Ammonia oxidation: Ecology, physiology, biochemistry and why they must all come together

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Abstract

Ammonia oxidation is a fundamental core process in the global biogeochemical nitrogen cycle. Oxidation of ammonia (NH_3) to nitrite (NO_2^-) is the first and rate-limiting step in nitrification and is carried out by distinct groups of microorganisms. Ammonia oxidation is essential for nutrient turnover in most terrestrial, aquatic and engineered ecosystems and plays a major role, both directly and indirectly, in greenhouse gas production and environmental damage. Although ammonia oxidation has been studied for over a century, this research field has been galvanised in the past decade by the surprising discoveries of novel ammonia oxidising microorganisms. This review reflects on the ammonia oxidation research to date and discusses the major gaps remaining in our knowledge of the biology of ammonia oxidation.

Introduction

Microbial oxidation of ammonia to nitrite is a critical part of the global biogeochemical nitrogen cycle and the first step in nitrification. Nitrification is responsible for nitrogen loss and environmental damage through production of nitrate which is subject to leaching and via production of nitrous oxide (N_2O), a potent greenhouse gas. Ammonia oxidation contributes to N_2O emission by both direct production and by fuelling denitrification which generates N_2O. Anthropogenic nitrogen input has had a profound impact on the global nitrogen cycle: Annually 120 Tg of ammonia-based fertilisers are

applied globally. Ammonia oxidising microorganisms are central to the fate of the nitrogen in the environment. There are three distinct groups of aerobic autotrophic microorganisms that oxidise ammonia: ammonia oxidising bacteria (AOB), ammonia oxidising archaea (AOA) and comammox bacteria (complete oxidation of ammonia to nitrate). This review focuses on aerobic ammonia oxidation. Ammonia can also be oxidised under anaerobic conditions by anammox (anaerobic ammonium oxidation) bacteria which are phylogenetically and biochemically distinct from aerobic ammonia oxidisers and have been reviewed separately in this issue.

The first evidence that ammonia oxidation is a biological rather than a chemical process originated in a 1877 study by Schloesing and Muntz (Schloesing 1877), who demonstrated that soil-based nitrification was inhibited by application of chloroform. Frankland and Frankland were the first to cultivate ammonia oxidising bacteria in 1890 (Frankland 1890), and their pioneering study was soon followed by Sergei Winogradsky's isolation of *Nitrosomonas europaea*, which still remains the model ammonia oxidising bacterium in research today (Winogradsky 1890). Canonical AOB are restricted to two phylogenetic groups, β-proteobacteria (*Nitrosomonas* and *Nitrosospira*) and γ-proteobacteria (*Nitrosococcus)* (Purkhold 2000).

For over a century, AOB were assumed to be the sole drivers of nitrification in the environment. This view was transformed by the cultivation of the first ammonia oxidising archaeon *Nitrosopumilus maritimus* from a marine aquarium in 2005 (Könneke 2005). The discovery of ammonia oxidation in an entirely different domain of life was unanticipated and prompted a major re-assessment of the paradigm for ammonia oxidation. Isolation of *N. maritimus* was followed by the cultivation of many AOA, including representatives from e.g. neutral pH soil (*Nitrososphaera viennensis*), acidic soil (*Nitrosotalea devanaterra*), hot springs (*Nitrosocaldus yellowstoneii, Nitrososphaera gargensis*), fresh and brackish water (*Nitrosoarchaeum* and *Nitrosotenuis*) and wastewater treatment plants (*Nitrosocosmicus exaquare*) (Tourna 2011; Lehtovirta-Morley 2011; de la Torre 2008; Hatzenpichler 2008; Blainey 2011; Lebedeva 2013; Sauder 2017). AOA belong to the phylum Thaumarchaeota (previously known as non-extremophilic Crenarchaeota) (Brochier-Armanet 2008; Spang 2010), although not all archaea of this phylum are ammonia oxidisers (Weber 2015).

Since Winogradsky's seminal work, the central dogma of nitrification has stated that nitrification is a two-step process, where metabolic labour is strictly divided between two distinct groups of microorganisms: ammonia is first oxidised to nitrite by ammonia oxidising microorganisms and the nitrite is subsequently oxidised to nitrate by a different group of microorganisms, the nitrite oxidisers. The notion that the complete oxidation of ammonia to nitrate (comammox) could be carried out by a single microorganism was previously predicted from kinetic modelling (Costa 2006), but this dual process was only demonstrated in 2015, when the microorganisms performing comammox were discovered and cultivated (Daims 2015; van Kessel 2015). Microorganisms responsible for comammox belong to *Nitrospira*, a bacterial genus once considered to be specialised in nitrite oxidation (for a recent review on the re-evaluation of metabolic potential in *Nitrospira,* see Daims (2016)).

The ecology

Ammonia oxidising microorganisms are ubiquitous in the environment, including soils, freshwater and marine habitats, engineered ecosystems such as wastewater treatment plants and even human skin (Leininger 2006; Wuchter 2006; Mussmann 2011; Koskinen 2017). The abundance and diversity of ammonia oxidisers varies widely across environments and this is important for ecosystem functioning as different ammonia oxidisers respond differently to changes in the environment and for instance make different contributions to greenhouse gas emissions. For example, AOB produce more of the greenhouse gas N_2O than AOA (Hink 2016). AOB are more sensitive than AOA to certain commercial nitrification inhibitors, e.g. allylthiourea, which has consequences for agricultural management (Shen 2013; Lehtovirta-Morley 2013). AOB are also more resistant to drought than AOA, and might therefore be more resilient to certain environmental changes (Placella 2013).

AOA vastly outnumber AOB in most soil and aquatic environments, often by orders of magnitude (Leininger 2006). It is estimated that there are $1x10^{28}$ AOA cells in the Earth's oceans and they are some of the most numerous living organisms on Earth, accounting for up to 40% of all prokaryotes in marine ecosystems and 1-5% in terrestrial ecosystems (Karner 2001; Leininger 2006). In contrast,

AOB usually dominate numerically over AOA in wastewater treatment plants and occasionally in fertilised soils (Mussmann 2011; Bates 2011). Little is known about the abundance and diversity of comammox *Nitrospira*, although their abundance has been reported to be comparable to, or higher than the abundance of other ammonia oxidisers at least in some engineered ecosystems and soil environments (Hu 2017; Pjevac 2017).

Nearly everything that is known about the ecology of ammonia oxidisers has been discovered by examining the 16S rRNA gene and the *amoA* gene, which encodes for AmoA subunit of the ammonia monooxygenase, a key enzyme shared by all three groups of ammonia oxidisers. In general, β-proteobacterial AOB are found in soil environments and wastewater treatment plants and γ-proteobacterial AOB are typically found in marine habitats. A recently discovered terrestrial *Nitrosococcus* strain TAO100 is a notable exception to this (Hayatsu 2017) and γ-proteobacterial AOB have also been recently detected in an acidic wastewater treatment plant (Fumasoli 2017). Strain TAO100 was cultivated from an acidic soil, although metagenomic and amplicon sequencing datasets suggest that γ-proteobacterial ammonia oxidisers are rare in soil environments. Microorganisms of the genus *Nitrosospira* tend to dominate soil AOB communities and although *Nitrosomonas europaea* was originally cultivated from soil, representatives of the genus *Nitrosomonas* are typically less common in soil that those of the genus *Nitrosospira*.

Five main clusters of AOA have been proposed: *Nitrososphaera*, *Nitrosocosmicus* (previously *Nitrososphaera*-sister lineage), *Nitrosocaldus*, *Nitrosotalea* and *Nitrosopumilus* (which also comprises the genera *Nitrosoarchaeum*, *Nitrosotenuis* and *Nitrosopelagicus*) (Pester 2012). AOA communities in neutral pH soils are typically dominated by the genus *Nitrososphaera*, although *Nitrosoarchaeum* and *Nitrosocosmicus* are also present (Gubry-Rangin 2011). *Nitrososphaera* and *Nitrosotalea* are the most abundant AOA in acidic soils. *Nitrosocaldus* and *Nitrososphaera* are found in hot springs. Of the AOA belonging to the *Nitrosopumilus* cluster, *Nitrosopumilus* and *Nitrosopelagicus* are found in marine environments and *Nitrosoarchaeum* and *Nitrosotenuis* predominantly in freshwater (Santoro 2015). Importantly, AOA thrive in habitats inaccessible for the AOB, including acidic and high temperature environments. The cultivation of the world's first

obligately acidophilic ammonia oxidiser *Nitrosotalea devanaterra* from an acidic agricultural soil, and the cultivation of the thermophilic *Nitrosocaldus yellowstoneii* from a hot spring provided previously unsuspected explanations for nitrification in conditions where no known AOB are able to grow (Lehtovirta-Morley 2011, de la Torre 2008).

Little is known about the ecology of comammox *Nitrospira* in different environments. *amoA* sequences related to these organisms are present in neutral and acidic pH soils, aquatic environments and engineered ecosystems, but further studies are required to assess both their contribution to nitrogen cycling and their environmental distribution (van Kessel 2015; Daims 2015; Lawson 2018).

Environmental drivers of ammonia oxidation

There has been an extensive investigation into environmental factors which determine the ecological niches of different ammonia oxidisers and their relative contributions to nitrogen fluxes in the environment. Environmental pH and ammonia concentration consistently emerge as the two key drivers of diversity and abundance of ammonia oxidisers. There are also additional factors which influence the environmental distribution of ammonia oxidising microorganisms, including their different responses to organic compounds, metals, temperature, salinity and inhibition by light (Merbt 2012). For example, activity of AOA has been reported to be greater at higher temperatures in both soil and freshwater and temperature optima of cultivated AOA are typically higher than those of cultivated AOB (Tourna 2008, Zeng 2014; Tourna 2011; Hatzenpichler 2008). Some, but not all AOB and AOA can grow on urea, and the AOA *N. gargensis* is also able to grow on cyanate (Palatinszky 2015). In contrast, AOB *N. europaea* grows mixotrophically on fructose and pyruvate (Hommes 2003). Small organic acids like pyruvate also stimulate the growth of catalase-negative AOA by alleviating oxidative stress (Kim 2016). The ecological relevance of these traits is largely unexplored.

Ammonia concentration is a strong selector between different ammonia oxidisers (Verhamme 2011; Bates 2011). It has been repeatedly demonstrated that in soil, AOB prefer high ammonia concentration whereas AOA dominate when ammonia concentration is low. In marine ecosystems, ammonia concentrations are generally very low, ~10 nM, and more favourable for growth of AOA

rather than AOB (Martens-Habbena 2009). In general, there is a strong trend suggesting that AOA rather than AOB prefer low ammonia concentration. This pattern however is not clear-cut: for example, the recently cultivated novel genus of AOA, *Nitrosocosmicus*, is adapted to high ammonia concentration unlike most AOA (Lehtovirta-Morley 2016a; Sauder 2017). Although further studies are required on the environmental distribution and physiology of different strains of comammox *Nitrospira*, the current knowledge strongly suggests that comammox *Nitrospira*, like AOA, are adapted to low ammonia concentrations (Kits 2017; Costa 2006).

The bioavailable ammonia concentration and environmental pH are connected: ammonia exists in two different forms in the environment, non-protonated ammonia (NH_3) and its protonated form ammonium (NH_4^+) which are in a pH-dependent equilibrium (pK_a=9.27). In acidic conditions, the amount of available NH_3 is extremely low. A 1974 study by Suzuki and colleagues demonstrated that the AOB *Nitrosomonas europaea* is only able to use ammonia, rather than ammonium, which has long been considered the key reason why AOB are unable to grow in acidic environments. This study is still the cornerstone of ammonia oxidation research although it has not been repeated with other strains of AOB, AOA nor comammox *Nitrospira*. However, the inability to use ammonia in low pH does not exclude survival and activity of AOB in these conditions: AOB are able to grow in acidic conditions using urea as substrate and by growing in biofilms and aggregates (de Boer 1991, Burton 2001). The notion that ammonia oxidation is problematic at low pH was overturned by the discovery of the first obligately acidophilic ammonia oxidiser, *Nitrosotalea devanaterra* (Lehtovirta-Morley 2011). Originally isolated from an acidic agricultural soil, this archaeon grows autotrophically in the pH range of 4-5.5 in laboratory culture with ammonium chloride as its sole energy source. Although *Nitrosotalea* is the only obligately acidophilic ammonia oxidiser described to date, it is unlikely to be the only microorganism performing nitrification in acidic soils. Cultivation of acid-tolerant γ- and β-proteobacterial AOB has been reported (Hayatsu 2017; de Boer 1991). In addition to AOB which tolerate acidic conditions and are found in acidic soils, the archaeal genus *Nitrososphaera* is abundant in many acidic soils globally and has been reported to grow in acidic soil microcosms (Gubry-Rangin 2011; Wang 2014). Surprisingly little is known about nitrification in alkaline environments: although

alkaline pH selects for specific lineages of ammonia oxidisers and nitrification occurs in alkaline soils, no obligately alkalinophilic ammonia oxidising microorganism has yet been obtained in culture (Gubry-Rangin 2011; Shen 2008).

Key physiological, structural and biochemical differences between ammonia oxidisers

Environmental factors determine the structure and function of ammonia oxidising communities, but the mechanisms underpinning this selection are relatively poorly understood. There are fundamental differences in the physiology and cellular architecture of ammonia oxidising microorganisms which are likely to influence their distribution, their ability to adapt to their respective environments and their capacity for nitrogen turnover, although the consequences of these differences remain largely untested.

The difference in the ammonia oxidation kinetics and substrate affinities between AOB, AOA and comammox *Nitrospira* is considered to be one of the key explanations as to why different ammonia oxidisers dominate in contrasting environments. AOB have higher half-saturation constants (K_m) and lower substrate affinities than AOA and comammox *Nitrospira*, with $K_m(NH_3)$ ranging between 6-11 µM for AOB, 3.6 nM – 4.4 µM for AOA and 49 nM for the comammox bacterium *N. inopinata* (Martens-Habbena 2009; Kits 2017; Prosser and Nicol 2012). Greater affinity is advantageous when substrate concentration is low, and this has been generally reflected by molecular ecological surveys: AOA dominate over AOB in the open ocean and in low nitrogen soils, whereas AOB dominate in wastewater treatment plants with a high ammonium content. The ammonia oxidation kinetics of comammox *Nitrospira* reflect the original prediction that any comammox organism would be highly oligotrophic. The kinetic theory of the optimal pathway length suggested that the comammox process would lead to a higher yield but lower growth rate than incomplete ammonia oxidation, giving comammox organisms a competitive advantage when ammonia concentration is low (Costa 2006). It is interesting to note that the half-saturation constants of many AOA and comammox are roughly within the same range. It is currently not known how this affects the competition between AOA and comammox in the environment and what other factors influence their environmental distribution.

All three groups of ammonia oxidisers (AOB, AOA and comammox *Nitrospira*) are autotrophic but their inorganic carbon assimilation pathways are different. Both AOB and comammox *Nitrospira* fix atmospheric CO_2, AOB by the Calvin cycle and the comammox *Nitrospira* by reductive tricarboxylic acid cycle, whereas AOA fix HCO_3^- by the hydroxypropionate-hydroxybutyrate cycle (Berg 2007). Energetic requirements of these pathways different and the thaumarchaeal hydroxypropionate-hydroxybutyrate cycle is the single most efficient aerobic inorganic carbon assimilation pathway known(Könneke 2014). In addition, CO_2 and HCO_3^- are in a pH-dependent equilibrium and the concentration of HCO_3^- decreases with a decreasing pH. This is rather paradoxical given the existence of acidophilic AOA, and potentially implies that acidophilic AOA have a very high affinity for HCO_3^- in order to grow at low pH. The ecological and physiological consequences of these differences are largely unexplored. All known AOB have extensive intracytoplasmic membrane structures, but both AOA and comammox lack these membrane structures and apparently only have the plasma membrane (although some AOA, e.g. *Nitrosoarchaeum koreensis* MY1 and *Nitrososphaera viennensis*, have an additional intracellular structure which may be membrane-bound (Jung 2011; Stieglmeier 2014)). The amount of membrane is very important for ammonia oxidation, because ammonia monooxygenase, the key enzyme of ammonia oxidation is membrane-bound. It has long been assumed that the purpose of the extensive membranes found in AOB is to accommodate the maximum amount of ammonia monooxygenase (Fiencke 2006). Although this implies that a single AOB cell may contain more ammonia monooxygenase than an AOA or a comammox cell, this has never been tested. This is potentially supported by the facts that the V_{max} for ammonia oxidation mg protein^{-1} h^{-1} is roughly within the same range for AOA and comammox, but greater in the AOB *N. europaea* (Martens-Habbena 2009; Kits 2017) and the rate per cell is 10-fold greater in the AOB *N. multiformis* compared to the AOA *N. viennensis* (Kozlowski 2016b). While bacterial ammonia oxidisers have iron-based respiratory chains, archaea lack the cytochrome c proteins and instead have copper-based respiratory chains where multicopper oxidases and small blue copper-containing proteins are predicted to substitute for the missing cytochromes (Walker 2010).

The biochemistry

During ammonia oxidation, ammonia is oxidised to hydroxylamine by ammonia monooxygenase (AMO), a membrane-bound enzyme that belongs to a superfamily of ammonia, methane and alkane monooxygenases. AMO is a fundamentally important enzyme in ammonia oxidation and the only enzyme of the ammonia oxidation pathway which is shared by all three major groups of ammonia oxidising microorganisms. Furthermore, *amoA* (the gene encoding of the A subunit of the AMO) is overwhelmingly the most extensively used functional marker gene for ammonia oxidation and has been indispensable in understanding the ecology of ammonia oxidisers (Rotthauwe 1997; Purkhold 2000; Leininger 2006; Pjevac 2017). Despite the enormous importance of the AMO for global nitrogen cycling, surprisingly little is known about this enzyme and extrapolations are usually made from the related particulate methane monooxygenase (pMMO) which has been characterised much more thoroughly. pMMO is a heterotrimer and has a di-nuclear copper centre at its active site on the PmoB subunit (Lieberman 2005). pMMO is also reported to bind zinc at the variable metal binding site, which is coordinated by amino acid residues on PmoC subunit and located at the interface of PmoB and PmoC subunits. The amino acid residues coordinating both metal centres are highly conserved in all ammonia oxidisers. The AMO is likely to be a copper-dependent enzyme and its activity in *N. europaea* is stimulated by copper (Ensign 1993). In addition, all known ammonia oxidisers are inhibited by the copper chelator allylthiourea (Hatzenpichler 2008; Taylor 2010; Shen 2013; Lehtovirta-Morley 2013). Interestingly, the PmoB subunit of pMMO from the methane oxidiser *M. capsulatus* Bath is active without any additional subunits, but AmoB subunit of AMO from the AOA *N. yellowstoneii* is not (Balasubramanian 2010; Lawton 2014).

Archaeal AMO is very divergent from that of either AOB or comammox *Nitrospira*. The AMO of the comammox bacterium *N. inopinata* is predicted to be acquired by horizontal gene transfer, suggested by aberrant tetranucleotide word frequencies and by the flanking transposons (Daims 2015; Palomo 2017), but this is not the case for other comammox strains. The AMO operon is in *amoCAB* arrangement in bacterial ammonia oxidisers and often multiple copies are present (Fig. 1) (Arp 2007). Unlike bacteria, archaea do not use operons nor produce polycistronic transcripts. In some AOA,

AMO subunits are located together in *amoAXCB* arrangement and in other archaea, e.g. *Nitrososphaera* and *Nitrosocosmicus* AMO subunits are interjected by many open reading frames (Fig. 1). Some AOA contain multiple copies of isolated *amoA*, *amoB* and *amoC* subunits (Spang 2012; Bayer 2016; Herbold 2017). Comammox *Nitrospira* also harbour multiple copies of *amoC*. Archaeal AMO AmoB and AmoC subunits are truncated compared to both bacterial ammonia and methane oxidisers (Fig. 1). Ever since the first AOA genomes became available, there has been a lasting fascination with the archaeal "*amoX*", a short open reading frame found adjacent to the *amoA* gene on all sequenced AOA genomes which has been speculated to encode an additional subunit of the archaeal AMO and substitute for the missing domains of other subunits (Treusch 2005; Kerou 2016).

The orientation of the AMO active site (facing the cytoplasm or extracellular space) is of critical importance as it determines the conditions the enzyme has to function in and affects the subsequent oxidation of intermediates. Location of the active site is not known, but the evidence strongly favours an extracellular active site (Fig. 2) (Walker 2010; Lehtovirta-Morley 2016b). The related pMMO has an outward-facing active site (Lieberman 2005) and *in silico* predictions of protein folding also favour this orientation. In addition, in bacteria the next enzyme downstream in the pathway, the hydroxylamine dehydrogenase, is periplasmic and it is advantageous to exclude toxic, reactive intermediates such as hydroxylamine from the cytoplasm. This orientation is significant also because there may be a difference in the pH between the cytoplasm and the (pseudo-)periplasm.

Hydroxylamine and nitric oxide (NO) are intermediates in both bacterial and archaeal ammonia oxidation, but the enzymology differs between bacteria and archaea (Vajrala 2013; Martens-Habbena 2015; Caranto 2017). Both AOB and comammox organisms oxidise hydroxylamine using hydroxylamine dehydrogenase (HAO, formerly known as hydroxylamine oxidoreductase). Although hydroxylamine is an intermediate in archaeal ammonia oxidation, archaeal genomes lack any recognisable HAO homologues, implying that a novel enzyme must be responsible for the hydroxylamine oxidation step.

For several decades it was assumed that bacterial ammonia oxidation is a two-step pathway where ammonia is first oxidised into hydroxylamine by AMO and then into nitrite by HAO. This view was recently challenged by the discovery that NO, not nitrite, is the reaction product of the bacterial HAO (Fig. 2a) (Caranto 2017). It is interesting to note that anammox bacteria also have a homologue of HAO, which performs oxidation of hydroxylamine to NO, just like that of AOB. The discovery of NO as an obligate intermediate in AOB means that ammonia oxidising bacteria use a three-step ammonia oxidation pathway and likely have a third, unidentified enzyme catalysing the final step, conversion of NO into nitrite. Caranto and colleagues (2017) proposed that this missing enzyme may be NirK, although this remains currently untested (Fig. 2a). Until recently, bacterial NirK was assumed to be a nitrite reductase involved in denitrification of nitrite into NO. However, *N. europaea nirK* deletion mutant is still able to reduce nitrite (Kozlowski 2014) and *nirK* homologues have been previously demonstrated to perform both oxidation of NO into nitrite as well as reduction of nitrite to NO *in vitro* (Wijma 2004). Furthermore, *N. europaea* a *nirK* deletion mutant has a lower ammonia oxidation rate and a higher N_2O production rate than the wild-type, and multiple genes involved in NO detoxification are upregulated in the transcriptome of the *nirK* mutant compared to the wild-type (Cantera 2007, Cho 2006). There are, however, some caveats to proposing NirK as the missing NO oxidase: Some AOB e.g. *N. communis* lack *nirK* (Kozlowski 2016a) and the phylogeny of NirK shows multiple evolutionary origins in AOB. Therefore, a potential role of other enzymes in the oxidation of NO should not be excluded. One potential candidate gene is ncyA, encoding for red copper protein nitrosocyanin, found in many, but not all AOB. It is likely, although unproven, that the pathway of ammonia oxidation is conserved between AOB and comammox *Nitrospira*, as the homologues for genes encoding for AMO, HAO and NirK in AOB are conserved in the published genomes of comammox *Nitrospira*. AOA are inhibited by NO scavengers including PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) which in the recent years has been interpreted as evidence that NO is an intermediate in AOA pathway, but not in AOB (Martens-Habbena 2015; Kozlowski 2016b). However, in line with the recent discovery of NO as an obligate intermediate in ammonia oxidation by AOB, AOB are also inhibited NO scavengers albeit at higher concentrations than AOA (Shen 2013; Sauder 2016).

Over the years since the discovery of AOA there have been several competing theories regarding the novel enzymology of archaeal ammonia oxidation. One of the most recent theories proposed the involvement of a novel copper-containing enzyme donating five electrons and an alternative order of the pathway (Fig. 2b) (Kozlowski 2016b). Another recent study suggested that within this model, hydroxylamine is oxidised using a multicopper oxidase (MCO1) (Kerou 2016). This is theoretically possible, although MCO1 homologues are missing in the core genome of *Nitrosotalea* (Herbold 2017) and this scenerio would therefore necessitate the loss and re-evolution of a different hydroxylamine oxidation mechanism in this archaeal genus. Key observations supporting this alternative order of the pathway were the production and consumption of NO during the oxidation of hydroxylamine to nitrite and the fact that the isotopic signature of N_2O produced by the AOA *N. viennensis* indicates that one nitrogen in the N_2O molecule originates from ammonium and the other from nitrite (Kozlowski 2016b; Stieglmeier 2014).

The recent re-evaluation of the bacterial ammonia oxidation enzymology raises a very important question whether the archaeal pathway may proceed in the same order as that of AOB (Fig. 2c). This three-step pathway could also result in accumulation (and consumption) of NO as previously reported (Martens-Habbena 2015; Kozlowski 2016b) if the rate of hydroxylamine oxidation exceeds that of NO oxidation. This would be the case if the NO oxidising enzyme needs to be induced by the presence of NO and the reaction is oxygen-dependent. Further, nitrous acid and hydroxylamine have been reported to react to produce hybrid N_2O (with one nitrogen sourced from ammonium and the other from nitrite) as observed in the AOA *N. viennensis*. Given that nitrite and nitrous acid are in a pH-dependent equilibrium, it is interesting to note that AOA strains grown in low pH media produce proportionally much more N_2O than AOA strains in neutral pH, although it is unclear whether this is due to effects of pH or strain physiology (Jung 2014). In addition, bidirectional catalysis by nitrite reductase has been previously reported *in vitro* (Wijma 2004). This could potentially explain both the participation of NirK in both NO oxidation to nitrite and nitrite reduction to NO although this has not been explored in ammonia oxidising microorganisms nor demonstrated *in vivo*. Both hypothetical models (Fig. 2bc) of the archaeal ammonia oxidation pathway are plausible in that they would explain

all the observations made so far and both models would also result in net acquisition of two electrons. However, one line of evidence that potentially favours the new three-step model proposed here is that although most AOA possess *nirK* homologues, thermophilic AOA *N. yellowstoneii, N. islandicus* and *N. cavascurensis* as well as the marine sponge symbiont *C. symbiosum* lack *nirK* (Kerou 2017; Daebeler 2017; Abby 2018). Without NirK, it is difficult to explain how these organisms would generate the NO required for the hydroxylamine oxidation as proposed by Kozlowski and colleagues (2016). In the new three-step model, the lack of NirK would merely mean that the net yield would be one rather than two electrons, which would still be energetically viable. However, this is currently only another hypothetical model of archaeal ammonia oxidation pathway, and both this and the two-step model remain to be tested.

Conclusions and outlook

Recent years have seen exciting discoveries of new organisms involved in ammonia oxidation and provided a thorough dissection of the ecology and genomics of ammonia oxidisers. However, the mechanisms underpinning the environmental distribution and activity of these microorganisms are largely unexplored and many hypotheses generated by genomic analyses remain untested (Table 1). This review has discussed the possibility of a novel ammonia oxidation pathway in AOA, which is another hypothesis for testing in future studies. The recently obtained culture models of AOA and comammox *Nitrospira* provide a starting point to address how ammonia oxidising microorganisms adapt and respond to their environment. A better coordination of studies on the ecology, physiology and biochemistry of ammonia oxidation is required for a detailed and systematic understanding of these mechanisms and how they shape ammonia oxidising communities. New methodological innovations, such as mutagenesis systems for AOA and comammox *Nitrospira*, could also rapidly enhance the mechanistic understanding and help bridge the gap between the biochemistry and ecology of ammonia oxidation. Elucidating what happens at the cellular and molecular level can help us address major environment questions about the diversity, abundance and activity of ammonia oxidisers as well as their contribution to nitrogen losses and greenhouse gas production throughout different environments.

Conflicts of interest

The author declares no conflict of interest.

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References

Abby S, Melcher M, Kerou M, Krupovich M, *et al*. *Candidatus* Nitrosocaldus cavascurensis, an ammonia oxidizing, extremely thermophilic archaeon with a highly mobile genome. *Front Microbiol* 2018 (in press).

Arp DJ, Chain PS, Klotz MG. The impact of genome analyses on our understanding of ammonia-oxidizing bacteria. *Annu Rev Microbiol* 2007;**61**:503–528.

Balasubramanian R, Smith SM, Rawat S, Yatsunyk LA, *et al*. 2010. Oxidation of methane by a biological dicopper centre. *Nature* 2010;**465**:115–119.

Bates ST, Berg-Lyons D, Caporaso JG, Walters WA, *et al*. Examining the global distribution of dominant archaeal populations in soil. *ISME J* 2011;**5**:908–917.

Bayer B, Vojvoda J, Offre P, Alves RJE, *et al.* Physiological and genomic characterization of two novel marine thaumarchaeal strains indicates niche differentiation. *ISME J 2016;***10***:*1051–1063.

Berg IA, Kockelkorn D, Buckel W, Fuchs G. A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in archaea. *Science* 2007;**318**:1782–1786.

Blainey PC, Mosier AC, Potanina A, Francis CA, *et al*. Genome of a low-salinity ammonia-oxidizing archaeon determined by single-cell and metagenomic analysis. *PLoS ONE* 2011;**6**:e16626.

Brochier-Armanet C, Boussau B, Gribaldo S, Forterre P. Mesophilic crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. *Nat Rev Microbiol* 2008;**6**:245–252.

Burton SAQ, Prosser JI. Autotrophic ammonia oxidation at low pH through urea hydrolysis. *Appl Environ Microbiol* 2001;**67**:2952–2957.

Cantera J, Stein LY. Role of nitrite reductase in the ammonia-oxidizing pathway of *Nitrosomonas europaea*. *Arch Microbiol* 2007;**188**:349–354.

Caranto JD, Lancaster KM. Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. *Proc Natl Acad Sci USA* 2017;**114**:8217–8222.

Cho CMH, Yan T, Liu X, Wu L, *et al*. Transcriptome of a *Nitrosomonas europaea* Mutant with a Disrupted Nitrite Reductase Gene (nirK). *Appl Environ Microbiol* 2006;**72**:4450-4454.

Costa E, Pérez J, Kreft JU. Why is metabolic labour divided in nitrification? *Trends Microbiol* 2006;**14**:213–9.

Daebeler A, Herbold C, Vierheilig J, Sedlacek CJ, *et al*. Cultivation and genomic analysis of *Candidatus* Nitrosocaldus islandicus, a novel obligately thermophilic ammonia-oxidizing Thaumarchaeon. bioRxiv 2017;235028.

Daims H, Lebedeva EV, Pjevac P, Han P, *et al*. Complete nitrification by *Nitrospira* bacteria. *Nature* 2015;**528**:504–509.

Daims H, Lucker S, Wagner M. A new perspective on microbes formerly known as nitrite-oxidizing bacteria. *Trends Microbiol* 2016;**24**:699-712.

De Boer W, Gunnewiek PJ, Veenhuis M, Bock E, *et al*. Nitrification at low pH by aggregated chemolithotrophic bacteria. *Appl Environ Microbiol* 1991;**57**:3600–3604.

de la Torre JR, Walker CB, Ingalls AE, Konneke M, *et al*. Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. *Environ Microbiol* 2008;**10**:810-818.

Ensign SA, Hyman MR,Arp DJ. *In vitro* activation of ammonia monooxygenase from *Nitrosomonas europaea* by copper. *J Bacteriol* 1993;**175**:1971‑1980.

Fiencke C, Bock E. Immunocytochemical localization of membrane-bound ammonia monooxygenase in cells of ammonia oxidizing bacteria. *Arch Microbiol* 2006;**185**:99-106.

Frankland PF, Frankland GC. The nitrifying process and its specific ferment. Part I *Philos T Roy Soc B* 1890;**181**:107-28.

Fumasoli A, Burgmann H, Weissbrodt DG, Wells GF, *et al*. Growth of *Nitrosococcus*-related ammonia oxidizing bacteria coincides with extremely low pH values in wastewater with high ammonia content. *Environ Sci Technol* 2017;**51**:6857-6866.

Gubry-Rangin C, Hai B, Quince C, Engel M, *et al.* Niche specialization of terrestrial archaeal ammonia oxidizer. *Proc Natl Acad Sci USA* 2011;**108**:21206-21211.

Hatzenpichler R, Lebedeva EV, Spieck E, Stoecker K, *et al.* A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proc Natl Acad Sci USA* 2008;**105**:2134-2139.

Hayatsu M, Tago K, Uchiyama I, Toyoda A, *et al*. An acid-tolerant ammonia-oxidizing γ-proteobacterium from soil. *ISME J* 2017;**11**:1130-1141.

Herbold CW, Lehtovirta-Morley LE, Jung MY, Jehmlich N, *et al.* Ammonia-oxidising archaea living at low pH: Insights from comparative genomics. *Environ Microbiol* 2017;**19**:4939-4952.

Hu HW, He JZ. Comammox—a newly discovered nitrification process in the terrestrial nitrogen cycle. *J Soils Sediments* 2017;**17**:2709–2717.

Jung MY, Park SJ, Min D, Kin JS, *et al.* Enrichment and Characterization of an Autotrophic Ammonia-Oxidizing Archaeon of Mesophilic Crenarchaeal Group I.1a from an Agricultural Soil. *Appl Environ Microbiol* 2011;**77**:8635-8647.

Jung MY, Well R, Min D, Giesemann A, *et al.* Isotopic signatures of N_2O produced by ammonia-oxidizing archaea from soils. *ISME J* 2014;**8**:1115-1125.

Karner MB, DeLong EF, Karl DM. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* 2001;**409**:507–510.

Kerou M, Offre P, Valledor L, Abby SS, *et al.* Proteomics and comparative genomics of *Nitrososphaera viennensis* reveal the core genome and adaptations of archaeal ammonia oxidizers. *Proc Natl Acad Sci USA* 2016;**113**:e7937-e7946.

Kim JG, Park SJ, Sinninghe Damste JS, Schouten S, *et al*. Hydrogen peroxide detoxification is a key mechanism for growth of ammonia-oxidizing archaea. *Proc Natl Acad Sci USA* 2016;**113**:7888-7893.

Kits KD, Sedlacek CJ, Lebedeva EV, Han P, *et al*. Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. *Nature* 2017;5**49**:269-272.

Könneke M, Bernhard AE, de la Torre JR, Walker CB, *et al.* Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 2005;**437**:543-546.

Konneke M, Schubert DM, Brown PC, Hugler M, *et al*. Ammonia-oxidizing archaea use the most energy-efficient aerobic pathway for CO_2 fixation. *Proc Natl Acad Sci USA* 2014;**111**:8239-8244.

Koskinen K, Pausan MR, Perras AK, Beck M, *et al.* First Insights into the Diverse Human Archaeome: Specific Detection of Archaea in the Gastrointestinal Tract, Lung, and Nose and on Skin. *mBio* 2017;**8**:00824-17.

Kozlowski JA, Price J, LY Stein. Revision of N_2O-Producing Pathways in the Ammonia-Oxidizing Bacterium *Nitrosomonas europaea* ATCC 19718. *Appl Environ Microbiol* 2014;**80**:4930-4935.

Kozlowski JA, Kits KD, Stein LY. Genome Sequence of *Nitrosomonas communis* Strain Nm2, a Mesophilic Ammonia-Oxidizing Bacterium Isolated from Mediterranean Soil. *Genome Announc* 2016;4:e01541-15.

Kozlowski JA, Stieglmeier M, Schleper C, Klotz MG, *et al*. Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota. *ISME J* 2016;**10**:1836-1845.

Lawson CE, Lücker S. Complete ammonia oxidation: an important control on nitrification in engineered ecosystems? *Curr Opin Biotechnol* 2018;**50**:158-165.

Lawton TJ, Ham J, Sun T, Rosenzweig AC. Structural conservation of the B subunit in the ammonia monooxygenase/particulate methane monooxygenase superfamily. *Proteins* 2014;**82**:2263–2267.

Lebedeva EV, Hatzenpichler R, Pelletier E, Schuster N, *et al*. Enrichment and genome sequence of the group I.1a ammonia-oxidizing Archaeon "*Ca*. Nitrosotenuis uzonensis" representing a clade globally distributed in thermal habitats. *PLoS ONE* 2013;**8**:e80835.

Lehtovirta-Morley LE, Ross J, Hink L, Weber, EB, Gubry-Rangin C, *et al*. Isolation of '*Candidatus* Nitrosocosmicus franklandus', a novel ureolytic soil archaeal ammonia oxidiser with tolerance to high ammonia concentration. *FEMS Microbiol Ecol* 2016;**92**:fiw057.

Lehtovirta-Morley LE, Sayavedra-Soto L, Gallois N, Schouten S, *et al*. Identifying potential mechanisms enabling acidophily in the ammonia-oxidizing archaeon '*Candidatus* Nitrosotalea devanaterra'. *Appl Environ Microbiol* 2016;**82**:2608-2619.

Lehtovirta-Morley LE, Stoecker K, Vilcinskas A, Prosser JI, *et al*. Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. *Proc Natl Acad Sci USA* 2011;**108**:15892-15897.

Lehtovirta-Morley LE, Verhamme D, Nicol GW, Prosser JI. Effect of nitrification inhibitors on the growth and activity of *Nitrosotalea devanaterra* in culture and soil. *Soil Biol Biochem* 2013;**62***: 129-133.*

Leininger S, Urich. T, Schloter M, Schwark L, *et al.* Archaea predominate among ammonia-oxidizing. prokaryotes in soils. *Nature* 2006;**442**:806-809.

Lieberman RL, Rosenzweig AC. Crystal structure of a membrane bound metalloenzyme that catalyses the biological oxidation of methane. *Nature* 2005;**434**:177–182.

Martens-Habbena W, Berube PM, Urakawa H, Torre JR, *et al*. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature* 2009;**461**:976–979.

Martens-Habbena W, Qin W, Horak REA, Urakawa H, *et al*. The production of nitric oxide by marine ammonia-oxidizing archaea and inhibition of archaeal ammonia oxidation by a nitric oxide scavenger. *Environ Microbiol* 2015;**17**:2261–2274.

Merbt SN, Stahl DA, Casamayor EO, Marti E, *et al*. Differential photoinhibition of bacterial and archaeal ammonia oxidation. *FEMS Microbiol Lett* 2012:**327**:41-46.

Mussmann M, Brito I, Pitcher A, Sinninghe Damste JS, *et al*. Thaumarchaeotes abundant in refinery nitrifying sludges express *amoA* but are not obligate autotrophic ammonia oxidizers. *Proc Natl Acad Sci USA* 2011;**108**:16771-16776.

Palatinszky M, Herbold CW, Jehmlich N, Pogoda M, *et al*. Cyanate as an energy source for nitrifiers. *Nature* 2015;**524**:105-108.

Palomo A, Pedersen AG, Fowler SJ, Dechesne A, *et al*. Comparative genomics sheds light on niche differentiation and the evolutionary history of comammox *Nitrospira*. *bioRxiv* 2017:138586.

Pester M, Rattei T, Flench S, Gröngröft A, *et al*. *amoA*-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of *amoA* genes from soils of four different geographic regions. *Environ Microbiol* 2012;**14**:525–539.

Pjevac P, Schauberger C, Poghosyan L, Herbold CW, *et al*. *AmoA*-Targeted Polymerase Chain Reaction Primers for the Specific Detection and Quantification of Comammox *Nitrospira* in the Environment. *Front Microbiol* 2017;**8**:1508.

Placella SA, Firestone MK. Transcriptional response of nitrifying communities to wetting of dry soil. *Appl Environ Microbiol* 2013*;***79***:*3294–3302.

Purkhold U, Pommerening-Roser A, Juretschko S, Schmid MC, *et al*. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: Implications for molecular diversity surveys. *Appl Environ Microbiol* 2000;**66**:5368-5382.

Rotthauwe JH, Witzel KP, Liesack W. The ammonia monooxygenase structural gene *amoa* as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microbiol* 1997;**63**:4704-4712.

Santoro AE, Dupont CL, Richter RA, Craig MT, *et al*. Genomic and proteomic characterisation of "*Candidatus* Nitrosopelagicus brevis": An ammonia-oxidizing archaeon from the open ocean. *Proc Natl Acad Sci USA* 2015;**112**:1173-1178.

Sauder LA, Ross AA, Neufeld JD. Nitric oxide scavengers differentially inhibit ammonia oxidation in ammonia-oxidizing archaea and bacteria. *FEMS Microbiol Lett* 2016;**363**;fnw052.

Sauder LA, Albertsen M, Engel K, Schwarz J, *et al*. Cultivation and characterization of *Candidatus* Nitrosocosmicus exaquare, an ammonia-oxidizing archaeon from a municipal wastewater treatment system. *ISME J* 2017;**5**:1142-1157.

Schloesing JJT, Muntz A (1877) Sur la nitrification par les ferments organises. *C R Acad Sci* 1877;**LXXXIV**:301–303.

Shen JP, Zhang LM, Zhu YG, Zhang JB, *et al*. Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. *Environ Microbiol* 2008;**10**:1601–1611.

Shen T, Stieglmeier M, Dai J, Urich T, *et al*. Responses of the terrestrial ammonia-oxidizing archaeon *Ca*. Nitrososphaera viennensis and the ammonia-oxidizing bacterium *Nitrosospira multiformis* to nitrification inhibitors. *FEMS Microbiol Lett* 2013;**344**:121-9.

Spang A, Hatzenpichler R, Brochier-Armanet C, Rattei T, *et al*. Distinct gene set in two different lineages of ammonia-oxidizing archaea supports the phylum Thaumarchaeota. *Trends Microbiol 2010;**18**:331-340.*

Spang A, Poehlein A, Offre P, Zumbrägel S, *et al.* The genome of the ammonia-oxidizing *Candidatus* Nitrososphaera gargensis: insights into metabolic versatility and environmental adaptations. *Environ Microbiol 2012;***14**:3122–3145.

Stieglmeier M, Klingl A, Alves RJE, Rittmann SKMR, *et al*. *Nitrososphaera viennensis* gen. nov., sp. nov., an aerobic and mesophilic, ammonia-oxidizing archaeon from soil and a member of the archaeal phylum *Thaumarchaeota*. *Int J Syst Evol Micr* 2014;**64**:2738-2752.

Stieglmeier M, Mooshammer M, Kitzler B, Wanek W, *et al*. Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea. *ISME J* 2014;**8**;1135–1146.

Suzuki I, Dular U, Kwok SC. Ammonia or ammonium ion as substrate for oxidation by *Nitrosomonas europaea* cells and extracts. *J Bacteriol* 1974;**120**:556–558.

Taylor AE, Zeglin LH, Dooley S, Myrold DD, *et al*. Evidence for different contributions of archaea and bacteria to the ammonia-oxidizing potential of diverse Oregon soils. *Appl Environ Microbiol* 2010;**76**:7691-7698.

Tourna M, Stieglmeier M, Spang A, Könneke M, *et al*. *Nitrososphaera viennensis*, an ammonia oxidizing archaeon from soil. *Proc Natl Acad Sci USA* 2011;**108**:8420-8425.

Treusch AH, Leininger S, Kletzin A, Schuster SC, *et al.* Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ Microbiol 2005;7:1985–1995.*

Vajrala N, Martens-Habbena W, Sayavedra-Soto LA, Schauer A, *et al*. Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea. *Proc Natl Acad Sci USA* 2013;**110**:1006–1011.

van Kessel MAHJ, Speth DR, Albertsen M, Nielsen PH, *et al*. Complete nitrification by a single microorganism. *Nature* 2015;**528**,555–559.

Verhamme DT, Prosser JI, Nicol GW. Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. *ISME J* 2011;**5**;1067-1071.

Walker CB, de la Torre JR, Klotz MG, Urakawa H, *et al*. *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proc Natl Acad Sci USA* 2010;**107**:8818–8823.

Wang B, Zheng Y, Huang R, Zhou X, et al. Active ammonia oxidizers in an acidic soil are phylogenetically closely related to neutrophilic archaeon. *Appl Environ Microbiol* 2014;**80**:1684-1691.

Weber EB, Lehtovirta-Morley LE, Prosser JI, Gubry-Rangin C. Ammonia oxidation is not required for growth of Group 1.1c soil Thaumarchaeota. *FEMS Microbiol Ecol* 2015;**91**:fiv001.

Wijma HJ, Canters GW, de Vries S, Verbeet MP. Bidirectional catalysis by copper-containing nitrite reductase. *Biochemistry* 2004;**43**:10467–10474.

Winogradsky S. Recherches sur les organismes de al nitrification. *Ann Inst Pasteur* 1890;**4**:213–331.

Wuchter C, Abbas B, Coolen MJL, Herfort L, *et al*. Archaeal nitrification in the ocean. *Proc Natl Acad Sci USA* 2006;**103**:12317-12322.

Zeng J, Zhao D, Yu Z, Huang R, *et al*. Temperature responses of ammonia-oxidizing prokaryotes in freshwater sediment microcosms. *PLoS ONE* 2014;**9**:e100653.

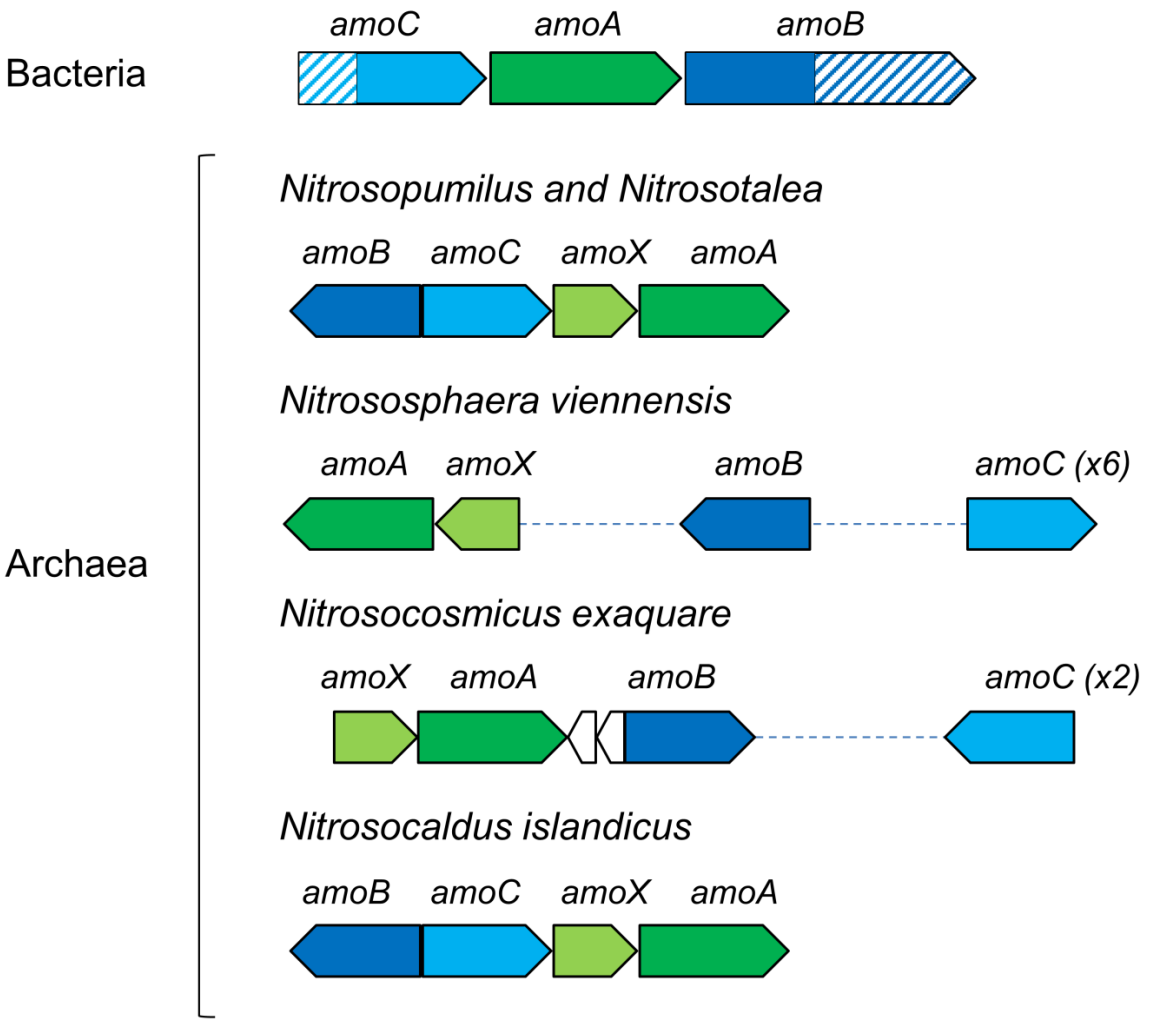

Figure 1. Organisation of the AMO gene clusters in bacteria and archaea. The N-terminus of AmoC and C-terminus of AmoB are truncated in archaea (indicated by stripes in bacteria).

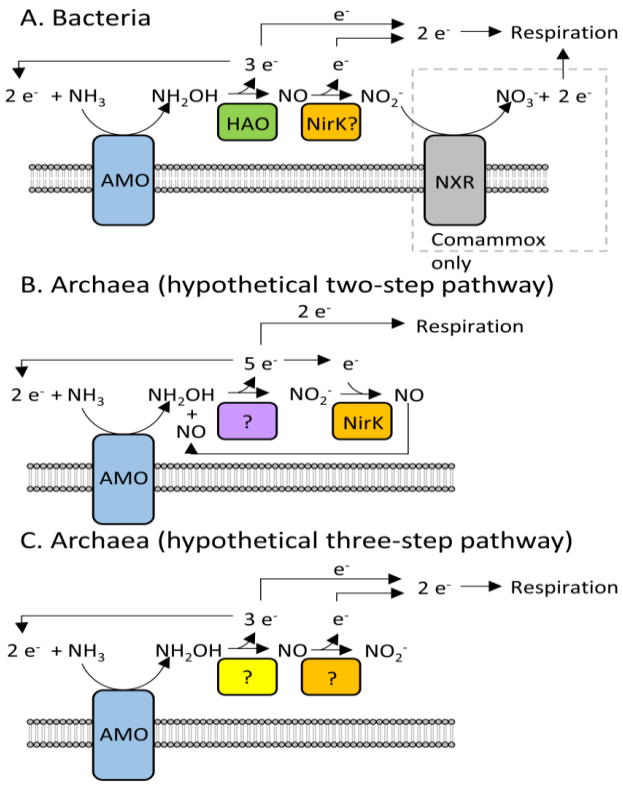

Figure 2. Pathways of ammonia oxidation in bacteria and archaea. A. AOB and comammox *Nitrospira* (based on Caranto (2017)), B. Hypothetical two-step model in AOA (proposed by Kozlowski (2016)), C. Hypothetical three-step model in AOA proposed by this study. The function of NirK has not been confirmed in any of the models.

Table 1.

Major questions

- ***How can such a great diversity of ammonia oxidising microorganisms coexist in a single ecosystem?*** The substrate affinity undoubtedly plays an important role in adaptation to the environment, but it is unlikely to be the only trait determining the niche specialisation.
- ***What gives rise to different substrate affinities?*** We know very little about structure, function and orientation of the AMO and mechanisms which control the local pH and substrate concentration within an ammonia oxidising cell.
- ***How do the abundance and diversity of ammonia oxidisers translate to nitrification rates in the environment?*** Pure culture studies show that V_{max} can be vastly different for different ammonia oxidisers. Sometimes the most abundant organisms may not be the most important contributors in nitrification.
- ***What role do the pathway intermediates and enzymology play in the production of climate active gases NO and N_2O?*** Very little is known about the rates of the enzymes in the pathway, how tightly the steps are coupled and controlled and how this affects the accumulation of intermediates and release of reactive nitrogen to the environment.

What is required?

- ***More tractable and environmentally relevant model organisms.*** Ecological surveys suggest that some of the most abundant and active ammonia oxidisers in the environment are yet to be cultivated.
- ***More physiology.*** To help us understand how ammonia oxidisers compete with each other, which factors determine their different ecological niches and how much different ammonia oxidisers contribute to the total nitrification.
- ***Higher biomass yields.*** Slow growth rates and low yields are currently a major bottleneck to biochemical studies of ammonia oxidisers.
- ***Identification and purification of key enzymes.*** These enzymes are critical for ammonia oxidation kinetics, for the accumulation and release of reactive nitrogen and for understanding how ammonia oxidisers fit into their ecological niches.
- ***Heterologous expression of key enzymes for biochemical studies.*** Heterologous expression could overcome the difficulty of obtaining sufficient biomass for protein characterisation.
- ***Genetic system.*** Mutagenesis approaches have already been established in AOB. Development of genetic systems for AOA and comammox *Nitrospira* will provide major advances for understanding the function and regulation of the key genes.
- ***Linking 'omics' to biochemistry.*** Combining culture-independent techniques e.g. single cell genomics, metagenomics and diversity studies to culture physiology, mutagenesis and protein characterisation to understand who is where and why.