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Dimethylpyrazole-based nitrification inhibitors have a dual role in N_2O emissions mitigation in forage systems under Atlantic climate conditions

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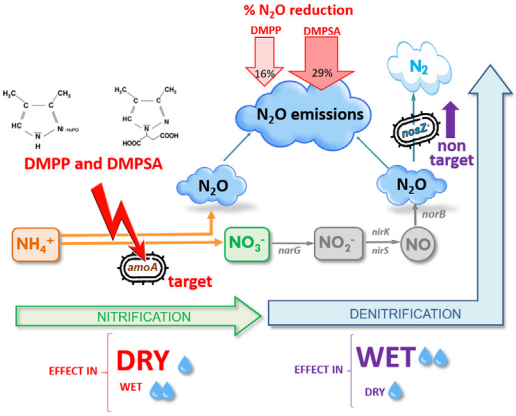
HIGHLIGHTS

- DMPP and DMPSA efficiency reducing N_2O emissions tested in a temperate grassland was 19% and 29%.
- NIs avoided the stimulation of nitrifying bacteria induced by fertilization.
- NIs promoted denitrifying bacteria reducing N_2O up to N_2 under soil high water and low nitrate conditions.
- DMPSA is a more promising NI mitigating N_2O emissions and increasing ryegrass yield.

GRAPHICAL ABSTRACT



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ABSTRACT

Nitrogen fertilization is the most important factor increasing nitrous oxide (N_2O) emissions from agriculture, which is a powerful greenhouse gas. These emissions are mainly produced by the soil microbial processes of nitrification and denitrification, and the application of nitrification inhibitors (NIs) together with an ammonium-based fertilizer has been proved as an efficient way to decrease them. In this work the NIs dimethylpyrazole phosphate (DMPP) and dimethylpyrazole succinic acid (DMPSA) were evaluated in a temperate grassland under environmental changing field conditions in terms of their efficiency reducing N_2O emissions and their effect on the amount of nitrifying and denitrifying bacterial populations responsible of these emissions. The stimulation of nitrifying bacteria induced by the application of ammonium sulphate as fertilizer was efficiently avoided by the application of both DMPP and DMPSA whatever the soil water content. The denitrifying bacteria population capable of reducing N_2O up to N_2 was also enhanced by both NIs provided that sufficiently high soil water conditions and low nitrate content were occurring. Therefore, both NIs showed the capacity to promote the denitrification process up to N_2 as a mechanism to mitigate N_2O emissions. DMPSA proved to be a promising NI, since it showed a more significant effect than DMPP in decreasing N_2O emissions and increasing ryegrass yield.

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1. Introduction

Grasslands, including permanent meadows and pastures, occupy 37% of the global terrestrial area and 69% of the available agricultural land (O'Mara, 2012). Nitrogen fertilization of grasslands has relevant productive and environmental consequences, with major effects on the nutritive value of forage (Lee, 2018). In intensive managed grasslands, fertilizer application can range from 200 up to 600 kg N ha^{-1} yr^{-1} (Galloway et al., 2009), but only around 50% of the N applied is assimilated by plants and enter into the animal system (Tilman et al., 2002; Robertson and Vitousek, 2009). The remaining 50% is lost to the environment in reactive N forms (NO_3^-, N_2O and NO) (Galloway et al., 2009). The agricultural sector is responsible of about 25% of total anthropogenic greenhouse-gases (GHG) emissions, having N_2O approximately 265 times the global warming potential of carbon dioxide (CO_2) on a mass basis (IPCC, 2014). Furthermore, it is expected that N_2O emissions from agriculture will account for 59% of global N_2O emission in 2030 (Reay et al., 2012). Additionally, N_2O is not only involved in the global warming effect, but also contributes to the depletion of stratospheric ozone layer (IPCC, 2014; Ravishankara et al., 2009).

N_2O is mainly produced through the microbial processes of autotrophic nitrification and heterotrophic denitrification in soils (Wrage et al., 2001; Philippot et al., 2011). In the nitrification process ammonium (NH_4^+) is oxidized to nitrate (NO_3^-) under aerobic conditions through ammonia monooxygenase (AMO), hydroxylamine oxidoreductase (HAO) and nitrite oxidoreductase (NXR) enzymes. The resulting nitrate can be subsequently denitrified under anaerobic conditions by nitrate reductase (encoded by *narG*/*napA* genes), nitrite reductase (encoded by *nirS*/*nirK* genes), nitric oxide reductase (encoded by *norB* and *norC* genes) (Richardson et al., 2001) and nitrous oxide reductase (encoded by *nosZ* gene, clades I and II), this last one being present in two-thirds of all denitrifiers (Jones et al., 2012). The predominance of nitrification or denitrification is closely related to soil conditions such as texture, pH, temperature, water availability, aeration, carbon availability or microbial activity. Under most soil conditions, both nitrification and denitrification occur simultaneously, although many studies suggest that denitrification is the dominant process responsible for the increase in atmospheric N_2O (Jia et al., 2013; Ji et al., 2015).

Given that the application of fertilizer is predicted to increase by 35 to 60% before 2030 (IPCC, 2014), it is mandatory to develop effective management strategies to mitigate the environmental impact and the economic loss derived from the use of N in agroecosystems. In this sense, the use of ammonium-based fertilizers accompanied by nitrification inhibitors has been proposed by the Intergovernmental Panel on Climate Change (IPCC, 2007) as a tool to reduce N losses from agriculture, therefore increasing N use efficiency. NIs are defined as compounds capable of retarding ammonia oxidation through the deactivation of AMO (Hatch et al., 2005; Chaves et al., 2006). 3,4-dimethylpyrazole phosphate (DMPP) is one of the most widely used NI. Its ability mitigating N_2O emissions has been reported in wheat (Huérfano et al., 2015; Huérfano et al., 2016; Guardia et al., 2019), maize (Huérfano et al., 2018; Guardia et al., 2017), pasture (Macadam et al., 2003; Di and Cameron, 2012; Huérfano et al., 2019) or vegetable production systems (Xu et al., 2005; Huérfano et al., 2016). In addition to the ability to mitigate N_2O emissions, several authors (Abalos et al., 2014; Huérfano et al., 2015; Linquist et al., 2013; Huérfano et al., 2018; Guardia et al., 2019) have confirmed that DMPP does not result in negative effects on yield and/or quality of different crops. Another dimethylpyrazole-based NI is the isomeric mixture of 2-(3,4-dimethyl-1H-pyrazol-1-yl) succinic acid and 2-(4,5-dimethyl-1H-pyrazol-1-yl) succinic acid (DMPSA) (CA 2933591 A1 2015/06/18 Patent), which has been developed to be combined with any mineral fertilizer due to its non-polarity (Pacholski et al., 2016). DMPSA has also been tested in wheat crops (Huérfano et al., 2016; Guardia et al., 2019; Recio et al., 2018; Recio et al., 2019; Corrochano-Monsalve et al., 2020; Herr et al., 2020), irrigated maize (Guardia et al., 2017) and forage systems (Huérfano et al., 2018 and 2019), showing efficiencies mitigating fertilizer-derived N_2O in a wide range between 0% and 86%. To our knowledge, only Huérfano et al. (2016 and 2018) have compared both DMPP and DMPSA in the same field experiment, obtaining similar results for both NIs, with a mitigation of N_2O emissions ranging from 33% to 86%.

Regarding the effect of dimethylpyrazole-based NIs on soil microbial community, no negative effect on non-target soil microorganisms (i.e. non-nitrifier microorganisms) has been reported in either laboratory (Kong et al., 2016; Barrena et al., 2017; Torralbo et al., 2017; Fuertes-Mendizábal et al., 2019; Bozal et al., 2021) or field (Montoya et al., 2021; Corrochano-Monsalve et al., 2020) conditions. By the contrary, several works have described a decrease in AOB abundance due to DMPP application (Di and Cameron, 2011; Chen et al., 2015; Duan et al., 2017, Barrena et al., 2017, Shi et al., 2017) and to DMPSA application (Torralbo et al., 2017; Corrochano-Monsalve et al., 2020). On the other hand, dimethylpyrazole-based NIs exert their effect not only on nitrification rates, by blocking the AMO enzyme, but also on denitrification rates, by reducing the nitrate available for denitrifiers (Florio et al., 2014; Menéndez et al., 2012). Moreover, recent studies under both field and laboratory conditions have shown an effect of dimethylpyrazole-based NIs presumably also stimulating the last step of the denitrification process that consists in the reduction of N_2O to N_2, which would decrease N_2O emissions. Thus, the increasing of *nosZ* gene bearing bacterial populations has been reported after the application of DMPP (Barrena et al., 2017; Torralbo et al., 2017) and DMPSA (Torralbo et al., 2017, Corrochano-Monsalve et al., 2020). However, this non-target effect on denitrifiers has not been monitored under changing field environmental conditions comparing simultaneously both kinds of dimethylpyrazole-based NIs. The Atlantic climate conditions of the coast of the Basque Country, although characterized by high precipitation rates during the whole year (amounts up to almost 1200 mm yr^{-1}), offer a scenario with a short-term relative variability in soil water content, which will presumably influence nitrifier and denitrifier populations. Since previous laboratory incubations studies have demonstrated the influence of water availability and temperature on NIs efficiency (Menéndez et al., 2012; Barrena et al., 2017; Torralbo et al., 2017; Fuertes-Mendizábal et al., 2019), our climate conditions, with soil water filled pore space (WFPS) values ranging from almost 100% down to 50% in the winter-spring period, provide the possibility to compare the efficiency of DMPP and DMPSA under changing soil water availability. In this scenario, the main objectives of this work were to study the efficiency of DMPSA compared to DMPP reducing N_2O emissions in a temperate grassland, as well as their effect on nitrifying and denitrifying bacterial populations responsible of N_2O emissions under environmental changing field conditions. The effect of both NIs on ryegrass yield and quality were also addressed.

2. Materials and methods

The work was conducted in an intensive grassland under Atlantic climate conditions in the north of Spain (43°17′22"N, 2°52′20"W) from September 2016 to May 2017. Daily precipitation and temperature during the assayed period are detailed in Fig. 1, where the irregular distribution of rainfall during the experiment can be appreciated. The texture of the top soil (0–30 cm) was silt loam, composed of 33% sand, 52% silt and 15% clay. The pH (1:2.5 H_2O) was 5.8, the soil organic matter content was 1.8% and the C/N ratio was 7.96. The experimental plots of 28m^2 (7 m × 4 m) were part of a forage maize-ryegrass crops rotation system where the same random distribution of fertilizer treatments (with or without NIs) with four replications was maintained along the time during the consecutive crops. Therefore, maize (*Zea mays* L. var. CisKo) was the previous crop for the present study, which had been sown in spring 2016, had received 80 and 100 kg N ha^{-1} in May and June 2016, respectively, and was harvested in September

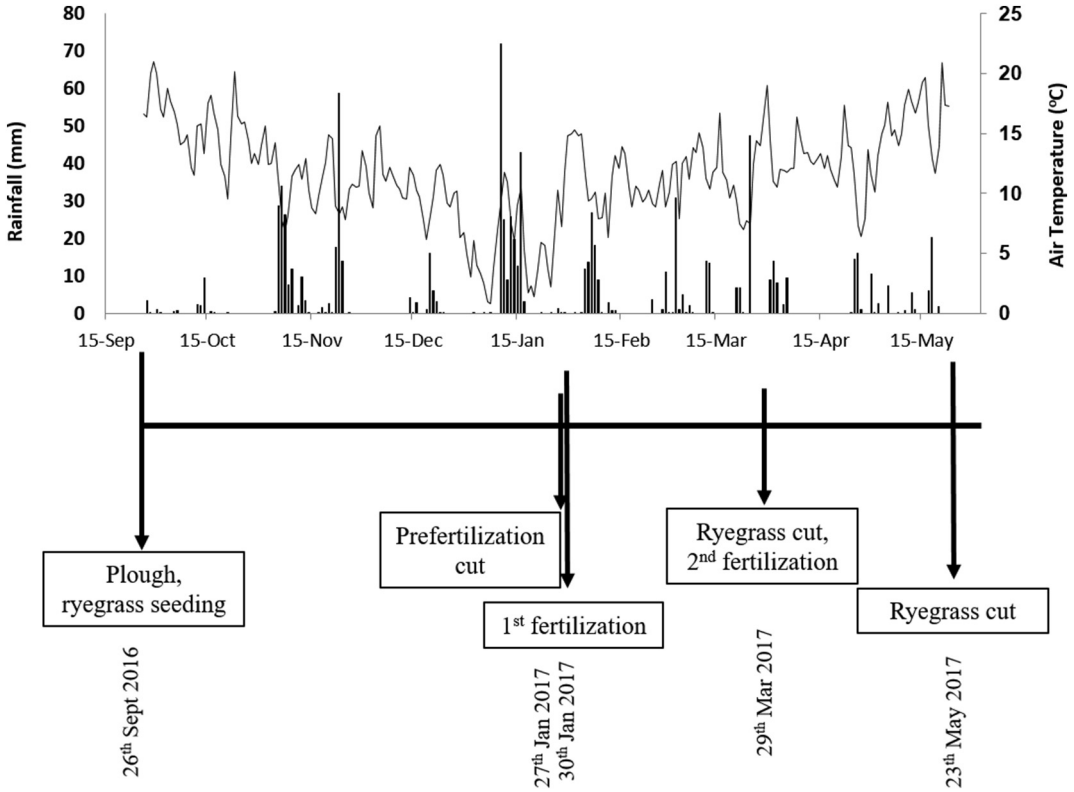

Fig. 1. Daily precipitation (bars) and mean air temperature (line) for the whole period of the study location (top) and schedule indicating the dates of the different managements performed during the measurement period (bottom).

2016 (Huérfano et al., 2018). After maize harvest the soil was ploughed, and the trial presented in this work consisted of an Italian ryegrass (*L. multiflorum* Lam. var. Westerwold Starter) crop sown at a density of 40 kg ha^{-1} on September 26th (Fig. 1). Ryegrass was harvested in January, March and May, and was fertilized just after the first and second harvests. The treatments were an unfertilized control and three fertilized treatments with the same fertilizer dose (Table 1). Fertilized treatments were defined as AS (ammonium sulphate, 21% N), DMPP (AS in combination with the NI DMPP) and DMPSA (AS in combination with the NI DMPSA). Both NIs were provided by EuroChem Agro GmbH, Germany, and were combined at a rate of 0.8% of the N-fertilizer in ammonium form.

2.1. Soil determinations

Soil determinations were made with three homogenized soil samples (2.5 cm diameter x 10 cm depth) taken in each plot at the beginning of the experiment, at 0, 15, 30 and 36 days after the first fertilization, and at 0, 15, 30 and 60 days after the second fertilization. NO_3^- and NH_4^+ soil contents were determined using subsamples of 100 g fresh soil mixed with 1 M KCl (1:2, w:v) and shaken for 1 h at 165 rpm. The soil solution was filtered through Whatman no. 1 filter paper (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and then through a Sep-Pak Classic C18 Cartridge (125 Å pore size; Waters, Milford, MA, USA) to eliminate organic carbon. NO_3^- content was determined as described by Cawse (1967) and NH_4^+ content by the Berthelot method (Patton and Crouch, 1977). Soil water content was determined 3 times per month during the pre-fertilization period, while after each fertilization 3 determinations per week were performed during 2 weeks, this frequency being later reduced to 2 determinations per week. Soil water content was expressed as the percentage of water filled pore space (WFPS) calculated as described by Linn and Doran (1984):

WFPS = (soil gravimetric water content × bulk density) x (1 – (bulk density / particle density)) x 100 by using a particle density of 2.65 Mg m^{-3} and a bulk density (0–10 cm) of 1.22 Mg m^{-3}.

Table 1
Fertilizer application rates (kg N ha^{-1}) and application dates.

	Total N	1st Fertilization (30-01-2017)	2nd Fertilization (29-03-2017)
Control	0	0	0
AS	140	80	60
DMPP	140	80	60
DMPSA	140	80	60

2.2. N_2O emissions

N_2O emissions were measured using the closed chamber technique as described by Chadwick et al. (2014). Linearity of N_2O emissions was checked out by determining this gas accumulation during 45 min. Four chambers were placed randomly in each plot, the fluxes of two of them being alternatively measured every sampling day. Emissions were determined 3 times per month during the pre-fertilization period. After each fertilization event 3 measurements per week were done during 2 weeks. The frequency was reduced to 2 measurements per week in the following 2 weeks and to 1 measurement per week afterwards. N_2O concentrations in the gas samples were determined by gas chromatography (GC) (Agilent, 7890A) with an electron capture detector (ECD). A capillary column (IA KRCIAES 6017: 240 °C, 30 m × 320 μm) was used and the samples were injected by means of a headspace autosampler (Teledyne Tekmar HT3) connected to the GC. Standards of N_2O were stored and analysed at the same time as the samples. Cumulative emissions during the sampling period were estimated using the trapezoidal rule integration (linear interpolation

and numerical integration between sampling times) (Levy et al., 2017). Yield-scaled N_2O emissions (YSNE) were calculated as follows: first, $YSNE_{DM}$ as the ratio between the amount of N emitted as N_2O and the yield (dry matter) of the crop (Venterea et al., 2011), and second, $YSNE_N$ as the ratio between the amount of N emitted as N_2O and the aboveground N uptake by the crop (Van Groenigen et al., 2010). The percentage of N applied as fertilizer that was lost to the atmosphere as N_2O (Emission Factor, EF%) was also calculated for the whole period following the equation: EF = [(Cumulative N_2O flux (kg N-N_2O) from the fertilized treatment – Cumulative N_2O flux (kg N-N_2O) from the unfertilized control treatment)] / [N applied as fertilizer (kg N)] x 100.

2.3. Yield and quality parameters

Forage yield was determined by harvesting the central 1.5 m along each plot on the dates detailed in Fig. 1. Ryegrass total N content was determined by the Kjeldhal procedure (AOAC, 1980) with a Kjeltec Auto sampler System 1035 analyzer (Foss Tecator, Hoganas, Sweden). Crude protein concentration was calculated as 6.25 times the total N concentration (Teller, 1932). The neutral-detergent fiber (NDF) and the acid-detergent fiber (ADF) were determined using ANKOM filter bag technology (Ankom, 2006a, 2006b).

2.4. DNA isolation and quantification of nitrifying and denitrifying gene abundance

Quantitative polymerase chain reaction (qPCR) was used to quantify the abundance of microbial nitrogen-cycling functional marker genes in three of the four replicates of the field assay. Soil samples were taken at 0–10 cm depth on days 15, 30 and 60 after each fertilization event. After soil homogenization, subsamples equivalent to 0.25 g of dry soil were weighted, frozen in liquid nitrogen and then stored at −80 °C until analysis. Total DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) with the following modifications: cell lysis was carried out in a homogenizer Precellys24 (Bertin, Montigny-le-Bretonneux, France), cooling incubations were increased to 15 min and, before the elution step, filter tubes were incubated at room temperature for 15 min. Soil DNA concentration and quality were determined spectrophotometrically (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA). Quantification of bacteria and archaea abundances (16S rRNA) and functional marker genes involved in nitrification (*amoA*) and denitrification (*nirK, nirS, nosZI* and *nosZII*) were amplified by qPCR using SYBR® Premix Ex Taq™ II (Takara-Bio Inc.) and gene-specific primers (Supplementary Table S1). Each sample was quantified in triplicate using the StepOnePlus™ Real-Time PCR System and data analysis was performed by StepOnePlus™ Software v2.3 (Thermo Scientific). Standard curves (log gene copy number per reaction volume versus log N) were prepared from serial dilutions from 10^7 to 10^2 gene copies μl^{-1} of linearized plasmids with insertions of target gene following the equations detailed in Torralbo et al. (2017). Thus, the copy number of target genes per gram of dry soil was calculated according to a modified equation detailed in Behrens et al. (2008): [(number of target gene copies per reaction x volume of DNA extracted) / (volume of DNA used per reaction x gram of dry soil extracted)] / DNA concentration.

2.5. Statistical analysis

Statistical analysis was conducted using the IBM SPSS statistics package (version 24.0; SPSS Inc., Chicago, IL, USA). To test normality of residuals and homogeneity of variances, the Kolmogorov-Smirnov and Levene tests were used. Variance was analysed by one-way ANOVA and the Student *t*-test. Significant differences between treatments were analysed using Duncan post hoc test, least significant difference (LSD) test and Kruskal-Wallis test. Relationships between variables were tested by Pearson's correlation. For correlations in Fig. 7, cumulative N_2O emissions within each post-fertilized period were divided in three sub-periods adjusted to the three sampling dates of soil physicochemical and microbial determinations.

3. Results

3.1. Soil temperature, water content and mineral N content

Soil WFPS and temperature values at 0–10 cm depth were, as expected, influenced by seasonal weather conditions along the crop cycle (Figs. 1 and 2). During the pre-fertilization period the average soil temperature was 11.6 °C and the average soil WFPS was 68.7%, ranging this last one between 50% and 100%. In the first and the second fertilization periods the mean soil temperatures were 9.3 °C and 13.7 °C respectively, and the mean WFPS values were 67.8% and 54.5%, ranging between 60% and 79% and between 39% and 74%, respectively.

At the beginning of the trial, in September, soil mineral N content reflected the residual effect of the fertilizer applied to the previous maize crop (Fig. 2 C and D), being both ammonium and nitrate soil contents higher in the plots having received N during the former maize crop period. Then, as expected, the application of fertilizer to ryegrass led to rises in soil mineral N content in the following weeks after both fertilization events. In the case of ammonium, this rise was more evident after the first fertilization, and declined after 14 days. Neither DMPSA nor DMPP increased significantly the soil ammonium content in comparison to AS. Nitrate content remained low after the first fertilization, regardless the treatment, and increased after the second fertilization to finally decrease during the next weeks, while no differences between treatments were observed.

3.2. N_2O emissions

N_2O fluxes from the unfertilized treatment varied from −1.51 to 7.34 g N-N_2O ha^{-1} day^{-1}, and the application of AS increased these fluxes up to a maximum of 27.12 g N-N_2O ha^{-1} day^{-1} after the first fertilization (Fig. 2, B). Daily N_2O emissions from DMPP and DMPSA treatments during this time period were reduced respecting to AS treatment, being of 19.92 and 11.40 g N-N_2O ha^{-1} day^{-1} for DMPP and DMPSA respectively. After the second fertilization period the application of AS induced fluxes of up to 21.5 g N-N_2O ha^{-1} day^{-1}, while those after DMPP and DMPSA application were again below that value, being of 11.57 and 7.35 g N-N_2O ha^{-1} day^{-1}, respectively. Cumulative N_2O losses during the 123 days before the first fertilization of the unfertilized control treatment were 413 g N-N_2O ha^{-1} (Fig. 3). Although no fertilizer had been yet applied to ryegrass, the residual effect of the previous maize crop fertilization significantly induced N_2O emissions in AS treatment by 55% respect to the unfertilized treatment (643 g N-N_2O ha^{-1}). Treatments with DMPP and DMPSA showed cumulative N_2O emissions 15% and 20% respectively lower (although non-significantly, $p < 0.05$) than AS treatment. These emissions from the precedent crop fertilized with both NIs were closer to the emission of the unfertilized control treatment. The first ryegrass fertilization induced an increase in N_2O losses, which were eight times higher in AS treatment (195 g N-N_2O ha^{-1}) than in the unfertilized control (25 g N-N_2O ha^{-1}), and no significant reduction in this emission was observed with the application of DMPP, and in a lesser degree in DMPSA. The second fertilization induced a four-fold increase in the cumulative N_2O emissions of AS treatment (243 g N-N_2O ha^{-1}) respecting to the unfertilized control. Reductions of 24% and 37% in N_2O emission were observed in this period of time compared to AS when DMPP and DMPSA were applied, being this reduction significant only in the case of DMPSA ($p < 0.05$). Regarding the total cumulative N_2O emissions of the whole 240 days period of study, the efficiency of NIs mitigating N_2O emissions was of 16% for DMPP and of 29% for DMPSA, being significant only for the latter ($p < 0.05$), with values dropping down towards the unfertilized control's level.

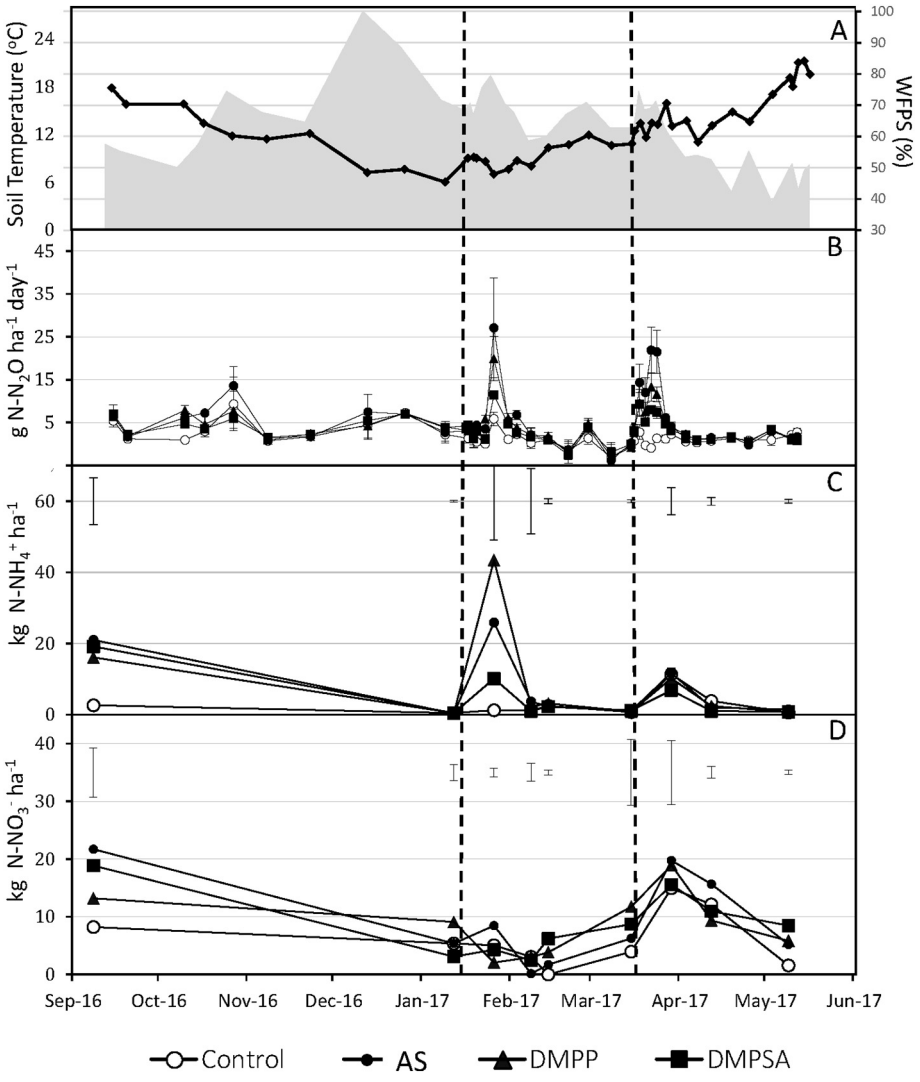

Fig. 2. Soil temperature (line) and WFPS (shaded) (A), daily N_2O emission (B), and evolution of soil ammonium (C) and nitrate (D) content during the field experiment. Vertical bars over soil nitrogen contents indicate least significant difference (LSD) ($p < 0.05$; $n = 4$) for each sampling moment; (○) = Control, (●) = AS, (▲) = DMPP, (■) = DMPSA, (♦) = soil temperature (0–10 cm depth), grey area = WFPS (0–30 cm depth).

3.3. Abundance of nitrogen cycle-related bacterial and archaeal populations

Total bacterial abundance, measured as 16S rRNA gene abundance, remained stable along the experiment with only slight changes over time due to environmental conditions, fluctuating between 1.2×10^9 and 2.2×10^9 gene copies per g dry soil (Fig. 4). By the contrary, significant changes in the ammonia oxidizing bacteria (AOB) abundance, measured as bacterial *amoA* gene copy number, were registered in response to fertilization treatments. During both fertilized periods, soils fertilized with AS showed 3 to 6 times significantly higher amounts of *amoA* gene copy number than the unfertilized control. The application of NIs significantly affected the AOB population, leading to a general decrease of *amoA* gene abundance down to the unfertilized control levels. The abundance of nitrous oxide-reducing bacteria was measured as *nosZI* (also called typical nosZ) and *nosZII* (atypical *nosZ*) genes copy numbers. The *nosZI* gene abundance remained stable between treatments, with values around 2.5×10^7–3.5×10^7 gene copies per g dry soil, during the two fertilization periods. However, a significant rise of 85% in the amount of *nosZI*-denitrifiers was registered in treatments fertilized with DMPP and DMPSA at the end of the first fertilization period. Regarding the denitrifiers bearing *nosZII* gene, they were less abundant than *nosZI*-denitrifiers and significantly decreased along the time during the two fertilization periods. In contrast to *nosZI, nosZII* gene abundance showed to be lower with the application of fertilizer, regardless the fertilization treatment. Other genes involved in denitrification, as *nirS* and *nirK*, showed no response to the treatments applied (Fig. 5), except for minor changes at the middle-end of the first fertilization period (on 28th February and 29th March) and at the end of the second one (23th May). It was remarkable that both gene abundances tended to be lower in the second fertilization period with respect to the first one. The ratio of the sum of *nosZI* and *nosZII* gene copies over the sum of *nirK* and *nirS* gene copies (*nosZI* + *nosZII*)/(*nirS* + *nirK*) shows the proportions between nitrous oxide-reducing bacteria and nitrite-reducing bacteria, suggesting a shift in the N_2 versus N_2O production ratio in the denitrification process. During the first fertilization period this ratio values were between 0.4 and 0.7, indicating that nitrite-reducing bacteria were more abundant, with the exception of DMPP and DMPSA treatments at the end of this period. In that case the ratio values increased up to 1.35, which implies a higher abundance of nitrous oxide-reducing bacteria when NIs were applied. After the second fertilization, the values of this ratio were among 0.5 and 0.9, with no noteworthy differences between treatments.

Total soil archaeal abundance, measured as archaeal 16S rRNA gene abundance, fluctuated between 1.6×10^8 and 4.4×10^8 gene copies per g dry soil during the experiment (Fig. 6), a five to ten-fold lower amount

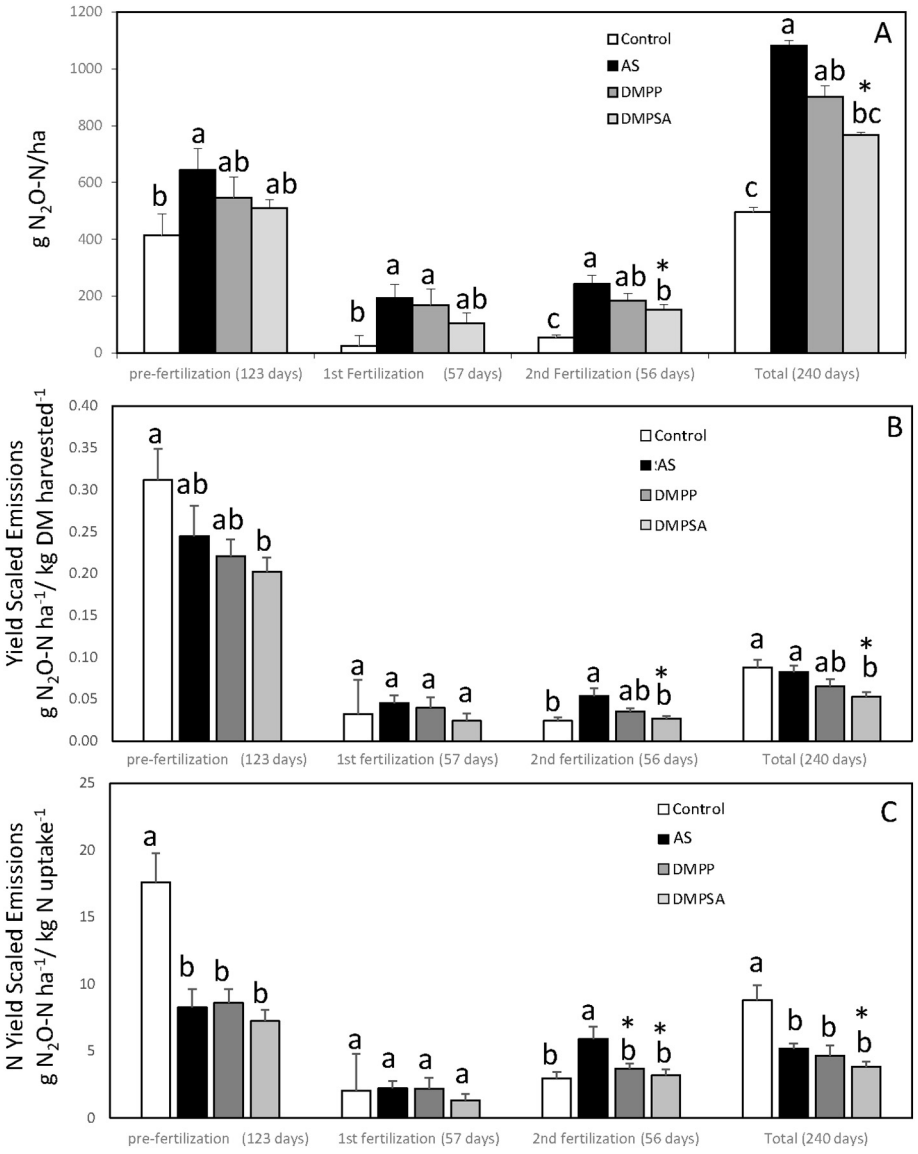

Fig. 3. Cumulative N_2O emission losses (A), yield scaled N_2O emission losses per kg of dry matter ($YSNE_{DM}$, B) and Nitrogen yield scaled emissions per kg of N uptaken ($YSNE_N$, C) in ryegrass. Control (white bars), AS (black bars), DMPP (dark grey bars) and DMPSA (light grey bars). Different letters indicate significant different rates using Duncan Test ($p < 0.05$; $n = 4$) within each period. Asterisks indicate a significant difference between the respective inhibitor and the AS treatment ($p < 0.05$; $n = 4$).

than total bacterial abundance extracted in this study. In general, archaeal populations did not change between fertilized treatments, being slightly lower in the unfertilized control treatment in some moments. The AOA gene copy number followed the same pattern, showing little or no changes whatever the treatment along the experiment. Thus, changes registered in the AOB/AOA ratio were governed almost exclusively by the differences exerted by the fertilized treatments on bacterial *amoA* gene abundances, leading to the same pattern for these two parameters.

3.4. Ryegrass yield and quality

In the pre-fertilization ryegrass cut, the plots having received fertilization seven months ago during the previous maize-crop period yielded a mean of 2548 kg ha^{-1} (double that of the unfertilized control), with no significant difference among them (Table 2). In the first fertilized cut, the fertilized treatments achieved a mean yield of 4279 kg ha^{-1} with no differences between them and significantly higher than the unfertilized treatment. However, after the second fertilization, the application of DMPP and DMPSA significantly increased the ryegrass yield by 12% and 23%, respectively, with regard to the AS treatment. Overall, the cumulative yield of the whole period of study was higher when NIs were applied, although this increase was only significant for DMPSA. Concerning the forage quality, while the crude protein content was increased by the application of fertilizer, the application of NIs showed no effect on this parameter, except in the pre-fertilization cut. In this first cut, the treatment having received DMPP few months ago during the previous maize-crop period showed a protein content 13% lower than in AS, which led to a 20% lower N extraction in that period. The application of NIs did not exert any effect in terms of forage fiber composition. Only in the pre-fertilization cut DMPP treatment showed a slight decrease in NDF.

3.5. Yield scaled N_2O emissions

When also taking into account the crop yield and the efficiency of the fertilizer applied, the yield scaled emission on a ryegrass dry matter basis ($YSNE_{DM}$) ranged from 0.20 to 0.31 g N_2O-N kg DM $harvested^{-1}$ in the pre-fertilization period, and around 0.03 g N_2O-N kg DM $harvested^{-1}$ post-fertilization (Fig. 3). Total yield scaled emissions referred to N uptake ($YSNE_N$) for the whole period of study resulted to

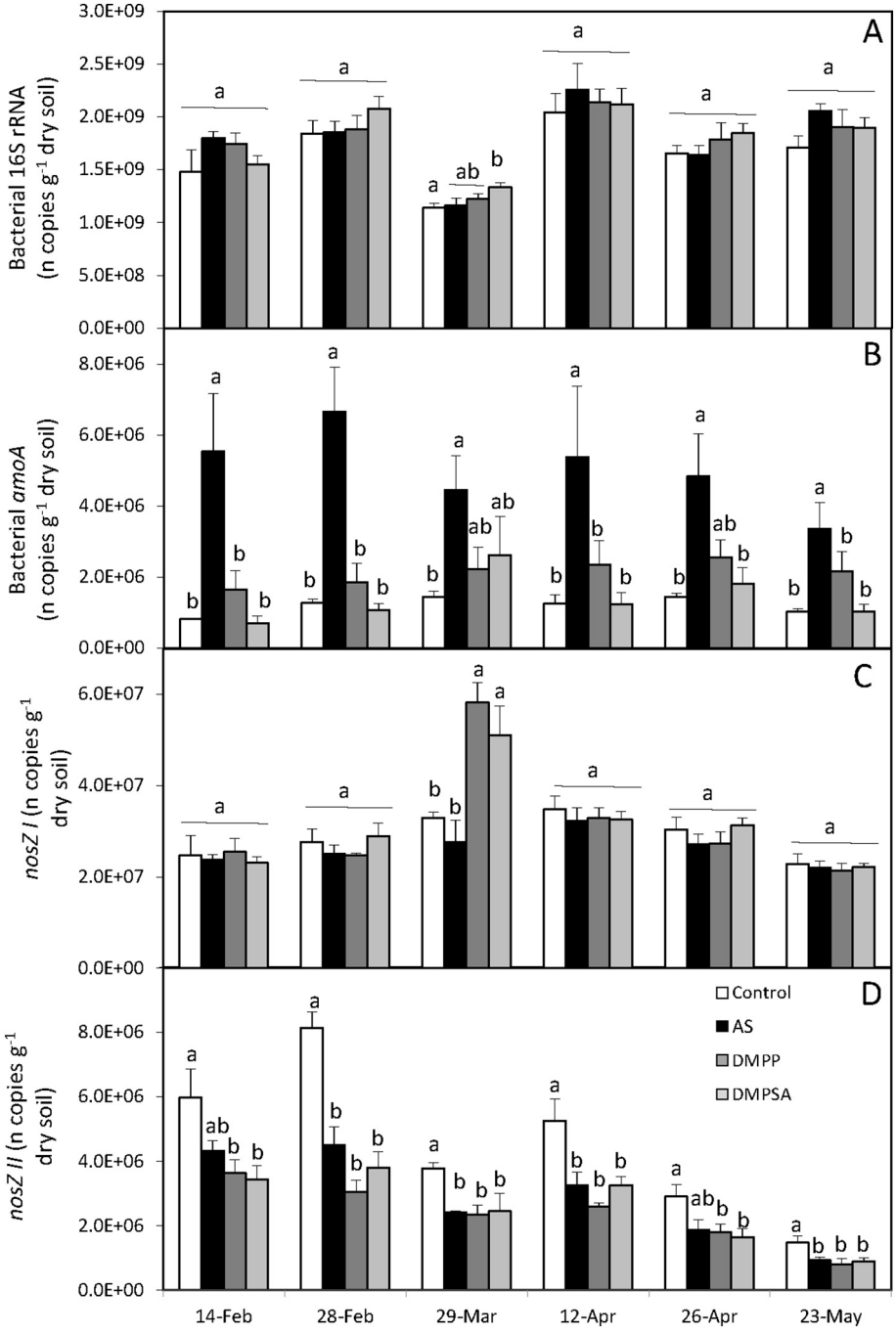

Fig. 4. Quantification of various key genes of microbial nitrogen transformation processes in the control (white bars), AS (black bars), DMPP (dark grey bars) and DMPSA (light grey bars). From top to bottom panel summarizes the gene copy numbers for 16S rRNA, *amoA*, *nosZI* and *nosZII* per gram dry soil. Treatments sharing the same letter within each period do not differ significantly at $p \leq 0.05$ using Duncan Test.

be <10 g N_2O-N kg N uptake^{-1} in any fertilized treatment assayed. In fact, after both fertilization events this parameter fell down to values ≤5 g N_2O-N kg N uptake^{-1} (Fig. 3). Both parameters, $YSNE_{DM}$ and $YSNE_N$ tended to decrease when NIs were applied. This trend was significant for DMPSA in the whole period of study.

4. Discussion

4.1. Influence of ploughing and edaphoclimatic conditions on N_2O emissions

The significantly 55% higher N_2O cumulative emissions of AS treatment respecting to the control during the 123 days of the pre-fertilization period suggested a noteworthy residual effect of the previous crop fertilization (Fig. 3). As observed in the same edaphoclimatic conditions by Huérfano et al. (2018), the mineralization process occurring as a consequence of ploughing (Goss et al., 1993) can provide the source of mineral N needed for N_2O emission in absence of fertilization. This presumed mineralization should have occurred in accordance with the increasing soil water content along this period from 57% to almost 100% of soil WFPS, since soil water content is the main factor regulating N_2O fluxes (Davidson, 1991; Dobbie and Smith, 2003) coming either from fertilization or from mineralization. In this sense, daily N_2O emission rates along the whole study were correlated with WFPS values both in the control ($r = 0.350^{**}$, $p < 0.01$) and AS

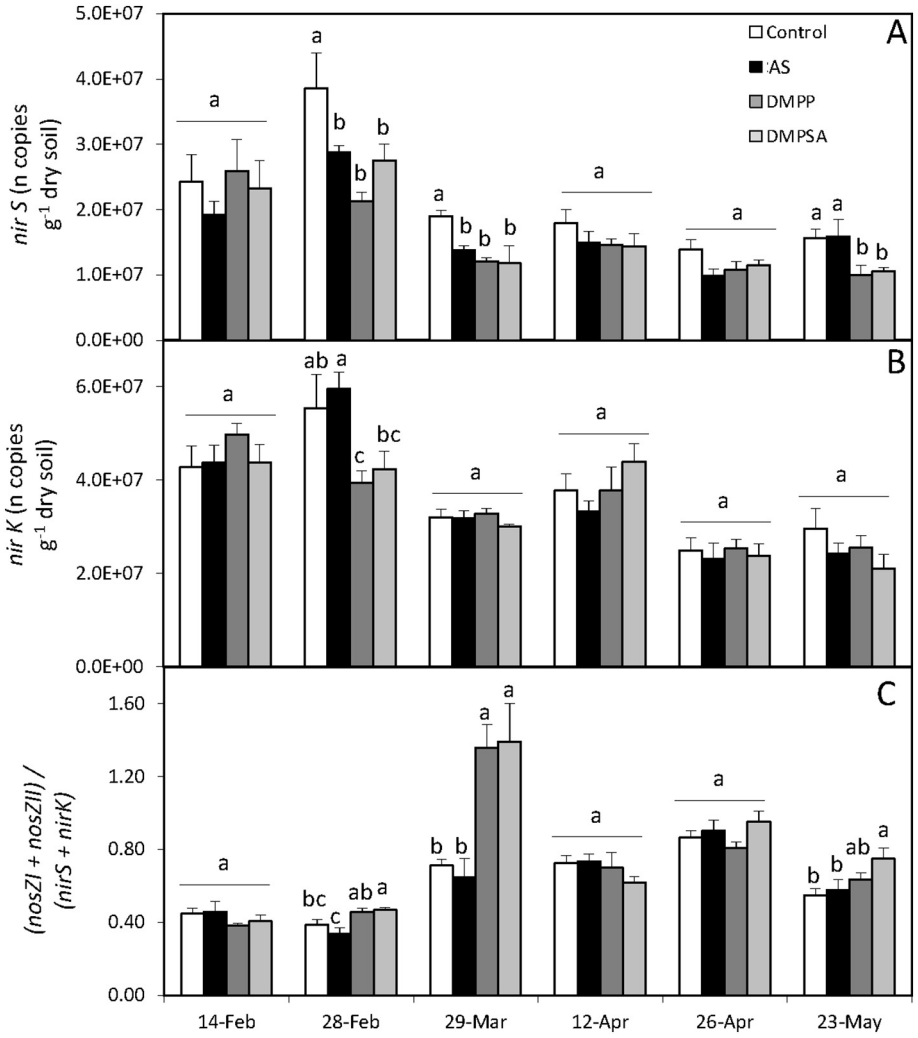

Fig. 5. Quantification of various key genes of microbial nitrogen transformation processes in the control (white bars), AS (black bars), DMPP (dark grey bars) and DMPSA (light grey bars). From top to bottom panel summarizes the gene copy numbers over time for *nirS, nirK* per gram dry soil and the ratio (*nosZI* + *nosZII*)/(*nirS* + *nirK*). Treatments sharing the same letter within each period do not differ significantly at $p \leq 0.05$ using Duncan Test.

($r = 0.271^{**}$; $p < 0.01$) treatments, the highest correlations between N_2O fluxes and WFPS being observed when fertilizer was applied after the first ($r = 0.537^{**}$) and the second ($r = 0.631^{**}$) AS applications. Attending to the mean soil WFPS values of each period of time (68% and 54%) both nitrification and denitrification would have been simultaneously occurring in both periods, as previously described (Kuenen and Robertson, 1994, Abassi and Adams, 1998). Taking into account that N_2O can be produced by nitrification and/or denitrification processes, the significant induction of AOB populations after AS application (Fig. 4) reveals that nitrifying bacteria have the potential to play an important role in N_2O emission, since this induction was observed in the wide range of soil WFPS values and no substantial changes were observed in AOB behavior along the two fertilization periods. Nevertheless, as expected, better correlations between soil ammonium and nitrate contents, and between AOB abundance and cumulative N_2O emissions were found in the second fertilization period (Fig. 7), when nitrification would be expected to be the dominant process responsible for N_2O production. By the contrary, better correlations between AOA abundance and both ammonium content and N_2O emissions were observed during the first fertilization period (Fig. 7). The low or absent response of AOA to fertilizer application has been previously addressed (Gong et al., 2013; Kleineidam et al., 2011; Hink et al., 2016; Yang et al., 2017; Lourenço et al., 2018). Differences between AOA and AOB in terms of specific metabolic traits related to ammonia oxidation explain why AOA have lower reaction rates and produce less N_2O per individual than AOB (He et al., 2012; Stieglmeier et al., 2014; Hink et al., 2018) even though being more abundant in grassland soils (Leininger et al., 2006; Duan et al., 2017), as we have also observed in this study (Figs. 4B and 6B).

Denitrification related gene abundances were well correlated with soil WFPS during the entire experiment, especially those of *nirS* and *nirK* ($r = 0.435^{**}$ and $r = 0.536^{**}$, respectively). Actually, *nirS* and *nirK* denitrifying bacteria populations tended to diminish during the second fertilization period (Fig. 5) concomitantly with the decrease in WFPS (Fig. 2). The ratio *niS* + *nirK*/AOB, which gives information about which process is being dominant, also showed a significant positive correlation ($r = 0.513^{**}$) with soil WFPS along the entire experiment. Thus, we can assume that denitrification contributed into a greater extent to N_2O emission in the first fertilization period compared to the second one. Furthermore, in the first fertilization period a significant negative correlation between *nosZI* and N_2O emission (Fig. 7) and also between the ratio (n*osZI* + *nosZII*)/(*nirS* + *nirK*) and N_2O emission ($r = -0.405^{**}$) was observed. This suggested that complete denitrification up to N_2 should be more favored during the first fertilization period. By the contrary, the significant correlation between soil nitrate content and N_2O emission observed in the second fertilization period (Fig. 7) suggested an incomplete denitrification process due to a suppression of nitrous

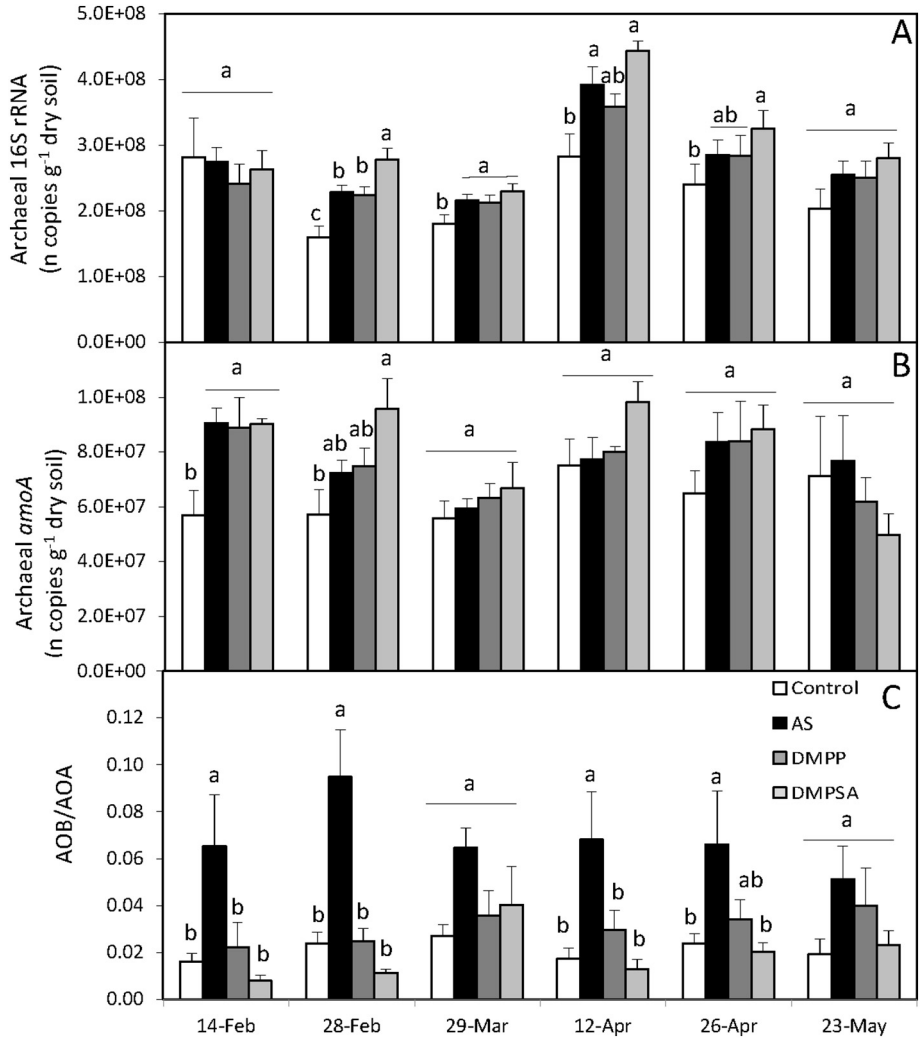

Fig. 6. Quantification of various key genes of microbial nitrogen transformation processes in the control (white bars), AS (black bars), DMPP (dark grey bars) and DMPSA (light grey bars). Panel summarizes the gene copy numbers over time for archaeal 16S rRNA, archaeal *amoA* (AOA) per gram dry soil and the ratio (AOB/AOA). Treatments sharing the same letter within each period do not differ significantly at $p \leq 0.05$ using Duncan Test.

Table 2
Yield production (dry matter), crude protein content, acid detergent fiber (ADF) content and neutral detergent fiber (NDF) content in ryegrass. Different letters indicate significant different rates using Duncan Test ($p < 0.05$; $n = 4$) within each period.

	Control	AS	DMPP	DMPSA
Pre-fertilization cut				
Yield (kg DM ha^{-1})	1308 b	2666 a	2449 a	2530 a
Protein (%)	11.1 c	18.6 a	16.3 b	17.7 ab
ADF (%)	20.0 b	22.4 a	21.3 ab	22.2 ab
NDF (%)	36.5 c	44.9 a	41.3 b	42.7 ab
N extraction (Kg N ha^{-1})	23,6 c	79.6 a	63.5 b	71.5 ab
1st fertilized cut				
Yield (kg DM ha^{-1})	990 b	4223 a	4229 a	4385 a
Protein (%)	9.0 b	12.9 a	12.1 a	11.1 ab
ADF (%)	23.3 b	26.1 a	26.0 a	26.7 a
NDF (%)	44.0 a	47.5 a	46.3 a	47.4 a
N extraction (Kg N ha^{-1})	14.2 c	86.6 a	81.9 ab	78.3 b
2nd fertilized cut				
Yield (kg DM ha^{-1})	2324 c	4629 b	5205 a	5712 a
Protein (%)	5.1 a	5.7 a	5.9 a	5.4 a
ADF (%)	30.3 b	33.3 a	33.4 a	32.1 ab
NDF (%)	51.9 b	56.4 a	55.9 a	54.5 ab
N extraction (Kg N ha^{-1})	19 b	41.9 a	49.2 a	48.9 a
Cummulative Yield	4622 c	11,518 b	11,883 ab	12,627 a
Total extraction (Kg N ha^{-1})	57 b	208 a	188 a	199 a

oxide reductase activity, as described by Saggar et al. (2013). Thus, a higher soil nitrate concentration typically results in a greater N_2O:N_2 ratio. Attending to this, the higher soil WFPS values of the first fertilization period would be responsible for the higher contribution of denitrification to N_2O emission compared to the second period, when WFPS decreased along the time.

4.2. NIs efficiency mitigating N_2O emissions

The fact that dimethylpyrazole-based inhibitors can effectively reduce N_2O emissions coming from fertilizationhas been widely observed (Xu et al., 2005; Di and Cameron, 2012; Guardia et al., 2019; Recio et al., 2019; Corrochano-Monsalve et al., 2020), even under our edaphoclimatic conditions (Huérfano et al., 2018). In this field trial, the efficiency of dimethylpyrazole-based NIs reducing N_2O emissions ranged between 10 and 25%, being only significant for DMPSA. These values are far from the almost 85% efficiency of DMPP observed by Fuertes-Mendizábal et al. (2019) in the same soil but in laboratory conditions. This high variability in N_2O emission reduction efficiencies has already been described in the literature for field studies, with values ranging between 0% and 95% (Misselbrook et al., 2014; Gilsanz et al., 2016; Soares et al., 2016; Marsden et al., 2017). The different success of NIs appears to be a function of soil properties, Cu-levels and

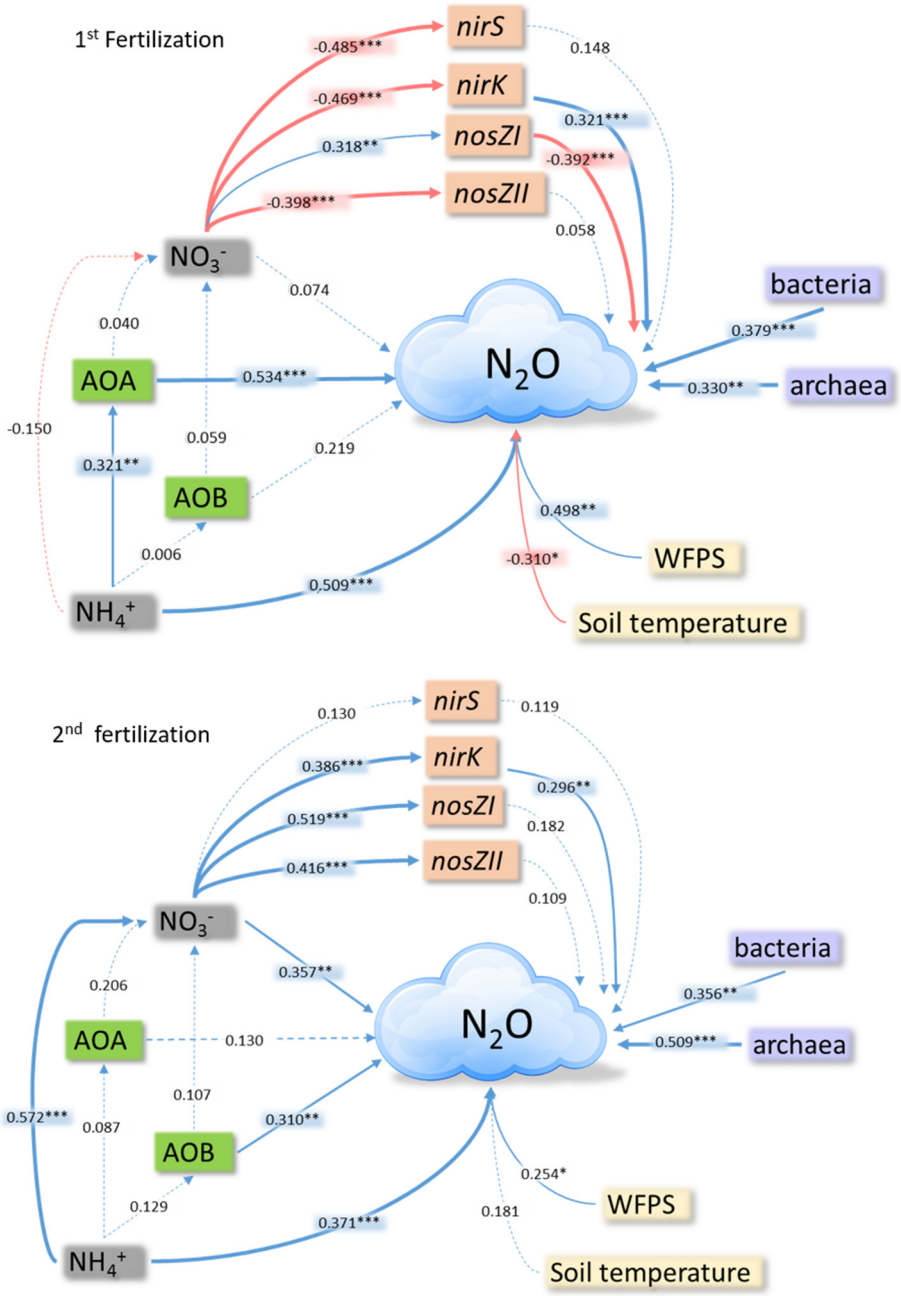

Fig. 7. Pearsons correlation coefficients between cumulative N_2O emissions and abundance of *amoA* (bacterial, AOB, and archaeal, AOA), *nirS, nirK, nosZ* (clades I and II), total *bacteria* and total *archaea* (by means of 16S rRNA), and abiotic factors: soil ammonium and nitrate contents, soil water filled pore space (WFPS), pH and temperature in first fertilization (from the begging of the experiment up to the first fertilized cut) (top) and second fertilization (from the first fertilized cut up to the end of the experiment) (bottom). Blue bold lines indicate significant positive correlations, red bold lines indicate significant negative correlations and dotted black lines indicate no correlation between variables (n = for each season). Significant difference: * $p \leq 0.1$, ** $p \leq 0.05$ and *** $p \leq 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

temperature, as well as of the variation in the abundance, genetic potential and/or expression levels of nitrifiers (Ruser and Schulz, 2015). Taking into account that, as discussed above, both nitrification and denitrification are co-occurring in the soil micro aggregates, and attending to the changes recorded in both nitrifier and denitrifier populations, we can suggest that in our study the effect of NIs is being exerted by means of two processes: 1) a reduction of N_2O emissions coming directly from nitrifiers and 2) a reduction of N_2O emissions coming directly from denitrifiers. Regarding the first process, the efficiency of both DMPP and DMPSA is related with their ability to avoid the rise of the AOB population after AS application in both fertilization periods, since blocking the AMO enzyme prevented the development of ammonium-oxidizing bacterial populations (Florio et al., 2014; Barrena et al., 2017; Duan et al., 2017; Shi et al., 2017; Torralbo et al., 2017; Fuertes-Mendizábal et al., 2019; Corrochano-Monsalve et al., 2020; Nair et al., 2021). In this sense, some works have described decreases in particular nitrifying bacterial populations such as *Nitrosomonadales* in a mediterranean agricultural soil (Corrochano-Monsalve et al., 2020) or *Nitrosospira* spp. in tropical agricultural soils (Lourenço et al., 2018; Cassman et al., 2019). Regarding the AOA response to NIs, some works have described a decrease in AOA after DMPP application (Dong et al., 2013; Liu et al., 2015; Quemada et al., 2019). However, other authors suggested that this response could be related to the rate of NI applied, being AOB more sensitive than AOA (Liu et al., 2019). Thus,

other authors did not observe any effect of NI on AOA population (Di and Cameron, 2011; Shen et al., 2013; Fuertes-Mendizábal et al., 2019), as was also the case in the present study. In fact, although in this grassland AOA was numerically dominant over AOB (Figs. 4B and 6B), it has been demonstrated that this numerical advantage at genomic level does not necessary equal the dominance at functional level (Tindaon et al., 2012). Thus, the decrease observed in AOB populations when applying NIs may explain the trend reducing N_2O emission, trend that reached higher significance when applying DMPSA compared to DMPP.

In relation to the second process mentioned above, in this field experiment dimethypyrazole-based NIs would reduce N_2O losses coming not only from nitrification, but also from denitrification, since decreasing ammonium oxidation would have also reduced nitrate availability for denitrification. Concerning the denitrification process, a non-target effect has been observed in this field trial by means of *nosZI* induction at the end of the first fertilization period, when soil water content favored denitrification. While there are studies reporting an absence of non-target effects of DMPP on soil microbial activity (Kong et al., 2016), several studies have recently demonstrated that, under certain conditions, dimethylpyrazole-based NIs would be able to induce the complete denitrification process up to N_2 by means of the induction of nitrous oxide reductase. Barrena et al. (2017), Torralbo et al. (2017) and Friedl et al. (2020) described inductions of typical *nosZ*-bearing denitrifiers in laboratory incubations under high soil water content conditions in response to dimethylpyrazole-based NIs application. Fuertes-Mendizábal et al. (2019) also reported the same induction in laboratory conditions after the application of DMPP, both under low and high soil water content conditions. Corrochano-Monsalve et al. (2020) reported the first data obtained in field conditions concerning an induction of *nosZ*-denitrifiers in response to DMPSA application, induction that was better observed in a non-tilled soil respecting to the same soil under conventional tillage management due to the higher soil WFPS of the former. As discussed above, in the first fertilization period of our ryegrass forage system, the higher presence of *nosZI*-bearing denitrifyiers was correlated with a lower N_2O emission in this period (Fig. 7). In this sense, the clear induction of *nosZI*-bearing denitrifiers observed 60 days after the application of both DMPP and DMPSA (Fig. 5C) confirms that the application of dimethylpyrazole-based NIs promotes the potentiality for a complete denitrification up to N_2, contributing to reduce as this, N_2O emission. Although still not directly proven, Wu et al. (2017) suggested that NIs application limited the nitrate supply to soil microsites, therefore decreasing the $N_2O/(N_2 + N_2O)$ ratio due to the competitive effect of NO_3^- and N_2O as terminal electron acceptors during denitrification. The results obtained under field conditions during the first fertilization period also suggest that, besides a lower soil nitrate content, it was necessary to maintain the high WFPS values for a certain period of time after both NIs application so that they were able to induce this significant increase in the denitrifying bacteria population bearing *nosZI* gene. According to this, when NIs were also applied at the beginning of the second fertilization period, no induction was observed in *nosZI*-bearing denitrifiers in this period, the only notable effect being the decrease in AOB population (Fig. 4). Provided that WFPS values at the end of the first period (March 29th) and at the first sampling date of the second fertilization (April 12th) were close to 60%, it might be expected that the induction of *nosZ* gene abundance remained throughout these periods. However, the absence of this non-target effect at the beginning of the second fertilization period could be related to the higher soil nitrate content observed in this second fertilization period, which would suppress nitrous oxide reductase activity thus altering the N_2O: N_2 ratio (Saggar et al., 2013; Senbayram et al., 2019).

4.3. NIs effect on forage yield and quality

Opposite to Yang et al. (2015), no negative effect exerted by NIs on yield was observed in our study (Table 2), which was in agreement with the results obtained by other authors (Merino et al., 2005; Menéndez et al., 2006; Huérfano et al., 2018). In the ryegrass pre-fertilization cut a residual effect of the fertilizer applied to the previous maize was observed in terms of ryegrass yield in all fertilized treatments. However, the protein content of DMPP treatment was lower in this pre-fertilization cut, leading to a lower quantity of N extracted. In the first fertilized cut, while no effect of NIs was observed regarding yield or protein content, a slight lower N extraction was observed for DMPSA. By the contrary, in the second fertilized cut a clear improvement in nitrogen use efficiency was observed in the plots receiving NIs, since the yields obtained after DMPP and DMPSA application increased significantly compared to AS plots (Table 2). The application of N fertilizer usually results in increased protein contents with reductions in other forage components such as fiber constituents (Van Soest, 1982). In this sense, NDF is used to predict intake potential by cattle and ADF is used to calculate digestibility. Thus, as fiber content increases, forage quality declines. Taking into account that N fertilizer effects on fiber components are usually lower than the effect on protein content (Park et al., 2017), together with the slight changes observed in both protein and fiber contents in DMPP and DMPSA treatments, we can conclude that, although in this study both NIs have demonstrated to be able to improve yield, they have a negligible impact on ryegrass quality.

4.4. Sustainability factors

In our Atlantic climate conditions, both for the entire experiment (8 months) and for each fertilization period, the N_2O emission factors (EF) of the treatments assayed were below the 1% proposed by the IPCC (2007) as a default value. Actually, the EF value of AS treatment for the whole experimental period was 0.42%, and DMPP and DMPSA were able to reduce it down to 0.29% and 0.20% respectively. When N_2O emissions were referred to the total N harvested (Yield Scaled N_2O emissions; YSNE, Fig. 3), the observed values were lower than 5 g N_2O-N kg aboveground N uptake^{-1} in any fertilized treatment, which, based on the 5 to 15 g N_2O-N kg N uptake^{-1} YSNE value range stablished by Van Groenigen et al. (2010), indicates that N management was at the agronomical optimal or even below. Hence, we can conclude that DMPP and DMPSA are capable of increasing ryegrass yield while maintaining the forage quality parameters at the same time they can reduce the N_2O emission factor.

5. Conclusions

This work provides valuable insights into the potential of dimethylpyrazole-based nitrification inhibitors increasing ryegrass yield while mitigating N_2O emissions due to a dual role over nitrification and denitrification processes throughout field-seasonal changes in soil water content and temperature. On one hand they avoid the stimulation of nitrifying bacteria, regardless the soil WFPS value, and, on the other hand they can also enhance the abundance of the denitrifying bacteria population capable of reducing N_2O up to N_2, provided that sufficiently high soil WFPS and low nitrate content conditions are occurring. Thus, after certain period of time under conditions of WFPS and soil nitrate content favoring the denitrification process in soil, both NIs show the capacity to promote the denitrification process up to N_2. Interestingly, DMPSA was found to be a promising NI, since it showed a more significant effect than DMPP reducing N_2O emissions and increasing ryegrass yield.

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CRediT authorship contribution statement

Ximena Huérfano: Methodology, Investigation, Data curation, Software, Writing – original draft. **José M. Estavillo:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition, Project

administration. **Fernando Torralbo:** Investigation, Visualization, Writing – review & editing. **Izargi Vega-Mas:** Investigation, Visualization, Writing – review & editing. **Carmen González-Murua:** Writing – review & editing, Funding acquisition. **Teresa Fuertes-Mendizábal:** Conceptualization, Validation, Data curation, Writing – original draft, Writing – review & editing, Supervision.

Declaration of competing interest

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

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