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

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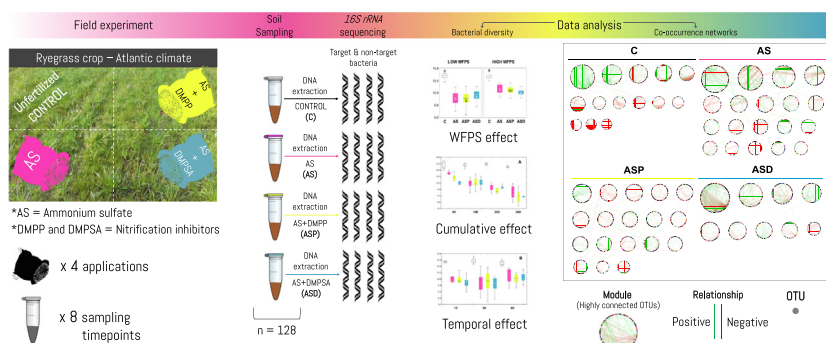[b] Department of Genetics, Physical Anthropology & Animal Physiology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Leioa, Spain
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HIGHLIGHTS

- Soil water content played a key role in the effect of DMPP and DMPSA on soil bacteria.
- Both nitrification inhibitors (NIs) shifted non-target bacteria abundances.
- The effect of NIs within nitrifiers was highly focused on *Nitrosomonas*.
- DMPSA decreased bacterial-community richness and evenness at high soil moisture.
- NIs shifted bacterial interaction networks by decreasing their complexity.

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%),

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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.

1. Introduction

Nitrogen (N) fertilizer application is necessary to achieve higher yields in crops and guarantee food security. However, it also modifies several biotic and abiotic parameters of agricultural soils. As a consequence, microbial diversity, structure and relationships are altered. The soil microbiome plays a key role in the maintenance of ecosystem functionality (Brussaard, 1997; Van der Heijden et al., 2008), and the fertilizer type, composition and dose applied all modulate the response of this community (Hartmann et al., 2015; Zhou et al., 2015; Tao et al., 2018). On the one hand, fertilizer use indirectly affects the bacterial community by changing soil carbon levels and chemical composition (Liu et al., 2016), root development (Peng et al., 2017) and soil pH (Guo et al., 2010). On the other hand, bacteria are directly affected by nutrient availability. In this manner, fertilization favors groups adapted to nutrient-rich environments (Fierer et al., 2007). Furthermore, the response of the bacterial consortia to N fertilization varies depending on the initial characteristics of the soil (Wang et al., 2018). Oligotrophic bacteria play a key role in the maintenance of system multifunctionality, which is described as the capability to conduct multiple functions simultaneously, and is critical to nutrient cycling (Wagg et al., 2014; Bender et al., 2016). Thus, multifunctionality is negatively affected by the external N input, which provides a less favorable environment for oligotrophic bacteria. In contrast, copiotrophic populations are promoted due to their fast growth rates (Fierer et al., 2007). The vast diversity of soil microorganisms is interconnected and maintain a complex network of relationships that encompasses predation, competition and mutualism, all driving biogeochemical cycles and ecosystem functioning (Montoya et al., 2006; Olesen et al., 2007). Thus, an alteration to part of the community, for instance due to external factors such as agricultural practices, can trigger a chain reaction in the rest of the associations. Co-occurrence network construction is a powerful tool to analyze these relationships (Deng et al., 2012). However, there are very few studies that have used this approach to analyze the response of the whole soil bacterial community to fertilizer (Barberán et al., 2012; Tao et al., 2018; Yu et al., 2019).

Synthetic nitrification inhibitors (NIs) can be applied alongside fertilizers to improve N use efficiency. These products delay NH_4^+ oxidation by the copper-dependent ammonia-oxidizing enzyme (AMO), which is harbored by ammonia-oxidizing bacteria (AOB) (Arp and Stein, 2003). By keeping N in the form of NH_4^+, NO_3^- production and N_2O emissions resulting from the nitrification process are reduced. As a consequence, leaching risk is diminished and less substrate is available for denitrifying bacteria, which sequentially reduce NO_3^- to NO, N_2O and N_2. Nevertheless, 40% of denitrifiers lack the copper-dependent nitrous-oxide reductase enzyme (N_2OR), capable of reducing N_2O to N_2 (Hallin et al., 2018). Thus, a great amount of N is lost as N_2O, which has a global warming potential 265 times higher than CO_2 over a 100-year time frame (IPCC, 2014). The use of NIs such as 3,4-dimethyl-1H-pyrazol dihydrogen phosphate (DMPP) and 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), two copper-chelating compounds (Corrochano-Monsalve et al., 2021a), has demonstrated great efficiency in reducing N_2O losses in many types of soils and crops (Pfab et al., 2012; Huérfano et al., 2018; Recio et al., 2019; Sha et al., 2020). The impact of DMPP and DMPSA is believed to be specific to AOB, since the abundance of this population decreases after NI application, while the total bacterial abundance is not affected (Ruser and Schulz, 2015;

Corrochano-Monsalve et al., 2020a; Luchibia et al., 2020). However, recent studies have found unexpected effects on the denitrifying pathway, since the abundance of *nosZI* genes (encoding for N_2OR) is increased after DMPP and DMPSA application (Torralbo et al., 2017; Corrochano-Monsalve et al., 2020a, 2021a). Moreover, the shifts in AOB can either directly or indirectly impact other bacteria, and therefore it is necessary to assess NI effects on the entire bacterial community. However, there are only three preliminary studies in the literature that address the effects of NIs on bacteria other than nitrifiers. While Zhang et al. (2017a) and Luchibia et al. (2020) reported almost no effects of DMPP on soil bacterial consortia, our earlier work (Corrochano-Monsalve et al., 2020b) documented that the application of DMPSA with ammonium sulfate as fertilizer affected both bacterial diversity and structure, a result that should be taken into account, since the application of fertilizer alone also decreases the microbial diversity (Wang et al., 2018). Furthermore, the impact of NIs on bacterial consortia was evidenced to be highly dependent on soil water content (Corrochano-Monsalve et al., 2020b). When the water-filled pore space (WFPS) in the soil reaches above 56%, the soil pores are connected, and this affects microbial distribution and facilitates the diffusion of substances such as NIs (Carson et al., 2010). In agreement, we previously observed a greater impact of DMPSA on the bacterial community when the WFPS was above 56% (Corrochano-Monsalve et al., 2020b), which was probably due to a greater diffusion of DMPSA into the soils studied. The same authors also evidenced a striking increase in the relative abundance of Cyanobacteria after DMPSA application, which deserves further analysis given its possible benefits for agriculture (Singh et al., 2016). Therefore, it is necessary to investigate whether these responses are replicated in soils with different physicochemical characteristics, different climates and different crops.

Thus, in this study, we analyzed the effect of both of the NIs, DMPP and DMPSA, on the entire bacterial community through *16S rRNA* amplicon sequencing. We provide the first comparison of the magnitude of the impact exerted by the fertilizer itself on soil bacterial consortia to that of fertilizers combined with the inhibitors. In addition, we evaluated the effects of their recurrent application on bacterial diversity, addressing whether these shifts revert over time or, on the contrary, whether the changes are stable/higher as fertilizer accumulates. In addition, NI modifications to microbial relationships (co-occurrence and co-exclusion) were studied. To the best of our knowledge, this is the first time that a co-occurrence network approach has been applied to assess the effect of NIs on the soil bacterial community. We hypothesized that i) the impact of the application of NIs on microbial diversity, structure and co-occurrence patterns would be subtle in comparison to the impact of the fertilizer alone, as NIs are presumably specific to nitrifying bacteria and are added at a rate of only 0.8% of the NH_4^+-N applied with the fertilizer; ii) the response of soil bacterial consortia to DMPP and DMPSA would be similar because both NIs are based on dimethylpyrazole (DMP), and iii) the microbial impact of DMPP and DMPSA will be WPFS dependent, because the diffusion of these compounds into the soil will be different depending on the moisture content.

The present work aims to enrich our understanding of the possible effects of NIs in microbial processes to ensure their application does not have harmful effects on soil health. The latter insights are of great value to respond to the major challenges currently faced by agriculture: increasing crop production for food and energy while preserving ecosystem functioning and soil quality.

2. 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 × 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} (1st application) and 60 kg N ha^{-1} (2nd, 3rd and 4th 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} (1st application) and 0.48 kg ha^{-1} (2nd, 3rd, and 4th applications). The timing and rates of application are detailed in Fig. 1.

2.2. Soil water content determination

Four soil samples (2 cm diameter × 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 × bulk density) × (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 × 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
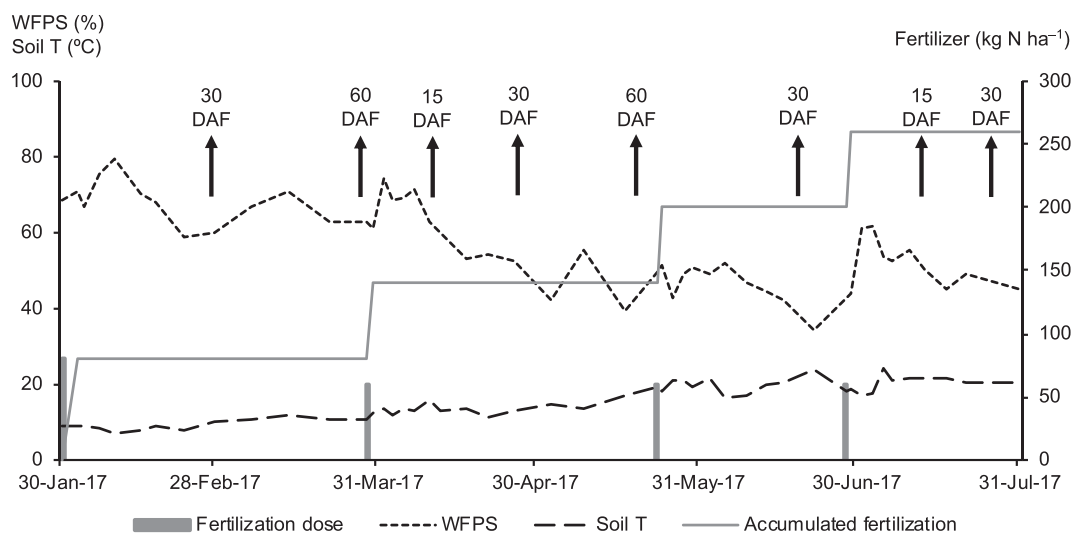

Fig. 1. Soil water content expressed as water-filled pore space (WFPS) and soil temperature (0–10 cm), fertilizer application rates and total cumulative fertilization. Arrows indicate soil sampling for DNA determinations and timing from last fertilizer application (Days after fertilization, DAF).

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 network 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. Results and discussion

3.1. Nitrification inhibitors intensified the impact of fertilizer 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). 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
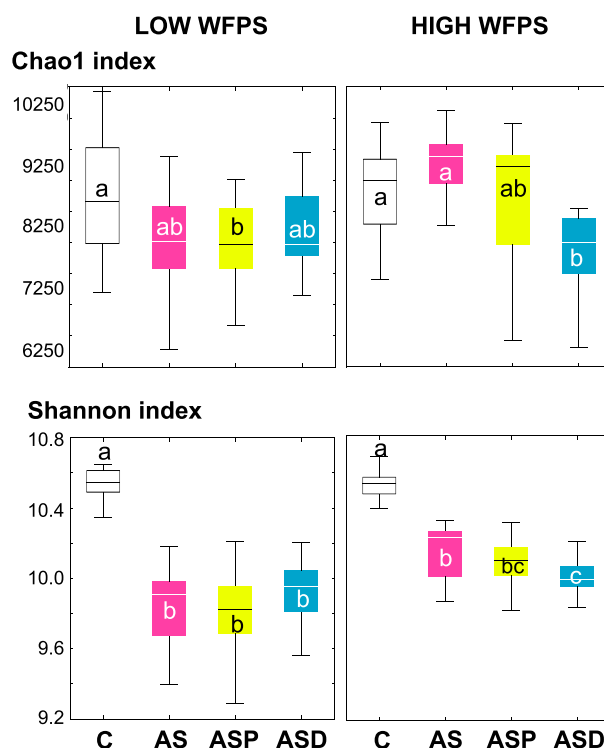

Fig. 2. Alpha diversity Chao1 (richness) and Shannon (evenness) indices by treatment at low and high water-filled pore space (WFPS). Different letters indicate significant differences (Monte Carlo, $P < 0.05$ based on 999 permutations). C = Unfertilized control; AS = Ammonium Sulfate 21%; ASP = Ammonium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

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
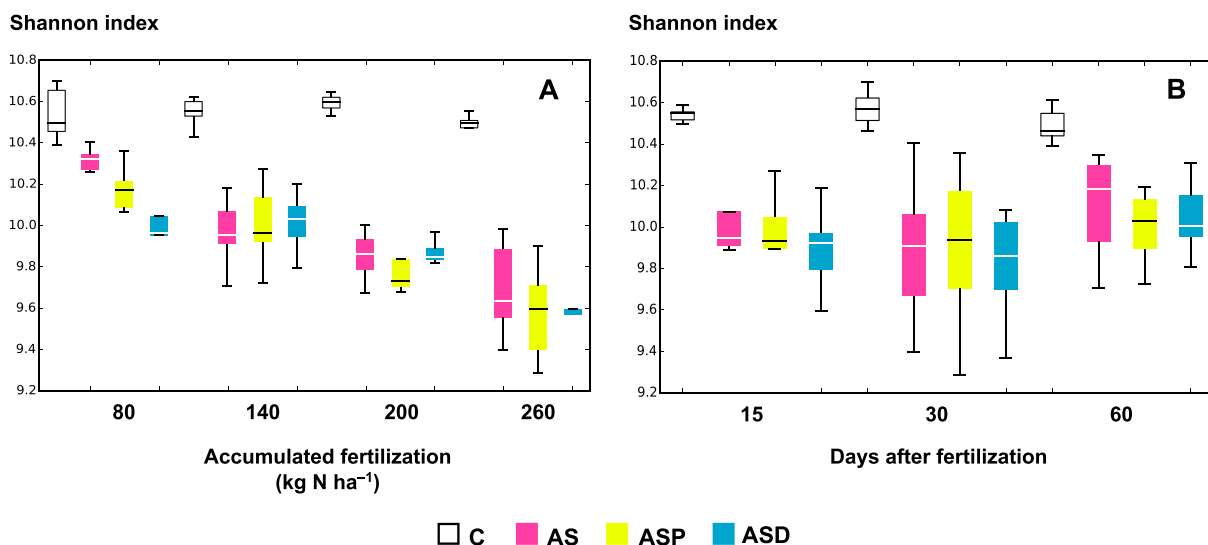
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Shannon index

Shannon index

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

related the effects of fertilization on alpha and beta diversity to soil acidification (Zeng et al., 2016; Ling et al., 2017; Zhang et al., 2017b; 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.

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. (2017a) 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

Shifts in the abundance of particular microbial groups as a consequence of NI application might cause (or be the result of) a change in

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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 and 0.77 at low WFPS and between 0.92 and 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 (Fig. 4) (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

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)	R² 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 within 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 Sulfate 21%; ASP = Ammonium Sulfate 21% + DMPP; ASD = Ammonium Sulfate 21% + DMPSA.
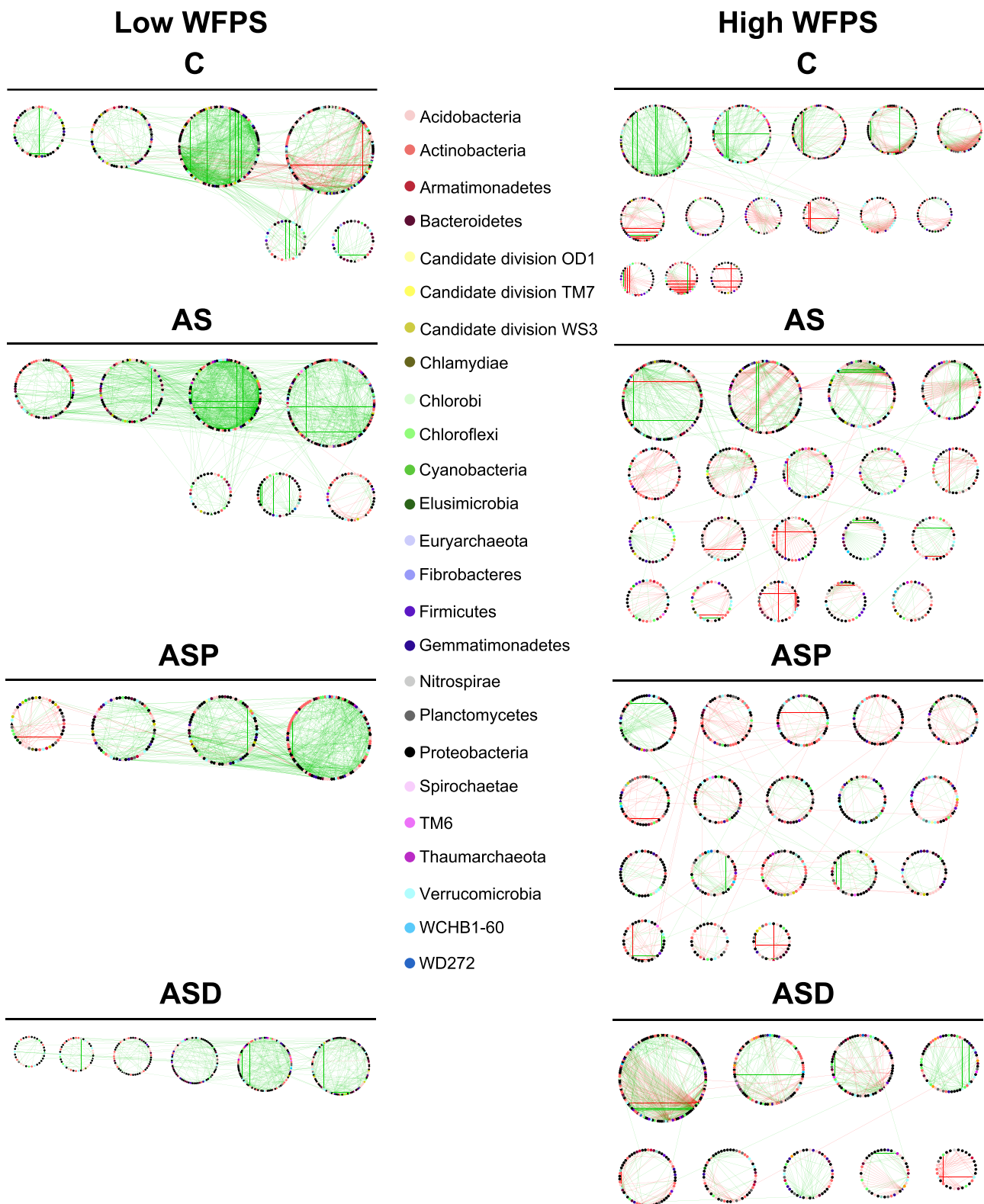
Low WFPS

C

AS

ASP

ASD

High WFPS

C

AS

ASP

ASD

- ● Acidobacteria
- ● Actinobacteria
- ● Armatimonadetes
- ● Bacteroidetes
- ● Candidate division OD1
- ● Candidate division TM7
- ● Candidate division WS3
- ● Chlamydiae
- ● Chlorobi
- ● Chloroflexi
- ● Cyanobacteria
- ● Elusimicrobia
- ● Euryarchaeota
- ● Fibrobacteres
- ● Firmicutes
- ● Gemmatimonadetes
- ● Nitrospirae
- ● Planctomycetes
- ● Proteobacteria
- ● Spirochaetae
- ● TM6
- ● Thaumarchaeota
- ● Verrucomicrobia
- ● WCHB1-60
- ● WD272

Fig. 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 Sulfate 21%; ASP = Ammonium Sulfate 21% + DMPP; ASD = Ammonium Sulfate 21% + DMPSA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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.

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

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., 2017b) , 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.

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

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	0.120
		AS vs DMPSA	−0.01	0.500
		DMPP vs DMPSA	0.06	0.100
	High	AS vs DMPP	0.03	0.250
		AS vs DMPSA	−0.11	0.050
		DMPP vs DMPSA	0.06	0.210

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

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. (2017a) 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
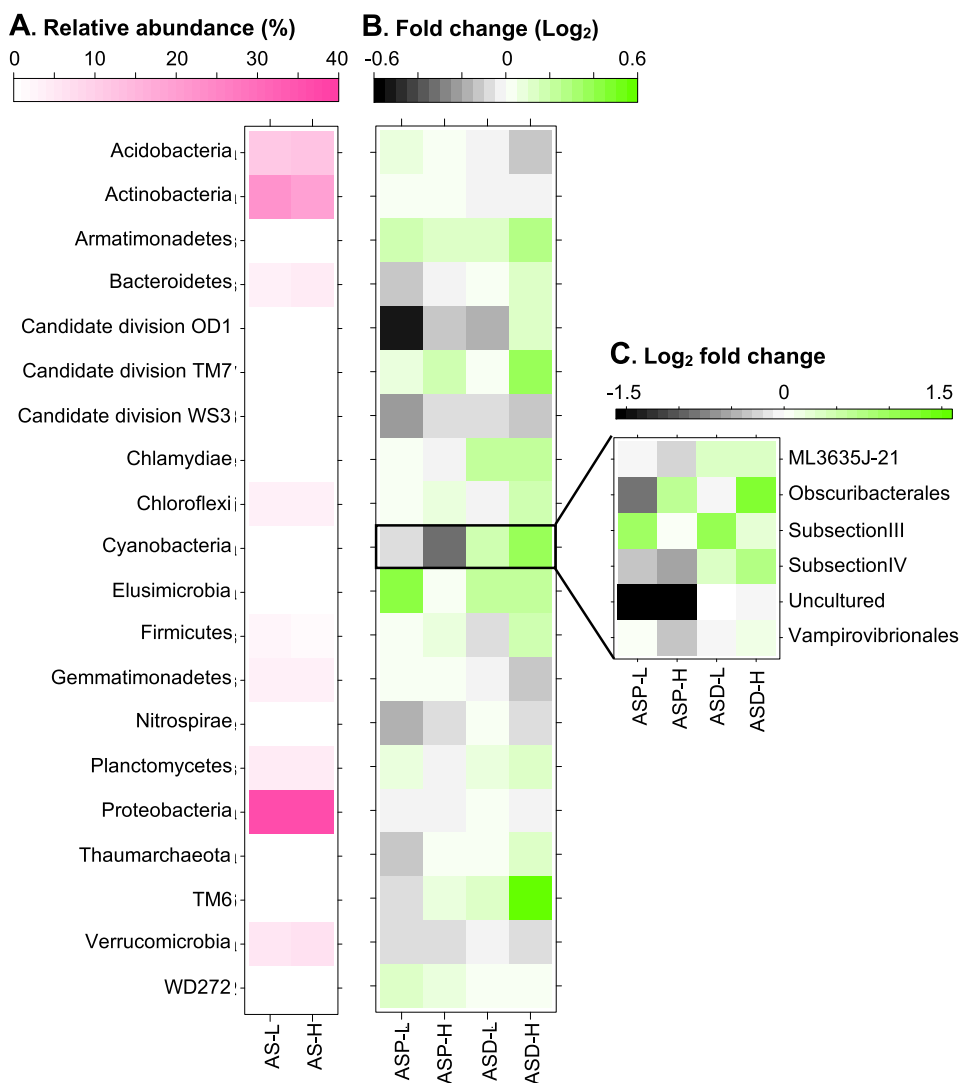
Fig. 5. A) Soil bacterial relative abundance at Phylum level in AS treatment. B) Variation of the abundance of the different phyla in soils treated with nitrification inhibitors with respect to AS. C) Variation of the abundance of Cyanobacteria's orders in sols treated with nitrification inhibitors with respect to AS. AS = Ammonium Sulfate 21%; ASP = Ammonium Sulfate 21% + DMPP; ASD = Ammonium Sulfate 21% + DMPSA; L = Low WFPS; H = High WFPS.

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),
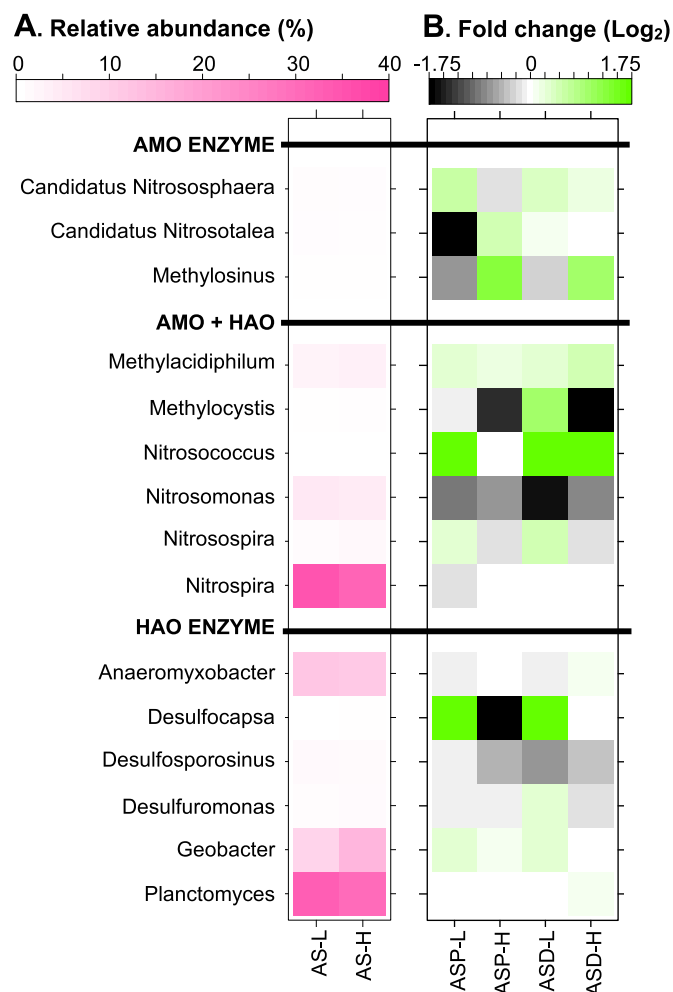
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Fig. 6. A) Relative abundance of identified nitrifying genera with respect to total nitrifiers. B) Variation of the abundance of the different genera in soils treated with nitrification inhibitors with respect to AS. AS = Ammonium Sulfate 21%; ASP = Ammonium Sulfate 21% + DMPP; ASD = Ammonium Sulfate 21% + DMPSA; L = Low WFPS; H = High WFPS.

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.

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.

4. Conclusions

The application of DMPP and DMPSA NIs had little impact on the abundance of the main phyla. Nonetheless, several significant shifts were detected in bacterial diversity, the abundance of particular taxa, and microbial co-occurrence patterns. Soil water content played a key role in the effect of the inhibitors 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* but not on other nitrifying bacteria, which needs to be further investigated to gain insights into the exact mode of action of these agrochemicals. However, the response of non-target bacteria was different for each of the NIs studied. Although both compounds are based on dimethylpyrazole, our results suggest that their individual behaviors differ in the soil. Indeed, DMPSA decreased the bacterial diversity to lower values than DMPP and was associated with greater changes in bacterial community structure. Notably, its application partially alleviated the negative effect of fertilizer on several oligotrophic taxa, which are intrinsic to soil multifunctionality, and interestingly, induced the enrichment of Cyanobacteria populations, a group with substantial agricultural potential. Our results show that NI application not only altered alpha and beta diversity, but also exerted considerable influence on interactions within microbial relationship by decreasing the complexity of co-occurrence networks.

In summary, this work provides experimental evidence that the impacts of DMPP and DMPSA on soil-borne bacteria are dissimilar, and not exclusive to their putative target taxonomic group (nitrifiers). Nonetheless, the changes observed do not seem to be of a sufficient magnitude to have a significant impact on soil health. These results, together with previous works that evidenced the ability of NIs to reduce N_2O emissions, suggest that DMP-based NIs are suitable tools in our pursuit of agricultural sustainability.

Availability of data

The datasets supporting the conclusions of this article are available in the Qiita repository, under study ID 13517 (https://qiita.ucsd.edu/study/description/13517).

CRediT authorship contribution statement

Mario Corrochano-Monsalve: Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft. **Carmen González-Murua:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **José-María Estavillo:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Andone Estonba:**

Supervision, Writing – review & editing. **Iratxe Zarraonaindia:** Formal analysis, Visualization, Supervision, Writing – review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.148374.

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