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# Approaches to understanding the ecology and evolution of understudied terrestrial archaeal ammonia-oxidisers

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**Key take-home messages:**

- Most abundant terrestrial AOA clades are understudied (uncultured and without genome representation)
- Environmental surveys, genomes and cultures are complementary approaches to study AOA eco-evo

**Abstract**

Ammonia oxidising archaea (AOA) form a phylogenetic group within the phylum Thaumarchaeota and are of ecological significance due to their role in nitrification, an important biogeochemical process. Previous research has provided information on their ecosystem role and potential physiological characteristics, for example, through analyses of their environmental distribution, ecological adaptation and evolutionary history. However, most AOA diversity, assessed using several environmental marker genes, is not represented in laboratory cultures, with consequent gaps in knowledge of their physiology and evolution. This article critically reviews existing and developing approaches for the assessment of AOA function and diversity and their potential to provide a deeper understanding of these ecologically important, but understudied microorganisms.

## Introduction

Nitrification, the conversion of ammonia ($NH_3$) to nitrate ($NO_3^-$), is one of the fundamental processes controlling the cycling of nitrogen. In aerobic environments, it is a two-step process consisting of ammonia oxidation to nitrite ($NO_2^-$), followed by nitrite oxidation to nitrate. Aerobic ammonia oxidation was considered to be restricted to ammonia-oxidising bacteria (AOB) prior to isolation of ammonia-oxidising archaea (AOA) [1], which are important nitrifiers in marine and terrestrial environments [2-4], and, the subsequent discovery of complete ammonia-oxidisers (comammox) [5,6].

Ammonia oxidation is generally the limiting step in soil nitrification and AOA therefore play a critical role in the soil nitrogen cycle [7], with important environmental consequences. Biologically available nitrogen (such as ammonia or ammonia precursors) is applied as nitrogen-based fertilisers to the soil by farmers, as soil N is a major limiting factor for crop production. The transformation of ammonia to the more mobile nitrate, via nitrification, results in leaching of this bio-available nitrogen from agricultural land into water systems, simultaneously reducing fertiliser utilisation efficiency and polluting water systems (see [8]); development of strategies is required to control this process and reduce environmental consequences. A further major environmental consequence is the production of nitrous oxide ($N_2O$), a potent greenhouse gas associated with climate change. While both AOA and AOB contribute to $N_2O$ production, AOA appear unable to perform nitrifier-denitrification [9,10] and their net contribution to global greenhouse gas emissions is much lower than that of AOB in some agricultural soils [11,12], but may be higher in the acid soils in which they dominate ammonia oxidation [13,14]. This difference between groups suggests the potential for nitrous oxide mitigation strategies through use of different land-use practices.

All known AOA belong to the class Nitrososphaeria [15], within the phylum Thaumarchaeota [16,17]. This phylum contains several distinct phylogenetic lineages [18], some of which, e.g.

Group 1.1c Thaumarchaeota, do not appear able to perform ammonia oxidation, due to their growth in soil in the presence of known nitrification inhibitors and without production of detectable nitrite or nitrate [19]. In addition, the only Group 1.1c Thaumarchaeota genome available contains no homologue of ammonia monooxygenase, the enzyme responsible for ammonia oxidation [20]. In mesophilic environments, three order-level phylogenetic lineages represent the majority of known AOA diversity and abundance (Fig. 1): the Nitrososphaerales [15], Nitrosopumilales [21] and *Candidatus* Nitrosotaleales [22], previously known as groups 1.1b, 1.1a and 1.1a-associated. Two of these three lineages (Nitrososphaerales and *Ca*. Nitrosotaleales) (Fig. 1) dominate archaea in terrestrial environments, suggesting that they are actively nitrifying and growing in these environments, and there is also evidence for activity of some organisms affiliated to Nitrosopumilales in soil [23,24]. A fourth and deeply-rooted AOA order, *Ca*. Nitrosocaldales [25], contains thermophilic AOA [26-28] and presents lower observed diversity than other AOA orders [29], although this may be an artefact of low sampling effort. Nine distinct genera have been either described or proposed as candidates within the AOA, with more than half falling within the Nitrosopumilales and only a single candidate genus in each of *Ca.* Nitrosotaleales and *Ca.* Nitrosocaldales [29].

All published large-scale archaeal ammonia monooxygenase subunit A (*amoA*) phylogenies identify diverse phylogenetic groups at the sub-order level with no cultivated representatives [29-32] (Fig. 1). Notably, analyses of these terrestrial *amoA* phylogenetic reconstructions identified C1/2 (or NS-Delta) and C11 (or NS-Gamma-2.32) as the two most abundant AOA lineages in mesophilic terrestrial environments, neither of which has a cultivated representative or associated complete genome (Fig. 1), defining them as understudied AOA lineages. As such, while these organisms contribute to a significant fraction of AOA in soil, our understanding of their overall ecological significance and ecosystem functioning is limited. An incomplete picture of their genomic content and diversity also hinders comprehensive understanding of the

evolutionary history of these AOA, whose genomic and ecological characteristics are largely unknown, and whose potential environmental importance is not reflected in their presence in cultivation or genome databases. Therefore, this review critically summarises the different approaches, with associated advantages and limitations, typically used to expand current AOA knowledge, especially in the context of the AOA ecology and evolution, and implications for their potential application to such 'understudied' lineages.

## Environmental surveys and microcosm incubations

Environmental surveys have a distinct advantage for studying understudied organisms: they can be conducted without *a priori* knowledge of or restrictions on the organisms under study. This type of approach has been used extensively to describe ammonia oxidiser distribution in soil ecosystems and differential growth and activity of AOA and AOB has been analysed in relation to various environmental factors in attempts to identify niche specialisation [33], including ammonia sources and concentration [11,12,34,35] and soil moisture [36,37]. Although these effects have been explained in terms of greater ammonia affinity of AOA, recent studies [38,39] failed to find evidence of major differences in ammonia affinity of soil AOA and AOB, with higher substrate affinity being demonstrated for the comammox bacteria than for AOA or AOB based on a limited number of isolates. Similarly, alleviation of competition between AOA and AOB using differential inhibitors, leads to growth of AOA at high ammonium concentration [11]. This suggests that niche specialisation between AOA and AOB may not be based, in soil, on substrate affinity or sensitivity and highlights the need for deeper understanding of their distribution and underlying physiology.

In most soils without artificial ammonia amendment (i.e. fertilisation), AOA dominate numerically over AOB, particularly in acidic soils [3,13,14,24,34,40,41]. However, the relative activities of these groups are not necessarily reflected in their relative abundance [40,42]. Their contributions are associated with a range of environmental factors: high pH correlates with

AOB, rather than AOA activity [40,43,44], high water content with AOA activity [45], high inorganic nitrogen availability with AOB activity [11,34,44,46] and low C:N ratio appears to be associated with AOA activity [47], possibly due to their preferential use of mineralized N from organic matter [35]. While these studies provide evidence for links between AOA growth and particular environmental factors, most of these environmental studies are observational surveys based on correlations and do not test potential physiological mechanisms experimentally. They are unable to distinguish cause and effect and ammonia oxidisers themselves will alter, for example, ammonia concentration and soil pH, confounding interpretation of correlations. In addition, such approaches involve autocorrelations, e.g. pH and irrigation [45], and many unknown effects prevent accurate analysis of individual environmental factors.

Among these environmental studies, incubation of soil under controlled conditions, using experimental model soil systems (microcosms), provides much greater control and improved monitoring than *in situ* studies, enabling analysis of individual factors, such as water content [37], ammonium source and concentration [11], oxygen concentration [48] and soil pH [49]. Microcosms provide many of the benefits of a controlled environment, including stability and manipulation of several factors, including temperature, pH, light and water content, under environmental conditions that are known to support growth of groups of AOA for which pure cultures are not available. This approach also allows inhibition of specific groups of nitrifiers, e.g., 1-octyne (to inhibit AOB) [50] or acetylene (to inhibit all ammonia oxidisers) [13,23,51]. However, complexity and logistics of experimental design can restrict analysis of environmental factors and their interactions.

**Phylogenetic Studies**

Phylogenetic reconstruction provides a powerful approach to detect understudied lineages, its chief advantage being a lack of requirement for detailed genomic information, but rather single sequences readily amplified from environmental DNA. Both ammonia monooxygenase subunit A (*amoA*) and 16S rRNA genes have been widely used for phylogenetic analysis of AOA. These two genes exist as a single copy in all genomes of cultivated AOA, except some *Ca*. Nitrosotalea genomes, which possesses two copies of *amoA* [52] and *Ca*. Nitrosocosmicus genomes, which possesses either two or three copies of the 16S rRNA gene [53,54]. However, differential phylogenetic approaches (such as Maximum Likelihood vs Bayesian), different substitution models, including different codon site and rate heterogeneity, and the inclusion or exclusion, in analyses, of detection of recombinant sequences or saturation in substitutions have provided several hypothetical frameworks of AOA evolution. To our knowledge, five phylogenetic analyses have focused on analysis of large numbers of *amoA* gene sequences [18,29-32]. These analyses led to similar sequence clustering at the order- and higher sub-order-levels (Fig. 1) while most differences are associated with phylogenetic placement of the clades formed at the sub-order level. Substitution saturation (evidenced on the third codon position of the *amoA* gene) was only removed in the two Bayesian phylogenetic trees [18,31] indicating that the effects of synonymous substitutions generate misleading and conflicting relationships in the other phylogenetic reconstructions by decreasing the accuracy of placement of deeper branches [55]. This is exemplified by the separation of a single cluster (C1/2; Fig. 1) [18,31] into 2 distinct clusters (C1 and C2) in other approaches [29,30].

Correlations between phylogenetic classification and several environmental factors (including pH or total soil nitrogen and carbon content) have been interpreted as evidence for niche specialisation of the different phylogenetic clusters [18,29-32]. In particular, two AOA lineages with no cultured representative have been identified with high abundance in soils (Fig. 1) [30,56]. The first dominates in neutral-alkalinophilic soils (pH>6) and forms the cluster C1/2

(37% of soil sequences [30,31]), equivalent to clade NS-Delta (39% of soil sequences [29]), with 77.7% sequences within this clade originating from soil with pH>6.5 [29]. The second dominates in neutral-acidic soils (pH<6) and forms the cluster C11 (27% of soil sequences [30,31]), equivalent to clade NS-Gamma-2.3.2 (27% of soil sequences [29]) with 97.1% sequences within this clade originating from soil with pH<7.5 [29]. Confirmation of the initial description of differential pH-associated distributions of soil AOA [30,56] therefore supports previously proposed hypotheses of pH-based links between phylogeny and function that are further supported by cultivation-based studies (described below). This example also demonstrates the potential advantages of this correlation-based approach where links between phylogeny and environmental characteristics can lead to predictions regarding phenotypic characteristics of understudied clusters that can be tested in laboratory cultures or through experimentation. A second example is the detection of different temperature optima in terrestrial acidophilic and neutrophilic lineages [57], facilitating better predictions about AOA community activities under different environmental conditions, but these effects have yet to be tested critically in independent experiments. These two environmental factors, pH and temperature, are widely recognised to influence microbial distribution by having not only direct effects on growth but also influencing many other physicochemical and biological characteristics of soil, making it difficult to link, directly, environmental characteristics and phylogeny.

Importantly, phylogenetic analysis not only generates hypotheses about phenotype and environmental preferences but also facilitates hypothetical scenarios concerning microbial evolutionary history, including those of understudied groups. In fact, the mechanisms and environmental factors influencing AOA evolutionary processes over deep-evolutionary time demonstrate many gaps in our understanding. However, cutting-edge comparative phylogenetic methods have recently enabled identification of pH as a probable crucial factor

for terrestrial AOA diversification [31], while lateral gene transfer events [52,58] and differential natural selective pressures across diverse AOA lineages [56] were suggested to be distinct mechanisms for environmental adaptation. Indeed, acquisition of acidophily in the two most abundant acidophilic AOA lineages, C14 and C11 (Fig. 1), probably occurred from independent evolution events through different selective pressures acting at the origin of these groups [56]. In turn, the evolutionary history of AOA is reflected in the phylogenetic classification of several genomic traits, such as GC content, effective number of codons or preferred codon usage [29]. Phylogenetic coherence of these traits with environmental factors reflects the habitat preference and niche adaptation of the organisms.

Phylogenetic approaches have also led to hypothetical predictions about the origin of ammonia oxidation [29,59], although clear resolution of the organismal origin and subsequent transfer to other ammonia oxidiser lineages is still required. Temporally, archaeal ammonia oxidation likely arose after the appearance of significant oxygen in the atmosphere [60] but precise dating of the emergence of microbial groups is limited due to scarcity of reliable fossils. Therefore, a lateral gene transfer-aware approach has been used and has constrained the last common ancestor of mesophilic AOA to have occurred between 750-1400 Mya, but innovative approaches are still required for dating of the last common ancestor of Thaumarchaeota [61].

The first major limitation of comparative phylogenetic studies lies in the inference of environmental preference, which is based upon the presence/absence or relative abundance of a given gene sequence in each habitat. Most of the comparative phylogenetic studies are not based on sampling methods targeting specific lineages of interest (based on their abundance or niche specialisation), and are instead highly dependent on sequences deposited in databases. In addition, dormancy is a common microbial strategy allowing survival in various environmental conditions, including those where their growth is not supported (see [62]). Another important limitation is that these phylogenies are based on a single gene marker, *amoA* [18,29-32],

210 although phylogenetic congruence with both 16S rRNA gene phylogenies [18,29] and
211 phylogenomic reconstructions using multiple single-copy markers [29,52] has been
212 demonstrated, suggesting that the *amoA* gene is a relevant marker for reconstructing AOA
213 evolutionary history. However, relations between these single marker genes and environmental
214 adaptation may only ever be correlative with environmental preference, as these *amoA* and 16S
215 rRNA genes are not known to be directly involved in environmental adaptation.
216
217 **Genome analysis**
218 Understanding AOA physiology has been facilitated by genome sequencing, which allows
219 prediction of potential metabolic pathways, including those for ammonia oxidation [10] and
220 carbon dioxide fixation [63]. Genome sequences are among the more powerful tools available
221 for studying new organisms as they allow detailed metabolic prediction, as well as being a
222 gateway to more detailed phylogenomic reconstruction of evolutionary history. Genomic data
223 have provided some key information in AOA, highlighting the lack of a hydroxylamine
224 dehydrogenase enzyme HAO similar to that in AOB [64-66] or the suggestion that nitrite
225 reductase gene *nirK* is related to the ammonia oxidation pathway [10] through formation of a
226 nitric oxide (NO) intermediate. In the two most recent models proposed, the protein NirK could
227 also be involved in the AOA ammonia oxidation pathway alongside two novel membrane-
228 bound, Cu-containing metalloproteins to oxidise hydroxylamine [67]. However, the absence of
229 *nirK* in the genomes of two recently analysed thermophilic AOA [27,28] suggests that these
230 proposed models may not be valid for *Ca*. Nitrosocaldales organisms. Genomic data have also
231 been useful in providing hypotheses of ecological relevance regarding the ammonia oxidation
232 process in several environments through comparisons of AOA and AOB. For example, two
233 types of ammonium/ammonia transport systems were described in AOA with putative low-
234 affinity and high-affinity systems (Amt1 and Amt2, respectively), while AOB possess only one

type (Rh type) [68,69]. The existence of both multiple ammonium/ammonia transporters and a charged S-layer (which itself increases substrate concentration in the pseudo-periplasmic compartment [68,70]) in AOA probably facilitates substrate acquisition in oligotrophic conditions and provides the AOA with a competitive advantage over AOB. Comparison of *amo* genes homologies and AMO operon structure between AOA and AOB led to the suggestions of *amoB* as a ligand site and pseudo-periplasmic localisation of the ammonia oxidation process [64,67,68,71]. Another useful genomic comparison concerns nitrous oxide production, which arises mainly from hybrid formation between hydroxylamine and nitric oxide in AOA, while production via nitrifier denitrification and incomplete hydroxylamine oxidation have additionally been demonstrated in AOB [10].

Discoveries of several genes and metabolic pathways of potential environmental relevance have relied on genomics approaches, for example methylphosphonate synthesis [72] and production of cobalamin (Vitamin B12) in marine AOA [73]. However, the dangers of over-interpreting genomics information are well recognised and, while such data may suggest potential phenotypic characteristics, they are not conclusive indicators of metabolic characteristics. For example, genomic information has not been very useful in identifying the ammonia oxidation pathway (see above). Under the assumption that missing steps are encoded by a conserved gene(s) within the AOA, characterisation of more diverse AOA may assist in restricting potential candidates for this gene. Identifying such a gene will assist in metabolic reconstruction of the entire pathway and hence facilitate predictions of, for example, greenhouse gas emissions.

With increasing numbers of AOA genome sequences (>35 from pure or enrichment cultures to date), comparative genomics has been applied to AOA at the phylum level [74] or to clades of interest, such as *Ca*. Nitrosotaleales [52] or *Ca*. Nitrosocaldes [27]. Such approaches allow delineation of gene sets shared between organisms (core genome) leading to hypothetical

prediction of metabolic pathways and identification of putative mechanisms behind AOA environmental adaptation. In particular, comparative genomics has been used to investigate obligate acidophily and has suggested the existence of several genes linked to pH homeostasis or detoxification of reactive nitrogen compounds [52]. In comparison to single genome analysis, comparative approaches have restricted the number of candidate genes with potential roles in environmental adaptation [52,68]. Despite the undeniable advantages of comparative genomics, it has several limitations. The first concerns the high proportion of genes with unknown function, which often account for nearly 50% of the predicted genes in AOA [52]. Another major limitation is confidence in predictions, as the presence of a gene does not necessarily mean that it is transcribed or translated under the relevant environmental conditions. Therefore, any genomic approach requires experimental testing of the resultant functional predictions. Despite these limitations, it is reasonable to assume that similar sequencing effort of understudied AOA lineages, facilitated by advances in metagenomics, may increase understanding of their environmental adaptation. These phylogenomic approaches have also clarified some aspects of Thaumarchaeota evolutionary history, with the existence of basal thaumarchaeotal thermophiles and a hypothesized thermophilic common ancestor with the Aigarchaeota, suggesting that the thaumarchaeotal ancestor originated in a thermal habitat and later colonised mesophilic environments [75].

**Enriched and isolated cultures**

Isolated or enriched thaumarchaeotal strains are essential to confirm physiology of different AOA phylotypes and cultivation approaches allow characterisation of a range of environmental adaptations to pH, temperature or oligotrophy, e.g. [63,76-78], or estimation of detailed metabolic information regarding substrate affinities or greenhouse gas production. They also serve as a platform for directly testing the physiological or functional hypotheses generated

from environmental and genomic observations. Indeed, experimentation in culture is used to test specific mechanistic responses to given perturbations, although care is required in relating laboratory conditions to those *in situ* about which inferences are being made. The major disadvantages of culture-based approach are difficulties in obtaining enrichment or pure cultures, especially for these slow-growing organisms. In addition, AOA growth is currently limited to liquid medium, in which optical density is low, even in fully grown cultures. Despite such limitations, more than 35 AOA belonging to 7 (out of 19) phylogenetic sub-orders are now cultivated [29], enabling their physiological characterisation.

One example of hypothesis-testing in AOA cultures is the long-standing notion that some AOA are mixotrophic [79] based on observations that several AOA were unable to grow in isolation without supplementation of growth media with organic acids such as pyruvate or α-ketoglutaric acid [78,80]. Physiological studies with laboratory isolates comprehensively demonstrated that dependence on organic acids was due to scavenging and consequent detoxification of toxic hydrogen peroxide by these compounds, rather than mixotrophy [81]. However, growth of some AOA possessing their own ROS-detoxification machinery is stimulated by organic acid supplementation [54], allowing the possibility that alternative mechanisms operate for utilisation of organic compounds by AOA.

Culture-based experimentation has clearly contributed to advances in knowledge of ammonia oxidation pathways, demonstrating the intermediary role of hydroxylamine ($NH_2OH$) and nitric oxide (NO) in AOA ammonia oxidation [9,10,66,82]. Characterisation of candidate genes derived from genomic investigations (see above) is initially likely to be through heterologous expression, especially for simple catalytic functions of individual genes, as for previous unknown AOA genes [63,72]. AOA are not an attractive target for development of a native genetic toolkit themselves due to their slow growth and requirement for growth in liquid medium; however, a reverse genetics and genetic manipulation toolkit would assist greatly in

studies of genes with environmental significance and allow exploration of potential interactions between such genes.

## Conclusion: the AOA investigative toolkit

The remaining questions on the ecology, evolution and physiology of AOA can be addressed using an array of methodologies, each of which has advantages and limitations (Table 1). Genomics tools are complementary to environment-based studies generating strong hypotheses and predictions surrounding physiological or environmental adaptation, which can then be tested using cultivation-based approaches or controlled microcosm experiments. Investigation of complex gene functions or interactions will hopefully benefit from future developments such as reverse and forward genetics. The current and future efforts to explore the significant underexplored diversity of terrestrial AOA (Fig. 1) will certainly yield disproportionate benefits in evolutionary understanding, but progression of this knowledge requires directed exploration using specific mechanistic-based approaches.

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**Figures legend:**

**Figure 1:** Phylogeny of class Nitrososphaeria, constructed using *amoA* gene sequences from soil environmental DNA. Names of the phylogenetic clusters are based on their initial terrestrial denomination [31] and more recent denominations of these clades [29] (based on a BLASTn approach) have been added into brackets to unify the various phylogenetic approaches. Line colour of the phylogenetic trees corresponds to inferred pH preference along a given branch [31]. Circle size is proportional to the relative abundance of each cluster among 48 soil samples representative of the mesophilic terrestrial AOA diversity [30]. Yellow stars indicate phylogenetic clusters containing a cultivated strain, while green stars indicate clusters containing an associated sequenced genome.

626 **Table 1:** Summary of some of the common approaches used to address the ecology and evolution of archaeal ammonia oxidisers (AOA), including

627 their potential advantages and limitations.

628 .

| *Approach* | **Environmental surveys and microcosms** | **Amplicon-based phylogenetics** | **Whole-genome sequencing** | **Pure cultures** |
|---|---|---|---|---|
| *Advantages* | • Relation of processes to real-world conditions<br>• No requirement for representative organisms<br>• Investigation of complex community interactions | • Relation of specific diversity to ecosystem function<br>• No requirement for representative organisms<br>• Exploration of evolutionary history | • Global metabolic investigation<br>• Identification of novel genes and potential metabolic pathways | • Detailed physiological investigations<br>• Experimental confirmation of the ecosystem function<br>• Controlled experimental conditions |
| *Limitations* | • Correlation-based approach without mechanistic inference<br>• Inter-correlation of variables and no causal information<br>• Linking ecosystem function to diverse communities | • Amplification biases with potential omission of unknown diversity<br>• No mechanistic information<br>• Correlation between phylogeny and environmental parameters | • Restricted to metabolic predictions<br>• Error-based sequencing technologies<br>• Mainly automated annotations | • Conditions restricted to laboratory conditions<br>• Significant time investment, especially for slow-growers and for isolation<br>• Unknown cultivation requirements |

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