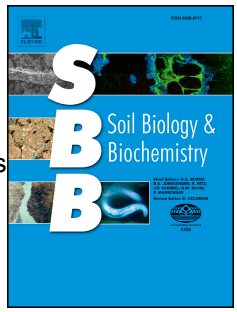

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Deciphering the functional importance of comammox *vs.* canonical ammonia oxidisers in nitrification and N_2O emissions in acidic agricultural soils

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Abstract

The discovery of comammox *Nitrospira* has altered our perception of the nitrogen biogeochemical cycle. However, their functional importance compared to canonical ammonia oxidisers (i.e., ammonia-oxidising bacteria (AOB) and archaea (AOA)) in agricultural soils remains elusive, especially in acidic soils. Here, we assessed the functional importance of these functional guilds in nitrification and nitrous oxide (N_2O) emissions in three acidic agricultural soils by using a range of nitrification inhibitors (acetylene, 3,4-dimethylpyrazole phosphate (DMPP) and different concentrations of 1-octyne) and monitored their community assemblage and population dynamics. The sensitivity of comammox *Nitrospira* clade A to 1-octyne varied across soils, highlighting that the inappropriate use of 1-octyne can lead to misestimation of comammox activity. AOA were key NH_3 oxidisers in the three soils, while AOB also contributed significantly to nitrification in one soil. In contrast, comammox *Nitrospira* always played a minor role in ammonia oxidation and N_2O emissions, likely due to their low abundances, restricted cellular kinetic properties and N_2O production mechanisms. Together, this study demonstrates that comammox *Nitrospira* play a less important role in ammonia oxidation and N_2O production in acidic agricultural soils than AOA and AOB, thereby providing important novel insights into the mitigation of nitrogen fertiliser loss and N_2O emissions.

Keywords: *Comammox Nitrospira; Nitrous oxide; 1-Octyne; 3,4-Dimethylpyrazole phosphate (DMPP); Acid soils.*

1. Introduction

The ongoing discovery of microorganisms has been refining our knowledge of the nitrogen biogeochemical cycle (Kuypers et al., 2018; Wu et al., 2021). Among these, comammox *Nitrospira* are of particular interest since they are equipped with all the enzymes needed for ammonia and nitrite oxidation, enabling the oxidation of ammonia (NH_3) to nitrate (NO_3^-) in a single cell (Daims et al., 2015; van Kessel et al., 2015). This significantly differs from their canonical counterparts (i.e., ammonia-oxidising archaea (AOA) and ammonia-oxidising bacteria (AOB)) in that the latter two guilds catalyse the conversion of NH_3 to nitrite (NO_2^-), the first step of nitrification. Meanwhile, mounting research suggests that these three guilds generally coexist in agricultural soils (Pjevac et al., 2017; Orellana et al., 2018; Xu et al., 2020), actively contributing to nitrification and likely nitrous oxide (N_2O) emissions (Hink et al., 2017; Wang et al., 2019b; Huang et al., 2021), a long-lived and ozone-depleting greenhouse gas (Ravishankara et al., 2009; Prather et al., 2015). In many cases, comammox *Nitrospira* outnumbered AOA and AOB (Li et al., 2019; Hu et al., 2021). These findings raise the speculation that comammox *Nitrospira* have an underappreciated role in the nitrogen cycling of agricultural soils. However, the ecological significance of comammox *vs.* canonical NH_3 oxidisers for NH_3 oxidation and N_2O emissions in agricultural soils is rarely empirically evidenced (Tan et al., 2022; Jiang et al., 2023).

The ecological importance of comammox *vs.* canonical NH_3 oxidisers in nitrification is expected to depend on the selected soils, as these guilds have been found to show distinct niche preferences (i.e., niche differentiation) for edaphic properties such as moisture, organic matter, nitrogen availability, and pH (Hatzenpichler, 2012; Prosser and Nicol, 2012). Of these, the overarching role of soil pH in driving the assemblage and activities of AOA and AOB has been

extensively studied. AOA often prefer to flourish in oligotrophic habitats with low pH, while AOB normally dominate nitrification in nitrogen-rich neutral and alkaline environments (Nicol et al., 2008; He et al., 2012), even if several studies reported the dominance of AOB in nitrification of some acidic soils (Dai et al., 2017; Huang et al., 2018; Lin et al., 2018; Zhang et al., 2019). In contrast, the pH preference of comammox *Nitrospira* is still inconclusive, and multiple lines of evidence support its activity in both high and low pH soils (Wang et al., 2019b; Zhao et al., 2020b; Hu et al., 2021; Yuan et al., 2021; Tan et al., 2022). These observations hint at a complex scenario in acidic soils, where comammox *Nitrospira* and AOB may play substantial roles in nitrification and associated N_2O emissions. Consequently, there is an urgent need to reassess the relative contribution of NH_3 oxidisers, particularly comammox *Nitrospira* and AOB, to nitrification and N_2O production in diverse acidic soils.

The niche differentiation among AOA, AOB and comammox *Nitrospira* is primarily driven by their distinct metabolic and physiological characteristics. AOB typically exhibit lower affinity for NH_3 and higher NH_3 tolerance and maximum oxidation rate (V_{max}) than AOA and comammox *Nitrospira* (Jung et al., 2022), reflecting a copiotrophic lifestyle. AOA possess a surprising variability of cellular kinetic properties (Jung et al., 2022), while comammox *Nitrospira* display extremely high NH_3 affinity, high growth yield, and low maximum oxidation rate (Kits et al., 2017; Sakoula et al., 2021), which are typical characteristics of an oligotrophic lifestyle. Recently, genome and *in vitro* analyses further indicated that comammox *Nitrospira* may have a broad ecological niche and thrive in high N-input agricultural soils (Li et al., 2023). In addition, the mechanisms of N_2O production differ substantially among these guilds. In comparison with the enzymatic pathways in AOB (Shaw et al., 2006; Zhu et al., 2013; Prosser et al., 2020), the

comammox bacterium *Nitrospira inopinata* only produces N_2O via the abiotic conversion of hydroxylamine (NH_2OH) (Kits et al., 2019), while AOA-associated N_2O production is derived from hybrid formation and NH_2OH oxidation (Stieglmeier et al., 2014; Kozlowski et al., 2016; Wan et al., 2023). Consequently, the N_2O yields of comammox *Nitrospira* and AOA are much lower than those of AOB (Hink et al., 2017; Kits et al., 2019; Han et al., 2021). Altogether, we hypothesised that comammox *Nitrospira* and AOA dominate NH_3 oxidation in acidic soils but produce less N_2O than AOB.

The ecological significance of these functional guilds was rarely explicitly assessed until the emergence of specific inhibitors such as 1-octyne, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3oxide (PTIO), 3,4-dimethylpyrazole phosphate (DMPP), simvastatin, and chlorate (Taylor et al., 2013; Martens-Habbena et al., 2015; Papadopoulou et al., 2020; Zhao et al., 2020a; Wang et al., 2021). Recently, based on the differential response patterns of NH_3 oxidisers to 1-octyne and DMPP, we developed and tested a novel method to parse out the contribution of comammox to nitrification and N_2O production (Tan et al., 2022), demonstrating that comammox played a minor role in N_2O production and nitrification in an alkaline soil. In contrast, comammox were found to cause significant NH_3 fertiliser loss comparable in magnitude to AOB in a global-scale survey using the same approach (Jiang et al., 2023). The specific inhibition efficacy of 1-octyne has been recently questioned, with several studies noting that 1-octyne can also inhibit comammox *Nitrospira* (Taylor et al., 2017; Lin et al., 2023). In addition, the ability of 1-octyne to inhibit AOB growth may be limited due to restricted diffusion in some soils (Yin et al., 2021). To prevent future misevaluation of the functional importance of NH_3 oxidisers, it is imperative to re-evaluate the inhibitory effect of 1-octyne on comammox *Nitrospira* and AOB in a range of soils, especially in

acidic soils where comammox *Nitrospira* are active.

Therefore, the main objectives of this study were to (i) decipher the contribution of comammox *Nitrospira vs.* AOA and AOB to NH_3 oxidation and N_2O emissions in acidic agricultural soils and (ii) reassess the inhibitory effect of different concentrations of 1-octyne on NH_3 oxidisers. For this, three different types of acidic agricultural soils were selected, and soil microcosms were conducted using a combined inhibitor method (acetylene, 1-octyne, and DMPP). The shift in the *amoA* gene abundance of NH_3 oxidisers was monitored by qPCR, while the community composition of canonical NH_3 oxidisers (AOA and AOB) and comammox *Nitrospira* was assessed by Illumina MiSeq sequencing.

2. Materials and methods

2.1 Study sites and soil sampling

The sampling sites, located in the southern region of China, were representative agricultural fields with distinct soil characteristics (Fig. S1). The specific locations were Xiantao city (30°32′N, 113°46′E) in Hubei Province (HB), Lingao County (19°94′N, 109°74′E) in Hainan Province (HN), and Guiyang city (26°44′N, 106°53′E) in Guizhou Province (GZ). In May 2022, three 100 m^2 plots were randomly selected at each site. Five soil cores were randomly taken from each plot's top layer (0–20 cm in depth) and combined. The samples were sieved (2 mm) to remove plant debris and stones and then mixed to generate each site's final composite soil samples. The collected soils were immediately transported to the laboratory on ice. The composite samples were divided into two parts: one subsample was used for microcosm experiments to determine microbial activity, and the other was air-dried for physicochemical analysis. Climate variables for each site, including mean

126 annual temperature (MAT) and mean annual precipitation (MAP), were obtained from the
127 WorldClim database (www.worldclim.org). Details of the study sites and soil physicochemical
128 properties are provided in Table S1.

129 ***2.2 Microcosm incubation using the optimised combined inhibitor method***

130 Preliminary experiments showed a decrease in N_2O emissions in 1-octyne-inhibited
131 microcosms as the concentration of 1-octyne increased. We proposed two possible explanations
132 for this phenomenon: 1) Low concentrations of 1-octyne only partially inhibited AOB activity, and
133 as the concentration of 1-octyne increased, AOB activity was completely inhibited; 2) High
134 concentrations of 1-octyne partially inhibited the activity of comammox *Nitrospira*. The
135 illustration is presented in Fig. S2. To test the above hypotheses, we incorporated a range of 1-
136 octyne concentrations (see below) into the previously established combined inhibitor method (Tan
137 et al., 2022).

138 Soil microcosms were established in 125-ml serum bottles filled with ~18 g of homogeneous
139 fresh soil (equivalent to 15 g of dry soil). The bottles were sealed with butyl rubber stoppers and
140 aluminium caps and preincubated at 25 °C in the dark for seven days with ventilation every three
141 days. Following preincubation, the soil moisture content was adjusted to 50% WHC (water holding
142 capacity) using an ammonium sulfate solution (150 μg $(NH_4)_2SO_4$-N g^{-1} $soil_{dw}$) in the absence or
143 presence of inhibitors acetylene (0.1%, v/v), 1-octyne (0.01%, 0.015%, 0.02%, 0.03%, 0.04% and
144 0.05%, v/v), or DMPP (1.5% of added NH_4^+-N). Microcosms were then incubated in the dark at
145 25 °C for 21 days, with aeration on Days 2, 5, 8, 11, 14, 17 and 21 to maintain oxic conditions.
146 After resealing, microcosms were evacuated using a vacuum pump and replenished with 125 ml

of air, air-diluted acetylene (0.1%, v/v), or air-diluted 1-octyne (0.01%, 0.015%, 0.02%, 0.03%, 0.04% and 0.05%, v/v) to maintain the inhibitor concentrations. Gas samples (12 ml) were taken before opening the microcosms using a syringe and injected into pre-evacuated 12-ml glass vials (Labco, Lampeter, UK) to determine N_2O and CO_2 concentrations. Triplicate microcosms were destructively sampled on Days 0, 5, 11 and 21, and the soil samples were immediately divided into two parts and stored at −20 °C for chemical analysis (storage ≤ 2 weeks) and at −80 °C for molecular analysis (storage ≤ 4 weeks).

2.3 Physicochemical analysis of soil and gas samples

Soil texture was measured using the laser diffraction method with a Mastersizer 2000 (Malvern, Worcestershire, UK). Soil moisture content was determined by measuring the weight loss after drying at 105 °C for 24 h. Soil pH was measured using an FE28-Standard pH meter (Mettler Toledo, Shanghai, China) with a soil-to-water ratio of 1:2.5 (w/v). Extractable NH_4^+ and combined NO_2^- and NO_3^- were measured using the indophenol blue and VCl_3/Griess methods (Kandeler and Gerber, 1988; Hink et al., 2018), respectively, after 6 g of wet soil was extracted with 30 ml of 2 M KCl. The detailed procedures were described elsewhere (Yin et al., 2021). Results of combined NO_2^- and NO_3^- analysis are expressed as NO_3^- concentration, as NO_2^- was below the detection level (0.15 μg g^{-1}) in all samples. Soil organic carbon (SOC) was measured by dichromate oxidation. Soil available phosphorus (AP) was determined by the molybdenum blue colorimetry after extraction with 0.5 M $NaHCO_3$. Soil available potassium (AK) was extracted with 1 M CH_3COONH_4 (pH 7.0) and determined with a flame photometer (Inesa Instrument, Shanghai, China). The N_2O concentrations in the gas samples were measured using Trace1300 and

1310 gas chromatographs equipped with a flame ionization detector (FID) and an electron capture detector (ECD) (Thermo Fisher Scientific, Rodano, Italy).

2.4 Rates, relative contributions and N_2O yield of ammonia oxidisers

Potential NH_3 oxidation rates for each treatment were calculated by performing a linear regression of NH_4^+ consumption over time, with a coefficient of determination (R^2) > 0.89 for all microcosms except those treated with acetylene. Potential N_2O production rates were determined by conducting a linear regression of N_2O accumulation over time, with R^2 > 0.75 for all microcosms.

As 1-octyne specifically inhibits the activity of AOB at specific concentrations (Taylor et al., 2013; Li et al., 2019), the AOB contribution can be assessed by subtracting the NH_3 oxidation (or N_2O production) rate of the 1-octyne-treated microcosms from the rate of non-inhibited microcosms. Since DMPP inhibits the activity of both AOB and comammox *Nitrospira* (Papadopoulou et al., 2020; Zhou et al., 2020), AOA is the only NH_3 oxidiser consuming NH_4^+ or producing N_2O in DMPP-treated microcosms. Therefore, the AOA rate can be obtained by subtracting the rate of acetylene-treated microcosms, which represents the non-NH_3 oxidation process rate, from the rate of DMPP-treated microcosms. The comammox rate can be calculated by subtracting the rate of DMPP-treated microcosms from the rate of 1-octyne-treated microcosms. We only selected the 1-octyne concentrations that achieved the ideal inhibitory effect (completely inhibiting AOB but not inhibiting comammox *Nitrospira* clade A growth) and calculated the average rate of NH_3 oxidisers and their relative contribution to total NH_3 oxidation and NH_3 oxidation-derived N_2O production.

189 The relative contributions of NH_3 oxidisers to NH_4^+ consumption or N_2O production were
190 calculated by dividing the potential rates of NH_3 oxidisers by the total NH_3 oxidation rate or the
191 total NH_3 oxidation-related N_2O production rate.

192 $$Contribution_{NH_4^+ \text{ or } N_2O\ (Comammox\ or\ AOA\ or\ AOB)} = \frac{Rate_{(Comammox\ or\ AOA\ or\ AOB)}}{Rate_{(Comammox\ +\ AOA\ +\ AOB)}} \times 100\%$$

193 The N_2O yields of AOA, AOB, and comammox *Nitrospira* were calculated as the amount of
194 N_2O-N produced per NH_4^+-N consumed, as shown in the following equation:

195 $$N_2O\ yield\ (\%) = \frac{Rate_{N_2O\ production}}{Rate_{NH_4^+\ consumption}} \times 100\%$$

196 ***2.5 Nucleic acid extraction and quantitative PCR***

197 Nucleic acid was extracted from 0.5 g of wet soil, equivalent to 0.39 g (HB), 0.36 g (HN),
198 and 0.35 g (GZ) of dry soil, using the Fast DNA SPIN Kit for Soil (MP Biomedicals, OH, USA)
199 according to the manufacturer's instructions. DNA quality and quantity were assessed using a
200 Colibri Microvolume Spectrometer (Titertek Berthold, Pforzheim, Germany). The abundances of
201 AOA, AOB, comammox *Nitrospira* clade A and B *amoA* genes were quantified by specific qPCR
202 assays on a LightCycler® 480II (Roche Diagnostics, Rotkreuz, Switzerland). Details of the primer
203 sets and qPCR conditions are provided in Table S2. The R^2 values were >0.99 for all genes, and
204 the amplification efficiencies were 79.4 to 91.0% for AOA, 90.4 to 96.5% for AOB, 79.8 to 86.9%
205 for comammox *Nitrospira* clade A and 78.6% for comammox *Nitrospira* clade B. The
206 amplification specificity was verified by melting curve analysis and agarose gel electrophoresis.
207 As we could not amplify the comammox *Nitrospira* clade B *amoA* gene in the HN and GZ soil
208 samples, we excluded clade B from the downstream analysis of these samples.

2.6 Illumina sequencing and phylogenetic analysis

DNA samples were sent to the Illumina MiSeq sequencing platform (Shanghai Majorbio Bio-Pharm Technology Co., Ltd., China) for high-throughput sequencing. The V4 region of the archaeal and bacterial 16S rRNA genes was amplified using the barcoded primer pair 515FmodF/806RmodR (Walters et al., 2016). In addition, the comammox *Nitrospira* clade A and clade B *amoA* genes were amplified using the primer pairs CA377f/C576r and CB377f/C576r (Jiang et al., 2020), respectively. The obtained raw sequences were quality-filtered and checked for chimaeras using Quantitative Insights into Microbial Ecology (QIIME) 2 with default criteria and UCHIME 4.2 (Edgar et al., 2011; Bolyen et al., 2019). The high-quality sequences were then classified into operational taxonomic units (OTUs) based on 97% similarity using Uparse 7.0.1090 (Edgar, 2013). The 16S rRNA OTUs were classified using RDP Classifier v. 2.11 against the SILVA database v. 138 (Quast et al., 2012) and comammox *Nitrospira* OTUs were classified using the nucleotide database (nt_v20210917) of the NCBI database. Representative AOA, AOB, and comammox *Nitrospira* sequences with identity values <90% from BLAST alignment or relative abundance <0.05% were excluded from the downstream analysis.

2.7 Statistical analysis

Statistical analysis was performed using R (R Core Team, 2021) by comparing the means from triplicate samples. Differences in *amoA* gene abundance were tested using a factorial two-way ANOVA with inhibitors and incubation time as fixed factors, followed by a Tukey HSD *post hoc* test. Temporal differences in NH_4^+, NO_3^- and N_2O concentrations were determined by comparing the slopes of the linear models using one-way ANOVA, followed by a Tukey HSD *post*

hoc test. Differences between the N_2O yields of AOA, AOB, and comammox *Nitrospira* were tested by one-way ANOVA. The absolute abundance of comammox *Nitrospira* OTUs was calculated as the comammox *Nitrospira* qPCR values multiplied by the corresponding relative abundance obtained from MiSeq sequencing. Differences in the relative and absolute abundance of OTUs between treatments were determined using one-way ANOVA.

3. Results

3.1 Sensitivity of ammonia oxidisers to inhibitors in different soils

In the absence of inhibitor, the *amoA* gene abundance of AOA, AOB, and comammox *Nitrospira* increased over time in response to NH_4^+ addition in HB and HN soils (Fig. 1a and b). In GZ soil, NH_4^+ amendment without inhibitor supplementation stimulated AOA growth but led to a decreased abundance of AOB ($P < 0.001$) and comammox *Nitrospira* clade A ($P < 0.001$) over time (Fig. 1c).

Acetylene inhibited the growth of AOA, AOB, and comammox *Nitrospira* in all soils during incubation (compared with initial abundance, two-way ANOVA; Table S4). In HN and GZ soils, AOA *amoA* gene abundance was significantly greater in the presence of 1-octyne or DMPP than in non-inhibited microcosms at the end of incubation (Table S5). 1-Octyne and DMPP application prevented the growth of AOB in all soils. DMPP inhibited the growth of comammox *Nitrospira* clade A, and different concentrations of 1-octyne differentially affected its growth in different soils. In HB soil, the growth of comammox *Nitrospira* clade A was unaffected by the presence of 0.01% and 0.015% 1-octyne, but higher concentrations of 1-octyne inhibited their growth (Fig. 1a; Table S6). In HN soil, none of the 1-octyne concentrations tested (0.01–0.05%) inhibited the growth of

comammox *Nitrospira* clade A. Instead, their growth was stimulated in the presence of 0.01% and 0.04% 1-octyne compared with that in non-inhibited microcosms (Fig. 1b; Table S6). In GZ soil, the abundance of comammox *Nitrospira* clade A decreased significantly ($P < 0.001$) in the presence of 0.01–0.05% 1-octyne, but that of the *amoA* gene at the last sampling point was greater than that in non-inhibited microcosms (Fig. 1c; Table S6).

3.2 Dynamics of nitrification and cumulative N_2O emissions

The differential growth patterns of NH_3 oxidisers in the absence or presence of inhibitors were ultimately reflected in the nitrification dynamics and N_2O emissions. In the absence of inhibitors, NH_4^+ was oxidised into NO_3^- at rates of ~5.4, 7.6 and 6.8 μg N g^{-1} $soil_{dw}$ d^{-1} in HB, HN and GZ soils, respectively (Fig. 2). In acetylene-treated microcosms, mineralisation-derived NH_4^+ did not accumulate over time, but NO_3^- concentrations decreased in HB and HN soils (Fig. 2a and b, $P < 0.05$), which may have resulted from microbial assimilation or denitrification. Nevertheless, N_2O produced via denitrification was negligible under oxic conditions, as NO_3^- addition did not affect N_2O emissions compared with those in acetylene-treated microcosms (Fig. S3).

Inhibition of AOB and comammox *Nitrospira* activity by DMPP reduced the nitrification rates by ~20%, 50% and 30% in HB, HN and GZ soils, respectively, and N_2O accumulation rates were also reduced by ~50%, 80% and 40% in HB, HN and GZ soils (Fig. 2). This suggests that AOA dominated NH_3 oxidation and associated N_2O production in HB and GZ soils, while N_2O emissions in HN soil resulted mainly from AOB and comammox *Nitrospira* activities. No significant difference in the nitrification and N_2O emissions rates was observed between the

different 1-octyne treatments (0.01–0.05%) in any of the soils tested (Fig. 2).

3.3 Ammonia oxidation and N_2O production rates, relative contributions and N_2O yields of ammonia oxidisers

In HN and GZ soils, the application of 1-octyne and DMPP did not significantly affect the growth of comammox *Nitrospira* and AOA (increases in *amoA* gene abundance) compared to non-inhibited microcosms during incubation for 11 days (Table S5–6), suggesting that NH_3 oxidation and N_2O production were also unaffected; thus, we calculated the NH_3 oxidation and N_2O production rates of NH_3 oxidisers within the initial 11 days of incubation. A similar assumption was made for HB soil after 21 days of incubation, enabling calculation of the NH_3 oxidation and N_2O production rates of NH_3 oxidisers throughout the entire period of incubation (21 days).

Comammox *Nitrospira* contributed to a small extent to NH_3 oxidation in the three acidic agricultural soils (3.3 to 8.5% of the total NH_3 oxidation rate) (Fig. 3a). The NH_3 oxidation rate of comammox was not significantly different among HB (0.17 ± 0.39 μg N g^{-1} $soil_{dw}$ d^{-1}), HN (0.39 ± 0.29 μg N g^{-1} $soil_{dw}$ d^{-1}) and GZ soils (0.50 ± 0.34 μg N g^{-1} $soil_{dw}$ d^{-1}). AOA dominated NH_3 oxidation in HB (4.41 ± 0.29 μg N g^{-1} $soil_{dw}$ d^{-1}, ~83.2%) and GZ soils (3.99 ± 0.34 μg N g^{-1} $soil_{dw}$ d^{-1}, ~67.4%). The NH_3 oxidation rate of AOB varied significantly between soils ($P < 0.001$); it was similar to the AOA rate in HN soil (AOB: 3.29 ± 0.34 μg N g^{-1} $soil_{dw}$ d^{-1}, ~47.0% and AOA: 3.32 ± 0.29 μg N g^{-1} $soil_{dw}$ d^{-1}, ~47.5%) but was significantly lower than the AOA rate in HB (0.71 ± 0.43 μg N g^{-1} $soil_{dw}$ d^{-1}, $P < 0.001$) and GZ (1.43 ± 0.34 μg N g^{-1} $soil_{dw}$ d^{-1}, $P < 0.001$) soils. These results indicate that AOA are key NH_3 oxidisers in all three acidic agricultural soils tested, with AOB also contributing significantly in one of these soils, while comammox *Nitrospira*

only play a limited role.

Consistent with their limited contribution to NH_3 oxidation, comammox *Nitrospira* contributed the lowest N_2O production among the three NH_3 oxidiser groups, producing 3.1 to 13.0% of NH_3 oxidation-associated N_2O emissions across the three soils (Fig. 3b). The N_2O production rates of comammox in HB (0.21 ± 0.48 ng N g^{-1} $soil_{dw}$ d^{-1}), HN (0.76 ± 0.04 ng N g^{-1} $soil_{dw}$ d^{-1}) and GZ soils (0.76 ± 0.19 ng N g^{-1} $soil_{dw}$ d^{-1}) were significantly lower than those of AOB (HB, 3.27 ± 0.57 ng N g^{-1} $soil_{dw}$ d^{-1}, $P < 0.01$; HN, 4.32 ± 0.19 ng N g^{-1} $soil_{dw}$ d^{-1}, $P < 0.001$; GZ, 2.93 ± 0.19 ng N g^{-1} $soil_{dw}$ d^{-1}, $P < 0.001$). AOB played a key role in N_2O production (32.8 to 73.5%), especially in HN soil, where AOB dominated N_2O production, producing ~73.5% of the NH_3 oxidation-associated N_2O generated (Fig. 3b). The AOA-associated N_2O production rate varied significantly among the soils ($P < 0.001$). In HN soil, the AOA rate (0.79 ± 0.02 ng N g^{-1} $soil_{dw}$ d^{-1}) was similar to the comammox rate, but it increased to 3.33 ± 0.33 ng N g^{-1} $soil_{dw}$ d^{-1} in HB soil and 5.25 ± 0.14 ng N g^{-1} $soil_{dw}$ d^{-1} in GZ soil.

N_2O yield, which incorporates NH_3 oxidation activity and N_2O production, enabled the assessment of the N_2O footprint of the three guilds of NH_3 oxidisers. N_2O yields for AOA ($P < 0.001$), AOB ($P < 0.001$) and comammox *Nitrospira* ($P < 0.05$) varied significantly between soil types. N_2O yields for AOB (0.13 to 0.46%) were higher than those for AOA (0.02 to 0.13%) and comammox *Nitrospira* (0.12 to 0.22%) (Fig. 3c).

3.4 Temporal changes in ammonia oxidiser community composition

The AOA community in HB and HN soils was dominated by OTUs within *Nitrososcosmicus* and *Nitrososphaera*, while GZ soil also contained a large proportion of *Ca*. Nitrosotaleales OTUs

(Fig. 4a). Most of the abundant OTUs responded to the soil treatments, with different relative abundances under the different inhibitor conditions. However, the common OTUs across the three soils (such as OTU8971 or OTU9644) did not respond similarly to the treatments across the three soils.

Two of the soils (HB and HN) showed a diverse AOB community containing OTUs affiliated with *Nitrosomonas, Nitrosospira*, the MND1 cluster and *Nitrosococcus*, while the GZ soil was less diverse (no *Nitrosomonas* and *Nitrosococcus* OTUs) (Fig. 4b). In contrast to AOA, many OTUs did not respond to the different treatments. Nevertheless, the relative abundance of some OTUs (e.g., *Nitrosospira* or *Nitrosomonas*) increased significantly in the absence of inhibitor in HB and HN soils ($P < 0.01$).

Four major phylogenetic clusters of comammox *Nitrospira* were detected in the tested soils, including clade A.2.2, A.2.1, A.3 and B (Fig. 4c). Clade A.2.2 was the most abundant cluster in all soils, accounting for 76.8%, 88.9% and 100% of the clade A sequences, respectively (Fig. 4c). Within each soil, the inhibitor treatments only affected the absolute abundance of a restricted number of OTUs. Similar to the other nitrifying guilds, the common OTUs across all soils did not respond consistently to the inhibitor treatments. Specifically, OTU31 grew in 1-octyne-treated microcosms of HB and HN soils but not in GZ soil; OTU10 grew in 1-octyne-treated microcosms of HN soil but not in HB and GZ soils (Fig. S4). These results indicate that in addition to the sensitivity to inhibitors, there are other factors affecting the growth of NH_3 oxidisers.

4. Discussion

This study demonstrates that comammox *Nitrospira* play a minor role in NH_3 oxidation and

335 N_2O emissions in all three fertilised acidic agricultural soils tested. Our results were consistent
336 with global-scale results, which show that comammox *Nitrospira* contribute ~11.7% to NH_3
337 oxidation-associated N_2O emissions (Jiang et al., 2023), indicating that comammox *Nitrospira* are
338 a minor but nonnegligible N_2O source in acidic agricultural soils. Comammox *Nitrospira* (0.12 to
339 0.22%) and AOA (0.02 to 0.13%) produced lower yields of N_2O than AOB (0.13 to 0.46%). These
340 results are generally consistent with previous pure culture, enrichment and microcosm studies
341 where yields of 0.07 to 0.20% and 0.05 to 0.09% were recorded for comammox *Nitrospira* and
342 AOA, respectively, while AOB were able to reach 0.51% (Stieglmeier et al., 2014; Hink et al.,
343 2017; Kits et al., 2019; Han et al., 2021). They are also aligned with current knowledge of the
344 mechanism of N_2O production in comammox *Nitrospira* and AOA, where production is restricted
345 to abiotic hybrid formation and NH_2OH oxidation (Stieglmeier et al., 2014; Kozlowski et al., 2016;
346 Kits et al., 2019; Wan et al., 2023), and in AOB, which can additionally produce N_2O via nitrifier
347 denitrification (Shaw et al., 2006). It should be noted that the mechanism for N_2O production in
348 comammox *Nitrospira* was derived from a single pure culture obtained from aquatic environments,
349 despite a vast phylogenetic diversity within comammox *Nitrospira* (Palomo et al., 2022). In the
350 tested soils, comammox *Nitrospira* exhibited higher N_2O yields compared to previous pure culture
351 studies (Kits et al., 2019; Han et al., 2021), suggesting that comammox *Nitrospira* may possess
352 other pathways for N_2O production.

353 The limited contribution of comammox *Nitrospira* to NH_3 oxidation and N_2O emissions
354 may result from their low abundance and cellular kinetic properties. In HB, HN, and GZ soils,
355 comammox *Nitrospira amoA* genes were 61-, 30-, and 6-fold lower than AOA *amoA* genes,
356 respectively. Despite AOB having a comparable or even lower abundance than comammox

Nitrospira, they contributed more to NH_3 oxidation and N_2O production. In addition to the low abundance, comammox *Nitrospira* had lower cell-specific NH_3 oxidation rates (0.44 to 0.58 fmol NH_4^+-N $cell^{-1}$ h^{-1}) than AOA (0.15 to 0.35 fmol NH_4^+-N $cell^{-1}$ h^{-1}) or AOB (0.36 to 15.80 fmol NH_4^+-N $cell^{-1}$ h^{-1}). These cell-specific rates are in the same range as those previously published for soils (Table S3), but comparisons of the cell-specific rates should be made with caution due to different methodologies. Consistently, the cell-specific NH_3 oxidation rate of AOB can be >10 times greater than that of AOA and comammox *Nitrospira* (Prosser and Nicol, 2012). Comammox *Nitrospira* were characterised as k-strategists adapted to slow growth in oligotrophic habitats (Costa et al., 2006; Daims et al., 2015). They exhibited low nutrient and energy requirements at low maximum growth rates (G_{max}) (Flynn et al., 2018), thus avoiding the need for rapid NH_3 oxidation. This is consistent with the lower V_{max} value for the comammox strain *N. inopinata* (~12 μmol N mg $protein^{-1}$ h^{-1}) compared with that for the AOB strains (38–84.2 μmol N mg $protein^{-1}$ h^{-1}) (Jung et al., 2022).

Our demonstration that the growth of comammox *Nitrospira* clade A was inhibited by higher concentrations of 1-octyne in HB and GZ soils but stimulated in HN soil indicates that the sensitivity of comammox *Nitrospira* to 1-octyne varies among soils. This can be attributed to the differences in sensitivity among different comammox *Nitrospira* clade A phylotypes, given the distinct comammox *Nitrospira* community composition in these soils. This hypothesis is reinforced by the growth of comammox *Nitrospira* in neutral or alkaline soils but inhibition in acidic soils, all supplemented with the same concentration of 1-octyne (Li et al., 2019; Tan et al., 2022; Lin et al., 2023). Earlier works on AOA and AOB pure cultures show that different phylotypes of AOA and AOB have different sensitivities to 1-octyne (Taylor et al., 2013; Taylor et

al., 2015), but there is currently limited information on the sensitivity of comammox pure cultures to 1-octyne (Taylor et al., 2017). An alternative explanation, presumably, is the varying degradation of 1-octyne in these soils. However, the stable and complete inhibition of AOB growth by 1-octyne in all soils, along with the regular replenishment of 1-octyne, question the strength of this argument. Although the inhibition of comammox *Nitrospira* growth by $\geqslant$ 0.02% 1-octyne in HB soil did not result in underestimation of the comammox rates (Fig. S5), possibly due to the low rate hindering the detection of changes, the results might be different in some habitats with high comammox activity. Therefore, when applying the combined inhibitor method in various soils, it is essential to evaluate the effectiveness of inhibitors on NH_3 oxidisers by measuring the growth of NH_3 oxidisers through their change in absolute abundance over time, as performed in some previous studies (Hink et al., 2017; Tan et al., 2022; Krüger et al., 2023).

This study confirms that AOA dominate NH_3 oxidation in fertilised acidic agricultural soils, while AOB also contribute significantly to NH_3 oxidation and NH_3 oxidation-related N_2O production in one of the soils tested. Chemolithotrophic NH_3 oxidisers were the primary drivers of NO_3^- and N_2O production in the tested soils, and the contribution of heterotrophic denitrifiers and heterotrophic nitrifiers was found to be negligible (Fig. S3), likely due to the oxic conditions and the limited availability of easily decomposable organic substrates (Bateman and Baggs, 2005; Martikainen, 2022). One previous hypothesis explaining the dominance of AOA in acidic soils was their high substrate affinity for NH_3 (Martens-Habbena et al., 2009; Jung et al., 2011), which confers a competitive advantage in acidic environments with low NH_3 availability, but this argument was questioned (Qin et al., 2024). In addition, most AOA sequences in HB and HN soils were affiliated with the *Nitrosocosmicus* cluster, and several *Nitrosocosmicus* species were

previously demonstrated to show similar substrate affinity to AOB (Jung et al., 2022). The function of V-type ATPases as proton pumps has been proposed, among others, as an adaptive mechanism in acidic conditions. *Nitrosocosmicus* species encode V-type ATPase to thrive at low pH (Wang et al., 2019a). This study also provides another piece of evidence that AOB can be important NH_3 oxidisers in acidic soils (Lin et al., 2021), contributing ~47.0% to NH_3 oxidation in HN soil, potentially due to the activity of acidic AOB organisms (Aigle et al., 2019). *Nitrosomonas* and *Nitrosospira* represented two AOB phylotypes promoted by NH_4^+ amendment in HN soil, suggesting an important role of these phylotypes in nitrification. This is consistent with earlier studies reporting that *Nitrosomonas communis* and *Nitrosospira multiformis* drive nitrification in acidic agricultural and forest soils, respectively (Huang et al., 2018; Yin et al., 2021).

5. Conclusion

Our study demonstrates that AOA dominate nitrifying activities in fertilised acidic agricultural soils, followed by AOB, while comammox *Nitrospira* only play a minor role in NH_3 oxidation and N_2O production. It also robustly illustrates that the inhibitory effect of 1-octyne on comammox *Nitrospira* clade A varies across soils, emphasising that the inappropriate use of 1-octyne may lead to misestimation of NH_3 oxidiser activity. This is fundamental for future studies using the combined inhibitor method to assess the functional importance of NH_3 oxidisers in diverse soils. Therefore, this work opens avenues of research to reduce nitrogen fertiliser loss and N_2O emissions by manipulating the role of comammox *Nitrospira* in cropland nitrification.

Data availability

The 16S rRNA sequence data obtained in this study have been deposited in the National Center for Biotechnology Information (NCBI) database under Bioproject accession number PRJNA975883. The *amoA* gene sequences of comammox *Nitrospira* have been uploaded to Genbank with accession numbers OR062097–OR062210. The shapefile of China is publicly obtained from https://www.csgpc.org/list/254.html (drawing approval no. GS(2020)4632). The cropland cover map of China is provided by Yang and Huang (2021) (https://zenodo.org/record/5816591#.ZAWM3BVBy5c).

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Figure legends

Figure 1 Changes in the *amoA* gene copies of AOA, AOB and comammox *Nitrospira* clades A and B during the 21-day incubation period of HB (Panels a), HN (Panels b) and GZ (Panels c) soils. Soil microcosms were amended with NH_4^+ in the absence or presence of inhibitors (acetylene, 0.01 to 0.05% 1-octyne, or DMPP). Error bars represent the standard errors of the mean (n = 3).

Figure 2 Temporal dynamics of NH_4^+, NO_3^- and cumulative N_2O emissions during the 21-day incubation period of HB (Panels a), HN (Panels b) and GZ (Panels c) soils. Soil microcosms were amended with NH_4^+ in the absence or presence of inhibitors (acetylene, 0.01 to 0.05% 1-octyne, or DMPP). Error bars represent the standard errors of the mean (n = 3).

Figure 3 NH_3 oxidation and N_2O production driven by NH_3 oxidisers in HB, HN and GZ soils. Average NH_3 oxidation (Panels a) and N_2O production (Panels b) rates and relative contributions

652 of AOA (orange), AOB (gray), and comammox *Nitrospira* (CMX, green) in HB, HN, and GZ soils.
653 The contribution of each group is displayed under the columns with the corresponding colours. **c**
654 The N_2O yield of AOA (orange), AOB (gray), and comammox *Nitrospira* (CMX, green) in
655 different soils. Error bars indicate the standard errors of triplicate microcosms. The average rates
656 and relative contributions in HB soil were calculated as the mean values based on the 0.01% and
657 0.015% 1-octyne-treated microcosms. The average rates and relative contributions in HN and GZ
658 soil were calculated as the mean values based on the 0.01 to 0.05% 1-octyne-treated microcosms.
659 **Figure 4** Ammonia oxidiser community analysis in HB, HN and GZ soils incubated in the initial
660 sample (Original), in the absence (N) or presence of acetylene (CH), DMPP (DP), or 0.01% to
661 0.05% 1-octyne (O1 to O5) for 21 days. **a** Relative abundance of AOA OTUs (based on MiSeq
662 sequencing using universal 16S rRNA gene primers). **b** Relative abundance of AOB OTUs (based
663 on MiSeq sequencing using universal 16S rRNA gene primers). **c** Relative abundance of the
664 comammox *Nitrospira* OTUs (relative abundance > 5%). Data are presented as the mean and
665 standard error of triplicate microcosms for each treatment. An asterisk (*) next to the OTU
666 indicates a significant difference in abundance between treatments ($p < 0.05$), based on a one-way
667 ANOVA.
668

669 **Supporting Information**

670 **Supplementary Tables S1–S6**

671 **Table S1** Locations, climatic parameters of the study sites, and physicochemical properties of the
672 soils.

673 **Table S2** Reaction systems and thermal cycling protocols used for target gene amplification in
674 qPCR.

675 **Table S3** Cell-specific NH_3 oxidation rates in soils. It is assumed that each cell of AOB, AOA and
676 comammox *Nitrospira* contains 2.5, 1.0 and 1.0 *amoA* gene copies, respectively (Huang et al.,
677 2021).

678 **Table S4** The *amoA* gene abundance of AOA, AOB and comammox *Nitrospira* in HB, HN and
679 GZ soils in the presence of acetylene (CH) statistical analyses (Figure 1).

680 **Table S5** The *amoA* gene abundance of AOA in HB, HN and GZ soils in the absence (N) or
681 presence of 1-octyne (OC) and DMPP (DP) statistical analyses (Figure 1).

682 **Table S6** The comammox *Nitrospira* clade A *amoA* gene abundance in HB, HN and GZ soils in
683 the absence (N) or presence of 1-octyne (OC) and DMPP (DP) statistical analyses (Figure 1).

684

685 **Supplementary Figures S1–S5**

686 **Figure S1** Geographical location of the sampling sites for agricultural soils across southern China.

687 **Figure S2** Schematic diagram of the inhibitory effects of inhibitors. Acetylene completely

688 inactivates AOA, AOB and comammox *Nitrospira* (CMX). 1-Octyne specifically inhibits AOB at

689 certain concentrations. Low concentrations of 1-octyne result in partial inhibition of AOB activity,

690 and high concentrations of 1-octyne partially inhibit the activity of comammox *Nitrospira*. Both

691 AOB and comammox *Nitrospira* are inhibited by DMPP.

692 **Figure S3** Temporal dynamics of cumulative N_2O emissions during the 5-day incubation period

693 of HB, HN and GZ soils. Soil microcosms were amended with NH_4^+ (150 μg N g^{-1} $soil_{dw}$) in the

694 absence of acetylene (0.01%, v/v) or amended with NO_3^- (150 μg N g^{-1} $soil_{dw}$) in the presence of

695 acetylene (0.01%, v/v). Error bars represent the standard errors of the mean (n = 3).

696 **Figure S4** Absolute abundance of comammox *Nitrospira* OTUs (relative abundance > 5%)

697 retrieved in HB, HN and GZ soils incubated in the initial sample (Original), in the absence (N) or

698 presence of acetylene (CH), DMPP (DP), or 0.01% to 0.05% 1-octyne (O1 to O5) for 21 days. The

699 absolute abundance of comammox *Nitrospira* OTUs was calculated as the product of comammox

700 *Nitrospira amoA* gene numbers (obtained by qPCR) and the relative abundance of the

701 corresponding OTUs (obtained by MiSeq sequencing using *amoA* gene primers). Data are

702 presented as the mean and standard error of triplicate microcosms for each treatment. An asterisk

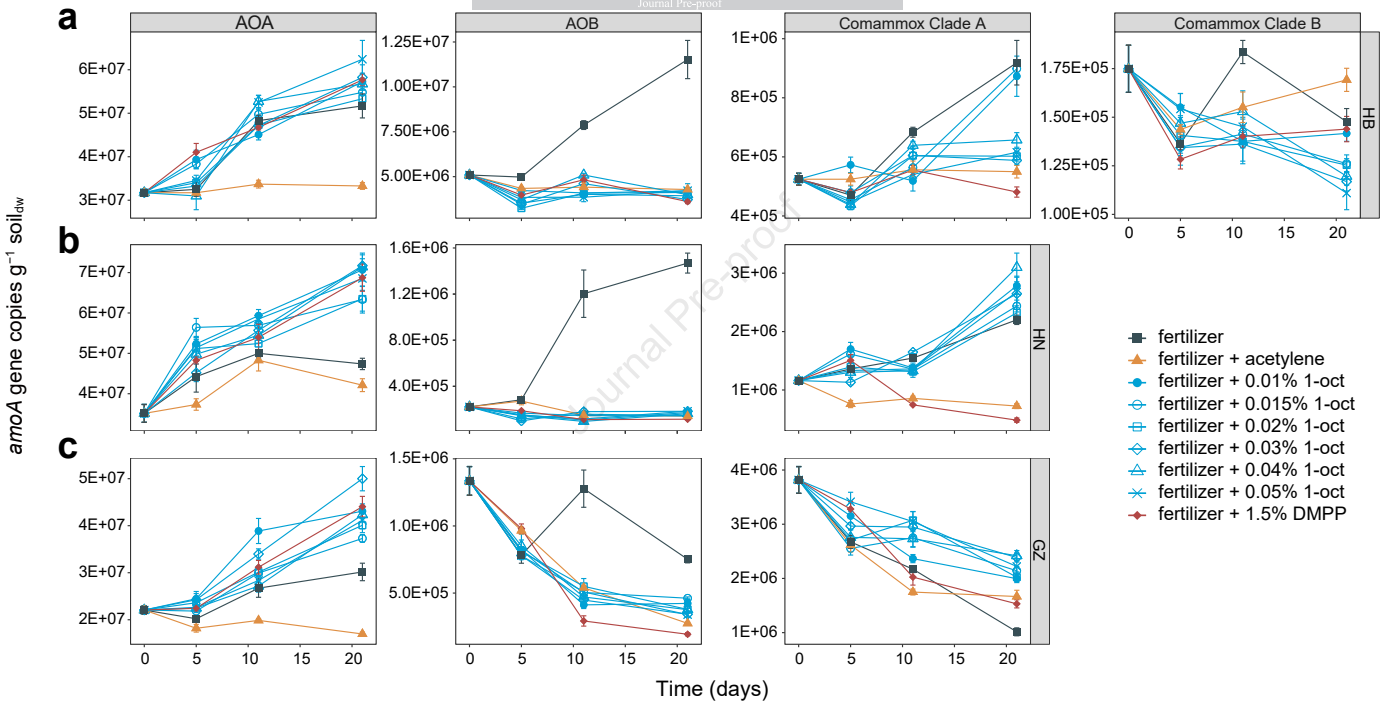
703 (*) next to the OTU indicates a significant difference in abundance between treatments ($p < 0.05$),

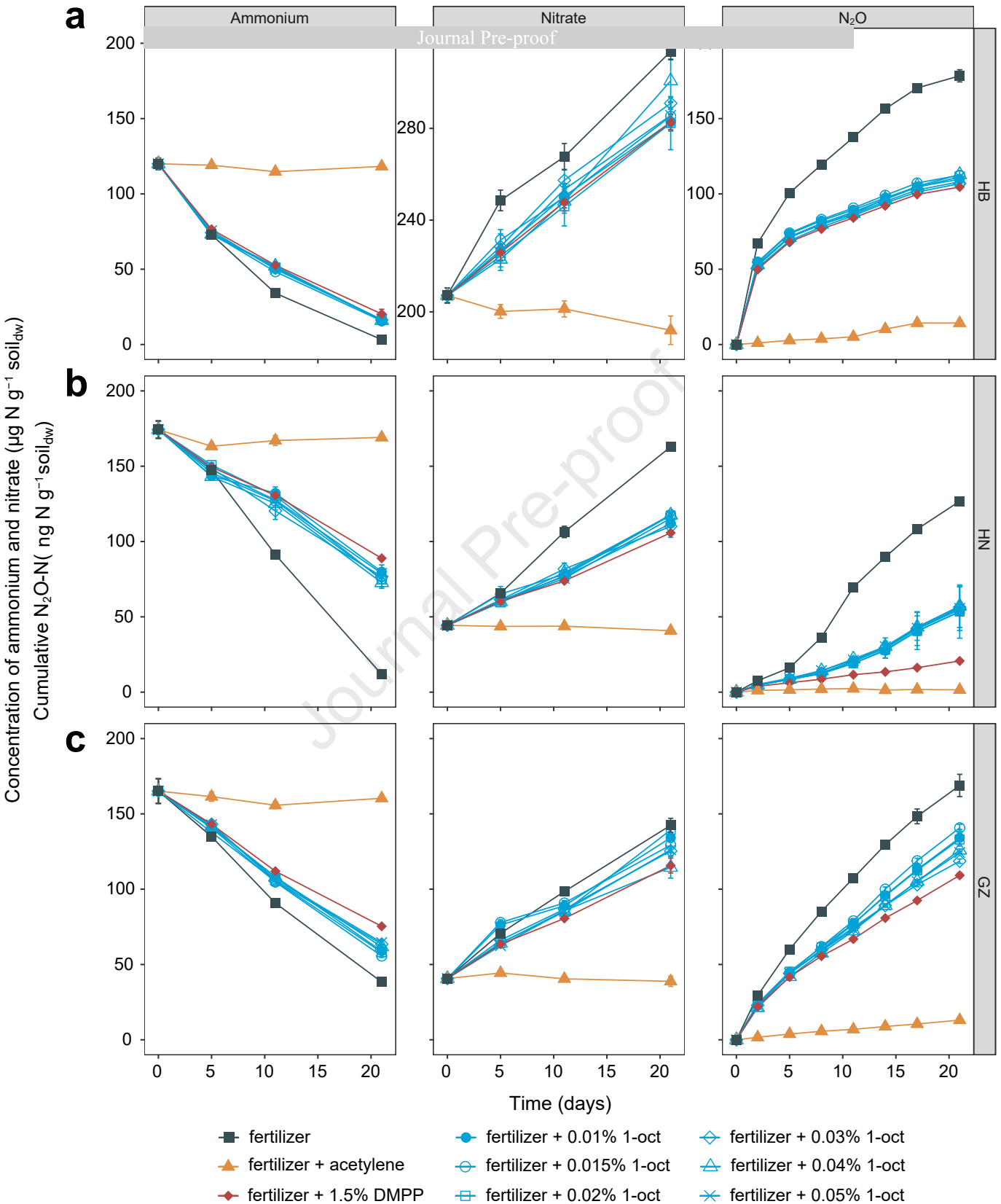
704 based on a one-way ANOVA.

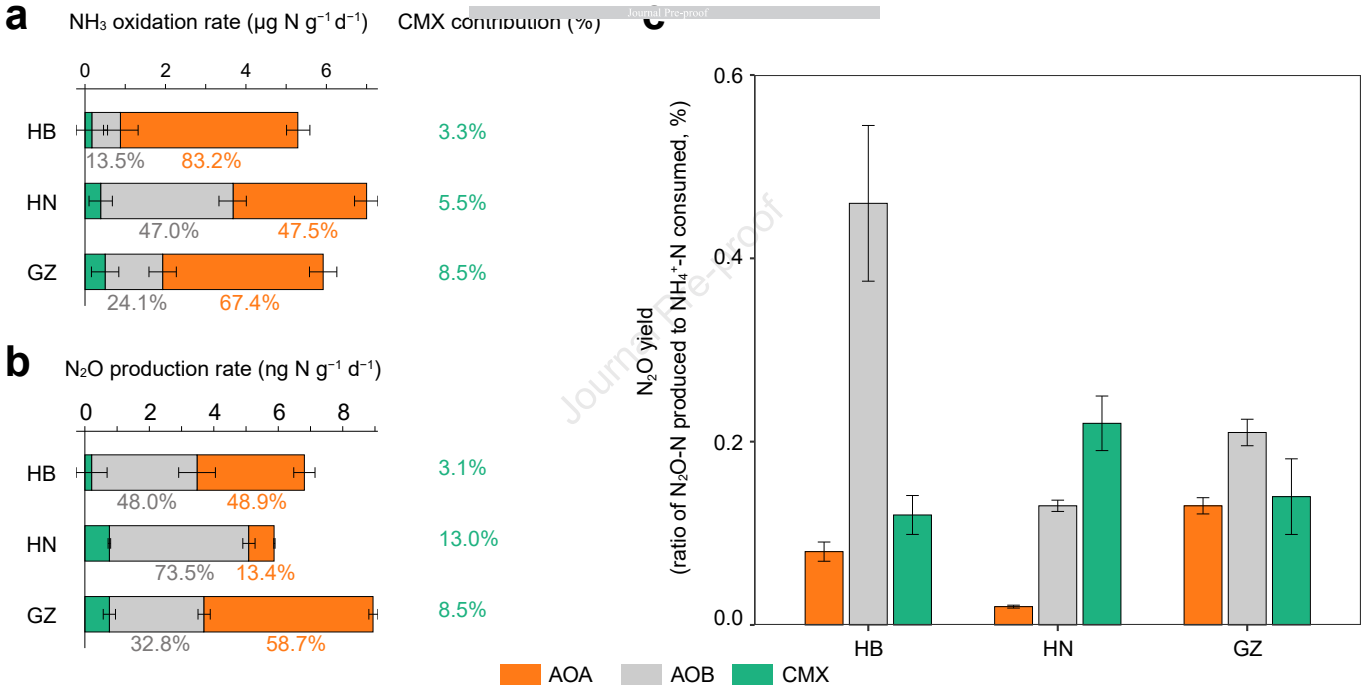
705 **Figure S5** Deviation of NH_3 oxidiser rates in microcosms treated with different concentrations of

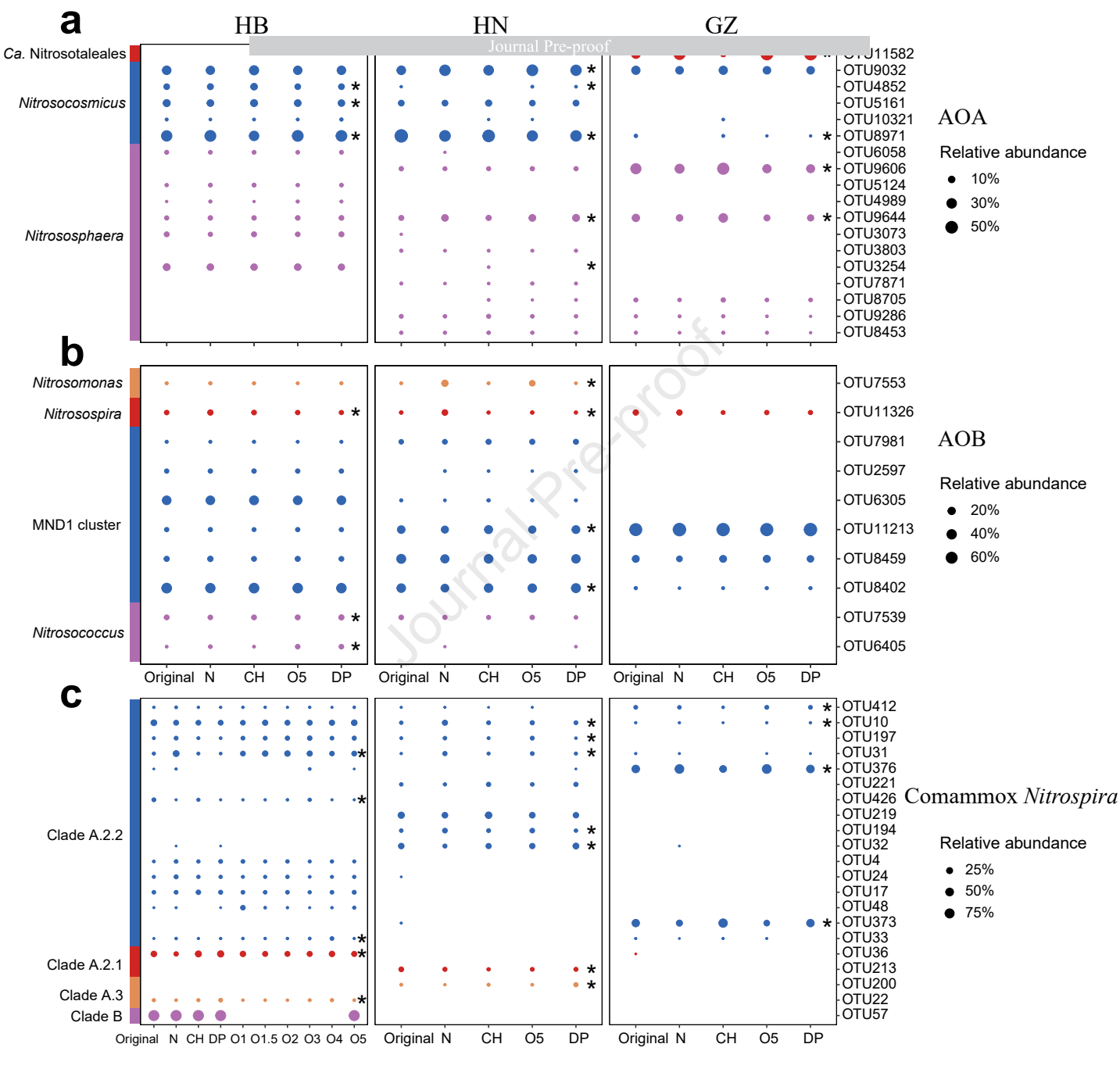
706 1-octyne. **a** NH_3 oxidation rates of AOA (orange), AOB (gray), and comammox *Nitrospira* (CMX,

707 green) calculated from all 1-octyne concentrations tested (0.01%–0.05%). **b** N_2O production rates
708 of AOA, AOB, and comammox *Nitrospira* calculated from all 1-octyne concentrations tested
709 (0.01%–0.05%).








a
HB
HN
GZ
Journal Pre-proof
Ca. Nitrosotaleales
Nitrososcosmicus
Nitrososphaera
OTU11582
OTU9032
OTU4852
OTU5161
OTU10321
OTU8971
OTU6058
OTU9606
OTU5124
OTU4989
OTU9644
OTU3073
OTU3803
OTU3254
OTU7871
OTU8705
OTU9286
OTU8453
AOA
Relative abundance
10%
30%
50%
b
Nitrosomonas
Nitrosospira
MND1 cluster
Nitrosococcus
OTU7553
OTU11326
OTU7981
OTU2597
OTU6305
OTU11213
OTU8459
OTU8402
OTU7539
OTU6405
AOB
Relative abundance
20%
40%
60%
Original N CH O5 DP
c
Clade A.2.2
Clade A.2.1
Clade A.3
Clade B
OTU412
OTU10
OTU197
OTU31
OTU376
OTU221
OTU426
OTU219
OTU194
OTU32
OTU4
OTU24
OTU17
OTU48
OTU373
OTU33
OTU36
OTU213
OTU200
OTU22
OTU57
Comammox Nitrospira
Relative abundance
25%
50%
75%
Original N CH DP O1 O1.5 O2 O3 O4 O5
Original N CH O5 DP

Highlights

- Comammox play a minor role in NH_3 oxidation and N_2O emissions in acidic arable soils;
- AOB are functionally active in acidic agricultural soils;
- The sensitivity of comammox *Nitrospira* clade A to 1-octyne varies across soils.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.