Long-term elevation of temperature affects organic N turnover and associated N$_2$O emissions in a permanent grassland soil

Anne B. Jansen-Willems$^{a,b}$, Gary J. Lanigan$^a$, Timothy J. Clough$^c$, Louise C. Andresen$^{b,e}$, and Christoph Müller$^{b,d}$

$^a$Teagasc Johnstown Castle, Wexford, Co. Wexford, Ireland
$^b$Institute for Plant Ecology, JLU Giessen, Heinrich-Buff-Ring 26-32, 35390 Giessen, Germany
$^c$Department of Soil and Physical Sciences, Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln 7647, New Zealand
$^d$School of Biology and Environmental Science, University College Dublin, Dublin, Ireland
$^e$Department of Earth Science, University of Gothenburg, Gothenburg, Sweden

Correspondence to: Anne Jansen-Willems

(anne.jansen@teagasc.ie, anne.willems@bot2.bio.uni-giessen.de)
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 (N₂O) 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. N₂O) 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 NO₃¹⁵NH₄ Gly,¹⁵NO₃NH₄ Gly or NO₃NH₄ ¹⁵N-Gly. Soil extractions and N₂O emissions were analysed using a ¹⁵N tracing 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-incubation). Analyses of N transformations using a ¹⁵N 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 N₂O 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 N₂O. 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.
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).
studies also found no effect of soil warming on total soil N (Bai et al., 2013) and inorganic N (Larsen et al., 2011).

N mineralisation follows a step-wise sequence of protein depolymerisation by extracellular activity to oligomers (e.g. peptides) and monomers (e.g. amino acids) and then uptake by microorganisms before mineralisation to NH$_4^+$ (Schimel and Bennett, 2004). Hence, production of peptides and amino acids as well as mineralisation of amino acids, affects the main fluxes regulating gross N mineralisation. Amino acids have a short residence time in the soil due to either rapid assimilation by soil microbes or mineralisation, which occurs within a few hours (Farrell et al., 2014). In heathland and grassland soils no effect of soil warming on the amino acid concentration has been observed (Chen et al., 2014; Andresen et al., 2015).

Nitrous oxide (N$_2$O), a potent greenhouse gas with a global warming potential of 298 on a 100 year basis, can be produced by several processes, such as nitrification, partial denitrification, co-denitrification and the oxidation of organic matter (Butterbach-Bahl et al., 2013; Zhang et al., 2015a) (Fig. 1). Laughlin and Stevens (2002) confirmed the importance of co-denitrification for N$_2$ production, a process that may comprise 25% of the total N balance in pastures (Selbie et al., 2015). Müller et al. (2014) found that, for the same grassland soil as used in this study, co-denitrification contributed 17.6% of the total N$_2$O production. N$_2$O emissions following fertilisation with ammonium nitrate (NH$_4$NO$_3$) may be greater than from urea fertiliser because of the greater susceptibility to denitrification (Harrison and Webb, 2001). The amount and form of N inputs primarily govern N$_2$O emissions with further impacts resulting from climatic factors, such as temperature and precipitation, and soil factors, such as C availability and microbial community structure (Harrison and Webb, 2001; Müller et al., 2003; Stark and Richards, 2008; Laughlin et al., 2009; Li and Lang, 2014). However, the
impact of elevated soil temperature on $\text{N}_2\text{O}$ production, in semi-natural grasslands is unclear (Peterjohn et al., 1994; Bijoor et al., 2008; Larsen et al., 2011). Furthermore, there has been very limited research into the effect of elevated soil temperature on the different $\text{N}_2\text{O}$ production processes. Maag and Vinther (1996) observed a decrease in nitrification associated $\text{N}_2\text{O}$ emissions and an increase in denitrification associated $\text{N}_2\text{O}$ with increasing soil temperature. It has been suggested that this was due to creation of anoxic conditions and the associated depletion of oxygen following the increase in microbial respiration with higher soil temperatures (Castaldi, 2000). Prolonged elevated soil temperatures, on the other hand, could also lead to changes in the microbial community (Avrahami and Conrad, 2003; French et al., 2009).

Several methods, such as source partitioning, have been used to quantify the contributions of individual N pools to $\text{N}_2\text{O}$ emissions (Stange et al., 2009; Rütting et al., 2010; Zhang et al., 2011; Zhu et al., 2011; Stange et al., 2013; Müller et al., 2014). However, one of the assumptions of the source partitioning method is the absence of hybrid reactions such as co-denitrification (Zhang et al., 2015a). Because of the potential importance of co-denitrification for the $\text{N}_2\text{O}$ production, it should not be omitted from the analysis of $\text{N}_2\text{O}$ sources. Currently, only one technique is available to identify several processes including a hybrid reaction, which is a full $^{15}\text{N}$ tracing approach (Müller et al., 2014). This approach however, requires data on $\text{NO}_2^-$; $\text{NO}_3^-; \text{NH}_4^+$ pool sizes and measurements at multiple time points. Furthermore, it requires at least multiple days of running the model to be able to distinguish the different processes. A straightforward method partitioning $\text{N}_2\text{O}$ fluxes into several pathways including a hybrid reaction, which does not rely on measurements of $\text{NO}_2^-$ and data at multiple time points, would therefore be very beneficial.
The objectives of this study were to quantify the legacy effects of six years of elevated temperature (via IR heaters) on soil N cycling dynamics, including (1) net and gross N transformation rates in the soil (2) N₂O fluxes immediately after fertilisation and (3) the processes responsible for these N₂O fluxes. 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. To determine the processes involved in N₂O 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 ¹⁵N 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. N₂O) 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 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. (2016). Briefly, the site had been managed as a meadow with two cuts per year and fertilised with 50-80 kg N ha⁻¹ year⁻¹ for the
last three decades. Since 1995, the N fertiliser input had been reduced to 40 kg N ha\(^{-1}\) year\(^{-1}\), as KAS (calcium-ammonium-nitrate). The mean annual temperature and precipitation were 9.5°C and 560 mm (observation period: 1995-2014) respectively.

The site had been divided into 16 plots, four rows of four plots. The 16 plots were, according to a Latin square design, assigned to one of four treatments. From January 28, 2008, the soil temperature of each plot, measured at 5 cm depth, was elevated by 0, 1 (mean 0.8 standard error 0.02), 2 (mean 1.9 standard error 0.03) or 3 (mean 2.6 standard error 0.03) °C above ambient temperature, using infrared heaters. The use of heaters will also affect the soil moisture content. The temperature treatments (including any moisture effect) are referred to as T\(_{\text{control}}\), T\(_1\), T\(_2\), and T\(_3\), respectively. The infrared heaters were installed at different heights to create the different temperature elevations (Jansen-Willems et al., 2016).

2.2. Incubation, labelling and extraction

On the day the heaters were turned off, all soil within a circular area of 318 cm\(^2\) directly underneath each infrared lamp was excavated to 7.5 cm for the tracing experiment. A small subsample of each plot was dried at 70°C for 48 hours, ground and analysed by a CNH Macro Elemental Analyser (Hanau, Germany) for total N content. A subsample of the soil for each plot was dried at 105°C for 24 hours to determine the soil gravimetric water content. The remaining field moist soil was kept at 4°C (for less than 60 hours) until further analysis whereupon the soil from each field plot was sieved through a 10 mm sieve, to homogenise it and to remove roots. Incubations were carried out in 750 ml jars (WECK GmbH u. Co. KG, Wehr, Germany). Thirteen jars per field plot were prepared each with an average of 67 (stdev 8.4) g dry soil per jar (except for plots 3, 5, 7, 11 and 14, where only 10 jars were prepared due to lack of soil). All jars were closed with glass lids that were fitted with septa to allow for gas
sampling. During gas flux analyses the jars were sealed using a clamp and a rubber ring between the jar and the lid. At other times a gap was left between the jar and the lid to allow air exchange while minimising water loss. Two days after soil sampling (day -55), all jars were put in a dark climate chamber at 20°C and 50% humidity and incubated for 55 days prior to $^{15}$N substrate addition (day 0).

Soil gravimetric moisture data were used to determine the exact amount of dry soil in each jar, and to calculate the amount of water to be added to ensure the same soil water content in each jar. On day -53 the soil moisture in each jar was adjusted to a water-filled pore space (WFPS) of 64%. On day -43 and -5 the jars were watered to replenish the water lost due to evaporation.

For the $^{15}$N tracing study three different labels were used, NO$_3$$^{15}$N$\text{H}_4$ Gly, $^{15}$NO$_3$NH$_4$ Gly and NO$_3$NH$_4$ $^{15}$N-Gly (at 60, 60 and 99 atm% $^{15}$N respectively). All solutions contained 50 µg NO$_3$-N, 50 µg NH$_4$-N, and 30 µg Gly-N g$^{-1}$ soil. On day 0, the substrate solution was added to each jar using a needle with side-ports, to inject the solution into the soil to minimise disturbance, while providing an equal distribution in the soil (Müller et al., 2007). For each field plot, jars were set up for four soil extractions, at day 0, 1, 3 and 6 after N application, and three labels, except for plot 3, 5, 7, 11 and 14, where due to the lack of soil no NO$_3$NH$_4$ $^{15}$N-Gly label addition was possible.

The soil in each jar was extracted with 2M KCl using the blending procedure of Stevens and Laughlin (1995). The $^{15}$N enrichments of NO$_3^-$ and NH$_4^+$ in the extracts were determined by converting NO$_3^-$ and NH$_4^+$ into N$_2$O following the procedures by Stevens and Laughlin (1994) for determination of the $^{15}$N enrichment in NO$_3^-$ and Laughlin et al. (1997) for the $^{15}$N enrichment in NH$_4^+$. The extraction of soil prior to $^{15}$N addition, took place on day -2. The
other extractions took place at 0.11 days (+/- 0.004), 1.02 days (+/- 0.001), 2.95 days (+/- 0.001) and 5.93 days (+/- 0.001) after \(^{15}\)N substrate addition, and are hereafter referred to as 0, 1, 3 and 6 days after \(^{15}\)N substrate addition, respectively.

2.3. Gas sampling

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

Gas samples were taken using a 60 ml syringe (Ecoject Plus, Gelnhausen, Germany). At time zero (t\(_0\)) 15 gas samples were taken from 15 different jars. Then at time 1 (t\(_1\)) a gas sample was taken through the rubber septum. At both t\(_0\) and t\(_1\) the syringe was flushed twice with headspace gas to ensure a representative sample was taken. The times between t\(_0\) and t\(_1\) during each of the seven different gas samplings (three before label addition and four immediately prior to extraction) were 120-129, 120, 180, 233, 240, 235 and 214 minutes, respectively. Gas samples were analysed within 24 h after sampling using a GC (Bruker) equipped with an electron capture detector (ECD) for N\(_2\)O analysis. An average of the concentrations measured in the 15 samples was used as the t\(_0\) concentration for all 43 jars. Fluxes were based on the ppm and time difference between t\(_0\) and t\(_1\). They were calculated using the constant gas law, with ambient pressure, and temperature was assumed to be 20\(^\circ\)C (the temperature of the incubation room). The fluxes were then converted to a per dry gram basis.

For the \(^{15}\)N abundance of N\(_2\)O, a 30 ml sample was taken at t\(_1\) and transferred to a 12 ml Exetainers\textsuperscript{®} 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 ventral needle submerged 3-4 mm in a beaker of water and the gas sample held upside down and pushed onto the dorsal needle. The excess pressure in the sample vial was thus released causing the water to bubble until the pressure inside the vial has equilibrated with the ambient atmospheric pressure. Cessation of bubbling implied equal pressure had been reached. The $^{15}$N enrichments of $^{15}$N$_2$O and $^{15}$N$_2$ were determined using an automated isotope ratio mass spectrometry (Sercon Ltd 20-20), as described by Stevens et al. (1993), interfaced to a TGII cryofocusing unit (Sercon Ltd 20-20). The detection limit for atom% $^{15}$N of a 50 ppm N$_2$O standard gas was 0.00003 (n= 10), stdev was 0.00009 atom% $^{15}$N. Respective values for a 0.4 ppm N$_2$O standard were higher (0.00084 (n= 10), stdev 0.003).

2.4. $^{15}$N tracing model

The $^{15}$N tracing analysis tool described by Müller et al. (2007) was used to quantify gross soil N transformations. In the current study, the only changes to the original model were the addition of an amino-acid (glycine) pool, and the transformations to and from this pool. The model (Fig. 2.) considered seven N pools and 13 N transformations. The N pools were NH$_4^+$, NO$_3^-$, amino acid glycine (AA), labile (N$_{lab}$) and recalcitrant (N$_{rec}$) organic N, adsorbed ammonium (NH$_4^+$$_{ads}$) and stored nitrate (NO$_3^-$$_{sto}$).

The initial NO$_3^-$ and NH$_4^+$ pool sizes were determined by extrapolating the first two extraction times back to time zero. The initial AA pool size was set to 30 µg N g$^{-1}$ soil, corresponding to the application of glycine (Gly). The initial NH$_4^+$$_{ads}$ and NO$_3^-$$_{sto}$ were based on the difference between the added and initial N (Müller et al., 2004). The initial pool sizes for organic N (N$_{rec}$ and N$_{lab}$) were based on previous field measurements. However, these organic N values were not critical because for N$_{rec}$, zero-order kinetics were used (independent of initial pool size),
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.

The $N$ transformations are described in Table 1. The $N$ transformations were calculated based
on zero or first order kinetics (Table 1). Whether $N_{lab}$ and $N_{rec}$ were transformed into AA or
$NH_4^+$ was determined by two factors, one for $M_{Nlab}$ and one for $M_{Nrec}$. This factor determines
the fraction of the $M_{Nlab}$ or $M_{Nrec}$ flowing into the AA pool with the remainder entering the
$NH_4^+$ pool. For each temperature treatment the kinetic parameters and the two split factors were
simultaneously optimised by minimising the misfit between the modelled and measured $NH_4^+$
and $NO_3^+$ concentrations and their respective $^{15}N$ enrichments (Müller et al., 2004). For
treatment $T_2$ the measurements of the $^{15}N$-Gly label were not included in the optimisation
because only one replicate was available for this label.

A Markov chain Monte Carlo Metropolis algorithm (MCMC-MA) was used for the
optimisation, which practices a random walk technique to find global minima (Müller et al.,
2007). The uncertainties (standard deviation) of the observations were taken into account by
the optimisation routine. The MCMC-MA routine was programmed in MatLab-Simulink
(Mathworks Inc) as described in Müller et al. (2007). The most suitable parameter set was
determined using the Akaiikes Information Criterion (AIC). Gross and net nitrification, and
gross and net mineralisation were calculated using equation 1 to 4 in which SF stands for split
factor. The combined standard deviation was calculated by $((stdev\ rate\ 1)^2+(stdev\ rate\ 2)^2+\ldotsd)^{0.5}$, in which the stdev of $M_{Nx}\cdot SF_{MNx}$ is the stdev of $M_{Nx}$ multiplied by the SF.

The following combined rates were calculated:

Gross nitrification: $O_{Nrec}+O_{NH4}$

\[(1)\]
Net nitrification: $O_{\text{Nrec}} + O_{\text{NH4}} - I_{\text{NO3}} - D_{\text{NO3}}$ (2)

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

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

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

The $N_2O$ fluxes, from the soil labelled with $NO_3^{15}NH_4$ Gly and $NO_3^{15}NO_3$ NH$_4$ Gly, were separated into four different processes. These were nitrification, denitrification, co-denitrification and oxidation of organic matter. The $N_2O$ was assumed to be derived from three uniformly distributed pools, and based on initial substrate $^{15}N$ enrichments, isotopic discrimination was considered negligible for all four processes. The pools and processes accounting for the $N_2O$ production are shown in Fig. 1. The $^{15}N$ content of the organic matter was considered to be at natural abundance (0.3663 atom%). The $N_2O$ produced via co-denitrification consists of one $N$ atom from the $NO_3^-$ pool, and one $N$ atom from the organic $N$ pool. The chance that the $N_2O$ produced via nitrification, denitrification or oxidation of organic $N$ contains zero, one or two $^{15}N$ enriched atoms can be described by equations 5, 6 and 7, respectively. Where $a_x$ (the $^{15}N$ fraction of the pool) is $a_n$ for nitrification, $a_d$ for denitrification and $a_o$ for the oxidation of organic $N$: $a_n$, $a_d$ and $a_o$ are explained in Fig. 1.

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

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

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

The chance that the $N_2O$ produced via co-denitrification consists of zero, one or two $^{15}N$ enriched atoms is described by equations 8, 9 and 10 respectively.
The chance that the N$_2$O in the gas sample contains zero, one or two $^{15}$N atoms is described by equations 11, 12 and 13 respectively. Where the subscripts $d$, $n$ and $o$ refer to the fractions of N$_2$O produced by denitrification, nitrification and oxidation of organic N, respectively. The fraction of N$_2$O produced by co-denitrification is 1-$d-n-o$ as all of the N$_2$O produced was assumed to come from one of the four processes.

The automated continuous-flow isotope-ratio mass spectrometer enabled the measurement of $^{45}$R ($^{45}$I/$^{44}$I) and $^{46}$R ($^{46}$I/$^{44}$I), where $^x$I is the ion currents at $m/z$ $x$. The $^{45}$R and $^{46}$R were corrected for the presence of $^{18}$O. This, therefore, means that $^{45}$R is the fraction of N$_2$O molecules containing one $^{15}$N atom divided by the fraction of N$_2$O molecules containing zero $^{15}$N atoms, and $^{46}$R is the fraction of N$_2$O molecules containing two $^{15}$N atoms divided by the fraction of N$_2$O molecules containing zero $^{15}$N atoms. The expected fractions are described by equations 11 to 13, where $a_0$ was set to 0.003663, $a_n$ and $a_d$ were considered to be the $^{15}$N content of NH$_4^+$ and NO$_3^-$ respectively, while $n$, $d$ and $o$ were quantified using the fminsearchbnd function in MatLab (The MathWorks Inc, Natick, MA). For this the $^{45}$R, $^{46}$R, $a_n$ and $a_d$ of soil labelled with NO$_3^{15}$NH$_4$ Gly and soil labelled with $^{15}$NO$_3$NH$_4$ Gly were used. The amount of N$_2$O produced via each process was calculated by multiplying the average N$_2$O flux from the jars labelled
with $\text{NO}_3^{15}\text{NH}_4$ Gly and $^{15}\text{NO}_3\text{NH}_4$ Gly with the fractions of $\text{N}_2\text{O}$ produced by the four different processes. This was carried out separately for each plot and time step. Because of missing $^{15}\text{NH}_4$ data, the different processes were not distinguished for plot 1 time step 3. Total $\text{N}_2\text{O}$ flux contributions were calculated using linear interpolations between time steps.

2.6. Statistical analyses

**Treatment differences in total soil N** were analysed with the non-parametric Kruskal-Wallis test using IBM SPSS statistics (version 22) because one sample per plot was taken, resulting in only four measurements per treatment. The effect of treatment $\text{N}_2\text{O}$ fluxes (including different processes), inorganic-N ($\text{NO}_3^{-}+\text{NH}_4^{+}$), $\text{NO}_3^{-}$ and $\text{NH}_4^{+}$ concentrations were analysed using the MIXED procedure in SAS (Version 9.3, SAS institute). The $\text{N}_2\text{O}$ fluxes were transformed using $\log(\text{flux}+10)$. The $\text{N}_2\text{O}$ fluxes via the different processes were transformed using $\text{flux}^{1/4}$. A Tukey-Kramer adjustment was used to correct for multiplicity effects in pairwise comparisons. Residual checks were made to ensure that the assumptions of the analysis were met. The effect of treatment on modelled N transformation rates were analysed using a one-way ANOVA based on the averages and standard deviations in Matlab (Version 2013b, The MathWorks Inc.). The pairwise comparisons were calculated with the Holm-Sidak test in SigmaPlot (Version 11.0, Systat Software Inc.).

3. Results

3.1. Soil nitrogen pool sizes

Total soil N content did not differ between soil warming treatments prior to the incubation study. A significant interaction between treatment and time affected soil $\text{NH}_4^{+}$ concentrations, thus, these results are therefore given separately for each time step. No such interaction was found for $\text{NO}_3^{-}$ or total inorganic N ($\text{NO}_3^{-}+\text{NH}_4^{+}$) 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: T1<Tcontrol<T3<T2.

Soil NH$_4^+$ concentrations increased from 2 µg N g$^{-1}$ soil to between 28 and 54 µg N g$^{-1}$ soil upon label addition, and subsequently decreased over the next five days to ca. 9 µg N g$^{-1}$ soil (Fig. 3a). Soil NH$_4^+$ concentrations did not differ as a result of the soil warming treatments on either days 0 or 6. However, on day 1, treatment T1 had a lower NH$_4^+$ concentration compared to all other treatments (p<0.029), while the soil NH$_4^+$ concentration in the T2 treatment was higher than in the Tcontrol or T1 treatments (p<0.001). Three days after label addition the NH$_4^+$ concentration in the T1 treatment remained lower compared to the T2 and T3 treatments (p respectively <0.001 and 0.044).

After the initial increase in NO$_3^-$ due to label addition, the NO$_3^-$ concentrations continued to slowly increase over the following six days (Fig. 3b). 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 Tcontrol, while the lowest NO$_3^-$ concentration was observed in the T1 treatment.

3.2. Soil N transformations

The modelled and observed concentrations and $^{15}$N enrichments were in good agreement with $R^2$>0.97 for all runs (Fig. 4). The gross rates of most N transformations did not differ as a result of the previously imposed soil warming treatment (Table 1). However, the rates of recalcitrant N mineralisation were reduced under the T2 and T3 treatments (p=0.040). Mineralisation of amino acids also became slower with increasing temperatures (p=0.045). However, the overall
gross mineralisation of organic N to NH$_4^+$ did not differ with the previously imposed warming
treatments. This was because the mineralisation of labile organic N was the major contributor
to total mineralisation, and this rate was not significantly affected by previous warming (Table
2). Net mineralisation did not differ as a result of the previously imposed warming treatments.
Despite the fact that the release of stored NO$_3^-$ tended to increase with warming (p=0.096), and
also that cumulative O$_{NH4}$ and O$_{Nrec}$ rates tended to be different (p=0.095), no significant effect
on net nitrification could be observed (Table 2).

3.3. $N_2O$ fluxes

In response to N supply, $N_2O$ emissions immediately increased, and decreased thereafter (Fig.
3c). While treatments T$_2$ and T$_3$ had lower $N_2O$ fluxes than the control treatment (p=0.004 and
p=0.036, respectively) no interaction between incubation time and treatment was observed.
The $N_2O$ fluxes from the T$_2$ treatment were also lower than those from the T$_1$ treatment
(p=0.016). However, observed fluxes from the T$_1$ treatment did not differ from the control
treatment and $N_2O$ fluxes from the T$_2$ treatment did not differ from the T$_3$ treatment.

The newly developed partitioning model was successful to identify cumulative $N_2O$ fluxes
(Fig. 5) and $N_2O$ contribution at each extraction time (Fig. 6) associated with nitrification,
denitrification, co-denitrification and the oxidation of organic N between 0.11 and 5.93 days
after N addition. The oxidation of organic N was the main source of $N_2O$ at all sampling dates,
comprising between 63 and 85% of the total $N_2O$ flux (Fig. 5). The percentage contribution
made by organic N to $N_2O$ fluxes increased over the sampling period, rising from a minimum
of 40% in the control treatment, to virtually 100% across all treatments by Day 6 (Fig. 6). The
fluxes from organic N oxidation were the highest in the control treatment, followed by T$_1$, and
lowest for T$_2$ and T$_3$. Significant differences were found between the control and the T$_2$ and T$_3
treatment (p=0.011 and p=0.002, respectively) and between T1 and T3 (p=0.039). The amount of N2O produced via denitrification was also the highest under the control treatment, followed 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). Co-denitrification 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.

4. Discussion

Prior to incubation the inorganic N, as well as the NO3− concentrations, were higher in the T2 and T3 treatments as a result of the six years warming treatment. This suggests that a sustained increase in temperature led to an increase in net mineralisation and net nitrification. This is in line with previous studies showing increases in net mineralisation in response to warming (Peterjohn et al., 1994; Rustad et al., 2001; Norby and Luo, 2004; Bai et al., 2013; Björsne et al., 2014; Zhang et al., 2015b). An increase in net nitrification in response to soil warming, while less common, has also been shown (Barnard et al., 2005; Bai et al., 2013; Björsne et al., 2014; Zhang et al., 2015b). Both could be due to infield temperatures being more favourable for optimal microbial activity. Concurring with previous research (Bai et al., 2013; Zhang et al., 2015b) the total soil N pool did not differ among warming treatments. This result may be due to the fact that the relative sizes of the N pools differ: since the total soil N pool is significantly larger than the inorganic N pool it may take longer to register a change (Galloway et al., 2008; Bai et al., 2013).
During incubation all soil was kept at 20°C, regardless of the in-field treatment, to investigate any legacy impacts of sustained soil warming on inherent soil N cycling. It has been suggested that changes in the microbial community structure could alter the sensitivity of the microbial community to temperature shifts (Balser et al., 2006). While both net and gross mineralisation rates did not differ as a result of the previously imposed soil warming treatments, the mineralisation of recalcitrant N and mineralisation of amino acids did differ. Lowest rates were found under T$_2$ ($M_{Nrec}$) and T$_3$ ($M_{Nrec}$ and $M_{AA}$). A similar effect to warming was found by Jamieson et al. (1998) who reported decreased gross N mineralisation rates in spring following winter warming of soil. Adaptation of the microbial community, altering the sensitivity to temperature shifts, could possibly provide an explanation why no differences in net and gross mineralisation, and even decreases in individual mineralisation rates were found. However, no data were available to test this hypothesis. Another possible explanation for the reduction in mineralisation rates could be a depletion of substrate due to the six years of elevated temperatures.

Previous research in heathland and grassland soils showed no significant effect of warming on amino acid mineralisation rates (Andresen et al., 2015). The lower rates in the current study, however, could be due to a change in amino-acid oxidase activity (Vranova et al., 2013). Another possible explanation for the lower amino acid mineralisation rates could be an increase in direct microbial assimilation of amino acids (Farrell et al., 2014), since direct assimilation of glycine and larger amino acids is well known (Barraclough, 1997; Andresen et al., 2009, 2011). Chen et al. (2015), however, did not show an effect of warming on the microbial uptake of amino acids. The fact that NH$_4^+$ immobilisation rates were not affected by previously imposed warming in the current study, is in line with previous research (Niboyet et al., 2011;
Bai et al., 2013; Björsne et al., 2014). It has been suggested that the depletion of labile C due to warming might initiate a decrease in immobilisation rates (Bai et al., 2013). In the current experiment a labile carbon source (Gly) was added to the soil, which could explain why no reduction in NH$_4^+$ immobilisation was found.

Nitrous oxide emissions were highest shortly after label addition and declined thereafter. Thus, initial higher rates from NH$_4^+$ and NO$_3^-$ were due to label addition. The higher absolute rate of organic N oxidation at the start of the incubation did not come solely from the Gly addition. If this had been the case, highest N$_2$O $^{15}$N enrichment would have been observed at the first measurement following addition of the NO$_3$NH$_4$$^{15}$N-gly label. However, for all treatments the highest $^{15}$N enrichment of N$_2$O was found in the second measurement after label addition. The lower net rates of N$_2$O production, at the end of incubation period could possibly have been caused by N$_2$O consumption, however, the consumption of pathway specific N$_2$O emissions cannot be evaluated with the current model. However, as WFPS was set to 64%, it is unlikely that N$_2$O consumption occurred, as this would predominantly occur only under fully reductive conditions (but see Goldberg and Gebauer (2009) for an exception).

Oxidation of organic N was found to be the main source of N$_2$O. The production of N$_2$O from an unlabelled organic source would most likely follow a combined process of organic N oxidation via heterotrophic nitrifiers to nitrite, followed by a reduction of nitrite to gaseous N products (Butterbach-Bahl et al., 2013). This process, where oxidation and reduction processes occur hand in hand would be conceptually similar to the nitrifier-denitrification process (Wrage et al., 2001). Most research, however, does not take the oxidation of organic N into account as a possible source of N$_2$O (Zhang et al., 2015a). Even though recent studies showed that this process contributed 54-85% of N$_2$O emissions in pastures (Rütting et al., 2010; Müller et al.,
2014). These contributions are in line with the current study. Müller et al. (2014) also showed that the fraction of N\textsubscript{2}O contributed via the oxidation of organic N was lowest immediately following NH\textsubscript{4}NO\textsubscript{3} addition, and that this fraction increased to over 80%, while the contribution of denitrification decreased with time even though NO\textsubscript{3}\textsuperscript{-} concentrations increased. Because of the large contribution of oxidation of organic N in N\textsubscript{2}O emissions, this pathway should not be omitted in future research.

A decrease in N\textsubscript{2}O produced via denitrification was found in soil previously subjected to higher temperature treatments. This could be due to a decrease in the rate of denitrification. However, though complete denitrification was likely not a dominant process in these aerobic soils, it is also possible that under treatment T\textsubscript{2} and T\textsubscript{3} more of the NO\textsubscript{3}\textsuperscript{-} underwent complete denitrification, forming N\textsubscript{2} as opposed to N\textsubscript{2}O. This highlights the importance of the gaseous N stoichiometries in particular the N\textsubscript{2}/N\textsubscript{2}O ratio. Stevens and Laughlin (2001) reported N\textsubscript{2}/N\textsubscript{2}O ratios in a fine loamy grassland soil of 2.2 and 0.5 from control and combined slurry plus NO\textsubscript{3}\textsuperscript{-} fertiliser treatments, respectively. However, Clough et al. (1998) showed that ratios can vary between 6.2 and 33.2 following \textsuperscript{15}N-labelled urine application to ryegrass (Loilum perenne)/white clover (Trifolium repens) pasture on four different soils (silt loam, sandy loam, peat and clay soils). Unfortunately, due to methodological restrictions were not able to detect significant N\textsubscript{2} fluxes, as they were <4 g N\textsubscript{2}-N ha\textsuperscript{-1} day\textsuperscript{-1} (Stevens and Laughlin, 1998).

Adaptation of microorganisms, to long-term elevated temperature treatments, might also provide an explanation for the decrease in N\textsubscript{2}O emissions during the incubation with soil previously subjected to increasing soil warming temperatures (Avrahami and Conrad, 2003; French et al., 2009; Pritchard, 2011). Enhanced NO\textsubscript{3}\textsuperscript{-} concentrations in the T\textsubscript{2} and T\textsubscript{3} treatments, at the end of the field experiment, also suggests an in situ reduction of
denitrification and/or co-denitrification. A possible explanation for the in situ reduction of denitrification could be the altered field soil moisture content. While during the incubation, soil moisture was purposely kept constant (WFPS of 64%), in the field however, moisture conditions were affected by the heating treatment, leading to generally drier, and thus more aerated, conditions in the heated plots (Jansen-Willems et al., 2016). Under low WFPS, nitrification is predominantly responsible for N$_2$O efflux (Bollmann and Conrad, 1998; Bateman and Baggs, 2005). This may be a consequence of altered soil moisture or changes in soil texture and physical soil structure. The reduction of NO$_3^-$ (denitrification) takes place under more anoxic to anaerobic conditions (Smith, 1997), because under aerobic conditions, denitrifiers reduce O$_2$ rather that NO$_3^-$ (Arah, 1997). Any reduction in soil moisture could therefore lead to a decrease in the in situ denitrification rate.

Co-denitrification was observed to be significant in T$_{control}$ and T$_1$ shortly after N addition. Rates were comparable with those from true denitrification. Co-denitrification is a co-metabolic process which uses inorganic and organic N compounds concurrently and converts it to the same end products as in denitrification. Gases produced in this process are a hybrid N-N species where one atom of N comes from NO$_2^-$ and the other one from a co-metabolised compound (Spott et al., 2011). The conditions for increased co-denitrification are still not fully understood, but the presence of fungi along with adequate amino acid pools appears to enhance losses via this pathway (Laughlin and Stevens, 2002; Spott et al., 2011).

Laughlin and Stevens (2002) found that fungi dominated denitrification and co-denitrification in grassland soils. It has been suggested that warming could increase the relative contribution of fungi to the soil microbial community (Zhang et al., 2005; Pritchard, 2011). Most fungi lack N$_2$O reductase, resulting in N$_2$O as the final denitrification product (Saggar et al., 2013). It can
therefore be expected that warming would lead to an increase in N\textsubscript{2}O produced via
denitrification and co-denitrification. However, the opposite was found in the current
experiment, although the changes in co-denitrification were not significant. The reduced co-
denitrification and total denitrification rates seem to indicate a reduction in fungal-mediated N
processes under elevated temperatures in these soils. Further research is required to elucidate
the effect of increased temperatures on N processes mediated by fungi.

5. Conclusion

Sustained increases in soil temperatures over 6 years (between 2 and 3°C) led to an increase in
both total inorganic soil N and NO\textsubscript{3}\textsuperscript{-} pools. Subsequent analyses of gross N transformations,
during an incubation of these soils under common temperature and moisture conditions to study
the legacy effect of increased temperatures, revealed that mineralisation of amino acids
(glycine) and recalcitrant organic N decreased with previously imposed elevated temperatures.
This decrease in mineralisation was also correlated with a decrease in N\textsubscript{2}O emissions from
organic N turnover. However, elevated temperature did not cause a significant change in
relative N\textsubscript{2}O emissions from the different pathways as hypothesised, but it led to an absolute
decrease in N\textsubscript{2}O emission rates. A new, easy to use, source partitioning method was developed
to determine the contribution of four different pathways to N\textsubscript{2}O emissions. Emissions of N\textsubscript{2}O
in the first six days after fertilisation were decreased for soils previously subjected to higher
temperatures as a consequence of a reduction in the rates of denitrification and the oxidation
of organic N. For all treatments, oxidation of organic N was the main contributor to N\textsubscript{2}O
emissions, and should therefore in future research not be omitted as a possible source of N\textsubscript{2}O.
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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 $^{15}$N atom fraction of $a_n$, a nitrate pool (NO₃⁻) with a $^{15}$N atom fraction of $a_d$, and an organic-N pool with a $^{15}$N 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. $^{15}$N tracing model for analyses of gross soil N transformation rates. Abbreviations of the transformations are explained in the Table 1. The pools are explained in section 2.4.
Fig. 3. NH₄-N content (a), NO₃-N content (b), and N₂O emissions (c) at the extraction times. Time point 0 is the time of label addition (¹⁵NH₄NO₃ Gly, NH₄¹⁵NO₃ Gly or NH₄NO₃ ¹⁵N-Gly). The ammonium and nitrate content at time point 0 is based on unlabelled soil. The N₂O 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 NH₄-N from T_control (p<0.03), # shows a significant difference in NO₃-N from T_control (p<0.0001), and Δ shows a significant difference in N₂O flux from T_control (p<0.05).
Fig. 4. Modelled vs measured data. The lines are modelled data, and the squares, circles and triangles are the measured data points. Error bars are standard deviations. Time is the time in days from the moment of label addition.
Fig. 5. Cumulative N$_2$O flux via four processes between 3 h and 6 days after labelling. N$_2$O fluxes based on average flux from soil labelled with $^{15}$NH$_4$NO$_3$ 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).
Fig. 6. N$_2$O flux divided into 4 processes at different time points after fertilisation. N$_2$O fluxes based on average flux from soil labelled with $^{15}$NH$_4$NO$_3$ Gly or NH$_4^{15}$NO$_3$ Gly. The portrayed flux per process is an average over the four plots per treatment. Error bars are standard error of the mean. The scale of the y-axis is different for each time point.
Table 1: Description of N transformations and average gross N fluxes per treatment (diagram shown in Fig. 2). Standard deviation between brackets. K stands for Kinetics were 0 implies the use of zero-order and 1 the use of first-order kinetics in the model. The p is the p-value of the one-way ANOVA, with ns (non-significant) if p > 0.1 (p value in bold if < 0.05). For the holm-sidak pairwise comparisons: † tends to be different from control (p<0.10).

| Transformation          | K | T_{control} | T_1   | T_2   | T_3   | p     |
|-------------------------|---|-------------|-------|-------|-------|-------|
| 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)† | **0.045** |
Table 2. Gross mineralisation (MinGross), net mineralisation (MinNet), gross nitrification (NitGross) and net nitrification (NitNet) rate in µg N g soil$^{-1}$ d$^{-1}$. Including the contributions from the different N pools for the gross transformations (italics), where $N_{lab}$ is a labile organic N pool, $N_{rec}$ is a recalcitrant organic N pool, $NH_4^+$ is the ammonium pool and $N_{AA}$ is the amino acid Gly pool. $^1$ one-way ANOVA tendency p<0.1

|          | T control | T1 | T2 | T3 |
|----------|-----------|----|----|----|
| MinGross | 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% |
| MinNet   | 6.78      | 6.32 | 2.29 | 4.30 |
| NitGross | 19.04     | 13.62 | 16.24 | 18.17 |
| $N_{rec}$ | 19%       | 15% | 12% | 16% |
| $NH_4^+$ | 81%       | 85% | 82% | 84% |
| NitNet   | 13.22     | 11.23 | 11.30 | 13.06 |