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Differential effects of warming and nitrogen fertilization on soil respiration and microbial dynamics in switchgrass croplands

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Abstract

The mechanistic understanding of warming and nitrogen (N) fertilization, alone or in combination, on microbially mediated decomposition is limited. In this study, soil samples were collected from previously harvested switchgrass (*Panicum virgatum* L.) plots that had been treated with high N fertilizer (HN: 67 kg N ha⁻¹) and those that had received no N fertilizer (NN) over a 3-year period. The samples were incubated for 180 days at 15 °C and 20 °C, during which heterotrophic respiration, $\delta^{13}\text{C}$ of CO₂, microbial biomass (MB), specific soil respiration rate (R_s: respiration per unit of microbial biomass), and exoenzyme activities were quantified at 10 different collections time. Employing switchgrass tissues (referred to as litter) with naturally abundant ¹³C allowed us to partition CO₂ respiration derived from soil and amended litter. Cumulative soil respiration increased significantly by 16.4% and 4.2% under warming and N fertilization, respectively. Respiration derived from soil was elevated significantly with warming, while oxidase, the agent for recalcitrant soil substrate decomposition, was not significantly affected by warming. Warming, however, significantly enhanced MB and R_s indicating a decrease in microbial growth efficiency (MGE). On the contrary, respiration derived from amended litter was elevated with N fertilization, which was consistent with the significantly elevated hydrolase. N fertilization, however, had little effect on MB and R_s, suggesting little change in microbial physiology. Temperature and N fertilization showed minimal interactive effects likely due to little differences in soil N availability between NN and HN samples, which is partly attributable to switchgrass biomass N accumulation (equivalent to ~53% of fertilizer N). Overall, the differential individual effects of warming and N fertilization may be driven by physiological adaptation and stimulated exoenzyme kinetics, respectively. The study shed insights on distinct microbial acquisition of different substrates under global temperature increase and N enrichment.

Keywords: exoenzyme activities, heterotrophic respiration, microbial biomass, microbial growth efficiency, nitrogen fertilization, soil warming, switchgrass

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Introduction

The mean surface temperature of the Earth is predicted to increase by 1.5 °C by the end of this century (Melillo *et al.*, 2014; Stocker *et al.*, 2014). Nitrogen fertilization is a major contributor to global reactive N, which is projected to increase from 86 Tg N in 1995 to 135 Tg N in 2050 (Galloway *et al.*, 2008; Fowler *et al.*, 2013). Given the fact that soils harbor the largest organic C pool in the terrestrial biosphere, both warming and enhanced N availability could affect the formation and decomposition of soil organic matter (SOM), resulting in potential positive feedback to climate change (Thornton *et al.*,

2007; Allison *et al.*, 2010; Suddick *et al.*, 2013). Switchgrass (*Panicum virgatum* L.), a model bioenergy crop, can mitigate climate change by reducing greenhouse gas emissions and enhancing C sequestration in soils via root and microbial biomass turnover (Follett *et al.*, 2012). Given its low fertilizer and irrigation requirements, switchgrass was studied to promote growth and achieve high dry matter yields (McLaughlin, 1992; Heaton *et al.*, 2004; Monti *et al.*, 2012). However, the mechanistic understanding of soil response to climate warming and N fertilization in bioenergy croplands remains strikingly elementary (Ma *et al.*, 2000b; Heaton *et al.*, 2004).

Warming can increase the rates of SOM mineralization and CO₂ respiration (Rustad *et al.*, 2001; Bergner *et al.*, 2004; Kirschbaum, 2004; Bradford *et al.*, 2008; Li *et al.*, 2012), and soil microbial biomass carbon (MBC; Li *et al.*, 2013; Ziegler *et al.*, 2013). The extracellular hydrolytic and oxidative enzymes facilitate microbial decomposition of labile and recalcitrant substrates in soils (Sinsabaugh & Shah, 2012; Burns *et al.*, 2013). Warming can enhance hydrolytic C acquisition enzymes (e.g., β -1,4-glucosidase and β -D-cellobiosidase) and phenol oxidase activities (Sowerby *et al.*, 2005; Li *et al.*, 2012), while hydrolytic and oxidative enzyme activities were reported to be unresponsive to warming (Bell *et al.*, 2010; Gutknecht *et al.*, 2010). Although there have been no reported warming effects on switchgrass soil C cycling, studies of switchgrass greenhouse gas fluxes have demonstrated that CO₂ flux is strongly associated with seasonal temperature variations, with flux rates being high in the summer and low in autumn and winter (Ma *et al.*, 2000a; Nikiema *et al.*, 2011). Also, indirect evidence from studies on C₄ prairie soils (dominated by switchgrass and other grasses) showed that warming significantly increased soil heterotrophic respiration (Luo *et al.*, 2009).

N fertilization elevated SOM mineralization and CO₂ respiration in croplands (Lu *et al.*, 2011) and significantly stimulated hydrolytic C acquisition enzyme activities but suppressed phenol oxidase and peroxidase activities across different ecosystems (Jian *et al.*, 2016; Chen *et al.*, 2017a). Soil MBC may decline with N fertilization due to depressed microbial growth at lower pH and the depletion of labile substrate (Treseder, 2008; Liu & Greaver, 2010; Jian *et al.*, 2016). In general, switchgrass soil CO₂ and CH₄ fluxes and soil total C and organic C content were not altered by N fertilization (Jung & Lal, 2011; Nikiema *et al.*, 2011; Mbonimpa *et al.*, 2015). According to a 3-year switchgrass study, soil microbial biomass and potential mineralizable C were not affected by NH₄NO₃ fertilization (Lee *et al.*, 2007). However, a recent study showed N fertilization decreased soil organic C and N pools in switchgrass

systems and moderated soil C sequestration potential (Valdez *et al.*, 2017). The differential effects of warming and N fertilization render it imperative to study their interactive effects on soil respiration and microbial dynamics.

Previous studies showed strong interactive effects of warming and N fertilization on soil respiration, microbial community composition, and oxidase activities in various soil and ecosystems (Liu *et al.*, 2011; Liang & Balser, 2012; Li *et al.*, 2013; Zhao *et al.*, 2014; Chen *et al.*, 2017b). Warming and N fertilization in combination increased the ratio of fungi to bacteria (F:B) but decreased total phospholipid fatty acid and phenol oxidase activity in forest soils (Zhao *et al.*, 2014) and the microbial contribution to soil C pool in a grassland soil (Liang & Balser, 2012). Warming, fertilization, and their interaction decreased soil MBC significantly but substantially increased soil microbial biomass nitrogen (MBN) in the subalpine coniferous forest ecosystem (Liu *et al.*, 2011). In boreal forest soils, higher N bioavailability enhanced the positive warming effects on soil phenol oxidase activity and lower N availability suppressed the warming-induced CO₂ derived from labile material (Li *et al.*, 2013). In a switchgrass cropland, N fertilization and high temperatures in summer resulted in the higher soil respiration and microbial biomass (Nikiema *et al.*, 2011). Given that the microbial mining of N and phosphorus (P) nutrients may vary widely under N fertilization (Saiya-Cork *et al.*, 2002; Marklein & Houlton, 2012; Deng *et al.*, 2017b) or warming (Bai *et al.*, 2013; Billings & Ballantyne, 2013), a potentially strong interaction between warming and N fertilization on the hydrolase associated with N and P acquisitions may be expected. On the other hand, global warming may increase soil N availability, which could have far-reaching impacts on soil respiration and microbial activities (Joseph & Henry, 2008; Dijkstra *et al.*, 2010; Turner & Henry, 2010; Melillo *et al.*, 2011). N fertilization may enhance N availability in soil and plant N uptake and biomass accumulation, resulting in changes in nutrient availability to plants (Jenkinson *et al.*, 1985), which in turn may alter plant, soil, and microbial responses to climate warming (Melillo *et al.*, 2011).

The effects of climate change on switchgrass have focused on aboveground crop yield responses (Hartman & Nippert, 2013; Palmer *et al.*, 2014; Deng *et al.*, 2017a; Zhu *et al.*, 2017). For instance, N fertilization has been shown to greatly increase biomass yield by 1.5-fold to 2.5-fold (Nikiema *et al.*, 2011; Qin *et al.*, 2015). Warming also increased biomass yield or had no effect (Hartman & Nippert, 2013). However, few studies have investigated belowground microbial and enzymatic activities under both warming and N fertilization conditions. The plant biomass N accumulation accounted for a

significant portion of fertilizer N in switchgrass croplands (Garten *et al.*, 2010; Owens *et al.*, 2013); however, how switchgrass and soil interact and mechanistically mediate climate change has not been addressed. Because climate warming and N fertilizer inputs appear to exert strong controls on soil C cycling and potentially positive feedback to climate change, lacking evidence on the interactive effects of warming and N fertilization prevents the prediction of soil C responses under multi-factor climate change scenarios.

In the established switchgrass stands subjected to N fertilization for 3 years in middle Tennessee, soil samples were collected from two N fertilization treatments (NN: no N input; HN: 67 kg N ha⁻¹) and incubated for 180 days at two temperatures (i.e., 15 °C and 20 °C) with or without amended switchgrass tissue materials (hereafter referred to as litter). Soil CO₂ emission, $\delta^{13}\text{C}$ of respired CO₂, microbial biomass, and exoenzyme activities were quantified at 10 different collections during the incubation. It was hypothesized that (1) warming would increase soil heterotrophic respiration associated with the elevated microbial biomass and oxidase activities; (2) N fertilization would increase soil heterotrophic respiration associated with the elevated microbial biomass and hydrolase activities; and (3) warming would stimulate soil respiration and microbial activities in the fertilized soils during incubation as a result of the strong interaction between warming and N fertilization. Alternatively, when there is no interaction between these two factors, this study explored how soil N availability may moderate their effects, because switchgrass biomass N accumulation usually represents a major portion of fertilizer N. By combining a switchgrass field experiment, laboratory incubation, and microbial activity assays, this study explored soil and microbial responses and offered insights into the underlying microbial processes and their interaction with soil and plants that govern these responses.

Materials and methods

Site description, plant and soil sampling, and chemical analysis

The switchgrass stands were located at the Tennessee State University Agricultural Research and Education Center, Ashland City, Tennessee. The site occupies Lindsie silt loam soil (fine-silty, mixed, mesic Fluvaquentic Eutrochrepts; de Koff & Allison, 2015). On the year prior to planting switchgrass in 2012, the field site was left fallow. The switchgrass was planted in 2012 in four blocks (3.2 m by 39 m) with a 2.4 m buffer between each block. Each block was divided into eight individual plots (3.2 m by 4.9 m). A full factorial experiment design was employed in which three different treatments (i.e., N fertilizer, biochar, and potassium fertilizer), and two levels of each

treatment were randomly assigned to the eight plots (2 × 2 × 2). This study focused on N fertilizer treatment, which included two levels: no N input (NN) and relatively high N input (HN, 67 kg N ha⁻¹). The N fertilizer (i.e., ammonium nitrate) was applied to the fertilized plots by hands on May 6, 2014, and March 26, 2015. Plant biomass above approximately 15 cm in height was harvested in December 2015 from all plots with a sickle bar mower. The samples were weighed and then dried in a forced-air oven at 60 °C until their weight decreased by 0.7% or less per day. After drying, subsamples of plant tissues were ground with a large Wiley Mill (Thomas Manufacturing, Hillside, NJ, USA) until they could pass through a 1 mm screen.

After switchgrass biomass was harvested, soil samples (0–15 cm) were collected from the mineral soil horizon in January 2016 by removing the surface litter layer. Five samples were collected from each of the NN and HN plots for a total of 40 samples (5 samples × 2 treatments × 4 replicates). All of the samples were stored in coolers and taken to the laboratory for analysis. After roots were removed from each core, soil samples collected from the same plot were homogenized into one single sample and sieved through a 2-mm soil sieve (Fisher Scientific, Hanover Park, IL, USA). Two composite soil samples obtained from the NN and HN plots were subjected to different temperatures (15 °C and 20 °C) in laboratory incubation to be conducted within 2 weeks after soil collection. Soil moisture was determined by oven-drying subsamples for 24 h at 105 °C. Air-dried soil subsamples were ground to fine powder for C and N analysis. Both switchgrass plant materials and soil samples were shipped to the University of North Carolina at Wilmington Center for Marine Science for analysis of total C and N, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ using a Thermo Scientific HT Plus elemental analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA) interfaced with a Thermo Scientific Delta V Plus stable isotope mass spectrometer. The switchgrass biomass N accumulation was calculated by multiplying the harvested switchgrass biomass by the biomass N concentration (i.e., 0.28%) obtained via the aforementioned N analysis.

Laboratory incubation

Field moist soil samples (10.0 g equivalent dry weight) were weighed in PVC cores (5 cm diameter, 7.5 cm tall) that had been sealed with glass fiber paper on the bottom. The PVC cores were placed in Mason jars (~1 L) lined with a bed of glass beads to ensure that the cores did not rest in any moisture. The ground dry switchgrass aboveground tissue material (1.0 g, $\delta^{13}\text{C} = -18.5\text{‰}$) was added to the cores and mixed well with soils, marking the initiation of incubation (day 0). This treatment hereafter is referred to as litter treatment. The added litter material is relatively more labile than soil samples due to its higher abundance of nonstructural compounds (Cotrufo *et al.*, 2015). An equivalent number of soil samples were incubated without litter addition (no litter treatment). A total of 240 jars underwent incubation based on 2 temperature treatments × 2 N fertilizer treatments × 2 litter treatments × 3 replicates × 10 sampling times. Water was added to the incubation vessel periodically to bring the soils to a 75% water-holding capacity (0.4 g_{H₂O} g_{soil}⁻¹), a moisture content expected to promote microbial activity (Linn & Doran, 1984). The weight

of each jar was monitored weekly to determine whether any water had been lost from its core, and an equivalent amount of water that was lost was added to each core to ensure that diffusion of substrates to enzymatic reaction sites was not limited. The jars were also aerated each week to prevent an anaerobic environment.

On days 1, 5, 10, 15, 30, 60, 90, 120, 150, and 180, soil respiration was measured and soil was destructively collected from 24 jars to measure microbial biomass C and N and extracellular enzyme activities. The total CO₂ concentration in the jars and $\delta^{13}\text{C}$ of CO₂ were measured by connecting the jars to a Picarro G2131-*i* analyzer (Picarro Inc., Santa Clara, CA, USA). This method took advantage of high-precision stable isotope ratio measurements with continuous time. Respiration rate was calculated using the amount of CO₂ that had accumulated in the circulation system over time and soil dry weight. The cumulative respiration calculation assumed the respiration rate was constant until the next measurement was made.

The effect of laboratory air on measured [CO₂] and $\delta^{13}\text{C}$ of CO₂ in each jar was corrected for using average values of laboratory air [CO₂] (500 ppm) and its $\delta^{13}\text{C}$ value (-11‰). These numbers represented the average of multiple samplings of laboratory air during the incubation. Based on a mixing model, $\delta^{13}\text{C}$ of CO₂ in the incubation jars (litter treatment) represented a mixture of CO₂ derived from laboratory air, respired SOM, and litter. The $\delta^{13}\text{C}$ of mixed CO₂-C was derived from respired SOM and litter (Eqn 1) by excluding the effect of laboratory CO₂-C.

$$\delta^{13}\text{C}_{\text{soil} + \text{litter}} = \frac{\delta^{13}\text{C}_{\text{soil} + \text{litter} + \text{air}} \times V_{\text{soil} + \text{litter} + \text{air}} - \delta^{13}\text{C}_{\text{air}} \times V_{\text{air}}}{V_{\text{soil} + \text{litter} + \text{air}} - V_{\text{air}}} \quad (1)$$

$\delta^{13}\text{C}_{\text{soil} + \text{litter} + \text{air}}$ and $\delta^{13}\text{C}_{\text{soil} + \text{litter}}$ denote $\delta^{13}\text{C}$ of CO₂-C from SOM, litter, and laboratory air and from SOM and litter, respectively. $V_{\text{soil} + \text{litter} + \text{air}}$ denotes the total concentration of CO₂ respired from SOM, the replaced litter, and ambient laboratory CO₂ introduced into the sample. V_{air} represents the CO₂ concentration of laboratory air (500 ppm). The proportion of respired CO₂-C was then derived from SOM in the total respiration from SOM and litter (Eqn 2).

$$P_{\text{soil}} = \frac{\delta^{13}\text{C}_{\text{soil} + \text{litter}} - \delta^{13}\text{C}_{\text{litter}}}{\delta^{13}\text{C}_{\text{soil}} - \delta^{13}\text{C}_{\text{litter}}}, \quad (2)$$

where P_{soil} denotes the proportion of respired CO₂-C from indigenous SOM, and $\delta^{13}\text{C}_{\text{soil}}$ and $\delta^{13}\text{C}_{\text{litter}}$ denote the $\delta^{13}\text{C}$ of SOM and litter, respectively. It was assumed that the difference between the $\delta^{13}\text{C}$ of respired CO₂ and the $\delta^{13}\text{C}$ of the substrate from which it is derived is negligible and that this offset is equivalent for both indigenous SOM and replaced litter. The most simplistic assumptions were adopted in accordance with established protocols (OMalley *et al.*, 1996; Phillips *et al.*, 2005; Li *et al.*, 2012).

Microbial biomass and biomass-specific soil respiration

A chloroform fumigation–K₂SO₄ extraction (Brookes *et al.*, 1985) and potassium persulfate (0.5 M K₂S₂O₈) digestion methods were used to quantify microbial biomass C and N (Paul,

2007). All K₂SO₄ soil extracts were shaken on a mechanical shaker for 1 h and then filtered through Whatman #40 filter paper. Extractable organic C or N in fumigated and unfumigated samples were analyzed on a Shimadzu analyzer (Shimadzu Corp., Kyoto, Japan), and the difference between fumigated and unfumigated treatments represented MBC or MBN. The ratio of MBC and MBN (C:N_{mb}) was also derived and analyzed. Biomass-specific soil respiration was derived by the ratio of soil respiration divided by microbial biomass in each collection and it was used to index microbial physiology (Bradford *et al.*, 2008).

Hydrolytic and oxidative extracellular enzyme activities

On days 1, 5, 10, 15, 30, 60, 90, 120, 150, and 180, hydrolytic and oxidative extracellular enzyme assays were performed according to protocols discussed in previous studies (Sinsabaugh *et al.*, 2000; Allison *et al.*, 2008; Li *et al.*, 2012). These measures represent potential enzyme activities indicative of overall enzyme concentrations (Wallenstein & Weintraub, 2008) and the potential microbial capacity to process labile and relatively slow-turnover SOM. Fluorescent-labeled substrates were used to index the enzymes α -1,4-glucosidase (AG), β -1,4-glucosidase (BG), cellobiohydrolase (CBH), β -1,4-xylosidase (BX), acid phosphatase (AP), β -1,4-N-acetyl-glucosaminidase (NAG), and leucine amino peptidase (LAP; Marx *et al.*, 2001; Li *et al.*, 2012). Colorimetric techniques were used to assess the potential activity of phenol oxidase (PHO), peroxidase (PER), and urease (UREA; Saiya-Cork *et al.*, 2002). In this study, labile C-acquiring enzymes (*C-acq*) were considered as the sum of AG, BG, CBH, and BX, N-acquiring enzymes (*N-acq*) as the sum of NAG and LAP, and oxidative enzymes (*OX*) as the sum of PHO and PER.

For these assays, a 1.0 g soil sample (fresh weight) was homogenized by mixing it with 125 mL of 50 mM sodium acetate buffer (pH 5.5) for 30 s with a hand blender. To quantify extracellular enzyme activities (EEA) for each soil sample, 16 replicate wells containing 200 μL soil slurry and 50 μL of substrate were used. To calculate the quench coefficient, eight wells were used containing 200 μL of soil slurry and 50 μL of standard (10 μM 4-methylumbelliferone (MUB)) for hydrolytic enzymes; an additional control (blank) was composed of eight wells pipetted with 200 μL of soil slurry. Negative controls consisted of eight wells with 50 μL of substrate and 200 μL of buffer. Eight wells with 50 μL of MUB or 7-amino 4-methylcoumarin (MC) and 200 μL buffer were used to derive the emission coefficient. L-3,4-dihydroxyphenylalanine (L-DOPA) was used as a substrate for PHO and PER. The plates were incubated at 15 °C or 20 °C, corresponding to their respective temperature treatments, for approximately 20 h. In each well of all fluorescence plates, 10 μL of 0.5 M NaOH was added to raise the MUB or MC emission coefficients to a detectable level. Fluorescence was assessed using a microliter plate fluorometer (Molecular Devices, Sunnyvale, CA, USA) set to an excitation wavelength of 365 nm and emission wavelength of 460 nm. Spectrophotometric activity was quantified with a spectrophotometer (Molecular Devices). The absorbance was measured at

460 nm for PHO and PER. Measurements are presented as $\mu\text{mol activity h}^{-1} \text{g}_{\text{soil}}^{-1}$.

Statistical analysis

Repeated-measure ANOVA (PROC MIXED, SAS, Cary, NC, USA) was used to assess the main effects of temperature, N fertilization, litter, and their interactions on soil respiration rate, $\delta^{13}\text{C}$ of respired CO_2 , proportion of CO_2 respired from indigenous SOM, microbial biomass, biomass-specific respiration, and EEAs during the incubation. *Post hoc* tests via Tukey-Kramer-adjusted *P*-values were also used to assess the effects of temperature, N fertilization, or their interaction on a day in which significant interaction was observed. A two-way ANOVA was also used to assess the major effects of temperature, N fertilization, and their interactions on cumulative respiration (as distinct from respiration rates) and differences between two temperatures (20 °C and 15 °C) on each day in no litter and litter treatments, respectively. The overall average of each EEA for all days was tested by two-way ANOVA to examine generalized temperature and N fertilization effects. All datasets are in Table S2.

Results

Switchgrass biomass yield and soil C and N contents

High N led to 30% higher biomass yield on average than NN ($1.3 \pm 0.1 \text{ kg m}^{-2}$ vs. $1.0 \pm 0.4 \text{ kg m}^{-2}$). The N removal via biomass was $36.4 \text{ kg N ha}^{-1}$ in the HN stands, which was equivalent to 54.3% of fertilizer N applied. There were no significant differences in either soil organic C (1.05% vs. 1.06%) or total N (0.099% vs. 0.102%) between NN and HN. The $\delta^{13}\text{C}$ of bulk SOM is -24.3‰ and -26.0‰ in NN and HN, respectively. The soil $\delta^{15}\text{N}$ is 4.5‰ and 4.6‰ in NN and HN, respectively.

Soil CO_2 efflux and its partitioning

Warming significantly increased the cumulative respiration by 16% over 180 days. The positive warming effects were revealed on days 30, 60, 90, 120, 150, and 180 in the no litter treatment and on days 15, 30, 60, 90, 120, 150, and 180 in the litter treatment (Table 1; Fig. 1). On day 60, the proportion of respired CO_2 derived from indigenous SOM was significantly enhanced with warming from 60% (15 °C) to 82% (20 °C; Fig. 2; $P < 0.05$). N fertilization significantly increased the cumulative respiration by 4.2% over 180 days. The positive N fertilization effects were revealed on days 120 and 180 in the litter treatment (Table 1; Fig. 1). On day 5, the proportion of respired CO_2 derived from indigenous SOM significantly decreased with N fertilization from 43% (NN) to 32% (HN; Fig. 2; $P < 0.05$). That is, the proportion of respired CO_2 derived from litter

Table 1 Summary of repeated-measure ANOVA test for the effects of temperature, N fertilization, and dates on heterotrophic soil respiration rate, cumulative respiration, biomass-specific respiration, MBC, MBN, C:N_{mb}, and exoenzyme activities

Soil variable	T	N	D	T*N	T*D	N*D	T*N*D
<i>No litter treatment</i>							
Respiration rate	**		***				
Cumulative respiration	***		***		***		
Specific respiration	* ^a		***				
MBC			***				
MBN			**				
C:N _{mb}							
AG		**	***	*	**		*
BG	*		***		**	***	
BX	*		***		*	*	***
CBH							
C-acq	*		**		*	*	
NAG			**			**	
LAP							
N-acq			***			**	
PHO			*				*
PER			***			**	*
OX			***			*	*
AP		*	***		*	*	**
UREA			*				
<i>Litter treatment</i>							
Respiration rate	***		***				
Cumulative respiration	***		***		***	*	
Specific respiration	*		***				
MBC	*		***				
MBN			***				
C:N _{mb}							
AG	*		***				
BG	***	**	***		***	***	
BX	***		***		***		
CBH							
C-acq	***		***		***	*	
NAG			***		*	***	
LAP			**				
N-acq			***		**	***	
PHO		**		*			
PER			***				
OX			***				
AP			***		***	**	
UREA			***				

T, temperature; N, fertilization, D, date.

Asterisks denote significance (*0.05–0.01, **0.01–0.001, ***0.001).

significantly increased with N fertilization from 57% (NN) to 68% (HN). In both litter treatments, ^{13}C of respired CO_2 was significantly depleted with warming

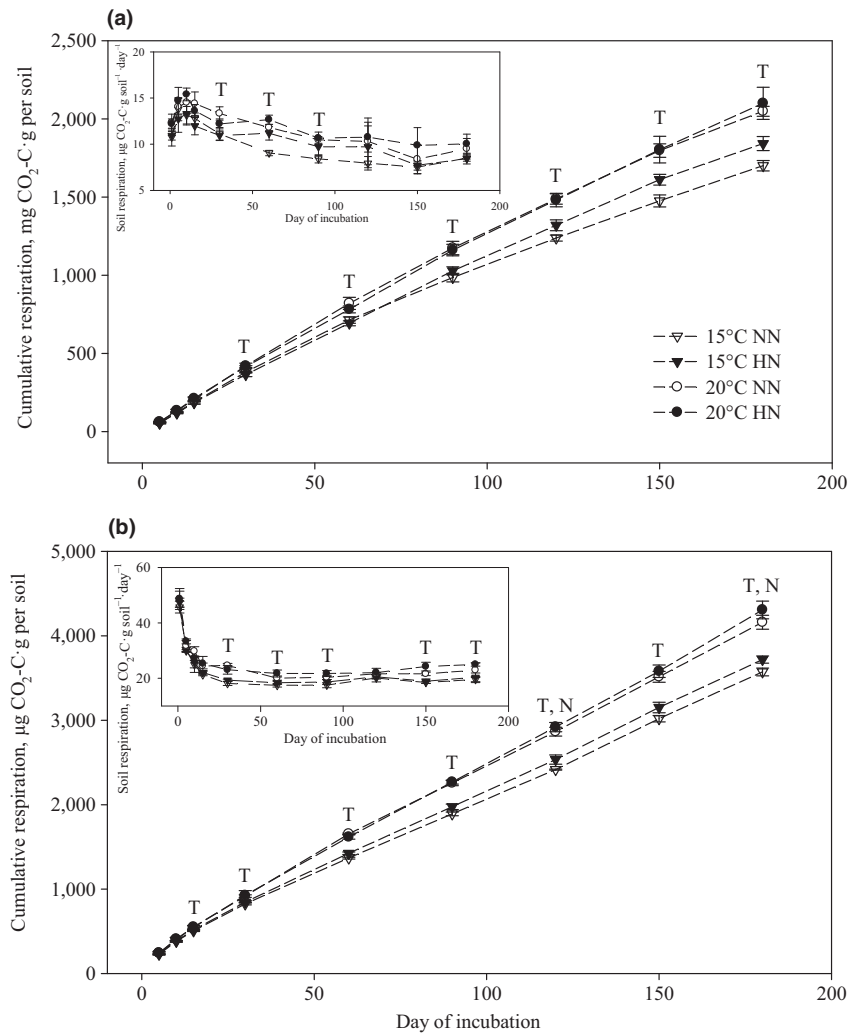


Fig. 1 Mean (\pm SE) cumulative soil CO_2 efflux in each collection date over 180-day incubation without litter addition (above) and with litter addition (below). The insets show soil respiration rates applied to estimate cumulative respiration, assuming the rate is applicable until the following measurement. Error bars indicate standard errors of the means ($n = 3$). T and N denote significant temperature and N fertilization effects on specific dates.

on days 15, 30, 60, 90, 120, and 150 and was enriched with N fertilization on days 1 and 5 (Table 2).

MBC, MBN, and biomass-specific soil respiration

Warming significantly increased MBC in the litter treatment, but N fertilization showed no significant effect on MBC (Table 1; Table S1). There was no significant warming, N fertilization, or their interactive effects on MBN or C:N_{mb} in both no litter and litter treatments (Table 1). As an index of microbial physiology, microbial biomass-specific respiration rates were relatively higher in the litter treatment than in the no litter treatment (Fig. 3). Warming significantly increased microbial biomass-specific respiration in the litter treatment

($P < 0.05$) and in the no litter treatment ($P = 0.057$; Fig. 3). There was no significant N fertilization effect on specific respiration rates (Table 1).

Hydrolytic and oxidative extracellular enzymes

Warming significantly increased AG, BG, BX, C-acq, AP, NAG, and N-acq in specific collection date (Table 1). For instance, warming significantly increased BG, BX, and C-acq on day 5 in both litter treatments (Fig. 4a, b). Warming showed no significant effects on CBH, LAP, PER, PHO, or OX (Table 1). N fertilization significantly increased BG, C-acq, NAG, N-acq, and AP on certain collection in both litter treatments (Table 1). In particular, N

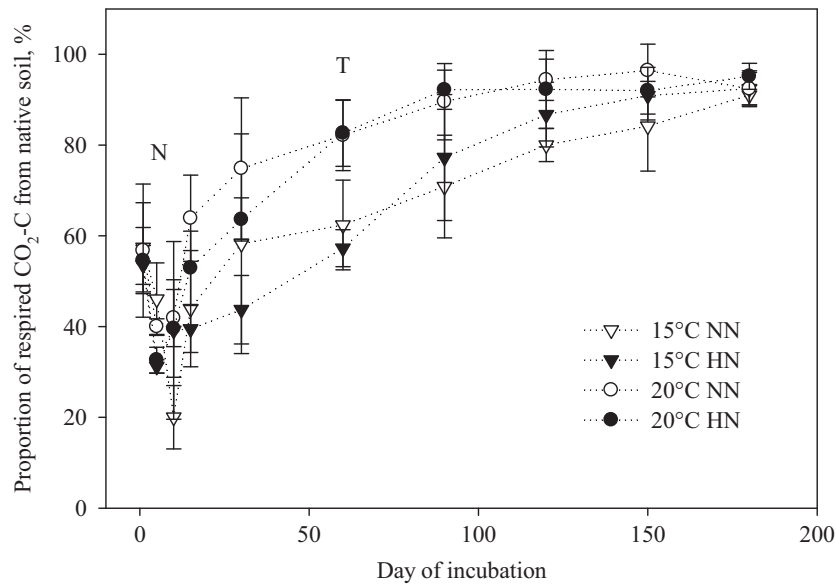


Fig. 2 Mean (\pm SE) percent (%) of respired CO_2 derived from the soil in litter addition treatments. Error bars indicate standard errors of the means ($n = 3$). T and N denote significant temperature and nitrogen fertilization effects on specific dates, respectively ($P < 0.05$).

fertilization increased C-acq on day 5 in both litter treatments and CBH in litter treatment (Fig. 4a, b). N fertilization also decreased OX and PER in no litter treatment but had no significant effects on OX, PHO, and PER (Table 1; Fig. 4c, d). The significant interactions between warming and N fertilization were found only in the no litter treatment on PHO and AG during the incubation (Table 1) and on BX on day 5 (Fig. 4a).

Discussion

Warming effects on soil respiration, microbial biomass, and enzyme activities

Warming increased soil respiration significantly in most collections during incubation. This supports the first hypothesis and is also consistent with former studies that reported positive warming effects driven by a rapid depletion of labile substrates (Xu *et al.*, 2012), microbial community change (Zhou *et al.*, 2012; DeAngelis *et al.*, 2015), and high temperature sensitivity of recalcitrant substrates (Davidson & Janssens, 2006). Warming not only increased microbial biomass, which has been reported in different types of soils (Ziegler *et al.*, 2013), but also elevated the specific respiration rate (i.e., respiration per unit microbial biomass), suggesting a decrease in microbial growth efficiency (MGE) of the microbial communities. This finding supports that rising soil temperatures are generally expected to reduce MGE, as warming limits microbial growth by increasing

the energy cost of maintaining the existing biomass (Manzoni *et al.*, 2012; Sinsabaugh *et al.*, 2013).

The results of this study also suggested that warming promoted the microbial substrate preference for relatively more recalcitrant substrates after 2 months of incubation. This finding is consistent with the warming-enhanced microbial preference for a relatively humified substrate in a boreal forest soil (Li *et al.*, 2012). Thus, more pronounced warming-induced increases in oxidase rather than hydrolase activities are expected, as revealed by Li *et al.* (2012). However, this study showed no significant increase in oxidases despite the increase in hydrolases with warming. This was likely attributable to the similar nature of soil and litter substrates in switchgrass cropland due to the large volume of root exudates and their contribution to SOM (Rovira, 1959). This similarity was supported by elevated soil labile C pools and N immobilization in matured switchgrass stands (Pryatel, 2015), but future studies should explicitly obtain the quality of switchgrass aboveground materials, root, and soil.

N fertilization effects on soil respiration, microbial biomass, and enzyme activities

Consistent with our second hypothesis, N fertilization increased soil respiration and hydrolase activities. The stimulatory N fertilization effect on both soil respiration and hydrolase activities was also revealed across broader spatiotemporal scales (Jian *et al.*, 2016; Chen *et al.*,

Table 2 Mean (\pm SE) stable C isotopic signature of respired CO₂ derived from two fertilizer treatment (Fert: NN and HN) soils under two temperature treatments (Temp: 15 °C, 20 °C) in no litter and litter addition treatments during a 180-day incubation

Litter	Temp, °C	Fert	Incubation time											
			Day 1 ^N	Day 5 ^N	Day 10	Day 15 ^T	Day 30 ^T	Day 60 ^T	Day 90 ^T	Day 120 ^T	Day 150 ^T	Day 180		
No litter	15	NN	-26.86 ± 0.8	-29.82 ± 1.0	-26.92 ± 4	-22.72 ± 0.5	-26.93 ± 0.8	-26.45 ± 1.8	-23.23 ± 0.9	-21.82 ± 0.8	-22.04 ± 0.9	-21.91 ± 0.8		
	15	HN	-22.46 ± 0.4	-26.27 ± 0.8	-25.24 ± 1.7	-23.32 ± 0.8	-25.94 ± 0.7	-23.98 ± 5.5	-23.87 ± 0.9	-23.13 ± 0.9	-23.57 ± 0.9	-23.92 ± 0.9		
	20	NN	-26.01 ± 2.3	-25.44 ± 0.9	-25.75 ± 1.4	-25.19 ± 0.4	-28.29 ± 0.3	-28.69 ± 3.0	-22.44 ± 0.9	-22.51 ± 0.9	-22.94 ± 0.9	-21.72 ± 0.8		
	20	HN	-24.07 ± 1.4	-24.71 ± 1.0	-24.33 ± 0.7	-25.88 ± 0.9	-27.11 ± 1.4	-28.51 ± 3.6	-23.61 ± 0.9	-22.4 ± 0.9	-22.82 ± 0.9	-24.03 ± 0.9		
	15	NN	-22.53 ± 0.6	-21.18 ± 0.8	-21.45 ± 2.5	-21.06 ± 1.3	-21.89 ± 2.4	-22.13 ± 1.0	-22.62 ± 1.1	-23.15 ± 0.4	-23.39 ± 1.0	-23.79 ± 0.2		
	15	HN	-21.84 ± 1.0	-20.85 ± 0.2	-19.67 ± 0.7	-21.47 ± 0.7	-21.79 ± 1.0	-23.47 ± 1.3	-23.7 ± 0.8	-23.98 ± 0.5	-24.1 ± 0.6	-25.44 ± 0.5		
Litter	20	NN	-22.6 ± 0.1.0	-20.83 ± 0.2	-21.48 ± 1.4	-22.22 ± 1.0	-22.85 ± 1.6	-23.27 ± 0.8	-24.3 ± 1.8	-25.01 ± 0.9	-25.33 ± 0.7	-23.87 ± 0.4		
	20	HN	-21.8 ± 1.5	-20.96 ± 0.4	-20.94 ± 0.6	-22.48 ± 1.0	-23.28 ± 0.6	-24.71 ± 1.0	-25.42 ± 0.6	-25.43 ± 1.1	-25.41 ± 0.7	-25.65 ± 0.4		

^TSignificant temperature effects for both no litter and litter addition treatments at $\alpha < 0.05$.

^NSignificant N fertilization effects for both no litter and litter addition treatments at $\alpha < 0.05$.

2017a). N fertilization generally depressed microbial biomass and oxidase activities in forests or grasslands (Lu *et al.*, 2011; Jian *et al.*, 2016) but increased microbial biomass in agricultural soils (Geisseler & Scow, 2014). In this bioenergy cropland, N fertilization had little effect on microbial biomass indicating distinct microbial responses from that in forests, grassland, and traditional croplands. N fertilization little changed the specific respiration rate despite elevated soil respiration levels, suggesting little change in MGE in response to N fertilization. On the other hand, significant changes in hydrolase activities induced by N fertilization were not accompanied by changes in either microbial biomass pool sizes or microbial physiology, consistent with increased hydrolytic enzyme activity under N fertilization (Stone *et al.*, 2012).

In the early stage of incubation, soil respiration from litter was particularly enhanced by N fertilization. Many studies found that N fertilization stimulated labile SOM decomposition (Gallo *et al.*, 2004; Sinsabaugh *et al.*, 2005) or decreased the decomposition of older SOM (Jung *et al.*, 2011). N fertilization provides bioavailable N for microbes to produce hydrolase to acquire the most feasible resources to achieve energy efficiency (Allison & Vitousek, 2005). However, decomposers may exhibit less demand for lignin to obtain N when mineral N is available from amended fertilizer, resulting in less oxidase production (Rinkes *et al.*, 2016). These results indicate that N fertilization caused greater decomposition of labile C derived from switchgrass litter material but lower decomposition of indigenous SOM.

Insignificant interaction between warming and N fertilization

Strong interactive effects of warming and N fertilization have been observed on soil respiration, microbial community composition, and exoenzyme activities in various soils and ecosystems (Liu *et al.*, 2011; Liang & Balser, 2012; Li *et al.*, 2013; Zhao *et al.*, 2014; Chen *et al.*, 2017b). Contrary to the former findings and our third hypothesis, this study found no significant interaction between warming and N fertilization effects on soil respiration, microbial biomass, oxidase activities, or most hydrolase in switchgrass cropland. The reason for the lack of interactive effects between warming and N fertilization in this study may be attributed to similar soil N availability in NN and HN plots, as driven by fertilization intensity, soil-plant interaction, and specific sampling times. In those studies, demonstrating the significant interaction effect of warming and N fertilization, soil total N was significantly higher in plots that had been fertilized than those that had not (Liu *et al.*, 2011; Li *et al.*, 2013; Zhao *et al.*,

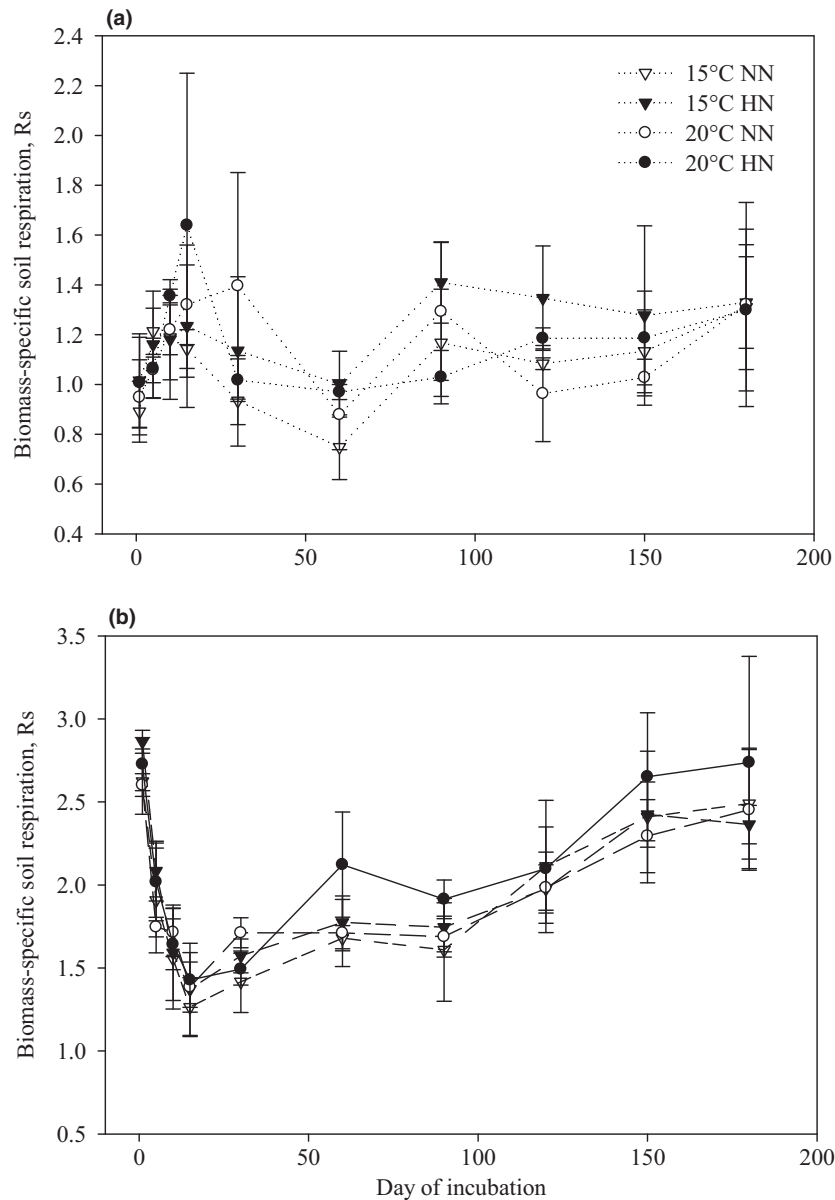


Fig. 3 Mean (\pm SE) specific soil CO_2 efflux in each collection date over 180-day incubation without litter addition (above) and with litter addition (below). Error bars indicate standard errors of the means ($n = 3$). The effects of temperature, nitrogen fertilizer and date were presented in Table 1.

2014). In general, N fertilizers increased the amount of readily available N (i.e., NO_3^-) for plant and microbial uptake (Yanai *et al.*, 1998). In switchgrass croplands, the lower the amount of N fertilizer used, the less positive the effect of N fertilization on soil C and N stocks (Rasmussen *et al.*, 1980; Heggenstaller *et al.*, 2009; Stewart *et al.*, 2016). The amount of fertilizer used in this study (i.e., 67 kg N ha^{-1}) should be regarded as the lower end of a wide spectrum of fertilization intensity of up to 300 kg N ha^{-1} (Potter *et al.*, 2011; Lu & Tian, 2017).

In addition, the harvested biomass N removal was equivalent to more than 53% of fertilizer N applied annually in our switchgrass cropland, which is lower than 68–94% found in other switchgrass croplands (Garten *et al.*, 2010; Owens *et al.*, 2013). Garten *et al.* (2011) found that during the growing season, the belowground biomass contained twice the amount of N stock in comparison with the aboveground biomass under 67 kg N ha^{-1} N fertilization. These results suggest that plant N uptake and accumulation moderated soil N availability, which may contribute to the weak or lack

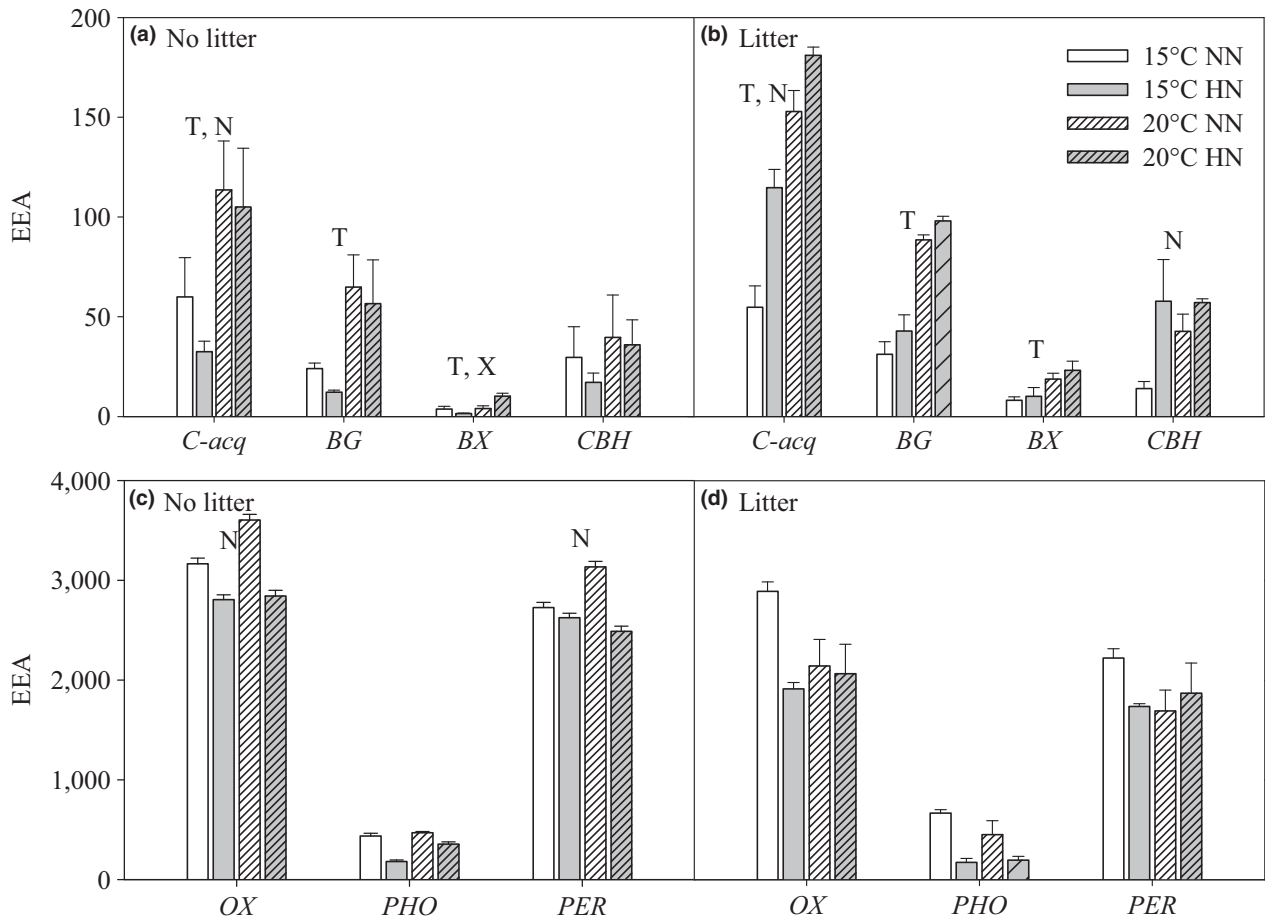


Fig. 4 Mean (\pm SE) exoenzyme activities (EEA, $\mu\text{mol activity h}^{-1} \text{gsoil}^{-1}$) of C-acq activities (a) without litter addition and (b) with litter addition and OX activities (c) without litter addition and (d) with litter addition. T, N and X denote significant temperature, nitrogen fertilization and their interactive effects, respectively ($P < 0.05$).

of warming and N fertilization interaction. Nevertheless, soil samples used in the study were collected during the winter season following harvesting of the biomass when environmental stress for soil microbial activity was high (Rustad *et al.*, 2001; Dessureault-Rompré *et al.*, 2010). To further explore whether interactive effects of warming and N fertilization exist in the switchgrass cropland, future studies should conduct soil collections prior to harvesting biomass (i.e., during the growing season or shortly after fertilization). Quantifying microbial community compositions via molecular and genomic analyses will shed new insights on microbial routing of different substrates under multiple climate change scenarios in the future.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Mean (\pm SE) of microbial biomass carbon (MBC, $\mu\text{g C g}^{-1}$ soil), microbial biomass nitrogen (MBN, $\mu\text{g N g}^{-1}$ soil) and the ratio of MBC and MBN (C:N_{mb}) of the NN and HN soils under 15°C or 20°C in no litter (a) and litter treatments (b) during a 180-day incubation.

Table S2. Dataset collected for the current study.