UNIVERSITY OF CALIFORNIA,
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Adaptation of soil fungi to warming and consequences for decomposition and the carbon cycle

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

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2017

DEDICATION

To

my mom & dad, and Ramón & Pedro, always

Jovani, forever

Para

mi mamá y papá, y Ramón y Pedro, siempre

Jovani, para siempre

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ABSTRACT OF THE DISSERTATION

Adaptation of soil fungi to warming and consequences for decomposition and the carbon cycle

By

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Doctor of Philosophy in Biological Sciences

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Professor Kathleen K. Treseder, Chair

Studying soil carbon (C) losses and carbon dioxide (CO_2) feedbacks to the atmosphere under global climate change allows us to quantify and understand how our ecosystems are responding to warming. To accurately project the fate of the terrestrial C, we need to incorporate processes that are pivotal in shaping microbial communities that are responsible of processing the C in the soil. One of these processes is the evolutionary adaptation to warming which has been difficult to study because it may only be noticeable on the long term. The goal of my dissertation was to examine soil microbes, their response and adaptation to warming, and consequences to the C cycle. In Chapter 1, I synthesized data from 25 field warming experiments to assess the effect of microbial responses —relevant to the C cycle— to warming over time. I found that the effect of soil respiration decreases as warming progresses and explored the potential microbial-related causes of this decrease. In my second chapter, I experimentally adapted the model fungus *Neurospora discreta* to warming and analyzed physiological traits important for the C cycle before and after adaptation. I discovered that when *N. discreta* adapts to warming it

allocates more resources to increase its fitness by producing more spores at the expense of biomass. I found that adaptation to warming is accompanied by increases in CO_2 respiration potentially due to higher production of energetically expensive spores. In this chapter, I discussed the potential consequences for the terrestrial C if the soil microbial community adapts in a similar manner as *N. discreta*. Finally, in my third chapter, I quantified decomposition of specific C fractions in litter in a long-term field warming experiment. I found that the proportional losses of recalcitrant vs non-recalcitrant C was higher in warmed plots compared to control plots. Similarly, the ratio of microbial extracellular enzyme activities responsible for breaking down recalcitrant C was higher under warming compared to enzymes that break down non-recalcitrant C. Collectively, in my dissertation research I integrated the process of evolutionary adaptation of microbes to warming, thus providing an overview of the potential long-term effects of warming to decomposition and the C cycle.

INTRODUCTION

Carbon dioxide (CO_2), a greenhouse gas, reached historical high levels in Earth's atmosphere in 2016 (Dlugokencky and Tans, 2017). Although anthropogenic CO_2 emissions have been increasing at unprecedented rates in the past century (IPCC, 2014), soils are a significant, natural, and constant source of CO_2 (Schimel et al., 1994). A portion of this natural source is the result of microbial decomposition of soil organic matter (SOM) in which soil carbon (C) is returned to the atmosphere as CO_2 (Schimel, 1995). This process is highly temperature sensitive and thus vulnerable to global warming (Davidson and Janssens, 2006; von Lützow and Kögel-Knabner, 2009). Because soils are the largest terrestrial carbon pool (Lal, 2004; Scharlemann et al., 2014), they represent a large C source. Under global warming, decomposition rates may increase and return soil C to the atmosphere as CO_2; these CO_2 emissions can add to anthropogenic CO_2 emissions and worsen global climate change (Davidson and Janssens, 2006; Rustad et al., 2000).

A central goal in ecosystem ecology is to develop a comprehensive understanding of how our ecosystems are responding to warming, and quantify decomposition rates and CO_2 feedbacks to the atmosphere (Rustad, 2008). To make progress towards this goal, in the past two decades, research efforts have focused on emulating global warming using warming experiments to monitor changes in soil respiration, microbial community composition, microbial biomass, litter decomposition, soil nutrients, among others (Cregger et al., 2014; Kirschbaum, 2004; Luo et al., 2001; Riikka Rinnan et al., 2007; Treseder et al., 2016; van Meeteren et al., 2008; Zelikova et al., 2012). Initial observations uncovered that warming can accelerate plant productivity, increases nitrogen

mineralization, and enhances soil respiration (Allison and Treseder, 2008; Arft et al., 1999; Rustad et al., 2001; Schaeffer et al., 2013; Xu et al., 2010).

However, inherent differences in warming experiments, including duration and intensity of warming, sampling efforts, and ecosystem characteristics hinder our ability to reach consensus on climate warming and feedbacks to the atmosphere on the long-term (Luo, 2007). For example, Luo and collaborators (2001) observed that soil respiration in a warming experiment decreased after short-term (i.e., ≤ 1 year) exposure to warming, but Flanagan and collaborators observed the opposite (2013). Moreover, after long-term (i.e., ≥ 5 years) experimental warming of soils, Schindlbacher et al. (2015) found no evidence of decreases in soil respiration, but Melillo and collaborators (2002) did. While these studies were aiming to characterize the response of ecosystems to global warming by using warming experiments, opposing results evidenced missing key elements that needed to be incorporated to better understand ecosystem-scale observations. In fact, further research suggested that differences in the composition of soil C and litter (i.e., labile vs recalcitrant content), as well as a closer look to the microbial community, might explain ecosystem-level responses to warming and provide insights into future climate under global climate change (Biasi et al., 2005; Bradford et al., 2008; Melillo et al., 2002).

Microorganisms, fungi particularly, are decomposition powerhouses. They have the molecular machinery to decompose all types of C, from simple sugars (i.e., labile) to complex macro-molecules such as lignin (i.e., recalcitrant) (Cooke and Rayner, 1984; Osono, 2007; Schneider et al., 2012). Fungi inhabit soils of all ecosystems in the world and are abundant in boreal forests (Hättenschwiler et al., 2005) which are threatened by global warming (Allison and Treseder, 2011; Bonan, 2008). Here, fungi are the main decomposers

of SOM (Hudson, 1968); they break down soil C into simple molecules that are easily assimilated by plants and other microorganisms (i.e., bacteria), and release CO_2 as a by-product of decomposition. Studying the response of fungi to warming will shed light on the ecosystem-level implications of climate change such as soil C loss and CO_2 feedbacks to the atmosphere.

The composition of soil C fractions is vital for understanding the susceptibility of soils to global warming and thus CO_2 feedbacks to the atmosphere. Soils of ecosystems vulnerable to global warming, such as boreal forests, store approximately 1700 billion tons of C (Allison and Treseder, 2011; Schlesinger, 1977), of which approximately 30% is recalcitrant (Boerjan et al., 2003). Recalcitrant C pools in boreal forests have been disconnected from the active C cycle, but changes in temperature might induce the availability of recalcitrant C (Arrhenius, 1896; Davidson and Janssens, 2006) and favor proliferation of recalcitrant C-decomposers (Pold et al., 2015; Treseder et al., 2016). Consequently, turnover rates of recalcitrant C may increase, so that recalcitrant C stocks are returned to the atmosphere as CO_2, thereby providing a positive feedback to global warming (Davidson and Janssens, 2006; IPCC, 2014).

A critical process that has been overlooked in global warming science is the evolutionary adaptation of microbes. This process may only be noticeable in the long term but it is required for projecting accurately ecosystems' long-term responses to warming. Fungal evolutionary adaptation is a time-consuming process that is difficult —if not impossible— to measure in the wild, thus is not well understood (Pringle and Taylor, 2002). Integrating the process of fungal evolutionary adaptation to warming to ecosystem-level ecology, such as soil respiration and decomposition, represents a critical challenge (Pastor,

2016). Given that fungi are inconspicuous organisms that inhabit soils, keeping track of their growth and reproduction using current technologies is not possible. However, disparities among global warming projections may be greatly reduced if studied under the light of evolution (i.e., Dobzhansky, 1973).

In my dissertation, I examined the response to warming at the organism- and ecosystem-level in the context of evolutionary adaptation, and assessed consequences to the terrestrial C cycle and feedbacks to global warming. My goal was to answer three main questions:

1. How do microbes respond to warming over time and what are the consequences to the C cycle?
2. What physiological changes coincide with fungal adaptation to warming and how can it feedback to global climate change?
3. How do decomposition patterns change in response to warming and which underlying mechanism might be driving this change?

To address the first question, Chapter 1 is a meta-analysis of 25 field warming experiments across 11 different types of ecosystems, where I examined how warming effects on soil respiration, microbial biomass, and soil C respond to the duration of warming. I synthesized the findings of 52 studies, ranging from 1 to 15 years, and analyzed if changes in the effect of soil respiration were similar to those of microbial biomass and soil C. This chapter provided an overview on how the effect of soil respiration changes across the duration of warming and how short-term warming responses are different from long-term responses. With this analysis, I reached consensus on the effect of soil respiration in warming experiments but found few data regarding microbial responses,

both on the short- and long-term. This chapter is published as a review in *Soil Biology & Biochemistry*.

In Chapter 2, I address the second question by experimentally adapting wild strains of the fungus *Neurospora discreta* to warm temperatures of 16 °C and 28 °C. I assessed physiological traits that can be important for C cycle dynamics under global warming, such as growth rate, biomass, spore production, and CO_2 respiration, before and after adaptation. I evaluated the fitness and the evolutionary tradeoffs as a result of adaptation to warming and proposed how adaptation of fungi might feedback to global climate change. In this chapter, I found evidence for adaptation to warming, based on increased fitness in the selective temperature and evolutionary tradeoffs as a result of adaptation. The implications of increased fitness and evolutionary tradeoffs have the potential to impact C cycle dynamics. The results of this chapter are published in *BMC Evolutionary Biology*.

Finally, in Chapter 3, I estimated the decomposition of recalcitrant C under warming in a long-term warming experiment in a boreal ecosystem in Alaska. Here, I used litterbags with senescent spruce needles to measure mass loss, recalcitrant (i.e., lignin) and non-recalcitrant C (i.e., soluble sugars, cellulose, and hemicellulose) loss, and C-degrading microbial extracellular enzyme activities (EEA). I measured changes in decomposition at 1, 2, 12, and 16 months to gain insights on decomposition dynamics under global warming to better assess soil C change in the next century. Here, I show that the loss of recalcitrant C fractions compared to non-recalcitrant C fractions might be higher under warming. In other words, warming may change decomposition dynamics and impact soil C stocks. This manuscript is currently in revision in *PLoS ONE*.

Taken together, my dissertation offers insights into ecosystems' responses to global warming from a microbial perspective. I integrated the process of evolutionary adaptation of fungi into C cycle dynamics, and provided an overview of the fate of the terrestrial C pools under global warming and potential CO_2 feedbacks to the atmosphere in the long-term. I show that under the light of evolution, global warming is an evolutionary force at the organism-level with implications at the ecosystem-level. Only by implementing long-term observations, when evolutionary adaptation is operating, we will obtain accurate climate change projections.

CHAPTER 1: Soil microbes and their response to experimental warming over time: a meta-analysis of field studies

INTRODUCTION

To predict the effects of global warming on ecosystems, researchers have manipulated soil and air temperatures in numerous field experiments (Carey et al., 2016). Although some warming experiments have lasted over a decade (Dorrepaal et al., 2009; Melillo et al., 2011, 2002; Rousk et al., 2013), the majority have been shorter. Therefore, the long-term effects of field experimental warming on ecosystem functions have been challenging to examine. Here we focus on microbial responses to warming, because their contributions to soil CO_2 respiration can influence future trajectories of climate change (Wieder et al., 2013). In an earlier meta-analysis, Rustad et al. (2001) noted that warming generally increased soil respiration across 16 field studies. Nevertheless, at that time, these studies represented relatively short warming periods of six years or less. Whether soil respiration remains elevated or returns to baseline levels under longer-term warming has been subject to debate. Some studies have reported a decrease in warming effects over time (Luo et al., 2001; Melillo et al., 2002), whereas others have documented no significant change (Schindlbacher et al., 2011). Thus, an examination of the temporal trends in responses of ecosystems to warming should shed light on long-term feedbacks between soils and climate (Allison and Treseder, 2011; Pold and DeAngelis, 2013).

Warming might initially stimulate decomposition by enhancing the metabolism of decomposers, provoking increases in microbial CO_2 production (Lloyd and Taylor, 1994). This could lead to soil C losses, higher soil respiration rates, and an overall positive feedback to global warming (Jenkinson et al., 1991). However, this response can be transient (Luo et al., 2001). For example, in Prospect Hill at Harvard Forest, soil respiration

rates in warmed plots were higher than those in the controls for the first few years, but the warming effect declined over time and eventually became non-significant (Giasson et al., 2013; Melillo et al., 2002). Several mechanisms could drive this pattern by altering microbial C use as warming proceeds (Allison et al., 2010b; Bradford et al., 2008; Frey et al., 2013; Pritchard, 2011; Rousk et al., 2012; Sierra et al., 2010). These include acclimation of individual microbes (Allison et al., 2010b; Crowther and Bradford, 2013; Malcolm et al., 2008; Tucker et al., 2013; Yuste et al., 2010), shifts in microbial communities (Bárcenas-Moreno et al., 2009; Luo et al., 2014; Rousk et al., 2012; Treseder et al., 2016; Wei et al., 2014), and evolutionary adaptation of microbial populations to higher temperatures (Romero-Olivares et al., 2015). In addition, labile C pools in the soils could become depleted owing to higher microbial activity (Bradford et al., 2008; Eliasson et al., 2005; Kirschbaum, 2004; McHale et al., 1998). These mechanisms are non-exclusive, and their influence may vary among seasons (Contosta et al., 2015), ecosystems, and across time scales.

To improve predictions of long-term consequences on soil C, we must determine whether warming effect sizes on soil respiration and microbial abundance diminish over time, and how quickly this occurs. Meta-analysis is a rigorous statistical tool that can address these questions; it combines quantitative data from previously published studies to reach conclusions with greater statistical power. For example, several meta-analyses have determined that experimental warming generally increases soil respiration, soil microbial abundance, net N mineralization, decomposition, soil microbial C and N, net primary production, and photosynthesis (García-Palacios et al., 2015; Lu et al., 2013; Rustad et al., 2001; Zhang et al., 2015). A recent meta-analysis also showed that the temperature sensitivity of soil respiration does not change with experimental warming in

many ecosystems (Carey et al., 2016). Although these meta-analyses have contributed greatly to our knowledge of the response of ecosystems to warming, none has focused on trends over time.

Toward this end, we used meta-analysis to analyze the effect of field experimental warming over time on soil respiration, fungal biomass, bacterial biomass, and soil microbial C. We chose these parameters because they govern large ecosystem-scale processes affected by global warming, such as CO_2 inputs to the atmosphere through soil C losses (Allison et al., 2010a; Šantručková and SiraŠicraba, 1991; Wang et al., 2003). We compiled data from field-based experimental warming studies that varied in duration from 1 to 15 years. We asked, how do warming effects change as duration of warming increases? We hypothesized that warming effects on each parameter would diminish as duration of warming increased.

MATERIALS AND METHODS

Literature survey

We searched the ISI Web of Science and Google Scholar for published papers reporting the response of soil fungal and bacterial biomass, soil respiration, and soil microbial C to experimentally warmed soils and its respective controls. We performed separate literature searches for each of the following terms: “soil microb* experimental warming”, “soil fung* experimental warming”, “soil bacter* experimental warming”, “soil resp* experimental warming”. In addition, we manually searched for papers published in previous meta-analyses (Arft et al., 1999; García-Palacios et al., 2015; Lu et al., 2013; Rustad et al., 2001; Wu et al., 2011; Zhang et al., 2015) and review papers (Allison and Treseder, 2011; Giasson et al., 2013; Pold and DeAngelis, 2013). To complete our data

collection, we used the geographic coordinates of the experimental plots as search terms, to account for all published studies conducted in the same experimental plots but missed by our initial search terms. Our literature search included papers published (or accepted for publication) between January 1994 and July 2015. We excluded studies manipulating factors other than temperature, unless a split-plot design was used and a single subplot for the temperature effect was present.

A total of 52 studies met our search criteria, representing 25 field warming experiments across 11 different types of ecosystems, and a total duration of warming ranging from 1 to 15 years (Table 1.1). Measurements that were taken from the same unique set of field plots were considered as belonging to the same experiment.

Data acquisition

For each experiment, we recorded the mean, standard deviation (SD), standard error (SE), and sample size (n), of both warmed and control plots, for fungal and bacterial biomass, soil respiration, and soil microbial C. The data were extracted directly from tables, published supplementary material, and from graphs using Plot Digitizer 2.6.6 (http://plotdigitizer.sourceforge.net). In addition, we recorded the type of warming (e.g., infrared heater, open top chamber, closed top chamber, buried heating cables), the duration of warming, and other information such as type of ecosystem, mean annual temperature, mean annual precipitation, magnitude of soil warming, change in soil moisture, and geographic coordinates (Table 1.1). If SEs were presented instead of SDs, we used the formula $SD = SE (n^{1/2})$ to obtain SDs. Any unidentified error bars were assumed to represent SE (Peng et al., 2014).

Soil respiration, fungal & bacterial biomass, and soil microbial C

Soil respiration was measured in all studies by an in situ CO_2 flux chamber, with one exception where authors used a gas headspace with isotope mass spectrometer. To measure fungal biomass, authors used a variety of techniques; total phospholipid fatty acids (PLFA) analysis was the most common (19 out of 21 experiments used this method). The remaining two experiments used either total fatty acids methyl esters (FAME) or microscopy (i.e.,hyphal lengths). Similarly, bacterial biomass was quantified through PLFA, in all but one experiment where microscopy was the preferred quantification method. Moreover, soil microbial C was measured through chloroform fumigation extraction in all studies.

Statistics

We used meta-analysis to determine warming effects on soil respiration, fungal biomass, bacterial biomass, and soil microbial C. For each experiment and each response variable, we calculated the effect size as the natural logarithm of the response ratio (lnR). First, we averaged all sampling time points per year within each experimental plot, to remove seasonal-level variation. Then, with the averaged data, we calculated the response ratio of the mean of the treatment group (warmed) divided by the mean of the control group (unwarmed). An lnR of 0 indicates that warming had no effect on the response variables. We also calculated the variance (V_R) using the means, n, and SD of both treatments (Table 1.2). To calculate lnR and V_R, we used MetaWin software (Rosenberg et al., 2001).

We tested our hypothesis for each soil parameter separately. In each case, we used a linear mixed-effects model fitted with a restricted maximal likelihood (REML) approach ("nlme" R package) (R Core Development Team, 2009). This structure allowed us to

account for non-independence of repeated measurements within experiments, by essentially nesting measurements within experiment. Experiments were defined as unique sets of field plots. For each test, warming effect size (lnR) of soil respiration (or fungal biomass, bacterial biomass, or microbial C) was the dependent variable, duration of warming was the independent variable, and experiment ID was a random effect. In separate analyses, we tested if the magnitude of soil warming (or change in soil moisture) also influenced the effect size of soil respiration. Specifically, we tested whether lnR (dependent variable) was significantly related to magnitude of warming, duration of warming, or the interaction between magnitude and duration (independent variables). Similarly, we tested for significant relationships between lnR (dependent variable) and change in soil moisture or duration of warming (independent variables). In the latter case, we did not test for an interaction between change in soil moisture and duration of warming, because substantial (>10%) declines in soil moisture were only reported for studies that lasted 6 years or less. For all analyses, data were weighted by the reciprocal of V_R, which is a standard approach for meta-analyses (Gurevitch and Hedges, 1999). Significant decreases in lnR with duration of warming would support our hypothesis.

RESULTS

Soil respiration was measured in 19 experiments. In support of our hypothesis, warming effect sizes declined significantly with duration of warming (Fig. 1.1, $t = -2.230$, $P = 0.031$). Initially, warming increased soil respiration by 46 ± 8% across studies (y-intercept of linear mixed-effects model, $P < 0.001$). Yet, the magnitude of this warming effect decreased over time, so that after 10 years, soil respiration in the warmed treatments was near that of the controls. The attenuation of the warming effect is also evident within

individual studies. Specifically, in all but one of the studies with ≥4 years of measurements (i.e.,MRS, Niinistö et al., 2004), warming effect sizes tended to decline over time (Fig. 1.2). When magnitude of warming and duration of warming were both included as independent variables, magnitude of warming was not significantly related to lnR of soil respiration ($t = -0.471$, $P = 0.640$), nor was there a significant interaction between magnitude and duration of warming ($t = 1.732$, $P = 0.091$); duration of warming remained significant ($t = -2.723$, $P = 0.010$). Likewise, change in soil moisture did not significantly influence lnR of soil respiration ($t = 1.507$, $P = 0.1514$), and duration of warming still had a significant effect ($t = -2.508$, $P = 0.016$) when soil moisture was included in the model.

Fungal and bacterial biomass was reported in 10 and 9 experiments respectively, ranging from 1 to 13 years after warming began. There was no indication, however, of significant declines in effect size with duration of warming for either fungi (Fig. 1.3, $t = -1.529$, $P = 0.157$) or bacteria (Fig. 1.4, $t = -0.109$, $P = 0.916$). Microbial C was measured at 1 to 15 years of warming, across nine experiments. Again, effect sizes of microbial C did not decrease with duration of warming (Fig. 1.5, $t = 1.464$, $P = 0.169$). As such, we rejected our hypothesis with respect to fungal biomass, bacterial biomass, and microbial C.

DISCUSSION

In our meta-analysis of field experiments, we found that warming effects on soil respiration diminished significantly over time, with declines most evident after a decade of warming (Fig. 1.1). Although increases in soil respiration are often observed within the first few years of warming (Flanagan et al., 2013; Melillo et al., 2002; Niinistö et al., 2004; Peng et al., 2015; Peterjohn et al., 1994), our results suggest that this response is transient. We found two lines of evidence for attenuation of warming effects on soil respiration. First,

effect sizes declined significantly with warming duration when data from all studies were combined. Second, this trend was apparent within individual studies in which soil respiration had been measured over four years or more (Fig. 1.2). In fact, despite temporal variations, in seven of the eight studies that met this criterion, the warming effect size on soil respiration tended to decrease with time. In fact, in the study in which soil respiration increased at 4 years (MRS), authors acknowledged that in the fourth year of warming, measurements were taken in warmer days compared to years two and three. This difference amplified the results between control and treatment "such that the response in the fourth year became equivalent to that of the first" (Niinistö et al., 2004). Altogether, our results suggest that long-term effects of warming on soil C dynamics may be weaker than suggested by initial responses.

Our meta-analysis is the first to focus on changes in warming effect sizes on soil respiration throughout the duration of field experiments lasting more than 10 years. Previously, Rustad et al. (2001) noted that the mean effect size of warming on soil respiration tended to be smaller (albeit non-significantly) in studies that lasted more than three years. Nevertheless, at that time, the longest studies included in that comparison were five years. Lu and collaborators (2013) contrasted effect sizes on soil respiration for short-term (<5 years) versus intermediate-term (5–10 years) studies. They reported that the mean effect size of soil respiration did not differ significantly between the two categories. Moreover, Zhou et al. (2016) found no significant relationship between warming duration and effect size of soil respiration in studies with ≤6 years of warming. In the current meta-analysis, the decrease in effect sizes for soil respiration was most striking after 10 years of warming (Fig. 1.1), which highlights the importance of longer-term

studies. The attenuation in effect size of warming is especially noticeable in the two longest studies, Prospect Hill (PH) (Melillo et al., 2002) and Kessler's Farm Field (KFF) (Belay-Tedla et al., 2009; Li et al., 2013; Luo et al., 2009; Wan et al., 2005; Zhang et al., 2005) (Fig. 1.2). In both cases, effect sizes remained positive during the first 10 years; negative effect sizes were only observed after 10 years. Regarding microbial abundance, meta-analyses by García-Palacios et al. (2015), Wang et al. (2014), and Zhang et al. (2015) detected no significant effects of duration on effect sizes for fungal abundance, bacterial abundance, microbial biomass, or microbial C. These findings are similar to ours.

What might have driven this attenuation of the warming effect on soil respiration? Researchers have previously suggested that acclimation of soil microbes (Bradford et al., 2010; Crowther and Bradford, 2013; Malcolm et al., 2008; Tucker et al., 2013; Yuste et al., 2010), shifts in microbial community composition (Luo et al., 2014; Treseder et al., 2016; Wei et al., 2014), evolutionary adaptation of microbes (Romero-Olivares et al., 2015; Wallenstein and Hall, 2012), or depletion of labile C (Bradford et al., 2008; Eliasson et al., 2005; Kirschbaum, 2004; McHale et al., 1998) can be responsible. Any combination of these mechanisms could have influenced the temporal trends in soil respiration. Even though mean effect sizes for fungal biomass, bacterial biomass, and microbial C did not shift significantly with warming duration (Figs. 3–5), we cannot rule out acclimation, community shifts, or evolutionary adaptation in the microbial community, since each could alter microbial respiration rates without changing biomass.

Root respiration is a component of soil respiration rates reported in studies in our meta-analysis. Although most studies do not isolate the response to warming of the different components of soil respiration (i.e., microbial respiration vs root respiration),

some short-term studies have reported decreases in root respiration rates in response to warming (Zong et al., 2013) or no significant responses (Vogel et al., 2014). Nevertheless, it is challenging to partition root versus microbial respiration in a manner consistent enough to support a meta-analysis (Kelting et al., 1998; Sapronov and Kuzyakov, 2007). Consequently, we cannot discard the possibility that changes in the response of root respiration might have contributed to decreases in the response of soil respiration to long-term warming.

Because warming can increase evapotranspiration, it is possible that soil respiration and microbial biomass responses were affected by soil drying (Verburg et al., 1999). Although effects of soil moisture on microbial community composition and functioning might be an important factor, we did not observe any significant relationships between soil moisture change under warming and the soil respiration response, either on average or over time. Several studies have suggested a link between warming, reductions in soil moisture, and reductions in soil respiration at specific sites (Allison and Treseder, 2008; Bronson et al., 2008; Liu et al., 2009; Suseela et al., 2012) but this mechanism was not consistent across our larger dataset. Therefore soil drying does not appear to play a major role in the attenuation of soil respiration response to warming.

Our meta-analysis demonstrates that the increases previously reported in soil respiration in response to short-term warming (Bokhorst et al., 2007; Contosta et al., 2011; Flanagan et al., 2013; Niinistö et al., 2004; Schindlbacher et al., 2012; Wan et al., 2005) might be ephemeral as previously suggested (Eliasson et al., 2005; Luo et al., 2001; Oechel et al., 2000). Collectively, our results and these ideas suggest that ecosystems will lose soil C most quickly in the first several years after warming, and more slowly thereafter.

Therefore, release of CO_2 to the atmosphere may not be as extreme as suggested by short-term warming experiments. Nevertheless, our study was restricted by the scarcity of long-term warming experiments and equivocal responses of microbial biomass. As current warming experiments progress, repeated measurements of soil respiration and microbial abundance would be highly valuable.

CONCLUSION

Our meta-analysis shows that soil respiration decreases after long-term warming and suggests that soil C losses might not be as substantial as previously suggested by short-term warming experiments. We suggest that microbial community shifts, evolutionary adaptation, and/or depletion of labile soil C might be contributing to the attenuation of the effect size on soil respiration over time. These mechanisms should be further explored in laboratory and field settings, especially in long-term field warming experiments. We emphasize the importance of long-term warming studies, because 1) declines in mean effect sizes on soil respiration were most evident after 10 years, 2) short-term studies might be sensitive to temporal variations, and 3) long-term studies provide more data to partition temporal variation from long-term trends. Future research should incorporate microbial parameters obtained from long-term warming experiments to provide concise projections of the effects of climate change on the global C cycle.

TABLES

Table 1.1. Locations and characteristics of the field warming experiments included in this meta-analysis.

ID	Location	Geographic coordinates	Ecosystem type	Total duration of warming (years)	Type of warming	Change in soil temperature with warming (+ °C)	Change in soil moisture with warming (%)	Mean annual precipitation (mm y^{-1})	Mean annual temperature (°C)	Studies
BR	Beilu River Research Station, China	34°49′N, 92°56′E	Alpine grassland	3	Infrared heater/open top chamber	1.5	-0.4	291	3.8	Zhang et al. 2011; Peng et al. 2014, 2015
BW	Barre Woods, Harvard Forest, Massachusetts, United States	42.48°N, 72.18°W	Deciduous forest	7	Buried heating cables	5	-0.04	1110	7.6	Melillo et al. 2011
C	CLIMAITE experiment, Copenhagen	55°53'N, 11°58'E	Boreal forest	6	Infrared reflective curtain	1	-1.9	600	8	Andresen et al. 2009, 2014; Reinsch et al. 2014
DC	Duolun County, Inner Mongolia, China	42°02'N, 116°17'E	Alpine grassland	5	Infrared heater	0.9	-2.5	386	2.1	Liu et al. 2009; Zhang et al. 2011, 2013; Song et al. 2012; Zhou et al. 2013b; Shen et al. 2014
DJ	Delta Junction, Alaska, United States	63°55'N, 145°44'W	Boreal forest	6	Greenhouse	0.5	-30	303	-2	Allison & Treseder 2008; Allison et al. 2010; German & Allison 2015
GP	Great Plains, Alberta, Canada	49°28'N, 112°56'W	Semiarid grassland	1	Open top chamber	0.8	+8	386	5.1	Flanagan et al. 2013
HI	Howland Integrated Forest Study, Maine, United States	45°10'N, 68°40'W	Deciduous forest	3	Buried heating cables	5	-30	1000	6	Rustad and Fernandez, 1998
HF	Huntington Forest, New York, United States	43°59′N, 74°14′W	Deciduous forest	2	Buried heating cables	2.5	-20	1010	4.4	McHale et al. 1998
I	INCREASE field experiments, Oldebroekse heide, Netherlands	52°24′N, 5°55′E	Temperate grassland	13	Field scale night time warming/Infrared reflective curtain	0.5	-1.3	946	10	van Meeteren et al. 2008; Rousk et al. 2013

JR	Jasper Ridge Biological Preserve, Stanford, United States	37°40'N, 122°22'W	Semiarid grassland	6	Infrared heater	1	NA	400	14	Gutknecht et al. 2012
KFF	Kessler's Farm Field Laboratory, Oklahoma, United States	34°58'N, 97°31'W	Tallgrass prairie	13	Infrared heater	2	-0.65	967	16.3	Wan et al. 2005; Zhang et al. 2005; Belay-Tedla et al. 2009; Luo et al. 2009, 2014a; Li et al. 2013
MAI	Maritime Antarctic Islands, Signy Island	60°71'S, 45°59'W	Tundra	2	Open top chamber	0.8	-0.3	400	-2	Bokhorst et al. 2007
MES	Maoxian Ecological Station, China	31°41'N, 103°53'E	Subalpine forest	5	Infrared heater	3.7	-1.8	920	8.9	Yin et al. 2012
MRS	Mekrijärvi Research Station, Finland	62°47'N, 30°58'E	Boreal forest	4	Greenhouse	3	-1	667	3.1	Niinistö et al. 2004
NM	Nyainqentanglha Mountains, Tibetan Plateau, China	30°51'N, 91°05'E	Alpine meadow	2	Open top chamber	1.6	-3.7	477	1.3	Zong et al. 2013, Fu et al. 2012
NTL	North Tyrolean Limestone Alps, Austria	47°34′N, 11°38′E	Subalpine forest	9	Buried heating cables	4	-4.5	1480	5.7	Schindlbacher et al. 2009, 2011, 2012, 2015
PHA, PHB	Prospect Hill, Harvard Forest, Massachusetts, United States	42.5°N, 72.18°W	Deciduous forest	10	Buried heating cables	5	-0.03	1110	7.6	Peterjohn et al. 1994; Melillo et al. 2002; Frey et al. 2008; Contosta et al. 2011; Giasson et al. 2013
RSS	Research Station Songnen, China	44°45'N, 123°45'E	Temperate grassland	2	Infrared radiation	3	-0.01	410	4.9	Ma et al. 2011
SBB	Subarctic blanket bog, Abisko, Sweden	68°21'N, 18°49'E	Tundra	15	Open top chamber	1	-8	240	7.7	Rinnan et al. 2007, 2008, 2009, Dorrepaal et al. 2009
TFS	Takayama Field Station, Japan	36°08'N, 135°05'E	Temperate grassland	3	Infrared heater	1.4	-0.8	2128	7.1	Yoshitake et al. 2015
TLA, TLB	Toolik Lake, Alaska, United States	68°38'N, 149°34'W	Boreal forest	8, 4	Greenhouse, open top chamber	5.5, 2	NA	309	-7	Jones et al. 1998; Arft et al. 1999; Johnson et al. 2000
TM	Thompson, Manitoba, Canada	55°53'N, 98°20'W	Boreal forest	3	Greenhouse	1	NA	510	2.4	Vogel et al. 2014

UCP	Upper Colorado Plateau, Utah, United States	38°40'N, 109°24'W	Semiarid desert	2	Infrared heater	2	NA	240	12.5	Zelikova et al. 2012

Table 1.2. Effect sizes of microbial and soil parameters per year of warming.

Factor	Id	Duration of warming (y)	ln(R)	V_R
Soil respiration	BR	1	0.586	0.005
		2	0.434	0.007
		3	0.193	0.076
	BW	1	0.213	0.095
		2	0.061	0.059
		3	0.087	0.107
		4	0.233	0.041
		5	0.082	0.112
		6	0.100	0.091
		7	0.029	0.123
	DC	1	0.016	0.003
		2	-0.077	0.003
	DJ	0	-0.010	0.184
		1	-0.162	0.115
		2	-0.614	0.060
		3	0.063	0.019
		6	-1.062	0.033
	GP	1	1.090	0.08
	HI	1	0.200	0.006
		2	0.318	0.014
		3	0.125	0.020
	HF	1	0.456	0.041
		2	0.258	0.021
	I	5	-0.073	0.028
	KFF	1	0.011	0.006
		2	0.152	0.008
		3	0.035	0.008
		6	0.072	0.001
		8	0.258	0.013
		11	-0.036	0.004
		12	-0.073	0.007
		13	-0.062	0.006
	MAI	2	0.358	0.090
	MRS	1	0.442	0.033
		2	0.406	0.035
		3	0.380	0.031
		4	0.446	0.044
	NM	2	-0.245	0.028
	NTL	1	0.438	0.127
		2	0.385	0.074
		3	0.286	0.050
		4	0284	0.029
		5	0.316	0.042
	PHA	1	0.352	0.006
		2	0.137	0.006
		3	0.184	0.006

		4	0.177	0.006
		5	0.189	0.016
		6	0.190	0.004
		7	0.076	0.003
		8	0.031	0.004
		9	-0.087	0.012
		10	0.019	0.014
	PHB	1	0.294	0.017
		2	0.398	0.018
	SBB	1	0.601	0.021
		2	0.264	0.034
		3	0.302	0.038
		4	0.357	0.039
	TLA	8	0.141	0.289
	TLB	2	0.486	0.039
		3	0.746	0.009
	TM	3	0.341	0.065
Fungal biomass	BR	3	0.080	0.047
	C	1	-0.357	0.497
		6	-0.134	0.187
	DC	1	-0.069	0.007
		2	-0.143	0.017
		4	0.202	0.087
	I	13	-0.063	0.017
	JR	1	0.000	0.068
		2	-0.013	0.013
		3	0.116	0.015
		4	0.216	0.096
		5	0.025	0.018
		6	-0.288	0.037
	NTL	5	-0.080	0.029
	PHA	12	-0.218	0.014
	RSS	1	-0.260	3.993
		2	-0.284	1.454
	TFS	1	0.069	0.036
		2	-0.239	0.039
		3	-0.172	0.036
	UCP	2	0.319	0.022
Bacterial biomass	BR	3	0.197	0.016
	C	1	-0.362	0.147
		6	-0.105	0.134
	DC	1	0.050	0.006
		2	-0.075	0.011
		4	0.007	0.002
	I	13	0.129	0.001
	JR	1	-0.044	0.043
		2	-0.082	0.012
		3	-0.081	0.036
		4	-0.137	0.091
		5	0.066	0.029
		6	-0.134	0.028
	NTL	5	-0.013	0.021
	PHA	12	-0.101	0.011

	TFS	1	-0.005 0.008	0.008
		2	-0.167	0.029
		3	-0.109	0.023
	UCP	2	0.188	0.031
Soil microbial C	C	0	0.175	0.105
	DC	1	-0.214	0.013
		2	-0.310	0.030
		3	-0.058	0.059
		4	-0.114	0.016
		5	-0.131	0.005
	I	5	0.199	0.079
	KFF	3	0.023	0.029
		4	0.570	0.023
		9	0.577	0.028
		10	0.410	0.044
	MES	5	0.016	0.003
	NM	2	-0.140	0.021
	NTL	3	0.051	0.001
		5	0.044	0.028
		9	-0.196	0.030
	PHA	12	-0.299	0.018
		15	-0.507	0.085
	SBB	4	-0.142	0.002
		6	-0.036	0.008
		7	0.021	0.012
		15	-0.175	0.013

FIGURES

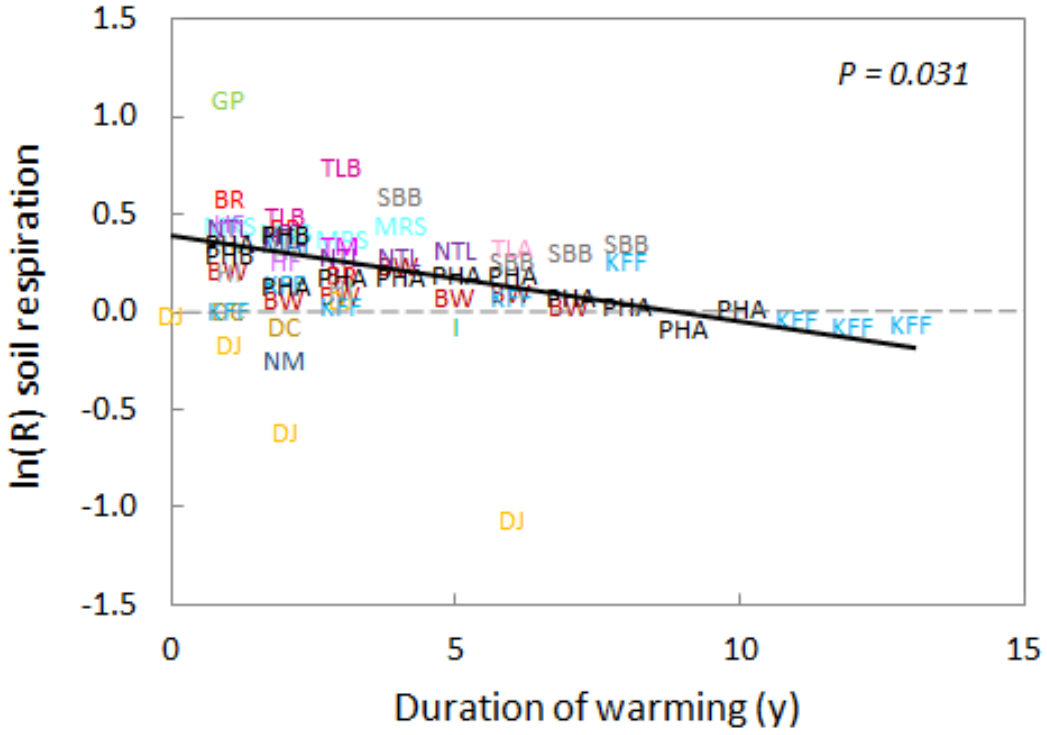

Figure 1.1. Effect sizes of soil respiration versus duration of warming, as the natural log of warming:control treatments (lnR). Where lnR is less than 0, soil respiration decreased with warming. Where lnR is greater than 0, soil respiration increased. Effect sizes decreased significantly with duration of warming, across all studies. Symbols are experiment IDs (Table 1). Line is best fit.

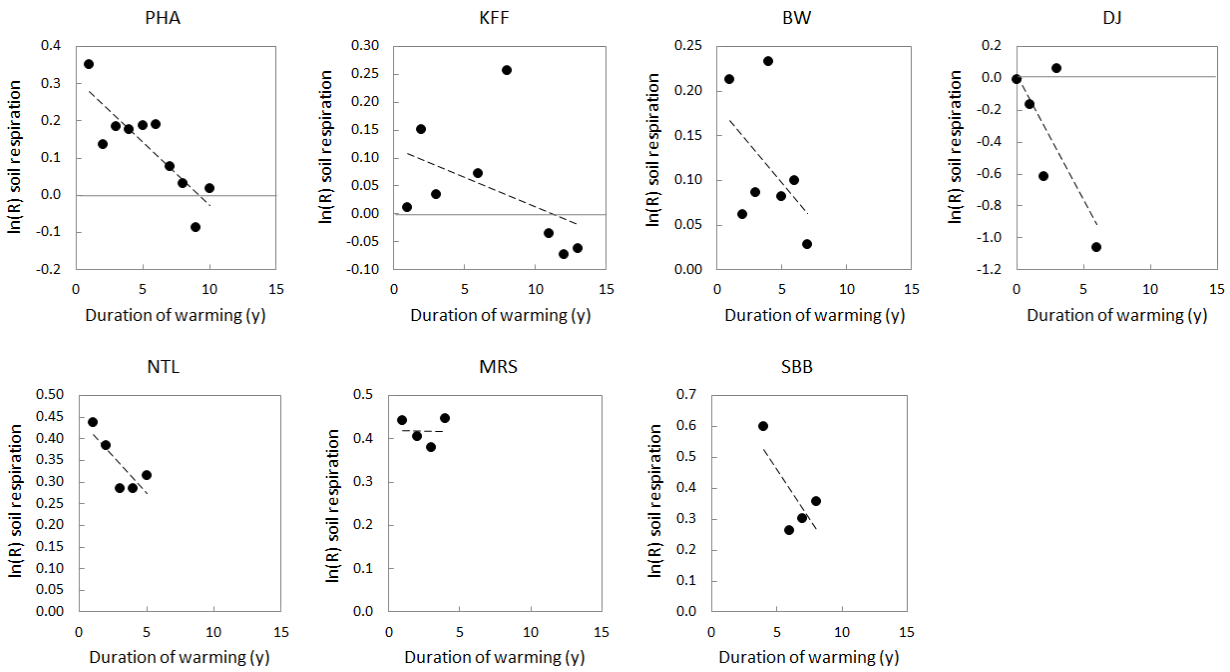

Figure 1.2. Effect sizes of soil respiration versus duration of warming for experiments with measurements in at least four years. Letters indicate experiment IDs (Table 1). Lines are best-fit.

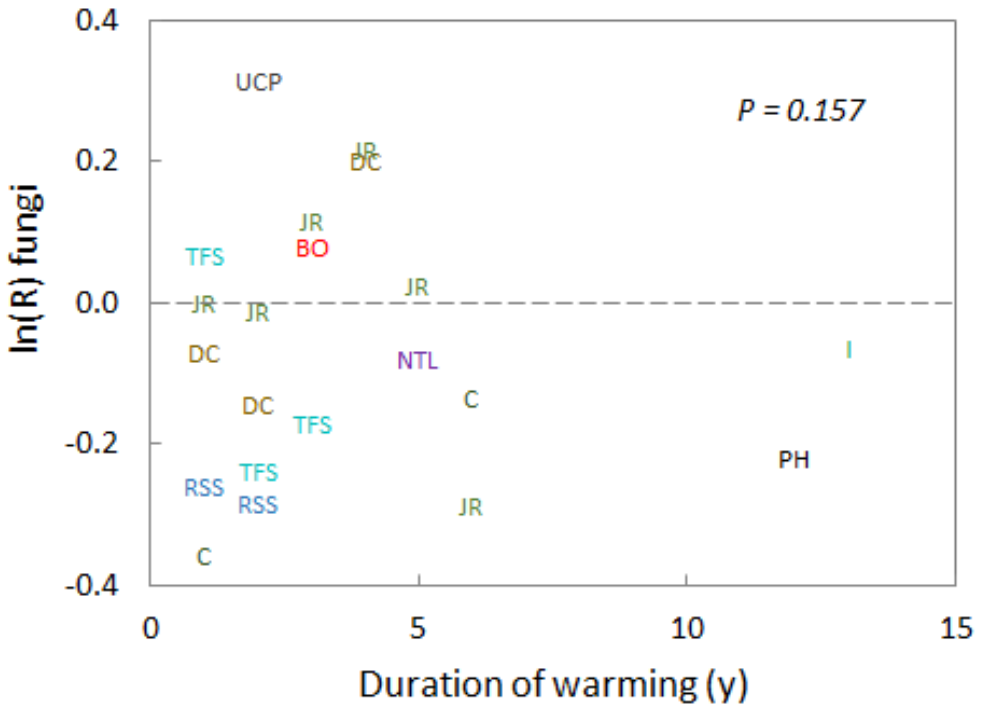

Figure 1.3. Effect sizes of fungal abundance versus duration of warming, as the natural log of warming:control treatments (lnR). Where lnR is less than 0, fungal abundance decreased with warming. Where lnR is greater than 0, fungal abundance increased. There was no significant relationship between effect size and duration of warming. Symbols are experiment IDs (Table 1).

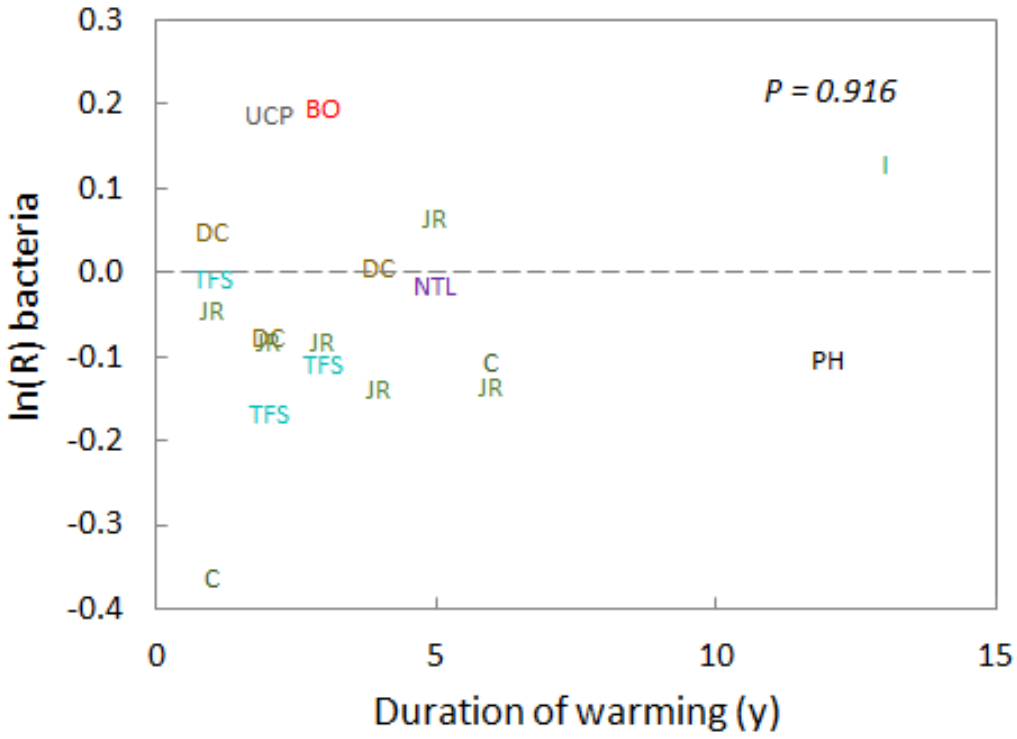

Figure 1.4. Effect sizes of bacterial abundance versus duration of warming, as the natural log of warming:control treatments (lnR). Where lnR is less than 0, bacterial abundance decreased with warming. Where lnR is greater than 0, bacterial abundance increased. There was no significant relationship between effect size and duration of warming. Symbols are experiment IDs (Table 1).

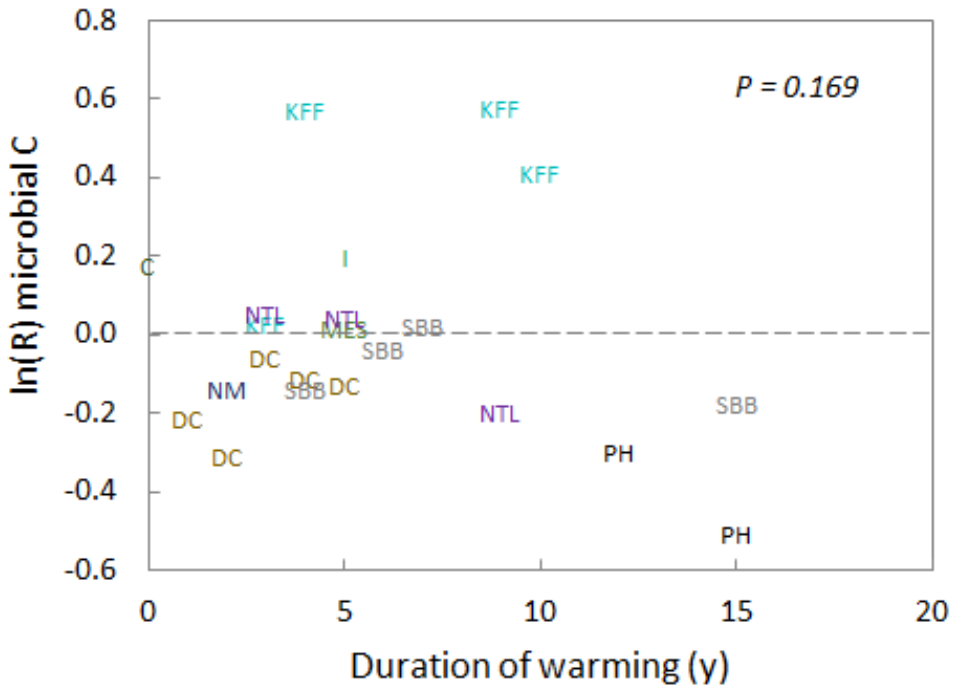

Figure 1.5. Effect sizes of microbial C versus duration of warming, as the natural log of warming:control treatments (lnR). Where lnR is less than 0, microbial C decreased with warming. Where lnR is greater than 0, microbial C increased. There was no significant relationship between effect size and duration of warming. Symbols are experiment IDs (Table 1).

CHAPTER 2: *Neurospora discreta* as a model to assess adaptation of soil fungi to warming

INTRODUCTION

It has been proposed that global warming will enhance metabolic activities of microbes, and in doing so, provide a positive feedback to global warming due to high rates of CO_2 production (Davidson and Janssens, 2006). Nevertheless, studies have shown contrasting results regarding the response of soil microbes to warming and C cycle feedbacks (e.g.,Bradford et al., 2008; Davidson and Janssens, 2006; Kirschbaum, 2004; Luo et al., 2001; Schindlbacher et al., 2011). Moreover, little is known about the capacity of fungi to evolve in response to warm conditions, and the potential consequences for the C cycle. Given the relatively short generation time of some fungi (*e.g.,Neurospora* spp., Dettman et al., 2008) it is possible that they can adapt to warming at ecologically-relevant timescales; recent studies support this idea (e.g.,Crowther and Bradford, 2013). However, the specific changes in physiology and function of adapted fungi remain unknown.

We can examine evolutionary responses of fungi to warming by conducting selection experiments on a model fungus with a particularly fast generation time. This approach should provide information that we can then compare with community and ecosystem level observations (Somero, 2010). Toward this end, we selected the renowned model fungus *Neurospora discreta*, because it is a globally-distributed saprotrophic fungus that persists in natural fungal communities (Frederick et al., 1969; Jacobson et al., 2004; Lee and Dighton, 2010; Palanivelu and Moherhwari, 1976; Powell et al., 2003). Moreover, it is readily manipulated under laboratory conditions, where it can complete thousands of generations within a few months (Dettman et al., 2008).

Laboratory studies on individual fungal species have already improved our knowledge regarding physiological responses to climate. For example, experimental work with *Neurospora crassa* showed that this fungus can acclimate to changes in temperature, suggesting that fungi can respond to seasonal changes and to different climates (Mohsenzadeh et al., 1998). Additionally, previous research using *N. crassa* (Mohsenzadeh et al., 1998) and *Saccharomyces cerevisiae* (Gasch et al., 2000) showed that under short-term temperature stress, fungi increase their metabolic activity (core metabolism) and reduce their growth. It also showed that yeasts and filamentous fungi can acclimate quickly to warming, reaching a steady-state which cannot be reverted (Gasch and Werner-Washburne, 2002). In contrast to these acclimation experiments, adaptation has rarely been specifically addressed. In our work, we examined the adaptation of *N. discreta* to warm temperatures. We chose three strains of *N. discreta* that had been isolated from relatively cool environments, and exposed them to 16 °C and 28 °C for 1500 mitotic generations. We then assessed changes in four physiological traits: mycelial growth rate (MGR), relative spore production (i.e., spores per unit biomass), mass specific respiration (MSR), and biomass production. To date, there is no standard measure of fungal fitness (Pringle and Taylor, 2002). We selected relative spore production as an indicator of reproductive fitness, because it has been suggested that high fitness in saprotrophic fungi is associated with the ability to quickly colonize new environments by allocating resources to spore production (Gilchrist et al., 2006; Pringle and Taylor, 2002). In addition, we used MGR as another fitness trait (Gilchrist et al., 2006), because fast mycelial growth could also improve colonization ability.

We also examined mass specific respiration (MSR) as a general measure of the efficiency with which fungi use carbon sources, as previously suggested (Leroi et al., 1994). MSR has been widely used by soil microbial ecologists as a proxy to measure adaptation (e.g.,Bradford et al., 2008; Hartley et al., 2007; Schindlbacher et al., 2011) and quantify carbon use efficiency. It has been hypothesized that carbon use efficiency will be greater as microorganisms adapt to warmer temperatures (i.e.,higher microbial biomass accompanied by lower MSR); several studies support this idea to some extent (e.g., Frey et al., 2013; Luo et al., 2001), but more recent studies have predicted that carbon use efficiency will decline with increasing temperature (e.g.,Allison, 2014).

We performed these selection experiments to address the question: "What physiological changes coincide with fungal adaptation to warming?" We hypothesized that the adapted strains will grow faster (Hypothesis 1) and have higher relative spore production (Hypothesis 2), than the parental strains in the selective temperature. In addition, we expected lower MSR by the adapted strains than the parental strains in the selective temperature, owing to more efficient carbon use metabolism (Hypothesis 3).

MATERIALS AND METHODS

Strains

We selected three strains of *N. discreta* isolated in 2000 and 2001 from AK (Tok, Alaska, FGSC 9979), MT (Perma, Montana, FGSC 8572), and NV (Wells, Nevada, FGSC 8565) (Jacobson et al., 2004). Mean annual temperatures at these sites is –4.7 °C in Alaska, 7.5˚C in Montana and 8.1 °C in Nevada (Table 2.1). In addition, the mean annual precipitation at

all sites was low (234 to 351 mm y^{-1}). Strains were maintained on VMM agar (Vogel's Minimum Medium: 1x Vogel's salt solution, 1.5% sucrose, 1.5% agar).

Experimental design

For each parental strain (AK, MT, and NV), we conducted three adaptations to 16 °C and another three to 28 °C. The selection experiment lasted 1500 mitotic generations. For each parental strain, we used these 3 x 16 °C adapted and 3 x 28 °C adapted strains plus 3 replicates of the parental strain to assess physiological traits at five temperatures (4, 10, 16, 22, 28 °C). The four measures by which we assessed physiological traits were: MGR, biomass, relative spore production (number of spores per unit biomass), and MSR. For each physiological trait, the total number of data points was 135: 3 geographic regions (AK, MT, NV) * 9 adapted and parental strains (3 x 16 °C + 3 x 28 °C + 3 x parental) * 5 temperatures (4, 10, 16, 22 or 28 °C).

Inoculum preparation

For all physiological measurements, our inoculum was 5 million spores in 5 ml VMM broth or agar. We prepared the inoculum by adding spores from mycelium (grown on VMM agar) after 5 days of growth, flooding the culture with 20 ml of 1M sorbitol, filtering the liquid, and recovering the spores by centrifuging for 5 minutes at 2,500 rpm. The spores were then washed in fresh sorbitol, recovered by centrifugation (3x), and stored at -20 °C. We used a Neubauer chamber to determine the spore concentrations in dilutions of the stored spores.

Adaptation and physiological tests

We initiated the adaptation process by adding 5 million spores to 20 ml VMM agar in 30 cm race tubes. Based on a total growth of mycelium over 450 cm, we estimate that the

adaptation process involved 1500 mitotic generations (briefly, 100 cell cycles are completed every 30 cm of growth based on a cell cycle time of 1.5 h and a mycelial extension rate of ~ 2 mm/h at 22 °C on VMM agar) [6]. To transfer mycelium between race tubes, we collected the last 2 cm of agar with mycelium and spores. The final collection was done in the same manner, except mycelium was transferred to a 50 ml tube and grown for one more week before harvesting spores.

Each physiological test was initiated by adding 5 million spores to 5 ml of VVM agar at 9:00 pm, incubating in darkness for 12 hours at 22 °C, and then shifting the culture to the treatment temperature (4, 10, 16, 22 or 28 °C) for an additional 48 hours, also in darkness. By standardizing inoculum, germination time, and germination temperature, we could assume that all cultures were at the same life stage when they were shifted to the treatment temperatures.

Spore production was quantified on VMM agar as described for inoculum preparation and reported as the number of spores per g of fungal biomass.

MGR was measured in the selective environment (race tubes with VMM agar) after growth for 48 hrs at the treatment temperature. MGR was reported as mm per hour on the average of all replicates divided by 48 hours of incubation time.

Biomass was determined in VMM agar after growth for 48 hrs at the treatment temperature by melting the agar (autoclaving at 121 °C for 10 minutes) and filtering through Whatman #1 filter paper (Crowther and Bradford, 2013). We then dried the mycelium for 48 hours at 60 °C and weighed it to determine fungal biomass (mg).

MSR was measured in VMM agar (same samples used to determine biomass) using septum vials and an infrared gas analyzer (PP Systems EGM-4, Amesbury, MA, USA). After

48 hours of incubation at the treatment temperature, we equilibrated gas concentration of all samples by opening the vials under a laminar flow hood for approximately 15 minutes. We proceeded to close the vials tightly and then incubated the samples for four more hours. We measured CO_2 concentrations in ppm before and after the four-hour incubation and, by calculating the difference in CO_2 concentration between the two time points and dividing by biomass, we could calculate MSR as mg CO_2 g^{-1} fungal biomass h^{-1}.

Statistical analyses

For each physiological test, we conducted a nested repeated measures analysis of variance (ANOVA). The independent variables were adapted state (parental, 16 °C-adapted, and 28 °C-adapted) and strain origin (AK, MT, and NV), with strain origin nested within adapted state. Incubation temperature was the repeated measure. The dependent variable was spore production, biomass, or MSR. Kolmogorov-Smirnov *post hoc* tests were used to assess pairwise differences. Significant interactions between adapted state and incubation temperature would support our hypotheses if the 16 °C- or 28 °C-adapted strains also displayed significantly higher sporulation, less biomass, and lower MSR than the parental strains when all were incubated at the selective temperature. Differences were considered significant when $P < 0.05$. We ranked all data, because they did not conform to assumptions for normality or homogeneity of variances. All statistical analyses were done using the statistical program R (www.R-project.org) and SYSTAT (SPSS, Evanston, IL).

RESULTS

Our adaptation experiments consisted of exposing three different strains of *N. discreta* to moderately warm (16 °C) and warm (28 °C) temperatures for 1500 generations.

These strains were originally isolated from Tok, Alaska; Perma, Montana; and Wells, Nevada (Table 2.1) (Jacobson et al., 2004). These sites were relatively cool, with mean annual temperatures ranging from –4.7 °C to 8.1 °C (Table 2.1). The strains grew along 30 cm race tubes while exposed to the selective temperature. We inoculated one end of the race tube with an initial population size of 5 million spores. When the strains had reached the opposite side of the tube (i.e.,that 100% of the area was colonized), we took the last 15 x 20 mm strip of culture (about 5 million spores, and 25 million nuclei) and transferred it to a new tube. These transfers continued until the strains had crossed 15 race tubes, for a total of 450 cm. Prior to starting the adaptation experiment, we assessed MGR, biomass production, relative spore production, and MSR of the parental strains at incubation temperatures ranging from 4 to 28 °C. After the adaptation regime was completed, we performed the same assessment on the 16 °C-adapted and 28 °C-adapted strains.

Parental strains

We incubated parental strains for 48 hours at 4, 10, 16, 22, and 28 °C, and then measured MGR, biomass production, relative spore production, and MSR; they grew faster as incubation temperature increased—a pattern typical of parental and adapted strains alike (Fig. 2.1 and 2.2, temperature effect MGR: $F_{4,8} = 190.87$, $P < 0.001$; temperature effect biomass: $F_{4,8} = 420.94$, $P < 0.001$). In contrast, relative spore production by the parental strains peaked at 10 °C (Fig. 2.3), the incubation temperature closest to the mean annual temperatures of the Montana and Nevada sites. Regardless of adaptation status, relative spore production displayed a unimodal-shaped relationship to incubation temperature (Fig. 2.3, temperature effect, $F_{4,8} = 141.87$, $P < 0.001$). MSR of the parental strains remained consistently low at the three coolest temperatures, increased three-fold at 22 °C, and

declined at the highest temperature (Fig. 2.4). Indeed, increasing temperatures (up to 22 °C) augmented MSR in parental as well as adapted strains (Fig. 2.4, temperature effect, $F_{4,8} = 20.87$, $P < 0.001$).

Overall, the parental strains appeared to be better adapted to cooler temperatures (10 and 16 °C) based on patterns of relative spore production (Fig. 2.3). MGR, biomass and MSR were sensitive to incubation temperature as well—they all increased markedly at higher temperatures, with the exception of MSR at 28 °C (Figs. 1, 2, & 3). How did the warm-adapted strains compare?

16 °C-adapted strains

Contrary to our expectations, MGR of the 16 °C-adapted strains was not higher than parental strains when both were incubated at 16 °C (Fig. 2.1). Neither was biomass (Fig. 2.2). In fact, across all incubation temperatures, we observed a significant interaction between adaptation state and incubation temperature (MGR $F_{9,16} = 3.4$, $P < 0.001$; biomass: $F_{9,16} = 310.98$, $P < 0.001$), but not in the expected direction at 16 °C. Instead, there was a trend toward a decline in MGR at 16 °C. With respect to the 16 °C adapted strains, we rejected Hypothesis 1, that adapted strains would have higher MGR than parental strains in the selective temperature.

Nevertheless, Hypothesis 2, which predicted that adapted strains would produce more spores per unit biomass compared to parental strains in the selective temperature, was supported. Specifically, 16 °C-adapted strains produced more spores per unit biomass than the parental strains at 16 °C (Fig. 2.3, $P < 0.001$). Moreover, relative spore production of the 16 °C-adapted strains peaked at 16 °C—at a warmer temperature than did the

parental strains (Fig. 2.3). Accordingly, the interaction between adaptation state and incubation temperature was significant ($F_{9,16} = 86.83$, $P < 0.001$).

Unexpectedly, MSR of 16 °C-adapted strains was significantly higher than that of parental strains in the selective temperature (Fig. 4, $P = 0.005$). This finding was contrary to Hypothesis 3, which predicted the opposite. The interaction between adaptation state and incubation temperature was significant (Fig. 2.4; $F_{9,16} = 24.33$, $P < 0.001$).

In summary, the 16 °C-adapted strains appeared to display higher fitness at 16 °C, in terms of relative spore production (Fig. 2.3). However, this adaptation was not accompanied by an increase in MGR and biomass (Fig. 2.1 & 2.2) as previously hypothesized. Additionally, this adaptation was also accompanied by an increase of MSR rather than a decrease. Did strains adapted to an even warmer temperature display similar physiological shifts?

28 °C-adapted strains

The 28 °C-adapted strains displayed physiological shifts that were very similar to the 16 °C-adapted strains (adaptation state*incubation temperature effect MGR: $F_{9,16} = 178.87$, $P < 0.001$; biomass: $F_{9,16} = 379.26$, $P < 0.001$). Specifically, MGR and biomass production by the 28 °C-adapted strains was significantly smaller than that of the parental strains when both were incubated at 28 °C (Fig. 2.1 & 2.2, MGR $P < 0.001$; biomass: $P < 0.001$). Furthermore, relative spore production at 28 °C increased significantly compared to the parental strain (Fig. 2.3, $P < 0.001$). We note that relative spore production by the 28 °C-adapted strain tended to peak at the 22 °C incubation temperature, but was not significantly different from the 28 °C incubation temperature ($P = 0.91$). Finally, MSR of the 28 °C-adapted strain was higher than the parental strain at 28 °C (Fig. 2.4, $P < 0.001$).

Accordingly, Hypothesis 2 was supported for adaptation to 28 °C, but not Hypotheses 1 and 3. Similar to the 16 °C-adapted strains, adaptation to 28 °C was accompanied by a reduction in MGR and biomass, as well as an increase in MSR.

Geographic origin

Strains isolated from the three field sites varied significantly in MGR, biomass, relative spore production, and MSR. Nevertheless, there was no noticeable relationship between mean annual temperature at the site of origin versus biomass, MGR, MSR, or relative spore production.

Mycelial growth rate versus biomass

Biomass and mycelial growth rate were each of interest in this study. We measured mycelial growth rate as a potential indicator of fitness (in addition to relative spore production). We quantified biomass because it was required to calculate relative spore production and MSR. In addition, microbial biomass is a common component of ecosystem models of soil dynamics (McGuire and Treseder, 2010); the biomass data from this study can be used by modelers for parameterization or validation. Mycelial growth rate and biomass were strongly-but not perfectly- correlated (2.5; Spearman's rank-order correlation $\rho = 0.75$, $P < 0.001$).

DISCUSSION

After adaptation to 16 and 28 °C, *N. discreta* displayed an enhanced metabolic rate at those temperatures, perhaps in order to support higher production rates of energetically-expensive spores. Accordingly, tradeoffs in resource allocation may have led to the slower MGR (Fig. 2.1) and lower biomass production (Fig. 2.2) in the adapted strains. We rejected

Hypothesis 1, because MGR in the warm-adapted strains was not higher at the selective temperature, compared to parental strains (Fig. 2.1). Nevertheless, Hypothesis 2 was supported because adapted strains of *N. discreta* produced significantly more spores per unit biomass at the selective temperature (Fig. 2.3). Finally, we rejected Hypothesis 3, because adapted strains produced higher MSR at the selective temperature (Fig. 2.4), suggesting that adaptation did not lead to greater efficiency of carbon use.

Higher relative spore production in the adapted strains may indicate greater fitness at the selective temperatures. Similarly, in experiments carried out using *Aspergillus niger*, the rate of spore production was used as a measure of fitness. In this case however, colony surface area was used as a unit of biomass, and contrary to our observations, there was a positive trend between biomass and number of spores (de Visser et al., 1997). More recently, mathematical models have shown that for asexual fungi in nature, higher production of spores facilitates the extension of the colony while avoiding the risk of extending the mycelium into a resource-poor area (Gilchrist et al., 2006). In addition, previous studies have reported that fungi that invest in dispersal structures (i.e.,spores) instead of vegetative structures (i.e.,hyphae) tend to colonize more litter patches and are more prevalent within the ecosystem (Peay et al., 2007).

The adapted strains of *N. discreta* may have grown more slowly because they were allocating a greater proportion of resources to spore production instead of biomass. Dettman and colleagues (Dettman et al., 2008) also performed temperature-selection experiments with *Neurospora* spp., but they adapted the strains to a cooler temperature of 12 °C. Their cold-adapted strains exhibited an increase in MGR at 12 °C, compared to the parental strains. However, the authors discussed that their method of propagation of

lineages (transferring the fastest-growing hyphal tips) might not be the best approach to allow efficient competition and adaptive response (Dettman et al., 2008). In fact, transferring the fastest growing mycelial sector has been identified as artificial selection, whereas transferring of random samples of mycelial-produced spores has been identified as a more accurate way of replicating natural selection (Schoustra et al., 2005). In our work, we transferred mycelia and spores together, rather than only the fastest growing hyphal tips.

The increase in MSR at high temperatures following adaptation was unexpected. This result might be explained by the increased production of spores in adapted strains (Fig. 2.2). Spores are known to have energetically-costly compounds such as nutrients for survival during dormancy and complex structural molecules for efficient spore dispersal. For example: the fibrous layer of "rodlets" in the surface of *Neurospora's* spores make them highly hydrophobic and readily dispersible through air; these compounds are not present in vegetative mycelium and are composed mostly of hydrophobic proteins (Beever et al., 1979; Hallett and Beever, 1981). In addition, studies in *Penicillium chrysogenum*, showed that production of spores is usually accompanied by thickening of the cell wall of the hyphae and reduced growth rate due to extensive vacuolation and plugging of the hyphal septum; these processes are metabolically costly (Righelato et al., 1968). In fact, they reported a positive relationship between production of spores and MSR.

Our findings contrast with those often observed in short-term acclimation experiments. Typically, MSR declines following acclimation of microbes to higher temperatures, although underlying mechanisms are a matter of debate. For example, Luo and collaborators (Luo et al., 2001) observed a decrease in soil respiration as temperature

increased. They suggested that this response was the result of acclimatization to warming by microbes, and concluded that acclimatization might weaken the positive feedbacks to global warming. However, Kirschbaum (Kirschbaum, 2004) argued that the apparent acclimatization was due to depletion of labile carbon in the soil. Later, Bradford and collaborators (Bradford et al., 2008) indicated that reduced MSR from microbes resulted from both mechanisms operating in concert. The observations of Schindlbacher and collaborators (Schindlbacher et al., 2011) were an exception; they found that acclimation to warming enhanced MSR. Crowther and Bradford (Crowther and Bradford, 2013) conducted one of the few studies that have assessed the temperature acclimation of fungal species, instead of the microbial community as a whole. They observed an increase in MSR and declines in growth efficiency at elevated temperatures (28°C) after 10 days of incubation. Since these studies were generally short-term, the fungi likely had minimal opportunity for adaptation.

The response of MSR to temperature in our adaptation experiment may have differed from that of the majority of the acclimation experiments because our experiment selected for higher relative spore production. Sporulation was likely to be less important in shorter-term studies with fewer generation cycles. In ecosystems exposed to global warming, selection for high sporulation rates may lead to increases in MSR at the evolutionary time scale, even though MSR may initially decline owing to short-term acclimation.

Evolution of natural populations occurs by many processes such as gene flow, genetic drift, sexual recombination, and mutation. Those natural processes were not necessarily replicated in our study. The adaptation process that we carried out was

performed under controlled conditions, and the sample size for each transfer was kept as consistent as possible. In addition, we focused on one fungal species, so it remains unknown whether similar adaptive responses will occur in other species. Nevertheless, we incorporated some genotypic variation in our study—we used strains of *N. discreta* that were collected from three different sites and that varied initially in spore production and biomass (Table 2.2). Moreover, for asexually reproducing strains of fungi, selection is easily affected by changes in temperature, because the direction of selection is strongly dependent upon changes in its environment, and not on sexual recombination (Ennos and McConnell, 1995). Therefore, our results should not be affected by the lack of sexual reproduction.

Temperature has a direct effect on microbial physiological processes that control decomposition (Davidson and Janssens, 2006). For example, Allison and collaborators (Allison et al., 2010b) simulated changes in soil carbon under global warming under three scenarios: MSR increases markedly with temperature; MSR increases with temperature, but only moderately; and MSR does not vary with temperature. Their model predicts that soil carbon losses are greatest when MSR remains constant, and smallest when MSR is most sensitive to temperature. This pattern occurs because high MSR leads to low microbial biomass and slower ecosystem-level CO_2 efflux. Our results are most in line with the first scenario, in which MSR increases in response to warming and is particularly sensitive to temperature following adaptation. This pattern may also be true for wild populations of *Heterobasidium parviporum* (a root-rot pathogenic fungus), since the annual respiration activity of this fungus is increasing with annual air temperature in boreal ecosystems of northern Finland (Müller et al., 2014). Based on the modeled predictions of Allison and

collaborators (Allison et al., 2010b), we might expect that the adaptation responses we observed in *N. discreta* would lead to a mitigation of soil carbon loss. If this response were widespread globally, it might slow the enrichment of atmospheric CO_2 under warmer conditions. Nevertheless, it remains to be seen whether other fungi will adapt to warming in similar ways.

CONCLUSION

In conclusion, our data show that fungi can adapt to warm temperatures. This adaptation might be accompanied by evolutionary tradeoffs such as increased allocation of resources to spore production but reduced MGR and higher MSR. Our results provide little support for the idea that adaptation to global warming will lead to increases in carbon use efficiency. Incorporating this information to climate change model simulations could help provide a more concise forecast on decomposition processes and carbon feedbacks to the atmosphere.

TABLES

Table 2.1. Location and climatic characteristics of the wild isolates of *Neurospora discreta* used in this work.

Location	Geographic coordinates[a]	Altitude (masl)[a]	Mean annual precipitation (mm y^{-1})[b]	Mean annual temperature (°C)[b]	FGSC id
Tok, AK	63° 21' N, 142° 60' W	515	234	-4.7	9979
Perma, MT	47° 23' N, 114° 35' W	930	351	7.5	8572
Wells, NV	41° 12' N, 114° 57' W	1952	248	8.1	8565

[a]From Jacobson *et al.* 2004.
[b]Data from Western Regional Climate Center, 2014, wrcc.dri.edu

Table 2.2. Physiological profile of strains from different geographic locations, averaged across incubation temperatures and adaptation state (means ±SE).

Site of origin	Mycelial growth rate (mm/h)	Fungal biomass (mg)	Spore production (# g^{-1})	Mass specific respiration (mg g^{-1} h^{-1})
AK	1.35 ±0.22	9.25 ±1.50	6420 ±521	7 ±1
MT	1.09 ±0.18	6.58 ±1.32	11141 ±1055	18 ±3
NV	1.40 ±0.18	9.96 ±1.68	7957 ±538	9 ±1
	$F_{2,8}$ = 6.28	$F_{2,8}$ = 7.64	$F_{2,8}$ = 7.35	$F_{2,8}$ = 11.36
	P = 0.002	P < 0.001	P < 0.001	P < 0.001

FIGURES

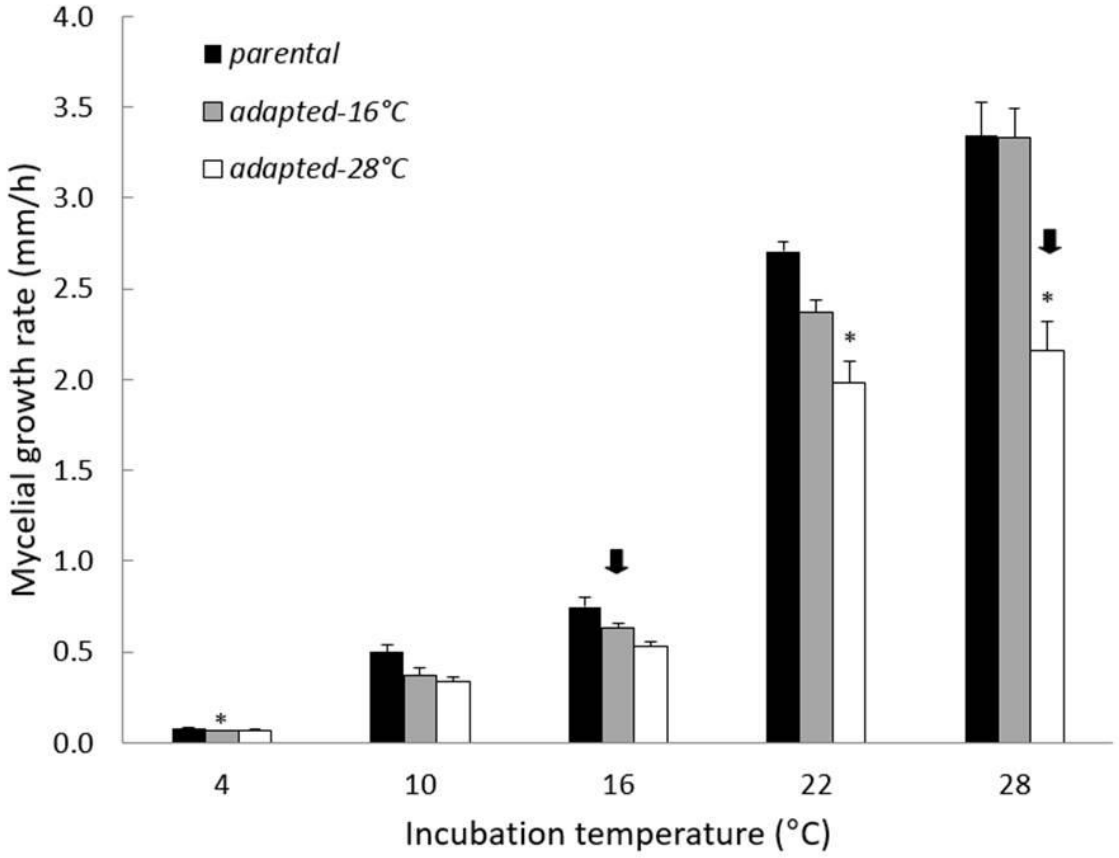

Figure 2.1. Mycelial growth rate of parental and adapted strains of *N. discreta* measured at different incubation temperatures. Bars are means of all three geographical strains and their replicates + 1SE (n=9). Asterisks indicate significant pairwise differences between adapted and parental strains within a given incubation temperature ($P < 0.05$). Arrows indicate the selective temperature for each adapted strain.

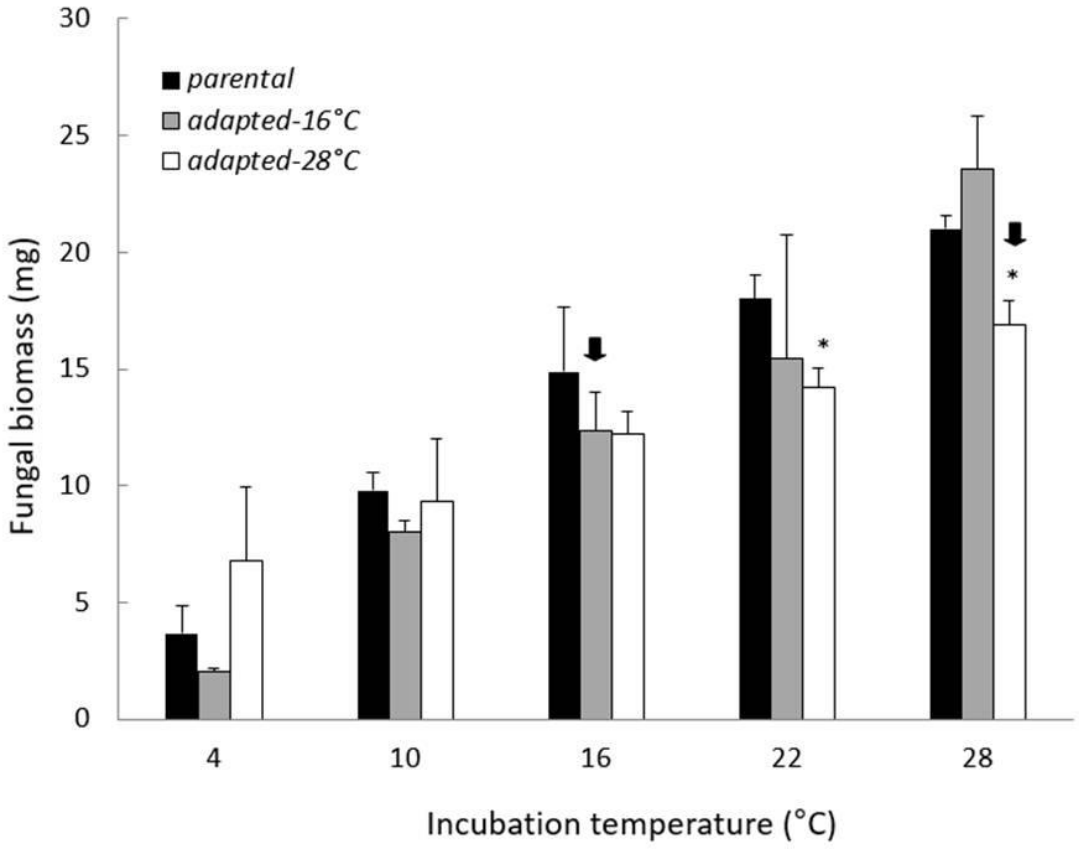

Figure 2.2. Fungal biomass of parental and adapted strains of *N. discreta* measured at different incubation temperatures. Bars are means of all three geographical strains and their replicates + 1SE (n=9). Asterisks indicate significant pairwise differences between adapted and parental strains within a given incubation temperature ($P < 0.05$). Arrows indicate the selective temperature for each adapted strain.

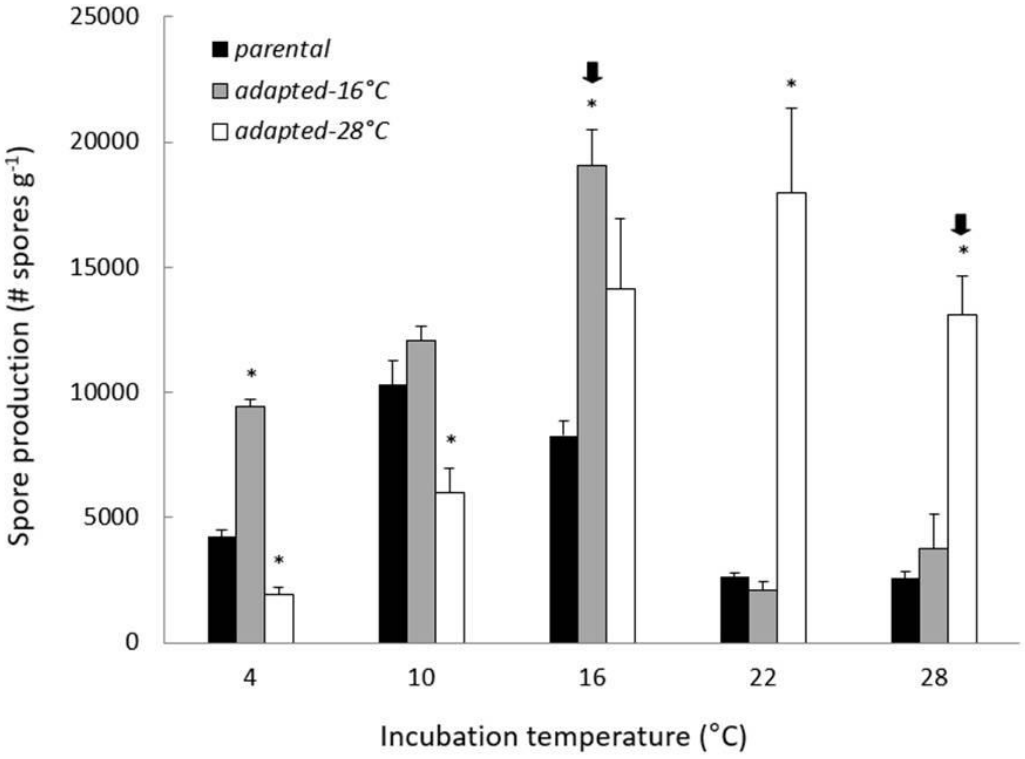

Figure 2.3. Spore production of parental and adapted strains of *N. discreta* measured at different incubation temperatures. Bars are means of all three geographical strains and their replicates + 1SE (n=9). Asterisks indicate significant pairwise differences between adapted and parental strains within a given incubation temperature ($P < 0.05$). Arrows indicate the selective temperature for each adapted strain.

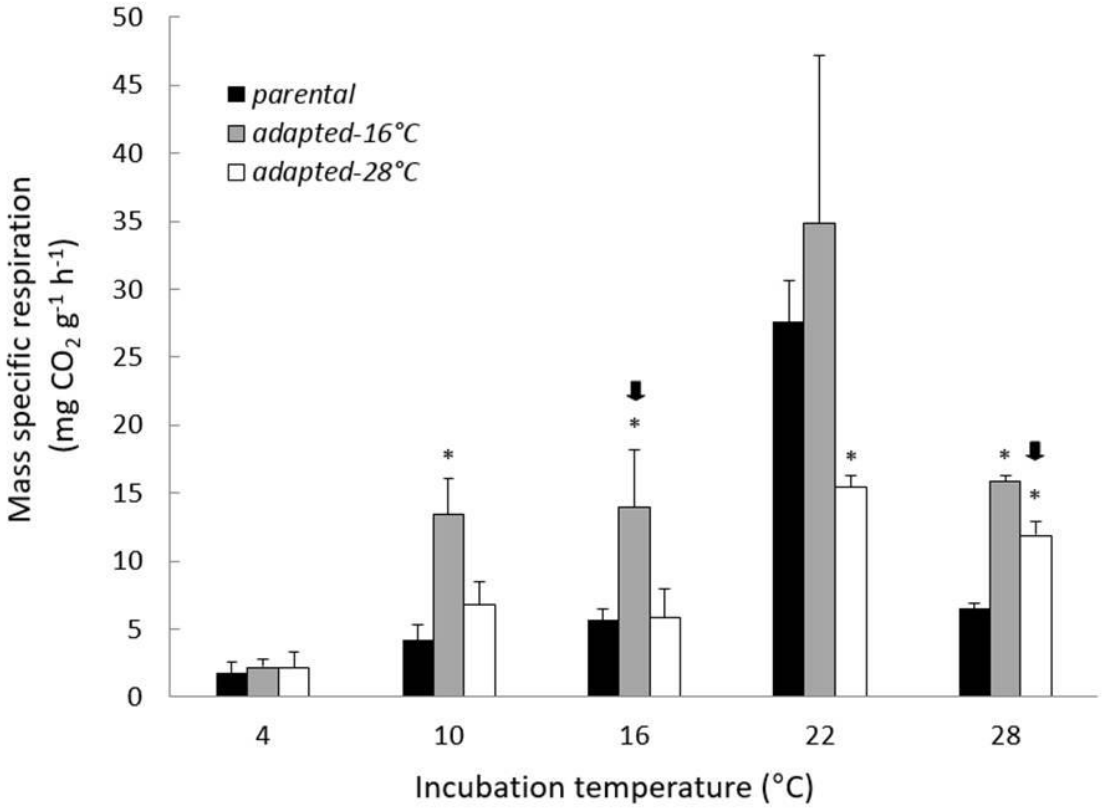

Figure 2.4. Mass specific respiration of parental and adapted strains of *N. discreta* measured at different incubation temperatures. Bars are means of all three geographical strains and their replicates + 1SE (n=9). Asterisks indicate significant pairwise differences between adapted and parental strains within a given incubation temperature ($P < 0.05$). Arrows indicate the selective temperature for each adapted strain.

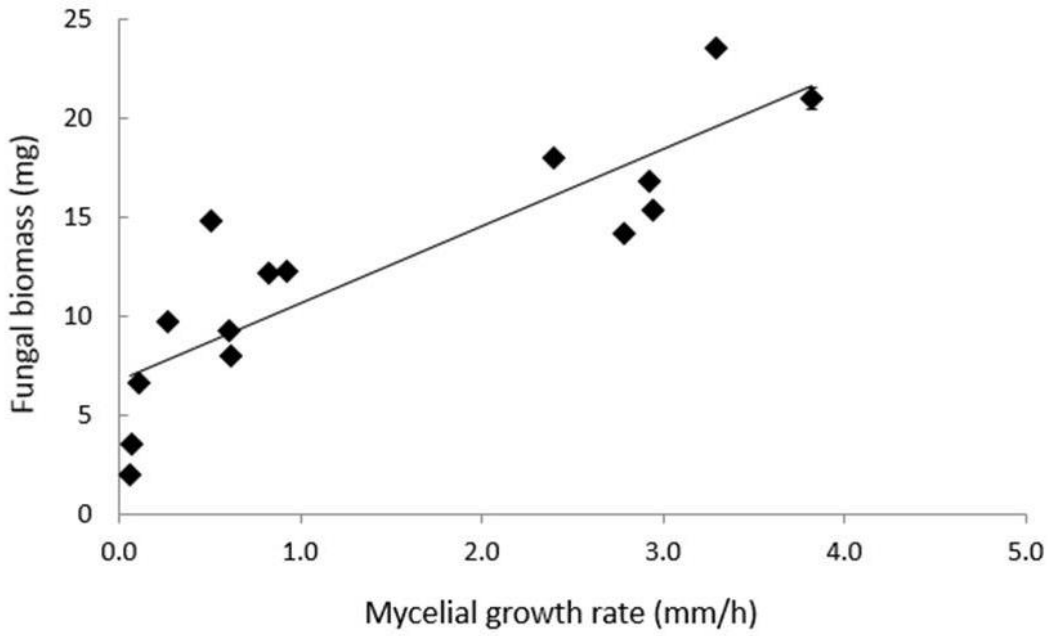

Figure 2.5. Correlation between biomass and mycelial growth rates of parental and adapted strains of *N. discreta* measured at different temperatures ($\rho = 0.75$, $P < 0.001$). Each symbol represents a different combination of incubation temperature and adapted state (parental, 16 °C-adapted, and 28 °C-adapted). Symbols are means ± 1SE of all three geographical strains and their replicates (n=9).

CHAPTER 3: Decomposition of recalcitrant carbon under experimental warming in boreal forest

INTRODUCTION

High-latitude soils store approximately 510 Pg of C, primarily owing to the buildup of recalcitrant C (Boerjan et al., 2003), such as lignin. Much of this soil C has decomposition rates of years to centuries, due to its complex chemical structure and exposure to cold temperatures (Biasi et al., 2005; Davidson and Janssens, 2006; Stokstad, 2004). Global warming is particularly rapid at high-latitudes (Aerts, 2006; IPCC, 2014; Schuur et al., 2008) and as a result, decomposition of soil C may increase, reducing high-latitude C stocks (Davidson and Janssens, 2006). If so, the CO_2 released from these soils might form a positive feedback to global warming (Allison and Treseder, 2011; Cox et al., 2000; Davidson and Janssens, 2006; Heimann and Reichstein, 2008; IPCC, 2014).

Moreover, recalcitrant C decomposition may be especially sensitive to temperature (Arrhenius, 1896; Davidson and Janssens, 2006). This idea is based on the theories of collision and enzyme kinetics, which imply that temperature sensitivity of decomposition is positively related to the complexity of the substrate (Arrhenius, 1896; Davidson and Janssens, 2006). In other words, the breakdown of complex recalcitrant C requires more enzymatic steps with higher activation energies (Arrhenius, 1896; Bosatta and Ågren, 1999; Xu et al., 2014). Accordingly, Davidson and Janssens (2006) predicted that 2 °C warming would increase decomposition of recalcitrant C by 21%, compared to only a 10% increase for non-recalcitrant C. The consequences of this difference should be exacerbated at high-latitudes, where a 2–5 °C warming is predicted by the end of this century (IPCC, 2014).

In addition to the direct kinetic effects of warming on decomposition, warming may also select for microbial communities that preferentially degrade recalcitrant C (DeAngelis et al., 2015; Pold et al., 2015). For example, some filamentous fungi are less tolerant to cold stress compared to yeasts (Treseder and Lennon, 2015) and might proliferate under warming; in addition, some of these filamentous fungi are better at decomposing recalcitrant C (Treseder et al., 2016). Variation in microbial breakdown of recalcitrant C compounds can disproportionately influence long-term C storage in soils (Davidson and Janssens, 2006; Parton et al., 1988). However, changes in decomposition of recalcitrant C, specifically, are rarely assessed in field-based warming experiments (von Lützow and Kögel-Knabner, 2009) and are thus challenging to predict. In this warming experiment, Treseder and collaborators (Treseder et al., 2016) reported that warming induced a shift in fungal community composition toward taxa that could break down recalcitrant C. Was there a concomitant shift in C use toward recalcitrant C under warming? Here, we tested this question by examining decomposition of recalcitrant C versus non-recalcitrant C in plant litter under experimental warming.

According to Hudson (1968), lignin is more recalcitrant than soluble sugars, hemicellulose, and cellulose due to its complex chemical structure. Although there is conflicting evidence regarding long-term lignin stability (Thevenot et al., 2010) and the definition of recalcitrance (Kleber, 2010), in this paper we refer to lignin as recalcitrant C. We grouped the less chemically-complex soluble sugars, hemicellulose, and cellulose, as non-recalcitrant C. We hypothesized that ratios of recalcitrant C to non-recalcitrant C (i.e. lignin: soluble sugars + hemicellulose + cellulose) remaining in decomposed litter would be lower in the warming treatment than in controls. In addition, we predicted that extracellular

enzymes produced by microbes would target recalcitrant C (relative to non-recalcitrant C) more under warming.

MATERIALS AND METHODS

Field site

The study area was located in a mature black spruce (Picea mariana) forest on the Fort Greely military base near Delta Junction, Alaska, USA (63°55'N, 145°44'W) (Treseder et al., 2004). At this site, the vegetation was dominated by black spruce with an understory of shrubs, mosses, and lichens. The climate was cold and dry, with approximately 303 mm y-1 of precipitation and a mean annual temperature of -2 °C. The growing season extends from mid-May to mid-September.

Warming experiment

In July 2005, a warming experiment was established as described in Allison & Treseder (2008). Five pairs of 2.5 x 2.5 m plots were marked in a 1 km2 area; one plot from each pair was assigned as the treatment while the other one was assigned as the control. Control plots were left under ambient conditions while treatment plots were warmed passively with greenhouses (closed-top chambers). Gutters and tubing were installed to direct precipitation into the greenhouses during the growing season. Greenhouses were left in place but the top plastic panels of the greenhouses were removed in mid-September and re-installed in mid-May to allow snowfall to enter the warmed plots. The warming treatment increased air temperature on average by 1.6 °C, and soil temperature (5 cm depth) by 0.5 °C. In addition, the warming treatment reduced soil moisture by 22% on average due to higher evapotranspiration (Allison and Treseder, 2008).

Litterbag experiment

On May 22, 2013, the warming experiment had been ongoing for eight years. On this date, we detached brown senescent spruce needles of living black spruce trees near the experimental plots by shaking branches lightly and/or by touching them and collecting the fallen needles in a plastic bag. Immediately after collection, we filled litterbags (10 x 10 cm, 1 mm mesh of nylon covered with a layer of 1 mm fiberglass mesh) with 2 g of spruce needles. We deployed four sets of two litterbags in the forest floor of each plot and took five subsamples of spruce needles for initial litter chemistry analysis.

We retrieved a set of litterbags after 1, 2, 12, and 16 months. We combined the contents of each of the two litter bags within each plot. Therefore, for each sampling time point we had five samples from control plots and five from warmed plots (n= 5). In the lab, we determined total fresh weight, then separated ~0.6 g for extracellular enzyme activity (EEA) measurements and ~0.5 g for litter chemistry. The EEA subsample was stored at -80 °C, and the litter chemistry subsample was stored at -20 °C. In addition, we used ~0.5 g from the first collection for fungal DNA sequencing; these findings are reported in Treseder et al. (2016). We determined fresh weight of the remaining litter, and dried it at 70 °C for two days to obtain percent dry weight. We calculated litter mass remaining as the product of total fresh weight and fraction dry weight.

We have permission from Ft. Greely to work in this study location. No specific permissions were required for the activities in the current study. All samplings took place within public space in the forest and no military areas were accessed. No endangered or protected species were involved in this research.

Litter chemistry

Litter samples were air dried for 48 hours and ground for 1 min in a Spex SamplePrep 8000D mixer/mill (Spex SamplePrep LLC, New Jersey) using stainless steel vials and grinding balls. To determine concentration of non-recalcitrant C (i.e. soluble sugars, cellulose, and hemicellulose) and recalcitrant C (i.e. lignin), we processed litter samples following Talbot et al. (2011). Samples were fractionated following the International Association of Analytical Communities (AOAC International) official Uppsala method (Theander et al., 1995). In all cases, we performed triplicate measurements of each of the five replicates. For each date and each assay, we used two blanks to account for background absorbance.

Soluble sugars. First, we extracted and discarded lipids, waxes, and pigments with 100% petroleum ether. Next, we extracted soluble sugars with 80% ethanol and removed starch by α-amylase digestion. The starch-less fraction was used to determine glucose concentration by the phenol-sulfuric acid method (Buysse and Merckx, 1993). We then washed the samples with 95% ethanol and 100% acetone, followed by drying at 70°C for 48 hours to obtain a lipid- and sugar-free fraction to quantify lignin, hemicellulose, and cellulose concentration.

Cellulose. We determined cellulose by the Updegraff method (Updegraff, 1969). This method consists of the removal of hemicellulose and lignin and the extraction of cellulose with acetic acid/nitric acid followed by solubilization of cellulose in 67% sulfuric acid. We quantified cellulose concentrations via the Anthrone reaction in sulfuric acid at 100 °C in a water bath, and measured absorbance at 620 nm. We used crystalline cellulose (MP biomedical cat. 02191499) as a standard.

Hemicellulose. Similarly, we measured hemicellulose in the acetic/nitric extracts of the Updegraff method by Hansen & Møller (1975) with modifications following Aravantinos-Zafiris et al. (1994). We used a mixture of 10:7.5:7.5:7.5:7.5:5:5 of glucose, xylose, arabinose, mannose, galactose, fucose, and rhamnose as a standard. We quantified sugar concentrations by measuring absorbance at 630 nm.

Lignin. Finally, we determined total lignin by the acetyl bromide method (Hatfield et al., 1999), in which lignin is solubilized in 1:4 acetyl bromide:acetic acid solution and quantified by measuring absorbance at 280 nm. We used alkali lignin (Sigma cat. 370959) as a standard.

Extracellular enzymes

To assess decomposer investment in recalcitrant C degradation under warming, we assayed the activities of four extracellular enzymes involved in decomposing different types of C, as previously described (German et al., 2012, 2011). We performed this assay on litter that had decomposed 12 months, since this was the timepoint with the highest enzyme activity (marginal enzyme activity was detected on previous dates and thus, not included in our analyses). Using pyrogallol as the substrate, we assayed polyphenol oxidase (PPO) that degrades lignin as an index of the enzymatic potential to decompose recalcitrant C. As an index of the enzymatic potential to degrade non-recalcitrant C we assayed cellobiohydrolase (CBH) that targets cellulose, β-xylosidase (BX) that targets xylose —a component of hemicellulose— and β-glucosidase (BG) that catalyzes the hydrolysis of glycosidic bonds in later steps of cellulose degradation (soluble sugars). Litter samples were homogenized in 50 mM maleate buffer, pH 6.0, and pipetted into microplates. We measured enzyme Vmax (nmol h-1 g-1 dry litter) at 4, 10, 16, 22, 28, and 34 °C either

colorimetrically (PPO), or fluorimetrically (CBH, BX, BG) on a microplate reader. Enzyme Vmax values were obtained by fitting the Michaelis-Menten equation to reaction velocities as a function of substrate concentration using the non-linear least squares (nls) method in R. Enzymes were assayed across a range of temperatures and substrate concentrations because these measurements were conducted as part of a separate study on the temperature sensitivity of enzyme Vmax and Km parameters. Fitted Vmax values were normalized to an overall mean of 1 for PPO and 1/3 for each of the other three enzymes. With the normalized values, we calculated activity ratios as PPO/(BG+BX+CBH) such that ratios >1 indicate greater relative investment in recalcitrant C degradation. Because there were no interactions between incubation temperature and the field warming treatment, activity ratios were averaged across incubation temperatures to obtain a single ratio for each experimental plot.

Statistical analysis

To test our hypothesis, we conducted repeated measures analyses of variance (ANOVAs). Our dependent variable was the ratio of recalcitrant to non-recalcitrant C remaining, and the independent variable was warming treatment. Sampling date was the temporal factor. We conducted the same tests for mass remaining of each C fraction and for overall mass loss. For statistically significant ANOVAs, we followed up with post hoc t-tests to compare means within each sampling date.

For our prediction that the EEA activity ratios of recalcitrant to non-recalcitrant C should increase with warming, we performed a mixed-model ANOVA with block as a random factor. Our independent variable was warming treatment, and our dependent variable was the EEA activity ratio of recalcitrant to non-recalcitrant C. We log-transformed

EEA activity ratios to meet assumptions of normality. All data were analyzed using R software (R Core Development Team, 2009).

RESULTS

The ratio of recalcitrant C to non-recalcitrant C remaining in decomposing litter was significantly lower in the warmed treatments than in the controls, but only after 12 months of decomposition (Fig 3.1, warming x date interaction, $F_{3,24} = 3.102$, $P = 0.046$). Interestingly, across all sampling dates, significantly more litter mass remained in the warmed treatment than in the control (Fig 3.2, $F_{1,8} = 11.91$, $P = 0.009$) and there was no significant interaction with sampling date ($F_{3,24} = 0.98$, $P = 0.419$).

The warming effect on recalcitrant versus non-recalcitrant C after 12 months was attributable to declines in the breakdown of cellulose and soluble sugars, but not lignin (Fig 3.3 and Table 3.1). Warming did not significantly alter lignin loss across sampling dates ($F_{1,8} = 3.28$, $P = 0.108$), but cellulose loss was significantly slower in the warmed plots than in the control plots ($F_{1,8} = 8.55$, $P = 0.019$). In addition, soluble sugar loss was marginally reduced by warming ($F_{1,8} = 4.121$, $P = 0.077$). Hemicellulose loss was not significantly altered by warming ($F_{1,8} = 2.17$, $P = 0.179$). There were no significant interactions between sampling date and treatment for any of the chemical fractions ($F_{3,24} < 1.89$, $P > 0.159$ for all). Moreover, warming nearly doubled the ratio of recalcitrant C-targeting enzymes (i.e., PPO) to non-recalcitrant C-targeting enzymes (i.e., sum of BG, CBH, and BX) (Fig 4) ($F_{1,4} = 46.86$, $P = 0.002$). Most of this change was driven by the non-recalcitrant enzymes whose normalized activities declined from 1.29 0.11 to 0.86 21 (mean SE) with

warming. In contrast, normalized recalcitrant enzyme activity remained similar (1.05 0.15 for control versus 0.94 0.22 with warming).

DISCUSSION

In our study, we found that warming affected degradation of recalcitrant C versus non-recalcitrant C. Specifically, ratios of recalcitrant to non-recalcitrant C remaining were lower in the warming treatment compared to controls (Fig 3.1) despite slower overall litter decay (Fig 3.2). This shift in C decay ratios occurred because decay of non-recalcitrant C declined significantly with warming, but decay of recalcitrant C did not (Fig 3.3). Moreover, under warming, microbes shifted their allocation away from extracellular enzymes that targeted non-recalcitrant C (Fig 3.4). Altogether, we accepted our hypothesis that warming would reduce the ratio of recalcitrant C to non-recalcitrant C remaining in decomposed litter, consistent with a potential direct kinetic effect of warming and/or a shift in the fungal community with increased ability to break down recalcitrant C, as previously reported (Treseder et al., 2016). However, indirect warming effects like drying might have also influenced our results. Below we will discuss this possibility.

Moisture is a major control over decomposition in cold biomes (Aerts, 2006; Bronson et al., 2008; Hicks Pries et al., 2013; Liu et al., 2009; Suseela et al., 2012; Verburg et al., 1999). Indeed, moisture constraints might have played an important role in decay dynamics in our experiment. Our warmed plots are on average 22% drier than control plots (Allison and Treseder, 2008). This drying effect likely contributed to declines in microbial biomass and soil respiration documented earlier in this field experiment (Allison and Treseder, 2008). In fact, this drying effect might be responsible for the overall slower

decomposition in warmed plots compared to controls (Fig 3.2). Another abiotic factor that might be exerting control over decomposition is nutrient availability. For example, increases of nitrogen in soils can reduce fungal diversity (Allison et al., 2007) and biomass of microbial decomposers (Treseder, 2008). In a previous study in our experimental warming plots, warmed plots had a slight increase in nitrogen availability compared to control plots (Allison and Treseder, 2008).

In addition to the indirect effect of drying on decomposition, we may have observed a direct warming effect. We found two lines of evidence for this effect. The first is the decline in recalcitrant: non-recalcitrant C ratios after 12 months of decomposition (Fig 3.1). The second is the shift in EEA away from enzymes that break down non-recalcitrant C (Fig 3.4). These responses are consistent with theory based on thermodynamics of chemical reactions—recalcitrant C is expected to be more temperature sensitive than non-recalcitrant C (Arrhenius, 1896; Davidson and Janssens, 2006). Even though microbial activity declined in general, warmer temperatures could have allowed those microbes that were active to better acquire energy from recalcitrant C. In addition, warming may have selected for microbial taxa that produce fewer non-recalcitrant-degrading enzymes because the resource returns from these enzymes were relatively lower under warming. However, this could be the effect of warming-induced drying. Previous research has shown that EEA of non-recalcitrant degrading enzymes (i.e. carbohydrate-degrading enzymes) decreases up to 63% with drying (Alster et al., 2013).

Treseder et al. (2016) examined the fungal community composition in litterbags from the current study. They reported that eight years of experimental warming had selected for recalcitrant C-decomposers, mostly represented by free-living filamentous

fungi. This finding mirrors earlier observations by McGuire et al. (2010), who found that the ability to use lignocellulose was positively related to warming responses of fungal taxa after one year of warming in this experiment. A warming experiment in Harvard Forest documented similar results with bacteria, where the warming treatment tended to enrich putatively lignin-using bacterial taxa (Pold et al., 2015). These previous studies suggest that the relative increase in recalcitrant C degradation that we found in our current study might be facilitated by community shifts toward microbial taxa with the capacity to enzymatically access and use these compounds.

If losses of recalcitrant C in litter increase under warming, what are the potential consequences for soil C storage? Decades- and century-old carbon is more temperature sensitive than months- and years-old carbon (Conant et al., 2011). In this sense, most soil organic matter in high latitude ecosystems is considered recalcitrant since it is decades old or older (Jones et al., 2005). Where soil moisture does not become more limiting with warming, an increase in recalcitrant C decay could reduce soil C storage. Nevertheless, the boreal forest we examined may not fit this scenario because warming-induced drying appeared to limit microbial activity, which could mitigate losses of soil C (Bradford et al., 2016). In our system, warming may serve to maintain the decomposition rates of recalcitrant C despite negative effects of moisture limitation on overall decomposition.

CONCLUSION

In conclusion, our data suggest that in boreal ecosystems, recalcitrant C loss from litter differed in sensitivity to warming compared to non-recalcitrant C loss. Altogether, we found that warming decreased the ratio of recalcitrant to non-recalcitrant C, accompanied

by a higher ratio of enzymes that target recalcitrant C. This change was consistent with previous observations of a shift in the fungal community toward lignin users. We stress the need to incorporate empirical measurements of recalcitrant C losses into field warming manipulations, along with assessments of microbial physiology like extracellular enzyme activities, to better assess the fate of litter inputs under warming in the next century.

TABLE

Table 3.1. Litter chemistry in litter decomposed in control and warmed plots.

C fraction	Sampling time (months)	Control (mg)	Warmed (mg)
Lignin	Initial	287 ±15	
	1	221 ±18	265 ±9
	2	261 ±6	297 ±9
	12	168 ±17	164 ±19
	16	189 ±21	170 ±12
Cellulose	Initial	312 ±14	
	1	153 ±17	160 ±9
	2	135 ±4	163 ±10†
	12	69 ±5	95 ±5*
	16	78 ±6	99 ±6*
Hemicellulose	Initial	115 ±8	
	1	19 ±2	29 ±3
	2	36 ±3	39 ±3
	12	14 ±2	12 ±3
	16	9 ±2	8 ±1
Soluble sugars	Initial	21 ±1	
	1	11 ±1	11 ±2
	2	7 ±0	10 ±0*
	12	3 ±0	4 ±0*
	16	1 ±0	2 ±0†

Values are mean ±SE, n = 5 plots. *P < 0.05, †P < 0.10 between treatments.

FIGURES

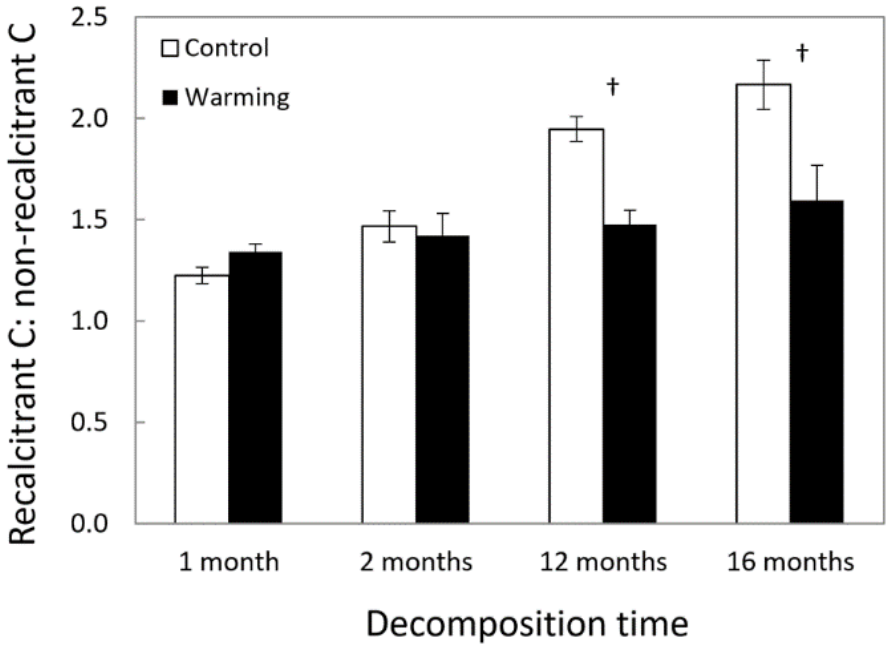

Figure 3.1. Mass remaining of recalcitrant (lignin) to non-recalcitrant (cellulose, hemicellulose, and soluble sugars) C over time. Across sampling times, the ratio was significantly lower in the warming treatment ($P = 0.032$), but there was a significant interaction between treatment and time ($P = 0.046$). Data are means ± SE, with $n = 5$ plots. †$P < 0.10$ for sampling date.

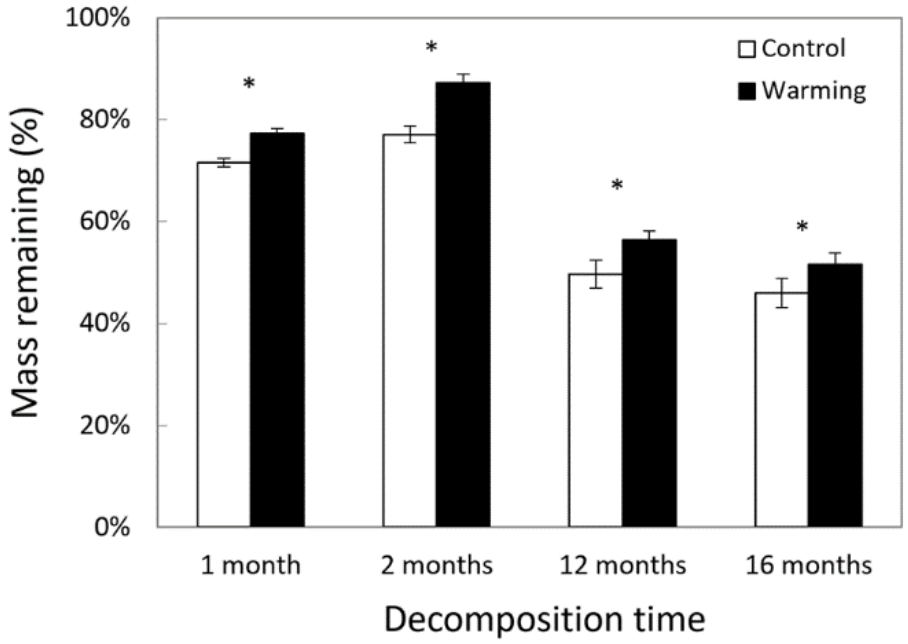

Figure 3.2. Percentage total mass remaining in spruce needles over time. Decomposition was significantly slower in the warming treatment compared to the control (P = 0.009). Data are means ± SE, with n = 5 plots. *P < 0.05 for sampling date.

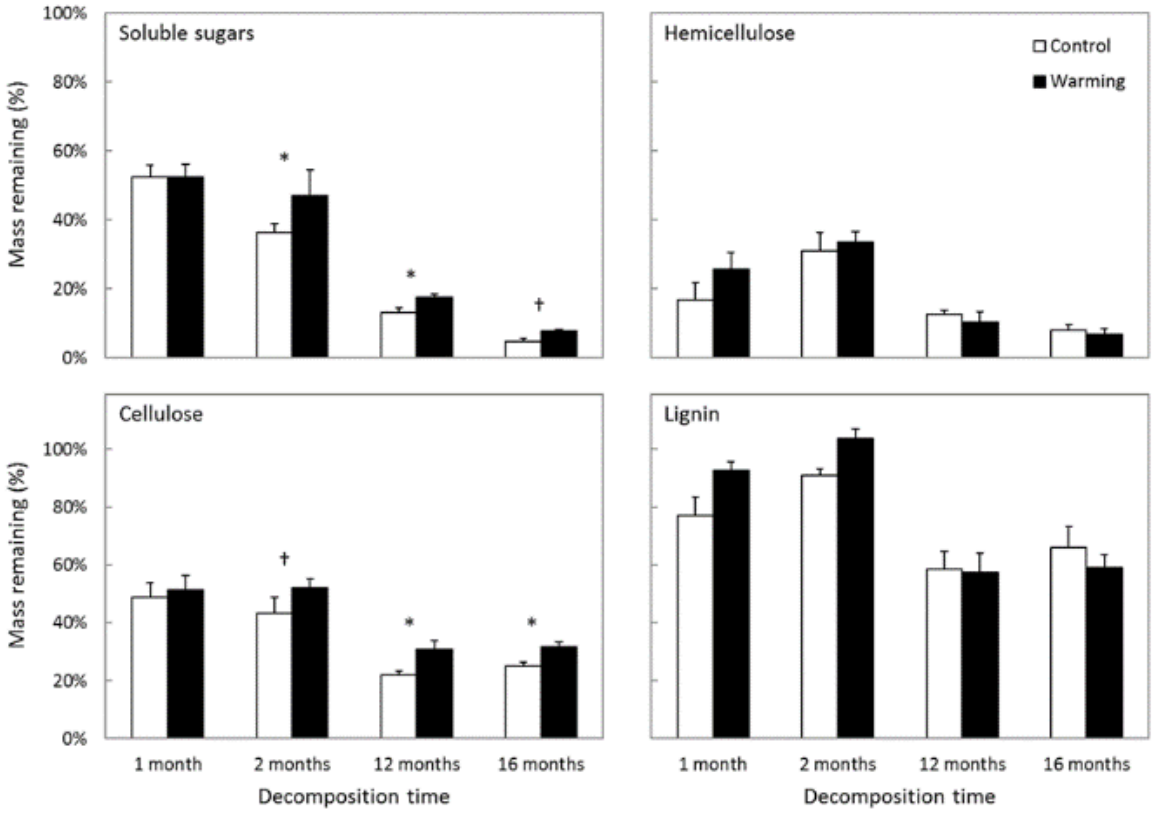

Figure 3.3. Percentage of mass remaining of lignin, cellulose, hemicellulose, and soluble sugar in spruce needles over time. Warming did not significantly affect lignin breakdown ($P = 0.108$) or hemicellulose breakdown ($P = 0.179$). In contrast, warming slowed the breakdown of cellulose significantly ($P = 0.019$) and soluble sugars marginally significantly ($P = 0.077$). Data are means ± SE, with $n = 5$ plots. *$P < 0.05$, †$P < 0.10$ for sampling date.

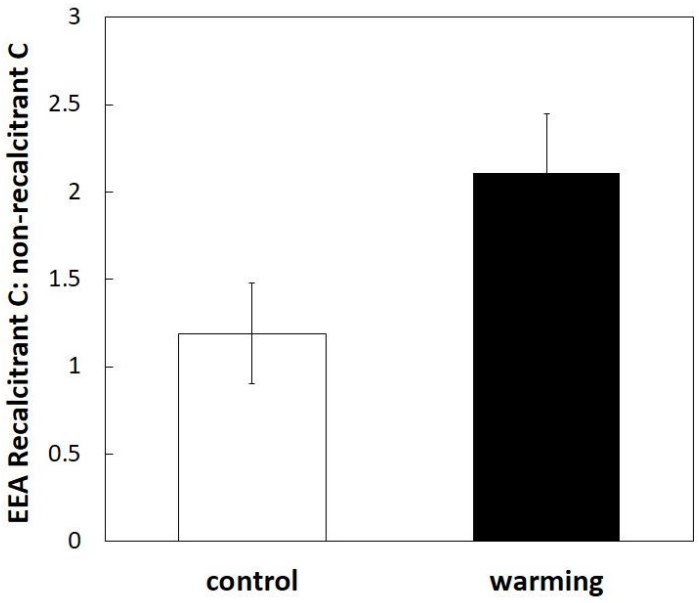

Figure 3.4. Extracellular enzyme activity (EEA) ratios of recalcitrant to non-recalcitrant C on litter retrieved at 12 months. Recalcitrant enzyme activity is polyphenol oxidase, while non-recalcitrant enzymes are the sum of cellobiohydrolase, β-xylosidase, and β-glucosidase. Warming significantly increased the ratio of recalcitrant C decay enzymes to non-recalcitrant C decay enzymes (P = 0.002). Activities were measured in units of nmol h-1 g-1 dry litter. Data are means ± SE, with n = 5 plots.

CONCLUSION

Understanding terrestrial carbon (C) dynamics under global warming from a soil microbial perspective is an area of great interest and debate in ecosystem ecology. Soil microbes are the main decomposers of C in terrestrial ecosystems and thus play an important role in soil C dynamics. If we can understand how microbes respond to warming, we can better predict the future of soil C stocks (Singh et al., 2010). Hence, to improve our understanding of warming effects on microbes and the environment, scientists have relied on warming experiments to foresee microbial community changes that might impact ecosystem processes (Rustad, 2008). However, the duration of various warming experiments has caused discrepancies in C-related observations. For example, the response of soil respiration in short-term warming experiments differs from the response in long-term warming experiments. In the latter, the evolutionary process of adaptation in microbes may be a strong determinant of warming effects (Pastor, 2016). Understanding how C dynamics will change under warming on the long-term is pivotal for developing accurate climate change projections; integrating the process of microbial evolutionary adaptation to warming will increase the precision of those projections. Thus, a current need in ecosystem ecology of climate change is to monitor long-term warming effects of C dynamics and integrate the process of microbial evolutionary adaptation to better assess those effects. Therefore, the overarching questions of my dissertation were 1) How do microbes respond to warming over time and what are the consequences to the C cycle? 2) What physiological changes coincide with fungal adaptation to warming and how can these responses feedback to global climate change? and 3) How do decomposition patterns

change in response to warming and what underlying mechanism might be driving this change?

In my first chapter, I addressed the first question using meta-analysis. I analyzed the results of 25 warming experiments ranging from 1 to 15 years with more than one sampling time point across the duration of the experiment. I synthesized data relevant for ecosystem C dynamics such as microbial biomass, soil C, and soil respiration. I found that the effect of soil respiration decreases as warming progresses and this effect is especially evident after 5 years. However, the effect of microbial biomass and soil C did not change throughout time. Nonetheless, these effects were not measured as often as the effects of soil respiration. Perhaps more rigorous monitoring on the effects of microbial biomass and soil C throughout time might change these observations. In fact, measuring microbial biomass in a reproducible manner has been listed as one of fifty important research questions in microbial ecology (Antwis et al., 2017). In summary, my meta-analysis highlighted the importance of long-term observations in warming experiments, as short-term observations might be biased by seasonal and or temporary (i.e., acclimation) effects. In fact, warming experiments might not represent well seasonal changes thus, short-term observations on warming experiments might have this additional potential error. My meta-analysis showed that long-term responses to warming of microbes and soil C require more attention thus, these two topics were further assessed in Chapters 2 and 3 in my dissertation.

The long-term responses to warming of microbes may be unnoticeable if measured as soil microbial biomass. Microbes might have ways of adapting to warming without altering their biomass production. For example, microbial communities might shift in

composition without altering the total microbial biomass in the soil or microbes might adapt their physiology to warming without altering their phenotype (e.g., biomass production). In fact, microbial community shifts to long-term warming have been reported by using PCR-based community analysis (i.e.,Pold et al., 2015; Treseder et al., 2016). However, assessing the adaptation of soil microbes to long-term warming is still a technical challenge. Therefore, in my second chapter I addressed the second question: what physiological changes coincide with fungal adaptation to warming and how can these responses feedback to global climate change? To this end, I adapted the fungus *Neurospora discreta* to warm temperatures of 16 °C and 28 °C for 1500. I measured physiological traits important to the C cycle, such as biomass production, CO_2 respiration, spore production, and growth rate, before and after adaptation. I found that after 1500 generations, *N. discreta* adapted to warm temperatures by producing more spores at the expense of biomass, therefore increasing its fitness. In other words, in the selective temperatures, *N. discreta* produced more spores but also produced less biomass. Thus, there was an increase in CO_2 respiration, potentially because the production of spores is more metabolically expensive than the production of biomass. This was an unexpected result. Based on my meta-analysis, I was expecting CO_2 respiration to decrease after long-term exposure to warming.

Although my results with *N. discreta* revealed evolutionary adaptation strategies of fungi under warming, they should be taken with caution when extrapolated to ecosystem-level ecology. In my evolution experiment, *N. discreta* had access to unlimited labile C, nutrients, and water. Without any other stress, except for warming and space availability (i.e., racing tubes), *N. discreta* increased its chances of survival by colonizing new space

through higher production of spores. However, in nature this might not be the best strategy. In the wild, fungi are not as limited by space as they are by resources, especially in tough environments with cold temperatures and recalcitrant C substrates. In this case, fungi might adapt using a more metabolically efficient strategy, perhaps by increasing growth rate to colonize space faster without investing in producing expensive spores. The results of my second chapter might be unique for *N. discreta* and thus, not a good representation of adaptation to warming of the overall soil microbial community. This type of experiment should be replicated with other microbes from diverse taxonomic backgrounds and diverse functional groups. For example, yeast and specialized fungi such as wood decomposers.

In Chapter 3, I addressed the third and final question of my dissertation. How do decomposition patterns change in response to long-term warming and what underlying mechanism might be driving this change? To answer this question, I measured overall decomposition (i.e., total mass loss) and decomposition of recalcitrant (i.e., lignin) and non-recalcitrant C (soluble sugars, cellulose, and hemicellulose). In addition, I measured microbial extracellular enzyme activities (EEA) involved in the breakdown of each type of C. I found that in warmed plots decomposition is slower compared to control plots. However, losses of recalcitrant C are higher compared to non-recalcitrant C in warmed plots vs control plots. In other words, the decomposition of recalcitrant C slows down in control plots but not in warmed plots. The microbial EEA supported this result, since the ratio of recalcitrant:non-recalcitrant EEA was higher in warmed plots. My results complement findings by Treseder and collaborators (2016) that show that warming induces the proliferation of recalcitrant decomposers in the same long-term warming

experiment where I conducted this decomposition experiment. Altogether, my results could support the idea that warming favors the decomposition of recalcitrant C over non-recalcitrant C, potentially due to increased availability of recalcitrant C under warming. However, these results should be taken with caution due to two characteristics specific of my study. First, even though EEA and mass loss of recalcitrant C showed that lignin was being broken down more than non-recalcitrant C under warming, I did not find direct physical or chemical evidence to support the claim that recalcitrant C is more temperature sensitive than other C fractions in litter. Second, warming experiments might not replicate accurately seasonal changes thus, our results might have a co-founding bias based on lack of seasonality. Third, my decomposition study was carried out in senescent litter, thus my results are unique to that type of C in an ecosystem. Decomposition of different C fractions in diverse soil horizons should be further explored in detail. Similarly, further research should focus on unearthing the mechanistic explanation behind temperature sensitivity of the different types of C in litter and soil.

In summary, in my dissertation I discovered that soil respiration decreases as warming progresses. However, elucidating the mechanisms behind this attenuation is an area of research that requires further investigation. Although my evolutionary adaptation experiment with *N. discreta* aimed to provide a mechanistic explanation to decreases in soil respiration after long-term exposure to warming, my results did not parallel the findings of my meta-analysis. Nonetheless, I provided an overview of an adaptation strategy certain fungi might utilize when adapting to warming. However, I urge further research on this area, as the microbial world is vast and diverse, and *N. discreta* does not represent the entire microbial community. Research on fungal ecology in the context of evolution had

never been easier. The 1000 fungal genomes project, as well as the Fungal Genetic Stock Center (FGSC), offer a broad collection of genomes and fungal cultures, respectively, that can be used to further our knowledge in evolution of fungi. Only by producing more data, we will be able to concisely conclude on the ecological consequences of the adaptation of fungi to warming.

I discovered that under warming, losses of recalcitrant C in litter are greater compared to non-recalcitrant C losses. Although, I provided indirect evidence (i.e., increased mass loss and EEA of recalcitrant:non-recalcitrant C under arming) to support the idea that decomposition of recalcitrant C will increase under warming, further research is needed to conclude if recalcitrant C is more temperature sensitive than other C fractions. Future research in this area must be multi-trophic. For example, we need to know if the composition of leaves is changing with changes in the environment. Are these changes in litter composition triggering changes in the microbial community and thus on patterns of decomposition? We must take advantage not only of long-term ecological field experiments, but also of microcosm and greenhouse experiments, where it is easier to monitor multi-trophic interactions (e.g. plant-microbe). In conclusion, by implementing the evolutionary adaptation of microbes from diverse taxonomic backgrounds to warming, complemented with genomic studies, together with multi-trophic observations (e.g. plant responses to global warming) and abiotic conditions (e.g. warming-induced drying), we will be able to accurately forecast the future of soil C pools and CO_2 feedbacks to the atmosphere under global climate change.

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