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Long-term elevation of temperature affects organic N turnover and associated N_2O emissions in a permanent grassland soil

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Abstract

Over the last century an increase in mean soil surface temperature has been observed and it is predicted to increase further in the future. To evaluate the legacy effects of increased temperature on both nitrogen (N) transformation rates in the soil and nitrous oxide (N_2O) emissions, an incubation experiment was conducted with soils taken from a long term in situ warming experiment on temperate permanent grassland. In this experiment the soil temperature was elevated by 0 (control), 1, 2 or 3°C (4 replicates per treatment) using IR-lamps over a period of 6 years. The soil was subsequently incubated under common conditions (20°C and 50% humidity) and labelled with NO_3**$^{15}NH_4$** Gly, **$^{15}NO_3$**NH_4 Gly or NO_3NH_4 **^{15}N-Gly**. Both inorganic N (NO_3^-+NH_4^+) and NO_3^- contents were higher in soil subjected to the +2 and +3°C temperature elevations. Analyses of N transformations using a ^{15}N tracing model, showed that, following incubation, gross organic (and not inorganic) N transformation rates decreased in response to the prior soil warming treatment. This was also reflected in reduced N_2O emissions associated with organic N oxidation and denitrification. A newly developed source partitioning model showed the importance of oxidation of organic N as a source of N_2O. Concluding, long term soil warming can cause a legacy effect which diminishes organic N turn over and the release of N_2O from organic N and denitrification.

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1. Introduction

Globally, managed pastures were estimated to occupy 34.7 million square kilometres in 2000 and this area is projected to increase by a further 13.4% by 2050 (Tilman et al., 2001). Concomitantly, the Earth's mean surface temperature has increased by 0.6°C in the past century with surface temperatures expected to increase by a further 1.5-4.5°C resulting from a doubling of the atmospheric carbon dioxide (CO_2) concentration (IPCC, 2013). Agricultural soils play a central role in the global carbon (C) and nitrogen (N) cycles (French et al., 2009), and C-N interactions are to a large extent affected by temperature (Luo, 2007). Thus, research into the effect of elevated soil temperatures is essential to better understand biogeochemical N cycling in grassland ecosystems.

Previous research generally showed an increase in both net (Peterjohn et al., 1994; Rustad et al., 2001; Norby and Luo, 2004; Butler et al., 2012; Bai et al., 2013; Björsne et al., 2014; Zhang et al., 2015b) and gross (Larsen et al., 2011; Björsne et al., 2014) mineralisation under elevated soil temperatures. However, not all studies found this effect (Emmett et al., 2004; Niboyet et al., 2011; Andresen et al., 2015). An effect on N immobilisation or nitrification was generally not observed (Emmett et al., 2004; Barnard et al., 2005; Andresen et al., 2010; (Niboyet et al., 2011; Bai et al., 2013; Björsne et al., 2014). Dijkstra et al. (2010) and Bai et al. (2013) identified, in their meta-analyses, increases in inorganic N under elevated soil temperatures. Most of this inorganic N increase occurred as nitrate (NO_3^-) (Dijkstra et al., 2010). Peterjohn et al. (1994) also found that average monthly ammonium (NH_4^+) concentrations increased in a mineral soil under forest, however, daily average concentrations did not differ. In the same study, no differences in NO_3^- concentrations were observed, and the amount of extractable NO_3^- was very small. Another meta-analysis showed no effect of soil warming on total soil N,

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56 NH_4^+ or NO_3^- in a Tibetan grassland (Zhang et al., 2015b). Which is in line with other studies
57 regarding total soil N (Bai et al., 2013) and inorganic N (Larsen et al., 2011).
58
59 N mineralisation follows a step-wise sequence of protein depolymerisation by extracellular
60 activity to oligomers (e.g. peptides) and monomers (e.g. amino acids) and then uptake by
61 microorganisms before mineralisation to NH_4^+ (Schimel and Bennett, 2004). Hence,
62 production of peptides and amino acids as well as mineralisation of amino acids, affects the
63 main fluxes regulating gross N mineralisation. Amino acids have a short residence time in the
64 soil due to either rapid assimilation by soil microbes or mineralisation, which occurs within a
65 few hours (Farrell et al., 2014). In heathland and grassland soils no effect of soil warming on
66 the amino acid concentration was observed (Chen et al., 2014; Andresen et al., 2015).
67
68 Nitrous oxide (N_2O), a potent greenhouse gas with a global warming potential of 298 on a 100
69 year basis, can be produced by several processes, such as nitrification, partial denitrification,
70 co-denitrification and the oxidation of organic matter (Butterbach-Bahl et al., 2013; Zhang et
71 al., 2015a) (Fig. 1). Laughlin and Stevens (2002) confirmed the importance of co-
72 denitrification for N_2 production, a process that may comprise 25% of the total N balance in
73 pastures (Selbie et al., 2015). Müller et al. (2014) quantified for the same grassland soil as used
74 in this study the contribution of co-denitrification to the total N_2O production. N_2O emissions
75 following fertilisation with ammonium nitrate (NH_4NO_3) may be greater than from urea
76 fertiliser because of the greater susceptibility to denitrification (Harrison and Webb, 2001). The
77 amount and form of N inputs primarily govern N_2O emissions with further impacts resulting
78 from climatic factors, such as temperature and precipitation, and soil factors, such as C
79 availability and microbial community structure (Harrison and Webb, 2001; Müller et al., 2003;
80 Stark and Richards, 2008; Laughlin et al., 2009; Li and Lang, 2014). However, the impact of

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elevated soil temperature on N_2O production, in semi-natural grasslands is unclear (Peterjohn et al., 1994; Bijoor et al., 2008; Larsen et al., 2011). Furthermore, there has been very limited research into the effect of elevated soil temperature on the different N_2O production processes. Maag and Vinther (1996) observed a decrease in nitrification associated N_2O emissions and an increase in denitrification associated N_2O with increasing soil temperature. It has been suggested that this was due to creation of anoxic conditions and the associated depletion of oxygen following the increase in microbial respiration with higher soil temperatures (Castaldi, 2000). Prolonged elevated soil temperatures, on the other hand, could also lead to changes in the microbial community (Avrahami and Conrad, 2003; French et al., 2009).

Several methods, such as source partitioning, have been used to quantify the contributions of individual N pools to N_2O emissions (Stange et al., 2009; Rütting et al., 2010; Zhang et al., 2011; Zhu et al., 2011; Stange et al., 2013; Müller et al., 2014). However, one of the assumptions of the source partitioning method is the absence of hybrid reactions such as co-denitrification (Zhang et al., 2015a). Because of the potential importance of co-denitrification for the N2O production, it should not be omitted from the analysis of N_2O sources. Currently, only one technique is available to identify several processes including a hybrid reaction, which is a full ^{15}N tracing approach (Müller et al., 2014). This approach however, requires data on NO_2^-; NO_3^-/NH_4^+ pool sizes and measurements at multiple time points. Furthermore, it requires at least multiple days of running the model to be able to distinguish the different processes. A straight forward method partitioning N_2O fluxes into several pathways including a hybrid reaction, which does not rely on measurements of NO_2^- and data at multiple time points, would therefore be very beneficial.

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105 The objectives of this study were to quantify the legacy effects of six years of elevated
106 temperature (via IR heaters) on soil N cycling dynamics, including (1) net and gross N
107 transformation rates in the soil (2) N_2O fluxes immediately after fertilisation and (3) the
108 processes responsible for these N_2O fluxes. To determine the processes involved in N_2O
109 production, a new source partitioning method was developed to allow the identification of
110 hybrid reactions. To identify the legacy effect of different in situ temperature treatments on the
111 internal N transformation processes, soil incubations were carried out under identical moisture
112 and temperature conditions in the laboratory. Based on previous observations that gross N
113 transformations in soils are affected by long-term elevated temperature treatments we
114 hypothesized that any associated effects on gaseous N emissions (e.g. N_2O) can be confirmed
115 by a change in the relative emission rates from various pathways. Thus, the newly developed
116 source partitioning method would be helpful to confirm such a change.
117

118 **2. Material and method**

119 *2.1. Site description and field treatment*

120 The 100 m^2 site was established on a permanent grassland of the 'Environmental Monitoring
121 and Climate Impact Research Station Linden' in Germany (50°31.6'N, 8°41.7'E). A full
122 description of the site can be found in Jansen-Willems et al. (in press). Briefly, the site had
123 been managed as a meadow with two cuts per year and fertilised with 50-80 kg N ha^{-1} $year^{-1}$
124 for the last three decades. Since 1995, the N fertiliser input had been reduced to 40 kg N ha^{-1}
125 $year^{-1}$, as KAS (calcium-ammonium-nitrate). The mean annual temperature and precipitation
126 were 9.5°C and 560 mm (observation period: 1995-2014) respectively.
127

128 The site had been divided into 16 plots, four rows of four plots. From January 28, 2008, the
129 soil temperature of each plot, measured at 5 cm depth, was elevated by 0, 1 (mean 0.8 standard

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130 error 0.02), 2 (mean 1.9 standard error 0.03) or 3 (mean 2.6 standard error 0.03) ºC above
131 ambient temperature, using infrared heaters. The use of heaters will also affect the soil moisture
132 content. The temperature treatments (including any moisture effect)are referred to as $T_{control}$,
133 T_1, T_2, and T_3, respectively. The infrared heaters were installed at different heights to create
134 the different temperature elevations (Jansen-Willems et al., in press).
135
136 *2.2. Incubation, labelling and extraction*
137 On the morning of May 12, 2014 the heaters were turned off. All the soil within a circular area
138 of 318 cm^2 directly underneath each infrared lamp was excavated to 7.5 cm for the tracing
139 experiment. A small subsample of each plot was dried at 70°C for 48 hours, ground and
140 analysed by a CNH Macro Elemental Analyser (Hanau, Germany) for total N content. A
141 subsample of the soil for each plot was dried at 105°C for 24 hours to determine the soil
142 gravimetric water content. The remaining field moist soil was kept at 4°C (for less than 60
143 hours) until further analysis whereupon the soil from each field plot was sieved through a 10
144 mm sieve, to homogenise it and to remove roots. Incubations were carried out in 750 ml jars
145 (WECK GmbH u. Co. KG, Wehr, Germany). Thirteen jars per field plot were prepared each
146 with an average of 67 (stdev 8.4) g dry soil per jar (except for plots 3, 5, 7, 11 and 14, where
147 only 10 jars were prepared due to lack of soil). All jars were closed with glass lids that were
148 fitted with septa to allow for gas sampling. During gas flux analysis the jars were sealed using
149 a clamp and a rubber ring between the jar and the lid. At other times a gap was left between
150 the jar and the lid to allow air exchange while minimising water loss. On May 14 (day 0) all
151 jars were put in a dark climate chamber at 20°C and 50% humidity and incubated for 55 days
152 prior to ^{15}N substrate addition.
153

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Soil gravimetric moisture data were used to determine the exact amount of dry soil in each jar, and to calculate the amount of water to be added to ensure the same soil water content in each jar. On day 2 the soil moisture in each jar was adjusted to a water-filled pore space (WFPS) of 64%. On day 12 and 50 the jars were watered to replenish the water lost due to evaporation.

For the ^{15}N tracing study three different labels were used, NO_3**$^{15}NH_4$** Gly, **$^{15}NO_3$**NH_4 Gly and NO_3NH_4 **^{15}N-Gly** (at 60, 60 and 99 atm% ^{15}N respectively). All solutions contained 50 µg NO_3-N, 50 µg NH_4-N, and 30 µg Gly-N g^{-1} soil. On day 55, the substrate solution was added to each jar using a needle with side-ports, to inject the solution into the soil to minimise disturbance, while providing an equal distribution in the soil (Müller et al., 2007). For each field plot, jars were set up for four soil extractions, at day 0, 1, 3 and 6 after N application, and three labels, except for plot 3, 5, 7, 11 and 14, where due to the lack of soil no NO_3NH_4 **^{15}N-Gly** label addition was possible.

The soil in each jar was extracted with 2M KCl using the blending procedure of Stevens and Laughlin (1995). The ^{15}N enrichments of NO_3^- and NH_4^+ in the extracts were determined by converting NO_3^- and NH_4^+ into N_2O following the procedures by Stevens and Laughlin (1994) for determination of the ^{15}N enrichment in NO_3^- and Laughlin et al. (1997) for the ^{15}N enrichment in NH_4^+. The extraction of soil prior to ^{15}N addition, took place on day 53. The other extractions took place at 0.11 days (+/- 0.004), 1.02 days (+/- 0.001), 2.95 days (+/- 0.001) and 5.93 days (+/- 0.001) after ^{15}N substrate addition, and are hereafter referred to as 0, 1, 3 and 6 days after ^{15}N substrate addition, respectively.

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177 *2.3. Gas sampling*

178 Gas samples were taken from 43 different jars, one jar per ^{15}N label, for each plot. During the
179 pre-incubation gas samples were taken 1, 46 and 48 days before label addition. After labelling,
180 gas samples were taken immediately prior to soil extractions.

181

182 Gas samples were taken using a 60 ml syringe (Ecoject Plus, Gelnhausen, Germany). At time
183 zero (t_0) 15 gas samples were taken from 15 different jars. Then at time 1 (t_1) a gas sample was
184 taken through the rubber septum. At both t_0 and t_1 the syringe was flushed twice with headspace
185 gas to ensure a representative sample was taken. The times between t_0 and t_1 during each of the
186 seven different gas samplings (three before label addition and four immediately prior to
187 extraction) were 120-129, 120, 180, 233, 240, 235 and 214 minutes, respectively. Gas samples
188 were analysed within 24 h after sampling using a GC (Bruker) equipped with an electron
189 capture detector (ECD) for N_2O analysis. An average of the concentrations measured in the 15
190 samples was used as the t_0 concentration for all 43 jars. Fluxes were calculated based on the
191 concentration difference between the two sample points.

192

193 For the ^{15}N abundance of N_2O, a 30 ml sample was taken at t_1 and transferred to a 12 ml
194 Exetainers® vial (Labco Ltd, High Wycombe, Buckinghamshire, UK). The over-pressurised
195 sample vials were returned to ambient pressure immediately before analyses of stable isotopes.
196 The ^{15}N enrichments of $^{15}N_2O$ and $^{15}N_2$ was determined using an automated isotope ratio mass
197 spectrometry (Sercon Ltd 20-20), as described by (Stevens et al., 1993), inter-faced to a TGII
198 cryfocusing unit (Sercon Ltd 20-20).

199

200 *2.4. ^{15}N tracing model*

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The ^{15}N tracing analysis tool described by Müller et al. (2007) was used to quantify gross soil N transformations. In the current study, the original model has been adapted to include an amino acid Gly pool. The model (Fig. 2.) considered seven N pools and 13 N transformations. The N pools were NH_4^+, NO_3^-, amino acids (AA), labile (N_{lab}) and recalcitrant (N_{rec}) organic N, adsorbed ammonium ($NH_4^+{}_{ads}$) and stored nitrate ($NO_3^-{}_{sto}$). The initial NO_3^- and NH_4^+ pool sizes were determined by extrapolating the first two extraction times back to time zero. The initial AA pool size was set to 30 µg N g^{-1} soil, corresponding to the application of Gly. The initial $NH_4^+{}_{ads}$ and $NO_3^-{}_{sto}$ were based on the difference between the added and initial N (Müller et al., 2004). The N transformations are described in Table 1. The N transformations were calculated based on zero or first order kinetics (Table 1). Whether N_{lab} and N_{rec} were transformed into AA or NH_4^+ was determined by two factors, one for M_{Nlab} and one for M_{Nrec}. This factor determines the fraction of the M_{Nlab} or M_{Nrec} flowing into the AA pool with the remainder entering the NH_4^+ pool. For each temperature treatment the kinetic parameters and the two split factors were simultaneously optimised by minimising the misfit between the modelled and measured NH_4^+ and NO_3^+ concentrations and their respective ^{15}N enrichments (Müller et al., 2004). For treatment T_2 the measurements of the ^{15}N-Gly label were not included in the optimisation because only one replicate was available for this label. A Markov chain Monte Carlo Metropolis algorithm (MCMC-MA) was used for the optimisation, which practices a random walk technique to find global minima (Müller et al., 2007). The uncertainties (standard deviation) of the observations were taken into account by the optimisation routine. The MCMC-MA routine was programmed in MatLab-Simulink (Mathworks Inc) as described in Müller et al. (2007). The most suitable parameter set was determined using the Akaikes Information Criterion (AIC). Gross and net nitrification, and gross and net mineralisation were calculated using equation 1 to 4 in which SF stands for split

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factor. The combined standard deviation was calculated by ((stdev rate 1)^2+(stdev rate 2)^2+……..)$^{0.5}$, in which the stdev of M_{Nx}·SF_{MNx} is the stdev of M_{Nx} multiplied by the SF.

The following combined rates were calculated:

Gross nitrification: $O_{Nrec}+O_{NH4}$ (1)

Net nitrification: $O_{Nrec}+O_{NH4}-I_{NO3}-D_{NO3}$ (2)

Gross mineralisation: $M_{Nlab}·SF_{MNlab} + M_{Nrec}·SF_{MNrec} + M_{AA}$ (3)

Net mineralisation: $M_{Nlab}·SF_{MNlab} + M_{Nrec}·SF_{MNrec} + M_{AA}-I_{NH4Nrec}-I_{NH4Nlab}-I_{NO3}$ (4)

2.5. *Determining contribution of different processes to N_2O flux*

The N_2O fluxes, from the soil labelled with NO_3**$^{15}NH_4$** Gly and **$^{15}NO_3$**NH_4 Gly, were separated into four different processes. These were nitrification, denitrification, co-denitrification and oxidation of organic matter. The N_2O was assumed to be derived from three uniformly distributed pools, and based on initial substrate ^{15}N enrichments, isotopic discrimination was considered negligible for all four processes. Fig. 1. shows the pools and processes accounting for the N_2O production. The ^{15}N content of the organic matter was considered to be at natural abundance (0.3663 atom%). The N_2O produced via co-denitrification consists of one N atom from the NO_3^- pool, and one N atom from the organic N pool. The chance that the N_2O produced via nitrification, denitrification or oxidation of organic N contains zero, one or two ^{15}N enriched atoms can be described by equations 5, 6 and 7, respectively. Where a_x (the ^{15}N fraction of the pool) is a_n for nitrification, a_d for denitrification and a_0 for the oxidation of organic N: a_n, a_d and a_o are explained in Fig. 1.

Chance of 0 ^{15}N atoms: $(1-a_x)^2$ (5)

Chance of 1 ^{15}N atom: $2(1-a_x)a_x$ (6)

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Chance of 2 ^{15}N atoms: a_x^2 (7)

The chance that the N_2O produced via co-denitrification consists of zero, one or two ^{15}N enriched atoms is described by equations 8, 9 and 10 respectively.

Chance of 0 ^{15}N atoms: $(1-a_d)(1-a_0)$ (8)

Chance of 1 ^{15}N atom: $a_d(1-a_0) + a_0(1-a_d)$ (9)

Chance of 2 ^{15}N atoms: $a_d a_0$ (10)

The chance that the N_2O in the gas sample contains zero, one or two ^{15}N atoms is described by equations 11, 12 and 13 respectively. Where the subscripts *d*, *n* and *o* refer to the fractions of N_2O produced by denitrification, nitrification and oxidation of organic N, respectively. The fraction of N_2O produced by co-denitrification is 1-*d*-*n*-*o* as all of the N_2O produced was assumed to come from one of the four processes.

Chance of 0 ^{15}N atoms: $n(1-a_n)^2 + d(1-a_d)^2 + o(1-a_o)^2 + (1-n-d-o)(1-a_d)(1-a_0)$ (11)

Chance of 1 ^{15}N atom: $2n(1-a_n)a_n + 2d(1-a_d)a_d + 2o(1-a_o)a_o + (1-n-d-o)(a_d(1-a_0)+a_0(1-a_d))$ (12)

Chance of 2 ^{15}N atoms: $na_n^2 + da_d^2 + oa_n^2 + (1-n-d-o)a_d a_0$ (13)

The automated continuous-flow isotope-ratio mass spectrometer enabled the measurement of ^{45}R ($^{45}I/^{44}I$) and ^{46}R ($^{46}I/^{44}I$), where ^xI is the ion currents at $m/z\ x$. The ^{45}R and ^{46}R were corrected for the presence of ^{18}O. This, therefore, means that ^{45}R is the fraction of N_2O molecules containing one ^{15}N atom divided by the fraction of N_2O molecules containing zero ^{15}N atoms, and ^{46}R is the fraction of N_2O molecules containing two ^{15}N atoms divided by the fraction of N_2O molecules containing zero ^{15}N atoms. The expected fractions are described by equations

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11 to 13, where a_o was set to 0.003663, a_n and a_d were considered to be the ^{15}N content of NH_4^+ and NO_3^- respectively, while *n*, *d* and *o* were quantified using the *fminsearchbnd* function in MatLab (The MathWorks Inc, Natick, MA). For this the ^{45}R, ^{46}R, a_n and a_d of soil labelled with NO_3**$^{15}NH_4$** Gly and soil labelled with **$^{15}NO_3$**NH_4 Gly were used. The amount of N_2O produced via each process was calculated by multiplying the average N_2O flux from the jars labelled with NO_3**$^{15}NH_4$** Gly and **$^{15}NO_3$**NH_4 Gly with the fractions of N_2O produced by the four different processes. This was carried out separately for each plot and time step. Because of missing $^{15}NH_4$ data, the different processes were not distinguished for plot 1 time step 3. Total N_2O flux contributions were calculated using linear interpolations between time steps.

2.6. Statistical analyses

Total soil N was analysed with the non-parametric Kruskal-Wallis test using IBM SPSS statistics (version 22) because one sample per plot was taken, resulting in only four measurements per treatment. The N_2O fluxes (including different processes), inorganic-N (NO_3^-+NH_4^+), NO_3^- and NH_4^+ concentrations were analysed using the MIXED procedure in SAS (Version 9.3, SAS institute). The N_2O fluxes were transformed using log(flux+10). The N_2O fluxes via the different processes were transformed using $flux^{1/4}$. A Tukey-Kramer adjustment was used to correct for multiplicity effects in pairwise comparisons. Residual checks were made to ensure that the assumptions of the analysis were met. The modelled N transformation rates were analysed using a one-way ANOVA based on the averages and standard deviations in Matlab (Version 2013b, The MathWorks Inc.). The pairwise comparisons were calculated with the Holm-Sidak test in SigmaPlot (Version 11.0, Systat Software Inc.).

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3. Results

3.1. Soil nitrogen pool sizes

Total soil N content did not differ between soil warming treatments prior to the incubation study. A significant interaction between treatment and time affected soil NH_4^+ concentrations, thus, these results are therefore given separately for each time step. No such interaction was found for NO_3^- or total inorganic N (NO_3^-+NH_4^+) concentrations. The total inorganic N content differed with temperature treatment ($p<0.0001$) (all pairwise comparisons were also significant; $p<0.0001$). The total inorganic N content was in the order: $T_1< T_{control}< T_3<T_2$.

Soil NH_4^+ concentrations increased from 2 µg N g^{-1} soil to between 28 and 54 µg N g^{-1} soil upon label addition, and subsequently decreased over the next five days to ca. 9 µg N g^{-1} soil (Fig. 3b.). Soil NH_4^+ concentrations did not differ as a result of the soil warming treatments on either days 0 or 6. However, on day 1, treatment T_1 had a lower NH_4^+ concentration compared to all other treatments ($p<0.029$), while the soil NH_4^+ concentration in the T_2 treatment was higher than in the $T_{control}$ or T_1 treatments ($p<0.001$). Three days after label addition the NH_4^+ concentration in the T_1 treatment remained lower compared to the T_2 and T_3 treatments (p respectively <0.001 and 0.044).

After the initial increase in NO_3^- due to label addition, the NO_3^- concentrations continued to slowly increase over the following six days (Fig.3c). NO_3^- concentrations were significantly different among the treatments ($p<0.001$), with differences also occurring with respect to the initial NO_3^- concentrations prior to label addition ($p<0.001$). The highest NO_3^- concentrations occurred in the T_2 treatment followed by the T_3 and $T_{control}$, while the lowest NO_3^- concentration was observed in the T_1 treatment.

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3.2. *Soil N transformations*

The modelled and observed concentrations and ^{15}N enrichments were in good agreement with R^2>0.97 for all runs (Fig. 4). The gross rates of most N transformations did not differ as a result of the previously imposed soil warming treatment (Table 1). However, the rates of recalcitrant N mineralisation were reduced under the T_2 and T_3 treatments (p=0.040). Mineralisation of amino acids also became slower with increasing temperatures (p=0.045). However, the overall gross mineralisation of organic N to NH_4^+ did not differ with the previously imposed warming treatments because the mineralisation of labile N was the major contributor to total mineralisation, and this rate was not significantly affected by previous warming (Table 2). Net mineralisation did not differ as a result of the previously imposed warming treatments. Despite the fact that the release of stored NO_3^- tended to increase with warming (p=0.096), and also that cumulative O_{NH4} and O_{Nrec} rates tended to be different (p=0.095), no significant effect on net nitrification could be observed (Table 2).

3.3. *N_2O fluxes*

In response to N supply, N_2O emissions immediately increased, and decreased thereafter (Fig. 3a). While treatments T_2 and T_3 had lower N_2O fluxes than the control treatment (p=0.004 and p=0.036, respectively) no interaction between incubation time and treatment was observed. The N_2O fluxes from the T_2 treatment were also lower than those from the T_1 treatment (p=0.016). However, observed fluxes from the T_1 treatment did not differ from the control treatment and N_2O fluxes from the T_2 treatment did not differ from the T_3 treatment.

The newly developed partitioning model was successful to identify cumulative N_2O fluxes (Fig. 5) and N_2O contribution at each extraction time (Fig. 6) associated with nitrification, denitrification, co-denitrification and the oxidation of organic N between 0.11 and 5.93 days

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after N addition. The oxidation of organic N was the main source of N_2O at all sampling dates, comprising between 63 and 85% of the total N_2O flux (Fig. 5). The percentage contribution made by organic N to N_2O fluxes increased over the sampling period, rising from a minimum of 40% in the control treatment, to virtually 100% across all treatments by Day 6 (Fig. 6). The fluxes from organic N oxidation were the highest in the control treatment, followed by T_1, and lowest for T_2 and T_3. Significant differences were found between the control and the T_2 and T_3 treatment ($p=0.011$ and $p=0.002$, respectively) and between T_1 and T_3 ($p=0.039$). The amount of N_2O produced via denitrification was also the highest under the control treatment, followed by T_1 and T_3. It was the lowest under T_2. Compared to the control treatment, denitrification contributed less to N_2O under the T_2 and T_3 treatments ($p<0.0001$ and $p=0.002$, respectively). The contribution of denitrification also differed between treatments T_2 and T_1 ($p=0.004$). Co-denitrification only contributed to the N_2O flux during the first day after substrate addition. The highest amount of N_2O produced via co-denitrification was found under the control treatment, followed by T_1. Under T_2 and T_3 treatments, the contribution of co-denitrification was minor. However, these differences were not significant. No significant differences were found in the amount of N_2O produced via nitrification.

4. Discussion

Prior to incubation the inorganic N, as well as the NO_3^- concentrations, were higher in the T_2 and T_3 treatments as a result of the six years warming treatment. This suggests that a sustained increase in temperature led to an increase in net mineralisation and net nitrification. This is in line with previous studies showing increases in net mineralisation in response to warming (Peterjohn et al., 1994; Rustad et al., 2001; Norby and Luo, 2004; Bai et al., 2013; Björsne et al., 2014; Zhang et al., 2015b). An increase in net nitrification in response to soil warming, while less common, has also been shown (Barnard et al., 2005; Bai et al., 2013; Björsne et al.,

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374 2014; Zhang et al., 2015b). Both could be due to infield temperatures being more favourable
375 for optimal microbial activity. Concurring with previous research (Bai et al., 2013; Zhang et
376 al., 2015b) the total soil N pool did not differ among warming treatments. This result may be
377 due to the fact that the relative sizes of the N pools differ: since the total soil N pool is
378 significantly larger than the inorganic N pool it may take longer to register a change (Galloway
379 et al., 2008; Bai et al., 2013).
380
381 During incubation all soil was kept at 20°C, regardless of the in-field treatment, to investigate
382 any legacy impacts of sustained soil warming on inherent soil N cycling. It has been suggested
383 that changes in the microbial community structure could alter the sensitivity of the microbial
384 community to temperature shifts (Balser et al., 2006). While both net and gross mineralisation
385 rates did not differ as a result of the previously imposed soil warming treatments, the
386 mineralisation of recalcitrant N and mineralisation of amino acids did differ. Lowest rates were
387 found under T_2 (M_{Nrec}) and T_3 (M_{Nrec} and M_{AA}). A similar effect to warming was found by
388 Jamieson et al. (1998) who reported decreased gross N mineralisation rates in spring following
389 winter warming of soil. Adaptation of the microbial community, altering the sensitivity to
390 temperature shifts, could possibly provide an explanation why no differences in net and gross
391 mineralisation, and even decreases in individual mineralisation rates were found. However, no
392 data were available to test this hypothesis. Another possible explanation for the reduction in
393 mineralisation rates could be a depletion of substrate due to the six years of elevated
394 temperatures.
395
396 Previous research in heathland and grassland soils showed no effect of warming on amino acid
397 mineralisation rates (Andresen et al., 2015). The lower rates in the current study, however,
398 suggest there was a change in amino-acid oxidase activity (Vranova et al., 2013). Another

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399 possible explanation for the lower amino acid mineralisation rates could be an increase in direct
400 microbial assimilation of amino acids (Farrell et al., 2014), since direct assimilation of glycine
401 and larger amino acids is well known (Barraclough, 1997; Andresen et al., 2009, 2011). Chen
402 et al. (2015), however, did not show an effect of warming on the microbial uptake of amino
403 acids. The fact that NH_4^+ immobilisation rates were not affected by previously imposed
404 warming in the current study, is in line with previous research (Niboyet et al., 2011; Bai et al.,
405 2013; Björsne et al., 2014). It has been suggested that the depletion of labile C due to warming
406 might initiate a decrease in immobilisation rates (Bai et al., 2013). In the current experiment a
407 labile carbon source (Gly) was added to the soil which could explain why no reduction in NH_4^+
408 immobilisation was found.
409
410 Oxidation of organic N was found to be the main source of N_2O. The production of N_2O from
411 an unlabelled organic source would most likely follow a combined process of organic N
412 oxidation via heterotrophic nitrifiers to nitrite, followed by a reduction of of nitrite to gaseous
413 N products (Butterbach-Bahl et al., 2013). This process, where oxidation and reduction
414 processes occur hand in hand would be conceptually similar to the nitrifier-denitrification
415 process (Wrage et al., 2001). Most research, however, does not take the oxidation of organic N
416 into account as a possible source of N_2O (Zhang et al., 2015a). Even though recent studies
417 showed that this process contributed 54-85% of N_2O emissions in pastures (Rütting et al., 2010;
418 Müller et al., 2014). These contributions are in line with the current study. Müller et al. (2014)
419 also showed that the fraction of N_2O contributed via the oxidation of organic N was lowest
420 immediately following NH_4NO_3 addition, and that this fraction increased to over 80%, while
421 the contribution of denitrification decreased with time even though NO_3^- concentrations
422 increased. Because of the large contribution of oxidation of organic N in N_2O emissions, this
423 pathway should not be omitted in future research.

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424

425 A decrease in N_2O produced via denitrification was found in soil previously subjected to higher
426 temperature treatments. This could be due to a decrease in the rate of denitrification. However,
427 it is also possible that under treatment T_2 and T_3 more of the NO_3^- was converted into N_2 as
428 opposed to N_2O as denitrification, in contrast to nitrification, can also lead to production of N_2
429 as well as N_2O. This highlights the importance of the gaseous N stoichiometries in particular
430 the N_2/N_2O ratio. Stevens and Laughlin (2001) reported N_2:N_2O ratios in a fine loamy grassland
431 soil of 2.2 and 0.5 from control and combined slurry plus NO_3^- fertiliser treatments,
432 respectively. However, Clough et al. (1998) showed that ratios can vary between 6.2 and 33.2
433 following ^{15}N-labelled urine application to ryegrass (*Loilum perenne*)/white clover (*Trifolium*
434 *repens*) pasture on four different soils (silt loam, sandy loam, peat and clay soils).
435 Unfortunately, due to methodological restrictions were not able to detect significant N_2 fluxes,
436 as they were <4 g N_2-N ha^{-1} day^{-1} (Stevens and Laughlin, 1998).
437
438 Adaptation of microorganisms, to long-term elevated temperature treatments, might also
439 provide an explanation for the decrease in N_2O emissions during the incubation with soil
440 previously subjected to increasing soil warming temperatures (Avrahami and Conrad, 2003;
441 French et al., 2009; Pritchard, 2011). Enhanced NO_3^- concentrations in the T_2 and T_3
442 treatments, at the end of the field experiment, also suggests an in situ reduction of
443 denitrification and/or co-denitrification. A possible explanation for the in situ reduction of
444 denitrification could be the altered field soil moisture content. While during the incubation, soil
445 moisture was purposely kept constant (WFPS of 64%), in the field however, moisture
446 conditions were affected by the heating treatment, leading to generally drier, and thus more
447 aerated, conditions in the heated plots (Jansen-Willems et al., in press). Under low WFPS,
448 nitrification is predominantly responsible for N_2O efflux (Bollmann and Conrad, 1998;

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Bateman and Baggs, 2005). This may be a consequence of altered soil moisture or changes in soil texture and physical soil structure. The reduction of NO_3^- (denitrification) takes place under more anoxic to anaerobic conditions (Smith, 1997), because under aerobic conditions, denitrifiers reduce O_2 rather that NO_3^- (Arah, 1997). Any reduction in soil moisture could therefore lead to a decrease in the in situ denitrification rate.

Co-denitrification was observed to be significant in $T_{control}$ and T_1 shortly after N addition. Rates were comparable with those from true denitrification. Co-denitrification is a co-metabolic process which uses inorganic and organic N compounds concurrently and converts it to the same end products as in denitrification. Gases produced in this process are a hybrid N-N species where one atom of N comes from NO_2^- and the other one from a co-metabolised compound (Spott et al., 2011). The conditions for increased co-denitrification are still not yet fully understood, but the presence of fungi along with adequate amino acid pools appears to enhance losses via this pathway (Laughlin and Stevens, 2002; Spott et al., 2011).

Laughlin and Stevens (2002) found that fungi dominated denitrification and co-denitrification in grassland soils. It has been suggested that warming could increase the relative contribution of fungi to the soil microbial community (Zhang et al., 2005; Pritchard, 2011). Most fungi lack N_2O reductase, resulting in N_2O as the final denitrification product (Saggar et al., 2013). It can therefore be expected that warming would lead to an increase in N_2O produced via denitrification and co-denitrification. However, the opposite was found in the current experiment, although the changes in co-denitrification were not significant. The reduced co-denitrification and total denitrification rates seem to indicate a reduction in fungal-mediated N processes under elevated temperatures in these soils. Further research is required to elucidate the effect of increased temperatures on N processes mediated by fungi

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474

475 **5. Conclusion**

476 Sustained increases in soil temperatures over 6 years (between 2 and 3°C) led to an increase in
477 both inorganic soil N and NO_3^- pools. Subsequent analyses of gross N transformations, during
478 an incubation of these soils under common temperature and moisture conditions to study the
479 legacy effect of increased temperatures, revealed that mineralisation of amino acids (glycine)
480 and recalcitrant organic N decreased with previously imposed elevated temperatures. A new,
481 easy to use, source partitioning method was developed to determine the contribution of four
482 different pathways to N_2O emissions. Emissions of N_2O in the first six days after fertilisation
483 was decreased for soils previously subjected to higher temperatures as a consequence of a
484 reduction in the rates of denitrification and the oxidation of organic N. For all treatments,
485 oxidation of organic N was the main contributor to N_2O emissions, and should therefore in
486 future research not be omitted as a possible source of N_2O.

487

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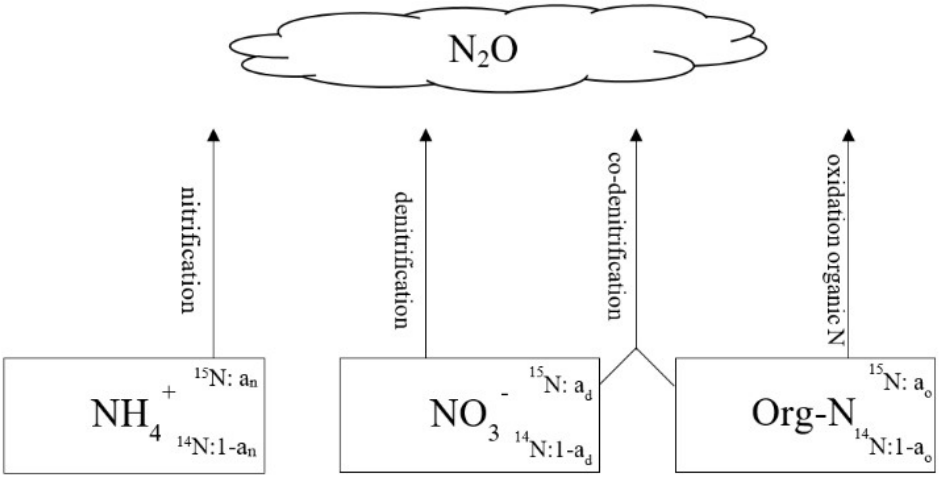
685 **Figures**



686

687 Fig. 1. N_2O production via four processes (nitrification, denitrification, co-denitrification and
688 oxidation of organic N). Three uniformly distributed pools were considered. These pools were
689 an ammonium pool (NH_4^+) with a ^{15}N atom fraction of a_n, a nitrate pool (NO_3^-) with a ^{15}N atom
690 fraction of a_d, and an organic-N pool with a ^{15}N atom fraction of a_o (=0.003663). The N_2O
691 produced via co-denitrification consists of one N atom from the nitrate pool, and one from the
692 organic N pool.

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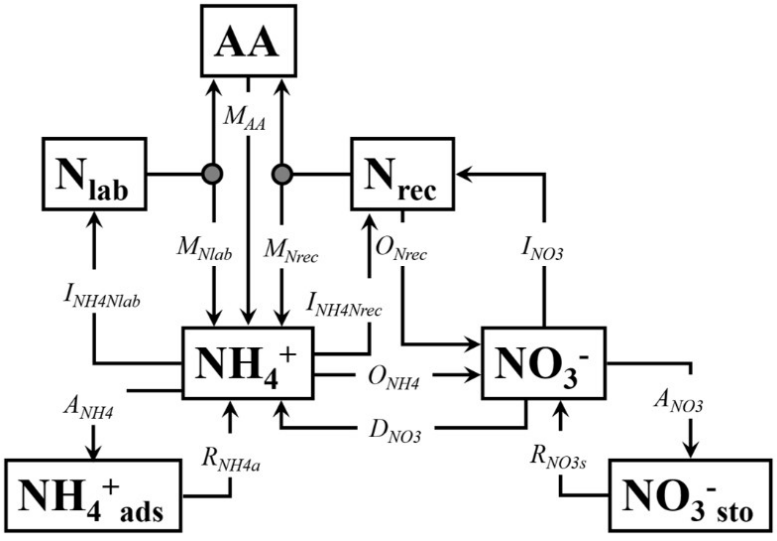

Fig. 2. ^{15}N tracing model for analyses of gross soil N transformation rates. Abbreviations of the transformations are explained in the table 1. The pools are explained in section 2.4.

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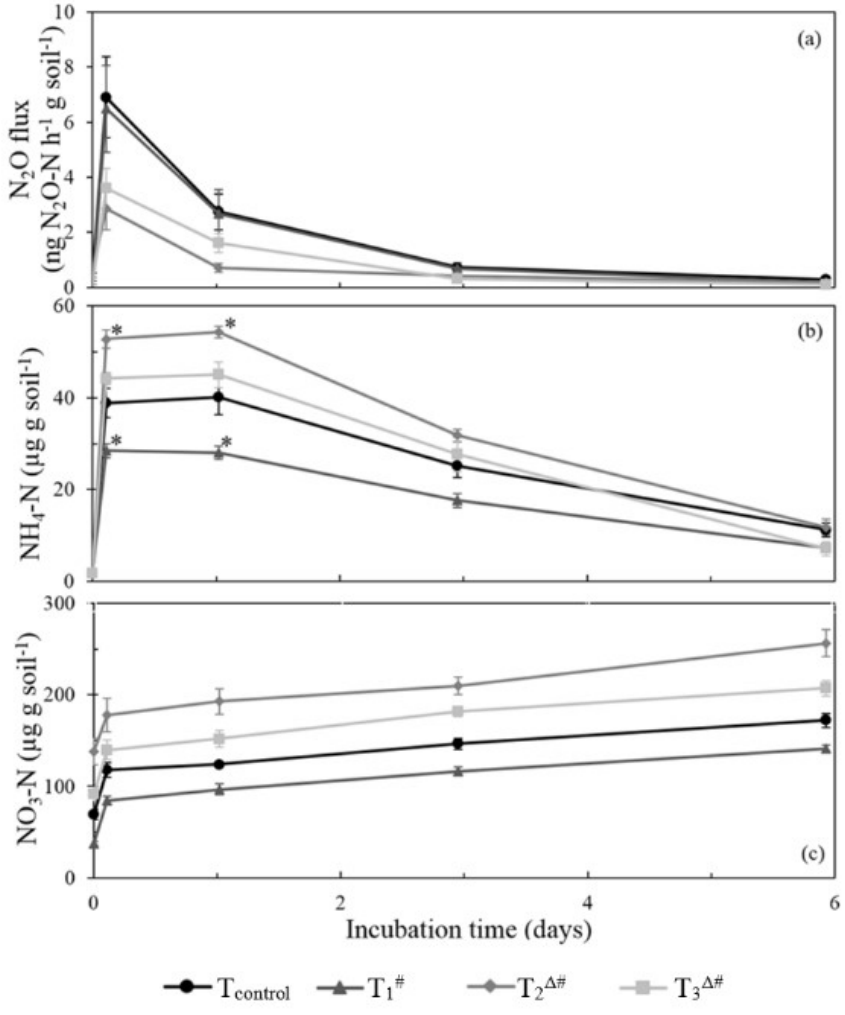

Fig. 3. N_2O emission (a), NH_4-N content (b) and NO_3-N content at the extraction times. Time point 0 is the time of label addition (**$^{15}NH_4NO_3$ Gly**, $NH_4{}^{15}NO_3$ Gly or NH_4NO_3 **^{15}N-Gly**). The N_2O flux at time point 0 is based on the average flux of the 3 gas samplings before label addition. The ammonium and nitrate content at time point 0 is based on unlabelled soil. The error bars are the standard error of the mean. $^{\Delta}$ shows a significant difference in N_2O flux from $T_{control}$ ($p<0.05$), * shows a significant difference in NH_4-N from $T_{control}$ ($p<0.03$), and $^{\#}$shows a significant difference in NO_3-N from $T_{control}$ ($p<0.0001$).

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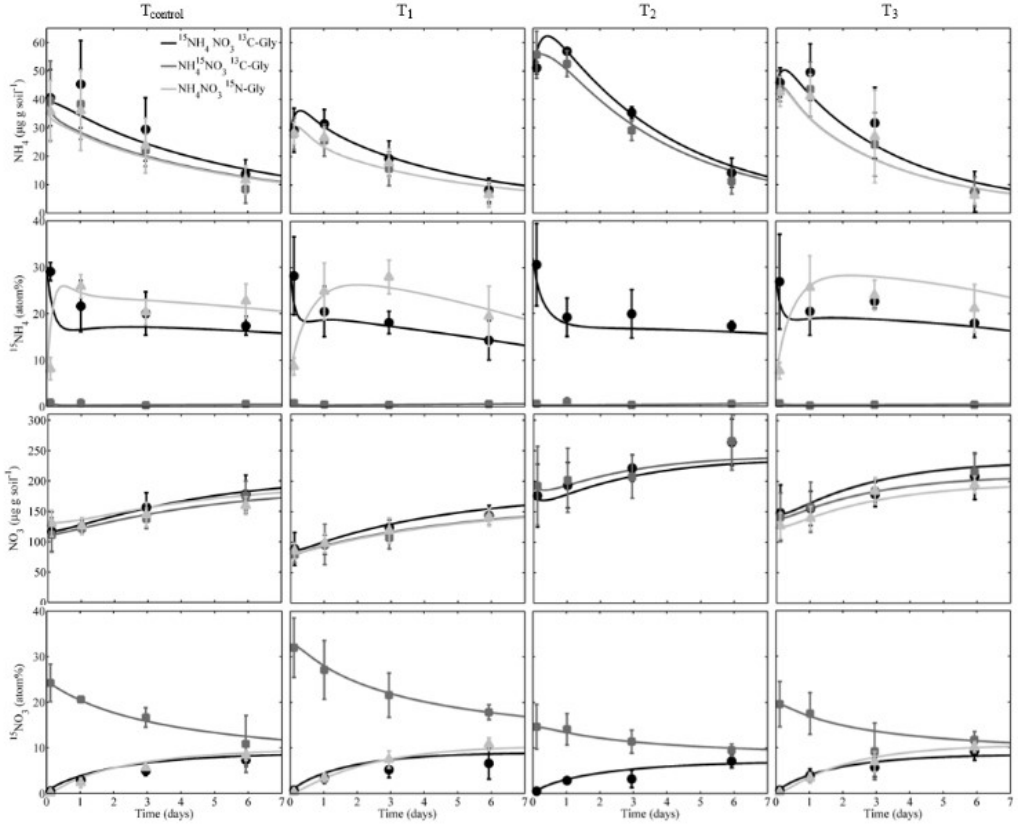

706

707 Fig. 4. Modelled vs measured data. Error bars are standard deviations. Time is the time in days

708 from the moment of label addition.

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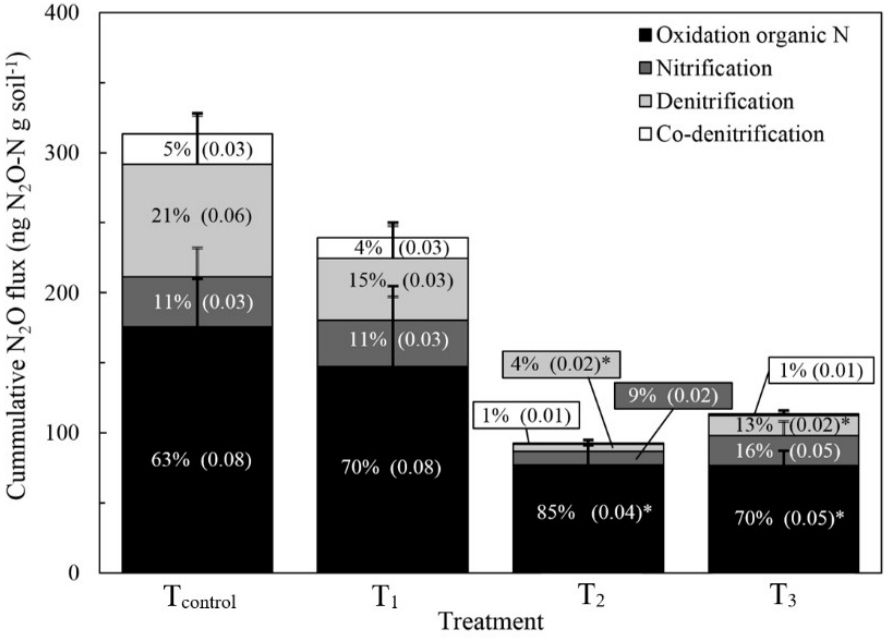

Fig. 5. Cumulative N_2O flux via four processes between 3 h and 6 days after labelling. N_2O fluxes based on average flux from soil labelled with $^{15}NH_4NO_3$ Gly or $NH_4{}^{15}NO_3$ Gly. The cumulative flux per process is an average over the four plots per treatment. Error bars are standard error of the mean (SEM). Percentages are the average percentage of flux produces via each process, SEM between brackets. *Significantly lower cumulative flux compared to the control ($p<0.05$).

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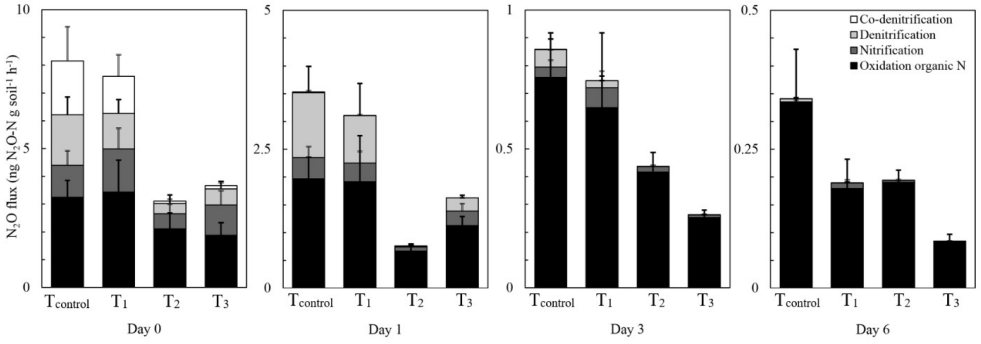

719

720 Fig. 6. N_2O flux divided into 4 processes at different time points after fertilisation. N_2O fluxes
721 based on average flux from soil labelled with $^{15}NH_4NO_3$ Gly or $NH_4{}^{15}NO_3$ Gly. The portrayed
722 flux per process is an average over the four plots per treatment. Error bars are standard error of
723 the mean. The scale of the y-axis is different for each time point.

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725 **Tables**

726 Table 1: Description of N transformations and average gross N fluxes per treatment (diagram
727 shown in Fig. 2). Standard deviation between brackets. K stands for Kinetics were 0 implies
728 the use of zero-order and 1 the use of first-order kinetics in the model. The p is the p-value of
729 the one-way ANOVA, with ns (non-significant) if p>0.1 (p value in bold if < 0.05). For the
730 holm-sidak pairwise comparisons: ^{t} tends to be different from control (p<0.10).

	Transformation	K	Average gross flux (µg N g soil^{-1} d^{-1})								p
			$T_{control}$		T_1		T_2		T_3		
M_{Nrec}	Mineralisation of N_{rec} to NH_4^+ or AA	0	3.18	(1.95)	5.42	(2.50)	0.91	(0.73)	1.35	(0.90)	**0.040**
$I_{NH4Nrec}$	Immobilisation of NH_4^+ to N_{rec}	1	16.12	(9.23)	13.43	(6.92)	17.45	(6.53)	4.72	(3.65)	ns
M_{Nlab}	Mineralisation of N_{lab} to NH_4^+ or AA	1	35.86	(16.49)	28.01	(8.92)	36.14	(10.17)	35.43	(8.78)	ns
$I_{NH4Nlab}$	Immobilisation of NH_4^+ to N_{lab}	1	30.59	(19.34)	22.28	(14.65)	30.54	(8.82)	29.59	(19.78)	ns
O_{Nrec}	Oxidation of N_{rec} to NO_3^-	0	3.64	(0.96)	1.99	(1.31)	2.02	(0.56)	2.92	(1.34)	ns
I_{NO3}	Immobilisation of NO_3^- to N_{rec}	1	5.64	(2.74)	2.15	(1.31)	4.57	(2.62)	4.97	(3.10)	ns
O_{NH4}	Oxidation of NH_4^+ to NO_3^-	1	15.40	(2.30)	11.64	(1.65)	14.21	(1.92)	15.26	(2.58)	ns
D_{NO3}	Dissimilatory NO_3^- reduction to NH_4^+	0	0.18	(0.05)	0.24	(0.12)	0.36	(0.12)	0.14	(0.10)	ns
A_{NH4}	Adsorption of NH_4^+	1	34.26	(19.67)	20.41	(19.61)	23.64	(11.50)	15.81	(12.84)	ns
R_{NH4a}	Release of adsorbed NH_4^+	1	33.22	(21.43)	20.51	(12.33)	24.77	(6.15)	16.41	(9.07)	ns
A_{NO3}	Adsorption of NO_3^-	1	28.08	(14.18)	55.23	(37.72)	82.39	(58.45)	62.99	(47.75)	ns
R_{NO3s}	Release of stored NO_3^-	1	23.70	(10.48)	53.23	(10.63)	78.49	(36.84)	59.96	(22.29)	0.096
M_{AA}	Mineralisation of AA to NH_4^+	1	32.21	(7.67)	17.40	(4.32)	27.29	(9.52)	15.32	$(3.63)^t$	**0.045**

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732 Table 2. Gross/net mineralisation (Min_{Gross}/Min_{Net}), gross/net nitrification (Nit_{Gross}/Nit_{Net}) rate
733 in µg N g $soil^{-1}$ d^{-1}. Including the contributions from the different N pools for the gross
734 transformations (italics), where N_{lab} is a labile organic N pool, N_{rec} is a recalcitrant organic N
735 pool, and NH_4^+ is the ammonium pool. ^{t} one-way ANOVA tendency $p<0.1$

	$T_{control}$	T_1	T_2	T_3
Min_{Gross}	59.13	44.18	54.86	43.58
N_{lab}	*44%*	*54%*	*50%*	*63%*
N_{rec}	*1%*	*6%*	*1%*	*2%*
N_{AA}	*54%*	*39%*	*50%*	*35%*
Min_{Net}	6.78	6.32	2.29	4.30
Nit_{Gross} ^{t}	19.04	13.62	16.24	18.17
N_{rec}	*19%*	*15%*	*12%*	*16%*
NH_4^+	*81%*	*85%*	*82%*	*84%*
Nit_{Net}	13.22	11.23	11.30	13.06

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