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Original article

Glycine uptake in heath plants and soil microbes responds to elevated temperature, CO_2 and drought☆

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ABSTRACT

Temperate terrestrial ecosystems are currently exposed to climatic and air quality changes with increased atmospheric CO_2, increased temperature and prolonged droughts. The responses of natural ecosystems to these changes are focus for research, due to the potential feedbacks to the climate. We here present results from a field experiment in which the effects of these three climate change factors are investigated solely and in all combinations at a temperate heath dominated by heather (*Calluna vulgaris*) and wavy hair-grass (*Deschampsia flexuosa*).

Climate induced increases in plant production may increase plant root exudation of dissolved organic compounds such as amino acids, and the release of amino acids during decomposition of organic matter. Such free amino acids in soil serve as substrates for soil microorganisms and are also acquired as nutrients directly by plants. We investigated the magnitude of the response to the potential climate change treatments on uptake of organic nitrogen in an *in situ* pulse labelling experiment with $^{15}N^{13}C_2$-labelled glycine (amino acid) injected into the soil.

In situ root nitrogen acquisition by grasses responded significantly to the climate change treatments, with larger ^{15}N uptake in response to warming and elevated CO_2 but not additively when the treatments were combined. Also, a larger grass leaf biomass in the combined *T* and CO_2 treatment than in individual treatments suggest that responses to combined climate change factors cannot be predicted from the responses to single factors treatments.

The soil microbes were superior to plants in the short-term competition for the added glycine, as indicated by an 18 times larger ^{15}N recovery in the microbial biomass compared to the plant biomass. The soil microbes acquired glycine largely as an intact compound (87%), with no effects of the multi factorial climate change treatment through one year.

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1. Introduction

Natural ecosystems respond to changes in air and soil temperature, atmospheric CO_2 concentration and drought, with consequences for biological processes and functioning of plants and soil microbes. According to extrapolations and models reported by IPCC, air temperatures may increase by 0.1 °C for each future decade, and the increase in CO_2 concentration of the atmosphere will depend on the actual stabilization scenarios achieved. Furthermore, the precipitation patterns will change, with expected extended summer drought periods in Denmark (IPCC, 2007; Danish Meteorological Institute, 2009). Investigations of the combined effects of increased temperature, CO_2 and drought are necessary to reveal the ecosystem responses to future climate changes (Beier et al., 2004; Finzi et al., 2006; Luo et al., 2008; Mikkelsen et al., 2008). There are few field experiments in which the combined effects of CO_2 and warming have been studied (Hovenden et al., 2008; Pregitzer et al., 2008; Ainsworth and Long, 2005), and none which combine these factors with drought.

Nitrogen availability may control plant biomass responses to climate changes, through the mechanism of increasing N demand by the increasing biomass, leading to possible progressive nitrogen limitation of the biomass response (Finzi et al., 2006; Hungate et al., 2006; Norby and Iversen, 2006; Sokolov et al., 2008). To study this, plant acquisition of N, soil nutrient availability (Hovenden et al., 2008) and mineralisation and nitrification (Emmett et al., 2004; Niklaus et al., 2007) must be investigated.

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The current study presents data on plant and microbial uptake of nitrogen from the amino acid glycine, and soil microbial and plant biomass responses to the factors warming, elevated CO_2 and drought in a temperate heathland after one year of climate treatments (Mikkelsen et al., 2008).

Soil microorganisms and plants acquire nitrogen from both inorganic (NO_3^- and NH_4^+) and organic sources (amino acids), and acquire intact amino acids (Näsholm et al., 1998; Bardgett et al., 2003; Andresen and Michelsen, 2005; Hofmockel et al., 2007; Bardgett et al., 2003; Hofmockel et al., 2007). The free amino acids in the soil pore water originate partly from plant root exudation (Lesuffleur et al., 2007; Ström and Christensen, 2007) and partly as leachates from decomposing organic matter (Bengtson and Bengtsson, 2007). Amino acids in the soil function both as nitrogen sources and as labile carbon substrates for soil microorganisms (Schimel and Bennett, 2004; Ström and Christensen, 2007; Vestergård et al., 2008). Many studies of short-term competition for labelled nitrogen sources have shown that soil microorganisms take up far more N than plants do, irrespective of N-form (Schimel and Chapin, 1996; Sorensen et al., 2008; Andresen et al., 2008).

Plant uptake of organic nitrogen has never been studied under the full factorial combination of the climatic changes: elevated CO_2, elevated temperature and drought. Single-factor investigations have shown highly variable responses in root nutrient uptake to elevated CO_2 reflecting differential responses in growth of the plants (Fig. 1), while plant physiological processes such as photosynthetic rate consistently increase and tissue N-concentration decrease in response to elevated CO_2 (Bassirirad, 2000; Paterson et al., 1999; Lutze and Gifford, 2000; van Heerwaarden et al., 2005; Chen et al., 2007). Plant nitrogen uptake responses to drought has to our knowledge not been investigated previously, but plant biomass and growth was reduced (Gordon et al., 1999; Peñuelas et al., 2004) or non-responsive (Britton et al., 2003) in field scale drought manipulations (Fig. 1).

Warming stimulates various parameters associated with root N uptake kinetics. Increased N acquisition may be a consequence of changed root transport properties (Clarkson and Warner, 1979), and changed fluidity of the phospholipids in root cell plasmalemma (Pike and Berry, 1980). Root biomass, depth distribution and morphology respond differentially to warming (Björk et al., 2007) and furthermore, NO_3^- uptake capacity is highly modulated by the N status of the roots or the whole plant (Bassirirad, 2000). Consequently, the response of the plant root N uptake to warming is a combined effect of changes in root properties, root biomass and root growth, combined with nutrient status and seasonal developmental stage.

Root exudation may respond to climate change in the same direction as photosynthesis and plant production (Gill et al., 2002; Johnson and Pregitzer, 2007; Lesuffleur et al., 2007) (Fig. 1) and soil concentrations of amino acids (e.g. glycine, one of the most abundant amino acids in heath soil (Abuarghub and Read, 1988)) may therefore increase as a consequence of elevated temperature and CO_2. Hence, in this experiment we were motivated to investigate the acquisition and partitioning of glycine between plants and soil microorganisms and responses to changes in atmospheric CO_2, temperature and prolonged drought. Glycine was dual-labelled with the stable isotopes ^{15}N and ^{13}C and injected into the soil *in situ* in the climate change experiment, and the uptake in plants, mosses and soil microorganisms was traced.

As direct and indirect responses to the climate change factors we hypothesised that:

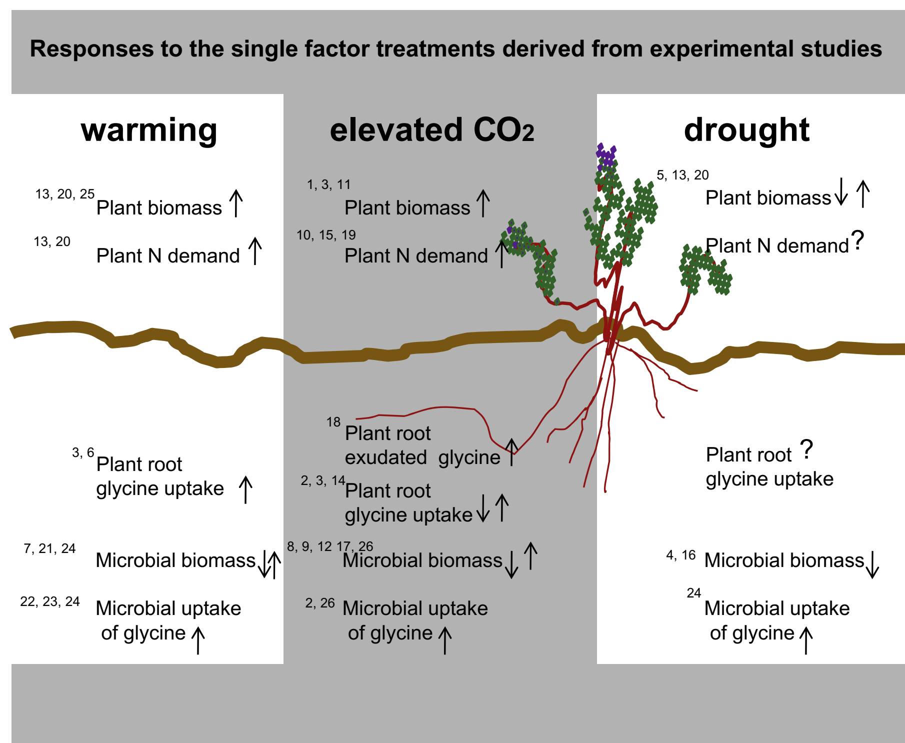

Fig. 1. Responses to single-factor treatments derived from experimental studies. Arrows indicate direction of response, ? indicates that no study was made. References: 1 Ainsworth and Long (2005); 2 Barnard et al. (2006); 3 Bassirirad (2000); 4 Bottner (1985); 5 Britton et al. (2003); 6 Clarkson and Warner (1979); 7 Clemmensen et al. (2006); 8 de Graaff et al. (2006); 9 Finzi and Schlesinger (2003); 10 Finzi et al. (2006); 11 Gill et al. (2002); 12 Gill et al. (2006); 13 Gordon et al. (1999); 14 Hofmockel et al. (2007); 15 Hungate et al. (2006); 16 Jensen et al. (2003); 17 Johnson et al. (2004); 18 Johnson and Pregitzer (2007); 19 Norby and Iversen (2006); 20 Peñuelas et al. (2004); 21 Rinnan et al. (2006); 22 Rinnan et al. (2007); 23 Sorensen et al. (2008); 24 Sowerby et al. (2005); 25 van Wijk et al. (2003); 26 Zak et al. (2000).

- Soil microorganisms would in the short term acquire more of the added glycine than plants, and microbial acquisition of glycine would increase in response to warming and elevated CO_2 and decrease in response to drought.
- Plant biomass and N demand would increase in response to warming and elevated CO_2. This would lead to increased ^{15}N uptake, increased N pool in the plant and to lower concentration of ammonium and nitrate in the soil. By contrast, drought would decrease plant N acquisition. DOC would increase in response to enhanced plant productivity caused by warming and elevated CO_2.
- Additive effects of treatment factors were expected. Hence, we expected that there would be interactive effects with responses in treatment combinations predictable from single-factor responses.

2. Methods

2.1. The field site

The experiment took place at the site of the CLIMAITE experiment (Mikkelsen et al., 2008) at Brandbjerg (55°53′N 11°58′E) c. 50 km NW of Copenhagen, Denmark. The site was a managed, dry, temperate heath on a hilly nutrient-poor sandy deposit, with an organic layer of c. 5 cm depth and a pH of about 5. The vegetation was dominated by *Calluna vulgaris*, *Deschampsia flexuosa* and *Festuca ovina* accompanied by heathland mosses and herbs. The average annual precipitation was about 600 mm and the average temperature was 8 °C (Danish Meteorological Institute, 2009).

2.2. The climate change manipulations

The climate manipulations started October 2005 (Mikkelsen et al., 2008) and consisted of eight treatments: plots with elevated temperature (*T*), extended summer drought (*D*), elevated atmospheric CO_2 concentration (CO_2), all combinations of these treatments (*TD*, *T*CO_2, *D*CO_2 and *TD*CO_2) and untreated reference plots (A). All treatments were replicated 6 times. The field site covered an area of about 2 ha and the experimental plots were distributed in 12 seven meter diameter octagons arranged pair-wise in six blocks, one octagon exposed to elevated CO_2 and one octagon at ambient CO_2 in each of the six blocks. Each octagon comprised four plots with the treatments drought or elevated temperature solely or in combination, and a non-warmed, non-drought plot. The temperature was increased by passive night-time warming, by means of low automatic curtains automatically removed during rain events. The drought was imposed also with automatic curtains that automatically unfold during rain events. The atmospheric CO_2 was increased by a regular FACE technique including feedback control on CO_2 concentrations, wind speed and wind direction. The temperature increase in 2 cm soil depth averaged 1 °C, and the CO_2 concentration in the FACE plots was 510 ppm. The drought period started in late June 2006 and continued for 5 weeks until early August when soil water reached c. 0.05 m^3 m^{-3} water in the top 20 cm of the soil. For further information about the experimental design see Mikkelsen et al. (2008).

Each of the 48 plots of the climate treatment experiment had temperature probes installed at 5 cm depth in the soil, at the soil surface, and in the vegetation canopy at 20 cm height, each recording temperature on an hourly basis. TDR probes were also installed at 0–20 cm depth and 0–60 cm depth for registration of soil water content on an hourly basis. In addition, the water content of the soil samples from the depths 0–5 cm, 5–10 cm and 10–15 was measured once, by drying the soil for two days at 80 °C. Cups for collection of precipitation water were installed on two masts at the field site.

2.3. In situ injection

In each of the 48 plots, an area of 80 × 80 cm was chosen prior to the start of the climate treatments to contain an approximately equal amount of *C. vulgaris* (evergreen dwarf shrub) and grasses (mainly *D. flexuosa*). Within each of these areas, a plot of 20 × 20 cm was labelled with stable isotope $^{15}N^{13}C_2$-glycine. The labelling solution was re-demineralised water with dual-labelled ^{15}N and ^{13}C (U–$^{13}C_2$, 98%; ^{15}N 98%) glycine, H_2NCH_2COOH. Each plot received 0.1 l of re-demineralised water with 0.027 g glycine, corresponding to 130 mg N m^{-2}. The label was injected into the soil just below the soil surface with a syringe at 20 grid points within the 20 × 20 cm plots. The glycine concentration abundant in the soil prior to labelling was presumably close to that measured one year earlier at the field site: 0.197 μg N g^{-1} SOM ± 0.052. Consequently, addition of glycine enhanced the soil concentration of glycine 200-fold, and increased total amino acid N five-fold (total amino acid N was 25.7 ± 4.5 g N m^{-2} in August 2005 at the field site ($n = 12$)), which is unavoidable if uptake of label in plants and microbes is to be determined with sufficient precision. For practical reasons the experimental work was divided in two series. The first series started September 26th using block 1–3 and the second series started October 3rd using block 4–6.

The local climate during the labelling experiment (September 23rd–October 3rd 2006) was not stable (Fig. 2). Small rain events after both series of labelling prior to harvest ensured equal conditions for label distribution in the soil, and caused a small drop in temperature and increases in soil water content. At the days of labelling, the warming treatment increased the canopy temperature by 0.7 °C (n.s.) and the soil temperatures at 0 cm and 5 cm depth by: 0.8 °C ($P < 0.0001$) and 0.8 °C ($P < 0.0001$), at first labelling series, and by: 0.3 °C (n.s.), 0.5 °C and 0.5 °C (both $P < 0.0001$) at the second labelling series. The soil water (TDR) showed marginal effects residing from the previously imposed summer drought at first labelling series with a decrease of 0.01 m^3 m^{-3} ($P < 0.0001$). The soil water in samples from 0 to 5, 5 to 10 and 10 to 15 cm depth (Fig. 2) was not significantly affected by the climate treatments.

2.4. Plant biomass and soil sampling

One day (24–26 h) after labelling with $^{15}N^{13}C$-glycine, representative shoots from aboveground (down to soil surface) vegetation was sampled within the 20 × 20 cm plots, of *Calluna*, *Deschampsia* (including leaf meristem) and mosses (mostly: *Hypnum cupressiforme*, *Pleurozium screberi* and *Dicranum scoparium*). Additionally, one day after labelling, soil cores were sampled from the soil surface (including the litter layer) and down to 15 cm depth. Three soil cores were taken from each plot and divided into three soil depths: 0–5 cm, 5–10 cm and 10–15 cm. The subsamples were mixed to a composite sample from each depth and immediately sorted into soil and roots. The samples were kept on ice until further processing. All plant material (roots and shoots) was washed with 0.5 mM $CaCl_2$, frozen and freeze dried. Within 48 h, a subsample of the fresh soil from each plot was extracted with re-demineralised water (1:5) on a shaker for 1 h and another set of subsamples was vacuum-incubated with chloroform for 24 h to release microbial C and N (Joergensen and Mueller, 1996; Brookes et al., 1985) before extraction with water as above. A third subsample of the sorted and sifted soil was freeze dried and weight difference was used for estimating soil water content. Just before the labelling was performed, additional soil samples and plant shoot samples were taken in adjacent subplots within the climate treated plots to obtain $\delta^{15}N$ and $\delta^{13}C$ natural abundances from all the investigated fractions. The same procedures as for the labelled samples were followed a few days

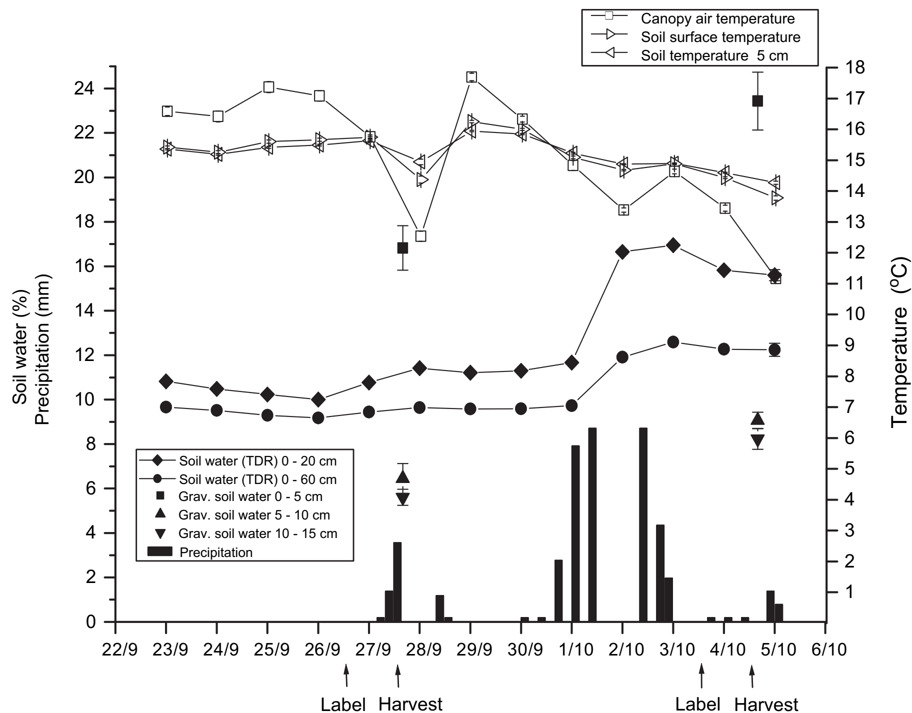

Fig. 2. Soil water and temperature as mean over all treatments (error bars are standard error) in late September and early October 2006. Canopy, soil surface and soil (5 cm depth) temperature (°C) measured with temperature probes. Soil water (%) in 0–20 and 0–60 cm depth was measured with TDR probes. Gravimetric soil water in 0–5 cm, 5–10 cm and 10–15 cm depth was measured in soil samples dried at 80 °C. Precipitation (mm) is by 4 h sums. The two series of the labelling experiment (divided equally with respect to treatment): Label and Harvest, are indicated with arrows. First series block 1–3 second series block 4–6.

earlier, before the isotope label was brought to the site, to avoid cross-contamination with ^{13}C and ^{15}N labelled samples.

One week after labelling, all the remaining aboveground plant material was sampled from the plots in order to obtain plant biomass. The *Calluna* material was sorted into green shoots with green leaves attached, coarse (non-green) branches, coarse roots (>0.5 mm) and fine roots (<0.5 mm) and the grasses were sorted into leaves, coarse (>1 mm), and fine roots (<1 mm). Mosses and aboveground litter (mainly of grasses, but also of *Calluna*) constituted additional fractions.

2.5. Chemical and isotopic analysis

The soil extracts were spectrophotometrically analyzed for NH_4^+ (indophenol-blue reaction) with a Hitachi U 2010 spectrophotometer and for NO_3^- with a Tecator FIAstar analyzer. Part of the extract was digested with H_2SeO_3, H_2SO_4 and H_2O_2 and analyzed as above to yield total dissolved N (TDN), with DON (dissolved organic nitrogen) = TDN − total mineral N. Total microbial N (MicN) was calculated as TDN in the fumigated samples minus TDN in the non-fumigated samples, using 0.4 as the extractability factor (Jonasson et al., 1996; Michelsen et al., 1999; Schmidt et al., 1999). Total microbial C (MicC) was calculated as DOC in the fumigated samples minus DOC in the non-fumigated samples, using 0.45 as the extractability factor (Schmidt et al., 2000).

For the $^{15}N/^{14}N$ and $^{13}C/^{12}C$ isotope ratio analysis of the fumigated and non-fumigated soil extracts, the extracts were freeze-dried in a small vial containing a quartz filter (Quartz microfiber filters QMA Whatman, free of C and N) used to collect the freeze-dried extract, and with a parafilm lid with a small hole. Filters, dried ground soil and plant material were analyzed with a Eurovector CN analyzer coupled to an Isoprime isotope ratio mass spectrometer. Plant material calibrated against certified IAEA standards was used as working standards.

2.6. Calculations and statistics

The ^{15}N enrichment of the plant material is reported as excess mole per gN of the material and ^{15}N and ^{13}C enrichments of the microbial biomass is reported as mole per m^2 in excess of natural abundance ^{15}N and ^{13}C (Fry, 2006). In particular the plots with elevated CO_2 exhibited a change in ^{13}C natural abundance due to different isotopic composition of CO_2 in air and in fumigation CO_2, thus for all treatment combinations and each plant or soil fraction, the measured ^{15}N or ^{13}C contents were subtracted with plot specific values for isotope abundance for each sample component. The ^{15}N recovery was calculated as the percentage of total added ^{15}N label per m^2 recovered in the total dissolved N (TDN), total microbial N (MicN), total soil N pool and in the plant biomass per m^2.

Linear mixed models were applied to analyse the treatment responses using SAS 8.0. Random effect terms were block, treatment plot and octagons, respecting the nested structure of the design. Main effects terms were the treatment factors: CO_2, *T*, and *D*. All interaction terms between the factors CO_2, *D* and *T* were included. Soil water content was included as a covariate in all proc mixed analysis of soil variables. The models were gradually simplified, starting with the third order interaction, taking out non-significant terms until only significant ($P < 0.05$) or close to significant ($0.05 < P < 0.10$) terms remained. Homogeneity of variances was investigated with residual plots but no transformations were necessary (SAS Institute Inc., 2003).

3. Results

A minor part (2.3 ± 0.6 to 4.5 ± 1.7%) of the added ^{15}N was recovered in plants one day after labelling, while the large part (44 ± 7 to 120 ± 67%) was recovered in soil microbes from top and down to 15 cm depth. The recovery of ^{15}N in the microbial biomass

overall decreased significantly (both $P < 0.0001$) with depth (Table 1). The largest ^{13}C enrichment was in the top 0–5 cm depth, 30-fold higher than at 10–15 cm depth. The treatments did not affect microbial ^{15}N and ^{13}C enrichment (Fig. 3), though tendencies to interactions were found in 5–10 cm depth ($T^*D^*CO_2$: $P = 0.0639$ and T^*D: $P = 0.0785$, data not shown).

The overall microbial enrichment with ^{15}N and ^{13}C correlated significantly in 0–5 cm depth, with $^{13}C = 1.74 \cdot {}^{15}N$, $R^2 = 0.92175$ and $P < 0.0001$ (Fig. 3), corresponding to a nitrogen uptake with 87% in form of intact glycine. Microbial C and N both decreased with depth (both $P < 0.0001$) (Fig. 6d and Table 2) while microbial C:N ratio increased with depth ($P = 0.0038$). The microbial biomass had a C:N ratio of 6.3 in 0–5 cm depth, 11.2 in 5–10 cm depth and 9.2 in 10–15 cm depth. There were no significant effects of treatment on microbial parameters, although a tendency to a reduced microbial C in response to CO_2 was observed, except when combined with T (T^*CO_2: $P = 0.0620$ in 10–15 cm depth; Table 2).

Increased grass fine root ^{15}N uptake in the top 5 cm soil in T and in CO_2 plots was non-additive (T^*CO_2: $P = 0.0886$ and $T^*D^*CO_2$: $P = 0.0486$; Fig. 4a). In 5–10 cm depth, ^{15}N acquisition was larger in the CO_2 plots alone and in the plots with all three treatments combined ($T^*D^*CO_2$: $P = 0.0527$, data not shown). The grass fine root ^{15}N enrichment showed no effect of depth (Fig. 6b).

Calluna fine root ^{15}N enrichment was non-significantly reduced by D and T in 0–5 cm depth (T^*D: $P = 0.0578$, Fig. 4c). In 5–10 cm depth ^{15}N enrichment showed the same pattern in CO_2 plots (T^*D^*CO2: $P = 0.0202$, data not shown). ^{15}N enrichment in *Calluna* fine roots was reduced (non-significantly) with depth (Fig. 6c).

The N pool in the whole *Deschampsia* plant tended to decrease in response to warming (T: $P = 0.0553$, Table 2). Grass fine root N concentration decreased with depth effect ($P < 0.0001$; Fig. 6e). *Calluna* fine root N concentration decreased ($P = 0.0392$, Fig. 4d), and coarse root N concentration tended to decrease ($P = 0.0769$, data not shown) by warming in 0–5 cm depth. Grass fine root N-concentration showed the same pattern, although the N dilution by warming was confined to non-drought plots (T^*D significant, Table 2). At the 10–15 cm depth, however, grass fine root N concentration increased by warming ($P = 0.0139$, Table 2). N concentration in moss and grass shoots was not significantly affected by treatment (Table 2), while N concentration was reduced in the green fraction of *Calluna* shoots in all CO_2 plots, except when all treatments were combined (T^*CO_2: $P = 0.0276$, D^*CO_2: $P = 0.0657$ and $T^*D^*CO_2$: $P = 0.0281$; Table 2).

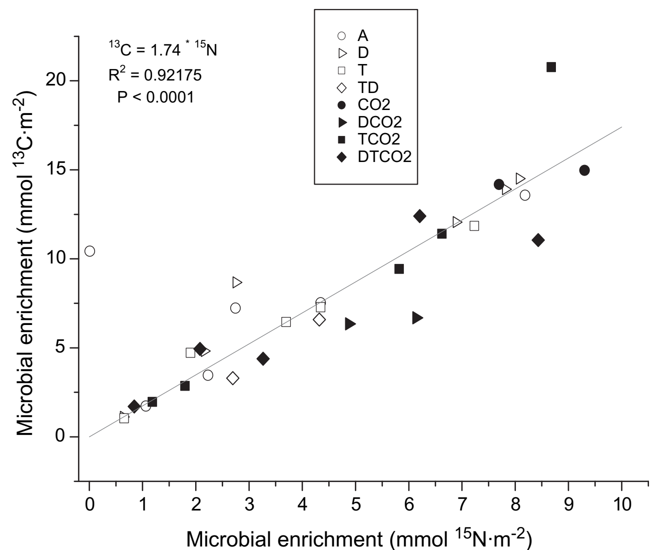

Fig. 3. ^{15}N enrichment (mmol^{15} N m^{-2}) versus ^{13}C enrichment (mmol^{13} C m^{-2}) in microbial biomass sampled at 0–5 cm depth one day after labelling with $^{15}N^{13}C_2$-glycine in the climate change treatments: A is ambient (no treatment), D is drought, CO_2 is elevated atmospheric CO_2 and T is warming. Linear regression is forced through zero for all treatments. No significant effects were found for the climate treatments.

The grass and *Calluna* root biomasses decreased significantly (both: $P < 0.0001$) by depth (Table 2). The fine root biomass of grasses summed for 0–15 cm depth was one order of magnitude larger than the *Calluna* fine root biomass summed for 0–15 cm depth, but the total (fine plus coarse, (data not shown)) root biomasses of the two species were approximately equal (Table 2), while the aboveground leaf biomass of *Calluna* in early October generally exceeded that of grass (Table 2). There was a main negative response in fine root biomass of grass to warming (in 0–5 cm depth, $P = 0.0107$ and $P = 0.0305$ in 0–15 cm depth), but no responses to treatment in *Calluna* fine root biomass (Table 2).

Leaf biomass of grasses was reduced by warming in non-CO_2 plots, while warming promoted grass leaf growth in plots with elevated CO_2 (T^*CO_2: $P = 0.0247$, Fig. 5a). *Calluna* green shoot biomass responded (non-significantly) in the opposite direction than grass leaf biomass (T^*CO_2: $P = 0.0578$, Table 2 and Fig. 5b). The ratio of leaf to branch in *Calluna*, which presumably is the most

Table 1
^{15}N recovery (%) in soil microbial biomass N, dissolved organic N and the whole plant (all shoot and root fractions and depths) one day after $^{15}N^{13}C_2$-glycine labelling. No statistical effects of treatments were found, except for moss ^{15}N recovery which showed a response to treatment (D: $P = 0.0006$ and D^*CO_2: $P = 0.0004$). Effect of depth is indicated with asterisks: ***for $P < 0.001$; *for $P < 0.05$. The treatments are: D for drought, T for elevated temperature, CO_2 for elevated CO_2; A is ambient conditions (control plots).

	Depth	Effect of depth	Treatment							
			A	*D*	*T*	*TD*	CO_2	DCO_2	TCO_2	$TDCO_2$
^{15}N recovery (%)										
Microbial N	0–5 cm	***	35.7 ± 13.7	54.5 ± 15.3	89.1 ± 49.1	36.9 ± 10.7	62.3 ± 16.0	59.3 ± 4.0	56.5 ± 13.6	110.2 ± 63.6
	5–10 cm		10.7 ± 5.6	10.7 ± 4.6	8.1 ± 3.2	6.3 ± 2.2	10.9 ± 4.8	5.7 ± 2.8	2.9 ± 1.1	8.9 ± 3.2
	10–15 cm		3.4 ± 2.2	1.9 ± 1.7	0.1 ± 0.1	0.7 ± 0.4	1.1 ± 1.1	0.6 ± 0.4	0.4 ± 0.3	0.8 ± 0.6
Total dissolved N	0–5 cm	*	0.13 ± 0.08	0.03 ± 0.02	0.03 ± 0.01	0.38 ± 0.37	0.10 ± 0.05	0.10 ± 0.09	0.05 ± 0.03	0.09 ± 0.05
	5–10 cm		0.00 ± 0.00	0.01 ± 0.01	0.16 ± 0.13	0.03 ± 0.03	0.02 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.03 ± 0.02
	10–15 cm		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Total soil	0–5 cm	***	43.0 ± 8.3	44.7 ± 13.7	64.6 ± 18.0	59.4 ± 22.2	73.0 ± 22.0	68.9 ± 6.3	60.3 ± 21.6	51.5 ± 8.0
	5–10 cm		5.5 ± 2.3	4.8 ± 1.8	7.0 ± 3.3	4.2 ± 1.8	7.3 ± 4.0	4.1 ± 0.9	3.6 ± 0.5	6.7 ± 1.9
	10–15 cm		6.8 ± 4.6	1.1 ± 0.9	1.2 ± 0.9	0.4 ± 0.2	3.3 ± 2.0	0.7 ± 0.3	0.3 ± 0.0	0.3 ± 0.2
Deschampsia		–	1.4 ± 0.4	2.5 ± 1.1	2.5 ± 1.0	2.0 ± 0.6	3.6 ± 0.7	3.4 ± 1.0	2.0 ± 0.4	2.5 ± 0.6
Calluna		–	0.8 ± 0.4	1.3 ± 0.5	1.4 ± 0.7	0.7 ± 0.2	0.8 ± 0.2	1.3 ± 0.4	0.6 ± 0.1	0.7 ± 0.3
Mosses		–	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Total experiment (soil + plant)		–	57.6 ± 15.9	54.4 ± 18.0	76.6 ± 23.9	66.6 ± 25.0	88.1 ± 28.8	78.2 ± 9.0	66.9 ± 22.7	61.6 ± 11.0

Table 2
Ecosystem properties (mean ± s.e.) after one year of climate treatments. Statistical significant effects from proc mixed model analysis of variances for the main effects: *D*, *T* and CO_2 and the interactions D^*T, D^*CO_2, T^*CO_2 and $D^*T^*CO_2$ are indicated if $P < 0.05$; and in brackets if $P < 0.1$. Effect of depth is indicated with asterisks: ***for $P < 0.001$; **for $P < 0.01$; n.s. is non-significant. The treatments are: *D* for drought, *T* for elevated temperature, CO_2 for elevated CO_2; *A* is ambient conditions (control plots).

	Depth	Effect of depth	Treatment								Main effects and interactions
			A	*D*	*T*	*TD*	CO_2	DCO_2	TCO_2	$TDCO_2$	
Biomass (g m^{-2})											
Calluna green shoot	Above gr.	–	286 ± 75	190 ± 47	378 ± 131	437 ± 135	305 ± 60	355 ± 94	290 ± 66	257 ± 46	(T^*CO_2)
Calluna coarse shoot	Above gr.	–	428 ± 139	342 ± 125	405 ± 110	552 ± 200	290 ± 83	418 ± 117	290 ± 76	393 ± 93	
Mosses	Above gr.	–	196 ± 51	141 ± 41	124 ± 65	204 ± 68	329 ± 144	131 ± 72	94 ± 82	120 ± 59	
Litter	Above gr.	–	637 ± 79	1030 ± 119	595 ± 140	646 ± 55	875 ± 280	605 ± 79	1149 ± 466	876 ± 145	
Deschampsia fine root	0–5 cm	***	266.0 ± 51.5	396.4 ± 46.4	185.6 ± 69.5	295.6 ± 8.9	451.3 ± 96.6	493.7 ± 114.9	318.4 ± 56.1	299.9 ± 45.0	*T*
	5–10 cm		11.0 ± 3.7	49.1 ± 17.7	47.7 ± 28.1	28.2 ± 4.6	36.1 ± 8.2	23.8 ± 4.0	53.7 ± 29.5	36.0 ± 8.7	
	10–15 cm		7.0 ± 3.0	4.7 ± 1.4	6.2 ± 4.7	6.1 ± 1.6	5.6 ± 1.2	3.4 ± 1.5	3.7 ± 1.0	6.2 ± 3.8	
Calluna fine root	0–5 cm	***	32.2 ± 9.7	27.8 ± 11.7	32.2 ± 11.2	30.0 ± 10.7	29.1 ± 7.4	35.7 ± 13.0	29.5 ± 6.9	17.8 ± 6.8	
	5–10 cm		4.3 ± 2.9	4.6 ± 1.7	6.3 ± 2.9	6.3 ± 2.7	3.6 ± 1.1	5.0 ± 1.4	8.2 ± 1.0	2.4 ± 0.7	
	10–15 cm		4.3 ± 2.4	0.7 ± 0.4	9.8 ± 8.4	1.6 ± 0.7	0.5 ± 0.4	1.4 ± 0.9	2.3 ± 1.3	0.6 ± 0.2	
Total plant (g N m^{-2})											
Calluna			9.4 ± 2.6	10.3 ± 3.2	11.4 ± 3.0	11.4 ± 3.6	8.3 ± 1.9	9.1 ± 1.8	6.9 ± 1.3	8.6 ± 1.7	
Deschampsia			7.3 ± 2.1	9.6 ± 2.0	5.5 ± 1.9	6.2 ± 0.7	8.9 ± 2.0	10.3 ± 2.4	8.4 ± 1.4	7.6 ± 1.2	(*T*)
Nitrogen (%)											
Deschampsia shoot	Above gr.	–	1.58 ± 0.16	1.81 ± 0.18	1.46 ± 0.13	1.47 ± 0.14	1.30 ± 0.12	1.64 ± 0.33	1.44 ± 0.06	1.55 ± 0.09	
Calluna green shoot	Above gr.	–	1.61 ± 0.10	1.73 ± 0.07	1.67 ± 0.09	1.39 ± 0.07	1.38 ± 0.06	1.45 ± 0.05	1.44 ± 0.12	1.68 ± 0.12	T^*CO_2 $T^*D^*CO_2$ (D^*CO_2)
Calluna coarse shoot	Above gr.	–	0.78 ± 0.05	0.87 ± 0.10	0.78 ± 0.10	0.72 ± 0.05	0.68 ± 0.01	0.72 ± 0.08	0.72 ± 0.06	0.72 ± 0.05	
Mosses	Above gr.	–	1.54 ± 0.17	1.42 ± 0.11	1.62 ± 0.20	1.54 ± 0.39	1.48 ± 0.29	1.39 ± 0.40	1.35 ± 0.19	1.89 ± 0.33	
Deschampsia fine root	5–10 cm	–	0.61 ± 0.08	0.67 ± 0.13	0.66 ± 0.16	0.64 ± 0.07	0.55 ± 0.02	0.65 ± 0.09	0.73 ± 0.11	0.64 ± 0.04	
	10–15 cm	–	0.59 ± 0.06	0.48 ± 0.01	0.73 ± 0.15	0.61 ± 0.09	0.56 ± 0.06	0.55 ± 0.09	0.73 ± 0.09	0.64 ± 0.08	*T*
Calluna fine root	5–10 cm	–	0.72 ± 0.11	0.75 ± 0.12	0.60 ± 0.05	0.60 ± 0.04	0.71 ± 0.07	0.71 ± 0.07	0.75 ± 0.07	0.67 ± 0.02	
	10–15 cm	–	0.67 ± 0.09	0.57 ± 0.05	0.63 ± 0.13	0.60 ± 0.06	0.47 ± 0.03	0.66 ± 0.14	0.61 ± 0.05	0.54 ± 0.08	$T^*D^*CO_2$ (T^*CO_2)
Soil properties (μg g^{-1} SOM)											
NO_3^-–N	0–5 cm	n.s.	3.4 ± 2.6	1.0 ± 0.6	2.7 ± 2.3	8.7 ± 8.3	3.9 ± 2.3	2.5 ± 2.2	0.6 ± 0.2	5.1 ± 2.0	(T^*D)
	5–10 cm		2.4 ± 1.1	2.7 ± 1.3	9.8 ± 5.3	7.6 ± 4.9	0.8 ± 0.3	1.5 ± 0.7	1.9 ± 0.6	2.8 ± 1.0	CO_2 *T*
	10–15 cm		2.2 ± 0.9	4.6 ± 2.7	7.4 ± 2.4	3.4 ± 2.0	4.5 ± 1.7	5.4 ± 3.6	2.9 ± 0.9	4.9 ± 1.0	
NH_4^+–N	0–5 cm	***	10.1 ± 4.2	8.9 ± 3.8	8.2 ± 3.7	12.3 ± 6.2	11.6 ± 5.8	8.2 ± 5.1	8.6 ± 3.2	26.8 ± 15.2	
	5–10 cm		31.1 ± 12.5	25.3 ± 6.7	25.7 ± 6.3	30.7 ± 9.0	22.3 ± 6.7	26.1 ± 5.2	22.5 ± 5.3	23.4 ± 5.1	
	10–15 cm		19.4 ± 6.0	19.5 ± 5.3	11.0 ± 1.1	16.0 ± 5.3	20.4 ± 2.6	17.3 ± 5.6	20.3 ± 2.3	16.8 ± 1.9	
DON	0–5 cm	***	54.2 ± 4.2	56.8 ± 8.9	53.0 ± 13.7	119.6 ± 56.1	60.3 ± 11.2	48.0 ± 2.9	55.0 ± 3.1	47.5 ± 12.9	(D^*CO_2)
	5–10 cm		135.7 ± 25.2	277.9 ± 140.8	153.0 ± 39.9	171.4 ± 39.5	166.0 ± 32.0	202.5 ± 62.5	149.6 ± 26.3	135.9 ± 31.0	
	10–15 cm		140.7 ± 36.1	235.6 ± 37.0	208.7 ± 59.3	308.9 ± 137.7	457.6 ± 140.8	242.3 ± 59.8	322.3 ± 62.1	284.8 ± 86.7	
DOC	0–5 cm	**	934 ± 53	994 ± 165	820 ± 55	1073 ± 143	1034 ± 87	907 ± 68	1038 ± 65	1003 ± 83	D^*CO_2
	5–10 cm		1602 ± 273	1519 ± 163	1146 ± 208	1289 ± 130	1469 ± 129	1525 ± 171	1582 ± 110	1513 ± 143	(T^*CO_2)
	10–15 cm		2033 ± 485	7126 ± 5128	1483 ± 196	1879 ± 184	2321 ± 358	4239 ± 2157	1187 ± 126	1909 ± 218	
Microbial N	0–5 cm	***	675 ± 186	842 ± 66	1147 ± 407	844 ± 162	1084 ± 236	950 ± 169	896 ± 92	1085 ± 263	
	5–10 cm		571 ± 156	348 ± 104	494 ± 114	484 ± 79	396 ± 76	597 ± 251	516 ± 120	463 ± 82	
	10–15 cm		355 ± 81	298 ± 139	207 ± 99	227 ± 80	121 ± 81	244 ± 64	150 ± 97	96 ± 68	
Microbial C	0–5 cm	***	6109 ± 1065	6009 ± 480	5210 ± 696	6435 ± 658	5635 ± 1004	4198 ± 674	6340 ± 771	4944 ± 499	(D^*CO_2)
	5–10 cm		4116 ± 1257	3788 ± 1159	4887 ± 698	4840 ± 654	4108 ± 663	4235 ± 625	4243 ± 1013	4178 ± 675	
	10–15 cm		1977 ± 491	3583 ± 635	2372 ± 350	1567 ± 361	1284 ± 575	2747 ± 990	3283 ± 1213	3245 ± 943	(T^*CO_2) (D^*CO_2)

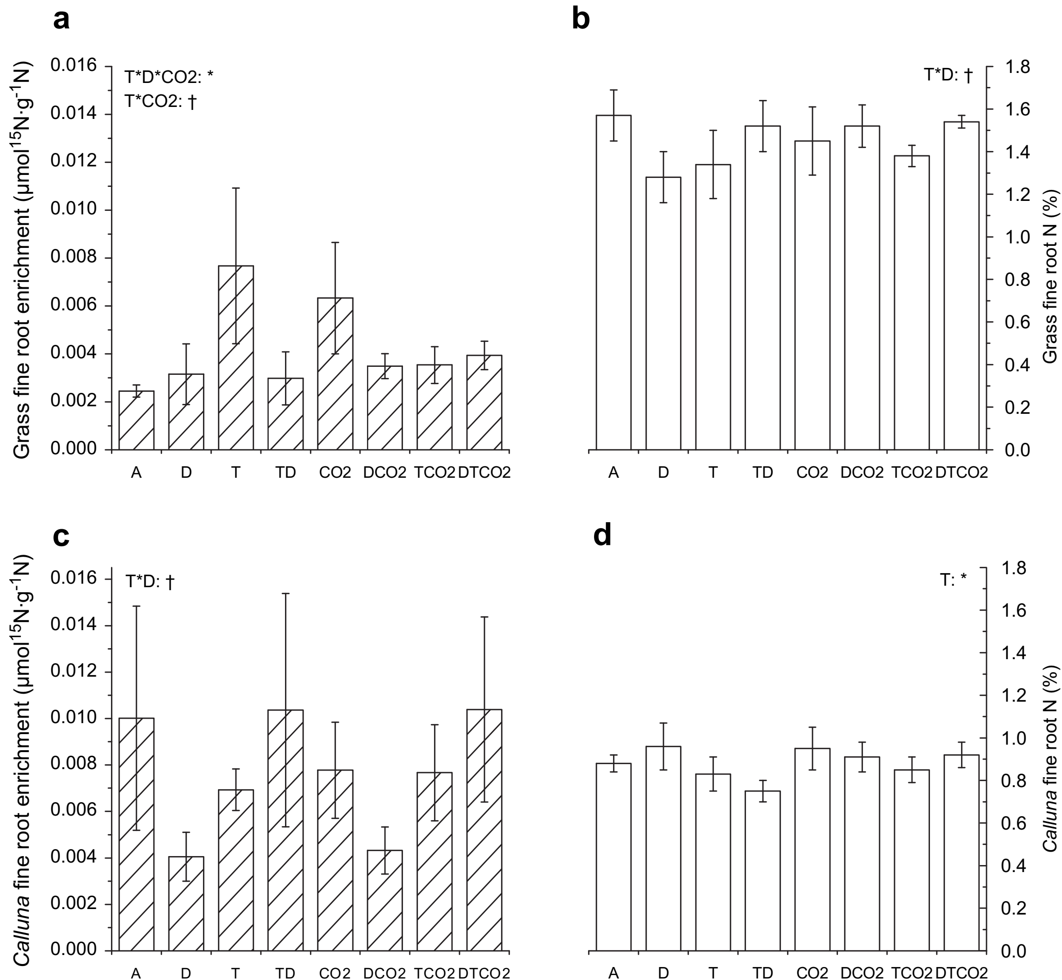

Fig. 4. a: Grass fine root ^{15}N enrichment (μmol ^{15}N g^{-1} N), b: grass fine root N (%), c: *Calluna* fine root ^{15}N enrichment (μmol ^{15}N g^{-1} N), d: *Calluna* fine root N (%) in 0–5 cm soil depth in the climate change treatments: *A* is ambient (no treatment), *D* is drought, CO_2 is elevated atmospheric CO_2 and *T* is warming. Statistical significant effects from proc mixed model analysis of variances for the main effects: *D*, *T* and CO_2 and the interactions D^*T, D^*CO_2, T^*CO_2 and $D^*T^*CO_2$ is indicated as follows: *: $P < 0.05$; †: $P < 0.1$.

response-sensitive variable for *Calluna* biomass, as it normalizes recent plant production relative to pre-treatment biomass, significantly showed larger production in warmed and in elevated CO_2 plots (T^*CO_2: $P = 0.0038$), but not an additive response in the combined *T* and CO_2 treatment (Fig. 5b).

Only moss ^{15}N recovery showed a response to treatment (*D*: $P = 0.0006$ and D^*CO_2: $P = 0.0004$; Table 1), with most ^{15}N in non-drought plots, perhaps because the mosses died during the imposed summer drought which caused a 25% lower moss biomass in drought than non-drought plots. Plant shoot and root samples also showed ^{13}C enrichment from the dual-labelled glycine, but overall this was non-significant and the ^{13}C results for plants are not presented.

Dissolved organic C (DOC) and N (DON) increased with depth and NH_4^+–N peaked in 5–10 cm depth (all $P < 0.05$; Table 2). DOC was significantly (and DON by tendency) increased by elevated CO_2 in 0–5 cm depth, except when CO_2 was combined with drought (DOC: D^*CO_2: $P = 0.0333$; DON: $P = 0.0710$). In 5–10 cm depth, there was a tendency to more DOC in CO_2 treatments when CO_2 was combined with *T* (T^*CO_2: $P = 0.0938$; Table 2). In 0–5 cm depth NO_3^-–N concentration was by tendency lower in response to *D* and *T* but larger when these treatments were combined and combined with CO_2 (T^*D: $P = 0.0929$, Table 2). In 5–10 cm depth NO_3^-–N concentration was lower in response to CO_2 (CO_2: $P = 0.0173$) and larger in response to warming (*T*: $P = 0.0490$; Table 2).

4. Discussion

The larger recovery of ^{15}N from the glycine label in soil microorganisms (36–110% ^{15}N recovery in 0–5 cm depth) compared to the low recovery in plants (2.4–4.7%) did indicate that the soil microorganisms in the short term acquired the added glycine more rapidly than the plants did. Similar results have been achieved in arctic heaths (Hobbie and Chapin, 1998; Andresen et al., 2008; Sorensen et al., 2008) and confirms our hypothesis that superior microbial N uptake also would be the case in temperate heaths. The ^{13}C and ^{15}N enrichment in microorganisms with the average ratio of 1.74 (Fig. 3), is close to the ratio of the labelled C and N in the injected glycine (which is 2) and corresponded to an 87% intact uptake. Hence, we conclude that soil microorganisms in this heath ecosystem acquire glycine as intact compounds, similar to findings in other ecosystem types (Nordin et al., 2004; Näsholm and Persson, 2001; Harrison et al., 2008).

The non-responsive microbial acquisition of the glycine label across treatments (Fig. 3), suggests that microbial glycine acquisition in this temperate heathland ecosystem was not affected by the climate change factors, and we could not confirm our hypothesis.

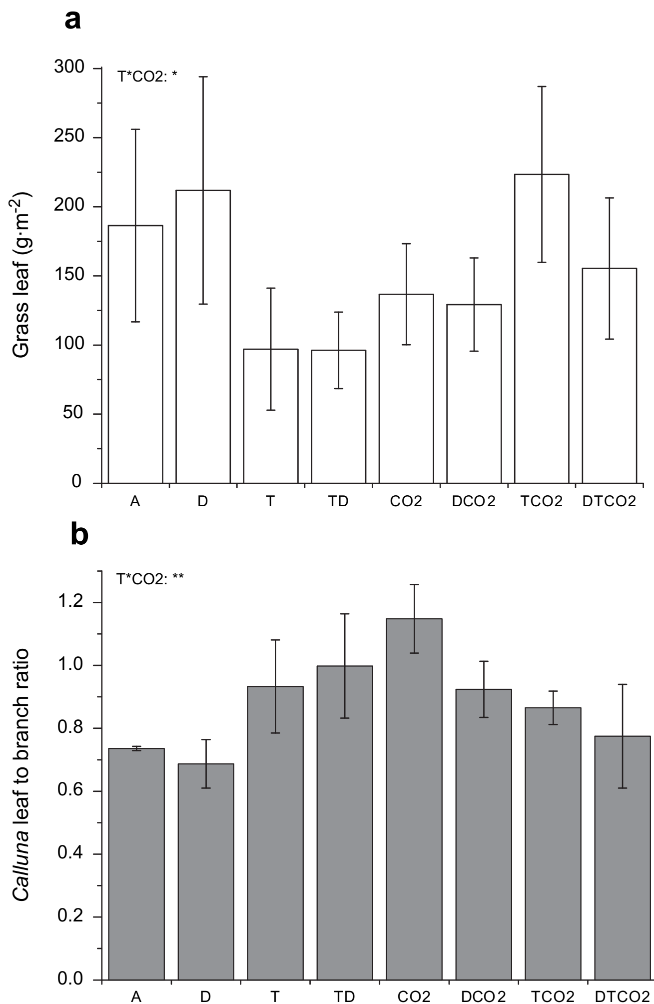

Fig. 5. Aboveground plant biomass 10th October 2006 harvested in 20 × 20 cm plots in the climate change treatments: *A* is ambient (no treatment), *D* is drought, CO_2 is elevated atmospheric CO_2 and *T* is warming. a: Grass green leaf and b: *Calluna* leaf to branch ratio. Statistical significant effects from proc mixed model analysis of variances for the main effects: *D*, *T* and CO_2 and the interactions $D*T$, $D*CO_2$, $T*CO_2$ and $D*T*CO_2$ is indicated as follows: ** indicates $P < 0.01$; *: $P < 0.05$.

This lack of microbe response to warming was also found in microbial uptake of $^{15}N^{13}C$-glycine in a subarctic heath (Sorensen et al., 2008), while microbial incorporation of leucine increased in warmed plots (Rinnan et al., 2007). However, the tendencies to decreasing microbial biomass C in response to elevated CO_2 in our study shows that the soil microorganisms did respond to the treatments, as has previously been observed in temperate soils exposed to drought and warming (Bottner, 1985; Jensen et al., 2003; Sowerby et al., 2005) and elevated CO_2 (Hu et al., 2001). Previously, no generality has been found for soil microbial biomass when exposed to elevated CO_2 alone (Zak et al., 2000; Finzi and Schlesinger, 2003; de Graaff et al., 2006; Gill et al., 2006; Johnson et al., 2004), but effects on community composition were suggested based on PLFA and DGGE assays (Chung et al., 2007; Drissner et al., 2007).

In contrast to microbes, there were no significant ^{15}N:^{13}C linear relation in grass and *Calluna* roots, suggesting that glycine was not acquired as an intact compound by plants, or that ^{13}C was so quickly respired that intact uptake could not be proven.

The *Calluna* aboveground biomass (Table 2) was within the range for *Calluna* in the mature phase in England and Wales (Milne et al., 2002). The observed increase in production in response to warming (Fig. 5b and Table 2) corresponded to observations of increased *Calluna* shoot length (Gordon et al., 1999) and *Calluna* growth in an investigation using the same methodological approach for warming and drought as in the current study (Peñuelas et al., 2004). In tundra ecosystems differential plant biomass responses to warming was found (van Wijk et al., 2003). For instance, in N. Sweden an increase of dwarf-shrub biomass, but not herb biomass, was observed in response to warming (Jonasson et al., 1999), while no such changes were observed in the Alaskan tundra (Hobbie and Chapin, 1998). Increased root biomass has previously been found in response to warming and to elevated CO_2 in temperate forests and grasslands (Volder et al., 2007; Pregitzer et al., 2008), leading to increased belowground carbon sequestration. Decreasing N concentration in leaves in response to warming was found in subarctic evergreen shrubs (Jonasson et al., 1999; Hansen et al., 2006), but in *Calluna* (Gordon et al., 1999; Peñuelas et al., 2004) an increase in shoot N concentration along with increased growth was found, this suggests an increased plant N demand. In the present study, the tendency to a decreased N pool in *Deschampsia* plants in response to warming may be a consequence of such an advancement of the inter-specific competition with *Calluna*. The larger nitrogen uptake potential by *Deschampsia* roots, traced by ^{15}N, in response to warming may then be a response to this directional competition (Britton et al., 2003) causing compensatory adjustments (Bassirirad, 2000).

The tendency to decreased *Calluna* root ^{15}N acquisition in response to drought reflects a decreased plant N demand (Gordon et al., 1999; Peñuelas et al., 2004), which was not reflected by biomass or N concentration. However, the absence of responses in biomass and nitrogen content may simply rely on the short duration of the climate change experiment.

C. vulgaris and *D. flexuosa* has not to our knowledge been investigated in field studies with elevated CO_2. The decrease in *Calluna* leaf N concentration in response to elevated CO_2 (Table 2) is probably a dilution effect (Paterson et al., 1999; Lutze and Gifford, 2000; van Heerwaarden et al., 2005; Chen et al., 2007), caused by increased photosynthetic carbon assimilation, as also suggested by the increase in *Calluna* leaf biomass and leaf to branch ratio in response to elevated CO_2. The observed soil NO_3^-–N decrease in response to CO_2 (Table 2) could reflect increased plant nitrogen N demand (Finzi et al., 2006; Hovenden et al., 2008), which is suggested by the large grass ^{15}N root acquisition in CO_2 plots (Fig. 4a). With the ^{15}N acquisition normalized to per gN in the root, the larger acquisition truly reflects a positive physiological response to warming and to elevated CO_2. In a pine forest ecosystem experiment, elevated CO_2 reduced the plant uptake of the amino acid alanine (Hofmockel et al., 2007), while in a mesocosm experiment with a grass, elevated CO_2 increased plant acquisition of ammonium ^{15}N (Barnard et al., 2006). Hence, elevated CO_2 may cause opposite and species specific changes in plant root nitrogen acquisition, dependent on plant nutrient status (Bassirirad, 2000).

The species specific plant biomass and N concentration responses to the climate change treatments may also reflect that the plants were in different stages of seasonal development at the end of the growing season, due to effects of the climate treatments. For instance, the autumnal decline in normalized difference vegetation index (NDVI) was delayed for poplar and aspen under elevated CO_2 (Taylor et al., 2008). The decrease in green leaf and root biomass and total plant N pool (Fig. 5a and Table 2) and the increase in deep root N concentration (Table 2) in grasses in response to warming, suggest such an advanced seasonal allocation and storage of N in the grass in response to warming. Consequently, the decrease in grass fine root biomass caused a displacement of functional rooting depth between the treatments.

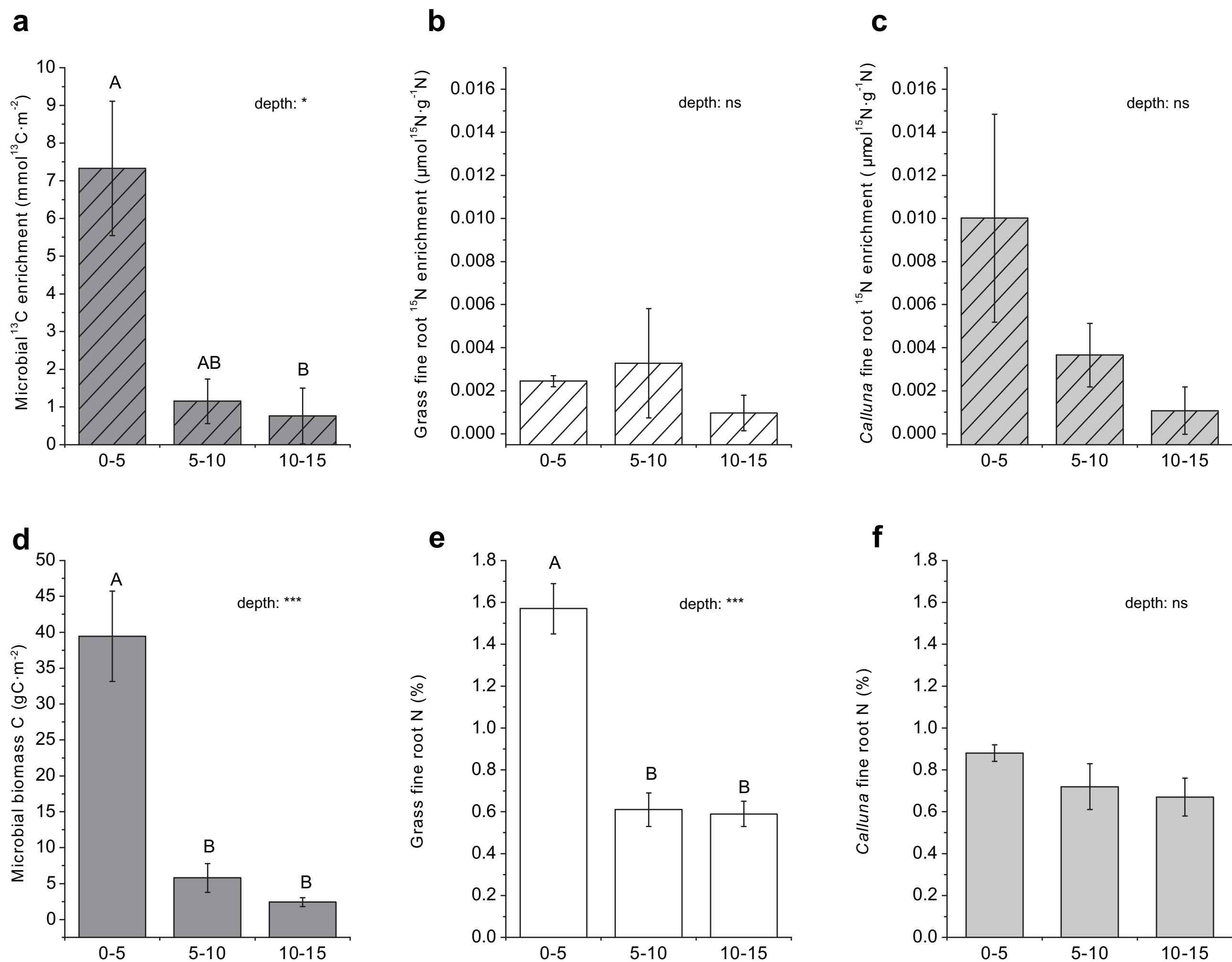

Fig. 6. The soil profile in 0–5 cm depth, 5–10 cm depth and 10–15 cm depth in ambient plots. Significant effects of depth are indicated: ns is non-significant, *for $P < 0.01$ and ***for $P < 0.001$. Different letters above bars indicate significant difference (Tukeys test, $P < 0.05$). a: microbial biomass ^{13}C enrichment (mmol ^{13}C m^{-2}), b: grass fine root ^{15}N enrichment (mmol ^{15}N g^{-1} DW), c: *Calluna* fine root ^{15}N enrichment (mmol ^{15}N g^{-1} DW), d: microbial biomass C (g C m^{-2}) e: Grass fine root N% and f: *Calluna* fine root N% one day after *in situ* labelling with $^{15}N^{13}C_2$-glycine.

The soil water content was stable over the period, and even over the different treated plots. Hence, it is reasonable to assume that the distribution and adsorption of the glycine label was even over all plots. The decreasing ^{15}N enrichment of plant roots with greater depth was accompanied by decreasing ^{13}C and ^{15}N enrichment of the microbial biomass and decreasing total soil ^{15}N recovery (Table 1), all indicating a lower concentration of the added label downwards, below the surface injection points. Furthermore, the decreasing plant root and soil microbial biomass, and the increasing microbial C:N ratio downwards, together with increasing dissolved organic C and N and NH_4^+–N concentration with greater depth, suggest a downwards decrease in live biomass and altered function of biota with decreased utilization of the labile substrates and nutrients (Andresen et al., 2008; Kemmitt et al., 2008).

The unavoidable dilution of the added isotope labelled glycine with abundant glycine in the soil solution may have distorted the responses, had these differed in the plots. Our hypothesis, that belowground carbon allocation in form of root exudation increased in response to elevated CO_2 (Johnson and Pregitzer, 2007), was supported by the increased DOC concentrations (Table 2). This, and the rapid and large microbial acquisition of the labelled glycine, supports that DOC is rapidly cycled under elevated CO_2 (Bengtson and Bengtsson, 2007).

5. Conclusions

The climate change factors caused changes in plant N concentration, biomass and plant N uptake patterns in the temperate heathland ecosystem.

- Soil microorganisms acquired the largest part of the added $^{15}N^{13}C_2$-glycine, largely as intact compounds (87%), with no significant effects of treatment.
- *Calluna* aboveground production increased in warmed plots and in elevated CO_2 plots, which caused a dilution of tissue nitrogen concentration.
- Grass fine root biomass, green leaf biomass and nitrogen pool decreased in warmed plots (without elevated CO_2).
- Increased plant N demand seen as larger grass fine root ^{15}N acquisition in warmed and in elevated CO_2 plots was non-additive in the combined treatment.
- The combined treatments in many cases responded in opposite directions to the single treatments: warming, CO_2

and drought. Hence, realistic climate change effects on important ecosystem properties are not predictable from single-factor experiments.

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