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# Warming alters potential enzyme activity but precipitation regulates chemical transformations in grass litter exposed to simulated climatic changes

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ABSTRACT

Warming generally accelerates the decomposition of plant litter. However, changes in precipitation could alter the sensitivity of litter decomposition to warming, thereby affecting the formation of litter-derived soil organic matter. As grassland soils store ~20% of Earth's soil carbon, understanding the effect of climatic changes on the decomposition dynamics of grasses is important. However, little is known about how projected changes in climate would affect litter microbial communities and enzyme activities, and the consequences of these changes for the mass loss and compound-specific degradation of grass litter that possess complex lignocellulosic chemistry. Over a period of two years, using litter of the grass *Poa trivialis*, we studied how mass loss, microbial enzyme activity and fine-level litter chemistry responded to a factorial combination of 4 levels of warming (up to ambient +~4 °C) and three levels of precipitation [ambient, wet (+50%) and dry (−50%)] at the Boston-Area Climate Experiment (BACE), in Massachusetts, USA. After 393 days of decomposition, supplemental precipitation accelerated mass loss compared to the dry treatment, as a consequence of faster loss of hydroxycinnamates, which protect carbohydrates through cross-linkages with lignins. Only a third as much of the cell wall-bound ferulic and *p*-coumaric acids remained in litter from the supplemental precipitation treatment compared to the ambient controls. In contrast, the warming treatments did not affect mass loss until later, after 740 days, when the litter in the warmest treatment (+~4 °C) had lost the most mass. Although warming significantly affected mass loss after 740 days, there was also a trend in the warmest treatments toward greater mass loss in the wet (78% mass loss) and ambient (68%) plots compared to dry plots (61%), possibly due to the higher activity of β-glucosidase. Though mass loss at this final time point varied with both warming and precipitation treatments, the compound-specific degradation of litter captured by diffuse reflectance infra-red Fourier transform (DRIFT) and $^{13}$C Nuclear Magnetic Resonance (NMR) spectroscopy revealed that only the precipitation treatments significantly altered the chemistry of carbon compounds in the decomposed tissue. Litter that decomposed in the dry treatment had a higher proportion of carbohydrates remaining than litter in the wet and ambient treatments. Similarly, although ergosterol content and potential activity of phenol oxidase decreased in the warmer treatments, the consequences of this response were not observed in the degradation of specific compounds in litter, which varied only with precipitation treatments. Our results suggests that mass loss and enzyme activities may not accurately capture the complexity of compound-specific degradation of litter during decomposition. Our results also identified non-linear responses of β-glucosidase and N-acetyl-β-D-glucosaminidase (NAG) activities to warming. These results thus emphasize the complexities of litter decomposition and suggest that similar changes in decomposition across other grass species could alter the carbon budget of grasslands.


## 1. Introduction

Grassland ecosystems store ~20% of Earth's soil carbon and occupy ~30% of its land surface (Asner et al., 2004; FAO, 2010), and

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thus strongly influence terrestrial carbon cycling. Rates of grassland carbon cycling respond to warming and changes in precipitation. For instance, the projected 4–6 °C increase in temperature by the turn of this century and associated changes in precipitation patterns (IPCC, 2007) would alter soil temperatures and moisture balances, changing the rates of litter decomposition (Adair et al., 2008; Suseela et al., 2013). Any associated increase in nutrient mineralization might help to sequester more atmospheric $CO_2$ through higher ecosystem productivity (Melillo et al., 2011), leading to a negative feedback to climate change. However, an accelerated release of $CO_2$ during litter decomposition would positively feed back to climate change. The magnitude and direction of any feedback effects would depend largely on the changes in climate, on litter chemistry, and on their interaction (Erhagen et al., 2013; Suseela et al., 2013).

In general, graminoids decompose much more slowly than forbs (Cornwell et al., 2008), largely because these two plant functional types differ in the chemical composition of their litters (Carpita, 1996). In the primary cell walls of grasses, aromatic heteropolymers such as lignin cross-link with proteinaceous compounds and polysaccharides such as cellulose to form a strong structural framework. During decomposition of grass litter, this structural framework makes the polysaccharides and proteins less accessible to microbes, ultimately slowing C and N mineralization. Most previous studies on the effect of climate on litter decomposition have focused on forest ecosystems, using foliar litter from trees (Gholz et al., 2000; Liski et al., 2003). As the chemistry of grass litter is substantially different from other herbaceous plants and trees, focusing on the effects of multiple climate factors on grass litter decomposition can provide new insights into carbon cycling in grassland ecosystems. To our knowledge, none of the previous studies on the effects of climate on grass litter have assessed the degradation of specific compounds, thus limiting our knowledge of the chemical transformations during decomposition that facilitate soil organic matter formation. Addressing this knowledge gap would provide a more complete understanding of the contribution of litter to soil organic matter formation (Wickings et al., 2012).

Warming generally increases the metabolic activity of microbes (Schindlbacher et al., 2011) and the rate of litter decomposition (Fierer et al., 2005). However, changes in precipitation regimes could alter the response of the microbial community to warming, affecting the rate and magnitude of litter decomposition. For example, in a long-term warming experiment, during normal precipitation years the population size of soil bacteria increased with warming, whereas under dry conditions warming led to significant reduction in bacterial population (Sheik et al., 2011). Similarly, in an annual grassland system, water additions enhanced the activity of phenol oxidase and peroxidase, which degrades lignin in plant litter (Henry et al., 2005). Although these studies suggest the significance of moisture in driving the responses of soil microbial communities and their functional activity, little is known about the concerted effects of warming and altered precipitation on the compound-specific degradation of litter via changes in microbial activity. Also, most studies that have measured responses of extracellular enzyme activities to climate manipulations have used only two levels of each climatic factor (Allison and Treseder, 2008; Bell et al., 2010; Kardol et al., 2010). However, microbial activity is known to respond to distinct moisture and/or temperature thresholds leading to non-linear responses (Suseela et al., 2012; Suseela and Dukes, 2013). To characterize any non-linear response of the extracellular enzyme activity (EEA) we need climate experiments that manipulate each factor at multiple levels (Henry, 2012). Although few studies have measured the EEA in soils exposed to multiple climatic factors (Brzostek et al., 2012; Steinweg et al., 2012), to our knowledge none of the previous studies have characterized EEA in litter decomposing at multifactor climatic conditions.

To test in unprecedented detail the degree to which changes in precipitation and temperature affect the rate and compound-specific degradation of grass litter, we decomposed stem litter of *Poa trivialis* for 740 days in 12 different combinations of warming and precipitation treatments at the Boston-Area Climate Experiment (BACE). We hypothesized that addition and removal of precipitation would alter the temperature responses of litter decomposition and enzyme activities. Specifically, we predicted that moisture stress induced by precipitation removal and/or warming would slow litter decomposition, but that warming would accelerate mass loss under ambient and wet precipitation treatments (i.e., conditions with less water stress). We also predicted that extracellular enzyme activity would respond non-linearly to climate treatments, as microbial activity would exhibit distinct temperature and/or soil moisture thresholds.

## 2. Materials and methods

### 2.1. Site description and experimental design

The litter decomposition experiment was conducted at the Boston-Area Climate Experiment (BACE), located at the University of Massachusetts Agricultural Experiment Station in Waltham, Massachusetts (42°23.1′N, 71°12.9′W). This mesic old-field system with ~40 species of grasses and forbs is comparable to temperate grasslands in plant community composition (Hoeppner and Dukes, 2012). Mean annual precipitation and temperature in nearby Boston are 1063 mm and 10.3 °C, respectively. The study site has a loamy topsoil (Mesic Typic Dystrudept; Haven series) with 45% sand, 46% silt and 9% clay (gravel content: 7%) and a gravelly sandy loam subsoil. BACE is a factorial split-plot experiment with three levels of precipitation [ambient (A), dry (D) and wet (W)] as main treatments and four levels of warming [unwarmed (0), low (L), medium (M) and high warming (H)] as subplot treatments (Suseela et al., 2012). There are three experimental blocks. The soil around each 2 × 2 m plot had been trenched to 60 cm and lined with polyethylene sheets to prevent the movement of water and nutrients between plots. The precipitation treatments were applied using rainout shelters and a sprinkler system. The dry section of each block was located under clear polycarbonate slats that intercepted 50% of the ambient rainfall, which was then diverted to storage tanks and applied immediately to the wet section using sprinklers. We used infrared heaters of different wattages (200 W for low, 600 W for medium and 1000 W for high warming) to apply the warming treatments. Heaters were mounted 1 m above each corner of each plot. Infrared radiometers above the ambient and high warming plots measured canopy temperatures every 10 s. The difference in canopy temperature readings within each group of four plots was used to achieve feedback control (target difference of 4 °C; Suseela and Dukes, 2013). Volumetric soil moisture (10 cm depth) was measured weakly using time-domain reflectometry (TDR) waveguides installed in each plot. In each plot, we also monitored soil temperature (2 cm depth) every 30 min using custom-made linear temperature sensors (Auyeung et al., 2013).

### 2.2. Litter placement, harvest and processing

We started the decomposition experiment at the BACE in October 2008, using the senesced litter of the grass *Poa trivialis* (hollow stem litter) collected from the old-fields adjacent to the BACE plots. Chemical analysis of initial undecomposed tissues showed that *P. trivialis* litter had a C:N ratio of 107. We put 2 ± 0.03 g of air-dried stem of *P. trivialis* in each 5-cm-diameter

litter bag; bags were made of 2 mm mesh window screening material. We placed 72 litter bags in the field (two bags per plot × 36 plots; six bags per treatment), and retrieved three bags from each treatment (one from each plot) after 393 and 740 days of field incubation (half on each date). At each retrieval time, a homogenized subsample from each bag was dried at 50 °C for 48 h. The dried samples were used for estimating mass loss, carbon and nitrogen content (with an elemental analyzer; ECS 4010 Elemental Combustion System, Costech Analytical Technologies, Valencia, California, USA), cell wall-bound phenolics, and carbon quality using Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectroscopy and $^{13}C$ Nuclear Magnetic Resonance (NMR) spectroscopy. After 740 days, half of the litter in each bag was used for enzyme assays and determination of ergosterol content within two to three days after retrieval. The litter samples for enzyme and ergosterol content were stored immediately at 4 °C until analysis.

### 2.3. Potential enzyme activity

We used 250 mg of the litter to determine the potential activity of two hydrolytic enzymes – β-1,4-glucosidase (βG) and N-acetyl-β-D-glucosaminidase (NAG; degrade chitin) – and the oxidative enzyme phenol oxidase. Beta-1,4-glucosidase and NAG were assayed using 4-methylumbelliferyl-β-D-glucopyranoside, and 4-methylumbelliferyl- N-acetyl-β-D-glucosaminide, respectively, as specific substrates. The hydrolase assays were conducted in black 96-well plates in replicates of eight, and the amount of methylumbelliferone produced was quantified using a microplate-fluorometer (Ex-355 nm, Em-450 nm; Saiya-Cork et al., 2002). Briefly, ~250 mg of litter was blended with 150 ml of 50 mM sodium acetate buffer (pH 5.5) in a high-speed blender for 1 min. The slurry was pipetted into the 96-well plates and incubated at 25 °C for 2 h. We used 250 μl of buffer as a blank, 200 μl of buffer and 50 μl of 4-methylumbelliferone (MUB; 10 μM) as a reference standard, 200 μl of buffer and 50 μl of 4- MUB-linked substrates (200 μM) as negative controls, 50 μl of buffer and 200 μl of slurry as a sample control and 200 μl of slurry and 50 μl of 4-MUB as a quench control. The sample assay wells received 200 μl of slurry and 50 μl of 4-MUB-linked substrates (200 μM). The emission from blanks, controls, and standards was also measured, and enzyme activity was calculated following DeForest (2009). The activity of phenol oxidase, which facilitates the degradation of polyphenol and lignocellulose, was estimated using L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate. For this assay, the sample tubes received 0.75 ml of buffer and 0.75 ml L-DOPA (25 mM). The controls for this assay included 0.75 ml each of slurry + buffer, 0.75 ml each of substrate + buffer, and 1.5 ml of buffer alone as a blank. The samples and controls were incubated in the dark at 25 °C for 2 h. The activity was quantified by measuring the absorbance at 450 nm using a spectrophotometer (Saiya-Cork et al., 2002; DeForest, 2009).

### 2.4. Ergosterol (fungal biomass indicator)

Ergosterol is the major sterol in fungal cell membranes; it is involved in permeability and micro-viscosity of the membrane and facilitates the activity of membrane-bound enzymes (Klamer and Baath, 2004; Joergensen and Wichern, 2008). Ergosterol is used as a biomarker for fungi in soils, as it is absent in vascular plants and most algae (Newell et al., 1988; Engelking et al., 2007). The litter harvested at 740 days from the high and unwarmed plots in all precipitation treatments was used for the determination of ergosterol content. Ergosterol was extracted from decomposing litter as per the method described in Gessner and Schmitt (1996). Briefly, lipids were extracted from 100 mg of litter by refluxing for 30 min in 10 ml of 0.14 M methanoic-KOH. The extract was further concentrated using solid-phase extraction (SPE) cartridges (C18, Hypersep, 500 mg sorbent weight; 3 ml column volume) as described below. The cartridges were initially conditioned with 5 ml methanol followed by 5 ml of the extraction solvent, to which 0.5 ml of 1 M HCl had been added. The samples were acidified by adding 0.5 ml of 1 M HCl, and were loaded in to the SPE cartridges and eluted under mild vacuum (2 ml/min). The cartridges were washed with 3 ml of 0.4 M KOH in 60% methanol and the sorbent bed was dried under vacuum for 35 min. The ergosterol was eluted with 1.5 ml of 0.4 M isopropanoic KOH. In preliminary studies we did not find any degradation of ergosterol in the elution matrix. The percent recovery was 88 ± 5%. The amount of ergosterol was quantified using high-pressure liquid chromatography (HPLC). All the samples were analyzed with a Shimadzu quaternary pump UFLC system equipped with an auto-sampler, inline degasser, and UV–visible diode array detector. Separation of ergosterol compounds was performed on an Onyx $C_{18}$ column (monolithic silica, 130 Å; 100 mm × 4.6 mm I.D.; Phenomenex, Torrance, CA). The ergosterol was separated by an isocratic elution of 99% methanol. This gave a minimum peak resolution (*Rs*) of 4. The limit of detection was defined as having a signal-to-noise (S/N) ratio of 10 and all values reported are based on the peak area at 282 nm. The identification and quantification of ergosterol was based on comparison of retention time and UV-spectra to those of an authentic standard.

### 2.5. Litter chemistry analyses

#### 2.5.1. Cell-wall bound phenolics

We used alkaline hydrolysis (Martens, 2002) to quantify total phenolic acids in the initial, undecomposed litter and in litter decomposed in the unwarmed and the high warming treatments in all precipitation treatments at each time of collection. Briefly, 75 mg of litter sample was extracted with 10 ml of 4 M NaOH at 121 °C for 2 h. After cooling, the samples were acidified to a pH of <1.5 using 50% HCl and centrifuged at 2000 rpm for 5 min. The supernatant was transferred to glass tubes and the phenolic compounds were extracted through liquid–liquid partitioning to ethyl acetate on a shaker at 4 °C. A subsample (200 μl) of the ethyl acetate fraction was transferred to vials with glass inserts and was dried completely under $N_2$. The samples were then silylated via reaction with 100 μl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) for 40 min at 40 °C. The samples were analyzed using GC–MS within 10 h after derivatization.

The GC–MS analysis was done using an Agilent 7980A GC system coupled with a 5975 C series mass detector. Compounds were separated in a DB-5 MS capillary column (30 m length × 0.25 mm internal diameter × 0.20 μm film thickness) using split (1:2) injection (1 μl) with the following temperature program: 80 °C for 2 min, followed by ramping at 10 °C per minute to 260 °C prior to cool down. The carrier gas (He) was maintained at a constant pressure; the injection port and the MS interphase were maintained at 270 °C; the MS quad temperature was maintained at 150 °C; and the MS source temperature was set at 230 °C. The electron multiplier was operated at a constant gain of 10 (EMV = 1478 V), and the scanning range was set at 100–400 amu, achieving 4.65 scans $s^{-1}$. Phenolic compounds were identified and quantified using authentic standards. We used the total phenolic acid content and the sum of ferulic and *p*-coumaric acid separately for statistical analyses. We calculated the amount of ferulic and *p*-coumaric acid lost at each time of collection by subtracting the amount of ferulic and *p*-coumaric acid in the remaining litter from the undecomposed initial litter. The percentage of ferulic and *p*-coumaric acid lost per gram of decomposed litter in each treatment was also calculated.

#### 2.5.2. Diffuse reflectance infra-red Fourier transform (DRIFT) spectroscopy

We used litter from the high and unwarmed treatments in the dry (dry + high warming, DH; dry + unwarmed, D0), ambient (ambient + high warming, AH; ambient + unwarmed, A0) and wet precipitation (wet + high warming, WH; wet + unwarmed, W0) treatments, after 393 and 740 days of field incubation, for the DRIFT analysis. DRIFT is a robust tool to identify the overall chemistry of the litter as it measures the vibrational frequencies of covalent bonds in compounds (Nault et al., 2009). We collected the DRIFT spectra in transmission mode using a Perkin–Elmer Spectrum One DRIFT spectrometer equipped with a deuterated triglycine sulfate detector. For obtaining the DRIFT spectra, we ball milled the litter samples and spectral grade KBr separately and then mixed them in a ratio of 1:50 using an agate mortar and pestle. We then gently packed the finely powdered mixture in a macrocup accessory. We acquired 40 interferograms per sample from 4000 to 650 $cm^{-1}$ at 4 $cm^{-1}$ resolution (Tharayil et al., 2011). We processed the spectra using Kubelka–Munk transformation and corrected the baseline using ACD Spec Manager (Advanced Chemistry Development, Ontario, Canada). The spectral assignment was made according to Silverstein et al. (2005) and based on pure standards. The peak interpretation was based on Lammers et al. (2009), Movasaghi et al. (2008) and Filley et al. (2008). For the interpretation of DRIFT spectrum, we used principal component analysis (PCA) of 13 identifiable DRIFT peaks that corresponded to the major carbon functional groups. In PCA, we used relative peak heights for comparison across different samples. We computed the relative peak heights as the ratio of the intensity of each individual peak to the sum of the intensities of all selected peaks (Haberhauer and Gerzabek, 1999).

#### 2.5.3. $^{13}C$ Cross-polarization magic angle spinning (CPMAS) Nuclear Magnetic Resonance (NMR) spectroscopy

Litter samples from treatments subjected to DRIFT analyses after 740 days of decomposition were also used for $^{13}C$ NMR analyses. The $^{13}C$ NMR analysis of the litter samples was done using a Chemagnetics CMX400 (Varian Inc., Palo Alto, CA, USA) spectrometer operating at a frequency of 100.6 MHz. For the NMR analysis, we packed the finely powdered litter samples in a 4 mm zirconium rotor and spun at 5 kHz using a standard cross-polarization pulse sequence. We used the following parameters to acquire the spectra: a contact time of 3.5 ms, an acquisition time of 20 ms, and a recycle delay of 3 s. Preliminary experiments were conducted with similar litter samples to optimize the NMR parameters. For each sample, we obtained about 4000 transients. The spectra were baseline corrected and processed using a line broadening of 22 Hz. We used glycine at 176 ppm as an external reference for the chemical shift values of the spectra. The relative intensities for the chemical shift regions were integrated using the ACD/Spectrus processor (Version 2012, Advanced Chemistry Development, Inc., Ontario, Canada). The major peaks identified in the $^{13}C$ NMR spectra are as follows (Preston et al., 2009): 23 ppm: acetate of hemicellulose; 56 ppm: methoxyl carbon of lignin; shoulder at 65 ppm: methoxyl group of hemicellulose; intense signal at 72 ppm: carbon 2–6 of cellulose and hemicellulose; 105 ppm: anomeric C1 of glucose in cellulose; 140–165 ppm: O substituted ring carbon from aromatic structures. The $^{13}C$ NMR spectra were divided into 7 chemical shift regions (Table 1). We analyzed the area under each of these chemical shift regions, expressed as the percentage of total intensity. We then used these percentage intensity values for calculating ratios such as CC: MC (carbohydrate C : methoxyl C; *O*-alkyl C : methoxyl and N-alkyl C), alkyl C: *O*-alkyl C (alkyl/alkyl + methoxyl + di-*O*-alkyl), and aryl + *O*-aryl C : *O*- alkyl C (Preston et al., 2009).

**Table 1**
Standard chemical shift regions and corresponding carbon functional group in a $^{13}C$ NMR spectrum (Preston et al., 2009).

| Chemical shift region (ppm) | Carbon functional group |
|---|---|
| 0–50 | Alkyl C |
| 50–60 | Methoxyl and N-alkylC |
| 60–93 | *O*-alkyl C |
| 93–112 | Di-*O*-alkyl & some aromatic C |
| 112–140 | Aromatic C |
| 140–165 | Phenolic C |
| 165–190 | Carboxyl/carbonyl C |

### 2.6. Data analysis

The effect of warming and precipitation treatments on soil moisture and temperature was analyzed using a mixed-model restricted maximum likelihood (REML) repeated measures ANOVA with warming, precipitation and year considered as fixed factors. Mass loss of the litter was first analyzed with warming, precipitation and time of collection as main effects using a mixed-model REML analysis (Suseela et al., 2013). The analyses of mass loss response variables separately at each time of collection would provide more explanatory power for the effect of climate on litter decomposition (Suseela et al., 2013). The effects of warming and precipitation treatments on litter mass loss, decomposition rate constants and phenolic acid contents at individual time of retrieval and enzyme activities and ergosterol content at 740 days of retrieval were analyzed using a mixed-model restricted maximum likelihood (REML) ANOVA. The differences among treatments were compared using Tukey's HSD multicomparison test. The relative heights of peaks from DRIFT spectra were analyzed using PCA and then ANOVA of PCA coordinate scores, followed by Tukey's HSD multicomparison test. All statistical analyses were performed using SAS version 9.2 (SAS Institute, Inc., 2002–2008, North Carolina, USA). Decomposition rate constant ($k$) of the litter at each harvest time was calculated using the equation

$$\ln\left(M_t/M_0\right) = y - kt,$$

where $M_t$ = litter mass remaining at time $t$, and $M_0$ = initial litter mass and $y$ is the intercept (Austin and Vitousek, 2000). The data were fitted using Sigmaplot (v12; Systat Software, Inc., San Jose, CA, USA).

## 3. Results

### 3.1. Soil microclimate

The soil moisture at 10 cm depth during the study period varied by month ($P < 0.0001$; Fig. 1a, b). The average soil moisture also varied between the two years of decomposition; soil moisture during the first year was 12% higher than in the second year of study ($P < 0.0001$). During the growing season of 2009, the volumetric soil water content in the dry treatment (average of 10%) was consistently lower than in the wet treatment (average >20%; Fig. 1a). However, during the growing season of 2010 the difference in soil moisture among the treatments diminished during the peak summer months of July and August, when volumetric soil moisture was <15% in all precipitation treatments (Fig. 1b). The effects of warming treatments on soil moisture varied by month ($P < 0.05$; Fig. S1). Over the two years of decomposition, soils in the high warming treatment were drier, on average, than soils in the unwarmed, low, and medium warming treatments (Fig. S2).

Warming treatments increased the soil temperature (2 cm depth) ($P < 0.001$; Fig. 1c, d). The soils were warmer in 2010 than

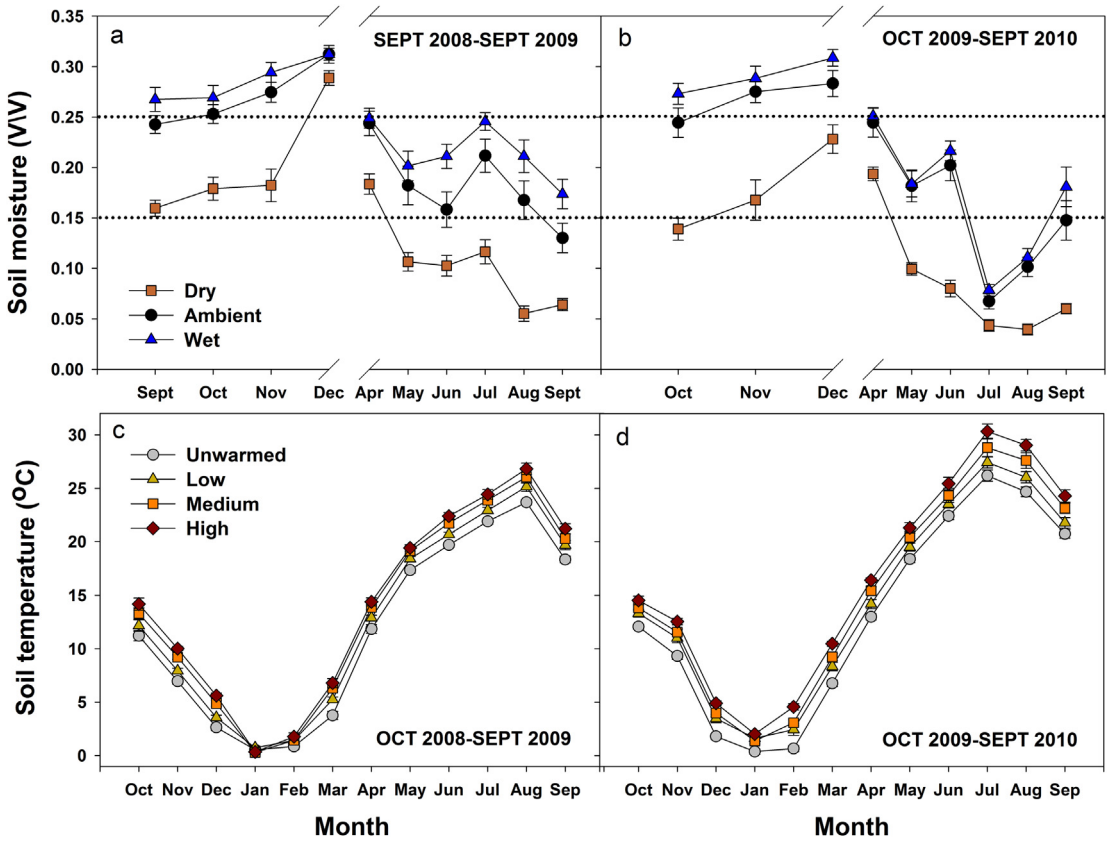


**Fig. 1.** Effect of climatic treatments on a & b) volumetric soil moisture ($n = 12$) at 10 cm depth and c & d) soil temperature ($n = 9$) at 2 cm depth during the study period. The dotted lines in panels a & b indicate the moisture thresholds for soil microbial activity. Volumetric soil moisture content below ~15% and above ~25% drastically reduced microbial respiration (Suseela et al., 2012).

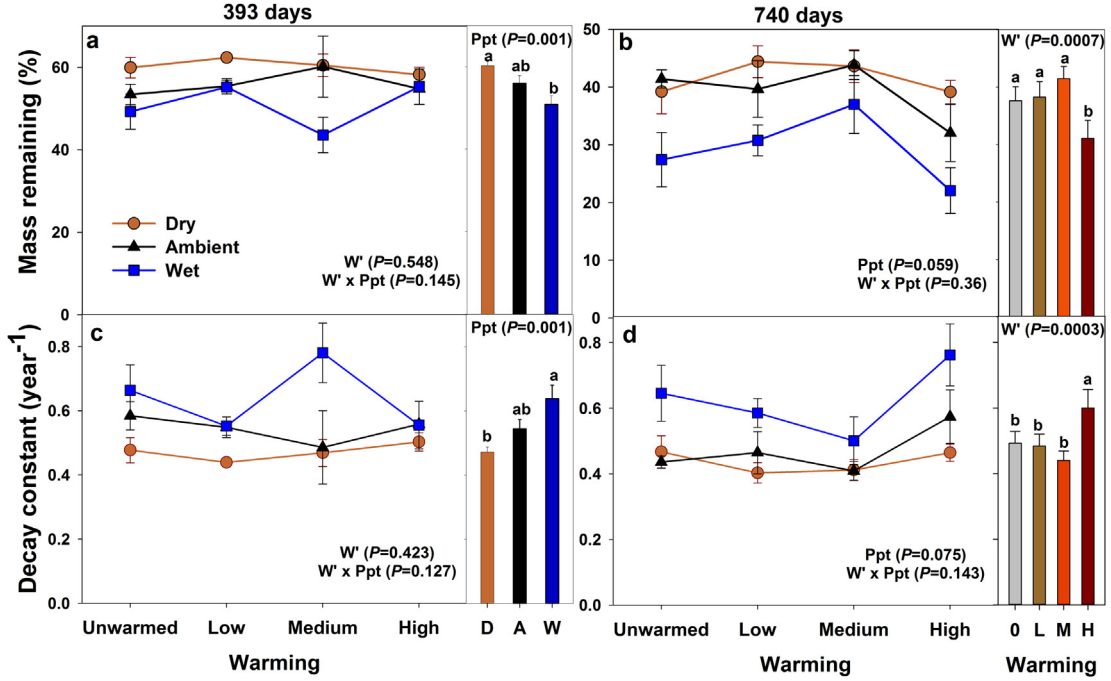


**Fig. 2.** Effect of climatic treatments on percent initial mass remaining (a & b) and decay constants (c & d) of *P. trivialis* litter at 393 days and 740 days. Values in the interaction graphs ($n = 3$) and vertical bars in the single factor graphs (a & c; $n = 12$; b & d; $n = 9$) represent means ± SE. Key: D, dry; A, ambient; W, wet; 0, unwarmed; L, low warming; M, medium warming; H, high warming; Ppt, precipitation and W′, warming.

2009. The average soil temperatures in the summer of 2009 were 21.7 °C and 24.5 °C in the unwarmed and high warming treatments, respectively (Fig. 1c). However, during the summer of 2010, the average soil temperatures were 24.4 °C and 28.2 °C in the unwarmed and the high warming treatments, respectively (Fig. 1d). Over the course of 393 days (October 2008 to September 2009), the low, medium and high warming treatments increased soil temperature by an average of 1, 1.8 and 2.4 °C, respectively, than unwarmed treatment. From 2009 (October) to 2010 (September), soils were warmed by an average of 1.4, 2.2 and 3.3 °C in the low, medium and high warming treatments, respectively, compared to unwarmed treatment.

### 3.2. Litter mass loss and decay constant

Analysis of mass loss with warming, precipitation and time of retrieval as main effects indicated that the climatic factors affecting mass loss depended on the incubation period in the field (precipitation × time; $P < 0.001$, warming × time; $P = 0.015$). Analyses of mass loss at each time of retrieval revealed that after 393 days of decomposition, mass loss varied only with the precipitation treatment (warming effect; $P = 0.548$); litter in the wet treatment lost more mass (49%) than litter in the dry treatment (40% mass loss; $P < 0.001$; Fig. 2a). Litter exposed to ambient precipitation showed a statistical trend of lower mass loss (44%) than that in the wet treatment ($P = 0.059$). However, after 740 days the high warming treatment (70% mass loss) accelerated decomposition rates compared to medium (59%), low (62%) and the unwarmed (63%) treatments ($P < 0.001$; Fig. 2b). At this stage of decomposition, precipitation treatments affected mass loss ($P = 0.059$) with greater loss in the wet treatment (70%) compared to the dry treatment (58%).

Decomposition rate constants ($k$) followed the same pattern as mass loss (Fig. 2c), with different climate change factors having the most statistically robust influence at different time periods. After 393 days of decomposition, we recorded a higher decay constant in the wet treatment ($P < 0.001$; $k = 0.63$) compared to the dry treatment ($k = 0.47$) and a statistical trend towards a higher decay constant in the wet treatment compared to the ambient treatment ($k = 0.54$; $P = 0.059$). After 740 days, the decay constant in the high warming treatment ($k = 0.6$) was greater than in the medium ($k = 0.44$), low ($k = 0.48$) and unwarmed ($k = 0.49$) treatments ($P < 0.001$; Fig. 2d). The effect of precipitation treatments on the decay constant showed a statistical trend ($P = 0.075$) after 740 days, with $k$ in the wet treatment exhibiting 13% and 30% increases over ambient and dry treatments, respectively (Fig. 2d).

### 3.3. Enzyme activity and ergosterol (fungal biomass indicator) content after 740 days of decomposition

Extracellular enzyme potential responded to the treatments in different ways, and sometimes non-linearly. Potential βG activity varied from about 1000 to 6000 nmol $g^{-1}$ dry litter $h^{-1}$ in the different treatments. The activity of βG responded to interactions of precipitation and warming treatments, with a distinct non-linear response to warming ($P = 0.048$; Fig. 3a). Potential βG activity was more than 50% lower in the dry treatments receiving low, medium and high warming than in the wet treatments receiving high or no warming (Table S1). Potential activity of NAG also responded non-linearly to warming, and similarly to that of βG, with the lowest activities in the medium warming treatment followed by the low warming treatment, and maximum activity in the high and unwarmed treatments ($P = 0.001$; Fig. 3b). However, precipitation did not affect the activity of NAG ($P = 0.6$). Potential phenol oxidase activity generally declined with warming ($P < 0.05$;

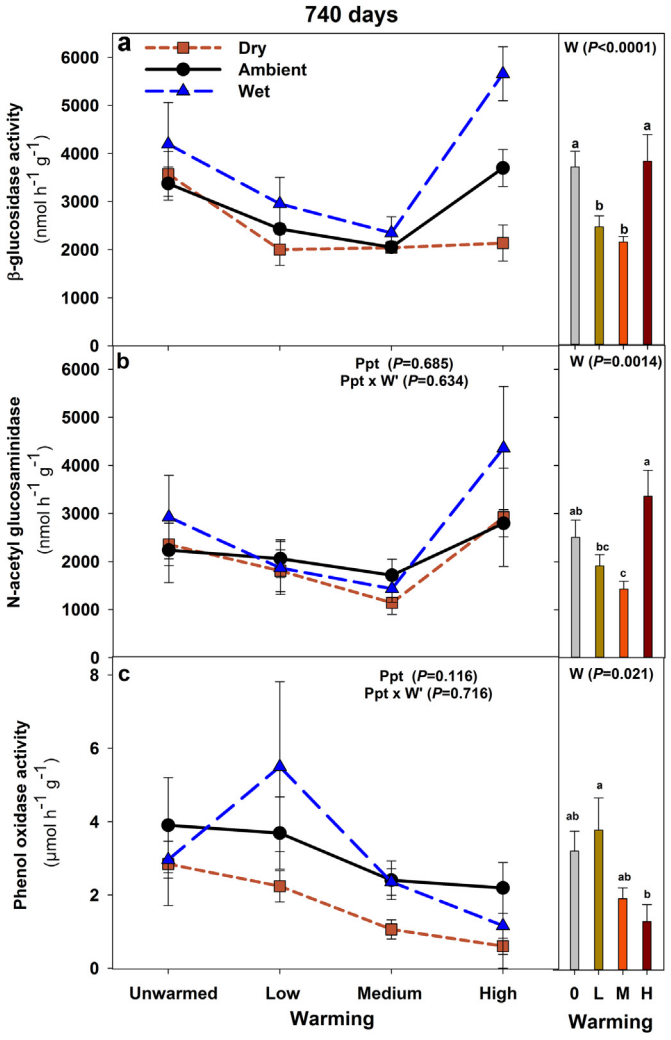


**Fig. 3.** Potential enzyme activity of a) β-glucosidase b) N-acetyl-glucosaminidase and c) phenol oxidase in *P. trivialis* litter after 740 days of decomposition. Values in the interaction graphs ($n = 3$) and vertical bars in the single factor graphs ($n = 9$) represent means ± SE. Key: 0, unwarmed; L, low warming; M, medium warming, H, high warming; Ppt, precipitation; W′, warming.

Fig. 3c), but did not respond to precipitation, and the ergosterol content of litter had a similar response. The high warming treatment decreased ergosterol content compared to the unwarmed treatment ($P = 0.002$; Fig. 4), but precipitation treatments had no effect ($P = 0.4$).

### 3.4. Litter chemical characteristics

Among the 14 phenolic acids identified (cinnamic acid, ethylvanillin, *p*-hydroxybenzaldehyde, *p*- hydroxyacetophenone, *p*-hydroxybenzoic acid, vanillin, acetovanillone, vanillic acid, syringaldehyde, acetosyringone, syringic acid, 3,5-dihydroxy-benzoic acid, *p*-coumaric acid and ferulic acid), *p*-coumaric acid and ferulic acid accounted for 45–50% of the total phenolic acid content in the initial undecomposed litter. After 393 days, the precipitation treatments had affected the rate of degradation of ferulic and *p*-coumaric acid ($P < 0.001$); litter in the wet treatment lost three and four times as much ferulic and *p*-coumaric acid per gram than litter in the ambient and dry treatments, respectively (Fig. 5). Total ferulic and *p*-coumaric acids also showed a trend of greater loss in the high warming treatment compared to the unwarmed treatment ($P = 0.069$). However, this effect subsequently disappeared; neither

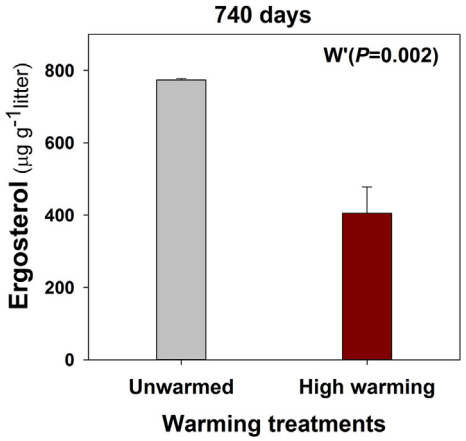


**Fig. 4.** Ergosterol content ($n = 9$) of *P. trivialis* litter after 740 days of decomposition. Values represent means ± SE. Key: W', warming.

total phenolic acids nor the sum of ferulic and *p*-coumaric acid varied with warming or precipitation treatments in litter retrieved after 740 days ($P > 0.05$).

Principal component analysis of the relative intensities of various DRIFT peaks of *P. trivialis* litter (Fig. 6a, b) revealed that the climate treatments affected the chemistry of decomposing litter. After 393 days of decomposition (Fig. 6a), litter from different precipitation treatments separated along PC axis 1 ($P = 0.037$), with wet treatments differing from dry treatments (Tukey's *post-hoc* analysis; $\alpha = 0.05$). The relative abundance of vibrations corresponding to lignins (1510 $cm^{-1}$) and aliphatic compounds (2925 $cm^{-1}$) was higher in litter exposed to wet treatments, whereas the litter decomposing in dry treatments was abundant in mixed carbohydrates (1248, 1050 $cm^{-1}$). There was no separation of the treatments along PC axis 2. After 740 days of decomposition, a similar analysis showed that effects of the precipitation treatments on litter chemistry persisted (Fig. 6b; Table 2). However, at this later date, litter in the dry treatments differed chemically from that in other treatments (Tukey's HSD analysis of treatments on PC axis 1; $P = 0.012$), and was characterized by greater abundance of carbohydrates (1248, 896 $cm^{-1}$) relative to polyphenolic compounds (1596, 1655, 1510 $cm^{-1}$). Again, PC axis 2 did not separate the treatments.

The $^{13}C$ NMR spectra of the litter after 740 days of decomposition (Fig. 7; Table 3) revealed changes in the carbon chemistry of litter decomposing in different climatic treatments. Litter in the wet, high warming treatment had the lowest intensity of peaks at 65, 72, 82 and 105 ppm compared to all other treatments (Fig 7). Thus, the ratio of carbohydrate carbon to methoxyl carbon (CC:MC; *O*- alkyl C : methoxyl and N-alkyl C) was smallest in the wet, high warming treatment and largest in the dry (unwarmed and high warming) treatments. The alkyl C: *O*-alkyl C ratio and the aryl + *O*-aryl C/*O*-alkyl C ratio did not differ by treatment.

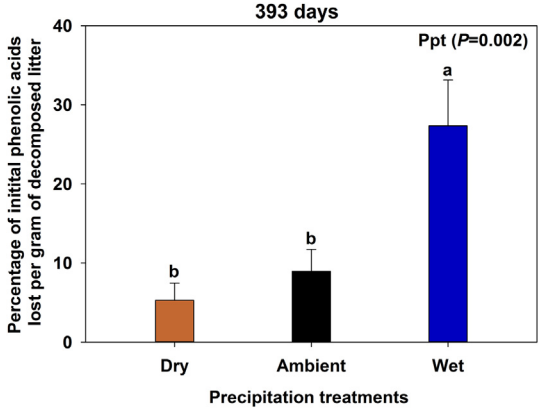


**Fig. 5.** Percentage of phenolic acids (ferulic and p-coumaric acid) lost per gram of decomposed litter after 393 days ($n = 6$). Values represent means ± SE. Key: Ppt, precipitation.

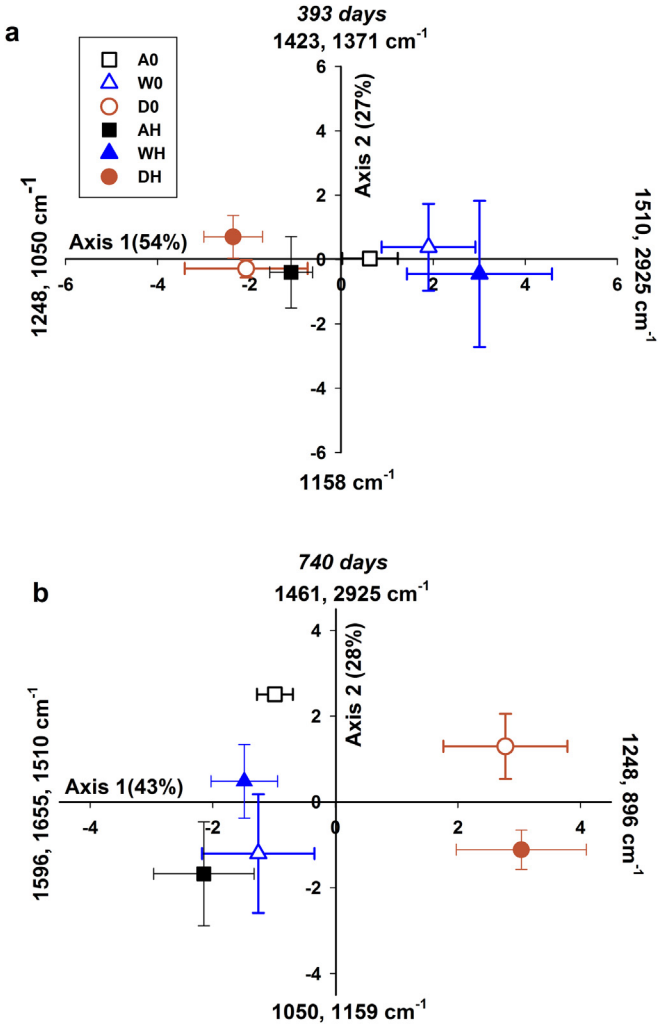


**Fig. 6.** Principal component analysis of the relative intensities of dominant DRIFT peaks of *P. trivialis* litter from different climatic treatments of a) after 393 days b) after 740 days. On each principal component axis the wave numbers with the highest eigenvector loading are listed. The values in parentheses on each axis label show how much of the variation in the dataset was accounted for by the PCA axis. Graphed values represent means ($n = 3$) ± SE. Key; A, ambient; W, wet; D, dry; 0, unwarmed; H, high warming.

## 4. Discussion

Our results clearly demonstrate the differential response of mass loss, enzyme activities and compound-specific degradation of grass litter to simulated climatic changes. Over the 740 days of decomposition, although both warming and precipitation affected the rate of mass loss, precipitation had an overriding influence on the compound-specific degradation of litter. This decoupling of mass loss and compound-specific degradation suggest that the rate of mass loss provide little insight into the chemical transformation in litter that ultimately aids in soil organic matter formation. Similarly, the compound-specific degradation in litter did not

**Table 2**
Results of post-hoc mean separation test (Tukey's HSD) from ANOVA of principal component axis coordinate scores of diffuse reflectance infra-red Fourier transform (DRIFT) peaks from *P. trivialis*. PC axis 1 is strongly related to the chemical changes in decomposed litter due to altered precipitation.

| *P. trivialis* (393 days) | | | | *P. trivialis* (740 days) | | | |
|---|---|---|---|---|---|---|---|
| Precipitation ($P = 0.037$) | Warming ($P = 0.758$) | Scores | Tukey group | Precipitation ($P = 0.012$) | Warming ($P = 0.526$) | Scores | Tukey group |
| PC axis 1 (54%) | | | | PC axis 1 (43%) | | | |
| Wet | High warming | 3.0779 | A | Wet | High warming | −1.4855 | B |
| Wet | Unwarmed | 2.0032 | AB | Wet | Unwarmed | −1.2577 | B |
| Ambient | Unwarmed | 0.6759 | ABC | Ambient | Unwarmed | −0.9221 | B |
| Ambient | High warming | −1.2307 | BC | Ambient | High warming | −2.1464 | B |
| Dry | High warming | −2.2548 | C | Dry | High warming | 3.0355 | A |
| Dry | Unwarmed | −2.2714 | C | Dry | Unwarmed | 2.7763 | A |

Treatments that do not share letters differ significantly ($\alpha = 0.05$).

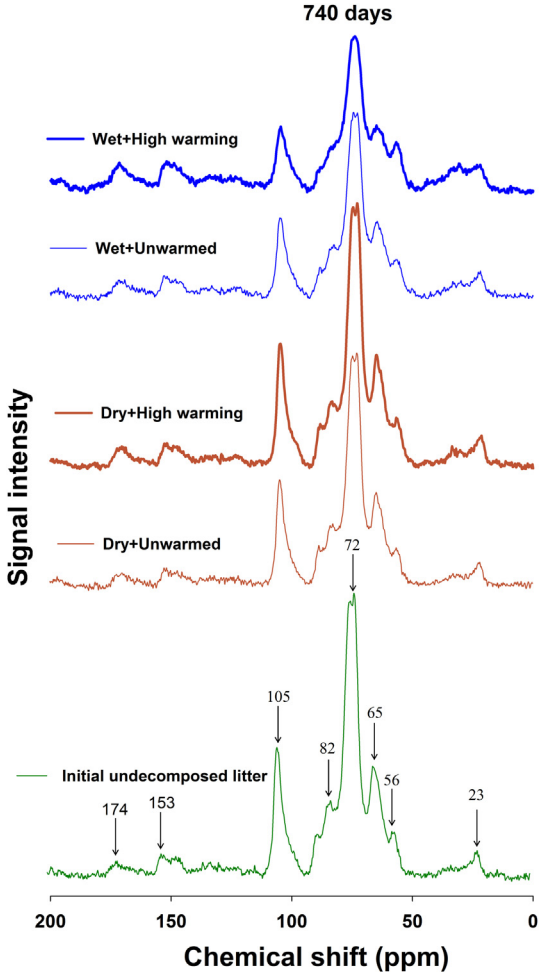


**Fig. 7.** $^{13}C$ NMR spectra of *P. trivialis* exposed to different climatic treatments after 740 days of decomposition. See Tables 1 and 3 for details on chemical shift regions and ratios.

**Table 3**
Ratios of the relative intensities of chemical shift regions from $^{13}C$ NMR of *P. trivialis* litter after 740 days of decomposition. Key: CC:MC – carbohydrate carbon: methoxyl carbon; A/*O*-A – alkyl carbon: *O*-alkyl carbon.

| Treatment | CC/MC | A/*O*-A | (Aryl + *O*-aryl C)/ *O*-alkyl C |
|---|---|---|---|
| Initial undecomposed | 20.08 | 0.077 | 0.112 |
| Dry + unwarmed | 21.18 | 0.071 | 0.108 |
| Dry + high warming | 19.45 | 0.076 | 0.124 |
| Ambient + unwarmed | 11.94 | 0.117 | 0.141 |
| Wet + unwarmed | 11.65 | 0.100 | 0.140 |
| Wet + high warming | 8.99 | 0.157 | 0.191 |

follow the pattern of enzyme activities suggesting that apart from substrate quality and availability, EEA could be influenced by other edaphic factors that affect their diffusion, stabilization and potential activity. Compounds that require non-enzymatic decomposition could also contribute to the decoupling of enzyme activities and compound-specific degradation in litter. To our knowledge, this study also captured for the first time the non-linear response of potential βG and NAG activities in decomposing litter which could be attributed to the effect of climatic factors on both microbial communities and/or the response of enzymes to temperature and moisture perturbations. Along with emphasizing the multifaceted nature of litter decomposition, our study also highlights that projected changes in climate could strongly influence carbon cycling in grasslands through changes in the rate and compound-specific degradation of litter.

### 4.1. Litter mass loss

The significant effect of precipitation on mass loss at the initial stage (393 days) and warming at the later stage (740 days) of decomposition suggests that the inter-annual variability in rainfall as well as the quality of litter remaining at each decay stage may have modified the effect of climatic treatments on decomposition. Variability in precipitation may regulate litter decomposition, particularly during the summer months (June–August; Fig. S3) when temperatures are the highest (Trofymow et al., 2002). Thus, after 393 days the greater mass loss in the wet treatment compared to the dry treatment have been caused by the difference in soil moisture between the treatments in summer 2009 (Fig. 1a). During the summer of 2010, soil moisture was lower across all treatments, and the effect of the precipitation treatments on soil moisture was less pronounced, potentially diminishing the influence of these treatments on decomposition (Fig. 2; $P = 0.059$). Concurrently, beyond 393 days, the higher average soil temperatures in 2010 compared to 2009, leading to a stronger moisture limitation (Fig. 1a), might have had an overall inhibitory effect on decay of litter across all the treatments. Although the high warming treatment increased the rate of mass loss at 740 days, there was also a trend in the warmest treatments toward greater mass loss in the wet (78% mass loss) and ambient (68%) plots compared to dry plots (61%), possibly due to the higher activity of βG (Fig. 3a) further indicating a greater influence of soil moisture in litter decomposition.

In our study, the differential responses of mass loss to climate treatments could also be due to the unique litter chemistry of grasses. Compared to non-graminaceous species, members of the family Poaceae have primary cell walls with different chemical constituents and architecture (Carpita, 1996). The stem litter of grasses has cell walls that are enriched in aromatic compounds such as esters of hydroxycinnamates, ferulate and *p*-coumarate that

often limit their biodegradation (Carpita, 1996). Most of the structural carbohydrates in grasses are cross-linked to lignin by hydroxycinnamic acids (ester linked to cellulose and ether linked to lignin; Iiyama et al., 1990; Ralph et al., 1995; Carpita, 1996), thus increasing the recalcitrance of the otherwise labile carbohydrates. The cleavage of these ester-ether linkages and in turn the degradation of this lignocellulose material requires an ensemble of microbial enzymes (Jeffries, 1990; Floudas et al., 2012). Thus, increased precipitation could accelerate mass loss rates of litter by providing favorable conditions for microbial biomass and activity (Allison and Vitousek, 2004), whereas drought could dehydrate litter (Manzoni et al., 2012), reducing microbial metabolic activity, and slow decomposition (Schimel et al., 2007). Thus, in the initial stages of decomposition (through 393 days), supplemental precipitation may have accelerated a preferential loss of hydroxycinnamic acids that cross link polysaccharides to lignin, in turn increasing the bioavailability of carbohydrates in litter exposed to the wet treatment. This is supported by the three times greater loss of *p*-coumaric acid and ferulic acid from litter (Fig. 5) in the wet treatment, and an accompanying greater mass loss of litter exposed to this treatment. Though this could be partly attributed to the increased leaching loss of compounds in wet treatments, given that a majority of hydroxycinnamates are cross linked to cell walls the enzymatic cleavage of the ester-ether linkages is a prerequisite for the disappearance of these compounds from litter. The drier and warmer climate during the second year of the experiment combined with the higher recalcitrance of the residual litter after 393 days decreased the overall mass loss across all the treatments in the second year. However, the supplemental precipitation treatment exhibited a trend of higher mass loss and *k* value at 740 days (Fig. 2b, d), even though the soil moisture of this treatment did not differ from that of the ambient precipitation treatment in 2010 (Fig. 1b). This increase in mass loss in the wet treatment during the second year and a greater response of βG activity (Fig. 3a) further supports the possibility that the residual litter in the wet treatment was less recalcitrant (due to the preferential loss of cross-linkages, which increases the exposure of carbohydrates to microbial attack) after 393 days of decomposition. Thus, the availability of more labile carbohydrates in the wet treatment compared to the ambient precipitation treatment after 393 days might have resulted in a marginally significant increase in mass loss in the wet treatment at the end of the study period (740 days).

### 4.2. Response of microbial extracellular enzyme activity (EEA) and fungal biomass

Extracellular enzymes are the key mediators of the degradation and transformation of plant litter and soil organic matter (Sinsabaugh, 2010; Burns et al., 2013). Thus, it is imperative to understand the effects of warming and altered soil moisture on microbial community and functional activity. We observed non-linear responses of βG and NAG to warming treatments (Fig. 3). In general, warming and reduced precipitation have been found to decrease rates of enzyme activity in soil. For example, proteolytic enzyme activity in soil was shown to decrease in dry grasslands in response to increases in temperature and reductions in precipitation (Brzostek et al., 2012). Similarly, a previous study at BACE found that the activity of βG in soil responded primarily as a function of temperature when soil moisture was not limiting (Steinweg et al., 2012). In our study, the potential activity of βG and NAG in litter decreased from the ambient to medium warming treatment and was upregulated only in the high warming treatment (Fig. 3a,b), with the greatest response in the wet, high warming treatment, suggesting the potential importance of temperature and moisture thresholds for the activity of microbial enzymes. In a previous study from the BACE site, we found threshold responses of microbial respiration to soil moisture, where microbial respiration decreased drastically when soil moisture dropped below ~15% or exceeded ~25% (Suseela et al., 2012). In soils, moisture below ~15% reduces microbial activity by limiting solute diffusion, while in litter the reduction in microbial activity could be due to the dehydration of litter (Manzoni et al., 2012). In our study, the high level of warming decreased soil moisture compared to the unwarmed, low and medium warming treatments (Fig. S2). Although we measured soil moisture at 10 cm depth, and did not measure litter moisture directly, the effect of the high warming treatment on moisture might be stronger in litter than in soil, as the litter holds less moisture and can dry out rapidly. Thus, high warming with more optimum moisture in the wet treatment might have accelerated the enzyme activities (Fig. 3a, b) thereby accelerating mass loss (Fig. 2b) while moisture limitation due to precipitation removal and high warming could dehydrate litter, decrease enzyme activity and reduce mass loss. In a previous litter decomposition study at the BACE site we have also observed increased decomposition of lignin and alkyl-carbon only in the wettest, warmest treatment, suggesting that these are the most optimal conditions for microbial activity (Suseela et al., 2013).

Although we observed non-linear responses of the potential activities of βG and NAG, we can only speculate about the possible mechanisms for this trend. As catalysts, enzymes lower the activation energy of biochemical reactions (Wallenstein et al., 2011). Temperature can influence the activation energy of enzymatic reactions by enabling more substrates to take part in the reaction and by altering the structural conformation of enzymes, thereby modifying the active site and in turn the enzyme-substrate affinity (Londesborough, 1980; Hall, 1985; Lonhienne et al., 2000). Temperature can also affect enzyme production leading to changes in potential enzyme activities (Henry, 2012). Extracellular enzyme activity could also be construed as a combined effect of both direct climatic effects on enzyme activity and the feedbacks resulting from the indirect effects of climate on other plant and soil processes (Sistla and Schimel, 2013). For example, increases in soil nitrogen mineralization due to high warming could increase the activity of hydrolytic enzymes. Previous studies have found that βG activity increases with nitrogen availability (Sistla and Schimel, 2013). Our results provide new insight into the potentially complex responses of enzyme activities in litter to warming that may influence the rate of litter decomposition under future climate scenarios.

Unlike β-glucosidase, for which the response to warming depended on the precipitation treatment, the activity of phenol oxidase in the decomposing litter generally decreased with warming (Fig. 3c). As fungi are the major producers of phenol oxidase, its activity is highly correlated with fungal biomass (Fioretto et al., 2000; Sinsabaugh, 2010). This is consistent in our study, where the high warming treatment decreased ergosterol (fungal biomass; Fig. 4), with a corresponding decrease in phenol oxidase activity. Apart from the direct effect of temperature in accelerating EEA, climate could thus affect EEA by altering the enzyme production through changes in microbial biomass and community composition (Henry, 2012). In a boreal forest ecosystem, a soil temperature increase of 0.5 °C reduced bacterial and fungal abundance, and led to changes in the fungal community structure (Allison and Treseder, 2008). Although we observed decreases in fungal biomass and phenol oxidase with high warming, we found an opposing trend in the potential activity of NAG, which increased. As NAG is an enzyme that catalyzes the degradation of chitin from fungal cell walls, the fungal death and turnover in the high warming treatment may have led to a corresponding increase in the activity of NAG (Lindahl and Finlay, 2006; Allison et al., 2009; Fig. 3b).

### 4.3. Litter chemical composition

Although precipitation and warming both affected mass loss and EEA (Figs. 2 and 3) over the 740 days of decomposition, the overall chemical composition of the remaining litter diverged only with precipitation treatments (Fig. 6), indicating that moisture had an overriding influence on the degradation of specific compounds in the litter. After 393 and 740 days, litter that decomposed in the dry treatment had a higher relative abundance of carbohydrates, whereas litter from the supplemental precipitation treatments was relatively enriched in lignin and aliphatic compounds as indicated by the PCA of the DRIFT peaks. As decomposition progresses through the initial preferential loss of labile carbohydrates, the remaining litter would become enriched in recalcitrant compounds, rendering it increasingly resistant to decomposition. In leaf litters of grasses, where a proportionately higher percent of carbohydrates remains protected through cross linkages by hydroxycinnamates to lignins, the overall recalcitrance of litter increases rapidly during the progression of decomposition. This preferential enrichment of recalcitrant compounds limits the climatic conditions in which the decomposition can further proceed (Suseela et al., 2013). This was further supported by the lower CC/MC ratio in the ambient and wet treatments that reflect an increase in the relative abundance of lignins, further revealing an advanced decomposition compared to litter from the dry treatment. Previous studies have found similar decreases in the CC/MC ratio with the progress of decomposition (Fig. 7; Almendros et al., 2000; Blumfield et al., 2004; Mathers et al., 2007). Thus, the greater abundance of carbohydrates in the dry treatment, along with the reduction in mass loss, indicates the slow decomposition of litter under moisture stress. After 740 days, although mass loss was greatest in the high warming treatment, the litter chemistry was influenced only by the precipitation treatments. Similarly, although EEA (phenol oxidase and NAG) and ergosterol content responded to warming, the degradation of compounds from litter varied only with precipitation treatments. This suggests that apart from substrate quality and availability, both edaphic and climatic factors that affects the diffusion, stabilization and potential activity of enzymes (Wallenstein et al., 2011) partly decouples EEA from compound-specific degradation. Also degradation of carbon compounds that can be readily utilized without enzymatic catalysis would contribute to the decoupling of EEA and compound-specific degradation (Allison and Vitousek, 2004). This suggests that mass loss and EEA may not accurately capture the complexity of compound-specific degradation of litter during decomposition.

### 4.4. Conclusion

Litter decomposition, a fundamental ecosystem process sustaining plant productivity, is influenced by an ensemble of factors including climate, litter chemistry, and microbial activity. This study used a grass litter with complex lignocellulose chemistry to examine how climatic factors affect mass loss and the microbially mediated chemical transformations that lead to soil organic matter formation. Our results emphasize that mass loss dynamics of litter may not track complex chemical transformations in litter during decomposition. Our results also suggest that precipitation regulates the preferential decomposition of compounds in litter, and that interannual variability in precipitation may modify the effects of warming on the rate of litter decomposition.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2014.03.022.

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