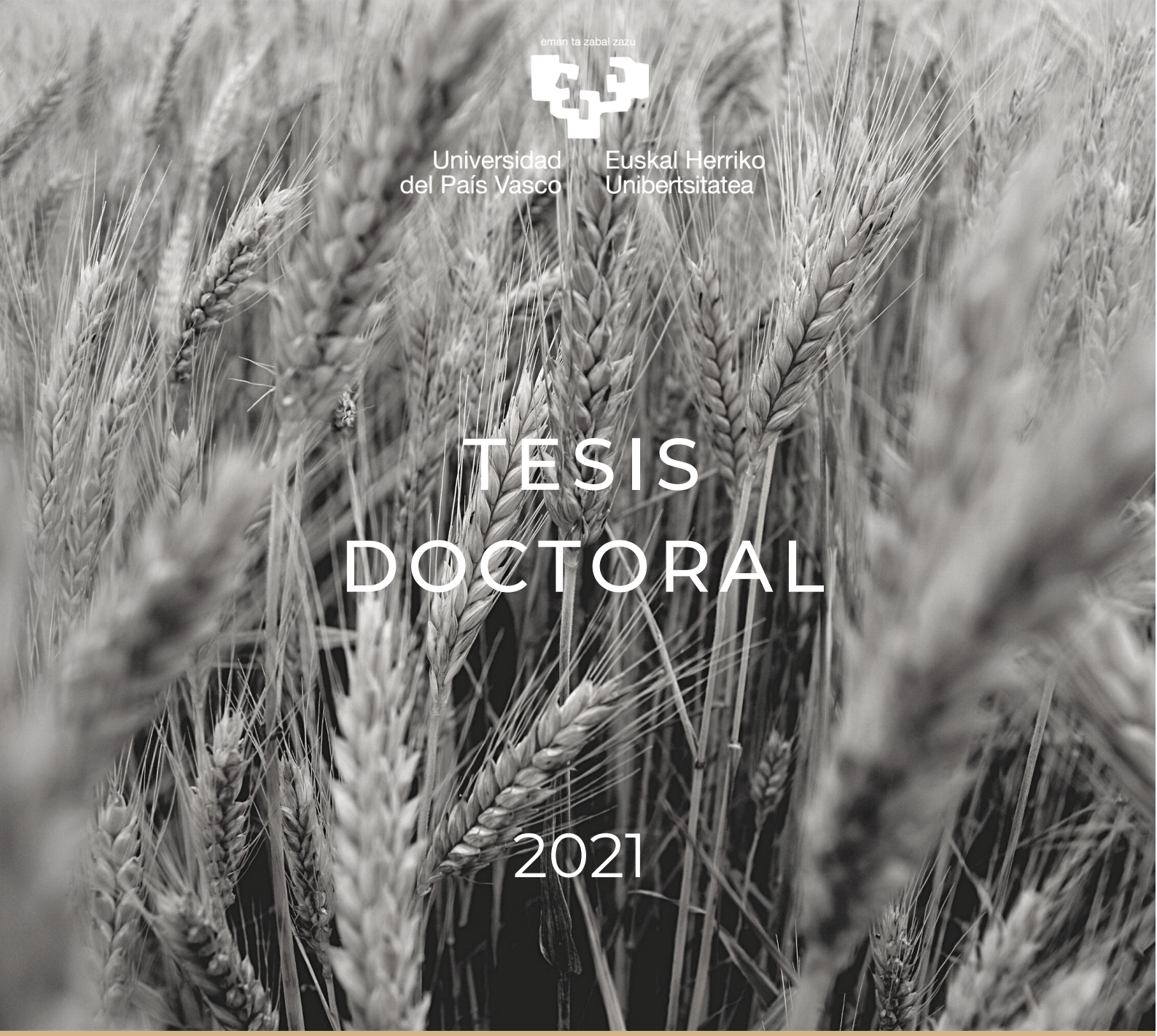

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TESIS DOCTORAL

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USE OF DMPSA NITRIFICATION INHIBITOR AND NO-TILLAGE SYSTEMS TO ENHANCE AGRICULTURAL SUSTAINABILITY: IMPACT ON SOIL NITROGEN GASEOUS EMISSIONS AND BACTERIAL POPULATIONS

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FACULTAD DE CIENCIA Y TECNOLOGÍA

Use of DMPSA nitrification inhibitor and no-tillage systems to enhance agricultural sustainability: effect on soil nitrogen gaseous emissions and bacterial populations

TESIS DOCTORAL

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DIRECTORES

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2021

No son pocos los que buscan por propia voluntad ese estado de muerte cerebral, ya que no hay duda de que así es más cómodo. No necesitan devanarse los sesos con complicaciones, sólo tienen que obedecer lo que les dicen los de arriba.

Haruki Murakami (1Q84)

Cuando el conocimiento gana en claridad y se incrementa la consciencia, también aumenta la angustia.

Arthur Schopenhauer (El mundo como voluntad y representación)

Uno será suficientemente afortunado si queda todavía algo por desear y anhelar, para que se mantenga el juego del perpetuo tránsito desde el deseo a la satisfacción y de ésta a un nuevo deseo -tránsito que se llama felicidad cuando es ágil y sufrimiento cuando es lento-

Arthur Schopenhauer (El mundo como voluntad y representación)

AGRADECIMIENTOS

Los trabajos realizados en esta tesis doctoral se han llevado a cabo mayoritariamente en el Laboratorio de Fisiología Vegetal de la Universidad del País Vasco (UPV/EHU). Para esta investigación, se ha operado con la financiación del Gobierno de España a través de los proyectos AGL2015-64582-C3-2-R (MINECO/FEDER) y RTI2018- 094623-B-C21 (MCIU/AEI/FEDER, UE), del Gobierno Vasco mediante el proyecto IT-932-16, y de la empresa EuroChem Agro Iberia S.L. El autor de esta tesis ha contado con un Contrato Predoctoral para la Formación de Doctores del Ministerio de Economía y Competitividad (BES-2016- 076725).

El carácter interdisciplinar de los estudios que se han planteado en esta tesis generó la oportunidad de establecer colaboraciones con otros grupos de investigación dentro y fuera de la UPV/EHU. Creo que el hecho de haber trabajado con grupos de diferentes campos de especialización es lo que realmente ha enriquecido esta tesis y es una de las cosas de las que me siento más orgulloso. No quería pasar por alto la oportunidad de resaltar a cada uno de ellos:

Para asesorarnos en el campo de la química, tuvimos el privilegio de colaborar con el Dr. Luis Lezama y el Dr. Beñat Artetxe, investigadores del departamento Química Inorgánica de la UPV/EHU. Los resultados de esta colaboración dieron lugar a la publicación de un artículo científico, que se recoge en el Capítulo 3 de esta tesis.

Con el fin de realizar análisis de secuenciación genética, tuvimos la fortuna de contar con la guía de la Dra. Andone Estonba y la Dra. Iratxe Zarraonaindia, investigadoras del departamento de Genética, Antropología Física y Fisiología Animal de la UPV/EHU. Esta colaboración resultó en la publicación de dos artículos científicos, que se exponen en el Capítulo 4 y el Capítulo 5.

Para mi estancia internacional, contamos con el entusiasmo de la Dra. Cristina Cruz, investigadora del departamento de Biología Vegetal de la Universidad de Lisboa, con quien diseñamos un experimento para analizar el efecto de los inhibidores de la nitrificación en los metabolitos del suelo, cuyos resultados esperamos publicar próximamente.

Además de las colaboraciones incluidas en este trabajo, tuve la suerte de poder participar en proyectos de otros grupos de prestigio internacional liderados por el Dr. Antonio Vallejo, de la Universidad Politécnica de Madrid, y los Dres. Tom Beeckman y Hans Motte, del Vlaams Instituut voor Biotechnologie (VIB) (Universidad de Gante), a los cuales no quiero olvidar agradecer que depositasen en mí su confianza. Aunque estas colaboraciones no hayan formado parte de esta tesis, han contribuido indirectamente a mi formación como investigador.

He optado por no incluir agradecimientos personales en esta tesis, pues la verdad es que nunca he creído en este tipo de demostraciones públicas y puntuales, sino en el valor de lo privado y el día a día. Si bien no quisiera que esto se entendiera como un acto de arrogancia por mi parte. Nada más lejos de la realidad pensar que esta tesis ha salido adelante gracias y únicamente a mi trabajo y capacidad. Esta tesis no habría sido en absoluto posible sin la inestimable ayuda de muchas personas a lo largo de estos cuatro años, lo cual tengo y tendré muy presente. Gracias a ellos, los momentos difíciles no lo han sido tanto, y puedo presumir de haber realmente disfrutado de este periodo hasta el punto de que se me haya hecho corto. Espero, sinceramente, haber sabido mostraros mi aprecio y agradecimiento a lo largo de todo este tiempo.

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RESUMEN

El incremento de la presión alimentaria como consecuencia del aumento y desarrollo de la población ha obligado a una intensificación de la agricultura. La necesidad de aplicar fertilizantes nitrogenados para obtener mayores producciones ha generado un aumento del nitrógeno reactivo presente en el medio, desencadenando problemas ambientales como el incremento del efecto invernadero, la reducción de la biodiversidad, la acidificación de los suelos y la contaminación de las aguas subterráneas. En un escenario global de emergencia climática, en el que las políticas se están enfocando hacia medidas para reducir el impacto ambiental de la actividad humana, el incremento de la sostenibilidad de la agricultura juega un papel fundamental.

El uso de inhibidores sintéticos de la nitrificación permite reducir las pérdidas de nitrógeno reactivo, disminuyendo la liberación de N_2O, uno de los gases que más contribuye al calentamiento global. Es necesario conocer la eficiencia de estos compuestos en conjunto con otros manejos para mejorar la sostenibilidad como son los sistemas de no laboreo, de gran interés en clima Mediterráneo, así como es imprescindible garantizar la seguridad de su aplicación para la salud del suelo.

En esta tesis se analiza la eficacia del inhibidor de la nitrificación 2-(3-4-dimetil-1H-pirazol-1-yl)-ácido succínico en mezcla isomérica (DMPSA), en combinación con sistemas de no laboreo para aumentar la sostenibilidad de la agricultura. Los resultados obtenidos demuestran que la aplicación de DMPSA evita la emisión de N_2O derivada de la fertilización nitrogenada en suelos no labrados, sin efectos negativos en la producción y sin causar un impacto profundo en la comunidad bacteriana. Sin embargo, su aplicación puede aumentar el riesgo de volatilización de NH_3 cuando se fertiliza con urea, lo cual puede ser evitado haciendo una aplicación conjunta con el inhibidor de la ureasa triamida N-(n-butil) tiofosfórica (NBPT). Nuestros estudios indican que la aplicación del DMPSA es especialmente recomendable cuando el suelo tiene contenidos relativamente altos de agua, cobre y/o zinc, condiciones que promueven las pérdidas de N_2O.

En conjunto, esta tesis concluye que la aplicación del inhibidor DMPSA es una técnica útil y segura para incrementar la sostenibilidad de la agricultura mediante la mitigación del impacto ambiental derivado de la fertilización nitrogenada.

ABSTRACT

Increased pressure for food production as a result of population growth and development has forced an intensification of agriculture. The need for applying nitrogen fertilizers to obtain higher yields has generated an increase in the reactive nitrogen present in the environment, triggering environmental issues such as the increase in the greenhouse effect, reduction of biodiversity, soil acidification, and groundwater contamination. In a global-climate-emergency scenario, in which policies are implementing measurements to reduce the environmental impact of human activities, increasing agriculture sustainability plays a key role.

The use of synthetic nitrification inhibitors reduces reactive nitrogen losses, diminishing N_2O releasing, one of the gases with greater contribution to global warming. It is necessary to assess the efficiency of these compounds in combination with other managements for improving sustainability such as no-tillage systems, of great interest in Mediterranean climate, as well as it is essential to guarantee the safety of their application for soil health.

This thesis analyzes de effectiveness of the nitrification inhibitor formed by the isomeric mixture of 2-(3,4-dimethyl-1H-pyrazol-1-yl)-succinic acid (DMPSA), in combination with no-tillage systems to increase the sustainability of agriculture. The results obtained show that DMPSA application avoids the N_2O emissions derived from nitrogen fertilization in non-tilled soils, without negative effects on crop yield and quality, and without causing a drastic impact on the bacterial community. However, its application can increase the risk of NH_3 volatilization when fertilizing with urea, which can be counteracted by co-applying the urease inhibitor N-(n-butyl) thiophosphoric triamide (NBPT). Our studies indicate that DMPSA application is especially advisable in soils with relatively-high water, copper and/or zinc contents, conditions that promote N_2O losses.

Overall, this thesis concludes that DMPSA application is a useful and safe practice for increasing agriculture sustainability by mitigating the environmental impact derived from nitrogen fertilization.

INTRODUCTION

1. GREAT NEEDS ENTAIL GREAT COMMITMENTS

The human population growth rate peaked in the 1960s, reaching an annual increase of 2%. Although the growing rate has declined to 1.2% per year, the world is heading towards a total population of 9.7 billion in 2050 and 11.2 billion in 2100 (UN, 2017).

To meet the growing demand for food, feed and biofuel, agriculture will need to produce almost 50% more than in 2012 (Alexandratos and Bruinsma, 2012). To do so, projections indicate a need of 100 million ha of additional agricultural soils (FAO, 2017). However, it will be very difficult to increase the agricultural area, since most of the available land is not suitable for agriculture and its use would imply great environmental, social and economic damage. Moreover, agriculture is responsible for about 80% of deforestation worldwide (FAO, 2014). This forest-land destruction entails the decrease of animal and plant biodiversity, increases soil erosion and is a source of greenhouse gases (GHG) emission. This means that future needs should be addressed by intensifying agricultural production on already-available lands. However, these lands are already in a compromised situation, with ~33% of the world's agricultural soils in moderately to highly degraded conditions due to the unsustainable competition for scarce resources. At the same time, this intensification can lead to water scarcity, since in some areas up to 80–90% of the water is used for agricultural purposes (FAO, 2011). This challenge is even exacerbated when considering the effects that climate change will have on agriculture, according to the projections of the IPCC. In this sense, it is expected a negative impact on agricultural yield, especially in low latitudes due to a higher frequency of extreme events (Fig. 1) (IPCC, 2007; Porter et al., 2014). Nonetheless, IPCC is moderately confident that agronomic adaptation can improve yields by about 15–18%.

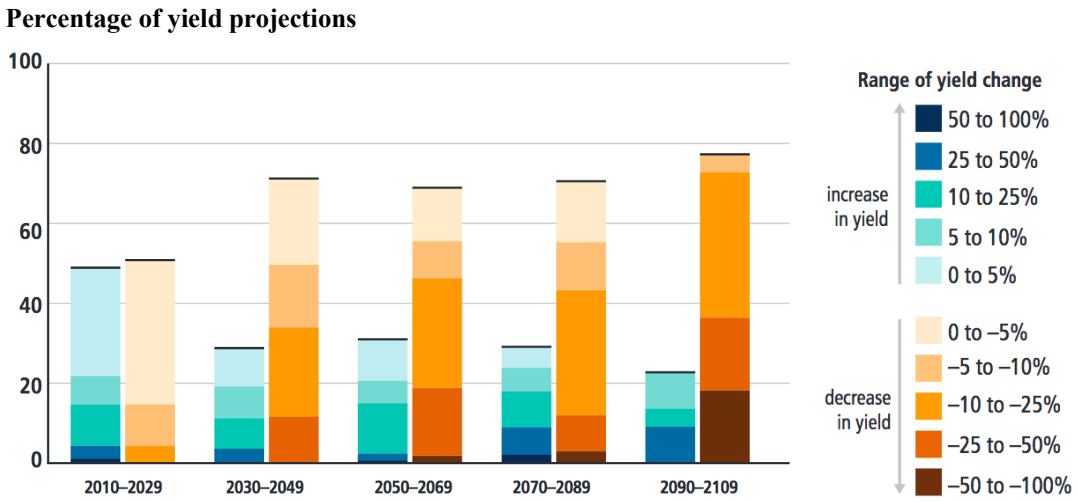


Figure 1. Projected changes in crop yields owing to climate change over the 21st century. *Changes in crop yields are relative to late-20th century-levels. The figure includes projections for different emission scenarios, different regions and adaptation and no-adaptation-policies cases combined (n = 1090) (Adapted from Porter et al., 2014).*

2. SO MUCH NITROGEN, SO DIFFICULT TO RETAIN

Crops yield depends to a long extent on the availability of nitrogen (N) for plants. Despite N_2 is the majority element in the atmosphere, many ecosystems are limited by N (Vitousek et al., 2002). N_2 is unavailable to most organisms because of the strength of the triple bond holding the N atoms, and it should be first converted to reactive N (Nr). Atmospheric deposition and biologic fixation are the only natural ways for N input to the soil (Fig. 2). The first accounts for a global average input of 1.8 kg N ha^{-1} $year^{-1}$ (Ackerman et al., 2016), while a total of 50–70 Tg N may be fixed annually by biological agents in agricultural systems (Herridge et al., 2008).

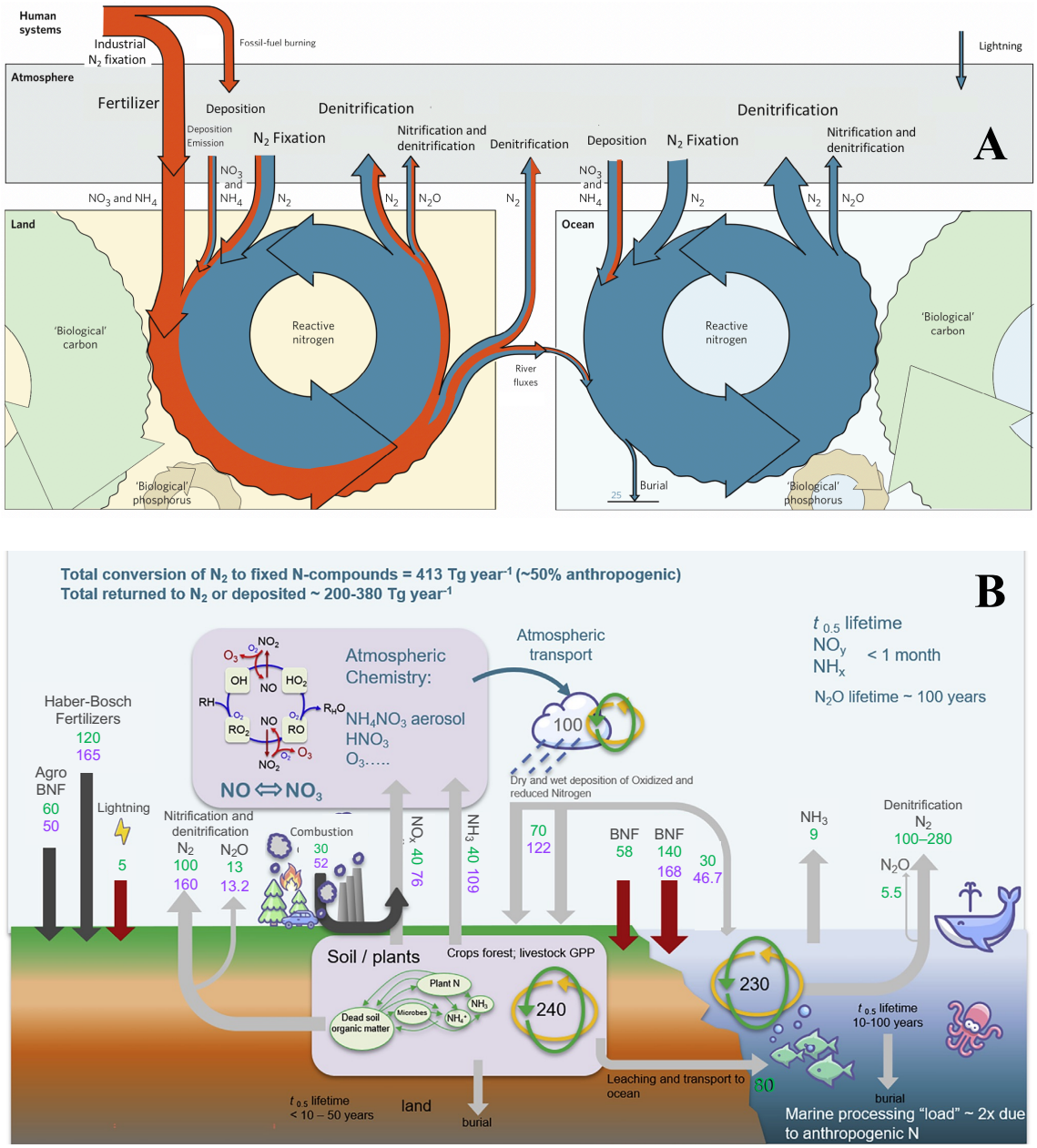
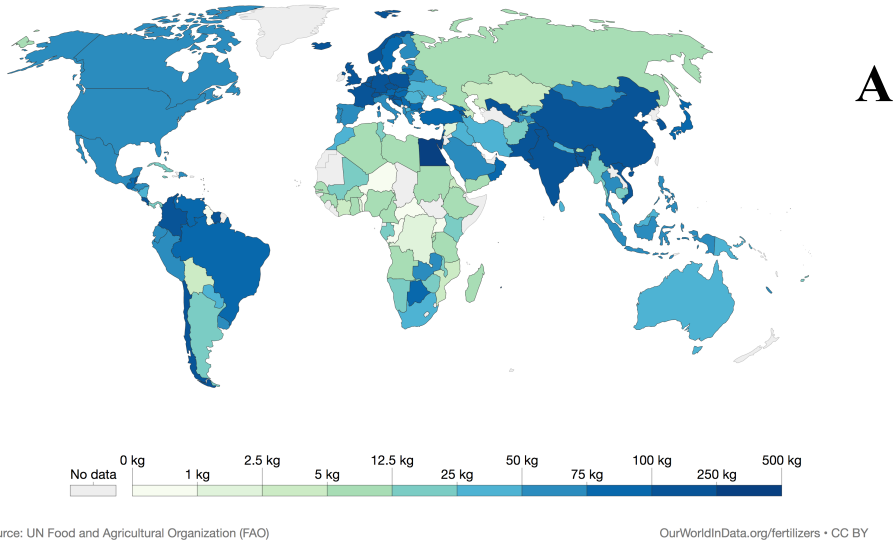


Figure 2. Components and fluxes of the global nitrogen cycle. A) Schematic representation of anthropogenic perturbation in relation to natural (unperturbed) fluxes*. Blue, natural fluxes; Orange, anthropogenically perturbed fluxes. Edited from Gruber and Galloway, 2008.* ***B) Estimation of fluxes in Tg N year^{-1}****. Numbers in green indicate fluxes in the early 2000s (Fowler et al., 2013). Numbers in purple indicate estimated fluxes for 2050 (Galloway et al., 2004). BNF, biological nitrogen fixation (Agro, BNF within agroecosystems); GPP, gross primary productivity. Edited from MacFarlane et al., 2020.*

It was in 1913 when a disruptive new development gave humanity the capacity to produce Nr in the form of ammonia (NH_3) from molecular nitrogen (N_2) and dihydrogen (H_2) through the Haber-Bosch process, which changed the history of food production. The system has evolved from the original capacity of 5 Mg NH_3 day^{-1} of a single-set equipment to the current 2200 Mg, consuming 2% of the total world energy supply and contributing to 1.6% of global CO_2 emissions (more than 400 Mt CO_2) (Chen et al., 2019). Since the development of the Haber-Bosch process, crop yields have increased, supporting further population growth.



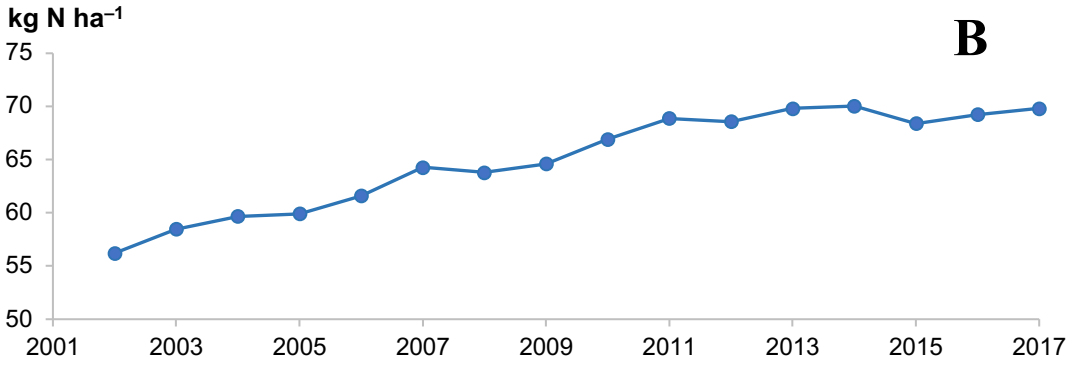


Figure 3. Nitrogen fertilizer use in 2017. A) Application per hectare of cropland. B) World average evolution from 2002 to 2017 *(FAO, 2020). (Adapted from www.ourworldindata.org).*

IN 2017, the global fertilizer application rate was about 70 kg N ha^{-1} $year^{-1}$. And the consumption rate will continue increasing in the next decades to reach 109 kg N ha^{-1} $year^{-1}$ in 2050 (Figs. 3 and 4) (Alexandratos and Bruinsma, 2012; FAO, 2020). Considering that their production is highly polluting, it will be necessary to use them efficiently. However, it is difficult to retain in the soil the N applied in agriculture.

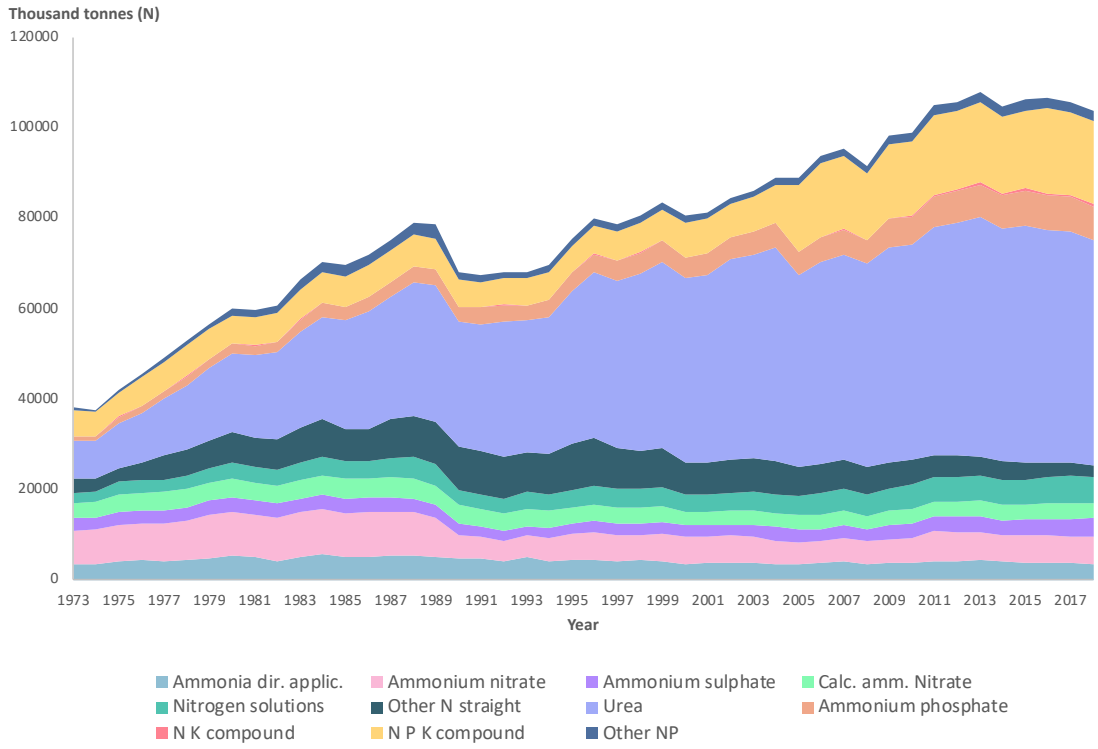


Figure 4. Evolution of world nitrogen-fertilizers consumption by product *(data source: IFA, 2021).*

3. PATHWAYS DETERMINING THE FATE OF NITROGEN IN SOILS

Nitrogen in the soil is subjected to a chain of redox reactions carried out by microorganisms, which obtain their energy through these processes (Fig. 5). These reactions are grouped into two main processes: nitrification, which encompasses oxidation reactions, and denitrification, during which reduction reactions take place. N fertilizers are also subjected to this microbial activity, entering into one step of the chain or another depending on the type of molecule applied. For instance, urea, the most-applied

fertilizer worldwide (Fig. 4), first undergoes the hydrolyzation of its molecule by the action of the extracellular enzyme urease, present in very diverse organisms (including several prokaryotes, fungi, algae and plants) (Mobley and Hausinger, 1989), to form ammonium (NH_4^+) (Table 1). In this reaction, protons are consumed, which triggers a rapid pH-increase in the area surrounding the urea granule (Ernst and Massey, 1960; Terman, 1979). The equilibrium between NH_3 and NH_4^+ in the soil depends on the pH and, in this manner, at higher pH it is driven to the form of NH_3.

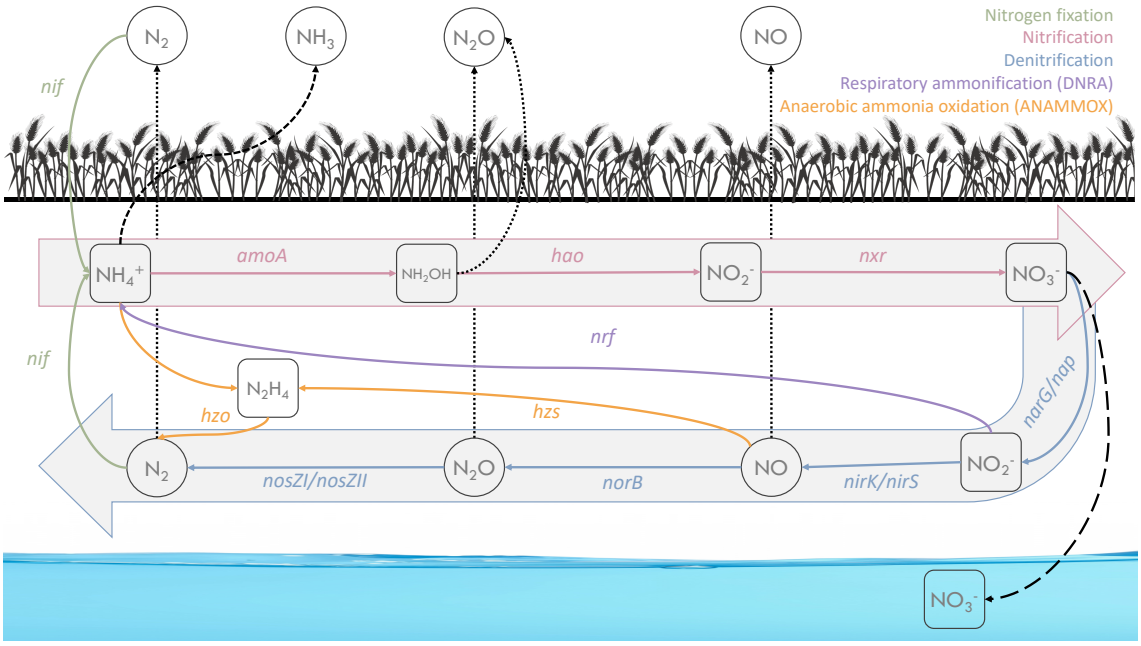


Figure 5. Main microbial nitrogen transformation pathways in the soil. *Italic text indicates the genes encoding for the enzyme involved in each respective stage.*

3.1. NITRIFICATION

$NH_3 \leftrightarrow NH_4^+$ is the initial substrate for nitrification, in which, under aerobic conditions, N is successively oxidized to finally form nitrate (NO_3^-). This process begins with the action of ammonia-oxidizing bacteria (AOB) and/or archaea (AOA), carrying out the oxidation of NH_3 to hydroxylamine (NH_2OH) and then to nitrite (NO_2^-) (Arp and Stein, 2003). The main genera involved in this process are *Nitrososphaera,* within AOA, and *Nitrosomonas* and *Nitrosospira* within AOB. In the case of AOB, the prevalence of

one or the other seems to be related with the soil pH. Thus, *Nitrosospira* is commonly predominant in acid soils while *Nitrosomonas* in alkaline (Li et al., 2017). AOB exhibit a greater response to fertilization by increasing their population and nitrification activity. On the contrary, AOA seem to be adapted to low-N availability and their abundance is less responsive. Although the knowledge about the peculiarities of AOA is still scarce, this suggests that nitrification is driven by AOB rather than AOA in the N-rich conditions of agricultural soils (Di et al., 2009; 2010). After the action of ammonia oxidizers, nitrite-oxidizing bacteria (NOB) such as *Nitrobacter* and *Nitrospira* (Daims et al., 2001), *Nitrotoga* (Alawi et al., 2007; Ishii et al., 2017) and *Nitrolancea* (Sorokin et al., 2012) oxidize NO_2^- to the final product of nitrification, NO_3^-. Within NOB, *Nitrospira* have a special relevance because they are able to perform both the oxidation of NH_3 and NO_2^- and, thus, perform the whole nitrification (complete NH_3 oxidation) through the pathway known as comammox (Daims et al., 2015; Van Kessel et al., 2015).

3.2. DENITRIFICATION

Unlike nitrification, in which only a few genera are involved, a large variety of organisms can participate in different steps of denitrification. The first step of this pathway, carried out under anaerobic conditions, is NO_3^- reduction to form NO_2^- and, afterward, NO_2^- is reduced to nitric oxide (NO). Due to its cytotoxicity, NO is rapidly transformed, giving rise to nitrous oxide (N_2O). The N_2O can be further reduced to form N_2 by complete denitrifying bacteria and archaea, but also by non-denitrifiers. This latest group encompasses a group of organisms, such as *Anaeromyxobacter*, which have been recently found to be capable of converting N_2O to N_2 but that lack other denitrification genes (Sanford et al., 2012; Graf et al., 2014).

3.3. OTHER OFFSHOOT PATHWAYS

In addition to these two great routes, there are other less studied processes modifying the state of N in the soil.

Low O_2 and C availability favor nitrifier denitrification, a pathway in which NO_2^- is reduced to NO and then to N_2O instead of being oxidized to NO_3^- (Wrage et al., 2001; Shaw et al., 2006).

NH_4^+ can be anaerobically oxidized by chemolithoautotrophic bacteria within *Planctomycetales* order in a process known as anammox (Kartal et al., 2011; 2013). By this way, NH_4^+ and NO_2^- are combined to form N_2, with hydrazine (N_2H_4) as intermediate. Although this process has a great importance in oceans, its contribution in agricultural is minor (Long et al., 2013; Zhu et al., 2018).

In addition to denitrification, NO_3^- can be also reduced through dissimilatory NO_3^- to NO_2^- and NH_4^+ (DNRA or respiratory ammonification). The relative importance of this process with respect to denitrification increases with higher C/NO_3^- ratios (Tiedje et al., 1983; Putz et al., 2018), although, generally, the contribution seems to be marginal (Rütting et al., 2011).

Additionally, in chemodenitrification, NO_2^- can react with reduced Fe to produce NO and N_2O (Coby et al., 2005; Buchwald et al., 2016).

Table 1. Main reactions within soil N cycle, enzymes and metal cofactors involved, cellular location and encoding gene.

Reaction	**Enzyme**	**Metal Cofactors**	**Location**	**Encoding gene**
$CO(NH_2)_2 + 3H_2O \rightarrow 2NH_4^+ + HCO_3^- + OH^-$	Urease[a]	2 Ni	Extracellular	*ureC*
$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$	Ammonia monooxygenase (AMO)[b]	9 Cu; 4 Fe; 3 Zn (*)	Membrane Cytoplasm	*amoA*
$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$	Hydroxylamine oxidoreductase (HAO)[c]	24 Fe	Periplasm	*hao*
$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$	Nitrite oxidoreductase (NXR)[d]	Fe; Mo (*)	Cytoplasm	*nxrA* *nxrB*
$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$	Nitrate reductase (Nar/Nap)[e]	21 Fe; 1 Mo	Membrane (Nar) Periplasm (Nap)	*narG*
$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$	Nitrite reductase (NirK/NirS)[f]	6–24 Cu (NirK) 4 Fe (NirS)	Periplasm	*nirK* *nirS*
$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$	Nitric oxide reductase (NorBC)[g]	3 Fe	Membrane	*norB*
$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$	Nitrous oxide reductase (N_2OR)[h]	12 Cu	Periplasm	*nosZI* *nosZII*

*a: Koper et al., 2004; Mazzei et al., 2020. b: Gilch et al., 2009a. c: Glass and Orphan, 2012; Caranto and Lancaster, 2017. d: Meincke et al., 1992. Sorokin et al., 2012. e: Glass and Oprhan, 2012; González et al., 2006. f: Glass and Orphan, 2012; Felgate et al., 2012. g: Hino et al., 2010. h: Richardson 2009; Glass and Orphan, 2012; Pauleta et al., 2013. *Molar stoichiometry*

4. MAIN ENZYMES INVOLVED IN THE NITROGEN CYCLE

Many of the enzymes involved in the N cycle contain transition metals as cofactors for electron transport or as catalytic centers at active sites (Glass and Orphan, 2012). Throughout nitrification and denitrification routes, iron (Fe), copper (Cu), molybdenum (Mo) and -probably- zinc (Zn) are required (Table 1). In addition, nickel (Ni) is essential for urease activity, which contain 2 Ni atoms linked by a carmate bridge, binding urea to only one of the Ni atoms (Mazzei et al., 2020).

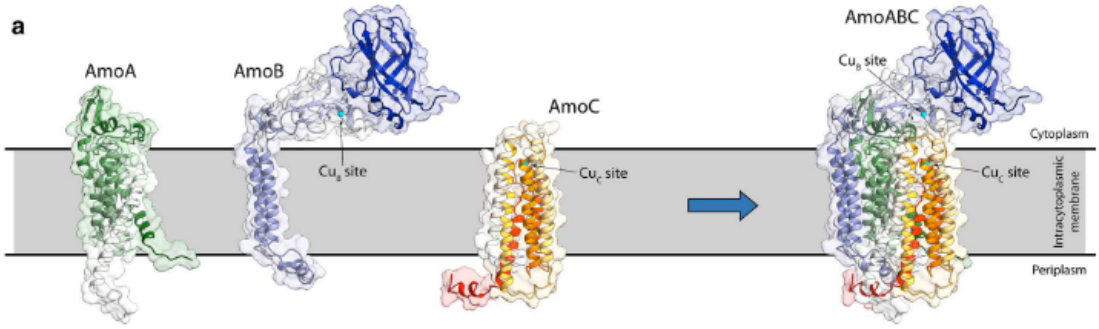


Fig. 6. Preliminary model of ammonia-monooxygenase enzyme (AMO) *(Musiani et al., 2020).*

Bacterial ammonia monooxygenase (AMO) is a membrane-bound enzyme (Fig. 6) that carries out the initial step of nitrification, in which NH_3 is converted to NH_2OH through the reductive insertion of a dioxygen-derived O atom in a N—H bond (Musiani et al., 2020). Its crystal structure has not been already solved, but it is known that this enzyme contains Cu (6 Cu^{2+} and 3 Cu^{+} ions), Fe (4 Fe^{3+}) and Zn (3 Zn^{2+}) (although it is not clear if the presence of Zn is just adventitious or it has any role in the enzymatic functioning) (Gilch et al., 2009a; 2010). Nonetheless, there seems to be differences between AOA, AOB and comammox AMO enzyme. For instance, archaeal AMO exhibits a higher NH_3 affinity and a lower maximum rate of NH_3 oxidation, resulting in a faster saturation under high NH_3 concentrations. Thus, although the abundance of AOA use to overpass AOB (Leininger et al., 2006), their contribution to nitrification is relatively low in agricultural soils (Kits et al., 2017). Furthermore, comammox AMO

might present a structural differentiation to conventional AOB displaying a higher substrate affinity, which provides advantage at very low NH_3 contents (Beeckman et al., 2018). Nitrification continues with NH_2OH oxidation to NO_2^- through the Fe-dependent hydroxylamine oxidoreductase (HAO). Two different models have been proposed for this step: in the 'single obligate intermediate model' AOB oxidize NH_3 to NO_2^- via a single intermediate, NH_2OH. However, this model is in doubt, since NO might be also an intermediate product ('NH_2OH/NO obligate intermediate model'), which would imply the existence of a third unknown enzymatic step (Caranto and Lancaster, 2017). Afterward, the formation of NO_3^- is catalyzed by the Fe-Mo nitrite oxidoreductase (NXR) harbored by nitrite-oxidizing bacteria (NOB) (Lücker et al., 2010).

When environmental conditions are oxygen-limited, some bacteria are able to respire NO_3^-, starting the denitrification route that will totally reduce NO_3^-, to the form of N_2, or partially, to an intermediate compound. NO_3^- is sequentially reduced first to NO_2^- by means of the nitrate-reductase enzyme (NarGH), which requires Fe and Mo, and then to NO by one of two different nitrite reductases, NirS (exhibits Fe cations) and NirK (displays Cu cations) (Fig. 7) (Glass and Orphan, 2012). Nir synthesis is co-regulated by the same transcription factor as nitric-oxide reductase enzyme (NorBC), to avoid NO cytotoxicity by accelerating its transformation into N_2O (Hutchings and Spiro, 2000; Bergaust et al., 2012).

Figure 7. Crystal structure of the hexameric Cu-Nitrite Reductase (NirK). *Purified from Hyphomicrobium denitrificans. Each monomer is represented by a discrete color. Spheres represent Cu atoms, accounting for a total of 18 atoms per enzyme (Nojiri et al., 2007).*

Although N_2O reduction to N_2 is highly favored thermodynamically, reduction-activation is difficult because N_2O binds very poorly to metal ions. This has been solved by nature towards the unique enzyme known to carry out this process: the nitrous-oxide reductase (N_2OR). N_2OR is a multi-copper enzyme (12 Cu ions) formed by two domains: an electron transferring domain that binds a binuclear Cu center (Cu_a) and a unique catalytic domain in biology, the Cu_z, which binds a tetranuclear Cu-S center (Fig. 8) (Richardson et al., 2009; Pomowski et al., 2011; Pauleta et al., 2013).

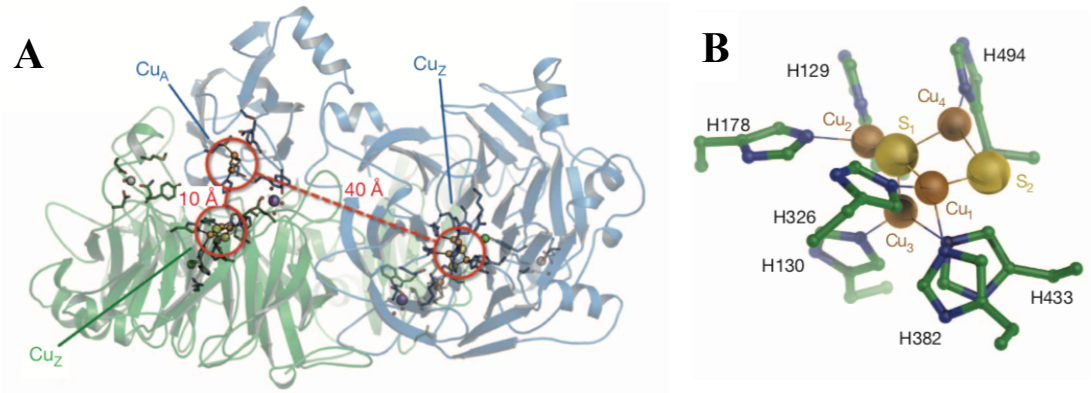


Figure 8. A) Crystal structure of the homodimeric nitrous-oxide-reductase enzyme (N_2OR). B) Detailed view of the tetranuclear Cu-site. *Purified from Pseudomonas stutzeri (Pomowski et al., 2011).*

It has been recently reported the existence of a so-called atypical N_2OR, harbored by denitrifying bacteria and archaea, but also by non-denitrifiers and organisms that perform DNRA. Although very little is still known about it, this atypical N_2OR may operate with greater efficiency than the typical N_2OR of complete denitrifiers (Sanford et al., 2012).

The genes encoding for these enzymes (Table 1) are used as molecular markers of the abundance (DNA quantification) and/or activity (RNA quantification) of the different organisms and steps within nitrification and denitrification.

4.1. DEPENDENCY ON RESOURCES AVAILABILITY

The need for metal cofactors makes these enzymes' activity dependent on the bioavailability of these elements. Some organisms have developed homologous proteins that can use different ions, such is the case of Fe-dependent Nir (NirS) and Cu-dependent Nir (NirK), which implies greater adaptability. However, other enzymes such as AMO and N_2OR are highly influenced by the availability of these ions, since there are non-known homologous.

Several factors determine metals availability. For instance, bioavailability decreases at alkaline pH and high organic C and clay content (Baker and Senft, 1995). About 40% of European arable soils present Cu deficiency (concentration < 2 mg Cu kg^{-1} soil), which can limit the activity of the enzymes involved in the N cycle (Thomson et al., 2012; Saggar et al., 2013). The correct functioning of AMO and, especially, N_2OR, demands high amounts of Cu, which has led some denitrifiers to develop mechanisms to even take up Cu strongly bound to natural ligands in very-poor-Cu environments (Twining et al., 2007). It has been reported and hormetic effect of Cu availability on AOB populations, which exhibit an increased growth up to contents of 300 mg Cu kg^{-1} soil, when Cu concentration becomes toxic (He et al., 2018; Liao et al., 2019). In the same manner, Cu addition exerts and hormetic effect on N_2OR synthesis and N_2O-reducers growth (Sullivan et al. 2013; Shen et al., 2020). Thus, it could be supposed that higher N_2O emissions as a result of an enhanced nitrification would be compensated by an also enhanced N_2O reduction to N_2.

Environmentally, it would be desirable a complete reduction of NO_3^- to N_2 to avoid leaks in form of NO and N_2O. However, 40% of denitrifiers lack N_2OR (Hallin et al., 2018), and N_2O reduction to N_2 is not always carried out -or is carried out without synchrony- even by organisms harboring this enzyme. This can imply higher N_2O emissions in soils with high Cu contents (He et al., 2018) (such as those receiving great amounts of Cu-based fungicides).

The complete reduction of NO_3^- to N_2 requires 10 electrons (8 e^- for NO_3^- reduction to N_2O and 2 e^- to reduce N_2O to N_2), generating a translocation of 30 protons for ATP synthesis (24 H^+ derived from NO_3^- to N_2O and 6 H^+ from N_2O to N_2) (Van Spaning et al., 2007). In some cases, bacteria performing denitrification prefer the use of NO_3^- over N_2O as an e^- acceptor. Thus, under rich electron-acceptor environments (>10 mg NO_3^- g^{-1} soil), such as some agricultural soils, there is little impact on the bioenergetics of the bacteria non-performing the last stage within denitrification, because NO_3^- can be further consumed to compensate the energy balance (Blackmer and Bremmer, 1978). Denitrifiers' metabolism might be further driven through this route in Cu-poor soils, as

this poses an additional difficulty for N_2OR functioning. As a consequence, in poor-Cu soils with great NO_3^- (conditions easily found in agricultural systems), there will be an imbalance between N_2O generation and N_2 reduction. On the contrary, in poor NO_3^- environments, there would be an advantage on reducing N_2O to N_2 if enough Cu is bioavailable (Felgate et al., 2012). Surprisingly, this decrease of N_2O reduction under high NO_3^- availability has not been observed in the case of atypical N_2OR, suggesting possible structural differences between typical and atypical N_2OR cluster (Sanford et al., 2012).

Less attention has been paid to the relationship between Fe availability and nitrifiers/denitrifiers growth, but preliminary studies suggest that Fe addition enhances nitrification (Glass and Orphan, 2012). Studies analyzing the importance of Zn content are also scarce (most analyzing inhibition by heavy metals in contaminated soils), since its implication within the N cycle is not clear. Some works have suggested that Cu and Zn may compete by the same binding site in AMO, which undergoes inhibition when Cu is displaced from its active site (Radniecki and Ely, 2008). However, related studies have reported hormetic effects on nitrification due to Zn application at low doses (Ruyters et al., 2010a; Chen et al., 2014), suggesting that it does seem to be necessary *per se* for AMO activity. On the contrary, Zn does not play any role in denitrification (Ruyters et al., 2010b; Chen et al., 2014). As a result, studies analyzing the effect of Zn addition to soils (a practice carried out for crop biofortification) reveal an increase in N_2O emissions (Montoya et al., 2021a), which implies that there may be a compromise between crop quality and the release of harmful gases.

5. ENVIRONMENTAL IMPACT DERIVED FROM GREENHOUSE GASES RELEASE AND NITROGEN LOSSES

The agricultural systems are facing a problem due to the low efficiency in the use of nitrogen (NUE) (a ratio between the crop N uptake and the total N supplied). The leaks during N transformation imply that not all the applied fertilizer is being assimilated by the crop, which is both economically and environmentally negative, especially

considering that most crops are overfertilized (i.e. receiving more N than they can actually use) (Anas et al., 2020). The greatest N losses take place in the form of NO_3^- and NH_3. NO_3^- can be easily lost through leaching due to its low adhesion to soil particles. Therefore, the losses are greater under humid climates than in drylands, but also depending on the season, irrigation, soil properties, plant species, fertilizer type and rate. Losses between 4 and 155 kg N ha^{-1} $year^{-1}$ have been reported in arable and horticultural crops (Cameron et al., 2013). When this NO_3^- reach lakes or rivers, it can contribute to eutrophication (Smith and Schindler, 2009).

NH_3 in the soil can easily scape to the atmosphere by volatilization as a consequence of the equilibrium between the gaseous phase of NH_3 and NH_3 in solution. When a higher NH_3 concentration is present in the soil solution (see section 3), also higher NH_3 is lost by volatilization (Harrison and Webb, 2001). The amount of N lost by this way largely depends on the environmental conditions, form of fertilizer application and soil properties. For instance, greater losses can be expected in compacted and alkaline soils, and windy and warm locations, reaching values ~30% of lost N (Silva et al., 2017; Cantarella et al., 2018). On average, it is estimate that about 18% of the N applied with fertilizers lost by NH_3 volatilization globally (Pan et al., 2016). This makes that agriculture accounts for about 75–85% of projected global NH_3 emissions throughout 2000–2050 (Reay et al., 2012). This is not only a damage for farmers economy, but also for the environment, since NH_3 is a precursor of fine particulate matter (Wang et al., 2016) and can cause acidification, eutrophication and N_2O emissions after deposition (Mosier et al., 1998; Galloway et al., 2008; Zhu et al., 2016). However, these indirect effects of NH_3 volatilization are rarely taking into account. The marginal human and environmental damage cost of the emission of NH_3 has been estimated in about 16–106 billion € only in the European Union (Van Grinsven et al., 2013).

During N transformation, N_2O can also be released to the atmosphere by nitrifiers denitrification, chemical decomposition of NH_2OH, and when it is not completely reduced to N_2 in denitrification (i.e. when N_2O is not processed by N_2OR). The magnitude of these losses depends on several factors (Cameron et al., 2013; Hu et al., 2015). For

instance, the soil water content, which is directly related with the O_2 availability in the soil, influences nitrification and denitrification functioning. In general, denitrification rates increase at higher soil moisture content, when O_2 becomes limited. Thus, the optimum condition for N_2O release from nitrification take place the when soil water-filled pore spaces (WFPS) is between 30–65% (Fig. 9). Above this level, N_2O production is dominate by denitrification (Braker and Conrad, 2011). In turn, different studies have indicate that N_2OR is more sensitive to O_2 than the previous enzymes within denitrification. In this manner, less N_2O is reduced to N_2 if conditions are not totally anoxic. In concordance, N_2O emissions are also related with the soil texture, so that denitrification use to be lower in soils with higher drainage (sandy soils) than in clay soils. Soil pH also displays an influence on N_2O denitrification but also in the ratio between N_2O and N_2. Denitrification rates are lower in agricultural soils with pH below 5 than in those with pH 6, and the $N_2O/(N_2O + N_2)$ ratio has a negative relationship at pH between 5 and 8. Thus, N_2O reduction to N_2 is favored in alkaline soils. In addition, there is also a relationship of nitrification and/or denitrification with the soil temperature and the organic carbon content.

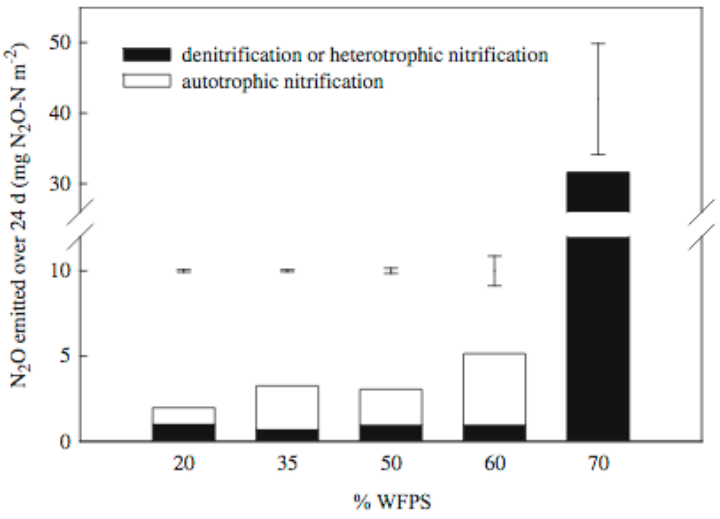


Figure 9. Relationship between soil water content and nitrification/denitrification contribution to N_2O emissions. *Cumulative N_2O-N emissions from soil at different water contents (expressed as water-filled pore spaces, WFPS) over 24 days after fertilization with $NH_4^+NO_3^-$ (Bateman and Bagss, 2005).*

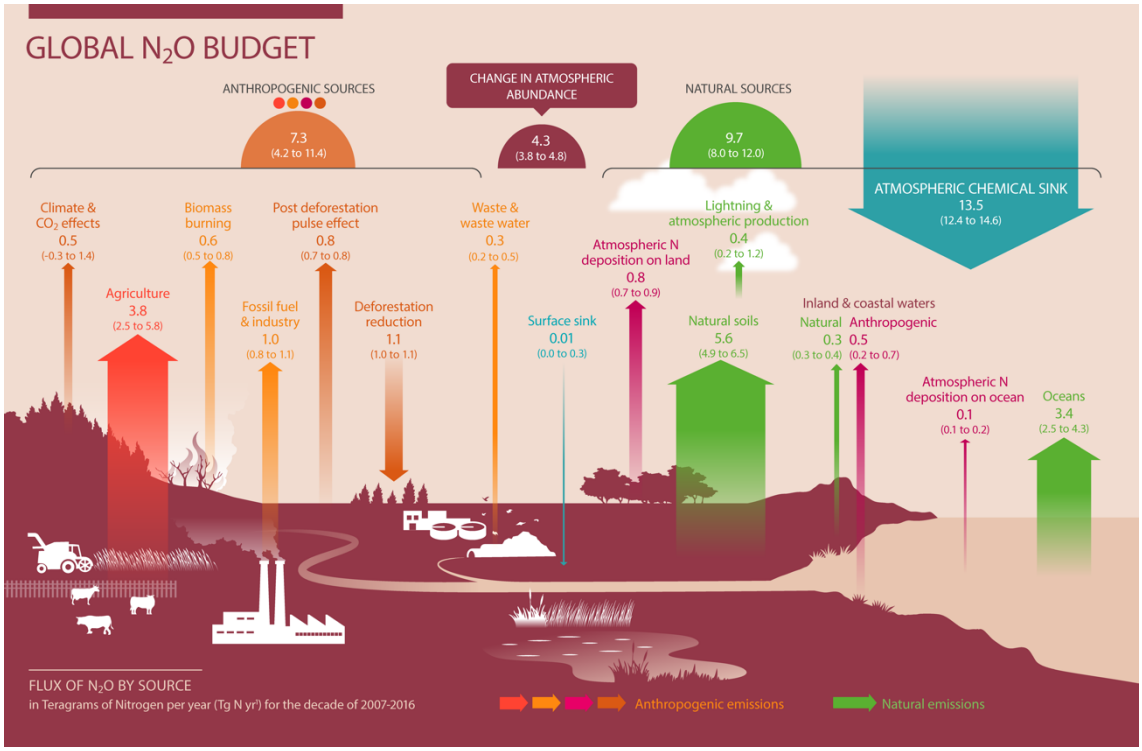


Figure 10. Flux of N_2O by source for the decade 2007-2016 *(Tg N year^{-1}) (Tian et al., 2020) (Source: The Global Carbon Project).*

The atmospheric concentration of N_2O has growth at a rate of 0.73 ppb per year over the last three decades (IPCC, 2014). Anthropogenic sources contribute to 43% of total N_2O emissions (Fig. 10) and, in the last decades, these emissions have increased by 30% (Fig. 11) (Reay et al., 2012; Tian et al., 2020). It is expected that N_2O emissions from agricultural soils will account for 59% of global anthropogenic N_2O emissions in 2030. For every 1000 kg of applied N fertilizers, it is estimated that 10-50 kg N are lost in form of N_2O (Hu et al., 2015). Although the amount of N that is lost by N_2O release is small compared to the losses via NH_3 volatilization, it encompasses a great radiative forcing because N_2O global warming potential (GWP) is 265 greater than that of CO_2 in a 100-year time horizon. Thus, it is estimated a marginal damage cost of 5–12€ kg N_2O-N (Van Grinsven et al., 2013). In addition, soils are also a major source of NO, which is mainly released during nitrification (Liu et al., 2017). Its liberation can promote tropospheric ozone production and acid rain (Crutzen, 1981).

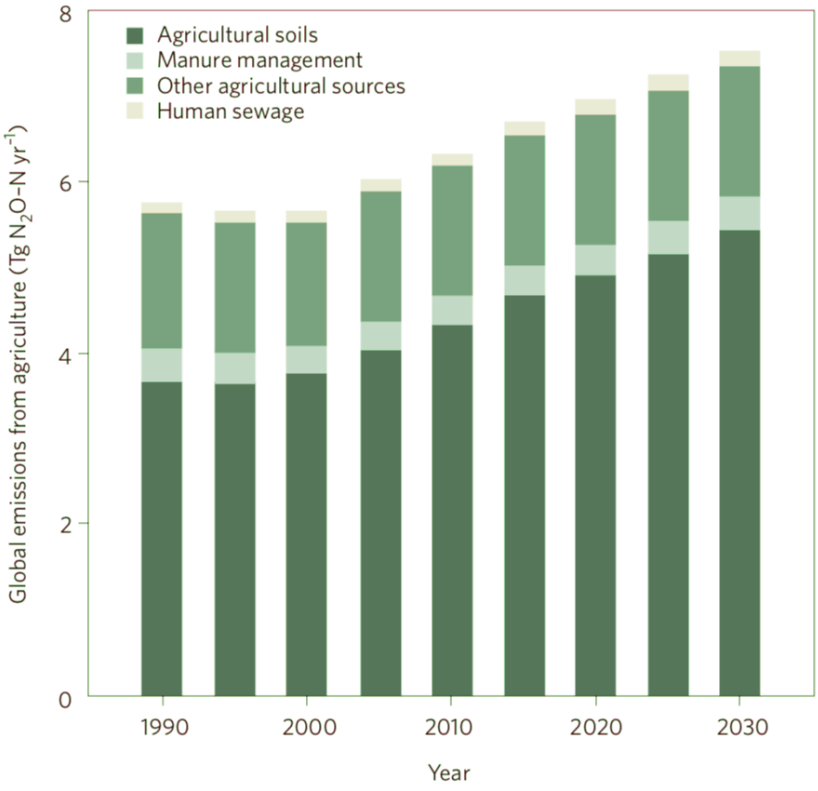


Figure 11. Evolution of global N_2O emissions from agriculture between 1990–2030 *(Reay et al., 2012).*

The Global Warming Potential is a factor to value the sustainability of the agroecosystems based on the GHGs emitted. For this calculation, CO_2 and methane (CH_4) fluxes are usually taking into account in addition to N_2O. Soils are the most important biological sources and sinks of atmospheric CH_4. CH_4 fluxes are greatly dependent on the soil water content. This determine de predominance of methanotrophy, carried out under oxic conditions by the bacterial-enzyme particulate methane monooxygenase (pMMO), which is highly similar to AMO (Glass and Orphan 2012), or methanotrogenesis (under anoxic conditions). In this manner, wetlands are a net source of atmospheric CH_4, while drylands act as sinks (Le Mer and Roger, 2001). Nitrogen fertilization may exert an effect on CH_4 fluxes because NH_4^+ can compete with CH_4 for the active site of pMMO (Dunfield and Knowles, 1995). However, the results from several studies indicate that no generalizations can be made with respect to the effect of ammonium-based fertilizers on CH_4 fluxes (Bodelier and Laanbroek, 2004). The

magnitude of CH_4 fluxes and N_2O emissions in agricultural systems is small compared to CO_2 emissions (g ha^{-1} *vs* Mg ha^{-1}). Meta-analysis indicate that N fertilization can reduce microbial biomass in many ecosystems, with corresponding declines in soil CO_2 emissions, being this effect more evident in the longer term and with larger amount of added N (Treseder, 2008). On the contrary, CO_2 is liberated from soil pores with plowing and microbial decomposition is increased (González-Sánchez et al., 2012; Soane et al., 2012). Implementing a no-tillage (NT) system can increase organic C storage and lead to lower CO_2 losses from soils under certain conditions compared to conventional tillage (CT). NT practices are increasing in south-western Europe because, under these climatic conditions, it improves water conservation, increases soil C stocks and maintains or increases crop yields (Soane et al., 2012; Plaza-Bonilla et al., 2015). The tillage management has also a strong relationship with the soil N_2O emissions because of its influence on water storage and soil compaction and, then, soil aeration. For instance, under rainfed Mediterranean systems, long-term NT can be a good practice to increase crop yields and reduce N_2O emissions (Cantero-Martinez et al., 2016; Lampurlanés et al., 2016; Plaza-Bonilla et al., 2018). Nevertheless, the response of the system is very variable depending on the climatic conditions, water content and time since the implantation of the system. (Holland, 2004; Rochette, 2005; Venterea et al., 2005). The studies regarding with soil CH_4 fluxes and tillage practices are scarcer, but there might be a trend to a greater CH_4 uptake under NT (Six et al., 2004); although most works indicate that the effects, in general, are weak (Soane et al., 2012).

6. ENHANCED NITROGEN RETENTION THROUGH THE USE OF INHIBITORS

6.1. BIOLOGICAL INHIBITORS

Plants have developed their own way to stabilize N in the soil and, thus, maintain it available to meet their needs for a longer time period. Some species are capable to delay the N cycle through root exudates that inhibit nitrification, known as biological

nitrification inhibitors (BNIs). These compounds reduce the mobility of N by avoiding NH_4^+ oxidation to NO_3^-, which might be an adaptation of plants living in natural systems where N is limiting. Thus, no BNI release has been observed under NO_3^-, while stronger BNI capacity has been observed in species adapted to poor-N environments (Subbarao et al., 2013a). Most plants are capable of having mixed NH_4^+ and NO_3^- nutrition (Haynes and Goh, 1978; Salsac et al., 1987) so the retention of NH_4^+ for longer does not affect N absorption. The release of different BNIs have been demonstrated in sorghum (Sorgoleone and Methyl 3-(4-hydroxyphenyl), MHPP) (Subbarao et al., 2013b), *Brachiaria humidicola* (Brachialactone) (Subbarao et al., 2009), rice (1,9-Decanediol) (Sun et al., 2016; Tanaka et al., 2010), wheat (O'Sullivan et al., 2016), and *Leymus racemosus* (wild wheat) (Subbarao et al., 2007). As a result of this inhibition, N_2O emissions from soil are reduced (Subbarao et al., 2013b; Lu et al., 2019). Although the mechanism of action of BNIs is still very unknown, it seems that most of them block the action of AMO, while some of them are also able to affect HAO (Coskun et al., 2017). Nonetheless, it is believed that there must be a great a great variety of compounds in a great variety of plans exerting different effects, since root exudates have a clear effect on the microbiome, as has been also observed in the case of denitrification (Bardon et al., 2014, 2016; Malique et al., 2019).

It would be desirable to obtain crops with the ability to manage nitrification themselves to reduce N leaking and the associate environmental impact. Nevertheless, the mechanisms determining the release of these compounds, their stability and the factors determining their effectivity are only just beginning to be understood. This makes it not a mitigation tool to be adopted by the moment, although, hopefully, the selection and development of new crop lineages would make this possible in the next decades. At present, it is necessary to intervene nitrification by another way.

6.2. SYNTHETIC INHIBITORS

Humans have imitated nature by synthetizing compounds that are also capable of acting as inhibitors. There are many compounds with inhibition potential (Subbarao et

al., 2006), but especially four compounds have been widely applied in the last decades: N-(n-butyl) thiophosphoric triamide (NBPT), 2-chloro-6-(trichloromethyl)-pyridine (nitrapyrin), Dicyandiamide (DCD) and 3,4-dimethyl-1H-pyrazol phosphate (DMPP).

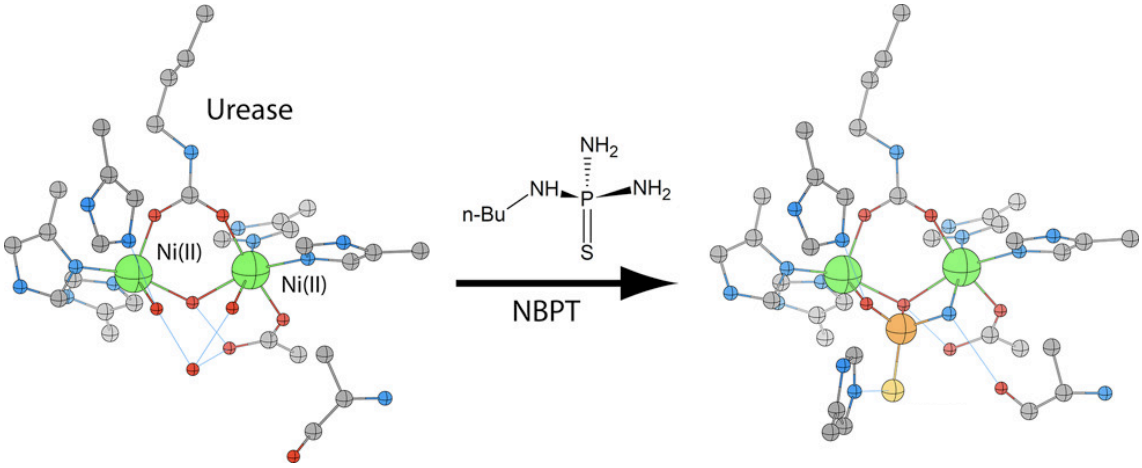


Figure 12. Crystallographic structure of the urease inhibitor NBPT binding to urease. Carbon atoms, gray; nitrogen, blue; oxygen, red; sulfur, yellow; phosphorus, orange; nickel, green *(Mazzei et al., 2017)*.

NBPT (Fig. 12) become to be applied in the mid-1990s to delay NH_3 hydrolysis by blocking urease enzyme. The application of urease inhibitors is an alternative to other methods to diminish NH_3 volatilization such as the mechanical incorporation of fertilizers into the soil, which requires specific machinery and cannot be carried out in combination with perennial crops and no-tillage managements. The efficiency of NBPT to reduce NH_3 volatilization is highly variable, from results showing great reductions of ~50% (Abalos et al., 2012; Lam et al., 2018, 2019; Noor Affendi et al., 2020), to medium reductions of ~35% (Martins et al., 2017) and negligible effects (Menéndez et al., 2009). Despite this reduction of N losses, the effects on crop yield are slighter, since meta-data reveals increments about 5–12% (Cantarella et al., 2018).

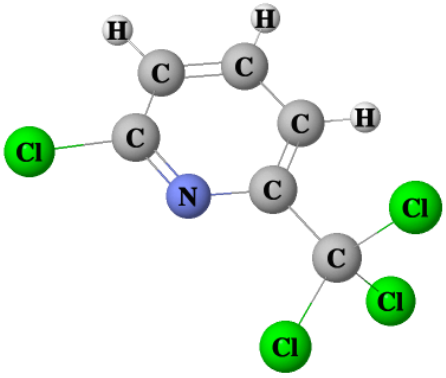


Figure 13. Atomic model of the nitrification inhibitor Nitrapyrin.

Nitrapyrin (Fig. 13) is a nitrification inhibitor that has been extensively applied in North America. In pure cultures, it deploys inhibition on *Nitrosomonas*, with smoother effects on *Nitrosospira*, *Nitrosolobus* and *Nitrososphaera* (Shen et al., 2013; O'Sullivan et al., 2017), although its mode of action is not clear. Its inconvenience is a high volatility, making necessary its incorporation into the soil. Nonetheless, meta-analysis showed a 16% decreasing of NO_3^- leaching, 51% lower N_2O emissions and 7% higher yield (Wolt, 2004).

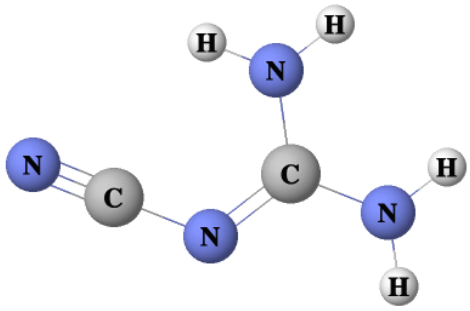


Figure 14. Atomic model of the nitrification inhibitor dicyandiamide (DCD).

Unlike nitrapyrin, DCD (Fig. 14) is not volatile, thus being suitable for use as coating on solid fertilizers. Its main weakness is its high water solubility, making it susceptible to leaching out of the rooting zone, thus reducing its effectiveness. DCD is able to reduce NO_3^- leaching (Di and Cameron, 2018) and diminish N_2O and NO losses

by ~35% and ~59% respectively (Akiyama et al., 2010; Ruser and Schulz, 2015), while increasing yield about 6%; although it has to be taking that an extra N input is applied with DCD, since it contains 67% N (Abalos et al., 2014). After DCD application, inhibition has been observed in AOB and also lower inhibition on AOA (Ruser and Schulz, 2015; O'Sullivan et al., 2017).

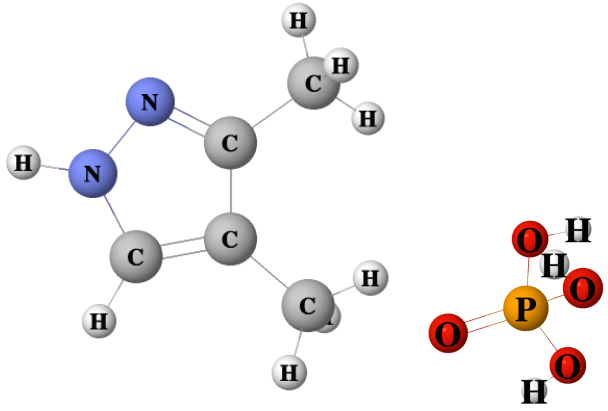


Figure 15. Atomic model of the nitrification inhibitor 3,4-dimethyl-1H-pyrazol phosphate (DMPP).

DMPP (Fig. 15) has lower vapor pressure than nitrapyrin and lower mobility than DCD, exhibiting a similar performance to DCD with a 10 times lower application rate (Weiske et al., 2001). Thus, N_2O emissions are diminished about 35-40% without exerting any positive or negative effect in crops yield (Abalos et al., 2014). New compounds are being developed with the aim of improving the inhibition efficiency and the combination with different types of fertilizers.

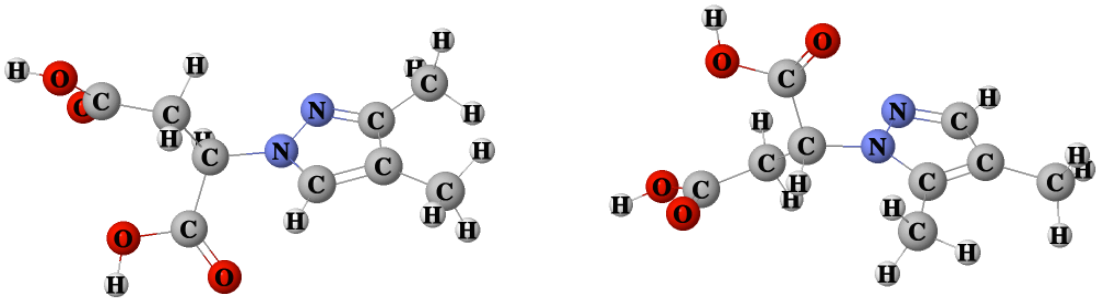


Figure 16. Atomic model of the nitrification inhibitor 2-(3,4-dimethyl-1H-pyrazol-1-yl)-succinic acid and 2-(4,5-dimethyl-1H-pyrazol-1-yl)-succinic acid (DMPSA).

In this sense, a new compound based on dimethylpyrazole, the isomeric mixture 2-(3,4-dimethyl-1H-pyrazol-1-yl)-succinic acid and 2-(4,5-dimethyl-1H-pyrazol-1-yl)-succinic acid (DMPSA) (Fig. 16), was registered in the European Chemical Agency in 2014 (EC number: 940-877-5) by Eurochem Agro GmbH (Mannheim, Germany). This product is not still on the market and, therefore, the number of articles about it is still very scarce. In turn, this represents an opportunity to investigate its behavior before it is commercialized, so as to guarantee the highest efficiency and safety in its application. The first studies have showed the capacity of DMPSA to delay NH_4^+ oxidation and reduce N_2O emissions under field conditions (Huérfano et al., 2018; Guardia et al., 2018a; Recio et al., 2019, 2020), inhibiting AOB growth in a microcosm experiment (Torralbo et al., 2017).

6.2.1. MODE OF ACTION OF SYNTHETIC INHIBITORS

Knowing the mode of action of these inhibitors can be a complex task. Nonetheless, However, finding this information would allow a better understanding of their behavior, as well as improve their management and the development of new products.

The mechanism of action of NBPT has been addressed in different works, finding that it acts as a tridentate ligand, coordinating both Ni centers and one oxygen from the carbamate bridge of urease, reducing the probability of urea to reach the Ni atom (Fig. 12) (Manunza et al., 1999).

Many assumptions have been made regarding the mechanism and mode of action of the main NIs. Nevertheless, the mode of action of Nitrapyrin, DCD and DMPP remains unknown (Wenderborn, 2020). There is hardly any evidence published in the literature, and most articles reference to statements made in the reviews of Subbarao et al. (2006) and Ruser and Schulz (2015). However, the reality is that these reviews do not refer to any article in which the mode of action of inhibitors had actually been empirically demonstrated. For instance, Subbarao et al. (2006) stated that nitrapyrin and DCD act by chelating Cu components of the cytochrome oxidase involved in AMO, based on the work of Powell and Prosser (1986). First of all, DCD was not even analyzed in the work of Powell and Prosser. In addition, what was stated regarding nitrapyrin is just the opposite, as described by the authors: "*To date the hypothesis of* Campbell and Aleem (1965) *that nitrapyrin acts by binding Cu centers of cytochrome oxidase components involved in ammonia oxidation has been generally accepted as the mechanisms of action of this inhibitor. Our data are not consistent with this hypothesis".* In Ruser and Schulz (2015), it is also indicated that nitrapyrin and DCD are Cu-chelating compounds, but this is based on the review of Subbarao et al. (2006).

With respect to NIs based on DMP, some authors reported that both DMPP and DMPSA act in the same manner because it is believed that DMPSA molecules need to be decomposed to DMP in order to be active as inhibitor (Pacholski et al., 2016a). However, the rupture of covalent C—N bonds is unlikely because its bond energy is as high as 305 kJ mol^{-1} (Luo, 2007). In addition, the registration dossier of DMPSA in the European Chemicals Agency (ECHA) reports no biodegradation in surface waters after 28 days. On the contrary, this same dossier also claims that the degradation of DMPSA into DMP does take place in soils, which may indicate i) the capacity of some microorganisms to carry out this rupture or ii) the strong effect of some other physical parameters such as ultraviolet radiation. Nevertheless, to the best of knowledge of the author, no studies have been published confirming this issue. Most publications dealing with DMPP and DMPSA refer again to the Ruser and Schulz (2015) in which the ability to chelate Cu^{2+} ions is supposed to be displayed by DMPP, but this is just based on a personal communication.

Furthermore, it has been proposed that this specific Cu-chelation capacity of DMP could hinder the activity of AMO by i) coordinating to the enzymatic active site, as it has been reported for some other inhibitors as acetylene and EDTA (Gilch et al., 2009b, 2010), or ii) reducing the bioavailability of Cu ions in soils (Duncan et al., 2017). On the other hand, if the mechanism of action of DMPs is based on a reduction of Cu availability, this raises the question of whether this ability might also affect other enzymes with Cu requirements, leading in non-expected side effects. Not knowing the exact mode of action supposes a great knowledge gap, which is a drawback when trying to further improve their use and, in many cases, means for researchers trying to explain some effects of inhibitors as if we were blindfolded.

6.2.2. EFFECTS OF NITRIFICATION INHIBITORS ON NON-TARGET ORGANISMS

Nitrogen fertilization causes a great disruption on the natural conditions of soils due to the extraordinary nutrient input. This modification of soil nutrients stock promotes a change in the soil dynamics, causing a direct impact on bacterial populations that, at the same time, might disrupt soil multifunctionality. Multifunctionality, which is positively related to microbial diversity, is described as the capability to role multiple functions simultaneously, critical to maintaining nutrient cycling (Wagg et al., 2014; Bender et al., 2016; Delgado-Baquerizo et al., 2017). *Armatimonadetes*, *Cyanobacteria* and *Fibrobacteres* are standing out taxa playing a key role within soil multifunctionally (Chen et al., 2020). Oligotrophic organisms (such as these latest taxa) are adapted to natural soils, where nutrients are scarce. Thus, although the specific response of bacterial consortia varies depending on the initial characteristics of the soil (Wang et al., 2018), the enhanced nutrient availability derived from fertilization is detrimental for oligotrophic bacteria, favoring copiotrophs (organisms better adapted to nutrient-rich environments) due to their fast growth rates (Fierer et al., 2007).

Although the amount of NIs applied to soils is much lower than fertilizers in absolute terms (for instance, 0.8% of the NH_4^+-N applied with the fertilizer, in the case

of DMPP and DMPSA), the possible side effects of these agrochemicals should not be ignored; especially when the mechanisms and mode of action, the routes and degradation periods of these compounds have not been deeply analyzed. Depending on the real mode of action of these inhibitors, we can speculate that -at least- the next hypothetical scenarios -or a combination of them- might take place within N cycle:

Scenario 1: DMPs are highly specific on AMO, not affecting directly any other organism within the N cycle.

- **1-A:** the lower growth of AOB increases the availability of scarce resources for competing organisms. For instance, this scenario could occur in a Cu-deficient soil, in which several organisms within the N cycle are competing for Cu. Thus, it might be observed an increase of *nosZI* and *nosZII* genes after DMPs application. The effect on *nirK* would be less evident, since some nitrifying bacteria also harbor *nirK* genes. In terms of N emissions, it would be expected a lower absolute N_2O emission and a lower N_2O:N_2 ratio.
- **1-B:** the decrease of nitrification rates results in less substrate available for denitrification (NO_3^-). Then, it might be expected a decrease of denitrifying genes such as *narG*, *nirK*/*nirS* and *norB*. On the other hand, two additional responses might be observed with respect to *nosZ* genes:
    - **i:** to follow the general trend of denitrification showing an abundance decrease due to lower substrate availability. In terms of N emissions, it would be expected a lower absolute N_2O emission with no changes in the N_2O:N_2 ratio.
    - **ii:** an increase of N_2O reduction to N_2 to compensate for the lower availability of NO_3^- as electron-acceptor (see section 4.1), then showing an increase of *nosZ* abundance. In terms of N emissions, it would be expected lower N_2O emissions and a lower N_2O:N_2 ratio.

Scenario 2: DMPs are general Cu-chelating compounds.

- **2-A:** as a result of Cu chelation by DMPs in the soil, Cu is less bioavailable for bacteria. Thus, every Cu-dependent enzyme reduces its activity. As a direct result, it would be expected an abundance decrease of every *amoA*, *nirK* and *nosZ* genes. As an indirect effect, the rest of the genes would also decrease their abundance; although it might be also observed an increase in the abundance of the *nirS* gene as a non-copper-dependent alternative to *nirK*. In terms of N emissions, lower absolute N_2O emissions might be observed, with no change in N_2O:N_2 ratio. It would be expected that there were no effects-reversion until the liberation of Cu (for instance, due to the degradation of the inhibitor) or until a Cu-addition to the soil.
- **2-B:** Cu is bound to the inhibitor; however, bacteria can take this chelate. Nevertheless, when Cu is attached to the active center of the enzymes, the presence of the DMP molecule hinders the enzymatic action. This might lead to three alternative results:
    - **i:** every Cu-dependent enzyme is negatively affected by the presence of the inhibitor in its active center. Therefore, the enzymatic activity is completely disabled. The expected results could be similar to those of scenario 2-A.
    - **ii:** the activity of Cu-containing enzymes is negatively affected. However, the activity is not completely inhibited, either because the functioning remains partial despite the presence of the inhibitor or because Cu can be substituted with an alternative metal cofactor within the active center. In this scenario, the result would be variable depending on the relative activity maintained by each enzyme or its ability to use alternative cofactors.
    - **iii:** only some enzymes are negatively affected while, in others, the presence of the inhibitor is not a problem for the conformation of

their active sites or because such organisms are able to break the bond between Cu and the inhibitor. In this scenario, the result would be variable depending on the ability of each organism to use the chelated-Cu.

Surprisingly, although several of these products have been widely applied along decades, the impact that they may have on the soil microbiome and their interactions has hardly been addressed. This could be attributed to three findings: i) the fact that DMPs deploy a very effective action on AOB (Ruser and Schulz, 2015; Torralbo et al., 2017); ii) no effect on total bacterial abundance (measured as *16S rRNA* gene abundance) has been observed (Barrena et al., 2017; Torralbo et al., 2017); iii) there is no detrimental effect on plant growth at agricultural dosage (Pasda et al., 2001; Rodrigues et al., 2018). However, these results should not be considered as sufficient evidence to demonstrate that non-target shifts are not taking place in the soil microorganisms after DMPs application.

The first studies analyzing the effect of DMPs on microorganisms (both at laboratory and field conditions), focused on the action deployed on nitrifying organisms (both archaea and bacteria). In this sense, most studies have shown the effectivity of DMPP to inhibit AOB growth (measured as *amoA* gene abundance), while there is not a significant effect on AOA population (Di and Cameron, 2011; Kleineidam et al., 2011; Duncan et al., 2017). Although to a lesser extent, the impact of DMPSA has been also compared between AOB and AOA, showing similar results to DMPP (Torralbo et al., 2017). On the other hand, more recent studies applying amplicon sequencing techniques, have found positive effects on the abundance of some archaeal taxa and variations within nitrifying-community composition after DMPP application (Cassman et al., 2019; Liu et al., 2020).

Since the oxidation of NH_4^+ initiates a cascade of redox reactions where, in each one, the substrate from a previous stage is used, it could not be ruled out that the alteration of the first step affected the rest of the reactions. Therefore, the analysis was expanded to

almost the entire soil-N cycle. In this manner, the effect on the entire nitrification pathway (not only NH_4^+ oxidation to NH_2OH) as well as denitrification and -to a lesser extend- N_2 fixation began to be considered. It was at this point that the first effects on non-target organisms were found. However, the studies are still scarce and most of them were performed on raw soils under laboratory conditions, showing variable results depending on different parameters and, therefore, making it difficult to draw conclusions. Only Dong et al. (2013) has addressed the interaction between nitrogen-fixers and DMPP application, showing an increase in *nifH* gene abundance, 7 and 14 days after fertilization; but with opposite results after 49 days. More studies have analyzed the effects on a part or the whole denitrification route. When it comes to DMPP, Fuertes-Mendizabal et al. (2019) analyzed the abundance of 6 of the main denitrification-enzymes encoding genes, showing variable results depending on the time from application and soil water content. For instance, at 40% WFPS, *narG* abundance decreased with DMPP at 11 and 31 days after application, but increased after 163 days; no differences were observed at 80% WFPS. A lower abundance of *nirK* genes was observed 31 days after application at 40% WFPS, but an increase was observed after 163 days both at 40 and 80% WFPS. There was a greater abundance of *nosZII* with DMPP application at 11 (80% WFPS), 31 and 163 (40% WFPS) days after application. On the contrary, almost no shifts were observed in the case of *nirS* and *nosZI*, in concordance with the results of Duan et al. (2017). However, two works in which only the last steps of denitrification were evaluated (NO and N_2O reduction), showed an induction of *norB* and *nosZI* 1 and 60 days after DMPP application at 50 and 80% WFPS (Castellano-Hinojosa et al., 2020) and both *nosZI* and *nosZII* in soils treated with DMPP and DMPSA after 51 days at 80% WFPS (Torralbo et al. (2017). In general, no convincing explanations could be found for these highly variable results. In a recent work under field conditions, Luchibia et al. (2020) reported that some effects exerted by DMPP depended on the total applied dose. In this manner, no shifts were observed in *amoA*, *comaA* and *comaB* (encoding genes for comammox AMO), *nirk* and *nosZ* with fertilization rates of 40 and 80 kg N ha^{-1} (therefore 0.32 and 0.64 kg DMPP

ha^{-1}). However, with a dose of 0.96 kg DMPP ha^{-1} (corresponding to 120 kg N ha^{-1}), it was observed a decrease of *amoA* and *nirK* genes.

Even fewer works have considered the impact exerted by DMPs on the whole soil microbiome, but a community approach is necessary to assess both the direct and/or indirect effects of NIs on non-target organisms. So far, only two preliminary studies have analyzed DMPP through this approach (no one with DMPSA) and there are almost no studies with any other of the most widely applied NIs (DCD and nitrapyrin). The results from a microcosm experiment performed by Zhang et al. (2017) showed no effects of DMPP on bacterial diversity nor community structure. No severe impact was found by Luchibia et al. (2020) at phylum level under field conditions, although some variations were found at higher taxonomical resolution. In any case, these two studies did not provide sufficient depth analysis and evidence to assume the immunity of the soil bacterial community to DMPP. The only study of this type that has carried out a more robust analysis is that presented by Suleiman et al. (2016) dealing with DCD. Although several shifts were observed at the genus level, these authors reported no significant effects of DCD in the overall microbial community structure, in accordance with the results of Morales et al. (2015).

OBJECTIVES

Nitrification inhibitors can be useful tools to reduce the environmental impact of agriculture by diminishing the reactive nitrogen losses derived from fertilizer application. However, it is necessary to expand the knowledge about their functioning and test their security for the maintenance of the soil health. The main objective of this thesis was to determine the suitability of synthetic nitrification inhibitors based on dimethylpyrazole for increasing agriculture sustainability by reducing the release of nitrous oxide emissions from soils.

The initial hypothesis of this thesis was: **nitrification inhibitors based on dimethylpyrazole are useful and safe tools for increasing agriculture sustainability by mitigating the environmental impact derived from nitrogen fertilization.**

To test this hypothesis, it was necessary to approach this work from different points of view:

- Determining the effectivity of their application to reduce reactive nitrogen losses in combination with other mitigating practices such as no-tillage management.
- Studying the effects of their application in the nitrogen fate within the soil-plant-atmosphere system.
- Analyzing the chemical properties of these compounds and making an approach to their mechanism of action.
- Addressing their impact on the target bacteria populations and on the whole microbiome to determine possible unexpected side effects in the soil.

With these four main objectives in mind, the research was divided into five experimental works, each one focused on trying to test a specific hypothesis:

Many studies in the literature report that N_2O fluxes can be modified because of the modifications in soil structure caused by tillage. Consequently:

- Hypothesis 1: **The structural differences between tilled and non-tilled soils might lead to a dissimilar performance of DMPSA to avoid N_2O emissions depending on the soil management practice.**

To test this hypothesis, the efficiency of the nitrification inhibitor DMPSA was analyzed in a wheat crop fertilized with ammonium sulfate under two systems of soil tillage: one based on conventional tillage (moldboard plow) and another based on direct seeding (no-tillage). The results are presented in Chapter 1.

Nitrification inhibitors increase the risk of NH_3 volatilization as a consequence of the greater soil ammonium content derived from nitrification inhibition. To avoid these negative effect, these inhibitors are being with urease inhibitors. Therefore, we hypothesize that:

- Hypothesis 2: **DMPSA will increase the risk of NH_3 volatilization. This risk could be greater in non-tilled soils because of a slower infiltration of urea as a consequence of higher soil density. The joint application of DMPSA with a urease inhibitor (NBPT) could counteract this effect and be especially advisable when applying a no-tillage management.**

To test this hypothesis, a rapeseed crop was established and fertilized with urea under conventional and no-tillage managements, determining the effect of the interaction of the different inhibitors on the N_2O and NH_3 volatilization. The results are presented in Chapter 2.

Since previous studies have shown no differences in the inhibition of nitrification deployed by DMPP and DMPSA nor in their efficiency in reducing N_2O emissions, the next hypothesis was raised:

- Hypothesis 3: **The action of both DMPP and DMPSA should be based on the same mechanism: copper-chelation. Thus, the efficiency of both inhibitors will vary depending on the soil copper content.** Two experiments were performed to address this hypothesis:

- Synthetic chemistry experiments were carried out on the lab-scale with the subsequent crystallographic analysis to elucidate the chelating capacity of the inhibitors.
- A pots experiment was carried out under greenhouse conditions to analyze the relationship between the efficiency of the inhibitors and the content of Cu and Zn in the soil.

The results are presented in Chapter 3.

For the sake of sustainable agriculture, soil health is an important value. Considering that previous works showed that the application of nitrification inhibitors based on dimethylpyrazole had no effect on total bacterial abundance, deleterious effects could be ruled out. However, it was hypothesized that:

- Hypothesis 4: **Since these inhibitors alter the nitrogen cycle and could deploy a chelating action, this can lead to side effects on non-target microorganisms. However, we expected the impact on the bacterial community to be subtle and reversible over time since these products are quickly degraded in the soil.** This hypothesis was tested based on two objectives:

- To make a first approximation to settle the **side effects** on non-target bacteria derived from the application of DMPSA compared with the application of ammonium sulfate. This was addressed by analyzing the impact on the entire bacterial community through amplicon sequencing techniques. Soil samples from conventional tilled and non-tilled soils from a wheat crop established under

Humid Mediterranean conditions were analyzed. This objective is addressed in Chapter 4.

- To elucidate the **magnitude of the impact** exerted on the microbiome by comparing the bacterial consortia of unfertilized soils, fertilized soils and soils fertilized recurrently with DMPP or DMPSA. Samples were collected from a ryegrass crop established under Atlantic conditions, at different times, and under several fertilizations, to give also the possibility to analyze the temporal effect. This objective is addressed in Chapter 5.

CHAPTER 1

Suitability of DMPSA application in no-tillage systems

Relationship between tillage management and DMPSA nitrification inhibitor efficiency

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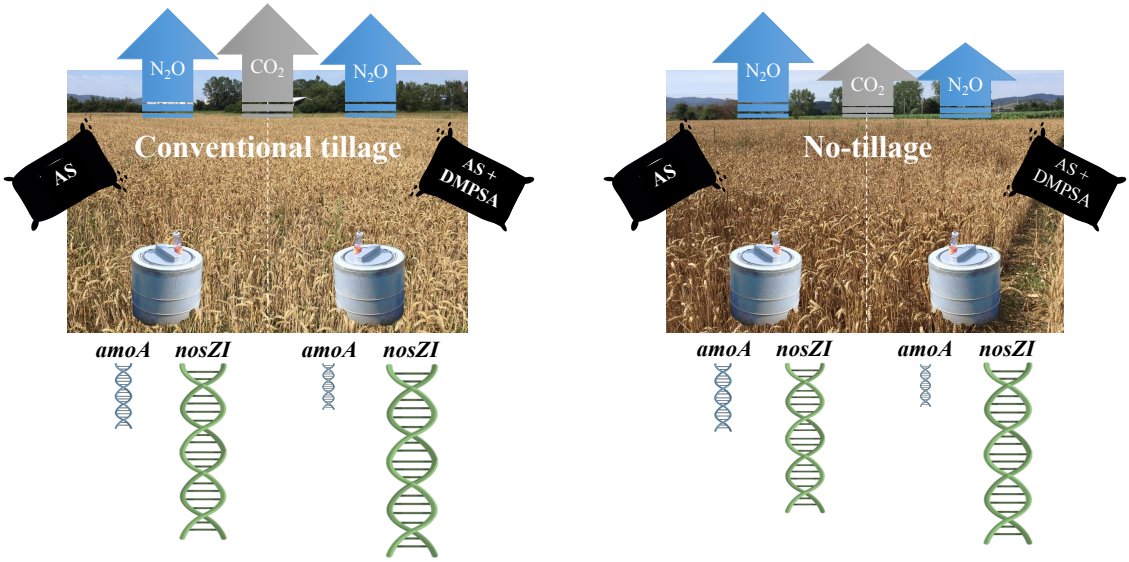
Soil no-tillage management can be an efficient tool to increase agriculture sustainability because it reduces the use of machinery and can generate positive effects such as reducing CO_2 and N_2O emissions, improving the water storage capacity, and increasing the organic matter content. The different structure between conventional tilled and non-tilled soils affect N_2O fluxes because nitrification and denitrification rates are determined by soil characteristics such as porosity, compaction, moisture and oxygen availability. In the same manner, it has been observed with several nitrification inhibitors that the efficiency of reducing N_2O emissions varies depending on different parameters.

The application of DMPSA nitrification inhibitor in no-tillage systems could be a good combination to reduce the environmental impact caused by agriculture, but it is necessary to check if its effectiveness is maintained in soils subjected to different managements. The suitability of applying DMPSA in non-tilled soils was assessed by analyzing its effects on greenhouse gases emissions as well as on grain yield and quality of a wheat crop grown under two different soil tillage systems.

GRAPHICAL ABSTRACT



ABSTRACT

Agricultural sustainability is compromised by nitrogen (N) losses caused by soil microbial activity. Nitrous oxide (N_2O) is a potent greenhouse gas (GHG) produced as a consequence of nitrification and denitrification processes in soils. Nitrification inhibitors (NI) as 3,4-dimethylpyrazole-succinic acid (DMPSA) are useful tools to reduce these N losses from fertilization. The objective of this work was to test the efficiency of DMPSA in two different tillage management systems, conventional tillage (CT) and no-tillage (NT), in a winter wheat crop under Humid Mediterranean conditions. N fertilizer was applied as ammonium sulfate (AS) with or without DMPSA in a single or split application, including an unfertilized treatment. GHG fluxes (N_2O, CO_2 and CH_4) were measured by the closed chamber method. *amoA* and *nosZI* genes were quantified by qPCR as indicators of nitrifying and denitrifying populations. Nitrification was inhibited by DMPSA in both CT and NT, while the higher water-filled pore space (WFPS) in NT

promoted a better efficiency of DMPSA in this system. This higher efficiency might be due to a greater N_2O reduction to N_2 as a result of the *nosZI* gene induction. Consequently, DMPSA was able to reduce N_2O emissions down to the unfertilized levels in NT. Provided that NT reduced CO_2 emissions and maintained crop yield compared to CT, the application DMPSA under NT management is a promising strategy to increase agro-systems sustainability under Humid Mediterranean conditions.

MATERIALS AND METHODS

2.1. EXPERIMENT SETUP

Wheat crop under conventional tillage (right) and no-tillage (left)

This work was conducted during a crop season (2016–2017) of winter wheat (*Triticum aestivum* L., var. Cezanne) in Arkaute, northern Spain (42º51´N, 2º37´W, 530 m above sea level). Soil characteristics of the upper horizon (0–30 cm) are compiled in Table 1. Daily precipitation and mean temperatures are shown in Figure S1.

***Table 1**. Physical and chemical properties of the soil (0 – 30 cm depth).*

Soil texture			Soil chemical properties								
Sand	Silt	Clay	pH[a]	C:N	N[b]	Organic matter[c]	Carbonate[e]	P[d]	Mg[e]	K[e]	Ca[e]
(%)					(g kg^{-1})			(mg kg^{-1})			
43.4	24.7	31.9	8.0	8.15	1.6	21.2	9.8	59.0	92.4	167	6,356

a: pH (1:2.5 soil:water). b: N Kjeldahl digestion (Keeney and Nelson, 1982). c: Organic matter (Walkley and Black, 1934). d: P (Watanabe and Olsen, 1965). e: CaCO3 , Mg, K (MAPA, 1994).

To compare conventional tillage (CT) and no-tillage (NT) managements, two randomized complete blocks designs were established with four replicates and an individual plot size of 40 m^2 (8 x 5 m). The plots, whose previous crop was also wheat, were conditioned with a seedbed preparation consisting of mechanical tillage (disk and moldboard plow) for CT. For NT plots, spontaneous weeds were desiccated with glyphosate-based herbicide before direct sowing. Wheat was sown at a density of 220 kg seeds ha^{-1} in both managements on December 1st, 2016 and harvested on July 18th, 2017. Disc harrow was applied again on CT treatments on August 29th, 2017 in order to prepare the soil for the next crop.

Fertilization rate was 180 kg N ha^{-1} applied as ammonium sulfate 21% (AS) split in two applications of 60 kg N ha^{-1} at beginning of tillering stage (GS21) and 120 kg N ha^{-1} at stem elongation stage (GS30) according to the Zadoks growth scale (Zadoks et al., 1974). Provided that Huérfano et al. (2015) showed that the application of nitrification inhibitors allows to apply the fertilizer in a single application without effects on yield, we also have included a single application treatment of 180 kg N ha^{-1} at beginning of tillering stage (GS21).

Within each design (CT or NT), five treatments were applied: I) Control without N fertilization (C); II) AS in a single application (1AS); III) AS in split application (2AS); IV) AS combined with the nitrification inhibitor DMPSA in a single application (1ASD) and V) AS combined with DMPSA in a split application (2ASD). All plots were supplied with 85 kg ha^{-1} K (K_2SO_4) at GS21 stage. Fertilizer combined with DMPSA inhibitor

was provided by EuroChem Agro Iberia S.L. DMPSA rate was 0.8% of the NH_4^+-N applied with the fertilizer. Treatments application dates and rates are detailed in Table 2.

***Table 2**. **Nitrogen application rates as ammonium sulphate (kg N ha^{-1}) and growth stage.** C = unfertilized control; 1AS = ammonium sulphate 21% (single application); 2AS = ammonium sulphate 21% (split application); 1ASD = ammonium sulphate 21% + 3,4-dimethylphyrazole succinate (DMPSA) (single application); 2ASD = ammonium sulphate 21% + 3,4-dimethylphyrazole succinate (DMPSA) (split application).*

Treatment	Tillering (GS21)[a]	Stem elongation (GS30)[a]
	(kg N ha^{-1})	(kg N ha^{-1})
C	0	0
1AS	180	0
1ASD	180	0
2AS	60	120
2ASD	60	120

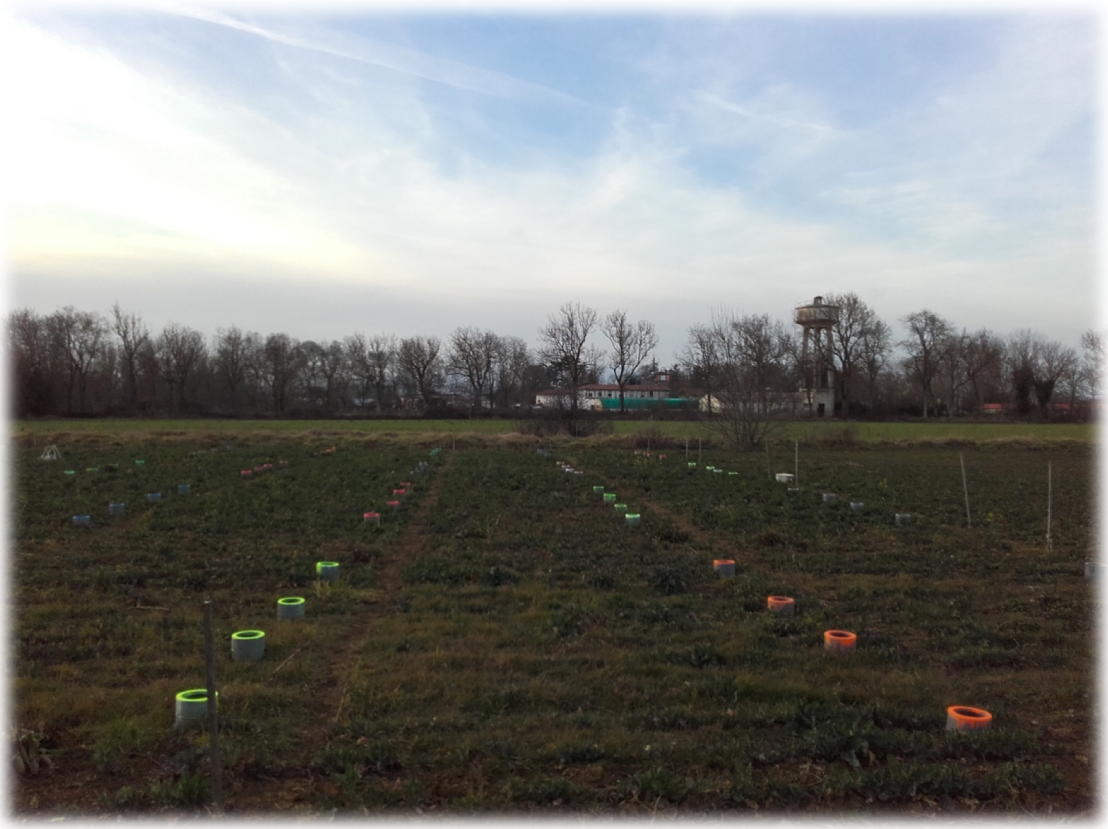
a: Zadoks growth scale (Zadoks et al., 1974)

2.2. SOIL MINERAL NITROGEN AND WATER CONTENTS

To measure ammonium (NH_4^+) and nitrate (NO_3^-) content, three soil subsamples (3 cm diameter x 30 cm depth) were taken randomly in each plot before sowing, every week during a month after fertilizer applications, and before harvesting. From the homogenized soil from each plot, 100 g fresh soil was extracted with 200 mL 1 M KCl. These extracts were filtered through Whatman nº1 filter papers (GE Healthcare, Little Chalfont, Buckinghamshire, UK) to remove particles and, secondly through Sep-Pak Classic C18 Cartridges 125 Å pore size (Waters, Milford, MA, USA) to eliminate organic matter. The resultant solutions were used to determine NO_3^- content by ultraviolet spectrophotometry as described by Cawse, (1967) and NH_4^+ by the Berthelot method (Patton and Crouch, 1977).

The rest of the soil sample was oven-dried to determine soil water content, which is expressed as the percentage of water-filled pore space (WFPS). WFPS was calculated as in Linn and Doran (1984): WFPS = (soil gravimetric water content x bulk density) $ (1 – bulk density/particle density))$^{-1}$, by using a particle density of 2.65 Mg m^{-3} and bulk densities determined for each tillage management resulting in values of 1.13 Mg m^{-3} and 1.35 Mg m^{-3} for CT and NT respectively.

2.3. GREENHOUSE GASES EMISSIONS MEASUREMENTS



Chambers inserted into the soil for sampling of GHG emissions.

N_2O, methane (CH_4) and CO_2 soil emissions were measured using the close chamber method (Chadwick et al., 2014). The chambers, with a diameter of 20 cm, were inserted into the soil at the beginning of the experiment and just removed to allow managements events. Sampling frequency was 3 times per week after each fertilization along 2 weeks, reducing frequency to 2 times per week in the next 2 weeks and 1 time

per week in the following 2 weeks. In the remaining time sampling was conducted every 2 weeks. Taking into account the diurnal variation of emissions (Baggs and Blum, 2004), sampling was performed between 10 a. m. and 13 p.m. To account for soil heterogeneity, four chambers were placed in each plot and two of them were sampled each day alternatively. Gas samples were taken just after closing the chambers and after 45 min. 20 mL of gas were taken from each chamber and stored at overpressure in pre-evacuated 12 mL glass vials. The linearity of the fluxes was checked regularly along the experiment. Samples were analyzed in a gas chromatograph (GC) (Agilent, 7890A) equipped with an electron capture detector for N_2O detection, with a methanizer to measure CO_2 by reducing it to CH_4, and a flame ionization detector for CH_4 determination. A capillary column (IA KRCIAES 6017:240 ºC, 30 m x 320 mm) was used, and samples were injected by means of a headspace auto-sampler (Teledyne Tekmar HT3) connected to the GC. Standards of N_2O, CO_2 and CH_4 were analyzed at the same time.

Gas emission rates were calculated taking into account the gas concentration variation from the beginning to the end of the 45 min. 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). Cumulative N_2O and CH_4 emissions were converted to CO_2 equivalents following the recommendations of IPCC (2007), using a global warming potential (GWP) factor of 265 for N_2O and 28 for CH_4.

Soil temperature (10 cm depth) was measured before sampling of gaseous emissions. Air temperature was measured 3 times during the 45 min. gas-sampling period to get the average.

2.4. NITROGEN-CYCLE-RELATED MICROBIAL ABUNDANCE

Soil cores from 2AS and 2ASD treatments of both CT and NT managements were collected at 0–30 cm depth 8 and 19 days after the first fertilization, and 12 and 31 days after the second. After homogenization, subsamples were weighted, frozen in liquid nitrogen and stored at –80 ºC until use. DNA was extracted from 0.35 g FW of soil using

the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) including some modifications described in Harter et al. (2014).

Extracted DNA concentration and quality were determined by spectrophotometry with a NanoDrop® 1000 (Thermo Scientific, Walthman, MA, USA). Quantitative polymerase chain reactions (qPCR) were performed using SYBR! Premix Ex TaqTM II (Takara-Bio Inc.) and gene-specific primers (Supplementary Table S1) to amplify and quantify total bacteria abundance (16S rRNA), nitrification-involved *amoA* gene and denitrification-involved *nosZI* gene. Each sample was quantified in triplicate using the StepOnePlusTM Real-Time PCR System and data analysis was carried out by StepOnePlusTM Software 2.3 (Thermo Scientific). Standard curves (log gene copies number per reaction volume versus log N) were prepared from serial dilutions of 10^7 to 10^2 gene copies mL^{-1} of linearized plasmids with insertions of the target genes, and 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. CROP YIELD PARAMETERS

A harvested surface of 12 m^2 (1.5 m x 8 m) per plot was used for grain yield determination, being adjusted to 12% moisture content. A surface of 0.45 m^2 per plot was measured to calculate the number of tillers per m^2, number of grains per ear and dry weight of 1000 grains. Total grain N content was analyzed by the Kjeldhal procedure (A.O.A.C., 1980) with a Kjeltec Autosampler System 1035 analyzer (Tecator) after grinding the grain through a 1 mm screen. Grain protein content was calculated as 5.7 times the total N content (Teller, 1932).

2.6. STATISTICAL ANALYSIS

Data were statistically evaluated by one-way ANOVA using Duncan's multiple-range test for separation of means between treatments, and the Student-T test or Mann-

Whitney-U test were carried out to specifically compare two treatments with the statistical software SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp). In all cases, significant differences are expressed at $P < 0.05$. Additional details have been included in figure legends.

RESULTS

3.1. SOIL MINERAL N

NH_4^+, NO_3^- contents and their ratio (NO_3^-/ NH_4^+) along all the experiment are shown in Table 3. After N fertilization, NH_4^+ content increased in both CT and NT systems. Although ASD treatments tended to show higher NH_4^+ content than AS in both CT and NT, only in three cases differences were statistically significant. DMPSA effects were much more obvious in the case of NO_3^- in NT management, where this content was significantly lower in both NT–2ASD and NT–1ASD treatments up to 30 days after fertilization (daf) and 50 daf respectively (Table 3). NO_3^- content tended to be lower in both CT–1ASD and CT–2ASD, although significant differences were only observed 30 daf in 2ASD. Most part of significant differences observed between CT and NT treatments are referred to NO_3^- content, usually showing CT higher soil NO_3^- content than NT.

Table 3. Soil NH_4^+ (kg NH_4^+-N ha^{-1}), NO_3^- (kg NO_3^--N ha^{-1}) content and ratio (NO_3^--N/NH_4^+-N) at 0-30 cm depth before fertilization and at different days after fertilization (daf) until harvest. *Hash (#) indicates significant differences (P < 0.05; n = 4; Mann-Whitney-U test) induced by DMPSA application respect to AS. Asterisk (*) indicates significant differences (P < 0.05; n = 4; Mann-Whitney-U test) between CT and NT within a fertilizer treatment. CT = conventional tillage; NT = no-tillage; C = unfertilized control; 1AS = ammonium sulphate (single application); 2AS = ammonium sulphate (split application); 1ASD = ammonium sulphate + DMPSA (single application); 2ASD = ammonium sulphate + DMPSA (split application).*

		Pre fertilization	First fertilization (15th March)		Second fertilization (4th April)			
		14th March	8 daf	19 daf	11 daf	21 daf	30 daf	72 daf
AMMONIUM								
	C	15	18	20	23	25	17	18
	1AS	15	81	56	47	41	24	18
CT	1ASD	14	111 #	90	73	73	36	22
	2AS	19	38	29	86	82	39	18
	2ASD	18	53	33	91	63	55 #	17
	C	28 *	29 *	23	31	26	33 *	16
	1AS	26 *	112 *	80	36	75	36 *	23
NT	1ASD	22 *	100	91	82 #	79	46	23 *
	2AS	28 *	35	32	89	66	58	22
	2ASD	24	44	40	73 *	106	88	25 *
NITRATE								
	C	26	21	20	31	38	28	27
	1AS	15	49	102	163	140	124	18
CT	1ASD	23	55	93	117	83	69	27
	2AS	21	30	92	124	79	144	26
	2ASD	20	29	71	89	68	83 #	17
	C	20	15	6 *	15	13 *	11 *	9.2
	1AS	13	29	56	46 *	49 *	44 *	13
NT	1ASD	9	24 *	39 # *	38 *	26 # *	21 # *	15
	2AS	18	28	41 *	57 *	58	51 *	22
	2ASD	15	22	27 *	23 # *	39 # *	30 # *	33
NO_3^-/NH_4^+								
	C	1.23	0.98	1.27	1.05	1.51	1.68	1.33
	1AS	0.75	0.62	1.96	3.98	3.40	4.81	0.96
CT	1ASD	1.23	0.49	1.06	1.65 #	1.11 #	1.94 #	1.30
	2AS	1.12	0.82	3.84	1.48	0.80	3.70	1.47
	2ASD	1.20	0.55	2.23	0.99 #	1.09	1.52 #	1.10
	C	0.69	0.54	0.26	0.47 *	0.52 *	0.33 *	0.55 *
	1AS	0.49	0.26 *	0.74 *	1.29 *	0.74 *	1.23 *	0.58
NT	1ASD	0.41	0.25 *	0.45 *	0.51 # *	0.34 # *	0.50 # *	0.84
	2AS	0.64	0.77	1.30 *	0.65 *	1.08	0.78 *	1.00
	2ASD	0.65 *	0.50	0.70 *	0.32 *	0.40 *	0.37 # *	1.36

3.2. GASEOUS EMISSIONS, SOIL WFPS AND TEMPERATURE

3.2.1. SOIL WFPS AND TEMPERATURE

Soil WFPS (30 cm depth) and soil temperature (10 cm depth) are shown in Fig. 1. WFPS ranged between a minimum of 31% and a maximum of 78% in CT, whereas values ranged between 41% and 100% in NT. WFPS was higher in NT than in CT in 40 of 42 sampling days, with an average value for the whole experimental period of 58% in NT, which was higher than the average value of 44% in CT. By periods (Table 5), soil water content was higher in the pre-fertilization and 1st fertilization, showing values above 60% WFPS, and it decreased below 50% in the 2nd fertilization and post-harvest periods. Soil temperature ranged between a minimum of 2 ºC and a maximum of 21 ºC without differences between CT and NT.

3.2.2. NITROUS OXIDE EMISSIONS

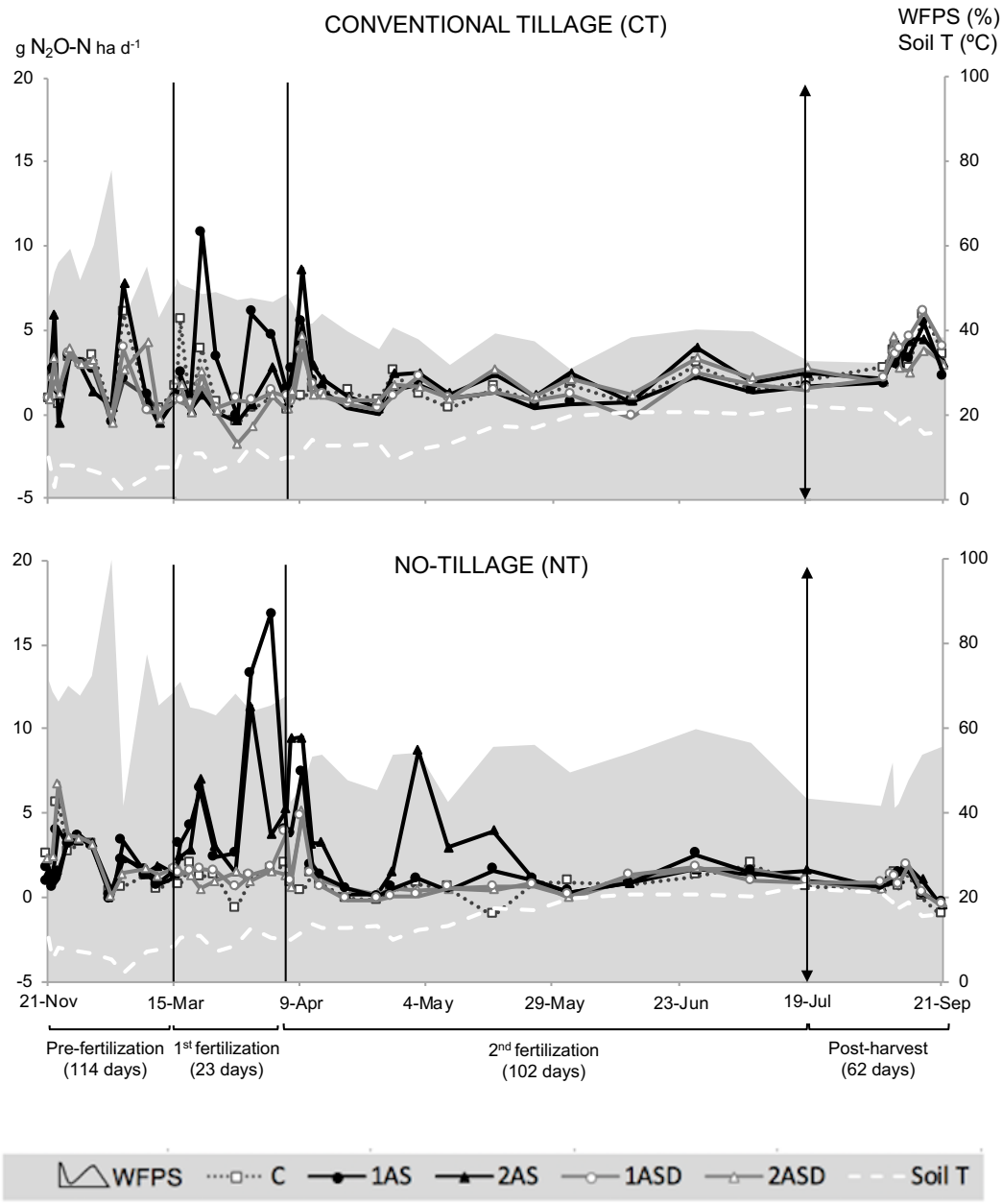


Figure 1. N_2O daily emissions, WFPS (0-30 cm depth) and soil temperature (0-10 cm depth) in conventional tillage (CT) and no-tillage (NT). *Vertical lines indicate fertilizer applications and harvest (arrows). C = unfertilized control; 1AS = ammonium sulphate (single application); 2AS = ammonium sulphate (split application); 1ASD = ammonium sulphate + DMPSA (single application); 2ASD = ammonium sulphate + DMPSA (split application).*

Daily N_2O emissions (Fig. 1) ranged from –1.77 to 10.84 g N_2O-N ha^{-1} d^{-1} in CT and from –1.14 to 16.75 g N_2O-N ha^{-1} d^{-1} in NT. These maximum daily rates occurred in 1AS treatment in both CT and NT managements. Nevertheless, maximums for DMPSA treatments only reached 6.09 g N_2O-N ha^{-1} d^{-1} in CT (CT-1ASD) and 6.79 g N_2O-N ha^{-1} d^{-1} in NT (NT-2ASD).

Differences between CT and NT in total cumulative N_2O emissions (from sowing to the last sampling day) (Table 4) were statistically significant in ASD and C treatments, being lower under NT. Total cumulative emissions values in AS treatments were similar between CT and NT. However, emissions in the C treatment were double in CT than in NT, and there were no differences between C and AS treatments in CT. Additionally, there were no significant differences due to DMPSA application in CT management, although, by periods, DMPSA showed an effect in the 1st fertilization decreasing CT-1AS N_2O losses by 76% (Table 5). As opposite, fertilization clearly increased N_2O losses in both NT-1AS and NT-2AS treatments with respect to the Control. However, DMPSA application significantly decreased N_2O emissions in both NT-1ASD and NT-2ASD in 24% and 31% respectively, being this reduction down to C levels. This reduction induced by DMPSA was higher when considering individual fertilization periods, with reductions of 79% in the 1st fertilization and 55% in the second one (Table 5).

Table 4. Total cumulative emissions (from sowing to the end of the experiment) of N_2O, CO_2 and CH_4; Emission Factor (EF) N yield scaled N_2O emissions (YSNE), Global Warming Potential (GWP) and Greenhouse Gas Intensity index (GHGI) from sowing until the end of the experiment. *Different letters within a column and management (CT = lowercase; NT= capital) indicate significant differences between treatments using the Duncan Test ($P < 0.05$; $n = 4$). Values in square brackets indicate significant differences in percentage between DMPSA and AS ($P < 0.05$; $n = 4$; Student-T test). Values in round brackets indicate significant differences in percentage between CT and NT within each treatment ($P < 0.05$; $n = 4$; Student-T test).*

CT = conventional tillage; NT = no-tillage; C = unfertilized control; 1AS = ammonium sulphate (single application); 2AS = ammonium sulphate (split application); 1ASD = ammonium sulphate + DMPSA (single application); 2ASD = ammonium sulphate + DMPSA (split application).

		N_2O (g N_2O-N ha^{-1})		EF (%)	YSNE (g N_2O-N/kg N uptake)	CO_2 (kg CO_2-C ha^{-1})	CH_4 (g CH_4-C ha^{-1})	GWP (Mg CO_2-eq ha^{-1})	GHGI (kg CO_2eq ha^{-1}/ kg yield ha^{-1})
	C	625 ab			17.7 a	6812 a	-432 a	6.97 a	3.36 a
	1AS	546 ab		0	6.6 b	5984 b	-512 a	6.12 b	1.28 b
CT	1ASD	521 ab		0	6.2 b	6171 b	-568 a	6.20 b	1.38 b
	2AS	659 a		0.02	5.2 b	6056 b	-459 a	6.22 b	1.35 b
	2ASD	619 ab		0	5.9 b	6424 ab	-493 a	6.58 ab	1.36 b
	C	328 B	(-47%)		7.2 A	(-59%)	4078 A (-40%)	-280 A (35%)	4.16 A (-40%)	1.65 A (-51%)
	1AS	547 A		0.12	5.4 AB		3814 A (-36%)	-206 A (60%)	3.95 A (-35%)	0.91 B (-29%)
NT	1ASD	396 B	[-28%] (-24%)	0.04	4.1 B	(-34%)	4002 A (-34%)	-386 A	4.10 A (-34%)	0.92 B (-33%)
	2AS	617 A		0.16	5.3 AB		4094 A (-32%)	-393 A	4.25 A (-32%)	0.84 B (-38%)
	2ASD	429 B	[-31%] (-31%)	0.06	4.6 B	(-21%)	4359 A (-32%)	-282 A	4.47 A (-32%)	1.00 B (-26%)

Table 5. Cumulative N_2O emissions (g N_2O-N ha^{-1}), mean Water Filled Pore Spaces (WFPS) (%) and mean Soil Temperature (ºC) for each period. *Different letters within a column and management (CT = lowercase; NT= capital) indicate significant differences using the Duncan Test (P < 0.05; n = 4). Values in square brackets indicate significant differences in percentage between DMPSA and AS (P < 0.05; n = 4; Student-T test). Values in round brackets indicate significant differences in percentage between CT and NT within each treatment (P < 0.05; n = 4; Student-T test).*

CT = conventional tillage; NT = no-tillage; C = unfertilized control; 1AS = ammonium sulphate (single application); 2AS = ammonium sulphate (split application); 1ASD = ammonium sulphate + DMPSA (single application); 2ASD = ammonium sulphate + DMPSA (split application).

		Pre-fertilization Sowing-Tillering (114 days)	1st fertilization Tillering-Stem elongation (23 days)	2nd fertilization Stem elongation-Harvest (102 days)	Post-harvest (62 days)
CT	C	266.8 a	25.1 b	145.3 ab	188.0 ab
	1AS	177.6 a	86.0 a	126.1 b	156.7 a
	1ASD	196.6 a	20.7 b [-76%]	126.1 b	177.3 a
	2AS	250.7 a	19.1 b	213.1 a	176.4 a
	2ASD	255.6 a	6.2 b	190.9 ab	166.7 a
	WFPS (%)	*55.3*	*48.4*	*38.5*	*35.0*
	Soil T (ºC)	*6.3*	*9.7*	*15.3*	*18.2*
NT	C	207.5 A	23.4 C	68.4 C	29.4 B (-84%)
	1AS	216.7 A	162.5 A (47%)	123.1 B	44.9 AB (-71%)
	1ASD	232.0 A	34.9 C [-79%]	80.1 C [-35%](-36%)	49.3 AB (-72%)
	2AS	234.1 A	108.6 B (82%)	213.3 A	61.4 A (-65%)
	2ASD	268.2 A	26.2 C [-76%]	96.3 C [-55%](-50%)	44.2 AB (-74%)
	WFPS (%)	*70.3*	*66.0*	*50.8*	*47.5*
	Soil T (ºC)	*6.5*	*10*	*15.4*	*17.9*

3.2.3. CARBON DIOXIDE EMISSIONS

In CT, CO_2 emissions ranged from 0.40 to 44.53 kg CO_2-C ha^{-1} d^{-1} (data not shown). Total cumulative CO_2 emissions (Table 4) were significantly higher in CT-C than in the rest of the CT-treatments except CT-2ASD. In NT, emissions ranged between 2.48 and 28.65 kg CO_2-C ha^{-1} d^{-1} (data not shown) and total cumulative emissions showed no significant differences between NT-treatments.

Comparison between CT and NT managements provided lower CO_2 emissions in NT than in CT, with these differences being statistically significant in all treatments with an average reduction of 35%. DMPSA application did not significantly affect cumulative CO_2 emissions in any case.

3.2.4. METHANE EMISSIONS

Methane daily fluxes in CT oscillated between –11.52 and 2.36 g CH_4-C ha^{-1} d^{-1} (data not shown). In the case of NT, fluxes ranged between –11.34 and 2.18 g CH_4-C ha^{-1} d^{-1} (data not shown). Total cumulative fluxes (Table 4) showed that both CT and NT managements acted as CH_4 sinks. Although, on average, soil CH_4 uptake was 37% higher in CT than in NT, only in CT-C and CT-1AS treatments the uptake of CH_4 was significantly higher than in NT-C and NT-1AS respectively (Table 4). DMPSA application did not significantly affect cumulative CH_4 fluxes in any case.

3.2.5. GLOBAL WARMING POTENTIAL

GWP ranged from 6.12 Mg CO_2-eq ha^{-1} in 1AS up to 6.97 Mg CO_2-eq ha^{-1} in the C treatment under the CT management. It was significantly higher in the CT-C treatment with respect to the rest, except CT-2ASD (Table 4). In the case of NT, GWP ranged from 3.95 Mg CO_2-eq ha^{-1} in 1AS up to 4.47 Mg CO_2-eq ha^{-1} in 2ASD. No significant differences between treatments were found in this management. On the other hand, GWP was significantly reduced in all treatments of NT with respect to CT by 35% on average (Table 4).

3.3. ABUNDANCE OF NITRIFICATION AND DENITRIFICATION GENES

No difference in total bacteria abundance (*16S rRNA* gene abundance) was observed due to DMPSA application in any sampling day (Fig. 2-A). DMPSA effect on AOB (in terms of absolute abundance of *amoA* gene) varied between managements and days (Fig. 2-B). After the 1st fertilization, DMPSA application significantly reduced *amoA* gene abundance 8 days after fertilization (daf) in CT management. After the 2nd fertilization, results were similar 12 dafs, when only CT-2ASD showed lower *amoA* gene abundance than CT-2AS, while the application of DMPSA significantly decreased *amoA* gene abundance in both CT and NT 31 daf. When analyzing the trend in time, AOB abundance clearly increased with time in both CT-2AS and NT-2AS treatments reaching a maximum on day 31 after the 2nd fertilization, while just a slight increase was observed in 2ASD treatments. DMPSA application also affected denitrifying bacteria, significantly increasing *nosZI* gene abundance (Fig. 2-C) 8 and 19 days after the 1st fertilization and 31 days after the 2nd fertilization in NT. This trend was only significant 19 days after 1st fertilization in the case of CT.

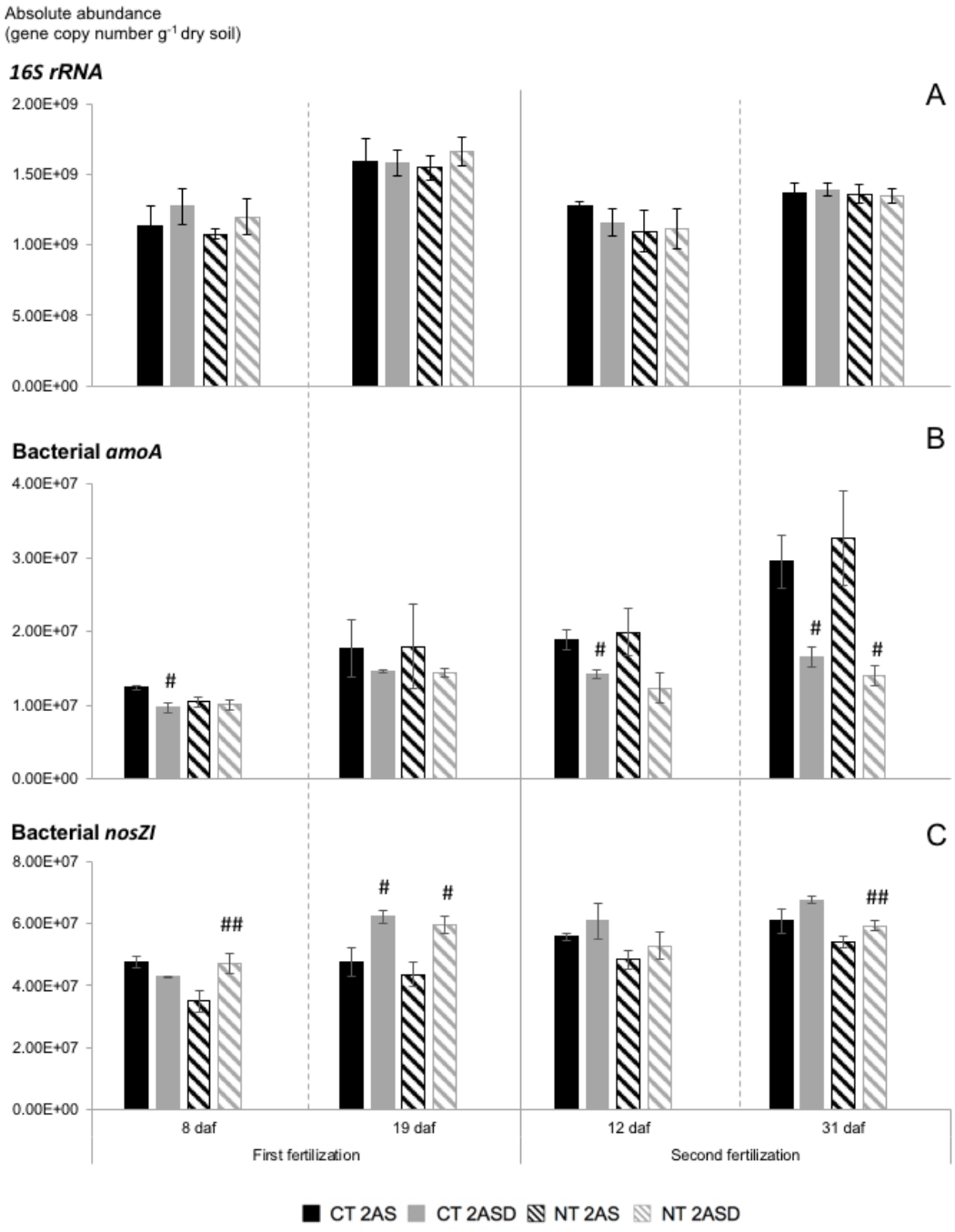


Figure 2. Total abundance of bacteria (16S rRNA) (A), amoA (B), and nosZI (C) genes, expressed as gene copy number per gram of dry soil, at different days after each fertilization (daf). *Hash (#) indicates significant effect of DMPSA application (P < 0.05 = #; P < 0.1 = ##; n = 3; Student-T test). 2AS = ammonium sulphate (split application); 2ASD = ammonium sulphate + DMPSA (split application).*

3.4. CROP YIELD PARAMETERS

Grain yield in the C treatments was 2162 and 2547 kg ha^{-1} for CT and NT respectively (Table 6). Yield was increased by fertilization to an average for all the fertilized treatments of 4713 kg ha^{-1} (CT) and 4617 kg ha^{-1} (NT). Neither fertilized treatments nor management (CT or NT) induced any difference in grain yield in any case. However, some yield components did show significant differences between CT and NT managements depending on treatments (Table 6). The number of tillers per m^2 was higher in NT than in CT, being these differences statistically significant in C, 1AS and 1ASD treatments. The dry weight of 1000 grains was lower in NT than in CT, with significant differences in all treatments except the Control. The harvest index also tended to be lower in NT than in CT, being significantly lower in 1AS and 2ASD. On the other hand, grain protein content was not significantly affected either by the fertilization treatments or soil management (Table 6), except by a slight increase in NT–1ASD with respect to CT–1ASD.

Table 6. Grain yield, yield components and grain protein content of wheat. *Different letters within a column and management (CT = lowercase; NT= capital) indicate significant differences using the Duncan Test (P < 0.05; n = 4). Significant differences between CT and NT within each treatment are represented by an asterisk (*) (P < 0.05 ; n = 4; Student-T test).*

CT = conventional tillage; NT = no-tillage; C = unfertilized control; 1AS = ammonium sulphate (single application); 2AS = ammonium sulphate (split application); 1ASD = ammonium sulphate + DMPSA (single application); 2ASD = ammonium sulphate + DMPSA (split application).

		Grain yield (kg ha^{-1})	Tillers m^{-2}	1000 grains DW (g)	Grains ear^{-1}	Harvest index	Grain protein (%)
	C	2162 b	316 b	37.5 a	30.6 b	0.67 a	9.6 b
	1AS	4781 a	439 a	36.5 a	39 ab	0.69 a	12.7 a
CT	1ASD	4542 a	419 ab	34.4 a	43.4 ab	0.67 a	11.2 ab
	2AS	4650 a	471 a	35.3 a	39.3 ab	0.68 a	12.5 a
	2ASD	4878 a	404 ab	37.5 a	41.2 ab	0.72 a	11.7 ab
	C	2547 B	469 B *	35.3 A	35.3 A	0.54 AB	10.6 B
	1AS	4356 A	772 A *	28.3 B *	39.4 A	0.49 B *	13.2 A
NT	1ASD	4542 A	770 A *	30.7 B *	35.7 A	0.54 AB	12.4 A *
	2AS	5103 A	721 A	27.9 B *	37.3 A	0.61 A	13.2 A
	2ASD	4467 A	615 AB	31.0 B *	37.1 A	0.58 A *	11.7 AB

DISCUSSION

4.1. NO-TILLAGE REDUCES CO_2 EMISSIONS

NT systems tend to decrease CO_2 emissions in dry climates (Álvaro-Fuentes et al., 2008), but not in humid (Huang et al., 2018). In our case, NT management was revealed as the better option in terms of reducing CO_2 emissions, with an average reduction of 35% with respect to CT (Table 4). This reduction in CO_2 emissions is in agreement with other studies carried out under Mediterranean conditions (Alvaro-Fuentes and Cantero-Martinez, 2010; Carbonell-Bojollo et al., 2011, 2015), although there are cases in which this effect was not observed (Guardia et al., 2016). Our results indicate that NT practices can effectively increase the soil carbon stock in our climate conditions, Humid Mediterranean. Different studies performed in Spain have shown that CT increases CO_2 emissions due to a greater root respiration, a higher microbial decomposition and liberation of the CO_2 accumulated in soil pores with plowing (González-Sánchez et al., 2012; Soane et al., 2012). Although higher CO_2 emissions could be expected just after plowing in CT, differences between CT and NT were constant along all periods (data not shown). Soil CO_2 efflux is a key indicator of both microbial and plant activity, being soil temperature and water content two main variables controlling soil CO_2 emissions (Morell et al., 2011). In order to know to what extent the tillage system could be inducing quantitative changes in the soil microbial population, soil bacterial abundance was quantified as the abundance of bacterial *16S rRNA* gene. The results showed that there were no differences between the total bacterial abundance of both tillage systems (Fig. 2). So, it could be inferred that the differences in soil CO_2 emissions between CT and NT systems could be more related to differences in plants' root respiration rather than differences in microbial activity.

With respect to NIs effects on CO_2 emissions, several studies have found variable effects of DMP based NIs. While Huérfano et al. (2016) found no effect of DMPSA on CO_2 fluxes, Guardia et al. (2018a) observed decreased respiration rates. Other studies

have demonstrated no effect (Menéndez et al., 2012; Huérfano et al., 2015; Florio et al., 2016) or a decrease (Weiske et al., 2001; Pfab et al., 2012) in CO2 emissions after DMPP application. In our case, we did not find any effect due to fertilization nor DMPSA application in NT or CT in terms of CO_2 emissions (Table 4) or bacteria population abundance (Fig. 2). The only remarkable difference was that CT fertilized treatments emitted less CO_2 than the unfertilized Control, as also described by Bowden et al. (2000) and Menéndez et al. (2006) after mineral fertilization, which was attributed to a different soil C/N ratio between fertilized and unfertilized treatments.

4.2. TILLAGE MASKS THE EFFECT OF FERTILIZATION ON N_2O EMISSIONS

Two different tillage managements (CT and NT) would result in different soil physicochemical conditions, inducing changes in soil porosity, compaction, moisture and O_2 availability. The differences generated by CT and NT managements in soil physical properties could lead to different gas fluxes. In terms of N_2O emissions, it has been described that variations due to NT practices as an alternative to CT can generate different results depending on other management practices, crop type, soil properties and climatology (Soane et al., 2012). In our study, the use of NT management as a replacement for CT was a valuable strategy to mitigate N_2O emissions, since a reduction of almost 50% in the total cumulative emissions was observed when C treatments were compared (Table 4). However, following fertilization, N_2O emissions were statistically equal between CT-AS and NT-AS treatments. Analyzing in more detail the data presented in Table 5, it is observed that the effect exerted by the tillage system on N_2O emissions was a function of the time period analyzed. Thereby, this effect was also dependent on the WFPS in each management and period. In fact, the tillage management did not exert any difference during the pre-fertilization period, when WFPS was > 55% in both management systems. After the 2nd fertilization and post-harvest, during spring and summer, soil moisture in the CT system remained below a mean WFPS value of 40–45%. In these conditions, CT-C showed soil NO_3^- contents which were around 50% higher than

soil NH_4^+ contents (i.e., NO_3^-/NH_4^+ values around 1.5) while in NT-C they were around 50% lower (i.e., NO_3^-/NH_4^+ values around 0.5) (Table 3). These higher NO_3^-/NH_4^+ values in the CT system could be observed as a general trend along the whole experimental period and also in the fertilized treatments when comparing CT with NT within each treatment. This suggests that in the CT system nitrification was taking place in a more efficient manner than in the NT system. In this sense, it has been described that the intensity of nitrification in soils is influenced by soil structure, which regulates aeration (Hoffman et al., 2007), being this structure more aerated in CT respecting to NT. At the low soil WFPS < 40–45% of the CT system in the 2nd fertilization and post-harvest periods, the basal NH_4^+ content of treatment CT-C seems to have been enough to produce relatively high N_2O emissions by means of an efficient nitrification process, which were of the same magnitude of those produced by a higher NH_4^+ quantity in the fertilized AS treatments (i.e., no effect of fertilization was observed on N_2O emissions). Therefore, the tillage management exerted a great effect on soil N emissions by means of lowering the soil water content in comparison to the NT system, thus masking the effect of fertilization on N_2O emissions. Contrarily, in the NT system, the > 40–45% WFPS resulted in conditions where denitrification was a process taking relevance as responsible for N_2O emissions. Under these more denitrifying conditions, lower basal N_2O emissions were observed in NT-C treatment than in CT-C treatment. This was especially well observed in the post-harvest period, when N_2O emissions in NT-C were 84% lower than in CT-C, when mean WFPS for that period was 47.5% in NT with respect to 35% in CT (Table 5). With these lower basal N_2O losses in the unfertilized NT-C, the increase in N_2O emissions induced by fertilization (both 1AS and 2AS treatments) was clearly observed both after the 1st and 2nd fertilizations in the NT system. Moreover, provided that after the 1st fertilization also in the CT system soil water content showed a mean WFPS > 40–45% (48.4% of WFPS), also under this tillage system an induction in N_2O emissions could be observed after fertilization over the low N_2O emissions of the unfertilized CT -C. This induction was observed when the whole N rate of 180 kg N ha^{-1} was applied (1AS treatment), while the application of 60 kg N ha^{-1} (2AS treatment) was not enough to

induce an increasing effect. Therefore, the induction of N_2O emissions after fertilizer application seems to be due to an induction of the denitrification process. On top of this, soil structure is an additional factor that influences the differential N_2O emissions observed under NT with respect to CT management. The N_2O produced in the NT system could be retained for more time within the more structured NT soil before reaching the soil surface. This will enhance the probability that N_2O could be reduced to N_2 in the microbial denitrification process. This helps to explain why, when WFPS is lower than 48% (i.e. after the 2nd fertilization and post-harvest periods), the N_2O emissions of NT-C (coming in a greater extent from denitrification) are lower than those of CT-C (predominantly coming from nitrification). Then, the effect of fertilization on N_2O emissions is more clearly observed in the NT system, because it is known that after fertilization the higher NO_3^- content enhances proportionally more the production of N_2O than its reduction to N_2 in the denitrification process (Saggar et al., 2013). Finally, the increase in N_2O emission observed after the application of 180 kg N ha^{-1} in the 1st fertilization of the CT system would also indicate that, above a threshold value of about 47% of WFPS, denitrification seems to have been the process responsible for the increase observed in N_2O emissions.

4.3. DMPSA SHOWS BETTER EFFICIENCY REDUCING N_2O EMISSIONS UNDER NO-TILLAGE CONDITIONS

Although a few studies have been already carried out to analyze the potential of DMPSA to reduce N losses, to our knowledge this is the first study analyzing DMPSA behavior under NT management. Our experiment demonstrated that the application of DMPSA in the NT system, both applied in a single or split application, effectively reduced N_2O emissions by 28% and 31%, respectively. Both NT-ASD treatments were able to reduce emissions down to Control levels. A more exhaustive analysis by periods (Table 5) showed that, in the NT system, the highest reductions occurred in the 23 days length period after 1st fertilization, when average WFPS was 66%, with a DMPSA reduction efficiency of 79% (single application) and 76% (split). After the 1st fertilization,

WFPS conditions were in a range between 60% and 70%. These values are related to high emissions episodes (Davidson, 1991; Del Prado et al., 2006), when denitrification should have been also taking place together with nitrification. Bearing in mind that the response to AS application was higher in NT, it is relevant that the application of DMPSA was able to reduce NT-ASD treatments emissions to the same level of CT-ASD treatments, so DMPSA demonstrating to be an efficient tool to offset the response of the NT system to fertilization in terms of N_2O. After the 2nd fertilization, in a 102 days cumulative emissions period, DMPSA reduction in NT-2ASD was 55%, when WFPS decreased down to an average of 51%, making thus conditions less suitable for denitrification. The DMPSA effect inhibiting nitrification was also reflected in NT in the lower soil NO_3^- contents during this 2nd fertilization period (Table 3) as well as in the lower nitrifier bacteria abundance (Fig. 2-B). In this sense, the decrease in *amoA* gene abundance observed 31 daf in NT-2ASD with respect to NT-2AS (Fig. 2-B) showed that the effect of DMPSA was stronger at the low WFPS values (< 51%) of the 2nd fertilization rather than at the higher WFPS values (> 60%) of the 1st fertilization. These results are in accordance with those of Torralbo et al. (2017), who found a decrease in AOB abundance at low soil water content values (40% of WFPS) 16 daf and 51 daf, while with higher soil water contents (80% of WFPS) they did find a decrease 16 daf but not 51 daf. Other studies with the other DMP-based nitrification inhibitor (DMPP) have reported similar effects, with a significant reduction in AOB abundance at 40% of WFPS, but no effect at 80% of WFPS (Barrena et al., 2017). DMPSA is considered as a highly specific NI that focuses its activity on nitrifying communities. So, it was expected to find a clear effect on *amoA* gene abundance without affecting total bacterial abundance (*16S rRNA* gene abundance). To our knowledge, this is the first study analyzing DMPSA effect on soil bacteria abundance in field conditions and, as expected, soil total bacterial abundance was not affected by DMPSA application along the field experiment (Fig. 2-A). In this same sense, no other microbial activity except ammonium monooxygenase of nitrifiers should be expected to be affected by the application of DMP-based Nis. However, previous works have reported different responses of *nosZI* gene abundance after DMPP application

depending on WFPS as a main factor. In a laboratory experiment, Barrena et al. (2017) found an induction at 80% WFPS that was not observed at 40% WFPS at the same incubation temperature. This induction at 80% WFPS was also observed by Torralbo et al. (2017) 51 daf but not at 40% WFPS in any case, and Duan et al. (2017) did neither find any effect at 50% WFPS. Regarding DMPSA, Torralbo et al. (2017) also demonstrated that DMPSA can stimulate *nosZI* expression 51 daf at 80% WFPS, while 16 daf was not enough time as to have induced a change in this bacterial population. In our case, it seems that a threshold value of around 48% of WFPS is necessary to induce an increase in *nosZI* gene abundance in the NT system, and also even in the CT system provided that a sufficient time is maintained under these soil water content conditions. Thus, an increase of *nosZI* gene copy number was observed 8, 19 and 31 daf in the case of NT, and also 19 daf in the case of CT when WFPS was higher than 48% (Fig. 2-C). So, *nosZI* induction was better observed at the higher WFPS levels present in the NT management after the 1st fertilization, which matches with the results of Barrena et al. (2017) and Torralbo et al. (2017). These results fully support the argument that in the case of the NT system the decrease observed in N_2O emissions after DMPSA application might be occurring by means of an induced reduction of N2O up to N_2 as a specific mechanism that is promoted due to the fact that NT induces a higher WFPS with respect to the CT system. It should be remarked that this is the first time that such a *nosZI* gene inducing effect by the application of DMPSA has been described to occur under field conditions instead of under the laboratory incubation studies previously described (Torralbo et al., 2017). It was also demonstrated that the DMPSA applied in the 1st fertilization was still effective after the 2nd fertilization, when NT-1ASD treatment was emitting 35% less N_2O than NT-1AS (Table 5). In fact, DMPSA mitigation effect was observable up to the end of May (43 days after the second application) (Fig. 1).

With respect to CT, other field experiments applying DMPSA in CT systems as Huérfano et al. (2018) found reductions up to 32% in a rainfed maize-ryegrass rotation, and reductions up to 58% have been reported in maize under irrigated conditions (Guardia et al., 2017, 2018a; Recio et al., 2018). In our case, and by the contrary to that observed

in NT, DMPSA did not cause a significant reduction in N_2O losses under CT conditions when taking into account the whole experimental period (Table 4). This was due to the induction of N_2O emissions caused by the tillage management, which masked the response of N_2O emissions to fertilization, as previously discussed. Recio et al. (2018) also found no significant reduction in total cumulative emissions when DMPSA was applied with CAN, and Huérfano et al. (2016), in our same edaphoclimatic conditions, found that DMPSA was not effective under a CT management in the second of two experimental years, attributing that lack of effectivity to environmental conditions less prone to N_2O losses. However, the mitigation of 56% reported by Huérfano et al. (2016) in the first experimental year of conventional tillage, at an average WFPS of 75% in their fertilizations period, was above ours (28–31% mitigation) under NT management at an average WFPS of 57% in our fertilizations period. This means that, under the same edaphoclimatic conditions, the efficiency of DMPSA reducing N_2O emissions depends on the WFPS level as a main factor, which, while being modulated by the tillage system, will also depend on the environmental conditions of each year and particular season. In fact, in the 23 days period after 1st fertilization, DMPSA strongly reduced N_2O emissions by 76% after applying 180 kg N ha^{-1} in a single application. Nevertheless, the emission levels in all fertilized treatments in CT were much lower than in NT, so, in absolute values, the reduction was not as high as in NT-ASD treatments. In the 102 days period after the 2nd fertilization, we did not observe any significant effect of DMPSA on N_2O emissions, not even in the split application, since the high unfertilized treatment emission was masking the effect of fertilization on N_2O emissions. This was already proposed by Menéndez et al. (2012), who described that the percentages of reduction induced by DMPP are proportional to the percentage of emissions-increasing induced by fertilizer application. Nevertheless, there was evidence indicating that DMPSA was inhibiting nitrification during this period of time, as shown by the lower NO_3^-/NH_4^+ values (Table 3) and lower *amoA* gene abundances (Fig. 2-C) 12 and 31 daf in treatment CT-2ASD with respect to CT-2AS.

4.4. EFFECT OF THE TILLAGE SYSTEM AND DMPSA APPLICATION ON CROP YIELD AND QUALITY

In our Humid Mediterranean conditions wheat can obtain higher yields with respect to other areas of Mediterranean climate due to the relatively high precipitation in spring (Fig. S1). In fact, in the humid Mediterranean conditions that prevail in our field site (Alava, Northern Spain), the average yield for winter wheat as reported by the Ministry of Agriculture, Food and Environment (MAGRAMA, 2014) is 5000 kg ha^{-1} approximately, being the usual N rate applied 180 to 220 kg N ha^{-1}, and 182 kg N ha^{-1} the agronomically optimum N fertilizer rate described for the region (Ortuzar-Iragorri et al., 2010). Maximum yields have been described for this region in a range as high as 9000 to 11000 kg grain ha^{-1} for Cezanne variety (Huérfano et al., 2015, 2016). The yield obtained in this field experiment under CT conditions was half of the yield registered by Huérfano et al. (2016) in the same location, with the same Cezanne variety and same CT management. This might be attributed to the fact that these authors registered a slightly higher precipitation (715 mm yr^{-1} compared to the 666 mm yr^{-1} in our case). Regarding yield components, in CT tillers per m^{-2} and grains per ear were in the same range as in Huérfano et al. (2016), being 1000 grains DW lower in our experiment, which is concomitant with the lower grain yield obtained. However, in our case, grain protein content was higher than in their case, pointing that the lower production favored grain N content. In this sense, it is well known that the increase in grain yield leads to a decrease in the protein to starch ratio in the grain (Triboi et al., 2006).

It is assumed that lower yields could be expected just after adopting NT, while equal yields with respect to a CT system can be achieved after a few years (Soane et al., 2012). Moreover, under Mediterranean conditions, low or NT practices usually generate an increase in crop yield (Sanz-Cobena et al., 2017). In our case, NT management induced the appearance of more tillers per m^{-2} than CT (Table 6), especially in the case when fertilizer was applied in a single application. The number of tillers per m^{-2} is determined at the beginning of tillering stage (GS21); thus, a higher soil water content in the NT

system in that period could have improved plant N absorption, causing the increase observed in the number of tillers, especially in the single fertilization treatment. This was compensated by an opposite effect in the 1000 grains dry weight, whose values are determined later, during the grain-filling period, and were lower in all fertilized treatments of the NT management compared to CT.

Until now, some studies have reported no effect of DMPSA on yield parameters and grain N content of wheat (Huérfano et al., 2015, 2016), except a decrease in grain N content in one of two field experimental years (Guardia et al., 2018b). In our experiment, we did not observe any detrimental or beneficial effect of DMPSA application on any yield component nor on grain protein content (Table 6). In the case of NT, a decrease in the harvest index was detected in the AS single application soil with respect to the split. In this point, it is interesting to observe that DMPSA was able to reverse that effect and reach the harvest index values of the split treatments, highlighting that DMPSA could be an efficient tool to join the split fertilizer applications into just one single application while maintaining yield.

4.5. SUSTAINABILITY FACTORS

When N_2O emissions are referred to the total N harvested (Yield Scaled N_2O emissions; YSNE), our study shows the advantages of the NT management in conjunction with the application of DMPSA, when YSNE resulted to be 21–34% lower than in the CT management (Table 4). While N_2O emissions factors were, in any case, much lower than the default value of 1% proposed by IPCC (2006), this approach (NT + DMPSA application) demonstrates to be able to reduce it even further in our edaphoclimatic conditions. CH_4 and, especially, CO_2 emissions have also a high importance in terms of sustainability. The Global Warming Potential (GWP) and Greenhouse Gas Intensity (GHGI) (Mosier et al., 2006) factors consider N_2O, CH_4 and CO_2 emissions together (in terms of CO_2-equivalent emissions) to give an account for the global impact of the system. Guardia et al. (2016), considering other associated costs and savings of different tillage systems, already indicated that the NT system decreases GWP under

Mediterranean conditions. In our experiment, NT management also showed a much lower GWP and GHGI, with an average reduction of 35% with respect to CT. This was mainly due to its capacity to maintain yield and a higher carbon stock, which added to the fact that NT usually leads to lower net costs (Sánchez-Girón et al., 2004) with savings in both labor time and fuel inputs in comparison to CT (Álvaro-Fuentes et al., 2014; Guardia et al., 2016) remarks NT management as a good mitigation strategy.

With respect to NIs effect on CH_4 fluxes, a higher content of NH_4^+ as a result of DMPSA application might derive in inhibition of CH_4 oxidation (Tlustos et al., 1998; Ullah et al., 2008) due to the competition between NH_3 and CH_4 for methane monooxygenase enzyme (Holmes et al., 1995), so increasing CH_4 emissions. Nevertheless, our results are in concordance with other studies showing no statistically significant effects of DMPSA application on CH_4 fluxes (Guardia et al., 2018a, 2018b; Huérfano et al., 2018).

C O N C L U S I O N S

The soil management system plays a key role in terms of GHG emissions, being no-tillage an environmentally efficient practice to reduce CO_2 and N_2O emissions, as shown in the unfertilized treatments. However, when fertilizing with ammonium sulfate, N_2O emissions in no-tillage increased up to the levels of conventional tillage. The higher soil WFPS in no-tillage resulted in DMPSA being more effective under this management, where its application was able to avoid fertilization-induced N_2O emissions. This higher efficiency might be due to a greater N_2O reduction to N_2 as a consequence of the *nosZI* gene induction observed. Therefore, we conclude that the use of a no-tillage management combined with DMPSA is a promising strategy to increase the sustainability of the rainfed agricultural systems under our Humid Mediterranean conditions.

SUPLEMENTARY MATERIAL

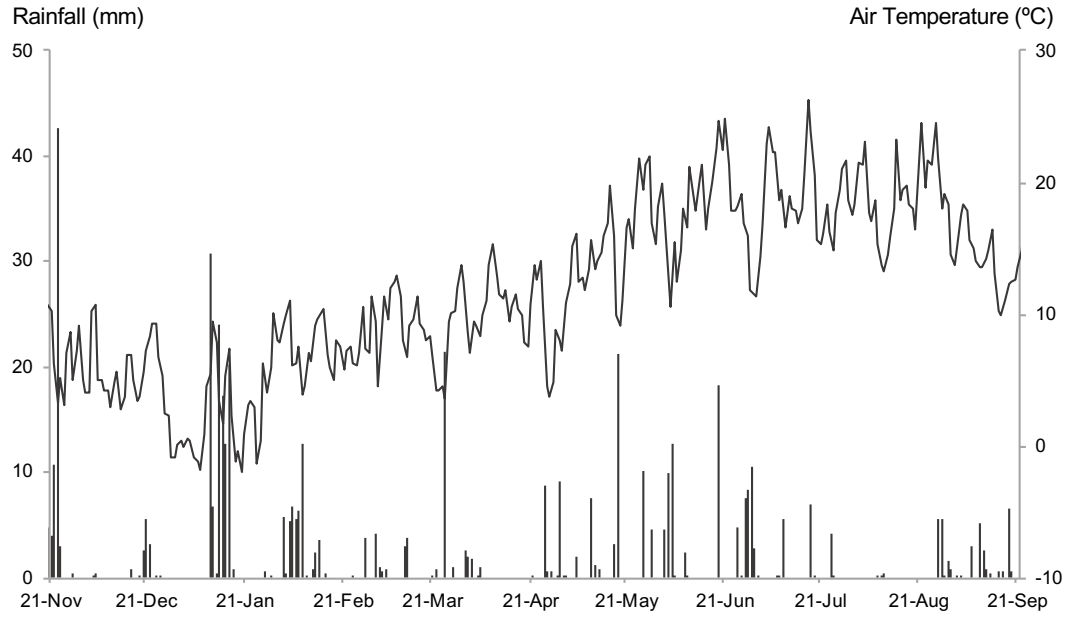


Figure S1. Daily precipitation (bars) and mean air temperature (line) for the whole period of study.

Table S1. Primers pairs and thermal conditions used in real-time qPCR.

Target group	Primer name	Sequence	Thermal profile	bp lenght	References
Bacterial *16S rRNA*	341F	5'-CCTACGGGAGGCAGCAG-3'	95ºC for 2 min - x 1 cycle 95ºC for 15 sec 60ºC for 30 sec 72ºC for 30 sec 80ºC for 30 sec - x 40 cycles	174	Lopez-Gutiérrez et al., 2004
	534R	5'-ATTACCGCGGCTGCTGGCA-3'			
Bacterial *amoA*	amoA1F	5'-GGGGTTTCTACTGGTGGT-3'	95ºC for 2 min - x 1 cycle 95ºC for 15 sec 54ºC for 60 sec 72ºC for 60 sec - x 40 cycles	491	Rotthauwe et al., 1997
	amoA2R	5'-CCCTCKGSAAAGCCTTCTTC-3'			
nosZI	nosZ-F	5'-CGCRACGGCAASAAGGTSMSSGT-3'	95ºC for 2 min - x 1 cycle 95ºC for 15 sec 63ºC for 30 sec (-1ºC/cycle), 72ºC for 30 sec 80ºC for 15 sec – x 6 cycles 95ºC for 15 sec 60ºC for 30 sec 72ºC for 30sec 80ºC for 30 sec - x 40 cycles	267	Henry et al., 2006
	nosZ-R	5'-CAKRTGCAKSGCRTGGCAGAA-3'			

CHAPTER 2

Influence on NH_3 volatilization

Joint application of urease and nitrification inhibitors to diminish gaseous nitrogen losses under different tillage systems

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The first test with DMPSA in combination with a no-tillage management was positive in different aspects, although it leaves several open questions. We found that a no-tillage management can be a good practice to increase the sustainability of the system under our conditions, but only when DMPSA is applied to avoid the N_2O emissions caused by fertilization.

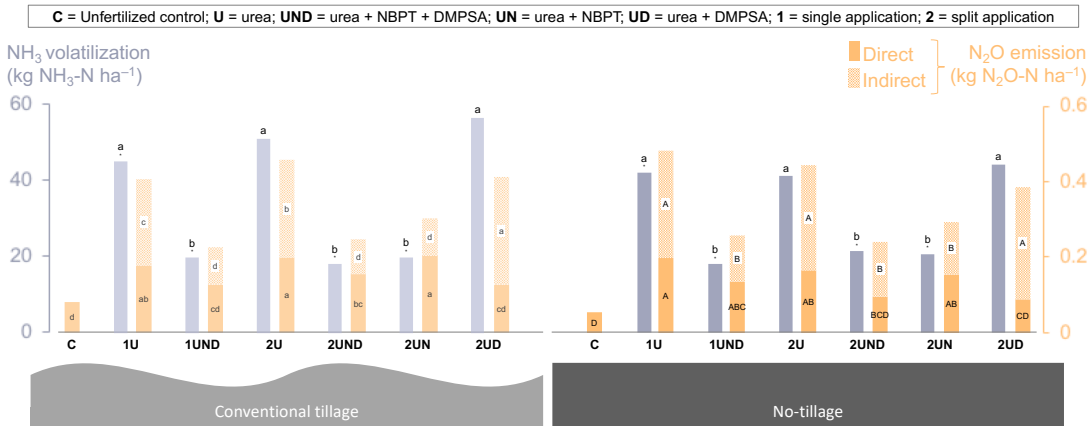
Although the measurement of soil mineral nitrogen did not exert many statistically significant differences, it was clear when observing the absolute values that a higher NH_4^+ content was present in both tillage systems when DMPSA was applied. This raised the question of whether the application of DMPSA could affect NH_3 volatilization. As it was commented in the introduction section, there is an equilibrium between NH_3 and NH_4^+ in the soil and, at the same time, NH_3 is in equilibrium between the aqueous and gaseous phases. In this manner, a greater NH_4^+ concentration can also result in a higher NH_3 content which, therefore, can increase the rate of nitrogen lost by volatilization. This could be an especially relevant problem in soils and climates prone to NH_3 volatilization; that is, those with an alkaline pH and receiving a low rainfall, as is the case of Mediterranean systems. Thus, we could be trying to avoid direct N_2O emissions but, as a counterpart, increasing the indirect N_2O emissions once the volatilized NH_3 is deposited, in addition to other problems associated with NH_3 volatilization, which imply a great economic and environmental cost. Therefore, we wanted to determine if the application of DMPSA together with a widely applied fertilizer prone to great volatilization losses (such as urea), could be advisable or counterproductive for the system's sustainability. It can be discussed that the easiest option to reduce volatilization when fertilizing with urea would be a subsurface application. However, this practice requires specific machinery and is not possible when adopting a no-tillage system. This latter is a problem because non-tilled soils are especially prone to present large volatilization rates due to the higher soil compaction (which difficult urea infiltration) and because of the great urease activity of the vegetal residues that are left on the surface from the previous crop. There is no other option than to opt for other solutions such as the application of urease inhibitors as

NBPT; but this does not solve the impact derived from nitrification. An equilibrium solution, therefore, could be the joint application of NBPT together with DMPSA.

One of the challenges we faced was the size of our experimental plots; too small for the traditional ammonia passive samplers, since large plots are necessary to avoid cross-contamination by NH_3 carried from other treatments by the wind. This forced us to look for alternative methods, adopting the Dräger-Tube Method (DTM) developed by Roelcke et al. (2002) and Pacholski et al. (2006, 2016b). This system allows the quantification of NH_3 volatilization by measuring in contact with the soil, avoiding interference from other treatments. The system was previously tested and its ability to avoid cross-contamination proven. We verified that the system is capable of obtaining results within the expected range, with little deviation between repeats and without cross-contamination between treatments even with a small plot size and relatively-high wind speeds.

In this manner, we proceeded to analyze how the application of DMPSA could affect NH_3 volatilization rates, with special interest not only in observing the interaction between the inhibition of urease activity and the inhibition of nitrification on NH_3 losses, but also in the effects exerted on the crop yield by the different treatments considering the huge nitrogen losses that can take place as a consequence of NH_3 volatilization.

GRAPHICAL ABSTRACT



ABSTRACT

Urea fertilization is a widely spread source of nitrogen (N) for agriculture because of its easy accessibility. However, its use is highly inefficient, since a large amount of the applied N is lost to the environment mainly in the form of ammonia (NH_3) volatilization, causing serious environmental and economic damages. Although there are simple strategies to reduce these losses, as the subsurface application, this practice cannot be adopted in all types of cultivations and tillage systems. To address this problem, combinations of urease and nitrification inhibitors (double inhibitors) have been developed to close all possible N escape routes, which is not achieved when these inhibitors are applied individually. The objective of this work was to evaluate the potential of a new double inhibitor combining N-(n-butyl) thiophosphoric triamide (NBPT) and 2-(3,4-dimethylpyrazole-1-YL)-succinic acid (DMPSA) to mitigate N losses in a rapeseed crop established in conventional tillage (CT) and no-tillage (NT) soils under

Humid Mediterranean conditions. To do so, N losses in form of NH_3 volatilization and nitrous oxide (N_2O) emissions were determined through the Dräger-Tube method (DTM) and closed chamber method respectively. The specific weather conditions present during the experiment promoted great losses, mostly in form of NH_3 volatilization. The treatments receiving urea without inhibitors or urea with DMPSA lost up to 57.3 and 59.9 kg N ha^{-1} in CT and NT respectively. Nonetheless, the application of the double inhibitor was able to diminish these losses by more than 50%, reducing the losses to a minimum of 18.8 kg N ha 1 (CT) and 25 kg N ha^{-1} (NT). As a whole, a CT system fertilized with a single application of urea with the double inhibitor showed the lowest global warming potential (GWP) (3.93 Mg CO2-eq ha^{-1}), at the same level of the unfertilized treatment.

MATERIALS AND METHODS

2.1. EXPERIMENT SETUP

This work was conducted in Vitoria, northern Spain (42º51´ N, 2º37´ W, 530 m above sea level), under Humid Mediterranean conditions. Soil characteristics of the upper horizon (0–30 cm) were: pH (1:2.5 soil:water), 8.0; C:N ratio, 8.15; total N (Keeney and Nelson, 1982), 1.6 g kg^{-1}; organic matter (Walkley and Black, 1934), 21.2 g kg^{-1} ; Carbonate (MAPA, 1994), 9.8 g kg^{-1}; Ca, 6.4 g kg^{-1}; P (Watanabe and Olsen, 1965), 59.0 mg kg^{-1}; Mg (MAPA, 1994), 92.4 mg kg^{-1}; K (MAPA, 1994)167 mg kg^{-1}. The soil texture consisted of 43.4% of sand, 24.7% silt and 31.9% clay.

The experimental area was divided in two tillage systems, conventional tillage (CT) and no-tillage (NT), with an interspacing of 25 m. In September, 2018, the CT plot was conditioned with a seedbed preparation consisting of mechanical tillage (disc harrow, chisel and moldboard plow). NT was conditioned with a superficial disc harrow due to the excessive compaction of the soil. Winter rapeseed (*Brassica napus*, var. *Arazzo*) was

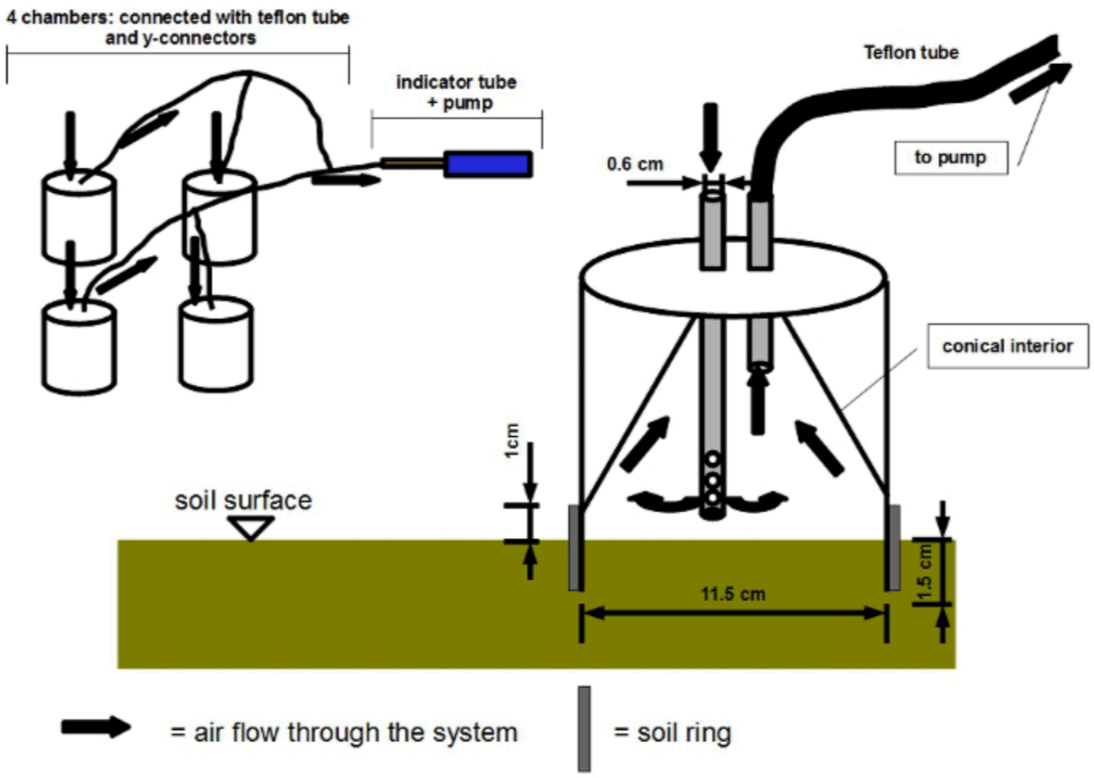
sown on September 13th, at a density of 4 kg seeds ha^{-1}. Due to the dry conditions, irrigation was necessary to ensure the proper establishment of the crop after sowing. Therefore, the experiment was maintained under irrigated conditions from September 13th to October 5th. Each tillage system was divided into four replicates of 38 x 28 m, separated by 2 m between them. Each replicate was subdivided into 7 plots of 8 x 4 m, being 6 fertilized and 1 unfertilized treatments randomly distributed between them. Treatments application dates and rates are detailed in Table 1. Fertilizers were applied on the surface in form of granules, as provided by EuroChem Agro Iberia S.L. The mixture of urea plus NBPT was supplied in the commercial form of UTEC® 46. All plots were also supplied with 60 kg K ha^{-1} (K_2SO_4), 50 kg P ha^{-1} (P_2O_5) and 55 kg S ha^{-1} (SO_3).

Table 1 Nitrogen application rates as urea (kg N ha^{-1}) in conventional tillage (CT) and no-tillage (NT) and rapeseed phenological stage.

Treatment	**BBCH21** (19-Feb-2019)	**BBCH57** (13-Mar-2019)
C	0	0
1U	150	0
1UND	150	0
2U	100	50
2UND	100	50
2UN	100	50
2UD	100	50

C = Unfertilized Control; 1U = Urea in a single application; 1UND = Urea + NBPT + DMPSA in a single application; 2U = Urea in a split application; 2UND = Urea + NBPT + DMPSA in a split application; 2UN = Urea + NBPT in a split application; 2UD = Urea + DMPSA in a split application.

2.2. NH_3 VOLATILIZATION



Set-up and application of dynamic chamber of Dynamic Tube Method (DTM). *Each system consists of 4 chambers connected by PTFE tubing, reduction connection are used to connect all chambers to one pump. Air is drawn through a copper tube perforated at the lower end and sealed at the very bottom, passed over the soil, and sucked at the top of the conical internal volume to another copper tube. The air which has passed through the system is then led via PTFE tubing to the indicator tube for determination of ammonia concentrations (Source: Pacholski, 2016b).*

The Dräger-Tube Method (DTM) (Roelcke et al., 2002) was used to determine NH_3 emissions from soils, including the modifications of the method and equations proposed in Pacholski et al. (2006) and Pacholski (2016b). Briefly, 4 stainless steel chambers (11.5 cm diameter) were placed in soil rings inserted into the soil. Perforated copper tubes allowed a forced airflow through each chamber. This airflow was forced with an automatic Dräger pump (Drägerwerk AG, Lübeck, Germany). Teflon tubes were used to connect the output flow of the 4 chambers and conduct this unified flow through

a Dräger sampling tube. Dräger tubes of two concentration ranges (0.25–3 and 2–30 ppm) were used depending on the expected NH_3 concentration of the flow. To account for the intra-diurnal variability, NH_3 emission was measured at dawn, midday and sunset. Likewise, at each sampling moment soil rings were differently positioned along the surface of each plot to account for the spatial variability. These measurements were carried out daily along 10 days after fertilization, decreasing the frequency to every 3 days when volatilization dropped.

Dräger-Tube Method for NH_3 emissions measurement. A) *Automatic Dräger pump to force airflow through the chambers connected by Teflon tubes.* ***B)*** *Dräger tubes for NH_3 detection.*

Air temperature and wind speed were registered at 0.2 and 2 m height with a frequency of 10 minutes through a meteorological station placed into the crop to account for the attenuation of wind speed by plants at 0.2 m height.

Nitrogen losses by NH_3 volatilization were calculated following the equations for high canopies of Pacholski (2016b). To obtain the daily and the along time cumulative

losses, the fluxes of the periods between two measurements were estimated by averaging the values obtained between two subsequent measurements, multiplying the obtained value by the duration of the interval and adding it to the previous cumulative value.

Due to the characteristic of the method, cross-contamination may occur in small plots with high wind speeds. To ensure that cross-contamination was not taking place, unfertilized treatments were measured every 3 fertilized-plots samplings. In the same way, after each measure, chambers were washed with water and sponges and completely dried with clean paper before the next measure.

2.3. GREENHOUSE GASES EMISSIONS

N_2O, CO_2 and CH_4 emissions were measured using the closed chamber method (Chadwick et al., 2014). Gas samples were collected every 3 days after sowing to account for the emissions from soil conditioning and irrigation (accounting for 4 samplings). Afterward, sampling was carried out every 15 days until fertilization. Samples were collected every 2 days after fertilizer application for one week. In the next three weeks, the frequency was diminished to 3, 2 and 1 day per week respectively. In the remaining time samples were collected every 15 days. Taking into account the diurnal variation of emissions (Baggs and Blum, 2004), sampling was performed between 10 a.m. and 1 p.m. To account for soil heterogeneity, four chambers (20 cm diameter x 18 cm height once inserted into the soil) were placed in each plot and two of them were sampled each day alternatively. Gas samples were taken just after closing the camber (t = 0) and after 45 minutes. 20 mL of gas were taken from each chamber and stored at overpressure in pre-evacuated 12 mL glass vials. Samples were analyzed by gas chromatography (GC) (Agilent, 7890A) equipped with an electron capture detector for N_2O detection, with a methanizer to measure CO_2 by reducing it to CH_4, and a flame ionization detector for CH_4 determination. A capillary column (IA KRCIAES 6017:240 ºC, 30 m x 320 µm) was used with Helium as the carrier gas. Samples were injected employing a headspace auto-sampler (Teledyne Tekmar HT3) connected to the GC. Certified standards of N_2O (in concentrations 0, 0.5, 0.7, 1, 3 and 5 ppm), CO_2 (0, 253, 256, 507, 984 and 2045 ppm)

and CH_4 (0, 3, 6, 12 and 26 ppm) were used to construct calibration curves. A certified mixture of these gases was also analyzed at the same time of samples. Gas emission rates were calculated taking into account the gas concentration variation from t = 0 to the end of the 45 min. 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).

Indirect N_2O emissions derived from the deposition of the volatilized NH_3-N were calculated as 0.5% of the volatilized NH_3, following the guidelines of IPCC for GHG inventories in dry climates (IPCC, 2019). Cumulative N_2O and CH_4 emissions were converted to CO_2 equivalents, using a GWP factor of 265 for N_2O and 28 for CH_4 (IPCC, 2014).

The cost of the damages derived from N losses has been estimated by applying the minimum and maximum values reported by Van Grinsven et al. (2013) for each emitted N form and human and/or environmental effect.

2.4. SOIL ANALYSIS

Soil NH_4^+ and NO_3^- contents were firstly determined 15 days before fertilization. Then, samples were taken 12 and 21 days after the 1st fertilization and 9, 19, 28 and 53 days after the 2nd fertilization. On each sampling day, three soil subsamples (3 cm diameter x 30 cm depth) were taken from each plot. Rocks and roots were removed and the soil homogenized. Next, 100 g of fresh soil were extracted with 200 mL 1 M KCl. The extracts were filtered through Whatman nº1 filter papers (GE Healthcare, Little Chalfont, Buckinghamshire, UK) to remove particles and, secondly through Sep-Pak Classic C18 Cartridges 125 Å pore size (Waters, Milford, MA, USA) to eliminate the organic matter. The resultant solutions were used to determine NO_3^- content by ultraviolet spectrophotometry as described by Cawse, (1967) and NH_4^+ by the Berthelot method (Patton and Crouch, 1977).

Soil water content was also determined each day of soil and/or greenhouse gases sampling. Two subsamples (3 cm diameter x 30 cm depth) were taken randomly from

each tillage system. Rocks were removed and the soil subsamples were oven-dried for 48 h at 80 ºC to determine the water content. It was expressed as the percentage of water-filled pore space (WFPS) following Linn and Doran (1984): WFPS = (soil gravimetric water content x bulk density) x (1 – (bulk density/particle density))$^{-1}$, by using a particle density of 2.65 Mg m^{-3}. Bulk densities were determined three times during the experiment: September 14th (after soil conditioning for sowing), February 5th (before 1st fertilization) and May 5th (after fertilizations). Bulk densities obtained for each tillage management resulted in 1.14, 1.13 and 1.11 Mg m^{-3} in the case of CT and 1.22, 1.26 and 1.26 Mg m^{-3} in NT.

2.5. CROP YIELD AND QUALITY PARAMETERS

A surface of 12 m^2 (1.5 x 8 m) was harvested in each plot. Grain yield was expressed at a 10% moisture content. Grain protein was calculated using a conversion factor of 5.35 times the total N content (Mariotti et al., 2008) determined by the Kjeldahl procedure (AOAC, 1980). Gross fat content was determined by acid hydrolysis (AOAC, 2006).

2.6. STATISTICAL ANALYSIS

Statistical analysis of the data was carried out with SPSS (IBM SPSS Statistics for macOS, version 25.0. Armonk, NY: IBM Corp). One-way ANOVA with Duncan's multiple-range test for separation of means ($P < 0.05$) was employed to test the differences in NH_3 volatilization, soil mineral N content, GHG emissions, GWP, N loss, crop yield and quality parameters among the treatments tested (C, 1U, 1UND, 2U, 2UND, 2UD, 2UN). Student's T-test ($P < 0.05$**)** was employed to analyze the differences between tillage systems (CT and NT) within a treatment. Additional details have been included in the captions of figures and tables.

RESULTS

3.1. ENVIRONMENTAL CONDITIONS

The experiment was carried out under similar climatic conditions after both fertilization events (Fig. 1A). The wind was constant all days and the highest intensities were reached frequently at midday. The mean wind speed increased slightly from 1st (1.0 m s^{-1}) to 2nd fertilization (1.3 m s^{-1}). In general, sunny days were frequent, with relatively warm temperatures but also with high thermal amplitude (temperatures usually around 0 ºC at dawn, with frost after 1st fertilization, and above 20 ºC at midday, even higher after the 2nd fertilization). 10.8 and 11.8 ºC were the mean air temperatures during the periods after 1st and 2nd fertilization. Precipitation events were very scarce, being total accumulated rainfall 9.5 mm during the 1st fertilization period and 21.3 mm in the 2nd. This resulted in mean WFPS values of 48.9% and 50.3% for CT and NT respectively in the 1st fertilization and 43.3% and 47.6% in the 2nd. Considering the whole experimental period, mean WFPS was very similar between CT (46.5%) and NT (49.8%).

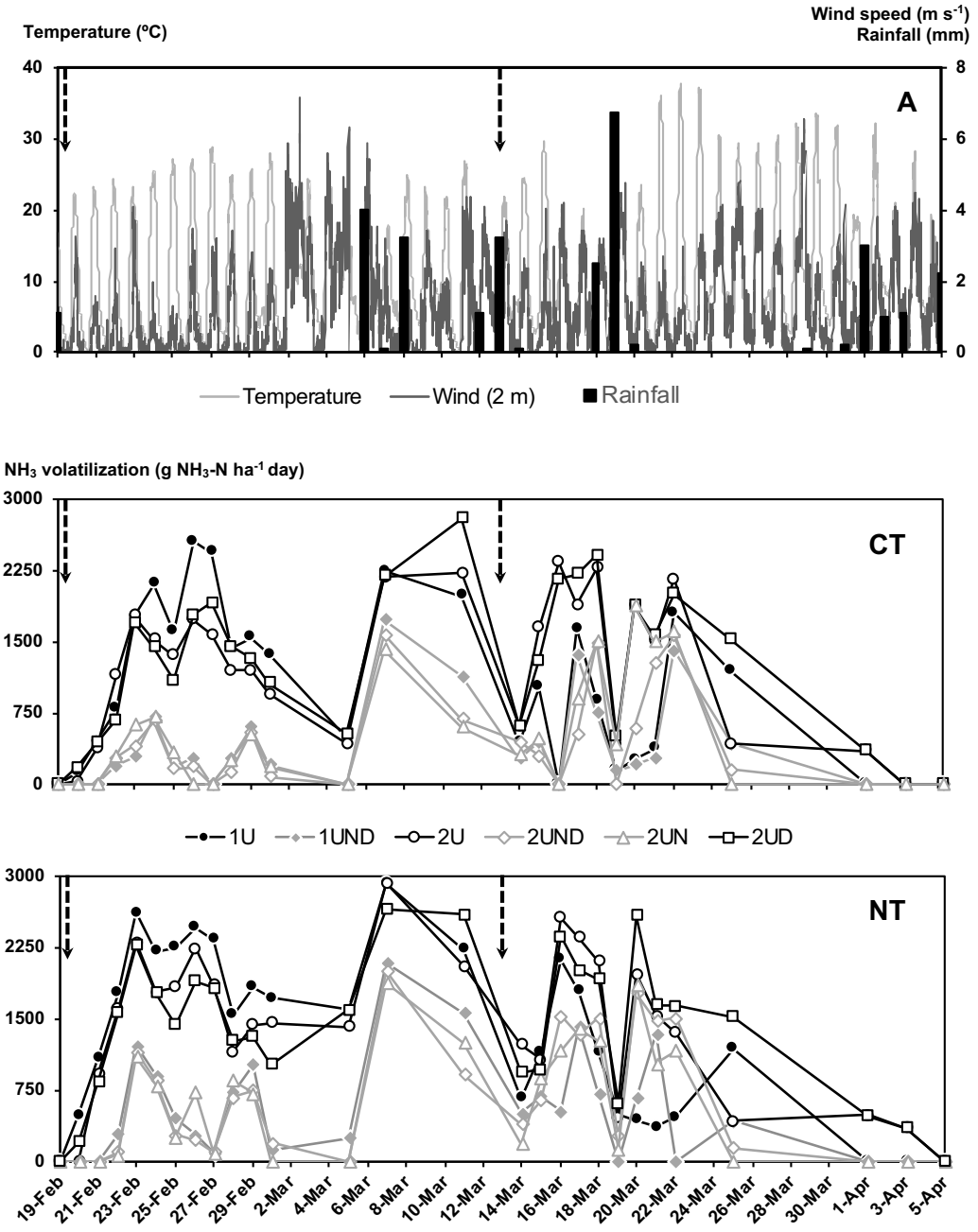


***Figure 1. A) Wind speed at 2 m height and air temperature. CT) Daily NH_3 volatilization in conventional tillage. NT) Daily NH_3 volatilization in no-tillage**. Arrows indicate fertilizer application*

3.2. AMMONIA VOLATILIZATION

Relatively warm temperatures and constant winds promoted high NH_3 volatilization rates (Fig. 1). The main differences in NH_3 volatilization were observed between the treatments receiving NBPT (UND and UN) and the ones which not (U and UD). The maximum daily NH_3 volatilization reached 2.8 and 2.9 kg NH_3-N ha^{-1} day^{-1} in CT-2UD and NT-2U respectively; both after the 1st fertilization. Contrarily, the treatments receiving NBPT did not exceed 1.7 and 2.1 kg NH_3-N ha^{-1} day^{-1} as maximum rates (CT-1UND and NT-1UND respectively). As a result, total cumulative losses in decreasing order of treatments were as follows: 2UD > 2U > 1U > 2UN = 1UND = 2UND (respectively 56.6 > 51.1 > 45.2 > 19.9 = 19.9 = 18. 3 kg NH_3-N ha^{-1}) in CT (Fig. 2A). In NT, the order was: 2UD = 1U = 2U > 2UND = 2UN = 1UND (respectively 58.8 = 56.1 = 54.8 > 28.6 = 27.4 = 24.0 kg NH_3-N ha^{-1}) (Fig. 3A). Total volatilization from treatments receiving NBPT was about 29 kg NH_3-N ha^{-1} lower than from treatments without NBPT. Moreover, there were no differences between the application of NBPT alone (2UN) or applied in combination with DMPSA (2UND), nor between the single (1UND) and the split (2UND) application. On the contrary, the application of DMPSA without NBPT (2UD) produced an 11% increase in NH_3 volatilization in CT with respect to 2U, which was also observed in NT during the period after the 2nd fertilization but not in the total losses. In general, NT tended to lose more N in the form of NH_3 than CT, being significantly different in 1U, 1UND and 2UND with 24%, 21% and 56% higher losses, respectively.

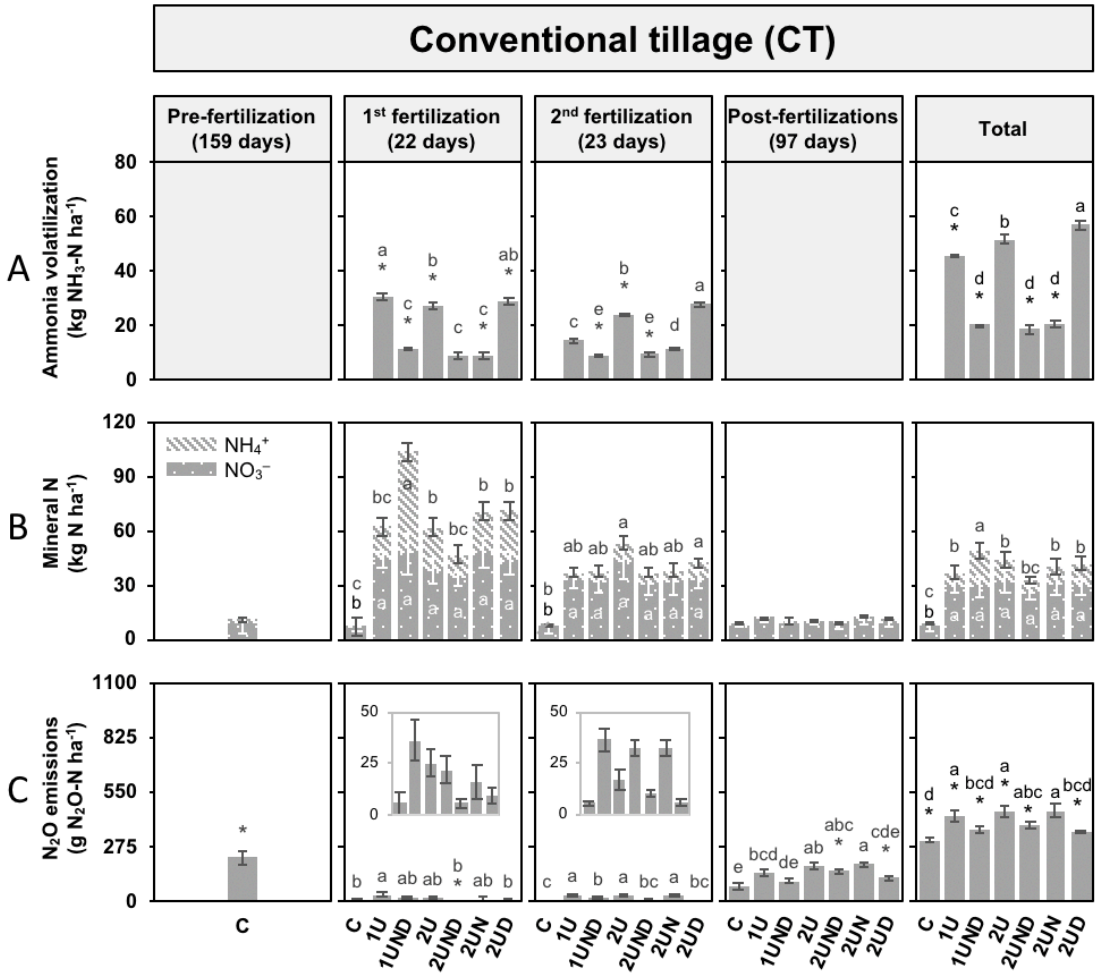


Figure 2. A) Cumulative NH_3 volatilization, B) Average content of NH_4^+ and NO_3^- and C) N_2O emissions for each period and treatment under conventional tillage. The inset graphs in sub-figures of (C) show an amplified view of the cumulative N_2O emissions for 1st and 2nd fertilization. *Different letters indicate significant differences between treatments using the Duncan test ($P < 0.05$; $n = 4$). Asterisk (*) indicates significant differences between conventional tillage and no-tillage (Fig. 3) within a treatment using the Student's T-test ($P < 0.05$; $n = 4$).*

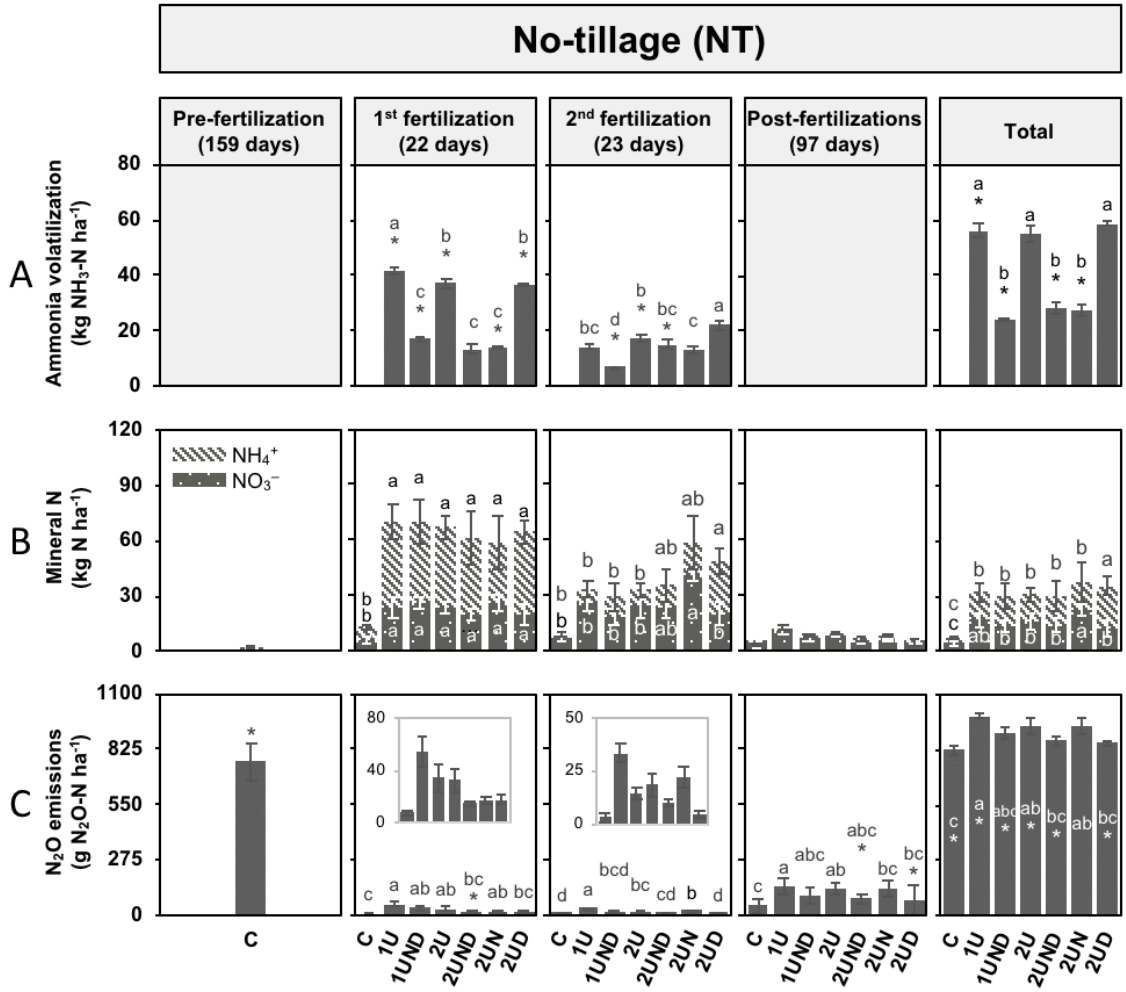


Figure 3. A) Cumulative NH_3 volatilization, B) Average content of NH_4^+ and NO_3^- and C) N_2O emissions for each period and treatment under no-tillage. The inset graphs in sub-figures of (C) show an amplified view of the cumulative N_2O emissions for 1st and 2nd fertilization. *Different letters indicate significant differences between treatments using the Duncan test ($P < 0.05$; $n = 4$). Asterisk (*) indicates significant differences between conventional tillage (Fig. 2) and no-tillage within a treatment using the Student's T-test ($P < 0.05$; $n = 4$).*

3.3. SOIL MINERAL NITROGEN

Total soil mineral N content (0–30 cm) increased drastically in response to fertilization (Supplemental figs. S1 and S2).

Considering the whole experimental period, the application of inhibitors had variable effects on NH_4^+ content (Figs. 2B and 3B). Within the fertilized treatments of

CT, 1UND showed the highest mean content (19.7 kg NH_4^+-N ha^{-1}), while 1U showed the lowest (7.4 kg NH_4^+-N ha^{-1}). However, this effect of inhibitors was not observed with the split application. On the contrary, there was an effect of the split application of DMPSA (2UD) in NT, which showed higher NH_4^+ content than 2U (25.5 and 16.7 kg NH_4^+-N ha^{-1} respectively).

Mean NO_3^- content presented fewer differences between treatments than NH_4^+ (Figs. 2B and 3B). In fact, there were no differences between the fertilized treatments of CT. With regard to NT, the treatment that received a split application of NBPT (2UN) showed higher mean NO_3^- content than the rest during the whole experiment (25.6 kg NO_3^--N ha^{-1}).

3.4. GREENHOUSE GASES EMISSIONS

The joint effect of irrigation and soil conditioning for sowing caused an important N_2O emission event, which reached 13.54 and 91.31 g N_2O-N ha^{-1} day^{-1} in CT and NT respectively (Supplemental fig. S3). In this pre-fertilization period, emissions were 3.5 times higher in NT than in CT. That emission was much higher than the one due to fertilization, whose maximum daily peak was 4.11 and 11.7 g N_2O-N ha^{-1} day^{-1} in CT and NT respectively (Supplemental fig. S4). Therefore, considering the emissions accumulated along the whole experiment, the amount due to fertilization accounted for a relatively small part (Figs. 2C and 3C). This meant that the differences between the different fertilized treatments were diluted by the initial emissions when considering the whole experimental period. However, an effect of inhibitors after their application was observed (Fig. 4). The range of direct N_2O emissions after fertilizations (from 1st fertilization to the end of the experiment) in decreasing order was as follows: 2UN = 2U ≥ 1U ≥ 2UND ≥ 1UND = 2UD ≥ C (respectively 0.20 = 0.20 ≥ 0.18 ≥ 0.15 ≥ 0.12 = 0.12 ≥ 0.08 g N_2O-N ha^{-1}) in CT (Fig. 4). In NT, the order was: 1U ≥ 2U = 2UN ≥ 1UND ≥ 2UND ≥ 2UD ≥ C (respectively 0.20 ≥ 0.16 = 0.15 ≥ 0.13 ≥ 0.10 ≥ 0.09 ≥ 0.05 g N_2O-N ha^{-1}). The application of DMPSA alone (2UD) reduced N_2O emissions under both managements and after both fertilizations (average reduction of 71% in CT and 56% in

NT), although the difference was statistically significant only after the 2nd fertilization (Figs. 2C and 3C). The joint application of DMPSA with NBPT (2UND) showed almost the same reduction in emissions than 2UD (average reduction of 71% in CT and 51% in NT). When the double inhibitor was applied in a single dose (1UND), the percentage of N_2O emissions reduction was lower (42% in NT and 44% in CT), although the reduction was statistically significant with respect to 1U and emissions were statistically equal to those in 2UND. In general, N_2O emissions due to fertilization application did not differ between CT and NT.

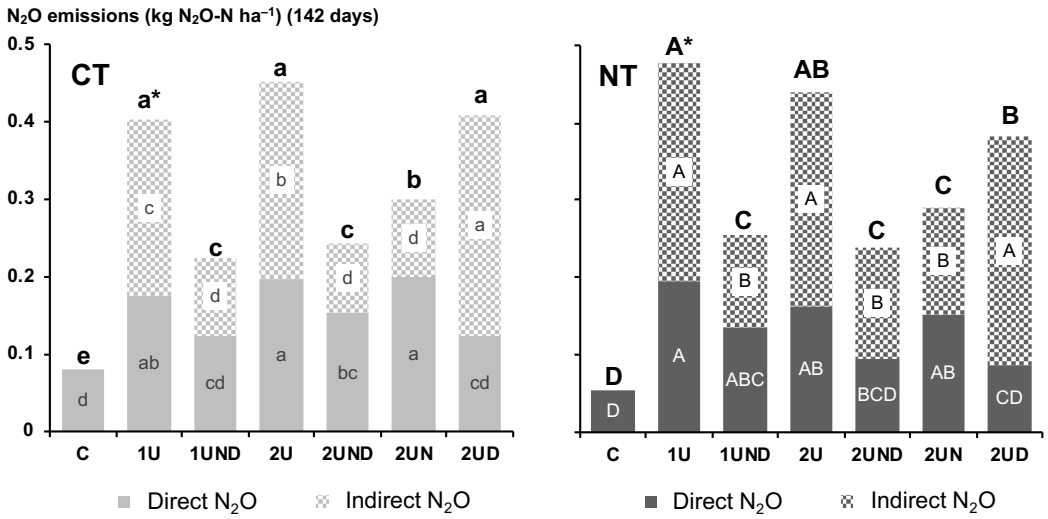


Figure 4. Cumulative direct and indirect N_2O emission in conventional tillage (CT) and no-tillage (NT) derived from fertilization (from 1st fertilization to the end of the experiment) (142 days). *Different letters within a management (CT = lowercase; NT = capital) indicate significant differences between treatments using the Duncan test ($P < 0.05$; $n = 4$). Asterisk (*) indicates significant differences between conventional tillage and no-tillage within a treatment using the Student's T-test ($P < 0.05$; $n = 4$).*

Direct N_2O emissions due to fertilization were lower than those derived from the atmospheric deposition of the volatilized NH_3 (estimated indirect N_2O emissions) (Fig. 4). When these indirect N_2O emissions were accounted, the total sum in decreasing order was as follows: 2U = 2UD = 1U > 2UN > 2UND = 1UND > C (respectively 0.45 = 0.41

= 0.40 > 0.30 > 0.24 = 0.22 > 0.08 g N_2O-N ha^{-1}) in CT. In NT, the order was: 1U ≥ 2U ≥ 2UD > 2UN = 2UND = 1UND > C (respectively 0.48 ≥ 0.44 ≥ 0.38 > 0.29 = 0.25 = 0.24 > 0.05 g N_2O-N ha^{-1}). Significant differences between tillage managements were only found in 1U.

Nitrogen fertilization increased CO_2 emissions in both management systems, but there were almost no differences between fertilized treatments nor between CT and NT (Table 2). Concerning CH_4 fluxes, both soils acted as sinks, being the absorption 2.5 higher in NT than in CT (Table 2).

Table 2. Total cumulative CH_4 flux, CO_2 emissions, Global Warming Potential (GWP), N losses and N-damage costs.

Treatment		CH_4 (g CH_4-C ha^{-1})	CO_2 (Mg CO_2-C ha^{-1})	GWP (Mg CO_2-eq ha^{-1})	N loss[a] (kg N ha^{-1})	N Damage costs[b] (€ ha^{-1})
CT	**C**	-197 ± 121 a*	3.74 ± 0.06 b	3.81 ± 0.06 c	0.3 ± 0.0 e*	2–6
	1U	-257 ± 117 a	4.01 ± 0.05 a	4.18 ± 0.06 a*	45.8 ± 0.7 c*	47–1365
	1UND	-212 ± 116 a*	3.81 ± 0.05 ab	3.93 ± 0.05 bc*	20.3 ± 0.2 d*	22–604
	2U	-219 ± 113 a*	3.95 ± 0.12 ab	4.13 ± 0.11 ab	51.8 ± 0.2 b	53–1542
	2UND	-273 ± 117 a	3.89 ± 0.03 ab	4.01 ± 0.04 abc	18.8 ± 1.8 d*	20–557
	2UN	-247 ± 113 a	3.93 ± 0.02 ab	4.07 ± 0.02 ab	20.4 ± 1.5 d*	22–606
	2UD	-229 ± 131 a	3.81 ± 0.08 ab	3.97 ± 0.08 abc	57.3 ± 1.1 a	58–1705
NT	**C**	-613 ± 25 A*	3.93 ± 0.14 A	4.13 ± 0.14 A	0.8 ± 0.0 C*	4–16
	1U	-596 ± 33 A	4.36 ± 0.16 A	4.69 ± 0.16 A*	57.3 ± 2.5 A*	61–1703
	1UND	-623 ± 61 A*	4.30 ± 0.26 A	4.56 ± 0.27 A*	25.0 ± 0.6 B*	29–738
	2U	-588 ± 30 A*	4.05 ± 0.22 A	4.35 ± 0.23 A	56.0 ± 3.0 A	60–1663
	2UND	-572 ± 4 A	3.90 ± 0.12 A	4.16 ± 0.12 A	29.6 ± 2.1 B*	33–875
	2UN	-568 ± 50 A	3.97 ± 0.20 A	4.24 ± 0.22 A	28.5 ± 1.6 B*	32–841
	2UD	-596 ± 33 A	4.03 ± 0.14 A	4.32 ± 0.14 A	59.9 ± 1.1 A	63–1781

[a] Sum of nitrogen lost by NH_3 volatilization, direct N_2O emissions and indirect N_2O emissions.

[b] Estimated following Van Grinsven et al. (2013)

Different letters within a column and management (CT = lowercase; NT = capital) indicate significant differences between treatments using the Duncan test ($P < 0.05$; n = 4). Significant differences between conventional tillage and no-tillage within a treatment are indicated with asterisks (*)

3.5. CROP YIELD AND QUALITY

Grain yield in the unfertilized treatments (C) was 913 (CT) and 772 (NT) kg ha^{-1} (Table 3). With the fertilizer application, it increased to an average of 1988 (CT) and 1877 (NT) kg ha^{-1}. These values were much lower than the mean yield of this crop in the area of the experiment. Differences were not observed between tillage systems nor within fertilizer treatments. Average protein contents were 14.7% (CT) and 14.4% (NT), whereas gross fat content averages of 43.6% (CT) and 43.7% (NT) were observed. The same as for yield, neither the protein content nor the gross fat content responded differently between tillage systems or between treatments.

Table 3. Yield, protein and gross fat content of rapeseed grain

Treatment		Grain yield (kg ha^{-1})	Protein content (%)	Gross fat (%)
CT	**C**	913 ± 64 b	14.2 ± 0.2 a	43.1 ± 0.9 a
	1U	1994 ± 124 a	14.4 ± 0.2 a	44.0 ± 0.8 a
	1UND	2039 ± 135 a	14.5 ± 0.3 a	43.9 ± 1.0 a
	2U	1995 ± 178 a	14.7 ± 0.4 a	43.9 ± 1.3 a
	2UND	1920 ± 69 a	14.8 ± 0.1 a*	43.8 ± 0.9 a
	2UN	2038 ± 168 a	15.0 ± 0.3 a	43.5 ± 0.3 a
	2UD	1942 ± 145 a	15.1 ± 0.3 a	42.9 ± 1.1 a
NT	**C**	772 ± 120 B	14.1 ± 0.3 A	42.9 ± 0.3 A
	1U	1993 ± 153 A	14.5 ± 0.3 A	43.5 ± 0.8 A
	1UND	1907 ± 103 A	14.5 ± 0.2 A	44.2 ± 0.4 A
	2U	1908 ± 157 A	14.3 ± 0.2 A	44.7 ± 0.6 A
	2UND	1892 ± 167 A	14.5 ± 0.1 A*	44.2 ± 0.2 A
	2UN	1897 ± 241 A	14.6 ± 0.1 A	43.6 ± 1.0 A
	2UD	1666 ± 113 A	14.3 ± 0.3 A	42.6 ± 0.3 A

Different letters within a column and management (CT = lowercase; NT = capital) indicate significant differences between treatments using the Duncan test ($P < 0.05$; $n = 3$). Significant differences between conventional tillage and no-tillage within a treatment are indicated with asterisks (*)

DISCUSSION

4.1. NITROGEN LOSSES

The application of urea in surface combined with the environmental conditions present during our experiment (relatively high temperatures at midday, a source of superficial moisture with the night frost and constant winds) promoted great NH_3 volatilization (Cantarella et al., 2018). The fertilizer was applied when rain was expected to facilitate urea dissolution. The precipitation of 1.13 mm on February 20th (Fig. 1A) was presumably enough to dissolve the urea granules, but scarce to allow the fertilizer to penetrate deep into the soil, since rainfalls of 7–14 mm are needed for this latter (Sanz-Cobena et al., 2011). During the 45 days after fertilizer application, rainfall events were very mild, which probably promoted the great volatilization observed in our study (Lam et al., 2019; Sha et al., 2020). Volatilization from the 1st fertilization was still going on after the 2nd fertilizer application (Fig. 1), since the single application of urea (1U) continued losing N in this second period. Finally, from April 3rd in CT and April 5th in NT (43 and 45 days after 1st fertilization respectively) volatilization was not observed in any treatment. In total, more than 30% and 36% of the applied N was lost from the U treatments of CT (Fig. 2A) and NT (Fig. 3A) respectively. These losses are above the global average (14%) (Bouwman and Boumans, 2002), although in the same range of other studies (Martins et al., 2017; Silva et al., 2017; Noor Affendi et al., 2020). Splitting of urea application does not produce a constant effect on NH_3 volatilization, since the response is variable depending on edaphoclimatic conditions (Pan et al., 2016). Thus, our conditions of relatively low soil moisture and high temperatures promoted an increase of NH_3 losses in CT and no effects in NT. Differences between tillage systems were found in 1U treatments but not in 2U, since NT-1U lost more NH_3 than CT-1U. Soil compaction is higher in NT systems, which difficult urea incorporation into the soils. Furthermore, residues from the previous crop are kept on the surface, and these residues present a high

urease activity (Malhi et al., 2001). Altogether might have increased the volatilization with respect to the CT system.

This higher N loss in NT resulted in a lower soil mineral N average content than in CT (~52 and ~47 kg N ha^{-1} respectively) during the 1st and 2nd fertilization periods (Figs. 2B and 3B), although this slight difference was not statistically significant. Nevertheless, it seems that nitrification was more intense in CT than in NT, since the difference in the NH_4^+:NO_3^- ratio was greater, being 0.31 in the case of CT and 0.86 in NT (Supplemental table S1). This might be explained by higher oxygen availability in CT soils. However, it was not accompanied by a difference in the direct N_2O emissions from both systems (Fig. 4). This may be interpreted as a higher ratio of N_2O coming from incomplete denitrifiers in NT, which would have been promoted by the higher WFPS in this system (49%) than in CT (45%) during this period (Supplemental fig. S3). On the other hand, in a previous work in the same location, Corrochano-Monsalve et al. (2020a) showed that *nosZI* abundance (encoding gene of the nitrous-oxide reductase enzyme) tended to be higher in CT than in NT, despite a lower WFPS in that tillage system. Therefore, although we did not study N_2O-reducing bacteria in this work, we hypothesize that these populations could be more abundant in CT soils. Thus, the N_2O from nitrification might have been denitrified to N_2 to a greater extent than in NT.

4.2. UREASE INHIBITOR EFFECT ON NITROGEN LOSSES

It has been reported a high variability on the efficiency of NBPT to reduce NH_3 volatilization, from results showing great reductions of ~50% (Abalos et al., 2012; Lam et al., 2018, 2019; Noor Affendi et al., 2020), to medium reductions of ~35% (Martins et al., 2017) and negligible effects (Menéndez et al., 2009). Nevertheless, NBPT shows the greater reduction the higher the NH_3 volatilization is, especially in rainfed systems with alkaline pH soils as ours (pH = 8) (Li et al., 2018; Cantarella et al., 2018). Thus, NBPT efficiency was promoted in our experiment, and N losses were decreased from 30% down to 13% (CT-2UN) and from 36% to 18% (NT-2UN), which means a total reduction of more than 50% (Figs. 2A and 3A). Two maximum peaks were observed in the case of

urea without inhibitors (the first within the 7 days after fertilization and the second almost 20 days after fertilization) (Fig. 1). The objective to be achieved with the UI application is to slow down urea hydrolysis. In this manner, the increment of pH is smoother, which reduces the possibility of NH_3 volatilization. This objective was accomplished in our experiment, since urea hydrolysis was avoided from the beginning, suggesting that NBPT was oxidized to its active form (NBPTO) rapidly (Manuenza et al., 1999). This has been previously observed in rainfed Mediterranean systems (Abalos et al., 2012). The effect of NBPT was maintained over time, especially during the first 16 days after 1st fertilization (Fig. 1). On day 17, a peak of volatilization was registered, probably as a response to the rain of the previous day (Lam et al., 2019). In this peak, the differences between 2U and 2UN treatments were minor, suggesting that NBPT had been almost completely degraded at that time. This persistence of the efficacy of NBPT is similar to that reported by Liu et al. (2019) and consistent with the degradation observed in template regions (10–15 days) (Cantarella et al., 2018). Whereas after the 1st fertilization NH_3 losses were decreased in more than 60% in both tillage systems, after the 2nd the performance of NBPT was more variable (Fig. 1). Its efficiency was maintained above 50% of reduction in CT, but it decreased down to 27% in NT (Figs. 2A and 3A). We attribute this decrease of efficiency in NT to the lower volatilization of NT-2U with respect to CT-2U (17.6 and 24.1 kg N ha^{-1} respectively), since NBPT efficiency increases as higher the baseline is, as has been previously described.

Depending on the conditions, the application of UIs can diminish N_2O emissions, since it can induce a smoother apparition of NH_4^+ (and, therefore, nitrification) (Guardia et al., 2017, 2018b; Zhao et al., 2017; Noor Affendi et al., 2020). This trend has been observed especially in basic soils (Fan et al., 2018). On the other hand, when its effectivity declines, the apparition of NH_4^+ is highly susceptible to be nitrified. Thus, its application would promote N_2O emissions (Lam et al., 2018; Mateo-Marín et al., 2020). In our experiment, the 2UN treatments showed a smoother apparition of NH_4^+ along time, as a result of the delay in urea hydrolysis (Supplemental fig. S1). However, there were almost no differences in soil mineral N content during fertilizing periods (Figs. 2B and 3B), in

spite that it was avoided the volatilization of 31 and 27 kg N ha^{-1} in 2UN treatments with respect to 2U. Nevertheless, NT-2UN tended to show higher NO_3^- content than NT-2U, especially after the 2nd fertilization (Supplemental fig. S2), suggesting that NH_4^+ was being rapidly converted to NO_3^- by nitrification (Fu et al., 2020). Furthermore, the NH_4^+:NO_3^- ratio was not modified in CT (0.31 in 2U *vs* 0.36 in 2UN) but it changed in NT (0.86 in 2U *vs* 0.68 in 2UN) (Supplemental table S1). In general, works reporting slight or no changes in soil mineral N in response to NBPT application also report no effects on direct N_2O emissions (Menéndez et al., 2009; Dougherty et al., 2016; Rose et al., 2018; Suter et al., 2020). The same event occurred in our case in both tillage systems (Fig. 4).

4.3. NITRIFICATION INHIBITOR EFFECT ON NITROGEN LOSSES

Metadata studies show that NIs tend to increase NH_3 volatilization (Li et al., 2018; Cantarella et al., 2018). The application of DMPSA can generate collateral effects on NH_3 volatilization due to its interference in the nitrification process. NIs delay nitrification by inhibiting ammonia-oxidizing bacteria. As a result of this inhibition, NH_4^+ stays for longer in soils when it is applied in combination with ammonium sulfate (Barrena et al., 2017; Torralbo et al., 2017; Corrochano-Monsalve et al., 2020a). Thus, with the urea hydrolysis not delayed by an UI, soil pH increases and the NH_3:NH_4^+ ratio remains high for a longer period, which altogether increases NH_3 volatilization (Kim et al., 2012; Cantarella et al., 2018); especially in alkaline soils. By counterpart, the effects of DMPSA on NH_3 volatilization might be lower due to its low pH (Lam et al., 2018). Here, we report that DMPSA showed no effects in the NT system, but it significantly increased N losses via NH_3 volatilization in CT by 11% (Figs. 2A and 3A). To our knowledge, only Recio et al. (2018; 2020) have analyzed the effects of DMPSA on NH_3 volatilization, reporting no effects in irrigated alkaline soils. Therefore, more studies are needed on this specific NI. It would be expected that the effect of DMPSA would be similar to that of DMPP because both are copper-chelating compounds based on dimethylpyrazole (Corrochano-Monsalve et al., 2021a). DMPP has been commercialized

for a long time and is widely applied. Hence, its effect on NH_3 losses has been analyzed to a longer extent, showing reductions (Lam et al., 2018), no effects (Menéndez et al., 2009) or an increase (Castellano-Hinojosa et al., 2020; Sha et al., 2020).

Although the averaged data showed small effects of DMPSA on soil mineral N (Figs. 2B and 3B), the NH_4^+:NO_3^- ratio was drastically increased in NT-2UD (1.71) with respect to NT-2U (0.88) (Supplemental table S1). Moreover, the daily evolution revealed high peaks of NH_4^+ in 2UD treatments (Supplemental fig. S1). However, when these peaks disappeared, it was not linked to high peaks of NO_3^- content (Supplemental fig. S2), which suggests that N was lost by volatilization rather than nitrification. N_2O emission data were consistent with this latter, since direct emissions were significantly lower in 2UD than in 2U treatments (Fig. 4). As well as in the case of NBPT, NIs efficiency increases as higher the baseline is (Menéndez et al., 2012; Li et al., 2018). Even though direct N_2O emissions were relatively low (Supplemental fig. S4), DMPSA was able to abate N_2O emissions by 36% (CT) and 47% (NT) with respect to 2U from its application to the end of the experiment. Similar (Guardia et al., 2018b) and higher (Recio et al., 2019) efficiencies have been shown under rainfed and irrigated conditions (Recio et al., 2020). Nevertheless, we observed higher values within the fertilizing periods; especially after the 2nd fertilization (81% CT; 76% NT) (Figs. 2C and 3C); similar to that described by Corrochano-Monsalve et al., (2020a) with ammonium sulfate as fertilizer. The effect of DMPSA was still observed in the emission peak of April 10th and 16th (Supplemental fig. S4), indicating that it was even active 35 days after fertilization. Analogous persistence was found by Corrochano-Monsalve et al., (2020a), whereas Recio et al. (2018) reported no effect after 3 weeks in irrigated conditions. Therefore, there are signs that DMPSA degradation might be slower in rainfed systems.

4.4. DOUBLE INHIBITOR EFFECT ON NITROGEN LOSSES

As we have shown, the application of urea is highly inefficient. Huge amounts of N are lost to the environment by different ways, causing an environmental impact. The sole application of UIs or NIs tackle part but not the whole problem. NBPT

is highly effective mitigating NH_3 volatilization, but is not able to abate N_2O emissions coming from nitrification and/or denitrification. On the contrary, DMPSA can decrease N_2O emissions down to the level of the unfertilized control, but NH_3 losses are increased with its application. In this context, the application of the double inhibitor NBPT + DMPSA (UND treatments) might achieve the desired full mitigation. Other combinations of NBPT with other NIs such as DMPP or DCD have been tested previously with variable results (Li et al., 2018; Cantarella et al., 2018; Castellano-Hinojosa et al., 2020; Mariano et al., 2019). Our results revealed that UND treatments were able to counteract the negative effect of DMPSA on NH_3 volatilization, maintaining the same levels of 2UN treatments (Figs. 1, 2A and 3A). Specifically, the reduction reached 56% (1UND) and 64% (2UND) in CT, whereas reductions of 57% (1UND) and 48% (2UND) were observed in NT. Although in the case of CT-U treatments we found higher volatilization in CT-2U than in CT-1U, this difference disappeared between the single or the split application of the double inhibitor. Up to date, DMPSA has not been still commercialized; and therefore, neither the double inhibitor. Consequently, there are almost no studies in this regard. In alkaline soils, Recio et al. (2020) reported an efficiency of 72%, whereas Nikolajsen et al. (2020) showed variable results in acids.

Total soil mineral N content in UND treatments was similar to the rest of the fertilized treatments (Figs. 2B and 3B), even though they tended to present higher NH_4^+ contents (Supplemental Table 2). Although there were no significant differences, NH_4^+ peaks tended to appear delayed with respect to 2UD treatments (Supplemental fig. S1), which matches with a slower hydrolysis of urea due to NBPT presence. Moreover, NO_3^- peaks also tended to be higher than in 2UD (Supplemental fig. S2), suggesting that DMPSA might have lost some of its effectiveness with the later release of NH_4^+.

This seems to be in line with N_2O emissions. As well as NBPT counteracted the effect of DMPSA on NH_3 volatilization, DMPSA was able to reduce direct N_2O emissions comparing to U and 2UN treatments (Figs. 2C and 3C). However, the effectivity reducing these losses decreased slightly in both CT (29%, 1UND; 22%, 2UND) and NT (42%, 1UND; 45% 2UND) comparing to 2UD (36%, CT; 47%, NT). Nevertheless, direct N_2O

losses in UND treatments were maintained at the levels of 2UD (Fig. 4). Also under rainfed conditions and similar soil pH, Guardia et al. (2018b) reported an average reduction of 33% of N_2O emissions by applying this double inhibitor, although showing a slightly lower efficiency than DMPSA itself. Comparing with other mixtures, NBPT + DMPP has shown similar reductions of 40% in basic soils (Zhao et al., 2017) and up to 53% in acids (Souza et al., 2019); whereas higher efficiencies up to 68% have been found with NBPT + DCD (Tosi et al., 2020).

4.5. CROP YIELD AND QUALITY PARAMETERS

Under our climatic conditions, this highly productive variety has shown a grain yield of approximately 4600 kg ha^{-1} (GENVCE, 2017), comparing to an average rapeseed production of 2600 kg ha^{-1} in the same area and year (MAPA, 2018). However, cumulative precipitation was unusually low during our crop development. From 1st fertilization (February 19th) to harvest (July 11th), 163 mm rainfall was accumulated, whereas the historical average is 335 mm. Therefore, grain harvest was negatively affected by the dryness (Rathke et al., 2006). Unfertilized treatments (C) showed grain yields of 913 (CT) and 772 (NT) kg ha^{-1}, whereas fertilized treatments only reached an average grain yield of 1988 (CT) and 1877 (NT) kg ha^{-1} (Table 3); far below the expected production. Despite treatments without UI lost ~29 kg N ha^{-1} more than NBPT treatments (Table 2), we did not found differences in grain yield within fertilized treatments. In this context, the crop seemed to be limited to a greater extent by water rather than N availability. Thus, the remaining N in the soil seemed to be enough in all the treatments for the growth, limited by these dry conditions. Nevertheless, the lowest yield was found in NT-2UD, which precisely was the treatment with the highest N-losses (Table 2). This suggests that, probably, we would have found yield differences within fertilized treatments in a scenario of average weather conditions. In general, most studies show that the potential of inhibitors to improve the crop yield and/or quality parameters is much weaker than its potential to reduce NH_3 volatilization and N_2O emissions, especially in the case of NIs. Meta-analysis studies reveal yield increases about 5–12% with the

application of UIs (Cantarella et al., 2018), whereas nitrification and double inhibitors have shown no effects (Abalos et al., 2014), especially in low-rainfall areas (< 800 mm) (Li et al., 2018). In the particular case of DMPSA (applied alone or in combination with NBPT) the trend is similar, since no effects have been found in different crops both under rainfed (Guardia et al., 2018b; Huérfano et al., 2016, 2018; Corrochano-Monsalve et al. 2020a) and irrigated conditions (Recio et al. 2018, 2020). Protein and fatty acids contents were also stable between all the treatments, including the unfertilized (Table 3).

4.6. SELECTING THE BEST COMBINATION FOR THE SAKE OF SUSTAINABILITY

The Global Warming Potential (GWP) is a factor of the sustainability of agro-systems based on the GHGs emitted (in this case, CO_2, CH_4 and direct/indirect N_2O). In a 100-year time horizon, the GWP of CH_4 and N_2O is 28 and 265 times greater than CO_2, respectively (IPCC, 2014). Nevertheless, the different magnitude of the soil emissions of CH_4 and N_2O with respect to CO_2 (g ha^{-1} *vs* Mg ha^{-1}), makes this latter the main contributor to the total GWP of the system in drylands. The adoption of a NT management can lead to lower CO_2 losses, both by diminishing the emissions from soils (Soane et al., 2012; Corrochano-Monsalve et al., 2020a) and by reducing the fuel consumption needed for a CT management (Guardia et al., 2016). Moreover, soil organic carbon content is also increased (Carbonell-Bojollo et al., 2015; Plaza-Bonilla et al., 2015). Unlike these studies, CT and NT showed the same CO_2 losses in our experiment (Table 2) probably because of the similar WFPS (46.5% in CT *vs* 49.8% in NT) induced by the scarce rainfall, since WFPS is the main source of variation in CO_2 production between tillage systems (Linn and Doran, 1984).

The information regarding the relationship between tillage management and CH_4 fluxes is scarcer, but most studies indicate that the effects are weak (Soane et al., 2012), with a slight trend of NT to more CH_4 uptake (Six et al., 2004). This trend was also observed in our work, since the uptake was more than 50% higher in NT (Table 2). Nevertheless, the CH_4 contribution to the total GWP was almost negligible.

There is evidence for a stronger relationship between the tillage system and N_2O emissions because of its influence on soil aeration (Soane et al., 2012). Tilting the balance to one side or the other depends on many factors such as rainfall regimens, water content and time since the implantation of the system. Long-term NT systems can reduce N_2O losses (Ussiri et al., 2009; Plaza-Bonilla et al., 2018). However, in the short-term, higher emissions may occur if WFPS levels in NT are in the range to promote nitrification and denitrification losses (Corrochano-Monsalve et al., 2020a). CT and NT did not show differences in N_2O emissions from the 1st fertilization to the end of the experiment (Fig. 4) because of the similar WFPS. However, total cumulative N_2O losses resulted higher in NT than in CT due to the emissions produced because of the soil conditioning (pre-fertilization period) (Figs. 2C and 3C). Alongside the usual labors for CT, it was necessary to carry out a superficial rupture of the soil in NT due to the excessive compaction, to favor correct implantation of the seed. At that moment (September 14th) the WFPS was quite different between CT (39.2%) and NT (50.2%) (Supplemental Fig. S3). This resulted in a massive N_2O emission peak in NT (Supplemental fig. S3), in which denitrification may have contributed significantly (Corrochano-Monsalve et al., 2020a). Therefore, it should be taken into consideration that small disruptions of soil in NT systems may lead to N_2O losses even higher than those derived from a CT.

To select the most sustainable combination, it is also necessary to consider the crop production. NT management can increase the yield in rainfed Mediterranean systems due to the higher water storage capacity (Lampurlanés et al., 2016). The absence of differences in water content between our tillage systems led to no differences in grain yield nor protein and fatty acids content (Table 3).

In summary, tillage management did not suppose the main differential factor in terms of GWP and crop production and quality. Therefore, the key to select the most sustainable combination would depend on the fertilizing treatment. In this aspect, there were also almost no differences in terms of CO_2 nor CH_4 emissions (Table 2), in agreement with other studies showing no effects of UIs nor NIs on these gases fluxes (Guardia et al., 2017, 2018b; Volpi et al., 2017; Mateo-Martín et al., 2020). However, the

sum of the direct and indirect N_2O emissions made the U treatments the ones with the highest GWP. Under these conditions, we conclude that the best selection would be a CT system together with a single application of the double inhibitor. This combination produced the lowest GWP and was able to reduce it down to the levels of the unfertilized control. Furthermore, it also showed the lowest total N losses (Table 2). This latter can lead to direct and indirect economic impacts. The marginal damage cost derived from N losses was estimated to be reduced in a range between 25–761 € ha^{-1} (Table 2). At the farm level, it might imply savings for farmers in the case that would allow a reduction of fertilizer needs; which would also depend on the market price the double inhibitor would have. Moreover, it should be also noted the savings in fuel consumption derived from making a single fertilization without affecting grain yield and quality (Table 3). In this work, provided the unusual scarce rainfalls which led to unusual low grain yields, we have not been able to properly evaluate these aspects, because (as previously discussed) we were not able to observe a better N use efficiency despite the lower N losses achieved by the application of the double inhibitor. With regard to the NT system, despite the efficiency of the double inhibitor to reduce N losses was similar to CT in percentage, the GWP of this treatment under NT was higher because of the higher baseline in U (Table 2). This was attributed to the higher urease activity in the residues from the previous crop (Malhi et al., 2001) and, probably, to a slower infiltration of urea in soils due to the higher compaction. Thus, the net N losses were higher, which makes adopting a NT system less recommended from this point of view and under these specific conditions.

CONCLUSIONS

Fertilizing with urea leads to the loss of large amounts of nitrogen, which derives in environmental and economic costs. In our work, more than 30% of the nitrogen applied was lost via NH_3 volatilization and N_2O emissions. The individual use of urease (NBPT) and nitrification (DMPSA) inhibitors allowed to mitigate only part of these losses. However, it was necessary the use of the double inhibitor (NBPT + DMPSA) to achieve a more complete mitigation by delaying both urea hydrolysis and nitrification without counterparts. Nonetheless, halving nitrogen losses was not translated into a higher nitrogen use efficiency in terms of a higher crop yield in a context in which nitrogen was not the limiting factor. Although the contribution of N_2O emissions to the global warming potential (GWP) of this system was minor in comparison with CO_2, our results show that nitrogen losses make the difference to choose the best strategy. In this aspect, indirect N_2O emissions derived from the deposition of the volatilized NH_3 should be taken into consideration when designing mitigation strategies for urea fertilization, since they have considerable importance in the balance of nitrogen losses. In this manner, a conventional tillage system fertilized with a single application of urea with the double inhibitor resulted in the most sustainable combination to be applied under our conditions, reducing the GWP down to the levels of unfertilized soils. Further studies would be necessary to confirm the performance of this new double inhibitor under a range of different edaphoclimatic conditions and crops.

SUPLEMENTARY MATERIAL

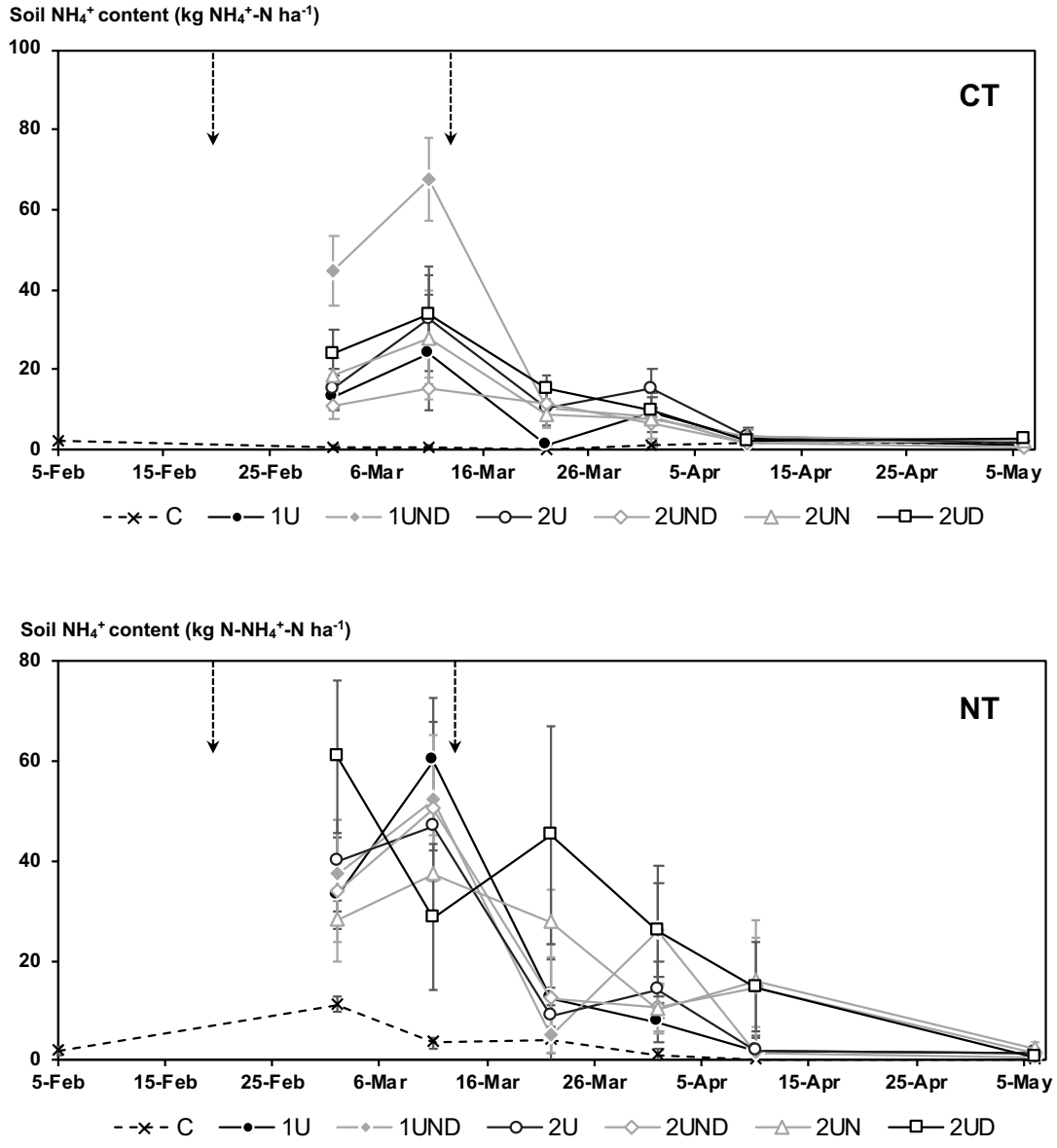


Figure S1. Soil NH_4^+ content in conventional tillage (CT) and no-tillage (NT). *Arrows indicate fertilizer application.*

C = Unfertilized control; 1U = Urea (single application); 1UND = Urea + NBPT + DMPSA (single application); 2U = Urea (split application); 2UND = Urea + NBPT + DMPSA (split application); 2UN = Urea + NBPT (split application); 2UD = Urea + DMPSA (split application).

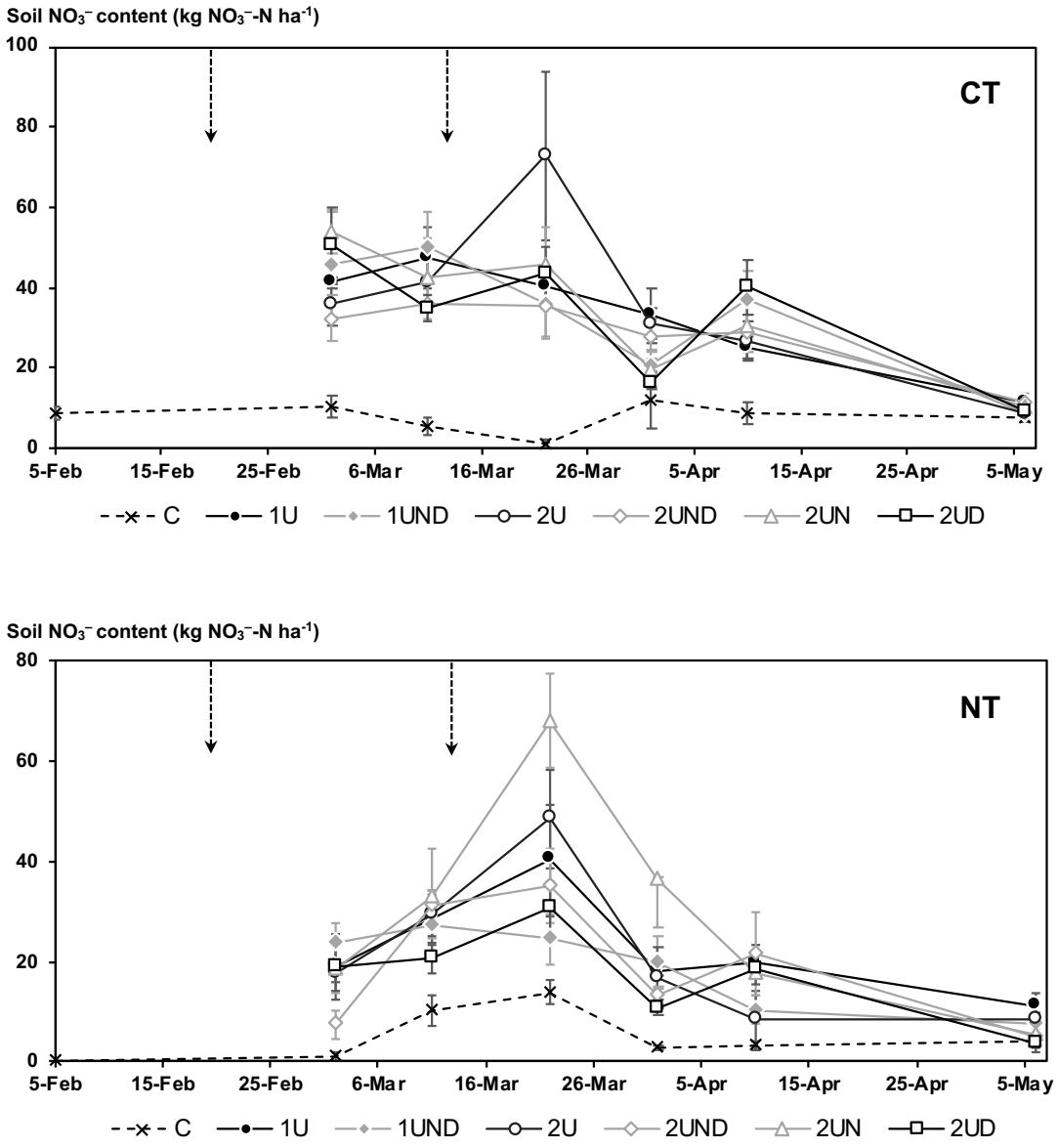


Figure S2. Soil NO_3^- content in conventional tillage (CT) and no-tillage (NT). *Arrows indicate fertilizer application.*

C = Unfertilized control; 1U = Urea (single application); 1UND = Urea + NBPT + DMPSA (single application); 2U = Urea (split application); 2UND = Urea + NBPT + DMPSA (split application); 2UN = Urea + NBPT (split application); 2UD = Urea + DMPSA (split application).

Table S1. Soil NH_4^+:NO_3^- average ratio from 1st fertilization

Treatment		NH_4^+:NO_3^-
CT	C	0.12
	1U	0.26
	1UND	0.68
	2U	0.36
	2UND	0.27
	2UN	0.33
	2UD	0.45
NT	C	0.57
	1U	0.85
	1UND	1.08
	2U	0.88
	2UND	1.09
	2UN	0.68
	2UD	1.71

CT = Conventional tillage; NT = No-tillage; C = Unfertilized control; 1U = Urea (single application); 1UND = Urea + NBPT + DMPSA (single application); 2U = Urea (split application); 2UND = Urea + NBPT + DMPSA (split application); 2UN = Urea + NBPT (split application); 2UD = Urea + DMPSA (split application).

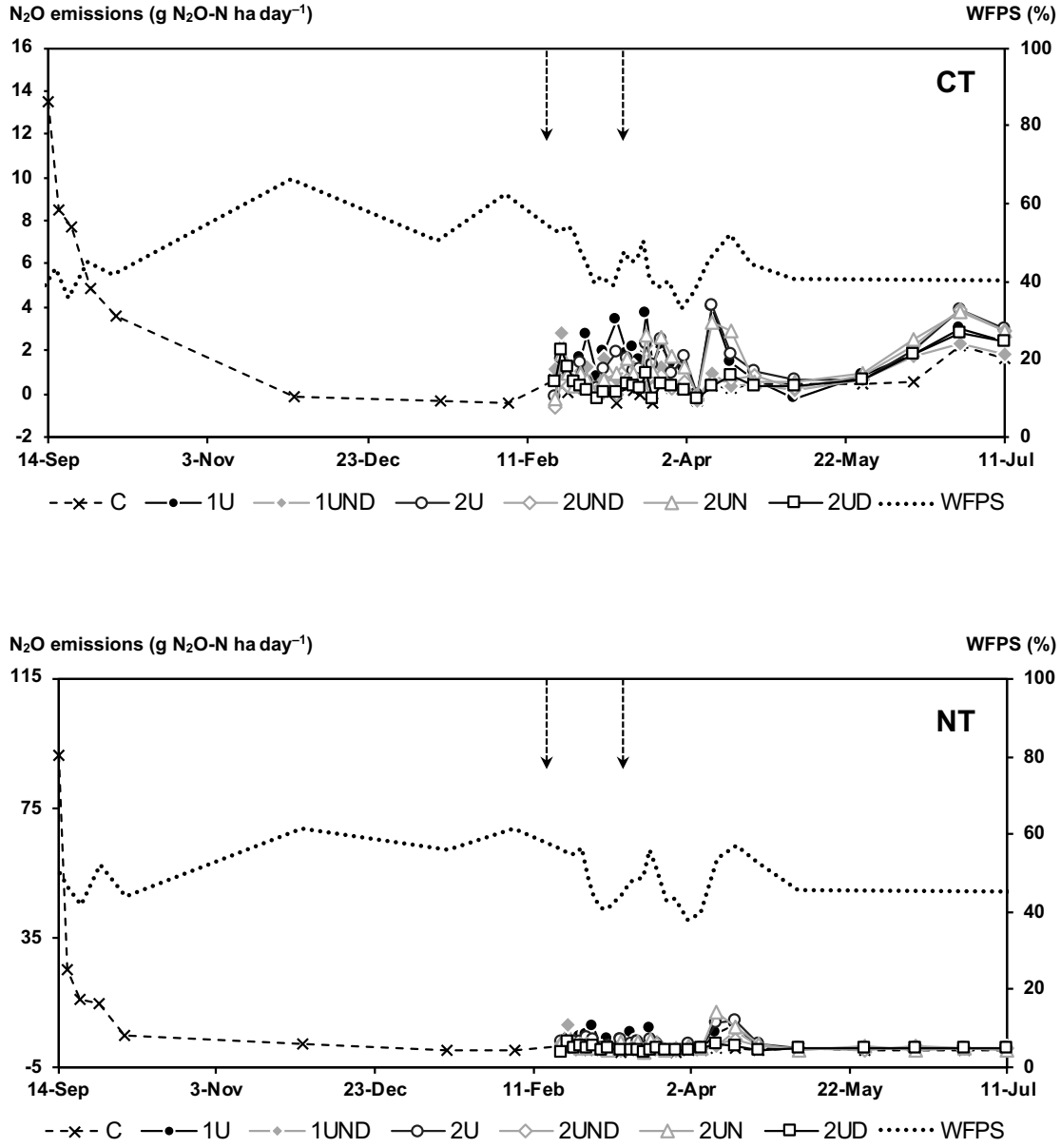


Figure S3. N_2O daily emissions and water filled pore space (WFPS) in conventional tillage (CT) and no-tillage (NT). *Arrows indicate fertilizer application.*

C = Unfertilized control; 1U = Urea (single application); 1UND = Urea + NBPT + DMPSA (single application); 2U = Urea (split application); 2UND = Urea + NBPT + DMPSA (split application); 2UN = Urea + NBPT (split application); 2UD = Urea + DMPSA (split application).

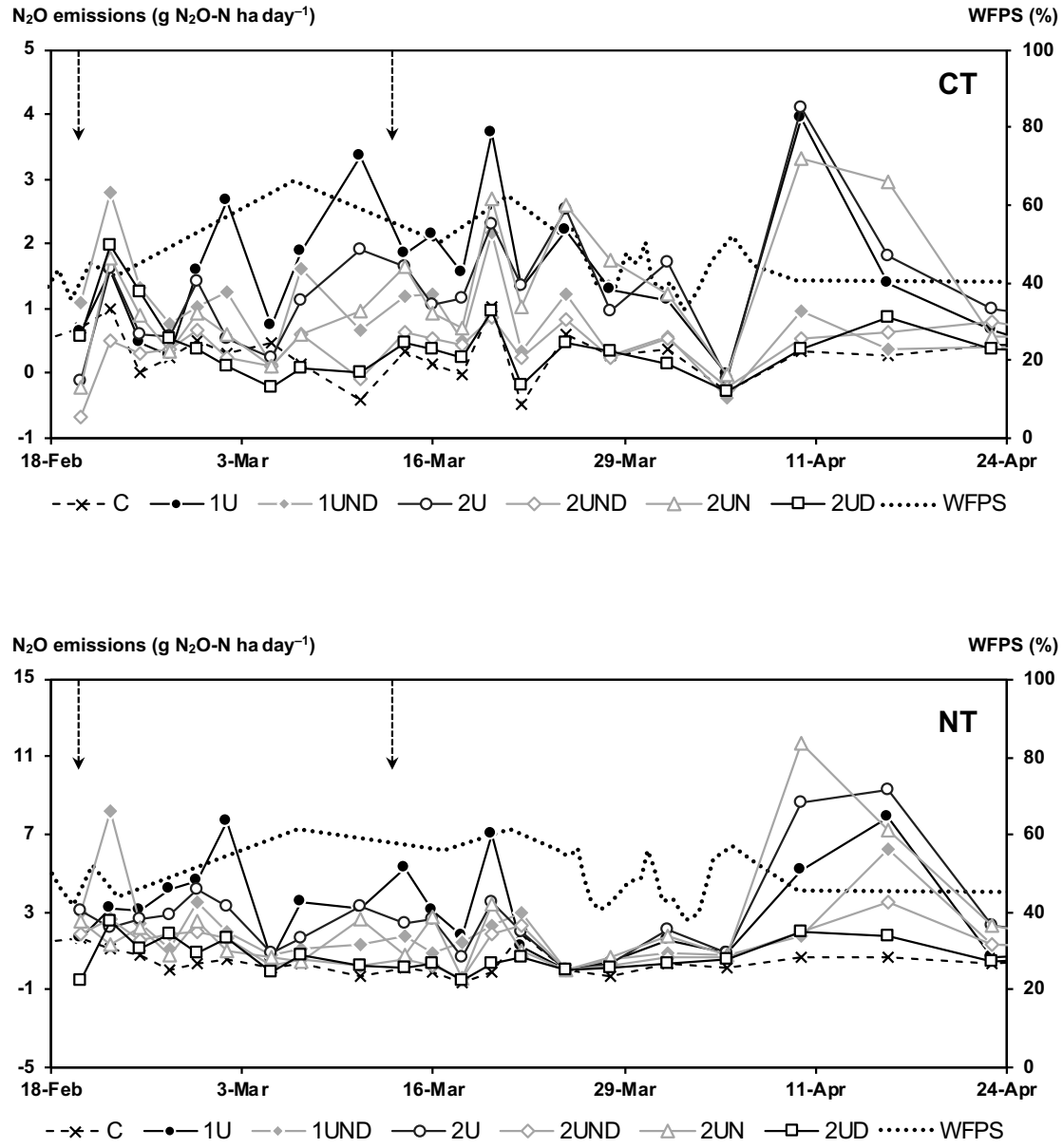


Figure S4. N_2O daily emissions and water filled pore space (WFPS) in conventional tillage (CT) and no-tillage (NT) during fertilization periods. *Arrows indicate fertilizer application.*

C = Unfertilized control; 1U = Urea (single application); 1UND = Urea + NBPT + DMPSA (single application); 2U = Urea (split application); 2UND = Urea + NBPT + DMPSA (split application); 2UN = Urea + NBPT (split application); 2UD = Urea + DMPSA (split application).

CHAPTER 3

Mechanism of action

Mechanism of action of nitrification inhibitors based on dimethylpyrazole: A matter of chelation

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When reviewing the literature, we realized that there was a great gap with respect to the mode of action of many inhibitors; such is the case of DMPP and DMPSA. Indeed, knowing the mode of action is not strictly necessary to meet the objective of a product, but it can be of great help to understand the exerted effects, improve their application procedure and for the development of new compounds.

In the case of DMPP and DMPSA, it has been assumed by a large part of the scientific community that they work as copper chelators and, thus, they should affect ammonia monooxygenase enzyme (AMO) because of the need for copper as a cofactor (see introduction section 4). In the same manner, many works state that this is more or less a direct action in the enzyme active site. The reality is that there is not any empirical demonstration giving support to any of these statements.

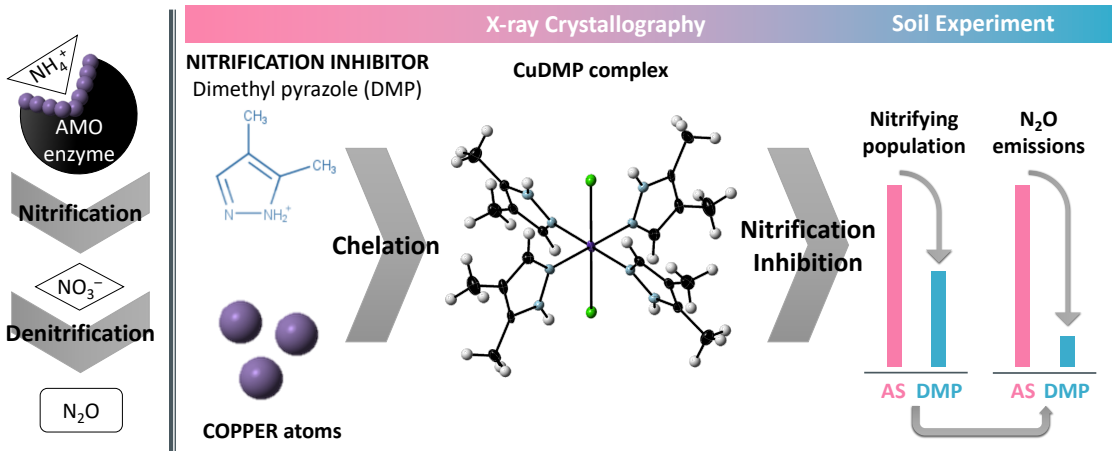
The fact that in Chapter 1 we observed that DMPSA (and, in the case of other works, DMPP as well) is capable of inducing the expression of *nosZI* gene, raised our doubts regarding the mode of action of these inhibitors. As described in the introduction (section 4), both the transcription of *nosZ* genes and the activity of its encoded enzyme (nitrous oxide reductase; N_2OR), are highly dependent on copper availability. However, the effects observed after the application of DMPSA were opposed between *amoA* and *nosZI* genes, making it difficult to maintain that DMPSA has some kind of interaction with copper.

Going back to the selection of hypothetical scenarios that were presented in section 6.2.2 of the introduction for nitrogen cycle organisms, these results would not fulfill the postulates exposed in the scenarios “1-B-i”, “2-A” and “2-B-I”, since the abundance of *nosZI* increased and not decreased after DMPSA application.

We decided that taking a first step towards the understating of the mode of action of these inhibitors could help to respond to some of the doubts regarding their effects; especially because of the great interest in the induction of N_2OR.

Encouraged by the results obtained, we also decided to apply this new knowledge to real conditions, seeking to find signs for a relationship between DMPP and DMPSA efficiency and copper levels in the soil.

GRAPHICAL ABSTRACT



ABSTRACT

In agriculture, the applied nitrogen (N) can be lost in the environment in different forms because of microbial transformations. It is of special concern the nitrate (NO_3^-) leaching and the nitrous oxide (N_2O) emissions, due to their negative environmental impacts. Nitrification inhibitors (NIs) based on dimethylpyrazole (DMP) are applied worldwide in order to reduce N losses. These compounds delay ammonium (NH_4^+) oxidation by inhibiting ammonia-oxidizing bacteria (AOB) growth. However, their mechanism of action has not been demonstrated, which represent an important lack of knowledge to use them correctly. In this work, through chemical and biological analysis, we unveil the mechanism of action of the commonly applied 3,4-dimethyl-1H-pyrazole dihydrogen phosphate (DMPP) and the new DMP-based NI, 2-(3,4-dimethyl-1H-pyrazol-1-yl)-succinic acid (DMPSA). Our results show that DMP and DMPSA form complexes with copper (Cu^{2+}) cations, an indispensable cofactor in the nitrification pathway. Three coordination compounds namely $[Cu(DMP)_4Cl_2]$ (**CuDMP1**),

$[Cu(DMP)_4SO_4]_n$ (**CuDMP2**) and $[Cu(DMPSA)_2]\cdot H_2O$ (**CuDMPSA**) have been synthesized and chemical and structurally characterized. The CuDMPSA complex is more stable than those containing DMP ligands; however, both NIs show the same nitrification inhibition efficiency in soils with different Cu contents, suggesting that the active specie in both cases is DMP. Our soil experiment reveals that the usual application dose is enough to inhibit nitrification within the range of Cu and Zn contents present in agricultural soils, although their effects vary depending on the content of these elements. As a result of AOB inhibition by these NIs, N_2O-reducing bacteria seem to be beneficed in Cu-limited soils due to a reduction in the competence. This opens up the possibility to induce N_2O reduction to N_2 through Cu fertilization. On the other hand, when fertilizing with micronutrients such as Cu and Zn, the use of NIs could be beneficial to counteract the increase of nitrification derived from their application.

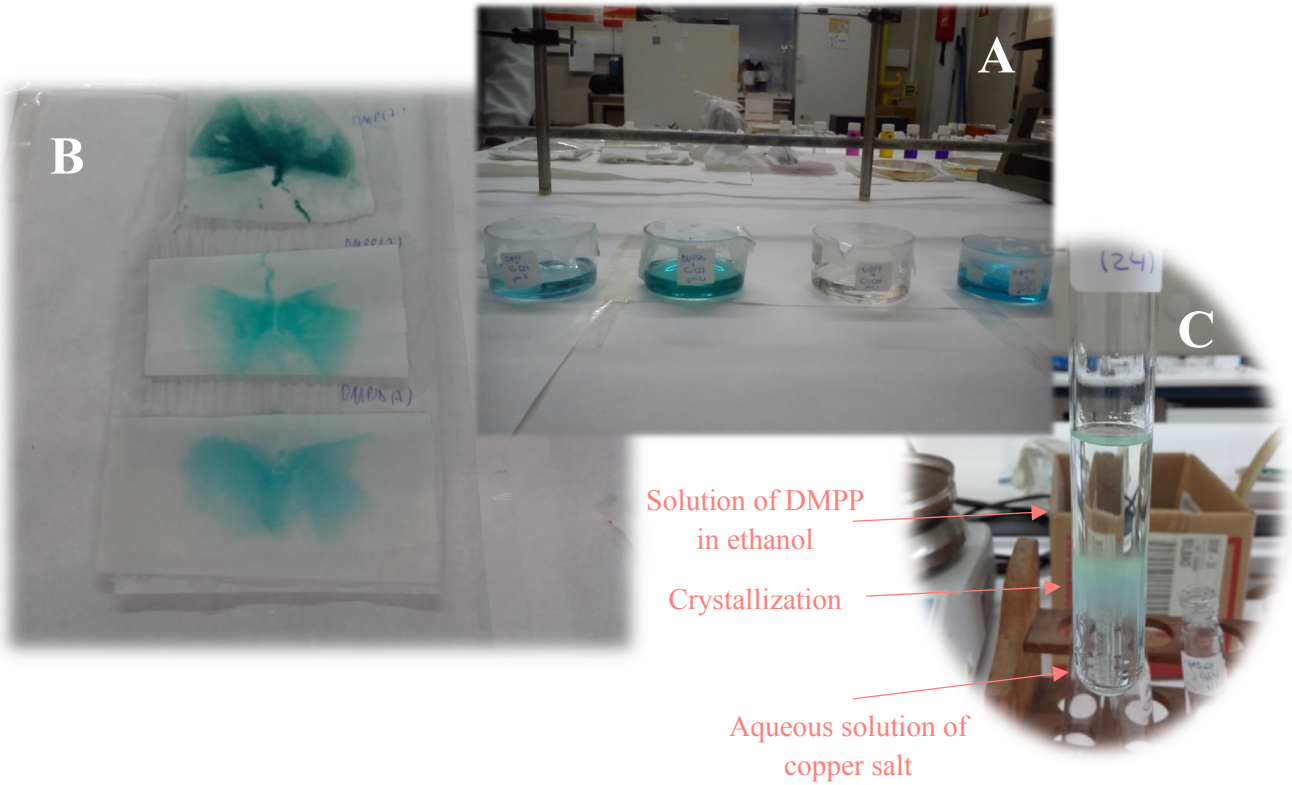
MATERIALS AND METHODS

2.1. EXPERIMENT 1 - SYNTHESIS AND STRUCTURAL CHARACTERIZATION OF METAL COMPLEXES

All the solvents and reagents were purchased from commercial sources and used without further purification. The 3,4-dimethyl-1H-pyrazol dihydrogen phosphate (DMPP) and 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) were supplied by EuroChem Agro Iberia S. L. and exhibited high purity (> 97%). The infrared spectra (FT-IR) were recorded as KBr pellets on a Shimadzu FTIR-8400S spectrophotometer in the 400–4000 cm^{-1} spectral range. Carbon, hydrogen and nitrogen contents were determined on a Perkin Elmer 2400 CHN analyzer. Thermogravimetric analyses (TGA) were carried out from room temperature to 600 °C at a rate of 5 °C min^{-1} on a Mettler Toledo TGA/ SDTA 851^e

thermobalance under a 50 cm^3 min^{-1} flow of synthetic air. Powder X-ray diffraction (PXRD) patterns were recorded from 2θ = 5 to 38° (0.038 step size, 30 s per step) using a Philips X'PERT PRO diffractometer operating at 40 kV/40 mA in *θ–θ* configuration with monochromated CuKα radiation (λ = 1.5418 Å) and a PIXcel detector. X-band EPR measurements were registered on a Bruker ELEXSYS 500 spectrometer equipped with a super-high-Q resonator ER-4123-SHQ and standard Oxford low-temperature devices. For Q-band studies, EPR spectra were recorded on a Bruker EMX system equipped with an ER-510-QT resonator. The magnetic field was calibrated by a NMR probe and the frequency inside the cavity was determined with a Hewlett-Packard 5352B microwave frequency counter. Computer simulation: WINEPR-Simfonia, version 1.5, Bruker Analytische Messtechnik GmbH.

2.1.1. SYNTHESIS OF COPPER(II) COMPLEXES WITH NIs AS LIGANDS



Synthesis of copper complexes with nitrification inhibitors as ligands. *A) Different prepared solutions. B) Filtered precipitated. C) Liquid-liquid diffusion tube.*

2.1.1.1 [Cu(DMP)$_4$Cl$_2$] (CuDMP1). A mixture of $CuCl_2 \cdot 2H_2O$ (17 mg, 0.1 mmol) and 3,4-dimethyl-1H-pyrazol dihydrogen phosphate (38 mg, 0.2 mmol) in water (20 mL) was stirred for 1 h at room temperature after adjusting the pH to ca. 5 with aqueous 1 M NaOH. Then, the solution was filtered and the blue precipitate formed can be identified as **CuDMP1** on the basis of FTIR spectroscopy. Blue prismatic single-crystals suitable for XRD experiments were only obtained by liquid-liquid diffusion procedure which involves an aqueous solution of $CuCl_2 \cdot 2H_2O$ (pH = 5) and a solution of DMPP in ethanol, using the same amounts of reactants described above. Yield: 18 mg, 70% based on DMPP. Anal. Calcd (Found): C, 46.29 (45.98); H, 6.27 (6.34); N, 21.59 (21.63). IR (cm−1): 1458(s), 1383(m), 1124(s), 1071(s), 998 (m), 972(m), 899(w), 875(w), 808(w), 714(w), 634(w), 602(w), 577 (w), 546(w).

2.1.1.2 [Cu(DMP)$_4$SO$_4$]$_n$ (CuDMP2). A hot solution of $CuSO_4 \cdot 5H_2O$ (50 mg, 0.2 mmol) and 3,4-dimethyl-1H-pyrazol dihydrogen phosphate (78 mg, 0.4 mmol) in water (20 mL) was stirred for 20 min and filtered. The blue precipitate formed can be identified as **CuDMP2** on the basis of FTIR spectroscopy and PXRD. Dark-blue prismatic single-crystals suitable for XRD experiments were only obtained by liquid-liquid diffusion procedure which involves an aqueous solution of $CuSO_4 \cdot 5H_2O$ and a solution of DMPP in ethanol, using the same amounts of reactants described above. Yield: 25 mg, 46% based on DMPP. Anal. Calcd (Found): C, 44.48 (44.92); H, 5.22 (5.10); N, 20.75 (20.69). IR (cm−1): 1593(w), 1528(w), 1448(w), 1389(w), 1350(w), 1317(m), 1126(s), 1061(m), 988(m), 951(w), 883(m), 808(w), 652(w), 633(m), 604(w).

2.1.1.3 [Cu(DMPSA)$_2$]·H$_2$O (CuDMPSA). To 10 mL of an aqueous solution (pH = 5) of $CuSO_4 \cdot 5H_2O$ (25 mg, 0.1 mmol), 2-(3,4-dimethyl-1H-pyrazol-1-yl)-succinic acid (42 mg, 0.2 mmol) dissolved in ethanol (10 mL) was added dropwise. This mixture was stirred for 30 min at room temperature, filtered and left to evaporate in an open container at room temperature. Light-blue prismatic crystals suitable for XRD experiments were obtained after two weeks. Yield: 28 mg, 56% based on Cu. Anal. Calcd (Found): C, 42.88

(42.90); H, 4.62 (4.80); N, 11.22 (11.12). IR (cm−1): 2926(s), 1692(s), 1585(s), 1448(w), 1413(m), 1363(w), 1267(w), 1206(m), 921(w), 834(w), 408(w).

2.1.2. X-RAY CRYSTALLOGRAPHY

Single-crystal X-ray diffraction data for **CuDMP1**, **CuDMP2**, **CuDMPSA** and **DMPSA** are given in the Supplementary Information (Table S1). Intensity data were collected at 100 K (293 K in the case of **CuDMP2** and **DMPSA**) on an Agilent Technologies Supernova single source diffractometer equipped with MoKα (0.71073 Å) radiation and Eos CCD detector. For **DMPSA** CuKα (1.54184 Å) radiation and Atlas CCD detector were used instead. Data frames were processed (unit cell determination, multi-scan absorption correction, intensity data integration and correction for Lorentz and polarization effects) using the CrysAlis Pro software package (CrysAlis Pro CCD V38.2 and RED; Oxford Diffraction, Ltd.: Oxford, UK, 2009). The structures were solved using OLEX2 (Dolomanov et al., 2009) and refined by full-matrix least-squares based on F^2 with SHELXL–2014/6 (Sheldrick, 2015) as integrated in WinGX (Farrugia, 1999). Thermal vibrations were treated anisotropically for non-H atoms. Hydrogen atoms from the organic ligands were placed in calculated positions and refined using a riding model with standard SHELXL parameters, whereas those belonging to the hydration water molecule in **CuDMPSA** were located in the Fourier map and O—H bond lengths were manually restrained to 0.84(2)Å (DFIX). For **CuDMP2**, sulfur atoms belonging to bridging sulfate groups were disordered over four crystallographic positions with 25% population factors, whereas oxygen atoms showed 50% occupancy. Organic DMP ligands exhibit statistical N/C disorder (50% occupancy) in 2 and 5 positions of the pyrazole ring. In the case of **CuDMPSA**, two isomers of the DMPSA ligand were found to coexist in the complex: the 2,3-di-methyl (25%) and 3,4-dimethyl (75%) forms as observed from the refinement of the occupancy of the methyl groups without restriction. These populations were fixed in the last refinement cycle.

CCDC1998718 **(CuDMP1)**, 1998719 **(CuDMP2)**, 1998720 **(CuDMPSA)** and 1998721 (**DMPSA**) contain the supplementary crystallographic data for this paper. These

data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

2.2. EXPERIMENT 2 - POTS EXPERIMENT

2.2.1. SOIL SAMPLING AND EXPERIMENTAL SETUP

Soil was collected in September 2019, from a 0–30 cm layer of clay loam soil from non-fertilized plots of a rapeseed crop in the Basque Country (Northern Spain), with 43% of sand, 25% of silt and 32% of clay and pH (1:2.5) of 8. The soil was air dried at room temperature. Roots and rocks were removed and the soil was sieved. In order to increase soil's porosity, it was mixed with sand in proportion of 3:1 soil: sand (v:v). After homogenization, it was stored until the start of the experiment. Each pot (21 cm diameter) was filled with a total of 3.5 kg of dry soil. In order to reactivate the soil microorganisms, pots were supplied with 500 mg of carbon in the form of glucose and 3 mg of NH_4NO_3 per kg of dry soil (Menéndez et al., 2012; Torralbo et al., 2017), the soil was rehydrated with deionized water and adjusted to a water-filled pore space (WFPS) (Linn and Doran, 1984) of 50% to provide mixed aerobic and anaerobic conditions. 8 mg Cu kg^{-1} dry soil were applied to Cu-added soils in the form of $CuSO_4$, content that has been already probed as non-toxic for nitrogen-cycle bacteria (He et al., 2018; Shen et al., 2020). In the same manner, 8 mg Zn kg^{-1} dry soil were applied to Zn-added soils in the form of $ZnSO_4$. 15 days after soil activation, deionized water was added to reach 60% WFPS to ensure both nitrification and denitrification (Davidson, 1991; Del Prado et al., 2006). Afterward, all pots were fertilized with ammonium sulfate $(NH_4)_2SO_4$ 21% (AS) at a rate of 63.11 mg N kg^{-1} dry soil (equivalent to 180 kg N ha^{-1}). NIs, 3,4-dimethyl-1H-pyrazol dihydrogen phosphate (DMPP) and 2-(3,4-dimethyl-1H-pyrazol-1-yl)-succinic acid (DMPSA), were applied to soil surface with the fertilizer in granular form, as supplied by the manufacturer, EuroChem Agro Iberia S. L (dose of 0.8% of the NH_4^+-N applied with fertilizer). Pots were placed in a greenhouse with conditions consisting in 25 °C and 50% relative humidity (RH) during daytime and 18 °C and 60% RH during nights, with natural environmental light conditions.

2.2.2. N_2O EMISSIONS MEASUREMENTS

Chambers introduced inside pots for GHG emissions sampling under controlled conditions.

N_2O soil emissions were measured using the close chamber method (Chadwick et al., 2014). Gas samples were collected along 22 days from fertilization. Sampling frequency was every two days after fertilization (DAF), increasing it to every day between days 5 and 13 for a more precise record of the emission peak. For the collection of the gas, chambers (20 cm diameter) were inserted inside the pot and they were hermetically sealed. Samples were taken just after closing the chamber (t = 0) and after 60 min. 20 mL of gas were taken from each chamber and stored at overpressure in pre-evacuated 12 mL glass vials. Then, they were analyzed in a gas chromatograph (GC) (Agilent, 7890A) equipped with an electron capture detector for N_2O analysis. A capillary column (IA KRCIAES 6017:240 °C, 30 m × 320 μm) was used, and samples were injected by means of a headspace auto-sampler (Teledyne Tekmar HT3) connected to the GC. Standards of

N_2O were analyzed at the same time. Gas emission rates were calculated taking into account the gas concentration variation from the beginning to the end of the 60 min. 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).

2.2.3. DNA EXTRACTION AND QUANTIFICATION OF NITRIFYING AND DENITRIFYING GENES

Soil cores from each treatment were collected at 0–15 cm depth just before fertilizer application and 15 DAF. After homogenization, subsamples were weighted, frozen in liquid nitrogen and stored at −80 °C until use. DNA was extracted from 0.35 g fresh weight of soil using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) including some modifications described in Harter et al. (2014). Extracted DNA concentration and quality were determined by spectrophotometry with a NanoDrop® 1000 (Thermo Scientific, Waltham, MA, USA). Quantitative polymerase chain reactions (qPCR) were performed using SYBR® Premix Ex TaqTM II (Takara-Bio Inc.) and gene-specific primers (Supplementary Table S2) to amplify and quantify total bacteria abundance (16S rRNA), AMO encoding gene (*amoA*) and N_2OR encoding gene (*nosZI*). Each sample was quantified in triplicate using the StepOnePlusTM. Real-Time PCR System and data analysis were carried out by StepOnePlusTM Software 2.3 (Thermo Scientific). Standard curves (log gene copies number per reaction volume versus log N) were prepared from serial dilutions of 10^7 to 10^3 gene copies μL^{-1} of linearized plasmids with insertions of the target genes, and the copy number of target genes per gram of dry soil was calculated according to a modified equation detailed by 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.

2.2.4. STATISTICAL ANALYSIS

Statistical evaluation of the data was carried out with SPSS (IBM SPSS Statistics for macOS, version 25.0. Armonk, NY: IBM Corp). One-way ANOVA with Duncan's multiple-range test for separation of means ($P < 0.05$) was employed to test the differences in N_2O emissions and genes abundances depending on the variables tested in this work (Soil Cu and Zn contents and nitrification inhibitors application).

RESULTS AND DISCUSSION

3.1. COORDINATING ABILITY OF INHIBITORS: SYNTHETIC ASPECTS

Focusing on our interest on DMPP and DMPSA inhibitors and their environmental implications, we decided to conduct chemical reactions in the lab-scale to prepare model systems for the activity of these nitrification inhibitors as chelators of metal ions such as Cu^{2+}. First, we tested the direct reaction between $CuCl_2 \cdot 2H_2O$ and a two-fold excess of DMPP in water at room temperature. From the initial clear solution, the formation of a dark blue precipitate is observed when adjusting the pH to 5 using aqueous 1 M NaOH. FT-IR spectroscopy (Supplementary Fig. S1A) reveals that this blue precipitate contains DMP molecules in its structure as evidenced by the most intense vibrational bands located at 500–1500 cm^{-1} region that could be ascribed to different stretching and bending modes within the dimethyl imidazole ring (Hasegawa et al., 2000). Coordination of DMP to copper centers can be clearly observed not only in its color, but also when comparing the IR spectrum with that of pure DMPP as observed in related methyl-imidazole systems (Di Santo et al., 2018). To get more insight about this complex, we performed different synthetic modifications for the $CuCl_2$/DMPP system including i) the screening of the pH of aqueous solutions between 3 and 7; ii) variation of the reaction

temperature (room temperature, 50 and 90 °C); and iii) the use of ethanol as co-solvent in liquid-liquid diffusion procedures. Fortunately, blue single-crystals (**CuDMP1**) suitable for X-ray diffraction experiments were obtained following the latter approach. In a test tube, a solution of DMPP in ethanol (10 mL) was slowly deposited over an aqueous solution of the Cu^{2+} salt (10 mL), in such a way that two phases are not mixed. Crystals grew in the interphase between both components. As can be observed by FT-IR spectroscopy, the spectrum of **CuDMP1** is virtually identical to that of the powdered sample isolated in the parent reaction. This indicates that DMP easily coordinates to Cu^{2+} in water and suggests that similar processes could be taking place in soils. We tried to confirm if both powder and crystals belong to the same phase but with no success. PXRD experiments carried out for the powdered sample revealed its amorphous nature because no well-defined diffraction maxima can be observed in the XRD pattern.

Analogous experiments to those carried out for the chloride salt were performed for $CuSO_4 \cdot 5H_2O$. The direct reaction between this Cu^{2+} salt and a two-fold excess of DMPP in water afforded a blue precipitate whose FTIR spectrum differs considerably from that of **CuDMP1** (Supplementary Fig. S1A). Thus, we tried to get crystals from this reaction by modifying synthetic conditions as described above for the previous system. Similar diffusion procedures employed for **CuDMP1** yielded crystals of **CuDMP2**, whose FTIR spectrum is virtually identical to that of the powdered, amorphous sample. These observations show that the counterion plays a key role in the formation of different complexes between Cu^{2+} and DMP. Encouraged by all these results, we decided to test whether the coordination of DMP could be observed in other metal ions of interest such as Cu^{+} and Zn^{2+}. A fast oxidation to Cu^{2+} is observed under similar synthetic conditions for the former ion as evidenced by the color change from colorless to blue observed in the aqueous reaction. For Zn^{2+}/DMP system, the IR of the white precipitate formed from the direct aqueous reaction suggests that the inhibitor coordinates to the metal center, because it is virtually identical to that of **CuDMP1**. Unfortunately, no crystal was obtained following similar reaction approaches.

When it comes to the DMPSA inhibitor, its lower solubility in aqueous acidic media (pH = 2 to 5) forced us to select ethanol:water (1:1) mixtures as reaction solvent. The addition of a two-fold excess of DMPSA dissolved in ethanol to an aqueous solution of $CuSO_4 \cdot 5H_2O$ yielded blue light crystals of **CuDMPSA**. The FTIR spectrum of **CuDMPSA** (Supplementary Fig. S1B) in comparison to that of pure DMPSA reveals the coordination of DMPSA to copper centers as observed in the blue shift of the most intense vibrational bands located at 1740 and 1648 cm^{-1} that appear at 1692 and 1585 cm^{-1} for **CuDMPSA** and could be ascribed to C—O stretching modes from carboxylate groups. It is worth mentioning that crystals of pure protonated **DMPSA** were isolated when the reaction took place in very acidic medium (pH = 2).

Thermal stability of all the three compounds synthesized was evaluated by TGA/SDTA analyses (Supplementary Fig. S2). The endothermic dehydration process for **CuDMPSA** is completed at ~140 °C [calcd (found) for 1 H_2O: 3.48 (3.39)], whereas complexes **CuDMP1** and **CuDMP2** are thermally stable up to ~105 °C and 185 °C respectively. This suggests that the later phases do not exhibit crystallization solvent molecules in their structures. Initial stages are followed by a three-step, highly exothermic ligand combustion, resulting in the final residue above 555 °C, 485 °C and 465 °C for **CuDMP1**, **CuDMP2** and **CuDMPSA**, respectively. The weight percentage of the final residues is in full agreement with the expected value [calcd (found) for CuO: 15.33 (16.11) for **CuDMP1**; 14.73 (16.08) for **CuDMP2**; 15.74 (15.48) for **CuDMPSA**] and the mass loss accounts for at least 4 DMP molecules in the case of **CuDMP1** and **CuDMP2** and 2 DMPSA units per copper center for **CuDMPSA**. This implies that the reaction yield of **CuDMP1** and **CuDMP2** could be considerably improved by adding a larger (four-fold) excess of DMPP instead of the 1:2 Cu^{2+}:DMPP ratio used in this work.

3.2. CRYSTAL STRUCTURES

Two different compounds were isolated in the reaction between Cu^{2+} salts and the DMPP inhibitor depending on the counterion of the metal source: $CuCl_2$ afforded **CuDMP1**, whereas $CuSO_4$ led to **CuDMP2**. To our knowledge, both represent the first

examples in the literature of transition metal complexes containing the DMP ligand. **CuDMP1** (Fig. 1) crystallizes in the triclinic $P-1$ space group and the complex contains one Cu^{2+} cation on an inversion center, exhibiting an axially elongated octahedral coordination geometry. The equatorial plane is formed by four N-donor DMP ligands and the axial positions are occupied by Cl atoms. The crystal packing is formed by monomeric units connected by weak Cl···Cl interactions (3.694(3)Å) that result in one-dimensional arrangements running parallel to the crystallographic *x-axis* (Supplementary Fig. S3). **CuDMP2** (Fig. 1) crystallizes in the monoclinic *C2*/*c* space group and it displays Cu^{2+} cations exhibiting axially elongated octahedral coordination geometry. The equatorial plane of each copper center is formed by four N-donor DMP ligands and the axial positions are occupied by O atoms from sulfate groups that act as bridging units between metal centers. These covalent chains run along the crystallographic *y-axis* and their packing strongly resembles that displayed by **CuDMP1** (Supplementary Fig. S4).

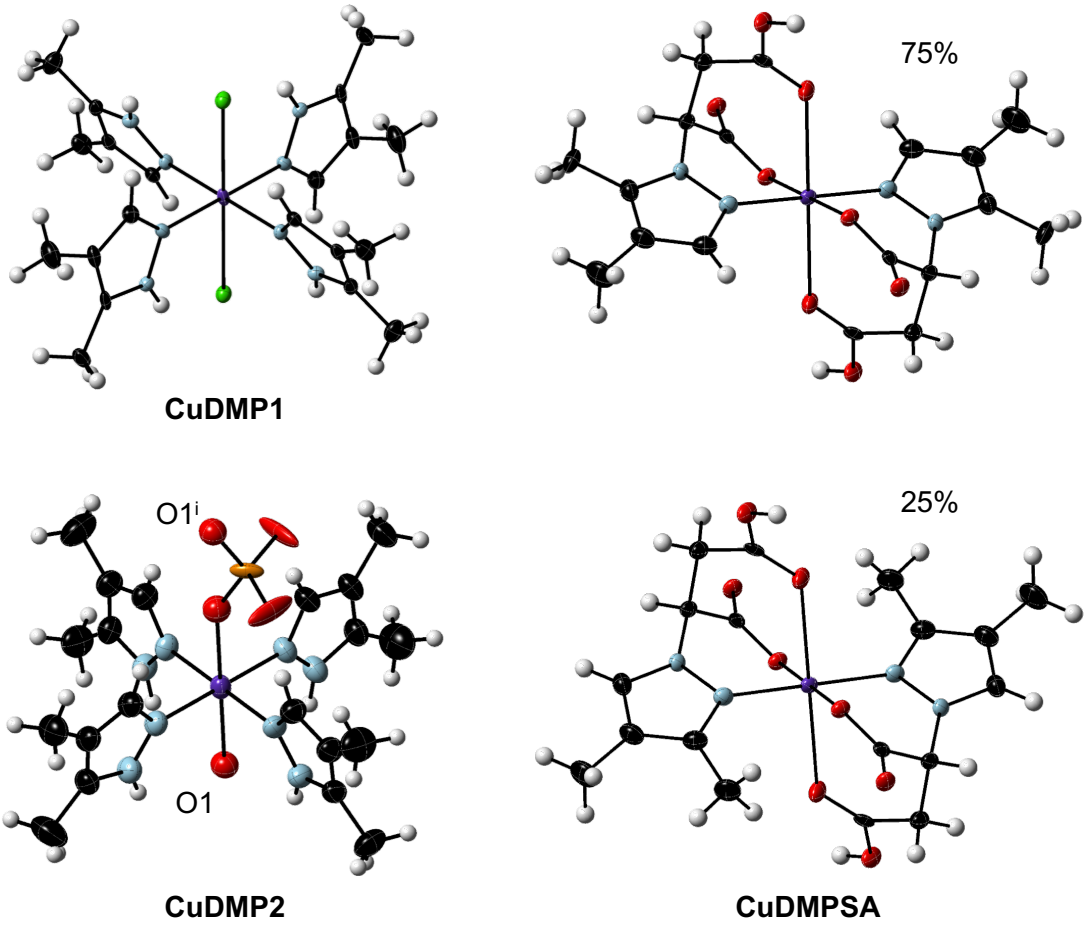


Figure 1. ORTEP views of CuDMP1, CuDMP2 and CuDMPSA showing 50% probability displacement ellipsoids*. The two isomers of DMPSA that coexist in the structure of* ***CuDMPSA*** *are represented independently. Color code: Cu, violet; C, black; Cl, green; H, white; N, blue; O, red; S, orange. Symmetry code: i) x, 1+y, z.*

When it comes to metal complexes containing DMPSA ligands, **CuDMPSA** is completely unprecedented because no examples of such kind of structures can be found in the CSD database. **CuDMPSA** (Fig. 1) crystallizes in the monoclinic *P21/c* space group and the complex contains one Cu^{2+} cation on an inversion center, exhibiting a highly distorted octahedral coordination geometry due to the geometrical restrictions arising from the two deprotonated, tridentate N,O,O′-donor DMPSA− ligands coordinated to the metal center in *fac*-mode. Equatorial positions are occupied by N atoms from pyrazole rings and O atoms belonging to the carboxylate groups, whereas axial positions are blocked by O atoms from protonated carboxylic groups. The cell unit also

contains one hydration water molecule disordered over two symmetry-related positions showing 50% occupancy. The crystal packing is constituted by supramolecular layers formed by hydrogen bonding parallel to the *yz* plane. Each carboxylic moiety establishes one strong O—H···O interaction (2.54(4)Å) with the analogous group from an adjacent complex, in such a way that each monomer is linked to four neighbors. Two weak C—H···O type contacts between complexes reinforce this arrangement. These layers stack along the crystallographic *x-axis* (Supplementary Fig. S5). It is worth mentioning that the DMPSA inhibitor provided by the supplier sources contains two different isomers of the DMPSA ligands: the 2,3-dimethyl and the 3,4-dimethyl forms. The presence of these two isomers can be clearly observed in the structure of **CuDMPSA** which shows 25% of the former and 75% of the latter configuration according to XRD data. In the course of the systematic synthetic studies, crystals of pure diprotonated DMPSA (**DMPSA**) were also isolated. The structure of **DMPSA** displays only the 2,3-dimethyl isomer of the inhibitor. These molecules are organized in double chains running along the crystallographic *z-axis*, in which monomers are connected to three neighbors through O—H···O and interaction O—H···N type interactions (2.605(4) and 2.631(%) Å, respectively) established between carboxylic groups and pyrazole rings (Supplementary Fig. S6).

3.3. EPR SPECTROSCOPY AND COMPETITIVE REACTIONS

EPR spectroscopy experiments were conducted for all the three compounds synthesized because this sensitive technique could be useful to tentatively identify the formation of these complexes in real samples such as soils. X and Q band EPR measurements for the three complexes were carried out on powdered samples at several temperatures in the range 4–300 K. All the spectra exhibit near axial symmetry for the g tensor in the $\Delta Ms = \pm 1$ region, but a slight degree of rhombicity can be deduced from the spectra recorded in Q band (Fig. 2A). The main components of the g tensors were determined by comparison of the experimental spectra with those calculated at a second order of the perturbation theory with a computer simulation program. Adjusting the observed signals by the trial and error method, the following values were obtained: g_1 =

2.306, g_2 = 2.065, g_3 = 2.061 (gII = 2.306, g⊥ = 2.063, <g> = 2.144) for compound **CuDMP1**; g_1 = 2.318, g_2 = 2.068, g_3 = 2.060 (gII = 2.318, g⊥ = 2.064, <g> = 2.149) for compound **CuDMP2**; g_1 = 2.314, g_2 = 2.071, g_3 = 2.065 (gII = 2.314, g⊥ = 2.068, <g> = 2.150) for compound **CuDMPSA**. These values were used to simulate the powder EPR spectrum in order to produce the dashed line in Fig. 2A. In all cases, the minor g value is higher than 2.04 as expected for mainly $d_{x^2-y^2}$ ground states derived from axially elongated octahedral geometries in Cu^{2+} ions (Hathaway and Billing, 1970). The spectrum of **CuDMP2** shows partially resolved hyperfine structure for the nuclear spin of copper (^{65}Cu, ^{63}Cu; I = 3/2) on the low field line (AII = 181 x 10^{-4} cm^{-1}), indicating that the intermolecular magnetic exchange pathway is less efficient in this compound in spite of the sulfate bridges. The G parameter as defined by Hathaway has been utilized to confirm the significance of the calculated g values to give any definitive information on the electronic ground state present. For the three complexes the G value lies in the 4.4–5.0 range, therefore the effect of the exchange coupling is negligible and the observed g values are meaningful. Moreover, the spectra remain practically unchanged over the temperature range 4.2–298 K, so the magnetic interactions should be of small magnitude.

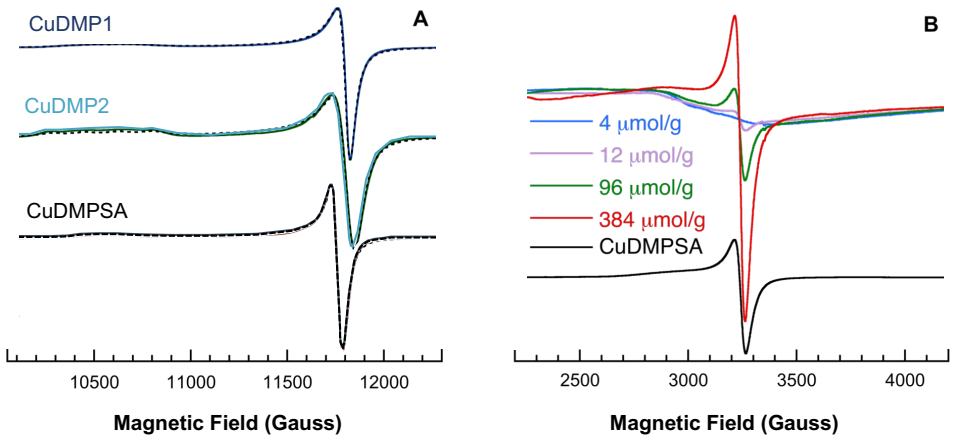


Figure 2. A) Experimental and simulated Q-band (33.9 GHz) EPR spectra of CuDMP1, CuDMP2 and CuDMPSA. B) Room temperature X-band (9.40 GHz) EPR spectra of soils with different Cu content.

Despite the high sensitivity of EPR spectroscopy to detect the presence of paramagnetic ions in different environments, no signal attributable to Cu^{2+} species could be observed in the EPR spectra recorded on as prepared soil samples, not even in the presence of DMPP or DMPSA. In order to increase its copper content to analyze the possible formation of chelates with nitrification inhibitors, a weighed amount (500 mg) of dry soil were rehydrated and mixed with different amounts of $CuSO_4$ (0.33, 1, 8 and 32 mg) and the proportional inhibitor amounts. After being stirred, the samples were filtered and dried. Fig. 2B displays the EPR spectra registered with these powders at room temperature. It can be seen that the signal of the **CuDMPSA** chelate can be easily detected for copper contents higher than 12 μmol g^{-1}. Furthermore, it was verified that the signal intensity remained practically constant for more than 72 h. All in all, taking into account the prepared model systems for the interaction between Cu^{2+} and NIs and the EPR experiments described above, we have confirmed that i) the DMP formed after the dissociation of DMPP in water and DMPSA can chelate Cu^{2+} ions in soils and ii) DMPSA do not need to decompose to DMP to coordinate to metal centers.

Furthermore, to determine the different kinetics/affinity of both inhibitors towards the formation of copper complexes, we decided to perform additional experiments in which one equivalent of $CuSO_4 \cdot 5H_2O$ (0.1 mmol) and two equivalents (0.2 mmol) of each inhibitor DMPP and DMPSA were mixed in 20 mL of water. The resulting clear solution was left to evaporate at room temperature and a blue powder precipitated out from the mother solution in three days. PXRD analyses of this crystalline powder and its comparison with the simulated patterns from single-crystal X-ray diffraction data for **CuDMP2** and **CuDMPSA** revealed that **CuDMPSA** was exclusively formed because the experimental profile is virtually identical to that of the simulated pattern (Supplementary Fig. S7).

Different reasons could be on the origin of this fact: i) Thermodynamics: the complex with the tridentate ligand DMPSA much more stable and hence more difficult to break in comparison to that of the monodentate DMP due to the well-known chelate effect. The chelate effect is the enhanced affinity of polydentate ligands for a metal ion

compared to the affinity of a collection of similar non-chelating (monodentate) ligands for the same metal (Martell, 1967). ii) Solubility: we experimentally observed that **CuDMPSA** complex was much more insoluble in water. This could be a major reason for the exclusive isolation of **CuDMPSA** in our experiment and strongly affects the availability of copper ions in soils. iii) Kinetics: although less probable, the formation of **CuDMPSA** complex could be faster than that of **CuDMP**.

3.4. EFFECTS OF COPPER AND ZINC ADDITION TO SOIL ON N_2O EMISSIONS

Once it has been demonstrated the ability of both DMPP and DMPSA (hereinafter DMPs) to chelate copper, we decided to conduct a soil experiment to test a possible relationship between the action of these NIs and the soil copper content. Moreover, supported by the different affinity showed through PXRD analysis and the different number of DMP/ DMPSA ligands found in the crystalline structures of **CuDMP** complexes and **CuDMPSA**, a differential inhibition efficiency between them was considered as a possibility in soils with high copper content. As our results suggested that DMPs may also be able to coordinate Zn^{2+}, we decided to go deeper into this question testing also the effect of the inhibitors under different zinc contents in soils.

Despite being necessary for the enzymatic activity of nitrogen cycle, Cu and Zn are toxic above certain concentrations. Nitrification and denitrification show inhibition with Cu concentrations of about 1000 mg kg^{-1} and 100 mg kg^{-1} respectively (Glass and Orphan, 2012; He et al., 2018). On the other hand, Zn concentrations of about 100 mg kg^{-1} and 230–1000 mg kg^{-1} have been reported as EC50 values (effective concentration to produce a 50% of reduction) for nitrification and denitrification. This tolerance varies with the soil NH_4^+-N content (De Brouwere et al., 2007; Ruyters et al., 2010b; Vasileiadis et al., 2012). Thus, Cu and Zn doses supplied in this work have been adjusted to values naturally present in European agricultural soils (Tóth et al., 2016; Ballabio et al., 2018) to avoid possible toxicity. Soils were incubated during 15 days after the addition of metal ions and before fertilization in order to allow microbial populations to adapt to the new

conditions (He et al., 2018). Based on *16S rRNA* gene abundance in the pre-fertilization period (Fig. 3), we can confirm that neither Cu nor Zn supply affected total bacterial abundance in our experiment.

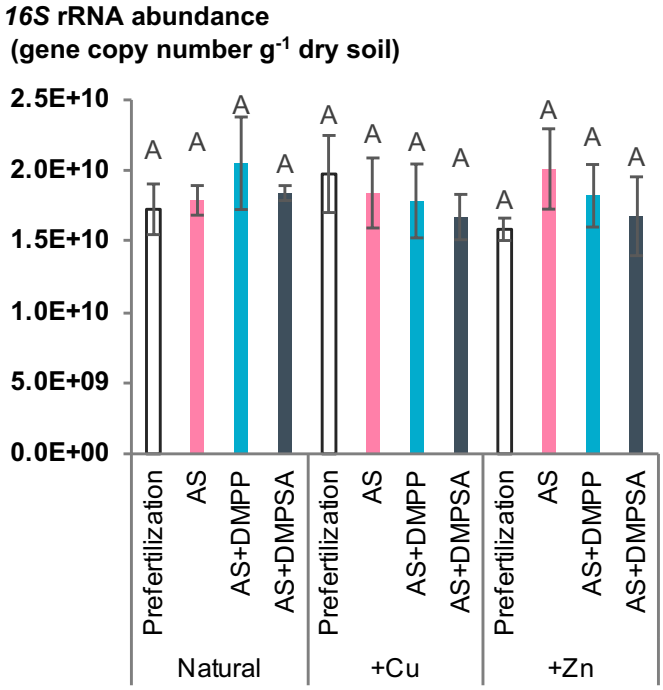


Figure 3. Total bacterial abundance expressed as 16S rRNA gene copy number per gram of dry soil. *Different letters indicate significant differences using the Duncan Test ($P < 0.05$; $n = 3$).*

AS = Ammonium sulphate 21%; DMPP = 3,4-dimethyl-1H-pyrazole phosphate; DMPSA = 2-(3,4-dimethyilpyrazole-1-YL)-succinic acid; Natural = soil with no-added metals; +Cu = natural soil + 8 mg Cu kg^{-1} dry soil; +Zn = natural soil + 8 mg Zn kg^{-1} dry soil.

The N_2O emissions of AS treatments showed clear differences when comparing natural and metal-added soils. Daily emissions disclosed that maximum peaks were reached 6 days after fertilization (DAF) in case of "AS" (~37 µg N_2O-N kg^{-1} dry soil day^{-1}) (Fig. 4). However, maximum emission was delayed to 11 DAF in case of "AS+Cu" and "AS+Zn" soils, reaching values of ~89 and 45 µg N_2O-N kg^{-1} dry soil day^{-1} respectively.

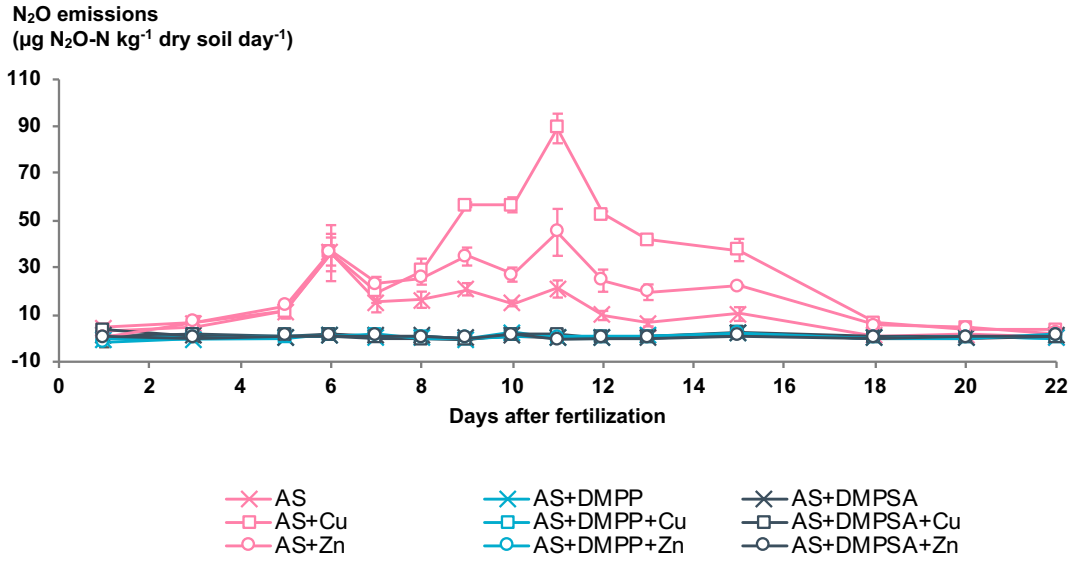


Figure 4. N_2O daily emissions from soil expressed as µg N_2O-N per kg of dry soil and day.

AS = Ammonium sulphate 21%; DMPP = 3,4-dimethyl-1H-pyrazole phosphate; DMPSA = 2-(3,4-dimethyilpyrazole-1-YL)-succinic acid; +Cu = natural soil + 8 mg Cu kg^{-1} dry soil; +Zn = natural soil + 8 mg Zn kg^{-1} dry soil.

Copper is one of the cofactors involved in the activity of AMO (Gilch et al., 2009a; Glass and Orphan, 2012) and therefore, there is a direct relationship between Cu availability and nitrification. Previous studies have reported a hormetic effect of Cu addition on nitrification, as Cu contents of ~100 mg kg^{-1} can increase potential nitrification (PNR) (Oorts et al., 2006; Sun et al., 2008; Ruyters et al., 2010a) and AOB abundance (He et al., 2018). This suggests that nitrifying activity may be limited by Cu availability. This seems to be the case in our soil, because the addition of Cu ("AS+Cu") produced a 2.7 fold increase in the total cumulative N_2O emissions with respect to "AS" (Fig. 5). The increase in N_2O emissions should be driven by the growth of AOB populations, as it was accompanied by a 3.6 fold increase in *amoA* gene abundance in "AS+Cu" (Fig. 6A).

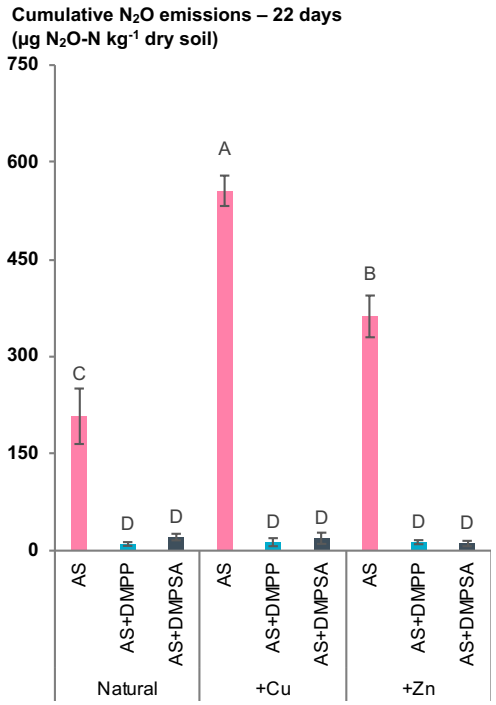


Figure 5. Total cumulative N_2O emissions from soil expressed as µg N_2O-N per kg of dry soil. *Different letters indicate significant differences using the Duncan Test ($P < 0.05$; $n = 3$).*

AS = Ammonium sulphate 21%; DMPP = 3,4-dimethyl-1H-pyrazole phosphate; DMPSA = 2-(3,4-dimethyilpyrazole-1-YL)-succinic acid; Natural = soil with no-added metals; +Cu = natural soil + 8 mg Cu kg^{-1} dry soil; +Zn = natural soil + 8 mg Zn kg^{-1} dry soil.

Some other nitrogen-cycle enzymes also require Cu as cofactor (Glass and Orphan, 2012). Therefore, other populations would be expected to be affected as well by Cu addition. Among them, N_2OR has a crucial importance because it is the only enzyme known to date able to reduce N_2O to innocuous N_2. The expression of the encoding gene of N_2OR, *nosZI*, depends on Cu availability (Sullivan et al., 2013) and hence, agricultural soils may present a Cu deficiency to maximize N_2O reduction to N_2 (Richardson et al., 2009; Thomson et al., 2012; Black et al., 2016; Shen et al., 2020). Our findings match with those studies because *nosZI* abundance also increased by 1.4 fold with Cu application (Fig. 6B). The differential increase between *amoA* (3.6 fold) and *nosZI* (1.4 fold) abundances seems to be the reason why emissions have increased in "AS+Cu" with

respect to "AS" (2.7 fold), as the enhanced growth of AOB population has not been counteracted with a sufficient increment in populations able to reduce the higher N_2O generation to N_2. We can propose that this smoother response of N_2O-reducers (*nosZI*-holding bacteria) may be explained by the stimulation of AOB population growth, which immobilized an important part of the applied Cu and, therefore, decreased its availability for N_2O-reducers.

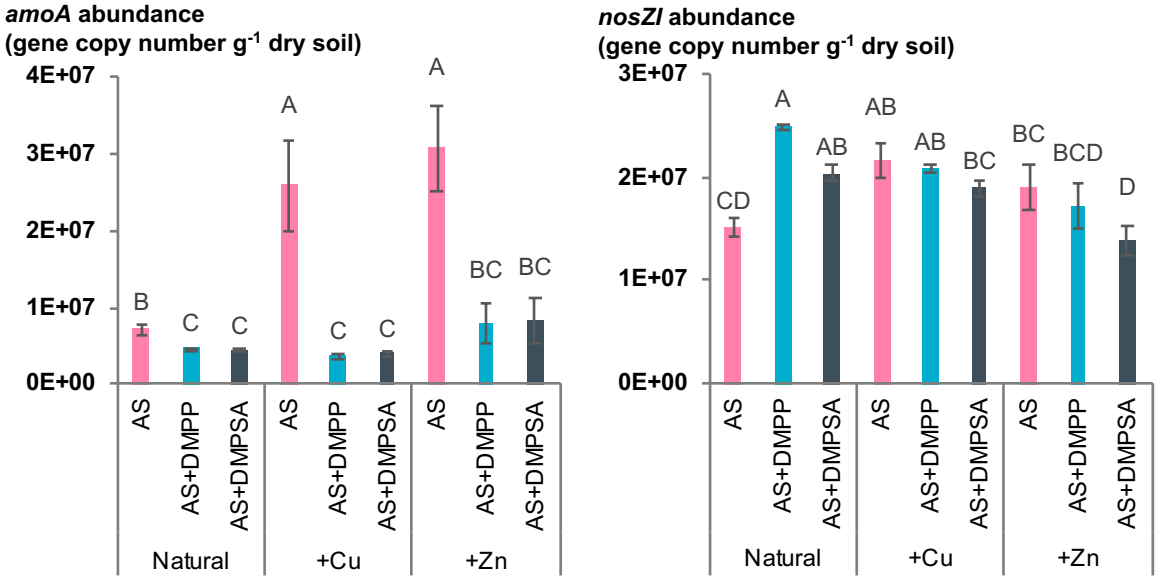


Figure 6. A) AOB abundance and B) N_2O-RD abundance, expressed respectively as amoA and nosZI gene copy number per gram of dry soil. *Different letters indicate significant differences using the Duncan Test ($P < 0.05$; $n = 3$).*
AS = Ammonium sulphate 21%; DMPP = 3,4-dimethyl-1H-pyrazole phosphate; DMPSA = 2-(3,4-dimethyilpyrazole-1-YL)-succinic acid; Natural = soil with no-added metals; +Cu = natural soil + 8 mg Cu kg^{-1} dry soil; +Zn = natural soil + 8 mg Zn kg^{-1} dry soil.

Although to a lesser extent than Cu, Zn also plays an important role within enzymatic activity of nitrogen cycle. It is believed that Zn may be necessary for nitrifiers denitrification (Glass and Orphan, 2012). Furthermore, it might also be present in AMO, although it is not clear if this presence is just accidental, because this site is occupied by

Fe in natural conditions (Gilch et al., 2009a, 2010). Some works have suggested that Cu and Zn may compete by the same binding site in AMO, which undergoes inhibition when Cu is displaced from its active site (Radniecki and Ely, 2008). However, related studies have reported hormetic effects on nitrification due to Zn application at low doses (Radniecki and Ely, 2008; Ruyters et al., 2010a; Chen et al., 2014), suggesting that it does seem to be necessary per se for AMO activity. This matches also with the 1.7 fold increase of N_2O emissions observed in "AS+Zn" with respect to "AS" (Fig. 5). The data acquired for the *amoA* gene abundance seem to be enough to link the increase in N_2O emissions with the 4.3 fold increase in "AS+Zn" AOB population with respect to "AS" (Fig. 6A). On the contrary, *nosZI* gene abundance was not statistically affected by Zn application (Fig. 6B). This coincides with previous studies that reported no hormetic effect on denitrification (Ruyters et al., 2010b; Chen et al., 2014) and confirms that Zn does not play any key role in the N_2OR activity.

3.5. RELATIONSHIP BETWEEN NITRIFICATION INHIBITORS AND SOIL COPPER AND ZINC CONTENTS

The application of NIs was highly effective, because almost a total elimination of N_2O emissions with respect to AS treatments was observed (Figs. 4 and 5). Both DMPs showed the same performance. The reduction capacity found in this work is much greater than the values observed for field experiments in the original location of these soils (Corrochano-Monsalve et al., 2020a), although it is in line with the values observed in previous experiments carried out in microcosms conditions with soils from the same location (Torralbo et al., 2017). The differences between field and controlled conditions could be attributed to the higher homogeneity of soils in laboratory conditions after all the conditioning processes (rock and root removal, drying, sieving, etc.), which probably facilitates a more homogeneous distribution of fertilizer, NIs and water.

Up to date, it has been assumed that DMPs inhibit nitrification through Cu chelation (Barrena et al., 2017; Duncan et al., 2017; Torralbo et al., 2017; Beeckman et al., 2018; Guardia et al., 2018c; Montoya et al., 2018; Cassman et al., 2019; Fuertes-

Mendizábal et al., 2019; Sheikhi et al., 2020), but this assumption was founded in a personal communication cited in the review of Ruser and Schulz (2015). However, there is no report in the literature that validates this experimentally. To our knowledge, our work is the first demonstration of the capacity of DMPP and DMPSA to chelate Cu (and most likely Zn). With this confirmation, we hypothesized that there could be a relation between the nitrification inhibition efficiency of these compounds and the soil Cu and Zn content, as it has been previously suggested by Duncan et al. (2017).

Our results indicate that N_2O emissions from all NIs-treated soils are statistically equal (Fig. 5). In fact, as emissions were higher in "AS+Cu" and "AS+Zn" soils, the percentage of emissions reduction by the NIs was even more drastic in both Cu-added soils (98% DMPP; 97% DMPSA) and Zn-added soils (96% DMPP; 97% DMPSA) in comparison to that observed in natural soils (95% DMPP; 90% DMPSA). This is in concordance with the general trend displayed by NIs which show greater emission reduction as higher is the baseline (Li et al., 2018). Moreover, *amoA* abundance was also statistically equal in all the NIs-treated soils (Fig. 6A), in spite of the addition of Cu or Zn addition. Interestingly, the percentage of inhibition of AOB population growth was considerably higher in Cu-added soils (86% DMPP; 85% DMPSA) and Zn-added soils (75% DMPP; 73% DMPSA) than in natural soils (35% DMPP; 40% DMPSA). Although the inhibition of N_2O emissions was similar in all the different inhibitor treatments, our results showed that there was a difference in the way it was achieved when comparing natural and metal-added soils. There was a clear increase of *nosZI* gene abundance in natural soils with NIs application (+65% DMPP; +35% DMPSA) with respect to "AS" (Fig. 6B). However, this induction was not replicated in the metal-added soils, but it exhibited an opposite trend because a slight decrease was observed in both Cu (+3% DMPP; −12% DMPSA) and Zn-added soils (−10% DMPP; −27% DMPSA) in comparison to "AS+Cu" and "AS+Zn" respectively. This observation indicates that the total reduction of N_2O emissions in natural soils was attributed to two simultaneous processes: 1) the decline of the size of AOB population, which means a decrease of N_2O generation and, 2) a higher reduction of N_2O to N_2 through the increase of the abundance

of N_2O-reducers. On the contrary, the reduction of emissions was based only on the decrease of AOB abundance in the case of metal-added soils.

Our results seem to support the theory of a chelation-based mechanism for nitrification inhibition. However, in this case, it would be expected that other biological processes with Cu and/or Zn requirements, such as NirK and N_2OR enzymatic activity, would be directly affected as well by DMPs application. In fact, Cu availability can be limiting for NirK activity (Zumft, 1997), although this limitation seems to occur only at very low Cu concentrations (0.9 mg kg^{-1}) (Yang et al., 2015). Previous studies showed that the application of DMPs produces no effects on *nirK* gene abundance (Duan et al., 2017; Torralbo et al., 2017), which could indicate that the remaining available Cu is enough to maintain the activity of NirK. Nevertheless, no convincing reason could be found to explain the observed induction of *nosZ* genes after DMPs application so far (Barrena et al., 2017; Torralbo et al., 2017; Fuertes-Mendizábal et al., 2019; Castellano-Hinojosa et al., 2020; Corrochano-Monsalve et al., 2020a), taking into account that the reduction of N_2O to N_2 is not carried out in absence of Cu (Glass and Orphan, 2012; Sullivan et al., 2013). Denitrifiers promote the consumption of NO_3^- rather than N_2O reduction in low Cu environments by shutting down N_2OR activity (Felgate et al., 2012). However, as it was observed for NirK, N_2OR could maintain its function (although slowed down) even at extremely low Cu concentrations, and may also be able to directly use that coordinated to organic ligands (Twining et al., 2007). Previous studies have proposed the addition of Cu with chelating ligands to enhance N_2OR activity when soil Cu content is below 150 mg kg^{-1} (Shen et al., 2020). Altogether, it could mean that Cu content is relatively low in "AS" soils, a great part of it is immobilized by AOB and hence, the N_2OR activity is limited. A similar argumentation has been proposed by Richardson et al. (2009). These observations would be supported by the higher *nosZI* abundance observed in "AS+Cu" in comparison to that of "AS". In the case of "AS+DMPP" and "AS+DMPSA" treatments, Cu is not completely immobilized because the growth of AOB is inhibited. In this manner, Cu remains available for N_2O-reducers, which take advantage of their capacity to use it even when it is bound to the inhibitors.

This induction of *nosZI* was not observed in "AS+DMPP+Cu" and "AS+DMPSA+Cu" because the growth of these bacteria was not limited by Cu availability in "AS+Cu".

We propose that the processes that took place in Zn-added soils were different: the application of Zn in "AS+Zn" implied a partial substitution of Cu by Zn in AMO (Radniecki and Ely, 2008) and as a consequence, more Cu remained available for N_2OR. This seems to be reflected in the slight increase of *nosZI* abundance in "AS+Zn" with respect to "AS". In the case of "AS+DMPP+Zn" and "AS+DMPSA+Zn" treatments, Cu and Zn were chelated by the NIs and thus, they were not available for AOB. As a result, nosZI abundance reflected a slight decrease in DMPs+Zn soils with respect to "AS+Zn" due to the negative effects of the higher Zn content in soils on denitrifiers (De Brouwere et al., 2007; Chen et al., 2014).

The unique features of N_2OR make this enzyme very attractive from both an ecological and economical point of view. The response of *nosZ* genes to DMPs application may pave the way for the development of inductors of N_2OR activity. Nevertheless, the information that has been obtained at this respect until now is still poor, as it has been focused especially in N_2O. However, there is evidence suggesting that *nos* genes expression is regulated by NO rather than N_2O (Pauleta et al., 2013). Considering that previous studies have shown no effects of DMPs over the abundance of *nirK* and *nirS* (Duan et al., 2017; Torralbo et al., 2017), *norB* should gain interest in future works as responsible for NO transformation to N_2O. In the same way, some other genes that seem to be involved in N_2OR synthesis (i.e. *nosR*, *nosC*, *senC2*, *pcuC*) (Richardson et al., 2009; Sullivan et al., 2013) should be considered to complete the scheme. Most of the studies so far have been developed for pure cultures, and works that analyze the relation between Cu content and N_2O reduction to N_2 in soils are still scarce (Richardson et al., 2009; Shen et al., 2020). Nevertheless, it has been already proposed the application of Cu to soils to favor N_2O reducers (Thomson et al., 2012). Our study confirms that Cu addition increases the abundance of N_2O reducers in soils not receiving NIs. However, Cu application increased even more AOB population, thus resulting in higher N_2O emissions due to enhanced nitrification. In addition, the application of Zn also resulted in higher N_2O

emissions, which has been previously reported when applying Zn fertilizers to achieve crop biofortification (Montoya et al., 2018). On the other hand, the application of DMPs can minimize the stimulation of nitrification in Cu/Zn-treated soils while maintaining *nosZI* abundance; thus minimizing N_2O emissions. Therefore, the application of these inhibitors would be especially advisable in soils with high Cu/Zn contents and/or when these metals are added with fertilizers. Nevertheless, it would be necessary to analyze whether the dynamics observed in our work take place in the same way in other soil-moisture conditions

Our work is the first approach to elucidate the mode of action of the NIs based on DMP. Herein, we have demonstrated the Cu^{2+} chelation capacity of DMPP and DMPSA through X-ray crystallography. We can conclude that the effectiveness of DMPs is constant within the thresholds of Cu and Zn contents typically present in agricultural soils (Tóth et al., 2016; Ballabio et al., 2018). Therefore, if the mechanism of action is effectively driven by their chelation capacity as it has been suggested, it would be displayed not only on Cu, but also on Zn. Furthermore, our results indicate that the applied concentration of the inhibitors is enough to neutralize a potential extra input of Cu and/or Zn. Besides, DMPs' capacity to counteract the emissions increase derived from Zn application, would support the essentiality of Zn for the activity of AMO.

The inhibitory effect of a Cu chelator on nitrification is related to the stability of the complex formed between the ligands and the Cu (Shi et al., 2015). The tridentate coordination of DMPSA to Cu is more stable than the monodentate bond between DMP and Cu; thus, DMPSA should be more effective. Nevertheless, the inhibitory performance of both DMPP and DMPSA in soils has shown no differences between them, suggesting that, i) DMPSA is really acting as DMP, which would imply that despite the high energy of the covalent C\\N bonds, DMPSA is somehow rapidly degraded to DMP in the soil (which would match with the half-life of 1.5–3.3 days registered in ECHA) and/or ii) DMPSA maintains its integrity and thus, its dose could be reduced in comparison to that of DMPP, as its chelation efficiency is higher according to the number of ligands per Cu^{2+} cation observed in XRD structures (Fig. 2).

CONCLUSIONS

This is the first demonstration of the capacity of DMPP and DMPSA to form complexes with Cu^{2+} cations. Although DMPSA is able to directly bond with Cu^{2+}, our results suggest that it might be degraded to DMP in soils. In any case, the nitrification inhibition displayed by these compounds seems to be driven by the interaction between them and Cu^{2+}, which is an essential cofactor for the activity of AMO. Moreover, there is evidence to suggest a relationship between DMP and Zn^{2+}, and also an essentiality of this element for AMO activity. Our results also indicate that N_2O-reducing bacteria growth seems to be limited by Cu availability. These bacteria are benefited by DMPs application, which might be due to a reduction in the competence when AOB growth is inhibited. In this manner, the reduction of N_2O to N_2 is promoted. Moreover, the addition of Cu and/or Zn to soils stimulates AOB growth, leading to an increase in N_2O emissions than can be counteracted by DMPs application. Therefore, further studies should be carried out in field conditions to consider the application of NIs when fertilizing with these micronutrients.

SUPLEMENTARY MATERIAL

Table S1. Crystallographic data for CuDMP1, CuDMP2 and CuDMPSA

	CuDMP1	**CuDMP2**	**CuDMPSA**
Formula	$C_{20}H_{32}Cl_2CuN_8$	$C_{20}H_{28}CuN_8O_4S$	$C_{18}H_{24}CuN_4O_9$
FW (g mol^{-1})	518.97	540.10	503.95
Crystal System	Triclinic	Monoclinic	Monoclinic
Space Group	*P*–1	*C*2/c	$P2_1/c$
a (Å)	7.3025(6)	26.548(4)	8.7385(4)
b (Å)	9.2925(6)	7.2600(3)	10.5590(3)
c (Å)	9.6423(8)	18.777(3)	11.4628(4)
α (˚)	90.356(6)	90	90
β (˚)	104.924(7)	134.99(3)	99.399(4)
γ (˚)	99.693(6)	90	90
V (Å^3)	622.37(9)	2559.6(11)	1043.48(6)
Z	1	4	2
ρ_{calcd} (g cm^{-3})	1.385	1.402	1.604
μ (mm^{-1})	1.116	0.976	1.106
Reflections Collected	3808	7414	7001
Unique Reflections (R_{int})	2251 (0.029)	2249 (0.027)	1843 (0.043)
Observed Reflections [$I>2\sigma(I)$]	1910	1785	1588
Parameters (Restraints)	146	182	165 (3)
$R(F)^a$ [$I>2\sigma(I)$]	0.040	0.069	-
$wR(F^2)^a$ [all data]	0.085	0.189	-
GoF	1.068	1.053	-

[a] $R(F) = \Sigma||F_o|-|F_c||/\Sigma|F_o|$; $wR(F^2) = \{\Sigma[w(F_o^2-F_c^2)^2]/\Sigma[w(F_o^2)^2]\}^{1/2}$

Table S2. Primers pairs and thermal conditions used in real-time qPCR

Target group	Primer name	Sequence	Thermal profile	bp lenght	References
Bacterial *16S rRNA*	341F	5'-CCTACGGGAGGCAGCAG-3'	95ºC for 2 min - x 1 cycle 95ºC for 15 sec 60ºC for 30 sec 72ºC for 30 sec 80ºC for 30 sec - x 40 cycles	174	Lopez-Gutiérrez et al., 2004
	534R	5'-ATTACCGCGGCTGCTGGCA-3'			
Bacterial *amoA*	amoA1F	5'-GGGGTTTCTACTGGTGGT-3'	95ºC for 2 min - x 1 cycle 95ºC for 15 sec 54ºC for 60 sec 72ºC for 60 sec - x 40 cycles	491	Rotthauwe et al., 1997
	amoA2R	5'-CCCTCKGSAAAGCCTTCTTC-3'			
nosZI	nosZ-F	5'-CGCRACGGCAASAAGGTSMSSGT-3'	95ºC for 2 min - x 1 cycle 95ºC for 15 sec 63ºC for 30 sec (-1ºC/cycle), 72ºC for 30 sec 80ºC for 15 sec – x 6 cycles 95ºC for 15 sec 60ºC for 30 sec 72ºC for 30sec 80ºC for 30 sec - x 40 cycles	267	Henry et al., 2006
	nosZ-R	5'-CAKRTGCAKSGCRTGGCAGAA-3'			

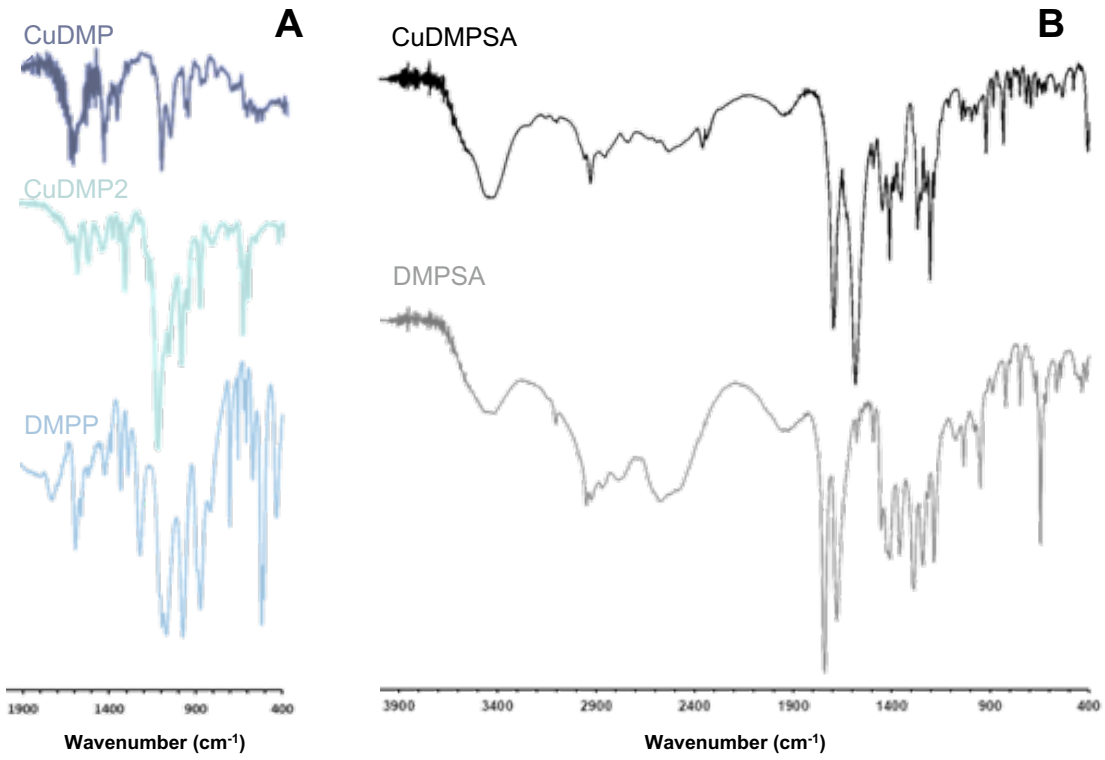


Figure S1. A) FT-IR spectra of CuDMP1, CuDMP2 and DMPP and B) CuDMPSA and DMPSA.

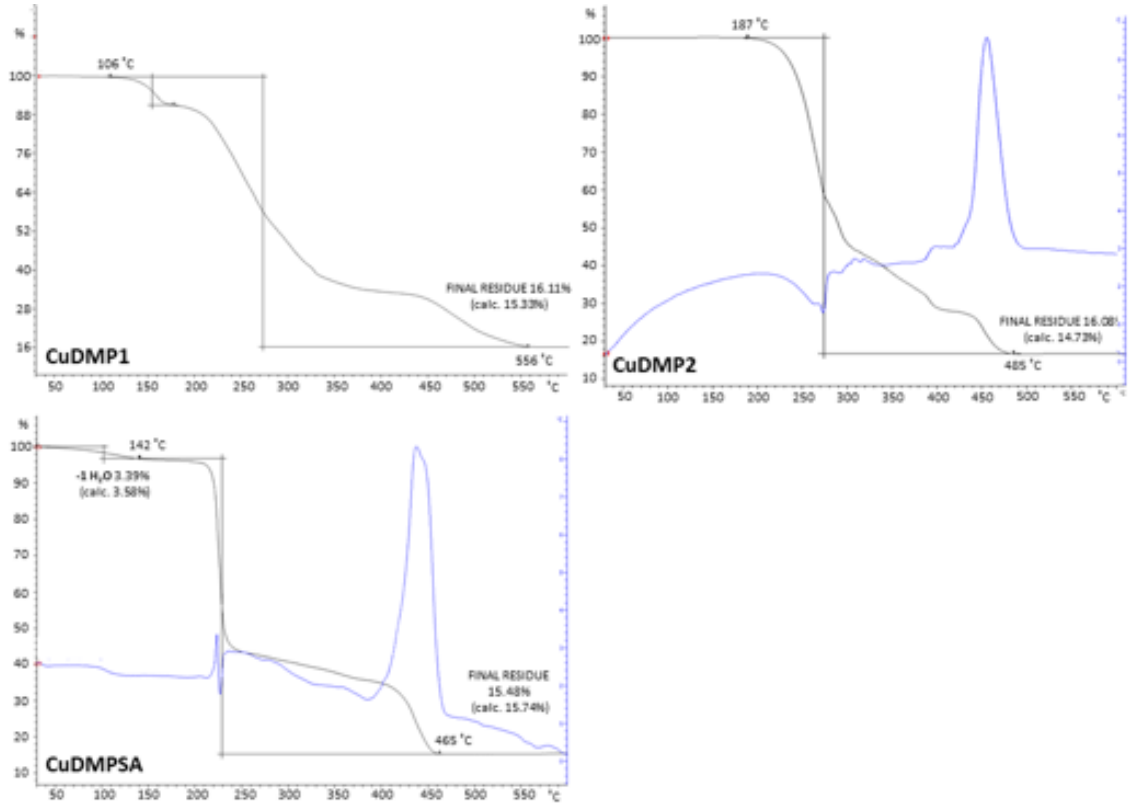


Figure S2. TGA curves for CuDMP1, CuDMP2 and CuDMPSA together with SDTA curves for CuDMP2 and CuDMPSA.

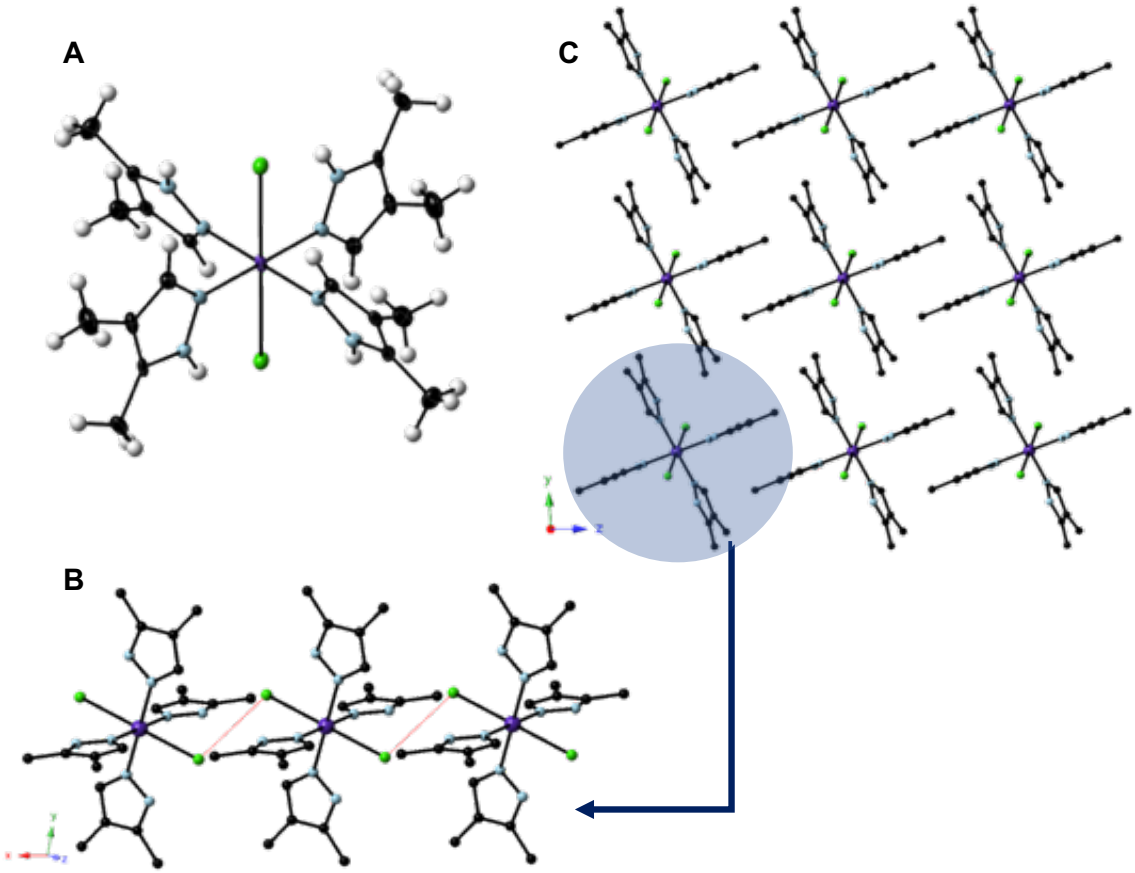


***Figure S3**. A) ORTEP view of CuDMP1 showing 50% probability displacement ellipsoids. B) Supramolecular one-dimensional arrangement running along the crystallographic x axis. The Cl···Cl contacts are represented as dashed red lines. C) View of the crystal packing along the crystallographic x axis.*

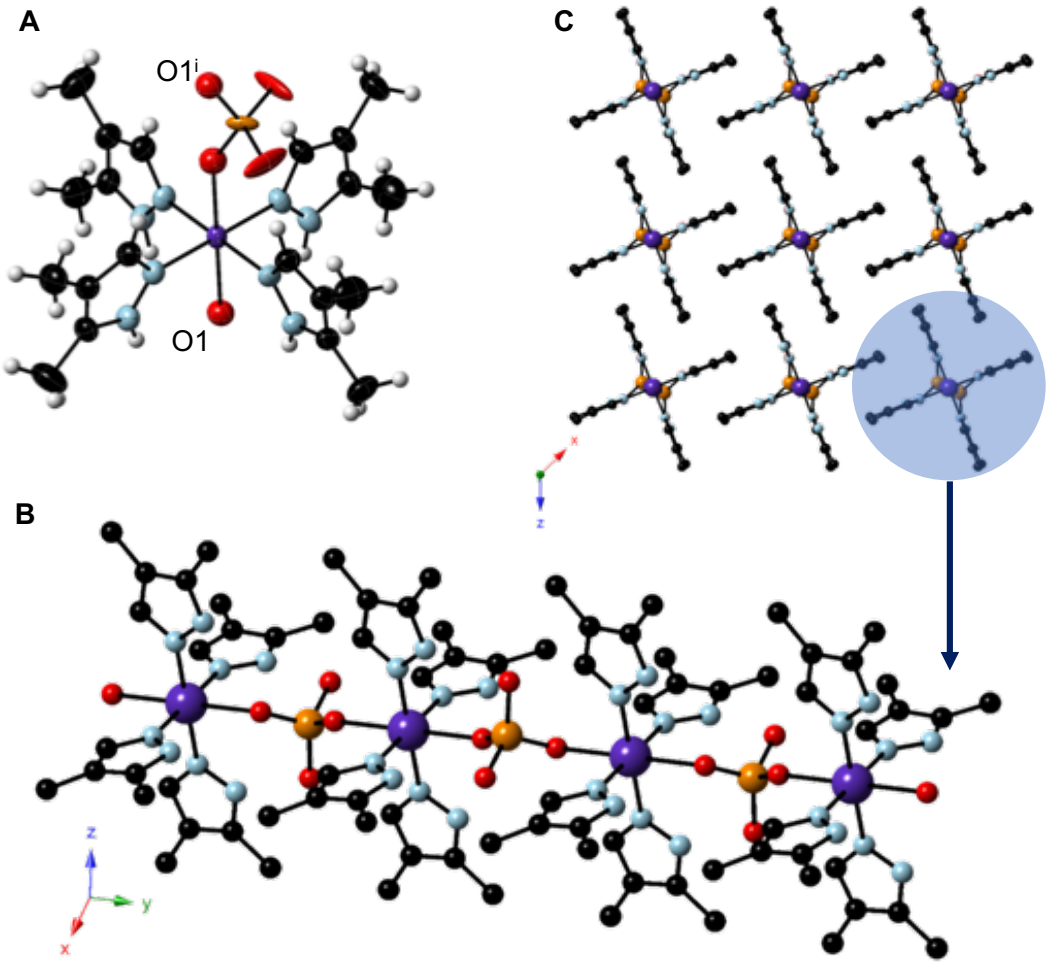


Figure S4. A) ORTEP view of CuDMP2 showing 50% probability displacement ellipsoids. B) Covalent chains running along the crystallographic y axis. C) View of the crystal packing along the crystallographic y axis. *Symmetry code: i) x, 1+y, z.*

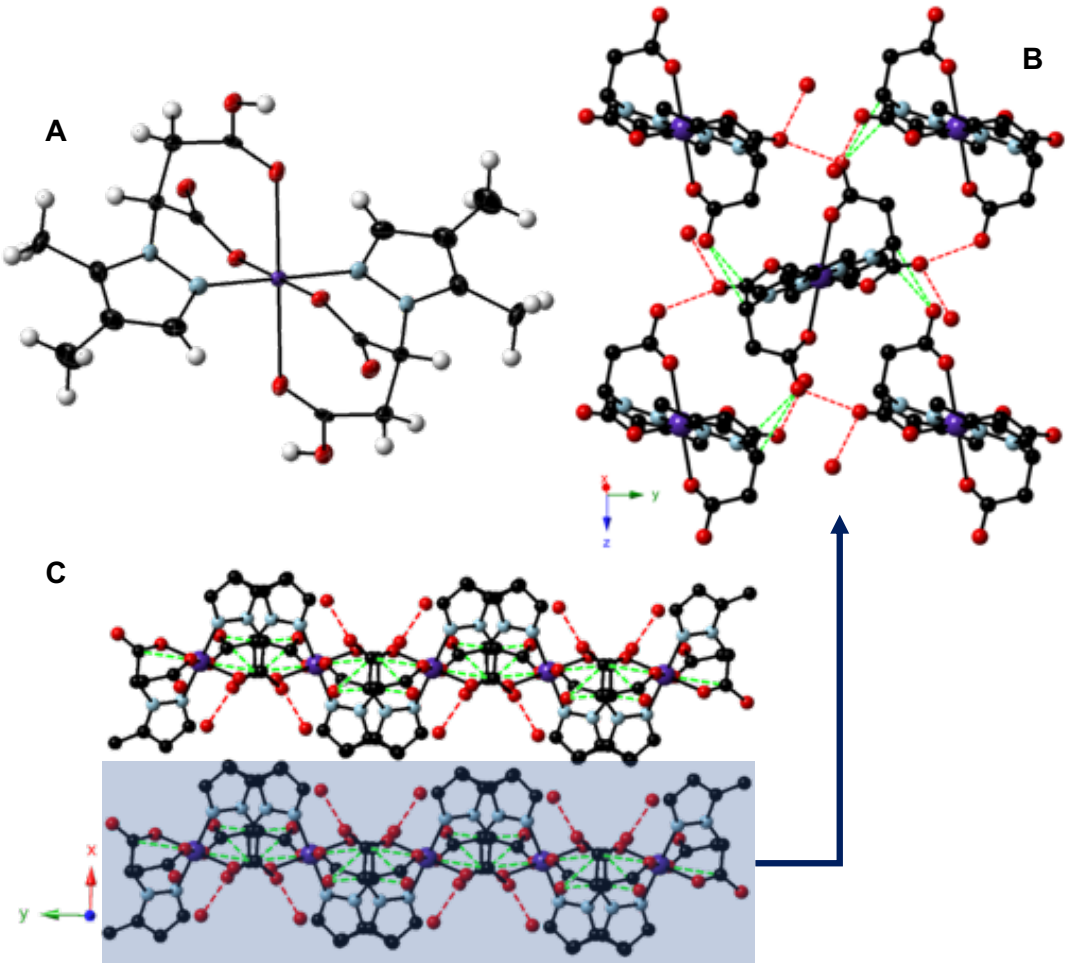


Figure S5. A) ORTEP view of CuDMPSA showing 50% probability displacement ellipsoids. B) Projection of the hydrogen-bonded two dimensional arrangement parallel to the yz plane. C) View of the crystal packing along the crystallographic z axis highlighting the stacking of the layers along the x axis*. The O–H·· O-type hydrogen bonds are depicted as red dashed lines; C–H·· O-type contacts as green dashed lines.*

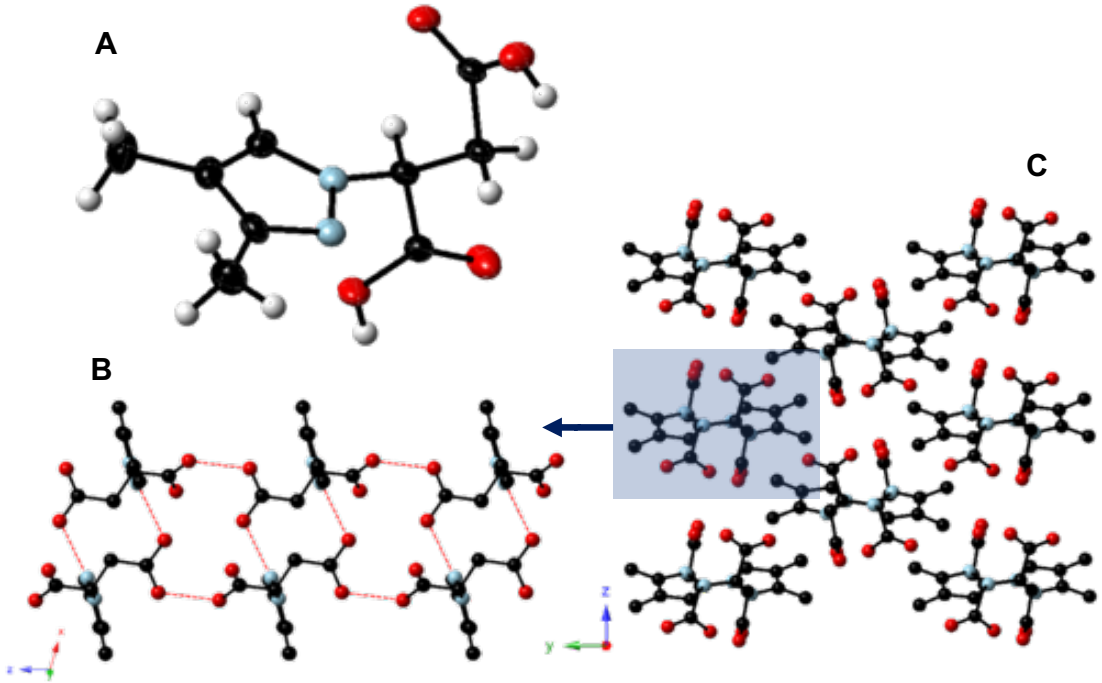


Figure S6. A) ORTEP view of DMPSA showing 50% probability displacement ellipsoids. B) Hydrogen-bonded double chains running along the crystallographic z axis. C) View of the crystal packing along the crystallographic x axis*. The O–H·· O and N–H·· O -type hydrogen bonds are depicted as red dashed lines.*

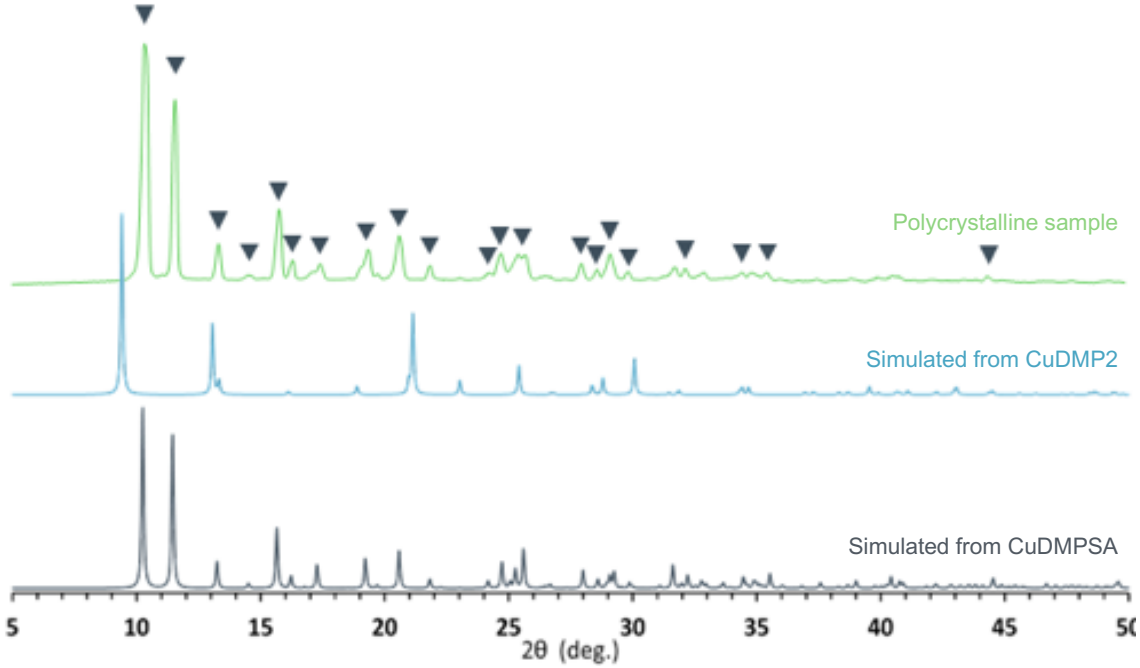


Figure S7. Powder XRD pattern of the polycrystalline sample formed in the competitive reaction between DMP and DMPSA (1Cu:2DMPP:2DMPSA) and its comparison with those simulated from single-crystal XRD data from CuDMP2 and CuDMPSA. *Arrowheads (▼) indicate positions of diffraction maxima that fit well with those of* ***CuDMPSA****.*

CHAPTER 4

Side effects on non-target bacteria

Unraveling DMPSA nitrification inhibitor impact on soil bacterial consortia under different tillage systems

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Finally, we had demonstrated that DMPP and DMPSA are able of chelating copper. These new findings reinforced the chances of some of our scenarios being correct; but did not allow us to completely rule out any of them.

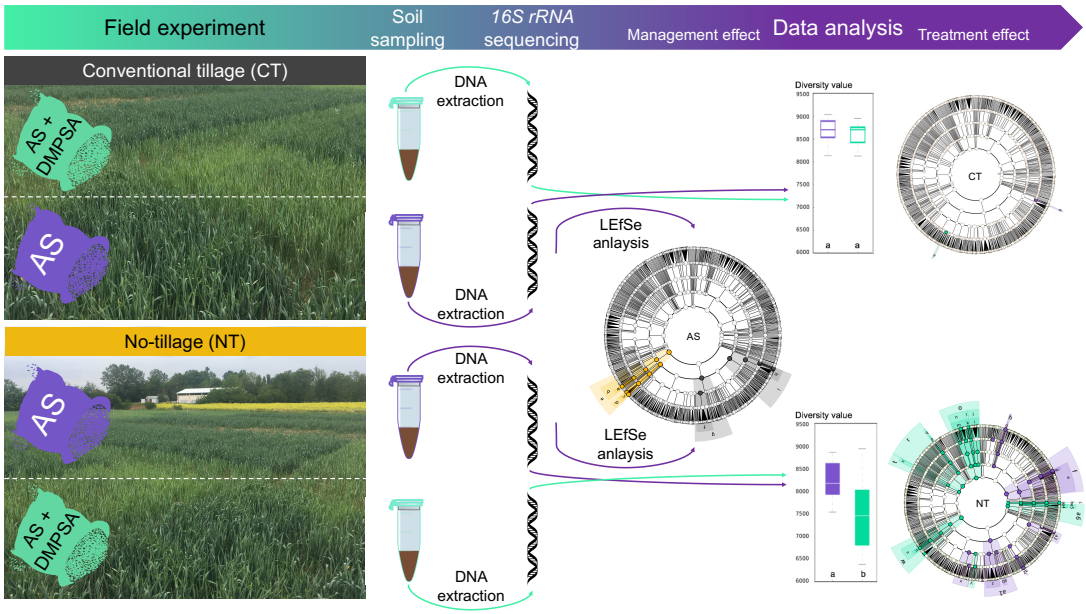
We should admit that this was just a first step but not the final answer, since we have not demonstrated unequivocally the mode of action. Even if the inhibition of nitrification is based on their capacity to chelate copper (and/or zinc?), we do not know how the action is exactly displayed. Among other options, copper atoms might be chelated directly in the soil and then cause a general decrease of availability and only some enzymes would have the ability to use them even when attached to an organic ligand. On the other hand, a more specific mechanism would be a direct bond to the copper of AMO within the active site; but it is complicated to think in the way of this mechanism to be so specific for this enzyme. Moreover, it could be even possible that the mode of action was not based on the chelation capacity. Therefore, although we had progressed in our objective, perhaps more new questions had been opened than solved.

One of the most interesting things about the chelation capacity of DMPSA is that many enzymes use copper as a cofactor. Thus, in the same manner that we observed an effect on complete denitrifiers, DMPSA may exert an impact on other organisms. But even beyond its chelation capacity, the higher NH_4^+ of the soil as a consequence of DMPSA might shift the bacterial community. Therefore, the next study should be a first approach to determine the non-target effects, considering the whole bacterial consortia and not only the organisms involved in nitrification and denitrification. To do so, soil samples from the experiment described in Chapter 1 were analyzed, giving us the possibility to go deeper into the dissimilar effect of DMPSA between tillage systems by analyzing what was happening at the bacterial level.

The amplicon sequencing technique that was used has some pitfalls. On the one hand, it is based on the amplification of a gene fragment based on universal primers. This step, as in any PCR-primer based investigation, can result in community biases where members may be omitted, distorted, and/or misrepresented, typically due to primer mismatches and preferential amplification. Moreover, the taxomic classification obtained

from the sequences is dependent on the diversity and accuracy of annotated sequences available in public repositories. Therefore, it is quite common in soil, due to its high diversity nature, not to be able to assign a high percentage of the reads farther than family/genera hierarchical taxonomic level, or even getting high number of unidentified reads. In addition, the microbial composition obtained represents relative abundances and not absolute values, in contrast to other quantitative techniques such as qPCR. However, the advantage of amplicon sequencing technique relies on the ability to analyze high numbers of samples at once, as well as obtaining a more holistic view of the entire microbial community (including abundant and rare taxonomic groups), rather than focusing into specific groups. In this work, this technique represented a great advance, since it allowed us to go much deeper into the effects of DMPSA on the microbiome and, furthermore, to identify many of the organisms whose abundances shifted due to NIs application.

GRAPHICAL ABSTRACT



ABSTRACT

Nitrogen (N) applied with fertilizers is not efficiently used in agriculture. In the soil, this N is transformed into different compounds by means of several biological processes. As a result, there is a negative economic and environmental impact due to water contamination, via nitrate (NO_3^-) leaching, and greenhouse gasses emission, via nitrous oxide (N_2O). To reverse this situation, nitrification inhibitors (NI) such as dicyandiamide (DCD), nitrapyrin and 3,4-dimethylpyrazole phosphate (DMPP) are widely applied to agricultural soils in order to delay ammonium (NH_4^+) transformation. A new NI, 3,4-dimethylpyrazole-succinic acid (DMPSA), has been recently developed with the aim of deploying a specific action on ammonium-oxidizing bacteria (AOB). However, previous studies have demonstrated that DMPSA application increases *nosZI*

gene abundance. Thus, non-target populations involved in N-cycle are also affected by its application. For better understanding the effects of DMPSA addition, this NI was applied with ammonium sulfate (AS) fertilizer in a winter wheat crop soil under Humid Mediterranean conditions, in two different soil tillage managements (conventional tillage, CT; and no-tillage, NT). Soil samples were then analyzed by *16S rRNA* amplicon sequencing. DMPSA application induced a decrease in bacterial alpha-diversity under the NT management, which showed higher water-filled pore space (WFPS) than the CT management. This suggests that water content played a key role in DMPSA effects. Even at the phyla level, the abundances of several non-target organisms, either involved or not in the N-cycle, were affected by DMPSA application. Within them, the biggest changes were found in Cyanobacteria (+48%) phylum (considered promising bio-agents for sustainable agriculture), which may have also triggered the increase of Bacteroidetes (+20%) and the decrease of certain phytopathogens. This decrease of phytopathogens may have also been helped by the great increase observed after DMPSA application in the genus *Vermamoeba vermiformis,* a protist known to control/regulate several soil-borne pathogens. Altogether, the results showed that DMPSA may lead to a reduction of the environmental impacts derived not only from the loss of reactive N, but also from the maintenance of a safer microbial community for plant health. However, further studies would be necessary to analyze the persistence and the consequences of all these effects in the long-term.

MATERIALS AND METHODS

2.1. EXPERIMENTAL DESIGN

This work was carried out with soils of a winter wheat crop (*Triticum aestivum* L., var. Cezanne) located in Arkaute, northern Spain (42°51'N, 2°37'W, 530 m above sea level) and sown on December 1st, 2016. Soil characteristics of the upper horizon (0-30 cm) are compiled in Supplementary Table S1.

To compare conventionally tillage (CT) and no-tillage (NT) managements, two randomized complete blocks designs were established with four replicates and an individual plot size of 40 m^2 (8 x 5 m). The plots were conditioned with a seedbed preparation consisting of mechanical tillage (disk and moldboard plow) for CT. For NT plots, spontaneous weeds were desiccated with glyphosate-based herbicide before direct sowing, thus the NT management being established for the first time in this growing season.

Within each design (CT and NT), two treatments were applied: ammonium sulfate (hereafter AS) and ammonium sulfate + DMPSA (hereafter ASD). Both were fertilized at the rate of 180 kg N ha^{-1} applied as ammonium sulfate 21% split in two applications of 60 kg N ha^{-1} at beginning of tillering stage (GS21) (Zadoks et al., 1974) and 120 kg N ha^{-1} at stem elongation stage (GS30). DMPSA was applied in ASD treatments in combination with ammonium sulfate 21%, as provided by Eurochem Agro Iberia S.L (rate of 0.8% of the NH_4^+-N content of fertilizer). Additionally, all plots were supplied with 85 kg ha^{-1} K (K_2SO_4) at GS21 stage.

Edaphoclimatic conditions along the whole crop season can be further consulted in Corrochano-Monsalve et al. (2020a).

2.1. SOIL WATER CONTENT DETERMINATION

Two soil samples (3 cm diameter x 30 cm depth) were collected randomly from each tillage system every two days along two weeks after fertilizer application. Sampling

was carried out two days per week in the remaining weeks. Rocks were removed and soil was oven-dried during 48 h at 80 ºC. Soil water content was calculated as the percentage of water-filled pore space (WFPS) as in Linn and Doran (1984): WFPS = (soil gravimetric water content x bulk density) x $(1 - (\text{bulk density} / \text{particle density}))^{-1}$, by using a particle density of 2.65 Mg m^{-3} and bulk densities determined for each tillage management resulting in values of 1.13 Mg m^{-3} and 1.35 Mg m^{-3} for CT and NT, respectively. WFPS value for each sampling day has been considered as the average WFPS of the previous 15 days.

2.2. SOIL SAMPLING AND DNA EXTRACTION

Three soil subsamples (3 cm diameter x 30 cm depth) were collected randomly and then mixed from each individual plot of each of three of four replicates. Samplings were carried out 8 and 19 days after the first fertilization, and 12 and 31 days after the second fertilization. In total, 48 samples were collected: 24 samples in CT (12 samples from AS treatment and 12 from ASD) and 24 in NT (12 from AS and 12 from ASD). After homogenization, DNA was extracted from 0.35 g FW of soil 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 and final elution incubation was performed as described by Harter et al. (2014).

Extracted DNA concentration and quality were determined by spectrophotometry with a NanoDrop® 1000 (Thermo Scientific, Walthman, MA, USA).

2.3. LIBRARY CONSTRUCTION AND DNA SEQUENCING

The V4 region of the bacterial *16S rRNA* gene was amplified with the primers 515F/806R as described by Caporaso et al. (2012) including Illumina barcodes and sequencing adaptors. The following PCR conditions were used for amplification: initial denaturation at 95 ºC for 4 min, 35 cycles at 95 ºC for 15 s, 50 ºC for 30 s and a final extension of 72 ºC for 30 s. PCR products were examined by electrophoresis in 1%

agarose gel. Amplicon purification was performed with the CleanPCR kit (Cleanna), using magnetic beads. Samples were quantified by Qubit™ v2.0 (ThermoFisher Scientific) and normalized in a 8 pM pool. A paired-end sequencing of the pool was carried out with the kit v3 PE 2 x 150bp (600 cycles) on an Illumina MiSeq modified to run for 300 cycles at the Sequencing and Genotyping Unit of the University of the Basque country (SGIKER).

2.4. QUALITY CHECKING, PROCESSING AND TAXONOMIC ASSIGNMENT

Forward and reverse raw sequences were quality checked with Sickle v1.33 (Joshi & Fass, 2011) using default parameters including a Phred score ≥ 20. The assembly of the pair-end sequences was conducted with Pear v0.9.10 (Zhang et al., 2014), with an overlap of 15 bp. Fastq-barcode.pl script (Smith, 2012) was used to remove non-existent (non-assembled) barcodes from the fastq files obtained in Pear. Seq_filter.pl was used to eliminate sequences by length, keeping sequences with a min and max length of 205-295 bp to avoid background noise in the subsequent analyses. An open reference OTU picking method was used in QIIME v1.9. OTUs were clustered against GreenGenes 13_8 at the 97% similarity level using uclust (Edgar 2010). OTU sequences were aligned using PYNAST (Caporaso et al., 2010a) and the ones failing to align were discarded. OTU taxonomy was determined using the RDP classifier (Wang et al., 2007) retrained towards the GreenGenes13_8 database (97% similarity). A final OTU table was created, excluding unaligned sequences and OTUs with less than 10 sequences. Finally, the OTU table was normalized using metagenomeSeq's CSS algorithm, which normalized sequences using the cumulative sum scaling transformation (Paulson et al., 2013).

2.5. STATISTICAL ANALYSIS

Statistical evaluation of the data was carried out with QIIME v1.9 (Caporaso et al., 2010b), SPSS (IBM SPSS Statistics for macOS, version 25.0. Armonk, NY: IBM Corp) and R version 3.1.2 (R core team, 2013) using Rstudio version 1.1.463 (Rstudio

Team, 2016). Soil samples community richness and evenness were calculated through Chao1 and Shannon indices, respectively, rarifying the original (not normalized by CSS) OTU table to 33000 sequencing depth. Student-T test and one-way ANOVA with Duncan's multiple-range test for separation of means ($P < 0.05$) were employed to test the differences in alpha diversity measurements depending on the variables tested in this study ("management practice", "WFPS" and "treatment"). Kruskal-Wallis test with Bonferroni correction ($P \leq 0.01$) was applied to the normalized OTU table to identify taxa whose relative abundances significantly differed between management practice: CT vs NT. Linear discriminant analysis effect size (LEfSe) method (Segata et al., 2010) was used to identify biomarkers of the different treatments (AS or ASD) within each management system (LDA > 2.0; $P < 0.05$). Additionally, Mann-Whitney-U test was applied to identify N-cycle taxa whose relative abundances differed between treatments (AS vs ASD) and/or managements (CT vs NT).

Community dissimilarity between samples was assessed with Bray-Curtis index and ANOSIM test was used to address whether microbial community composition changed significantly according to the exploratory variables "management practice" and "treatment."

RESULTS

3.1. BACTERIAL COMMUNITY DIFFERENCES BETWEEN SOIL MANAGEMENTS

3.1.1. SOIL MANAGEMENT EFFECT ON ALPHA DIVERSITY

Significant lower alpha-diversities were observed in NT than in CT (Chao1= 8654, CT; 7858, NT and Shannon= 10.51, CT; 10.41, NT) (Student-T test; $P = 0.001$) (Fig. 1, A^1, B^1). WFPS difference between CT and NT would explain this. In fact, the highest WFPS values were reached in NT coupled to the lowest alpha diversity values.

Analyzing the range of variation of the WFPS values in this study we found that, as they increased, alpha-diversity decreased in richness (Fig. 1, A^2) and evenness (Fig. 1, B^2) (Duncan, $P < 0.05$).

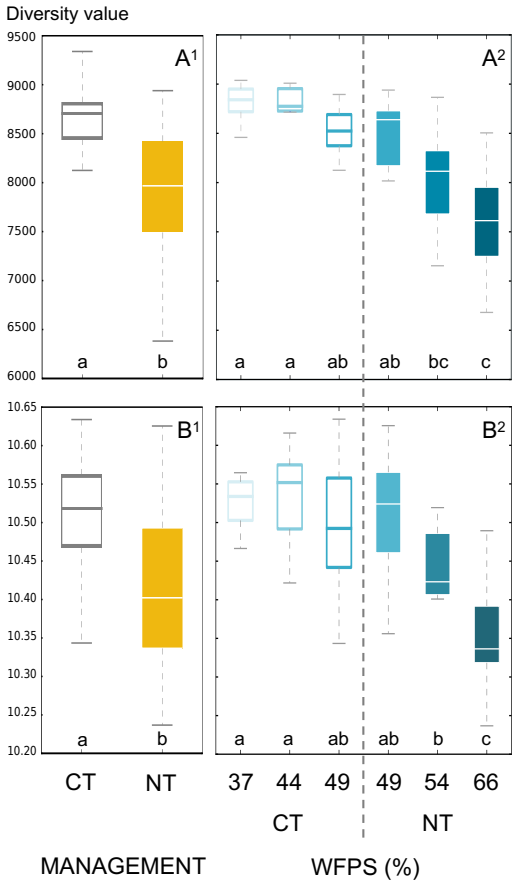


Figure 1. Alpha diversity Chao1 (A) and Shannon (B) indices by ([1]) management system and ([2]) water-filled pore space (WFPS). *Different letters indicate significant differences (Student-T test, P = 0.001; management) (Duncan, P < 0.05; WFPS). WFPS value for each sampling day has been considered as the average WFPS of the previous 15 days. CT = Conventional Tillage; NT = No- Tillage.*

3.1.2. SOIL MANAGEMENT EFFECT ON COMMUNITY COMPOSITION

ANOSIM analysis revealed that soil community composition differed between CT and NT managements (ANOSIM for both treatments $R = 0.50$; $P = 0.001$) (Table 1). Moreover, the treatment factor (AS or ASD) affected differently in CT or NT. Assuming that common agricultural practices rely on the application of fertilizer without

nitrification inhibitors, further analysis to explore microbial composition differences between the two management systems (CT vs NT) were conducted on AS samples (ANOSIM $R = 0.33$; $P = 0.001$ (CT-AS *vs.* NT-AS).

Table 1. Differences in beta diversity of bacterial community based on analysis of similarity (ANOSIM) test (Bray-Curtis distances)*. P-values are based on 999 permutations.*

Factor	Groups	*R*	*P*-value
Management	All (AS + ASD)	0.50	0.001
Management	AS	0.33	0.001
Management	ASD	0.77	0.001
Treatment	All (CT + NT)	0.07	0.024
Treatment	CT	-0.01	0.548
Treatment	NT	0.28	0.001

CT = Conventional tillage; NT = No-tillage; AS = ammonium sulphate 21%; ASD = ammonium sulphate 21% + DMPSA

The most abundant phyla found in AS treated soils were Proteobacteria, Actinobacteria, Acidobacteria, Planctomycetes, Bacteroidetes, Chloroflexi, Gemmatimonadetes, Verrucomicrobia, Firmicutes, Thaumarchaeota and Nitrospirae (Fig. 2). LEfSE analysis showed that the main differences between CT and NT were located in phylum Planctomycetes and orders Burkholderiales, Xanthomonadales and WD2101 ($P < 0.05$) (Figs. 3 and S1).

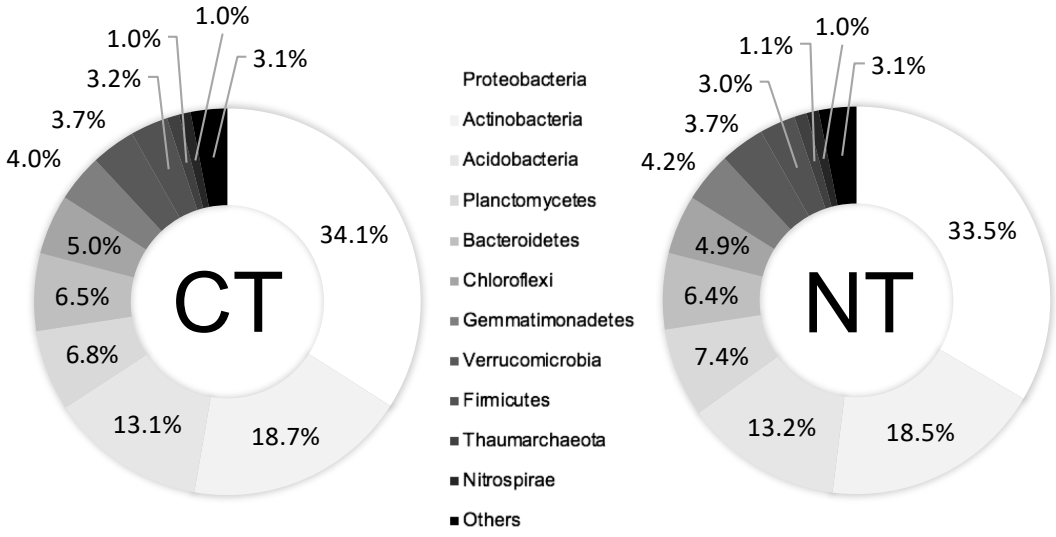


Figure 2. Soil bacterial taxonomic distribution at the phylum level for each management system within AS treatment*. CT = Conventional Tillage; NT = No-Tillage.*

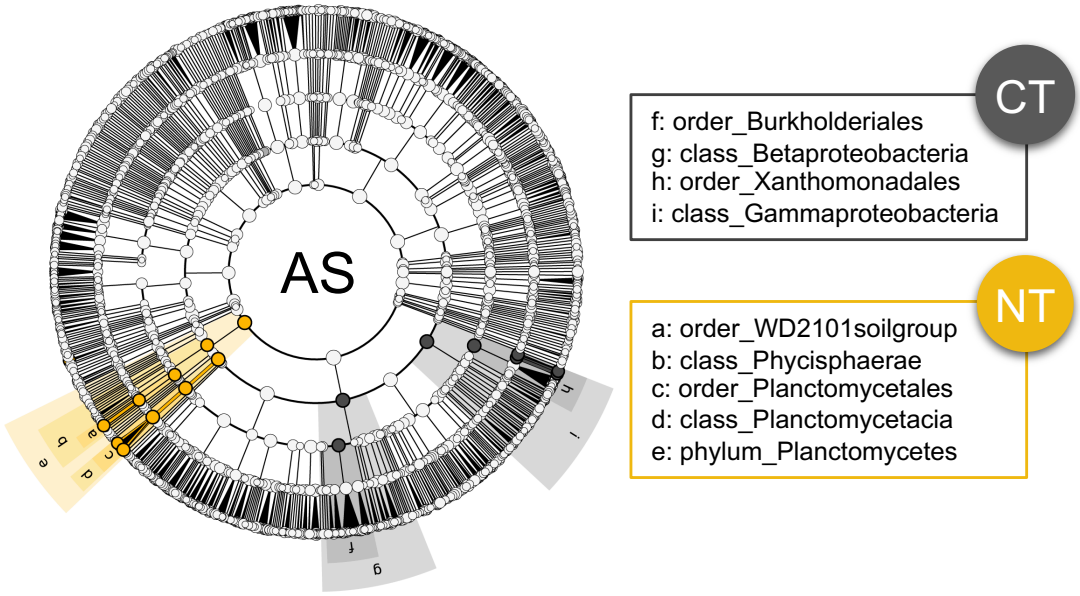


Figure 3. LEfSe analysis of bacterial abundance within AS treatment. *Colored circles represent differentially abundant bacteria among managements (LDA score > 2.0; P < 0.05). Uncolored circles represent bacteria with no statistical differences. Tags have been included only for certain levels; the rest of taxonomic levels can be consulted in Supplementary Fig. S1. CT = Conventional Tillage; NT = No-Tillage.*

The relative abundance of bacteria known to participate in N-cycle was around 4% in both managements, being denitrifiers more abundant than nitrifiers in all cases (Figs. 4 and S7). When comparing CT and NT within AS treatment, nitrifiers did not show differential abundances (Mann-Whitney-U test; $P < 0.05$) (Fig. S3), while complete denitrifiers (taxa with *nosZI* and *nosZII genes*) tended to be more abundant in NT (Figs. S4 and S5).

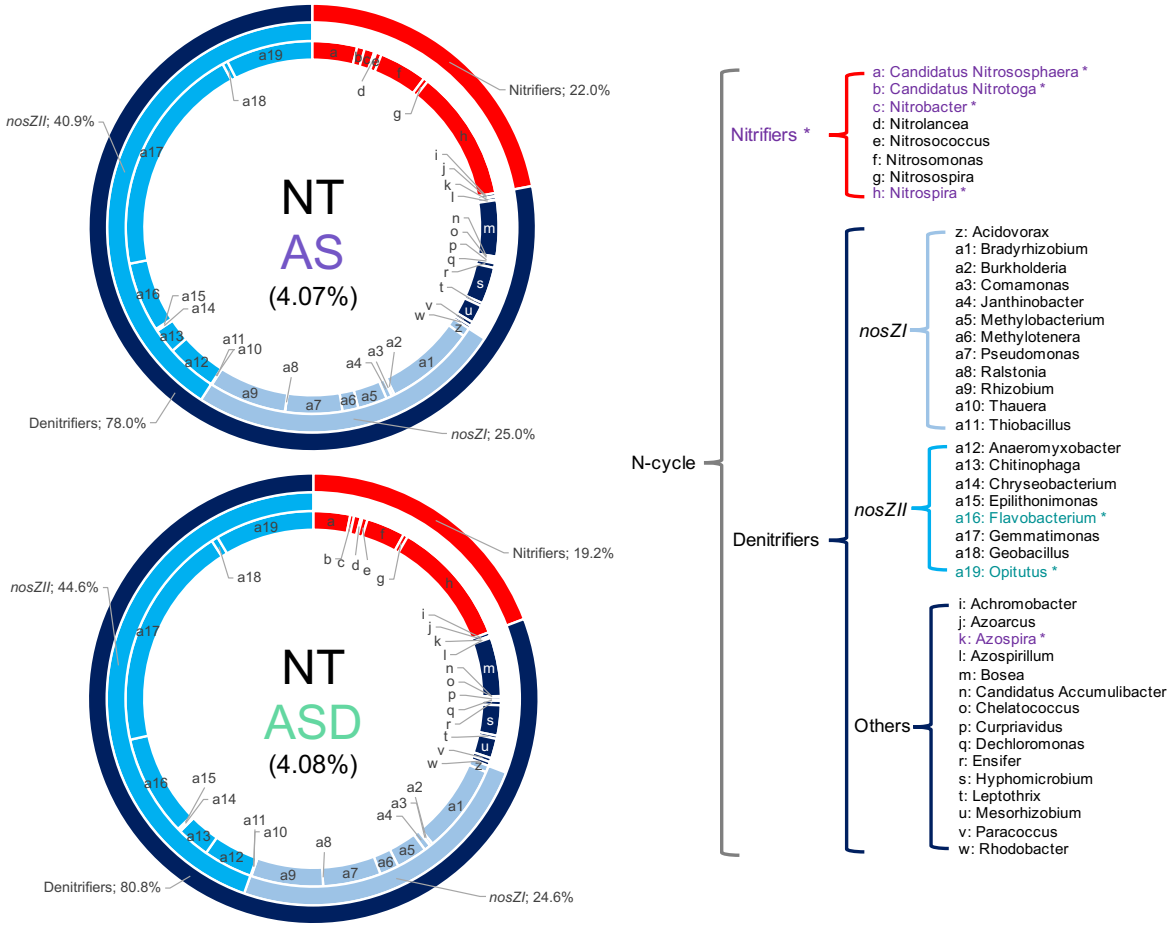


***Figure 4*. Distribution of identified N-cycle genera within NT.** *Total N-cycle bacteria relative abundance is indicated for each treatment in parenthesis. Significant differences between treatments are indicated with asterisks (*) (Mann-Whitney-U; $P < 0.05$). Purple text indicates significantly higher abundance in AS; Green text indicates significantly higher abundance in ASD. NT = No-Tillage; AS = ammonium sulphate 21%; ASD = ammonium sulphate 21% + DMPSA.*

3.2. COMPARISON OF BACTERIAL COMMUNITY DIVERSITY AND STRUCTURE BETWEEN FERTILIZER-TREATMENTS

3.2.1. DMPSA EFFECTS ON BACTERIAL DIVERSITY

The impact of DMPSA on bacterial diversity varied according to the soil management system (Fig. 5). CT system's soil diversity did not change with DMPSA application, while it reduced the alpha-diversity under the NT system. In fact, in NT soils richness decreased significantly (Chao1 = 8230 in AS *vs*. Chao1 = 7485 in ASD) (Duncan; $P < 0.05$) (Fig. 5, A), while evenness values also declined although not significantly (Shannon index = 10.43 in AS *vs*. Shannon index = 10.40 in ASD) (Fig. 5, B). Moreover, DMPSA enhanced richness differences between CT and NT, as higher differences in mean Chao1 values were detected between managements within ASD treated soils in comparison to AS soils (CT-ASD *vs*. NT-ASD, $P = 0.012$; CT-AS *vs*. NT-AS, $P = 0.054$; Student-T test).

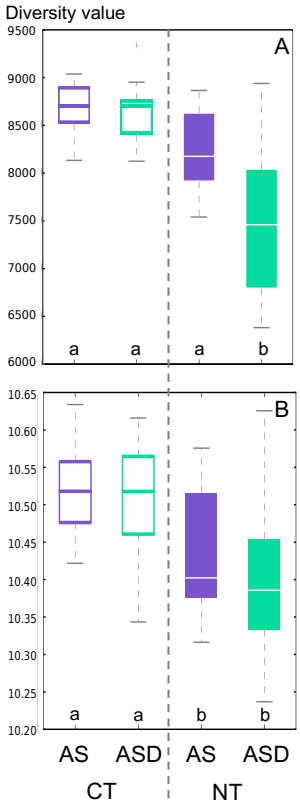


Figure 5. Alpha diversity Chao1 (A) and Shannon (B) indices by treatment. *Different letters indicate significant differences (Duncan, P < 0.05). CT = Conventional Tillage; NT = No-Tillage; AS = ammonium sulphate 21%; ASD = ammonium sulphate 21% + DMPSA.*

3.2.2. DMPSA EFFECTS ON COMMUNITY COMPOSITION

When taking into account the combined dataset including CT and NT samples, none of the phyla was identified to be significantly affected by DMPSA application, and only *Vermamoeba vermiformis* was significantly enriched in the DMPSA treatment at genus level (Kruskal-Wallis, Bonferroni; $P = 0.007$) (data not shown).

Similar to alpha-diversity results, DMPSA enhanced community composition differences between CT and NT (ANOSIM for ASD $R = 0.77$ (CT-ASD *vs*. NT-ASD); ANOSIM for AS $R = 0.33$ (CT-AS *vs*. NT-AS) (Table 1). Results evidenced that both systems responded in a very different manner to the inhibitor application. Furthermore, the application of DMPSA induced significant changes only within the NT management system ($R = 0.28$; $P = 0.001$) while no significant difference was observed within the CT management ($P = 0.548$) (Table 1).

The latter was also evidenced when carrying out LEfSE analysis, which showed almost no effect of DMPSA application in CT soil's microbial composition, while various taxa abundances were statistically differentiated between AS and ASD in NT soils ($P < 0.05$) (Figs. 6 and S2) even at phyla level. In NT, Acidobacteria were enriched in soils without DMPSA application (AS), while Bacteroidetes, Chloroflexi, Cyanobacteria, Planctomycetes and Verrucomicrobia were enriched in soils that received the inhibitor (ASD). Among them, DMPSA induced the biggest abundance changes in Cyanobacteria phylum (an abundance increase of 48%) and Bacteroidetes (+20%). Within Cyanobacteria, SubsectionIII (+91%) and Vampirovibrionales (+62%) were the most enriched orders. Similarly, Flavobacteriales (+41%) and Sphingobacteriales (+22%) were the orders showing the highest abundance differences within Bacteroidetes.

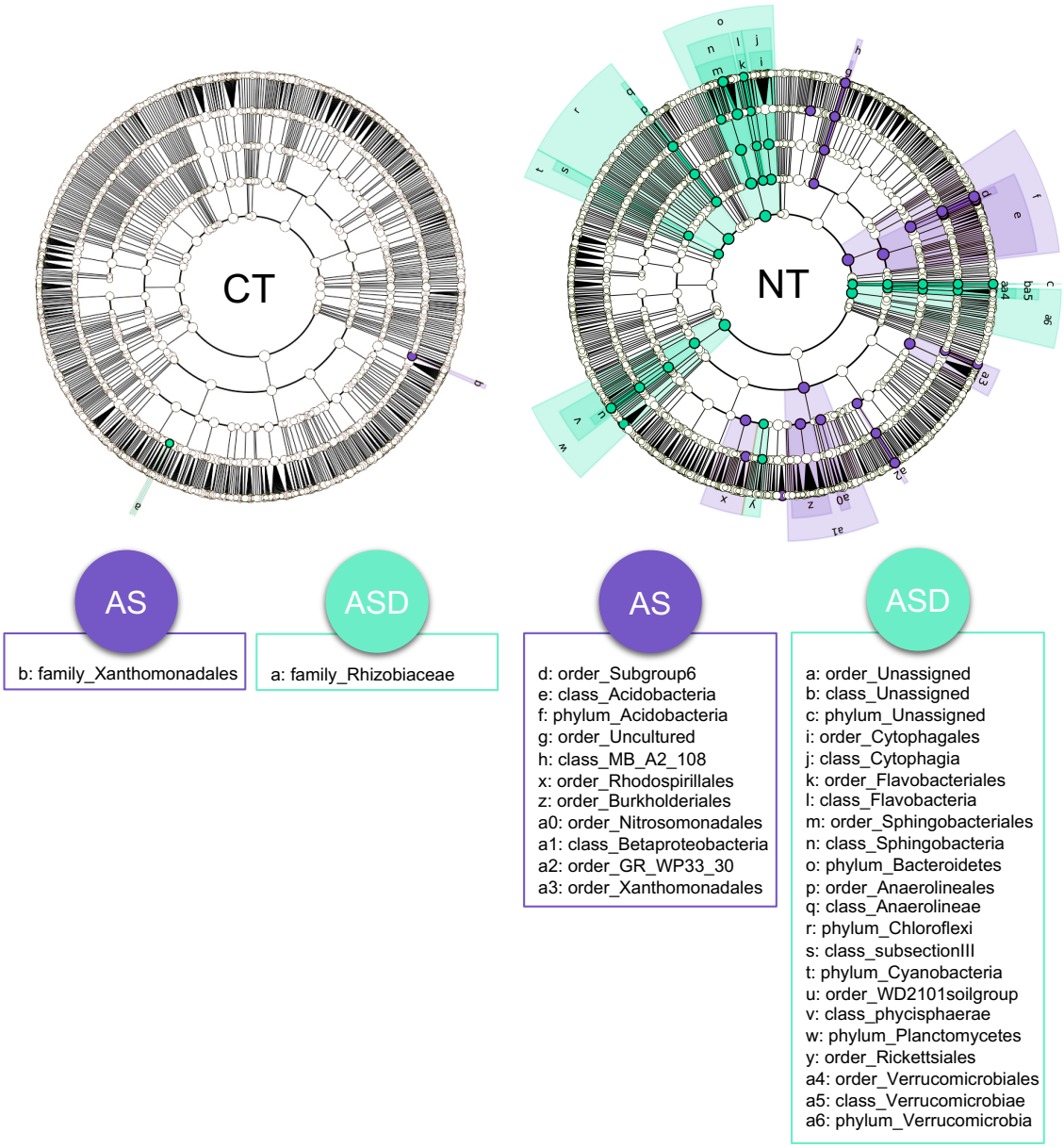


Figure 6. LEfSe analysis of bacterial abundance. *Colored circles represent differentially abundant bacteria among treatments (LDA score > 2.0; P < 0.05). Uncolored circles represent bacteria with no statistical differences. Tags have been included only for certain levels; the rest of taxonomic levels can be consulted in Supplementary fig. S2. CT = Conventional tillage; NT = No-tillage; AS = ammonium sulphate 21%; ASD = ammonium sulphate 21% + DMPSA.*

Xanthomonadales were over-represented in both CT and NT management within AS treated soils and thus depleted with DMPSA application. In total, 26 genera of Xanthomonadales were detected. Of them, 8 genera showed an abundance decrease of more than 10% in CT-ASD treatment, being the average reduction within this 8 genera group of 37%. *Pseudofulvimonas* (–71%) and *Luteimonas* (–69%) were the genera with the highest abundance shift, both members of the Xanthomonadaceae family (data not shown). 9 genera showed an abundance reduction of more than 10% in NT-ASD, being the average reduction of this 9 genera of 28%. *Luteibacter* (–81%) and *Stenotrophomonas* (–35%) were the most shifted genera (data not shown), both also members of the Xanthomonadaceae family.

DMPSA-target bacteria (AOB population) were negatively affected by its application, as demonstrated by the decline in the relative abundances of nitrifiers in both NT and CT soils (Figs. 6 and S7). This was particularly evident for *Nitrosomonas* genera, although its reduction was statistically significant only in CT (Mann-Whitney U; $P < 0.05$) (Fig. S7).

Interestingly, DMPSA also shifted the abundances of other genera involved in the N-cycle in both managements, with several non-target taxa abundances showing either an increase or decrease. Within nitrifiers, AOA *Candidatus Nitrososphaera* abundance was statistically reduced with DMPSA application in NT soils. Similarly, NOB genera *Candidatus Nitrotoga, Nitrobacter* and *Nitrospira* showed a decrease in their abundances in NT (Fig. 4). It is remarkable that, apart from nitrifiers, several other N-cycle genera abundances were also shifted. In fact, total N-cycle relative abundance declined with DMPSA application in CT (Mann-Whitney U; $P = 0.1$) (Fig. S7). DMPSA's impact on other N-cycle bacteria, apart from nitrifiers, also differed between managements. In this sense, while the abundance of *nosZII* holding taxa was stable regardless the fertilization treatment in CT (40.7% in AS *vs.* 40% in ASD) (Fig. S7), DMPSA induced an abundance increase of this group under NT management (*nosZII* holding taxa abundance was 40.9% in AS and 44.6% in ASD) (Mann-Whitney U, $P = 0.1$) (Fig. 4), being *Flavobacterium* and *Opitutus* the main responsible for this increase.

DISCUSSION

4.1. NO-TILLAGE DECREASED RICHNESS AND EVENNESS

Previous works have shown that different soil management practices can exert changes in bacterial diversity, although the trends of these changes have been variable. Most studies have indicated that NT management increases richness (Chao1 and/or observed species indices) and evenness (Shannon index) (Silva et al., 2013; Miura et al., 2016; Dong et al., 2017), while others as Pastorelli et al. (2013) reported lower soil microbial diversities under NT soil systems. Our results in this particular experimental site clearly showed a reduction of soil bacterial richness (Fig. 1, A^1) and evenness (Fig. 1, B^1) in NT soils, which hold significantly higher average WFPS (44.3% CT *vs.* 57.6% NT) along the whole sampling period. Interestingly, a negative relationship between WFPS and alpha-diversity was evidenced in this study. Richness and evenness tended to decrease with higher WFPS values, and a pronounced loss of biodiversity was found when water content increased up to 66% WFPS (Fig. 1 A^2, B^2). According to the pore connectivity theory, low water content would promote spatial isolation of bacterial communities, then allowing less competitive organisms to persist (Tiedje et al., 2001; Treves et al., 2003). CT soils in the present experiment would be characterized by lower pore connectivity than NT soils, as they remained in an average WFPS of 44.3% in all the sampling period, which is under the previously proposed threshold of 56% WFPS below which soil pores become isolated and diversity tends to increase (Carson et al. 2010). Therefore, the lower pore connectivity of CT soils might have provided opportunities for less competitive organisms to colonize and grow (Zhou et al. 2002, 2004) promoting higher alpha-diversities in these soils.

Both soil managements presented a similar bacterial-community structure, overall, at high taxonomic ranks, although they differed slightly in the abundance of phyla Proteobacteria (Fig. 2) and statistically in Planctomycetes (Fig. 3). Proteobacteria is the largest and most diverse bacterial group taking part in carbon (C), sulfur and N cycles

(Kersters et al., 2006). Larger abundances of Proteobacteria under CT management have been previously associated with the positive correlation between β-Proteobacteria abundance and the C addition (Fierer et al., 2007) resultant from the incorporation of plant residues into the soil with plowing (Souza et al., 2013). In line with it, in this study β-Proteobacteria showed higher significant abundances in CT soils (+12%). However, Dong et al. (2017) showed a contrary result (more abundance of Proteobacteria under NT management). Planctomycetes phylum seems to be adapted to anoxic environments (Derakshani et al., 2001; Fuerst and Sagulenko, 2011), which would give them an advantage to live in the higher water contents of NT soils studied here. Similarly, the more anoxic conditions of the NT system could also explain why complete denitrifiers abundances (*nosZI* and *nosZII*) tended to be higher in NT than in CT (Mann-Whitney U; $P = 0.1$) (Figs. S4 and S5). However, this trend was not followed by the *nosZI* holding Burkholderiales (Fig. 3), a free-living diazotroph group (Graf et al., 2019).

4.2. DMPSA APPLICATION EXERTED DIFFERENT RESPONSES DEPENDING ON TILLAGE MANAGEMENT

Corrochano-Monsalve et al. (2020a) showed that the reduction of N_2O emissions by DMPSA was more pronounced under NT than CT. Interestingly, while CT and NT microbial alpha (Fig. 5) and beta (Table 1) diversities were similar under AS treatment, the present work assessing the overall bacterial community, show a different response of bacterial populations to DMPSA application depending on the management. This result confirmed our hypothesis that the different efficiency of DMPSA to reduce N_2O emissions in each management system is related to a dissimilar response of the soil bacterial community to the inhibitor (i).

DMPSA did not exert any change in alpha-diversity in the case of CT soils (Fig. 5), while it is remarkable that bacterial richness decreased in NT when DMPSA was applied (ASD soils) (Fig. 5, A). The observed impact in NT is not in concordance with other studies where different NIs were applied. For instance, analysis with an alternative DMP-based NI (3,4-dimethylpyrazole phosphate, DMPP) has reported no effect on

alpha-diversity under laboratory conditions (Zhang et al., 2017). Similarly, Suleiman et al. (2016) did not found effects of dicyandiamide (DCD) on bacterial alpha-diversity in a field experiment.

The trend observed in richness was also confirmed in terms of community composition (Table 1 and Fig. 6). Our results clearly show that DMPSA shapes microbial composition differently between management practices. The bacterial community composition of NT soils that received AS treatment was statistically different to those that received ASD treatment, but this was not the case in CT soils, suggesting a higher impact of the inhibitor under NT soils.

Order level is believed to be a good indicator of the response of bacterial communities to a stressor (Salis et al., 2017). In this sense, in NT 8 orders showed higher abundances in ASD treatment, while other 7 different orders were enriched in AS (Fig. 6). This ratified that, aside from the AOB population, non-target organisms were also affected by DMPSA application, thus confirming our hypothesis (ii). The taxa enriched in soils that received DMPSA included several groups: Verrucomicrobiales order encompasses from strict aerobes organisms up to strict anaerobes (Janssen et al., 2011), with some genera that seem to be relatively abundant in anoxic soils carrying out fermentation metabolism (Schlesner et al., 2006). Almost no information is available about the ecology of other enriched orders such as WD2101 (now renamed as Tepidisphaerales) –heterotrophic aerobes or facultative anaerobes (Kovaleva et al., 2015)– and Rickettsiales –obligate intracellular bacteria usually associated to arthropods (Yu and Walker, 2006)–.

Several other groups were enriched in soils that did not receive the DMPSA inhibitor (AS treatment). For instance, Rhodospirillales, a photoheterotroph that includes anaerobic and aerobic families (Garrity et al., 2005). It is to be highlighted that Xanthomonadales was the only taxa overrepresented in both CT and NT management within AS treated soils (Fig. 6). Interestingly, many taxa of this group, particularly members of the Xanthomonadacea family, are phytopathogens (Ryan et al., 2011). Furthermore, we found that *Vermamoeba vermiformis* was the only genus enriched by

DMPSA in both managements (+662% in CT and +1756% in NT) (data not shown). Although this amoeba can harbor pathogenic bacteria and viruses (Delafont et al., 2018), it also secretes bactericide or bacteriostatic compounds. Interestingly, this genus has been reported to be bactericide against the rice pathogens *Xanthomonas oryzae* and *oryzicola* (Long et al., 2018). Therefore, the decrease in the relative abundance of Xanthomonadales could have been a consequence of the increase of *Vermamoeba vermiformis*. These results highlight the need of conducting further analysis to unravel the interactions of DMPSA and putative pathogenic organisms.

4.2.1. RESPONSE OF N-CYCLE TAXA TO DMPSA

DMPSA is believed to deploy a specific action on AOB population, delaying NH_4^+ conversion to NO_3^-. In the present study, as expected, nitrifiers were negatively affected by its application, in both CT and NT soils. Nitrosomonadales order, which includes AOB, were more abundant under AS treatment and depleted in soils treated with DMPSA (Fig. 6). Further analysis within this order showed that 3 genera were identified (*Nitrosomonas, Nitrosospira* and *Nitrosococcus*) (Figs. 4 and S7). The neutral-basic characteristics of the soil from the present work are more adequate for *Nitrosomonas* (which showed the highest abundance within AOB genera), while *Nitrosospira* is more adapted to acid environments (Li et al., 2017). While nitrifiers' population decreased in both CT and NT soils, within AOB, only *Nitrosomonas* abundance was statistically lower in CT (Fig. S7). On the other hand, although this effect was only observed under NT, it is interesting to highlight that AOA *Nitrososphaera* abundance decreased with DMPSA application. This result shows that DMPSA might influence AOA populations, which deserves to be further investigated, since previous results assessing DMPSA impact (Torralbo et al., 2017), or other nitrification inhibitors (Shen et al., 2013), have shown almost no effect on the total abundance of AOA populations in comparison with their effects on AOB. We also observed a reduction in the abundance of NOB genera such as *Candidatus Nitrotoga*, *Nitrobacter* and *Nitrospira* (Figs. 4 and S7), probably due to the lower availability of their substrate (NO_2^-) by the inhibition of AOB populations.

Previous works suggested that NIs application decrease N_2O emissions due to a reduction in both nitrification and denitrification rates (Nguyen et al., 2017; Wu et al., 2017). These authors hypothesize that the lower growth of nitrifiers reduces oxygen consumption, then promoting a more aerobic condition in soil. Therefore, denitrification, which is promoted under anoxic conditions, is collaterally reduced. However, other works have found that, while total *amoA* gene abundance decreased with DMPP or DMPSA application, *nosZI* gene abundance increased (Barrena et al., 2017; Corrochano-Monsalve et al., 2020a). This was associated with a shift in the (*nosZI*+*nosZII)*:(*nirK*+*nirS)* genes ratio and, thus, denitrification was leaned to a complete reduction of N_2O to N_2 (Torralbo et al., 2017). However, further research would be necessary to definitively elucidate the reason for the *nosZ* induction. In the present work we did not found a general increase in *nosZI* genera after the application of DMPSA, which might be due to the fact that the analyses performed only included the taxa that were confidently assigned to genera. Nevertheless, we were able to observe an increase in *Rhizobium* in the CT management system (Fig. S7). However, in concordance with previous results of Torralbo et al. (2017) under laboratory conditions, *nosZII* holding bacteria did increase with DMPSA in NT soils (40.9% in AS *vs* 44.6% in ASD in NT) ($P = 0.1$) (Fig. 4), but not in CT (Fig. S7). *Flavobacterium* and *Opitutus* genera were more abundant in NT-ASD than in NT-AS, suggesting that these groups would also be contributing to the depletion of N_2O emissions by promoting its complete reduction to N_2 when DMPSA is applied in the NT management system. Similarly, Bacteroidetes phylum showed higher abundance under ASD treatment (Fig. 6). These bacteria are known to play an important role in soil organic matter degradation (Thomas et al., 2011) and, interestingly, account for a high proportion of *nosZII* gene holding bacteria (Jones et al., 2013), of which a majority are non-denitrifying N_2O reducers (Sanford et al., 2012; Graf et al., 2014). Within Bacteroidetes, 3 orders (Cytophagales, Sphingobacteriales and Flavobacteriales) showed larger abundances in ASD treatments. Interestingly, an increase in the abundance of Cytophagales has previously been found after the application of DMPP (Zhang et al., 2017). The higher NH_4^+ soil content in the soils with ASD treatments (Corrochano-

Monsalve et al., 2020a) may have benefited them, as Cytophagales have been reported to grow better under NH_4^+ rather than NO_3^- (Reichenbach, 2006). A decrease in O_2 consumption due to nitrification inhibition by DMPSA could benefit Sphingobacteriales, which are known to be very sensitive to O_2 availability (Schellenberger et al., 2010). Similarly, *Flavobacterium* (Flavobacteriales), known to hold the *nosZII* gene (Graf et al., 2019), showed higher relative abundance in ASD soils (Fig. 4). On the contrary, Acidobacteria phyla were more abundant under AS treatment (Fig. 6). They are considered oligotrophic bacteria typical of poor-C soils (Fierer et al., 2007) and are related to N-cycle (Eichorst et al., 2018). Interestingly, within them, we identified higher abundance of members of subgroup6 in AS, which are known to contain NO-reductase (NOR) and N_2OR enzymes (Huang et al., 2016). Hester et al. (2018) showed that this subgroup is more adapted to low-N environments, and may not be able to compete with other denitrifiers more specialized in higher-N inputs. Therefore, the enhancement of other denitrifiers after DMPSA application, such as the aforementioned *nosZII* gene holding bacteria (e.g members of Bacteroidetes phyla), could have exerted a negative effect on this Acidobacteria subgroup6 population. It should be noted that Cyanobacteria phylum was largely over-represented in ASD treatment (+48%) (Fig. 6; data not shown). These bacteria undergo substantial adaptations to adjust their machinery to N availability and are capable to assimilate it in the form of NO_3^-, NO_2^-, and even urea, cyanate and amino acids (Bolay et al., 2018). However, the preferred source of N for Cyanobacteria is NH_4^+. Therefore, this group would have been presumably favored by the increase in soil NH_4^+ content as a consequence of DMPSA application. Cyanobacteria are gaining interest from an agricultural sustainability point of view due to several of their beneficial aspects, which include: the capability of atmospheric N_2-fixation, improvement of soil physicochemical characteristics, stimulation of plant growth and protection against plants diseases (Singh et al., 2016). Therefore, the decrease of Xanthomonadales (mostly phytopathogens, as has been previously discussed in section 4.2) and the increase of Cyanobacteria could be also related in ASD soils. It has been found that rice lineages with high nitrogen-use efficiency (NUE) show rhizo-compartmental microbial communities

with a high abundance of Cyanobacteria when are compared to the wild type rice (Khan et al., 2019). Moreover, close relationships are established between Cyanobacteria and other groups such as Bacteroidetes and Chloroflexi (Abed et al., 2018). Interestingly, Khan et al. (2019) found that a greater abundance of Cyanobacteria was also associated with a higher abundance of Chloroflexi and a lower abundance of Acidobacteria. These same facts were exactly replicated in our study, since we found a higher abundance of Bacteroidetes and Chloroflexi in ASD and a greater abundance of Acidobacteria in AS (therefore, lower abundance in ASD). All these results suggest that the effects observed in those bacterial groups might have been triggered by the induction of Cyanobacteria.

The different response of bacterial community (both involved or not in N-cycle) to DMPSA in CT and NT could be associated with a dissimilar diffusion of the inhibitor in the soils in different management systems. Barth et al. (2008) already related higher DMPP efficiency with higher water contents, which will facilitate DMPP dissolution. We can hypothesize that the differential response found in the present study relies on the lower diffusion of DMPSA in CT soils due to its lower water content and pore connectivity. This hypothesis seems to be supported by the fact that, 1) nitrifiers abundance did decrease in both CT and NT (Figs. 4 and S7). As nitrifiers are expected to be more abundant in soil surface layers (characterized by more aerobic conditions are present), DMPSA could reach them easily, no matter the management. 2) A different response of *nosZII* holding genera to DMPSA was observed according to the management. *nosZII* taxa are expected to be more abundant in deeper soil surface layers (more anaerobic conditions). Several *nosZII* bacteria were enriched in NT-ASD, while none in CT-ASD, suggesting that DMPSA might only be reaching to further depth in NT soils, but not in CT. Finally, further works would be necessary to determine the persistence of the effects observed in this study in the long-term.

CONCLUSIONS

DMPSA exerted effects on target and non-target organisms. A diversity decrease was produced by DMPSA, especially when it was applied under no-tillage management, which also decreased the diversity with respect to the conventional tillage. However, the interpretation of the influence of DMPSA on soil should be made carefully, as 1) it varied depending on the management, 2) it shaped the abundance of varying microorganisms even at phylum level, but the disrupted organisms did not seem to share peculiarities, and 3) the effects on non-target organisms might be a mix of direct and indirect effects.

Microbial communities are characterized by complex networks of microbial populations interacting with each other in different ways. In this regard, results from the present work suggest that the increase in the abundance of certain taxa such as Bacteroidetes, as well as the decrease of certain phytopathogens, could have been triggered by the increase of Cyanobacteria. This decrease of phytopathogens may have also been helped by the great increase observed in the genus *Vermamoeba vermiformis,* a protist known to control/regulate several soil-borne pathogens, when DMPSA was applied.

In conclusion, the application of DMPSA implies not only environmental benefits due to the reduction of reactive-nitrogen losses, but also because DMPSA induced the abundance of agronomically beneficial organisms.

SUPLEMENTARY MATERIAL

Table S1. Physical and chemical properties of the soil *(0 –30 cm depth).*

Soil texture			Soil chemical properties								
Sand	Silt	Clay	pH[a]	C:N	N[b]	Organic matter[c]	Carbonate[d]	P[e]	Mg[d]	K[d]	Ca[d]
(%)					(g kg^{-1})			(mg kg^{-1})			
43.4	24.7	31.9	8.0	8.15	1.6	21.2	9.8	59.0	92.4	167	6,356

a: pH (1:2.5 soil:water). b: N Kjeldahl digestion (Keeney and Nelson, 1982). c: Organic matter (Walkley and Black, 1934). d: CaCO3, Mg, K (MAPA, 1994). e: P (Watanabe and Olsen, 1965).

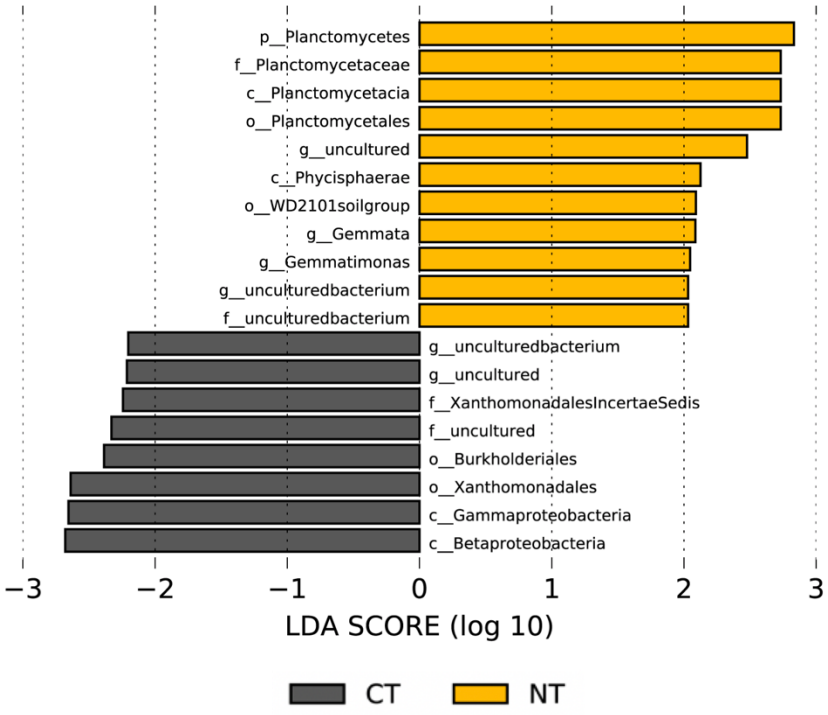


Figure S1. LEfSe analysis of bacterial abundance within AS treatment. *LDA scores of the taxa with different abundances between managements (LDA threshold > 2). (CT = conventional tillage; NT = no-tillage).*

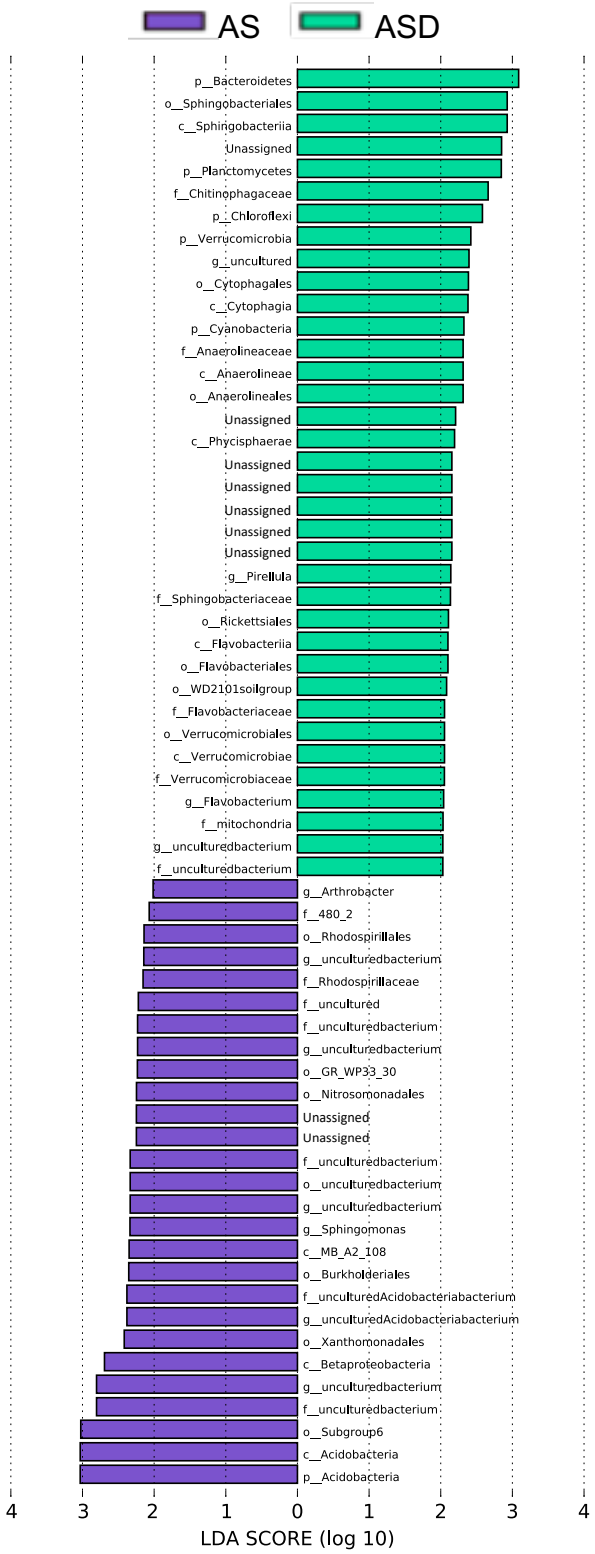


Figure S2. LEfSe analysis of bacterial abundance within no-tillage system. *LDA scores of the taxa with different abundances between treatments (LDA threshold > 2).*
AS = ammonium sulfate 21%; ASD = ammonium sulfate 21% + DMPSA.

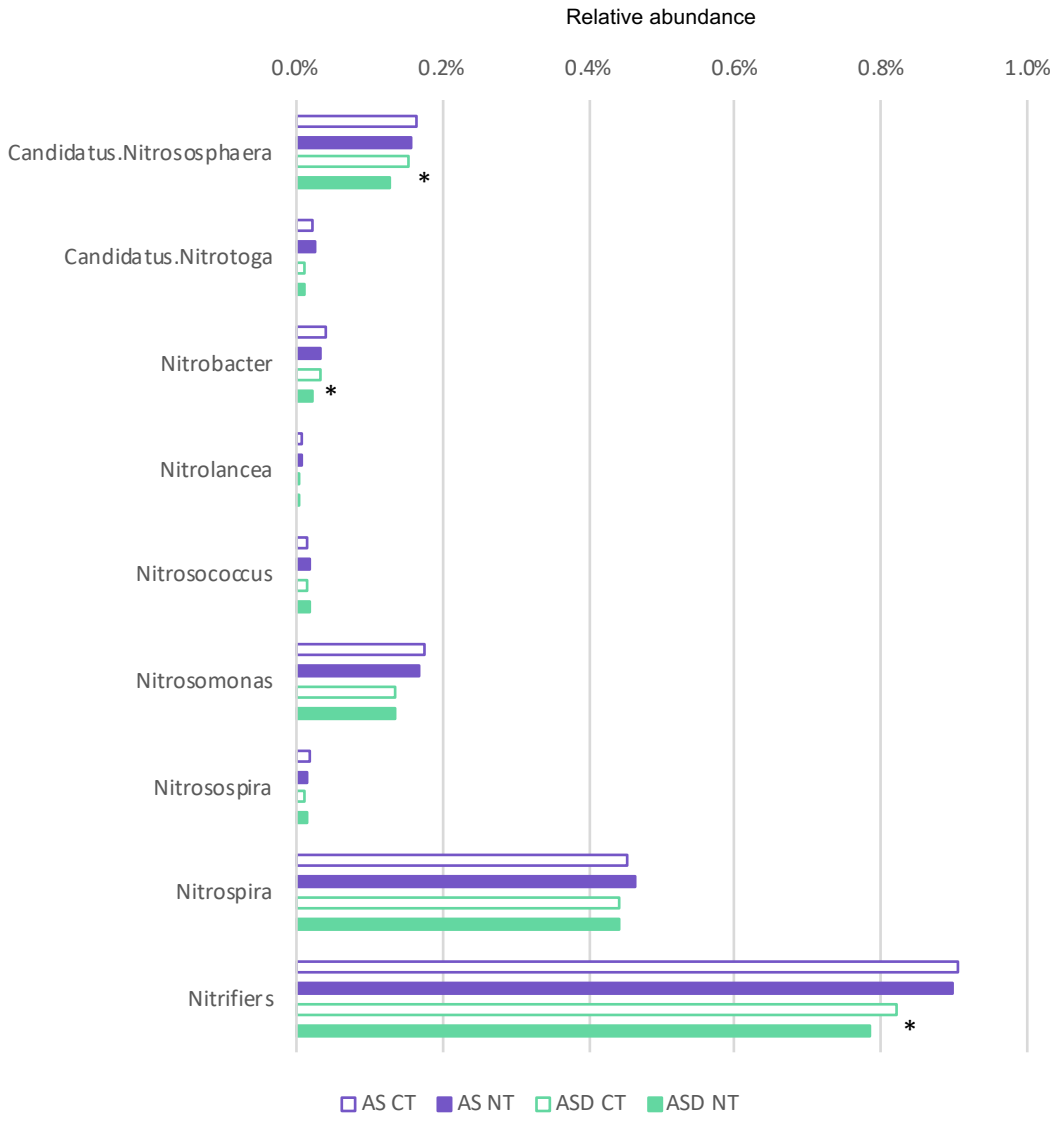


Figure S3. Relative abundance of identified nitrifying genera by management ant treatment. *Significant differences between managements within a treatment are indicated with asterisks (*) (Mann-Whitney-U; P < 0.05).*

CT = conventional tillage; NT = no-tillage; AS = ammonium sulfate 21%; ASD = ammonium sulfate 21% + DMPSA.

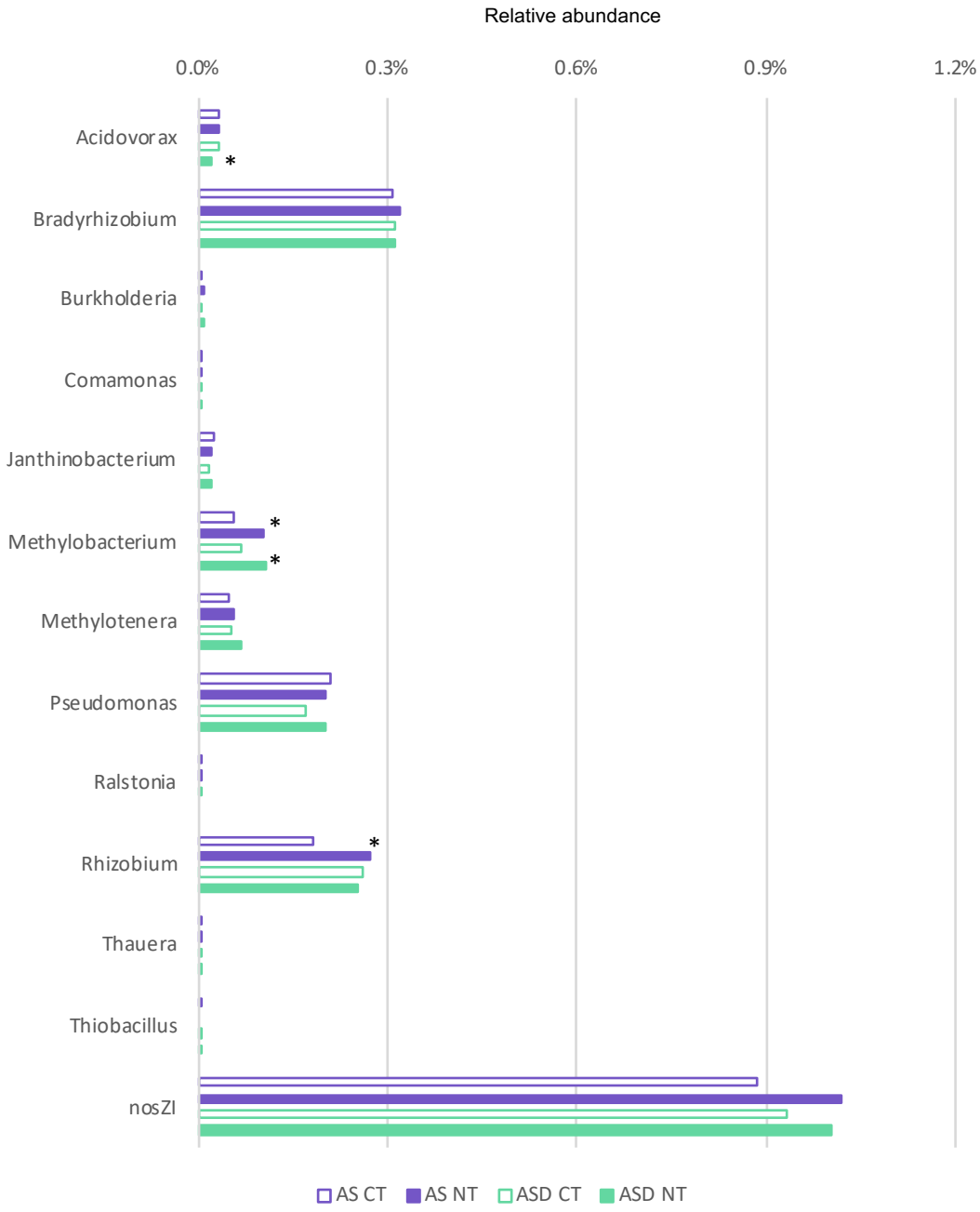


Figure S4. Relative abundance of identified nosZI genera by management and treatment. *Significant differences between managements within a treatment are indicated with asterisks (*) (Mann-Whitney-U; $P < 0.05$).*

CT = Conventional tillage; NT = No-tillage; AS = ammonium sulfate 21%; ASD = ammonium sulfate 21% + DMPSA.

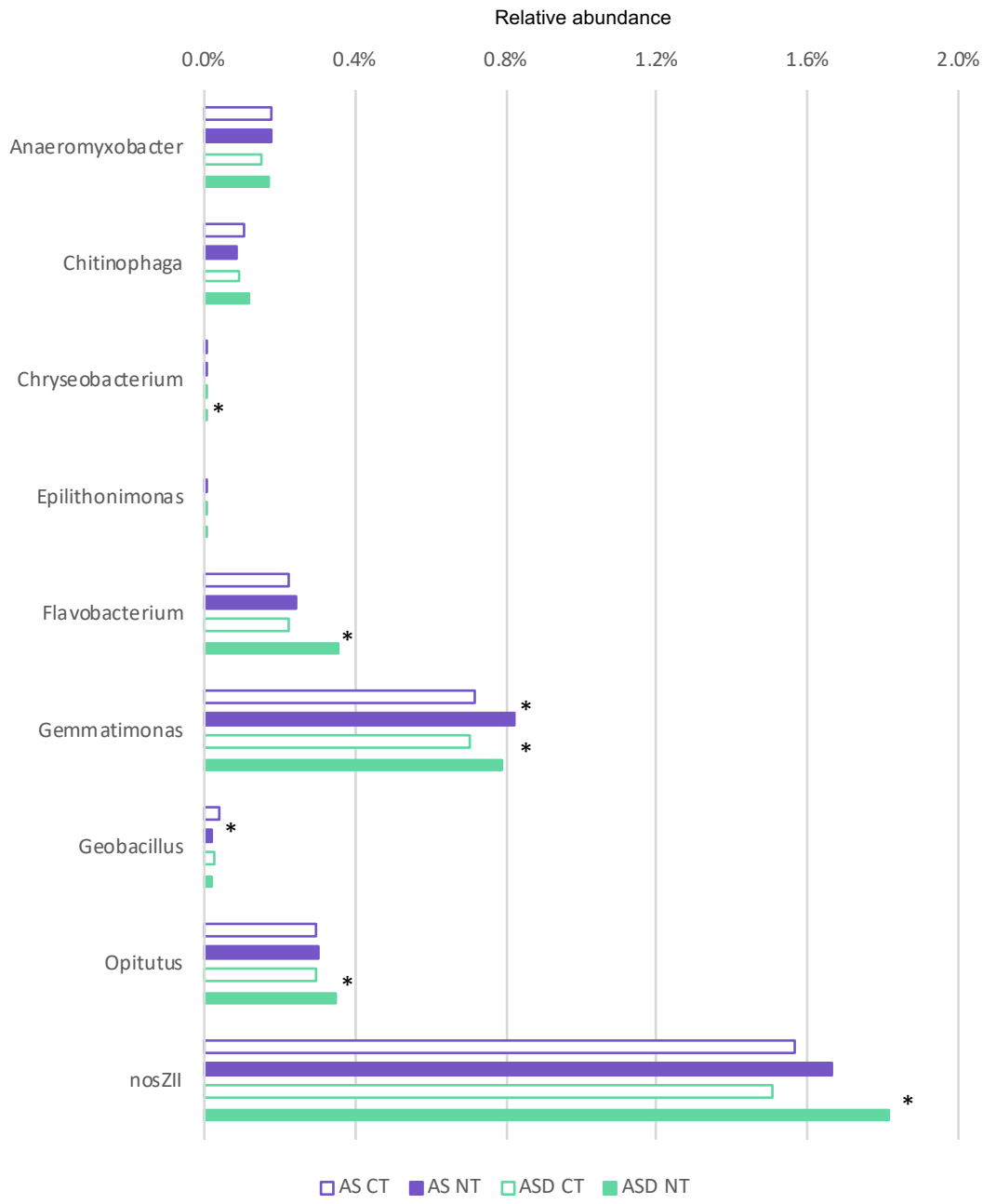


Figure S5. Relative abundance of identified nosZII genera by management and treatment. *Significant differences between managements within a treatment are indicated with asterisks (*) (Mann-Whitney-U; P < 0.05).*

CT = Conventional tillage; NT = No-tillage; AS = ammonium sulfate 21%; ASD = ammonium sulfate 21% + DMPSA.

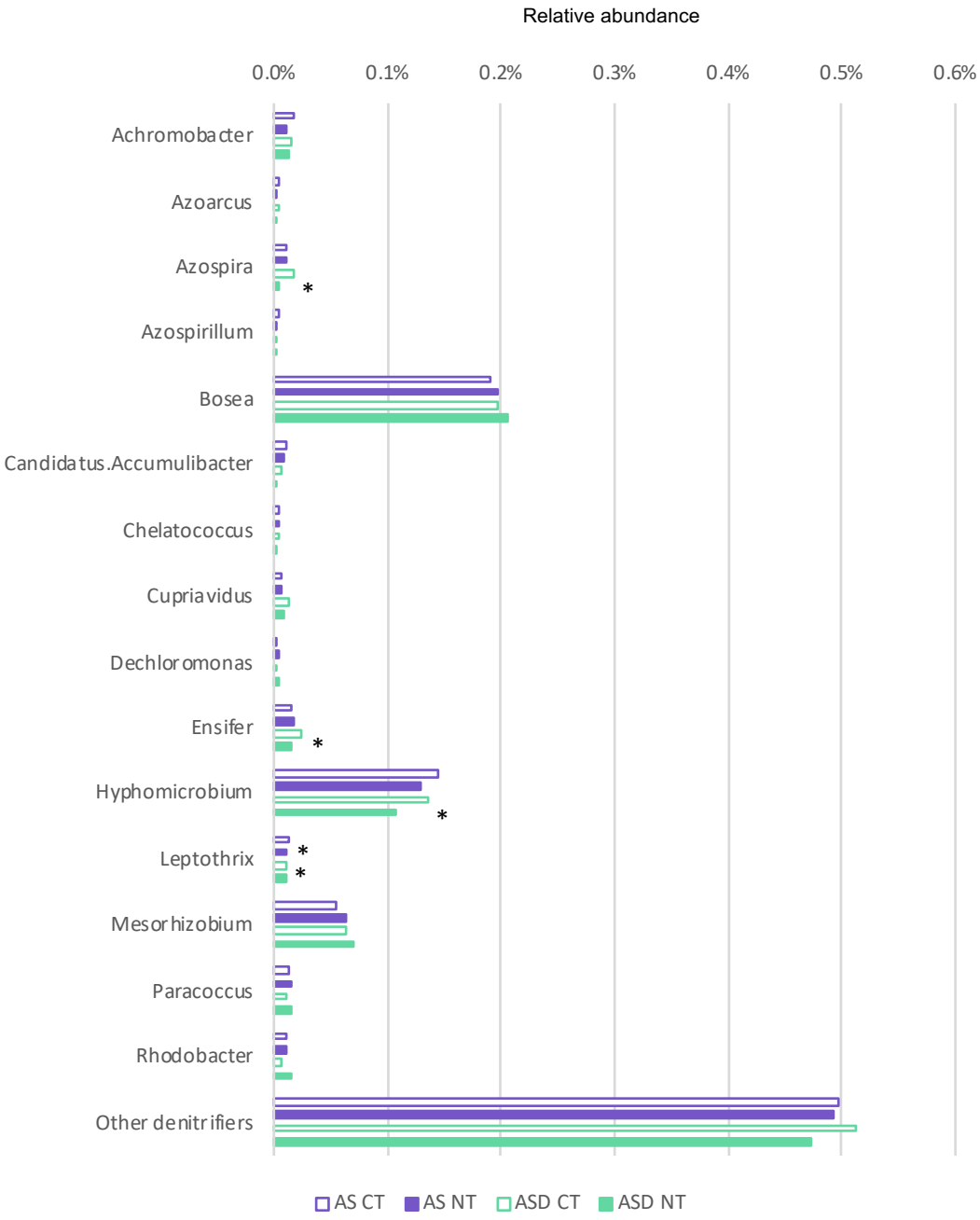


Figure S6. Relative abundance of other identified denitrifiers genera by management and treatment. *Significant differences between managements within a treatment are indicated with asterisks (*) (Mann-Whitney-U; P < 0.05).*

CT = Conventional tillage; NT = No-tillage; AS = ammonium sulfate 21%; ASD = ammonium sulfate 21% + DMPSA.

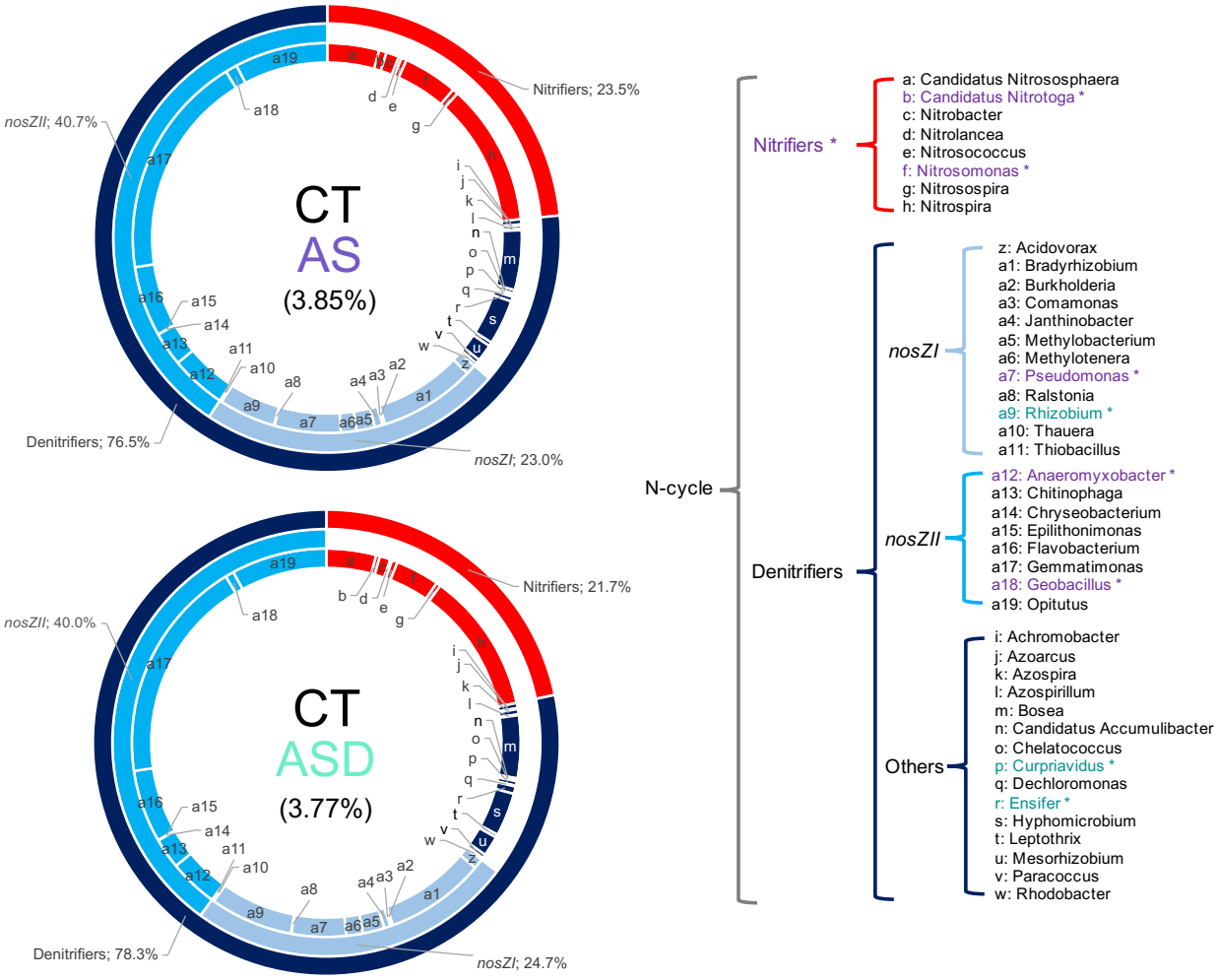


Figure S7. Distribution of identified N-cycle genera within conventional tillage system.

Total N-cycle bacteria relative abundance is indicated for each treatment in parenthesis. Significant differences between treatments are indicated with asterisks (*) (Mann-Whitney-U; P < 0.05). Text in purple indicates significant higher abundance in AS; text in green indicates significant higher abundance in ASD.

CT = Conventional tillage; NT = No-tillage; AS = ammonium sulfate 21%; ASD = ammonium sulfate 21% + DMPSA.

CHAPTER 5

Magnitude of the impact on bacterial community

Impact of dimethylpyrazole-based nitrification inhibitors on soil-borne bacteria

SCIENCE OF THE TOTAL ENVIRONMENT

UNDER REVIEW (2021)

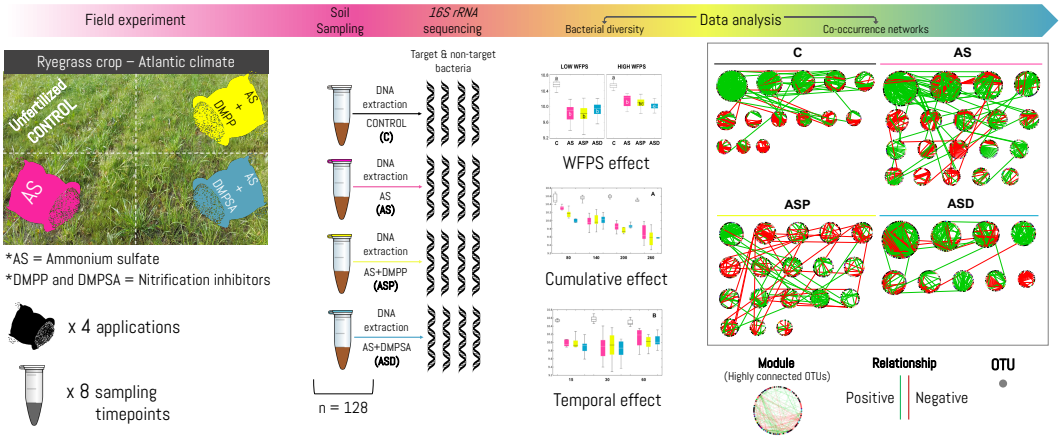
The first approach to analyze the impact of DMPSA on the soil microbiome confirmed that there are indeed non-target effects, that is, its application causes shifts beyond nitrifying organisms. This indicated that our hypothetical scenarios were too conservative, since the effects of DMPSA not only extended to the nitrogen cycle, but also reached more parts of the bacterial consortia. Attending to the affected taxa, there were signs to think that several changes might be related to a greater NH_4^+ availability; and, on the other hand, it seemed that oligotrophic organisms were favored after DMPSA application.

Nevertheless, the study presented in Chapter 4 had some limitations. We did not know whether the characteristics of the bacterial community after DMPSA application were more similar to unfertilized soils (i.e. like a reversion of the effects exerted by fertilization itself) or, on the contrary, DMPSA led to a third state or condition different from fertilized soils without inhibitor and different from unfertilized ones. Furthermore, the obtained data did not allow us to assess the magnitude of the impact exerted by DMPSA to determine how severe were the changes compared with fertilization itself. Nitrogen fertilizer application causes a drastic impact on soils because of the great nutrients input, so we would expect a much slighter impact of the inhibitor. In addition, it was not possible to determine either the remanence of these shifts, so another question to be addressed was whether they would be durable over time or quickly reversed. In the same manner, as that was the first approximation to determine the effects of DMPSA on the whole bacterial consortia, we could not assume that the effects would be the same in another type of crop and different edaphoclimatic conditions.

Finally, despite DMPP and DMPSA are based on the same compound, we found many more side effects caused by DMPSA than that found by other authors after DMPP application, which might be a consequence of the differences found between both inhibitors in some of the aspects analyzed in Chapter 3. Therefore, it seemed interesting to us to investigate a direct comparison between both inhibitors.

We tried to address all these questions in this last chapter of the thesis.

GRAPHICAL ABSTRACT



ABSTRACT

Nitrogen (N) input from fertilizers modifies the properties of agricultural soils as well as bacterial community diversity, composition and relationships. This can lead to negative impacts such as the deterioration of system multifunctionality, whose maintenance is critical to normal nutrient cycling. Synthetic nitrification inhibitors (NIs) can be combined with fertilizers to improve the efficiency of N use by reducing N losses. However, analysis of their effects on non-target bacteria are scarce. This study aimed to analyze the effect of applying the NIs DMPP and DMPSA on the whole bacterial community. Through *16S rRNA* amplicon sequencing we determined the differences between samples in terms of microbial diversity, composition and co-occurrence networks.

The application of DMPP and DMPSA exerted little impact on the abundance of the dominant phyla. Nevertheless, several significant shifts were detected in bacterial

diversity, co-occurrence networks, and the abundance of particular taxa, where soil water content played a key role. For instance, the application of NIs intensified the negative impact of N fertilization on bacterial diversity under high water-filled pore spaces (WFPS) (> 64%), reducing community diversity, whereas alpha-diversity was not affected at low WFPS (< 55%). Interestingly, despite NIs are known to inhibit ammonia monooxygenase (AMO) enzyme, both NIs almost exclusively inhibited *Nitrosomonas* genera among AMO holding nitrifiers. Thus, *Nitrosomonas* showed abundance reductions of up to 47% (DMPP) and 66% (DMPSA). Nonetheless, non-target bacterial abundances also shifted with NI application. Notably, DMPSA application partially alleviated the negative effect of fertilization on soil multifunctionality. A remarkable increase in populations related to system multifunctionality, such as *Armatimonadetes* (up to +21%), *Cyanobacteria* (up to +30%) and *Fibrobacteres* (up to +25%) was observed when DMPSA was applied. NI application substantially influenced microbial associations by decreasing the complexity of co-occurrence networks, decreasing the total edges and node connectivity, and increasing path distances.

MATERIALS AND METHODS

2.1. EXPERIMENTAL DESIGN

This research was conducted under Atlantic climate conditions, on an experimental field located in Zamudio (Basque Country, northern Spain). Soil was collected from the 0–10 cm layer of a ryegrass crop (*Lolium multiflorum* Lam. Var. Westerwold). At the beginning of the experiment, the soil upper horizon (0–30 cm) presented a silt loam texture (33.4% of sand, 52.6% of silt and 15.0% of clay), an organic matter content of 1.8%, a C/N ratio of 7.9 and pH 5.6 (1:2.5 H_2O; 0–10 cm).

The experimental design consisted of a completely randomized block with four replicates and an individual plot size of 28 m^2 (7 m x 4 m). The soil was plowed and sown

in September 2016 at a density of 40 kg ryegrass seeds ha^{-1}. The treatments studied consisted of control soils with no fertilizer (C), soils amended with ammonium sulfate (AS), soils with ammonium sulfate + DMPP (ASP) and soils with ammonium sulfate + DMPSA (ASD). Ammonium sulfate was applied in granular form at a rate of 80 kg N ha^{-1} (1^{st} application) and 60 kg N ha^{-1} (2^{nd}, 3^{rd} and 4^{th} applications). NIs were applied at a rate of 0.8% of the NH_4^+-N, as supplied by Eurochem Agro Iberia S.L., accounting for 0.64 kg ha^{-1} (1^{st} application) and 0.48 kg ha^{-1} (2^{nd}, 3^{rd}, and 4^{th} applications). The timing and rates of application are detailed in Fig. 1.

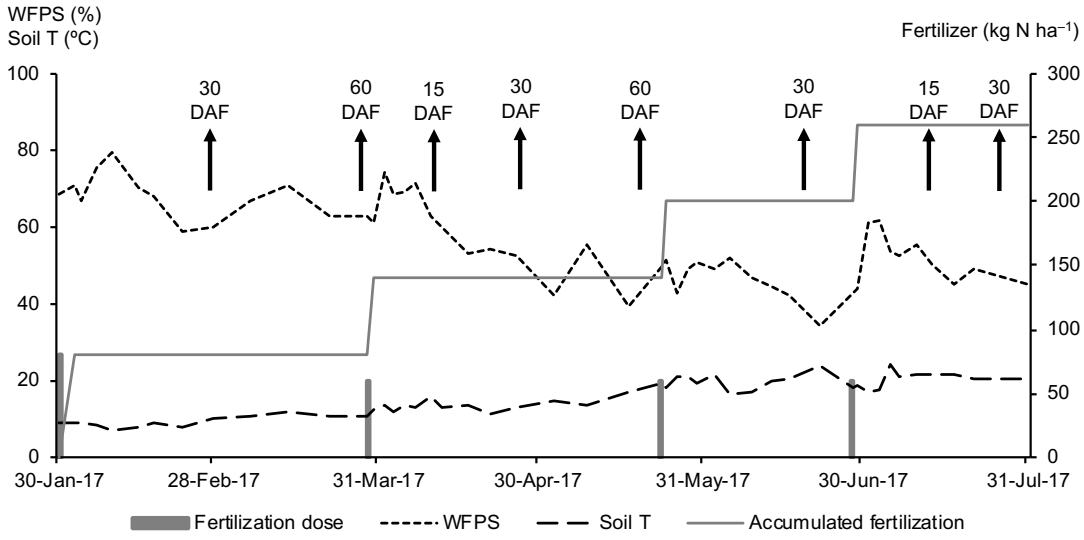


Figure 1. Soil water content expressed as water-filled pore space (WFPS) and soil temperature (0–10 cm), fertilizer application rates and total cumulative fertilization. *Arrows indicate soil sampling for DNA determinations and timing from last fertilizer application (Days after fertilization, DAF).*

2.2. SOIL WATER CONTENT DETERMINATION

Four soil samples (2 cm diameter x 10 cm depth) were collected randomly from each plot every two days for two weeks after fertilizer application. In the remaining time, sampling was carried out two days per week. Rocks were removed and the soil was oven-dried for 48 h at 80 °C. Soil water content was calculated as the percentage of water-filled

pore space (WFPS) according to Linn and Doran (1984): WFPS = (soil gravimetric water content x bulk density) x (1 - (bulk density/particle density))$^{-1}$, by using a particle density of 2.65 Mg m^{-3}, while bulk density resulted in a value of 1.22 Mg m^{-3}. For statistical analysis, samples were grouped into low WFPS (ranging from 41.9% to 55.0%; 80 samples) and high WFPS (from 64.2% to 66.8%; 48 samples) conditions, following the Carson et al., (2010) pore connectivity theory, which specifies that below WPFS 56% soil pores become disconnected. To do this clustering, mean WFPS values from the previous 15 days were used because the bacterial community is modulated by conditions maintained for several days before the sampling.

2.3. SOIL SAMPLING AND DNA EXTRACTION

Five soil subsamples (2 cm diameter x 10 cm depth) were collected randomly from each individual plot and then mixed. Sampling times are shown in Fig. 1. In total, 128 samples were collected. After homogenization, DNA was extracted from 0.35 g soil fresh weight using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) with the following modifications: cell lysis was carried out in a Precellys24 homogenizer (Bertin, Montigny-le-Bretonneux, France), and cooling incubations and the final elution incubation were performed as described by Harter et al. (2014). Extracted DNA concentration and quality were determined by spectrophotometry with a NanoDrop® 1000 (Thermo Scientific, Walthman, MA, USA).

2.4. LIBRARY CONSTRUCTION AND DNA SEQUENCING

The V4 region of the bacterial *16S rRNA* gene was amplified with 515F/806R primers as described by Caporaso et al. (2012) including Illumina barcodes and sequencing adaptors. The following PCR conditions were used for amplification: initial denaturation at 95 °C for 4 min, 35 cycles at 95 °C for 15 s, 50 °C for 30 s and a final extension of 72 °C for 30 s. PCR products were examined by electrophoresis in a 1% agarose gel. Amplicon purification was performed with the CleanPCR kit (Cleanna), using magnetic beads. Samples were quantified with QubitTM v2.0 (ThermoFisher

Scientific) and normalized in an 8 pM pool. A paired-end sequencing of the pool was carried out with the kit v3 PE 2 × 150 bp (600 cycles) on an Illumina MiSeq modified to run for 300 cycles at the Sequencing and Genotyping Unit of the University of the Basque Country (SGIKER).

2.5. QUALITY CHECKING, PROCESSING AND TAXONOMIC ASSIGNMENT

Forward and reverse raw sequences were quality checked with Sickle v1.33 (Joshi and Fass, 2011) using default parameters including a Phred score ≥ 20. The assembly of the pair-end sequences was conducted with Pear v0.9.10 (Zhang et al., 2014), with an overlap of 15 bp. Fastq-barcode.pl script (Smith, 2012) was used to remove non-existent (non-assembled) barcodes from the fastq files obtained in Pear. Seq_filter.pl was used to eliminate sequences by length, keeping sequences with a min and max length of 205–295 bp to avoid background noise in the subsequent analyses. An open reference Operational-Taxonomic-Unit (OTU) picking method was used in QIIME v1.9. OTUs were clustered against the GreenGenes 13_8 database at the 97% similarity level using uclust (Edgar, 2010). OTU sequences were aligned using PYNAST (Caporaso et al., 2010a) and the ones that failed to align were discarded. OTU taxonomy was determined using the RDP classifier (Wang et al., 2007) retrained towards GreenGenes13_8 (97% similarity). A final OTU table was created, excluding unaligned sequences and OTUs with less than 10 sequences. Finally, the OTU table was normalized using the metagenomeSeq CSS algorithm, which normalized sequences using the cumulative sum scaling transformation (Paulson et al., 2013).

2.6. CO-OCCURRENCE NETWORKS CONSTRUCTION AND VISUALIZATION

To explore the response of bacterial relationships to fertilizer and NI application, we constructed eight co-occurrence networks (four networks for low WFPS conditions and four for high WFPS). Only OTUs that appeared in more than 50% of the

samples were considered for the construction of each network. Networks were constructed on the MENAP website (http://ieg4.rccc.ou.edu/mena/main.cgi) following the developers' recommendations (Zhou et al., 2010; 2011; Deng et al., 2012). A simulated annealing algorithm was used for better separation of the modules (Guimerà and Amaral, 2005; Tao et al., 2018). Networks were visualized with Cytoscape v3.8.2 (Shannon et al., 2003). The networks were assessed based on their topological features determining the network size, number of edges (connectivity), percentage of positive edges, average connectivity (avgK), average path distance (GD), average clustering coefficient (avgCC), modularity (M), and number of modules (Table 2).

2.7. STATISTICAL ANALYSIS

Statistical evaluation of the data was carried out with QIIME v1.9 (Caporaso et al., 2010b) and R version 3.1.2 (R core team, 2013) using Rstudio version 1.1.463 (R studio team, 2016). Community richness and evenness were calculated through Chao1 and Shannon indices, respectively, rarefying the original (not normalized by CSS) OTU table to 27,000 sequencing depth. The Monte-Carlo test was applied to test the differences in alpha-diversity measurements between treatments, accumulated fertilizer treatments and days after fertilizer application ($P < 0.05$ based on 999 permutations). To test the significance of the differences between treatments of the parameters describing the topology of each co-occurrence network, 100 random networks were generated to use the standard deviation for the Student-T test. Community dissimilarity between samples was assessed with the Bray-Curtis index and the ANOSIM test was used to address whether microbial community composition changed significantly according to the exploratory variables "Fertilization" and "Nitrification inhibitor". The linear discriminant analysis effect size (LEfSe) method (Segata et al., 2011) was used to identify biomarkers of the different treatments (Kruskal-Wallis $P < 0.05$ and LDA $Log_{10} > 2$) in the Galaxy online tool (http://huttenhower.sph.harvard.edu/galaxy).

3.1. NITRIFICATION INHIBITORS INTENSIFIED THE IMPACT OF FERTILIZATION ON ALPHA-DIVERSITY AT HIGH WFPS

3.1.1. IMPACT OF FERTILIZER ON BACTERIAL DIVERSITY WITH RESPECT TO UNFERTILIZED SOILS

When comparing unfertilized and AS soils, we showed that fertilizer decreased the Shannon evenness index at both low (C = 10.54; AS = 9.84; Monte-Carlo test, P = 0.006) and high WFPS (C = 10.54; AS = 10.22; Monte-Carlo test, P = 0.006), but that was not the case for the Chao1 richness index, especially at high WFPS (low WFPS, C = 8757; AS = 8018; High WFPS, C = 8829; AS = 9180) (Fig. 2).

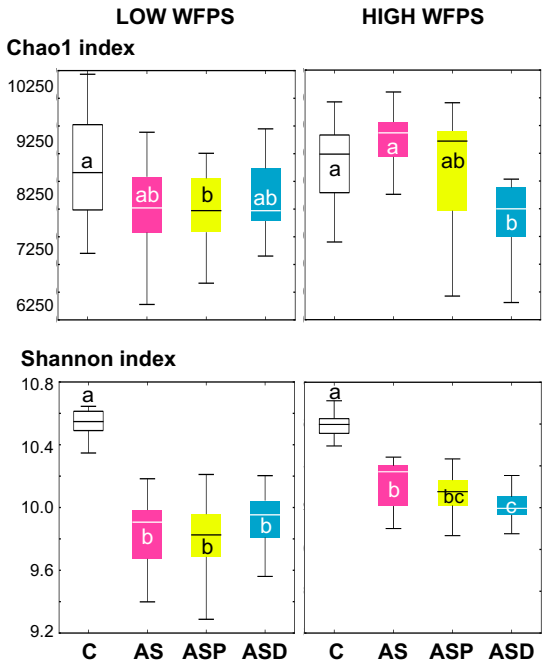


Figure 2. Alpha diversity Chao1 (richness) and Shannon (evenness) indices by treatment at low and high water-filled pore space (WFPS). *Different letters indicate significant differences (Monte Carlo, $P < 0.05$ based on 999 permutations). C = Unfertilized control; AS = Ammonium Sulphate 21%; ASP = Ammonium Sulphate 21% + DMPP; ASD = Ammonium Sulphate 21% + DMPSA.*

This suggests that adding ammonium sulfate fertilizer alone did not cause adverse effects on relatively-rare taxa, but it made the abundance of the taxa found more similar. Most previous studies have reported a decrease in bacterial alpha-diversity after N application. N addition through fertilizers shifts soil conditions, thus modifying soil bacterial consortia. This large N input favors copiotrophic taxa over oligotrophs (Leff et al., 2015), with fast growth rates of the former (Fierer et al., 2007). Besides this, applying fertilizer indirectly affects carbon pools (Liu et al., 2016) and diminishes soil pH in the long term as a consequence of H^+ release through nitrification (Pierre, 1928; Tian and Niu, 2015). The reason behind not observing a significant decrease in Chao1 in the present experiment (P = 0.12 at low WFPS) may be related to several factors identified by Wang et al. (2018) that influence the magnitude of the impact of fertilizer applications. i) The negative effect of fertilizer on alpha-diversity tends to be smoother in grassland systems, as in the present case. ii) The impact is influenced by the initial conditions in the soils before fertilization. Thus, effects on alpha-diversity are not significant when the initial soil pH is below 7 (in the present study, the initial pH was 5.6). iii) There is a greater accumulation of these effects the longer the soils have been fertilized and the higher the dosage of fertilizer received. Therefore, smaller diversity drifts should be expected in the present study due to the relatively short time period of the experiment (four fertilizer applications in six months).

Unfertilized soils maintained constant evenness values throughout the experiment (C Shannon = 10.54, 10.55, 10.59, 10.48), but in AS it dropped from 10.29 (when only 80 kg N ha^{-1} had been applied) down to 9.97 (a total of 140 kg N ha^{-1} applied), 9.85 (200 kg N ha^{-1}) and 9.69 (when a total dose of 260 kg N ha^{-1} had been accumulated). Thus, fertilizer accumulation was clearly accompanied by a reduction in the Shannon index (Fig. 3A). In contrast, this trend was not observed in terms of the Chao1 values (Supplemental Fig. S1A). Nonetheless, AS soils tended to recover higher diversities 60 days after fertilizer application (DAF) (AS Shannon, 15 DAF = 10.03, 30 DAF = 9.90, 60 DAF = 10.10), approaching C levels (Shannon = 10.54, 10.56, and 10.49 respectively) (Fig. 3B and Supplemental Fig. S1B). Many studies have related the effects of

fertilization on alpha and beta diversity to soil acidification (Zeng et al., 2016; Ling et al., 2017; Zhang et al., 2017; Bei et al., 2018, Li et al., 2020). We also observed that soil pH decreased as fertilizer accumulated, from 5.6 at the beginning of the experiment to 4.4 at the end (with no difference between AS, ASP and ASD), which could have been harmful to some bacteria. Nevertheless, the meta-analysis of Wang et al. (2018) suggests that acidification is not the main direct driving factor influencing alpha-diversity, but rather the changes in N availability and soil organic carbon.

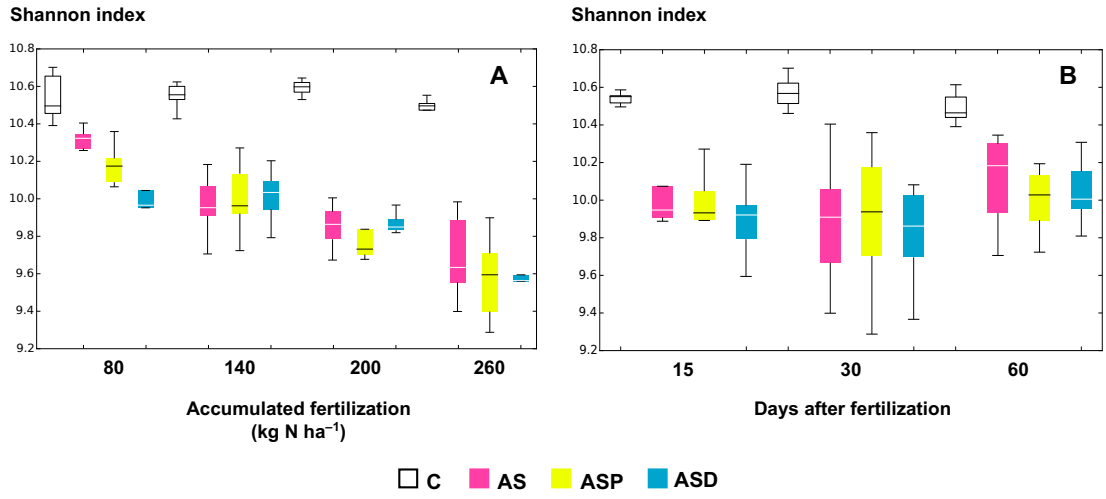


Figure 3. Shannon alpha diversity index by A) accumulated fertilization and B) days after fertilization. *C = Unfertilized control; AS = Ammonium Sulphate 21%; ASP = Ammonium Sulphate 21% + DMPP; ASD = Ammonium Sulphate 21% + DMPSA.*

3.1.2. ASSESSING THE EFFECT OF DMPP AND DMPSA ON BACTERIAL DIVERSITY WITH RESPECT TO CONVENTIONAL FERTILIZER

Both NIs tended to decrease bacterial richness (Chao1) at high WFPS (AS = 9180; ASP = 8718; ASD = 7784), although the decrease with respect to AS was significant only in the case of ASD (Monte-Carlo test, $P = 0.036$). However, the NIs did not affect Chao1 at low WFPS (AS = 8018; ASP = 7991; ASD = 8217). Further, the application of both inhibitors also tended to decrease the evenness (Shannon) to lower values than in the AS

samples at high WFPS (AS = 10.22; ASP = 10.11; ASD = 9.98), again this was significant only in the case of ASD (Monte-Carlo test, $P = 0.047$). As before, there was no effect of NIs on the Shannon index at low WFPS (AS = 9.84; ASP = 9.81; ASD = 9.88). These results are in concordance with Zhang et al. (2017) and Corrochano-Monsalve et al. (2020b). The former reported a non-significant trend of DMPP to decrease richness and almost no effects on the Shannon index, while our earlier work indicated that DMPSA clearly decreased Chao1 in soils with high WFPS, but did not affect the Shannon index. Therefore, despite dimethylpyrazole (DMP) being the supposed active compound in both of the inhibitors tested, they do not have the same impact on soil bacterial consortia, suggesting that there should be some differences in the behavior of these inhibitors in the soil. It is considered that the mechanism of action of these DMPs relies on their chelation capacity, since AMO needs copper as a cofactor. Nevertheless, previous studies have demonstrated differences in their copper-chelation mechanism, because DMPSA can chelate copper without being degraded to DMP and it demonstrates a greater chelation efficiency (Corrochano-Monsalve et al., 2021a). Therefore, these NIs differential microbial impact might be ascribed to their chelation singularities. In addition, this chelation ability might be extended to other metallic cofactors, thus imposing effects on organisms requiring these metals.

As the number of fertilizer applications increased, the ASP and ASD treatments followed a similar trend to AS. While no clear trend was observed in Chao1 values (Supplemental Fig. S1A), after the 1st fertilizer application the Shannon indexes in the ASP and ASD treatments were 10.15 and 9.99, respectively, and in the subsequent 2nd, 3rd, and 4th additions decreased to 10.01, 9.81 and 9.58 (in ASP) and 10.02, 9.87 and 9.62 (in ASD) respectively (Fig. 3A). Soil conditions were wetter in the period after the 1st (68% average WFPS) and 2nd (58% WFPS) applications than after the 3rd (46% WFPS) and 4th (51% WFPS) applications (Fig. 1). It is noteworthy that the greatest differences between the treatments receiving NIs and AS were found in the 1st fertilizer application. The reason why NIs induced the biggest changes after the 1st application is not clear. It might be related to the higher WFPS present after the 1st fertilization, which allowed a

greater pore connectivity, as Carson et al. (2010) described, which could facilitate the diffusion of substances such as NIs. On the other hand, it has been observed that dependent on the WFPS, the application rate of DMPSA affects its performance in reducing N_2O emissions (Lin and Hernandez-Ramirez, 2020). Although the application rate relative to the amount of N applied with the fertilizer was always the same in our experiment (0.8% of the applied NH_4^+-N), there could also be a relationship between the total NI applied dose and the bacterial response, because the highest fertilizer dose (and thus the highest net NI amount) was applied in the 1st application (80 kg N ha^{-1} and 0.64 kg NI ha^{-1}), diminishing to 60 kg N ha^{-1} (0.48 kg NI ha^{-1}) in the next applications. This might suggest a dose-dependent impact on the bacterial community, which should be considered for further studies.

This is the first time that the impact exerted by DMPP and DMPSA on the bacterial diversity has been analyzed in a temporal framework. Our results indicate that 60 DAF, the soils that received NIs still tended to maintain lower Shannon values than AS (AS = 10.10; ASP = 10.00; ASD = 10.05) (Fig. 3B). This is in agreement with previous work demonstrating the presence of DMP in the soil 50 DAF (Menéndez et al., 2012). There are no conclusive studies regarding the degradation of DMPSA, but our earlier research indicated a capacity to reduce N_2O emissions up to 35 and 42 DAF (Corrochano-Monsalve et al., 2020a, 2021b). Therefore, a longer degradation time than 42 days for this compound can be speculated, coinciding with the results from the current work.

3.2. SOIL WATER CONTENT AND NITRIFICATION INHIBITORS INFLUENCED MICROBIAL CO-OCCURRENCE NETWORKS

Table 1. Topological properties of soil bacterial networks within each treatment at low and high water-filled pore space (WFPS).

WFPS	Treatment	No. of original OTUs	Similarity threshold (St)	R^2 of power law	Network Size (Total nodes)[a]	Percentage of original OTUs[b]	Total edges[c]	Percentage of positive edges	Avg. connectivity (avgK)[d]	Avg. path distance (GD)[e]	Avg. Clustering coefficient (avgCC)[f]	Modularity (M)[g]	No. Modules[h]
Low	C	4969	0.91	0.90	794	15.98	1836	85.24	4.62	5.46	0.13	0.62	115
	AS	3659	0.87	0.94	857	23.42	2033	95.82	4.74	5.34*	0.15*	0.60*	108
	ASP	4094	0.89	0.91	724	17.68	1298	86.29	3.59	**4.84***	**0.12***	**0.64***	121
	ASD	3911	0.89	0.93	691	17.67	1116	97.67	3.23	**7.75*#**	**0.14*#**	**0.77*#**	87
High	C	4054	0.94	0.87	2167	53.45	3851	34.72	3.55	9.59	0.08	0.95	240
	AS	3284	0.94	0.95	1996	60.78	2800	48.54	2.81	11.21*	0.10*	0.94*	229
	ASP	3254	0.88	0.93	1844	56.67	1871	51.04	2.02	**12.96***	**0.05***	**0.95**	285
	ASD	2848	0.94	0.87	1604	56.32	2210	54.70	2.76	**12.15*#**	**0.11*#**	**0.92*#**	198

Indexes legend (Zhou et al., 2011; Deng et al., 2012):

a. *Number of OTUs included in the network.*
b. *Percentage of OTUs included in the network with respect to the number of original OTUs.*
c. *Number of pairwise correlations between OTUs obtained by Pearson's correlation analysis.*
d. *Higher avgK means a more complex network.*
e. *A lower GD means that nodes in the network are closer.*
f. *How well a node is connected with its neighborhood. A value close to 0 means that there are hardly any connections.*
g. *Capability of the nodes to form modules.*
h. *Group of OTUs highly connected among themselves (high density edges) but less linked with OTUs outside the module.*

Bold values indicate significant differences between AS and ASP or ASD (Student-T; $P < 0.0003$). Significant differences between the unfertilized treatment (C) and the fertilized treatments (AS, ASP, ASD) are indicated by asterisk (Student-T; $P < 0.0002$). Significant differences between ASP and ASD are indicated by hash (#) (Student-T; $P < 0.0001$).
C = Unfertilized control; AS = Ammonium Sulphate 21%; ASP = Ammonium Sulphate 21% + DMPP; ASD = Ammonium Sulphate 21% + DMPSA.

Shifts in the abundance of particular microbial groups as a consequence of NI application might cause (or be the result of) a change in microbial relationships beyond compositional aspects. Here we have implemented for the first time a microbial co-occurrence network approach to analyze the modifications in the soil microbe-microbe association patterns as mediated by the application of NIs.

All the soils analyzed showed modularity (M) values above 0.4 (M ranged between 0.60 to 0.77 at low WFPS and between 0.92 to 0.95 at high WFPS) (Table 1), indicating that the networks from the soils studied were modularly structured (Newman, 2006). Modularity measures the degree to which a network is organized into clearly delimited modules, a group of OTUs highly connected among themselves (which might have similar ecological niches, but not necessarily physical interaction) but less linked to OTUs outside the module (Zhou et al., 2011).

Major topological indexes analysis showed significant differences, indicating different patterns of community interaction depending on the WFPS and treatment (Table 1). For instance, the proportion of initial OTUs that were present within the correlation network (i.e. total nodes with respect to original OTUs) was considerably lower under low WFPS (average of 18.4%) than at high WFPS (average of 56.6%), suggesting a highly disconnected microbial community overall under low WFPS conditions. Thus, the network size was considerably larger at high WFPS (average network size, low WFPS = 766 nodes; high WFPS = 1903 nodes). Consequently, the network presented more edges at high WFPS (average number of edges, low WFPS = 1571; high WFPS = 2683), which seems to agree with a greater connection of the soil pores (and thus communities) at high WFPS, in favor to pore connectivity theory (Carson et al., 2010). In the same manner, more modules formed the network at high WFPS (average number of modules, low WFPS = 108; high WFPS = 238), although the relation between the number of modules with respect to total nodes was almost the same at both WFPS conditions (0.14 at low WFPS and 0.12 at high WFPS). In addition, the percentage of positive interactions (positive edges) decreased drastically at high WFPS (average positive edges, low WFPS

= 91.26%; high WFPS = 47.25%), which might be driven by the higher pore connection allowing more adapted bacteria to negatively affect the less competitive.

Our results showed that unfertilized soils (C) presented the lowest percentage of positive edges (85.24% and 34.72% at low and high WFPS respectively), typical of a co-exclusion network in which resources are scarce (Deng et al., 2012). In contrast, AS fertilization increased the percentage of positive edges (95.82% and 48.54% at low and high WFPS respectively), which matches a lower competition for resources due to the addition of nutrients. The co-occurrence network in AS had greater similarity to C at low WFPS, presenting a slight increase in total edges (C = 1836; AS = 2033) and avgK (C = 4.62; AS = 4.74), lower GD (C = 5.46; AS = 5.34; $P < 0.0002$) and lower M (C = 0.62; AS = 0.60; $P < 0.0002$). In contrast, at high WFPS the difference was greater and opposite, showing fewer edges than C (C = 3851; AS = 2800), lower avgK (C = 3.55; AS = 2.81), larger GD (C = 9.59; AS = 11.21; $P < 0.0002$) and lower M (C = 0.95; AS = 0.94; $P < 0.0002$).

When compared with AS, the ASP and ASD treatments had a lower number of nodes (low WFPS, AS = 857; ASP = 724; ASD = 691; high WFPS, AS = 1996; ASP = 1844; ASD = 1604), lower number of edges (low WFPS, AS = 2033; ASP = 1298; ASD = 1116; high WFPS, AS = 2800; ASP = 1871; ASD = 2210), lower avgK (low WFPS, AS = 4.74; ASP = 3.59; ASD = 3.23; high WFPS, AS = 2.81; ASP = 2.02; ASD = 2.76) and greater GD (low WFPS, AS = 5.34; ASP = 4.84; ASD = 7.75; high WFPS, AS = 11.21; ASP = 12.96; ASD = 12.15; $P < 0.0003$) under both levels of soil water content (Table 1). Thus, our results indicate that NI application reduced the network complexity with respect to the fertilizer alone (AS), which is of high relevance because previous studies have suggested that crops might benefit from a more complex microbial network through a greater ability to cope with environmental changes or suppress soil-borne pathogens (Berry and Widder, 2014; Yang et al., 2017; Tao et al., 2018). In the ASP and ASD treatments, the larger GD might hinder rapid communication among different members of the microbial community, and thus the system would respond more slowly to environmental changes (Zhou et al., 2010). On the other hand, the greater M in soils

that receive NIs (not in the case for ASD at high WFPS) (low WFPS, AS = 0.60; ASP = 0.64; ASD = 0.77; high WFPS, AS = 0.94; ASP = 0.95; ASD = 0.92; $P < 0.0003$) is thought to restrict and localize the effects of a disturbance within compartments in the network (Ruiz-Moreno et al., 2006; Zhou et al., 2010). At the same time, short path distances (faster communication) also allow local perturbations to reach the whole network quickly, which could alter the system (Kitano, 2004; Zhou et al., 2010). These changes in the complexity of microbial relationships might also affect the endophytic bacterial community and have an impact on their capacity to cope with stressors. Therefore, future studies should explore this question further.

Interestingly, the ASP and ASD treatments also showed differences between them (Student-T test, $P < 0.0001$). At low WFPS, ASD had a lower avgK, higher GD and clustering coefficients (how well a node is connected with its neighbors), and greater M. At high WFPS, the trend was opposite, because ASD had a higher avgK, lower GD and lower M. When the water content was high, ASD had fewer nodes than ASP but more edges, indicating greater numbers of connections within the community. Moreover, at both low and high WFPS, ASD tended to show a greater percentage of positive edges than the rest of the treatments, which might suggest a lower degree of competition within the ASD soil community.

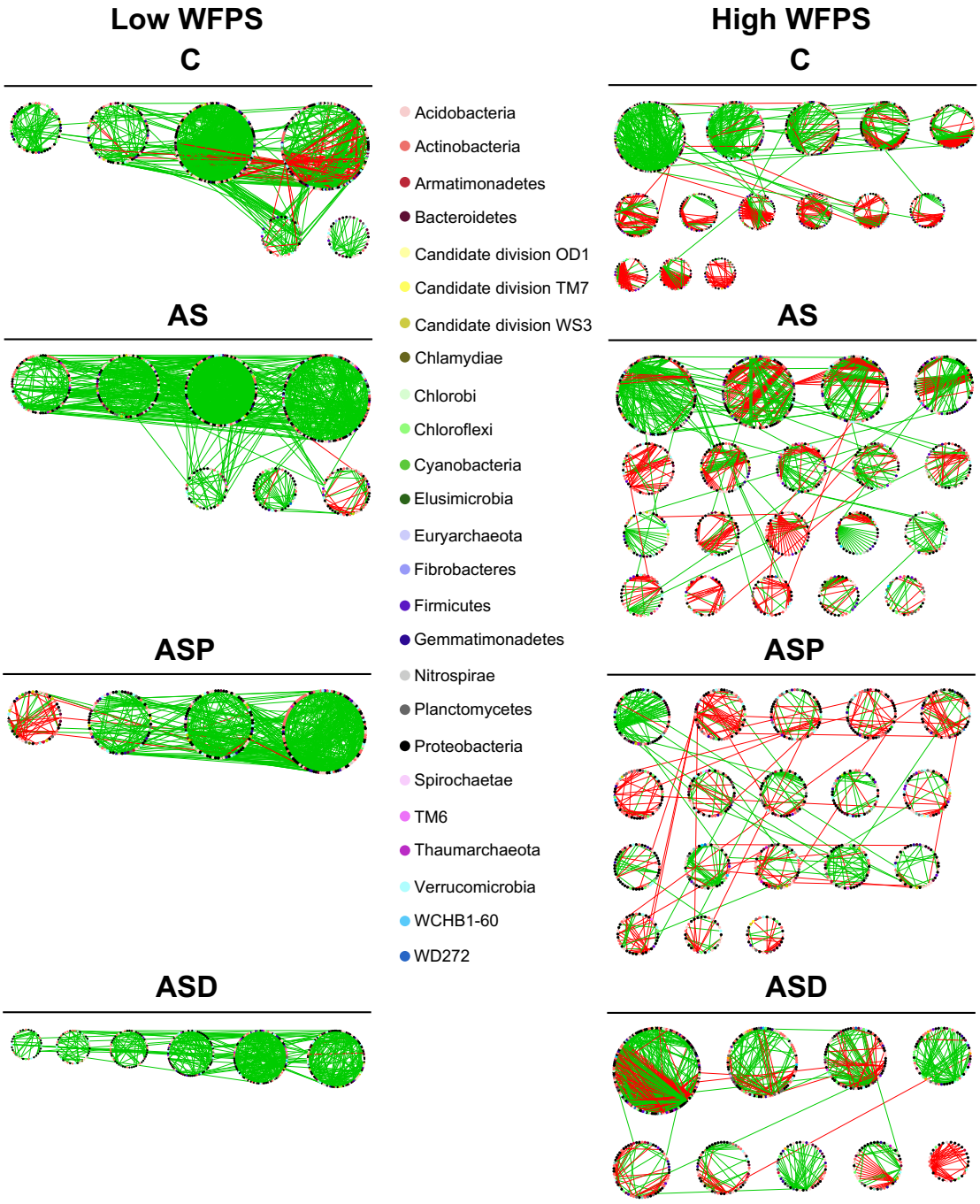


Figure 4. Co-occurrence networks analysis of bacterial community based on the 16S rRNA gene. *Each node represent an OTU colored according to taxonomy (Phylum). Only majority modules (≥ 30 nodes) have been represented. Edges represent significant Pearson correlations (P < 0.05). Green edges correspond to positive relationship between nodes; Red edges correspond to negative relationship between nodes.*

C = Unfertilized Control; AS = Ammonium Sulphate 21%; ASP = Ammonium Sulphate 21% + DMPP; ASD = Ammonium Sulphate 21% + DMPSA.

3.3. FERTILIZATION SURPASSED THE EFFECT OF NITRIFICATION INHIBITORS ON BACTERIAL COMMUNITY STRUCTURE

3.3.1. SHIFTS IN COMMUNITY COMPOSITION EXERTED BY FERTILIZER WITH RESPECT TO UNFERTILIZED SOILS

Table 2. Differences in beta diversity of bacterial community based on analysis of similarity (ANOSIM) test (Bray-Curtis distances). *P-values are based on 999 permutations.*

Factor	Soil WFPS	Groups	*R*	*P*-value
Fertilization	Low	C *vs* AS	0.90	0.001
		C *vs* DMPP	0.88	0.001
		C *vs* DMPSA	0.89	0.001
	High	C *vs* AS	0.82	0.001
		C *vs* DMPP	0.88	0.001
		C *vs* DMPSA	0.79	0.001
Nitrification inhibitor	Low	AS *vs* DMPP	0.04	0.12
		AS *vs* DMPSA	-0.01	0.50
		DMPP *vs* DMPSA	0.06	0.10
	High	AS *vs* DMPP	0.03	0.25
		AS *vs* DMPSA	-0.11	0.05
		DMPP *vs* DMPSA	0.06	0.21

C = Unfertilized control; AS = Ammonium Sulphate 21%; ASP = Ammonium Sulphate 21% + DMPP; ASD = Ammonium Sulphate 21% + DMPSA.

ANOSIM analysis showed significant differences when comparing the C and AS treatments, both at low ($R = 0.90$; $P = 0.001$) and high ($R = 0.82$; $P = 0.001$) WFPS (Table 2), indicating that fertilizer application induced drastic changes in bacterial community composition, as previously suggested by other authors (Pan et al., 2014; Leff et al., 2015). *Proteobacteria* (36% relative abundance), *Actinobacteria* (19%) and *Acidobacteria* (12%) were the dominant phyla in AS soils (Fig. 5A), with less than 4% variation between low and high WFPS conditions. *Verrucomicrobia*, *Planctomycetes*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes*, *Firmicutes* and *Cyanobacteria* comprised the rest of the

phyla, representing more than 1% of the total bacterial abundance. Surprisingly, the *Acidobacteria* population, which is adapted to low pH environments (Lauber et al., 2009), exhibited 18% lower abundance in AS than in C. This same trend was observed in the metadata of Wang et al. (2018), who used this fact to support the concept that soil acidification due to fertilizer would not be the main factor that alters bacterial biomass. The *Acidobacteria* include many oligotrophic members (Fierer et al. 2007), so N addition could work against them. Therefore, relying on this group to determine the role of acidification might not be the most robust option. In contrast, phylum TM7 (currently *Saccharibacteria*), which is also associated with low pH (Zhou et al., 2015; Zhang et al., 2017), showed the greatest shift (+600%) when fertilizer was applied. *Cyanobacteria*, *Armatimonadetes* and *Fibrobacteres* are considered to be the most important taxa for predicting the multifunctionality of an ecosystem (Chen et al., 2020). Multifunctionality is described as the capability to play multiple functions simultaneously, which is critical to maintaining nutrient cycling, and positively correlated with microbial diversity (Wagg et al., 2014; Bender et al., 2016; Delgado-Baquerizo et al., 2017). These three phyla suffered abundance reductions (–32%, *Armatimonadetes*; –59%, *Cyanobacteria*; –55%, *Fibrobacteres*) as a consequence of fertilizer applications, thus suggesting an important degradation of system multifunctionality.

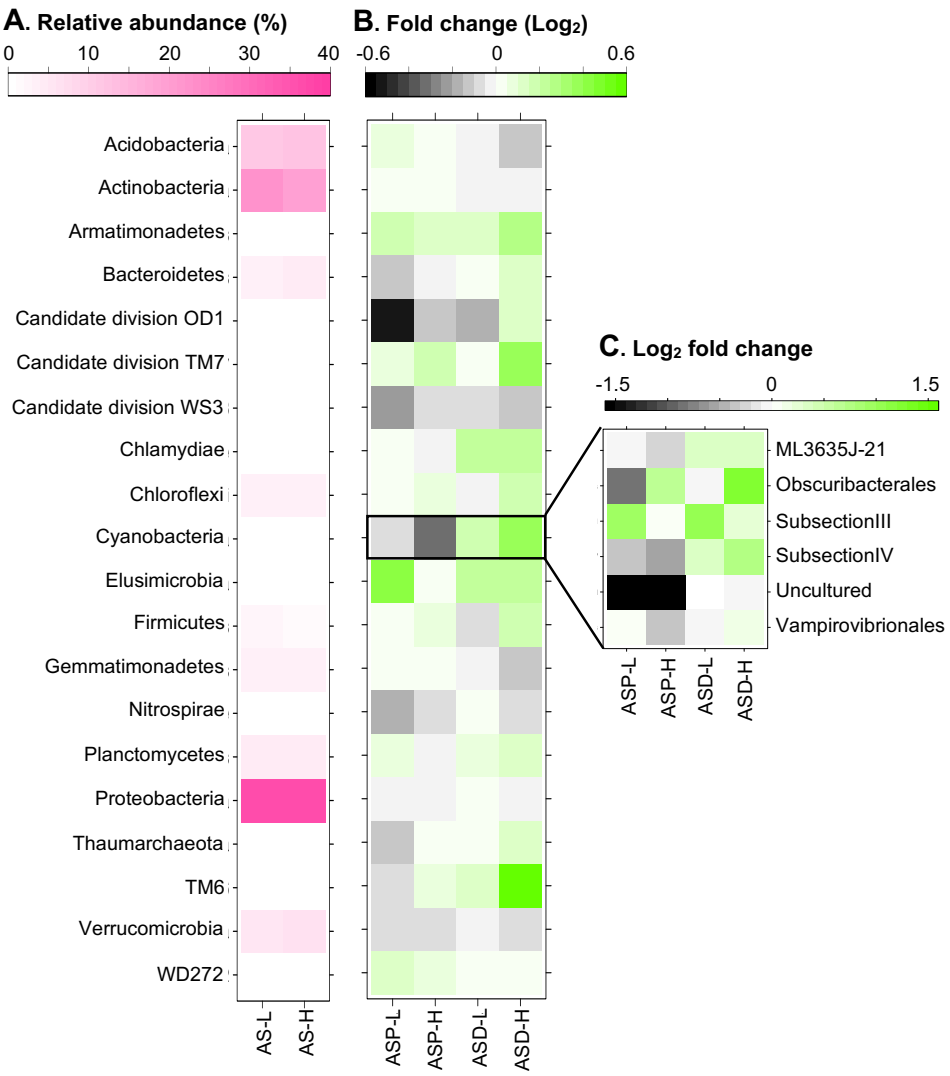


Figure 5. A) Soil bacterial relative abundance at Phylum level in AS treatment. B) Variation of the abundance of the different phyla in soils treated with nitrification inhibitors with respect to AS. C) Variation of the abundance of Cyanobacteria's orders in sols treated with nitrification inhibitors with respect to *AS. AS = Ammonium Sulphate 21%; ASP = Ammonium Sulphate 21% + DMPP; ASD = Ammonium Sulphate 21% + DMPSA; L = Low WFPS; H = High WFPS.*

3.3.2. IMPACT OF NITRIFICATION INHIBITORS ON BACTERIAL COMMUNITY STRUCTURE AND DOMINANT PHYLA

Unlike the results observed for alpha-diversity and co-occurrence networks, almost negligible differences in community composition were detected when comparing the treatments receiving NIs relative to AS. Indeed, the greatest effect was observed in the comparison between the AS and ASD communities at high WFPS (ANOSIM R = –0.11; P = 0.05), but no significant effects were observed in any other statistical comparison (Table 2). The impact of DMPSA on the beta-diversity observed here was smoother than the values reported previously (Corrochano-Monsalve et al. 2020b) at high WFPS. This leads us again to hypothesize that the marked differences between studies might be related to the inhibitor dose applied, since in our earlier work up to 0.96 kg of DMPSA ha^{-1} were provided in a single application (the maximum dose applied in this work was 0.64 kg NI ha^{-1}), which could have generated a greater microbial response. This theory is also supported by Luchibia et al. (2020), who found that DMPP tended to induce more changes at an application rate of 0.96 than at 0.64 or 0.32 kg DMPP ha^{-1}.

The application of NIs did not produce significant changes in the abundance of the main dominating phyla. Nevertheless, it is interesting that the abundance of *Armatimonadetes*, *Cyanobacteria* and *Fibrobacteres* was significantly higher in ASD than in AS (Fig. 5B), suggesting that the negative impact of fertilization on the main phyla related to multifunctionality was partially alleviated by DMPSA application. This shift in bacterial abundance was greater at high WFPS (+21%, *Armatimonadetes*; +30%, *Cyanobacteria*; and +25%, *Fibrobacteres*) than low WFPS (+12%, +10% and +16% respectively). However, while the same trend was observed in the ASP treatment at low WFPS for *Armatimonadetes* (+15%) and *Fibrobacteres* (+19%), there was an opposite effect of DMPP on *Cyanobacteria*, especially at high WFPS (–19% with respect to AS). *Cyanobacteria* represent a very interesting taxon from an agronomic point of view due to their implication in N_2 fixation, their contribution to improving soil physicochemical characteristics, the protection they provide against diseases, and their stimulation of plant growth (Singh et al., 2016). In this study, all orders within the *Cyanobacteria* followed

the same response to DMPSA (Fig. 5C), including a 106% increase in the *Nostoc* genus. Induction of the *Cyanobacteria* population by DMPSA has also been reported under different conditions (wheat crops in non-tilled alkaline soil), suggesting an intrinsic impact of DMPSA on this taxon that might be driven by a higher NH_4^+ content in the soil due to nitrification inhibition (Corrochano-Monsalve et al., 2020b). However, DMPP also maintains NH_4^+ for a longer period (Huérfano et al., 2016; Guardia et al., 2018) and no *Cyanobacteria* induction was observed in the ASP treatment. Therefore, the reasons behind the specific induction of *Cyanobacteria* by DMPSA remains unclear and needs further investigation.

LEfSe analysis also evidenced that the impact of DMPSA was highly dependent on WFPS (Supplemental Fig. S2). While no significant effects were found at low WFPS, several shifts in bacterial abundance were observed at high WFPS (LDA > 2; $P < 0.05$), although the response was smoother overall than observed before (Corrochano-Monsalve et al. 2020b). At the phylum level, *Armatimonadetes* (+21%), *Chloroflexi* (+11%) and TM6 (+53%) abundances were promoted by DMPSA application at high WFPS. Indeed, there seems to be an interrelation between the induced groups. *Armatimonadetes* are a fairly unknown oligotrophic group, but they are usually detected alongside photosynthetic organisms such as *Cyanobacteria* (+30%; LDA = 1.63; $P = 0.1$), *Chloroflexi* and others, suggesting that they take advantage of exudates of these organisms (Dunfield et al., 2012). In turn, *Chloroflexi* is usually associated with *Cyanobacteria* (Abed et al., 2018). In general, *Chloroflexi* encompasses oligotrophic organisms, which are negatively affected by fertilizer (Zeng et al., 2016; Sun et al., 2019). Therefore, our results suggest that the negative impact of fertilizer on oligotrophic and N-fixing bacteria (Ramirez et al., 2010; Chen et al., 2016) seems to be partially alleviated by DMPSA application. It is relevant that Zhang et al. (2017) discussed the same question in relation to DMPP, although we have not found the same trend in our study. TM6, which is a phylum that includes endosymbionts of free-living amoebas such as *Vermamoeba vermiformis* (Delafont et al., 2015; Yeoh et al., 2016), was the most promoted taxa in ASD among the 20 dominant phyla under high WFPS (Fig. 5B and Supplemental Fig. S2). The increase in TM6 might

therefore be related to the observed 63% increase in the abundance of *Vermamoeba vermiformis* in the soils that received DMPSA, which reiterated earlier observations (Corrochano-Monsalve et al. 2020b). Similarly, the *Chlamydiae,* which also encompass endosymbionts of free-living amoebas (Ishida et al., 2014; Yeoh et al., 2016), were also enriched in ASD (Fig. 5B),

3.4. ACTION OF DMPP AND DMPSA ON NITRIFYING AND DENITRIFYING TAXA

3.4.1. AMONG THE NITRIFIERS, THE INHIBITION DEPLOYED BY NIs WAS ALMOST EXCLUSIVE ON NITROSOMONAS

The ASP treatment resulted in a lower abundance of the *Nitrospirae* phylum than the AS treatment at low WFPS (–13%), and a decrease in the *Nitrosomonadales* order was observed in ASD under high WFPS (–18%) (LDA > 2; $P < 0.05$) (Fig. 5B and Supplemental Fig. S2). The identified nitrifying genera represented between 1.24% (low WFPS) and 1.47% (high WFPS) of the total bacterial abundance in AS. Among them, *Planctomycetes*, *Nitrospira*, *Geobacter* and *Anaeromyxobacter* were the dominant groups (all of them involved in NH_2OH conversion to NO_2^- by HAO enzymes) (Kanehisa and Goto, 2000) (Fig. 6A). Almost negligible effects were observed on the most abundant nitrifying genera as a consequence of NI applications to soils (Fig. 6B). In total, the relative abundance of nitrifiers was similar to AS soils at low WFPS (ASP = 1.19%; ASD = 1.24%) and high WFPS (ASP = 1.44%; ASD = 1.49%). Within the genera harboring AMO enzyme (the target of NIs), *Nitrosomonas* presented a higher abundance than *Nitrosospira* (Fig. 6A), even though *Nitrosospira* is often predominant in acid soils (Li et al., 2017). In fact, *Nitrosomonas* underwent the greatest inhibition following application of DMPP or DMPSA (Fig. 6B). This inhibition was observed under both moisture conditions, although it was greater at low WFPS (ASP = 47% of inhibition; ASD = 66%) than at high WFPS (ASP = 38% of inhibition; ASD = 44%). It was notable that other AMO-possessing genera such as *Nitrospira*, *Nitrosospira*, *Nitrosococcus* and *Nitrososphaera* (archaea) did not show the same depletion trend, suggesting that the

action of these NIs to be specific to *Nitrosomonas* and no other nitrifiers. *Nitrosococcus* might have benefited from a reduction in competence due to the decline in *Nitrosomonas*. However, a negligible effect on the nitrification rate of the soil would be expected, since the relative abundance of *Nitrosococcus* in ASP and ASD (~0.001%) was much lower than that of *Nitrosomonas* in AS (~0.06%). No studies have analyzed the direct effect of DMPSA on pure bacterial and archaeal cultures, and only a few have been carried out with the most common NIs (Nitrapyrin, DCD and DMPP). In this sense, similar responses have been observed for Nitrapyrin (Belser and Schmidt, 1981) and DCD (Shen et al., 2013; O'Sullivan et al., 2017), which has a strong capacity to inhibit nitrification by *Nitrosomonas*, but it only causes a slight inhibition of *Nitrosospira*, *Nitrosolobus* and *Nitrososphaera.* Conversely, O'Sullivan et al. (2017) reported a sensitivity of *Nitrosomonas* and *Nitrosospira* to DMPP. Further studies analyzing the effects of DMPP and DMPSA on pure nitrifying bacteria cultures would be essential to determine the exact mode of action of these compounds.

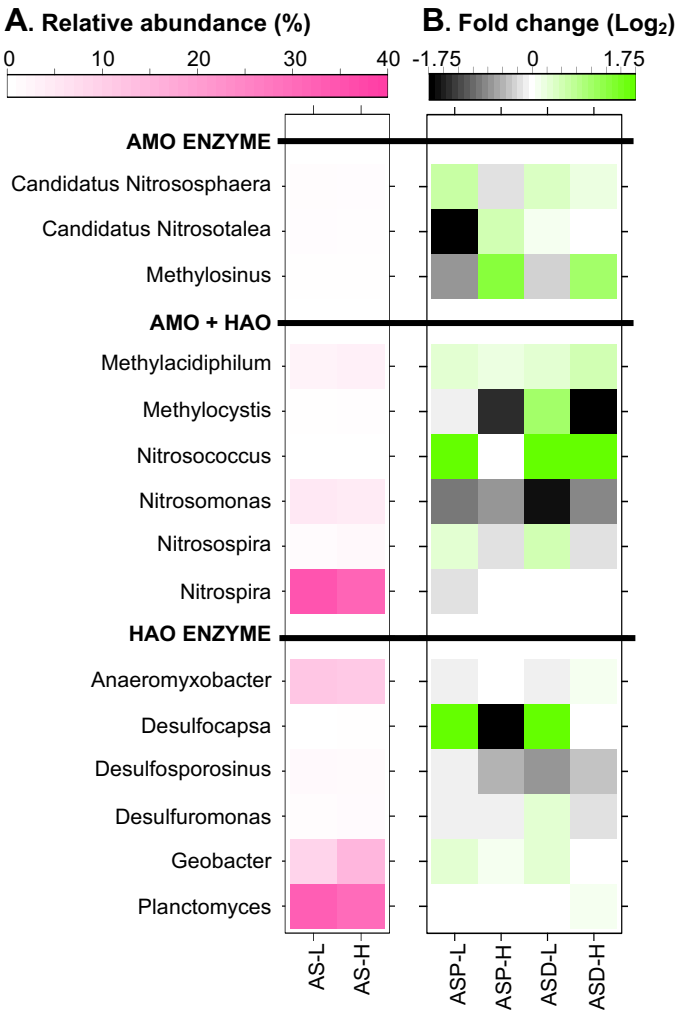


***Figure 6**. A) Relative abundance of identified nitrifying genera with respect to total nitrifiers. B) Variation of the abundance of the different genera in soils treated with nitrification inhibitors with respect to AS.*

AS. AS = Ammonium Sulphate 21%; ASP = Ammonium Sulphate 21% + DMPP; ASD = Ammonium Sulphate 21% + DMPSA; L = Low WFPS; H = High WFPS.

3.4.2. NO GENERAL EFFECT ON DENITRIFYING BACTERIA WAS FOUND

Bacteria harboring one or more denitrifying gene (*nirK*, *nirS*, *norB* or *nosZ*) (Kanehisa and Goto, 2000) accounted for 11.5% (low WFPS) and 11.7% (high WFPS) of the total bacterial abundance. Previous studies have reported effects of both DMPP and DMPSA on denitrifying bacteria harboring *nosZI* genes. These organisms, which are capable of reducing N_2O to N_2, seem to be promoted after fertilizer applications including

DMPP or DMPSA (Torralbo et al., 2017; Corrochano-Monsalve et al., 2020a, 2021a), and this contributes to reducing the negative environmental impact of gaseous N emissions. In the present study, two genera stood out in their abundance: *Gemmatimonas* (harboring *nirK* and *nosZI* genes) and *Sphingomonas* (*nirK* and *norB* genes) (Supplemental Fig. S3A). There were no substantial changes in the abundances of these two genera in either ASP- or ASD-treated soils (Supplemental Fig. S3B).

The diversity of the bacteria involved in the denitrification route resulted in a highly variable response of these genera to NI applications (Supplemental Fig. S3B). For instance, the abundance of three genera harboring both *nirK* and *norB* (*Nitrosococcus*, *Polaromonas* and *Propionibacterium*) tended to be increased by NI application, but the inverse trend was found in *Pseudoxanthomonas* (which harbor the same genes). Furthermore, two genera possessing only *nosZ* genes (*Comamonas* and *Epilithonimonas*) also showed the opposite trend. Therefore, it was not possible to assume a general response of the denitrifying pathway based on these results.

CONCLUSIONS

Soil water content played a key role in the effect of DMPP and DMPSA nitrification inhibitors (NI) on the soil bacterial community. For instance, the application of NIs amplified the negative impact of nitrogen fertilization on bacterial diversity under high moisture conditions, reducing community diversity. Both NIs exhibited a highly specific action on *Nitrosomonas* within the nitrifying organisms, which needs to be further investigated to determine their exact mode of action. However, the response of non-target bacteria to NIs was dissimilar. Although both compounds are based on dimethylpyrazole, our results suggest that their behavior differs in the soil. In this manner, DMPSA decreased to lower rates the bacterial diversity and exerted higher shifts on bacterial community structure. Notably, its application partially alleviated the negative effect of the fertilization on several oligotrophic taxa, which are basic to maintain the soil multifunctionality and, interestingly, induced the enrichment of *Cyanobacteria* populations, a group with agriculturally important potentialities. Our results show that NIs application not only influenced alpha and beta diversity, but also exerted considerable influence on interaction networks by decreasing their complexity.

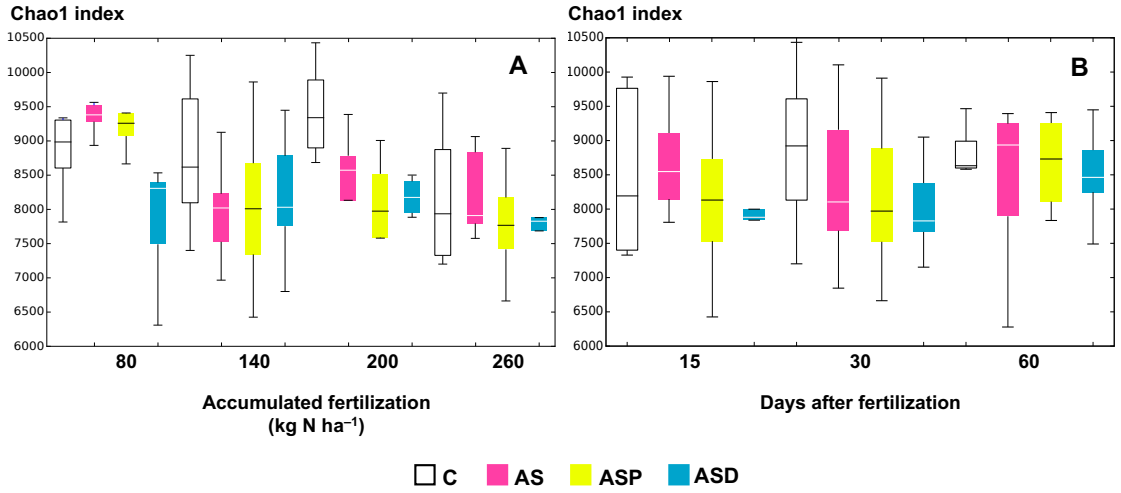


Figure S1. Chao1 alpha diversity index by A) accumulated fertilization and B) days after fertilization. *C = Unfertilized control; AS = Ammonium Sulphate 21%; ASP = Ammonium Sulphate 21% + DMPP; ASD = Ammonium Sulphate 21% + DMPSA.*

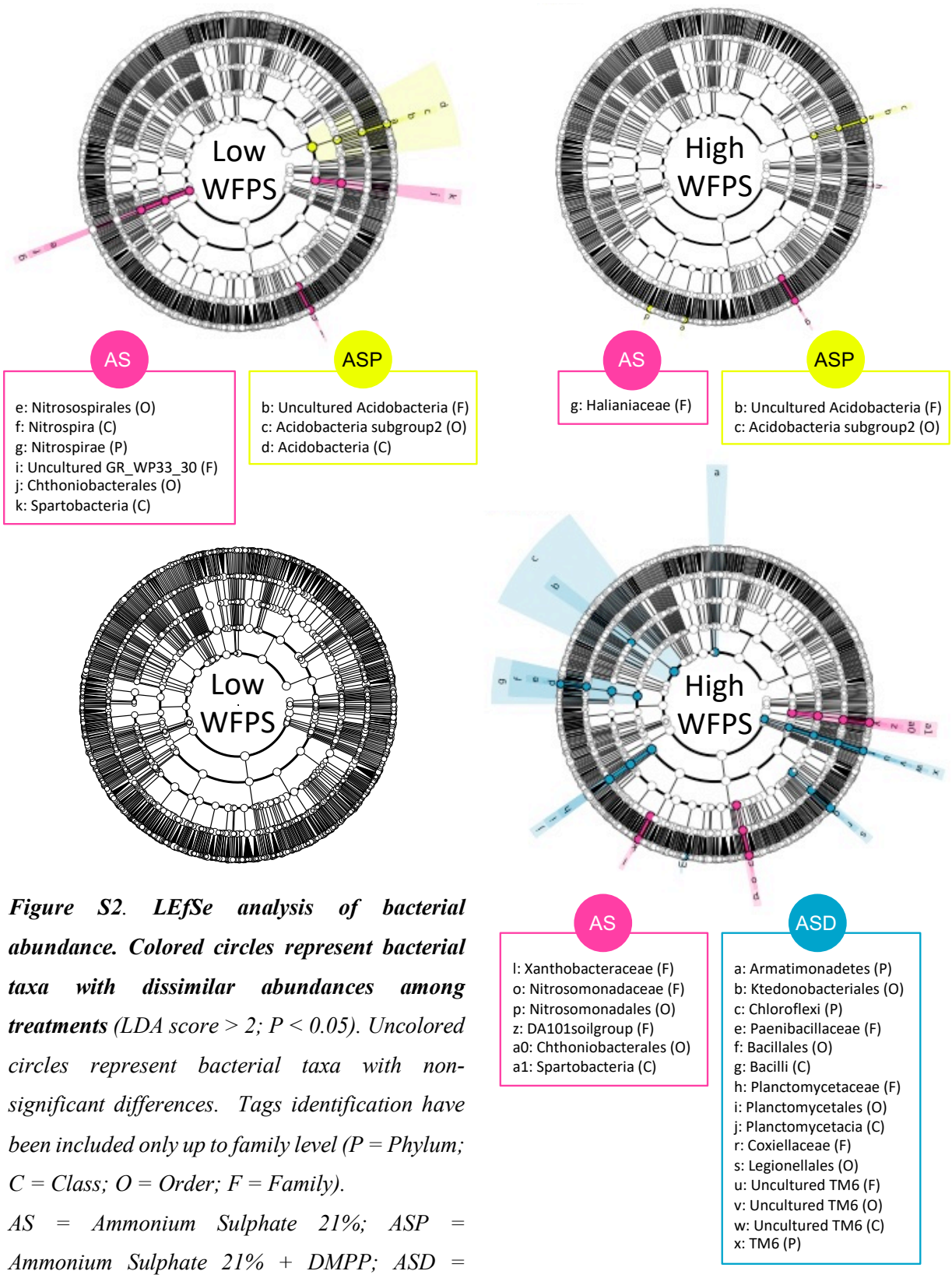


Figure S2. LEfSe analysis of bacterial abundance. Colored circles represent bacterial taxa with dissimilar abundances among treatments *(LDA score > 2; P < 0.05). Uncolored circles represent bacterial taxa with non-significant differences. Tags identification have been included only up to family level (P = Phylum; C = Class; O = Order; F = Family).*

AS = Ammonium Sulphate 21%; ASP = Ammonium Sulphate 21% + DMPP; ASD = Ammonium Sulphate 21% + DMPSA.

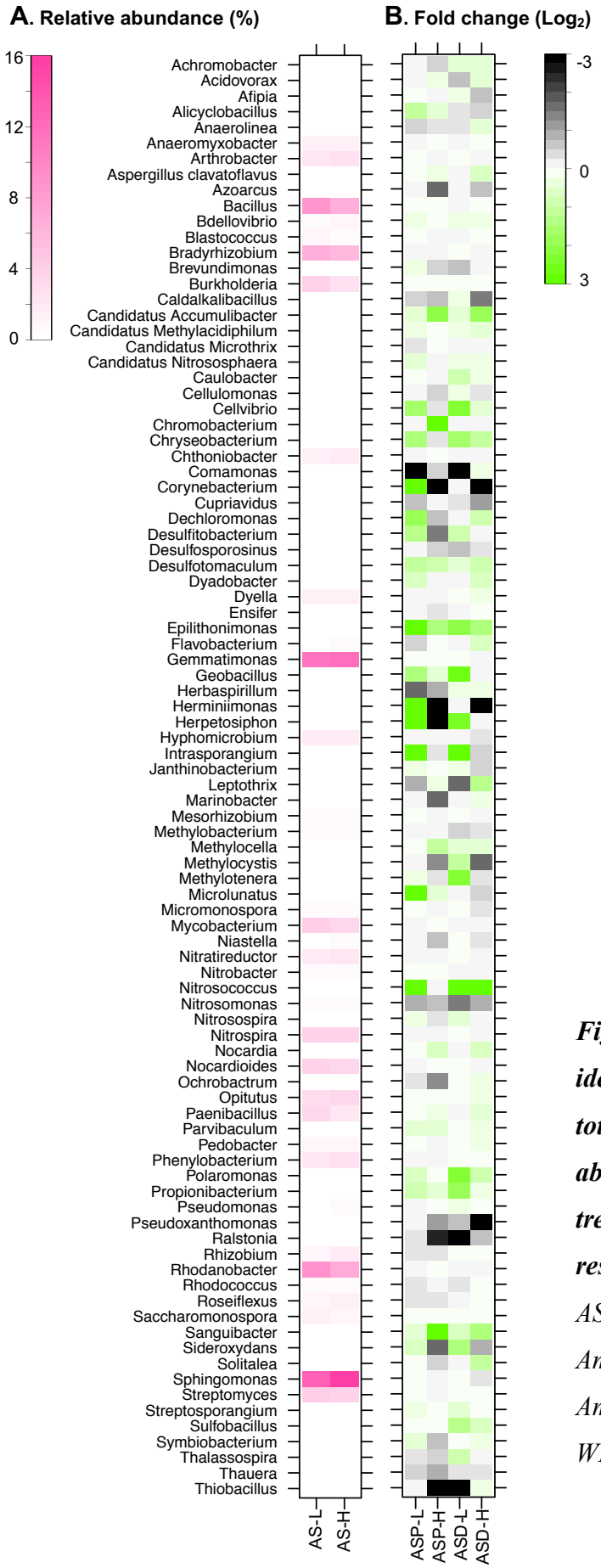


Figure S3. A) Relative abundance of identified denitrifying genera with respect to total denitrifiers. B) Variation of the abundance of the different genera in soils treated with nitrification inhibitors with respect to AS.

AS. AS = Ammonium Sulphate 21%; ASP = Ammonium Sulphate 21% + DMPP; ASD = Ammonium Sulphate 21% + DMPSA; L = Low WFPS; H = High WFPS.

GENERAL DISCUSSION

1. INFLUENCE OF SOIL MANAGEMENT IN THE SUSTAINABILITY OF AGROECOSYSTEMS

Crop yield from no-tillage (NT) systems tends to approach or slightly exceed the yield obtained from conventional tillage (CT), but this is greatly influenced by soil characteristics, crop and weather. In this manner, more positive effects on yield are found as the rainfall decreases from northern to southwestern Europe (Soane et al., 2012). In this work, soil tillage management did not influence the yield of wheat nor rapeseed crops (Chapters 1 and 2). However, tillage management had a marked influence on soil CO_2 and N_2O emissions. This is a consequence of the interaction between different factors, such as crop residues, organic matter content, physical disturbance, root growth, soil density, water content and aeration, which trigger different responses (Ruser et al., 2006; Rochette, 2008; Soane et al., 2012; Plaza-Bonilla et al., 2015). In consonance, the results obtained in this work were not identical between the two field experiments. Total cumulative N_2O emissions in the wheat cropping season (Chapter 1) were equal between both tillage systems. This confirmed the greater sustainability of the NT management, which showed much lower CO_2 emissions (–34%). Oppositely, in the rapeseed season (Chapter 2) the adoption of a NT system seemed less advisable since it tended to a greater global warming potential (GWP). This was as a consequence of the lack of differences in CO_2 fluxes, likely due to the similar water-filled pore spaces (WFPS) between both managements (Linn and Doran, 1984), and because the NT system doubled N_2O emissions and partially increased NH_3 volatilization with respect to CT soils. The analysis of the differences between soil managements was much more revealing when different periods were considered instead of the total accumulated N_2O emissions. In this manner, we realized the great importance of the periods in which soil is tilled (both before sowing and after harvesting). Works prior to sowing, such as tilling or irrigation for soil conditioning, had a drastic impact on N_2O emissions. Thus, in Chapter 1 we observed that a CT conditioning increased N_2O emissions with respect to NT soils, probably because of the soil oxygenation. This same effect could be found when the soil was tilled after harvesting to prepare it for the next cropping season. In contrast, the soil that was

not tilled at all maintained much lower emissions (–70%) after harvesting. However, it was very remarkable to observe that small soil disruption in non-tilled soils (such as the surface disc harrow applied in Chapter 2) can lead to much higher N_2O emissions than those caused by a CT. This can be especially worrisome in soils prone to excessive compaction, since non-tilled soils will require surface-works after a certain time to ensure the correct establishment of the crop. These results indicate that: 1) A tillage system can be more or less sustainable depending on the edaphoclimatic conditions, which means that the most appropriate option has to be determined locally. 2) The emissions derived from the periods in which the fertilizer has not been yet applied can be as important, or even more important, than the emissions derived from nitrogen fertilization to determine the sustainability of the systems in the base of N_2O emissions.

Consequently, it would be interesting to evaluate the ability of nitrification inhibitors (NIs) to avoid N_2O emissions due to soil conditioning, by applying the inhibitor prior to fertilizer application. In general, with these data, we suggest that the application of DMPSA whenever the soil is going to be tilled in some way (being it a moldboard plow in conventional tillage or just a small shallow plow in no-tillage) could be addressed, because these are events that generate large emissions peaks. The feasibility of this mitigating practice would be awkward, since the inhibitor should be applied at the same time as the tilling to avoid an increase in the environmental impact caused by the machinery. Furthermore, it would entail an additional cost for farmers, so it would require awareness campaigns and, probably, incentives for farmers adopting this practice.

In addition to variable effects on N_2O emissions, NT systems also exhibit different effects on soil health. Generally, the effects on the abundance of the main bacterial phyla are subtle, but the effects on bacterial diversity are greater. Some studies indicate that NT management increases bacterial richness and evenness (Silva et al., 2013; Miura et al., 2016; Dong et al., 2017), although there are also examples showing negative effects (Pastorelli et al., 2013; Li et al., 2021; Tyler, 2021; Wipf et al., 2021). In this work, NT soils tended to present a lower bacterial diversity than CT soils. The key might be related to the magnitude of the difference in terms of WFPS between management systems,

especially when one of the systems exceeds a threshold of 56% WFPS promoting the soil pore connectivity (Carson et al., 2010). Thus, in soils where tillage management does not generate differences in WFPS, it would not be expected to find a significant impact on bacterial diversity and structure. On the contrary, bacterial communities in soils with higher WFPS use to present negative correlations (Chapter 5) and promote the dominance of the most competitive organisms as a consequence of the greater soil pore connection (Zhou et al., 2002, 2004; Treves et al., 2003) thus reducing bacterial biodiversity (Chapter 4). In the same manner, there are also dissimilar responses of soil multifunctionality to soil tillage management (Miura et al., 2016; Zhang et al., 2019; Nazaries et al., 2021); so this question has to be further studied.

2. DMPSA'S SUITABILITY TO IMPROVE THE SUSTAINABILITY OF AGROECOSYSTEMS

An important point to note when recommending the application of NIs is that their purpose is to reduce the environmental impact and not to increase the crop yield; at least under our edaphoclimatic conditions. DMPSA did not have any negative effect on wheat (Chapter 1) nor rapeseed (Chapter 2) yield under the two different soil tillage managements, but it did not increase it either. Metadata indicates that NIs such as nitrapyrin, DCD, or DMPP, have almost no effect on crop yield and, if any, tend to increase it slightly (Abalos et al., 2014; Thapa et al., 2016; Li et al., 2018; Gao et al., 2020). The original objective of the application of NIs looks for an increase in the N retention in the soil. By maintaining N for longer in the form of NH_4^+ it is possible to reduce N losses in the form of NO_3^-. However, in areas with a low risk of leaching, the application of NIs does not suppose a great N-saving by this way. Consequently, it is unlikely to find enhanced yields. On the contrary, this benefit could be found in tropical climates and irrigation agriculture, systems susceptible to high leaching rates (Abalos et al., 2014). Although NIs are able to reduce N losses in form of N_2O, the total N amount lost by this way is too small to find a benefit in crop yield as a consequence of the emissions reduction. Moreover, it should be taken into account that in some parts of the

world crops are receiving a N surplus in many occasions (Sun et al., 2012). Furthermore, crop yield was not increased even when applying urease inhibitors to reduce NH_3 volatilization from urea, considering that this did suppose the saving of many kilograms of N. It seems that this was because water was limiting for crop growth, noting that, even with a higher N availability, crop yield can be limited by other factors.

Thanks to the application of DMPSA, it is possible to maintain N_2O emissions at the levels of unfertilized soils, reaching a complete mitigation of the emissions derived from fertilization both when applied with ammonium sulfate (Chapters 1 and 3) or with urea (Chapter 2). Moreover, the application of DMPSA would be especially advisable if irrigation is established or in non-tilled soils, since we have observed a clear interaction between soil water content, N_2O emissions and DMPSA efficiency. A NT management generates a benefit in Mediterranean agroecosystems because of the higher water storage capacity (Moreno et al., 2010; Lampurlanés et al., 2016; Cantero-Martínez et al., 2016). However, under the higher moisture conditions of NT, greater N_2O emissions coming from fertilization take place due to the promotion of simultaneous nitrifying and denitrifying activity. Under these conditions is when DMPSA shows a greater efficiency, probably because it is able to reach deeper soil layers and be distributed in a more homogeneous way (Chapters 4 and 5). In this sense, studies about the distribution of DMPSA along the soil profile, similar to that carried out by Marsden et al. (2016) with DCD and DMPP, could be interesting. As a counterpart, DMPSA application can increase the risk of NH_3 volatilization, which is an effect derived from the use of most NIs, as indicated by meta-analysis (Kim et al., 2012; Pan et al., 2016; Li et al., 2018; Cantarella et al., 2018; Gao et al., 2020). As a consequence of the enhanced NH_3 volatilization, indirect N_2O emissions after NH_3 deposition are also increased. Future studies should give more importance to the measurement of NH_3 volatilization after NIs application and associated indirect N_2O emissions, as their magnitude may exceed that of direct N_2O emissions (Chapter 2). The joint application of NBPT and DMPSA can partially avoid NH_3 volatilization and, consequently, indirect N_2O emissions. However, direct N_2O

emissions are not reduced to the levels of unfertilized soils when applying this double inhibitor.

2.1. ACTION DEPLOYED BY DMPSA TO AVOID N_2O EMISSIONS

DMPSA exhibited a great performance to inhibit the growth of AOB (Chapters 1 and 3). Strikingly, somehow, this inhibition appeared very specific on *Nitrosomonas* and showed almost no effect on other AMO-holding bacteria (Chapter 5). In addition, DMPSA might display different ways to reduce N_2O emissions: by inhibiting nitrification, but also by promoting N_2O reduction to N_2 under certain conditions. The analysis of the data collected along this thesis showed that *amoA*/*nosZI* ratio was lower when DMPSA was applied, both under field (Fig. 1A) and controlled conditions (Fig. 1B). Interestingly, in soils with moisture conditions suitable for denitrification and low copper levels, this shift in the ratio was not only because of the decrease of *amoA* abundance, but also because of an increase of *nosZI* genes. Even more, in some cases the effect of DMPSA was more strongly displayed on *nosZI* genes than on AOB, highlighting the key role of *nosZI* induction for the mitigation of N_2O emissions. Thus, a greater proportion of the N_2O produced by AOB would be completely reduced to N_2. On the contrary, in soils with higher copper or zinc content, the *amoA/nosZI* ratio decreased only due to the inhibition of AOB growth, but not as a consequence of a greater abundance of *nosZI* genes (Fig. 1B).

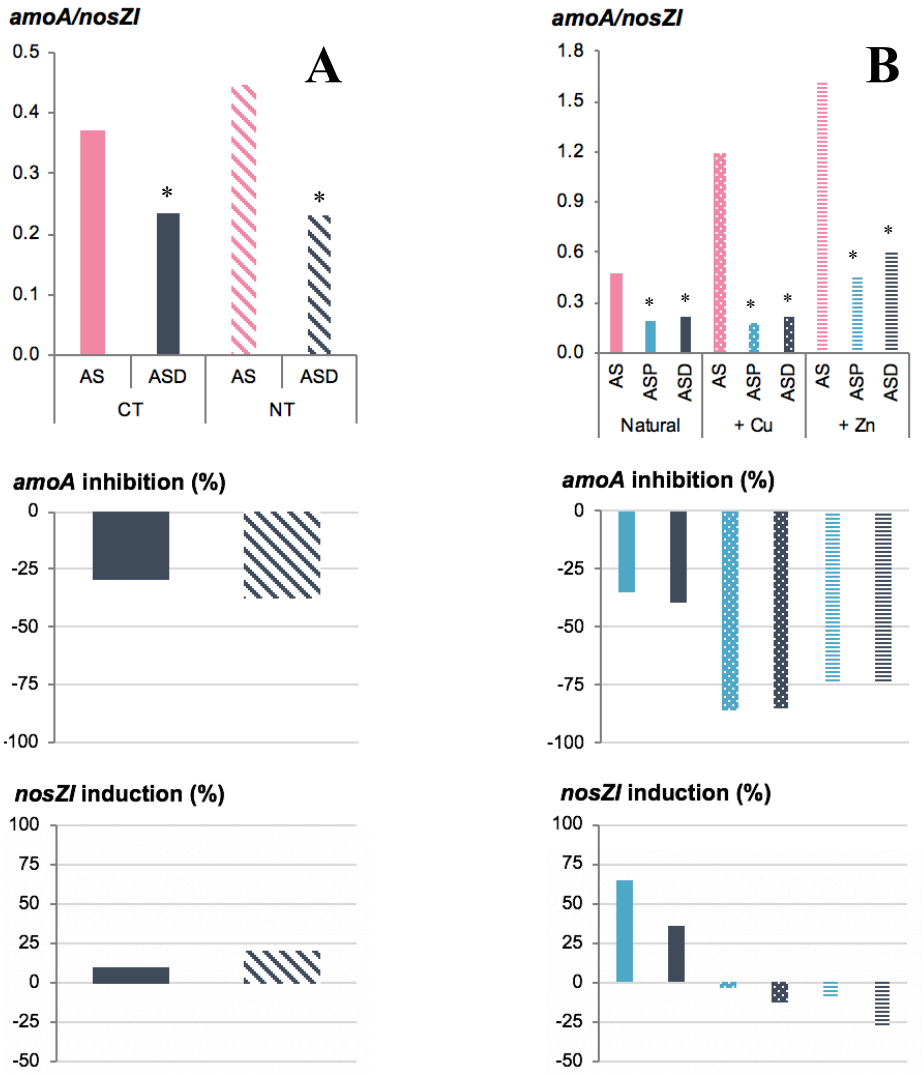


Figure 1. amoA/nosZI genes ratio, amoA inhibition and nosZI induction refers to the gene-abundance variation in soils receiving inhibitors with respect to soils fertilized with ammonium sulfate. A) *Data from Chapter 1 (average data from different sampling timepoints).* ***B)*** *Data from Chapter 3. AS = ammonium sulfate; ASP = ammonium sulfate + DMPP; ASD = ammonium sulfate + DMPSA; Natural = soil with no-added metals; +Cu = natural soil + 8 mg Cu kg^{-1} dry soil; +Zn = natural soil + 8 mg Zn kg^{-1} dry soil. CT = conventional tillage; NT = no-tillage. Asterisks (*) indicate significant differences between AS treatments and ASP or ASD treatments (Student-T test; $P < 0.05$).*

Further experiments will be necessary to elucidate the mechanism for *nosZI* induction, since it can be of huge environmental interest not only for agriculture, but also for some wastewater—treatment systems. It could be related to the preference of complete denitrifiers for further using NO_3^- as an electron acceptor over N_2O in nitrate-rich environments (Blackmer and Bremmer, 1978; Saggar et al., 2013). Thus, under conditions with lower NO_3^- availability as a consequence of nitrification inhibition, complete denitrifiers will find an energetic advantage on reducing N_2O to N_2 and the N_2O/N_2 ratio will be lower (Ruser et al., 2006; Felgate et al., 2012). With these preliminary data, we propose a model based on the competence for Cu (Fig. 2), whose availability is scarce in some agricultural soils (Thomson et al., 2012; Saggar et al., 2013; Ballabio et al., 2018).

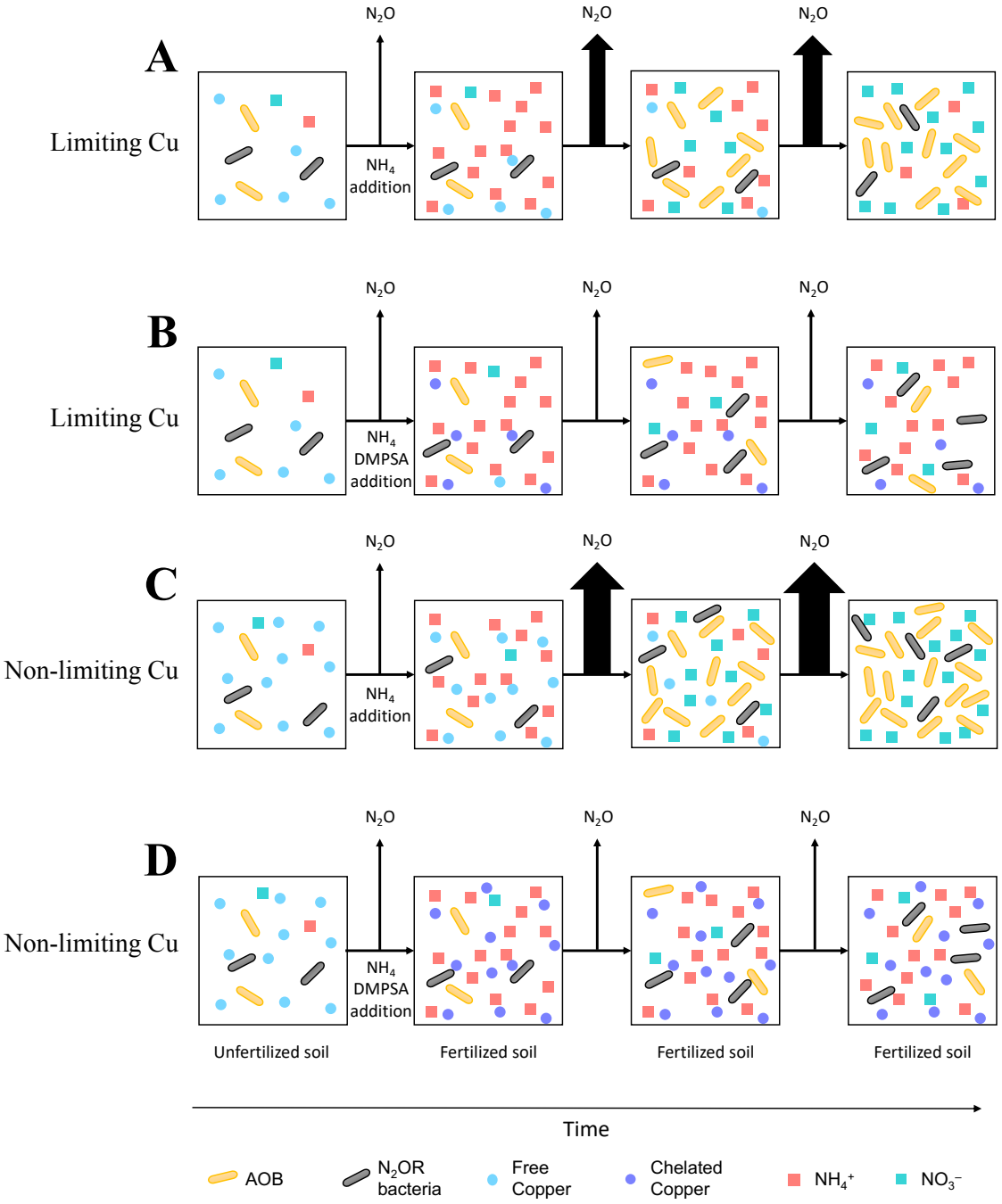


Figure 2. Hypothetical model for DMPSA induction on nosZI genes. A) *Soil with low copper content fertilized with NH_4^+.* ***B)*** *Soil with low copper content fertilized with NH_4^+ and DMPSA. C) Soil with higher copper content fertilized with NH_4^+. D) Soil with higher copper content fertilized with NH_4^+ and DMPSA.*

A) When soils are fertilized with NH_4^+ in copper-limiting soils, AOB population increases because of the higher nutrient availability. During this growth, N_2O is released and Cu is immobilized to satisfy their enzymatic demands. Nonetheless, Cu availability is low, so the increment of the population is limited by Cu. In this manner, when more NO_3^- appears as a consequence of nitrification, denitrifying bacteria also increase their activity, producing more N_2O. Due to the high growth of AOB, almost no Cu is available for the synthesis of N_2OR and, thus, denitrifiers lean towards the use of the abundant NO_3^- as an electron acceptor rather than N_2O, which is not reduced to N_2.

B) When NH_4^+ is applied with DMPSA, Cu is chelated. If the soil presents a low Cu content, AOB population does not increase despite the NH_4^+ input, because of the scarcity of free Cu for the synthesis of AMO. Nevertheless, the already established AOB colonies can nitrify this NH_4^+. As the population size is small, NO_3^- will appear little by little. In this case, N_2OR is synthetized because, due to the low NO_3^- availability, there is an advantage on N_2O reduction to N_2. This implies assuming that Cu remains still available for N_2OR somehow. We speculate that it could be because they can use Cu even bonded to organic ligands such as DMPSA (Twining et al., 2007). In this manner, the ratio *amoA*/*nosZI* decreases, and also the ratio N_2O/N_2 (Fig. 1).

C) In soils with a higher Cu content, AOB exhibit a greater growth when fertilizer is applied because Cu is not limiting for AMO activity. Despite this, there is still enough Cu for the synthesis of N_2OR and, therefore, for N_2O reduction to N_2. In this case, as both nitrifying and complete denitrifying communities have grown, the N_2O/N_2 ratio would depend on which community has grown the most. Since AOB are the first in the chain of reactions, it would make sense a greater and earlier growth of this community taking further advantage of available Cu. Once NO_3^- content increases, less Cu is available for complete denitrifiers, which therefore grow less, which, overall, results in high N_2O losses.

D) If DMPSA is applied in soils with higher Cu content, nitrifying activity is slight because Cu is chelated. Although in this case, the Cu availability is higher than in poor-Cu soils, complete-denitrifiers growth will be limited because N_2O production would be low due to low nitrification and low NO_3^- availability.

2.2. DMPSA EFFECTS ON SOIL MICROBIOME

The effect exerted by DMPSA on N_2O-reducers was not the unique side effect on non-target bacteria. The shifts in the bacterial community were greatly dependent on the soil water content but also on the application dose (Chapters 4 and 5). The impact was larger at high soil moisture, probably because of a greater diffusion of the NI into the soil when WFPS was above 56%, according to the pore connectivity theory (Carson et al., 2010). Thus, bacterial communities from NT soils would be more susceptible to being affected by the application of NIs. On the other hand, the greater effects were observed at the highest application dosages of 0.96 kg DMPSA ha^{-1} (Chapter 4) and 0.64 kg DMPSA ha^{-1} (Chapter 5). Alike other works (Torralbo et al., 2017; Montoya et al., 2021b), our analysis on *16S rRNA* gene abundance and soil CO_2 emissions indicated that DMPSA does not exert a general detrimental effect on soil organisms (Chapters 1, 2 and 3). However, DMPSA promoted a decrease of alpha-diversity metrics at high WFPS with respect to soils fertilized without DMPSA (Chapters 4 and 5). This is of special concern because fertilizer application already encompassed the decreasing of soil diversity compared to unfertilized soils, as is also denoted by metadata analysis (Wang et al., 2018). The decrease of diversity with respect to conventionally fertilized soils was not recovered 60 days after application, so it would be interesting to investigate how long it would take to recover the same diversity. Nonetheless, this effect tended to be greater in terms of evenness (Shannon index) rather than richness (Chao1 index), indicating that the inhibitor is promoting the dominance of some organisms (in terms of abundance) but the system is not losing rare organisms. Although we observed significant shifts in the abundance of some taxa, it must be recognized that there were almost no effects at phylum level, indicating the safety of NIs application for soil health, since they did not exert drastic

consequences. Moreover, our results indicate that DMPSA seems to partially alleviate the negative effect of fertilizer on soil multifunctionality by maintaining a higher abundance of key taxa such as *Cyanobacteria*, *Armatimonadetes*, *Fibrobacteres* and *Chloroflexi*, driving the system to more oligotrophic conditions compared to soils fertilized without the inhibitor. This is a very positive point for the sustainability of the system, but it should be taken into account that it did not reach the levels of unfertilized soils. Surprisingly, this same effect was not observed in the case of DMPP despite their chemical similarity. Although DMPP and DMPSA show similar effects on AOB and N_2O emissions (Torralbo et al., 2017; Huérfano et al., 2016, 2018), they differ in their way of chelating copper (Chapter 3) and also in the effects exerted on the bacterial community (Chapter 5). In this aspect, DMPSA had a greater impact than DMPP on community evenness. But, on the other hand, DMPP did not exhibit positive effects for soil multifunctionality.

It is complicated to set the reason for explaining the effects of the NI on non-target organisms, which probably relies on an union of several factors. Presumably, since all organisms are interconnected, abundance-variations in some of them may affect the rest. Part of these effects could be due to a greater preponderance of NH_4^+ in specific moments, although the data from soil mineral N indicated that differences in NH_4^+ content between soils receiving ammonium sulfate and ammonium sulfate with DMPSA were minor overall. Furthermore, this explanation does not agree with the fact that DMPP did not exert the same effects on the community as DMPSA, which was also surprising (Chapter 5).

It is difficult to think that these impacts could be a direct effect from the inhibitors instead of side effects. Despite in this thesis we have taken steps to resolve some issues with respect to the mode of action of NIs, it is likely that we have opened more hypotheses that were not previously contemplated. The obtained results showed some contradictions. Some of them suggest that DMPP and DMPSA might display a general Cu-chelating capacity. On the other hand, it is complex to understand why other enzymes with Cu needs such as archaeal AMO, pMMO, NirK and N_2OR –at least– are not negatively affected by these NIs. It would be really interesting to study the effects in pure cultures

of organisms harboring these enzymes. Furthermore, additional chemical experiments with different metallic salts revealed that the chelating capacity of DMPP is not copper-specific and exhibits affinity for other divalent cations. In this manner, we were able to isolate single–crystals from cadmium—DMP and cobalt—DMP complexes (Fig. 3). We do not rule out that this affinity might be displayed also by DMPSA and with other cations and, thus, the effect on other organisms might be related to the chelation of other metals by NIs.

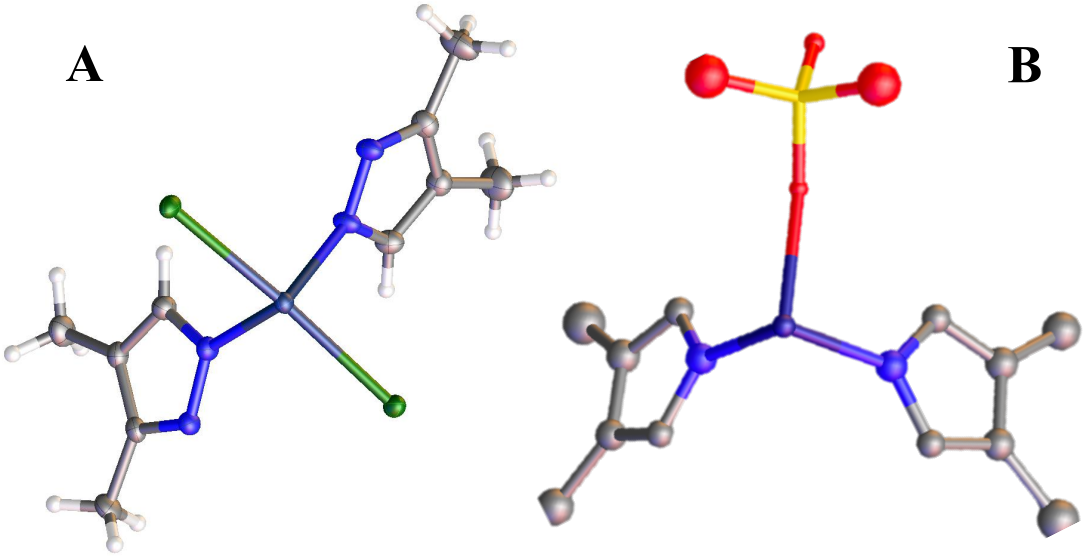


Figure 3. Single-crystal structures isolated from solutions of DMPP with metallic salts ***A)*** *CdDMP complex.* ***B)*** *CoDMP complex. Color code: C, gray; N, light blue; O, red; H, white; Cl, Green; S, yellow; Cd, dark blue; Co, Purple.*

Consequently, it is not dismissed that the application of NIs could interact with the absorption of some elements by plants. Therefore, we highlight the interest for further studies in analyzing the microelements' contents in plant tissues and grain when NIs are applied.

The recommendation for applying NIs in an agricultural system seeking improving sustainability depends on the balance between the associate risks and the achieved benefits. The main risks to be ruled out were negative effects on crops yield and

quality parameters, an amplified environmental impact of fertilizer application due to an increase of NH_3 volatilization, and a detrimental impact on soil health. This thesis demonstrates that DMPSA does not have a negative effect on crops and that the risk for soil health is low since there are no deleterious effects on bacterial consortia nor a drastic impact on community structure. Although DMPSA application does carry the risk for increasing NH_3 losses after fertilization with urea, this can be fought with the joint application with urease inhibitors. Considering the benefits of applying DMPSA: greater N retention by a higher NH_4^+/NO_3^- ratio, decrease in direct N_2O emissions, and a partial remediation of the impact of N fertilizer application on oligotrophic bacteria, the balance between risks and benefits leans towards the recommendation of applying DMPSA for enhancing agriculture sustainability.

CONCLUSIONS

A. INFLUENCE OF SOIL MANAGEMENT IN THE SUSTAINABILITY OF AGROECOSYSTEMS

1. The differences in N_2O emissions between conventional tillage (CT) and no-tillage (NT) systems seem to rely on the differences in the water-filled pore spaces levels (WFPS) between them, which modulate the nitrifying and denitrifying activity. As a consequence of the higher soil compaction and the higher water storage capacity, NT soils tend to present a higher WFPS.
2. The works for soil conditioning before sowing accounts for a large part of the N_2O emitted during a cropping season, and can be even greater than the emissions derived from fertilizer application. In this sense, CT promotes higher N_2O emissions when plowing, but this can be compensated by the usual lower WFPS, which reduces emissions during crop growing. On the other hand, a NT management avoids the emissions derived from plowing, but can increase direct N_2O release (because of the higher WFPS promoting higher rates of nitrification and denitrification) and indirect N_2O emissions (due to a greater vulnerability to NH_3 volatilization).
3. The sustainability of the agroecosystems can be improved by choosing the most suitable management for each particular edaphoclimatic condition. In this manner, a NT system can be an efficient management to be adopted under Humid Mediterranean conditions. However, it should be accompanied by the application of nitrification inhibitors and also by urease inhibitors when fertilizing with urea. In addition, this management may not be the most appropriate solution for soils with a tendency to compact, since the soil disruption with a superficial disc harrow to facilitate the implantation of the seed caused a drastic release of N_2O.
4. The tillage management can also exert an impact on the soil microbiome, which is greatly determined by the differences in soil WFPS between tillage systems. NT system exhibits a lower microbial diversity probably because of the higher WFPS connecting soil pores, which favors the most competitive organisms.

B. DMPSA NITRIFICATION INHIBITOR SUITABILITY TO IMPROVE THE SUSTAINABILITY OF AGROECOSYSTEMS

1. DMPSA does not produce negative nor positive effects on wheat and rapeseed yield nor quality parameters.
2. The best option to reduce nitrogen losses caused by urea application and the associated environmental impact is the joint application of NBPT and DMPSA since, in this way, both NH_3 volatilization and N_2O emissions are reduced. Indirect N_2O emissions derived from NH_3 volatilization play a key role in determining the sustainability of the system, and should be further considered. Individually, DMPSA is able to avoid the increase of N_2O emissions derived from ammonium sulfate and urea fertilization to the levels of unfertilized soils, but does not reduce NH_3 volatilization, and can even increase it.
3. WFPS plays a key role in determining DMPSA performance. Its application is more efficient in reducing N_2O losses at higher WFPS, probably because of a greater infiltration and more homogeneous distribution into the soil. In this manner, DMPSA can be especially effective in NT systems.
4. DMPSA application is especially advisable in soils with relatively high copper and zinc contents since these soils promote greater N_2O emissions.
5. Although DMPSA does not exert general deleterious effects, it cannot be considered as an inhibitor with an exclusive effect on nitrifying bacteria, since an impact is exerted on non-target bacteria, the magnitude of which depends on the WFPS. While at low WFPS the impact is negligible, at high WFPS there is a detrimental effect on bacterial diversity that exceeds that caused by nitrogen fertilizer.
6. Nitrogen fertilization benefits copiotrophic bacterial populations rather than oligotrophic, but DMPSA can partially alleviate this effect by promoting oligotrophic bacteria and favoring key taxa associated with the maintenance of the system multifunctionality.

C. MECHANISM OF ACTION OF NITRIFICATION INHIBITORS BASED ON DIMETHYLPYRAZOLE - THE ROLE OF COPPER

1. In copper-poor soils, the way for reducing N_2O emissions by DMPP and DMPSA relies on both the inhibition of ammonia-oxidizing bacteria but also in the induction of bacteria capable to reduce N_2O to N_2. This might be related to the ability of DMPP and DMPSA for copper chelation.
2. Chemical analysis suggests that DMPP and DMPSA might be acting in different modes and/or as different compounds in the soil, which might explain the dissimilar effects exerted on the bacterial community.

VALIDATION OF HYPOTHESIS

- Initial Hypothesis 1: The structural differences between tilled and non-tilled soils might lead to a dissimilar performance of DMPSA to avoid N_2O emissions depending on the soil management practice.

We corroborated that **tillage influences both the soil N_2O emissions and bacterial communities as a consequence of the variations in soil WFPS. The application of DMPSA is even more necessary and can reach a higher mitigation of N_2O emissions under non-tilled systems.**

- Initial Hypothesis 2: DMPSA will increase the risk of NH_3 volatilization. This risk could be greater in non-tilled soils because of a slower infiltration of urea as a consequence of higher soil density. The joint application of DMPSA with a urease inhibitor (NBPT) could counteract this effect and be especially advisable when applying a no-tillage management.

Our results confirmed that **DMPSA can increase NH_3 volatilization but, contrarily to our initial hypothesis, this was the case under conventional tilled but not in non-tilled soils. As expected, the joint application of DMPSA with NBPT was able to counteract this effect.**

- Initial Hypothesis 3: The action of both DMPP and DMPSA should be based on the same mechanism: copper-chelation. Thus, the efficiency of both inhibitors will vary depending on the soil copper content.

It was proved that both **DMPP and DMPSA have the ability to chelate copper and that their percentage of nitrifying inhibition increase with higher soil copper contents. However, our experiment was not far-reaching enough to unequivocally confirm that nitrification inhibition is based on this mechanism. Moreover, our results indicated that DMPP and DMPSA can be acting as different compounds and/or in different modes in the soil.**

- Initial Hypothesis 4: Since these inhibitors alter the nitrogen cycle and could deploy a chelating action, this can lead to side effects on non-target microorganisms. However, we expected the impact on the bacterial community to be subtle and reversible over time since these products are quickly degraded in the soil.

We demonstrated that **DMPP and DMPSA can exert side effects on non-target bacteria. The impact was subtle in the abundance of the main phyla. However, the effect on bacterial diversity was greater than expected.**

As a global result of this thesis, we are able to confirm our main initial hypothesis: **NITRIFICATION INHIBITORS BASED ON DIMETHYLPYRAZOLE ARE USEFUL AND SAFE TOOLS FOR INCREASING AGRICULTURE SUSTAINABILITY BY MITIGATING THE ENVIRONMENTAL IMPACT DERIVED FROM NITROGEN FERTILIZATION.**

CONCLUSIONES

A. INFLUENCIA DEL MANEJO DEL SUELO EN LA SOSTENIBILIDAD DE LOS AGROECOSISTEMAS

1. La diferencia de emisiones de N_2O entre sistemas de laboreo convencional (LC) y no-laboreo (NL) parecen depender de las diferencias en el nivel de espacios porosos del suelo rellenos de agua (EPRA) entre ambos manejos, lo cual modula la actividad nitrificante y desnitrificante. Como consecuencia de una mayor compactación y una mejor capacidad de almacenamiento de agua, el LC tiende a presentar un mayor porcentaje de EPRA.
2. Las labores para acondicionar el suelo antes de la siembra son precursoras de una gran parte de las emisiones de N_2O acumuladas a lo largo de una temporada de cultivo, pudiendo sobrepasar incluso las emisiones totales derivadas de la fertilización nitrogenada. En este sentido, el LC promueve mayores emisiones de N_2O cuando el suelo es labrado, pero esto puede ser compensado por unos niveles de EPRA usualmente más bajos que hacen que las emisiones durante el crecimiento del cultivo sean menores.
3. La sostenibilidad de los agroecosistemas puede ser mejorada eligiendo el manejo más adecuado para cada condición edafoclimática específica. El sistema de NL puede ser un manejo recomendable para ser adoptado bajo condiciones de clima Mediterráneo Húmedo. Sin embargo, debe ser acompañado por la aplicación de inhibidores de la nitrificación, y también por inhibidores de la ureasa cuando se fertiliza con urea. Por otro lado, este manejo puede no ser la opción más apropiada en suelos que tiendan a tener una compactación excesiva, ya que las pequeñas labores superficiales que serán necesarias para el correcto establecimiento de la semilla causan una drástica liberación de N_2O.
4. El manejo del laboreo también puede generar un impacto en el microbioma del suelo, lo cual está altamente determinado por las diferencias de EPRA entre los manejos. Los suelos sometidos a un NL presentan una menor diversidad

microbiana, probablemente por el mayor EPRA aumentando la conexión entre los poros del suelo, lo cual favorece a los organismos más competitivos.

B. IDONEIDAD DEL INHIBIDOR DE LA NITRIFICACIÓN DMPSA PARA MEJORAR LA SOSTENIBILIDAD DE LOS AGROECOSISTEMAS

1. El DMPSA no produce efectos negativos ni positivos en el rendimiento y parámetros de calidad del trigo ni de la colza.
2. La mejor opción para reducir las pérdidas de nitrógeno y el impacto ambiental causado por la fertilización con urea, es la aplicación conjunta de DMPSA y el inhibidor de la ureasa NBPT. De esta manera, se consiguen reducir tanto las emisiones de N_2O como la volatilización de NH_3. Las emisiones indirectas de N_2O derivadas de la volatilización de NH_3 juegan un papel clave para determinar la sostenibilidad del sistema y deberían, por ello, ser más tenidas en cuenta. Individualmente, el DMPSA es capaz de evitar las emisiones de N_2O producidas por la fertilización con sulfato amónico o urea, pero no reduce la volatilización de NH_3, pudiendo incluso incrementarla.
3. El EPRA es un factor determinante para el rendimiento del DMPSA. La aplicación de este inhibidor para reducir las emisiones de N_2O es más eficiente a mayor EPRA, probablemente como consecuencia de una mejor infiltración y una distribución más homogénea en el suelo. Por ello, el DMPSA puede ser especialmente efectivo en sistemas de NL.
4. La aplicación de DMPSA es especialmente recomendable en suelos con un contenido relativamente alto de cobre y/o zinc, ya que estos suelos presentan mayores tasas de emisión de N_2O.
5. Aunque el DMPSA no produce un efecto deletéreo generalizado, no puede considerarse como un inhibidor con efecto exclusivo en las bacterias nitrificantes, ya que existe un impacto en bacterias no-objetivo. La magnitud de este impacto

depende del nivel de EPRA. Mientras que a bajo EPRA el impacto es insignificante, a alto EPRA hay un efecto perjudicial sobre la diversidad bacteriana, que excede al causado por el fertilizante nitrogenado.

6. La fertilización nitrogenada beneficia a las bacterias copiotrofas por encima de las oligotrofas, pero el DMPSA puede aliviar parcialmente este efecto promoviendo la abundancia de bacterias oligotrofas y favoreciendo taxones asociados con el mantenimiento de la multifuncionalidad del sistema.

C. MECANISMO DE ACCIÓN DE LOS INHIBIDORES DE LA NITRIFICACIÓN BASADOS EN DIMETILPIRAZOL – EL ROL DEL COBRE

1. En suelos pobres en cobre, la vía para reducir las emisiones de N_2O por parte del DMPP y el DMPSA se basa tanto en la inhibición de las bacterias oxidantes del amonio como en la inducción de las bacterias capaces de reducir el N_2O hasta N_2. Esto podría estar relacionado con la capacidad de estos inhibidores para quelar cobre.
2. Los análisis químicos sugieren que el DMPP y el DMPSA podrían actuar de modo diferente y/o en forma de diferentes compuestos en el suelo, lo cual podría explicar los diferentes efectos producidos por cada uno de ellos en la comunidad bacteriana.

VALIDACIÓN DE HIPÓTESIS

- Hipótesis inicial 1: las diferencias estructurales ente suelos labrados y no labrados podría derivar en una variación del rendimiento del DMPSA para evitar las emisiones de N_2O dependiendo del tipo de manejo del suelo.

Hemos corroborado que **el laboreo del suelo influye tanto en las emisiones de N_2O como en las comunidades bacterias a consecuencia de las variaciones en el porcentaje de espacios porosos del suelo rellenos de agua. La aplicación de DMPSA es incluso más necesaria, y puede alcanzar una mayor mitigación de emisiones de N_2O, en sistemas de no-laboreo.**

- Hipótesis inicial 2: el DMPSA incrementará el riesgo de volatilización de NH_3. Este riesgo podría ser mayor en suelos no labrados debido a una infiltración de la urea más lenta como consecuencia de una mayor densidad del suelo. La aplicación conjunta de DMPSA con el inhibidor de la ureasa NBPT podría contrarrestar este efecto y ser especialmente recomendable en sistemas de no-laboreo.

Nuestros resultados confirman que **el DMPSA puede incrementar la volatilización de NH_3, pero, al contrario de nuestra hipótesis inicial, esto tuvo lugar en suelos convencionalmente labrados y no en suelos no labrados. Como se esperaba, la aplicación de DMPSA junto con NBPT fue capaz de contrarrestar este efecto.**

- Hipótesis inicial 3: la acción tanto del inhibidor DMPP como del DMPSA debe estar basada en el mismo mecanismo: la quelación de cobre. Así, la eficiencia de ambos inhibidores variará dependiendo del contenido de cobre del suelo.

Se ha probado que **tanto el DMPP como el DMPSA tienen la capacidad para quelar cobre, y que su porcentaje de inhibición de la nitrificación se incrementa cuando el contenido de cobre en el suelo es mayor. Sin embargo, nuestros experimentos no tuvieron el alcance suficiente para confirmar con rotundidad que la inhibición de la nitrificación se basa en este mecanismo. De hecho, nuestros resultados indicaron que el DMPP y el DMPSA podrían actuar como diferentes compuestos y/o en diferentes modos en el suelo.**

- Hipótesis inicial 4: dado que estos inhibidores alteran el ciclo del nitrógeno y pueden ejercer una acción quelante, esto podría derivar en efectos colaterales en organismos no-objetivo. Sin embargo, nosotros esperamos que el impacto en la comunidad bacteriana sea ligero y reversible en el tiempo, ya que estos productos son rápidamente degradados en el suelo.

Hemos demostrado que **el DMPP y el DMPSA puede generar efectos colaterales en bacterias no-objetivo. Aunque el impacto producido en la abundancia de los principales filos bacterianos fue ligero, el efecto en la diversidad bacteriana fue mayor de lo esperado.**

Como resultado global de esta tesis, podemos confirmar nuestra principal hipótesis inicial: **LOS INHIBIDORES DE LA NITRIFICACIÓN BASADOS EN DIMETILPIRAZOL SON HERRAMIENTAS ÚTILES Y SEGURAS PARA INCREMENTAR LA SOSTENIBILIDAD DE LA AGRICULTURA MEDIANTE LA MITIGACIÓN DEL IMPACTO AMBIENTAL DERIVADO DE LA FERTILIZACIÓN NITROGENADA.**

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