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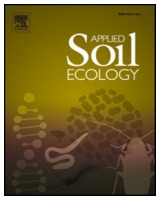

Research paper

Nitrogen rate drives the effectiveness of 3,4-Dimethylpyrazole phosphate (DMPP) in reducing N_2O emissions from limed soil subjected to temporary waterlogging

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ABSTRACT

Soil pH, moisture, and nitrogen (N) rate may affect the effectiveness of the nitrification inhibitor 3,4-Dimethylpyrazole phosphate (DMPP) in mitigating N_2O release. Thus, the present study raised the question: do soil pH and N rate regulate the effect of DMPP on mitigating N_2O production and promoting N_2O reduction to N_2 from soils subjected to temporary waterlogging? This was studied in a greenhouse experiment under semi-controlled conditions in which wheat was cultivated under aerobic conditions for 30 days, followed by a temporary waterlogging period of 5 days. The study investigated the factors of soil pH, which was manipulated by liming (unlimed and limed), application of the nitrification inhibitor DMPP, and N application rate (60 and 180 mg N kg soil^{-1}), referred to as N60 and N180. The N source was ammonium sulfate. DMPP reduced the relative abundance of nitrifying bacteria such as *Nitrospira* and *Nitrosospira* and, therefore, suppressed nitrification in all treatments. After waterlogging, DMPP efficiently mitigated N_2O-N release from unlimed soil (80 %), probably by suppressing fungal (*Cladosporium*, *Chaetomium*, and *Fusarium*) and bacterial (*Bradyrhizobium*) denitrifiers due to DMPP-induced lower NO_3^- concentrations. In addition, DMPP reduced cumulative N_2O-N release by 94 % at the N180 rate but was ineffective at the N60 rate in the limed soil. In conclusion, DMPP was confirmed as an excellent mitigation measure for N_2O release from unlimed soils subjected to waterlogging. However, our study demonstrated that relatively low N rates can lead to DMPP ineffectiveness in reducing N_2O release from limed soil subjected to temporary waterlogging.

1. Introduction

High crop yields are strongly dependent on the application of nitrogen (N)-based fertilizers (Zamanian et al., 2024). However, N fertilizers are subjected to several loss pathways, such as nitrate (NO_3^-) leaching, ammonia (NH_3) volatilization, and nitrous oxide (N_2O) emission, which is a potent greenhouse gas (Han et al., 2024; Ribeiro et al., 2024a). Therefore, mitigation measures for NO_3^- leaching and N_2O emissions, such as the application of ammonium (NH_4^+) N-based fertilizers treated with nitrification inhibitors (NIs), have been developed and studied worldwide (Lei et al., 2025; Ottaiano et al., 2020; Ribeiro et al., 2023; Vitale et al., 2017; Yang et al., 2016).

Applying NH_4^+-N fertilizers with NIs in agricultural soils suppresses the ammonium (NH_4^+) oxidation to NO_3^-. This occurs because NIs such as 3,4-Dimethylpyrazole phosphate (DMPP) can block the first step of nitrification due to the inhibition of the ammonium monooxygenase enzyme (Bozal-Leorri et al., 2022). DMPP is one of the most studied and utilized nitrification inhibitors worldwide. For instance, in a meta-analysis of field experiments, Yang et al. (2016) showed that DMPP reduced the overall N_2O emissions by 47.6 %. Additionally, DMPP has been demonstrated to be more effective than other common NIs, such as Dicyandiamide (DCD), especially in coarse-textured soils (Barth et al., 2019; Ribeiro et al., 2024b; Zerulla et al., 2001).

Some field experiments have shown that DMPP may be ineffective in reducing N_2O release. For example, in a study comprising a series of field trials, Nauer et al. (2018) found that DMPP did not reduce N_2O

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emissions from urea. Similarly, Rose et al. (2020) indicated the low effectiveness of DMPP in abating N_2O emissions from urea applied in a Gleysol. Both above-mentioned studies suggested that acidic soil pH may contribute to the poor performance of DMPP. Soil pH drives the deprotonation of NH_4^+ to NH_3, and it affects the activity of different soil ammonia-oxidizers because of their different affinities for NH_3 (Jung et al., 2022; Rojas-Pinzon et al., 2024). In this sense, ammonia-oxidizing archaea (AOA) is usually the main ammonia-oxidizer in acidic soils (Li et al., 2018). Moreover, there is evidence that DMPP targets ammonia-oxidizing bacteria (AOB) rather than ammonia-oxidizing archaea (AOA) (Shi et al., 2016b), so its efficiency in acidic soils might be hampered. Moreover, acidic soils commonly present inherent low nitrification rates, which might lead to unnoticeable differences between soils treated or untreated with NIs (Rose et al., 2020). In contrast, other researchers reported that NIs were effective in acidic soils (Oliveira et al., 2022). Huérfano et al. (2022) indicated that AOA was numerically dominant over AOB in an acidic grassland soil; however, DMPP reduced N_2O emissions by targeting only AOB, likely because it was dominant at the functional level.

Under conditions conducive to denitrification, soil pH is an important factor in controlling N_2O reduction to N_2 because low pH hampers the assembly of the nitrous oxide reductase enzyme (Wang et al., 2021). Thus, liming has been suggested as an alternative to mitigate N_2O emissions owing to enhanced N_2O reduction to N_2. Application of DMPP can also reduce N_2O emissions from denitrification by decreasing the substrates (NO_3^-) for denitrification, and reducing the $N_2O/(N_2O + N_2)$ product ratio of denitrification because NO_3^- and N_2O compete as electron acceptors (Huérfano et al., 2022; Torralbo et al., 2017). In addition, the N application rate may also impact N_2O production by influencing soil NO_3^- accumulation (Senbayram et al., 2019).

While DMPP effectiveness influenced by soil pH (e.g., liming) has been investigated elsewhere under aerobic conditions (Das et al., 2022; Kaveney et al., 2020; Li et al., 2024; Shi et al., 2016a, 2016b), it has not been studied under temporary waterlogging. Moreover, several studies evaluated DMPP effectiveness under semi-anaerobic conditions (e.g., 80 % water-filled pore space, WFPS) (Guo et al., 2022b; Ribeiro et al., 2024c; Torralbo et al., 2017). However, a comparison simulating an extreme weather event, such as heavy rainfall and, consequently, soil waterlogging, is lacking. Thus, it is unknown if the effect of DMPP on mitigating soil N_2O production and/or enhancing N_2O reduction N_2 relies on pH and N rate under such circumstances. Temporary waterlogging after heavy rainfall events is becoming more common due to climate change (Zhao et al., 2024). Moreover, most yearly N_2O emissions may originate from periods after rainfall because of intense nitrification and denitrification rates (Li et al., 2015). For instance, Zhao et al. (2024) indicated that high-frequency precipitation events were the main driver of N_2O emissions during the maize growing season in China.

Therefore, to the best of our knowledge, this study is the first to investigate the interactive effect of N rate and soil pH on DMPP effectiveness influenced by changes in soil moisture simulating temporary waterlogging. We hypothesized that DMPP effectively suppresses nitrification by reducing the relative abundance of nitrifying bacteria irrespective of soil pH and N rate. In addition, this manuscript raised the question: Do soil pH and N rate regulate the effect of DMPP on mitigating N_2O production and promoting N_2O reduction to N_2 from soils subjected to temporary waterlogging? To address the working hypothesis and research question, the following variables were measured: (i) daily and cumulative N_2O-N emissions, (ii) soil NH_4^+-N and NO_3^--N concentrations, (iii) relative abundance of nitrifying and denitrifying soil microorganisms, and (iv) the $N_2O/(N_2O + N_2)$ product ratio of denitrification.

2. Material and methods

2.1. Site and soil description

The soil was collected with a mechanized caterpillar excavator at a depth of 0 to 30 cm from an agricultural site in Grevenkrug, Schleswig-Holstein, Northern Germany (54°12′45“ N, 10°0’37” E). The site is located in the geological region “Lower Geest”, which is a sandy outwash region characterized by Gleyic Podzols and Brunic Arenosols (World Reference Base for Soil Resources, WRB) (FAO, 2015; Mordhorst et al., 2021). The “Lower Geest” region contains sandy soils with low fertility and water-holding capacity (Peters et al., 2022). In addition, Schleswig-Holstein has a temperate maritime climate, with an average annual precipitation of 833 mm and an average temperature of 8.9 °C (1981–2010) (Peters et al., 2022). After collection, the soil was air-dried, passed through a 2 mm sieve, and homogenized. The main properties of the sandy soil were: sand, 908 g kg^{-1}; silt, 59 g kg^{-1}; clay, 33 g kg^{-1}; SOC, 9.7 g kg^{-1}; total N, 0.7 g kg^{-1}; NH_4^+-N, 3.16 mg kg^{-1}; NO_3^--N, 9.00 mg kg^{-1}; pH_{CaCl2}, 5.4; water-holding capacity (WHC), 0.27 g g^{-1}.

2.2. Experimental setup

A greenhouse experiment was carried out at the Institute of Plant Nutrition and Soil Science at Kiel University, Germany, from May to July 2023. A completely randomized design with four replicates was utilized, and the investigated factors were liming (unlimed and limed), application of the inhibitor DMPP (with and without DMPP), and N application rate (60 and 180 mg N kg $soil^{-1}$) referred to as N60 and N180. These N rates were chosen based on previous studies in which N60 represents common fertilization practices of ca. 150 kg ha^{-1}, and N180 represents very high N concentrations, which may occur shortly after N application by spot or band techniques (Yang et al., 2021).

Liming was performed by applying calcium carbonate ($CaCO_3$) at a rate of 4 g $CaCO_3$ kg $soil^{-1}$. The $CaCO_3$ application rate was based on trials aiming to reach a pH_{CaCl2} of 7 and cause significant changes in soil NH_4 oxidation rates. $CaCO_3$ was incorporated into the soil with a cement mixer. At this step, the soil also received basic fertilization, except for N (Table S1).

Subsequently, thirty-two cylindrical pots (15 cm diameter and 33 cm height) were filled with 6 kg soil (dry weight) up to 25 cm height. After filling the pots with soil, ammonium sulfate ($(NH_4)_2SO_4$) was applied at rates of 60 and 180 mg N kg $soil^{-1}$. DMPP was applied as 1 % of the applied NH_4^+-N according to the European Union fertilizer regulations (European Commission, 2014). The N fertilizer and DMPP were dissolved in deionized water, and a 100 mL solution per pot was applied to the surface. Afterward, the soil was removed from the pots and homogenized in a bucket before filling the pots again. This step was done to avoid ammonia volatilization, especially from the limed soil, and to ensure uniform incorporation of the treatments into the soil.

Wheat (*Triticum aestivum* L.) cultivar KWS Scirocco, with three plants per pot, was sown and cultivated for 45 days under a day/night temperature of 21/15 °C, a photoperiod of 14/10 h (day/night) and relative humidity between 62 and 70 %. According to previous trials, such settings were chosen because they are favorable to the cultivation of the summer wheat cultivar utilized in this study. Wheat biomass data were measured and are presented in supplementary fig. S7.

Soil water content was monitored every one or two days with a time domain reflectometry (TDR) sensor (*E*-Test, Lublin, Poland) placed into the 5 to 15 cm soil layer. Subsequently, irrigation was performed with deionized water to keep soil moisture at 65 % of the WHC during the first 30 days. Subsequently, as the pots had an opening in their bottom, they were placed in 20 L buckets filled with deionized water to stepwise cause waterlogging by capillarity. Waterlogging was kept for 5 days, and later, the pots were removed from the buckets to allow drainage for a further 10 days (Fig. 1). At the end of the experiment, the soil moisture in the pots was 90 % WHC. There are several published field studies on the

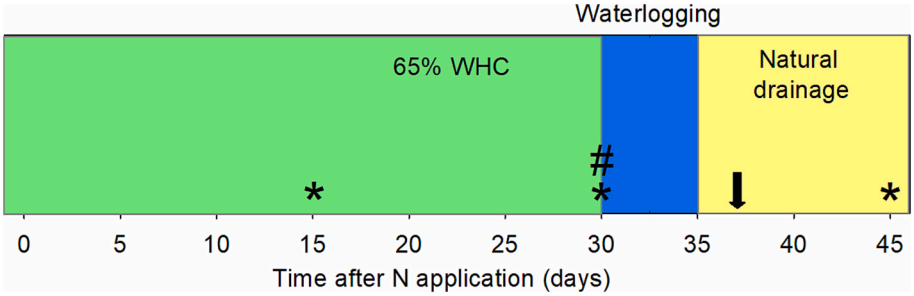

Fig. 1. – Representation of the soil moisture over the experiment. * represent the sampling days for soil pH_{CaCl2} and mineral N measurements. # shows the sampling day for the $N_2O/(N_2O + N_2)$ product ratio of denitrification measurement. The arrow indicates the sampling day for microbiological analysis. WHC = water-holding capacity.

effectiveness of DMPP, but they failed to identify the exact underlying mechanisms regulating the results. Thus, it is noteworthy that our study was carried out in a greenhouse because it is impractical to precisely manipulate the described soil moisture conditions in the field. Similarly, this setup enabled us to avoid other influencing factors, such as temperature, that are not controllable under field conditions.

2.3. Sampling, measurement, and calculation of N_2O-N emissions

Wheat plants were carefully folded, and air-tight lids were placed on the pots for gas sampling following the static chamber method (Guo et al., 2022a, 2022b). Gas samples were collected from the pots' headspace with 10-mL syringes at 0, 20, 40, and 60 min daily during the first five days and, subsequently, every two or three days. During waterlogging, gas samples were taken daily and, after waterlogging, every two or three days. The gas sampling was stopped when N_2O-N release decreased to low values in all treatments. Gas samples were utilized to measure N_2O concentrations by gas chromatography (Agilent 7890A GC, Agilent) using an electron capture detector. The same samples were also used to measure CO_2-C and CH_4-C fluxes (Figs. S1, S2). Except for the sampling period, the pots were kept open. The slope of a linear function representing the relationship between N_2O concentrations and pot enclosure time was used to estimate the rate of increase in N_2O concentration in the pot headspace. Subsequently, the equation described by Guo et al. (2022a) was used to calculate the N_2O-N emissions from soil. Linear interpolation was utilized to estimate emissions for days when gas sampling was not performed. Finally, the cumulative N_2O-N emissions were estimated by integrating daily emissions.

2.4. $N_2O/(N_2O + N_2)$ product ratio of denitrification

The $N_2O/(N_2O + N_2)$ product ratio of denitrification was estimated by the acetylene inhibition technique. For this purpose, this study adapted the methodology presented by Qin et al. (2012). Briefly, 10 g of fresh soil sampled before waterlogging at 30 days of the experiment was added to 100 mL glass vials. Afterward, 5 mL of deionized water was added to the same vials. The vials were air-tight sealed with a cap and a butyl rubber septum. Then, they were made anoxic by vacuuming and filling them with pure helium. This setup enabled mimicking the soil waterlogging conditions.

The samples were divided into two sets (with and without acetylene addition) in triplicate. The samples with acetylene received 10 mL acetylene after extracting 10 mL headspace gas from the vials. After incubation for 2 h at 18 °C, a gas sample was taken using a 10 mL syringe for measuring N_2O concentrations by gas chromatography (Agilent 7890A GC, Agilent) using an electron capture detector. Since acetylene blocks the reduction of N_2O to N_2 by inhibiting the nitrous-oxide reductase enzyme, the N_2O production in the acetylene-added vials represents the total production of N_2O and N_2. Therefore, we estimated the $N_2O/(N_2O + N_2)$ product ratio of denitrification by dividing the N_2O concentration in vials without acetylene by the N_2O concentration in vials with acetylene.

The acetylene inhibition technique for estimating the $N_2O/(N_2O + N_2)$ product ratio of denitrification utilized in this study has been extensively evaluated and drawbacks have been reported (Felber et al., 2012). Most drawbacks are related to the presence of oxygen in soil (Felber et al., 2012), which was not the case in our study. We used the AIT to mimic anoxic conditions during waterlogging; thus, oxygen and other gases in the soil were replaced by helium. In this case, the main limitations are associated with possible acetylene decomposition by soil microbes and incomplete inhibition of the N_2O reductase (Felber et al., 2012; Qin et al., 2012). Even though, the AIT technique has been utilized in recent studies and was efficient in demonstrating how the product $N_2O/(N_2O + N_2)$ product ratio of denitrification is impacted by factors such as soil pH (Wu et al., 2024; Xu et al., 2023).

2.5. Sampling and analysis of soil pH_{CaCl2}, NH_4^+-N, and NO_3^--N concentrations

Soil samples were collected after 15, 30, and 45 days from the 0–20 cm layer for NH_4^+-N and NO_3^--N measurements. For these measurements, soil samples were mixed with 1 M KCl solution (1:10) and shaken for 1 h. Subsequently, the samples were filtered, and the extracts were analyzed for NH_4^+-N and NO_3^--N concentrations using a continuous flow analyzer (San^{++} Continuous Flow Analyzer, Skalar). Additionally, a sub-sample was oven-dried at 105 °C for 24 h to calculate water content. Soil samples were also used to measure pH_{CaCl2} by using 10 g of air-dried soil mixed with 25 mL of 0.01 M $CaCl_2$ solution, measured in a pH meter (inoLab® Multi 9310 IDS).

2.6. Soil DNA extraction, 16S rRNA gene, and ITS amplicon sequencing and analysis

Soil samples collected after waterlogging on the 37th day of the experiment were used for microbial (bacterial and fungal) community analysis. Genomic DNA extraction was done for 0.5 g of soil using the Fast DNA SPIN Kit for Soil (MP Biomedicals). For bacterial sequencing library preparation (in three technical replicates), primer pair targeting 16S rRNA gene V3-V4 region (341F 5'-CCTACGGGAGGCAGCAG-3′ and 805R 5'-GGACTACHVGGGTWTCTAAT-3′) was used for amplicon sequencing (Hugerth et al., 2014). Similarly, for fungal sequencing library preparation (in three technical replicates) and amplicon sequencing, primer pair targeting ITS2 region (5.8S-Fun 5'-AACTTTYRRCAAYGGATCWCT-3′ and ITS4-Fun 5'-AGCCTCCGCTTATTGATATGCTTAART-3′) was used (Taylor et al., 2016). Sequencing library preparation and MiSeq paired-end (2 × 300 bp) sequencing (Illumina, v3 chemistry) on the sample pool was performed at the Center of Molecular Life Sciences (ZMB), Kiel University. ZymoBIOMICS™ Microbial Community DNA Standard was used for sequencing, data analysis, and quality control for the amplicon sequencing. 16S rRNA gene and ITS2

region amplicon sequencing data were analyzed on the de-multiplexed fastq reads. Cutadapt (v3.5) (Martin, 2011) was used for the adapter and primer removal and quality control (Q-score > 20). The quality-controlled paired end reads were merged using VSEARCH (v2.21.1) (Rognes et al., 2016). Chimeric sequence removal and generation of amplicon sequence variants (ASV) were performed on the merged sequences using package *dada2* (v1.22.0) (Callahan et al., 2016) in RStudio (v2021.09.0 + 351) (RStudio Team, 2020) running R (v4.1.3) (R Core Team, 2022). Non-chimeric reads were used to analyze sequence variants. Taxonomic annotations of bacterial ASVs were performed using the 16S rRNA database formatted for DADA2 with Genome Taxonomy Database taxonomies (v202) (Alishum, 2021). For the taxonomic annotations of fungal ASVs, UNITE database (v10.0, RefS) was used (Abarenkov et al., 2010). For the respective bacterial and fungal datasets, the abundance table, taxonomy table, sample metadata, and phylogenetic tree were merged into a single object and used for visualization and statistical analysis using packages *phyloseq* (v1.38.0) (McMurdie and Holmes, 2013), *vegan* (v2.6.2) (Oksanen et al., 2019), *ggplot2* (v3.3.6) (Wickham, 2016). Only bacterial ASVs were considered for further analysis, and the archaeal ASVs were discarded. All fungal ASVs were considered for the analysis. The differential abundance testing was done and visualized using package *DESeq2* (v1.34.0) (Love et al., 2014), stats (v4.2.2), *pheatmap* (1.0.12) (Kolde, 2012). Bacterial and fungal MiSeq sequencing de-multiplexed paired-end fastq reads were deposited in SRA at NCBI under accession numbers PRJNA1214020 and PRJNA1214021, respectively.

2.7. Statistical analyses

The data for cumulative N_2O-N emissions and $N_2O/(N_2O + N_2)$ product ratio of denitrification were subjected to the Shapiro-Wilk normality test. Subsequently, a three-way analysis of variance (F test) was performed for the factors liming, DMPP addition, and N rate. The response variables that presented significant treatment effects were submitted to the Tukey test ($p < 0.05$) using the R statistical software version 4.3.1 (R Development Core Team, 2022) using the package *agricolae* (de Mendiburu, 2023). Additionally, linear correlation among the response variables was tested using the Pearson correlation coefficient with the R package *ggcorrplot2* (Cai et al., 2022). The dataset used for the correlations considered only results obtained from samples collected at (soil pH, mineral N, and $N_2O/(N_2O + N_2)$) or after (N_2O-N cumulative fluxes and soil microbiological data) waterlogging deployment. This dataset was split into unlimed and limed soil for correlation analysis due to their divergent responses to waterlogging.

3. Results

3.1. Soil pH_{CaCl2}

The overall pH_{CaCl2} was 5.4 and 6.9 in unlimed and limed soils at the beginning of the experiment, respectively. In addition, the treatments N60 and N180 acidified the unlimed soil by 0.23 and 0.5 units over the trial (Fig. 2). In contrast, the pH_{CaCl2} from the limed soil was steady during the experiment and did not indicate any significant difference between treatments.

3.2. Soil N_2O-N emissions and the $N_2O/(N_2O + N_2)$ product ratio of denitrification

The application of DMPP mitigated daily N_2O-N emissions from unlimed and limed soils under both studied N rates during the first 30 experimental days (Fig. 3). After waterlogging deployment on day 30, N_2O-N release peaked in all treatments. The highest N_2O-N peaks occurred in the unlimed soil without DMPP application in which N60 and N180 showed similar values. For the limed soil, the treatment N180 without DMPP showed the highest N_2O-N emissions after waterlogging. Furthermore, the mean N_2O-N release ranged between 0.01 and 0.26 $\mu g\,h^{-1}\,kg\ soil^{-1}$ for unlimed and between 0.01 and 1.35 $\mu g\,h^{-1}\,kg\ soil^{-1}$ for limed treatments before waterlogging. After waterlogging, N_2O-N emissions ranged between 0.45 and 44.92 $\mu g\,h^{-1}\,kg\ soil^{-1}$ for unlimed and between 0.14 and 13.82 $\mu g\,h^{-1}\,kg\ soil^{-1}$ for limed treatments.

Cumulative N_2O-N emissions indicated the same pattern described for daily fluxes (Fig. 4). Likewise, DMPP decreased cumulative emissions by 78 % on average under all tested treatments before waterlogging (Fig. 4). Considering emissions after waterlogging, DMPP efficiently mitigated N_2O-N release from unlimed soil (80 %), and no differences between N rates were found. In addition, the unlimed soil showed the highest total N_2O-N emission. Contrasting to the results found for the unlimed soil, the N rate had a significant effect on the limed soil after waterlogging. In this case, the rate N180 showed higher emissions than the rate N60 for treatments without DMPP. Moreover, DMPP reduced cumulative N_2O-N release by 94 % at the rate of N180, but the inhibitor did not significantly affect N_2O-N efflux at the rate of N60.

Greater values of the $N_2O/(N_2O + N_2)$ product ratio of denitrification were observed in the unlimed soil (Fig. 5). Additionally, the N180 further increased the $N_2O/(N_2O + N_2)$ relative to N60. Application of DMPP did not change the $N_2O/(N_2O + N_2)$ product ratio in the unlimed soil. In contrast, DMPP decreased the $N_2O/(N_2O + N_2)$ product ratio from the limed soil by 26 % and 85 % in the N60 and N180, respectively.

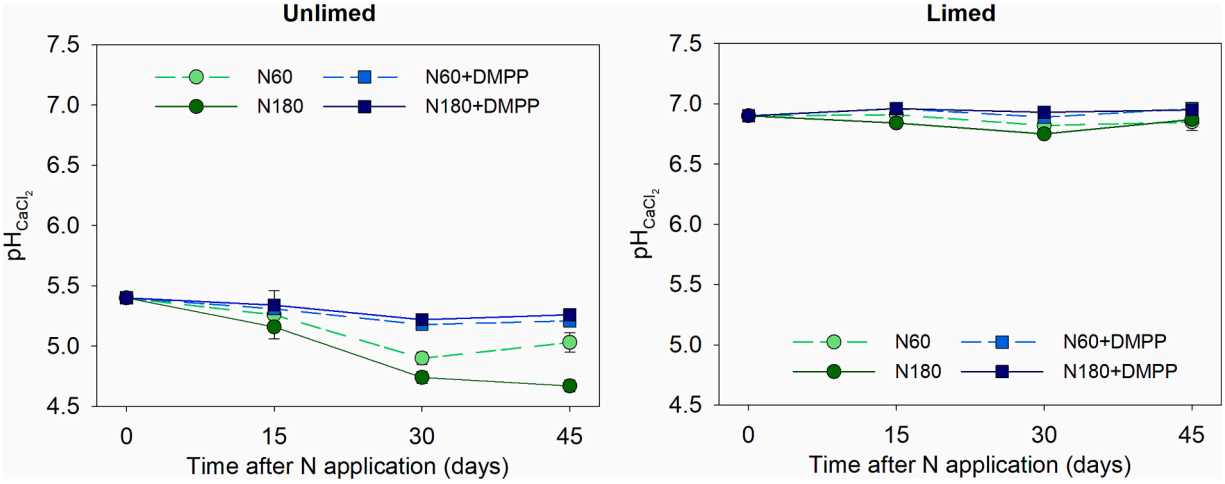

Fig. 2. – Soil pH_{CaCl2} from unlimed and limed soils subjected to the application of 60 and 180 mg N kg $soil^{-1}$ with and without adding 3,4-Dimethylpyrazole phosphate (DMPP). Vertical bars represent the mean standard deviation.

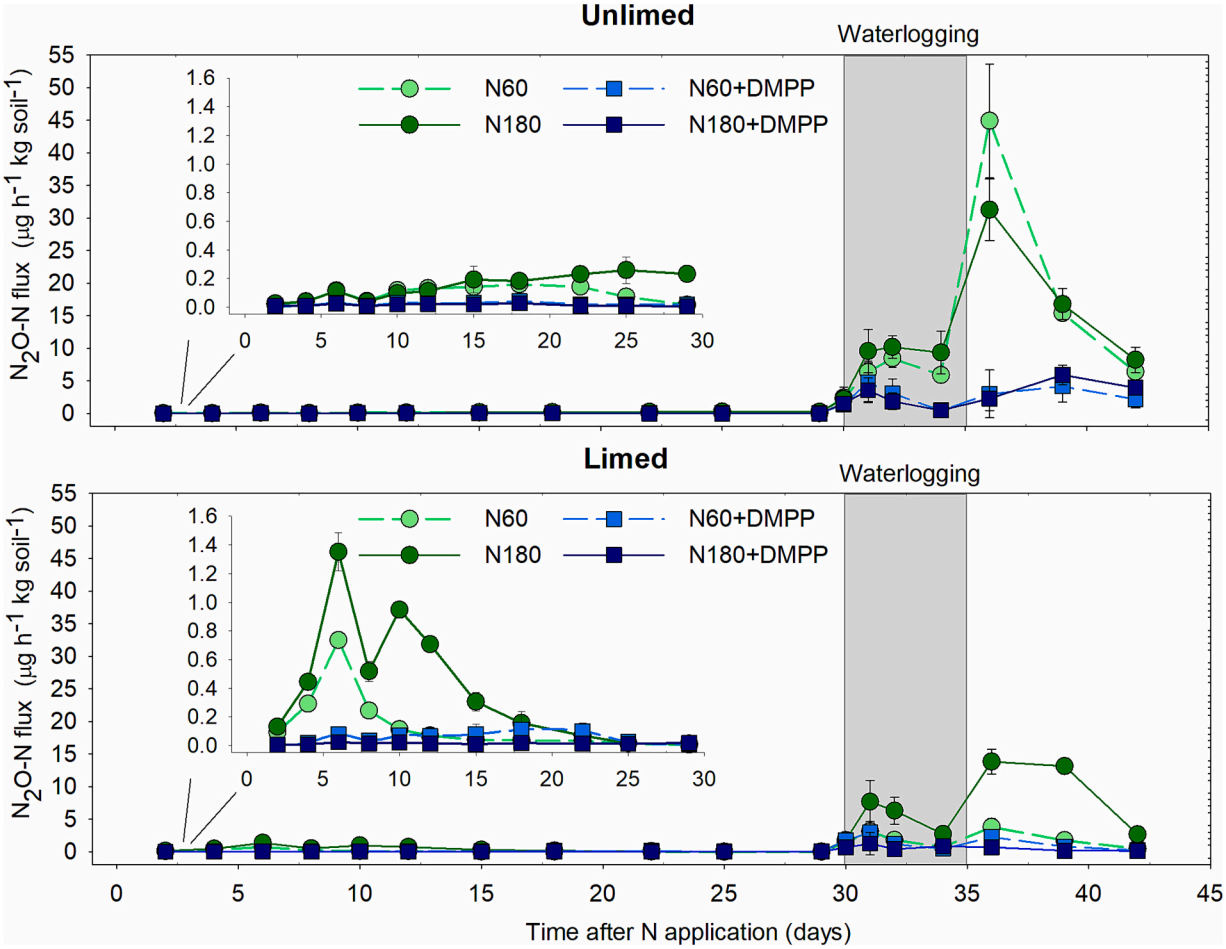

Fig. 3. – Daily N_2O-N emissions from unlimed and limed soils subjected to the application of 60 and 180 mg N kg soil^{-1} with and without adding 3,4-Dimethylpyrazole phosphate (DMPP). The grayish area indicates the deployment of temporary waterlogging between days 30 and 35. Vertical bars represent the mean standard deviation.

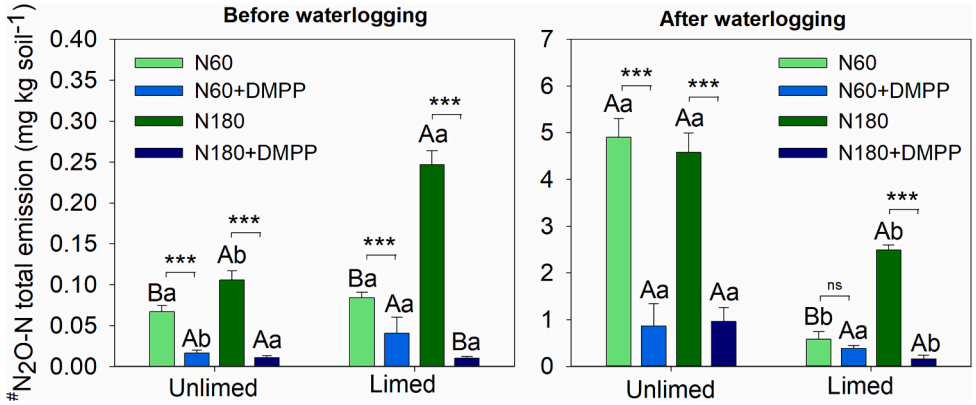

Fig. 4. – Cumulative N_2O-N emissions from unlimed and limed soils subjected to the application of 60 and 180 mg N kg soil^{-1} with and without adding 3,4-Dimethylpyrazole phosphate (DMPP). Vertical bars represent the mean standard deviation. Means followed by different uppercase letters within the factors liming and inhibitor represent significant differences between N rates. Means followed by different lowercase letters within the factors N rate and inhibitor denote significant differences between unlimed and limed treatments. *** and ns represent significant ($p \leq 0.001$) and non-significant differences, respectively, of DMPP application within the factors liming and N rate according to the analysis of variance (F test). #Note that the y-axis of the graphics presents different scales.

3.3. Soil NH_4^+-N and NO_3^--N concentrations and relative abundance of selected nitrifying and denitrifying microorganisms

Soil NH_4^+-N and NO_3^--N concentrations over the experimental period are shown in Fig. 6. The concentration of NH_4^+-N decreased, while that of NO_3^--N increased more quickly in the limed than in the unlimed soil. For instance, NH_4^+-N concentrations decreased to values <15 mg kg^{-1} on day 15 in the limed soil at the N180 rate without DMPP, while 130 mg kg^{-1} of NH_4^+-N was detected in the unlimed soil under the same circumstances. Furthermore, DMPP effectively delayed NH_4^+-N

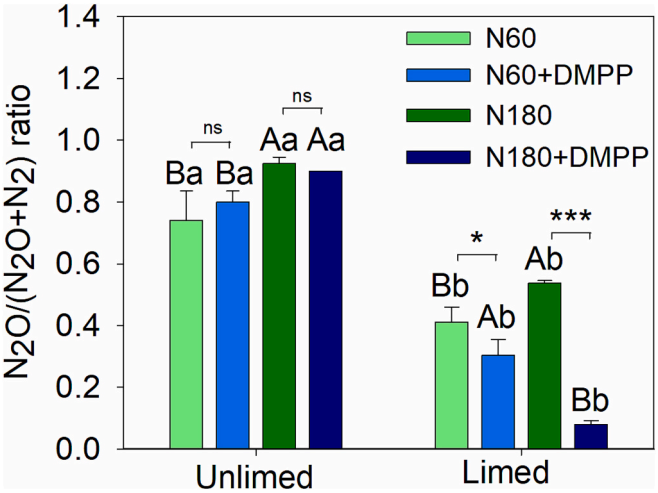

Fig. 5. – The $N_2O/(N_2O + N_2)$ product ratio of denitrification from unlimed and limed soils subjected to the application of 60 and 180 mg N kg soil^{-1} with and without adding of 3,4-Dimethylpyrazole phosphate (DMPP). Vertical bars represent the mean standard deviation. Means followed by different uppercase letters within the factors liming and inhibitor represent significant differences between N rates. Means followed by different lowercase letters within the factors N rate and inhibitor denote significant differences between unlimed and limed treatments. *, ***, and ns represent significant at $p \leq 0.05$, $p \leq 0.001$, and non-significant differences, respectively, of DMPP application within the factors liming and N rate treatments according to the analysis of variance (F test).

consumption and NO_3^--N accumulation under all studied conditions.

Application of DMPP reduced the relative abundance of *Nitrospira* and *Nitrosospira* in limed and unlimed soils (Fig. 7a). Furthermore, *Nitrosomonas* was found only in the limed soils, whereas DMPP application diminished its relative abundance. *Nitrospiraceae* was also detected in the studied soils; however, no consistent effect of treatments was observed. Considering denitrifying fungi, DMPP reduced the relative abundance of *Cladosporium* and *Chaetomium* while increasing *Aspergillus* (Fig. 7b).

Considering the limed soil (Fig. 8b), the N_2O-N cumulative efflux was positively correlated with $N_2O/(N_2O + N_2)$, NO_3^--N, *Chaetomium*, *Nitrospira*, *Nitrosospira*, *Nitrosomonas*, *Bradyrhizobium*, *Mesorhizobium*, *Acidovorax*, and negatively correlated with NH_4^+-N, pH_{CaCl2}, *Achromobacter*, *Mortierella*, *Aspergillus*, and *Fusarium* ($p < 0.05$).

For the unlimed soil, N_2O-N was not correlated with $N_2O/(N_2O + N_2)$ and showed a positive correlation with NO_3^--N, *Nitrospira*, *Nitrosospira*, Bradyrhizobium, *Chaetomium*, and *Fusarium* ($p < 0.05$). Moreover, N_2O-N presented a strong negative correlation with soil pH_{CaCl2} ($p < 0.05$).

4. Discussion

Importantly, periods of transient waterlogging are becoming more common due to climate change (Zhao et al., 2024). In addition, previous research on soil pH and DMPP effectiveness did not consider waterlogging (Das et al., 2022; Guo et al., 2022b; Kaveney et al., 2020; Ribeiro et al., 2024c; Shi et al., 2016a, 2016b). Likewise, it is unknown if the effect of DMPP on reducing N_2O emissions from denitrification by altering NO_3^--N availability and end-product ratio depends on soil pH and N rate. Our results showed, for the first time, that the N rate drives the DMPP effectiveness in reducing N_2O release from limed soil, whereas the inhibitor was effective at the N180 rate, but ineffective at the N60 rate that represents a N fertilizer rate of ca. 150 kg N ha^{-1}. Finally, this study also provided new information indicating that the underlying mechanisms by which DMPP reduces N_2O emissions differ in unlimed and limed soils under temporary waterlogging.

4.1. DMPP reduces the relative abundance of nitrifying bacteria and suppresses nitrification irrespective of soil pH and N rate

The application of DMPP effectively suppressed nitrification in unlimed and limed soils (Fig. 6) because of the reduced relative abundance of nitrifying bacteria such as *Nitrospira*, *Nitrosospira,* and *Nitrosomonas* (Fig. 7). These results corroborate the findings from Oliveira et al. (2022), which demonstrated that DMPP was effective for both acidic and near-neutral tropical soils. Das et al. (2022) also found that DMPP was effective in unlimed and limed soils. Huérfano et al. (2022) indicated that AOA outnumbered AOB in an acidic grassland soil and DMPP targeted AOB rather than AOA; however, nitrification rates were suppressed by DMPP because the AOB was likely dominant at the functional level. There is evidence that AOA is the main ammonia-oxidizer in acidic soils and that DMPP targets AOB rather than AOA (Li et al., 2018). This was unlikely in our study because N_2O production was strongly correlated with *Nitrospira* and *Nitrosospira*, and DMPP reduced their relative abundances and nitrification rates in the unlimed soil (Figs. 6, 7, and 8). The reason might be that AOA prevails under conditions of low NH_4^+-N concentrations, such as in unfertilized acidic soils when slow soil organic matter degradation is the main NH_4^+-N source, while AOB thrives under high NH_4^+-N inputs, such as in fertilized soils with slightly acidic pH (Li et al., 2018). In other studies where AOA was the main ammonia-oxidizer, DMPP ineffectiveness was reported (Shi et al., 2016b), but some studies also indicated that DMPP reduced AOA (Lan et al., 2022). Therefore, this topic still requires further research. Notably, we did not find consistent effects of DMPP on non-target microbial communities (Figs. S5 and S6); however, other authors indicated that DMPP might reduce microbial activity, especially at high concentrations (Tedeschi et al., 2020), and affect non-target microbial communities (Corrochano-Monsalve et al., 2021).

In addition to the possible DMPP ineffectiveness in suppressing AOA growth and activity, inherent low nitrification rates in acidic soils have been suggested to cause unnoticeable differences between soils treated or untreated with DMPP (Kaveney et al., 2020; Rose et al., 2020). Indeed, in our study, while differences between NO_3^--N concentrations were significant for the limed soil, they were insignificant for the unlimed soil on day 15. However, on day 30, visible differences between NO_3^--N concentrations between DMPP-treated and untreated soils were also observed for the unlimed soil. Thus, it is possible that in field experiments where the data variability is much larger, the low nitrification rates of acidic soils may lead to statistically insignificant differences and explain the limited effectiveness of DMPP in these soils reported elsewhere (Nauer et al., 2018; Rose et al., 2020).

4.2. Soil moisture drives the relevance of DMPP application for mitigating N_2O release from unlimed soil but not its effectiveness

Adding DMPP reduced N_2O emissions from the unlimed soil before (82 %) and after (80 %) waterlogging (Fig. 4). This reduction in N_2O release before waterlogging is ascribed to the relative abundance mitigation of *Nitrospira* and *Nitrosospira* and, thus, suppressed nitrification. Furthermore, in the present study, soils untreated and treated with DMPP showed 70 and 21 mg kg^{-1} of NO_3^--N when waterlogging was implemented, respectively. Thus, the effect of DMPP on mitigating N_2O emissions after waterlogging is solely attributed to reduced NO_3^--N availability for denitrification because no DMPP effects were found for the $N_2O/(N_2O + N_2)$ product ratio of denitrification in the unlimed soil (Fig. 5).

This contrasts with the results observed for the limed soil in which DMPP reduced the $N_2O/(N_2O + N_2)$ product ratio of denitrification. Previous studies have already shown that nitrification inhibitors can reduce the $N_2O/(N_2O + N_2)$ product ratio because NO_3^--N and N_2O compete as electron acceptors during denitrification, and soils treated with DMPP show lower NO_3^--N (Huérfano et al., 2022; Torralbo et al., 2017). However, to our knowledge, our study is the first to show that

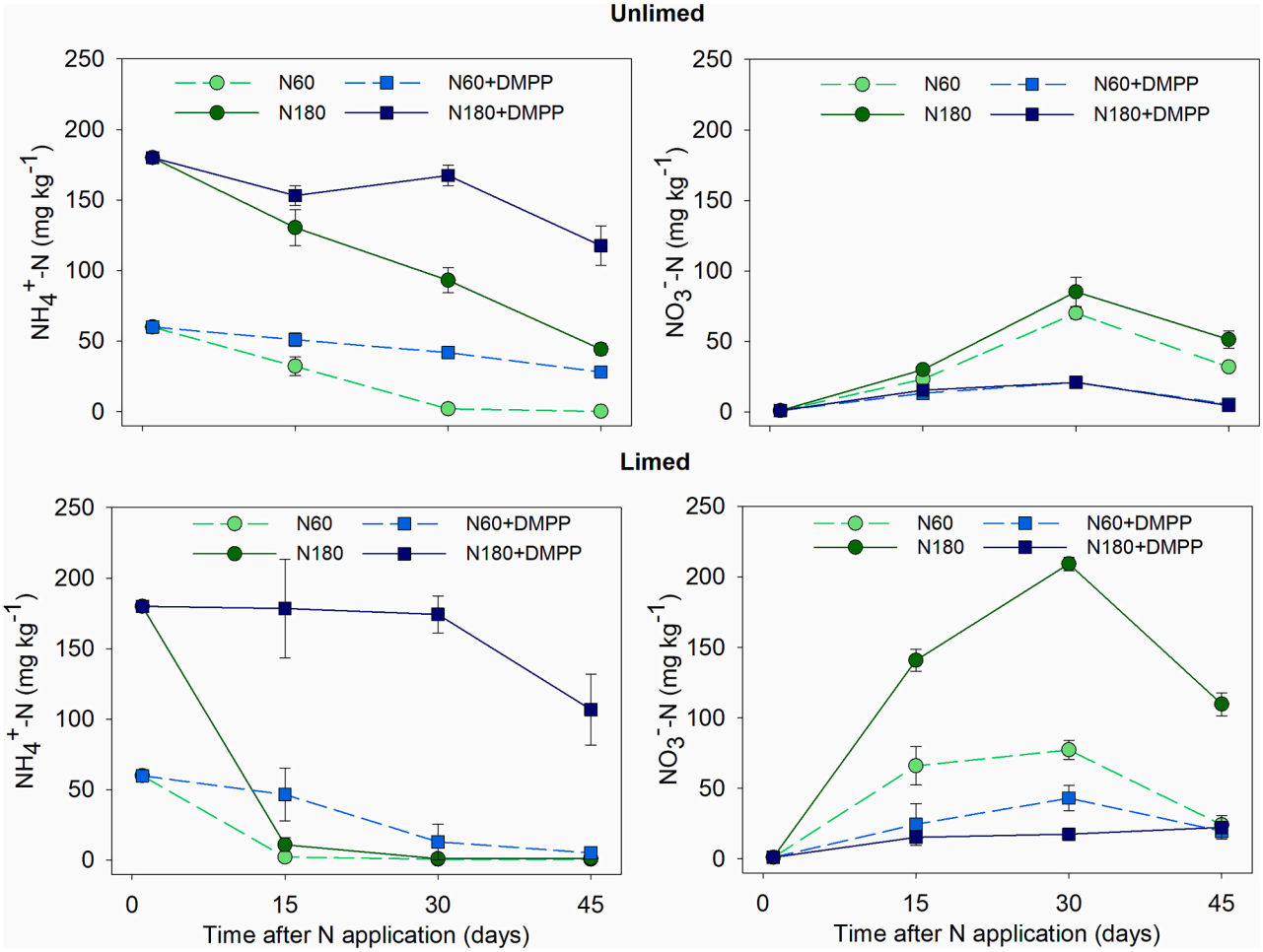

Fig. 6. – Time-related changes of NH_4^+-N and NO_3^--N concentrations from unlimed and limed soils subjected to the application of 60 and 180 mg N kg soil^{-1} with and without adding of 3,4-Dimethylpyrazole phosphate (DMPP).

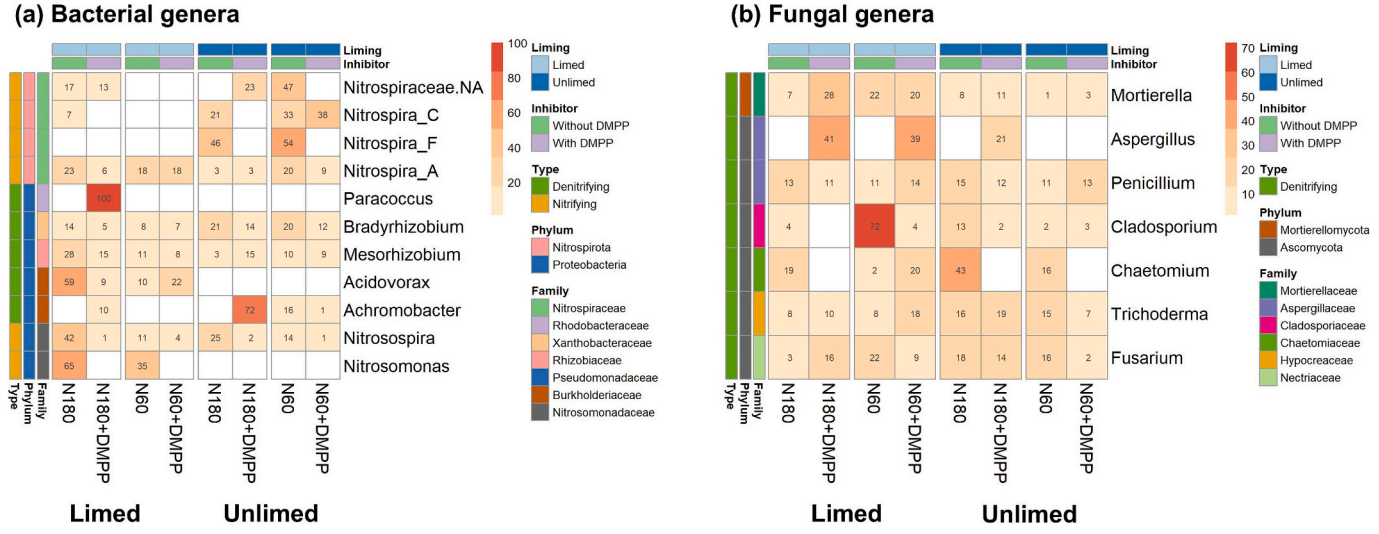

Fig. 7. – Heatmap indicating the relation among the relative abundance of selected (a) nitrifying and denitrifying bacterial and (b) denitrifying fungal genera from unlimed and limed soils subjected to the application of 60 and 180 mg N kg soil^{-1} with and without adding 3,4-Dimethylpyrazole phosphate (DMPP).

this effect depends on soil pH. Therefore, it is likely that low soil pH impairs the assembly of the N_2O reductase enzyme and overcomes the effects of DMPP on NO_3^--N concentration at low pH. The Pearson correlation matrix corroborates this explanation because the $N_2O/(N_2O + N_2)$ product ratio of denitrification was not significantly correlated with N_2O release in the unlimed soil, while N_2O emission was negatively and positively correlated with soil pH and NO_3^--N, respectively ($p < 0.05$) (Fig. 8). Supporting this explanation, Liu et al. (2014) found reduced

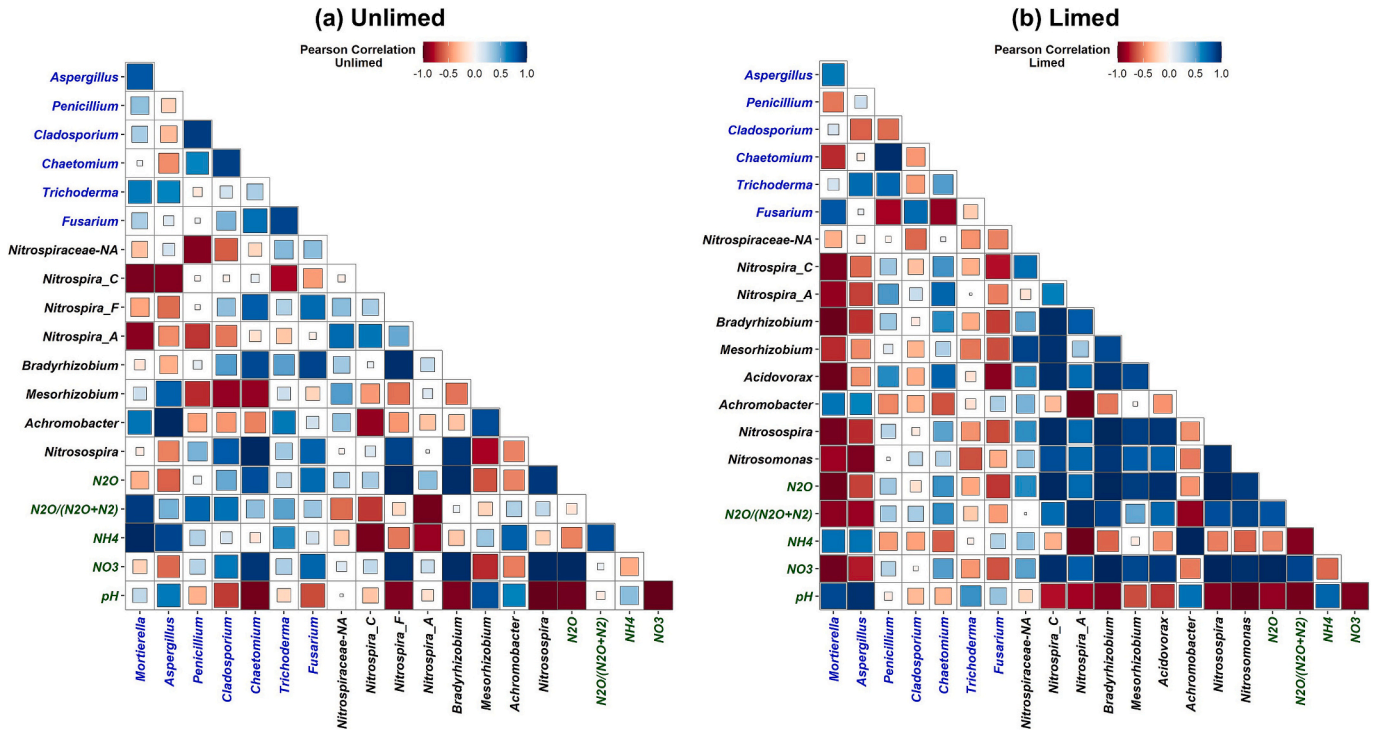

Fig. 8. – Pearson correlation coefficients for cumulative N_2O-N emission, $N_2O/(N_2O + N_2)$ product ratio, soil NH_4^+-N and NO_3^--N concentrations, pH_{CaCl2}, and the relative abundance of selected nitrifying and denitrifying bacterial and denitrifying fungal genera in unlimed (a) and limed (b) soils.

complete denitrification in denitrifying bacteria cultures when pH decreased from 7 to 6. The authors did not find evidence that low pH impairs enzyme production at the transcriptional level; thus, they suggested that the low pH interferes with N_2O reductase assembly because it occurs in the bacterial periplasm, where pH is probably less controlled than in the cytoplasm. Alternatively, Senbayram et al. (2019) suggested that N_2O reductase can be functional even at low pH levels, and found evidence that high soil pH causes NO_3^- depletion more quickly due to higher denitrification rates compared to acidic soils. Consequently, as N_2O and NO_3^- compete as electron acceptors, more N_2O is reduced to N_2 under these circumstances (Senbayram et al., 2019).

Our data suggested that DMPP application resulted in a significant reduction of N_2O emissions during aerobic conditions. However, this reduction is of relatively low importance because the emissions from N60 and N180 were 0.11 and 0.06 % of the applied N rate in the unlimed soil. This is in agreement with Rose et al. (2020), who reported the limited effectiveness of DMPP on high-carbon Gleysol, suggesting that inhibitors should not be applied to this type of soil due to its low N_2O production potential. However, when acidic soils reach high soil moisture, such as the temporary waterlogging simulated in our study, they can produce extreme N_2O peaks. For example, considering the period after waterlogging, 8.18 and 2.54 % of the applied N was emitted as N_2O from N60 and N180 treatments, respectively. Thus, it is evident that the DMPP reduction in N_2O release of ca. 80 % found in this study for the unlimed soil is particularly relevant under high soil moisture conditions. Therefore, the likelihood of heavy rainfall and anoxic soil conditions should be considered when recommending nitrification inhibitors for low-pH soils.

4.3. N rate regulates DMPP effectiveness in reducing N_2O emissions from limed soil subjected to temporary waterlogging

Considering the limed soil, DMPP mitigated N_2O emissions before (74 %) and after (65 %) waterlogging. Exceptionally, DMPP did not result in a significant decrease in N_2O emissions at the N60 rate in the limed soil after waterlogging. At the same time, DMPP caused a highly significant reduction (94 %) in cumulative N_2O flux at the N180 rate. Such results clearly indicated that the N rate drove DMPP effectiveness under temporary waterlogging in our study. Das et al. (2022) also found that DMPP was ineffective in reducing N_2O emissions when the soil moisture was raised from 55 to 90 % WHC in both unlimed and limed soils; however, the authors did not investigate the product ratio of denitrification or denitrification functional genes; thus, the underlying mechanisms remained unclear.

Considering our results, a possible explanation for the above-mentioned effect is proposed here. At the N60, NO_3^--N concentrations were 77 and 43 mg kg^{-1} for soil without and with DMPP at the waterlogging implementation. At the same time, NO_3^--N concentrations were 209 and 15 mg kg^{-1} at N180. Consequently, the DMPP effect on reducing N_2O release was weakened at the lower N rate due to the smaller difference between NO_3^--N values from limed soils without and with DMPP.

This also led to the failure of DMPP to decrease the relative abundance of denitrifying microbes such as *Acidovorax*, *Bradyrhizobium*, and *Chaetomium* at N60 (Fig. 7), which were positively correlated with N_2O-N efflux (Fig. 8b). In this context, the results concerning *Chaetomium* are especially important because fungal denitrifiers generally do not encode the N_2O reductase gene (NosZ) and therefore are unable to reduce N_2O to N_2 (Mothapo et al., 2015), which may explain the weaker effect of DMPP on reducing $N_2O/(N_2O + N_2)$ product ratio at N60 relative to N180 found in our study (Fig. 5). This might have occurred because DMPP is applied at a steady ratio of 1 % of the added NH_4^+-N or urea-N. Thus, more DMPP is applied at higher N rates. Therefore, it might be possible that at relatively low N rates, when less DMPP is applied, soil biodegradation of the inhibitor may cause lower efficiency, and, in the case of waterlogging, when only indirect DMPP effects on denitrification occur, the inhibitor may be ineffective. The lower N_2O reduction by DMPP at N60 (52 %) than at N180 (96 %) before waterlogging supports this rationale. This occurred because the N_2O-N release from the treatment N60 + DMPP slightly increased after 15 days of the experiments, suggesting the DMPP effect was weakened in this treatment (Fig. 3). Moreover, this effect was only observed in the limed soil in our study,

probably because liming stimulates microbial growth and activity (Zhang et al., 2022). The biomass production of plants was similar among treatments (Fig. S7); therefore, it is unlikely that differences in plant N uptake would explain these results.

It is important to note that we considered the effectiveness of DMPP in mitigating N_2O-N emissions to draw conclusions from the present study. The other measured variables, including soil mineral N, nitrifying and denitrifying microbes, and $N_2O/(N_2O + N_2)$ ratio, were measured to provide insight into underlying mechanisms. For instance, DMPP significantly reduced NO_3^--N concentrations and $N_2O/(N_2O + N_2)$ ratio in the limed soil at N60 during waterlogging implementation. Thus, the interpretation that DMPP is ineffective in the limed soil subject to waterlogging at N60 is based mainly on the N_2O-N cumulative emissions. In addition, DMPP was equally and highly effective in the first two weeks for all treatments. Exceptionally for N60, this effect weakened between 15 and 30 days of the experiment and was insignificant after waterlogging deployment.

4.4. Study limitations

Genomic DNA utilized to analyze the relative abundance of nitrifying and denitrifying microorganisms in the present study may present DNA from dead, inactive, or low-active microbes. Therefore, it may produce biased information on the microbial genera driving the nitrification process (Nannipieri et al., 2019). Consequently, the interpretation of the data presented in this study should consider the methodological limitations. To minimize this limitation, we also monitored soil NH_4^+-N and NO_3^--N concentrations, N_2O fluxes, and the $N_2O/(N_2O + N_2)$ product ratio of denitrification to comprehensively assess DMPP effects impacted by soil pH and N rates.

5. Conclusions

This study confirmed the hypothesis that DMPP suppresses nitrification by reducing the relative abundance of nitrifying bacteria such as *Nitrosospira* and *Nitrospira*, irrespective of soil pH and N rate. Such results support a better understanding of DMPP effects, especially in acidic soils where previous studies indicated variable results. Furthermore, N_2O emissions from unlimed soil under aerobic conditions were minimal, and DMPP application might not be worth it in this situation. However, the unlimed soil presented the highest N_2O release when subjected to waterlogging, and DMPP was confirmed as an excellent mitigation measure for such emissions.

Finally, our study demonstrated that relatively low N rates can lead to insignificant effects of DMPP on N_2O release in limed soil subjected to temporary waterlogging. Importantly, the N rate referred to as N60 in this study is equivalent to ca. 150 kg N ha^{-1}, a N rate commonly utilized for many cultivated crops. These results support understanding why nitrification inhibitors might be ineffective in some field conditions. Emissions of N_2O after heavy rainfalls may account for most of the yearly emissions; therefore, it is crucial that nitrification inhibitors are also efficient during these periods. Thus, further research is required to address this limitation of nitrification inhibitors such as DMPP.

CRediT authorship contribution statement

Pablo Lacerda Ribeiro: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Abhijeet Singh:** Writing – review & editing, Visualization, Methodology. **Amit Sagervanshi:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Karl Hermann Mühling:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2025.106287.

Data availability

Data will be made available on request.

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