Impact of nitrogen fertilization on the soil microbiome and nitrous oxide emissions

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Chapter 4

Nitrous oxide emission related to ammonia-oxidizing bacteria and mitigation options from N fertilization in a tropical soil

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Abstract

Nitrous oxide (N_2O) from nitrogen fertilizers applied to sugarcane has high environmental impact on ethanol production. This study aimed to determine the main microbial processes responsible for the N_2O emissions from soil fertilized with different N sources, to identify options to mitigate N_2O emissions, and to determine the impacts of the N sources on the soil microbiome. In a field experiment, nitrogen was applied as calcium nitrate, urea, urea with dicyandiamide or 3,4 dimethylpyrazone phosphate nitrification inhibitors (NIs), and urea coated with polymer and sulfur (PSCU). Urea caused the highest N_2O emissions (1.7% of N applied) and PSCU did not reduce cumulative N_2O emissions compared to urea. NIs reduced N_2O emissions (95%) compared to urea and had emissions comparable to those of the control (no N). Similarly, calcium nitrate resulted in very low N_2O emissions. Interestingly, N_2O emissions were significantly correlated only with bacterial *amoA*, but not with denitrification gene (*nirK*, *nirS*, *nosZ*) abundances, suggesting that ammonia-oxidizing bacteria, via the nitrification pathway, were the main contributors to N_2O emissions. Moreover, the treatments had little effect on microbial composition or diversity. We suggest nitrate-based fertilizers or the addition of NIs in NH_4^+-N based fertilizers as viable options for reducing N_2O emissions in tropical soils and lessening the environmental impact of biofuel produced from sugarcane.

4.1 Introduction

Agriculture is the main anthropogenic source of N_2O emissions, which are predicted to increase as nitrogen fertilizer use increases worldwide to meet the global food demand[1]. Currently, N_2O emissions derived from N fertilizers account for up to 40% of total greenhouse gases (GHG) emissions in ethanol production from sugarcane[2]. High N_2O emissions can negate the benefits of GHG reduction of biofuels used to replace fossil fuels[3].

Emissions of N_2O from soils occur mainly through nitrification and denitrification processes. These processes are carried out by autotrophic and heterotrophic microorganisms belonging to Bacteria, Archaea and Fungi divisions[4-6]. Other N transformations such as nitrifier denitrification, dissimilatory reduction of NO_3^- to NH_4^+, chemo-denitrification and co-denitrification may also produce N_2O. Despite considerable knowledge of the processes evolving N_2O, the prevalence of these processes in tropical soils has only begun to be addressed.

The denitrification process has been demonstrated to contribute more to N_2O emissions than nitrification at soil moisture levels above 75% of the water-filled pore space (WFPS); however, nitrification has been observed to be more prevalent in soil at 60% WFPS[7]. High correlation between N_2O emissions and bacterial *amoA* and *nirK* abundances are observed[8], suggesting that both nitrification and denitrification and/or nitrifier denitrification processes are responsible for N_2O emissions when cattle urine is applied to soils with 100 and 130% of water-holding capacity.

In the central-west and southeast regions of Brazil, about 80% of the land area is cultivated with sugarcane[9]. The dominant soils in these regions are Red Latosols (Hapludox), which are highly weathered, deep and well-drained soils[10]. Here, we expected that denitrification would be low because the optimal conditions are at least 60% WFPS. Though high levels of rainfall and anaerobic conditions in soil micropores may increase the contribution of denitrification to N_2O emissions[11], we predicted based on the high soil drainage that nitrification would be the major pathway contributing to N_2O emissions. In this case NH_4^+-based fertilizer would result in higher N_2O emissions than those from NO_3^--based fertilizers in these soils. Up to date, this process has not been shown for these types of soils grown with sugarcane.

The Intergovernmental Panel on Climate Change (IPCC) estimates that 1% of N applied is emitted as N_2O as default value[12-14]. However, in practice, different amounts of N_2O are emitted depending on N fertilizers and soil types, and environmental conditions[12-14]. Therefore, experiment-based nitrogen manage-

ment is an important tool to decrease N_2O emissions and to reduce the environmental impact of agricultural practices[14].

Urea is the most widely used fertilizer in the world, and generally has been linked to higher N_2O emissions compared with other N sources[13]. One way to reduce N_2O emissions is the addition of specific nitrification inhibitors (NIs) such as dicyandiamide (DCD), 3,4 dimethylpyrazone phosphate (DMPP), nitrapyrin, and others with urea fertilization[14, 15]. These nitrification inhibitors block the enzyme ammonia monooxygenase in the first step of nitrification[16]. The gene encoding this enzyme is *amoA*, present in ammonia-oxidizing bacteria (AOB) and archaea (AOA). Several studies indicate that DCD and DMPP reduce AOB or AOA gene abundances, depending on which microorganism was prevalent[8, 17-19]. DCD has been reported to reduce also the abundance of *nirK*, probably because AOB abundances are correlated with *nirK* abundances, implying reduced nitrifier denitrification abundances[8].

To our knowledge, there are no studies identifying the main microbial processes, the effect of different fertilizers on N_2O emissions, and the impact of different N fertilizers on the microbial community in tropical soils grown with sugarcane. Therefore, the goals of this study were to (i) determine the main microbial process responsible for the N_2O emissions, (ii) evaluate the efficacy of enhanced-efficiency fertilizers, including nitrification inhibitors, in reducing N_2O emissions, and (iii) determine the short-term effects of the fertilizer treatments on bacterial community composition and diversity.

4.2 Methods

4.2.1 Experimental set up

The present experiment was carried out in the 2013/14 season, corresponding to the third ratoon cycle of sugarcane, the variety SP791011, in the experimental area of the Agronomic Institute in Campinas, Brazil (22º52'15" S, 47º04'57" W). The soil in the area was classified as Typic Hapludox or Red Latosol[10, 21]. The same experiment was carried out during the seasons of 2011/12 and 2012/13[15]. However, in the 2013/14 season an extra treatment with calcium nitrate was included to consider N_2O emissions due to nitrification or denitrification processes. Here, soil samples were collected in order to associate greenhouse gases (GHG) emissions with the microbial processes that were involved. The treatments were: 1) Control plot without N fertilization (control); 2) urea (UR); 3) UR + DCD; 4) UR + DMPP; 5) Polymer and Sulphur Coated Urea (PSCU); 6) UR + DCD-R; 7) UR + DMPP-R; 8) Calcium Nitrate. R stands for reapplication of in-

hibitors in the same plots during the previous two cycles of the experiment. The fertilizers were applied on 19 December 2013, 20 days after the harvest of the previous cycle. Phosphorus and potassium were concurrently applied to all plots at rates of 20 and 100 kg ha^{-1} of P and K, respectively.

Nitrogen was applied at a rate of 120 kg ha^{-1}; the nitrification inhibitor DCD (Sigma Aldrich) was added in a dose of 5% DCD-N in relation to urea-N whereas DMPP (powder form) was added as 1% DMPP (w/w) to urea-N; PSCU was produced by Produquímica (Produquímica Ltda, Brazil) and calcium nitrate by Yara (Yara International ASA). Fertilizers were incorporated at a 5 cm soil depth to avoid NH_3 volatilization from urea and the effect of NIs on this N loss[35]. The fertilizers were applied on either side of the plant row, 10 cm away from the recently harvested sugarcane plants. On one side of the plant row the greenhouse gases were measured; on the opposite side of the same plant, soil for chemical and molecular microbial analyses was collected.

Sugarcane yields were not measured in this study because the amount of N lost as N_2O is generally much too low to affect yields. Furthermore, the plot size necessary to evaluate yields usually exceeds 100 m^2. Because our focus was on GHGs emissions, which are dependent on localized soil conditions, small plots were chosen. In our study, large plots were not only unnecessary but would contribute to noise in the gas flux data.

4.2.2 Greenhouse gases analysis

Greenhouse gases were collected using static chambers[15]. Chambers were fixed in the soil 5 cm deep along two 25-m long rows of sugarcane. In total, 32 chambers were used, with four replicates per treatment, in a completely randomised design. Gases were sampled in the morning and three times per week during the first three months after fertilizer application, then biweekly as previously done[15]. In each sampling date, gas samples were taken at three time intervals: 1, 15, and 30 minutes.

After sampling, the gases were immediately stored in pre-evacuated Extainers vials (Labco Limited, Ceredigion, United Kingdom) and analysed in a Shimadzu gas chromatograph (GC-2014). Cumulated gas emissions were calculated by linear interpolation between gas samplings periods. Details of the procedures used for gas analysis and calculations are described elsewhere[14, 20].

4.2.3 Soil chemical analysis

Soil samples (0-10 cm depth) were collected more intensively in the first two months after fertilizer application, a period corresponding to higher N_2O emissions. Using an auger, three subsamples were collected as a composite sample per experimental plot. In total, eight soil sampling campaigns were collected at 7, 16, 18, 27, 35, 42, 82 and 158 days after fertilizer application. The soil samples were stored in plastic bags at -20ºC. Gravimetric moisture after constant weight was attained at 105 ºC. The water-filled pore space (WFPS) was calculated considering soil bulk density and porosity determined at the beginning of the experiment. Soil pH was measured in $CaCl_2$ (0.0125mol L^{-1}) and NH_4^+-N and NO_3^--N contents were determined by steam distillation after soil extraction in 2 mol L^{-1} KCl solution[36].

4.2.4 Real-time PCR analysis

Soil subsamples (20 g) were stored at -80ºC for molecular analyses. Total soil DNA was extracted from 0.25 g of soil using the Power Soil kit (Mobio, Carlsbad, CA USA) following the manufacturer's instructions. The quantity and quality of DNA was measured by NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Montchanin, USA). The DNA samples were diluted in water free of DNase and RNase (Sigma Aldrich) and the abundance of the genes encoding for nitrification and for denitrification processes were quantified by quantitative real-time PCR with a Qiagen Rotor-Gene Q6000 cycler (RO212226). A reaction was performed in total volume of 12 µl, containing 6 µl Sybrgreen Bioline SensiFAST SYBR non-rox mix, 0.5 µl of each primer (5 pmol) and 5 µl of DNA (3 ng). Exceptions were the reaction for the *nirK* amplification, for which the Sybrgreen Qiagen Rotor-Gene SYBR Green PCR Kit was used, and the *nosZ* amplification, for which the starting DNA concentration was 30 ng. Reactions were performed by a QIAgility robot (003516).

The thermal conditions of each gene amplification are listed in Supplementary Table S5. Acquisition was done at 72°C (cycle A) or 82-86°C (cycle B) to avoid primer dimers. Melt curve analysis was done at 55-99°C to confirm specificity; the qPCR products were checked by agarose gel electrophoresis to confirm the desired amplicon size. Plasmid DNA from microorganisms containing the gene of interest or from environmental samples were used for the standard curve and then cloned into vectors as described in Table S5. Normal PCR reactions were carried out with similar thermal conditions as qPCR to confirm the fragment size of interest, then cloned and transformed into JM109 High Efficiency Competent Cells (Promega, In Vitro Technologies, Auckland, New Zealand). After overnight bacterial growth in LB medium with ampicillin at 37ºC, plasmids were extracted

using the PureLin Quick Plasmid Miniprep Kit (Life Technologies, Auckland, New Zealand). The quantity and quality of plasmid DNA were checked by spectrophotometer (NanoDrop ND-1000 Technologies, Montchanin, USA). Standard dilutions were obtained from 10 to 10^8 copies/µl of each gene. Each run included a DNA template, standard, and a no-DNA control – water free of DNase and RNase (Sigma Aldrich) – done in duplicate. Reaction efficiency was 89-105% and R^2 values ranged from 0.94 to 0.99.

4.2.5 16S rRNA partial gene sequencing

To assess the impact of the treatments on the bacterial community, we sequenced the 16S rRNA gene marker from total DNA extracted from the soil samples. The V4 region of the 16S rRNA gene was amplified by using archaeal/bacterial primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGAC-TACVSGGGTATCTAAT-3'). The samples were PCR-amplified using barcoded primers linked with the Ion adapter "A" sequence (5′-CCATCTCATCCCT-GCGTGTCTCCGACTCAG-3′) and Ion adapter "P1" sequence (5′-CCTCTC-TATGGGCAGTCGGTGAT-3′) to obtain a sequence of primer composed for A-barcode-806R and P1-515F adapter and primers. The 16S rRNA gene amplifications for library preparation were performed on the C1000 thermocycler (Biorad, Hercules, CA, USA) with thermal conditions of 95°C-5 min.; 35x 95°C-30s, 53°C-30s, 72°C-60s; 72°C-10 min. A reaction of 25 µl in total was done, including 2.5 µl of 10X PCR Buffer, 2.5 µl dNTPs (200 µM), 0.25 µl of each primer (0.1 pmol/µl), 0.2 µl of fast startExp-Polymerase (0.056 U) and 1 µl of DNA (0.6 ng). The reactions were carried out in duplicate and included a negative control. The amplicons were checked by gel electrophoresis. The PCR products were purified by Agencourt AMPURE XP to remove primer dimers, then quantified by Quant-iT PicoGreen and equimolar mixed for sequencing using the PGM Ion Torrent (Life Technologies).

4.2.6 16S rRNA amplicon sequences processing

MOTHUR Version 1.34.2 was used to process the 16S rRNA partial genes sequences, implemented using a Snakemake workflow on a 32-node server running Linux Ubuntu 14.4[37]. Forward and reverse primer sequences were removed from each sample FASTQ file using Flexbar version 2.5[38]. Reads were filtered based on sequence quality by running the Sickle tool (minimum quality score 25, minimum length 150). Filtered reads were converted to FASTA format and concatenated into a single file, then clustered into OTUs using the UPARSE

strategy of dereplication, sorting by abundance with at least two sequences and clustering using the UCLUST smallmem algorithm[39]. These steps were performed with VSEARCH version 1.0.10, which is an open-source and 64-bit multi-threaded compatible alternative to USEARCH. Chimeric sequences were detected using the UCHIME algorithm[40] implemented in VSEARCH. All reads before the dereplication step were mapped to OTUs using the USEARCH_global method implemented in VSEARCH to create an OTU table and then converted to the BIOM-Format 1.3.1[41]. Last, taxonomic information for each OTU was added to the BIOM file using the RDP Classifier version 2.10[42].

4.2.7 Statistical analysis – gas fluxes and gene abundances

Daily GHG fluxes, cumulated emissions of N_2O, CO_2, CH_4 and gene abundance values were checked for normal distribution of residues by Shapiro-Wilk test, and then submitted to variance analysis (ANOVA) and the means compared by Tukey's test at $P \leq 0.05$. Soil pH was transformed to H^+: 10^{-pH} before statistical analysis. Linear correlations between N_2O fluxes and environmental variables were evaluated at the 5% level of significance. Multiple linear regressions, which were selected by the stepwise process at $p \leq 0.05$, were fitted between N_2O fluxes and environmental variables. When necessary, the N_2O flux values were log(x) transformed and rechecked to obtain a normal distribution of residues and variance stability[43]. The calculations were performed with the SISVAR statistical software[43] and graphics plotted using Sigma Plot[43].

Cumulative N_2O emissions as a function of time were fitted by sigmoidal or exponential equations, for which the sigmoid equation was:

$$N_2O = \frac{a}{1 + e^{-\left(\frac{t-t_0}{b}\right)}} \tag{1}$$

where N_2O is the cumulative N_2O-N emission, g ha-1, t is the time in days after fertilizer application, and a, t0 and b are equation parameters, where a is the maximum loss and t0 is the time in which 50% of maximum loss occurs.

The exponential rise to the maximum model had the following equation:

$$N_2O = a(1 - e^{-bt}) \tag{2}$$

where N_2O is the cumulative N_2O-N emission, g ha-1, t is the time in days after fertilizer application, and a and b are equation parameters, where a is the maximum loss and b is the rate of rise.

4.2.8 Statistical analysis – 16S rRNA amplicon sequence data

The 16S rRNA samples were analysed to compare bacterial community alpha diversity and composition across treatments and time point (days 7, 16, 18, 27, 35, 42, 82 and 158). The BIOM files were handled with the "phyloseq" package[44] in R[50]. Rarefaction curves were generated to ensure adequate sequencing depth across samples. Discarding undersequenced samples, the minimum sample size was 2000. For alpha diversity analyses, the 16S rRNA samples were rarefed to 2000 sequences using the "vegan" package[51]. Renyi diversities at alpha level 1, corresponding to the Shannon diversity index were kept ("BiodiversityR" R package). The Shannon diversity data was furthermore subjected to Kruskal-Wallis tests among treatments and the Kruskal-Wallis multiple comparison test between treatments using the "pgirmess" R package.

Comparisons of bacterial community compositions were evaluated using the Statistical Analysis of Metagenomic Profiles (STAMP) software[52]. The top nine Bacterial phyla based on relative abundances across all samples were compared among and between treatments for each time point. The unclassified sequences were removed prior to analysis. The ANOVA statistical and Tukey-Kramer post-hoc tests (CI 95%) were applied using the Benjamini Hochberg multiple test correction. To explore beta diversity (treatment differences) of the bacterial communities, Between-Class Analysis (BCA) of the non-rarefied 16S rRNA samples grouped by treatment was performed using the "ade4" R package[53]. First, with unclassified sequences removed, correspondence analyses of the compositional data agglomerated at the rank of Phylum and Genus were conducted, followed by BCA. Further, the BCA groups for the phyla and genera analyses were tested using the Monte-Carlo permutation method with 999 repetitions.

4.3 Results

4.3.1 Greenhouse gases emissions

Emissions of N_2O were low in the first 10 days after fertilizer application, with less than 10 g ha^{-1} day^{-1} of N emitted (**Figure 1**). High N_2O emission followed rain events coupled with high soil inorganic N availability (**Figure 2**). The UR treatment had the highest peak of N_2O emission, on the 14th day, which corresponded to a loss rate of more than 200 g ha^{-1} day^{-1} of N. On the 29th day, another high emission peak of about 170 g ha^{-1} day^{-1} occurred. Between these peaks, N_2O emissions were still relatively high ranging from 15 to 70 g ha^{-1} day^{-1} in the UR treatment. The treatments UR+DCD, UR+DMPP, and calcium nitrate had smaller N_2O fluxes than those of UR, showing emission levels similar to the control

treatment (around 5 g ha^{-1} day^{-1} of N). Urea containing NIs (UR+DCD-R, UR+DMPP-R) had been reapplied in the same plots in the previous two years[15]. Emissions in the plots with repeated application of NIs were also low at $\leq$ 5 g ha^{-1} day-1 of N (**Figure 1**). The controlled release fertilizer PSCU treatment showed lower N_2O emission (80 g ha^{-1} day^{-1}) compared to the UR treatment on the 14th and 29th days, but was similar to UR treatment levels afterwards, until the 50th day. Between 70 and 120 days after fertilizer application, N_2O emissions were greater in the PSCU treatment (between 10 – 20 g ha^{-1} day^{-1}) compared to the other treatments (2 g ha^{-1} day^{-1}) (**Figure 1**).

Cumulated N_2O emissions in the control treatment were equivalent to 0.3 kg ha^{-1} after 278 days. The UR treatment emitted more than 2.3 kg ha^{-1} of N_2O-N, which corresponded to 1.7% of total N applied. The UR+DCD, UR+DMPP treatments resulted in considerable reductions in cumulated N_2O emissions compared to UR, with emissions that did not differ from those of the control (**Table 1**). The reduction of N_2O emissions by addition of NIs to UR varied from 88 to 97% (95% in average). The PSCU treatment resulted in cumulative emissions similar to those of UR. Calcium nitrate resulted in low N_2O emissions that did not differ from those of the UR+DCD and UR+DMPP treatments or the control (**Table 1**).

Cumulative N_2O emissions data were fit with sigmoidal or exponential equations for the control, UR and PSCU treatments. These models were not applied to the UR+DCD, UR+DMPP, and calcium nitrate treatments because in these treatments the N_2O emissions did not differ from the control. The N_2O emissions in the control treatment were low and whether included or excluded did not affect the model equations. The UR treatment achieved 90% of maximum N_2O loss 40 days after fertilizer application. The N_2O emission lasted longer with the PSCU treatment than with UR; 90% of total N_2O emission was achieved 187 days after fertilization with PSCU (**Figure 3**).

The total CO_2 and CH_4 emissions were around 6 t ha^{-1} and -600 g ha^{-1}, respectively. For both CO_2 emissions and CH_4 consumption, no differences between the treatments were observed (**Table 1**).
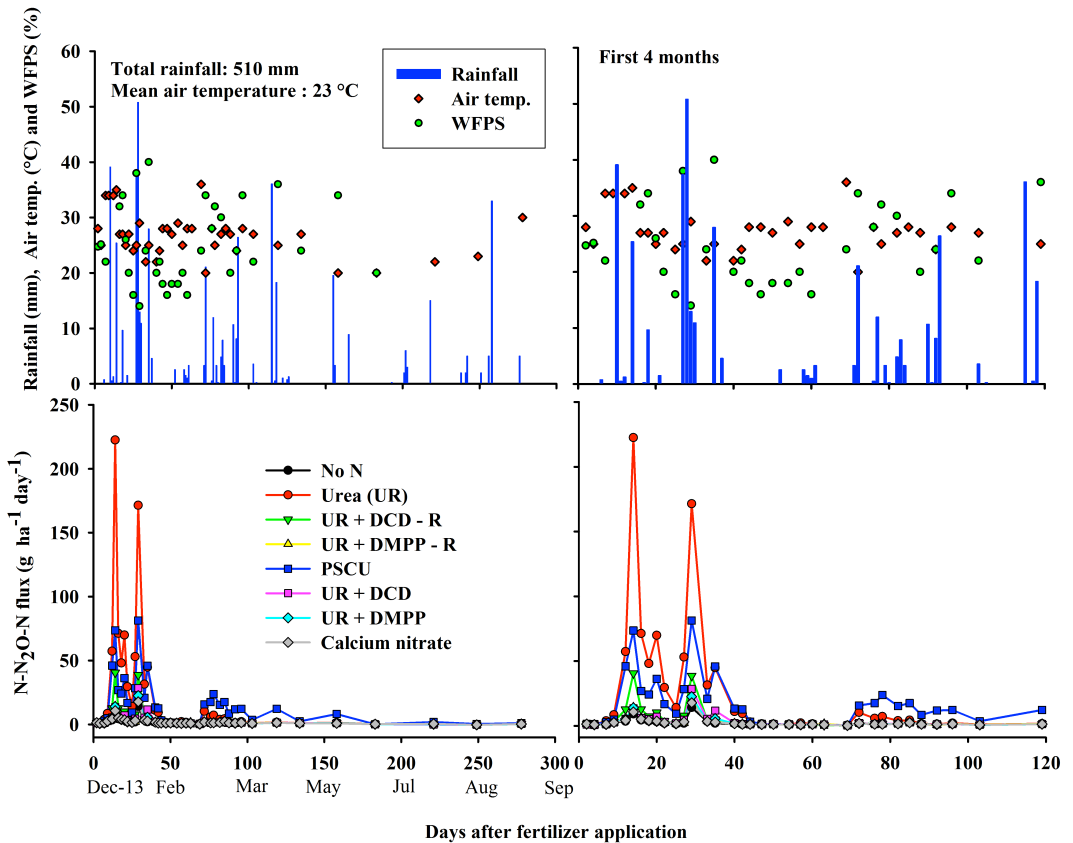
Figure 1. Rainfall, air temperature, water-filled pore space (WFPS) and nitrous oxide fluxes from Control (No N), urea (UR) with or without nitrification inhibitors (DCD and DMPP), polymer sulphur coated urea (PSCU) and calcium nitrate applied to sugarcane. R: reapplication of inhibitors in the same plots during the previous two cycles of the experiment. N fertilizers were applied on 13 December 2013.
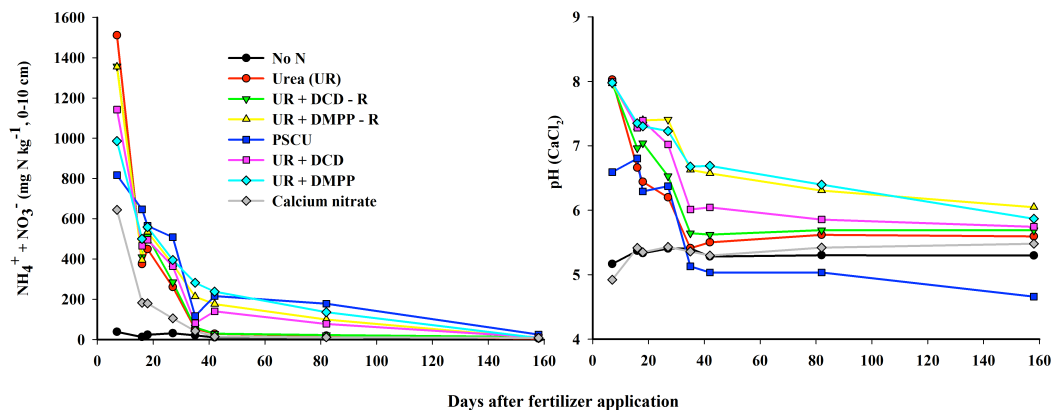
Figure 2. Soil concentration of NH4+-N + NO3--N and pH from Control (No N), urea (UR) with or without nitrification inhibitors (DCD and DMPP), polymer sulphur coated urea (PSCU) and calcium nitrate applied to sugarcane. R: reapplication of inhibitors in the same plots during the previous two cycles of the experiment.

Table 1. Cumulated nitrous oxide, carbon dioxide and methane emissions from Red Latosol soil during 278 days after application of urea with or without nitrification inhibitors (DCD and DMPP), polymer sulphur coated urea (PSCU) and calcium nitrate applied to sugarcane.

Treatments	N_2O-N					CO_2-C		CH_4-C	
	g ha^{-1}	log*		% N applied†	Reduction (%)	kg ha^{-1}*		g ha^{-1}*	
Control	286	2.4	C	-	-	5835	ns	-598	ns
UR	2301	3.4	A	1.68	-	5933		-633	
UR+DCD-R	531	2.7	B	0.20	88	5883		-612	
UR+DMPP-R	350	2.5	C	0.05	97	5871		-532	
PSCU	2165	3.3	A	1.57	7	5912		-648	
UR+DCD	410	2.6	Bc	0.10	94	5859		-633	
UR+DMPP	353	2.5	C	0.06	97	5897		-656	
Calcium nitrate	329	2.5	C	0.04	98	5973		-600	
P value		<0.00001				0.9769		0.9328	

* Tukey test, p ≤ 0.05; ns: no significant; N_2O-N: g ha^{-1} transformed in log(X) † Results from treatment without N were subtracted for this calculation. - R means reapplication of inhibitors in the same plot in the two preceding years. Different characteristics in the column of N_2O mean significant differences (p<0.05) between the values.

4.3.2 Soil analysis

In the first soil sampling seven days after fertilizer application, the calcium nitrate treatment showed N inorganic content (NH_4^+ + NO_3^-) around 600 mg kg^{-1}, which was lower than the 1000 mg kg^{-1} of N in the 10 cm soil layer found in the UR treatment (**Figure 2**). Afterwards the N content in soil decreased exponentially; during this time, nitrification inhibitors in the UR+DCD and UR+DMPP treatments maintained soil N mostly in the NH_4^+ form (**Supplementary Figure S1**). At the 82th day, soil treated with PSCU showed N content higher than other treatments, near 200 mg kg^{-1} of N, as opposed to 100 mg kg^{-1} from the UR treatment.

The original soil pH (control treatment) was around 5.1 but increased to 8 in the UR, UR+DCD and UR+DMPP treatments by seven days after fertilizer application, because of urea hydrolysis. PSCU showed pH value 1.4 units lower (pH 6.6) but treatments with calcium nitrate did not affect soil pH. In the 42th day, the soil pH of the UR+DCD and UR+DMPP treatments had dropped to values around 7 (**Figure 2**).
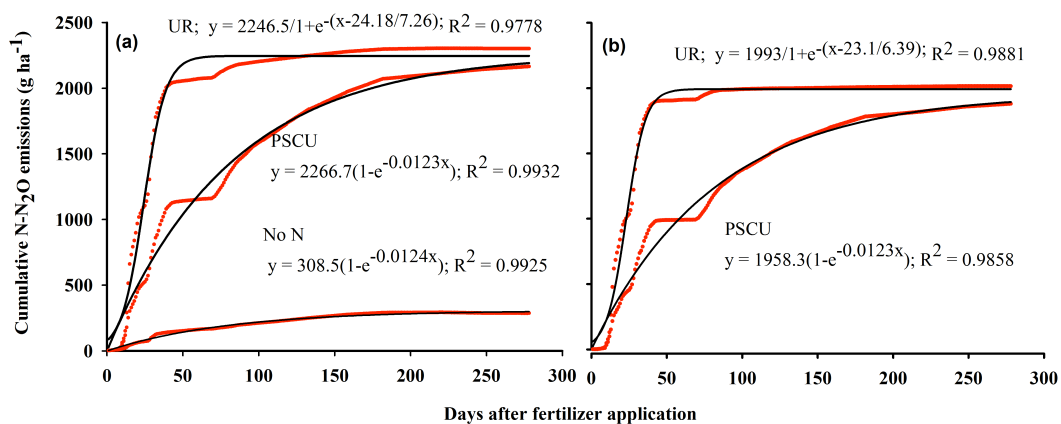
Figure 3. Cumulative N_2O emission (red dots) and sigmoidal or exponential equations fitted (black lines) to data of urea (UR) and polymer sulphur coated urea (PSCU) applied to sugarcane. (b) net UR and PSCU N_2O emissions calculated by subtracting data of the control treatment.

4.3.3 Nitrogen cycle gene abundances

The abundances of N cycling genes related to N_2O emissions are depicted in **Figure 4** for one timepoint sampling that featured high N_2O emissions: 16 days after fertilizer application, corresponding to the second soil sampling. The qPCR results from all data sampling timepoints are available in **Supplementary Table S1**. The abundance of *amoA* belonging to ammonia-oxidizing archaea (AOA) was lower in treatments with N sources than in the control plot and did not show significant differences among the N sources across nearly all data sampling points. The correlation between AOA *amoA* abundance and N_2O emissions was negative (**Supplementary Table S2**). The gene abundance representing total archaea showed a similar pattern as the AOA *amoA* abundance (**Figure 4** and **Supplementary Table S1**). On the other hand, the *amoA* abundance of ammonia-oxidizing bacteria (AOB) was best correlated with N_2O emissions, showing a coefficient (R^2) of 0.18 ($p \leq 0.05$) (**Supplementary Table S2**). Over almost all data sampling points, AOB *amoA* abundances were higher in the UR treatment than in other treatments, following the data from N_2O emissions (**Figure 4** and **Supplementary Table S1**). For example, concurrent to high N_2O emissions at day 16 after fertilizer application, the coefficient of correlation (R^2) between N_2O and AOB *amoA* was 0.53 (**Supplementary Figure S2**).

The denitrification genes *nirS* and *nosZ* as well as the 16S rRNA gene of total bacteria did not show differences in abundance between treatments over almost all data samplings. The *nirK* occurring in both ammonia-oxidizing and denitrification microorganisms had a negative correlation with N_2O emissions, while

the total bacteria abundance resulted in a positive correlation with this emission (**Supplementary Table S2**). In some data samplings, the abundance of the *nosZ* was higher in treatment with N than the control treatment, but no differences in *nosZ* abundance were observed between the N sources treatments (**Figure 4** and **Supplementary Table S1**).

A good fit, with an R^2 of 0.47, was obtained by stepwise regression model relating N_2O emissions to environmental variables, including the AOB *amoA* abundance, rain amount accumulated one week before GHGs measurement, NH_4^+-N and NO_3-N contents, total bacteria abundance, pH, and CO_2 emission (**Supplementary Table S3**). Removing the treatments without nitrification inhibitors, NH_4^+-N content in soil was correlated with N_2O, while NO_3^--N was not (**Supplementary Tables S2 and S3**).

4.3.4 Bacterial community composition and diversity

Because the AOB *AmoA* abundance was correlated with N_2O emissions, we sequenced the 16S rRNA genes from our samples to ascertain the effect of the treatments on the entire microbial (bacterial and archaeal) community. After processing the 16S rRNA amplicon sequences, the 177 samples (8 treatments x 8 timepoints x 3 replicates, excluding undersampled samples and outliers) contained between 2,000 and 56,638 sequences, with a total of 3,607,143 sequences distributed into 9,267 Operational Taxonomic Units (OTUs). Rarefaction curves indicated that most of the community diversity was captured with our sequencing depth (**Supplementary Figure S3**). The top nine bacterial phyla across the samples were *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Gemmatimonadetes*, *Proteobacteria* and *Verrucomicrobia* (**Supplementary Table S4**). The top phyla that differed between the treatments within at least one timepoint were *Firmicutes*, *Bacteroidetes*, *Nitrospira*, *Proteobacteria*, *Verrucomicrobia* and *Acidobacteria*. Shannon diversity indices of the bacterial communities ranged between 5.3 and 6.3 in treatments over all time points and were significantly different between treatments only for days 27 (Control *versus* UR) and 82 (PSCU *versus* UR+DMPP; **Table 2**).

Based on phylum-level relative abundances, the samples were significantly grouped by treatment on days 7, 27, 35, 42, 85 and 158 (permutation test, P < 0.100; **Table 2**) with a range of observed values between 0.83 and 0.44. At day 7, three separate clusters were formed respectively with the Control and calcium nitrate treatments, the PSCU treatment and the other treatments (**Supplementary Table S5**). However, this clustering pattern was not observed during the remain-

ing other days, indicating no differences in the bacterial communities between treatments. When bacterial community compositions were compared at the taxonomical level of genera, the samples were significantly grouped by treatment and the grouping was characterized by low observation values for all days except day 16 (P=0.119; **Table 2** and **Supplementary Table S5**). The sample treatment grouping pattern from day 7 was seen in the genus-level comparisons (**Supplementary Table S5**).

Table 2. Shannon indices and Between-Class Analysis (BCA) ordinations of the bacterial communities present in the Red Latosol soil under treatments with urea with or without nitrification inhibitors (DCD and DMPP); polymer sulphur coated urea (PSCU) or calcium nitrate applied to sugarcane.

Treatments	16S rRNA gene diversity (Shannon index)															
	7 DAF*		16		18		27		35		42		85		158	
Control	6.2	ns	6.1	ns	6.0	ns	6.2	a	6.1	ns	6.2	ns	6.0	ab	6.1	ns
UR	5.8		6.0		6.0		5.3	b	5.7		5.8		5.6	ab	6.0	
UR+DCD-R†	5.9		6.2		5.8		6.0	ab	6.0		6.1		5.8	ab	6.2	
UR+DMPP-R	5.6		6.1		5.9		5.8	ab	6.1		6.2		6.1	ab	6.2	
PSCU	5.9		5.9		5.8		5.8	ab	5.1		5.2		5.4	a	5.8	
UR+DCD	5.7		6.1		5.7		5.8	ab	5.9		6.0		6.0	ab	6.2	
UR+DMPP	5.7		6.0		5.9		5.8	ab	5.5		6.1		6.1	b	6.1	
Calcium nitrate	6.3		6.0		5.7		5.9	ab	5.9		6.2		6.0	ab	6.2	

*DAF: Days after fertilizer application. Means in column followed by same letter did not differ; ns: no significant. † R means reapplication of inhibitors in same plots.
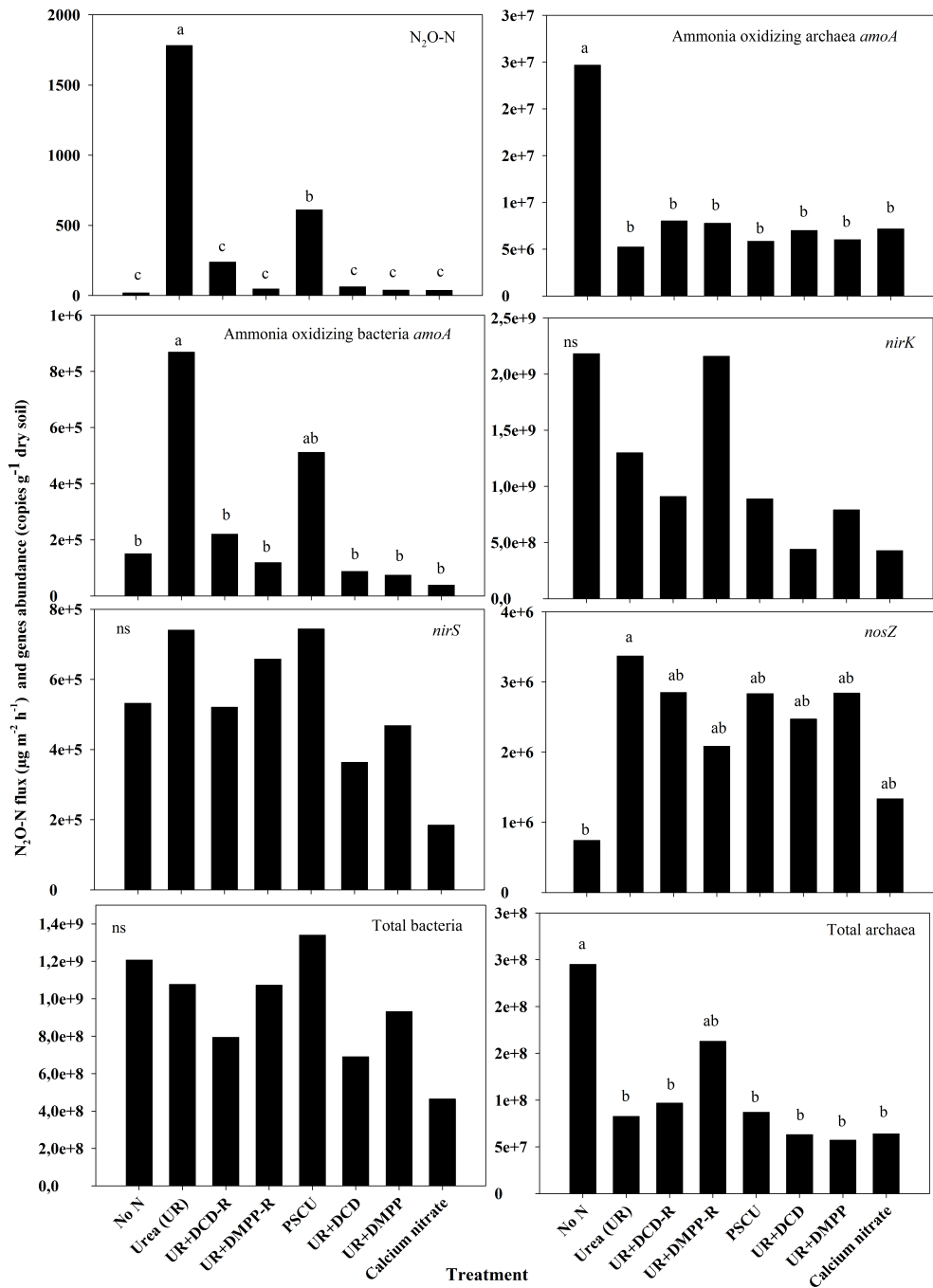
Figure 4. Nitrous oxide fluxes, nitrogen cycle genes (*amoA* bacteria, *amoA* archaea, *nirK*, *nirS*, *nosZ*) abundances and total bacteria and total archaea abundances in the Red Latosol soil 16 days after fertilizer application of urea with or without nitrification inhibitors (DCD and DMPP); polymer sulphur coated urea (PSCU) or calcium nitrate applied to sugarcane. R: reapplication of inhibitors in the same plots during the previous two cycles of the experiment.

4.4 Discussion

Nitrous oxide emissions from the urea treatment were higher than the emissions found in two previous sugarcane cycles in this experimental area[15]. Here, the emission factor was 1.7% of N applied, which is greater than the emission factors found in other studies of sugarcane soils in Brazil, around $0.7 - 1\%$ of N applied as urea[12, 15, 20]. The sugarcane plant phenology may give insight into higher emissions. The N fertilizer treatments were applied 20 days after the previous sugarcane ratoon was harvested. At the time of fertilization, the soil was relatively dry – below 30% of the WFPS (Figure 1) and sugarcane plants were still beginning to sprout. At this stage the root system was being reformed and nutrient uptake was slow, as the high soil N concentration indicated (Figure 2). This probably led to the greater N_2O emissions than expected. The high peaks of N_2O emission occurred after two high rain events in the first 35 days (total 65 mm and 90 mm) on a mostly dry soil (15 – 20% WFPS) but with high air temperatures as shown in Figure 1. Moreover, high correlation between N_2O emission and accumulated rain in the week was found (Supplementary Table S2). Thus, the climatic conditions in this season contributed to the high N_2O emission values.

A strong reduction in N_2O emissions due to the addition of nitrification inhibitors to urea was found, as well as a lack of beneficial effects of the controlled release fertilizer PSCU, supporting similar observations in the same area[15]. The N_2O emissions from PSCU were lower than those from UR in the first 30 days after N application, as expected from a slow-release fertilizer (Figure 2). There was a dry spell from mid-January to the end of March (Figure 1), which may have slowed down the release of N from PSCU. Subsequent release of N from the PSCU pellets likely led to the observed increase in N_2O emissions (Figure 1). In this way, the N_2O emission from PSCU had lower peaks than those of UR but lasted longer (Figure 3). Thus, in the end of the experiment cumulated N_2O of UR and PSCU emissions were similar, suggesting that PSCU is not an environmentally friendly N source during one cycle of sugarcane.

In the present study, the calcium nitrate treatment showed very small N_2O emissions that were similar to those of the control plots or plots with urea and nitrification inhibitors (NIs). In the present study intensive GHG measurements under field condition were performed over a whole yearly cycle of sugarcane. We maintain that this is the first field study demonstrating much lower N_2O emissions of a nitrate-N source in comparison to high emissions with urea-N or NH_4^+-N sources; the reduction in N_2O emissions were 98% when compared to urea. This reduction in emissions might be attributed to the high drainage capacity of the soil of the present study, which was classified as Typic Hapludox[21] or Red

Latosol[10]. Water accumulation does not tend to occur in these soil profiles, and consequently, favourable conditions for denitrifiers are avoided.

Under controlled conditions with ^{15}N-labeled sources, denitrification was more important at the high soil moisture (75% WFPS), while N_2O emissions with NH_4^+ fertilizers were two times higher than with NO_3^- fertilization at 60% WFPS[7]. In our study WFPS reached a maximum of 40% (Figure 1), which is more favourable for nitrification. The O_2 concentrations were likely not low enough to favour the denitrification process in relation to nitrification.

An alternate explanation for the low N_2O emissions in the calcium nitrate treatment could be NO_3^- leaching. Indeed, the N concentration in the 0-10 cm soil layer of the calcium nitrate treatment was lower than that observed with the other N sources (Figure 2). However, with only 70 mm of rain on a dry soil in 15 days, nitrate is unlikely to have moved beyond 30 cm. Moreover studies with ^{15}N labelling showed little NO_3^- leaching in sugarcane fields in Brazil[22, 23]. Therefore, NO_3^- leaching was not expected to explain the small amount of N_2O emission found with calcium nitrate in the present study. However, further studies should include NO_3^- leaching measurements to confirm the present data.

Another aspect that may have contributed to the small N_2O emission in the calcium nitrate treatment was the relatively low organic carbon content in the soil, approximately 1%[15]. Sugarcane trash and vinasse have been reported to increase N_2O emissions[24], especially under high soil moisture conditions[24]. Here, we did not include in our treatments C sources such as vinasse, filter cake or sugarcane trash, common sugarcane residues or by-products. These residues can favour not only denitrifiers but also nitrifiers and other microorganisms related to the N cycle[25]. Application of exclusively NO_3^--N sources with the addition of C sources, as commonly applied during sugarcane production, may result in N_2O emissions different from those observed here and deserves further attention.

Smaller N_2O emissions from calcium nitrate as compared to UR or NH_4-based fertilizer have been previously reported[26, 27]. However, in one study, N_2O emissions observed with all the studied N sources were low (around 0.5% of the N applied), which makes it difficult to compare the treatments[27]. In a field grown with maize in Brazil, no differences were reported between UR and calcium nitrate in N_2O emissions, but the emission factor was 0.2% of the N applied[11]. In our study, the N_2O emissions from UR were high at 1.7 % of N applied; the low N_2O emission under calcium nitrate occurred concurrently to high N_2O emissions from UR treatment, which highlights the relevance of the present study.

If soil moisture conditions are favourable to denitrification, nitrate-based N fertilizers may produce higher N_2O emissions than urea or ammonium fertilizers. That is the case of the study conducted in a Gleysol soil in which the WFPS was above 60% during most of the experimental period[28].

Based on Between-Class ordinations of the 16S rRNA compositional data as well as the total 16S rRNA gene copy numbers, the bacterial community appeared to be more affected by sampling day than by treatment. This suggests overall a minimal impact of the treatments on bacterial community composition and diversity. Though further work should examine the long-term impacts, there appears to be a low short-term impact of NIs on the bacterial community. Culturing or shotgun metagenomic and metatranscriptomic techniques may provide future avenues to illuminate the activity of specific nitrifiers under the environmental conditions in this study and to enhance predictions of N_2O emissions due to nitrification in tropical soils.

Archaeal *amoA* abundances were highest in the control treatment compared to the treatments with any N sources. Elevated ammonia concentrations and higher soil pH are suggested to favour bacteria compared to archaea[8, 17, 29]. Interestingly, the plots with calcium nitrate also showed a reduction in archaeal *amoA* abundance compared to the control. This may reflect the accumulated effect of ammonium nitrate applied in the two previous cycles as this was the N source previously used in a separate study[15].

Significant correlations between N_2O emissions and *amoA* abundances were found for ammonia-oxidizing bacteria (AOB), indicating that in our study, N_2O emissions occurred via nitrification. During the first month after UR application, a peak in the AOB *amoA* abundances was observed. Concurrently, the nitrate content in soil increased whereas ammonium decreased, soil pH decreased from 8 to around 6.3, and the soil temperature was 25ºC (Supplementary Figure S5). Thus, the soil and climatic conditions were favourable to AOB and N_2O emissions from nitrification.

Apart from identifying nitrification as the likely source of N_2O emissions here, the data also suggest that denitrification was very low. No differences among treatments nor significant correlations between N_2O emissions and the genes encoding denitrification process as *nirK*, *nirS* and *nosZ* abundances were observed. Further, the model that best estimated N_2O emissions included bacterial *amoA* abundances and N present in the NH_4^+ form but not in NO_3^-. Thus, our gene abundance data supported the results of the low N_2O emission data obtained from application of nitrate as the N source.

Significant correlation between AOB *amoA* abundance and N_2O emissions was also shown. Under controlled conditions, Venterea et al.[30] found a high correlation of N_2O emissions from urea and NO_2^--N content in soil resulting from increased bacterial *amoA* abundance with no increase in the abundance of the *nxr* gene, which encodes for nitrite oxidation. The authors discussed that N_2O emissions occurred more during nitrification than denitrification, similar to the results found here. Dicyandiamide (DCD) application with cattle urine effectively inhibited the growth of AOB and reduced N_2O emissions as well as the numbers of the *nirK* gene, which encodes for a nitrate reductase[8]. Since DCD did not affect the abundance of other denitrification genes, the authors concluded that AOB, including nitrifier denitrifiers containing *nirK*, were the main contributors to N_2O emissions[8]. In the present study no evidences relating *nirK* and N_2O emissions was found, but the nitrifier denitrification process could have great contribution to N_2O released due the presence of the gene *norB* in AOB [4,5,32]. Besides nitrifier denitrification, nitrous oxide could be emitted during oxidation of hydroxylamine by ammonia-oxidizing bacteria[31], heterotrophic nitrifiers[32, 33], and/or abiotic chemodenitrification[34]. Abiotic N_2O emissions also occur due nitrite reduction by organic and inorganic compounds as amine, copper and iron[4,37]. Others processes that could be involved in N_2O emissions are abiotic or biotic co-denitrification, by archaea, bacteria or fungi. In co-denitrification, a reducing compound as NO^-, NO_2^- or NO_3^- combined with organic N, hydroxylamine or ammonium generates N_2O emissions in oxic and anoxic conditions[6, 34]. More studies targeting these reactions can pin down the relative contribution of factors explaining N_2O fluxes from nitrification. The present study showed high N_2O losses from urea, but very small from a nitrate fertilizer source and nitrification was the most relevant microbial process associated with such losses, which has not been reported in soil with sugarcane. The relationship between N_2O emissions and bacterial *amoA* abundances may, therefore be a useful indicator for N management strategies to mitigate N_2O emissions in tropical soils. Other classes of soils and N sources are necessary to confirm our data.

4.5 Declarations

Acknowledgments

Author contribution statement

J.R.S., J.B.C., H.C. and E.E.K. designed research; J.R.S. and K.S.L. conducted the experiment; J.R.S., N.A.C., A.M.K., A.P., H.J.L. and E.E.K. conducted the qPCR and the sequencing analyses; J.R.S. and N.A.C. performed the statistical analyses; J.R.S., N.A.C., H.J.L., H.C. and E.E.K. wrote the paper. All authors reviewed the manuscript.

Competing interest statement

The authors declare no conflict of interest.

Accession codes

European Nucleotide Archive study accession number PRJEB13027.

4.6 Supplementary Material

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Table S1. Nitrous oxide fluxes, nitrogen cycling gene abundances and total bacterial and total archaea abundance in a Red Latosol soil as affected by treatments with urea with or without nitrification inhibitors (DCD and DMPP); polymer sulfur coated urea (PSCU) or calcium nitrate applied to sugarcane. The R represents the second application of the nitrification inhibitors.

Treatment	N_2O-N	AOA *amoA*	AOB *amoA*	*nirK*	*nirS*	*nosZ*	Total archaea	Toral bacteria
7 DAF†	$\mu g\ m^{-2}\ h^{-1}$	10^6 copies g^{-1}	10^5 copies g^{-1}	10^8 copies g^{-1}	10^5 copies g^{-1}	10^6 copies g^{-1}	10^7 copies g^{-1}	10^9 copies g^{-1}
No N	3.8 c	7.5 a	2.3 b	7.8 ns	4.2 ns	15.9 ns	9.7 ns	2.6 abc
UR	82.0 a	2.0 b	12.2 a	6.8	4.6	21.3	4.5	5.9 a
UR+DCD - R‡	12.9 c	3.5 b	2.8 b	7.1	3.9	13.9	5.1	4.9 abc
UR+DMPP - R	5.8 c	3.2 b	3.0 b	8.5	4.8	18.7	8.6	5.6 ab
PSCU	55.5 b	1.9 b	2.6 b	4.0	1.4	6.7	2.9	1.3 bc
UR+DCD	8.9 c	3.5 b	0.6 b	2.0	0.8	5.3	2.3	1.2 c
UR+DMPP	8.3 c	2.5 b	1.4 b	3.7	2.7	10.2	3.1	2.0 abc
Calcium Nitrate	12.8 c	1.6 b	0.5 b	1.1	1.7	3.7	1.5	0.8 c
16 DAF								
No N	18.7 c	41.1 a	1.5 b	2.2 ns	5.3 ns	0.7 b	24.5 a	1.2 ns
UR	1781.3 a	8.7 b	8.7 a	1.3	7.4	3.4 a	8.3 b	1.1
UR+DCD - R	238.4 c	13.4 b	2.2 b	0.9	5.2	2.9 ab	9.7 b	0.8
UR+DMPP - R	47.7 c	12.9 b	1.2 b	2.2	6.6	2.1 ab	16.3 ab	1.1
PSCU	609.8 b	9.8 b	5.1 ab	0.9	7.4	2.8 ab	8.7 b	1.3
UR+DCD	62.2 c	11.7 b	0.9 b	0.4	3.6	2.5 ab	6.3 b	0.7
UR+DMPP	39.3 c	10.0 b	0.7 b	0.8	4.7	2.8 ab	5.7 b	0.9
Calcium Nitrate	36.9 c	11.9 b	0.4 b	0.4	1.8	1.3 ab	6.4 b	0.5
18 DAF								
No N	15.3 c	23.6 a	2.8 b	14.5 ns	35.2 ns	0.7 b	18.0 a	1.0 ns
UR	1187.0 a	8.5 b	10.7 ab	7.9	14.2	1.6 a	3.2 b	0.7
UR+DCD - R	110.6 c	4.3 b	16.7 a	10.0	20.0	1.0 ab	5.1 b	1.1
UR+DMPP - R	27.6 c	6.4 b	1.7 b	9.3	27.7	1.2 ab	5.2 b	1.0
PSCU	558.0 b	2.2 b	6.6 ab	2.8	6.0	0.8 b	2.0 b	0.3
UR+DCD	53.8 c	7.4 b	4.4 b	5.8	9.6	1.1 ab	7.3 b	0.9
UR+DMPP	20.9 c	3.5 b	1.9 b	5.9	6.4	1.0 ab	4.7 b	0.5
Calcium Nitrate	27.4 c	9.0 b	1.1 b	4.1	10.7	0.5 b	9.9 ab	0.5

Table S1 cont'd.

Treatment	N_2O-N	AOA *amoA*	AOB *amoA*	*nirK*	*nirS*	*nosZ*	Total archaea	Total bacteria
27 DAF	$\mu g\ m^{-2}\ h^{-1}$	10^6 copies g^{-1}	10^5 copies g^{-1}	10^8 copies g^{-1}	10^5 copies g^{-1}	10^6 copies g^{-1}	10^7 copies g^{-1}	10^9 copies g^{-1}
No N	8.1 c	27.8 a	6.8 bc	15.4 a	5.3 a	1.8 abc	14.4 a	1.3 ns
UR	1356.3 a	2.1 bc	53.8 a	7.3 ab	4.1 ab	2.2 a	2.0 c	0.9
UR+DCD - R	163.8 c	6.0 bc	31.3 ab	7.3 ab	4.9 ab	1.7 abc	4.1 bc	0.7
UR+DMPP - R	10.7 c	8.3 b	3.7 c	11.1 ab	6.5 a	2.1 ab	8.3 ab	1.4
PSCU	704.8 b	1.5 c	17.9 bc	3.9 b	2.1 b	1.3 bc	2.4 bc	0.8
UR+DCD	77.3 c	5.0 bc	10.2 bc	5.2 ab	2.2 b	1.4 abc	3.2 bc	0.8
UR+DMPP	39.3 c	6.0 bc	4.4 bc	7.1 ab	2.6 b	1.7 abc	3.1 bc	0.9
Calcium Nitrate	36.9 c	6.8 bc	1.8 c	3.6 b	0.8 b	0.9 c	3.0 bc	0.4
35 DAF								
No N	8.4 b	12.4 a	1.2 b	5.5 ns	1.2 ns	2.1 ns	3.7 ns	0.3 ns
UR	1137.0 a	4.0 ab	11.8 a	6.2	1.3	2.0	2.4	0.5
UR+DCD - R	83.9 b	4.4 ab	5.7 b	2.6	0.9	2.2	2.0	0.3
UR+DMPP - R	42.4 b	3.8 ab	1.6 b	5.6	1.3	1.3	3,0	0.5
PSCU	1167.5 a	1.4 b	2.8 b	2.5	0.3	1.2	1,0	0.2
UR+DCD	264.5 b	4.8 ab	3.4 b	3.0	0.6	1.9	1,7	0.3
UR+DMPP	105.4 b	9.1 ab	1.6 b	4.3	1.4	3.1	2,1	0.4
Calcium Nitrate	28.7 b	9.4 ab	1.0 b	4.7	0.7	2.0	2,8	0.3
42 DAF								
No N	4.0 b	28.1 a	1.6 ns	13.5 ns	3.4 ns	2.5 ns	7,9 a	0.7 ns
UR	225.3 ab	1.4 b	8.5	4.5	2.0	2.4	0,8 b	0.4
UR+DCD - R	10.8 b	3.3 b	8.0	10.2	2.8	1.7	3,6 ab	0.6
UR+DMPP - R	22.1 b	5.7 b	3.0	11.9	4.2	2.1	3,5 ab	0.7
PSCU	312.9 a	1.8 b	6.5	4.0	1.6	1.7	1,0 b	0.3
UR+DCD	15.3 b	6.1 b	9.7	6.0	1.4	1.7	3,3 ab	0.5
UR+DMPP	10.7 b	5.5 b	1.6	7.7	2.4	1.6	3,3 ab	0.6
Calcium Nitrate	6.5 b	7.0 b	1.2	5.0	1.0	1.1	2,7 ab	0.4

Table S1 cont'd.

Treatment	N_2O-N	AOA *amoA*	AOB *amoA*	*nirK*	*nirS*	*nosZ*	Total archaea	Total bacteria
82 DAF	$\mu g\ m^{-2}\ h^{-1}$	10^6 copies g^{-1}	10^5 copies g^{-1}	10^8 copies g^{-1}	10^5 copies g^{-1}	10^6 copies g^{-1}	10^7 copies g^{-1}	10^9 copies g^{-1}
No N	0.2 b	40.7 a	1.7 b	22.1 ns	4.6 ab	0.6 c	13.8 ns	1.3 ns
UR	1.6 b	1.0 b	10.7 a	2.9	1.1 b	1.7 abc	0.8	0.2
UR+DCD - R	0.1 b	1.9 b	2.8 b	13.4	9.2 ab	1.1 bc	6.2	0.8
UR+DMPP - R	0.2 b	6.2 b	0.8 b	3.0	3.1 ab	0.8 bc	4.0	0.1
PSCU	8.8 a	1.4 b	4.8 ab	1.9	1.4 b	0.7 c	1.0	0.1
UR+DCD	0.2 b	22.4 ab	3.7 b	19.5	8.8 ab	2.2 ab	9.8	2.2
UR+DMPP	0.1 b	23.2 ab	2.4 b	29.6	13.0 ab	3.1 a	12.6	2.4
Calcium Nitrate	0.1 b	51.3 a	2.4 b	19.7	17.4 a	2.0 abc	20.8	2.6
158 DAF								
No N	0.1 b	35.3 a	2.9 ab	23.9 ns	14.7 ns	1.0 ns	9.2 a	1.1 ns
UR	0.3 b	2.8 b	4.2 ab	6.3	5.2	1.0	2.4 ab	0.5
UR+DCD - R	0.2 b	10.1 b	5.7 a	13.8	16.5	0.9	6.2 ab	2.0
UR+DMPP - R	0.1 b	5.2 b	1.3 b	7.0	5.1	0.8	3.7 ab	0.5
PSCU	4.6 a	7.7 b	4.1 ab	6.6	4.0	0.8	2.4 ab	0.7
UR+DCD	0.1 b	6.5 b	1.7 b	5.5	4.2	0.7	0.5 b	0.5
UR+DMPP	0.2 b	10.0 b	0.9 b	5.5	4.7	0.9	0.3 b	0.4
Calcium Nitrate	0.0 b	11.9 b	1.3 b	13.1	4.4	0.7	0.5 b	0.6

Means followed by same letter in column per DAF did not differ by Tukey test 5%. ns: no significant. †Days after fertilizer application. ‡ R means reapplication of inhibitors in same plot.

Table S2. Linear regression and coefficients (R^2) relating daily N_2O flux to environmental variables from Red Latosol soil after application of urea with or without nitrification inhibitors (DCD and DMPP), polymer sulfur coated urea (PSCU) and calcium nitrate applied to sugarcane. (n = 256).

Variable (x)†	All treatments Regression*	R^2	Without nitrification inhibitors Regression	R^2
NH_4^+-N	ns	-	y = 1.87 + 0.00073x	0.0879
NO_3^--N	y = 1.64 + 0.0016x	0.0389	ns	-
WFPS	y = 0.74 + 0.031x	0.1021	y = 0.82 + 0.034x	0.0864
Temp. air	ns	-	ns	-
Temp. soil	ns	-	ns	-
pH	y = 1.70 + 2.4e4x	0.0214	ns	-
Rain day	y = 1.58 + 0.013x	0.0818	y = 1.77 + 0.013x	0.0609
Rain week	y = 1.40 + 0.0081x	0.1589	y = 1.60 + 0.0078x	0.1017
CO_2-C	ns	-	ns	-
CH_4-C	y = 1.78 + 0.0073x	0.0243	y = 2.02 + 0.011x	0.0422
AOA *amoA*	y = 1.88 - 2.83e-8x	0.0990	y = 2.18 - 3.83e-8x	0.2107
AOB *amoA*	y = 1.56 + 2.94e-7x	0.1786	y = 1.71 + 3.02e-7x	0.1944
nirK	y = 1.82 - 1.32e-11x	0.0405	y = 2.03 - 1.55e-11x	0.0485
nirS	ns	-	ns	-
nosZ	ns	-	ns	-
Total bacteria	y = 1.80 + 7.83e-11x	0.0297	ns	-
Total archaea	y = 1.84 - 2.28e-9x	0.0532	y = 2.10 - 3.24e-9x	0.1076

* $p \leq 0.05$. ns: no significant. † N_2O-N: $\mu g\ m^{-2}\ h^{-1}$ transformed in log(X+10); CO_2-C: $mg\ m^{-2}\ h^{-1}$; CH_4-C: $\mu g\ m^{-2}\ h^{-1}$; NH_4^+-N and NO_3^--N: $mg\ kg^{-1}$ 0-10 cm soil; Rainweek and Rainday: mm, Temp. air and soil: ºC; WFPS: Water-filled pore space, %; AOB *amoA*, AOA *amoA*, *nir*K, *nir*S, *nos*Z, total bacteria and total archaea: copies gene g^{-1} dry soil; pH-CaCl$_2$: transformed in H$^+$, 10^{-pH}.

Table S3. Multiple linear regression parameters (β) and coefficients (R^2) relating daily N_2O flux to environmental variables after fertilizer application to sugarcane (n = 256).

Model*	Parameters	R^2
All treatments†		
$N_2O\text{-}N = \beta0 + \beta AOB + \beta Rainweek + \beta pH + \beta NH_4^+\text{-}N + \beta Bacteria + \beta CO_2 + \beta NO_3^-\text{-}N$ ‡	$0.84 + 2.96e\text{-}7AOB + 0.0097Rainweek + 3.3e4pH + 0.0050NH_4^+\text{-}N - 0.9e\text{-}10Bacteria + 0.0019NO_3^-\text{-}N + 0.00093CO_2$	0.4741
Without nitrification inhibitors		
$N_2O\text{-}N = \beta0 + \beta AOB + \beta Rainweek + \beta NH_4^+\text{-}N + \beta Bacteria + \beta pH + \beta CO_2$	$0.96 + 2.93e\text{-}7AOB + 0.0096Rainweek + 0.0012NH_4^+\text{-}N - 1.8e\text{-}10Bacteria + 3.0e4pH + 0.0023CO_2$	0.5267

* Stepwise selection, $p \leq 0.05$. † Treatments: No N – control, Urea (UR), UR+DCD, UR+DMPP, Polymer Sulfur Coated Urea and calcium nitrate. ‡ $N_2O\text{-}N$: µg m^{-2} h^{-1} transformed in log(X+10); AOB and Bacteria: *amoA* from ammonia oxidizing bacteria and all bacteria, copies gene g^{-1} dry soil; $NH_4^+\text{-}N$ and $NO_3^-\text{-}N$: mg kg^{-1} 0-10 cm soil; Rainweek: mm; pH-CaCl$_2$: transformed in H$^+$,

Table S4. Top-nine bacterial phyla presented in the microbial communities in the Red Latosol soil under treatments with urea with or without nitrification inhibitors (DCD and DMPP); polymer sulfur coated urea (PSCU) or calcium nitrate applied to sugarcane.

Treatment	Mean relative abundance (%) of Bacterial Phyla, or effect size(corrected p-value)								
	Proteo	Firm	Actino	Acido	Bacter	Verruco	Plancto	Gemmati	Nitro
7 DAF†	ns	0.73 (0.07)	ns	ns	ns	ns	ns	ns	ns
No N	24	4	20	21	3	5	2	1	1
UR	27	24	23	5	4	2	1	1	0
UR+DCD - R‡	26	26	14	12	4	3	2	1	1
UR+DMPP – R	25	28	12	12	7	2	2	1	0
PSCU	44	6	24	7	3	2	2	1	0
UR+DCD	34	20	13	8	5	3	1	1	0
UR+DMPP	23	32	16	7	4	2	1	1	0
Calcium Nitrate	30	9	22	13	4	4	2	1	1
16 DAF†	ns	ns	ns	ns	0.65(0.09)	ns	ns	ns	0.63(0.09)
No N	28	10	31	11	3	3	1	1	1
UR	36	15	23	4	8	1	1	1	0
UR+DCD - R‡	30	12	19	12	6	4	3	1	0
UR+DMPP – R	36	13	20	11	4	2	2	1	0
PSCU	35	18	23	5	7	1	1	1	0
UR+DCD	32	15	22	7	7	3	2	1	0
UR+DMPP	30	11	21	10	4	5	3	1	0
Calcium Nitrate	22	21	24	12	2	3	2	1	0
18 DAF†	ns	ns	ns	ns	ns	ns	ns	ns	ns
No N	25	9	24	15	3	5	2	1	1
UR	34	14	24	6	6	2	2	1	0
UR+DCD - R‡	41	13	17	2	12	1	3	1	0
UR+DMPP – R	43	10	17	5	10	2	1	2	0
PSCU	38	13	23	6	6	1	1	1	0
UR+DCD	31	20	17	5	11	2	2	1	0
UR+DMPP	34	17	24	5	5	2	1	1	0
Calcium Nitrate	35	18	23	6	5	2	1	1	0
27 DAF†	ns	ns	ns	ns	ns	ns	ns	ns	ns
No N	42	3	23	8	6	2	0	2	1
UR	49	7	13	3	17	1	1	2	0
UR+DCD - R‡	49	5	16	5	7	3	1	2	0
UR+DMPP – R	48	4	12	6	13	3	1	2	0
PSCU	49	6	20	3	10	1	1	2	0
UR+DCD	48	5	16	3	14	2	1	2	0
UR+DMPP	53	6	18	3	9	1	0	1	0
Calcium Nitrate	45	7	19	6	6	2	1	2	0

Significance from Tukey-Kramer post-hoc test based on Benjamini-Hochberg corrected p-values above 0.1 from STAMP analysis. ns: not significant. †Days after fertilizer application. ‡ R means reapplication of inhibitors in same plot. Proteo=Proteobacteria, Firm=Firmicutes, Actino=Actinobacteria, Bacter=Bacteroidetes, Verruco=Verrucomicrobia, Planct=Planctomycetes, Gemmati=Gemmatimonadetes, Nitro=Nitrospira.

Table S4 cont'd.

Treatment	Mean relative abundance (%) of Bacterial Phyla, or effect size(corrected p-value)								
	Proteo	Firm	Actino	Acido	Bacter	Verruco	Planct	Gemmati	Nitro
35 DAF†	0.71(0.02)	ns	ns	ns	ns	0.67(0.04)	ns	ns	ns
No N	32	2	15	18	6	5	1	2	1
UR	48	3	15	5	12	1	1	2	0
UR+DCD - R‡	46	5	14	8	12	2	1	2	0
UR+DMPP – R	45	6	14	7	12	3	1	2	0
PSCU	57	3	12	5	13	1	1	1	0
UR+DCD	42	5	13	8	12	2	1	3	0
UR+DMPP	43	3	11	8	18	2	1	2	0
Calcium Nitrate	42	10	20	5	8	2	1	3	0
42 DAF†	0.60(0.07)	ns	ns	0.60(0.09)	0.64(0.08)	ns	ns	ns	0.74(0.02)
No N	31	2	19	17	8	6	1	1	1
UR	39	5	17	7	16	3	1	2	0
UR+DCD - R‡	43	4	15	6	15	3	1	2	1
UR+DMPP – R	37	6	19	10	11	2	2	2	0
PSCU	53	2	17	4	11	1	1	1	0
UR+DCD	40	4	18	7	13	5	1	2	0
UR+DMPP	38	4	18	11	9	4	2	2	0
Calcium Nitrate	37	4	19	8	13	4	1	2	0
82 DAF†	ns	0.80(0.003)	ns	ns	0.65(0.03)	ns	ns	0.72(0.01)	0.77(0.004)
No N	37	4	13	14	9	5	1	2	1
UR	47	3	15	5	16	1	1	3	0
UR+DCD - R‡	37	22	22	2	9	1	0	1	0
UR+DMPP – R	48	15	18	5	7	1	0	1	0
PSCU	50	4	20	3	14	0	0	1	0
UR+DCD	45	3	16	8	11	3	1	3	0
UR+DMPP	42	6	18	9	10	2	1	2	1
Calcium Nitrate	30	20	30	4	4	2	1	1	0
158DAF†	ns	ns	ns	ns	ns	ns	ns	ns	0.70(0.06)
No N	39	3	21	9	11	3	1	2	0
UR	47	3	15	6	12	3	1	3	0
UR+DCD - R‡	42	6	22	5	11	1	0	3	0
UR+DMPP – R	43	5	23	7	9	2	1	2	0
PSCU	47	3	20	5	9	2	1	2	0
UR+DCD	43	5	18	8	10	3	1	2	0
UR+DMPP	46	3	17	8	10	2	1	3	0
Calcium Nitrate	40	5	24	5	9	2	1	3	0

Significance from Tukey-Kramer post-hoc test based on Benjamini-Hochberg corrected p-values above 0.1 from STAMP analysis. ns: not significant. †Days after fertilizer application. ‡ R means reapplication of inhibitors in same plot. Proteo=Proteobacteria, Firm=Firmicutes, Actino=Actinobacteria, Bacter=Bacteroidetes, Verruco=Verrucomicrobia, Planct=Planctomycetes, Gemmati=Gemmatimonadetes, Nitro=Nitrospira.

Table S5. Primers and thermocycler conditions used in gene abundance analysis by real time qPCR

| Target gene	Primer	Primer Sequence	Size (bp)	Thermal profile	Reference	Source of Standard plasmid	Vector
AOA *amoA*	Arch-amoAF	5'-STAATGGTCTG GCTTAGACG-3'	635	95°C-5 min.; 95°C-30s, 55°C-45s, 72°C-45s, 82°C-15s	40xFrancis et al.[54]	Environmental Archaea	pGEM®-T Systems Promega
	Arch-amoAR	5'-GCGGCCATCC ATCTGTATGT-3'					
AOB *amoA*	amoA1F	5'-GGGGTTTCT ACTGGTGGT-3'	491	95°C-5 min.; 95°C-30s, 56°C-45s, 72°C-45s, 82°C-15s	40xRotthauwe et al.[55]	*Nitrosomonas europaea*	pGEM®-T Systems Promega
	amoA2R	5'-CCCCTCKGSA AAGCCTTCTTC-3'					
nosZ	nosZ2F	5'-CGCRACGGCAA SAAGGTSMSSGT-3'	267	95°C-5 min.; 95°C-15s, 60°C-15s, 72°C-30s, 82°C-15s	40xHenry et al.[56]	*Pseudomonas stutzeri* (M13R/F)	Dh5alpha p PCR4-topo
	nosZ2R	5'-CAKRTGCAKSG CRTGGCAGAA-3'					
*nir*K	NirK876	5'-ATYGGCGG VAYGGCGA-3'	165	95°C-5 min.; 95°C-15s, 63°C-30s, 72°C-30s, 82°C-15s	40xHenry et al.[57]	*Paracoccus denitrificans* (DSM 413)	Dh5alpha p PCR4-topo
	NirK1040	5'-GCCTCGATCA GRTTRTGGTT-3'					
*nir*S	nirScd3aF	5'-GTSAACGTSA AGGARACSGG-3'	425	95°C-5 min.; 95°C-10s, 60°C-10s, 72°C-20s, 86°C-5s	40xThrobäck et al.[58]	*Pseudomonas stutzeri* (M13R/F)	PCR produ
	nirSR3cd	5'-GASTTCGGRT GSGTCTTGA-3'					
Total bacteria	Eub338	5'-ACTCCTACGG GAGGCAGCAG-3'	200	95°C-5 min.; 95°C-5s, 53°C-10s, 72°C-20s	40xFierer et al.[59]	Firmicutes	Dh5alpha p PCR4-topo
	Eub518	5'-ATTACCGC GGCTGCTGG-3'					
Total archaea	Arch1017R	5'-AGGAATTGGC GGGGGAGCAC-3'	112	95°C-10 min.; 95°C-10s, 60°C-10s, 72°C-20s	40xKlindworth et al.[60]	Environmental Archaea	Dh5alpha p PCR4-topo
	Arch915F	5'-GGCCATGCA CCWCCTCTC-3'					
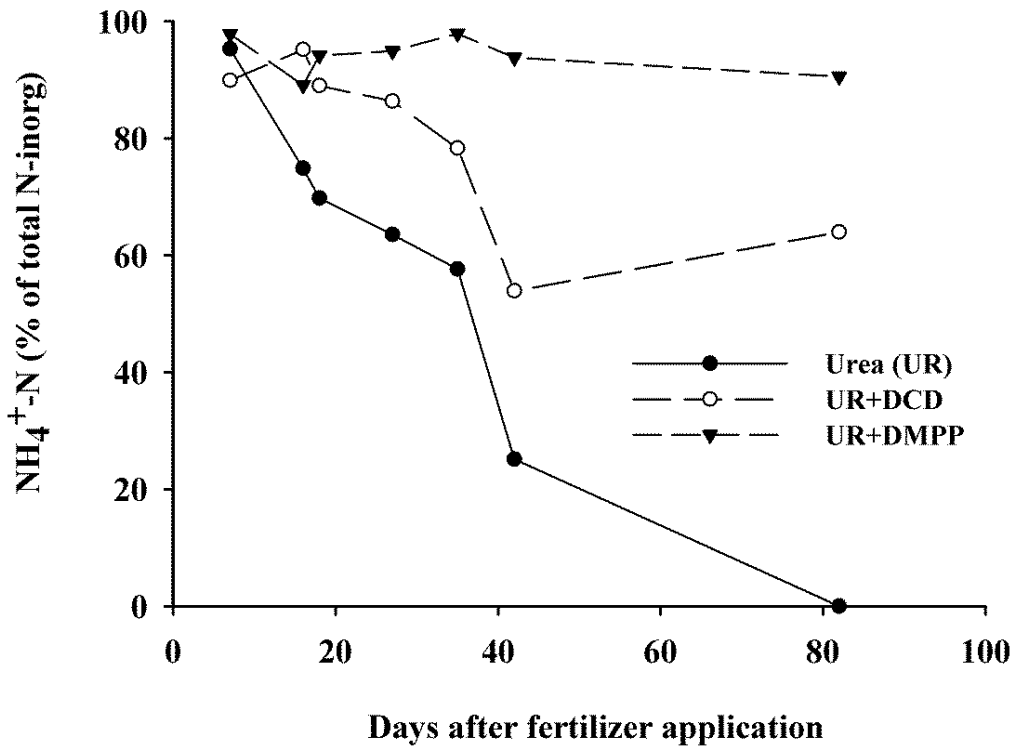
Figure S1. Percentage of NH4+ in relation to total inorganic N (NH_4^+ + NO_3^-) at 0 - 10 cm soil depth from application of urea with or without nitrification inhibitors (DCD and DMPP) to sugarcane.

$$y = 43.63 + 1.20e\text{-}3x$$
$$R^2 = 0.5323; \ p < 0.001$$
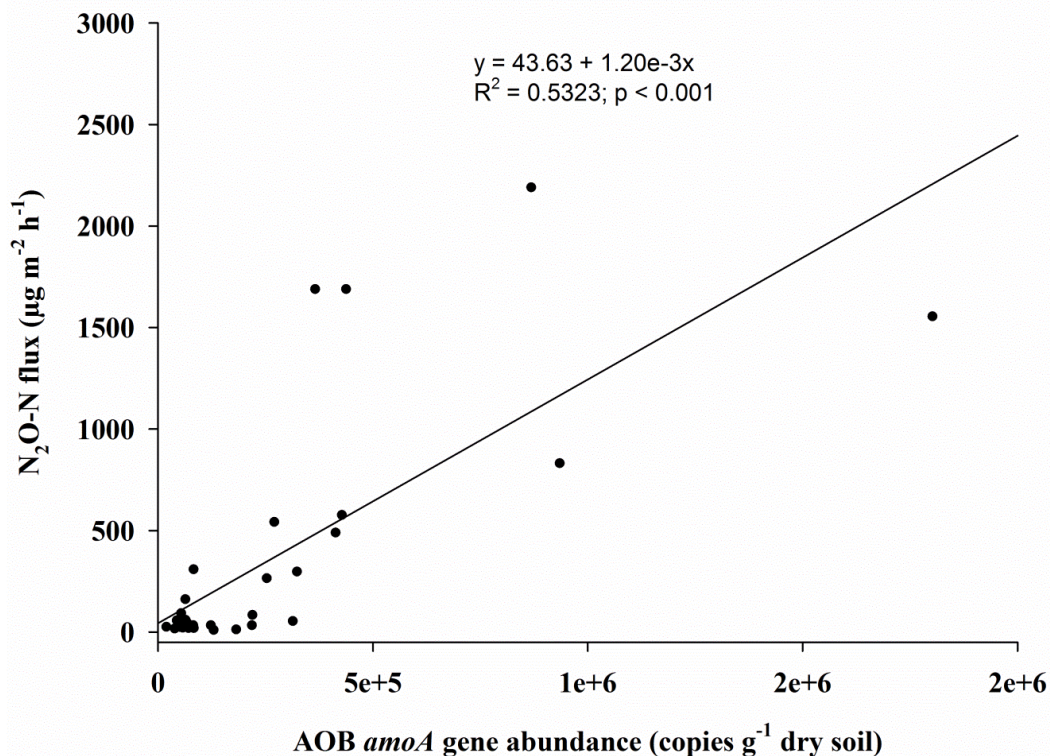
Figure S2. Correlation between ammonia oxidizing bacteria (AOB) *amoA* gene abundance and nitrous oxide emission from Red Latosol soil 16 days after application of urea with or without nitrification inhibitors (DCD and DMPP), polymer sulfur coated urea (PSCU) and calcium nitrate applied to sugarcane (n = 32).
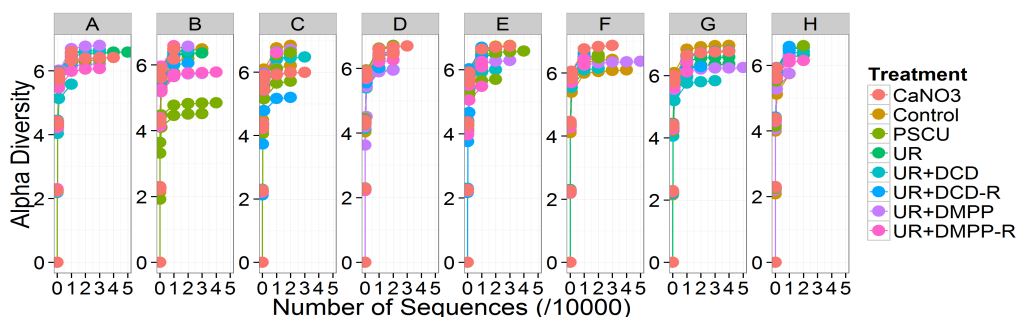
Figure S3. Rarefaction curves of the microbial communities present in the Red Latosol under treatments with urea, incorporated, with or without nitrification inhibitors (DCD and DMPP); polymer sulfur coated urea (PSCU) and calcium nitrate applied to sugarcane. Time points are separated such that A, B, C, D, E, F, G and H correspond to days 7, 16, 18, 27, 35, 42, 82 and 158 of the experiment.

Table S4. Shannon indices and Between-Class Analysis (BCA) ordinations of the bacterial communities under treatments of urea, incorporated in the Red Latosol, with or without nitrification inhibitors (DCD and DMPP); polymer sulfur coated urea (PSCU) or calcium nitrate applied to ratoon sugarcane. Continued on next four pages. DAF = days after fertilization.

Treatment	16s rRNA gene diversity		BCA ordination based on 16S gene abundances within Phyla	BCA ordination based on 16S gene abundances within Genus
7 DAF†				
No N	6.2	ns		
UR	5.8			
UR+DCD - R	5.9			
UR+DMPP – R	5.6			
PSCU	5.9			
UR+DCD	5.7			
UR+DMPP	5.7			
Calcium Nitrate	6.3			
16 DAF				
No N	6.1	ns		
UR	6.0			
UR+DCD - R	6.2			
UR+DMPP – R	6.1			
PSCU	5.9			
UR+DCD	6.1			
UR+DMPP	6.0			
Calcium Nitrate	6.0			
18 DAF				
No N	6.0	ns		
UR	6.0			
UR+DCD - R	5.8			
UR+DMPP – R	5.9			
PSCU	5.8			
UR+DCD	5.7			
UR+DMPP	5.9			
Calcium Nitrate	5.7			

Table S4 con't.

Treatment	16s rRNA gene diversity		BCA ordination based on 16S gene abundances within Phyla	BCA ordination based on 16S gene abundances within Genus
27 DAF				
No N	6.2	a		
UR	5.3	b		
UR+DCD - R	6.0	ab		
UR+DMPP – R	5.8	ab		
PSCU	5.8	ab		
UR+DCD	5.8	ab		
UR+DMPP	5.8	ab		
Calcium Nitrate		ab		
	5.9			
35 DAF				
No N	6.1	ns		
UR	5.7			
UR+DCD – R	6.0			
UR+DMPP – R	6.1			
PSCU	5.1			
UR+DCD	5.9			
UR+DMPP	5.5			
Calcium Nitrate	5.9			
42 DAF				
No N	6.2	ns		
UR	5.8			
UR+DCD - R	6.1			
UR+DMPP – R	6.2			
PSCU	5.2			
UR+DCD	6.0			
UR+DMPP	6.1			
Calcium Nitrate	6.2			

BCA Day 27, Obs. 0.61, P = 0.001 · BCA Day 27, Obs. 0.48, P = 0.017

BCA Day 35, Obs. 0.68, P = 0.002 · BCA Day 35, Obs. 0.52, P = 0.001

BCA Day 42, Obs. 0.54, P = 0.012 · BCA Day 42, Obs. 0.69, P = 0.001

Table S4 con

Treatment	16S rRNA gene diversity	BCA ordination based on 16S gene abundances within Phyla	BCA ordination based on 16S gene abundances within Genus
85 DAF			
No N	6.0 ab		
UR	5.6 ab		
UR+DCD – R	5.8 ab		
UR+DMPP – R	6.1 ab		
PSCU	5.4 a		
UR+DCD	6.0 ab		
UR+DMPP	6.1 b		
Calcium Nitrate	6.0 ab		
158DAF			
No N	6.1 ns		
UR	6.0		
UR+DCD – R	6.2		
UR+DMPP – R	6.2		
PSCU	5.8		
UR+DCD	6.2		
UR+DMPP	6.1		
Calcium Nitrate	6.2		

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