# Reduced N cycling in response to elevated $CO_2$, warming, and drought in a Danish heathland: Synthesizing results of the CLIMAITE project after two years of treatments

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## Abstract

Field-scale experiments simulating realistic future climate scenarios are important tools for investigating the effects of current and future climate changes on ecosystem functioning and biogeochemical cycling. We exposed a seminatural Danish heathland ecosystem to elevated atmospheric carbon dioxide ($CO_2$), warming, and extended summer drought in all combinations. Here, we report on the short-term responses of the nitrogen (N) cycle after 2 years of treatments. Elevated $CO_2$ significantly affected aboveground stoichiometry by increasing the carbon to nitrogen (C/N) ratios in the leaves of both co-dominant species (*Calluna vulgaris* and *Deschampsia flexuosa*), as well as the C/N ratios of *Calluna* flowers and by reducing the N concentration of *Deschampsia* litter. Belowground, elevated $CO_2$ had only minor effects, whereas warming increased N turnover, as indicated by increased rates of microbial $NH_4^+$ consumption, gross mineralization, potential nitrification, denitrification and $N_2O$ emissions. Drought reduced belowground gross N mineralization and decreased fauna N mass and fauna N mineralization. Leaching was unaffected by treatments but was significantly higher across all treatments in the second year than in the much drier first year indicating that ecosystem N loss is highly sensitive to changes and variability in amount and timing of precipitation. Interactions between treatments were common and although some synergistic effects were observed, antagonism dominated the interactive responses in treatment combinations, i.e. responses were smaller in combinations than in single treatments. Nonetheless, increased C/N ratios of photosynthetic tissue in response to elevated $CO_2$, as well as drought-induced decreases in litter N production and fauna N mineralization prevailed in the full treatment combination. Overall, the simulated future climate scenario therefore lead to reduced N turnover, which could act to reduce the potential growth response of plants to elevated atmospheric $CO_2$ concentration.

*Keywords:* climate driver interactions, C/N ratio, multifactor climate change experiment, $N_2O$, nitrogen cycling, nitrogen mineralization, soil fauna

*Received 16 July 2010 and accepted 14 August 2010*

## Introduction

Climate change with elevated atmospheric dioxide ($CO_2$), increased temperature and altered precipitation will fundamentally affect key drivers for ecosystem functioning and lead to adverse effects in terrestrial ecosystems across the globe (IPCC, 2007). These climate-driven changes may affect ecosystem functioning directly, e.g. through $CO_2$-stimulated photosynthesis and growth (de Graaff et al., 2006; Luo et al., 2006), warming-induced increased nutrient mineralization (Emmett et al., 2004) or drought-induced growth limitation (Penuelas et al., 2007). They may also act indirectly e.g. through impacts on species composition (Penuelas et al., 2007).

Growth in many natural and seminatural terrestrial ecosystems is limited by nitrogen (N) and characterized

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by strong competition for N between plants and the soil communities of fungi and bacteria (Marion et al., 1989; Jonasson et al., 1996). Consequently, ecosystem N availability is an important factor controlling the carbon (C) uptake in terrestrial ecosystems and therefore also plays a dominant role in controlling the feedback between the biosphere and the atmosphere in a warmer and more $CO_2$ enriched world. The theory of N as a limiting factor constraining the C uptake in terrestrial ecosystems in response to elevated $CO_2$ is conceptualized in the Progressive Nitrogen Limitation (PNL) framework (Luo et al., 2004) describing how increased C sequestration driven by elevated $CO_2$ requires increased access to N in order to balance the C input. Accordingly, without additional input of plant-available N, nitrogen will progressively limit further C uptake. But increased N availability has also been shown as a consequence of increased availability of labile soil C substrate (Zak et al., 1993) and increased soil moisture content due to improved plant water use efficiency (WUE) (Zak et al., 1993; Hungate, 1999), as well as increased N mineralization in response to warming (Emmett et al., 2004; Schmidt et al., 2004). The interaction between the C and N cycles clearly illustrates how sensitive ecosystems are to changes in input, pools and turnover of N and other nutrients, as well as changes in the water regime. This is particularly important for N-limited systems, such as natural and seminatural ecosystems with relatively low N input and internal turnover rates of the mineral N pool as fast as a few days (Davidson et al., 1990; Lipson & Näsholm, 2001; Schimel & Bennett, 2004; Vervaet et al., 2004). In systems with very high internal turnover rates of N, the N turnover has also been shown to be very sensitive to changes in natural climatic variations such as reduced water availability (Jamieson et al., 1999), potentially leading to a high sensitivity of these systems to climate-driven changes in the internal processes regulating the demand or supply of N.

Most of the experimental evidence of PNL and the role of N in constraining C uptake is based on modeling studies (Rastetter et al., 1997) or on experiments involving elevated $CO_2$ alone or in combination with various levels of N addition to demonstrate the role of N in stimulating $CO_2$ sequestration responses (Lutze & Gifford, 2000; Lutze et al., 2000; van Heerwaarden et al., 2005; de Graaff et al., 2006; Reich et al., 2006). However, in a future $CO_2$-enriched world, the N limitation and the $CO_2$ response in N-limited systems will to a large extent be regulated by the climate change-associated responses of the internal N cycle, which are not well captured in an experiment with elevated $CO_2$ alone. For instance, it has been demonstrated that warming can stimulate internal N mineralization, leading to in-

creased N availability (Emmett et al., 2004) and even increased N leaching (Lukewille & Wright, 1997; Schmidt et al., 2004). Correspondingly, changes in water availability, such as water limitations by drought, can reduce N mineralization and N availability (Emmett et al., 2004). On the other hand, increased water availability due to increased precipitation or to $CO_2$-stimulated increase in WUE can increase N mineralization and N availability under water-limited conditions (Hungate, 1999). Since future climate change will involve changes in all of these factors, it is important to understand the possible interactions between different climate drivers on N availability and limitation. Therefore, studies involving all factors simultaneously are needed.

We exposed a seminatural Danish heathland to elevated atmospheric $CO_2$ concentration, extended summer drought and increased temperature. These treatments served to mimic climate predictions for Denmark year 2075 as closely as possible (http://www.dmi.dk; Christensen & Christensen, 2007), except that our summer drought treatment is not accompanied by increased winter precipitation as the predictions suggest. In the present study, we aim to synthesize the overall impact on the N cycle after 2 years of treatments. We compare the responses of the three-factor treatment with responses to treatments including one, or pairwise combinations of the factors.

We anticipated an increased C to N ratio in plant tissue and litter in response to elevated $CO_2$ (Paterson et al., 1999; Lutze & Gifford, 2000; van Heerwaarden et al., 2005; Finzi et al., 2006; Chen et al., 2007; Hovenden et al., 2008). In the short term, we further expected that the increased water use efficiency under elevated $CO_2$ (Ainsworth & Long, 2005; Ainsworth & Rogers, 2007; Leuzinger & Körner, 2007; Albert, 2009) would stimulate plant growth and increase plant N demand and biomass, and that concomitant increased rhizodeposition would lead to increased microbial biomass, activity and mineralization (Hamer & Marschner, 2002; de Graaff et al., 2007). Warming was expected to increase plant growth and increase mineralization (Rustad et al., 2001), whereas extended summer drought was expected to reduce plant N demand and decrease N mineralization and nitrification rates (Emmett et al., 2004; Schmidt et al., 2004; Sowerby et al., 2008) as a product of direct or indirect effects on microbial activity. Assuming similar response strengths, as well as simple addition of the single treatment effects, we expected that drought effects would be reduced in combination with both warming and elevated $CO_2$. Consequently, increased N mineralization and plant growth in response to both warming and elevated $CO_2$ would dominate over decreases in N mineralization and plant

growth caused by drought in the full combination of treatments.

## Materials and methods

### Experimental site and climate change manipulations

The CLIMAITE experimental site is a dry, temperate heathland situated approximately 50 km NW of Copenhagen, Denmark (55°53′N, 11°58′E). The vegetation is dominated by the evergreen dwarf shrub *Calluna vulgaris* (L.), the perennial grass *Deschampsia flexuosa* (L.) and various mosses and herbs. The soil is a well-drained, nutrient-poor sandy deposit with a pH of 4–5 and an organic top layer ranging from 2 to 5 cm in depth. Long-term annual mean air temperature is 8.0 °C, annual mean precipitation is 613 mm (Danish Meteorological Institute, 2009, http://www.dmi.dk) and the total N bulk deposition at the site was $1.27 \pm 0.07$ and $1.35 \pm 0.04 \, \mathrm{g \, N \, m^{-2} \, yr^{-1}}$ in 2006 and 2007, respectively. The experimental area covers approximately 2 ha and consists of twelve 7 m diameter octagons laid out pair-wise in six blocks, i.e. $n = 6$. Each block consists of two octagons, one with ambient (A) atmospheric $CO_2$ and one receiving elevated atmospheric $CO_2$ concentration ($CO_2$, 510 ppm in a free air $CO_2$ enrichment (FACE) setup). Each octagon is separated into four plots receiving in addition to the ambient or elevated $CO_2$ either (1) 1 month summer drought (D) by rain-out shelters, (2) passive warming (T) of air and soil by nighttime warming curtains 50 cm above ground, (3) a combination of drought and warming (TD) or (4) no drought or temperature treatment. In total, the experiment provides a full-factorial design with all eight combinations of D, T and $CO_2$ and an untreated control for reference (A), i.e. $N = 48$. Details on the experimental setup are given by Mikkelsen *et al.* (2008).

The elevated $CO_2$ and warming treatments were initiated in October 2005 and the first summer drought was applied in July/August 2006. The temperature elevation, of warmed vs. un-warmed plots, achieved overnight in ideal conditions of low wind and a clear sky sometimes reached 3–5 °C in the air (20 cm) for periods of a few hours. Since the nighttime temperature elevation only partially persists into the daytime, the maximum temperature elevation on a daily average was lower, peaking at 1.5, 1.8 and 3.2 °C at 5 cm depth (soil), soil surface (litter layer) and 20 cm height (air), respectively (4 October 2005–31 December 2007). The passive nighttime warming method relies on capturing incoming energy and therefore the warming effect also varies with season. Consequently, the overall seasonal range of temperature elevation, given as the mean to maximum at 5 cm depth was higher in spring (0.6–1.5 °C), summer (0.5–1.3 °C) and autumn (0.3–1.0 °C), than in the winter (0.2–0.7 °C). The drought campaigns removed 52 and 94 mm of precipitation, resulting in peak reductions of soil water content of 11% and 13% v/v over 0–20 cm soil depth in 2006 (3–20 July and 26 July–4 August) and 2007 (21 May–22 June), respectively. The drought campaigns were stopped when soil water content reached about 5% v/v over 0–20 cm soil depth.

### N stocks in plants, litter, microbes and soil, and C/N ratios

The amount of aboveground plant dry matter was estimated by nondestructive point intercept measurements in two $50 \times 50$ cm subplots within each plot combined with a linear regression model linking point intercept measurements and biomass obtained from destructive harvesting of similar plots outside the treatment plots (Jonasson, 1988). To compensate for pretreatment differences between the initial biomass values in individual subplots and the subsequent development during the experiment, treatment effects on aboveground plant biomasses were normalized and scaled to the average biomass development in the ambient plots while maintaining the observed relative variance from the plot-scale point intercept measurements. Briefly, a regression was fitted between the pretreatment plant biomass and the treatment biomass at a given time point, within each treatment. The slope of the regression in the ambient treatment defined the ambient or control response; the normalized treatment effect was determined as the difference between the biomass predicted by the regression to the control response and the observed biomass for a given treatment.

All aboveground *Calluna* and *Deschampsia* plants, as well as mosses, were harvested from $20 \times 20$ cm subplots within the experimental plots in August 2007. The C and N concentrations measured in green tissue, flowers, wood/stem and roots or whole plants (mosses) (Andresen *et al.*, 2010a) were used to calculate C to N ratios. The N concentrations were used in combination with the nondestructive aboveground biomass estimates to calculate the total N stock of these pools. Concurrent with plant harvest, soil samples from each plot (0–10 cm soil depth) were taken to measure plant root biomasses, microbial biomass N and, soil organic matter N (SOM-N) and soil extractable $NO_3^-$, $NH_4^+$ and dissolved organic nitrogen (DON) (Andresen *et al.*, 2010a). Litter production by *Deschampsia* was estimated from point intercept-based difference between seasonal maximum and minimum biomasses of live *Deschampsia* leaves. Reproductive shoots were harvested after senescence and included. Litter N production was calculated by multiplying with the litter N concentration.

### Soil fauna

Soil cores within each plot (0–10 cm soil depth) were sampled in October and November 2007 for biomass estimation of protozoa/nematodes and enchytraeids/microarthropods, respectively. For estimation of protozoan biomass, a soil suspension was prepared by blending 5 g of soil with 100 mL of Amoeba Saline (AS, Page, 1988) for 1 min. Three-fold dilution series of the soil suspension were prepared in microtiter plates being inspected for protozoa after 1 and 3 weeks of growth at 15 °C with a modified version of the Most Probable Number method (Darbyshire *et al.*, 1974; Rønn *et al.*, 1995). Nematodes were extracted from between 10 and 11 g (fresh weight) of soil by a modified Baermann tray extraction method (Georgieva *et al.*, 2005). Samples were extracted for 48 h, and nematodes were then counted at $\times 40$ magnification using a dissecting microscope. Biomass N of protozoa and bacterivorous nema-

todes were calculated according to Stout & Heal (1967) and Sohlenius & Sandor (1987), respectively. N mineralization (excretion and turnover) by microfauna was calculated assuming bacterivore turnover rate of $0.16\,day^{-1}$ (Zwart *et al.*, 1994) for both nematodes and protozoans and setting yield ratios of protozoans and nematodes on bacteria to 0.4 and 0.04, respectively (Coleman *et al.*, 1978). The total biomass of enchytraeids was determined as described by Maraldo *et al.* (2008). The density of microarthropods was determined as described by Holmstrup *et al.* (2007), and the biomass of dominating taxonomic groups was estimated using representative values from Petersen & Luxton (1982). The pool of N contained in the biomass was calculated assuming N concentrations of 11.5% and 10% of dry weight in enchytraeids and microarthropods, respectively (Persson, 1983). The annual turnover rate of biomass N was assumed to be 1.2 for enchytraeids (Standen, 1973), and 1.8 for microarthropods (De Ruiter *et al.*, 1993). The annual mineralization (excretion and turnover) of $NH_4^+$-N by enchytraeids and microarthropods was estimated based on biomass as described by Persson (1983).

## N2O emissions and N2O reductase

Fluxes of $N_2O$ were measured with a static chamber method using white PVC chambers (height 15 or 45 cm depending on seasonal vegetation height) placed on metal collars ($60 \times 60$ cm) permanently installed in the soil. Enclosure times were 2–2.5 h, during which headspace air was sampled four times and later analyzed for $N_2O$ concentration by gas chromatography. The $N_2O$ flux was calculated using linear regression. Nine $N_2O$ measuring campaigns including all eight treatments were carried out from June 2006 to March 2007, and from April to June 2007 five campaigns were conducted in five of the treatments, viz. A, $CO_2$, D, $DCO_2$ and $TDCO_2$. Annual cumulative $N_2O$ fluxes were derived by interpolation between measurement days and by extrapolating the emission from the treatments T, TD and $TCO_2$ for the period March–June 2007.

To determine $N_2O$ reductase activity, nitrate was removed from sub samples of the soil cores taken in November 2007 for fauna biomass estimation by vortexing 10 g fresh soil and 30 mL of phosphate-buffered saline (PBS) for 5 s followed by centrifugation for 10 min at $3500 \times g$ and 5 °C. The supernatant was discarded and the pellet resuspended in 30 mL PBS. After the third centrifugation the pellet was resuspended in 15 mL of 0.5 mM glucose, 0.5 mM sodium acetate, and 0.5 mM sodium succinate and transferred to a 100 mL incubation bottle. The bottle was sealed with a butyl rubber stopper, flushed with $N_2$ for 2 min, added 100 ppm $N_2O$ (final concentration), placed horizontally on a shaker (200 rpm), and incubated at 22 °C. After 0, 1, 3, 6, and 24 h, 3 mL of headspace was transferred to a pre-evacuated 3 mL Venoject vial before analysis of $N_2O$ by gas chromatography. The $N_2O$ reductase activity was calculated from the consumption of $N_2O$ during the incubation.

## N mineralization and immobilization

Gross rates of N mineralization and $NH_4^+$ consumption were determined by the isotope pool-dilution approach applied in laboratory incubations. Soil cores within each plot were sampled in September 2007 (0–5 and 5–10 cm soil depth, respectively), sieved and stored at 5 °C for about 1 week until incubations were initiated. Fresh samples (10 g) were weighed in 250 mL poly-ethylene bottles and mixed thoroughly with 400 μL of a $^{15}N$-$NH_4Cl$ solution (5 atm% $^{15}N$) to provide $5\,\mu g\,N\,g^{-1}$ soil. One set of samples was analyzed immediately upon substrate application, and a second set was analyzed after 7 days of incubation at 20 °C. Contents of mineral N and the $^{15}N$ contents were determined upon extraction and micro-diffusion (Sørensen & Jensen, 1991). Gross daily rates of mineralization and $NH_4^+$ consumption were calculated according to equations given by Kirkham & Bartholomew (1954). Upscaling to annual rates was done by running a $Q_{10}$ function on the mean daily soil temperatures in 5 cm soil depth from each treatment and assuming a $Q_{10}$ of 2.

## Potential nitrification and denitrification

Subsamples from the soil cores taken in November 2007 were gently homogenized by hand and major roots were removed. Soil samples were stored at 4 °C until analysis (less than 24 h). Potential nitrification was determined in 3 g soil samples by a modification of the method described by Belser & Mays (1980) but without adding chlorate. Potential nitrification rates were estimated from the increase in nitrite plus nitrate concentrations. Potential denitrification rates (Wolsing & Prieme, 2004) in 10 g soil samples were estimated from linear regression of increase in headspace $N_2O$ concentration during 180 min incubation. Potential nitrification and denitrification rates were upscaled to annual rates in the same way as gross mineralization and $NH_4^+$ consumption.

## Bulk deposition, water percolation and N leaching

Rain water was collected monthly for determination of bulk N deposition from three PVC rain water collectors placed randomly within the experimental area. Each rain water collector was placed 2 m above the soil surface and connected to a collection bottle buried in the soil. Leachate water from each experimental plot was collected monthly from passive PVC soil water draining collectors below the organic soil layer (approximately 5 cm depth) and polytetrafluoroethylene suction cups with continuous sub-atmospheric pressure (Prenart Super Quartz, Frederikberg, DK) below the main rooting zone (60 cm depth). Concentrations of $NH_4^+$, $NO_3^-$, and total N (by UV-persulphate oxidation) were analyzed on an Autoanalyzer 3 (Bran + Luebbe Gmbh, Germany). DON was calculated as total N minus inorganic N. Precipitation was measured at the site and the volume of percolating water was estimated by simulation with the CoupModel model (Jansson & Moon, 2001; Svensson *et al.*, 2008) – a coupled ecosystem model with a balance of abiotic and biotic processes in the soil–plant–atmosphere system. The basic structure of the model is a depth profile of the soil, and calculations of water and heat flows are based on common soil properties. The model was parameterized using measured soil layer properties and against time series of soil temperature and moisture content, using on site

measurements of radiation, precipitation and wind speed as driving data and a generalized likelihood uncertainty evaluation approach. Percolation of water below 60 cm was calculated as an output of the simulations and leaching of N at that depth was calculated as the product of the sum of water percolation and the concentration measured in the soil water at 60 cm soil depth for each water collection period (Schmidt *et al.*, 2004).

### Statistical analyses

Statistical analyses were conducted using the multiple linear regression procedure PROC MIXED of SAS (SAS Institute, 2003). The statistical model included a random statement that accounted for the experimental design (*Random* Block octagon octagon × D octagon × T). The same model was used for all tested variables and included the three main factor effects ($CO_2$, D, and T) as well as all possible interactions (D × $CO_2$, T × $CO_2$, T × D, and T × D × $CO_2$). *P*-values $\leq 0.05$ were considered significant. Data were transformed when necessary to obtain normality and homogeneity of variance. Differences of least squares means (DLSM) were used to interpret significant treatment interactions. During the DLSM evaluation, each significant interaction was categorized as either *antagonistic*, i.e. the combined effect led to a reduction of the observed individual effects or *synergistic*, i.e. the combined effect led to either (a) an amplification of the observed individual effect(s) or (b) to a significant effect only when treatments were combined. The DLSM evaluation also revealed if interactions were due to significant single treatment effects, i.e. effects observe in single treatment plots only, which disappear in combination with other treatments. Furthermore, DLSM evaluation of significant interactions revealed if significant main factor effects were effects observed only when in combination with other treatments or if it was significant also as a single treatment effect. Finally, we noted simple additive effects when two of the treatments both showed significant main factor effects but with no interactions.

## Results

### Ecosystem N balance

The N cycle at the site is dominated by higher internal N turnover in comparison with the inputs and losses by leaching and gaseous emission to the atmosphere (Fig. 1). Gross mineralization ($7.0 \pm 1.0\,g\,N\,m^{-2}\,yr^{-1}$) is five times higher than bulk deposition ($1.35 \pm 0.04\,g\,N\,m^{-2}\,yr^{-1}$), an order of magnitude higher than losses by leaching ($0.80 \pm 0.37\,g\,N\,m^{-2}\,yr^{-1}$) and two orders of magnitude higher than gaseous losses in form of $N_2O$ ($0.031 \pm 0.006\,g\,N\,m^{-2}\,yr^{-1}$). Negligible $N_2O$ reductase activity was observed in the soil samples with $N_2O$ uptake rates $<1\,ng\,N\,g^{-1}\,dw\,h^{-1}$ in all samples and $N_2$ production is therefore assumed to be negligible (data not shown). Aboveground, about 90%

of N is found in the two dominant species *Calluna* (~60%) and *Deschampsia* (~30%). Belowground, most N is stored in the SOM pool (~90%) with *Deschampsia* roots (~4%), *Calluna* roots (~3%) and soil microbial (~1.5%) pools dominating the remaining fraction. Soil fauna biomass was ~10% of microbial biomass but their mineralization of N is estimated to $5.0 \pm 0.6\,g\,NH_4^+$-$N\,m^{-2}\,yr^{-1}$, i.e. an amount of labile N much larger than the N input to the system by bulk deposition. The system retained $0.53 \pm 0.42\,g\,N\,m^{-2}\,yr^{-1}$ of bulk deposition in the ambient, non-manipulated plots in 2007 as estimated by difference between bulk deposition and sum of total N leaching below 60 cm soil depth and $N_2O$ emission. While bulk deposition in 2006 ($1.27 \pm 0.07\,g\,N\,m^{-2}\,yr^{-1}$) was similar to the deposition in 2007 ($1.35 \pm 0.04\,g\,N\,m^{-2}\,yr^{-1}$), total N leaching across all treatments was significantly lower ($P < 0.0001$) in 2006 ($0.13 \pm 0.06\,g\,N\,m^{-2}\,yr^{-1}$) than in 2007 ($0.56 \pm 0.20\,g\,N\,m^{-2}\,yr^{-1}$) with no significant treatment effects in either 2006 (data not shown) or in 2007 (Table 1). The major cause of this interannual difference was the wet summer (June–August) in 2007 when precipitation was 433 mm and 46% of annual leaching occurred compared with the dry summer in 2006 when precipitation was much lower at 204 mm and leaching was only 1% of annual leaching.

### Plant responses to climate manipulations

The aboveground biomass N of the dominant plant species *Calluna* and *Deschampsia* did not respond to the climate manipulations (Table 2). Root biomass N of *Deschampsia* was also unaffected by the treatments, whereas root biomass N of *Calluna* increased in response to drought (Table 2, $P = 0.04$) indicating a change in the root/shoot N allocation pattern. While N in aboveground biomasses were unaffected by treatments, the C/N ratios of *Calluna* flowers, as well as leaves from both *Calluna* and *Deschampsia*, increased in response to elevated $CO_2$ (Table 3, $P = 0.02$, 0.04, and 0.01, respectively). The evaluation by DLSM of the three-way interaction for the C/N ratio of mosses (Table 3, $P = 0.003$) showed that the interaction was due to significantly higher C/N ratios in the single treatments of drought and elevated $CO_2$, while ratios were unchanged when drought and elevated $CO_2$ were combined with each other as well as when they were combined with warming. The observed increases in C/N ratios in new plant tissue of leaves and flowers were not seen in whole-plant C/N ratios of *Deschampsia* or in the green tissue or wood fractions of *Calluna*. Yet, the N concentration of *Deschampsia* litter decreased under elevated $CO_2$ (Table 3, $P = 0.05$), indicating that
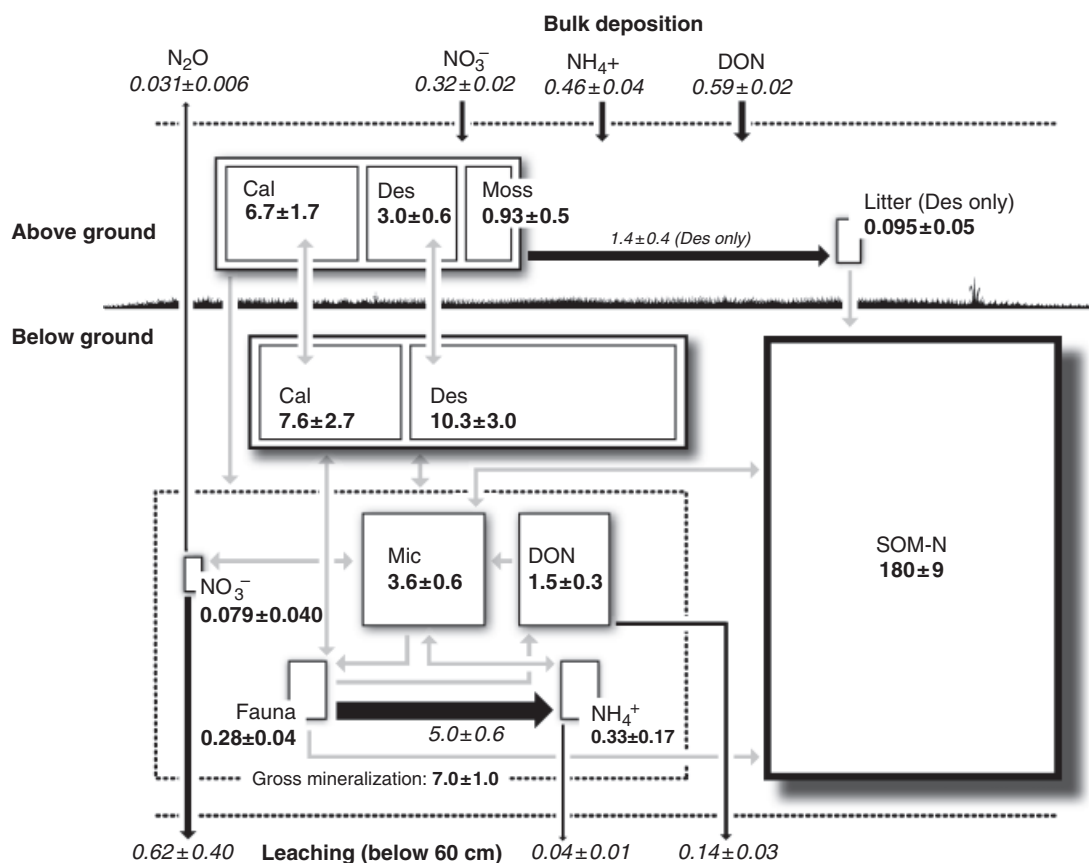
**Fig. 1** Measured N pools in August 2007 (values in bold, $g N m^{-2}$) and annual fluxes (values in italic, $g N m^{-2} y^{-1}$) in ambient plots down to 10 cm soil depth (leaching below 60 cm depth) in 2007. *Calluna vulgaris* (Cal), *Deschampsia flexuosa* (Des), dissolved organic nitrogen (DON), microbial biomass nitrogen (Mic), soil organic matter nitrogen (SOM-N). Data of litter N mass and litter N production was only available for *Deschampsia* (Des only). Size of boxes and arrow widths are scaled non-linearly to indicate size of measured pools and fluxes, respectively. Black arrows indicate measured/estimated fluxes while gray arrows indicate unmeasured fluxes. Dashed lines at top and bottom indicate ecosystem boundaries while the dashed square encompasses the major components involved in N mineralization. Estimates of fauna biomass and mineralization include enchytraeids, microarthropods, nematodes, and protozoans. The annual N balance for the ambient plots shown here was $+ 0.53 \pm 0.42 g N m^{-2} yr^{-1}$.

the changes in C/N ratios starting in the leaves, are already beginning to cascade through the system, after only 2 years of treatments (Fig. 2a).

*Deschampsia* leaves and also mosses (single treatment only) responded to drought with increased C/N ratios (Table 3, $P = 0.002$ and $P \leq 0.05$, respectively) whereas *Calluna* leaves were unaffected (Table 3, Fig. 2b). Also, the pool of N in the annually produced *Deschampsia* litter decreased in response to drought (Table 2, $P = 0.01$). The warming treatment had fewer effects on aboveground processes than the other treatments, showing only an increase of N concentration in *Deschampsia* litter (Table 3, $P = 0.03$, Fig. 2c).

When all treatments were combined (Fig. 2d), the lack of interactions between single treatment effects for leaf level C/N ratios indicates that the effect is found also in treatment combinations. Consequently, increased C/N

ratios of leaves, as well as decreased *Deschampsia* litter N production, are found in the full combination of treatments, i.e. in the simulation of the projected future climate scenario. In contrast, the C/N ratio of mosses increases in two of the three single treatments and N concentrations in *Deschampsia* litter either increase or decrease depending on treatment, however, these effects disappear when all treatments are combined.

*Responses of belowground processes to climate manipulations*

The belowground N turnover was significantly affected by the climate manipulations, primarily by the warming and drought treatments (Fig. 3, Tables 1–3) showing changes cascading through pools and processes. Elevated $CO_2$ as a main factor had no effects below ground

**Table 1** Annual Ecosystem N flux rates of N mineralization and immobilization, potential nitrification and denitrification, litter production, ecosystem N losses and bulk N deposition in a Danish heathland in 2007

| Annual ecosystem N flux rates ($g\,N\,m^{-2}\,yr^{-1}$) | A | $CO_2$ | D | T | $DCO_2$ | $TCO_2$ | TD | $TDCO_2$ | Main treatment effects[1] — $CO_2$ | D | T | Interactions[1] — $D\times CO_2$ | $T\times CO_2$ | $T\times D$ | $T\times D\times CO_2$ | Single treatment effects[2] — $CO_2$ | D | T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Total immobilization and mineralization rates (0–5 cm)* | | | | | | | | | | | | | | | | | | |
| $NH_4^+$ consumption | 3.36 ± 0.65 | 3.95 ± 0.59 | 3.94 ± 0.54 | 8.71 ± 1.88 | 5.94 ± 0.63 | 7.11 ± 1.36 | 6.42 ± 0.50 | 3.11 ± 0.22 | | | 0.03 ↑ | | | | | | | 0.03 ↑ |
| Gross mineralization | 4.46 ± 0.42 | 4.41 ± 0.43 | 4.11 ± 0.49 | 6.70 ± 1.02 | 6.34 ± 0.90 | 6.65 ± 0.70 | 5.70 ± 0.37 | 3.64 ± 0.16 | | 0.02 ↓ | | | 0.04§ | 0.01§ | | 0.02 ↓ | | 0.05 ↑ |
| *Total immobilization and mineralization rates (5–10 cm)* | | | | | | | | | | | | | | | | | | |
| $NH_4^+$ consumption | 5.88 ± 4.12 | 4.75 ± 0.77 | 11.00 ± 4.27 | 6.16 ± 1.94 | 2.66 ± 1.69 | 0.94 ± 0.24 | 1.03 ± 0.25 | 3.38 ± 0.69 | | | | | 0.02§ | 0.02§ | 0.01‡ | 0.05 ↑ | | |
| Gross mineralization | 2.51 ± 0.59 | 5.52 ± 1.56 | 6.84 ± 4.22 | 4.01 ± 1.24 | 4.39 ± 1.24 | 2.40 ± 0.93 | 2.23 ± 0.53 | 3.60 ± 0.94 | | | | | | | 0.03§ | | | |
| *Fauna-related part of total mineralization (0–10 cm)* | | | | | | | | | | | | | | | | | | |
| Enchytraeids | 0.592 ± 0.061 | 0.959 ± 0.294 | 0.418 ± 0.086 | 0.590 ± 0.151 | 0.702 ± 0.222 | 0.604 ± 0.088 | 0.315 ± 0.058 | 0.675 ± 0.231 | 0.03 ↓ | | | | | | | 0.03 ↑ | | |
| Microarthropods | 0.191 ± 0.054 | 0.416 ± 0.156 | 0.292 ± 0.072 | 0.259 ± 0.093 | 0.351 ± 0.102 | 0.308 ± 0.063 | 0.367 ± 0.090 | 0.347 ± 0.090 | | | | | | | | | | |
| Nematodes | 2.63 ± 0.33 | 3.84 ± 0.84 | 2.22 ± 0.48 | 3.45 ± 1.25 | 3.18 ± 0.70 | 2.50 ± 0.30 | 2.77 ± 0.50 | 2.56 ± 0.42 | | | | | | | | | | |
| Protozoans | 1.58 ± 0.69 | 0.86 ± 0.21 | 1.30 ± 0.30 | 0.56 ± 0.17 | 2.33 ± 0.74 | 2.03 ± 1.29 | 0.96 ± 0.23 | 0.59 ± 0.14 | | | 0.04 ↓ | | | | | | 0.04 ↓ | |
| *Potential nitrification and denitrification (0–10 cm)* | | | | | | | | | | | | | | | | | | |
| Potential nitrification | 3.82 ± 1.63 | 0.71 ± 0.65 | 3.37 ± 1.68 | 5.77 ± 2.16 | 4.44 ± 2.84 | 4.95 ± 1.96 | 11.37 ± 3.77 | 3.38 ± 1.58 | | | 0.0002 ↑ | | | | 0.02§‡ | | | 0.0002 ↑ |
| Potential denitrification | 19.0 ± 5.8 | 17.8 ± 4.6 | 21.5 ± 7.1 | 29.9 ± 12.3 | 17.9 ± 6.6 | 18.7 ± 6.7 | 36.4 ± 8.2 | 25.6 ± 7.2 | | | 0.02 ↑ | | | | | | | 0.02 ↑ |
| *Litter N production* | | | | | | | | | | | | | | | | | | |
| *Deschampsia* | 1.42 ± 0.40 | 1.32 ± 0.32 | 0.99 ± 0.40 | 0.91 ± 0.25 | 1.50 ± 0.72 | 1.66 ± 0.28 | 1.04 ± 0.35 | 1.10 ± 0.19 | 0.01 ↓ | | | | | | | 0.01 ↓ | | |
| *Ecosystem N losses (leaching below 60 cm soil depth)* | | | | | | | | | | | | | | | | | | |
| $N_2O$ emission | 0.031 ± 0.006 | 0.024 ± 0.011 | 0.028 ± 0.008 | 0.016 ± 0.005 | 0.030 ± 0.006 | 0.051 ± 0.007 | 0.033 ± 0.006 | 0.033 ± 0.006 | | | 0.05 ↓ | 0.04‡ | | | | | | |
| $NO_3^-$ leaching | 0.619 ± 0.385 | 0.194 ± 0.099 | 0.453 ± 0.195 | 0.244 ± 0.123 | 0.196 ± 0.108 | 0.401 ± 0.174 | 0.616 ± 0.233 | 0.170 ± 0.054 | | | 0.05 ↑ | | | | | | ns | 0.05 ↑ |
| $NH_4^+$ leaching | 0.045 ± 0.012 | 0.031 ± 0.003 | 0.067 ± 0.017 | 0.091 ± 0.052 | 0.090 ± 0.021 | 0.125 ± 0.064 | 0.033 ± 0.005 | 0.056 ± 0.022 | | | | | | + | | | | |
| DON leaching | 0.144 ± 0.034 | 0.135 ± 0.034 | 0.147 ± 0.032 | 0.194 ± 0.104 | 0.123 ± 0.042 | 0.210 ± 0.056 | 0.130 ± 0.054 | 0.091 ± 0.016 | | | | | | | | | | |
| Total N leaching | 0.804 ± 0.426 | 0.353 ± 0.127 | 0.663 ± 0.214 | 0.518 ± 0.227 | 0.387 ± 0.164 | 0.721 ± 0.220 | 0.763 ± 0.280 | 0.310 ± 0.067 | | | | | | | | | | |
| *Bulk N deposition* | | | | | | | | | | | | | | | | | | |
| $NO_3^-$ | 0.322 ± 0.016 | | | | | | | | | | | | | | | | | |
| $NH_4^+$ | 0.456 ± 0.041 | | | | | | | | | | | | | | | | | |
| DON | 0.588 ± 0.023 | | | | | | | | | | | | | | | | | |
| Total N | 1.35 ± 0.04 | | | | | | | | | | | | | | | | | |

Soil depth is indicated in brackets. Data are means with SE, $n = 6$, except for bulk N deposition ($n = 3$, data only available at site level).

[1] Significant P values ($P \leq 0.05$) from statistical analysis. Direction of main treatment effects is indicated with arrows. Significant interactions are indicated as antagonistic (§), or synergistic (‡). Additive effects of multiple significant main effects are also indicated (+).

[2] Significant single treatment effects as indicated by evaluation of main effects plus interaction(s) by analysis of DLSM (see text for further details). Direction of effects is indicated with arrows. ns = not significant.

**Table 2** Ecosystem N pools in a Danish heathland in 2007

| Ecosystem N pools (g N m⁻²) | A | CO₂ | D | T | DCO₂ | TCO₂ | TD | TDCO₂ | Main treatment effects[1] CO₂ | D | T | Interactions[1] D×CO₂ | T | T×CO₂ | T×D | T×D×CO₂ | Single treatment effects[2] CO₂ | D | T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Aboveground plant biomass N* | | | | | | | | | | | | | | | | | | | |
| Calluna | 6.74 ± 1.65 | 6.84 ± 0.59 | 5.92 ± 0.60 | 6.48 ± 1.40 | 5.36 ± 1.29 | 6.55 ± 1.38 | 5.65 ± 0.86 | 4.50 ± 0.95 | | | | | | | | | | | |
| Deschampsia | 2.99 ± 0.61 | 2.67 ± 0.22 | 3.58 ± 0.81 | 2.90 ± 0.81 | 2.81 ± 0.62 | 3.15 ± 0.52 | 2.19 ± 0.51 | 2.35 ± 0.17 | | | | | | | | | | | |
| Mosses | 0.93 ± 0.50 | 2.97 ± 1.03 | 3.67 ± 1.53 | 2.39 ± 0.82 | 1.44 ± 0.68 | 1.81 ± 0.62 | 2.31 ± 1.11 | 1.64 ± 1.14 | | | | | | | | | | | |
| *Belowground plant biomass N (0–10 cm)* | | | | | | | | | | | | | | | | | | | |
| Calluna | 7.56 ± 2.66 | 7.43 ± 1.79 | 13.09 ± 2.54 | 11.07 ± 2.62 | 14.39 ± 3.16 | 6.53 ± 2.17 | 12.93 ± 2.37 | 9.33 ± 2.85 | | 0.04 | | | | | | | | 0.04 ↑ | |
| Deschampsia | 10.25 ± 2.95 | 6.83 ± 2.25 | 6.16 ± 1.41 | 5.77 ± 1.04 | 6.87 ± 1.83 | 6.63 ± 2.22 | 4.59 ± 1.05 | 6.07 ± 1.58 | | | | | | | | | | | |
| *Litter N mass* | | | | | | | | | | | | | | | | | | | |
| Deschampsia | 0.095 ± 0.049 | 0.053 ± 0.018 | 0.018 ± 0.004 | 0.014 ± 0.004 | 0.061 ± 0.031 | 0.044 ± 0.016 | 0.020 ± 0.014 | 0.055 ± 0.016 | | | | | | | | | | | |
| *Soil microbial biomass N and soil fauna biomass N (0–10 cm)* | | | | | | | | | | | | | | | | | | | |
| Microbes | 3.55 ± 0.58 | 5.20 ± 1.40 | 4.70 ± 0.72 | 5.19 ± 0.72 | 4.50 ± 1.00 | 4.12 ± 1.00 | 3.67 ± 0.54 | 3.62 ± 0.77 | | | | | | | | | | | |
| Enchytraeids | 0.197 ± 0.020 | 0.320 ± 0.098 | 0.139 ± 0.029 | 0.234 ± 0.074 | 0.197 ± 0.029 | 0.201 ± 0.029 | 0.105 ± 0.019 | 0.225 ± 0.077 | | 0.03 | | | | | | | | 0.03 ↓ | |
| Microarthropods | 0.0637 ± 0.0180 | 0.1386 ± 0.0520 | 0.0974 ± 0.0240 | 0.1170 ± 0.0339 | 0.0864 ± 0.0310 | 0.1028 ± 0.0211 | 0.1222 ± 0.0171 | 0.1158 ± 0.0300 | | | | | | | | | | | |
| Nematodes | 0.0072 ± 0.0009 | 0.0105 ± 0.0023 | 0.0061 ± 0.0013 | 0.0087 ± 0.0019 | 0.0094 ± 0.0034 | 0.0068 ± 0.0008 | 0.0076 ± 0.0014 | 0.0070 ± 0.0012 | | | | | | | | | | | |
| Protozoans | 0.0108 ± 0.0047 | 0.0059 ± 0.0014 | 0.0089 ± 0.0020 | 0.0159 ± 0.0051 | 0.0039 ± 0.0012 | 0.0139 ± 0.0088 | 0.0066 ± 0.0016 | 0.0040 ± 0.0010 | | 0.04 | | | | | | | | 0.04 ↓ | |
| *Extractable soil organic and inorganic N pools (0–10 cm)* | | | | | | | | | | | | | | | | | | | |
| SOM-N | 180.2 ± 8.6 | 213.8 ± 25.7 | 193.9 ± 19.3 | 146.6 ± 7.3 | 234.1 ± 64.5 | 153.8 ± 12.0 | 154.7 ± 15.5 | 200.7 ± 28.0 | | | | | | | | | | | |
| NO₃⁻ | 0.0793 ± 0.0403 | 0.0339 ± 0.0156 | 0.0640 ± 0.0578 | 0.2290 ± 0.1329 | 0.1759 ± 0.0973 | 0.2809 ± 0.1375 | 0.0870 ± 0.0834 | 0.0415 ± 0.0279 | | | | | | | | | | | |
| NH₄⁺ | 0.330 ± 0.171 | 0.251 ± 0.154 | 0.190 ± 0.166 | 0.286 ± 0.110 | 0.237 ± 0.123 | 1.087 ± 0.328 | 0.063 ± 0.021 | 0.062 ± 0.017 | | 0.02 | | | | | | | | 0.02 ↓ | |
| DON | 1.50 ± 0.27 | 1.65 ± 0.30 | 1.35 ± 0.22 | 1.67 ± 0.16 | 1.72 ± 0.36 | 0.94 ± 0.21 | 1.36 ± 0.19 | 1.54 ± 0.21 | | | | | | | | 0.05§‡ | | | |

Data are means with SE, $n = 6$.

[1] Significant $P$ values ($P \leq 0.05$) from statistical analysis. Direction of main treatment effects is indicated with arrows. The significant three-way interaction consisted of both an antagonistic (§) and a synergistic (‡) effect.

[2] Significant single treatment effects as indicated by evaluation of main effects plus interaction(s) by analysis of DLSM (see text for further details). Direction of effects is indicated with arrows.

**Table 3** Ecosystem N characteristics, i.e. C/N ratios of above ground plant components, N concentration of litter and N concentrations of leachate water below the organic soil layer (5 cm depth) and below the main root zone (60 cm depth) in a Danish heathland in 2007

| Ecosystem N characteristics | A | CO$_2$ | D | T | DCO$_2$ | TCO$_2$ | TD | TDCO$_2$ | Main CO$_2$ | Main D | Main T | D×CO$_2$ | T×CO$_2$ | T×D | T×D×CO$_2$ | Single CO$_2$ | Single D | Single T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *C/N ratios* | | | | | | | | | | | | | | | | | | |
| *Cal* leaves | 26.4 ± 1.9 | 30.9 ± 1.4 | 28.2 ± 1.2 | 27.2 ± 2.3 | 30.1 ± 2.1 | 30.2 ± 3.3 | 25.5 ± 0.7 | 30.2 ± 2.4 | 0.02 ↑ | | | | | | | 0.02 ↑ | | |
| *Cal* flowers | 29.5 ± 1.3 | 34.6 ± 2.6 | 32.2 ± 1.3 | 30.8 ± 2.1 | 34.2 ± 1.9 | 33.9 ± 1.6 | 32.2 ± 1.2 | 35.8 ± 1.3 | 0.04 ↑ | | | | | | | 0.04 ↑ | | |
| *Cal* green tissue | 28.1 ± 1.4 | 26.5 ± 2.2 | 26.4 ± 2.3 | 25.2 ± 2.1 | 30.6 ± 1.1 | 24.9 ± 1.4 | 27.3 ± 1.4 | 27.7 ± 1.6 | | | | | | | | | | |
| *Cal* wood | 56.7 ± 5.0 | 55.1 ± 4.5 | 56.7 ± 5.6 | 53.3 ± 3.9 | 58.5 ± 3.3 | 53.8 ± 4.8 | 60.0 ± 4.8 | 65.8 ± 1.4 | | | | | | | | | | |
| *Des* green leaves | 22.2 ± 1.3 | 25.8 ± 0.4 | 25.8 ± 2.6 | 23.4 ± 1.6 | 30.8 ± 1.4 | 28.2 ± 2.4 | 26.2 ± 1.2 | 31.1 ± 1.5 | 0.01 ↑ | 0.002 ↑ | | | | | | 0.01 ↑ | 0.002 ↑ | |
| *Des* whole plant | 23.3 ± 1.9 | 25.3 ± 1.4 | 21.5 ± 2.0 | 25.0 ± 3.2 | 29.1 ± 3.6 | 20.9 ± 0.9 | 24.9 ± 2.3 | 26.2 ± 1.5 | | | | + | | | | 0.05 ↑ | 0.05 ↑ | |
| Mosses | 19.4 ± 1.0 | 28.0 ± 3.0 | 25.4 ± 2.8 | 24.1 ± 1.8 | 21.7 ± 3.5 | 18.9 ± 2.5 | 22.5 ± 2.8 | 19.9 ± 2.1 | | | | | | | 0.003§ | | | |
| *Litter N conc.* | | | | | | | | | | | | | | | | | | |
| *Des* litter N conc. | 0.936 ± 0.113 | 0.714 ± 0.100 | 0.982 ± 0.074 | 1.006 ± 0.115 | 0.702 ± 0.110 | 0.820 ± 0.088 | 1.079 ± 0.140 | 0.991 ± 0.077 | 0.05 ↓ | | 0.03 ↑ | | + | | | 0.05 ↓ | | 0.03 ↑ |
| *Mean annual leachate N concentrations* (mg N L$^{-1}$) | | | | | | | | | | | | | | | | | | |
| L.NO$_3^-$ (5 cm) | 7.39 ± 1.59 | 1.78 ± 0.07 | 4.14 ± 0.55 | 7.48 ± 0.73 | 5.03 ± 0.62 | 6.84 ± 1.73 | 5.90 ± 0.82 | 5.28 ± 0.82 | | | 0.01 ↑ | 0.002§ | | | | 0.05 ↓ | 0.05 ↓ | |
| NH$_4^+$ (5 cm) | 6.87 ± 1.21 | 7.54 ± 0.62 | 12.06 ± 3.31 | 8.30 ± 0.93 | 7.19 ± 1.29 | 14.61 ± 3.50 | 9.30 ± 1.13 | 7.29 ± 0.64 | | | | 0.004§ | | | 0.03§ | | 0.05 ↓ | ns |
| DON (5 cm) | 21.4 ± 3.4 | 15.1 ± 1.0 | 26.8 ± 5.4 | 23.3 ± 1.7 | 19.1 ± 2.4 | 30.7 ± 6.0 | 22.6 ± 1.0 | 22.9 ± 1.9 | | 0.01 ↑ | | | 0.02§ | | | 0.05 ↓ | 0.01 ↑ | |
| Total N (5 cm) | 7.23 ± 0.74 | 5.77 ± 0.30 | 10.50 ± 1.78 | 7.50 ± 0.35 | 6.93 ± 0.92 | 9.26 ± 0.92 | 7.44 ± 0.26 | 10.30 ± 1.06 | | | | | 0.05§ | | | 0.05 ↓ | | |
| NO$_3^-$ (60 cm) | 2.04 ± 1.39 | 0.55 ± 0.24 | 1.47 ± 0.61 | 1.16 ± 0.79 | 0.92 ± 0.47 | 1.18 ± 0.58 | 2.49 ± 1.04 | 0.62 ± 0.21 | | | | | | | | | | |
| NH$_4^+$ (60 cm) | 0.14 ± 0.03 | 0.09 ± 0.01 | 0.20 ± 0.05 | 0.23 ± 0.09 | 0.25 ± 0.12 | 0.37 ± 0.22 | 0.11 ± 0.02 | 0.15 ± 0.05 | | | | | | | | | | |
| DON (60 cm) | 2.60 ± 1.52 | 0.96 ± 0.26 | 2.07 ± 0.64 | 1.64 ± 0.94 | 1.56 ± 0.63 | 1.92 ± 0.59 | 2.93 ± 1.18 | 1.09 ± 0.22 | | | | | | | | | | |
| Total N (60 cm) | 0.44 ± 0.13 | 0.33 ± 0.08 | 0.42 ± 0.09 | 0.35 ± 0.14 | 0.42 ± 0.15 | 0.41 ± 0.09 | 0.43 ± 0.18 | 0.31 ± 0.05 | | | | | | | | | | |

Data are means with SE, *n* = 6.

[1] Significant *P* values (*P* ≤ 0.05) from statistical analysis. Direction of main treatment effects is indicated with arrows. Significant interactions are indicated as antagonistic (§), or synergistic (‡). Additive effects of multiple significant main effects are also indicated ( + ).

[2] Significant single treatment effects as indicated by evaluation of main effects plus interaction(s) by analysis of DLSM (see text for further details). Direction of effects is indicated with arrows. ns = not significant. *Cal, Calluna; Des, Deschampsia.*
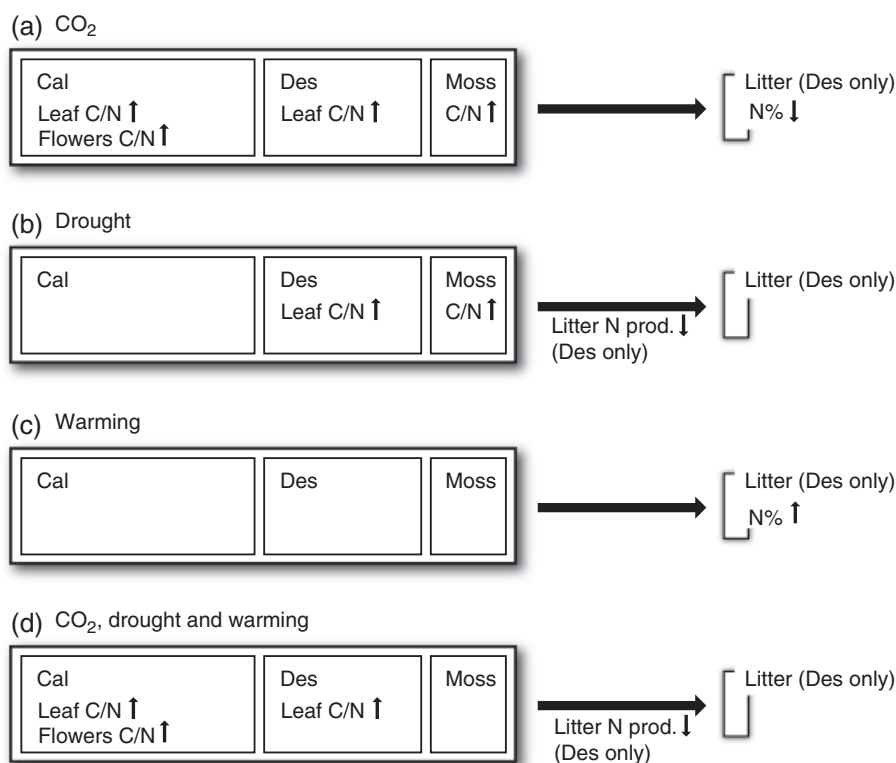
**Fig. 2** Observed aboveground changes in N pools, C/N ratios, litter N production of *Deschampsia* and percentage N in litter of *Deschampsia* caused by single treatments factors (a–c) and when all treatments are combined (d). *Calluna vulgaris* (Cal), *Deschampsia flexuosa* (Des). Small arrows next to pool and flux names indicate direction of change. See Tables 1–3 for statistical significance of observed changes.

but the analysis of DLSM, when evaluating the statistical model interactions, revealed reductions in leachate concentrations in 5 cm soil depth of $NO_3^-$-N and DON (Table 3, Fig. 3a) in the single $CO_2$ treatment. This effect was reduced when elevated $CO_2$ was combined with other treatments causing the significant interactions for these three variables (Table 3).

The drought treatment reduced the N pools of enchytraeids and protozoans and their mineralization (Tables 1 and 2, $P = 0.03$ and $0.04$, respectively), decreased gross mineralization (Table 1, $P = 0.02$), and reduced the pool of $NH_4^+$-N (Table 2, $P = 0.02$), thus indicating an overall decrease in the N turnover in response to drought (Fig. 3b). In contrast, warming stimulated microbial ammonium consumption in the top 5 cm of soil (Table 1, $P = 0.03$) as well as the gross mineralization rate (single treatment only, Table 1, $P \leq 0.05$) and the potential nitrification rate (Table 1, $P = 0.0002$), leading to both increased denitrification (Table 1, $P = 0.02$) and $N_2O$ emission (Table 1, $P = 0.05$). Overall, the belowground response to the warming treatments was therefore an increase in N turnover (Fig. 3c).

While no direct main effects of elevated $CO_2$ were observed, this treatment influenced the responses to drought and warming primarily by reducing some of the significant individual effects in the three-factor combination. Still, the drought-induced decrease of fauna N biomass and fauna N mineralization, as well as the warming-induced increased potential denitrification rate, did not interact significantly with the other treatments and consequently remained in the full treatment combination (Fig. 3d).

*Interactions between climate manipulations*

Across the 47 response variables reported (Tables 1–3) the drought treatment lead to the highest number of significant main effects (11) followed by warming and $CO_2$ treatments (6 and 4, respectively). Simultaneously there were a large number of interactions (15) between the three treatments. Interestingly, the analysis of the interactions revealed a dominance of antagonistic effects (Fig. 4) with approximately three times more antagonistic than synergistic effects. Furthermore, sim-
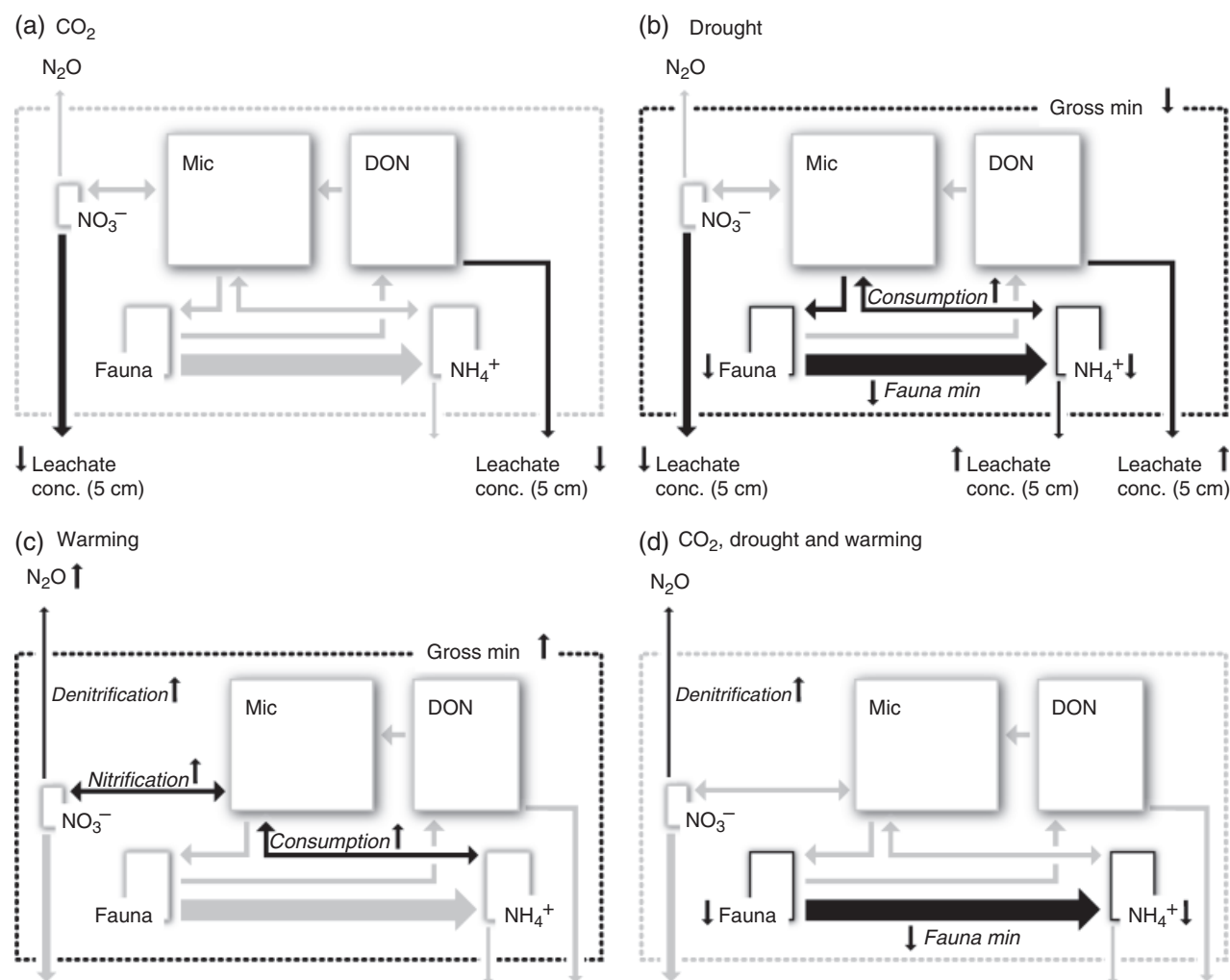
**Fig. 3** Observed belowground changes in N pools, N fluxes/processes and N concentrations caused by single treatments factors (a–c) and when all treatments are combined (d). Dissolved organic nitrogen (DON), microbial biomass nitrogen (Mic), mineralization (min). Black arrows indicate significantly changed fluxes while gray arrows indicate no change or unmeasured fluxes. Short arrows next to pool and flux names indicate direction of change. See Tables 1–3 for statistical significance of observed changes.

ple additive effects were less frequent than both antagonistic and synergistic effects.

## Discussion

### Overall ecosystem N turnover

The N cycle at the experimental site is characterized by a greater flux of N within the system than arrives in bulk N deposition or leaves via leaching and $N_2O$ production as generally seen in natural and seminatural ecosystems. In comparison with our estimated annual gross mineralization rate ($7.0 \pm 1.0 \, g \, N \, m^{-2} \, yr^{-1}$), Emmett *et al.* (2004) reported net mineralization rates of $3.8$–$6.6 \, g \, N \, m^{-2} \, yr^{-1}$ from another Danish dry heathland

and Aerts (1993) reported rates ranging from $4.4$ to $12.6 \, g \, N \, m^{-2} \, yr^{-1}$ under different plant species in wet and dry Dutch heathlands, where atmospheric deposition is two to three times higher than in Denmark. Interannual variability in N leaching was significant, while treatment differences after 2 years of treatments were not significant. The observed difference in leaching between 2006 and 2007 shows that the ecosystem N loss by leaching is highly dependent on the amount and timing of precipitation. In a future climate with more precipitation distributed in fewer, but more severe rain events, N leaching is likely to increase in this ecosystem. The interannual variability in leaching may also indicate that the ecosystem is close to N saturation as also suggested from an adjacent short-term experiment
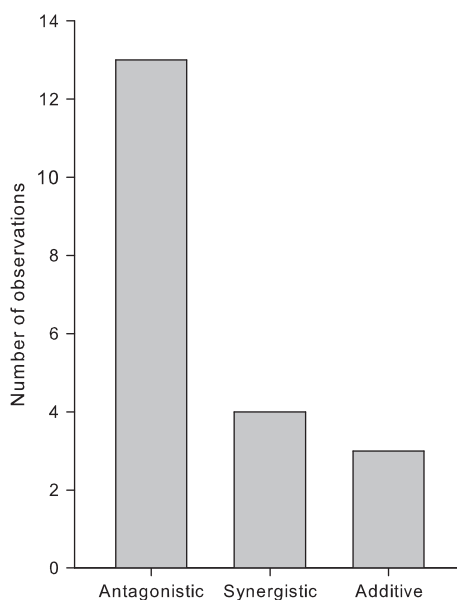
**Fig. 4** Observed significant interactions ($P \leq 0.05$) from the PROC MIXED analysis of 47 individually tested response variables (Tables 1–3) followed by analysis of differences of least squares means (DLSM) were categorized as antagonistic, i.e. combination lead to reduction of effects, or as synergistic, i.e. combination lead to amplification of single effects or significant effect was only observed in combination. Number of observed simple additive effects is also shown, i.e. cases where two significant individual effects were observed without significant interaction. Note that the sum of antagonistic and synergistic effects are 17 because two three-way interactions (potential nitrification and extractable DON) were caused by both antagonistic and synergistic effects. See Tables 1–3 for further statistical details.

where N and phosphorus (P) addition had no effect in *Calluna* root biomass and only moderately increased the fine root biomass of *Deschampsia* (Nielsen *et al.*, 2009). Furthermore, *Deschampsia* responded more when both N and P were added indicating that this species is currently co-limited by N and P availability. If N availability should decrease due to progressive N limitation as the experiment continues, this, in combination with the N and P co-limitation for *Deschampsia*, would be beneficial to the slower growing *Calluna*. However, other climate factors, i.e. water availability and temperature increase, are also likely to influence the overall competitive relationship, potentially disturbing the balance of the equilibrium, especially if the rate of mortality of *Calluna* is increased.

The mineralization of $NH_4^+$-N by soil fauna is often neglected but contributes significantly to the pool of plant available N in the soil (Osler & Sommerkorn, 2007). In this study, we calculated fauna N mineralization from estimated biomasses and literature values of the turnover times and yields of microfauna and mesofauna. Obviously, upscaling of e.g. enumeration of bacterivorous protozoa and nematodes to gross N mineralization should be evaluated with caution. Values for bacterivore biomass, turnover, and growth yield on bacteria are all needed to perform the upscaling and very few references exist in the literature on such values. Despite this fact, the value for fauna-related N mineralization of $5.0 \pm 0.6\,\text{g N m}^{-2}\,\text{yr}^{-1}$ in the ambient treatment based on this calculation seems trustworthy when compared with the gross N mineralization from the pool dilution assay of $7.0 \pm 1.0\,\text{g N m}^{-2}\,\text{yr}^{-1}$. The assumption that most bacterial mineralization is facilitated through predation and mineralization by the soil fauna implies that N mineralization by bacteria and archaea is responsible for approximately 75% of the total activity, the rest being due to fungi.

*Responses to elevated $CO_2$, warming and drought*

Elevated atmospheric $CO_2$ concentration is known to induce downregulation of photosynthetic capacity, e.g. via reduced content of Rubisco in the leaves (Ainsworth & Long, 2005; Ainsworth & Rogers, 2007) and reduction of leaf N content and increased C/N ratios of leaves (Paterson *et al.*, 1999; Lutze & Gifford, 2000; van Heerwaarden *et al.*, 2005; Finzi *et al.*, 2006; Chen *et al.*, 2007; Hovenden *et al.*, 2008). At our experimental site, we observed reduced photosynthetic capacity (Albert, 2009) and leaf N under elevated $CO_2$ for the dominant species, *Calluna* and *Deschampsia*, as well as increased leaf C/N ratios. However, the increased C/N ratios observed in leaves, and for *Calluna* also observed in flower biomass, were after 2 years of treatments not large enough to affect whole plant biomass C/N ratios, which remained unaffected by the treatments. Previous studies have found increasing C/N ratios in other plant tissues than leaves (McGuire *et al.*, 1995; Cotrufo *et al.*, 1998), but the change is often much weaker than that observed for leaves (McGuire *et al.*, 1995), indicating that the C/N effect of elevated $CO_2$ is closely linked to the processes involved in the C sequestration in the photosynthetic apparatus (Körner, 2000). As hypothesized, however, the N concentration in *Deschampsia* litter after 2 years of treatments was also reduced by elevated $CO_2$, possibly showing that the effect is beginning to cascade from leaf level to other ecosystem N pools, although the effect disappears in the full treatment combination. Together with the observed increased concentration of tannins (Schmidt *et al.*, 2007) in plant tissues, we anticipate that the changes in C/N ratios will affect litter decomposition rates as the experimental treatments continue.

In contrast to our expectations, the above- and below-ground plant N pools were not increased by elevated $CO_2$. However, total root biomass N of *Calluna* increased unexpectedly in response to drought. It is inherently difficult to estimate total roots from small soil samples due to the large spatial heterogeneity in root distribution and, at the same time, collection of larger samples is not possible as it would rapidly result in the destruction of the experimental plots. We are therefore reluctant to put too much emphasis on this result and conclude overall that total biomass N of both dominant species, as well as mosses, after 2 years of treatments were only marginally affected by the applied climate drivers.

Increased rhizodeposition is a common plant response to elevated $CO_2$ (Pendall *et al.*, 2004; Allard *et al.*, 2006), and strong effects on belowground C cycling have been observed in response to elevated $CO_2$ in the current experiment, e.g. increased soil respiration rates (Selsted *et al.*, 2010) and increased production of dissolved organic C (Andresen *et al.*, 2010a). However, in contrast to our expectation, we observed very few direct effects of elevated $CO_2$ on belowground N processes. Still, the decreased concentrations of organic and inorganic N leachate water at 5 cm soil depth imply that mineralization processes are affected or possibly that root N uptake has increased (Andresen *et al.*, 2009). In contrast to the lack of direct responses to elevated $CO_2$, soil processes were more sensitive to the drought and warming treatments. Our study supports previous observations of increased belowground N turnover in response to warming (Rustad *et al.*, 2001; Schmidt *et al.*, 2004). We observed increased microbial $NH_4^+$-N consumption, gross mineralization, and potential nitrification and denitrification rates from lab incubations of soils, as well as increased *in situ* $N_2O$ emissions. Soil and litter incubations *in situ* also showed that warming promoted N cycling (Andresen *et al.*, 2010b). Previous field studies with artificial warming showed no warming effect on $N_2O$ emission rates (Peterjohn *et al.*, 1994; Mchale *et al.*, 1998). However, we interpret our observation of five variables that all responded in the same direction to warming as a strong indicator that the warming effect on N turnover was real.

The belowground N turnover was reduced by drought as observed by reduced gross N mineralization and fauna N mineralization. Reduced N mineralization in response to drought has previously been observed (Emmett *et al.*, 2004; Schmidt *et al.*, 2004; Sowerby *et al.*, 2008) but the negative effects of drought on N mineralization are often attributed directly to reduced microbial activity. The importance of soil microfauna to the N mineralization shown in this study, together with the negative effects of drought on top soil layer populations of enchytraeids (Maraldo *et al.*, 2010) and other fauna groups and their decreased N mineralization rates, indicate that the fauna response plays a significant role in the observed decrease in N turnover in drought-exposed plots. Furthermore, while the warming effects were generally reduced in combination with drought and elevated $CO_2$, the negative effect of drought on fauna N biomass and N mineralization was not reduced by interactions. Consequently, the effect prevailed in the full combination of treatments, indicating that N mineralization will be reduced in the full future climate scenario. Therefore, despite the fact that this ecosystem currently shows no clear sign of N limitation of plant growth, we cannot rule out the possibility that progressive N limitation will play a role in the longer term, as the impacts of increased $CO_2$ on C/N ratios of leaves combined with the drought-induced reduction of mineralization and litter N production continues to reduce the availability of N in the ecosystem.

## Importance of climate driver interactions

We thoroughly investigated the nature of all significant interactions observed in the 47 response variables reported in Tables 1–3 by evaluating the DLSM from the mixed model statistical output. This analysis revealed a clear dominance of antagonistic effects over synergistic and simple additive effects when several treatments were combined (Fig. 4), i.e. the ecosystem is more robust when more than one factor is changed. For instance, the C/N ratio of mosses increased in both single treatments of drought and elevated $CO_2$ but was unaffected when treatments were combined. Similarly, the increased gross mineralization rate observed in the single warming treatment was reduced especially in combination with elevated $CO_2$. Furthermore, two of the three additive effects observed (*Deschampsia* litter N concentration and $N_2O$ emissions) were additive responses with opposing directions, which therefore acts to reduce the observed effects in treatment combinations.

Previous studies have investigated interactions between elevated $CO_2$ and e.g. N supply (Lutze & Gifford, 2000; Lutze *et al.*, 2000; van Heerwaarden *et al.*, 2005; Reich *et al.*, 2006), warming (Peltola *et al.*, 2002; Tingey *et al.*, 2003; Hovenden *et al.*, 2008; Dijkstra *et al.*, 2010), biodiversity (Niklaus *et al.*, 2001; Reich *et al.*, 2001) and water relations (Morgan *et al.*, 2004; Nowak *et al.*, 2004). A review by Barnard *et al.* (2005) of 25 multifactor studies concluded that interactions were generally rare. These 25 studies were dominated by experimentally elevated $CO_2$ combined with increased N supply. The lack of interactions could be due to the common trend

that most ecosystems respond strongly to increased N supply, which hides weaker interactive responses. Luo *et al.* (2008) analyzed and modeled interactions between elevated $CO_2$, warming and changes in precipitation in seven multifactor studies and found that three-way interactions were rare while two-way interactions were more common. Generally, interactions were positive between elevated $CO_2$ and warming and negative between elevated $CO_2$ and reduced precipitation with respect to net primary production (NPP).

A general pattern of most published studies is the focus on a few important variables, i.e. overall changes in NPP or biomasses or specific studies focusing on processes, e.g. nitrification and denitrification. As interactions are only sometimes significant, investigating a small number of variables prevents a more general interpretation of the importance of interactions. Furthermore, assuming that three-way interactions are more uncommon than two-way interactions, important three-way interactions could be missed when fewer variables are analyzed. Out of 15 interactions observed across 47 N-related variables in the present study, six were three-way interactions. In contrast to the model results from Luo *et al.* (2008), our results reinforce the value of multifactor ecosystem manipulation experiments, which are able to identify and characterize complex interactive effects.

When analyzing the interactions between our three climate drivers, we found a predominance of antagonism in the combined responses, which implies that ecosystem changes caused by climate change may be less pronounced than judged from single factor experiments. Based on these results we raise the hypothesis that combined effects of all future climate change are dampened compared with expected effects based on simple addition of single treatment responses. If this pattern prevails for ecosystem responses to future climate change in general it highlights (1) that single factor studies are likely to overestimate responses and should be evaluated with caution, and (2) that multifactor field-scale ecosystem experiments are critical in order to be able to predict realistic response strengths as well as for developing a conceptual framework for understanding interactions among climate drivers. Our multivariable approach may aid the latter point if tested in other multifactor studies and by including variables not only constrained to the N cycle.

Large areas of Europe are covered by seminatural shrublands and grasslands with no or insignificant management similar to our study site. Furthermore, the applied treatments are assumed to be relatively realistic according to model predictions (Christensen & Christensen, 2007). We therefore assume that our findings shed light not only on effects and interactions on individual biological processes, but also on expected short-term effects at the ecosystem scale at our study site as well as more generally on a larger scale. Relative to the existing climatic conditions our drought treatment increases the frequency (but not the length) of periods with little or no rain during summer. Therefore, the clear response to the drought treatment observed in this study exemplifies the effects of an amplification of the water cycle. On the other hand, the comparatively small effects of the warming treatment may partly be a consequence of the relatively small degree of warming obtained by the applied passive warming technique.

## Conclusions

The drought treatment effects dominated over effects of warming and elevated $CO_2$ in observed responses of the N cycle after 2 years of treatments. In contrast to our expectations, we did not observe increased growth of the N pools of the dominant plant species, *Calluna* and *Deschampsia*, in response to elevated $CO_2$. While previous studies have usually found interactions to be rare, interactions were commonly observed and antagonism dominated over synergism in combined responses, i.e. the ecosystem response to the combination of several climate drivers is dampened compared with single treatment responses. Still, the observed negative response to drought of soil fauna biomass and N mineralization and *Deschampsia* litter N production prevailed in the full treatment combination and so did the increased leaf C/N ratios of the dominant species *Calluna* and *Deschampsia*. If persistent, these changes are likely to lead to reduced N availability and possibly to progressive N limitation as the experimental treatments continue.

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