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Klaus Steenberg Larsen, Louise C. Andresen, Claus Beier, Sven Jonasson, Kristian R. Albert, Per Ambus, Karen Stevnbak, Marie Frost Arndal, Mette S. Carter, Soren Christensen, et al.

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Reduced N cycling in response to drought, warming, and elevated CO₂ in a Danish heathland: Synthesizing results of the CLIMAITE project after two years of treatments

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Keywords:	<p>Climate driver interactions, C/N ratio, multi-factor climate change experiment, nitrogen cycling, nitrogen mineralization, N₂O, soil fauna</p>
Abstract:	<p>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 semi-natural Danish heathland ecosystem to elevated atmospheric carbon dioxide (CO₂), warming, and extended summer drought in all combinations. Here, we report on the short-term responses of the nitrogen (N) cycle after two years of treatments. Elevated CO₂ significantly affected above-ground stoichiometry by increasing the carbon to nitrogen (C/N) ratios in the leaves of both co-dominant species (<i>Calluna vulgaris</i> and <i>Deschampsia flexuosa</i>), as well as the C/N ratios of <i>Calluna</i> flowers and by reducing the N concentration of <i>Deschampsia</i> litter. Below-ground, elevated CO₂ had only minor effects, whereas warming increased N turnover, as indicated by increased rates of microbial NH₄⁺-N consumption, gross mineralization, potential nitrification, denitrification and N₂O emissions. Drought reduced below-ground gross N mineralization and decreased fauna N mass and 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, decreased litter N production, and decreased fauna N mineralization prevailed in the full treatment combination indicating reduced N turnover in the simulated future climate scenario, which could act to reduce the potential growth response of plants to elevated atmospheric CO₂ concentration. In conclusion, effects observed in single-factor studies should be evaluated with caution. Multi-factor climate experiments are needed for improving realistic estimation of the combined ecosystem responses to future climate changes.</p>

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For Review Only

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6 2 heathland: Synthesizing results of the CLIMAITE project after two years of treatments
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10 4 **Running title:** Effects of climate change on N cycling
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55 23 **Keywords:** Climate driver interactions, C/N ratio, multi-factor climate change experiment,
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57 24 nitrogen cycling, nitrogen mineralization, N₂O, soil fauna
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67 **Abstract**

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10 27 Field-scale experiments simulating realistic future climate scenarios are important tools for
11
12 28 investigating the effects of current and future climate changes on ecosystem functioning and
13
14 29 biogeochemical cycling. We exposed a semi-natural Danish heathland ecosystem to elevated
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16 30 atmospheric carbon dioxide (CO₂), warming, and extended summer drought in all
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18 31 combinations. Here, we report on the short-term responses of the nitrogen (N) cycle after two
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20 32 years of treatments.
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24 33 Elevated CO₂ significantly affected above-ground stoichiometry by increasing the
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26 34 carbon to nitrogen (C/N) ratios in the leaves of both co-dominant species (*Calluna vulgaris*
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28 35 and *Deschampsia flexuosa*), as well as the C/N ratios of *Calluna* flowers and by reducing the
29
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32 37 whereas warming increased N turnover, as indicated by increased rates of microbial NH₄⁺-N
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34 38 consumption, gross mineralization, potential nitrification, denitrification and N₂O emissions.
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67 **54 Introduction**

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10 55 Climate change with elevated atmospheric CO₂, increased temperature and altered
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12 56 precipitation will fundamentally affect key drivers for ecosystem functioning and lead to
13
14 57 adverse effects in terrestrial ecosystems across the globe (IPCC, 2007). These climate-driven
15
16 58 changes may affect ecosystem functioning directly, *e.g.* through CO₂-stimulated
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18 59 photosynthesis and growth (Luo *et al.* 2006; de Graaff *et al.* 2006), warming-induced
19
20 60 increased nutrient mineralization (Emmett *et al.* 2004) or drought-induced growth limitation
21
22 61 (Penuelas *et al.* 2007). They may also act indirectly *e.g.* through impacts on species
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24 62 composition (Penuelas *et al.* 2007).

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28 63 Growth in many natural and semi-natural terrestrial ecosystems is limited by nitrogen
29
30 64 and characterized by strong competition for nitrogen between plants and the soil communities
31
32 65 of fungi and bacteria (Marion *et al.* 1989; Jonasson *et al.* 1996). Consequently, ecosystem N
33
34 66 availability is an important factor controlling the carbon uptake in terrestrial ecosystems and
35
36 67 therefore also plays a dominant role in controlling the feedback between the biosphere and the
37
38 68 atmosphere in a warmer and more CO₂ enriched world. The theory of nitrogen as a limiting
39
40 69 factor constraining the carbon uptake in terrestrial ecosystems in response to elevated CO₂ is
41
42 70 conceptualized in the PNL framework (Progressive Nitrogen Limitation) (Luo *et al.* 2004)
43
44 71 describing how increased carbon sequestration driven by elevated CO₂ requires increased
45
46 72 access to nitrogen in order to balance the carbon input. Accordingly, without additional inputs
47
48 73 of plant-available N, nitrogen will progressively limit further carbon uptake. But increased N
49
50 74 availability has also been shown as a consequence of increased availability of labile soil
51
52 75 carbon substrate (Zak *et al.* 1993) and increased soil moisture content due to improved plant
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54 76 water use efficiency (WUE) (Zak *et al.* 1993; Hungate 1999), as well as increased N
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56 77 mineralization in response to warming (Schmidt *et al.* 2004; Emmett *et al.* 2004). The

1
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3 78 interaction between the carbon and nitrogen cycles clearly illustrates how sensitive
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5 79 ecosystems are to changes in nitrogen pools and turnover, current input of N, and other
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8 80 nutrients, and changes in the water regime. This is particularly important for N limited
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10 81 systems, such as natural and semi-natural ecosystems with relatively low nitrogen inputs and
11
12 82 internal N turnover rates of the mineral N pool as fast as a few days (Davidson *et al.* 1990;
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14 83 Lipson & Näsholm 2001; Schimel & Bennett 2004; Vervaet *et al.* 2004). In systems with very
15
16 84 high internal turnover rates of nitrogen, the N turnover has also been shown to be very
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18 85 sensitive to changes in natural climatic variations such as reduced water availability
19
20 86 (Jamieson *et al.* 1999), potentially leading to a high sensitivity of these systems to climate-
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22 87 driven changes in the internal processes regulating the demand or supply of nitrogen.
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27 88 Most of the experimental evidence of PNL and the role of nitrogen in constraining
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29 89 carbon uptake is based on modeling studies (Rastetter *et al.* 1997) or on experiments
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31 90 involving elevated CO₂ alone or in combination with various levels of nitrogen addition to
32
33 91 demonstrate the role of N in stimulating CO₂ sequestration responses (Lutze & Gifford 2000;
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35 92 Lutze *et al.* 2000; van Heerwaarden *et al.* 2005; de Graaff *et al.* 2006; Reich *et al.* 2006).
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38 93 However, in a future CO₂ enriched world, the nitrogen limitation and the CO₂ response in N
39
40 94 limited systems will to a large extent be regulated by the climate change-associated responses
41
42 95 of the internal nitrogen cycle, which are not well captured in a CO₂ alone experiment. For
43
44 96 instance, it has been demonstrated that warming can stimulate internal nitrogen
45
46 97 mineralization, leading to increased N availability (Emmett *et al.* 2004) and even increased N
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48 98 leaching (Lukewille & Wright 1997; Schmidt *et al.* 2004). Correspondingly, changes in water
49
50 99 availability, such as water limitations by droughts, can reduce N mineralization and N
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52 100 availability (Emmett *et al.* 2004). On the other hand, increased water availability due to
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54 101 increased precipitation or to CO₂ stimulated increase in WUE can increase N mineralization
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56 102 and N availability under water limited conditions (Hungate 1999). Since the future climate
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3 103 change will involve changes in all of these factors, it is important to understand the possible
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5 104 interactions between different climate drivers on N availability and nitrogen limitation.
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8 105 Therefore, studies involving all factors simultaneously are needed.
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10 106 In the present study, we aim to synthesize the overall impacts of changes in the nitrogen
11
12 107 cycle in response to warming, elevated atmospheric CO₂ concentration and increased summer
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14 108 drought events after two years of treatments. We compare the responses of the three-factor
15
16 109 treatment with responses to treatments including only one, or pair wise combinations of the
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20 110 factors.

21
22 111 In response to elevated CO₂, we expected to observe increased plant tissue and litter
23
24 112 C/N ratios (Paterson *et al.* 1999; Lutze & Gifford 2000; van Heerwaarden *et al.* 2005; Finzi *et*
25
26 113 *al.* 2006; Chen *et al.* 2007; Hovenden *et al.* 2008). In the short term, we further expected that
27
28 114 the increased water use efficiency under elevated CO₂ (Ainsworth & Long 2005; Leuzinger &
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30 115 Körner 2007; Ainsworth & Rogers 2007; Albert *et al.* 2010) would stimulate plant growth,
31
32 116 and increase plant N demand and biomass, and that concomitant increased rhizodeposition
33
34 117 would lead to increased microbial biomass activity and mineralization. Warming was
35
36 118 expected to increase plant growth and increase mineralization (Rustad *et al.* 2001), whereas
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38 119 extended summer drought was expected to reduce plant N demand and decrease N
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40 120 mineralization and nitrification rates (Emmett *et al.* 2004; Schmidt *et al.* 2004; Sowerby *et al.*
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42 121 2008) as a product of direct or indirect effects on microbial activity. Assuming simple
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44 122 addition of these single treatment effects, we expected that drought effects would be reduced
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46 123 in combination with both warming and elevated CO₂ and, consequently, that increased N
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48 124 mineralization and plant growth in response to both warming and elevated CO₂ would
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50 125 dominate over decreases caused by drought in the full combination of treatments.
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127 Materials and methods*128 Experimental site and climate change manipulations*

129 The CLIMAITE experimental site is a dry, temperate heathland situated approximately 50 km
130 NW of Copenhagen, Denmark (55°53' N, 11°58'E). The vegetation is dominated by the
131 evergreen dwarf shrub *Calluna vulgaris* (L.), the perennial grass *Deschampsia flexuosa* (L.)
132 and various mosses and herbs. The soil is a well-drained, nutrient-poor sandy deposit with a
133 pH of 4-5 and an organic top layer ranging from 2 to 5 cm in depth. Long-term annual mean
134 air temperature is 8.0 °C, annual mean precipitation is 613 mm (Danish Meteorological
135 Institute 2009) and the total N bulk deposition at the site was 1.27 ± 0.07 and 1.35 ± 0.04 g N m⁻²
136 y⁻¹ in 2006 and 2007, respectively (Larsen KS, unpublished data). The experimental area
137 covers approximately 2 ha and consists of twelve 7m diameter octagons laid out pair-wise in 6
138 blocks (= 6 replicates). Each block consists of two octagons, one with ambient (A)
139 atmospheric CO₂ and one receiving elevated atmospheric CO₂ concentration (CO₂) (510 ppm
140 in a free air CO₂ enrichment setup (FACE)). Each octagon is separated into 4 subplots
141 receiving in addition to the ambient or elevated CO₂ 1) one month summer drought (D) by
142 rain-out shelters, 2) passive warming (T) of air and soil by night time warming curtains 50 cm
143 above ground, 3) a combination of drought and warming (TD) or 4) no drought or
144 temperature treatment. In total, the experiment provides a full factorial design with all 7
145 combinations of D, T and CO₂ and an untreated control for reference (A). Details on the
146 experimental setup are described by Mikkelsen *et al.* (2008).

147 The treatments were initiated in October 2005 and the first summer drought was
148 applied in July/August 2006. The warming treatment elevated temperature at 5 cm depth by
149 0.3 °C in the winter to 0.7 °C in the summer months, on average. The maximum mean daily
150 temperature elevation was 1.2, 2.1 and 2.8 °C in the 5 cm depth, soil surface and 20 cm height

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3 151 sensors, respectively (4 October 2005 - 31 December 2007). The drought campaigns removed
4
5 152 52 mm and 94 mm of precipitation, resulting in peak reductions of soil water content of 11
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8 153 and 13 % v/v over 0-20 cm soil depth in 2006 (3-20 July and 26 July-4 August) and 2007 (21
9
10 154 May-22 June), respectively . The drought campaigns were stopped when soil water content
11
12 155 reached about 5 % v/v over 0-20 cm soil depth.

156

157 *N stocks in plants, litter, microbes and soil, C/N ratios, extractable N concentrations*

158 Above-ground biomasses N of *Calluna* and *Deschampsia* were estimated from non-
159 destructive point intercept measurements in two 50 x 50 cm subplots within each plot
160 combined with a linear regression model linking point intercept measurements and biomass
161 obtained from destructive harvesting of similar plots outside the treatment plots (Jonasson
162 1988; Kongstad *et al.* 2010; Riis-Nielsen & Schmidt 2010). To compensate for pre-treatment
163 differences between the initial biomass values in individual sub-plots and the subsequent
164 development during the experiment, treatment effects on above-ground plant biomasses were
165 normalized and scaled to the average biomass development in the ambient plots while
166 maintaining the observed relative variance. Briefly, a regression was fitted between the pre-
167 treatment plant biomass and the treatment biomass at a given time point, within each
168 treatment. The slope of the regression in the ambient treatment defined the ambient or control
169 response; the normalized treatment effect was determined as the difference between the
170 predicted control response and the predicted treatment response for a given treatment. See van
171 der Linden *et al.* (2010) for further description.

172 *Calluna* and *Deschampsia* plants as well as mosses from the experimental plots were
173 harvested in August 2007. The C and N concentrations measured in green tissue, flowers,
174 wood/stem and roots or whole plants (mosses) (Andresen *et al.* 2010a) were used to calculate

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3 175 C/N ratios. The N concentrations were used in combination with the non-destructive above-
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6 176 ground biomass estimates to calculate the total N stock of these pools. Concurrent with plant
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8 177 harvest, soil samples from each plot (0-10 cm soil depth) were taken to measure plant root
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10 178 biomasses, microbial biomass N and, soil organic matter N (SOM-N) and soil extractable
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13 179 $\text{NO}_3\text{-N}$, $\text{NH}_4\text{-N}$ and dissolved organic nitrogen (DON) (Andresen *et al.* 2010a). Litter
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15 180 production by *Deschampsia* was estimated from point intercept-based difference between
16
17 181 seasonal minimum and maximum biomass of live and dead fractions of *Deschampsia* leaves
18
19 182 multiplied with N concentrations in the litter (Kongstad *et al.* 2010; Riis-Nielsen & Schmidt
20
21 183 2010).

184

185 *Soil fauna*

186 Soil cores within each plot (0-10 cm soil depth) were sampled in October and November 2007
187 for biomass estimation of protozoa/nematodes and enchytraeids/microarthropodes,
188 respectively. For estimation of protozoan biomass, a soil suspension was prepared by
189 blending 5 g of soil with 100 ml of Amoeba Saline (AS, Page 1988) for 1 min. Three-fold
190 dilution series of the soil suspension were prepared in microtiter plates being inspected for
191 protozoa after one and three weeks of growth at 15°C with a modified version of the Most
192 Probable Number method (Darbyshire *et al.* 1974; Rønn *et al.* 1995). Nematodes were
193 extracted from between 10 and 11 g (fresh weight) of soil by a modified Baermann tray
194 extraction method (Georgieva *et al.* 2005). Samples were extracted for 48 h, and nematodes
195 were then counted at 40× magnification using a dissecting microscope. Biomass N of
196 protozoa and bacterivorous nematodes were calculated according to Stout & Heal (1967) and
197 Sohlenius & Sandor (1987), respectively. N mineralization (excretion and turnover) of
198 microfauna was calculated assuming bacterivore turnover rate of 0.16 d⁻¹ (Zwart *et al.* 1994)

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3 199 for both nematodes and protozoans and setting yield ratios of protozoans and nematodes on
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5 200 bacteria to 0.4 and 0.04 (Coleman *et al.* 1978). The total biomass of enchytraeids was
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7 201 determined as described by Maraldo *et al.* (2008). The density of microarthropods was
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9 202 determined as described by Holmstrup *et al.* (2007), and the biomass of dominating
10
11 203 taxonomic groups was estimated using representative values from Petersen & Luxton (1982).
12
13 204 The pool of N contained in the biomass was calculated assuming nitrogen concentrations of
14
15 205 11.5 % and 10 % of dry weight in enchytraeids and microarthropods, respectively (Persson
16
17 206 1983). The yearly turnover rate of biomass N was assumed to be 1.2 for enchytraeids
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19 207 (Standen 1973), and 1.8 for microarthropods (De Ruiter *et al.* 1993). The annual
20
21 208 mineralization (excretion and turnover) of NH_4^+ -N by enchytraeids and microarthropods was
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23 209 estimated based on biomass as described by Persson (1983).
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211 *N₂O emissions and N₂O reductase*

212 Fluxes of N_2O were measured with a static chamber method using white PVC chambers
213 (height 15 or 45 cm depending on vegetation height) placed on metal collars (60 × 60 cm)
214 permanently installed in the soil. Enclosure times were 2 to 2.5 h, during which headspace air
215 was sampled four times and later analyzed for N_2O concentration by gas chromatography.
216 The N_2O flux was calculated using linear regression. Nine N_2O measuring campaigns
217 including all eight treatments were carried out from June 2006 to March 2007, and from April
218 to June 2007 five campaigns were conducted in five of the treatments, *viz.* A, CO_2 , D, DCO_2
219 and TDCO_2 . Annual cumulative N_2O fluxes were derived by interpolation between
220 measurement days and by extrapolating the emission from the treatments T, TD and TCO_2 for
221 the period March- June 2007. Further methodological description is found in Carter *et al.*
222 (2010).

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3 223 To determine N₂O reductase activity, nitrate was removed from sub samples of the
4
5 224 soil cores taken in November 2007 for fauna biomass estimation by vortexing 10 g fresh soil
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8 225 and 30 ml of phosphate buffered saline (PBS) for 5 sec followed by centrifugation for 10 min
9
10 226 at 3500 x G and 5 °C. The supernatant was discarded and the pellet resuspended in 30 ml
11
12 227 PBS. After the third centrifugation the pellet was resuspended in 15 ml of 0.5 mM glucose,
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14
15 228 0.5 mM sodium acetate, and 0.5 mM sodium succinate and transferred to a 100 ml incubation
16
17 229 bottle. The bottle was sealed with a butyl rubber stopper, flushed with N₂ for 2 min, added
18
19 230 100 ppm N₂O (final concentration), placed horizontally on a shaker (200 rpm), and incubated
20
21 231 at 22 °C. After 0, 1, 3, 6 and 24 h, 3 ml of headspace was transferred to a pre-evacuated 3 ml
22
23 232 Venoject vial before analysis of N₂O by gas chromatography. The N₂O reductase activity was
24
25 233 calculated from the consumption of N₂O during the incubation.
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33 235 *Nitrogen mineralization and immobilization*

36 236 Gross rates of N mineralization and immobilization were determined by the isotope pool-
37
38 237 dilution approach applied in laboratory incubations. Soil cores within each plot were sampled
39
40 238 in September 2007 (0-5 and 5-10 cm soil depth, respectively), sieved and stored at 5 °C for
41
42 239 about one week until incubations were initiated. Fresh samples (10 g) were weighed in 250 ml
43
44 240 poly-ethylene bottles and mixed thoroughly with 400 µl of a ¹⁵N-NH₄Cl solution (5 atm %
45
46 241 ¹⁵N) to provide 5 µg N g⁻¹ soil. One set of samples was analyzed immediately upon substrate
47
48 242 application, and a second set was analyzed after 7 days of incubation at 20 °C. Contents of
49
50 243 mineral N and the ¹⁵N contents were determined upon extraction and micro-diffusion
51
52 244 (Sørensen & Jensen 1991). Gross daily rates of mineralization and immobilization were
53
54 245 calculated according to equations given by Kirkham & Bartholomew (1954). Upscaling to
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3 246 annual rates was done using the mean annual soil temperatures in 5 cm depth (10 °C) at the
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6 247 experimental site and assuming a Q_{10} of 2.
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12 249 *Potential nitrification and denitrification*
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15 250 Subsamples from the soil cores taken in November 2007 were gently homogenized by hand
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17 251 and major roots were removed. Soil samples were stored at 4 °C until analysis (less than 24
18
19 252 h). Potential nitrification was determined in 3 g soil samples by a modification of the method
20
21 253 described by Belser & Mays (1980) but without adding chlorate. Potential nitrification rates
22
23 254 were estimated from the increase in nitrite+nitrate concentrations. Potential denitrification
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25 255 rates (Wolsing & Prieme 2004) in 10 g soil samples were estimated from linear regression of
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27 256 increase in headspace N_2O concentration during 180 min incubation.
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36 258 *Water percolation and nitrogen leaching*
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39 259 Leachate water was collected monthly from passive PVC soil water draining collectors below
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41 260 the organic soil layer (approximately 5 cm depth) and polytetrafluoroethylene (PTFE) suction
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43 261 cups with continuous sub-atmospheric pressure (Prenart Super Quarz, Frederikberg, DK)
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45 262 below the main rooting zone (60 cm depth). Concentrations of NH_4^+ -N, NO_3^- -N, and total N
46
47 263 (by UV-persulphate oxidation) were analyzed on an Autoanalyzer 3 (Bran+Luebbe GmbH,
48
49 264 Germany). DON was calculated as total N minus inorganic N. Precipitation was measured at
50
51 265 the site and the volume of percolating water was estimated by simulation with the CoupModel
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53 266 model (Jansson & Moon 2001; Svensson *et al.* 2008) – a coupled ecosystem model with a
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55 267 balance of abiotic and biotic processes in the soil-plant-atmosphere system. The basic
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57 268 structure of the model is a depth profile of the soil, and calculations of water and heat flows
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3 269 are based on common soil properties. The model was parameterised using measured soil layer
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5 270 properties and against time series of soil temperature and moisture content, using on site
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8 271 measurements of radiation, precipitation and wind speed as driving data and a generalised
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10 272 likelihood uncertainty evaluation approach. Percolation of water below 60 cm was calculated
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12 273 as an output of the simulations and leaching of nitrogen at that depth was calculated as the
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15 274 product of the sum of water percolation and the concentration measured in the soil water for
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17 275 each water collection period (Schmidt *et al.* 2004).
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24 277 *Statistical analyses*
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27 278 Statistical analyses were conducted using the multiple linear regression procedure PROC
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29 279 MIXED of SAS (SAS Institute 2003). The statistical model included a random statement that
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31 280 accounted for the experimental design (*Random* Block octagon octagon*D octagon*T). The
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33 281 same model was used for all tested variables and included the three main factor effects (CO₂,
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35 282 D, and T) as well as all possible interactions (D*CO₂, T*CO₂, T*D, and T*D*CO₂). P-values
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37 283 ≤ 0.05 were considered significant. Data were transformed when necessary to obtain
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39 284 normality and homogeneity of variance. Differences of Least Squares Means (DLSM) were
40
41 285 used to interpret significant treatment interactions. During the DLSM evaluation, each
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43 286 significant interaction was categorized as either *antagonistic*, *i.e.* the combined effect led to a
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45 287 reduction of the observed individual effects or *synergistic*, *i.e.* the combined effect led to
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47 288 either a) an amplification of the observed individual effect(s) or b) to a significant effect only
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49 289 when treatments were combined. The DLSM evaluation also revealed if interactions were due
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51 290 to significant single treatment effects, *i.e.* effects observe in single treatment plots only, which
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53 291 disappear in combination with other treatments. Furthermore, DLSM evaluation of significant
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55 292 interactions revealed if significant main factor effects were effects observed only when in
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3 293 combination with other treatments or if it was significant also as a single treatment effect.
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5 294 Finally, we noted simple additive effects when two of the treatments both showed significant
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8 295 main factor effects but with no interactions.
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For Review Only

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297 **Results**298 *Ecosystem N balance*

299 The N cycle at the site is dominated by higher internal N turnover in comparison to the inputs
300 and losses by leaching and gaseous emission to the atmosphere (Fig. 1). Gross mineralization
301 ($6.7 \pm 0.96 \text{ g N m}^{-2}\text{y}^{-1}$) is five times higher than bulk deposition ($1.35 \pm 0.04 \text{ g N m}^{-2}\text{y}^{-1}$), an
302 order of magnitude higher than losses by leaching ($0.80 \pm 0.37 \text{ g N m}^{-2}\text{y}^{-1}$) and two orders of
303 magnitude higher than gaseous losses in form of N_2O ($0.031 \pm 0.006 \text{ g N m}^{-2}\text{y}^{-1}$). Negligible
304 N_2O reductase activity was observed in the soil samples with N_2O uptake rates less than 1 ng
305 $\text{N g}^{-1} \text{ dw h}^{-1}$ in all samples and N_2 production is therefore assumed to be negligible (data not
306 shown). Above ground, about 90 % of N is found in the two dominant species *Calluna* (~60
307 %) and *Deschampsia* (~30 %). Below ground, most N is stored in the SOM pool (~90 %) with
308 *Deschampsia* roots (~4 %), *Calluna* roots (~3 %) and soil microbial (~1.5 %) pools
309 dominating the remaining fraction. Soil fauna biomass was ~10 % of microbial biomass but
310 their mineralization of N is estimated to $5.0 \pm 0.6 \text{ g NH}_4^+\text{-N m}^{-2}\text{y}^{-1}$, *i.e.* an amount of labile N
311 much larger than the N input to the system by N bulk deposition. The system retained 0.53 g
312 $\text{N m}^{-2}\text{y}^{-1}$ of bulk deposition in the ambient, non-manipulated plots in 2007 as estimated by
313 difference between bulk deposition and sum of total N leaching below 60 cm soil depth and
314 N_2O emission. While bulk deposition in 2006 ($1.27 \pm 0.07 \text{ g N m}^{-2}\text{y}^{-1}$) was similar to the
315 deposition in 2007 ($1.35 \pm 0.04 \text{ g N m}^{-2}\text{y}^{-1}$), total N leaching across all treatments was
316 significantly lower ($P < 0.0001$) in 2006 ($0.13 \pm 0.06 \text{ g N m}^{-2}\text{y}^{-1}$) than in 2007 ($0.56 \pm 0.20 \text{ g}$
317 $\text{N m}^{-2}\text{y}^{-1}$) with no significant treatment effects in either 2006 (data not shown) or in 2007
318 (Table 1). The major cause of this interannual difference was a wet summer in 2007 (202 mm

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3 319 precipitation in July) with extensive leaching compared to a dry summer in 2006 (60 mm
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6 320 precipitation in July) when summer leaching was absent.
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11 322 *Plant responses to climate manipulations*
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15 323 The above-ground biomass N of the dominant plant species *Calluna* and *Deschampsia* did not
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17 324 respond to the climate manipulations (Table 1). Root biomass N of *Deschampsia* was also
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19 325 unaffected by the treatments, whereas root biomass N of *Calluna* increased in response to
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21 326 drought (Table 1, $P = 0.04$) indicating a change in the root:shoot N allocation pattern. While
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23 327 N in above-ground biomasses were unaffected by treatments, the C/N ratios of *Calluna*
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25 328 flowers as well as leaves from both *Calluna* and *Deschampsia* increased in response to
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27 329 elevated CO₂ (Table 1, $P = 0.02$, 0.04 , and 0.01 , respectively). The evaluation by DLSM of
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29 330 the three-way interaction for the C/N ratio of mosses (Table 1, $P = 0.003$) showed that the
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31 331 interaction was due to significantly higher C/N ratios in the single treatments of drought and
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33 332 elevated CO₂, while ratios were unchanged when drought and elevated CO₂ were combined
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35 333 with each other as well as when they were combined with warming. The observed increases in
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37 334 C/N ratios in new plant tissue of leaves and flowers were not seen in whole-plant C/N ratios
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39 335 of *Deschampsia* or in the green tissue or wood fractions of *Calluna*. Yet, the N concentration
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41 336 of *Deschampsia* litter decreased under elevated CO₂ (Table 1, $P = 0.05$), indicating that the
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43 337 changes in C/N ratios starting in the leaves, are already beginning to cascade through the
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45 338 system, after only two years of treatments (Fig. 2a).
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53 339 *Deschampsia* leaves and also mosses (single treatment only) responded to drought
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55 340 with increased C/N ratios (Table 1, $P = 0.002$ and $P \leq 0.05$, respectively) whereas *Calluna*
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57 341 leaves were unaffected (Table 1, Fig. 2b). Also, the pool of N in the annually produced
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59 342 *Deschampsia* litter decreased in response to drought (Table 1, $P = 0.01$). The warming
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3 343 treatment had fewer effects on above-ground processes than the other treatments, showing
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5 344 only an increase of N concentration in *Deschampsia* litter (Table 1, P = 0.03, Fig 2c).
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9 345 When all treatments were combined (Fig. 2d), the lack of interactions between single
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11 346 treatment effects for leaf level C/N ratios indicates that the effect is found also in treatment
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13 347 combinations. Consequently, increased C/N ratios of leaves as well as decreased
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15 348 *Deschampsia* litter N production are found in the full combination of treatments, *i.e.* in the
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17 349 simulation of the projected future climate scenario. In contrast, the C/N ratio of mosses
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19 350 increases in two of the three single treatments and N concentrations in *Deschampsia* litter
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21 351 either increase or decrease depending on treatment but these effects disappear when all
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23 352 treatments are combined.
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29 354 *Responses of below-ground processes to climate manipulations*

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32 355 The below-ground N turnover was significantly affected by the climate manipulations,
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34 356 primarily by the warming and drought treatments (Table 1, Fig. 3) showing changes
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36 357 cascading through pools and processes. Elevated CO₂ as a main factor had no effects below
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38 358 ground but the analysis of DLSP when evaluating the interactions revealed reductions in
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40 359 leachate concentrations in 5 cm soil depth of NO₃⁻-N, NH₄⁺-N and DON (Table 1, Fig. 3a) in
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42 360 the single CO₂ treatment. These effects were reduced when elevated CO₂ was combined with
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44 361 other treatments causing the significant interactions for these three variables (Table 1).
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52 362 The drought treatment reduced the N pools of enchytraeids and protozoans and their
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54 363 NH₄⁺-N mineralization (Table 1, P = 0.03 and 0.04, respectively), decreased gross
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56 364 mineralization (Table 1, P = 0.03), and reduced the pool of NH₄⁺-N (Table 1, P = 0.02), thus
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58 365 indicating an overall decrease in the N turnover in response to drought (Fig. 3b). In contrast,
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60 366 warming stimulated microbial ammonium consumption in the top 5 cm of soil (Table 1, P =

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3 367 0.04) as well as the gross mineralization rate (single treatment only, Table 1, $P \leq 0.05$) and
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5 368 nitrification rates (Table 1, $P = 0.004$), leading to both increased denitrification (Table 1, $P =$
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8 369 0.02) and N_2O emission (Table 1, $P = 0.05$). Overall, the below-ground response to the
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10 370 warming treatments was therefore an increase in N turnover (Fig. 3c).
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13 371 While no direct main effects of elevated CO_2 were observed, it influenced the
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15 372 responses to drought and warming primarily by reducing some of the significant individual
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17 373 effects in the three-factor combination. Still, the drought-induced decrease of fauna-N and
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19 374 fauna N mineralization as well as the warming-induced increased denitrification did not
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21 375 interact significantly with the other treatments and consequently remained in the full
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23 376 treatment combination.
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30 31 32 378 *Interactions between climate manipulations*

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35 379 Across the 47 variables reported (Table 1) the drought treatment lead to the highest number of
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37 380 significant main effects (11) followed by warming and CO_2 treatments (6 and 4, respectively).
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39 381 Simultaneously there were a large number of interactions between the three treatments (14).
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41 382 Interestingly, the analysis of the interactions revealed a dominance of antagonistic effects
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43 383 (Fig. 4) with more than twice as many antagonistic effects as synergistic effects. Furthermore,
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45 384 simple additive effects were less frequent than both antagonistic and synergistic effects.
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386 **Discussion**387 *Overall ecosystem N turnover*

388 The N cycle at the experimental site is characterized by higher internal N turnover than bulk
389 N deposition and losses by leaching and N₂O production as generally seen in natural and
390 semi-natural ecosystems. In comparison to our estimated annual gross mineralization rate (6.7
391 ± 0.96 g N m⁻²y⁻¹), Emmett *et al.* (2004) reported net mineralization rates of 3.8 to 6.6 g N m⁻²
392 y⁻¹ from another Danish dry heathland and Aerts (1993) reported rates ranging from 4.4 to
393 12.6 g N m⁻²y⁻¹ under different plant species in wet and dry Dutch heathlands, where
394 atmospheric deposition is 2-3 times higher than in Denmark. Inter-annual variability in N
395 leaching was significant, while treatment differences after two years of treatments were not
396 significant. The observed difference in leaching between 2006 and 2007 shows that the
397 ecosystem N loss by leaching is highly dependent on the amount and timing of precipitation.
398 In a future climate with more precipitation distributed in fewer, but more severe rain events, N
399 leaching is likely to increase in this ecosystem. The inter-annual variability in leaching may
400 also indicate that the ecosystem is close to N saturation as also suggested from an adjacent
401 short-term experiment where N and phosphorus (P) addition resulted in only a modest
402 increase in fine root biomass of *Deschampsia*, and no effects on *Calluna* roots (Nielsen *et al.*
403 2009). Furthermore, *Deschampsia* responded more when both N and P were added indicating
404 that this species is currently more limited by P than N availability. If nitrogen availability
405 should decrease due to progressive nitrogen limitation as the treatment exposure continues,
406 this, in combination with the P limitation for *Deschampsia*, would be beneficial to the slower
407 growing *Calluna*. However, other climate factors, *i.e.* water availability and temperature

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3 408 increase, are likely to influence the overall competitive relationship, potentially disturbing the
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5 409 balance of the equilibrium, especially if the rates of mortality of *Calluna* are increased.
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9 410 The mineralization of $\text{NH}_4^+\text{-N}$ by soil fauna is often neglected but contributes
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11 411 significantly to the pool of plant available N in the soil (Osler & Sommerkorn 2007). In the
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13 412 present study, we calculated fauna N mineralization from estimated biomasses and literature
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15 413 values of the turnover times and yields of microfauna and mesofauna. Obviously, upscaling of
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17 414 *e.g.* enumeration of bacterivorous protozoa and nematodes to gross nitrogen mineralization
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19 415 should be evaluated with caution. Values for bacterivore biomass, turnover, and growth yield
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21 416 on bacteria are all needed to perform the upscaling and very few references exist in the
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23 417 literature on such values. This said, the value for fauna-related N mineralization in the
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25 418 ambient treatment based on this calculation seems trustworthy based on the gross N
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27 419 mineralization from the pool dilution assay of $6.7 \text{ g N m}^{-2}\text{y}^{-1}$. The assumption that most
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29 420 bacterial mineralization is facilitated through predation and mineralization by the soil fauna
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31 421 implies that N mineralization by bacteria is responsible for approximately 75% of the total
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33 422 activity, the rest being due to fungi.
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43 424 *Responses to elevated CO₂, warming and drought*

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46 425 Elevated atmospheric CO₂ concentration is known to induce down-regulation of
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48 426 photosynthetic capacity, *e.g.* via reduced content of Rubisco in the leaves (Ainsworth & Long
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50 427 2005; Ainsworth & Rogers 2007) and reduction of leaf nitrogen content and increased C/N
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52 428 ratios of leaves (Paterson *et al.* 1999; Lutze & Gifford 2000; van Heerwaarden *et al.* 2005;
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54 429 Finzi *et al.* 2006; Chen *et al.* 2007; Hovenden *et al.* 2008). At our experimental site, we
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56 430 observed reduced photosynthetic capacity (Albert *et al.* 2010) and leaf nitrogen under
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58 431 elevated CO₂ for the dominant species, *Calluna* and *Deschampsia*, as well as increased leaf
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3 432 C/N ratios. However, the increased C/N ratios observed in leaves, and for *Calluna* also
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5 433 observed in flower biomass were after two years of treatments not large enough to affect
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8 434 whole plant biomass C/N ratios, which were not affected by the treatments. Previous studies
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10 435 have found increasing C/N ratios in other plant tissues than leaves (McGuire *et al.* 1995;
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12 436 Cotrufo *et al.* 1998), but the change often is much weaker than that observed for leaves
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15 437 (McGuire *et al.* 1995), indicating that the C/N effect of elevated CO₂ is closely linked to the
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17 438 processes involved in the carbon sequestration in the photosynthetic apparatus (Körner 2000).
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19 439 As hypothesized, however, N concentration in *Deschampsia* litter after two years of
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21 440 treatments was also reduced by elevated CO₂, possibly showing that the effect is beginning to
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23 441 cascade from leaf level to other ecosystem N pools, although the effect disappears in the full
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25 442 treatment combination. Together with the observed increased concentration of tannins
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27 443 (Schmidt *et al.* 2007) in plant tissues, we anticipate that the changes in C/N ratios will affect
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29 444 litter decomposition rates as the experimental treatments continue.
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34 445 In contrast to our expectations, the above- and below-ground plant N pools were not
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36 446 increased by elevated CO₂. However, total root biomass N of *Calluna* increased unexpectedly
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38 447 in response to drought. It is inherently difficult to estimate total roots from small soil samples
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40 448 due to the large spatial heterogeneity in root distribution and, at the same time, collection of
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42 449 larger samples is problematic due to the destruction of the experimental plots. We are
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44 450 therefore reluctant to put too much emphasis on this result and conclude overall that total
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46 451 biomass N of both dominant species as well as mosses after two years of treatments were only
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48 452 marginally affected by the applied climate drivers.
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54 453 Increased rhizodeposition is a common plant response to elevated CO₂ (Pendall *et al.*
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56 454 2004; Allard *et al.* 2006), and strong effects on below-ground C cycling have been observed
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58 455 in response to elevated CO₂ in the current experiment, *e.g.* increased soil respiration rates
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60 456 (Selsted *et al.* 2010) and increased production of dissolved organic carbon (Andresen *et al.*

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3 457 2010a). However, in contrast to our expectation, we observed very few direct effects of
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5 458 elevated CO₂ on below-ground N processes. Still, the decreased concentrations of organic and
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7 459 inorganic N leachate water at 5 cm soil depth imply that mineralization processes are affected
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10 460 or possibly that root N uptake has increased. In contrast to the lack of direct changes in
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12 461 response to elevated CO₂, soil processes were more sensitive to the drought and warming
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14 462 treatments. Our study supports previous observations of increased below-ground N turnover
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16 463 in response to warming (Rustad *et al.* 2001; Schmidt *et al.* 2004). We observed increased
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18 464 microbial NH₄⁺-N consumption, gross mineralization, potential nitrification, and
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20 465 denitrification from lab incubations of soils, as well as increased *in situ* N₂O emissions. Soil
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22 466 and litter incubations *in situ* also showed that warming promoted N cycling (Andresen *et al.*
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24 467 2010b). Previous field studies with artificial warming also showed no warming effect on N₂O
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26 468 emission rates (Peterjohn *et al.* 1994; Mchale *et al.* 1998). However, we interpret our
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28 469 observation of five variables that all responded in the same direction to warming as a strong
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30 470 indicator that the warming effect on N turnover was real.
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37 471 The below-ground N turnover was reduced by drought as observed by reduced gross
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39 472 N mineralization and fauna N mineralization. Reduced N mineralization in response to
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41 473 drought has previously been observed (Emmett *et al.* 2004; Schmidt *et al.* 2004; Sowerby *et*
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43 474 *al.* 2008) but the negative effects of drought on N mineralization are often attributed directly
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45 475 to reduced microbial activity. The importance of soil fauna to the N mineralization shown in
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47 476 this study, together with the negative effects of drought on top soil layer populations of
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49 477 enchytraeids (Maraldo *et al.* 2010) and other fauna groups and their decreased N
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51 478 mineralization rates, indicate that the fauna response plays a significant role in the observed
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53 479 decrease in N turnover in drought-exposed plots. Furthermore, while the warming effects
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55 480 were generally reduced in combination with drought and elevated CO₂, the negative effect of
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57 481 drought on fauna N biomass and N mineralization was not reduced by interactions.
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3 482 Consequently, the effect prevailed in the full combination of treatments, indicating that N
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5 483 mineralization will be reduced in the full future climate scenario. Therefore, despite the fact
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8 484 that this ecosystem currently show no clear sign of N limitation of plant growth, we cannot
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10 485 rule out the possibility that progressive nitrogen limitation will play a role in the longer term,
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12 486 as the impacts of increased CO₂ on C/N ratios of leaves combined with the drought induced
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15 487 reduction of mineralization and litter N production continues to reduce the availability of N in
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17 488 the ecosystem.
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22 23 24 490 *Importance of climate driver interactions*

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27 491 We thoroughly investigated the background for all significant interactions observed in the 47
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29 492 variables reported in Table 1 by evaluating the DLSP from the mixed model statistical
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31 493 output. This analysis revealed a clear dominance of antagonistic effects over synergistic and
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33 494 simple additive effects when several treatments were combined (Table 1 and Fig. 4), *i.e.* the
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35 495 ecosystem is more robust when more than one factor is changed. For instance, the C/N ratio
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37 496 of mosses increased in both single treatments of drought and elevated CO₂ but was unaffected
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39 497 when treatments were combined. Similarly, the increased gross mineralization rate observed
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41 498 in the single warming treatment was reduced especially in combination with elevated CO₂.
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43 499 Furthermore, two of the three additive effects observed (*Deschampsia* litter N concentration
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45 500 and N₂O emissions) were additive responses with opposing directions, which therefore acts to
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47 501 reduce the observed effects in treatment combinations.
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54 502 Previous studies have investigated interactions between elevated CO₂ and *e.g.* N
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56 503 supply (Lutze & Gifford 2000; Lutze *et al.* 2000; van Heerwaarden *et al.* 2005; Reich *et al.*
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58 504 2006), warming (Peltola *et al.* 2002; Tingey *et al.* 2003; Hovenden *et al.* 2008; Dijkstra *et al.*
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60 505 2010), biodiversity (Reich *et al.* 2001; Niklaus *et al.* 2001) and water relations (Morgan *et al.*

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3 506 2004; Nowak *et al.* 2004). A review by Barnard *et al.* (2005) of 25 multi-factor studies
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5 507 concluded that interactions were generally rare. These 25 studies were dominated by
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7 508 experimentally elevated CO₂ combined with increased N supply. The lack of interactions
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9 509 could be due to the common trend that most ecosystems respond strongly to increased N
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11 510 supply, which hides weaker interactive responses. Luo *et al.* (2008) analyzed and modeled
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13 511 interactions between elevated CO₂, warming and changes in precipitation in 7 multi-factor
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15 512 studies and found that three-way interactions were rare while two-way interactions were more
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17 513 common. Generally, interactions were positive between elevated CO₂ and warming and
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19 514 negative between elevated CO₂ and reduced precipitation with respect to net primary
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21 515 production (NPP).
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28 516 A general pattern of most published studies is the focus on a few important variables,
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30 517 *i.e.* overall changes in NPP or biomasses or specific studies focusing on processes, *e.g.*
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32 518 nitrification and denitrification. As interactions are only sometimes significant, investigating a
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34 519 small number of variables prevents a more general interpretation of the importance of
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36 520 interactions. Furthermore, assuming that three-way interactions are more uncommon than
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38 521 two-way interactions, important three-way interactions could be missed when fewer variables
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40 522 are analyzed. Out of 14 interactions observed across 47 N related variables in the present
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42 523 study, 5 were three-way interactions. This suggests that insufficient interactions between
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44 524 elevated CO₂, warming and changes in precipitation were included in the ecosystem models
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46 525 applied by Luo *et al.* (2008) and reinforces the need for further multi-factor ecosystem
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48 526 manipulation experiments focusing on interactive effects.
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54 527 When analyzing the interactions, we found a predominance of antagonism in the
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56 528 combined responses. The consequence of the dominance of antagonistic interactions in
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58 529 combined climate driver responses is that ecosystem changes caused by climate change may
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60 530 be less pronounced than judged from single factor experiments. Based on these results we

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3 531 raise the hypothesis that combined effects of all future climate change are dampened
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6 532 compared to expected effects based on simple addition of single treatment responses. If this
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8 533 pattern prevails for ecosystem responses to future climate change in general it highlights 1)
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10 534 that single factor studies are likely to overestimate responses and should be evaluated with
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12 535 caution, and 2) that multi-factor field-scale ecosystem experiments are critical in order to be
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15 536 able to predict realistic response strengths as well as for developing a conceptual framework
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17 537 for understanding interactions among climate drivers. Our multi-variable approach may aid
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20 538 the latter point if tested in other multi-factor studies and by including variables not only
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22 539 constraint to the nitrogen cycle.
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56 541 **Conclusions**
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10 542 The drought treatment effects dominated over effects of warming and elevated CO₂ in
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12 543 observed responses of the nitrogen cycle after two years of treatments. In contrast to our
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14 544 expectations, we did not observe increased growth of the N pools of the dominant plant
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16 545 species, *Calluna* and *Deschampsia* in response to elevated CO₂. While previous studies have
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18 546 usually found interactions to be rare, interactions were commonly observed and antagonism
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20 547 dominated over synergism in combined responses, *i.e.* the ecosystem response to the
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22 548 combination of several climate drivers is dampened compared to single treatment responses.
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24 549 Still, the observed negative response to drought of soil fauna biomass and N mineralization
25
26 550 and *Deschampsia* litter N production prevailed in the full treatment combination and so did
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28 551 the increased leaf C/N ratios of the dominant species *Calluna* and *Deschampsia*. If persistent,
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30 552 these changes are likely to lead to reduced N availability and possibly to progressive nitrogen
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32 553 limitation as the experimental treatments continue.
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21 562 Commission.
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788 **Figure captions**

789 Figure 1

790 Measured N pools in August 2007 (**bold**, g N m⁻²) and annual fluxes (*italic*, g N m⁻²y⁻¹) in
791 ambient plots down to 10 cm soil depth (leaching below 60 cm depth) in 2007. Abbreviations:
792 *Calluna vulgaris* (Cal), *Deschampsia flexuosa* (Des), dissolved organic nitrogen (DON),
793 microbial biomass nitrogen (Mic-N), soil organic matter nitrogen (SOM-N), Gross
794 mineralization (Gross min). Data of litter N mass and litter N production was only available
795 for *Deschampsia*. Estimates of fauna biomass and mineralization includes enchytraeids,
796 microarthropods, nematodes, and protozoans. Dashed line indicates the major components
797 involved in N mineralization. The annual N balance was +0.53 g N m⁻²y⁻¹.

798

799 Figure 2

800 Observed above-ground changes in N pools, C/N ratios, litter N production of *Deschampsia*
801 and percentage N in litter of *Deschampsia* caused by single treatments factors (a – c) and
802 when all treatments are combined (d). *Calluna vulgaris* (Cal), *Deschampsia flexuosa* (Des).
803 See Table 1 for statistical significance of observed changes.

804

805 Figure 3

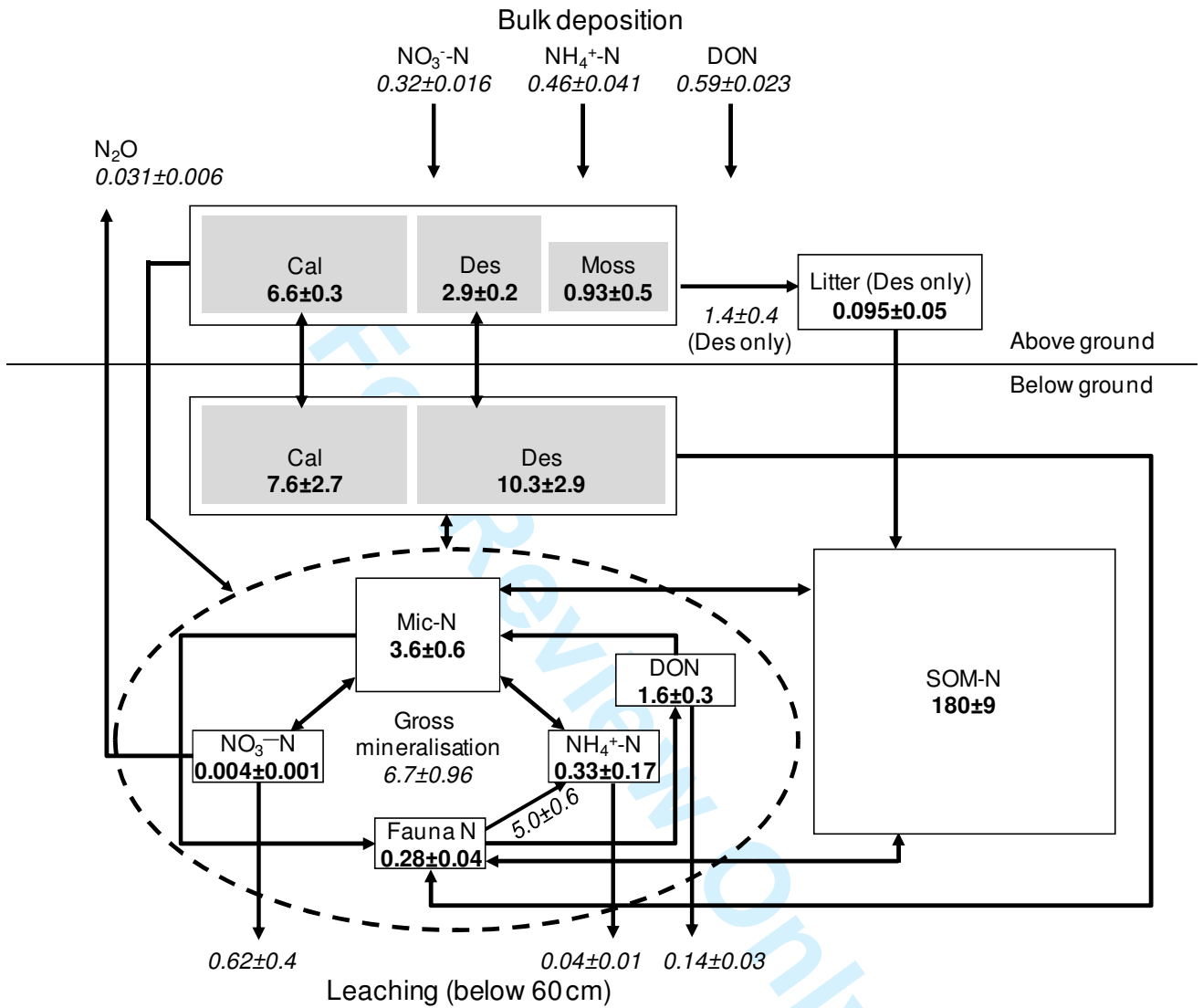
806 Observed below-ground changes in N pools, N fluxes/processes and N concentrations caused
807 by single treatments factors (a – c) and when all treatments are combined (d). Dissolved
808 organic nitrogen (DON), microbial biomass nitrogen (Mic-N), mineralization (min). See

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3 809 Table 1 for statistical significance of observed changes.
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For Review Only

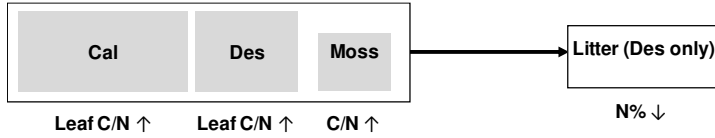
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10 812 Observed significant interactions ($P \leq 0.05$) from the PROC MIXED analysis of 47
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12 813 individually tested variables (see Table 1) followed by analysis of Differences of Least
13
14 814 Squares Means (DLSM) were categorized as antagonistic, *i.e.* combination leads to reduction
15
16 815 of effects, or as synergistic, *i.e.* combination leads to amplification of single effects or
17
18 816 significant effect is only observed in combination. Number of observed simple additive
19
20 817 effects is also shown, *i.e.* cases where two significant individual effects were observed
21
22 818 without significant interaction. Note that the sum of antagonistic and synergistic effects are 15
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24 819 because one three-way interaction (potential nitrification – see Table 1) was caused by both
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26 820 antagonistic and synergistic effects.
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Figure 1

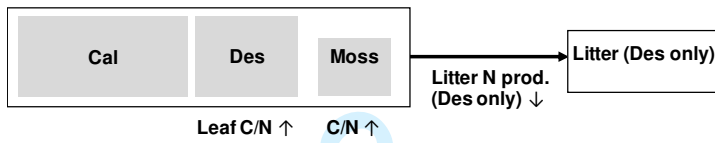


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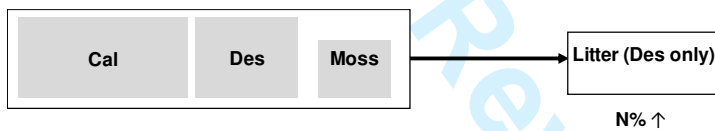
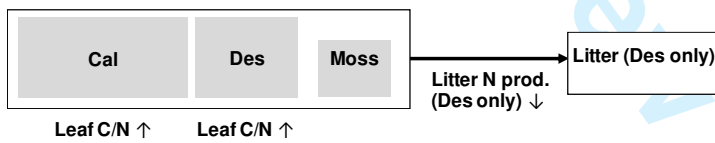
Figure 2

(a) CO₂

(b) Drought



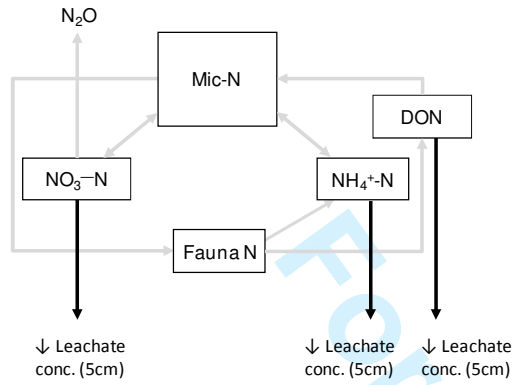
(c) Warming

(d) CO₂, drought and warming1
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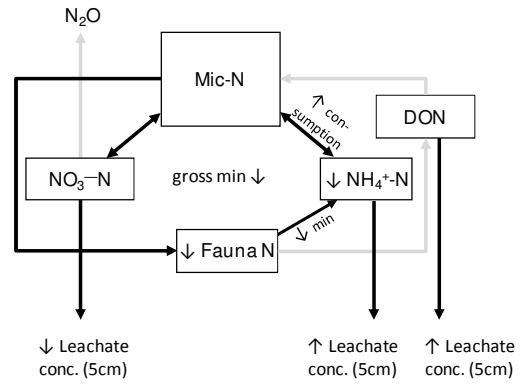
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Figure 3

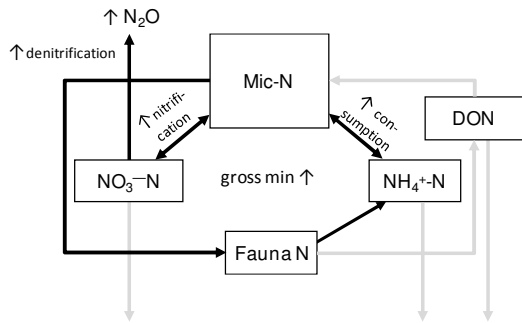
(a) CO₂



(b) Drought



(c) Warming



(d) CO₂, drought and warming

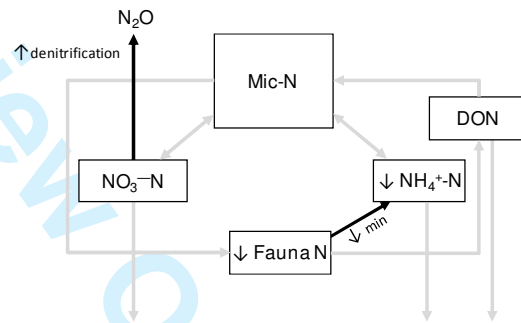
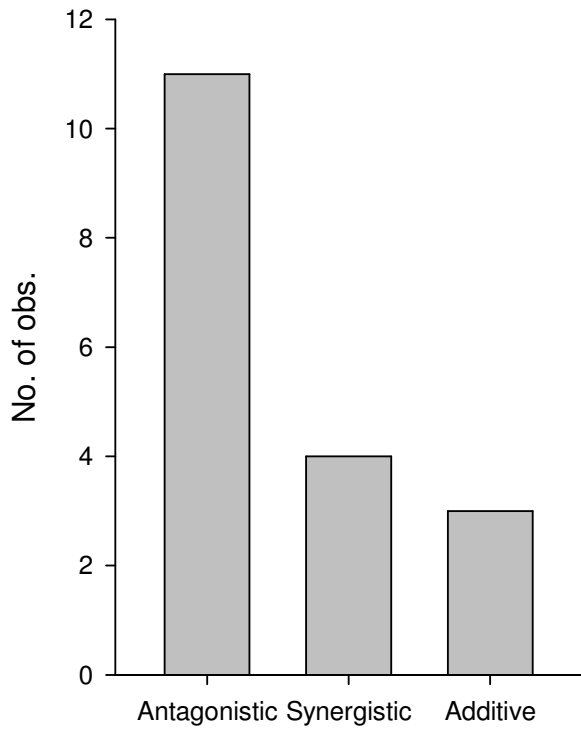


Figure 4



view Only