Effect of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on N-turnover, the N\textsubscript{2}O reductase-gene \textit{nosZ} and N\textsubscript{2}O:N\textsubscript{2} partitioning from agricultural soils

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Nitrification inhibitors (NIs) have been shown to reduce emissions of the greenhouse gas nitrous oxide (N\textsubscript{2}O) from agricultural soils. However, their N\textsubscript{2}O reduction efficacy varies widely across different agro-ecosystems, and underlying mechanisms remain poorly understood. To investigate effects of the NI 3,4-dimethylpyrazole-phosphate (DMPP) on N-turnover from a pasture and a horticultural soil, we combined the quantification of N\textsubscript{2} and N\textsubscript{2}O emissions with \textsuperscript{15}N tracing analysis and the quantification of the N\textsubscript{2}O-reductase gene (\textit{nosZ}) in a soil microcosm study. Nitrogen fertilization suppressed \textit{nosZ} abundance in both soils, showing that high nitrate availability and the preferential reduction of nitrate over N\textsubscript{2}O is responsible for large pulses of N\textsubscript{2}O after the fertilization of agricultural soils. DMPP attenuated this effect only in the horticultural soil, reducing nitrification while increasing \textit{nosZ} abundance. DMPP reduced N\textsubscript{2}O emissions from the horticultural soil by >50% but did not affect overall N\textsubscript{2} + N\textsubscript{2}O losses, demonstrating the shift in the N\textsubscript{2}O:N\textsubscript{2} ratio towards N\textsubscript{2} as a key mechanism of N\textsubscript{2}O mitigation by NIs. Under non-limiting NO\textsubscript{3}\textsuperscript{−} availability, the efficacy of NIs to mitigate N\textsubscript{2}O emissions therefore depends on their ability to reduce the suppression of the N\textsubscript{2}O reductase by high NO\textsubscript{3}\textsuperscript{−} concentrations in the soil, enabling complete denitrification to N\textsubscript{2}.

Agricultural soils have become the main source of anthropogenic nitrous oxide (N\textsubscript{2}O), a powerful greenhouse gas and the single most important substance depleting stratospheric ozone\textsuperscript{1}. Delaying the conversion of ammonium (NH\textsubscript{4}\textsuperscript{+}) to nitrate (NO\textsubscript{3}\textsuperscript{−}), nitrification inhibitors (NIs) have been suggested as a means to reduce N\textsubscript{2}O emissions from agricultural soils. NIs demonstrated their efficacy across different cropping soils\textsuperscript{2}, but results vary widely, and in particular in pasture soils the use of NIs had no or little effect on N\textsubscript{2}O emissions\textsuperscript{3–5}. Despite a growing body of research on NIs, mechanisms and factors determining their efficacy to reduce N\textsubscript{2}O emission remain poorly understood\textsuperscript{6}. The challenges to understand these mechanisms derive from the fact that N\textsubscript{2}O is formed via several different pathways in the soil matrix\textsuperscript{7}, tightly coupled to different processes of N supply and consumption\textsuperscript{8}. Critically, N\textsubscript{2}O can be further reduced to N\textsubscript{2} via the microbial-mediated process of denitrification, and the sole quantification of N\textsubscript{2}O as affected by NIs provides therefore only a limited insight into mechanisms of N\textsubscript{2}O mitigation using NIs.

Microbial metabolic pathways can contribute via a wealth of different processes to N\textsubscript{2}O production and consumption, i.e. the reduction to N\textsubscript{2} in soils. Apart from abiotic processes, N\textsubscript{2}O formation can be categorized into

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nitrification-mediated pathways, denitrification and biotic formation of hybrid N₂O⁹. Denitrification is generally assumed to be the main process contributing to overall N₂O production from agricultural soils⁷⁻¹² and is also the main process reducing N₂O into environmentally benign N₂ via the N₂O reductase, the enzyme encoded by the functional nosZ gene. The reduction of N₂O to N₂ does not reduce overall N losses but limits the environmental impact of denitrification losses from agricultural soils. A reduction of N₂O emissions by NIs can be attributed to (a) reduced N₂O production via nitrification mediated pathways, (b) reduced N₂O production via denitrification (c) increased consumption of N₂O via denitrification, i.e., a shift in the N₂O:N₂ ratio towards N₂. As these effects may overlap, a mechanistic understanding of the effects of NIs on N₂O production and consumption processes needs to be based on N₂O source partitioning, and the direct quantification of N₂.

Most of the NIs inhibit the first and rate-limiting enzymatic step of nitrification, the conversion of NH₄⁺ to hydroxylamine (NH₂OH) via the ammonia monoxygenase¹³. The inhibition of nitrification means a reduced supply of N into the NO₃⁻ pool as the source pool of denitrification, but also an increase in NH₂OH availability, leading to an increase of fertilizer N immobilization¹¹ and mineralization/immobilization turnover rates¹⁴,¹⁵. Availability of N for N₂O producing processes determines both production, but also consumption of N₂O, as high NO₃⁻ availability shifts the N₂O:N₂ ratio of denitrification towards N₂O¹⁶. The link between N transformation rates and N₂O and N₂ emissions is therefore critical to understand the effects of NIs in agricultural soils.

Typically, pulses of N₂O are observed after fertilization and irrigation events. These pulses are short-lived and can account for more than 90% of cumulative N₂O emissions from agro-ecosystems⁵⁻⁷, defining the critical time-window which determines the efficacy of NIs to mitigate N₂O emission. Building on extensive research at the field scale conducted across different agro-ecosystems⁵⁻¹⁷,¹⁸⁻²⁰, this study investigated the short-term effect of 3,4-dimethylpyrazole phosphate (DMPP) on N-turnover and N₂O and N₂ emissions from two contrasting agricultural soils in response to N-fertilization. We combined a ¹⁵N tracing analysis with the direct quantification of N₂O and N₂ emissions using the ¹⁵N flux technique, complemented with the quantification of the nosZ gene via quantitative polymerase chain reaction (qPCR) in a soil microcosm study to constrain factors determining the efficacy of the NI DMPP to mitigate N₂O emissions from agricultural soils.

**Results**

Physical and chemical properties for the two soils used in this experiment are shown in Table 1. The contrasting soils, a horticultural and a pasture soil, are henceforth referred to as sandy clay loam (sandy CL) and loam, according to their texture from 0–10 cm.

| Soil property          | Sandy clay loam | Horticulture soil | Loam - Pasture soil |
|------------------------|----------------|-------------------|---------------------|
| Texture (USDA) (0–10 cm) | Sandy clay loam | Loam              |
| Site                   | Gatton         | Gympie            |
| Latitude               | −27.54         | −26.19            |
| Longitude              | 152.32         | 152.74            |
| Mean annual rainfall   | 773 mm         | 1127 mm           |
| Soil type (ASC)        | Dermosol       | Dermosol          |
| Soil type (FAO)        | Udic Argustoll | Ferric Acrisol    |
| Sand (%)               | 50.5           | 47.2              |
| Silt (%)               | 23.8           | 38.8              |
| Clay (%)               | 20.7           | 20.4              |
| pH                     | 7.4            | 6.2               |
| Organic Carbon (%)     | 1.0            | 4.9               |
| Total Nitrogen (%)     | 0.08           | 0.5               |
| C:N ratio              | 12.5           | 9.8               |

Table 1. Selected soil characteristics (0–10 cm) for a horticultural (Sandy clay loam) and a pasture soil (Loam) from subtropical Australia.
Effect of DMPP on N-transformations and soil parameters. The application of N-fertilizer with DMPP had no significant effect on N transformations in the loam but changed N-turnover dynamics in the sandy CL (Table 2). DMPP reduced ONH4 only by 6% in the loam, but reduced ONO3 by more than 60% in the sandy CL. In the sandy CL, both Mtot and INH4tot increased, as well as the relative contribution of Mrec to Mtot accounting for 80% of Mtot, INH3 decreased by 31%, while DNO3 increased by a factor of >5. DMPP did not affect the soil microbial biomass (SMB) but increased dissolved organic carbon (DOC) by 50% and 32% in the sandy CL and loam, respectively (Table 3).

Emissions of N2O and N2. The dominant N2O production pathway in both soils was denitrification, accounting for more than 90% of the N2O produced (Fig. 2). Over 48 hours, 0.24 ± 0.03 and 1.46 ± 0.38 μg N2O - N g−1 soil were emitted from the sandy CL and the loam, respectively. Both N2O emissions via denitrification (N2OΔ) and nitrification (N2OΔ) were higher from the loam, exceeding those from the sandy CL by a factor of >8 (Fig. 2). Over the two day incubation period, 0.47 ± 0.09 μg N2 - N g−1 soil and 0.87 ± 0.11 μg N2 - N g−1 soil were emitted as N2 from the sandy CL and the loam, respectively. The main product of denitrification (N2OΔ + N2) from the sandy CL was N2, with N2OΔ accounting for 36% of total denitrification losses. Denitrification losses from the loam however were dominated by N2OΔ, accounting for 75% of total denitrification. There was no induction for hybrid production of N2O or N2.

The response of the N2O reductase gene nosZ to fertilization and the use of DMPP. The abundance of nosZ prior to fertilization differed markedly between soils (Fig. 2). Copy numbers of nosZ in the loam exceeded those in the sandy CL by a factor of 6. After fertilization and the increase in soil moisture from 50% to 75% water-filled pore space (WFPS), nosZ copy numbers decreased in both soils, with a reduction by 77% and 32% for the sandy CL and the loam, respectively. DMPP did not affect nosZ abundance in the loam, but increased nosZ copy numbers by 227% compared to the fertilizer only treatment in the sandy CL.

Effect of DMPP on N2O and N2 emissions. DMPP significantly reduced N2O emission from the sandy CL, but had no effect on N2O emissions from the loam (Table 4). DMPP reduced N2OΔ from the sandy CL by 46% (P < 0.05), but did not affect N2OΔ (Fig. 2). There was no effect of DMPP on N2 emissions from the two soils. In the sandy CL, DMPP shifted the product ratio of denitrification (N2OΔ / (N2OΔ + N2)) to N2, decreasing the percentage of denitrification emitted as N2OΔ from 36% to 20%.

Discussion

The fertilization and irrigation of agricultural soils triggers a cascade of N transformations associated with pulses of N2O and N2 emissions. These short-term events are critical to understand the effects of NIs on N2O production and consumption in agricultural soils. Linking N turnover to emissions of N2O and N2 and the abundance of the N2O reductase gene nosZ in a short-term incubation demonstrated (a) that increasing NO3 − availability after fertilization suppressed nosZ abundance, (b) that nosZ abundance, nitrification and N2 + N2O emissions remained largely unaffected by DMPP in the loam and (c) that DMPP decreased nitrification and increased nosZ abundance in the sandy CL, shifting the N2: N2O ratio towards N2. Our findings highlight the short-term effect of DMPP as highly soil specific, and show that reduced nitrification by DMPP can limit the suppression of the N2O reductase gene nosZ during immobilization of mineral N (INH4tot and IMNO3) of more than 8 μg N g−1 soil day−1 together with a low immobilization of mineral N (INH4tot and IMNO3) denote high mineral N availability due to the rapid mineralization of organic N. This is further supported by the dominant contribution of the labile organic N pool to mineralization (Mlab), representing the microbial biomass and low molecular organic N compounds with a fast turnover. The high nitrification rates in the loam (>18 μg N g−1 soil day−1) denote rapid conversion of mineralized N to NO3 − and show the dominant role of NH4 + oxidation for N-turnover in this soil. Gross mineralization was markedly lower in the sandy CL with Mtot at only 0.21 μg N g−1 soil day−1 and dominated by the mineralization of recalcitrant organic N, indicating limited and slower supply of mineral N via mineralization. Mineralization accounted for only 4% of nitrified N in the sandy CL, as compared to 45% in the loam, implying a rapid depletion of the NH4 + pool in the sandy CL. The observed differences between soils are consistent with microbial C and N contents (Table 3), indicating a larger soil microbial biomass in the loam and reflect the impact of perennial
Mineralisation of $\mu g$ N g$^{-1}$ soil day$^{-1}$

| N transformation         | Sandy clay loam - Horticulture soil | Loam - Pasture soil |
|--------------------------|-------------------------------------|---------------------|
|                          | $+DMPP$                             | $-DMPP$             |
|                          | $+DMPP$                             | $-DMPP$             |
|                          | $+DMPP$                             | $-DMPP$             |
| Mineralisation of $N_\text{N}^+$ to $NH_3^+$ | $M_{\text{ads}}$ | $0.12 \pm 0.04$ | c | $1.03 \pm 0.11^*$ | b | $+77.6^*$ | $2.59 \pm 0.09$ | a | $2.50 \pm 0.11$ | a |
| Immobilisation of $NH_3^-$ to $N_{\text{N}}^-$ | $I_{\text{Nit-NH3}}$ | $0.16 \pm 0.04$ | b | $0.79 \pm 0.22^*$ | a | $+397^*$ | $0.002 \pm 0.0005$ | c | $0.0020 \pm 0.00005$ | c |
| Mineralisation of $N_{\text{N}}$ to $NH_3^+$ | $M_{\text{tot}}$ | $0.10 \pm 0.03$ | d | $0.25 \pm 0.05^*$ | c | $+166^*$ | $5.80 \pm 0.28^*$ | a | $5.37 \pm 0.07^*$ | b $-7^%$ |
| Immobilisation of $NH_3^+$ to $N_{\text{N}}$ | $I_{\text{Nit-NH3}}$ | $0.81 \pm 0.26$ | b | $2.59 \pm 0.50^*$ | a | $+220^*$ | $0.002 \pm 0.0002$ | c | $0.002 \pm 0.0002$ | c |
| Oxidation of $NO_3^-$ to $NO_2^-$ | $O_{\text{N2O}}$ | $0.38 \pm 0.11$ | a | $0.34 \pm 0.22$ | a | 0 | 0 |
| Immobilisation of $NO_3^-$ to $N_{\text{N}}$ | $I_{\text{Nit-NO3}}$ | $9.48 \pm 0.12$ | a | $6.55 \pm 0.31^*$ | b | $-31^%$ | $0.017 \pm 0.0005$ | c | $0.016 \pm 0.0005$ | c |
| Oxidation of $NH_3^+$ to $NO_3^-$ | $O_{\text{NO2}}$ | $5.44 \pm 0.28$ | c | $2.04 \pm 0.20^*$ | d | $-63^%$ | $18.64 \pm 0.24^*$ | a | $17.46 \pm 0.37^*$ | b $-6^%$ |
| Dissimilatory $NO_2^-$ reduction to $NH_4^+$ | $D_{\text{N2O}}$ | $0.026 \pm 0.003$ | d | $0.14 \pm 0.01^*$ | c | $+431^%$ | $2.14 \pm 0.05$ | a | $2.02 \pm 0.08$ | a |
| Adsorption of adsorbed $NH_3^+$ to $NH_3^+$ | $A_{\text{N2}}$ | $1.18 \pm 0.22$ | a | $0.87 \pm 0.75$ | a | 0 | 0 |
| Release of adsorbed $NH_3^+$ to $NH_4^+$ | $R_{\text{N2}}$ | $0.08 \pm 0.02$ | b | $0.68 \pm 0.07^*$ | a | $+71^%$ | 0 | 0 |
| Total mineralisation $M_{\text{ads}} + M_{\text{tot}}$ | $M_{\text{tot}}$ | $0.21 \pm 0.05$ | d | $1.29 \pm 0.12^*$ | c | $-502^%$ | $8.39 \pm 0.29$ | a | $7.87 \pm 0.13^*$ | b $-6^%$ |
| Total nitrification $O_{\text{N2O}} + O_{\text{NO2}}$ | $O_{\text{N2O}}$ | $5.82 \pm 0.30$ | c | $2.39 \pm 0.30^*$ | d | $-59^%$ | $18.64 \pm 0.24$ | a | $17.46 \pm 0.37^*$ | b $-6^%$ |
| Total $NH_3^+$ immobilisation $I_{\text{Nit-NH3}}$ | $I_{\text{Nit-NH3}}$ | $0.97 \pm 0.31$ | b | $3.37 \pm 0.72^*$ | a | $+249^%$ | $0.004 \pm 0.001$ | c | $0.004 \pm 0.0001$ | c |
| Contribution of $M_{\text{ads}}$ to $M_{\text{tot}}$ | $O_{\text{N2O}}/M_{\text{ads}}$ | $45^%$ | 20^% | 69^% | 68^% |
| Contribution of $O_{\text{N2O}}$ to $N_{\text{N}}$ | $O_{\text{N2O}}/N_{\text{N}}$ | $93^%$ | 86^% | 100^% | 100^% |

Table 2. Gross soil N transformations (average ± standard deviation) in a horticultural (Sandy clay loam) and a pasture soil (Loam) after the application of NH$_4$NO$_3$ with and without the nitrification inhibitor DMPP. Means denoted by a different letter indicate significant differences for a specific N transformation across soils and treatments (i.e. no overlap of 95% confidence intervals). * denotes a significant effect of DMPP. Letters denote significant differences for a specific N transformation across soils and treatments (i.e. no overlap of 95% confidence intervals).
of denitrification. In the study presented here, DMPP reduced the N₂O:N₂ ratio by 44% in the sandy CL, demonstrating a significant shift towards N₂ (Fig. 2). These results link the increase of \( \text{no}_{2}/\text{N}_2 \) abundance in response to DMPP in the sandy CL to a shift in the N₂O:N₂ ratio towards N₂, based on direct measurements of N₂ and N₂O using the \(^{15}\text{N} \) gas flux method. In contrast to previous incubation studies investigating N₂O:N₂ partitioning in response to DMPP, emissions of N₂O and N₂ were quantified after incubation under atmospheric O₂ conditions and without adding an easily available C source to stimulate denitrification, as these conditions would have altered short-term N dynamics in response to DMPP. Importantly, the shift towards N₂ was not observed for the loam, where DMPP had a negligible effect on nitrification. Our findings indicate that the reduction of nitrification by DMPP in the sandy CL reduced the suppression of the N₂O reductase after fertilization, enabling complete denitrification to N₂. Emissions of N₂O produced via nitrification mediated pathways were not affected by DMPP in this soil, showing the reduction of N₂O emissions by DMPP as an indirect effect limiting NO\(_3^-\) availability for denitrification.

The spatial coverage of nitrifying microsites by the inhibitor is critical for efficient inhibition of nitrification. Limited diffusion of DMPP may explain the he observed inefficacy of DMPP to reduce autotrophic nitrification in the loam, which is consistent with reports from other pasture soils. The amount of DMPP applied with N fertilizer is small, and the initial sorption to organic matter and uneven distribution of DMPP may hinder its short-term effectiveness to reduce nitrification in specific micro sites. Sorption of DMPP is likely to be more pronounced in the loam as a pasture soil with higher organic matter content as compared to the sandy CL owing to the positive correlation of DMPP sorption with organic C. The high microbial activity in the loam also infers a larger number of microsites with nitrifying activity compared to the sandy CL, suggesting the spatial separation of DMPP from nitrifiers may be responsible for the short-term ineffectiveness of DMPP to reduce autotrophic nitrification in the loam. This theory is further supported by a study where DMPP did not affect the initial pulse of denitrification in response to DMPP. Importantly, the shift towards N₂ was not observed for the loam, where DMPP had a negligible effect on nitrification. Our findings indicate that the reduction of nitrification by DMPP in the sandy CL reduced the suppression of the N₂O reductase after fertilization, enabling complete denitrification to N₂. Emissions of N₂O produced via nitrification mediated pathways were not affected by DMPP in this soil, showing the reduction of N₂O emissions by DMPP as an indirect effect limiting NO\(_3^-\) availability for denitrification.

Table 3. Soil mineral N concentrations 30 minutes and 48 hours after N fertilizer application with and without the nitrification inhibitor DMPP; and dissolved organic C and soil microbial C and N prior and 48 hours after fertilizer application with and without DMPP in a horticulture and a pasture soil. Letters denote significant differences between treatments within a soil. * denote significant differences (P < 0.05) between soils within a treatment.

|                | time | Sandy clay loam Horticulture soil | Loam Pasture soil | DMPP effect |
|----------------|------|----------------------------------|-------------------|-------------|
|                |      |                                  |                   | +34%        |
| NH\(_4^+\)     | 30 min after fertilization | 17.0 ± 0.1 a | 18.2 ± 0.2 a | *           |
|                | 48 h Fertilizer | 9.9 ± 0.5 c | 2.1 ± 0.1 c | *           |
|                | 48 h Fertilizer + DMPP | 14.4 ± 0.2 b | 7.1 ± 0.8 b | *           |
|                | DMPP effect | +42% | +233% |           |
| NO\(_3^-\)     | 30 min after fertilization | 70.9 ± 2.2 a | 135.2 ± 1.4 b | *           |
|                | 48 h Fertilizer | 70.2 ± 2.1 a | 175.0 ± 3.3 a | *           |
|                | 48 h Fertilizer + DMPP | 61.9 ± 1.4 b | 171.0 ± 4.0 a | *           |
|                | DMPP effect | −12% | — |           |
| DOC            | 0 prior fertilization | 37.7 ± 1.3 c | 146.1 ± 2.0 c | *           |
|                | 48 h Fertilizer | 71.3 ± 4.1 b | 197.9 ± 9.4 b | *           |
|                | 48 h Fertilizer + DMPP | 107.3 ± 12.0 a | 261.3 ± 6.5 a | *           |
|                | DMPP effect | +50% | +32% |           |
| Microbial C    | 0 prior fertilization | 93.6 ± 18.7 a | 433.0 ± 34.4 b | *           |
|                | 48 h Fertilizer | 61.5 ± 13.9 b | 471.8 ± 13.5 a | *           |
|                | 48 h Fertilizer + DMPP | 66.3 ± 7.4 b | 480.3 ± 7.5 a | *           |
|                | DMPP effect | — | — |           |
| Microbial N    | 0 prior fertilization | 11.9 ± 0.6 a | 89.1 ± 16.8 a | *           |
|                | 48 h Fertilizer | 13.9 ± 1.9 a | 82.3 ± 2.5 a | *           |
|                | 48 h Fertilizer + DMPP | 11.9 ± 0.8 a | 92.7 ± 4.3 a | *           |
|                | DMPP effect | — | — |           |
organic matter induced by DMPP contributed to higher DOC availability, but no such effect was observed for MNrec in the loam. Based on the data available, it remains unclear what caused the increase in DOC in response to DMPP. This increase has however important implications for N-turnover, in particular for the sandy CL as soil with limited labile C availability. DMPP increased DNRA by a factor >5 in the sandy CL, suggesting labile C promoted NO$_3^-$ consumption via DNRA\(^{19,23}\). DNRA competes with denitrification for available NO$_3^-$, but the magnitude of DNRA in the sandy CL was insignificant regarding NO$_3^-$ availability for denitrification. More importantly, labile C affects denitrification\(^{23}\), by supplying a reductant for denitrifiers, or through the stimulation of heterotrophic soil respiration, decreasing soil O$_2$ levels and thus promoting denitrification. Furthermore, readily decomposable C can decrease the N$_2$O/(N$_2$O + N$_2$) ratio of denitrification\(^{23}\). The increase in DOC observed in this study demonstrates an important non-targeted effect of DMPP, which can alter both rate and N$_2$O:N$_2$ partitioning of denitrification losses and therefore warrants further research.

Figure 2. Cumulative emissions of N$_2$O derived from nitrification (N$_2$O$_n$) and denitrification (N$_2$O$_d$), cumulative N$_2$ emissions, the product ratio of denitrification (N$_2$O/(N$_2$O$_d$ + N$_2$)) and the abundance of the nosZ gene encoding the N$_2$O reductase from a horticultural soil (Sandy clay loam) and a pasture soil (Loam) after the application of NH$_4$NO$_3$ with and without the nitrification inhibitor DMPP.
Table 4. Cumulative emissions of N₂, N₂O and CO₂ from a horticultural soil (Sandy clay loam) and a pasture soil (Loam) after the application of NH₄NO₃ with and without the nitrification inhibitor DMPP.

| Soil        | Material and Methods | Fertilizer | Fertilizer + DMPP | DMPP effect |
|-------------|----------------------|------------|-------------------|-------------|
| Sandy clay loam | Loam                | Denitrification | N₂ emissions     | N₂O emissions | CO₂ emissions |
| Horticulture soil | Pasture soil         | μg N₂ + N₂O₃ - N g⁻¹ soil | 0.73 ± 0.13      | 0.47 ± 0.09   | 6.55 ± 0.52   |
| N₂ emissions | μg N₂ - N g⁻¹ soil  | 3.08 ± 0.87 | 0.87 ± 0.11       | 1.46 ± 0.38   | 44.66 ± 1.73  |
| N₂O emissions | μg N₂O - N g⁻¹ soil | 0.04 ± 0.05 | 0.03 ± 0.04       | 0.01 ± 0.01   | 0.01 ± 0.01   |
| CO₂ emissions | μg CO₂ - C g⁻¹ soil | 2.83 ± 1.02 | 1.20 ± 0.20       | 1.80 ± 0.52   | 46.27 ± 1.35  |

Nitrification activity during pre-incubation increased NO₃⁻ levels in both soils. In the loam, NO₃⁻ levels were above those measured at the respective field site, which is also reflected in higher N₂O/(N₂O₃ + N₂) ratios. This phenomenon often occurs in incubation studies, where the absence of plant uptake, pre-incubation, and the addition of glucose increases NO₃⁻ levels in the soil. It is therefore important to consider N substrate availability when interpreting the effects of NIs on rate and N₂O:N₂ partitioning of denitrification losses. The mineral N levels in both soils indicate no N substrate limitation for denitrification regardless of the treatment. Under these conditions, DMPP had no effect on overall denitrification losses in both soils. The minor reduction of nitrification by DMPP in the loam did not reduce NO₃⁻ availability to a degree that limited preferential reduction of NO₂⁻. The high initial NO₂⁻ values in the loam are also likely to have over-written a significant reduction of nitrification. The reduction of N₂O emissions, together with the increase of nosZ abundance in the sandy CL suggests however that DMPP lowered NO₃⁻ availability below a soil specific threshold, limiting the preferential reduction of NO₂⁻ over N₂O. The results from the sandy CL confirm the proposed mechanism of N₂O reduction via a shift in the N₂:N₂O ratio, and show that DMPPs inhibitory effect on nitrification can limit the sumption of the N₂O reductase, promoting complete denitrification to N₂.

The demonstrated link between nosZ and directly measured N₂O and N₂ emissions suggests that DMPP promotes the abundance of nosZ carrying denitrifiers. Including a comprehensive assessment of abundance and activity of nitrifying and denitrifying microbial communities in future research could further help to understand mechanisms of N₂O mitigation by DMPP. Our study shows N dynamics in response to DMPP on a soil microcosm scale. This approach does not account for plant-microbe interactions and plant N uptake under field conditions but enables to isolate effects of NIs on key N transformations, with practical implications for the use of NIs in different agricultural soils. The relative magnitude of N₂O emissions reflects cumulative losses observed from the same soils in the field, demonstrating a larger N₂O mitigation potential for the pasture soil as compared to the horticultural soil. The short term inefficacy of DMPP to reduce nitrification in the pasture soils demands therefore improved strategies regarding rate and application of NIs. In soils with high organic matter content, and high soil intrinsic N turnover, repeated applications of DMPP, increasing the rate of DMPP, and/or the application of DMPP prior to fertilization may increase DMPPs efficacy, limiting the effect of N fertilizer priming on N₂O emissions. Decreased nosZ abundance after fertilization and irrigation indicates suppression of the N₂O reductase by increased NO₃⁻ availability, identifying NO₃⁻ availability as the control for the reduction of NO₂⁻ vs. N₂O, which determines the magnitude of N₂O losses. These findings apply to conditions of non-limiting NO₃⁻ availability for overall denitrification, which can be found in agricultural soils after N fertilization and irrigation when plant N uptake is limited. Under these conditions, the efficacy of NIs to mitigate N₂O emissions depends on their ability to limit the suppression of the N₂O reductase by high NO₃⁻ concentrations in the soil, enabling complete denitrification to N₂.

Material and Methods
Soils and site. Soil samples (0–10 cm) were collected randomly (n = 4) from a vegetable cropping site (Gatton, Qld) and an intensively managed dairy pasture (Gympie, Qld) in subtropical Australia, referred to according to their texture in the first 10 cm as sandy clay loam (sandy CL) and loam, respectively. Site characteristics including physical and chemical soil properties are shown in Table 1. Soil samples were bulked, air dried and sieved to <4 mm and stored in a cold room at 4°C.

Soil microcosms. Before treatment application, the soils were incubated in bulk for 4 days at a gravimetric water content of 30%. The experimental design consisted of two soils and two treatments: ammonium nitrate (NH₄NO₃) and NH₄NO₃ with DMPP (DMPP), each with four different ¹⁵N label combinations and four replicates. The NH₄NO₃ was applied with either (a) the NH₄⁺ (¹⁵NH₄NO₃⁻) or (b) the NO₃⁻ (NH₄⁺¹⁵NO₃⁻) labeled at 10 atom %. NH₄¹⁵NO₃⁻ at 60 atom % (c) was used to quantify N emissions, while non-labeled NH₄NO₃ (d) was used for the quantification of the SMB, DOC, and nosZ abundance. For the incubation, soil microcosms were established in centrifuge tubes (50 ml) using the equivalent of 8 g oven dry soil at a soil bulk density of 1 g cm⁻³. NH₄NO₃ equivalent to 35 µg N g⁻¹ soil was applied in solution (1 ml) with 0.6% DMPP (w/w) added for the DMPP treatment. Additional water was applied to achieve the water-filled pore space (WFPS) of 75%. Water and fertilizer solutions were applied dropwise on two layers of 4 g of soil to ensure homogenous ¹⁵N labeling. After fertilization, centrifuge tubes were closed with Suba-seals (Sigma Aldrich) and were kept closed in an incubator at a constant temperature of 25°C between gas sampling events. Additional soil microcosms (a and b, n = 4) were established for destructive sampling 30 minutes after fertilizer application.
Soil analysis. Soil mineral N. All soil mineral N extractions were conducted in the centrifuge tubes to avoid subsampling errors using 40 ml 2 M KCl (1:5 w/v ratio). Four soil microcosms per soil were extracted before fertilizer application to determine initial conditions. Soil microcosms a and b were extracted with 40 ml 2 M KCl, 30 min (t = 0) and 48 h (t = 2 days) after N fertilizer application. The centrifuge tubes were shaken with a horizontal shaker (150 rpm) for one hour, and extracts were filtered through Whatman no. 42 filter paper. After sample dilution, concentrations of NH$_4^+$ and NO$_3^-$ were determined using colorimetric methods. NH$_4^+$ with a modified indophenol reaction and NO$_3^-$ with the VCL3/Griess assay. The $^{15}$N enrichments of the NH$_4^+$ and NO$_3^-$ pool were determined for soil microcosms a and b by the diffusion method.

Quantitative PCR analyses. For qPCR analysis, subsamples of 0.25 g of soil were taken prior to fertilizer application, and 48 h after (t = 2 days) from soil microcosms d and extracted immediately for total DNA using the PowerLyzer® PowerSoil® DNA Isolation Kit from MoBio (Mobio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions, with some minor modifications. Briefly, the soil was extracted twice by using the same soil and PowerBead Tubes to increase recovery of DNA. DNA concentration and quality were determined spectrophotometrically (NanoDrop 2000, Thermofisher, MA, USA). The total DNA aliquots from each sample were pooled before qPCR. The real-time PCR assay was carried out in a volume of 10 µl, and the assay mixture contained GoTaq® qPCR Master Mix (Promega, USA), 10 µM of each nosZ primer and 1 µl of pooled template DNA. Thermal cycling conditions for the nosZ2F (CGCAGCCGCAAAAGGTSMSSTG) and nosZ2R (CASRGCAAKSGCRTGGCAGAA) were as follows: an initial cycle of 95°C for 3 min, 39 cycles of 95°C for 15 s, 39 cycles of 60°C for 45 s, 39 cycles of 72°C for 45 s and 95°C for 5 s. Each sample was quantified in triplicates using the iCycler iQ Real-Time PCR Detection System and the iQ 5 Optical System software (Bio-Rad Laboratories, Hercules, CA, USA).

Soil microbial biomass. Microbial C ($C_{mic}$) and N ($N_{mic}$) were quantified before and two days after fertilizer application using the chloroform fumigation-extraction. Two aliquots of 3.5 g soil were sampled from each soil microcosm (d) with one aliquot subsequently fumigated with chloroform for 24 h. Fumigated and non-fumigated samples were extracted with 2 M KCl (1:10 w/v) and stored frozen until further analyses. Samples were acidified to remove inorganic C and analyzed for total N and organic C with an automated TOC/TN analyzer (TOC-V CPH200V) linked with a TN-unit (TNM-1 220 V, Shimadzu Corporation, Kyoto, Japan). $C_{mic}$ and $N_{mic}$ were calculated as the difference in N and C between fumigated and non-fumigated samples without using a correction factor. Dissolved organic C (DOC) was quantified as the amount of total C in the extracts of the non-fumigated samples. Gross N transformations. Gross N transformations were quantified using a $^{15}$N tracing model (Fig. 1), which uses a Markov Chain Monte Carlo method optimizing the kinetic parameters for the various N transformations by minimizing the mismatch between modeled and observed NH$_4^+$ and NO$_3^-$ concentrations and their respective $^{15}$N enrichments (soil microcosms a and b). The model considers five N pools including the NH$_4^+$ and NO$_3^-$ pool, a labile ($N_{lab}$) and a recalcitrant ($N_{rec}$) organic N pool, and a pool for NH$_4^+$ adsorbed to cation exchange sites ($NH_4^{+}_{ads}$). These pools are defined by 10 simultaneous occurring gross N transformations calculated by zero-, first-order or Michaelis-Menten kinetics (Table 2): The mineralization of $N_{lab}$ and $N_{rec}$ to NH$_4^+$ was assumed at 20 atom %.
(Mlab, Mrec), the immobilization of NH4+ to Nlab and Nrec (ςH4N-Nlab, ςH4N-Nrec), the adsorption (A15N) and release (RNH4a)
the oxidation of NH4+ from NH4+ ads, the oxidation of NH4+ to NO3− (O15N), referred to as autotrophic nitrification; the oxidation of Nrec to NO3− (O15N), referred to as heterotrophic nitrification; dissimilatory NO3− reduction to NH4+ (O15N) and RNH4a, the immobilisation of NO3− to Nrec. Total mineralization was calculated as the sum of Mlab and Mrec total nitrification as the sum of O15N and O15N and total immobilization of NH4+ as the sum of ςH4N-Nlab and ςH4N-Nrec.

Calculations and statistical analysis. The optimization routine used for the 15N tracing model gives a probability density function for each model parameter, which is used to calculate average values and standard errors of the mean. Average gross N transformation rates are obtained by integrating these values over the incubation period. Differences between N-transformations were assessed testing whether the 95% confidence intervals overlap45. The Benjamini Hochberg (BH) adjustment46 was performed to assess the effect of the different fertilization strategies on microbial C and N, DOC and nosZ gene abundance for each soil type. Analysis of variance was performed to assess differences in cumulative emissions of N2, N2O, total denitrification (N2 + N2O) and CO2 between soils within treatments and within soils between fertilization strategies. All values unless otherwise stated are given as mean ± standard error of the mean.

Data availability
All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Author contributions

J.F., C.S., D.R., P.G. and K.K. designed the experimental setup. J.F. and K.K. conducted the experiment. J.F. performed 15N isotope analysis and C.M. analyzed the 15N tracing data. E.D. and M.G. performed the molecular analysis. D.D.R. conducted the statistical analysis. All authors interpreted the data and contributed to the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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