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MINIREVIEW

The History of Aerobic Ammonia Oxidizers: from the First Discoveries to Today

Maria Monteiro, Joana Séneca, and Catarina Magalhães*

EcoBioTec Laboratory, Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto, Rua dos Bragas 289, P 4050-123 Porto, Portugal

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Nitrification, the oxidation of ammonia to nitrite and nitrate, has long been considered a central biological process in the global nitrogen cycle, with its first description dated 133 years ago. Until 2005, bacteria were considered the only organisms capable of nitrification. However, the recent discovery of a chemoautotrophic ammonia-oxidizing archaeon, *Nitrosopumilus maritimus*, changed our concept of the range of organisms involved in nitrification, highlighting the importance of ammonia-oxidizing archaea (AOA) as potential players in global biogeochemical nitrogen transformations. The uniqueness of these archaea justified the creation of a novel archaeal phylum, Thaumarchaeota. These recent discoveries increased the global scientific interest within the microbial ecology society and have triggered an analysis of the importance of bacterial *vs* archaeal ammonia oxidation in a wide range of natural ecosystems. In this mini review we provide a chronological perspective of the current knowledge on the ammonia oxidation pathway of nitrification, based on the main physiological, ecological and genomic discoveries.

***Keywords*:** archaea, bacteria, ammonia-oxidizers, AOA, AOB

Introduction

Nitrification represents the oxidative part of the nitrogen (N) cycle and refers to the two-step process where ammonia is oxidized to nitrite and subsequently to nitrate (Fig. 1). This process completes the redox cycle of N, from the most reduced to the most oxidized form and plays a key role in the global N budget of Earth ecosystems.

The first step of nitrification, was described by Houzeau in 1872, and later attributed to the action of fermentative microorganisms (Müller, 1873; Schloesing and Muntz, 1877). Only thirteen years later, with the isolation of an ammonia oxidizing bacterium, the role of bacteria in mediating the initial step of the nitrification pathway (Winogradsky, 1890) was confirmed. From 1890 until 2004, scientists believed that only bacteria mediated aerobic ammonia oxidation. However, our knowledge about nitrification and the organisms involved changed greatly in the last few years, with the identification of a set of genes predicted to encode ammonia monooxygenase (AMO) in marine group I Crenarchaeota (Venter *et al.*, 2004; Treusch *et al.*, 2005) and by the cultivation of the first ammonia-oxidizing archaeon *Nitrosopumilus maritimus* (Könneke *et al.*, 2005), now placed in a novel archaeal phylum, Thaumarchaeota (Brochier-Armanet *et al.*, 2008). The involvement of Thaumarchaeota in ammonia oxidation has attracted the attention of numerous research groups that recognize Thaumarchaeota as a major archaeal lineage, comprising a large group of ubiquitous organisms (Hallam *et al.*, 2006a; Brochier-Armanet *et al.*, 2008; de la Torre *et al.*, 2008; Pester *et al.*, 2011, 2012). Beyond that, a considerable diversity and dispersion of ammonia oxidizing archaea (AOA) was demonstrated to occur worldwide, and the idea that the activity of this group of organisms contribute to the global N-cycle is generally accepted (Francis *et al.*, 2005; Hallam *et al.*, 2006b; Brochier-Armanet *et al.*, 2008; de la Torre *et al.*, 2008; Biller *et al.*, 2012; Pester *et al.*, 2012; Stahl and de la Torre, 2012). The current explosion of studies focusing on the cellular physiology, ecology, biogeochemistry, ecophysiology, genomics (Martens-Habbena and Stahl, 2011; Biller *et al.*, 2012; Mosier *et al.*, 2012; Stahl and de la Torre, 2012; Vajrala *et al.*, 2013; Jung *et al.*, 2014) and, more recently, proteomics (Santoro A., communication at Ocean Sciences Meeting 2014) of these new AOA is opening new doors of exciting research. In this mini review, we provide a chronological perspective of the knowledge regarding the ammonia oxidation pathway of nitrification, going back to the time when the process was initially described, and following up into the current molecular era, highlighting the main scientific discoveries, which are important for those who pursue research in this field.

The first insights on ammonia oxidation

The initial step of nitrification, the biological oxidation of ammonia into nitrite (Fig. 1), was first described 133 years ago by Houzeau (1872) in low pH soils and by Müller (1873),

*For correspondence. E-mail: cmagalhaes@ciimar.up.pt; Tel.: 00351-223 4018

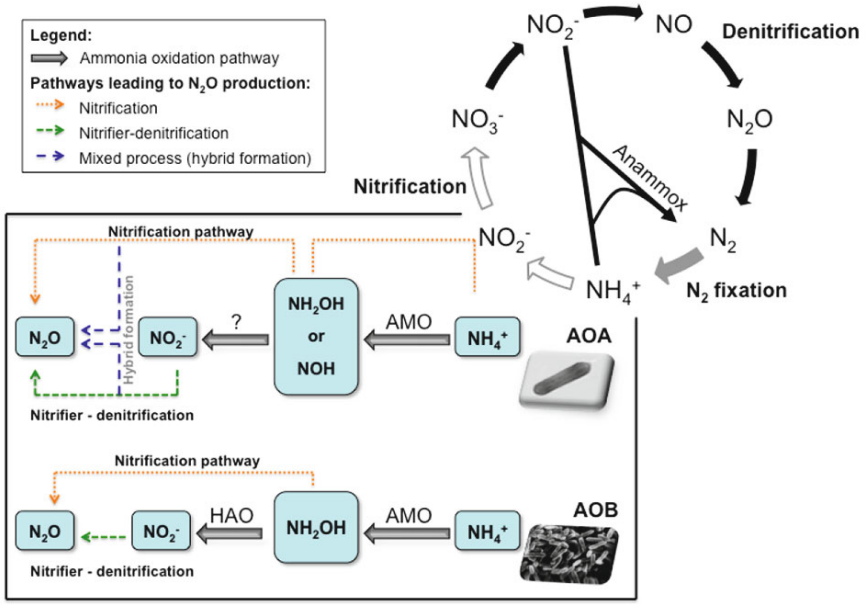

Fig. 1. Schematic illustration of the key processes involved in the nitrogen cycle emphasizing the AOA and AOB aerobic ammonia oxidation pathway within nitrification. While bacterial ammonia oxidation is performed by the oxidation of ammonia into hydroxylamine by AMO activity and then re-oxidized into nitrite by HAO enzyme (hydroxylamine oxireductase), the archaeal one is still in debate, once some enzymes (eg, HAO homologue) are still unidentified. However, recent studies demonstrated the formation of hydroxylamine (NH_2OH) by archaeal AMO enzyme, being the process coupled to an O_2 uptake. Once no HAO homologue was identified until now, the question marks indicate the still unknown pathway. N_2O production was shown to occur in AOA, however many ways of this production are under debate. Recently, three ways were proposed: through the oxidation of NH_4^+ or another intermediate (nitrification pathway); through nitrifier-denitrification pathway and through a mixed process between the two last pathways. In bacteria, this step can occur by a nitrifier-denitrification pathway or by the oxidation of hydroxylamine. Adapted from Jung *et al.* (2013).

who observed a rapid decrease of ammonia concentrations in sewage solutions (Fig. 2). At this time the idea that nitrification was due to the action of ferment was advanced (Müller, 1873) and further confirmed by Schloesing and Müntz (1877). In 1878, more exhaustive experiments (Warington, 1878) provided the first insights on how the rates of nitrification are affected by oxygen ("*air*"), light and temperature. Additionally, this author showed that nitrification was a two-step process (Fig. 2; Warington, 1891). In 1890, Winogradsky confirmed the role of bacteria on this process by the isolation of an ammonia oxidizing organism, and ascertained the chemoautotrophic mode of life (Winogradsky, 1890). Despite the difficulties in growing pure cultures of these organisms, several ammonia oxidizing bacteria (AOB) were isolated from soil samples of different continents, including the isolation of *Nitrosomonas europaea* (Winogradsky, 1890; Omeliansky, 1899; Winogradsky, 1904). Today *N. europaea* is the most studied ammonia-oxidizing species and has been used for decades as an important model organism to understand aerobic ammonia oxidation at physiological, biochemical, taxonomical, molecular and phylogenetic levels (e.g. Clark and Schmidt, 1967; Ritchie and Nicholas, 1974; Bhandari and Nicholas, 1981; Abeliovich and Vonshak, 1992; Juliette *et al.*, 1993; McTavish *et al.*, 1993; Bergmann and Hooper, 1994; Hommes *et al.*, 1994; Kowalchuk and Stephen, 2001; Beaumont *et al.*, 2002; Bock and Wagner, 2006; Koops *et al.*, 2006; Arp *et al.*, 2007).

AOB were initially classified based on morphological criteria, particularly on the arrangement of internal membranes of the available isolates (Bock *et al.*, 1986). Head *et al.* (1993) were the first to classify AOB from PCR-amplified 16S rRNA gene sequences from pure cultures of representative strains (Fig. 2). Since then, the 16S rRNA gene has been widely used to assess the phylogeny of AOB. So far, there are two recognized lineages of AOB within the Proteobacteria class. One of the lineages is affiliated with the Betaproteobacteria subdivision and includes genera *Nitrosospira*, *Nitrosomonas*, and one representative of the *Nitrosococcus* genera (*N. mobilis*). The other lineage is placed within the Gammaproteobacteria subdivision, and includes the remaining members of the *Nitrosococcus* genera (Teske *et al.*, 1994; Pommerening-Röser *et al.*, 1996; Stephen *et al.*, 1996; Purkhold *et al.*, 2000).

Even though ammonia oxidizing isolates were available since the end of the 19th century, studies on their diversity within natural nitrifying populations only began when culture-independent molecular technologies became available for microbial environmental studies (Fig. 2). The use of the 16S rRNA gene to analyse AOB communities was initially reported in water samples from estuarine, lacustrine and basin systems (Stehr *et al.*, 1995; Voytek and Ward, 1995). This achievement was possible through the development of group-specific primers for the 16S rRNA gene, which enabled the detection and characterization of natural ammonia-oxidizing communities (Fig. 2) (McCaig *et al.*, 1999; Bano and Hollibaugh, 2000; Bothe *et al.*, 2000; Burrell *et al.*, 2001; Hollibaugh *et al.*, 2002; Freitag and Prosser, 2003; Magalhaes *et al.*, 2007). Following studies expanded the number of different AOB 16S rRNA gene sequences, allowing the establishment of more refined and robust evolutionary relationships between species (Purkhold *et al.*, 2003). The surprising prevalence of uncultured AOB species in natural environments (Zehr and Ward, 2002) raised great curiosity concerning the actual diversity and function of these organisms in nitrification. Thus, besides the use of the 16S rRNA gene, other molecular markers started to be employed (Rotthauwe *et al.*, 1997; Purkhold *et al.*, 2000, 2003). The increasing interest in linking nitrification with diversity of ammonia-oxidizing microorganisms encouraged the use of probes based on enzymes or genes directly involved in ammonia oxidation (Gieseke *et al.*, 2001; Cebron *et al.*, 2003; Francis *et al.*, 2003; Horz *et al.*, 2004; O'Mullan and Ward, 2005). Ammonia monooxygenase (AMO) is a transmembrane enzyme responsible for the conversion of am-

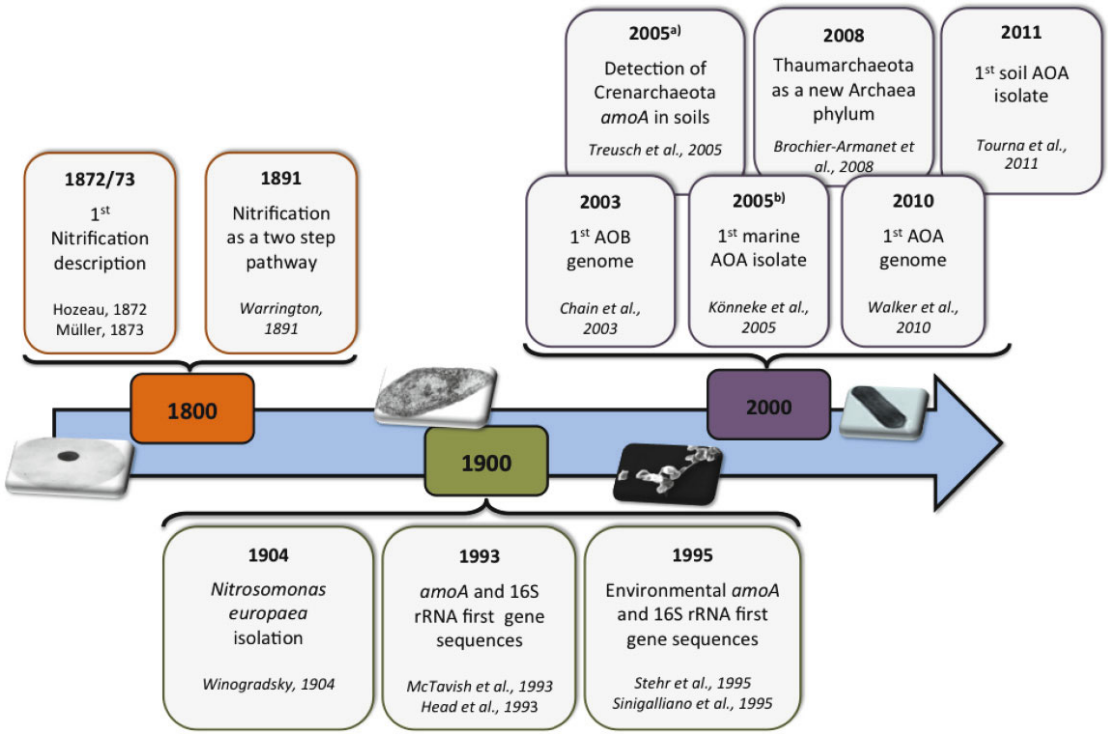

Fig. 2. Historical timeline with the important discoveries on nitrification. Figure highlights the discoveries made on aerobic ammonia oxidation and its respective microorganisms.

monia into hydroxylamine (Hyman and Arp, 1992), with a relevant role in the metabolism of AOB. The operon encoding this enzyme comprises three genes (*amoC*, *amoA*, and *amoB*) (Chain *et al.*, 2003), which exist in 2–3 copies in all the ammonia-oxidizing Betaproteobacteria strains and only in a single copy in Gammaproteobacteria (Norton *et al.*, 2002). After being sequenced (McTavish *et al.*, 1993) and retrieved from natural ammonia-oxidizing populations (Sinigalliano *et al.*, 1995) (Fig. 2), the gene coding for the AMO active site (*amoA*) in *Nitrosomonas europaea*, has been greatly exploited as a molecular marker for investigating AOB diversity in natural systems (Rotthauwe *et al.*, 1997; Gieseke *et al.*, 2001; Cebron *et al.*, 2003; Horz *et al.*, 2004; O'Mullan and Ward, 2005). The fact that this gene encodes a part of a key enzyme for AOB metabolism, displaying a high specificity and diversity, promotes a fine-scale resolution analysis within closely related AOB populations (Rotthauwe *et al.*, 1997). In fact, the presence of a non-conserved region in *amoA* peptides is suitable to discriminate the two different classes of AOB as well as ammonia and methane oxidizers (Alzerreca *et al.*, 1999). Nevertheless, although the phylogeny based on 16S rRNA and *amoA* genes sequences were similar (Purkhold *et al.*, 2000, 2003), the tree topology based on 16S rRNA gene sequences was found to have greater resolution (Purkhold *et al.*, 2003). Besides *amoA*, other genes have been proposed as potential molecular markers for AOB, including the gene encoding for hydroxylamine oxidoreductase enzyme (*hao*) (Schmid *et al.*, 2008), the *amoB* sub-unit of ammonia monooxygenase (Calvo and Garcia-Gil, 2004), and the intergenic spaces between the *amo* genes subunits (Alzerreca *et al.*, 1999). Unfortunately, the *hao* gene is not exclusive of ammonia oxidizers (Bergmann *et al.*, 2005), and the still low availability of *amoB* and *amoC* gene sequences on genome databases (Junier *et al.*, 2010) discourage the use of those genes as AOB molecular markers. The use of functional genes as molecular markers in environmental studies enables the establishment of connections between the presence of a given organism/community with *in situ* measurements of biogeochemical transformations. In fact, the necessity of combining biogeochemical measurements with functional molecular markers, by integrating multiple disciplines and methodologies, became a priority to fully understand the ecosystem-level importance of the different taxa responsible for nitrification (McCaig *et al.*, 1999; Caffrey *et al.*, 2003; Cebron *et al.*, 2003; O'Mullan and Ward, 2005; Fernandez-Guerra and Casamayor, 2012).

The attempts to directly quantify AOB have been initiated with *in situ* fluorescence hybridization (FISH) (Schramm *et al.*, 1999) and results revealed that AOB represented only a small fraction (0.1–2%) of the total microscopic bacterial counts (Altmann *et al.*, 2003; Schramm, 2003; Urakawa *et al.*, 2006). Subsequently, real-time PCR has proved to provide a highly sensitive method for enumerating the relatively low AOB numbers in natural environments and has become widely used to quantify AOB *amoA* in complex microbial communities (Hermansson and Lindgren, 2001; Harms *et al.*, 2003; Limpiyakorn *et al.*, 2005).

However, these more precise quantification studies reinforced the still unanswered question regarding the sole res-

ponsibility of AOB for such a critical biogeochemical process (Hermansson and Lindgren, 2001; Harms *et al.*, 2003; Schramm, 2003; Limpiyakorn *et al.*, 2005; Urakawa *et al.*, 2006). In addition, the fact that ammonia oxidation was even measured in oligotrophic environments where NH_4^+ concentrations were below the affinity threshold expected from kinetic studies of AOB in pure cultures (Olson, 1981; Hashimoto *et al.*, 1983), raised many questions regarding how these rare and slow growing bacteria could be the only ones responsible for this nitrification pathway, in environments with limiting concentrations of ammonia (Auguet *et al.*, 2011).

New discoveries on archaeal *vs* bacterial ammonia oxidation

The low abundance of AOB in natural environments, contrasting with the prevalence of nitrification rates in a wide range of environments (including oligotrophic environments) presented a paradigm, that was only solved when pioneering studies confirmed the nitrifying potential of non-thermophilic Crenarchaeota. Sets of genes encoding ammonia monooxygenase (AMO) were detected in a 1.2 Gb fosmid library of a sandy soil ecosystem (Treusch *et al.*, 2005). The parallel identification of these *amoA*-like genes in the Sargasso Sea metagenomic database (Venter *et al.*, 2004; Treusch *et al.*, 2005), showing high protein level similarities to those of soil Crenarchaeota, suggested that both soil and marine non-thermophilic Crenarchaeota might use ammonia as their primary energy source (Schleper *et al.*, 2005; Treusch *et al.*, 2005). Those studies were followed by the isolation of the first ammonia-oxidizing MG-1 archaeon, *Nitrosopumilus maritimus*, from a seawater aquarium (Konneke *et al.*, 2005) (Fig. 2). The discovery of chemoautotrophic AOA highlighted the possible importance of these microorganisms in the biogeochemical transformation of N, triggering a global scientific interest.

These recent discoveries led to the rearrangement of the previous established evolutionary relationships between archaeal species by considering a third archaeal phylum, the Thaumarchaeota, comprising all the ammonia-oxidizing archaea (Brochier-Armanet *et al.*, 2008). The addition of this phylum to the Archaeal domain was reinforced by the analysis of ribosomal protein markers of the *Cenarchaeum symbiosum* genome (Brochier-Armanet *et al.*, 2008), which so far can only be cultivated in association with its host, under controlled conditions (Hallam *et al.*, 2006a; Schleper and Nicol, 2010). High resolution analysis of ribosomal proteins indicated a new phylogenetic position for *C. symbiosum* and its mesophilic Crenarchaeota relatives in a robust branch located prior to separation between Euryarchaeota and Crenarchaeota (Brochier-Armanet *et al.*, 2008). The genome sequences of *N. maritimus* (Walker *et al.*, 2010) and *Nitrososphaera gargensis* (Hatzenpichler *et al.*, 2008; Spang *et al.*, 2012) supported the establishment of this new phylum as an ancient lineage of the Archaea domain (Spang *et al.*, 2010, 2012), confirming the rearrangement of the Archaea phylogenetic tree (Spang *et al.*, 2010; Brochier-Armanet *et al.*, 2012). In addition to the genomic differentiation, the presence of a unique membrane lipid-crenarchaeol- in all Thaumarchaeota representatives is a good biomarker for AOA (Zhang *et al.*, 2006; de la Torre *et al.*, 2008; Schouten *et al.*, 2008; Pitcher *et al.*, 2009; Pester *et al.*, 2011; Damste *et al.*, 2012).

Meanwhile, the first AOA genome (*Nitrosopumilus maritimus*) was published (Walker *et al.*, 2010) (Fig. 2) and followed by genome sequences of new isolates and enriched cultures (de la Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008; Blainey *et al.*, 2011; Kim *et al.*, 2011; Tourna *et al.*, 2011; Mosier *et al.*, 2012), which provide important insights to characterize the AOA physiological pathway. All together, these studies suggest a significant difference in the pathway of ammonia oxidation between AOA and AOB, which was confirmed by the dissimilarity of their ammonia monooxygenase (AMO) gene sequences (Walker *et al.*, 2010). While AOB oxidizes ammonia to hydroxylamine (NH_2OH) by the AMO enzyme, and reoxidizes it to NO_2^-, through the hydroxylamine oxidoreductase (HAO) enzyme, there is no evidence of genes encoding the latter enzyme in archaeal ammonia oxidation. Hence, either a novel uncharacterized enzyme is responsible for archaeal ammonia oxidation to hydroxylamine (Schleper and Nicol, 2010; Hatzenpichler, 2012), or the oxidation of ammonia would result in other product

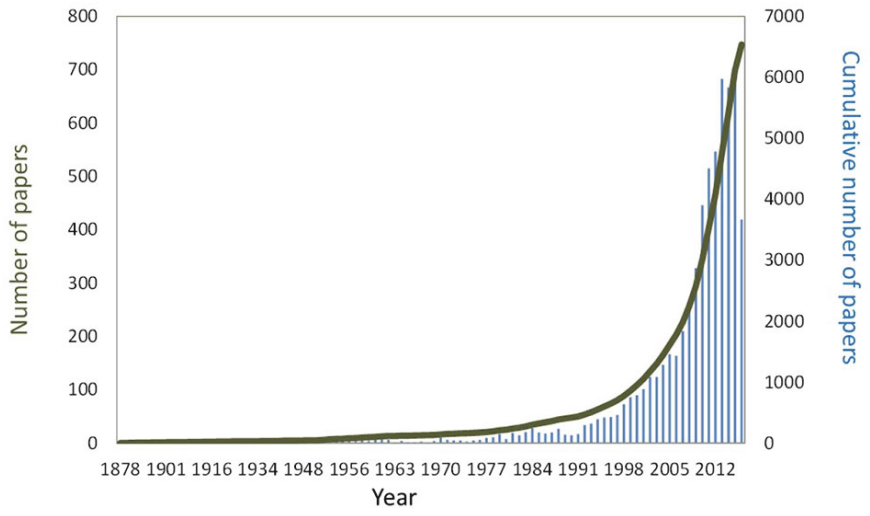

Fig. 3. Evolution of the number of papers published per year from 1872 to 31st of May, 2014. The graph was constructed using the ISI Web of Knowledge, Science Direct, Pub Med, ProQuest Research, B-on and Scopus platforms, with the topics ammonia oxidizing archaea, ammonia-oxidizing bacteria, microbial nitrification, archaeal ammonia oxidation and bacterial ammonia oxidation. Overlapping papers were cleaned from the database.

than hydroxylamine. Nitroxyl hydride (HNO) was proposed as a candidate product by Walker *et al.* (2010), but recent studies using combined physiological and stable isotope tracer analysis confirmed not only the production of hydroxylamine, but also its consumption during the oxidation of NH_4^+ to NO_2^- by *N. maritimus* (Vajrala *et al.*, 2013). Hydroxylamine is then the most likely product of the archaeal AMO homolog, despite the phylogenetic divergence that separates these organisms from AOB (Vajrala *et al.*, 2013). Other metabolic differences between AOA and AOB rely on a high number of copper-containing proteins in AOA, as well as an absence of cytochrome c proteins (Walker *et al.*, 2010). All together, these findings reinforce the fact that these two groups possess distinct metabolic pathways regarding ammonia oxidation (Walker *et al.*, 2010; Blainey *et al.*, 2011). Ecophysiological studies of ammonia-oxidizing Thaumarchaeota also suggest that substrates other than ammonia may be used as an energy source by some members of the phylum (Hallam *et al.*, 2006b; Tourna *et al.*, 2011; Alonso-Saez *et al.*, 2012; Hatzenpichler, 2012). In fact, the *N. maritimus* genome encodes genetic machinery allowing it to use organic compounds as energy source (Walker *et al.*, 2010). This is in agreement with studies performed in soil and WWTPs (waste water treatment plants), showing that not all Thaumarchaeota are ammonia-oxidizing chemoautotrophs (besides carrying *amoA*) and proposing the existence of a heterotrophic or mixotrophic metabolism (Jia and Conrad, 2009; Mußmann *et al.*, 2011).

The discovery of a chemoautotrophic archaeon with the capacity to promote the first and rate limiting step of nitrification (Konneke *et al.*, 2005) led to a reassessment of bacterial *vs* archaeal ammonia oxidation in a wide range of natural ecosystems, supporting a rapid increase in the number of published papers on this topic (Fig. 3) and an unprecedented explosion in available *amoA* sequences in databases (Fig. 4). These studies documented a widespread distribution of AOA in natural and managed soils (Leininger *et al.*, 2006), in diverse marine and estuarine waters and sediments (Beman and Francis, 2006; Dang *et al.*, 2008; Magalhães *et al.*, 2009), in wastewater treatment plant bioreactors (Park *et al.*, 2006), polar environments (Kalanetra *et al.*, 2009; Magalhães *et al.*, unpublished), hot springs (Hatzenpichler *et al.*, 2008) and many other environments. Recent studies focusing on the global diversity of AOA revealed a surprising differentiation of these organisms within different habitats (Biller *et al.*, 2012; Fernandez-Guerra and Casamayor, 2012; Stahl and de la Torre, 2012). These studies indicated that selective pressures might be responsible for the differentiation of AOA populations among different environments (Biller *et al.*, 2012), highlighting a specific habitat phylogeny association within AOA (Fernandez-Guerra and Casamayor, 2012).

Studies focusing on the quantification of bacterial *vs* archaeal *amoA* genes and on their relative contributions to nitrification are also emerging (Leininger *et al.*, 2006; Caffrey *et al.*, 2007; Nicol *et al.*, 2008; Santoro *et al.*, 2008; Jung *et al.*, 2014). High numbers of AOA have been observed in many systems (up to 20% of the total bacteria plus archaea in a sample), with a prevalence of AOA over AOB *amoA* gene copy numbers (Betaproteobacteria AOB) (Leininger *et al.*, 2006; Wuchter *et al.*, 2006; Nicol *et al.*, 2008). However, some researchers have reported a dominance of AOB in different agricultural soils and in coastal and estuarine sediments (Mosier and Francis, 2008; Santoro *et al.*, 2008; Magalhães *et al.*, 2009; Zhang *et al.*, 2009). It is important to note that most of these studies were based on AOA and AOB *amoA* quantifications, and these abundances alone did not provide information about the relative contribution of AOA and AOB for ammonia oxidation, since the genes might not have been expressed or its transcript/enzyme might have been inactivated (Di *et al.*, 2009; Mußmann *et al.*, 2011; Prosser and Nicol, 2012). Thus, inferences stating a higher contribution of archaea or bacteria for nitrification, based only in *amoA* quantifications, should be carefully analysed. Other approaches are required to ascertain the activity of ammonia oxidizers, such as quantification of *amoA* transcripts (Gubry-Rangin *et al.*, 2010; Baker *et al.*, 2012; Pedneault *et al.*, 2014), the use of DNA-stable isotope probing (SIP) (Pratscher *et al.*, 2011; Lu and Jia, 2012; Zhang *et al.*, 2012), CARD-FISH targeting *amoA* mRNA (Pratscher *et al.*, 2011) or the recent developed NanoSIMS (Wagner, 2009; Tourna *et al.*, 2011)

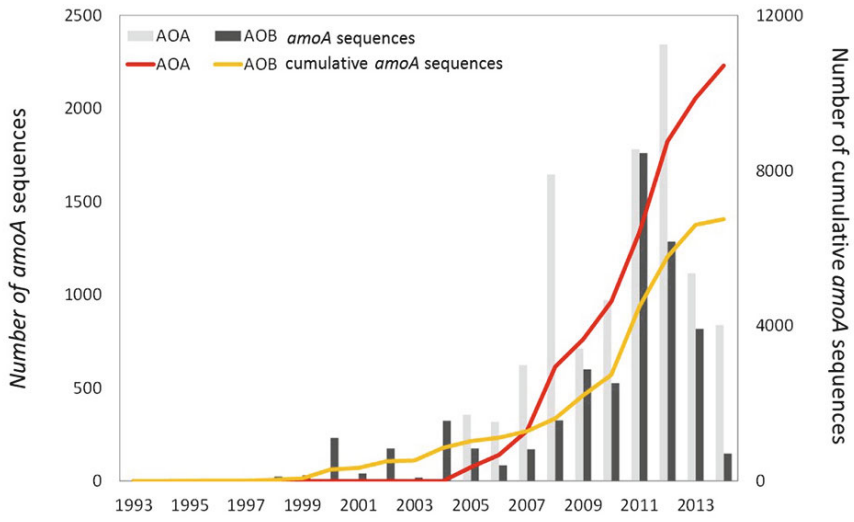

Fig. 4. Evolution of the number of bacteria and archaeal *amoA* sequences that have been submitted to the National Center for Biotechnology Information (NCBI) database. Topics used for the advance search were: "ammonia oxidizing archaea" or "ammonia oxidizing bacteria" (all fields), *amoA* (gene name) and archaea or bacteria (organism).

and single-cell sequencing approaches for microbial studies (Blainey *et al.*, 2011; Luo *et al.*, 2014). The potential use of selective AOA and/or AOB inhibitors to distinguish AOA *vs* AOB activity has been proposed (Santoro *et al.*, 2010; Martens-Habbena and Stahl, 2011; Yan *et al.*, 2012; Shen *et al.*, 2013), but the efficiency of the inhibitors in natural complex samples requires further validation, since they were mostly tested in pure cultures (Martens-Habbena and Stahl, 2011; Shen *et al.*, 2013). Acetylene, a suppressor of total nitrification, was found to cause total inhibition of AOA *in situ* due to its inhibitory action on the AMO enzyme (Offre *et al.*, 2011). Other studies have provided direct evidence of the nitrogen radical scavenger 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), the antibiotics sulfathiazole and sufadiazine, N1-guanyl-1,7-diaminoheptane (GC7) and allylthiourea as selective inhibitors of AOA and AOB (Schauss *et al.*, 2009; Santoro *et al.*, 2010; Martens-Habbena and Stahl, 2011; Löscher *et al.*, 2012; Yan *et al.*, 2012; Shen *et al.*, 2013).

The fact that AOB and AOA coexist and have to compete for the same energy source, promoted an ecological segregation of those two domains. Environmental factors are the main forces that dictate AOA and AOB population dynamics, with a major role in shaping the niche distribution and functionality of these communities (Biller *et al.*, 2012; Fernandez-Guerra and Casamayor, 2012). Substrate concentration is known to play an important role in the relative abundance and distribution of AOA *vs* AOB, with a dominance of AOA in low NH_4^+ concentration and oligotrophic environments (Wuchter *et al.*, 2006; Martens-Habbena *et al.*, 2009; Santoro *et al.*, 2010; Martens-Habbena and Stahl, 2011). Pure culture studies showed a higher affinity for NH_4^+ by *N. maritimus* SCM1 AMO enzyme and a higher capacity to grow in depleted NH_4^+ environments (Martens-Habbena *et al.*, 2009). Other AOA enriched cultures have shown tolerance for higher NH_4^+ concentrations (Tourna *et al.*, 2011); however, those concentrations still remain low for the growth of most AOB (Koops *et al.*, 2006). It is also well accepted that specific AOA ecotypes may prefer terrestrial systems with low pH (Gubry-Rangin *et al.*, 2010; Yao *et al.*, 2011; He *et al.*, 2012), being more transcriptionally active than AOB and thus the major contributors for nitrification (Zhang *et al.*, 2012). The fact that Thaumarchaeota dominate ammonia oxidation in acidic soils goes along with the low ammonia concentrations that characterize those soils (He *et al.*, 2012). Moreover, the recent identification of genes coding for ureases in AOA could afford greater advantages for ammonia oxidation in those types of soils (Lu and Jia, 2012). In addition to pH, salinity has been identified as a potential environmental regulator of AOA and AOB habitat selectivity, with higher salinities favoring numerical dominance of AOB over AOA in coastal and estuarine sediments (Santoro *et al.*, 2008; Magalhães *et al.*, 2009). Oxygen concentration may also play a key role in shaping the distribution of AOB and AOA. Besides the identification of AOA in the oxygen minimum zone (OMZ) (Lam *et al.*, 2007) and suboxic water columns (Francis *et al.*, 2005), dominance of AOA over AOB was also reported in waters with lower levels of oxygen (<10 μM) (Molina *et al.*, 2010). Moreover, kinetic studies in pure and enrichment cultures confirmed that the archaeal AMO had higher affinities to O_2 than AOB, indicating a better adaptation by AOA to environments with low O_2 availability (Park *et al.*, 2006, 2010; Martens-Habbena and Stahl, 2011).

The prevalence of AOA over AOB in the oceanic OMZ of Eastern Tropical South Pacific (ESTP), was also found to be strongly related to the production of the greenhouse gas nitrous oxide (N_2O) a by-product of archaeal nitrification (Santoro *et al.*, 2011; Löscher *et al.*, 2012).

The suggestion that AOA is largely responsible for marine N_2O emissions was extended to terrestrial ecosystems by identifying the role of AOA in N_2O production in these environments (Jung *et al.*, 2011; Ali *et al.*, 2013; Jung *et al.*, 2014). Thus, the recent implication of AOA in N_2O production should now be taken in account when predicting the contribution of natural ecosystems to global atmospheric N_2O fluxes (Jung *et al.*, 2014). Nevertheless, there is still little knowledge regarding the relative contribution of AOA *vs* AOB for N_2O production. Therefore, the use of selective inhibitors for AOB and AOA could provide an important tool for differentiating the activity of both groups regarding N_2O emissions (Santoro *et al.*, 2011; Löscher *et al.*, 2012; Ali *et al.*, 2013; Jung *et al.*, 2014). Unlike AOB, the AOA metabolic pathway for N_2O production is still under debate. Genomic studies in *N. maritimus*, as well as in environmental samples, identified the presence of *nirK* genes (Bartossek *et al.*, 2010; Walker *et al.*, 2010) which, along with isotopic studies, led to the hypothesis that in AOA N_2O was produced by the nitrifier-denitrification pathway (Jung *et al.*, 2011; Santoro *et al.*, 2011). However, AOA lacks nitric oxide reductase (NOR), involved in nitrifier-denitrification, and *Nitrosopumilus maritimus* and *Nitrososphaera viennensis* showed no increase in N_2O production under different oxygen levels, indicating that AOA is probably not capable of nitrifier-denitrification (Stieglmeier *et al.*, 2014). On the other hand, the results of Löscher *et al.* (2012) point to the production of N_2O through the oxidation of NH_4^+ to NO_2^-, probably through an unknown intermediate. Later, with the identification of NH_2OH as an intermediate in the NH_4^+ oxidation process in *N. maritimus* (Vajrala *et al.*, 2013), it was again proposed that *N. maritimus* would produce N_2O through NH_4^+ oxidation, but not from the reduction of NO_2^- (Vajrala *et al.*, 2013). Jung *et al.* (2014) reinforced, through the use of combined isotopic signatures, that N_2O production was due to both ammonia oxidation and nitrifier-denitrification metabolic pathways. However, recent stable isotope experiments with AOA pure cultures showed that N_2O production might occur during aerobic ammonia oxidation, from nitrite and an intermediate of ammonia oxidation, probably through a hybrid formation mechanism (i.e. the two nitrogen atoms of N_2O originate from distinct N sources) (Stieglmeier *et al.*, 2014).

Although there have been some attempts to evaluate the ecological variables that shape the dynamics of natural AOA and AOB populations, there are still large gaps concerning the factors that control selection of AOA *vs* AOB in diverse ecosystems (Hatzenpichler, 2012). Filling these gaps should help in predicting how future environmental changes could affect the distribution and metabolic function of the aerobic ammonia oxidizing bacteria and archaea, and how these

changes might alter the overall N cycle. Multidisciplinary studies are starting to appear, making use of genome and metagenomic analyses with contextualized environmental data as well as controlled environmental experiments (mesocosms). These new frameworks, together with newly cultivated representatives and enrichment cultures from different environments (de la Torre *et al.*, 2008; Blainey *et al.*, 2011; Jung *et al.*, 2011; Tourna *et al.*, 2011; Mosier *et al.*, 2012; Lebedeva *et al.*, 2013) will provide a more robust basis to identify key environmental drivers of AOA and AOB distribution, speciation and activity (Prosser and Nicol, 2012).

Concluding remarks and future perspectives

In this mini-review we aimed to succinctly highlight the most relevant milestones regarding the knowledge of aerobic ammonia-oxidizers. A chronological perspective of our knowledge on the aerobic ammonia oxidation pathway is given, highlighting the role of culture-independent methods in our understanding of these organisms. Nevertheless, some major issues still need to be addressed, especially regarding the metabolic machinery of AOA and the genes encoding a bacterial HAO-like enzyme. The development of accurate methodologies to differentiate AOA and AOB activities in natural habitats, and the isolation of new AOA and AOB are also urgently needed, to provide information about the effect of environmental factors driving AOA and AOB speciation and activity worldwide. A great improvement is expected in the promising field of metagenomics and metatranscriptomics by retrieving and analysing massive sequencing data from environmental samples and characterizing novel species and functions. As an example of the effort that is being invested in the potential of metagenomics to unravel the "known unknowns" in the microbial world, the Ocean Sampling Day (OSD), integrated in the Micro B3 Project, coordinate a massive global sampling event spatially, chronologically and environmentally synchronized. Metagenomic approaches provide an inventory of all genes present in a given habitat, and thus, combined with proteomic and transcriptomic studies, will certainly provide better resolution to infer the functionality and/or adaptation of natural ammonia oxidizing communities. In conclusion, the recent scientific effort coupled with a fast growing array of technologies currently being applied to this area of research, are expected to provide new insights in order to solve questions related to the unique intrinsic features of AOA and AOB, and their implication in niche differentiation and relative contribution to the global nitrogen cycle.

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