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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. 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 hypothesized 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 (four replicates per treatment) using IR (infrared) lamps over a period of 6 years. The soil was subsequently incubated under common conditions (20 °C and 50 % humidity) and labelled as $NO_3^{15}NH_4$ Gly, $^{15}NO_3NH_4$ 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 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. In conclusion, long-term soil warming can cause a legacy effect which diminishes organic N turnover 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 km^2 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; X. Z. Zhang et al., 2015) and gross (Larsen et al., 2011; Björsne et al., 2014) N mineralization 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 immobilization 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). In their meta-analyses, Dijkstra et al. (2010) and Bai et al. (2013) identified 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 (X. Z. Zhang et al., 2015). 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 mineralization follows a step-wise sequence of protein depolymerization by extracellular activity to oligomers (e.g. peptides) and monomers (e.g. amino acids) and then uptake by microorganisms before mineralization to NH_4^+ (Schimel and Bennett, 2004). Hence, the production of peptides and amino acids as well as the mineralization of amino acids, affects the main fluxes regulating gross N mineralization. Amino acids have a short residence time in the soil due to either rapid assimilation by soil microbes or mineralization, 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; J. Zhang et al., 2015) (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 fertilization with ammonium nitrate (NH_4NO_3) may be greater than from urea fertilizer 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 the 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, 2013; Rütting et al., 2010; Zhang et al., 2011; Zhu et al., 2011; Müller et al., 2014). However, one of the assumptions of the source-partitioning method is the absence of hybrid reactions such as co-denitrification (J. Zhang et al., 2015). 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^- and 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 straightforward 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 6 years of elevated temperature (via IR heaters) on soil N cycling dynamics, including (1) net and gross N trans-

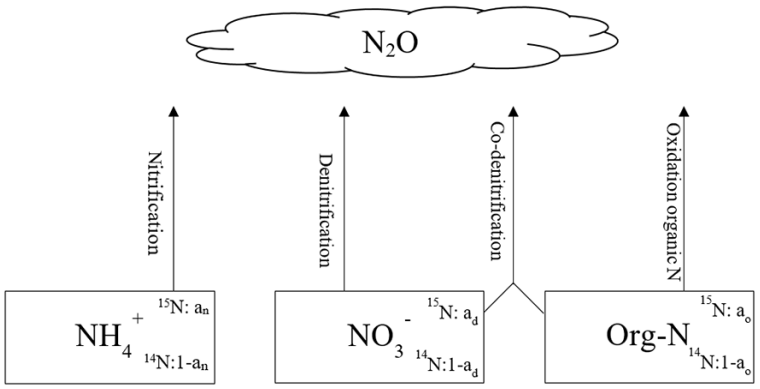

Figure 1. N_2O production via four processes (nitrification, denitrification, co-denitrification and oxidation of organic N). Three uniformly distributed pools were considered. These pools were ammonium (NH_4^+) with a ^{15}N atom fraction of a_n, nitrate (NO_3^-) with a ^{15}N atom fraction of a_d and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

formation rates in the soil, (2) N_2O fluxes immediately after fertilization 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 the 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 hypothesized 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 has been managed as a meadow with two cuts per year and fertilized with 50–80 kg N ha^{-1} yr^{-1} for the last 3 decades. Since 1995, the N fertilizer input has been reduced to 40 kg N ha^{-1} yr^{-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 was 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 28 January 2008, the soil temperature of each plot, measured at 5 cm depth, was elevated by 0, 1 (mean 0.8 standard error (SE) 0.02), 2 (mean 1.9 SE 0.03) or 3 (mean 2.6 SE 0.03) °C 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 h, 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 h to determine the soil gravimetric water content. The remaining field-moist soil was kept at 4 °C (for less than 60 h) until further analysis whereupon the soil from each field plot was sieved through a 10 mm sieve to homogenize 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 (SD 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 sam-

pling. 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 minimizing 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 labelling (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, $^{15}NO_3NH_4$ 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 label was added to each jar using a needle with side ports to inject the solution into the soil to minimize 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, 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 2 M 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 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 day 0, 1, 3 and 6.

2.3 Gas sampling

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

Gas samples were taken using a 60 mL syringe (Ecoject Plus, Gelnhausen, Germany). At time zero (t_0) 15 gas samples were taken from 15 different jars. Then at time 1 (t_1) a gas sample was taken through the rubber septum. At both t_0 and t_1 the syringe was flushed twice with headspace gas to ensure a representative sample was taken. The times between t_0 and t_1 during each of the seven different gas samplings (three before label addition and four immediately prior to extraction) were 120–129, 120, 180, 233, 240, 235 and 214 min, respectively. Gas samples were analysed within 24 h after sampling using a gas chromatograph (GC; Bruker) equipped with an electron capture detector (ECD) for N_2O analysis. An average of the concentrations measured in the 15 samples was used as the t_0 concentration for all 43 jars. Fluxes were based on the parts per million and time difference between t_0 and t_1. They were calculated using the constant gas law, with ambient pressure, and temperature was assumed to be 20 °C (the temperature of the incubation room). The fluxes were then converted to a per dry gram basis.

For the ^{15}N abundance of N_2O, a 30 mL sample was taken at t_1 and transferred to a 12 mL Exetainer® vial (Labco Ltd, High Wycombe, Buckinghamshire, UK). The overpressurized 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 had 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 spectrometer (Sercon Ltd 20-20), as described by Stevens et al. (1993), interfaced to a TGII cryofocusing unit (Sercon Ltd 20-20). The detection limit for atom % ^{15}N of a 50 ppm N_2O standard gas was 0.00003 ($n = 10$); SD was 0.00009 atom % ^{15}N. Respective values for a 0.4 ppm N_2O standard were higher (0.00084 ($n = 10$); SD 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 7 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_{4ads}^+), and stored nitrate (NO_{3sto}^-).

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_{4ads}^+ and NO_{sto3}^- 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), and for N_{lab}, the quick turnover time ensures that a small pool will be governed quickly by the dynamics of the in- and out-flowing rates.

Table 1. Description of N transformations and average gross N fluxes per treatment (diagram shown in Fig. 2). Standard deviation in brackets. K stands for kinetics, where 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–Šídák pairwise comparisons, ^{t} indicates a tendency to be different from control ($p < 0.10$).

	Transformation	K	Average gross flux ($\mu g\,N\,g\,soil^{-1}\,day^{-1}$)								p
			$T_{control}$		T_1		T_2		T_3		
$M_{N_{rec}}$	Mineralization 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**
$I_{NH_4N_{rec}}$	Immobilization of NH_4^+ to N_{rec}	1	16.12	(9.23)	13.43	(6.92)	17.45	(6.53)	4.72	(3.65)	ns
$M_{N_{lab}}$	Mineralization 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
$I_{NH_4N_{lab}}$	Immobilization of NH_4^+ to N_{lab}	1	30.59	(19.34)	22.28	(14.65)	30.54	(8.82)	29.59	(19.78)	ns
$O_{N_{rec}}$	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_{NO_3}	Immobilization 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_{NH_4}	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_{NO_3}	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_{NH_4}	Adsorption of NH_4^+	1	34.26	(19.67)	20.41	(19.61)	23.64	(11.50)	15.81	(12.84)	ns
R_{NH_4a}	Release of adsorbed NH_4^+	1	33.22	(21.43)	20.51	(12.33)	24.77	(6.15)	16.41	(9.07)	ns
A_{NO_3}	Adsorption of NO_3^-	1	28.08	(14.18)	55.23	(37.72)	82.39	(58.45)	62.99	(47.75)	ns
R_{NO_3s}	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}	Mineralization 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**

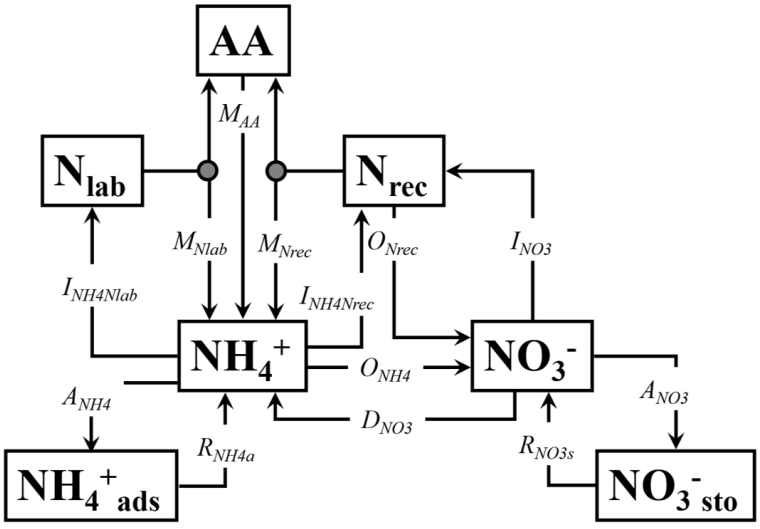

Figure 2. ^{15}N tracing model for analyses of gross soil N transformation rates. Abbreviations of the transformations are explained in Table 1. The pools are explained in Sect. 2.4.

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 $M_{N_{lab}}$ and one for $M_{N_{rec}}$. These factors determine the fraction of the $M_{N_{lab}}$ or $M_{N_{rec}}$ 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 optimized by minimizing 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 optimization because only one replicate was available for this label.

A Markov chain Monte Carlo Metropolis algorithm (MCMC-MA) was used for the optimization, 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 optimization 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 Akaike information criterion (AIC). Gross and net nitrification and gross and net mineralization were calculated using Eqs. (1)–(4), in which SF stands for

split factor. The combined standard deviation was calculated by $((\text{SD rate 1})^2 + (\text{SD rate 2})^2 + \ldots)^{0.5}$, in which the SD of M_{N_x}. $\mathrm{SF}_{M_{\mathrm{N}_x}}$ is the SD of M_{N_x} multiplied by the SF.

The following combined rates were calculated.

$$\text{Gross nitrification}: \; O_{\mathrm{N_{rec}}} + O_{\mathrm{NH_4}} \quad (1)$$

$$\text{Net nitrification}: \; O_{\mathrm{N_{rec}}} + O_{\mathrm{NH_4}} - I_{\mathrm{NO_3}} - D_{\mathrm{NO_3}} \quad (2)$$

$$\text{Gross mineralization}: M_{\mathrm{N_{lab}}} \cdot \mathrm{SF}_{M_{\mathrm{N_{lab}}}} + M_{\mathrm{N_{rec}}} \cdot \mathrm{SF}_{M_{\mathrm{N_{rec}}}} + M_{\mathrm{AA}} \quad (3)$$

$$\text{Net mineralization}: M_{\mathrm{N}lab} \cdot \mathrm{SF}_{M_{\mathrm{N_{lab}}}} + M_{\mathrm{N_{rec}}} \cdot \mathrm{SF}_{M_{\mathrm{N_{rec}}}} + M_{\mathrm{AA}} - I_{\mathrm{NH_4N_{rec}}} - I_{\mathrm{NH_4N_{lab}}} - I_{\mathrm{NO_3}} \quad (4)$$

2.5 Determining the contribution of different processes to N_2O flux

The N_2O fluxes, from the soil labelled with $NO_3^{15}NH_4$ Gly and $^{15}NO_3NH_4$ 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. The pools and processes accounting for the N_2O production are shown in Fig. 1. 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 Eqs. (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_o for the oxidation of organic N; a_n, a_d and a_o are explained in Fig. 1.

$$\text{Chance of } zero^{15}\text{N atoms}: \; (1-a_x)^2 \quad (5)$$

$$\text{Chance of } one^{15}\text{N atom}: \; 2(1-a_x)a_x \quad (6)$$

$$\text{Chance of } two^{15}\text{N atoms}: \; a_x^2 \quad (7)$$

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

$$\text{Chance of } zero^{15}\text{N atoms}: \; (1-a_\mathrm{d})(1-a_0) \quad (8)$$

$$\text{Chance of } one^{15}\text{N atom}: \; a_\mathrm{d}(1-a_0) + a_0(1-a_\mathrm{d}) \quad (9)$$

$$\text{Chance of } two^{15}\text{N atoms}: \; a_\mathrm{d}a_0 \quad (10)$$

The chance that the N_2O in the gas sample contains zero, one or two ^{15}N atoms is described by Eqs. (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.

$$\text{Chance of } zero^{15}\text{N atoms}: n(1-a_\mathrm{n})^2 + d(1-a_\mathrm{d})^2 + o(1-a_\mathrm{o})^2 + (1-n-d-o)(1-a_\mathrm{d})(1-a_0) \quad (11)$$

$$\text{Chance of } one^{15}\text{N atom}: 2n(1-a_\mathrm{n})a_\mathrm{n} + 2d(1-a_\mathrm{d})a_\mathrm{d} + 2o(1-a_\mathrm{o})a_\mathrm{o} + (1-n-d-o)(a_\mathrm{d}(1-a_0) + a_0(1-a_\mathrm{d})) \quad (12)$$

$$\text{Chance of } two^{15}\text{N atoms}: na_\mathrm{n}^2 + da_\mathrm{d}^2 + oa_\mathrm{n}^2 + (1-n-d-o)a_\mathrm{d}a_0 \quad (13)$$

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 ^{x}I 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 Eq. (11)–(13), where a_o was set to 0.003663 and a_n and a_d were considered to be the ^{15}N abundance 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 $^{15}NO_3NH_4$ 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 $^{15}NO_3NH_4$ 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) and inorganic N (NO_3^-+NH_4^+), NO_3^- and NH_4^+ concentrations was analysed using the MIXED procedure in SAS (Version 9.3, SAS institute). The N_2O fluxes were transformed using $\log(\text{flux}+10)$. The N_2O fluxes via the different processes were transformed using $\text{flux}^{1/4}$. A Tukey–Kramer adjustment was used to correct for multiplicity effects in pairwise comparisons. Residual checks were made to ensure that

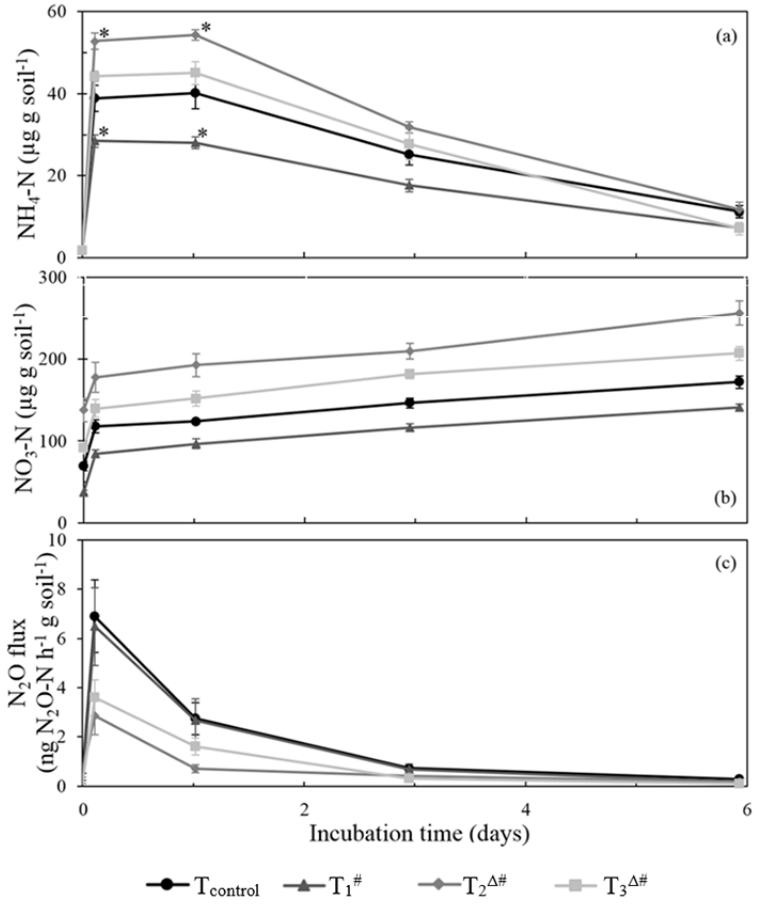

Figure 3. NH_4-N content **(a)**, NO_3-N content **(b)** and N_2O emissions **(c)** at the extraction times. Time point 0 is the time of label addition ($^{15}NH_4NO_3$ Gly, $NH_4^{15}NO_3$ Gly or $NH_4NO_3^{15}N$ Gly). The ammonium and nitrate content at time point 0 is based on unlabelled soil. The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

the assumptions of the analysis were met. The effect of treatment on modelled N transformation rates was 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–Šídák 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 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 of $T_1 < T_{control} < T_3 < T_2$.

Soil NH_4^+ concentrations increased from $2\,\mu g\,N\,g^{-1}$ soil to between 28 and $54\,\mu g\,N\,g^{-1}$ soil upon label addition, and subsequently decreased over the next 5 days to ca. $9\,\mu g\,N\,g^{-1}$ soil (Fig. 3a). 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 ($p < 0.001$ and 0.044, respectively).

After the initial increase in NO_3^- due to label addition, the NO_3^- concentrations continued to slowly increase over the following 6 days (Fig. 3b). NO_3^- concentrations were significantly different among the treatments ($p < 0.001$), with dif-

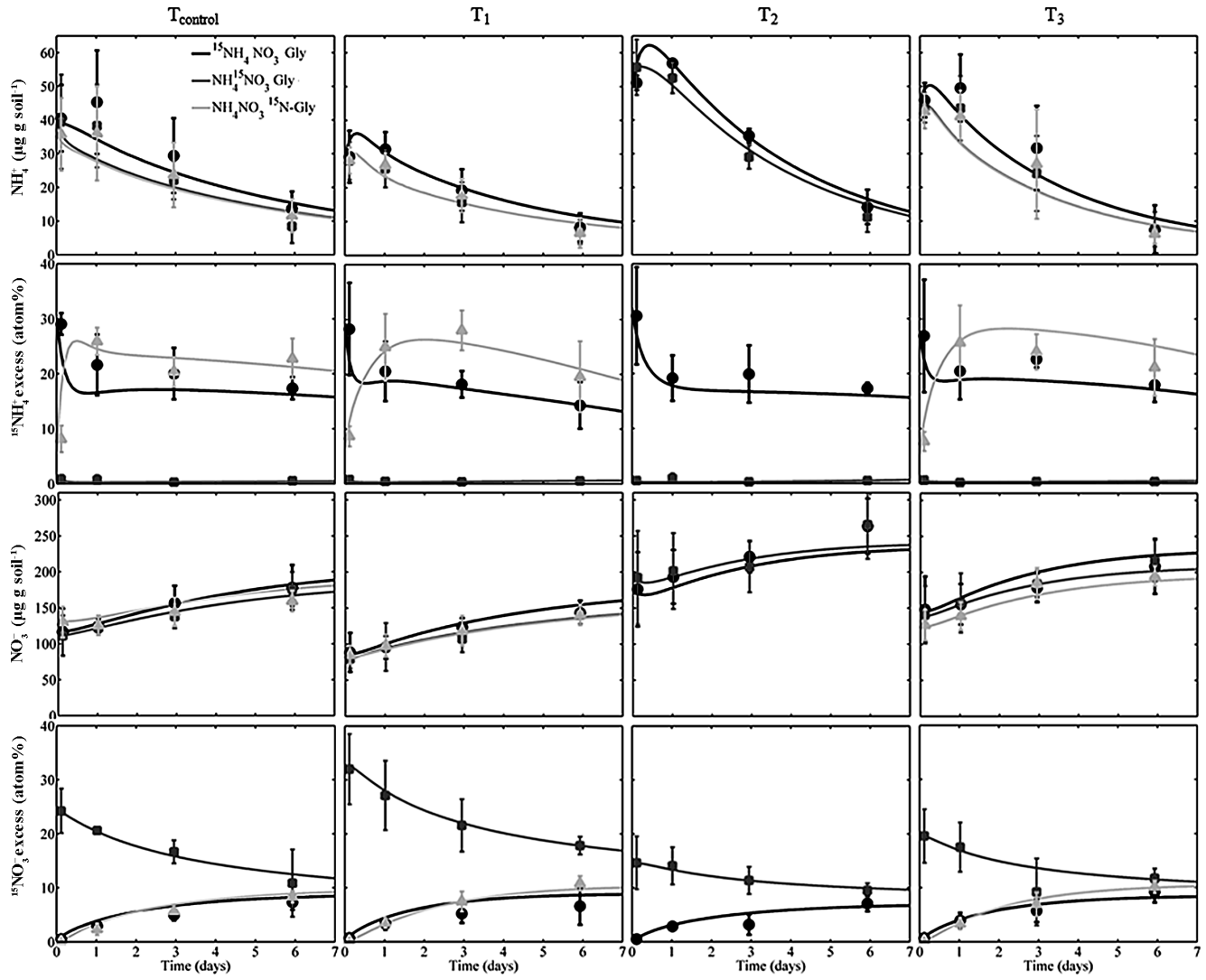

Figure 4. Modelled vs. measured data. The lines are modelled data, and the squares, circles and triangles are the measured data points. Error bars are standard deviations. Time is the time in days from the moment of label addition.

ferences 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.

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 mineralization were reduced under the T_2 and T_3 treatments ($p = 0.040$). Mineralization of amino acids also became slower with increasing temperatures ($p = 0.045$). However, the overall gross mineralization of organic N to NH_4^+ did not differ with the previously imposed warming treatments. This was because the mineralization of labile organic N was the major contributor to total mineralization, and this rate was not significantly affected by previous warming (Table 2). Net mineralization 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_{NH_4} and $O_{N_{rec}}$ 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

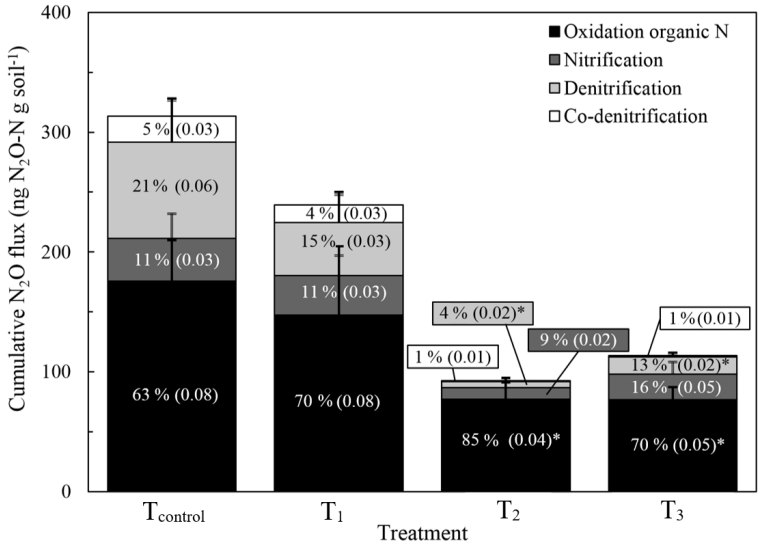

Figure 5. Cumulative N_2O flux via four processes between 3 h and 6 days after labelling. N_2O fluxes based on average flux from soil labelled with $^{15}NH_4NO_3$ Gly or $NH_4^{15}NO_3$ Gly. The cumulative flux per process is an average over the four plots per treatment. Error bars are standard error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

Table 2. Gross mineralization (Min_{Gross}), net mineralization (Min_{Net}), gross nitrification (Nit_{Gross}) and net nitrification (Nit_{Net}) rate in $\mu g\,N\,g\,soil^{-1}\,day^{-1}$. 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} indicates 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

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 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.

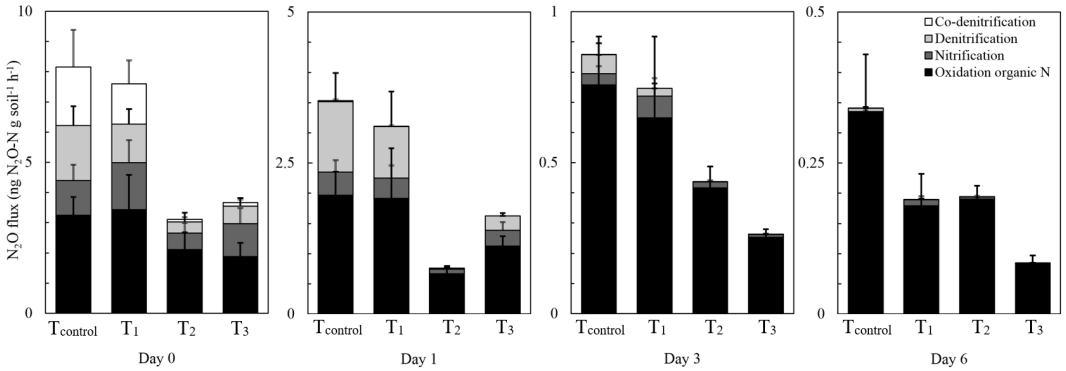

Figure 6. N_2O flux divided into four processes at different time points after fertilization. N_2O fluxes based on average flux from soil labelled with $^{15}NH_4NO_3$ Gly or $NH_4^{15}NO_3$ Gly. The portrayed flux per process is an average over the four plots per treatment. Error bars are standard error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

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 6-year warming treatment. This suggests that a sustained increase in temperature led to an increase in net mineralization and net nitrification. This is in line with previous studies showing increases in net mineralization in response to warming (Peterjohn et al., 1994; Rustad et al., 2001; Norby and Luo, 2004; Bai et al., 2013; Björsne et al., 2014; X. Z. Zhang et al., 2015). 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., 2014; X. Z. Zhang et al., 2015). Both could be due to infield temperatures being more favourable for optimal microbial activity. In agreement with previous research (Bai et al., 2013; X. Z. Zhang et al., 2015), the total soil N pool did not differ among warming treatments. This result may be due to the fact that the relative sizes of the N pools differ: since the total soil N pool is significantly larger than the inorganic N pool, it may take longer to register a change (Galloway 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 mineralization rates did not differ as a result of the previously imposed soil warming treatments, the mineralization of recalcitrant N and mineralization of amino acids did differ. Lowest rates were found under T_2 ($M_{N_{rec}}$) and T_3 ($M_{N_{rec}}$ and M_{AA}). A similar effect to warming was found by Jamieson et al. (1998), who reported decreased gross N mineralization 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 mineralization and even decreases in individual mineralization rates were found. However, no data were available to test this hypothesis. Another possible explanation for the reduction in mineralization rates could be a depletion of substrate due to the 6 years of elevated temperatures.

Previous research in heathland and grassland soils showed no significant effect of warming on amino acid mineralization 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 mineralization 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^+ immobilization rates were not affected by previously imposed warming in the current study is in line with previous research (Niboyet et al., 2011; Bai et al., 2013; Björsne et al., 2014). It has been suggested that the depletion of labile C due to warming might initiate a decrease in immobilization rates (Bai et al., 2013). In the current experiment a labile carbon source (Gly) was added to the soil, which could explain why no reduction in NH_4^+ immobilization was found.

Nitrous oxide emissions were highest shortly after label addition and declined thereafter. Thus, initial higher rates from NH_4^+ and NO_3^- were due to label addition. The higher absolute rate of organic N oxidation at the start of the incubation did not come solely from the Gly addition. If this had been the case, highest N_2O ^{15}N enrichment would have been observed at the first measurement following addition of the NO_3NH_4 ^{15}N-Gly label. However, for all treatments the highest ^{15}N enrichment of N_2O was found in the second measurement after label addition. The lower net rates of N_2O production at the end of incubation period could pos-

sibly have been caused by N_2O consumption; however, the consumption of pathway-specific N_2O emissions cannot be evaluated with the current model. However, as WFPS was set to 64 %, it is unlikely that N_2O consumption occurred, as this would predominantly occur only under fully reductive conditions (but see Goldberg and Gebauer, 2009, for an exception).

Oxidation of organic N was found to be the main source of N_2O. The production of N_2O from an unlabelled organic source would most likely follow a combined process of organic N oxidation via heterotrophic nitrifiers to nitrite, followed by a reduction of nitrite to gaseous N products (Butterbach-Bahl et al., 2013). This process, where oxidation and reduction processes occur hand in hand, would be conceptually similar to the nitrifier-denitrification process (Wrage et al., 2001). Most research, however, does not take the oxidation of organic N into account as a possible source of N_2O (J. Zhang et al., 2015) even though recent studies showed that this process contributed 54–85 % of N_2O emissions in pastures (Rütting et al., 2010; Müller et al., 2014). These contributions are in line with the current study. Müller et al. (2014) also showed that the fraction of N_2O contributed via the oxidation of organic N was lowest immediately following NH_4NO_3 addition and that this fraction increased to over 80 %, while the contribution of denitrification decreased with time even though NO_3^- concentrations increased. Because of the large contribution of oxidation of organic N in N_2O emissions, this pathway should not be omitted in future research.

A decrease in N_2O produced via denitrification was found in soil previously subjected to higher-temperature treatments. This could be due to a decrease in the rate of denitrification. However, though complete denitrification was likely not a dominant process in these aerobic soils, it is also possible that under treatment T_2 and T_3 more of the NO_3^- underwent complete denitrification, forming N_2 as opposed to N_2O. This highlights the importance of the gaseous N stoichiometries, in particular the N_2 / N_2O ratio. Stevens and Laughlin (2001) reported N_2 / N_2O ratios in a fine loamy grassland soil of 2.2 and 0.5 from control and combined slurry plus NO_3^- fertilizer treatments, respectively. However, Clough et al. (1998) showed that ratios can vary between 6.2 and 33.2 following ^{15}N-labelled urine application to ryegrass (*Lolium perenne*)–white clover (*Trifolium repens*) pasture on four different soils (silt loam, sandy loam, peat and clay soils). Unfortunately, due to methodological restrictions we were not able to detect significant N_2 fluxes, as they were $< 4\,g\,N_2\text{-}N\,ha^{-1}\,day^{-1}$ (Stevens and Laughlin, 1998).

The adaptation of microorganisms to long-term elevated-temperature treatments might also provide an explanation for the decrease in N_2O emissions during the incubation with soil previously subjected to increasing soil warming temperatures (Avrahami and Conrad, 2003; French et al., 2009; Pritchard, 2011). Enhanced NO_3^- concentrations in the T_2 and T_3 treatments, at the end of the field experiment, also suggest an in situ reduction of denitrification and/or co-denitrification. A possible explanation for the in situ reduction of denitrification could be the altered field soil moisture content. While during the incubation, soil moisture was purposely kept constant (WFPS of 64 %); in the field, however, moisture conditions were affected by the heating treatment, leading to generally drier, and thus more aerated, conditions in the heated plots (Jansen-Willems et al., 2016). Under low WFPS, nitrification is predominantly responsible for N_2O efflux (Bollmann and Conrad, 1998; 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 them to the same end products as in denitrification. Gases produced via this process are a hybrid N–N species where one atom of N comes from NO_2^- and the other one from a co-metabolized compound (Spott et al., 2011). The conditions for increased co-denitrification are still not 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.

5 Conclusion

Sustained increases in soil temperatures over 6 years (between 2 and 3 °C) led to an increase in both total inorganic soil N and NO_3^- pools. Subsequent analyses of gross N transformations, during an incubation of these soils under common temperature and moisture conditions to study the legacy effect of increased temperatures, revealed that mineralization of amino acids (glycine) and recalcitrant organic

N decreased with previously imposed elevated temperatures. This decrease in mineralization was also correlated with a decrease in N_2O emissions from organic N turnover. However, elevated temperature did not cause a significant change in relative N_2O emissions from the different pathways as hypothesized, but it led to an absolute decrease in N_2O emission rates. A new, easy to use, source-partitioning method was developed to determine the contribution of four different pathways to N_2O emissions. Emissions of N_2O in the first 6 days after fertilization were decreased for soils previously subjected to higher temperatures as a consequence of a reduction in the rates of denitrification and the oxidation of organic N. For all treatments, oxidation of organic N was the main contributor to N_2O emissions and should therefore in future research not be omitted as a possible source of N_2O.

6 Data availability

The data will be made available via the following database: http://www.face2face.center.

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