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Activity of ammonia-oxidizing bacteria in enriched cultures exposed to 3,4-dimethyl-1H-pyrazole dihydrogen phosphate nitrification inhibitor

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ABSTRACT

The use of nitrification inhibitors is an interesting tool to achieve a higher N efficiency in plants while decreasing the environmental impact of N fertilization. However, an integrated evaluation of the efficiency of nitrification inhibitors over time, understood as the period in which the nitrifying activity is inhibited or slows down, is necessary to assess whether their use is ecofriendly and sustainable.

To test the direct efficiency of 3,4-dimethyl-1H-pyrazole dihydrogen phosphate (DMPP) on nitrification, a study has been carried out in two cultures enriched with ammonia-oxidizing bacteria (AOB) obtained from a soil with continuous N fertilization (80 kg N ha^{-1} $year^{-1}$ as NH_4NO_3) and from soil without N fertilization. In addition, Cu has been evaluated as a cofactor of ammonia monoxygenase, a key enzyme in the nitrifying activity of AOBs. On the other hand, the stability of DMP has been studied both in the cultivation system enriched in AOBs and in soil to assess the efficiency of the inhibitor due to its persistence over time.

Our work reveals that nitrification rates observed in cultures enriched in AOBs from genus *Nitrosospira* isolated from soils with continuous N fertilization were not higher than those of cultures without N fertilization. In AOB cultures, DMPP was a very efficient inhibitor of nitrification (> 50 % inhibition of integrated AMO activity), mainly due to the stability of DMP (3,4-dimethyl-1 H-pyrazole) in the cultures. However, DMP stability was significantly lower under soil conditions (> 90 % of DMP was degraded in the first 30 days of incubation). Other metals are suggested as cofactors of the enzyme ammonia monooxygenase alternatively to Cu.

1. Introduction

Until the "Green Revolution", characterized by the extensive use of synthetic nitrogenous fertilizers in agriculture, nitrification was considered as a minor component of the Nitrogen (N) Cycle. Nitrification is a process mediated by chemolithoautotrophic microorganisms that obtain energy from the oxidation of N and fix inorganic C (from carbon dioxide - CO_2 or bicarbonate - HCO_3). Among these microorganisms there are ammonia-oxidizing bacteria and archaea (AOB and AOA, respectively), nitrite oxidizing bacteria (NOB), and complete ammonia-oxidizing bacteria able to perform complete oxidation of ammonium to nitrate (Daims et al., 2015; van Kessel et al., 2015). Most of the AOB are currently included in the genera *Nitrosomonas, Nitrosospira* and *Nitrosococcus* (Norton, 2011)), while the best well known AOA in nitrification processes belong to the genus *Nitrosopumilus* (Prosser and Nicol, 2012). Although the enzymatic machinery responsible for nitrification differs between AOB and AOA, the mechanism of action used for the first step of NH_3 oxidation is similar between AOA and AOB (Shen et al., 2013). This step is catalyzed by the enzyme ammonia-monooxygenase (AMO) producing hydroxylamine, which is in turn oxidized to nitrite (at least in AOB) by the enzyme hydroxylamine oxidoreductase (HAO) (Bock and Wagner, 2006; Hatzenpichler, 2012) and quickly converted to nitrate by the action of NOB bacteria. The nitrate obtained can be immobilized by microorganisms in the soil, absorbed by plants or denitrified leading to the emission of NO, N_2O and N_2.

The bacterial population involved in the nitrification process is determined by the concentration of NH_4^+. At low NH_4^+ concentrations the nitrifying population is dominated by bacteria of the genus *Nitrosospira*, while at high NH_4^+ concentrations the population is dominated by

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bacteria of the genus *Nitrosomonas* (Kox and Jetten, 2015).

Due to the high nitrification rates observed in agricultural soils, and despite advances in the management of N fertilizers, a high percentage of the N applied as NH_4^+ and urea (or as nitrate) is lost via nitrate leaching (Zhou et al., 2016) and via N gaseous emissions in the form of N_2O, NO and N_2 (Skiba and Smith, 2000; Ussiri and Lal, 2013; Loick et al., 2016) with the consequent reduction of N use efficiency and environmental impacts.

Nitrification inhibitors (NIs) are biological or chemically synthesized chemicals that lower nitrification rates and, as a result, N losses are minimized through nitrate leaching and gaseous emissions. Thus, nitrification inhibition is a first step towards the reduction of N oxides emissions and the increase of nitrogen fertilizer use efficiency (Linzmeier et al., 2001; Akiyama et al., 2010; Quemada et al., 2013).

The nitrification inhibitors, 2-chloro-6-(trichloromethyl) pyridine (Nitrapyrin), dicyandiamide (DCD) and 3,4-dimethyl-1H-pyrazole dihydrogen phosphate (DMPP) are the most commercialized NIs worldwide. DCD appeared as an alternative to Nitrapyrin, after the description of Nitrapyrin phytotoxic effects. However, recent studies detected traces of DCD in the milk of cows grazing on pastures treated with fertilizers associated with DCD (Kim et al., 2012; Chen et al., 2014). DMPP is used at concentrations 10 times lower than DCD and presents a greater persistence in the maintenance of the inhibition of the nitrification activity (Zerulla et al., 2001).

Many physical and/or biochemical processes such as mineralization, microbial absorption and water availability can influence the efficacy of nitrification inhibition (Barth et al., 2008; McGeough et al., 2016). The adsorption of NIs to the soil matrix (i.e., clays and organic matter) and the persistence of these compounds in the soil influence the efficiency in the control of nitrification (Benckiser et al., 2013). To date, few studies have evaluated these processes (Marsden et al., 2016), therefore, it is important to understand the key variables that affect the effectiveness and evolution of the NIs, to choose the most effective product in each situation, according to points of view from the sustainability between agricultural production, health, economy and environmental implications.

DMPP has a high inhibition potential of AOB (Benckiser et al., 2013; Zerulla et al., 2001), but the mechanism of action of this nitrification inhibitor derived from dimethylpyrazole is not yet clear.

Our first goal is to study the nitrification activities in cultures enriched with ammonia-oxidizing bacteria isolated from two soils, one that historically has received continuous N fertilization and the other without N fertilization, exposed to a DMPP, nitrification inhibitor derived from dimethylpyrazole. Our second goal is to study the persistence of the DMPP in cultures enriched in ammonia-oxidizing bacteria and in soil solution.

2. Materials and methods

2.1. Obtaining and characterizing cultures enriched with AOBs

Soil samples were collected from a long-term trial started in 2007 in the Natural Park of Arrábida, Natura 2000 south of Lisbon, Portugal (PTCON0010 Arrábida / Espichel), established to determine the effect of nitrogen fertilization on ecosystem functioning and services. The area is part of high nature value farmland and was not disturbed for the last 15 years. The natural vegetation is dominated by Cistaceae. The trial was located at (38º 29'N - 9º 01'W) and corresponds to a thermo-mediterranean subhumid bioclimatic domain (Dias et al., 2012).

The inocula used in this experiment were obtained from the unfertilized plots (NF) throughout the whole trial and from plots fertilized with N (F), where a dose of 80 kg N ha^{-1} $year^{-1}$ was applied in the form of NH_4NO_3.

Bacterial populations enriched in AOB were obtained through 6 sequential enrichment events performed in vitro conditions. Each soil sample was diluted 5 % (w/v) in 20 mL of Synthetic Freshwater Crenoarchaeota medium (SFC) containing 1 mM NH_4Cl (Konneke et al., 2005; De la Torre et al., 2008), supplemented with calcium carbonate (7.5 g L^{-1}), to buffer the medium; pimaricine (0.04 g L^{-1}) to prevent fungi growth; and sodium chlorate (5 mg L^{-1}) to inhibit growth of nitrite oxidizing bacteria. Cultures were incubated at 28 ºC for 30 days. After which the bacterial cultures were sub-cultured into the following sequential enrichment by transferring 5 % of the previous culture (v/v) to fresh medium.

To confirm the presence of AOBs at the end of the last sequential enrichment, an identification strategy based on 16 S rRNA gene amplification, sequencing and phylogenetic analysis was applied.

DNA was extracted from the corresponding stock experimental cultures using a Tris-EDTA buffer-based boiling method of DNA extraction (Sambrook et al., 2001) The samples were centrifuged at 2000 rpm for 10 min to remove precipitated $CaCO_3$ and the supernatant was centrifuged at 4000 rpm for 10 min. The pellet was re-suspended in Tris-EDTA buffer (pH 8) and centrifuged at 13 000 rpm for 5 min. The supernatant was discarded, and the pellet was re-suspended in Tris-EDTA buffer supplemented with 0.1 % Tween 80. Samples were incubated at 100 ºC for 10 min

16 S rRNA gene amplification was performed with primers CTO189F and CTO654r targeting betaproteobacterial ammonia oxidizers (Kowalchuk et al., 1997). All PCR products were verified on 1 % agarose gels stained with ethidium bromide under UV light. The 16 S rRNA gene amplicon was purified (GeneJet Quick Prep) and sequenced.The 16 S rRNA partial gene sequences were automatically edited with Phred-Phrap and blasting was performed with WebBLAST from NCBI using the nucleotide database.

Phylogenetic analysis was performed on the Phylogeny.fr platform, using complete 16 S rRNA gene sequences from 30 betaproteobacterial AOBs from *Nitrosomonas* and *Nitrosospira* genera, to compare with the sequences obtained from the experimental cultures. Gammaproteobacterial AOBs from *Nitrosococcus* genus were used as outgroup. After alignment with ClustalW, ambiguous regions were removed with Gblocks (v0.91b) using the default parameters. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT), using the default parameters. Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3).

2.2. Effect of DMPP on cultures enriched with AOBs

The ammonia-oxidising bacteria (AOBs) of the two plots (NF or F) and 9 mL of growth medium 240 with a concentration of 1 ppm of DMPP were used in the experiment, which was set up in sterile falcon tubes (15 mL). Simultaneously, a control was tested with the inocula of both origins, cultures NF and F, without the inhibitor.

The concentration of DMPP was set at 1 ppm, based on the commercial recommendation that is 0.8 % of applied N applied as ammonium. Medium 240 (NBRC culture collection; https://www.nite.go.jp/nbrc) contained $(NH_4)_2SO_4$ (0.5 g L^{-1}), NaCl (0.3 g L^{-1}), K_2HPO_4 (1 g L^{-1}), $MgSO_4 0.7\ H_2O$ (0.3 g L^{-1}), $FeSO_4 0.7\ H_2O$ (0.03 g L^{-1}), $CaCO_3$ (7.5 g L^{-1}). The medium was prepared with deionized water and sterilized in an autoclave (121 °C, for 30 min), the final pH ranged between 7.9 and 8.2. Since medium 240 does not contain Cu and this appears to be a cofactor of the target enzyme of the AMO nitrification inhibitors, the same assay (described above) was performed in parallel by adding 1 ppm of $CuSO_4$ to the medium.

The falcon tubes with the different treatments were incubated during 63 days, in a horizontal agitator at a speed of 100 rpm, in darkness at 22 °C. The sampling for evaluation of nitrification activity was carried out weekly, except for the first week when a sample was also taken at 72 h from the start of the assay. Samples of 200 µl of each culture were taken under sterile conditions within a laminar flow hood.

The AMO activity was estimated by quantifying the disappearance of ammonium in the medium, and the ammonium was quantified by the

Solorzano method (Solórzano, 1969). The HAO activity was determined based on the nitrite accumulation profile, using the Griess method (Hood-Nowotny et al., 2010). In addition, to verify that the culture was enriched in AOB bacteria and not in NOB bacteria, the accumulation of nitrate was determined at the end of the test period by ion chromatography, using a 940 Professional IC Vario 2, Metrohm, Metrosep A Supp7 column, 150 / 4.0 and pre-column Metrosep A Supp 4/5 Guard 4.0, equipped with a conductivity detector, flow 0.7 mL min^{-1}, 45 ºC, eluent 3.6 mM $NaCO_3$.

2.3. DMP content in the cultures enriched with AOBs

DMP content was determined at different times in the culture medium (3, 14, 27, 41, 56 and 63 days). For this purpose, 0.5 mL of the AOB cultures were taken and sonicated for 20 min, centrifuged for 15 min at 10,000 *g* and 4 °C, then the supernatant was filtered using a nylon syringe filter of 0.2 μm to remove the carbonate from the medium and the remains of bacteria. In the supernatants obtained, the content of DMPP was measured by HPLC as described in Rodrigues et al. (2018).

2.4. DMP content in soil solution

A sample of soil was taken from the first top 20 cm of an agricultural land located in the Navarra Public University practices farm (42º 47'N - 1º 37'W), air dried, sieved with a 2 mm sieve and moistened to field capacity using funnels buchner. The humidity at field capacity was estimated by drying some subsamples to 34 % at a temperature of 105 °C. The soil used was silty clay loam in texture, with a pH of 7.9, 3.1 % organic matter and 0.14 % total nitrogen. Fifty grams of soil samples at field capacity were placed in Falcon tubes with perforated caps to facilitate aeration, DMPP inhibitor was added to the soil dissolved in 1 mL of water so that the concentration in the total water volume of the samples was 1, 10 and 100 ppm. Falcon tube batteries were placed in triplicate for the inhibitor to sample it independently at different incubation times until the DMP content was less than 10 % initially applied. Every three days the humidity of the samples was controlled and corrected by the addition of deionized water.

For each time the corresponding falcons were taken and centrifuged at 500 g for 30 min. The soil solution (supernatant) was collected, sonicated and filtered using a nylon syringe filter of 0.2 μm, the DMPP concentration was determined from the filtrate in HPLC as described in Rodrigues et al. (2018).

2.5. Data analysis

To obtain an overall assessment of the effects of DMPP on AMO and HAO activities, an integration of the rates of ammonium consumption (for AMO) or nitrite production (for HAO) along the 63 days of assay was performed for each treatment by calculating the respective area under curve (AUC) using trapezoidal rule, followed by normalization (AUCr) with the maximum AUC (area assuming no NH_4 consumption).

AMO AUCr (%) = [1 - (AUC / AUC max)] * 100.

HAO AUCr (%) = (AUC / AUC max) * 100.

The percentage of ammonium use for energy was calculated based on the following equations.

[NH_4 consumed] = [NH_4 initial] - [NH_4 minimum].

% NH_4 for energy = [NO_2 produced] / [NH_4 consumed] * 100.

Data on AMO, HAO and NOR activity and DMPP contents was analysed using a one-way analysis of variance (ANOVA) and Duncańs Multiple Range Test was used. Statistical analyses were conducted at a significance level of 5 % ($P \leq 0.05$), using Statistical Product and Service Solutions (SPSS) for Windows, version 15.0.

3. Results and discussion

3.1. Characterization of the cultures enriched in AOBs

Following six months of sequential enrichment, each culture from an initial soil inoculum of an unfertilized plot (culture NF) or of a fertilized plot with 80 Kg N ha^{-1} $year^{-1}$ (culture F), showed to harbor only a single dominant population of ammonia oxidizing bacteria. In fact, a unique and unambiguous sequence of 16 S rRNA gene was obtained for each culture, one with 462 bp for NF and another with 389 bp for the F culture, after PCR amplification with primers targeting the majority of betaproteobacterial AOB (Kowalchuk et al., 1997). Such trend for homogeneity has already been described by other authors during soil enrichments and microcosm experiments (Stephen et al., 1996; Webster et al., 2005).

As shown in Fig. 1, both cultures are affiliated with *Nitrosospira* genus, although belonging to different currently accepted phylogenetic clusters and subclusters (Purkhold et al., 2012; Webster et al., 2005).

The retrieval of *Nitrosospira* spp. cultures from the Arrábida soil samples was somehow expected, considering that this AOB genus has been demonstrated by 16S rRNA gene sequencing to be the most abundant one in soils of the Iberian Peninsula (Calleja-Cervantes et al., 2015).

Culture F belongs to cluster 0 and shows 99.74 % 16SrRNA identity with the type strain of *Nitrosospira lacus*. Culture NF belongs to cluster 3, being more related with *Nitrosospira* spp. strains from subcluster 3a (99.78 % identity with strain 24 C) than to *Nitrosospira tenuis* and *Nitrosospira briensis* of subcluster 3b (less than 99 % identity).

Although *Nitrosospira* spp. from cluster 3 have been described as dominant in grasslands or arable soil with neutral pH that receive high fertilizer inputs (Ciccolini et al., 2016), strains from cluster 3a are inhibited at higher ammonia concentrations while strains from cluster 3b remain unaffected (Webster et al., 2005).

Regarding *Nitrosospira* spp. from cluster 0, that seem more likely to appear in low intensity agriculture or unmanaged soils with no fertilization (Ciccolini et al., 2016), *Nitrosospira lacus* strains $APG3^T$ and Nsp5 have a maximum ammonium tolerance of 100 mM NH_4Cl similarly to strains from cluster 3b (Urakawa et al., 2015).

Since the serial enrichments for fertilized and unfertilized soil samples were performed with 1 mM NH_4Cl and identical procedures, the different phylogenetic clustering of cultures F and NF reveals a change in the evolution pattern of the original AOB soil community (that included strains from clusters 0 and 3a). Because of the long-term N fertilization, the more ammonium tolerant strain from cluster 0 was adaptively selected and became the dominant species, being selected along the laboratory enrichment (culture F). In the absence of fertilization, the strain from cluster 3a became the dominant species being therefore selected along the laboratory enrichment (culture NF).

3.2. Effectiveness of DMPP in cultures enriched with ammonia-oxidizing bacteria

Taking into account that AOB are organisms of small size and that the cellular concentrations reached in the culture medium are quite low, these bacteria are very difficult to quantify with classical cell counting techniques (Farges et al., 2012), so most of the growth and activity studies of AOB are based on quantifying the production of nitrite in the culture medium.

Indirect measurements of ammonia oxidation (AMO) and nitrite production (HAO) have been proposed as indicators of the potential nitrifying activity of the soil (Norton and Stark, 2011) and as a proxy for the population size of AOB (HesselsØe et al., 2001).

Fig. 2 shows the decrease in the ammonium concentration of AOB cultures according to the different treatments. A sharp decrease of ammonium concentration was observed during the first three days for all treatments. However, from this time point onwards, AOB cultures

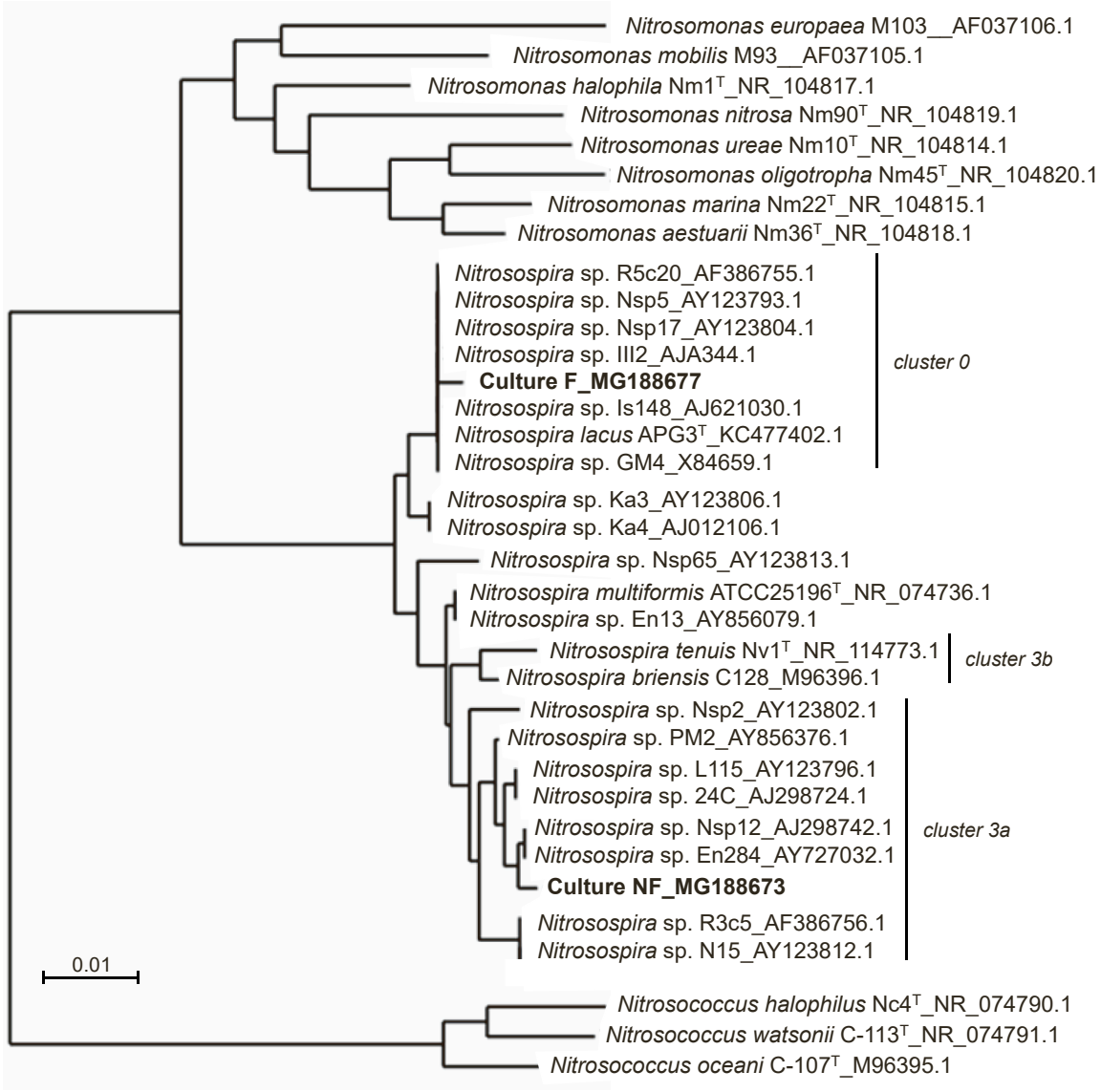

Fig. 1. Maximum likelihood partial 16S rRNA phylogenetic tree displaying the relationships of enriched cultures F and NF with reference strains and enrichment cultures of betaproteobacterial AOBs from *Nitrosomonas* and *Nitrosospira* genera. The gammaproteobacterial *Nitrosococcus* genus was used as outgroup. The scale bar represents 1 % estimated substitutions. Cluster numbers are according to Purkhold et al. (2012) and Webster et al. (2005).

treated with DMPP maintained their ammonium concentration, while control cultures without inhibitor showed no nitrification inhibition and the ammonium concentration decreased until levels very similar to zero. Integrating the rates of ammonium oxidation along the 63 days of the experiment for each treatment clearly showed that DMPP was effective in reducing the AMO activity by 50 % or more in both NF and F isolates (Fig. 2B).

The initial sharp decrease of ammonium was a very surprising result and although no immediate explanation can be provided several factors may be evoked (Robertson, 1989). In fact, some NI such as Nitrapyrin (2-chloro-6- (trichloromethylpyridine) are not soluble in water and the commercialized product needs to the stabilized with organic solvents, which may interfere with its nitrification efficiency (McCarty and Bremner, 1990). However, DMPP has the advantage of being soluble in water, which decreases the contribution of confounding effects to its nitrification inhibition efficiency.

The environmental concentration of Cu is also known to affect the nitrification rate of the AOB treated with DMPP (Corrochano-Monsalve et al., 2021). At the end of the assay (63 days), ammonium consumption by DMPP treated AOB cultures was between 15 % and 29 % of the initial ammonium concentration in the NF and NF+Cu cultures respectively and between 24 % and 37 % in the F and F+Cu cultures respectively, mainly occurring this decrease in the first three days of incubation. Moreover, in the AOB cultures without DMPP, the addition of Cu slightly decreased the disappearance of ammonium with respect to their controls without Cu, while in the cultures with DMPP the opposite was observed (Fig. 2B).

In Fig. 3 we can observe the changes of the nitrite concentration along the time for all treatments. From the third day of incubation until day 35 the production of nitrite significantly increased in control AOB cultures but not in those treated with DMPP regardless of the origin of the culture (NF or F) or the addition of Cu (Fig. 3A).

These huge differences in the proportion of ammonium used for biomass accumulation does not imply a sharp increase in the AOB growth because it is estimated that 35 mol of ammonium are needed to fix one mole of CO_2 (Bock and Wagner, 2006), a very low energy use efficiency that justifies the slow growth rate of these organisms.

The production of nitrite, both in bacterial cultures from NF and F soil isolates, without and with Cu addition treated with DMPP were significantly lower than in the control AOB cultures, not exceeding

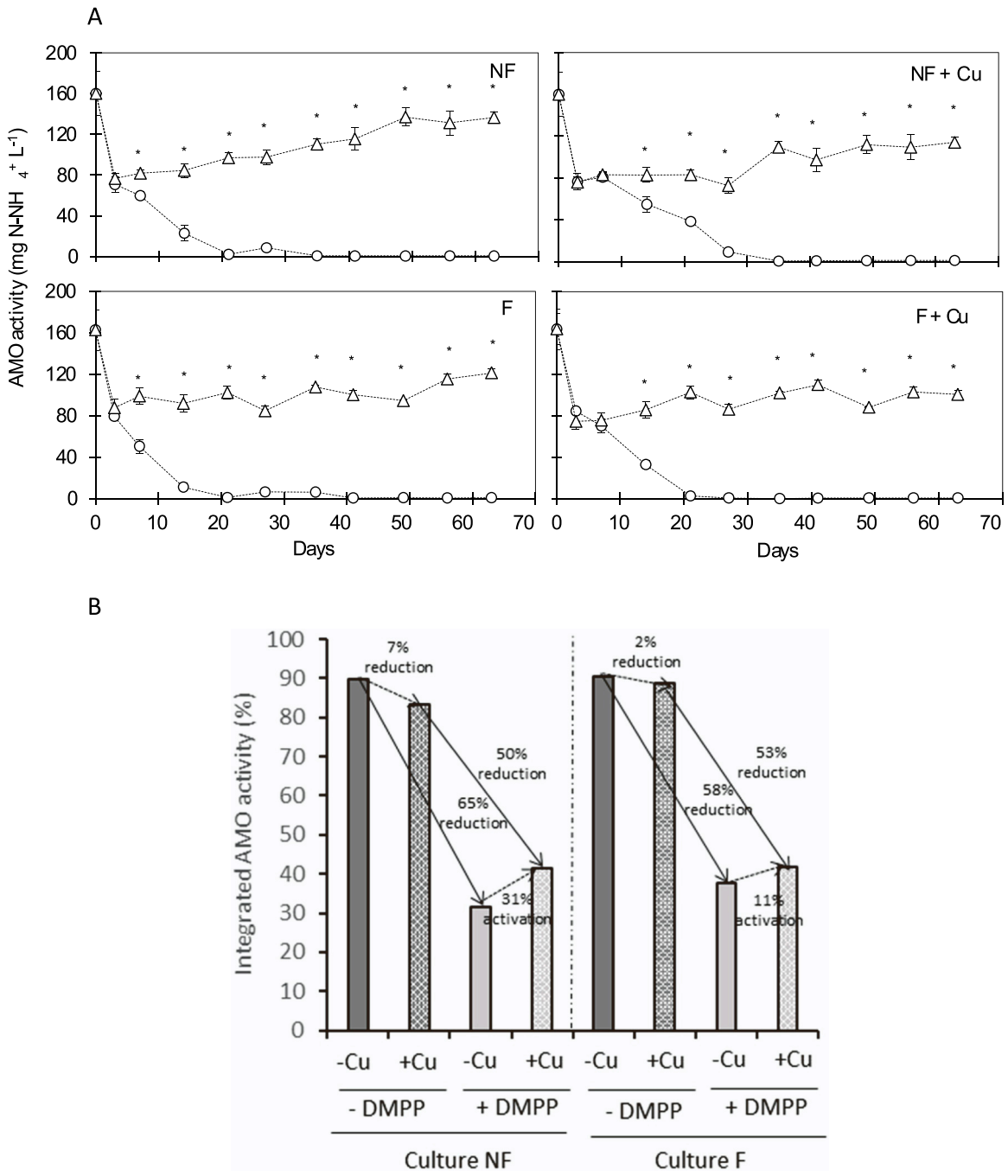

Fig. 2. (A) AMO activity expressed by consumption of ammonium in cultures of AOB isolated from a soil without fertilization (NF) and a similar soil with fertilization (F), without and with Cu addition. Data represents the mean and standard error (n = 3) for the treatments of 1 ppm of DMPP (Δ) and control without inhibitor (○). (n = 3). (B) Effect of DMPP, without and with Cu addition, on the integrated AMO activity of culture NF and culture F estimated by relative AUCs.

10 mg $N\text{-}NO_2$ L^{-1} at the end of the assays.

It was interesting to note that during the first 3 days of incubation the observed high ammonium oxidation rates were not accompanied by high HAO rates, since the sharp decrease in ammonium concentration was not accompanied by a sharp increase of nitrite accumulation in the medium of AOB cultures (Figs. 2B and Fig. 3B). This discrepancy between the rate of "substrate disappearance and product accumulation" may be explained if part of the substrate (ammonium) was used for biomass production (Junier et al., 2010; Farges et al., 2012). In fact, using this rational, it was possible to demonstrate that the presence of DMPP drastically changed the balance between ammonium consumed for energy production and for biomass accumulation. All the AOB cultures without DMPP used 87–88 % of the ammonium for energy production (high levels of nitrite accumulation in the medium), while in presence of DMPP only 19 % of the ammonium consumed was used for energy production (Fig. 4).

From day 35–47, nitrite production stabilized in the control treatments. The differences found in these treatments for the consumption of ammonium at day 14, coincide with trends contrary to the accumulation of nitrite. With the results obtained, we can conclude that the AOB cultures from the soil with N-fertilization did not have a greater nitrifying capacity than those from soil without N-fertilization, in disagreement with the finding of Di et al. (2009) and Di et al. (2010), that fertilization greatly favors the activity of nitrifying bacteria.

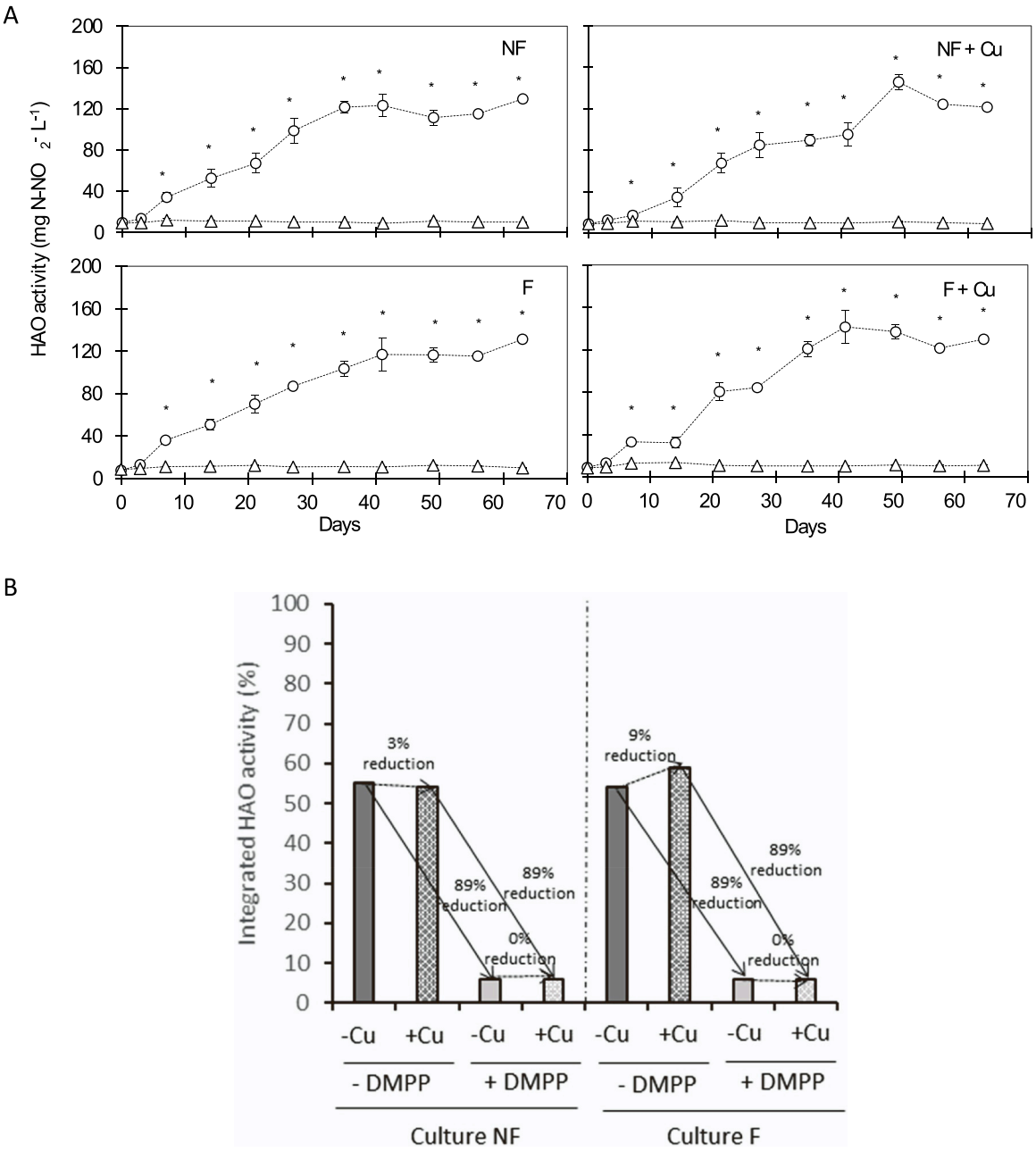

Fig. 3. (A) HAO activity expressed by nitrite production in cultures of AOB isolated from a soil without fertilization (NF) and a similar soil with fertilization (F), without and with Cu addition. Data represents the mean and standard error (n = 3) for the treatments of 1 ppm of DMPP (Δ) and control without inhibitor (○). (B) Effect of DMPP, without and with Cu addition, on the integrated HAO activity of culture NF and culture F estimated by relative AUCs.

During the first 14 days in control treatments the addition of Cu slowed down the consumption of ammonium and the production of nitrite, therefore nitrification. It is possible that in the culture media without Cu, the function of Cu as a cofactor of amoA (Arp et al., 2002; Corrochano-Monsalve et al., 2021) is being performed by Fe, which was present in culture medium as $FeSO_4$. In fact, several authors describe Fe as a possible cofactor of amoA acting in substitution of Cu (Zahn et, 1996).

With respect to NOR activity, nitrate accumulation at the end of the experiment (day 63) was very low for all AOB cultures (Table 1), revealing that our cultures were enriched in ammonia-oxidizing but not in nitrite-oxidizing bacteria. While nitrate accumulation was small for all treatments, DMPP was able to reduce it to 16 % of the accumulation present in cultures without DMPP. The origin of the culture (F and NF) did not affect the minimum NOR activity detected, while the presence of Cu slightly decreased the NOR activity in control AOB cultures (Table 1). These data suggest an inhibitory effect by Cu on NOR activity, there is much bibliography that highlights the role of metals such as Cu, Fe, Zn. as cofactors of oxidation-reduction enzymes, but there are no works that specifically indicate Cu as a factor of inhibition of NOR activity. Therefore, this result may be of great interest in future research.

The ability of DMPP to inhibit nitrifying bacteria is well documented: Benckiser et al. (2013) using nitrifying bacteria isolated from soil, showed that in the presence of 5 μg L^{-1} DMPP, nitrite production was totally inhibited for 75 days; Dong et al. (2013) observed a decrease of almost 100 % in the expression of the amoA gene compared to control conditions without inhibitor; Li et al. (2008) found that DMPP caused a decrease of 30 % in the AOB without affecting the population of nitrite-oxidizing bacteria (NOB). These observations support the specificity of the action of DMPP, which like DCD, is considered to specifically

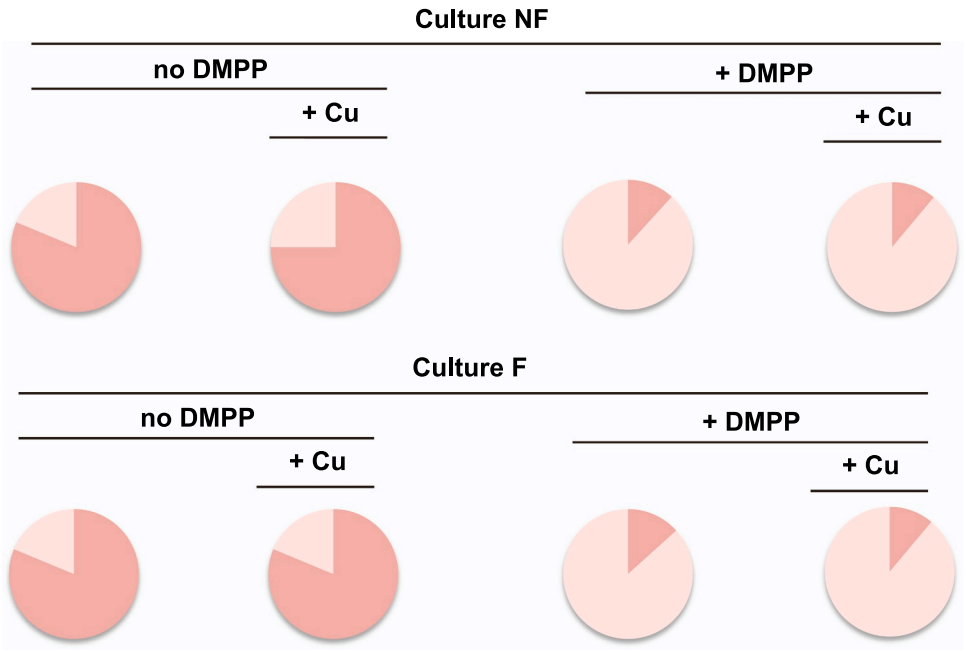

Fig. 4. Partition of NH_4^+ for energy (dark sector) and biomass production (light sector) in cultures NF and F without and with DMPP, in the absence or presence of Cu.

Table 1
NOR activity, expressed by accumulation of nitrate (at day 63) in cultures of AOB isolated from a soil without fertilization (NF) and a similar soil with fertilization (F), without and with Cu addition. Data represents the mean and standard error (n = 3) for the treatments of control without inhibitor and DMPP (1 ppm). Significant differences between control and DMPP are shown in capital letters and significant differences between the different cultures are shown in lower case letters.

	Final accumulated $N\text{-}NO_3^-$ (mg L^{-1})			
	Culture NF	Culture F	Culture NF+Cu	Culture F+Cu
Control	2.4 ± 0.4^{Aa}	3.3 ± 0.4^{Aa}	1.8 ± 0.0^{Ab}	1.5 ± 0.2^{Ab}
DMPP	0.4 ± 0.0^{Ba}	0.4 ± 0.0^{Ba}	0.4 ± 0.0^{Ba}	0.4 ± 0.0^{Ba}

inhibit the activity of the enzyme ammonia monooxygenase. Di and Cameron (2004) found that with the application of the nitrification inhibitor DCD, nitrate leaching was reduced by 76 % (from 85 to 20 kg N ha^{-1} $year^{-1}$) in grasslands receiving cow urine. Similarly, DCD can significantly reduce nitrate leaching from grazed pastures (Monaghan et al., 2009; Dennis et al., 2012). However, DCD performance may present a seasonal effect with higher improvements in N plant uptake (46 %) in spring when compared to autumn (27–37 %) (Moir et al., 2012). DMPP has some advantages over DCD. It is equally efficient in doses ten times lower than those of DCD (Zerulla et al., 2001); and it decreases N losses by reducing NO_3^- leaching and N_2O emissions (Weiske et al., 2001; Macadam et al., 2003; Liu et al., 2013). Although, there are controversial conclusions on this topic, such that some authors find that DMPP is more effective in inhibiting nitrification than DCD, but only on AOBs, although it has no inhibitory effect on AOAs (Chen et al., 2015), while other authors observe a similar inhibition of nitrification of the two inhibitors for both AOBs and AOAs (Di and Cameron, 2011). Our results show, like these authors, a high effectiveness of DMPP inhibiting the nitrifying activity in cultures enriched in AOBs.

The mechanism of amoA activity inhibition by DMPP is not clear, supposedly it acts as a Cu (or Fe) chelator (Ruser and Schulz, 2015), reducing the Cu available to act as an AMO cofactor, since metal chelating agents, in particular Cu chelators, inhibit AMO activity.

3.3. Persistence of DMP

The DMP persistence term in a medium has been defined by Vilas et al. (2019) as, the DMP content in the medium studied over a period of time. The efficiency of a nitrification inhibitor will depend, to some extent, on its persistence over time in the soil and of its partitioning between the soil solution and soil particles. Once in solution, DMPP dissociates in DMP and Pi, and DMP is the active molecule of nitrification inhibitors based in dimethylpyrazole (Benckiser et al., 2013). The stability of DMP, from the dissociation of DMPP into DMP + Pi in the AOB cultures was high, since after 63 days of incubation the concentration of DMP in the culture media was similar to the initial one regardless of the origin of the culture (NF and F) and the copper addiction or not (Table 2). DMP stably in the culture medium was high because the culture corresponds to a culture enriched in AOBs selected from the soil in a specific AOBs medium. In these cultures and according to the data obtained, it does not seem to have bacteria that degrade DMP.

In the soil solution (Fig. 5), DMP (originated from DMPP) was not as stable as it was in AOB cultures. Highlighting that in one hand, its effectiveness under field conditions may be time dependent, but on the other hand its degradation may avoid its accumulation in the soil and decrease possible phytotoxicity effects (Rodrigues et al., 2018). The stability that the inhibitor presents in AOB cultures allows evaluating

Table 2
Content of DMP nitrification inhibitor in cultures of AOB isolated from a soil without fertilization (NF) and a similar soil with fertilization (F), without and with Cu addition. Data represents the mean and standard error (n = 3) for the treatments of control without inhibitor and DMPP (1 ppm). Significant differences between different incubation times are shown in capital letters and significant differences between the different cultures are shown in lower case letters.

Days	NF culture	F culture	NF culture + Cu	F culture + Cu
3	0.87 ± 0.00^{Ab}	0.86 ± 0.00^{Bb}	0.80 ± 0.01^{Bc}	0.90 ± 0.00^{Aa}
14	0.85 ± 0.01^{Bb}	0.85 ± 0.00^{Cb}	0.90 ± 0.00^{Aa}	0.90 ± 0.01^{Aa}
27	0.86 ± 0.00^{Bb}	0.85 ± 0.00^{Cb}	0.90 ± 0.00^{Aa}	0.90 ± 0.01^{Aa}
41	0.83 ± 0.02^{Bb}	0.86 ± 0.00^{Bb}	0.90 ± 0.00^{Aa}	0.90 ± 0.00^{Aa}
56	0.87 ± 0.00^{Ab}	0.87 ± 0.00^{Ab}	0.90 ± 0.01^{Aa}	0.90 ± 0.02^{Aa}
63	0.87 ± 0.00^{Ab}	0.87 ± 0.00^{Ab}	0.90 ± 0.00^{Aa}	0.90 ± 0.00^{Aa}

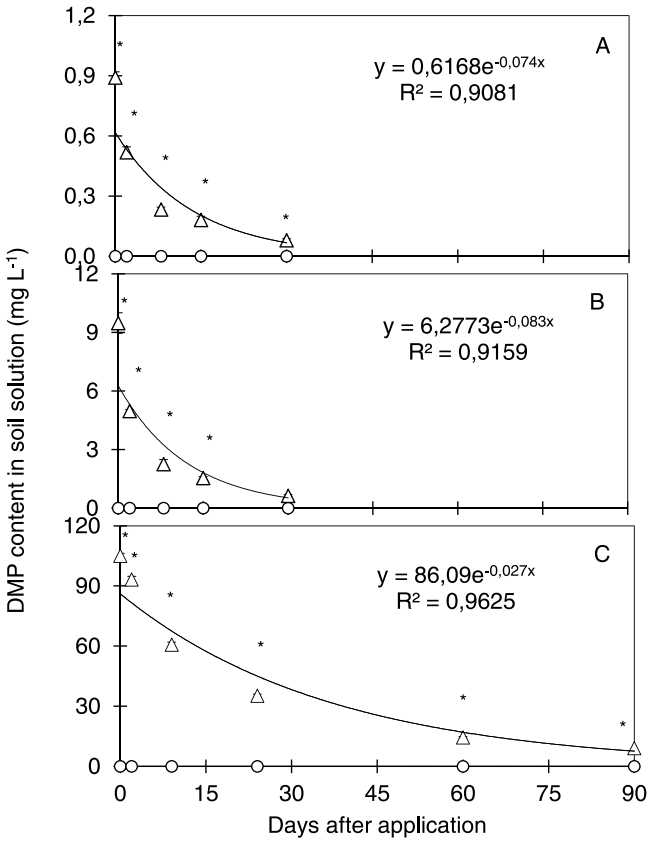

Fig. 5. Evolution of DMP in soil solution of soil treated with 1 ppm (A), 10 ppm (B) and 100 ppm of DMPP (Δ) and without inhibitor (○) with exponential function adjustment. Data represents the mean and standard error (n = 3).

the sensitivity of AOB to DMP but not its efficiency in soil conditions where the biota is more diverse than in enriched cultures and DMP is more susceptible to be degraded.

The instability of DMP in soil may be associated with the presence of microorganisms able to decompose DMP such as Burkholderia spp.

During the first 15 days of DMP incubation in soil between 79 % and 84 % of DMP was degraded; and by day 30, 91–94 % when the DMPP applied to the soil was 1 and 10 ppm respectively (Fig. 5A, B). The low stability of DMP in soils is interesting, on the one hand, to know the limited time which DMP can exercise the nitrification inhibition, so that there is no risk of a complete or lasting inhibition of natural nitrifying activity of soil. On the other hand, the chances decrease of DMP being absorbed by crops, such as grasslands (Rodrigues et al., 2018) and entering the animal trophic chain are decreased.

DMP was degraded if applied to the soil in higher doses (100 ppm), reaching 90 % of product degradation only on day 90 (Fig. 5C). Which can be quantified by the factor (f) of the exponential function that determines DMP degradation over time, which is lower for 1 (f = −0.074) and 10 (f = −0.083) ppm treatments than for the 100 (f = −0.027) ppm treatment. Lower rate of degradation at high concentrations of DMP (x100 the recommended dose) would indicate a longer period of permanence of DMP in the soil, although these concentrations of the inhibitor are unlikely to be found in soils of agricultural systems even with repeated applications in different crop season.

4. Conclusions

The dominant strains of AOB present in the enrichments of original F and NF soils belonged to the *Nitrosospira* genus, the most common genus of AOB found in Mediterranean ecosystems.

The nitrification rates observed in cultures enriched in AOBs isolated from soils with continuous N fertilization were similar than those of cultures without N fertilization.

In AOB cultures DMPP was a very efficient inhibitor of nitrification (> 50 % inhibition of integrated AMO activity), mainly due to the stability of DMP in the cultures. However, the stability of DMP was drastically reduced under soil conditions, indicating that the study of the efficiency of this nitrification inhibitor in cultures enriched with AOBs could be overvalued.

The Cu addition to the culture medium did not enhance nitrification rates, which suggests that AMO may use alternative co-factors, or that distinct organisms may have AMO enzymes using distinct co-factors.

CRediT authorship contribution statement

Berta Lasa: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Writing - Original Draft, Supervision, Project Administration, Funding Acquisition. **Janaina M. Rodrigues:** Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing - Original Draft. **Catarina A. Gouveia:** Methodology, Formal Analysis, Investigation, Resources, Data Curation, Writing - Original Draft. **Rogerio Tenreiro:** Validation, Writing - Review & Editing, Visualization, Resources. **Cristina Cruz:** Validation, Writing - Review & Editing, Visualization, Resources.

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.

Data Availability

No data was used for the research described in the article.

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