# Nitrous oxide production by ammonia oxidisers: physiological diversity, niche differentiation and potential mitigation strategies

Running head – $N_2O$ production by ammonia oxidisers

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**Abstract**

Oxidation of ammonia to nitrite by bacteria and archaea is responsible for global emissions of nitrous oxide directly and indirectly through provision of nitrite and, after further oxidation, nitrate to denitrifiers. Their contributions to increasing $N_2O$ emissions are greatest in terrestrial environments, due to the dramatic and continuing increases in use of ammonia-based fertilisers, which have been driven by requirement for increased food production, but which also provide a source of energy for ammonia oxidisers, leading to an imbalance in the terrestrial nitrogen cycle. Direct $N_2O$ production by ammonia oxidisers results from several metabolic processes, sometimes combined with abiotic reactions. Physiological characteristics, including mechanisms for $N_2O$ production, vary within and between ammonia oxidising archaea (AOA) and bacteria (AOB) and comammox bacteria and $N_2O$ yield of AOB is higher than in the last two groups. There is also strong evidence for niche differentiation between AOA and AOB with respect to environmental conditions in natural and engineered environments. In particular, AOA are favoured by low soil pH and AOA and AOB are respectively favoured by low rates of ammonium supply, equivalent to application of slow-release fertiliser, or high rates of supply, equivalent to addition of high concentrations of inorganic ammonium or urea. These differences between AOA and AOB provide the potential for better fertilisation strategies that could both increase fertiliser use efficiency and reduce $N_2O$ emissions from agricultural soils. This article reviews research on the biochemistry, physiology and ecology of ammonia oxidisers and discusses the consequences for ammonia oxidiser communities subjected to different agricultural practices and the ways in which this knowledge, coupled with improved methods for characterising communities, might lead to improved fertiliser use efficiency and mitigation of $N_2O$ emissions.

## Introduction

Nitrous oxide ($N_2O$) is an important greenhouse gas produced by ammonia oxidisers (AO) and denitrifiers. It ranks third behind carbon dioxide ($CO_2$) and methane ($CH_4$) in terms of radiative forcing, with estimated global $N_2O$ emission rates of 11 Tg $y^{-1}$ (range 8.1 - 30.7 Tg $y^{-1}$) (IPCC, 2013). However, $N_2O$ has a global warming potential that is 265- and 10-fold greater than those of $CO_2$ and $CH_4$, respectively, has a long atmospheric lifetime (109 - 125 years, Prather et al., 2015), contributed 17% to radiative forcing in 2005 (IPCC, 2013) and is predicted to be the primary contributor to ozone depletion in the stratosphere in the 21$^{st}$ Century (Ravishankara et al., 2009). Atmospheric $N_2O$ levels are 20% greater than in pre-industrial times (MacFarling-Meure et al., 2006), largely through increases in reactive nitrogen, resulting from increased nitrogen fertilisation as manure, inorganic nitrogen and urea (Smil, 1999). $N_2O$ levels are increasing by 0.73 ± 0.03 ppb $y^{-1}$ (~0.3% $y^{-1}$) and are projected to increase significantly through this century, due to food and animal feed demands of an increasing global population (Tilman et al., 2001; Alexandratos & Bruinsma, 2012).

Nitrification, the sequential oxidation of ammonia ($NH_3$) to nitrite ($NO_2^-$) and nitrate ($NO_3^-$) in the presence of oxygen, can contribute indirectly to $N_2O$ emissions, providing nitrogen oxides to denitrifying microorganisms for energy conservation under reduced oxygen conditions and anoxia (Kuypers et al., 2018). Denitrifiers are traditionally considered major producers of $N_2O$, but $N_2O$ is also emitted by organisms that oxidise $NH_3$ to $NO_2^-$. This direct contribution to emissions is particularly important given projected increases in N fertiliser applications. Traditionally ammonia oxidation was thought to be performed by bacterial ammonia oxidisers (AOB), but marine ammonia oxidation is now known to be dominated by archaeal ammonia oxidisers (AOA) and both groups are important in other ecosystems. Recently discovered complete ammonia oxidisers (comammox bacteria) oxidise both $NH_3$ and $NO_2^-$ within the same

cell (Daims et al., 2015; van Kessel et al., 2015), but little is currently known of their ecology. Cellular rates of $N_2O$ production by AOA and AOB differ, leading to different contributions to emissions, and there is evidence for niche differentiation between AOA and AOB with respect to environmental factors and land use strategies. This article reviews evidence for differences in $N_2O$ emissions by AO in culture and in natural ecosystems and assesses potential links between niche differentiation, $N_2O$ emissions and agricultural systems and potential mitigation strategies.

## The role of nitrifiers in biogeochemical cycling

In both terrestrial and aquatic ecosystems, the major source of bioavailable nitrogen is fixation of atmospheric dinitrogen ($N_2$) by free-living or plant-associated $N_2$-fixing bacteria, photosynthetic cyanobacteria and hydrogenotrophic methanogenic archaea (Boyd & Peters, 2013). Following consumption by higher trophic levels, excretion and death, mineralisation of organic N by microbes releases inorganic ammonium ($NH_4^+$), which can be assimilated by plants and microorganisms. Ammonium also provides an essential source of energy for obligate aerobic, lithotrophic microorganisms, the ammonia oxidisers. These organisms oxidise $NH_3$ to $NO_2^-$, which is then oxidised to $NO_3^-$, before dissimilatory reduction under anaerobic/microaerobic conditions. Facultative denitrifiers can return $N_2$ to the atmosphere (Kuypers et al., 2018). These nitrogen cycling processes are supplemented by a network of other biological and physicochemical processes.

In aerobic marine ecosystems, nitrification is highly efficient and results in concentrations of $NH_4^+$ in the nanomolar range (Horak et al., 2013), but can be limited under reduced oxygen concentration in oxygen minimum zones (OMZ; Ward, 2011) and within decaying particulate organic matter or polluted environments, where oxygen demand can be high. These conditions favour denitrification.

In natural terrestrial ecosystems, in which $NH_4^+$ is provided via mineralisation and $NO_3^-$ is available, the balance between nitrification and denitrification is again controlled largely by oxygen concentration, which is itself controlled by soil moisture content. At high moisture content, the potential for aerobic nitrification will be low and denitrification will dominate. The situation reverses as soil moisture decreases, and oxygen concentration increases (Bateman & Baggs, 2005). These potential rates are moderated by other factors, e.g. rates of $NH_3$ supply from mineralisation or supply of organic carbon and nitrate for denitrification (Booth et al., 2005). This leads to variability in relative concentrations of $NH_4^+$ and $NO_3^-$ and in relative activities of nitrifiers and denitrifiers at both 'bulk' and microscale levels. For example, even in 'aerobic' soils, denitrification may occur in microenvironments, where oxygen diffusion is limited, or in regions of high decomposition, where oxygen demand is high.

Despite spatial heterogeneity, and resultant complexity, soils that have not been subjected to modern agricultural developments have a 'closed' nitrogen cycle in which most $NH_4^+$ generated from mineralisation is assimilated by plants and soil microbes and <10% is oxidised to $NO_3^-$ (Haynes, 1986). The closed nature of this cycle is facilitated further by the production of biological nitrification inhibitors (BNI) by some plants and $NH_3$ concentrations in such soils are relatively high and $NO_3^-$ concentrations low (Subbarao et al., 2012).

This situation is reversed in managed, agricultural soils due to application of inorganic, $NH_3$- and urea-based fertilisers. Over the past 50 years, N fertiliser use increased in Western Europe until stabilisation in the 1990s, but continues to accelerate globally through increased usage elsewhere, with further increases projected to support future increases in world population (FAO, 2017; Lassaletta et al., 2014; Tilman et al., 2001). N entering terrestrial ecosystems produced via the Haber-Bosch process (170 Tg annually; FAO, 2017) exceeds that produced naturally and this energy-intensive process consumes >1% of the global energy demand (IFA-

UNEP, 1998). For AO, this represents an injection of energy and has created an imbalance in the nitrogen cycle, with high $NO_3^-$ production due to high nitrification rates. These effects appear to have been exacerbated by crop breeding programmes developed on the assumption of high levels of inorganic nitrogen fertilisation, decreasing $NH_3$ competition between nitrifiers and plants and reducing the requirement for BNI, such that these are not produced by modern crop varieties (Subbarao et al., 2017). This has significantly reduced N fertiliser use efficiency to approximately 47% (Lassaletta et al., 2014), through leaching of anionic $NO_3^-$, in contrast to cationic $NH_4^+$ that is bound to negatively charged particles. It has also led to greatly elevated $N_2O$ emissions.

**Diversity of ammonia oxidising microorganisms**

Three main groups of aerobic ammonia oxidising prokaryotes have been described: AOB, AOA and comammox bacteria. AOB belong to beta- and gammaproteobacteria classes, with two (*Nitrosomonas* and *Nitrosospira*) and one (*Nitrosococcus*) genera, respectively (Purkhold et al., 2000). These genera display different environmental distributions indicating distinct physiological characteristics. *Nitrosococcus* is mainly found in marine environments and salt lakes (Campbell et al., 2011), although one strain was recently enriched from an acidic soil (Hayatsu et al., 2017). *Nitrosomonas* has been found in similar environments but also in engineered systems, such as wastewater treatment systems (Mobarry et al., 1996; Schramm et al., 1996). *Nitrosospira* organisms dominate soil AOB but are found in a range of ecosystems and are genetically diverse, in terms of both 16S rRNA and *amoA* gene markers (Aigle et al., 2019; Purkhold et al., 2000, 2003).

AOA belong to the class *Nitrososphaeria* within the phylum Thaumarchaeota and known AOA diversity is represented by four order-level phylogenetic lineages: the *Nitrososphaerales*, *Nitrosopumilales*, *Ca.* Nitrosotaleales and the thermophilic *Ca*. Nitrosocaldales (Brochier-

Armanet et al., 2008; Kerou et al., 2016; Stieglmeier et al., 2014a). AOA are ubiquitous and mesophilic lineages and are genetically diverse, based on *amoA* and 16S rRNA gene phylogenies, with higher diversity described for terrestrial than marine lineages (Alves et al., 2018; Gubry-Rangin et al., 2011; Pester et al., 2011). Thermophilic AOA diversity is lower, possibly reflecting sampling bias (Alves et al., 2018; Gubry-Rangin et al., 2018).

All comammox bacteria belong to the genus *Nitrospira* of the class Nitrospira, which also include canonical nitrite oxidizing bacteria (Daims et al., 2016), and have been detected in a range of natural and engineered environments (Fowler et al., 2018; Palomo et al., 2018; Pjevac et al., 2017; Wang et al., 2019; Zheng et al., 2019), with the possible exception of oceanic environments. Available *amoA* gene sequences suggest two phylogenetic groups (clades A and B) of similar diversity with potential environmental specialisations. Currently cultivated representatives belong to clade A (Daims et al. 2015; van Kessel et al. 2015) and while both clades A and B are found in soil, conditions under which comammox bacteria are active in soil have only been demonstrated for clade B (Shi et al., 2018; Wang et al., 2019).

## Mechanisms of ammonia oxidation

### *Ammonia oxidation to hydroxylamine*

The first step in ammonia oxidation in all currently characterised aerobic, autotrophic AO is oxidation of $NH_3$ to hydroxylamine by $NH_3$ monooxygenase (AMO), a copper-based, broad-range, membrane-bound oxygenase. Although studied in detail in relatively few organisms (Arp and Stein, 2003; Kozlowski et al., 2016b), AMO has similar characteristics in AOA and AOB. It contains three major structural subunits (Tolar et al., 2017) and although the active enzyme complex has not been identified, comparison with the particulate methane monooxygenase suggests the β-subunit as the active site (Tolar et al., 2017). The structural gene for the α-subunit, *amoA*, has become the target gene for detection and distinction of AOA

166 and AOB in natural environments (e.g. Francis et al., 2003; Rotthauwe et al., 1997; Tourna et
167 al., 2008) and, with 16S rRNA genes, for phylogenetic analysis (Alves et al., 2018; Gubry-
168 Rangin et al. 2011; Pester et al., 2011; Purkhold et al., 2000; Stephen et al., 1996). Comparative
169 genomic analysis of comammox *Nitrospira* indicates that their ammonia oxidation genomic
170 repertoire is more similar to AOB than AOA, with homologues of *amoA* genes found in all
171 autotrophic AO and of AOB-like hydroxylamine dehydrogenase and c-type cytochromes,
172 which are responsible for transferring electrons to the quinone pool (Daims et al., 2015).

173 A potential difference between AO is substrate affinity. A report of high $NH_3$ affinity in
174 *Nitrosopumilus maritimus* provided a convincing explanation for the dominance of AOA in
175 marine environments, where $NH_3$ is present at nanomolar concentrations, and led to the
176 suggestion that all AOA had higher affinity than AOB (Martens-Habbena et al., 2009). The
177 generality of this finding was challenged by two studies (Hink et al., 2017a; Kits et al., 2017),
178 which questioned the high affinity in *N. maritimus* and the clear distinction between AOA and
179 AOB. The comammox strain, *Nitrospira inopinata*, also has high $NH_3$ affinity, leading to the
180 view that comammox bacteria are oligotrophs with a competitive advantage in low $NH_3$
181 environments (Kits et al., 2017).

182 These studies raise a number of general points. Caution is required in generalising from results
183 obtained from a small number (often one) laboratory cultures whose properties will differ from
184 those of the 'natural' strain, through uncharacterised genetic and physiological changes during
185 selective isolation. $K_m$ values for whole cells, rather than individual enzymes, do not identify
186 the processes (e.g. ammonia oxidation *vs.* ammonia uptake) that are limiting activity and
187 affinity may be determined by non-enzymatic 'characteristics', e.g. cell surface area:volume
188 ratio. In addition, affinity constants for activity ($K_m$) will differ from those for growth ($K_s$),
189 which are more relevant for outcomes of competition (see Prosser, 2012). The relevance and

significance of substrate affinity for organisms in spatially complex and heterogeneous environments such as biofilms and soil, in which $NH_4^+$ exchanges with surfaces on which cells are attached, is also unclear.

*Hydroxylamine oxidation*

The conversion of hydroxylamine to $NO_2^-$ is much less understood and leads to many of the difficulties in assessing contributions of different processes to $N_2O$ production in natural environments. Until recently, it was commonly accepted that hydroxylamine is oxidised to $NO_2^-$ by hydroxylamine dehydrogenase (previously referred to as hydroxylamine oxidoreductase) in AOB (Arp & Stein, 2003). This reaction generates four electrons, two of which fuel ammonia oxidation, while two enter the electron transport chain to generate ATP and reducing equivalents. This mechanism failed to explain fully a number of experimental observations and is now challenged by enzymatic studies that suggest that the direct product of the hydroxylamine dehydrogenase reaction is not $NO_2^-$ but nitric oxide (NO) (Caranto & Lancaster, 2017). This reaction generates only three electrons and NO may then be converted abiotically to $NO_2^-$. Alternatively, generation of a fourth electron is possible, and likely, through enzymatic oxidation to $NO_2^-$. Caranto et al. (2016) suggested that under aerobic conditions NO could be rapidly oxidised to $NO_2^-$ by $NO_2^-$ reductase, encoded by *nirK*, as this enzyme can act reversibly. However, *nirK* expression is not high in AOB, expression is not coordinate with other ammonia oxidation genes, *nirK* is not found in genomes of all AOB and growth of *Nitrosomonas europaea* was unaffected by deletion of *nirK* (Cantera & Stein, 2007; Kozlowski *et al.*, 2014). Caranto et al. (2016) also proposed oxidation of NO by an as-yet uncharacterised NO oxidoreductase (NOO). A potential candidate is a red Cu protein, nitrosocyanin, encoded by *ncyA*, which is co-ordinately expressed with other ammonia

oxidation genes. Although this may fulfil this role in some AOB, *nycA* homologues are not present in all AOB genomes or in comammox bacteria (Kits et al., 2019).

Hydroxylamine oxidation in AOA is less well characterised. AOA (*N. maritimus*) can grow on hydroxylamine, producing $NO_2^-$ (Vajrala et al., 2013), and ammonia oxidation is inhibited by the NO-quenching agent PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; Jung et al., 2014; Kozlowski et al., 2016b; Shen et al., 2013; Yan et al., 2012). There is currently no obvious candidate enzyme for hydroxylamine oxidation to NO in AOA and genomes contain no gene homologous to those encoding heme-based enzymes. The current model for hydroxylamine oxidation proposes hydroxylamine and NO as co-substrates for a currently unidentified, putative hydroxylamine dehydrogenase enzyme complex that produces two molecules of $NO_2^-$, generating 5 electrons, and that *nirK* (which is expressed highly in AOA) reduces one of these molecules to NO, for further oxidation (Kozlowski et al., 2016b). The absence of heme-based enzymes in AOA suggests that the enzyme may be copper-based.

Comammox bacteria contain hydroxylamine dehydrogenase and produce NO, but lack *ncyA* gene homologues. PTIO inhibition is similar to that in AOA, suggesting similar tight regulation of NO production, although a NO reductase has yet to be identified (Kits et al., 2019).

## Mechanisms of nitrous oxide production during nitrification

### *Nitrifier denitrification*

Classical heterotrophic denitrifiers rely on nitrifiers for $NO_2^-$ or $NO_3^-$ and are responsible for $N_2O$ emissions under anaerobic conditions (coupled nitrification-denitrification). Ammonia oxidisers also produce $N_2O$ directly through a number of partially understood mechanisms. Traditionally, the most important was considered to be nitrifier denitrification, the reduction of $NO_2^-$ to $N_2O$ via NO, mainly under reduced oxygen concentrations (Goreau et al., 1980;

Kozlowski et al., 2014; Poth and Focht, 1985; Shaw et al., 2006; Wrage-Mönnig et al., 2018; Zhu et al., 2013). There is currently no evidence of $N_2O$ reductase gene homologues in AO genomes (e.g. Campbell et al., 2011; Chain et al., 2003; Norton et al., 2008; Spang et al., 2012; Tourna et al., 2011; Walker et al., 2010) and reports of reduction of $N_2O$ to $N_2$ by AO are rare, although nitrosocyanin may have this function (Arciero et al., 2002; Beyer et al., 2009; Schmidt, 2009; Todt and Dörsch, 2016; Whittaker et al., 2000).

In denitrifiers, $NO_2^-$ or $NO_3^-$ acts as a terminal electron acceptor and is linked to growth and energy production from organic carbon (Kuypers et al., 2018). In AO, the most likely role of denitrification enzymes is the removal of excess electrons at high $NH_3$ concentration (Hink et al., 2017a) and possibly detoxification of any accumulated $NO_2^-$ (Beaumont et al 2002, Stein and Arp 1998).

Nitrifier denitrification has been studied most in *N. europaea*, which possesses genes homologous to those of classical heterotrophic denitrifiers: *nirK*, encoding a Cu-containing nitrite reductase, and *norB*, encoding NO reductase (Chain et al., 2003; Kozlowski et al., 2014). While NorB is obligatory for nitrifier denitrification, NirK may be involved in oxidation of hydroxylamine rather than $NO_2^-$ reduction, possibly involving an alternative nitrite reductase (Kozlowski et al., 2014). While studies have focused on *N. europaea*, there is evidence for genetic and physiological diversity within AOB, for example differences in gene content and responses to anoxia of oligotrophic and non-oligotrophic strains (Kozlowski et al., 2016a).

There is currently no evidence for nitrifier denitrification in AOA or comammox bacteria as both groups lack NO reductase genes (e.g. Daims et al., 2015; Kim et al., 2011; Kits et al., 2017; Spang et al., 2012; Tourna et al., 2011; Walker et al., 2010), although the neutrophilic soil AOA *Nitrosocosmicus oleophilus* may be capable of enzymatically denitrifying nitrite to $N_2O$ at low pH (Jung et al., 2019). Furthermore, $N_2O$ produced by both AOA and comammox

bacteria possesses a site preference (difference in $\delta^{15}N$ of the $\alpha$ and $\beta$ N atoms of $N_2O$) which is distinct from that produced via nitrifier denitrification (Jung et al., 2014; Kits et al. 2019; Löscher et al., 2012; Santoro et al., 2011) and production is independent of oxygen availability (Hink et al., 2017b; Stieglmeier et al., 2014b).

*$N_2O$ production through hydroxylamine oxidation*

Hooper and Terry (1979) provided evidence that NO and $N_2O$ are also produced through incomplete oxidation of hydroxylamine. NO was considered to result from incomplete oxidation of hydroxylamine, with $N_2O$ produced through the activity of NO reductase (Kozlowski et al., 2014; Pacheco et al., 2011). This mechanism is compatible with the revised, 2-intermediate model for ammonia oxidation (Caranto and Lancaster, 2017). Furthermore, there is evidence that cytochrome P460 converts hydroxylamine to $N_2O$ with NO as an intermediate under anaerobic conditions enabling detoxification of hydroxylamine and NO and, in the presence of oxygen, NO would then abiotically dissociate to $N_2O$ (Caranto et al., 2016).

Hydroxylamine oxidation in AOA differs from that in AOB with cycling of NO, which reacts with hydroxylamine to form $NO_2^-$ (Kozlowski et al., 2016b), and there is currently no evidence for enzymatic $N_2O$ production from either hydroxylamine or NO. However, $N_2O$ may be produced through abiotic reactions linked to hydroxylamine oxidation (see below). Production mechanisms in *N. inopinata* are suggested to be similar to those of AOA, associated with abiotic conversion of hydroxylamine only, with no evidence for denitrification activity producing $N_2O$ with decreasing oxygen availability (Kits et al. 2019).

*Linked biotic and abiotic processes*

A range of abiotic processes exists by which hydroxylamine, $NO_2^-$ and NO can be converted to $N_2O$. The chemical reactions responsible, and their consequences for global emissions, have recently been reviewed (Heil et al., 2016; Zhu-Barker et al., 2015). They involve non-enzymatic conversions of products and intermediates of the nitrification process and therefore combine abiotic and biotic processes. $NO_2^-$ may be reduced, by a range of reducing agents, and fixed by soil organic matter (SOM). The significance of abiotic processes is rarely considered, through difficulties in detection and measurement in natural environments, and measurement of $NO_2^-$ concentration, rather than flux, but there is some evidence for $NO_2^-$ accumulation in terrestrial and aquatic ecosystems (Shen et al., 2003; van Cleemput & Samater, 1995).

Chemical decomposition of hydroxylamine generates $N_2O$ and small amounts of $N_2$ (Bremner et al., 1980). More importantly, hydroxylamine and nitrous acid (rather than $NO_2^-$) react to generate $N_2O$, particularly at low pH (Nelson, 1977). Detailed mechanisms are poorly understood and hydroxylamine can also be oxidised and, like $NO_2^-$, may form complexes with SOM (Heil et al., 2016). Abiotic production of $N_2O$ from hydroxylamine has rarely been considered seriously because of lack of evidence for release of hydroxylamine from AO cells and low or undetectable levels in natural environments. Concentrations in marine environments are in the nM range, and highest when nitrification is active, and improved techniques have now led to its detection in soil at 0.3 – 35 µg N $kg^{-1}$ dry soil (Liu et al., 2014). Low environmental concentrations may, in fact, reflect the high and diverse reactivity of hydroxylamine rather than low flux and Liu et al. (2017) demonstrated release of hydroxylamine from several AO in laboratory culture. Within AOB, production was greatest in *Nitrosospira multiformis* and *N. europaea*, but undetectable in *Nitrosomonas nitrosa* or *Nitrosomonas communis*, with low levels produced by the comammox strain *N. inopinata*.

Within AOA, hydroxylamine was produced by *Nitrososphaera gargensis* and *Nitrosotenuis uzonensis*, but not by *Nitrososphaera viennensis* or *Nitrosotalea devanaterra*. $NO_2^-$ reduced chemical decomposition of hydroxylamine and proportions of $NH_3$ converted to $N_2O$ were similar to those found previously in AOA and AOB.

Although AOB produce $N_2O$ enzymatically, smaller contributions via abiotic reactions with hydroxylamine, NO or $NO_2^-$, and the observed influence of NO-quenching agents on AOA and comammox bacteria activity suggest leakage of NO (Jung et al., 2014; Kits et al., 2019; Kozlowski et al., 2016b; Shen et al., 2013; Yan et al., 2012). In the presence of oxygen, NO and hydroxylamine can be rapidly converted abiotically to $N_2O$ (Kozlowski et al., 2016b; Stieglmeier et al., 2014b). Although it has implicitly been assumed that abiotic conversion is intracellular, direct evidence is lacking and it is possible that some or all abiotic production occurs after export or leakage of hydroxylamine from AO.

The situation is therefore complex. The two enzymatic pathways for $N_2O$ production in AOB, from hydroxylamine and $NO_2^-$, are treated as distinct pathways but share NO as an intermediate. $NO_2^-$, NO and hydroxylamine are also intermediates in abiotic processes, which may require inorganic reducing agents, in solution or in solid form. Other factors can also influence production, e.g. storage compounds, including intracellular hydroxylamine, may be responsible for apparent anoxic production. In addition, physiological studies are generally performed in well-mixed systems in which environmental conditions are constant. In natural environments, conditions will be transient, potentially leading to metabolic imbalance that may result in accumulation of intermediates involved in $N_2O$ production. It is difficult to assess the specific roles of abiotic reactions but there is sufficient evidence to merit further research into abiotic conversion of hydroxylamine and its relative contribution to emissions in comparison with nitrifier denitrification.

Assessment of global rates and contributions of abiotic processes is technically difficult and requires development and improvement of approaches for application of stable isotope tracing methods, including analysis of $^{15}N$ site preferences of $N_2O$ for the different processes. Estimates for the formation of $N_2O$ in sterile soils were suggested to be 31 - 75% of total $N_2O$ production in non-sterile agricultural soils (Venterea et al., 2007). However, there are difficulties in studying sterilised soil including incomplete sterilisation, alteration of soil properties and an absence of biotic production of hydroxylamine or $NO_2^-$, which are precursors for abiotic production (Lotrario et al., 1995; McNamara et al., 2003; Nowak et al., 1987).

**Distinction of archaeal and bacterial $N_2O$ production**

Biochemical and physiological studies of individual organisms provide a basis for the assessment of differences between AO and have been used in developing techniques to distinguish growth and activity of different groups in natural communities, e.g. differential inhibitors or isotopic methods. $^{15}N$- and $^{18}O$-enriched substrates can distinguish $N_2O$ production from nitrification and denitrification processes in the environment (see Ostrom and Ostrom, 2017; Wrage- Mönnig et al., 2018), although their ability to distinguish autotrophic and heterotrophic processes has been questioned (Bakken and Frostegård, 2017). Importantly, however, these techniques cannot differentiate emissions associated with AOA, AOB and comammox bacteria activities, which have common substrates and products. Attempts to distinguish these activities involve correlation of nitrification activity and $N_2O$ production with growth or transcriptional activity of different AO, usually via quantification of temporal changes in group-specific *amoA* genes or transcripts, respectively. There are significant limitations to this approach, but their value is increased when used in conjunction with inhibitors targeting specific groups in short term assays. Alkynes can inhibit monooxygenases through irreversible covalent binding of the active site. Acetylene is a potent inhibitor of AMO

of all AO, but 1-octyne has recently been established as a specific inhibitor of AOB (Taylor et al., 2013; Hink et al., 2017b). The NO-scavenger PTIO has been used as a specific inhibitor of AOA (Yan et al., 2012; Shen et al., 2013; Kozlowski et al., 2016b), but also inhibits comammox bacteria (Kits et al., 2019), and simvastatin can specifically inhibit AOA (Zhao et al., in revision).

*$N_2O$ yield*

$N_2O$ production associated with nitrification is quantified as the ratio of $N_2O$ to $NO_x^-$ ($NO_2^-$ and/or $NO_3^-$) produced or $NH_3$ consumed, and is termed $N_2O$ yield. Yields vary significantly within AO through differences in the physiology, and comparison with yields in environmental samples provides information on likely sources of production. $N_2O$ yield of AOB cultures ranges from 0.1 to 8% (Anderson et al., 1993; Hink et al., 2017a; Jiang & Bakken, 1999; Shaw et al., 2006), due to differences in available $NH_4^+$ and oxygen concentrations that affect enzymatic reactions. Higher yields are observed with increasing $NH_4^+$ concentration, potentially due to a lower reaction rate of hydroxylamine dehydrogenase than AMO, leading to accumulation of hydroxylamine, which is subsequently transformed abiotically to $N_2O$ (Hink et al., 2017a). An alternative explanation is redox balancing, in which electrons generated by hydroxylamine dehydrogenase are shuttled to denitrification enzymes when they exceed the capacity of terminal oxidases (Hink et al., 2017a). $N_2O$ yields from AOA cultures are below or in the lower range of those observed for AOB i.e. 0.04 - 0.3%, and $NH_4^+$ and oxygen concentration has no or little effect on yield (Jung et al., 2011; Hink et al., 2017a; 2014; Kim et al., 2012; Löscher et al., 2012; Santoro et al., 2011; Stieglmeier et al., 2014b; Qin et al., 2017). This is consistent with the assumption that $N_2O$ is produced only from abiotic reactions of intermediate compounds (Kozlowski et al., 2016b), although enzymatic production has been reported in *Nitrosocosmicus oleophilus* (Jung et al., 2019). Comammox bacteria and AOA lack

377 homologues of AOB NO reductase, suggesting low yields of $N_2O$, and abiotic production with
378 a yield of 0.07% has been reported in *N. inopinata* (Kits et al., 2019).

379 **Niche specialisation and differentiation in ammonia oxidisers**

380 AOA and AOB belong to different domains of life and differ considerably in cellular structure
381 and fundamental aspects of metabolism and physiology. This suggests the potential for niche
382 differentiation associated with different physiologies and has fuelled the search for associations
383 between environmental characteristics and AOA and AOB abundance and community
384 composition. Comammox studies are in their infancy but all current evidence indicates that
385 they are oligotrophs (Kits et al., 2017).

386 Marine AO communities are dominated by AOA (Beman et al., 2008), AOA are often absent
387 in wastewater treatment plants (Mussmann et al., 2011) and investigation of niche
388 differentiation between AO has focussed on terrestrial environments, with two factors
389 attracting particular attention: pH and $NH_4^+$. In laboratory culture, many AO can grow at pH
390 ≥6.5 (Hatzenpichler et al., 2008; Lehtovirta-Morley et al., 2016; Tourna et al., 2011), but
391 obligate autotrophic growth in acidic liquid batch culture is restricted to acidophilic AOA
392 belonging to the *Nitrosotalea* group, which grow in the pH range 4 - 6 (Jung et al., 2014;
393 Lehtovirta-Morley et al., 2011, 2014). This is reflected in their global presence and activity in
394 acidic soils (Gubry-Rangin et al., 2011), which contain AOA clades (particularly
395 *Nitrososphaera* clade C11) that are currently not represented in culture, restricting
396 physiological studies (Gubry-Rangin et al., 2018). AOA dominate ammonia oxidation in acidic
397 soils, which comprise 30% of soils globally, some of which are heavily fertilised arable soils
398 (von Uexküll and Mutert, 1995). Betaproteobacterial AOB activity has been observed at pH as
399 low as 5.5 in biofilms (Allison & Prosser, 1993), but AOA and AOB activities are found in
400 soils down to pH 4.5. AOB activity may be linked to an uncharacterised *Nitrosospira* clade

active in acidic soils (Aigle et al., 2019) and growth of ureolytic AO at low pH is also possible, if $NH_3$ is supplied as urea, as ureolytic activity is not pH-dependent (Burton et al., 2001). Evidence for this mechanism in soil is lacking and the role of AOB and *Nitrososphaera* clade C11 in acid soils, and potential metabolisms, require further study.

There is also evidence for niche differentiation associated with $NH_3$ supply. AOB growth is favoured in soils fertilised by single additions of high levels of inorganic $NH_3$, while AOA grow preferentially when $NH_3$ is produced through mineralisation of organic N (Hink et al., 2017b, 2018; Verhamme et al., 2011). AOA and AOB relative activities may therefore be influenced by different N fertilisation strategies, with subsequent effects on $N_2O$ yields.

**Terrestrial ecosystems**

Terrestrial environments contribute 56 - 70% of $N_2O$ emissions globally with agricultural systems contributing ~40% of that derived from soils (Davidson, 2009; Syakila & Kroeze, 2011). As described above, AO activity contributes directly to $N_2O$ emissions through both biotic and linked abiotic processes. However, coupled with the activity of NOB, they also contribute indirectly to $N_2O$ emissions by producing $NO_3^-$ for heterotrophic denitrifiers and subsequent reduction of NO to $N_2O$ as part of denitrification. The application of AO inhibitors has therefore been considered as a method to inhibit $N_2O$ emissions directly or indirectly (Ruser & Schulz, 2015). Although $NH_3$ can be lost through volatilisation, reduction of ammonia oxidation is generally considered beneficial for plant uptake and decreased fertilisation loss, and niche differentiation of AOA and AOB suggests that controlling the activities of each group may also have substantial impacts on reducing $N_2O$ emissions. This leads to the hypothesis that inorganic fertilisation will benefit AOB, and high $N_2O$ yield and emissions, while slow release of ammonia from native organic N, organic fertiliser or slow-release fertiliser will favour AOA and lower emissions (Figure 1).

425 This hypothesis has been tested most critically in microcosms in which AOA and AOB $N_2O$
426 production is distinguished using differential inhibitors or comparison of isotopic signatures
427 with those obtained in AOA or AOB cultures. The inhibitor approach was first used by Hink
428 et al. (2017b) in soil microcosms in which $N_2O$ production was dominated by AO and $N_2O$
429 emissions by AOA and AOB were discriminated by differential inhibition of AOB using 1-
430 octyne. Inorganic N fertilisation stimulated $N_2O$ production, which was reduced in the presence
431 of 1-octyne, and estimated $N_2O$ yields associated with AOA and AOB activity were
432 comparable to those obtained in pure cultures, i.e. the $N_2O$ yield of AOA was approximately
433 half that of AOB. AOA-associated $N_2O$ emission was also determined in unfertilised soils, in
434 which $NH_3$ is supplied through mineralisation of organic N, and $N_2O$ yields were again similar
435 to those reported for AOA cultures. These results therefore indicate that the relative
436 contribution to ammonia oxidation and $N_2O$ generation by AOB may be greatly reduced by
437 controlling the rate of $NH_4^+$ supply.

438 To test the potential for reduction in $N_2O$ through use of different fertiliser strategies, a similar
439 approach compared single addition of urea-N at high concentration and continuous low
440 production from a slow-release urea-N fertiliser (polymethylene urea) or organic N
441 mineralisation (Hink et al., 2018). When fertiliser was supplied at high concentration, AOB
442 dominated activity and $N_2O$ production, with high yield (Figure 2).

443 Inhibition of AOB activity by 1-octyne reduced $N_2O$ production and yield but AOA activity
444 increased, demonstrating the ability of AOA to grow at high $NH_4^+$ concentration in the absence
445 of AOB. Low $NH_4^+$ supply, through slow release, led to dominance of activity by AOA and
446 low $N_2O$ yield.

447 AOB-dominated nitrification and high AOB-associated $N_2O$ production have also been
448 observed after N fertilisation of alluvial (pH 8) and red (pH 6) soils (Wang et al. 2016).

Fertilisation did not result in AOA growth and, in fact, led to a decrease in AOA abundance in the red soil. AOA did not grow in the alluvial soil but grew in control and 1-octyne-treated, unfertilised red soils, but not following acetylene inhibition. AOA growth was not associated with detectable $N_2O$ production. In fertilised soils, proportions of emissions associated with AOB and AOA were similar (70.5 - 78.1% and 18.7 - 19.7%, respectively) to those observed by Hink et al. (2017b). Meinhardt et al. (2018) complemented this approach by additional use of PTIO, to inhibit AOA, and isotopic analysis, to distinguish $N_2O$ arising from hydroxylamine oxidation and nitrifier denitrification in an alkaline switch-grass soil. Nitrification, AOA and AOB growth and activity and $N_2O$ production were low in control soil microcosms. Inorganic N fertilisation favoured AOB growth and increased $N_2O$ production, and isotopic analysis indicated that $N_2O$ production was associated with AOB activity, while production in unfertilised soil was similar to that from AOA cultures. These results were consistent with field data, in which AOB abundance correlated with $N_2O$ emissions.

While $NO_2^-$ concentration in soil is typically low, accumulation of $NO_2^-$ and its influence on $N_2O$ emissions has also been investigated in microcosms. Venterea et al. (2015) and Breuillin-Sessoms et al. (2017) reported $NO_2^-$ accumulation and associated $N_2O$ production in bovine urine- and urea-fertilised soil. Increased $N_2O$ production after fertilisation was thought to be due to nitrifier denitrification of accumulated $NO_2^-$. Interestingly, Giguere et al. (2017) reported $NO_2^-$ accumulation in three non-cropped soils that was not associated with increased N fertilisation. This led to increased $N_2O$ production in soil in which ammonia oxidation was dominated by AOA or shared between AOB and AOA and production was stimulated by addition of $NO_2^-$. While this can be explained by nitrifier denitrification by AOB, some of the activity was associated with AOA. The mechanism for this is unclear, but could be through abiotic reaction between $NO_2^-$ and leaked hydroxylamine or currently uncharacterized AOA

473 nitrifier denitrification. Regardless of the mechanism, the authors highlight the potential for
474 nitrite accumulation to influence $N_2O$ yields in soil studies.

475 Duan et al. (2019) used 1-octyne to distinguish AOA- and AOB-associated $N_2O$ emissions in
476 urea-amended microcosms using several Chinese soils cultivated with vegetables in
477 greenhouses and previously treated with different levels of urea fertiliser. AOA dominated $N_2O$
478 production in microcosms containing previously unfertilised soil and AOA and AOB
479 contributed to production in soil with a history of intermediate fertilisation. AOA abundance
480 increased with historically high urea fertilisation levels and dominated nitrification and $N_2O$
481 production in the two most heavily fertilised soils. The authors also reported a correlation
482 between $N_2O$ production and accumulated $NO_2^-$, suggesting differential inhibition of NOB at
483 high $NH_4^+$. These, and other studies of complex soil ecosystems, indicate that the potential for
484 localised depletion of oxygen and nitrate accumulation must also be considered, through
485 ammonia oxidation following high levels of fertilisation, that will provide conditions
486 favourable for $N_2O$ production by heterotrophic nitrifiers.

487 In addition, an increasing number of microcosm and field studies have generated data on
488 correlations between $N_2O$ and AOA or AOB *amoA* gene or transcript abundance. These studies
489 have generally been carried out to assess the effects of fertiliser on production rates and AO
490 abundances, rather than to test the above hypotheses, and suffer from the fundamental
491 limitations of correlation-based studies and use of gene abundance as a measure of activity.
492 They frequently report AOB growth only, following fertilisation, and therefore find
493 correlations between $N_2O$ production and AOB, rather than AOA abundance, e.g. studying
494 fertilised paddy soil subjected to wetting and drying (Abid et al., 2018), biochar-treated wheat-
495 maize soil (Liu et al., 2019), arable soil (Song et al., 2018) and forest soil (Martins et al., 2017).
496 Several studies, however, reported a greater role for AOA in nitrification and correlation

between AOA and $N_2O$, e.g. in tropical rainforest soil (Soper et al., 2018) and Tibetan alpine soil (Peng et al. 2018).

Directed microcosm studies, using inhibitor and isotopic approaches to distinguish AOA and AOB activities, therefore support proposed links between niche specialisation and the consequences for $N_2O$ production and yield. Care must be taken in interpreting results, inhibitor specificity may require more rigorous testing and additional inhibitors may be required to differentiate comammox bacteria. PTIO inhibits *N. inopinata* and *N. maritimus* (Martens-Habbena et al., 2015; Kits et al., 2019) and is therefore not specific for AOA activity, and both PTIO and 1-octyne can inhibit both AOA and AOB at sufficiently high concentrations (Shen et al., 2013; Taylor et al., 2013). They must therefore be used with care with complex natural communities. In addition, greater activity of AOA when AOB are inhibited may lead to overestimation of $N_2O$ production by AOA when both groups are present and potentially active. Studies based only on AO abundances produce a range of effects with results that are difficult to interpret because of a number of limitations, including lack of control, inability to distinguish effects of different factors and difficulty in relating abundance to activity.

**Aquatic ecosystems**

The major source of nitrogen in marine and freshwater ecosystems is biological nitrogen fixation. AO therefore obtain $NH_3$ through mineralisation of organic N and $N_2O$ is produced by both ammonia oxidisers and traditional denitrifiers, the latter also providing a sink for $N_2O$. Production is greatest in regions of equatorial and coastal upwelling (transporting $N_2O$ produced in deep sediments), OMZ and areas of high productivity, with hot-spots in the Arabian Sea and East tropical South Pacific (ETSP) (Nevison et al., 1995). Marine systems are estimated to contribute ~3.8 Tg $N_2O$ $y^{-1}$, equivalent to 21% of global emissions, although the range of estimates is large (1.8 – 9.4 Tg $N^{-1}$) (IPCC, 2013). This high range is due to

uncertainties in methodology, reliance on assumed correlations between oxygen utilisation and $N_2O$ production and lack of information for parametrisation of simulation models, in addition to uncertainties regarding biophysical processes, such as transfer functions from surface waters to atmosphere, wind dispersal and hydrogeography.

AOA dominate AO communities in the open ocean and are the major contributors to marine ammonia oxidation and AO-associated $N_2O$ production (Santoro et al., 2011), although a minority of studies report similar abundances for AOA and AOB. AOA abundance is low in surface waters, except in the Arctic (Müller et al., 2018), through photoinhibition and low $NH_3$ flux, but increases between ~100 - 1000 m, before decreasing at greater depths. AOA communities are dominated by two phylogenetic groups within the *Nitrosopumilales* (Santoro et al., 2019). The water column A (WCA) or high $NH_4^+$ (HAC) group is found in polar regions, contains the cultivated AOA *Nitrosopelagicus brevis* and dominates when total AOA abundance is high. The water column B (WCB) or low $NH_4^+$ (LAC) group dominates at depths >300 m and currently has no cultivated representative. Correlations of gene and transcript abundances and nitrification rates with environmental conditions suggest that WCA has a broad environmental niche, but WCB genes correlate with lower temperature, higher nutrients and low chlorophyll (Smith et al., 2014). The basis of niche differentiation between these two groups, however, remains uncertain.

A role for ammonia oxidation and $N_2O$ production by AOA is based on correlations between gene and transcript abundances and $N_2O$ production rates and isotopic methods. For example, Löscher et al. (2012) reported $N_2O$ production in the East Tropical North Atlantic (ETNA) (oxygen concentration >40 µmol $l^{-1}$) and the OMZ of ETSP waters. In the ETSP, $N_2O$ emissions correlated with both archaeal *amoA* gene abundance and those involved in denitrification, suggesting a mixed origin for $N_2O$, while ETNA production correlated with

archaeal *amoA* gene abundance only. AOA outnumbered AOB by 1 - 2 orders of magnitude. $N_2O$ production was also inhibited when sea water was incubated with the inhibitor N1-guanyl-1,7-diaminoheptane (GC7), which inhibits synthesis of hypusine, required for protein synthesis in archaea. Peng et al. (2015) used on-board incubations of ETNP (East tropical North Pacific) water to assess depth-related nitrification potential, *amoA* gene abundance, $N_2O$ production and selective inhibition of AOB and AOA by ATU and PTIO, respectively. AOA outnumbered AOB by approximately one order of magnitude and estimated $K_m$ values were similar to those of *N. maritimus*, confirming the potential for activity in the OMZ. Horak et al. (2013) also measured a $K_m$ of 98 nmol $l^{-1}$ in incubations of Puget Sound sea water where, again, AOA outnumbered AOB by 1 – 2 orders of magnitude. In contrast, Ji et al. (2015) measured rates and gene abundances in ETSP water and suggested nitrification and associated $N_2O$ production were greatest between the euphotic zone and the OMZ, but that denitrification dominate production in the OMZ. Effects of climate change have also been investigated by Rees et al. (2016), who found that acidification (pH reduction by 0.06 – 0.4) of polar and subpolar Atlantic Ocean waters did not affect AOA community composition but reduced $N_2O$ production in proportion to the reduction in $NH_3$ availability (vs. $NH_4^+$) due to the pH reduction.

Many simulation models estimate $N_2O$ production rate from measurements of oxygen concentration, assuming a linear negative relationship. Trimmer et al. (2016) tested this assumption by determining relationships between AOA *amoA* and *nirK* gene abundances (AOB were below the detection limit), oxygen concentrations and $N_2O$ production in an ETNP oxycline. Correlations between oxygen and molecular data were similar to those from low-oxygen regions of the Baltic Sea (Berg et al., 2015), supporting AOA activity at low oxygen concentration. $N_2O$ production increased exponentially as oxygen concentration decreased from 30 – 1 µmol $O_2$ $l^{-1}$ and there was no evidence for denitrification or nitrifier denitrification.

570 This confirmed early work of Goreau et al. (1980) and the improved information on dynamics
571 improved modelling of $N_2O$ emissions in OMZs. This led to estimates of 17 µmol $N_2O$ $m^{-2}$ $d^{-1}$,
572 which agree with previous estimates, and predictions that ETNP and global OMZ production
573 generates 2.1 Tg N $y^{-1}$ and 5.1 Tg N $y^{-1}$ as $N_2O$, respectively.

574 *Lakes and coastal regions*

575 AOB appear to dominate ammonia oxidation in freshwater environments and Wenk et al.
576 (2016) used isotope measurements to distinguish different sources of $N_2O$ production in two
577 basins (North and South) of Lake Lugano. Production was higher in the holomictic lake and
578 was associated with denitrification and bacterial (rather than archaeal) ammonia oxidation in
579 the redox transition zone. $N_2O$ production in the meromictic North Basin was lower and was
580 due to nitrifier denitrification. Frame et al. (2017) compared $N_2O$ production in Lake Lugano
581 and Namibian coastal waters. In the former, AOB outnumbered AOA and isotope methods
582 indicated AOA or AOB production by abiotic conversion of hydroxylamine and insignificant
583 nitrifier denitrification. In Namibian seawater, AOA outnumbered AOB but the mechanism of
584 production was not clear. Angell et al. (2018) correlated abundance of functional genes
585 associated with $N_2O$ production with process data in salt marshes subjected to different levels
586 of N fertilisation. N fertilisation increased rates of production and consumption of $N_2O$,
587 increased the contribution of denitrification, had no effect on AOA composition but changed
588 AOB community composition, which contributed more than AOA to $N_2O$ production.

589 *Wastewater treatment systems*

590 Wastewater treatment encompasses a wide range of aerobic and anaerobic processes in which
591 $N_2O$ is generated by ammonia oxidisers and/or denitrifiers, contributing to 1.3% of global
592 anthropogenic emissions (Kampschreur et al., 2009). AO will generate $N_2O$ in aerobic
593 processes but their contributions will be influenced by spatial heterogeneity, with anoxic

conditions within flocs of activated sludge processes and biofilms formed in trickling filters. The impact of these conditions and of pH are discussed in recent reviews (Blum et al., 2018; Sabba et al., 2018; Todt & Dörsch, 2016). AOB are generally considered to dominate AO communities but Yin et al. (2018) found that AOB dominated 13 of 23 wastewater treatment plants, including 3 in which AOA were not detectable. AOA outnumbered AOB by ~1 – 2 orders of magnitude in 10 plants but, if cellular ammonia oxidation and $N_2O$ rates are lower for AOA, AOA will not necessarily dominate activity. These findings do, however, suggest that AOA should not be considered insignificant, but it is currently not clear which conditions influence relative abundances of AOA and AOB. Mitigation of $N_2O$ emissions may therefore be possible, but requires investigation of niche specialisation and sources of emissions.

## Potential strategies for mitigation of $N_2O$ emissions

$N_2O$ emissions in open oceans are from natural, rather than anthropogenic sources, and mitigation in coastal regions and freshwater environments is achieved by reducing N run-off. Soils are the main source of $N_2O$ globally and advances in the understanding of the ecology of the organisms involved presents an opportunity to influence agricultural practices and decrease global emissions.

Ammonia oxidation directly and indirectly leads to all microbially-mediated $N_2O$ production. Ammonia oxidation can reduce volatilisation of $NH_3$ in alkaline soils but reduction of AO activity in non-alkaline agricultural soils will not only improve fertiliser use efficiency, by increasing the residence time of $NH_4^+$ in soil and the opportunity for plant uptake, but will also decrease AO-associated $N_2O$ emissions. This can be achieved by use of synthetic nitrification inhibitors, whose benefits and limitations have been reviewed elsewhere (Coskun et al., 2017). BNIs associated with arable crops may provide a more efficient approach to nitrification inhibition (Subbarao et al., 2012) and Byrnes et al. (2017) demonstrated the use of forage

grasses with high BNI activity in decreasing $N_2O$ emissions in urine bovine pasture. Both synthetic inhibitors and BNIs have been developed against AOB and, while some also inhibit AOA, others have not been tested. Future studies should therefore assess inhibition of a range of both AOB and AOA, given increasing appreciation of diversity within these groups.

Increased understanding of niche differentiation within AO, and within AOA and AOB, and significant improvements in the ability to quantify these organisms, adds a further dimension and presents new opportunities. For example, fertiliser use efficiency will be greater in soils dominated by AOA or AOB, following application of inorganic or slow-release fertilisers, respectively, given their different $NH_3$ supply preferences. Ammonia oxidation by either group will result in $N_2O$ emissions, but management strategies that favour AOA would have an additive effect in reducing $N_2O$ emissions, as $N_2O$ yield from AOB activity is double that of AOA (Hink et al., 2017b, 2018). In contrast, liming of acidic soil is likely to increase AOB, rather than AOA, increasing $N_2O$ emissions associated with AOB activity. This highlights the need to consider the impact on relevant and important microbial communities, in addition to benefits for plant productivity and is consistent with calls for greater consideration of sustainability in agricultural practice (Rockström et al., 2017).

Realisation of this potential requires a broader range of microcosm studies with a greater range of soil types and conditions and fertilisation strategies to confirm the relatively limited data currently available. Importantly, it also requires well-designed field experiments to translate laboratory findings to agricultural soils and to assess impacts of mitigation strategies on microbial communities, plant productivity, fertiliser use efficiency and $N_2O$ emissions under field conditions. To date, field studies have focused largely on inorganic N fertilisers and few have considered diversity within AO and the consequences for $N_2O$ emissions.

## Conclusion

Research over the last 25 years has dramatically increased our understanding of AO diversity and the importance of AO community composition in determining rates of ammonia oxidation, the influence of environmental factors and important consequences for $N_2O$ emissions. This provides the basis for better quantitative information required for parameterisation of biogeochemical models to improve predictions and assess impacts of different management strategies. Physiological studies should consider these requirements but care should also be taken in generalising and extrapolating findings from single isolates, single genomes and single studies and information is required to determine the degree to which isolates represent active members of natural communities. Ecological studies require greater focus on structured experiments that identify the combinations of factors that determine the activity of different AO groups based on niche specialisation, with less emphasis on descriptive studies. Research would also benefit from development of better techniques to distinguish the many different biotic and abiotic processes contributing to $N_2O$ production and to distinguish activity of different functional microbial groups *in situ*. A critical requirement, however, is for studies that test the predictions of physiological and microcosm experiments ultimately to inform mitigation strategies under field conditions.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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**Figure legends**

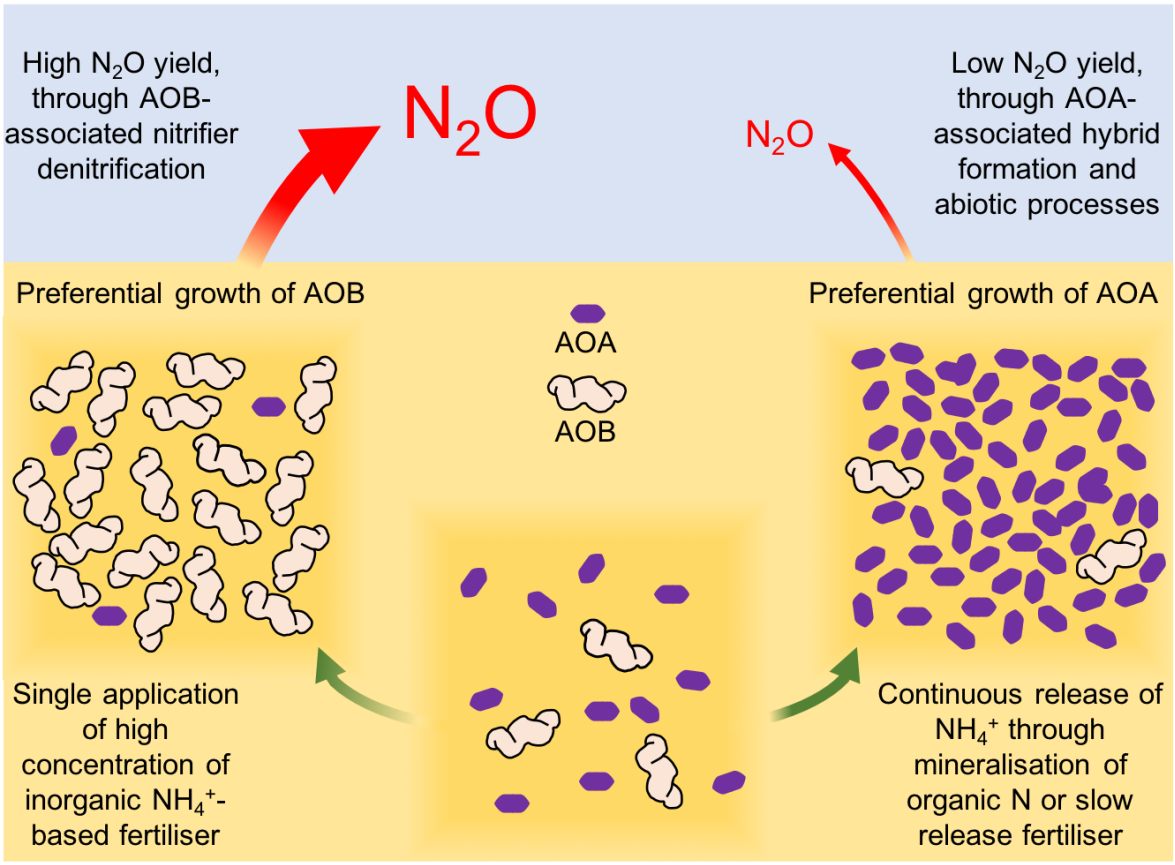
Figure 1. A schematic representation of changes in AO communities and directly AO-associated $N_2O$ emissions during incubation of soil after addition of single application of high inorganic $NH_4^+$-based fertiliser or with slow release of $NH_4^+$ from soil organic nitrogen or a slow release fertiliser. The initial AO community, prior to incubation, is dominated by AOA, which are generally assumed to be smaller than AOB.

Figure 2. Changes in (a) $NH_4^+$ and (b) $NO_3^-$ concentrations, (c) AOA and AOB *amoA gene* abundances and (d) $N_2O$ emissions from soil microcosms incubated after addition of water (green triangle, blue cross) or under conditions of high $NH_4^+$ supply (0 – 10 days) or low $NH_4^+$ supply (10 – 25 days) (black circle, red square). Ammonium was supplied as a slow-release, urea-based fertiliser that contained residual free urea. (Urea was converted rapidly to $NH_4^+$.) This generated high initial concentrations of available $NH_4^+$, which was oxidised within 10 days. Thereafter, $NH_4^+$ was generated by mineralisation of native organic nitrogen or of the slow-release fertiliser. In addition, microcosms were incubated with (black circle, green triangle) or without (red square, blue cross) 1-octyne, a specific inhibitor of AOB.

AO growth in the absence of fertiliser (green triangle, blue cross) was due to mineralisation of native organic nitrogen. Under these conditions, AOB growth was not detectable, AOA grew slowly and $N_2O$ emissions were low and unaffected by the 1-octyne. Initial high inorganic $NH_4^+$ after fertiliser addition (1 – 10 days) resulted in greater growth of AOB, increases in the AOB:AOA ratio and substantial $N_2O$ emissions that were significantly reduced, almost to those of control microcosms, in the presence of 1-octyne. During slow release of $NH_4^+$ (10 – 25 days), growth was dominated by AOA, $N_2O$ emissions were significantly reduced and

emissions were much less affected by the AOB inhibitor, 1-octyne. Note, however, that AOA growth was stimulated to some extent when AOB were inhibited. (See Hink et al. 2018 for experimental details and further discussion of results.)

1147 Figure 1

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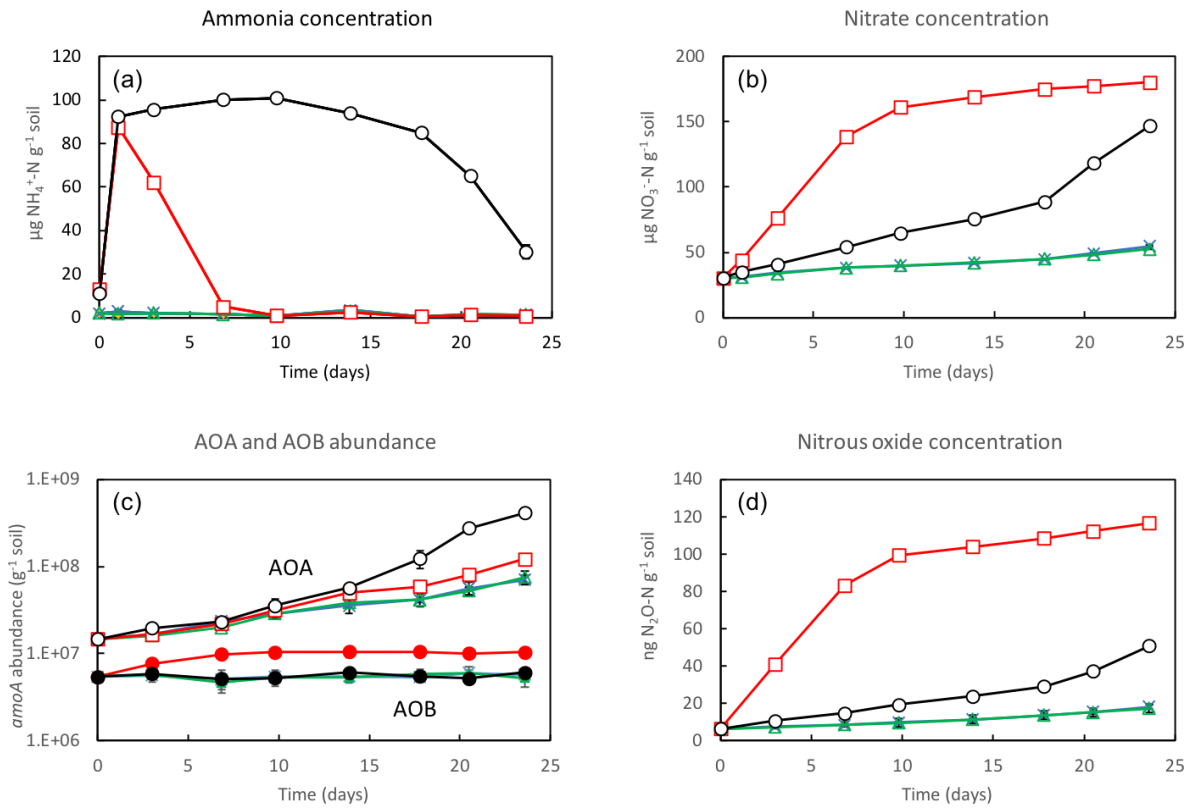
1151 Figure 2

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