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

Johnny R. Soares[1,2*], Noriko A. Cassman[1*], Anna M. Kielak[1], Agata Pijl[1], Janaína B. Carmo[3], Kesia S. Lourenço[1,2], Hendrikus J. Laanbroek[1,4], Heitor Cantarella[2] & Eiko E. Kuramae[1§]

[1]Department of Microbial Ecology, Netherlands Institute of Ecology, 6708 PB, Wageningen, Netherlands.

[2]Soils and Environmental Resources Center, Agronomic Institute of Campinas, P.O. Box 28, 13012-970, Campinas, SP, Brazil.

[3]Environmental Science Department, Federal University of São Carlos, 1852-780, Sorocaba, SP, Brazil.

[4]Institute of Environmental Biology, Utrecht University, Netherlands

[*]Equal contribution

[§]Corresponding author: e.kuramae@nioo.knaw.nl

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.

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

60 N_2O emissions[11], we predicted based on the high soil drainage that nitrification would be the
61 major pathway contributing to N_2O emissions. In this case NH_4^+-based fertilizer would result
62 in higher N_2O emissions than those from NO_3^--based fertilizers in these soils. Up to date, this
63 process has not been shown for these types of soils grown with sugarcane.

64 The Intergovernmental Panel on Climate Change (IPCC) estimates that 1% of N applied is
65 emitted as N_2O as default value[12]. However, in practice, different amounts of N_2O are emitted
66 depending on N fertilizers and soil types, and environmental conditions[13-15]. Therefore,
67 experiment-based nitrogen management is an important tool to decrease N_2O emissions and to
68 reduce the environmental impact of agricultural practices[15].

69 Urea is the most widely used fertilizer in the world, and generally has been linked to higher
70 N_2O emissions compared with other N sources[14]. One way to reduce N_2O emissions is the
71 addition of specific nitrification inhibitors (NIs) such as dicyandiamide (DCD), 3,4
72 dimethylpyrazone phosphate (DMPP), nitrapyrin, and others with urea fertilization[15,16]. These
73 nitrification inhibitors block the enzyme ammonia monooxygenase in the first step of
74 nitrification[17]. The gene encoding this enzyme is *amoA*, present in ammonia-oxidizing
75 bacteria (AOB) and archaea (AOA). Several studies indicate that DCD and DMPP reduce
76 AOB or AOA gene abundances, depending on which microorganism was prevalent[8,18-20].
77 DCD has been reported to reduce also the abundance of *nirK*, probably because AOB
78 abundances are correlated with *nirK* abundances, implying reduced nitrifier denitrification
79 abundances[8].

80 To our knowledge, there are no studies identifying the main microbial processes, the effect
81 of different fertilizers on N_2O emissions, and the impact of different N fertilizers on the
82 microbial community in tropical soils grown with sugarcane. Therefore, the goals of this
83 study were to (i) determine the main microbial process responsible for the N_2O emissions, (ii)
84 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.

Results

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 (Fig. 1). High N_2O emission followed rain events coupled with high soil inorganic N availability (Fig. 2). The UR treatment had the highest peak of N_2O emission, on the 14^{th} day, which corresponded to a loss rate of more than 200 g ha^{-1} day^{-1} of N. On the 29^{th} 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[16]. Emissions in the plots with repeated application of NIs were also low at $\leq$ 5 g ha^{-1} day-1 of N (Fig.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 14^{th} and 29^{th} days, but was similar to UR treatment levels afterwards, until the 50^{th} 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}) (Fig. 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 (Fig. 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).

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 (Fig. 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 Fig. S1). At the 82^{th} 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.

134 The original soil pH (control treatment) was around 5.1 but increased to 8 in the UR,
135 UR+DCD and UR+DMPP treatments by seven days after fertilizer application, because of
136 urea hydrolysis. PSCU showed pH value 1.4 units lower (pH 6.6) but treatments with calcium
137 nitrate did not affect soil pH. In the 42^{th} day, the soil pH of the UR+DCD and UR+DMPP
138 treatments had dropped to values around 7 (Fig. 2).
139
140 *Nitrogen cycle gene abundances.*
141 The abundances of N cycling genes related to N_2O emissions are depicted in Fig. 4 for one
142 timepoint sampling that featured high N_2O emissions: 16 days after fertilizer application,
143 corresponding to the second soil sampling. The qPCR results from all data sampling
144 timepoints are available in Supplementary Table S1. The abundance of *amoA* belonging to
145 ammonia-oxidizing archaea (AOA) was lower in treatments with N sources than in the
146 control plot, and did not show significant differences among the N sources across nearly all
147 data sampling points. The correlation between AOA *amoA* abundance and N_2O emissions was
148 negative (Supplementary Table S2). The gene abundance representing total archaea showed a
149 similar pattern as the AOA *amoA* abundance (Fig. 4 and Supplementary Table S1). On the
150 other hand, the *amoA* abundance of ammonia-oxidizing bacteria (AOB) was best correlated
151 with N_2O emissions, showing a coefficient (R^2) of 0.18 ($p \leq 0.05$) (Supplementary Table S2).
152 Over almost all data sampling points, AOB *amoA* abundances were higher in the UR
153 treatment than in other treatments, following the data from N_2O emissions (Fig. 4 and
154 Supplementary Table S1). For example, concurrent to high N_2O emissions at day 16 after
155 fertilizer application, the coefficient of correlation (R^2) between N_2O and AOB *amoA* was
156 0.53 (Supplementary Fig. S2).
157 The denitrification genes *nirS* and *nosZ* as well as the 16S rRNA gene of total bacteria did
158 not show differences in abundance between treatments over almost all data samplings. The

159 *nirK* occurring in both ammonia-oxidizing and denitrification microorganisms had a negative
160 correlation with N_2O emissions, while the total bacteria abundance resulted in a positive
161 correlation with this emission (Supplementary Table S2). In some data samplings, the
162 abundance of the *nosZ* was higher in treatment with N than the control treatment, but no
163 differences in *nosZ* abundance were observed between the N sources treatments (Fig. 4 and
164 Supplementary Table S1).

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

171

172 *Bacterial community composition and diversity.*

173 Because the AOB *AmoA* abundance was correlated with N_2O emissions, we sequenced the
174 16S rRNA genes from our samples to ascertain the effect of the treatments on the entire
175 microbial (bacterial and archaeal) community. After processing the 16S rRNA amplicon
176 sequences, the 177 samples (8 treatments x 8 timepoints x 3 replicates, excluding
177 undersampled samples and outliers) contained between 2,000 and 56,638 sequences, with a
178 total of 3,607,143 sequences distributed into 9,267 Operational Taxonomic Units (OTUs).
179 Rarefaction curves indicated that most of the community diversity was captured with our
180 sequencing depth (Supplementary Fig. S3). The top nine bacterial phyla across the samples
181 were *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Gemmatimonadetes*,
182 *Proteobacteria* and *Verrucomicrobia* (Supplementary Table S4). The top phyla that differed
183 between the treatments within at least one timepoint were *Firmicutes*, *Bacteroidetes*,

184 *Nitrospira*, *Proteobacteria*, *Verrucomicrobia* and *Acidobacteria*. Shannon diversity indices of
185 the bacterial communities ranged between 5.3 and 6.3 in treatments over all time points, and
186 were significantly different between treatments only for days 27 (Control *versus* UR) and 82
187 (PSCU *versus* UR+DMPP; Table 2).

188 Based on phylum-level relative abundances, the samples were significantly grouped by
189 treatment on days 7, 27, 35, 42, 85 and 158 (permutation test, $P < 0.100$; Table 2) with a
190 range of observed values between 0.83 and 0.44. At day 7, three separate clusters were
191 formed respectively with the Control and calcium nitrate treatments, the PSCU treatment and
192 the other treatments (Supplementary Table S5). However, this clustering pattern was not
193 observed during the remaining other days, indicating no differences in the bacterial
194 communities between treatments. When bacterial community compositions were compared at
195 the taxonomical level of genera, the samples were significantly grouped by treatment and the
196 grouping was characterized by low observation values for all days except day 16 ($P=0.119$;
197 Table 2 and Supplementary Table S5). The sample treatment grouping pattern from day 7 was
198 seen in the genus-level comparisons (Supplementary Table S5).

199

200 **Discussion**

201 Nitrous oxide emissions from the urea treatment were higher than the emissions found in two
202 previous sugarcane cycles in this experimental area[16]. Here, the emission factor was 1.7% of
203 N applied, which is greater than the emission factors found in other studies of sugarcane soils
204 in Brazil, around 0.7 – 1% of N applied as urea[13,16,21]. The sugarcane plant phenology may
205 give insight into higher emissions. The N fertilizer treatments were applied 20 days after the
206 previous sugarcane ratoon was harvested. At the time of fertilization, the soil was relatively
207 dry – below 30% of the WFPS (Fig. 1) and sugarcane plants were still beginning to sprout. At
208 this stage the root system was being reformed and nutrient uptake was slow, as the high soil N

209 concentration indicated (Fig. 2). This probably led to the greater N_2O emissions than
210 expected. The high peaks of N_2O emission occurred after two high rain events in the first 35
211 days (total 65 mm and 90 mm) on a mostly dry soil (15 – 20% WFPS) but with high air
212 temperatures as shown in Fig. 1. Moreover, high correlation between N_2O emission and
213 accumulated rain in the week was found (Supplementary Table S2). Thus, the climatic
214 conditions in this season contributed to the high N_2O emission values.

215 A strong reduction in N_2O emissions due to the addition of nitrification inhibitors to urea
216 was found, as well as a lack of beneficial effects of the controlled release fertilizer PSCU,
217 supporting similar observations in the same area[16]. The N_2O emissions from PSCU were
218 lower than those from UR in the first 30 days after N application, as expected from a slow-
219 release fertilizer (Fig. 2). There was a dry spell from mid-January to the end of March (Fig.
220 1), which may have slowed down the release of N from PSCU. Subsequent release of N from
221 the PSCU pellets likely led to the observed increase in N_2O emissions (Fig. 1). In this way,
222 the N_2O emission from PSCU had lower peaks than those of UR but lasted longer (Fig. 3).
223 Thus, in the end of the experiment cumulated N_2O of UR and PSCU emissions were similar,
224 suggesting that PSCU is not an environmentally friendly N source during one cycle of
225 sugarcane.

226 In the present study, the calcium nitrate treatment showed very small N_2O emissions that
227 were similar to those of the control plots or plots with urea and nitrification inhibitors (NIs).
228 In the present study intensive GHG measurements under field condition were performed over
229 a whole yearly cycle of sugarcane. We maintain that this is the first field study demonstrating
230 much lower N_2O emissions of a nitrate-N source in comparison to high emissions with urea-N
231 or NH_4^+-N sources; the reduction in N_2O emissions were 98% when compared to urea. This
232 reduction in emissions might be attributed to the high drainage capacity of the soil of the
233 present study, which was classified as Typic Hapludox[22] or Red Latosol[10]. Water

234 accumulation does not tend to occur in these soil profiles, and consequently, favourable
235 conditions for denitrifiers are avoided.

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

241 An alternate explanation for the low N_2O emissions in the calcium nitrate treatment could
242 be NO_3^- leaching. Indeed, the N concentration in the 0-10 cm soil layer of the calcium nitrate
243 treatment was lower than that observed with the other N sources (Fig. 2). However, with only
244 70 mm of rain on a dry soil in 15 days, nitrate is unlikely to have moved beyond 30 cm.
245 Moreover studies with ^{15}N labelling showed little NO_3^- leaching in sugarcane fields in
246 Brazil[23,24]. Therefore, NO_3^- leaching was not expected to explain the small amount of N_2O
247 emission found with calcium nitrate in the present study. However, further studies should
248 include NO_3^- leaching measurements to confirm the present data.

249 Another aspect that may have contributed to the small N_2O emission in the calcium nitrate
250 treatment was the relatively low organic carbon content in the soil, approximately 1%[16].
251 Sugarcane trash and vinasse have been reported to increase N_2O emissions[25], especially under
252 high soil moisture conditions[25]. Here, we did not include in our treatments C sources such as
253 vinasse, filter cake or sugarcane trash, common sugarcane residues or by-products. These
254 residues can favour not only denitrifiers but also nitrifiers and other microorganisms related to
255 the N cycle[26]. Application of exclusively NO_3^--N sources with the addition of C sources, as
256 commonly applied during sugarcane production, may result in N_2O emissions different from
257 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[27,28]. 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[28]. 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[29].

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[8,18,30]. Interestingly, the plots with calcium nitrate also showed a reduction in archaeal *amoA* abundance compared to the control. This

283 may reflect the accumulated effect of ammonium nitrate applied in the two previous cycles as
284 this was the N source previously used in a separate study[16].

285 Significant correlations between N_2O emissions and *amoA* abundances were found for
286 ammonia-oxidizing bacteria (AOB), indicating that in our study, N_2O emissions occurred via
287 nitrification. During the first month after UR application, a peak in the AOB *amoA*
288 abundances was observed. Concurrently, the nitrate content in soil increased whereas
289 ammonium decreased, soil pH decreased from 8 to around 6.3, and the soil temperature was
290 25ºC (Supplementary Figure S5). Thus, the soil and climatic conditions were favourable to
291 AOB and N_2O emissions from nitrification.

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

299 Significant correlation between AOB *amoA* abundance and N_2O emissions was also
300 shown. Under controlled conditions, Venterea et al.[31] found a high correlation of N_2O
301 emissions from urea and NO_2^--N content in soil resulting from increased bacterial *amoA*
302 abundance with no increase in the abundance of the *nxr* gene, which encodes for nitrite
303 oxidation. The authors discussed that N_2O emissions occurred more during nitrification than
304 denitrification, similar to the results found here. Dicyandiamide (DCD) application with cattle
305 urine effectively inhibited the growth of AOB and reduced N_2O emissions as well as the
306 numbers of the *nirK* gene, which encodes for a nitrate reductase[8]. Since DCD did not affect
307 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[33], heterotrophic nitrifiers[34,35], and/or abiotic chemodenitrification[36]. 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[6,36]. 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.

Methods

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[10,22]. The same experiment was carried out during the

seasons of 2011/12 and 2012/13[16]. 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 inhibitors 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[38]. 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.

Greenhouse gases analysis.

Greenhouse gases were collected using static chambers[16]. 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[16]. 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[16,21.]

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[39].

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 (NanoDrop 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.

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'-GGACTACVSGGGTATCTAAT-3'). The samples were PCR-amplified using barcoded primers linked with the Ion adapter "A" sequence (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′) and Ion adapter "P1" sequence (5′-CCTCTCTATGGGCAGTCGGTGAT-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).

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[40]. Forward and reverse primer sequences were removed from each sample FASTQ file using Flexbar version 2.5[41]. 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[42]. These steps were performed with VSEARCH version 1.0.10, which is an open-source and 64-bit multithreaded compatible alternative to USEARCH. Chimeric sequences were detected using the UCHIME algorithm[43] 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[44]. Last, taxonomic information for each OTU was added to the BIOM file using the RDP Classifier version 2.10[45].

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

457 stability[46]. The calculations were performed with the SISVAR statistical software[47] and
458 graphics plotted using Sigma Plot[48].

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

461 $$N_2O = \frac{a}{1+e^{-(\frac{t-t_0}{b})}} \quad (1)$$

462 where N_2O is the cumulative N_2O-N emission, g ha^{-1}, t is the time in days after fertilizer
463 application, and a, t_0 and b are equation parameters, where a is the maximum loss and t_0 is the
464 time in which 50% of maximum loss occurs.

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

466 $$N_2O = a(1 - e^{-bt}) \quad (2)$$

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

470

471 *Statistical analysis – 16S rRNA amplicon sequence data.*

472 The 16S rRNA samples were analysed to compare bacterial community alpha diversity and
473 composition across treatments and time point (days 7, 16, 18, 27, 35, 42, 82 and 158). The
474 BIOM files were handled with the "phyloseq" package[49] in R[50]. Rarefaction curves were
475 generated to ensure adequate sequencing depth across samples. Discarding undersequenced
476 samples, the minimum sample size was 2000. For alpha diversity analyses, the 16S rRNA
477 samples were rarefied to 2000 sequences using the "vegan" package[51]. Renyi diversities at
478 alpha level 1, corresponding to the Shannon diversity index were kept ("BiodiversityR" R
479 package). The Shannon diversity data was furthermore subjected to Kruskal-Wallis tests
480 among treatments and the Kruskal-Wallis multiple comparison test between treatments using
481 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.

References

1 Erisman, J. W., Galloway, J., Seitzinger, S., Bleeker, A. & Butterbach-Bahl, K. Reactive nitrogen in the environment and its effect on climate change. *Cur. Opin. Environ. Sus.* **3**, 281-290 (2011).

2 Egeskog, A., Freitas, F., Berndes, G., Sparouek, G. & Wirsenius, S. Greenhouse gas balances and land use changes associated with the planned expansion (to 2020) of the sugarcane ethanol industry in Sao Paulo, Brazil. *Biomass Bioenerg.* **63**, 280-290, d (2014).

3 Crutzen, P. J., Mosier, A. R., Smith, K. A. & Winiwarter, W. N_2O release from agro-biofuel production negates global warming reduction by replacing fossil fuels. *Atmos. Chem. Phys.* **8**, 389-395 (2008).

4 Braker, G. & Conrad, R. Diversity, structure, and size of N_2O-producing microbial communities in soils – what matters for their functioning? in *Adv. App. Microbiol.*, Vol. 75 (eds Laskin, A. I., Sariaslani, S., Gadd, G. M.) 33–70 (Elsevier Academic Press Inc., 2011).

5 Hayatsu, M., Tago, K. & Saito, M. Various players in the nitrogen cycle: Diversity and functions of the microorganisms involved in nitrification and denitrification. *Soil Sci. Plant Nutr.* **54**, 33-45 (2008).

6 Muller, C., Laughlin, R. J., Spott, O. & Rutting, T. Quantification of N_2O emission pathways via a N-15 tracing model. *Soil Biol. Biochem.* **72**, 44-54, (2014).

7 Liu, X. J. J., Mosier, A. R., Halvorson, A. D., Reule, C. A. & Zhang, F. S. Dinitrogen and N_2O emissions in arable soils: Effect of tillage, N source and soil moisture. *Soil Biol. Biochem.* **39**, 2362-2370 (2007).

8 Di, H. J., Cameron, K. C., Podolyan, A. & Robinson, A. Effect of soil moisture status and a nitrification inhibitor, dicyandiamide, on ammonia oxidizer and denitrifier growth and nitrous oxide emissions in a grassland soil. *Soil Biol. Biochem.* **73**, 59-68, (2014).

9 CONAB - Companhia Nacional de Abastecimento 'National Supply Company'. *Quarto levantamento de safra de cana-de-açúca,* abril/2015 [In Portuguese]. *Technical Report* (2015). Avaliable at: http://www.conab.gov.br/OlalaCMS/uploads/arquivos/15_04_13_08_45_51_boletim_cana_portugues_-_4o_lev_-_14-15.pdf. Accessed: 8th October 2015.

10 EMBRAPA - Empresa Brasileira de Pesquisa Agropecuária 'National Agriculture Research Company'. in *Sistema brasileiro de classificação do solo 2nd edn* [In Portuguese] (eds Santos, H.G. *et al.*) Ch. 3, 66-92 (EMBRAPA-SPI, 2006).

11 Martins, M. R. *et al.* Nitrous oxide and ammonia emissions from N fertilization of maize crop under no-till in a Cerrado soil. *Soil Tillage Res.* **151**, 75-81, (2015).

12 IPCC - Intergovernmental Panel on Climate Change, in *IPCC Guidelines for National Greenhouse Gas Inventories: Prepared by the National Greenhouse Gas Inventories Programme* (eds Eggleston, H. S., Buendia, L., Miwa, K., Ngara, T. & Tanabe, K.) (Institute for Global Environmental Strategies, 2006).

13 Filoso, S. *et al.* Reassessing the environmental impacts of sugarcane ethanol production in Brazil to help meet sustainability goals. *Ren. Sus. Energ. Rev.* **52**, 1847-1856 (2015).

14 Snyder, C. S., Bruulsema, T. W., Jensen, T. L. & Fixen, P. E. Review of greenhouse gas emissions from crop production systems and fertilizer management effects. *Agric. Ecosyst. Environ.* **133**, 247-266 (2009).

15 Snyder, C. S., Davidson, E. A., Smith, P. & Venterea, R. T. Agriculture: sustainable crop and animal production to help mitigate nitrous oxide emissions. *Cur. Opin. Environ. Sus.* **9-10**, 46-54 (2014).

16 Soares, J. R. *et al.* Enhanced-efficiency fertilizers in nitrous oxide emissions from urea applied to sugarcane. *J. Environ. Qual.* **44**, 423-430 (2015).

17 Ruser, R. & Schulz, R. The effect of nitrification inhibitors on the nitrous oxide (N_2O) release from agricultural soils-a review. *J. Plant Nutr. Soil Sci.* **178**, 171-188, (2015).

18 O'Callaghan, M. *et al.* Effect of the nitrification inhibitor dicyandiamide (DCD) on microbial communities in a pasture soil amended with bovine urine. *Soil Biol. Biochem.* **42**, 1425-1436 (2010).

19 Yang, J. B. *et al.* Influence of the nitrification inhibitor DMPP on the community composition of ammonia-oxidizing bacteria at microsites with increasing distance from the fertilizer zone. *Biol. Fertil. Soils* **49**, 23-30 (2013).

20 Zhang, L. M., Hu, H. W., Shen, J. P. & He, J. Z. Ammonia-oxidizing archaea have more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly acidic soils. *ISME J.* **6**, 1032-1045 (2012).

21 Carmo J. B. *et al.* Infield greenhouse gas emissions from sugarcane soils in Brazil: effects from synthetic and organic fertilizer application and crop trash accumulation. *Glob. Change Biol. Bioenergy* **5**, 267-280 (2013).

22 Staff, S. S. in *Soil taxonomy: A basic system of soil classification for making and interpreting soil surveys. 2nd edn. Natural Resources Conservation Service. U.S. Department of Agriculture Handbook* 436 (1999).

23 Cantarella, H. Nitrogênio in: *Fertilidade do Solo* 'Nitrogen in Soil Fertility' [In Portuguese] (eds Novais, R. F. *et al*.) Ch. 7, 375-470 (Sociedade Brasileira de Ciência do Solo, 2007).

24 Ghiberto, P. J., Libardi, P. L., Brito, A. D. & Trivelin, P. C. O. Nitrogen fertilizer leaching in an oxisol cultivated with sugarcane. *Sci. Agr.* **68**, 86-93 (2011).
25 Vargas, V. P. *et al.* Sugarcane Crop Residue Increases N_2O and CO_2 emissions under high soil moisture conditions. *Sugar Tech* **16**, 174-179 (2014).
26 Pitombo, L. M. *et al.* Exploring soil microbial 16S rRNA sequence data to increase carbon yield and nitrogen efficiency of a bioenergy crop. *Glob. Change Bio. Bioenergy*, doi:10.1111/gcbb.12284 (2015).
27 Abalos, D., Sanchez-Martin, L., Garcia-Torres, L., van Groenigen, J. W. & Vallejo, A. Management of irrigation frequency and nitrogen fertilization to mitigate GHG and NO emissions from drip-fertigated crops. *Sci. Total Environ.* **490**, 880-888, (2014).
28 Tenuta, M. & Beauchamp, E. G. Nitrous oxide production from granular nitrogen fertilizers applied to a silt loam. *Can. J. Soil Sci.* **83**, 521-532 (2003).
29 Zanatta, J. A., Bayer, C., Vieira, F. C. B., Gomes, J. & Tomazi, M. Nitrous oxide and methane fluxes in south brazilian gleysol as affected by nitrogen fertilizers. *Rev. Bras. Cie. Solo* **34**, 1653-1665 (2010).
30 Nicol, G. W., Leininger, S., Schleper, C. & Prosser, J. I. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. *Environ. Microbiol.* **10**, 2966-2978 (2008).
31 Venterea, R. T., Clough, T. J., Coulter, J. A. & Breuillin-Sessoms, F. Ammonium sorption and ammonia inhibition of nitrite-oxidizing bacteria explain contrasting soil N_2O production. *Sci. Rep.* **5**, 12153; DOI:10.1038/srep12153 (2015).
32 Kozlowski, J. A., Price, J. & Stein, L. Y. Revision of N_2O-producing pathways in the ammonia-oxidizing bacterium Nitrosomonas europaea ATCC 19718. *Appl. Environ. Microbiol.* **80,** 4930–4935 (2014).
33 Frame, C. H. & Casciotti, K. L. Biogeochemical controls and isotopic signatures of nitrous oxide production by a marine ammonia-oxidizing bacterium. *Biogeosciences* **7**, 2695-2709 (2010).
34 Joo, H. S., Hirai, M. & Shoda, M. Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* no. 4. *J. Biosci. Bioeng.* **100**, 184-191 (2005).
35 Zhao, B., An, Q., He, Y. L. & Guo, J. S. N_2O and N_2 production during heterotrophic nitrification by Alcaligenes faecalis strain NR. *Bioresour. Technol.* **116**, 379-385 (2012).
36 Spott, O., Russow, R. & Stange, C. F. Formation of hybrid N_2O and hybrid N_2 due to codenitrification: First review of a barely considered process of microbially mediated N-nitrosation. *Soil Biol. Biochem.* **43**, 1995-2011 (2011).
37 Jones, L. C., Peters, B., Lezama Pacheco, J. S., Casciotti, K. L. & Fendorf, S. Stable isotopes and iron oxide mineral products as markers of chemodenitrification. *Environ. Sci. Technol.* **49,** 3444–3452 (2015).
38 Soares, J. R., Cantarella, H. & Menegale, M. C. L. Ammonia volatilization losses of surface-applied urea with urease and nitrification inhibitors. *Soil Biol. Biochem.* **52**, 82-89 (2012).
39 Cantarella, H. & Trivelin, P. C. O. Determinação de nitrogênio inorgânico em solo pelo método da destilação a vapor in *Análise química para avaliação da fertilidade de solos tropicais* 'Chemistry analysis for tropical soils fertility evaluation' [In Portuguese] (eds Raij, B. van, Andrade, J. C., Cantarella, H., Quaggio, J. A.) Ch. 19, 271–276 (Instituto Agronômico, 2001).
40 Koster, J. & Rahmann, S. Snakemake-a scalable bioinformatics workflow engine. *Bioinformatics* **28**, 2520-2522 (2012).

41 Dodt, M., Roehr, J., Ahmed, R. & Dieterich, C. FLEXBAR—Flexible barcode and adapter processing for next-generationsequencing platforms. *Biology* **1**, 895 (2012).
42 Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460-2461 (2010).
43 Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194-2200, (2011).
44 McDonald, D. *et al.* The biological observation matrix (BIOM) format or: how I learned to stop worrying and love the ome-ome. *GigaScience* **1**, 7 (2012).
45 Cole, J. R. *et al.* Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* **42**, D633-D642 (2014).
46 Rawlings, J. O., Pantula, S. G. & Dickey, D. A. Transformation of variables. in *Applied Regression Analysis: A Research Tool 2nd edn*, (eds Rawlings, J. O., Pantula, S. G., Dickey, D. A.) Ch. 12, 397-432 (Springer-Verlag New York, Inc. 1998).
47 Ferreira, D. F. Sisvar: a computer statistical analysis system. *Ciência e agrotecnologia* **35**, 1039-1042 (2011).
48 Systat Software. 2006. SSI. Sigmaplot for Windows, version 12.5. Systat Software, San Jose, CA.
49 McMurdie, P. J. & Holmes, S. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *Plos One* **8**, doi:10.1371/journal.pone.0061217 (2013).
50 R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.
51 Oksanen, J. *et al.* Package 'vegan'. Community ecology package, version, 2.2-1. (2015). URL http://www.R-project.org/".
52 Parks, D. H. & Beiko, R. G. Identifying biologically relevant differences between metagenomic communities. *Bioinformatics* **26**, 715-721, (2010).
53 Dray, S. & Dufour, A.-B. The ade4 package: implementing the duality diagram for ecologists. *J. Stat. Softw.* **22**, 1-20 (2007).

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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.

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

675

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	

676 * Tukey test, $p \leq 0.05$; ns: no significant; N_2O-N: g ha^{-1} transformed in log(X) † Results from treatment without
677 N were subtracted for this calculation. - R means reapplication of inhibitors in the same plot in the two preceding
678 years. Different characteristics in the column of N_2O mean significant differences ($p<0.05$) between the values.
679

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

684

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	

685 *DAF: Days after fertilizer application. Means in column followed by same letter did not differ; ns: no
686 significant. † R means reapplication of inhibitors in same plots.
687
688
689
690
691
692

693 **Figure legends**

694 **Figure 1.** Rainfall, air temperature, water-filled pore space (WFPS) and nitrous oxide fluxes
695 from Control (No N), urea (UR) with or without nitrification inhibitors (DCD and DMPP),
696 polymer sulphur coated urea (PSCU) and calcium nitrate applied to sugarcane. R:
697 reapplication of inhibitors in the same plots during the previous two cycles of the experiment.
698 N fertilizers were applied on 13 December 2013.

699

700 **Figure 2.** Soil concentration of NH_4^+-N + NO_3^--N and pH from Control (No N), urea (UR)
701 with or without nitrification inhibitors (DCD and DMPP), polymer sulphur coated urea
702 (PSCU) and calcium nitrate applied to sugarcane. R: reapplication of inhibitors in the same
703 plots during the previous two cycles of the experiment.

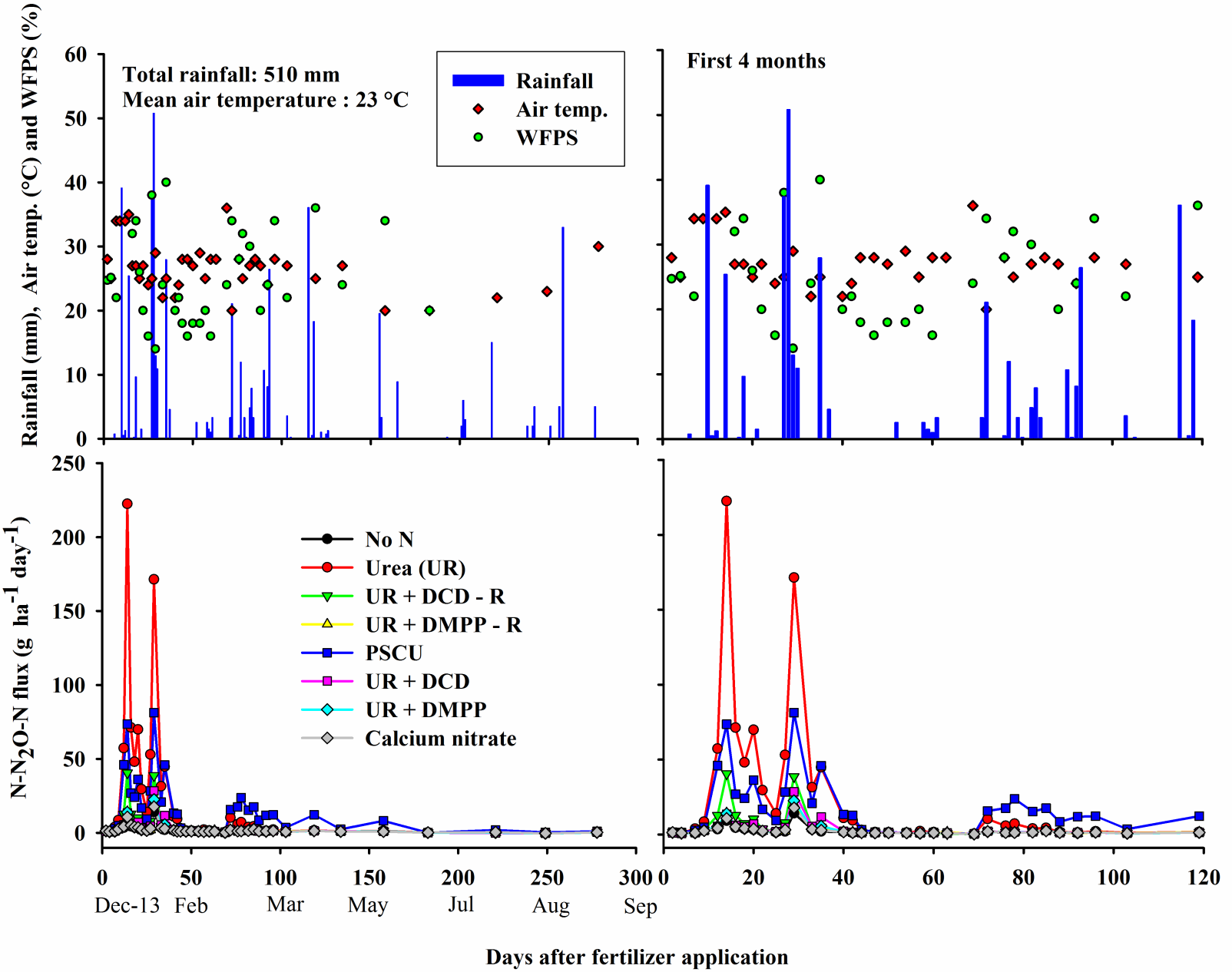
704

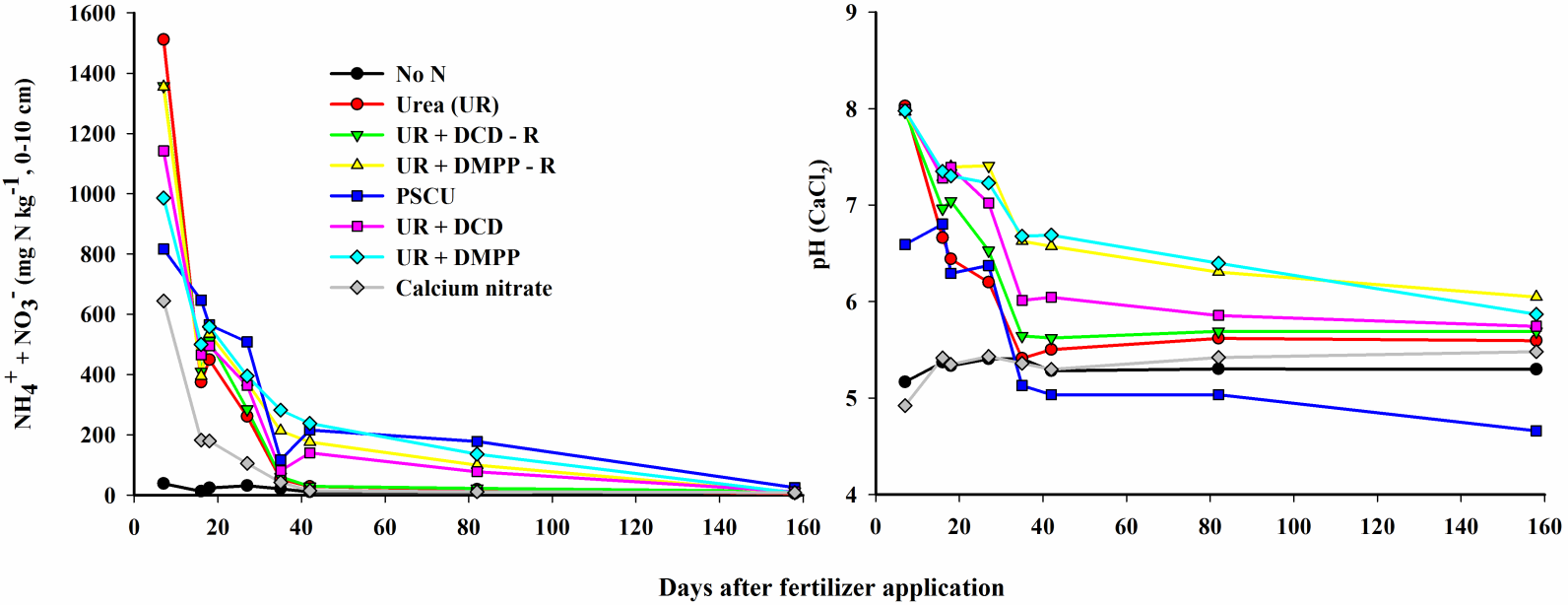
705 **Figure 3.** Cumulative N_2O emission (red dots) and sigmoidal or exponential equations fitted
706 (black lines) to data of urea (UR) and polymer sulphur coated urea (PSCU) applied to
707 sugarcane. (b) net UR and PSCU N_2O emissions calculated by subtracting data of the control
708 treatment.

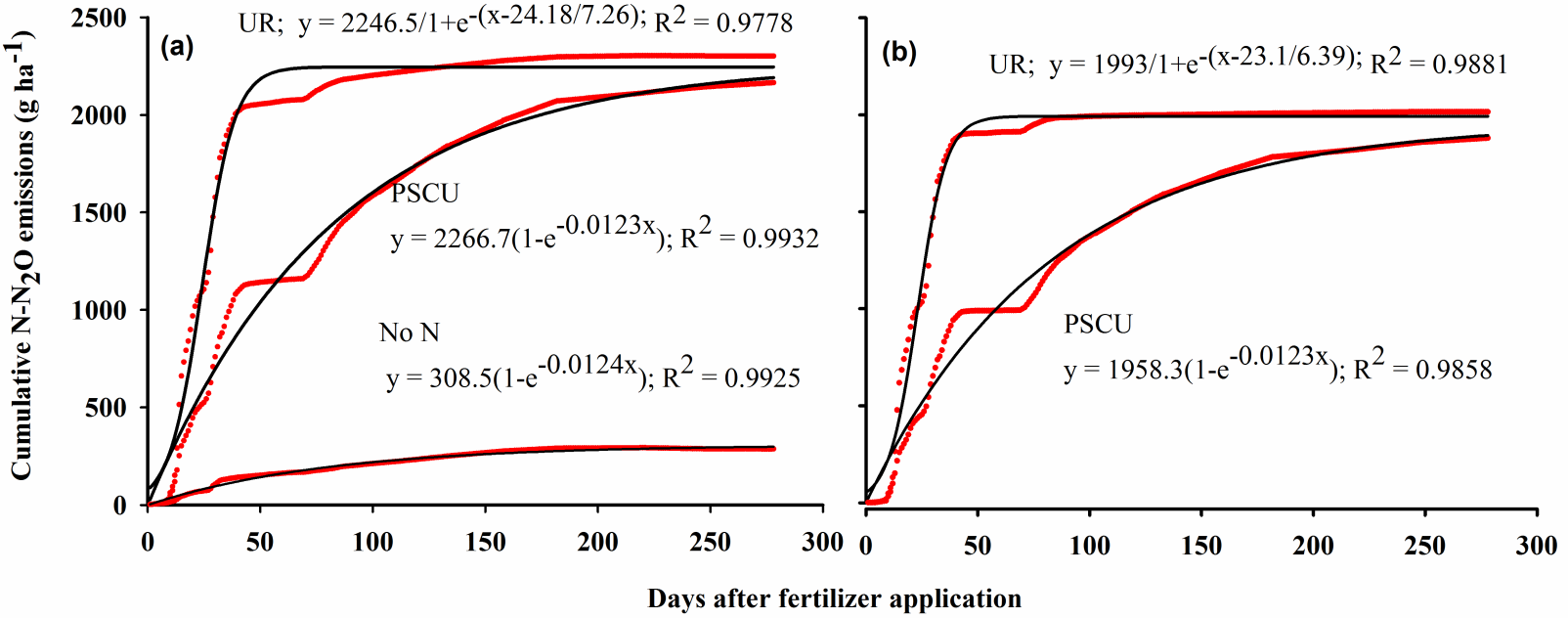
709

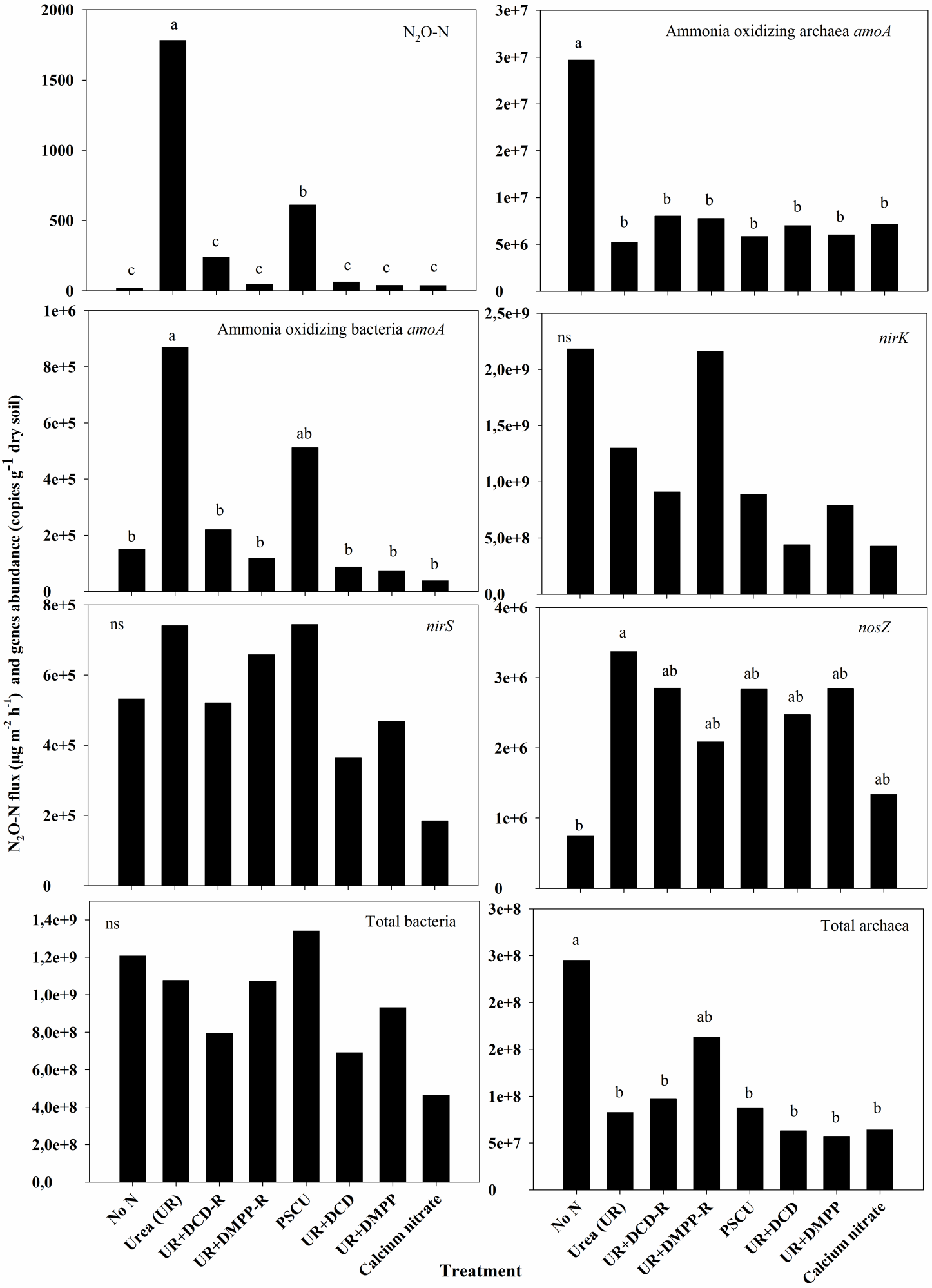
710 **Figure 4.** Nitrous oxide fluxes, nitrogen cycle genes (*amoA* bacteria, *amoA* archaea, *nirK*,
711 *nirS*, *nosZ*) abundances and total bacteria and total archaea abundances in the Red Latosol soil
712 16 days after fertilizer application of urea with or without nitrification inhibitors (DCD and
713 DMPP); polymer sulphur coated urea (PSCU) or calcium nitrate applied to sugarcane. R:
714 reapplication of inhibitors in the same plots during the previous two cycles of the experiment.

715


Total rainfall: 510 mm
Mean air temperature : 23 °C
Rainfall
Air temp.
WFPS
First 4 months
Rainfall (mm), Air temp. (°C) and WFPS (%)
N-N2O-N flux (g ha-1 day-1)
No N
Urea (UR)
UR + DCD - R
UR + DMPP - R
PSCU
UR + DCD
UR + DMPP
Calcium nitrate
Dec-13
Feb
Mar
May
Jul
Aug
Sep
Days after fertilizer application


No N
Urea (UR)
UR + DCD - R
UR + DMPP - R
PSCU
UR + DCD
UR + DMPP
Calcium nitrate
$NH_4^+ + NO_3^-$ (mg N kg^{-1}, 0-10 cm)
pH ($CaCl_2$)
Days after fertilizer application


(a)
UR; y = 2246.5/1+e-(x-24.18/7.26); R2 = 0.9778
PSCU
y = 2266.7(1-e-0.0123x); R2 = 0.9932
No N
y = 308.5(1-e-0.0124x); R2 = 0.9925
(b)
UR; y = 1993/1+e-(x-23.1/6.39); R2 = 0.9881
PSCU
y = 1958.3(1-e-0.0123x); R2 = 0.9858
Cumulative N-N2O emissions (g ha-1)
Days after fertilizer application
0
500
1000
1500
2000
2500
0
50
100
150
200
250
300


N2O-N
Ammonia oxidizing archaea amoA
Ammonia oxidizing bacteria amoA
nirK
nirS
nosZ
Total bacteria
Total archaea
ns
N2O-N flux (μg m-2 h-1) and genes abundance (copies g-1 dry soil)
Treatment
No N
Urea (UR)
UR+DCD-R
UR+DMPP-R
PSCU
UR+DCD
UR+DMPP
Calcium nitrate