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Seasonal, biogeochemical, and microbial response of soils to simultaneous warming and nitrogen additions

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SEASONAL, BIOGEOCHEMICAL, AND MICROBIAL RESPONSE OF SOILS TO SIMULTANEOUS WARMING AND NITROGEN ADDITIONS

BY

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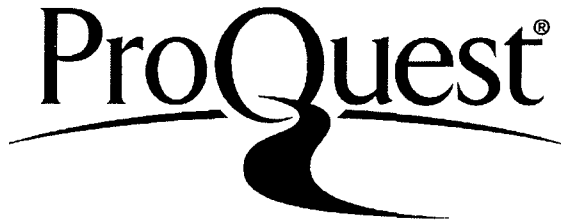

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DEDICATION

I dedicate this dissertation to winter, to deep, starry nights and ice blue days, to snowflakes floating to the ground, to snow that warms roots and soil, that recharges aquifers and streams, and that calls me outside to play like a child. I dedicate this dissertation to winter in the hopes that human folly and shortsightedness does not turn snow to rain, ice to water, sleeping earth to flowing mud.

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TABLE OF CONTENTS

LIST OF TABLES

Table 4.6. r^2 and P values resulting from permutation tests of experimental treatments (factors) and decomposition gradients (vectors) fit onto spatial ordinations of fungal community structure at increasing levels of sequence similarity.

LIST OF FIGURES

Figure 4.2. Rarefaction curves of number of sequences sampled versus number of sequences observed at ≥70, 80, 90, 96, or 99 % sequence similarity in control (C), warming (W), warming × N (WN) and N addition (N) plots. Error bars represent 95% confidence intervals. Data were pooled across studies of different durations but with the same experimental manipulation.

Figure 4.3. Non-metric multidimensional scaling plots of fungal community structure at the 96% OTU cutoff level. Distances represent rank dissimilarity, not actual distances. Although treatments and decomposition vectors were fit simultaneously onto the spatial ordination, they are shown separately here for visual clarity. Ellipses indicate the 95% confidence interval of the standard error of the mean for each treatment. Treatments are control (C), warming (W), warming × N (WN) and N additions (N). Arrows indicate the correlation between community structure and decomposition vectors. Only vectors with $P \leq 0.20$ were included.

ABSTRACT

SEASONAL, BIOGEOCHEMICAL, AND MICROBIAL RESPONSE OF SOILS TO SIMULTANEOUS WARMING AND NITROGEN ADDITIONS

by

Alexandra R. Contosta

University of New Hampshire, May, 2011

Climate warming and nitrogen deposition are global environmental threats that could alter soil microbial communities and the biogeochemical processes they perform. Few studies have examined interactive effects of elevated temperatures and nitrogen inputs. Many studies have also not considered the role that season plays in mediating the response of soils to warming and nitrogen. Finally, most research has not linked changes in the soil microbial community with ecosystem-scale dynamics. One objective of this dissertation was to examine season-specific microbial and biogeochemical responses to simultaneous warming and nitrogen additions. Another aim was to investigate whether warming and nitrogen can restructure microbial communities in such a way as to alter ecosystem processes. The research occurred at the Soil Warming × Nitrogen Addition study at the Harvard Forest, and included four treatments: control, warming, warming × nitrogen, and nitrogen additions. Soil respiration and nitrogen mineralization were measured continuously for two years. During winter, spring, summer, and fall of a single year, labile carbon, enzyme activity, microbial biomass, and microbial community structure were quantified. Finally, a wood decomposition study was conducted at the field site to examine changes in both wood decay and the fungi performing the decay.

Results indicated season-specific responses of soil respiration, nitrogen mineralization, and microbial biomass to the experimental manipulations. Soil respiration and nitrogen mineralization increased with warming and nitrogen additions, even during winter. Soil respiration in the warming treatment also displayed heightened temperature sensitivity during winter months. By contrast, microbial biomass declined with warming and nitrogen and this decline primarily occurred in autumn. Where warming × nitrogen occurred together, warming appeared to moderate the negative effect of nitrogen additions on soil respiration and microbial biomass. Regarding the decomposition experiment, nitrogen additions suppressed wood decay while warming had no effect. The combination of warming × nitrogen was synergistic, accelerating wood decay beyond either treatment on its own. Lower decay rates in fertilized plots were not associated with a concomitant change in the structure of the fungal community colonizing the wood. Overall, the findings suggest that anthropogenic stressors and seasonal changes can interact to affect soil microbial communities and biogeochemical cycles.

INTRODUCTION

Mean global temperature is projected to increase between 0.3 and 6.4°C by the year 2100 (IPCC 2007). Over the same period, global deposition of reactive nitrogen (N) into terrestrial ecosystems is predicted to double (Galloway *et al.* 2004). These increases in temperature and N deposition could dramatically alter microbially-mediated soil carbon (C) and N cycling, with consequences for water quality, air quality, and greenhouse gas emissions. For example, elevated temperatures can accelerate microbial decomposition of soil organic C, resulting in a positive feedback to climate change (Kirschbaum 1995). By contrast, N deposition can suppress microbial respiration (Bowden *et al.* 2004), resulting in net C storage. Both soil warming and N additions can also increase soil N availability (Aber *et al.* 1993, Peterjohn *et al.* 1994), which, if in excess of demand, could leach from soil and become a pollutant in surface water (Aber *et al.* 1989).

Over the past several decades, numerous studies have examined the long-term effects of either warming or N additions on soil microbial communities and the biogeochemical processes they perform (*e.g.*, Aber *et al.* 1993, Peterjohn *et al.* 1994,Frey *et al.* 2004, Bradford *et al.* 2008, Frey *et al.* 2008, Burton *et al.* 2009). Most of these studies have occurred in single-factor experiments that have manipulated either temperature or N inputs. The separation of these two global change drivers does not represent a scenario where warming and N deposition occur simultaneously, interacting in ways that may be difficult to predict. Because both increased temperatures and N inputs can affect microbially-mediated processes similarly, *e.g.*, by boosting N mineralization rates (Aber *et al.* 1993, Peterjohn *et al.* 1994), the response to simultaneous warming and N additions may be additive. Alternatively, warming and N

additions could interact to multiply their largely negative impact on the soil microbial community, since both can decrease microbial biomass (Compton *et al.* 2004, Bradford *et al.* 2008). The combination of warming and N addition could also cancel one another; the negative effect of N additions on soil respiration (Bowden *et al.* 2004) could annul the positive impact warming has on soil CO_2 flux (Peterjohn *et al.* 1994). Whether soil biogeochemical and microbial processes in temperate forests will show an additive, annulling, or synergistic response to concurrent warming and N fertilization is unknown. Such multifactor studies have typically occurred in grassland and tundra ecosystems (Chapin *et al.* 1995, Christensen *et al.* 1997, Zavaleta *et al.* 2003, Rinnan *et al.* 2007, Turner and Henry 2010), and their findings may not translate to temperate, deciduous forests.

Most investigations of soil warming and N fertilization have also not explicitly addressed the role that season plays in mediating the response of soils to warming and N deposition. Yet numerous studies have demonstrated seasonal shifts in microbial community structure and function that mirror seasonal patterns in temperature, moisture, plant productivity, and soil C and N availability. For example, soil respiration during summer may be primarily due to roots and rhizosphere microbes that utilize recent plant photosynthate (Boone *et al.* 1998, Epron *et al.* 2001, Hogberg *et al.* 2001, Lipson *et al.* 2002, Schindlbacher *et al.* 2009). Respiration outside of this period, during the early spring, late fall, and winter, likely comes from saprotrophic microorganisms consuming recalcitrant substrates like cellulose and lignin (Lipson *et al.* 2002, Schmidt *et al.* 2004). Environmental changes such as warming and N fertilization may differentially impact these components of the soil ecosystem at different parts of the year. Failure to address

season-specific changes in soil C and N cycling may neglect to identify certain organisms and processes affected by increased temperatures and N inputs.

Winter biogeochemical dynamics may be especially important to consider when evaluating the seasonal response of soils to warming and N fertilization. Until recently, winter has been perceived as a "dormant" period based on the belief that biological activity declines when temperatures grow cold (Campbell *et al.* 2005). However, research in areas with seasonal snowpack has demonstrated significant contributions of winter C and N fluxes to the total annual flux (Knoepp and Swank 2002, Groffman *et al.* 2001, Mo *et al.* 2005, Groffman *et al.* 2006, Kielland *et al.* 2006, Groffman *et al.* 2009). Likewise, both total soil microbial biomass and fungal biomass can be twice as high in winter as during the height of the growing season (Schadt *et al.* 2003), and the functional capacity of the microbial community to utilize organic matter can vary from winter to summer (Schmidt *et al.* 2004). As a result, changes in microbially-mediated processes in winder due to higher temperatures and / or N inputs may have negative ecosystem consequences. Selection against lignin and cellulose degrading fungi, which are believed to be most active during winter (Lipson *et al.* 2002, Schmidt *et al.* 2004), could alter soil C storage. Increased rates of winter N mineralization in the absence of plant demand could result in N leaching or gas loss (Fisk and Schmidt 1996, Turner and Henry 2010).

The effect of increased temperatures on winter soil respiration is an especially important topic. In ambient conditions, winter soil respiration accounts for 15 to 30% of C fixed during the previous growing season (Goulden *et al.* 1996). Because the temperature sensitivity of soil respiration can be extremely high at cold temperatures (Kirschbaum 1995, Janssens and Pilegaard 2003, Monson *et al.* 2006*a*, Miller *et al.*

2007), winter soil warming could substantially increase this loss of carbon from the system. Higher temperature dependence of CO_2 flux in warmed winter soils may also counterbalance the lower temperature sensitivity of soil respiration reported for other chronically warmed sites. Many soil warming studies have reported an initial increase followed by an ultimate decline in soil respiration with warming (Oechel *et al.* 2000, Luo *et al.* 2001, Melillo *et al.* 2002, Strömgren 2001), and have postulated that microbial acclimatization to higher temperatures (*i.e.*, reduced temperature sensitivity) is responsible for lower soil respiration rates (McHale *et al.* 1998, Luo *et al.* 2001, Melillo *et al.* 2004, Bronson *et al.* 2008, Reth *et al.* 2009). However, these studies have typically measured soil respiration from April through November. Thus the hypothesis that the temperature dependence of soil respiration declines with warming is based on measurements and models representing only the warmer part of the year. Including winter measurements in these models could show accelerated or constant temperature sensitivity of soil respiration to chronic warming, indicating strong feedbacks between climate warming and soil carbon that would be overlooked when modeling respiration only during the growing season.

In addition to examining the seasonal response of soils to warming and N deposition, it is equally important to study interactions and feedbacks among global change drivers, soil microbial community structure, and ecosystem function. Many studies have examined how microbial community structure (*e.g.*, fungal diversity) or ecosystem function (*e.g.*, litter decomposition) has shifted with elevated temperatures and / or N inputs. Fewer have directly quantified structure-function linkages. A fundamental question is whether warming and N deposition can restructure microbial communities in

such a way as to alter ecosystem-scale nutrient cycles. The loss of a specific group of soil microbes, such as fungi that decompose lignin, could change ecosystem carbon storage if the function these fungi perform also disappears. Lignolytic fungi are some of the only organisms believed to be able to completely decompose the lignin polymer (Kirk and Farrell 1987, Hammel *et al.* 1997), which is one of the primary constituents of plant litter and woody tissue (Rayner and Boddy 1988, Aber and Melillo, 2001). It has long been recognized that increased N availability alters the activity lignin degrading fungi, suppressing lignin decay and lignolytic enzymes (Magill and Aber 1998, Carreiro *et al.* 2000, Frey *et al.* 2004). At the same time, N additions can decrease fungal biomass and reduce fungal diversity in soils (Frey *et al.* 2004, Allison *et al.* 2007). Whether suppressed lignin decay is related to shifts in fungal community structure and biomass is unknown.

The purpose of this dissertation was to examine the seasonal, microbial, and biogeochemical response of soils exposed to simultaneous warming and N additions. One objective was to study season-specific soil microbial and biogeochemical responses to simultaneous warming and N additions. A second aim was to investigate whether warming and N deposition can restructure microbial communities in such a way as to alter ecosystem-scale nutrient cycles. To this end, a multifactorial experiment was installed at the Harvard Forest Long Term Ecological Research (LTER) site in Petersham, Massachusetts. The experimental treatments were: control, warming, N additions, and warming × N. Four different studies were conducted within the context of this experiment, presented in this dissertation as four distinct chapters. Chapter 1, **Seasonal dynamics of soil respiration and N mineralization in chronically warmed**

and fertilized soils, addresses how soil warming and N additions, alone and together, affect soil respiration and N mineralization. It also examines seasonal changes in the response of soil CO_2 flux and N turnover to the experimental manipulations. This chapter is currently in press for publication in the journal, Ecosphere. Chapter 2, **Acclimatization or acceleration? Seasonal changes in the temperature sensitivity of soil respiration in chronically warmed soils**, addresses changes in the temperature dependence of soil respiration with warming and among seasons. It discusses how soil respiration in chronically warmed sites can show characteristics of acclimatized, accelerated, or constant temperature sensitivity depending on the timeframe (annual, growing season, season-specific) and function (exponential or flexible) used to construct models of the temperature-respiration relationship. Chapter 3, **Soil microbial response to chronic warming and nitrogen additions is mediated by season**, examines how elevated temperatures and / or N inputs affect labile C availability, extracellular enzyme activity, soil microbial biomass, and microbial community composition. As with Chapter 1, it also investigates how these responses might vary among seasons. Chapter 4, **Soil warming and nitrogen additions alter wood decomposition without changing fungal community structure**, addresses how soil warming and N additions, together and separately, influence the diversity and community composition of fungi colonizing woody litter. In addition, it quantifies how shifts in fungal community structure might be related to changes in wood decay with elevated temperatures and N inputs.

CHAPTER 1

SEASONAL DYNAMICS OF SOIL RESPIRATION AND N MINERALIZATION IN CHRONICALLY WARMED AND FERTILIZED SOILS

Abstract

Although numerous studies have examined the individual effects of increased temperatures and N deposition on soil biogeochemical cycling, few have considered how these disturbances interact to impact soil C and N dynamics. Likewise, many have not assessed season-specific responses to warming and N inputs despite seasonal variability in soil processes. We studied interactions among season, warming, and N additions on soil respiration and N mineralization at the Soil Warming × Nitrogen Addition Study at the Harvard Forest. Of particular interest were wintertime fluxes of C and N typically excluded from investigations of soils and global change. Soils were warmed to 5°C above ambient, and N was applied at a rate of 5 g m^{-2} y^{-1}. Soil respiration and N mineralization were sampled over two years between 2007 and 2009 and showed strong seasonal patterns that mirrored changes in soil temperature. Winter fluxes of C and N contributed between 2 and 17% to the total annual flux. Net N mineralization increased in response to the experimental manipulations across all seasons, and was 8% higher in fertilized plots and 83% higher in warmed plots over the duration of the study. Soil respiration showed a more season-specific response. Nitrogen additions enhanced soil respiration by 14%, but this increase was significant only in summer and fall. Likewise, warming increased soil respiration by 44% over the whole study period, but the effect of warming was most pronounced in spring and fall. The only interaction between warming × N additions took place in autumn, when N availability likely diminished the

positive effect of warming on soil respiration. Our results suggest that winter measurements of C and N are necessary to accurately describe winter biogeochemical processes. In addition, season-specific responses to the experimental treatments suggest that some components of the belowground community may be more susceptible to warming and N additions than others. Seasonal changes in the abiotic environment may have also interacted with the experimental manipulations to evoke biogeochemical responses at certain times of year.

Introduction

Mean global temperature is projected to increase between 0.3 and 6.4°C by the year 2100 (IPCC 2007). Over the same period, global deposition of reactive N into terrestrial ecosystems is predicted to double (Galloway *et al.* 2004). These increases in temperature and N deposition could dramatically alter terrestrial C and N cycling, with consequences for water quality, air quality, and greenhouse gas emissions. Soil respiration comprises the majority of the C flux from terrestrial ecosystems to the atmosphere (Raich and Schlesinger 1992). Even a 10% loss in soil organic C worldwide is comparable to 30 years worth of anthropogenically emitted CO_2 (Kirschbaum 2000). The ability of soils to lose or retain N is equally important. Nitrogen can limit primary productivity, act as a pollutant in surface waters as NO_3^-, contribute to the formation of tropospheric ozone as NO, and behave as a powerful greenhouse gas as N_2O (Vitousek and Howarth 1991, Fenn *et al.* 1998).

Numerous studies over the past two decades have examined the long-term effects of either warming or N additions on soil biogeochemical processes (*e.g.*, Aber *et al.*

1993, Peterjohn *et al.* 1994, McNulty *et al.* 1996, Magill *et al.* 1996, McHale *et al.* 1998, Rustad and Fernandez1998*a*). In the short term, soil warming has generally stimulated soil respiration and N mineralization (Peterjohn *et al.* 1994, McHale *et al.* 1998, Rustad and Fernandez 1998 *a*). Over time, warming has often ceased to stimulate soil CO_2 flux, even while maintaining high N mineralization rates (Luo *et al.* 2001, Melillo *et al.* 2004). Nitrogen fertilization has shown small, initial increases in soil respiration, but has generally suppressed soil CO_2 flux in chronically amended plots (Butnor *et al.* 2003, Bowden *et al.* 2004, Burton *et al.* 2004). Likewise, N additions have initially increased N mineralization, but elevated rates of N cycling have either returned to control levels (Aber *et al.* 1993, Magill *et al.* 1996) or declined after a few months or years of fertilization (McNulty *et al.* 1996). Further, many N amendment experiments have reported that between 85-100% of the added N is retained in the soil, even after 15-20 years of elevated N inputs (Christ *et al.* 1995, Magill *et al.* 2000). Thus where they occur separately, soil warming and N additions appear to have an opposite effect on soil C and N dynamics, with warming causing long term soil C losses and high N turnover, and N deposition resulting in soil C gain and low rates of N cycling.

While both increased temperatures and N fertilization can impact soil C and N cycles, much of the work to date that quantifies these effects have existed independently of one another. The segregation of these two ecological disturbances does not represent a real-world scenario where increased temperatures and N loads occur simultaneously, interacting in ways that may be difficult to predict. Warming can negate the positive effect of nutrient additions on plant and microbial biomass (Chapin *et al.* 1995, Christensen *et al.*, 1997, Rinnan *et al.* 2007). Increased temperatures and N inputs can

also interact to synergistically increase soil N availability (Chapin *et al.* 1995, Turner and Henry 2010). Alternatively, the combined effect of warming and N additions can be purely additive and can be extrapolated from each treatment on its own (Zavaleta *et al.* 2003). Whether soil biogeochemical cycling in temperate forests will show an additive, annulling, or synergistic response to concurrent warming and N fertilization is unknown. Such multifactor studies have typically occurred in grassland and tundra ecosystems, and their findings may not be applicable to temperate, deciduous forests.

Most investigations of soil warming and N fertilization have also not explicitly addressed the role that season plays in mediating the response of soils to warming and N deposition. Yet numerous studies have demonstrated strong seasonal differences in soil C and N cycling that mirror patterns in temperature and plant productivity (Davidson *et al.* 1998, Knoepp and Swank 2002, Jaeger *et al.* 1999, Bohlen *et al.* 2001, Groffman *et al.* 2001, Hogberg *et al.* 2001, Bowden *et al.* 2004, Mo *et al.* 2005, DeForest *et al.* 2006). That is, soil respiration and N mineralization tend to be greatest at the height of the growing season, when temperatures are warmest and root exudates of labile C are greatest. In contrast, soil C and N fluxes tend to be lowest when temperatures are cold and labile C is scarce. These seasonal differences in soil C and N cycling may be linked to compositional and functional differences in the belowground community. For example, soil respiration during summer may be primarily due to roots and rhizosphere microbes that utilize recent plant photosynthate (Boone *et al.* 1998, Epron *et al.* 2001, Högberg *et al.* 2001, Lipson *et al.* 2002, Schindlbacher *et al.* 2009). Respiration outside of this period, *i.e.*, during the early spring, late fall, and winter, likely comes from saprotrophic microorganisms consuming recalcitrant substrates like cellulose and lignin

(Lipson *et al.* 2002, Schmidt *et al.* 2004). Likewise, N mineralization and uptake during the spring and summer may be driven primarily by plant demand, while immobilization in fall and winter may be due to microbial N requirements for decomposition (Jaeger *et al.* 1999, Lipson *et al.* 1999). Environmental changes such as warming and N fertilization may differentially impact these components of the soil ecosystem at different parts of the year. Failure to address season-specific changes in soil C and N cycling may neglect to identify certain organisms and processes affected by increased temperatures and N inputs.

Winter soil C and N cycling may be especially important to consider when evaluating the seasonal response of soils to warming and N fertilization. Until recently, winter has been perceived as a "dormant" period based on the belief that biological activity declines when temperatures grow cold (Campbell *et al.* 2005). However, research in areas with seasonal snowpack has demonstrated significant contributions of winter C and N fluxes to the total annual flux (Knoepp and Swank 2002, Groffman *et al.* 2001, Mo *et al.* 2005, Groffman *et al.* 2006, Kielland *et al.* 2006, Groffman *et al.* 2009). As a result, changes in winter soil C and N cycling due to higher temperatures and / or N inputs may have negative ecosystem consequences. Increased rates of winter N mineralization in the absence of plant demand could result in N leaching or gas loss (Fisk and Schmidt 1996, Turner and Henry 2010). Already winter soil respiration in temperate forests accounts for 15 to 30% of C fixed during the previous growing season (Goulden *et al.* 1996); larger winter CO_2 fluxes due to increased temperatures could augment that amount.

Most experimental and observational studies in temperate forests have not measured soil C and N fluxes during winter, often because of the logistical complications of sampling in snow. Instead they rely on over-winter incubations to measure winter N mineralization (*e.g.*, Magill *et al.* 2000, Melillo *et al.* 2004, Tuner and Henry 2010), and either linear interpolation or empirical models of the temperature-respiration relationship to estimate winter soil CO_2 fluxes (*e.g.*, Savage and Davidson 2001, Melillo *et al.* 2004). These methods may not adequately capture winter responses to warming and N fertilization, and consequently may underestimate the impact of these disturbances on soil biogeochemical cycling.

The purpose of this study was to examine the interactive effects of soil warming and N additions on soil C and N fluxes, with particular emphasis on the role that season plays in mediating this response. We hypothesized that: (1) both warming and N additions would stimulate soil respiration and N mineralization, but that the effect of warming would be much greater than that of N additions, (2) where warming and N additions occurred simultaneously, N fertilization would negate the positive effect of warming on soil respiration and N mineralization, (3) the impact of warming and N additions on soil respiration and N mineralization would vary seasonally, and (4) traditional methods of calculating winter fluxes would undervalue both total annual and winter soil respiration and N mineralization, thereby underestimating the effect of warming and N additions on C and N processes.

Methods

Site description and experimental design

This study was conducted at the Soil Warming × Nitrogen Addition Study at the Prospect Hill Tract of the Harvard Forest Long Term Ecological Research Site in Petersham, Massachusetts, USA (42°50' N, 72°18'W). The forest at the site is comprised of even-aged, mixed hardwoods, including red oak (*Quercus rubra*), black oak (*Quercus velutina*), red maple (*Acer rubrum*), striped maple (*Acer pensylvanicum*), American beech (*Fagus grandifolia*), white birch (*Betula papyrifera*), and American chestnut (*Castanea dentata*). Soils are of the Gloucester series (fine loamy, mixed, mesic, Typic Dystrochrepts; Peterjohn *et al.* 1994). Mean annual air temperature at the Harvard Forest is 7°C, with summer temperatures as high as 32°C and winter temperatures as low as -25°C. Average total annual precipitation, including water equivalent of snow, is 1100 mm (Boose *et al.* 2002). Mean annual snowfall is 1700 mm and occurs primarily from December through February (NOAA 2009).

Using a completely randomized design, twenty-four 3×3 m plots were assigned one of four experimental treatments with six replicates per treatment: control (C), nitrogen addition (N), warming (W), and warming × N (WN). Average soil temperature in the heated plots (warming and warming × N) was continuously elevated 5°C above ambient using buried heating cables placed at 10 cm depth below the soil surface and spaced 20 cm apart. The 5°C temperature differential is the same as other soil warming studies at the Harvard Forest (Peterjohn *et al.* 1993) and falls within the range of worst-case-scenario model projections for increased global air temperature by the year 2100 (IPCC 2007). Cables were installed in the heated plots in October 2005, with the cables

extending 10 cm outside each plot perimeter to minimize heat loss at the plot edge. Cables were not buried in unheated plots. Disturbance controls in an adjacent soil warming experiment showed no differences in fluxes of C and N as compared to undisturbed plots (Peterjohn *et al.* 1994), suggesting that the initial disturbance of burying the cables did not have lasting effects on soil processes. After recovering for ten months from cable installation, the system was activated in August 2006. The soil warming treatment resulted in an average 5°C temperature difference between heated and unheated plots (Figure 1.1). Mean soil temperature for both sampling years was 9.9°C in unheated plots and 14.7°C in heated treatments. Minimum temperatures were 1.6°C to 4.7°C, and maximum temperatures were 21°C to 26°C for unheated and heated plots respectively.

Nitrogen additions were also initiated in August 2006. Nitrogen was applied in equal doses during the May to October growing season as an aqueous solution of NH_4NO_3 at a rate equivalent to the low N plots at the Harvard Forest Chronic Nitrogen Addition Study (5 g N m^{-2} y^{-1}). This fertilization rate was about eight times that of ambient N deposition, which has been calculated at 0.66 g m^{-2} y^{-1} from eddy covariance estimates of wet and dry deposition at the Harvard Forest (Munger *et al.* 1998). As with the heating cables, the fertilizer is applied 10 cm outside of the plot boundary to reduce plot edge effects.

Soil sampling and analysis

Soils were sampled monthly between May 2007 and April 2009. On each sampling date, two 8 cm wide and 10 cm long cores were removed from each plot, separated into

mineral and organic fractions, and bulked by soil horizon. Two more soil cores previously taken from an adjacent area were used to back-fill the holes created by the sampling and marked with the date to prevent re-sampling in that location. During the growing season, sampling occurred at least two weeks following fertilizer application. In winter, a narrow hole was dug into the snowpack to access the soil. This hole was refilled with snow following soil sampling. The cores were transported back to the University of New Hampshire, passed through a 2 mm sieve to remove rocks and roots, and stored at 4°C for less than 48 hours prior to analysis. Gravimetric moisture was determined by drying the organic fraction at 60°C and the mineral soil at 105°C for 24 hours.

Net N mineralization was determined using the buried bag technique outlined by Westermann and Crothers (1980) and Eno (1960), where the values of extractable NO_3-N and NH_4-N were compared in initial and incubated soil cores. Incubations occurred *in situ* for approximately one month. While taking two sample cores for immediate analysis, a third core was removed, placed into an air-permeable plastic bag nested inside a 1-mm fiberglass mesh sac, and re-situated into the soil. During the following soil sampling four to five weeks later, the incubated core was removed, and the hole was back-filled and marked with a soil core from off plot. Within 48 hours of sampling, the mineral and organic fractions of both initial and incubated cores were extracted using 2M KCl, filtered, and stored at -20°C. A BioTek Synergy HT microplate reader (Winooski, Vermont, USA) was used to analyze NH_4 and NO_3 in the soil extracts. Ammonium was quantified using the indophenol-blue method adapted for microtiter plates (Sims *et al.* 1995). Nitrate was assessed by the vanadium (III) reduction reaction (Braman and

Hendrix 1989) modified for microplate assays (J.L. DeForest, *personal communication*). The detection limits for both NH_4 and NO_3 was 0.1 ppm.

Soil respiration

Net CO_2 flux was measured bi-monthly from May 2007 through April 2009 using a static chamber technique (Raich *et al.* 1990, Peterjohn *et al.* 1993). In April 2006, permanent collars were situated into the soil, leaving about 10 cm above the soil surface. There was one collar per plot, or 24 collars total. While spatial heterogeneity of soil respiration can be high (*e.g.*, Rayment and Jarvis 2000), we believe that installing a single collar in each 3×3 m plot adequately captured the spatial variability of the forest floor. Davidson *et al.* (2002) estimated that as few as 3 collars could be sampled per day in a several hundred square meter field site and be within ± 40% of the full site population mean. Here we have measured six collars per treatment per day in a field site whose plots total 216 m^2.

All sampling occurred when the average of the diel flux takes place: between 10:00 and 13:00 local time (Davidson *et al.* 1998). Before each sampling, the depth of the collar was measured to account for changes in chamber volume with litter inputs and decay. In winter, snow was removed as carefully as possible from collars, and was replaced upon completion of sampling to minimize disturbance. Once excavated, collars equilibrated with the atmosphere for one hour prior to sampling to release CO_2 that had accumulated in the surrounding snow. Immediately prior to measurement, lids were placed over the pre-installed collars to create respiration chambers. Ten ml headspace samples were taken from these chambers using air-tight, plastic syringes at zero, five,

ten, and 15 minutes. Air temperature and pressure were measured concurrent with sampling. Following sampling, gas samples were immediately transported back to the University of New Hampshire and analyzed using a LI-COR LI-6252 infrared gas analyzer (LI-COR Biosciences, Lincoln, Nebraska, USA). The air temperature and pressure of the room in which the IRGA was located were also recorded. Fluxes were calculated from the linear increase in gas concentration over the 15 minute incubation, the volume of the chamber, and the surface area of the soil within the chamber. In addition, the rate calculations were corrected for differences between *in situ* air temperature and pressure and the atmospheric conditions of the lab where the gas analyzer was located. In the summer of 2008, we compared our static chamber measurements with those made on a dynamic, portable IRGA system and found fluxes to be well-correlated ($r^2 = 0.86$) between the two techniques (A. Contosta, *unpublished data*). As a result, we believe that our static chamber method provided reliable, *in situ* flux measurements.

There were two reasons why we opted for snow removal over other winter respiration sampling strategies such as placing a lid on top of the snow (*e.g.*, Savage and Davidson 2001, Groffman *et al.* 2006) or inserting CO_2 sensors into the snowpack (*e.g.*, Monson *et al.* 2006*b*, Schindlbacher *et al.* 2007). First, placing a lid on top on the snowpack to create a respiration chamber can substantially underestimate soil CO_2 flux due to lateral diffusion (Schindlbacher *et al.* 2007). Second, both the snow-top chamber and the CO_2 sensor approach require the presence of snow at all sampling sites. This was not the case in the present study, where heated plots were often snow free even when unheated plots were covered with as much as 20 cm of snow. Because the snowpack

can trap gases emitted from soil (Monson *et al.* 2006*b*), our measurements of winter flux did not necessarily represent CO_2 diffusing through the snow into the atmosphere at the time of sampling. However, since the CO_2 that accumulates in the snowpack is eventually released to the atmosphere, we believe that our winter soil respiration measurements are valid for comparing respiration rates across seasons.

Statistical analysis

All statistical analyses were conducted in R 2.9.2 (R Development Core Team 2009). We used a mixed model approach with the *nlme* package (Pinhiero *et al.* 2009) to examine differences among experimental treatments and across seasons for soil CO_2 flux, net N mineralization, and soil moisture. Each dependent variable (soil CO_2 flux, net N mineralization, and soil moisture) was log-transformed to meet the normality and homoscedasticity assumptions of the mixed-effects model. Fixed effects included the warming and N addition treatments and their interaction with one another and with season, which was defined as both a categorical and continuous variable as described below. Soil horizon was also included as a fixed effect for net N mineralization and soil moisture, which were measured in both the mineral and organic fractions.

Seasons were defined as winter, spring, summer or autumn based on the phenology of the site using twenty-year records of bud-break, leaf-out, senescence, and leaf-fall for the Harvard Forest (O'Keefe 2000). Spring was delineated as the time between the initial swelling of buds and when more than 80% of leaves had reached their full size. For the three dominant canopy species on the site—red maple, red oak, and black oak—this approximated the period between April 1 and May 30. With the same

dataset, the start of autumn was determined as the time when greater than 20% of leaves had changed color, which was around September 1. Although most trees had lost all of their leaves by October 31, autumn actually extended to late November, after which soil temperatures precipitously declined (Figure 1.1). Thus spring occurred from April 1 to May 31, summer from June 1 to August 30, autumn from September 1 to November 30, and winter from December 1 to March 31. Winter also coincided with daily air temperatures at or below 2.2°C (Boose *et al.* 2002), which typically defines the "dormant" period outside the growing season (Schwartz *et al.* 2006).

In addition to defining season as a categorical variable, we also explicitly modeled the seasonal cycle as the periodic function:

$$\sin(2 \times \pi \times \text{adjusted Julian day}) + \cos(2 \times \pi \times \text{adjusted Julian day}) \qquad \text{Eq. 1.1}$$

Adjusted Julian days were the sampling dates scaled between 0 and 3 to represent the three calendar years over which soil respiration and N mineralization measurements occurred. These were obtained by assigning Julian days to the sampling date for 2007, Julian days + 365 for 2008, Julian days + 730 for 2009, and dividing all values by 365. The inclusion of this periodic function both explicitly modeled the temporal autocorrelation structure of the data (Crawley 2007) and also allowed for the examination of whether the experimental treatments shifted the seasonal cycle.

Models were chosen with backward selection, starting with a beyond optimal model containing all hypothesized fixed effects and their interactions as per Sour *et al.* (2009). After creating the beyond optimum model of fixed effects, random effects were

chosen by comparing models constructed with no random component, a random intercept (plot), and a random intercept (plot) plus a random slope (warming and / or N addition). Models containing no random effects were fit as generalized linear models (*gls* in R), and models with random effects were fit as linear mixed-effects models (*lme* in R) (Pinhiero and Bates 2000). For the variables soil moisture and net N mineralization, soil horizon was nested within plot as part of the random intercept. The optimal random effects structure was determined with the likelihood ratio test of restricted maximum likelihood (REML) estimates for each model (Zuur *et al.* 2009). The resulting *P*-values were corrected to account for the fact that this test occurred on the boundary and therefore violated the assumption that the likelihood values compared followed a χ^2 distribution. After choosing the random effects, the autocorrelation structure was selected using Akaike's Information Criteria (AIC). Fixed model components were subsequently selected in a backwards selection process by examining *P*-values for all fixed effects and interactions estimated with a maximum likelihood (ML) fit as recommended by Zuur *et al.* (2009). All statistical tests were performed at $\alpha = 0.05$ level. After completing model selection, the final model was refit with the REML method for reporting significant differences among group means. Model verification consisted of visually inspecting residuals for normality and homoscedasticity.

Seasonal and annual soil CO_2 respiration was estimated by linearly interpolating fluxes between two sampling dates, and then adding these values to obtain the total flux for the year, or for winter, spring, summer, and autumn. That is:

$$R = \Sigma F_{m,k} \Delta t_k \qquad \text{Eq. 1.2}$$

where, R represents either the annual, winter, spring, summer, or fall estimate of soil respiration, $\Delta t_k = (t_k - t_{k-1})$ is the difference in days between adjacent field sampling dates, and $F_{m,k}$ is the average CO_2 flux, in units of mg C m^{-2} d^{-1}, for the (t_k, t_{k-1}) sampling interval (Wang *et al.* 2010*b*). In order to obtain daily CO_2 flux rates for linear interpolation, we multiplied our hourly flux measurements by 24 hours. Because winter measurements of soil respiration are not usually taken into account when calculating annual and winter fluxes, we also used Eq. 2 to estimate annual and winter CO_2 respiration using data collected during the growing season (*i.e.*, April through November). Finally, we used linear regressions to compare field-season only estimates of winter and annul flux with those obtained from the entire data set.

Although our daily, seasonal, and annual estimates were based on hourly measurements made once per week, we believe that our approach adequately captured temporal variability of soil respiration on diel and weekly timescales. Savage and Davidson (2003) reported that daily soil respiration rates extrapolated from single, hourly measurements made between 10:00 and 13:00 were in strong agreement with daily respiration rates obtained from hourly autochamber measurements added together for the same timeframe. In addition, Savage and Davidson (2003) showed that over a 58-day span, linear interpolation of hourly fluxes, made once per week and with a manual system, could produce identical estimates of total soil respiration when compared to the sum of hourly fluxes made continuously with an autochamber.

Seasonal totals of N mineralization were determined by adding the net N mineralized over the incubation period for winter, spring, summer and fall for each sampling year. An ice storm in December of 2008 prevented incubating cores from

December 2008 to January 2009. The amount of N mineralized during this period was estimated by interpolating N fluxes between December and January using the method outlined above for soil respiration.

Relative treatment effects were described as percentages, and were calculated as follows:

% effect = ((treatment – control) / control) × 100.

Results

Soil respiration, N mineralization, and soil moisture data are presented within the context of our hypotheses, and are thus organized into categories of season, annual fluxes, N additions, warming, and warming × N. Final model output statistics for showing the effect of season, warming and / or N additions on these variables are located in Tables 1.1-1.3. Model selection statistics are presented in Appendix A.

Seasonal patterns of soil moisture, nitrogen mineralization, and soil respiration

Soils showed pronounced seasonal differences in moisture, N mineralization, and soil respiration. The forest floor was wettest in winter and early spring and significantly drier in summer ($P = 0.01$) and autumn ($P < 0.0001$) (Table 1.1, Figure 1.2). A drought in the summer of 2007 caused soil moisture values in the O horizon to drop in August in all the experimental manipulations. The same seasonal pattern occurred in the mineral fraction, with significantly drier soils in summer ($P < 0.0001$) and autumn ($P < 0.0001$) as compared to winter. In addition, soil moisture varied between mineral and organic soil fractions ($P < 0.0001$), and the effect of warming on soil moisture was horizon dependent

(warming × horizon: $P < 0.05$). As a result, differences in gravimetric soil moisture among treatments and sampling dates were measured separately for mineral and organic fractions (Appendix A).

Unlike moisture, N mineralization and soil respiration were lowest in winter and spring and highest in summer and fall. Starting from a winter minimum, N mineralization increased through April and May (spring: $P < 0.0001$) to reach an annual maximum from mid July to mid August (summer: $P < 0.0001$) (Table 1.2, Figure 1.3). Although N fluxes subsequently declined during September and October, they remained higher in autumn than they were in winter (autumn: $P = 0.01$). In addition to these seasonal differences in N mineralization, the sin ($P = 0.007$) and cos ($P < 0.0001$) models of the seasonal cycle indicated a strong, periodic trend in N cycling. January 2007 appeared to deviate from this trend when a mid-winter thaw increased net N mineralization to rates typical of late spring and early autumn. Following this mid-winter boost, N mineralization returned to amounts near zero for the subsequent two months. For the 2007-2008 sampling year, the lowest fluxes were between 2 and 10% of the largest mineralization rates, while in 2008-2009, the lowest mineralization rates were less than 1% of the highest annual fluxes. Since NO_3 concentrations were rarely above the detection limit of 0.1ppm in both initial and incubated cores, net N mineralization represented ammonification and not nitrification. While the organic fraction had significantly higher rates of mineralization than the mineral soil (horizon: $P < 0.0001$), there were no treatment by horizon interactions. Consequently, N flux rates in mineral and organic fractions were analyzed together, with horizon as both a fixed effect and nested within plot in the temporal autocorrelation structure of the model (Appendix A).

Soil respiration also displayed a distinct seasonal cycle ($P < 0.0001$ for the sin and cos models) (Table 1.3, Figure 1.4). Fluxes in summer and autumn were significantly higher than fluxes in winter and spring (summer: $P < 0.001$, autumn: $P < 0.0001$). The lowest fluxes of the year were about 10% of the largest respiration rates.

Estimates of seasonal and annual fluxes

Winter N mineralization comprised 2 to 17% of the total annual mineralization, with much higher winter fluxes in 2007-2008 than in 2008-2009. Spring made up 5-18%, summer 29-66%, and fall 20-50% of net annual N mineralization (Table 1.4). Winter CO_2 fluxes comprised 10-14% of the total annual soil respiration, spring 12-16%, summer 46-50%, and fall 20-32% (Table 1.5). Total annual and seasonal fluxes of C and N varied between the two sampling years. Annual net N mineralization was higher in the control plots in 2007-2008 as compared to 2008-2009. By contrast, annual soil respiration was larger in 2008-2009 than it was in 2007-2008.

Soil respiration measurements taken during the field season consistently underestimated winter fluxes, particularly in the unheated plots (Figure 1.5). Linear interpolation of fluxes between the end of November and the beginning of April were an average 35% lower in the control plots ($r^2 = 0.56$, $P = 0.005$) and 25% lower in the N addition treatment ($r^2 = 0.07$, $P = 0.41$) compared to interpolation of fluxes between bi-monthly winter sampling dates. Winter flux estimates were only 13% lower in the warming plots ($r^2 = 0.73$, $P = 0.001$), and 5% lower in the warming x N treatment ($r^2 = 0.72$, $P = 0.001$) when using field-season data as compared to the year-round data set. Because interpolation of field-season data across the winter months disproportionately

underestimated winter respiration in unheated plots, the apparent treatment effect of warming during winter would be greater when using this approach. However, linear interpolation of field season data over the entire year generated almost identical estimates of annual flux as data taken year-round ($r^2 = 0.99$, $P < 0.0001$ for all four treatments, Figure 1.5). The small contribution of winter respiration to the total annual flux meant that even large shortfalls in winter estimates did not translate to large deficits in annual estimates.

Nitrogen additions

Nitrogen amendments increased both N mineralization and soil respiration relative to the control treatment. Over the entire study period, N additions increased N mineralization by an average of 8% irrespective of season ($P = 0.03$) (Table 1.2, Figure 1.3). By contrast, the increase in CO_2 flux in N amended plots occurred only in summer and autumn (N addition: $P > 0.05$, N addition × summer: $P = 0.06$, N addition × autumn: $P = 0.02$) (Table 1.3, Figure 1.4). Nitrogen additions neither altered the seasonal cycle of N mineralization nor that of soil respiration ($P > 0.05$ for interactions between N additions and sin and cos models of both variables, Tables 1.2 and 1.3). There was no effect of N additions on soil moisture ($P > 0.05$, Table 1.1).

Soil warming

Soil warming decreased soil moisture but increased both N mineralization and soil respiration. Over the entire study period, moisture was an average 9% lower in the O horizon ($P > 0.05$) and 19% lower in the mineral soil ($P = 0.02$) of heated plots (Table

1.1, Figure 1.2). Despite this lower moisture, net N mineralization rates were an average 83% higher in warmed plots ($P < 0.0001$) both during the height of the growing season and during winter ($P > 0.05$ for warming × season) (Table 1.2, Figure 1.3). Warming also increased soil CO_2 flux an average of 44% over the control plots throughout the study (Figure 1.4). However, the effect of warming varied seasonally, and was least pronounced during winter (warming × winter: $P = 0.001$) and summer (warming × summer: $P = 0.04$, Table 1.3).

In addition, warming altered the seasonal cycle of soil respiration ($P = 0.007$ for warming × cos model), decreasing its periodicity (Table 1.3, Figure 1.4). Average respiration in the warming only plots on April 22, 2008 was 38 mg C m^{-2} h^{-1}—a value that the control treatment did not reach until two weeks later, when average respiration was 33 mg C m^{-2} h^{-1}. Likewise, average respiration in the warming treatment on September 23, 2008 was 111 mg C m^{-2} h^{-1}—a rate that the control plots had not exhibited since August 25, when fluxes were 112 mg C m^{-2} h^{-1}. Warming did not alter the seasonal cycle of N mineralization ($P > 0.05$, Table 1.2).

Soil warming × nitrogen additions

There were no interactive effects of combined soil warming × N additions on N mineralization or soil respiration ($P > 0.05$, Tables 1.2 and 1.3). Simultaneous warming x N had a marginally significant effect on soil respiration, but only in autumn (warming × N x autumn: $P = 0.06$, Table 1.3). Since average soil respiration in the warming × N plots was as much as 70% lower than fluxes in the warming only treatment during this

season, this interaction was likely the result of an annulling effect of N additions on soil warming.

Discussion

Seasonal patterns of soil moisture, soil respiration and net N mineralization

Soils showed strong seasonal differences in moisture, soil CO_2 flux, and net N mineralization. Seasonal cycles of soil respiration and N mineralization largely followed changes in soil temperatures, with CO_2 and N fluxes highest in summer and lowest during the coldest parts of the year. This pattern has been reported in other seasonal studies of soil C and N dynamics at the Harvard Forest and at similar temperate forest ecosystems (Davidson *et al.* 1998, Knoepp and Swank 2002, Jaeger *et al.* 1999, Bohlen *et al.* 2001, Groffman *et al.* 2001, Savage and Davidson 2001, Bowden *et al.* 2004, Mo *et al.* 2005, DeForest *et al.* 2006, Groffman *et al.* 2009). Soil moisture did not show this pattern, but exhibited the seasonal characteristics typical of forests in the region, with less moisture in summer and early autumn, and more moisture in winter, spring, and late fall (Davidson *et al.* 1998, Savage and Davidson 2001, DeForest *et al.* 2006, Turner and Henry 2010).

Like other systems with seasonal snow cover, our data suggest that soils are active in winter, and that winter transformations of soil C and N can contribute substantially to the total annual flux. While winter net N mineralization was low compared to summer rates, it was similar to that of spring, and comprised 2 to17% of the total annual mineralization. The contribution of winter N mineralization to the annual amount was similar to other studies in temperate regions (Knoepp and Swank 2002,

Groffman *et al.* 2009, Turner and Henry 2010). Winter N mineralization also showed high interannual variation, with winter contributing 11 to 17% of annual net N mineralization in 2007-2008 and 2 to 5% of annual mineralization in 2008-2009. The very low N fluxes in the winter of 2008-2009 may have resulted from a later onset of snowfall, which may have suppressed N mineralization due to soil freezing. Groffman *et al.* (2009) reported a fourfold difference in winter mineralization between two sampling years and attributed the much lower N flux in the second year to earlier, deeper soil frost and thinner snow pack. Alternatively, a mid-winter thaw may have caused the much higher N mineralization in 2007-2008. In January 2008, air temperatures the week prior to sampling were 8.5°C compared to the average -7°C for the rest of the month. The higher temperatures may have stimulated mineralization, freed N from microbial cells lysed during freeze-thaw, released N trapped in the snow, or all of the above. In any case, this mid-winter increase in N mineralization may be overlooked in studies that allow buried bags to incubate from late fall through April or May (*e.g.*, Keilland *et al.* 2006, Miller *et al.* 2009, Turner and Henry 2010). Such a sampling scheme may provide an estimate of total winter N mineralization, but it may not capture the response of soils to fluctuating winter temperatures that are expected to be more common as climate changes.

As with N mineralization, soil respiration during the coldest months of the year contributed substantially to the total annual flux. Winter respiration comprised between 10-14% of the total CO_2 respired throughout the year. Other studies in temperate forests show comparable winter respiration rates and winter contributions to annual respiration (Mo *et al.* 2005, De Forest *et al.* 2006). In addition, CO_2 fluxes quantified beneath the

snowpack approximated eddy covariance measurements at the Harvard Forest for daily ecosystem respiration during the leafless part of the year: 42 to 84 mg C m^{-2} h^{-1} (Munger *et al.* 2004). Consistent with our hypothesis, our data also indicated that obtaining accurate estimates of winter soil respiration requires sampling throughout the winter months, not interpolating the winter flux between November and April. When we used field-season only data to determine winter respiration, we underestimated total winter flux by 5 to 35%. This was especially the case for unheated plots. However, annual flux calculations based on field-season data were almost identical to those generated with both year-round and field-season measurements. Consequently, a sampling campaign conducted during the April through November field season typical for the northeastern US should be adequate when the objective is to determine annual soil respiration. When the purpose of a study is to more closely examine winter soil respiration dynamics, researchers should consider measuring fluxes during the winter season. This may be especially important when evaluating the effects of increased temperatures on winter soil respiration; the apparent treatment effect of warming on soil respiration was 40 to 72% higher when using field-season only data to estimate total winter flux than when using measurements taken year-round.

Seasonal response of soils to nitrogen additions

As we predicted, N fertilization generally accelerated CO_2 respiration and N mineralization, though the stimulatory effect was less than that of soil warming. Over the entire study period, N additions increased net N mineralization an average 8% over the control treatment. The small overall increase in N mineralization was similar to the

findings for the first few years of other N fertilization experiments in the northeastern US (*e.g.*, Aber *et al.* 1993, Christ *et al.* 1995, McNulty *et al.* 1996, Magill *et al.* 1996). In addition, N fertilization boosted N mineralization rates in all seasons, with N fluxes as much as 70% higher than the control treatment during winter.

Winter increases in N mineralization in the absence of plant demand could result in large N losses from the system as leachate (Fisk and Schmidt 1996) or as N_2O flux (Groffman *et al.* 2006). However, lysimeter samples taken at 60 cm depth during the growing season showed that dissolved inorganic N levels in the N addition plots were no different from the controls (A. Contosta, *unpublished data*), suggesting that these soils exhibit the same high N retention observed in the Harvard Forest Chronic Nitrogen Addition Experiment (Aber *et al.* 1998). Higher rates of winter N mineralization in the N addition treatment could also stimulate N_2O flux, but since the small amount of nitrification that occurred in this study took place during the growing season, denitrification probably did not release the mineralized N to the atmosphere during winter. In fact, the higher winter N mineralization rates in fertilized plots only occurred during the 2007-2008 mid-winter thaw, and may have resulted from the release of N sequestered in microbial biomass. Schmidt *et al.* (2004) showed that both microbial biomass N and total microbial biomass can be higher in winter in fertilized plots as compared to unfertilized ones. This could allow for higher rates of N mineralization as microbial cells lyse during a melt event.

In addition to accelerating N mineralization, N additions increased soil CO_2 flux. Several studies have also reported short-term increases in soil respiration in response to N additions (Thirukkumaran and Parkinson 2000, Bowden *et al.* 2004, Waldrop *et al.*

2004*a*), which may result from higher microbial utilization of labile carbon (Aber *et al.* 1998), increased microbial access to plant litter compounds (Craine *et al.* 2007), and / or increased root productivity (Bowden *et al.* 2004). In the long term, a significant decrease in CO_2 flux from chronically amended soils is typically observed (*e.g.*, Nohrstedt *et al.* 1989, Butnor *et al.* 2003, Bowden *et al.* 2004, Burton *et al.* 2004), which could be due to labile C depletion (Neff *et al.* 2002, Frey *et al.* 2004), reduced microbial biomass (Compton *et al.* 2004, DeForest *et al.* 2004, Frey *et al.* 2004, Allison *et al.* 2007), and / or reduced root biomass (Bowden *et al.* 2004). The stimulation in soil respiration that we observed likely represents the initial phase of the response, which may be followed by longer-term declines in CO_2 flux in the N addition treatment.

Consistent with our hypothesis, the increased rates of soil respiration with N fertilization were seasonally dependent, occurring only in summer and autumn. The lack of response in winter and spring may have been due to the experimental manipulation since fertilization only occurred between May and October. However, N fertilization accelerated N mineralization throughout the year, suggesting that the season-specific response in soil respiration was not an experimental artifact. Rather, the treatment by season interaction may be related to phenological differences in soil C and N availability and microbial community dynamics. For example, N additions could stimulate C acquisition in order to balance the C:N ratio of microbial biomass (Schimel 1988, Hart *et al.* 1994). Yet labile C from root exudates and litter leachate may not become available for this purpose until summer and fall, which could account for why N fertilization only stimulated soil respiration during this time.

Seasonal response of soils to warming

As we predicted, warming reduced soil moisture, increased both soil respiration and N mineralization, and the effect of warming was larger than that of N additions. Lower soil moisture is typical for many heating experiments (*e.g.*, Peterjohn *et al.* 1993, and Fernandez 1998*a*, Allison and Treseder 2008, Hagedorn *et al.* 2010) and may inhibit root and microbial activity if it falls below a certain threshold (*e.g.*, 0.12 g m^3 m^{-3} volumetric water content, Davidson *et al.* 1998). The lower effect of warming on soil respiration in summer suggests that drier conditions in heated plots may have limited C cycling during this season.

Over both sampling years and across all seasons, heating accelerated N mineralization rates by an average of 83%. This increase is of the same magnitude as other investigations showing a doubling of N mineralization within the first few years of a warming experiment (*e.g.*, Peterjohn *et al.* 1994, Hartley *et al.* 1999). The stimulatory effect of warming on N mineralization showed no treatment by season interactions, occurring throughout the year and even during winter. Increased rates of winter net N mineralization in the warming treatment could result in the same N losses as higher rates of winter N mineralization in the fertilized plots: denitrification, N_2O flux, and / or export of inorganic N below the rooting zone. As with the N addition treatment, the lack of difference in DIN in lysimeter water between control and warming plots and the almost nonexistent nitrification in any of the treatments during winter render each of these potential fates of the mineralized N unlikely. However, increased rates of winter N mineralization were much more common in the warming treatment than they were in the

N addition plots, occurring both during the winter thaw of 2008 and during January and March of 2009.

In addition to accelerating N mineralization, soil warming increased soil CO_2 flux by an average 44%. This was slightly less than the 60% increase that Peterjohn *et al.* (1994) reported for the first year of experimental warming, but is equivalent to values reported for the first few years of other soil warming studies (*e.g.*, Rustad and Fernandez 1998 *a*, Schindlbacher *et al.* 2009, Hagedorn *et al.* 2010). The positive response of soil respiration to warming varied seasonally, and was higher in spring and fall than it was during winter and summer (Table 1.3). During summer, lower soil moisture in heated plots may have limited both root and microbial respiration. During winter, the absence of root respiration may account for the lower response of CO_2 flux to warming. Nevertheless, enhanced heterotrophic respiration in winter could augment the 15-30% of C already lost from the system that was fixed during the previous growing season (Goulden *et al.* 1996), and could result from greater microbial access to C substrates due to the presence of liquid water.

Soil warming also decreased the periodicity of the soil respiration seasonal cycle, in particular showing higher soil respiration rates earlier in spring. The doubling of soil respiration in the warming plots in late March and April as compared to the controls may have been due to the earlier disappearance of snowpack and soil temperatures well above freezing, both of which could have deepened the active layer responsible for soil respiration sooner (Rayment and Jarvis 2000, Mo *et al.* 2005). The pulse of water from snowmelt may also have prompted soil microbes to substantially increase respiration rates (Monson *et al.* 2006*a*). Whatever the mechanism, increased springtime respiration

rates are more likely to occur as climate warming extends spring several weeks into what was historically the winter season (Hayhoe *et al.* 2007). The accompanying shifts in spring respiration could both alter soil respiration dynamics throughout the year and contribute to interannual variability in CO_2 flux (Goulden *et al.* 1996, Savage and Davidson 2001).

Interactions among season, soil warming, and N additions

Contrary to our hypothesis, this study showed no interactive effects of simultaneous warming and N fertilization on soil respiration and N mineralization. We observed a marginally significant interaction between warming × N on soil respiration. This occurred in autumn, overlapped with one of the two seasons in which N additions impacted soil respiration (summer and fall), and indicated an annulling effect of N additions on the warming treatment. On several dates in the autumns of 2007 and 2008, average soil respiration in the warming × N plots was as much as 76% lower than fluxes in the warming only plots. Environmental conditions may have been conducive on these occasions for producing the lower fluxes in the warming × N plots. Several months of fertilization, higher rates of N mineralization, decreased plant N demand, and N inputs in senescing litter may have increased soil N levels high enough to partially suppress the stimulatory effect of warming on soil respiration.

Instead of showing a synergistic response, the warming × N plots generally displayed the same rates of soil respiration and N mineralization as the warming only treatment. The lack of interaction between warming and N additions may have resulted from differences in the magnitude of the treatment effects, a lag time between the

initiation of the treatments and the ecosystem response, and / or divergent pathways through which the treatments affected the system. A 5°C increase in soil temperature may perturb soil processes much more strongly than an eightfold increase in atmospheric N deposition. Indeed, the relative changes in soil respiration and N mineralization in response to warming and N additions support this idea. Over the course of the study, the warming plots increased N mineralization by 83% and soil respiration by 44%, whereas the N addition plots only boosted N mineralization by 8% and soil CO_2 flux by 14%. However, the smaller impact in the N fertilized plots may not just be the result of a smaller disturbance to the system, but could be due to a lag between the initiation of fertilizer application and the ecosystem response. After all, soils at the Harvard Forest Chronic Nitrogen Addition experiment showed small immediate reactions to N fertilization, such as slight increases in N mineralization and CO_2 respiration (Aber *et al.* 1993, Bowden *et al.* 2004). Other responses, such as suppression of litter decay and export of DIN below the rooting zone, took three to five years to manifest (Magill *et al.* 1998, Magill *et al.* 2000).

Alternatively, the disparity between the effects of warming and N additions on soil C and N cycling may be related to different ways in which they trigger the system. Increased temperatures change the kinetics of microbial and root activity, at least initially speeding up the rates of many biochemical reactions and thus accelerating soil respiration and N mineralization. Nitrogen additions can also stimulate these processes in the short term, but for stoichiometric reasons related to the C:N ratio of microbial biomass, plant litter, and soil organic matter that may not produce as dramatic an effect as warming. The long term effects of warming and N fertilization on soil biogeochemical processes

can also differ. The reduced soil respiration rates often observed in chronically warmed plots can be attributed to thermal acclimatization of soil microbes to higher temperatures (Bradford *et al.* 2008) and / or depletion of labile C (Melillo *et al.* 2002, Bradford *et al.* 2008). Lower CO_2 fluxes in chronically fertilized plots can also result from diminished labile C supplies (Frey *et al.* 2004). However, suppressed decomposition of litter and SOM in N amended soils (Magill and Aber 1998, Frey *et al.* 2004, Knorr *et al.* 2005*a*) may play just as important a role. If the short and long term pathways through which soil warming and N fertilization affect soil processes diverge too widely, then they may not be able to overlap to interactively affect soil biogeochemical processes.

Because this study represents two and a half years of soil warming and N additions, we are not in a position to scale up our finding to make long-term forecasts of regional C and N dynamics. Results from longer-running soil warming and N fertilization studies show that the response of soils to global change drivers can vary over time, such that initial response differs from the response after ten or twenty years (*e.g.*, Melillo *et al.* 2002, Magill *et al.* 2004). With this in mind, concurrent warming and N deposition may interact over longer timescales despite the lack of significant, short-term interactions. Likewise, the interface between season, warming, and N additions may evolve with sustained elevated temperatures and N inputs. A combination of long-term, manipulative experiments and modeling studies are needed to address future seasonal changes in soil C and N budgets with simultaneous warming and N deposition.

Conclusion

The objective of this study was to examine interactions among season, soil warming, and N additions on soil C and N cycling. Of particular interest were wintertime responses that often are excluded from investigations of soils and global change. We determined that traditional approaches of estimating winter N mineralization may fail to capture dynamic shifts in soil N cycling that occur during freeze-thaw. Likewise, common methods for calculating soil CO_2 fluxes between November and April significantly underestimated winter respiration, particularly in unheated soils. As a result, we recommend that measurements of winter N mineralization and soil respiration become research priorities for examining small scale temporal dynamics of winter nutrient cycling. This is especially important since global change drivers such as warming and N additions increased CO_2 and N fluxes during the winter months. Elevated rates of winter N mineralization in the absence of plant demand may result in the loss of this N from the system. In addition, higher rates of winter soil respiration signal larger losses of CO_2 fixed during the previous growing season, as well as enhanced microbial access to previously unavailable substrates. Season-specific responses also suggest that certain components of the belowground community are more responsive to warming and N additions than others. They also indicate that the environmental conditions necessary for a biogeochemical response may only occur at certain times of the year. The marginally significant interaction between warming and N took place only in autumn, when the combination of six months of N additions, elevated rates of N mineralization, decreased plant N demand, and an influx of N-rich plant litter may have raised soil N levels high enough to dampen the positive response of soil respiration to warming. The lack of

interaction between warming × N may also have resulted from differences in the magnitude of their disturbance, lags between the initiation of the treatments and ecosystem response, and / or disparate pathways through they disrupted the system. In combination with modeling studies, additional research at this and other multiple-manipulation experiments will be necessary to determine long-term, seasonal changes in soil C and N dynamics with warming and N deposition.

Table 1.1. Final model output for gravimetric soil moisture (g H_20 g^{-1} soil) for the organic and mineral soil horizons.

Fixed Effect	Estimate	SE	DF	*t*-value	*P*-value
Organic Horizon					
Winter	0.340	0.057	494	5.947	<0.0001
Spring	-0.097	0.056	494	-1.746	0.082
Summer	-0.126	0.051	494	-2.480	0.014
Fall	-0.606	0.054	494	-11.282	<0.0001
Warming	-0.091	0.069	22	-1.308	0.204
Mineral Horizon					
Winter	-0.569	0.062	490	-9.228	<0.0001
Spring	-0.050	0.040	490	-1.247	0.213
Summer	-0.176	0.036	490	-4.920	<0.0001
Fall	-0.286	0.038	490	-7.571	<0.0001
Warming	-0.198	0.082	22	-2.418	0.024

Notes: The structure of the final models for both the organic horizon and the mineral fraction was: ln (Moisture) ~ Warming + Season. Model output includes parameter estimates, standard errors of the estimates (SE), degrees of freedom (DF), *t*-values and *P*-values for fixed effects.

Table 1.2. Final model output for net N mineralization (μg N g^{-1} soil d^{-1}).

Fixed Effect	Estimate	SE	DF	*t*-value	*P*-value
Winter	-0.964	0.125	933	-7.725	< 0.0001
Spring	-1.363	0.162	933	-8.412	< 0.0001
Summer	-1.087	0.203	933	-5.353	< 0.0001
Fall	-0.427	0.164	933	-2.598	0.010
sin (2πAJD)	-0.251	0.093	933	-2.709	0.007
cos (2πAJD)	-1.196	0.110	933	-10.897	< 0.0001
Warming	0.336	0.070	22	4.802	< 0.0001
N Additions	0.148	0.070	22	2.114	0.035
O Horizon	0.978	0.070	22	13.983	< 0.0001

Notes: The structure of the final model was: ln (N Mineralization) ~ Warming + N Additions + Season + Horizon + (sin (2πAJD) + cos (2πAJD)). The abbreviation 2πAJD denotes 2 × π × adjusted Julian day, the explicit model of seasonal cycle. Model output is as in Table 1.1.

Table 1.3. Final model output for soil respiration (mg CO_2-C m^{-2} h^{-1}).

Fixed Effects	Estimate	SE	DF	*t*-value	*P*-value
Winter	3.177	0.092	900	34.396	< 0.0001
Spring	0.155	0.112	900	1.377	0.169
Summer	1.040	0.145	900	7.151	< 0.0001
Fall	0.777	0.123	900	6.325	< 0.0001
sin (2πAJD)	-0.177	0.058	900	-3.041	0.002
cos (2πAJD)	-0.378	0.072	900	-5.258	< 0.0001
Warming × sin (2πAJD)	-0.108	0.082	900	-1.315	0.189
Warming × cos (2πAJD)	-0.278	0.102	900	-2.723	0.007
Warming × Winter	0.486	0.142	900	3.415	0.001
Warming × Spring	-0.094	0.160	900	-0.592	0.554
Warming × Summer	-0.417	0.206	900	-2.022	0.04
Warming × Fall	-0.148	0.174	900	-0.851	0.395
N Additions × Winter	0.012	0.097	900	0.120	0.905
N Additions × Spring	0.139	0.120	900	1.152	0.250
N Additions × Summer	0.199	0.105	900	1.891	0.059
N Additions × Fall	0.254	0.110	900	2.316	0.021
Warming × N × Winter	0.028	0.159	22	0.178	0.860
Warming × N × Spring	-0.040	0.172	900	-0.234	0.815
Warming × N × Summer	-0.219	0.149	900	-1.475	0.141
Warming × N × Fall	-0.296	0.155	900	-1.908	0.057

Notes: The structure of the final model was: ln (Soil Respiration) ~ Warming × N Additions × Season + Warming × (sin (2πAJD) + cos (2πAJD)). Model output is as in Table 1.1. The abbreviation of 2πAJD is as in Table 1.2.

Table 1.4. Net N mineralization (μg N g^{-1} soil y^{-1} or $season^{-1}$) for control, warming, warming x N, and N addition plots.

Year	Treatment	Annual	Winter	Spring	Summer	Autumn
2007-2008	Control	482 ± 55	55 ± 14	26 ± 9	197 ± 50	204 ± 49
	Warming	565 ± 42	96 ± 15	59 ± 16	230 ± 34	179 ± 57
	Warming × N	633 ± 91	70 ± 20	68 ± 10	185 ± 39	309 ± 72
	N Addition	430 ± 51	59 ± 10	41 ± 11	167 ± 26	162 ± 43
2008-2009	Control	242 ± 50	7 ± 3	44 ± 12	136 ± 27	57 ± 15
	Warming	466 ± 92	25 ± 9	54 ± 12	242 ± 63	146 ± 35
	Warming × N	531 ± 130	21 ± 4	65 ± 11	313 ± 110	133 ± 26
	N Addition	354 ± 55	18 ± 5	39 ± 7	234 ± 46	69 ± 13

Note: Data represent mean of six replicates ± 1 SE.

Table 1.5. Soil respiration (g C m^{-2} y^{-1} or $season^{-1}$) for control, warming, warming × N, and N addition plots.

Year	Treatment	Annual	Winter	Spring	Summer	Autumn
2007-2008	Control	409 ± 38	55 ± 2	58 ± 4	188 ± 9	127 ± 6
	Warming	549 ± 51	75 ± 5	87 ± 7	277 ± 23	179 ± 13
	Warming × N	565 ± 31	74 ± 5	82 ± 7	271 ± 21	181 ± 7
	N Addition	466 ± 17	62 ± 3	69 ± 4	227 ± 15	142 ± 8
2008-2009	Control	475 ± 28	52 ± 3	62 ± 3	218 ± 15	97 ± 5
	Warming	707 ± 86	72 ± 5	85 ± 7	322 ± 28	148 ± 11
	Warming × N	711 ± 71	70 ± 7	84 ± 6	333 ± 30	142 ± 16
	N Addition	588 ± 22	63 ± 3	76 ± 5	273 ± 23	125 ± 9

Note: Data represent mean of six replicates ± 1 SE.

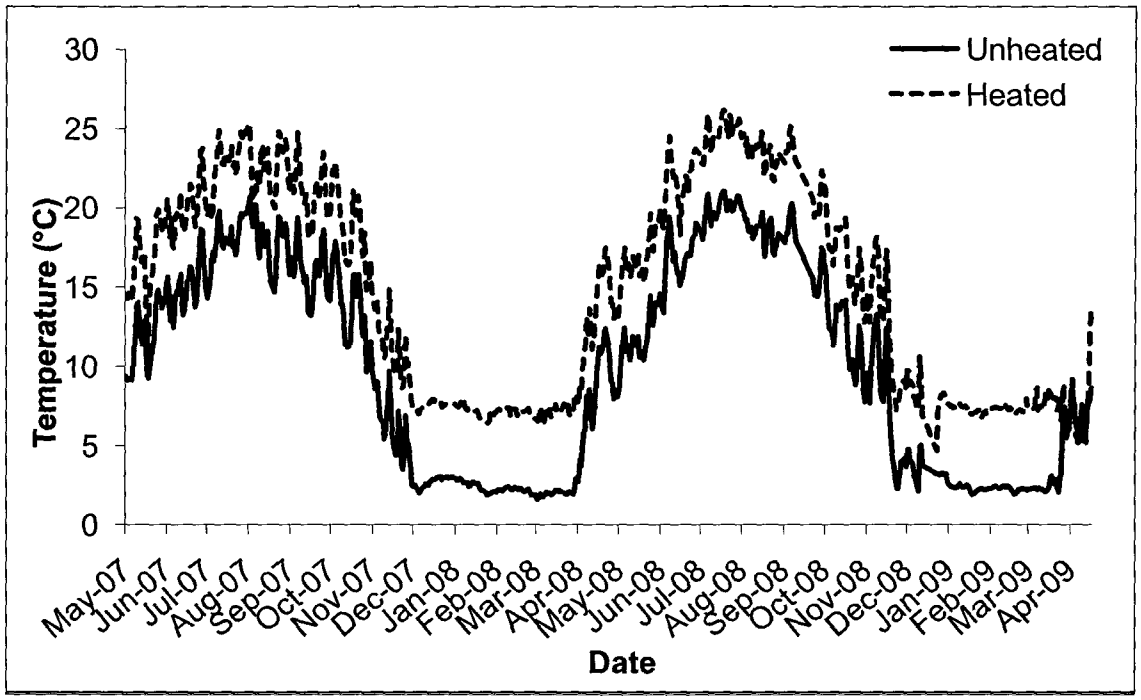

Figure 1.1. Daily soil temperatures (°C) for unheated (control, N addition) and heated (warming, warming × N) plots.

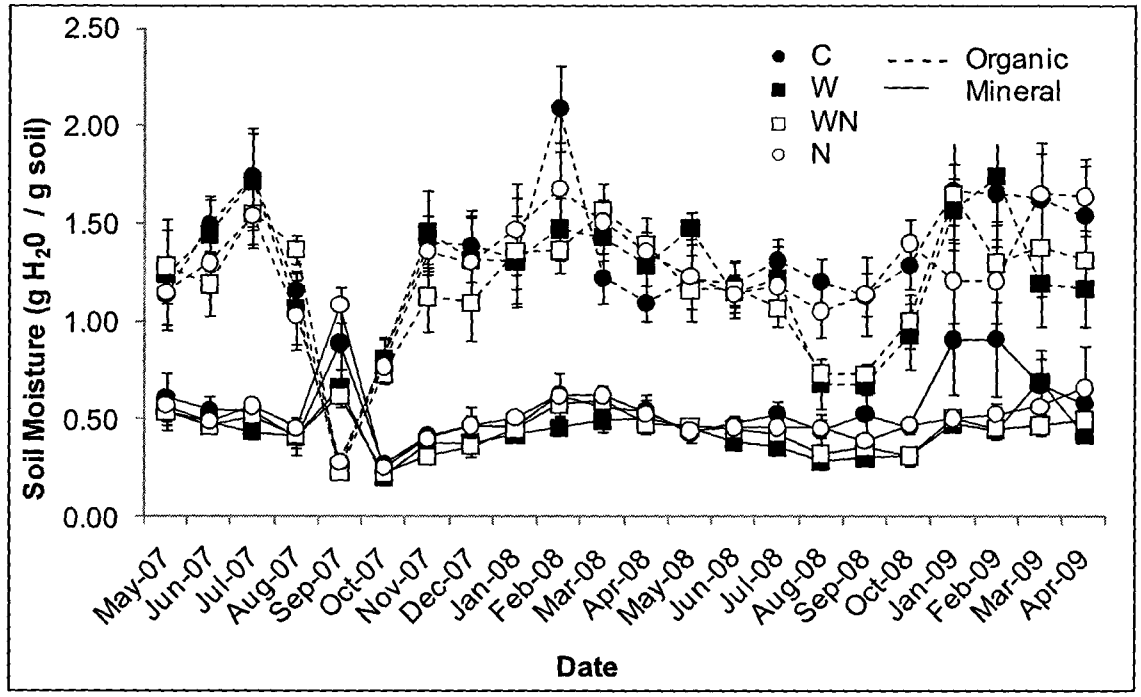

Figure 1.2. Soil moisture (g H_20 g^{-1} soil) for mineral and organic fractions in control(C), warming (W), warming × N (WN), and N addition (N) plots. Each data point represents the mean of six replicates, and error bars indicate ± 1 SE.

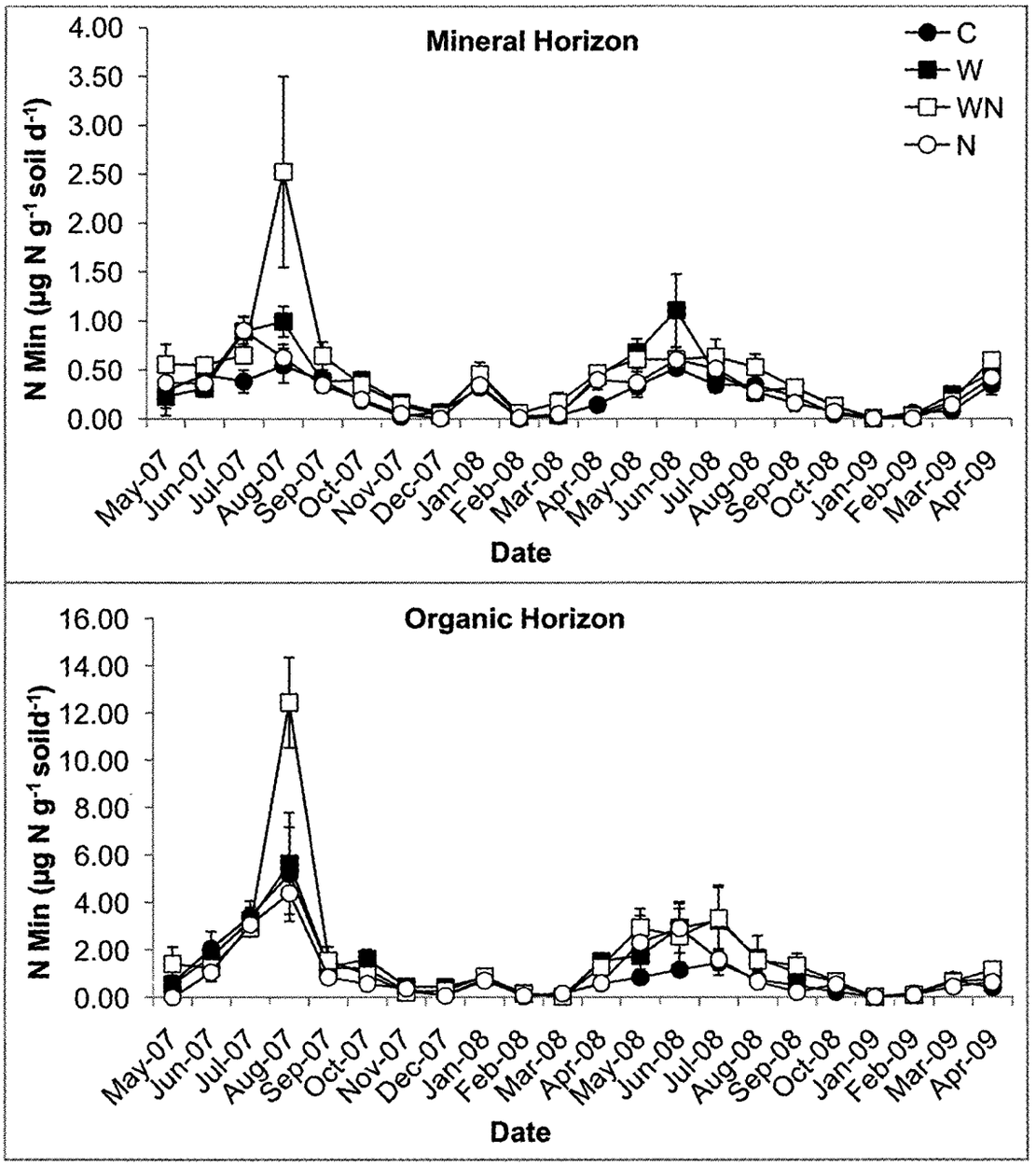

Figure 1.3. Net N mineralization (µg N g^{-1} soil d^{-1}) for mineral and organic soil horizons in control (C), warming (W), warming × N (WN), and N addition (N) plots. Each data point represents the mean of six replicates, and error bars indicate ± 1 SE.

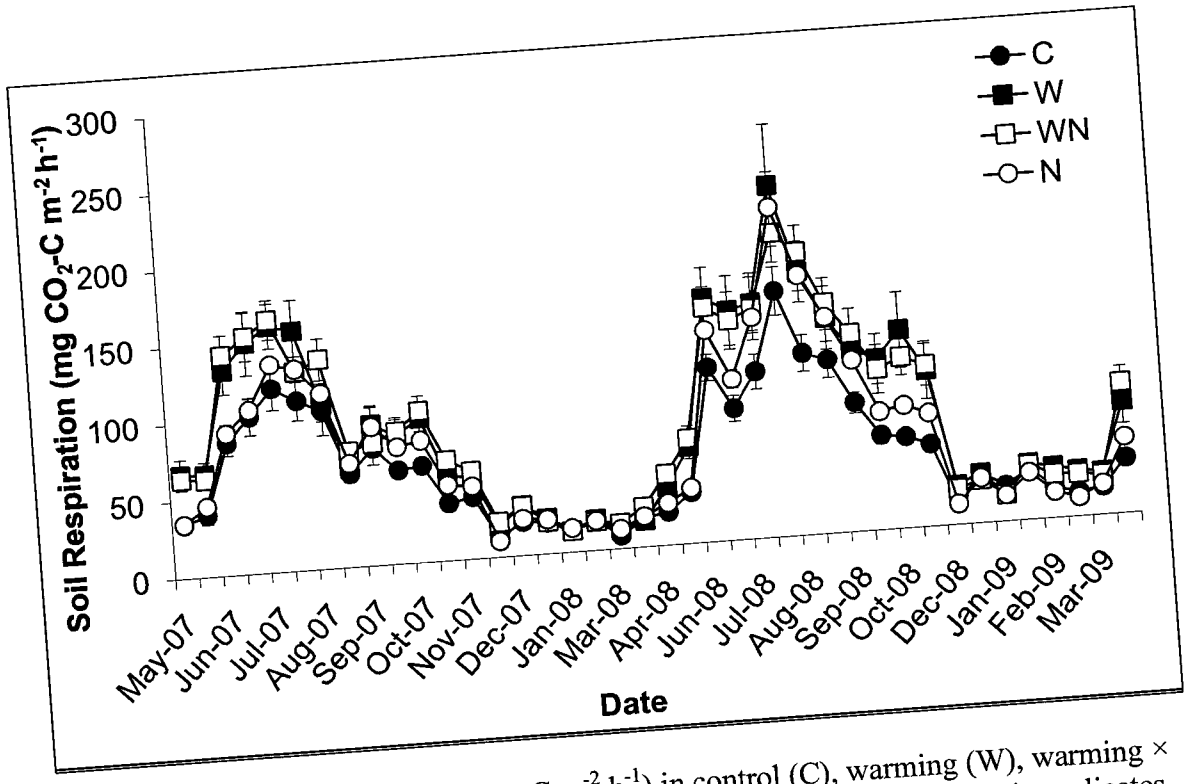

Figure 1.4. Soil respiration (mg CO_2-C m^{-2} h^{-1}) in control (C), warming (W), warming × N (WN), and N addition (N) plots. Each data point represents the mean of six replicates, and error bars indicate ± 1 SE.

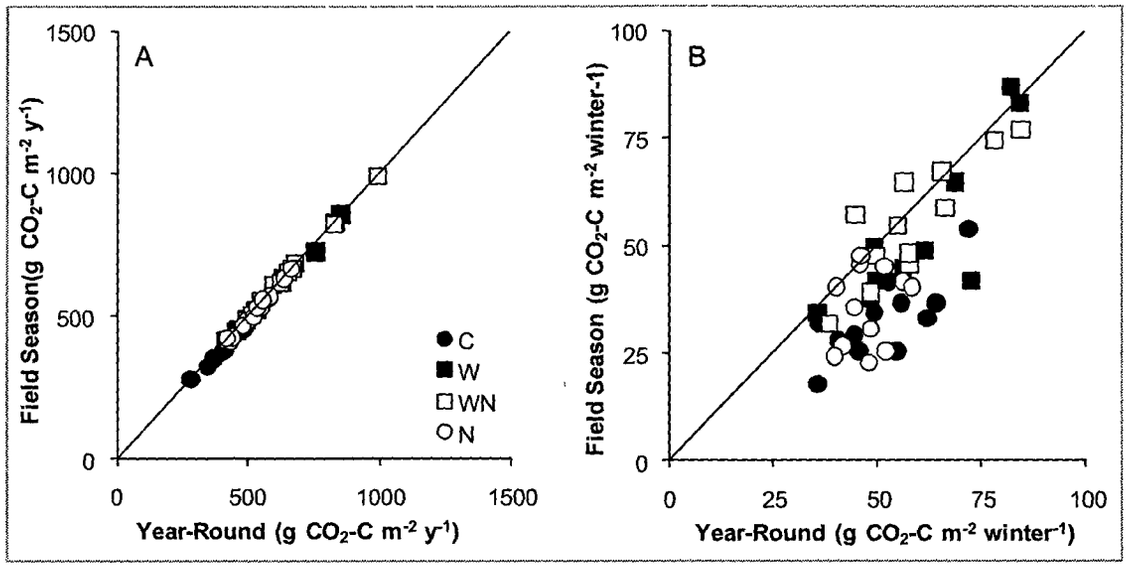

Figure 1.5. Comparison of soil respiration (mg CO_2-C m^{-2} h^{-1}) estimates using measurements taken year-round versus during the field season only for (A) annual fluxes and (B) winter fluxes in control (C), warming (W), warming × N (WN), and N addition (N) plots.

CHAPTER 2

ACCLIMATIZATION OR ACCELERATION? SEASONAL CHANGES IN THE TEMPERATURE SENSITIVITY OF CHRONICALLY WARMED SOILS

Abstract

Long-term soil warming studies have generally demonstrated an ephemeral response of soil respiration to increased temperatures, suggesting that the temperature sensitivity of soil respiration acclimatizes to the new temperature regime. Many of these studies depict acclimatization as an empirical temperature-respiration model with data collected from late spring through early autumn. Yet winter respiration can contribute substantially to the total annual CO_2 flux. Winter soils can also exhibit extremely high temperature sensitivity, leading to considerable carbon losses not captured in traditional temperature-respiration models. We compared the apparent temperature sensitivity of soil respiration between chronically warmed and unwarmed soils over three different timescales: throughout the calendar year (annual), during April through November (growing season), and seasonally during winter, spring, summer, and fall. Temperature sensitivity was assessed by fitting exponential and flexible temperature functions as mixed effects models. From the model parameters, we also estimated annual, growing season, and season-specific Q_{10} values. Our results indicate that soil respiration in chronically warmed plots can exhibit characteristics of acclimated, accelerated, or constant temperature sensitivity depending on the timeframe and the function (exponential or flexible) used to construct the model. Growing season models suggested acclimatization, while winter and spring models indicated enhanced temperature sensitivity with 5°C of

warming. These differences in temperature sensitivity affected the predicted response of soil respiration to elevated temperatures, particularly in winter and spring. Failure to use season-specific models to depict seasonal changes in temperature sensitivity may underestimate carbon losses due to climate warming, especially during the colder months of the year.

Introduction

Soils worldwide contain between 2100 and 2400 Gt of carbon (Kirschbaum 2000, Davidson and Janssens 2006, Wang *et al.* 2010*a*). Climate warming could result in a substantial loss of this carbon, but the magnitude of the loss depends, in part, on the temperature sensitivity of soil respiration to elevated temperatures. Coupled climate-carbon cycle models often assume a Q_{10} of 2, such that respiration doubles for every 10°C increase in temperature (*e.g.*, Cox *et al.* 2000, Friedlingstein *et al.* 2006). However, soils globally can exhibit Q_{10} values as low as 1.3 and as high as 3.3 (Raich and Schlesinger 1992), and projections for global carbon loss by the year 2100 can vary from 50 Gt to 300 Gt depending on which value of Q_{10} is chosen (Jones *et al.* 2003).

Many climate-carbon models also assume that the temperature sensitivity of soil respiration, or Q_{10}, remains constant even as the climate warms (Friedlingstein *et al.* 2006). Evidence from numerous soil warming studies contradicts this assumption. Although elevated temperatures can initially stimulate respiration, many researchers have reported long-term declines in CO_2 flux in chronically warmed soils (Oechel *et al.* 2000, Melillo *et al.* 2002, Strömgren 2001). In some cases, warming has even reduced soil

respiration within the first year or two of the study (McHale *et al.* 1998, Luo *et al.* 2001, Bronson *et al.* 2008, Allison and Treseder 2008).

One of the most common hypotheses used to explain decreased soil respiration in chronically warmed sites is that soil organisms acclimatize to the new temperature regime. Empirical models of the relationship between temperature and respiration have shown lower model slopes (using exponential functions), higher activation energies (using Arrhenius functions), and lower Q_{10} values, all of which suggest that soil organisms have adjusted to warmer conditions (McHale *et al.* 1998, Luo *et al.* 2001, Melillo *et al.* 2004, Bronson *et al.* 2008, Reth *et al.* 2009).

Depletion of the labile fraction of soil organic carbon (SOC) may also be responsible for depressed CO_2 fluxes in soil warming experiments. Models of SOC dynamics have indicated that warming quickly depletes the fast-cycling, labile pool, leaving behind slow and passive pools (Kirschbaum 2004, Eliasson *et al.* 2005, Knorr *et al.* 2005*b*). While the topic is under considerable debate (Kirschbaum 2006), it is likely that both acclimatization and labile C depletion contribute to the transient response of soil respiration to elevated temperatures. Bradford *et al.* (2008) reported both lower soil respiration per unit microbial biomass (suggesting acclimatization) and lower labile C availability (suggesting substrate depletion) in heated versus control soils sampled from a 15 year-old warming experiment. In any case, both the acclimatization and substrate depletion theories suggest that climate-carbon cycle feedbacks may not be as positive as they were once believed to be (Luo *et al.* 2001, Melillo *et al.* 2002).

A common feature of most studies reporting the ephemeral response of soil respiration to elevated temperatures is the timeframe used to conduct the research. In

temperate and boreal biomes, field measurements of CO_2 flux are typically made from April or May through October or November. These growing-season data are then used to create a single, empirical model of the relationship between temperature and respiration. As a result, the hypothesis that soil respiration acclimatizes to elevated temperatures depends upon observations made during only the warmer half of the year. However, a substantial portion of total annual CO_2 flux can occur in winter (Mo *et al.* 2005, Wang *et al.* 2010*b*) when cold conditions result in high temperature sensitivity of organic matter decomposition. Q_{10}values can range over orders of magnitude, from 8 to 23 to 1,000 to 1.25×10^6 (Kirschbaum 1995, Janssens and Pilegaard 2003, Monson *et al.* 2006*a*, Miller *et al.* 2007), implying that small increases in winter soil temperatures could lead to substantially higher rates of soil respiration. Because winter temperatures are expected to increase between 1.1 and 5.4 °C in the Northeastern US by the year 2100 (Hayhoe *et al.* 2007), it is crucial to develop a more sophisticated understanding of the temperature sensitivity of CO_2 flux in cold soils and how this sensitivity might change with winter warming.

Empirical models constructed only during the growing season may not be able to capture such a change in winter temperature sensitivity. The temperature dependence of soil respiration can vary widely with seasonal shifts in water and substrate availability (Kirschbaum 1995, Lavigne *et al.* 2004, Davidson *et al.* 2006). A model constructed over an entire growing season may be appropriate for predicting fluxes over the same timeframe because the confounding influences of temperature, moisture, and carbon inputs will be similar for both the modeled and the predicted fluxes. However, this same model may over- or under- estimate soil respiration when making predictions over

shorter or longer timescales due to asynchronous changes in temperature, water, and substrate availability (Janssens and Pilegaard 2003, Curiel-Yuste *et al.* 2004, Mo *et al.* 2005). To adequately capture the temperature dependence of soil respiration in warmed winter soils, it may therefore be necessary to measure and model fluxes in winter.

The purpose of this study was to evaluate the temperature dependence of soil respiration in chronically warmed soils at several different timescales: annually, during April through November (growing season), and seasonally during winter, spring, summer, and fall. For the purposes of this study, we use the term, "temperature sensitivity," to refer to apparent, not intrinsic, temperature sensitivity (Davidson and Janssens 2006), and soil respiration comprises both autotrophic and heterotrophic fluxes. We hypothesized that the temperature sensitivity of soil respiration would be lower in warmed soils when the empirical model contained data collected only from April through November. By contrast, we expected that the temperature sensitivity of respiration would be higher in warmed soils as compared to unwarmed ones when the model contained data collected throughout the calendar year. We anticipated that the difference in temperature dependence between the annual and growing season timeframes would result from higher sensitivity in warmed winter plots and lower sensitivity in warmed summer soils. For this reason, we hypothesized that using growing season models to predict changes in CO_2 flux with elevated temperatures would underestimate increases in soil respiration during winter and over-estimate changes in soil respiration during the growing season.

Methods

Site description and experimental design

This research took place at the Soil Warming × Nitrogen Addition Study, which has been described in detail by Contosta *et al.* (in press). It is located at the Harvard Forest Long Term Ecological Research (LTER) site in Petersham, Massachusetts (42°50' N, 72°18'W). The forest in the study area is comprised of even-aged, mixed hardwoods. Soils are of the Gloucester series (fine loamy, mixed, mesic, Typic Dystrochrepts; Peterjohn *et al.* 1994). Mean annual air temperature is 7°C, with summer temperatures as high as 32°C and winter as low as -25°C. Average total annual precipitation, including water equivalent of snow, is 1100 mm (Boose *et al.* 2002). The experimental design consists of twenty-four, 3 x 3 m plots randomly assigned to one of four treatments: control, N addition (N), warming (W), and warming × N (WN). Average soil temperature in the heated plots (W and WN) was continuously elevated 5°C above ambient using heating cables placed at 10 cm depth. Nitrogen fertilization in the N and WN plots occurred as equal doses of NH_4NO_3 applied throughout the growing season at a rate of 5 g N m^{-2} y^{-1}.

Soil respiration

Soil respiration measurements were previously described in Contosta *et al.* (in press). Briefly, net CO_2 flux was measured bi-monthly from May 2007 through April 2009 using a static chamber technique (Raich *et al.* 1990, Peterjohn *et al.* 1993). All sampling occurred between 10:00 and 13:00, when the average of the diel flux takes place (Davidson *et al.* 1998). Before each sampling, the depth of the collar was measured to

account for changes in chamber volume with litter inputs and decay. In winter, snow was removed as carefully as possible from collars, and was replaced upon completion of sampling to minimize disturbance. Once excavated, collars equilibrated with the atmosphere for one hour prior to sampling to release CO_2 that had accumulated in the surrounding snow. Immediately prior to measurement, lids were fit over pre-installed collars to create static chambers. There was one chamber per plot. Ten ml headspace samples were taken from each chamber using air-tight, plastic syringes at zero, five, ten, and 15 minutes. During sampling, soil temperature was measured adjacent to the chamber at 5 cm depth in order to model the relationship between temperature and respiration. Following sampling, gas samples were immediately transported back to the University of New Hampshire and analyzed using a LI-COR LI-6252 infrared gas analyzer (LI-COR Biosciences, Lincoln, Nebraska, USA). In the summer of 2008, we compared our static chamber measurements with those made on a dynamic, portable IRGA system and found fluxes to be well-correlated ($r^2 = 0.86$) between the two techniques (A. Contosta, *unpublished data*). As a result, we believe that our static chamber method provided reliable, *in situ* flux measurements.

In winter, we opted for snow removal for equally practical reasons. Placing a lid on top of the snowpack can substantially underestimate flux due to lateral diffusion (Schindlbacher *et al.* 2007). Inserting CO_2 sensors into the snow (*e.g.* Monson *et al.* 2006*b*, Schindlbacher *et al.* 2007) was not feasible because heated plots were often snow free even when unheated plots were covered in 20 cm of snow. Because the snowpack can trap gases emitted from soil (Monson *et al.* 2006*b*), our measurements of winter flux did not necessarily represent CO_2 diffusing through the snow into the atmosphere at the

time of sampling. However, since the CO_2 that accumulates in the snowpack is eventually released to the atmosphere, we believe that our winter soil respiration measurements were valid for comparing respiration rates across seasons.

Modeling and statistical analysis

We modeled the apparent temperature sensitivity of soil respiration in chronically warmed soils at several different timescales, annual, growing season, and season-specific, and with two different functions, exponential and flexible (Table 2.1). The annual model included all the months during which we measured soil respiration (April 2007 through March 2009). The growing season model also contained these months, but included a categorical variable that partitioned the data into measurements taken during the winters (December through March) and those obtained from the April through November periods that comprise field sampling in areas with prolonged winter snow cover. The season-specific model went a step further and contained a categorical variable that separated the data into individual seasons of winter, spring, summer, and fall. Seasons were delineated based on criteria previously defined in Contosta *et al.* (in press) for the research site. According to these criteria, winters occurred from December 1 to March 31, springs from April 1 to May 31, summers from June 1 to August 30, and autumns from September 1 to November 30. Initial data exploration indicated that N additions at the experimental plots did not alter the temperature-respiration relationship. As a result, the N addition plots were grouped with the controls, and the warming treatment included both warming and warming × N plots.

For all three timescales, we fit two different types of models to the data: a first-order exponential function and flexible temperature-respiration function. The first-order, exponential model (EXP) is one of the most frequently applied for examining the temperature-respiration relationship:

$$R = \beta e^{kT} \qquad \text{Eq. 2.1}$$

For this model, β is the exponential constant, or flux at 273 K, in units of mg CO_2-C m^{-2} h^{-1}, k is the exponential parameter, and T is soil temperature (K) at 5 cm. Since the EXP model often underestimates flux at low temperatures and overestimates flux at high ones (Lloyd and Taylor 1994), we also applied the flexible temperature function (LT) designed to overcome this bias:

$$R = Ae^{(-E0/(T-T0))} \qquad \text{Eq.2.2}$$

For eq. 2, A is a dataset-specific variable in units of mg CO_2-C m^{-2} h^{-1} that accounts for inter site variation in soil respiration. The E_0 parameter is similar to an activation energy, and represents temperature (K) when $R \rightarrow \infty$. The T_0 parameter is temperature (K) when $R \rightarrow 0$, and T represents the soil temperature (K) at 5 cm depth.

A mixed effect modeling approach was applied when fitting both the EXP and LT functions. The mixed effects strategy allowed for the partitioning of random errors associated with measurement, instrumentation, and site variability not typically addressed in least-square regression models. It also allowed for the specification of temporal

autocorrelation between measurements taken on the same plot that are usually ignored in linear and non-linear curve fitting (Hess and Schmidt 1995, Peek *et al.* 2002).

For the exponential model (EXP, eq. 1), we used the *lme* function in the *nlme* package in R 2.9.2 (Pinhiero *et al.* 2009, R Core Development Team 2009) to fit three linear mixed effects models: annual, growing season, and season-specific. Prior to model fitting, fluxes were log-transformed to ensure homoscedasticity and normality of residuals. Fixed effects included the EXP parameters β, k, and T. Warming was included as a categorical variable for all three models. A dummy variable specifying the categories of winter and growing season was also included for the growing season model. Likewise, the season-specific model contained a dummy variable indicating the categories winter, spring, summer, and fall. All three mixed models (annual, growing season, and season-specific) included plot as a random intercept. Differences in temperature sensitivity, seasonally and / or with warming, were assessed by examining 95% confidence intervals (CIs) of estimated model parameters. A lack of overlap in the 95% CI for model intercepts (different β's for experimental treatments and / or seasons) and / or slope (different k's for treatment, season, and treatment × season) indicated a difference in the temperature dependence of soil respiration.

For the flexible temperature function (LT, eq. 2), we used the *nlme* function in the *nlme* package of R (Pinhiero *et al.* 2009) to fit annual, growing season, and season-specific models of the temperature-respiration relationship. To fit the LT model, we first built a function specifying the LT model formula and model parameters: A, E_0, and T and T_0. The site-specific constant, A, was calculated by averaging flux for the entire data set, multiplying it by 1,000 (Savage and Davidson 2001), and then taking the natural log of

the result. As with EXP model, log-transforming A allowed us to run the nonlinear model with log-transformed soil CO_2 fluxes, thus ensuring that the model met the homoscedasticity assumption. Initial values for E_0 and T_0 were determined by plotting log-transformed soil respiration versus temperature. Using the *curve* command, the LT function was fit onto the plot using a range of E_0 and T_0 values. The E_0 and T_0 combination that produced the best visual fit to the data points were chosen to initialize the model, such that E_0 = 450 K and T_0 = 223.8 K. A self-starting, nonlinear model was subsequently written using these initial values and the protocol outlined in Pinhiero and Bates (2000).

The self-starting model was then implemented as a nonlinear mixed effects model (*nlme*) for all three timeframes. The parameters E_0 and T_0 were identified as fixed effects. Plot was included as a random intercept for E_0 only; modeling plot as a random effect for both E_0 and T_0 resulted in overparameterization of the fit. For the annual, growing season, and season-specific models, the warming treatment was included as a hypothesized categorical variable for the E_0 and T_0 parameters. As with the EXP function, a dummy variable specifying the categories of winter and growing season was included for the growing season model. The season-specific model contained a dummy variable indicating the categories winter, spring, summer, and fall. Significant differences in temperature sensitivity for with warming and / or season was determined by comparing the estimated 95% CI for E_0 and T_0.

For all of the EXP and LT fits, model verification consisted of visually inspecting residuals for normality and homoscedasticity. Since *lme* and *nlme* approaches employed likelihood-based instead of least-squares estimation, true r^2 values were not available to

describe goodness-of-fit for any of the models. Instead, models were assessed for their ability to fit the data with an information-theoretic approach. Briefly, we used Akaike's Information Criteria (AIC) modified for small sample sizes (AICc) to rank the EXP and LT models of the temperature-respiration relationship. The AICc evaluates which model in an *a priori* set of models best approximates the unknown truth. The model with the lowest AICc in the set has the most support and comes closest to describing the true pattern in the data (Burnham and Anderson 2000). From the AICc values, we also determined Δi for each model (Δi = AICc – $AICc_{min}$, where AIC_{min} is the AICc of the best model). The Δi for the best model equals 0. Models that have Δi values from 0-2 have substantial support from the data. Those with Δi values from 4 to 7 have considerably less support from the data, while those with $\Delta i > 10$ have essentially none (Burnham and Anderson 2000). We also calculated Akaike weights, or w_i, for determining the relative probability of the models given an independent data set and the same group of *a priori* models.

After fitting, verifying, and ranking each of the annual, growing season, and season-specific models, we calculated Q_{10} values from the resulting parameter estimates. These Q_{10} values were used as summary variables for qualitatively comparing temperature sensitivity among all the models. We defined Q_{10} as:

$$Q_{10} = R_{293} / R_{283} \qquad \text{Eq. 2.3}$$

where R_{293} and R_{283} are estimated rates of soil respiration at 293 and 283K, respectively.

Model parameters for the EXP and LT functions were also used to examine the response of soil respiration to elevated temperatures over annual, growing season, and season-specific timescales. For each model timeframe (annual, growing season, and season-specific), hourly fluxes were estimated for each plot with model parameters and *in situ*, hourly temperature data recorded from plot thermistors. For the growing season timeframe, which contained both winter and growing season parameter estimates, only those for the growing season were used in predicting fluxes. Estimated fluxes were then added together within each sampling year to represent total annual flux, or within winter, spring, summer, and autumn for the total seasonal flux. Estimated total annual, winter, spring, summer, and autumn fluxes were averaged by treatment (warmed versus unwarmed). The response of soil respiration to warming within each timeframe was subsequently determined as: % effect = ((warmed – unwarmed) / unwarmed) x 100.

Results

Both the exponential (EXP) and Lloyd and Taylor (LT) model fits indicated that the temperature sensitivity of soil respiration differed between warmed and unwarmed soils. However, this difference shifted with the timeframe used to model the data: annual (January through December), growing season (December through March versus April through November), or season specific (winter, spring, summer, and fall).

For the annual timeframe, temperature sensitivity, or Q_{10}, was either higher or similar in warmed versus unwarmed soils depending on whether the EXP or LT function was used to fit the data (Figure 2.1, Tables 2.2 and 2.3). The EXP fit showed similar Q_{10}s for warmed and unwarmed soils, though Q_{10} for the warmed treatment, 2.91, was

slightly higher than that of the unwarmed plots, 2.83. The LT function resulted in higher temperature dependence of soil respiration in warmed plots ($Q_{10} = 2.80$) as compared to unwarmed ones ($Q_{10} = 2.38$). Q_{10} values changed with warming if the model parameters used to calculate them also differed between warmed and unwarmed treatments. According to pairwise comparisons of estimated 95% CIs, the EXP model showed similar intercept (β values) and slope (k values) in the warmed plots as compared to the unwarmed soils. By contrast, the LT model produced both a lower E_0 and a higher T_0 value in warmed soils as opposed to unwarmed ones.

Allowing the temperature dependence of soil respiration to vary between winter and the growing season produced a different outcome from the simple, annual model. During winter, temperature sensitivity was higher in the heated plots as compared to unheated ones. During the growing season, temperature sensitivity was either similar or lower in warmed plots as compared to unwarmed ones (Figure 2.2, Tables 2.2 and 2.3). In winter, both the EXP and the LT models showed higher Q_{10} values in the warming treatment versus unwarmed soils. Values were 1.97 (EXP) and 2.08 (LT) in the warmed soils and 0.89 (EXP) and 1.12 (LT) in unheated ones. During the growing season, the EXP fit showed a lower Q_{10} value, 2.05, in the warmed plots versus the Q_{10} for unwarmed soils, which was 3.13. The LT function produced growing season Q_{10} values that were similar between the heated and unheated treatments: 2.86 for the warmed plots and 2.77 for the unwarmed ones. Any shifts in temperature sensitivity, or Q_{10}, between warmed and unwarmed soils resulted from significant differences in model parameter estimates, as indicated by pairwise comparisons of the 95% CI. For the EXP fit, intercept, or β, was lower and slope, or k, was higher in warmed plots during winter as

compared to unwarmed plots. The reverse was true during the growing season, and intercept was higher and slope was lower in the warmed soils versus the unwarmed treatment. For the LT model, E_0 and T_0 model parameters were similar for both treatments during winter. In the growing season, only E_0 was higher in the warmed plots, while T_0 was similar for both treatments.

Season-specific fits indicated higher temperature sensitivity in warmed plots during winter and spring and constant temperature sensitivity in summer and fall (Figure 2.3, Tables 2.2 and 2.3). For both season-specific EXP and LT models, Q_{10}s were higher for warmed plots in winter and spring as compared to unwarmed ones. In winter, Q_{10}s were 1.97 (EXP) and 1.89 (LT) in warmed plots versus Q_{10}s of 0.89 (EXP) and 1.18 (LT) in unwarmed soils. By spring, Q_{10} had increased to 2.48 (EXP) and 2.28 (LT) in the heated treatment and had increased to 1.89 (EXP) and 1.85 (LT) in the unheated sites. As with the annual and field season timescales, changes in Q_{10} values with warming resulted from differences in model parameter estimates in heated versus unheated plots. According to pairwise comparisons of the 95% CI, the EXP model parameters β and k differed between treatments in winter, but did not during spring. While the LT model parameters E_0 and T_0 differed between warmed and unwarmed plots in winter, an overlap of the 95% CIs indicated that this difference was not substantial. In spring, both E_0 and T_0 were nearly identical in heated versus unheated soils.

During summer and fall, temperature sensitivity was lower than during winter and spring, and did not vary between heated and unheated plots (Figure 2.3, Tables 2.2 and 2.3). For the EXP function, parameter estimates (β and k values for intercept and slope) were very similar. As a result, Q_{10} values were alike in the warmed and unwarmed

treatment. In summer, Q_{10}s from the EXP fit were 1.50 in the warming treatment and 1.51 for the unwarmed plots. By fall, Q_{10}s had increased to 1.79 in heated soils and 1.96 in unheated ones. The LT fit also produced no difference in model parameter estimates (E_0 and T_0) between heated and unheated soils for summer and fall. Consequently, Q_{10}s calculated from LT model parameters were nearly identical between heated and unheated plots. In summer they were 2.18 and 2.09 in warmed and unwarmed soils, respectively. In autumn, they decreased to 1.94 in the warming treatment and 1.87 in the unwarmed soils.

Ranking of models using AICc indicated that the season-specific LT model had the most support from the data, while the annual, EXP model had the least (Table 2.4). Compared to the season-specific LT model, none of the other models in the set exhibited Δi values < 10. Even the model that scored second best, the season-specific EXP fit, had a Δi of 90.61. However, the two models that appeared to have the best support from the data, the season-specific LT and EXP fits, also had the largest uncertainty associated with their parameter estimates (Tables 2.2 and 2.3). Standard errors for β and k were as much as 100% greater than the estimates themselves, while those for E_0 and T_0 were as much as 480% greater.

The predicted response of soil respiration to warming varied depending on timeframe (annual, growing season, or season-specific) used to calculate fluxes (Figure 2.4). When estimating the impact of warming on total annual flux, annual and growing season models showed similar effect sizes, while season-specific models displayed a larger effect size than either. Estimates of the impact of warming on winter fluxes were similar for growing season and season-specific models (38-89%), both of which were

much higher than the effect predicted by annual models (23-27%). In spring, the annual and growing season timeframes showed the lowest effects of warming on soil respiration (30-66%), while the largest responses occurred with the season-specific parameter estimates (57-66%). The predicted responses of summer and autumn respiration to warming were fairly similar among annual, growing season, and season-specific timeframes. However, growing season generally predicted a stronger response to increased temperatures during summer and fall than season-specific models.

Discussion

We used a combination of mixed effects modeling and Q_{10} values to assess the temperature sensitivity of soil respiration in chronically warmed soils. Our results indicate that soil respiration can show characteristics of acclimatized or accelerated temperature dependence depending on the timeframe used to model the data. Annual EXP model parameters and Q_{10}s indicated no difference in temperature sensitivity between warmed and unwarmed soils, while annual LT model output showed enhanced temperature sensitivity in chronically warmed plots. Growing season models suggested either acclimatization (EXP) or no shift (LT) in temperature sensitivity, as indicated by model parameters and Q_{10} values. While β, k, E_0, and T_0 parameters cannot easily be compared to other studies, the Q_{10} values we calculated for both the annual and the growing season models fit within the range of those reported in other forest ecosystems (*e.g.*, Curiel-Yuste *et al.* 2004, Lavigne *et al.* 2004, DeForest *et al.* 2006).

The disparity between the results of the EXP and LT models of temperature sensitivity may be related to their inherent ability to model soil respiration across a range

of temperatures. The EXP model is known to underestimate CO_2 fluxes at low temperatures and to overestimate fluxes at higher ones (Lloyd and Taylor 1994, Kirschbaum 1995), resulting in potentially biased model parameters and Q_{10} estimates. By contrast, the LT model has often performed better across a range of temperatures (Lloyd and Taylor 1994, Richardson and Hollinger 2005, Richardson *et al.* 2006), suggesting that it is better able to portray temperature sensitivity over annual timescales. It is not within the scope of this paper to discuss the merits of the EXP versus the LT function in depicting the temperature sensitivity of soil respiration, though LT models showed lower AICc values with a given model timeframe (annual, growing season, season-specific) as compared to EXP fits. However, studies showing the transient response of soil respiration to elevated temperatures have typically employed the EXP model to depict acclimatization (McHale *et al.* 1998, Luo *et al.* 2000, Strömgren 2001, Melillo *et al.* 2004), which may have shaped inferences about the temperature sensitivity of soil respiration as much as the timeframe used to model the relationship.

Regardless of the different findings of the EXP and LT models, both showed an overall trend toward increased temperature sensitivity for unwarmed plots and decreased sensitivity for warmed plots when using a growing season timeframe instead of an annual one. As hypothesized, this shift in apparent temperature sensitivities between annual and growing season models was likely due to the differences in the temperature dependence of soil respiration in winter. The actual range of temperatures over which fluxes were measured may have influenced the apparent temperature sensitivity during this season. In the unwarmed treatment, soil temperatures were below 0°C until late February in the winter of 2007-2008 and late March in winter of 2008-2009. Such low temperatures may

have directly inhibited soil respiration in unwarmed plots, and may also have indirectly limited microbial activity due to lack of available water in frozen soils. Warmed plots likely did not experience these temperature or diffusion limitations, since winter soil temperatures in the warming treatments were always above 0°C. In this way, higher soil temperatures on the warming plots during winter may have allowed microbial access to substrates that normally would not be available at that time of year. Since labile C availability tends to be low during winter (Contosta *et al.* in prep), these substrates likely represent more recalcitrant pools of SOM that can show equal or higher sensitivity to temperature than the more labile fraction (Knorr *et al.* 2005*b*, Conen *et al.* 2006, Fierer *et al.* 2006).

Although the higher temperature sensitivity in warmed winter soils suggests a positive feedback to climate change, both warmed and unwarmed plots displayed Q_{10}s that were much lower than those described in the literature for cold soils (*e.g.*, Kirschbaum 1995, Janssens and Pilegaard 2003, Monson *et al.* 2006*b*, Miller *et al.* 2007). The disparity between the Q_{10} values reported here and those cited in other studies was not likely due to methodological differences for measuring flux, since our raw winter respiration values were similar to those described for temperate forest ecosystems (*e.g.*, Davidson *et al.* 1998, Savage and Davidson 2001, Bowden *et al.* 2004, Mo *et al.* 2005, De Forest *et al.* 2006, Groffman *et al.* 2006). Rather, the discrepancy may be due to the depth of the probe used for measuring soil temperature and its relation to the active layer, or location of the highest respiration activity in the soil profile (Janssens and Pilegaard 2003, Mo *et al.* 2005). We measured soil temperatures at 5 cm, which was the same depth at which we continuously monitored temperatures for

maintaining the warming treatment. However, winter respiration may come from slightly deeper in the soil profile in both warmed and unwarmed plots since soils can be partially frozen near the surface. In fact, when taking soil cores in winter at the Soil Warming x N Addition experiment, we routinely observed ice lenses in the O horizon, particularly in the unwarmed plots. As a result, measuring soil temperature slightly deeper in the soil profile may have resulted in higher Q_{10}s, while temperatures taken closer to the soil surface, at 1 or 2 cm, may have produced lower ones (Davidson *et al.* 1998).

In addition to higher temperature dependence in winter, warmed soils also exhibited somewhat enhanced temperature sensitivity in spring as compared to unwarmed plots. This higher spring temperature dependence could result from accelerated phenological changes in warmed soils, such as earlier snowmelt, earlier root growth and activity (Mo *et al.* 2005), and deepening of the active layer responsible for soil respiration (Rayment and Jarvis 2000). Like winter, higher springtime sensitivity of soil respiration could also be related to the quality of the substrate available. Labile C resources can be limited in spring (Contosta *et al.* in prep, Bradford *et al.* 2008), such that the more recalcitrant SOM displayed higher sensitivity to increased temperatures at this time of year.

In comparison to winter and spring, summer and autumn displayed low temperature dependence of soil respiration in both the warmed and unwarmed soils as indicated by lower Q_{10} values. Other researchers have also measured low Q_{10} values for these two seasons (*e.g.* Janssens and Pilegaard 2003, Curiel-Yuste *et al.* 2004, DeForest *et al.* 2006). The depth of the active layer of soil respiration may account for low temperature sensitivity during summer and fall. The active layer for soil CO_2 flux in

summer extends deep enough that small, surficial changes in soil temperature will not cause large fluctuations in soil respiration. In autumn, soils cool from the top down, such that lower soil horizons contribute more to respiratory activity and are not as sensitive to changes in surface temperatures (Mo *et al.* 2005, Rayment and Jarvis 2000). Soil moisture may also have moderated the relationship between temperature and respiration in both warmed and unwarmed treatments. Low soil moisture due to summer drought can limit the ability of soils to respire CO_2, even when soil temperatures are very warm (Davidson *et al.* 1998, Savage and Davidson 2001, Janssens and Pilegaard 2003, Lavigne *et al.* 2004). This may have especially been the case in warmed plots, where soil moisture was an average 13% lower during summer as compared to unwarmed plots (Contosta *et al.* in press). Finally, substantial belowground allocation of labile C during summer and fall may influence soil respiration more than temperature during these seasons (Curiel-Yuste *et al.* 2004, Sampson *et al.* 2007). As a result, labile C depletion during these summer and fall may be important in explaining the ephemeral response of soil respiration to increased temperatures.

Ranking the six models used to examine the temperature sensitivity of soil respiration showed that the season-specific LT and EXP models enjoyed the most support in the data. These models also had the highest levels of uncertainty. This was likely due to the large standard errors associated with the base case of season-specific model, the unwarmed treatment in winter, which showed no relationship between temperature and respiration. If we refit the season-specific models and make the warmed plots in spring the base case, the standards errors of model estimates decrease, but the AICc still ranks these two models as having the most support in the data.

Therefore we conclude that that the season-specific models best capture changes in the temperature-respiration relationship at our study site. Our season-specific models indicate that warming enhances temperature sensitivity in winter and spring but has less of an effect on temperature dependence in summer and fall. We attribute these changes in temperature sensitivity to both seasonal and warming-induced shifts in water and carbon availability. In winter and spring, increased moisture due to warming coupled with a shortage of labile C makes the apparent temperature dependence of soil respiration high. In summer and fall, lower moisture, due to both natural drought and increased temperatures, obscures the stimulatory effect of increased temperatures on soil respiration. Depletion of more readily available labile C in summer and fall may also moderate the effect of higher temperatures on CO_2 flux, making it appear that temperature sensitivity has not changed.

We believe that modeling such season-specific shifts in the temperature-respiration relationship with warming is important in understanding feedbacks between soil respiration and climate warming. An annual model that does not specify for the differences in temperature sensitivity among seasons would show no overall difference in warmed versus unwarmed plots, despite the fact that temperature dependence is enhanced during half of the year. A growing season model would display constant or reduced sensitivity, suggesting that climate change feedbacks from soil respiration are not as positive as they were once believed to be (*e.g.*, Luo *et al.* 2001, Melillo *et al.* 2002).

Choosing an annual or growing season model over a season-specific one could also influence predicted responses of soil respiration to 5°C of warming. In summer and

fall, predicted increases from the growing season timeframe were slightly higher than those obtained from season-specific parameters, suggesting that growing season models inflate the effect of warming on soil respiration during these two seasons. Because summer and autumn fluxes together comprise 60 to 80% of the total annual flux (Contosta *et al.* in press), even slightly overestimating the response of soils to warming would indicate a much larger annual soil C loss than actually occurs. Conversely, accelerated temperature sensitivity in warmed soils during winter and spring may result in a much greater treatment effect than would be detected with annual or growing season models of the temperature-respiration relationship. The largest increases in soil respiration during both seasons were predicted with season-specific model parameters. This was especially the case for spring, when the difference between season-specific predictions and those from longer timescales (annual and growing season) differed by as much as 30%. In fact, the season-specific 57-66% increases in springtime fluxes with warming were much closer to actual increases in CO_2 flux reported for this site (Contosta *et al.* in press) compared annual and growing season model predictions. Such an undetected loss of carbon could result in a significant feedback to climate change since respiration during winter and spring comprises between 22 and 40% of the total annual flux (Contosta *et al.* in press). Further, underestimating the effect of warming on soil respiration in winter and spring could result in underestimating the effect of warming for the entire year. When fluxes for winter, spring, summer, and fall were combined to predict total annual flux, the impact of heating on soil respiration was 10% higher than when annual models were used for the same purpose.

It is important to note that the models and predictions reported in this study were based on the temperature dependence of soil respiration following one to three years of warming. Consequently, our models and predictions may represent an initial phase of stimulated respiration with increased temperatures that may decline over time. However, even in this "initial" stage, we have detected slight decreases in labile C and microbial biomass (Contosta *et al.* in prep) that may moderate the effects of warming on CO_2 flux. Modeling the temperature respiration relationship with growing season data would also suggest acclimatization to elevated temperatures. However, models constructed over shorter or longer timescales indicated enhanced or constant temperature sensitivity. Overall, our findings show that traditional, growing season models of the temperature-respiration relationship may be insufficient for describing changes in temperature sensitivity in chronically warmed soils. Instead, season-specific models better portray the ways that warming alters temperature sensitivity during certain times of year. Enhanced sensitivity in winter and spring could result from increased access to C substrates (winter), accelerated snowmelt and rhizosphere activity (spring), and higher temperature dependence of recalcitrant organic matter decay during both seasons. Diminished or constant sensitivity in summer and fall may result from a combination of moisture limitation, labile C depletion, and the depth of the active layer of soil respiration. Failure to consider these season-specific responses, particularly during winter and spring, could underestimate the positive feedback between soils and climate change.

Table 2.1. Exponential (EXP) and flexible (LT) models of the temperature-respiration relationship during annual, growing season, and season-specific timeframes.

Model	Timeframe	Model Form
EXP	Annual	$\ln R \sim \beta \times W + ((k \times W) \times T)$
	Growing Season	$\ln R \sim \beta \times W \times GS + ((k \times W \times GS) \times T))$
	Season Specific	$\ln R \sim \beta \times W \times SS + ((k \times W \times SS) \times T))$
LT	Annual	$\ln R \sim A + ((-E_0 \times W) / (T - (T_0 \times W)))$
	Growing Season	$\ln R \sim A + ((-E_0 \times W \times GS) / (T - (T_0 \times W \times GS)))$
	Season Specific	$\ln R \sim A + ((-E_0 \times W \times SS) / (T - (T_0 \times W \times SS)))$

Notes: "W," "GS" and "SS" indicate warming, growing season, and season-specific variables, respectively. Models were of log-transformed fluxes.

Table 2.2. Estimated β and k parameters, 95% CI of the estimates, and calculated Q_{10} values from exponential (EXP) models of the temperature-respiration relationship during annual, growing season, and season-specific timeframes.

Model	Timeframe	Trt	β	±95% CI	k	±95% CI	Q_{10}
Annual	Annual	C	-25.62	1.31	0.104	0.005	2.83
		W	-26.73	1.90	0.107	0.007	2.91
GS	Winter	C	5.89	11.82	-0.011	0.043	0.89
		W	-17.01	14.69	0.072	0.053	2.05
	GS	C	-28.56	3.58	0.114	0.009	3.13
		W	-23.99	4.99	0.098	0.013	2.65
SS	Winter	C	5.99	10.28	-0.012	0.038	0.89
		W	-15.90	12.73	0.068	0.046	1.97
	Spring	C	-14.61	11.52	0.064	0.042	1.89
		W	-22.24	14.53	0.091	0.052	2.48
	Summer	C	-7.19	13.81	0.041	0.049	1.51
		W	-7.07	17.85	0.041	0.063	1.50
	Fall	C	-15.04	11.68	0.067	0.042	1.96
		W	-12.46	14.73	0.058	0.053	1.79

Notes: "GS" refers to growing season, and "SS" indicates season-specific models. Parameter estimates are based on log-transformed fluxes.

Table 2.3. Estimated E_0 and T_0 parameters, 95% CI of the estimates, and calculated Q_{10} values from flexible (LT) models of the temperature-respiration relationship during annual, field campaign, and seasonal timeframes.

Model	Timeframe	Trt	E_0	±95% CI	T_0	±95% CI	Q_{10}
Annual	Annual	C	547.71	25.65	208.28	3.31	2.38
		W	485.61	34.33	219.14	4.48	2.80
GS	Winter	C	5783.68	16247.62	-417.78	1942.23	1.12
		W	731.30	16249.19	187.98	1942.43	2.08
	SS	C	450.80	35.60	222.28	4.98	2.86
		W	489.45	54.45	218.45	7.83	2.77
SS	Winter	C	4072.39	5670.00	-213.21	677.78	1.18
		W	880.73	5675.27	170.47	678.46	1.89
	Spring	C	906.43	5668.40	166.25	677.62	1.85
		W	635.41	5674.17	200.14	678.38	2.28
	Summer	C	605.08	5659.06	197.18	676.24	2.09
		W	596.16	5665.37	200.40	677.23	2.18
	Fall	C	757.95	5664.50	178.08	677.09	1.87
		W	728.35	5671.33	183.14	678.08	1.94

Notes: "GS" refers to growing season, and "SS" indicates season-specific. Parameter estimates are based on log-transformed fluxes.

Table 2.4. Ranking of annual, growing season, and season-specific models fit with EXP and LT functions.

Model	N	AICc	Δi	w_i
LT Season-Specific	914	587.07	0.00	1
EXP Season-Specific	914	677.68	90.61	2.11E-20
LT Growing Season	914	785.29	198.22	9.05E-44
EXP Growing Season	914	826.29	239.22	1.13E-52
LT Annual	914	843.24	256.17	2.37E-56
EXP Annual	914	856.24	269.17	3.55E-59

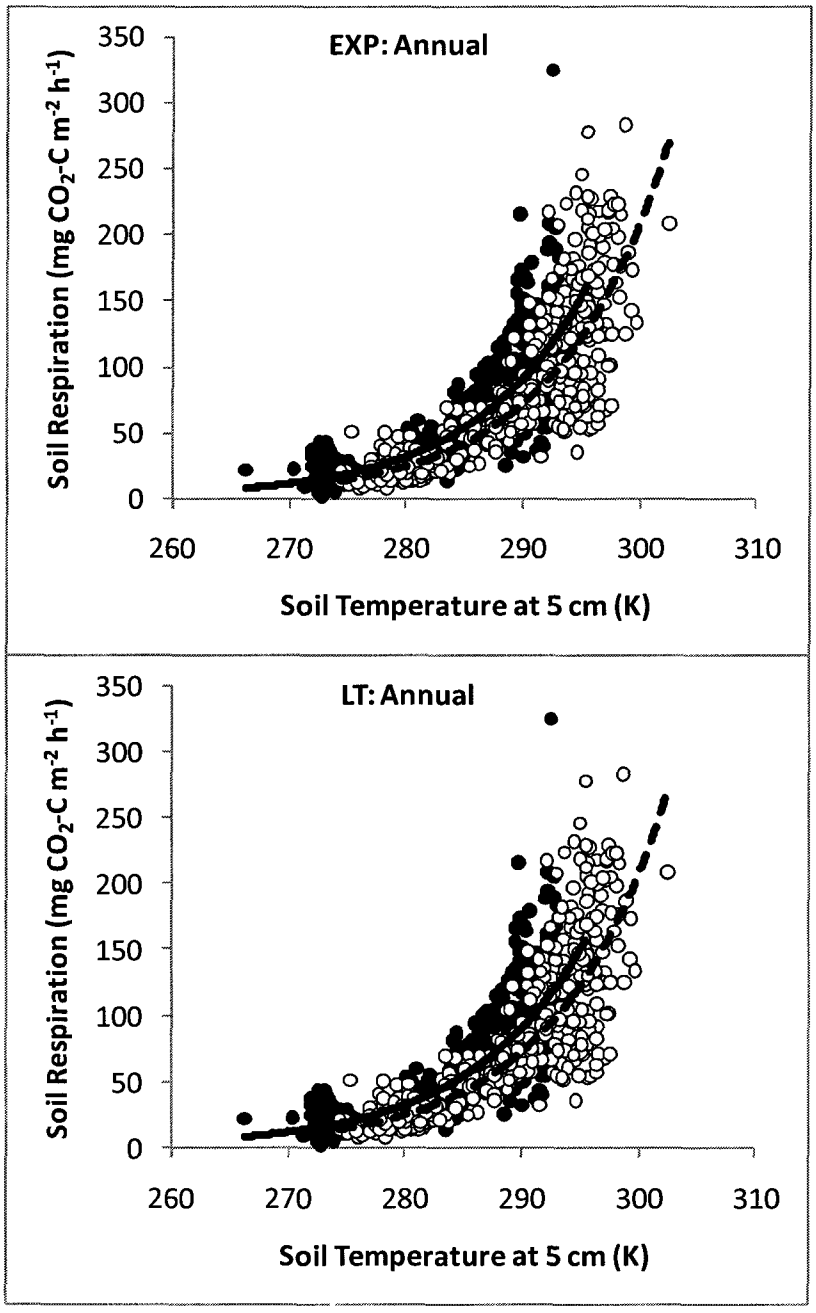

Figure 2.1. Relationship between soil temperature at 5 cm (K) and soil respiration (mg CO_2-C m^{-2} h^{-1}) in warmed and unwarmed plots over an annual timescale for exponential (EXP) and Lloyd and Taylor (LT) models. Each circle represents an hourly flux. The solid line and the solid circles depict unwarmed plots, while the dashed line and open circles show warmed plots.

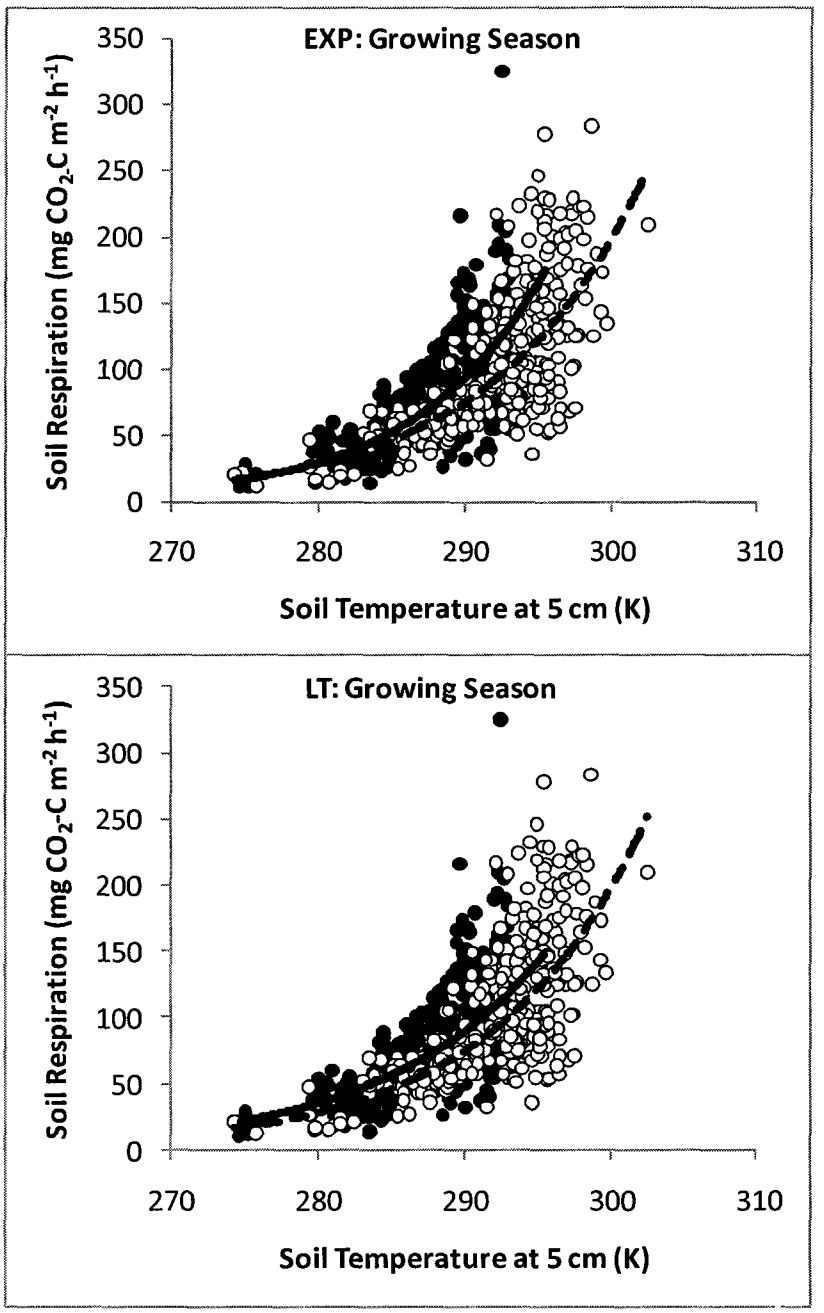

Figure 2.2. Figure 2.2. Relationship between soil temperature at 5 cm (K) and soil respiration (mg CO_2-C m^{-2} h^{-1}) in warmed and unwarmed plots during the growing season for exponential (EXP) and Lloyd and Taylor (LT) models. Lines and data points are as in Fig. 2.1.

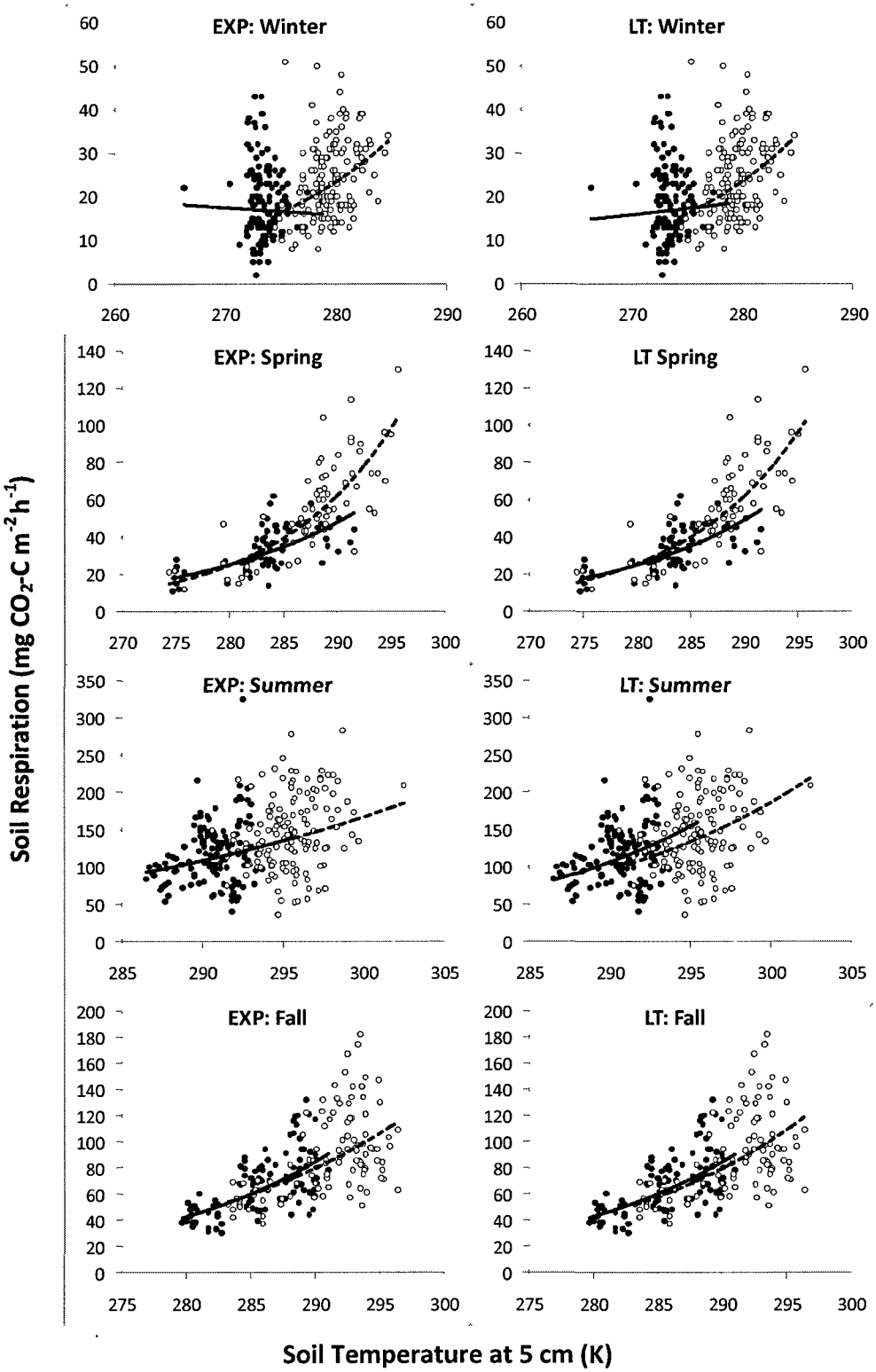

Figure 2.3. Relationship between soil temperature at 5 cm (K) and soil respiration (mg CO_2-C m^{-2} h^{-1}) in warmed and unwarmed plots over seasonal timescales for exponential (EXP) and Lloyd and Taylor (LT) models. Lines and data points are as in Fig. 2.1

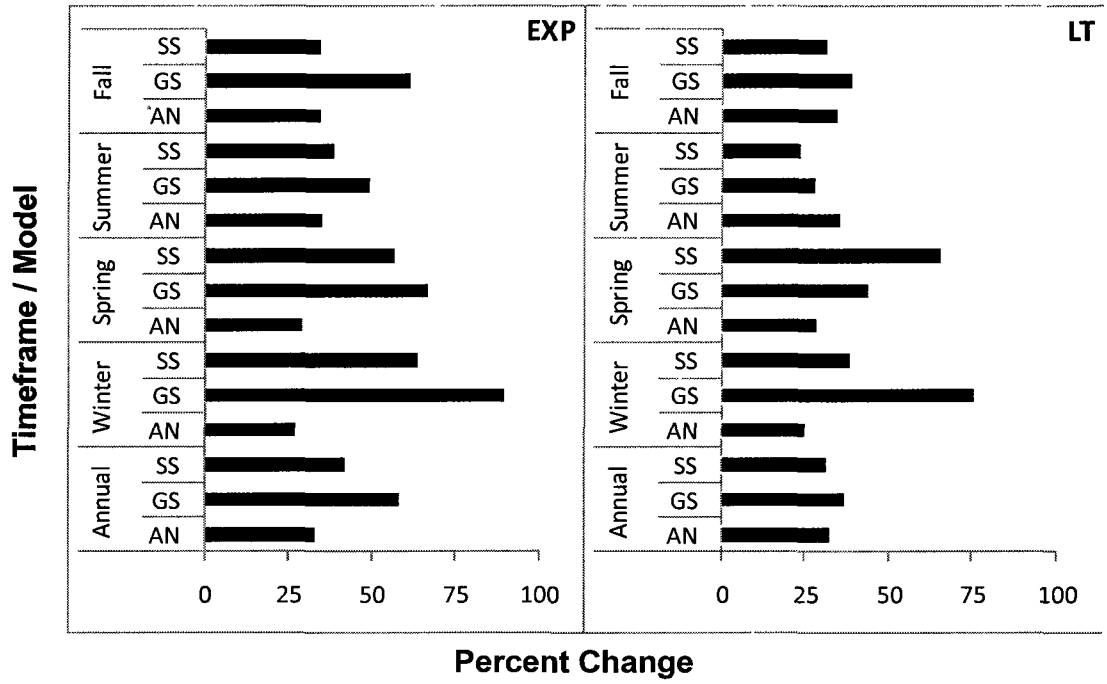

Figure 2.4. Predicted response of soil respiration to warming shown as average percent change for annual and winter, spring, summer, and fall fluxes when estimated with parameters from annual (AN), growing season (GS) and season-specific (SS) models. The EXP panel shows predicted responses from exponential model parameters, while the LT panel depicts predicted response from Lloyd and Taylor model parameters.

CHAPTER 3

SOIL MICROBIAL RESPONSE TO CHRONIC WARMING AND N ADDITIONS IS MEDIATED BY SEASON

Abstract

Although both increased temperatures and N deposition can negatively impact the soil microbial community, few studies have examined how simultaneous warming and N additions affect microbial community dynamics. Many researchers have also not assessed season-specific responses to warming and N fertilization despite evidence that the microbial community is attuned to seasonal shifts in C and nutrient availability. We studied interactions among season, warming, and N additions on microbial composition, biomass, and function at a combined Soil Warming × N Addition experiment. Soils were warmed to 5°C above ambient, and N was applied at a rate of 5 g m^{-2} y^{-1}. Labile C availability, extracellular enzyme activity, microbial biomass, and microbial community composition were measured during winter, spring, summer, and autumn of 2008. Labile C, β-glucosidase, total biomass, and bacterial biomass were highest in autumn, and community composition also shifted during this month. By contrast, the F:B ratio was highest and labile C was lowest in winter, suggesting that fungi can outcompete bacteria when labile C is scarce. Warming and N additions, acting alone and together, generally depleted C supplies and suppressed microbial biomass. Many of these adverse effects were season specific, highlighting the role that season plays in mediating the response of soils to disturbance. Simultaneous warming and N fertilization also impacted microbial biomass in unexpected ways. For example, warming alleviated the negative effect of N

additions in October, despite the fact that reductions in the warming only and N addition only treatment made an additive or synergistic response more likely. Surprising interactions between the warming × N treatments underscore the importance of multifactor studies in understanding how soils respond to global change.

Introduction

Global changes such as climate warming and N deposition can alter microbial regulation of soil biogeochemical cycles. Numerous studies have documented the effects of increased temperatures or N additions on microbial resource availability, process rates, biomass, and / or community structure. Both warming and N fertilization can reduce labile C availability (Frey *et al.* 2004, Bradford *et al.* 2008) and alter extracellular enzyme activity (Saiya-Cork *et al.* 2002, DeForest *et al.* 2004, Waldrop and Firestone 2004, Allison and Treseder 2008). Both can also decrease microbial biomass (Arnold *et al.* 1999, Compton *et al.* 2004, Frey *et al.* 2004, Bradford *et al.* 2008, Frey *et al.* 2008), shift microbial community composition (Zogg *et al.* 1997, Allison *et al.* 2007), and reduce the relative abundance of fungi (Waldrop *et al.* 2004*a*, Frey *et al.* 2008).

To date, much of the research quantifying the effects of warming and N deposition on soil microbial communities has occurred separately. Segregating these two disturbances does not represent a real-world situation where increased temperatures and N deposition occur at the same time, altering microbial community dynamics in a way that may be difficult to predict. Because both increased temperatures and N inputs can affect the microbial community in similar ways, *e.g.*, by decreasing labile C availability, the response to simultaneous warming and N additions may be purely additive.

Alternatively, warming and N additions could interact to multiply their largely negative effect on the soil microbial community, or could annul one another's impact on microbial dynamics. The few studies that have examined the interactive effects of warming and N additions on the soil microbial community have reported conflicting results. In some cases, warming has canceled the positive effect of N additions on microbial biomass (Rinnan *et al.* 2007). In others, only one of the experimental manipulations has provoked a response (Henry *et al.* 2005, Horz *et al.* 2008, Bell *et al.* 2010). Furthermore, these studies may not apply to temperate forests since they have occurred in grassland or arctic ecosystems.

Most of the work examining the response of soils to warming and / or N additions has also not explicitly addressed the role that season plays in influencing the response. However, research from both temperate and alpine ecosystems suggests a seasonally dynamic soil microbial community attuned to temporal shifts in soil C, N, temperature, and moisture (Jaeger *et al.* 1999, Lipson *et al.* 1999, Bohlen *et al.* 2001, Lipson *et al.* 2002, Schadt *et al.* 2003, Schmidt *et al.* 2004). For example, labile C availability tends to be highest in summer and autumn, when inputs from rood exudates (summer) and litter leachate (autumn) are at their peak (Bohlen *et al.* 2001). By contrast, recalcitrant C resources such as lignins and phenols are most abundant in fall and winter (Schmidt *et al.* 2004). These differences in belowground C resources may seasonally partition the microbial community, such that bacteria dominate in summer when labile C is plentiful, and fungi prevail in winter when labile C is scarce (Lipson *et al.* 2002, Schadt *et al.* 2003). Failure to address season-specific shifts in soil microbial dynamics may neglect to identify organisms and processes affected by elevated temperatures and N inputs.

Already numerous studies indicate that both warming and N fertilization select against fungi and fungal enzymes in favor of bacteria (DeForest *et al.* 2004, Gallo *et al.* 2004, Waldrop and Firestone 2004, Frey *et al.* 2008). This selection pressure may be most pronounced in summer when higher temperatures and a more palatable C supply put fungi at a disadvantage. Alternatively, fungi may suffer more from warming and N additions in winter when their enzymatic systems are believed to be most active.

The purpose of this study was to examine the effects of simultaneous warming and N additions on soil microbial community dynamics, emphasizing the role that season plays in mediating the response. We hypothesized that labile C availability, microbial community structure, and extracellular enzyme activity would show a strong seasonal pattern, with labile C and bacterial biomass highest in summer and fall, and fungal biomass and lignocellulose enzyme activity highest in fall and winter. We expected that warming and N additions would reduce labile C availability and microbial biomass, and that N fertilization would decrease lignolytic enzyme activity. At the same time, we predicted that both experimental manipulations would enhance cellulolytic enzyme activity and would shift microbial community composition toward bacterial dominance. Further, we predicted that the combination of warming and N additions would negatively impact soils, either additively or synergistically. Finally, we expected that the effect of warming and / or N additions on soils would vary throughout the year, impacting the microbial community most when is already stressed biochemically (*e.g.*, by nutrient shortages) and / or physically (*e.g.*, by freeze-thaw).

Methods

Site description and experimental design

This research took place at the Soil Warming × Nitrogen Addition Study, which has been described in detail by Contosta *et al.* (in press). It is located at the Harvard Forest Long Term Ecological Research site in Petersham, Massachusetts (42°50' N, 72°18'W). The forest in the study area is comprised of even-aged, mixed hardwoods. Soils are of the Gloucester series (fine loamy, mixed, mesic, Typic Dystrochrepts; Peterjohn *et al.* 1994). Mean annual air temperature is 7°C, with summer temperatures as high as 32°C and winter as low as -25°C. Average total annual precipitation, including water equivalent of snow, is 1100 mm (Boose *et al.* 2002). The experimental design consists of twenty-four, 3 x 3 m plots randomly assigned to one of four treatments: control, N addition (N), warming (W), and warming × N (WN). Average soil temperature in the heated plots (W and WN) was continuously elevated 5°C above ambient using heating cables placed at 10 cm depth. Nitrogen fertilization in the N and WN plots occurred as equal doses of NH_4NO_3 applied throughout the growing season at a rate of 5 g N m^{-2} y^{-1}.

Soil sampling

Soils were sampled four times in 2008: during January or February, April, July, and October. These dates were chosen to coincide with environmental conditions typical of winter, spring, summer, and autumn in forests of the northeastern US. After brushing aside the litter layer, two 8 cm wide by 10 cm deep cores were removed from each plot, separated into mineral and organic fractions, and bulked by soil horizon. Two more soil cores previously taken from an adjacent area were used to back-fill the holes created by

the sampling and marked with the date to prevent re-sampling in that location. During the growing season, sampling occurred at least two weeks following fertilizer application. In winter, a narrow hole was dug into the snowpack to access the soil. This hole was refilled with snow following soil sampling. The cores were transported back to the University of New Hampshire, passed through a 2mm sieve to remove rocks and roots, and stored at 4°C until analysis.

Labile C availability

Labile C was assessed by incubating 10 g field fresh mineral soil in plastic cups nested inside quart-sized mason jars. Soils were stored for approximately one week prior to the incubation. Soil moisture was adjusted to field capacity. Then 1 ml of water was added to the bottom of each jar to ensure that samples remained moist throughout the 30 day incubation, during which they were stored at 20°C. Carbon dioxide concentrations were measured every two to four days using a LI-COR LI-6252 infrared gas analyzer (LI-COR Biosciences, Lincoln, Nebraska, USA), after which jars were flushed with ambient air and resealed. The labile C fraction was calculated as the total CO_2 respired over the 30 day period ($\mu g\ C\ g^{-1}$ soil).

Extracellular enzyme activity

The activities of β-1,4-glucosidase and phenol peroxidase, representing cellulose and lignin degrading enzymes, respectively (Sinsabaugh *et al.* 1992), were assessed for the mineral soil. Enzyme assays occurred on field fresh soils within 48 hours of sampling and were based on the methods outlined by Marx *et al.* (2001), Saiya-Cork *et al.* (2002),

and DeForest *et al.* (2009). Briefly, 10 g of soil was added to 500 ml of 0.5 mM sodium acetate buffer (pH 4.5) and mixed on high speed in a blender for one minute. The resulting slurry was poured into a ceramic bowl and stirred on low speed while pipetting aliquots into 96-well, 300 µl microplates. The addition of soil slurries, substrates, quench standards, reference standards, and blanks followed the same order and occurred in the same configuration as described by DeForest (2009). A 200 µM solution of 4-methylumbelliferyl (MUB)-β-D-glucopyranoside was used as the substrate for β-glucosidase, and a combination of 25 mM L-3,4-dihydroxyphenylalanine and 0.3% hydrogen peroxide was the substrate for phenol peroxidase. After adding substrates, plates were incubated in the dark for two hours in the case of β-glucosidase and four hours for phenol peroxidase. In order to measure enzyme activity at *in situ* temperatures, incubations were conducted simultaneously at 0, 15, and 25°C instead of the 20°C typical for many enzyme studies. This configuration allowed for the measurement of enzyme activity at seasonally optimal temperatures (*e.g.*, Fenner *et al.* 2005), as well as the comparison of activity across sampling dates. Following incubation, a Biotek Synergy HT microplate reader (Winooski, Vermont, USA) was used to quantify absorbance for the peroxidase plates at 450 nm and fluorescence for the β-glucosidase plates using 365 nm excitation and 450 emission filters.

Raw fluorescence and absorbance readings were converted into β-glucosidase (nmol h^{-1} g^{-1} soil) and phenol peroxidase (µmol h^{-1} g^{-1} soil) activities with the equations outlined in DeForest (2009). Instead of using the emission coefficient (*i.e.*, fluorescence of 10 µM 4-MUB divided by 0.5 nmol), a standard line was constructed and validated with solutions between 1 and 10 µM 4-MUB. During method optimization, similar

standards were attempted for oxidative enzymes using horseradish peroxidase, but these were found to be neither reliable nor repeatable. As a result, the 7.9 μmol^{-1} extinction coefficient applied in many other studies (*e.g.*, Saiya-Cork *et al.* 2002, Gallo *et al.* 2004, DeForest *et al.* 2009) was used in determining peroxidase activity. In addition, phenol peroxidase was considered to be a combination of both phenol oxidase and peroxidase, instead of subtracting phenol oxidase from phenol peroxidase to obtain rates of peroxidase alone.

Microbial biomass and community composition

Microbial biomass and community composition was measured in both the organic horizon and mineral soil using phospholipid fatty acid analysis (PLFA). For each sampling date, PLFA was performed on the same subset of plots: three from each experimental treatment for a total of 12 plots and 24 samples (mineral and organic horizons). Sieved, root-free soil was freeze-dried within 24 h of sampling, and was stored at -80°C until analysis. Microbial lipids were extracted from one gram of the freeze-dried soil using a modified Bligh and Dyer (1959) procedure (White *et al.* 1979, Guckert *et al.* 1985) in which a single phase solvent system (chloroform) was adapted to include a phosphate buffer. This modification allowed for lipid extraction from only viable microorganisms captured at the time of sampling. Lipid extracts were then fractionated on silicic acid columns into neutral, glyco- and polar lipids. Only polar lipids were collected and then methylated with 0.2 M methanolic KOH to form fatty acid methyl esters (FAMEs). Purified FAMEs were then brought to volume with hexane containing an internal FAME standard, $C_{19:0}$, and were injected into a Varian 3800 gas

chromatograph (Varian, Inc, Santa Clara, California, USA) equipped with an FID detector. Chromatographic peaks of individual FAMEs were identified, quantified, and converted to units of nmol g^{-1} soil with the use of the $C_{19:0}$ internal standard. Additional confirmation of FAME peak identities by double bond and mid-chain branching positions was obtained by GC-mass spectrometry (GC-MS) by the commercial laboratory, Microbial Insights (Microbial Insights, Inc., Rockford, Tennessee, USA).

The polyenoic unsaturated fatty acids, 18:2ω6 and 18:1ω9c, were considered fungal biomarkers (Bardgett *et al.* 1996, Bååth, 2003). Gram positive fatty acids included the branched, saturated markers i15:0, a15:0, i16:0, i17:0, a17:0, and10Me16:0. Gram negative fatty acids consisted of the monoenoic and cyclopropane unsaturated markers cy17:0, 18:1ω7c and cy19:0 (Ekelund *et al.* 2003). Along with these gram positive and gram negative makers, the remaining fatty acids of 15:0, 16:1ω7c, and 16:1ω7t, or general bacterial markers, completed the bacterial assessment (Leckie *et al.* 2004). Total microbial biomass was estimated by summing the concentrations (nmol g^{-1}) of all fatty acids measured. Fungal biomass was determined by adding the fungal markers, and bacterial biomass was calculating by totaling all of the bacterial ones. While this method for estimating microbial biomass does not directly measure soil microbial biomass, it is able to capture the relative trends in fungal and bacterial abundance among treatments and seasons (Zelles *et al.* 1997).

Statistical analysis

All statistical analyses were conducted in R 2.9.2 (R Core Development Team 2009). We used a mixed model approach with the *nlme* package (Pinhiero *et al.* 2009) to

examine differences among experimental treatments and across seasons. Each dependent variable (labile C, enzyme activity, total microbial biomass, fungal biomass, bacterial biomass, and the fungal to bacterial, or F:B, ratio) was log-transformed to meet the normality and homoscedasticity assumptions of the mixed-effects model. Fixed effects included the warming and N addition treatments and their interaction with one another and with season. Soil horizon was included as a fixed effect for variables that were measured in both the organic horizon and mineral soil: total microbial biomass, fungal biomass, bacterial biomass, and the F:B ratio. Incubation temperature was also a fixed effect when comparing extracellular enzyme activity among treatments and seasons. Season was defined as winter, spring, summer, or autumn, which were the four 2008 sampling dates from which the data were derived.

Random effects were chosen by comparing models constructed with no random component, a random intercept (plot), and a random intercept (plot) plus a random slope (warming and/or N additions). For the variables fungal biomass, bacterial biomass, and the F:B ratio, soil horizon was nested within plot as part of the random intercept. Optimal random effects, autocorrelation, and variance structures were selected using the protocol recommended by Zuur *et al.* (2009) and described by Contosta *et al.* (in press). All statistical tests were performed at $\alpha = 0.05$ level. If neither variance covariates nor autocorrelation structures improved model fit, then the data were considered to have met the assumptions of equal variance and equal correlation necessary for linear modeling (Littel *et al.* 1998).

A backward selection procedure was subsequently used to examine the significance of three-way interactions between warming, N additions, and season as per

Zuur *et al.* (2009). First, a model containing all three fixed effects and their interactions was fit (*i.e.*, warming + N + season + warming × N × season). A second model was then fit that did not contain the three way interaction, but instead included all three main effects and two-way interactions between them (warming + N + season + warming × N + warming × season + N × season). Both the first and second models were fit using maximum likelihood (ML) as the estimation method. Using the *anova* command, the two models were tested against one another with the likelihood ratio (LR) test. If P was < 0.05, then the three-way interaction was considered significant and was kept in the model. If the three-way interaction was not significant, then it was removed. The significance of the remaining two-way interaction terms was determined by omitting each of the two-way interactions in turn, and comparing the each of the reduced models to the one containing all the two way interactions. Again, any two-way interactions that were not significant were removed. If the resulting model retained any two-way interactions, they were re-evaluated by removing them from the model, refitting it, and performing the LR test between the model containing the interactions and the one without them. The significance of interaction terms are reported in the results section as P-values from the LR tests.

Once all interactions had been tested, the significance of main effects in the new, full model was determined by examining t-statistics and P-values for all fixed effects using the *summary* command (Zuur *et al.* 2009, Contosta *et al.* in press). Any main effects that: 1) were not included in a significant interaction and 2) were not significant at $P < 0.05$ on their own were removed from the model. Again, the reduced model was compared to the new, full model with the LR test. Once the final model was chosen, it

was refit with REML for reporting significant differences between group means. Model verification consisted of visually inspecting residuals for normality and homoscedasticity. *P*-values and *t* statistics that describe differences between group means from the final model were obtained with the *summary* command. These *P*-values were relative to baseline, or model intercept, which was either the control treatment or the control treatment in winter if there was a significant treatment × season interaction in the model. Thus a significant *P*-value for a specific treatment × season interaction (*e.g.*, N × April) indicated that the effect of N additions in January differed from the effect of N inputs April, not that the effect of N additions was significant in April. To evaluate differences between control and treated plots during specific months (*i.e.*, pairwise differences), the final model was refit without an intercept, and 95% confidence intervals (CI) were calculated from the resulting estimated group means and standard errors. A lack of overlap in the 95% CI indicated a significant difference between two means.

Changes in microbial community composition, as represented by PLFA biomarkers, were assessed with a combination of spatial ordinations and permutation tests of community dissimilarity. All multivariate analyses were conducted with the *vegan* package in R (Oksanen *et al.* 2010) and were performed separately for organic and mineral horizons. Data were mole percentages, or relative abundances, of each marker relative to total PLFA and were arranged in a matrix with relative abundances as columns and samples as rows. Non-metric multidimensional scaling was done using the *metaMDS* function (Oksanen *et al.* 2010) and the Gower dissimilarity index of the PLFA data matrix. The dimensionality of the NMDS was chosen by adding dimensions

(starting with one) to the *metaMDS* ordination until they ceased to reduce the stress (or "badness-of-fit") statistic.

Following NMDS, the effects of warming, N additions, and season on community dissimilarity were examined by superimposing these factors onto the ordination using the *envfit* function. This projection allowed for the scores of each dimension of the NMDS to be averaged for each factor. Then a permutation test of 999 randomized trials was performed to determine whether differences among mean NMDS scores were greater than by chance alone. A P value ≤ 0.05 indicated a significant difference in community composition. Since this process only tested main effects (warming, N additions, season), a non-parametric MANOVA (*e.g.*, Anderson 2001) was subsequently applied that included both main effects and interactions (warming × N, warming × season, N additions × season, warming × N × season). Called *adonis* in R, this test also relied on the permutations of observations to examine differences in community composition. Again, dissimilarity was determined using the Gower metric, and 999 permutations were run to obtain significance values. Since this test is especially sensitive to differences in dispersion (Anderson 2001), post-hoc comparisons of among-group heterogeneity were examined for each main effect using the *betadisper* function, which calculated the average distance of each group to its centroid.

Results

Mixed effects model selection parameters and final model output for labile C, enzyme activity, and total, fungal, and bacterial biomass are located in Appendix B.

Labile C availability varied throughout the year and with N additions (Figure 3.1, Appendix B1). In the control plots, it was lowest in January, increased in April and July, and reached a maximum in October (October: $t = 6.40$, $P < 0.0001$). Labile C in the N addition plots slightly deviated from this trend, decreasing from January through July, and then peaking in October. There was no effect of warming or warming × N on labile C. By contrast, N additions altered labile C availability, though the effect of N on labile C differed among seasons (N × season: $L = 7.64$, $P = 0.05$). The N × season interaction indicated that N additions increased labile C by 43% in January. By contrast, N fertilization reduced labile C by 18% in April, 21% in July, and 3% in October. Pairwise differences in labile C availability between control and N addition treatments, as indicated by estimated means and 95% CIs, indicated that the increase in labile C in January was marginally significant, but that the decreases in April, July, and October were not.

Extracellular enzyme activity also shifted seasonally (Figures 3.2 and3.3, Appendices B2 and B3). In the case of β-glucosidase, activity was significantly lower in February and April than it was in July and October (February: $t = 12.86$, $P < 0.0001$; April: $t = -5.50$, $P < 0.0001$). However, activity did not change with warming, N additions, or temperature, nor did interactions among these parameters significantly affect β-glucosidase ($P > 0.05$). By contrast, peroxidase activity varied with season, temperature, and warming. Seasonal patterns in peroxidase were linked to incubation temperature (season × temperature: $L = 22.46$, $P < 0.0001$). Peroxidase activity was generally highest when incubated at 25°C, intermediate at 15 °C and lowest at 0 °C (incubation temperature: $t = 6.61$, $P < 0.0001$), though rates at 0 and 25°C were similar in

October. Across all temperatures, peroxidase rates were highest in April compared to the other three months during which activity was measured. Peroxidase was also higher in April (t = -3.56, $P < 0.0001$), July (t = -4.38, $P < 0.0001$) and October (t = -4.53, $P < 0.0001$) as compared to rates in February. In addition, peroxidase showed small but significant changes with warming, though the effect of warming was dependent on incubation temperature (warming × temperature: $L = 5.99$, $P = 0.01$).

The soil microbial community shifted seasonally and with experimental treatment (Figures 3.4 and 3.5, Appendices B4-B11). Because differences were influenced by soil horizon ($P < 0.05$ for treatment × season × horizon interactions), microbial community data were examined separately for the O-horizon and mineral soil. In the O-horizon, total microbial biomass gradually increased from January through July in the control plots, and then reached an annual maximum in October ($t = 3.38$, $P = 0.002$, Figure 3.4, Appendix B4). Both warming and N additions, alone and in combination, decreased total biomass. The effect of warming, N, and warming × N on total biomass significantly interacted with season ($L = 12.39$, $P = 0.006$). According to pairwise differences in the 95% CI for the three-way interaction, N additions reduced total biomass by 82% in October. Warming marginally decreased total biomass by 64% in July and by 68% in October. The combination of warming × N reduced total biomass by 71% in October only. Since this value fell between the reduction for warming only and N additions only, it seemed that warming moderated the effect of N additions on total biomass.

Fungal biomass in the O-horizon followed the same seasonal trajectory as total biomass, showing the lowest values in January and April, increasing in July, and peaking in October. However, these seasonal differences were not significant ($P > 0.05$, Figure

3.4, Appendix B5). Warming and / or N additions affected fungal biomass differently throughout the year, as indicated by the significant three-way interaction for warming × N x season ($L = 9.06$, $P = 0.03$). Nitrogen additions reduced fungal biomass by 92% in October (95% CI). Both warming and warming × N also lowered fungal biomass during this month, but since estimated 95% CIs overlapped between control and manipulated plots, these differences were not significant.

Like total and fungal biomass, bacterial biomass in the O-horizon was highest in October ($t = 3.95$, $P = 0.0004$, Figure 3.4, Appendix B6). Warming, N additions, and the interaction between the two, tended to reduce bacterial biomass. The effect of the experimental manipulations differed among seasons, as evidenced by the significant, three-way interaction for warming × N × season ($L = 12.44$, $P = 0.006$). In October, bacterial biomass was 83% lower in the N addition treatment (95% CI) and was 67% lower in the warming only plots, though this reduction was not significant (95% CI). As with total biomass, the 72% reduction in the warming × N treatment (95% CI) fell between that of the N addition only and the warming only plots, suggesting an annulling effect of warming on N inputs.

The ratio of fungi to bacteria (F:B) in the O-horizon was highest in January, decreased in April ($t = -3.26$, $P = 0.002$), recovered in July ($t = -0.85$, $P = 0.37$), and then reached its annual minimum in October ($t = -4.09$, $P = 0.0002$, Figure 3.5, Appendix B7). Warming reduced F:B by 11% ($t = -2.36$, $P = 0.02$), and this decrease was independent of season (warming × season: $P > 0.05$). Nitrogen additions, either alone or in combination with warming, had no effect on the F:B in the organic soil.

In the mineral horizon, total biomass in the control plots was highest in January and October and was lowest in July (t = -2.55, P = 0.01, Figure 3.4, Appendix B8). Neither N additions nor warming × N had an effect on total biomass in the mineral horizon. Warming alone reduced fungal biomass, and this effect was seasonally dependent (L = 9.61, P = 0.02). Pairwise comparisons of 95% CIs in the warming × season model indicated that warming marginally reduced total biomass by 34% in January but marginally increased biomass by 42% in July.

Fungal biomass in the mineral horizon showed a similar seasonal trajectory as total biomass, with lower values in July as compared to the rest of the year (July: t = -3.17, P = 0.003, Figure 3.4, Appendix B9). Nitrogen additions, either alone or in combination with warming, had no effect on fungal biomass. The impact of warming on fungal biomass was seasonally dependent (L = 10.28, P = 0.02), decreasing it by 40% in January (95% CI), and increasing it, though not significantly (95% CI) by 40% in July.

Like total and fungal biomass, bacterial biomass in the mineral horizon decreased from January through April to reach a minimum value in July (July: t = -2.30, P = 0.03, Figure 3.4, Appendix B10). Nitrogen additions, either alone or in combination with warming, had no effect on bacterial biomass. However, the warming only treatment altered bacterial biomass, but this effect was season-dependent (L = 11.36, P = 0.01). Using the model with the warming × season interaction, pairwise comparisons of 95% CI estimates indicated that warming decreased bacterial biomass by 30% in January and marginally increased it by 68% in July.

The F:B ratio in the mineral horizon was highest in January across all treatments, and was significantly lower in April (t = -3.50, P = 0.001, Figure 3.5,

Appendix B11), July (t = -4.06, P = 0.0002), and October (t = -3.94, P = 0.0003). Both warming and N additions reduced the F:B ratio, and this effect was independent of season. Overall, warming reduced the F:B by 17% (P = 0.0002), and N additions lowered F:B by 10% (P = 0.03). Pairwise differences in the 95% CI indicate that the F:B in the warming only plots was lower than that of the N addition plots.

In the mineral fraction, NMDS resulted in an ordination with three dimensions and a stress statistic of 13.1. Ordination scores of PLFA biomarkers along the first axis indicated that the gram negative bacterial marker, 16:1ω7t, was responsible for most of the dissimilarity among observations, followed by the gram positive i16:0 and gram negative 16:1ω7c markers. Fitting the effects of warming, N additions, and season onto the ordination showed a significant effect of season (r^2 = 0.39, P = 0.001), but no relationship between community composition and experimental treatment (r^2 = 0.03, P = 0.86). The non-parametric MANOVA produced slightly different results, with community composition changing both among seasons (F = 7.60, P = 0.001) and with warming (F = 2.78, P = 0.03). Visual inspection of the dissimilarity among treatments showed that the largest differentiation between heated and unheated plots occurred along the second axis. Here the biomarkers most responsible for driving the difference were 15:0, 18:2ω6, and 18:1ω7c. Post-hoc comparisons of among group heterogeneity suggested equal multivariate dispersion between heated and unheated plots and among seasons. There were no effects of N additions, either alone or interacting with warming and / or season (P > 0.05).

Discussion

We hypothesized that peak labile C availability would coincide with summer inputs of plant photosynthate and autumn inputs of plant litter and litter leachate. While labile C was slightly higher in July as compared to January, it reached its maximum concentration in October only. This finding coincides with Bohlen *et al.* (2001) and Bradford *et al.* (2008), both of whom reported the largest pools of easily mineralizable C in autumn in temperate, deciduous forests. We also expected that seasonal shifts in labile C availability would interact with the warming and N addition treatments. As predicted, N additions impacted labile C availability differently throughout the year. The smallest decline occurred in October, when the influx of plant litter and litter leachate likely overwhelmed the negative impact of N additions on labile C supplies. Nitrogen additions also reduced labile C by 21% in July, which was comparable to the 20-30% reductions that Frey *et al.* (2004) cited for the Harvard Forest Chronic N Addition experiment. This was surprising given our prediction that N additions would have the greatest effect on labile C availability when it was most scarce, *i.e.*, in January and April. In fact, the largest effect of N additions on labile C availability occurred in January, where N amendments increased labile C 43% relative to the controls. Seasonally divergent effects of N additions on labile C availability may account for this disparate response. During winter, elevated N inputs may have allowed microbes to access C resources that were previously unavailable, augmenting the "labile" C pool. During summer, microbial uptake of N fertilizer may have consumed recent plant photosynthate to balance C:N ratios, drawing down the labile fraction.

Unlike the N addition plots, we did not see significant reductions in labile C in the heated treatment, regardless of season. Depletion of labile C due to soil warming was hypothesized as one of the explanations for long-term decreases in soil respiration in experimentally heated plots (Luo *et al.* 2001, Melillo *et al.* 2002), and has been validated by several researchers (Bradford *et al.* 2008, Frey *et al.* 2008). It may be that it takes longer than two years of warming to detect substantial changes in labile C, and indeed both the Bradford *et al.* (2008) and Frey *et al.* (2008) studies occurred following more than ten years of elevated temperatures. The slightly lower labile C availability we noted in heated plots during July and October suggests that soils are beginning to lose C, and may eventually show the larger deficits documented for other studies.

Like labile C availability, we predicted that seasonal changes in extracellular enzyme activity would, in part, follow patterns in plant available C. We based this hypothesis on reports of maximum annual rates of soil phenol mineralization in October and February (Schmidt *et al.* 2004) and of 60-90% loss of cellulose and phenol in leaf litter between autumn and the following spring (Moore 1983, Uchida *et al.* 2005). In concert with our hypotheses, β-glucosidase activity was highest in October and was lowest in April, perhaps due to a depletion of cellulose. The maximum rates of peroxidase activity in April suggest that unlignified cellulose had indeed been consumed during the winter.

In addition, we expected that β-glucosidase activity would be stimulated in N addition plots, particularly in fall and winter when cellulose resources would be most available. We also predicted that N fertilization would suppress peroxidase activity, especially in summer when recalcitrant substances would be in shortest supply.

However, N additions had no effect on enzyme activity, which differs from reports of enhanced β-glucosidase and suppressed peroxidase in chronically fertilized soils (Saiya-Cork *et al.* 2002, Frey *et al.* 2004, Sinsabaugh *et al.* 2005). However, the Saiya-Cork *et al.* (2002), Frey *et al.* (2004), and Sinsabaugh *et al.* (2005) studies occurred following three to ten years of N fertilization, and thus represent a longer-term response to elevated N inputs. By contrast, one to three years of fertilizer application has not shown significant shifts in either soil β-glucosidase or peroxidase activity (Gallo *et al.* 2004, Waldrop *et al.* 2004*a*, Waldrop *et al.* 2004*b*). Our study occurred following one to two years of N amendments, and displays the same lack of treatment effect as other investigations over the same time frame.

We anticipated that warming would have a slightly different effect on soil enzymes, accelerating β-glucosidase while having no effect on peroxidase activity. Both field and laboratory investigations of litter decay suggest higher rates of cellulose decomposition and no change in lignin degradation with elevated temperatures (Rustad and Fernandez 1998*b*, Osono and Takeda 2006). The lack of increase in β-glucosidase activity, either with soil warming or elevated incubation temperatures, contradicts the results of Koch *et al.* (2007) and Trasar-Cepeda *et al.* (2007). We also found that peroxidase showed a slight response to warming, which differs from reports of unchanged peroxidase in experimentally warmed plots (Waldrop and Firestone 2004, Allison and Treseder 2008, Bell *et al.* 2010). However, the effect of warming on peroxidase activity was dependent on the incubation temperature of the assay, and at any given incubation temperature (0, 15, 25°C), warming altered peroxidase rates no more than 8%. Much larger than the effect of warming *in situ* on peroxidase activity was that

of incubation temperature. With the exception of October, peroxidase activity was highest when incubated at 25°C and lowest when incubated at 0°C. The strong response to incubation temperature and the weak response to chronic, *in situ* warming suggests that temperature can accelerate peroxidase rates, but that two years of warming has not altered the pool size of the peroxidase enzyme in the soil.

Based on seasonal shifts in C quality, we postulated that bacterial biomass would be highest in summer and autumn, while total and fungal biomass, though high in autumn, would peak in winter. As predicted, both fungal biomass and bacterial biomass were higher in October than in April or July. Many other studies have reported peak microbial biomass in autumn as compared to spring and summer (Holmes and Zak 1994, Maxwell and Coleman 1994, Fisk and Schmidt 1996, Jaeger *et al.* 1999, Lipson *et al.* 1999), which may result from a simultaneous influx of labile C and decreased plant N demand (Maxwell and Coleman 1994, Wardle 1998, Jaeger *et al.* 1999). The peak in labile C availability and β-glucosidase activity in October in the current study suggests this scenario. However, other investigations of microbial biomass during the April through November field season have reported maximum biomass occurring in summer (Bradford *et al.* 2008, Bohlen *et al.* 2001). Environmental factors such as soil N availability and soil moisture may explain discrepancies between our data and those of other investigations, since both can influence temporal patterns of microbial biomass (Wardle 1998).

As hypothesized, both total microbial biomass and fungal biomass were also higher in winter than they were in summer, though only in the mineral horizon. Microbial biomass may have remained low in the O horizon because it was closer to the

soil surface and thus was less insulated from freeze-thaw. During winter sampling, we often observed ice lenses in the O-horizon. Nevertheless, both the mineral and the organic fractions showed significantly higher F:B ratios in January as compared to the rest of the year. The higher fungal biomass in winter in the mineral fraction and the higher F:B in both horizons suggest that fungi are relatively more dominant underneath snowpack in the Harvard Forest plots, a finding that has been reported for winter soils in alpine regions (Lipson *et al.* 1999, Schadt *et al.* 2003) and overwintering leaf litter in temperate, deciduous forests (Uchida *et al.* 2005). Because fungal biomass did not significantly differ among sampling dates, this relative fungal dominance in winter appears to be the result of a seasonally shifting bacterial community less able to compete with fungi when labile C is scarce (Lipson *et al.* 2002).

In addition to these seasonal patterns in fungal and bacterial biomass, the relative abundance of PLFA biomarkers changed during October for both the mineral and organic fractions. The general bacterial marker, 16:1ω7t, and the gram positive marker, i16:0, explained most of the variation in community composition among sampling dates, increasing in relative abundance in both horizons. These increases occurred as the fungal marker 18:2ω6 decreased in the O-horizon, while the general bacterial marker, 16:1ω7c, decreased in the mineral horizon. Other researchers have also reported shifts in PLFA among sampling dates (Gallo *et al.* 2004, Moore-Krucera and Dick 2008), though the specific markers causing the change in community composition were not reported. It is not clear whether the changes we observed in community composition carry any ecological significance, though the decline in the 18:2ω6 marker in the organic fraction could help to explain the low F:B ratio observed for that horizon during autumn.

As hypothesized, N fertilization suppressed total, fungal, and bacterial biomass, though only in October, and primarily in the organic fraction. Other studies have also reported lower microbial biomass in chronically fertilized soils (Thirukkumaran and Parkinson 2000, Compton *et al.* 2004, DeForest *et al.* 2004, Frey *et al.*, 2004, Schmidt *et al.* 2004), which may be due depleted labile C supplies (Frey *et al.* 2004). This could have been the case in the current study, since N additions reduced labile C in April, July, and October. However, labile C availability reached its seasonal maximum in October, even in N treated plots. As a result, readily utilizable C may not have limited microbial growth in N addition plots at this time. Alternatively, N additions may have had to accumulate over the entire growing season before adversely affecting the microbial community. Whether from depleted labile C or accretion of the N fertilizer, the magnitude of the effect of N additions on microbial biomass in October was surprising given that environmental conditions seemed optimal for microbial growth during this month. Data from this and other studies (Holmes and Zak 1994, Jaeger *et al.* 1999, Bohlen *et al.* 2001) suggest that soil C, N, and moisture are plentiful in autumn in temperate forests, and competition with aboveground plants for resources is low. Thus the large, negative impact of N additions on microbial biomass in autumn is noteworthy since both fungi and bacteria should be immobilizing N, decomposing litter, and accumulating biomass during this season.

In addition to reducing biomass overall, we predicted that N fertilization would select against fungi in favor of bacteria (*e.g.*, DeForest *et al.* 2004, Frey *et al.* 2004, Pietikäinen *et al.* 2005, Rinnan *et al.* 2007). Lower F:B ratios in the mineral soil of N amended plots supported this prediction, with potential ecosystem consequences. Fungi

are believed to be more responsible than bacteria for the decay of recalcitrant compounds in plant litter (Horwath 2007), such that a reduction in the F:B ratio could alter the dominant C utilization pathways in the system. Lower wintertime F:B ratios in N addition plots may be especially important in this regard. Both the current study and the findings of Schadt *et al.* (2003) indicate that fungi are relatively more dominant in winter under snowpacks. If decay of recalcitrant litter compounds is also highest in winter (Moore 1983, Schmidt *et al.* 2004, Uchida *et al.* 2005), then a decrease in wintertime F:B ratio may substantially alter soil C cycling.

Although N additions altered the ratio of fungi to bacteria, there was no shift in overall community composition as expressed by PLFA. This contrasts with the findings of Gallo *et al.* (2004) and Rinnan *et al.* (2007), both of whom reported significant changes in PLFA markers in N amended soils, but fits with the report of DeForest *et al.* (2004), who cited no change in the relative abundance of PLFAs in N-fertilized plots. The shift that Gallo *et al.* (2004) reported only occurred in plots that had received 80 kg NO_3 ha^{-1} y^{-1}–a fertilization rate that is 60% higher than the one in the current study. The ones that received a lower dose (30 kg NO_3 ha^{-1} y^{-1} in both DeForest *et al.* 2004, Gallo *et al.* 2004) showed no change in community composition. The duration of the study may also play a role; Rinnan *et al.* (2007) reported that soils receiving as much as 100 kg NH_4NO_3 ha^{-1} y^{-1} took 15 years to show significant changes in microbial community composition. Thus it may be that three years of fertilization at a rate of 5 g NH_4NO_3 m^{-2} $y^{-1}$is not enough to alter microbial community structure.

Unlike the N addition treatment, the effects of increased temperatures on soil microbial biomass were not limited to October. Consistent with the findings of several

other studies (Arnold *et al.* 1999, Waldrop *et al.* 2006, Rinnan *et al.* 2007, Bradford *et al.* 2008, Frey *et al.* 2008), warming reduced total, fungal, and bacterial biomass in July and October in the organic fraction, and in January in the mineral horizon. Since warming did not significantly affect labile C availability, these decreases were not likely due to reduced labile C supplies. Lower soil moisture in July and October may have caused the lower biomass in the organic horizon of warmed plots (Rinnan *et al.* 2007). Moisture was between 12 to 26% lower in July and 25 to 33% lower in October in the warming and warming x N treatments as compared to unheated ones (Contosta *et al.* in press). However, reduced microbial biomass in January was not likely due to a moisture limitation, since moisture did not differ between heated and unheated plots during this time. Instead, lower biomass may be related to a higher temperature sensitivity of the microbial community to warming at low temperatures that drastically altered the physiological capacity of the community to utilize C (Monson *et al.* 2006*b*). In any case, reduced microbial biomass in winter is of interest given the importance of microbial decomposition and N immobilization during this season.

As with the N addition plots, soil warming reduced the F:B ratio in both the organic and mineral horizons across all sampling dates. The F:B ratio in the mineral soil of the warming plots was also significantly lower than it was in the N addition treatment, suggesting that the shift toward bacterial dominance is more likely with warming than with N additions. Further, warming shifted the relative abundance of all PLFA markers in the mineral soil. Zogg *et al.* (1997) reported a broad-spectrum shift in microbial community composition under elevated temperature incubations, where biomarkers for several gram-negative bacteria decreased with rising temperatures. Likewise, Frey *et al.*

(2008) showed a shift in the microbial community fingerprint toward gram-positive bacteria and actinomycetes at a long-term warming study adjacent to our experimental plots. In the case of the current study, there was no overall trend toward gram positive or gram negative bacteria. The markers 15:0, 18:1ω7c, and 18:2ω6 that were most responsible for the shift in community composition represent each of three types of fatty acids: gram positive, gram negative, and fungal, respectively. However, the relative abundance of the 18:2ω6 fungal marker was 7% lower in the warming only and 19% lower in the warming x N plots compared to the controls, again suggesting a shift in the warming treatment toward greater bacterial dominance.

Significant interactions between warming × N occurred only in October and only in the O horizon. That the interaction was limited to October was not surprising since the impacts of N additions on microbial biomass only occurred during that month. Contrary to our hypothesis, the interaction was an annulling one, where warming dampened the adverse effect of N additions on microbial biomass. This result was curious since warming alone suppressed total, fungal, and bacterial biomass, making it logical to deduce that the combination of warming × N would reduce biomass at least as much as each treatment had on its own. However, increased temperatures may have stimulated the ecosystem in ways that helped to moderate the large reductions in biomass in the N addition plots. Heating in our experiment has resulted in elevated rates of soil respiration, suggesting that warming enhances the activity of roots, rhizosophere and / or free-living microbes even as it suppresses microbial biomass (Contosta *et al.* in press). In any case, the unexpected annulling effect of warming on N fertilization suggests that these two global changes interacted in ways that would be difficult to predict using

single-factor experiments. Further, the treatment × date interaction highlights the role that season played in mediating the microbial response to environmental disturbance. Failure to examine such interactions could distort our view of how warming and N additions impact soil microbial communities.

Conclusion

The purpose of this study was to examine the interactive effects of warming and N additions on the soil microbial community with particular emphasis on the role that season played in mediating the response. We found that labile C availability, extracellular enzyme activity, microbial biomass, and microbial community structure were seasonally dynamic. Labile C, β-glucosidase, total biomass, and bacterial biomass reached their peak in autumn, while community composition shifted during this month. By contrast, the F:B ratio was highest in winter, suggesting that fungi can outcompete bacteria when labile C is scarce. We also detected some of the largest changes to the microbial community during these autumn and winter, with potential ecosystem consequences. Both warming and N inputs decreased the F:B ratio in winter, a season when fungi typically decompose cellulose and lignin in plant litter. In autumn, when microbes should be accumulating biomass, warming and N fertilization reduced total, fungal, and bacterial biomass between 50 and 90%. Interactions between the warming × N addition treatments were also seasonally dependent; warming alleviated the negative effect of N additions on microbial biomass only in October. This annulling effect was unexpected given that the warming only treatment also reduced microbial biomass, implying that the warming × N combination would be additive or multiplicative.

Overall, our findings demonstrate the importance of studying multiple drivers of change, both seasonal and anthropogenic, in order to understand how they impact soil microbial communities and the ecosystem processes they perform.

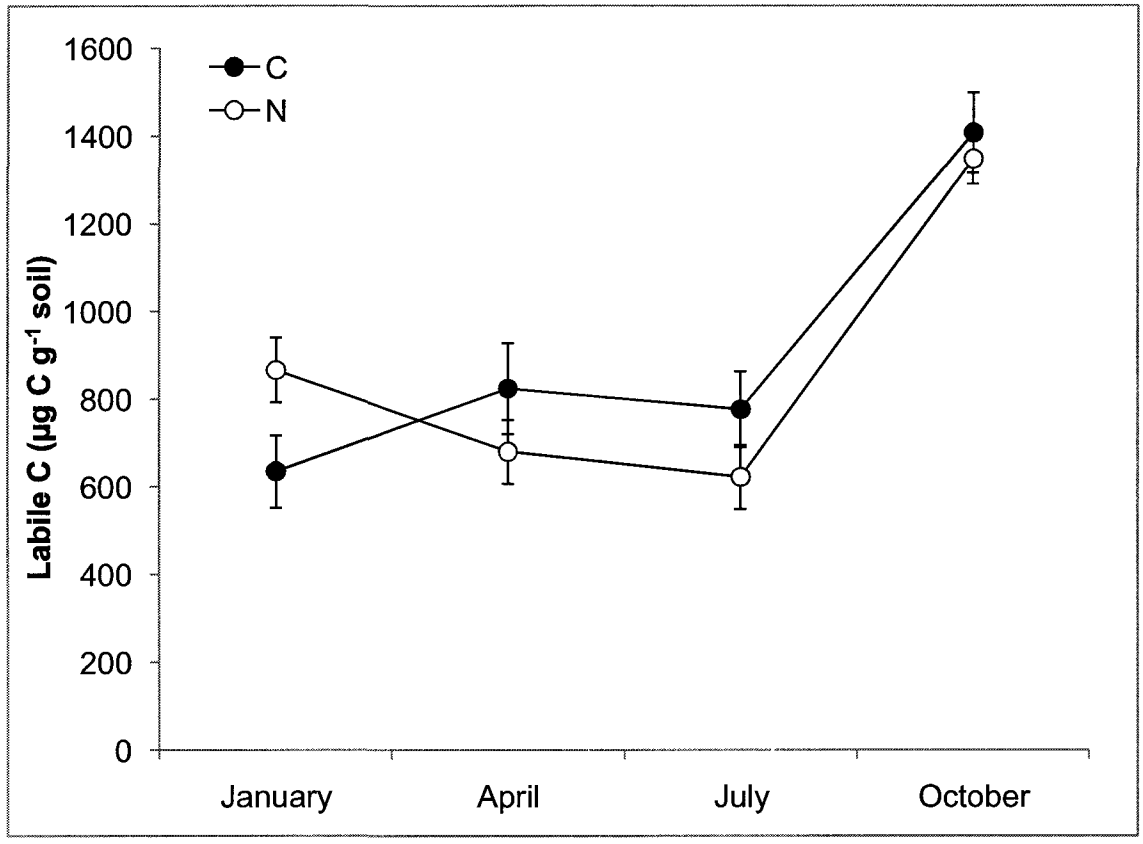

Figure 3.1. Seasonal trends in labile C availability in control (C) and N addition (N) plots. Data were pooled across warming treatments. Error bars represent ± 1 SE.

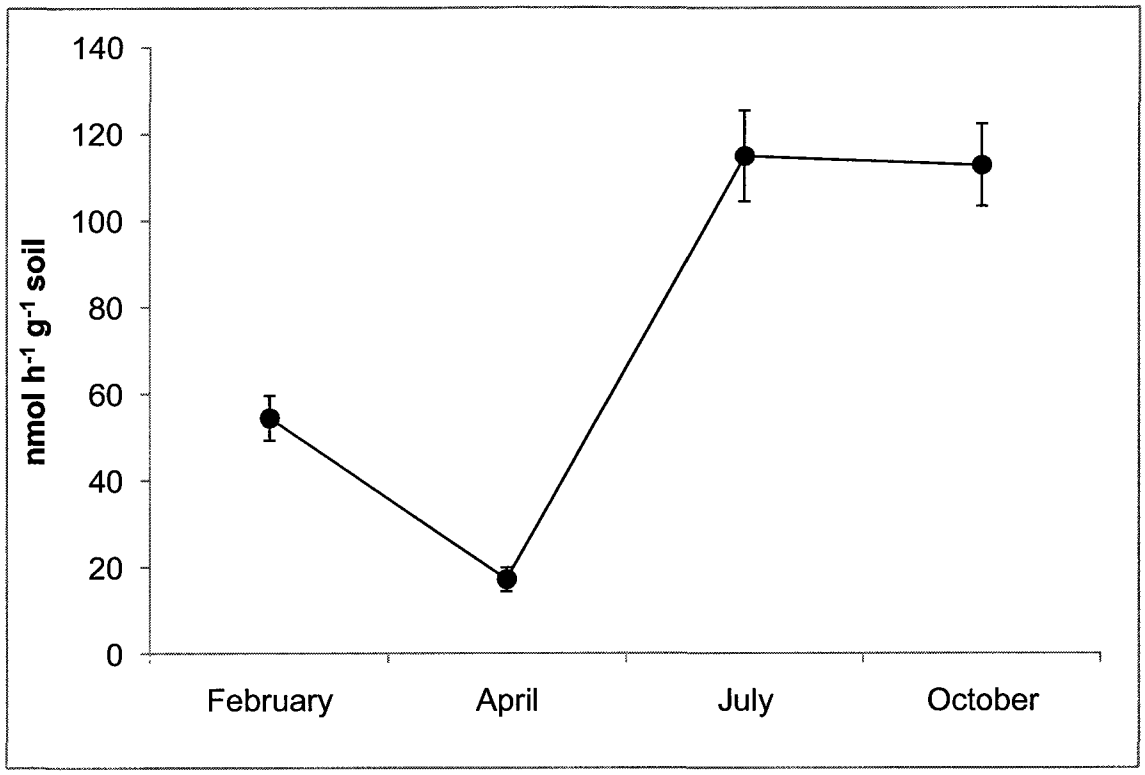

Figure 3.2. Seasonal trends in β-glucosidase activity (nmol h^{-1} g^{-1} soil). Data were pooled across incubation temperatures and treatments. Error bars represent ± 1 SE.

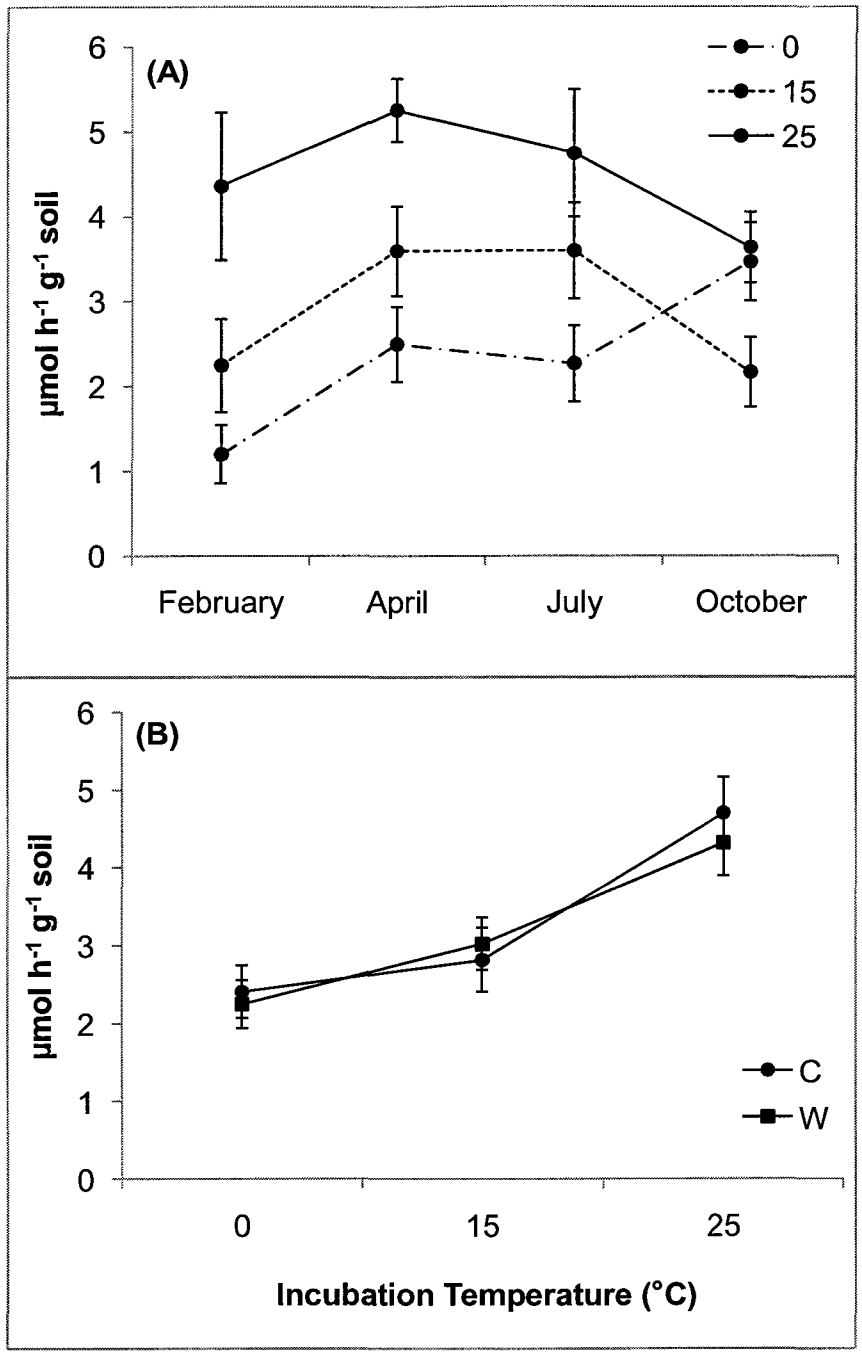

Figure 3.3. (A) Seasonal trends in phenol peroxidase activity at 0, 15, and 25°C. Data were pooled across treatments. The solid line indicates activity at 0°C, the line with small dashes activity at 15°C, and the line with large dashes activity at 25°C. (B) Phenol peroxidase activity in warmed and unwarmed plots at 0, 15, and 25°C. Data were pooled across seasons and N addition treatments. Solid circles represent unwarmed plots, and solid squares indicate warmed plots. Error bars represent ± 1 SE.

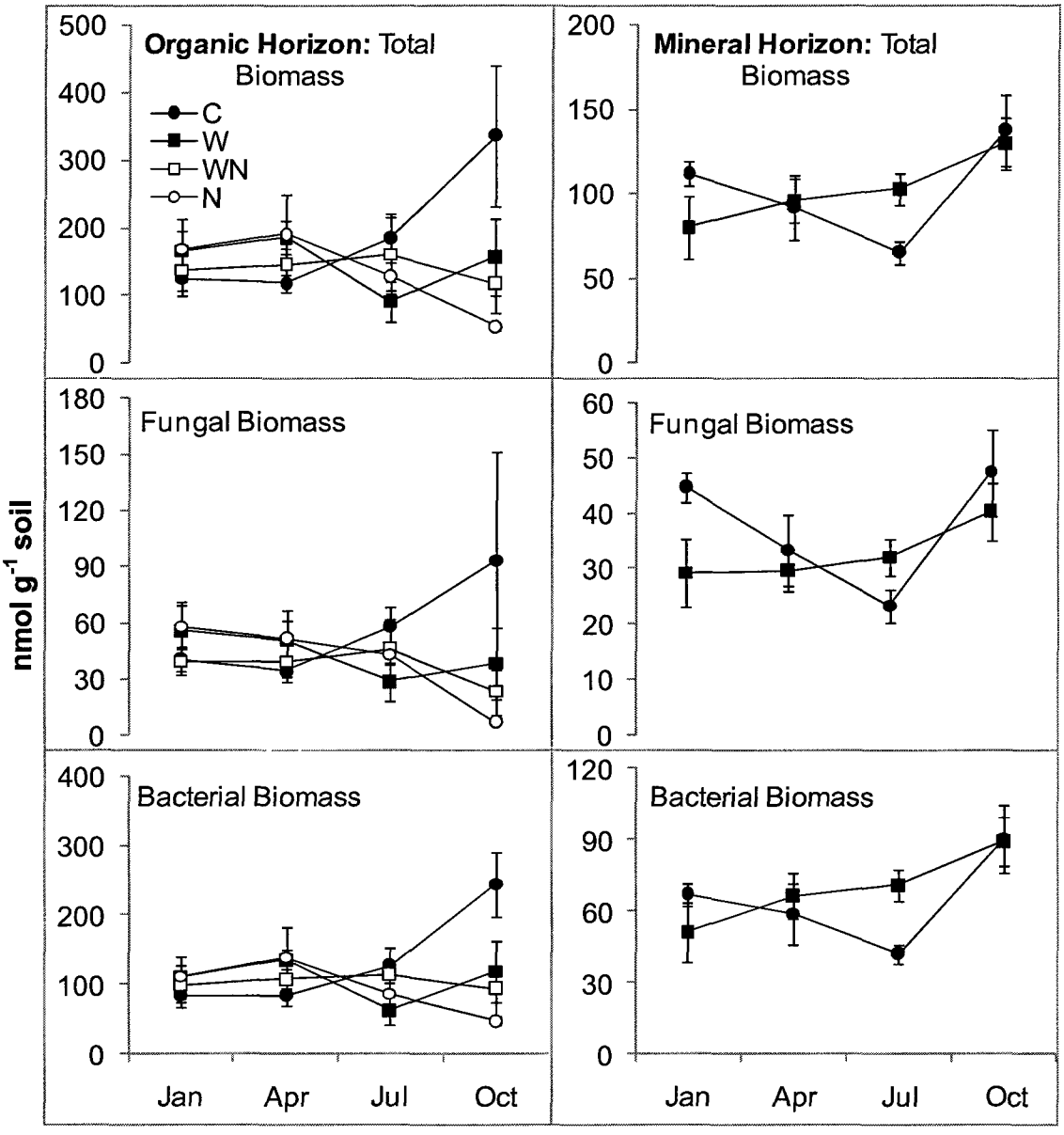

Figure 3.4. Seasonal trends in microbial biomass (nmol g^{-1} soil) in control (C), warming (W), warming × N (WN), and N addition (N) plots. Data were pooled across N addition treatments when N fertilization did not significantly affect microbial biomass. Error bars represent ± 1 SE.

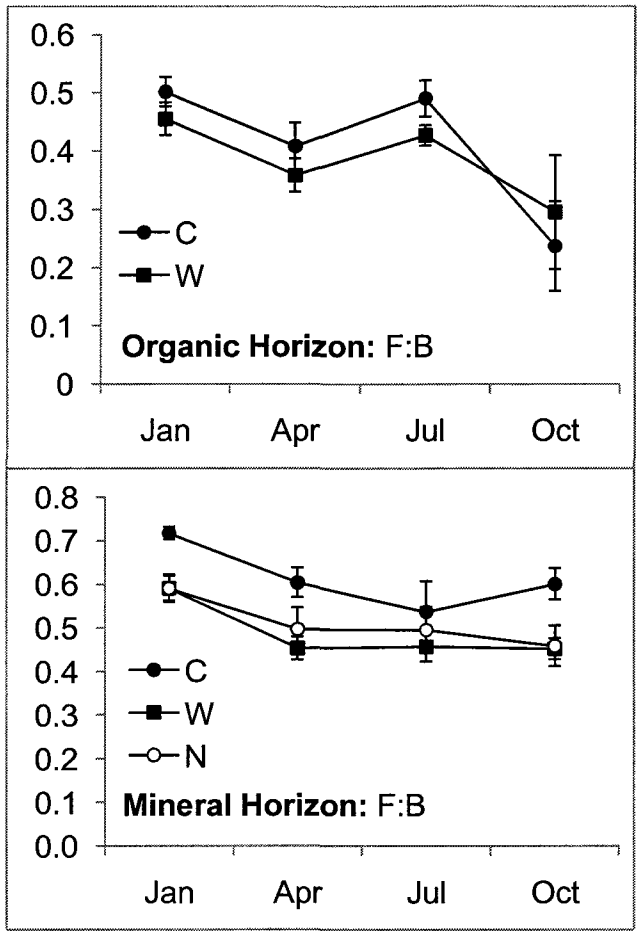

Figure 3.5. Seasonal trends in the F:B in control (C), warming (W), warming × N (WN), and N addition (N) plots. Data were pooled across N addition treatments when N fertilization did not significantly affect microbial biomass. Error bars represent ± 1 SE.

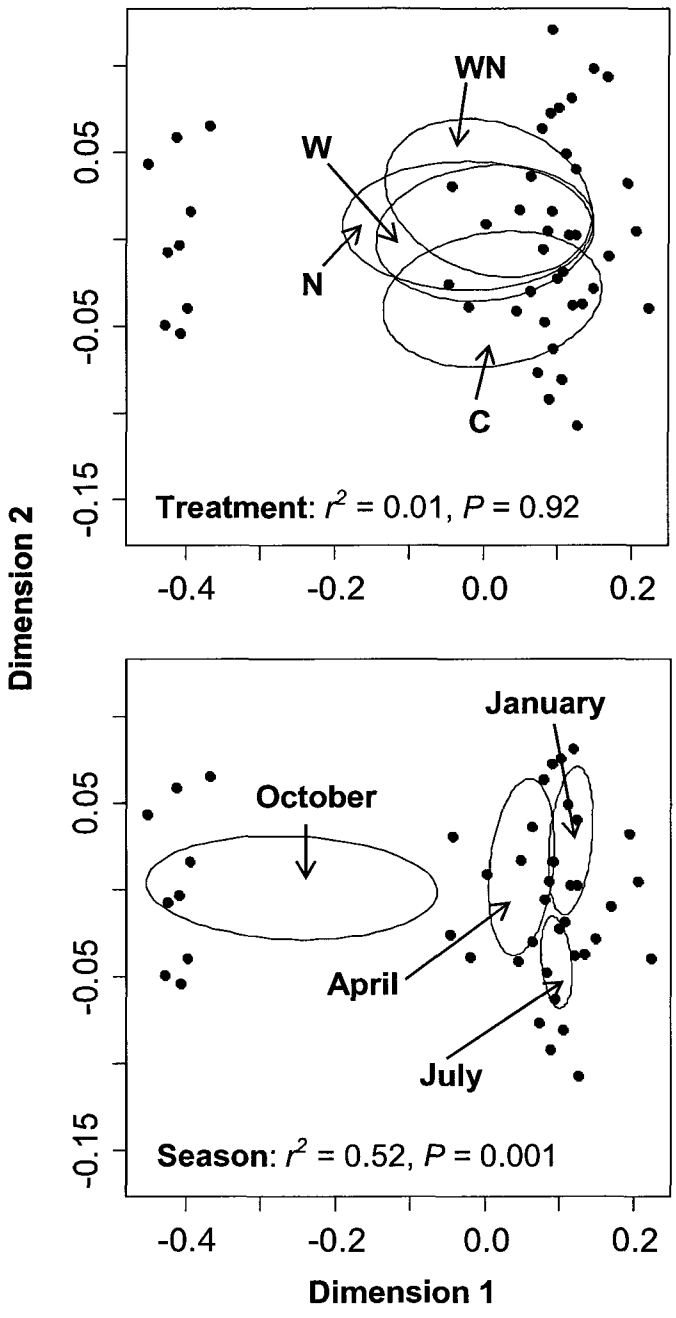

Figure 3.6. Spatial ordination plots showing microbial community dissimilarity for the organic soil horizon. Distances between points on a plot represent rank dissimilarity, not actual distances. Ellipses indicate the 95% confidence interval of the standard error of the mean for each grouping (treatment and season), and r^2 and P-values were generated by fitting these groupings onto the spatial ordination. For treatments, "C" indicates control, "W" warming, "WN" warming × N, and "N" N additions. Although season and experimental treatment were fit simultaneously for the permutation test, they are presented separately here for visual clarity.

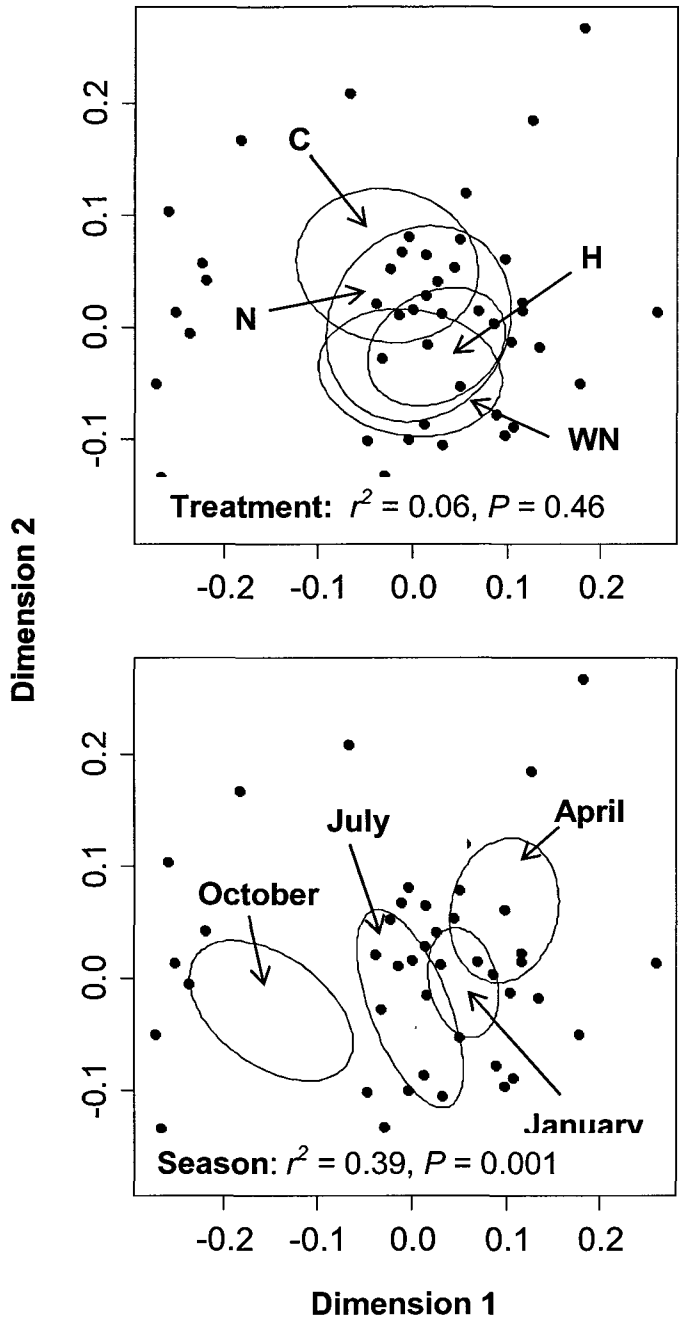

Figure 3.7. Spatial ordination plots showing microbial community dissimilarity for the mineral soil horizon. Distances between points on a plot represent rank dissimilarity, not actual distances. Ellipses indicate the 95% confidence interval of the standard error of the mean for each grouping (treatment and season), and r^2 and P-values were generated by fitting these groupings onto the spatial ordination. For treatments, "C" indicates control, "W" warming, "WN" warming × N, and "N" N additions. Although season and experimental treatment were fit simultaneously for the permutation test, they are presented separately here for visual clarity.

CHAPTER 4

SOIL WARMING AND NITROGEN ADDITIONS ALTER LITTER DECOMPOSITION WITHOUT CHANGING FUNGAL COMMUNITY STRUCTURE

Abstract

Fungal decomposition of lignin and cellulose is essential to soil carbon cycling given the abundance of these two compounds in plant litter. While many studies have investigated litter degradation with soil warming or N fertilization, few have examined how they interact to influence litter decay. Many researchers have also not explicitly quantified the fungal community responsible for lignocellulose decomposition. The goal of this work was to examine how soil warming and N additions, alone and together, influence the diversity and community composition of fungi colonizing woody litter. In addition, we studied how shifts in fungal community structure might be related to changes in wood decay with elevated temperatures and N inputs. The research took place in four global change experiments that have similarly manipulated soil temperature and N inputs but have been operating for different lengths of time. Nitrogen amendments suppressed decomposition, resulting in 53% more mass remaining in N fertilized plots than the controls. The effect of N additions on mass loss depended on the duration of the field study, with plots treated for 2 years having slower decay than plots manipulated for 20 years. Warming alone did not affect wood decay. By contrast, warming × N accelerated mass loss 28% beyond the control treatment, suggesting a synergistic interaction between the experimental manipulations. Except for lignin degradation, fungal community structure was generally not correlated with vectors of decay. Likewise, changes in wood

decomposition with warming and / or N additions were largely not linked to shifts in fungal community structure. However, reduced fungal diversity in warmed plots may have moderated the otherwise stimulatory effect of increase temperatures on decomposition. The overall lack of a relationship between fungal community structure and wood decomposition suggests that global change factors such as warming and N additions alter fungal activity without restructuring the community.

Introduction

Fungi are the primary decomposers of cellulose and lignin, two of the most abundant compounds in plant litter (Dix and Webster 1995, de Boer *et al.* 2005). Together, lignocellulose can comprises 50 to 70% of leaf litter and 60 to 90% of woody litter in woody plants (Rayner and Boddy 1988, Aber and Melillo, 2001). Cellulose, a relatively labile substrate when free of lignin, can be degraded by a wide variety of Basidiomycota and Ascomycota (de Boer *et al.* 2005). By contrast, a narrow suite of Basidiomycota fungi, often classified as "white rot," are largely responsible for the complete degradation of the more recalcitrant lignin polymer (Kirk and Farrell 1987, Hammel *et al.* 1997). Because they decompose such widespread and abundant plant compounds, the combined activities of lignocellulytic fungi are central to the terrestrial carbon cycle. Any environmental disturbance that impacts these fungi, such as climate warming or atmospheric N deposition, could influence soil carbon storage.

It has long been recognized that increased N availability through atmospheric N deposition alters the activities of cellulose and lignin degrading fungi. Fog (1988) hypothesized that a high N environment inhibits lignin degradation and selects against

lignolytic fungi in favor of cellulose decomposers. A hypothesized reason for this shift is that most fungi do not decompose lignin until they have encountered N limitations and have moved on to secondary stages of metabolism (Hammel *et al.* 1997). In an early demonstration of this concept, Keyser *et al.* (1978) reported that lignin degrading enzymes were only produced in the model fungus *Phanerochaete chryosporium* when N supplies were exhausted. Nitrogen availability has also been shown to decrease *in situ* rates of lignin decay and ligninolytic enzyme activity while at the same time boosting cellulose decomposition. Magill and Aber (1998) reported that N additions suppressed both lignin and cellulose decomposition at Harvard Forest, while Frey *et al.* (2004) reported higher rates of β-glucosidase and lower rates of phenol oxidase activity at the same site. Similar changes in hydrolytic and oxidative enzyme activity have been cited in other N fertilized forests (Carreiro *et al.* 2000, DeForest *et al.* 2004). Elsewhere, however, N additions have accelerated decomposition and boosted the production of both cellulose and lignin degrading enzymes (Saiya-Cork *et al.* 2002, Sinsabaugh *et al.* 2002). In a meta-analysis of N fertilization experiments, Knorr *et al.* (2005*a*) concluded that decomposition of low quality, high-lignin litters tended to be suppressed by N additions, while high-quality, low-lignin litters were stimulated.

In comparison to the many studies that have linked N deposition with litter decomposition, fewer efforts have directly examined the relationship between elevated temperatures and litter decay. Based on the idea that recalcitrant litter shows a high temperature sensitivity to decomposition (*e.g.*, Q_{10}, Fierer *et al.* 2005, Conant *et al.* 2008), both cellulose and lignin degradation should be expected to accelerate with warming. Several studies have supported this idea and have reported accelerated mass

loss in response to elevated temperatures (Hobbie *et al.* 1996, Rustad and Fernandez 1998*b*). Others have shown increased cellulolytic enzyme activity with warming, but no change in either lignin decay or lignolytic enzymes (Verburg *et al.* 1999, Waldrop and Firestone 2004, Cheng *et al.* 2010). In some cases, experimental warming has even reduced litter decomposition (McHale *et al.* 1998). These contradictory findings suggest that additional factors may moderate the stimulatory effect warming on litter decay. For example, elevated temperatures can significantly speed up N mineralization rates (Hartley *et al.* 1999, Melillo *et al.* 2004, Contosta *et al.* in press). The subsequent increase in soil N availability could have the same effect on litter decay as direct N fertilization.

Most of the work to date that examines how lignocellulytic fungi respond to increased temperatures and N inputs has relied on indirect evidence of microbial enzyme activity or has occurred in pure cultures. Consequently, very little research exists linking *in situ* increases in temperature or N availability with fungal decomposer community structure and function. The few studies that have examined the relationship between decomposer fungi and global change are inconclusive. Using laccase genes to select for lignin degrading fungi, Blackwood *et al.* (2007) and Lauber *et al.* (2009) found no relationship between fungal community structure and soil N additions. However, the lack of a correlation between potential soil phenol oxidase activity and laccase gene expression suggests that the abundance of organisms expressing laccase genes may not represent the actual capacity of the microbial community to degrade lignin (Hofmockel *et al.* 2007). McGuire *et al.* (2010) reported that fungal taxa with an affinity for lignocellulose substrates responded positively to warming. However, these taxa

represented only nine species (> 97% sequence similarity) that had incorporated lignocelluloses over a 48 hour incubation period, and thus may not represent a more diverse group of fungi decomposing plant litter in chronically warmed sites.

One of the largest gaps in understanding the relationship between fungal decomposition and global change is how multiple stressors may produce a response that neither would elicit on its own. Other multifactor experiments have demonstrated that the interactive effects of two or more global change drivers can be unexpected and non-linear. For example, in an experiment that simultaneously manipulated temperature, precipitation, CO_2, and N availability, Horz *et al.* (2008) observed a significant change in ammonia-oxidizing bacterial community composition with N fertilization. This change was less pronounced when temperature and precipitation were also elevated, despite the fact that neither precipitation nor temperature caused a change in community structure on their own. Several studies have examined the effects of simultaneous warming and N additions on soil N cycling (*e.g.*, Turner and Henry 2010) and microbial biomass (*e.g.*, Rinnan *et al.* 2007). To our knowledge, however, only two have targeted the decomposition of lignocellulytic compounds (Henry *et al.* 2005, Papanikolaou *et al.* 2010). Both of these studies reported no interactive effects of elevated temperature and N inputs on lignocellulytic enzymes, litter decay, or the structure of the fungal community colonizing decomposing litter. Indeed, the combination of increased temperatures and N amendments could cancel one another's effects on litter decomposition, resulting in no net response. Since both N fertilization and warming can enhance soil N availability, the combination of both factors could also inhibit litter decay more than either treatment separately. Likewise, concomitant warming and N

fertilization may annul one another's impacts on fungal community structure, or they may result in drastically reduced diversity and a dramatic shift in community composition.

Finally, research that quantifies the fungal decomposer community response to warming or N deposition also has not directly addressed how this response changes over time. On the scale of weeks to months, elevated temperatures and N inputs may affect decomposer fungi in contrasting ways as plant litter degrades and substrate quality declines. Ruderal species that colonize litter earlier in the decay sequence may respond more positively to warming and N additions than lignocellulytic species that dominate later in the decomposition trajectory (Moorehead and Sinsabaugh 2006). Over longer timescales, decomposer fungi may react differently to one year of elevated temperatures and N inputs than they would to a decade of the same disturbances. For example, Gallo *et al.* (2004) observed an increase in litter phenol oxidase activity in three forest sites receiving their first dose of NO_3^- additions. Two years later in the same locations, Sinsabaugh *et al.* (2005) reported that litter phenol oxidase production in fertilized red maple (*Acer rubrum*) and red oak (*Quercus rubra*) stands were 15 and 23% lower respectively than unmanipulated soils. Similarly, while five years of soil warming had no effect on total microbial biomass in a subalpine heath (Jonasson *et al.* 1999), 15 years of warming at that site significantly reduced microbial biomass C (Rinnan *et al.* 2007). These disparate responses may reflect lagging, indirect effects of global change that may be just as detrimental to the decomposer fungi as the direct impact of warming and N additions.

The objective of this study was to determine how soil warming and N additions, alone and together, influence the structure and function of decomposer fungi, as well as how this response changes over time. To this end, we extracted, cloned, and sequenced fungal DNA from decaying woody debris incubated in four global change experiments that had implemented similar manipulations for different periods. From the decomposing wood, we also measured changes in mass, N, and lignin contents so we could link changes in fungal community structure with the functions these fungi perform. We hypothesized that N additions would suppress decomposition and enhance N immobilization. By contrast, we expected that warming would accelerate mass loss and result in lower N accumulation. However, we thought that lignin decomposition would be suppressed with warming due to increased in soil N availability in heated soils. We predicted that the combination of warming and N additions on wood decomposition would cancel one another's effects. We also anticipated that the effect of warming and / or N additions would be more pronounced during the later stages of decomposition and with longer-running experiments. Finally, we hypothesized that any changes we observed with warming and /or N amendments would be accompanied by shifts in fungal diversity and community composition, and that community composition in particular would be correlated with the decay vectors of mass loss, and C, N, and lignin dynamics.

Methods

Site description and experimental design

This study took place in four global change experiments located at the Harvard Forest Long Term Ecological Research (LTER) site, Petersham, Massachusetts, USA (42°50' N,

72°18'W) (Table 4.1). Mean annual air temperature at the Harvard Forest is 7°C, with summer temperatures as high as 32°C and winter temperatures as low as -25°C. Average total annual precipitation, including water equivalent of snow, is 1100 mm (Boose *et al.* 2002). Tree species composition is similar among study areas, and consists primarily of red oak (*Quercus rubra*), black oak (*Quercus velutina*), red maple (*Acer rubrum*), and American beech (*Fagus grandifolia*).

The four experiments included in the study have similarly manipulated soil temperature and N inputs but have been operating for different periods of time. This allowed for the examination of shorter-term (2-5 years) versus longer-term (17- 20 years) effects of warming and/or N fertilization on fungal community structure and wood decay. The most recently initiated experiment, the combined Soil Warming × Nitrogen Addition study (SWNA), began in 2006. It consists of twenty-four 3 x 3 m plots randomly assigned to control, warming, warming plus N, and N additions treatments, with six replicates per treatment (Contosta *et al.* in press). The Soil Warming experiment in the Barre Woods tract (SW2) has been operating since 2003, and is comprised of two unreplicated 30 × 30 m plots, one disturbance control (see below) and one warming plot (Burton *et al.* 2008). Each 30 × 30 m mega-plot is divided into thirty six 5 × 5 m subplots. A longer-running Soil Warming experiment (SW1) located in the Prospect Hill tract has been functioning since 1991. It has eighteen 6 × 6 m plots assigned as control, disturbance control, and warming, for a total of six replicates per treatment (Peterjohn *et al.* 1993). The oldest study, the Chronic Nitrogen Amendment Study (CNA), began in 1988 and consists of eight unreplicated 30 × 30 m plots, four of which occur in red pine (*Pinus resinosa*) stands and four of which are located in a mixed hardwood forest. The

plots in each forest type are assigned as control, low N fertilization, high N fertilization, and N + S fertilization (Aber *et al.* 1993). Like the Soil Warming experiment at Barre Woods (SW2), the mega-plots at CNA are partitioned into thirty six 5 × 5 m subplots. Only subplots in the low N and the control treatments in the hardwood forest were used for this study.

At each of the soil warming experiments (SWNA, SW1, SW2), warmed plots are continuously elevated 5°C above ambient using cables buried at 10 cm depth below the soil surface and spaced 20 cm apart (Peterjohn *et al.* 1993). Disturbance control plots at the SW1 and SW2 contain unpowered cables. Because disturbance controls have shown no difference in soil moisture and soil biogeochemical fluxes (Peterjohn *et al.* 1994), cables were not buried in the unwarmed plots of the SWNA study. Each of the N addition experiments (SWNA and CNA) amend fertilized plots with an aqueous solution of NH_4NO_3 applied at a rate of 5 g N m^{-2} y^{-1}. The high N plots at the CNA study, which are fertilized with NH_4NO_3 at a rate of 15 g N m^{-2} y^{-1}, were not used for this study.

Wood decomposition

Wood decay was assessed by measuring changes in mass, lignin, C, and N in red maple woody litter over a two year period at the four Harvard Forest experiments. Red maple was chosen because it is one of the most dominant canopy species at all four studies. Woody litter was used instead of leaf litter because its higher initial C:N and lignin:N ratio should select for primarily lignocellulytic fungi. Samples were obtained from a single red maple tree harvested from the Massabessic Experimental Forest in Alfred and Lyman, Maine in June 2007. Wood blocks approximately 1 × 2 × 5 cm were cut from

two to three year old sapwood, with reference blocks cut for each sample block incubated in the field. The fresh weights of both blocks were recorded. The reference blocks were then dried at 105°C for 48 h. The wet-to-dry weight conversion factor of the reference blocks was used to determine the initial dry weight of the sample blocks. The sample blocks were subsequently autoclaved and stored at 4°C before deploying them to the study sites.

In July of 2007, two weeks after cutting the red maple tree from which they originated, autoclaved blocks were inserted horizontally between the Oe and Oa horizons of soil at each of the Harvard Forest global change experiments described previously. Three replicate blocks were installed in each of the 24 plots in the SWNA study (n = 72). Three replicate blocks were also buried in each of six subplots at the SW2 experiment: three control and three warming subplots (n = 18). At SW1, we added three replicate blocks to each of three warming plots (n = 9), but did not add blocks to any control plots. Since the SW1 experiment is directly adjacent to the SWNA study, we used the control plots at the SWNA site as the control treatment for SW1. Finally, we installed three replicate blocks in each of eight selected subplots at the CNA study: four control and four N addition subplots (n = 24). We installed a total of 123 blocks.

One block was removed from each plot in July 2008, November 2008, and July 2009, representing 12, 16, and 24 months since installation. Collection times were scheduled to coincide with mass loss of ~20, 40, and 60% of the original woody debris mass in the control treatments. Extra blocks were buried in control plots and were destructively sampled every few months to determine percent decomposition. Once sampled, wood blocks were processed within 24 h for wood decay and fungal community

structure. To determine wood decay, soil and fungal mycelia were first scraped from the surface of the block. Based on the method outlined by Jasalavich *et al.* (2000), the block was then weighed, and three holes were drilled at low speed through the wood perpendicular to the sides that had been parallel to the soil profile. The resulting wood plus fungus powder was placed in a 1.5 ml microcentrifuge tube, flash frozen in liquid N, freeze-dried, and placed in a -80°C freezer pending molecular analysis. The drill bit was cleaned with 97% ethanol and flamed between samples. After drilling, the blocks were weighed again and dried at 105°C for 48 h. The dry weight was adjusted to account for the approximately 250 mg removed from the sample with the drill. Mass loss was then calculated as the difference in weight between initial and incubated woody debris and is expressed as percentage of original mass.

Dried samples were then ground to a fine power with a SPEX Sample Prep Mixer Mill (SPEX Sample Prep, Metuchen, New Jersey, USA) and used for C, N, and lignin analysis. Total C and N were determined by dry combustion using a Perkin-Elmer elemental analyzer (Perkin-Elmer, Waltham, Massachusetts, USA). Percent lignin was assessed with sulfuric acid digestion of acid detergent fiber using the method outlined in Goering and VanSoest (1970) at the Cumberland Valley Analytical Services Lab (Hagerstown, Maryland, USA). Nine reference blocks were randomly selected for C, N, and lignin analysis and were averaged to estimate initial C, N, and lignin in all sample blocks (Table 4.2). As with mass loss, changes in C, N, and lignin were calculated as the difference between initial and incubated samples and are expressed as percent remaining.

Fungal community structure

The diversity and composition of the fungal decomposer community was determined by extracting, cloning, and sequencing DNA of fungi colonizing the woody debris. Using ~ 50 mg of wood plus fungus power drilled from the samples, DNA was extracted with a MO BIO PowerPlant DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA). Extracted DNA was purified with a MO BIO PowerClean DNA Clean-Up Kit (MO BIO Laboratories) to remove polyphenols and other PCR inhibitors. Once purified, DNA extracts were diluted in a 1:100 solution of DNA and ultrapure water. The ITS region of nuclear ribosomal DNA was then amplified using the fungal specific primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White *et al.* 1990). The PCR reaction contained 25 mM $MgCl_2$, 200 μM of each dNTP, 50 μM of each primer, DMSO ($(CH_3)2SO$), 10 x standard *Taq* reaction buffer (New England Biolabs, Ipswich, Massachusetts, USA), 5 units μL^{-1} *Taq* DNA polymerase (New England Biolabs), and 1 μL of DNA template μL^{-1} reaction mixture. The PCR reaction was performed with an initial denaturation at 94°C for 85 s, followed by 35 cycles of denaturation, annealing, and elongation. The first 13 cycles had 35 s of denaturation at 95°C, 55 s of annealing at 55°C, and 45 s of extension at 72°C. The extension step was lengthened to 120 s for cycles 14 to 26 and to 180 s for cycles 27 to 35. After completing 35 cycles, there was a final extension step for 10 min at 72°C. Reaction products were electrophoresed on 1% agarose gels to screen PCR products for positive amplicons. Positive PCR amplicons were then used to create clone libraries. Using the Invitrogen TOPO TA Cloning Kit for sequencing (Invitrogen Corporation, Carlsbad, California, USA), PCR products were mixed with the TOPO vector and incubated for one hour at room temperature. The

cloning reaction was then transformed into TOP10 competent *E. coli* cells following the manufacturer's instructions. Transformed cells incubated overnight at 37°C on agar media containing 50 μg ml $^{-1}$ ampicillin. As many as ten colonies per sample were picked with sterile toothpicks. If the clone library contained fewer than ten colonies, then all of them were selected. To perform PCR, chosen colonies were mixed with 25 μL ultrapure water. The reaction proceeded as above, using the ITS-1F and ITS4 primers instead of the M13 primers typically applied to PCR for colonies. M13 primers resulted in PCR products that were often too diluted to use for sequencing. After running the PCR products on 1% agarose gels, positive amplicons were purified with the QIAGEN QIAquick PCR Purification Kit (QIAGEN Incorporated, Valencia, California, USA). Purified samples were sent to Cornell University Life Sciences Core Laboratories Center (Ithaca, New York, USA) for sequencing using the ITS1-F primer.

Sequence processing

Sequences were manually edited using the Finch TV 1.4 DNA chromatogram viewer (Geospiza, Incorporated, Seattle, Washington, USA). Remnants of primers and low-quality ends were clipped, leaving 400 to 600 bp. Sequences were then evaluated for potential chimeras using Chimera Checker, an open source program that targets the fungal ITS region (Nilsson *et al.* 2010). Chimeric sequences were removed, and the remaining sequences were then queried against specimens in the National Center for Biotechnology Information database using the BLAST search algorithm. BLAST hits that had ≥ 96% sequence similarity were identified to order when this taxonomic information was available. We limited our identification to order level because finer

scale taxonomic resolutions have not yet been classified on a phylogenetic basis (Hibbett *et al.* 2007). Sequences were classified as unknown when order-level taxonomy was not available for a ≥ 96% match.

Edited sequences were then aligned using the ClustalW progressive multiple alignment method (Chenna *et al.* 2003) in BioEdit software (Hall 1999) using default parameters. Using MOTHUR software (Schloss *et al.* 2009), aligned sequences were used to generate distance matrices, which, in turn, were clustered into OTUs. Clustering was performed with the neighbor-joining algorithm to generate OTUs at five levels of sequence similarity: ≥70, 80, 90, 96, or 99 %. This allowed for the examination of how warming and N fertilization affected fungal diversity at a range of broadly to narrowly defined levels of taxonomic relatedness (Allison *et al.* 2007). While percent sequence similarity does not necessarily refer to the Linnaean system of classification, species are generally thought to be between 95 and 99% similar in the overlap of nucleotide sequences (Konstantinidis and Tiejde 2010).

Statistical analysis

We used a mixed modeling approach to determine changes in wood decay with warming and/or N additions. For each dependent variable (percent mass, N, and lignin remaining) hypothesized fixed effects included warming and N addition treatments, woody litter incubation time, and the duration of the study. Incubation times were 12, 16, and 24 months. Study duration was set to coincide with the sample collection in 2008, such that SWNA was 2 years, SW2 was 5 years, SW1 was 17 years, and CNA was 20 years. Since models produced unrealistic confidence intervals (CI) when plot was nested within study

as a random effect, plot only was included as a hypothesized random intercept. Warming, N additions, and warming plus N additions were also hypothesized as random slope effects. Selection of fixed and random effects was based on the methods recommended by Zuur *et al.* (2009) and outlined in Contosta *et al.* (in press). Mixed effects modeling was performed with the *nlme* package (Pinhiero *et al.*, 2009) in R 2.9.2 (R Development Core Team, 2009). Post-hoc tests examining pairwise differences between means were performed by refitting the final model without an intercept, and then calculating 95% CIs from the resulting estimated group means and standard errors. A lack of overlap in the 95% CI indicated a significant difference between two means.

Differences in fungal community diversity among treatments were first analyzed by calculating rarefaction curves of OTU richness at ≥70, 80, 90, 96, or 99 % sequence similarity. Using MOTHUR software (Schloss *et al.* 2009), rarefaction curves were generated for each level of sequence similarity by randomly sampling all sequences within treatment groups without replacement. Study duration and woody litter incubation time were pooled within treatment groups due to low sample sizes. This process was performed in 1,000 randomizations to produce mean OTU richness and 95 % CIs. We then used to MOTHUR to estimate mean and 95 % CIs of Chao1 diversity (Chao 1987), Shannon diversity (Magurran 1988), and Simpson diversity (Magurran 1988) for each of the experimental treatments at each level of OTU richness. As with the rarefaction analysis, study duration and incubation time were pooled within each treatment group when calculating diversity indices.

A combination of non-metric multidimensional scaling (NMDS) and permutation testing was used to examine relationships among fungal community structure, wood

decay, and the experimental manipulations for each level of sequence similarity. All multivariate analyses were conducted with the *vegan* package in R (Oksanen *et al.* 2010). Data were relative abundances of OTUs relative to total OTUs for a certain level of richness (*e.g.*, $\geq$ 96%) and were arranged in a matrix with relative abundances as columns and wood samples as rows. NMDS was performed using the *isoMDS* function and the Gower dissimilarity index of the OTU data matrix. Following NMDS, the relationship among OTU diversity, the experimental manipulations, and wood decay was examined with permutation testing. Using the *envfit* function in *vegan*, treatment, incubation time, study duration, and decomposition parameters (percent mass, C, N, and lignin remaining) were superimposed onto the ordination. This projection allowed for the scores of each dimension of the NMDS to be averaged for categorical variables (warming and / or N additions). It also fit continuous variables (incubation time, study duration, and decomposition parameters) onto the ordination to maximize their correlation with NMDS scores. A permutation test of 999 randomized trials was then performed to determine whether correlations (continuous variables) and differences among group means (categorical variables) were greater than by chance alone. A P value ≤ 0.05 indicated a significant difference in community composition among treatments, over time, or along gradients in wood decay.

Results

Wood decomposition

Of the 123 wood blocks buried, 106 were recovered from the study sites. The majority of the wood debris that was not recovered had been installed in the SW1 and SW2

experiments, represented both control and warming treatments, and could not be located during the 24-month sampling. The remaining wood that was not retrieved was so degraded that it could not be removed from the ground. Mixed effects models were used to analyze changes in decomposition among treatments, across study durations, and over the incubation period. Model selection statistics and fixed effects included in the final models are presented in Appendix C.

Nitrogen amendments suppressed wood decay over the entire study period (N additions: $P = 0.002$), resulting in 53% more mass remaining in N fertilized plots than the controls at the end of the incubation (Table 4.3, Figure 4.1). However, the effect of N additions on mass loss depended on the duration of the study (N × study duration: $P = 0.009$), with plots treated for 2 years having slower decay than plots manipulated for 20 years. Warming alone did not affect mass loss (warming: $P = 0.28$). Instead, it followed the same decomposition trajectory as the control plots. However, the combination of warming × N accelerated mass loss beyond the combination of warming and N additions on their own (warming × N: $P = 0.02$). At the end of 24 months, mass remaining in the warming x N plots was 28% lower than the control treatment. Both the warming only and the N addition only plots showed higher mass remaining than the simultaneous warming x N treatment (95% CI). The N addition plots also had higher mass remaining than the warming only treatment (95% CI). While percent original mass remaining decreased over the course of the incubation (incubation time: $P < 0.0001$), there were no treatment by incubation time interactions.

Nitrogen additions enhanced N immobilization (N additions: $P = 0.05$), but the effect of N fertilization on N immobilization varied among incubation times (N ×

incubation time: $P = 0.01$) (Table 4.3, Figure 4.1). Wood N increased throughout the study for both treatments, but was 22% lower in the N amended plots after 12 months of decay, 42% higher after 16 months, and 26% higher after 24 months as compared to the control plots. There was neither an effect of warming alone, nor of warming × N additions, on N immobilization.

Lignin decay was suppressed in N additions plots regardless of incubation time or study duration (N additions: $P = 0.03$) (Table 4.3, Figure 4.1). After 24 months of decay, 69% of the original lignin persisted in wood incubated in N fertilized plots as compared to 52% remaining in the control treatment. Warming did not alter lignin decomposition, either alone or in combination with N additions. The warming only treatment had less lignin remaining than the N fertilized plots, as estimated by the 95% CI. Both the warming only and the N addition only plots had higher percent lignin remaining than the warming × N treatment (95 % CI). All four treatments lost lignin as the incubation progressed (incubation time: $P < 0.0001$), and lignin decay changed marginally with study duration ($P = 0.06$).

Fungal community structure

The 106 wood samples recovered from the forest floor were equally distributed across treatments. Of these, approximately 50% produced positive PCR amplicons, primarily from the control, warming only, and N addition only plots. Clone libraries were successfully constructed from 60% of these positive amplicons and evenly represented all four experimental manipulations. The clone libraries resulted in 141 total sequences, three of which were identified as chimeric and removed. Of the 138 remaining, 70%

originated from wood blocks buried in control or N addition only plots, while 30% came from warming and warming x N plots. Each clone library produced an average of four sequences, with a minimum of one and a maximum of eight. Operational taxonomic units were identified at ≥70, 80, 90, 96, or 99 % sequence similarity. The number of OTUs at each level of sequence similarity ranged from 20 at the ≥ 70% to 69 at the ≥ 99% cutoff.

Warming decreased the diversity of operational taxonomic units (OTUs) while N additions had no effect. Rarefaction curves diverged between the control and the warming only treatments at all levels of sequence similarity after sampling a maximum of 15 sequences (Figure 4.2). The asymptotic shape of the warming only curve suggested that diversity was saturated after sampling only 10 sequences. By contrast, rarefaction curves for control, N addition, and warming × N treatments were much steeper, suggesting that additional sampling would increase diversity. Further, the N addition and warming × N curves overlapped with the control curve at all levels of sequence similarity, showing no change in diversity with either treatment.

Shannon, Chao, and Simpson's indices also indicated lower diversity with soil warming at all OTU cutoff levels (Table 4.4). At the 96% sequence similarity cutoff, Shannon diversity was 2.97 [95% CI = (2.71, 3.22)] in the control treatment and 1.33 [95% CI = (1.02, 1.64)] in warming only plots. Likewise, Chao diversity was 35.38 [95% CI = (27.25, 63.84)] in the control plots and 5.00 [95% CI = (0.00, 5.00)] in the warming only treatment. Simpson's diversity went from 0.04 [95% CI = (0.02, 0.07)] in the control treatment to 0.28 [95% CI = (0.17, 0.38)]. Because values closer to zero with the Simpson's index indicate higher diversity, the increase in the Shannon index in the

warmed plots represented a decline in richness. As with rarefaction analysis of OTUs, N additions alone did not affect diversity as measured by Shannon, Chao, and Simpson's indices. However, the combination of warming × N decreased diversity at all levels of sequence similarity. Because the 95% CIs of estimated diversity overlapped between control and warming × N treatments, these decreases were not significant.

According to BLAST searches, sequences that produced a match of ≥ 96% similarity primarily consisted of fungi from the Basidiomycota phylum, with only 10 to 25% of clone libraries comprised of Ascomycota. The most dominant fungal order represented in the clone libraries was the Agaricales, comprising 20 to 38% of all identified sequences in control, warming, and N addition plots. The other major orders present within the Basidiomycota included the Polyporales and Russulales. The two most dominant orders in the Ascomycota were Helotiales and Chaestosphaeriales. The combination of warming × N seemed to reduce the dominance of Basidiomycota as compared to the other three experimental manipulations. Nitrogen fertilization, both alone and in combination with warming, also appeared to increase the relative abundance of clones from the Ascomycota, boosting the relative dominance of the Helotiales and Chaestosphaeriales orders (Table 4.5).

These increases and decreases in the relative dominance of certain phyla and orders did not represent a significant change in fungal community composition with warming and/or N additions. Ordinations of community dissimilarity at each level of OTU richness showed no spatial separation of the fungal community among treatment groups. These NMDS ordinations all had two dimensions, except the ordination for the 99% OTU cutoff level, which had three. Experimental treatments fit onto NMDS spatial

ordinations showed no difference in community composition for all levels of sequence similarity (Table 4.6). Fungal community composition was also not correlated with study duration, incubation time, or vectors of decomposition such as mass loss or N dynamics. The exception was a significant relationship between community composition and percent lignin remaining at the 96% level of sequence similarity ($R^2 = 0.22$, $P = 0.045$, Figure 4.3).

Discussion

Wood decomposition

The purpose of this research was to determine how soil warming and N additions, acting alone and together, influence wood decay dynamics and the structure of the fungal community decaying that wood. Red maple wood blocks were used as a substrate for fungal decomposition, which was measured in four different global change experiments over a 48 month period. The wood itself had a much larger initial C:N ratio (*i.e.*, ~670:1) than reported elsewhere for this species (Downs *et al.*1996), though such high values have been occasionally observed (Clinton *et al.* 1996). Despite the low proportion of C to N in the woody debris used, decomposition in unmanipulated soils proceeded similarly to other studies examining woody litter decay (Downs *et al.* 1996, Micks *et al.* 2004). After 24 months of decomposition, wood in the control plots had lost ~60% of its original mass and had accumulated ~275% more N than was present initially.

As hypothesized, N additions suppressed wood decomposition, slowing mass loss and lignin decay At the same time, N fertilization increased wood N uptake, resulting in 26% more N in fertilized plots at the end of the 48 month incubation as compared to

controls. These findings fit with other reports of slower decomposition and higher N immobilization in leaf litter incubated in a high N environment (Magill *et al.* 1998, Sinsabaugh *et al.* 2005). Other studies that have examined wood decay in response to N inputs have found a different result, showing that N fertilization enhanced wood decomposition (Downs *et al.* 1996, Micks *et al.* 2004, van der Wal *et al.* 2007). Nitrogen additions could initially accelerate wood decay because the added N relieves limitations in litter with a high initial C:N ratio (Berg 2000). In fact, N amendments have often been observed to stimulate cellulose decay and the production of celluloytic enzymes (Carreiro *et al.* 2000, Sinsabaugh *et al.* 2002, Gallo *et al.* 2004). This did not occur in the current study, perhaps due to a difference in the position in the soil profile where decomposition occurred. In the case of Downs *et al.* (1996) and Micks *et al.* (2004), woody debris was added to the Oi horizon. Here, wood was incubated between the Oe and the Oa horizons where microbes may have responded to the N additions differently than in the litter layer.

Contrary to our prediction, N fertilization for 2 years at the SWN site suppressed overall mass loss and C decay more than 20 years of N additions at the CNA study. Hobbie (2008) also reported that the negative effect of N additions on litter decay was mediated by site, where site × substrate combinations with the highest initial decay rates showed a more negative reaction to N inputs. Hobbie (2008) speculated that litter with faster initial decay more rapidly formed insoluble complexes with N additions that then frustrated further decomposition. Substrates that were slower to degrade produced less secondary decay products to interact with the N fertilizer, and thus were less negatively impacted. In our study, the substrate for all the sites was the same, but 20 years of N fertilization at the CNA site, which has caused significant reductions in soil microbial

biomass and extracellular enzyme activity (Frey *et al.* 2004), may have slowed decomposition enough to prevent the formation of recalcitrant molecules. By contrast, the two years of fertilization that took place in the SWN study may have created conditions for slightly faster, initial decay, high rates of N immobilization, and the subsequent formation of refractory, secondary compounds.

The effect of warming on wood decay also did not fit with our original hypotheses. In keeping with the findings of both natural gradient and manipulative studies (Hobbie 1996, Rustad and Fernandez 1998*b*, Trofymow *et al.* 2002, Mackensen *et al.* 2003), we predicted that increased temperatures would result in higher rates of overall mass loss, but that lignin decay would be suppressed due to increased N availability in warmer soils. Instead, we found that warming did not alter the degradation of any of the decay variables measured. Other studies have also reported no response of litter decomposition to elevated temperatures (Verburg *et al.* 1999, Cheng *et al.* 2010) or have cited suppressed litter decay with warming (McHale *et al.* 1998). In a meta-analysis of litter decomposition and experimental warming studies, Aerts (2006) showed that increased temperatures had either small positive or negative responses depending on the type of manipulation used and the extent to which warming caused soil drying. Lower soil moisture in mesic and xeric sites counterbalanced the positive effect of increased temperatures on litter decay. In the current study, warming did not affect moisture in woody debris (A. Contosta, *unpublished data*), but it significantly lowered the moisture of the soil in which the wood was buried (Contosta *et al.* in press). Drier soil conditions may have inhibited fungal colonization of the woody substrate, which in turn, could explain decreased fungal diversity in wood incubated in the warming plots.

However, if lower soil moisture dampened the positive effects of increased temperatures on wood decay, then we should have observed a similar phenomenon with the warming × N treatment.

Contrary to our prediction that simultaneous warming × N would cancel one another's effects on decomposition, the combined warming × N fertilization accelerated decay beyond either treatment on its own. This result also contrasts the findings of Papanikolaou *et al.* (2010), who reported no response of both litter decay and litter decaying enzymes to simultaneous warming and N additions. Likewise, Henry *et al.* (2005) cited no interactive effects of increased temperatures plus N amendments on oxidative enzyme activity. Since both of these studies reported few effects of either warming or N additions alone on litter decay or enzyme activity, it was not surprising that there were no interactions between the experimental manipulations. However, the unexpected interactive effect we observed in this study highlights the importance of conducting multifactor global change experiments whose outcomes would be difficult to predict from single factor studies.

Fungal community structure

Our analytical efforts to isolate, clone, and sequence fungal DNA were comparable to other research using genomic approaches to study fungi in woody litter. If a "species" is defined as sharing between 95 to 99% nucleotide sequence similarity (Konstantinidis and Tiejde 2010), then our average four "species" per sample was well within the range of values reported elsewhere for woody debris (Allmér *et al.* 2006, Kuhlhànkovnà *et al.* 2006, Kubartovnà *et al.* 2007, Meier *et al.* 2010). It was, however, low compared to

efforts to quantify fungal diversity in soil as well as leaf litter (Allison *et al.* 2007, Allison *et al.* 2008, Castro *et al.* 2010). This may be due to the antagonistic relationships between fungal wood decomposers that often result in few species occupying a single woody substrate (Rayner and Boddy 1988, Rayner 1994, Fukami *et al.* 2010). The relative proportion of Ascomycota and Basidiomycota was also consistent with other investigations of fungi decaying woody litter, with Basidiomycota typically dominant in temperate and boreal forests (Nordén *et al.* 2004, Allmér *et al.* 2006).

We expected that fungal community structure would change over time, both with the length of the experimental manipulation and over the course of the incubation. For the 48 month timeframe of our study, we expected that fungal populations would succeed one another as decomposition progressed and substrate quality changed (Rayner and Boddy 1988, Moorehead and Sinsabaugh 2006). As a result, we thought that fungi present during the final sampling would be primarily lignin decomposers most impacted by warming and/or N additions. Contrary to the findings of Kulhànkovnà *et al.* (2006) and Kubartovà *et al.* (2007), we detected no difference in fungal community structure among our three sampling dates, such that the fungal population on any given date was not disproportionately affected by our experimental manipulations. As with the low diversity of organisms per sample, the lack of change in the fungal community over time may be due to the early colonization of combative organisms.

Because lignolytic capabilities in particular are believed to be limited to a specific suite of fungi (Kirk and Farrell 1987, Hammel 1997), we also predicted that we would see a correlation between fungal community structure and vectors of decay. The data partially supported our hypothesis, in that we found a significant relationship between

lignin decomposition and community composition. However, this result should be interpreted with caution since it only occurred at the 96% OTU cutoff for sequence similarity, indicating that it applied to neither broad nor very narrow taxonomic groups. It also was not highly significant, having a *P*-value close to 0.05 and an r^2 of 0.22. Nevertheless, if lignin decay was indeed primarily performed by a unique set of fungal species, these organisms were not affected by warming and/or N additions since we saw no evidence that any of the experimental manipulations affected fungal community composition.

We predicted that N fertilization would reduce diversity and shift community composition of wood decaying fungi. While we observed that the relative dominance of Ascomycota increased with N additions, neither diversity nor community composition changed. The evidence that N additions affects fungal community structure is mixed, with some studies finding no effect (Blackwood *et al.* 2007, Hassett *et al.* 2008 Lauber *et al.* 2009, Papanikolaou *et al.* 2010), while others report both lowered diversity and altered community composition (Edwards *et al.* 2004, Allison *et al.* 2007). A shared feature of research citing shifts in the fungal community with N additions is that they utilized PCR primers universal to all fungi or specific to basidiomycetes. By contrast, studies that have reported no shift in fungal community composition with N fertilization have either used primers targeting laccase genes believed to be involved in the production of lignolytic enzymes or they have isolated fungi directly from decomposing wood. In the current study, we used the universal fungal primers ITS-1F and ITS-4. However, we also limited our analysis to decomposing woody litter that should attract primarily lignocellulytic fungi. Thus our results fit with other research showing no

change in the structure of the fungal community responsible for litter decomposition in general and lignin decay in particular.

As with N additions, we also expected that increased temperatures would alter fungal diversity and shift community composition. The data partially supported our hypothesis in that heating decreased diversity but had no effect on the composition of the fungal community. We do not believe that reduced diversity with elevated temperatures was an analytical artifact, despite the fact that the number of sequences obtained from wood decaying in warming plots was much lower than that of other treatments. Rarefaction analysis indicated that species richness in the warming treatment reached an asymptote after only 15 sequences, suggesting that more sampling in heated plots would not have boosted OTU richness. In addition, we obtained a similar number of total sequences from the warming × N treatment but observed much higher diversity from these samples. As a result, we believe that warming in fact decreased diversity of wood decaying fungi. Again, this decrease occurred in the absence of a significant change in community composition, suggesting that the loss of some taxa was not accompanied by the appearance of others.

These findings add to contradictory reports of how elevated temperatures affect fungal community structure. Some researchers have cited no change in fungal diversity or community composition with experimental warming (Castro *et al.* 2009). Others have shown altered fungal community composition but no difference in diversity (Papanikolaou *et al.* 2010), or have observed both a shift in composition and an increase in diversity (Allison *et al.* 2008). As mentioned above, dry soil conditions likely did not decrease diversity in warming plots since lower moisture should have similarly affected

fungi in the warming × N treatment. Likewise, selection against fungi that prefer cooler conditions should have equally reduced diversity in the warming × N and the warming treatments. Regardless of the mechanism, the decrease in diversity in warmed plots may have moderated an otherwise positive effect of increased temperatures on wood decomposition. There is some evidence to suggest a direct correlation between fungal diversity and litter decay (*e.g.*, Robinson *et al.* 1993), though Setälä and McLean (2004) showed that the benefit of increased diversity may saturate when the number of species reaches six to 12. Average OTU richness at the 96 to 99 cutoff levels (indicating species) was much lower than this for all the treatments, ranging from 3.7 to 4.4 taxa for the control, N addition, and warming x N plots. However, the warming only plots had even lower OTU richness, averaging only 2.6 taxa per block. As a result, the complementary effects of a diverse community of fungal decomposers may have been lost in wood incubated in warmed plots, slowing overall decay.

Finally, we found no evidence that the warming × N treatment altered fungal community composition. This finding was consistent with another report of no change in decomposer fungal community structure with simultaneous warming × N (Papanikolaou *et al.* 2010) and was not surprising given the lack of change in community composition with either elevated temperatures or N inputs. However, we did observe that Shannon, Chao, and Simpson diversity indices for the warming × N plots consistently fell between values for the warming only and the N addition only plots. Because diversity significantly decreased with warming alone, the rebound to higher diversity with simultaneous warming and N inputs suggests that N additions alleviated the negative effect of warming on fungal diversity.

Environmental change, decomposer fungi, and wood decay

This study produced little evidence that changes in wood decomposition with warming and/or N additions are related to shifts in fungal diversity or community composition. Yet both N additions and the warming × N treatment significantly affected wood decay, either suppressing or accelerating mass loss and influencing the accumulation of N in woody debris. How could the experimental manipulations have impacted wood decomposition in this way without causing a concomitant shift in the structure of the fungal community mediating the decay?

It is possible that the changes we observed in wood decay with warming and / or N additions occurred without an accompanying shift in microbial community structure. That is, changes in the physical environment (*e.g.*, increased temperature) or resource availability (*e.g.*, N additions) could alter microbial process rates without actually restructuring the microbial community itself (Balser *et al.* 2006). The question of whether microbial community structure shifts in response to changing environmental conditions may ultimately depend on their physiological capacity to deal with specific stressors (Schimel *et al.* 2007). In the case of a 5°C increase in soil temperature and 5 g N m^{-2} y^{-1} of inorganic N inputs, it appears that the fungal decomposer community responds by changing the rates at which it performs certain processes, not by shifting its fundamental structure.

Even if the fungal community was unaffected, shifts in the structure or function of bacterial decomposers may account for the changes we observed in wood decomposition with warming and/or N inputs. For example, Eisenlord and Zak (2010) reported that N additions reduced the abundance (as number of gene copies) and altered

the composition of the actinobacterial community in litter and surface soils. Like many fungi, *Actinobacteria* can produce extracellular enzymes that attack lignin (Godden *et al.* 2002). While these enzymes cannot completely decompose lignin (Godden *et al.* 2002), a decline in actinobacterial abundance or activity could nevertheless suppress litter decay independently of the fungal community.

N additions may have suppressed wood decomposition in a variety of ways other than by selecting against lignocellulytic fungi and lignin-degrading *Actinobacteria*. Increased recalcitrance of plant litter with N additions, which may account for the slower decomposition in plots fertilized for 2 years as compared to 20, may also explain overall slower decomposition with N additions. Theory states that at late stages of decay, N randomizes bond structures in litter when amino acids and ammonium interact with decay byproducts to form humid complexes that ligninolytic enzymes are less effective at attacking (Berg 1986, Fog 1988). This process could occur with either exogenous N fertilizers or with N immobilization during the normal course of decomposition, and would not require a shift in the fungal community. The empirical evidence for whether this actually occurs is mixed, and both supports (Gallo *et al.* 2005) and refutes (Knickers and Lederman 1995, Grandy and Neff 2007) the idea that N increases litter recalcitrance. Besides increasing refractory compounds in plant litter, N additions may also suppress decomposition by reducing fungal biomass, primarily through reductions in labile C availability that have been observed in chronically fertilized sites (Frey *et al.* 2004). Both Frey *et al.* (2004) and Contosta *et al.* (in prep) have documented depressed soil fungal biomass in N amended plots at the CNA and SWN studies respectively, which, in turn, may have simply left fewer fungi to secrete enzymes.

As with N additions, it may be that wood decomposition in heated soils has more to do with kinetic and stoichiometric constraints on decay and less to do with an actual shift in fungal community structure. In terms of enzyme kinetics, it was surprising that our low quality red maple wood did not respond more strongly to warming given that recalcitrant litter is believed to show much higher temperature sensitivity to decomposition (*e.g.*, Q_{10}) than labile materials (Fierer *et al.* 2005, Conant *et al.* 2008). However, the intrinsic temperature sensitivity of litter decay (*sensu* Davidson and Janssens 2006) may be obscured by stoichiometric limitations to decomposition. In other words, the high initial C:N and lignin:N in the red maple wood may have prevented it from decaying more quickly in warmed plots. Low quality litter has been documented to show less of a response to elevated temperatures as more palatable substrates (Hobbie 1996, Rustad and Fernandez 1998*b*), which simply may result from N shortages.

The synergistic response of warming × N may also have been related to microbially-mediated biogeochemical processes but not a change in microbial community structure per se. Higher temperature sensitivity, due to warming, and an alleviation of N limitations, due to fertilization, may have conspired to stimulate decomposition in a way that neither could alone. It would be logical to expect that longer-running soil warming experiments, such as the SW1 and the SW2, would function in the same way as the warming × N plots since each has shown substantially higher N mineralization rates in the heated plots as compared to controls (Melillo *et al.* 2002, Melillo *et al.* 2003). However, the deleterious effects of five to 17 years of warming on the soil microbial community (Bradford *et al.* 2008, Frey *et al.* 2008) may have counteracted the potentially stimulatory effect of the mineralized N at these sites.

Conclusion

The purpose of this research was to determine how soil warming and N additions, acting alone and together, influence wood decay dynamics and community structure of decomposer fungi. Overall, we observed almost no relationship between fungi and the decay of the woody substrate they decomposed. Similarly, we found that while global change drivers such as climate warming and N deposition can affect wood decomposition rates, these impacts seem largely divorced of any concomitant shift in fungal community structure. We can only speculate that factors such as increased recalcitrance of plant litter or stoichiometric constraints on litter decay contributed to the shifts in decomposition we observed with soil warming and N additions. However, we can state with confidence that changes in wood decay with elevated temperatures and N inputs carry important ecosystem consequences. Suppressed decomposition in N fertilized plots may result in higher soil C storage. By contrast, accelerated decay in warming × N soils suggests net soil C loss. These changes in soil C cycling likely involved alterations in fungal enzyme expression and enzyme kinetics. Elucidating what drives fungal enzymatic responses may therefore be central to understanding how litter decay interfaces with global environmental change.

Table 4.1. Summary of ongoing global change experiments at Harvard Forest LTER.

	CNA[†]	SW2	SW1	SWNA
Year started	1988	1991	2003	2006
Plot size	30 × 30 m	5 × 5 m	30 × 30 m	3 × 3 m
Treatments	0, 5, 15 g N m^{-2} y^{-1}	ambient, +5°C	ambient, +5°C	ambient, +5°C, 5 g N m^{-2} y^{-1}, warming × N
Replication	1 plot / treatment; 36 5 × 5 m subplots per megaplot	6 replicate plots / treatment	1 plot / treatment; 36 5 × 5 m subplots per megaplot	6 replicate plots / treatment

[†]CNA = Chronic Nitrogen Addition Study; SW1 = Soil Warming at Prospect Hill; SW2 = Soil Warming at Barre Woods; SWNA = Soil Warming × Nitrogen Addition Study.

Table 4.2. Initial chemistry of red maple wood blocks.

Carbon (%)	46.2 (0.1)
Nitrogen (%)	0.07 (0.0)
Lignin (%)	14.7 (0.1)
C:N	673 (9)
Lignin:N	214 (3)

Note: Values are averages (± 1 SE) of nine reference blocks randomly selected for chemical analysis.

Table 4.3. Final mixed model output for percent mass, nitrogen, and lignin remaining in red maple woody litter.

Variable	Fixed Effect	Estimate	SE	DF	*t*-value	*P*-value
Mass	Intercept	72.23	7.99	66	9.04	< 0.0001
	W	7.68	6.99	66	1.10	0.276
	N	30.47	9.56	66	3.19	0.002
	Inc	-2.15	0.32	33	-6.65	< 0.0001
	Study	1.37	0.47	66	2.92	0.006
	W × N	-25.69	11.12	66	-2.31	0.024
	N × Study	-1.56	0.58	66	-2.69	0.009
Nitrogen	Intercept	267.74	47.69	88	5.61	<0.0001
	N	-147.50	72.73	88	-2.03	0.046
	Inc	-0.46	2.74	88	-0.17	0.868
	N × Inc	10.58	4.20	88	2.52	0.014
Lignin	Intercept	82.45	10.66	57	7.74	< 0.0001
	W	9.71	9.06	57	1.07	0.288
	N	27.60	12.43	57	2.22	0.030
	Inc Time	-2.01	0.45	33	-4.47	< 0.0001
	Study	1.20	0.61	57	1.99	0.055
	W × N	-23.36	14.44	57	-1.62	0.111
	N × Study	-1.27	0.76	57	-1.67	0.100

Notes: Model output includes parameter estimates, standard errors of the estimates (SE), degrees of freedom (DF), *t*-values and *P*-values for fixed effects. "Intercept" represents the base case of the model, or the control plots. "W" refers to warming, "N" to N additions, "Study" to study duration, and "Inc" to length of incubation of decomposing litter.

Table 4.4. Shannon, Chao1, and Simpson's diversity indices (95% CI) of DNA sequences at increasing levels of sequence similarity in control (C), warming (W), warming × N (WN) and N addition (N) treatments.

	Sequence Similarity		
	≥ 70%	≥ 80%	≥ 90%
Shannon			
C	1.93 (1.64, 2.21)	2.34 (2.03, 2.64)	2.64 (2.34, 2.94)
W	1.33 (1.02, 1.64)	1.33 (1.02, 1.64)	1.33 (1.02, 1.64)
WN	1.64 (1.23, 2.06)	2.19 (1.85, 2.54)	2.40 (2.06, 2.73)
N	2.21 (1.92, 2.50)	2.55 (2.27, 2.83)	2.64 (2.35, 2.93)
Chao1			
C	14.33 (11.50, 33.07)	25.00 (18.00, 56.45)	33.20 (23.44, 70.60)
W	5.00 (5.00, 5.00)	5.00 (5.00, 5.00)	5.00 (5.00, 5.00)
WN	16.50 (10.322, 51.53)	19.00 (13.34, 48.54)	26.00 (16.66, 68.20)
N	24.33 (16.94, 59.97)	37.33 (23.48, 94.08)	66.50 (33.09, 192.30)
Simpsons			
C	0.18 (0.11, 0.24)	0.12 (0.06, 0.17)	0.08 (0.03, 0.13)
W	0.28 (0.17, 0.38)	0.28 (0.17, 0.38)	0.28 (0.17, 0.38)
WN	0.25 (0.11, 0.39)	0.11 (0.04, 0.18)	0.08 (0.02, 0.14)
N	0.14 (0.08, 0.20)	0.09 (0.05, 0.13)	0.08 (0.04, 0.13)

Table 4.4, contd.

	Sequence Similarity	
	≥ 96%	≥ 99%
Shannon		
C	2.97 (2.71, 3.22)	3.28 (3.03, 3.54)
W	1.33 (1.02, 1.64)	2.11 (1.78, 2.44)
WN	2.56 (2.22, 2.90)	2.56 (2.22, 2.90)
N	2.70 (2.40, 2.99)	3.22 (2.96, 3.47)
Chao1		
C	35.38 (27.25, 63.84)	77.00 (47.05, 162.85)
W	5.00 (5.00, 5.00)	13.33 (10.50, 32.07)
WN	29.75 (19.40, 71.55)	29.75 (19.40, 71.55)
N	74.50 (36.27, 215.10)	169.00 (73.68, 477.24)
Simpsons		
C	0.04 (0.02, 0.07)	0.02 (0.00, 0.04)
W	0.28 (0.17, 0.38)	0.10 (0.01, 0.12)
WN	0.06 (0.01, 0.12)	0.06 (0.01, 0.12)
N	0.08 (0.04, 0.12)	0.03 (0.01, 0.06)

Note: Data were pooled across both sampling dates and studies of different durations that had the same experimental manipulation.

Table 4.5. Relative abundance (% sequences in the clone library) of dominant fungal phyla and orders based on NCBI BLAST searches in control (C), warming (W), warming × N (WN) and N addition (N) treatments.

Trt	Total Seqs	Ascomycota	
		Chaetosphaeriales	Helotiales
C	44	2	9
W	13	0	10
WN	20	10	15
N	53	8	19

Table 4.5, Contd.

Trt	Total Seqs	Basidiomycota			Unknown
		Agaricales	Polyporales	Russulales	
C	44	20	9	7	48
W	13	38	10	5	38
WN	20	0	5	10	55
N	53	25	15	2	32

Notes: Unkown sequences are those that could not be identified to order level. Data were pooled across both sampling dates and studies of different durations that had the same experimental manipulation.

Table 4.6. r^2 and P values resulting from permutation tests of experimental treatments (factors) and decomposition gradients (vectors) fit onto spatial ordinations of fungal community structure at increasing levels of sequence similarity.

	≥ 70%		≥ 80%		≥ 90%		≥ 96%		≥ 99%	
Variable	r^2	P	r^2	P	r^2	P	r^2	P	r^2	P
Treatment	0.06	0.801	0.08	0.631	0.11	0.394	0.12	0.379	0.04	0.911
Inc Time	0.08	0.361	0.06	0.440	0.01	0.932	0.07	0.406	0.03	0.690
Study	0.03	0.673	0.01	0.862	0.02	0.756	0.01	0.860	0.01	0.875
Mass	0.10	0.278	0.09	0.296	0.04	0.623	0.14	0.138	0.04	0.617
Nitrogen	0.15	0.129	0.09	0.316	0.05	0.547	0.15	0.111	0.06	0.462
Lignin	0.08	0.346	0.15	0.171	0.04	0.654	0.22	0.045	0.03	0.690

Note: Statistics are based on 999 permutations.

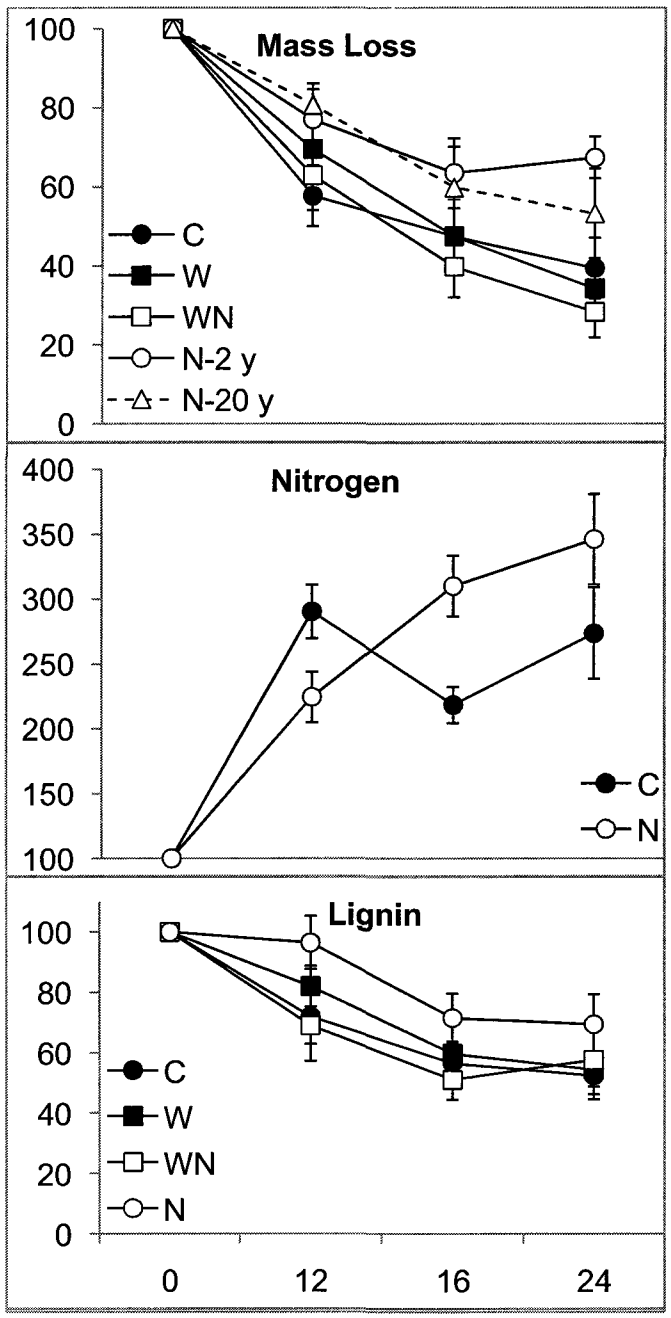

Figure 4.1. Percent original mass, N, and lignin remaining in red maple wood blocks buried in control (C), warming (W), warming × N (WN) and N addition (N) plots after 0, 12, 16, and 24 months. Error bars represent ±1 SE. Data were pooled across study duration and/or experimental treatment when they did not signifcantly affect decomposition.

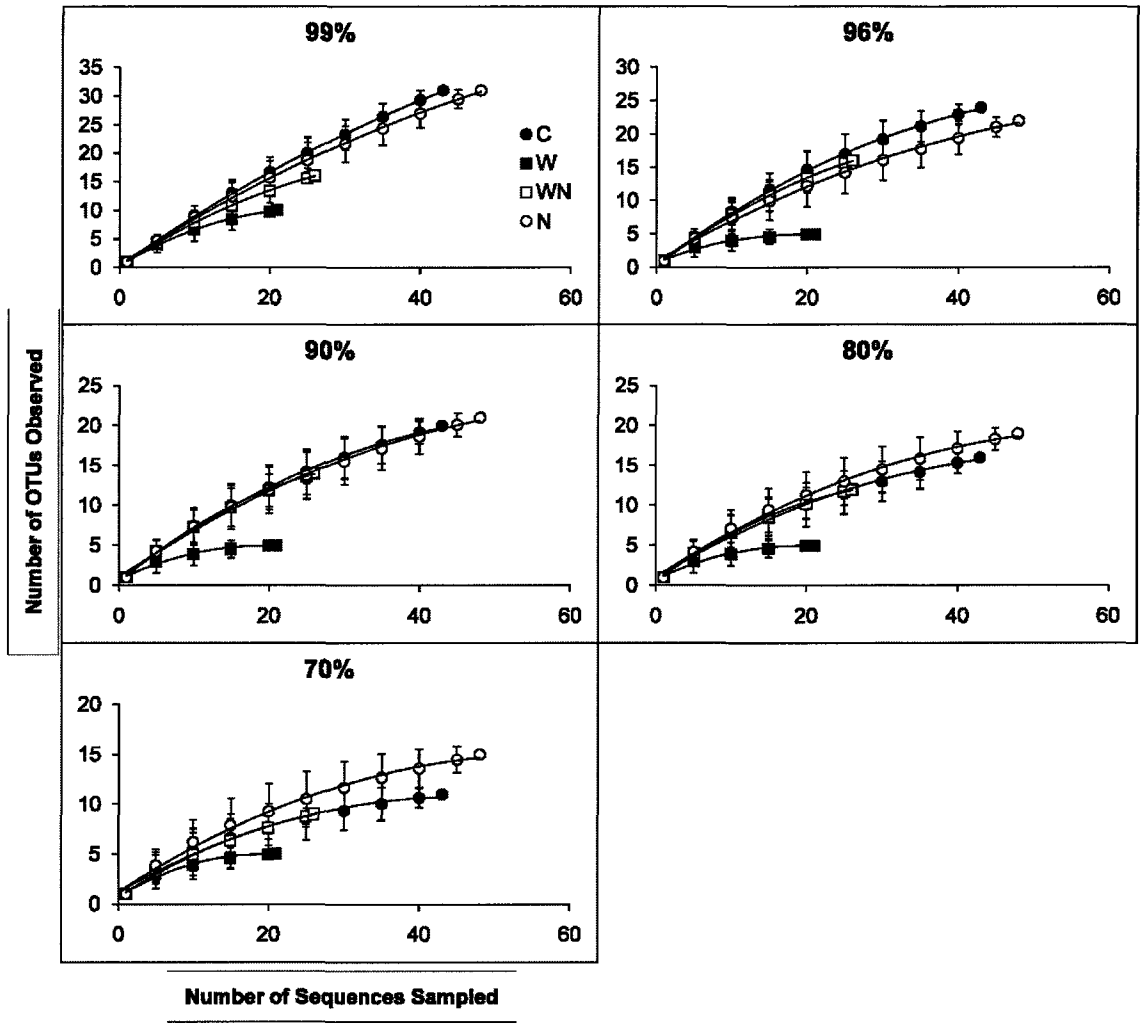

Figure 4.2. Rarefaction curves of number of sequences sampled versus number of OTUs observed at ≥70, 80, 90, 96, or 99 % sequence similarity in control (C), warming (W), warming × N (WN) and N addition (N) plots. Error bars represent 95% confidence intervals. Data were pooled across both sampling dates and studies of different durations but with the same experimental manipulation.

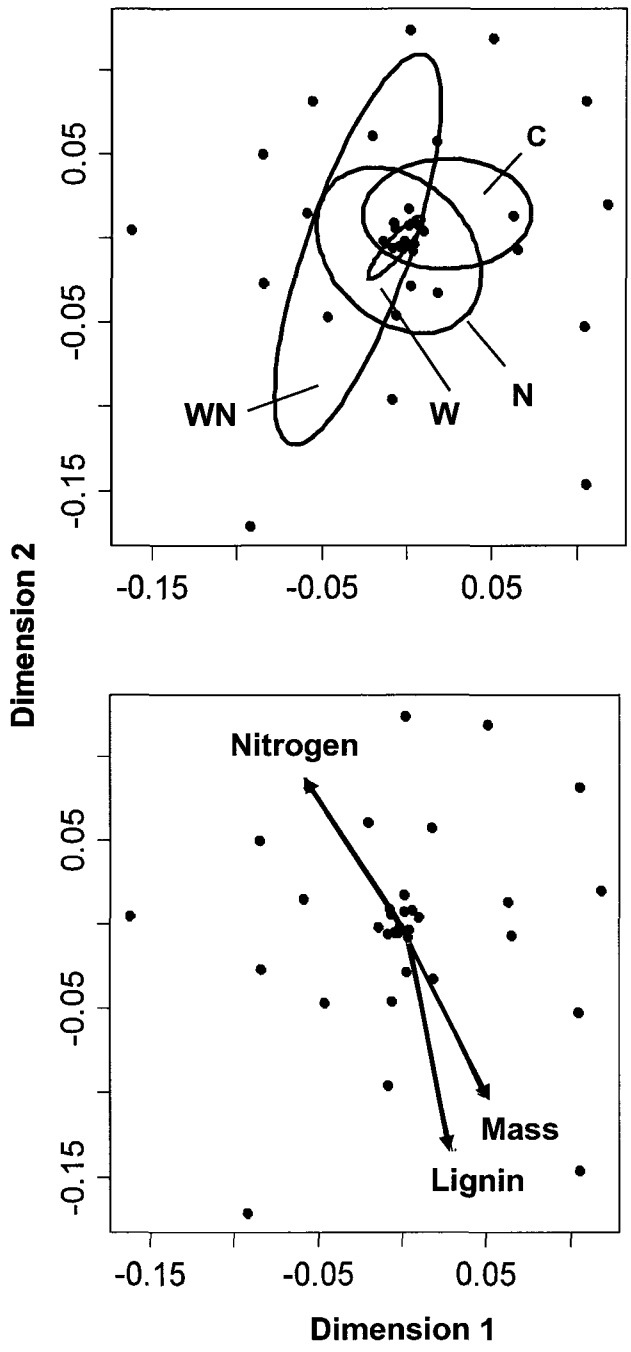

Figure 4.3. Non-metric multidimensional scaling plots of fungal community structure at the 96% OTU cutoff level. Distances represent rank dissimilarity, not actual distances. Although treatments and decomposition vectors were fit simultaneously onto the spatial ordination, they are shown separately here for visual clarity. Ellipses indicate the 95% confidence interval of the standard error of the mean for each treatment. Treatments are control (C), warming (W), warming × N (WN) and N additions (N). Arrows indicate the correlation between community structure and decomposition vectors. Only vectors with $P \leq 0.20$ were included.

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APPENDICES

APPENDIX A

Random effects and autocorrelations model specification for gravimetric soil moisture, net N mineralization, and soil respiration.

Variable	Random effects	*L*	*Δ* DF	*P*	Autocorrelation	*Δ* AIC	*Δ* DF
Moisture							
O Horizon	Plot	25.50	1	<0.0001	ARMA	26.76	2
M Horizon	Plot	103.68	1	<0.0001	None	0	0
N min	None	1.36	0	0.51	ARMA	2.54	2
Respiration	Plot, W	110.43	3	<0.0001	ARMA	25.76	2

Notes: For the random effects, plot was modeled as a random intercept and warming as a random slope. The likelihood ratio (*L*) and *P*-values refer to the random effects structure in a linear mixed effects (*lme*) model tested against a generalized least squares (*gls*) model, with *P*-values shown in their uncorrected form for testing on the boundary. The *Δ* degrees of freedom (*Δ* DF) column for random effects indicates the increase in degrees of freedom by adding random effects to a *gls* model, while the *Δ* DF column for autocorrelations refers to the increase in degrees of freedom from adding an autocorrelation structure. The *Δ* Akaike's Information Criterion (*Δ* AIC) column indicates the decrease in AIC by adding an autocorrelation structure. The ARMA autocorrelation structure indicates an autoregressive moving average. The names O Horizon and M Horizon represent organic and mineral soil horizons.

APPENDIX B

Model selection and final model output for labile C availability, β-glucosidase, phenol peroxidase, total biomass, fungal biomass, bacterial biomass, and the F:B ratio.

Model selection for random effects included testing for significance of random intercepts, random slopes, autocorrelation structures, and variance structures. Selection of fixed effects involved a backward selection procedure for determining the significance of three-way and two-way interactions among warming, N additions, and season. In the case of enzyme activity, backward selection involved examining the significance of four-way, three-way, and two-way interactions among warming, N, season, and incubation temperature. Final model output includes parameter estimates, standard errors of the estimates (SE), degrees of freedom (DF), *t*-statistics and *P*-values for fixed effects. All parameter estimates and statistics in the model output are relative to baseline, or model intercept. To evaluate pairwise differences between two means, the final model was refit without and intercept, and 95% confidence intervals (CI) were calculated from the resulting estimated group means and standard errors. A lack of overlap in the 95% CI indicated a significant difference between two means.

B1. Labile C availability

There was no difference among models when comparing a generalized least squares (gls) regression fit to mixed models containing random intercepts (*i.e.* plot) or random intercepts plus random slopes (*i.e.* plot and warming, plot and N additions). Adding an autocorrelation structure to the gls model did not improve model fit, as indicated by higher AIC values for all candidate autocorrelation structures. However, adding a constant variance structure, with season as the grouping factor, decreased AIC (ΔAIC = 6.75). Selection for fixed effects indicated that the three-way interaction among warming × N × season was not significant ($L = 3.99$, $P = 0.26$). Two-way interactions between warming × N and warming × season were also not significant and were omitted from the model (warming × N: $L = 0.12$, $P = 0.73$; warming × season: $L = 0.26$, $P = 0.97$). However, the interaction between N × season was significant ($L = 7.64$, $P = 0.05$). Testing for the significance of the main effect of warming showed that it did not improve model fit ($L = 1.95$, $P = 0.16$). The final model included fixed effects for N, season, and the interaction between them.

ln (Labile C) ~ N × Season

Fixed Effect	Estimate	SE	DF	*t*-value	*P*-value
Intercept	6.36	0.12	86	51.47	< 0.0001
April	0.28	0.17	86	1.73	0.09
July	0.23	0.18	86	1.28	0.21
October	0.87	0.14	86	6.40	< 0.0001
Nitrogen	0.36	0.17	86	2.06	0.04
Nitrogen × April	-0.55	0.23	86	-2.35	0.02
Nitrogen × July	-0.60	0.25	86	-2.42	0.02
Nitrogen × October	-0.39	0.19	86	-2.03	0.05

B2. Beta-glucosidase

A random intercept model showed a significantly better fit than a gls model (ΔAIC = 4.04, $P = 0.014$). Adding a first-order autoregressive autocorrelation structure to the random intercept model improved model fit (ΔAIC = 174.17). A constant variance structure, with season as the grouping factor further decreased AIC (ΔAIC = 75.66). In the fixed effects portion of the model, none of the interaction terms were significant. The four-way interaction between warming × N × season × temperature was not significantly different from a model fit with three-way interactions among these variables ($P > 0.05$). Backward selection of three-way versus two-way interactions, and subsequently two-way versus one-way models also indicated no significant differences in model fits. The main effects of warming, N additions, and temperature were also removed from the model, such that the final model included only the effect of season.

ln (β-Glucosidase) ~ Season

Fixed Effect	Estimate	SE	DF	*t*-value	*P*-value
Intercept	3.82	0.30	215	12.86	< 0.0001
April	-1.16	0.21	215	-5.50	< 0.0001
July	-0.04	0.07	215	-0.61	0.54
October	0.15	0.10	215	1.51	0.13

B3. Phenol peroxidase

Compared to a gls model, a random intercept model with plot as the random effect substantially improved mode fit ($P < 0.0001$, ΔAIC = 41.90). A first-order autoregressive autocorrelation (corAR1) structure showed a better fit than the random intercept model (ΔAIC = 241.18). A constant variance structure, with season as the grouping factor further decreased AIC (ΔAIC = 2.04). Neither the four-way nor the three-way interactions were significant ($P > 0.05$). However, there were significant two-way interactions between warming × temperature ($L = 5.99$, $P = 0.01$) and season × temperature ($L = 22.46$, $P < 0.0001$) as compared to a model without these interactions. Selection of main effects resulted in the deletion of N from the model ($L = 0.04$, $P = 0.84$). The final model contained main effects of warming, season, temperature, and interactions between warming × temperature and season × temperature.

ln (Phenol Peroxidase) ~ Warming × Temperature + Season × Temperature

Fixed Effect	Estimate	SE	DF	*t*-value	*P*-value
Intercept	0.47	0.22	241	2.14	0.03
April	0.30	0.10	241	2.90	0.004
July	0.44	0.11	241	4.12	< 0.0001
October	0.35	0.11	241	3.34	0.001
Temperature	0.03	0.00	241	6.61	< 0.0001
Warming	0.25	0.27	22	0.93	0.36
Warming × Temperature	-0.01	0.00	241	-2.43	0.02
April × Temperature	-0.02	0.01	241	-3.56	< 0.0001
July × Temperature	-0.03	0.01	241	-4.38	< 0.0001
October × Temperature	-0.03	0.01	241	-4.53	< 0.0001

B4. Total Biomass, O Horizon

There was no difference among models when comparing a gls regression fit to mixed models containing random intercepts or random intercepts plus random slopes. While adding a first-order autoregressive autocorrelation (corAR1) slightly improved model fit (ΔAIC = 1.23), the lowest AIC was obtained when a constant variance, with warming as the grouping factor, was included without an accompanying autocorrelation structure (ΔAIC = 2.96). The three-way interaction, warming × N × season, was significant (L = 12.39, p = 0.006), and was preserved in the final model.

ln (Total Biomass) ~ Warming × N × Season

Fixed Effect	Estimate	SE	DF	*t*-value	*P*-value
Intercept	4.78	0.20	32	24.12	< 0.0001
April	-0.02	0.28	32	-0.06	0.95
July	0.40	0.28	32	1.44	0.16
October	0.95	0.28	32	3.38	0.002
Warming	0.24	0.40	32	0.59	0.56
Warming x April	0.20	0.20	32	0.36	0.72
Warming × July	-1.04	0.57	32	-1.82	0.08
Warming × October	-1.13	0.57	32	-1.98	0.06
Nitrogen	0.31	0.28	32	1.11	0.28
Nitrogen × April	0.09	0.40	32	0.22	0.83
Nitrogen × July	-0.66	0.40	32	-1.67	0.10
Nitrogen × October	-2.06	0.40	32	-5.20	< 0.0001
Warming × N	-0.45	0.57	32	-0.80	0.43
Warming × N × April	-0.19	0.81	32	-0.24	0.81
Warming × N × July	1.34	0.81	32	1.66	0.11
Warming × N × October	1.94	0.81	32	2.41	0.02

B5. Fungal Biomass, O Horizon

Neither random intercept nor random slope models improved fit compared to a gls model. The inclusion of a first-order autoregressive autocorrelation slightly improved model fit (ΔAIC = 2.60). However, the best model fit occurred with no autocorrelation structure, but a constant variance structure with season as the grouping factor (ΔAIC = 5.90). The three-way interaction among warming, N additions, and season was significant (L = 9.06, P = 0.03), and was included in the final model.

ln (Fungal Biomass) ~ Warming × N × Season

Fixed Effect	Estimate	SE	DF	*t*-value	*P*-value
Intercept	3.67	0.26	32	13.96	< 0.0001
April	-0.14	0.29	32	-0.49	0.63
July	0.36	0.38	32	0.95	0.35
October	0.49	0.62	32	0.78	0.44
Warming	0.25	0.37	32	0.67	0.63
Warming × April	0.09	0.41	32	0.22	0.82
Warming × July	-1.07	0.54	32	-1.97	0.06
Warming × October	-1.26	0.88	32	-1.43	0.16
Nitrogen	0.33	0.37	32	0.90	0.38
Nitrogen × April	0.01	0.58	32	0.02	0.99
Nitrogen × July	-0.62	0.54	32	-1.15	0.26
Nitrogen × October	-2.55	0.88	32	-2.89	0.01
Warming × N	-0.61	0.53	32	-1.16	0.26
Warming × N × April	0.00	0.57	32	0.01	0.99
Warming × N × July	1.36	0.77	32	1.77	0.09
Warming × N × October	2.47	1.25	32	1.98	0.06

B6. Bacterial Biomass, O Horizon

Random intercept and random slope models did not improve model fit, nor did the addition of an autocorrelation structure. The inclusion of a constant variance structure, with warming as the grouping factor, produced the best model fit (ΔAIC = 2.71). There was a significant interaction for warming × N × season (L = 12.44, P = 0.006), which was included in the final model.

ln (Bacterial Biomass) ~ Warming × N × Season

Fixed Effect	Estimate	SE	DF	t-value	P-value
Intercept	4.38	0.19	32	22.76	< 0.0001
April	0.02	0.27	32	0.09	0.93
July	0.42	0.27	32	1.55	0.131
October	1.08	0.27	32	3.95	< 0.001
Warming	0.23	0.39	32	0.60	0.55
Warming × April	0.26	0.55	32	0.47	0.64
Warming × July	-1.02	0.55	32	-1.86	0.07
Warming × October	-1.10	0.55	32	-2.01	0.05
Nitrogen	0.30	0.27	32	1.09	0.28
Nitrogen × April	0.14	0.39	32	0.36	0.72
Nitrogen × July	-0.68	0.39	32	-1.77	0.09
Nitrogen × October	-1.92	0.39	32	-4.98	< 0.0001
Warming × N	-0.38	0.55	32	-0.70	0.45
Warming × N × April	-0.29	0.77	32	-0.38	0.71
Warming × N × July	1.33	0.77	32	1.72	0.10
Warming × N × October	1.75	0.77	32	2.26	0.03

B7. F:B Ratio, O Horizon

There was no difference among models when comparing a gls fit to models containing either a random intercept or a random intercept and random slope. Including an autocorrelation structure did not improve model fit, but a constant variance structure, with season as the grouping factor, reduced AIC (ΔAIC = 27.55). Selection for fixed effects indicated that the three-way interaction among warming × N × season was not significant ($L = 2.23$, $P = 0.53$). Two-way interactions between warming × season and N × season were also no significant ($P > 0.05$). While a two-way interaction between warming x N was marginally significant ($L = 3.71$, $P = 0.05$), t-values and P-values obtained for a model with this interaction were also only of marginal significance ($t = -1.92$, $P = 0.06$). As a result, this interaction was removed from the final model. The main effect of N was also omitted for lack of significance ($P > 0.05$). The final model contained fixed effects for season and warming, but no interaction between them.

ln (F:B) ~ Warming + Season

Fixed Effect	Estimate	SE	DF	t-value	P-value
Intercept	-0.69	0.05	43	-15.1026	< 0.0001
Warming	-0.11	0.05	43	-2.3638	0.02
April	-0.24	0.07	43	-3.25541	0.002
July	-0.04	0.05	43	-0.84653	0.40
October	-0.80	0.20	43	-4.08823	0.0002

B8. Total Biomass, Mineral Horizon

Neither random intercept nor random slope models improved model fit when compared to a gls model. Likewise, adding a covariance structure did not improve model fit, but a compound symmetry autocorrelation structure lowered AIC (ΔAIC = 1.02). The three-way interaction among warming, N additions, and season was not significant ($L = 3.39$, $P = 0.34$), nor was the two-way interaction between N × season ($L = 3.46$, $P = 0.33$). The two-way interactions between warming × season and warming x N were both significant (warming × season: $L = 9.61$, $P = 0.02$; warming × N: $L = 3.94$, $P = 0.05$). However, the warming × N interaction was only marginal, and t-values and P-values obtained for a model with this interaction were also marginal ($t = -1.92$, $P = 0.06$). As a result, this interaction was removed from the final model. The main effect of N was also omitted ($L = 0.33$, $P = 0.56$). The final model included fixed effects for season and warming, plus an interaction between them.

ln (Total Biomass) ~ Warming × Season

Fixed Effect	Estimate	SE	DF	t-value	P-value
Intercept	4.71	0.14	40	33.46	< 0.0001
April	-0.30	0.22	40	-1.38	0.18
July	-0.55	0.22	40	-2.55	0.01
October	0.16	0.22	40	0.74	0.46
Warming	-0.41	0.20	40	-2.07	0.05
Warming × April	0.52	0.31	40	1.70	0.10
Warming × July	0.87	0.31	40	2.85	0.007
Warming × October	0.37	0.31	40	1.23	0.23

B9. Fungal Biomass, Mineral Horizon

Random intercept and random slope models did not improve overall fit as compared to a gls model. While the inclusion on an autocorrelation structure did not enhance model fit, a constant variance structure, with warming as the grouping factor, lowered AIC (ΔAIC = 0.97). Selection for fixed effects indicated that the three-way interaction among warming × N × season was not significant ($L = 3.05$, $P = 0.56$). Two-way interactions between warming × N and N × season were also not significant ($P > 0.05$), and therefore were removed from the model. However, the two-way interaction between warming × season was significant ($L = 10.28$, $P = 0.02$). The main effect of N was omitted from the final model due to lack of significance. The final model included fixed effects for warming and season and the interaction between them.

ln (Fungal Biomass) ~ Warming × Season

Fixed Effect	Estimate	SE	DF	*t*-value	*P*-value
Intercept	3.79	0.15	40	24.69	< 0.0001
April	-0.40	0.22	40	-1.84	0.07
July	-0.69	0.22	40	-3.17	0.003
October	0.00	0.22	40	0.02	0.98
Warming	-0.49	0.20	40	-2.45	0.02
Warming × April	0.45	0.29	40	1.58	0.12
Warming × July	0.83	0.29	40	2.92	0.006
Warming × October	0.35	0.29	40	1.23	0.23

B10. Bacterial Biomass, Mineral Horizon

Including either a random intercept or a random slope did not improve overall model fit. The addition of an autocorrelation structure or a variance also did not improve the fit of the model. As a result, the data were considered to have met the assumptions of equal variance and equal correlation necessary for linear modeling. The three-way interaction, warming × N × season, was not significant ($L = 4.37$, $P = 0.22$). Two-way interactions between warming × N and N × season were also not significant ($P > 0.05$) and were omitted. The two-way interaction between warming × season was significant ($L = 11.36$, $P = 0.01$), and was included in the final model. The main effect of N was removed due to lack of significance, such that the final model contained fixed effects for warming, season, and the interaction between the two.

ln (Bacterial Biomass) ~ Warming × Season

Fixed Effect	Estimate	SE	DF	*t*-value	*P*-value
Intercept	4.19	0.15	40	28.76	< 0.0001
April	-0.24	0.21	40	-1.16	0.26
July	-0.47	0.21	40	-2.30	0.03
October	0.24	0.21	40	1.18	0.25
Warming	-0.36	0.21	40	-1.75	0.09
Warming × April	0.55	0.29	40	1.88	0.07
Warming × July	0.88	0.29	40	3.02	0.004
Warming × October	0.38	0.29	40	1.32	0.20

B11. F:B Ratio, Mineral Horizon

There was no difference among models that contained random intercepts and /or random slopes when compared to a gls model. Adding an autocorrelation structure did not improve model fit, but a constant variance structure, with N as the grouping factor, reduced AIC (ΔAIC = 1.00). Neither three-way nor two-way interactions were significant ($P > 0.05$), and were removed from the model. The final model contained fixed effects for warming, N additions, and season, but no interactions among them.

ln (F:B) ~ Warming + N + Season

Fixed Effect	Estimate	SE	DF	*t*-value	*P*-value
Intercept	-0.32	0.05	42	-6.22	< 0.0001
April	-0.22	0.06	42	-3.50	0.001
July	-0.25	0.06	42	-4.06	0.002
October	-0.25	0.06	42	-3.94	0.003
Warming	-0.18	0.04	42	-4.08	0.0002
Nitrogen	-0.11	0.05	42	-2.23	0.03

APPENDIX C

Random effects, autocorrelation structures, and final models selected for percent original mass, carbon, nitrogen, and lignin remaining in decomposing woody litter.

Variable	Random effects	*L*	Δ DF	*P*	Autocorrelation	Δ AIC	Δ DF
Mass	Intercept	14.49	1	0.00	none	—	—
Nitrogen	None	—	—	—	corAR1	0.98	1
Lignin	Intercept	8.96	1	0.00	none	—	—

Appendix C, contd.

Variable	Final Model
Mass	Mass ~ W × N + Inc + Time + N × Time
Nitrogen	Nitrogen ~ N × Inc
Lignin	Lignin ~ W × N + Inc + Time + N x Time

Notes: For the random effects, plot was modeled as a random intercept and warming and / or N additions as a random slope. The likelihood ratio (*L*) and *P*-values refer to the random effects structure in *lme* tested against the *gls* model, with *P*-values shown in their uncorrected form. The delta degrees of freedom (Δ DF) column for random effects indicates the increase in degrees of freedom by adding random effects to a *gls* model. The Δ DF column for autocorrelations refers to the increase in degrees of freedom from adding an autocorrelation structure. The Δ AIC column indicates the decrease in AIC when adding an autocorrelation structure. The corAR1 abbreviation indicates a first-order autoregressive autocorrelation structure. The "W" refers to warming, "N" to N additions, "Study" to study duration, and "Inc" to length of incubation of decomposing litter.