1 **Increased winter soil temperature variability enhances nitrogen cycling and soil biotic**
2 **activity in temperate heathland and grassland mesocosms**

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ABSTRACT

Winter air temperatures are projected to increase in the temperate zone, whereas snow cover is projected to decrease, leading to increased soil temperature variability, and potentially to changes in nutrient cycling. Here, we experimentally evaluated the effects of increased winter soil temperature variability on selected aspects of the N-cycle in mesocosms containing different plant community compositions. The experiment was replicated at two sites, a colder mountainous upland site with high snow accumulation and a warmer and dryer lowland site.

Increased soil temperature variability enhanced soil biotic activity for both sites during winter, as indicated by 35% higher nitrogen (N) availability in the soil solution, 40% higher belowground decomposition and a 25% increase in the potential activity of the enzyme cellobiohydrolase. The mobilization of N differed between sites, and the incorporation of ^{15}N into leaves was reduced by 31% in response to winter warming pulses, but only at the cold site, with significant reductions occurring for three of four tested plant species at this site. Furthermore, there was a trend of increased N leaching in response to the recurrent winter warming pulses.

Overall, projected winter climate change in the temperate zone, with less snow and more variable soil temperatures, appears important for shifts in ecosystem functioning (i.e. nutrient cycling). While the effects of warming pulses on plant N mobilization did not differ among sites, reduced plant ^{15}N incorporation at the colder temperate site suggests that frost damage may reduce plant N uptake in a warmer world, with important implications for nitrogen cycling and nitrogen losses from ecosystems.

1   Introduction

Winter soil temperature is an important driver for many ecological and biogeochemical processes in the cold-temperate and boreal zone, and it can influence the activity of plants and soil biota (Matzner and Borken, 2008; Kreyling, 2010). While microbial activity and nitrogen (N) cycling continue below freezing (Clein and Schimel, 1995; Mikan et al., 2002), higher mean soil temperatures are generally expected to cause exponentially higher soil biotic activity (Rustad et al., 2001; Melillo et al., 2002). Consequently, winter warming can result in increased N mineralization and N availability in the soil solution in the following growing season (Turner and Henry, 2010). Warmer soils over winter increase soil biotic activity, e.g. soil respiration, decomposition by soil fauna and microbes, higher enzymatic activity, higher N mineralization, etc. This holds true especially towards the end of winter, and can accelerate plant productivity (Schuerings et al., 2013). Since plants are capable of winter N uptake (Grogan et al., 2004; Andresen and Michelsen, 2005), their activity could counteract N leaching (Patil et al., 2010). The general effectiveness of plants in taking up N over winter, however, is not fully clear until now. Comparable N uptake rates over winter and summer have been reported for some species (Nasholm et al., 2000; Bardgett et al., 2003), but there is also evidence that cold acclimation reduces the potential for N uptake (Malyshev and Henry, 2012a).

Due to increased winter air temperatures, snow cover will decrease in many regions of the temperate zone (Christensen et al., 2007; Kreyling and Henry, 2011). However, air frost events will still occur with unchanged magnitude and duration as nowadays in many temperate regions (Kodra et al., 2011), and with less insulating snow cover, winter soil temperatures can become more variable, particularly in upland and cold temperate regions (Henry, 2008; Brown and DeGaetano, 2011). The resulting more variable soil temperature conditions with frequent soil frost and freeze-thaw cycles (FTC) can affect N cycling. Soil frost and FTC can physically damage plant roots (Tierney et al., 2001) and therefore reduce the plants ability to take up N (Campbell et al., 2014), break up soil aggregates (Oztas and Fayetorbay, 2003), and lyse microbial cells what enlarges the easily available N pool (Skogland et al., 1988), thereby affecting N cycling and leading to N losses in dissolved (Boutin and Robitaille, 1995; Brooks et al., 1998; Joseph and Henry, 2008) or gaseous forms (Matzner and Borken, 2008). For warmer, lowland temperate regions, however, although soil temperature variability might still increase (Kreyling, 2010), an increase in winter air temperatures could lead to fewer soil FTC due to less frost (e.g. lowland Germany, Kreyling and Henry, 2011). Contrasting effects of winter climate change can therefore be expected for colder (stronger effects due to greater increase in soil temperature variability) versus warmer (naturally higher soil temperature variability) temperate regions, and studies of biogeochemical responses to increased soil temperature variability should be designed to account for these differences.

Finally, plant species and vegetation types are known to influence N cycling (Hooper and Vitousek, 1998; Knops et al., 2002). Different plant species and communities further show different reactions to increased winter temperature variability in the temperate zone, with grasses appearing more responsive than dwarf shrubs (Kreyling et al., 2010; Schuerings et al., 2014) regarding their productivity, probably due to their faster life-cycle. However, this increased responsiveness in productivity of grasses can either be beneficial (Kreyling et al., 2008), or detrimental (Schuerings et al., 2014), probably depending on whether the minimum

82 temperatures experienced after warm phases induce frost damage. Altered plant productivity
83 can therefore indirectly affect N cycling. Generally, stress resistance is linked to nitrogen or
84 nutrient stress tolerance (Macgillivray et al., 1995). Moreover, increased N availability over
85 winter can increase the risk of frost damage to plants (Malyshev and Henry, 2012b).

86 In this experiment we tested the effects of more variable winter temperature conditions, i.e.
87 recurrent, short winter warming pulses, on soil biotic and potential extracellular enzyme
88 activity, N availability in the soil solution, and N uptake by plants in different plant
89 communities (grassland, heathland; same communities as in Schuerings et al., 2014) at two
90 sites with contrasting winter climate (a warm, snow-poor lowland and a cold, snow-rich
91 upland site). We hypothesised that (1) recurrent winter warming pulses would enhance N-
92 cycling (i.e. increased N availability, soil biotic activity and N uptake into plants). (2) We
93 further expected different responsiveness to the recurrent warming pulses at the two sites,
94 with more variable soil temperatures and stronger frost, therefore frost damage negatively
95 affecting plant N uptake at the colder upland site. (3) Finally, we expected differences among
96 the plant communities in the response of N cycling to the recurrent warming pulses, with a
97 higher ability for winter N uptake in grassland than in heathland plants.

98

99 **2   METHODS**

100 2.1   Experimental design and site description

101 This research is part of the EVENT IV experiment, testing the effects of increased winter
102 temperature variability on temperate heath and grassland communities. The effects of the
103 recurrent warming pulses on plant growth (above- and below-ground) are summarized in
104 Schuerings et al. (2014), whereas here we concentrate on nitrogen cycling. The experiment
105 was replicated at two sites: the warm site was located in the Ecological-Botanical Garden of
106 the University of Bayreuth (49° 55' 36.32" N, 11° 34' 57.28" E, 358 m a.s.l.) and the cold site
107 was located at the Waldstein mountain in the Fichtelgebirge (50° 8' 35.81" N, 11° 51' 50.92"
108 E, 781 m a.s.l.). The cold site generally experiences more precipitation and harsher winter
109 conditions (Table 1).

110 The experiment consisted of three fully crossed factors: (1) increased winter temperature
111 variability by application of winter warming pulses versus ambient reference conditions, (2)
112 two experimental sites with naturally different winter climate, (3) six different plant
113 communities and an additional bare ground control. The plant communities consisted of
114 three grassland communities (monocultures of the grass *Holcus lanatus* (L.) and the herb
115 *Plantago lanceolata* (L.), and a community with a mix of both species) and three heathland
116 communities (monocultures of the dwarf shrub *Calluna vulgaris* (L.) and the grass
117 *Deschampsia flexuosa* (L.) and a community with a mix of both species). All species present
118 in this experiment are very common perennial species in Central Europe. In addition, there
119 was a bare ground control in every block. Plant communities were blocked and randomly
120 assigned to the winter warming pulses manipulation and ambient reference. Temperature
121 manipulation blocks, and therefore each factorial combination, were replicated five times.
122 This setup was fully replicated at both experimental sites. For the 140 plots, plastic barrels
123 with 0.2 m^2 surface (50 cm diameter) and 80 cm depth were used as mesocosms. Each of
124 the six mesocosms per treatment was placed in a corner of a hexagon, with 30 cm distance

125 between mesocosms and at least 50 cm separation from the hexagon edge. The bare
126 ground control was placed in the middle of the hexagons. All space between the mesocosms
127 was filled with the same substrate as used within the mescosms. The soil substrate was
128 homogenized loamy sand (77% sand, 16% silt, 7% clay) from a nearby sand quarry (where
129 all used plant species naturally occur), with a pH=7.35 (measured in 1 M KCl) and a total
130 carbon content of 2.37%. The barrels were attached with outlet hoses at the bottom of each
131 mesocosm, so that the mesocosms functioned as zero tension lysimeters. Sixteen plants per
132 mesocosm were planted in a systematic grid in May 2010. All plants were grown from seed
133 in January 2010, except for the dwarf-shrub *C. vulgaris*, which was obtained as 2-year old
134 individuals in February 2010.

135 2.2   Manipulation of winter temperature variability

136 Winter warming pulses were applied with six IR-heating lamps (250 W) located in between
137 the mesocosms at a height of 60 cm and surface heating wires (distance 20 cm, 400 W per
138 block), which resulted in 1900 W per block (7 mesocosms). The ambient reference
139 mesocosms were equipped with dummy lamps. Six warming pulses were administered
140 simultaneously for both sites between 15 December 2010 and 28 February 2011 (see Fig. 1).
141 Warming pulses were administered when there was soil frost at both sites and weather
142 forecast predicted further air frost for at least the next 48 h.

143 Soil temperature (-2 cm; once in every treatment and reference block; 10 measurements per
144 site and 20 in total) and air temperature (+5 cm; one treatment and reference block per site;
145 2 measurements per site and 4 in total) were measured hourly by thermistors (B57863-S302-
146 F40, EPCOS AG, Germany) connected to a datalogger (dl2, Delta-T Devices Ltd, UK). To
147 quantify the effect of the warming pulses treatment on soil temperature variability, we
148 calculated the coefficient of variation (CV = standard deviation x hourly mean^{-1} x 100;
149 temperatures were converted to K for this). Snow height was measured each morning via a
150 webcam picture of a measuring stick.

151 2.3   Response parameters

152 Plant available N was measured via the resin stick method (Plant-root-simulator (PRS^{TM})-
153 probes; Western Ag Innovations Inc., Canada). Two cation and two anion PRS^{TM}-probes
154 were installed vertically with a distance of 20 cm to each other (0 -15 cm depth) per
155 mesocosm prior to the warming pulse manipulation on 18 December 2010 and collected on
156 17 March 2011 after the winter warming pulses treatment. PRS^{TM}-probes were cleaned and
157 kept in a fridge until being sent to Western Ag Innovations Inc. (Canada) in a cool box for
158 analysis. For the statistical analysis, nitrate and ammonium were pooled due to low
159 ammonium concentrations. The maximum ion capacity of the probes for nitrate is 2088 µg 10
160 cm^{-2}. The values in our study are far lower, showing that the system was not saturated. Since
161 NO_3/NH_4 uptake by resin sticks is not a linear process, values are given for the whole burial
162 period.

163 Soil biotic activity, i.e. decomposition by microorganisms and feeding by soil fauna, was
164 measured via bait-lamina sticks (terra protecta GmbH, Germany) (Kratz, 1998). One bait-
165 lamina stick containing 16 baits was inserted vertically in the top soil layer of every
166 mesocosm prior to the warming pulses treatment on 18 December. The baits consisted of a
167 mixture of powdered cellulose, bran flakes and active coal. These baits are potentially eaten

by earthworms, macro- to micro arthropods and additionally are decomposed by soil
microorganisms. The sticks were collected after the winter warming pulses treatment on 17
March, cleaned, and the number of eaten baits was counted. For the latter, sticks were
placed on a light bench and when light shined through the baits they were counted as eaten.
This analysis was done by a single person who was blind to the factors.

For the potential extracellular enzymatic activity (PEEA), which we used as another proxy for
soil biotic activity and decomposition, three soil samples (2 cm diameter, 10 cm depth) per
mesocosm were collected and mixed for assays of potential extracellular enzyme activity in
soil on 21 February 2011. Soil samples were stored in airtight plastic zip-bags at 4°C and
were analysed within 3 days. PEEA assays were carried out with Methylumbelliferone
substrates (MUF) (Pritsch et al., 2004; Pritsch et al., 2005). The following PEEAs were
measured: MU-β-D-glucopyranoside (MU-G), for β-glucosidase, MU-β-cellobioside (MU-C)
for cellobiohydrolase, MU-β-D-xylopyranoside (MU-X) for xylosidase, MU-phosphate (MU-P)
for acid phosphatase. Substrates and calibration saturation and incubation times were
determined in pre-experiments (data not shown) as follows: MU-G and MU-X each 500 μM
incubating for 60 min, MU-C 500 μM incubating for 120 min, MU-P 800 μM incubating for 40
min. Fluorescence was detected at an excitation wavelength of 360 nm and an emission
wavelength of 450 nm with a Gemini EM Fluorescence Microplate Reader from Molecular
Device, California.

Prior to the warming pulses treatment (18 December 2010), plots were labelled with 0.02 g
Potassium Nitrate-^{15}N (min. 99.19 atom % 15N; Campro Scientific GmbH, Germany),
dissolved in 250 ml deionized water, resulting in 0.1 g ^{15}N m^{-2}. Leaf (2-3 medium aged leaves
per plot and species, randomly chosen), root (fine roots from a soil sample taken directly next
to a randomly chosen plant per mesocosm and species) and soil samples (3 soil samples per
plot were mixed; 2 cm diameter, 10 cm depth) were taken on 17 March 2011, after the winter
warming pulses treatment. The samples were kept frozen until they were cleaned, dried (48
h at 50° C) and ball milled. Mass spectroscopy analysis was done at the laboratory of Isotope
Biogeochemistry, BayCEER, University of Bayreuth, with a combination of an elemental
analyzer (Carlo Erba NC 2500, CE Instruments, Italy) and an isotope mass spectrometer
(delta plus, Thermo Fisher Scientific, Germany). Atom % increase values for plant and soil
material collected after the winter warming pulses treatment were calculated by comparing to
values obtained from unlabelled reference plants (n = 5 per species) and soil material taken
prior to the winter warming pulses treatment (n = 3 per experimental site). Due to missing
volume readings, the isotopic signature of leachate could only be determined and related to
volume of leachate for four mesocosms (*Holcus lanatus* and *Plantago lanceolata* mixed
mesocosms at both sites for both winter warming pulses treatments), which were
permanently equipped by tipping buckets (7041.3000X, Theodor Friedrichs & Co., Germany).
Therefore, no mass balancing of the label was possible, and we report ^{15}N-atom% here. It is
important to note that above-ground biomass remained stable or decreased due to the
warming pulses treatment (Schuerings et al., 2014),

2.4   Data analyses

Linear mixed-effect models combined with analysis of variance (ANOVA) were applied to test
for significant winter warming pulses treatment, site and plant community effects. All possible
interactions of community or species and site with the warming pulses treatment were

212 included as fixed effects (s. Table 2 & 3 for all tested interactions). For the analysis of [15]N
213 content in plants, species identity was included as a fixed factor instead of community
214 composition, whereas community was included as a random effect. Block identity was set as
215 a random effect in all models, thereby accounting for the blocked design. Before statistical
216 analysis, we tested for normality and homogeneity of variance by examining the residuals
217 versus fitted plots and the normal qq-plots of the linear models (Faraway, 2005). If conditions
218 were not satisfactorily met, we applied log(x)- (plant available N; ^{15}N atom% increase of
219 leaves and roots; PEEA of beta-glucosidase, cellobiohydrolase, xylosidase), log(x+1)- ([15]N
220 atom% increase in soil), or sqrt(x)- (PEEA acid phosphatase) transformation. Significance
221 level was set to $p < 0.05$. All statistical analyses were performed using R 2.12.2 (R
222 Development Core Team 2011) and additional packages nlme (Version 3.1-98, 2011) and
223 sciplot (Version 1.0-9, 2011) for graphical illustrations.

224

225 **3   RESULTS**

226 The winter warming pulses manipulation successfully decreased snow cover and resulted in
227 increased soil temperature variability (Fig. 1). At the warm site, variation in soil temperature
228 during the manipulation period (15 December 2010 to 28 February 2011) was increased to
229 CV = 0.99 in comparison to CV = 0.66 in the reference mesocosms. Mean soil temperature
230 increased to 1.8°C in the manipulation as compared to 0.1°C in the ambient reference.
231 Minimum temperature reached -4.2 °C and -4.0 °C, respectively. For the cold site, variation
232 in soil temperature during the manipulation period increased to CV= 0.68 in comparison to
233 CV= 0.43 in the reference mesocosms. Mean soil temperature was almost unchanged with -
234 0.1°C in the warming pulses manipulation and -0.3°C under ambient reference conditions.
235 However, minimum temperature was considerably lower in the warming pulses mesocosms,
236 reaching -4.7 °C, as compared to -2.6 °C in the reference mesocosms. The number of soil
237 freeze thaw cycles was not altered noticeably at any site (warm site: 7 vs. 8, cold site: 6 vs.
238 5).

239 Plant available nitrate and ammonium significantly increased by 34.5% in response to the
240 winter warming pulses treatment (F=14.2, p<0.001; Table 2, Fig. 2). The cold site overall had
241 a 48.4% higher amount of N available than the warm site (F=20.2, p<0.001; Table 2, Fig. 2).
242 Plant community composition also influenced plant available N (F=15.4, p<0.001; Table 2,
243 Fig. 2). Bare ground control mesocosms had the highest N values, followed by the heathland
244 communities and then the grassland communities, with only monocultures of *H. lanatus*
245 reaching levels of the heathland communities. Winter warming pulse effects were not
246 influenced by site or plant community (no significant interactions, Table 2).

247 Soil biotic activity, i.e. the number of eaten baits, increased by 40% (F=17.5, p<0.001; Table
248 2, Fig. 2) due to the winter warming pulses treatment in comparison to reference conditions.
249 Soil biotic activity did not significantly differ between sites or plant communities. The warming
250 pulses effect, however, was influenced by the plant communities (F=2.3, p=0.037), with
251 slightly decreasing activities in monocultures of *P. lanceolata* and mixed communities of *C.*
252 *vulgaris* & *D. flexuosa* due to the warming pulses (Fig. 2). All other communities showed an
253 increase in soil biotic activity due to the warming pulses. No other interaction with the
254 warming pulses treatment yielded significance for soil biotic activity (Table 2).

255 Regarding PEEA there was a general trend towards higher values under the winter warming
256 pulses treatment, yet only for cellobiohydrolase was this effect statistically significant (F=5.3,
257 p=0.035). For the other three tested enzymes no significant effect of the winter warming
258 pulses treatment was observed. Generally, there were significantly higher PEEAs at the cold
259 site than at the warm site (Table 2, Fig. 3) and plant community composition effects differed
260 such that, except for acid phosphatase, grassland communities showed higher PEEA than
261 heathland communities (Table 2, Fig. 3). No significant interactions between the warming
262 pulses treatment and site or plant community were observed (Table 2).

263 The uptake and incorporation of ^{15}N into leaves was significantly reduced by 21.7% (relative
264 difference) under the winter warming pulses treatment in comparison to reference conditions
265 (F=5.9, p=0.016), whereas for root and soil material no significant winter warming pulse
266 effect was observed (Table 3, Fig. 4). For leachate, no statistical analysis was performed due
267 to the low replication, but for the existing samples (n=2 per winter warming pulses treatment),
268 a clear trend towards increased leaching of the ^{15}N-tracer was observed (Fig. 4). Generally,
269 the cold site showed significantly higher plant ^{15}N incorporation than the warm site (Table 3,
270 Fig. 4). *D. flexuosa* exhibited the highest ^{15}N incorporation, followed by *P. lanceolata*, with
271 the same pattern observed for leaves and roots. Significant decreases in the ^{15}N signal in
272 plant leaves (-30.7%) in response to warming pulses only occurred at the cold site (winter
273 warming pulses treatment x site interaction: F=8.6, p=0.004; Table 3, Fig. 4). The significant
274 three-way interaction between warming pulses treatment, site, and species identity (F=3.4,
275 p=0.004) indicated that the decrease in ^{15}N values only happened at the cold site and only
276 for three of the four species (*C. vulgaris*, *D. flexuosa* and *H. lanatus*; Fig. 4).

277

278 **4   DISCUSSION**

279 Recurrent winter warming pulses led to increased soil temperature variability and influenced
280 N cycling in our experiment. As expected, N availability was increased (+35%) in the
281 mesocosms which received the winter warming pulses treatment. Increased N availability
282 during winter/early spring is often explained by freeze-thaw events resulting in increased
283 biological and physical decomposition of soil organic matter (SOM) (Matzner and Borken,
284 2008) and increased N mineralization (Rustad et al., 2001; Melillo et al., 2002). Yet, in our
285 study FTC frequency was merely changed between winter warming pulses and references
286 plots (±1), implying that the warming pulses treatment affected N availability either through
287 increased temperature variability or the increase in mean temperature. Due to the winter
288 warming pulses soil biotic activity increased by 40%. This increase in soil biotic activity is in
289 line with results from other winter warming experiments which measured soil respiration as
290 an index of soil biotic activity (Davidson and Janssens, 2006; Allison and Treseder, 2011).
291 The soil enzymes we examined play a major role in the decomposition of biological material
292 (Marx et al., 2001). We observed significantly increased PEEA for cellobiohydrolase,
293 whereas for the other three tested enzymes the observed increases were not significant.
294 Therefore in our experiment, increased soil temperature variability led to increased biotic
295 decomposition as indicated by increased soil biotic activity and increased PEEA of
296 cellobiohydrolase. In winter warming experiments, increased N cycling is often attributed to
297 changes in the frequency of soil FTC (Mikan et al., 2002). Despite only small changes in FTC
298 frequency in our mesocosms, however, we observed increased N availability, increased soil

299   biotic and soil potential enzymatic activity. However, for the cold site, where it is important to
300   note that mean soil temperature only increased by 0.2 °C, minimum temperature was
301   considerably lower in the warming pulses mesocosms, reaching -4.7 °C, as compared to -2.6
302   °C. Since we found lowered N incorporation into plants (see discussion further down) and
303   stable or lower plant biomass (Schuerings et al., 2014) at the cold site, this could have
304   lowered N immobilization by plants. The temporal dynamics of soil temperature, in particular
305   the intensity of freezing right after warming pulses, is therefore another important
306   determinant of N cycling responses, possibly leading to frost damaging of dehardend plants.
307   While changed FTCs (Joseph and Henry, 2008), warmer mean soil temperatures (Rustad et
308   al., 2001; Melillo et al., 2002) and single extreme frost events (Elliott and Henry, 2009) are
309   known to be important drivers of N cycling, our results imply that soil temperature variability,
310   i.e. temperature dynamics, can also affect N availability and soil biotic activity.

311   We found significantly higher N availability and potential activity of all four tested potential
312   soil enzymes for the cold site despite lower mean temperatures at the site. Groffman et al.
313   (2009) found the same pattern along an altitudinal gradient in a northern hardwood forest.
314   This suggests that the local climate may have an important influence on the magnitude of N
315   mobilization processes. However, since we found no significant interaction between winter
316   warming pulses treatment and site, the effects of winter warming pulses on N availability, soil
317   biotic activity and potential soil enzymatic activity therefore appear independent of the local
318   climate.

319   The mobilization of N was influenced by the plant community composition, with the bare
320   ground control showing highest levels of available N. Since there were no roots in the bare
321   ground plots competing with the PRS[TM]-probes for N, this result is not surprising. Regarding
322   plant communities, there was no clear pattern in N availability, although the heathland
323   communities showed higher values than grassland communities with the exception of
324   monocultures of *H. lanatus*, which showed similar values as the heathland communities. The
325   interaction between the warming pulses treatment and plant community indicated that plant
326   species composition influenced soil biotic activity differently under winter warming pulses.
327   However, there was no clear pattern, since all communities showed increased soil biotic
328   activity in response to the winter warming pulses, except for monocultures of *P. lanceolata*
329   and mixed cultures of *C. vulgaris* and *D. flexuosa*. Potential soil enzymatic activity was
330   generally higher in grassland mesocosms in comparison to heathland mesocosms, with the
331   exception of acid phosphatase.

332   [15]N incorporation by plants was, contrary to our expectations, decreased by the winter
333   warming pulses treatment. Plants can lose their cold hardiness within hours in response to
334   elevated temperatures (Kalberer et al., 2006), and subsequent frost events after a winter
335   warm spell can thus damage plants substantially (Bokhorst et al., 2009). Freezing intensity is
336   also an important determinant of plant frost damage, and while most temperate species can
337   tolerate temperatures at or below freezing, there is often a threshold subfreezing temperature
338   where damage intensifies (Malyshev and Henry, 2012a). Notably, the minimum temperatures
339   reached in the reference mesocosms at the cold site were the least severe, and the greatest
340   [15]N incorporation was observed in these plots, whereas minimum soil temperatures of at
341   least -4 °C were reached in the treatment plots at the cold site and in all of the warm site
342   mesocosms, all of which featured relatively low ^{15}N incorporation. Similarly, in other systems,
343   grass ecotypes located at northern sites that are protected from cold air by thick snow cover

have developed lower frost tolerance than conspecific ecotypes located in warmer locations that feature less snow cover, because the latter ecotypes experience more intense frost (Dionne et al., 2010).

We also observed significant differences among the tested species in ^{15}N incorporation, which is not surprising, given that species exhibit wide variation in their nutrient uptake capacities (Hooper and Vitousek, 1998; Knops et al., 2002). The interesting point is that the reduction in ^{15}N incorporation only happened at the cold site and only for *C. vulgaris*, *D. flexuosa* and *H. lanatus* (interaction: winter warming pulses treatment x site x species). Above-ground biomass of all tested species remained stable or decreased in response to the winter warming pulses treatment for these species (Schuerings et al., 2014), thus dilution effects on N-tracer uptake can be excluded. Such differences among species in frost susceptibility could have important consequences for competitive balances and shifts in community composition over the long term (Joseph and Henry, 2008; Cornelissen and Makoto, 2014).

Chronic winter warming can increase above-ground biomass (Hutchison and Henry, 2010; Natali et al., 2012; Schuerings et al., 2013). This additional growth may be fuelled by increased N mobilization in early spring. Pulsed winter warming increasing the risk of frost damage, however, complicates this simple expectation of increased plant growth under winter climate change. The inability of frost-damaged plants to take up the available N in the soil solution might trigger N losses from ecosystems by N leaching or gaseous losses (Ineson et al., 1998; Campbell et al., 2014). In this experiment we also found species-specific responses in above-ground biomass production due to the winter warming pulses (Schuerings et al., 2014); only *H. lanatus* showed a decrease in above-ground biomass, whereas the other tested species remained unaffected by the winter warming pulses treatment in their above-ground productivity. Taken together, species- or vegetation type-specific responses have to be taken into account when forecasting effects of climate change on N-cycling (Makoto et al., 2014). Furthermore, regarding winter climate change, pulsed warming events can result in opposing effects on N cycling and biomass accumulation than chronic warming.

5   CONCLUSIONS

Future winters in the temperate zone are expected to be characterized by more variable soil temperatures due to increasing air temperature variability and due to missing insulation by snow. Our experiment implies that more variable soil temperatures enhance nitrogen mobilization in the soil independent from vegetation types and the local climate. Plant performance, however, depended on local climate, with plant ^{15}N immobilization during winter and early spring after exposure to winter warming pulses being reduced at colder sites, probably due to frost damage after the warming pulses. This pattern implies increased risk for nitrogen leaching at colder temperate sites in response to increased winter temperature variability. Taken together, our findings emphasize the importance of temperature variability, plant performance, and frost damage in a warmer world for nitrogen cycling and nitrogen losses from ecosystems.

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TABLES

519

520 Table 1: Climate characteristics of the two experimental sites, measured on site by the
521 department of Micrometeorology until 2008; University of Bayreuth, Prof. T. Foken
522 (Schuerings et al., 2014)
523

| Parameter (Unit; start of measurements warm site / cold site)	Warm site	Cold site
Mean annual temperature (°C; 1998 / 1994)	8.8	5.0
Mean winter temperature (DJF; °C; 1998 / 1994)	0.6	-2.0
Mean annual precipitation (mm; 1998 /1994)	717	1002
Mean winter precipitation (DJF; mm; 1998 / 1994)	158	237
Mean # of days with soil frost (-5 cm) (2003 / 1999)	19	31

524 Table 2: ANOVA-results of all tested main and interaction effects for N mobilization, i.e. N availability in the soil solution (NH_4^+ and NO_3^-), soil biotic
525 activity (bait-lamina test), and the four tested potential soil enzyme activities. Warming pulses: Winter warming pulses treatment.
526

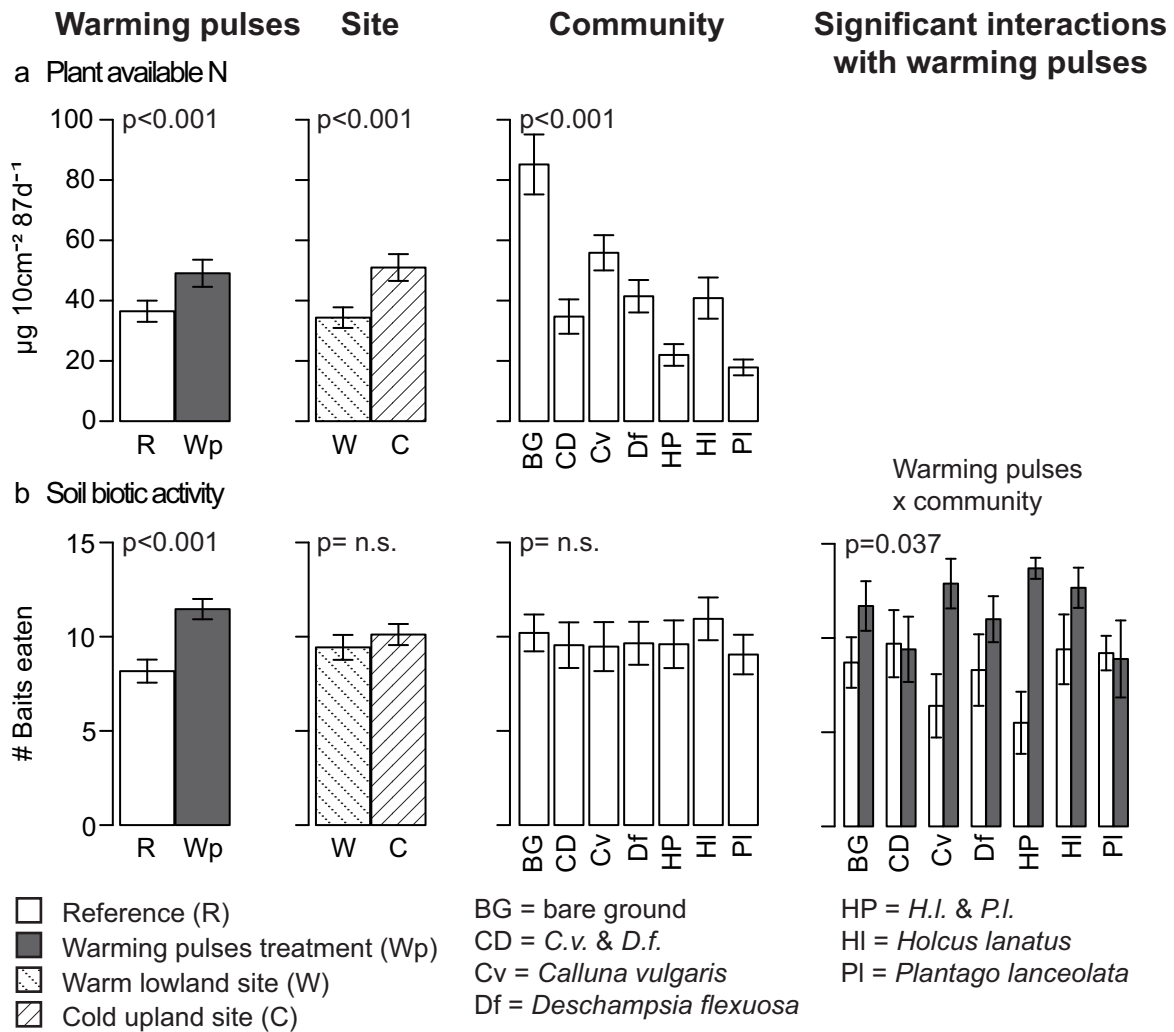| Factor	N availability in soil solution		Soil biotic activity		Beta-glucosidase activity		Cellobiohydrolase activity		Acid phosphatase activity		Xylosidase activity	
	F	P	F	P	F	P	F	P	F	P	F	P
Warming pulses	**14.2**	**<0.001**	**17.5**	**<0.001**	1.8	0.199	**5.3**	**0.035**	2.6	0.127	2.0	0.173
Site	**20.2**	**<0.001**	0.6	0.441	**67.2**	**<0.001**	**69.2**	**<0.001**	**12.6**	**0.003**	**33.6**	**<0.001**
Community	**15.4**	**<0.001**	0.3	0.912	**23.5**	**<0.001**	**16.2**	**<0.001**	**32.5**	**<0.001**	**44.5**	**<0.001**
Warming pulses x Site	0.7	0.415	0.9	0.358	3.2	0.094	1.3	0.266	0.9	0.359	3.8	0.068
Warming pulses x Community	0.3	0.949	**2.3**	**0.037**	1.4	0.213	0.7	0.663	1.1	0.388	0.6	0.694
Warming pulses x Site x Community	0.9	0.547	1.1	0.370	0.7	0.685	1.0	0.400	0.9	0.500	1.4	0.212

527

528 Table 3: ANOVA-results of all tested main and interaction effects for the fate of a ^{15}N label
529 (increase in atom % ^{15}N in the compartments leaves, fine roots, and bulk soil). Warming
530 pulses: Winter warming pulses treatment.
531

	^{15}N atom % increase					
Factor	Leaves		Roots		Bulk soil	
	F	P	F	P	F	P
Warming pulses	**5.9**	**0.016**	1.5	0.228	0.9	0.331
Site	**144.5**	**<0.001**	**19.3**	**<0.001**	**29.9**	**<0.001**
Species/Community (Soil)	**7.4**	**<0.001**	**9.6**	**<0.001**	1.7	0.134
Warming pulses x Site	**8.6**	**0.004**	2.1	0.153	2.0	0.162
Warming pulses x Species	1.2	0.313	0.5	0.695	0.7	0.647
Warming pulses x Site x Species	**3.4**	**0.004**	1.0	0.422	1.2	0.292

FIGURES
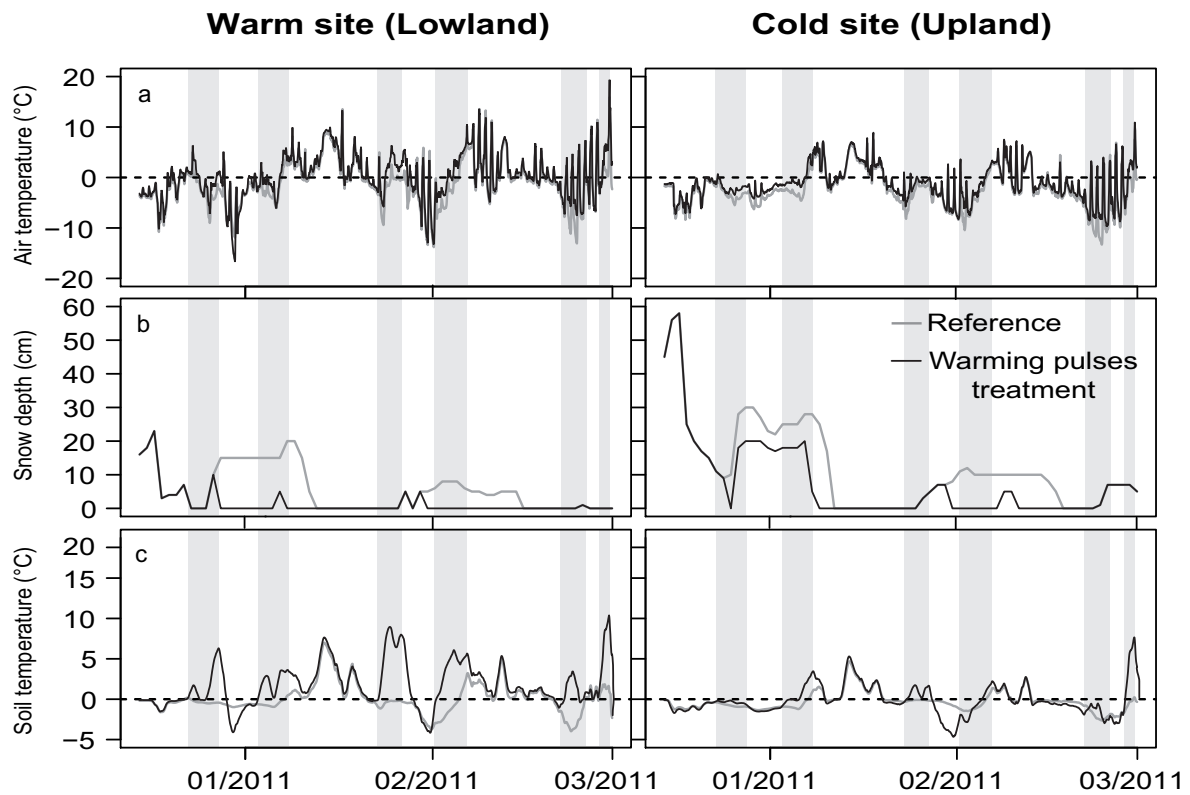

Fig. 1 Mean daily air temperature at +5 cm (a), snow depth (b) and mean daily soil
temperature at -2 cm (c) at the two experimental sites for the winter warming pulses
treatment (black line) and reference conditions (grey line). Warming pulses (grey boxes)
were applied between 15th December 2010 and 28th February 2011 (Schuerings et al.,
2014).

Warming pulses **Site** **Community** **Significant interactions with warming pulses**

a Plant available N

b Soil biotic activity

Reference (R)
Warming pulses treatment (Wp)
Warm lowland site (W)
Cold upland site (C)

BG = bare ground
CD = *C.v.* & *D.f.*
Cv = *Calluna vulgaris*
Df = *Deschampsia flexuosa*

HP = *H.l.* & *P.l.*
HI = *Holcus lanatus*
Pl = *Plantago lanceolata*

539

540   Fig. 2 (a) Plant available nitrogen (nitrate and ammonium; PRS^TM-probes) and (b) soil biotic
541   activity (bait-lamina test) during the manipulation period (18 December 2010 - 17 March
542   2011). Main winter warming pulses treatment, site and community effects and all significant
543   interactions between the winter warming pulses treatment with site and community are
544   shown. Mean (± S.E.) values are shown (n=140).
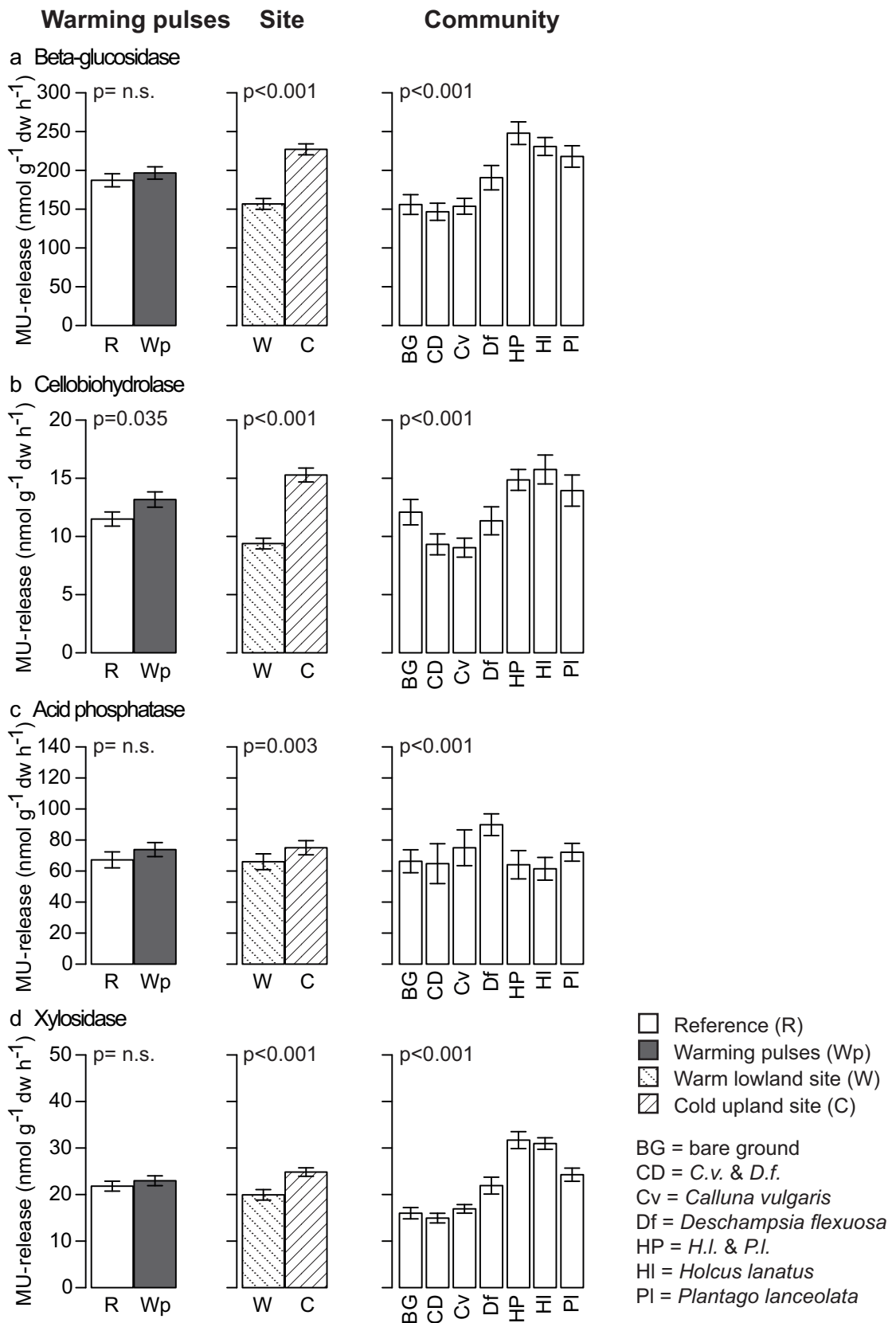
Fig. 3 Mean potential soil enzymatic activity for the four tested enzymes (a) β-glucosidase,
(b) cellobiohydrolase, (c) acid phosphatase and (d) xylosidase (all ± S.E.) during the
manipulation period (18 December 2010 - 17 March 2011). Main winter warming pulses

549   treatment, site and community effects are shown. No significant interactions between the
550   winter warming pulses treatment with site and community were detected.
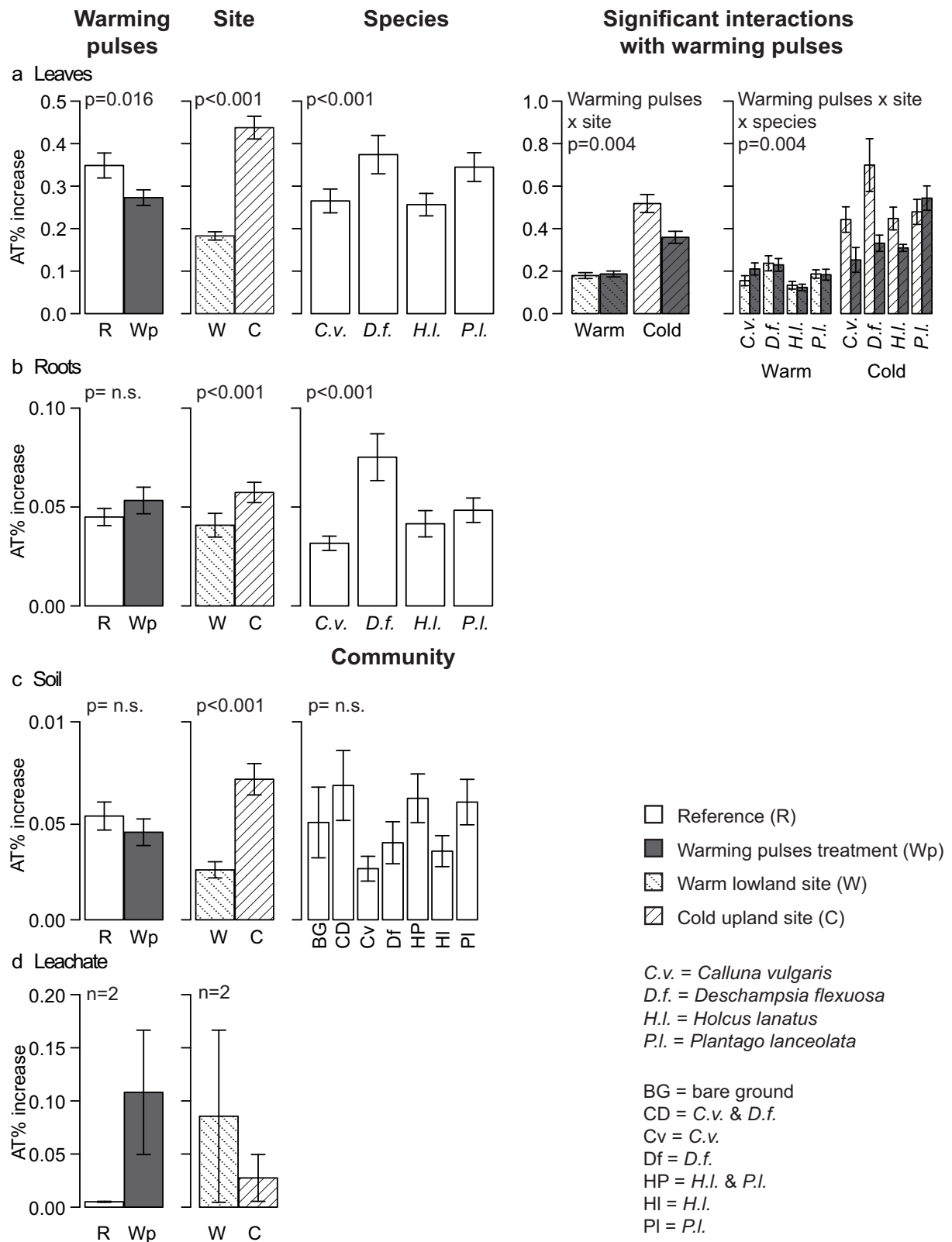
Fig. 4 Mean increase in atom% values (± S.E.) for leaves (n=80), roots (n=80), bulk soil (n=70) and leachate (n=2). Before the warming pulses treatment all plots were watered with 0.25 l of water with 0.02 g Potassium Nitrate-^{15}N (min. 99.19 atom % ^{15}N). Main winter warming pulses treatment, site and community effects and all significant interactions between the winter warming pulses treatment with site and community are shown.