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Evaluation of a crop rotation with biological inhibition potential to avoid N_2O emissions in comparison with synthetic nitrification inhibition

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ABSTRACT

Agriculture has increased the release of reactive nitrogen to the environment due to crops' low nitrogen-use efficiency (NUE) after the application of nitrogen-fertilisers. Practices like the use of stabilized-fertilisers with nitrification inhibitors such as DMPP (3,4-dimethylpyrazole phosphate) have been adopted to reduce nitrogen losses. Otherwise, cover crops can be used in crop-rotation-strategies to reduce soil nitrogen pollution and benefit the following culture. Sorghum (*Sorghum bicolor*) could be a good candidate as it is drought tolerant and its culture can reduce nitrogen losses derived from nitrification because it exudates biological nitrification inhibitors (BNIs). This work aimed to evaluate the effect of fallow-wheat and sorghum cover crop-wheat rotations on N_2O emissions and the grain yield of winter wheat crop. In addition, the suitability of DMPP addition was also analyzed. The use of sorghum as a cover crop might not be a suitable option to mitigate nitrogen losses in the subsequent crop. Although sorghum–wheat rotation was able to reduce 22% the abundance of *amo*A, it presented an increment of 77% in cumulative N_2O emissions compared to fallow–wheat rotation, which was probably related to a greater abundance of heterotrophic-denitrification genes. On the other hand, the application of DMPP avoided the growth of ammonia-oxidizing bacteria and maintained the N_2O emissions at the levels of unfertilized-soils in both rotations. As a conclusion, the use of DMPP would be recommendable regardless of the rotation since it maintains NH_4^+ in the soil for longer and mitigates the impact of the crop residues on nitrogen soil dynamics.

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Introduction

Since the beginning of the green revolution, the application of nitrogen (N) fertilisers to agricultural crops has increased the level of reactive nitrogen present in the biosphere (Subbarao et al., 2017). Ammonium (NH_4^+) can be present in the soil after being applied with fertilisers, but also be released from organic matter thanks to microbial activity in a process called "mineralisation" (Coskun et al., 2017). In aerobic soils, NH_4^+ is oxidised by chemolithoautotrophic ammonia-oxidising bacteria (AOB) and archaea (AOA) in what is known as nitrification. Nonetheless, nitrification is mainly driven by AOB rather than AOA in soils receiving nitrogen-fertilisers (Di et al., 2009, 2010). First, nitrifiers oxidise NH_4^+ to hydroxylamine (NH_2OH) through the enzyme ammonium monooxygenase (AMO) which is encoded by the *amoA* gene (Arp and Stein, 2003). NH_2OH is then converted to nitrite (NO_2^-) and finally nitrite-oxidising bacteria (NOB) oxidise it to nitrate (NO_3^-) (Könneke et al., 2005). As an anion, NO_3^- is susceptible to be lost through leaching because its negative charge is repelled by negatively charged soil colloids (Fiencke et al., 2005). In anoxic conditions, NO_3^- is the substrate for the denitrification process. During denitrification, NO_3^- is sequentially reduced to NO_2^-, nitric oxide (NO) and nitrous oxide (N_2O) by enzymes encoded by the genes *narG, nirK, nirS* and *norB* (Hochstein and Tomlinson, 1988). In this process, the emission of N_2O is a harmful threat for the environment since N_2O is a gas with a global warming potential (GWP) that is 265 time greater than that of CO_2 in a 100-year period (IPCC, 2014). Finally, bacteria harbouring *nosZI* or *nosZII* genes can carry out a complete reduction of N_2O to N_2. Nonetheless, 40% of denitrifiers lack of the genes to perform this last step (Hallin et al., 2018).

Because of these nitrogen leaks and transformations, agriculture presents a low nitrogen-use-efficiency (NUE), since crops only assimilate an average of 30%–50% of the nitrogen applied with fertilisers (Wendeborn, 2020). This leads field-crop agriculture, such as wheat, to be responsible for more than 61% of total global anthropogenic N_2O emissions (Montzka et al., 2011). Therefore, we must guide agricultural systems towards sustainability in order to maintain adequate production levels while reducing the amount of reactive nitrogen lost to the environment. Some of the options to achieve this goal and reduce nitrogen-oxide emissions are the optimisation of nitrogen supply and synchronisation with crop demand or the application of stabilised nitrogen fertilisers with synthetic nitrification inhibitors (SNIs) (Thapa et al., 2016). SNIs inhibit the AMO enzyme delaying the oxidation of NH_4^+ to NO_2^-, giving plants more time to absorb the NH_4^+ (Keeney, 1983; Ruser and Schulz, 2015). Several chemical compounds with nitrification inhibition activity have been developed (Subbarao et al., 2006). The 3,4-dimethylpyrazole phosphate (DMPP) is one of the most worldwide used SNIs (Gilsanz et al., 2016). In a 10 times lower application rate, DMPP presents similar efficiency to another worldwide used SNI, dicyandiamide (DCD) (Ruser and Schulz, 2015). In microcosm experiments, DMPP ability to decrease N_2O emissions increase up to 90% (Bozal-Leorri et al., 2021; Corrochano-Monsalve et al., 2021a). In field conditions, reductions between 35% and 50% in N_2O emissions are reported with a higher maintenance of soil NH_4^+ content for longer period, without any deleterious effects on the yields of different crops such as wheat (Huérfano et al., 2015; Duncan et al., 2017), pasture and corn (Huérfano et al., 2018; Nair et al., 2020).

Nevertheless, recent studies have shown an impact of dimethylpyrazole-based inhibitors on non-target organisms (Corrochano-Monsalve et al., 2020, 2021a). Therefore, the potential risks for soil health of using SNIs in the long term should be considered. As an alternative, the use of cover crops, in a crop rotation, with the ability to modify the soil nitrogen cycle through the release of root exudates is also considered a good strategy to reduce nitrogen pollution (Subbarao et al., 2013). This allelopathy, which is known as biological nitrification inhibition (BNI), is highlighted in the framework of sustainable agriculture based on the use of environmentally friendly agronomic practices (Subbarao et al., 2013; Zhang et al., 2015). Some of these biological nitrification inhibitors (BNIs) have the potential to inhibit not just AOB but also AOA (Byrnes et al., 2017), and thus improve the nitrogen retention in soils, influencing positively crop NUE and mitigate GHG emissions. Among crops, sorghum (*Sorghum bicolor* L.) has the greatest BNI-releasing capacity (Subbarao et al., 2017), which makes it advisable to use it in crop rotations as a cover crop. Thus, because of a wakened nitrification, N pollution in the following crop should be reduced. Sorghum is drought tolerant (Hadebe et al., 2017), so it can develop despite the dry summer climate of areas with humid Mediterranean conditions. Moreover, it has a short growth cycle, which provides winter crops enough time to settle. In addition, the use of cover crops also bring multiple environmental benefits such as an improved soil structure and fertility, weed control and a reduction in nutrient leaching and soil erosion (Muhammad et al., 2019; Garland et al., 2021). Furthermore, the use of a cover crop is a very efficient tool in reducing the amount of leachable NO_3^- in soil (Constantin et al., 2010; Plaza-Bonilla et al., 2015).

The present work aimed to evaluate the effect of two different no-till crop rotations: (1) a sorghum-wheat rotation, in which the possible benefits of a cover crop with BNI potential will be analysed in terms of soil mineral nitrogen content, N_2O emissions and grain yield of winter wheat; in comparison to (2) a conventional fallow-wheat rotation. In this study, the application of a synthetic nitrification inhibitor (DMPP) will be also considered as (1) a control of nitrification inhibition to compare with the potential BNI activity of sorghum, and (2) a complement for increasing the sustainability of these wheat rotations in terms of N_2O emissions.

1. Materials and methods

1.1. Experimental design

This work was conducted in Pamplona, northern Spain (42°47'N, 1°37'W, 450 m above sea level) during the 2019/2020 growing season. The soil characteristics of the upper horizon before the start of the experiment are given in Table 1, while daily precipitation and mean temperatures are shown

Table 1 – Physical and chemical properties of the soil collected in 0 – 30 cm depth layer in Pamplona before the start of the experiment (42°47′N, 1°37′W, 450 m above sea level, Navarre, Spain).

Soil texture		Soil chemical properties	
Sand	38.6%	pH[a]	8.3
Silt	31.8	C:N ratio	8.9
Clay	29.6	N[b] (g/kg)	1.4
		Organic matter[c] (g/kg)	21.5
		$CaCO_3$[d] (g/kg)	20.3
		Mg[d] (mg/kg)	53.5
		K[d] (mg/kg)	270.0
		Ca[d] (mg/kg)	2735.7
		P[e] (mg/kg)	11.5

[a] 1:2.5 (*m*/*V*) soil:water
[b] Kjeldahl digestion (Keeney, 1983);
[c] Walkley and Black, 1934
[d] NH_4AcO, MAPA, 1994;
[e] Watanabe and Olsen, 1965.

in Appendix A Fig. S1. A bifactorial experimental design (crop rotation and type of wheat fertilisation) was implemented. The crop rotations were: (1) fallow–wheat rotation (fallow–wheat); and (2) sorghum cover crop without nitrogen fertilisation–wheat rotation (sorghum–wheat). The soil treatments were arranged in two blocks. In the first block, adventitious plants were desiccated on May 20, 2019 using RoundUp (a glyphosate-based herbicide) (36% W/V, Fertiberia, Spain) at a rate of 1.5 L/ha, dose that is routinely applied in no-till systems from this region. In the second one, sorghum (*Sorghum bicolor* L. var. PR88P68 Pioneer Corteva Agriscience®, USA) was sown under no-till conditions at a rate of 15 kg/ha on May 20, 2019. The sorghum cover crop was crushed on October 14, 2019 and left on the soil surface. One month before sorghum termination, soil NH_4^+-N content from fallow and sorghum plots were 2.0 and 1.7 kg N/ha, respectively, while the soil NO_3^--N content was 43.7 and 7.0 kg N/ha for fallow and sorghum plots. Winter wheat (*Triticum aestivum* L. cv. Marcopolo RAGT®, Spain) was sown under no-till conditions and over sorghum stover at a rate of 220 kg/ha on October 31, 2019. Within each block of crop rotation (fallow–wheat and sorghum–wheat), three wheat fertiliser treatments were applied in four random plots replications of individual size of 25 m^2 (5 m × 5 m): (1) control without fertilisation (Control); (2) fertilised with ammonium sulphate 21%-Nitrogen (AS); and (3) fertilised with ammonium 21%-Nitrogen sulphate combined with DMPP (AS+DMPP). The fertilisation rate was 90 kg N/ha applied in a single dose on February 28, 2020 at the beginning of stem elongation (Z30) according to the Zadoks scale (Zadoks et al., 1974). The AS treatment used ammonium sulphate (99%, Agro Iberia S.L., Spain) as fertiliser, and AS+DMPP treatment used ENTEC® (Agro Iberia S.L., Spain), which contains ammonium sulphate (99%), and DMPP (99%) at a rate of 0.8% of the NH_4^+-N present in the ENTEC®. The wheat was harvested on July 24, 2020.

1.2. *Wheat soil geochemical analysis and water content*

Soil NH_4^+ and NO_3^- contents were first determined the day before applying the treatments. Samples were then taken 10, 30 and 60 days post-fertilisation. Three soil subsamples (3 cm diameter × 0.3 m deep) were taken from each plot, rocks and stones were removed and finally they were homogenised. Next, 100 g fresh weight of the homogenised subsamples were mixed with 200 mL of 1 mol/L KCl (99%, PanReac Química, Spain) and shaken for 1 hr at 165 r/min. The soil solution was filtered through Whatman No. 1 filter paper (GE Healthcare, Spain) to remove particles and then through Sep-Pak Classic C18 125 Å cartridges (Waters, USA) to eliminate organic matter. The NH_4^+ content of the filtered solution was determined using the Berthelot method (Patton and Crouch, 1977) and the NO_3^- content as described by Cawse (1967).

The soil water content was also measured each time the soil and/or GHG were sampled. Two subsamples (3 cm diameter × 0.3 m deep) were taken randomly from the field. Rocks were removed and the soil subsamples were dried at 80°C in a circulation oven for 72 hr until they reached a constant dry weight. Water content was expressed as the percentage of water-filled pore space (WFPS, %) as per Linn and Doran (1984), Eq. (1):

$$\mathrm{WFPS} = (C \times D_b) \times (1 - (D_b/D_p))^{-1} \quad (1)$$

where, C (g) is the soil gravimetric water content, D_b (Mg/m^3) is the bulk density; D_p (Mg/m^3) is the particle density.

D_p was taken as 2.65 Mg/m^3. D_b was measured at the beginning of the experiment and was found to be 1.0 Mg/m^3.

1.3. *Measurement of N_2O emissions from wheat soils*

N_2O soil emissions were measured using the closed chamber method (Chadwick et al., 2014). Samples were collected 3 times/week for 2 weeks after wheat fertilisation, then 2 times/week for the next 2 weeks and 1 times/week up to day 60. Considering the diurnal variation of emissions (Baggs and Blum, 2004), sampling was performed between 10:00 a.m. and 1:00 p.m. To account for soil heterogeneity, four chambers (20 cm diameter × 16 cm high once inserted in the soil) were placed in each plot and two were sampled on alternate days. Linearity was checked and gas samples were taken just after closing the chambers and then 45 min later. 20 mL of gas was taken from each chamber and stored at overpressure in pre-evacuated 12 mL glass vials. Samples were analysed in a gas chromatograph (GC) (7890A, Agilent, USA) equipped with an electron capture detector for N_2O detection. A capillary column (IA KRCIAES 6017: 240°C, 30 m × 320 µm) was used and the samples were injected using a headspace autosampler (HT3, Teledyne Tekmar, USA). N_2O standards were analysed at the same time as the samples. Gas emission rates were calculated by considering the variation in gas concentration from the beginning to the end of the 45 min (Menéndez et al., 2008). Cumulative N_2O emissions during the sampling period were estimated by averaging the rate of loss between two successive determinations, multiplying that average rate by the length of the period between the measurements, and adding that amount to the previous cumulative total (Menéndez et al., 2008). Soil temperature (at a depth of 10 cm) was measured

before taking the gas samples. The air temperature was measured 3 times during the 45 min gas sampling period to get the average.

1.4. Abundance of N-cycle related microorganisms in wheat soils

Quantitative polymerase chain reaction (qPCR) was used to quantify the abundance of nitrifying and denitrifying genes. Soil DNA was isolated from 0 to 30 cm soil samples (three subsamples per plot) collected at 10 days post-fertilisation. DNA was extracted from 0.25 g of dry soil using the PowerSoil® DNA Isolation Kit (Qiagen, Germany) with the modifications described in Harter et al. (2014). The concentration and quality of DNA extracts were determined by means of spectrophotometry (NanoDrop® 1000, Thermo Scientific, USA).

The *16S rRNA* gene (for quantification of total bacterial abundance) and functional marker genes involved in bacterial nitrification (*amoA*) and denitrification (*nirK*, *nirS*, *nosZI* and *nosZII*) were amplified by means of qPCR using SYBR® Premix Ex TaqTM II (Takara-Bio Inc., Japan) and gene-specific primers (Appendix A Table S1) in the StepOnePlus™ Real-Time PCR System. Each qPCR reaction was performed in triplicate for each sample. Data analysis was carried out with StepOnePlus™ Software 2.3 (Thermo Scientific, USA). Standard curves were prepared from serial dilutions of linearised plasmids with insertions of the target gene ranging from 10^7 to 10^3 gene copies/μL. The number of copies of target gene/gram of dry soil (*N*) was calculated according to a modified equation (Eq. (2)) detailed in Behrens et al. (2008):

$$N = (N_{\text{per reaction}} \times V_{\text{DNA extracted}}/V_{\text{DNA used}} \times W)/[\text{DNA}_{\text{extracted}}] \quad (2)$$

where, $N_{\text{per reaction}}$ is the number of target gene copies/reaction; $V_{\text{DNA extracted}}$ is the volume of DNA extracted; $V_{\text{DNA used}}$ is the volume of DNA used per reaction; *W* (g) is the weight of dry soil extracted. $[\text{DNA}_{\text{extracted}}]$ is the extracted DNA concentration.

1.5. Wheat crop yield parameters

Grain yields were calculated based on a harvested area of 7.5 m^2 (1.5 m × 5 m) per plot and adjusted for a moisture content of 12%. An area of 0.36 m^2 per plot was measured to calculate the number of spikes/m^2 and dry weight of 1000 grains. The total grain nitrogen content was analysed by applying the Kjeldhal procedure (AOAC, 1980) with a Kjeltec Autosampler System 1035 (Tecator, Spain) after grinding the grain and passing it through a 1 mm screen. Grain protein content was taken as 5.7 times the total nitrogen content (Teller, 1932). The yield-scaled N_2O emissions (YSNE) were expressed as the ratio between the amount of N emitted as N_2O and the aboveground nitrogen uptake (van Groenigen et al., 2010). The nitrogen use efficiency (NUE) (kg dry matter/kg N) was determined as, Eq. (3):

$$\text{NUE} = (W_{Nx} - W_{N0})/W_{\text{nitrogen}} \quad (3)$$

where, W_{Nx} (kg) is the dry matter obtained when 90 kg N/ha were added; W_{N0} (kg) is the dry matter obtained with no fertiliser application. W_{nitrogen} (kg) is the weight of applied nitrogen.

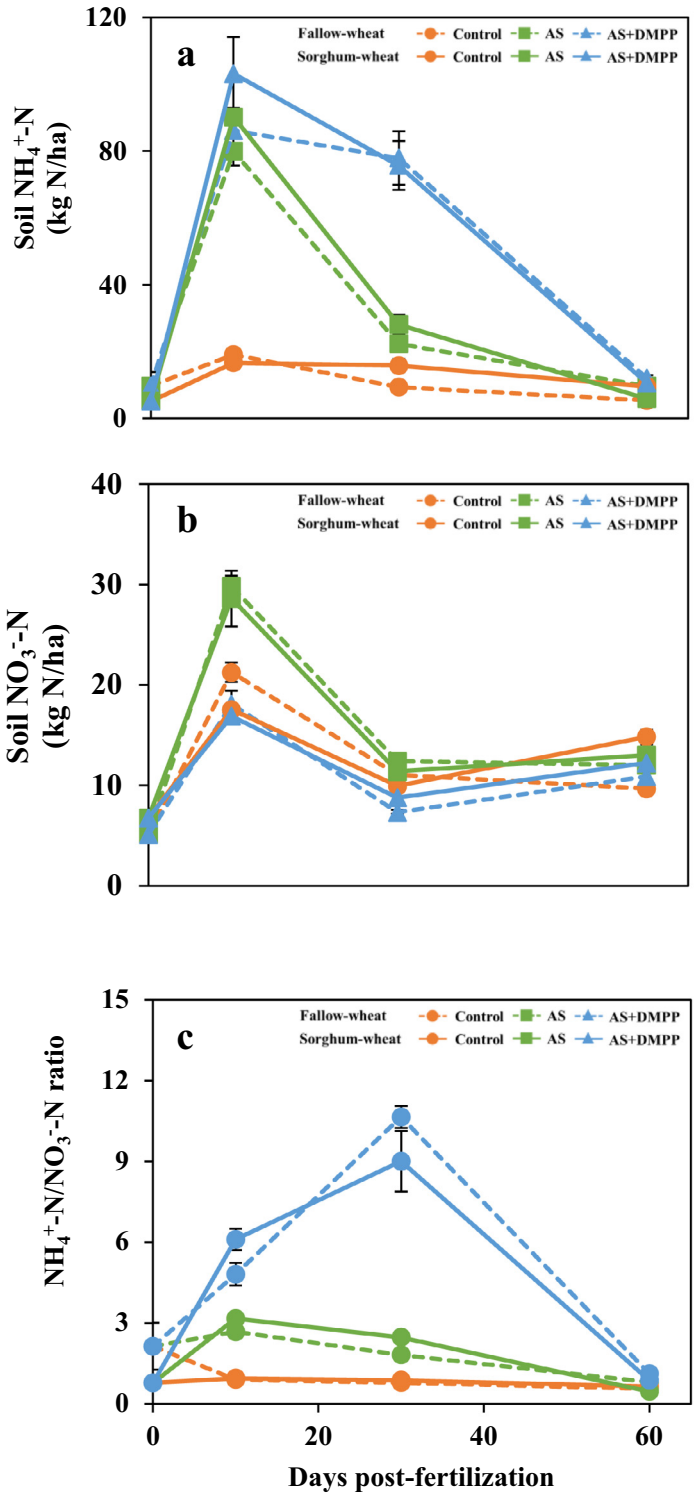
1.6. Statistical analysis

The results from soil mineral nitrogen content determinations were subject to a two-way (crop rotation, "R"; and fertiliser treatment, "F") analysis of variance statistical analysis. The results of N_2O measurements, microbial quantification and grain yield parameters were analysed by one-way ANOVA using Duncan's multiple range test for separation of means between treatments and the Mann–Whitney U test was used to compare the two treatments with the SPSS statistical software package (2016, IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY, IBM Corp, USA). *p*-Values < 0.05 were considered to be statistically significant differences.

2. Results and discussion

2.1. Nitrifying microorganisms are reduced in sorghum-wheat crop rotation

Fertilisation had a strong effect on soil mineral nitrogen content ($p < 0.01$). Soil from both crop rotations started with a similar soil NH_4^+ content before fertiliser application to the wheat crop (Fig. 1a). After the addition of the nitrogen fertiliser, soil NH_4^+ content increased to the same level in the AS and AS+DMPP treatments for both crop rotations, but there was a significant decrease in the AS treatment at 30 days post-fertilisation (DPF). However, at the same time, the use of DMPP meant the soil retained twice the amount of NH_4^+ compared to the AS treatment. Although the NH_4^+ content for the AS and AS+DMPP treatments dropped to the level of the Control treatment at 60 DPF, DMPP was able to prolong the availability of NH_4^+ at least until 30 DPF. These results are comparable to other studies that applied DMPP as a SNI (Huérfano et al., 2015, 2016; Liu et al., 2020). On the contrary, AS+DMPP reduced NO_3^- content to 40% at 10 DPF and to 30% at 30 DPF compared to the AS treatment (Fig. 1b). The Control treatment also maintained low soil NO_3^- values throughout the experiment. As NH_4^+ content remained high and NO_3^- content was low due to the delay in NH_4^+ oxidation (Ruser and Schulz, 2015), AS+DMPP presented the highest NH_4^+/NO_3^- ratio of all the fertilised treatments (Fig. 1c). AS treatment showed a higher NH_4^+/NO_3^- ratio than Control treatment but it was only able to maintain 53% and 22% of AS+DMPP ratio at 10 and 30 DPF, respectively. Nevertheless, although the use of DMPP treatment was able to maintain more NH_4^+ in the soil, it can present some undesired effects. A higher retention of soil NH_4^+ content could lead to an increase on NH_3 volatilization. Even though we did not measure NH_3 volatilization in our experiments, meta-analysis estimate that the use of SNIs can increase NH_3 volatilization around 20% (Qiao et al., 2015). Therefore, despite the fact that SNIs application alleviates global warming potential derived from direct N_2O emissions, their potential negative side effects should be fully considered. On the other hand, we did not find any differences between the two crop rotations in the maintenance of soil mineral nitrogen, in terms of soil NH_4^+ and soil NO_3^- content, during wheat development (Fig. 1a and b). This may indicate that, even though cover crops carry benefits for the following culture such as improved soil physicochemical prop-



	10	30	60
R	*	NS	NS
F	**	**	**
R×F	*	NS	NS

	10	30	60
R	*	NS	NS
F	**	**	NS
R×F	NS	NS	NS

	10	30	60
R	NS	NS	NS
F	**	**	NS
R×F	NS	NS	NS

Fig. 1 – Wheat crop soil mineral nitrogen (0 – 30 cm) evolution during 60 days post-fertilisation in form of NH_4^+ (a), NO_3^- (b) and the ratio of NH_4^+-N/NO_3^--N (c). Control: control without fertilization; AS: fertilised with ammonium sulphate; AS+DMPP: fertilised with ammonium sulphate + 3,4-dimethylpyrazole phosphate. Statistical analysis was made through analysis of variance (two-way ANOVA) showing the effect of crop rotation (R), fertilizer treatment (F) and their interaction (R × F). Significant differences are marked with an asterisk (*) when $p < 0.05$ and double asterisk (**) when $p < 0.01$.

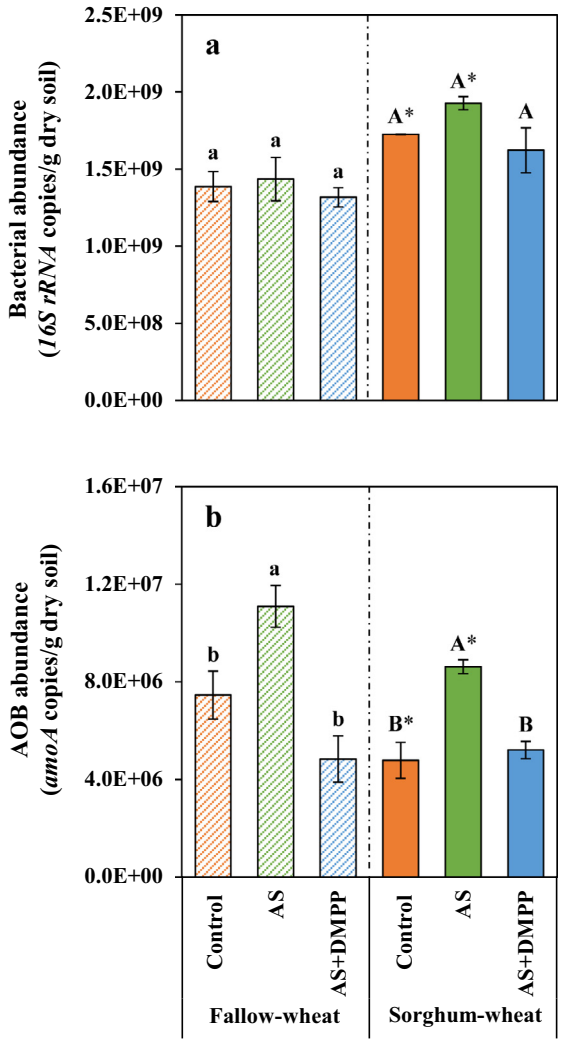


Fig. 2 – Abundance of total bacteria (a) and ammonia oxidizing bacteria (AOB) (b) at 10 days post-fertilisation (DPF) on soil of wheat crop. Significant differences between treatments of "Fallow-wheat" rotation are marked with lowercase letters. Significant differences between treatments of "Sorghum-wheat" rotation are marked with capital letters. For both ANOVA, the Duncan Test was used ($p < 0.05$; $n = 4$). The Mann-Whitney U test was used for the comparison between crop rotations within the same fertilization treatment. Significant differences at $p < 0.05$ are marked with an asterisk (*).

erties (such as water holding capacity, aggregate stability and C stock) (Buyer et al., 2010; Lal, 2015; Poeplau and Don, 2015) or reduced nitrogen losses (Kaye and Quemada, 2017), in this case, the use of sorghum as a summer cover plantation did not affect the evolution of soil mineral nitrogen during the following crop. Nonetheless, the analysis of soil mineral nitrogen in the subsequent culture may not be sufficiently sensitive to detect the effects of using sorghum as a cover crop.

Fertiliser treatment did not affect the total bacterial abundance (measured as *16S rRNA* gene abundance) (Fig. 2a). However, the AS treatment greatly enhanced nitrification (in terms of bacterial *amoA* gene abundance), especially in fallow–wheat rotation (Fig. 2b). Even though adventitious plants were desiccated with glyphosate-based herbicide to create a fallow plot, the great AOB growth in AS treatment in fallow–wheat rotation indicated the lack of deleterious effects of glyphosate on nitrifying microorganisms. Our results agree with previous studies where was demonstrated that higher doses of glyphosate or repeated exposure did not affect nitrifying populations (Allegrini et al., 2017; Zabaloy et al., 2017). The application of the DMPP was very effective at reducing AOB abundance in soils from both crop rotations, even down to the levels of the unfertilised Control, with reductions of 56% and 40% compared to AS in fallow–wheat and sorghum–wheat rotations, respectively. Notwithstanding that some studies in microcosms have reached an AOB inhibition of over 85% with the use of DMP-based inhibitors (Torralbo et al., 2017; Bozal-Leorri et al., 2021; Corrochano-Monsalve et al., 2021b), our results are similar to those obtained in field studies where the AOB inhibition was efficient but at lower percentages (Kleineidam et al., 2011; Duncan et al., 2017). Although the different crop rotations did not affect the soil nitrogen content, it did influence soil microbial populations. We observed a significant increase in the total bacterial abundance in soil of sorghum–wheat rotation, as it was 24% and 34% higher compared to fallow–wheat for the Control and AS treatments, respectively (Fig. 2a). Furthermore, the type of crop rotation also affected AOB abundance, as the levels for the Control and AS treatments for the sorghum–wheat rotation were 35 and 22% lower than the fallow–wheat plots (Fig. 2b). This reduction indicates that, during its development, sorghum might have exuded BNIs that can keep nitrifiers inhibited until the next crop. Dayan et al. (2010) indicated that the inhibitory effect of sorghum could persist for at least 60 days after the harvest was removed. In our case, the BNIs may had a more enduring effect (140 days) because the sorghum was not eliminated from the cultivation soil and the experiment was carried out under no-tillage conditions, which retards BNI degradation (Roth et al., 2000). Thus, leaving the sorghum stover in the soil under no-tillage conditions ensures slower root degradation and the consequent release of exudates with a BNI capacity because such compounds are produced exclusively in the roots (Baerson et al., 2008).

2.2. N_2O emissions are affected by the type of rotation

Daily N_2O emissions ranged from 0.89 to 13.74 g N_2O-N/(ha•day) in fallow–wheat rotation and from 0.38 to 24.58 g N_2O-N/(ha•day) in sorghum–wheat rotation (Fig. 3a). The cumulative N_2O emissions from the AS treatments were the highest of all the fertiliser treatments with 382.9 and 678.3 g N_2O-N/ha in soil from the fallow–wheat and sorghum–wheat rotations, respectively (Fig. 3b). As is well supported elsewhere (Ruser and Schulz, 2015), DMPP reduced cumulative N_2O emissions to values akin to the Control treatment, corresponding to reductions of 79% and 86% compared to the AS treatment for the fallow–wheat and sorghum–wheat rotations, respectively. Since AOB populations were reduced in the wheat crop (see Section 2.1), probably because of the BNIs released from sorghum, we also expected a decrease in N_2O emissions (either due to a reduction in N_2O emitted by nitrifiers

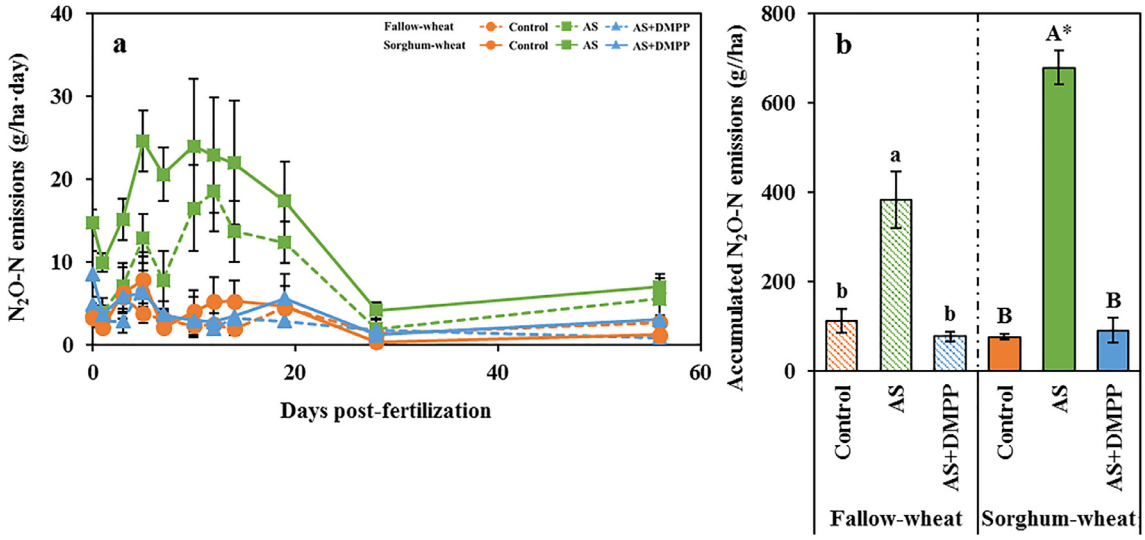


Fig. 3 – Daily (a) and cumulative (b) N_2O emission during 56 days post-fertilisation on soil of wheat crop. Significant differences between treatments of "Fallow-wheat" rotation are marked with lowercase letters. Significant differences between treatments of "Sorghum-wheat" rotation are marked with capital letters. For both ANOVA, the Duncan Test was used ($p < 0.05$; $n = 4$). The Mann-Whitney U test was used for the comparison between crop rotations within the same fertilization treatment. Significant differences at $p < 0.05$ are marked with an asterisk (*).

or a decrease in denitrifying activity because of a delay in the transformation of NH_4^+ into NO_3^-). However, as shown in Fig. 3a, N_2O emissions from the AS treatment of sorghum–wheat rotation were higher than those for the fallow–wheat rotation throughout the entire experiment, resulting in a 77% increase in cumulative N_2O emissions (Fig. 3b). It should be remarked that sorghum stover is an extra carbon source in the soil, and the main mechanism connecting carbon cycling with nitrogen gas emissions is the carbon availability in the soil that enhances heterotrophic denitrification, which is one of the main processes responsible for N_2O production (Davidson et al., 2000). Furthermore, N_2O emissions are related to soil water content (Davidson, 1991), with a threshold of 60% WFPS between water-limited and aeration-limited microbial processes. In the present work, during N_2O measurements, the soil WFPS remained between 45% and 60% (Appendix A Fig. S2), a range in which denitrifying microorganisms become more relevant for N_2O release. Therefore, we argue that the increment in N_2O emissions from soils in the sorghum–wheat rotation is due to an enhanced heterotrophic denitrification because of a greater carbon availability, as larger portions of labile carbon substrates promote denitrification reactions (Surey et al., 2020). This greater abundance of heterotrophic denitrifiers in sorghum–wheat rotations was evidenced by the increased abundance of nitrite reductase (NIR) enzyme containing denitrifying bacteria (Fig. 4a and b). The Control and AS treatments from the sorghum–wheat rotation showed a 56% and 73% increase in *nirK* abundance compared to the equivalent treatments on the fallow–wheat rotation (Fig. 4a). On the other hand, the abundance of *nirK* was not affected by any of the treatments (with or without nitrogen) on the fallow–wheat rotation, but the AS+DMPP treatment on the sorghum–wheat rotation had a lower *nirK* abundance than the Control and AS treatments. Comparing crop rotations in the case of *nirS* abundance, an increase of 30% was only significant for the AS treatment (Fig. 4b). Similarly to *nirK*, *nirS* abundance was not affected by fertiliser treatment in the fallow–wheat rotation, but the AS+DMPP treatment presented a lower level than the Control and AS treatments for the sorghum–wheat rotation. In this case, regarding N_2O-reducing bacteria, neither the crop rotations nor the N treatments affected both *nosZI* and *nosZII* genes abundances (Fig. 4c and d).

In contrast to the soil mineral nitrogen, the changes in the abundances of N-related microorganisms were sensitive enough to detect the effects of using a cover crop. In soil with fallow–wheat rotation, AS treatment presented the highest *amoA*/*nirK* and *amoA*/*nirK*+*nirS* ratios of all three treatments (Table 2). This means that those soils were more balanced towards nitrification since the addition of N-fertilisation increases the level of nitrification genes (Ouyang et al., 2018). In the same manner, there was a higher ratio between N_2O production in denitrification and N_2O reduction (*nirK*+*nirS*)/(*nosZI*+*nosZII*). On the other hand, the addition of DMPP decreased *amoA* abundance, which is why this treatment presented the lowest *amoA*/*nirK* and *amoA*/*nirK*+*nirS* ratios. Fertiliser treatment did not affect the nitrifying/denitrifying ratios in the sorghum–wheat rotation. Nevertheless, the type of crop rotation influenced these ratios, as there were significant differences between them. As has been mentioned before, the higher availability of soil carbon due to sorghum cover crop residues might be responsible for an increase in denitrification reactions (Palmer and Horn, 2015; Surey et al., 2020). Then, the alleged increase of denitrifying microorganisms containing NIR enzyme because of sorghum stover (Fig. 4a and b), balanced the *amoA*/*nirK* and *amoA*/*nirK*+*nirS* ratios towards denitrification. This produced a 60% reduction in the *amoA*/*nirK* ratio and 55% in the *amoA*/*nirK*+*nirS* ratio for the Control treatment on the

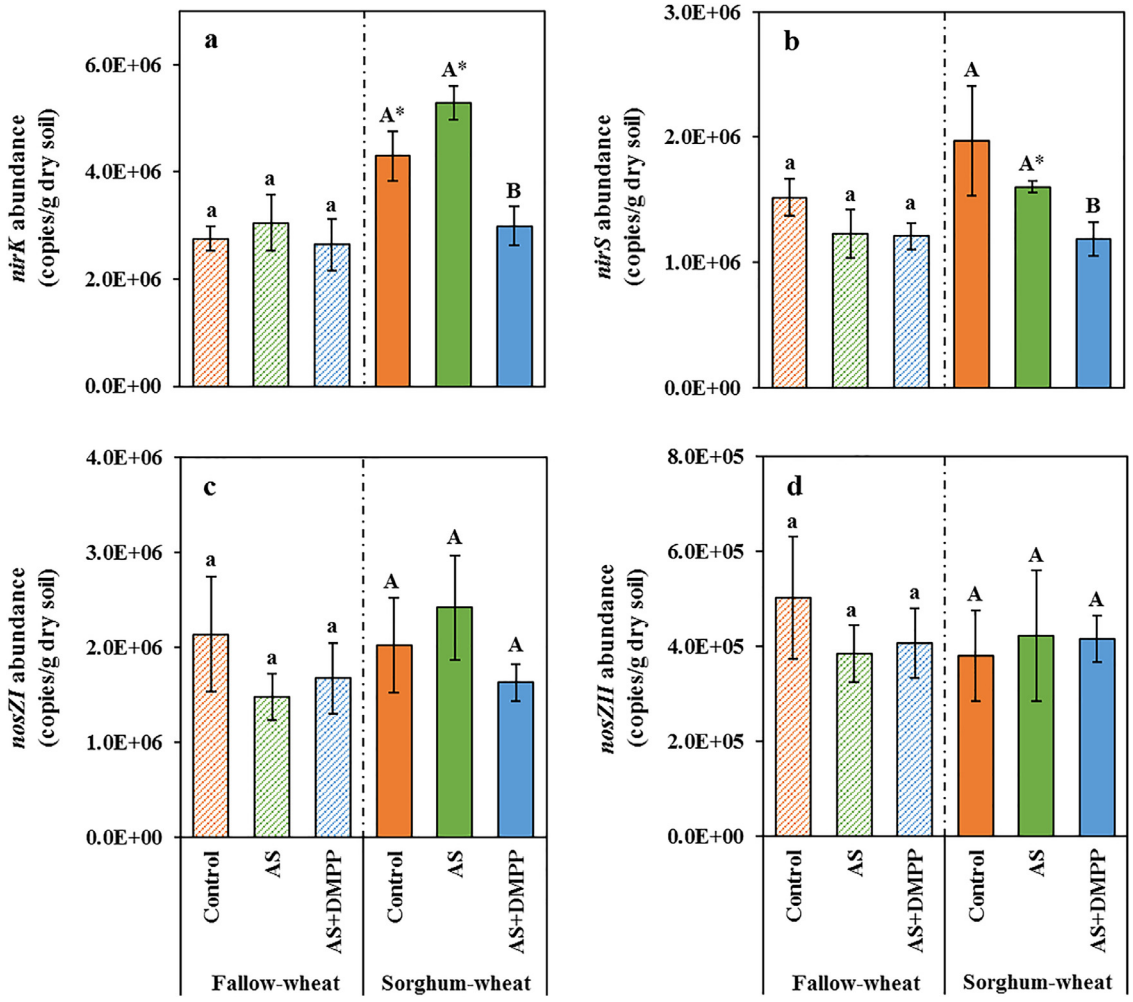


Fig. 4 – Abundance of denitrifying bacteria measured as the abundance of *nirK* (a), *nirS* (b), *nosZI* (c) and *nosZII* (d) genes at 10 days post-fertilisation (DPF) on soil of wheat crop. Significant differences between treatments of "Fallow-wheat" rotation are marked with lowercase letters. Significant differences between treatments of "Sorghum-wheat" rotation are marked with capital letters. For both ANOVA, the Duncan Test was used ($p < 0.05$; $n = 4$). The Mann-Whitney U test was used for the comparison between crop rotations within the same fertilization treatment. Significant differences at $p < 0.05$ are marked with an asterisk (*).

Table 2 – The *amoA/nirK*, *amoA/(nirK+nirS)* and *(nirK+nirS)/(nosZI+nosZII)* ratio on soil of wheat crop.

		amoA/nirK		*amoA/(nirK+nirS)*		*(nirK+nirS)/(nosZI+nosZII)*	
Fallow-wheat	Control	2.76 ± 0.44	ab	1.77 ± 0.24	ab	1.49 ± 0.25	b
	AS	3.37 ± 0.45	a	2.79 ± 0.61	a	2.27 ± 0.15	a
	AS+DMPP	1.86 ± 0.29	b	1.25 ± 0.19	b	2.36 ± 0.18	a
Sorghum-wheat	Control	1.11 ± 0.14	A*	0.78 ± 0.14	A*	2.45 ± 0.14	B*
	AS	1.64 ± 0.09	A*	1.26 ± 0.05	A*	3.00 ± 0.24	A*
	AS+DMPP	1.82 ± 0.32	A	1.29 ± 0.21	A	2.32 ± 0.13	B

Significant differences between treatments of "Fallow-wheat" rotation are marked with lowercase letters. Significant differences between treatments of "Sorghum-wheat" rotation are marked with capital letters. For both ANOVA, the Duncan Test was used ($p < 0.05$; $n = 4$). The Mann-Whitney U test was used for the comparison between crop rotations within the same fertilization treatment. Significant differences at $p < 0.05$ are marked with an asterisk (*).

sorghum–wheat rotation compared to fallow–wheat. Furthermore, the reduction of the AOB population in the AS treatment (Fig. 2b) due to the potential release of BNIs from sorghum roots also contributed to the decrease in *amoA*/*nirK* and *amoA*/*nirK*+*nirS* ratios with a 51% and 54% reduction, respectively, compared to the fallow–wheat rotation. Moreover, although the abundances of *nirK* and *nirS* might increase due to the extra carbon, no effects could be observed on the abundance of *nosZI* and *nosZII* genes. We theorize that, somehow, the use of sorghum as a cover crop favoured a scenario of incomplete denitrification, which increased the emission of N_2O due to the lack of increase of the microorganisms that could reduce it completely to N_2.

Consequently, focusing on the capacity to modify the nitrifying/denitrifying ratio of the different crop rotations, we might develop a better understanding of how soil N emissions respond, such as the different N_2O emissions for the AS treatment. However, since the AS+DMPP treatment significantly inhibited AOB growth (Fig. 2b), soil NO_3^- formation diminished compared to the AS treatment and there was no increase in *nirK* and *nirS* genes (Fig. 4a and b), ultimately resulting in similar nitrifying/denitrifying ratios between crop rotations. In addition, AS+DMPP treatment showed the lower (*nirK*+*nirS*)/(*nosZI*+*nosZII*) ratio, resulting in a better balance between N_2O production/N_2O reduction. We therefore suggest the use of synthetic NIs such as DMPP to reduce the pollution derived from the use of sorghum as a cover crop. Moreover, Menéndez et al. (2012) reported that the reduction in N_2O emissions induced by DMPP is conditioned by the magnitude of the losses from the fertiliser without NIs. Thus, DMPP can counteract higher N_2O emissions with greater efficiency, as can be observed in our experiment, with a 79% reduction of N_2O emissions with respect to AS in the fallow–wheat rotation versus 86% in the sorghum–wheat rotation (Fig. 3b).

2.3. *Yield parameters are not affected by the type of rotation*

Planting winter wheat after a sorghum crop is a common practice, yet it can affect the settlement of wheat seed. Guenzi et al. (1967) demonstrated that water extracts from sorghum residues could inhibit corn and wheat seed germination. This effect is due to the allelopathic substances, such as sorgoleone, that sorghum releases through its roots. Roth et al. (2000) suggested that soil management is the key to counteracting these effects. In soils with conventional tillage management, sorghum exudates are quickly solubilised and degraded. In no-tillage soils, by contrast, sorghum remains releasing their degradation compounds gradually and therefore affect crop yield. Even so, the effects of sorghum residues on wheat seed germination can be mitigated by increasing the seeding rate or delaying the planting of subsequent crops until the residues have decomposed or weathered (Weston et al., 2013). In our experiment, the wheat sowing density was 220 kg/ha, which seems high enough to palliate the effects of the previous sorghum crop since the type of crop rotation did not affect the wheat grain yield of fertilised treatments. As expected, wheat grain yield was much higher for the AS and AS+DMPP treatments with 6616 and 6133 kg/ha, respectively, for the fallow–wheat rotation and 6230 and 6543 kg/ha for the sorghum–wheat rotation (Appendix A Table S2), surpassing the average for that region in 2019, which was 5000 kg/ha (MAGRAMA, 2019). Similarly, the use of SNIs did not affect grain yield, which was in line with other works where DMPP maintained the grain yield of rainfed winter cereals compared to fertiliser treatments without inhibitor (Arregui and Quemada, 2008; Huérfano et al., 2016). Furthermore, the number of spikes/m^2 was also higher in the AS and AS+DMPP treatments in both crop rotations than in the Control treatments. However, even though crop rotations did not influence the grain yield of fertilised treatments, this was not the case for the Control treatment, which presented a decrease in wheat grain yield in the sorghum–wheat rotation (Appendix A Table S2). This decrease may be due to the competition for nutrients between plants and soil microbes in soils with a low nitrogen content. It is assumed that heterotrophic soil microorganisms are stronger competitors for inorganic N than plants (Kaye and Hart, 1997). Moreover, the growth of these microorganisms is carbon-limited. Thus, when soil mineral nitrogen increased in the Control treatments, such as between 0 and 10 DPF when mineralisation can be observed (Fig. 1a and b), the additional C from sorghum stover led to an increase in the total bacterial abundance compared to the fallow–wheat rotation (Fig. 2a), and therefore to a greater competition against the plants for soil nitrogen uptake. In addition, at these stages for wheat plants grown in a sorghum–wheat rotation, the reduction in N uptake was evident as the number of spikes/m^2 in the Control treatment of the sorghum–wheat rotation was lower than those for the fallow–wheat rotation. Despite this, there were no significant differences between fertiliser treatments or between crop rotations on the number of grains/spike and the percentage of grain protein (Appendix A Table S2). Furthermore, attending to the nitrogen use efficiency (NUE), there were also no differences between fertilisation treatments or crop rotations. Finally, to evaluate the N_2O efficiency of cropping systems and develop strategies for optimal crop productivity, and hence minimise environmental contamination, it may be more informative to express N_2O emissions in relation to crop productivity (YSNE) (van Groenigen et al., 2010; Schwenke and Haigh, 2016). The AS treatment applied to both crop rotations presented an increased YSNE compared to the Control and AS+DMPP treatments, which were equally low. These treatments did not show any differences between the two crop rotations, but fertilising wheat with AS in the sorghum–wheat rotation had a 93% higher YSNE than wheat fertilised with AS in the fallow–wheat rotation. Thus, when sorghum was used as a cover crop, it produced twice the N_2O emissions/kg of nitrogen uptake because it increased the gaseous nitrogen losses.

3. Conclusions

The use of sorghum in a crop rotation might not be a suitable option to mitigate the nitrogen losses, such as N_2O emissions, derived from the nitrogen fertiliser application in the subsequent culture. Sorghum–wheat rotation did not present any effect on the maintenance of soil NH_4^+ content during wheat crop development, as the levels were the same as the fallow–wheat rotation. Although the potential release of BNIs

from sorghum roots produced a 22% decrease in the growth of AOB in the AS treatment compared to the fallow–wheat rotation, this did not lead to lower the nitrogen losses through N_2O emissions. We theorize that the 77% increase in cumulative N_2O emissions for the AS treatment applied to the sorghum–wheat rotation was the result of the increase of heterotrophic denitrification due to a higher carbon availability from sorghum stover, since *nirK* and *nirS* genes were 73% and 30% more abundant compared to the fallow–wheat rotation. Moreover, while the type of crop rotation did not affect wheat grain yields, the higher cumulative N_2O emissions of the AS treatment on the sorghum–wheat rotation produced a 93% increase in the emissions of N_2O/kg of nitrogen uptake compared to the fallow–wheat rotation. However, we suggest the use of synthetic NIs such as DMPP to avoid the greater N_2O release derived from the use of sorghum as a cover crop. The application of DMPP maintained AOB growth at the levels of the unfertilised soils, thereby delaying soil NH_4^+ oxidation. As more soil NH_4^+ content was maintained, soil NO_3^- formation was diminished compared to AS, thus mitigating the increase of *nirK* and *nirS* genes resulting from the higher carbon availability in the sorghum–wheat rotation. The cumulative N_2O emissions were also maintained at the levels of the unfertilised soils. Therefore, as grain yield was not affected by the use of the synthetic NI, the AS+DMPP treatment yielded reductions of 73% and 86% in the emissions of N_2O/kg of nitrogen uptake compared to the AS treatment in the fallow–wheat and sorghum–wheat rotations, respectively.

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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Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.jes.2022.04.035.

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