Impact of nitrogen compounds on fungal and bacterial contributions to codenitrification in a pasture soil

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Ruminant urine patches on grazed grassland are a significant source of agricultural nitrous oxide (N₂O) emissions. Of the many biotic and abiotic N₂O production mechanisms initiated following urine-urea deposition, codenitrification resulting in the formation of hybrid N₂O, is one of the least understood. Codenitrification forms hybrid N₂O via biotic N-nitrosation, co-metabolising organic and inorganic N compounds (N substrates) to produce N₂O. The objective of this study was to assess the relative significance of different N substrates on codenitrification and to determine the contributions of fungi and bacteria to codenitrification.

15N-labelled ammonium, hydroxylamine (NH₂OH) and two amino acids (phenylalanine or glycine) were applied, separately, to sieved soil mesocosms eight days after a simulated urine event, in the absence or presence of bacterial and fungal inhibitors. Soil chemical variables and N₂O fluxes were monitored and the codenitrified N₂O fluxes determined. Fungal inhibition decreased N₂O fluxes by ca. 40% for both amino acid treatments, while bacterial inhibition only decreased the N₂O flux of the glycine treatment, by 14%. Hydroxylamine (NH₂OH) generated the highest N₂O fluxes which declined with either fungal or bacterial inhibition alone, while combined inhibition resulted in a 60% decrease in the N₂O flux. All the N substrates examined participated to some extent in codenitrification. Trends for codenitrification under the NH₂OH substrate treatment followed those of total N₂O fluxes (85.7% of total N₂O flux). Codenitrification fluxes under non-NH₂OH substrate treatments (0.7–1.2% of total N₂O flux) were two orders of magnitude lower, and significant decreases in these treatments only occurred with fungal inhibition in the amino acid substrate treatments. These results demonstrate that in situ studies are required to better understand the dynamics of codenitrification substrates in grazed pasture soils and the associated role that fungi have with respect to codenitrification.

The nitrous oxide (N₂O) molecule is a potent greenhouse gas, with a global warming potential 298 times that of carbon dioxide over a 100 year time period1. It is also a precursor to reactions involved in the depletion of stratospheric ozone2. A major source of anthropogenic N₂O emissions is the intensive grazing of grasslands and the resulting ruminant urine deposition that occurs3,4. Thus, in order to achieve mitigation of N₂O emissions from intensively managed pasture soils it is important to identify and understand the processes that lead to N₂O formation and consumption within ruminant urine-affected soil.

Typically, ruminant urine-N deposited onto pasture soil is comprised of >70% urea-N. Upon contact with the soil the urea begins to hydrolyse, forming ammonium (NH₄⁺) resulting in a rapid elevation of soil pH to 8.0 or higher5. The equilibrium between NH₄⁺ and ammonia (NH₃) is pH driven6,7. Soil pH > 7.0 leads to elevated NH₃ concentrations in the soil, that not only result in NH₃ volatilization8 but which can also inhibit the microbial oxidation of nitrite (NO₂⁻) by Nitrobacter sp.9,10. As the pH decreases to ca. <7.0, the equilibrium between NH₄⁺ and NH₃ shifts in favour of NH₃, which may undergo clay mineral fixation, plant uptake, immobilization or nitrification11.

Production of N₂O may occur via the microbial pathways of nitrification, denitrification, and nitrifier-denitrification12. However, under ruminant urine-affected soil it is bacteria, not archaea, that respond to the high concentration of NH₄⁺.
substrate that forms in the soil following ruminant urine deposition\textsuperscript{13,14}, since bacterial nitrifiers operate under conditions of high inorganic NH\textsubscript{4}\textsuperscript{+} inputs\textsuperscript{12,13}. During the conventional nitrification process bacteria produce NO\textsubscript{3}\textsuperscript{−} as a by-product of NH\textsubscript{4}OH oxidation\textsuperscript{17} or during nitrifier-denitrification following nitric oxide (NO) reduction\textsuperscript{21}. However, the major source of NO\textsubscript{3}\textsuperscript{−} emissions from ruminant urine-affected soil occurs as a result of the NO\textsubscript{3}\textsuperscript{−} formed, as a consequence of nitrification. Under anaerobic conditions microbes denitrify NO\textsubscript{3}\textsuperscript{−} to sequentially form NO\textsubscript{2}\textsuperscript{−}, NO and N\textsubscript{2}O, which are all obligate intermediaries of the denitrification pathway\textsuperscript{13,18-20} to finally create dinitrogen (N\textsubscript{2}).

In order to conserve both energy and oxygen, nitrifier-denitrification may occur in response to limited soil oxygen conditions\textsuperscript{21}, whereupon nitrifiers convert NO\textsubscript{2}\textsuperscript{−} to NO\textsubscript{3}\textsuperscript{−} and N\textsubscript{2}O, although the significance of this process may have been overstated in some studies\textsuperscript{21}. In addition to these NO\textsubscript{3}\textsuperscript{−} production pathways, NO\textsubscript{3}\textsuperscript{−} may also be produced as 'hybrid' N\textsubscript{2}O via codenitrification, a process involving two different N pools\textsuperscript{20,22}. Spott et al.\textsuperscript{22} reviewed possible biotic and abiotic reactions that may be included under the term 'odenitrification'. For example, abiotic reactions involving reduced iron (Fe\textsuperscript{2+}) and NO\textsubscript{2}\textsuperscript{−}, may occur at the interface between an aerobic zone overlying an anaerobic zone when NO\textsubscript{2}\textsuperscript{−} diffusing downwards meets Fe\textsuperscript{2+}\textsuperscript{24,25}. However, this process is unlikely to contribute significantly to NO\textsubscript{3}\textsuperscript{−} emissions due to insufficient Fe\textsuperscript{2+} ion concentrations in most soils\textsuperscript{26,27}. A more common abiotic reaction that occurs in acidic soil (pH < 5.0) is that of chemodenitrification (abiotic-nitrosation), whereby NO\textsubscript{3}\textsuperscript{−} and H\textsuperscript{+} react to form nitrous acid (HNO\textsubscript{2}), which can then react with amino compounds, NH\textsubscript{3}OH, NH\textsubscript{4}\textsuperscript{+} or other organic N compounds resulting in the formation of N\textsubscript{2}O-NO\textsubscript{3}\textsuperscript{−}. However, under alkaline conditions when oxygen is depleted codenitrification may occur via biologically mediated nitrosation\textsuperscript{28-30}. Under such conditions the hydrogen atom in an organic compound is replaced with a nitroso group (−N=O). Enzymatic nitrosyl compounds attract nucleophile compounds (e.g. NH\textsubscript{3}OH, NH\textsubscript{4}\textsuperscript{+}, hydrazine (N\textsubscript{2}H\textsubscript{4}), amino compounds and NH\textsubscript{3}) resulting in hybrid NO\textsubscript{3}\textsuperscript{−} or N\textsubscript{2}O species, containing one N atom derived from the nucleophile and one N atom derived from the nitrosyl compound\textsuperscript{30,31}. Recent studies have revealed the significant contribution of codenitrification to NO\textsubscript{3}\textsuperscript{−} emissions where 92% of the N\textsubscript{2}O evolved was derived from codenitrification. Selbie et al.\textsuperscript{32} confirmed, in-situ, the dominance of codenitrification derived N\textsubscript{2}O under urine patch conditions when 56% of applied urine was codenitrified. Recently, studies have found further evidence for NO\textsubscript{3} \textsuperscript{−} production via codenitrification under simulated ruminant urine patch conditions\textsuperscript{33,34}. However, knowledge about the nucleophile species that potentially partake in codenitrification under ruminant urine patch conditions is still lacking. Different N substrates (as potential nucleophiles) such as amino acids, NH\textsubscript{4}\textsuperscript{+} and NH\textsubscript{3}OH have been previously proven to be capable of generating hybrid N\textsubscript{2}O/N\textsubscript{2}O in vitro when utilized by one microbial species in combination with either NO\textsubscript{3}\textsuperscript{−} or NO\textsubscript{2}\textsuperscript{−}\textsuperscript{34-37}. Amino acids have been reported to be freely available within the soil solution, for example, phenylalanine (8–50 µg N g\textsuperscript{−1} soil) and glycine (35–193 µg N g\textsuperscript{−1} soil) were measured in long-term agricultural land on a Stagnic-Haplic Luvisol\textsuperscript{38} and in different cattle manure treated crop fields on a sandy Orthic Luvisol\textsuperscript{39}. Reported concentrations of NH\textsubscript{3}OH are orders of magnitude lower, for example, Liu et al.\textsuperscript{40} reported concentrations of <0.0348 µg N g\textsuperscript{−1} in a forest soil, while NH\textsubscript{4}\textsuperscript{+} and NH\textsubscript{3} are routinely reported following ruminant urine deposition events\textsuperscript{41}. Therefore, we hypothesise that in a soil matrix under simulated ruminant urine deposition the N substrates applied in this study will be utilized for codenitrification reactions, with a microbial preference for NH\textsubscript{3}OH and that these reactions would be mainly fungi driven.

Results

Soil pH\textsubscript{4} and mineral N. Within 6 h of applying the urea solution to the soil surface pH values increased uniformly in all treatments from an average of 5.6 ± 0.2 on Day –2 to >7.6 on Day 0. The surface soil pH peaked 30 h after the urea application, at 7.9, followed by a steady decline to 4.8 ± 0.1 on Day 9 (Fig. 1) in the positive control and all treatments. The surface pH in the negative control ranged from 5.4 ± 0.05 to 5.6 ± 0.06 over the course of the experiment (Fig. 1).

Soil NO\textsubscript{2}\textsuperscript{−} concentrations were significantly elevated within the first 4 days following urea application (p < 0.05). Soil NO\textsubscript{2}\textsuperscript{−} concentrations peaked at 1.5 ± 0.2 µg NO\textsubscript{2}\textsuperscript{−} N g\textsuperscript{−1} soil on Day 9, subsequent to the physical mixing and then decreased to 0.6 ± 0.1 µg NO\textsubscript{2}\textsuperscript{−} N g\textsuperscript{−1} soil on Day 11 (Fig. 1b).

Both the soil NO\textsubscript{3}\textsuperscript{−} and NH\textsubscript{4}\textsuperscript{+} concentrations were higher (p < 0.01) in the positive control at Day 12 compared with the negative control. The NO\textsubscript{3}\textsuperscript{−} concentrations in the positive control were in the range of 366 ± 122 µg NO\textsubscript{3}\textsuperscript{−} N g\textsuperscript{−1} soil while NH\textsubscript{4}\textsuperscript{+} concentrations were 174 ± 7 µg NH\textsubscript{4}\textsuperscript{+} N g\textsuperscript{−1} soil. The soil NO\textsubscript{3}\textsuperscript{−} and NH\textsubscript{4}\textsuperscript{+} concentrations in the negative control were 64 ± 23 µg NO\textsubscript{3}\textsuperscript{−} N g\textsuperscript{−1} soil and 22 ± 1 µg NH\textsubscript{4}\textsuperscript{+} N g\textsuperscript{−1} soil, respectively.

N\textsubscript{2}O fluxes. Initially NO\textsubscript{3} \textsuperscript{−} fluxes increased within the first 48 h following urea application, with treatments and positive controls emitting 100–200 µg N\textsubscript{2}O-N m\textsuperscript{−2} h\textsuperscript{−1}. From Day 4 to Day 8, the NO\textsubscript{3} \textsuperscript{−} fluxes from the urea-treated soil were <100 µg N\textsubscript{2}O-N m\textsuperscript{−2} h\textsuperscript{−1} across all treatments. Following NO\textsubscript{3} \textsuperscript{−} flux measurement on Day 8, the process of mixing the soil and/or the addition of N substrates increased NO\textsubscript{3} \textsuperscript{−} fluxes at Day 9 (Fig. 1). In the absence of microbial inhibition, the addition of the NH\textsubscript{3}OH substrate resulted in higher NO\textsubscript{3} \textsuperscript{−} fluxes (4496 µg N\textsubscript{2}O-N m\textsuperscript{−2} h\textsuperscript{−1}) when compared to the amino acid (1796 to 2130 µg N\textsubscript{2}O-N m\textsuperscript{−2} h\textsuperscript{−1}) treatments on Day 9 (p < 0.001), 24 h after N substrate addition.

The magnitude of the decrease in the NO\textsubscript{3} \textsuperscript{−} fluxes, following inhibition treatment, varied due to inhibitor type and N substrate applied (Table 1). The NO\textsubscript{3} \textsuperscript{−} emissions were lower under fungal inhibition by 46, 34 and 21% in the glycine, phenylalanine, and NH\textsubscript{3}OH treatments, respectively, while fungal inhibition did not affect fluxes from the NH\textsubscript{4}\textsuperscript{+} treatment. Bacterial inhibition decreased NO\textsubscript{3} \textsuperscript{−} fluxes by 14, and 26% in the glycine and NH\textsubscript{3}OH treatments, respectively, while fluxes from the phenylalanine and NH\textsubscript{4}\textsuperscript{+} treatments were unaffected by bacterial inhibition (Table 1). Applying both inhibitors simultaneously (combined inhibition) resulted in NO\textsubscript{3} \textsuperscript{−} fluxes decreasing by 29–41% in all N substrate treatments (Table 1). In the glycine treatment fungal inhibition decreased NO\textsubscript{3} \textsuperscript{−} fluxes more than bacterial inhibition, but this decrease was not enhanced when the two inhibitors were combined (Table 1). While bacterial inhibition did not significantly lower NO\textsubscript{3} \textsuperscript{−} fluxes in the phenylalanine treatment,
the fungal inhibition either alone or within the combined inhibition did decrease N₂O fluxes (Table 1). Sterilizing effectively eliminated N₂O fluxes in both the amino acid treatments, and the NH₄⁺ treatment (Table 1). However, this was not the case when NH₂OH was applied, where emissions decreased by 72% (Table 1).

Table 1. Emission rates of total N₂O (µg N₂O-N m⁻² h⁻¹) of the inhibitor × N substrate treatments on Day 9. As taken 24 h after the microbial inhibition, these data represent the N₂O emissions during the overlapping time of N substrates starting to contribute to N₂O emissions and still working microbial inhibitors. Different statistical analyses have been used to determine differences, dependent on normal or non-normal distributed data and homogeneous or inhomogeneous variances. Values are means (n = 3) with standard deviation, different letters indicate the level of significance based on the mentioned test, where all inhibition treatments for each N substrate are tested against each other. Level of significance: *p < 0.05, **p = 0.001.

| N substrate | no inhibition | fungal inhibition | bacterial inhibition | combined inhibition | sterilized soil | test & significance |
|-------------|---------------|-------------------|---------------------|--------------------|----------------|--------------------|
| Glycine     | 2130 ± 134    | 1144 c ± 177      | 1830 b ± 163        | 1331 c ± 114       | 2 d ± 0         | Holm-Sidak*        |
| Phenyl      | 1796 ± 333    | 1182 b ± 66       | 1705 a ± 36         | 1267 b ± 93        | 1 c ± 1         | t-tests*           |
| Ammonium    | 1405 ± 49     | 1142 ab ± 301     | 1010 ab ± 873       | 904 b ± 111        | 3 ± 0           | Tukey**            |
| Hydroxy     | 4496 ± 467    | 3563 b ± 358      | 3324 bc ± 240       | 2671 c ± 253       | 1246 d ± 21     | Holm-Sidak*        |

Figure 1. Soil response to urea and treatment application. The N₂O fluxes over time (a) of the no inhibition treatments. Before Day 9 the N₂O fluxes did not significantly differ between the positive control and the treatments. On Day 9, the N₂O fluxes of all treatments and the positive control increased as listed in Table 1, for simplicity only the non-inhibition treatments are depicted in Fig. 1 to visualize the range of increase. Below the NO₂⁻ concentration in the soils as measured in the NO₂⁻ control. (b) These partially destructive analysis was not performed within the treatment soils and the positive controls, but depicts the assumed NO₂⁻ concentration development within these soils. The soil surface pH was measured in all jars, however, all treatment soil surface pH values did not differ from the depicted positive control, in contrast to the negative control. (c) Each symbol represents a mean of n = 3, all error bars are ± SD.
While NH3 can inhibit NO2− dependent on normal or non-normal distributed data and homogeneous or inhomogeneous variances.

9, 24 h after the treatment application. Different statistical analyses have been used to determine differences, significance: the mentioned test where all inhibition treatments for each N substrate are tested against each other. Level of inhibition present. Sterilizing the soil significantly lowered NH2OH derived codenitrification fluxes to 617 ± 3034 µg N2O-N m⁻² h⁻¹. Under NH2OH, hybrid N2O fluxes equalled 3851 ± 3432 µg N2O-N m⁻² h⁻¹. With NH4⁺ the N2O fluxes from the positive control (urea only at natural abundance) had a N2O-15N enrichment of 0.363 ± 0.004 (SD) on Day 9. At the same time, the addition of an N substrate resulted in small increases in the N2O-15N enrichments in all treatments with the following exceptions (Table 2): the phenylalanine treatment with inhibition treatments occurred in the NH2OH treatment where applying bacterial inhibition, either alone or within the combined inhibition treatment, caused significant decreases in N2O-15N enrichment relative to the no inhibition treatment (Table 2). Applying phenylalanine also resulted in enhanced N2O-15N enrichment, mostly when applied to the sterilized soil but this was not statistically different from the no inhibition treatment (Table 2). With NH4⁺ as the N substrate the N2O-15N enrichment was again highest in the sterilized soil treatment, but none of the inhibitor treatments caused N2O-15N enrichment to differ from the no inhibitor treatment (Table 2). The biggest shifts in N2O-15N enrichment with inhibition treatments occurred in the NH4OH treatment where applying bacterial inhibition, either alone or within the combined inhibition treatment, caused significant decreases in N2O-15N enrichment relative to the no inhibition treatment (Table 2).

**Discussion**

The hydrolysis of urea and its resulting products increases NH4⁺ and OH⁻ concentrations in the soil9, with the latter responsible for the elevated soil surface pH observed in treatments containing urea. Urea application elevated soil NH4⁺-N concentrations, as evidenced by the higher concentrations in the positive control when compared with the negative control. Elevated soil pH will have resulted in the NH4⁺/NH3 equilibrium shifting towards NH4⁺. However, by Day 8 the concentration of NH4⁺ will have been relatively low based on soil pH values at this time7. While NH4⁺ can inhibit NO3⁻ oxidisers under urea-affected soil10 the elevated soil NO3⁻-N concentrations at the end of the experiment and the decline in NO3⁻ from Day 1 to 7 demonstrates NO3⁻ oxidisers were functioning. The soil NO3⁻-N concentration on Day 9 was higher when compared to a previous study by Rex et al.38, at a similar time following urea application. This higher soil NO3⁻-N concentration is likely to have occurred due to

### Table 2. N2O-15N enrichment (atm%) of the inhibitor × N substrate treatments on Day 9, 24 h after the treatment application.

| N substrate | no inhibition | fungal inhibition | bacterial inhibition | combined inhibition | sterilized soil | test & significance |
|--------------|---------------|-------------------|----------------------|---------------------|----------------|---------------------|
| Glycine      | 0.370 ± 0.001 | 0.380 ± 0.001     | 0.373 ± 0.002        | 0.375 ± 0.006       | 1.211 ± 0.104  | Tukey*              |
| Phenyl      | 0.363 ± 0.003 | 0.377 ± 0.003     | 0.360 ± 0.003        | 0.378 ± 0.011       | 0.900 ± 0.170  | Tukey*              |
| Ammonium     | 0.481 ± 0.034 | 0.374 ± 0.003     | 0.475 ± 0.026        | 0.384 ± 0.003       | 0.896 ± 0.088  | Tukey*              |
| Hydroxyl     | 41.587 ± 1.414| 43.147 ± 4.055    | 27.163 ± 1.555       | 30.384 ± 3.499      | 44.219 ± 4.625 | Dunnett’s Method*   |

### Table 3. Codenitrification fluxes (N2O-N, µg N2O-N m⁻² h⁻¹) of the inhibitor × N substrate treatments on Day 9, 24 h after the treatment application.

| N substrate | no inhibition | fungal inhibition | bacterial inhibition | combined inhibition | sterilized soil | test & significance |
|--------------|---------------|-------------------|----------------------|---------------------|----------------|---------------------|
| Glycine      | 16 ± 0        | 9 ± 0             | 14 ± 0               | 10 ± 0              | 0 ± 0          | Holm-Sidak*         |
| Phenyl      | 13 ± 0        | 9 ± 0             | 12 ± 0               | 10 ± 0              | 0 ± 0          | Holm-Sidak*         |
| Ammonium     | 17 ± 0        | 9 ± 0             | 12 ± 0               | 7 ± 0               | 0 ± 0          | Tukey*              |
| Hydroxyl     | 3851 ± 365    | 3432 ± 717        | 3034 ± 190           | 2196 ± 853         | 617 ± 138      | Holm-Sidak*         |

**N2O-15N enrichment.** The positive control (urea only at natural abundance) had a N2O-15N enrichment of 0.363 ± 0.004 (SD) on Day 9. At the same time, the addition of an N substrate resulted in small increases in the N2O-15N enrichments in all treatments with the following exceptions (Table 2): the phenylalanine treatment with either no inhibition or bacterial inhibition, and the NH4⁺ treatment with bacterial inhibition (Table 2). Within a given N substrate treatment, when comparing the N2O-15N enrichment of the no inhibition treatment and a specific inhibitor treatment, few treatment differences occurred. Under glycine only the sterilized soil treatment varied, with a higher N2O-15N enrichment relative to the no inhibition treatment (Table 2). Applying phenylalanine also resulted in enhanced N2O-15N enrichment, mostly when applied to the sterilized soil but this was not statistically different from the no inhibition treatment (Table 2). With NH4⁺ as the N substrate the N2O-15N enrichment was again highest in the sterilized soil treatment, but none of the inhibitor treatments caused N2O-15N enrichment to differ from the no inhibitor treatment (Table 2). The biggest shifts in N2O-15N enrichment with inhibition treatments occurred in the NH4OH treatment where applying bacterial inhibition, either alone or within the combined inhibition treatment, caused significant decreases in N2O-15N enrichment relative to the no inhibition treatment (Table 2).
the reduced potential for nitrifier inhibition\(^9,10\), a consequence of the lower urea-N rate used in the current study. Considering the soil pH and inorganic-N dynamics it can be concluded that the application of urea was representative of conditions under a typical urine patch\(^41,42\), and that the N substrate treatments were applied during a period of relatively rapid inorganic-N transformation.

The rapid increase in \(\text{N}_2\text{O}\) fluxes following inhibitor application was partially the result of physically mixing the soil in order to distribute the inhibitors, which resulted in entrapped \(\text{N}_2\text{O}\), in the soil, being released\(^43\). Furthermore, soil, not previously exposed to oxygen, would have become exposed and thus there is also the possibility that inhibition of \(\text{N}_2\text{O}\) reductase occurred, preventing complete denitrification\(^44\). However, the application of substrate-N also contributed to the \(\text{N}_2\text{O}\) flux as demonstrated by the increased \(\text{N}_2\text{O}\)\(^{15}\text{N}\) enrichments, particularly in the case of the \(\text{NH}_2\text{OH}\) treatment (Fig. 1a).

Soil \(\text{N}_2\text{O}\) emissions are strongly driven by the presence and turn-over of \(\text{NO}_2^-\) which is the ‘gate-way molecule’ for \(\text{N}_2\text{O}\) production\(^6,47\). In the current study soil \(\text{NO}_2^-\) concentrations were elevated on Day 9 but at concentrations lower than previously observed (e.g. Clough et al.\(^31\)) due to the lower urea application rate in the current study preventing \(\text{NH}_3\) inhibition of \(\text{NO}_2^-\) oxidation\(^48\). Hence, the ensuing \(\text{N}_2\text{O}\) emissions most likely result from the net effects of microbial processes utilising \(\text{NO}_3^-\) and/or the N substrate added.

The effects of the microbial inhibitors, cycloheximide, streptomycin and heat sterilization on \(\text{N}_2\text{O}\) production were assessed 12 h after inhibitor application since maximum efficacy is reported within 24 h of application\(^46\). The decline in the \(\text{N}_2\text{O}\) fluxes following fungal inhibition within the amino acid and \(\text{NH}_2\text{OH}\) treatments demonstrates fungal mechanisms were responsible for a portion of the \(\text{N}_2\text{O}\) produced (21–46%). Previous studies have shown fungi are able to produce \(\text{N}_2\text{O}\)\(^32,33,47,48\). Nitric oxide reductase (\(\text{P}450\text{nor}\)), is a key feature of fungal denitrification and has been observed to require hypoxia and either \(\text{NO}_2^-\) or \(\text{NO}_3^-\) substrate to generate \(\text{N}_2\text{O}\),\(^47,48\); these conditions occurred within the current study. Biotic \(\text{N}_2\text{O}\) emissions from non- autoclaved soil suspensions can be stimulated by the presence of both \(\text{NH}_2\text{OH}\) and \(\text{NO}_3^-\), as was the case in the \(\text{NH}_2\text{OH}\) treatment of the current study. Thus, the decline in \(\text{N}_2\text{O}\) emissions in the \(\text{NH}_2\text{OH}\) treatment, with fungal inhibition, implies a fungal mechanism was partially responsible for the \(\text{N}_2\text{O}\) flux, via \(\text{NH}_2\text{OH}\) utilisation.

With bacterial inhibition, the decline in the \(\text{N}_2\text{O}\) flux under the \(\text{NH}_2\text{OH}\) treatment likely occurred due to the bacterial inhibitor preventing the function of the ammonia oxidising bacteria (AOB), which utilise \(\text{NH}_3\) of \(\text{NH}_2\text{OH}\) to gain energy\(^9\). Increased mRNA transcription levels of the functional genes present in AOB that encode for \(\text{NH}_2\text{OH}\) oxidoreductase (\(\text{haoA}\)), and the reductases for \(\text{NO}_2^-\) and \(\text{NO}_3^-\), which are \(\text{nirK}\) and \(\text{norB}\), respectively, become elevated following \(\text{NH}_2\text{OH}\) application\(^6,40\). A similar result and explanation might have been expected following bacterial inhibition in the \(\text{NH}_4^+\) treatment, given that \(\text{NH}_2\text{OH}\) is an intermediate in the nitrification pathway, however the result was not statistically significant (Table 1). Lower \(\text{N}_2\text{O}\) fluxes from the glycine treatment under bacterial inhibition may have also resulted from a diminished nitrification rate of the \(\text{NH}_4^+\) derived from the mineralized glycine-N, and thus delivering less \(\text{NO}_2^-\) to the soil pool. However, this did not occur under the phenylalanine treatment possibly because it is a more complex molecule and potentially slower to be mineralized, and thus potentially bacteria played less of a role in the \(\text{N}_2\text{O}\) fluxes derived from phenylalanine. Again, with glycine the combined inhibition treatment demonstrated the role of fungi in generating \(\text{N}_2\text{O}\). This was also the case with phenylalanine where the combined inhibition cut \(\text{N}_2\text{O}\) emissions to a level comparable to fungal inhibition alone.

The near complete suppression of \(\text{N}_2\text{O}\) emissions in the amino acid and \(\text{NH}_4^+\) treatments, under the combined inhibition treatment, demonstrates that the observed \(\text{N}_2\text{O}\) fluxes were almost entirely from biologically driven processes. As previously shown, from the \(\delta^{13}\text{C}\) signatures of respired amino acid–\(\text{CO}_2\)-C, amino acids are readily mineralized, forming \(\text{NH}_4\)\(^43\). Consequently, amino acids will contribute to \(\text{N}_2\text{O}\) fluxes if this \(\text{NH}_4^+\) is nitrified, or via the denitrification of the nitrification products\(^41\). The residence time of amino acids in soils is generally reported in hours and depends on soil type\(^41-43\). However, the lack of a significant \(\text{N}_2\text{O}\) flux response to amino acid and \(\text{NH}_4^+\) substrate additions at Day 9, relative to the positive control (Fig. 1), is most likely due to the large background \(\text{NH}_4^+\) pool present at the time of N substrate addition, derived from the urea addition. Hence, the \(\text{NH}_4^+\) formed from either amino acid mineralization or direct \(\text{NH}_4^+\) addition will have been diluted by at least 10-fold, assuming all substrate-N was immediately available. Furthermore, it is likely other amino acids were also present to further dilute the amino acid additions. For example, after extracting three soils McLain and Martens\(^44\) found the sum of 18 amino acids to range from 9 to 20 g kg\(^-1\) of soil, when examining an arid grassland (Well-drained Typic Torrifluvents of the Pima series). In contrast to the soil used in this study, these amino acid concentrations referred to a non-irrigated soil with an expected lower microbial abundance.

With the exception of \(\text{NH}_2\text{OH}\), the near-zero \(\text{N}_2\text{O}\) emissions after applying the N substrates to the sterilized soils indicated that the \(\text{N}_2\text{O}\) fluxes were dominated by biotic processes. This was not the case for \(\text{NH}_2\text{OH}\) where the \(\text{N}_2\text{O}\) flux from the sterilized soil was ~28% that of the no inhibition treatment. It has previously been shown that the \(\text{NH}_2\text{OH}\) molecule may decompose abiotically to produce \(\text{N}_2\text{O}\)\(^48,56\). The lack of any corresponding shifts in the relatively low \(\delta^{15}\text{N}\) enrichments of the \(\text{N}_2\text{O}\) evolved from the amino acid treatments, under the various inhibition treatments, suggests fungi were not directly utilising the amino acids for \(\text{N}_2\text{O}\) production. The codenitrification product depends on the redox state of the N-donor, and prior studies have shown amines (-R-NH\(_2\)) to be codenitrified to \(\text{N}_2\)\(^47\). Thus, the lack of any corresponding shifts in the relatively low \(\delta^{15}\text{N}\) enrichments of the \(\text{N}_2\text{O}\) evolved from the amino acid treatments, under the various inhibition treatments, suggests fungi were not directly utilising the amino acids for \(\text{N}_2\text{O}\) production. The codenitrification product depends on the redox state of the N-donor, and prior studies have shown amines (-R-NH\(_2\)) to be codenitrified to \(\text{N}_2\text{O}\). Thus, the lack of any corresponding shifts in the relatively low \(\delta^{15}\text{N}\) enrichments of the \(\text{N}_2\text{O}\) evolved from the amino acid treatments, under the various inhibition treatments, suggests fungi were not directly utilising the amino acids for \(\text{N}_2\text{O}\) production. The codenitrification product depends on the redox state of the N-donor, and prior studies have shown amines (-R-NH\(_2\)) to be codenitrified to \(\text{N}_2\text{O}\). Thus, the lack of any corresponding shifts in the relatively low \(\delta^{15}\text{N}\) enrichments of the \(\text{N}_2\text{O}\) evolved from the amino acid treatments, under the various inhibition treatments, suggests fungi were not directly utilising the amino acids for \(\text{N}_2\text{O}\) production. The codenitrification product depends on the redox state of the N-donor, and prior studies have shown amines (-R-NH\(_2\)) to be codenitrified to \(\text{N}_2\text{O}\). Thus, the lack of any corresponding shifts in the relatively low \(\delta^{15}\text{N}\) enrichments of the \(\text{N}_2\text{O}\) evolved from the amino acid treatments, under the various inhibition treatments, suggests fungi were not directly utilising the amino acids for \(\text{N}_2\text{O}\) production.
Using soil suspensions Spott and Stange concluded N₂O production from NH₂OH in soil was complex due to the interaction of production pathways involving both abiotic formation and biogenic formation, resulting from both codenitrification and denitrification. Adding the NH₂OH substrate to the sterilized soil (abiotic conditions) the 1⁵N enrichment of the N₂O (~44 atom%) aligned closely with the calculated 1⁵N enrichment of 49 atom% that indicates hybrid N₂O production via abiotic N-nitrification. The formation of N₂O via NH₂OH reacting with NO⁻ occurs due to abiotic nitrosation processes, and has been previously observed in sterilized soils. The NH₂OH compound has also been reported to decay abiotically to form N₂O with the process slowed down when NO⁻ is present. However, had this been the main process for N₂O formation the 1⁵N enrichment of the N₂O evolved would have aligned more with the applied NH₂OH-¹⁵N enrichment. The combined inhibition treatment significantly decreased the N₂O codenitrification flux by 50% (Table 3) compared to the no inhibition treatment (Table 2) indicating abiotic reactions were also contributing substantially to the observed N₂O flux.

Fungi contributed to N₂O production when NH₂OH was applied, as indicated by the flux decrease under the fungal inhibition treatment, however, the lack of any change in the N₂O-¹⁵N enrichment indicates fungal inhibition was not affecting the process generating ¹⁵N enriched N₂O. Conversely, the further decrease in both the N₂O and N₂O-¹⁵N enrichment in the bacterial inhibition and the combined inhibition treatments, showed that the N₂O production process was inhibited, and that less ¹⁵N enriched NH₂OH contributed to the N₂O flux produced. Therefore, the codenitrification flux also tended to decline in the presence of the bacterial inhibitor. Bacterial inhibition diminishes, amongst others, the activity of AOB and thus (i) lowers the consumption of NH₂OH via bacterial nitrification, (ii) lowers the enrichment of the nitrification products derived from ¹⁵N enriched NH₂OH, and thus (iii) the formation of ¹⁵N enriched nitrification intermediaries NO₂⁻ and NO declines. Since NO₂⁻ and NO have been shown to be involved in codenitrification, decreases in the concentration of these molecules would lead to lower N₂O fluxes with lower ¹⁵N enrichment. Furthermore, had NH₂OH progressed to NO⁻ then any denitrification of this NO⁻ that contributed to the ¹⁵N enriched N₂O pool, would also have occurred at a slower rate or been prevented with inhibition of bacterial denitrifiers.

Conclusions
Codenitrification occurs when N-donors, such as those studied here (NH₄⁺, glycine, phenylalanine and NH₂OH) react with a nitrosyl compound, to form hybrid N₂O. Using selective microbial inhibition treatments, and simulating a ruminant urine patch environment, we demonstrated that all the used ¹⁵N-labelled N substrates contributed to codenitrification in a soil matrix. Hydroxylamine was the most important N substrate with respect to increasing the N₂O flux and contributing to codenitrification (85.7% of total flux), likely because of its more reactive character compared to the other N substrates. The codenitrification N₂O fluxes following amino acid-¹⁵N addition were orders of magnitude lower (0.7–1.2% of total flux), potentially due to dilution from antecedent amino acids or their break down products, which in turn means that a contribution of these natural amino acids could be assumed under the experimental conditions. Fungal inhibition resulted in a significant decline in the formation of amino acid derived codenitrification fluxes, underlining once more the importance of fungal code-nitrification vs. bacterial codenitrification. The relatively lower codenitrification N₂O fluxes with amino acids may also be a result of the microbial community structure that is present. Alternatively, codenitrification of NH₂OH to form N₂O has been reported in the absence of organic electron donors hence, given that codenitrification is in principle dependent on organic carbon respiration a lack of organic substrate or variations in its form may have favoured codenitrification of NH₂OH. The results of this study, demonstrated that codenitrification occurs via multiple pathways in a pasture soil following a simulated bovine urine event. Codenitrification resulting from the presence of NH₂OH is likely to be the dominant process, in the short-term following the deposition of ruminant urine with its relatively high urea-N loading. The results warrant further in situ investigation of the dynamics of potential N-donors, in conjunction with N₂O fluxes, under ruminant urine patches.

Materials and Methods
Experimental design. A bulked soil sample was taken from a sandy loam pasture soil on the Lincoln University dairy farm (0–10 cm). New Zealand (43°38′25.23″S, 172°27′24.71″E, Typic Immature Pallic Soil, (USDA: Udic Haplustalf)). The pasture consisted of perennial rye grass (Lolium perenne L.) and white clover (Trifolium repens L.). Field moist soil was sieved (4 mm) to remove stones and plants and then placed into jars (250 mL, Ø 8.1 cm), corresponding to 100 g dry weight (ca. 82 cm³), and moistened to 50% of water-holding capacity (ca. 83% water-filled pore space).

Initially the jars, with soil, were placed in an incubator, in the dark, at 23 °C and wetted-up daily to preincubation weight. After four days, any germinated weed seedlings were removed and the experimental period of 14 days commenced (Day 0 to Day 11). An aqueous urea solution (500 µg urea-N g dry soil⁻¹) was applied on Day 0 in order to simulate a bovine urine deposition event. On Day 8, microbial inhibition treatments were applied with the N substrate treatments applied immediately after this in an aqueous solution (4 mL) as noted below.

Treatments consisted of ¹⁵N enriched N substrate species (glycine (98), L-phenylalanine (98), NH₄⁺ (99) and NH₂OH (98); atom% ¹⁵N enrichment in bracket) with each N substrate treatment further split into five microbial inhibition treatments (no inhibition, fungal inhibition, bacterial inhibition, fungal and bacterial inhibition ('combined inhibition') and soil total microbial inhibition (heat sterilised soil)). Treatments were replicated thrice. The amino acid-N concentrations were based on the findings of Scheller and Raupp and in order to apply a realistic concentration, these were applied at equal ¹⁵N enrichment. Hydroxylamine and NH₄⁺ were applied at equal N rates for comparative purposes.

According to Anderson and Domsch, cycloheximide, a fungal inhibitor, was applied at a rate of 8 mg g⁻¹ soil and streptomycin, a bacterial inhibitor, at a rate of 5 mg g⁻¹ soil. Both chemicals were applied as a dry powder on to the soil surface and subsequently mixed into the soil with a spatula for 1 min. The combined inhibition
included the simultaneous application of cycloheximide and streptomycin and was designed to inhibit both bacteria and fungi. Sterilizing (as complete microbial inhibition) was performed by heating the soil. This was achieved by microwaving the soil in the jars for 4 minutes, remoistening the dry soil, and then microwaving the jars for another 3 minutes, as microwave heating is a proven method to stop microbial activities\(^{62,63}\). Thereafter, the microwaved soils were readjusted to 50% water-holding-capacity and also mixed for 1 minute. The control treatment contained urea, but no inhibitors were applied, and the soil was mixed to replicate the physical disturbance of the other treatments. Immediately after application of the inhibitor treatments the N substrate treatments were applied according to treatment at a rate of 20µg N g\(^{-1}\) dry soil, without subsequent mixing.

In addition, three further control treatments were set up; a positive control (soil with urea but no N substrate or inhibitor addition (n = 3), also physically mixed on Day 8; a negative control (n = 3) consisting of soil without urea, inhibitors, or N substrates, also physically mixed on Day 8; and a separate NO\(_2\) control (soil with urea but no N substrate addition, physically mixed on Day 8) for soil NO\(_2\)−N sampling at 4 different times over the duration of the experiment.

**Gas sampling and analysis.** On Day −2, −1, 0, 1, 2, 4, 6, 7, 8 (before inhibitor application), 9, 10 and 11, the jars were sealed with lids equipped with rubber septa. Jar headspace gas samples were taken with a plastic syringe, fitted with a three-way-stop cock and a 25G hypodermic needle, and injected into a previously evacuated Exetainer\(^{64}\) vials (Labco Ltd., High Wycombe, UK). The first gas sample (12 mL) was taken immediately after sealing the jar headspace. The second gas sample was taken after 1 h, only from the positive control to verify the linearity of the increase in the headspace gas concentration, and the third gas sample was taken after a 2 h incubation time (12 mL, all jars). On Days 8, 9, 10 and 11, the third gas sample (30 mL), was split between a 6 mL Exetainer\(^{64}\) that received 12 mL, and an evacuated and helium flushed 12 mL Exetainer\(^{64}\) that received 18 mL for \(^{15}\)N-N\(_2\)O determination.

Nitrous oxide concentrations were determined using a gas chromatograph (SRI-8610, SRI Instruments, Torrance, CA) coupled to an autosampler (Gilson 222XL; Gilson, Middleton, WI) equipped with a \(^{63}\)Ni electron capture detector\(^{64}\). PeakSimple 4.44 software (SRI Instruments, Torrance, CA) and several N\(_2\)O standards (range 0–100 µL L\(^{-1}\)) were used to determine the N\(_2\)O concentrations. The N\(_2\)O fluxes (µg N\(_2\)O-N m\(^{-2}\) h\(^{-1}\)) were determined using the following equation:

\[
N_2O\text{ flux} = \frac{V \times \Delta N_2O \times P \times R \times T}{m_N \times t^{-1} \times A^{-1}}
\]

\(V\) = headspace volume (L). \(\Delta N_2O\) = change in headspace N\(_2\)O concentration during sampling (µL L\(^{-1}\)). \(P\) = pressure (atm). \(R\) = gas constant (0.08206 L atm K\(^{-1}\) mol\(^{-1}\)). \(T\) = temperature (K). \(m_N\) = mass of N per mole of N\(_2\)O (g mol\(^{-1}\)). \(t\) = time (h). \(A\) = soil surface area (m\(^2\)).

The \(^{15}\)N enrichment of the N\(_2\)O evolved was determined by analysing the gas samples with a continuous-flow-isotope ratio mass spectrometry CFIRMS (Sercon 20/20; Sercon, Cheshire, UK) inter-faced with a TGII cryofocusing unit (Sercon, Chesire, UK). If required, gas samples were diluted by injecting 4 mL of sample gas into a helium-filled 12 mL Exetainer\(^{64}\) (1:4 dilution).

The measured \(^{15}\)N concentration of the headspace N\(_2\) was close to natural abundance thus a determination of the N\(_2\) flux was not possible, hence, the N\(_2\) emissions were not considered further.

**Codenitrification calculations.** As previously reported\(^{20}\) conventional denitrification produces N\(_2\)O (non-hybrid N\(_2\)O) while N\(_2\)O produced via codenitrification results in an N atom from NO\(_2\). The following calculations determine the codenitrification flux, assuming that hybrid N\(_2\)O only arises from codenitrification. We do not distinguish between the roles of biotic and abiotic reactions in this process. However, the use of biological inhibitors and soil sterilization indicate the relative roles of abiotic and biotic processes in producing hybrid N\(_2\)O.

For the N\(_2\)O evolved it was assumed that this was generated from one \(^{15}\)N enriched pool-fraction (\(d'_i\)\() with \(^{15}\)N enriched N (\(d_N\)) and a fraction (\(d'_N\)) equal to 1 − \(d'_i\) derived from a pool or pools at natural abundance (\(d_N\)).

The ratios \(r'_1\) and \(r'_2\) were determined from the N\(_2\)O m/z ion currents at m/z 44, 45 and 46\(^{65}\):

\[
\begin{align*}
\frac{r'_1}{r'_2} &= \frac{45}{44} \quad (1) \\
\frac{r'_2}{r'_3} &= \frac{46}{44} \quad (2)
\end{align*}
\]

where, \(44_i\), \(45_i\) and \(46_i\) represent the ion-currents of the N\(_2\)O mass fractions 44, 45 and 46.

Then, following Arah\(^{65}\) (equations 22 and 23), the values of the \(^{15}\)N atom fraction of the sample (\(a'_i\)) and the \(46\)N\(_2\)O component of the molecular fraction, of the N\(_2\)O molecule, in the sample (\(x'_i\)) were calculated using \(r'_i\) and \(r'_3\), while allowing for the presence of oxygen isotopes.

In Arah\(^{65}\) \(a'_i\) and \(x'_i\) are defined as follows:

\[
a'_i = (1 - d'_D - d'_N) \ast a'_A + d'_D \ast a'_D + d'_N \ast a'_N
\]

\[
x'_i = (1 - d'_D - d'_N) \ast a'_2 + d'_D \ast a'_D + d'_N \ast a'_N
\]
When letting $d'_s$ equal $(1 - d'_p)$ and $a'_s$ equal the $^{15}$N enrichment at natural abundance (0.003663) Eqs 3 and 4, when set to equal zero, become:

$$0 = d'_D + a'_D + (1 - d'_D) \cdot 0.003663 - a'_s$$

$$0 = d'_D + a'_D + (1 - d'_D) \cdot 0.003663^2 - x'_s$$

Since $x'_s$ and $x'_P$ are known the values of $d'_D$ and $a'_s$ can be determined using the Solver function in Microsoft Excel \textsuperscript{TM}, while setting the target value at zero, with the result accepted when the target value is $< 1 \times 10^{-5}$.

Then the codenitrification flux was calculated according to Clough et al. (2001) as:

$$d_{CD} = -\Delta^{15}R_p^2/(-\Delta^{15}R_p^2 + \Delta^{45}R_p^2 + qP_2 - qP_1)$$

were $d_{CD}$ is the fraction of $N_2O$ within the headspace derived from codenitrification and $\Delta^{45}R$ is the $^{15}N/^{14}N$ ratio, while $P_1$ (0.9963) and $P_2$ (0.0037) are fractions of $^{14}N$ and $^{15}N$ in the natural abundance pool, and where $q_1$ equals $a_1D$, derived above, with $p_2$ equal to $1 - q_2$.

Finally the codenitrification flux was determined as:

$$N_2O_{CD} = d_{CD} \times (\text{total } N_2O \text{ flux})$$

### Surface pH and inorganic-N measurement.

Surface pH was measured on Days 2, 0, 1, 3, 5, 7, 9 and 11, by adding one drop of deionised water to the soil surface and then placing a flat surface pH probe (Broadley James Corp., Irvine, California) onto the soil surface. The $NO_3^-$ concentration in the unmixed $NO_2^-$ control (soil + urea solution) was determined by subsampling soil with a corer (diameter 1.6 cm, depth 1.5 cm). The soil was then blended with 2 M potassium chloride (KCl), adjusted to pH 8 with potassium hydroxide \textsuperscript{66} at a 1:6 ratio. This procedure was performed on Days 1, 4, 6 and 10. Subsamples of moist soil (4 g dry weight) were taken after Day 11, from the positive and negative controls, and extracted with 2 M KCl in order to determine the $NH_4^+$ and $NO_3^-$ concentrations at the end of the experiment \textsuperscript{66}. Inorganic-N concentrations in the extracts were determined using Flow Injection Analysis \textsuperscript{66}.

### Statistics.

The single jars were defined as experimental units by the independent applications of treatments. The experiment focused on achieving the most sensitive test of treatment differences and inference is not claimed for a population wider than the paddock, used for sampling. All statistical analyses were performed using SigmaPlot 13.0 (Systat Software Inc., Chicago). For each variable of interest a general linear model (ANOVA) was fitted with $N$ substrate treatment or a factorial combination of $N$ substrate treatment and inhibition method as explanatory variables. Using this method, the different inhibition treatments within each $N$ substrate treatment were compared. Tests for normality (Shapiro-Wilk test) and variance (Brown-Forsythe test) were used to evaluate the residuals and define the most powerful test for each comparison of means. Hence, means comparisons were adjusted for multiplicity using Tukey, Holm-Sidak, Dunn’s or Student’s t-test adjustments to $p$ values.

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Author Contributions

K.R. and G.L. were the principal investigators for the project funding. D.R., T.C., K.R. and G.L. designed the experiment. D.R. conducted the laboratory work related to this experiment and conducted the analysis. D.R., T.C. and G.L. carried out the calculations. K.R., T.C. and D.R. outlined the manuscript and completed it with help of L.C., C.D.K. and S.M.

Additional Information

Compelling Interests: The authors declare no competing interests.

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