Soil Biology and Biochemistry 166 (2022) 108563

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Ammonia-oxidizing bacteria and fungal denitrifier diversity are associated with N_2O production in tropical soils

Késia Silva Lourenço [a,b,*], Ohana Yonara de Assis Costa [a], Heitor Cantarella [b], Eiko Eurya Kuramae [a,c,**]

[a] Microbial Ecology Department, Netherlands Institute of Ecology (NIOO), Droevendaalsesteeg 10, 6708, PB Wageningen, the Netherlands
[b] Soils and Environmental Resources Center, Agronomic Institute of Campinas (IAC), Av. Barão de Itapura 1481, 13020-902, Campinas, SP, Brazil
[c] Ecology and Biodiversity, Institute of Environmental Biology, Utrecht University, Padualaan 8, 10 3584, CH, Utrecht, the Netherlands

ARTICLE INFO

Keywords:
Concentrated vinasse
Fungi
Nitrous oxide
Greenhouse gas emission
amoA
nirK
AOB

ABSTRACT

Nitrous oxide (N_2O) production in tropical soils cultivated with sugarcane is associated with ammonia-oxidizing bacteria (AOB) and fungal denitrifiers. However, the taxonomic identities and the community diversities, compositions, and structures of AOB and fungal denitrifiers in these soils are not known. Here, we examined the effects of applying different concentrations of an organic recycled residue (vinasse: regular non-concentrated or 5.8-fold concentrated) on the dynamics of AOB and fungal denitrifier community diversity and composition and greenhouse gas emissions during the sugarcane cycle in two different seasons, rainy and dry. DNA was extracted from soil samples collected at six timepoints to determine the dynamics of *amoA*-AOB and *nirK*-fungal community diversity and composition by amplicon sequencing with gene-specific primers. Bacterial and archaeal *amoA*, fungal and bacterial *nirK,* bacterial *nirS* and *nosZ*, total bacteria (*16S rRNA*) and total fungi (*18S rRNA*) were quantified by real-time PCR, and N_2O and CO_2 emissions were measured. The genes *amoA*-AOB and bacterial *nirK* clade II correlated with N_2O emissions, followed by fungal *nirK*. The application of inorganic nitrogen fertilizer combined with organic residue, regardless of concentration, did not affect the diversity and structure of the AOB and fungal denitrifier communities but increased their abundances and N_2O emissions. *Nitrosospira* sp. was the dominant AOB, while unclassified fungi were the dominant fungal denitrifiers. Furthermore, the community structures of AOB and fungal denitrifiers were affected by season, with dominance of uncultured *Nitrosospira* and unclassified fungi in the rainy season and the genera *Nitrosospira* and *Chaetomium* in the dry season. *Nitrosospira*, *Chaetomium, Talaromyces purpureogenus*, and *Fusarium* seemed to be the main genera governing N_2O production in the studied tropical soils. These results highlight the importance of deciphering the main players in N_2O production and demonstrate the impact of fertilization on soil microbial N functions.

1. Introduction

Nitrous oxide (N_2O) emissions from agricultural systems account for approximately 60% of total anthropogenic N_2O emissions (IPCC, 2013). These high emissions are a major concern, as N_2O is both a greenhouse gas (GHG) with a global warming potential 298 times greater than that of carbon dioxide (CO_2) (IPCC, 2013) and an ozone-depleting substance (Ravishankara et al., 2009). N_2O production is the result of both abiotic processes involving chemical reactions and biotic processes via reactions mediated by microbes (Butterbach-Bahl et al., 2013; Hu et al., 2015). In agricultural soils, nitrification and denitrification are the main biotic processes of N_2O production (Goreau et al., 1980; Wrage et al., 2001).

Nitrification reactions are mediated by autotrophic ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). Ammonia oxidation is catalyzed by the enzyme ammonia monooxygenase, which is encoded by the *amoA* gene of β- and γ-proteobacteria (AOB), and by the newly described phylum *Thaumarchaeota* (AOA) (Prosser et al., 2020). However, a recently described nitrite-oxidizing bacterium from the genus *Nitrospira* can perform nitrification in a single step by oxidizing not only NO_2^- to NO_3^- but also NH_4^+ to NO_3^- (Daims et al., 2015; van Kessel et al., 2015). Under laboratory conditions, *Nitrospira inopinata* emits N_2O during nitrification at levels similar

* Corresponding author. Microbial Ecology Department, Netherlands Institute of Ecology (NIOO), Droevendaalsesteeg 10, 6708, PB Wageningen, the Netherlands.
** Corresponding author. Microbial Ecology Department, Netherlands Institute of Ecology (NIOO), Droevendaalsesteeg 10, 6708, PB Wageningen, the Netherlands.
E-mail addresses: lourencokesia@gmail.com (K.S. Lourenço), e.kuramae@nioo.knaw.nl (E.E. Kuramae).

https://doi.org/10.1016/j.soilbio.2022.108563
Received 13 August 2021; Received in revised form 12 January 2022; Accepted 14 January 2022
Available online 17 January 2022

to those of AOA but much lower than those of AOB (Kits et al., 2019). Denitrification is performed by a variety of bacteria (Philippot et al., 2007; Canfield et al., 2010; Maeda et al., 2015; Nelson et al., 2016) and produces N_2O as a byproduct (Butterbach-Bahl et al., 2013). These reduction reactions are catalyzed by a set of enzymes encoded by distinct functional genes, such as nitrate reductases encoded by *narG* or *nap*A; two entirely different types of nitrite reductases encoded by *nirK* or *nirS*; nitric oxide reductases encoded by *cnor*B or *qnor*B; and a nitrous oxide reductase encoded by *nos*Z (Philippot et al., 2007; Jones et al., 2013).

Fungi are also correlated with the production of N_2O (Maeda et al., 2015; Higgins et al., 2016) and, depending on agriculture management, can be one of the main players in N_2O production (Xu et al., 2017, 2019b; Lourenço et al., 2018b), accounting for up to 89% of emitted N_2O (Laughlin and Stevens, 2002; Chen et al., 2014; Xu et al., 2017, 2019b; Zhong et al., 2018). Fungal denitrifiers can use both NO_2^- and NO_3^- as electron acceptors, with a preference for NO_2^- (Maeda et al., 2015). Fungal enzymes responsible for reducing NO_2^- to N_2O are encoded by *nirK* and *P450nor* (a cytochrome P450 nitric oxide reductase), which differ from the bacterial *nirK* and *nor* genes (Shoun et al., 2012; Wei et al., 2015b). Fungal and bacterial denitrifying genes are closely related, but with differences in the conserved amino acids (Wei et al., 2015b), indicating that fungi most likely acquired *nirK* by gene transfer, from protomitochondrion (Kim et al., 2009). On the other hand, *P450nor* gene is unique to the fungi (Shoun et al., 2012). Denitrifying fungi generally lack the *nosZ* gene, and thus the final product is N_2O (Laughlin and Stevens, 2002; Shoun et al., 2012), making fungi significant contributors to N_2O production (Crenshaw et al., 2008; Herold et al., 2012; Mothapo et al., 2013, 2015; Chen et al., 2014), even though there is little evidence of their contribution in tropical systems. Moreover, studies that aim to quantify and identify fungi with denitrifying capacity, as well as studies of the associations of fungal denitrifiers with environmental factors are scarce. Considering recent literature by Keuschnig et al. (2020) and hypotheses on the role of *p450nor*-containing fungi e.g., secondary metabolism (Higgins et al. (2018), it is highly likely that "most fungi" are not denitrifiers. The overestimation of the fungal contribution for N_2O production is related with culture media preparation problems and biases associated with the use of antibacterial and antifungal inhibitors. In consequence, more studies are needed.

Nitrification and denitrification rates are regulated by critical factors such as soil organic carbon (C) availability and nitrogen and oxygen concentrations (Morley and Baggs, 2010; Liang et al., 2015). In sugarcane production systems, combining labile organic C with mineral N increases N_2O production (Carmo et al., 2013). Specifically, applying vinasse, an organic residue of sugarcane ethanol production that is rich in organic C (10,000–20,000 mg C L^{-1}) and organic N (357 mg N L^{-1}), with inorganic N fertilizer has been shown to drastically increase N_2O emissions from soil (Carmo et al., 2013; Pitombo et al., 2016; Lourenço et al., 2019). These increased emissions are mainly correlated to changes in the abundances of *amoA*-AOB and *nirK*-fungal genes (Lourenço et al., 2018b) as well as soil microbial community composition (Lourenço et al., 2018c, 2020). Nonetheless, recycling of vinasse, whether regular (non-concentrated, V) or concentrated (CV), is essential as a nutrient source and to alleviate the environmental impacts (water pollution) of large-scale bioethanol production from sugarcane in Brazil. Identifying the bacterial and fungal functional genes associated with N_2O production would support the development of strategies to increase the environmental sustainability of bioenergy production worldwide, by mitigation of N_2O emissions such as management practices and use of nitrification inhibitors in sugarcane cultivation system (Carvalho et al., 2021). In addition, information is needed on the dynamics of microbes related to N_2O production, such as AOB and fungal denitrifiers, in soils amended with V or CV combined with inorganic N under different weather conditions and sugarcane cropping seasons. Unlike AOB, which are mainly linked to nitrification, fungi with denitrifying capacity are usually related to multiple functions in the soil (Aldossari and Ishii, 2021), including organic matter decomposition (Higgins et al., 2016) and production of compounds such as NO, a gas used as a signal for plant invasion (Ding et al., 2020). Accordingly, the main goal of this study was to decipher the diversity of the main bacterial ammonium-oxidizers and fungal denitrifiers related to the production of N_2O in an agricultural tropical soil amended with V or CV combined with inorganic N under different weather conditions (fertilization in the spring-rainy vs. winter-dry).

2. Material and methods

2.1. Experimental setup

In the current study, a subset of samples from former experiments (Lourenço et al. (2018b) located at APTA (Paulista Agency for Agribusiness Technology), Piracicaba, Brazil, was used. The climate is humid tropical with a rainy summer and dry winters, a Cwa type according to the Köppen classification system (Critchfield, 1960). Briefly, the experiments were conducted in a rainy (RS) and in a dry season (DS) (spring-rainy vs. winter-dry). RS experiment started on December 13, 2013, while DS experiment started on August 15, 2014. Precipitation and daily temperature measurements were obtained from a meteorological station near by (~500 m) the experimental fields (Fig. S1). The soil is Ferrasol (FAO, 2015) or oxisol (USDA, 2014), and the soil properties (Camargo et al., 1986; Van Raij et al., 2001) of 0–20 cm and 20–40 cm soil layers are listed in Table 1. Sugarcane was mechanically harvested and the straw was left on top of the soil (RS: 12 Mg ha^{-1} and DS: 16 Mg ha^{-1}). Treatments in both experiments were similar: (1) Control: plot without inorganic N fertilization or vinasse; (2) N: inorganic N fertilizer; (3) V + N: regular vinasse plus inorganic N fertilizer; and (4) CV + N: concentrated vinasse plus inorganic N fertilizer. The experiment was conducted in a randomized block design with three replicate blocks (n = 3).

In both seasons, N fertilizer rate was 100 kg N ha^{-1} applied as ammonium nitrate (NH_4NO_3). Regular vinasse (V) was sprayed (1.0×10^5 L ha^{-1} or 10 L m^2) over the experimental plots following the legislation for sugarcane plantations in Sao Paulo state in Brazil (CETESB, 2014). V rate is based on the quantity of potassium uptaken by sugarcane plants (185 kg K_2O ha^{-1}) and average of potassium concentration in V (2 g K_2O L^{-1}). N fertilizer and concentrated vinasse (CV) were surface-applied (1.7×10^4 L ha^{-1}) in bands close to the plant (0.1 m), in agreement with standard practices in commercial sugarcane production. The actual vinasse rate on the fertilized band (2000 m^2

Table 1
Properties of the soils in the two seasons.

	Cm	Rainy season (RS)		Dry season (DS)	
Soil layer		0–20	20–40	0–20	20–40
Bulk density	g cm^{-3}	1.42	1.25	1.48	1.38
pH [a]	–	5.3	4.9	5.0	4.9
OM [b]	g dm^{-3}	23	19	21	15
P [c]	mg dm^{-3}	10	5	15	10
K	$mmol_c dm^{-3}$	0.5	0.4	0.7	0.4
Ca	$mmol_c dm^{-3}$	45	27	17	12
Mg	$mmol_c dm^{-3}$	20	10	12	6
H + Al [d]	$mmol_c dm^{-3}$	31	38	35	36
CEC[e]	$mmol_c dm^{-3}$	98	76	65	54
Soil texture [f]					
Clay	g kg^{-1}	619	668	631	703
Silt	g kg^{-1}	145	125	151	123
Sand	g kg^{-1}	236	207	218	174

[a] ($CaCl_2$; 0.0125 mol L^{-1}).
[b] Organic matter.
[c] Available phosphorus, K, Ca, and Mg were extracted with ion exchange resin.
[d] Buffer solution (pH 7.0).
[e] Cation exchange capacity.
[f] Soil texture determined by the densimeter method.

ha^{-1}, disregarding the interrow space, which was not fertilized) was 8.5 L m^2. The chemical characteristics of the V and CV are listed in the Supplementary Table S1.

Data of soil chemical parameters, CO_2 and N_2O gas fluxes, and qPCR data are fully described in Lourenço et al. (2018b). The methodologies used are summarized below.

2.2. Soil sampling

In both experiments, soil samples (each sample composed of six soil samplings per plot) collected throughout the season were stored at −20 °C for chemical analyses (~200 g), and soil subsamples (30 g) were stored at −80 °C for molecular analyses. As the goal of this study was to identify AOB and fungal denitrifying community members related to N_2O production, the soil samples from six timepoints (three per season) were chosen based on N_2O emissions, selecting dates when emissions were contrasting - high and low. In the RS, soil samples were collected at 7, 22 and 24 days and in DS at 11, 19, and 45 days after N fertilizer and vinasse application. For each treatment, samples were collected from 0 to 10 cm soil layer in the fertilized band close to the plant for determination of NH_4^+-N and NO_3^--N concentrations, moisture content, and pH. For each timepoint, soil temperature was measured with a digital thermometer. Water-filled pore space (WFPS) was calculated considering soil moisture and bulk density. NH_4^+-N and NO_3^--N concentrations were measured with a continuous flow analytical system (FIAlab-2500 System) (Kamphake et al., 1967; Krom, 1980).

2.3. Carbon dioxide and nitrous oxide measurements

Fluxes of CO_2 and N_2O for each timepoint were evaluated according to Lourenço et al. (2018b). Briefly, gas samples were collected in the morning between 7:00 and 13:00 a.m. GMT-3, from polyvinyl chloride (PVC) static chambers measuring 0.20 m in height and 0.30 m in diameter, inserted 0.05 m into the soil. At 1, 15, and 30 min after the chambers were closed, the gases were sampled with 60-mL plastic syringes, transferred and stored in pre-evacuated 12 mL exetainers (glass vials). Gas samples were analyzed in a gas chromatograph with a flame ionization detector for CO_2 determination and an electron capture detector for N_2O (model GC-2014, Shimadzu Co.). CO_2 and N_2O fluxes were calculated by linear interpolation of the three sampling times and adjusted by taking into account the chamber volume, air temperature, and atmospheric pressure using the "Ideal gas law" to calculate the number of moles inside each chamber and the CO_2 and N_2O concentration [1]:

$$GHG\ Flux = a^* \frac{Vm}{A} * MM \quad [1]$$

where GHG flux is CO_2 or N_2O flux (g m^{-2} h^{-1}), a ($\Delta C/\Delta t$) is the rate of change of the gas concentration inside the chamber (ppb h^{-1} or ppm h^{-1}) during the incubation time. Vm is the molar volume of the gas at the sampling temperature (L mol^{-1}), MM is the molecular mass of C in CO_2 or N in N_2O, and A is the area of soil covered by the chamber (m^2).

2.4. DNA extraction and real-time PCR

Soil samples (0.30 g) were subjected to total DNA extraction with MoBio PowerSoil DNA Isolation Kit's following the manufacturer's instructions (MoBio, Solana Beach, CA, United States). Quality and quantity of DNA were checked and quantified using a 1% (w/v) agarose gel under UV light and Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States). The abundance of the *amoA*-AOB, *nirK*-fungi genes, and total bacterial and fungal communities were quantified in duplicate by real-time PCR with a BIORAD CFX96 TouchTM Real-Time PCR Detection System. Amplification and thermal cycler conditions are described in Supplementary Table S2. It is worth to mention that there is variability of primers efficiency in different soils. For the soil of the current study, the primers designed by Long et al. (2015) for *nirK*-fungi were the most suitable. The qPCR amplicon products were checked by agarose gel electrophoresis. The efficiencies of the *amoA*-AOB, *16S rRNA*, *nirK*-fungi, and *18S rRNA* qPCR were 88% (R^2 = 0.98), 96% (R^2 = 0.99), 85% (R^2 = 0.98), and 88% (R^2 = 1.00), respectively (Table S3). Each qPCR run included a DNA template, a standard positive control and a negative control. Plasmid DNA containing fragments of bacterial *amoA* and fungi *nirK* genes and *16S rRNA* and *18 rRNA* were used as standards (Soares et al., 2016). The primers used for archaea *amoA* and bacteria *nirS*, *nirK*, and *nosZ* amplification and the PCR conditions are listed in Supplementary Table S2.

2.5. Amplicon sequence processing of amoA-ammonia-oxidizing Bacteria and nirK-Fungal denitrifiers

The total soil DNA used for qPCR was also used for amplification of *amoA*-AOB and *nirK*-fungal genes using the same primers, the conditions of amplification are shown in Supplementary Table S2. Dual-index and Illumina sequencing adapters were attached to the amplicons. After library quantification, normalization and pooling, MiSeq V3 reagent kits were used to prepare the samples for MiSeq sequencing. The samples were amplified and sequenced on the Illumina MiSeq System at Genome Québec, Montréal, Canada.

Raw data of bacterial *amo*A-AOB and fungal *nirK* sequences were preprocessed using Mothur v 1.3.3 (Schloss et al., 2009). Raw sequences were merged (make.contigs command), then trimmed and sorted simultaneously (trim.seqs). Sequences were filtered out if average read quality was lower than 25, there were more than two N's or if the read length was shorter than 150 bp; remaining sequences were filtered based on primer quality (≤2 errors), spacers (≤2 errors), and barcodes (≤1 error). Barcodes and primers were removed. Further, the sample reads were processed using the UCLUST pipeline implemented in a Snakemake workflow available upon request (Edgar, 2010). In summary, the bacterial *amo*A and fungal *nirK* sequences were truncated to 480 bp and singletons and chimeras were removed (Norton et al., 2002). OTU tables were created at the 90% and 97% cut-off level (phylum = 75%, class = 78.5%, order = 82%, family = 86.5%, genus = 94.5%, species/OTU = 90% for *amoA* and 97% for *nirK*), similar to other studies (Lourenço et al., 2018a; Xu et al., 2019a). Biom-format OTU tables and representative OTU sequences were imported into QIIME 2 (Bolyen et al., 2019). The plugin classify-sklearn (Pedregosa et al., 2011) was used to classify the taxonomic lineage of the representative sequences of each specific gene based on the corresponding database. Custom bacterial *amoA* and fungal *nirK* databases were built using NCBI sequences (NCBI Resource Coordinators, 2016) and an in-house pipeline. For the fungal *nirK* dataset, contaminating bacterial sequences were removed using the plugin "qiime taxa filter-table". In average, 68% of the total reads belonged to bacteria and they were removed from the dataset. The OTU tables were converted into tab separated values (tsv) format and exported using the BIOM package (McMurdie and Paulson, 2020). In addition, a second OTU table was build using all the sequences to explore the *nirK* from bacteria, and the sequences were clustered against taxonomic lineage of the representative sequences of bacterial *nirK* clades I, II and III.

2.6. Phylogenetic trees

Phylogenetic trees were constructed for the representative sequences of the 30 most abundant since they represent 99.5 and 91% of the bacterial *amoA* and fungal *nirK* OTUs, respectively. Reference sequences from close neighbors were downloaded from NCBI (NCBI Resource Coordinators, 2016) and included in the analysis. The sequences were aligned using the software MAFFT v7.455 (Katoh and Standley, 2013), using the L-INS-I strategy (Katoh et al., 2005). Maximum likelihood phylogenetic inference was performed in IQ-TREE 1.6.12 (Nguyen et al.,

2015), with automatic selection of the best-fit nucleotide evolution model (Kalyaanamoorthy et al., 2017). The best-fitted model parameters defined by Bayesian Information Criterion were K3Pu + F + G4 for bacterial *amoA* and TPM3u + F + G4 for fungal *nirK* datasets. Edge support was estimated with an ultrafast bootstrap test (UFBoot2, 1000 standard replicates) (Hoang et al., 2018). Archaeal *Nitrososphaera viennensis* amoA gene (CP007174) was used as an outgroup for bacterial *amoA* sequences, and bacterial *Nitrosomonas europaea* nirK gene (EF016124) was used as an outgroup for the nirK gene dataset. The Interactive Tree of Life (iTOL) (Letunic and Bork, 2016) platform was used to visualize and annotate the trees.

2.7. Statistical analyses

Generalized linear models (Bolker et al., 2009) were used to test the effect of different treatments on N_2O fluxes, *amoA,* and *nirK*-fungi gene copy numbers using the "multcomp" package (Hothorn et al., 2008) in R. Differences between treatments were analyzed within each sampling event ($p < 0.01$). As criteria to the generalized linear models, to account for the increasing variation with the increase in mean, we used Gamma (N_2O emission) and Poisson family (*amoA*-AOB, *nirK*-Fungi, 16S rRNA, and 18S rRNA genes copy number) distributions. Subsequently, the "glht" function was used to evaluate the differences among treatments (Tukey $p < 0.01$). The correlation between N_2O flux and gene abundances was calculated by Spearman correlation analysis in SigmaPlot, version 13.0 (SystatSoftware, 2014). ANOVA and Tukey test ($p < 0.05$) were used to test differences between treatments in each sampling event on CO_2-C, NH_4^+-N, NO_3^- -N, and pH. Primers for fungal denitrifier quantification are degenerated and can also amplify bacterial *nirK* (Ma et al., 2019). Accordingly, abundance of the fungi-*nirK* from qPCR analysis was normalized based on the relative abundance of *nirK* fungi gene from the sequence samples, a procedure similar to that of Cassman et al. (2018). We assumed that the sequence data represented the proportions of individuals in each sample and that qPCR determined the total number of copies of the target gene. Based on these assumptions, a phylogenetic tree was inferred using the sequence data, and the proportions of *nirK* from bacteria and fungi were estimated, after that the total number of copies of *nirK*-fungi and *nirK*-bacteria were adjusted for each sample.

Rarefaction curves from non-rarefied data using the sequence sample size and number of different OTUs were used to indicate whether the measurement depth had met the requirements (Fig. S2). Estimates of alpha and beta diversities were calculated in RStudio version 1.0.136 running R version 3.3.1. using the "phyloseq" package (McMurdie and Holmes, 2013). Estimates for alpha-diversity included Richness, Chao1, Simpson, and Shannon diversity indices (Chao, 1984). ANOVA and Tukey test ($p < 0.05$) were used to test the differences among treatments. We rarefied the samples to 1253 and 111 reads for AOB and fungal denitrifying communities due to the low abundance of reads. However, for both datasets, the analyses were done using rarefied and non-rarefied data, that resulted in similar results. Therefore, we presented the results from rarefied data, and all data was Hellinger transformed. Hellinger-transformed data was used to test the effect of season and treatments on AOB bacterial and fungal denitrifying community compositions.

3. Results

3.1. Weather conditions, greenhouse gas emissions, and soil analysis

The climatic conditions during the RS and DS experiments are shown in Supplementary Fig. S1. The air temperature reached lows of 14.7 °C and 7.4 °C and highs of 36.2 °C and 35.7 °C in RS and DS, respectively, and the mean temperature was 25.9 °C in the 30-day RS experiment and 21.7 °C in the 50-day DS experiment (Fig. S1). The soil temperatures on the sampling days ranged from 24.0 to 26.2 °C in RS and 19.0–22.7 °C in DS. The water-filled pore space (WFPS) values were similar between the two seasons, with the low WFPS on the first sampling day varying from 63.4 to 87.5% in RS and 61.9–86.9% in DS.

Compared with the treatment with only inorganic N and the control, both CO_2 and N_2O emissions were higher in the treatments with vinasse (V or CV) plus inorganic N (Figs. S3a and b). In both seasons, the N_2O emission rate was low on the first sampling day (day 7 in RS and day 11 in DS) (Table 2, Figs. S3c and d) due to the lack of rain during the previous period (Fig. S1). The mineral soil N patterns differed between the seasons: in RS, both total NH_4^+-N and NO_3^--N content decreased as the experiment progressed (Figs. S3e and g), whereas in DS, total NH_4^+-N content decreased while NO_3^--N content increased (Figs. S3f and h). The variation of soil pH among the treatments was low and similar in the two seasons; over time, pH decreased in the inorganic N and V + N treatments but increased slightly in the CV + N treatment (Figs. S3i and j). N_2O emissions were correlated with CO_2 emissions ($r_s = 0.69$) and various environmental parameters, including WFPS, pH, NH_4^+-N content and NO_3^--N content ($r_s = 0.51$, −0.20, 0.20, and 0.51, respectively) (Fig. 1, Table S4).

3.2. Correlations of N_2O production with abundances of bacterial and fungal N-Cycle genes

At the timepoints analyzed in this study, the abundances of *amoA*-AOB ($r_s = 0.38$) and *nirK*-fungi ($r_s = 0.24$) were significantly correlated with N_2O emissions ($r_s = 0.38$ and 0.24, respectively) and WFPS ($r_s = 0.35$ and 0.20, respectively) (Fig. 1). Furthermore, exploring the *nirK* sequence data and qPCR analyses, the N_2O emission was also correlated with *nirK*-bacteria clade II ($r_s = 0.39$) (Fig. 1 and Fig. S4). Regardless of concentration, the addition of vinasse plus inorganic N (CV + N or V + N) significantly increased *amoA*-AOB copy numbers by more than 1-, 7-, and 6-fold at days 7, 22, and 24 in RS and 2-, 8-, and 50-fold at days 11, 19, and 45 in DS compared with the control (Table 2). However, the total bacterial abundance (*16S rRNA* copy number) was similar in all treatments and both seasons. The ratio of bacterial abundance (16S rRNA gene) to *amoA*-AOB gene copy number differed among the treatments and was the lowest in the V + N and CV + N treatments (Table 3), suggesting an increase in *amoA*-AOB gene copy number in these treatments. The *nirK*-fungi gene abundance also increased in both treatments with vinasse and inorganic N (V + N and CV + N, Table 3), as did the ratio of fungal abundance (18S rRNA gene) to *nirK*-fungi gene abundance. The increase in this ratio was due to the higher 18S rRNA gene copies in these treatments.

We obtained a total of 1,365,783 high-quality *amoA*-AOB sequences from 72 samples (2 seasons x 4 treatments x 3 timepoints x 3 replicates), with an average of 18,969 reads per sample clustered into 83 OTUs. Rarefaction curves indicated that the *amoA*-AOB community diversity was adequately captured by our sequencing depth (Fig. S2a). Season but not treatments or timepoints affected AOB alpha-diversity and community structure (OTU richness, Chao1, Simpson, and Shannon) (Table S5, Fig. S5). PERMANOVA ($p = 0.00$) revealed a clear separation at the OTU level by season (Table S6), but no interaction was found between treatment and time (PERMANOVA, RS: $p = 0.73$ and $p = 0.89$, DS: $p = 0.65$ and $p = 0.09$, respectively) (Table S6). The AOB community present in the soil was composed mainly of the class β-*Proteobacteria*, family *Nitrosomonadaceae* and genus *Nitrosospira* (Fig. 2). In the phylogenetic tree, 91% of *amoA* OTUs clustered with the genera *Nitrosospira*, *Nitrosolobus,* and *Nitrosovibrio* (now included in the genus *Nitrosospira*) (Fig. 3).

A total of 168,366 high-quality *nirK*-fungi sequences were obtained, with an average of 2200 reads per sample clustered into 252 OTUs. Despite the low number of reads, treatments but not season or time affected the alpha-diversity of the fungal *nirK* community (OTU richness, Chao1, Simpson, and Shannon) (Table S5, Fig. S6). PERMANOVA ($p = 0.00$) and ANOSIM ($p = 0.00$) revealed a separation at the OTU level by season (Table S6), but no difference was found among

Table 2
Abundances of ammonia-oxidizing bacterial (*amoA*-AOB) and fungal denitrifier (*nirK*) genes and nitrous oxide fluxes in the different treatments: Control; N, inorganic fertilization with ammonium nitrate; V + N, regular vinasse plus ammonium nitrate; and CV + N, concentrated vinasse plus ammonium nitrate.

Treatments	Rainy season (RS)						Dry season (DS)					
	N_2O-N[a]		*amoA-AOB*[b]		*nirK*-fungi[c]		N_2O-N		*amoA-AOB*		*nirK*-fungi	
	Day 7						Day 11					
Control	0.26	±0.28 ab	2.4	±3.0c	14.5	±4.1c	−0.07	±0.12b	7.1	±2.8d	87.2	±61.6b
N	0.09	±0.31b	4.3	±3.7a	3.4	±2.0d	0.11	±0.03b	12.8	±6.5b	29.5	±7.1d
V + N	0.39	±0.52 ab	3.0	±2.1b	19.1	±9.5b	0.70	±0.09a	12.3	±5.1c	49.8	±17.2c
CV + N	3.77	±3.44b	0.5	±0.5d	55.1	±16.1a	0.33	±0.05b	15.0	±6.6a	143.0	±88.1a
	Day 22						*Day 19*					
Control	0.08	±0.01b	3.8	±1.5c	5.2	±2.7c	0.11	±0.03b	2.8	±1.1d	36.7	±6.3d
N	4.22	±0.62a	8.9	±12.0b	13.7	±6.9b	0.35	±0.09b	38.6	±12.4a	66.3	±39.7b
V + N	3.53	±2.76a	2.0	±1.6d	4.1	±1.6d	23.71	±7.95a	11.6	±3.5c	178.8	±58.0a
CV + N	11.98	±7.87a	72.0	±75.0a	16.5	±3.2a	40.22	±7.04a	15.4	±8.6b	64.0	±33.9c
	Day 24						*Day 45*					
Control	0.13	±0.12c	9.4	±8.9c	15.5	±10.5b	0.24	±0.10b	2.4	±0.5d	28.0	±10.8c
N	3.79	±0.21b	6.1	±6.3d	4.9	±3.4c	8.34	±2.60a	41.4	±22.1c	231.5	±195.7a
V + N	3.67	±1.85b	32.0	±44.0b	15.2	±12.3b	8.93	±1.09a	71.5	±14.0b	61.9	±19.5b
CV + N	21.12	±13.55a	140.0	±213a	45.5	±19.7a	27.54	±14.65a	247.4	±146.9a	25.8	±12.3d

[a] mg N m^{-2} d^{-1}; Values followed by different letters are significantly different at $p \leq 0.05$ using the Tukey test.
[b] x10^6 gene copies g^{-1} dry soil.
[c] x10^2 gene copies g^{-1} dry soil. Fungi-*nirK* abundance was normalized based on the relative abundance of *nirK* fungi gene from the sequence samples.

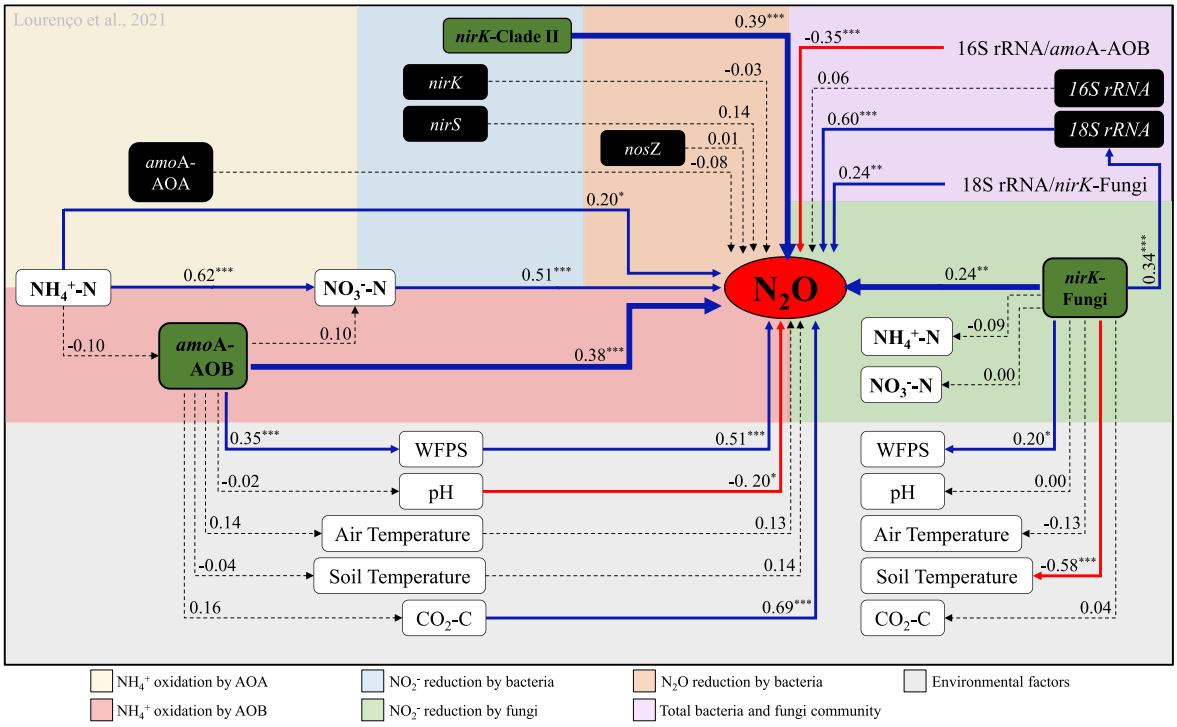

Fig. 1. Spearman's correlation coefficients (r_s) (neglecting season and sampling time) among N_2O emission fluxes (mg m^{-2} day^{-1}); abundances of *amoA* (bacterial and archaeal), *nirK* (fungal and bacterial clade I and II), *nirS* and *nosZ* (bacterial), total bacterial 16S rRNA and total fungal 18S rRNA (gene copies per g dry soil); and abiotic factors, mineral N, pH, air and soil temperatures, and CO_2-C emissions. Abbreviations: WFPS: water-filled pore space; NH_4^+-N: ammonium; NO_3^--N: nitrate; AOB: *amoA* of ammonia-oxidizing bacteria; AOA: *amoA* of ammonia-oxidizing archaea. Blue bold lines indicate significant positive correlations, red bold lines indicate significant negative correlations, and dotted lines indicate no correlations between variables (n = 72). Significant differences: *$p \leq 0.10$, **$p \leq 0.05$, and ***$p \leq 0.01$. The data are taken in part from Lourenço et al. (2018b). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

treatments (PERMANOVA: p = 0.12 and p = 0.15, respectively) or timepoints (PERMANOVA: p = 0.67 and p = 0.15) in RS and DS, respectively. No interaction was observed between treatment and time in RS and DS (PERMANOVA: p = 0.13 and p = 0.40, respectively) (Table S6). The fungal denitrifying community present in the soil was mainly dominated by members of the Ascomycota phylum belonging to the families *Chaetomiaceae, Trichocomaceae, Aspergillaceae,* and *Nectriaceae*, with a predominance of the genera *Chaetomium* and *Talaromyces purpureogenus* (Fig. 4). The majority of the OTUs were affiliated with environmental fungi with no taxonomic classification; the 30 most abundant fungal *nirK* OTUs are depicted in the phylogenetic tree in Fig. 5. The relative abundances of the genera *Talaromyces purpureogenus* ($r_s = 0.20$; p = 0.09) and *Fusarium* ($r_s = 0.27$; p = 0.02) were positively correlated with N_2O emissions, whereas the abundances of *nirK*-fungi from environmental samples ($r_s = -0.20$; p = 0.09) and the genus *Aspergillus* ($r_s = -0.19$; p = 0.11) were negatively correlated with

Table 3
Comparison of the ratios of the abundances of *16S rRNA* to ammonia-oxidizing bacteria (*amoA*-AOB) and of *18S rRNA* to fungal denitrifiers (*nirK*) in different time points and treatments: Control; N, inorganic fertilization with ammonium nitrate; V + N, regular vinasse plus ammonium nitrate; and CV + N, concentrated vinasse plus ammonium nitrate.

Treatments[a]	Rainy season (RS)						Dry season (DS)					
	Day 7		Day 22		Day 24		Day 11		Day 19		Day 45	
	*16S rRNA/amo*A-AOB						*16S rRNA/amo*A-AOB					
Control	1328	±1059c	1439	±527c	1071	±1164b	13891	±23017b	4565	±3514a	478	±292a
N	2320	±3579b	2742	±3507b	1831	±2097a	18332	±30151a	2974	±4889b	180	±212b
V + N	1234	±1373d	5734	±7133a	466	±376d	998	±1008d	1197	±1378c	26	±5d
CV + N	9421	±7656a	239	±236d	650	±965c	1351	±1466c	2005	±1498d	89	±75c
	18S rRNA/nirK-Fungi						*18S rRNA/nirK*-Fungi					
Control	4058	±421d	83507	±61485b	14372	±5261d	2183	±969d	3129	±908c	5789	±4631b
N	46754	±23929a	374611	±367816a	68836	±36839a	4941	±3048c	7939	±7225b	2368	±1618d
V + N	12731	±7264c	27739	±12023d	29075	±21038c	5136	±895b	2216	±979d	4698	±812c
CV + N	24975	±14172b	75028	±27344c	32629	±25461b	12259	±7186a	31037	±10473a	27437	±6314a

[a] Values followed by the same lowercase letter in the column are not significantly different at $p \leq 0.05$ using the Tukey test.

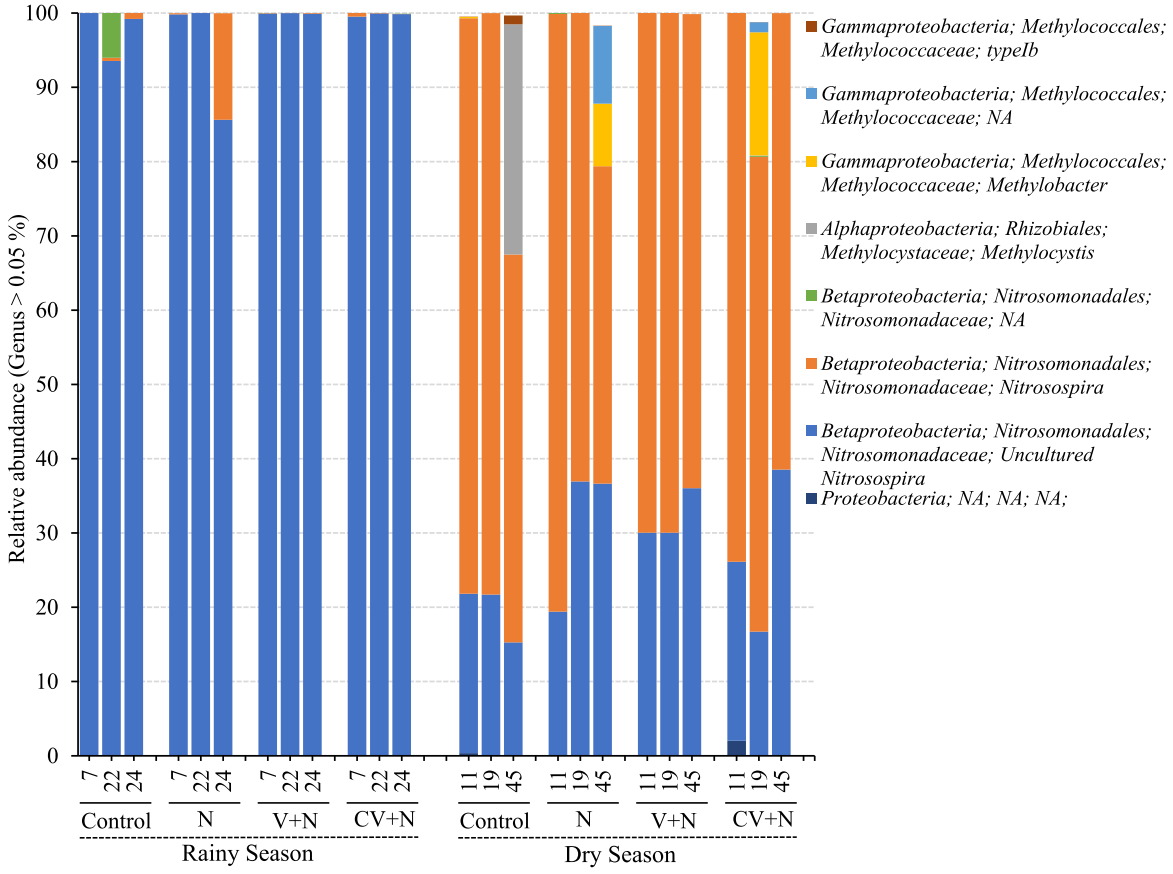

Fig. 2. Sequence abundances of *amoA* genes of ammonia-oxidizing bacteria (AOB) (>0.05%) using normalized values between 0 and 100 in different time points and treatments: Control; N, inorganic fertilization with ammonium nitrate; V + N, regular vinasse plus ammonium nitrate; and CV + N, concentrated vinasse plus ammonium nitrate.

N_2O emissions. However, the same primer amplified 429,485 high-quality *nirK*-bacterial sequences, with an average of 6049 reads per sample clustered into 867 OTUs. From the total of OTUs, 532 were from the bacterial *nirK* clade II, one from bacterial *nirK* clade Ic (*Pseudomonas* sp.), and 334 OTUs are an unidentified bacterial *nirK* (Fig. S7).

4. Discussion

Nitrification and denitrification processes are mediated by several microorganisms carrying a variety of genes that encode different enzymes. Depending on the N_2O production pathway, N_2O can be an indirect (nitrification) or direct product (denitrification). Our results showed that the abundances of the AOB *amoA* gene, bacteria clade II *nirK* gene and fungal *nirK* gene were significantly correlated with N_2O emissions ($r_s = 0.38$, $p \leq 0.01$, $r_s = 0.39$, $p \leq 0.01$, and $r_s = 0.24$, $p \leq 0.05$, respectively) and WFPS ($r_s = 0.35$, $p \leq 0.01$ and $r_s = 0.20$, $p \leq 0.10$, respectively) (Fig. 1), indicating that AOB, denitrifier bacteria clade II and fungal denitrifiers are related with N_2O production in tropical soil. Lourenço et al. (2018b) study on a sugarcane field using 4.5 times more soil samples (n = 324), showed that the impact of each process (AOB and AOA nitrification, bacterial and fungal denitrification) differed and contributed to N_2O production, depending on soil pH, soil

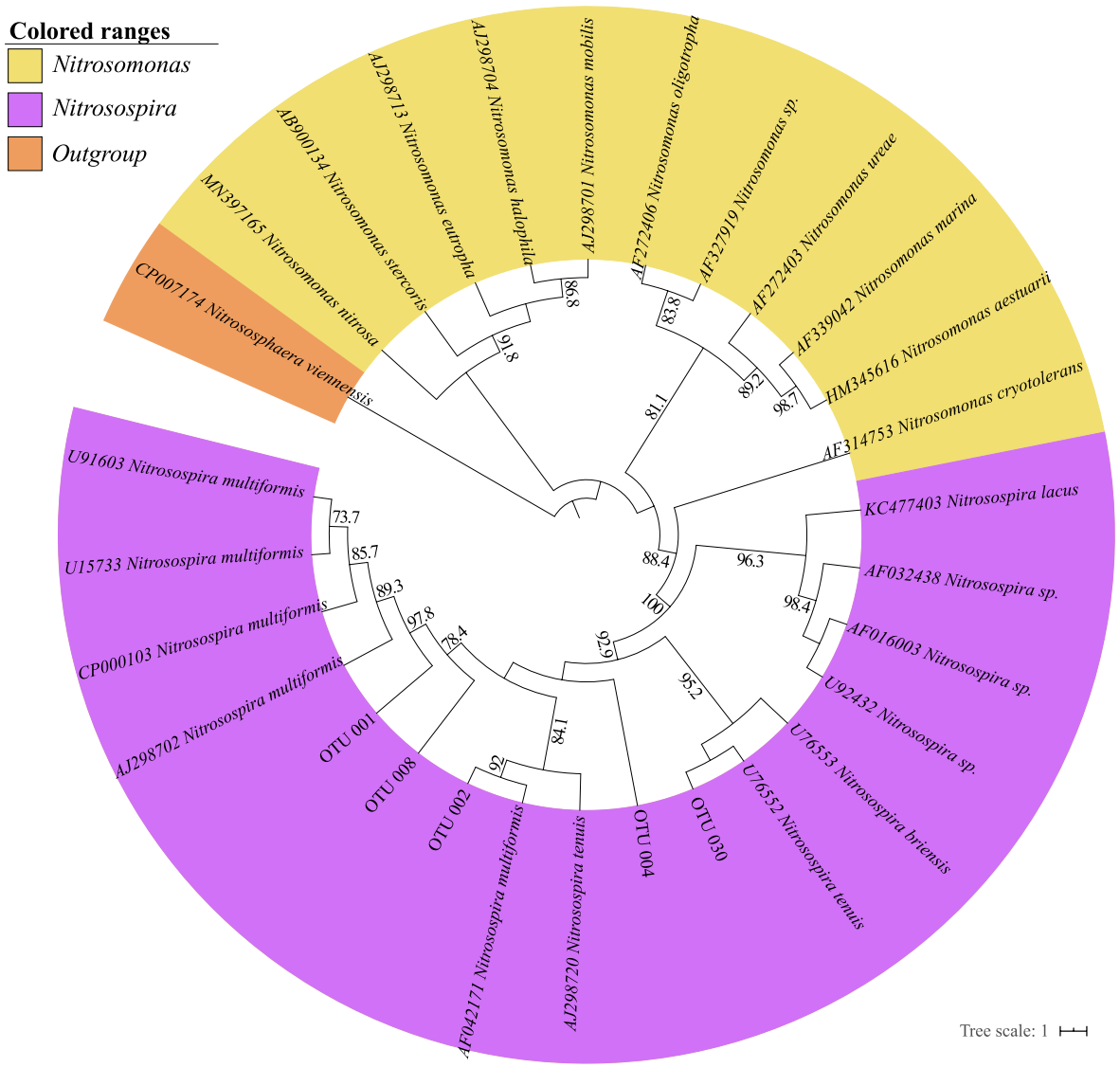

Fig. 3. Maximum-likelihood phylogenetic tree based on the top 30 *amoA*-AOB OTUs (91% of the community) and representative sequences and reference sequences from the NCBI database, with *Nitrososphaera viennensis* as an outgroup. The sequences were aligned using the software MAFFT v7.455 (Katoh and Standley, 2013) employing the L-INS-I strategy (Katoh et al., 2005). The software IQ-TREE 1.6.12 (Nguyen et al., 2015) was used to build the dendrogram based on maximum-likelihood inference with 1000 Ultrafast bootstraps (Hoang et al., 2018). Ultrafast bootstrap values > 0.70 are shown.

moisture and N sources. However, *amoA*-AOB and fungal *nirK* were the most important genes related to N_2O emissions. It is worth to mention that the fungal *nirK* primers used by Lourenço et al. (2018b) for the qPCR analyses might have generated biased results due bacterial *nirK* clade II amplification. In the current study, using the same primers but with an improved approach by combining qPCR and sequence data, we showed that fungi as well as denitrifier bacteria can be related with N_2O production in the same tropical soils.

Well-drained soils such as oxisols, which are common in areas of sugarcane production in Brazil, favor N_2O production by AOB during nitrification (Soares et al., 2016). However, a cascade of events occurs leading to N_2O production also by denitrification. After the first harvest, sugarcane plants can regrow up to five times (ratoon stage), leaving large amounts of straw (Carvalho et al., 2017). Thus, areas with sugarcane in the ratoon stage, similar to the current study site (straw levels of 12 t ha^{-1} and 16 t ha^{-1} in RS and DS), promote high fungal growth due to the functions of these microorganisms in organic matter decomposition (Lourenço et al., 2018b, 2020; Morais et al., 2019), including fungi related to N_2O production (Senbayram et al., 2018; Lourenço et al., 2020). Although denitrification rather than nitrification predominates in environments with low O_2 concentrations, fungal denitrifiers can release N_2O under both anaerobic and aerobic conditions (Shoun et al., 2012). The majority of denitrifier microbes are facultative aerobic heterotrophs that switch from aerobic respiration to denitrification under low oxygen, during which NO_3^- and NO_2^- are used as electron acceptors. Accordingly, it is possible that under high NO_2^- accumulation during nitrification, fungal denitrifiers can increase N_2O production. Fungi denitrifiers seem to prefer NO_2^- over NO_3^- (Maeda et al., 2015). Further evidence of the importance of fungi for N_2O production is provided by the effects on N_2O emissions of adding organic C in the form of vinasse. Unlike fungi, AOB are generally autotrophic microbes and do not use the organic C from vinasse as a source of C. Fujimura et al. (2020) evaluated the community composition of denitrifying bacteria and fungi across different land-use types (paddy, cropland, and forest soils) using the *nirK* gene as a target and concluded that *nirK*-fungi were not found in paddy soils, suggesting a preference of fungal denitrifiers for soils under O_2 limited condition. The positive correlations between N_2O emissions and the abundances of *amoA*-AOB and *nirK*-fungi in the present study, although not strong, are an indication that the AOB and fungal denitrifying communities are responsible for N_2O production in

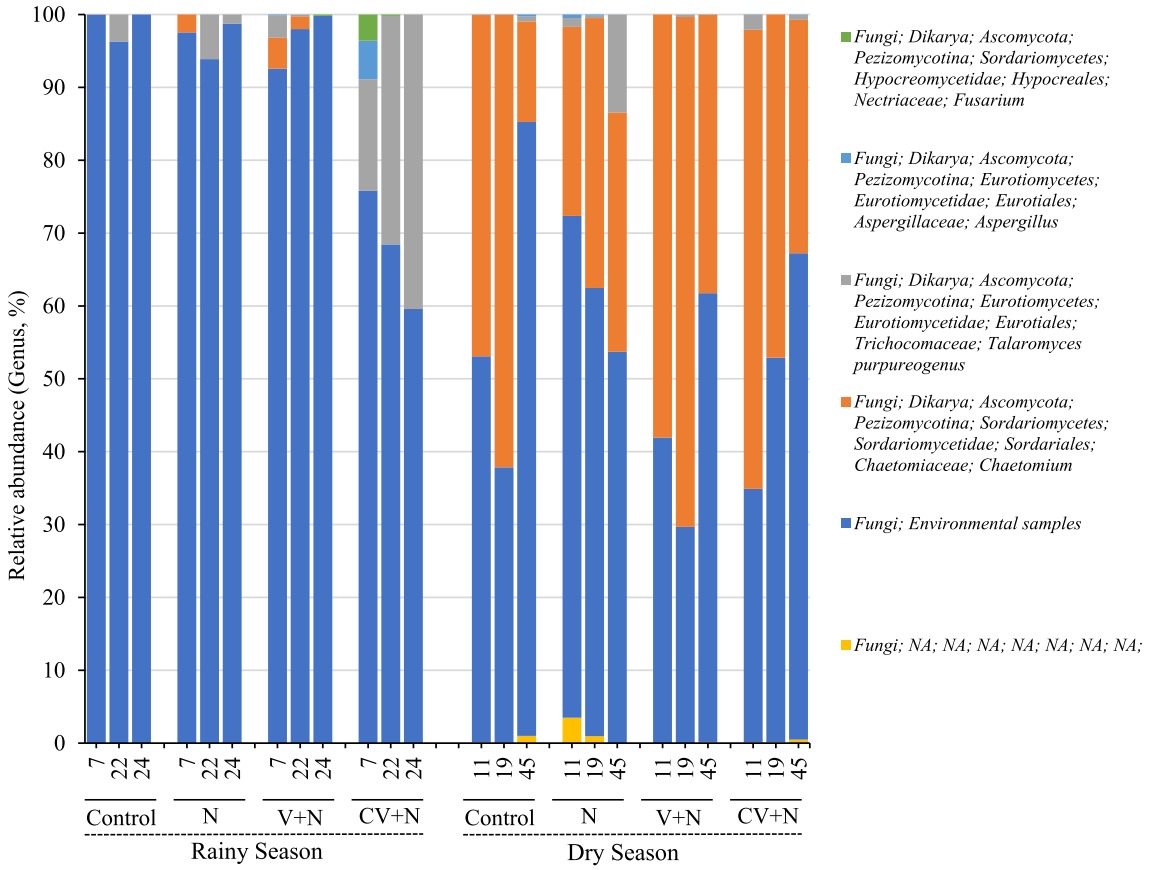

Fig. 4. Sequence abundances of *nirK* fungal denitrifiers (%) at the genus level using normalized values between 0 and 100 in different time points and treatments: Control; N, inorganic fertilization with ammonium nitrate; V + N, regular vinasse plus ammonium nitrate; and CV + N, concentrated vinasse plus ammonium nitrate.

the well-drained soils. Interestingly, the primer used to amplify the *nirK*-fungi also amplified the *nirK* gene from bacteria with majority from the *nirK* clade II gene (Fig. S7). Bacterial *nirK* clade II usually is not amplified by the common primers used to quantify the bacterial denitrifier community (Wei et al., 2015a). It seems that not only denitrifying fungi, but also denitrifying bacteria clade II can play an important role on the N_2O production.

To our knowledge, this study is the first to investigate the diversity and community structure of AOB and fungal denitrifiers in tropical soils under two different weather conditions (dry and rainy seasons). N_2O emissions and the abundances of both communities were dependent on the N source. However, the diversity and community structure of AOB and fungal denitrifiers were not affected by organic residue application, regardless of the type of vinasse, and inorganic N fertilization (Tables S5 and S6). The details of fertigation with vinasse depend on whether regular (non-concentrated) or concentrated vinasse is applied. Regular vinasse is spread over the whole field at a rate of approximately 100 m^3 ha^{-1}, whereas concentrated vinasse is applied at lower rates close to the plants, in bands that represent only 20% of the field area (~2000 m^2 ha^{-1}). Thus, in this study, the total amount of C and other nutrients (Table S1) applied in the bands close to the plants was twice as high in the CV + N treatments than in the V + N treatments (Table S1). Based on our previous work (Lourenço et al., 2018c, 2020), we expected differences in the AOB and fungal denitrifier communities, as well as the soil microbiome, between soil with and without vinasse application. However, it seems that different short-term fertilization regimes affect the abundance but not the structure of microbial communities related to specific pathways of the N cycle, such as nitrification and denitrification. In contrast to the lack of effect of the fertilization treatments, differences in weather conditions did affect the AOB and fungal denitrifier communities (Tables S5 and S6). The central-southern region of Brazil has two defined seasons: dry winters with mild temperatures and rainy summers with high temperatures. Changes in temperature (Lipson, 2007) and water content (Stark and Firestone, 1995; Valverde et al., 2014) are important factors regulating the activity and composition of the total microbial communities in soils (Bell et al., 2008; Lourenço et al., 2018c), and the community structure data in the present study indicate that these factors also impact AOB and fungal denitrifiers in soils.

Conflicting effects of N fertilization on AOB community composition have been reported in previous work, with some long-term experiments reporting changes (Shen et al., 2008; Glaser et al., 2010; Ouyang et al., 2016; Xiang et al., 2017) or no effects (Phillips et al., 2000; He et al., 2007). All of the AOB OTUs detected in the present study belonged to the genus *Nitrosospira* of the family *Nitrosomonadaceae*. In general, soil AOB belong to the genera *Nitrosospira* and *Nitrosomonas* (Prosser et al., 2020). Despite the lower capacity of *Nitrosospira* spp. to produce N_2O compared with *Nitrosomonas* sp., *Nitrosospira* spp. are K-strategists and have high N_2O production potential at lower NH_4^+-N concentrations (Shaw et al., 2006; Chandran et al., 2011). In addition, *Nitrosospira* spp. are more abundant in low-pH soils like that in our experiments (average pH of 5.1). It is possible that the local conditions selected for AOB from the genus *Nitrosospira*; the study area has been cultivated for more than 20 years with sugarcane, and the AOB community may have already adapted to the annual application of N fertilizers. In addition, some AOB have the capacity to produce N_2O through nitrifier-denitrification under NO_2^- accumulation and low O_2 concentrations, conditions that may occur after organic fertilization and rain events (Wrage-Mönnig et al., 2018). Due to its C content, applying vinasse may increase heterotrophic microbial activity, leading to a decrease in O_2 availability, creation of anaerobic conditions, and accumulation of NO_2^-, which is toxic to microbes, increasing the use of NO_2^- by AOB. Fujimura et al. (2020)

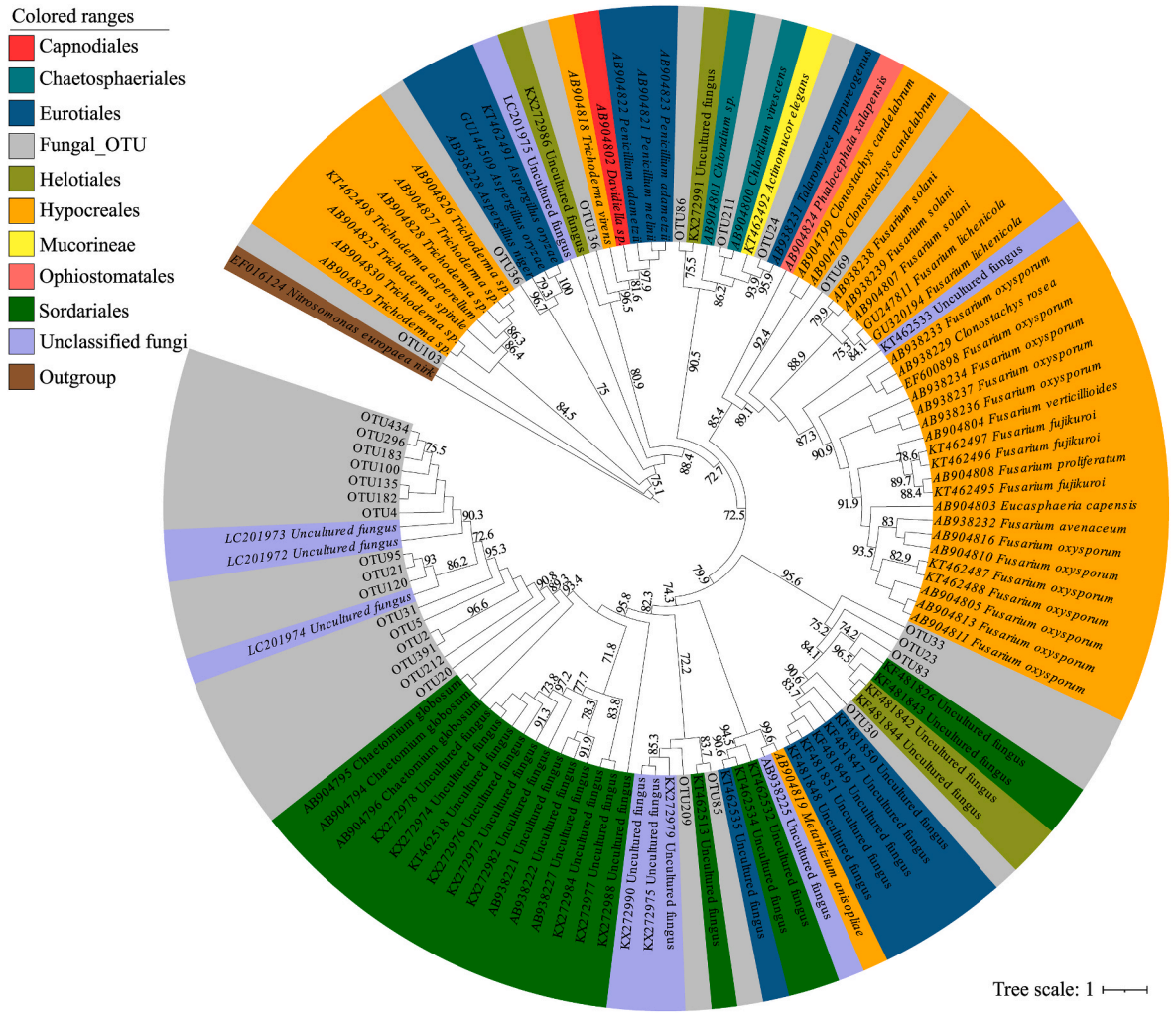

Fig. 5. Maximum-likelihood phylogenetic tree based on the top 30 *nirK*-Fungi OTUs and representative sequences and reference sequences from the NCBI database, with *Nitrosomonas europaea nirK* as an outgroup. The sequences were aligned using the software MAFFT v7.455 (Katoh and Standley, 2013) employing the L-INS-I strategy (Katoh et al., 2005). The software IQ-TREE 1.6.12 (Nguyen et al., 2015) was used to build the dendrogram based on maximum-likelihood inference with 1000 Ultrafast bootstraps (Hoang et al., 2018). Ultrafast bootstrap values > 0.70 are shown.

investigated the taxonomic composition of different *nirK* genes in soil and found a high proportion of *nirK* genes from the genus *Nitrosospira* in cropland soils in Japan. Thus, not only nitrification but also nitrifier-denitrification can drive N_2O production. Moreover, the differences in the community composition of these nitrifying genera between seasons might lead to changes in nitrifying activity and N_2O production, explaining in part the variations in N_2O emissions between seasons.

Fungal denitrifiers play an important role in N_2O production in soils, contributing from 17 to 89% of emissions (Laughlin and Stevens, 2002; Crenshaw et al., 2008; Xu et al., 2017, 2019b; Jiang et al., 2020), but a better understanding of their identities, distribution and functions in these ecosystems has been hampered by technical limitations (Higgins et al., 2016; Aldossari and Ishii, 2021). Most of the studies with soils use antibiotics to selectively inhibit bacteria to quantify the role of fungi in the N_2O production (Crenshaw et al., 2008; Wei et al., 2014); however, antibiotics select specific microbes, including fungi, resulting in ineffective results (Rousk et al., 2008; Ladan and Jacinthe, 2016). The bias of bacterial and fungal antibiotics inhibition in soil has not been sufficiently addressed (Ladan and Jacinthe, 2016) suggesting an overestimation of the contributions of fungi to N_2O production. An alternative approach to assess fungal N_2O production in soil is to determine the abundance of genes that encode enzymes related with N_2O production. The recently designed fungal nirK and P450nor PCR primers are beginning to resolve these limitations (Shoun et al., 2012; Long et al., 2015; Maeda et al., 2015; Wei et al., 2015b; Chen et al., 2016), but issues with specificity remain. Although we observed a positive correlation of N_2O emissions with *nirK*-fungi abundance, we acknowledge that the primers for fungal denitrifiers are degenerate and could also amplify bacterial *nirK* (Ma et al., 2019), mainly from clade II. To address the potential for biased results, we corrected the number of *nirK* copies by the relative abundance of fungal denitrifiers in each sample, similar to the methodology used by Cassman et al. (2018). We assumed that the sequence data represented the proportions of individuals in each sample and that qPCR determined the total number of copies of the target gene. Based on these assumptions, a phylogenetic tree was inferred using the sequence data, and the proportions of *nirK* genes from bacteria and fungi were estimated. This is a methodological limitation, but both sets of data were obtained from the same DNA pool, and similar amplification conditions were used.

In this study, a high diversity of nirK-containing fungal communities was detected. However, most of the sequences were unclassified (72% of total OTUs), suggesting a black box of unknown diversity of fungal denitrifiers. The identified fungi belonged to the phylum Ascomycota, including the families *Chaetomiaceae* (*Chaetomium* genus), *Trichocomaceae* (*Talaromyces purpureogenus* genus), *Aspergillaceae* (*Aspergillus*

genus) and *Nectriaceae* (*Fusarium* genus). The main identified fungi belonged to *Trichocomaceae* in RS and *Chaetomiaceae* in DS. *Trichocomaceae* contains many species known to live saprotrophically on decomposing plant residues in soils (Hibbett et al., 2007). Moreover, *Trichocomaceae* have the potential to utilize complex sources of carbon and phosphorus (Daynes et al., 2012). Our results show that this family may play an important role in N_2O production in tropical soils with high amounts of straw on the soil under wet-weather conditions. Interestingly, Costa et al. (2015) identified this family as one of the main contaminants of sugar fermentation to produce ethanol. It is possible that members of *Trichocomaceae* survive the process of sugar fermentation and are introduced in the soil with vinasse, but further studies are necessary. *Chaetomiaceae* includes numerous soil-borne, saprotrophic, endophytic, and pathogenic fungi (Zámocký et al., 2016; Clocchiatti et al., 2020), but our results indicated that this family is probably associated with N_2O production under dry-weather conditions. Incomplete sets of nitrate reductase genes have been detected in pure culture isolates of the genus *Chaetomium* sp. of this family (Gorfer et al., 2014). Members of *Trichocomaceae* and *Chaetomiaceae* containing *nirK* genes were also found by Xu et al. (2019b) and Fujimura et al. (2020) in soils from China and Japan. The enrichment of N_2O-producing fungi and the high density of N suggests that organic fertilizers on the soil surface might be a hot spot for fungal growth and N_2O production. Although results are an indication of fungal role on N_2O production, further studies with a broad approach must be conducted to determine their N_2O production capacity. An alternative approach is to combine qPCR and abundance of N_2O isotopomer analyses, since fungal and bacterial denitrification enzymes have different active site preferences (Yamamoto et al., 2017; Senbayram et al., 2018).

Despite the lower abundance of *Fusarium* in the *nirK*-fungal soil community (0.16% on average) compared with other genera, *Fusarium* and *Talaromyces purpureogenus* were the main fungi correlated with N_2O production ($r_s = 0.27$ and $r_s = 0.20$, respectively). These fungi were found in the CV + N and V + N treatments in RS, which had the highest N_2O emissions among the treatments. *Fusarium* is one of the main fungal genera related to N_2O production in soil (Maeda et al., 2015; Higgins et al., 2016) and is the most frequently isolated fungus with denitrifying capacity (Aldossari and Ishii, 2021). Recently, Keuschnig et al. (2020) provided solid evidence that *Fusarium* is capable of performing respiratory denitrification (*sensu stricto*). In addition, this genus harbors species capable of N_2O production in culture medium (Mothapo et al., 2013; Higgins et al., 2016). Consequently, the growth and activity of these fungi are probably favored in soils to which vinasse is applied, leading to increased N_2O production. Vinasse is a source of labile organic C, and its application selected members of *Fusarium* in RS. In addition, the treatments that received higher amounts of C, such as the treatments with CV, had higher relative abundances of *Fusarium*.

Taken together, our results indicate that the combination of vinasse (which is rich in organic C, organic N, and K) with mineral N fertilizer favors increases in the abundances of AOB and fungal denitrifiers without changing the structures of these two communities. The sugarcane field is a complex environment featuring inorganic fertilizer, vinasse (CV or V), and straw with a high C:N ratio (~100:1). Immediately after application, inorganic N increases microbes related to the N cycle, including AOB and fungi responsible for N_2O production. Vinasse has likely a priming effect by creating specific conditions for N_2O production. Furthermore, the presence of straw could select fungal members related to C compound degradation and N_2O production. The present findings thus provide relevant information on the interactions of organic residues that are recycled in sugarcane production systems (vinasse and straw) and inorganic (mineral N) amendments with the AOB and fungal denitrifier communities. Most importantly, this study unravels the main AOB and fungal denitrifiers native to tropical soils that influence N_2O production in two different seasons (dry- and wet-weather conditions). Further studies are necessary to determine the N_2O production capacity of these fungi under laboratory and field conditions.

5. Conclusion

Our aim in this work was to close the N_2O emission cycle by providing a comprehensive understanding of the impact of biofertilization with vinasse on AOB and fungal denitrifier community diversity and composition. We found that application of N fertilizer together with vinasse (CV or V) increased N_2O emissions and the abundances of AOB and fungi with denitrifying capacity. *Nitrosospira* sp. was the dominant AOB, while unclassified fungi from environmental samples and the genera *Chaetomium, Talaromyces purpureogenus,* and *Fusarium* were the dominant fungal denitrifiers. Amendment with vinasse and N fertilizer did not change AOB and fungal diversity and community structure, but both communities were impacted by weather conditions, with dominance of uncultured *Nitrosospira* and unclassified fungi in the rainy season and *Nitrosospira* and *Chaetomium* in the dry season. These results contribute to the elucidation of the main players in N_2O production and demonstrate the impact of fertilization on soil microbial N functions. Further studies are needed to obtain a better understanding of the roles of AOB and fungal denitrifiers in soil and in N_2O production.

Funding

This research was supported by The Netherlands Organization for Scientific Research (NWO) grant number 729.004.003, FAPESP-Brazil grant numbers 2013/50365-5, 2014/24141-5, 2018/20698-6, 18/2079-0, and CNPq grant number 310.478/2017-0. Publication number 7361 of The Netherlands Institute of Ecology (NIOO-KNAW).

Availability of data and materials

The raw sequences were submitted to the European Nucleotide Archive (ENA) under study accession number PRJEB44846.

Authors' contributions

K.S.L., E.E.K. and H.C designed the research; K.S.L. conducted the experiment, DNA extraction, qPCR analyses and performed the statistical analyses; O.Y.A.C. performed the bioinformatics. K.S.L. and O.Y.A.C. wrote the manuscript. E.E.K. and H.C. critically reviewed the manuscript. All authors reviewed the manuscript.

Ethics approval and consent to participate

Not applicable.

Conflicts of interest

The authors declare that they have no competing interests.

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.

Acknowledgments

The authors thank André C. Vitti (APTA), Raffaella Rossetto (APTA), Rafael M. Sousa, Zaqueu F. Montezano (IAC) for field assistance, Agata S. Pijl for molecular laboratory technical assistance, Marcio F. A. Leite for statistical assistance and Noriko A. Cassman for bioinformatics assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2022.108563.

References

Aldossari, N., Ishii, S., 2021. Fungal denitrification revisited – recent advancements and future opportunities. Soil Biology and Biochemistry 157, 108250. https://doi.org/10.1016/j.soilbio.2021.108250.

Bell, C., McIntyre, N., Cox, S., Tissue, D., Zak, J., 2008. Soil microbial responses to temporal variations of moisture and temperature in a Chihuahuan desert grassland. Microbial Ecology 56, 153–167. https://doi.org/10.1007/s00248-007-9333-z.

Bolker, B.M., Brooks, M.E., Clark, C.J., Geange, S.W., Poulsen, J.R., Stevens, M.H.H., White, J.-S.S., 2009. Generalized linear mixed models: a practical guide for ecology and evolution. Trends in Ecology & Evolution 24, 127–135. https://doi.org/10.1016/j.tree.2008.10.008.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., et al., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Zechmeister-Boltenstern, S., 2013. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? Philosophical Transactions of the Royal Society B: Biological Sciences 368, 20130122. https://doi.org/10.1098/rstb.2013.0122, 20130122.

Camargo, O.A., Moniz, A.C., Jorge, J.A., Valadares, J.M., 1986. Methods of Soil Chemical, Physical, and Mineralogical Analysis of the Agronomic Institute in Campinas. Instituto Agronômico, Campinas.

Canfield, D.E., Glazer, A.N., Falkowski, P.G., 2010. The evolution and future of earth's nitrogen cycle. Science 330, 192–196. https://doi.org/10.1126/science.1186120.

Carmo, J.B.d., Filoso, S., Zotelli, L.C., de Sousa Neto, E.R., Pitombo, L.M., Duarte-Neto, P. J., Vargas, V.P., Andrade, C.A., Gava, G.J.C., Rossetto, R., et al., 2013. Infield greenhouse gas emissions from sugarcane soils in Brazil: effects from synthetic and organic fertilizer application and crop trash accumulation. GCB Bioenergy 5, 267–280. https://doi.org/10.1111/j.1757-1707.2012.01199.x.

Carvalho, J.L.N., Nogueirol, R.C., Menandro, L.M.S., Bordonal, R.d.O., Borges, C.D., Cantarella, H., Franco, H.C.J., 2017. Agronomic and environmental implications of sugarcane straw removal: a major review. GCB Bioenergy 9, 1181–1195. https://doi.org/10.1111/gcbb.12410.

Carvalho, J.L.N., Oliveira, B.G., Cantarella, H., Chagas, M.F., Gonzaga, L.C., Lourenço, K. S., Bordonal, R.O., Bonomi, A., 2021. Implications of regional N_2O–N emission factors on sugarcane ethanol emissions and granted decarbonization certificates. Renewable and Sustainable Energy Reviews 149, 111423. https://doi.org/10.1016/j.rser.2021.111423.

Cassman, N.A., Lourenço, K.S., do Carmo, J.B., Cantarella, H., Kuramae, E.E., 2018. Genome-resolved metagenomics of sugarcane vinasse bacteria. Biotechnology for Biofuels 11, 48. https://doi.org/10.1186/s13068-018-1036-9.

CETESB, 2014. Norma Técnica P4.231 – Stillage - Criteria and Procedures for Agricultural Soil Application. Companhia Ambiental do Estado de São Paulo, São Paulo, p. 15.

Chandran, K., Stein, L.Y., Klotz, M.G., van Loosdrecht, M.C.M., 2011. Nitrous oxide production by lithotrophic ammonia-oxidizing bacteria and implications for engineered nitrogen-removal systems. Biochemical Society Transactions 39, 1832–1837. https://doi.org/10.1042/BST20110717.

Chao, A., 1984. Nonparametric estimation of the number of classes in a population. Scandinavian Journal of Statistics 11, 265–270. https://doi.org/10.2307/4615964.

Chen, H., Mothapo, N.V., Shi, W., 2014. The significant contribution of fungi to soil N_2O production across diverse ecosystems. Applied Soil Ecology 73, 70–77. https://doi.org/10.1016/j.apsoil.2013.08.011.

Chen, H., Yu, F., Shi, W., 2016. Detection of N_2O-producing fungi in environment using nitrite reductase gene (*nirK*)-targeting primers. Fungal Biology 120, 1479–1492. https://doi.org/10.1016/j.funbio.2016.07.012.

Clocchiatti, A., Hannula, S.E., van den Berg, M., Korthals, G., de Boer, W., 2020. The hidden potential of saprotrophic fungi in arable soil: patterns of short-term stimulation by organic amendments. Applied Soil Ecology 147, 103434. https://doi.org/10.1016/j.apsoil.2019.103434.

Costa, O.Y.A., Souto, B.M., Tupinambá, D.D., Bergmann, J.C., Kyaw, C.M., Kruger, R.H., Barreto, C.C., Quirino, B.F., 2015. Microbial diversity in sugarcane ethanol production in a Brazilian distillery using a culture-independent method. Journal of Industrial Microbiology and Biotechnology 42, 73–84. https://doi.org/10.1007/s10295-014-1533-1.

Crenshaw, C.L., Lauber, C., Sinsabaugh, R.L., Stavely, L.K., 2008. Fungal control of nitrous oxide production in semiarid grassland. Biogeochemistry 87, 17–27. https://doi.org/10.1007/s10533-007-9165-4.

Critchfield, H.J., 1960. General Climatology. Prentice-Hall, Englewood Cliffs.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., et al., 2015. Complete nitrification by *Nitrospira* bacteria. Nature 528, 504–509. https://doi.org/10.1038/nature16461.

Daynes, C.N., Zhang, N., Saleeba, J.A., McGee, P.A., 2012. Soil aggregates formed *in vitro* by saprotrophic Trichocomaceae have transient water-stability. Soil Biology and Biochemistry 48, 151–161. https://doi.org/10.1016/j.soilbio.2012.01.010.

Ding, Y., Gardiner, D.M., Xiao, D., Kazan, K., 2020. Regulators of nitric oxide signaling triggered by host perception in a plant pathogen. Proceedings of the National Academy of Sciences 117, 11147–11157. https://doi.org/10.1073/pnas.1918977117.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461. https://doi.org/10.1093/bioinformatics/btq461.

FAO, 2015. World Reference Base for Soil Resources 2014, Update 2015. International Soil Classification System for Naming Soils and Creating Legends for Soil Maps. World Soil Resources Reports No. 106. Food and Agriculture Organization of the United Nations: The State of Food and Agriculture, Rome.

Fujimura, R., Azegami, Y., Wei, W., Kakuta, H., Shiratori, Y., Ohte, N., Senoo, K., Otsuka, S., Isobe, K., 2020. Distinct community composition of previously uncharacterized denitrifying bacteria and fungi across different land-use types. Microbes and Environments 35, ME19064. https://doi.org/10.1264/jsme2.ME19064.

Glaser, K., Hackl, E., Inselsbacher, E., Strauss, J., Wanek, W., Zechmeister-Boltenstern, S., Sessitsch, A., 2010. Dynamics of ammonia-oxidizing communities in barley-planted bulk soil and rhizosphere following nitrate and ammonium fertilizer amendment. FEMS Microbiology Ecology 74, 575–591. https://doi.org/10.1111/j.1574-6941.2010.00970.x.

Goreau, T.J., Kaplan, W.A., Wofsy, S.C., McElroy, M.B., Valois, F.W., Watson, S.W., 1980. Production of NO_2^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen. Applied and Environmental Microbiology 40, 526–532.

Gorfer, M., Klaubauf, S., Berger, H., Strauss, J., 2014. The fungal contribution to the nitrogen cycle in agricultural soils. In: Marco, D. (Ed.), Metagenomics of the Microbial Nitrogen Cycle: Theory, Methods Applications. Caister Academic Press, UK, pp. 209–225.

He, J.-z., Shen, J.-p., Zhang, L.-m., Zhu, Y.-g., Zheng, Y.-m., Xu, M.-g., Di, H., 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. Environmental Microbiology 9, 2364–2374. https://doi.org/10.1111/j.1462-2920.2007.01358.x.

Herold, M.B., Baggs, E.M., Daniell, T.J., 2012. Fungal and bacterial denitrification are differently affected by long-term pH amendment and cultivation of arable soil. Soil Biology and Biochemistry 54, 25–35. https://doi.org/10.1016/j.soilbio.2012.04.031.

Hibbett, D.S., Binder, M., Bischoff, J.F., Blackwell, M., Cannon, P.F., Eriksson, O.E., Huhndorf, S., James, T., Kirk, P.M., Lücking, R., et al., 2007. A higher-level phylogenetic classification of the Fungi. Mycological Research 111, 509–547. https://doi.org/10.1016/j.mycres.2007.03.004.

Higgins, S.A., Schadt, C.W., Matheny, P.B., Löffler, F.E., 2018. Phylogenomics reveal the dynamic evolution of fungal nitric oxide reductases and their relationship to secondary metabolism. Genome Biology and Evolution 10, 2474–2489. https://doi.org/10.1093/gbe/evy187.

Higgins, S.A., Welsh, A., Orellana, L.H., Konstantinidis, K.T., Chee-Sanford, J.C., Sanford, R.A., Schadt, C.W., Löffler, F.E., 2016. Detection and diversity of fungal nitric oxide reductase genes (p450*nor*) in agricultural soils. Applied and Environmental Microbiology 82, 2919–2928. https://doi.org/10.1128/AEM.00243-16.

Hoang, D.T., Chernomor, O., von Haeseler, A., Minh, B.Q., Vinh, L.S., 2018. UFBoot2: improving the ultrafast bootstrap approximation. Molecular Biology and Evolution 35, 518–522. https://doi.org/10.1093/molbev/msx281.

Hothorn, T.H., Hornik, K., van De Wiel, M.A., Zeileis, A., 2008. Implementing a class of permutation tests: the coin package. Journal of Statistical Software 28, 1–23. https://doi.org/10.18637/jss.v028.i08.

Hu, H.-W., Chen, D., He, J.-Z., 2015. Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. FEMS Microbiology Reviews 39, 729–749. https://doi.org/10.1093/femsre/fuv021.

IPCC, 2013. Anthropogenic and natural radiative forcing. In: News, R. (Ed.), Climate Change 2013: the Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, United Kingdom and New York, NY, USA.

Jiang, M., Zhang, L., Liu, M., Qiu, H., Zhou, S., 2020. Fungi dominate denitrification when Chinese milk vetch green manure is used in paddy soil. Soil Ecology Letters. https://doi.org/10.1007/s42832-020-0064-0.

Jones, C.M., Graf, D.R.H., Bru, D., Philippot, L., Hallin, S., 2013. The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. The ISME Journal 7, 417–426. https://doi.org/10.1038/ismej.2012.125.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nature Methods 14, 587–589. https://doi.org/10.1038/nmeth.4285.

Kamphake, L.J., Hannah, S.A., Cohen, J.M., 1967. Automated analysis for nitrate by hydrazine reduction. Water Research 1, 205–216. https://doi.org/10.1016/0043-1354(67)90011-5.

Katoh, K., Kuma, K., Miyata, T., Toh, H., 2005. Improvement in the accuracy of multiple sequence alignment program MAFFT. Genome Inform 16, 22–33.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution 30, 772–780. https://doi.org/10.1093/molbev/mst010.

Keuschnig, C., Gorfer, M., Li, G., Mania Frostegrd, D., Bakken, L., Larose, C., 2020. NO and N_2O transformations of diverse fungi in hypoxia: evidence for anaerobic respiration only in Fusarium strains. Environmental Microbiology 22, 2182–2195. https://doi.org/10.1111/1462-2920.14980.

Kim, S.W., Fushinobu, S., Zhou, S., Wakagi, T., Shoun, H., 2009. Eukaryotic *nirK* genes encoding copper-containing nitrite reductase: originating from the protomitochondrion? Applied and Environmental Microbiology 75, 2652–2658. https://doi.org/10.1128/aem.02536-08.

Kits, K.D., Jung, M.-Y., Vierheilig, J., Pjevac, P., Sedlacek, C.J., Liu, S., Herbold, C., Stein, L.Y., Richter, A., Wissel, H., et al., 2019. Low yield and abiotic origin of N_2O formed by the complete nitrifier *Nitrospira inopinata*. Nature Communications 10, 1836. https://doi.org/10.1038/s41467-019-09790-x.

Krom, M.D., 1980. Spectrophotometric determination of ammonia: a study of a modified Berthelot reaction using salicylate and dichloroisocyanurate. Analyst 105, 305–316. https://doi.org/10.1039/AN9800500305.

Ladan, S., Jacinthe, P.-A., 2016. Evaluation of antibacterial and antifungal compounds for selective inhibition of denitrification in soils. Environmental Sciences: Processes & Impacts 18, 1519–1529. https://doi.org/10.1039/C6EM00456C.

Laughlin, R.J., Stevens, R.J., 2002. Evidence for fungal dominance of denitrification and codenitrification in a grassland soil. Soil Science Society of America Journal 66, 1540–1548. https://doi.org/10.2136/sssaj2002.1540.

Letunic, I., Bork, P., 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Research 44, W242–W245. https://doi.org/10.1093/nar/gkw290.

Liang, L.L., Eberwein, J.R., Allsman, L.A., Grantz, D.A., Jenerette, G.D., 2015. Regulation of $CO_2$and N_2O fluxes by coupled carbon and nitrogen availability. Environmental Research Letters 10, 034008. https://doi.org/10.1088/1748-9326/10/3/034008.

Lipson, D.A., 2007. Relationships between temperature responses and bacterial community structure along seasonal and altitudinal gradients. FEMS Microbiology Ecology 59, 418–427. https://doi.org/10.1111/j.1574-6941.2006.00240.x.

Long, A., Song, B., Fridey, K., Silva, A., 2015. Detection and diversity of copper containing nitrite reductase genes (*nirK*) in prokaryotic and fungal communities of agricultural soils. FEMS Microbiology Ecology 91, 1–9. https://doi.org/10.1093/femsec/fiu004.

Lourenço, K.S., Cassman, N.A., Pijl, A.S., van Veen, J.A., Cantarella, H., Kuramae, E.E., 2018a. *Nitrosospira sp.* govern nitrous oxide emissions in a tropical soil amended with residues of bioenergy crop. Frontiers in Microbiology 9. https://doi.org/10.3389/fmicb.2018.00674.

Lourenço, K.S., Dimitrov, M.R., Pijl, A., Soares, J.R., do Carmo, J.B., van Veen, J.A., Cantarella, H., Kuramae, E.E., 2018b. Dominance of bacterial ammonium oxidizers and fungal denitrifiers in the complex nitrogen cycle pathways related to nitrous oxide emission. GCB Bioenergy 10, 645–660. https://doi.org/10.1111/gcbb.12519.

Lourenço, K.S., Rossetto, R., Vitti, A.C., Montezano, Z.F., Soares, J.R., de Melo Sousa, R., do Carmo, J.B., Kuramae, E.E., Cantarella, H., 2019. Strategies to mitigate the nitrous oxide emissions from nitrogen fertilizer applied with organic fertilizers in sugarcane. The Science of the Total Environment 650, 1476–1486. https://doi.org/10.1016/j.scitotenv.2018.09.037.

Lourenço, K.S., Suleiman, A.K.A., Pijl, A., Cantarella, H., Kuramae, E.E., 2020. Dynamics and resilience of soil mycobiome under multiple organic and inorganic pulse disturbances. Science of The Total Environment, 139173. https://doi.org/10.1016/j.scitotenv.2020.139173.

Lourenço, K.S., Suleiman, A.K.A., Pijl, A., van Veen, J.A., Cantarella, H., Kuramae, E.E., 2018c. Resilience of the resident soil microbiome to organic and inorganic amendment disturbances and to temporary bacterial invasion. Microbiome 6. https://doi.org/10.1186/s40168-018-0525-1.

Ma, Y., Zilles, J.L., Kent, A.D., 2019. An evaluation of primers for detecting denitrifiers via their functional genes. Environmental Microbiology 21, 1196–1210. https://doi.org/10.1111/1462-2920.14555.

Maeda, K., Spor, A., Edel-Hermann, V., Heraud, C., Breuil, M.-C., Bizouard, F., Toyoda, S., Yoshida, N., Steinberg, C., Philippot, L., 2015. N_2O production, a widespread trait in fungi. Scientific Reports 5, 9697. https://doi.org/10.1038/srep09697.

McMurdie, P., Paulson, J., 2020. Biomformat: an Interface Package for the BIOM File Format.

McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217.

Morais, M.C., Ferrari, B.M., Borges, C.D., Cherubin, M.R., Tsai, S.M., Cerri, C.C., Cerri, C. E.P., Feigl, B.J., 2019. Does sugarcane straw removal change the abundance of soil microbes? BioEnergy Research 12, 901–908. https://doi.org/10.1007/s12155-019-10018-5.

Morley, N., Baggs, E.M., 2010. Carbon and oxygen controls on N_2O and N_2 production during nitrate reduction. Soil Biology and Biochemistry 42, 1864–1871. https://doi.org/10.1016/j.soilbio.2010.07.008.

Mothapo, N., Chen, H., Cubeta, M.A., Grossman, J.M., Fuller, F., Shi, W., 2015. Phylogenetic, taxonomic and functional diversity of fungal denitrifiers and associated N_2O production efficacy. Soil Biology and Biochemistry 83, 160–175. https://doi.org/10.1016/j.soilbio.2015.02.001.

Mothapo, N.V., Chen, H., Cubeta, M.A., Shi, W., 2013. Nitrous oxide producing activity of diverse fungi from distinct agroecosystems. Soil Biology and Biochemistry 66, 94–101. https://doi.org/10.1016/j.soilbio.2013.07.004.

NCBI Resource Coordinators, 2016. Database resources of the National center for biotechnology information. Nucleic Acids Research 44, D7–D19. https://doi.org/10.1093/nar/gkv1290.

Nelson, M.B., Martiny, A.C., Martiny, J.B.H., 2016. Global biogeography of microbial nitrogen-cycling traits in soil. Proceedings of the National Academy of Sciences 113, 8033–8040. https://doi.org/10.1073/pnas.1601070113.

Nguyen, L.-T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Molecular Biology and Evolution 32, 268–274. https://doi.org/10.1093/molbev/msu300.

Norton, J.M., Alzerreca, J.J., Suwa, Y., Klotz, M.G., 2002. Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. Archives of Microbiology 177, 139–149. https://doi.org/10.1007/s00203-001-0369-z.

Ouyang, Y., Norton, J.M., Stark, J.M., Reeve, J.R., Habteselassie, M.Y., 2016. Ammonia-oxidizing bacteria are more responsive than archaea to nitrogen source in an agricultural soil. Soil Biology and Biochemistry 96, 4–15. https://doi.org/10.1016/j.soilbio.2016.01.012.

Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., Blondel, M., Prettenhofer, P., Weiss, R., Dubourg, V., et al., 2011. Scikit-learn: Machine learning in Python. Journal of Machine Learning Research 12, 2825–2830.

Philippot, L., Hallin, S., Schloter, M., 2007. Ecology of denitrifying prokaryotes in agricultural soil. Advances in Agronomy 96, 249–305. https://doi.org/10.1016/S0065-2113(07)96003-4.

Phillips, C.J., Harris, D., Dollhopf, S.L., Gross, K.L., Prosser, J.I., Paul, E.A., 2000. Effects of agronomic treatments on structure and function of ammonia-oxidizing communities. Applied and Environmental Microbiology 66, 5410–5418. https://doi.org/10.1128/AEM.66.12.5410-5418.2000.

Pitombo, L.M., do Carmo, J.B., de Hollander, M., Rossetto, R., López, M.V., Cantarella, H., Kuramae, E.E., 2016. Exploring soil microbial 16S rRNA sequence data to increase carbon yield and nitrogen efficiency of a bioenergy crop. GCB Bioenergy 8, 867–879. https://doi.org/10.1111/gcbb.12284.

Prosser, J.I., Hink, L., Gubry-Rangin, C., Nicol, G.W., 2020. Nitrous oxide production by ammonia oxidizers: Physiological diversity, niche differentiation and potential mitigation strategies. Global Change Biology 26, 103–118. https://doi.org/10.1111/gcb.14877.

Ravishankara, A.R., Daniel, J.S., Portmann, R.W., 2009. Nitrous Oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century. Science 326, 123–125. https://doi.org/10.1126/science.1176985.

Rousk, J., Demoling, L.A., Bahr, A., Bååth, E., 2008. Examining the fungal and bacterial niche overlap using selective inhibitors in soil. FEMS Microbiology Ecology 63, 350–358. https://doi.org/10.1111/j.1574-6941.2008.00440.x.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., et al., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75, 7537–7541. https://doi.org/10.1128/aem.01541-09.

Senbayram, M., Well, R., Bol, R., Chadwick, D.R., Jones, D.L., Wu, D., 2018. Interaction of straw amendment and soil NO_3^- content controls fungal denitrification and denitrification product stoichiometry in a sandy soil. Soil Biology and Biochemistry 126, 204–212. https://doi.org/10.1016/j.soilbio.2018.09.005.

Shaw, L.J., Nicol, G.W., Smith, Z., Fear, J., Prosser, J.I., Baggs, E.M., 2006. *Nitrosospira spp.* can produce nitrous oxide via a nitrifier denitrification pathway. Environmental Microbiology 8, 214–222. https://doi.org/10.1111/j.1462-2920.2005.00882.x.

Shen, J.-p., Zhang, L.-m., Zhu, Y.-g., Zhang, J.-b., He, J.-z., 2008. Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. Environmental Microbiology 10, 1601–1611. https://doi.org/10.1111/j.1462-2920.2008.01578.x.

Shoun, H., Fushinobu, S., Jiang, L., Kim, S.-W., Wakagi, T., 2012. Fungal denitrification and nitric oxide reductase cytochrome P450*nor*. Philosophical Transactions of the Royal Society B: Biological Sciences 367, 1186–1194. https://doi.org/10.1098/rstb.2011.0335.

Soares, J.R., Cassman, N.A., Kielak, A.M., Pijl, A., Carmo, J.B., Lourenço, K.S., Laanbroek, H.J., Cantarella, H., Kuramae, E.E., 2016. Nitrous oxide emission related to ammonia-oxidizing bacteria and mitigation options from N fertilization in a tropical soil. Scientific Reports 6, 30349. https://doi.org/10.1038/srep30349.

Stark, J.M., Firestone, M.K., 1995. Mechanisms for soil moisture effects on activity of nitrifying bacteria. Applied and Environmental Microbiology 61, 218–221.

SystatSoftware, 2014. SSI. Sigmaplot for Windows, Version 13.0. Systat Software San Jose, California, USA.

USDA, 2014. Keys to soil taxonomy. In: Staff, S.S. (Ed.), Soil Survey Staff, twelfth ed. ed. Natural Resources Conservation Service, Washington, DC, p. 372.

Valverde, A., Makhalanyane, T.P., Cowan, D.A., 2014. Contrasting assembly processes in a bacterial metacommunity along a desiccation gradient. Frontiers in Microbiology 5, 668. https://doi.org/10.3389/fmicb.2014.00668.

van Kessel, M.A.H.J., Speth, D.R., Albertsen, M., Nielsen, P.H., Op den Camp, H.J.M., Kartal, B., Jetten, M.S.M., Lücker, S., 2015. Complete nitrification by a single microorganism. Nature 528, 555–559. https://doi.org/10.1038/nature16459.

Van Raij, B., Andrade, J.C., Cantarella, H., Quaggio, J.A., 2001. Chemical Analysis for Evaluation of Fertility of Tropical Soils. Instituto Agronômico, Campinas, Brazil.

Wei, W., Isobe, K., Nishizawa, T., Zhu, L., Shiratori, Y., Ohte, N., Koba, K., Otsuka, S., Senoo, K., 2015a. Higher diversity and abundance of denitrifying microorganisms in environments than considered previously. The ISME Journal 9, 1954–1965. https://doi.org/10.1038/ismej.2015.9.

Wei, W., Isobe, K., Shiratori, Y., Nishizawa, T., Ohte, N., Ise, Y., Otsuka, S., Senoo, K., 2015b. Development of PCR primers targeting fungal *nirK* to study fungal denitrification in the environment. Soil Biology and Biochemistry 81, 282–286. https://doi.org/10.1016/j.soilbio.2014.11.026.

Wei, W., Isobe, K., Shiratori, Y., Nishizawa, T., Ohte, N., Otsuka, S., Senoo, K., 2014. N_2O emission from cropland field soil through fungal denitrification after surface applications of organic fertilizer. Soil Biology and Biochemistry 69, 157–167. https://doi.org/10.1016/j.soilbio.2013.10.044.

Wrage-Mönnig, N., Horn, M.A., Well, R., Müller, C., Velthof, G., Oenema, O., 2018. The role of nitrifier denitrification in the production of nitrous oxide revisited. Soil Biology and Biochemistry 123, A3–A16. https://doi.org/10.1016/j.soilbio.2018.03.020.

Wrage, N., Velthof, G.L., van Beusichem, M.L., Oenema, O., 2001. Role of nitrifier denitrification in the production of nitrous oxide. Soil Biology and Biochemistry 33, 1723–1732. https://doi.org/10.1016/S0038-0717(01)00096-7.

Xiang, X., He, D., He, J.-S., Myrold, D.D., Chu, H., 2017. Ammonia-oxidizing bacteria rather than archaea respond to short-term urea amendment in an alpine grassland. Soil Biology and Biochemistry 107, 218–225. https://doi.org/10.1016/j.soilbio.2017.01.012.

Xu, H., Sheng, R., Xing, X., Zhang, W., Hou, H., Liu, Y., Qin, H., Chen, C., Wei, W., 2019a. Characterization of fungal *nirK*-containing communities and N_2O emission from fungal denitrification in arable soils. Frontiers in Microbiology 10. https://doi.org/10.3389/fmicb.2019.00117.

Xu, H., Sheng, R., Xing, X., Zhang, W., Hou, H., Liu, Y., Qin, H., Chen, C., Wei, W., 2019b. Characterization of fungal *nirK*-containing communities and N_2O emission from fungal denitrification in arable soils. Frontiers in Microbiology 10, 1–12. https://doi.org/10.3389/fmicb.2019.00117.

Xu, X., Liu, X., Li, Y., Ran, Y., Liu, Y., Zhang, Q., Li, Z., He, Y., Xu, J., Di, H., 2017. High temperatures inhibited the growth of soil bacteria and archaea but not that of fungi and altered nitrous oxide production mechanisms from different nitrogen sources in an acidic soil. Soil Biology and Biochemistry 107, 168–179. https://doi.org/10.1016/j.soilbio.2017.01.003.

Yamamoto, A., Akiyama, H., Nakajima, Y., Hoshino, Y.T., 2017. Estimate of bacterial and fungal N_2O production processes after crop residue input and fertilizer application to an agricultural field by ^{15}N isotopomer analysis. Soil Biology and Biochemistry 108, 9–16. https://doi.org/10.1016/j.soilbio.2017.01.015.

Zámocký, M., Tafer, H., Chovanová, K., Lopandic, K., Kamlárová, A., Obinger, C., 2016. Genome sequence of the filamentous soil fungus Chaetomium cochliodes reveals abundance of genes for heme enzymes from all peroxidase and catalase superfamilies. BMC Genomics 17, 763. https://doi.org/10.1186/s12864-016-3111-6.

Zhong, L., Bowatte, S., Newton, P.C.D., Hoogendoorn, C.J., Luo, D., 2018. An increased ratio of fungi to bacteria indicates greater potential for N_2O production in a grazed grassland exposed to elevated CO_2. Agriculture, Ecosystems & Environment 254, 111–116. https://doi.org/10.1016/j.agee.2017.11.027.