1 **Title: AOB *Nitrosospira* cluster 3a.2 (D11) dominates N$_2$O emissions in fertilised**

2 **agricultural soils**

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ABSTRACT

Ammonia-oxidation process directly contribute to soil nitrous oxide (N_2O) emissions in agricultural soils. However, taxonomy of the key nitrifiers (within ammonia oxidising bacteria (AOB), archaea (AOA) and complete ammonia oxidisers (comammox *Nitrospira*)) responsible for substantial N_2O emissions in agricultural soils is unknown, as is their regulation by soil biotic and abiotic factors. In this study, cumulative N_2O emissions, nitrification rates, abundance and community structure of nitrifiers were investigated in 16 agricultural soils from major crop production regions of China using microcosm experiments with amended nitrogen (N) supplemented or not with a nitrification inhibitor (nitrapyrin). Key nitrifier groups involved in N_2O emissions were identified by comparative analyses of the different treatments, combining sequencing and random forest analyses. Soil cumulative N_2O emissions significantly increased with soil pH in all agricultural soils. However, they decreased with soil organic carbon (SOC) in alkaline soils. Nitrapyrin significantly inhibited soil cumulative N_2O emissions and AOB growth, with a significant inhibition of the AOB *Nitrosospira* cluster 3a.2 (D11) abundance. One *Nitrosospira multiformis*-like OTU phylotype (OTU34), which classified within the AOB *Nitrosospira* cluster 3a.2 (D11), had the greatest importance on cumulative N_2O emissions and its growth significantly depended on soil pH and SOC contents, with higher growth at high pH and low SOC conditions. Collectively, our results demonstrate that alkaline soils with low SOC contents have high N_2O emissions, which were mainly driven by AOB *Nitrosospira* cluster 3a.2 (D11). Nitrapyrin can efficiently reduce nitrification-related N_2O emissions

42   by inhibiting the activity of AOB *Nitrosospira* cluster 3a.2 (D11). This study advances

43   our understanding of key nitrifiers responsible for high N_2O emissions in agricultural

44   soils and their controlling factors, and provides vital knowledge for N_2O emission

45   mitigation in agricultural ecosystems.

46   ***Keywords:*** Nitrification; N_2O emission; Nitrapyrin; *Nitrosospira*; Alkaline soil

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49   **1 Introduction**

50   Nitrous oxide (N_2O) is the third most important greenhouse gas, with a global

51   warming potential 273 times larger than carbon dioxide (CO_2) (IPCC, 2021), and is a

52   major stratospheric ozone-depleting substance (Ravishankara et al., 2009).

53   Anthropogenic N_2O emissions have increased by 30% over the last four decades,

54   mainly resulting from nitrogen (N) fertilisation in croplands (Tian et al., 2020). Soil

55   nitrification and denitrification are the two main sources of N_2O production, and

56   nitrification-related pathways (including ammonia oxidation and nitrifier

57   denitrification) dominates N_2O production in aerobic agricultural soils while

58   denitrification dominates N_2O production in anaerobic microsites of agricultural soils

59   (Butterbach-Bahl et al., 2013; Hu et al., 2017). Soil nitrification-related pathways

60   contributed 46.4% - 96.7% of total N_2O emissions in agricultural soils (Wrage et al.,

61   2005; Mørkved et al., 2007; Liu et al., 2016; Shi et al., 2017), as agricultural soils have

62   been shown to be aerobic at most time (Song et al., 2019). Unraveling the microbial

63   mechanisms responsible for fertiliser-induced N_2O emission via nitrification-related

64   pathways in agricultural soils is thus critical to mitigate and predict global N_2O

65   emissions.

66   Nitrification-related pathways, mainly performed by autotrophic ammonia-

67   oxidising bacteria (AOB), archaea (AOA) and complete ammonia oxidisers

68   (comammox *Nitrospira*) under aerobic conditions, can produce N_2O enzymatically via

69   incomplete hydroxylamine to N_2O (rather than nitrite (NO_2^-)) via nitric oxide (NO), and

70   via nitrifier denitrification, the sequential reduction of NO_2^- to NO and N_2O (Hu et al.,

71   2015; Hink et al., 2018). AOB, AOA and comammox *Nitrospira* physiologies differ, as

72   evidenced by different N forms preferences, niche differentiation and N_2O yields.

73   Generally, AOB and comammox *Nitrospira* clade A prefer high concentrations of

74   inorganic ammonium (NH_4^+) or urea and dominate nitrification in neutral, alkaline and

75   N-rich soils (Di et al., 2009; Xia et al., 2011; Hink et al., 2017; Li et al., 2019a), while

76   AOA and comammox *Nitrospira* clade B prefer organic N or slow-release fertiliser and

77   play dominant roles in nitrification in acid and N-poor soils (Gubry-Rangin et al., 2010;

78   Zhang et al., 2010; Zhang et al., 2012; Hink et al., 2018; Wang et al., 2019). AOB are

79   described as the main contributors to soil N_2O emissions in high-fertility, neutral and

80   alkaline soils (Liu et al., 2016; Linton et al., 2020; Hu et al., 2022; Lourenço et al.,

81   2022), while AOA compete over AOB and are the major N_2O-producer in soils without

82   NH_4^+ addition and in acid soils (Hink et al., 2017; Tzanakakis et al., 2019; Wu et al.,

83   2020; Hu et al., 2022). Still, some AOA species with high ammonia (NH_3) tolerance,

84   such as those within the *Candidatus Nitrosocosmicus* (NS) clade (Lehtovirta-Morley et

85   al., 2016), can contribute to N_2O production to the same extent as AOB under high

86   NH_4^+ inputs, as described for *Candidatus Nitrosocosmicus agrestis* (Liu et al., 2021;

87   Jiang et al., 2023b). In addition, comammox *Nitrospira* clade A.2 and clade B also

88   participate in nitrification in fertilised and unfertilised soils with NH_4^+ and manure

89   addition (Li et al., 2019a; Wang et al., 2019; Xu et al., 2020; Lin et al., 2022), but there

90   is rare empirical evidence of their contribution to N_2O emissions in agricultural soils

91   (Tan et al., 2022; Jiang et al., 2023a). Altogether, more knowledge of the key nitrifiers

92   responsible for N_2O emissions is required over a large spatial scale region, especially

93   considering the high diversity of soil conditions and nitrifier taxa.

94   Furthermore, fertilisation-induced N_2O emission displays noticeable regional

95   heterogeneities in global agricultural soils, and is primarily influenced by soil properties,

96   fertiliser management, climate and microbial community (Gerber et al., 2016; Aliyu et

97   al., 2019; Cui et al., 2021; Ginebra et al., 2022). Among these influential factors of N_2O

98   emission, soil pH has strong effects on the activities of nitrifiers and denitrifiers, and

99   thus further affects N_2O production (Wang et al., 2017; Zhu et al., 2019). For example,

100   it is frequently observed that acid soils emit much higher N_2O than alkaline soils, due

101   to the inhibition of bacterial N_2O-reductase enzyme and the activation of fungal

102   denitrification under acidic conditions (Ji et al., 2022; Yin et al., 2023). In contrast,

103   lower N_2O emissions were observed in acid soils than alkaline soils using microcosm

104   incubations, and this was attributed to the increase of nitrification and nitrifier

105   denitrification rates with soil pH increase (Shi et al., 2017; Wrage-Mönnig et al., 2018;

106   Tzanakakis et al., 2019). Nitrification-related N_2O emissions in microcosm-incubated

107   agricultural soils increase with soil pH within a pH range of 5.4-8.7 (Zhu et al., 2019),

108   but low N_2O emissions in alkaline soils with high nitrification rates have also been

109   reported (Zhu et al., 2019; Zhang et al., 2023). These discrepancies might be attributed

110   to the variation in the metabolism of different nitrifier groups related to N_2O emission,

111   reflecting the need to identify the specific nitrifiers associated with high N_2O emissions

112   in agricultural soils. Meanwhile, ^{15}N tracing based studies have demonstrated that

113   denitrification, induced by rapid oxgen consumption during high ammonia oxidation,

114   also contributes to the high N_2O emissions in alkaline soils (Zhu et al., 2013; Yang et

115   al., 2021; Wei et al., 2023), indicating the complexity of nitrification-related N_2O

116   emissions. More efforts are thus required to unveil the underlying mechanism.

117   Nitrification   inhibitors   (NIs),   such   as   dicyandiamide   (DCD),   3,4-

118   dimethylpyrazole   phosphate   (DMPP)   and   2-chloro-6-(trichloromethyl)-pyridine

119   (nitrapyrin), are commonly used for reducing N loss and improving N use efficiency in

120   agricultural soil (Qiao et al., 2015; Fan et al., 2022), by retarding nitrification through

121   interfering with the ammonia monooxygenase (AMO) enzyme of nitrifiers (Cui et al.,

122   2013; Beeckman et al., 2018; Zhao et al., 2020). However, the NIs efficacy on inhibiting

123   nitrification and soil N_2O emission vastly vary in different agricultural soils (Zhang et

124   al., 2012; Sha et al., 2020; Pokharel and Chang, 2021; Fan et al., 2022). In several

125   agricultural soils in which AOB dominated nitrification, DCD, DMPP and nitrapyrin

126   significantly reduced nitrification by AOB inhibition but without any significant effects

127   on AOA abundance (Cui et al., 2013; Soares et al., 2016; Zhou et al., 2020). However,

128   DCD and nitrapyrin also significantly inhibited the AOA abundance in some acid (pH

4.72) and alkaline (pH 7.91) paddy soils where AOA dominated nitrification (Gu et al., 2018; Li et al., 2019b; Meng et al., 2020). Futhermore, nitrapyrin reduced comammox *Nitrospira* clade A abundance in an arable soil in which comammox *Nitrospira* clade A was the dominant nitrifier (Li et al., 2019a; Li et al., 2019b). These findings suggest that the efficiency of NIs in inhibiting soil nitrification and N_2O emission greatly depends on the active nitrifier groups (Zhang et al., 2012). However, the response of soil nitrification and N_2O emission to NIs in various soils remains elusive.

In this study, soils with a range of pH were sampled across major cropland regions of China to establish microcosm incubations with nitrogen and nitrapyrin additions. The response of N_2O emission, nitrification, abundance and community structure of AOB, AOA and comammox *Nitrospira* to N fertilisation and nitrapyrin were investigated. We aimed to: (1) explore the nitrification-related N_2O emissions and its main controlling factors in agricultural soils; (2) identify the active nitrifier groups and phylogenetic clusters responsible for nitrification-related N_2O emissions in agricultural soils, especially in alkaline soils. We hypothesised that (1) soil properties determine the distribution and activity of different nitrifier groups with varied capabilities of N_2O production, causing the different N_2O emission among different soils and regions; (2) some nitrifiers possess high N_2O production potential, influencing soil N_2O emission, and they can be inhibited by application of NIs, contributing the N_2O emission mitigation.

2 Materials and methods

2.1 Sampling design and soil characterisation

Agricultural soils spanning a range of altitudes over 100 km were collected from 16 geographically dispersed sites across the major crop regions of China (Fig. S1, Table S1), including Changsha (CS), Laiyang (LY), Yushu (YS), Gongzhuling (GZL), Qujing (QJ), Xinzheng (XZ), Wuchang (WC), Hengyang (HY), Fengqiu (FQ), Zibo (ZB), Shijiazhuang (SJZ), Yongcheng (YC), Xuchang (XC), Mianyang (MY), Luancheng (LC) and Yuanyang (YY). All 16 agricultural soils were collected during July to September in 2020. Three replicate samples were included for each site, and each sample consisted five soil cores (0-20 cm depth; 5 cm in diameter) taken from a ~ 100 m^2 field plot and pooled as one replicate. After removing visible roots and stones, the samples were transported to the laboratory on ice. Samples were subsequently divided and stored at 4 °C for physicochemical analyses and for the incubation experiment or frozen at -80 °C for molecular analyses.

Soil water content was measured by the oven-drying method at 105 °C to constant weight. Soil pH was measured on air-dried using a soil:water ratio of 1:2.5 with a pH meter (DELTA-320, China). Soil NO_3^--N was extracted with 2 M KCl solution and determined with a continuous flow analyser (AA3, Bran + Luebbe, Germany). Soil organic carbon (SOC) was estimated after removal of the inorganic carbon with HCl. SOC, total carbon (TC) and total nitrogen (TN) were determined by the Dumas method with Element Analyzer (Vario EL III-Elementar, Germany).

2.2 Soil microcosm incubation

Microcosm experiments were conducted in 250-ml serum bottles containing 15 g equivalent dry mass of fresh soil. Each soil had two treatments: N (N fertilisation) and NI (N fertilisation and nitrapyrin amendment). To avoid the bias of urea hydrolysis rate among different soils, $(NH_4)_2SO_4$, rather than urea was applied as the N fertiliser at a rate of 100 mg N kg^{-1} dry soil (Faeflen et al., 2016; Li et al., 2019a), and nitrapyrin was applied as nitrification inhibitor at a rate of 1 mg kg^{-1} dry soil based on the recommended dose in previous literatures (Burzaco et al., 2014; Xi et al., 2017; Gu et al., 2018) and our preliminary experiments (data were not shown). Before microcosm experiment start, all soil samples were preincubated for one week at a unified moisture condition at about 30% WFPS by adding sterilized water or air drying in a well-ventilated environment with room temperature (~25 °C). Then, each fresh soil (15 g equivalent dry mass) was weighted into sterilized bottle, and equivalent volumes of $(NH_4)_2SO_4$ solution and nitrapyrin suspension (under stirring) were added into the corrresponding treatments. To mix well the reagents and soil particles, the reagents or sterilized water was sprinkled into soil with 100 μl-pipette and shaked after waiting for 5 minutes for fully diffusing, and the reagents sprinkling and shaking processes were repetively operated for 2 times for each reagent. The soil moisture in microcosms was finally adjusted to 60% WFPS by adding sterilized water. Serum bottles were sealed and incubated at 28 °C in the dark. Soil water content was maintained by resupplying the lost water every 2 or 3 days (after each gas sampling). Aerobic conditions were maintained before gas sampling by sealing with sterilized breathable rubber plugs or

192 opening the serum bottles in continuous air-flow to refresh the air in the headspace

193 every 1, 2 or 3 days. The headspace gas was collected at days 1, 3, 5, 7, 10, 13, 16, 19,

194 22, 25 and 27 after incubation. At each gas sampling time point, the headspace gas were

195 collected using 20 ml sterile syringes with triple valve at 0 and 4 hours after sealing the

196 serum bottles with rubber stoppers and aluminum caps. Before the gas sampling, 20 ml

197 sterile syringe with triple valve was checked for gas tight and flushed with pure N_2 three

198 times and with chamber air three times. And then, 20 ml gas was collected and saved

199 in the syrings, and 10 ml gas was injected into gas chromatography (GC) equipped with

200 the ECD detector (Agilent 7890B, USA) for N_2O concentration measurements within

201 1 weeks (Song et al., 2018). The N_2O emission rate was determined by multiplying the

202 increase in N_2O concentrations within 4 hours by the headspace volume and dividing it

203 by the soil dry weight. The cumulative N_2O emissions were calculated by multiplying

204 each N_2O emission rate by the respective sampling interval time and summing up all

205 estimated N_2O emissions.

206   Three replicates of each treatment were destructively sampled at days 0, 7 and 28

207 and some subsamples were frozen immediately at -80 °C for molecular analyses. The

208 other subsamples was stored in 4 °C for soil NO_3^--N and water content analyses. Soil

209 NO_3^--N was extracted from 4 g fresh soils with 2 M KCl and determined by a

210 continuous flow analyser (AA3, Bran + Luebbe, Germany). Soil water content was

211 measured using 1 g fresh soil by the oven-drying method. Nitrification rates were

212 calculated by dividing the increment of NO_3^--N between two consecutive sampling

213 dates (day 0 and day 7) , as the change of NO_3^--N was much faster in the initial period

214   than in the later period (day 7-28 and day 0-28) in all soils, thus reflecting the highest

215   nitrification rate for each soil.

216   **2.3 DNA extraction and quantitative PCR of *amoA* genes**

217   To quantify the abundances of AOB, AOA and comammox *Nitrospira* among 16

218   sites and their response to nitrapyrin amendment, DNA was extracted from 0.5 g of

219   frozen soil samples from day 28 using a DNeasy® PowerSoil® Pro Kit (QIAGEN

220   GmbH, Germany) following the manual's instructions. The concentration and purity of

221   DNA extracts were determined using a Nanodrop ND-2000c UV-Vis

222   spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

223   The abundances of AOB, AOA and comammox *Nitrospira* clade A *amoA* gene

224   were determined with real-time quantitative PCR (qPCR), with the primer set amoA-

225   1F/amoA-1R (Rotthauwe et al., 1997), Arch-amoAF/Arch-amoAR (Francis et al., 2005)

226   and comaA244f_a-e/comaA659r_a-e (Pjevac et al., 2017), respectively. The qPCRs

227   were conducted on a Roche LightCycler® 480 system (Roche Life Science, USA). The

228   20 μl reaction volume contained 2 μl DNA template (1-10 ng), 10 μl 2× Supermix (Bio-

229   Rad, Hurcules, CA, USA), 1 μl (10 μM) of each primer and 6 μl ddH$_2$O. Thermal cycle

230   program was 95 °C for 5 min, followed by 40 (AOB and AOA) / 45 (comammox

231   *Nitrospira* clade A) cycles of 95 °C for 30 s, 55 °C(AOB) / 53 °C(AOA) / 48 °C

232   (comammox *Nitrospira* clade A) for 30 s (AOB and AOA)/ 20s (comammox *Nitrospira*

233   clade A), 72 °C for 20 s, and a final extension of 10 min at 72 °C. The standard curves

234   were composed of tenfold serial dilutions of plasmid DNA containing the qPCR gene

235 fragments, and they were developed using the previously described procedure (Hu et

236 al., 2017). All amplification efficiencies ranged between 80% and 100% ($R^2 > 0.99$).

237 Comammox *Nitrospira* clade B was not amplified successfully with the primer set

238 comaB244f_a-e/comaB659r_a-e, so it was not included in the subsequent analysis.

2.4 Cloning library and phylogenetic analysis

240   The AOB and AOA community structure under the different treatments (N

241 fertilisation and nitrapyrin amendment) was analysed at day 28, using the AOB and

242 AOA *amoA* gene amplified with the same primers and thermal cycling condition

243 described above. The PCR reactions were conducted in 50 μl mixtures containing 25 μl

244 2×Premix (TaKaRa Bio Inc., Shiga, Japan), 1μl (10 μM) of each primer, 2 μl of DNA

245 template (1-10 ng) and 21 μl ddH$_2$O. PCR products were purified using the Agarose

246 Gel DNA purification kit (TaKaRa Bio), and were then sequenced on the MiSeq

247 Sequencing Platform (Illumina, San Diego, CA, USA).

248   Illumina sequence data were analysed in USEARCH v10.0.240 software (Edgar,

249 2010). Barcodes and primer sequences were trimmed, and then forward and reverse

250 reads of AOB *amoA* gene sequences were merged to create consensus sequences in

251 USEARCH v10.0.240 software, while only the forward reads of AOA *amoA* sequences

252 were used for subsequent analysis due to the long fragment length (~ 635 bp). Chimeras

253 were removed by performing the *chimera_uchime* algorithm (Edgar et al., 2011). After

254 filtering out chimeras, operational taxonomic units (OTUs) were clustered at 97%

255 similarity, and their corresponding representative sequences were acquired using the

256   UPARSE pipeline (Edgar, 2013). Sequences were then assigned to an OTU table for

257   each dataset and a clustered phylogenetic tree among all OTUs was created by using -

258   *usearch_global* and *-cluster_agg* commands, respectively. The obtained sequences

259   were rarefied to 33,957 reads for AOB and 12,145 reads for AOA per sample. The

260   representative sequences of each AOB *amoA* gene OTU were annotated with the

261   National Center for Biotechnology Information (NCBI) database using BLASTn to

262   obtain the taxonomy information based on the best match of Query coverage, Identity

263   and E-value, and the sequences that match targeted *amoA* genes with the highest

264   similarity were used for the phylogenetic tree construction. Some reference sequences

265   with known taxonomy information were also included for phylogenetic analysis. The

266   Neighbor-joining method with 1000 bootstraps was used for phylogenetic tree

267   construction in MEGA 7 (Kumar et al., 2016). For AOA *amoA* gene, all representative

268   sequences of each OTU were assigned and blasted to the database constructed by Alves

269   et al. (2018). All raw sequencing data have been submitted to the NCBI Sequence Read

270   Archive (SRA) database under the accession numbers PRJNA1038698 (AOB) and

271   PRJNA1039022 (AOA).

272   **2.5 Statistical analysis**

273   Cumulative N_2O emissions, nitrification rates and abundances of AOB, AOA and

274   comammox *Nitrospira* were compared between N and NI treatments across the 16 sites

275   by paired *t*-test in SPSS version 20 (IBM Co., Armonk, NY, USA). The significant

276   difference in the abundances of AOB, AOA and comammox *Nitrospira* clade A among

277   16 sites were tested by One-way analysis of variance (ANOVA) followed by Duncan

278   (equal variance) or Games-Howell (unequal variance) post-hoc tests in SPSS version

279   20 (IBM Co., Armonk, NY, USA). Statistical comparisons were considered significant

280   when P values were < 0.05. Pearson's correlation analysis was used to determine

281   relationships between cumulative N_2O emissions, nitrification rates, soil properties and

282   the abundances of AOB, AOA and comammox *Nitrospira* clade A, and the abundance

283   of AOB *Nitrosospira* cluster 3a.2 (D11) in SPSS version 20 (IBM Co., Armonk, NY,

284   USA). STAMP analysis was used to identify the differential phylogenetic clusters and

285   OTUs between N and NI treatments in STAMP *v* 2.1.3 (Parks et al., 2014). Random

286   forest analysis was used to estimate the key OTUs in predicting cumulative N_2O

287   emissions using the "randomForest" and "rfPermute" packages in R software (V 4.2.0).

288   The abundances of AOB *Nitrosospira* cluster 3a.2 (D11) were calculated by multiplying

289   the relative abundances of *Nitrosospira* cluster 3a.2 (D11) in each soil by its

290   corresponding AOB *amoA* gene abundances. All graphs were created using OriginPro

291   2021 (OriginLab, USA).

292   **3 Results**

293   **3.1 Cumulative N_2O emissions, nitrification rates and the abundances of nitrifiers**

294   **among 16 sites and their response to nitrapyrin amendment**

295   Soil cumulative N_2O emissions showed large variations among the 16 sites under

296   N treatment, from 17.3 to 182.0 μg N kg^{-1}, with a relatively low but consistent

297   cumulative N_2O emissions (17.7 - 52.8 μg N kg^{-1}) in acid and neutral soils (pH < 7.5, 8

298   sites) (Fig. 1a). The cumulative N_2O emissions were positively correlated to soil pH

299   and nitrification rates under N treatment in all tested soils (Table 1). In addition, soil

300   cumulative N_2O emissions were significantly correlated with soil TN and SOC contents

301   in alkaline soils (8 sites), without any significant relationships with soil properties in

302   acid soils (Table 1). In comparison to the N treatment, the NI treatment significantly

303   decreased soil cumulative N_2O emissions in 10 sites with high N_2O emissions, but

304   showed no significant inhibitory effect on the other 6 sites which mainly have low N_2O

305   emissions (< 60 μg N kg^{-1}) (Fig. 1a).

306   Soil nitrification rates were calculated in the initial period (day 0-7), as the changes

307   of NO_3^--N were much faster in the initial period than in the other periods, and can reflect

308   the highest nitrification rate for each soil. Soil nitrification rates ranged from 1.3 to 17.1

309   mg N kg^{-1} in N treatment among 16 sites (Fig. 1b). These rates were significantly

310   correlated to soil pH and TC contents across the 16 sites (Table 1). NI treatment

311   significantly decreased soil nitrification rates in 13 sites, but showed no significant

312   effects in 3 sites with either low nitrification rates (CS and LY sites) or low SOC

313   contents (XC site) (Fig. 1b).

314   The abundances of AOB, AOA and comammox *Nitrospira* clade A *amoA* gene

315   varied by several orders of magnitude among the different sites, within the range of

316   1.04×10^7 - 9.16×10^8, 4.18×10^6 - 8.45×10^8, 1.49×10^6 - 8.08×10^8 copies g^{-1} dry soil for

317   AOB, AOA and comammox *Nitrospira* clade A respectively (Fig. S2). In the majority

318   of sites (14 of 16), comammox *Nitrospira* clade A abundances were significantly lower

319   than AOB and AOA abundances. The three nitrifier group abundances significantly

increased with soil pH in all tested sites, even for AOA (Fig. 1c-e, Fig. S2). The NI

treatment significantly decreased the AOB abundances in 13 sites, but increased AOA

abundances in most of the acid and neutral soils (6 out of 7 sites; Fig. 1c, d). There is

no significant change of comammox *Nitrospira* clade A in the NI treatment compared

to the N treatment, except in a single site (Fig. 1e).

In N treatment, the soil cumulative N_2O emissions significantly increased with

AOB and AOA abundances (Fig. 2a, c). In contrast, the three nitrifier groups were

positively correlated with nitrification rates (Fig. 2b, d, f). AOB showed more robust

relationship coefficients with cumulative N_2O emissions and nitrification rates than the

other nitrifier groups (Fig. 2).

3.2 AOB and AOA community structures across different sites and their response
to nitrapyrin amendment

As soil cumulative N_2O emissions were positively related with AOB and AOA

abundances but not with comammox *Nitrospira* clade A abundances, the AOB and AOA

community structures were further characterised under N and NI treatments at the end

of the incubation by high-throughput sequencing of *amoA* genes. All AOB sequences

were grouped into *Nitrosospira* (50 OTUs at 97% similarity), and further classified into

cluster 3a.2 (D11) (44.0%), cluster 3b (D1-D7) (20.0%), cluster 10/11 (D9-D10)

(12.0%), Nsp65 (D12) (12.0%), cluster 2/4 (D13-D17) (6.0%) and cluster 3a.1 (D8)

(6.0%), as classified by Phillips et al. (2000) and Aigle et al. (2019) (Fig. S3).

Nitrosospira cluster 3a.2 (D11) was found in all soils, with high relative abundances in

most sites (17.5% - 95.3%) except for a strongly acid soil (CS site, 0.8%; Fig. 3a). *Nitrosospira* cluster 3a.1 (D8) and cluster 3b (D1-D6) were more abundant in the alkaline soils, with higher relative abundances (21.7%-24.5%) in alkaline soils than in acid soils (1.0%-1.2%; Fig. 3a). In contrast, *Nitrosospira* cluster 10/11 (D9-D10) and Nsp65 (D12) were primarily detected in acid soils (2.3%-98.1%) (Fig. 3a).

All AOA sequences (241 OTUs at 97% similarity) were grouped into clade *Nitrososphaerales* (NS, 89.2%), Ca. *Nitrosotaleales* (NT, 2.9%), *Nitrosopumilales* (NP, 1.6%) and unclassified (6.3%). Clade NS was present in all sites and further classified into NS-γ (29.1%), NS-α (NS-α-3.2.1; *Nitrososphaera viennensis* EN76, 29.1%), NS-δ (Fosmid clone 54d9, 15.7%), NS-β (8.2%) and NS-ζ (6.7%), as described by Alves et al. (2018). Clade NT was mainly composed of NT-α clade (to which Ca. *Nitrosostalea devanaterra* Nd1 and Ca. *Nitrosotalea* sp. Nd2 belong) and was only detected in acid soils with relative abundances varying between 0.02% and 31.6% (Fig. 3b).

As NI treatment significantly decreased AOB abundances and increased AOA abundances, the depleted AOB taxa and enriched AOA taxa under NI treatment were further characterized. Compared to the N treatment, the NI treatment significantly decreased the relative abundances of AOB *Nitrosospira* cluster 3a.2 (D11) in 9 of the 16 sites in which high N_2O emissions were recorded under N fertilisation (Fig. 1 and Fig. 3). Furthermore, NI treatment also reduced the relative abundances of AOB *Nitrosospira* Nsp65 (D12) in one site (QJ), and *Nitrosospira* cluster 3a.1 (D8) in another site (YY) (Fig. 3 and Table S2). In contrast to that, NI treatment significantly enriched AOA NS-α in 6 sites (mainly acid and neutral soils), AOA NS-δ in two sites (YS and

363   MY) and AOA NT-α in one site (QJ) (Fig. 3 and Table S2).

364   **3.3 keystone taxa associated with N$_2$O emissions in different sites**

365   As NI amendment significantly reduced AOB *amoA* gene abundances and

366   *Nitrosospira* cluster 3a.2 (D11) abundances (Fig. 1c and Fig. 3a), correlation analyses

367   were further performed to estimate the correlations between the cumulative N$_2$O

368   emissions, nitrification rates and the AOB *Nitrosospira* cluster 3a.2 (D11) abundances

369   (Fig. 4). AOB *Nitrosospira* cluster 3a.2 (D11) abundances were positively correlated

370   with soil cumulative N$_2$O emissions and with nitrification rates (16 sites) (Fig. 4a, b).

371   Random forest analysis was then used to estimate the importance of OTUs

372   belonging to AOB *Nitrosospira* cluster 3a.2 (D11) in predicting cumulative N$_2$O

373   emissions under N treatment (Fig. 5a). It showed that OTU34, OTU23, OTU2, OTU15,

374   OTU17, OTU24, OTU33, OTU36, OTU10, OTU31 and OTU5 were important for

375   predicting soil cumulative N$_2$O emissions (16 sites), with OTU34 playing the most

376   important role among those OTUs (Fig. 5a). STAMP analysis revealed that the

377   abundances of OTU34 were significantly lower in NI treatment than in N treatment

378   soils, especially in alkaline soils (7 of 8 sites) (Fig. 5b and 5c). The phylogenetic

379   analysis revealed that OTU34 was closely related to *Nitrosospira* sp. TCH716 and

380   *Nitrosospira multiformis* (Fig. 5d). In addition, the OTU34 abundances were positively

381   correlated with the cumulative N$_2$O emissions, the nitrification rates, and the soil pH,

382   but negatively correlated with soil SOC contents (Fig. 6).

4 Discussion

4.1 Soil organic carbon determines higher N₂O emissions driven by strong nitrification in alkaline soils

Based on a series of microcosm experiments, our study examined the response of three nitrifier groups to N fertilisation and nitrapyrin amendment and explored their association with N₂O emissions across 16 cropland soils with distinct physiochemical properties. We demonstrated that soil cumulative N₂O emissions showed significantly positive correlations with soil pH and nitrification rates under N treatment in the studied soils (Table 1, Fig. 2a and c), highlighting the importance of soil pH and nitrification as driving factors of soil N₂O emissions in agricultural soils under aerobic conditions (Hu et al., 2017; Zhu et al., 2019; Cui et al., 2021). NH₃, rather than NH₄⁺, is the direct substrate for nitrifier groups in nitrification, while NH₃ availability is highly pH-dependent and exponentially declines with decreasing pH, thus resulting in a higher nitrification activity with soil pH increase (Hu et al., 2014; Jung et al., 2022). Meanwhile, nitrification-related pathways are considered the primary sources of soil N₂O emission under aerobic conditions in fertilised agricultural soils (Liu et al., 2016; Hink et al., 2017; Zhu et al., 2019), which consequently resulted in the positive relationships among soil pH, nitrification rates and soil N₂O emissions.

Nonetheless, our results showed that not all alkaline soils had high N₂O emissions and that soil cumulative N₂O emissions in alkaline soils were negatively correlated with soil SOC contents (Fig. 1a and Table 1). Consistently, high N₂O emission via

404 nitrification-related pathways was also observed in the calcareous Fluvo-aquic soil with

405 high pH and low SOC content (Huang et al., 2014; Ju and Zhang, 2017). It was

406 previously supposed that soils with high SOC and TN contents have high microbial N

407 immobilisations and heterotrophic nitrification rates (Elrys et al., 2021a; Elrys et al.,

408 2021b; Chen et al., 2022; Tang et al., 2024). When NH_4^+ is applied to soils with high

409 SOC and TN, microbial N immobilisation could compete for NH_4^+ substrates with

410 autotrophic nitrification (Zhu et al., 2019). This competition would promote the

411 heterotrophic nitrification process, which produce less N_2O than nitrifier denitrification

412 (Prosser et al., 2020; Elrys et al., 2021b), resulting in lower nitrification-related N_2O

413 emission. Therefore, our findings highlight the importance of SOC in regulating

414 nitrification-related N_2O emissions. It also strengthens the need to develop management

415 strategies to promote organic C and N retention in agricultural soils to mitigate N_2O

416 emissions.

417 **4.2 NIs reduces N_2O emissions by impairing AOB abundances**

418 Our study further showed that nitrapyrin amendment significantly reduces N_2O

419 emissions and nitrification rates in the majority of tested soils, particularly in the

420 alkaline soils with high N_2O emissions (Fig. 1a), supporting the view that NIs can be

421 applied to decrease the nitrification and nitrification-related N_2O emissions in

422 agricultural soils (Soares et al., 2016; Cassman et al., 2019; Cowan et al., 2020; Lyu et

423 al., 2021). NIs were reported to reduce the nitrification and nitrification-related N_2O

424 emission through inhibiting the growth and activity of AOB in alkaline soils (Shi et al.,

425   2017). Consistently, we found that nitrapyrin amendment significantly reduced AOB

426   abundances in the majority of tested soils and increased AOA abundances in most acid

427   soils, with no significant influence on comammox *Nitrospira* clade A (Fig. 1 and Fig.

428   3). These results support previous findings that NIs can effectively inhibit AOB

429   abundances and nitrification activity (Cui et al., 2013; Xi et al., 2017; Duan et al., 2019;

430   Hayden et al., 2021), and suggest that AOB are the main active nitrifier responsible for

431   nitrification-related N_2O emissions in agricultural soils (Lourenço et al., 2018; Prosser

432   et al., 2020). Accordingly, previous studies showed that AOB isolates generated higher

433   N_2O emission via nitrification-related pathways than AOA and comammox *Nitrospira*

434   (Hu et al., 2015; Hink et al., 2017; Prosser et al., 2020), which could be attributed to

435   the nitrifier denitrification pathway present in AOB. In contrast to AOA and comammox

436   *Nitrospira*, AOB generally possess some copper-containing nitrite reductase (*nirK*) and

437   nitric oxide reductase (*norCBOD*) but no dissimilatory nitrate and nitrous oxide

438   reductases (*nosZ*), thus contributing to high N_2O emission in agricultural soils via

439   nitrifier denitrification (Norton, 2008; Hu et al., 2015). Nitrifier denitrification could be

440   a detoxifying process for AOB to counteract the toxic effects of NO_2^- accumulation

441   during nitrification (Beaumont et al., 2002; Beaumont et al., 2004; Hu et al., 2015;

442   Prosser et al., 2020). It was reported that remarkable NO_2^- accumulation caused by rapid

443   O_2 consumption coincided with strong emission peaks of N_2O during strong ammonia

444   oxidation, which was mainly attributed to nitrifier denitrification as evidenced by ^{15}N

445   tracing (Huang et al., 2014; Yang et al., 2021; Li et al., 2023). Together with our current

446   results, this suggests that the nitrifier denitrification driven by AOB might be the

447 dominant pathway of N_2O production rather than the direct emission in hydroxylamine

448 oxidation in alkaline agricultural soils with strong nitrification. AOA and comammox

449 *Nitrospira* have distinct niche differentiation with AOB in terms of NH_4^+ availability,

450 soil pH, etc., with high N and pH favoring AOB (Hu et al., 2013; Hink et al., 2018;

451 Aigle et al., 2020; Prosser et al., 2020), which explained well the AOB-dominated

452 nitrification and N_2O emission in fertilised alkaline soils.

453 We further showed that the abundance of AOA significantly increased when AOB

454 was inhibited in the majority of tested soils (Fig. 1 and Fig. 3), further confirming the

455 trade-off of activities of between AOA and AOB (Zhao et al., 2020). However,

456 nitrapyrin amendment also significantly reduced AOA abundances and N_2O emissions

457 in an acid soil from LY site in our study (Fig. 1d). Considering that AOA generally

458 dominated nitrification over AOB in acid soils and their sensitive response to NIs

459 amendment have been frequently recorded in soils where AOA or both AOA and AOB

460 are active (Zhang et al., 2012; Faeflen et al., 2016; Gu et al., 2018). These results

461 suggested that the inhibitory effect of NIs such as nitrapyrin is not specific for AOB,

462 AOA or comammox *Nitrospira*, but rather more dependent on the dominant active

463 nitrifier groups.

464 **4.3 AOB *Nitrosospira* cluster 3a.2 (D11) dominated nitrification-derived N_2O**

465 **production in alkaline soils**

466 Our study demonstrated that nitrapyrin significantly reduced the relative

467 abundances of AOB *Nitrosospira* cluster 3a.2 (D11) in most alkaline soils and in some

468　acid soils (Fig. 3). This phylogenetic cluster was previously found to be mostly present

469　in alkaline soils (Aigle et al., 2019). The abundance of this phylogenetic cluster was

470　also strongly correlated with soil cumulative N_2O emissions and nitrification rates (Fig.

471　4). Consistently, AOB *Nitrosospira* cluser 3a.2 (D11) is likely the primary N_2O-

472　producing phylogenetic cluster in an alkaline soils in a recent study (Bai et al., 2023).

473　We revealed that not all OTUs affiliating within this cluster 3a.2 (D11) are associated

474　with high N_2O production, but a restricted number of OTUs, are important to predict

475　soil cumulative N_2O emissions, with the greatest effect from OTU34 (Fig. 5a). This

476　OTU was widely detected in all tested alkaline soils and was also detected in two acid

477　soils, and its abundance decreased with nitrapyrin amendment (Fig. 5b and 5c),

478　indicating its important role in N_2O emissions in the tested soils. Further, the OTU34

479　showed high *amoA* gene identity with several active AOB nitrifiers strains (e.g. strains

480　*Nitrosopira* sp. TCH716, RY3C, PJA1 and *Nitrosospira multiformis*, Fig. 5d and Fig.

481　S5), which isolated from diverse soil conditions (Takahashi et al., 2001; Satoh et al.,

482　2003) and with an uncultured OTU previously identified as a key nitrifier in alkaline

483　soil (Xia et al., 2011). In addition, the abundance of several of these strains (e.g.

484　*Nitrosopira* sp. PJA1 and RY3C) were reported to be significantly positive correlated

485　with soil N_2O fluxes in tropical soils (Lourenço et al., 2018). *Nitrosospira multiformis*

486　was also demonstrated to have higher capability of N_2O production via ammonia

487　oxidation and nitrifier denitrification than other *Nitrosospira* isolates in laboratory

488　incubations (Shaw et al., 2006), and is highly susceptible to nitrapyrin (Matsuba et al.,

489　2002). Therefore, the OTU34-like AOB cells are widely present and active in various

490   soils and play a key role in N_2O emissions in agricultural soils. This suggested that the

491   OTU34-like AOB cells could be used as a molecular biomarker of predicting soil N_2O

492   emissions. However, the abundances of OTU34 were not significant different between

493   N and NI treatment (Fig. 5c), though nitrapyrin significantly reduced the cumulative

494   N_2O emission, nitrification rate and the relative abundance of *Nitrosospira* cluster 3a.2

495   (D11) in the YY site. This suggests there might be other OTUs/species within

496   *Nitrosospira* cluster 3a.2 (D11) responsible for N_2O production in some soils.

497   The abundances of OTU34 significantly increased with soil pH but decreased with

498   soil SOC contents (Fig. 6), coinciding with the trend of nitrification-derived N_2O

499   emissions. Indeed, high soil pH and low SOC content seem favorable for nitrification-

500   derived N_2O emission in fertilised upland soils, which contrasts to the higher N_2O

501   emission in soils with low pH and high SOC via conventional denitrification under high

502   soil moisture conditions (Wang et al., 2017; Yin et al., 2023). These results highlighted

503   the important roles of soil pH and SOC in regulating nitrification and denitrification

504   related N_2O emission through different mechanisms in agricultural soils. However, the

505   genetic mechanism of nitrifier denitrification driving by AOB *Nitrosospira* cluster 3a.2

506   (D11), particularly in OTU34-like cells including *Nitrosospira* sp. TCH716,

507   *Nitrosospira* sp. RY3C and *Nitrosospira* sp. PJA1, are still unknown, and require future

508   investigation.

509   **5 Conclusion**

510   Our study demonstrated that soil cumulative N_2O emissions increase with soil pH

and nitrification rates in N fertilised agricultural soils, while nitrapyrin amendment can significantly reduce N_2O emissions, accompanying with the inhibition of nitrification rates and AOB activity. High N_2O emissions in alkaline fertilised cropland soils are mainly derived from AOB dominated nitrification and nitrifier denitrification. Those nitrification-related N_2O emissions in alkaline soils are negatively correlated with soil SOC contents, highlighting the importance of SOC in regulating nitrification-related N_2O emission. Our study further revealed that the *Nitrosospira multiformis*-like OTU (OTU34), belonging to AOB *Nitrosospira* cluster 3a.2 (D11), is the main AOB taxa responsible of high N_2O emission in the tested soils. The widespread distribution and N_2O-emission role of these OTU34-like AOB cells place them as good candidate for biomarker for predicting nitrification-derived N_2O emissions in fertilised agricultural soils. Together, our study provided important knowledge for developing effective soil management and N fertilisation strategy for crop N use efficiency enhancement and N_2O emission mitigation.

Credit author statement

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Declaration of competing interest

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.

Data availability

Data will be made available on request.

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Applendix A. Supplementary data

Supplementary data to this article can be found online at

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836

Figure Captions

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839 **Fig. 1** Soil cumulative N_2O emissions (a) and nitrification rates (b), the abundances of

840 AOB (c), AOA (d) and comammox *Nitrospira* clade A (e) across 16 sites with pH

841 gradient under N addition (N treatment) and N plus nitrapyrin addition (NI treatment).

842 The numbers labeled at the top of the graph are soil pH values. The asterisks indicate

843 significant difference ($p < 0.05$) between N and NI treatments.

844

845 **Fig. 2** Correlations between the abundances of AOB (a, b), AOA (c, d), comammox

846 *Nitrospira* clade A (e, f) and cumulative N_2O emissions, nitrification rates in N

847 treatment among 16 sites. Pearson's correlation coefficients (r) and the associated p

848 values are shown.

849

850 **Fig. 3** Community compositions of AOB (a) and AOA (b) in N and NI treatments across

851 16 sites with pH gradient. The pH values are labeled at the top of the graph. The black

852 arrow indicated a significant decrease in AOB clusters between the N and NI treatments,

853 while the red arrow showed a significant increase in AOA clusters between the N and

854 NI treatments.

855

856 **Fig. 4** Correlations between AOB *Nitrosospira* cluster 3a.2 (D11) abundances and

857 cumulative N_2O emissions (a), nitrification rates (b) in N treatment among 16 sites.

858 Pearson's correlation coefficients (r) and the associated p values are shown.
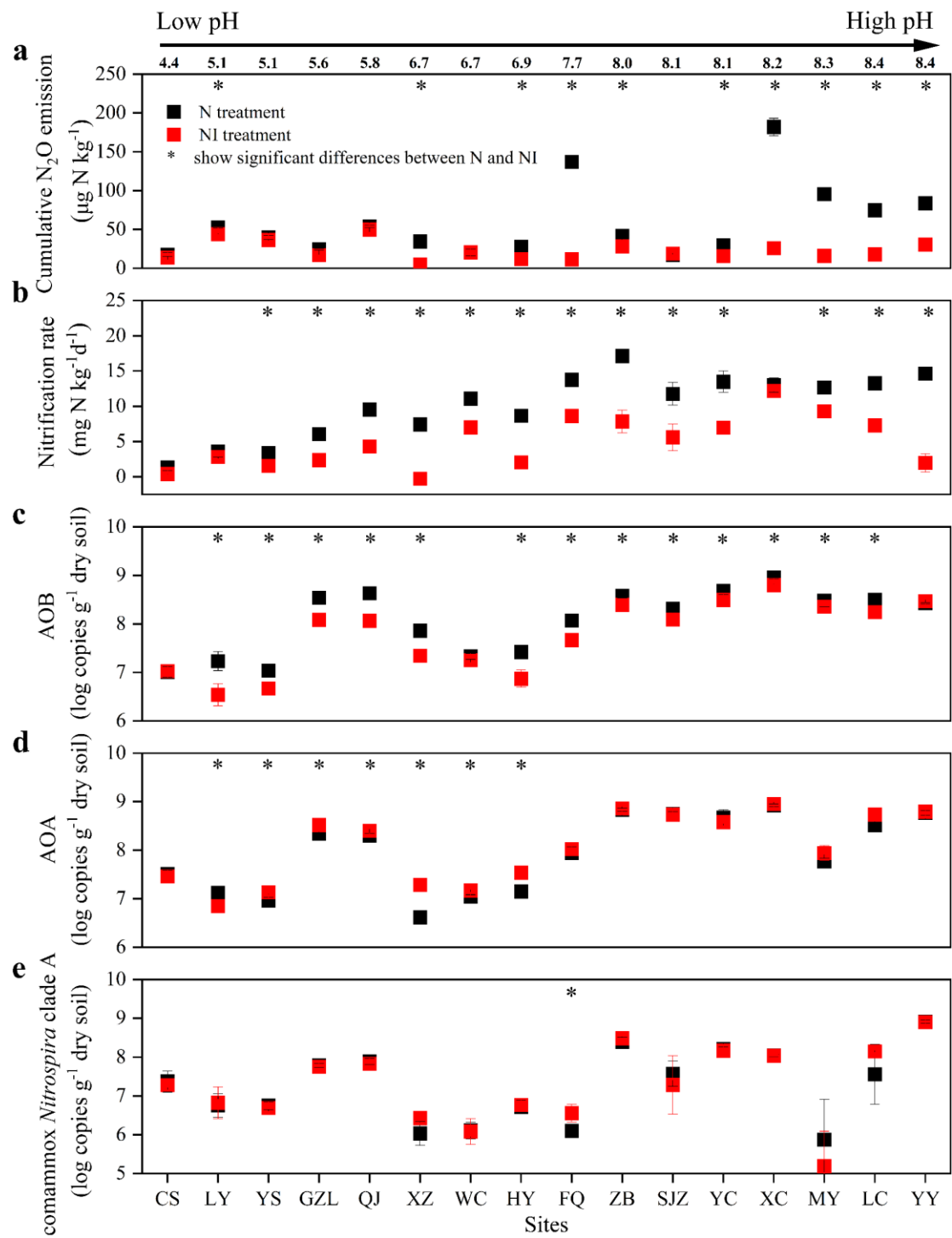
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Fig. 5 Identifying key AOB *Nitrosospira* cluster 3a.2 (D11) OTUs related to cumulative N$_2$O emissions across 16 sites. Predicting importance of OTUs (belong to AOB *Nitrosospira* cluster 3a.2 (D11)) on cumulative N$_2$O emissions by Random Forest analysis (%increase in MSE, %IncMSE; a), and **, $p < 0.01$; *, $p < 0.05$. The differences of abundant AOB *Nitrosospira* cluster 3a.2 (D11) OTUs between N and NI treatments by STAMP analysis (b). The OTU34 abundances between N and NI treatments (c), and the asterisks indicate significant difference ($p < 0.05$) between N and NI treatments. Neighbor-joining phylogenetic tree of the key OTU of AOB *Nitrosospira* cluster 3a.2 (D11) *amoA* gene detected in our study and representative sequences of major clades (d).

870

Fig. 6 Correlations between OTU34 abundances and cumulative N$_2$O emissions (a), nitrification rates (b), pH (c), SOC (d), TN (e) among 16 sites in N treatment. Pearson's correlation coefficients (r) and the associated p values are shown.

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877   Fig.1

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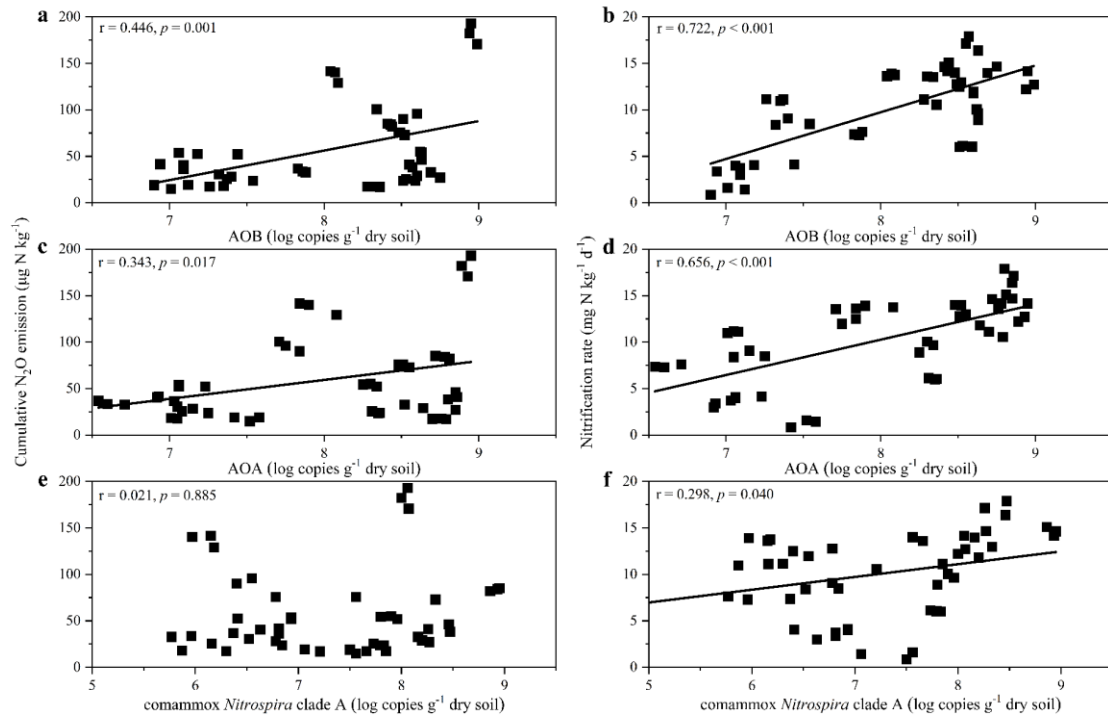
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880   Fig. 2

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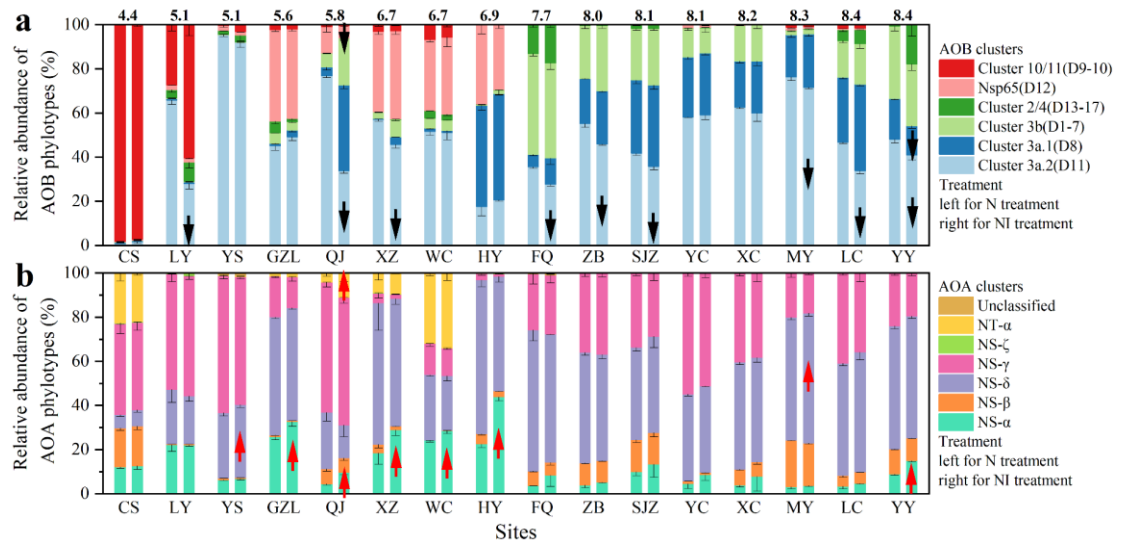
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883   Fig. 3

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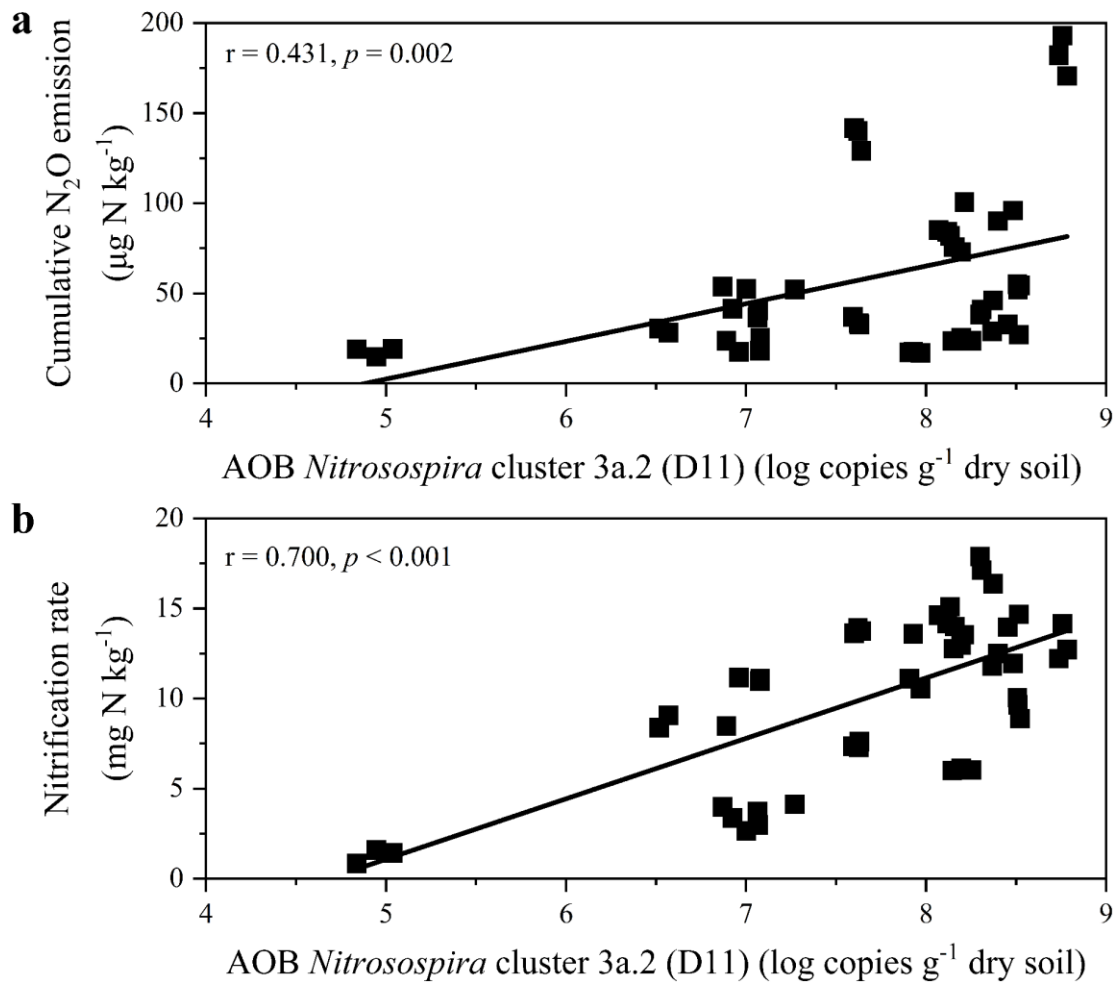
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a

Cumulative N_2O emission ($\mu g\ N\ kg^{-1}$)

r = 0.431, p = 0.002

AOB *Nitrosospira* cluster 3a.2 (D11) (log copies g^{-1} dry soil)

b

Nitrification rate ($mg\ N\ kg^{-1}$)

r = 0.700, p < 0.001

AOB *Nitrosospira* cluster 3a.2 (D11) (log copies g^{-1} dry soil)

886

887   Fig. 4

888

889

Fig. 5

891

a — Cumulative N$_2$O emission (μg N kg^{-1})
r = 0.552, *p* < 0.001

b — Nitrification rate (mg N kg^{-1} d^{-1})
r = 0.750, *p* < 0.001

c — pH
r = 0.755, *p* < 0.001

d — SOC (g kg^{-1})
r = 0.344, *p* = 0.022

e — TN (g kg^{-1})
r = -0.279, *p* = 0.066

OTU34 abundance (log copies g^{-1} dry soil)

892

893   Fig. 6

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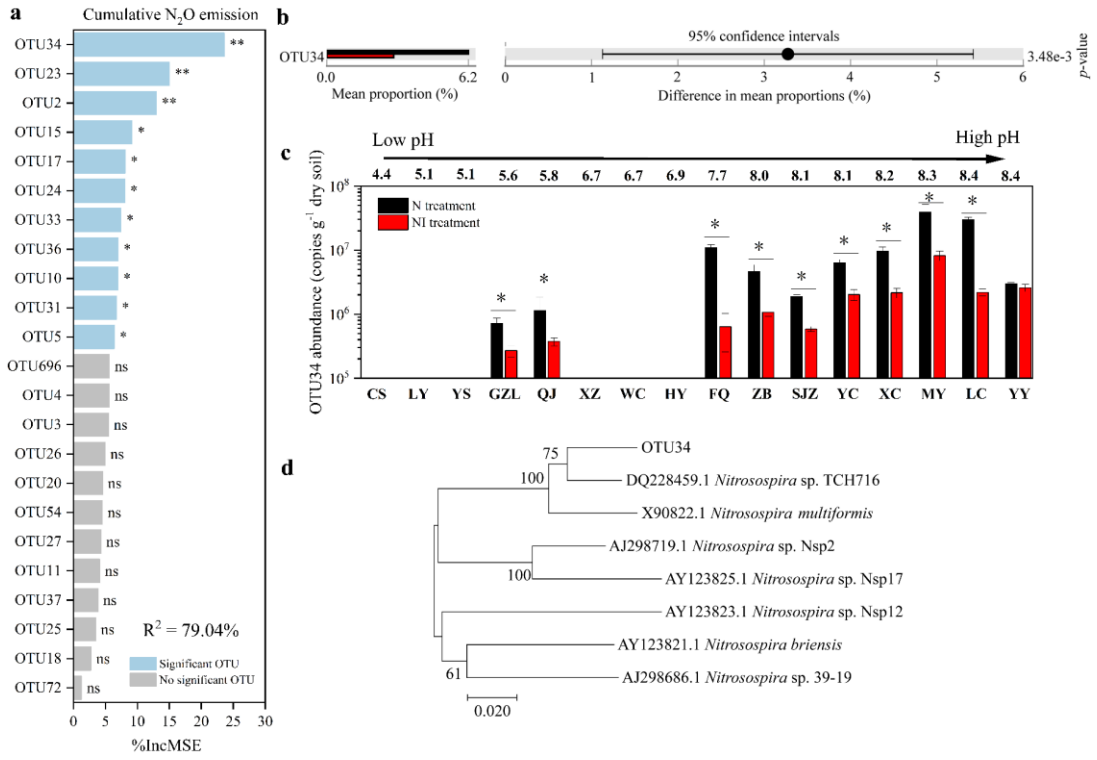
Table Captions

Table 1 Pearson's correlations between cumulative N_2O emissions, nitrification rates and soil properties in N treatment.

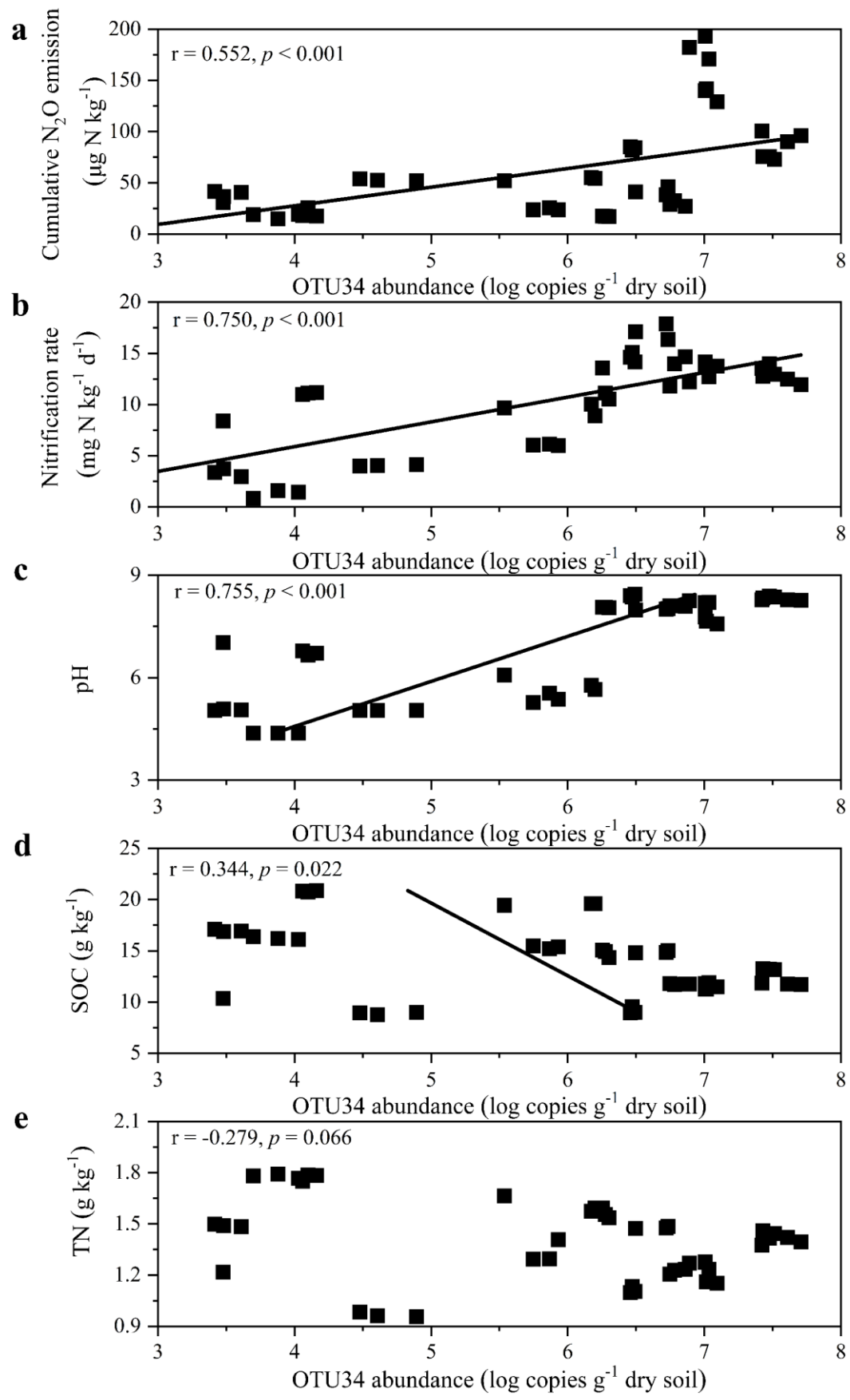| Parameter	Types	Nitrification rates	pH	TC	TN	SOC
Cumulative N_2O emissions	All soils (16 sites)	0.424**	0.458**	0.165	-0.195	-0.267
	Acid and neutral soils (pH < 7.5, 8 sites)	0.028	-0.108	-0.162	-0.310	-0.162
	Alkaline soils (pH >7.5, 8 sites)	-0.094	-0.046	-0.129	-0.475*	-0.478*
Nitrification rates	All soils (16 sites)		0.918**	0.380**	-0.013	-0.089
	Acid and neutral soils (pH < 7.5, 8 sites)		0.865**	0.166	-0.007	0.166
	Alkaline soils (pH > 7.5, 8 sites)		-0.124	-0.383	-0.076	0.085

Note: Cumulative N_2O emissions, Nitrification rates, TC, TN and SOC represents: cumulative N_2O emissions (μg N kg^{-1}), nitrification rates (mg N $kg^{-1}d^{-1}$), total carbon (g kg^{-1}), total nitrogen (g kg^{-1}), soil organic carbon (g kg^{-1}), respectively. Pearson's correlation coefficients (r) are shown. **, $p < 0.01$; *, $p < 0.05$.

905 **Supplementary materials**

906

907 **Fig. S1** Location of sampling sites.

908

909 **Fig. S2** The abundances of AOB, AOA and comammox *Nitrospira* clade A across 16 sites at the end

910 of microcosm incubation (28 days). Different lowercase letters indicate significant difference among

911 AOB, AOA and comammox *Nitrospira* clade A in each site at $P < 0.05$.

912

913 **Fig. S3** Correlations between the abundances of AOB (a), AOA (b), comammox *Nitrospira* clade A

914 (c) and soil pH under N treatments among 16 sites. Pearson's correlation coefficients (r) and the

915 associated *p* values are shown.

916

917 **Fig. S4** Neighbor-joining phylogenetic tree of AOB *amoA* gene showing representatives of OTUs

918 detected in our study and representative sequences of major clades. Sequences were retrieved form 16

919 sites under N and NI treatments.

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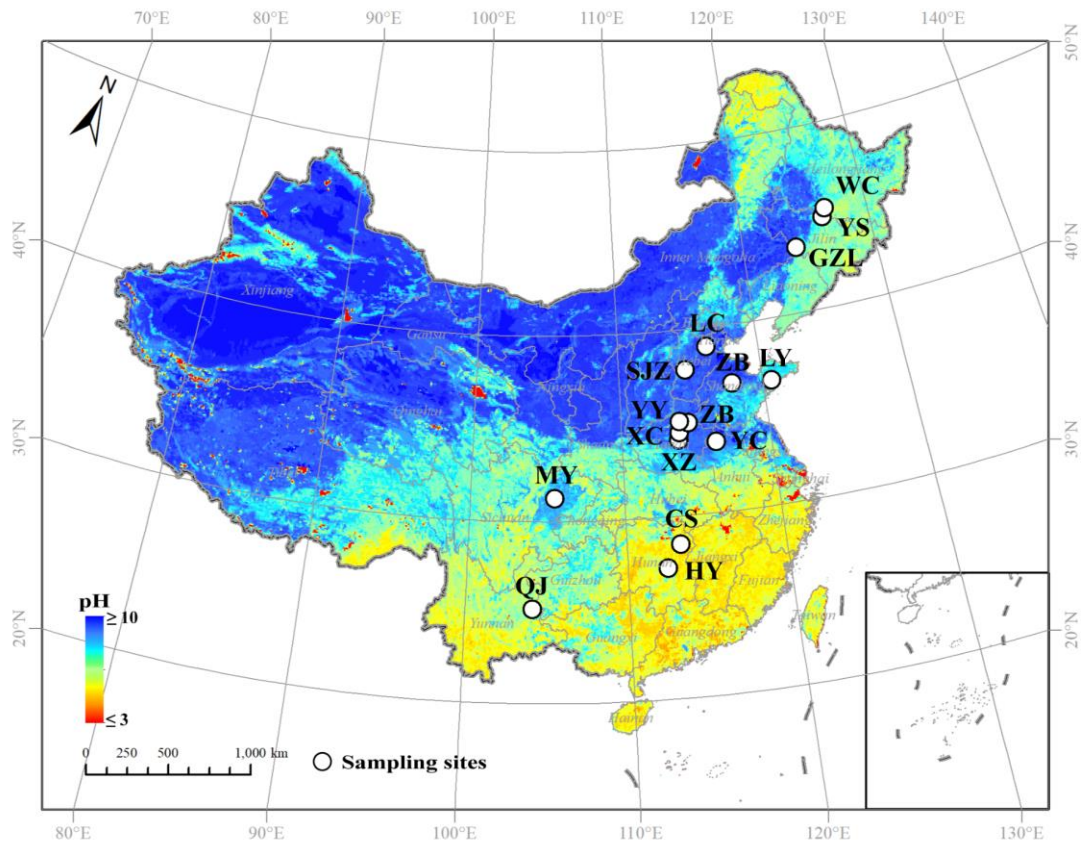
921 **Fig. S5** Neighbor-joining phylogenetic tree of AOB *amoA* gene showing OTU34 detected in our study

922 and representative sequences of major clades. Sequences were retrieved form 16 sites under N and NI
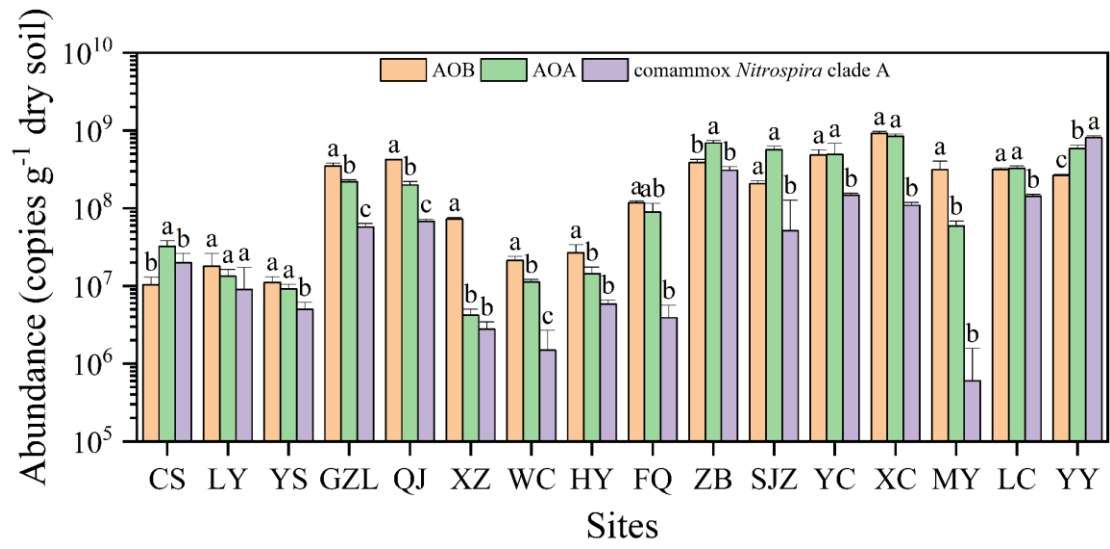
923 treatments.
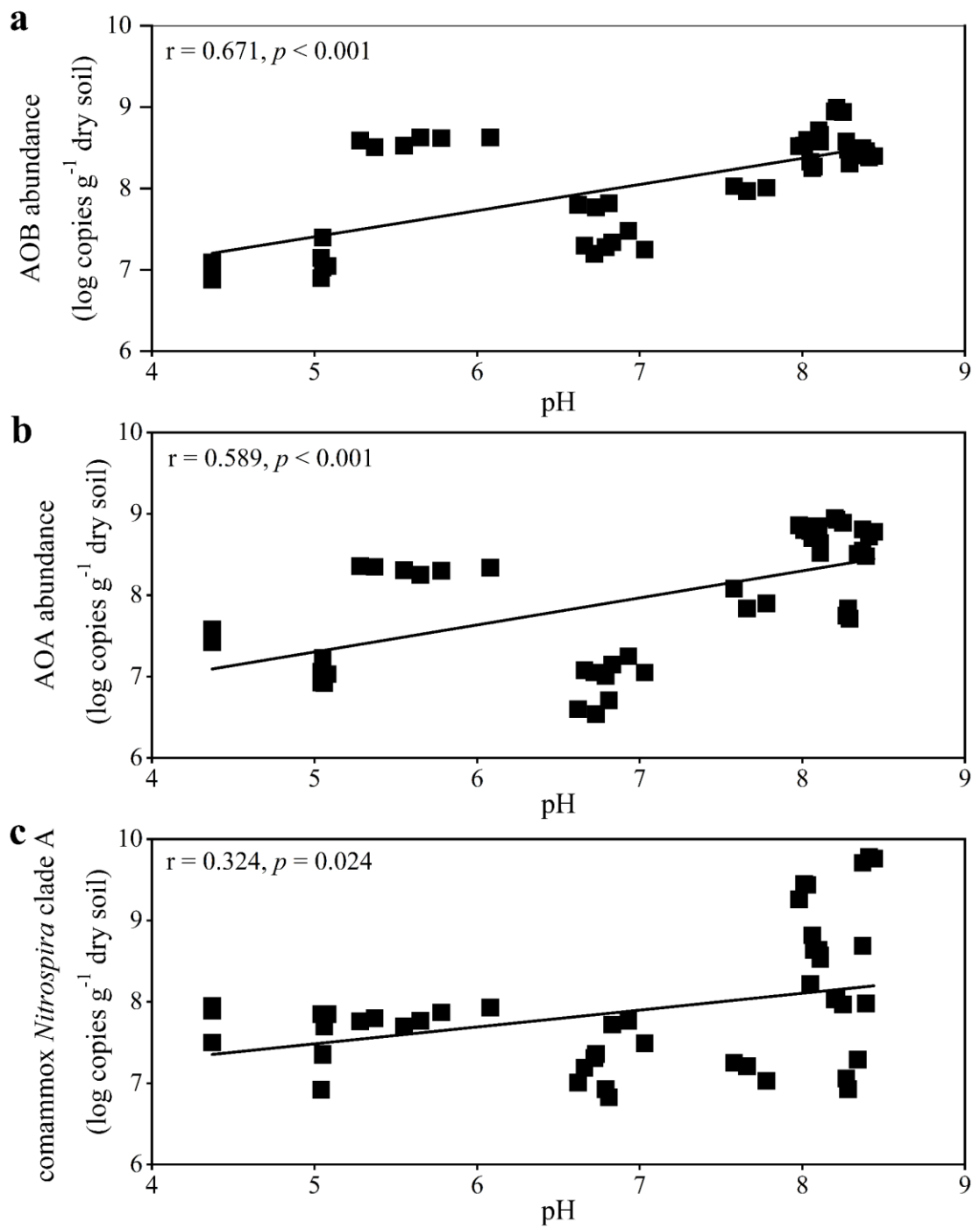
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Fig. S1
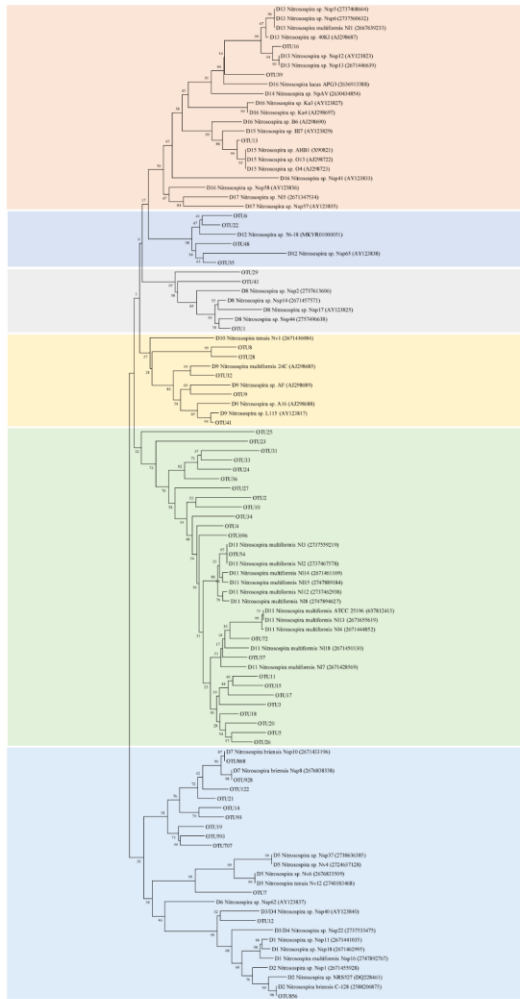
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Fig. S2

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Fig. S3

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Nitrosospira Cluster 2/4 (D13-17)

Nitrosospira Nsp65 (D12)

Nitrosospira Cluster 3a.1 (D8)
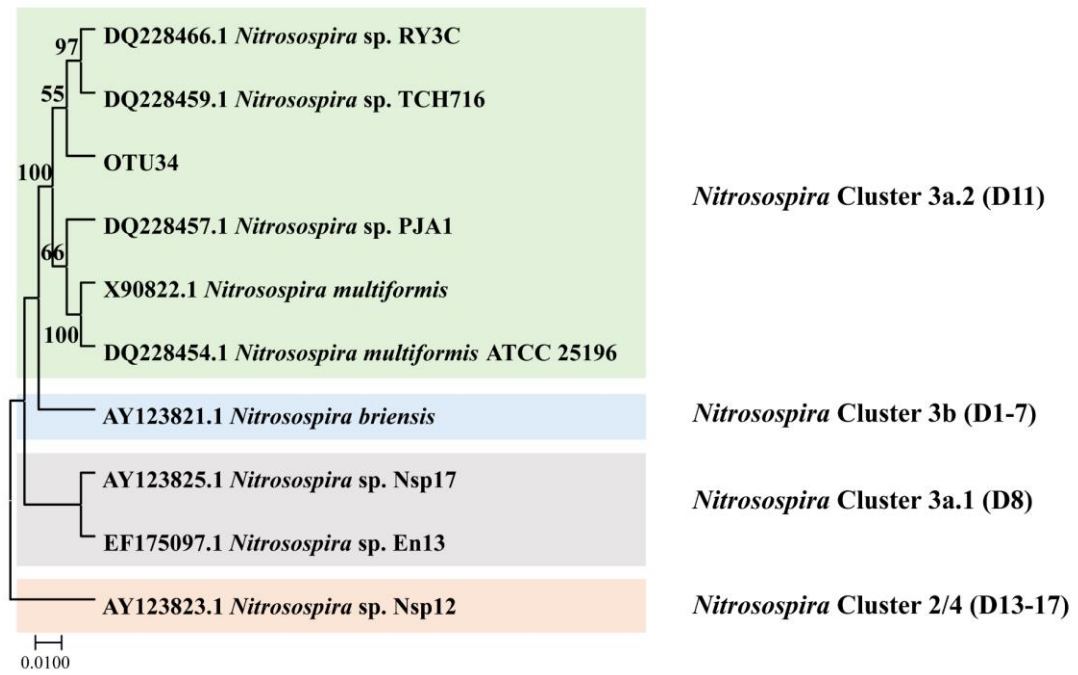
Nitrosospira Cluster 10/11(D9-10)

Nitrosospira Cluster 3a.2 (D11)

Nitrosospira Cluster 3b (D1-7)

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Fig. S4

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939   0.0100

940   **Fig. S5**

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Table S1 Sample sites description

Sample code	Site	Soil type	pH	Crop	TC (g kg^{-1})	TN (g kg^{-1})	SOC (g kg^{-1})
CS	Changsha, Hunan	Red soil	4.37	Summer maize-Winter rape	16.23±0.13[a]	1.78±0.01	14.83±0.48
LY	Laiyang, Shandong	Brown Soil	5.05	Summer maize-Winter wheat	8.92±0.12	0.97±0.01	7.94±0.42
YS	Yushu, Jilin	Black Soil	5.06	Summer maize only	16.96±0.11	1.49±0.01	16.61±0.05
GZL	Siping, Jilin	Black Soil	5.40	Summer maize only	15.37±0.15	1.33±0.07	14.34±0.27
QJ	Qujing, Yunnan	Red Soil	5.84	Summer maize-Winter barley	19.56±0.10	1.61±0.04	14.14±0.00
XZ	Xinzheng, Henan	Fluvo-aquic Soil	6.72	Summer maize-Winter wheat	4.28±0.10	0.51±0.03	5.34±1.06
WC	Wuchang, Heilongjiang	Meadow Soil	6.72	Summer maize only	20.83±0.06	1.78±0.02	19.28±0.49
HY	Hengyang, Hunan	Purple Soil	6.93	Summer maize-Winter rape	10.39±0.20	1.22±0.01	9.25±0.15
FQ	Fengqiu, Henan	Fluvo-aquic Soil	7.67	Summer maize-Winter wheat	18.06±0.15	1.20±0.07	8.89±0.69
ZB	Zibo, Shandong	Cinnamon Soil	8.01	Summer maize-Winter wheat	17.27±0.12	1.48±0.01	14.67±0.53
SJZ	Shijiangzhuang, Hebei	Fluvo-aquic Soil	8.06	Summer maize-Winter wheat	24.07±0.39	1.56±0.03	14.69±0.76
YC	Yongcheng, Henan	Mortar Black Soil	8.11	Summer maize-Winter wheat	13.07±0.04	1.22±0.01	11.12±0.10
XC	Xuchang, Henan	Fluvo-aquic Soil	8.22	Summer maize-Winter wheat	16.89±0.06	1.26±0.02	10.80±0.27
MY	Mianyang, Sichuan	Purple Soil	8.28	Summer maize-Winter wheat	21.66±0.07	1.40±0.02	11.07±0.32
LC	Luancheng, Hebei	Cinnamon Soil	8.37	Summer maize-Winter wheat	17.95±0.05	1.44±0.02	12.89±0.09
YY	Yuanyang, Henan	Fluvo-aquic Soil	8.41	Summer maize-Winter wheat	17.79±0.34	1.11±0.02	9.24±1.93

943   a. mean values ± standard error (n=3).

Table S2 The relative abundance of AOB and AOA phylogenetic clusters among 16 sites at the end of microsocm incubation (28 days)

Sample code	Treatment[a]	AOB (%)						AOA (%)				
		Cluster 3a.2 (D11)	Cluster 3a.1 (D8)	Cluster 3b (D1-7)	Cluster 2/4 (D13-17)	Nsp65 (D12)	Cluster 10/11 (D9-10)	NS-α	NS-β	NS-δ	NS-γ	NT-α
CS	N	0.9±0.0a[b]	~[c]	~	~	~	98.2±0.1a	12.2±0.7a	17.6±1.1a	6.0±0.7a	~	22.6±3.6a
	NI	1.7±0.8a	~	~	~	~	97.2±0.8a	12.6±1.6a	18.1±1.6a	7.4±1.1a	~	22.0±0.9a
LY	N	**65.9±2.1a**	~	~	**3.6±1.1b**	2.3±0.3a	**27.2±2.8b**	22.2±3.0a	~	24.6±5.9a	0.4±0.5a	~
	NI	**28.1±2.5b**	~	~	**8.8±2.7a**	1.8±0.3a	**60.4±5.1a**	22.1±0.6a	~	21.8±2.5a	1.1±1.3a	~
YS	N	95.3±0.7a	~	~	1.7±0.2a	2.2±0.8a	**0.3±0.0b**	6.4±0.3a	~	**29.5±1.5b**	**62.2±1.5a**	~
	NI	91.9±2.3a	~	~	3.0±2.6a	1.7±0.3a	**3.1±0.2a**	6.8±0.6a	~	**33.0±1.4a**	**57.9±1.1b**	~
GZL	N	45.1±2.0a	**1.2±0.4b**	**4.8±0.1a**	**5.3±1.3a**	41.4±1.0a	2.2±0.3a	**25.9±1.4b**	~	**53.6±0.8a**	**18.3±0.6a**	1.6±0.2a
	NI	49.3±1.8a	**2.9±0.8a**	**3.9±0.6b**	**1.4±0.7b**	40.6±1.0a	1.9±0.1a	**32.7±2.1a**	~	**51.0±0.7b**	**14.1±2.0b**	1.7±0.1a
QJ	N	**76.8±0.8a**	4.0±1.4b	6.3±0.3b	~	**11.8±2.4a**	~	**4.4±0.5b**	6.8±1.1a	**25.6±4.0a**	59.1±2.2a	**4.1±1.4b**
	NI	**33.9±1.1b**	38.7±1.7a	21.5±1.2a	~	**5.3±0.3b**	~	**9.7±1.0a**	6.5±1.2a	**14.9±5.2b**	57.9±2.6a	**11.0±3.8a**
XZ	N	**57.2±0.9a**	0.3±0.0b	2.9±0.2b	~	36.6±1.3b	3.0±0.7a	18.6±5.3b	3.7±1.5a	64.2±12.5a	4.4±2.3a	8.9±3.2a
	NI	**45.8±1.6b**	3.5±0.1a	7.8±0.5a	~	39.9±1.5a	2.8±0.4a	28.9±2.5a	1.9±0.4a	57.6±2.5a	2.1±0.1a	9.3±0.2a
WC	N	51.6±1.7a	1.5±0.4a	4.6±0.3a	3.4±0.2a	32.1±1.2a	6.8±0.1a	**24.2±0.8b**	~	**29.7±0.9a**	14.1±1.3a	31.6±0.9a
	NI	51.2±3.3a	0.8±0.4a	4.9±0.3a	2.5±0.5a	34.9±4.3a	5.7±0.9a	**28.6±1.4a**	~	**24.8±2.3b**	12.6±0.7a	33.7±3.0a
HY	N	17.6±4.1a	45.8±2.2a	0.5±0.4a	~	35.9±4.0a	~	**22.6±1.7b**	**4.7±0.7a**	**69.6±3.1a**	**2.9±0.8a**	~
	NI	20.6±0.5a	48.2±0.8a	1.5±2.0a	~	29.5±1.6a	~	**43.9±1.9a**	**2.7±0.4b**	**51.9±2.5b**	**1.1±0.3b**	~
FQ	N	**58.1±0.2a**	27.3±1.0b	13.1±1.1a	~	1.5±0.1a	~	3.7±0.2a	6.6±0.4a	63.9±5.1a	25.3±4.5a	~
	NI	**59.1±2.0b**	28.1±0.8a	11.6±2.2a	~	1.3±0.5a	~	8.5±5.2a	5.8±1.4a	58.1±0.3a	26.6±3.5a	~
ZB	N	**35.6±0.6a**	5.6±0.6b	**45.6±1.1b**	12.9±0.9a	~	~	3.9±1.2a	10.0±0.3a	50.2±1.4a	35.8±1.5a	~
	NI	**27.8±1.0b**	11.9±2.9a	**43.0±3.0a**	17.1±1.1a	~	~	5.2±0.4a	9.9±0.4a	48.2±2.1a	36.6±1.6a	~
SJZ	N	**55.1±1.7a**	20.5±0.2a	**24.0±1.4b**	~	~	~	10.0±1.9a	14.5±1.3a	41.9±1.8a	33.5±1.1a	~
	NI	**46.0±0.7b**	24.0±0.7a	**29.5±1.2a**	~	~	~	13.3±5.8a	14.3±1.9a	43.7±5.2a	28.5±3.3a	~
YC	N	41.9±1.1a	33.1±1.7a	23.4±1.0a	**1.4±0.2b**	~	~	4.6±2.2a	1.2±0.1a	39.5±0.9a	54.6±1.3a	~
	NI	35.8±1.5a	36.8±1.9a	25.8±1.0a	**1.5±0.2a**	~	~	8.6±2.5a	1.1±0.2a	39.2±0.5a	51.0±2.0a	~
XC	N	62.7±0.7a	20.9±0.9a	16.2±0.2a	~	~	~	3.9±0.9a	7.3±0.5a	48.6±1.2a	40.2±1.3a	~
	NI	60.0±3.7a	23.6±2.2a	16.2±1.5a	~	~	~	8.0±6.9a	6.4±1.1a	47.2±2.1a	38.2±3.8a	~
MY	N	**76.3±1.7a**	19.2±1.2b	1.9±0.5a	~	1.3±0.3a	1.1±0.1a	3.3±1.2a	**21.0±0.2a**	55.7±1.2b	19.9±1.8a	~
	NI	**71.7±0.8b**	24.1±0.9a	2.2±0.3a	~	0.9±0.1a	0.9±0.2a	3.8±0.6a	**19.2±0.5b**	58.8±1.4a	18.0±0.5a	~
LC	N	**46.7±0.6a**	29.5±0.8b	16.6±1.0a	**4.9±0.7b**	~	1.7±0.3a	3.4±1.2a	4.9±0.9a	50.8±1.0a	40.8±0.7a	~
	NI	**33.9±1.4b**	39.2±0.8a	18.3±2.1a	**6.5±0.2a**	~	1.5±0.2a	4.7±0.5a	5.2±0.3a	54.4±3.6a	35.7±3.9a	~
YY	N	**48.2±1.8a**	**18.3±0.5a**	32.8±2.1a	**0.7±0.2b**	~	~	**8.9±0.5b**	**11.5±0.4a**	55.7±1.4a	23.7±1.0a	~
	NI	**41.0±2.4b**	**13.2±1.2b**	28.0±2.9a	**17.7±5.2a**	~	~	**15±0.6a**	**10.3±0.3b**	55.3±1.2a	19.4±1.1b	~

945   a. N, N fertilisation; NI, N fertilisation and nitrapyrin amendment.

946   b. mean values ± standard error (n=3). Different lowercase letters indicate significant difference between N and NI treatments in each site at $P < 0.05$. The values in blue bold

947   indicate the clusters significantly lower in NI treatment than in N treatment, and the values in black bold indicate reverse cases.

948   c. "~" indicates the relative abundance of corresponding cluster is lower than 1%.

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