). The gene *accB* encoding the biotin carboxylase, one of the three subunits of the ACCase, has been proposed as a potential molecular marker to investigate CO_2 assimilation linked to archaeal autotrophy (Auguet et al. 2008).

In anammox, the complete acetyl CoA pathway was detected in the genome of Candidatus *K. stuttgartiensis* (Strous et al. 2006). However, so far, none of the genes involved in CO_2 assimilation have been used as molecular markers for ecological studies of anammox.

Application examples from different environments

Ammonia-oxidizing bacteria

The community structure of AOB has been investigated in a wide spectrum of environments. Most studied environments are soils, where pH was found to have a considerable effect on the activity of AOB communities and on the biogeochemical processes that they mediate. Experimentally, it has been demonstrated that ammonia oxidation rarely occurs at pH values lower than 7 (Burton and Prosser 2001; Gieseke et al. 2006). However, a series of studies revealed different niches for different groups of AOB (and also AOA) in soils with pH between 4.5 and 7.5 (Nicol et al. 2008). For example, a tendency toward the dominance of *Nitrosospira* cluster 3 in agricultural soils with neutral pH versus *Nitrosospira* cluster 2 in acidic soils has been observed using the 16S rRNA gene (Kowalchuk et al. 1997; Stephen et al. 1996) or *amoA* (Nugroho et al. 2007). A combination of 16S rRNA gene and *amoA* profiling has been also used to characterize the structure and abundance of AOB in response to the variation of soil management. Using this approach, the dominance of *Nitrosospira* cluster 3 and 4 was found in soils regardless of the amendment with low, medium, or high concentrations of ammonia (Avrahami et al. 2002; Schmidt et al. 2007).

The study of AOB in marine ecosystems initially carried out using 16S rRNA gene markers has shown that these microorganisms are widespread in coastal, open ocean, and even polar environments (Hollibaugh et al. 2002; O'Mullan and Ward 2005; Phillips et al. 1999), including oxygen-deficient water columns and anoxic sediments (Kim et al. 2006; Molina et al. 2007). 16S rRNA gene surveys in the water column reveal a niche differentiation of certain genera within the beta-AOB with the predominance of *Nitrosomonas* spp. in attached surfaces (particle-associated) and of *Nitrosospira* spp. as free-living microorganisms (Phillips et al. 1999). Studies using *amoA* as molecular marker also indicate the dominance of sequences associated to *Nitrosospira* spp. and particularly affiliated to a yet-uncultured Clade 1 (Freitag and Prosser 2004; Hollibaugh et al. 2002; Kim et al. 2008; Molina et al. 2007).

AOB also have been studied in extreme environments. Culture-based studies indicate a broad distribution of salt-tolerant and halophilic species of AOB. Molecular studies based on 16S rRNA and *amoA* in different hypersaline environments revealed communities dominated by *N. europaea* and *N. eutropha* in Mono Lake (Carini and Joye 2008; Ward et al. 2000), *Nitrosomonas halophila*, *Nitrosomonas marina*, *Nitrosomonas communis* in Salar de Huasco (Dorador et al. 2008), and by different *Nitrosomonas* and *Nitrosospira* species in Qinghai Lake (Jiang et al. 2009). Gamma-AOB have not been found in hypersaline environments using the described 16S rRNA gene primers, which could indicate the unspecific nature of these primers for nonmarine environmental samples. Since decades, it is well known that nitrification can occur at low temperatures in natural environments (Jones and Morita 1985; Jones et al. 1988). AOB communities in Antarctica analyzed by use of the 16S rRNA gene revealed a community composed by mainly two clusters: *Nitrosomonas*-like sequences and *Nitrosospira*-like sequences (Bano and Hollibaugh 2000; Hollibaugh et al. 2002). Also, pioneering studies on nitrification at high temperatures described a community dominated by *Nitrosospira*-like sequences identified by immunofluorescence labeling (Lebedeva et al. 2005). The recovery of *amo*A sequences from an acidic hot spring in a Japanese gold mine revealed the broad adaptability of certain AOB, highly similar to *Nitrosomonas* sp. Nm107 and *N. nitrosa*, to extreme conditions (Hirayama et al. 2005).

Anaerobic ammonia-oxidizing bacteria (anammox)

Using the 16S RNA phylogenetic marker by means of FISH and PCR-dependent techniques, researchers detected anammox in freshwater and marine environments, in soils, wetlands, and wastewater treatment plants. Despite the ubiquity of anammox in nature, a very low diversity has been reported in suboxic and anoxic aquatic ecosystems being mostly dominated by organisms belonging to Candidatus *Scalindua* spp. (Penton et al. 2006). However, a higher microdiversity was observed in oxygen minimum zones of the ocean (Galan et al. 2009; Woebken et al. 2009) and in man-made environments, where most candidate genera have been discovered, i.e., Candidatus *Brocadia*, Candidatus *Kuenenia*, Candidatus *Anammoxoglobus* (Schmid et al. 2005).

The anammox process is probably widespread in aquatic systems and is not limited to marine or brackish environments. In stratified lake chemoclines, sediment–water

interfaces and benthic boundary layers, the co-existence of ammonia, nitrite, and nitrate creates potentially suitable niches for anammox bacteria. Schubert et al. (2006) were the first to demonstrate the anammox process in the suboxic water column of Lake Tanganyika, incubating $^{15}NO_3$-labeled water samples in situ. The $^{29}N_2$ (anammox product) was recorded at all anoxic depths corresponding to 9–13% of total N_2 production in the lake. Presence of anammox bacteria (0.1–1.4% of total 4′,6-diamidino-2-phenylindole-stained bacteria) was confirmed at maximum anammox activity depths using FISH. Phylogenetic analysis of partial 16S rRNA gene sequences resulted in hits close to Candidatus *Scalindua brodae* and Candidatus *Scalindua sorokinii* (Schubert et al. 2006). The presence and distribution of anammox 16S rRNA gene sequences in shallow organic rich freshwater sediments from Wintergreen Lake and from wetlands in the Florida Everglades were demonstrated using specific primers (Brod541F/Brod 1260R and An7F/An1388R) serving as molecular markers for better tracking of anammox bacterial populations. The results suggest that anammox bacteria are agents of nitrogen removal from freshwater lakes besides denitrification (Penton et al. 2006).

Although many scientists have contributed to the knowledge of anammox in aquatic systems, so far they have been less studied in soils and extreme environments. There is a single report of anammox in mangrove soil (Wickramasinghe et al. 2009). 16S rRNA gene clonal sequences analyzed in this study represented novel lineages related to anammox. A less significant role of this process was suggested in saline tidal marsh sediments compared to a freshwater marsh (Koop-Jakobsen and Giblin 2009). Candidatus *Brocadia anammoxidans* is capable of growing at 43°C in the laboratory (Strous et al. 1999); nevertheless, there is little evidence of anammox in hot natural environments. Recently, the occurrence of anammox in hydrothermal vents was established using 16S rRNA gene sequences and lipids as markers (Byrne et al. 2009). Also, 16S rRNA gene sequences recovered from an acidic hot spring were highly similar to those of Candidatus *Brocadia anammooxidans* (Hirayama et al. 2005).

Ammonia-oxidizing archaea

The recovery of putative *amo* genes in crenarchaeal DNA from genomic surveys (Treusch et al. 2005; Venter et al. 2004) and the detection of the genes *amoA*, *amoB*, and *amoC* in the uncultivated crenarchaeal symbiont *Cenarchaeum symbiosum* (Hallam et al. 2006b) open the possibility for a potential role in autotrophic oxidation of ammonia for the highly abundant Crenarchaeota inhabiting the oceans (DeLong 1992, 1998; Fuhrman et al. 1992; Karner et al. 2001), terrestrial (Ochsenreiter et al. 2003), and several other nonextreme environments (Auguet and Casamayor 2008; Perreault et al. 2007). In addition, studies dealing with the amplification of archaeal *amoA* demonstrated the ubiquitous presence of AOA in marine, freshwater, and terrestrial environments showing an apparent niche adaptation to different habitats (Erguder et al. 2009; Francis et al. 2005). The quantification of bacterial and archaeal *amoA* gene copies indicated that AOA outnumbered AOB in most marine and terrestrial ecosystems by a factor of 10 to 1,000 (Beman et al. 2008; Leininger et al. 2006; Wuchter et al. 2006), suggesting a possible higher contribution to nitrification.

AOA also have been detected in extreme environments: 16S rRNA gene and crenarchaeal *amoA* sequences related to *N. maritimus* have been recovered from different cold environments like mountain lakes (Dorador 2007; Jiang et al. 2009) and deep-sea waters (Tamegai et al. 2007). Using *amoA*, archaeal nitrification capability was detected in a series of terrestrial hot springs of Kamchatka, Siberia, and Iceland at temperatures between 82°C and 97°C and pH below 7 (Reigstad et al. 2008). The recent cultivation of a thermophilic nitrifier (Candidatus *N. yellowstonii*), an autotrophic crenarchaeote growing at up to 74°C by aerobic ammonia oxidation (de la Torre et al. 2008), as well as the moderate thermophilic Candidatus *N. gargensis* (Hatzenpichler et al. 2008), confirms the possible presence of nitrifying activity due to AOA at high temperature habitats. In recent studies, sequences related to AOA were also found in saline systems like the Salar de Huasco in the Chilean Altiplano (Dorador 2007) and Qinghai Lake in the Tibet (Jiang et al. 2009). In some of these extreme habitats, AOA can be more abundant than AOB (Jiang et al. 2009).

Ammonia-oxidizing microorganisms in biotechnological applications

Deterioration of water quality by excessive input of organic nitrogen is a very serious environmental concern. The removal of ammonium is of special interest because it can be toxic to aquatic species (Khin and Annachhatre 2004). Because biological nitrogen removal is very cost-effective, the use of AOM for biotechnological applications involving the removal of ammonium has been widely adopted.

Conventional microbial nitrogen removal is based on the oxidation of ammonia and nitrite by AOB and nitrite-oxidizing bacteria, respectively, followed by the reduction of nitrate by heterotrophic denitrifying bacteria. Community analyses based on 16S rRNA and *amoA* genes to detect ammonia-oxidizing bacteria in wastewater treatment plants (WWTPs) have demonstrated that populations related to *Nitrosomonas*, including phylogenetic lineages with no cultured representatives, can be responsible for ammonia

oxidation in these systems (Hallin et al. 2005; Siripong and Rittmann 2007; Wagner and Loy 2002). Quantitative approaches have been implemented using *amoA* as target to quantify specific populations of AOB in WWTP (Dionisi et al. 2002a, b) and to study the dynamics of different AOB populations overtime (Layton et al. 2005). Quantification based on FISH has also been used to estimate the active populations of AOB in bioreactors (Gieseke et al. 2001).

The effects of environmental conditions such as high ammonium concentrations, different pH values, and different oxygen concentrations on nitrification and the community structure of AOB in wastewater have also been investigated (Princic et al. 1998). The results indicated that a high ammonium level result in a greater change in community structure, whereas the smaller changes in community structure originated by pH changes were irreversible. Sequence analysis revealed that *N. eutropha*-like AOB were stimulated at the highest ammonium concentration (Princic et al. 1998).

Anammox represents an alternative N_2-producing process to oxidize ammonium under strict anaerobic conditions. The anammox reaction has as advantages the reduction in energy requirements (e.g., to supply oxygen for nitrification), total reduction of carbon requirements, and a decreased biomass yield (Sliekers et al. 2003; van Dongen et al. 2001). All these make anammox a cost-effective and environmentally friendly N-removal system that can reach maximal removal rates of 9 kg Nm^{-3} day^{-1} (Jetten et al. 2005; Kartal et al. 2006; Op den Camp et al. 2006). Anammox bacteria were initially and are still enriched from many types of wastewater sludge and probably are indigenous to treatment plants worldwide (Schmid et al. 2005; Op den Camp et al. 2006). The application of enrichment techniques coupled to FISH using specific 16S rRNA gene probes and clone libraries has been essential to reveal the diversity of anammox bacteria in man-made systems (Op den Camp et al. 2006).

In WWTPs, anammox have the potential to replace the conventional denitrification step if preceded by partial nitrification to nitrite as was demonstrated by the implementation of the single reactor system for high ammonium removal over nitrite and completely autotrophic N-removal over nitrite (CANON) bioreactors (Hellinga et al. 1998; Mulder et al. 2001; Sliekers et al. 2003; van der Star et al. 2007). The CANON reactor utilizes the activity of autotrophic aerobic and anaerobic ammonium-oxidizing bacteria to remove ammonium. The nitrite that is produced from the activity of aerobic ammonium oxidizers could be used to remove ammonium from groundwater or other water sources that are low in organic carbon (Third et al. 2001, 2005). Other systems that take advantage of the metabolism of anammox bacteria are the upflow anaerobic sludge bed and sequencing batch reactors (Ahn et al. 2004; Kartal et al. 2006). In all the cases, the pitfall of the process is the slow growth of the anammox bacteria and the slow startup of anammox process in new reactors.

Another application of the anammox process is in treating recirculating water systems with the potential of providing significant oxygen and energy savings (van Rijn et al. 2006). The presence of organisms related to anammox has been demonstrated in biofilters from marine and freshwater recirculating aquaculture systems (Tal et al. 2003).

Members of Crenarchaeota that can perform ammonia oxidation have been also found in several man-made environments. Normally, nitrifying bacteria are largely responsible for the oxidation of ammonia in aquaria biofilters and recirculation aquaculture (Burrell et al. 2001). However, recently, it has been demonstrated that the nitrifying community in a marine aquarium biofilter was dominated by AOA instead of AOB, with a ratio of archaeal *amoA* to bacterial *amoA* ranging from 1.8 to 3.0 (Urakawa et al. 2008). Furthermore, it was demonstrated that temperature was the most important factor controlling the growth and diversity of AOA and AOB in aquaria biofilters. The role of AOA in this kind of system is still unknown.

AOA have also been detected in groundwater treatment plants and associated distribution systems (de Vet et al. 2009; van der Wielen et al. 2009). In one study, it was demonstrated that AOA were responsible for the removal of nitrogen in at least one of the plants studied, suggesting that high DOC levels might reduce growth of AOA (van der Wielen et al. 2009).

More detailed studies concerning to the role of AOA in nitrogen removal from engineered systems have been conducted in wastewater treatment bioreactors (Park et al. 2006; Zhang et al. 2009). AOA appear to be spread in WWTPs: Their dependence upon oxygen concentration and solid retention time was demonstrated in a study examining five plants through the US (Park et al. 2006). It was recently demonstrated using qPCR analyses of the *amoA* gene that the AOB community was more than three orders of magnitude more abundant than the AOA community in an activated sludge bioreactor (Wells et al. 2009). This study suggests that AOA have only a minor role in ammonia oxidation in highly aerated activated sludge.

Outlook

Molecular approaches have contributed significantly to our present knowledge on ecology, distribution, and phylogeny of ammonia-oxidizing microorganisms and on the contribution of AOB, AOA, and anammox to ammonia oxidation. These methods also made possible the study of a largely diverse array of habitats, such as soils, fresh waters, marine habitats,

and extreme habitats, in particular regarding temperature and salinity. Nevertheless, we expect many challenges to be waiting ahead. In an attempt to understand the ecosystem function, the relative contributions of different AOM to ammonia oxidation and the quantitative determination of their metabolic rates need to be studied and compared in different environments and ecological niches. Some studies with focus on the relative importance of aerobic ammonia oxidizers suggest the numerical dominance of AOA over AOB (Leininger et al. 2006; Mincer et al. 2007; Wuchter et al. 2006). However, recent evidence supports the view that numerical dominance is not necessarily a reliable indicator of activity (Jia and Conrad 2009). Similarly, aerobic and anaerobic ammonia oxidizers are expected to inhabit different ecological niches governed by the availability of oxygen, yet there is evidence that in nature the two groups cooperate (Lam et al. 2007, 2009). In addition, the analysis of the available genome sequences from AOM has highlighted their metabolic complexity and suggests the possibility of different functions of individual AOM under different environmental conditions. These data also suggest a multiple evolutionary origin of ammonia oxidation and imply that a universal molecular marker to study ammonia oxidation in the environment does not exist. Multiple complementary approaches are probably our best bet to unveil the natural diversity of AOM and their ecological function.

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