Pedosphere ISSN 1002-0160/CN 32-1315/P doi:10.1016/S1002-0160(19)60803-9

Running title: Niche differentiation of active soil nitrifiers

Evidence for Niche Differentiation of Nitrifying Communities in a Grassland Soil After 44 Years of Different Field Fertilization Scenarios

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ABSTRACT

Long-term nitrogen fertilization imposes strong selection on nitrifying communities in agricultural soil, but it remains poorly understood how a progressively changing niche will affect potentially active nitrifiers in field. Using a 44-year-old grassland fertilization experiment, we investigated community shift of active nitrifiers by DNA-based stable isotope probing (SIP) of field soils that received no fertilization (CK), high levels of organic cattle manure (HC) and chemical N fertilization (CF). Incubation of DNA-SIP microcosms showed significant nitrification activities in CF and HC soils, whereas no activity occurred in CK soils. 44 years of inorganic N fertilization selected for only ^{13}C-AOB, whereas cattle slurry applications created a niche in which both AOA and AOB could be actively ^{13}C-labeled. Phylogenetic analysis indicated that *Nitrosospira* sp. 62-like AOB dominated inorganically fertilized CF soils, and *Nitrosospira* sp. 41-like AOB were abundant in organically fertilized HC soils. ^{13}C-AOA in HC soil were affiliated with the 29i4 lineage. ^{13}C-NOB were dominated by both *Nitrobacter*- and *Nitrospira*-like communities in CF soils, whereas the latter was overwhelmingly abundant in HC soil. The ^{13}C-labeled nitrifying communities in SIP microcosms of CF and HC soils were largely similar to those predominant under field conditions. These results provide direct evidence for strong selection of distinctly active nitrifiers after 44 years of different fertilization regimes in the field. Our finding implies that niche differentiation of nitrifying communities could be assessed as a net result of microbial adaption over 44 years to inorganic and organic N fertilizations in the field, and distinct nitrifiers have been shaped by intensified anthropogenic N input.

Key Words: Ammonia oxidizers, niche differentiation, nitrogen enrichment, soil microbial communities, stable isotope probing

INTRODUCTION

Agriculture dominates landscapes across whole continents and annually receives 210 Tg reactive nitrogen, almost doubling the global cycling of nitrogen over the last century (Fowler et al., 2013). Nitrification is a key step in the nitrogen cycle, and is characterized by the oxidation of ammonia to nitrate via nitrite which is consecutively catalyzed by ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). The most reactive N in soils is usually applied in form of ammonium, which is then oxidized at least once by nitrifying communities before return to the

atmosphere as N_2 (Vitousek et al., 2009). Increasing lines of evidences have suggested significant shift of nitrifying communities in response to long-term field fertilization (He et al., 2007; Wessén et al., 2010; Wu et al., 2011). This can be explained in part by the increased availability of ammonium substrates in soils. Indeed, ammonia oxidation is considered the rate limiting step of nitrification and, AOA and AOB obtain energy solely from ammonia oxidation while assimilating CO_2 as carbon source for growth. In human-managed systems, chronic N inputs could thus have profound impacts on the activities of nitrifying communities and subsequent environmental concerns such as ground water contamination of nitrate and greenhouse gas emission of N_2O. However, the mechanisms that have structured nitrifying communities and determined its functional significance remain poorly understood.

Niche differentiation is a key concept of ecological theory and has been suggested as different patterns of resource utilization for AOA and AOB communities (Prosser and Nicol, 2012). We propose that niche differentiation represents an ecological and/or evolutionary process of microbial adaption to particular sets of abiotic and biotic characteristics over certain time scales, and consequently can be evaluated technically by the difference in ecophysiological features of resulting AOA and AOB in an environment. We speculate that there are three mechanisms driving niche differentiation of nitrifying communities in the field under different fertilization regimes. (1) Niche-based deterministic process. The increased availability of ammonium substrates generates a favorable niche, leading to the expansion in population size of a particular nitrifier in soils; (2) Dispersal-based stochastic process. Particular nitrifers from outside immigrated in fertilized and/or background soils, generating dissimilar communities among soils under different fertilization strategies. (3) Diversification-based evolutionary process. Field fertilization facilitates genetic mutation and/or drift of already present nitrifiers, evolving new nitrifiers with genetic variants while driving some of once-existing nitrifiers into extinction (Nemergut et al., 2013). Nevertheless, tracking population dynamics *in situ* of microbial fecundity, survival and mortality is an insurmountable task (Blazewicz et al., 2013; Blazewicz et al., 2014), given the fact that soil is arguably the most heterogeneous entity on Earth (Young and Crawford, 2004). Meanwhile, the activities of nitrifying communities are not always positively correlated to the net changes in total abundance and compositional shift in soils (Erguder et al., 2009; Hatzenpichler, 2012). It thus poses great challenges to differentiate the mechanisms for the selection of dominant nitrifying communities in field soils.

DNA-based stable isotope probing (DNA-SIP) can serve as a powerful means to assess niche differentiation of nitrifying communities under different long-term N fertilization regimes in field soils, in which the factors other than soil ammonium substrate have been minimized. DNA-SIP enables the recovery of ^{13}C-DNA produced during active cell division and proliferation of autotrophic nitrifying communities grown on $^{13}CO_2$, thereby providing direct evidence for potentially active AOB and AOA in nitrifying agricultural soils (Jia and Conrad, 2009; Zhang et al., 2012; Lu and Jia, 2013). A meta-analysis of 12 DNA-SIP experiments in the literature showed physiologically distinct AOA ecotypes across physio-chemically contrasting habitats (Alves et al., 2018). However, it remains elusive why distinct ^{13}C-labeled nitrifiers have emerged in different environments and, most importantly, each independent SIP sample was site-specific with little merit for comparative analysis (Alves et al., 2018). Furthermore, numerous studies also showed significant stimulation of nitrifying communities in total abundance in response to long-term field N fertilization (Freitag et al., 2005; He et al., 2007; Wessén et al., 2010; Wu et al., 2011), but none has employed DNA-SIP to assess whether the now-dominant nitrifiers are potentially capable of active proliferation in fertilized relative to control soils.

Therefore, in a 44-year-old grassland fertilization experiment we hypothesize that slow accumulation and gradual extinction of particular nitrifiers could have occurred continually in response to repeated N input, thereby leading to stabilized nitrifying communities as a seed bank of intact cells in field soils that received no fertilization (CK), inorganic chemical fertilizers (CF) and high levels of cattle manure (HC). These background nitrifers can represent the legacy effect of community composition after 44 years of long-term N fertilization. DNA-SIP for an incubation period of 8 weeks was employed to investigate the potentially active nitrifiers in fertilized and control soils, and we hypothesize that phylogenetically distinct nitrifiers would have been actively proliferated and become ^{13}C-labeled, representing niche differentiation of nitrifying communities after 44 years of different field N fertilizations.

MATERIALS AND METHODS

Site description and soil sampling

We used soils collected from a long-term grassland nutrient fertilization experiment that was established

at Hillsborough, UK (54°27′N, 6°4′W) in 1970 (Muller et al., 2011). This grassland site has a clay loam soil, is located at an elevation of 120 m above sea level, experiences ~900 mm in annual precipitation and has a mean annual temperature of 9 ℃ (www.metoffice.gov.uk). The experiment was originally designed to measure the potential effects of repeated applications of organic animal slurries and synthetic fertilizer on grass yields and on multiple soil physical and chemical properties.

In this study, we focus on three nutrient treatments in particular: (1) control (CK: no fertilizer or animal slurry applied); (2) NPK fertilizer control levels of inorganic fertilizer, CF, with NPK at rates of 200 kg N ha^{-1} y^{-1} (ammonium nitrate until June 1974 and subsequently as urea), 32 kg P as superphosphate, and 160 kg K ha^{-1} $year^{-1}$ as potassium chloride; and (3) a high rate of cattle slurry application (HC: 200 m^3 ha^{-1} yr^{-1}, which corresponds to 640 ± 88 kg N ha^{-1}). Each of the treatments is arranged in three randomized blocks with two replicates of each treatment fully randomized within each block. Compound NPK fertilizer and the cattle slurry have been applied every year since 1970 in three equal dressings, first in the spring and then in July and September. Previous botanical surveys in 1982 and 2006 (Christie, 1987; Liu et al., 2010) show common C3-grass species (*Lolium perenne*, *Agrostis stolonifera* and *Poa* spp.), which account for ~70% of aboveground plant biomass in plots receiving either inorganic or organic fertilizer nutrients. Legume species, mainly represented by *Trifolium repens*, account for ~5% of aboveground biomass in the control plots and ~0.05% or less in the nutrient fertilized plots (Liu et al., 2010). The grass was cut three times each year approximately at the end of May, July and September. The fertilizer and cattle slurries were applied in three equal dressings, first in the spring and again immediately after the first cut and second cut. Soil sampling was performed in February 2012. Three soil cores were taken from each plot using augers and these soil samples were mixed and homogenized into a composite sample. Soil from each plot was ground to <2 mm, air-dried and analyzed for soil properties including pH, Olsen-P, extractable K, Mg and S as described previously (Zhou et al., 2015). Physicochemical properties of the grassland soils used in this study are shown in Table S1.

DNA-SIP microcosm and gradient fractionation

SIP microcosms were established to link ammonia oxidation activity with the taxonomic identity of nitrifying communities and to assess the activity of phylogenetically distinct phylotypes of ammonia oxidizers in CK (control), CF (NPK) and HC (cattle slurry) soils. All treatments were set up in triplicate microcosms and incubated at 60% of the soil maximum water-holding capacity at 28 ℃ in the dark. Two treatments were established, namely ^{13}C-labeled receiving $^{13}CO_2$ and ^{13}C-urea and control ($^{12}CO_2$ and unlabeled urea) microcosms, as previously described (Wang et al., 2015). Briefly, fresh soil (equivalent to 5.0 g dry weight soil) was incubated in 120-ml serum bottles capped with black butyl stoppers for 8 weeks at 28 ℃ in the dark. The headspace of the bottles was renewed on a weekly basis with pressurized synthetic air (20% O_2, 80% N_2), and 100 μg urea-N g^{-1} soil was added once a week. The labeled $^{13}CO_2$ was injected in the bottles resulting a final concentration of 5% in the headspace, and $^{13}CO_2$ was renewed once a week. The labeled $^{13}CO_2$ (99 atom% ^{13}C) and ^{13}C-urea were purchased from the Shanghai Stable Isotope Engineering Research Center and $^{12}CO_2$ was prepared by acidifying sodium carbonate. The ^{13}C-urea was applied to avoid dilution of $^{13}CO_2$ with CO_2 emerging from urea hydrolysis. Soil samples were pre-incubated for 7 days at ~40% WHC, and this pre-incubation reduced the rates of heterotrophic CO_2 respiration, so that a subsequent treatment with 5% CO_2 in the headspace could be maintained at a constant level during the incubation period. Destructive sampling of 2.0 g fresh soil in each microcosm was performed at days 0 and 56, and frozen immediately at -80 ℃. The remainder of each soil was mixed with 15 ml of 2 M KCl and passed through filter paper after shaking at 200 rpm for 60 min. The concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N were determined with a Skalar SAN Plus segmented flow analyzer (Skalar, Inc., Breda, The Netherlands). Soil NO_2^--N was below the detection limit and nitrification was determined by the changes in the concentrations of NO_3^--N.

Soil samples were used for DNA extraction in triplicate after incubation with ^{13}C-label and ^{12}C-control for 8 weeks. Soils at Day 0 were considered as field soil samples, and it was subjected to a 7-day pre-incubation before SIP microcosms incubation as described above. Soil nucleic acid was extracted from 0.5 g soil using the FastDNA spin kit for soil (Qbiogene, Inc., Irvine, CA) according to the manufacturer's protocol. DNA purification was conducted with 5.5 M guanidine thiocyanate solution to remove contaminating humic substances. The quality and concentration of the extracted DNA was measured by gel electrophoresis (0.8% agarose) and a spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE), and stored at -80 ℃.

The total DNA was then fractionated in triplicate as described previously (Xia et al., 2011) to resolve

^{13}C-DNA from ^{12}C-DNA in soils from the three treatments. In detail, about 2.0 μg DNA from each soil microcosm was mixed with CsCl stock solution to form a CsCl buoyant density of 1.725 g ml^{-1}, and spun in 5.1-ml Beckman polyallomer ultracentrifuge tubes in a Vti65.2 vertical rotor (Beckman Coulter, Palo Alto, CA) at 177,000 × g for 44 h at 20 ℃. DNA fractions with different densities were retrieved by displacing the gradient medium from the top of the ultracentrifuge tube with sterile water using an NE-1000 single syringe pump (New Era Pump Systems Inc., Farmingdale, NY) with a precisely controlled flow rate of 0.38 ml min^{-1}. Up to 15 gradient fractions were generated, and the nucleic acids were separated from CsCl by PEG 6000 precipitation and dissolved in 30 μl TE buffer and stored at -20 ℃ (Jia and Conrad, 2009).

Quantitative PCR of amoA genes

The abundance of archaeal and bacterial *amoA* genes in the total DNA and fractionated DNA across the buoyant density gradients from DNA-SIP microcosms was assessed by quantification of *amoA* gene copies in soil samples using the primer pairs Arch-amoAF/Arch-amoAR (Francis et al., 2005) for AOA and amoA-1F/amoA-2R (Rotthauwe et al., 1997) for AOB (Table S5). Real-time quantitative PCR was carried out with a CFX96 Optical Real-Time Detection System (Bio-Rad Laboratories, Inc. Hercules, CA). Quantitative PCR reactions were performed in 20-μL reaction mixtures containing 10 μL 2x SYBR Premix Ex Taq (Takara Biotech, Dalian, China), 200 nM of each primer, and 20 ng DNA template. The thermal program for the real-time PCR assay is shown in Table S2 (Jia and Conrad, 2009; Xia et al., 2011). Blanks were always run with water as the template instead of soil DNA extract as a negative control. Standard curves spanning 10^7-10^1 gene copies per microliter were constructed by dilution series of plasmids harboring the *amoA* gene. The plasmid was extracted with a Plasmid Purification Kit (Takara) and the concentration was measured with a NanoDrop ND-1000 UV-Vis spectrophotometer and used for the calculation of standard copy numbers. The amplification efficiency ranged from 98 to 110% with R^2 values of approximately 0.99 in each reaction. Specific amplification of *amoA* genes was confirmed by agarose gel electrophoresis and melting curve analysis always resulted in a single peak.

Illumina sequencing of 16S rRNA gene amplicons

Universal primer pair Tag-515F and Tag-907R (Stubner, 2002) were used to amplify soil bacterial 16S rRNA gene fragments for the Illumina MiSeq platform (PE 300). PCR was carried out in 50-μL reaction mixtures each containing deoxynucleoside triphosphate at a concentration of 1.25 mM, 1 μl of forward and reverse primers (20 mM), 2U of Taq DNA polymerase (TaKaRa, Japan), and 50 ng of DNA. The following cycling parameters were used: 5 min of incubation at 94 ℃, followed by 32 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 45 s, and a 10 min extension at 72 ℃ (Table S5). Triplicate reaction mixtures per sample were pooled together and purified using an agarose gel DNA purification kit (TaKaRa) and quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). The bar-coded PCR products were pooled in equimolar amounts (10 pg in each sample) before sequencing as described previously (Xiang et al., 2016).

Sequences were merged with FLASH (Magoc and Salzberg, 2011) and then processed using Quantitative Insights Into Microbial Ecology (QIIME; http://www.qiime.org/) (Caporaso et al., 2011). Poor-quality sequences (below an average quality score of 25) and short sequences (< 200 bp) were removed. Sequences of each replicate were merged and then clustered into Operational Taxonomic Units (OTUs) using a 97% identity threshold (default QIIME settings) with UCLUST (Edgar, 2010) and all singleton OTUs were deleted. Approximately 1,364,221 high-quality sequences were obtained of the V4 region of the 16S rRNA gene (Table S3 and S4) and these were subjected to taxonomic classification. The nitrifying phylotypes were then selected for further analysis. The nitrifying sequences were screened at the phylum level for thaumarchaea (Fig. S1) and at the genus levels for both AOB (*Nitrosomonas* and *Nitrosospira*) (Fig. S2) and NOB (*Nitrobacter* and *Nitrospira*) (Fig. S3), and the representative sequences were extracted for phylogenetic analysis (Table S5). No nitrification activity was observed in the control (CK) soil, thus sequencing analysis was not performed.

Phylogenetic analysis

Phylogenetic analysis of the nitrifying 16S rRNA gene sequenced in this study was performed using the Molecular Evolutionary Genetics Analysis (MEGA 4.0) software package (Kumar et al., 2004). The basic tree of sequences from known AOA, AOB and NOB cultures was constructed using a neighbor-joining algorithm. The tree topology was checked using the neighbor-joining algorithm and the minimum evolution

method. The nucleotide sequences of the representative OTUs have been deposited in the NCBI (National Center for Biotechnology Information) under the accession numbers KX701984 to KX701990 (AOA) KX701991 to KX701992 (AOB) and KX701993 to KX701994 (NOB) for the 16S rRNA genes from the DNA-SIP experiment. The sequence reads of all 16S rRNA genes have been deposited in the NCBI under the accession number PRJNA344004.

RESULTS

Soil nitrification activity

Two types of microcosms were established including a SIP treatment labeled with 5% $^{13}CO_2$ plus ^{13}C-urea and a control treatment with 5% $^{12}CO_2$ plus ^{12}C-urea (Fig. 1). There was no significant difference in the concentration of inorganic N between ^{13}C-labeled and ^{12}C-control microcosms on day 56 (Fig. 1ab). During SIP microcosm incubation for a 56-day period, no significant increase in NO_3^- was detected in non-fertilized control soils (CK) (Fig. 1a), whereas significant nitrification occurred in the CF (1.1 μg NO_3^--N g^{-1} soil day^{-1}) and HC (6.8 μg NO_3^--N g^{-1} soil day^{-1}) soils (Fig. 1a). Soil NH_4^+ concentration at day 56 was higher in CF (728 μg N g^{-1}soil) than in HC (535 μg N g^{-1} soil.) soils (Fig. 1b), consistent with the difference in nitrification between the two soils. The increase in soil nitrate content corresponded well with the stoichiometric decrease of NH_4^+-N (Fig. 1b), suggesting a predominance of microbial nitrification in the urea-amended microcosms of CF and HC soils.

Fig. 1

Fig. 1 Changes in the concentration of soil NO_3^--N (a), NH_4^+-N (b), *amoA* gene abundance of ammonia-oxidizing archaea (AOA) (c), and ammonia-oxidizing bacteria (AOB) (d) over a 56-day microcosm incubation of soils, which either received no fertilization (CK), chemical fertilizer (CF) or high cattle slurry applications (HC) for 44 years. Day 56-$^{13}CO_2$ and Day 56-$^{12}CO_2$ represent soil microcosms incubated with 5% of $^{13}CO_2$ and $^{12}CO_2$ in the headspace, respectively. Day 0 is also considered to represent soil sample under field conditions. Bars represent standard errors of mean values of triplicate microcosms. Different letters indicate a significant difference ($P<0.05$).

Soils at Day 0 were considered as samples under field conditions, and soil inorganic N concentrations increased with repeated N input in the field. Background soil NO_3^--N concentrations were 6.21, 10.2 and 20.2 μg NO_3^--N g^{-1} soil in CK, CF and HC soils, respectively (Fig. 1a). Soil NH_4^+-N concentrations were 7.6, 12.0 and 50.0 μg N g^{-1} soil in CK, CF and HC soils, respectively (Fig. 1b). It indicates that chronic N fertilization over 44 years in the field could have resulted in higher NH_4^+-N concentrations, simulated ammonia oxidation, and generated significantly higher contents of soil NO_3^--N in the fertilized CF and HC soils than CK controls soils.

Abundance changes of soil nitrifying communities

Population sizes of AOA and AOB communities were measured using quantitative PCR of archaeal and bacterial *amoA* genes. At Day 0 presumably under field conditions, the archaeal *amoA* genes increased significantly from 22.8 x 10^3 copies g^{-1} soil in CK soils to 7.3 x 10^4 copies g^{-1} soil in HC soil (Fig.1c). No significant difference of archaeal *amoA* genes were observed between CK and CF soils. As for AOB, *amoA* gene abundances ranged two-fold, from 2.6 x 10^3 to 5.1 x 10^4 copies g^{-1} soil, but these were not significantly different (Fig. 1d).

During SIP incubation period of 56 days, archaeal *amoA* gene abundance remained largely stable in all three soils (ANOVA, $P>0.05$) (Fig. 1c). By contrast, the copy number of bacterial *amoA* genes (AOB) *increased* significantly ($P<0.01$) from 1.3 x 10^4 to 6.7 x 10^4 copies g^{-1} in CF soils and from 5.1 x 10^4 to 5.7 x 10^5 copies g^{-1} in HC soils, thus representing 5- and 11-fold increases, respectively (Fig.1d). The abundance of AOB did not change significantly in CK soils.

Labeling of active nitrifying communities

Quantification of *amoA* genes as a function of the buoyant density of the DNA gradient was conducted to assess the labeling of AOA (Fig. 2a) and AOB (Fig. 2b) in SIP microcosms. The highest *amoA* gene copy numbers of both AOA and AOB in the microcosms of the $^{12}CO_2$-control treatment were detected in the 'light'

fractions typical of the unlabeled DNA, with a buoyant density of 1.710-1.720 g ml^{-1} for all treatments of CK, CF and HF soils. In CK soil, there was no apparent difference in the *amoA* gene copies in the heavy DNA fractions between the ^{13}C-labeled and ^{12}C-control microcosms, suggesting no labeling of AOA and AOB, which is consistent with the lack of nitrification (Fig. 1). Only in HC soil,was there a shift towards heavy fractions of archaeal *amoA* gene abundance in the $^{13}CO_2$-labeled treatment compared to fractions in the $^{12}CO_2$-controls (Fig. 2a). In contrast, high abundance of bacterial *amoA* genes in the heavy fractions was detected in labeled microcosms of both CF and HC soils when compared to the controls (Fig. 2b). These results indicated labeling of AOA occurred only in HC soils, whereas AOB were labeled in both CF and HC soils.

Fig. 2

Fig. 2 The labeling patterns of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) from soil microcosms incubated with $^{13}CO_2$ or $^{12}CO_2$ in grassland soils. CK = control soils, CF = NPK fertilized soils and HC = cattle slurry fertilized soils. Quantitative distribution of bacterial *amoA* genes (a) and archaeal *amoA* genes (b) across the entire buoyant density gradient of the DNA fractions. Normalized data are the ratio between gene copy numbers for each DNA gradient and maximum quantities for each treatment. Bars represent standard errors of means of triplicate microcosms each containing three technical replicates. The relative frequency of AOA (c), AOB (d) and NOB (e) across the entire buoyant density gradient of the DNA fractions, which was determined as the proportion of target nitrifying reads to all high-quality 16S rRNA gene reads in each fraction. The total microbial communities in each DNA fraction were analyzed by MiSeq illumine-sequencing of 16S rRNA genes using universal primer pairs 515F-907R (Supplementary Table S2), and the nitrifying reads were sorted out for subsequent analysis.

Similar results were obtained by determining the relative abundance of AOA, AOB and NOB by Illumina sequencing of total 16S rRNA genes in the fractionated DNA across a wide range of buoyant *densities* (Fig. 2). Nitrifying communities were significantly enriched in the heavy DNA fractions from $^{13}CO_2$-labeled microcosms compared to $^{12}CO_2$-control microcosms of CF and HC soils (Fig.2). AOA were significantly enriched only in HC soils up to 1.3% of the total microbial communities in the ^{13}C-labeled 'heavy' DNA, when compared to those from ^{13}C-labeled CF soil and $^{12}CO_2$-control treatments (Fig. 2c). Significant enrichment of AOB occurred in the ^{13}C-labeled 'heavy' DNA of SIP microcosms of CF and HC soils, accounting for up to 17% and 23% of the total microbial communities, respectively (Fig. 2d). The ^{13}C-labeled NOB accounted for up to 8.6% of the total communities in the 'heavy' DNA fractions of HC soils but no difference was observed in the ^{13}C-labeled 'heavy' DNA fractions of CF soils relative to ^{12}C-control treatments (Fig. 2e).

Dynamics of nitrifying communities

Phylogenetic analysis was conducted for AOA (Fig. S1), AOB (Fig. S2) and NOB (Fig. S3) in field soils and in ^{13}C-DNA from SIP microcosms. The results clearly showed a shift in nitrifying communities in the field soil DNA (Day 0) under different fertilization regimes for 44 years, and this shift was overall well matched with the ^{13}C-labeled AOA and AOB at Day 56 after incubation for an 8-week period, while the NOB were surprisingly different (Fig. 3).

Fig. 3

Fig. 3 Proportional changes of nitrifying phylotypes in response to 44 years of inorganic *vs*. organic N fertilizations in field soils and in SIP microcosms. CK = control soils, CF = NPK fertilized soils and HC = cattle slurry fertilized soils, ND = not detected. Field soil DNA refers to data obtained from soil samples in the field, i.e., Day-0 during an 8-week incubation of SIP microcosms; Microcosm ^{13}C-DNA indicates the density-resolved ^{13}C-DNA from SIP microcosms incubated with $^{13}CO_2$ and urea. The 16S rRNA genes of nitrifying phylotypes were screened first by RDP classifier of the total 16S rRNA gene reads. The phylotype assignments of AOA, AOB and NOB are shown in Supplementary Figures S1, S2, and S3, respectively. The proportional changes were then analyzed for nitrifying phylotypes in each treatment. The error bars were removed for clarity. All other abbreviations are the same as those in Fig. 1 and Fig. 2.

In the field soil DNA (Day 0), AOA of the CK soil were exclusively affiliated with the 54d9 lineage (Fig. 3a). However, AOA within the 29i4 *lineage* dominated archaeal ammonia oxidizers in CF (73%) and

HC soils (83%) after 44 years of N fertilization. Intriguingly, SIP incubation indicated that AOA were labeled only in HC soils, and 29i4-like AOA accounted for 99% of the ^{13}C-labeled archaeal ammonia oxidizers in the HC soil, while 1% of the ^{13}C-AOA could be assigned to the marine group 1.1a lineage (Fig. 3a).

AOB in CK soils were phylogenetically most closely affiliated with *Nitrosospira* sp 62 (98%) and *Nitrosospira* sp.41 (2%) at Day 0 under field condition (Fig. 3b). It is interesting to note that AOB in CF soils were solely affiliated with the *Nitrosospira* sp 62, where in HC soil Nitrosospira sp.41- and *Nitrosomonas* Is86-like AOB account for 99% and 1% of AOB communities respectively (Fig. 3b). In SIP microcosms of CF and HC soils at Day 56, *Nitrosomonas*-like AOB were not detected. The *Nitrosospira* sp.62-like AOB contributed for 90% of ^{13}C-active AOB communities in CF soils, and in HC soil microcosms 99% of the ^{13}C-labeled AOB were phylogenetically most closely related to the *Nitrosospira* sp. 41 (Fig. 3b). Hence these data suggest that the predominant AOB at the beginning of the incubation also became the most active component of the ammonia oxidizing community during the incubations.

For NOB, the comparison between background communities under field conditions at Day 0 and ^{13}C-labeled communities at Day 56 suggests a different picture in that minor components of the community became highly active. *Nitrospira*- and *Nitrobacter*-like NOB were found in the field soils at Day 0 with 29% and 71% of the total nitrite oxidizer communities in the CK soils, respectively (Fig. 3c). *Nitrospira*-like NOB was significantly higher, amounting up to 90% in the CF soils and up to 99% in HC soils. However, SIP microcosm incubation at Day 56 indicated 99% of the ^{13}C-labeled NOB were affiliated with the *Nitrobacter* lineage in HC soils. In CF soils, 60% and 40% of ^{13}C-labeled NOB communities could be assigned to the *Nitrospira* and *Nitrobacter* lineages, respectively (Fig. 3c).

DISCUSSION

Using three soils from a 44-year-old long-term field fertilization experiment, we conducted SIP microcosm incubations for an 8-week period in order to assess the taxonomic identities of potentially active nitrifiers and its response to different field fertilization regimes. We consider that the shift of nitrifying communities under field conditions could be represented by soil samples at Day 0, and the difference in ^{13}C-labeled nitrifiers at Day 56 in SIP microcosms could largely reflect niche differentiation of potentially active nitrifying communities as a result of the 44-year-adaption to different field fertilization strategies. Our study suggests that different soil fertilizing regimes applied over 44 years have led to a shift in soil nitrifying communities under field conditions at Day 0 and that the largely similar nitrifying populations are ^{13}C-labeled at Day 56. The molecular survey demonstrates considerable changes of AOA, AOB and NOB communities in fertilized soils when compared to control soils under field conditions at Day 0. Cattle slurry application led to the labeling of both AOA and AOB, while inorganic N fertilizers selected only for AOB. Our study clearly showed that phylogenetically distinct nitrifiers emerged as the dominant communities in organically HC and inorganically fertilized CF soils when compared to non-fertilized CK soils. It provides evidences of how anthropogenic N input to grassland soils has led to the emergence and maintenance of distinct active nitrifying communities.

Our findings show that changes of nitrification activities under different field fertilization regimes are controlled by niche differentiation of nitrifying communities, a long presumed but rarely demonstrated mechanisms (Erguder et al., 2009; Martiny et al., 2011; Prosser and Nicol, 2012). At the continental scale, molecular surveys have indicated biogeographic distribution patterns of AOA and AOB communities, implying selection of particular nitirifers by physio-chemically contrasting environments (Gubry-Rangin et al., 2011; Alves et al., 2018). However, the remarkable heterogeneities among these environments prevent reasonable inference of abiotic factors that determine community assembly of nitrifers in different habitats (Martiny et al., 2011; Alves et al., 2018). In 9 field plots with a total area of 268-square meters, the environmental variables other than ammonium concentrations could be minimized in order to investigate niche differentiation of nitrifies in soils under different fertilization regimes. DNA-SIP indicated that active proliferation of ^{13}C-AOB occurred only in inorganically fertilized HC soils, whereas active growth of both ^{13}C-AOA and AOB were found in organically HC soils. It shows phylogenetically distinct nitrifiers have been selected after 44 years of different field fertilizations, although exact mechanisms underlying niche differentiation remains uncertain.

Soil ammonia-oxidizing communities were enriched to high levels over 44 years of nitrogen fertilizations as evident by the ability of fertilizer treatments to nitrify while control plot samples were unable to significantly metabolize ammonia during the 56-day incubation period. Indeed, potential nitrification activity was below detection limits in unfertilized soils (Fig. 1a), suggesting that the metabolic flexibility of the ammonia-oxidizing populations remained low. On the other hand, HC soils with organic manure

application showed much higher activity than CF soils with chemical fertilizers application. This is consistent with the findings from a previous ^{15}N tracer study from the same long-term grassland experiment (Müller et al., 2011). Addition of $^{15}NH_4$-N tracer revealed no production of $^{15}NO_3$-N in CK soils while significant amounts of $^{15}NO_3$-N were produced in CF and HC soils (Müller et al., 2011). This activity change was further supported by the observed stimulation of nitrifying communities in fertilized soils (Fig. S4)..

The results indicated that AOA represented by the fosmid 54d9 lineage are adapted to lower ammonia concentrations than other AOA (Fig. 3a). The fosmid 54d9 lineage was the only AOA in CK soils under field conditions and was also originally discovered in a metagenomic library from a German grassland soil, representing the first *amoA* gene discovered in Thaumarchaeota (Treusch et al., 2005). However, 54d9-like AOA were largely replaced by 29i4-like AOA in soils fertilized with inorganic CF (54.5%) and organic HC (83.0%) N inputs (Fig. 3a). Moreover, DNA-SIP incubation revealed autotrophic growth of 29i4-like AOA only in HC soils, and no labeling of 54d9-like AOA was detected across all soil samples studied. This is consistent with a study on Chinese grass lands showing that labeling of the 54d9 lineage was much less than the 29i4-like AOA (Pan et al., 2018). The 29i4 lineage was originally detected in Arctic soils (Alves et al., 2013) and appears to be capable of actively nitrifying among a wider variety of habitats including cold regions (Alves et al., 2013; Zhao et al., 2015), aerobic upland soils (Pan et al., 2018) and paddy soils (Wang et al., 2015). It is noteworthy that a tiny fraction of ^{13}C-AOA was phylogenetically closely associated with acidophilic AOA of the group 1.1a-associated lineage (Fig. 3a), although neutral soils were used in this study with pH ranging from 6.6 to 7.1 (Supplementary Table 1). It is thus possible that the 29i4- and group 1.1a-associated AOA are naturally adapted to higher concentrations of ammonia, or that they had stronger adaptive ability than 54d9-like AOA, enabling their gradual adaption to specific characteristics of grassland soils with chronic N input.

Dynamic changes of AOA communities showed that relatively closely related phylotypes can apparently possess different physiology. AOA within the 29i4 lineage were detected in field soils under both CF and HC treatments (Fig. 3a), but the labeling and active growth were observed only in HC soils, despite the fact that both soils were incubated under the same conditions in the SIP microcosms. It seems unlikely that the emergence of active 29i4-like AOA in HC soils could be attributed to the contamination of cattle slurry manure amendment because these AOA were also detected in CF soils that received only chemical N fertilizers. In fact, why the lineage dominates AOA in both fertilization treatments but is only stimulated in the HC treatment remains unknown. It is possible that there are additional chemical limitations in the artificial fertilizer treatments and that the 29i4 lineage is completely outcompeted by AOB in these samples while in the manure fertilized samples, limitations and/or competition from AOB might be less severe. Nevertheless, 44 years of organic *vs* inorganic fertilization have resulted in sufficient differences in soil physiochemical properties, which subsequently favored active growth of 29i4-like AOA in HC soils but not in CF soils, although the exact mechanism of niche construction remains unknown. Meanwhile, it has been shown that urease –containing AOA dominated ammonia oxidation in nitrifying acid soils (Lu and Jia, 2013) and marine environment (Kitzinger et al., 2019), but its ecophysiological characteristics remain elusive in neutral soils tested in this study. Cultivation of the representative AOA would help elucidate adaptive mechanisms under chronic N inputs in agricultural systems.

The population dynamics of AOB remained largely similar between field conditions and microcosm incubations, while different results were observed for NOB. *Nitrobacter* and *Nitrospira* are often found to be the major functional groups in soil for nitrite oxidation (Attard et al., 2010), whereas NOB is composed of five genera including another three genera of *Nitrococcus*, *Nitrospina* and *Nitrotoga* (Bock and Wagner, 2006; Kitzinger et al., 2018). *Nitrobacter-like* NOB is often characterized as *r*-strategists with higher growth rate and lower substrate affinity when compared to *Nitrospira*-like NOB, defined as *K*-strategist (Schramm et al., 1999; Ke et al., 2013). Although nitrite oxidizers in CH and HC soils were predominated by *Nitrospira*-like NOB under field conditions, Nitrobacter-like NOB overwhelmingly dominated the ^{13}C-labeled communities of nitrite oxidizers in HC soils. This discrepancy might be explained by the *K*- and *r*-strategies of soil NOB (Ke et al., 2013). It should be emphasized that SIP microcosms had exceptionally high NH_4-N levels with concentrations of 104 mM in CF and 76.4 mM in HC soils, assuming all ammonium was dissolved in soil water. The NO_3-N concentrations were estimated to be 10.3 and 57.3 mM in CF and HC soils. The amount of ammonium and nitrate far exceeded the reported concentrations for culture-dependent studies of AOA (Jung et al., 2016), and often favored AOB growth of *Nitrosomonas* rather than *Nitrosospira* species (Webster et al., 2005), and it might have had adverse impacts on nitrifying communities. This might in part explain the discrepancy of nitrifying communitiy dyanmics between field conditions and microcosm study. However, in our study, soil samples from different field plots were subjected to identical manipulations and distinct growth of different nitrifying populations could be most likely attributed to the legacy effect of long-term

conditioning by different N inputs. Taken together, our results suggest that 44 years of N enrichment have contributed to select for distinct nitrifying communities in permanent grassland soils. Nitrification in organically fertilized soils appears to be primarily catalyzed by 29i4-like AOA, *Nitrosospira* sp. 41-like AOB and *Nitrobacter*-like NOB. Instead, *Nitrosospira* sp. 62-like AOB and *Nitrospira*-like NOB dominated nitrification in inorganically fertilized soils. Shifts in nitrifying communities under field conditions could be largely represented by SIP microcosm incubations, particularly for AOB. The distinct labelling patterns indicate that niche differentiation of ammonia oxidizers and nitrite oxidizers could have occurred over 44 years of inorganic *vs.* organic nitrogen fertilization. Our results indicate that particular nitrifiers have been selected and enriched in field soils during a 44-year adaption to different N fertilization regimes. Niche differentiation can be evaluated by stable isotope probing of potentially active nitrifiers in field soils under chronic N enrichments.

ACKNOWLEDGEMENTS

This work was financially supported by the National Science Foundation of China (41530857, 41471208), the Department of Agriculture, Environment and Rural Affairs (DAERA) in Northern Ireland (UK) – project number 7001 (41499), and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB15040000) and the Startup Foundation for Introducing Talent of NUIST (S8113117001). We thank Zhiying Guo for technical support on data analysis and our laboratory colleagues for helpful discussions.

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Figures and Table

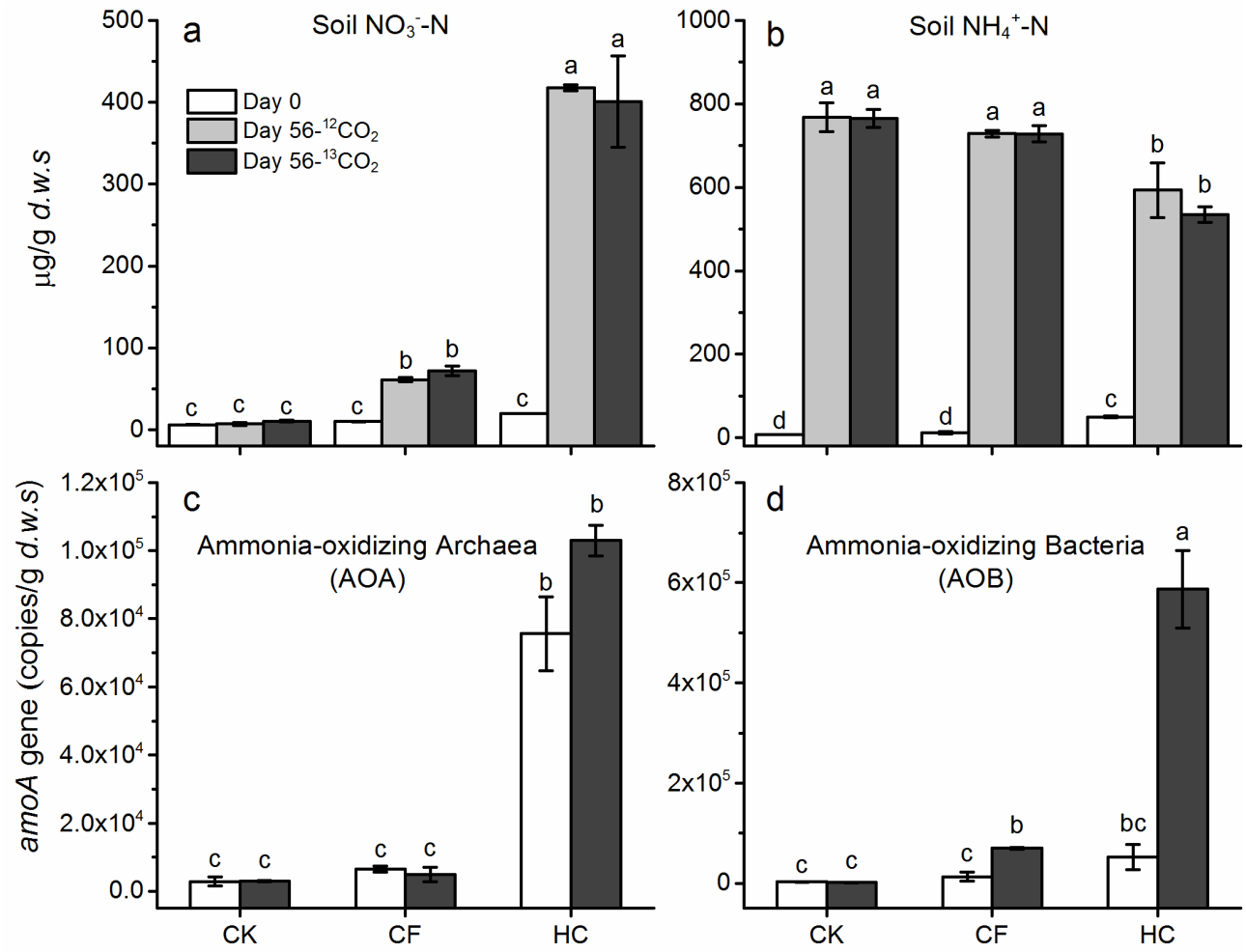

Fig. 1. Changes in the concentration of soil NO_3^--N (a), NH_4^+-N (b), *amoA* gene abundance of ammonia-oxidizing archaea (AOA) (c), and ammonia-oxidizing bacteria (AOB) (d) over a 56-day microcosm incubation of soils, which either received no fertilization (CK), chemical fertilizer (CF) or high cattle slurry applications (HC) for 44 years. Day 56-$^{13}CO_2$ and Day 56-$^{12}CO_2$ represent soil microcosms incubated with 5% of $^{13}CO_2$ and $^{12}CO_2$ in the headspace, respectively. Day 0 is also considered to represent soil sample under field conditions. Bars represent standard errors of mean values of triplicate microcosms. Different letters indicate a significant difference ($P<0.05$).

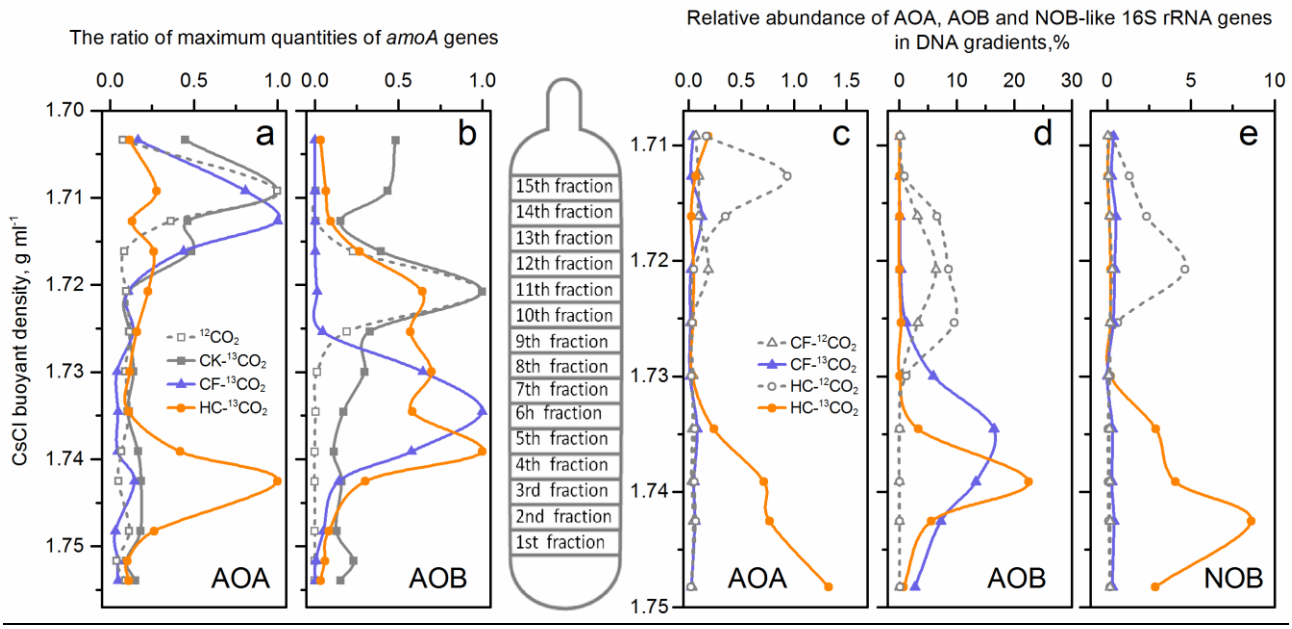

Fig. 2. The labeling patterns of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) from soil microcosms incubated with $^{13}CO_2$ or $^{12}CO_2$ in grassland soils. CK = control soils, CF = NPK fertilized soils and HC = cattle slurry fertilized soils. Quantitative distribution of bacterial *amoA* genes (a) and archaeal *amoA* genes (b) across the entire buoyant density gradient of the DNA fractions. Normalized data are the ratio between gene copy numbers for each DNA gradient and maximum quantities for each treatment. Bars represent standard errors of means of triplicate microcosms each containing three technical replicates. The relative frequency of AOA (c), AOB (d) and NOB (e) across the entire buoyant density gradient of the DNA fractions, which was determined as the proportion of target nitrifying reads to all high-quality 16S rRNA gene reads in each fraction. The total microbial communities in each DNA fraction were analyzed by MiSeq illumine-sequencing of 16S rRNA genes using universal primer pairs 515F-907R (Supplementary Table S2), and the nitrifying reads were sorted out for subsequent analysis.

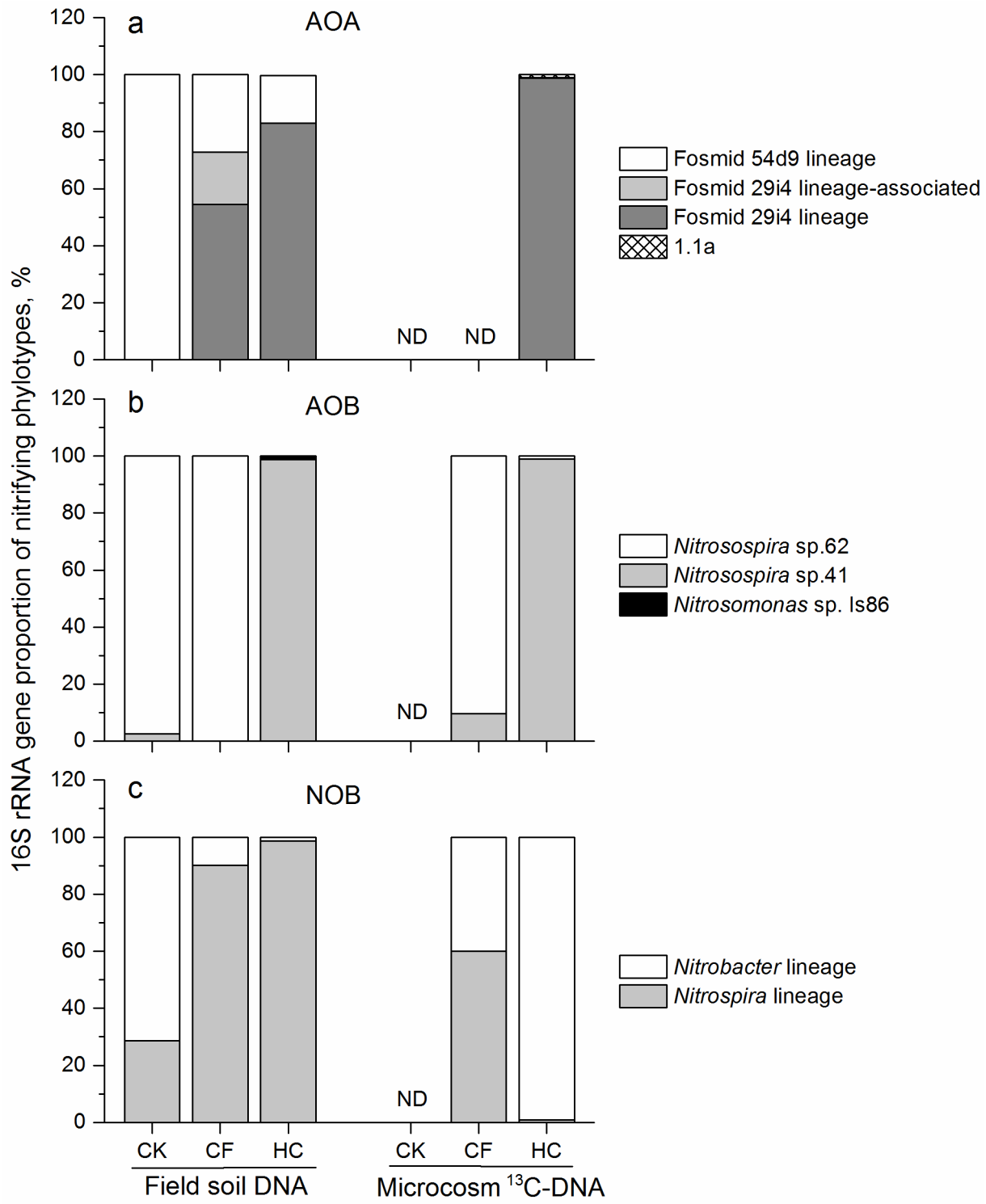

Fig. 3. Proportional changes of nitrifying phylotypes in response to 44 years of inorganic *vs.* organic N fertilizations in field soils and in SIP microcosms. CK = control soils, CF = NPK fertilized soils and HC = cattle slurry fertilized soils, ND = not detected. Field soil DNA refers to data obtained from soil samples in the field, i.e., Day-0 during an 8-week incubation of SIP microcosms; Microcosm ^{13}C-DNA indicates the density-resolved ^{13}C-DNA from SIP microcosms incubated with $^{13}CO_2$ and urea. The 16S rRNA genes of nitrifying phylotypes were screened first by RDP classifier of the total 16S rRNA gene reads. The phylotype assignments of AOA, AOB and

NOB are shown in Supplementary Figures S1, S2, and S3, respectively. The proportional changes were then analyzed for nitrifying phylotypes in each treatment. The error bars were removed for clarity. All other abbreviations are the same as those in Fig. 1 and Fig. 2.

Supporting Information

Evidence for niche differentiation of nitrifying communities in a grassland soil after 44 years of different field fertilization scenarios

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This SI file contains

1. Supporting Table S1 to S5
2. Supporting Figures S1 to Figure S4
3. Supporting References

Table S1. Physicochemical properties of the grassland soils used in this study.

Treatment	CK	CF	HC
pH	7.12±0.03	7.09±0.03	6.61±0.01
P (mg kg^{-1})	15.6±1.3	40.5±1.0	110±3
K (mg kg^{-1})	139±23	147±13	1133±34
Mg (mg kg^{-1})	85±10	48±5	740±31
S (mg kg^{-1})	12.4±0.3	14.5±0.3	17.7±0.5
Total N (%)	0.47±0.01	0.52±0.02	0.82±0.03
Total C (%)	6.73±0.30	6.43±0.20	8.94±0.34

Table S2. Primers and conditions used in this study.

Primer Name	Primer sequence (5'-3')	Target gene	Thermal Profile	Molecular analysis	Reference
Arch-*amo*AF	STA ATG GTC TGG CTT AGA CG	archaeal *amoA* gene	95°C, 3min; 35×(95°C, 30s; 55°C, 30s; 72°C, 30s with plate read); Melt curve 65.0°C to 95.0°C, increment 0.5℃, 0:05+ plate read	Real-Time PCR in Fig. 2 and Fig. 3	(Francis et al., 2005)
Arch-*amo*AR	GCG GCC ATC CAT CTG TAT GT				
amoA-1F	GGG GTT TCT ACT GGT GGT	bacterial *amoA* gene	95°C, 3min; 35×(95°C, 30s; 55°C, 30s; 72°C, 30s with plate read); Melt curve 65.0°C to 95.0°C, increment 0.5℃, 0:05+ plate read	Real-Time PCR in Fig. 2 and Fig. 3	(Rotthauwe et al., 1997)
amoA-2R	CCC CTC KGS AAA GCC TTC TTC				
515F	GTG CCA GCM GCC GCG G	universal 16S rRNA genes	94°C, 5min; 32×(94°C, 30s; 54°C, 30s; 72°C, 45s); 72℃,10min; hold at 4℃	454 Pyrosequencing	(Stubner, 2002)
907R	CCG TCA ATT CMT TTR AGT TT				

Table S3. Illumina-sequencing summary of the total 16S rRNA genes in the total DNA in CF and HC soils tested.

Treatment	Replicate	High Quality Read Number	Thaumarchaeota Read Number	AOB Read number	NOB Read Number
CF Day-0	1	16611	6	29	16
	2	12696	5	25	33
	3	26041	7	45	87
CF Day-56-$^{13}CO_2$	1	15813	2	71	41
	2	15895	4	56	47
	3	18745	6	88	56
HC Day-0	1	15441	168	57	107
	2	4201	39	7	21
	3	20887	220	63	150
HC Day-56-$^{13}CO_2$	1	24060	281	241	217
	2	5688	66	45	38
	3	14160	170	121	104

Table S4. MiSeq Illumina-sequencing summary of 16S rRNA genes in SIP fractionated DNA of ^{13}C-labeled and ^{12}C-control treatments in CF and HC soils tested.

Treatment	DNA Fraction	Replicate	High Quality Read Number	Crenarchaeota Read Number	AOB Read number	NOB Read Number
CF Day-56-$^{13}CO_2$	4	1	32429	10	907	100
		2	18482	5	483	60
	5	1	24260	16	1744	100
		2	6762	5	505	28
	6	1	23056	13	3163	70
		2	8605	3	1055	20
	7	1	4758	2	787	14
		2	23193	21	3818	80
	8	1	22702	3	1360	4
		2	20840	4	1217	4
	9	1	22942	3	268	60
		2	25302	6	318	120
	10	1	34151	8	91	190
		2	23225	7	75	80
	11	1	30299	43	41	176
		2	21073	23	37	105
	12	1	18756	3	15	51
		2	22326	8	26	63
	13	1	19847	16	18	85
		2	16780	9	21	55
CF Day-56-$^{12}CO_2$	4	1	16168	7	9	31
		2	15143	4	5	22
	5	1	18677	9	8	70
		2	14454	6	6	40
	6	1	27723	8	2	45
		2	20558	6	7	25
	7	1	15189	7	15	18
		2	17895	5	12	26
	8	1	8465	4	70	7
		2	11500	5	9	14
	9	1	25638	8	1507	44
		2	9919	2	62	17
	10	1	14596	4	739	4
		2	18620	7	1425	6
	11	1	11119	12	374	3
		2	25961	22	761	1
	12	1	17867	18	20	10

		2	33444	32	11	32
	13	1	16092	10	29	4
		2	17733	13	26	7
	4	1	6916	40	70	211
		2	4024	13	9	103
	5	1	26558	350	1507	2301
		2	1482	23	62	115
	6	1	3447	30	739	141
		2	6169	44	1425	250
	7	1	11305	55	374	513
		2	22925	190	761	483
	8	1	26220	56	20	50
HC		2	12118	37	11	29
Day-56-$^{13}CO_2$	9	1	9141	0	29	22
		2	7570	6	26	13
	10	1	14976	9	9	38
		2	9873	4	5	17
	11	1	15240	4	8	20
		2	19296	6	6	31
	12	1	10658	7	2	5
		2	26134	17	7	13
	13	1	41538	83	15	52
		2	16048	25	12	17
	4	1	16954	1	23	9
		2	13328	6	21	47
	5	1	18791	23	18	23
		2	13428	10	9	11
	6	1	28825	9	14	18
		2	14207	15	4	11
	7	1	13947	12	6	15
		2	18179	7	12	9
	8	1	14823	3	214	16
HC		2	9862	4	94	32
Day-56-$^{12}CO_2$	9	1	9855	4	865	56
		2	8948	3	937	64
	10	1	19087	7	1778	933
		2	13380	9	1042	621
	11	1	7684	34	518	183
		2	14466	36	931	324
	12	1	12683	122	111	161
		2	22923	203	213	317
	13	1	13924	3	26	33
		2	10740	34	14	31

Table S5. Phylogenetic classification of dominant nitrifying phylotypes in the heavy fractions (Fractions 4 to 9) of the $^{13}CO_2$-labeled and $^{12}CO_2$-control.

Treatment	DNA Fraction	High Quality Read Number	Crenarchaeota Read Number							AOB Read number		NOB Read Number	
			29i4	29i4 -associated	54d9	N. viennensis	1.1a -associated	1.1a	1.1c	Nitrosospira sp.41	Nitrosospira sp.62	Nitrospira	Nitrobacter
CF Day-56-$^{13}CO_2$	4	50911	7	2	2	4	0	0	0	55	1335	3	157
	5	31022	6	3	1	3	2	0	4	737	1512	34	94
	6	31661	3	0	0	13	0	0	1	503	3715	54	36
	7	27951	3	2	0	17	0	0	1	441	4164	30	64
	8	43542	2	2	0	3	0	0	0	298	2279	3	5
	9	48244	3	2	0	3	0	0	1	55	531	1	179
CF Day-56-$^{12}CO_2$	4	31311	1	1	3	3	0	0	3	3	11	0	53
	5	33131	1	0	0	7	0	1	6	0	14	9	101
	6	48281	2	0	3	1	0	3	5	2	7	12	58
	7	33084	4	0	0	8	0	0	0	2	25	5	39
	8	19965	3	0	0	4	1	0	1	0	79	2	19
	9	35557	0	1	1	8	0	0	0	1	1568	36	25
HC Day-56-$^{13}CO_2$	4	10940	27	0	5	2	0	15	4	77	2	5	309
	5	28040	368	1	0	0	0	4	0	1558	11	22	2394
	6	9616	58	2	0	3	0	5	6	2139	25	7	384
	7	34230	233	0	5	3	0	0	4	946	189	15	981
	8	38338	68	7	2	5	2	0	8	27	4	30	47
	9	16711	3	0	3	0	0	0	0	52	3	12	23
HC Day-56-$^{12}CO_2$	4	30282	7	0	0	0	0	0	0	44	0	1	55
	5	32219	21	0	0	0	0	0	0	25	2	8	26
	6	43032	21	2	0	0	0	0	1	17	1	7	22
	7	32126	15	0	0	0	0	4	0	18	0	11	13
	8	24685	5	1	0	0	0	0	1	296	12	18	30
	9	18803	2	0	3	0	0	0	2	1800	2	27	93

Fig. S1. Phylogenetic trees of Thaumarchaeal 16S rRNA genes in the fractioned DNA. All archaeal 16S rRNA sequence reads from the two replicates of each fraction were pooled and classified into different OTUs using mothur software, and one representative sequence from each OTU was selected for phylogenetic analysis. Phylogenetic analysis was performed by neighbor-joining analysis using 1,000 bootstrap replicates to infer tree topology. Bootstrap values higher than 60% are indicated at branch nodes. The scale bar represents nucleotide acid substitution percentage.

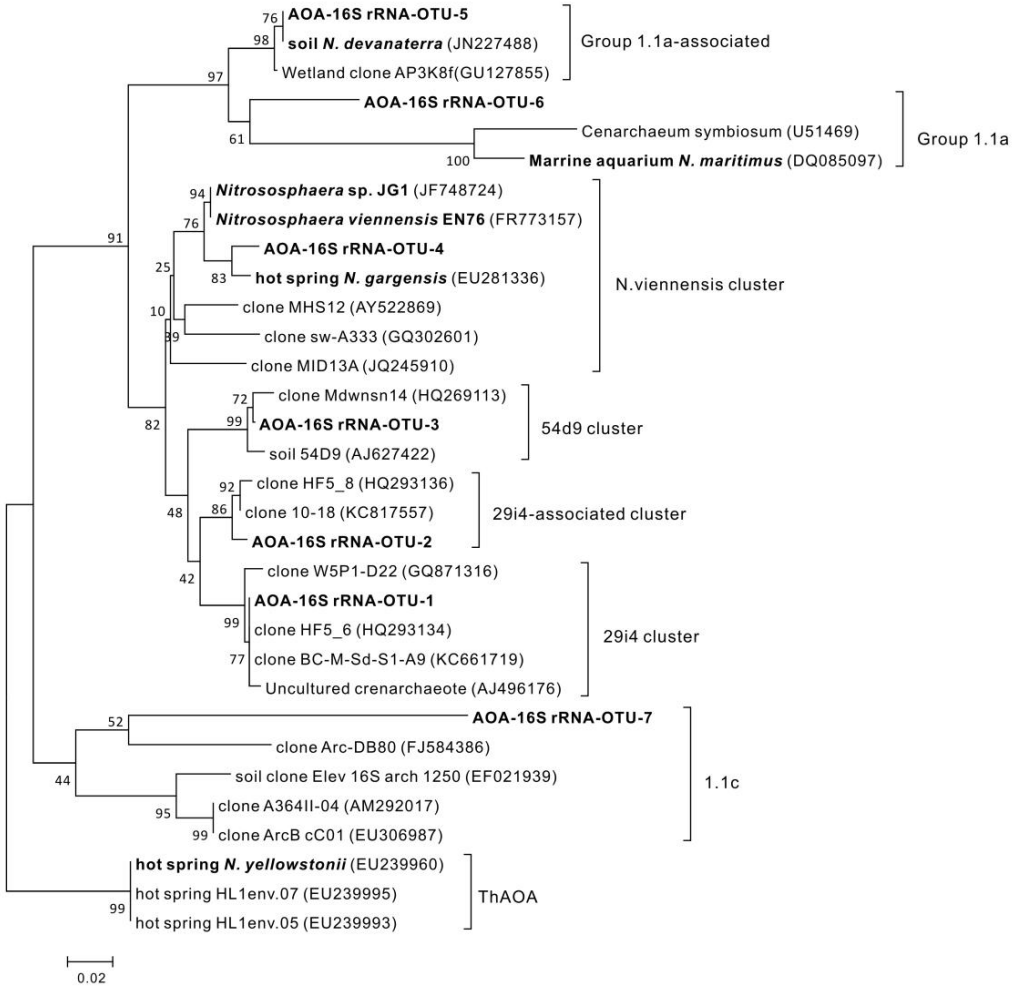


Fig. S2. Phylogenetic trees of 16S rRNA genes affiliated with ammonia-oxidizing bacteria (AOB) in the fractioned DNA. All AOB 16S rRNA sequence reads from the two replicates of each fraction were pooled and classified into different OTUs using mothur software, and one representative sequence from each OTU was selected for phylogenetic analysis.

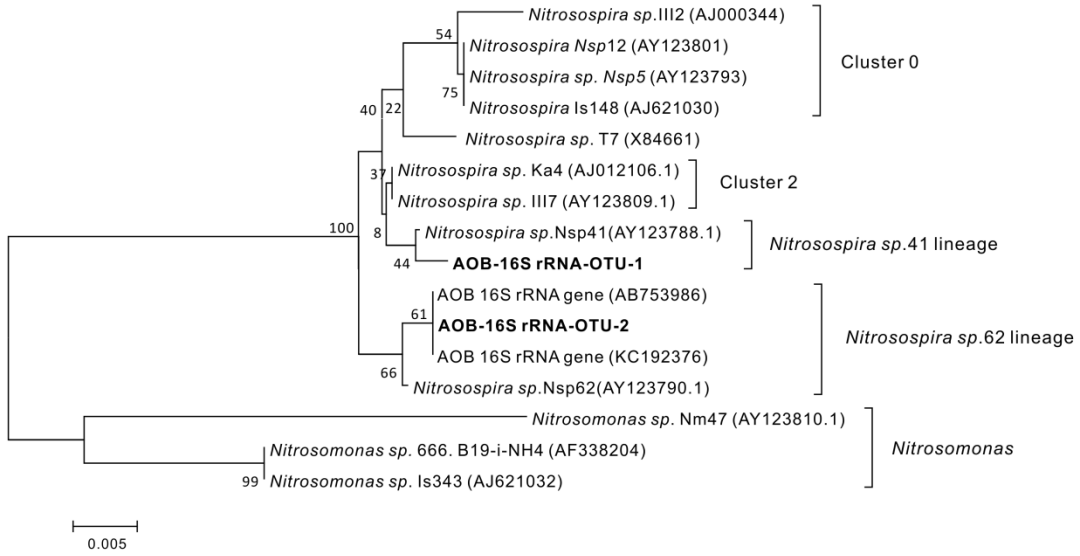


Fig. S3. Phylogenetic trees of 16S rRNA genes affiliated with nitrite-oxidizing bacteria (NOB) in the fractioned DNA. All NOB 16S rRNA sequence reads from the two replicates of each fraction were pooled and classified into different OTUs using mothur software, and one representative sequence from each OTU was selected for phylogenetic analysis.

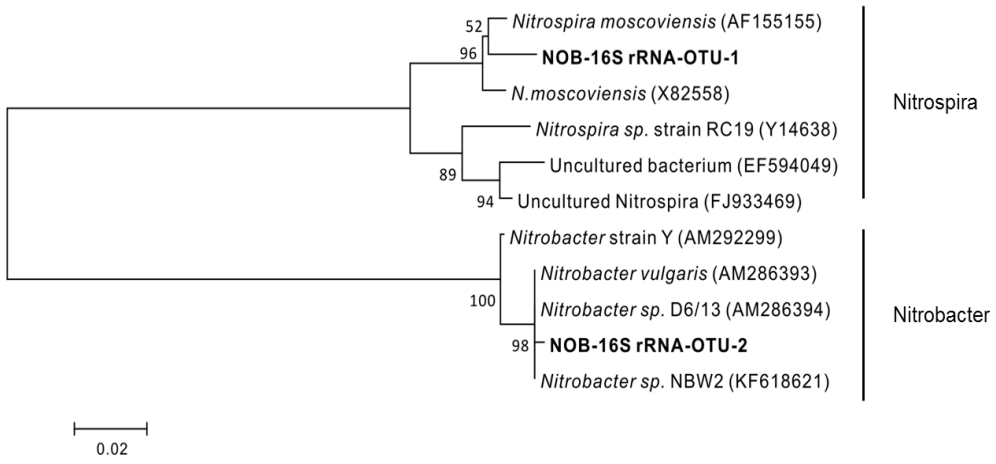
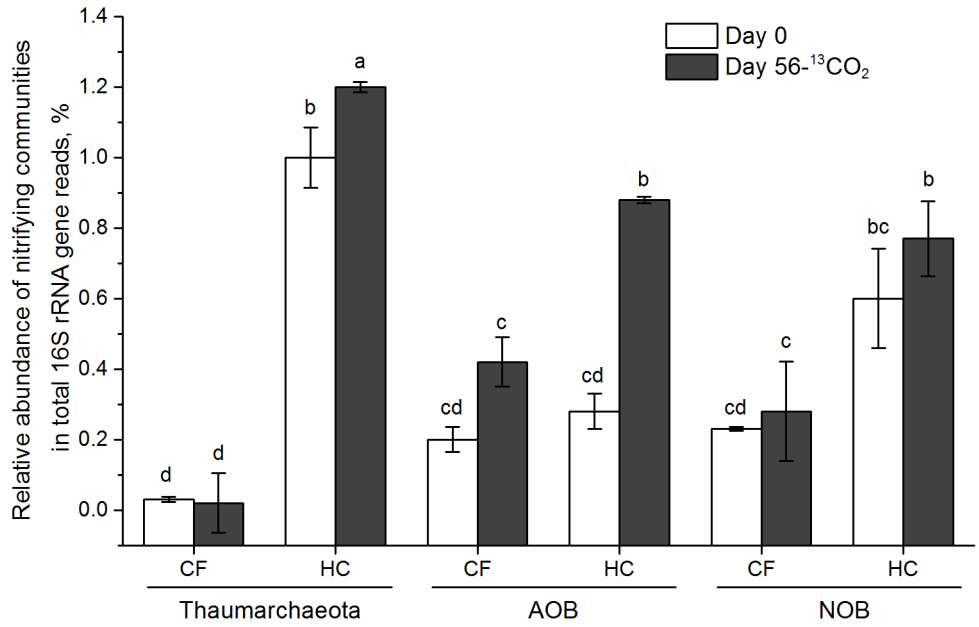


Fig. S4. Changes in relative frequencies of 16S rRNA genes of nitrifiers in total MiSeq Illumina-sequencing reads over a 56-day microcosm incubation of both CF and HC soils. Day 56-$^{13}CO_2$ represents soil microcosms incubated with 5% of $^{13}CO_2$ in the headspace. The abbreviations of CF and HC represent long-term field fertilizations with inorganic chemical fertilizers and organic cattle slurries, respectively. Bars represent standard errors of means of triplicate microcosms. Different letters indicate a significant difference ($P<0.05$).



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