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Nitrous oxide production by ammonia oxidizers: Physiological diversity, niche differentiation and potential mitigation strategies

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23 Abstract

24 Oxidation of ammonia to nitrite by bacteria and archaea is responsible for global emissions of
25 nitrous oxide directly and indirectly through provision of nitrite and, after further oxidation,
26 nitrate to denitrifiers. Their contributions to increasing N₂O emissions are greatest in terrestrial
27 environments, due to the dramatic and continuing increases in use of ammonia-based fertilisers,
28 which have been driven by requirement for increased food production, but which also provide
29 a source of energy for ammonia oxidisers, leading to an imbalance in the terrestrial nitrogen
30 cycle. Direct N₂O production by ammonia oxidisers results from several metabolic processes,
31 sometimes combined with abiotic reactions. Physiological characteristics, including
32 mechanisms for N₂O production, vary within and between ammonia oxidising archaea (AOA)
33 and bacteria (AOB) and comammox bacteria and N₂O yield of AOB is higher than in the last
34 two groups. There is also strong evidence for niche differentiation between AOA and AOB
35 with respect to environmental conditions in natural and engineered environments. In particular,
36 AOA are favoured by low soil pH and AOA and AOB are respectively favoured by low rates
37 of ammonium supply, equivalent to application of slow-release fertiliser, or high rates of
38 supply, equivalent to addition of high concentrations of inorganic ammonium or urea. These
39 differences between AOA and AOB provide the potential for better fertilisation strategies that
40 could both increase fertiliser use efficiency and reduce N₂O emissions from agricultural soils.
41 This article reviews research on the biochemistry, physiology and ecology of ammonia
42 oxidisers and discusses the consequences for ammonia oxidiser communities subjected to
43 different agricultural practices and the ways in which this knowledge, coupled with improved
44 methods for characterising communities, might lead to improved fertiliser use efficiency and
45 mitigation of N₂O emissions.

46 **Introduction**

47 Nitrous oxide (N₂O) is an important greenhouse gas produced by ammonia oxidisers (AO) and
48 denitrifiers. It ranks third behind carbon dioxide (CO₂) and methane (CH₄) in terms of radiative
49 forcing, with estimated global N₂O emission rates of 11 Tg y⁻¹ (range 8.1 - 30.7 Tg y⁻¹) (IPCC,
50 2013). However, N₂O has a global warming potential that is 265- and 10-fold greater than those
51 of CO₂ and CH₄, respectively, has a long atmospheric lifetime (109 - 125 years, Prather et al.,
52 2015), contributed 17% to radiative forcing in 2005 (IPCC, 2013) and is predicted to be the
53 primary contributor to ozone depletion in the stratosphere in the 21st Century (Ravishankara et
54 al., 2009). Atmospheric N₂O levels are 20% greater than in pre-industrial times (MacFarling-
55 Meure et al., 2006), largely through increases in reactive nitrogen, resulting from increased
56 nitrogen fertilisation as manure, inorganic nitrogen and urea (Smil, 1999). N₂O levels are
57 increasing by 0.73 ± 0.03 ppb y⁻¹ (~0.3% y⁻¹) and are projected to increase significantly through
58 this century, due to food and animal feed demands of an increasing global population (Tilman
59 et al., 2001; Alexandratos & Bruinsma, 2012).

60 Nitrification, the sequential oxidation of ammonia (NH₃) to nitrite (NO₂⁻) and nitrate (NO₃⁻) in
61 the presence of oxygen, can contribute indirectly to N₂O emissions, providing nitrogen oxides
62 to denitrifying microorganisms for energy conservation under reduced oxygen conditions and
63 anoxia (Kuypers et al., 2018). Denitrifiers are traditionally considered major producers of N₂O,
64 but N₂O is also emitted by organisms that oxidise NH₃ to NO₂⁻. This direct contribution to
65 emissions is particularly important given projected increases in N fertiliser applications.
66 Traditionally ammonia oxidation was thought to be performed by bacterial ammonia oxidisers
67 (AOB), but marine ammonia oxidation is now known to be dominated by archaeal ammonia
68 oxidisers (AOA) and both groups are important in other ecosystems. Recently discovered
69 complete ammonia oxidisers (comammox bacteria) oxidise both NH₃ and NO₂⁻ within the same

70 cell (Daims et al., 2015; van Kessel et al., 2015), but little is currently known of their ecology.
71 Cellular rates of N₂O production by AOA and AOB differ, leading to different contributions
72 to emissions, and there is evidence for niche differentiation between AOA and AOB with
73 respect to environmental factors and land use strategies. This article reviews evidence for
74 differences in N₂O emissions by AO in culture and in natural ecosystems and assesses potential
75 links between niche differentiation, N₂O emissions and agricultural systems and potential
76 mitigation strategies.

77 **The role of nitrifiers in biogeochemical cycling**

78 In both terrestrial and aquatic ecosystems, the major source of bioavailable nitrogen is fixation
79 of atmospheric dinitrogen (N₂) by free-living or plant-associated N₂-fixing bacteria,
80 photosynthetic cyanobacteria and hydrogenotrophic methanogenic archaea (Boyd & Peters,
81 2013). Following consumption by higher trophic levels, excretion and death, mineralisation of
82 organic N by microbes releases inorganic ammonium (NH₄⁺), which can be assimilated by
83 plants and microorganisms. Ammonium also provides an essential source of energy for
84 obligate aerobic, lithotrophic microorganisms, the ammonia oxidisers. These organisms
85 oxidise NH₃ to NO₂⁻, which is then oxidised to NO₃⁻, before dissimilatory reduction under
86 anaerobic/microaerobic conditions. Facultative denitrifiers can return N₂ to the atmosphere
87 (Kuypers et al., 2018). These nitrogen cycling processes are supplemented by a network of
88 other biological and physicochemical processes.

89 In aerobic marine ecosystems, nitrification is highly efficient and results in concentrations of
90 NH₄⁺ in the nanomolar range (Horak et al., 2013), but can be limited under reduced oxygen
91 concentration in oxygen minimum zones (OMZ; Ward, 2011) and within decaying particulate
92 organic matter or polluted environments, where oxygen demand can be high. These conditions
93 favour denitrification.

94 In natural terrestrial ecosystems, in which NH_4^+ is provided via mineralisation and NO_3^- is
95 available, the balance between nitrification and denitrification is again controlled largely by
96 oxygen concentration, which is itself controlled by soil moisture content. At high moisture
97 content, the potential for aerobic nitrification will be low and denitrification will dominate. The
98 situation reverses as soil moisture decreases, and oxygen concentration increases (Bateman &
99 Baggs, 2005). These potential rates are moderated by other factors, e.g. rates of NH_3 supply
100 from mineralisation or supply of organic carbon and nitrate for denitrification (Booth et al.,
101 2005). This leads to variability in relative concentrations of NH_4^+ and NO_3^- and in relative
102 activities of nitrifiers and denitrifiers at both ‘bulk’ and microscale levels. For example, even
103 in ‘aerobic’ soils, denitrification may occur in microenvironments, where oxygen diffusion is
104 limited, or in regions of high decomposition, where oxygen demand is high.

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

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

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

127 **Diversity of ammonia oxidising microorganisms**

128 Three main groups of aerobic ammonia oxidising prokaryotes have been described: AOB,
129 AOA and comammox bacteria. AOB belong to beta- and gammaproteobacteria classes, with
130 two (*Nitrosomonas* and *Nitrospira*) and one (*Nitrosococcus*) genera, respectively (Purkhold
131 et al., 2000). These genera display different environmental distributions indicating distinct
132 physiological characteristics. *Nitrosococcus* is mainly found in marine environments and salt
133 lakes (Campbell et al., 2011), although one strain was recently enriched from an acidic soil
134 (Hayatsu et al., 2017). *Nitrosomonas* has been found in similar environments but also in
135 engineered systems, such as wastewater treatment systems (Mobarry et al., 1996; Schramm et
136 al., 1996). *Nitrospira* organisms dominate soil AOB but are found in a range of ecosystems
137 and are genetically diverse, in terms of both 16S rRNA and *amoA* gene markers (Aigle et al.,
138 2019; Purkhold et al., 2000, 2003).

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

142 Armanet et al., 2008; Kerou et al., 2016; Stieglmeier et al., 2014a). AOA are ubiquitous and
143 mesophilic lineages and are genetically diverse, based on *amoA* and 16S rRNA gene
144 phylogenies, with higher diversity described for terrestrial than marine lineages (Alves et al.,
145 2018; Gubry-Rangin et al., 2011; Pester et al., 2011). Thermophilic AOA diversity is lower,
146 possibly reflecting sampling bias (Alves et al., 2018; Gubry-Rangin et al., 2018).

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

156 **Mechanisms of ammonia oxidation**

157 *Ammonia oxidation to hydroxylamine*

158 The first step in ammonia oxidation in all currently characterised aerobic, autotrophic AO is
159 oxidation of NH₃ to hydroxylamine by NH₃ monooxygenase (AMO), a copper-based, broad-
160 range, membrane-bound oxygenase. Although studied in detail in relatively few organisms
161 (Arp and Stein, 2003; Kozlowski et al., 2016b), AMO has similar characteristics in AOA and
162 AOB. It contains three major structural subunits (Tolar et al., 2017) and although the active
163 enzyme complex has not been identified, comparison with the particulate methane
164 monooxygenase suggests the β -subunit as the active site (Tolar et al., 2017). The structural
165 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 NH₃ affinity in
174 *Nitrosopumilus maritimus* provided a convincing explanation for the dominance of AOA in
175 marine environments, where NH₃ 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 NH₃ affinity, leading to the
180 view that comammox bacteria are oligotrophs with a competitive advantage in low NH₃
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

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

193 *Hydroxylamine oxidation*

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

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

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

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

229 **Mechanisms of nitrous oxide production during nitrification**

230 *Nitrifier denitrification*

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

236 Kozłowski et al., 2014; Poth and Focht, 1985; Shaw et al., 2006; Wrage-Mönnig et al., 2018;
237 Zhu et al., 2013). There is currently no evidence of N₂O reductase gene homologues in AO
238 genomes (e.g. Campbell et al., 2011; Chain et al., 2003; Norton et al., 2008; Spang et al., 2012;
239 Tourna et al., 2011; Walker et al., 2010) and reports of reduction of N₂O to N₂ by AO are rare,
240 although nitrosocyanin may have this function (Arciero et al., 2002; Beyer et al., 2009;
241 Schmidt, 2009; Todt and Dörsch, 2016; Whittaker et al., 2000).

242 In denitrifiers, NO₂⁻ or NO₃⁻ acts as a terminal electron acceptor and is linked to growth and
243 energy production from organic carbon (Kuypers et al., 2018). In AO, the most likely role of
244 denitrification enzymes is the removal of excess electrons at high NH₃ concentration (Hink et
245 al., 2017a) and possibly detoxification of any accumulated NO₂⁻ (Beaumont et al 2002, Stein
246 and Arp 1998).

247 Nitrifier denitrification has been studied most in *N. europaea*, which possesses genes
248 homologous to those of classical heterotrophic denitrifiers: *nirK*, encoding a Cu-containing
249 nitrite reductase, and *norB*, encoding NO reductase (Chain et al., 2003; Kozłowski et al., 2014).
250 While NorB is obligatory for nitrifier denitrification, NirK may be involved in oxidation of
251 hydroxylamine rather than NO₂⁻ reduction, possibly involving an alternative nitrite reductase
252 (Kozłowski et al., 2014). While studies have focused on *N. europaea*, there is evidence for
253 genetic and physiological diversity within AOB, for example differences in gene content and
254 responses to anoxia of oligotrophic and non-oligotrophic strains (Kozłowski et al., 2016a).

255 There is currently no evidence for nitrifier denitrification in AOA or comammox bacteria as
256 both groups lack NO reductase genes (e.g. Daims et al., 2015; Kim et al., 2011; Kits et al.,
257 2017; Spang et al., 2012; Tourna et al., 2011; Walker et al., 2010), although the neutrophilic
258 soil AOA *Nitrosocosmicus oleophilus* may be capable of enzymatically denitrifying nitrite to
259 N₂O at low pH (Jung et al., 2019). Furthermore, N₂O produced by both AOA and comammox

260 bacteria possesses a site preference (difference in $\delta^{15}\text{N}$ of the α and β N atoms of N_2O) which
261 is distinct from that produced via nitrifier denitrification (Jung et al., 2014; Kits et al.
262 2019; Löscher et al., 2012; Santoro et al., 2011) and production is independent of oxygen
263 availability (Hink et al., 2017b; Stieglmeier et al., 2014b).

264 *N₂O production through hydroxylamine oxidation*

265 Hooper and Terry (1979) provided evidence that NO and N_2O are also produced through
266 incomplete oxidation of hydroxylamine. NO was considered to result from incomplete
267 oxidation of hydroxylamine, with N_2O produced through the activity of NO reductase
268 (Kozłowski et al., 2014; Pacheco et al., 2011). This mechanism is compatible with the revised,
269 2-intermediate model for ammonia oxidation (Caranto and Lancaster, 2017). Furthermore,
270 there is evidence that cytochrome P460 converts hydroxylamine to N_2O with NO as an
271 intermediate under anaerobic conditions enabling detoxification of hydroxylamine and NO
272 and, in the presence of oxygen, NO would then abiotically dissociate to N_2O (Caranto et al.,
273 2016).

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

281 *Linked biotic and abiotic processes*

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

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

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

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

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

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

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

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

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

358 *N₂O yield*

359 N₂O production associated with nitrification is quantified as the ratio of N₂O to NO_x⁻ (NO₂⁻
360 and/or NO₃⁻) produced or NH₃ consumed, and is termed N₂O yield. Yields vary significantly
361 within AO through differences in the physiology, and comparison with yields in environmental
362 samples provides information on likely sources of production. N₂O yield of AOB cultures
363 ranges from 0.1 to 8% (Anderson et al., 1993; Hink et al., 2017a; Jiang & Bakken, 1999; Shaw
364 et al., 2006), due to differences in available NH₄⁺ and oxygen concentrations that affect
365 enzymatic reactions. Higher yields are observed with increasing NH₄⁺ concentration,
366 potentially due to a lower reaction rate of hydroxylamine dehydrogenase than AMO, leading
367 to accumulation of hydroxylamine, which is subsequently transformed abiotically to N₂O
368 (Hink et al., 2017a). An alternative explanation is redox balancing, in which electrons
369 generated by hydroxylamine dehydrogenase are shuttled to denitrification enzymes when they
370 exceed the capacity of terminal oxidases (Hink et al., 2017a). N₂O yields from AOA cultures
371 are below or in the lower range of those observed for AOB i.e. 0.04 - 0.3%, and NH₄⁺ and
372 oxygen concentration has no or little effect on yield (Jung et al., 2011; Hink et al., 2017a; 2014;
373 Kim et al., 2012; Löscher et al., 2012; Santoro et al., 2011; Stieglmeier et al., 2014b; Qin et al.,
374 2017). This is consistent with the assumption that N₂O is produced only from abiotic reactions
375 of intermediate compounds (Kozłowski et al., 2016b), although enzymatic production has been
376 reported in *Nitrosocosmicus oleophilus* (Jung et al., 2019). Comammox bacteria and AOA lack

377 homologues of AOB NO reductase, suggesting low yields of N₂O, 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 NH₄⁺. 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

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

405 There is also evidence for niche differentiation associated with NH_3 supply. AOB growth is
406 favoured in soils fertilised by single additions of high levels of inorganic NH_3 , while AOA
407 grow preferentially when NH_3 is produced through mineralisation of organic N (Hink et al.,
408 2017b, 2018; Verhamme et al., 2011). AOA and AOB relative activities may therefore be
409 influenced by different N fertilisation strategies, with subsequent effects on N_2O yields.

410 **Terrestrial ecosystems**

411 Terrestrial environments contribute 56 - 70% of N_2O emissions globally with agricultural
412 systems contributing ~40% of that derived from soils (Davidson, 2009; Syakila & Kroeze,
413 2011). As described above, AO activity contributes directly to N_2O emissions through both
414 biotic and linked abiotic processes. However, coupled with the activity of NOB, they also
415 contribute indirectly to N_2O emissions by producing NO_3^- for heterotrophic denitrifiers and
416 subsequent reduction of NO to N_2O as part of denitrification. The application of AO inhibitors
417 has therefore been considered as a method to inhibit N_2O emissions directly or indirectly (Ruser
418 & Schulz, 2015). Although NH_3 can be lost through volatilisation, reduction of ammonia
419 oxidation is generally considered beneficial for plant uptake and decreased fertilisation loss,
420 and niche differentiation of AOA and AOB suggests that controlling the activities of each
421 group may also have substantial impacts on reducing N_2O emissions. This leads to the
422 hypothesis that inorganic fertilisation will benefit AOB, and high N_2O yield and emissions,
423 while slow release of ammonia from native organic N, organic fertiliser or slow-release
424 fertiliser will favour AOA and lower emissions (Figure 1).

425 This hypothesis has been tested most critically in microcosms in which AOA and AOB N₂O
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 N₂O production was dominated by AO and N₂O
429 emissions by AOA and AOB were discriminated by differential inhibition of AOB using 1-
430 octyne. Inorganic N fertilisation stimulated N₂O production, which was reduced in the presence
431 of 1-octyne, and estimated N₂O yields associated with AOA and AOB activity were
432 comparable to those obtained in pure cultures, i.e. the N₂O yield of AOA was approximately
433 half that of AOB. AOA-associated N₂O emission was also determined in unfertilised soils, in
434 which NH₃ is supplied through mineralisation of organic N, and N₂O yields were again similar
435 to those reported for AOA cultures. These results therefore indicate that the relative
436 contribution to ammonia oxidation and N₂O generation by AOB may be greatly reduced by
437 controlling the rate of NH₄⁺ supply.

438 To test the potential for reduction in N₂O 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 N₂O production, with high yield (Figure 2).

443 Inhibition of AOB activity by 1-octyne reduced N₂O production and yield but AOA activity
444 increased, demonstrating the ability of AOA to grow at high NH₄⁺ concentration in the absence
445 of AOB. Low NH₄⁺ supply, through slow release, led to dominance of activity by AOA and
446 low N₂O yield.

447 AOB-dominated nitrification and high AOB-associated N₂O production have also been
448 observed after N fertilisation of alluvial (pH 8) and red (pH 6) soils (Wang et al. 2016).

449 Fertilisation did not result in AOA growth and, in fact, led to a decrease in AOA abundance in
450 the red soil. AOA did not grow in the alluvial soil but grew in control and 1-octyne-treated,
451 unfertilised red soils, but not following acetylene inhibition. AOA growth was not associated
452 with detectable N₂O production. In fertilised soils, proportions of emissions associated with
453 AOB and AOA were similar (70.5 - 78.1% and 18.7 - 19.7%, respectively) to those observed
454 by Hink et al. (2017b). Meinhardt et al. (2018) complemented this approach by additional use
455 of PTIO, to inhibit AOA, and isotopic analysis, to distinguish N₂O arising from hydroxylamine
456 oxidation and nitrifier denitrification in an alkaline switch-grass soil. Nitrification, AOA and
457 AOB growth and activity and N₂O production were low in control soil microcosms. Inorganic
458 N fertilisation favoured AOB growth and increased N₂O production, and isotopic analysis
459 indicated that N₂O production was associated with AOB activity, while production in
460 unfertilised soil was similar to that from AOA cultures. These results were consistent with field
461 data, in which AOB abundance correlated with N₂O emissions.

462 While NO₂⁻ concentration in soil is typically low, accumulation of NO₂⁻ and its influence on
463 N₂O emissions has also been investigated in microcosms. Venterea et al. (2015) and Breuillin-
464 Sessoms et al. (2017) reported NO₂⁻ accumulation and associated N₂O production in bovine
465 urine- and urea-fertilised soil. Increased N₂O production after fertilisation was thought to be
466 due to nitrifier denitrification of accumulated NO₂⁻. Interestingly, Giguere et al. (2017) reported
467 NO₂⁻ accumulation in three non-cropped soils that was not associated with increased N
468 fertilisation. This led to increased N₂O production in soil in which ammonia oxidation was
469 dominated by AOA or shared between AOB and AOA and production was stimulated by
470 addition of NO₂⁻. While this can be explained by nitrifier denitrification by AOB, some of the
471 activity was associated with AOA. The mechanism for this is unclear, but could be through
472 abiotic reaction between NO₂⁻ 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 N₂O yields in soil studies.

475 Duan et al. (2019) used 1-octyne to distinguish AOA- and AOB-associated N₂O 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 N₂O
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 N₂O
481 production in the two most heavily fertilised soils. The authors also reported a correlation
482 between N₂O production and accumulated NO₂⁻, suggesting differential inhibition of NOB at
483 high NH₄⁺. 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 N₂O production by heterotrophic nitrifiers.

487 In addition, an increasing number of microcosm and field studies have generated data on
488 correlations between N₂O 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 N₂O 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

497 between AOA and N₂O, e.g. in tropical rainforest soil (Soper et al., 2018) and Tibetan alpine
498 soil (Peng et al. 2018).

499 Directed microcosm studies, using inhibitor and isotopic approaches to distinguish AOA and
500 AOB activities, therefore support proposed links between niche specialisation and the
501 consequences for N₂O production and yield. Care must be taken in interpreting results,
502 inhibitor specificity may require more rigorous testing and additional inhibitors may be
503 required to differentiate comammox bacteria. PTIO inhibits *N. inopinata* and *N. maritimus*
504 (Martens-Habbena et al., 2015; Kits et al., 2019) and is therefore not specific for AOA activity,
505 and both PTIO and 1-octyne can inhibit both AOA and AOB at sufficiently high concentrations
506 (Shen et al., 2013; Taylor et al., 2013). They must therefore be used with care with complex
507 natural communities. In addition, greater activity of AOA when AOB are inhibited may lead
508 to overestimation of N₂O production by AOA when both groups are present and potentially
509 active. Studies based only on AO abundances produce a range of effects with results that are
510 difficult to interpret because of a number of limitations, including lack of control, inability to
511 distinguish effects of different factors and difficulty in relating abundance to activity.

512 **Aquatic ecosystems**

513 The major source of nitrogen in marine and freshwater ecosystems is biological nitrogen
514 fixation. AO therefore obtain NH₃ through mineralisation of organic N and N₂O is produced
515 by both ammonia oxidisers and traditional denitrifiers, the latter also providing a sink for N₂O.
516 Production is greatest in regions of equatorial and coastal upwelling (transporting N₂O
517 produced in deep sediments), OMZ and areas of high productivity, with hot-spots in the
518 Arabian Sea and East tropical South Pacific (ETSP) (Nevison et al., 1995). Marine systems are
519 estimated to contribute ~3.8 Tg N₂O y⁻¹, equivalent to 21% of global emissions, although the
520 range of estimates is large (1.8 – 9.4 Tg N⁻¹) (IPCC, 2013). This high range is due to

521 uncertainties in methodology, reliance on assumed correlations between oxygen utilisation and
522 N₂O production and lack of information for parametrisation of simulation models, in addition
523 to uncertainties regarding biophysical processes, such as transfer functions from surface waters
524 to atmosphere, wind dispersal and hydrogeography.

525 AOA dominate AO communities in the open ocean and are the major contributors to marine
526 ammonia oxidation and AO-associated N₂O production (Santoro et al., 2011), although a
527 minority of studies report similar abundances for AOA and AOB. AOA abundance is low in
528 surface waters, except in the Arctic (Müller et al., 2018), through photoinhibition and low NH₃
529 flux, but increases between ~100 - 1000 m, before decreasing at greater depths. AOA
530 communities are dominated by two phylogenetic groups within the *Nitrosopumilales* (Santoro
531 et al., 2019). The water column A (WCA) or high NH₄⁺ (HAC) group is found in polar regions,
532 contains the cultivated AOA *Nitrosopelagicus brevis* and dominates when total AOA
533 abundance is high. The water column B (WCB) or low NH₄⁺ (LAC) group dominates at depths
534 >300 m and currently has no cultivated representative. Correlations of gene and transcript
535 abundances and nitrification rates with environmental conditions suggest that WCA has a broad
536 environmental niche, but WCB genes correlate with lower temperature, higher nutrients and
537 low chlorophyll (Smith et al., 2014). The basis of niche differentiation between these two
538 groups, however, remains uncertain.

539 A role for ammonia oxidation and N₂O production by AOA is based on correlations between
540 gene and transcript abundances and N₂O production rates and isotopic methods. For example,
541 Löscher et al. (2012) reported N₂O production in the East Tropical North Atlantic (ETNA)
542 (oxygen concentration >40 μmol l⁻¹) and the OMZ of ETSP waters. In the ETSP, N₂O
543 emissions correlated with both archaeal *amoA* gene abundance and those involved in
544 denitrification, suggesting a mixed origin for N₂O, while ETNA production correlated with

545 archaeal *amoA* gene abundance only. AOA outnumbered AOB by 1 - 2 orders of magnitude.
546 N₂O production was also inhibited when sea water was incubated with the inhibitor N1-guanyl-
547 1,7-diaminoheptane (GC7), which inhibits synthesis of hypusine, required for protein
548 synthesis in archaea. Peng et al. (2015) used on-board incubations of ETNP (East tropical
549 North Pacific) water to assess depth-related nitrification potential, *amoA* gene abundance, N₂O
550 production and selective inhibition of AOB and AOA by ATU and PTIO, respectively. AOA
551 outnumbered AOB by approximately one order of magnitude and estimated K_m values were
552 similar to those of *N. maritimus*, confirming the potential for activity in the OMZ. Horak et al.
553 (2013) also measured a K_m of 98 nmol l⁻¹ in incubations of Puget Sound sea water where, again,
554 AOA outnumbered AOB by 1 – 2 orders of magnitude. In contrast, Ji et al. (2015) measured
555 rates and gene abundances in ETSP water and suggested nitrification and associated N₂O
556 production were greatest between the euphotic zone and the OMZ, but that denitrification
557 dominate production in the OMZ. Effects of climate change have also been investigated by
558 Rees et al. (2016), who found that acidification (pH reduction by 0.06 – 0.4) of polar and
559 subpolar Atlantic Ocean waters did not affect AOA community composition but reduced N₂O
560 production in proportion to the reduction in NH₃ availability (vs. NH₄⁺) due to the pH
561 reduction.

562 Many simulation models estimate N₂O production rate from measurements of oxygen
563 concentration, assuming a linear negative relationship. Trimmer et al. (2016) tested this
564 assumption by determining relationships between AOA *amoA* and *nirK* gene abundances
565 (AOB were below the detection limit), oxygen concentrations and N₂O production in an ETNP
566 oxycline. Correlations between oxygen and molecular data were similar to those from low-
567 oxygen regions of the Baltic Sea (Berg et al., 2015), supporting AOA activity at low oxygen
568 concentration. N₂O production increased exponentially as oxygen concentration decreased
569 from 30 – 1 μmol O₂ 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 N₂O emissions in OMZs. This led to estimates of 17 μmol N₂O 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 N₂O, 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 N₂O 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. N₂O production in the meromictic North Basin was lower and was
580 due to nitrifier denitrification. Frame et al. (2017) compared N₂O 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 N₂O production with process data in salt marshes subjected to different levels
586 of N fertilisation. N fertilisation increased rates of production and consumption of N₂O,
587 increased the contribution of denitrification, had no effect on AOA composition but changed
588 AOB community composition, which contributed more than AOA to N₂O production.

589 *Wastewater treatment systems*

590 Wastewater treatment encompasses a wide range of aerobic and anaerobic processes in which
591 N₂O is generated by ammonia oxidisers and/or denitrifiers, contributing to 1.3% of global
592 anthropogenic emissions (Kampschreur et al., 2009). AO will generate N₂O in aerobic
593 processes but their contributions will be influenced by spatial heterogeneity, with anoxic

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

604 **Potential strategies for mitigation of N_2O emissions**

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

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

618 grasses with high BNI activity in decreasing N₂O emissions in urine bovine pasture. Both
619 synthetic inhibitors and BNIs have been developed against AOB and, while some also inhibit
620 AOA, others have not been tested. Future studies should therefore assess inhibition of a range
621 of both AOB and AOA, given increasing appreciation of diversity within these groups.

622 Increased understanding of niche differentiation within AO, and within AOA and AOB, and
623 significant improvements in the ability to quantify these organisms, adds a further dimension
624 and presents new opportunities. For example, fertiliser use efficiency will be greater in soils
625 dominated by AOA or AOB, following application of inorganic or slow-release fertilisers,
626 respectively, given their different NH₃ supply preferences. Ammonia oxidation by either group
627 will result in N₂O emissions, but management strategies that favour AOA would have an
628 additive effect in reducing N₂O emissions, as N₂O yield from AOB activity is double that of
629 AOA (Hink et al., 2017b, 2018). In contrast, liming of acidic soil is likely to increase AOB,
630 rather than AOA, increasing N₂O emissions associated with AOB activity. This highlights the
631 need to consider the impact on relevant and important microbial communities, in addition to
632 benefits for plant productivity and is consistent with calls for greater consideration of
633 sustainability in agricultural practice (Rockström et al., 2017).

634 Realisation of this potential requires a broader range of microcosm studies with a greater range
635 of soil types and conditions and fertilisation strategies to confirm the relatively limited data
636 currently available. Importantly, it also requires well-designed field experiments to translate
637 laboratory findings to agricultural soils and to assess impacts of mitigation strategies on
638 microbial communities, plant productivity, fertiliser use efficiency and N₂O emissions under
639 field conditions. To date, field studies have focused largely on inorganic N fertilisers and few
640 have considered diversity within AO and the consequences for N₂O emissions.

641 **Conclusion**

642 Research over the last 25 years has dramatically increased our understanding of AO diversity
643 and the importance of AO community composition in determining rates of ammonia oxidation,
644 the influence of environmental factors and important consequences for N₂O emissions. This
645 provides the basis for better quantitative information required for parameterisation of
646 biogeochemical models to improve predictions and assess impacts of different management
647 strategies. Physiological studies should consider these requirements but care should also be
648 taken in generalising and extrapolating findings from single isolates, single genomes and single
649 studies and information is required to determine the degree to which isolates represent active
650 members of natural communities. Ecological studies require greater focus on structured
651 experiments that identify the combinations of factors that determine the activity of different
652 AO groups based on niche specialisation, with less emphasis on descriptive studies. Research
653 would also benefit from development of better techniques to distinguish the many different
654 biotic and abiotic processes contributing to N₂O production and to distinguish activity of
655 different functional microbial groups *in situ*. A critical requirement, however, is for studies that
656 test the predictions of physiological and microcosm experiments ultimately to inform
657 mitigation strategies under field conditions.

658 **Conflicts of interest**

659 The authors declare that there are no conflicts of interest.

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- 1118

1119 **Figure legends**

1120

1121 Figure 1. A schematic representation of changes in AO communities and directly AO-
1122 associated N₂O emissions during incubation of soil after addition of single application of high
1123 inorganic NH₄⁺-based fertiliser or with slow release of NH₄⁺ from soil organic nitrogen or a
1124 slow release fertiliser. The initial AO community, prior to incubation, is dominated by AOA,
1125 which are generally assumed to be smaller than AOB.

1126

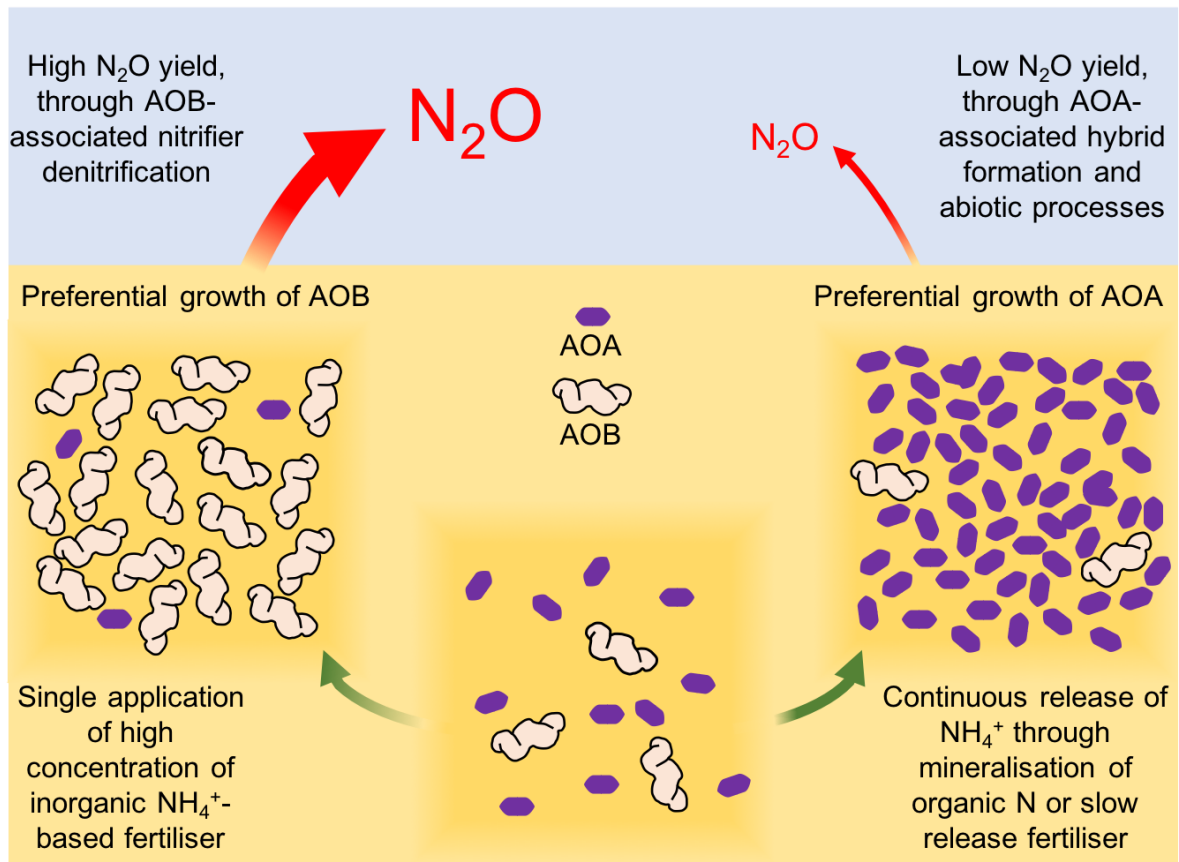
1127 Figure 2. Changes in (a) NH₄⁺ and (b) NO₃⁻ concentrations, (c) AOA and AOB *amoA* gene
1128 abundances and (d) N₂O emissions from soil microcosms incubated after addition of water
1129 (green triangle, blue cross) or under conditions of high NH₄⁺ supply (0 – 10 days) or low NH₄⁺
1130 supply (10 – 25 days) (black circle, red square). Ammonium was supplied as a slow-release,
1131 urea-based fertiliser that contained residual free urea. (Urea was converted rapidly to NH₄⁺.)
1132 This generated high initial concentrations of available NH₄⁺, which was oxidised within 10
1133 days. Thereafter, NH₄⁺ was generated by mineralisation of native organic nitrogen or of the
1134 slow-release fertiliser. In addition, microcosms were incubated with (black circle, green
1135 triangle) or without (red square, blue cross) 1-octyne, a specific inhibitor of AOB.

1136 AO growth in the absence of fertiliser (green triangle, blue cross) was due to mineralisation of
1137 native organic nitrogen. Under these conditions, AOB growth was not detectable, AOA grew
1138 slowly and N₂O emissions were low and unaffected by the 1-octyne. Initial high inorganic
1139 NH₄⁺ after fertiliser addition (1 – 10 days) resulted in greater growth of AOB, increases in the
1140 AOB:AOA ratio and substantial N₂O emissions that were significantly reduced, almost to those
1141 of control microcosms, in the presence of 1-octyne. During slow release of NH₄⁺ (10 – 25
1142 days), growth was dominated by AOA, N₂O emissions were significantly reduced and

1143 emissions were much less affected by the AOB inhibitor, 1-octyne. Note, however, that AOA
1144 growth was stimulated to some extent when AOB were inhibited. (See Hink et al. 2018 for
1145 experimental details and further discussion of results.)

1146

1147 Figure 1

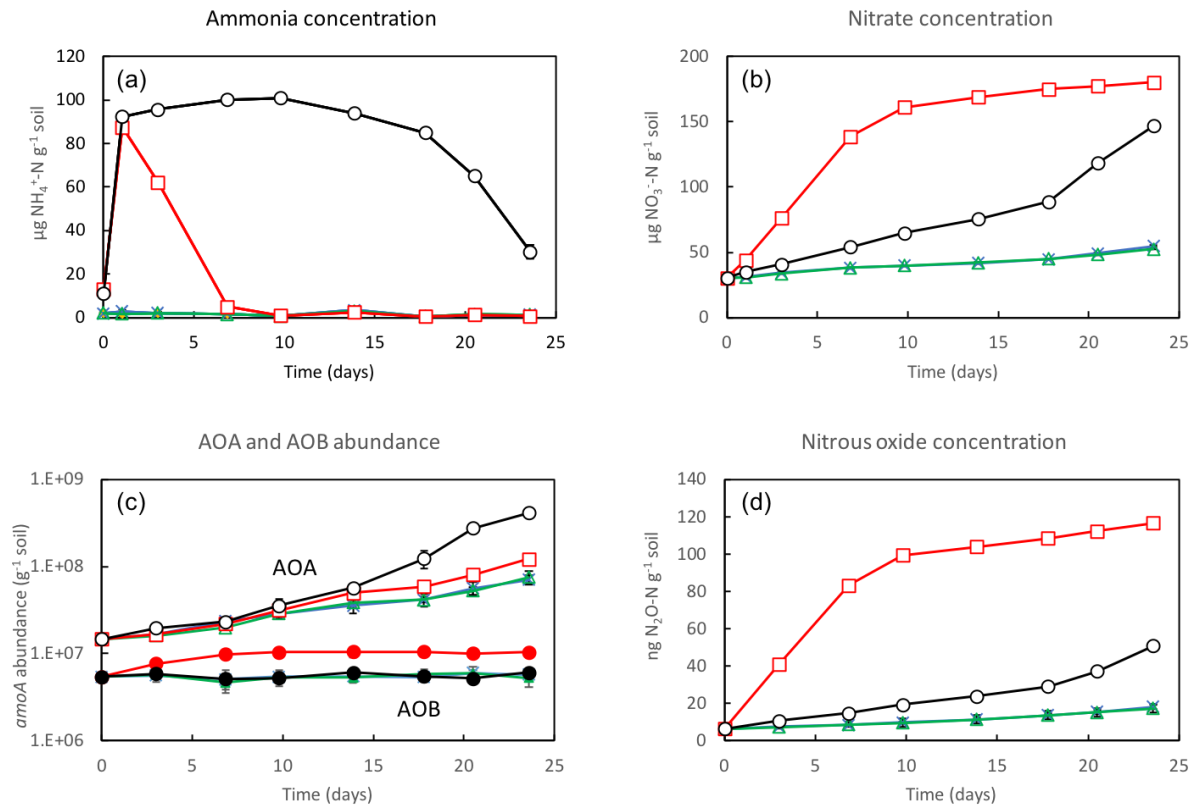


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1151 Figure 2



1152