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Changes in the activity and abundance of the soil microbial community in response to the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP)

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Abstract

Purpose: The application of organic and inorganic fertilizers to soil can result in increased gaseous emissions, such as NH_3, N_2O, CO_2 and CH_4, as well as nitrate leaching, contributing to climate warming and ground and surface water pollution, particularly in regions with hot climates, where high temperatures and high soil nitrification rates often occur. The use of nitrification inhibitors (NIs) has been shown to effectively decrease nitrogen (N) losses from the soil-plant system.

Materials and methods: Non-disruptive laboratory incubation experiments were conducted to assess the extent to which temperature (20°C and 30°C) and nutrient source (mineral and organic fertilizers) influence the rate of carbon (C)- and N-related microbial processes in soil in response to the NI 3,4-dimethylpyrazole phosphate (DMPP). Furthermore, short-term changes in the ability of microbes to degrade C substrates were evaluated in disruptive soil microcosms using microbial community-level physiological profiling and the abundance of the bacterial *16S rRNA* gene as a measure of total bacterial population size.

Results and discussion: DMPP reduced net nitrification after two and four weeks of incubation at 30°C and 20°C by an average of 78.3% and 84.5%, respectively, and with similar dynamics for mineral or organic fertilization. The addition of labile organic matter with cattle effluent led to a rapid increase in C mineralization that was significantly reduced by DMPP at both temperatures, whereas no changes could be detected after the addition of mineral fertilizer. The culturable heterotrophic microorganisms showed metabolic diversification in the oxidation of C sources, with organic fertilizer playing a major role in the substrate utilization patterns during the first week of incubation and the DMPP effects prevailing from day 14 until day 28. Furthermore, the copy number of the bacterial *16S rRNA* gene was reduced by the application of DMPP and organic fertilizer after 28 days.

Conclusions: Our results show the marked efficiency of DMPP as an NI at elevated temperatures of incubation and when associated with both mineral and organic fertilization, providing support for its use as a tool to mitigate N losses in Mediterranean ecosystems. However, we also observed impaired C respiration rates and bacterial abundances, as well as shifts in community-level physiological profiles in soil, possibly indicating a short-term effect of DMPP and organic fertilizers on non-target C-related processes and microorganisms.

Keywords 3,4-Dimethylpyrazol phosphate (DMPP) • Community-level physiological profiling (CLPP) • Nitrification • Nitrification inhibitor • N cycle • Soil microbial ecology

1 Introduction

Organic and inorganic fertilizers have had a significant impact on food production in the recent past and are currently an indispensable part of modern agriculture. However, the additional costs to environmental degradation and affects of human health pose a major limitation on their excessive use; thus, careful design of their application is needed.

The high rates of fertilizer application to crops, particularly in regions with a Mediterranean climate that experience high temperatures and thus high nitrification rates, could generate severe environmental consequences. Up to 30-50% of the nitrogen (N) provided to the soil may be lost to the atmosphere (Kroeze et al. 1999), and up to 30% may be leached (Ishikawa et al. 2003; Zhao et al. 2010) through nitrification and denitrification processes. Furthermore, increased nitrification rates due to annual N fertilizer inputs and water irrigation, along with high temperature, can result in significant shifts in the composition and activity of the microbial community (Lin et al. 2004; Shen et al. 2011; Sheng et al. 2013).

One of the management practices that has been shown to reduce the risk of N leaching and emissions without necessarily reducing N inputs or crop yield is the use of nitrification inhibitors (NIs), which are natural or synthetic compounds that delay microbial oxidation of NH_4^+ to NO_2^-, the first step of nitrification, for a certain period of time (Zerulla et al. 2001). 3,4-Dimethyl pyrazole phosphate (DMPP) is one of the major commercial NIs (Subbarao et al. 2006). To a large extent, the efficiency of these compounds largely depends on soil N status, soil physiochemical and biological factors (texture, temperature, moisture, organic matter, and pH; soil microbial activity and biomass, respectively) and climate factors (temperature, rainfall intensity and frequency) (Barth et al. 2001), which, on one hand, determine the size of these losses and, on the other hand, influence the dynamics of the inhibitors in the soil (Adair and Schwartz 2008).

DMPP has been shown to be effective in inhibiting nitrification in both field (Merino et al. 2005; Li et al. 2008; De Antoni Migliorati et al. 2014) and laboratory (Hatch et al. 2005; Barth et al. 2008; Di and Cameron, 2011; Huang et al. 2013) conditions when it is added to inorganic (Weiske et al. 2001; Linzmeier et al. 2001) or organic (Dittert et al. 2001; Macadam et al. 2003) fertilizer. Although its effectiveness is limited at high environmental temperatures (Irigoyen et al. 2003; Chen et al. 2010; Mahmood et al. 2011).

In soil, DMPP primarily interacts with the ammonia-oxidizing populations on which many other organisms are dependent. Although reductions in soil ammonia-oxidizing bacterial and, to a lesser extent, ammonia-oxidizing archaeal gene copy numbers (Kleineidam et al. 2011; Yang et al. 2012; Liu et al. 2015) and transcripts (Florio et al. 2014) have been reported, less information about presumed DMPP-induced changes in non-target soil microbial processes and activity is available. Contrasting evidence has been reported on the effect of DMPP on

soil respiration (Weiske et al. 2001; Menéndez et al. 2012) and on soil enzymatic activity (Tindaon et al. 2012; Guo et al. 2014). Therefore, non-target side effects of DMPP on general microbial activities in soils should not be neglected.

Our previous studies attempted to unravel the role of DMPP in both target and non-target processes and its effects on microorganisms in soil, showing marked inhibition of ammonia-oxidizing bacterial and archaeal transcriptional activity one week after the application of treatments and moderate non-target influence on the structure of the soil microbial community (Florio et al. 2014; Maienza et al. 2014). In the present study, we aimed to assess the extent to which different temperatures (20 and 30°C) and nutrient sources (mineral and organic fertilizers) influence the rate of Carbon (C)- and N-related microbial processes in soil in response to DMPP in non-disruptive laboratory incubation experiments. Furthermore, we evaluated short-term changes in the ability of microbes to degrade C substrates in disruptive soil microcosms using microbial community-level physiological profiling (CLPP) for 28 days after the amendments on selected treatments, to provide insights into the diversification of the culturable heterotrophic microbial metabolism. Quantitative PCR (qPCR) assay of the bacterial *16S rRNA* gene was also included as a measure of total bacterial population size in the microcosms at the end of incubation.

2 Materials and methods

2.1 Soil, nutrient sources and DMPP formulation

The soil used (Casalotti soil) was collected from a Eucalyptus, short rotation, high-density plantation field managed by the research unit for intensive wood production (CREA-PLF), located in Rome (Italy) (41°54'N, 12°21'E). The soil is classified as Luvisols (WRB 2006) and has a sandy loam texture. Six samples of soils from the top 30 cm were collected in June 2009 and stored in sterile plastic bags. The soil was air-dried, homogenized by sieving (2-mm mesh size), pooled and stored at room temperature. The physico-chemical properties of the soil were 63% sand, 16% clay, 21% silt, pH (H_2O 1:2.5): 7.5, organic C: 10.6 g Kg^{-1} and total N: 0.6 g Kg^{-1}.

Two types of nutrient sources were applied, ammonium sulfate as the mineral and cattle effluent as an organic fertilizer. The bovine effluent used was obtained from a dairy farm adjacent to the CREA-PLF. Sampling was performed in June 2009, and the sample was stored in a PVC barrel at 4°C until further analysis. On day 0, it was sampled after thorough stirring and blending, and the following physico-chemical properties were analyzed using standard laboratory methods: moisture (88.9%), dry matter (11.1%), N_{tot} (0.32%), $N\text{-}NH_4^+$ (0.17%), and TOC (5.97%).

A liquid formulation of DMPP (25%, provided by K+S Nitrogen, Italy) was added to either the mineral or the organic fertilizer as a mixed solution at a final concentration of 1%, according to the NH_4^+-N content and the manufacturer's recommendations (Zerulla et al. 2001).

2.2 Experimental design

Two non-disruptive laboratory incubation experiments were performed to evaluate the effects of DMPP on soil potential N mineralization and nitrification and soil C mineralization, as well as the influence of temperature (30°C or 20°C) and nutrient source (mineral or organic fertilizer). The following treatments, with three replicates, were compared in these experiments: soil+ammonium sulfate (AS), soil+ammonium sulfate+DMPP (ASD), soil+organic fertilizer (OF), and soil+organic fertilizer+DMPP (OFD). Soil+DMPP (D) and soil-only (C) control treatments were also included.

Furthermore, the short-term changes in the heterotrophic microbial dynamics in disruptive soil microcosms were determined in organically amended soils (OF, OFD) and in control soils (C, D) at the temperature of 30°C. In particular, microbial community-level physiological profiling (CLPP) and the abundance of the bacterial *16S rRNA* gene (quantitative PCR) were investigated.

2.3 Non-disruptive laboratory incubations for potential N and C mineralization

Nitrogen (250 mg N Kg $soil^{-1}$) from mineral (ammonium sulfate) or organic fertilizer (cattle effluent) and DMPP were added as mixed solutions to 50 g of air-dried soil mixed with quartz sand in a 1:1 ratio to determine the potential N mineralization (Stanford and Smith 1972). The mixture was incubated at a 60% water holding capacity in the dark at 30°C or 20°C for 12 weeks (Benedetti et al. 1994; Dell'Abate et al. 2003). The amounts of nitrite-N + NO_3^--N and NH_4^+-N produced during the incubation were monitored at 1, 2, 4, 8 and 12 weeks. The soils were eluted with 900 ml of a 0.01 M $CaSO_4$ solution and then with 100 ml of N-free solution [0.002 M $CaSO_4$, 0.005 M $Ca(H_2PO_4)_2$, 0.0025 M K_2SO_4, and 0.002 M $MgSO_4$] to reintegrate the nutrient elements. The nitrogen forms in the eluate were determined colorimetrically by a continuous flux analyzer (Autoanalyzer Technicon II), according to the methods described by Wall et al. (1975) for NH_4^+-N and Kamshake et al. (1967) for (NO_2^-+NO_3^-)-N. Cumulative net nitrification and net N mineralization were expressed as (NO_2^-+NO_3^-)-N (milligrams per N kilogram dry soil) and (NH_4^++NO_2^-+NO_3^-)-N (milligrams per N kilogram dry soil), respectively.

Soil respiration was measured in separate microcosms for 35 days at 30°C and 20°C, with three replicates for each treatment in 25 g (oven-dry basis) of moist sample placed in 1 L of stoppered glass jars that were treated with the same amounts of fertilizers and DMPP reported above. The evolved CO_2 was trapped after 1, 2, 4, 7, 10, 14, 21, 28 days of incubation in 5 ml of 1 M NaOH, and the amount was determined by titrating the excess NaOH with 0.1 M HCl (Badalucco et al. 1992). The CO_2 emitted in 35 days of incubation was reported as cumulative respiration (C_{cum}).

2.4 Disruptive microcosm study of the microbial community

The disruptive microcosm experiment was established as previously described (Florio et al. 2014; Maienza et al. 2014); briefly, three replicates per treatment of soil microcosms containing 1.1 Kg of homogenized soil were incubated at a 60% water holding capacity at 30°C in the dark for 28 days. Three gram subsamples were removed at days 1, 4, 7, 14 and 28 and used for CLPP analysis; furthermore, 10 g subsamples of the microcosm were stored at -20°C for qPCR analysis of the bacterial *16S rRNA* gene at the end of incubation (day 28).

2.5 Microbial community-level physiological profiling (CLPP)

The metabolic profiles of the microbial communities were generated by the Biolog® Microstation System 4.2 (Biolog Inc., Hayward, CA, USA) using ECOPlates, which are specifically designed for community analyses and microbial ecological studies. The ECOPlate contains 31 of the most useful carbon sources for soil community analyses, and the sources are repeated 3 times to provide more replicates for the data. The oxidation wells contain a redox indicator, tetrazolium violet, which undergoes a color change (from colorless to dark violet) whose intensity is proportional to the intensity of microbial metabolism (which in turn is due to the number and/or species involved). Three gram soil subsamples from each microcosm were mixed with 30 ml of sterile physiological solution (NaCl 9 g l^{-1}), stirred with 10 g of glass beads for 30 min, and centrifuged at 3,000 rpm for 3 min. Each plate was inoculated with 120 µl of supernatant, according to the method described by Torsvik (1995). The absorbance values corresponding to color changes were read by the E-MAX reader at 590 nm three times per day for 10 days. Each well of the ECOPlate contains the redox dye tetrazolium, which is reduced by the NADH produced by the respiration pathways. The rate and extent of color formation indicate the rate and extent to which respiration occurs with the substrate present in that well (Garland and Mills 1991; Garland 1996a, b).

2.6 Quantification of gene abundance using qPCR

DNA was extracted from 0.25 g of each soil sample using a DNA PowerSoil® Total DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's instructions, with a slight modification, in which the 10 min shaking on a flatbed vortex was replaced by a 30 s bead beating step (5.5 m s^{-1}, Fastprep). The DNA concentrations were determined using the Qubit quantification platform with a Quant-iT™ dsDNA high sensitivity (HS) Assay Kit (Invitrogen UK). The DNA was diluted to 5 ng/µl and stored in an -80°C freezer prior to qPCR analysis.

The abundance of the bacterial *16S rRNA* gene in the soil samples was quantified by real-time PCR using the primers Muyzer for/Muyzer rev (Nadkarni et al. 2002; Muyzer et al. 1993) and the conditions described by Clark et al. (2012). The standards were generated from PCR products that had been obtained from soil DNA extracts, gel purified, quantified, and diluted accordingly (Töwe et al. 2010) to give a concentration range from 0 to 10^9 gene copies μl^{-1}. All DNA preparations were checked for the absence of inhibitors prior to PCR, and all results were analyzed using the LinRegPCR program version 11.1 (Ramakers et al. 2003; Ruijter et al. 2009) to confirm the efficiency of amplification and the absence of inhibition.

2.7 Data analysis

Significant differences between the treatments during the experiment were detected using one-way ANOVA and a post hoc (Duncan) test at a level of $P<0.05$. Statistical analyses were performed using the SPSS 11 software package.

CLPP results were organized with Biolog MicroLog System 4.2 software. The raw OD data were corrected by blanking each response well against its own first reading (immediately after inoculation). The absorbance profile obtained for each trial at each reading time was transformed into the average well color development (AWCD) index (Garland 1997) with the formula $AWCD = \sum ab_t/96$ (ab_t is the absorbance value at a certain reading time, calculated as previously described, and 96 is the total number of wells), and the temporal evolution of these profiles was plotted as AWCD curves. Data analysis has been further elaborated by calculating the area under the curve for each well OD for the entire period of incubation and by estimating the kinetic parameters (*K*, *r*, *s*) by fitting the curve of OD versus time into a density dependent logistic growth equation $Y = OD_{592} = K/(1 + e^{-r(t-s)})$, where *K* is the asymptote (or carrying capacity), *r* determines the exponential rate of OD change, *t* is the time after the inoculation of the microplates, and *s* is the time when the midpoint of the exponential portion of

the curve (i.e., when y = *K*/2) is reached (Insam and Goberna 2004), using STATISTICA 6.0 (StatSoft Inc., Italia).

Principal component analysis (PCA) was performed on the correlation matrix of the variables, and the kinetic parameter *s* was used for well comparison. Single data points were corrected using the blank cell divided by the AWCD of the respective plate and then log-transformed according to the method described by Weber et al. (2007). The principal component data were analyzed using ANOVA.

3 Results

3.1 Potential N mineralization and nitrification

The values of net nitrification and the cumulative percentages of N mineralization recorded after the addition of DMPP and either mineral or organic fertilizer to the soil at both 30°C and 20°C are reported in Fig. 1. The N sources applied to the soil similarly influence the ammonium oxidation dynamics throughout the incubation period. DMPP significantly reduced nitrification starting from the second week of incubation (Fig. 1a); at 14 days, lower net nitrification was observed in ASD and OFD (1.6±0.1 and 1.4±0.3 mg N Kg^{-1} dry soil, respectively) than in AS and OF (7.2±0.1 and 10.4±5.7 mg N Kg^{-1} dry soil, respectively). A similar trend was recorded at 28 days (7.9±0.1 and 6.7±0.8 mg N Kg^{-1} dry soil in ASD and OFD, respectively, *vs* 37.7±0.5 and 29.5±2.5 mg N Kg^{-1} dry soil in AS and OF, respectively, $P<0.05$). After 8 and 12 weeks, the net nitrification values for each treatment were not different from the control (Fig. 1a), and inhibition decreased.

The cumulative mineral N concentrations in soil did not vary significantly among treatments throughout the incubation period (Fig. 1b), and, at the end of incubation, 201.8±13.8 and 188.1±10.6 mg N from mineral fertilizer per Kg^{-1} dry soil were mineralized (AS and ASD, respectively), whereas 155.0±15.3 and 138.2±10.8 mg N from organic fertilizer per Kg^{-1} dry soil were mineralized (OF and OFD, respectively) (Fig. 1b). No differences were detected between the control and DMPP-only treatment, and the values of both N mineralization and nitrification were consistently lower than those of the amended treatments (data not shown).

The cumulative N mineralization and net nitrification dynamics at 20°C varied similarly to those at 30°C (Fig. 1c, 1d), with gradually increasing N mineralization throughout the incubation period. At the fourth week of incubation, the nitrification rates were significantly reduced by the presence of DMPP (4.5±1.0 and 3.8±0.7 mg N per Kg^{-1} dry soil in ASD and OFD, respectively, *vs* 29.2±3.9 and 24.9±3.7 mg N per Kg^{-1} dry soil in AS and OF, respectively, $P<0.05$) (Fig. 1c), confirming the trend at 30°C.

3.2 Microbial respiration

Cumulative C mineralization was computed by adding the amount of respired C after the addition of DMPP and mineral or organic fertilizer to the soil over the 35 days of incubation at 30°C or 20°C and is reported in Fig. 2. A significant increase ($P<0.05$) in soil respiration rates was observed in the organically amended soils compared to the mineral amended soils at both temperatures, although it was more pronounced at 30°C (Fig. 2a) and less evident at 20°C (Fig. 2b). As expected, soil C mineralization increased with increasing temperature; the addition of DMPP significantly reduced cumulative C mineralization when combined with organic fertilizer (922.1±25.8 and 798.50±27.8 mg CO_2-C Kg^{-1} dry soil in OF at 30°C and 20°C, respectively, *vs* 546.82±27.1 and 409.11±27.0 mg CO_2-C Kg^{-1} dry soil in OFD at 30°C and 20°C, respectively), but not when combined with mineral fertilizer (Fig. 2).

3.3 Microbial heterotrophic metabolism and size of the bacterial community

Table 1 reports the CLPP inflection point (kinetic parameter "*s*") as a measure of the potential culturable heterotrophic metabolism in the microcosms after 1, 4, 7, 14 and 28 days of incubation at 30°C. Significantly ($P<0.05$) lower inflection was detected in the organic fertilizer-containing treatments than in the DMPP treatments during the first 7 days of incubation. Soil microbial communities showed metabolic diversification in the oxidation of the C sources, as shown in the PCA biplots of the first two principal components (PCs) in Fig. 3. Overall, the first two PCs accounted for more than 60% of the total variance for bacterial CLPP; PC1 accounted for approximately 50% of the variance, and PC2 accounted for 9.68% (day 28) to 12.23% (day 14) of the variance. PC1 did not produce a net separation between the treatments at all sample times, but they were separated along the PC2 axis. On day 1, no clear differences between the treatments could be detected (Fig. 3a), but on day 4, the soils amended with cattle effluent grouped well with each other and were separated from the soils that did not receive organic fertilizer (Fig. 3b). This finding was also confirmed on day 7 (Fig. 3c), although the DMPP-only soils showed some variability along the PC2 axis. Some separation among soils amended with cattle effluent, regardless of the presence of DMPP, was observed on day 4 ($P<0.05$), but not on day 7. On day 14, the soils that did not receive DMPP were significantly and positively affected by PC2 and were separated from the OF and OFD soils (Fig. 3d). Separation between the DMPP treatments and cattle effluent treatments was also observed on day 28 (Fig. 3e).

Table 2 shows the relative total bacterial abundance at the beginning and end of the experiment at a temperature of 30°C, as quantified using qPCR assays. The copy numbers of the bacterial *16S* gene were in the range of 4.01

x 10^8 to 2.36 x 10^9 copies g^{-1} dry soil. On day 0, the copy numbers of the *16S rRNA* gene in the organically amended soils were significantly ($P<0.05$) higher than those in the non-amended soils. Significantly ($P<0.05$) reduced levels of the *16S rRNA* were observed in the microcosms in which DMPP was applied at the end of incubation.

4 Discussion

Slowing of the nitrification rates is achieved by the addition of NIs to the soil, resulting in a longer-lasting supply of N for plants and limited N losses through leaching and denitrification. Among soil environmental parameters, soil temperature is thought to have a marked effect on the persistence of many NIs, including DMPP. Nitrification rates increase linearly with temperatures, reaching an optimum from 25 to 35°C (Justice and Smith 1962; Stark 1996); however, the efficiency of most NIs has been shown to decrease with temperature. The inverse relationship between temperature and the effectiveness of DMPP has been reported at different temperatures ranging from 5 to 25°C (Zerulla et al. 2001; Irigoyen et al. 2003; Chen et al. 2010). Incubation experiments at constant soil temperatures have shown that at 5°C, there was practically no nitrification of the NH_4^+ from the ammonium nitrate to which DMPP had been added (Zerulla et al. 2001), whereas at 10°C, the addition of DMPP stabilized the NH_4^+ content in soil over a period of more than 100 days (Irigoyen et al. 2003). At 20°C and moreso at 25°C, NH_4^+ degradation markedly accelerated, with half-lives of NH_4^+-N of 18 and 8 days, respectively (Chen et al. 2010), and the inhibitory effect lasted 2-3 weeks at temperatures ≥ 25°C (Zerulla et al. 2001). Under a warm climate regime, some NIs other than DMPP have been shown to even increase N losses (Mahmood et al. 2011). In this study, DMPP strongly inhibited nitrification at 20°C and even at 30°C after 14 and 28 days of incubation by an average 84.5% and 78.3%, respectively (Fig. 1a, 1c). There were no significant differences in nitrate concentrations between the fertilizer and fertilizer+DMPP treatments after 8 and 12 weeks, and the inhibitory effect decreased. Because the optimum temperature for nitrification in soil is a function of the native environment of the ammonia oxidizing community, ranging from 25°C in temperate regions to 30-35°C in Mediterranean and tropical climate soils (Justice and Smith 1962; Myers 1975), the temperatures of 30 and 20°C were chosen to represent the temperatures in the spring and summer in most Mediterranean ecosystems, respectively, when high nitrification rates are experienced, and the use of nitrification inhibitors is recommended to guarantee that the plant will have an adequate N supply throughout the entire vegetative cycle and to reduce NO_3^- leaching and N_2O emissions.

As expected, carbon mineralization increased with raising temperatures over the 35 days of incubation because high soil temperatures accelerate C degradation rates (Lloyd and Taylor 1994; Davidson et al. 1998; Bond and Thomson 2010). Moreover, C mineralization was reduced by DMPP by an average of 15.9% and 12.6% at 20 and 30°C, respectively. The influence of DMPP on soil respiration has previously been addressed in some studies using incubation at different temperatures. Nevertheless, the results of those studies are highly variable, which leads to substantial discrepancies in their subsequent conclusions. For example, Menéndez et al. (2012) did not observe any reduction in CO_2 release at temperatures ranging from 10 to 20°C, whereas a 10-28% decrease in CO_2 release was reported in other studies (Weiske et al. 2001; Maienza et al. 2014).

Nitrogen supplied as mineral or organic fertilizer would be expected to affect N mineralization and nitrification by increasing the availability of N for these microbial processes. In our experiment, the dynamics of N mineralization and nitrification in the presence or absence of DMPP were highly comparable between the two fertilizers used. These findings agree with previous studies (Weiske et al. 2001; Macadam et al. 2003), confirming the suitability of DMPP as a nitrification inhibitor in both inorganic and organic fertilizers. After 12 weeks of incubation, averages of 80.7% and 75.3% of the total N added as mineral fertilizer were mineralized (AS and ASD, respectively), whereas 62.0% and 55.3% of N from organic fertilizer were mineralized (OF and OFD, respectively) (Fig. 1b). Because there were no significant differences in total mineral N leaching between the two treatments (Fig. 1b, 1d), the reduction in the nitrate concentration in the presence of DMPP can be attributed to the inhibition of nitrification rather than the stimulation of denitrification. During the first 7 days, nitrification was not inhibited (Fig. 1a, 1c), and relatively low nitrification rates were recorded, regardless of the presence of DMPP. In this experiment, the addition of mineral or organic nutrients to low fertility soil may have stimulated an initial proliferation of the microbes in the soil and thus the immobilization of N compounds in the soil microbial biomass because the nutrients provided by the fertilizer may have been insufficient to fulfill N needs for the formation of cellular organic N constituents during growth of soil microbial populations (Jarvis et al. 1996). With certain amounts of nutrients added to the soil, the reproductive rates of the microbes were expected to increase; thus, high competition for the nutrients and subsequent immobilization occurred.

DMPP had no influence on soil respiration when added in combination with mineral fertilizer (Fig. 2a, 2b); conversely, the addition of labile organic matter in cattle effluent led to a rapid increase in C mineralization that was significantly ($P<0.001$) reduced by DMPP by an average of 19% (Fig. 2a, 2b). There have been only a few studies that determined the differential effects of DMPP in combination with organic and inorganic fertilization on soil CO_2 production. Nevertheless, either no effect (Ménendez et al. 2006) or an inhibitory effect (Weiske et al.

2001) was observed. It is known that the incorporation of organic matter in soil can increase microbial activity and biomass (Gonzales et al. 2010; Marinari et al. 2000) after both long- and short-term applications, even when applied in small quantities (Arancon et al. 2006; Florio et al. 2015). However, the reduction of soil respiration induced by DMPP raises questions about the specificity of the target process of the molecule in the soil. Considering that DMPP may have an indirect effect on soil respiration by affecting the consumption and/or production of CO_2 by ammonia oxidizers, it would be unlikely that we would observe any effect of DMPP on overall soil respiration because this microbial group represents only a very small proportion of the total soil microorganisms. For this reason, the physiological profiling of soil microbial communities capable of degrading C sources and the bacterial population size were determined in a study using organic fertilization as a sole nutrient source and incubation at a temperature of 30°C.

Cattle effluent and DMPP induced rapid changes in culturable microbial heterotrophic metabolism, even after 24 h of incubation. A significantly lower inflection ("s" value, Tab. 1) was detected in the organic fertilizer-containing treatments than in the DMPP treatments during the first 7 days of incubation, indicating that heterotrophic culturable microorganisms were significantly more active in treatments in which cattle effluent was added. Furthermore, PCA of the CLPP data showed a shift in the pattern of C sources used by the heterotrophic microbial community, which was evident on day 4 and, to a lesser extent, on day 7 (Fig. 3). On day 14, we observed a significantly lower inflection in soils where DMPP was added, suggesting that the NI, rather than the organic fertilizer, was the main driver of heterotrophic culturable microbial metabolism. This trend was also observed at the end of the incubation (day 28), although it was not significant (Tab. 1); however, clear differences across treatments could be detected in the PCA patterns on both day 14 and 28, and a separation between the DMPP treatments and cattle effluent treatments occurred. There are still some criticisms about the utilization of the Biolog method because it does not reflect the functional abilities of the entire soil microbial community but only those of a limited subset of microbial genera (Smalla et al. 1998). Moreover, incubating soil extracts with high concentrations of readily decomposable organic substrates may favor the growth of a few copiotrophic microbes that are able to grow rapidly and explosively (Buyer et al. 2002), thus outcompeting the slow-growing oligotrophic species in the wells. However, it has proven to be a useful tool to differentiate disturbances in soil microbial functional diversity and communities in response to different environmental stresses (Du Plessis et al. 2005; Hayyis-Hellal et al. 2009; Lupwayi et al. 2009).

The copy numbers of the bacterial *16S rRNA* gene were used as a measure of bacterial community size in the microcosms and decreased after 28 days of incubation in the presence of DMPP (Tab. 2), confirming a

generalized non-target effect of DMPP on microbial abundance and function. Therefore, although the copy numbers of the soil bacterial *16S rRNA* were reduced following the addition of DMPP, it is not possible to determine if DMPP differentially affected the organisms in the original soil and in the effluent itself.

Organic fertilizers and NIs are used to improve plant growth, increase C inputs to soil and limit N losses from the soil-plant system but can have indirect effects on the microbial community, as well as specific impacts on N-cycling microorganisms. However, to date, there is no clear evidence indicating whether NIs have a negative effect on non-target processes and microbes in soil. Little or no significant effects on soil microbial C and N levels nor on the abundance of bacteria and archaea were observed using dicyandiamide (DCD) as an NI (O'Callaghan et al. 2010; Guo et al. 2014). However, the mechanism of action of DMPP has been shown to differ from that of DCD (McCarty 1999) and has not been completely elucidated; thus, it presumably has different impacts on soil microbial processes and microbes. While Tindaon et al. (2012) failed to detect inhibitory effects on general soil microbial activity in soils, Maienza et al. (2014) found decreased bacterial and fungal growth, but not decreased soil microbial biomass, in the microcosms after the application of DMPP and bovine effluent, but the inhibitory effects disappeared after 28 days of incubation. However, Florio et al. (2014) found that the application of DMPP and organic fertilizer inhibited bacterial transcriptional activity but did not decrease the copy numbers of the *16S rRNA* gene during the first 7 days of incubation. Together, these results indicate that the cattle effluent played a major role in substrate utilization patterns during the first 7 days because its own microbiota may have provided specific strains responsible for the degradation of C sources, whereas from day 14 onwards, the effect of DMPP prevailed, and the influence of the organic fertilizer became less evident, suggesting that the compound may affect the metabolism of microbes that degrade the C substrates and decrease the soil respiration rates and the size of the bacterial community.

5 Conclusions

This laboratory study shows that NI DMPP inhibited nitrification in the experimental conditions tested using both mineral and organic fertilizers at 20°C and even 30°C, thus providing support for the use of DMPP as a tool to mitigate N losses in Mediterranean ecosystems. However, the labile organic matter in cattle effluent led to a rapid increase in C mineralization that was significantly reduced by DMPP at both temperatures. Furthermore, both culturable-dependent and -independent techniques revealed a moderate short-term effect on heterotrophic metabolism, as well as on the size of bacterial populations, at the temperature of 30°C, providing evidence of a non-target effect of DMPP on microbial activity and abundance over 28 days. However, because DMPP

performance is highly site-specific and primarily depends on the soil biotic and abiotic statuses, as well as the environmental conditions, further studies using soils with different properties, longer incubation periods and different field conditions in the presence of plants are needed to better understand whether the changes in the activity and abundance of the soil microbial community induced by DMPP and organic fertilization will be maintained. Therefore, the present study highlights the importance of evaluating the overall soil microbial response to the application and design of this agricultural practice, which merits further investigation and should not be neglected.

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Figure Captions

Fig. 1. Weekly net nitrification and cumulative net N mineralization as mg of nitrites+nitrates-N or total mineral N leached (as the sum of ammonium+nitrites+nitrates-N) per Kg of dry soil after incubation at 30°C (a, b) and 20°C (c, d). Significant effects of the inhibitor are indicated by asterisks, Duncan's test, $P<0.05$. Post hoc comparisons were performed to determine the effect of DMPP at each temperature. Treatments: ammonium sulfate (AS), ammonium sulfate plus DMPP (ASD), cattle effluent as organic fertilizer (OF), and cattle effluent plus DMPP (OFD).

Fig. 2. Cumulative soil respiration as mg of CO_2-C per Kg of dry soil over the 35 days of incubation at 30°C (a) and 20°C (b). Significant effects of the inhibitor are indicated by asterisks, Duncan's test, $P<0.05$. Post hoc comparisons were performed to determine the effect of DMPP at each temperature. Treatments: ammonium sulfate (AS), ammonium sulfate plus DMPP (ASD), cattle effluent as organic fertilizer (OF), and cattle effluent plus DMPP (OFD).

Fig. 3. Principal component analysis of the absorbance data at the inflection point of the AWCD curve after (a) 1, (b) 4 (c) 7, (d) 14 and (e) 28 days of incubation at 30°C. Treatments: ●, soil only (C); ○, soil + DMPP (D); ▲, soil + cattle effluent (OF); and Δ, soil + cattle effluent + DMPP (OFD). Principal components marked with an asterisk indicate a significant treatment effect as determined by ANOVA, $P<0.05$.