1 **Long-term elevation of temperature affects organic N turnover and**

2 **associated N$_2$O emissions in a permanent grassland soil**

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Abstract

Over the last century an increase in mean soil surface temperature has been observed and it is predicted to increase further in the future. In order to evaluate the legacy effects of increased temperature on both nitrogen (N) transformation rates in the soil and nitrous oxide (N_2O) emissions, an incubation experiment and modelling approaches were combined. Based on previous observations that gross N transformations in soils are affected by long-term elevated temperature treatments we hypothesised that any associated effects on gaseous N emissions (e.g. N_2O) can be confirmed by a change in the relative emission rates from various pathways. Soils were taken from a long term in situ warming experiment on temperate permanent grassland. In this experiment the soil temperature was elevated by 0 (control), 1, 2 or 3°C (4 replicates per treatment) using IR-lamps over a period of 6 years. The soil was subsequently incubated under common conditions (20 °C and 50 % humidity) and labelled with NO_3**^{15}NH$_4$** Gly, **^{15}NO$_3$**NH$_4$ Gly or NO_3NH$_4$ **^{15}N-Gly**. Soil extractions and N_2O emissions were analysed using a ^{15}N tracing model and source partitioning model. Both total inorganic N (NO_3^-+NH_4^+) and NO_3^- contents were higher in soil subjected to the +2 °C and +3 °C temperature elevations (pre- and post-incubation). Analyses of N transformations using a ^{15}N tracing model, showed that, following incubation, gross organic (but not inorganic) N transformation rates decreased in response to the prior soil warming treatment. This was also reflected in reduced N_2O emissions associated with organic N oxidation and denitrification. Furthermore, a newly developed source partitioning model showed the importance of oxidation of organic N as a source of N_2O. Concluding, long term soil warming can cause a legacy effect which diminishes organic N turn over and the release of N_2O from organic N and denitrification.

1. Introduction

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

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

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

65 (Larsen et al., 2011).

66

67 N mineralisation follows a step-wise sequence of protein depolymerisation by extracellular

68 activity to oligomers (e.g. peptides) and monomers (e.g. amino acids) and then uptake by

69 microorganisms before mineralisation to NH_4^+ (Schimel and Bennett, 2004). Hence,

70 production of peptides and amino acids as well as mineralisation of amino acids, affects the

71 main fluxes regulating gross N mineralisation. Amino acids have a short residence time in the

72 soil due to either rapid assimilation by soil microbes or mineralisation, which occurs within a

73 few hours (Farrell et al., 2014). In heathland and grassland soils no effect of soil warming on

74 the amino acid concentration has been observed (Chen et al., 2014; Andresen et al., 2015).

75

76 Nitrous oxide (N_2O), a potent greenhouse gas with a global warming potential of 298 on a 100

77 year basis, can be produced by several processes, such as nitrification, partial denitrification,

78 co-denitrification and the oxidation of organic matter (Butterbach-Bahl et al., 2013; Zhang et

79 al., 2015a) (Fig. 1). Laughlin and Stevens (2002) confirmed the importance of co-

80 denitrification for N_2 production, a process that may comprise 25% of the total N balance in

81 pastures (Selbie et al., 2015). Müller et al. (2014) found that, for the same grassland soil as

82 used in this study, co-denitrification contributed 17.6% of the total N_2O production. N_2O

83 emissions following fertilisation with ammonium nitrate (NH_4NO_3) may be greater than from

84 urea fertiliser because of the greater susceptibility to denitrification (Harrison and Webb,

85 2001). The amount and form of N inputs primarily govern N_2O emissions with further impacts

86 resulting from climatic factors, such as temperature and precipitation, and soil factors, such as

87 C availability and microbial community structure (Harrison and Webb, 2001; Müller et al.,

88 2003; Stark and Richards, 2008; Laughlin et al., 2009; Li and Lang, 2014). However, the

89   impact of elevated soil temperature on N_2O production, in semi-natural grasslands is unclear

90   (Peterjohn et al., 1994; Bijoor et al., 2008; Larsen et al., 2011). Furthermore, there has been

91   very limited research into the effect of elevated soil temperature on the different N_2O

92   production processes. Maag and Vinther (1996) observed a decrease in nitrification associated

93   N_2O emissions and an increase in denitrification associated N_2O with increasing soil

94   temperature. It has been suggested that this was due to creation of anoxic conditions and the

95   associated depletion of oxygen following the increase in microbial respiration with higher soil

96   temperatures (Castaldi, 2000). Prolonged elevated soil temperatures, on the other hand, could

97   also lead to changes in the microbial community (Avrahami and Conrad, 2003; French et al.,

98   2009).

99

100   Several methods, such as source partitioning, have been used to quantify the contributions of

101   individual N pools to N_2O emissions (Stange et al., 2009; Rütting et al., 2010; Zhang et al.,

102   2011; Zhu et al., 2011; Stange et al., 2013; Müller et al., 2014). However, one of the

103   assumptions of the source partitioning method is the absence of hybrid reactions such as co-

104   denitrification (Zhang et al., 2015a). Because of the potential importance of co-denitrification

105   for the N_2O production, it should not be omitted from the analysis of N_2O sources. Currently,

106   only one technique is available to identify several processes including a hybrid reaction, which

107   is a full ^{15}N tracing approach (Müller et al., 2014). This approach however, requires data on

108   NO_2^-; NO_3^-/NH_4^+ pool sizes and measurements at multiple time points. Furthermore, it requires

109   at least multiple days of running the model to be able to distinguish the different processes. A

110   straight forward method partitioning N_2O fluxes into several pathways including a hybrid

111   reaction, which does not rely on measurements of NO_2^- and data at multiple time points, would

112   therefore be very beneficial.

113

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

2.   Material and method

2.1. Site description and field treatment

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

139   last three decades. Since 1995, the N fertiliser input had been reduced to 40 kg N ha^{-1} year^{-1},

140   as KAS (calcium-ammonium-nitrate). The mean annual temperature and precipitation were

141   9.5°C and 560 mm (observation period: 1995-2014) respectively.

142

143   The site had been divided into 16 plots, four rows of four plots. The 16 plots were, according

144   to a Latin square design, assigned to one of four treatments. From January 28, 2008, the soil

145   temperature of each plot, measured at 5 cm depth, was elevated by 0, 1 (mean 0.8 standard

146   error 0.02), 2 (mean 1.9 standard error 0.03) or 3 (mean 2.6 standard error 0.03) °C above

147   ambient temperature, using infrared heaters. The use of heaters will also affect the soil moisture

148   content. The temperature treatments (including any moisture effect) are referred to as $T_{control}$,

149   T_1, T_2, and T_3, respectively. The infrared heaters were installed at different heights to create

150   the different temperature elevations (Jansen-Willems et al., 2016).

151

152   *2.2. Incubation, labelling and extraction*

153   On the day the heaters were turned off, all soil within a circular area of 318 cm^2 directly

154   underneath each infrared lamp was excavated to 7.5 cm for the tracing experiment. A small

155   subsample of each plot was dried at 70°C for 48 hours, ground and analysed by a CNH Macro

156   Elemental Analyser (Hanau, Germany) for total N content. A subsample of the soil for each

157   plot was dried at 105°C for 24 hours to determine the soil gravimetric water content. The

158   remaining field moist soil was kept at 4°C (for less than 60 hours) until further analysis

159   whereupon the soil from each field plot was sieved through a 10 mm sieve, to homogenise it

160   and to remove roots. Incubations were carried out in 750 ml jars (WECK GmbH u. Co. KG,

161   Wehr, Germany). Thirteen jars per field plot were prepared each with an average of 67 (stdev

162   8.4) g dry soil per jar (except for plots 3, 5, 7, 11 and 14, where only 10 jars were prepared due

163   to lack of soil). All jars were closed with glass lids that were fitted with septa to allow for gas

164   sampling. During gas flux analyses the jars were sealed using a clamp and a rubber ring

165   between the jar and the lid. At other times a gap was left between the jar and the lid to allow

166   air exchange while minimising water loss. Two days after soil sampling (day -55), all jars were

167   put in a dark climate chamber at 20°C and 50% humidity and incubated for 55 days prior to

168   ^{15}N substrate addition (day 0).

169

170   Soil gravimetric moisture data were used to determine the exact amount of dry soil in each jar,

171   and to calculate the amount of water to be added to ensure the same soil water content in each

172   jar. On day -53 the soil moisture in each jar was adjusted to a water-filled pore space (WFPS)

173   of 64%. On day -43 and -5 the jars were watered to replenish the water lost due to evaporation.

174

175   For the ^{15}N tracing study three different labels were used, NO_3**$^{15}NH_4$ Gly**, **$^{15}NO_3NH_4$ Gly** and

176   NO_3NH_4 **^{15}N-Gly** (at 60, 60 and 99 atm% ^{15}N respectively). All solutions contained 50 µg NO_3-

177   N, 50 µg NH_4-N, and 30 µg Gly-N g^{-1} soil. On day 0, the substrate solution was added to each

178   jar using a needle with side-ports, to inject the solution into the soil to minimise disturbance,

179   while providing an equal distribution in the soil (Müller et al., 2007). For each field plot, jars

180   were set up for four soil extractions, at day 0, 1, 3 and 6 after N application, and three labels,

181   except for plot 3, 5, 7, 11 and 14, where due to the lack of soil no NO_3NH_4 **^{15}N-Gly** label

182   addition was possible.

183

184   The soil in each jar was extracted with 2M KCl using the blending procedure of Stevens and

185   Laughlin (1995). The ^{15}N enrichments of NO_3^- and NH_4^+ in the extracts were determined by

186   converting NO_3^- and NH_4^+ into N_2O following the procedures by Stevens and Laughlin (1994)

187   for determination of the ^{15}N enrichment in NO_3^- and Laughlin et al. (1997) for the ^{15}N

188   enrichment in NH_4^+. The extraction of soil prior to ^{15}N addition, took place on day -2. The

189   other extractions took place at 0.11 days (+/- 0.004), 1.02 days (+/- 0.001), 2.95 days (+/- 0.001)

190   and 5.93 days (+/- 0.001) after ^{15}N substrate addition, and are hereafter referred to as 0, 1, 3

191   and 6 days after ^{15}N substrate addition, respectively.

192

193   *2.3. Gas sampling*

194   Gas samples were taken from 43 different jars, one jar per ^{15}N label, for each plot. During the

195   pre-incubation gas samples were taken 1, 46 and 48 days before label addition. After labelling,

196   gas samples were taken immediately prior to soil extractions.

197

198   Gas samples were taken using a 60 ml syringe (Ecoject Plus, Gelnhausen, Germany). At time

199   zero (t_0) 15 gas samples were taken from 15 different jars. Then at time 1 (t_1) a gas sample was

200   taken through the rubber septum. At both t_0 and t_1 the syringe was flushed twice with headspace

201   gas to ensure a representative sample was taken. The times between t_0 and t_1 during each of the

202   seven different gas samplings (three before label addition and four immediately prior to

203   extraction) were 120-129, 120, 180, 233, 240, 235 and 214 minutes, respectively. Gas samples

204   were analysed within 24 h after sampling using a GC (Bruker) equipped with an electron

205   capture detector (ECD) for N_2O analysis. An average of the concentrations measured in the 15

206   samples was used as the t_0 concentration for all 43 jars. Fluxes were based on the ppm and time

207   difference between t_0 and t_1. They were calculated using the constant gas law, with ambient

208   pressure, and temperature was assumed to be 20°C (the temperature of the incubation room).

209   The fluxes were then converted to a per dry gram basis.

210

211   For the ^{15}N abundance of N_2O, a 30 ml sample was taken at t_1 and transferred to a 12 ml

212   Exetainers® vial (Labco Ltd, High Wycombe, Buckinghamshire, UK). The over-pressurised

213   sample vials were returned to ambient pressure immediately before analyses of stable isotopes.

214   This was performed using a double ended needle fixed vertically in a clamp stand with the

215   ventral needle submerged 3-4 mm in a beaker of water and the gas sample held upside down

216   and pushed onto the dorsal needle. The excess pressure in the sample vial was thus released

217   causing the water to bubble until the pressure inside the vial has equilibrated with the ambient

218   atmospheric pressure. Cessation of bubbling implied equal pressure had been reached. The ^{15}N

219   enrichments of $^{15}N_2O$ and $^{15}N_2$ were determined using an automated isotope ratio mass

220   spectrometry (Sercon Ltd 20-20), as described by Stevens et al. (1993), inter-faced to a TGII

221   cryfocusing unit (Sercon Ltd 20-20). The detection limit for atom% ^{15}N of a 50 ppm N_2O

222   standard gas was 0.00003 (n= 10), stdev was 0.00009 atom% ^{15}N. Respective values for a 0.4

223   ppm N_2O standard were higher (0.00084 (n= 10), stdev 0.003).

224

225   *2.4. ^{15}N tracing model*

226   The ^{15}N tracing analysis tool described by Müller et al. (2007) was used to quantify gross soil

227   N transformations. In the current study, the only changes to the original model were the

228   addition of an amino-acid (glycine) pool, and the transformations to and from this pool. The

229   model (Fig. 2.) considered seven N pools and 13 N transformations. The N pools were NH_4^+,

230   NO_3^-, amino acid glycine (AA), labile (N_{lab}) and recalcitrant (N_{rec}) organic N, adsorbed

231   ammonium ($NH_4^+{}_{ads}$) and stored nitrate ($NO_3^-{}_{sto}$).

232

233   The initial NO_3^- and NH_4^+ pool sizes were determined by extrapolating the first two extraction

234   times back to time zero. The initial AA pool size was set to 30 μg N g^{-1} soil, corresponding to

235   the application of glycine (Gly). The initial $NH_4^+{}_{ads}$ and $NO_3^-{}_{sto}$ were based on the difference

236   between the added and initial N (Müller et al., 2004). The initial pool sizes for organic N (N_{rec}

237   and N_{lab}) were based on previous field measurements. However, these organic N values were

238   not critical because for N_{rec}, zero-order kinetics were used (independent of initial pool size),

239   and for N_{lab}, the quick turnover time ensures that a small pool will be governed quickly by the

240   dynamics of the in- and out-flowing rates.

241

242   The N transformations are described in Table 1. The N transformations were calculated based

243   on zero or first order kinetics (Table 1). Whether N_{lab} and N_{rec} were transformed into AA or

244   NH_4^+ was determined by two factors, one for M_{Nlab} and one for M_{Nrec}. This factor determines

245   the fraction of the M_{Nlab} or M_{Nrec} flowing into the AA pool with the remainder entering the

246   NH_4^+ pool. For each temperature treatment the kinetic parameters and the two split factors were

247   simultaneously optimised by minimising the misfit between the modelled and measured NH_4^+

248   and NO_3^+ concentrations and their respective ^{15}N enrichments (Müller et al., 2004). For

249   treatment T_2 the measurements of the ^{15}N-Gly label were not included in the optimisation

250   because only one replicate was available for this label.

251

252   A Markov chain Monte Carlo Metropolis algorithm (MCMC-MA) was used for the

253   optimisation, which practices a random walk technique to find global minima (Müller et al.,

254   2007). The uncertainties (standard deviation) of the observations were taken into account by

255   the optimisation routine. The MCMC-MA routine was programmed in MatLab-Simulink

256   (Mathworks Inc) as described in Müller et al. (2007). The most suitable parameter set was

257   determined using the Akaikes Information Criterion (AIC). Gross and net nitrification, and

258   gross and net mineralisation were calculated using equation 1 to 4 in which SF stands for split

259   factor. The combined standard deviation was calculated by $((stdev\ rate\ 1)^2+(stdev\ rate$

260   $2)^2+........)^{0.5}$, in which the stdev of $M_{Nx} \cdot SF_{MNx}$ is the stdev of M_{Nx} multiplied by the SF.

261

262   The following combined rates were calculated:

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

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

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

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

267

268   *2.5. Determining contribution of different processes to N$_2$O flux*

269   The N$_2$O fluxes, from the soil labelled with NO$_3$**^{15}NH$_4$** Gly and **^{15}NO$_3$NH$_4$** Gly, were separated

270   into four different processes. These were nitrification, denitrification, co-denitrification and

271   oxidation of organic matter. The N$_2$O was assumed to be derived from three uniformly

272   distributed pools, and based on initial substrate ^{15}N enrichments, isotopic discrimination was

273   considered negligible for all four processes. The pools and processes accounting for the N$_2$O

274   production are shown in Fig. 1. The ^{15}N content of the organic matter was considered to be at

275   natural abundance (0.3663 atom%). The N$_2$O produced via co-denitrification consists of one N

276   atom from the NO$_3^-$ pool, and one N atom from the organic N pool. The chance that the N$_2$O

277   produced via nitrification, denitrification or oxidation of organic N contains zero, one or two

278   ^{15}N enriched atoms can be described by equations 5, 6 and 7, respectively. Where a_x (the ^{15}N

279   fraction of the pool) is a_n for nitrification, a_d for denitrification and a_0 for the oxidation of

280   organic N: a_n, a_d and a_o are explained in Fig. 1.

281

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

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

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

285

286   The chance that the N$_2$O produced via co-denitrification consists of zero, one or two ^{15}N

287   enriched atoms is described by equations 8, 9 and 10 respectively.

288

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

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

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

292

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

294   equations 11, 12 and 13 respectively. Where the subscripts *d*, *n* and *o* refer to the fractions of

295   N_2O produced by denitrification, nitrification and oxidation of organic N, respectively. The

296   fraction of N_2O produced by co-denitrification is 1-*d*-*n*-*o* as all of the N_2O produced was

297   assumed to come from one of the four processes.

298

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

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

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

302

303   The automated continuous-flow isotope-ratio mass spectrometer enabled the measurement of

304   ^{45}R ($^{45}I/^{44}I$) and ^{46}R ($^{46}I/^{44}I$), where ^xI is the ion currents at $m/z\ x$. The ^{45}R and ^{46}R were corrected

305   for the presence of ^{18}O. This, therefore, means that ^{45}R is the fraction of N_2O molecules

306   containing one ^{15}N atom divided by the fraction of N_2O molecules containing zero ^{15}N atoms,

307   and ^{46}R is the fraction of N_2O molecules containing two ^{15}N atoms divided by the fraction of

308   N_2O molecules containing zero ^{15}N atoms. The expected fractions are described by equations

309   11 to 13, where a_o was set to 0.003663, a_n and a_d were considered to be the ^{15}N content of NH_4^+

310   and NO_3^- respectively, while *n*, *d* and *o* were quantified using the *fminsearchbnd* function in

311   MatLab (The MathWorks Inc, Natick, MA). For this the ^{45}R, ^{46}R, a_n and a_d of soil labelled with

312   NO_3**^{15}NH$_4$** Gly and soil labelled with **^{15}NO$_3$**NH$_4$ Gly were used. The amount of N_2O produced

313   via each process was calculated by multiplying the average N_2O flux from the jars labelled

314   with $NO_3{}^{15}NH_4$ Gly and $^{15}NO_3NH_4$ Gly with the fractions of N_2O produced by the four

315   different processes. This was carried out separately for each plot and time step. Because of

316   missing $^{15}NH_4$ data, the different processes were not distinguished for plot 1 time step 3. Total

317   N_2O flux contributions were calculated using linear interpolations between time steps.

318

319   *2.6. Statistical analyses*

320   Treatment differences in total soil N were analysed with the non-parametric Kruskal-Wallis

321   test using IBM SPSS statistics (version 22) because one sample per plot was taken, resulting

322   in only four measurements per treatment. The effect of treatment N_2O fluxes (including

323   different processes), inorganic-N ($NO_3{}^-+NH_4{}^+$), $NO_3{}^-$ and $NH_4{}^+$ concentrations were analysed

324   using the MIXED procedure in SAS (Version 9.3, SAS institute). The N_2O fluxes were

325   transformed using log(flux+10). The N_2O fluxes via the different processes were transformed

326   using flux$^{1/4}$. A Tukey-Kramer adjustment was used to correct for multiplicity effects in

327   pairwise comparisons. Residual checks were made to ensure that the assumptions of the

328   analysis were met. The effect of treatment on modelled N transformation rates were analysed

329   using a one-way ANOVA based on the averages and standard deviations in Matlab (Version

330   2013b, The MathWorks Inc.). The pairwise comparisons were calculated with the Holm-Sidak

331   test in SigmaPlot (Version 11.0, Systat Software Inc.).

332

333   **3.   Results**

334   *3.1. Soil nitrogen pool sizes*

335   Total soil N content did not differ between soil warming treatments prior to the incubation

336   study. A significant interaction between treatment and time affected soil $NH_4{}^+$ concentrations,

337   thus, these results are therefore given separately for each time step. No such interaction was

338   found for $NO_3{}^-$ or total inorganic N ($NO_3{}^-+NH_4{}^+$) concentrations. The total inorganic N content

339 differed with temperature treatment ($p<0.0001$) (all pairwise comparisons were also

340 significant; $p<0.0001$). The total inorganic N content was in the order: $T_1 < T_{control} < T_3 < T_2$.

341

342 Soil NH_4^+ concentrations increased from 2 μg N g^{-1} soil to between 28 and 54 μg N g^{-1} soil

343 upon label addition, and subsequently decreased over the next five days to ca. 9 μg N g^{-1} soil

344 (Fig. 3a). Soil NH_4^+ concentrations did not differ as a result of the soil warming treatments on

345 either days 0 or 6. However, on day 1, treatment T_1 had a lower NH_4^+ concentration compared

346 to all other treatments ($p<0.029$), while the soil NH_4^+ concentration in the T_2 treatment was

347 higher than in the $T_{control}$ or T_1 treatments ($p<0.001$). Three days after label addition the NH_4^+

348 concentration in the T_1 treatment remained lower compared to the T_2 and T_3 treatments (p

349 respectively <0.001 and 0.044).

350

351 After the initial increase in NO_3^- due to label addition, the NO_3^- concentrations continued to

352 slowly increase over the following six days (Fig. 3b). NO_3^- concentrations were significantly

353 different among the treatments ($p<0.001$), with differences also occurring with respect to the

354 initial NO_3^- concentrations prior to label addition ($p<0.001$). The highest NO_3^- concentrations

355 occurred in the T_2 treatment followed by the T_3 and $T_{control}$, while the lowest NO_3^- concentration

356 was observed in the T_1 treatment.

357

358 *3.2. Soil N transformations*

359 The modelled and observed concentrations and ^{15}N enrichments were in good agreement with

360 $R^2 > 0.97$ for all runs (Fig. 4). The gross rates of most N transformations did not differ as a result

361 of the previously imposed soil warming treatment (Table 1). However, the rates of recalcitrant

362 N mineralisation were reduced under the T_2 and T_3 treatments ($p=0.040$). Mineralisation of

363 amino acids also became slower with increasing temperatures ($p=0.045$). However, the overall

364   gross mineralisation of organic N to NH_4^+ did not differ with the previously imposed warming

365   treatments. This was because the mineralisation of labile organic N was the major contributor

366   to total mineralisation, and this rate was not significantly affected by previous warming (Table

367   2). Net mineralisation did not differ as a result of the previously imposed warming treatments.

368   Despite the fact that the release of stored NO_3^- tended to increase with warming (p=0.096), and

369   also that cumulative O_{NH4} and O_{Nrec} rates tended to be different (p=0.095), no significant effect

370   on net nitrification could be observed (Table 2).

371

372   *3.3. N_2O fluxes*

373   In response to N supply, N_2O emissions immediately increased, and decreased thereafter (Fig.

374   3c). While treatments T_2 and T_3 had lower N_2O fluxes than the control treatment (p=0.004 and

375   p=0.036, respectively) no interaction between incubation time and treatment was observed.

376   The N_2O fluxes from the T_2 treatment were also lower than those from the T_1 treatment

377   (p=0.016). However, observed fluxes from the T_1 treatment did not differ from the control

378   treatment and N_2O fluxes from the T_2 treatment did not differ from the T_3 treatment.

379

380   The newly developed partitioning model was successful to identify cumulative N_2O fluxes

381   (Fig. 5) and N_2O contribution at each extraction time (Fig. 6) associated with nitrification,

382   denitrification, co-denitrification and the oxidation of organic N between 0.11 and 5.93 days

383   after N addition. The oxidation of organic N was the main source of N_2O at all sampling dates,

384   comprising between 63 and 85% of the total N_2O flux (Fig. 5). The percentage contribution

385   made by organic N to N_2O fluxes increased over the sampling period, rising from a minimum

386   of 40% in the control treatment, to virtually 100% across all treatments by Day 6 (Fig. 6). The

387   fluxes from organic N oxidation were the highest in the control treatment, followed by T_1, and

388   lowest for T_2 and T_3. Significant differences were found between the control and the T_2 and T_3

389   treatment (p=0.011 and p=0.002, respectively) and between T_1 and T_3 (p=0.039). The amount

390   of N_2O produced via denitrification was also the highest under the control treatment, followed

391   by T_1 and T_3. It was the lowest under T_2. Compared to the control treatment, denitrification

392   contributed less to N_2O under the T_2 and T_3 treatments (p <0.0001 and p=0.002, respectively).

393   The contribution of denitrification also differed between treatments T_2 and T_1 (p=0.004). Co-

394   denitrification only contributed to the N_2O flux during the first day after substrate addition. The

395   highest amount of N_2O produced via co-denitrification was found under the control treatment,

396   followed by T_1. Under T_2 and T_3 treatments, the contribution of co-denitrification was minor.

397   However, these differences were not significant. No significant differences were found in the

398   amount of N_2O produced via nitrification.

399

400   **4.   Discussion**

401   Prior to incubation the inorganic N, as well as the NO_3^- concentrations, were higher in the T_2

402   and T_3 treatments as a result of the six years warming treatment. This suggests that a sustained

403   increase in temperature led to an increase in net mineralisation and net nitrification. This is in

404   line with previous studies showing increases in net mineralisation in response to warming

405   (Peterjohn et al., 1994; Rustad et al., 2001; Norby and Luo, 2004; Bai et al., 2013; Björsne et

406   al., 2014; Zhang et al., 2015b). An increase in net nitrification in response to soil warming,

407   while less common, has also been shown (Barnard et al., 2005; Bai et al., 2013; Björsne et al.,

408   2014; Zhang et al., 2015b). Both could be due to infield temperatures being more favourable

409   for optimal microbial activity. Concurring with previous research (Bai et al., 2013; Zhang et

410   al., 2015b) the total soil N pool did not differ among warming treatments. This result may be

411   due to the fact that the relative sizes of the N pools differ: since the total soil N pool is

412   significantly larger than the inorganic N pool it may take longer to register a change (Galloway

413   et al., 2008; Bai et al., 2013).

414

During incubation all soil was kept at 20°C, regardless of the in-field treatment, to investigate

any legacy impacts of sustained soil warming on inherent soil N cycling. It has been suggested

that changes in the microbial community structure could alter the sensitivity of the microbial

community to temperature shifts (Balser et al., 2006). While both net and gross mineralisation

rates did not differ as a result of the previously imposed soil warming treatments, the

mineralisation of recalcitrant N and mineralisation of amino acids did differ. Lowest rates were

found under T_2 (M_{Nrec}) and T_3 (M_{Nrec} and M_{AA}). A similar effect to warming was found by

Jamieson et al. (1998) who reported decreased gross N mineralisation rates in spring following

winter warming of soil. Adaptation of the microbial community, altering the sensitivity to

temperature shifts, could possibly provide an explanation why no differences in net and gross

mineralisation, and even decreases in individual mineralisation rates were found. However, no

data were available to test this hypothesis. Another possible explanation for the reduction in

mineralisation rates could be a depletion of substrate due to the six years of elevated

temperatures.

429

Previous research in heathland and grassland soils showed no significant effect of warming on

amino acid mineralisation rates (Andresen et al., 2015). The lower rates in the current study,

however, could be due to a change in amino-acid oxidase activity (Vranova et al., 2013).

Another possible explanation for the lower amino acid mineralisation rates could be an increase

in direct microbial assimilation of amino acids (Farrell et al., 2014), since direct assimilation

of glycine and larger amino acids is well known (Barraclough, 1997; Andresen et al., 2009,

2011). Chen et al. (2015), however, did not show an effect of warming on the microbial uptake

of amino acids. The fact that NH_4^+ immobilisation rates were not affected by previously

imposed warming in the current study, is in line with previous research (Niboyet et al., 2011;

439   Bai et al., 2013; Björsne et al., 2014). It has been suggested that the depletion of labile C due

440   to warming might initiate a decrease in immobilisation rates (Bai et al., 2013). In the current

441   experiment a labile carbon source (Gly) was added to the soil, which could explain why no

442   reduction in NH_4^+ immobilisation was found.

443

444   Nitrous oxide emissions were highest shortly after label addition and declined thereafter. Thus,

445   initial higher rates from NH_4^+ and NO_3^- were due to label addition. The higher absolute rate of

446   organic N oxidation at the start of the incubation did not come solely from the Gly addition. If

447   this had been the case, highest N_2O ^{15}N enrichment would have been observed at the first

448   measurement following addition of the NO_3NH_4 ^{15}N-gly label. However, for all treatments the

449   highest ^{15}N enrichment of N_2O was found in the second measurement after label addition. The

450   lower net rates of N_2O production, at the end of incubation period could possibly have been

451   caused by N_2O consumption, however, the consumption of pathway specific N_2O emissions

452   cannot be evaluated with the current model. However, as WFPS was set to 64%, it is unlikely

453   that N_2O consumption occurred, as this would predominantly occur only under fully reductive

454   conditions (but see Goldberg and Gebauer (2009) for an exception).

455

456   Oxidation of organic N was found to be the main source of N_2O. The production of N_2O from

457   an unlabelled organic source would most likely follow a combined process of organic N

458   oxidation via heterotrophic nitrifiers to nitrite, followed by a reduction of nitrite to gaseous N

459   products (Butterbach-Bahl et al., 2013). This process, where oxidation and reduction processes

460   occur hand in hand would be conceptually similar to the nitrifier-denitrification process (Wrage

461   et al., 2001). Most research, however, does not take the oxidation of organic N into account as

462   a possible source of N_2O (Zhang et al., 2015a). Even though recent studies showed that this

463   process contributed 54-85% of N_2O emissions in pastures (Rütting et al., 2010; Müller et al.,

464   2014). These contributions are in line with the current study. Müller et al. (2014) also showed

465   that the fraction of N_2O contributed via the oxidation of organic N was lowest immediately

466   following NH_4NO_3 addition, and that this fraction increased to over 80%, while the

467   contribution of denitrification decreased with time even though NO_3^- concentrations increased.

468   Because of the large contribution of oxidation of organic N in N_2O emissions, this pathway

469   should not be omitted in future research.

470

471   A decrease in N_2O produced via denitrification was found in soil previously subjected to higher

472   temperature treatments. This could be due to a decrease in the rate of denitrification. However,

473   though complete denitrification was likely not a dominant process in these aerobic soils, it is

474   also possible that under treatment T_2 and T_3 more of the NO_3^- underwent complete

475   denitrification, forming N_2 as opposed to N_2O. This highlights the importance of the gaseous

476   N stoichiometries in particular the N_2/N_2O ratio. Stevens and Laughlin (2001) reported $N_2:N_2O$

477   ratios in a fine loamy grassland soil of 2.2 and 0.5 from control and combined slurry plus NO_3^-

478   fertiliser treatments, respectively. However, Clough et al. (1998) showed that ratios can vary

479   between 6.2 and 33.2 following ^{15}N-labelled urine application to ryegrass (*Loilum*

480   *perenne*)/white clover (*Trifolium repens*) pasture on four different soils (silt loam, sandy loam,

481   peat and clay soils). Unfortunately, due to methodological restrictions were not able to detect

482   significant N_2 fluxes, as they were <4 g N_2-N ha^{-1} day^{-1} (Stevens and Laughlin, 1998).

483

484   Adaptation of microorganisms, to long-term elevated temperature treatments, might also

485   provide an explanation for the decrease in N_2O emissions during the incubation with soil

486   previously subjected to increasing soil warming temperatures (Avrahami and Conrad, 2003;

487   French et al., 2009; Pritchard, 2011). Enhanced NO_3^- concentrations in the T_2 and T_3

488   treatments, at the end of the field experiment, also suggests an in situ reduction of

489   denitrification and/or co-denitrification. A possible explanation for the in situ reduction of

490   denitrification could be the altered field soil moisture content. While during the incubation, soil

491   moisture was purposely kept constant (WFPS of 64%), in the field however, moisture

492   conditions were affected by the heating treatment, leading to generally drier, and thus more

493   aerated, conditions in the heated plots (Jansen-Willems et al., 2016). Under low WFPS,

494   nitrification is predominantly responsible for N_2O efflux (Bollmann and Conrad, 1998;

495   Bateman and Baggs, 2005). This may be a consequence of altered soil moisture or changes in

496   soil texture and physical soil structure. The reduction of NO_3^- (denitrification) takes place under

497   more anoxic to anaerobic conditions (Smith, 1997), because under aerobic conditions,

498   denitrifiers reduce O_2 rather that NO_3^- (Arah, 1997). Any reduction in soil moisture could

499   therefore lead to a decrease in the in situ denitrification rate.

500

501   Co-denitrification was observed to be significant in $T_{control}$ and T_1 shortly after N addition.

502   Rates were comparable with those from true denitrification. Co-denitrification is a co-

503   metabolic process which uses inorganic and organic N compounds concurrently and converts

504   it to the same end products as in denitrification. Gases produced in this process are a hybrid N-

505   N species where one atom of N comes from NO_2^- and the other one from a co-metabolised

506   compound (Spott et al., 2011). The conditions for increased co-denitrification are still not fully

507   understood, but the presence of fungi along with adequate amino acid pools appears to enhance

508   losses via this pathway (Laughlin and Stevens, 2002; Spott et al., 2011).

509

510   Laughlin and Stevens (2002) found that fungi dominated denitrification and co-denitrification

511   in grassland soils. It has been suggested that warming could increase the relative contribution

512   of fungi to the soil microbial community (Zhang et al., 2005; Pritchard, 2011). Most fungi lack

513   N_2O reductase, resulting in N_2O as the final denitrification product (Saggar et al., 2013). It can

514   therefore be expected that warming would lead to an increase in N_2O produced via

515   denitrification and co-denitrification. However, the opposite was found in the current

516   experiment, although the changes in co-denitrification were not significant. The reduced co-

517   denitrification and total denitrification rates seem to indicate a reduction in fungal-mediated N

518   processes under elevated temperatures in these soils. Further research is required to elucidate

519   the effect of increased temperatures on N processes mediated by fungi.

520

521   **5.   Conclusion**

522   Sustained increases in soil temperatures over 6 years (between 2 and 3°C) led to an increase in

523   both total inorganic soil N and NO_3^- pools. Subsequent analyses of gross N transformations,

524   during an incubation of these soils under common temperature and moisture conditions to study

525   the legacy effect of increased temperatures, revealed that mineralisation of amino acids

526   (glycine) and recalcitrant organic N decreased with previously imposed elevated temperatures.

527   This decrease in mineralisation was also correlated with a decrease in N_2O emissions from

528   organic N turnover. However, elevated temperature did not cause a significant change in

529   relative N_2O emissions from the different pathways as hypothesised, but it lead to an absolute

530   decrease in N_2O emission rates. A new, easy to use, source partitioning method was developed

531   to determine the contribution of four different pathways to N_2O emissions. Emissions of N_2O

532   in the first six days after fertilisation were decreased for soils previously subjected to higher

533   temperatures as a consequence of a reduction in the rates of denitrification and the oxidation

534   of organic N. For all treatments, oxidation of organic N was the main contributor to N_2O

535   emissions, and should therefore in future research not be omitted as a possible source of N_2O.

536

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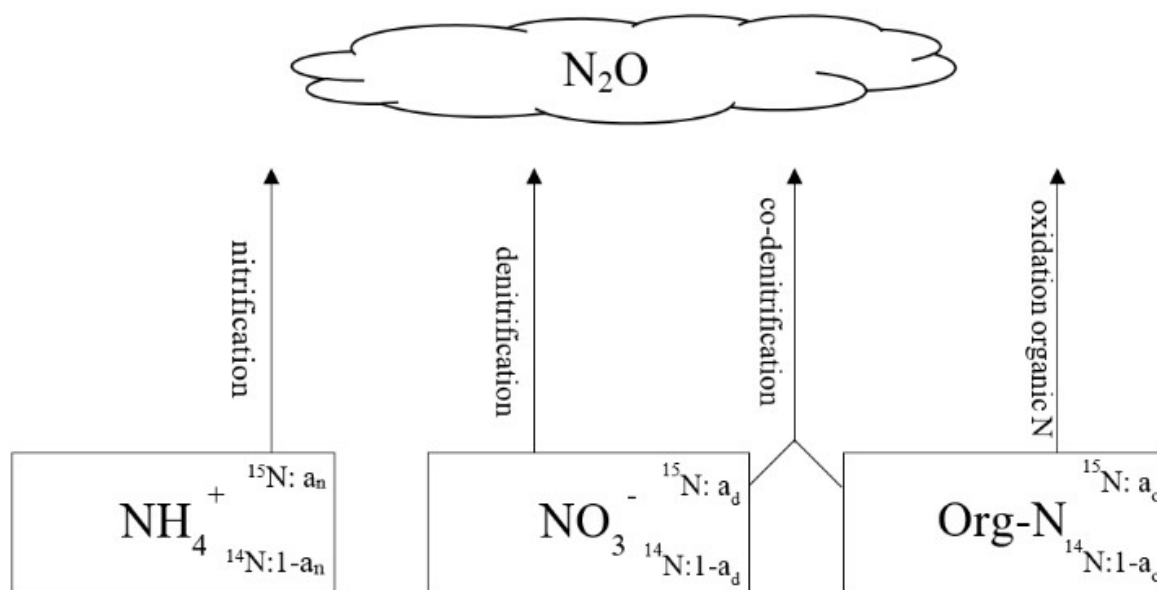
737

738 **Figures**

739

740 Fig. 1. N_2O production via four processes (nitrification, denitrification, co-denitrification and

741 oxidation of organic N). Three uniformly distributed pools were considered. These pools were

742 an ammonium pool (NH_4^+) with a ^{15}N atom fraction of a_n, a nitrate pool (NO_3^-) with a ^{15}N atom

743 fraction of a_d, and an organic-N pool with a ^{15}N atom fraction of a_o (=0.003663). The N_2O

744 produced via co-denitrification consists of one N atom from the nitrate pool, and one from the

745 organic N pool.

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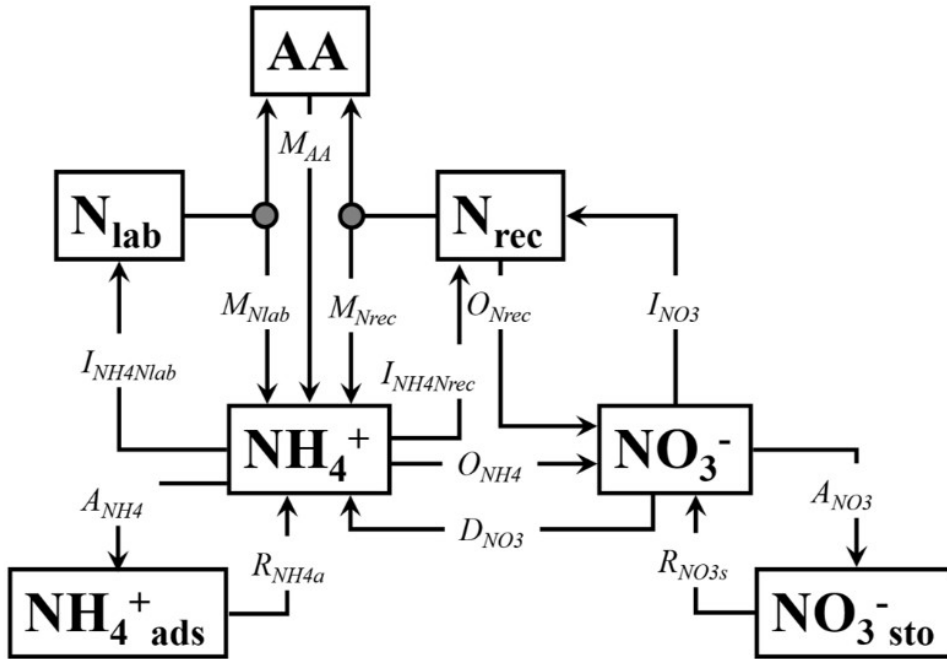
748   Fig. 2. ^{15}N tracing model for analyses of gross soil N transformation rates. Abbreviations of

749   the transformations are explained in the Table 1. The pools are explained in section 2.4.

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751

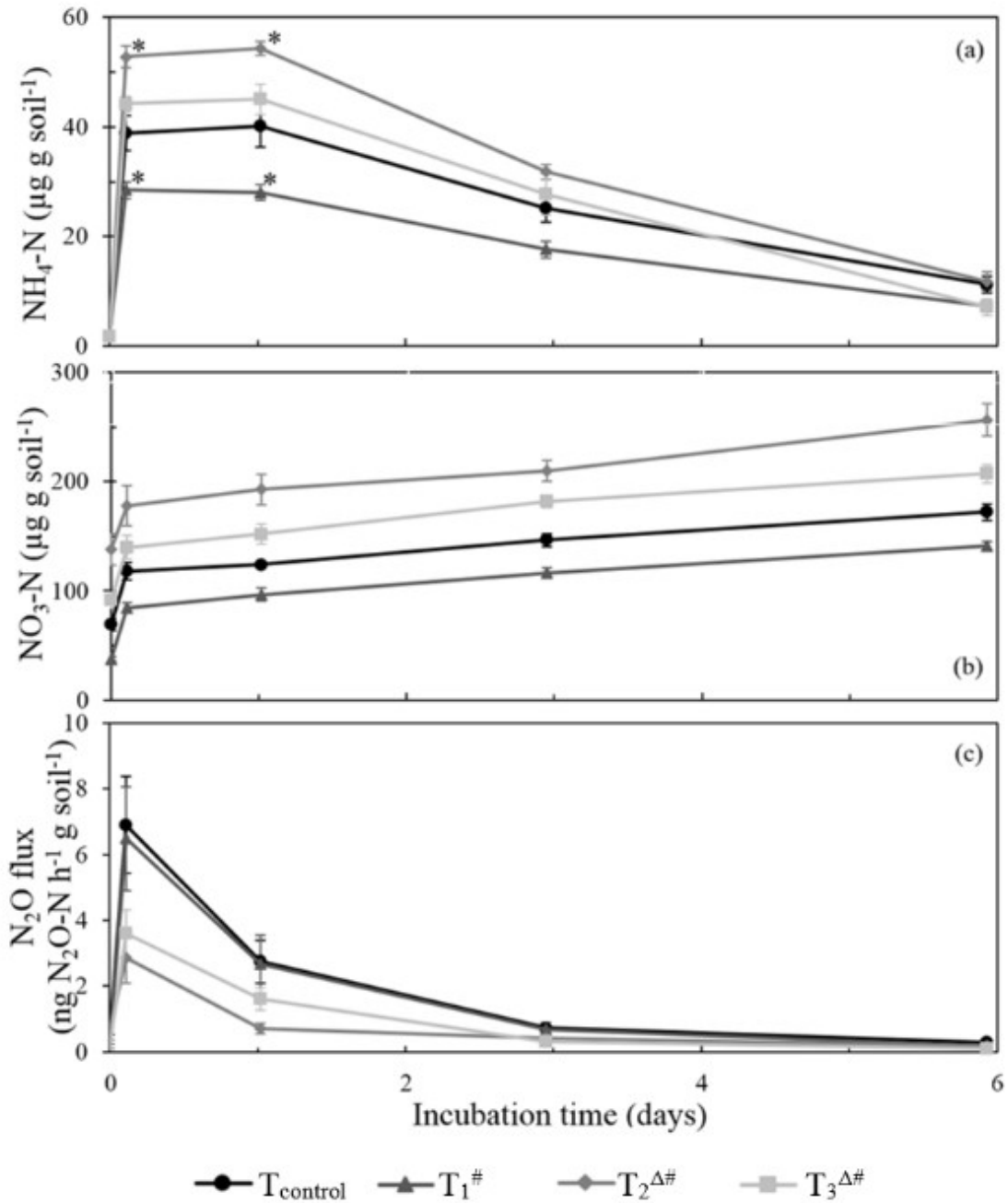
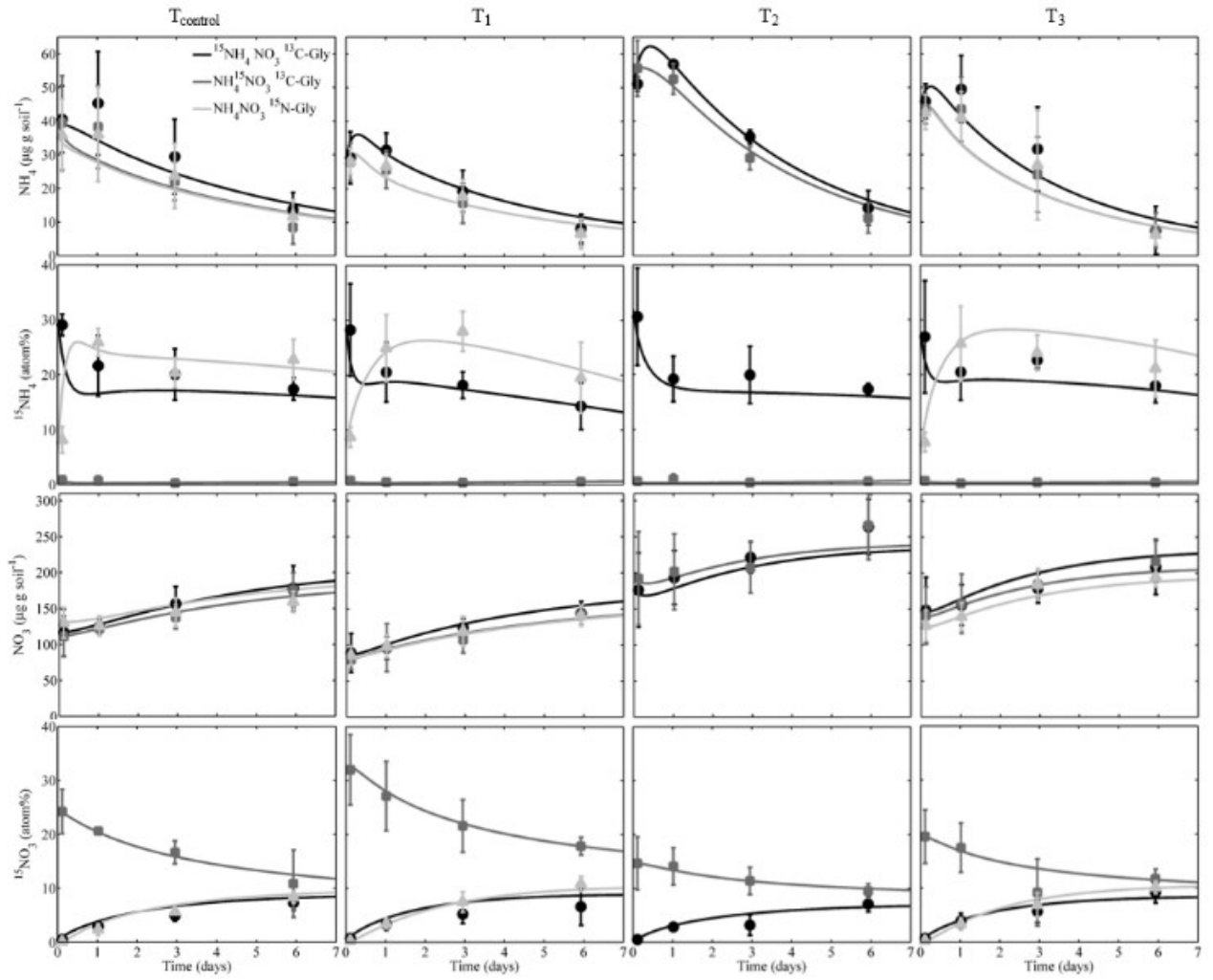



Fig. 3. NH$_4$-N content (a), NO$_3$-N content (b), and N$_2$O emissions (c) at the extraction times. Time point 0 is the time of label addition (^{15}NH$_4$NO$_3$ Gly, NH$_4$^{15}NO$_3$ Gly or NH$_4$NO$_3$ ^{15}N-Gly). The ammonium and nitrate content at time point 0 is based on unlabelled soil. The N$_2$O flux at time point 0 is based on the average flux of the 3 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 Δ shows a significant difference in N$_2$O flux from T$_{control}$ ($p<0.05$).

759

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

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

762 days from the moment of label addition.

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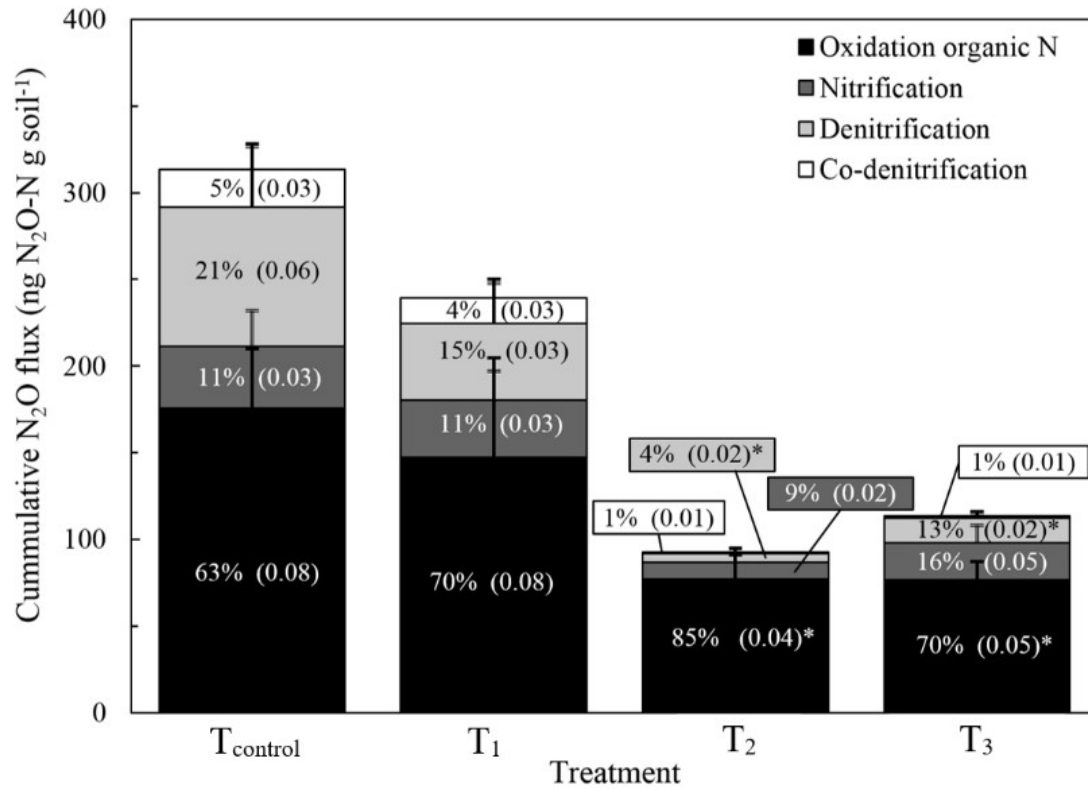
Fig. 5. Cumulative N_2O flux via four processes between 3 h and 6 days after labelling. N_2O fluxes based on average flux from soil labelled with **$^{15}NH_4NO_3$** Gly or **$NH_4^{15}NO_3$** Gly. The cumulative flux per process is an average over the four plots per treatment. Error bars are standard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

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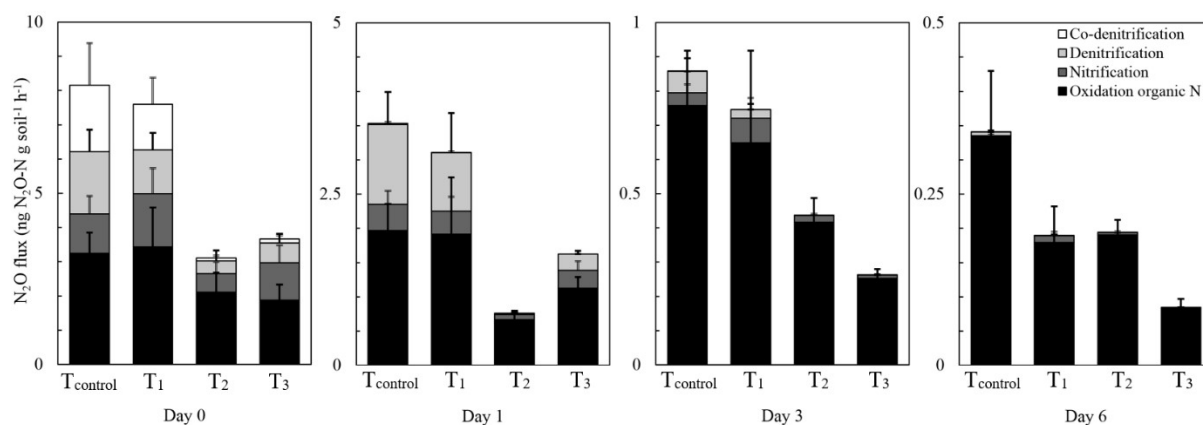
774   Fig. 6. N₂O flux divided into 4 processes at different time points after fertilisation. N₂O fluxes

775   based on average flux from soil labelled with **¹⁵NH₄NO₃** Gly or NH₄**¹⁵NO₃** Gly. The portrayed

776   flux per process is an average over the four plots per treatment. Error bars are standard error of

777   the mean. The scale of the y-axis is different for each time point.

Tables

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

780   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

781   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

782   from control ($p<0.10$).

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

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784

785 Table 2. Gross mineralisation (Min_{Gross}), net mineralisation (Min_{Net}), gross nitrification

786 (Nit_{Gross}) and net nitrification (Nit_{Net}) rate in µg N g soil^{-1} d^{-1}. Including the contributions from

787 the different N pools for the gross transformations (italics), where N_{lab} is a labile organic N

788 pool, N_{rec} is a recalcitrant organic N pool, NH_4^+ is the ammonium pool and N_{AA} is the amino

789 acid Gly pool. [t] one-way ANOVA tendency $p < 0.1$

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

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