Degradation of the Nitrification Inhibitor 3,4-Dimethylpyrazole Phosphate (DMPP) in Soils: Indication of Chemical Pathways

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Abstract

Nitrogen fertilizers amended with nitrification inhibitors (NIs) are used to increase nitrogen use efficiencies in agricultural systems. 3,4-Dimethylpyrazole phosphate (DMPP) is the most successful commercial NI to date but has a highly variable efficacy. To explore whether degradation could contribute to its inconsistent performance, incubation studies were performed with DMPP and 3,4-dimethylpyrazole glycolate (DMPG) in two alkaline clay soils that were treated with the fertilizer ammonium sulfate ($(NH_4)_2SO_4$). Analysis of the soil extracts revealed a qualitative correlation between the amount of NI present in the soil and inhibition efficiency as well as several degradation products resulting from oxidation of a methyl side chain and dimerization. A similar outcome was obtained for the degradation in sterilized soil and in accelerated weathering studies in the absence of soil. Our data suggest that chemical and not microbiological pathways are primarily responsible for the degradation of this inhibitor, which could potentially be initiated by reactive oxygen species (ROS) resulting from both biotic and abiotic processes in soils.

Keywords

Accelerated Weathering; Degradation; 3,4-Dimethylpyrazole Phosphate; Nitrification Inhibitor; Soil Incubation; Soil Extraction; Product Study

Introduction

Providing food for a constantly increasing world population has become a major challenge for society. To ensure adequate food resources by 2050, it is expected that the annual crop production needs to increase by almost 40%.[1] Nitrogen (N) fertilization is a common practice to maximize crop yield in agricultural systems.[2,3] However, globally the N use efficiencies (NUEs) have only remained at around 50% since the 1980's.[4,5] Two important pathways responsible for the undesired loss of N from the plant/soil system include leaching of nitrate (NO_3^-), which causes damaging eutrophication of surface waters and groundwater pollution, and nitrification-denitrification processes, which can result in emission of nitrous oxide (N_2O), a potent greenhouse gas, into the atmosphere.

One of the current approaches to reduce these N losses is by increasing NUEs through the use of fertilizers amended with nitrification inhibitors (NIs). These compounds are designed to increase the residence time of ammonium (NH_4^+) by inhibiting nitrifying microbes in the soil that are responsible for the $NH_4^+ \rightarrow NO_3^-$ conversion. 3,4-Dimethylpyrazole phosphate (DMPP or ENTEC®, BASF Ag, Figure 1) is the most promising commercial NI to date, which has undergone extensive toxicological testing and is effective at low concentrations of 0.5 – 1.5 kg ha^{-1}.[6] The active ingredient is 3,4-dimethyl-1H-pyrazole (DMP), which is applied as the water-soluble phosphate salt to increase its lifetime in soils by reducing both volatility and mobility due to the positive charge.[6] Similarly, in 3,4-dimethylpyrazole glycolate (DMPG or eNpower™ 18:20, Incitec Pivot Fertilizers Ltd.) glycolic acid is used to protonate the DMP core. Other approaches to lower the volatility involve derivatization with succinic acid to form a mixture of isomeric 2-(*N*-3,4-dimethylpyrazole)succinic acid and 2-(*N*-4,5-dimethylpyrazole)succinic acid (DMPSA, not shown),[7] but this product is currently not used commercially.

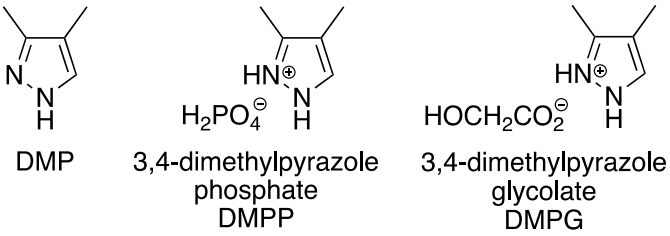


Figure 1. The nitrification inhibitor 3,4-dimethyl-1H-pyrazole (DMP) and commercial formulations DMPP and DMPG.

It is believed that DMP inhibits the first step of the ammonia oxidation process, which is catalyzed by ammonia monooxygenase (AMO).[8] While detailed studies of the mode of action of this membrane-bound enzyme are not possible, as its isolation results in loss of its structural organization and function, it is known that copper (Cu), and possibly also non-heme iron (Fe), are involved in the enzymatic functionality, indicating that DMP might inhibit AMO through (reversible) complexation of the Cu center via its adjacent N atoms in the pyrazole ring.[9]

Unfortunately, field studies with DMPP have shown highly unreliable inhibitory activity with regards to reducing NO_3^- leaching and N_2O emissions, ranging from no effect to inhibition as high as 90%.[10,11,12] Furthermore, the inhibitory activity of DMPP varies greatly with soil type,[13] moisture content,[14,15] soil pH,[15] soil temperature[16,17] and mode of application[18] for reasons not well understood. A possible rationale for the inconsistent performance of DMPP could be due to the varying stability of this molecule, which may be influenced by different soil characteristics and environmental conditions. The few available studies on the fate of DMPP in soils focus on its loss by measuring the amount of DMPP over time, revealing that the decrease in DMPP concentration can be described by first-order kinetics.[19,20,21] Apart from leaching,[22,23] microbiological degradation[20,24] has been proposed as a loss pathway for DMPP, however, so far products resulting from such degradation that would support this suggestion have not been identified. It was further shown that with increasing temperature mineralisation

of DMPP also increases.[25] Other factors affecting the stability of DMPP are heavy metal ions in soils, such as Cu^{2+} or Cd^{2+}, which, when present at a low concentration, lead to an increase of the DMPP degradation rate. This finding was rationalized by the stimulating effect that both Cu^{2+} or Cd^{2+} in low concentrations have on microorganisms.[25] On the other hand, DMPP decomposition was retarded at higher concentration of these heavy metal ions, possibly due to their biotoxicity.[25] While it is important that synthetic NIs applied onto soils should eventually decompose to products that are environmentally non-problematic, the loss of inhibitory function through premature degradation in soils during the cropping period is highly undesirable. Furthermore, as NIs are commonly supplied as coatings in commercial fertilizer granules, the usual application through top-dressing at the start of the cropping season, for example in cereal systems, exposes NIs to considerable environmental stress for some time, such as light, moisture and environmental pollutants. Identification of structural motifs in DMP (and in its formulations DMPP/G) vulnerable to degradation in soils and in the atmospheric environment is essential to enable the development of more efficient NIs in the future.

In this work we have performed soil incubation studies in both non-sterilized and sterilized soil to investigate the degradation of DMP in the two formulations DMPP and DMPG (the abbreviation DMP(P/G) will be used here for all three compounds). The experiments were conducted under 'forced' conditions, an approach used in drug discovery to test compound stability,[26] which enabled formation of products in sufficient amount to allow their isolation by high-performance liquid chromatography (HPLC) and identification by high-resolution mass spectrometry (HRMS), as well as nuclear magnetic resonance (NMR) spectroscopy. An improved procedure was developed for the extraction of DMPP/G from soils with excellent recovery rates, allowing quantification with high reproducibility. In addition, accelerated weathering studies in the absence of soils, which mimics exposure of DMP(P/G) to the

environment after top-dressed application, were performed to investigate chemical degradation products formed upon exposure to UV light at controlled temperature and humidity.

Materials and Methods

Chemicals

3,4-Dimethyl-1H-pyrazole (DMP; 98%) was provided by Incitec Pivot Ltd. 3,4-Dimethylpyrazole phosphate (DMPP) was prepared using DMP (98%, 10.00 g, 104.0 mmol) in phosphoric acid (85%, 47.14 g); actual [DMP] = 17.2% (w/w). 3,4-Dimethylpyrazole glycolate (DMPG) was prepared using DMP (98%, 4.96 g, 51.6 mmol) in glycolic acid (70%, 7.52 g) and sulfolane (7.52 g); actual [DMP] = 24.3% (w/w). 3-Methylpyrazole-4-carboxylic acid (97%) was obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia) and was used as received.

Soil Incubation Studies

The soils for the incubation studies were collected from the Wimmera region in Victoria, Australia: (i) a wheat cropping soil (clay, pH 8.8, organic carbon 0.73%) from Horsham (36º45' S, 142º07' E) and (ii) a winter cropping soil (clay, pH 8.4, organic carbon 1.90%) from Dooen (36°69' S, 142°24' E). The soils were sieved (2 mm) to remove any plant residues and stones and to increase homogeneity, followed by air-drying. The soil water content was determined by oven drying until a constant weight was reached. The required amount of water to achieve a water filled pore space (WFPS) of 60%[27] was calculated from the soil bulk density, which was determined from the volume of 20 g (dry weight) in 250 mL polypropylene containers (Sarstedt, Germany). The soil microorganisms were reactivated by rehydrating the soil with about half of the calculated water amount followed by incubation at the desired temperature (25°C or 35°C) for seven days.

After this pre-incubation period, the soil samples were treated with the fertiliser ammonium sulphate ($(NH_4)_2SO_4$) alone (control experiments) or in combination with the NI (DMPP or DMPG, respectively). An application rate of $(NH_4)_2SO_4$ of 100 mg N kg^{-1} of soil was used, while the NIs were applied at a rate of up to 500 mg kg^{-1} dry soil (corresponding to 73 mol% of fertiliser N). Each treatment was run in triplicate. Following addition of the remaining volume of water to achieve a WFPS of 60%, the treated soil samples were incubated at a temperature of either 25°C or 35°C for up to 63 days (no direct UV/vis exposure). Throughout the incubation period, soil samples were kept aerated (loosely placed lids for air exchange) and soil-moisture levels were maintained by the addition of H_2O based on weight loss every 3-4 days.

For the analysis of the NI concentration and water-soluble degradation products, soil samples were destructively sampled at chosen time points depending on the experiment (details are provided in Results and Discussion) using the optimized extraction methods, which will be described below. In the case of multiple analyses, subsampling was performed based on weight after thorough mixing of the soil samples. The extracts were stored at -20ºC until completion of the incubation test, when all samples were analysed.

Soil Extractions and Analyses

1. DMP: The extraction method was developed by modifying a previously published procedure.[28] Homogenized air-dried soil (20 g) was treated with $(NH_4)_2SO_4$ without or with amended DMPP/G. The DMPP/G solutions were prepared with active [DMP] in the range of 5.3 – 457.5 mg L^{-1}, corresponding to application rates of 0.8 – 68.6 mg kg^{-1} soil (0.12 – 10 mol% of fertilizer N). For the extraction of DMPP/G from the soil (which comprises the solid matrix and the soil solution), water (20 mL) and aqueous tribasic potassium phosphate (K_3PO_4) solution (1M, 0.4 mL) were added, and the samples were shaken for 2 hours, followed by

addition of aqueous calcium chloride solution (1M, 0.4 mL) and 1 hour of shaking. To convert DMPP/G back to uncharged DMP, aqueous sodium hydroxide (1M, 2 mL) was added, and the mixture was shaken for 30 mins. DMP was extracted with methyl *tert*-butyl ether (MTBE, 60 mL) after 1 hour of shaking, followed by centrifugation (5 minutes, 3000 rpm, 25°C). The samples were frozen at -20°C to separate the organic from the aqueous phase and the soil. The MTBE extracts were filtered (Whatman no 42) and stored at -20°C until analysis by HPLC.

The quantitative HPLC analysis was performed with an Agilent 1100 HPLC instrument with UV detector (at λ = 220 nm) and an ACE Excel Super C18 column (5 µm; 150 x 4.6 mm). A freshly prepared solution of aqueous phosphate buffer (pH 3; 788 mL) and methanol (212 mL) was used as the mobile phase, which was operated in isocratic mode with a flow rate of 1 mL min^{-1} at 40°C. The injection volume was 20 µL, and each sample was analysed at least three times. The instrument was calibrated for DMP on the day of the analysis using standard solutions, which were obtained by diluting a freshly prepared stock solution of DMP (100 µg mL^{-1}; prepared by dissolving 2.55 mg of DMP (98%) in 25 mL of the HPLC eluent). A calibration curve was obtained by measuring the peak area of the DMP signal in the HPLC chromatogram in the range 2.5 – 50 µg mL^{-1} DMP.

The DMP-containing MTBE extracts were analysed by pipetting 1 mL of the solution into a 15 mL polypropylene tube containing 0.2 mL of phosphate buffer. Where samples were too dilute to be detected/quantified, the amount of buffer was either reduced to 0.1 mL or measured quantities of the MTBE extract were added to 0.2 mL of buffer until HPLC detection was possible. The MTBE phase was evaporated *in vacuo* at constant temperature, pressure and time for all samples (usually 10 minutes), and the remaining aqueous solution was transferred into a micro-vial.

The recovery of DMP from the soil was calculated according to equation (1):

$$\text{DMP recovery (\%)} = \frac{\text{DMP extracted (mg kg}^{-1}\text{ dry soil)}}{\text{DMP applied (mg kg}^{-1}\text{ dry soil)}} \times 100 \qquad (1)$$

The concentrations of NH_4^+-N and NO_3^--N were determined by extracting the soil samples with an aqueous potassium chloride solution (2M, 50 mL KCl per 10 g dry soil) and shaking for 1 hour, followed by filtration (Whatman no. 42). The filtrates from each time-point were stored at -20°C until completion of the experiment, when all samples were analysed by Segmented Flow Analysis (San ++, Skalar, Breda, The Netherlands) after appropriate dilutions, as described previously.[29]

2. Water-Soluble Degradation Products: To 20 g of homogenized dry soil, which was treated with fertilizer and NIs as described above, milli-Q water was added (40 mL) and the sample was shaken for 1 hour, followed by sonication for 15 minutes to assist extraction of water-soluble degradation products that could be adsorbed on the soil matrix. The samples were centrifuged at 3000 rpm (3 minutes, 25°C), filtered through a paper filter (Whatman 42) and stored at -20°C. Before HPLC analysis, the samples were further purified by filtration through a 0.45 μm PTFE syringe filter.

The HPLC analysis was performed with the same C18 column as for the DMP extracts, using water/0.1% trifluoroacetic acid (TFA) as mobile phase A and acetonitrile/0.1% TFA as mobile phase B with an elution gradient of 2% B → 100% B in 20 min (holding time 3 min) → 2% B in 1.1 min (holding time 3 min) at a flow rate of 1 mL min^{-1} and 40°C.

Soil sterilization

To enable differentiation between chemical and microbiological degradation products, soils were sterilized by autoclaving using two cycles at a sterilizing temperature of 121.0°C for 20 minutes (Atherton Laboratory Sterilizer, Cyber series, model Jerboa).[30,31]

Accelerated Weathering Studies

Accelerated weathering was conducted in a Q-LAB QUV-se Accelerated Weathering Tester, which uses UVA-340 fluorescent lamps to emit UV radiation at wavelengths that simulate natural sunlight. The samples, typically ~2 mg NI (DMP, DMPP or DMPG, respectively) dissolved in 1 mL of aqueous $(NH_4)_2SO_4$ solution (3.14 g L^{-1}; comparable with the application rate of 100 mg N kg^{-1} used in the soil incubation studies) were exposed to weathering cycles comprising of 0.68 W m^{-2} UV irradiance for 8 h at a temperature of 50°C, followed by 4 h of condensing humidity. The reaction mixtures, which included unconsumed inhibitor and the degradation products, were directly analysed by HPLC using the same method as for the water-soluble degradation products, where collection of the fractions was also performed for identification by HRMS and, where possible, ^{1}H and ^{13}C NMR.

Results and Discussion

1. Optimization of the Extraction and Analytical Method

A major aspect of this work involved determination of the amount of DMP present in the soils over the duration of the incubation experiments. Currently, only two procedures have been reported on the quantification of soil DMP(P). Doran *et al.* developed a protocol to analyse the mobility of DMP in soils using liquid chromatography – tandem mass spectrometry (LC/MS-MS).[20] This highly sensitive and selective technique was designed to enable detection of the lowest levels of DMP in soils but requires synthesis of isotopically labelled internal standards for quantification. As the intention of this work was to investigate the chemical fate of DMPP/G in soils, a method was required that allowed for both quantification of unconsumed NI and extraction of the degradation products from the soil matrix for spectroscopic identification.

High-performance liquid chromatography (HPLC) is a well-established chromatographic method for the analysis, separation and quantification of compound mixtures using calibration standards. The recovery rates of the HPLC method reported in the literature varied between 50-80% for the concentrations tested (0.01 – 2 mg DMPP kg^{-1} dry soil).[28] Unfortunately, application of this method to the soils used in this study gave highly inconsistent recovery rates with large standard errors, particularly for concentrations < 1 mg DMP kg^{-1} dry soil. We therefore developed an improved procedure that provided consistent recovery values over the larger range of DMPP/G application rates that were used in this work.

The DMP quantification consisted of two steps, *i.e.,* (i) extraction of DMPP/G from the soil and conversion into DMP, and (ii) manipulation of the extracts for the HPLC analysis. The reported procedure[28] for the DMPP extraction uses 15 mL of methyl *tert*.-butyl ether (MTBE) per 10 g of dry soil. Using the clay soil from Horsham (pH 8.8) for the optimisation studies, it was found that for application rates of DMPP/G over a wide range of about 1 – 70 mg kg^{-1} dry soil, the recovery of DMP could be increased considerably from 65-75% to around 80-90% by doubling the amount of MTBE for the extraction. Details are outlined in the Materials and Methods section.

The subsequent preparation of the samples for the HPLC analysis was simplified. Rather than transferring the DMP-containing MTBE extracts into the actual HPLC eluent consisting of a mixture of phosphate buffer and methanol,[28] adding the MTBE phase to a defined amount of the buffer solution, followed by selective evaporation of MTBE *in vacuo* (under controlled temperature and pressure) gave the aqueous buffer solution containing DMP. This process avoided co-evaporation of some of the methanol, where samples with DMP dissolved in different amount of solvent are obtained that require subsequent weighing to determine the concentration.[28] Furthermore, reduction of the injection amount to 20 µL permitted repeat measurements to ensure consistency, contrary to the reported procedure,

where two third of the sample (100 of ~160 μL) was used for one HPLC run and repeat analyses could not be made.[28] Table 1 compiles the recovery data for DMP at different application rates using the optimised extraction and HPLC sample preparation method, clearly revealing the high consistency of the data and critical role of a higher amount of MTBE to maximise DMP recovery from the soil.

Table 1. Recovery rate of DMP from soil extractions (Horsham soil, pH 8.8; 10 g dry soil weight) using different amounts of MTBE.

DMP application rate (mg kg^{-1} dry soil)	**DMP recovery (%)**[a]		
	15 mL MTBE	**22.5 mL MTBE**	**30 mL MTBE**
0.8	~65[b]	n.d.[c]	81.9 ± 0.1
1.6	~65[b]	n.d.	83.3 ± 0.1
6.9	69.4 ± 0.7	89.2 ± 2.8	79.5 ± 2.9
34.3	69.2 ± 0.5	80.1 ± 0.5	84.6 ± 1.4
68.6	74.8 ± 0.9	67.4 ± 2.6	90.0 ± 1.5

[a]Determined by HPLC, using the optimised method; standard error from three replicates per concentration. [b]From ref. 28. [c]n.d. = not determined.

This extraction/analysis method worked similarly well with the clay soil from Dooen (pH 8.4) and also enabled extraction of DMP from an irrigated perennial dairy pasture soil (pH 6.8) from Casino, New South Wales (Australia), with recovery rates of around 77% at application rates of 1.3 – 3.8 mg kg^{-1} dry soil (see Table S1 in the Supplementary Information (SI)).

2. Soil Incubation Studies

Soil incubation studies in containers have the unique advantage over field studies as loss of DMP(P/G) must be caused by degradation (this includes mineralisation) since leaching cannot occur. In this work, the concentration-time profile for DMP was studied in soil incubation experiments in two clay soils with comparable characteristics; from Horsham (pH 8.8, moisture content 8.0%) and from Dooen (pH 8.4, moisture content 7.6%). The soils were treated with the fertilizer $(NH_4)_2SO_4$ and different concentrations of DMPP/G, depending on the experiment, which will be described in the following.

The concentration-time profiles of DMP obtained from 28-day incubation studies at 25°C in both soils using three different DMPP application rates of 6.9, 34.3 and 68.6 mg kg^{-1} dry soil (corresponding to 1 mol%, 5 mol% and 10 mol% of fertilizer N, respectively) are shown in Figure 2. DMP was recovered from the soil (*i.e.*, soil matrix and soil solution) using the optimized extraction procedure followed by HPLC analysis, as described in the previous section.

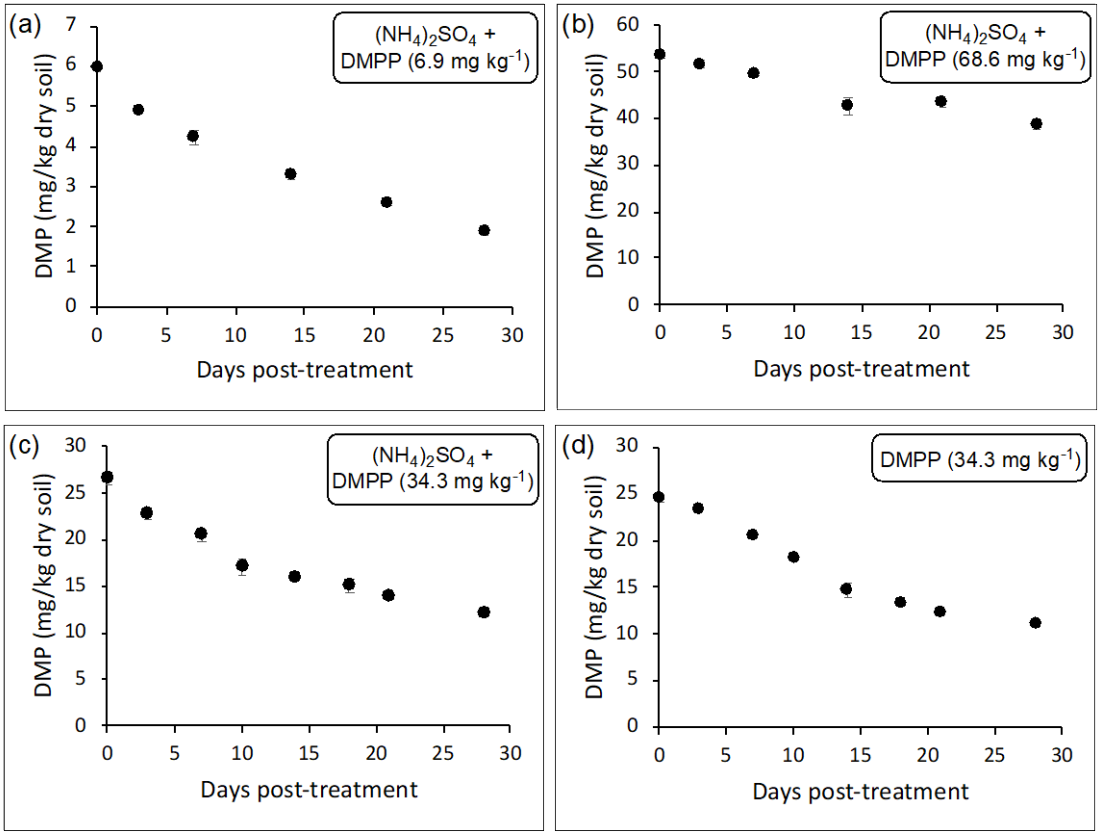


Figure 2. DMP concentration profiles over a 28-day incubation study at 25°C (trends are highlighted by variable Y-axis scales). (a) and (b): Horsham soil (clay, pH 8.8) with (a) $(NH_4)_2SO_4$ + DMPP (6.9 mg kg^{-1} dry soil; 1 mol% of fertilizer N) and (b) $(NH_4)_2SO_4$ + DMPP (68.6 mg kg^{-1} dry soil, 10 mol% of fertilizer N); extraction on day 0, 3, 7, 14, 21 and 28 post-treatment. (c) and (d): Dooen (clay, pH 8.4) with (c) $(NH_4)_2SO_4$ and DMPP (34.3 mg kg^{-1} dry soil; 5 mol% of fertilizer N) and (d) water and DMPP (34.3 mg kg^{-1} dry soil), no $(NH_4)_2SO_4$ (control); extraction on day 0, 3, 7, 10, 14, 18, 21 and 28 post-treatment. Mean values (n=3); errors are standard errors of the mean.

Interestingly, the graphs clearly reveal that the decrease of the DMP concentration over time depended on the application rate of the inhibitor, with considerably higher loss rates at lower concentrations. All decay profiles followed first-order kinetics, in agreement with literature,[19-21] which enabled determination of the rate coefficients, *k*, and half-lives, $t_{1/2}$.[34] The data are

provided in Figure S1 in the Supporting Information (SI). Thus, in the Horsham soil, only 30% of DMP was still present in the soil at the end of the incubation period when a DMPP application rate of 6.9 mg kg^{-1} soil was used (Figure 2a), corresponding to $k = 3.9 \times 10^{-2}$ day^{-1} and $t_{1/2}$ = 17.6 days. In the case of a 68.6 mg kg^{-1} application rate, the DMP concentration dropped to only about 72% over the same time span (Figure 2b; $k = 1.1 \times 10^{-2}$ day^{-1}, $t_{1/2}$ = 61.3 days). An analogous outcome was obtained for the Dooen soil, where $t_{1/2}$ for DMP was 25.1 days ($k = 2.8 \times 10^{-2}$ day^{-1}) following an application of 34.3 mg kg^{-1} soil (5 mol% of fertilizer N; Figure 2c). These data reveal that the residence time of DMP is very similar in these two alkaline clay soils, which is not unexpected given their similar characteristics and proximity of collection (see Materials and Methods). Figure 2d shows the profile for DMP for a control experiment, where the soil was treated only with DMPP (34.3 mg kg^{-1} soil) in the absence of fertilizer. The rate coefficient for the DMP decay of $k = 3.2 \times 10^{-2}$ day^{-1} ($t_{1/2}$ = 21.9 days) is close to that in the presence of $(NH_4)_2SO_4$ in Figure 2c, indicating that DMP loss occurs irrespective of whether the fertilizer is present in the soil or not. Furthermore, the formulation of the NI has no influence on the loss rate, as comparable DMP concentration-time profiles were obtained using DMPG under otherwise identical conditions to those in Figure 2c/d (the profile is shown in Figure S2). Overall, however, it is evident from these data that at higher application rates of DMPP/G, the absolute loss of the NI from the soil in terms of mass, *i.e.*, mg of DMP lost per kg of soil, is also higher.

It is worth highlighting that the loss of DMP from the soil, as shown in Figure 2, was found to qualitatively correlate with a reduction of inhibitory performance, as revealed by the loss of NH_4^+-N (Figure 3a and 3b) and accumulation of NO_3^--N (Figure 3c and 3d) under comparable conditions.[29]

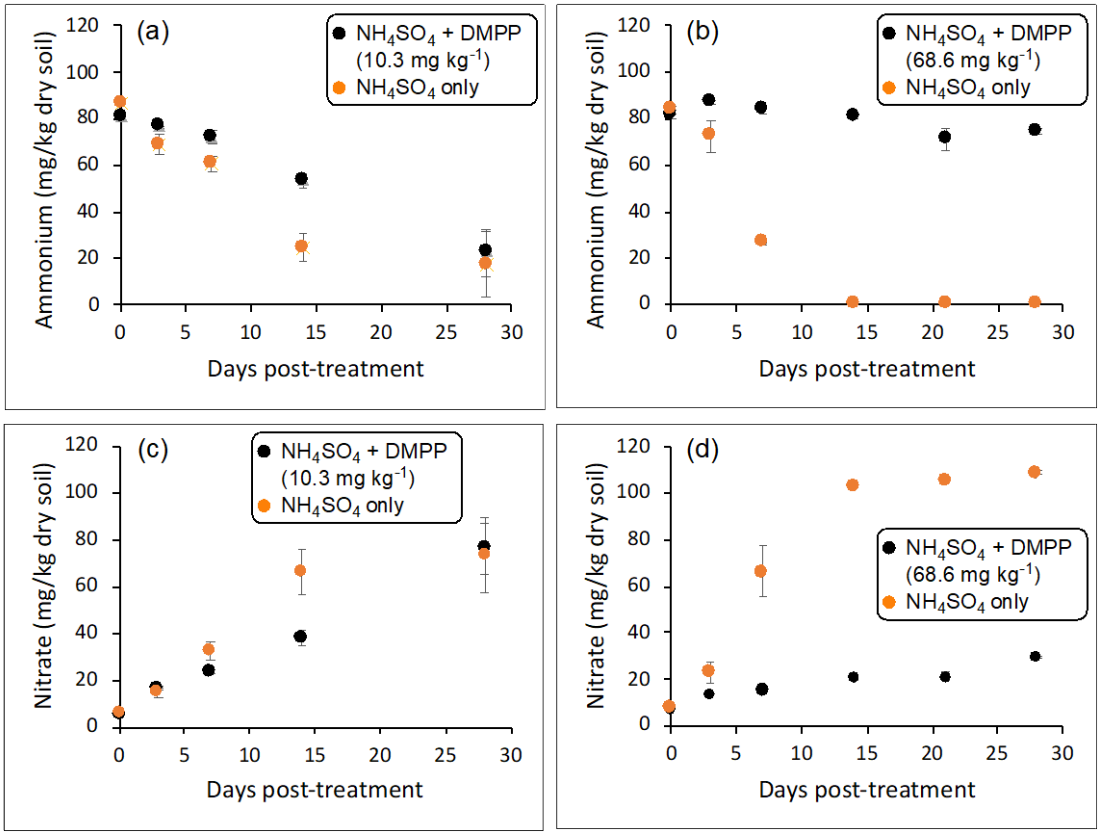


Figure 3. NH_4^+-N loss (a and b) and NO_3^--N accumulation (c and d) over 28 days in Horsham soil at 25°C for DMPP application rates of 10.3 and 68.6 mg kg^{-1} (1.5 mol% and 10 mol% of fertilizer N, respectively; black circles); extraction on day 0, 3, 7, 14, (21) and 28 post-treatment. In the control incubation experiments only the fertilizer was applied (orange circles). Mean values (n = 3); errors are standard errors of the mean.[28]

Compared with the control experiments, where only the fertilizer was applied without DMPP, in the presence of DMPP nitrification activity slowed down, as revealed by the rate of NH_4^+ loss and NO_3^- accumulation, particularly in the case of the higher DMPP application rate, where substantial amounts of the inhibitor were still present in the soil over the entire 28-day testing period.[29] On the other hand, for the given experimental conditions, at application rates below 10 mg kg^{-1} dry soil (<1.5 mol% of fertilizer N) the rate of DMP disappearance from the soil is so fast that no inhibition occurs after about 15 days.

The HPLC chromatograms of the soil extracts did not reveal, apart from DMP, any additional compounds, suggesting that products resulting from degradation of DMP were either not UV active or were water-soluble and therefore not extracted from the soil using the organic solvent MTBE. The soil samples were therefore extracted with water, followed by HPLC analysis (see Materials and Methods). Under the conditions of the experiments shown in Figure 2c with DMPP/G application rates of 34.3 mg kg^{-1} dry soil (5 mol% of fertilizer N) small amounts of products could be detected in the HPLC chromatogram, however, their concentration was too low to enable determination of their molecular masses by liquid chromatography mass spectrometry (LC/MS) analysis of the aqueous extract, and/or by HRMS following isolation by preparative HPLC and concentrating the samples.

We therefore performed soil incubation studies under forced conditions using a considerably higher application rate of DMPP/G of 500 mg kg^{-1} dry soil (corresponding to 73 mol% of fertilizer N) and an extended incubation time of 63 days at 25°C to generate degradation products in sufficient amount for isolation and characterisation by HRMS. Figure 4 shows the HPLC chromatogram of the aqueous soil extract for the treatment with DMPP, which was recorded at 220 nm. The chromatogram for the DMPG treatment is very similar and is shown in Figure S3.

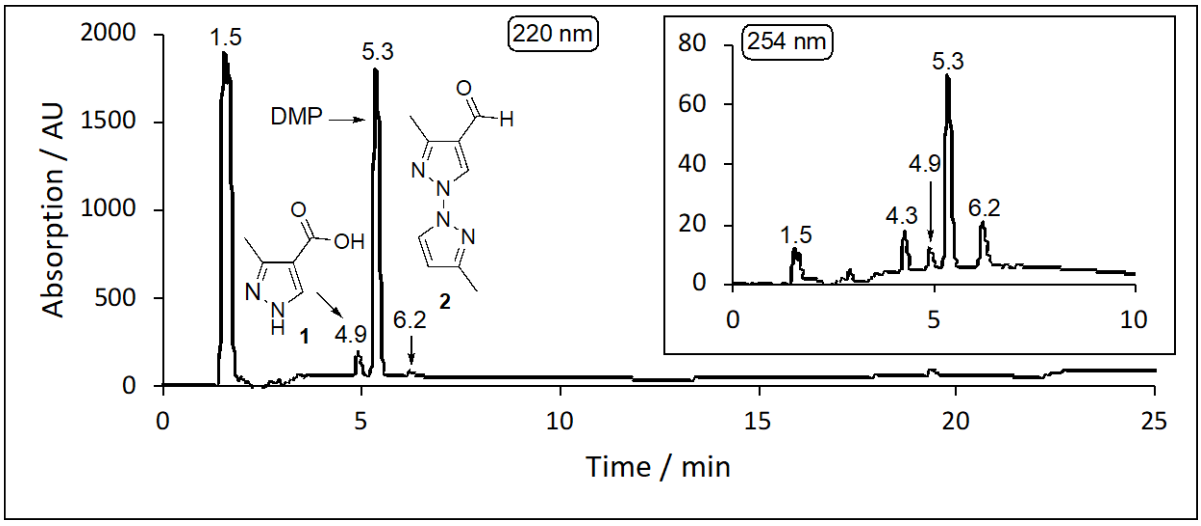
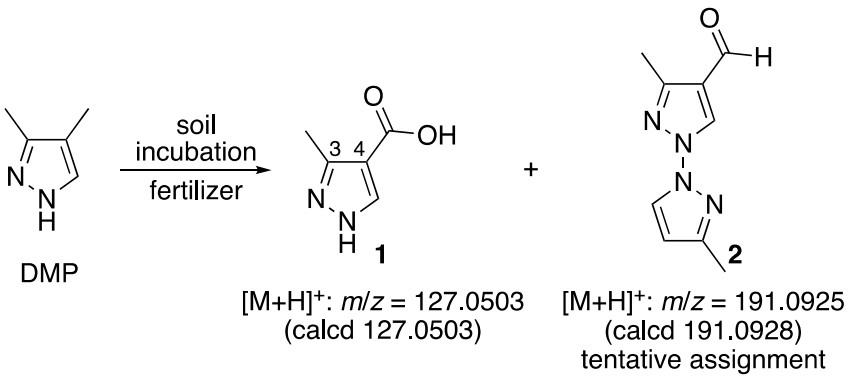


Figure 4. HPLC chromatogram of the aqueous soil extract (Dooen soil) for the treatment $(NH_4)_2SO_4$ and DMPP (500 mg kg^{-1} dry soil; 73 mol% of applied fertilizer N) after an incubation time of 63 days at 25°C, recorded at 220 nm. The numbers on the peaks are retention times (in minutes). The inset shows a section of the chromatogram recorded at 254 nm. Assignment of the peaks was performed through HRMS after separation of the products by HPLC.

The aqueous extracts from the experiments with high DMPP/G application rate showed the same peaks that were observed in the experiments with lower inhibitor application rates and no additional products, clearly indicating that the high inhibitor loading had no noticeable impact on the degradation process. The peak at 5.3 min can be assigned to unreacted DMP, which was extracted to some extent into the aqueous phase (the estimated recovery rate of DMP, which has a limited water-solubility, is approximately 50% under these conditions). The peak at *ca.* 1.5 mins was also observed in extracts of untreated soil and was therefore not further analysed. On the other hand, the peaks at 4.9 and 6.2 mins were only formed in soil incubations in the presence of DMPP/G. Their intensity gradually increased over the incubation time, suggesting that both could result from DMP degradation. The HPLC chromatogram recorded at 254 nm (see inset in Figure 4) revealed the same products with different signal intensities (due to their different absorption characteristics), in addition to a peak at 4.3 min. The intensity of the latter signal did not increase to a significant extent over the incubation period, suggesting that it likely does not result from DMP degradation. It should be noted that HPLC chromatograms recorded at 260 nm (not shown) did not reveal any additional peaks, indicating that no other products were formed in detectable amounts.

Separation of the products at 4.9 and 6.2 mins by preparative HPLC followed by HRMS analysis revealed a mass of m/z = 127.0503 for the product at 4.9 mins, which is in excellent

agreement with the molecular formula $C_5H_7N_2O_2$ ($[M+H]^+$: calcd. m/z = 127.0503), suggesting formation of the carboxylic acid derivative **1** (Scheme 1). This assignment, as well as the selective oxidation of the methyl group at C-4, was verified by comparison with an authentic sample (see below). Quantitative HPLC analysis for **1** provided a yield of about 8-10% with regards to consumed DMP as the lower limit (based on a DMP recovery rate during soil extraction with water of ~ 50%).



Scheme 1. Degradation products of DMP from soil incubation studies, according to HRMS analysis after purification by preparative HPLC.

The other product at 6.2 min has a mass of m/z = 191.0925, which supports a molecular formula of $C_9H_{10}N_4O$ ($[M+H]^+$: calcd m/z = 191.0928) and suggests formation of a dimer of DMP, in which one methyl group is oxidized to an aldehyde and one methyl group has been lost. The structure **2** shown in Scheme 1 for this dimeric product should be regarded as a tentative assignment only as further characterisation, for example by ^{1}H NMR, was not possible due to the small amount obtained. Comparison of the relative peak intensities in the HPLC chromatograms obtained for different DMPP/G application rates confirmed that formation of the dimeric product **2** is not an artefact of the high DMP concentration in the experiment shown in Figure 4 but also occurs at low inhibitor concentrations in the soil. A possible mechanism for the formation of the products will be outlined in section 4 below.

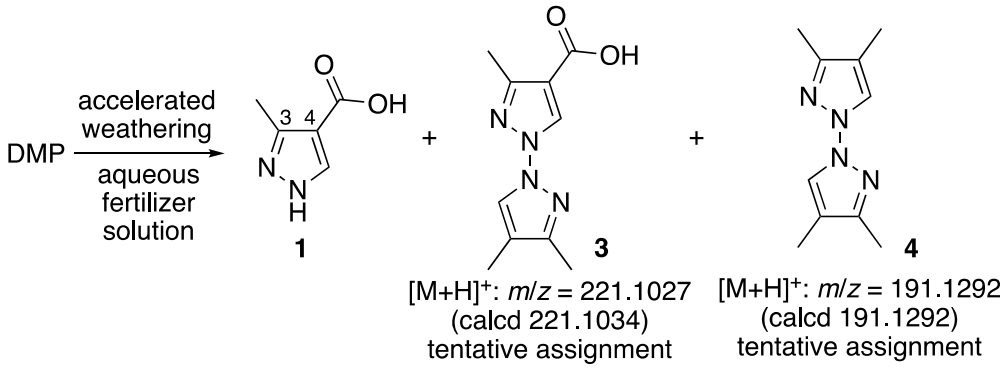
Since the active site of AMO is uncharacterized and details of the action of NIs on this enzymatic pathway are not known, it is not possible to unambiguously state whether the decomposition products of DMP are likely to have an inhibitory effect or not. However, given the decrease of inhibitory activity over the duration of the soil incubation, as revealed by the loss of NH_4^+ and accumulation of NO_3^- (see Figure 3) and which qualitatively correlates with the DMP concentration-time profile (see Figure 2), it is reasonable to suggest that both products **1** and **2**, despite their intact pyrazole framework, do not function as nitrification inhibitors, at least not in the soils explored in this study. Further work (outside the scope of the present study) is clearly required to assess whether unfavourable electrostatic interactions in the case of **1**, steric hindrance in the case of **2** or other factors are responsible for the lack of inhibitory activity of these compounds.

To investigate whether the degradation of DMP in soils to products **1** and **2** is due to chemical or microbiological processes, incubation studies were performed with sterilized Dooen soil (see: Materials and Methods). The experiments were conducted over 56 days using DMPG at an application rate of 500 mg kg^{-1} dry soil (73 mol% of fertilizer N) and at a temperature of 35°C to increase the rate of product formation. The HPLC chromatogram obtained after aqueous extraction is provided in Figure S4, alongside the control experiment using non-sterilized soil under otherwise identical conditions. Interestingly, the HPLC data and relative peak areas clearly reveal that in sterilized soil the same products were formed in a similar ratio as in non-sterilized soil, *i.e.*, the carboxylic acid **1** and the dimeric compound **2**. These findings provide a strong indication that, contrary to the perceived knowledge, DMP degradation in soils is caused by chemical processes and not by direct microbiological activity.

3. Accelerated Weathering Studies

Accelerated weathering is a commonly used technique in the coatings industry to investigate the durability of polymers in the environment on a short time scale.[35] The material is exposed to alternating cycles of UV light that simulates natural sunlight and moisture at controlled (usually elevated) temperatures. Water spray and/or condensing humidity are used to simulate rain and dew. In this work we have used this technique to explore the environmental degradation of DMP(P/G) in the absence of the soil matrix, which mimics exposure after topdressing the inhibitor-coated fertilizer.

Scheme 2 shows the products after 168 hours of accelerated weathering, where an aqueous fertilizer solution containing the inhibitor (*ca*. 2 mg DMP(P/G) mL^{-1}; see Materials and Methods) was exposed to a cycle of 0.68 W m^{-2} UV irradiance for 8 h at 50°C, followed by 4 h of condensing humidity (the HPLC chromatogram is shown in Figure S5). The same products were also formed at shorter exposure times, but in too low amounts to provide reliable HRMS data.



Scheme 2. Products resulting from accelerated weathering of DMP (assignment according to HRMS analysis after purification by preparative HPLC, formation of the carboxylic acid derivative **1** was also confirmed by ^{1}H NMR analysis). DMP(P/G) was dissolved in aqueous fertilizer solution (~ 2 mg mL^{-1}) and exposed to a weathering cycle of 0.68 W m^{-2} UV irradiance for 8 h at 50°C, followed by 4 h of condensing humidity; total exposure time 168 hours.

Similar to the soil incubation studies, the major product is the carboxylic acid derivative of DMP **1**. In addition to the HRMS data, we were also able to obtain the ^{1}H and ^{13}C NMR spectra for **1** from these experiments, which are in excellent agreement with those of an authentic sample. These data unequivocally confirmed the identity of this compound, in particular the selective oxidation of the methyl group at C-4 in DMP, and that formation of **1** does not require a soil environment. The two dimeric products **3** and **4** could only be tentatively assigned based on their HRMS data (^{1}H NMR spectra could not be obtained because of the low amounts formed): Product **3** has a mass of *m*/*z* = 221.1027, which supports a molecular formula of $C_{10}H_{12}N_4O_2$ ($[M+H]^+$: calcd *m*/*z* = 221.1034) and suggests a dimer of DMP and the carboxylic acid derivative **1**. The other product (**4**) has a mass of *m*/*z* = 191.1292, which is in accordance with the molecular formula $C_{10}H_{14}N_4$ ($[M+H]^+$: calcd *m*/*z* = 191.1292) and could be assigned to dimeric DMP. It was confirmed that the same products were formed during accelerated weathering of DMP, DMPP and DMPG, supporting the findings from the soil incubations that the formulation does not alter the degradation pathways of this nitrification inhibitor.

4. Mechanistic considerations

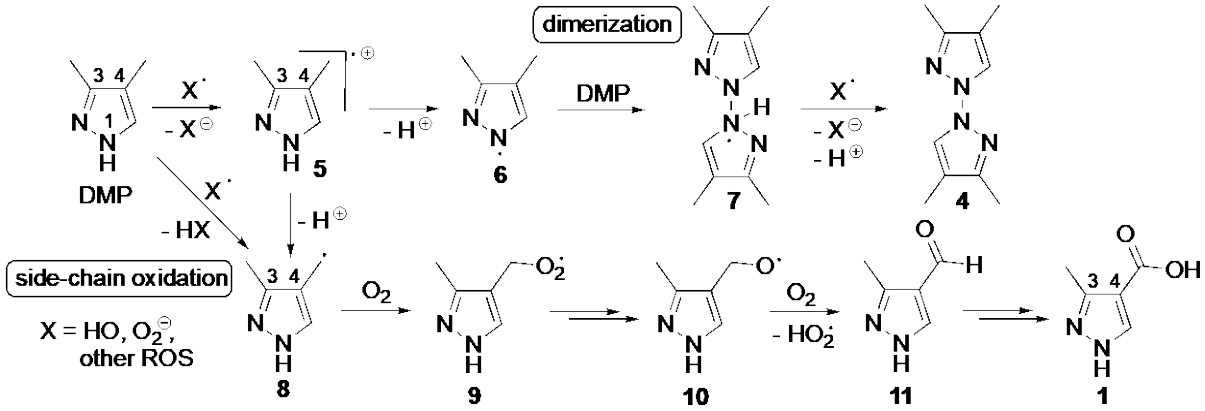
The closely related structures or even identical products formed during accelerated weathering of DMP(P/G) and in the incubation studies using both non-sterilized and sterilized soil provide additional support for the suggestion that these products result from chemical rather than direct microbiological degradation. In fact, the proposed side-chain oxidation in DMP to an aldehyde in dimer **2** (see Scheme 1) or to a carboxylic acid in products **1** and **3** (see Scheme 2), as well formation of dimeric products suggests a mechanism involving free radical species. Although free radicals, or more generally reactive oxygen species (ROS) are ubiquitous in soils, their role in chemical transformations in soils has only recently received attention.[36,37,38] For example, oxidase enzymes metabolically produce hydrogen peroxide (H_2O_2) that can be

transformed by iron (oxyhydr)oxides, which are typical soil minerals, into highly reactive hydroxyl radicals (HO$^{\bullet}$) through the Fenton reaction, according to equation (2):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet} \qquad (2)$$

The Fenton reaction is not restricted to Fe^{2+},[37] and is therefore not unreasonable to propose that the diminished inhibitory activity of DMPP in soils in the presence of low concentrations of copper or cadmium ions reported in the literature[25] could be due to ROS mediated degradation. On the other hand, the increased stability of DMPP at higher concentrations of these heavy metal ions[25] could be due to their toxicity for microorganisms and a decrease in the production of ROS precursors. Interestingly, ROS could also be generated through abiotic soil processes, for example through UV irradiation of biochar, a product resulting from biomass pyrolysis, which could produce superoxide ($O_2^{\bullet-}$, a precursor of H_2O_2) through a photocatalytic reaction in soils exposed to sunlight.[36] Another abiotic source of $O_2^{\bullet-}$ proceeds through photoinduced electron transfer (PET) between transition metal oxides in soils and surface-oxygen.[36] It can be assumed that the various ROS precursors should, at least to a certain extent, resist the temperature in the autoclave required for soil sterilization,[39] so that DMP continues to undergo degradation even in microorganism-free soils, as observed in this study.

A tentative mechanism for the side-chain oxidation and dimerization of DMP mediated by ROS (generalized as X$^{\bullet}$) is outlined in Scheme 3.



Scheme 3. Proposed mechanism for the ROS-induced transformation of DMP to the carboxylic acid derivative **1** and the dimer **4**.

Reaction of DMP could occur through oxidation of the aromatic ring by X•, leading to the radical cation **5**, which could subsequently deprotonate to give either the N-centered heteroarene radical **6** or the 'benzylic' radical **8**. The N-radical **6** is an electrophilic species that could subsequently undergo addition to one of the nucleophilic sites in DMP, for example N-1,[40,41] to give the dimeric radical intermediate **7**, which, after a second oxidation and deprotonation step, leads to the DMP dimer **4**. The radical intermediate **8** could also be formed directly from DMP through hydrogen abstraction by X•. As ROS are usually electrophilic species, the observed regioselective reaction with the methyl group at C-4 could be rationalised by the higher electron density at that site, compared with that at C-3.[40] In soil, **8** would be expected to react with residual oxygen to produce the peroxyl radical **9**. Subsequent transformations, possibly via an intermediate alkoxyl radical **10**, produces aldehyde **11**, which could be further oxidized to a carboxylic acid through both ionic as well as radical processes.[42]

To conclude, our experimental data from soil incubation and accelerated weathering studies suggest that DMPP/G undergoes degradation in soil through chemical reaction steps, potentially involving ROS generated through abiotic and/or biotic processes. Thus, in the latter

case, rather than directly, microorganisms would only be indirectly responsible for the inhibitor degradation, for example by providing the ROS precursor to the soil matrix. Despite its hypothetical nature at this stage, the mechanism proposed in Scheme 3 could provide useful guidelines for future mechanistic studies of inhibitor transformations in soils. Our suggestion that abiotic degradation pathways via soil ROS could be an important factor contributing to the highly variable inhibitory performance of DMPP in the field is supported by previous findings that soil organic matter stabilizes ROS[39] and the observation that DMPP is less effective in soils with a high clay/organic matter content.[21,43] Further investigations of the role of different soil properties, such as organic matter, water content, mineral composition, texture and pH as well as temperature, on the degradation of DMP and other NIs are therefore required to enable the development of more efficient inhibitors that are needed to increase NUEs in agricultural systems and to reduce the adverse environmental impact of N fertilization.

Abbreviations Used

AMO – ammonia monooxygenase

DMP – 3,4-dimethyl-1H-pyrazole

DMPG – 3,4-dimethylpyrazole glycolate

DMPP – 3,4-dimethylpyrazole phosphate

DMPP/G – 3,4-dimethylpyrazole phosphate/glycolate

DMP(P/G) – 3,4-dimethylpyrazole (phosphate/glycolate)

HPLC – high-performance liquid chromatography

HRMS – high-resolution mass spectrometry

LC/MS – liquid chromatography – mass spectrometry

LC/MS-MS – liquid chromatography – tandem mass spectrometry

MTBE – methyl *tert*-butyl ether

NI – nitrification inhibitor

NUE – nitrogen use efficiency

PET – photoinduced electron transfer

PTFE – polytetrafluoroethylene

ROS – reactive oxygen species

Rpm – rounds per minute

TFA – trifluoroacetic acid

UV – ultraviolet

WFPS – water filled pore space

Supporting Information

Supplementary Table S1 (extraction of DMP from pasture soil); Figures S1 (DMP kinetic plots) and S2 (DMP decay profiles); Figures S3 – S5 (HPLC chromatograms of DMPG exposure studies); characterization of reaction products (^{1}H and ^{13}C NMR data and spectra for compound **1**, HRMS data and spectra for compounds **1** – **4**).

Author Contributions

P. K. S. and B. I. T. performed the experiments, processed the experimental data and performed the analysis. P. K. S. worked out all technical details of the soil extraction and product studies. D. C. and U. W. designed, planned and supervised the work. All authors aided in interpreting the results. U. W. wrote the manuscript and designed the figures with input of all authors.

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