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Simultaneous measurement of net nitrogen mineralization and denitrification rates in soil using nitrification inhibitor 3,5-dimethylpyrazole (DMP)

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A practical means to quantify the response of the rates of net N mineralization and denitrification over a wide range of soil water contents is generally lacking. This study examined the potential to use a nitrification inhibitor (NI) assay system to simultaneously estimate the rates of net N mineralization and denitrification; and applied the NI assay to assess the effect of water content on net N mineralization and denitrification rates in two soils with contrasting soil texture. The compound 3,5-dimethylpyrazole (DMP) applied at a rate of 200 mg kg\(^{-1}\) was found to provide essentially complete inhibition of nitrification over the duration of the soil incubation for two soils with contrasting soil texture (clay loam vs sandy loam) and over a range of soil water contents (35, 55 and 85 %WFPS). This allowed net N mineralization to be estimated as the accumulation of soil ammonium (NH\(_4^+\)), and of denitrification as the disappearance of added nitrate (NO\(_3^-\)). Addition of DMP resulted in a small increase in soil respiration rate but did not appear to influence the rate of net soil N mineralization. The NI assay provides a practical means to quantify the rates of net N mineralization and denitrification simultaneously over a wide range of soil water contents. The assay can be readily scaled up to routinely test multiple soils in an efficient manner, has limited material costs, and is also relatively simple to perform.
**Introduction**

Nitrogen (N) is commonly the most limiting plant nutrient for non-leguminous agricultural crops, and consequently most crops receive N inputs as manure or mineral fertilizer (Zebarth et al. 2009). These N inputs supplement N released from soil organic N pools, which can supply 30 to 100% of the N required by the growing crop (Drury et al. 2003). However, the supply of N from these organic N pools is highly variable among fields and years and is therefore difficult to predict (Zebarth et al. 2009). Crop recovery of applied N is often not greater than 60%, and consequently a large proportion of the applied N is lost, primarily by leaching and denitrification (Janzen et al. 2003). Thus, from an economic and environmental perspective, it is necessary to improve the understanding and prediction of soil N availability within agricultural cropping systems.

The biochemical processes that transform soil mineral N are controlled by the nature, distribution and activity of soil microorganisms (Robertson and Groffman 2007). The processes of N mineralization, nitrification and denitrification are the major microbe facilitated processes governing soil mineral N availability (St. Luce et al. 2011; Curtin and Campbell 2008). As these processes are facilitated by factors that influence microbial growth and activity, such as substrate availability, soil water content and temperature (Zak et al. 1999), it is important to determine how microbial activity is affected by these factors. In humid climates with high annual precipitation, it is important to examine how soil water content regulates nitrate ($\text{NO}_3^-$) accumulation and losses during net N mineralization and denitrification over a wide range of soil water contents (Dessureault-Rompré et al. 2011b; Georgallas et al. 2012).

The response of net N mineralization to soil water content has been widely studied (e.g., Myers et al. 1982; Mikha et al. 2005), where net N mineralization is commonly assessed as the accumulation of soil mineral N. This is effective at low and intermediate values of soil water content, when denitrification is small relative to net N mineralization. At high water content, high denitrification rates commonly
result in a net loss of soil mineral N, and thus net N mineralization cannot easily be measured as accumulation of soil mineral N (Drury et al. 2003). While there is the potential to estimate the net N mineralization of an added substrate, for example $^{15}$N labelled plant residue, there is currently no practical means of labelling the soil organic N pool. The rate of gross net N mineralization can be estimated at any soil water content using the isotope pool dilution method (Davidson et al 1991; Burger and Jackson 2003), however it is difficult to estimate the net N mineralization rate, the method is inherently variable, and the assay is not amenable to routine use. Similarly, the rate of denitrification can be difficult to measure. Perhaps the most reliable method is the gaseous loss of $^{15}$N labeled nitrate added to soil (Groffman et al. 2006). This method is also difficult to perform on a routine basis.

The addition of a nitrification inhibitor (NI) to the assay system provides an opportunity to measure both net N mineralization and denitrification processes simultaneously. A NI temporarily blocks the conversion of NH$_4^+$ to NO$_3^-$ (Zerulla et al. 2001), allowing for concurrent measurements of net N mineralization as the accumulation of NH$_4^+$, and of denitrification as the disappearance of added NO$_3^-$ (Khosa et al. 2012). This study will 1) examine the potential to use a NI assay system to simultaneously estimate the rates of net N mineralization and denitrification; and 2) apply the NI assay to assess the effect of water content on net N mineralization and denitrification rates in two soils with contrasting soil texture.

Materials and Methods

A series of four experiments were conducted. These experiments were aimed at screening for a candidate NI (Expt. 1), selection of an appropriate NI application rate (Expt. 2), validating an experimental system for the NI assay (Expt. 3), and application of the NI assay to determine the effect of soil water content on net N mineralization and denitrification rates for two soils contrasting in texture (Expt. 4).

Experiment 1
This experiment compared three candidate NI compounds for use in the NI assay. The experiment used a completely randomized design (CRD) with four NI treatments, 4 incubation lengths (0, 14, 28 or 42 d) and 4 replicates. The NI treatments included: 1) a no NI control; 2) 25 mg NI kg\(^{-1}\) soil as 2-chloro-6-(trichloromethyl) pyridine (Nitrapyrin); 3) 50 mg NI kg\(^{-1}\) soil as 3,5-dimethylpyrazole (DMP); or 4) 50 mg NI kg\(^{-1}\) soil \(\text{C}_6\text{H}_5\text{C≡CH}\) (phenylacetylene; PA). It was not possible to obtain Nitrapyrin as a pure product and as a result this treatment was applied as the commercially available NI product “N-serve”. The lower rate of Nitrapyrin was necessary due to the low concentration of Nitrapyrin in the commercial product used. The DMP and phenylacetylene were previously shown to be effective as a NI (McCarty and Bremner 1986; 1989).

Soil (Research Station L; Table 1) was obtained from 0-15 cm depth from a field cropped to barley and passed through a 4.75 mm sieve. Moist soil, equivalent to 100 g of oven dry soil, was placed in 1 L Mason jars, and sufficient water was added to achieve the target water content [equivalent to 45 percent water-filled pore space (%WFPS) after packing at time zero]. The jars were covered with Parafilm with five pinholes to enhance gas exchange, and the jars pre-incubated at 25 °C for 10 days to condition the soil prior to the start of the experiment. Following pre-incubation (time zero), all jars received 50 mg N kg\(^{-1}\) soil as an NH\(_4\)Cl solution, and either distilled water or a solution containing the appropriate NI, and packed to constant bulk density (1.0 Mg m\(^{-3}\)), where the volume of solutions added was chosen to achieve 50 %WFPS. The soil bulk density was selected through a pre-trial and was chosen to be the lowest soil bulk density at which the soil could be maintained when incubated across a wide range of soil water contents (i.e., air dry to saturated). The Parafilm was then replaced and each jar incubated at 25 °C for the designated time period (0, 14, 28 or 42 d). Water content was maintained by weighing the jars twice per week and adding the requisite amount of water. Soil mineral N was determined at the end of the incubation period. Moist soil (20 g) was shaken for 30 min with 50 mL of 1.7 M KCl and vacuum filtered. Concentrations of NH\(_4\)-N and NO\(_3\)-N plus NO\(_2\)-N in the extract were
determined colorimetrically using a Technicon TRAACS 800 autoanalyzer (Zebarth and Milburn 2003). Please note that hereafter, NO$_3$-N plus NO$_2$-N concentrations will be referred to as NO$_3$-N concentrations.

Analysis of Variance was performed separately for each incubation length using the General Linear Model of SAS (SAS Institute Inc., Cary, NC, Version 9.4). Treatment means were compared using a protected LSD test. The percent inhibition was calculated as (McCarty and Bremner 1986):

\[
\text{Percent inhibition} = 100\% \times \frac{(C-T)}{C}
\]

where C is the increase in NO$_3$-N concentration in the control treatment, and T is the increase in NO$_3$-N concentration in the NI treatment, since time zero. For this calculation, a value of zero was assigned to C or T when there was a net decrease in in NO$_3$-N concentration.

Experiment 2

This experiment compared different rates of DMP addition to assist in selection of an optimal rate for use in the NI assay. The experiment used a CRD with six rates of DMP (0, 5, 10, 25, 50 or 100 mg kg$^{-1}$), two soil water contents (50 and 90 %WFPS), 4 incubation lengths (0, 14, 28 or 42 d) and 4 replicates.

Soil (Research Station L; Table 1) was obtained from 0-15 cm depth from the same field as the soil used for Experiment 1, but where the field had been cropped to red clover to have a greater potential for net N mineralization then in Experiment 1. The soil was air-dried and passed through a 2 mm sieve. Air-dry soil (72 g) was weighed into plastic cups (120 mL screw-cap sample container). The soil was moistened to 45 and 85 %WFPS (i.e., 5 %WFPS less than the target water content) and packed to constant bulk density (1.2 Mg m$^{-3}$) where the soil bulk density was chosen using a pre-trial as described in Experiment 1. A screw top cap with a pre-drilled hole to enhance gas exchange was placed on the cups, and the soil conditioned by pre-incubation at 25°C for 10 days to allow the flush of N
mineralization that occurs after an air-dried soil undergoes rewetting (Stanford et al. 1974). At time zero, 50 mg N kg\(^{-1}\) as NH\(_4\)Cl plus the appropriate quantity of DMP were added to the soil in sufficient water to achieve the target water content, the soil was thoroughly mixed and repacked to constant bulk density, and the screw caps replaced. The samples were incubated at 25 °C for the designated time period (0, 14, 28 or 42 d). Water content was maintained by weighing the jars twice per week and adding the requisite amount of water. Concentrations of soil mineral N were determined as described for Experiment 1.

Analysis of Variance was performed separately for each combination of incubation length and soil water content as described for Experiment 1. The percent inhibition was calculated as described in Experiment 1 only for 50 %WFPS, as the loss of NO\(_3\)-N through denitrification at 90 %WFPS does not provide meaningful results.

Experiment 3

This experiment was a test of a modified experimental system for the NI assay. In particular, the experiment examined the efficacy of nitrification inhibition by DMP as indicated by the lack of the appearance of NO\(_3\); and the effect of DMP on soil microbial activity as indicated by soil respiration to ensure there was no adverse effect of the DMP on soil microbial activity.

The experiment used a factorial arrangement of treatments in a CRD with four replicates. Treatments included two levels of N inhibitor [with (DMP+) or without (DMP-) at a rate of 200 mg DMP kg\(^{-1}\)], two soil types [Brookston clay loam (Brookston CL) or Fox sandy loam (Fox SL), Table 1], three water contents (35, 50 or 85 %WFPS), and three incubation lengths (0, 14 or 28 d). Soils were collected from 0-15 cm depth from fields cropped to corn (Zea mays L.) and were chosen to vary in clay content (Table 3), as this is the factor most likely to influence the efficacy of nitrification inhibition (Barth et al. 2001). An increased rate of DMP application, addition of DMP 10 days in advance of time zero, and a
shorter incubation length were used to improve the efficacy of the nitrification inhibitor. Water contents were chosen to represent the full range of water contents at which net N mineralization, nitrification and denitrification occur (Linn and Doran 1984).

One bulk soil sample was prepared for each combination of soil type and water content. The DMP plus sufficient water was added such that the water content was equivalent to 30, 45 and 45 %WFPS for the 35, 50 and 85 %WFPS treatments, based on a target soil bulk density of 1.15 and 1.30 Mg m\(^{-3}\) for the Brookston CL and Fox SL soils, respectively, where the target soil bulk densities were determined using a pre-trial as described in Experiment 1. The low (45 %WFPS) water content for the 85 %WFPS treatment during the pre-incubation period was selected to avoid rapid denitrification during the pre-incubation period. Each bulk soil sample was pre-incubated in polyethylene bags for 5 days at 25 °C. The appropriate mass of soil (equivalent to 70 g oven-dry soil) was then weighed into plastic 100 mL polypropylene tubes (SCP Science, cat # 010-501-028), but not packed to the target density, and the tubes capped with Parafilm. The tubes were incubated for an additional 5 days at 25 °C to complete the pre-incubation period. At time zero, 20 mg N kg\(^{-1}\) as NH\(_4\)Cl was added to each tube by pipette, the soil mixed, the soil packed to the target bulk density, and the tubes capped with Parafilm. Water content was monitored and maintained throughout the incubation by the addition of water if loss was more than 1 g.

Soil respiration was assessed at three time periods: at two hours after the soil was packed to the target bulk density at time zero, and at 14 and 28 d, just prior to destructive sampling of soils. The Parafilm was removed one hour prior to gas sampling to allow the headspace gases to equilibrate, and the headspace volume was flushed with compressed air for 5 seconds prior to capping the tube with a cap fitted with a rubber septum. Compressed air (30 mL) was injected into the tube to maintain positive pressure inside the tube for subsequent gas sampling. Headspace gas samples (10 mL) were taken using a disposable syringe at 10, 20 and 30 min after capping the tube and stored in 6 mL pre-evacuated
exetainers. Headspace gas samples (0.5 mL) were analyzed for CO₂ concentration using a Varian Star 3800 Gas Chromatograph with an attached thermal conductivity detector (Varian, Mississauga, ON) as described by Burton et al. (2008). The respiration rate was calculated as the slope of a regression of the mass of CO₂-C in the headspace per unit weight of oven dry soil over time (Miller et al. 2008).

Immediately after gas sampling, 75 mL of 0.5 M K₂SO₄ was added to the tube, which was then capped and shaken for one hour on a lateral shaker and filtered through a Whatman no. 40 filter paper. Extracts were frozen at -20°C until analyses. Concentrations of NH₄⁺-N and NO₃-N were determined using a Technicon AutoAnalyzer II system using Technicon Industrial Method #98-70W and Technicon Industrial Method #100-70W, respectively.

Analysis of Variance was performed on the complete data set using the General Linear Model of SAS (SAS Institute Inc., Cary, NC, Version 9.4). Subsequently, Analysis of Variance was performed separately for each incubation length for the DMP+ treatment only. The percent inhibition was calculated as described in Experiment 2.

**Experiment 4**

Experiment 4 applied the NI assay to determine the effect of soil water content on net N mineralization and denitrification rates for two soils contrasting in texture but with similar pedogenic and agronomic history. The experiment used a factorial arrangement of treatments in a CRD with four replicates. Treatments included two soil types [Brookston CL or Harrow sandy loam (Harrow SL), Table 1], ten water contents (20, 35, 50, 65, 75, 80, 85, 90, 95 and 100 %WFPS), and three incubation lengths (0, 14 and 28 d). Note that for the 100 %WFPS, the quantity of water added was equivalent to 110% of the soil pore space, such that standing water was present on the surface of the soil in order to reflect a flooded soil. Soils were obtained from 0-15 cm depth of fields cropped to corn.
The protocol used was similar to that of Experiment 3 except as described below. All soils received 200 mg DMP kg\(^{-1}\) prior to the pre-incubation. Soil water content during the pre-incubation period was 5 %WFPS below the target water content for target water contents of 50 %WFPS or less, and was 45 %WFPS for other water contents to avoid rapid denitrification during the pre-incubation period. At time zero, no NH\(_4\)^+ was added to soils to allow for better quantification of net N mineralization, whereas 100 mg NO\(_3\)-N kg\(^{-1}\) was added as KNO\(_3\) to allow for a non-limiting NO\(_3\)-N supply such that denitrification rate could be quantified. It was discovered after completion of the experiment that the oven dry water content of the soil had been determined incorrectly, and consequently the actual %WFPS values differed slightly between soil and from the target %WFPS values. The actual water contents were 17, 32, 47, 62, 72, 77, 82, 87, 92 and 100 %WFPS for the Brookston CL and 21, 36, 51, 66, 76, 81, 86, 91, 96 and 100 %WFPS for the Fox SL.

Analysis of Variance was performed separately for each soil, due to the differences in actual %WFPS) and for each incubation length using the General Linear Model of SAS (SAS Institute Inc., Cary, NC, Version 9.4). Comparisons among treatment means were performed using a protected LSD test. The net N mineralization rate and denitrification rate was calculated for each combination of soil type and water content using treatment means.

Results and Discussion

Experiment 1

Soil NH\(_4\)-N concentration in the control treatment decreased rapidly, reaching very low (< 1 mg N kg\(^{-1}\)) concentrations after 28 d of incubation (Table 2). The corresponding rapid increase in soil NO\(_3\)-N concentrations in the control reflects rapid nitrification during the incubation. In contrast, soil NH\(_4\)-N and NO\(_3\)-N concentrations were relatively consistent over time during the 42 d incubation when a NI product was added, providing evidence of limited nitrification during this time. The exception was the PA
treatment, which had a large decrease in soil NH$_4$-N concentration, and a large increase in soil NO$_3$-N concentration, between 28 and 42 d. After 42 d of incubation, the DMP treatment had the greatest soil NH$_4$-N concentration, and the Nitrapyrin and DMP treatments had the lowest soil NO$_3$-N concentrations. The percent inhibition was 98, 33, and 100% after 42 d of incubation for the Nitrapyrin, PA and DMP treatments, respectively. When averaged across treatments, soil mineral N increased from 52 to 59 kg N kg$^{-1}$ over the 42 d incubation (data not shown), indicating that the chosen soil had a low potential for net soil N mineralization.

The PA treatment did not inhibit nitrification over the 42-day duration of the experiment, and consequently is not a suitable NI for use in a NI assay. While both Nitrapyrin and DMP provided good inhibition of nitrification, DMP was chosen for use in this study due to the greater availability of the active ingredient and the essentially complete inhibition of nitrification. Similarly, McCarty and Bremner (1989) found DMP to be an effective nitrification inhibitor.

**Experiment 2**

Similar to Experiment 1, soil NH$_4$-N concentration in the control treatment at 50 %WFPS decreased rapidly, reaching very low (< 1 mg N kg$^{-1}$) concentrations after 28 d of incubation (Table 3). Soil NH$_4$-N concentration was decreased when compared with the 100 mg DMP kg$^{-1}$ rate, for DMP rates below 25, 50 and 50 mg kg$^{-1}$ after 14, 28 and 42 d of incubation, respectively. Corresponding increases in soil NO$_3$-N concentrations occurred, consistent with nitrification. The percent inhibition calculated after 42 d of incubation was 8, 10, 65, 92 and 95% for the 5, 10, 25, 50 and 100 mg DMP kg$^{-1}$ rates, respectively.

Interestingly, soil NH$_4$-N concentration in the control treatment at 90 %WFPS also decreased rapidly, reaching low (< 2 mg N kg$^{-1}$) concentrations after 28 d of incubation (Table 3). The response of soil NH$_4$-N concentration for DMP treated soil followed a similar pattern to that observed at 50 %WFPS,
except that the magnitude of the decrease was generally less for 90%WFPS than for 50%WFPS. In contrast, soil NO$_3$-N concentrations were generally lower for soils at 90%WFPS than 50%WFPS, presumably reflecting significant loss of NO$_3$-N through denitrification.

When averaged across treatments at 50%WFPS, soil mineral N increased from 76 to 97 kg N kg$^{-1}$ over the 42 d incubation (data not shown), indicating that the chosen soil had an increased potential for net soil N mineralization compared with the soil used for Experiment 1. In comparison, a similar increase in soil mineral N (62 to 82 kg N kg$^{-1}$) occurred at 90%WFPS when averaged over the 50 and 100 mg DMP kg$^{-1}$ rates for which there was limited evidence of denitrification.

In this experiment, nitrification was strongly inhibited for rates of 50 and 100 mg DMP kg$^{-1}$ over a 42 d period. While there was no statistical difference between these two rates in terms of soil NH$_4$-N and NO$_3$-N concentrations, the greater DMP rate performed slightly better when compared numerically. In addition, this greater rate of DMP had no adverse effect on net soil N mineralization. Thus, the 100 mg DMP kg$^{-1}$ should be considered a minimum application rate to inhibit nitrification.

Unlike Experiment 1 where nitrification was essentially completely inhibited by DMP, inhibition was not complete in this experiment. It is possible that this may reflect incomplete distribution of the DMP within the soil, or a delay in the action of the DMP in inhibiting nitrification. As a result, DMP was added prior to time zero in subsequent experiments to mitigate these possibilities.

Few previous studies have evaluated DMP as a nitrification inhibitor. Ali et al. (2008) did not achieve inhibition of nitrification at a rate of 3.6 mg DMP kg$^{-1}$ soil when incubated for 4 weeks at 35 °C. McCarty and Bremner (1989) had 90-99% inhibition across three soils at their highest rate of 48 mg DMP kg$^{-1}$ soil when incubated for 3 weeks at 25 °C.
Experiment 3

In the absence of DMP addition, soil NH$_4$-N concentration decreased rapidly over the first 14 d of the incubation, and soil NO$_3$-N increased throughout the 28 d incubation, when averaged across soil type and soil water content (Fig. 1). In contrast, soil NH$_4$-N concentration increased over time, and soil NO$_3$-N was stable over time, in the presence of DMP. This is consistent with rapid nitrification in the absence of DMP, and effective nitrification inhibition in the presence of DMP.

In the presence of DMP, soil NH$_4$-N concentration was greater for the Brookston CL than the Fox SL at all sampling times (Table 4). At time zero, soil water content had no significant effect on NH$_4$-N concentration for either soil type. At 28 d, soil water content had a limited effect on soil NH$_4$-N concentration for the Brookston CL, with slightly greater NH$_4$-N concentration for 35 %WFPS than for 85 %WFPS. In contrast, soil NH$_4$-N concentration at 28 d increased with increasing soil water content for the Fox SL.

In the presence of DMP, soil NO$_3$-N concentration was greater for the Brookston CL than the Fox SL at all sampling times (Table 4). In contrast to soil NH$_4$-N, soil NO$_3$-N concentration varied with water content at time zero for both soils, indicating that measurable denitrification occurred during the pre-incubation period. Soil NO$_3$-N concentration was lower for 85 %WFPS than for 35 and 50 %WFPS for the Brookston CL, and lower for the 85 and 50 %WFPS than 35 %WFPS for the Fox SL. Thus, greater denitrification occurred at increased water content as would be expected. The calculated % inhibition of nitrification was 99 and 98% for the Brookston CL, and 100 and 101% for the Fox SL, for 35 and 50 %WFPS, respectively (data not shown).

Soil respiration rate, averaged across soil type and water content, was significantly greater for the DMP+ than the DMP- treatment at time zero and at 28 d, but not at 14 d (Fig. 1C). The increase in soil respiration rate may reflect the addition of 125 mg C kg$^{-1}$ soil as DMP. Cumulative respiration from soil incubated under anoxic conditions was shown to be increased with increasing rate of C addition.
following amendment with glucose and plant residues (Miller et al. 2012), and it is likely that greater respiration would occur under the oxic conditions in this experiment. However, it is unclear how readily decomposable the DMP molecule is given that the nitrification inhibition effect persisted over the duration of the incubation. Alternatively, it is possible that the DMP was toxic to some microorganisms, and their death and decomposition stimulated increased respiration. For the 35 and 50 %WFPS treatments, which would be expected to have limited loss of nitrate by denitrification, the average increase in soil mineral N from 0 to 28 d was 27 and 26 mg N kg\(^{-1}\) for the DMP- and DMP+ treatments, respectively (data not shown). This suggests that any effect of the DMP on soil respiration did not appear to have a significant effect on net soil N mineralization.

This experiment demonstrated that the experimental system was effective in inhibiting the nitrification process across soils with contrasting texture and across a wide range of soil water contents. Addition of DMP resulted in a small increase in soil respiration rate, but perhaps more importantly, the addition of DMP did not decrease soil respiration rate, which would be an indicator of an adverse effect of the DMP on soil microbial processes.

Experiment 4

For the Brookston CL soil, soil NH\(_4\)-N concentrations either were not significantly affected by water content, or the differences were significant but small in magnitude, on all three sampling dates (Table 5). The exception was for the 17 %WFPS treatment, which had significantly lower soil NH\(_4\)-N concentration compared with all other water contents. It is possible that the very low soil NH\(_4\)-N concentration at day 0 may reflect that the water content during the pre-incubation period inhibited the mineralization process, and consequently the results from this water content are of questionable value and are not discussed further. When the rate of net mineralization over time was estimated, the net
The mineralization rate for this soil was generally insensitive to soil water content and averaged 0.43 mg N kg\(^{-1}\) d\(^{-1}\) (Fig. 2A).

In contrast, significant differences in soil NH\(_4\)-N concentration were measured at all three sampling times for the Fox SL soil (Table 5). This suggests that the net N mineralization rate differed during the pre-incubation period as well as during the incubation. The estimated net N mineralization was greatest at 66 %WFPS at 0.60 mg N kg\(^{-1}\) d\(^{-1}\) and decreased to less than 0.30 mg N kg\(^{-1}\) d\(^{-1}\) for soil water contents of 36, 96 and 100 %WFPS (Fig. 2A).

The pattern of increasing net N mineralization rate from dry to optimal water content for the Fox SL soil is consistent with the results of many soil incubation studies (Dessureault-Rompré et al. 2011). The general insensitivity of net N mineralization rate to water content observed for the Brookston CL soil is less common, but has been reported in some cases (Drury et al. 2003). The response of net N mineralization rate at above optimal soil water contents has generally not been examined because the potential for N loss by denitrification prevents the estimate of net N mineralization rate through the accumulation of soil mineral N.

For the Brookston CL soil, soil NO\(_3\)-N at time zero did not vary with water content, with the exception of the 17 %WFPS which is not being considered further (Table 5). As would be expected, soil NO\(_3\)-N concentration decreased over time, and the decrease was greater at high soil water contents. The calculated denitrification rate was low (< 0.5 mg N kg\(^{-1}\) d\(^{-1}\)) for water contents of 72 %WFPS and lower and increased to a maximum at 2.2 mg N kg\(^{-1}\) d\(^{-1}\) at 100 %WFPS (Fig. 2B).

For the Fox SL soil, some significant differences in soil NO\(_3\)-N concentration were observed at time zero, even though the nitrate was added at time zero, suggesting some loss of background nitrate occurred during the pre-incubation period (Table 4). The pattern of changes in soil NO\(_3\)-N over time were generally similar to that observed for the Brookston CL soil, but the magnitude of the decrease in soil NO\(_3\)-N was greater at high soil water content. For example, mean soil NO\(_3\)-N at 100 %WFPS for the
Fox SL was 9.4 mg N kg\(^{-1}\), and it is possible that denitrification may have become nitrate limited in this treatment. The calculated denitrification rate was low (< 0.5 mg N kg\(^{-1}\) d\(^{-1}\)) for water contents of 81 %WFPS and lower and increased to a maximum at 3.0 mg N kg\(^{-1}\) d\(^{-1}\) at 100 %WFPS (Fig. 2B).

A limitation of this method is that it does not provide explicit tracking of the fate of nitrate disappearance and thus cannot discriminate between pathways of denitrification or dissimilatory nitrate reduction. In situations where DNRA is of concern, namely C rich soils (SOC > 4%) at low redox potentials (Friedl et al., 2018), nitrate disappearance would be the result of the combined processes of denitrification and DNRA. Ammonium accumulation would be the result of net N mineralization and DNRA.

**Conclusion**

This study demonstrated the potential to use a NI assay to quantify the rates of net N mineralization and denitrification simultaneously over a wide range of soil water contents. The approach has several practical benefits. The assay is one that can be readily scaled up to routinely test multiple soils in an efficient manner. The assay allows for quantification of net N mineralization rate at high soil water contents. This is important as the potential for nitrate loss at high water content due to denitrification prevents assessment of net N mineralization rate through the measurement of the accumulation of mineral N. The assay has limited material costs; the primary costs are for soil mineral N analyses, which are low in comparison with costs for isotopic analyses. The assay is also relatively simple to perform and avoids some of the technical challenges for implementation of techniques as isotope pool dilution. The results of the NI assay may be of value as input data for simulation modeling or decision support systems.
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Figure Captions

**Figure 1** Effect of 3,5-dimethylpyrazole (DMP) nitrification inhibitor on A) soil NH$_4$-N, B) soil NO$_3$-N, and C) soil respiration rate over a 42 d incubation for soil amended with 20 mg N kg$^{-1}$ as NH$_4$Cl when averaged across two soil types (Brookston CL and Fox SL) and three water contents (35, 50 and 85 %WFPS). Error bars represent ± 1 SEM.

**Figure 2** Effect of water content on net N mineralization rate and denitrification for two soil types as measured over a 28-day incubation period and calculated from treatment means. Note that the lowest water content was excluded for each soil.
Table 1 Properties of experimental soils used in four experiments.

| Soil type          | Soil pH<sup>a</sup> | Clay<sup>b</sup> (g kg<sup>-1</sup>) | Sand<sup>b</sup> (g kg<sup>-1</sup>) | SOC<sup>c</sup> (g kg<sup>-1</sup>) | TN<sup>c</sup> (g kg<sup>-1</sup>) |
|--------------------|---------------------|------------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Experiment 1 & 2   |                     |                                    |                                  |                                  |                                  |
| Research Station L<sup>d</sup> | 6.2                 | 11                                 | 49                               | 19.2                             | 1.67                             |
| Experiment 3       |                     |                                    |                                  |                                  |                                  |
| Brookston CL       | 6.0                 | 23                                 | 43                               | 1.53                             | 0.17                             |
| Fox SL             | 5.0                 | 12                                 | 71                               | 1.19                             | 0.12                             |
| Experiment 4       |                     |                                    |                                  |                                  |                                  |
| Brookston CL       | 6.6                 | 20                                 | 47                               | 1.25                             | 0.13                             |
| Harrow SL          | 6.6                 | 11                                 | 54                               | 1.29                             | 0.13                             |

<sup>a</sup>CaCl<sub>2</sub> using 1:5 soil:solution ratio (Hendershot et al. 2008).

<sup>b</sup>Hydrometer method (Sheldrick and Wang 1993).

<sup>c</sup>Soil organic carbon (SOC) and soil total nitrogen (TN) measured by dry combustion using an Elementar VarioMax carbon and nitrogen analyzer (Skjemstad and Baldock 2008).

<sup>d</sup>L refers to loam, CL to clay loam, SL to sandy loam.
Table 2  Effect of four nitrification inhibitor treatments on soil NH$_4$-N and NO$_3$-N concentrations in soils over a 42 d incubation in soils amended with 50 mg N kg$^{-1}$ as NH$_4$Cl in Experiment 1.

| Time (d) | Control | Nitrapyrin | PA | DMP |
|----------|---------|------------|----|-----|
| 0        | 49.3    | 42.2       | 51.2| 55.7|
| 14       | 11.2 $a$| 46.7 $b$   | 50.9| 50.3 $b$|
| 28       | 0.5 $a$ | 55.0 $bc$  | 52.7| 56.5 $c$|
| 42       | 0.5 $a$ | 53.5 $c$   | 23.7| 59.8 $d$|

| NH$_4$-N (mg N kg$^{-1}$) |
|---------------------------|

| NO$_3$-N (mg N kg$^{-1}$) |
|---------------------------|

Note: Means in the same row followed by the same letter are not significantly different based on a protected LSD test (P < 0.05).

$^a$PA, phenylacetylene; DMP, 3,5-dimethylpyrazole.
Table 3: Effect of rate of 3,5-dimethylpyrazole (DMP) nitrification inhibitor on soil NH$_4$-N and NO$_3$-N concentrations over a 42 d incubation in soils at two water contents and amended with 50 mg N kg$^{-1}$ as NH$_4$Cl in Experiment 2.

| Time (d) | 0     | 5     | 10    | 25    | 50    | 100   |
|---------|-------|-------|-------|-------|-------|-------|
|         | 50 %WFPS |      |       |       |       |       |
|         | NH$_4$-N (mg N kg$^{-1}$) |      |       |       |       |       |
| 0       | 56.7  | 57.7  | 49.6  | 52.1  | 59.4  | 51.0  |
| 14      | 5.1 a | 50.7 b| 54.8 b| 64.0 c| 64.5 c| 64.3 c|
| 28      | 0.1 a | 19.7 b| 33.2 c| 59.8 d| 67.9 e| 69.3 e|
| 42      | 0.1 a | 0.3 a | 5.0 b | 44.6 c| 64.1 d| 67.0 d|
|         | NO$_3$-N (mg N kg$^{-1}$) |      |       |       |       |       |
| 0       | 20.4 a| 23.8 c| 22.5 bc|20.6 ab|21.5 ab|21.9 abc|
| 14      | 87.2 e| 41.0 d| 36.5 c| 27.5 b| 25.0 a| 25.0 a|
| 28      | 109.0 e|87.2 d| 71.2 c| 38.4 b| 28.3 a| 30.5 a|
| 42      | 101.7 d|98.6 cd|95.8 c| 48.7 b| 28.0 a| 25.6 a|
|         | 90 %WFPS |      |       |       |       |       |
|         | NH$_4$-N (mg N kg$^{-1}$) |      |       |       |       |       |
| 0       | 66.0  | 78.6  | 72.5  | 66.3  | 62.1  | 60.5  |
| 14      | 11.0 a| 76.9 b| 83.2 b| 77.3 b| 81.0 b| 82.0 b|
| 28      | 1.4 a | 61.5 b| 77.8 c| 85.4 cd|85.0 cd|88.4 d |
| 42      | 1.0 a | 55.0 b| 65.8 c| 75.0 cd|81.8 d |82.6 d |
|         | NO$_3$-N (mg N kg$^{-1}$) |      |       |       |       |       |
| 0       | 0.2   | 0.3   | 0.3   | 0.3   | 1.0   | 0.3   |
| 14      | 23.4 b| 1.3 a | 0.4 a | 0.0 a | 0.0 a | 0.0 a |
| 28      | 18.0 a| 3.0 a | 0.7 a | 0.2 a | 0.2 a | 0.3 a |
| 42      | 9.1 b | 1.2 a | 0.5 a | 0.1 a | 0.2 a | 0.2 a |

Note: Means in the same row followed by the same letter are not significantly different based on a protected LSD test (P < 0.05).
Table 4 Change in soil NH$_4$-N and NO$_3$-N concentrations over a 28 d incubation in two soils amended 3,5-dimethylpyrazole (DMP) nitrification inhibitor (200 mg DMP kg$^{-1}$) and 20 mg NH$_4$-N kg$^{-1}$ in Experiment 3.

| Time (d) | Brookston CL$^a$ | Fox SL |
|----------|-------------------|--------|
|          | Water content (%WFPS) | NH$_4$-N (mg N kg$^{-1}$) | Water content (%WFPS) | NO$_3$-N (mg N kg$^{-1}$) |
| 0        | 28.3 $b$ | 27.2 $b$ | 28.7 $b$ | 21.7 $a$ | 25.6 $a$ | 25.6 a |
| 14       | 43.4 $d$ | 43.1 $d$ | 43.4 $d$ | 32.5 $b$ | 26.3 $a$ | 34.8 c |
| 28       | 59.5 $e$ | 58.3 $de$ | 56.3 $d$ | 41.5 $a$ | 46.5 $b$ | 50.7 c |
|          | 5.2 $f$  | 4.3 $d$  | 4.7 $e$  | 2.1 $c$  | 0.6 $b$  | 0.2 $a$ |
| 14       | 6.1 $e$  | 5.5 $d$  | 5.5 $de$ | 2.4 $c$  | 0.6 $b$  | 0.1 $a$ |
| 28       | 5.6 $c$  | 5.0 $c$  | 2.8 $b$  | 2.0 $b$  | 0.2 $a$  | 0.2 $a$ |

Note: Means in the same row followed by the same letter are not significantly different based on a protected LSD test (P < 0.05).

$^a$L refers to loam, CL to clay loam, SL to sandy loam.
Table 5 Change in soil NH$_4$-N and NO$_3$-N concentrations over a 28 d incubation as affected by soil water content in two soils amended 3,5-dimethylpyrazole (DMP) nitrification inhibitor (200 mg DMP kg$^{-1}$) in Experiment 4.

| Water content (%WFPS) | NH$_4$-N concentration (mg N kg$^{-1}$) | Incubation length (d) | NO$_3$-N concentration (mg N kg$^{-1}$) | Incubation length (d) |
|-----------------------|----------------------------------------|-----------------------|----------------------------------------|-----------------------|
|                       |                                        | 0                     | 14                                     | 28                    |
|                       |                                        | 0                     | 14                                     | 28                    |
| Brookston CL$^a$       |                                        |                       |                                        |                       |
| 17                    | 0.3 a                                  | 9.0 a                 | 8.4 a                                  | 108.7 b               | 108.0 g               | 105.7 g               |
| 32                    | 9.2 bcde                               | 18.9 b                | 21.1 b                                 | 102.9 ab              | 105.4 g               | 100.4 g               |
| 47                    | 10.5 e                                 | 24.3 de               | 20.6 b                                 | 96.1 a                | 97.6 ef               | 87.1 f                |
| 62                    | 9.7 cde                                | 22.3 cd               | 22.0 b                                 | 94.7 a                | 99.0 f                | 89.3 f                |
| 72                    | 10.0 de                                | 22.0 cd               | 21.1 b                                 | 97.2 a                | 93.5 e                | 84.9 ef               |
| 77                    | 8.3 b                                  | 22.2 cd               | 22.4 b                                 | 99.2 a                | 83.1 d                | 78.4 de               |
| 82                    | 8.0 b                                  | 21.4 bc               | 20.3 b                                 | 96.0 a                | 80.1 cd               | 72.3 cd               |
| 87                    | 8.9 bcd                                | 23.6 cde              | 20.5 b                                 | 95.6 a                | 75.9 bc               | 69.7 c                |
| 92                    | 8.6 bc                                 | 22.8 cde              | 20.7 b                                 | 98.5 a                | 74.7 b                | 61.0 b                |
| 100                   | 8.9 bcd                                | 25.5 e                | 22.2 b                                 | 97.6 a                | 62.5 a                | 35.2 a                |
| Fox SL                |                                        |                       |                                        |                       |
| 21                    | 26.3 b                                 | 28.6 d                | 35.6 c                                 | NA                    | 108.8 f               | 108.1 f               |
| 36                    | 34.9 d                                 | 31.8 e                | 41.9 d                                 | 112.7 d               | 107.5 f               | 106.8 f               |
| 51                    | 30.1 c                                 | 32.9 e                | 43.5 d                                 | 101.0 c               | 97.2 e                | 96.1 ef               |
| 66                    | 24.8 ab                                | 28.2 cd               | 41.5 d                                 | 92.7 ab               | 87.7 d                | 85.4 de               |
| 76                    | 25.5 b                                 | 28.8 d                | 40.2 d                                 | 90.0 a                | 86.6 d                | 86.7 de               |
| 81                    | 21.4 a                                 | 24.9 a                | 34.5 bc                                | 91.4 ab               | 68.9 c                | 78.5 cd               |
| 86                    | 23.8 ab                                | 26.4 ab               | 35.8 c                                 | 92.3 ab               | 76.0 c                | 68.9 c                |
| 91                    | 23.0 ab                                | 26.7 bc               | 31.6 ab                                | 89.2 a                | 58.9 b                | 21.9 ab               |
| 96                    | 23.8 ab                                | 26.4 ab               | 29.9 a                                 | 97.0 bc               | 48.0 a                | 30.4 b                |
| 100                   | 24.6 ab                                | 26.2 ab               | 29.6 a                                 | 93.9 ab               | 46.0 a                | 9.4 a                 |

Note: Means in the same column followed by the same letter are not significantly different based on a protected LSD test (P < 0.05).

$^a$L refers to loam, CL to clay loam, SL to sandy loam

NA – Not available due to an error in nitrate addition to these incubation tubes.
Figure 1 Effect of 3,5-dimethylpyrazole (DMP) nitrification inhibitor on A) soil NH4-N, B) soil NO3-N, and C) soil respiration rate over a 42 d incubation for soil amended with 20 mg N kg⁻¹ as NH4Cl when averaged across two soil types (Brookston CL and Fox SL) and three water contents (35, 50 and 85 %WFPS). Error bars represent ± 1 SEM.
Figure 2 Effect of water content on net N mineralization rate and denitrification for two soil types as measured over a 28-day incubation period and calculated from treatment means. Note that the lowest water content was excluded for each soil.