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Effect of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on N-turnover, the N_2O reductase-gene *nosZ* and N_2O:N_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_2O) from agricultural soils. However, their N_2O 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_2 and N_2O emissions with ^{15}N tracing analysis and the quantification of the N_2O-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_2O is responsible for large pulses of N_2O after the fertilization of agricultural soils. DMPP attenuated this effect only in the horticultural soil, reducing nitrification while increasing *nosZ* abundance. DMPP reduced N_2O emissions from the horticultural soil by >50% but did not affect overall $N_2 + N_2O$ losses, demonstrating the shift in the N_2O:N_2 ratio towards N_2 as a key mechanism of N_2O mitigation by NIs. Under non-limiting NO_3^- availability, the efficacy of NIs to mitigate N_2O emissions therefore depends on their ability to reduce the suppression of the N_2O reductase by high NO_3^- concentrations in the soil, enabling complete denitrification to N_2.

Agricultural soils have become the main source of anthropogenic nitrous oxide (N_2O), a powerful greenhouse gas and the single most important substance depleting stratospheric ozone[1]. Delaying the conversion of ammonium (NH_4^+) to nitrate (NO_3^-), nitrification inhibitors (NIs) have been suggested as a means to reduce N_2O emissions from agricultural soils. NIs demonstrated their efficacy across different cropping soils[2], but results vary widely, and in particular in pasture soils the use of NIs had no or little effect on N_2O emissions[3–5]. Despite a growing body of research on NIs, mechanisms and factors determining their efficacy to reduce N_2O emission remain poorly understood[6]. The challenges to understand these mechanisms derive from the fact that N_2O is formed via several different pathways in the soil matrix[7], tightly coupled to different processes of N supply and consumption[8]. Critically, N_2O can be further reduced to N_2 via the microbial-mediated process of denitrification, and the sole quantification of N_2O as affected by NIs provides therefore only a limited insight into mechanisms of N_2O mitigation using NIs.

Microbial metabolic pathways can contribute via a wealth of different processes to N_2O production and consumption, i.e. the reduction to N_2 in soils. Apart from abiotic processes, N_2O 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[9]. Denitrification is generally assumed to be the main process contributing to overall N_2O production from agricultural soils[7,10–12] 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.

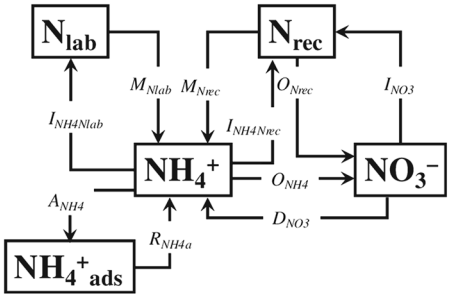
Most of the NIs inhibit the first and rate-limiting enzymatic step of nitrification, the conversion of NH_4^+ to hydroxylamine (NH_2OH) via the ammonia monooxygenase[13]. The inhibition of nitrification means a reduced supply of N into the NO_3^- pool as the source pool of denitrification, but also an increase in NH_4^+ availability, leading to an increase of fertilizer N immobilization[11] and mineralization/immobilization turnover rates[14,15]. 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[16]. 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[17], 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[5,11,18–20], 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 ^{15}N tracing analysis with the direct quantification of N_2 and N_2O emissions using the ^{15}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 ^{15}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_{rec}) dominated in the sandy CL. Gross nitrification (Nit_{tot}) was higher in the loam with 18.7 ± 0.03 μg N g^{-1} soil day^{-1} compared to 5.8 ± 0.03 μg N g^{-1} soil day^{-1} in the sandy CL. Autotrophic nitrification (O_{NH4}) 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_{NH4tot}) and NO_3^- (I_{NO3}) was higher in the sandy CL compared to the loam, and was dominated by I_{NO3}. 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_{NO3} in the ^{15}N tracing model), while D_{NO3} contributed with more than 2 μg 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).



	Transformation
O_{NH4}	Oxidation of NH_4^+ to NO_3^- -
O_{Nrec}	Oxidation of N_{rec} to NO_3^- -
I_{NO3}	Immobilisation of NO_3^- to N_{rec}
D_{NO3}	Dissimilatory NO_3^- reduction to NH_4^+
M_{Nrec}	Mineralisation of N_{rec} to NH_4^+
M_{Nlab}	Mineralisation of N_{lab} to NH_4^+
$I_{NH4Nlab}$	Immobilisation of NH_4^+ to N_{lab}
$I_{NH4Nrec}$	Immobilisation of NH_4^+ to N_{rec}
R_{NH4a}	Release of adsorbed NH_4^+ to NH_4^+
A_{NH4}	Adsorption of adsorbed NH_4^+ to NH_4^+

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 ± 0.03 and 1.46 ± 0.38 μg 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 ± 0.09 μg N_2 - N g^{-1} soil and 0.87 ± 0.11 μg 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 μg 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 μg 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 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 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 μg N g^{-1} soil day^{-1}		Sandy clay loam - Horticulture soil					Loam -Pasture soil				
		−DMPP		+DMPP		DMPP effect	−DMPP		+DMPP		DMPP effect
			↔		↔			↔		↔	
Mineralisation of N_{rec} to 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 NH_4^+ to N_{rec}	$I_{NH4\text{-}Nrec}$	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 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	−7%
Immobilisation of NH_4^+ to N_{lab}	$I_{NH4\text{-}Nlab}$	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 NO_3^-	O_{Nrec}	0.38 ± 0.11	a	0.34 ± 0.22	a		0		0		
Immobilisation of 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 NH_4^+ to 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	−6%
Dissimilatory NO_3^- reduction to 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 NH_4^+ to $NH_4^+{}_{ads}$	A_{NH4}	1.18 ± 0.22	a	0.87 ± 0.75	a		0		0		
Release of adsorbed NH_4^+ to 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	−6%
Total nitrification $O_{Nrec+}O_{NH4}$	Nit_{tot}	5.82 ± 0.30	c	2.39 ± 0.30*	d	−59%	18.64 ± 0.24	a	17.46 ± 0.37*	b	−6%
Total 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_{ntot}	45%		20%			69%		68%		
Contribution of O_{NH4} to Nit_{tot}	O_{NH4}/Nit_{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 NH_4NO_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[21], 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 N_2O in both soils was denitrification, accounting for more than 90% of N_2O produced (Fig. 2), which is in line with previous results from both field[11] and laboratory studies[10,12]. The ability of soils to act as an N_2O sink, i.e. the trait to reduce N_2O to N_2 has been linked to the abundance of *nosZ*, used as proxy for microorganisms involved in the reduction of N_2O. In the study presented here, we compared *nosZ* abundance with direct measurements of N_2 and N_2O, evaluating the influence of DMPP on of microorganisms reducing N_2O. 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[22]. The synthesis of the N_2O reductase is promoted by anoxic conditions[23], 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 NO_3^- availability due to fertilization and nitrification promoted the reduction of NO_3^- rather than N_2O, shifting the $N_2O_d/(N_2O_d + N_2)$ ratio towards N_2O. The magnitude and N_2O:N_2 partitioning of denitrification losses is consistent with the nitrification rates in both soils and as such shows the $N_2O_d/(N_2O_d + N_2)$ ratio as a function of soil intrinsic N – turnover. Cumulative N_2O_d losses of >2 μg N g^{-1} soil and 75% of denitrification ($N_2O_d + N_2$) emitted as N_2O from the loam show increased substrate availability for denitrification and simultaneous suppression of *nosZ* abundance by high NO_3^- availability (Fig. 2). In turn, lower denitrification losses with only 36% emitted as N_2O_d reflect slower N turnover in the sandy CL. These findings suggest that the suppression of the N_2O reductase and increased N substrate availability are responsible for the large pulses of N_2O from agricultural soils observed after fertilization and irrigation. Our results denote an increased risk of N_2O loss from highly productive agricultural soils[19], where increased mineralization of soil organic N due to fertilization, i.e., priming is likely to amplify the preferential reduction of NO_3^-, and as such the production of N_2O via denitrification.

DMPP reduced N_2O 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 N_2O emissions from the loam. In both soils, N_2O derived from nitrification mediated pathways accounted for less than 15% of overall N_2O, showing no response to the DMPP treatment. For the sandy CL, this suggests that DMPP primarily affected N_2O production pathways indirectly, that is by reducing NO_3^- availability for denitrification, demonstrated by the reduction of N_2O 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 N_2 measurements, this effect has been interpreted as a shift of denitrification losses towards N_2[24]. Experimental evidence linking increased *nosZ* abundance with DMPP to N_2 and N_2O emissions[25] is based on the acetylene inhibition method, which has been shown to lead to an irreproducible underestimation of denitrification rates[9]. Furthermore, acetylene itself is a potent NI, questioning the use of this method when investigating the effects of NIs on the magnitude and the $N_2O_d/(N_2O_d + N_2)$ ratio

		time		Sandy clay loam Horticulture soil	↓	Loam Pasture soil	↓	↔
NH_4^+	μg N g^{-1} 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	*
			DMPP effect	**+42%**		**+223%**		
NO_3^-	μg N g^{-1} 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	*
			DMPP effect	**−12%**		—		
DOC	μg C g^{-1} 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	*
			DMPP effect	**+50%**		**+32%**		
Microbial C	μg C_{mic} g^{-1} soil	0	prior fertilization	93.6 ± 18.7	a 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	*
			DMPP effect	—		—		
Microbial N	μg N_{mic} g^{-1} 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	*
			DMPP effect	—		—		

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 ^{15}N gas flux method. In contrast to previous incubation studies investigating N_2O:N_2 partitioning in response to DMPP[26,27], 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 he observed inefficacy of DMPP to reduce autotrophic nitrification in the loam, which is consistent with reports from other pasture soils[15]. 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[28,29]. 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[11]. 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 ($I_{NH4rec} + I_{NH4lab}$) by a factor > 2 (Table 2). Increased mineralization/immobilization turnover has been reported after the application of DMPP[15] and dicyandiamide (DCD)[14] and can be attributed to higher NH_4^+ availability, stimulating microbial immobilization of NH_4 (I_{NH4lab}) 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[30]. Interestingly, DMPP increased DOC availability in both soils, confirming previous results from a wheat-maize cropping system[31] (Table 3). Increased M_{Nrec} in the sandy CL indicates mineralization of

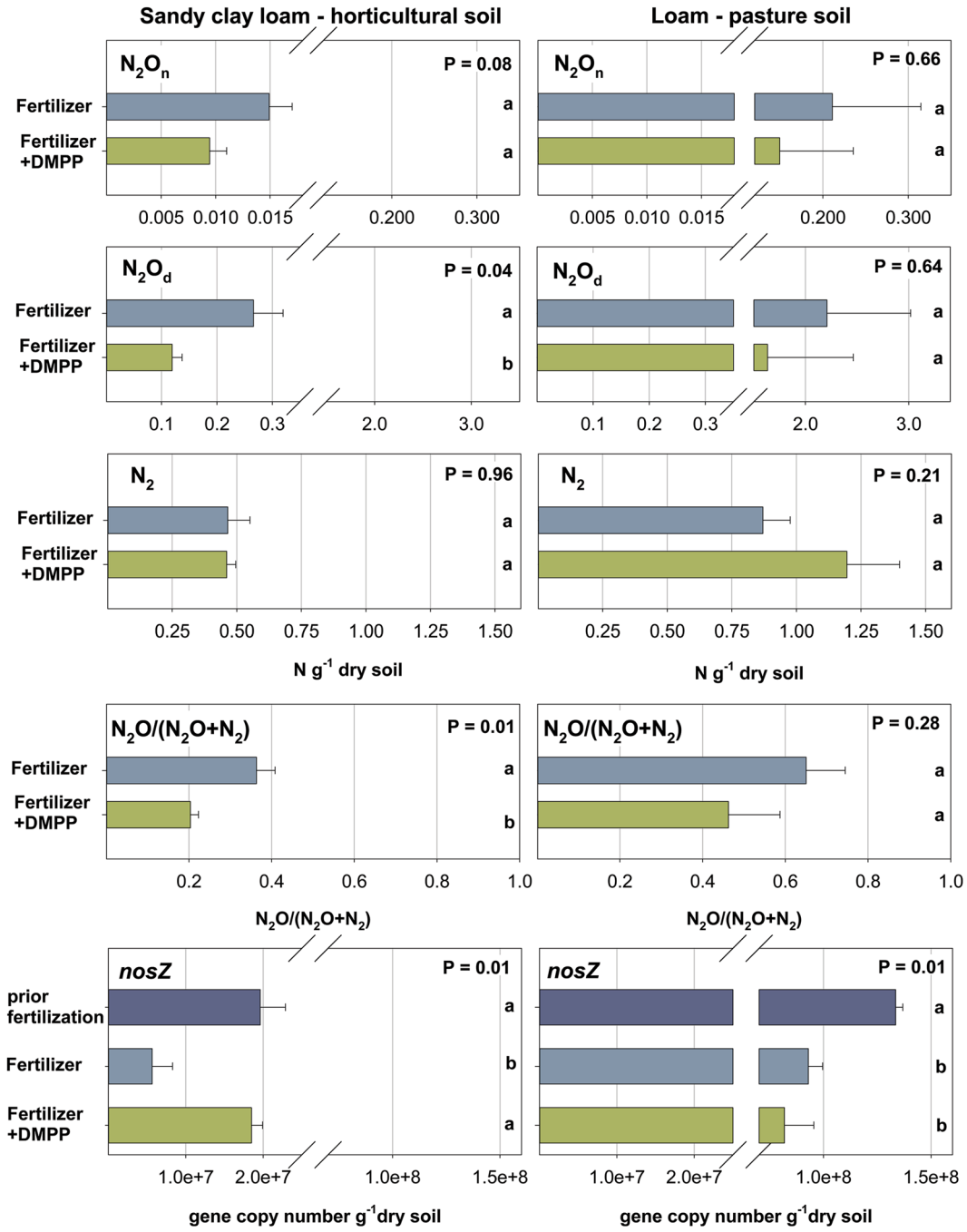


Figure 2. Cumulative emissions of N_2O derived from nitrification (N_2O_n) and denitrification (N_2O_d), cumulative N_2 emissions, the product ratio of denitrification ($N_2O/(N_2O_d + N_2)$) and the abundance of the *nosZ* gene encoding the N_2O reductase from a horticultural soil (Sandy clay loam) 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[10,23]. 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[32], 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[23]. 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	$\mu g\ N_2 + N_2O_d$ - N g^{-1} soil	0.73 ± 0.13	3.08 ± 0.87	P = 0.04	0.58 ± 0.05	2.83 ± 1.02	P = 0.07	P = 0.32	P = 0.86
N_2 emissions	$\mu g\ N_2$ - N g^{-1} soil	0.47 ± 0.09	0.87 ± 0.11	P = 0.03	0.46 ± 0.04	1.20 ± 0.20	P = 0.04	P = 0.96	P = 0.21
N_2O emissions	$\mu g\ N_2O$ - N g^{-1} soil	0.24 ± 0.03	1.46 ± 0.38	P = 0.01	0.14 ± 0.02	1.80 ± 0.52	P = 0.01	**−54%** /P = 0.01	P = 0.60
CO_2 emissions	$\mu g\ CO_2$ - C g^{-1} soil	6.55 ± 0.52	44.66 ± 1.73	P = 0.01	5.99 ± 0.18	46.27 ± 1.35	P < 0.01	P = 0.35	P = 0.47

Table 4. Cumulative emissions of N_2, N_2O and CO_2 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.

Nitrification activity during pre-incubation increased NO_3^- levels in both soils. In the loam, NO_3^- levels were above those measured at the respective field site, which is also reflected in higher $N_2O_d/(N_2O_d + N_2)$ ratios[11]. This phenomenon often occurs in incubation studies, where the absence of plant uptake, pre-incubation[33,34], and the addition of glucose[26] increases NO_3^- levels in the soil. It is therefore important to consider N substrate availability when interpreting the effects of NIs on rate and N_2O: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 NO_3^- availability to a degree that limited preferential reduction of NO_3^-. The high initial NO_3^- values in the loam are also likely to have overwritten a significant reduction of nitrification. The reduction of N_2O emissions, together with the increase of *nosZ* abundance in the sandy CL suggests however that DMPP lowered NO_3- availability below a soil specific treshold[35], limiting the preferential reduction of NO_3^- over N_2O. The results from the sandy CL confirm the proposed mechanism of N_2O reduction via a shift in the N_2:N_2O ratio[26], and show that DMPPs inhibitory effect on nitrification can limit the suppression of the N_2O reductase, promoting complete denitrification to N_2.

The demonstrated link between *nosZ* and directly measured N_2O and 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 N_2O 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 N_2O emissions reflects cumulative losses observed from the same soils in the field, demonstrating a larger N_2O 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 DMMP prior to fertilization may increase DMPPs efficacy, limiting the effect of N fertilizer priming on N_2O emissions. Decreased *nosZ* abundance after fertilization and irrigation indicates suppression of the N_2O reductase by increased NO_3^- availability, identifying NO_3^- availability as the control for the reduction of NO_3^- vs. N_2O, which determines the magnitude of N_2O losses. These findings apply to conditions of non-limiting 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 N_2O emissions depends on their ability to limit the suppression of the N_2O reductase by high NO_3^- concentrations in the soil, enabling complete denitrification to 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)[20] and an intensively managed dairy pasture (Gympie, Qld)[11] 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 (NH_4NO_3) and NH_4NO_3 with DMPP (DMPP), each with four different ^{15}N label combinations and four replicates. The NH_4NO_3 was applied with either (a) the NH_4^+ ($^{15}NH_4NO_3^-$) or (b) the NO_3^- ($NH_4{}^{15}NO_3^-$) labeled at 10 atom %. $NH_4{}^{15}NO_3^-$ at 60 atom % (c) was used to quantify N_2 emissions[36], while non-labeled NH_4NO_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 g cm^{-3}. NH_4NO_3 equivalent to 35 $\mu g\ N\ g^{-1}$ 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 ^{15}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 °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 NH_4^+ and NO_3^- were determined using colorimetric methods, NH_4^+ with a modified indophenol reaction[37] and NO_3^- with the VCL3/Griess assay[38]. The ^{15}N enrichments of the NH_4^+ and NO_3^- pool were determined for soil microcosms a and b by the diffusion method[39].

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 µl, and the assay mixture contained GoTaq® qPCR Master Mix (Promega, USA), 10 µM of each *nosZ* primer[40] and 1 µl of pooled template DNA. Thermal cycling conditions for the nosZ2F (CGCRACGGCAASAAGGTSMSSGT) and *nos*Z2R (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_{mic}) and N (N_{mic}) were quantified before and two days after fertilizer application using the chloroform fumigation-extraction[41]. 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_{mic} and N_{mic} were calculated as the difference in N and C between fumigated and non-fumigated samples without using a correction factor[42]. 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 N_2O concentrations. Specific background samples were taken above the respective soil microcosms treated with $NH_4{}^{15}NO_3$ at 60 atom % (c) for $^{15}N_2$ 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[10]. 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 N_2O and CO_2 analysis by gas chromatography (Shimadzu GC-2014). Gas samples from soil microcosms c were also analyzed for the isotopologues of N_2 ($^{15}N^{14}N$, $^{15}N^{15}N$) and N_2O ($[^{14}N^{15}N^{16}O + {}^{15}N^{14}N^{16}O]$ and $^{15}N^{15}N^{16}O$) using an automated isotope ratio mass spectrometer (IRMS) coupled to a trace gas preparation unit (Sercon Limited, 20–20, UK).

Fluxes of N_2, N_2O and CO_2. The triple labelling approach generates gas samples from three ^{15}N fertilizer treatments with four replicates: a,b and c. Cumulative N_2O and CO_2 fluxes given in Table 4, were calculated based on gas samples from ^{15}N fertilizer treatments a,b and c. Fluxes of N_2 and N_2O_d, as well as denitrification losses ($N_2 + N_2O_d$), were calculated based on the gas samples from treatment c. Calculating cumulative N_2O fluxes based on ^{15}N fertilizer treatments a, b and c or c alone did not result in significant differences. The reduction of N_2O by DMPP in the sandy CL was significant regardless of the calculation chosen.

The flux rates of N_2O and 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[20]. The ^{15}N enrichment of the NO_3^- pool undergoing denitrification (a_p) and the fraction of N_2 and N_2O emitted from this pool (f_p) were calculated following the equations given by Spott, *et al.*[43] detailed in the supplementary material. The headspace concentrations of N_2O and N_2 were multiplied by the respective f_p values giving N_2 and N_2O produced via denitrification (referred to as N_2 and N_2O_d), with their respective fluxes expressed in g N_2 or N_2O_d –N emitted g^{-1} soil day^{-1}. Potential hybrid formation of N_2 and N_2O was found to be irrelevant[30]. The precision of the IRMS for N_2 based on the standard deviation of atmospheric air samples (n = 18) at 95% confidence interval was 4.4×10^{-7} and 6.0×10^{-7} for ^{29}R and ^{30}R, respectively. The corresponding method detection limit ranged from 0.005 µg N_2-N g^{-1} soil with a_p assumed at 50 atom % to 0.014 µg N_2-N g^{-1} soil with a_p assumed at 20 atom %.

Gross N transformations. Gross N transformations were quantified using a ^{15}N tracing model[44] (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 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^+

(M_{nlab}, M_{Nrec}), the immobilization of NH_4^+ to N_{lab} and N_{rec} ($I_{NH4\text{-}Nrec}$, $I_{NH4\text{-}Nlab}$), the adsorption (A_{NH4}) and release (R_{NH4a}) of NH_4^+ from $NH_4^+{}_{ads}$, the oxidation of NH_4^+ to NO_3^- (O_{NH4}), referred to as autotrophic nitrification; the oxidation of N_{rec} to NO_3^- (O_{Nrec}), referred to as heterotrophic nitrification; dissimilatory NO_3^- reduction to NH_4^+ (D_{NO3}) and I_{NO3}, the immobilisation of 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 NH_4^+ as the sum of $I_{NH4\text{-}Nrec}$ and $I_{NH4\text{-}Nlab}$.

Calculations and statistical analysis. The optimization routine used for the ^{15}N tracing model gives a probability density function for each model parameter, which is used to calculated 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[45]. The Benjamini Horchberg (BH) adjustment[46] 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 N_2, N_2O, total denitrification ($N_2 + N_2O$) and CO_2 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 ^{15}N isotope analysis and C.M. analyzed the ^{15}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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