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Absence of soil frost affects plant-soil interactions in temperate grasslands

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Abstract

Background and aims Intermittently frozen ground in winter is expected to disappear over large areas in the temperate zone due to ongoing climate warming. The lack of soil frost influences plant soil interactions and needs to be studied in more detail.

Methods Winter soil frost was avoided by belowground heating wires in a field experiment over two subsequent winters in a temperate grassland. Soil respiration, soil nitrogen availability and plant performance (aboveground biomass, root length at two depth levels, greenness, nutrient content) were compared between "no-frost" and reference plots which underwent repeated freeze-thaw cycles in both winters.

Results Soil respiration increased in the "no-frost" treatment during the warming phase (+291 %). N-availability in the upper 10 cm of the soil profile was not affected, possibly due to increased plant N accumulation during winter (+163 %), increased plant N concentration (+18 %) and increased biomass production (+31.5 %) in the growing season. Translocation of roots into deeper soil layers without changes in total root length in response to the "no-frost" treatment, however, may be a sign of nutrient leaching.

Conclusions The cumulative effect on carbon cycling due to warmer soils therefore depends on the balance between increased winter carbon loss due to higher soil biotic activity and enhanced plant productivity with higher nutrient accumulation in the growing season.

Keywords Winter warming · Climate change · Freeze-thaw · Plant productivity · Nutrient cycling · Soil biotic activity

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Climate is changing, with observed warming over the last 30 years being greatest at higher northern latitudes and in winter (Christensen et al. 2007). In Germany, winter temperatures increased by 2.3 ° C between 1981 and 2000 (German Weather Service). Due to ongoing global warming, regions with no or rare soil frost are going to expand (Kreyling and Henry 2011). A lack of soil frost could directly or indirectly lead to changes in carbon (C) and nutrient cycling, with

implications for ecosystem functioning such as decomposition, primary production or carbon sequestration. However, knowledge about the effects of soil frost absence on plant-soil-interactions is sparse, particularly in the temperate zone (Kreyling 2010).

As temperature is an important driver for metabolic reactions, absent soil frost in winter can increase soil biotic activity if soil moisture does not become limiting (Davidson and Janssens 2006; Allison and Treseder 2011). While soil frost reduces the CO_2 emissions via soil respiration, thawing of frozen soil can lead to strong CO_2 pulses from the soil (Muhr et al. 2009). Carbon losses from soils that are currently exposed to extended frost periods are expected to increase as the soil frost duration subsides and increased soil biotic activity accelerates soil respiration and C loss from ecosystems (Rustad et al. 2001; Melillo et al. 2002). Generalisation across systems and temporal extent of such reactions, however, are unclear (Luo et al. 2001; Melillo et al. 2002; Wan et al. 2007).

Soil C and nitrogen (N) concentrations increase after soil freeze-thaw cycles (FTC) due to microbial lyses, death of roots, and changes in soil structure (Matzner and Borken 2008). Soil warming also increases N mineralization (Rustad et al. 2001; Melillo et al. 2002). Many plants remain photosynthetically active in winter (Larsen et al. 2007) and are capable of winter N uptake (Laine et al. 1994; Grogan et al. 2004; Andresen and Michelsen 2005; Malyshev and Henry 2012). It is unclear whether N mobilization due to increased mineralization will lead to N-leaching and loss from the ecosystem, or if the vegetation is capable of increased winter N uptake resulting in increased primary production (Ineson et al. 1998; Kreyling et al. 2008).

Observations of ecosystems in northern latitudes show an earlier start and increase in photosynthetic activity in spring with rising temperatures (Zhou et al. 2001; Loik et al. 2004). Greenness was used as a surrogate for photosynthetic activity in this study. Continuous winter air warming increases aboveground net primary production (ANPP) (Hutchison and Henry 2010; Kardol et al. 2010). Likewise winter soil warming pulses leading to additional FTC have been shown to increase ANPP in temperate grasslands (Kreyling et al. 2008). However, as plants become more active over winter they also loose frost hardiness, making them vulnerable to frost events in winter (Bokhorst et al. 2009) or spring (Kreyling et al. 2012). Root length decreases with winter soil warming pulses (Kreyling et al. 2008), likely due to frost damage to dehardened plant tissue while warming throughout winter increases root length (Hutchison and Henry 2010). Artificial spring soil warming prior to a natural spring thaw in a boreal forest also leads to an increase in root length (Majdi and Ohrvik 2004). The role of soil frost and freeze-thaw events versus the role of warmer mean soil temperatures remains unclear, however. No studies to our knowledge have administered warming to a level where soil frost does not occur at all.

Warmer soils increase soil enzyme activities leading to higher soil organic matter decomposition and changing N, P and K availabilities (Sardans et al. 2012a, b), potentially leading to higher plant nutrient accumulation and changing stoichiometric relationships. Changes in microbial community, root length and in soil structure affect plant-soil nutrient cycles by variable solubility and chemical traits of the respective elements. Warming and drought have been proven to asymmetrically affect soil nutrient status (e.g. Sardans et al. 2008a) and plant elemental composition (e.g. Sardans et al. 2008b, c) in Mediterranean ecosystems as well as in other biomes (Sardans and Peñuelas 2012). Changes in stoichiometric relationships can change plant metabolome, production and growth rate in turn affecting ecosystem structure and function (Rivas-Ubach et al. 2012; Sardans et al. 2012a). The kinds of plant species present also modifies plant elemental composition because each plant species tends to have a particular elemental composition such as projected by the biogeochemical niche hypothesis (Peñuelas et al. 2008). Different shifts in species biogeochemical niche have been observed under climate change (Peñuelas et al. 2008). However, very little is known about the effects of winter warming on nutrient cycling and stoichiometry in plant-soil systems (Sardans and Peñuelas 2012).

Here, we investigated how the absence of winter soil frost affected plant-soil interactions in two artificial temperate grassland communities over two winters and into the following growing seasons. We hypothesised that the absence of soil frost would lead to (1) increased soil biotic activity in winter, leading to (2) increased nutrient availability, and consequently to (3) increased winter activity of plants. The increase in plant winter activity should lead to earlier greening, higher ANPP and increased root growth. We further hypothesised that changes in microbial community,

root length and soil structure due to absence of soil frost would (4) asymmetrically affect different nutrients and different species, thereby producing changes in plant elemental concentrations and stoichiometry.

Materials and methods

Experimental design and site description

The research is part of the EVENT I -experiment (Jentsch et al. 2007) where the effects of climate change such as drought, heavy rain or winter warming on temperate plant communities are studied. The experimental site is located at the Ecological-Botanical Garden of the University of Bayreuth, Germany (49°55′19″N, 365 m asl). Mean annual air temperature at the site is 8.2 °C and mean annual precipitation is 724 mm (data: German Weather Service, 1971–2000). With average January air temperatures of −1.0 °C, the site is located at the transition between oceanic and continental climates. Winter soil frost depends on site conditions in the vicinity of the experimental site: early snow-pack or energy fluxes from ascending ground water can prevent soil frost completely, while well-drained, open sites such as our experimental site may freeze for several weeks.

All plots consisted of homogenized soil of 80 cm depth including 20 cm topsoil. The soil was taken from a nearby sand quarry. Topsoil carbon content totalled 2 % and pH=4.5 (measured in 1 M KCl), whereas the lower soil layer had 0.2 % total carbon and pH=6.2. The texture of the soil body was loamy sand (82 % sand, 13 % silt, 5 % clay). Bulk density for both soil layers was 1.6 g cm^{-3}.

In the "no frost"-treatment, soil temperature was manipulated by buried heating wires (deviflex DTIP, DEVI, Vejle, Denmark) to avoid soil frost completely. The wires were located at a depth of 7 cm and 20 cm apart from each other, resulting in 100 W m^{-2}. Installation was finished in the year prior to planting. Soil temperature manipulations were conducted from 1 December to 28 February during the winters 2009/2010 and 2010/2011. The reference plots did not receive any treatments. An artefact control with heating wires installed the same way as in our "no-frost" plots showed no difference for plant growth in comparison to untreated controls at the same site in a previous experiment (Kreyling et al. 2008).

Grassland communities of two different functional compositions were studied: one community consisted of two grasses and thus only one plant functional group (Arrhenatherum elatius and Holcus lanatus) (grasses-only), whereas the other community consisted of the same two grass species and two additional herbs (Geranium pratense and Plantago lanceolata) (grasses&herbs).

The plant communities were blocked and randomly assigned within the "no-frost" and reference plots. Each factorial combination was replicated five times resulting in 20 plots (2×2 m in size). The plants were grown from seeds in autumn 2004 and planted in April 2005. One hundred individuals per plot were planted in a hexagonal grid with a distance of 20 cm between neighbours. All species are perennial and original composition was maintained by periodical weeding. An analysis of species compositions (Kreyling et al. 2011) and above-and belowground biomass (Kreyling et al. 2010) showed no significant difference between the treatments prior to the first soil warming manipulation in winter 2009/2010.

Response parameters

Soil (−2 cm) and air temperature (+5 cm) were measured hourly in every plot by thermistores (B57863-S302-F40, EPCOS) connected to a datalogger (dl2, Delta). Snow height was manually measured each morning.

Soil respiration was measured biweekly or monthly (30 dates) from 22 March 2010 until 11 April 2011 on each plot of the four species community. Measurements were carried out with a respiration chamber connected to a non-dispersive infrared gas analyzer (SPC-1 & EGM-4, PP-systems, USA). The respiration chamber was placed on PVC-collars to get a closed system. The collars (10 cm in diameter, 5 cm in height) were installed into the soil one month before the start of measurements at a depth of 4 cm. The day before each measurement all aboveground plant material was clipped from the collar. The CO_2 fluxes were measured for four minutes, only analysing the last soil respiration rate values. Mixed soil samples of the upper layer (0–10 cm) of every plot were taken on six dates to quantify plant-available nitrate and ammonium content over winter and into spring (17 December 2009, 20 January 2010, 18 February 2010, 24 March 2010, 11 March 2011 and 23 March 2011). The samples were sieved (2 mm), extracted to a 1 M KCl solution and then filtered (Roth, Typ 15 A Blauband). Quantification was done by flow injection analysis (FIA,

measurements conducted at BayCEER Analytical Chemistry, Bayreuth, device: MLE Dresden FIA-LAB).

To measure plant activity early in the growing season, phenology of greenness was quantified by digital pictures, taken under standardized light conditions biweekly from 1 March 2011 till 14 April 2011. For this purpose, a portable light-tight box (56×55× 75 cm) with a camera (Nikon D2x) and artificial lighting (a flash) was used. The calculation of the greenness was based on Marchand et al. (2004), using a transformation from the RGB-photos to the HSL colour space. The determination of threshold values of the HSL-bands for the "greenness" was performed with remote sensing software ENVI 4.7 (Exelis Visual Information Solutions, Boulder, Colorado, USA) and ArcGIS 10 (Environmental Systems Research Institute, Redlands, California, USA). The processing and calculation of the percentage of greenness was done with the same parameters for all photos and all time steps with ImageMagick 6.7.6-5 (ImageMagick Studio LLC, Landenberg, Pennsylvania, USA).

Above-ground net primary productivity (ANPP) was measured by complete above-ground harvests of the central 1 m^2 of the plots. Harvests were done on 28 June 2010, 13 September 2010 and on May 26 2011. Harvested biomass was sorted by species, dried to constant weight at 70 °C and weighed.

A minirhizotron technique was used to determine root length. A clear plastic tube (5 cm diameter) was installed at a 45° angle to a depth of 45 cm in each plot before planting. Above-ground parts of the tubes were covered with adhesive aluminium foil and the tubes were capped to prevent entry of water, dust, light and heat. Images of 4 cm^2 were taken at a depth of 5 and 15 cm with a digital camera (Nikon Coolpix E995) mounted on an endoscope. A line intersection method (Tennant 1975) within a systematic grid of 10×10 (grid unit: 0.2 cm × 0.2 cm) was used to quantify root length. Sampling was done on 20 April 2010, 30 June 2010, 17 September 2010 and 28 March 2011.

Foliar C and N concentrations were determined by the combustion of 1–2 mg of pulverized dried sample mixed with 2 mg of V_2O_5 as oxidant. We coupled the combustion to gas chromatography using a Thermo Electron Gas Chromatograph model NA 2100 (C.E. instruments-Thermo Electron, Milan, Italy). For analyses of other elements (Ca, Fe, K, Mn, P, S), dried and ground samples were digested with concentrated HNO_3 and H_2O_2 (30 %, p/v) (MERCK, Darmstadt,

Germany) in a microwave oven. Measurements were regularly standardized with blank solutions. To assess the accuracy of digestion and the analytical biomass procedures, standard certified biomass (NIST 1573a, leaf tomato, NIST, Gaitherburg, MD) was used. After digestion, the contents of Ca, Fe, K, Mg, Mn, P and S were determined using ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma). By multiplying the elemental concentrations by the aboveground biomass per soil surface area, we obtained the mineralomass of each element, which meant the mass of each element accumulated in biomass per unit of soil surface.

Data analysis

Linear mixed-effect models combined with analysis of variance (ANOVA) were applied to test for significant differences between the "no-frost" and the reference plots. Community composition of the two grassland communities was used as a covariate, after confirming that no significant interaction between the soil frost manipulations and the community composition occurred. Replication was set as a random factor, thereby accounting for the block design. Before statistical analysis, we tested for normality and homogeneity of variance by examining the residuals versus fitted plots and the normal qq-plots of the linear models (Faraway 2005). If conditions were not met or to improve heterogeneity of variance, data was log(x+1)-(soil ammonium and nitrate), square-root-(ANPP) or square-root(x+1)-(root length) transformed. Significance level was set to p<0.05. All statistical analyses were performed using R 2.12.2 (R Development Core Team 2011) and additional packages sciplot (Morales 2011) and nlme (Pinheiro et al. 2013). To test for shifts in plant leaf composition after the winter warming treatment we conducted a principal component analyses (PCA). This analysis was performed with all leaf chemical variables (elemental concentrations and their ratios) to analyse differences between treatments and species. Those analyses were performed using Statistica 8.0 (StatSoft Inc., Tulsa, Oklahoma, USA).

Results

The soil warming manipulation successfully prevented soil frost during both winters, while the reference plots

experienced 46 days of soil frost (32 freeze-thaw cycles) in the first winter and 13 days of soil frost (15 freeze-thaw cycles) in the second winter (Fig. 1c). Mean soil temperature in the "no-frost"-plots was higher than in the reference plots by 6.4 °C and 6.3 °C in the first and second winters respectively. The soil warming led to reduced snow cover in the "no-frost" manipulation (Fig. 1b), yet air temperature was not affected by the warming (Fig. 1a).

Soil respiration rate was increased by 291 % (relative difference) in the "no-frost"-treatment during the second winter (1 December 2010–28 February 2011) (F=7.99, P=0.006, Fig. 2), but was unaffected outside the warming phase (F=2.35, P=0.127). The relative increase over the whole observation period (22 March 2010 – 11 April 2011) was 17 % (non-significant). Soil nitrogen availability at the upper 10 cm of the soil profile did not differ between the "no-frost"-treatment and the reference plots (ammonium: F=0.11, P=0.737; nitrate: F=2.75, P=0.100) (Table 1).

Plant activity, expressed as aboveground greenness, increased early in the growing season in the "no-frost"-treatment in comparison to the reference plots (F=685.71, P<0.001, Fig. 3). Despite the difference decreasing over time, greenness increased by 195.6 % (relative difference) over the observation period.

Total biomass production was increased by 31.5 % in the "no-frost"-treatment in comparison to the reference plots (F=5.50, P=0.024). The effect was greatest after the first manipulation in June 2010 and no longer visible in September 2010. In May 2011, ANPP increased in the "no-frost"-treatment again, but not as much as in June 2010 (Fig. 4).

The depth distribution of the roots was affected by the "no frost"-treatment (interaction between treatment and depth: F=4.35, P=0.039): Root length was reduced at −5 cm depth but increased at −15 cm in the "no-frost"-plots compared to the reference plots (Fig. 5). This translocation of roots had no effect on total root length (F=0.03, P=0.853).

"No-frost" treatment increased foliar C concentrations (F=186, P<0.001 in grasses-only and F=115, P<0.001 in grasses&herbs) (Table 2a, b). In the grasses-only community, in addition to having greater plant biomass, the nutrient concentrations were also generally higher in "no-frost" plots than in reference plots. Moreover, in grasses-only plots "no-frost"-treatment increased foliar N (F=10.4, P=0.007), P (F=6.88, P=0.022), K (F=5.37, P=0.039) and S (F=7.28, P=0.019) concentrations (Table 2a). Contrarily in grasses&herbs community "no-frost" treatment decreased K concentrations (F=17.2, P<0.001) (Table 2b). The mineralomass
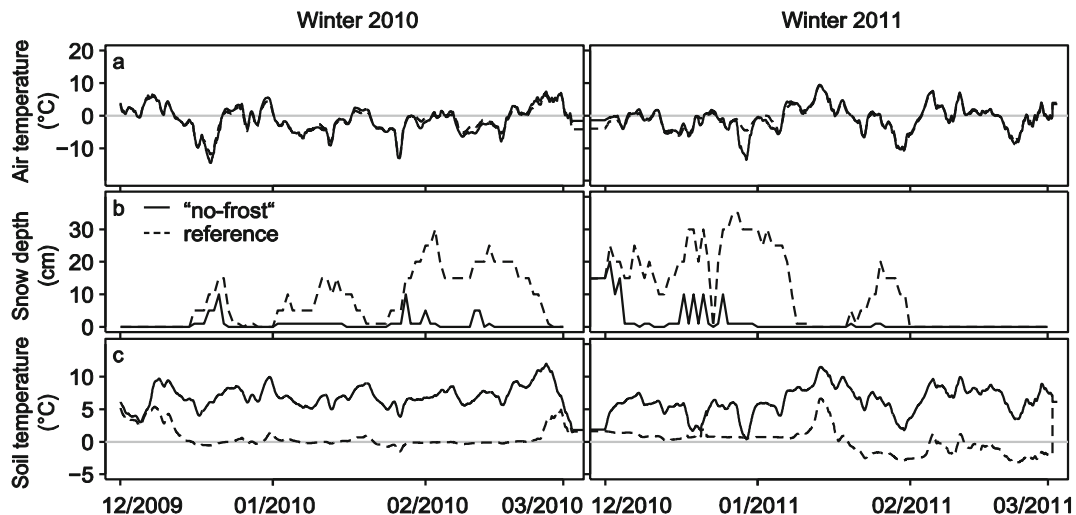

Fig. 1 Mean daily air temperatures (at +5 cm), snow depth and soil temperatures (at −2 cm) for the "no-frost"-treatment (*black line*) and reference plots (*dotted line*) during winter 2009/2010 and winter 2010/2011. Manipulations took place between 1 December 2009 and 1 March 2010 and between 1 December 2010 and 1 March 2011. Mean values over all plots are shown (n=10). Mean values for winter 2010: air temperature −1.3 ° C, reference soil temperature 0.6 ° C with 32 FTCs and "no-frost" soil temperature 7.0 ° C. Mean values for winter 2011: air temperature −0.9 ° C, reference soil temperature 0 ° C with 15 FTCs and "no-frost" soil temperature 6.3 ° C
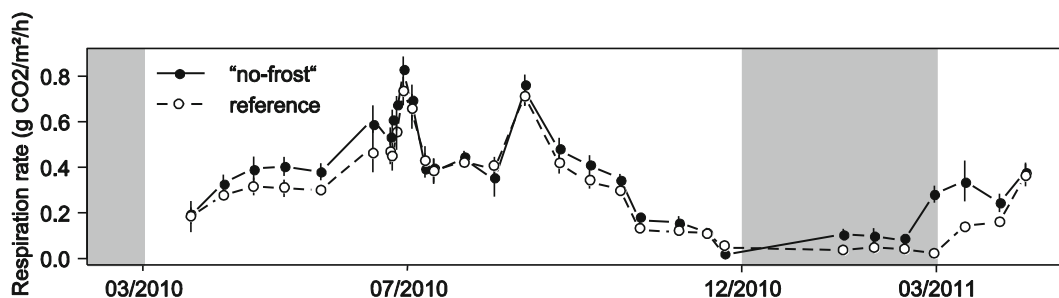
Fig. 2 Soil respiration for "no-frost"-plots (*black dots*, *solid line*) in comparison to reference plots (*open dots*, *dotted line*) from 22 March 2010 (after first manipulation) till 11 April 2011.

During the "no-frost"-manipulation (*grey area*) soil respiration was significantly increased ($F=7.99$, $P=0.006$). Mean values and standard error are shown, n=5 for each point

of K in "no-frost" treatment was higher than in the reference plots ($P<0.05$) while the mineralomasses of N, P, S and Fe were higher but marginally not significant ($P<0.1$). In grasses-only and in grasses&herbs communities the PC1 axis separated the scores of the reference samples from those of the "no-frost" treatment (Fig. 6). However, the loading variables were very different in both cases. In grasses-only the PC1 was mainly loaded by larger C, N, P, K and S concentrations in "no-frost" samples whereas in grasses&herbs the PC1 was mainly loaded by the concentrations of K and by its ratios with other elements. Thus, the changes in the elemental concentrations due to the "no-frost" treatment were different in each species when growing in different communities, e.g. in grasses&herbs community, *H. lanatus* plants growing in "no-frost" treatments occupied a PC space towards higher N, P and K concentrations than reference plants, whereas in grasses-only community the contrary was observed (Fig. 6).

Discussion

Soil respiration increased by 291 % in the "no-frost"-manipulation during the second winter. Yet the effect lasted for only two weeks after the warming phase in our study. From then on, we did not observe any difference in comparison to reference conditions. This implies a fast, yet, transient increase in soil biotic activity, which is in line with previous findings (Sharma et al. 2006). The increase in soil respiration in the "no-frost" treatment over the whole observation period is 17 % (non-significant). Rustad et al. (2001) found a mean increase of 20 % in a meta-analysis of 17 warming experiments with different warming methods and in different biomes. Increased soil biotic activity and soil respiration due to climate warming is
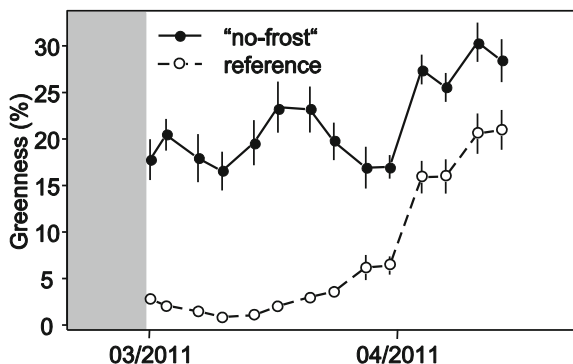
Table 1 Mean values (±S.E.) of plant-available soil NH4+ and NO3- concentrations in the "no-frost" and reference treatment at the different sampling dates (one mixed sample from 0 to −10 cm per plot)

Date	NH4+ (mg/l)		NO3- (mg/l)	
	"no-frost"	Reference	"no-frost"	Reference
17.12.09	5.43(1.17)	6.32(2.07)	0.20(0.05)	0.24(0.08)
20.01.10	1.98(0.25)	1.71(0.18)	0.15(0.02)	0.15(0.09)
18.02.10	1.96(0.33)	2.02(0.75)	0.23(0.07)	0.25(0.11)
24.03.10	2.68(0.22)	2.69(0.33)	0.25(0.15)	0.35(0.09)
11.03.11	2.50(1.1)	2.75(1.0)	0.16(0.04)	0.15(0.08)
23.03.11	2.04(0.88)	1.49(0.16)	0.18(0.04)	0.18(0.03)

Fig. 3 Phenology of greenness for "no-frost"-plots (*black dots*, *solid line*) in comparison to reference plots (*open dots*, *dotted line*) at the start of the growing season after the second manipulation (*grey box*) from 1 March 2011 till 14 April 2011. Standardized digital pictures were analysed for their content of green pixels. Highly significant differences in greenness were found ($F=685.71$, $P<0.001$). Mean values and standard errors are shown, n=10 for each point
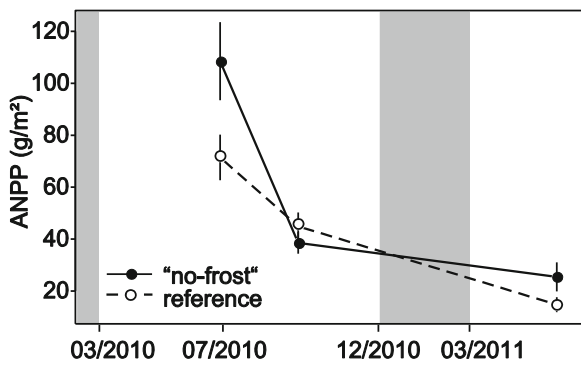
Fig. 4 Above-ground net primary production (ANPP) over the growing season in 2010 following the first "no-frost"-treatment and in May 2011 after the second "no-frost"-treatment (*grey box*). Significant differences between the "no-frost"-treatment (*black dots*, *solid line*) and reference (*open dots*, *dotted line*) were found ($F=5.50$, $P=0.024$). Mean values and standard errors are shown, n=10 for each point

viewed as one of the most important positive feedbacks in the climate system (Schlesinger and Andrews 2000). Recently, it has been suggested that such kinds of positive feedbacks were overestimated because water availability will limit soil biotic activity in many systems (Davidson and Janssens 2006; Bontti et al. 2009; Allison and Treseder 2011). Winters in temperate regions, however, do not pose any water limitations on soil biotic activities. In fact, they are projected to become even wetter (Christensen et al. 2007). With ongoing climate warming, winter conditions in the

southern temperate zone are expected to reach a point where soil frost gets very rare (Kreyling and Henry 2011), which is in contrast to more northern regions (Henry 2008; Brown and DeGaetano 2011) where decreasing snow cover is projected to lead to more soil frost. It is therefore likely that an acceleration of decomposition is more likely to take place in southern temperate regions than in northern temperate regions.

However, complexities of C dynamics make extrapolation of long-term trends difficult since soil carbon stocks are comprised of strongly contrasting C pools with turnover rates from years to centuries. Understanding the specific responses of different C pools to climate change will be essential for a realistic projection of warming impacts on the carbon cycle (Davidson and Janssens 2006; Conant et al. 2011). It is not certain that increased soil biotic activity and mineralization rates can be sustained by continuous carbon input via primary production (Ineson et al. 1998). Furthermore, species compositions might change because of altered competitive balance in response to winter climate change (Kreyling et al. 2011), and these changes are inherently slow but potentially important for nutrient cycling (Hollister et al. 2005).

Surprisingly, we did not detect increased N availability in the upper 10 cm of the soil during and shortly after the "no-frost"-treatment. Based on increased soil respiration rates, which indicate higher activity of decomposers and N-fixing bacteria, we
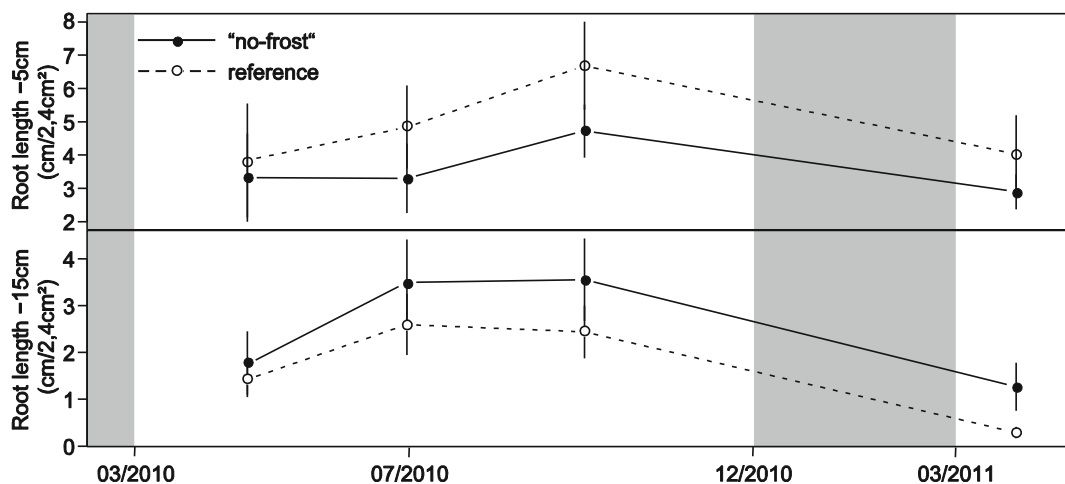
Fig. 5 Root length at −5 cm depth and at −15 cm depth measured by minirhizotron technique for "no-frost"-treatment (*black dots*, *solid line*) and reference (*open dots*, *dotted line*) during the growing season in 2010 and at the start of the growing season in 2011 after the second "no-frost"-treatment (*grey box*). Total root length did not differ between treatments but root distribution changed (interaction: treatment * depth, F=4.35, P=0.039). Mean values and standard errors are shown, n=10 for each point

Table 2 Mean±S.E. of the elemental concentrations and ratios in different plant species that underwent the "no-frost" treatment or were exposed to reference conditions. (a) Grasses-only community, (b) grasses&herbs community. Different bold letters indicate statistically significant differences (P<0.05). Ae=*Arrhenatherum elatius*. Hl=*Holcus lanatus*. Pl=*Plantago lanceolata*

b Grasses & herbs — Element contents (mg m^{-2})

Factor		C	N	P	K	Ca	Mg	S	Fe	Mn
Treatment	Reference	**43.1b (0.1)**	**1.42b (0.06)**	**0.209b (0.011)**	**1.11b (0.10)**	0.744 (0.056)	0.235 (0.012)	**0.187b (0.017)**	0.019 (0.003)	**0.084a (0.006)**
	No-frost	**45.2a (0.2)**	**1.67a (0.05)**	**0.242a (0.011)**	**1.37a (0.09)**	0.746 (0.048)	0.238 (0.013)	**0.218a (0.017)**	0.035 (0.008)	**0.049b (0.007)**
		F=186 P<0.001	**F=10.4 P=0.007**	**F=6.88 P=0.022**	**F=5.37 P=0.039**	F=0.000 P=0.99	F=0.0065 P=0.94	**F=7.28 P=0.019**	*F=4.01 P=0.07*	**F=14.0 P=0.0028**
Species	*Ae*	**44.6a (0.4)**	1.51 (0.08)	0.218 (0.015)	**1.09b (0.08)**	0.681 (0.048)	0.230 (0.014)	**0.161b (0.009)**	0.027 (0.008)	0.055 (0.009)
	Hl	**43.8b (0.4)**	1.59 (0.06)	0.233 (0.008)	**1.39a (0.10)**	0.802 (0.047)	0.242 (0.011)	**0.241a (0.013)**	0.028 (0.007)	0.070 (0.007)
		F=17.8 P<0.001	F=1.20 P=0.30	F=1.79 P=0.21	**F=6.62 P=0.024**	F=2.22 P=0.16	F=0.28 P=0.61	**F=45.5 P<0.0001**	F=0.059 P=0.81	*F=3.48 P=0.09*

Element contents (mg m^{-2})

Factor		C	N	P	K	Ca	Mg	S	Fe	Mn
Treatment	Reference	385 (90.7)	12.7 (3.0)	1.91 (0.47)	**10.7b (2.7)**	7.0 (1.7)	2.15 (0.50)	1.76 (0.43)	0.163 (0.041)	0.703 (0.147)
	No-frost	891 (256)	33.4 (9.4)	4.87 (1.44)	**26.3a (6.76)**	14.2 (4.0)	4.25 (1.16)	4.58 (1.38)	0.492 (0.150)	0.120 (0.435)
		F=3.15 P=0.095	*F=3.99 P=0.060*	*F=3.49 P=0.080*	**F=4.57 P=0.048**	F=2.53 P=0.13	F=2.62 P=0.13	*F=3.80 P=0.069*	*F=4.49 P=0.050*	F=1.10 P=0.31
Species	*Ae*	582 (239)	21.0 (0.9)	3.00 (1.30)	14.7 (6.2)	9.26 (3.65)	2.80 (1.04)	2.37 (1.04)	0.236 (0.076)	0.828 (0.380)
	Hl	714 (197)	26.9 (7.1)	3.89 (1.15)	22.6 (5.5)	12.2 (3.2)	3.67 (0.93)	4.03 (1.21)	0.426 (0.152)	1.09 (0.32)
		F=0.305 P=0.59	F=0.338 P=0.57	F=0.427 P=0.52	F=1.36 P=0.26	F=0.53 P=0.48	F=0.56 P=0.47	F=1.46 P=0.24	F=1.71 P=0.21	F=0.346 P=0.56

Stoichiometry

Factor		C:N	C:P	N:P	C:K	N:K	P:K
Treatment	Reference	30.8 (1.6)	211 (12)	6.88 (0.31)	41.7 (4.1)	1.35 (0.10)	0.196 (0.012)
	No-frost	27.3 (0.8)	190 (8)	6.98 (0.25)	34.1 (2.0)	1.25 (0.07)	0.182 (0.013)
		F=4.63 P=0.053	*F=3.27 P=0.096*	F=0.006 P=0.94	*F=4.04 P=0.07*	F=1.59 P=0.23	F=0.97 P=0.34
Species	*Ae*	30.2 (1.7)	212 (14)	7.05 (0.38)	**43.0a (3.6)**	**1.42a (0.08)**	0.204 (0.012)
	Hl	27.8 (0.8)	190 (6)	6.83 (0.15)	**32.9b (2.2)**	**1.18b (0.07)**	0.175 (0.202)
		F=2.29 P=0.16	*F=3.80 P=0.075*	F=0.45 P=0.52	**F=6.77 P=0.023**	**F=6.75 P=0.023**	*F=3.51 P=0.09*

b Grasses&herbs — Element concentrations (%, DW)

Factor		C	N	P	K	Ca	Mg	S	Fe	Mn
Treatment	Reference	**42.4b (0.1)**	1.56 (0.06)	0.261 (0.012)	**1.73a (0.15)**	1.14 (0.17)	0.255 (0.020)	0.248 (0.019)	0.020 (0.002)	0.035 (0.007)
	No-frost	**44.8a (0.2)**	1.44 (0.05)	0.241 (0.010)	**1.28b (0.12)**	1.31 (0.19)	0.256 (0.023)	0.248 (0.018)	0.029 (0.007)	0.046 (0.008)
		F=115 P<0.001	*F=4.0 P=0.06*	*F=3.7 P=0.07*	**F=17.2 P<0.001**	F=1.24 P=0.28	F=0.0001 P=0.99	F=0.059 P=0.81	F=1.11 P=0.31	F=1.02 P=0.33
Species	*Pl*	**44.0a (0.4)**	1.47 (0.05)	0.251 (0.014)	**1.13c (0.07)**	**1.91a (0.12)**	**0.333a (0.018)**	**0.311a (0.011)**	0.018 (0.003)	0.027 (0.01)
	Hl	**43.1b (0.4)**	1.56 (0.08)	0.259 (0.013)	**1.89a (0.17)**	**0.883b (0.112)**	**0.212b (0.010)**	**0.239b (0.007)**	0.035 (0.009)	0.053 (0.038)
	Ae	**43.8ab (0.6)**	1.54 (0.06)	0.236 (0.016)	**1.49b (0.18)**	**0.658b (0.040)**	**0.199b (0.015)**	**0.161c (0.010)**	0.021 (0.005)	0.044 (0.008)
		F=7.0 P<0.01	F=0.93 P=0.41	F=0.979 P=0.39	**F=19.9 P<0.001**	**F=30.2 P<0.001**	**F=24.8 P<0.001**	**F=48.0 P<0.001**	*F=2.95 P=0.08*	*F=3.39 P=0.06*

Table 2 (continued)

b Grasses & herbs — Element contents (mg m^{-2})

		C	N	P	K	Ca	Mg	S	Fe	Mn
Treatment	Reference	656 (128)	24 (4)	3.98 (0.7)	24.5 (4.2)	22.0 (6.4)	4.50 (1.1)	4.2 (0.9)	0.315 (0.067)	0.341 (0.090)
	No-frost	1000 (380)	31 (1.2)	5.31 (2.2)	24.8 (8.2)	38.0 (19.6)	6.36 (2.62)	6.1 (2.5)	0.707 (0.392)	0.611 (0.229)
		F=0.930 P=0.35	F=0.461 P=0.50	F=0.403 P=0.53	F=0.002 P=0.97	F=0.786 P=0.38	F=0.604 P=0.45	F=0.660 P=0.43	F=0.991 P=0.33	F=1.30 P=0.27
Species	Pl	**1443a (442)**	**46.3a (13.5)**	**8.01a (2.58)**	34.2 (8.95)	**64.7a (22.9)**	**10.6a (2.97)**	**9.78a (2.87)**	0.859 (0.491)	0.563 (0.237)
	Hl	**619ab (133)**	**22.4b (4.8)**	**3.55b (0.68)**	26.5 (5.7)	**11.9b (2.6)**	**3.10b (0.72)**	**3.31b (0.65)**	0.423 (0.126)	0.653 (0.194)
	Ae	**158b (34.4)**	**5.26c (1.2)**	**0.849c (0.220)**	5.47 (1.37)	**2.37c (0.61)**	**0.711c (0.150)**	**0.578c (0.146)**	0.075 (0.032)	0.036 (0.008)
		F=4.12 P=0.030	**F=4.09 P=0.031**	**F=3.71 P=0.041**	*F=3.40 P=0.052*	**F=4.75 P=0.019**	**F=6.08 P=0.008**	**F=5.43 P=0.012**	F=1.21 P=0.32	F=2.14 P=0.14

Stoichiometry

|		C:N	C:P	N:P	C:K	N:K	P:K
Treatment	Reference	**27.6b (1.1)**	**167b (8)**	6.07 (0.23)	**30.0a (2.5)**	**0.964c (0.069)**	**0.163c (0.015)**
	No-frost	**31.6a (1.1)**	**190a (8)**	6.04 (0.19)	**6.04b (0.19)**	**1.24a (0.11)**	**0.206a (0.017)**
		F=9.26 P=0.007	**F=7.63 P=0.013**	F=0.03 P=0.87	**F=14.0 P=0.0015**	**F=8.04 P=0.011**	**F=6.14 P=0.023**
Species	Pl	30.2 (1.0)	180 (10)	5.93 (0.20)	**40.6a (2.7)**	**1.34a (0.08)**	**0.228a (0.014)**
	Hl	28.5 (1.9)	171 (10)	6.05 (0.25)	**26.0b (4.3)**	**0.892b (0.098)**	**0.149b (0.017)**
	Ae	30.4 (1.2)	190 (12)	6.26 (0.36)	**32.5b (5.3)**	**1.05ab (0.14)**	**0.171ab (0.023)**
		F=0.767 P=0.49	F=1.13 P=0.35	F=0.496 P=0.62	**F=8.10 P=0.0031**	**F=9.39 P=0.0016**	**F=11.3 P<0.001**
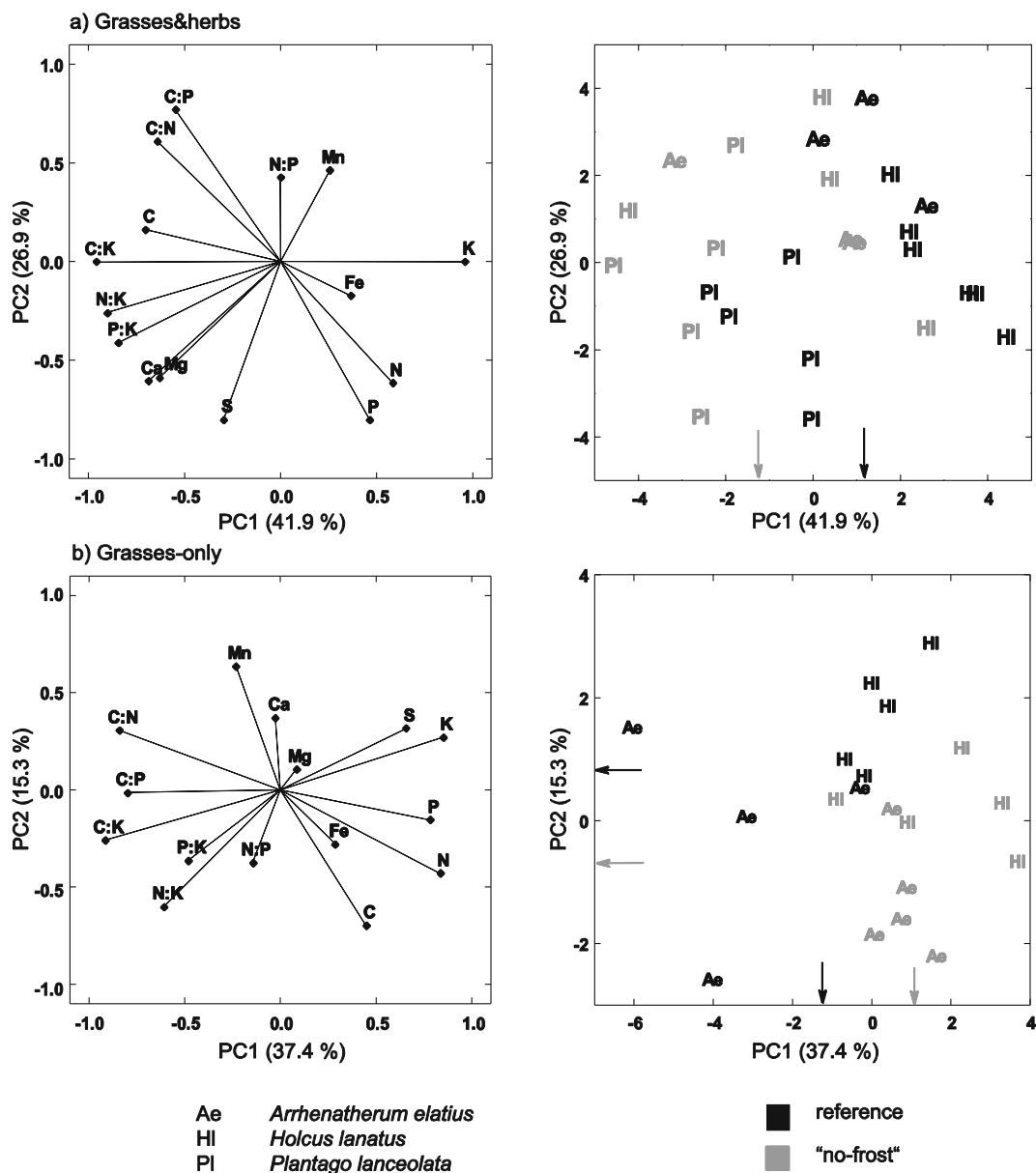
Fig 6 Principal Component Analysis (PCA) conducted with elemental concentrations and ratios as variables and plant samples of different species **a)** grasses&herbs and **b)** grasses-only as cases. (*Geranium pratense* was not included because of lack of data due to high mortality). Arrows indicate the mean of sample scores of PC1 axis in references (*black*) and in "no frost"(*grey*) that were significantly different (grasses&herbs: F=18.7, P=0.0004; grasses-only: F=12.8, P=0.004 and F=4.95, P=0.046, respectively)

would have expected increases in decomposition and mineralization, providing more ammonium and nitrate in the soil. We see three logical explanations for this finding: (1) Above-ground greenness of the vegetation early in the growing season suggests increased plant activity already during winter in response to the "no-frost"-treatment. Mineralized nitrogen may therefore have been taken up by the plants in order to fuel their enhanced greenness and growth. (2) Mineralized N may have quickly leached downward in the soil profile in the presence of sufficient moisture with downward flow. (3) Gaseous N could have left the plant-soil system due to increased nitrification or denitrification rates.

The first explanation is based on the fact that plants can maintain photosynthetic activity (Larsen et al.

2007) and N uptake (Grogan et al. 2004; Andresen and Michelsen 2005; Malyshev and Henry 2012) during winter, sometimes comparable to summer N uptake (Nasholm et al. 2000; Bardgett et al. 2003). Unfortunately, we lack samples from deeper soil layers to tackle the second explanation. However, we did observe a shift in rooting depth. Without changes in total root length, significantly more roots occurred in deeper soil layers in the "no-frost"-treatment compared to references, which could be a hint for downward leaching nutrients. Taken together, we see hints supporting both mechanisms. For the third explanation we lack measurements of N_2, N_2O and NO. Especially in the thawing period after freeze-thaw events there are often gaseous N_2O and NO fluxes, but their magnitude differs strongly (Matzner and Borken 2008). In a snow removal experiment, only frozen plots showed N_2O fluxes whereas unfrozen controls only showed a much smaller flux in spring (Goldberg et al. 2010). Since in our experiment we excluded soil frost, it is not likely that strong N_2O fluxes occurred. The relative contribution of the three mechanisms, however, is of high ecological importance with regard to nutrient loss and ground water quality and should be investigated in more detail.

The strong increase in aboveground primary production in early summer was not present anymore by autumn. Such stabilisation of ANPP has also been reported in other warming studies (Kardol et al. 2010; Kreyling et al. 2010). During the growing season, depleted nutrient pools could have been the limiting factor for ANPP in "no-frost"-plants. The detected increase in primary production due to winter warming had to be expected and it agrees with previous findings from temperate ecosystems (Hutchison and Henry 2010), although increased total root length was not supported in our data. Most warming manipulations in high-latitude ecosystems have been conducted during the growing season only (Elmendorf et al. 2012), so we lack studies to compare our results to.

Nutrient composition differed between reference and "no-frost" plants. Most plant nutrients increased their concentrations in "no-frost" plants in spite of higher biomass accumulation, indicating an increased accumulation of nutrients. These results are in accordance with previous reports of higher nutrient accumulation in response to warming in temperate ecosystems (Sardans et al. 2012b). The "Biogeochemical niche" hypothesis proposes that plants competing in the same

community use the nutrients in different amounts and proportions, which should diminish the competition for resources among them, such as observed in different Mediterranean plants growing in different climatic conditions (Peñuelas et al. 2008). Plant elemental compositions were affected by the "no-frost" treatment as well as by the plant community composition. In the grasses-only communities, the "no-frost" treatment had a stronger effect on the overall plant elemental concentrations than in the grasses&herbs communities. K concentration in *Holcus lanatus* increased within grasses-only communities but decreased within grasses&herbs communities. The observed shifts in plant elemental composition in response to winter warming deserve further study because stoichiometric changes in plants impact ecosystem trophic webs by favouring herbivores and decomposers with specific nourishment preferences (Sterner and Elser 2002; Sardans et al. 2012a).

Here, we investigated the extreme case of the complete absence of soil frost. Based on climate time series and projections, this is a realistic scenario for Central Europe and large parts of the southern temperate zone (Kreyling and Henry 2011). The mean soil temperature increased by +6.4 °C during the "no-frost"-manipulation in comparison to reference conditions, which is at the upper limit of current temperature projections for the end of this century (Christensen et al. 2007). It is important to note that we did not exclude air frost and, consequently direct frost stress to the above-ground parts of the plants. Avoiding air frost completely would represent a highly unrealistic scenario, as temperature fluctuations and minima are projected to occur in the future with persistent magnitudes despite reduced frequencies (Kodra et al. 2011).

During autumn, temperate plants gradually acquire freezing tolerance as temperature and photoperiod decline. The hardening period lasts from days to weeks, dependent on the species and is characterized by increased content of soluble sugars and specific cryoprotective amino acids, as starch content is decreased (Thomashow 1999). Earlier snowmelt (Fig. 1b) and increasing winter/spring temperatures have been shown to advance phenology in many plant species (Ahas et al. 2002; Dunne et al. 2003), even leading to winter growth (Kalberer et al. 2006). Winter growth of plants, indirectly shown by greenness in our data, probably reduces frost hardiness, thereby enhancing the risk of frost damage (Kalberer et al. 2006; Rigby and Porporato 2008; Bokhorst et al. 2009). With vanishing winters plant

dormancy can be disrupted altogether, paradoxically causing extended plant dormancy and delayed phenology in spring (Yu et al. 2010). It becomes evident that plant responses to winter warming are complex. To tackle the connected processes and mechanisms in more detail will be an important task in order to identify ecological implications with regard to nutrient leaching or carbon sequestration.

The projected loss of soil frost under future climate conditions over large parts of the temperate zone (Kreyling and Henry 2011) is expected to increase soil respiration, in particular as water availability will not become a limiting factor for biotic activity during winter in these regions. Yet, plant response appears crucial with regard to nutrient leaching and carbon sequestration, as enhanced nutrient uptake and primary productivity may keep nutrient cycles closed (Ineson et al. 1998) and provide additional organic matter to compensate for increased decomposition. Reduced frost hardiness combined with a potential increase in frost damage, however, may counteract this buffering feedback loop and make southern temperate regions prone to increased carbon and nutrient losses in future winters.

Conclusions

Warmer soils enhanced soil respiration, soil biotic activity, phenology, nutrient accumulation and primary production over winter in our temperate grassland communities. Plant nutrient content and stoichiometry were also altered differently by the absence of soil frost, depending on the species composition of the plant community, indicating that the interaction between climate change and changes in biodiversity is of high ecological importance. In addition, there was an indication of nutrient leaching (i.e. shifts in rooting depth), which demands quantification in relation to soil nutrient cycles. Furthermore, potential negative feedbacks between winter activity and frost tolerance of the plants require further investigation.

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