Dear Sharon Billings,

On behalf of myself and all the co-authors, I would to thank you, and the two reviewers for the time taken to review our manuscript. We have incorporated all suggested changes, and believe this has improved our manuscript. Below you can find the reviewers comments, including our response in blue. The line numbers given in blue refer to the line numbers in the revised manuscript.

Kind regards, Anne Jansen-Willems

Referee #1

"I found the approach very interesting, but I thought a little bit more experimental detail should be provided"

To accommodate this, the following sentence was added:

• The 16 plots were, according to a Latin square design, assigned to one of four treatments (L138-139)

And also the additions given in the following three comments

"In my opinion the authors nicely combined filed/lab work and modelling, however, when I read through the article at first I was a bit confused about the modelling component, because I found it presented as an "add on". I think it could be a bit more put forward in the abstract already'

We agree with the reviewer that it would be better to make this already clearer in the abstract as the modelling is a major component of this paper. We therefore made the following changes:

- Sentence in abstract starting with "To evaluate " has been replaced with the following sentence: In order to evaluate the legacy effects of increased temperature on both nitrogen (N) transformation rates in the soil and nitrous oxide (N₂O) emissions, an incubation experiment and modelling approaches were combined. Soils were taken from a long term in situ warming experiment on temperate permanent grassland. (L19-22)
- Following sentence is added: Soil extractions and N₂O emissions were analysed using a ¹⁵N tracing model and source partitioning model. (L26-27)

The following has been added to the end of the introduction

Net and gross transformation rates were determined using an extended version of a basic ¹⁵N 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. (L113-118)

"I would also have liked to be provided with more detail on the calculation of N_2O fluxes (which factors were taken into account for the calculation, which equations were used?)"

The following sentence has been added to L201-204

Fluxes were based on the ppm and time difference between t₀ and t₁. 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.

"Moreover, I would be interested in a little bit more detail on the IRMS analysis (e.g.: how were samples returned to ambient pressure?, what was the precision of your IRMS analyser?, how were δ -values defined?"

The following sentences have been added

- 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. (L209-213)
- The detection limit for atom% ¹⁵N of a 50 ppm N₂O standard gas was 0.00003 (n= 10), stdev was 0.00009 atom% ¹⁵N. Respective values for a 0.4 ppm N₂O standard were higher (0.00084 (n= 10), stdev 0.003). (L216-218)

"My last specific comment refers to figure 4. Maybe it's just me but I did not really get figure 4. In the figure caption is says: "Modelled vs measured data", but, to me it seems like either modelled or measured data is presented. I would be very grateful for an explanation."

Figure 4, does provide both the modelled and measured data. To make this clearer the figure caption has been changed to the following:

• 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.

Technical corrections

- L48 (has been removed (L50)
- L96 N2O has been changed to N₂O (L101)
- L132 space added (L143)
- L137 Sentence has been changed, see next correction
- L137&150 Sentences have been changed to:
 - On the day the heaters were turned off, all soil within a circular area of 318..... (L148)
 - Two days after soil sampling (day -55), all jars were put in... (L161).
- L329-332 Changed to
 - However, the overall gross mineralisation of organic N to NH4⁺ 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). (L354-358)
- L407 comma added between soil and which (L432)
- L426-429 Changed to:
 - \circ This could be due to a decrease in the rate of denitrification. However, it is also possible that under treatment T₂ and T₃ more of the NO₃⁻ underwent complete denitrification, forming N₂ as opposed to N₂O. (L463-465)
- L460 Still not yet changed to still not (L496)
- L473 dot added to sentence (L509)
- L483 Was decreased changed to were decreased (L519)
- L704 Space added (L740)
- L 716 Space added (L753)

Referee #2

"The summary of my suggestion for the authors is to clarify the role and importance of the model and the source partitioning in the hypothesis and discussion. Like the first reviewer, I find that the important role that the model and the math play in this paper is not sufficiently emphasized"

Changes have been made as described in comment 2 of the first reviewer.

"Regarding the model: I found it difficult from the included text to understand how this model differs from the one published in Müller 2014. I would be helpful to the reader is more detail were provided here."

The following sentence has been added

• In the current study, the only changes to original model were the addition of an amino-acid (glycine) pool, and the transformations to and from this pool. (L222-223)

"I am also not certain if those differences require further support; there is an addition of amino acids in the model (which is based on glycine additions, not the extant AA pool, unlike the NH3 and NO3 calculations), there are less time points, no nitrite measurements and no N gas output. I not that previous iterations of this model have received extensive verification. How can we evaluate the robustness of this new model?"

Evaluations of the robustness of the model are explained in the introduction (L113-118) To make clear that the AA pool refers to the glycine we changed the description of AA to amino acid glycine (L225)

"It is not clear how the starting point recalcitrant and labile N parameters were determined (it is probably in Muller 2014, or 2004 or 2007, but would like to see it in here), or even what these pools include (chemically/physically, because the functional implications are self-evident).

To explain the starting points, the following sentences have been included

 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. (L230-234)

"Regarding the source partitioning: I really enjoyed this method and results (fig. 5), a very nice illustration of the importance of organic N as a source of N2O, which in turn supports the model results. However, since the Ngas process has been removed in the model, and N2O consumption is not in the source partitioning (see my comment for Fig 6 below), could the authors please comment on whether the portioning math is sensitive to N2O consumption?"

Our response regarding the discussion of Fig. 6 does include this. (see number 6 of the comments by line from reviewer 2)

Comments by line

- L24 (pre- and post-incubation) added (L29)
- L74 sentence change to

- \circ Müller et al. (2014) found that, for the same grassland soil as used in this study, codenitrification contributed 17.6% of the total N₂O production. (L77-78)
- L130 changes made as described in comment 1 from reviewer 1
- L136-166 Day numbers changed, label addition was set as day 0
- L204 as explained in comment 4 of reviewer 2
- L331 labile N changed to labile organic N (L356)
- Fig 4. New caption given as described for reviewer 1
- Discussion Fig 6. The following has been added
 - Nitrous oxide emissions were highest shortly after label addition and declined thereafter. Thus, initial higher rates from NH₄⁺ and NO₃⁻ 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 N₂O ¹⁵N enrichment would have been observed at the first measurement following addition of the NO₃NH₄ ¹⁵N-gly label. However, for all treatments the highest ¹⁵N enrichment of N₂O was found in the second measurement after label addition. The lower net rates of N₂O production, at the end of incubation period could possibly have been caused by N₂O consumption, however, the consumption of pathway specific N₂O emissions cannot be evaluated with the current model. However, as WFPS was set to 64%, it is unlikely that N₂O consumption occurred, as this would predominantly occur only under fully reductive conditions. (L435-445).
- Table 2 Figure caption changed to:
 - \circ 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, NH₄⁺ is the ammonium pool and N_{AA} is the amino acid Gly pool. ^t one-way ANOVA tendency p<0.1

1	Long-term elevation of temperature affects organic N turnover and
2	associated N ₂ O emissions in a permanent grassland soil
3	
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17 Abstract

18 Over the last century an increase in mean soil surface temperature has been observed and it is 19 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 (N₂O) 20 emissions, an incubation experiment and modelling approaches were combined. Soils were 21 taken from a long term in situ warming experiment on temperate permanent grassland. In this 22 experiment the soil temperature was elevated by 0 (control), 1, 2 or 3°C (4 replicates per 23 treatment) using IR-lamps over a period of 6 years. The soil was subsequently incubated under 24 common conditions (20 °C and 50 % humidity) and labelled with NO3¹⁵NH4 Gly, ¹⁵NO3NH4 25 Gly or NO₃NH₄¹⁵N-Gly. Soil extractions and N₂O emissions were analysed using a ¹⁵N tracing 26 27 model and source partitioning model. Both total inorganic N (NO₃⁻⁺NH₄⁺) and NO₃⁻ contents were higher in soil subjected to the +2 °C and +3 °C temperature elevations (pre- and post-28 29 incubation). Analyses of N transformations using a ¹⁵N tracing model, showed that, following 30 incubation, gross organic (but not inorganic) N transformation rates decreased in response to 31 the prior soil warming treatment. This was also reflected in reduced N₂O emissions associated 32 with organic N oxidation and denitrification. Furthermore, a newly developed source 33 partitioning model showed the importance of oxidation of organic N as a source of N₂O. 34 Concluding, long term soil warming can cause a legacy effect which diminishes organic N turn over and the release of N₂O from organic N and denitrification. 35

36 1. Introduction

37 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). 38 39 Concomitantly, the Earth's mean surface temperature has increased by 0.6°C in the past century 40 with surface temperatures expected to increase by a further 1.5-4.5°C resulting from a doubling 41 of the atmospheric carbon dioxide (CO₂) 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 42 43 interactions are to a large extent affected by temperature (Luo, 2007). Thus, research into the 44 effect of elevated soil temperatures is essential to better understand biogeochemical N cycling 45 in grassland ecosystems.

46

47 Previous research generally showed an increase in both net (Peterjohn et al., 1994; Rustad et 48 al., 2001; Norby and Luo, 2004; Butler et al., 2012; Bai et al., 2013; Björsne et al., 2014; Zhang 49 et al., 2015b) and gross (Larsen et al., 2011; Björsne et al., 2014) mineralisation under elevated 50 soil temperatures. However, not all studies found this effect (Emmett et al., 2004; Niboyet et 51 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., 52 53 2011; Bai et al., 2013; Björsne et al., 2014). Dijkstra et al. (2010) and Bai et al. (2013) 54 identified, in their meta-analyses, increases in inorganic N under elevated soil temperatures. 55 Most of this inorganic N increase occurred as nitrate (NO_3^{-}) (Dijkstra et al., 2010). Peterjohn 56 et al. (1994) also found that average monthly ammonium (NH₄⁺) concentrations increased in a 57 mineral soil under forest, however, daily average concentrations did not differ. In the same study, no differences in NO3⁻ concentrations were observed, and the amount of extractable 58 59 NO₃⁻ 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). Which is in line with other studies regarding total soil N (Bai et al., 2013) and inorganic N (Larsen et al., 2011).

62

63 N mineralisation follows a step-wise sequence of protein depolymerisation by extracellular 64 activity to oligomers (e.g. peptides) and monomers (e.g. amino acids) and then uptake by microorganisms before mineralisation to NH4⁺ (Schimel and Bennett, 2004). Hence, 65 production of peptides and amino acids as well as mineralisation of amino acids, affects the 66 67 main fluxes regulating gross N mineralisation. Amino acids have a short residence time in the 68 soil due to either rapid assimilation by soil microbes or mineralisation, which occurs within a 69 few hours (Farrell et al., 2014). In heathland and grassland soils no effect of soil warming on 70 the amino acid concentration was observed (Chen et al., 2014; Andresen et al., 2015).

71

72 Nitrous oxide (N₂O), a potent greenhouse gas with a global warming potential of 298 on a 100 73 year basis, can be produced by several processes, such as nitrification, partial denitrification, 74 co-denitrification and the oxidation of organic matter (Butterbach-Bahl et al., 2013; Zhang et 75 al., 2015a) (Fig. 1). Laughlin and Stevens (2002) confirmed the importance of co-76 denitrification for N₂ 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 77 78 used in this study, co-denitrification contributed 17.6% of the total N_2O production. N_2O 79 emissions following fertilisation with ammonium nitrate (NH4NO3) may be greater than from 80 urea fertiliser because of the greater susceptibility to denitrification (Harrison and Webb, 81 2001). The amount and form of N inputs primarily govern N₂O emissions with further impacts 82 resulting from climatic factors, such as temperature and precipitation, and soil factors, such as 83 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 84

85 impact of elevated soil temperature on N₂O production, in semi-natural grasslands is unclear (Peterjohn et al., 1994; Bijoor et al., 2008; Larsen et al., 2011). Furthermore, there has been 86 87 very limited research into the effect of elevated soil temperature on the different N2O 88 production processes. Maag and Vinther (1996) observed a decrease in nitrification associated 89 N₂O emissions and an increase in denitrification associated N₂O with increasing soil 90 temperature. It has been suggested that this was due to creation of anoxic conditions and the 91 associated depletion of oxygen following the increase in microbial respiration with higher soil 92 temperatures (Castaldi, 2000). Prolonged elevated soil temperatures, on the other hand, could 93 also lead to changes in the microbial community (Avrahami and Conrad, 2003; French et al., 94 2009).

95

96 Several methods, such as source partitioning, have been used to quantify the contributions of 97 individual N pools to N₂O emissions (Stange et al., 2009; Rütting et al., 2010; Zhang et al., 98 2011; Zhu et al., 2011; Stange et al., 2013; Müller et al., 2014). However, one of the 99 assumptions of the source partitioning method is the absence of hybrid reactions such as co-100 denitrification (Zhang et al., 2015a). Because of the potential importance of co-denitrification 101 for the N_2O production, it should not be omitted from the analysis of N_2O sources. Currently, 102 only one technique is available to identify several processes including a hybrid reaction, which is a full ¹⁵N tracing approach (Müller et al., 2014). This approach however, requires data on 103 104 NO_2 ; NO_3 / NH_4^+ pool sizes and measurements at multiple time points. Furthermore, it requires 105 at least multiple days of running the model to be able to distinguish the different processes. A 106 straight forward method partitioning N₂O fluxes into several pathways including a hybrid 107 reaction, which does not rely on measurements of NO₂⁻ and data at multiple time points, would 108 therefore be very beneficial.

110 The objectives of this study were to quantify the legacy effects of six years of elevated 111 temperature (via IR heaters) on soil N cycling dynamics, including (1) net and gross N 112 transformation rates in the soil (2) N₂O fluxes immediately after fertilisation and (3) the processes responsible for these N₂O fluxes. Net and gross transformation rates were determined 113 using an extended version of a basic ¹⁵N tracing model described by Müller et al. (2007). Since 114 the publication of this basic model in 2007, more than 50 peer-reviewed papers have been 115 published, where the basic model or modifications of the basic model have been used, 116 117 demonstrating its robustness of the approach in various soils, ecosystems and climatic 118 conditions. To determine the processes involved in N₂O production, a new source partitioning 119 method was developed to allow the identification of hybrid reactions. To identify the legacy 120 effect of different in situ temperature treatments on the internal N transformation processes, 121 soil incubations were carried out under identical moisture and temperature conditions in the 122 laboratory. Based on previous observations that gross N transformations in soils are affected 123 by long-term elevated temperature treatments we hypothesized that any associated effects on 124 gaseous N emissions (e.g. N₂O) can be confirmed by a change in the relative emission rates 125 from various pathways. Thus, the newly developed source partitioning method would be 126 helpful to confirm such a change.

127

128 **2.** Material and method

129 2.1. Site description and field treatment

The 100 m² 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. (in press). 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.

137

138 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 139 140 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) °C above 141 142 ambient temperature, using infrared heaters. The use of heaters will also affect the soil moisture 143 content. The temperature treatments (including any moisture effect) are referred to as T_{control}, 144 T₁, T₂, and T₃, respectively. The infrared heaters were installed at different heights to create 145 the different temperature elevations (Jansen-Willems et al., in press).

- 146
- 147 2.2. Incubation, labelling and extraction

148 On the day the heaters were turned off, all soil within a circular area of 318 cm² directly 149 underneath each infrared lamp was excavated to 7.5 cm for the tracing experiment. A small 150 subsample of each plot was dried at 70°C for 48 hours, ground and analysed by a CNH Macro 151 Elemental Analyser (Hanau, Germany) for total N content. A subsample of the soil for each 152 plot was dried at 105°C for 24 hours to determine the soil gravimetric water content. The 153 remaining field moist soil was kept at 4°C (for less than 60 hours) until further analysis 154 whereupon the soil from each field plot was sieved through a 10 mm sieve, to homogenise it 155 and to remove roots. Incubations were carried out in 750 ml jars (WECK GmbH u. Co. KG, 156 Wehr, Germany). Thirteen jars per field plot were prepared each with an average of 67 (stdev 157 8.4) g dry soil per jar (except for plots 3, 5, 7, 11 and 14, where only 10 jars were prepared due 158 to lack of soil). All jars were closed with glass lids that were fitted with septa to allow for gas 159 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 ¹⁵N substrate addition (day 0).

164

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 ¹⁵N tracing study three different labels were used, NO₃¹⁵NH₄ Gly, ¹⁵NO₃NH₄ Gly and 170 NO₃NH₄¹⁵N-Gly (at 60, 60 and 99 atm%¹⁵N respectively). All solutions contained 50 µg NO₃-171 N, 50 μ g NH₄-N, and 30 μ g Gly-N g⁻¹ soil. On day 0, the substrate solution was added to each 172 173 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 174 175 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 NO₃NH₄ ¹⁵N-Gly label 176 177 addition was possible.

178

The soil in each jar was extracted with 2M KCl using the blending procedure of Stevens and Laughlin (1995). The ¹⁵N 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 ¹⁵N enrichment in NO_3^- and Laughlin et al. (1997) for the ¹⁵N enrichment in NH_4^+ . The extraction of soil prior to ¹⁵N 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 ¹⁵N substrate addition, and are hereafter referred to as 0, 1, 3
and 6 days after ¹⁵N substrate addition, respectively.

187

188 2.3. Gas sampling

Gas samples were taken from 43 different jars, one jar per ¹⁵N 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.

192

193 Gas samples were taken using a 60 ml syringe (Ecoject Plus, Gelnhausen, Germany). At time 194 zero (t_0) 15 gas samples were taken from 15 different jars. Then at time 1 (t_1) a gas sample was 195 taken through the rubber septum. At both t_0 and t_1 the syringe was flushed twice with headspace 196 gas to ensure a representative sample was taken. The times between t_0 and t_1 during each of the 197 seven different gas samplings (three before label addition and four immediately prior to 198 extraction) were 120-129, 120, 180, 233, 240, 235 and 214 minutes, respectively. Gas samples 199 were analysed within 24 h after sampling using a GC (Bruker) equipped with an electron 200 capture detector (ECD) for N₂O analysis. An average of the concentrations measured in the 15 201 samples was used as the t₀ concentration for all 43 jars. Fluxes were based on the ppm and time 202 difference between t₀ and t₁. They were calculated using the constant gas law, with ambient 203 pressure, and temperature was assumed to be 20°C (the temperature of the incubation room). 204 The fluxes were then converted to a per dry gram basis.

205

For the ¹⁵N abundance of N₂O, a 30 ml sample was taken at t_1 and transferred to a 12 ml Exetainers[®] vial (Labco Ltd, High Wycombe, Buckinghamshire, UK). The over-pressurised 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

210	ventral needle submerged 3-4 mm in a beaker of water and the gas sample held upside down
211	and pushed onto the dorsal needle. The excess pressure in the sample vial was thus released
212	causing the water to bubble until the pressure inside the vial has equilibrated with the ambient
213	atmospheric pressure. Cessation of bubbling implied equal pressure had been reached. The ¹⁵ N
214	enrichments of ${}^{15}N_2O$ and ${}^{15}N_2$ were determined using an automated isotope ratio mass
215	spectrometry (Sercon Ltd 20-20), as described by Stevens et al. (1993), inter-faced to a TGII
216	cryfocusing unit (Sercon Ltd 20-20). The detection limit for atom% ^{15}N of a 50 ppm N ₂ O
217	standard gas was 0.00003 (n= 10), stdev was 0.00009 atom% ¹⁵ N. Respective values for a 0.4
218	ppm N ₂ O standard were higher (0.00084 (n= 10), stdev 0.003).
219	
220	2.4. ^{15}N tracing model
221	The ¹⁵ N tracing analysis tool described by Müller et al. (2007) was used to quantify gross soil
222	N transformations. In the current study, the only changes to the original model were the
223	addition of an amino-acid (glycine) pool, and the transformations to and from this pool. The
224	model (Fig. 2.) considered seven N pools and 13 N transformations. The N pools were NH4 ⁺ ,
225	NO3 ⁻ , amino acid glycine (AA), labile (N _{lab}) and recalcitrant (N _{rec}) organic N, adsorbed
226	ammonium (NH ₄ ⁺ _{ads}) and stored nitrate (NO ₃ ⁻ _{sto}). The initial NO ₃ ⁻ and NH ₄ ⁺ pool sizes were
227	determined by extrapolating the first two extraction times back to time zero. The initial AA
228	pool size was set to 30 μ g N g ⁻¹ soil, corresponding to the application of glycine (Gly). The
229	initial $NH_4^+_{ads}$ and NO_3^{sto} were based on the difference between the added and initial N (Müller
230	et al., 2004). The initial pool sizes for organic N (Nrec and Nlab) were based on previous field
231	measurements. However, these organic N values were not critical because for N _{rec} , zero-order

- 232 kinetics were used (independent of initial pool size), and for N_{lab}, the quick turnover time
- 233 ensures that a small pool will be governed quickly by the dynamics of the in- and out-flowing
- 234 rates. The N transformations are described in Table 1. The N transformations were calculated

235 based on zero or first order kinetics (Table 1). Whether Nlab and Nrec were transformed into AA or NH4⁺ was determined by two factors, one for M_{Nlab} and one for M_{Nrec}. This factor determines 236 237 the fraction of the M_{Nlab} or M_{Nrec} flowing into the AA pool with the remainder entering the 238 NH₄⁺ pool. For each temperature treatment the kinetic parameters and the two split factors were simultaneously optimised by minimising the misfit between the modelled and measured NH4⁺ 239 and NO3⁺ concentrations and their respective ¹⁵N enrichments (Müller et al., 2004). For 240 treatment T₂ the measurements of the ¹⁵N-Gly label were not included in the optimisation 241 because only one replicate was available for this label. A Markov chain Monte Carlo 242 Metropolis algorithm (MCMC-MA) was used for the optimisation, which practices a random 243 244 walk technique to find global minima (Müller et al., 2007). The uncertainties (standard 245 deviation) of the observations were taken into account by the optimisation routine. The 246 MCMC-MA routine was programmed in MatLab-Simulink (Mathworks Inc) as described in 247 Müller et al. (2007). The most suitable parameter set was determined using the Akaikes 248 Information Criterion (AIC). Gross and net nitrification, and gross and net mineralisation were 249 calculated using equation 1 to 4 in which SF stands for split factor. The combined standard deviation was calculated by ((stdev rate 1)²+(stdev rate 2)²+.....)^{0.5}, in which the stdev of 250 M_{Nx} SF_{MNx} is the stdev of M_{Nx} multiplied by the SF. 251

- 253 The following combined rates were calculated:
- 254 Gross nitrification: $O_{Nrec}+O_{NH4}$ (1)
- 255 Net nitrification: $O_{\text{Nrec}} + O_{\text{NH4}} I_{\text{NO3}} D_{\text{NO3}}$ (2)
- 256 Gross mineralisation: M_{Nlab} SF_{MNlab} + M_{Nrec} SF_{MNrec} + M_{AA} (3)
- 257 Net mineralisation: M_{Nlab} ·SF_{MNlab} + M_{Nrec} ·SF_{MNrec} + M_{AA} -I_{NH4Nrec}-I_{NH4Nlab}-I_{NO3} (4)
- 258

259 2.5. Determining contribution of different processes to N₂O flux

The N₂O fluxes, from the soil labelled with NO₃¹⁵NH₄ Gly and ¹⁵NO₃NH₄ Gly, were separated 260 into four different processes. These were nitrification, denitrification, co-denitrification and 261 oxidation of organic matter. The N₂O was assumed to be derived from three uniformly 262 distributed pools, and based on initial substrate ¹⁵N enrichments, isotopic discrimination was 263 considered negligible for all four processes. The pools and processes accounting for the N₂O 264 production are shown in Fig. 1. The ¹⁵N content of the organic matter was considered to be at 265 266 natural abundance (0.3663 atom%). The N₂O produced via co-denitrification consists of one N 267 atom from the NO₃⁻ pool, and one N atom from the organic N pool. The chance that the N₂O 268 produced via nitrification, denitrification or oxidation of organic N contains zero, one or two 15 N enriched atoms can be described by equations 5, 6 and 7, respectively. Where a_x (the 15 N 269 fraction of the pool) is an for nitrification, ad for denitrification and ao for the oxidation of 270 271 organic N: a_n, a_d and a_o are explained in Fig. 1.

272

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

274 Chance of 1 ¹⁵N atom:
$$2(1-a_x)a_x$$
 (6)

275 Chance of 2 ¹⁵N atoms:
$$a_x^2$$
 (7)

276

The chance that the N₂O produced via co-denitrification consists of zero, one or two ¹⁵N enriched atoms is described by equations 8, 9 and 10 respectively.

- 280 Chance of 0^{15} N atoms: $(1-a_0)(1-a_0)$ (8)
- 281 Chance of 1 ¹⁵N atom: $a_d(1-a_0) + a_0(1-a_d)$ (9)
- 282 Chance of 2¹⁵N atoms: $a_d a_0$ (10)
- 283

The chance that the N₂O in the gas sample contains zero, one or two ¹⁵N atoms is described by equations 11, 12 and 13 respectively. Where the subscripts *d*, *n* and *o* refer to the fractions of N₂O produced by denitrification, nitrification and oxidation of organic N, respectively. The fraction of N₂O produced by co-denitrification is 1-*d*-*n*-*o* as all of the N₂O produced was assumed to come from one of the four processes.

290 Chance of 0¹⁵N 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)

291 Chance of 1¹⁵N 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)

292 Chance of 2¹⁵N atoms:
$$na_n^2 + da_d^2 + oa_n^2 + (1-n-d-o)a_da_0$$
 (13)

293

294 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 295 for the presence of ¹⁸O. This, therefore, means that ⁴⁵R is the fraction of N₂O molecules 296 containing one ¹⁵N atom divided by the fraction of N₂O molecules containing zero ¹⁵N atoms, 297 298 and ⁴⁶R is the fraction of N₂O molecules containing two ¹⁵N atoms divided by the fraction of N₂O molecules containing zero ¹⁵N atoms. The expected fractions are described by equations 299 11 to 13, where a_0 was set to 0.003663, a_n and a_d were considered to be the ¹⁵N content of NH₄⁺ 300 301 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 ⁴⁵R, ⁴⁶R, a_n and a_d of soil labelled with 302 NO₃¹⁵NH₄ Gly and soil labelled with ¹⁵NO₃NH₄ Gly were used. The amount of N₂O produced 303 304 via each process was calculated by multiplying the average N₂O flux from the jars labelled with NO₃¹⁵NH₄ Gly and ¹⁵NO₃NH₄ Gly with the fractions of N₂O produced by the four 305 306 different processes. This was carried out separately for each plot and time step. Because of missing ¹⁵NH₄ data, the different processes were not distinguished for plot 1 time step 3. Total 307 308 N₂O flux contributions were calculated using linear interpolations between time steps.

310 2.6. Statistical analyses

311 Total soil N was analysed with the non-parametric Kruskal-Wallis test using IBM SPSS 312 statistics (version 22) because one sample per plot was taken, resulting in only four 313 measurements per treatment. The N₂O fluxes (including different processes), inorganic-N 314 $(NO_3^++NH_4^+)$, NO_3^- and NH_4^+ concentrations were analysed using the MIXED procedure in SAS (Version 9.3, SAS institute). The N₂O fluxes were transformed using log(flux+10). The 315 N₂O fluxes via the different processes were transformed using flux^{1/4}. A Tukey-Kramer 316 adjustment was used to correct for multiplicity effects in pairwise comparisons. Residual 317 318 checks were made to ensure that the assumptions of the analysis were met. The modelled N 319 transformation rates were analysed using a one-way ANOVA based on the averages and 320 standard deviations in Matlab (Version 2013b, The MathWorks Inc.). The pairwise 321 comparisons were calculated with the Holm-Sidak test in SigmaPlot (Version 11.0, Systat 322 Software Inc.).

323

324 **3.** Results

325 3.1. Soil nitrogen pool sizes

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

Soil NH₄⁺ concentrations increased from 2 μ g N g⁻¹ soil to between 28 and 54 μ g N g⁻¹ soil 333 upon label addition, and subsequently decreased over the next five days to ca. 9 μ g N g⁻¹ soil 334 (Fig. 3b.). Soil NH₄⁺ concentrations did not differ as a result of the soil warming treatments on 335 either days 0 or 6. However, on day 1, treatment T₁ had a lower NH₄⁺ concentration compared 336 to all other treatments (p<0.029), while the soil NH_4^+ concentration in the T₂ treatment was 337 338 higher than in the $T_{control}$ or T_1 treatments (p<0.001). Three days after label addition the NH₄⁺ 339 concentration in the T₁ treatment remained lower compared to the T₂ and T₃ treatments (p respectively < 0.001 and 0.044). 340

341

After the initial increase in NO_3^- due to label addition, the NO_3^- concentrations continued to slowly increase over the following six days (Fig.3c). NO_3^- concentrations were significantly different among the treatments (p<0.001), with differences also occurring with respect to the initial NO_3^- concentrations prior to label addition (p<0.001). The highest NO_3^- concentrations occurred in the T₂ treatment followed by the T₃ and T_{control}, while the lowest NO_3^- concentration was observed in the T₁ treatment.

348

349 3.2. Soil N transformations

The modelled and observed concentrations and ¹⁵N enrichments were in good agreement with 350 R^{2} >0.97 for all runs (Fig. 4). The gross rates of most N transformations did not differ as a result 351 of the previously imposed soil warming treatment (Table 1). However, the rates of recalcitrant 352 N mineralisation were reduced under the T₂ and T₃ treatments (p=0.040). Mineralisation of 353 354 amino acids also became slower with increasing temperatures (p=0.045). However, the overall gross mineralisation of organic N to NH4⁺ did not differ with the previously imposed warming 355 356 treatments. This was because the mineralisation of labile organic N was the major contributor 357 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).

362

363 3.3. N_2O fluxes

In response to N supply, N₂O emissions immediately increased, and decreased thereafter (Fig. 3a). While treatments T₂ and T₃ had lower N₂O fluxes than the control treatment (p=0.004 and p=0.036, respectively) no interaction between incubation time and treatment was observed. The N₂O fluxes from the T₂ treatment were also lower than those from the T₁ treatment (p=0.016). However, observed fluxes from the T₁ treatment did not differ from the control treatment and N₂O fluxes from the T₂ treatment did not differ from the T₃ treatment.

370

371 The newly developed partitioning model was successful to identify cumulative N₂O fluxes (Fig. 5) and N₂O contribution at each extraction time (Fig. 6) associated with nitrification, 372 373 denitrification, co-denitrification and the oxidation of organic N between 0.11 and 5.93 days 374 after N addition. The oxidation of organic N was the main source of N₂O at all sampling dates, 375 comprising between 63 and 85% of the total N₂O flux (Fig. 5). The percentage contribution 376 made by organic N to N₂O 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 377 378 fluxes from organic N oxidation were the highest in the control treatment, followed by T₁, and 379 lowest for T₂ and T₃. Significant differences were found between the control and the T₂ and T₃ 380 treatment (p=0.011 and p=0.002, respectively) and between T_1 and T_3 (p=0.039). The amount 381 of N₂O produced via denitrification was also the highest under the control treatment, followed 382 by T₁ and T₃. It was the lowest under T₂. Compared to the control treatment, denitrification contributed less to N₂O under the T₂ and T₃ treatments (p < 0.0001 and p=0.002, respectively). The contribution of denitrification also differed between treatments T₂ and T₁ (p=0.004). Codenitrification only contributed to the N₂O flux during the first day after substrate addition. The highest amount of N₂O produced via co-denitrification was found under the control treatment, followed by T₁. Under T₂ and T₃ treatments, the contribution of co-denitrification was minor. However, these differences were not significant. No significant differences were found in the amount of N₂O produced via nitrification.

390

391 4. Discussion

392 Prior to incubation the inorganic N, as well as the NO_3^- concentrations, were higher in the T_2 393 and T₃ treatments as a result of the six years warming treatment. This suggests that a sustained 394 increase in temperature led to an increase in net mineralisation and net nitrification. This is in 395 line with previous studies showing increases in net mineralisation in response to warming 396 (Peterjohn et al., 1994; Rustad et al., 2001; Norby and Luo, 2004; Bai et al., 2013; Björsne et 397 al., 2014; Zhang et al., 2015b). An increase in net nitrification in response to soil warming, 398 while less common, has also been shown (Barnard et al., 2005; Bai et al., 2013; Björsne et al., 399 2014; Zhang et al., 2015b). Both could be due to infield temperatures being more favourable 400 for optimal microbial activity. Concurring with previous research (Bai et al., 2013; Zhang et 401 al., 2015b) the total soil N pool did not differ among warming treatments. This result may be 402 due to the fact that the relative sizes of the N pools differ: since the total soil N pool is 403 significantly larger than the inorganic N pool it may take longer to register a change (Galloway 404 et al., 2008; Bai et al., 2013).

405

406 During incubation all soil was kept at 20°C, regardless of the in-field treatment, to investigate 407 any legacy impacts of sustained soil warming on inherent soil N cycling. It has been suggested 408 that changes in the microbial community structure could alter the sensitivity of the microbial 409 community to temperature shifts (Balser et al., 2006). While both net and gross mineralisation 410 rates did not differ as a result of the previously imposed soil warming treatments, the 411 mineralisation of recalcitrant N and mineralisation of amino acids did differ. Lowest rates were 412 found under T₂ (M_{Nrec}) and T₃ (M_{Nrec} and M_{AA}). A similar effect to warming was found by 413 Jamieson et al. (1998) who reported decreased gross N mineralisation rates in spring following 414 winter warming of soil. Adaptation of the microbial community, altering the sensitivity to 415 temperature shifts, could possibly provide an explanation why no differences in net and gross 416 mineralisation, and even decreases in individual mineralisation rates were found. However, no 417 data were available to test this hypothesis. Another possible explanation for the reduction in 418 mineralisation rates could be a depletion of substrate due to the six years of elevated 419 temperatures.

420

421 Previous research in heathland and grassland soils showed no significant effect of warming on 422 amino acid mineralisation rates (Andresen et al., 2015). The lower rates in the current study, 423 however, could be due to a change in amino-acid oxidase activity (Vranova et al., 2013). 424 Another possible explanation for the lower amino acid mineralisation rates could be an increase 425 in direct microbial assimilation of amino acids (Farrell et al., 2014), since direct assimilation 426 of glycine and larger amino acids is well known (Barraclough, 1997; Andresen et al., 2009, 427 2011). Chen et al. (2015), however, did not show an effect of warming on the microbial uptake 428 of amino acids. The fact that NH₄⁺ immobilisation rates were not affected by previously 429 imposed warming in the current study, is in line with previous research (Niboyet et al., 2011; 430 Bai et al., 2013; Björsne et al., 2014). It has been suggested that the depletion of labile C due 431 to warming might initiate a decrease in immobilisation rates (Bai et al., 2013). In the current 432 experiment a labile carbon source (Gly) was added to the soil, which could explain why no
433 reduction in NH4⁺ immobilisation was found.

434



446

447 Oxidation of organic N was found to be the main source of N₂O. The production of N₂O from 448 an unlabelled organic source would most likely follow a combined process of organic N 449 oxidation via heterotrophic nitrifiers to nitrite, followed by a reduction of nitrite to gaseous N 450 products (Butterbach-Bahl et al., 2013). This process, where oxidation and reduction processes 451 occur hand in hand would be conceptually similar to the nitrifier-denitrification process (Wrage 452 et al., 2001). Most research, however, does not take the oxidation of organic N into account as 453 a possible source of N₂O (Zhang et al., 2015a). Even though recent studies showed that this 454 process contributed 54-85% of N₂O emissions in pastures (Rütting et al., 2010; Müller et al., 455 2014). These contributions are in line with the current study. Müller et al. (2014) also showed 456 that the fraction of N₂O contributed via the oxidation of organic N was lowest immediately 457 following NH_4NO_3 addition, and that this fraction increased to over 80%, while the 458 contribution of denitrification decreased with time even though NO_3^- concentrations increased. 459 Because of the large contribution of oxidation of organic N in N₂O emissions, this pathway 460 should not be omitted in future research.

461

462 A decrease in N₂O produced via denitrification was found in soil previously subjected to higher 463 temperature treatments. This could be due to a decrease in the rate of denitrification. However, it is also possible that under treatment T_2 and T_3 more of the NO₃⁻ underwent complete 464 denitrification, forming N₂ as opposed to N₂O. This highlights the importance of the gaseous 465 466 N stoichiometries in particular the N₂/N₂O ratio. Stevens and Laughlin (2001) reported N₂:N₂O 467 ratios in a fine loamy grassland soil of 2.2 and 0.5 from control and combined slurry plus NO3⁻ 468 fertiliser treatments, respectively. However, Clough et al. (1998) showed that ratios can vary 469 between 6.2 and 33.2 following ¹⁵N-labelled urine application to ryegrass (Loilum 470 perenne)/white clover (Trifolium repens) pasture on four different soils (silt loam, sandy loam, 471 peat and clay soils). Unfortunately, due to methodological restrictions were not able to detect significant N₂ fluxes, as they were ≤ 4 g N₂-N ha⁻¹ day⁻¹ (Stevens and Laughlin, 1998). 472

473

474 Adaptation of microorganisms, to long-term elevated temperature treatments, might also 475 provide an explanation for the decrease in N₂O emissions during the incubation with soil 476 previously subjected to increasing soil warming temperatures (Avrahami and Conrad, 2003; 477 French et al., 2009; Pritchard, 2011). Enhanced NO_3^- concentrations in the T₂ and T₃ 478 treatments, at the end of the field experiment, also suggests an in situ reduction of 479 denitrification and/or co-denitrification. A possible explanation for the in situ reduction of 480 denitrification could be the altered field soil moisture content. While during the incubation, soil 481 moisture was purposely kept constant (WFPS of 64%), in the field however, moisture

482 conditions were affected by the heating treatment, leading to generally drier, and thus more 483 aerated, conditions in the heated plots (Jansen-Willems et al., in press). Under low WFPS, 484 nitrification is predominantly responsible for N₂O efflux (Bollmann and Conrad, 1998; 485 Bateman and Baggs, 2005). This may be a consequence of altered soil moisture or changes in 486 soil texture and physical soil structure. The reduction of NO_3^- (denitrification) takes place under 487 more anoxic to anaerobic conditions (Smith, 1997), because under aerobic conditions, 488 denitrifiers reduce O₂ rather that NO₃⁻ (Arah, 1997). Any reduction in soil moisture could 489 therefore lead to a decrease in the in situ denitrification rate.

490

491 Co-denitrification was observed to be significant in T_{control} and T₁ shortly after N addition. 492 Rates were comparable with those from true denitrification. Co-denitrification is a co-493 metabolic process which uses inorganic and organic N compounds concurrently and converts 494 it to the same end products as in denitrification. Gases produced in this process are a hybrid N-495 N species where one atom of N comes from NO_2^- and the other one from a co-metabolised 496 compound (Spott et al., 2011). The conditions for increased co-denitrification are still not fully 497 understood, but the presence of fungi along with adequate amino acid pools appears to enhance 498 losses via this pathway (Laughlin and Stevens, 2002; Spott et al., 2011).

499

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 codenitrification 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.

510

511 **5.** Conclusion

512 Sustained increases in soil temperatures over 6 years (between 2 and 3°C) led to an increase in 513 both inorganic soil N and NO₃⁻ pools. Subsequent analyses of gross N transformations, during 514 an incubation of these soils under common temperature and moisture conditions to study the 515 legacy effect of increased temperatures, revealed that mineralisation of amino acids (glycine) 516 and recalcitrant organic N decreased with previously imposed elevated temperatures. A new, 517 easy to use, source partitioning method was developed to determine the contribution of four 518 different pathways to N₂O emissions. Emissions of N₂O in the first six days after fertilisation 519 were decreased for soils previously subjected to higher temperatures as a consequence of a 520 reduction in the rates of denitrification and the oxidation of organic N. For all treatments, 521 oxidation of organic N was the main contributor to N₂O emissions, and should therefore in 522 future research not be omitted as a possible source of N₂O.

523

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533 **References**

- Andresen, L., Bode, S., Tietema, A., Boeckx, P. and Rütting, T.: Amino acid and N
 mineralization dynamics in heathland soil after long-term warming and repetitive
 drought. Soil 1, 341-349, 2015.
- Andresen, L.C., Michelsen, A., Jonasson, S., Beier, C. and Ambus, P.: Glycine uptake in heath
 plants and soil microbes s responds to elevated temperature, CO₂ and drought. Acta
 Oecol 313, 283-295, 2009.
- Andresen, L.C., Michelsen, A., Jonasson, S. and Ström, L.: Seasonal changes in nitrogen
 availability, and root and microbial uptake of ¹⁵N¹³C₉-phenylalanine and ¹⁵Nammonium in situ at a temperate heath. Appl Soil Ecol 51, 94-101, 2011.
- Andresen, L.C., Michelsen, A., Jonasson, S., Schmidt, I.K., Mikkelsen, T.N., Ambus, P. and
 Beier, C.: Plant nutrient mobilization in temperate heathland responds to elevated CO₂,
 temperature and drought. Plant Soil 328, 381-396, 2010.
- Arah, J.: Apportioning nitrous oxide fluxes between nitrification and denitrification using gasphase mass spectrometry. Soil Biol Biochem 29, 1295-1299, 1997.
- Avrahami, S. and Conrad, R.: Patterns of community change among ammonia oxidizers in
 meadow soils upon long-term incubation at different temperatures. Appl Environ
 Microb 69, 6152-6164, 2003.
- Bai, E., Li, S., Xu, W., Li, W., Dai, W. and Jiang, P.: A meta-analysis of experimental warming
 effects on terrestrial nitrogen pools and dynamics. New Phytol 199, 441-451, 2013.
- 553 Balser, T.C., McMahon, K., Bart, D., Bronson, D., Coyle, D., Craig, N., Flores-Mangual, M.,
- Forshay, K., Jones, S. and Kent, A.: Bridging the gap between micro-and macro-scale
 perspectives on the role of microbial communities in global change ecology. Plant Soil
 289, 59-70, 2006.

- Barnard, R., Leadley, P.W. and Hungate, B.A.: Global change, nitrification, and denitrification:
 a review. Global Biogeochemical Cy 19, 2005.
- Barraclough, D.: The direct or MIT route for nitrogen immobilization: a ¹⁵N mirror image study
 with leucine and glycine. Soil Biol Biochem 29, 101-108, 1997.
- Bateman, E. and Baggs, E.: Contributions of nitrification and denitrification to N₂O emissions
 from soils at different water-filled pore space. Biol Fert Soils 41, 379-388, 2005.
- Bijoor, N.S., Czimczik, C.I., Pataki, D.E. and Billings, S.A.: Effects of temperature and
 fertilization on nitrogen cycling and community composition of an urban lawn. Glob
 Change Biol 14, 2119-2131, 2008.
- Björsne, A.-K., Rütting, T. and Ambus, P.: Combined climate factors alleviate changes in gross
 soil nitrogen dynamics in heathlands. Biogeochemistry 120, 191-201, 2014.
- Bollmann, A. and Conrad, R.: Influence of O₂ availability on NO and N₂O release by
 nitrification and denitrification in soils. Glob Change Biol 4, 387-396, 1998.
- 570 Butler, S.M., Melillo, J.M., Johnson, J., Mohan, J., Steudler, P.A., Lux, H., Burrows, E., Smith,
- 571 R., Vario and C., Scott, L.: Soil warming alters nitrogen cycling in a New England
 572 forest: implications for ecosystem function and structure. Oecologia 168, 819-828,
 573 2012.
- Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R. and Zechmeister-Boltenstern,
 S.: Nitrous oxide emissions from soils: how well do we understand the processes and
 their controls? Philosophical Transactions of the Royal Society of London B:
 Biological Sciences 368, 20130122, 2013.
- 578 Castaldi, S.: Responses of nitrous oxide, dinitrogen and carbon dioxide production and oxygen
 579 consumption to temperature in forest and agricultural light-textured soils determined
 580 by model experiment. Biol Fert Soils 32, 67-72, 2000.

581	Chen, J., Carrillo, Y., Pendall, E., Dijkstra, F.A., Evans, R.D., Morgan, J.A. and Williams,
582	D.G.: Soil microbes compete strongly with plants for soil inorganic and amino acid
583	nitrogen in a semiarid grassland exposed to elevated CO2 and warming. Ecosystems, 1-
584	14, 2015.

- Chen, J., Zelikova, T.J., Pendall, E., Morgan, J.A. and Williams, D.G.: Daily and seasonal
 changes in soil amino acid composition in a semiarid grassland exposed to elevated
 CO₂ and warming. Biogeochemistry 123, 135-146, 2014.
- 588 Clough, T., Ledgard, S., Sprosen, M. and Kear, M.: Fate of ¹⁵N labelled urine on four soil types.
 589 Plant Soil, 195-203, 1998.
- 590 Dijkstra, F.A., Blumenthal, D., Morgan, J.A., Pendall, E., Carrillo, Y. and Follett, R.F.:
 591 Contrasting effects of elevated CO₂ and warming on nitrogen cycling in a semiarid
 592 grassland. New Phytol 187, 426-437, 2010.
- 593 Emmett, B.A., Beier, C., Estiarte, M., Tietema, A., Kristensen, H.L., Williams, D., Penuelas,
 594 J., Schmidt, I. and Sowerby, A.: The response of soil processes to climate change:
 595 results from manipulation studies of shrublands across an environmental gradient.
 596 Ecosystems 7, 625-637, 2004.
- Farrell, M., Macdonald, L.M., Hill, P.W., Wanniarachchi, S.D., Farrar, J., Bardgett, R.D. and
 Jones, D.L.: Amino acid dynamics across a grassland altitudinal gradient. Soil Biol
 Biochem 76, 179-182, 2014.
- French, S., Levy-Booth, D., Samarajeewa, A., Shannon, K., Smith, J. and Trevors, J.: Elevated
 temperatures and carbon dioxide concentrations: effects on selected microbial activities
 in temperate agricultural soils. World J Microb Biot 25, 1887-1900, 2009.
- Galloway, J.N., Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli,
 L.A., Seitzinger, S.P. and Sutton, M.A.: Transformation of the nitrogen cycle: recent
 trends, questions, and potential solutions. Science 320, 889-892, 2008.

Harrison, R. and Webb, J.: A review of the effect of N fertilizer type on gaseous emissions.
Adv Agron 73, 65-108, 2001.

608 IPCC: Summary for policymakers, In: The physical science basis. Stocker, T.F., Qin, D., 609 Plattner, G.-K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, J., Bex, V.,

610 Midgley, P.M. (Eds.), Contribution of Working Group I to the Fifth Assessment Report

- 611 of the Intergovernmental Panel on Climate change, Cambridge, United Kingdom and612 New York, NY, USA, 2013.
- Jamieson, N., Barraclough, D., Unkovich, M. and Monaghan, R.: Soil N dynamics in a natural
 calcareous grassland under a changing climate. Biol Fert Soils 27, 267-273, 1998.
- Jansen-Willems, A.B., Lanigan, G.J., Grünhage, L. and Müller, C.: Carbon cycling in
 temperate grassland under elevated temperature. Ecol. Evol. In press.
- Larsen, K.S., Andresen, L.C., Beier, C., Jonasson, S., Albert, K.R., Ambus, P., Arndal, M.F.,
 Carter, M.S., Christensen, S. and Holmstrup, M.: Reduced N cycling in response to
 elevated CO₂, warming, and drought in a Danish heathland: synthesizing results of the
 CLIMAITE project after two years of treatments. Glob Change Biol 17, 1884-1899,
 2011.
- Laughlin, R., Stevens, R. and Zhuo, S.: Determining nitrogen-15 in ammonium by producing
 nitrous oxide. Soil Sci Soc Am J 61, 462-465, 1997.
- Laughlin, R.J., Rütting, T., Müller, C., Watson, C.J., Stevens, R.: Effect of acetate on soil
 respiration, N₂O emissions and gross N transformations related to fungi and bacteria in
 a grassland soil. Appl Soil Ecol 42, 25-30, 2009.
- Laughlin, R.J. and Stevens, R.J.: Evidence for fungal dominance of denitrification and
 codenitrification in a grassland soil. Soil Sci Soc Am J 66, 1540-1548, 2002.
- Li, P. and Lang, M.: Gross nitrogen transformations and related N₂O emissions in uncultivated
 and cultivated black soil. Biol Fert Soils 50, 197-206, 2014.

- Luo, Y.: Terrestrial carbon-cycle feedback to climate warming. Annual Review of Ecology,
 Evolution, and Systematics, 683-712, 2007.
- Maag, M. and Vinther, F.P.: Nitrous oxide emission by nitrification and denitrification in
 different soil types and at different soil moisture contents and temperatures. Appl Soil
 Ecol 4, 5-14, 1996.
- Müller, C., Kammann, C., Ottow, J. and Jäger, H.J.: Nitrous oxide emission from frozen
 grassland soil and during thawing periods. J Plant Nutr Soil Sc 166, 46-53, 2003.
- Müller, C., Laughlin, R.J., Spott, O. and Rütting, T.: Quantification of N₂O emission pathways
 via a ¹⁵N tracing model. Soil Biol Biochem 72, 44-54, 2014.
- Müller, C., Rütting, T., Kattge, J., Laughlin, R. and Stevens, R.: Estimation of parameters in
 complex ¹⁵N tracing models by Monte Carlo sampling. Soil Biol Biochem 39, 715-726,
 2007.
- Müller, C., Stevens, R. and Laughlin, R.: A ¹⁵N tracing model to analyse N transformations in
 old grassland soil. Soil Biol Biochem 36, 619-632, 2004.
- 645 Niboyet, A., Le Roux, X., Dijkstra, P., Hungate, B., Barthes, L., Blankinship, J., Brown, J.,
- Field, C. and Leadley, P.: Testing interactive effects of global environmental changes
 on soil nitrogen cycling. Ecosphere 2, art56, 2011.
- Norby, R.J. and Luo, Y.: Evaluating ecosystem responses to rising atmospheric CO₂ and global
 warming in a multi-factor world. New Phytol 162, 281-293, 2004.
- 650 Peterjohn, W.T., Melillo, J.M., Steudler, P.A., Newkirk, K.M., Bowles, F.P. and Aber, J.D.:
- Responses of trace gas fluxes and N availability to experimentally elevated soil
 temperatures. Ecol Appl 4, 617-625, 1994.
- Pritchard, S., 2011. Soil organisms and global climate change. Plant Pathology 60, 82-99, 2011.
- Rustad, L., Campbell, J., Marion, G., Norby, R., Mitchell, M., Hartley, A., Cornelissen, J. and
- 655 Gurevitch, J.: A meta-analysis of the response of soil respiration, net nitrogen

- mineralization, and aboveground plant growth to experimental ecosystem warming.
 Oecologia 126, 543-562, 2001.
- Rütting, T., Clough, T.J., Müller, C., Lieffering, M. and Newton, P.C.: Ten years of elevated
 atmospheric carbon dioxide alters soil nitrogen transformations in a sheep-grazed
 pasture. Glob Change Biol 16, 2530-2542, 2010.
- Saggar, S., Jha, N., Deslippe, J., Bolan, N., Luo, J., Giltrap, D., Kim, D.-G., Zaman, M. and
 Tillman, R.: Denitrification and N₂O:N₂ production in temperate grasslands: processes,
 measurements, modelling and mitigating negative impacts. Sci Total Environ 465, 173-
- 664195, 2013.
- Schimel, J.P. and Bennett, J.: Nitrogen mineralization: challenges of a changing paradigm.
 Ecology 85, 591-602, 2004.
- Selbie, D.R., Lanigan, G.J., Laughlin, R.J., Di, H.J., Moir, J.L., Cameron, K.C., Clough, T.J.,
 Watson, C.J., Grant, J., Somers, C. and Richards, K.G.: Confirmation of codenitrification in grazed grassland. Scientific reports, 5, 2015.
- 670 Seitzinger, S., Harrison, J.A., Böhlke, J., Bouwman, A., Lowrance, R., Peterson, B., Tobias, C.
- and Drecht, G.V.: Denitrification across landscapes and waterscapes: a synthesis. Ecol
 Appl 16, 2064-2090, 2006.
- 673 Smith, K.: The potential for feedback effects induced by global warming on emissions of
 674 nitrous oxide by soils. Glob Change Biol 3, 327-338, 1997.
- Spott, O., Russow, R. and Stange, C.F.: Formation of hybrid N₂O and hybrid N₂ due to
 codenitrification: First review of a barely considered process of microbially mediated
 N-nitrosation. Soil Biol Bioch 43, 1995-2011, 2011.
- Stange, C., Spott, O., Arriaga, H., Menéndez, S., Estavillo, J.M. and Merino, P.: Use of the
 inverse abundance approach to identify the sources of NO and N₂O release from

- 680 Spanish forest soils under oxic and hypoxic conditions. Soil Biol Biochem 57, 451-458,
 681 2013.
- Stange, C., Spott, O. and Müller, C.: An inverse abundance approach to separate soil nitrogen
 pools and gaseous nitrogen fluxes into fractions related to ammonium, nitrate and soil
 organic nitrogen. Eur J Soil Sci 60, 907-915, 2009.
- Stark, C.H. and Richards, K.G.: The continuing challenge of agricultural nitrogen loss to the
 environment in the context of global change and advancing research. Dynamic Soil,
 Dynamic Plant 2, 1-12, 2008.
- Stevens, R. and Laughlin, R.: Determining nitrogen-15 in nitrite or nitrate by producing nitrous
 oxide. Soil Sci Soc Am J 58, 1108-1116, 1994.
- 690 Stevens, R. and Laughlin, R.: Nitrite transformations during soil extraction with potassium
 691 chloride. Soil Sci Soc Am J 59, 933-938, 1995.
- 692 Stevens, R. and Laughlin, R.: Measurement of nitrous oxide and di-nitrogen emissions from
 693 agricultural soils. Nutr Cycl Agoecosys 52, 131-139, 1998.
- Stevens, R., Laughlin, R., Atkins, G. and Prosser, S.: Automated determination of nitrogen-15labeled dinitrogen and nitrous oxide by mass spectrometry. Soil Sci Soc Am J 57, 981988, 1993.
- 697 Stevens, R.J. and Laughlin, R.J.: Cattle slurry affects nitrous oxide and dinitrogen emissions
 698 from fertilizer nitrate. Soil Sci Soc Am J 65, 1307-1314, 2001.
- Tilman, D., Fargione, J., Wolff, B., D'Antonio, C., Dobson, A., Howarth, R., Schindler, D.,
 Schlesinger, W.H., Simberloff, D. and Swackhamer, D.: Forecasting agriculturally
 driven global environmental change. Science 292, 281-284, 2001.
- Vranova, V., Rejsek, K. and Formanek, P.: Proteolytic activity in soil: a review. Appl Soil Ecol
 703 70, 23-32, 2013.

704	Wrage, N., Velthof, G.L., Van Beusichem, M.L. and Oenema, O.: Role of nitrifier
705	denitrification in the production of nitrous oxide. Soil Biol Biochem 33, 1723-1732,
706	2001.

- Zhang, J., Cai, Z. and Zhu, T.: N₂O production pathways in the subtropical acid forest soils in
 China. Environ Res 111, 643-649, 2011.
- Zhang, J., Müller, C. and Cai, Z.: Heterotrophic nitrification of organic N and its contribution
 to nitrous oxide emissions in soils. Soil Biol Biochem 84, 199-209, 2015a.
- Zhang, W., Parker, K., Luo, Y., Wan, S., Wallace, L. and Hu, S.: Soil microbial responses to
 experimental warming and clipping in a tallgrass prairie. Glob Change Biol 11, 266277, 2005.
- Zhang, X.-Z., Shen, Z.-X. and Fu, G.: A meta-analysis of the effects of experimental warming
 on soil carbon and nitrogen dynamics on the Tibetan Plateau. Appl Soil Ecol 87, 32-38,
 2015b.
- 717 Zhu, T., Zhang, J. and Cai, Z.: The contribution of nitrogen transformation processes to total
 718 N2O emissions from soils used for intensive vegetable cultivation. Plant Soil 343, 313719 327, 2011.

721 Figures



722

Fig. 1. N₂O production via four processes (nitrification, denitrification, co-denitrification and oxidation of organic N). Three uniformly distributed pools were considered. These pools were an ammonium pool (NH₄⁺) with a ¹⁵N atom fraction of a_n , a nitrate pool (NO₃⁻) with a ¹⁵N atom fraction of a_d , and an organic-N pool with a ¹⁵N atom fraction of a_o (=0.003663). The N₂O produced via co-denitrification consists of one N atom from the nitrate pool, and one from the organic N pool.





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



Fig. 3. N₂O emission (a), NH₄-N content (b) and NO₃-N content at the extraction times. Time point 0 is the time of label addition (¹⁵NH₄NO₃ Gly, NH₄¹⁵NO₃ Gly or NH₄NO₃ ¹⁵N-Gly). The N₂O flux at time point 0 is based on the average flux of the 3 gas samplings before label addition. The ammonium and nitrate content at time point 0 is based on unlabelled soil. The error bars are the standard error of the mean. ^{Δ} shows a significant difference in N₂O flux from T_{control} (p<0.05), * shows a significant difference in NH₄-N from T_{control} (p<0.03), and [#] shows a significant difference in NO₃-N from T_{control} (p<0.0001).





Fig. 4. Modelled vs measured data. The lines are modelled data, and the squares, circles and

744 triangles are the measured data points. Error bars are standard deviations. Time is the time in

- 745 days from the moment of label addition.
- 746
- 747



Fig. 5. Cumulative N₂O flux via four processes between 3 h and 6 days after labelling. N₂O fluxes based on average flux from soil labelled with ¹⁵NH₄NO₃ Gly or NH₄¹⁵NO₃ Gly. The cumulative flux per process is an average over the four plots per treatment. Error bars are standard error of the mean (SEM). Percentages are the average percentage of flux produces via each process, SEM between brackets. * Significantly lower cumulative flux compared to the control (p<0.05).



Fig. 6. N₂O flux divided into 4 processes at different time points after fertilisation. N₂O fluxes based on average flux from soil labelled with $^{15}NH_4NO_3$ Gly or NH₄ $^{15}NO_3$ 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.

761 Tables

762 Table 1: Description of N transformations and average gross N fluxes per treatment (diagram shown in Fig. 2). Standard deviation between

763 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	V		A	verage g	gross flux ((µg N g s	soil ⁻¹ d^{-1})			2
Transformation	K	T _{con}	ıtrol	T	1	T	2	T	3	р
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 NH4 ⁺ 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
$I_{NH4Nlab}Immobilisation of NH4^+$ 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
$R_{\rm NH4a}$ Release of adsorbed ${\rm NH_4^+}$	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

767	
768	Table 2. Gross mineralisation (Min _{Gross}), net mineralisation (Min _{Net}), gross nitrification
769	(Nit _{Gross}) and net nitrification (Nit _{Net}) rate in μ g N g soil ⁻¹ d ⁻¹ . Including the contributions from
770	the different N pools for the gross transformations (italics), where N_{lab} is a labile organic N
771	pool, N_{rec} is a recalcitrant organic N pool, NH_4^+ is the ammonium pool and N_{AA} is the amino
772	acid Gly pool. ^t one-way ANOVA tendency p<0.1

	T_{control}	T_1	T_2	T ₃
MinGross	59.13	44.18	54.86	43.58
N _{lab}	44%	54%	50%	63%
Nrec	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
Nrec	19%	15%	12%	16%
NH_4^+	81%	85%	82%	84%
Nit _{Net}	13.22	11.23	11.30	13.06