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The Ability of Nitrification Inhibitors to Decrease Denitrification Rates in an Arable Soil

Jie Li [1,*], Wenyu Wang [1,2], Wei Wang [1] and Yaqun Li [1,2]

[1] Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110164, China
[2] University of Chinese Academy of Sciences, Beijing 100049, China
* Correspondence: jieli@iae.ac.cn; Tel.: +86-18840608623

Abstract: A nitrification inhibitor is an effective tool that can be used to reduce the loss of nitrogen (N) and improve crop yields. Most studies have focused on the changes in the soil N mineralization process that may influence the dynamics of soil inorganic N and the soil N cycle. However, the effects of the inhibitors on denitrification rates remain largely unclarified. Therefore, in this study, we monitored the dynamics in annual denitrification rates affected by nitrification inhibitors from a maize field for the first time. Treatments included inorganic fertilizer (NPK), cattle manure, a combination of NPK and DMPP (3,4-dimethylpyrazole phosphate), and a combination of manure and DMPP, applied to brown soils in a no-tillage maize field. The findings demonstrated that the denitrification rate and denitrifying enzyme activity (DEA) were highly variable and there were no significant decreases in all treatment groups after the addition of DMPP. Compared to the control soils, the ammonium (NH_4^+-N) concentration was significantly increased, while the nitrate (NO_3^--N) level was significantly decreased in the DMPP-amended soils less than 30 days after treatment application, indicating that nitrification was partially inhibited. The formation of NO_3^--N and the nitrification rates could be markedly reduced by DMPP, while NO_3^--N availability did not affect the denitrification rates. Complete degradation of DMPP was observed in the soil on day 70 after DMPP addition, and its half-life was 10 days. Our study may ultimately help to clarify the characteristics of denitrification rates affected by nitrification inhibitors from different N fertilizer types applied to soils and explore the influencing factors of the dynamics in annual denitrification rates. However, more field studies evaluating the effectiveness of nitrification inhibitors in reducing denitrification under different sites and climate conditions, and the molecular mechanisms driving denitrification rate changes, need to be performed in the future.

Keywords: denitrification process; inhibitors; N transformation; nitrification rates

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1. Introduction

In agricultural systems, nitrogen (N) is a critical nutrient that can lead to greater crop yields and higher production of wool, eggs, milk, and animal tissues [1]. The improved N availability has allowed farmers to intensify production and to cultivate low productive soils. However, N may not be fully utilized in soil, as the plant uptake of fertilizer N does not exceed half of the N applied [2]. A significant amount of excess N in the environment originating from fertilizer N is lost from the soil and plant systems through volatilization and denitrification [3]. Thus, considerable attention has been paid to the impact of N on the environment.

As an important anthropogenic greenhouse gas, nitrous oxide (N_2O) has a global warming effect approximately 300 times that of CO_2 [4,5]. N_2O is typically formed in the soils via nitrification–denitrification processes. Nitrification (the oxidation of NH_4^+-N (ammonium) to NO_3^--N (nitrate)) and denitrification (the reduction of NO_3^--N to dinitrogen gas) occur under aerobic and anaerobic conditions, respectively [6]. Denitrification not only leads to the generation of N_2O, but also represents a possible mechanism for the loss of

available N in plants [6]. Thus, the development of a novel strategy is needed to protect the environment and guarantee the production of crop products.

Nitrification inhibitors are a potential strategy that can fulfill both environmental and productivity goals [7,8]. By disrupting the roles of *Nitrosomonas bacteria*, nitrification inhibitors can inhibit the conversion of soil NH_4^+-N to NO_3^--N, leading to a decrease in the soil pool of NO_3^- [9]. NH_4^+-N can be more easily absorbed to the soil than NO_3^--N, resulting in the better uptake of NH_4^+-N by plants or immobilization with dissolved organic matter, instead of leaching losses [10]. Most studies have demonstrated that nitrification inhibitors can attenuate nitrate leaching and N_2O lost to the environment, thereby improving N usage and leading to better plant growth [11,12]. By reducing NO_3^--N concentrations, nitrification inhibitors can indirectly regulate other microbial processes (e.g., denitrification rates) [13]. Denitrification can be affected by a variety of factors, such as NO_3^--N concentrations, soil moisture, soil pH, available C, and temperature [14]. However, contradictory results have been obtained regarding the ability of nitrification inhibitors to affect denitrification. It has been reported that nitrification inhibitors can inhibit the oxidation of NH_4^+ to NO_3^-, but do not reduce denitrification rates [15]. On the contrary, nitrification inhibitors have been shown to reduce both nitrification and denitrification in soils after the application of cattle slurry [14]. However, when an inhibitor was added into mineral-fertilized soil, there was no significant decrease in N_2O emissions [16]. It remains unknown whether nitrification inhibitors can decrease denitrification rates in arable soils, where N fertilizer and cattle manure are the major sources of N.

Among the most widely used NIs, 3,4-dimethylpyrazole phosphate (DMPP) is one of the most effective at improving N retention and reducing losses [17]. It has been indicated that DMPP has the advantages of a long-lasting inhibitory effect, low application rate, high persistence, and minor eco-toxicological side effects on plants [18,19]. Previous studies have indicated that the performance of DMPP in reducing the nitrification rate is not constant, as the persistence and effectiveness of DMPP in soil are strongly influenced by the environmental conditions (e.g., soil temperature) [20]. If the widespread use of DMPP is to be encouraged to reduce N losses, it is important to know which other aspects of the N cycle are affected by this compound. Various studies have demonstrated that animal manure applications would input large amounts of metabolizable C, mineral N, and water into soil simultaneously, which may favor both the nitrification and denitrification processes [21]. Previous researchers have mainly focused on the effects of nitrification inhibitors (e.g., DCD, nitripyrin) on changes in the soil N mineralization process that may influence the dynamics of soil inorganic N and the soil N cycle, lacking comparison with denitrification rates from other N fertilizers and nitrification inhibitors (DMPP) [22,23]. Moreover, observations of the denitrification process under various fertilizers with DMPP application during the whole maize growing period are lacking. Hence, a field experiment was performed to determine whether DMPP can affect denitrification rates by limiting NO_3^- availability. Therefore, the specific objectives of this study were to (1) clarify the characteristics of denitrification rates affected by nitrification inhibitors from different N fertilizer types applied to soils; (2) explore the influencing factors of the dynamics in annual denitrification rates (e.g., DMPP concentrations, soil properties). This will provide insightful information for our understanding of the achievement of inhibitors on the mitigation of N losses in arable soil under field conditions.

2. Materials and Methods

2.1. Experimental Fields

The field study was carried out at the Shenyang Experimental Station of the Institute of Applied Ecology, Chinese Academy of Sciences (41°32′ N, 123°23′ E) in Liaoning province. The average annual temperature and precipitation are 7.0–8.0 °C and 700 mm, respectively, with 147–164 frost-free days. According to the soil taxonomy classification, the soil can be classified as Alfisols. The soil properties at 0–20 cm depth are shown in Table 1. Maize (*Zea mays* L.) is continuously planted in early May and harvested in late September.

Table 1. Characteristics of the soils (0–20 cm) and manure used in this study.

Types	pH (H_2O)	Total P (g kg^{-1})	Total N (g kg^{-1})	Total K (g kg^{-1})	Available N (mg kg^{-1})	Available P (mg kg^{-1})	Available K (mg kg^{-1})	Organic Carbon (g kg^{-1})
Soil	6.8	0.59	1.49	16.8	91.1	14.7	90.7	12.2
Manure	7.2	6.9	22.35	16.7	-	-	-	2.54

2.2. Experimental Design and Field Management

Five treatments were included and organized in a randomized design with 3 replicates in this study: unfertilized controls (CK); NPK (T2); NPK + DMPP (T3); cattle manure (T4); manure + DMPP (T5). The plots were 20 m^2 (4 m × 5 m) and randomly placed at a distance of 1.5 m apart. In this study, urea was applied at a rate of 200 kg/K/ha; phosphorus (P) and potassium (K) fertilizers, as K_2SO_4 and KH_2PO_4, were applied at 58 kg/K/ha and 30 kg/P/ha. The cattle manure was applied at 2 Mg/ha. The manure characteristics are shown in Table 1. The inhibitor DMPP was applied at the rate of 1% of urea N. All fertilizers and inhibitors were added to the topsoil (0–10 cm) before maize sowing. The maize hybrid 'Dandong 1501′ was sown on 4 May 2021 at a 25-cm distance between seeds within rows and 60-cm row spacing to reach a target density of 6.67 plants/m^2. All crops were harvested on 28 September. The field management was in accordance with the routine cultivation practices of the local farmers.

2.3. Soil Sampling and Measurement

The soils were sampled on days 0, 5, 10, 15, 20, 30, 45, 70, 100, 130, and 160 after fertilizer application. Four individual samples at each plot were collected using a 3-cm soil auger and then mixed thoroughly to obtain individual bulked plot soil samples. The mineral N concentration (NO_3^--N and NH_4^+-N) was detected after sieving the fresh soils (<2 mm) and extraction with 2 M KCl. A combined electrode pH meter was employed to assess soil pH at a soil: water ratio of 1:2.5. Soil microbial biomass (SMB) was evaluated by substrate-induced respiration. The soil carbon availability was subsequently assessed. Briefly, the soil sample (35 g) was placed in a 1.8-L glass preserving jar, which was then sealed with a septum stopper-fitted lid. After incubation at 25 °C for 7 days, the accumulation of CO_2 in the headspace atmosphere of the preserving jar was evaluated as an indicator of soil respiration.

2.4. In Situ Denitrification Rates

The static soil core incubation system was used to measure the in situ denitrification rates according to the acetylene inhibition method [24,25]. Briefly, the intact soil cores consisting of PVC pipes with uniformly distributed holes were isolated and transferred into a glass preserving jar sealed with a septum stopper-fitted lid. Then, 120 mL acetylene (10% *v*/*v* of headspace) was placed in the jar, mixed thoroughly, and kept in a temperature-controlled room. Gases (22 mL) were collected from the jars at 30 min and 3, 6, and 24 h following acetylene addition, and then injected into a vacutainer until further use. All specimens were analyzed using a gas chromatography system (Philips, Cambridge, MA, USA) equipped with an electron capture detector at 350 °C. A porous packed column was used to separate the gas samples at 80 °C. The temperature at the injection port was 120 °C. To calculate the total denitrification rate, the solubility of N_2O in the soil water was taken into account using the temperature-dependent Bunsen absorption coefficient.

2.5. Denitrifying Enzyme Activity

To measure DEA, 10 g soil samples were weighed into a 100-mL Schott bottle [26,27]. Then, 20 mL nitrate glucose solution (0.1 g KNO_3 and 0.2 g glucose in 1 L water) containing 0.125 g chloramphenicol was placed into the Schott bottle. After sealing with rubber septum-fitted lids, the bottles were flushed with N gas for 2 min. Then, 10 mL acetylene was used to prevent the conversion of dinitrogen gas from N_2O. All specimens were incubated at

25 °C with shaking. After the removal of 5 mL headspace at 15- and 75-min intervals, the samples were kept in a 3-mL evacuated vacutainer until further use. Finally, all specimens were analyzed using the gas chromatography system (Philips, Cambridge, USA).

2.6. DMPP Extraction and Its Qualification

DMPP extraction and qualification was adapted from previous studies by Benckiser et al. (2013) and Chen et al. (2019) [20,28]. To extract DMPP from the soil sample, 10 g field fresh soil, 10 mL distilled water, and 0.2 mL 1 M K_3PO_4 were mixed together and shaken for 2 h at 30 rpm. Then, 0.2 mL 1 M $CaCl_2$ was added to the soil suspensions and samples were shaken for another 30 min at 30 rmp. Afterwards, 1 mL 1 M NaOH was added and samples were further shaken for 1 h. For transferring DMP into the t-butyl-methyl-ether phase (MTBE), 15 mL of MTBE was added and samples shaken for 1 h. The extract was then centrifuged at 3000 rmp for 5 min. The supernatant was evaporated and filtered through a 0.45-µm Millipore filter. The DMPP concentration was quantified with a Shimadzu HPLC device (Shimadzu, Kyoto, Japan) using a 5 µm, 4.6 × 150 mm Shiseido Spolar C18 column (Shiseido, Tokyo, Japan).

2.7. Statistical Analysis

The differences in soil biochemical parameters were analyzed by two-way ANOVA using SPSS Statistics 16.0 (SPSS Inc., Chicago, IL, USA). The 5% confidence level ($p < 0.05$) was considered statistically different. Pearson correlation analysis was employed to analyze the relationships between soil properties and denitrification rates.

3. Results

3.1. Soil Denitrification Rates

A remarkable seasonal effect was observed for the denitrification rates. The highest denitrification rate was observed in summer, while the lowest denitrification rate was detected in spring and autumn (Figure 1a). The application of urea and manure could lead to an increase in denitrification compared with the control treatment. There was no marked difference between urea- or manure-only soils and DMPP-amended soils, indicating that DMPP may not inhibit denitrification rates (Figure 1a).

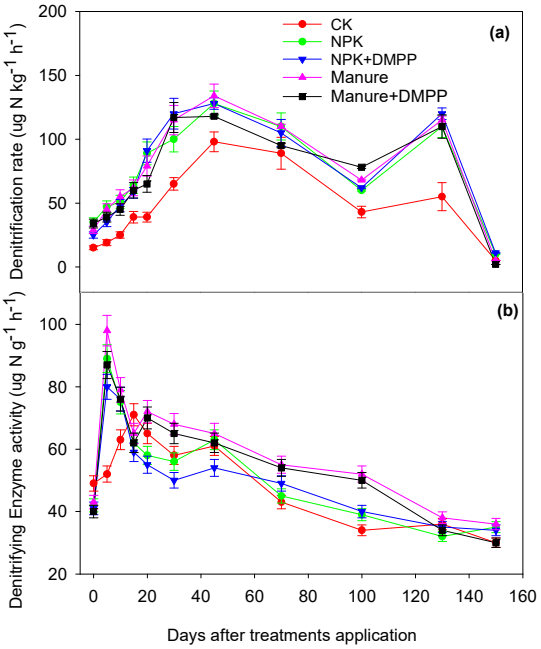

Figure 1. Denitrification rate (**a**) and denitrifying enzyme activity (**b**) at different days following application of different treatments. Data were presented as mean values with standard errors (n = 3).

As with denitrification rates, the highest DEA was found in the spring and summer months, while the lowest DEA was found in the autumn months. The increased DEA in the urea- or manure-amended soils was most obvious on day 5 after the fertilizer application and decreased with time ($p < 0.05$, Figure 1b). DEA was higher in manure-amended soils than other treatments ($p < 0.05$). Application of DMPP to soils could reduce DEA compared to urea- or manure-only treatment, but this difference was not significant (Figure 1b).

3.2. Soil Microbial Mass, C Availability, pH, NH_4^+-N, and NO_3^--N Concentration

The manure-amended soils had an increase in SMB compared with the urea-amended soils on day 5 after manure application ($p < 0.05$). However, the difference was negligible on day 30 (Figure 2a). SMB was not influenced by the application of DMPP in all treatment groups.

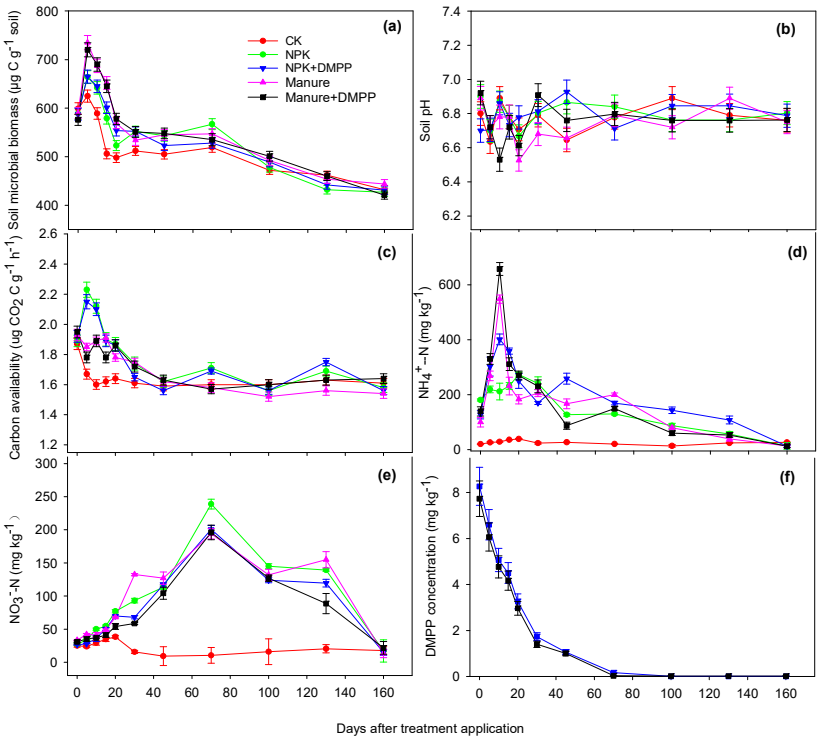

Figure 2. Soil microbial biomass (**a**), soil pH (**b**), carbon availability (**c**), NH_4^+-N concentration (**d**), NO_3^--N concentration (**e**), and DMPP concentration (**f**) at different days following application of different treatments. Data were presented as mean values with standard errors (n = 3).

The manure-amended soils demonstrated an obvious reduction in C availability over the sampling period ($p < 0.05$). However, the C availability was not decreased in both control and urea-amended soils (Figure 2b). No significant differences in carbon availability were observed among all treatment groups, indicating that C availability was not influenced by the addition of nitrification inhibitors in all treatment groups. Likewise, no significant differences in soil pH and C availability were observed between DMPP-amended soils and urea or manure soils (Figure 2c).

A similar variation trend was observed in all the fertilization treatments. After the application of NPK and manure soils, the concentrations of NH_4^+-N and NO_3^--N were first increased and then declined. In the urea and manure treatments, the addition of inhibitors could lead to higher soil NH_4^+-N concentrations compared to the soil without inhibitors (Figure 2d, $p < 0.05$). However, this was only significant before 20 days after inhibitor application, and, by day 30, the difference was negligible (Figure 2d,e). The manure-amended soils with inhibitors also had lower NO_3^--N content compared to those without inhibitors between 45 and 70 days after fertilization ($p < 0.05$).

In this study, Pearson correlation analysis was employed to analyze the relationships between soil properties and denitrification rates. The results revealed that the effect of inhibitors on denitrification rates was not markedly associated with DEA, soil NH_4^+-N, NO_3^--N, soil pH, and carbon availability (Table 2).

Table 2. Pearson correlation analysis of denitrification rates and soil properties.

Variable Factors	NPK		NPK + DMPP		Manure		Manure + DMPP	
	R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*
DEA	0.121	0.532	0.247	0.064	0.178	0.074	0.674	0.421
NH_4^+-N	0.378	0.126	0.498	0.079	0.452	0.145	0.546	0.178
NO_3^--N	0.236	0.214	0.216	0.142	0.312	0.126	0.201	0.097
pH	0.145	0.145	0.347	0.078	0.147	0.245	0.394	0.076
Carbon availability	0.189	0.321	0.421	0.231	0.325	0.365	0.414	0.069

3.3. Rate of Inhibitor Loss in the Soils

The degradation rate of DMPP was dramatically increased (Figure 2f). On day 5 after DMPP application, 81% of the applied DMPP was degraded. DMPP was not detectable in the soil on day 70. A half-life of 10 days was detected for DMPP.

4. Discussion

4.1. Impact of DMPP on Soil Denitrification Rates

In all the treatment groups, the denitrification rates increased progressively during the first 40 days after fertilization, with a progressive decrease observed on the following days. Previous research has shown that the denitrification rate is largely dependent on enzyme activity. The reduced denitrification rate over time in each treatment group may be a consequence of the decay of denitrifying microbes—that is, the DEA of soil [29] (Figure 1b). The application of inhibitors did not decrease the denitrification rates compared to soils treated with urea or manure only (Figure 1a). This may be due to the fact that the concentration of NO_3^--N exceeded 5 mg NO_3^-/kg soil, which is regarded as a threshold for denitrification [26]. The high concentration of NO_3^--N in the soil may limit the role of nitrification in supplying nitrate to denitrifiers [30]. Nitrification has become less crucial to ensure the adequate denitrification of NO_3^--N in denitrifying microbes. However, nitrification inhibitors can attenuate the losses of denitrification in soils with low initial NO_3^--N concentrations by affecting the availability of the nitrate pool in denitrifying microorganisms [30]. The limited denitrification by NO_3^- could also be attributed to the rapid degradation of inhibitors in the soil, thus decreasing the effectiveness of the inhibitors, which was supported by the inhibitor degradation data in our study. A remarkable decline (81%) in the concentrations of inhibitors was observed on day 5 after inhibitor application, which was probably due to microbial degradation, leaching, or sorption to soil organic matter (Figure 2f).

Furthermore, our results showed that the application of inhibitors did not affect soil DEA in all treatment groups (Figure 1b). DEA can be used to reflect the population size of denitrifying microorganisms, which is an indicator of the optimum conditions for denitrification [24,26]. The inability of inhibitors to regulate DAE in the soil can explain why the denitrification rates are not influenced by inhibitor application, as the denitrifying microbial communities were not inhibited. Similarly, previous work found that the application of nitrification inhibitors to urea did not inhibit denitrification [26]. Another study demonstrated that the effects of nitrification inhibitors on denitrification rates relied on the levels of available C [31]. The application of manure to the soil slightly increased the quantity of C present in the soil, and therefore the effects of DMPP in manure-amended soils were expected to improve slightly [32]. In our study, the manure-amended soils had higher DEA than the other two treatment groups (Figure 1b). The increased microbial

population might be attributed to the manure containing additional sources of nitrate and soluble C for microbial utilization [33].

4.2. Impacts of DMPP on Carbon Availability, Soil Microbial Biomass, and pH

It is crucial to assess whether the application of inhibitors can adversely influence the growth of soil microbial populations, as DMPP can specifically target the nitrifying bacteria [28]. As shown in Figure 2, the application of inhibitors did not affect SMB. These results are in agreement with previous findings reporting that DMPP did not affect the microbial biomass [28]. The non-significant effect of DMPP on SMB may be attributed to the fact that DMPP is bacteriostatic rather than bactericidal in its action [20].

In general, the application of DMPP had no effect on soil pH. A decline in soil pH was noted in all treatment groups between days 5 and 15, which may be related to the nitrification process, as nitrification is a major cause of soil acidification [34]. In this study, multiple stepwise regression analysis revealed that the effect of inhibitors on denitrification rates was not markedly associated with DEA, soil NH_4^+-N, NO_3^--N soil pH, or carbon availability. More studies are needed to clarify the molecular mechanisms underlying the inhibitory effects of nitrification inhibitors on denitrification rates.

5. Summary

The results showed that the denitrification rates and denitrifying enzyme activities were highly variable in different growing periods, but were not affected by the application of inhibitors. Partial inhibition of the nitrification process was observed, as revealed by an increase in the NH_4^+-N concentration and a decrease in the NO_3^--N concentration in the inhibitor treatments compared with the urea- or manure-only treatments. However, the decrease in NO_3^--N was not sufficient to limit NO_3^--N availability to denitrifiers, and, thus, the denitrification rates were found to not decrease. SMB, soil pH, and microbial respiration were not affected by nitrification inhibitors, regardless of whether manure or urea was applied in the soil. Our results concluded that the formation of NO_3^--N and the nitrification rates could be markedly reduced by DMPP, while NO_3^--N availability did not affect the denitrification rates. Furthermore, to confirm the findings of this study, field studies under different sites to explore additional mechanisms driving changes over longer time periods are needed.

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