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. In order 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 and modelling approaches were combined. Based on previous observations that gross N transformations in soils are affected by long-term elevated temperature treatments we hypothesised that any associated effects on gaseous N emissions (e.g. N_2O) can be confirmed by a change in the relative emission rates from various pathways. Soils were 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**. Soil extractions and N_2O emissions were analysed using a ^{15}N tracing model and source partitioning model. Both total inorganic N (NO_3^-+NH_4^+) and NO_3^- contents were higher in soil subjected to the +2 °C and +3 °C temperature elevations (pre- and post-incubation). Analyses of N transformations using a ^{15}N tracing model, showed that, following incubation, gross organic (but 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. Furthermore, 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.

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) N 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, NH_4^+ or NO_3^- in a Tibetan grassland (Zhang et al., 2015b). Other

studies also found no effect of soil warming on total soil N (Bai et al., 2013) and inorganic N (Larsen et al., 2011).

N mineralisation follows a step-wise sequence of protein depolymerisation by extracellular activity to oligomers (e.g. peptides) and monomers (e.g. amino acids) and then uptake by microorganisms before mineralisation to NH_4^+ (Schimel and Bennett, 2004). Hence, production of peptides and amino acids as well as mineralisation of amino acids, affects the main fluxes regulating gross N mineralisation. Amino acids have a short residence time in the soil due to either rapid assimilation by soil microbes or mineralisation, which occurs within a few hours (Farrell et al., 2014). In heathland and grassland soils no effect of soil warming on the amino acid concentration has been observed (Chen et al., 2014; Andresen et al., 2015).

Nitrous oxide (N_2O), a potent greenhouse gas with a global warming potential of 298 on a 100 year basis, can be produced by several processes, such as nitrification, partial denitrification, co-denitrification and the oxidation of organic matter (Butterbach-Bahl et al., 2013; Zhang et al., 2015a) (Fig. 1). Laughlin and Stevens (2002) confirmed the importance of co-denitrification for N_2 production, a process that may comprise 25% of the total N balance in pastures (Selbie et al., 2015). Müller et al. (2014) found that, for the same grassland soil as used in this study, co-denitrification contributed 17.6% of the total N_2O production. N_2O emissions following fertilisation with ammonium nitrate (NH_4NO_3) may be greater than from urea fertiliser because of the greater susceptibility to denitrification (Harrison and Webb, 2001). The amount and form of N inputs primarily govern N_2O emissions with further impacts resulting from climatic factors, such as temperature and precipitation, and soil factors, such as C availability and microbial community structure (Harrison and Webb, 2001; Müller et al., 2003; Stark and Richards, 2008; Laughlin et al., 2009; Li and Lang, 2014). However, the

impact of 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 N_2O 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.

The objectives of this study were to quantify the legacy effects of six years of elevated temperature (via IR heaters) on soil N cycling dynamics, including (1) net and gross N transformation rates in the soil (2) N_2O fluxes immediately after fertilisation and (3) the processes responsible for these N_2O fluxes. Net and gross transformation rates were determined using an extended version of a basic ^{15}N tracing model described by Müller et al. (2007). Since the publication of this basic model in 2007, more than 50 peer-reviewed papers have been published, where the basic model or modifications of the basic model have been used, demonstrating its robustness of the approach in various soils, ecosystems and climatic conditions. To determine the processes involved in N_2O production, a new source partitioning method was developed to allow the identification of hybrid reactions. This source partitioning method is a newly developed method, and not a modification of the ^{15}N tracing model. To identify the legacy effect of different in situ temperature treatments on the internal N transformation processes, soil incubations were carried out under identical moisture and temperature conditions in the laboratory. Based on previous observations that gross N transformations in soils are affected by long-term elevated temperature treatments we hypothesised that any associated effects on gaseous N emissions (e.g. N_2O) can be confirmed by a change in the relative emission rates from various pathways. Thus, the newly developed source partitioning method would be helpful to confirm such a change.

2. Material and method

2.1. Site description and field treatment

The 100 m^2 site was established on a permanent grassland of the 'Environmental Monitoring and Climate Impact Research Station Linden' in Germany (50°31.6'N, 8°41.7'E). A full description of the site can be found in Jansen-Willems et al. (2016). Briefly, the site had been managed as a meadow with two cuts per year and fertilised with 50-80 kg N ha^{-1} $year^{-1}$ for the

last three decades. Since 1995, the N fertiliser input had been reduced to 40 kg N ha^{-1} $year^{-1}$, as KAS (calcium-ammonium-nitrate). The mean annual temperature and precipitation were 9.5°C and 560 mm (observation period: 1995-2014) respectively.

The site had been divided into 16 plots, four rows of four plots. The 16 plots were, according to a Latin square design, assigned to one of four treatments. From January 28, 2008, the soil temperature of each plot, measured at 5 cm depth, was elevated by 0, 1 (mean 0.8 standard error 0.02), 2 (mean 1.9 standard error 0.03) or 3 (mean 2.6 standard error 0.03) ºC above ambient temperature, using infrared heaters. The use of heaters will also affect the soil moisture content. The temperature treatments (including any moisture effect) are referred to as $T_{control}$, T_1, T_2, and T_3, respectively. The infrared heaters were installed at different heights to create the different temperature elevations (Jansen-Willems et al., 2016).

2.2. Incubation, labelling and extraction

On the day the heaters were turned off, all soil within a circular area of 318 cm^2 directly underneath each infrared lamp was excavated to 7.5 cm for the tracing experiment. A small subsample of each plot was dried at 70°C for 48 hours, ground and analysed by a CNH Macro Elemental Analyser (Hanau, Germany) for total N content. A subsample of the soil for each plot was dried at 105°C for 24 hours to determine the soil gravimetric water content. The remaining field moist soil was kept at 4°C (for less than 60 hours) until further analysis whereupon the soil from each field plot was sieved through a 10 mm sieve, to homogenise it and to remove roots. Incubations were carried out in 750 ml jars (WECK GmbH u. Co. KG, Wehr, Germany). Thirteen jars per field plot were prepared each with an average of 67 (stdev 8.4) g dry soil per jar (except for plots 3, 5, 7, 11 and 14, where only 10 jars were prepared due to lack of soil). All jars were closed with glass lids that were fitted with septa to allow for gas

sampling. During gas flux analyses the jars were sealed using a clamp and a rubber ring between the jar and the lid. At other times a gap was left between the jar and the lid to allow air exchange while minimising water loss. Two days after soil sampling (day -55), all jars were put in a dark climate chamber at 20°C and 50% humidity and incubated for 55 days prior to ^{15}N substrate addition (day 0).

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 -53 the soil moisture in each jar was adjusted to a water-filled pore space (WFPS) of 64%. On day -43 and -5 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 0, 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 -2. The

189 other extractions took place at 0.11 days (+/- 0.004), 1.02 days (+/- 0.001), 2.95 days (+/- 0.001)
190 and 5.93 days (+/- 0.001) after ^{15}N substrate addition, and are hereafter referred to as 0, 1, 3
191 and 6 days after ^{15}N substrate addition, respectively.
192
193 *2.3. Gas sampling*
194 Gas samples were taken from 43 different jars, one jar per ^{15}N label, for each plot. During the
195 pre-incubation gas samples were taken 1, 46 and 48 days before label addition. After labelling,
196 gas samples were taken immediately prior to soil extractions.
197
198 Gas samples were taken using a 60 ml syringe (Ecoject Plus, Gelnhausen, Germany). At time
199 zero (t_0) 15 gas samples were taken from 15 different jars. Then at time 1 (t_1) a gas sample was
200 taken through the rubber septum. At both t_0 and t_1 the syringe was flushed twice with headspace
201 gas to ensure a representative sample was taken. The times between t_0 and t_1 during each of the
202 seven different gas samplings (three before label addition and four immediately prior to
203 extraction) were 120-129, 120, 180, 233, 240, 235 and 214 minutes, respectively. Gas samples
204 were analysed within 24 h after sampling using a GC (Bruker) equipped with an electron
205 capture detector (ECD) for N_2O analysis. An average of the concentrations measured in the 15
206 samples was used as the t_0 concentration for all 43 jars. Fluxes were based on the ppm and time
207 difference between t_0 and t_1. They were calculated using the constant gas law, with ambient
208 pressure, and temperature was assumed to be 20°C (the temperature of the incubation room).
209 The fluxes were then converted to a per dry gram basis.
210
211 For the ^{15}N abundance of N_2O, a 30 ml sample was taken at t_1 and transferred to a 12 ml
212 Exetainers® vial (Labco Ltd, High Wycombe, Buckinghamshire, UK). The over-pressurised
213 sample vials were returned to ambient pressure immediately before analyses of stable isotopes.

This was performed using a double ended needle fixed vertically in a clamp stand with the ventral needle submerged 3-4 mm in a beaker of water and the gas sample held upside down and pushed onto the dorsal needle. The excess pressure in the sample vial was thus released causing the water to bubble until the pressure inside the vial has equilibrated with the ambient atmospheric pressure. Cessation of bubbling implied equal pressure had been reached. The ^{15}N enrichments of $^{15}N_2O$ and $^{15}N_2$ were determined using an automated isotope ratio mass spectrometry (Sercon Ltd 20-20), as described by Stevens et al. (1993), inter-faced to a TGII cryfocusing unit (Sercon Ltd 20-20). The detection limit for atom% ^{15}N of a 50 ppm N_2O standard gas was 0.00003 (n= 10), stdev was 0.00009 atom% ^{15}N. Respective values for a 0.4 ppm N_2O standard were higher (0.00084 (n= 10), stdev 0.003).

2.4. ^{15}N tracing model

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 only changes to the original model were the addition of an amino-acid (glycine) pool, and the transformations to and from this pool. The model (Fig. 2.) considered seven N pools and 13 N transformations. The N pools were NH_4^+, NO_3^-, amino acid glycine (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 glycine (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 initial pool sizes for organic N (N_{rec} and N_{lab}) were based on previous field measurements. However, these organic N values were not critical because for N_{rec}, zero-order kinetics were used (independent of initial pool size),

239 and for N_{lab}, the quick turnover time ensures that a small pool will be governed quickly by the
240 dynamics of the in- and out-flowing rates.
241
242 The N transformations are described in Table 1. The N transformations were calculated based
243 on zero or first order kinetics (Table 1). Whether N_{lab} and N_{rec} were transformed into AA or
244 NH_4^+ was determined by two factors, one for M_{Nlab} and one for M_{Nrec}. This factor determines
245 the fraction of the M_{Nlab} or M_{Nrec} flowing into the AA pool with the remainder entering the
246 NH_4^+ pool. For each temperature treatment the kinetic parameters and the two split factors were
247 simultaneously optimised by minimising the misfit between the modelled and measured NH_4^+
248 and NO_3^+ concentrations and their respective ^{15}N enrichments (Müller et al., 2004). For
249 treatment T_2 the measurements of the ^{15}N-Gly label were not included in the optimisation
250 because only one replicate was available for this label.
251
252 A Markov chain Monte Carlo Metropolis algorithm (MCMC-MA) was used for the
253 optimisation, which practices a random walk technique to find global minima (Müller et al.,
254 2007). The uncertainties (standard deviation) of the observations were taken into account by
255 the optimisation routine. The MCMC-MA routine was programmed in MatLab-Simulink
256 (Mathworks Inc) as described in Müller et al. (2007). The most suitable parameter set was
257 determined using the Akaikes Information Criterion (AIC). Gross and net nitrification, and
258 gross and net mineralisation were calculated using equation 1 to 4 in which SF stands for split
259 factor. The combined standard deviation was calculated by $((\text{stdev rate } 1)^2+(\text{stdev rate }$
260 $2)^2+\ldots\ldots..)^{0.5}$, in which the stdev of $M_{Nx}{\cdot}SF_{MNx}$ is the stdev of M_{Nx} multiplied by the SF.
261
262 The following combined rates were calculated:
263 Gross nitrification: $O_{Nrec}+O_{NH4}$ (1)

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

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

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

267

268 *2.5. Determining contribution of different processes to N_2O flux*

269 The N_2O fluxes, from the soil labelled with NO_3**$^{15}NH_4$** Gly and **$^{15}NO_3$**NH_4 Gly, were separated
270 into four different processes. These were nitrification, denitrification, co-denitrification and
271 oxidation of organic matter. The N_2O was assumed to be derived from three uniformly
272 distributed pools, and based on initial substrate ^{15}N enrichments, isotopic discrimination was
273 considered negligible for all four processes. The pools and processes accounting for the N_2O
274 production are shown in Fig. 1. The ^{15}N content of the organic matter was considered to be at
275 natural abundance (0.3663 atom%). The N_2O produced via co-denitrification consists of one N
276 atom from the NO_3^- pool, and one N atom from the organic N pool. The chance that the N_2O
277 produced via nitrification, denitrification or oxidation of organic N contains zero, one or two
278 ^{15}N enriched atoms can be described by equations 5, 6 and 7, respectively. Where a_x (the ^{15}N
279 fraction of the pool) is a_n for nitrification, a_d for denitrification and a_0 for the oxidation of
280 organic N: a_n, a_d and a_o are explained in Fig. 1.

281

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

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

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

285

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

288

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

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

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

292

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

298

299 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)

300 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)

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

302

303 The automated continuous-flow isotope-ratio mass spectrometer enabled the measurement of
304 ^{45}R ($^{45}I/^{44}I$) and ^{46}R ($^{46}I/^{44}I$), where ^{x}I is the ion currents at *m*/*z x*. The ^{45}R and ^{46}R were corrected
305 for the presence of ^{18}O. This, therefore, means that ^{45}R is the fraction of N_2O molecules
306 containing one ^{15}N atom divided by the fraction of N_2O molecules containing zero ^{15}N atoms,
307 and ^{46}R is the fraction of N_2O molecules containing two ^{15}N atoms divided by the fraction of
308 N_2O molecules containing zero ^{15}N atoms. The expected fractions are described by equations
309 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^+
310 and NO_3^- respectively, while *n*, *d* and *o* were quantified using the *fminsearchbnd* function in
311 MatLab (The MathWorks Inc, Natick, MA). For this the ^{45}R, ^{46}R, a_n and a_d of soil labelled with
312 NO_3**$^{15}NH_4$** Gly and soil labelled with **$^{15}NO_3$**NH_4 Gly were used. The amount of N_2O produced
313 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

Treatment differences in total soil N were 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 effect of treatment 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 effect of treatment on 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.).

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

339 differed with temperature treatment ($p<0.0001$) (all pairwise comparisons were also
340 significant; $p<0.0001$). The total inorganic N content was in the order: $T_1 < T_{control} < T_3 < T_2$.
341
342 Soil NH_4^+ concentrations increased from 2 µg N g^{-1} soil to between 28 and 54 µg N g^{-1} soil
343 upon label addition, and subsequently decreased over the next five days to ca. 9 µg N g^{-1} soil
344 (Fig. 3a). Soil NH_4^+ concentrations did not differ as a result of the soil warming treatments on
345 either days 0 or 6. However, on day 1, treatment T_1 had a lower NH_4^+ concentration compared
346 to all other treatments ($p<0.029$), while the soil NH_4^+ concentration in the T_2 treatment was
347 higher than in the $T_{control}$ or T_1 treatments ($p<0.001$). Three days after label addition the NH_4^+
348 concentration in the T_1 treatment remained lower compared to the T_2 and T_3 treatments (p
349 respectively <0.001 and 0.044).
350
351 After the initial increase in NO_3^- due to label addition, the NO_3^- concentrations continued to
352 slowly increase over the following six days (Fig. 3b). NO_3^- concentrations were significantly
353 different among the treatments ($p<0.001$), with differences also occurring with respect to the
354 initial NO_3^- concentrations prior to label addition ($p<0.001$). The highest NO_3^- concentrations
355 occurred in the T_2 treatment followed by the T_3 and $T_{control}$, while the lowest NO_3^- concentration
356 was observed in the T_1 treatment.
357
358 *3.2. Soil N transformations*
359 The modelled and observed concentrations and ^{15}N enrichments were in good agreement with
360 $R^2>0.97$ for all runs (Fig. 4). The gross rates of most N transformations did not differ as a result
361 of the previously imposed soil warming treatment (Table 1). However, the rates of recalcitrant
362 N mineralisation were reduced under the T_2 and T_3 treatments ($p=0.040$). Mineralisation of
363 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. This was because the mineralisation of labile organic 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. 3c). 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 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

389 treatment (p=0.011 and p=0.002, respectively) and between T_1 and T_3 (p=0.039). The amount
390 of N_2O produced via denitrification was also the highest under the control treatment, followed
391 by T_1 and T_3. It was the lowest under T_2. Compared to the control treatment, denitrification
392 contributed less to N_2O under the T_2 and T_3 treatments ($p < 0.0001$ and p=0.002, respectively).
393 The contribution of denitrification also differed between treatments T_2 and T_1 (p=0.004). Co-
394 denitrification only contributed to the N_2O flux during the first day after substrate addition. The
395 highest amount of N_2O produced via co-denitrification was found under the control treatment,
396 followed by T_1. Under T_2 and T_3 treatments, the contribution of co-denitrification was minor.
397 However, these differences were not significant. No significant differences were found in the
398 amount of N_2O produced via nitrification.
399

400 **4. Discussion**

401 Prior to incubation the inorganic N, as well as the NO_3^- concentrations, were higher in the T_2
402 and T_3 treatments as a result of the six years warming treatment. This suggests that a sustained
403 increase in temperature led to an increase in net mineralisation and net nitrification. This is in
404 line with previous studies showing increases in net mineralisation in response to warming
405 (Peterjohn et al., 1994; Rustad et al., 2001; Norby and Luo, 2004; Bai et al., 2013; Björsne et
406 al., 2014; Zhang et al., 2015b). An increase in net nitrification in response to soil warming,
407 while less common, has also been shown (Barnard et al., 2005; Bai et al., 2013; Björsne et al.,
408 2014; Zhang et al., 2015b). Both could be due to infield temperatures being more favourable
409 for optimal microbial activity. Concurring with previous research (Bai et al., 2013; Zhang et
410 al., 2015b) the total soil N pool did not differ among warming treatments. This result may be
411 due to the fact that the relative sizes of the N pools differ: since the total soil N pool is
412 significantly larger than the inorganic N pool it may take longer to register a change (Galloway
413 et al., 2008; Bai et al., 2013).

During incubation all soil was kept at 20°C, regardless of the in-field treatment, to investigate any legacy impacts of sustained soil warming on inherent soil N cycling. It has been suggested that changes in the microbial community structure could alter the sensitivity of the microbial community to temperature shifts (Balser et al., 2006). While both net and gross mineralisation rates did not differ as a result of the previously imposed soil warming treatments, the mineralisation of recalcitrant N and mineralisation of amino acids did differ. Lowest rates were found under T_2 (M_{Nrec}) and T_3 (M_{Nrec} and M_{AA}). A similar effect to warming was found by Jamieson et al. (1998) who reported decreased gross N mineralisation rates in spring following winter warming of soil. Adaptation of the microbial community, altering the sensitivity to temperature shifts, could possibly provide an explanation why no differences in net and gross mineralisation, and even decreases in individual mineralisation rates were found. However, no data were available to test this hypothesis. Another possible explanation for the reduction in mineralisation rates could be a depletion of substrate due to the six years of elevated temperatures.

Previous research in heathland and grassland soils showed no significant effect of warming on amino acid mineralisation rates (Andresen et al., 2015). The lower rates in the current study, however, could be due to a change in amino-acid oxidase activity (Vranova et al., 2013). Another possible explanation for the lower amino acid mineralisation rates could be an increase in direct microbial assimilation of amino acids (Farrell et al., 2014), since direct assimilation of glycine and larger amino acids is well known (Barraclough, 1997; Andresen et al., 2009, 2011). Chen et al. (2015), however, did not show an effect of warming on the microbial uptake of amino acids. The fact that NH_4^+ immobilisation rates were not affected by previously imposed warming in the current study, is in line with previous research (Niboyet et al., 2011;

439 Bai et al., 2013; Björsne et al., 2014). It has been suggested that the depletion of labile C due
440 to warming might initiate a decrease in immobilisation rates (Bai et al., 2013). In the current
441 experiment a labile carbon source (Gly) was added to the soil, which could explain why no
442 reduction in NH_4^+ immobilisation was found.
443
444 Nitrous oxide emissions were highest shortly after label addition and declined thereafter. Thus,
445 initial higher rates from NH_4^+ and NO_3^- were due to label addition. The higher absolute rate of
446 organic N oxidation at the start of the incubation did not come solely from the Gly addition. If
447 this had been the case, highest N_2O ^{15}N enrichment would have been observed at the first
448 measurement following addition of the NO_3NH_4 ^{15}N-gly label. However, for all treatments the
449 highest ^{15}N enrichment of N_2O was found in the second measurement after label addition. The
450 lower net rates of N_2O production, at the end of incubation period could possibly have been
451 caused by N_2O consumption, however, the consumption of pathway specific N_2O emissions
452 cannot be evaluated with the current model. However, as WFPS was set to 64%, it is unlikely
453 that N_2O consumption occurred, as this would predominantly occur only under fully reductive
454 conditions (but see Goldberg and Gebauer (2009) for an exception).
455
456 Oxidation of organic N was found to be the main source of N_2O. The production of N_2O from
457 an unlabelled organic source would most likely follow a combined process of organic N
458 oxidation via heterotrophic nitrifiers to nitrite, followed by a reduction of nitrite to gaseous N
459 products (Butterbach-Bahl et al., 2013). This process, where oxidation and reduction processes
460 occur hand in hand would be conceptually similar to the nitrifier-denitrification process (Wrage
461 et al., 2001). Most research, however, does not take the oxidation of organic N into account as
462 a possible source of N_2O (Zhang et al., 2015a). Even though recent studies showed that this
463 process contributed 54-85% of N_2O emissions in pastures (Rütting et al., 2010; Müller et al.,

464 2014). These contributions are in line with the current study. Müller et al. (2014) also showed
465 that the fraction of N_2O contributed via the oxidation of organic N was lowest immediately
466 following NH_4NO_3 addition, and that this fraction increased to over 80%, while the
467 contribution of denitrification decreased with time even though NO_3^- concentrations increased.
468 Because of the large contribution of oxidation of organic N in N_2O emissions, this pathway
469 should not be omitted in future research.
470
471 A decrease in N_2O produced via denitrification was found in soil previously subjected to higher
472 temperature treatments. This could be due to a decrease in the rate of denitrification. However,
473 though complete denitrification was likely not a dominant process in these aerobic soils, it is
474 also possible that under treatment T_2 and T_3 more of the NO_3^- underwent complete
475 denitrification, forming N_2 as opposed to N_2O. This highlights the importance of the gaseous
476 N stoichiometries in particular the N_2/N_2O ratio. Stevens and Laughlin (2001) reported N_2:N_2O
477 ratios in a fine loamy grassland soil of 2.2 and 0.5 from control and combined slurry plus NO_3^-
478 fertiliser treatments, respectively. However, Clough et al. (1998) showed that ratios can vary
479 between 6.2 and 33.2 following ^{15}N-labelled urine application to ryegrass (*Loilum*
480 *perenne*)/white clover (*Trifolium repens*) pasture on four different soils (silt loam, sandy loam,
481 peat and clay soils). Unfortunately, due to methodological restrictions were not able to detect
482 significant N_2 fluxes, as they were <4 g N_2-N ha^{-1} day^{-1} (Stevens and Laughlin, 1998).
483
484 Adaptation of microorganisms, to long-term elevated temperature treatments, might also
485 provide an explanation for the decrease in N_2O emissions during the incubation with soil
486 previously subjected to increasing soil warming temperatures (Avrahami and Conrad, 2003;
487 French et al., 2009; Pritchard, 2011). Enhanced NO_3^- concentrations in the T_2 and T_3
488 treatments, at the end of the field experiment, also suggests an in situ reduction of

489 denitrification and/or co-denitrification. A possible explanation for the in situ reduction of
490 denitrification could be the altered field soil moisture content. While during the incubation, soil
491 moisture was purposely kept constant (WFPS of 64%), in the field however, moisture
492 conditions were affected by the heating treatment, leading to generally drier, and thus more
493 aerated, conditions in the heated plots (Jansen-Willems et al., 2016). Under low WFPS,
494 nitrification is predominantly responsible for N_2O efflux (Bollmann and Conrad, 1998;
495 Bateman and Baggs, 2005). This may be a consequence of altered soil moisture or changes in
496 soil texture and physical soil structure. The reduction of NO_3^- (denitrification) takes place under
497 more anoxic to anaerobic conditions (Smith, 1997), because under aerobic conditions,
498 denitrifiers reduce O_2 rather that NO_3^- (Arah, 1997). Any reduction in soil moisture could
499 therefore lead to a decrease in the in situ denitrification rate.
500
501 Co-denitrification was observed to be significant in $T_{control}$ and T_1 shortly after N addition.
502 Rates were comparable with those from true denitrification. Co-denitrification is a co-
503 metabolic process which uses inorganic and organic N compounds concurrently and converts
504 it to the same end products as in denitrification. Gases produced in this process are a hybrid N-
505 N species where one atom of N comes from NO_2^- and the other one from a co-metabolised
506 compound (Spott et al., 2011). The conditions for increased co-denitrification are still not fully
507 understood, but the presence of fungi along with adequate amino acid pools appears to enhance
508 losses via this pathway (Laughlin and Stevens, 2002; Spott et al., 2011).
509
510 Laughlin and Stevens (2002) found that fungi dominated denitrification and co-denitrification
511 in grassland soils. It has been suggested that warming could increase the relative contribution
512 of fungi to the soil microbial community (Zhang et al., 2005; Pritchard, 2011). Most fungi lack
513 N_2O reductase, resulting in N_2O as the final denitrification product (Saggar et al., 2013). It can

514 therefore be expected that warming would lead to an increase in N_2O produced via
515 denitrification and co-denitrification. However, the opposite was found in the current
516 experiment, although the changes in co-denitrification were not significant. The reduced co-
517 denitrification and total denitrification rates seem to indicate a reduction in fungal-mediated N
518 processes under elevated temperatures in these soils. Further research is required to elucidate
519 the effect of increased temperatures on N processes mediated by fungi.
520

521 **5. Conclusion**

522 Sustained increases in soil temperatures over 6 years (between 2 and 3°C) led to an increase in
523 both total inorganic soil N and NO_3^- pools. Subsequent analyses of gross N transformations,
524 during an incubation of these soils under common temperature and moisture conditions to study
525 the legacy effect of increased temperatures, revealed that mineralisation of amino acids
526 (glycine) and recalcitrant organic N decreased with previously imposed elevated temperatures.
527 This decrease in mineralisation was also correlated with a decrease in N_2O emissions from
528 organic N turnover. However, elevated temperature did not cause a significant change in
529 relative N_2O emissions from the different pathways as hypothesised, but it led to an absolute
530 decrease in N_2O emission rates. A new, easy to use, source partitioning method was developed
531 to determine the contribution of four different pathways to N_2O emissions. Emissions of N_2O
532 in the first six days after fertilisation were decreased for soils previously subjected to higher
533 temperatures as a consequence of a reduction in the rates of denitrification and the oxidation
534 of organic N. For all treatments, oxidation of organic N was the main contributor to N_2O
535 emissions, and should therefore in future research not be omitted as a possible source of N_2O.
536

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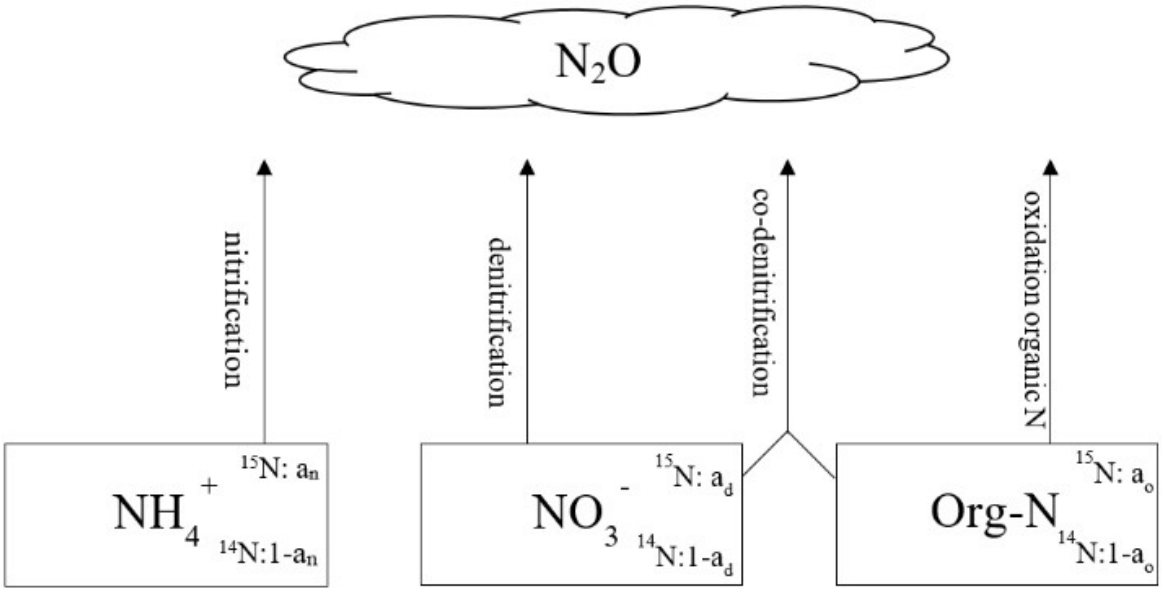
737

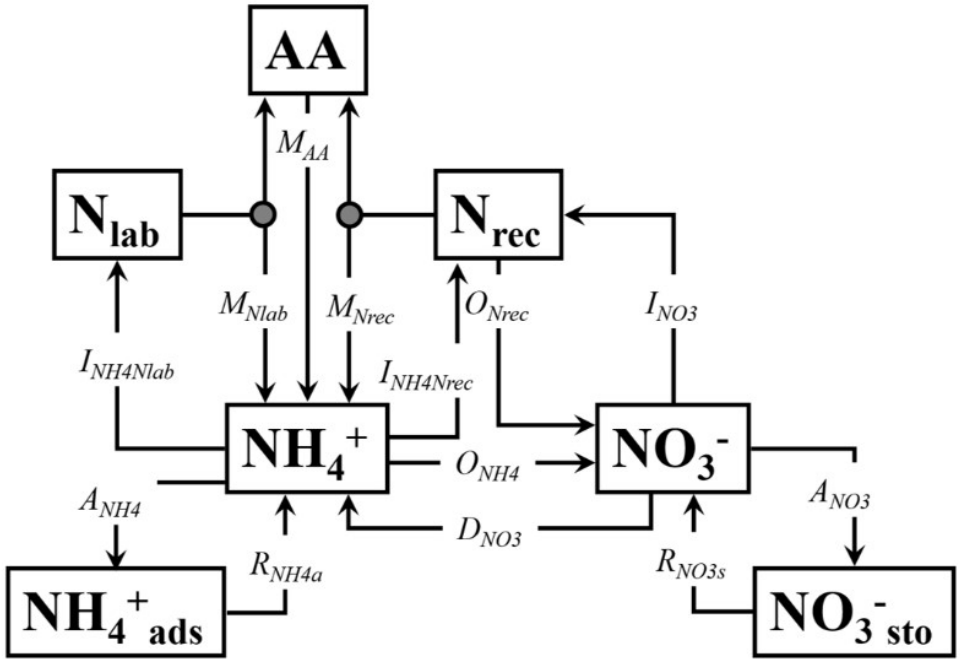
738 **Figures**



739

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

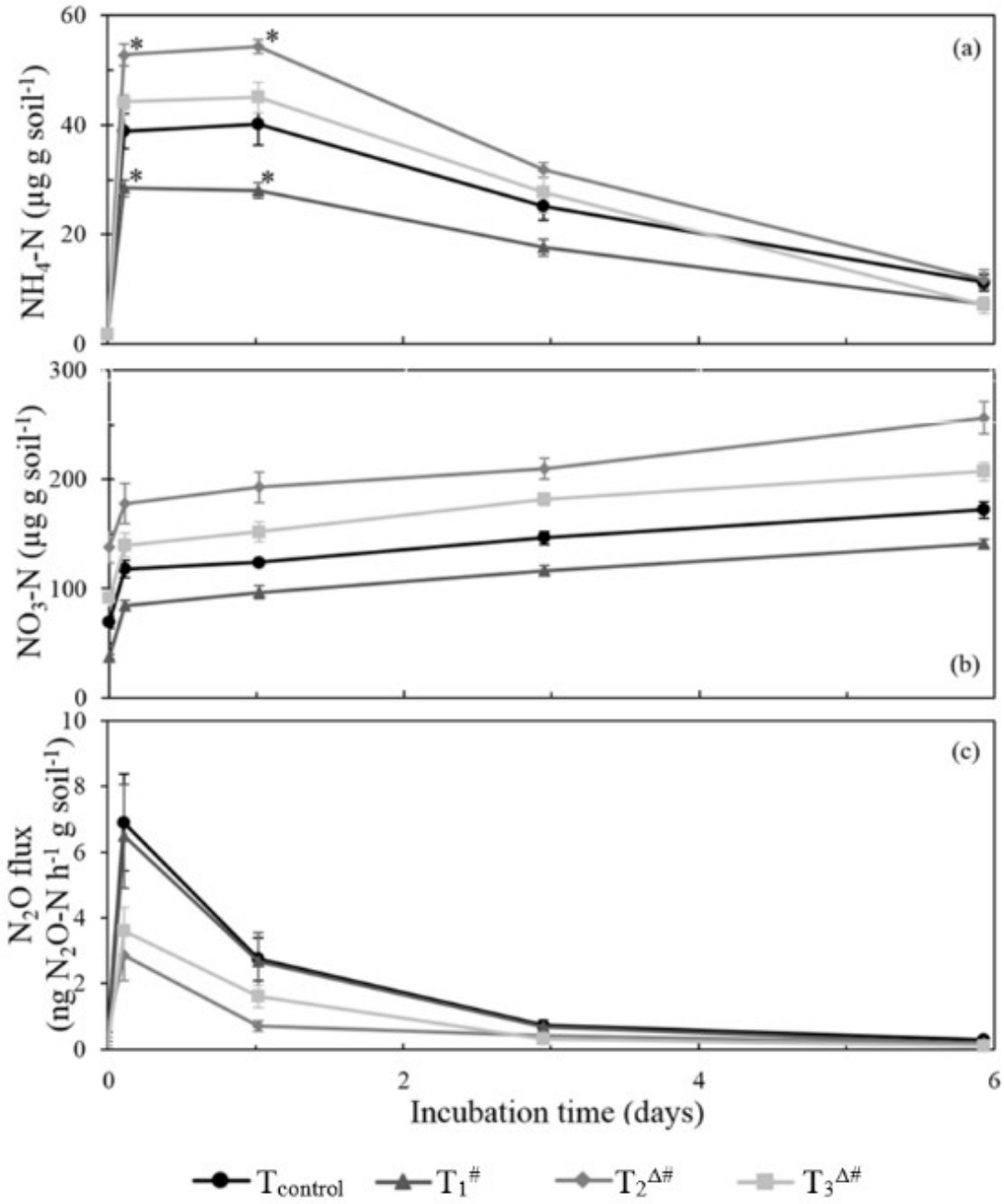
746



747

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

750



751

752 Fig. 3. NH_4-N content (a), NO_3-N content (b), and N_2O emissions (c) at the extraction times.
753 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-
754 **Gly**). The ammonium and nitrate content at time point 0 is based on unlabelled soil. The N_2O
755 flux at time point 0 is based on the average flux of the 3 gas samplings before label addition.
756 The error bars are the standard error of the mean. * shows a significant difference in NH_4-N
757 from $T_{control}$ ($p<0.03$), $^{\#}$ shows a significant difference in NO_3-N from $T_{control}$ ($p<0.0001$), and $^{\Delta}$
758 shows a significant difference in N_2O flux from $T_{control}$ ($p<0.05$).

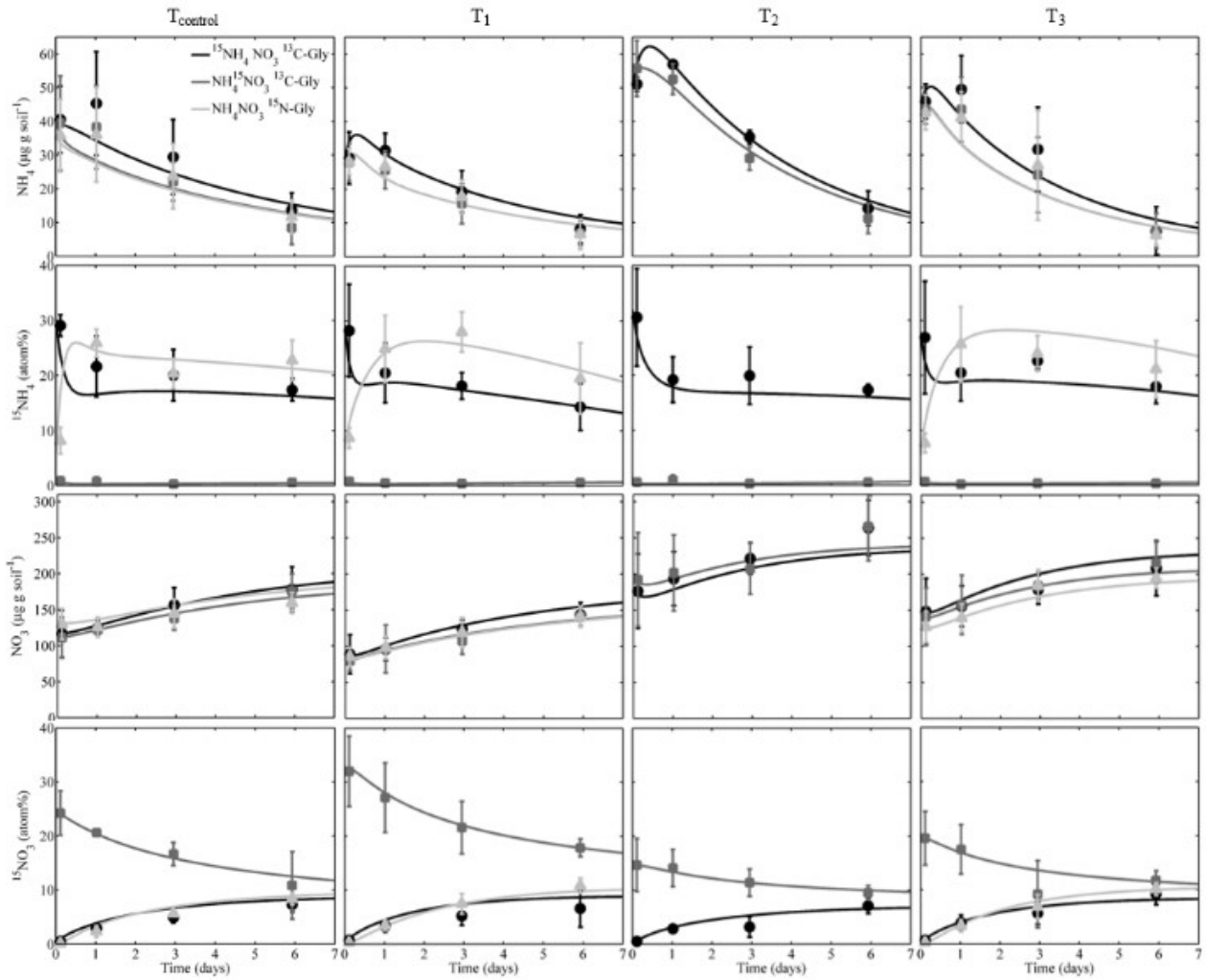
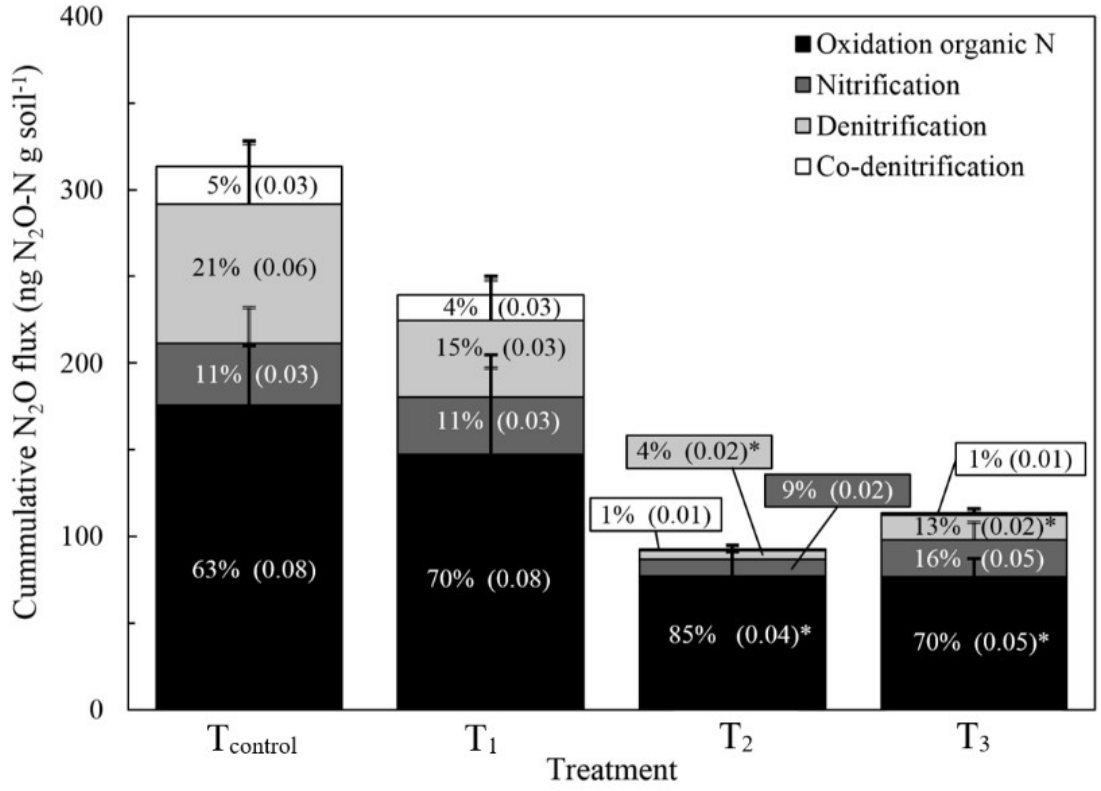


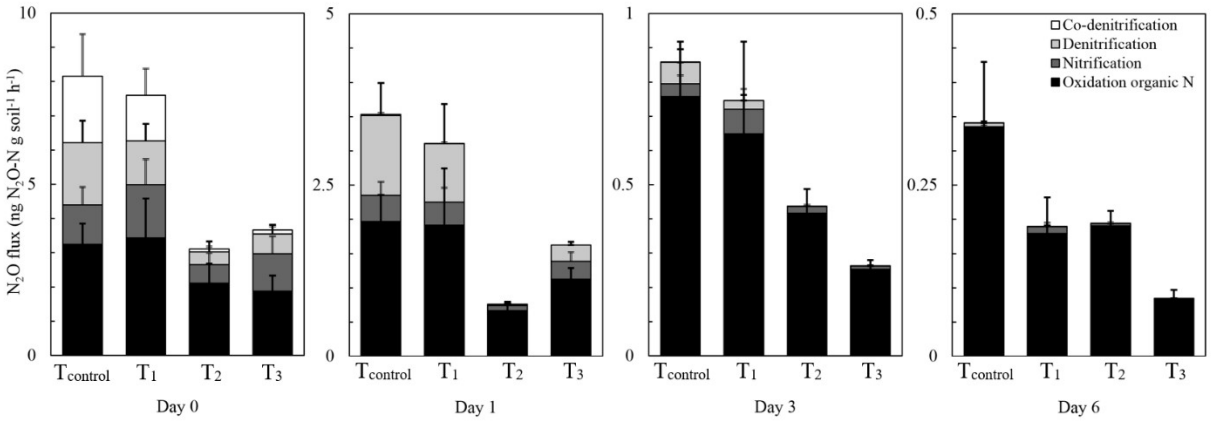
Fig. 4. Modelled vs measured data. The lines are modelled data, and the squares, circles and triangles are the measured data points. Error bars are standard deviations. Time is the time in days from the moment of label addition.



765

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

772



773

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

Tables

Table 1: Description of N transformations and average gross N fluxes per treatment (diagram shown in Fig. 2). Standard deviation between brackets. K stands for Kinetics were 0 implies the use of zero-order and 1 the use of first-order kinetics in the model. The p is the p-value of the one-way ANOVA, with ns (non-significant) if p>0.1 (p value in bold if < 0.05). For the 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**

Table 2. Gross mineralisation (Min_{Gross}), net mineralisation (Min_{Net}), gross nitrification (Nit_{Gross}) and net nitrification (Nit_{Net}) rate in µg N g $soil^{-1}$ d^{-1}. Including the contributions from the different N pools for the gross transformations (italics), where N_{lab} is a labile organic N pool, N_{rec} is a recalcitrant organic N pool, NH_4^+ is the ammonium pool and N_{AA} is the amino acid Gly 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