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Archaea produce lower yields of N_2O than bacteria during aerobic ammonia oxidation in soil

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Summary

Nitrogen fertilisation of agricultural soil contributes significantly to emissions of the potent greenhouse gas nitrous oxide (N_2O), which is generated during denitrification and, in oxic soils, mainly by ammonia oxidisers. Although laboratory cultures of ammonia oxidising bacteria (AOB) and archaea (AOA) produce N_2O, their relative activities in soil are unknown. This work tested the hypothesis that AOB dominate ammonia oxidation and N_2O production under conditions of high inorganic ammonia (NH_3) input, but result mainly from the activity of AOA when NH_3 is derived from mineralisation. 1-octyne, a recently discovered inhibitor of AOB, was used to distinguish N_2O production resulting from archaeal and bacterial ammonia oxidation in soil microcosms, and specifically inhibited AOB growth, activity and N_2O production. In unamended soils, ammonia oxidation and N_2O production were lower and resulted mainly from ammonia oxidation by AOA. The AOA N_2O yield relative to nitrite produced was half that of AOB, likely due to additional enzymatic mechanisms in the latter, but ammonia oxidation and N_2O production were directly linked in all treatments. Relative contributions of AOA and AOB to N_2O production, therefore, reflect their respective contributions to ammonia oxidation. These results suggest potential mitigation strategies for N_2O emissions from fertilised agricultural soils.

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Introduction

Nitrous oxide (N_2O) is a trace gas that possesses a global warming potential greater than that of carbon dioxide and contributes significantly to stratospheric ozone layer depletion (Ravishankara *et al.*, 2009). Annual N_2O emissions from agricultural (4–5 Tg N y^{-1}) and natural (6–7 Tg N y^{-1}) soils account for 56%–70% of total global N_2O emissions (Davidson *et al.*, 2009; Syakila and Kroeze, 2011). Anthropogenic sources of N_2O, in particular from N fertilisation, generate more than 40% of global N_2O emissions (Davidson *et al.*, 2009; Syakila and Kroeze, 2011; Prather *et al.*, 2012) and the increasing rate of N_2O emission associated with increasing rates of N fertilisation demands new mitigation strategies. These, in turn, require improved understanding of the biological processes and the organisms generating and consuming N_2O.

Under suboxic or anoxic conditions, N_2O production is dominated by facultative heterotrophic denitrifiers performing sequential enzymatic reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), N_2O and finally, in some denitrifiers under microaerobic or anaerobic conditions, dinitrogen gas (N_2) (Tiedje *et al.*, 1982; Zumft *et al.*, 1997). In oxic soils, typical of agricultural soils with relatively low moisture content [$\leq$60% water-filled pore space (WFPS)], N_2O is mainly produced by ammonia oxidisers (Bollmann and Conrad, 1998; Bateman and Baggs, 2005; Baggs *et al.*, 2010). These organisms oxidise NH_3 to NO_2^- via hydroxylamine (NH_2OH) under aerobic conditions (Prosser, 1989; Vajrala *et al.*, 2013). Ammonia oxidising bacteria (AOB) generate N_2O directly through incomplete NH_2OH oxidation to NO and further to N_2O and through nitrifier denitrification (sequential enzymatic reduction of NO_2^- to NO and N_2O, mainly under reduced oxygen conditions) (Arp and Stein, 2003; Shaw *et al.*, 2006). However, these dissimilatory processes can occur simultaneously in different microsites within the same soil (Stevens *et al.*, 1997), due to spatial heterogeneity in oxygen concentration.

The role of AOB in soil nitrification has been reassessed following realisation of the existence, ubiquity and activity of terrestrial ammonia oxidising archaea (AOA) (Prosser and Nicol, 2012). AOA frequently outnumber AOB and evidence suggests their dominance in abundance and

ammonia oxidising activity in soils with low NH_3 supply, such as unfertilised (Di *et al.*, 2010; Verhamme *et al.*, 2011) or acidic soils (Gubry-Rangin *et al.*, 2010; Zhang *et al.*, 2011).

N_2O production has been demonstrated during ammonia oxidation by pure and enrichment cultures of marine and soil AOA (Jung *et al.*, 2011; Jung *et al.*, 2013 Stieglmeier *et al.*, 2015) and in ocean samples (Santoro *et al.*, 2011; Löscher *et al.*, 2012), but the mechanism of N_2O production by AOA is unclear. There is currently no physiological or genomic evidence for enzymatic processes leading to N_2O production by AOA (Walker *et al.*, 2010; Tourna *et al.*, 2011; Spang *et al.*, 2012), but N_2O may be produced abiotically from a range of compounds (Harper *et al.*, 2015; Zhu-Barker *et al.*, 2015). These include NH_2OH (Vajrala *et al.*, 2013) and NO (Martens-Habenna *et al.*, 2015), which are intermediates of archaeal ammonia oxidation, and NO_2^-. For example, stable isotope analysis provides evidence for N_2O production by hybrid formation of NO_2^- and an intermediate of ammonia oxidation (possibly NH_2OH, HNO or NO) either biochemically or abiotically via a N-nitrosation reaction (Stieglmeier *et al.*, 2014). Abiotic processes may also contribute to N_2O production during ammonia oxidation by AOB, but biotic production is likely to be significantly greater.

In marine environments, both ammonia oxidation (Wuchter *et al.*, 2006) and N_2O production (Santoro *et al.*, 2011) result mainly from the activity of AOA, but the inability to distinguish AOA and AOB activity has prevented assessment of their relative contributions to terrestrial N_2O emissions. The importance of AOA in soil ammonia oxidation and consequent production of N_2O necessitate reassessment of the role of ammonia oxidisers in up to 90% of total N_2O emissions from soil (Bateman and Baggs, 2005). This is particularly pertinent given the strong evidence for AOA-driven ammonia oxidation in many soils, notably acid soils. In addition, microcosm studies indicate that AOB and AOA, respectively, favour high, inorganic NH_4^+ input and NH_4^+ derived from mineralisation of organic N amendment (Di *et al.*, 2010; Stopnišek, *et al.*, 2010; Verhamme *et al.*, 2011; Levičnik-Höfferle *et al.*, 2012; Zhou *et al.*, 2015), suggesting potential differences in N_2O production. Given, also, the distinct pathways in AOB and AOA leading to N_2O, the study aimed to test the hypothesis that AOB dominate both ammonia oxidation and N_2O emission in an NH_4^+-fertilised soil, where both AOB and AOA are present and potentially active, but that N_2O emission results mainly from the activity of AOA when nitrification is fuelled from NH_3 derived from native organic N. Recently, 1-octyne was proposed as a specific inhibitor of AOB, based on the responses to *n*-alkynes of AOA and AOB cultures and of soils dominated by either group (Taylor *et al.*, 2013). Here, the efficiency of 1-octyne as a differential inhibitor of both ammonia oxidiser growth and transcriptional activity was tested directly in a soil where both are active and was used to determine the role of AOA in soil N_2O production and, specifically, the influence of inorganic N amendment.

Results

Kinetics of nitrification and N_2O production

Nitrification activity and N_2O production associated with ammonia oxidation were determined in microcosms containing arable soil (free-draining sandy loam, pH 6.5) incubated for 20 days, either unamended or supplemented with NH_4^+ or NO_3^-, to assess the role of ammonia oxidisers and potential N_2O production by heterotrophic denitrifiers. Microcosms were also amended with or without 1-octyne or acetylene. Acetylene was added to the headspace at a concentration (0.01% v/v) that specifically inhibits ammonia oxidation by both AOB and AOA, by irreversible inactivation of the ammonia monooxygenase through covalent binding, but not production or reduction of N_2O by heterotrophic denitrifiers (Walter *et al.*, 1979; McCarty, 1999; Offre *et al.*, 2009). 1-octyne, as a specific inhibitor of AOB, was used to distinguish N_2O production by AOA and AOB.

Oxidation of NH_3 derived from mineralisation of native organic N was determined in control microcosms, without addition of inorganic N or ammonia oxidiser inhibitors (Fig. 1). NH_4^+ concentration was consistently low, while NO_3^- concentration increased continuously due to immediate nitrification of mineralisation-derived NH_4^+. Nitrification was associated with a small decrease in pH ($P < 0.001$; Supporting Information Fig. S1), a frequent consequence of nitrification, which is associated with the release of protons and acid production. Inhibition of ammonia oxidation by acetylene led to accumulation of mineralisation-derived NH_4^+, no production of NO_3^-, but net production of approximately 15 ng N_2O–N g^{-1} $soil_{dw}$ that was not associated with nitrification. In the absence of acetylene, an additional production of N_2O ($\sim$15 ng N_2O-N g^{-1} $soil_{dw}$) was associated with ammonia oxidation. 1-octyne did not influence ammonia oxidation or N_2O production, indicating that AOA, which are not inhibited by 1-octyne, dominate oxidation of mineralisation-derived NH_3 and associated N_2O production in native soil.

Amendment with NH_4^+ greatly increased nitrification rate and acidification (Figs. 1a and Supporting Information Fig. S1). NH_4^+ was converted stoichiometrically to NO_3^-, after accounting for NO_3^- derived from oxidation of mineralised NH_3. NH_4^+ amendment also increased N_2O production 5-fold after incubation for 20 days. Inhibition of AOB activity by 1-octyne reduced ammonia oxidation and N_2O production by $\sim$80% and $\sim$90%, respectively, after incubation for 13 days, and $\sim$60% and $\sim$80% after 20 days, indicating that N_2O production after NH_4^+

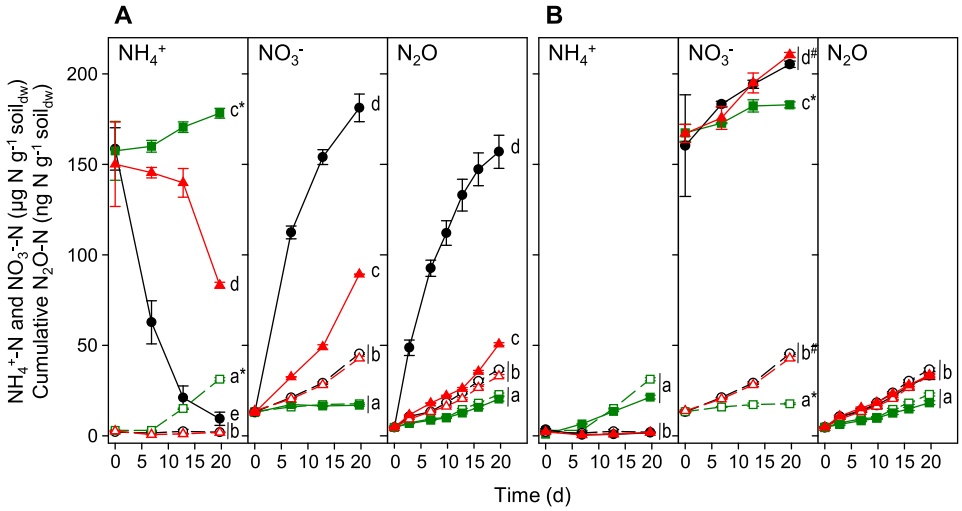

Fig. 1. Changes in NH_4^+, NO_3^- and N_2O during incubation of soil microcosms for 20 days. Microcosms were amended with NH_4^+ (A) or NO_3^- (B) (filled symbols, solid lines) or water only (A and B; open circles, dashed lines) in combination with 1-octyne (red triangles), acetylene (green squares) or without inhibitor (black circles). Concentrations of NH_4^+ and NO_3^- were determined after destructive sampling of triplicate microcosms. N_2O was analysed before opening and after recapping microcosms and cumulative values are presented. Mean concentration and standard errors of triplicate microcosms are plotted. The effects of inhibitors on consumed NH_4^+, produced NO_3^- and N_2O production rate (see Supporting Information Table S1) were analysed statistically to assess differences within treatments. Significant differences in absolute values are indicated by different lower case letters ($P < 0.05$), and * and # indicate no significant difference in net production of either NH_4^+ (A) or NO_3^- (B) ($P > 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com]

amendment was produced by AOB, rather than associated with AOA. Amendment of NH_4^+ also increased ammonia oxidation in the presence of 1-octyne ($P < 0.001$) and N_2O production was stimulated during the final week of incubation ($P < 0.001$; Supporting Information Table S1). Again, acetylene completely inhibited nitrification and reduced N_2O production, with no significant difference in N_2O production in acetylene-treated microcosms with or without added NH_4^+.

Amendment with NO_3^- did not significantly affect net changes in NH_4^+, NO_3^- and N_2O (Fig. 1B) compared with those in non-inorganic N-amended, control microcosms, indicating negligible NO_3^- reduction and N_2O production by heterotrophic denitrifiers due to added NO_3^- or NO_3^- produced via nitrification.

Ammonia oxidiser growth and transcriptional activity

Inhibition by acetylene and selective inhibition of AOB by 1-octyne were assessed by quantification of growth and activities of AOA and AOB by determining changes in abundance and expression (transcript abundance) of the ammonia monooxygenase subunit A (*amoA*) gene, a key functional gene in ammonia oxidisers (Rotthauwe *et al.*, 1997), by quantitative PCR (qPCR) during microcosm incubation (Fig. 2). *amoA* gene and transcript abundances of AOB did not increase in the presence of either inhibitor (Fig. 2B and D), but increases in AOA *amoA* gene and transcript abundances were prevented only by acetylene and not 1-octyne (Fig. 2A and C), confirming selective inhibition of AOB. In the absence of inhibitors, and after amendment with NH_4^+, there was no significant increase in AOB gene abundance, but day 20 values were significantly greater than those in inhibitor treatments. Transcript abundance increased initially, but then decreased, potentially due to a decrease in pH and in NH_3 availability, following its oxidation. In unamended microcosms, 1-octyne had no effect on increases in AOA gene abundance, compared with those in the absence of inhibitors, and no effect on increases in transcript abundance (Fig. 2A and C), suggesting that, under these conditions, AOA, but not AOB, were responsible for ammonia oxidation and associated N_2O production. Equal transcriptional activity of the AOA community in these treatments was confirmed by denaturing gradient gel electrophoresis (DGGE) of AOA *amoA* transcripts, with similar changes in DGGE profiles after incubation for 20 days (Supporting Information Fig. S2). NH_4^+ amendment did not affect changes in AOA *amoA* gene and transcript abundances (Fig. 2A and C) or DGGE profiles of *amoA* transcripts (Supporting Information Fig. S2). In the presence of 1-octyne, NH_4^+ amendment did not influence increases in AOA gene or transcript abundances, except for an increase in transcript abundance at 20 days. This is most likely due to a selection for AOA populations that could only grow in the absence of competition with AOB, and was confirmed by changes in DGGE profiles in samples from day 20 (Supporting Information Fig. S2).

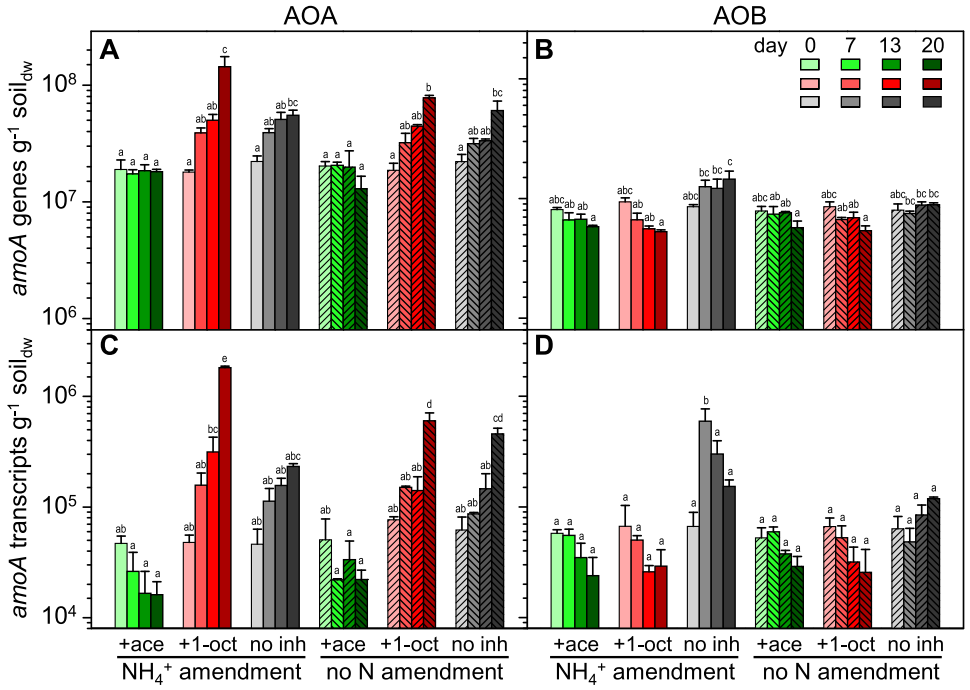

Fig. 2. Changes in the abundance of archaeal (A, C) and bacterial (B, D) *amoA* genes (A, B) and transcripts (C, D) during incubation of soil microcosms. Quantification was performed on DNA or cDNA from destructively sampled soil microcosms incubated for 0, 7, 13 and 20 days that were amended with NH_4^+ (filled) or water only (no N amendment; striped) in combination with 1-octyne (+1-oct; red), acetylene (+ace; green) or without inhibitor (no inh; grey). Means and standard errors of triplicate microcosms are plotted. Significant differences in means are indicated by different lower case letters above bars ($P < 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com]

Although AOA growth was greater than that of AOB following NH_4^+-amendment, AOA ammonia oxidation rate was lower due to a smaller cell specific activity (Jung *et al.*, 2011; Prosser and Nicol, 2012). In general, changes in transcript abundance were a more sensitive measure of effects of inhibitors and N amendment than changes in gene abundance.

Relationship between nitrification activity and N_2O production in AOA and AOB

N_2O yield (N_2O-N per NO_3^--N produced) by ammonia oxidisers was quantified after amounts produced in acetylene-treated microcosms were subtracted from those in which ammonia oxidation was not inhibited (Fig. 3). In general, differences in N_2O yield within treatments were not significant, indicating that N_2O production was directly related to ammonia oxidation. In non-amended microcosms and/or in the presence of 1-octyne, where ammonia oxidation and N_2O production resulted mainly from activity of AOA, the N_2O yield was $0.4 - 0.6 \times 10^{-3}$. As ammonia oxidation by AOB is assumed to be negligible in these microcosms, AOA-associated production may be considered to have a N_2O yield of approximately 0.5×10^{-3}. In NH_4^+-amended, non-inhibited microcosms, where both AOA and AOB were active, the N_2O yield was stable at $\sim 0.85 \times 10^{-3}$. NO_3^--production in these microcosms was twice that in unamended microcosms, suggesting an N_2O yield of approximately 0.95×10^{-3} for AOB.

Relative contributions to total N_2O emissions

1-octyne did not affect growth and transcriptional activity (increases in *amoA* gene and transcript abundance, respectively) of AOA (Fig. 2) or DGGE profiles of AOA

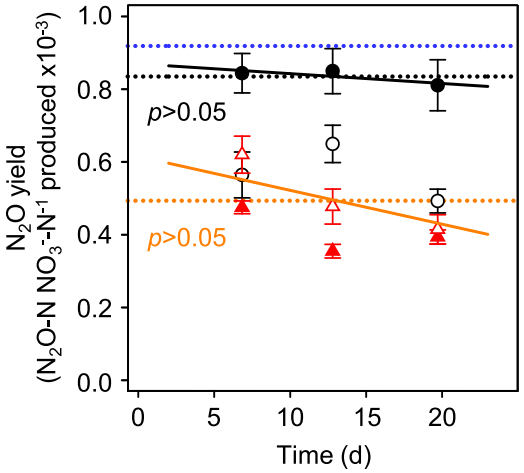

Fig. 3. N_2O yield in soil microcosms incubated for 20 days in the presence and absence of ammonia oxidiser inhibitors. Microcosms were amended with NH_4^+ (filled symbols) or water only (open symbols) in combination with 1-octyne (red triangles) or without inhibitor (black circles). N_2O yield is expressed as N_2O-N per NO_3^--N produced for treatments in which ammonia oxidation was not inhibited by acetylene. Means and standard errors of triplicate microcosms are plotted. Regression lines (solid) and means (dotted) of the overall N_2O yield are presented for AOA (orange) and for AOB plus AOA (black). Insignificant *P*-values ($P > 0.05$) suggest that the N_2O yield is independent of the incubation time. A mean overall N_2O yield for AOB only was calculated (blue). [Colour figure can be viewed at wileyonlinelibrary.com]

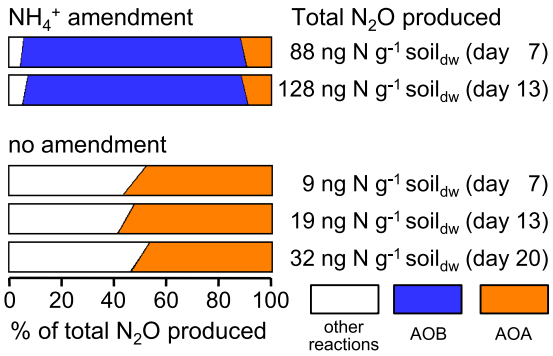

Fig. 4. Relative contributions of archaeal and bacterial ammonia oxidation to total N_2O production in microcosms amended with NH_4^+ after incubation for 7 and 13 days and with no N amendment after 7, 13 and 20 days. The contributions associated with AOB (blue), AOA (orange) and other non-characterized organisms and reactions (white) are shown as percentages of total N_2O. Minima and maxima are derived from standard errors of triplicate samples. [Colour figure can be viewed at wileyonlinelibrary.com]

amoA transcripts (Supporting Information Fig. S2) during incubation for 13 days with NH_4^+, suggesting that N_2O production was also not affected. A similar assumption was made for treatments without NH_4^+ amendment after incubation for 20 days (Fig. 2 and Supporting Information Fig. S2), enabling calculation of the relative contributions of AOB and AOA activity to total N_2O produced in NH_4^+-amended and unamended microcosms (Fig. 4). AOB dominated N_2O production following NH_4^+ amendment, producing 81%–86% of the N_2O generated. In contrast, in unamended soil, production of N_2O by AOB was negligible, and AOA were responsible for 47%–58% of the N_2O produced. Total amounts of N_2O produced were, however, low in unamended soils and other non-characterised reactions, responsible for N_2O production in acetylene treatments, contributed the remainder.

Discussion

This study is the first to distinguish ammonia oxidising activity and N_2O production resulting from the activity of AOA and AOB in soil, using 1-octyne to inhibit AOB. Its specificity was demonstrated in a soil containing active and abundant AOA and AOB by analysis of both growth and transcriptional activity. Tolerance of AOA to 1-octyne has been demonstrated in only three AOA pure cultures (Taylor *et al.*, 2013; Taylor *et al.*, 2015), but the lack of inhibition of ammonia oxidation dominated by AOA suggests tolerance by diverse natural communities as also shown in Taylor *et al.* (2013) and Lu *et al.* (2015).

NH_3 was derived from either added inorganic NH_4^+ or mineralisation of native organic N and observed rates of nitrification were similar to those observed previously in this soil (Tourna *et al.*, 2008; Baggs *et al.*, 2010; Gubry-Rangin *et al.*, 2010; Verhamme *et al.*, 2011). N_2O was derived from several sources. Non-ammonia oxidiser N_2O production was low and was not investigated further. There was no evidence of production by heterotrophic denitrifiers, as expected given the relatively low WFPS of 60% and oxic conditions. Previous studies have shown optimal N_2O production via ammonia oxidation at ~60% WPFS and increasing denitrifier N_2O production above 65% WFPS (Bateman and Baggs, 2005). Acetylene and 1-octyne may act as a C substrate for denitrification, potentially leading to N_2O production, but concentrations were low and denitrifier N_2O formation is likely to be negligible. N_2O production via chemical N transformations in the absence of nitrification cannot be excluded, but was likely negligible, as chemically produced N_2O was not detectable previously when NO_2^- and NH_2OH concentrations were low (Mørkved *et al.*, 2007; Heil *et al.* 2015), that is, when ammonia oxidisers are inactive.

The difference between N_2O produced in the presence and absence of acetylene represents that exclusively derived from ammonia oxidation (although the possibility of production by other, acetylene-insensitive organisms cannot be excluded) and, in general, N_2O was produced coordinately with NO_3^- during nitrification. Production following oxidation of ammonia by AOA and AOB was distinguished in two ways. The first, selective inhibition of AOB by 1-octyne, led to indirect stimulation of AOA in NH_4^+-amended microcosms by relieving competition for NH_3 and shifting the active AOA community. This suggests niche specialisation within AOA through adaptation to low NH_3 concentration produced during mineralisation, through greater NH_3 affinity (Martens-Habbena *et al.*, 2009) or close proximity to mineralisers (Prosser and Nicol, 2012), or through adaptation to high NH_3 concentration, with greater direct competition with AOB. This is consistent with stimulation of AOA by organic, but not inorganic N (Levičnik-Höfferle *et al.*, 2012; Zhou *et al.*, 2015), and stimulation of other AOA by high levels of inorganic N (Verhamme *et al.*, 2011; Lu *et al.*, 2015). Although indirect stimulation of AOA could lead to overestimation of their contribution to N_2O production in long-term incubations, *amoA* transcript abundance and DGGE transcript profiles were not affected by 1-octyne following NH_4^+-amendment for almost two weeks of incubation. In the second approach, ammonia oxidation was assumed to be driven by AOA in unamended soils, where NH_3 was generated from mineralised organic N. This was confirmed by stimulation of growth and transcriptional activity of AOA, but not AOB in unamended microcosms, as found previously (Tourna *et al.*, 2008; Offre *et al.*, 2009; Gubry-Rangin *et al.*, 2010; Verhamme *et al.*, 2011) and by tolerance to 1-octyne.

N_2O yields were $\sim 0.5 \times 10^{-3}$ and $\sim 0.95 \times 10^{-3}$ following ammonia oxidation by AOA and AOB, respectively. The AOA value is slightly lower than those of cultivated soil AOA ($0.8–2.3 \times 10^{-3}$) (Jung *et al.*, 2011; Jung *et al.*,

2013; Stieglmeier *et al*., 2014) and similar to those of marine AOA (0.04–1.1 $\times$ 10^{-3}) (Santoro *et al*., 2011; Stieglmeier *et al*., 2014). AOB N_2O yield also fell within the range (0.3–7 $\times$ 10^{-3}) for AOB cultures belonging to the *Nitrosospira* lineage (Shaw *et al*., 2006), which dominates soil AOB communities (Smith *et al*., 2001). Similar values (0.2–0.9 $\times$ 10^{-3}) were found in aerobic soil-slurries amended with NH_4^+ at neutral pH (Mørkved *et al*., 2007), similar to our incubation conditions.

The higher N_2O yield for AOB is consistent with their possession of two enzymatic mechanisms for N_2O production, incomplete NH_2OH oxidation and nitrifier denitrification. This contrasts with cultivated AOA, which appear to lack a known NO reductase (Walker *et al*., 2010; Tourna *et al*., 2011; Spang *et al*., 2012), preventing nitrifier denitrification, and where N_2O production is independent of oxygen concentration (Stieglmeier *et al*., 2014). AOA-associated N_2O production, therefore, appears to be restricted to hybrid formation (Stieglmeier *et al*., 2014). It is not known whether this process is biotic or abiotic, but abiotic N_2O production from hybridisation of NH_2OH and NO_2^- is even more thermodynamically favourable than the hydroxylamine dehydrogenase-mediated reaction (Harper *et al*., 2015). Further abiotic reactions of nitrification intermediates leading to N_2O have been reviewed in Zhu-Barker *et al*. (2015), including decomposition of NH_2OH by oxidised iron or manganese and reaction of NO_2^- with reduced iron.

Differences in N_2O yield associated with ammonia oxidation by AOA and AOB present a potential strategy for mitigation of N_2O emissions. NH_4^+-fertilisation of agricultural land dominated by AOA, for example in acid soils, will lead to lower N_2O emissions than from soils dominated by AOB. There is evidence that AOA are stimulated by organic N but not inorganic N fertiliser, unlike AOB (Levičnik-Höfferle *et al*., 2012; Zhou *et al*., 2015). Decreases in N_2O emissions have been linked to the application of organic, rather than inorganic, N-fertilisers (Ball *et al*., 2004), and organic-N fertilisation may, therefore, promote AOA activity with an associated decrease in N_2O production in oxic soils.

In summary, this study demonstrates production of N_2O associated with ammonia oxidation by both AOA and AOB in soil. AOB dominate both ammonia oxidation and N_2O production in soils amended with NH_4^+ at levels equivalent to fertiliser applications. N_2O production associated with AOA under these conditions is low, but their relative contribution to N_2O emission is higher when NH_3 availability is limited, and total emissions are low. N_2O yield is lower following ammonia oxidation by AOA, where N_2O production is potentially restricted to non-enzymatic reactions of nitrification intermediates, unlike AOB, which possess additional enzymatic mechanisms for N_2O production. These findings provide the potential for mitigation of N_2O emission through management regimes in agricultural ecosystems and under other conditions that favour ammonia oxidation by AOA rather than AOB, including the use of organic rather than inorganic N fertilisation.

Experimental procedures

Microcosm construction and incubation

Soil (pH 6.5, sandy loamy texture) was collected from the upper 10 cm of a crop-rotating agricultural plot that was being used for growing potatoes (SRUC, Craibstone, Scotland; grid reference NJ872104; for soil characteristics see Kemp *et al*. (2012)). Soil was sieved (3.35-mm mesh size) and stored at 4°C before use. Water content was determined by weight loss after drying at 100°C for 24 h. Soil microcosms consisted of 120-ml serum bottles containing 13.97 g $\pm$ 0.02 g of fresh soil at 55% WFPS (equivalent to 10 g dry weight soil), tightly capped with butyl rubber stoppers and metal crimp tops before pre-incubation for 5 days at 30°C in darkness. Soil was adjusted to 60% WFPS following amendment with water only (control, no N amendment) or inorganic nitrogen solution (150 μg NH_4Cl-N or $NaNO_3$-N g^{-1} $soil_{dw}$) in the presence or absence of the potential ammonia oxidiser inhibitors acetylene (0.01% v/v) or 1-octyne (0.03% v/v, prepared as described in Taylor *et al*., 2013). Triplicate microcosms were destructively sampled after incubation for 0, 7, 13 or 20 days and soil was stored at −20°C or −80°C for chemical or molecular analysis, respectively. Oxic conditions were maintained by opening microcosms twice weekly and allowing exchange of the gas phase before re-capping and re-establishing the partial pressure of acetylene and 1-octyne. Gas samples (5 ml) were taken before opening and after re-closure and transferred into pre-evacuated 3-ml glass vials (Labco, Lampeter, UK) for measurement of N_2O.

Measurement of nitrification kinetics, soil pH and N_2O production

Nitrification kinetics were determined by measuring NH_4^+ and combined NO_2^- and NO_3^- concentrations colorimetrically by flow injection analysis (FIA star 5010 Analyzer, Foss Tecator AB, Höganäs, Sweden) after extraction from 2 g soil (wet weight) in 10 ml 1 M KCl solution. Results of combined NO_2^- and NO_3^- analysis are expressed as NO_3^- concentration, as NO_2^- concentration was below the level of detection in all samples. pH was measured in 2 g soil homogenized in 4 ml deionised water. N_2O concentration in gas samples was analysed with an Agilent 6890 gas chromatograph equipped with a ^{63}Ni electron capture detector (Santa Clara, CA, USA) using N_2 as carrier gas and connected to an automatic sample-injection system (HT280T, HTA, Brescia, Italy).

Nucleic acid extraction and analysis of amoA *genes and transcripts*

Nucleic acid extraction and purification from 0.5 g soil were performed as described in Nicol and Prosser (2011), a modification of Griffiths *et al*. (2000). DNA and RNA co-extracts were verified by agarose gel electrophoresis and quantified

using a Nanodrop-Spectrophotometer (Thermo Scientific, Wilmington, USA). Nucleic acid concentrations between 450 and 550 ng μl^{-1} were obtained. For cDNA synthesis, a 10-μl subsample of the nucleic acid extract was treated with RQ1 DNase (Promega, Southampton, UK) according to the manufacturer's instructions. Reverse transcription (RT) of 8 μl template RNA into cDNA was performed using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Hemel Hempstead, UK) according to the manufacturer's instructions and with addition of 8 units Recombinant RNasin Ribonuclease Inhibitor (Promega, Southampton, UK). Two negative controls were performed with all reactions (no template (water treated with DNAse) and soil RNA with all DNase/RT reagents except for the final addition of RT enzyme).

Archaeal and bacterial *amoA* genes and transcripts were quantified by qPCR using domain-specific primers, crenamoA23F/crenamoA616F (Tourna *et al*., 2008) and amoA-1F/amoA-2R (Rotthauwe *et al*., 1997), respectively. Each amplification was performed in 20-μl reactions containing 10 μL of QuantiTect SYBR Green PCR Master Mix (Qiagen, Crawley, UK), 0.6 μM of each primer, 4 μg BSA and 5 μL of 200x diluted nucleic acid extract or 5× diluted cDNA. Amplification of AOA *amoA* was performed in a BioRad MyIQ Single-Color Real-Time PCR Detection System (Hertfordshire, UK) using the following cycling-parameters: 15 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 54°C, 50 s at 72°C and a plate read after incubation for 10 s at 78°C. Amplification of AOB *amoA* was performed in an Eppendorf Mastercycler Realplex Real-Time PCR System (Hamburg, Germany) using cycling-parameters described by Gubry-Rangin *et al.* (2010). Standards consisted of a dilution series (10^1-10^6 *amoA* gene copies) of a PCR product containing the *amo* operon of *Nitrosospira multiformis* ATCC 25196 (AOB) or gene cluster of *Nitrosotalea devanaterra* (AOA) amplified with the primers NmAOB-amoC-305F (TCCCAGCTGCCGGAGATGTTCATCC)/NmAOB-amoB-308R (GTCGTCTGGAACGGCCAGAGCAAA; adapted from Norton *et al*. (2002) with degeneracies removed to be specific for *N. multiformis*) and Ndev-amoF2/Ndev-amoR (Thion and Prosser, 2014), respectively. *The* r^2 values were >0.99 and the efficiencies of amplification ranged from 93% to 103% and 88% to 96% for archaeal and bacterial *amoA* gene qPCR assays, respectively. Specific amplification was verified by melting curve analysis and agarose gel electrophoresis. Amplicons of an end point PCR targeting cDNA of AOA *amoA* gene transcripts were analysed by DGGE as described previously (Tourna *et al*., 2008).

Statistical analysis

Statistical analysis was performed using R 3.1.1 (http://www.r-project.org/). Data were tested for normal distribution before further statistical analysis. The effects of substrate (NH_4^+, NO_3^- or water) and inhibitors (acetylene, 1-octyne or no inhibitor) on NH_4^+ consumption, NO_3^- production and change of pH at the end of the incubation and on N_2O production rates during the first, second and third week of incubation were tested by a factorial two-way ANOVA. The effects of substrate, inhibitors and incubation time on *amoA* gene and transcript abundances were tested by a factorial three-way ANOVA. Tukey *post hoc* tests were used to assess significant differences in means. The effect of time on AOA and AOB N_2O yields was tested by linear regression analysis.

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Supporting information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Changes in pH during incubation of soil microcosms amended with NH_4^+ or NO_3^- (filled symbols, solid lines) or water (no N amendment; open symbols, dashed lines) for 20 days. Microcosms were additionally treated with 1-octyne (red triangles), acetylene (green squares), or without (black circles) inhibitor. Means and standard errors of triplicate microcosms sampled destructively are plotted. Significant differences between the treatments are indicated by different letters ($P < 0.05$).
Fig. S2. Changes in DGGE profiles of AOA *amoA* transcripts during incubation. Each lane represents one of three replicate microcosms amended with water (no amendment) or NH_4^+ in the absence (no inhibitor) or presence of 1-octyne (+1-octyne) and sampled after incubation for 0, 7, 13 or 20 days.
Table. S1. Analysis of significant differences in means of N_2O production rates between differently treated microcosms during the first, second and third week of incubation.