Nitrous oxide emissions and microbial communities during the transition to conservation agriculture using N-enhanced efficiency fertilisers in a semiarid climate

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Abstract

The transition year from tillage to no tillage in semiarid areas and its effects on nitrous oxide (N_2O) emissions and related microbial communities, as well as the potential interaction with N management, including enhanced-efficiency fertilisers, are not well studied despite their economic and environmental implications. In tilled and nontilled plots, the effectiveness of the double DMPSA+NBPT inhibitor (applied with urea at basal fertilisation) and that of DMPSA (applied with calcium ammonium nitrate at top-dressing) in the mitigation of N_2O emissions were evaluated in a rainfed barley (*Hordeum vulgare* L.) crop in central Spain. Crop yield, nitrogen (N) uptake, the abundances of key genes involved in nitrification and denitrification processes and meteorological conditions and soil ancillary properties were monitored. In addition, the composition of bacterial communities was determined by sequencing the 16S rRNA gene. Fertilisers with inhibitors decreased cumulative N_2O emissions and yield-scaled N_2O emissions by 53% and 56%, respectively, with respect to those without inhibitors, which coincided with a trend of increasing grain and biomass yield and aboveground N uptake (by 11.3%, 9.2% and 7.2%, respectively). The highest N_2O emissions were measured 49 days after harvest (immediately after a rainfall event that like reactivated soil microorganisms), reaching 15 mg N m^{-2} d^{-1} for the treatment with fertiliser without inhibitor combined with tillage. This peak was linked to a remarkable increase in the abundance of denitrifiers. The abundance of nitrifiers and denitrifiers successfully explained the N_2O dynamics observed after basal fertilisation (i.e. an increase in the *amoA*/*nosZ* ratio in fertilised plots with inhibitors, where the highest emissions were observed). Our results also showed a reduction in the abundance of the phylum *Nitrospirae* throughout the cropping period in the plots that received inhibitors. No tillage led to a higher abundance of *Cyanobacteria*, *Verrucomicrobia* and *Bacteroidetes* and resulted in better implantation of the crop and

higher plant density compared with tillage, thus increasing yields and N use efficiency and decreasing N_2O emissions. Under the conditions of our study, shifting from conventional tillage to no tillage enhanced the balance between N use efficiency and yield-scaled N_2O emissions in the first year of conversion, particularity with the use of the double inhibitor with urea at basal fertilisation and DMPSA with CAN at dressing.

Keywords: Greenhouse gas emissions, Global warming potential, Drying-rewetting pulses, Urease inhibitor, Nitrogen use efficiency, Microbial diversity

1. Introduction

Crop residue can improve soil quality and enhance soil organic carbon (SOC) stocks (García-Ruiz et al., 2019; Soussana et al., 2019), particularly in the context of sustainable intensification (Bais-Moleman et al., 2019). The retention of crop residues requires optimum nitrogen (N) fertilisation management to prevent N immobilisation (Yansheng et al., 2020), particularly during early crop stages. However, the addition of a mineral N source to prevent immobilisation combined with high C:N residues can stimulate denitrification (Li et al., 2016) and increase the emissions of the potent greenhouse gas nitrous oxide (N_2O) (Duan et al., 2018), thus raising the global warming potential of agro-ecosystems (IPCC, 2019; Li et al., 2021).

Nitrogen losses from crop residues and fertilisation practices are closely linked to tillage management, i.e. tillage (T) or no tillage (NT) (van Kessel et al., 2013). In nontilled soils, soil organic C (SOC) accumulates in the topsoil, increasing the availability of labile organic C for denitrifiers and mineral N for both nitrifiers and denitrifiers (Shakoor et al., 2021). In addition, the reduction in porosity due to the higher soil compaction with NT may favour anoxic conditions and the consumption of nitrous oxide (N_2O) (Huang et al., 2018). Tillage management has also been shown to change the microbial functional

groups involved in N cycling. For instance, Wang et al. (2019) observed that in comparison with conventional tillage (i.e. chisel plough), long-term conservation tillage (i.e. NT) decreased the abundances of archaeal ammonia oxidisers and denitrifiers but increased the abundance of the *nosZ* gene, which is involved in the reduction of N_2O to dinitrogen (N_2), thus resulting in overall improved mitigation of N_2O emissions. It has also been demonstrated that crop residues, long-term NT and fertilisation management modify the microbial community composition (Suleiman et al., 2018; Li et al., 2020; Wang et al. 2021) due to the increase in soil C and N availability. However, little is known regarding microbial diversity and abundance changes during the transition year from T to NT particularly in semiarid cropping systems. Under these conditions, N_2O emissions are highly dependent on N addition and soil moisture and major N_2O peaks typically occur after several weeks of dry conditions, usually after harvest in summer, and subsequent soil rewetting (Guardia et al., 2018a; Montoya et al., 2021b). A robust temporal resolution of N_2O emissions and microbial community dynamics (including basal and dressing fertilisation and postharvest period) is lacking under semiarid conditions. In addition, in order to develop cost-effective mitigation strategies, it is necessary to determine the relative contribution of nitrification and denitrification to critical N_2O pulses (including those after rewetting) and whether tillage management alleviates these N_2O peaks.

Tillage management also influences the emission of ammonia (NH_3). The "mulch effect" occurring when residues are not being incorporated in nontilled soils (Pinheiro et al., 2018) causes higher volatilisation rates, as recently reported by the global meta-analysis of Ma et al. (2021). Volatilisation losses when urea is applied can be significantly reduced by the use of urease inhibitors (Ti et al., 2019) such as N-butyl thiophosphorictriamide (NBPT), while nitrification inhibitors such as 2-(3,4-dimethyl-1H-pyrazol-1-yl) succinic acid isomeric mixture (DMPSA) are the most effective

97 enhanced-efficiency N form to decrease N_2O (Thapa et al., 2016). As a result, the
98 combined use of nitrification and a urease inhibitor (named a "double inhibitor") can be
99 considered a promising strategy to mitigate gaseous N losses (Corrochano-Monsalve et
100 al., 2021a; Guardia et al., 2021; Souza et al., 2021). Corrochano-Monsalve et al. (2020a,
101 2020b) showed that DMPSA minimised N_2O losses by decreasing the abundance of
102 ammonia oxidisers while increasing that of N_2O reducers in a nontilled wheat crop under
103 humid Mediterranean conditions. However, this "tillage x inhibitors" interaction effect
104 on microbial communities and gaseous N losses is still poorly understood in arid or
105 semiarid climates, particularly regarding the combination of DMPSA with the urease
106 inhibitor NBPT and during the transition from T to NT.

107 This study aimed to determine potential N_2O mitigation strategies in a rainfed
108 semiarid agroecosystem based on a combination of two contrasting tillage intensities (T
109 and NT) and the use of nitrification and/or urease inhibitors. Another objective was to
110 assess whether nitrifying and denitrifying communities (represented by the abundances
111 of the functional genes from these pathways) and microbial diversity are useful indicators
112 of N_2O emissions during the first year of NT adoption. Soil mineral N, crop biomass and
113 N uptake were also included as ancillary measurements to explain N_2O fluxes and
114 differences in microbial communities. It was hypothesised that i) short-term NT would
115 result in changes in the abundance of genes involved in N cycling and the diversity of the
116 bacterial community, thus affecting N_2O fluxes; and ii) the use of inhibitors would
117 decrease N_2O losses in both T and particularly NT systems, in line with the findings of
118 Corrochano-Monsalve et al. (2020a, 2020b).

119 **2. Materials and Methods**

120 *2.1 Experimental site*

121 The experiment was conducted in the Madrid region in Spain from October 2018 to
122 November at the *Centro Nacional de Tecnología de Regadíos* "CENTER". The soil was
123 a silty loam (9% clay, 60% silt, 31% sand) *Typic xerofluvent* (Soil Survey Staff, 2017).
124 The main soil (0-20 cm) properties measured at the beginning of the experiment by
125 conventional methods were as follows: bulk density, 1.23 ± 0.08 g cm^{-3}; electric
126 conductivity, 0.49 ± 0.02 mS cm^{-1}; pH (water), 8.16 ± 0.02; total organic matter, 14.43 ±
127 0.17 g kg^{-1}; and $CaCO_3$, 9.87 ± 0.92 %. The last 10-year average mean temperatures and
128 yearly rainfall were 14.2 °C and 384.4 mm, respectively. Considering the wheat cropping
129 cycle, the average air temperatures were 6.9 °C and 19.4 °C in the November-March and
130 March-November periods, respectively. The accumulated rainfall in the same periods was
131 204.7 mm and 180.7 mm, respectively. The meteorological data (from the last 10 years
132 and during the experiment) were obtained from the Spanish "Sistema de información
133 agroclimática para el regadío, SIAR", http://eportal.mapama.gob.es/websiar/Inicio.aspx),
134 which collects data from a meteorological station that was set up at CENTER station.

135 *2.2 Experimental design and management*

136 The experimental design consisted of a split plot, with tillage as the main factor
137 (arranged in a randomised three-replicated block design) and fertiliser as the second factor
138 (arranged in a completely randomised design within each tillage plot). All tillage-fertilizer
139 combinations were established in 8 m x 8 m subplots (n = 3). The experiment was split
140 into two periods due to the differences in the meteorological conditions and crop
141 development, the latter as a result of the contrasting N management (N rate and N source,
142 Table 1) at each fertilisation event. The first period was from October 2018 until March
143 2019 (including basal fertilisation on 27 November), and the second period was from top-
144 dressing fertilisation on 14 March (beginning of stem elongation stage) until the end of
145 the experiment (including two irrigation events on 26 March and 13 May, due to severe

drought conditions, and the postharvest period). All fertilisers were broadcasted over the soil surface in granular form by hand. More information about the treatments and management in each period can be found in Table 1.

Table 1 Description of tillage and fertilisation factors

Treatments		
Tillage factor	**Description**	
NT	No tillage and herbicide (glyphosate 36% p/v at) spraying	
T	Tillage (disc harrow and cultivator)	
Fertilisation factor	**Description**	
	First period	**Second Period**
N0	Unfertilised control	
FER	urea 40 kg N ha^{-1}	calcium ammonium nitrate 80 kg N ha^{-1}
INH	urea + DMPSA[1]+ NBPT[2]40 kg N ha^{-1}	calcium ammonium nitrate + DMPSA[1]80 kg N ha^{-1}

[1]DMPSA was applied at 0.8% of the NH_4^+-N

[2]NBPT was applied at 0.13% of ureic N

Before the beginning of the experiment, an oilseed rape (*Brassica napus* L.) crop was planted in the same experimental area. After rape harvesting, the residues were left on the field and then managed for the different tillage systems, as explained in Table 1. Excluding grain, the average aboveground biomass of the rape crop was 4492 kg ha^{-1}, with an average C:N ratio of 22.7. The field was seeded with barley (*Hordeum vulgare* L. var. 'Esterel R1') on 17 December 2018 at 200 kg seed ha^{-1}. During the last decade, wheat (*Triticum aestivum* L., 2015-2017), maize (*Zea mays* L., 2012-2015), barley (*Hordeum vulgare* L., 2010-2012) and maize (2009-2010) were grown in the plots and the soil was managed through conventional tillage.

2.3 Sampling and measurement of N_2O

Gas samples were taken 2-3 times per week during the first month after both N applications. The sampling frequency decreased (i.e. weekly or fortnightly and even monthly during summer due to the lack of rainfall events), particularly during dry periods with low (winter) and high (summer) soil temperatures, but ensuring that all soil rewetting

events were covered. Nitrous oxide fluxes were measured using closed opaque chambers (one chamber per plot) with a volume of 19.3 L (diameter 35 cm, height 20 cm) placed over stainless rings inserted 5-10 cm into the soil. Chambers were closed during one hour, during which three gas samples were taken with 30-minute intervals at times t_0, t_{30} and t_{60} after closure from the headspaces of each chamber with 20 mL syringes fitted with 3-way stopcocks. The gas samples were then, transferred to vials sealed with a gas-tight neoprene septum in preparation for posterior analysis by gas chromatography. The concentration of N_2O was determined with a gas chromatograph equipped with an electron-capture detector (HP-6890, Agilent Technologies).

Cumulative gas emissions during the experimental period were calculated by trapezoidal integration by linear interpolation of daily fluxes (Cowan et al., 2019), i.e. by multiplying the average flux of two successive determinations by the length of the period between sampling and adding that amount to the previous cumulative total. Yield-scaled N_2O emissions were calculated as follows:

$$Yield-scaled\ N_2O\ emissions\ (g\ N\cdot\ kg\ grain^{-1}) = \frac{Cumulative\ N_2O\ emissions}{Grain\ yield} \quad \text{(Eq. 1)}$$

For which cumulative emissions were expressed in g N ha^{-1}.

2.4 Soil and plant analyses

Three 0.25 m x 0.25 m squares per subplot were harvested on 9 July to determine grain and biomass (i.e., aboveground biomass except for grain) yields and total N concentrations in grain and straw, analysed by Dumas's method with an elemental analyser (Association of Official Agricultural Chemists, 1975). Three soil cores (0-10 cm) per plot were taken at the same dates of gas sampling to determine the moisture content (expressed as water-filled pore space, WFPS), while NH_4^+-N and NO_3^--N

concentrations were generally determined in half of the gas sampling events using a similar frequency to that of Guardia et al. (2021; 2018b). After dressing fertilisation, the soil sampling frequency was initially decreased because of dry conditions that maintained the fertiliser granules over the soil surface, but was increased after both irrigation events. The soil NH_4^+-N and NO_3^--N contents were extracted using KCl 1 M (1:6.25 soil:extractant solution) and analysed by a colourimetric method (UV-V spectrophotometry) using a flow injection analyser (FIAS 400 Perkin Elmer), as explained in Montoya et al. (2018). The soil WFPS was estimated using the bulk density data of the T and NT plots as explained in Abalos et al. (2012), previously measuring the gravimetric water content by oven-drying the soil samples at 105 °C.

2.5 Extraction of DNA and quantification of 16S rRNA and N-cycling genes

Three soil samples per plot for DNA extraction and subsequent microbial analyses were taken, pooled and mixed after basal fertilisation but before the sowing of barley (7 December), during the period between fertilisations (21 February), after top-dressing fertilisation (22 April), before harvest (20 May) and after the rewetting event in summer (27 August). The DNA was extracted from 0.5 g of soil using the commercial PowerSoil® DNA isolation kit (Qiagen). The DNA quality was determined on an agarose gel and the quantity was measured using a Qubit fluorimeter (Invitrogen, Carlsbad, CA, USA). To estimate the abundances of the microbial communities involved in specific processes in the N cycle, quantitative PCR (qPCR) was conducted using primers (Table S1) for the *amoA* gene from ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Rotthauwe et al., 1997; Tourna et al., 2008, respectively) in nitrification, the nitrite reductase genes *nirK* (Hallin and Lindgren, 1999) and *nirS* (Throbäck et al., 2004) in denitrification, and the N_2O reductase genes *nosZI* (Henry et al., 2006) and *nosZII* (Jones et al., 2013). In addition, the 16S rRNA gene was quantified as a proxy for the total microbial

214 communities using taxa-specific primers (Quince et al., 2011; Parada et al., 2016). All
215 quantifications were performed in duplicate on two independent runs in a total reaction
216 volume of 15 μL using iQ™ SYBR Green Supermix (Bio–Rad, Hercules, CA, USA),
217 0.05% BSA, primer concentrations of 0.5-2 μM and 5 ng DNA on a CFX Connect Real-
218 Time System (Bio–Rad, Hercules, CA, USA). Thermal cycling conditions, primer
219 sequences and concentrations are shown in Table S1. Standard curves were obtained by
220 serial dilutions of linearised plasmids with cloned fragments of the specific genes and the
221 curves were linear in the range used (R^2=0.997–0.999). Electrophoresis in agarose and a
222 melting curve analysis verified the quality of qPCR amplification. To detect potential
223 inhibition of the PCR, a known amount of pGEMt plasmid was amplified using M13
224 primers where reactions with plasmid only and reactions containing the plasmid and 5 ng
225 of the DNA were compared. We could not detect inhibition of the PCRs with the DNA
226 concentrations used for any of the samples. The copy numbers of the target genes per
227 gram of dry soil were calculated according to Montoya et al. (2021b).

228 *2.6 Amplicon sequencing of 16S rRNA genes*

229 The composition of the bacterial and archaeal communities was determined by
230 amplicon sequencing of the V4-V5 region within the 16S rRNA gene using a two-step
231 PCR protocol (Berry et al., 2011), and the primers 515F (Parada et al., 2016) and 926R
232 (Quince et al., 2011) with Nextera adaptor sequences (Illumina, Inc.). The preparation of
233 amplicons is described in Hellman et al. (2019). Sequencing was performed by the
234 SNP&SEQ Technology Platform in Uppsala, Sweden on the MiSeq platform (Illumina)
235 using 2 × 250 bp paired-end chemistry. The sequences obtained in this study were
236 submitted to the NCBI short read archive and are available under Bioproject accession
237 number PRJNA761562.

The 16S rRNA gene sequences were trimmed with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) and paired-end reads were merged with PEAR (Zhang et al., 2014) using a minimum overlap of 30 bp. Further quality filtering was performed using VSEARCH (Rognes et al., 2016). The sequences were clustered at 97% nucleotide similarity into operational taxonomic units (OTUs), followed by de novo and reference-based (SILVA nonredundant reference database release 132 from 2017) chimaera checking using the UCHIME algorithm (Edgar et al., 2011). The resulting OTUs were assigned to taxonomic ranks using SINA (Pruesse et al., 2012) with the SILVA 132 database as a reference. OTUs classified as "Chloroplast" or "Mitochondria", and OTUs that composed less than 1% of all sequences within each sample were removed from the dataset. This resulted in a total of 4,186,126 sequences in 9,670 OTUs.

2.7 Calculations and statistical analyses

Analyses of variance were performed by using the *Statgraphics 18-X64* program individually for periods I and II (see section 2.2) and the entire experimental period for average mineral N and DOC concentrations and cumulative N_2O emissions. Data distribution normality and variance uniformity were previously assessed by the Shapiro–Wilk test and Levene´s statistic, respectively, and they were log-transformed before analysis when necessary. Means were separated by the LSD test at $P < 0.05$. For nonnormally distributed data, the Kruskal–Wallis test was used on nontransformed data to evaluate differences at $P < 0.05$. Simple linear regression analyses were performed to determine the relationships between N_2O-N, WFPS, mineral N (NH_4^+-N and NO_3^--N) and soil temperature, as well as between yield variables. Two types of correlation analyses were carried out: from the data of average contents and cumulative fluxes (correlations per subplot) and considering the average daily values (correlation per sampling date). Additionally, principal component analyses (PCAs) were performed to explore the

263 relationships between gene abundances, soil variables and N_2O fluxes based on Euclidian
264 distances using CANOCO for Windows version 5 (Wageningen, Netherlands) for the
265 different sampling dates used in the DNA extractions (see Section 2.5).

266 Statistical evaluation of the sequencing data was carried out with R version 4.0.5 (R
267 Core Team, 2013) using Rstudio version 1.1.456 (RStudio Team, 2018). The data were
268 processed using the 'phyloseq' package (McMurdie and Holmes, 2013), and the 'vegan'
269 package (Oksanen et al., 2015) was applied for the Shannon alpha diversity indices. In
270 addition, nonmetric multidimensional scaling (NMDS) using weighted phylogenetic
271 distances (weighted UniFrac) to visualise community patterns was generated with the
272 packages 'ape' (Paradis et al., 2004) and 'GUniFrac' (Chen and Chen, 2018), and plotted
273 using 'ggplot2' (Wickham, 2011). The effect of 'treatment' and 'time', and 'fertilisation'
274 and 'tillage' was tested by permutational multivariate analysis of variance
275 (PERMANOVA) using the function 'adonis'. The characteristic taxa for each specific
276 treatment throughout all sampling dates were tested using an indicator species analysis
277 for the core microbial OTUs (Jeanbille et al., 2016) with the packages 'indicspecies'
278 (Cáceres et al., 2010) and 'labdsv' (Roberts and Roberts, 2016).

279 **3. Results**

280 *3.1 Environmental conditions, mineral N and DOC*

281 During the first period (November-March), the mean air temperature, soil
282 temperature (10 cm) and cumulative rainfall were 6.1 °C, 8.1 °C and 91 mm, respectively
283 (Fig. 1a). In the March-July period (after dressing fertilisation), the mean air temperature,
284 soil temperature (10 cm) and cumulative rainfall were 15.7 °C, 15.8 °C and 96 mm,
285 respectively (136 mm considering the two irrigation events, see Section 2.2). Compared
286 with the 10-year average values, the total rainfall in the November-March and March-
287 July periods was 38 % and 39% lower, respectively. After harvest, the climatic conditions

were hot and dry, and the first precipitation event above 2 mm of effective rainfall occurred on 27 August (Fig. 1a). The soil WFPS ranged from 10% to 69% (Fig. 1b). Some values within the 50%-70% range were reached after both fertilisation events (particularly after basal fertilisation). After harvest, the soil WFPS values were low (< 15%) until the late-August rainfall event.

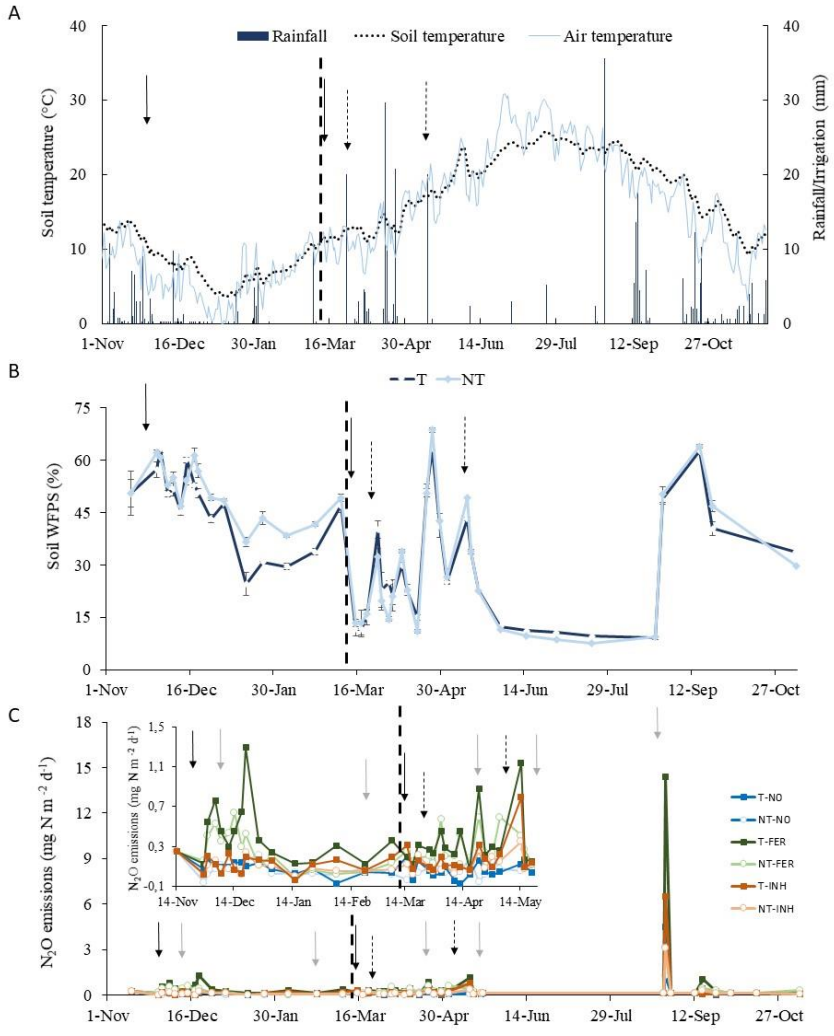
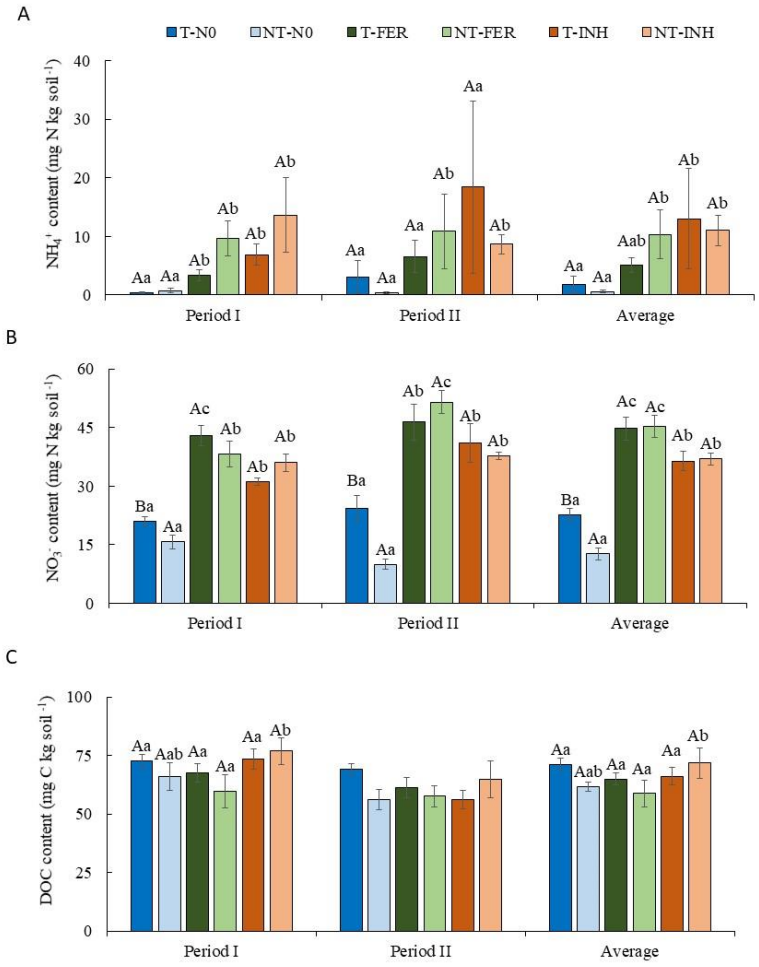


Figure. 1. (A) Daily mean temperatures in air and soil (10 cm depth) and daily rainfall/irrigation; (B) evolution of soil WFPS; and (C) daily N_2O emissions during the whole experimental period for the different treatments (see Table 1). The black and dotted arrows denote fertilisation and irrigation events, respectively. The grey arrows denote soil samplings for microbial community analyses. Dotted vertical line indicate the division into the two time periods. Error bars show standard error.

300 The application of DMPSA tended to increase the soil NH_4^+content during Period I
301 (Fig. S1a). After dressing fertilisation (Period II), the increase in average NH_4^+
302 concentration was observed in the T plots. Regarding NO_3^- contents (Fig. S1b), the use
303 of inhibitors decreased these concentrations compared to FER subplots in both periods.
304 No major differences between tillage treatments were observed for DOC content, apart
305 from the significantly higher levels of INH concerning FER in Period I (Fig. S1c).

306



307

308 **Fig. S1**. Average ammonium (N-NH_4^+) (A), nitrate (N-NO_3^-) (B) and DOC (C) contents in topsoil (0-10
309 cm) during Periods I and II and throughout the experimental period for the different treatments (see Table
310 1). Error bars indicate standard error. Different capital letters indicate differences ($P < 0.05$) between tillage
311 treatments within each fertilizer, while different lowercase letters indicate significant differences ($P < 0.05$)
312 between fertilizers within each tillage treatment.

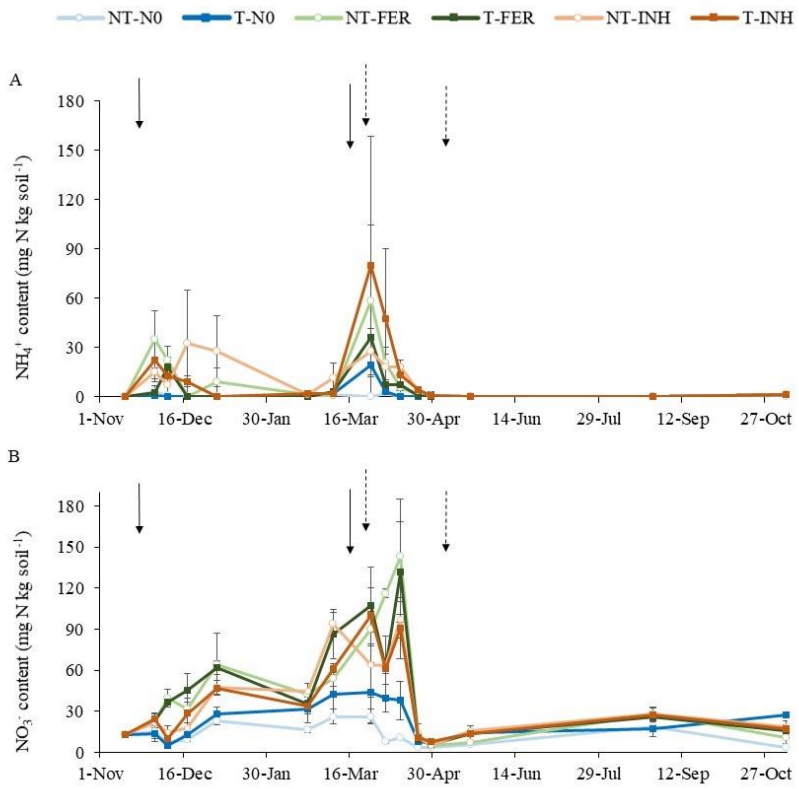


Fig. S2. Ammonium (N-NH_4^+) (A) and nitrate (N-NO_3^-) (B) contents topsoil (0-10 cm) during the entire experimental period for the different treatments (see Table 1). The black and dotted arrows denote fertilisation and irrigation events, respectively.

3.2 Agronomic parameters

The NT plots showed trends of higher grain ($0.05 < P < 0.10$) and biomass yields ($0.05 < P < 0.10$) and aboveground N uptake, but lower grain and biomass N contents ($0.05 < P < 0.10$) than T (Table S2). Fertilising with N increased biomass yield and aboveground N uptake compared to the N0 treatment ($P < 0.05$). Grain yields increased in the order N0 < FER < INH (Table S2). Grain N content was positively correlated with

biomass N content ($P < 0.001$, n = 18, r = 0.73), but negatively correlated with grain yield ($P < 0.05$, n = 18, r = -0.56).

3.3 Nitrous oxide and yield-scaled emissions

Nitrous oxide peaked during the first month after basal fertilisation, reaching 1.3 mg N m^{-2} d^{-1} in T-FER (Fig. 1c). The cumulative fluxes during this first fertilisation decreased in the order FER > INH > N0 (Table 2), while T increased N_2O emissions by 55% compared to NT. After dressing fertilisation, the main N_2O emissions occurred 39 and 61 days after N application (Fig. 1c). As in the previous period, the maximum values (0.9 and 1.1 mg N m^{-2} d^{-1}) were observed for T-FER. After harvest, emissions were generally low when effective rainfall was below 5 mm. However, after a rainfall event of 35 mm N_2O fluxes peaked (27 August,) ranging from 1.3 (in NT-N0) to 15.0 mg N m^{-2} d^{-1} (in T-FER) (Fig. 1c). At the end of Period II, NT and INH mitigated N_2O emissions by 32% and 45%, respectively, compared with T and FER (Table 2). Cumulative N_2O emissions and average soil NO_3^- content were positively correlated ($P < 0.001$, n = 18, r = 0.74). The yield-scaled emissions, expressed as emissions of N-N_2O per kilogram of grain yield, were similar to those of area-scaled N_2O emissions for both tillage (NT mitigated yield-scaled emissions by 57%, $P < 0.05$) and the fertiliser effect (in this case, the unfertilised control was not significantly different from INH, Table 2).

Table 2 Cumulative area-scaled and yield-scaled N_2O emissions at the end of each period (see Section 2.2) in the different treatments (see Table 1). Cumulative emissions at the end of period II are equivalent to the cumulative emissions at the end of the experimental period and are the values used to obtain the yield-scaled N_2O emissions.

Effect	N-N_2O (g ha^{-1})		Yield-scaled N_2O emissions (g N kg $grain^{-1}$)
	End of Period I	End of Period II	
Tillage			
NT	122.9 a	491.5 a	0.20 a

T	189.9 b	719.8 b	0.46 b
S.E.	12.4	53.2	0.07
***P* value**	**0.042**	**0.045**	**0.047**
Fertilizer			
N0	80.2 a	362.2 a	0.24 a
INH	115.0 b	515.8 b	0.25 a
FER	273.9 c	939.1 c	0.50 b
S.E.	15.1	40.7	0.05
***P* value**	**0.000**	**0.000**	**0.000**
Tillage x Fertilizer			
NT-N0	80.1	317.0	0.13
NT-INH	96.7	444.4	0.15
NT-FER	191.7	713.2	0.31
T-N0	80.3	407.4	0.34
T-INH	133.3	587.1	0.34
T-FER	356.1	1165.0	0.69
S.E.	21.4	66.0	0.08
***P* value**	**0.308**	**0.289**	**0.717**

Different letters within columns indicate significant differences by applying the LSD test at $P < 0.05$. The standard error (S.E., n=3) is given for each effect.

3.4 The dynamics of 16S rRNA genes, and functional genes involved in nitrification and denitrification.

Differences in the abundances of 16S rRNA genes (a proxy for the size of the total bacterial community) between sampling times were generally not significant (Fig. 2a, Table S3), except in e.g. NT-FER or T-INH treatments which showed greater abundances in spring than in December or August. The T plots showed higher abundances of the 16S rRNA gene than NT for all fertiliser treatments (Figs. 2a, Table S3), except in the FER treatment.

The highest abundance of the *amoA* genes from AOB and AOA was detected in May (Figs. 2b and 2c). In August, the rain causing the rewetting event increased the abundance of AOA communities (Fig. 2c, Table S4) in all treatments except NT-FER and T-INH, and the abundance of 16S rRNA gene and AOB in NT-N0. The AOA and AOB were more abundant in the T plots than NT for all fertiliser treatments (Figs. 2b and 2c, Tables

S3 and S4). However, in August, they increased in the NT treatment and were significantly higher in the N0 plots (Figs. 2b and 2c, Table S4). An effect of fertilisation was observed on AOA and AOB abundances, especially in Period II. In the NT treatments, the copy number of AOB and AOA decreased in the order FER > INH > N0 from February to May. The abundance of AOA in August was higher in the N0 plots (Fig. 2c) than in the rest of the fertiliser treatments ($P < 0.05$). For the T plots, the INH treatment significantly increased the abundances of AOB concerning N0 the following 40 days after the second fertilisation (April). The same tendency was observed for AOA abundance, although differences were significant only for the May sampling date.

The abundances of the functional genes involved in denitrification (*nirK*, *nirS*, *nosZI*, *nosZII*) were increased in the last timepoint that coincided with the heavy rainfall event (Figs. 2d-g, Tables S5 and S6), showing that the NT treatment supported a higher abundance of these in comparison with T plots (Fig. 2d-g) (in FER subplots, this was observed only for *nosZI* genes, $P > 0.05$, Table S6). During the crop period, the tillage factor did not affect the abundance of denitrifiers, except NT-FER and T-INH. In May, the INH treatment (for the T plot) showed significantly higher abundances for all denitrifiers than N0 and FER. However, FER significantly increased the abundances of all denitrifiers in the NT treatment compared to N0 from February to May, showing intermediate values in the INH treatment.

The *amoA*/*nosZ* ratio ranged from 0.08 to 0.37%, showing the highest and lowest average values of the treatments, in May and August, respectively (Table S7). Regarding the differences between treatments, FER resulted in the highest values of this ratio in December ($P < 0.05$, Fig. 2h, Table S7), compared to the INH subplots. In April and May, NT-N0 yielded the lowest values. In August, no differences were noticed except for the N0 treatment (Table S7).

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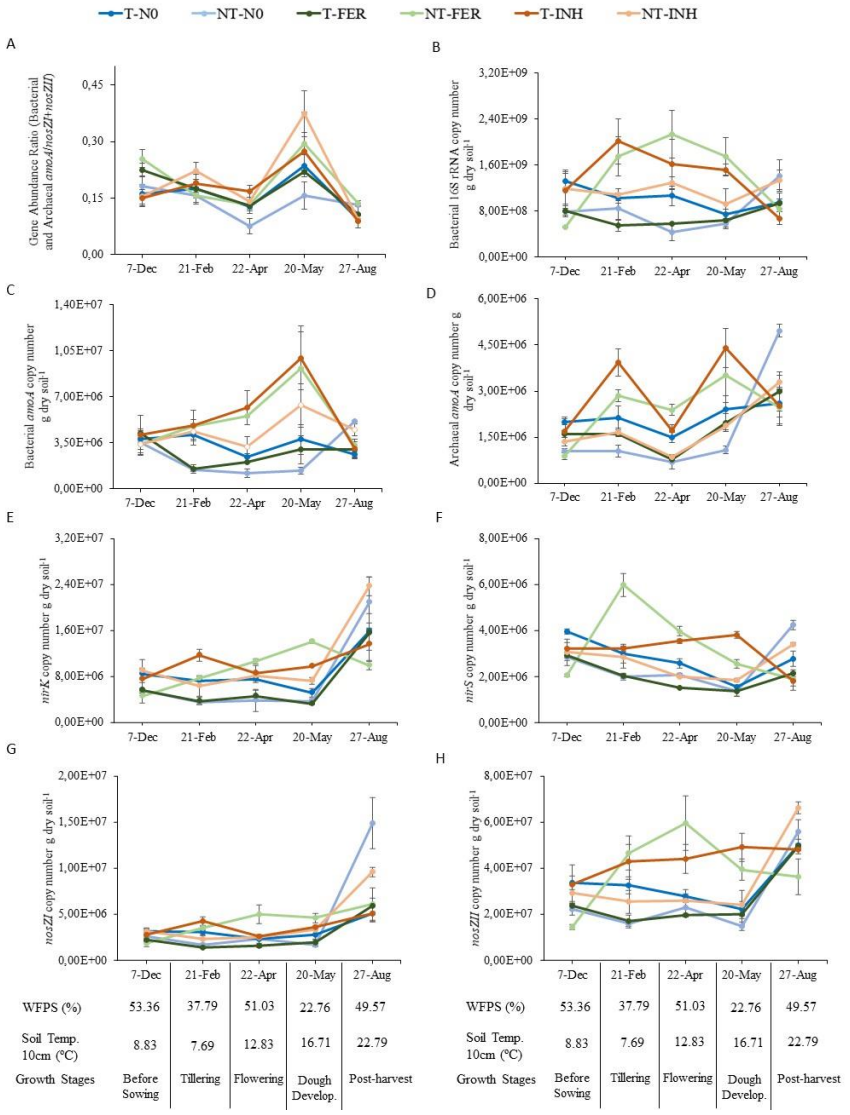


386

387 **Figure. 2.** Seasonal dynamics of the copy numbers of 16S rRNA (A), the *amoA* gene from AOB (B) and
388 AOA (C), *nirK* (D), *nirS* (E), *nosZI*(F), *nosZII* (G) genes and the abundance ratio of functional genes
389 involved nitrification to those involved in N_2O reduction (*amoA*/*nosZ* ratio) (H) for the different treatments
390 (see Table 1). The scale of the Y-axes has been adapted in each case to improve the visualisation of the

data. Error bars indicate standard error. The significant differences between sampling dates and treatments are shown in Tables S3-S7.

3.5 Microbial community composition, diversity and indicator taxa

The structure of the microbial communities from different treatments was influenced by the sampling time and fertilisation (Fig. S3, Table 3). Similar results were observed when time was used as a blocking variable in the permanova model, although the significance of the fertilisation effect increased (P = 0.0001) in both tillage systems (T and NT). The separation of the communities was significantly correlated with WFPS and soil NH_4^+ content, which characterised samples obtained during the high N_2O emission peaks (December and April) while the rest of the samples were characterised by higher abundances of AOA and *amoA*/*nosZ* ratios.

Table 3 Differences in the phylogenetic structure of microbial communities in soil samples with different treatments tested by permutational multivariate analysis of variance (PERMANOVA). *P* values are based on 1000 permutations. Significant effects are indicated in bold.

Factor	Groups	R	*P* value
Tillage	All	0.132	0.8032
Tillage	N0	0.058	0.9890
Tillage	INH	0.123	0.3387
Tillage	FER	0.088	0.7772
Fertilisation	All	**0.212**	**0.0190**
Fertilisation	T	**0.211**	**0.0159**
Fertilisation	NT	**0.211**	**0.0159**
Tillage x Fertilisation	-	0.161	0.4615
Time	All	**0.664**	**0.0009**
Treatment x Time	All	0.357	0.2208

Time: the four different sampling dates (7 December, N_2O peak in Period I;21 February, between both fertilisations; 22 April, N_2O peak in Period II; and 20 May, before harvest). For the different treatments see Table 1.

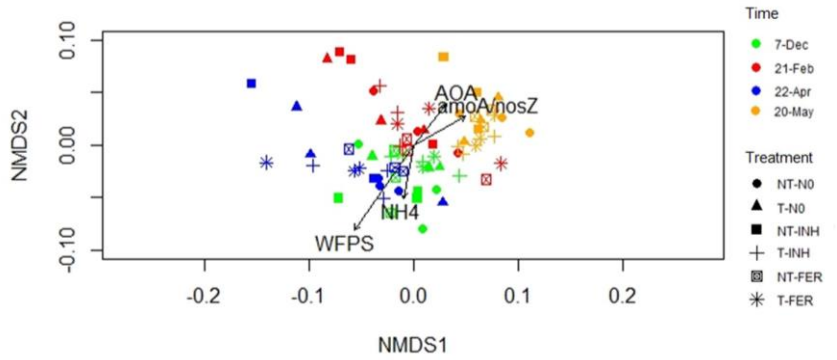


Fig. S3. Structure of microbial communities in the soil samples from different time points and treatments. Ordination is based on nonmetric multidimensional scaling (NMDS) of generalised UniFrac distances. Significant ($P < 0.05$) correlations between ordination axes and soil abiotic and biotic variables are shown as vectors whose length is proportional to the strength of the correlation. Timepoints: 7 December, N_2O peak in Period I; 21 February, between both fertilisations; 22 April, N_2O peak in Period II; and 20 May, before harvest.

Neither tillage nor fertiliser treatments, including inhibitors, had significant effects on the diversity of the soil microbial communities across all timepoints (Fig.3a). Diversity was highest in the samples taken before the harvest (20 May) irrespective of tillage or fertiliser treatment (Fig. 3b). In addition, T (with and without fertiliser treatments) demonstrated higher diversity at the time of the first N_2O peak (7 December) compared to NT and the lowest Shannon diversity at the time of the second N_2O peak (22 April) compared to the rest of the time points within the T plots (Fig. 3b).

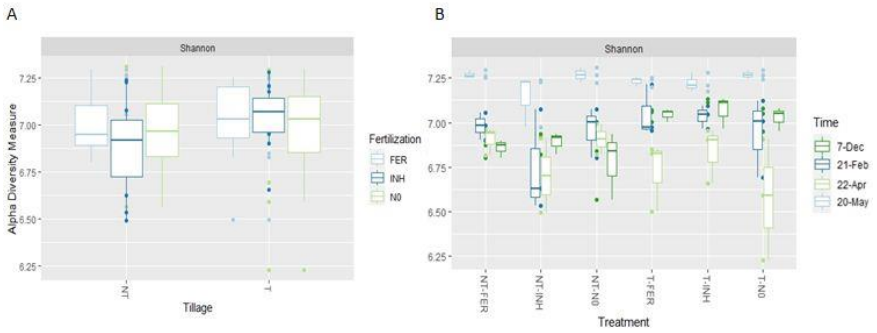


Figure. 3. Shannon's alpha diversity index. A) Bacterial diversity for the tillage management and fertiliser factors. B) Bacterial diversity for the different treatments (see Table 1) on the sampling dates: 7 December, N_2O peak in Period I; 21 February, between both fertilisations; 22 April, N_2O peak in Period II; and 20 May, before harvest.

The communities were dominated by the phyla *Bacteroidetes*, *Proteobacteria*, *Acidobacteria*, *Cyanobacteria*, *Gemmatimonadetes* and *Actinobacteria* (Fig. 4). Regardless of fertiliser, NT management increased the abundances of *Verrucomicrobia*, *Bacteroidetes*, FBP and *Cyanobacteria*. In contrast, the abundances of *Nitrospirae*, *Armatimonadetes*, *Chloroflexi* and *Firmicutes* (this one only for FER and INH) increased in T plots. In addition, the abundances of *Acidobacteria*, *Gemmatimonadetes* and *Planctomycetes* decreased by the inhibitor amendment regardless of the tillage management (Fig. 4). *Nitrospirae* and *Acidobacteria* were identified to be negatively affected in the NT-INH treatment (Fig. 4), and were significantly enriched in *Actinobacteria*, *Verrucomicrobia* and *Proteobacteria*. Furthermore, T-FER had the highest abundance of *Firmicutes* and *Nitrospirae* and showed a higher abundance of *Actinobacteria* than NT-FER did, indicating a tillage effect on this phylum.

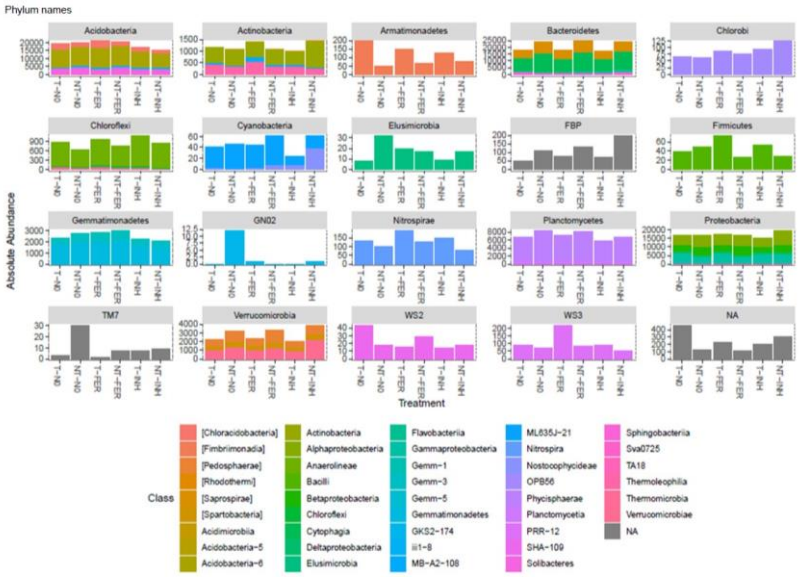


Figure. 4. Taxonomic composition of the bacterial communities in the different treatments. Relative abundance at the class level and taxa contributing more than 1% in each sample type are included.

4. Discussion

4.1 Tillage and fertilisation management effects on area-scaled and yield-scaled N_2O emissions

Avoiding or reducing basal fertilisation has been highlighted as a recommended practice to decrease N losses (Abalos et al., 2017; Xia et al., 2017), but may be necessary to prevent N immobilisation when crop residues with high or medium C:N ratios are left on the soil, particularly during the transition to no-till systems (Alijani et al., 2019). During the period following N addition at basal fertilisation, N_2O fluxes would be expected to be low due to the soil temperatures in late autumn (Fig. 1a).

The highest N_2O fluxes for all treatments were observed immediately after a rainfall event. The magnitude of the rewetting peak depended on tillage since higher N_2O fluxes

were observed in T soils with fertiliser treatments than in NT plots, particularly in T-FER compared with T-INH (Fig. 1c). This suggests that the emissions could be linked to a potential residual effect of the inhibitor and the soil N that was not efficiently taken-up by the crop (particularly in T plots) and to possible changes in soil microbiota (as explained below in Section 4.2). This high and short lived peak resulted from the pulse effect, which could be due to the reactivation of soil microbiota after several weeks during which the soil was dry (Fig. 1b) (Bergstermann et al., 2011; Leitner et al., 2017). Our results suggest that measuring N_2O emissions after the first rainfall event following long dry periods is essential, regardless of whether several weeks/months may have passed since N fertilisation or harvest, to obtain reliable emission factors in semiarid areas. This effect can be enhanced in dry years with low N uptake and/or N leaching and low crop yields, as observed in this study and in one of the two cropping seasons in Guardia et al. (2019). Our results confirmed that rainfall distribution and soil WFPS (as well as N availability) are limiting and pivotal factors driving N_2O losses in rainfed semiarid areas (Abalos et al., 2017; García- Marco et al., 2014). In the November-May period, a positive and significant correlation between N_2O fluxes and WFPS was found ($P < 0.05$, n = 38, r = 0.38). It has been suggested that these peaks are short-lived (e.g. Barrat et al., 2020), in agreement with what we also observed in other experiment under similar soil and climatic conditions (Guardia et al., 2021). This short-lived nature must be considered for adequately capturing the pulses and for an appropriate calculation of cumulative emissions. We recommend that these rewetting pulses should be explored in detail in future experiments under field conditions to develop specific sampling protocols for these events.

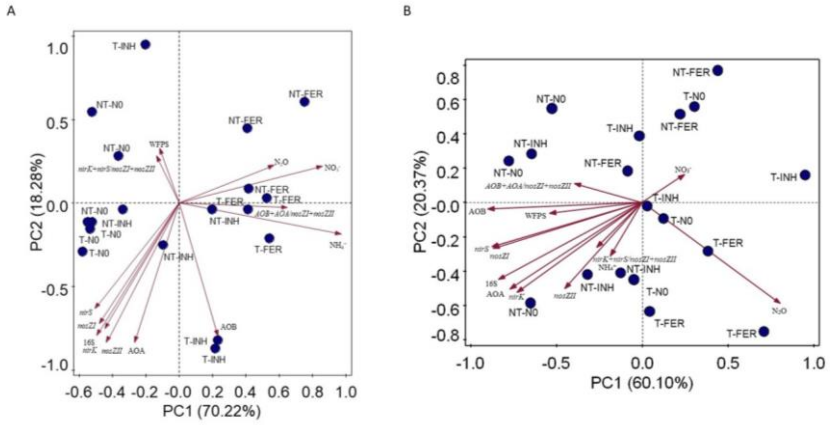
After both basal and dressing fertilisation, the cumulative N_2O emissions from the T plots were higher than those from the NT plots (Table 2). According to the recent meta-

481 analysis of Huang et al. (2018), the NT plots generally lead to higher N_2O losses than
482 conventional tillage, but this effect is not significant for short-term experiments or dry
483 climates. This increase in N_2O emissions is generally associated with an enhanced
484 denitrifying activity due to higher soil moisture and, therefore, less oxygen availability
485 (Wang and Zou, 2020). In our case, even though higher WFPS values were reached in
486 NT than in T plots during Period I (Fig. 1b), an opposite tendency was obtained for the
487 N_2O emissions. We hypothesised that the main reasons for greater N_2O losses in T were:
488 i) the higher soil disturbance after labour (i.e. Period I), thus favouring N_2O diffusion
489 from the soil to the atmosphere in Period I, and particularly ii) the better implantation of
490 the barley crop in NT which resulted in higher plant densities (Fig. S5) and higher yields
491 (Table S2). This effect was also suggested by the lower NO_3^- contents in NT-N0
492 compared to T-N0 (Fig. S1b). Therefore, the "tillage management" effect may have been
493 masked by the "plant density and development" effect, thus resulting in improved N
494 acquisition by the crop and a lower amount of N available for nitrifying or denitrifying
495 microorganisms. The potential of improving N uptake by plants (i.e., through crop
496 breeding, selection of acquisitive species in crop mixtures or increasing plant density) as
497 a strategy for N_2O mitigation has been suggested by several authors (e.g., Abalos et al.,
498 2018; Bowatte et al., 2018).

499 In Period I the double inhibitor reduced N_2O emissions, thus supporting the findings
500 by Guardia et al. (2018) and Montoya et al. (2021b) about the efficacy of this treatment
501 in rainfed conditions in calcareous low organic C content soils. Our results suggest that
502 the potential negative impacts of applying N at basal fertilisation can be minimised by
503 choosing appropriate N source, such as a nitrification + urease inhibitor. Regarding the
504 dressing application of CAN+DMPSA, our results supported those obtained by Guardia
505 et al. (2017) or Montoya et al. (2021b). However, the effectiveness of DMPSA with CAN

may be masked when environmental conditions lead to low N_2O fluxes from CAN, as those in our study were in the March-June period (Fig. 1c). In our experiment, the effectiveness of DMPSA (dressing fertilisation) and DMPSA+NBPT (basal fertilisation) was supported by the lower average NO_3^- concentrations (Fig.S1b) in the INH group than in the FER group. In this sense, during Period I, the PCA (Fig. S4a) showed a grouping of FER (but not INH or N0) treatments close to N_2O emissions, which were positively correlated with mineral N contents (particularly $N\text{-}NO_3^-$).

As reported by Corrochano-Monsalve et al. (2020b) and in agreement with our hypothesis, the combination of NT and INH at the conditions of our study was the optimum choice to minimise yield-scaled N_2O emissions (Table 2), without yield penalties (Table S2). The tendency to enhance yields and aboveground N uptake in the INH subplots compared with the FER subplots (Table S2) agreed with the global meta-analysis of Abalos et al. (2014). This tendency of increasing (in particular) grain yield was not associated with a significant decrease in the grain N concentration, contrary to Savin et al. (2019). The higher yields obtained in NT versus T result from the improved implantation of the crop under NT, as explained above. However, in semiarid Mediterranean areas, the potential of conservation tillage for increasing crop yields and/or decreasing yield-scaled emissions in Mediterranean regions has been pointed out, particularly in dry cropping seasons (Morell et al., 2011; Plaza-Bonilla et al., 2014). On a global scale, a negative effect of NT on yield was described by Pittelkow et al. (2015), while Shakoor et al. (2021) obtained a significant increment in barley.



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528 **Fig. S4.** Principal component analysis (PCA) of the effects of the different treatments (see Table 1) on the
529 total abundances of targeted genes, abundance ratios, N_2O emissions and soil properties at the sampling
530 occasions of A) 7 December (N_2O peak in Period I) and B) 27 August (N_2O peak after rewetting). The
531 percentage of variance in the dataset explained by Axis 1 and Axis 2 is indicated.

532

533 **Fig. S5.** Pictures of the experiments were taken on 4 April in the T (A) and NT (B) plots and on 6 February
534 in the T (C) and NT (D) plots.

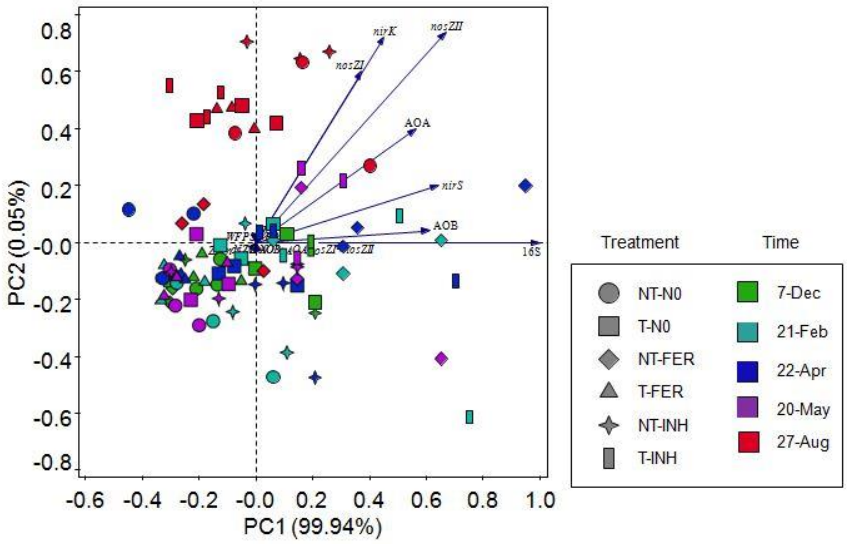

Fig. S6. Principal component analysis (PCA) of the effects of the different treatments (see Table 1) on the total abundances of targeted genes, abundance ratio, N_2O emissions and soil properties at five sampling occasions: (1) 7 December (N_2O peak in Period I), (2) 21 February (between both fertilisation events), (3) 22 April (small N_2O peak in Period II), (4) 20 May (before harvest) and (5) 27 August (N_2O peak after rewetting). The percentage of variance in the dataset explained by Axis 1 and Axis 2 is indicated.

4.2 Tillage and fertilisation effects on microbial communities and abundances of nitrifiers and denitrifiers

As N_2O can be formed during nitrification and consumed by denitrification, the *amoA*/*nosZ* ratio (Fig. 2h) can be a useful proxy coupling the microbial genetic potential of both processes (Breuillin-Sessoms et al., 2017; Montoya et al., 2021a) and explain N_2O emissions during Period I. In December, after basal fertilisation, the FER treatment, showing ~~(which led to~~ the highest N_2O emissions~~,)~~ caused a significant increase in the *amoA*/*nosZ* ratio compared to INH. Our results were supported by Montoya et al. (2021b) in a rainfed oilseed rape crop and by Barrena et al. (2017), who suggested that the effect of applying 3,4-dimethylpyrazole phosphate (DMPP) was explained by the increase in

551 *nosZ* gene abundance rather than by the direct inhibition of nitrification. Torralbo et al.
552 (2017) indicated that nitrification inhibitors (DMPP and DMPSA) decreased bacterial
553 nitrification rates and increased *nosZ* abundance under 40% and 80% WFPS conditions,
554 although the mechanism for increased *nosZ* abundance is not known. The effects on
555 bacterial diversity may also contribute to explaining our results. In this sense, the
556 *Nitrospirae* phylum, despite showing low abundance, were favoured under FER treatment
557 (especially in the T-FER treatment; Fig. 4) compared with INH. Staley et al. (2018)
558 indicated that soils amended with urea favored nitrifying genera such as *Nitrospira* or
559 *Nitrosospira*. Corrochano-Monsalve et al. (2020) also observed a lower abundance of
560 *Nitrospirae* in soils that received DMPSA inhibitor than those amended with ammonium
561 sulfate under humid rainfed conditions.

562 Regarding the tillage effect after basal fertilisation, the lower abundances of most
563 key N-cycling genes in NT than T were also found by Wang et al. (2019) in a winter
564 wheat crop. Our results also showed a tendency of decreased microbial diversity in the
565 INH treatment combined with NT that was not observed for T. This was also reported by
566 Corrochano-Monsalve et al. (2021, 2020), who suggested that water content played an
567 important role for the effect of the nitrification inhibitor. The crop residue on the surface
568 may prevent water losses and may provide anaerobic microsites that could support
569 denitrification (Suleiman et al., 2018). The abundance of *Nitrospirae* involved in
570 nitrification was significantly higher in T (higher N_2O emissions) than in NT soils (lower
571 N_2O emissions) throughout the cropping period (Fig. 4). The NT treatments instead
572 resulted in a higher abundance of *Bacteroidetes*, which represent an important community
573 of bacteria harbouring the *nosZII* gene (Jones et al., 2013) and are involved in soil organic
574 matter degradation (Thomas et al., 2011). These NT treatments also showed a greater
575 abundance of *Cyanobacteria*, which supportseveral beneficial aspects in agricultural

576 sustainability, e.g., stimulation of plant growth and protection against plant diseases
577 (Singh et al., 2016), although their abundance was low, and *Verrucomicrobia* (anaerobic
578 degradation of cellulose, Bao et al., 2019). Likewise, Navarrete et al. (2015) and Navarro-
579 Noya et al. (2013) indicated that straw surface application caused a significant increase
580 in *Verrucomicrobia*. Thus, our results suggest that conservation tillage selected for
581 microbes specialised in degrading organic compounds, which agree with finding reported
582 by Kielak et al. (2016) after straw surface application.

583 During most of Period II (except the rewetting event in August), conditions were dry,
584 and WFPS values were more favourable for nitrification than for denitrification, even
585 being close to the minimum threshold for the activity of nitrifying microorganisms
586 (Pilegaard, 2013; Ussiri and Lal, 2012). This assumption, coupled with the fact that the
587 abundances of AOB (May) and AOA, as well as the *amoA*/*nosZ* ratio, significantly
588 increased in Period II (Figs. 2b and c), may suggest that nitrification was the main process
589 for N_2O production during these sampling dates. In agreement, the ratio between bacterial
590 and archaeal *amoA* and all denitrifying genes was higher after dressing fertilisation (0.15
591 on average for the samplings in April and May) than in August (0.08) or December (0.14)
592 (data not shown). A short-lived contribution of denitrification in Period II after rainfall or
593 irrigation events, when small N_2O peaks were observed (Fig. 1c), could have been
594 supported by the presence of crop residues (oilseed rape) that can stimulate this process
595 even at medium WFPS values (Li et al., 2016). The bacterial communities in soil samples
596 from February and April clustered together and were positively correlated with AOA and
597 the *amoA*/*nosZ* ratio (Fig. S3).

598 At the rewetting peak in August, most of the treatments were grouped and separated
599 from other sampling dates (Fig, S6), confirming that the conditions of this period could

600 be of pivotal importance in the cumulative fluxes and the mitigation potential of the
601 different strategies. The average mineral N contents at the rewetting episode were 0.1 and
602 24.4 mg N kg soil^{-1} for NH_4^+ and NO_3^-, respectively (Fig. S2), possibly indicating that
603 this pulse was driven by denitrification rather than nitrification, as found by Bowen et al.
604 (2020) and Vázquez et al. (2020). This hypothesis was also supported by the grouping of
605 August values close to some key denitrifying genes (e.g., *nosZI*, *nosZII*, and *nirK*, Fig.
606 S6), or by the lower values of the *amoA*/*nosZ* ratio (Fig. 2h), in comparison with the
607 values in December. The rewetting event caused a significant increase in the abundances
608 of denitrifiers (*nirK*, *nosZI* and *nosZII*) compared to those in December and April, and
609 the opposite tendency occurred for the AOB abundance. The abundance of AOA also
610 increased, probably due to the low NH_4^+ contents after summer (Hink et al., 2017) and
611 the proposed greater tolerance of AOA to high temperatures (Duan et al., 2018) or other
612 stress factors, (He et al., 2018; Tao et al., 2021). A significant effect was observed for the
613 tillage management after rewetting. Nontilled plots (lower N_2O emissions) resulted in a
614 higher abundance of nitrifiers than T soils, suggesting that nitrification was less important
615 in this pulse. In addition, our results showed higher abundances of the 16S rRNA gene,
616 *nirS*, *nosZI* and *nosZII* in NT concerning T for those of N0 and INH (Fig. 2). During the
617 N_2O pulse in August, the abundance of N cycling genes was not a good indicator of the
618 amount of N_2O released from the different treatments. This could be due to the DNA-
619 based approach to quantify nitrifying and denitrifying genes, which cannot discriminate
620 between living or dead microbiota. This could be relevant under conditions of severe
621 drought lasting several weeks. Due to the importance of the postharvest rewetting peaks
622 in semiarid areas, further research should consider also RNA-based quantification. In
623 addition, the relationships between the diversity of microbial communities and N_2O
624 emissions during the critical rewetting pulses should be explored in future experiments.

5. Conclusions

The highest N_2O pulse occurred after a rewetting episode after several weeks of dry conditions and after barley harvesting, driven by a significant increase in the abundances of AOA and key genes involved in denitrification. N_2O emissions after applying N at basal fertilization were not negligible, particularly in T plots (despite the low temperatures of late autumn) and using the double inhibitor (DMPSA+NBPT) with urea can effectively mitigate these losses due to a possible stimulation of nitrous oxide reducing microorganism. This effective N_2O mitigation should be considered in addition to well-known side effects regarding the mitigation of other gaseous N losses, particularly NH_3 volatilisation, due to the use of the urease inhibitor NBPT. The application of fertilisers with inhibitor (INH) caused a decrease in the abundances of the phylum *Nitrospirae* during the cropping period, thus supporting its well-known potential to decrease N_2O emissions when conditions are favourable for nitrification. It was observed that tillage may have less influence than fertiliser management on the composition of soil microbial communities. However, NT management may have contributed to regulating the intensity of soil N_2O emissions, possibly due to a reduction in the abundance of *Nitrospirae* and a higher abundance of *Bacteroidetes*, which harbours the non-denitrifying N_2O reducers. These results demonstrate that changes in the N-cycling microbiota can be observed even during short-term changes in NT management and should be further explored under semiarid conditions. We also recommend exploring the potential effect of N management (including the use of enhanced-efficiency fertilisers) and proper crop density and development on optimising the plant uptake of N for reducing reactive N losses.

Acknowledgements

This research was funded by Agencia Estatal de Investigación (AEI)-Ministerio de Ciencia, Innovación y Competitividad and Fondo Europeo de Desarrollo Regional (RTI2018-096267-B-I00 MCIU/AEI/FEDER, UE), the Comunidad de Madrid (Spain) and Structural Funds 2014–2020 (ERDF and ESF) (project AGRISOST-CM S2018/BAA-4330). Montoya is the recipient of the FPI grant BES-2016-076712. García-Gutiérrez is the recipient of the Comunidad de Madrid contract number PEJD-2018-PRE/BIO-877. Funding for this research was also provided by EuroChemAgro GmbH. The funders had no role in the data collection and analysis or manuscript preparation. Sequencing was performed by the SNP&SEQ Technology Platform in Uppsala. The facility is part of the National Genomics Infrastructure (NGI) Sweden and Science for Life Laboratory. The SNP&SEQ Platform is also supported by the Swedish Research Council and the Knut and Alice Wallenberg Foundation. Special thanks are given to the field assistants working with us at Centro Nacional de Tecnología de Regadíos (CENTER), particularly to Alejandro Sánchez de Ribera. We also thank the technicians and researchers Guadalupe Álvarez, Rocío Rodríguez, Eduardo Aguilera, Alberto Sanz and Celia Ginés at the Department of Chemistry and Food Technology of the ETSIAAB. This work was done within the framework of the Moncloa Campus of International Excellence (UCM-UPM).

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Nitrous oxide emissions and microbial communities during the transition to conservation agriculture using N-enhanced efficiency fertilisers in a semiarid climate

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Abstract

The transition year from tillage to no tillage in semiarid areas and its effects on nitrous oxide (N_2O) emissions and related microbial communities, as well as the potential interaction with N management, including enhanced-efficiency fertilisers, are not well studied despite their economic and environmental implications. In tilled and nontilled plots, the effectiveness of the double DMPSA+NBPT inhibitor (applied with urea at basal fertilisation~~seeding~~) and that of DMPSA (applied with calcium ammonium nitrate at top-dressing) in the mitigation of N_2O emissions were evaluated in a rainfed barley (*Hordeum vulgare* L.) crop in central Spain. Crop yield, nitrogen (N) uptake, the abundances of key genes involved in nitrification and denitrification processes and ~~other~~ meteorological conditions and soil ancillary properties~~measurements~~ were monitored. In addition, the composition of~~distinct~~ bacterial communities was~~ere~~ determined by sequencing the 16S rRNA gene~~ fragments~~. Fertilisers with~~including~~ inhibitors decreased cumulative N_2O emissions and yield-scaled N_2O emissions by 53% and 56%, respectively, with respect to~~compared to~~ those without inhibitors, which coincided with a trend of~~ile~~ increasing grain and biomass yield and aboveground N uptake (by 11.3%, 9.2% and 7.2%, respectively). The highest N_2O emissions were measured 49 days after harvest (immediately after a rainfall event that like reactivated soil microorganisms), reaching 15 mg N m^{-2} d^{-1} for the treatment with fertiliser without inhibitor combined with tillage. This peak was linked to a remarkable increase in the abundance of denitrifiers. The abundance of nitrifiers and denitrifiers successfully explained the N_2O dynamics observed after basal~~seeding~~ fertilisation (i.e.~~,~~ an increase in the *amoA*/*nosZ* ratio in fertilised plots with inhibitors, where the highest emissions were observed). Our results also showed a reduction in the abundance of the phylum *Nitrospirae*~~ phyla~~ throughout the cropping period in the plots that received inhibitors. No tillage led to a higher abundance of

Cyanobacteria, *Verrucomicrobia* and *Bacteroidetes* and resulted in better implantation of the crop and higher plant density compared with~~to~~ tillage, thus increasing yields and N use efficiency and decreasing N_2O emissions. Under the conditions of our study, shifting from conventional tillage to no~~n~~ tillage ~~resulted in an~~ enhanced~~optimum~~ the balance between N use efficiency and yield-scaled N_2O emissions in the first year of conversion, particularity with the use of the double inhibitor with urea at basal fertilisation~~seeding~~ and DMPSA with CAN at dressing.

Keywords: Greenhouse gas emissions, ~~Illumina~~Global warming potential, Drying-rewetting pulses, Urease inhibitor, Nitrogen use efficiency, Microbial diversity

1. Introduction

~~The retention of c~~Crop residue~~s~~ can~~is a promising strategy to~~ improve soil quality and enhance soil organic carbon (SOC) stocks (García-Ruiz et al., 2019; Soussana et al., 2019), particularly in the context of sustainable intensification (Bais-Moleman et al., 2019). The retention ~~Retaining~~ of crop residues requires optimum nitrogen (N) fertilisation management to prevent N immobilisation (Yansheng et al., 2020), particularly during early crop stages. However, the addition of a mineral N source to prevent immobilisation combined with high C:N residues can stimulate denitrification (Li et al., 2016) and increase the emissions of the potent greenhouse gas nitrous oxide (N_2O) (Duan et al., 2018), thus raising the global warming potential of agro-ecosystems (IPCC, 2019; Li et al., 2021).

Nitrogen losses from crop residues and fertilisation practices ~~The retention of crop residues and the negative side effects of the addition of fertilisers at seeding concerning N loss (Xia et al., 2017)~~ are closely linked to tillage management, i.e.~~,~~ tillage (T) or no tillage (NT) (van Kessel et al., 2013). In nontilled soils, soil organic C (SOC) accumulates

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in the topsoil, increasing the availability of labile organic C for denitrifiers and mineral N for both nitrifiers and denitrifiers (Shakoor et al., 2021). In addition, the reduction in porosity due to the higher soil compaction with NT may ~~also~~ favour anoxic conditions and the consumption of nitrous oxide (N_2O) ~~by denitrifiers~~ (Huang et al., 2018). Tillage management has also been shown to change the microbial functional groups involved in N cycling. For instance, Wang et al. (2019) observed that in comparison with conventional tillage (i.e. chisel plough), long-term conservation tillage (i.e. NT) decreased the abundances of archaeal ammonia oxidisers and denitrifiers but increased the abundance of the *nosZ* gene, which is involved in the reduction of N_2O to dinitrogen (N_2), thus resulting in overall improved mitigation of N_2O emissions. It has also been demonstrated that crop residues, long-term NT and fertilisation management modify the microbial community composition (Suleiman et al., 2018; Li et al., 2020; Wang et al. 2021) due to the increase in soil C and N availability. However, little is known regarding microbial diversity and abundance changes during~~in~~ the ~~short term~~ transition year from T to NT ~~period before the NT system reaches equilibrium,~~ particularly in semiarid cropping systems. Under these conditions, N_2O emissions are highly dependent on N addition and soil moisture~~,~~, and m~~M~~ajor N_2O peaks typically occur after several weeks of dry conditions, usually after harvest in summer, and subsequent soil rewetting (Guardia et al., 2018a; Montoya et al., 2021b). A robust temporal resolution of N_2O emissions and microbial community dynamics (including basal~~seeding~~ and dressing fertilisation and postharvest period) is lacking under semiarid conditions. In addition, in order to develop cost-effective mitigation strategies, it is necessary to determine the relative contribution of nitrification and denitrification to critical N_2O pulses (including those after rewetting) and whether tillage management alleviates these N_2O peaks.

Tillage management also influences the emission of ammonia (NH_3). The "mulch effect" occurring when ~~caused by the nonincorporation of~~ residues are not being incorporated in nontilled soils (Pinheiro et al., 2018) causes higher volatilisation rates, as recently reported by the global meta-analysis of Ma et al. (2021). Volatilisation losses when urea is applied can be significantly reduced by the use of urease inhibitors (Ti et al., 2019) such as N-butyl thiophosphorictriamide (NBPT), while nitrification inhibitors such as 2-(3,4-dimethyl-1H-pyrazol-1-yl) succinic acid isomeric mixture (DMPSA) are the most effective enhanced-efficiency N form to decrease N_2O (Thapa et al., 2016). As a result, the combined use of nitrification and a urease inhibitor (named a "double inhibitor") can be considered a promising strategy to mitigate gaseous N losses (Corrochano-Monsalve et al., 2021a; Guardia et al., 2021; Souza et al., 2021~~; Zaman and Nguyen, 2012~~). Corrochano-Monsalve et al. (2020a, 2020b) showed that DMPSA minimised N_2O losses by decreasing the abundance of ammonia oxidisers while increasing that of N_2O reducers in a nontilled wheat crop under humid Mediterranean conditions. However, this "tillage x inhibitors" interaction effect on microbial communities and gaseous N losses is still poorly understood in arid or semiarid climates, particularly regarding the combination of DMPSA with the urease inhibitor NBPT and during the transition from T to NT.

T~~herefore, t~~his study aimed to determine potential N_2O mitigation strategies in a rainfed semiarid agroecosystem based on a combination of two contrasting tillage intensities (T and NT) and the use of nitrification and/or urease inhibitors. Another objective was to assess whether nitrifying and denitrifying communities (represented by the abundances of the functional genes from these pathways) and microbial diversity are useful indicators of N_2O emissions during the first year of NT adoption. Soil mineral N, crop biomass and N uptake were also included as ancillary measurements to explain N_2O

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fluxes and differences in microbial communities. It was hypothesised that i) short-term NT would result in changes in the abundance of genes involved in N cycling and the diversity of the bacterial community, thus affecting N_2O fluxes; and ii) the use of inhibitors would decrease N_2O losses in both T and particularly NT systems, in line with the findings of Corrochano-Monsalve et al. (2020a, 2020b).

2. Materials and Methods

2.1 Experimental site

The experiment was conducted in the Madrid region in Spain from October 2018 to November at the *Centro Nacional de Tecnología de Regadíos* "CENTER". The soil was a silty loam (9% clay, 60% silt, 31% sand) *Typic xerofluvent* (Soil Survey Staff, 2017). The main soil (0-20 cm) properties measured at the beginning of the experiment by conventional methods were as follows: bulk density, 1.23 ± 0.08 g cm^{-3}; electric conductivity, 0.49 ± 0.02 mS cm^{-1}; pH (water), 8.16 ± 0.02; total organic matter, 14.43 ± 0.17 g kg^{-1}; and $CaCO_3$, 9.87 ± 0.92 %~~g kg^{-1}~~. The last 10-year average mean temperatures and yearly rainfall were 14.2 °C and 384.4 mm, respectively. Considering the wheat cropping cycle, the average air temperatures were 6.9 °C and 19.4 °C in the November-March and March-November periods, respectively. The accumulated rainfall in the same periods was 204.7 mm and 180.7 mm, respectively. The meteorological data (from the last 10 years and during the experiment) were obtained from the Spanish "Sistema de información agroclimática para el regadío, SIAR", http://eportal.mapama.gob.es/websiar/Inicio.aspx), which collects data from a meteorological station that was set up at CENTER station.

2.2 Experimental design and management

The experimental design consisted of a split plot, with tillage as the main factor (arranged in a randomised three-replicated block design) and fertiliser as the second factor (arranged in a completely randomised design within each tillage plot). All tillage-fertilizer combinations were established in 8 m x 8 m subplots (n = 3). The experiment was split into two periods due to the differences in the meteorological conditions and crop development; the latter as a result of the contrasting N management (N rate and N source, Table 1) at each fertilisation event.~~:~~ The first period was from October 2018 until March 2019 (including basal fertilisation on 27 November), and the second period was from top-dressing fertilisation on 14 March (beginning of stem elongation stage) until the end of the experiment (including two irrigation events on 26 March and 13 May, due to severe drought conditions, and the postharvest period). All fertilisers were broadcasted over the soil surface in granular form by hand. More information about the treatments and management in each period can be found in Table 1.

Table 1 Description of tillage and fertilisation factors

Treatments		
Tillage factor	**Description**	
NT	No tillage and herbicide (glyphosate 36% p/v at) spraying	
T	Tillage (disc harrow and cultivator)	
Fertilisation factor	**Description**	
	First period	**Second Period**
N0	Unfertilised control	
FER	urea 40 kg N ha^{-1}	calcium ammonium nitrate 80 kg N ha^{-1}
INH	urea + DMPSA[1]+ NBPT[2]40 kg N ha^{-1}	calcium ammonium nitrate + DMPSA[1]80 kg N ha^{-1}

[1]DMPSA was applied at 0.8% of the NH_4^+-N

[2]NBPT was applied at 0.13% of ureic N

Before the beginning of the experiment, an oilseed rape (*Brassica napus* L.) crop was planted in the same experimental area. After rape harvesting, the residues were left on the field and then managed for the different tillage systems, as explained in Table 1. Excluding grain, the average aboveground biomass of the rape crop was 4492 kg ha^{-1}, with an average C:N ratio of 22.7. The field was seeded with barley (*Hordeum vulgare*

L. var. 'Esterel R1') on 17 December 2018 at 200 kg seed ha^{-1}. During the last decade, wheat (*Triticum aestivum* L., 2015-2017), maize (*Zea mays* L., 2012-2015), barley (*Hordeum vulgare* L., 2010-2012) and maize (2009-2010) were grown in the plots and the soil was managed through conventional tillage.

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2.3 Sampling and measurement of N_2O

Gas samples were taken 2-3 times per week during the first month after both N applications. The sampling frequency decreased (i.e., weekly or fortnightly and even monthly during summer due to the lack of rainfall events), particularly during dry periods with low (winter) and high (summer) soil temperatures, but ensuring that all soil rewetting events were covered. ~~The number of sampling events intensified after rainfall/irrigation events.~~ Nitrous oxide fluxes were measured using closed opaque chambers (one chamber per plot) with a volume of 19.3 L (diameter 35 cm, height 20 cm) placed over stainless rings inserted 5-10 cm into the soil. Chambers were closed during one hour, during which three gas samples were taken with 30-minute intervals at times t_0, t_{30} and t_{60} after closure from the headspaces of each chamber with 20 mL syringes fitted with 3-way stopcocks. The gas samples were then, transferred to vials sealed with a gas-tight neoprene septum in preparation for posterior analysis by gas chromatography. The concentration of N_2O was determined with a gas chromatograph equipped with an electron-capture detector (HP-6890, Agilent Technologies). ~~For detailed information about gas sampling and analyses, see Montoya et al. (2018).~~

Cumulative gas emissions during the experimental period were calculated by trapezoidal integration by linear interpolation of daily fluxes (Cowan et al., 2019), i.e. ~~as proposed~~ by multiplying the average flux of two successive determinations by the length of the period between sampling and adding that amount to the previous cumulative total.

~~Yield-scaled N_2O emissions were calculated as the ratio between cumulative N N_2O emissions and grain yield. Emission factors (EFs) were estimated as the ratio of cumulative N N_2O emissions (subtracting those of the corresponding control, i.e., T-N0 or NT-N0) to the total amount of synthetic N applied. The mitigation percentages, when given, were calculated from the EFs.~~ Yield-scaled N_2O emissions were calculated as follows:

$$Yield-scaled\ N_2O\ emissions\ (g\ N \cdot kg\ grain^{-1}) = \frac{Cumulative\ N_2O\ emissions}{Grain\ yield} \quad \text{(Eq. 1)}$$

For which cumulative emissions were expressed in g N ha^{-1}.

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2.4 Soil and plant analyses

Three 0.25 m x 0.25 m squares per subplot were harvested on 9 July to determine grain and biomass (i.e., aboveground biomass except for grain) yields and total N concentrations in grain and straw, analysed by Dumas's method with an elemental analyser (Association of Official Agricultural Chemists, 1975). Three soil cores (0-10 cm) per plot were taken at the same dates of gas sampling to determine the moisture content (expressed as water-filled pore space, WFPS), while NH_4^+-N and NO_3^--N concentrations were generally determined in half of the gas sampling events using a similar frequency to that of (Guardia et al., (2021; 2018b). After dressing fertilisation, the soil sampling frequency was initially decreased because of dry conditions that maintained the fertiliser granules over the soil surface, but was increased after both irrigation events. The soil NH_4^+-N and NO_3^--N contents were extracted using KCl 1 M (1:6.25 soil:extractant solution) and analysed by a colourimetric method (UV-V spectrophotometry) using a flow injection analyser (FIAS 400 Perkin Elmer), as explained in Montoya et al. (2018). The soil WFPS was estimated using the bulk density

data of the T and NT plots as explained in Abalos et al. (2012), previously measuring the gravimetric water content by oven-drying the soil samples at 105 °C.

2.5 Extraction of DNA and quantification of 16S rRNA and N-cycling genes

Three soil samples per plot for DNA extraction and subsequent microbial analyses were taken, pooled and mixed after basal~~seeding~~ fertilisation but before the sowing of barley (7 December), during the period between fertilisations (21 February), after top-dressing fertilisation (22 April), before harvest (20 May) and after the rewetting event in summer (27 August). The DNA was extracted from 0.5 g of soil using the commercial PowerSoil® DNA isolation kit (Qiagen). The DNA quality was determined on an agarose gel and the quantity was measured using a Qubit fluorimeter (Invitrogen, Carlsbad, CA, USA). To estimate the abundances of the microbial communities involved in specific processes in the N cycle, quantitative PCR (qPCR) was conducted using primers (Table S1) for the *amoA* gene from ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Rotthauwe et al., 1997; Tourna et al., 2008, respectively) in nitrification, the nitrite reductase genes *nirK* (Hallin and Lindgren, 1999) and *nirS* (Throbäck et al., 2004) in denitrification, and the N_2O reductase genes *nosZI* (Henry et al., 2006) and *nosZII* (Jones et al., 2013). In addition, the 16S rRNA gene was quantified as a proxy for the total microbial communities using taxa-specific primers (Quince et al., 2011; Parada et al., 2016) ~~(López-Gutiérrez et al., 2004)~~. All quantifications were performed in duplicate on two independent runs in a total reaction volume of 15 µL using iQ™ SYBR Green Supermix (Bio–Rad, Hercules, CA, USA), 0.05% BSA, primer~~s~~ concentrations of ~~(~~0.5-2 µM ~~of each primer for 16S rRNA, AOB and AOA, 0.8 µM of each primer for *nirK*, *nirS* and *nosZI* and 2 µM for *nosZII*)~~ and 5 ng DNA on a CFX Connect Real-Time System (Bio–Rad, Hercules, CA, USA). Thermal cycling conditions, primer sequences and concentrations are shown in Table S1. Standard curves were obtained by serial dilutions

of linearised plasmids with cloned fragments of the specific genes and the curves were linear in the range used (R^2=0.997–0.999). Electrophoresis in agarose and a melting curve analysis verified the quality of qPCR amplification. To detect potential inhibition of the PCR, a known amount of pGEMt plasmid was amplified using M13 primers where reactions with plasmid only and reactions containing the plasmid and 5 ng of the DNA were compared. We could not detect inhibition of the PCRs with~~by any of~~ the DNA concentrations used for any of the samples~~extracts~~. The copy numbers of the target genes per gram of dry soil were calculated according to Montoya et al. (2021b).

2.6 Amplicon sequencing of 16S rRNA genes

The composition of the bacterial and archaeal communities was determined by amplicon sequencing of the V4-V5 region within the 16S rRNA gene using a two-step PCR protocol (Berry et al., 2011), and the primers 515F (Parada et al., 2016) and 926R (Quince et al., 2011) with Nextera adaptor sequences (Illumina, Inc.). The preparation of amplicons is described in Hellman et al. (2019). Sequencing was performed by the SNP&SEQ Technology Platform in Uppsala, Sweden on the MiSeq platform (Illumina) using 2 × 250 bp paired-end chemistry. The sequences obtained in this study were submitted to the NCBI short read archive and are available under Bioproject accession number PRJNA761562.

The 16S rRNA gene sequences were trimmed with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) and paired-end reads were merged with PEAR (Zhang et al., 2014) using a minimum overlap of 30 bp. Further quality filtering was performed using VSEARCH (Rognes et al., 2016). The sequences were clustered at 97% nucleotide similarity into operational taxonomic units (OTUs), followed by de novo and reference-based (SILVA nonredundant reference database release 132 from 2017)

chimaera checking using the UCHIME algorithm (Edgar et al., 2011). The resulting OTUs were assigned to taxonomic ranks using SINA (Pruesse et al., 2012) with the SILVA 132 database as a reference. OTUs classified as "Chloroplast" or "Mitochondria", and OTUs that composed less than 1% of all sequences within each sample were removed from the dataset. This resulted in a total of 4,186,126 sequences in 9,670 OTUs.

2.7 Calculations and statistical analyses

Analyses of variance were ~~The analysis of data was~~ performed by using the *Statgraphics 18-X64* program individually for periods I and II (see section 2.2) and the entire~~whole~~ experimental period for average mineral N and ~~,~~ DOC concentrations and cumulative N_2O emissions. ~~Analyses of variance were performed for almost all variables over the experiment (except for climatic variables).~~ Data distribution normality and variance uniformity were previously assessed by the Shapiro–Wilk test and Levene´s statistic, respectively, and they were log-transformed before analysis when necessary. Means were separated by the LSD test at $P < 0.05$. For nonnormally distributed data, the Kruskal–Wallis test was used on nontransformed data to evaluate differences at $P < 0.05$. Simple linear regression analyses were performed to determine the relationships between N_2O-N, WFPS, mineral N (NH_4^+-N and NO_3^--N) and soil temperature, as well as between yield variables. Two types of correlation analyses were carried out: from the data of average contents and cumulative fluxes (correlations per subplot) and considering the average daily values (correlation per sampling date). Additionally, principal component analyses (PCAs) were performed to explore the relationships between gene abundances, soil variables and N_2O fluxes based on Euclidian distances using CANOCO for Windows version 5 (Wageningen, Netherlands) for the different sampling dates used in the DNA extractions (see Section 2.5).

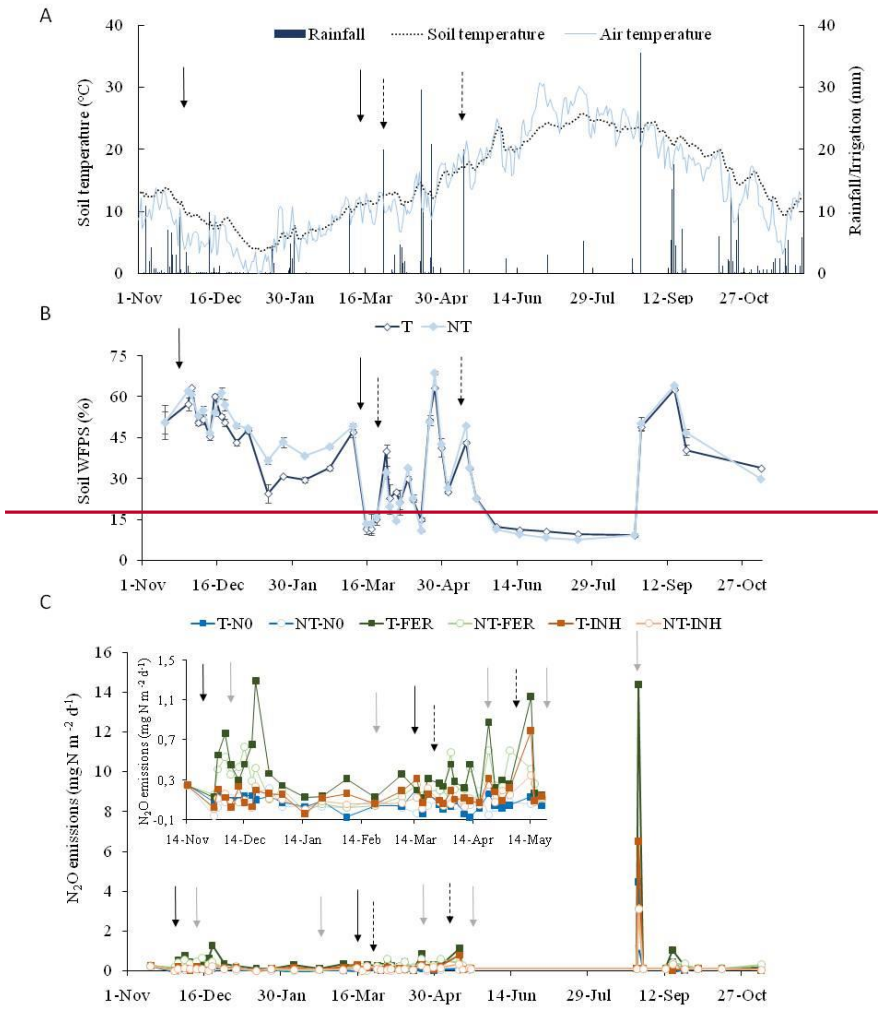
Statistical evaluation of the sequencing data was carried out with R version 4.0.5 (R Core Team, 2013) using Rstudio version 1.1.456 (RStudio Team, 2018). The data were processed using the 'phyloseq' package (McMurdie and Holmes, 2013), and the 'vegan' package (Oksanen et al., 2015) was applied for the Shannon alpha diversity indices. In addition, nonmetric multidimensional scaling (NMDS) using weighted phylogenetic distances (weighted UniFrac) to visualise community patterns was generated with the packages 'ape' (Paradis et al., 2004) and 'GUniFrac' (Chen and Chen, 2018), and plotted using 'ggplot2' (Wickham, 2011). The effect of 'treatment' and 'time', and 'fertilisation' and 'tillage' was tested by permutational multivariate analysis of variance (PERMANOVA) using the function 'adonis'. The characteristic taxa for each specific treatment throughout all sampling dates were tested using an indicator species analysis for the core microbial OTUs (Jeanbille et al., 2016) with the packages 'indicspecies' (Cáceres et al., 2010) and 'labdsv' (Roberts and Roberts, 2016).

3. Results

3.1 Environmental conditions, mineral N and DOC

During the first period (November-March), the mean air temperature, soil temperature (10 cm) and cumulative rainfall were 6.1 °C, 8.1 °C and 91 mm, respectively (Fig. 1a). In the March-July period (after dressing fertilisation), the mean air temperature, soil temperature (10 cm) and cumulative rainfall were 15.7 °C, 15.8 °C and 96 mm, respectively (136 mm considering the two irrigation events, see Section 2.2). Compared with the 10-year average values, the total rainfall in the November-March and March-July periods was 38 % and 39% lower, respectively. After harvest, the climatic conditions were hot and dry, and the first precipitation event above 2 mm of effective rainfall occurred on 27 August (Fig. 1a). The soil WFPS ranged from 10% to 69% (Fig. 1b). Some values within the 50%-70% range were reached after both fertilisation events

310 (particularly after basal fertilisation). After harvest, the soil WFPS values were low (<
311 15%) until the late-August rainfall event.



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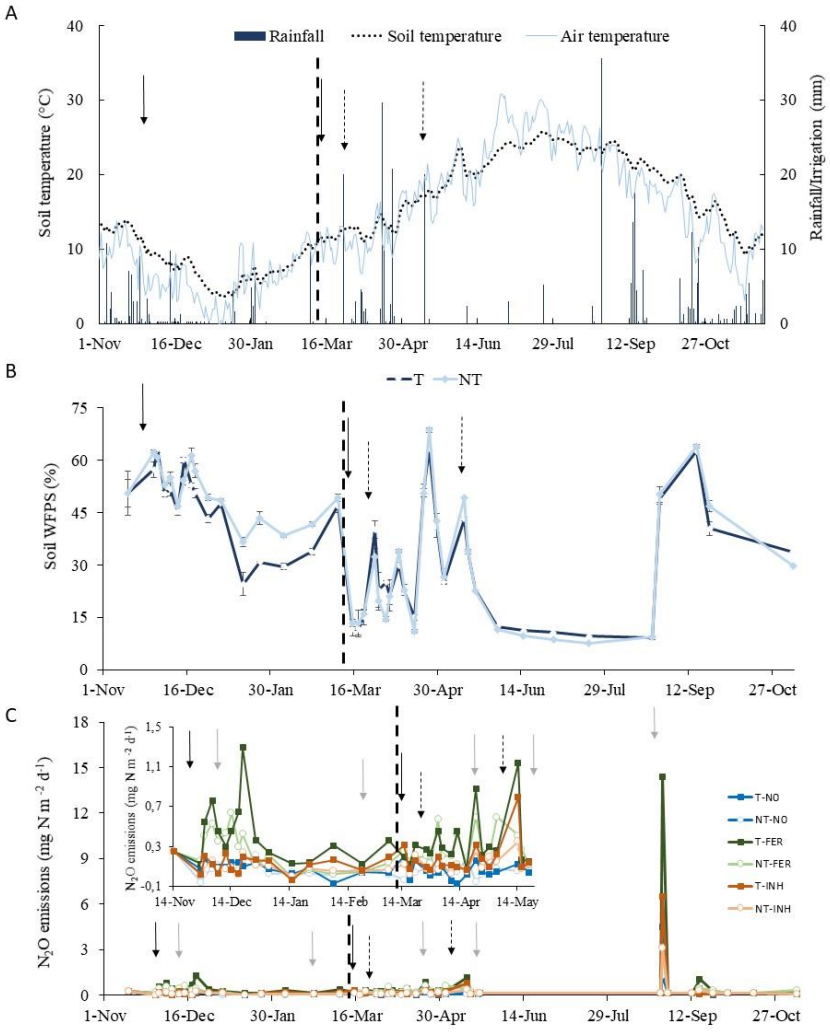
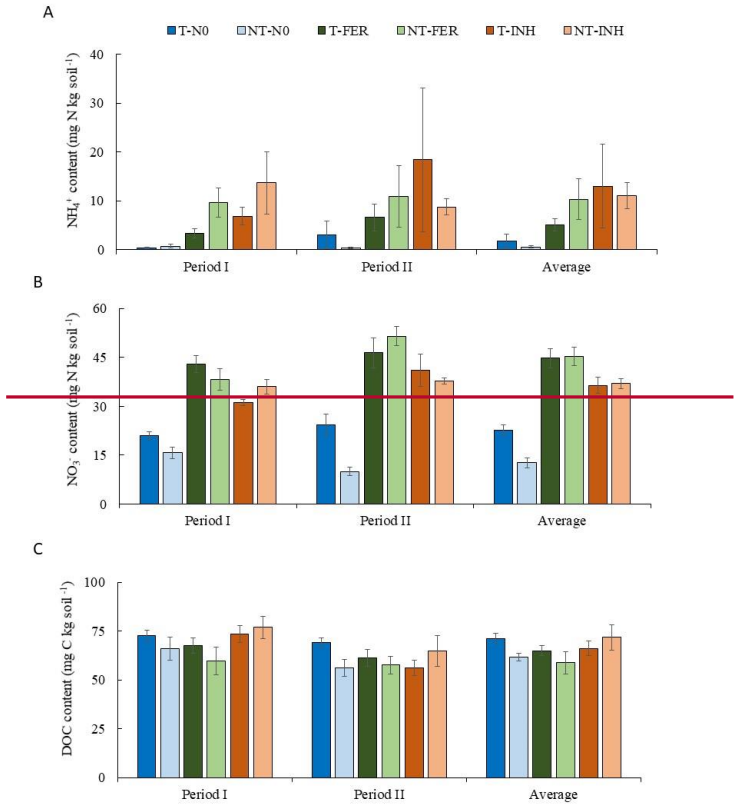


Figure. 1. (A) Daily mean temperatures in air and soil (10 cm depth) and daily rainfall/irrigation; (B) evolution of soil WFPS; and (C) daily N_2O emissions during the whole experimental period for the different treatments (see Table 1). The black and dotted arrows denote fertilisation and irrigation events, respectively. The grey arrows denote soil samplings for microbial community analyses. Dotted vertical line indicate the division into the two time periods. Error bars show~~Vertical lines indicate~~ standard error~~s~~.

The application of DMPSA tended to increase the soil NH_4^+content during Period I (Fig. S1a). After dressing fertilisation (Period II), the increase in average NH_4^+

concentration was observed in the T plots. Regarding NO_3^- contents (Fig. S1b), the use of inhibitors decreased these concentrations compared to FER subplots in both periods. No major differences between tillage treatments were observed for DOC content, apart from the significantly higher levels of INH concerning FER in Period I (Fig. S1c).


A
T-N0
NT-N0
T-FER
NT-FER
T-INH
NT-INH
NH_4^+ content (mg N kg soil^{-1})
40
30
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10
0
Period I
Period II
Average
B
NO_3^- content (mg N kg soil^{-1})
60
45
30
15
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Period I
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DOC content (mg C kg soil^{-1})
100
75
50
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Period II
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325

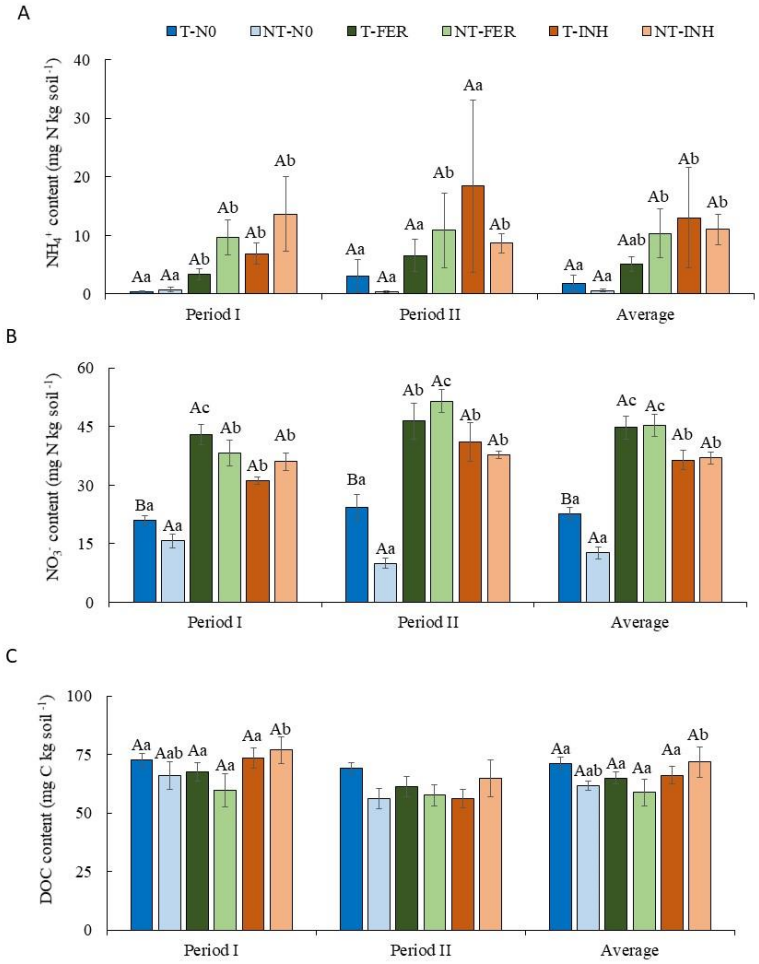

Fig. S1. Average ammonium ($N\text{-}NH_4^+$) (A), nitrate ($N\text{-}NO_3^-$) (B) and DOC (C) ~~topsoil (0-10 cm)~~ contents in topsoil (0-10 cm) during Periods I and II and throughout the experimental period for the different treatments (see Table 1). Error bars~~Vertical lines~~ indicate standard error~~s~~. Different capital letters indicate differences ($P < 0.05$) between tillage treatments within each fertilizer, while different lowercase letters indicate significant differences ($P < 0.05$) between fertilizers within each tillage treatment.

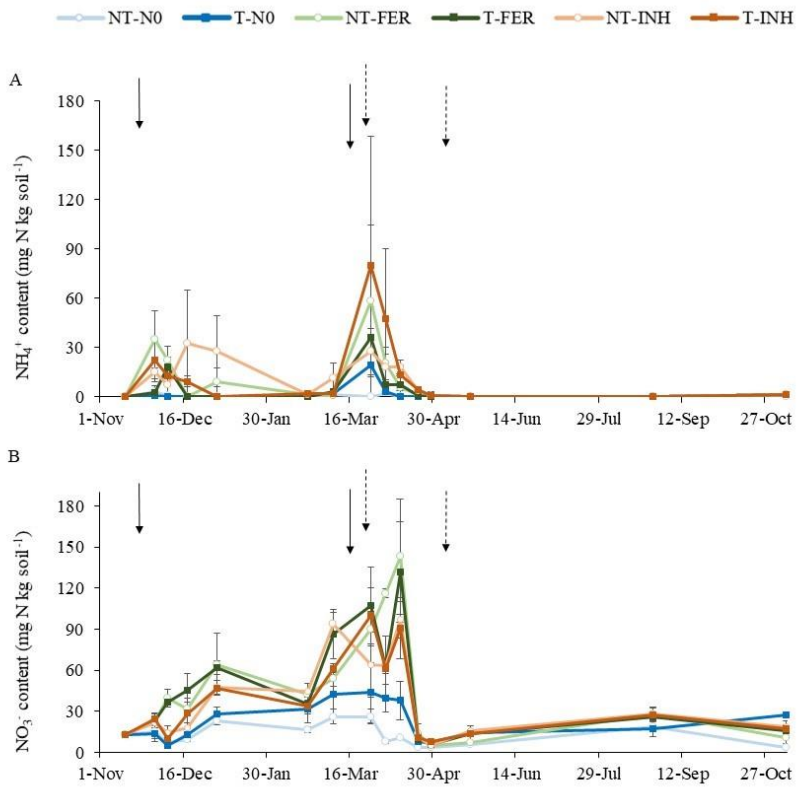

Fig. S2. Ammonium ($N\text{-}NH_4^+$) (A) and nitrate ($N\text{-}NO_3^-$) (B) contents topsoil (0-10 cm) during the entire experimental period for the different treatments (see Table 1). The black and dotted arrows denote fertilisation and irrigation events, respectively.

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3.2 Agronomic parameters

~~Both tillage and fertilisation influenced the agronomic parameters of barley.~~ The NT plots showed trends of~~resulted in numerically~~ higher grain ($0.05 < P < 0.10$) and biomass yields ($0.05 < P < 0.10$) and aboveground N uptake, but lower grain and biomass N contents ($0.05 < P < 0.10$) than T (Table S2~~1~~). Fertilising with N increased biomass yield and aboveground N uptake compared to the N0 treatment ($P < 0.05$). Grain yields increased in the order N0 < FER < INH (Table S2). ~~Only the INH subplots increased~~

~~grain yields concerning N0 (Table S1), showing that FER was an intermediate result~~. Grain N content was positively correlated with biomass N content ($P < 0.001$, n = 18, r = 0.73), but negatively correlated with grain yield ($P < 0.05$, n = 18, r = -0.56).

3.3 Nitrous oxide and yield-scaled emissions

Nitrous oxide peaked during the first month after basal fertilisation, reaching 1.3 mg N m^{-2} d^{-1} in T-FER (Fig. 1c). The cumulative fluxes during this first fertilisation decreased in the order FER > INH > N0 (Table 2), while T increased N_2O emissions by 55% compared to NT. After dressing fertilisation, the main N_2O emissions occurred 39 and 61 days after N application (Fig. 1c). As in the previous period, the maximum values (0.9 and 1.1 mg N m^{-2} d^{-1}) were observed for T-FER. After harvest, emissions were generally low when effective rainfall was below 5 mm. However, after a rainfall event of 35 mm N_2O fluxes peaked (27 August,) ranging from 1.3 (in NT-N0) to 15.0 mg N m^{-2} d^{-1} (in T-FER) (Fig. 1c). At the end of Period II, NT and INH mitigated N_2O emissions by 32% and 45%, respectively, compared with T and FER (Table 2). Cumulative N_2O emissions and average soil NO_3^- content were positively correlated ($P < 0.001$, n = 18, r = 0.74). The yield-scaled emissions, expressed as emissions of N-N_2O per kilogram of grain yield, were similar to those of area-scaled N_2O emissions for both tillage (NT mitigated yield-scaled emissions by 57%, $P < 0.05$) and the fertiliser effect (in this case, the unfertilised control was not significantly different from INH, Table 2).

Table 2 Cumulative area-scaled and yield-scaled N_2O emissions at the end of each period (see Section 2.2) in the different treatments (see Table 1). Cumulative emissions at the end of period II are equivalent to the cumulative emissions at the end of the experimental period and are the values used to obtain the yield-scaled N_2O emissions.

Effect	N-N_2O (g ha^{-1})		Yield-scaled N_2O emissions (g N kg $grain^{-1}$)
	End of Period I	End of Period II	

Tillage			
NT	122.9 a	491.5 a	0.20 a
T	189.9 b	719.8 b	0.46 b
S.E.	12.4	53.2	0.07
***P* value**	**0.042**	**0.045**	**0.047**
Fertilizer			
N0	80.2 a	362.2 a	0.24 a
INH	115.0 b	515.8 b	0.25 a
FER	273.9 c	939.1 c	0.50 b
S.E.	15.1	40.7	0.05
***P* value**	**0.000**	**0.000**	**0.000**
Tillage x Fertilizer			
NT-N0	80.1	317.0	0.13
NT-INH	96.7	444.4	0.15
NT-FER	191.7	713.2	0.31
T-N0	80.3	407.4	0.34
T-INH	133.3	587.1	0.34
T-FER	356.1	1165.0	0.69
S.E.	21.4	66.0	0.08
***P* value**	**0.308**	**0.289**	**0.717**

Different letters within columns indicate significant differences by applying the LSD test at $P < 0.05$. The standard error (S.E., n=3) is given for each effect.

3.4 The dynamics of 16S rRNA genes, and functional genes involved in nitrification and denitrification.

Differences in the abundances of 16S rRNA genes (a proxy for the size of the total bacterial community) between sampling times were generally not significant (Fig. 2a, Table S3~~2~~), except in e.g.~~,~~ NT-FER or T-INH treatments which showed greater abundances in spring than in December or August. The T plots showed higher abundances of the 16S rRNA gene than NT for all fertiliser treatments (Figs. 2a, Table~~s~~ S3~~2~~), except in the FER treatment.

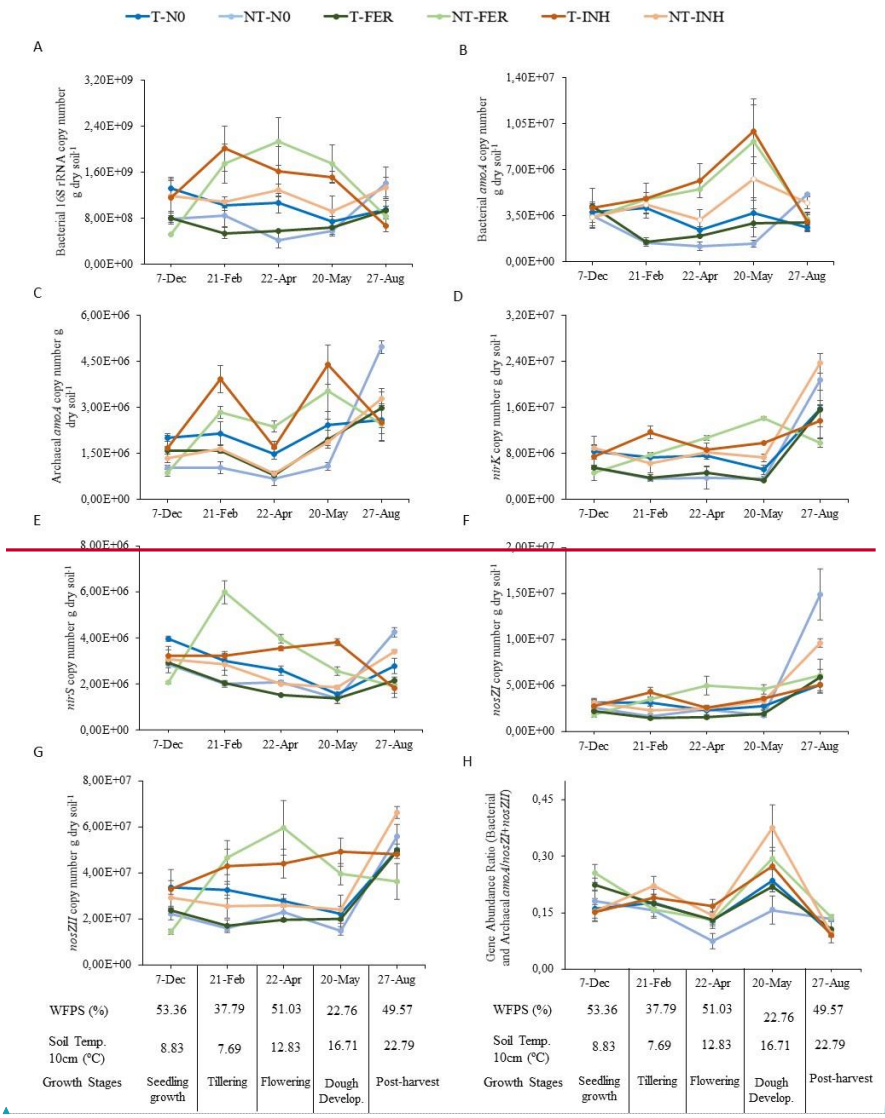
The highest abundance of the *amoA* genes from AOB and AOA was detected in May (Figs. 2b and 2c). In August, the rain causing the rewetting event increased the abundance of AOA communities (Fig. 2c, Table S3~~4~~) in all treatments except NT-FER and T-INH, and the abundance of 16S rRNA gene and AOB in NT-N0. The AOA and AOB were

more abundant in the T plots than NT for all fertiliser treatments (Figs. 2b and 2c, Tables S3~~2~~ and S4~~3~~). However, in August, they increased in the NT treatment and were significantly higher in the N0 plots (Figs. 2b and 2c, Table S4~~3~~). An effect of fertilisation was observed on AOA and AOB abundances, especially in Period II. In the NT treatments, the copy number of AOB and AOA decreased in the order FER > INH > N0 from February to May. The abundance of AOA in August was higher in the N0 plots (Fig. 2c) than in the rest of the fertiliser treatments ($P < 0.05$). For the T plots, the INH treatment significantly increased the abundances of AOB concerning N0 the following 40 days after the second fertilisation (April). The same tendency was observed for AOA abundance, although differences were significant only for the May sampling date.

The abundances of the functional genes involved in denitrification (*nirK*, *nirS*, *nosZI*, *nosZII*) were increased in the last timepoint that coincided with the heavy rainfall event (Figs. 2d-g, Tables S5 and S6), showing that the NT treatment supported a higher abundance of these in comparison with T plots (Fig. 2d-g) (in FER subplots, this was observed only for *nosZI* genes, $P > 0.05$, Table S6~~5~~). During the crop period, the tillage factor did not affect the abundance of denitrifiers, except NT-FER and T-INH. In May, the INH treatment (for the T plot) showed significantly higher abundances for all denitrifiers than N0 and FER. However, FER significantly increased the abundances of all denitrifiers in the NT treatment compared to N0 from February to May, showing intermediate values in the INH treatment.

The *amoA*/*nosZ* ratio ranged from 0.08 to 0.37%, showing the highest and lowest average values of the treatments, in May and August, respectively (Table S7~~6~~). Regarding the differences between treatments, FER resulted in the highest values of this ratio in December ($P < 0.05$, Fig. 2h, Table S7~~6~~), compared to the INH subplots. In April and

May, NT-N0 yielded the lowest values. In August, no differences were noticed except for the N0 treatment (Table S~~7~~6).



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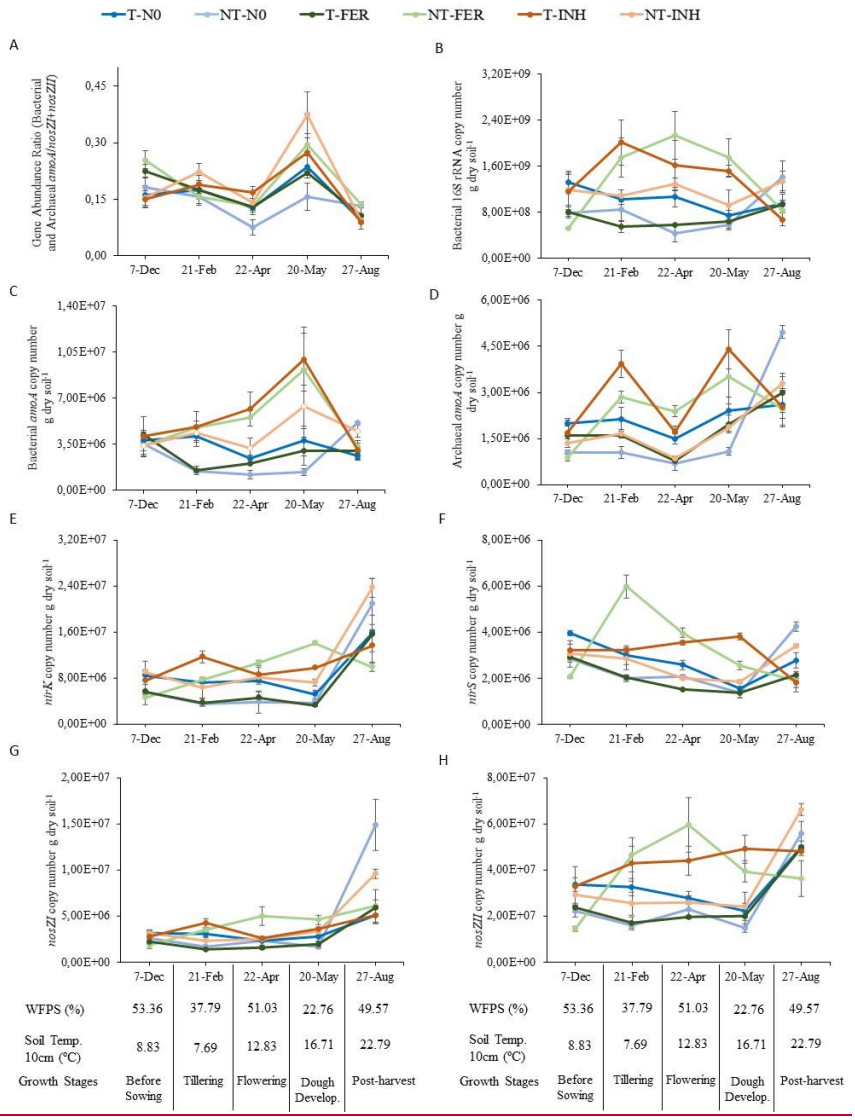

Figure. 2. Seasonal dynamics of the copy numbers of 16S rRNA (A), the *amoA* gene from AOB (B) and AOA (C), *nirK* (D), *nirS* (E), *nosZI*(F), *nosZII* (G) genes and the abundance ratio of functional genes involved nitrification to those involved in N_2O reduction (*amoA*/*nosZ* ratio) (H) for the different treatments (see Table 1). The scale of the Y-axes has been adapted in each case to improve the visualisation of the data. Error bars~~Vertical lines~~ indicate ~~the~~ standard error~~s of the mean~~. The significant differences between sampling dates and treatments are shown in Tables S3~~2~~-S7~~6~~.

3.5 Microbial community composition, diversity and indicator taxa

The ~~phylogenetic~~ structure of the microbial communities from different treatments was influenced by the sampling time and fertilisation (Fig. S3~~2~~, Table 3). Similar results were observed when time was used as a blocking variable in the permanova model, although the significance of the fertilisation effect increased (P = 0.0001) in both tillage systems (T and NT). The separation of the communities was significantly correlated with WFPS and soil NH_4^+ content, which characterised~~ing~~ samples obtained during~~from~~ the high ~~peak~~ N_2O emission peaks~~time of sampling~~ (December and April) while~~compared to~~ the rest of the samples were characterised by higher abundances of AOA and ~~the~~ *amoA*/*nosZ* ratios.

Table 3 Differences in the phylogenetic structure of microbial communities in soil samples with different treatments tested by permutational multivariate analysis of variance (PERMANOVA). *P* values are based on 1000 permutations. Significant effects are indicated in bold.

Factor	Groups	R	*P* value
Tillage	All	0.132	0.8032
Tillage	N0	0.058	0.9890
Tillage	INH	0.123	0.3387
Tillage	FER	0.088	0.7772
Fertilisation	All	**0.212**	**0.0190**
Fertilisation	T	**0.211**	**0.0159**
Fertilisation	NT	**0.211**	**0.0159**
Tillage x Fertilisation	-	0.161	0.4615
Time	All	**0.664**	**0.0009**
Treatment x Time	All	0.357	0.2208

Time: the four different sampling dates (7 December, N_2O peak in Period I;21 February, between both fertilisations; 22 April, N_2O peak in Period II; and 20 May, before harvest). For the different treatments see Table 1.

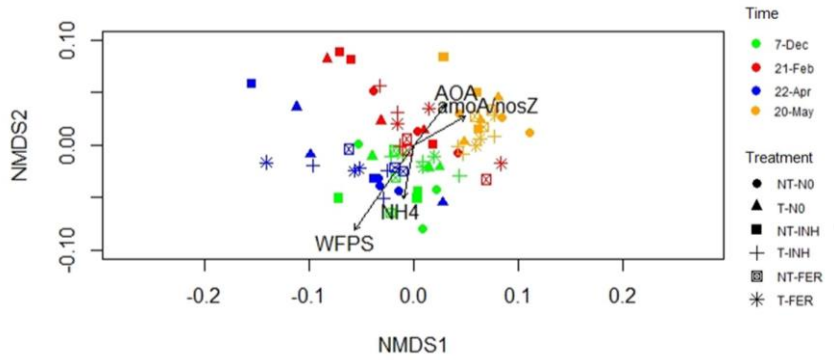

Fig. S3. ~~Phylogenetic s~~Structure of microbial communities in the soil samples from different time points and treatments. Ordination is based on nonmetric multidimensional scaling (NMDS) of generalised UniFrac distances. Significant ($P < 0.05$) correlations between ordination axes and soil abiotic and biotic variables are shown as vectors whose length is proportional to the strength of the correlation. Timepoints: 7 December, N_2O peak in Period I; 21 February, between both fertilisations; 22 April, N_2O peak in Period II; and 20 May, before harvest.

Neither tillage nor fertiliser treatments, including inhibitors, had significant effects on the diversity of the soil microbial communities across all timepoints (Fig.3a). Diversity was highest in the samples taken before the harvest (20 May) irrespective of tillage or fertiliser treatment (Fig. 3b). In addition, T (with and without fertiliser treatments) demonstrated higher diversity at the time of the first N_2O peak (7 December) compared to NT and the lowest Shannon diversity at the time of the second N_2O peak (22 April) compared to the rest of the time points within the T plots (Fig. 3b).

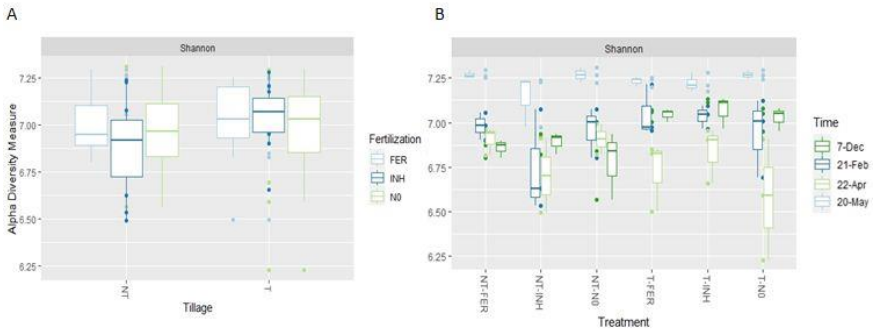

Figure. 3. Shannon's alpha diversity index. A) Bacterial diversity for the tillage management and fertiliser factors. B) Bacterial diversity for the different treatments (see Table 1) on the sampling dates: 7 December, N_2O peak in Period I; 21 February, between both fertilisations; 22 April, N_2O peak in Period II; and 20 May, before harvest.

The communities were ~~form~~dominated by the phyla *Bacteroidetes*, *Proteobacteria*, *Acidobacteria*, *Cyanobacteria*, *Gemmatimonadetes* and *Actinobacteria* (Fig. 4). Regardless of fertiliser, NT management increased the abundances of ~~the bacterial phyla~~ *Verrucomicrobia*, *Bacteroidetes*, FBP and *Cyanobacteria*. In contrast, the abundances of *Nitrospirae*, *Armatimonadetes*, *Chloroflexi* and *Firmicutes* (this one only for FER and INH) ~~were enhanc~~increased in T plots. In addition, the abundances of *Acidobacteria*, *Gemmatimonadetes* and *Planctomycetes* ~~were~~ decreased by the inhibitor amendment regardless of the tillage management (Fig. 4). *Nitrospirae* and *Acidobacteria* were identified to be negatively affected in the NT-INH treatment (Fig. 4), and were significantly enriched in *Actinobacteria*, *Verrucomicrobia* and *Proteobacteria*. Furthermore, T-FER had the highest abundance of *Firmicutes* and *Nitrospirae* and showed a higher abundance of *Actinobacteria* than NT-FER did, indicating a tillage effect on this phylum.

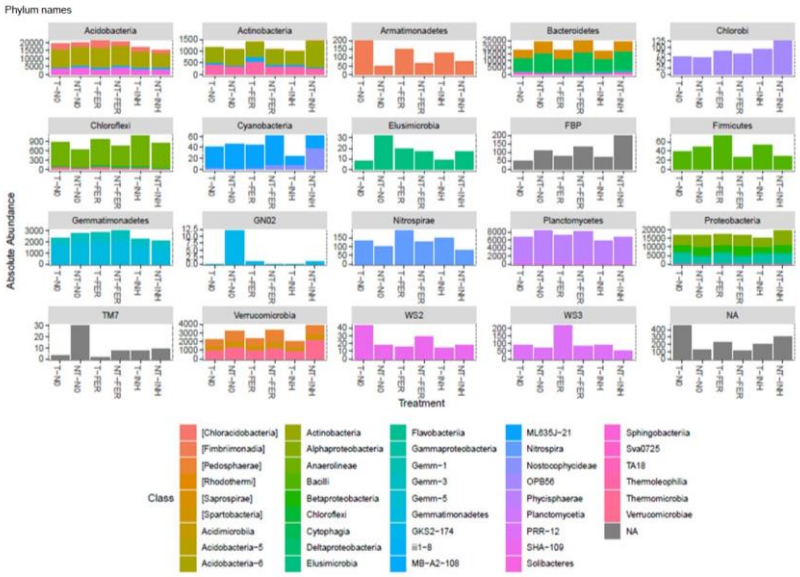

Figure. 4. Taxonomic composition of the bacterial communities in the different treatments. Relative abundance at the class level and taxa contributing more than 1% in each sample type are included.

4. Discussion

4.1 Tillage and fertilisation management effects on area-scaled and yield-scaled N_2O emissions

Avoiding or reducing basal~~seeding~~ fertilisation has been highlighted as a recommended practice to decrease N losses (Abalos et al., 2017; Xia et al., 2017), but may be necessary to prevent N immobilisation when crop residues with high or medium C:N ratios are left on the soil, particularly during the transition to no-till systems (Alijani et al., 2019). During the period following N addition at basal fertilisation~~seeding~~, N_2O fluxes would be expected to ~~increase~~ be low due to the ~~low~~ soil temperatures in late autumn (Fig. 1a). ~~The N_2O EFs after the first N application were, however, above the average EF for rainfed or cereal crops in Mediterranean areas (Cayuela et al., 2017). The lower N_2O emissions after dressing fertilisation with CAN support previous findings~~

~~under Mediterranean conditions (Guardia et al., 2018; Montoya et al., 2021b) when comparing both N sources (urea and CAN).~~

The highest N_2O fluxes for all treatments were observed immediately after a rainfall event. The magnitude of the rewetting peak depended on tillage since higher N_2O fluxes were observed in T soils with fertiliser treatments than in NT plots, particularly in T-FER compared with T-INH (Fig. 1c). This suggests that the emissions could be linked to a potential residual effect of the inhibitor and the soil N that was not efficiently taken-up by the crop (particularly in T plots) and to possible changes in soil microbiota (as explained below in Section 4.2). This high and short lived peak resulted from the pulse effect, which could be due to the reactivation of soil microbiota after several weeks during which the soil was dry (Fig. 1b) (Bergstermann et al., 2011; Leitner et al., 2017). Our results suggest that measuring N_2O emissions after the first rainfall event following long dry periods is essential, regardless of whether several weeks/months may have passed since N fertilisation or harvest, to obtain reliable emission factors~~EFs~~ in semiarid areas. This effect can be enhanced in dry years with low N uptake and/or N leaching and low crop yields, as observed in this study and in one of the two cropping seasons in Guardia et al. (2019). Our results confirmed that rainfall distribution and soil WFPS (as well as N availability) are limiting and pivotal factors driving N_2O losses in rainfed semiarid areas (Abalos et al., 2017; García- Marco et al., 2014). In the November-May period, a positive and significant correlation between N_2O fluxes and WFPS was found ($P < 0.05$, n = 38, r = 0.38). It has been suggested that these peaks are short-lived (e.g.~~,~~ Barrat et al., 2020), in agreement with what ~~as~~ we also observed in other experiment under similar soil and climatic conditions (Guardia et al., 2021). This short-lived nature must be considered for adequately capturing the pulses and for an appropriate ~~fair~~ calculations of cumulative emissions. We recommend that these rewetting pulses should be explored in detail in

future experiments under field conditions to develop specific sampling protocols for these events.

After both basal and dressing fertilisation, the cumulative N_2O emissions from the T plots were higher than those from the NT plots (Table 2). According to the recent meta-analysis of Huang et al. (2018), the NT plots generally lead to higher N_2O losses than conventional tillage, but this effect is not significant for short-term experiments or dry climates. This increase in N_2O emissions is generally associated with an enhanced denitrifying activity due to higher soil moisture and, therefore, less oxygen availability (Wang and Zou, 2020). In our case, even though higher WFPS values were reached in NT than in T plots during Period I (Fig. 1b), an opposite tendency was obtained for the N_2O emissions. We hypothesised that the main reasons for greater N_2O losses in T were: i) the higher soil disturbance after labour (i.e., Period I), thus favouring N_2O diffusion from the soil to the atmosphere in Period I, and particularly ii) the better implantation of the barley crop in NT which resulted in higher plant densities (Fig. S5) and higher yields (Table S2). This effect was also suggested by the lower NO_3^- contents in NT-N0 compared to T-N0 (Fig. S1b). Therefore, the "tillage management" effect may have been masked by the "plant density and development" effect, thus resulting in improved N acquisition by the crop and a lower amount of N available for nitrifying or denitrifying microorganisms. The potential of improving N uptake by plants (i.e., through crop breeding, selection of acquisitive species in crop mixtures or increasing plant density) as a strategy for N_2O mitigation has been suggested by several authors (e.g., Abalos et al., 2018; Bowatte et al., 2018).

In Period I the double inhibitor reduced N_2O emissions, thus supporting the findings by Guardia et al. (2018) and Montoya et al. (2021b) about the efficacy of this treatment in rainfed conditions in calcareous low organic C content soils. Our results suggest that

the potential negative impacts of applying N at ~~seeding~~ basal fertilisation can be minimised by choosing appropriate N source, such as a nitrification + urease inhibitor. Regarding the dressing application of CAN+DMPSA, our results supported those obtained by Guardia et al. (2017) or Montoya et al. (2021b). However, the effectiveness of DMPSA with CAN may be masked when environmental conditions lead to low N_2O fluxes from CAN, as those in our study were in the March-June period (Fig. 1c). In our experiment, the effectiveness of DMPSA (dressing fertilisation) and DMPSA+NBPT (basal fertilisation) was supported by the lower average NO_3^- concentrations (Fig.S1b) in the INH group than in the FER group. In this sense, during Period I, the PCA (Fig. S~~3~~4a) showed a grouping of FER (but not INH or N0) treatments close to N_2O emissions, which were positively correlated with mineral N contents (particularly $N\text{-}NO_3^-$).

As reported~~found~~ by Corrochano-Monsalve et al. (2020b) and in agreement with our hypothesis, the combination of NT and INH at the conditions of our study was the optimum choice to minimise yield-scaled N_2O emissions (Table 2), without yield penalties (Table S2~~1~~). The tendency to enhance yields and aboveground N uptake in the INH subplots compared with the FER subplots (Table S2~~1~~) agreed with the global meta-analysis of Abalos et al. (2014). This tendency of increasing (in particular) grain yield was not associated with a significant decrease in the grain N concentration, contrary to Savin et al. (2019). The higher yields obtained in NT versus T result from the improved implantation of the crop under NT, as explained above. However, in semiarid Mediterranean areas, the potential of conservation tillage for increasing crop yields and/or decreasing yield-scaled emissions in Mediterranean regions has been pointed out, particularly in dry cropping seasons (Morell et al., 2011; Plaza-Bonilla et al., 2014). On a global scale, a negative effect of NT on yield was described by Pittelkow et al. (2015), while Shakoor et al. (2021) obtained a significant increment in barley.

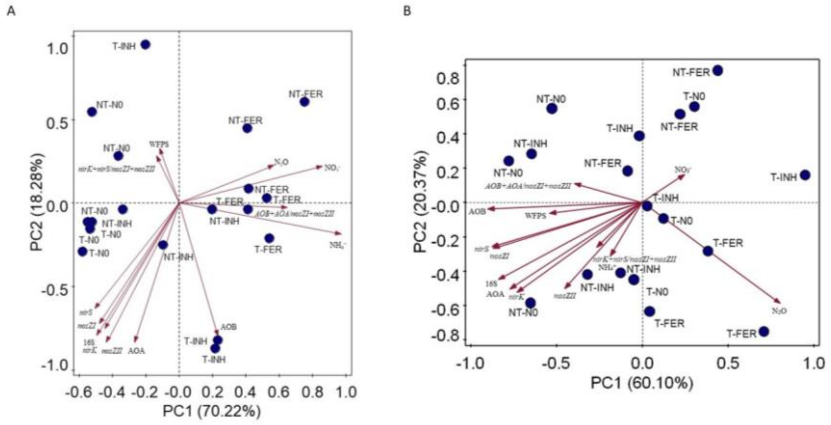

Fig. S4. Principal component analysis (PCA) of the effects of the different treatments (see Table 1) on the total abundances of targeted genes, abundance ratios, N_2O emissions and soil properties at~~in~~ the sampling occasions~~periods~~ of A) 7 December (N_2O peak in Period I) and B) 27 August (N_2O peak after rewetting). The percentage of variance in the dataset explained by Axis 1 and Axis 2 is indicated ~~in the figure~~.

Fig. S5. Pictures of the experiments were taken on 4 April in the T (A) and NT (B) plots and on 6 February in the T (C) and NT (D) plots.

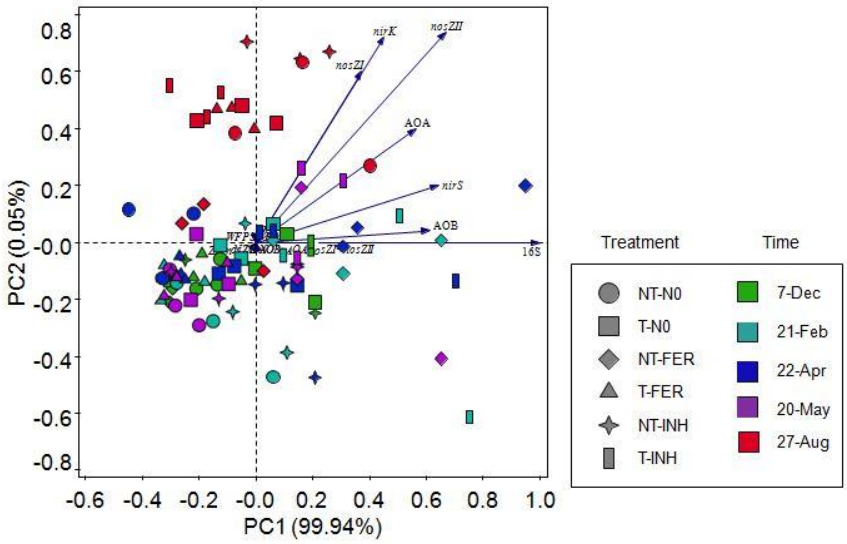

Fig. S6. Principal component analysis (PCA) of the effects of the different treatments (see Table 1) on the total abundances of targeted genes, abundance ratio, N_2O emissions and soil properties at~~in~~ five sampling ~~period~~occasions: (1) 7 December (N_2O peak in Period I), (2) 21 February (between both fertilisation events), (3) 22 April (small N_2O peak in Period II), (4) 20 May (before harvest) and (5) 27 August (N_2O peak after rewetting). The percentage of variance in the dataset explained by Axis 1 and Axis 2 is indicated ~~in the figure~~.

4.2 Tillage and fertilisation effects on microbial communities and abundances of nitrifiers and denitrifiers

As N_2O can be formed during nitrification and consumed by denitrification, the *amoA*/*nosZ* ratio (Fig. 2h) can be a useful proxy coupling the microbial genetic potential of both processes (Breuillin-Sessoms et al., 2017; Montoya et al., 2021a) and explain N_2O emissions during Period I. In December, after basal fertilisation, the FER treatment, showing ~~(which led to~~ the highest N_2O emissions,~~)~~ caused a significant increase in the *amoA*/*nosZ* ratio compared to INH. Our results were supported by Montoya et al. (2021b) in a rainfed oilseed rape crop and by Barrena et al. (2017), who suggested that the effect

of applying 3,4-dimethylpyrazole phosphate (DMPP) was explained by the increase in *nosZ* gene abundance rather than by the direct inhibition of nitrification. Torralbo et al. (2017) indicated that nitrification inhibitors (DMPP and DMPSA) decreased bacterial nitrification rates and increased *nosZ* abundance under 40% and 80% WFPS conditions~~.~~, although the mechanism for increased *nosZ* abundance is not known. The effects on bacterial diversity may also contribute to explaining our results. In this sense, the *Nitrospirae* phyluma ~~(including AOB)~~, despite showing low abundance, were favoured under FER treatment (especially in the T-FER treatment; Fig. 4) compared with~~to~~ INH. Staley et al. (2018) indicated that soils amended with urea favored nitrifying genera such as *Nitrospira* or *Nitrosospira*. Corrochano-Monsalve et al. (2020) also observed a lower abundance of *Nitrospirae* ~~phyla~~ in soils that received DMPSA inhibitor than those amended with ammonium sulfate ~~only~~ under humid rainfed conditions.

Regarding the tillage effect after basal~~seeding~~ fertilisation, the lower abundances of most key N-cycling genes in NT than T were also found by Wang et al. (2019) in a winter wheat crop. Our results also showed a tendency of~~to~~ decreased microbial~~Shannon's~~ diversity ~~of microbial communities~~ in the INH treatment combined with NT that was not observed for T. This ~~finding~~ was also reported by Corrochano-Monsalve et al. (2021, 2020), who~~ich~~ suggested that water content played an important role for~~in~~ the effect of the nitrification inhibitor. The crop residue on the surface may prevent ~~reducing the~~ water losses and may provide anaerobic microsites that could support~~be appropriate for the activity of denitrifiers~~ denitrification (Suleiman et al., 2018). ~~We also found that t~~The abundance of *Nitrospirae* ~~phyla~~ involved in ~~soil~~ nitrification was significantly higher in T (higher N_2O emissions) than in NT soils (lower N_2O emissions) throughout the cropping period (Fig. 4). The NT treatments instead resulted in a higher abundance of *Bacteroidetes*, which represent an important community of bacteria harbouring the *nosZII*

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gene (Jones et al., 2013) and are involved in soil organic matter degradation (Thomas et al., 2011). These NT treatments also showed a greater abundance of *Cyanobacteria*, which support ~~(~~several beneficial aspects in agricultural sustainability, e.g., stimulation of plant growth and protection against plant diseases~~;~~ (Singh et al., 2016), although their abundance was low, and *Verrucomicrobia* (anaerobic degradation of cellulose, Bao et al., 2019). Likewise, Navarrete et al. (2015) and Navarro-Noya et al. (2013) indicated that straw surface application caused a significant increase in *Verrucomicrobia*. Thus, ~~O~~our results suggest~~indicated~~ that conservation tillage selected for microbes specialised in degrading organic compounds, which agree with~~. This~~ finding ~~has also been~~ reported by Kielak et al. (2016) after straw surface application.

During most of Period II (except the rewetting event in August), conditions were dry, and WFPS values were more favourable for nitrification than for denitrification, even being close to the minimum threshold for the activity of nitrifying microorganisms (Pilegaard, 2013; Ussiri and Lal, 2012). This assumption, coupled with the fact that the abundances of AOB (May) and AOA, as well as the *amoA*/*nosZ* ratio, significantly increased in Period II (Figs. 2b and c), may suggest that nitrification was the main process for N_2O production during these sampling dates. In agreement ~~with this~~, the ratio between bacterial and archaeal *amoA* and all denitrifying genes was higher after dressing fertilisation (0.15 on average for the samplings in April and May) than in August (0.08) or December (0.14) (data not shown). A short-lived contribution~~relevance~~ of denitrification in Period II after rainfall or irrigation events, when small N_2O peaks were observed (Fig. 1c), could have been supported by the presence of crop residues (~~those of the previous crop, which was~~ oilseed rape) that can stimulate this process even at medium WFPS values (Li et al., 2016). The bacterial communities in soil samples ~~o~~from February

and April ~~for beta diversity~~ clustered together and were positively correlated with AOA and the *amoA*/*nosZ* ratio (Fig. S~~2~~3).

At the rewetting peak in August, most of the treatments were grouped and separated from other sampling dates (Fig, S~~6~~5), confirming that the conditions of this period could be of pivotal importance in the cumulative fluxes and the mitigation potential of the different strategies. The average mineral N contents at the rewetting episode were 0.1 and 24.4 mg N kg soil^{-1} for NH_4^+ and NO_3^-, respectively (~~data not shown~~Fig. S2), possibly indicating that this pulse was driven by denitrification rather than nitrification, as found by Bowen et al. (2020) and Vázquez et al. (2020). This hypothesis was also supported by the grouping of August values close to some key denitrifying genes (e.g., *nosZI*, *nosZII*, and *nirK*, Fig. S~~6~~5), or by the lower values of the *amoA*/*nosZ* ratio (Fig. 2h), in comparison with the values in December. The rewetting event caused a significant increase in the abundances of denitrifiers~~ying communities~~ (*nirK*, *nosZI* and *nosZII*) compared to those in December and April, and the opposite tendency occurred for the AOB abundance. The abundance of AOA also increased, probably due to the low NH_4^+ contents after summer (Hink et al., 2017) and the proposed greater tolerance of ~~archaeal communities (in comparison with nitrifying bacteria)~~AOA to high temperatures (Duan et al., 2018) or other stress factors, ~~as reported by, e.g.,~~ (He et al., ~~(~~2018~~);~~ ~~and~~ Tao et al., ~~(~~2021). A significant effect was observed for the tillage management after rewetting. Nontilled plots (lower N_2O emissions) resulted in a higher abundance of nitrifi~~yersing communities~~ than T soils, suggesting that nitrification was less important in this pulse. In addition, our results showed higher abundances of the 16S rRNA gene, *nirS*, *nosZI* and *nosZII* in NT concerning T for those of N0 and INH (Fig. 2). During the ~~August~~ N_2O pulse in August, the abundance of N cycling~~key~~ genes was not a good indicator of the amount of N_2O released from~~or~~ the different treatments. This could be due to the DNA-

based approach ~~Our methodology~~ to quantify~~estimate the size of~~ nitrifying and denitrifying ~~communiti~~genes, which cannot discriminate between living or dead microbiota. ~~(t~~This ~~effect~~ could be relevant under conditions of severe drought lasting several weeks~~)~~. Due to the ~~relevan~~importance of the~~se~~ postharvest rewetting peaks in semiarid areas, further research should be consider also ~~combining the DNA-~~RNA-based quantification ~~approach should be performed to obtain detailed information on the spatiotemporal evolution of N in the soil~~. In addition, the relationships between the diversity of microbial communities and N_2O emissions during the critical rewetting pulses should be explored ~~and~~ in future experiments. ~~its relation to microbial communities and N_2O emissions.~~

5. Conclusions

The highest N_2O pulse occurred after a rewetting episode after several weeks of dry conditions and after barley harvesting, driven by a significant increase in the abundances of AOA and ~~some~~ key genes involved in denitrification. N_2O emissions after applying N at ~~seeding~~ basal fertilization were not negligible, particularly in T plots (despite the low temperatures of late autumn), and using the double inhibitor (DMPSA+NBPT) with urea can effectively mitigate these losses due to a possible stimulation of nitrous oxide reducing microorganism~~the *nosZI* and *nosZII* genes~~. This effective N_2O mitigation should be considered in addition to well-known side effects regarding the mitigation of other gaseous N ~~oxide~~ losses, particularly NH_3 volatilisation, due to the use of the urease inhibitor NBPT. The application of fertilisers with inhibitor (INH) caused a decrease in the abundances of the phylum *Nitrospirae* ~~phyla~~ during the cropping period, thus supporting its well-known potential to decrease N_2O emissions when conditions are favourable for nitrification. It was observed that ~~the~~ tillage ~~factor~~ may have less influence

than fertiliser management on the composition of soil microbial communities~~beta diversity~~. However, NT management may have contributed to regulating the intensity of soil N_2O emissions, possibly~~maybe~~ due to a reduction in the abundance of *Nitrospirae* ~~bacteria phyla~~ and a higher abundance of *Bacteroidetes*, ~~(~~which harbours the non-denitrifying N_2O reducers. ~~*nosZII* gene, thus possibly stimulating complete denitrification), *Verrucomicrobia* and *Cyanobacteria* phyla~~. These results demonstrate that changes in the N-cycling microbiota can be observed even during~~in~~ short-term changes in NT management and should be further explored under semiarid conditions. We also recommend exploring the potential effect of N management (including the use of enhanced-efficiency fertilisers) and proper crop density and development on optimising the plant uptake of N for~~and~~ reducing reactive N losses.

Acknowledgements

This research was funded by Agencia Estatal de Investigación (AEI)-Ministerio de Ciencia, Innovación y Competitividad and Fondo Europeo de Desarrollo Regional (RTI2018-096267-B-I00 MCIU/AEI/FEDER, UE), the Comunidad de Madrid (Spain) and Structural Funds 2014–2020 (ERDF and ESF) (project AGRISOST-CM S2018/BAA-4330). Montoya is the recipient of the FPI grant BES-2016-076712. García-Gutiérrez is the recipient of the Comunidad de Madrid contract number PEJD-2018-PRE/BIO-877. Funding for this research was also provided by EuroChemAgro GmbH. The funders had no role in the data collection and analysis or manuscript preparation. Sequencing was performed by the SNP&SEQ Technology Platform in Uppsala. The facility is part of the National Genomics Infrastructure (NGI) Sweden and Science for Life Laboratory. The SNP&SEQ Platform is also supported by the Swedish Research Council and the Knut and Alice Wallenberg Foundation. Special thanks are given to the field assistants working with us at Centro Nacional de Tecnología de Regadíos

(CENTER), particularly to Alejandro Sánchez de Ribera. We also thank the technicians and researchers Guadalupe Álvarez, Rocío Rodríguez, Eduardo Aguilera, Alberto Sanz and Celia Ginés at the Department of Chemistry and Food Technology of the ETSIAAB. This work was done within the framework of the Moncloa Campus of International Excellence (UCM-UPM).

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Table S1 Primers, amplification efficiency and thermal cycling conditions for quantification of marker genes

Primer names	Sequences (5'-3')	Final primer concentration (μM)	Amplification efficiency (%)	Thermal cycling protocol[1]
16S rRNA				
515F	GTGYCAGCMGCCGCGGTAA	0.50	85.5	(95°C, 15 s; 50°C, 30 s; 72°C, 45 s; 80°C, 5 s) x 35
926R	CCGYCAATTYMTTTRAGTTT			
AOB				
amoA-1F	GGGGTTTCTACTGGTGGT	0.50	80.7	(95°C, 15 s; 55°C, 30 s; 72°C, 40 s; 77°C, 5 s) x 40
amoA-2R	CCCCTCKGSAAAGCCTTCTTC			
AOA				
CrenamoA23f	ATGGTCTGGCTWAGACG	0.50	84.4	(95°C, 15 s; 55°C, 30 s; 72°C, 40 s; 77°C, 5 s) x 40
CrenamoA616R	GCCATCCATCTGTATGTCCA			
nirK				
F1aCu	ATCATGGTSCTGCCGCG	0.80	84.5	(95°C, 15 s;(63°C, -1°/cycle), 30 s; 72°C, 35 s) x 6 (95°C, 15 s; 58°C, 30 s; 72°C, 35 s; 80°C, 5 s) x 35
1040R	GCCTCGATCAGRTTRTGGTT			
nirS				
cd3aFm	AACGYSAAGGARACSGG	0.80	76.7	(95°C, 15 s;(65°C, -1°/cycle), 30 s; 72°C, 30 s) x 6 (95°C, 15 s; 60°C, 30 s; 72°C, 30 s; 80°C, 5 s) x 35
R3cdm	GASTTCGGRTGSGTCTTSAYGAA			
nosZI				
1840F	CGCRACGGCAASAAGGTSMSSGT	0.80	76.6	(95°C, 15 s;(65°C, -1°/cycle), 30 s; 72°C, 30 s) x 6 (95°C, 15 s; 60°C, 30 s; 72°C, 30 s; 80°C, 5 s) x 35
2090R	CAKRTGCAKSGCRTGGCAGAA			
nosZII				
nosZII-F	CTIGGICCIYTKCAYAC	2.0	75.7	(95°C, 15 s; 54°C, 30 s; 72°C, 45 s; 77°C, 5 s) x 40
nosZII-R	GCIGARCARAAITCBGTRC			

[1] All protocols started with 5 min at 95°C. All protocols ended with a melt curve: (95°C, 15 s;(60 to 95° C, 10 s, increment 0.5°)).

Table S2 Grain and biomass yield, N concentration in grain and biomass and aboveground N uptake in the different treatments (see Table 1).

Effect	Grain yield (kg ha^{-1})	Biomass yield (kg ha^{-1})	Grain N content (%)	Biomass N content (%)	Aboveground N uptake (kg N ha^{-1})
Tillage					
NT	2552	3534	2.44	1.05	99.5
T	1596	2141	2.51	1.21	66.4
S.E.	85	111	0.01	0.04	4.3
***P* value**	**0.072**	**0.053**	**0.066**	**0.098**	**0.101**
Fertilizer					
N0	1787 a	2424 a	2.45	1.04	67.7 a
INH	2337 b	3179 b	2.47	1.16	93.7 b
FER	2099 ab	2911 b	2.51	1.18	87.4 b
S.E.	104	135	0.01	0.04	5.3
***P* value**	**0.017**	**0.012**	**0.126**	**0.096**	**0.020**
Tillage x Fertilizer					
NT-N0	2374	3358	2.39	0.93	88.0
NT-INH	2895	3922	2.41	1.03	110.9
NT-FER	2388	3321	2.50	1.18	99.7
T-N0	1199	1489	2.51	1.14	47.4
T-INH	1780	2436	2.53	1.30	76.6
T-FER	1809	2500	2.52	1.17	75.2
S.E.	147	191	0.03	0.06	7.5
***P* value**	**0.144**	**0.068**	**0.120**	**0.133**	**0.578**

Different letters within columns indicate significant differences by applying the LSD test at $P < 0.05$. Standard Error (S.E., n=3) is given for each effect.

Table S3 Average copy numbers of the 16S rRNA gene for the different treatments (see Table 1) and sampling dates. Different uppercase letters indicate significant differences between sampling dates within a "Tillage x Fertilizer" treatment. Different lowercase letters denote significant differences between "Tillage x Fertilizer" treatments within each sampling date. Standard errors of the mean (SE) for sampling date and treatment effects are given.

Treatment	**16S rRNA**					
	7th December[1]	21st February[1]	22nd April[1]	20th May[1]	27th August[1]	SE date
NT-N0	7.87 Aab	8.47 Aa	4.23 Aa	5.73 Aa	14.10 Bb	1.68
T-N0	13.00 Cb	10.20 A-Ca	10.60 BCb	7.43 ABa	7.01 Aa	1.10
NT-FER	5.10 Aa	17.70 Cbc	21.70 Cb	17.30 BC b	8.23 ABa	2.96
T-FER	7.93 Aab	5.40 Aa	5.77 Aab	6.30 Aa	7.85 Aa	0.94
NT-INH	11.70 Ab	10.80 Aab	13.00 Ab	9.13 Aa	13.30 Ab	2.03
T-INH	11.60 ABb	20.00 Bc	16.00 ABb	15.00 ABb	7.24 Aa	3.08
SE treatment	1.94	2.36	2.71	1.91	1.54	-

[1] $x10^8$ copies / g soil dw

Table S4 Average copy numbers of the *amoA* gene from ammonium oxidising bacteria (AOB) and ammonium oxidising archaea (AOA)for the different treatments (see Table 1) and sampling dates. Different uppercase letters indicate significant differences between sampling dates within a "Tillage x Fertilizer" treatment. Different lowercase letters denote significant differences between "Tillage x Fertilizer" treatments within each sampling date. Standard errors of the mean (SE) for sampling date and treatment effects are given.

Treatment	AOB						AOA					
	7th December[1]	21st February[1]	22nd April[1]	20th May[1]	27th August[1]	SE date	7th December[1]	21st February[1]	22nd April[1]	20th May[1]	27th August[1]	SE date
NT-N0	3.53 Ba	1.47Aa	1.16Aa	1.38Aa	5.12Cc	0.46	1.04Aab	1.03Aa	0.68Aa	1.08Aa	4.96Bb	0.17
T-N0	3.77Aa	4.13Ab	2.40Ab	3.73Aa	2.63Aa	0.67	2.00ABc	2.13ABbc	1.47 Ab	2.40ABbc	2.60 Ba	0.34
NT-FER	3.40Aa	4.70 Ab	5.53ABc	9.33Bb	3.29Aab	1.38	0.89Aa	2.8Bc	2.40Bc	3.53Bcd	2.46 Ba	0.47
T-FER	4.23 Ba	1.50Aa	2.00 Ab	2.97ABa	2.99ABa	0.58	1.60Bbc	1.60Bab	0.78Aa	1.93BCb	2.99 Ca	0.32
NT-INH	3.37ABa	4.33ABb	3.17 Ab	6.33Bab	4.47ABbc	0. 91	1.37Babc	1.67BCab	0.85Aa	1.87Cb	3.30 Da	0.14
T-INH	4.07Aa	4.80 Ab	6.17EABc	9.83Bb	3.05Aab	0. 15	1.69Abc	3.93BCd	1.73 Ab	4.40 Cd	2.51ABa	0.50
SE treatment	0.82	0.67	0.65	1.80	0.47	-	0.21	2.70E+05	0.16	0.50	0.48	-

[1] $x10^6$ copies / g soil dw

Table S5 Average copy numbers of the *nirK* and *nirS* genes for the different treatments (see Table 1) and sampling dates. Different uppercase letters indicate significant differences between sampling dates within a "Tillage x Fertilizer" treatment. Different lowercase letters denote significant differences between "Tillage x Fertilizer" treatments within each sampling date. Standard errors of the mean (SE) for sampling date and treatment effects are given.

Treatment	***nirK***						***nirS***					
	7th December[1]	21st February[1]	22nd April[1]	20th May[1]	27th August[1]	SE date	7th December[1]	21st February[1]	22nd April[1]	20th May[1]	27th August[1]	SE date
NT-N0	5.60Aab	3.53Aa	3.83Aa	3.70Aa	20.80Bbc	2.21	2.87Bab	2.00Aa	2.07 Ab	1.37Aa	4.24 Cd	0. 22
T-N0	8.37Bb	7.30ABb	7.60ABbc	5.17 Ab	15.80Cabc	1.58	3.97Cc	3.00Bc	2.57Bc	1.53Aa	2.78Bbc	0.25
NT-FER	4.60Aa	7.77Bbc	11.00Cc	14.30 De	9.90BCa	0.75	2.10Aa	6.00 Cd	3.97 Be	2.57 Ab	1.89Aa	0.29
T-FER	5.60Bab	3.77Aa	4.67ABab	3.23Aa	15.50 Ca-c	0.95	2.93Cab	2.03Bab	1.50Aa	1.39Aa	2.15Bab	0.14
NT-INH	9.00 Ab	6.37 Ab	8.20 Ac	7.27 Ac	23.70Bc	1.46	3.10Cbc	2.83BCbc	2.00ABb	1.87Aa	3.43Cc	0.28
T-INH	7.50Aab	11.70ABc	8.50ABc	9.80ABd	13.60Bab	1.74	3.20Bbc	3.20Bc	3.57Bd	3.83Bc	1.80Aa	0.26
SE treatment	1.34	0.97	1.11	0.47	2.73	-	0.28	0.33	0.13	0.17	0.26	-

[1] $x10^6$ copies / g soil dw

Table S6 Average copy numbers of the *nosZI* and *nosZII* genes for the different treatments (see Table 1) and sampling dates. Different uppercase letters indicate significant differences between sampling dates within a "Tillage x Fertilizer" treatment. Different lowercase letters denote significant differences between "Tillage x Fertilizer" treatments within each sampling date. Standard errors of the mean (SE) for sampling date and treatment effects are given.

Treatment	***nosZI***						***nosZII***					
	7th December[1]	21st February[1]	22nd April[1]	20th May[1]	27th August[1]	SE date	7th December[1]	21st February[1]	22nd April[1]	20th May[1]	27th August[1]	SE date
NT-N0	2.60Aab	1.67Aab	2.37Aab	1.73Aa	14.90Bc	0.13	22.3 Ab	16.0Aa	23.0Aa	14.7Aa	56.0Bb	3.30
T-N0	3.17 Ab	3.07Acd	2.33Aab	2.80Aab	5.11 Ba	0.54	34.0Bb	32.7Bbc	28.0ABa	22.3Aa	50.2Cb	3.03
NT-FER	1.87Aa	3.50Bde	5.03Bc	4.63Bc	6.14Bab	0.94	15.0Aa	47.0Cc	59.7Cb	39.7BCb	28.8 Ba	6.67
T-FER	2.23Aab	1.43Aa	1.57Aa	1.97Aa	5.91Bab	0.45	23.7 Ab	17.0Aa	19.7Aa	19.7Aa	49.8Bb	2.48
NT-INH	3.17 Ab	2.33Abc	2.53 Ab	3.37 Ab	9.62Bbc	0.38	29.0 Ab	25.3Aab	26.0Aa	24.0Aa	66.3Bc	4.33
T-INH	2.80Aab	4.33ABe	2.63 Ab	3.60ABbc	5.10 Ba	0.53	33.0Ab	42.7Ac	44.0Ab	49.0Ab	48.1Ab	6.54
SE treatment	0.40	0.31	0.48	0.38	1.50	-	4.29	5.18	5.99	4.62	2.78	-

[1] $x10^6$ copies / g soil dw

Table S7 Average values of the (AOB+AOA)/(*nosZI*+*nosZII*) ratio for the different treatments (see Table 1) and sampling dates. Different uppercase letters indicate significant differences between sampling dates within a "Tillage x Fertilizer" treatment. Different lowercase letters denote significant differences between "Tillage x Fertilizer" treatments within each sampling date. Standard errors of the mean (SE) for sampling date and treatment effects are given.

Treatment	**(AOB+AOA)/(*nosZI*+*nosZII*) ratio**					
	7th December	21st February	22nd April	20th May	27th August	SE date
NT-N0	0.18 Bab	0.16 Ba	0.08 Aa	0.16 Ba	0.13 ABb	0.02
T-N0	0.16 Ba	0.17 BCa	0.13 ABab	0.24 Cab	0.09 Aa	0.02
NT-FER	0.25BCc	0.16ABa	0.13 Ab	0.29 Cab	0.14 Ab	0.04
T-FER	0.22 Cab	0.17BCa	0.13ABab	0.22 Ca	0.11Aab	0.02
NT-INH	0.16ABa	0.22 Ba	0.14ABb	0.37Cb	0.09Aa	0.03
T-INH	0.15ABa	0.19BCa	0.17ABb	0.28Cab	0.09Aa	0.03
SE treatment	0.02	0.02	0.02	0.05	0.01	-

Figure 1

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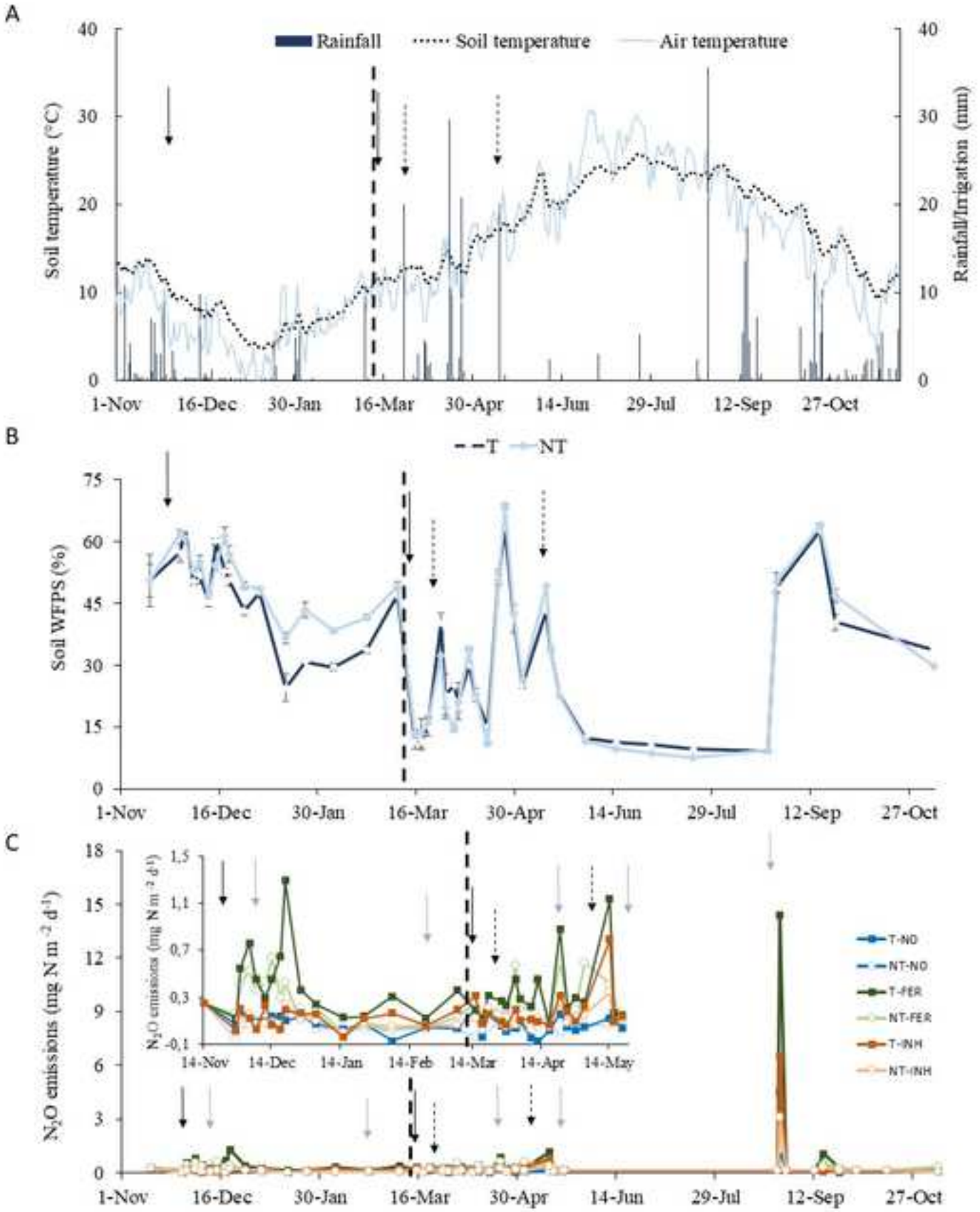

Figure 2

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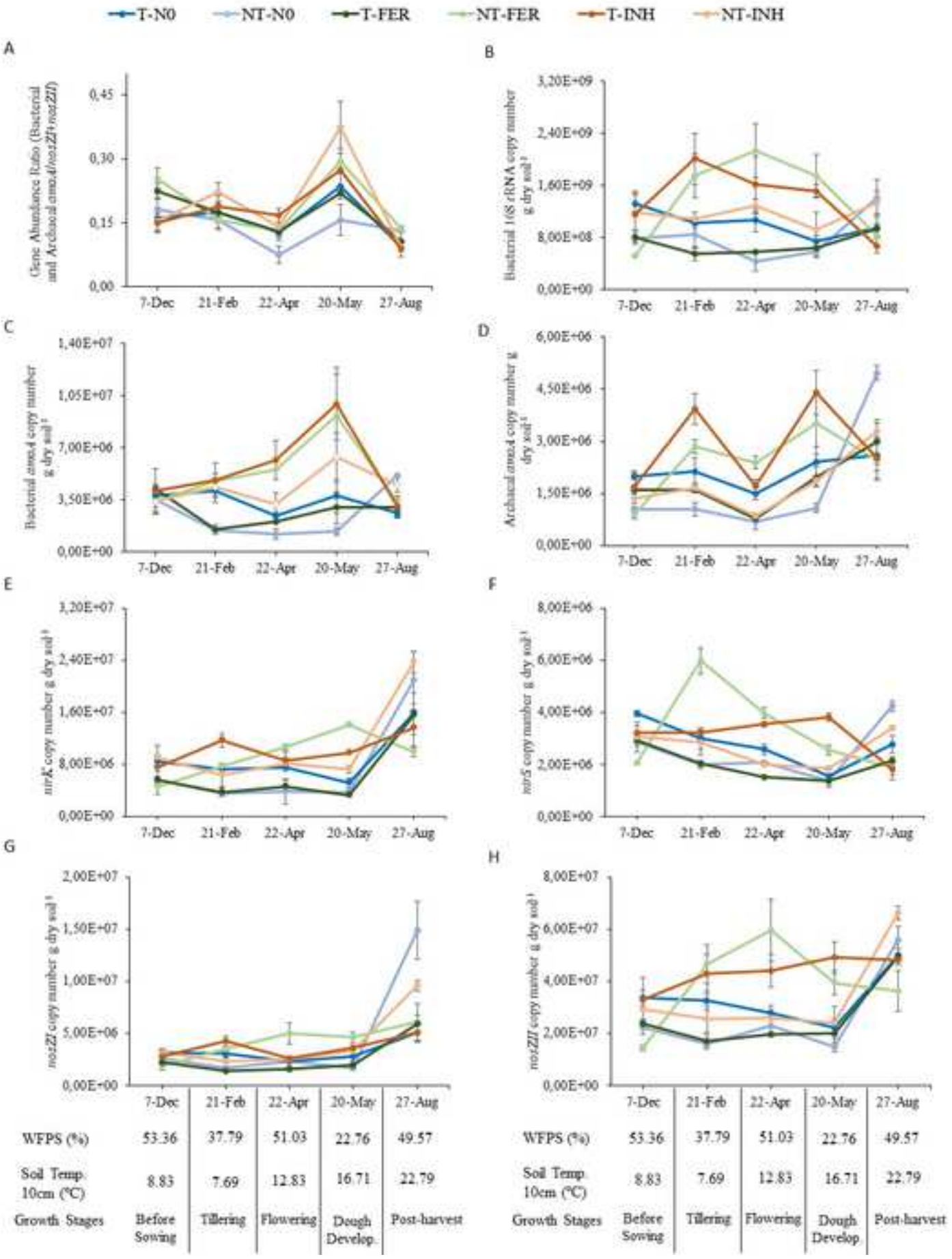

Figure 3

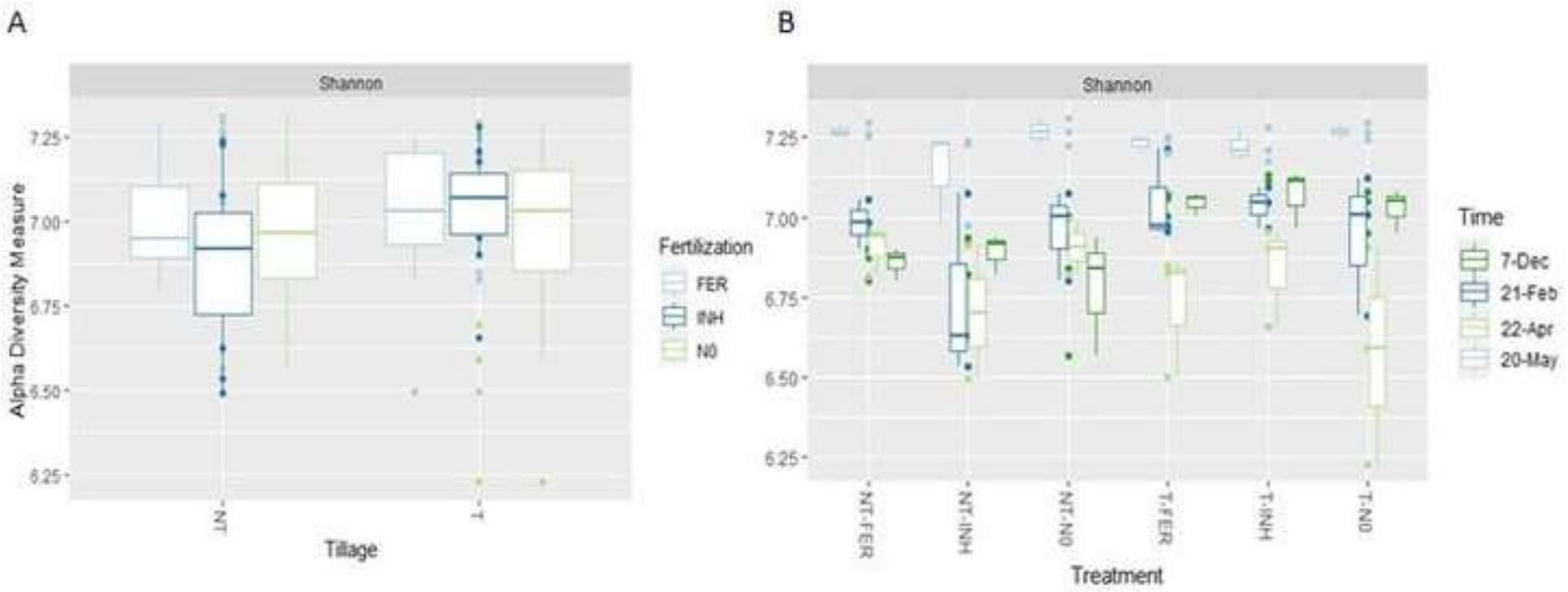

Figure 4

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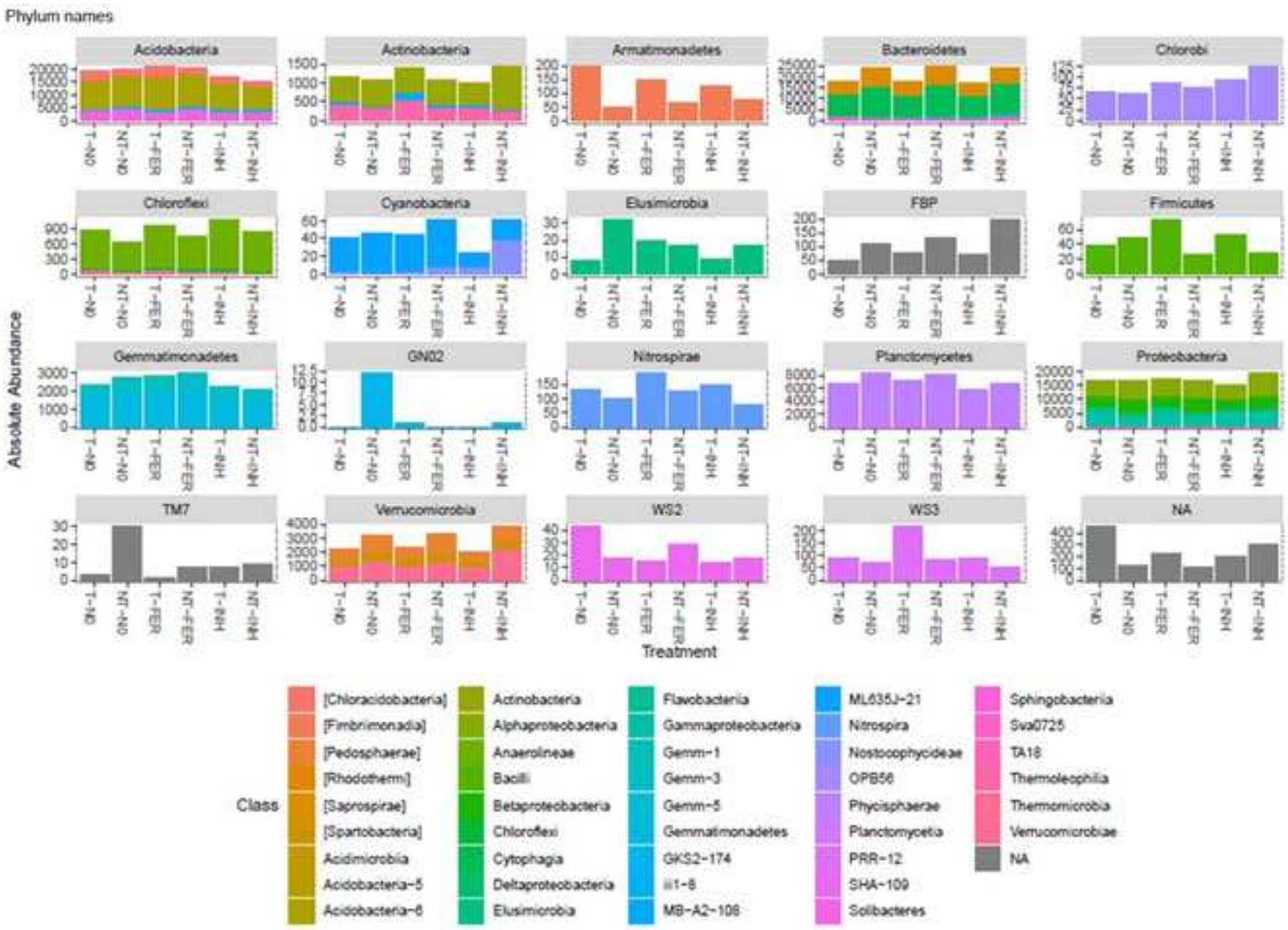

Fig. S3

Fig. S4

Fig. S5

Fig. S6

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: