Supplementary Information

Soil microbial communities as potential regulators of N_2O sources in highly acidic soils

Soil sampling

Four topsoil samples (0-20 cm) from different locations in China were sampled in the current study (Table S1). One pooled sample from five random samples were collected from each plot. The sampled soil was air-dried through a 2 mm sieve, and visible plant residues were removed manually for incubation experiments. Fresh soil samples were stored at -80 °C until needed for DNA extraction. Fresh soil samples (sieved 2 mm) were stored at 4 °C or air-dried (sieved 0.25 and 2.00 mm) for soil properties analysis.

The isotope signatures calculation

Bulk N isotopic ratios ($\delta^{15}N^{bulk}$) and intramolecular ^{15}N SP were calculated as follows:

$$\delta^{15}N^{i}=({}^{15}R^{i}_{sample}/{}^{15}R^{i}_{standard}-1)\times 1000\ (‰)\quad (i=bulk,\ \alpha) \qquad (1)$$

$$\delta^{15}N^{bulk}=(\delta^{15}N^{\alpha}+\delta^{15}N^{\beta})/2 \qquad (2)$$

$$SP=\delta^{15}N^{\alpha}-\delta^{15}N^{\beta} \qquad (3)$$

where $^{15}R^{\alpha}$ and $^{15}R^{\beta}$ represent the $^{15}N/^{14}N$ ratios at the center and ends of N_2O, respectively. SP denotes the different values of $^{15}N/^{14}N$ between central and terminal N position.

Microbial DNA extraction and real-time quantitative PCR

Soil samples were stored at -80 °C pending DNA extraction. The genomic DNA was extracted from 0.25 g fresh soil samples using FastDNA® Spin Kit for Soil (MP Biomedicals, U.S.) according to the manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA).

Quantitative PCR assays were performed using an ABI GeneAmp® 7300 PCR thermocycler (ABI, CA, USA) to estimate microbial abundances of 16S rRNA, ITS, AOA *amoA*, AOB *amoA*, *nirK*, fungi nirK, *nosZ*I and *nosZ*II. For details of gene-specific primers refer to Table S2. The qPCR was performed as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturing at 95 °C for 5 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. Each qPCR assay was carried out in a volume of 20 μl containing 10 μl 2X ChamQ SYBR Color qPCR Master Mix (Vazyme, China), 0.8 μl of each (forward and reverse) primer (5 μM), 2 μl of DNA template, 6 μl of ddH2O, and 0.4 μl of 50 X ROX Reference Dye 1. The standard curves were created with 10-fold dilution series of plasmids containing the target gene, and the R^2 of the standard curves ranged from 0.992 to 1.000. The threshold cycle values obtained for each sample were compared with the standard curve to calculate the copy number of the target gene. Sterile water was used as a negative control to replace the template. All amplifications were performed in triplicate.

Illumina MiSeq sequencing and bioinformatics analysis

Bacterial and fungal taxa were identified by amplicon sequencing of the 16S rRNA gene and the ITS fragment, respectively. To amplify the V3–V4 hypervariable region of bacteria and the ITS1 fragment of fungi, primer pairs 338F/806R and ITS1F/ITS2R were used, respectively. Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Raw sequencing data were analyzed as described before (Chen et al. 2018, Li et al. 2022). Briefly, the raw sequences were split, assembled, and processed using FLASH (Magoč & Salzberg 2011), and clustered at 97 % sequence similarity for Operational taxonomic UNITE (OTUs) with USEARCH (Edgar 2013). Then, the OTUs of bacterial and fungal were classified through BLAST in the Ribosomal Database Project database (Wang et al. 2007) and UNITE database (Kõljalg et al. 2013), respectively. To avoid bias due to sequencing depth, all samples were subsampled based on the minimum number of bacterial and fungal sequencing depths of this study. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP414779).

Table S1. Soil physical and chemical properties

Soil	Location	pH	Clay (%)	Silt (%)	Sand (%)	TN (g kg^{-1})	SOC (g kg^{-1})	C/N	Olsen-P (mg kg^{-1})	Available K (mg kg^{-1})
TP	119°10′7″E, 31°56′41″N	4.04	11.2	49.5	39.4	1.69	16.0	9.46	6.95	48
VF1	113°8′41″E, 27°47′27″N	3.51	14.4	70.1	15.4	1.89	19.0	10.0	128	125
VF2	113°26′8″E, 27°48′46″N	5.42	13.5	57.3	29.1	2.05	18.6	9.05	40.7	47
VF3	115°30′40″E, 25°47′40″N	7.95	11.8	53.6	34.6	1.28	14.7	11.5	18.8	92

Table S2. The primers, procedures and reaction systems of the PCR amplification.

Genes	**Primer**	**Primer sequence (5’→3’)**	**Product size**	**Reference**
16S rRNA	338F	ACTCCTACGGGAGGCAGCAG	470bp	(Mori et al. 2014)
	806R	GGACTACHVGGGTWTCTAAT		
ITS	ITS1F	CTTGGTCATTTAGAGGAAGTAA	268bp	(Yao et al. 2017)
	ITS2R	GCTGCGTTCTTCATCGATGC		
AOA-*amoA*	Arch-amoAF	STAATGGTCTGGCTTAGACG	636bp	(Francis et al. 2005)
	Arch-amoAR	GCGGCCATCCATCTGTATGT		
AOB-*amoA*	amoA-1F	GGGGTTTCTACTGGTGGT	492bp	(Rotthauwe et al. 1997)
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC		
nirK	nirK876C	ATYGGCGGVCAYGGCGA[a]	164bp	(Harter et al. 2014)
	nirK1040	GCCTCGATCAGRTTRTGG		
nosZ I	nosLb	CCCGCTGCACACCRCCTTCGA	309bp	(Chèneby et al. 1998)
	nosRb	CGTCGCCSGAGATGTCGATCA		
nosZ II	NosZ912F	CGTCCCCGGCCTCGTGTA	911bp	(Sanford et al. 2012)
	NosZ1853R	GAGCAGAAGTTCGTGCAGTAGTAGGG		
Fungi *nirK*	fnirK2F	GTYCAYATYGCYAACGGSATGTACGG	468bp	(Long et al. 2014)
	fnirK1R	GCRTGRTCNACMAGNGTRCGTCCC		

[a] Insertion of a cytosine (underlined) in order to increase target gene coverage.

Table S3. Published $\delta^{15}N^{bulk}$, $\delta^{18}O_{N2O}$, and SP values of N_2O involved in different N_2O production processes (Yamamoto et al. 2017).

Process	δ^{15}N (‰)	δ^{18}O (‰)	SP (‰)	Reference
Nitrification	−68.4 ~ −60.4			(Yoshida 1988)
	−67.2 ~ -13.8		35.5 ~38.9	(Yamazaki et al. 2014)
	−57.5 ~ −13.7		13.9 ~ 29.8	(Reinhard et al. 2008)
	-57.5 ~ -13.7	15.0 ~ 28.4	13.9 ~ 29.8	(Reinhard et al. 2008)
	−54.9 ~ −6.6	40.0	33.9 ~ 38.7	(Frame & Casciotti 2010)
	−46.9 ~ 5.1	22.2 ~ 45.7	27.5 ~ 37.5	(Sutka et al. 2006)
	−37.4 ~ −19.7		9.2 ~ 21.1	(Sutka et al. 2003)
	−26.9 ~ 1.4	33.9 ~ 60.2	33.9 ~ 35.6	(Heil et al. 2014)
	−19.88		27.9 ~ 29.9	(Jung et al. 2014)
	−0.3 ~ 4.8		24.9 ~ 36.7	(Sutka et al. 2003)
	-55.0 ~ -48.0	19.0 ~ 27.0		(Mandernack et al. 2009)
		13.0 ~ 35.0		(Snider et al. 2012)
Range of values	−68.4 ~ 5.1	13.0 ~ 60.2	9.2 ~ 38.9	
Bacterial denitrification	−37.0 ~ −33			(Yoshida et al. 1984)
	12.9 ~ 28.6			(Barford et al. 1999)
		40.0		(Casciotti et al. 2018)
	−37.2 ~ −7.9	4.0 ~ 23.0	−5 ~ 23.3	(Toyoda et al. 2005)
	-25 ~ 2.0	25.0 ~ 26.0	−2.5 ~ 3.7	(Sutka et al. 2006)
	−4.1 ~ −6.6		−6.8 ~ −5.0	(Ostrom et al. 2007)
	−29 ~ −20	17.0 ~ 43.0		(Snider 2011)
	11 ~ 17.1		−9.0 ~ 16.8	(Yamazaki et al. 2014)
	−23.9 ~ 2.3	22.9 ~ 47.9	−7.1 ~ −2.3	(Rohe et al. 2017)
Range of values	−37.2 ~ 28.6	4.0 ~ 47.9	−9 ~ 16.8	
Fungal denitrification	−20.0 ~ −2.6	30.2-38.6	22.8 ~ 40	(Sutka et al. 2008)
	−9.4 ~ 5.9	29.6 ~ 39		(Maeda et al. 2015)
		37.1 ~ 57.7	33.1 ~ 38.6	(Rohe et al. 2014)
		44.5 ~ 51.9		(Lewicka-Szczebak et al. 2016)
	-27.1~-0.6	33.01~38.6	31.2~35.3	(Rohe et al. 2017)
Range of values	−27.1 ~ −2.6	29.6 ~ 57.7	22.8 ~ 40	
Nitrifier denitrification	−36.2 ~ −34.9			(Yoshida 1988)
	−38.4 ~ −30.3	7.0 ~ 12.0	−8.7 ~ 8.5	(Sutka et al. 2004)
	−24.2 ~ −21.5	9.8-11.5	−4.0 ~ 1.9	(Sutka et al. 2006)
	−56.9	7.0 ~ 9.8	-10.7	(Frame & Casciotti 2010)
		13.0 ~ 35.0		(Snider et al. 2012)
Range of values	−56.9 ~ −21.5	7.0 ~ 35.0	-10.7 ~ 8.5	

Fig. S1

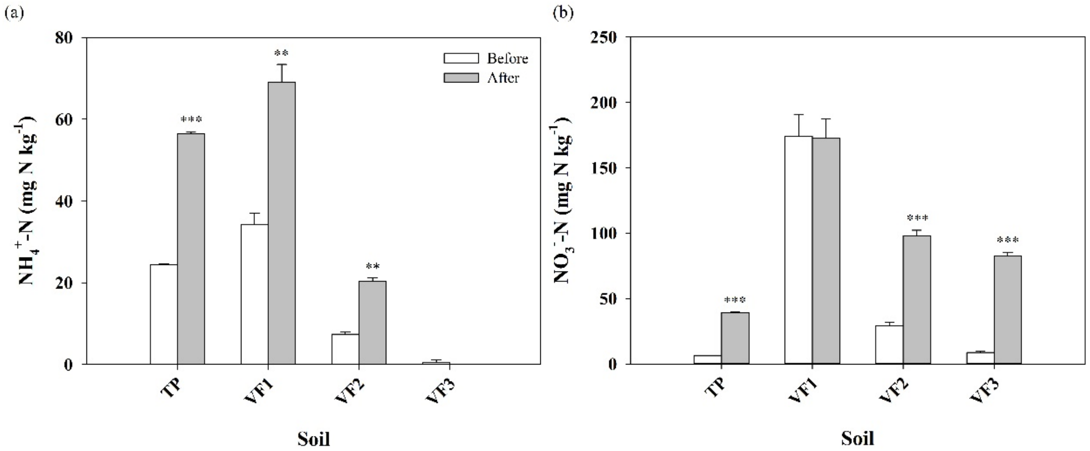

Fig. S1. Soil NH_4^+-N (a) and NO_3^--N (b) concentrations before and after incubation in different soil. **, $P<0.05$; ***, $P<0.001$.

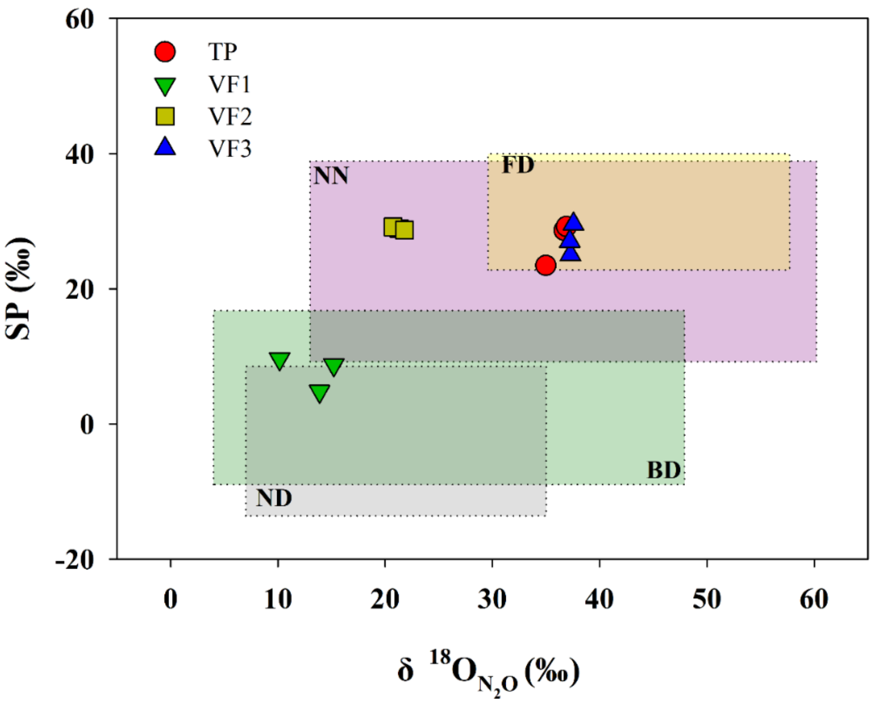

Fig. S2. SP vs. $\delta^{18}O$ of N_2O. The boxes with different colors indicate the expected ranges of different microbial processes according to published data (Table S1). The grey, green, purple, and yellow box represent ND (nitrifier denitrification), BD (bacterial denitrification), NN (nitrification), and FD (fungal denitrification) processes, respectively.

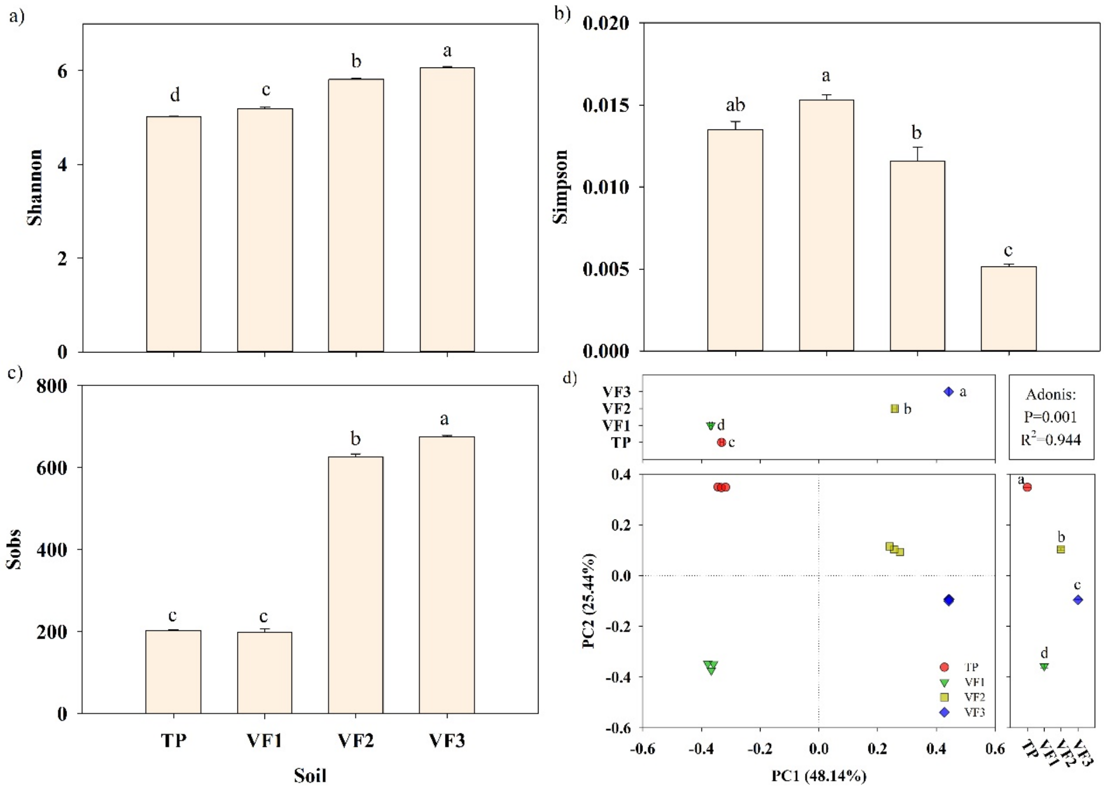

Fig. S3. The index of shannon (a), simpson (b), and sobs (c) of bacterial community in different soils. (d) Principal co-ordinates analysis (PCoA) ordination of soils bacterial community. All ordinations were performed at the OTU level. Error bars represent standard errors for n = 3. Different letters indicate significant differences at α = 0.05.

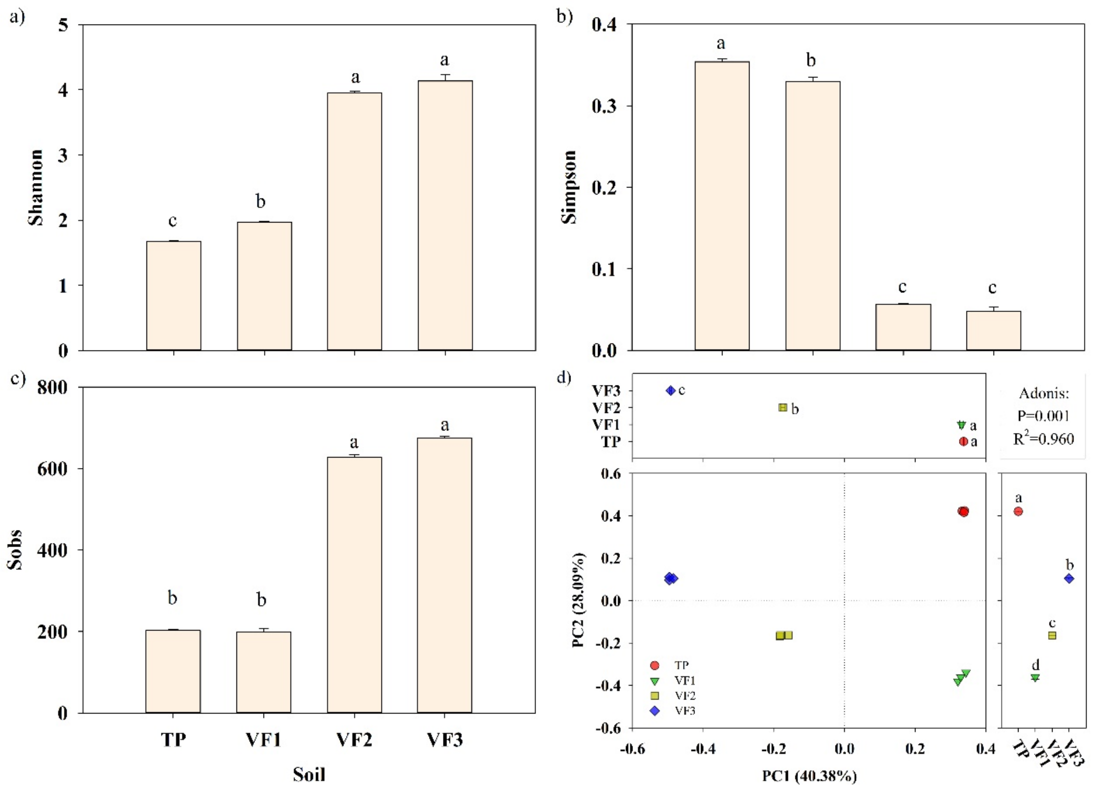

Fig. S4. The index of shannon (a), simpson (b), and sobs (c) of fungal community in different soils. (d) Principal co-ordinates analysis (PCoA) ordination of soils fungal community. All ordinations were performed at the OTU level. Error bars represent standard errors for n = 3. Different letters indicate significant differences at α = 0.05.

References

Barford CC, Montoya JP, Altabet MA, Mitchell R (1999) Steady-state nitrogen isotope effects of N_2 and N_2O production in Paracoccus denitrificans. Appl Environ Microb 65, 989-94

Casciotti KL, Forbes M, Vedamati J, Peters BD, Martin TS, Mordy CW (2018) Nitrous oxide cycling in the Eastern Tropical South Pacific as inferred from isotopic and isotopomeric data. Deep Sea Research Part II: Topical Studies in Oceanography 156, 155-167

Chen S, Zhou Y, Chen Y, Gu J (2018) fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34, i884--i890

Chèneby D, Hartmann A, H E Nault C, Topp E, Germon JC (1998) Diversity of denitrifying microflora and ability to reduce N_2O in two soils. Biol Fert Soils 28, 19--26

Edgar RC (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 10, 996--998

Frame CH, Casciotti KL (2010) Biogeochemical controls and isotopic signatures of nitrous oxide production by a marine ammonia-oxidizing bacterium. Biogeosciences 7, 2695-2709

Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB (2005) Ubiquity and Diversity of Ammonia-Oxidizing Archaea in Water Columns and Sediments of the Ocean. Proceedings of the National Academy of Sciences - PNAS 102, 14683-14688

Harter J, Krause H, Schuettler S, Ruser R, Fromme M, Scholten T, Kappler A, Behrens S (2014) Linking N2O emissions from biochar-amended soil to the structure and function of the N-cycling microbial community. The ISME journal 8, 660--674

Heil J, Wolf B, Brüggemann N, Emmenegger L, Tuzson B, Vereecken H, Mohn J (2014) Site-specific ^{15}N isotopic signatures of abiotically produced N_2O. Geochim Cosmochim Ac 139, 72-82

Jung M, Well R, Min D, Giesemann A, Park S, Kim J, Kim S, Rhee S (2014) Isotopic signatures of N_2O produced by ammonia-oxidizing archaea from soils. The ISME journal 8, 1115--1125

Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AF, Bahram M, Bates ST, Bruns TD, Bengtsson-Palme J, Callaghan TM, Others (2013) Towards a unified paradigm for sequence-based identification of fungi. Wiley Online Library

Lewicka-Szczebak D, Dyckmans J, Kaiser J, Marca A, Augustin J, Well R (2016) Oxygen isotope fractionation during N_2O production by soil denitrification. Biogeosciences 13, 1129-1144

Li N, Chang R, Chen S, Lei J, Liu Y, Cui W, Chen Q, Wu F (2022) The role of the biogas slurry microbial communities in suppressing fusarium wilt of cucumber. Waste Manage 151, 142--153

Long A, Song B, Fridey K, Silva A (2014) Detection and diversity of copper containing nitrite reductase genes (*nirK*) in prokaryotic and fungal communities of agricultural soils. Fems Microbiol Ecol 91, 1-9

Maeda K, Spor A, Edel-Hermann V, Heraud C, Breuil M, Bizouard F, Toyoda S, Yoshida N, Steinberg C, Philippot L (2015) N_2O production, a widespread trait in fungi. Sci Rep-Uk 5

Magoč T, Salzberg SL (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957--2963

Mandernack KW, Mills CT, Johnson CA, Rahn T, Kinney C (2009) The δ^{15}N and δ^{18}O values of N_2O produced during the co-oxidation of ammonia by methanotrophic bacteria. Chem Geol 267, 96-107

Mori H, Maruyama F, Kato H, Toyoda A, Dozono A, Ohtsubo Y, Nagata Y, Fujiyama A, Tsuda M, Kurokawa K (2014) Design and Experimental Application of a Novel Non-Degenerate Universal Primer Set that Amplifies Prokaryotic 16S rRNA Genes with a Low Possibility to Amplify Eukaryotic rRNA Genes. Dna Res 21, 217-227

Ostrom NE, Pitt A, Sutka R, Ostrom PH, Grandy AS, Huizinga KM, Robertson GP (2007) Isotopologue effects during N_2O reduction in soils and in pure cultures of denitrifiers. Journal of Geophysical Research 112

Reinhard W, Flessa H, Xing L, Xiaotang J, Römheld V (2008) Isotopologue ratios of N_2O emitted from microcosms with NH_4^+ fertilized arable soils under conditions favoring nitrification. Soil Biol Biochem 40, 2416-2426

Rohe L, Anderson T, Braker G, Flessa H, Giesemann A, Lewicka-Szczebak D, Wrage-Mönnig N, Well R (2014) Dual isotope and isotopomer signatures of nitrous oxide from fungal denitrification - a pure culture study. Rapid Commun Mass Sp 28, 1893-1903

Rohe L, Well R, Lewicka-Szczebak D (2017) Use of oxygen isotopes to differentiate between nitrous oxide produced by fungi or bacteria during denitrification. Rapid Commun Mass Sp 31, 1297-1312

Rotthauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microb 63, 4704-12

Sanford RA, Wagner DD, Wu Q, Chee-Sanford JC, Thomas SH, Cruz-García C, Rodríguez G, Massol-

Deyá A, Krishnani KK, Ritalahti KM, Nissen S, Konstantinidis KT, Löffler FE (2012) Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proceedings of the National Academy of Sciences 109, 19709-19714

Snider D (2011) A characterization of the controls of the nitrogen and oxygen isotope ratios of biologically-produced nitrous oxide and nitrate in soils.

Snider DM, Venkiteswaran JJ, Schiff SL, Spoelstra J (2012) Deciphering the oxygen isotope composition of nitrous oxide produced by nitrification. Global Change Biol 18, 356-370

Sutka RL, Ostrom NE, Ostrom PH, Gandhi H, Breznak JA (2003) Nitrogen isotopomer site preference of N_2O produced by Nitrosomonas europaea andMethylococcus capsulatus Bath. Rapid Commun Mass Sp 17, 738-745

Sutka RL, Ostrom NE, Ostrom PH, Gandhi H, Breznak JA (2004) Nitrogen isotopomer site preference of N_2O produced by Nitrosomonas europaea andMethylococcus capsulatus Bath. Rapid Commun Mass Sp 18, 1411-1412

Sutka RL, Ostrom NE, Ostrom PH, Breznak JA, Gandhi H, Pitt AJ, Li F (2006) Distinguishing nitrous oxide production from nitrification and denitrification on the basis of isotopomer abundances. Appl Environ Microb 72, 638--644

Sutka RL, Adams GC, Ostrom NE, Ostrom PH (2008) Isotopologue fractionation during N_2O production by fungal denitrification. Rapid Commun Mass Sp 22, 3989-3996

Toyoda S, Mutobe H, Yamagishi H, Yoshida N, Tanji Y (2005) Fractionation of N_2O isotopomers during production by denitrifier. Soil Biol Biochem 37, 1535-1545

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microb 73, 5261--5267

Yamazaki T, Hozuki T, Arai K, Toyoda S, Koba K, Fujiwara T, Yoshida N (2014) Isotopomeric characterization of nitrous oxide produced by reaction of enzymes extracted from nitrifying and denitrifying bacteria. Biogeosciences 11, 2679-2689

Yao Q, Liu J, Yu Z, Li Y, Jin J, Liu X, Wang G (2017) Three years of biochar amendment alters soil physiochemical properties and fungal community composition in a black soil of northeast China. Soil Biol Biochem 110, 56-67

Yoshida N, Hattori A, Saino T, Matsuo S, Wada E (1984) $^{15}N/^{14}N$ ratio of dissolved N_2O in the eastern tropical Pacific Ocean. Nature 307, 442--444

Yoshida N (1988) ^{15}N-depleted N_2O as a product of nitrification. Nature 335, 528--529