# Amino acid and N mineralization dynamics in heathland soil after long-term warming and repetitive drought

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## Abstract

Monomeric organic nitrogen (N) compounds such as free amino acids (~~fAA~~FAA) ~~is~~ are an important resource for both plants and soil microorganisms and ~~is, furthermore,~~ a source of ammonium ($NH_4^+$) via microbial ~~fAA~~FAA mineralization. We compared gross ~~fAA~~FAA dynamics with gross N mineralization in a Dutch heathland soil using a $^{15}N$ tracing technique. A special focus was made on the effects of climate change factors warming and drought, followed by rewetting. Our aims were to: 1) compare ~~fAA~~FAA mineralization ($NH_4^+$ production from ~~fAA~~FAAs) with gross N mineralization, 2) assess gross ~~fAA~~FAA production rate (depolymerization) and turnover time relative to gross N mineralization rate, and 3) assess the effects of a 14 years warming and drought treatment on these rates.

The turnover of ~~fAA~~FAA in the soil was ca. 3 hours, which is almost two orders of magnitude faster than that of $NH_4^+$ (i.e. ca. 4 days). This suggests that ~~fAA~~FAAs is an extensively used resource by soil microorganisms. In control soil (i.e. no climatic treatment), the gross N mineralization rate (10 ± 2.9 µg N $g^{-1}day^{-1}$) was eight-times smaller than the total gross ~~fAA~~FAA production rate of five AAs (alanine, valine, leucine, isoleucine, proline: 127.4 to 25.0 µg N $g^{-1}$ $day^{-1}$). Gross ~~fAA~~FAA mineralization (3.4 ± 0.2 µg N $g^{-1}$ $day^{-1}$) contributed by 34 % to the gross N mineralization rate and is therefore an important component of N mineralization. In the drought treatment, a 6-29% reduction in annual precipitation, caused a decrease of gross ~~fAA~~FAA production ~~was reduced~~ by 65% and of gross ~~fAA~~FAA mineralization by 41%, compared to control. On the other hand, gross N mineralization was unaffected by drought, indicating an increased mineralization of other soil organic nitrogen (SON) components. A 0.5-1.5°C warming did not significantly affect N transformations, even though ~~that~~ gross F~~f~~AA production ~~was more than halved~~declined.

Overall our results suggest that in heathland soil exposed to droughts a different type of SON pools are mineralized. Furthermore, compared to agricultural soils, ~~fAA~~FAA mineralization was relatively less important in the investigated heathland. This indicates ~~a~~ more complex mineralization dynamics in semi-natural ecosystems.

**Keywords:** depolymerization, ammonification, N cycle, $^{15}$N pool dilution, amino acid mineralization, mirror experiment

## 1. Introduction

Heathlands are protected under the European Union Habitats Directive (Directive (92/43/EEC); EUR-Lex) as this ecosystem type has declined throughout Europe (Fagundez, 2013). In the Netherlands, the heathland area has declined by 95% since the year 1900 (Fagundez, 2013). Sustaining this characteristic ecosystem type requires management of the vegetation (Webb, 1998; von Oheimb, 2009; Garcia et al., 2013) to mitigate the effects of the major present-day threats; climate change and increased nitrogen (N) deposition (Aerts et al., 1995; Fagundez, 2013). Most heathlands and shrublands are developed on nutrient poor soil, hence available N is a limited resource. Plant and microbial use of both inorganic N (IN; mainly ammonium ($NH_4^+$) and nitrate ($NO_3^-$)) and organic N (ON; *e.g.* free amino acids, ~~fAA~~FAA) is dependent on the availability and production of the different N moieties (Nordin et al., 2004; Jones and Kielland, 2012). However, knowledge of the relative importance of IN and ~~fAA~~FAAs for plants and microbes in heathlands is fragmented and variable (Nordin et al., 2004; Andresen et al., 2005, 2011; Clemmensen et al., 2008), and we need to understand how the interplay of ~~plant~~ available N and ~~effects from~~ global change factors threaten this ecosystem type.

Ammonium ($NH_4^+$) is produced during mineralization of ~~organic~~ ON. ~~Two alternative pathways for N mineralization have been discussed, 'direct route' and mineralization-immobilization turnover: MIT (Barraclough, 1997). In the former, organic N such as fAA is taken up by microorganism followed by excretion of $NH_4^+$ from excess N not needed for microbial assimilation. In contrast, MIT refers to the mineralization of the organic N by exo-enzymes, followed by microbial immobilization of released $NH_4^+$ (Barraclough, 1997). Direct microbial uptake of intact fAAs was evident in a Danish heathland from dual labelled fAA tracing ($^{13}C$ and $^{15}N$; Andresen et al., 2009; 2011), suggesting that the fAA mineralization took place inside bacterial cells (direct route). However, this does not rule out the possibility~~

~~of a simultaneous $NH_4^+$ production via extracellular fAAs mineralization (MIT). For instance, a gradual change from direct route dominance to MIT was observed during wheat residue decomposition (Barraclough, 1997; Giesseler et al., 2009).~~ Gross mineralization is depending on the availability of ~~fAA~~FAAs, because FAA mineralization is the main pathway of ammonium production (Barraclough 1997; Stange & Döhling 2005; Geisseler et al. 2012)~~and~~, hence, gross N mineralization depends on the ~~fAA~~FAA production rate. However, FAAs are not the only source of gross N mineralization. ~~fAA~~FAAs are produced in the soil during depolymerization of peptides, proteins and other components of detritus and litter (Weintraub and Schimel, 2005; Wanek et al., 2010; Mooshammer et al., 2012). The quantification of ~~fAA~~FAA production and ~~fAA~~FAA mineralization is until now poorly investigated and is one of the major knowledge gaps in soil N cycle (Gärdenäs et al., 2011). Methodologies using $^{14}C$ to study FAA turnover have revealed that the transformation of N from proteins to ammonium was much slower than from amino acid to ammonium, which suggest that the depolymerization rate is the main important constraining factor of N availability in forest ecosystems (Jones and Kielland, 2002; 2012). Carbon (C) to N ratio of amino acids is not a good predictor of ~~fAA~~FAA mineralization rates (Roberts et al., 2009; Rothstein, 2010), because microbial assimilation of ~~fAA~~FAAs differs between small C-poor and large C-rich amino acids (Knowles et al., 2010; Mooshammer et al., 2014). Recent developments of a $^{15}N$-AA pool dilution assay (Wanek et al., 2010; Wild et al., 2013) now enables us to study simultaneously gross ~~fAA~~FAA production rates (depolymerization rates), gross ~~fAA~~FAA mineralization and gross N mineralization rates. ~~Thereby, investigating the relative importance of direct mineralization versus MIT is now possible by focussing on the fAA nitrogen fluxes.~~

For NW Europe (including the Netherlands) it is expected that the future climate will be characterized by longer dry periods during summer and 1 to 2 °C warmer air temperatures

(IPCC 2013). Changes in soil N dynamics occurring in response to these conditions diverge for the two factors warming and drought. At experiments using field scale future climate change scenarios, net production of IN increased in response to warming (Emmett et al., 2004; Andresen et al., 2010; Bai et al., 2013). Furthermore, results studying *Calluna* litter mineralization rates suggested a positive correlation of IN production with temperature and moisture (van Meeteren et al., 2007). Likewise, in response to warming, gross mineralization rate increased in a *Calluna* - *Deschampsia* dominated heathland (Björsne et al., 2014). Contrastingly, drought events decreased gross mineralization rates (Björsne et al., 2014) and net mineralization rates (Emmett et al., 2004; Andresen et al., 2010). However, in the event of re-wetting following drought stress, gross N mineraliz~~s~~ation may rapidly increase to compensate the drought response (Pulleman and Tietema 1999; Chen et al., 2011). Overall, effects from changes in microclimate may increase (warming treatment) or decrease (drought treatment) enzymatic activity (Sardans et al., 2008; Vranova et al., 2013).

By experimental manipulation of rainfall and temperature at heathlands and shrublands across Europe, the field site ‘Oldebroek’ took part in investigating the research question: ‘Are heathlands vulnerable or resilient to climate change’. The effect of drought during growing season and passive night-time warming was followed since 1999 (van Meeteren et al., 2007; Kopittke et al., 2012). The present study aimed to investigate gross N dynamics in the heathland soil, especially, ~~fAA~~FAA mineralization, total N mineralization, and ~~fAA~~FAA production, and how it is affected by climate change. We hypothes~~s~~iz~~s~~ed that: i) drought would decrease gross rates and ii) warming would increase gross rates of all investigated N transformations.

# 2. Methods and Calculations

## 2.1 Field site

The study was conducted at the experimental site Oldebroek (52°24’N 5°55’E), which is part of the Oldebroekse heide, a large native heathland c. 25 m above sea level. The vegetation is dominated by the evergreen shrub *Calluna vulgaris*, the grass *Molinia caerulea* and mosses (mainly *Hypnum cupressiforme*). The *Calluna v.* plants were 28 year old (in 2012), which is at the end of their lifespan (Gimmingham, 1972). The soil is a Haplic Podzol and the parent material cover sand, a fluvioglacial deposit from the Saalien. Soil pH is 4.3. Nitrogen deposition is 23 kg N ha$^{-1}$ yr$^{-1}$ and N leaching is 29 kg N ha$^{-1}$ yr$^{-1}$ ~~(Kopittke et al., 2012)~~. Annual rainfall was 1072 mm and the annual average temperature 10.1˚C (Kopittke et al., 2012).

Climate change manipulations are conducted since 1999, including: 1) continuous passive night time warming by automated curtains and 2) sequential growing season drought by precipitation removal (Beier et al., 2004). Each plot (three per treatment) is 5 × 4 m with a 0.5 m buffer strip at the margin. Light galvanized steel tube scaffold structures were constructed over all the plots. The warming curtains are IR reflecting and they are pulled over the plots after sunset when light intensity is lower than 200 lux, and removed at sunrise when the light intensity increased and also during night rain-events. During heavy winds (wind speeds over 10 m s$^{-1}$) or frost and snowfall the curtains were not active. Throughout the 14 years, the warming treatments increased the top soil temperature by 0.5 to 1.5 °C. The drought treatment ~~is~~was applied each year in early growing season (April to July). Precipitation ~~is~~ was excluded for two to three months which reduced precipitation by a PVC curtain that ~~is~~ was automatically drawn over the vegetation during rain events. Throughout the 14 years, the treatment reduced precipitation by 6-29% annually (Kopittke et al., 2012). The recent drought period in 2013 started on April 15 and ended the day before soil sampling (June 22). Rainfall ~~is~~ was recorded in all plots by funnels (75 mm diameter at 1 m height). Soil moisture and soil temperature (at a soil depth of 4-7 cm) ~~is~~ was recorded in each plot by Decagon~~e~~ sensors. Soil moisture index was calculated for each probe relative to an average obtained from the wettest month (December 2012), where no drought treatment was active and all soils were water saturated.

## 2.2 Soil sampling and soil handling

Soil sampling was conducted on the 23rd of June 2013. Vegetation and loose litter were pushed gently aside. From three locations within each plot, three soil cores were sampled with a corer of 4.5 cm diameter to a depth of 5 cm~~depth~~. The 9 soil cores from each plot were mixed to a composite sample and stored until further processing within 48 hours. Roots were discarded and the remaining soil homogenized by hand. Gravimetric soil moisture content was determined by drying 10 g soil for 24 hours at 100 °C. Because of the extreme dryness of the drought soil (partly hydrophobic) the drought soils were adjusted to the same water content  as the control plots, 12 hours before the isotope labels were amended. This enabled homogenous mixing of the isotope label solution with the soil. Soil organic matter was determined ~~by glowing~~ on 2 g of dried soil ~~for~~ samples by loss of ignition (4 hours at 500° C). Total N and C was determined with an elemental analy~~s~~zer (ANCA SerCon, Crew, UK).

## 2.3 $^{15}$N pool dilution method

The set-up consisted of two isotope ($^{15}$N) dilution experiments conducted with the ‘mirror approach’ (Barraclough, 1997; Rütting et al., 2011; Figure 1). ~~fAA~~FAA production rate and ~~fAA~~FAA mineralization rate were determined by adding a $^{15}$N labelled amino acid mixture ($^{15}$N-AA mix; ‘Cell Free’ amino acid mix (20 AA) U-$^{15}$N 96-98%, Cambridge Isotope laboratories, USA) and gross N mineralization rate was determined by adding $^{15}$N labelled $(^{15}NH_4)_2SO_4$ (98% $^{15}$N). Both isotope experiments received both N moieties, in which one was labelled, the other unlabelled. In total 9.1 µg AA-N g$^{-1}$dry soil and 60.4 µg $NH_4$-N g$^{-1}$dry soil was added. For the $^{15}$N-AA labelling experiment 4 mL of the label solution was added to 40 g wet soil and stirred with a clean glass rod. Immediately after labelling the soil was evenly divided in two bottles for two parallel extractions after incubation. For the $^{15}$N-$NH_4$ labelling experiment, 2 mL of the label solution was added to 20 g of this soil. Incubation took place at room temperature (18-20˚C).

The soil was extracted after 10 min, 30 min ~~or~~ and 7 hours of incubation. A sub-sample of the $^{15}$N-AA labelled soil was extracted with 10 mM $CaSO_4$ containing 3.4% formaldehyde to stop the microbial activity. The subsample was hand-shaken, sonicated by ultra sound (20 mW cm$^{-3}$ by Elma S 100 H) during 30 sec, and then shaken for 30 min at 100 rpm and centrifuged for 10 min at 3500 rpm. Finally the supernatant was filtrated (0.45 µm). Samples for $^{15}$N-AA analysis were purified using cation-exchange cartridges (OnGuard II H, 1 cc, Dionex), conditioned with ultrapure water (> 18.2 MΩ), 3M $NH_3$ and 1M HCl. After loading the extract on the cation-exchange resin, the cartridge was washed with 10 mL of water and amino acids were eluted with 30 mL 3 M $NH_3$. The purified sample was dried under reduced pressure at 35°C, and finally derivatized using ethanol-pyridine and ethylchloroformate (Wanek et al., 2010). The other sub-sample (20 g wet soil) of the $^{15}$N-AA label, as well as the $^{15}NH_4^+$ labelled soil were extracted with 40 mL 1M KCl, then shaken for 30 min at 100 rpm, centrifuged for 10 min at 3500 rpm and finally filtrated.

## 2.4 $^{15}$N Amino acid analysis

The internal standard added to samples during purification was a mixture of two non-biological amino acids~~s~~: nor-valine and nor-leucine. The method described by Wanek et al., 2010 was developed further for our instrumentation at ISOFYS, Ghent University. Concentration and $^{15}$N enrichment w~~h~~ere determined using gas chromatography - mass spectrometry (GC - MS, Trace GC - DSQ, Thermo Fisher). Separation was done on a VF 5-

MScolumn (30m x 0.25mm ID x 0.25µm film). We focu~~s~~sed on five detectable amino acids (alanine mz: 116/117, valine mz: 144/145, leucine mz: 158/159, isoleucine mz: 158/159, and proline mz: 142/143).

### 2.5 $^{15}$N-NH$_4$ determination

The $^{15}$N enrichment of $NH_4^+$ in the KCl soil extracts was determined, using an ANCATGII Automated Nitrogen Carbon (Trace Gas) Analyzer (PDZ Europa, UK) coupled to a 20-20 Isotope Ratio Mass Spectrometer (IRMS; SerCon, UK), after conversion ~~converted~~ to nitrous oxide (Hauck, 1982; Saghir et al., 1993). For this ammonia ($NH_3$) was liberated from the sample extracts by adding magnesium oxide (MgO), and absorbed by an acid solution. Nitrous oxide is produced by reaction with sodium hypobromite (NaOBr).

### 2.6 Data analysis and calculations

Gross mineralization and gross ~~fAA~~FAA production rates (for each individual AA) were estimated by using time steps 10 min and 7 h or 10 and 30 min, respectively, using analytical equations (Kirkham and Bartholomew, 1954).

***Equation I*** $$m = \frac{N_t - N_0}{t} * \frac{\ln(a'_0 / a'_t)}{\ln(N_t / N_0)} \quad [\mu g\ N\ g^{-1}\ day^{-1}]$$

For the few cases with (nearly) constant $NH_4^+$ concentration throughout the incubation time of 7 h, the gross N mineralization was calculated as follows (Kirkham and Bartholomew, 1954):

***Equation II*** $$m = \frac{N_{av}}{t} * \ln\left(\frac{a'_0 * N_0}{a'_t * N_t}\right) \quad [\mu g\ N\ g^{-1}\ day^{-1}]$$

$N_0$ and $N_t$ are the concentrations of the respective N pool (i.e. $NH_4^+$ or AA) at time 0 and t, respectively; $N_{av}$ is the average of $N_t$ and $N_0$.

$a'_0$ and $a'_t$ are the excess $^{15}$N abundances at time 0 and t, respectively. Gross N mineralization had three replicates per treatment analyzed at each time step.

Total ~~fAA~~FAA production rate was equal to the sum of the individual ~~fAA~~FAA production rate. ~~Each ~~Some treatments had only ~~2~~two replicates successfully analyzed at each time step, both numbers are reported in addition to the average. The turnover time (mean residence time) was calculated as $N_0 / m$.

The fraction of mineralization derived from ~~fAA~~FAA mineralization (α) from the $^{15}$N-AA mixture was obtained by measuring $^{15}$N-NH$_4^+$ production in three replicates per treatment at the time steps 10 min and 7 hours, and was calculated according to Watkins and Barraclough, 1996:

**Equation III**

$$\alpha = \frac{a'_t * (N_t / N_0)^{\frac{m}{\theta}} - a'_0}{a'_{aa} * (N_t / N_0)^{\frac{m}{\theta}} - a'_{aa}}$$

Hereafter ~~fAA~~FAA mineralization was calculated as

**Equation IV** $m_{AA} = \alpha * m$ [µg N g$^{-1}$ day$^{-1}$]

$a'_{aa}$ is the excess $^{15}$N abundance of AA calculated for the total AA pool, averaged for the two time steps;

$a'_0$ and $a'_t$ are the excess $^{15}$N abundances of the NH$_4^+$ pool at time 0 and t, respectively

$\theta = (N_t - N_0) / t$, where $N$ refers to NH$_4^+$ concentration.

$m$ is the gross NH$_4^+$ production (gross minerali~~s~~zation) calculated from equation I or II

Statistical analysis ~~of effect of climatic treatment ~~was conducted using SigmaPlot 11; t-test, by comparing drought (D) or warming (T) to control (C).

# 3. Results

## 3.1 Climate and soil properties

The efficiency of the climatic treatments varied between years since 1999 when the manipulations started. The drought treatment ~~conducted with~~imposed via precipitation reduction was within 6-29 % of annual precipitation from 1999 till 2011 (Kopittke et al., 2012). The precipitation exclusion in 2013 (from April 15 - June 28) prior to soil sampling reduced the annual accumulated precipitation till June 28 by 43% (Figure 2a). Furthermore, soil moisture index (average volumetric moisture content at 4-7 soil depth, relative to the wet month December 2012), decreased most during early summer ~~most~~ in drought treatment (Figure 2b). The gravimetric soil moisture of the sampled soil was significantly reduced in the drought treatment (P = 0.007; Table 1). Soil temperature in the top layer (0-7 cm) was enhanced during the ~~ose~~ 14~~5~~ years by 0.5 °C in the warming treatment compared to control (Figure 2c).

Total soil N was decreased by the drought treatment (P = 0.012; Table 1) and soil organic matter content tended to be reduced by drought, while both factors were unaffected by warming (Table 1). The soil ~~carbon~~ C content and C to N ratio was unaffected by climatic treatments (Table 1). The initial soil concentration of the sum of the five considered AAs was 0.0024 ± 0.0006 µg N $g^{-1}$ and total AA and individual AA concentrations were not significantly affected by climate manipulation (Figure 3).

### 3.2 N transformations

In control soil, gross ~~fAA~~FAA production was 76.2 µg AA-N $g^{-1}$ $day^{-1}$, which was ca. eight-fold larger than the gross N mineralization rate of 10.0 µg N $g^{-1}$ $day^{-1}$ (Table 2). ~~fAA~~FAA mineralization ($NH_4$-N production rate directly from ~~fAA~~FAAs) was 3.4 µg N $g^{-1}$ $day^{-1}$ in control, representing 34% of the total gross N mineralization (Table 2). In drought treatment FAA mineralization was reduced (P = 0.006; Table 2), and gross FAA production seemed to decline with drought and warming (Table 2) though, due to limited amount of replicates this could not be test statistically.~~In the drought treatments only fAA mineralization was significantly reduced (P = 0.006; Figure 3), but also gross fAA production declined at drought and warming (Table 2).~~ AA turnover time ranged between 1 h (valine, C) and 32 h (leucine, T), while turnover time for $NH_4^+$ was 4.3 days in control (Table 2). ~~Turnover times were not significantly affected by climate manipulations.~~

## 4. Discussion

In heathland ecosystems, ~~fAA~~FAAs are only sparsely studied, but previous research suggested a wide concentration range in soil of 0.02 to 36 μg N g$^{-1}$ (Abuarghub and Read, 1988a; 1988b; Kielland, 1995; Finzi and Berthrong, 2005; Andresen et al., 2008; 2011). These studies ~~furthermore~~ showed that the ~~standing fAA~~FAA-N pool was in general smaller than the $NH_4^+$-N pool. The turnover time of ~~fAAs~~ FAAs in our study indicate a longer residence time compared with results from $^{15}$N labelling studies in forest litter and agricultural soils (0.5 to 1.5 h in Wanek et al., 2010; 3.5 h in Geisseler et al., 2012). Moreover, studies using $^{14}$C methodologies suggest a maximum turnover of 12 h (Jones et al., 2009; Farrell et al., 2014; Wilkinson et al., 2014).

The gross ~~fAA~~FAA production rates quantified in the current study~~-~~ can be considered as ~~have to be seen as~~ an indicator for total depolymerization, as the rates are only based on five AAs~~ only~~. Nevertheless, total ~~fAA~~FAA production rate was ca. 8 times larger than gross N mineralization (control plots), which ~~was also observed~~ is in line with observations by Wanek et al., (2010) and Wild et al., (2013). ~~Immobilization of fAAs via a direct uptake pathway by microbes is evident from previous dual-labelling studies at similar~~ ~~*Calluna*~~ ~~heathlands (Andresen et al., 2009; 2011), and is from our study further supported by the high fAA production and mineralization rates, and short AA turnover times. This supports the paradigm that the 'direct route' is the main pathway for N mineralization, whereby microorganisms take up fAAs and excrete excess N as~~ ~~$NH_4^+$~~ ~~(Barraclough, 1997; Schimel and Bennett, 2004).~~

~~fAA~~FAA mineralization, which was calculated based on the addition of 20 $^{15}$N-labelled AAs, was an important component of gross N mineralization. However, due to the addition of a large amount of AA-N, the ~~fAA~~FAA mineralization rates are potentially overestimated. Nevertheless, the contribution of ~~fAA~~FAA mineralization to total gross N mineralization of 18-41% was smaller than what has been found in other ‘mirror $^{15}$N experiments’ (39-100%; Table 3) in agricultural systems. This indicates a more complex mineralization dynamics in semi-natural ecosystems. ~~However, the fAA turnover was a dominant N flux also for this temperate heathland, as also suggested from $^{14}$C studies of fAA turnover in forest systems (Jones and Kielland, 2002; 2012).~~

Warming had, unlike our hypotheses, ~~only small~~no significant effect~~s~~ on any of the measured~~on gross~~ N ~~transformation~~ rates~~s~~, ~~however, the treatment was only aminimal~~ ~~w~~which may also be related to the warming of 0.5 °C, which~~-~~ was possibly too low to have a significant impact. ~~is probably below the impact temperature~~. One the other hand, this small warming was continuous~~ce 1999, so it was a long-term, consistent climatic warming~~ and long-term, climatic warming over more than 14 years. In contrast at a Danish heathland, using the same techniques of passive warming and precipitation removal, drought evidently reduced and warming increased gross N ~~transformation~~ mineralization and nitrification rates, (Larsen et al., 2011; Björsne et al., 2014). The weak responses of ~~N transformations~~rates in the present study, were obscured by the low number of replicates~~ion~~ and the ~~large~~ observed large variability. Moreover, for the drought treatment a rewetting of the soil was necessary prior to $^{15}$N label addition. Consequently, the findings for that treatment reflect~~s~~ the effect at the moment of re-wetting after severe drought rather than a direct drought effect.

An interesting contrasting response of the N cycle to drought and warming was related to the relative importance of ~~fAA~~FAA mineralization for gross N mineralization rate. While under drought ~~fAA~~FAA mineralization became relatively less important for total N mineralization, its importance was unchanged by warming. This implies that the various proteolytic enzymes involved in N mineralization are inhibited in the drought affected soil, and as the observed gross mineralization was not affected, other sources for ammonium became relatively more important than the amino acids. This together with the fact that drought treated soils had markedly smaller amount of total soil N percentage, reflected the many years of soil disturbance by severe droughts (Sowerby et al., 2008), combined with smaller organic matter input to the soils from the drought-inhibited vegetation. Down-regulation of N dynamics in drought treated ecosystems can be a temporary phenomenon, which is alleviated by peak rain events (Pulleman and Tietema, 1999; Chen et al., 2011) or by simultaneous warming (Björsne et al., 2014). However, changes in the N availability ~~for plants~~, as ~~we have~~ observed in this study, ~~potentially occurring at droughts, followed by sudden rain events during the main growing season,~~ is a potential realistic effect ~~could be part of a negative feedback effect during climate change~~during droughts, followed by sudden rain events in the main growing season ~~that threatens the heathland ecosystem functioning and diversity~~.

Overall, we conclude that N transformation processes ~~during~~ in response to drought ~~events~~ will shift towards a dominance of inorganic N ~~production~~ rather than organic N (*e.g.* FAA) production. ~~, with consequences for the N availability for vegetation at future frequent drought events.~~ We suggest further analysis of seasonal effects on these production rates, and a look into the combination of drought and warming treatments. ~~A resource based N niche differentiation of co-occurring species would result in a drought induced shift from species relying on free amino acid N uptake to species relying on inorganic N uptake (McKane et al., 2001; Nordin et al., 2004), which may threaten the heathland ecosystem.~~

## Author contributions

AT set up and maintained the long term field ~~manipulation~~ experiment; LCA carried out the fieldwork together with TR; LCA ~~made~~ carried out the $^{15}N$ labelling laboratory experiment; SB and LCA set up the GC-MS method under supervision of PB; calculations by TR, PB and LCA; LCA wrote the first draft of the paper, while all authors contributed to writing and interpretation of results.

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**Table 1**: Soil properties. Total soil nitrogen (N) and Carbon (C), soil organic matter (SOM) and gravimetric soil water content (GWC) in percentage of dry weight (%). Significant effect ($P < 0.05$) of treatment is indicated by asterisk (*) whereas ns is non-significant.

| | **Control** | **Drought** | **Warming** | **stat.** |
|---|---|---|---|---|
| Total N (%) | 0.38 ± 0.05 | 0.21 ± 0.03 | 0.43 ± 0.04 | D: *; T: ns |
| SOM (%) | 12.4 ± 2.8 | 7.6 ± 2.2 | 12.7 ± 2.1 | D: ns; T: ns |
| Total C (%) | 6.0 ± 1.0 | 3.9 ± 1.1 | 6.8 ± 1.0 | D: ns; T: ns |
| C/N | 19.3 ± 0.5 | 19.7 ± 0.6 | 19.2 ± 0.2 | D: ns; T: ns |
| GWC (%) | 5.4 ± 0.9 | 1.3 ± 0.4 | 6.5 ± 0.7 | D: *; T: ns |

**Table 2**: Nitrogen transformation rates and turnover times. Gross mineralization rate (*Eq. I* & *II*), free amino acid (~~fAA~~FAA) mineralization (*Eq. IV*), represented by average ± standard error. ~~fAA~~FAA production rate is the sum of the five AA, the two measurement points in square brackets, $NH_4^+$ turnover time (days) and turnover time of amino acids (hours). Significant effect ($P < 0.01$) of treatment is indicated by asterisk (**), ns. is non-significant, and nd. is non-determined.

| | **Control** | **Drought** | **Warming** | **stat.** |
|---|---|---|---|---|
| ~~fAA~~FAA production rate ($\mu g\ N\ g^{-1}\ day^{-1}$) | 76.2 [127.4; 25.0] | 27.0 [17.3; 36.6] | 43.4 [34.6; 52.1] | nd. |
| Gross mineralization rate ($\mu g\ N\ g^{-1}\ day^{-1}$) | 10.0 ± 2.9 | 11.2 ± 1.6 | 9.3 ± 4.1 | T: ns; D: ns |
| ~~fAA~~FAA mineralization ($\mu g\ N\ g^{-1}\ day^{-1}$) | 3.4 ± 0.2 | 2.0 ± 0.3 | 3.8 ± 0.2 | D:**; T: ns |
| Turnover time $NH_4$ (days) | 4.3 ± 2.2 | 3.2 ± 0.5 | 5.2 ± 3.1 | T: ns; D: ns |
| Turnover time ~~fAA~~FAA (hours) | 2.9 | 11.5 | 7.2 | nd. |

**Table 3**: Mirror $^{15}$N labelling experiments in literature recording proportion of gross N mineralization directly from amino acids. Reference to paper, ecosystem type, soil type, pH (all in $H_2O$), soil total C and *N*, type of amino acid label ($^{15}$N-enriched) in the experiment, incubation time, free amino acid mineralization rate, gross mineralization rate and proportion (α) of N mineralization from free amino acids.

| **Reference** | **Ecosystem** | **Soil (pH)** | **soil C and *N* (%)** | **$^{15}$N labelled amino acid** | **t (hour)** | **~~fAA~~FAA min. rate (μgN $g^{-1}$ $h^{-1}$)** | **gross min. rate (μgN $g^{-1}$ $h^{-1}$)** | **α (%)** |
|---|---|---|---|---|---|---|---|---|
| Barraclough 1997 | Agri. (wheat) | Sandy loam (6.0) | 1.05 ***0.07*** | Leucine | 6 | 1.61 | 2.28 | 71 |
| Barraclough 1997 | Agri. (wheat) | Sandy loam (6.0) | 1.05 ***0.07*** | Glycine | 6 | 6.24 | 8.94 | 70 |
| Hadas et al.1992 | Agri. | Chromoexert (7.8) | 0.82 ***0.08*** | Alanine | 7 | 4.93 | 5.59 | 88 |
| Hadas et al.1992 | Agri. | Camborthid (8.1) | 1.31 ***0.12*** | Alanine | 7 | 7.59 | 10.08 | 75 |
| Stange& Döhling 2005 | Agri. | Haplic Phaeozem | 2.1 ***1.7*** | Glycine | 6 | 3.50 | 5.10 | 69 |
| Geisseler et al. 2012 | Pasture~~, straw~~ | Anthrosol (7.2) | 1.44 ***0.15*** | Gly&Leu | 168 ~~(week)~~ | 0.62 | 1.60 | 39 |
| Geisseler et al. 2012 | Pasture~~, straw~~ | Cambisol (8.0) | 1.13 ***0.11*** | Gly&Leu | 168 ~~(week)~~ | 0.42 | 0.90 | 47 |
| Current study | Heathland | Haplic Podzol (3.9) | 6.0 ***0.31*** | AA mix | 7 | 0.140 | 0.420 | 34 |

## Figure Captions

**Figure 1:** Con~~s~~cept model of investigated N transformations in heathland soil by $^{15}$N tracer techniques; 1) free amino acid (~~fAA~~FAA) production ($^{15}$N-AA pool dilution), 2) ~~fAA~~FAA mineralization ($^{15}$N-$NH^+_4$ production from $^{15}$N-AA, $^{15}$N-tracing), 3) mineralization from other soil organic matter (not measured directly), 4) gross N mineralization ($^{15}$N- $NH^+_4$ pool dilution).

**Figure 2:** Climatic data for the treated plots control (black), drought (blue) and warming (red) **A**. precipitation (accumulated mm rainfall for the given month) **B**: soil moisture (index) for 0 to 5 cm depth; and **C**: temperature in the soil for 0 to 5 cm depth. This year drought treatment started April 15 and ended June 22 2013. Soil was sampled from all plots on June 23.

**Figure 3** Initial amino acid concentrations for: a) alanine, b) valine, c) leucine, d) isoleucine and e) proline; in a heathland soil exposed to the climatic manipulations T = warming treatment,  D = drought treatment and C = control.