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# Effect of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on N-turnover, the N<sub>2</sub>O reductase-gene *nosZ* and N<sub>2</sub>O:N<sub>2</sub> partitioning from agricultural soils

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Nitrification inhibitors (NIs) have been shown to reduce emissions of the greenhouse gas nitrous oxide (N<sub>2</sub>O) from agricultural soils. However, their N<sub>2</sub>O reduction efficacy varies widely across different agroecosystems, 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<sub>2</sub> and N<sub>2</sub>O emissions with <sup>15</sup>N tracing analysis and the quantification of the N<sub>2</sub>O-reductase gene (*nosZ*) in a soil microcosm study. Nitrogen fertilization suppressed *nosZ* abundance in both soils, showing that high nitrate availability and the preferential reduction of nitrate over N<sub>2</sub>O is responsible for large pulses of N<sub>2</sub>O after the fertilization of agricultural soils. DMPP attenuated this effect only in the horticultural soil, reducing nitrification while increasing *nosZ* abundance. DMPP reduced N<sub>2</sub>O emissions from the horticultural soil by >50% but did not affect overall N<sub>2</sub> + N<sub>2</sub>O losses, demonstrating the shift in the N<sub>2</sub>O:N<sub>2</sub> ratio towards N<sub>2</sub> as a key mechanism of N<sub>2</sub>O mitigation by NIs. Under non-limiting NO<sub>3</sub><sup>-</sup> availability, the efficacy of NIs to mitigate N<sub>2</sub>O emissions therefore depends on their ability to reduce the suppression of the N<sub>2</sub>O reductase by high NO<sub>3</sub><sup>-</sup> concentrations in the soil, enabling complete denitrification to N<sub>2</sub>.

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

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

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| 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 Argiustoll                    | Ferric Acrisol      |
| Sand (%)                 | 50.5                               | 47.2                |
| Silt (%)                 | 22.8                               | 38.8                |
| Clay (%)                 | 30.7                               | 20.4                |
| pH                       | 7.4                                | 6.1                 |
| 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.

nitrification-mediated pathways, denitrification and biotic formation of hybrid  $N_2O$ <sup>9</sup>. Denitrification is generally assumed to be the main process contributing to overall  $N_2O$  production from agricultural soils<sup>7,10–12</sup> and is also the main process reducing  $N_2O$  into environmentally benign  $N_2$  via the  $N_2O$  reductase, the enzyme encoded by the functional *nosZ* gene. The reduction of  $N_2O$  to  $N_2$  does not reduce overall N losses but limits the environmental impact of denitrification losses from agricultural soils. A reduction of  $N_2O$  emissions by NIs can be attributed to (a) reduced  $N_2O$  production via nitrification mediated pathways, (b) reduced  $N_2O$  production via denitrification (c) increased consumption of  $N_2O$  via denitrification, i.e., a shift in the  $N_2O:N_2$  ratio towards  $N_2$ . As these effects may overlap, a mechanistic understanding of the effects of NIs on  $N_2O$  production and consumption processes needs to be based on  $N_2O$  source partitioning, and the direct quantification of  $N_2$ .

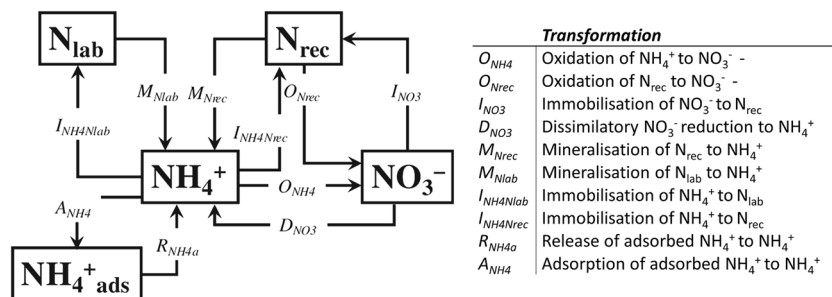
Most of the NIs inhibit the first and rate-limiting enzymatic step of nitrification, the conversion of  $NH_4^+$  to hydroxylamine ( $NH_2OH$ ) via the ammonia monooxygenase<sup>13</sup>. The inhibition of nitrification means a reduced supply of N into the  $NO_3^-$  pool as the source pool of denitrification, but also an increase in  $NH_4^+$  availability, leading to an increase of fertilizer N immobilization<sup>11</sup> and mineralization/immobilization turnover rates<sup>14,15</sup>. Availability of N for  $N_2O$  producing processes determines both production, but also consumption of  $N_2O$ , as high  $NO_3^-$  availability shifts the  $N_2O:N_2$  ratio of denitrification towards  $N_2O$ <sup>16</sup>. The link between N transformation rates and  $N_2O$  and  $N_2$  emissions is therefore critical to understand the effects of NIs in agricultural soils.

Typically, pulses of  $N_2O$  are observed after fertilization and irrigation events. These pulses are short-lived and can account for more than 90% of cumulative  $N_2O$  emissions from agro-ecosystems<sup>17</sup>, defining the critical time-window which determines the efficacy of NIs to mitigate  $N_2O$  emission. Building on extensive research at the field scale conducted across different agro-ecosystems<sup>5,11,18–20</sup>, this study investigated the short-term effect of 3,4-dimethylpyrazole phosphate (DMPP) on N-turnover and  $N_2O$  and  $N_2$  emissions from two contrasting agricultural soils in response to N-fertilization. We combined a <sup>15</sup>N tracing analysis with the direct quantification of  $N_2$  and  $N_2O$  emissions using the <sup>15</sup>N gas flux method, 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  $N_2O$  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.

**Nitrogen transformations and soil microbial parameters.** Gross N transformation rates were quantified with a <sup>15</sup>N tracing model (Fig. 1) and differed markedly between soils when N-fertilizer was applied without the NI DMPP, referred to as the fertilizer only treatment (Table 2). Gross mineralization rates ( $M_{tot}$ ) in the loam exceeded those in the sandy CL by a factor of 39. In the loam,  $M_{tot}$  was dominated by the mineralization of labile N ( $M_{Nlab}$ ), while the mineralization of recalcitrant organic N ( $M_{Nrec}$ ) dominated in the sandy CL. Gross nitrification ( $Nit_{tot}$ ) was higher in the loam with  $18.7 \pm 0.03 \mu g N g^{-1} soil day^{-1}$  compared to  $5.8 \pm 0.03 \mu g N g^{-1} soil day^{-1}$  in the sandy CL. Autotrophic nitrification ( $O_{NH_4}$ ) was the main pathway of  $NO_3^-$  production in both soils, as heterotrophic nitrification of organic N ( $O_{Nrec}$ ) accounted for only 7% of  $Nit_{tot}$  in the sandy CL, and was negligible for  $Nit_{tot}$  in the loam. Immobilization of  $NH_4^+$  ( $I_{NH_4tot}$ ) and  $NO_3^-$  ( $I_{NO_3}$ ) was higher in the sandy CL compared to the loam, and was dominated by  $I_{NO_3}$ . In the sandy CL, only minor amounts of  $NO_3^-$  were recycled in the  $NH_4^+$  pool via dissimilatory  $NO_3^-$  reduction to  $NH_4^+$  (DNRA, referred to as  $D_{NO_3}$  in the <sup>15</sup>N tracing model), while  $D_{NO_3}$  contributed with more than  $2 \mu g N g^{-1} soil day^{-1}$  to  $NH_4^+$  production in the loam. Microbial C ( $C_{mic}$ ) and N ( $N_{mic}$ ) as indicators for the size of the soil microbial biomass (SMB) were higher in the loam, exceeding  $C_{mic}$  and  $N_{mic}$  in the sandy CL by a factor of 5 and 7, respectively (Table 3).



**Figure 1.** Conceptual  $^{15}N$  tracing model for the analysis of N gross transformations with the respective N transformations.

**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  $O_{NH4}$  only by 6% in the loam, but reduced  $O_{NH4}$  by more than 60% in the sandy CL. In the sandy CL, both  $M_{tot}$  and  $I_{NH4tot}$  increased, as well as the relative contribution of  $M_{Nrec}$  to  $M_{tot}$ , accounting for 80% of  $M_{tot}$ .  $I_{NO3}$  decreased by 31%, while  $D_{NO3}$  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  $N_2O$  and  $N_2$ .** The dominant  $N_2O$  production pathway in both soils was denitrification, accounting for more than 90% of the  $N_2O$  produced (Fig. 2). Over 48 hours,  $0.24 \pm 0.03$  and  $1.46 \pm 0.38 \mu g N_2O - N g^{-1}$  soil were emitted from the sandy CL and the loam, respectively. Both  $N_2O$  emissions via denitrification ( $N_2O_d$ ) and nitrification ( $N_2O_n$ ) were higher from the loam, exceeding those from the sandy CL by a factor of >8 (Fig. 2). Over the two day incubation period,  $0.47 \pm 0.09 \mu g N_2 - N g^{-1}$  soil and  $0.87 \pm 0.11 \mu g N_2 - N g^{-1}$  soil were emitted as  $N_2$  from the sandy CL and the loam, respectively. The main product of denitrification ( $N_2O_d + N_2$ ) from the sandy CL was  $N_2$ , with  $N_2O_d$  accounting for 36% of total denitrification losses. Denitrification losses from the loam however were dominated by  $N_2O_d$ , accounting for 75% of total denitrification. There was no indication for hybrid production of  $N_2O$  or  $N_2$ .

**The response of the  $N_2O$  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. DMPP however increased *nosZ* copy numbers by 227% compared to the fertilizer only treatment in the sandy CL.

**Effect of DMPP on  $N_2O$  and  $N_2$  emissions.** DMPP significantly reduced  $N_2O$  emission from the sandy CL but had no effect on  $N_2O$  emissions from the loam (Table 4). DMPP reduced  $N_2O_d$  from the sandy CL by 46% ( $P < 0.05$ ), but did not affect  $N_2O_n$  (Fig. 2). There was no effect of DMPP on  $N_2$  emissions from the two soils. In the sandy CL, DMPP shifted the product ratio of denitrification ( $N_2O_d / (N_2O_d + N_2)$ ) to  $N_2$ , decreasing the percentage of denitrification emitted as  $N_2O_d$  from 36% to 20%.

## Discussion

The fertilization and irrigation of agricultural soils triggers a cascade of N transformations associated with pulses of  $N_2O$  and  $N_2$  emissions. These short-term events are critical to understand the effects of NIs on  $N_2O$  production and consumption in agricultural soils. Linking N turnover to emissions of  $N_2O$  and  $N_2$  and the abundance of the  $N_2O$  reductase gene *nosZ* in a short-term incubation demonstrated (a) that increasing  $NO_3^-$  availability after fertilization suppressed *nosZ* abundance, (b) that *nosZ* abundance, nitrification and  $N_2 + N_2O$  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  $N_2:N_2O$  ratio towards  $N_2$ . 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  $N_2O$  reductase by high  $NO_3^-$  concentrations in the soil, enabling complete denitrification to  $N_2$ .

Nitrogen transformation rates identified the loam as the more active soil regarding N turnover compared to the sandy CL (Table 2). Gross mineralization rates ( $M_{tot}$ ) of more than  $8 \mu g N g^{-1}$  soil day $^{-1}$  together with a low immobilization of mineral N ( $I_{NH4tot}$  and  $I_{NO3}$ ) 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 ( $M_{Nlab}$ ), representing the microbial biomass and low molecular organic N compounds with a fast turnover. The high nitrification rates in the loam ( $>18 \mu g N g^{-1}$  soil day $^{-1}$ ) denote rapid conversion of mineralized N to  $NO_3^-$  and show the dominant role of  $NH_4^+$  oxidation for N-turnover in this soil. Gross mineralization was markedly lower in the sandy CL with  $M_{tot}$  at only  $0.21 \mu g N g^{-1}$  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  $NH_4^+$  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

| N - transformation $\mu\text{g N g}^{-1} \text{ soil day}^{-1}$ |                    | Sandy clay loam - Horticulture soil |   |              |   | Loam - Pasture soil |   |                |   | DMPP effect      |   |
|---|--------------------|-------------------------------------|---|--------------|---|---------------------|---|----------------|---|------------------|---|
|   |                    | -DMPP                               |   | +DMPP        |   | -DMPP               |   | +DMPP          |   |                  |   |
|   |                    |                                     | ↔ |              | ↔ |                     | ↔ |                | ↔ |                  |   |
| Mineralisation of $N_{rec}$ to $\text{NH}_4^+$                  | $M_{Nrec}$         | 0.12 ± 0.04                         | c | 1.03 ± 0.11* | b | +776%               |   | 2.59 ± 0.09    | a | 2.50 ± 0.11      | a |
| Immobilisation of $\text{NH}_4^+$ to $N_{rec}$                  | $I_{NH4-Nrec}$     | 0.16 ± 0.04                         | b | 0.79 ± 0.22* | a | +397%               |   | 0.002 ± 0.0005 | c | 0.0020 ± 0.00005 | c |
| Mineralisation of $N_{lab}$ to $\text{NH}_4^+$                  | $M_{Nlab}$         | 0.10 ± 0.03                         | d | 0.25 ± 0.05* | c | +166%               |   | 5.80 ± 0.28*   | a | 5.37 ± 0.07      | b |
| Immobilisation of $\text{NH}_4^+$ to $N_{lab}$                  | $I_{NH4-Nlab}$     | 0.81 ± 0.26                         | b | 2.59 ± 0.50* | a | +220%               |   | 0.002 ± 0.0002 | c | 0.002 ± 0.0002   | c |
| Oxidation of $N_{rec}$ to $\text{NO}_3^-$                       | $O_{Nrec}$         | 0.38 ± 0.11                         | a | 0.34 ± 0.22  | a |                     |   | 0              |   | 0                |   |
| Immobilisation of $\text{NO}_3^-$ to $N_{rec}$                  | $I_{NO3}$          | 9.48 ± 0.12                         | a | 6.55 ± 0.31* | b | -31%                |   | 0.017 ± 0.0005 | c | 0.016 ± 0.0005   | c |
| Oxidation of $\text{NH}_4^+$ to $\text{NO}_3^-$                 | $O_{NH4}$          | 5.44 ± 0.28                         | c | 2.04 ± 0.20* | d | -63%                |   | 18.64 ± 0.24*  | a | 17.46 ± 0.37     | b |
| Dissimilatory $\text{NO}_3^-$ reduction to $\text{NH}_4^+$      | $D_{NO3}$          | 0.026 ± 0.003                       | d | 0.14 ± 0.01* | c | +431%               |   | 2.14 ± 0.05    | a | 2.02 ± 0.08      | a |
| Adsorption of adsorbed $\text{NH}_4^+$ to $\text{NH}_4^+$ ads   | $A_{NH4}$          | 1.18 ± 0.22                         | a | 0.87 ± 0.75  | a |                     |   | 0              |   | 0                |   |
| Release of adsorbed $\text{NH}_4^+$ to $\text{NH}_4^+$          | $R_{NH4a}$         | 0.08 ± 0.02                         | b | 0.68 ± 0.07* | a | +714%               |   | 0              |   | 0                |   |
| Total mineralisation $M_{Nrec} + M_{Nlab}$                      | $M_{tot}$          | 0.21 ± 0.05                         | d | 1.29 ± 0.12* | c | +502%               |   | 8.39 ± 0.29    | a | 7.87 ± 0.13*     | b |
| Total nitrification $O_{Nrec} + O_{NH4}$                        | $Ni_{tot}$         | 5.82 ± 0.30                         | c | 2.39 ± 0.30* | d | -59%                |   | 18.64 ± 0.24   | a | 17.46 ± 0.37*    | b |
| Total $\text{NH}_4^+$ immobilisation                            | $I_{NH4tot}$       | 0.97 ± 0.31                         | b | 3.37 ± 0.72* | a | +249%               |   | 0.004 ± 0.001  | c | 0.004 ± 0.0001   | c |
| Contribution of $M_{Nlab}$ to $M_{tot}$                         | $M_{Nlab}/M_{tot}$ | 45%                                 |   | 20%          |   |                     |   | 69%            |   | 68%              |   |
| Contribution of $O_{NH4}$ to $Ni_{tot}$                         | $O_{NH4}/Ni_{tot}$ | 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  $\text{NH}_4\text{NO}_3$  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).

versus short term/annual and tilled versus undisturbed plant-systems on soil organic matter and microbial activity: Intensive tillage and irrigation in horticultural systems lead to loss of soil organic C<sup>21</sup>, while an extensive root system under permanent pasture is likely to promote microbial activity through constant inputs of C and N. These findings establish the differences in magnitude and relative importance of N transformations and microbial activity between the two contrasting soils.

The main source of  $\text{N}_2\text{O}$  in both soils was denitrification, accounting for more than 90% of  $\text{N}_2\text{O}$  produced (Fig. 2), which is in line with previous results from both field<sup>11</sup> and laboratory studies<sup>10,12</sup>. The ability of soils to act as an  $\text{N}_2\text{O}$  sink, i.e. the trait to reduce  $\text{N}_2\text{O}$  to  $\text{N}_2$  has been linked to the abundance of *nosZ*, used as proxy for microorganisms involved in the reduction of  $\text{N}_2\text{O}$ . In the study presented here, we compared *nosZ* abundance with direct measurements of  $\text{N}_2$  and  $\text{N}_2\text{O}$ , evaluating the influence of DMPP on of microorganisms reducing  $\text{N}_2\text{O}$ . The abundance of *nosZ* prior to fertilizer addition was higher in the loam, which is consistent with the reported positive correlation of *nosZ* copy numbers with soil organic C<sup>22</sup>. The synthesis of the  $\text{N}_2\text{O}$  reductase is promoted by anoxic conditions<sup>23</sup>, and the increase in soil moisture together with the addition of fertilizer should have increased *nosZ* abundance. However, *nosZ* abundance decreased in both soils in the fertilizer only treatment (Fig. 2), indicating that increased  $\text{NO}_3^-$  availability due to fertilization and nitrification promoted the reduction of  $\text{NO}_3^-$  rather than  $\text{N}_2\text{O}$ , shifting the  $\text{N}_2\text{O}_d/(\text{N}_2\text{O}_d + \text{N}_2)$  ratio towards  $\text{N}_2\text{O}$ . The magnitude and  $\text{N}_2\text{O}:\text{N}_2$  partitioning of denitrification losses is consistent with the nitrification rates in both soils and as such shows the  $\text{N}_2\text{O}_d/(\text{N}_2\text{O}_d + \text{N}_2)$  ratio as a function of soil intrinsic N - turnover. Cumulative  $\text{N}_2\text{O}_d$  losses of  $>2 \mu\text{g N g}^{-1}$  soil and 75% of denitrification ( $\text{N}_2\text{O}_d + \text{N}_2$ ) emitted as  $\text{N}_2\text{O}$  from the loam show increased substrate availability for denitrification and simultaneous suppression of *nosZ* abundance by high  $\text{NO}_3^-$  availability (Fig. 2). In turn, lower denitrification losses with only 36% emitted as  $\text{N}_2\text{O}_d$  reflect slower N turnover in the sandy CL. These findings suggest that the suppression of the  $\text{N}_2\text{O}$  reductase and increased N substrate availability are responsible for the large pulses of  $\text{N}_2\text{O}$  from agricultural soils observed after fertilization and irrigation. Our results denote an increased risk of  $\text{N}_2\text{O}$  loss from highly productive agricultural soils<sup>19</sup>, where increased mineralization of soil organic N due to fertilization, i.e., priming is likely to amplify the preferential reduction of  $\text{NO}_3^-$ , and as such the production of  $\text{N}_2\text{O}$  via denitrification.

DMPP reduced  $\text{N}_2\text{O}$  emissions from the sandy CL by more than 54% (Table 4). This is reflected in DMPP's effect on autotrophic nitrification ( $O_{NH4}$ ) showing a reduction of 63% in the sandy CL (Table 2). The minor reduction of  $O_{NH4}$  by DMPP had however no effect on  $\text{N}_2\text{O}$  emissions from the loam. In both soils,  $\text{N}_2\text{O}$  derived from nitrification mediated pathways accounted for less than 15% of overall  $\text{N}_2\text{O}$ , showing no response to the DMPP treatment. For the sandy CL, this suggests that DMPP primarily affected  $\text{N}_2\text{O}$  production pathways indirectly, that is by reducing  $\text{NO}_3^-$  availability for denitrification, demonstrated by the reduction of  $\text{N}_2\text{O}$  derived from denitrification by 46%. DMPP increased *nosZ* abundance in the sandy CL by a factor  $>2$  compared to the fertilizer only treatment (Fig. 2). In the absence of direct  $\text{N}_2$  measurements, this effect has been interpreted as a shift of denitrification losses towards  $\text{N}_2$ <sup>24</sup>. Experimental evidence linking increased *nosZ* abundance with DMPP to  $\text{N}_2$  and  $\text{N}_2\text{O}$  emissions<sup>25</sup> is based on the acetylene inhibition method, which has been shown to lead to an irreproducible underestimation of denitrification rates<sup>9</sup>. Furthermore, acetylene itself is a potent NI, questioning the use of this method when investigating the effects of NIs on the magnitude and the  $\text{N}_2\text{O}_d/(\text{N}_2\text{O}_d + \text{N}_2)$  ratio

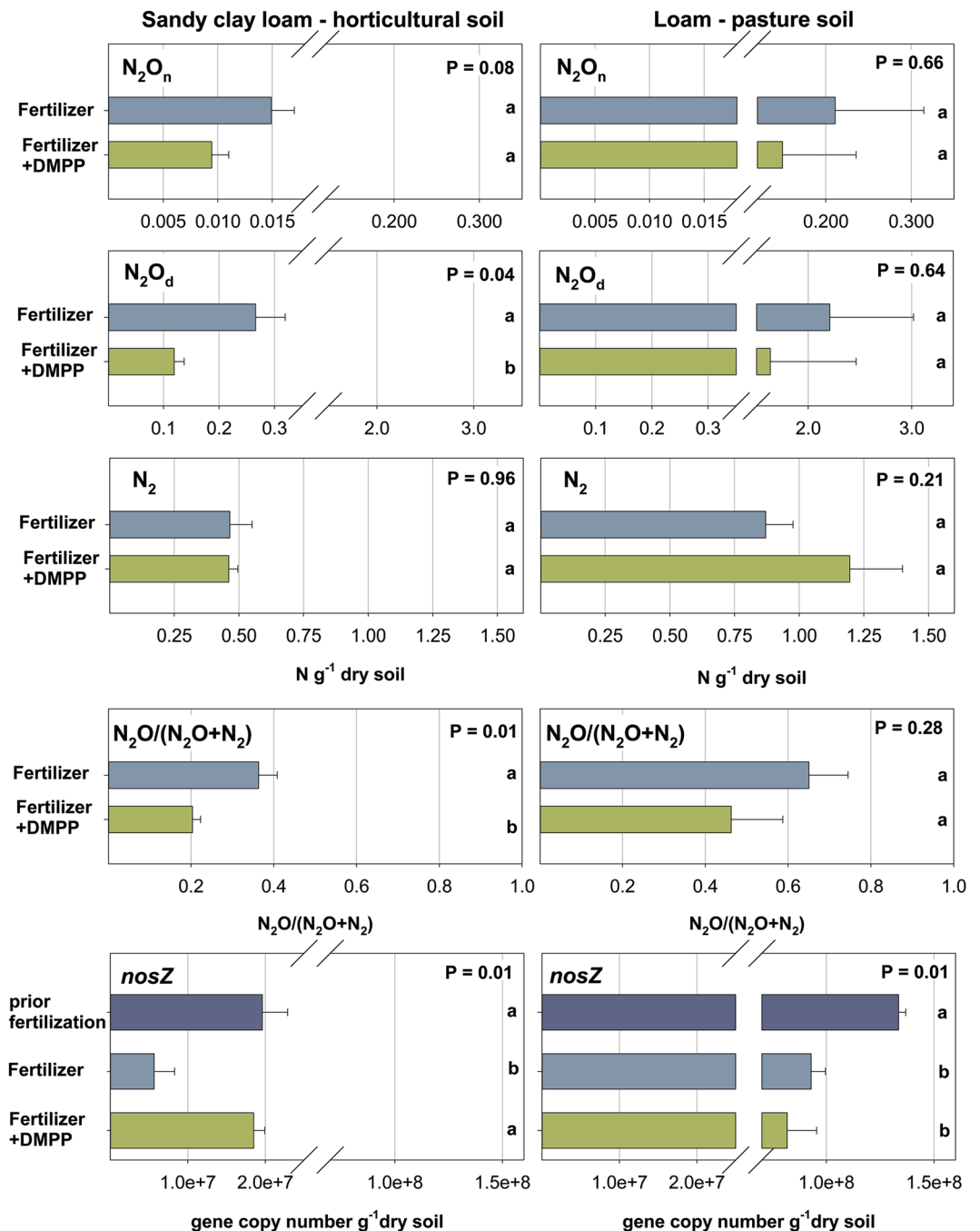
|                              |  | time   |                     | Sandy clay loam Horticulture soil | Loam Pasture soil | ↓            | ↔   |
|------------------------------|--|--------|---------------------|-----------------------------------|-------------------|--------------|-----|
|                              |  |        |                     | ↓                                 | ↓                 |              |     |
| NH <sub>4</sub> <sup>+</sup> | μg N g <sup>-1</sup> soil                | 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 * |
|                              |  |        | <b>DMPP effect</b>  | <b>+42%</b>                       |                   | <b>+223%</b> |     |
| NO <sub>3</sub> <sup>-</sup> | μg N g <sup>-1</sup> soil                | 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 * |
|                              |  |        | <b>DMPP effect</b>  | <b>-12%</b>                       |                   | —            |     |
| DOC                          | μg C g <sup>-1</sup> soil                | 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 * |
|                              |  |        | <b>DMPP effect</b>  | <b>+50%</b>                       |                   | <b>+32%</b>  |     |
| Microbial C                  | μg C <sub>mic</sub> g <sup>-1</sup> soil | 0      | prior fertilization | 93.6 ± 18.7                       | a a               | 433.0 ± 34.4 | b * |
|                              |  | 48 h   | Fertilizer          | 61.5 ± 13.9                       | b b               | 471.8 ± 13.5 | a * |
|                              |  | 48 h   | Fertilizer + DMPP   | 66.3 ± 7.4                        | b                 | 480.3 ± 7.5  | a * |
|                              |  |        | <b>DMPP effect</b>  | —                                 |                   | —            |     |
| Microbial N                  | μg N <sub>mic</sub> g <sup>-1</sup> soil | 0      | prior fertilization | 11.9 ± 0.6                        | a 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 * |
|                              |  |        | <b>DMPP effect</b>  | —                                 |                   | —            |     |

**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.

of denitrification. In the study presented here, DMPP reduced the  $N_2O_d/(N_2O_d + N_2)$  ratio by 44% in the sandy CL, demonstrating a significant shift towards  $N_2$  (Fig. 2). These results link the increase of *nosZ* abundance in response to DMPP in the sandy CL to a shift in the  $N_2O_d:N_2$  ratio towards  $N_2$ , based on direct measurements of  $N_2$  and  $N_2O_d$  using the <sup>15</sup>N gas flux method. In contrast to previous incubation studies investigating  $N_2O:N_2$  partitioning in response to DMPP<sup>26,27</sup>, emissions of  $N_2O$  and  $N_2$  were quantified after incubation under atmospheric  $O_2$  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  $N_2$  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  $N_2O$  reductase after fertilization, enabling complete denitrification to  $N_2$ . Emissions of  $N_2O$  produced via nitrification mediated pathways were not affected by DMPP in this soil, showing the reduction of  $N_2O$  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 observed inefficacy of DMPP to reduce autotrophic nitrification in the loam, which is consistent with reports from other pasture soils<sup>15</sup>. 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<sup>28,29</sup>. 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 inefficacy 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  $N_2O$  after fertilization and irrigation from the loam, but reduced denitrification losses after that initial period<sup>11</sup>. This shows a delayed effect of DMPP in this soil, demanding further research on how diffusion in the soil matrix, sorption and distribution affects DMPPs efficacy to reduce autotrophic nitrification.

DMPP also affected non-targeted N transformation in the sandy CL: Mineralization and immobilization turnover was stimulated by DMPP, demonstrated by the five-fold increase of total mineralization ( $M_{nrec} + M_{nlab}$ ) and the simultaneous increase of  $NH_4^+$  immobilization ( $I_{NH_4rec} + I_{NH_4lab}$ ) by a factor  $> 2$  (Table 2). Increased mineralization/immobilization turnover has been reported after the application of DMPP<sup>15</sup> and dicyandiamide (DCD)<sup>14</sup> and can be attributed to higher  $NH_4^+$  availability, stimulating microbial immobilization of  $NH_4$  ( $I_{NH_4lab}$ ) and mineralization of labile  $N_{org}$  ( $M_{Nlab}$ ) to  $NH_4^+$ . This effect may further prime the mineralization of recalcitrant N ( $M_{Nrec}$ ) in response to DMPP<sup>30</sup>. Interestingly, DMPP increased DOC availability in both soils, confirming previous results from a wheat-maize cropping system<sup>31</sup> (Table 3). Increased  $M_{Nrec}$  in the sandy CL indicates mineralization of



**Figure 2.** Cumulative emissions of  $N_2O$  derived from nitrification ( $N_2O_n$ ) and denitrification ( $N_2O_d$ ), cumulative  $N_2$  emissions, the product ratio of denitrification ( $N_2O/(N_2O+N_2)$ ) and the abundance of the *nosZ* gene encoding the  $N_2O$  reductase from a horticultural soil (Sandy clay loam) and a pasture soil (Loam) after the application of  $NH_4NO_3$  with and without the nitrification inhibitor DMPP.

organic matter induced by DMPP contributed to higher DOC availability, but no such effect was observed for  $M_{Nrec}$  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<sup>10,23</sup>. 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<sup>32</sup>, 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  $N_2O_d/(N_2O_d+N_2)$  ratio of denitrification<sup>23</sup>. The increase in DOC observed in this study demonstrates an important non-targeted effect of DMPP, which can alter both rate and  $N_2O:N_2$  partitioning of denitrification losses and therefore warrants further research.

|                                |   | Fertilizer        |                  |            | Fertilizer + DMPP |                  |            | DMPP effect        |              |
|--------------------------------|---|-------------------|------------------|------------|-------------------|------------------|------------|--------------------|--------------|
|                                |   | Sandy clay loam   | Loam             |            | Sandy clay loam   | Loam             |            | Sandy clay loam    | Loam         |
|                                |   | Horticulture soil | Pasture soil     |            | Horticulture soil | Pasture soil     |            | Horticulture soil  | Pasture soil |
| Denitrification                | $\mu\text{g N}_2 + \text{N}_2\text{O}_4 - \text{N g}^{-1} \text{ soil}$ | $0.73 \pm 0.13$   | $3.08 \pm 0.87$  | $P = 0.04$ | $0.58 \pm 0.05$   | $2.83 \pm 1.02$  | $P = 0.07$ | $P = 0.32$         | $P = 0.86$   |
| $\text{N}_2$ emissions         | $\mu\text{g N}_2 - \text{N g}^{-1} \text{ soil}$                        | $0.47 \pm 0.09$   | $0.87 \pm 0.11$  | $P = 0.03$ | $0.46 \pm 0.04$   | $1.20 \pm 0.20$  | $P = 0.04$ | $P = 0.96$         | $P = 0.21$   |
| $\text{N}_2\text{O}$ emissions | $\mu\text{g N}_2\text{O} - \text{N g}^{-1} \text{ soil}$                | $0.24 \pm 0.03$   | $1.46 \pm 0.38$  | $P = 0.01$ | $0.14 \pm 0.02$   | $1.80 \pm 0.52$  | $P = 0.01$ | $-54\% / P = 0.01$ | $P = 0.60$   |
| $\text{CO}_2$ emissions        | $\mu\text{g CO}_2 - \text{C g}^{-1} \text{ soil}$                       | $6.55 \pm 0.52$   | $44.66 \pm 1.73$ | $P = 0.01$ | $5.99 \pm 0.18$   | $46.27 \pm 1.35$ | $P < 0.01$ | $P = 0.35$         | $P = 0.47$   |

**Table 4.** Cumulative emissions of  $\text{N}_2$ ,  $\text{N}_2\text{O}$  and  $\text{CO}_2$  from a horticultural soil (Sandy clay loam) and a pasture soil (Loam) after the application of  $\text{NH}_4\text{NO}_3$  with and without the nitrification inhibitor DMPP.

Nitrification activity during pre-incubation increased  $\text{NO}_3^-$  levels in both soils. In the loam,  $\text{NO}_3^-$  levels were above those measured at the respective field site, which is also reflected in higher  $\text{N}_2\text{O}_d/(\text{N}_2\text{O}_d + \text{N}_2)$  ratios<sup>11</sup>. This phenomenon often occurs in incubation studies, where the absence of plant uptake, pre-incubation<sup>33,34</sup>, and the addition of glucose<sup>26</sup> increases  $\text{NO}_3^-$  levels in the soil. It is therefore important to consider N substrate availability when interpreting the effects of NIs on rate and  $\text{N}_2\text{O}:\text{N}_2$  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  $\text{NO}_3^-$  availability to a degree that limited preferential reduction of  $\text{NO}_3^-$ . The high initial  $\text{NO}_3^-$  values in the loam are also likely to have overwritten a significant reduction of nitrification. The reduction of  $\text{N}_2\text{O}$  emissions, together with the increase of *nosZ* abundance in the sandy CL suggests however that DMPP lowered  $\text{NO}_3^-$  availability below a soil specific threshold<sup>35</sup>, limiting the preferential reduction of  $\text{NO}_3^-$  over  $\text{N}_2\text{O}$ . The results from the sandy CL confirm the proposed mechanism of  $\text{N}_2\text{O}$  reduction via a shift in the  $\text{N}_2:\text{N}_2\text{O}$  ratio<sup>26</sup>, and show that DMPPs inhibitory effect on nitrification can limit the suppression of the  $\text{N}_2\text{O}$  reductase, promoting complete denitrification to  $\text{N}_2$ .

The demonstrated link between *nosZ* and directly measured  $\text{N}_2\text{O}$  and  $\text{N}_2$  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  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  emissions reflects cumulative losses observed from the same soils in the field, demonstrating a larger  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  emissions. Decreased *nosZ* abundance after fertilization and irrigation indicates suppression of the  $\text{N}_2\text{O}$  reductase by increased  $\text{NO}_3^-$  availability, identifying  $\text{NO}_3^-$  availability as the control for the reduction of  $\text{NO}_3^-$  vs.  $\text{N}_2\text{O}$ , which determines the magnitude of  $\text{N}_2\text{O}$  losses. These findings apply to conditions of non-limiting  $\text{NO}_3^-$  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  $\text{N}_2\text{O}$  emissions depends on their ability to limit the suppression of the  $\text{N}_2\text{O}$  reductase by high  $\text{NO}_3^-$  concentrations in the soil, enabling complete denitrification to  $\text{N}_2$ .

## Material and Methods

**Soils and site.** Soil samples (0–10 cm) were collected randomly ( $n = 4$ ) from a vegetable cropping site (Gatton, Qld)<sup>20</sup> and an intensively managed dairy pasture (Gympie, Qld)<sup>11</sup> 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  $4^\circ\text{C}$ .

**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 ( $\text{NH}_4\text{NO}_3$ ) and  $\text{NH}_4\text{NO}_3$  with DMPP (DMPP), each with four different  $^{15}\text{N}$  label combinations and four replicates. The  $\text{NH}_4\text{NO}_3$  was applied with either (a) the  $\text{NH}_4^+$  ( $^{15}\text{NH}_4\text{NO}_3^-$ ) or (b) the  $\text{NO}_3^-$  ( $\text{NH}_4^{15}\text{NO}_3^-$ ) labeled at 10 atom %.  $\text{NH}_4^{15}\text{NO}_3^-$  at 60 atom % (c) was used to quantify  $\text{N}_2$  emissions<sup>36</sup>, while non-labeled  $\text{NH}_4\text{NO}_3$  (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  $1 \text{ g cm}^{-3}$ .  $\text{NH}_4\text{NO}_3$  equivalent to  $35 \mu\text{g N g}^{-1} \text{ soil}$  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  $^{15}\text{N}$  labeling. After fertilization, centrifuge tubes were closed with Suba-seals (Sigma Aldrich) and were kept closed in an incubator at a constant temperature of  $25^\circ\text{C}$  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 minutes ( $t=0$ ) 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  $\text{NH}_4^+$  and  $\text{NO}_3^-$  were determined using colorimetric methods,  $\text{NH}_4^+$  with a modified indophenol reaction<sup>37</sup> and  $\text{NO}_3^-$  with the VCL3/Griess assay<sup>38</sup>. The  $^{15}\text{N}$  enrichments of the  $\text{NH}_4^+$  and  $\text{NO}_3^-$  pool were determined for soil microcosms a and b by the diffusion method<sup>39</sup>.

**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 two DNA aliquots from each sample were pooled before qPCR. The real-time PCR assay was carried out in a volume of 10  $\mu\text{l}$ , and the assay mixture contained GoTaq® qPCR Master Mix (Promega, USA), 10  $\mu\text{M}$  of each *nosZ* primer<sup>40</sup> and 1  $\mu\text{l}$  of pooled template DNA. Thermal cycling conditions for the *nosZ2F* (CGCRACGGCAASAAGGTSMSSGT) and *nosZ2R* (CAKRTGCAKSGCRTGGCAGAA) 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 65 °C 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 ( $C_{\text{mic}}$ ) and N ( $N_{\text{mic}}$ ) were quantified before and two days after fertilizer application using the chloroform fumigation-extraction<sup>41</sup>. 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 CPHE200V) linked with a TN-unit (TNM-1 220 V, Shimadzu Corporation, Kyoto, Japan).  $C_{\text{mic}}$  and  $N_{\text{mic}}$  were calculated as the difference in N and C between fumigated and non-fumigated samples without using a correction factor<sup>42</sup>. Dissolved organic C (DOC) was quantified as the amount of total C in the extracts of the non-fumigated samples.

**Gas sampling and analysis.** Air samples ( $n=4$ ) were taken daily before closing the centrifuge tubes to quantify ambient  $\text{N}_2\text{O}$  concentrations. Specific background samples were taken above the respective soil microcosms treated with  $\text{NH}_4^{15}\text{NO}_3$  at 60 atom % (c) for  $^{15}\text{N}_2$  analysis before closing the tubes. The entire headspace atmosphere was sampled 24 and 48 h after closure using a gas-tight syringe from soil microcosms a, b and c. After the 24 h gas sampling, the Suba-seals were removed for 10 minutes, allowing the headspace atmosphere to equilibrate<sup>10</sup>. Gas samples were transferred into pre-evacuated 12 ml exetainer tubes with a double wadded Teflon/silicon septa cap (Labco Ltd, Buckinghamshire, UK) and stored until  $\text{N}_2\text{O}$  and  $\text{CO}_2$  analysis by gas chromatography (Shimadzu GC-2014). Gas samples from soil microcosms c were also analyzed for the isotopologues of  $\text{N}_2$  ( $^{15}\text{N}^{14}\text{N}$ ,  $^{15}\text{N}^{15}\text{N}$ ) and  $\text{N}_2\text{O}$  ( $^{14}\text{N}^{15}\text{N}^{16}\text{O} + ^{15}\text{N}^{14}\text{N}^{16}\text{O}$ ) and  $^{15}\text{N}^{15}\text{N}^{16}\text{O}$ ) using an automated isotope ratio mass spectrometer (IRMS) coupled to a trace gas preparation unit (Sercon Limited, 20–20, UK).

**Fluxes of  $\text{N}_2$ ,  $\text{N}_2\text{O}$  and  $\text{CO}_2$ .** The triple labelling approach generates gas samples from three  $^{15}\text{N}$  fertilizer treatments with four replicates: a, b and c. Cumulative  $\text{N}_2\text{O}$  and  $\text{CO}_2$  fluxes given in Table 4, were calculated based on gas samples from  $^{15}\text{N}$  fertilizer treatments a, b and c. Fluxes of  $\text{N}_2$  and  $\text{N}_2\text{O}_d$ , as well as denitrification losses ( $\text{N}_2 + \text{N}_2\text{O}_d$ ), were calculated based on the gas samples from treatment c. Calculating cumulative  $\text{N}_2\text{O}$  fluxes based on  $^{15}\text{N}$  fertilizer treatments a, b and c or c alone did not result in significant differences. The reduction of  $\text{N}_2\text{O}$  by DMPP in the sandy CL was significant regardless of the calculation chosen.

The flux rates of  $\text{N}_2\text{O}$  and  $\text{CO}_2$  were calculated from the slope of the linear increase in gas concentration during the closure period and were corrected for temperature and air pressure<sup>20</sup>. The  $^{15}\text{N}$  enrichment of the  $\text{NO}_3^-$  pool undergoing denitrification ( $a_p$ ) and the fraction of  $\text{N}_2$  and  $\text{N}_2\text{O}$  emitted from this pool ( $f_p$ ) were calculated following the equations given by Spott, *et al.*<sup>43</sup> detailed in the supplementary material. The headspace concentrations of  $\text{N}_2\text{O}$  and  $\text{N}_2$  were multiplied by the respective  $f_p$  values giving  $\text{N}_2$  and  $\text{N}_2\text{O}$  produced via denitrification (referred to as  $\text{N}_2$  and  $\text{N}_2\text{O}_d$ ), with their respective fluxes expressed in g  $\text{N}_2$  or  $\text{N}_2\text{O}_d$  -N emitted  $\text{g}^{-1}$  soil  $\text{day}^{-1}$ . Potential hybrid formation of  $\text{N}_2$  and  $\text{N}_2\text{O}$  was found to be irrelevant<sup>30</sup>. The precision of the IRMS for  $\text{N}_2$  based on the standard deviation of atmospheric air samples ( $n=18$ ) at 95% confidence interval was  $4.4 \times 10^{-7}$  and  $6.0 \times 10^{-7}$  for  $^{29}\text{R}$  and  $^{30}\text{R}$ , respectively. The corresponding method detection limit ranged from 0.005  $\mu\text{g N}_2\text{-N g}^{-1}$  soil with  $a_p$  assumed at 50 atom % to 0.014  $\mu\text{g N}_2\text{-N g}^{-1}$  soil with  $a_p$  assumed at 20 atom %.

**Gross N transformations.** Gross N transformations were quantified using a  $^{15}\text{N}$  tracing model<sup>44</sup> (Fig. 1), which uses a Markov Chain Monte Carlo method optimizing the kinetic parameters for the various N transformations by minimizing the misfit between modeled and observed  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations and their respective  $^{15}\text{N}$  enrichments (soil microcosms a and b). The model considers five N pools including the  $\text{NH}_4^+$  and  $\text{NO}_3^-$  pool, a labile ( $N_{\text{lab}}$ ) and a recalcitrant ( $N_{\text{rec}}$ ) organic N pool, and a pool for  $\text{NH}_4^+$  adsorbed to cation exchange sites ( $\text{NH}_4^+_{\text{ads}}$ ). 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  $N_{\text{lab}}$  and  $N_{\text{rec}}$  to  $\text{NH}_4^+$



( $M_{nlab}$ ,  $M_{Nrec}$ ), the immobilization of  $\text{NH}_4^+$  to  $N_{lab}$  and  $N_{rec}$  ( $I_{NH4-Nrec}$ ,  $I_{NH4-Nlab}$ ), the adsorption ( $A_{NH4}$ ) and release ( $R_{NH4a}$ ) of  $\text{NH}_4^+$  from  $\text{NH}_4^+$  ads, the oxidation of  $\text{NH}_4^+$  to  $\text{NO}_3^-$  ( $O_{NH4}$ ), referred to as autotrophic nitrification; the oxidation of  $N_{rec}$  to  $\text{NO}_3^-$  ( $O_{Nrec}$ ), referred to as heterotrophic nitrification; dissimilatory  $\text{NO}_3^-$  reduction to  $\text{NH}_4^+$  ( $D_{NO3}$ ) and  $I_{NO3}$ , the immobilisation of  $\text{NO}_3^-$  to  $N_{rec}$ . Total mineralization was calculated as the sum of  $M_{nlab}$  and  $M_{Nrec}$ ; total nitrification as the sum of  $O_{Nrec}$  and  $O_{NH4}$  and total immobilization of  $\text{NH}_4^+$  as the sum of  $I_{NH4-Nrec}$  and  $I_{NH4-Nlab}$ .

**Calculations and statistical analysis.** The optimization routine used for the  $^{15}\text{N}$  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 overlap<sup>45</sup>. The Benjamini Horchberg (BH) adjustment<sup>46</sup> 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  $\text{N}_2$ ,  $\text{N}_2\text{O}$ , total denitrification ( $\text{N}_2 + \text{N}_2\text{O}$ ) and  $\text{CO}_2$  between soils within treatments and within soils between fertilization strategies. All values unless otherwise stated are given as mean  $\pm$  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 <sup>15</sup>N isotope analysis and C.M. analyzed the <sup>15</sup>N 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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