SHORT AND LONG-TERM EFFECTS OF NITROGEN FERTILIZATION ON CARBON AND NITROGEN CYCLES IN NORTHEASTERN FOREST SOILS

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Humans now dominate the nitrogen cycle by producing more biologically available nitrogen (N) than all natural processes combined. Over the past 150 years, northeastern forests have seen a four to ten-fold increase in N deposition as a result of anthropogenic activities such as agriculture and industry. Most of this added N is retained in surface soils. N deposition has the potential to alter atmospheric carbon dioxide (CO_2) by influencing rates of decomposition of the vast reservoir of soil organic matter. N deposition may also change the soil nitrogen cycle, influencing the ability of soils to retain N. The objective of this work was to measure the response of decomposition and N retention to added N in northeastern forest soils.

I used a cross-site lab experiment with soils from five long-term N addition experiments to measure the effects of long-term N fertilization on CO_2 release, microbial biomass, heavy soil C (ie: mineral-associated), light soil C (ie: not mineral-associated), dissolved organic carbon (DOC), and the activities of four C-degrading enzymes. In response to a short-term pulse of N applied to soils in the lab, I focused on changes in CO_2 and C degrading enzymes. To study the effects of N addition on N retention, I added a ^{15}N tracer to incubating soils, and measured the partitioning of the tracer into several soil N pools.

I found evidence to support the hypothesis that N fertilization can stimulate decomposition of easily degraded labile C such as sugars and starches; I measured

transient increases in CO_2 respiration and a cellulose-degrading enzyme with a short-term pulse of N at one site. N inhibited decomposition of harder to degrade, lignin-based C compounds, decreasing CO_2 respired during the first week of a year long incubation at four sites, along with microbial biomass and the lignin-degrading enzyme phenol oxidase. Soils with more organic matter appear to retain more N; light fraction ^{15}N retention was positively correlated with soil %C. N addition reduced ^{15}N retention in mineral soils.

BIOGRAPHICAL SKETCH

Marissa Sage Weiss grew up in Rockville Centre, New York, and from an early age gravitated to environmental science. Her favorite class trip in grade school was to the New York Museum of Natural History. Her formative outdoor experiences were hiking in the White Mountains of New Hampshire on summer vacations with her family. As a teenager, she learned about field biology from a National Geographic article, and immediately thought, "That's what I want to be when I grow up."

In high school, Marissa had two important mentors. Mrs. Goldie Seiderman taught history, English, and social studies. She encouraged and modeled intellectual curiosity, and has continued to be an inspiration, mentor, and friend to this day. Mr. Stephen Mutz taught biology, and Marissa was fortunate to take both his 9th and 12th grade classes. He also taught an elective called Research Skills, where he mentored Marissa for a semester, designing and carrying out a science fair type project.

Mr. Mutz's classes inspired Marissa to seek a summer science research program. In July and August 1996, she participated in the Juneau Icefield Research Program, where she worked with another high school student to study glacial retreat, using lichens on exposed rock surfaces to determine the relative duration of exposure from the ice.

Marissa was an undergraduate at Cornell University, where she took as many field courses as she could. She was hired by Dr. Joe von Fisher, who was then a graduate student, to work as a general lab assistant in Dr. Lars Hedin's ecology lab. Her first assignment was to process root and soil samples for Dr. Steve Perakis who was also a graduate student in Dr. Hedin's lab at the time. Marissa was mentored in the Hedin lab by the legendary and talented Mike Brown, who did analytical chemistry and general lab management in the Hedin lab. Dr. von Fisher and Dr. Perakis encouraged Marissa to take Dr. Hedin's Ecosystem Ecology class, saying it

would explain the basis for everything happening in the lab. In Dr. Hedin's class, Marissa met Dr. Gretchen Gettel, then the graduate TA of the class, who became another influential mentor. She suggested Marissa contact Dr. Sarah Hobbie of the University of Minnesota about a summer Research Experiences for Undergraduates (REU) internship at the Arctic Long Term Ecological Research Site, Toolik Lake, Alaska. Marissa's first field season at Toolik Lake was in the summer of 2000 as an REU with Dr. Hobbie, where Dr. Gettel also was doing her graduate research, and both collaborated to mentor Marissa who studied nitrogen fixation in Arctic lichens.

Upon graduating from Cornell in 2001, Marissa moved to St. Paul, MN, to be a graduate student with Dr. Hobbie at the University of Minnesota. She was so taken with the Arctic, the lichens, and working with Dr. Hobbie and Dr. Gettel that she returned to Toolik Lake for two more field seasons for her masters research.

Marissa completed her M.S. in 2003. She took a job with Dr. Laurie Drinkwater in Cornell's Department of Horticulture. She worked as a technician and lab manager studying agricultural ecology and biogeochemistry. There, her interest in and appreciation for soils deepened. She learned many soil sampling and analysis skills which would come in handy as a Ph.D. student.

In the fall of 2004, Marissa returned to graduate school, as a Ph.D. student in Dr. Christine Goodale's forest ecology lab, where she studied the effects of nitrogen fertilization on carbon and nitrogen cycling in northeastern forest soils. The project was inspired by nitrogen fertilization studies in arctic and alpine systems, agricultural ecology, and Dr. Goodale's work on nitrogen deposition in northeastern forests.

During graduate school, Marissa collaborated with then fellow graduate student Dr. Dana Warren to coordinate a science communication class for graduate students in the sciences. Marissa's interest in communication grew, and upon completing her thesis she will be an American Association for the Advancement of

Science (AAAS) Science and Technology Mass Media Fellow at KUNC public radio in Greeley, Colorado.

For my grandparents, Toby and Irving Sternman and Harriet and Emeric Weiss

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I am grateful to the many mentors, collaborators, friends and family who have helped me complete this thesis. My advisor, Christy Goodale encouraged me to push my thinking about soil carbon and nitrogen cycling further and deeper, and gave me helpful suggestions and constructive feedback along the way. My committee members, Eric Davidson, Laurie Drinkwater, and Tim Fahey have all offered sound advice and valuable insights. Each of you helped me to distil pages of data reports into three cohesive stories around which to develop these thesis chapters.

Field site collaborators Mary Beth Adams, Serita Fry, Scott Ollinger, Gary Lovett, Steve McNulty, Johnny Boggs, and Ivan Fernandez welcomed me to their field sites and generously offered soil, and field assistance. I was 9 years old when John Aber initiated the N fertilization experiment at Harvard Forest; the ability to study treatments established while I was still playing with backyard science kits was a tremendous opportunity.

The members of the Goodale lab, April Melvin, Quinn Thomas, Christina Tonitto, and Guin Fredriksen have provided a collaborative work environment and were always first to offer help. Thank you to the BEB grads for providing helpful critiques of my work over the years, and to Suzanne Wapner for being there with answers.

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Rachel Hatch, Oliver Schaufelberger, and Max Kraft. Maddie Stone, you are the champion of the enzyme assays, and I am continuing to enjoy and learn from our collaboration.

Past mentors, Lars Hedin, Mike Brown, Gretchen Gettel, and Sarah Hobbie, I continue to carry lessons from each of you with me. To my mentor-friends, Dana Warren, Neil Bettez, Kathy Crowley, and Meagan Schipanski who recently traveled this road, you generously offered advice and encouraging dispatches from the other side, thank you.

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PREFACE

In the northeastern United States, studies to examine the long-term consequences of anthropogenic nitrogen (N) deposition to forested ecosystems began in 1988 (Harvard Forest, MA and Mt. Ascutney, VT) and 1989 (Fernow Forest , WV and Bear Brook, ME). At first, most researchers had one of three primary interests: acidification, consequences of the added N for N retention and loss within the forest, and consequences of the N for tree growth and mortality. The effects of the added N on the soil carbon (C) cycle were not initially a high priority. However, interest in consequences of added N for soils grew when early studies at Harvard Forest and Bear Brook showed that the bulk of N fertilizer applied to the forest is retained in the soil. Further work at Harvard Forest showed that following N fertilization, a very transitory pulse of carbon dioxide (CO_2) was released from some soils, though over time soil respiration ultimately declined in all soils with added N. European ecosystem ecologists were independently studying the response of soil C to N fertilization, and led the way in advancing hypotheses about the mechanisms underlying the response of soil C to N fertilization. Among their ideas was the hypothesis that N can physically stabilize particular decomposition-resistant compounds, while stimulating decomposers to degrade easily metabolized C more quickly. Though these mechanisms have been examined in the literature, definitively discerning the mechanisms underlying the response of soil C to N fertilization remains elusive.

The motivating question for this thesis is: How does N fertilization affect soil C and N dynamics? My goal was to study the magnitude and mechanisms of the response of soil C to N fertilization, and to study the fate of added N in soil N pools. I have addressed this question with a cross-site lab experiment using soils from five long-term N addition experiments. I used two main approaches. One approach was to examine the response of soil C pools and fluxes to a short-term pulse of added N as

well as to long-term N addition. In response to a short-term pulse of N applied to soils in the lab, I focused on changes in CO_2 emission and C degrading enzymes. I measured the effects of long-term N fertilization on CO_2 release, microbial biomass, heavy soil C (ie: mineral-associated), light soil C (ie: not mineral-associated), dissolved organic carbon (DOC), and the activities of four C-degrading enzymes. The second approach was to add a ^{15}N tracer to incubating soils, and to measure the partitioning of the tracer into various soil N pools. I looked for evidence of a relationship between N retention and SOM, as evidence that SOM plays an important role in N retention.

Chapter 1, "Effects of Long-Term Nitrogen Addition on Carbon Fluxes and Pools in Northeastern Forest Soils," reports the effects of long-term N fertilization on soil C pools and fluxes from the six long-running N addition studies. I found that long-term N fertilization inhibited CO_2 release from the forest floor in the first week of the soil incubation, corresponding with a decrease in microbial biomass and lignin-degrading enzymes. However, this inhibition disappeared later in the long-term incubation, such that there was no effect on long-term CO_2 release over the course of the year.

My objective in Chapter 2, "The response of soil C decomposition to short-term and long-term N fertilization" was to assess the conceptual model that early in decomposition, N fertilization can stimulate decomposition of a small pool of labile C, such as sugars and starches. As N fertilization persists, N will stabilize lignin-based C compounds, decreasing decomposition rate. Over time, decomposers will ultimately become increasingly C limited, and C limitation will exceed the N fertilization effect. I measured changes in CO_2 and C-degrading enzyme activity after adding a pulse of N to Harvard Forest Hardwood and Harvard Forest Pine soils. Within a day of the N fertilizer addition in the lab, Harvard Forest Hardwood soils first showed a transient

pulse of CO_2 release, which was followed by inhibition of CO_2 production in N-fertilized soils. Those same N-fertilized soils also showed elevated levels of cellulose degrading enzyme activity and diminished levels of lignin-degrading enzyme activity at the conclusion of the year-long soil incubation, affirming the conceptual model. Harvard Forest Pine soils did not show any stimulation of decomposition with N fertilization. But decreases in both CO_2 release and lignin-degrading enzyme activity with long-term N fertilization suggest that the Pine soils were lacking a small pool of labile C to be mobilized by the added N. The decreases support the conceptual model of N effects on the later stages of decomposition.

Chapter 3, "Effects of long-term nitrogen addition on nitrogen retention in three soil nitrogen pools from six northeastern forest soils: A ^{15}N tracer approach," reports the results of the ^{15}N tracer addition. I measured the fate of the recovered ^{15}N in the bulk soil, heavy fraction, light fraction, and microbial biomass in forest floor and mineral soil. I found that across sites, N fertilization decreased ^{15}N retention in mineral soils. In the forest floor, most ^{15}N was recovered in the light fraction, whereas in the mineral soil, most ^{15}N was recovered in the mineral-associated N pool (heavy fraction). This indicates that new N can be retained in association with a relatively old and slow cycling SOM pool. There was a positive relationship between soil C and ^{15}N recovery in the light fraction SOM for both forest floor and mineral soil, suggesting that C could be important for N retention. An important next step is to discern whether the associations measured here between added N and SOM result in SOM stabilization, on what timescales, and by what mechanisms.

The results presented in this thesis show that in soils with a pool of easily degraded C compounds, N addition can stimulate decomposition of that labile fraction. N fertilization also slows decomposition of lignin-based C compounds by reducing microbial biomass and lignin degradation. Without new inputs of C, the N effect on

decomposition disappears over time. Thus, the magnitude of the suppression of decomposition over time relies at least in part on the effects of N on C sources to soils, such as trees. The ^{15}N tracer study suggests that SOM may play a role in N retention in soils. The observations in Chapters 1 and 2 that N fertilization can inhibit forest floor decomposition, and the ^{15}N tracer result in Chapter 3 that most of the retained ^{15}N in the forest floor was recovered in the light SOM fraction, taken together, indicate that an important next step is to determine whether added N is stabilizing SOM.

CHAPTER 1

Effects of long-term nitrogen addition on carbon fluxes and pools in northeastern forest soils

ABSTRACT

Atmospheric nitrogen deposition can drive an increase in soil carbon storage in temperate forest ecosystems, yet the magnitude of carbon sequestration and the mechanisms underlying the response of soil carbon to nitrogen addition remain poorly understood. Here, we conducted a suite of measurements of forest floor and surface mineral soils from six forests in the northeastern U.S. with long-term nitrogen addition experiments; the goal of this experiment was to measure the effects of nitrogen addition on pools and fluxes of soil carbon. We quantified the response of four soil carbon pools to long-term nitrogen addition: light fraction, heavy fraction, leachable organic carbon, and microbial biomass carbon in forest floor and mineral horizon soil. We also measured carbon-degrading enzyme activities at all sites and microbial respiration of carbon dioxide. All measurements were made at the start of a year-long soil incubation experiment in which of carbon dioxide production was monitored over the course of a year. Across sites, the four carbon pools were largely resistant to detectable change from long-term nitrogen addition in both forest floor and mineral soil. Forest floor microbial biomass and oxidative enzyme activity decreased in response to long-term N addition which, taken together, indicate that nitrogen addition decreases microbial population size and activity, thus decreasing the decomposition rate of soil organic matter in the forest floor. Mineral soil microbial biomass and enzyme activity were generally unresponsive to nitrogen addition. Heterotrophic respiration in the forest floor declined significantly in response to nitrogen addition at four sites, averaging a 28% decrease over the first week. Over the course of a year,

the nitrogen effect on forest floor respiration disappeared as respiration slowed, presumably because the microbial decomposers became limited by degradable carbon substrates; this substrate limitation may have superseded the nitrogen inhibition detected earlier in the incubation. Nitrogen addition did not significantly change respiration in mineral soils. Although we did find that long-term nitrogen addition inhibited heterotrophic respiration during the first week of measurements in the forest floor, the inhibition was transient; by the end of one year nitrogen addition no longer inhibited decomposition, indicating that soils are not likely to sustain rapid increases in carbon sequestration in response to nitrogen addition.

Keywords:
decomposition, soil carbon, nitrogen addition, density fractions, soil respiration, extracellular enzyme, microbial biomass

INTRODUCTION

Human production of biologically available nitrogen (N) now exceeds N fixation by all natural terrestrial processes combined (Vitousek et al. 1997, Galloway and Cowling 2002). Over the past 150 years, northeastern forests have seen a 4 to 10-fold increase in N deposition as a result of anthropogenic activities such as agriculture and industry (Galloway et al. 2004, Elliott et al. 2007). N deposition has the potential to alter atmospheric carbon dioxide (CO_2) by influencing rates of decomposition of soil organic matter. With regard to effects on the ecosystem carbon (C) balance, N deposition first received attention for its potential to stimulate plant growth (e.g., LeBauer and Treseder 2008, Thomas et al., 2010, Goodale et al. submitted). Only recently have the effects of N deposition on soil C begun to receive comparable

attention (Janssens et al. 2010). Understanding the effects of N deposition on soil C is important for discerning the effects of N pollution on sequestering atmospheric CO_2, however we cannot yet reliably predict how much C a particular soil may sequester with N addition, and we do not fully understand the mechanisms underlying soil C sequestration with N addition.

Forest soils contain a large reservoir of organic C, and added N may decrease decomposition rates and stabilize the large soil C pools. In the Northern Hemisphere alone, forests contain around 288 Pg C in the forest floor and top 1 m of mineral soil, or nearly three times the amount of C stored in forest vegetation (97 Pg C; including dead wood) (Goodale et al. 2002). Soil C is consumed by heterotrophic microbes which produce CO_2; this pathway moves C from the soil pool to the atmosphere (Schlesinger and Andrews 2000). N deposition may be changing both the size of the soil C pool and the rate of CO_2 flux from soils to the atmosphere.

A new consensus is emerging that in temperate forests, N addition typically leads to a decrease in the CO_2 flux from soils to the atmosphere, increasing soil C accumulation (Fog 1988, Berg and Matzner 1997, Magill and Aber 1998, Agren et al. 2001, Franklin et al. 2003, Hagedorn et al. 2003, Bowden et al. 2004, Micks et al. 2004, Pregitzer et al. 2008, Zak et al. 2008, Janssens et al. 2010, Goodale et al. Submitted). However, other ecosystems, such as Arctic tundra show the opposite result (Mack et al. 2004, Janssens et al. 2010). The magnitudes and exact mechanisms behind the response of soil C to N addition is open to lively debate.

Across northern temperate forests, several studies estimate C sequestration in soils to be 5 to 30 kg C per kg of N added (De Vries et al. 2006, Hogberg 2007, Janssens et al. 2010, Goodale et al. Submitted). Increasing the accuracy of models and large-scale estimates of the soil C response to N ultimately requires a better understanding of the underlying microbial mechanisms coupled with detailed

measurements to link microbial mechanisms to changes in soil C pools.

The goal of this work was to link microbial mechanisms to changes in soil C pools in response to long-term N addition. To that end, we examined three metrics of microbial activity (CO_2 flux, microbial biomass, and extracellular enzyme activity), and two operationally defined soil organic matter fractions (light and heavy). CO_2 fluxes give a direct measure of microbial activity, which is generally correlated with microbial biomass (Treseder 2008), although microbial biomass could theoretically increase in the absence of a change in CO_2 if the microbes simultaneously increase C use efficiency (Schimel and Weintraub 2003). Thus, knowing both respiration rate and biomass is useful for inferring changes in microbial C use efficiency and for drawing mechanistic conclusions about the response of microbial communities to N addition. Extracellular enzymes are the primary means by which soil microbes degrade soil organic matter; examining the response of soil enzymes to added N is a useful way to mechanistically evaluate the response of soil C processes to N addition. This approach has been applied to forest soils, and these studies report that N addition often corresponds with a decrease in the production of phenol oxidase, a lignin-degrading enzyme (Carreiro et al. 2000, Saiya-Cork et al. 2002, Frey et al. 2004, Waldrop et al. 2004, Sinsabaugh et al. 2005). N addition has also been associated with the stimulation of two cellulolytic enzymes that degrade labile organic matter, b-glucosidase and cellobiohydrolase (Carreiro et al. 2000, Saiya-Cork et al. 2002, Sinsabaugh et al. 2005). These studies have documented the impacts of N addition on enzyme activity at individual sites. Our study contributes new information by applying a standard suite of C decomposition measurements across six long-term N addition experiments, expanding the range and geographic scope of our knowledge. Also, here we connect enzymatic mechanisms with C fluxes and C pools to understand the effect of long-term N addition on soil organic matter decomposition at six

northeastern forested sites.

To connect the microbial activity responses to soil C pools, we used density fractionation of two operationally defined soil organic matter fractions, heavy C and light C. These two fractions are distinguished by flotation; the heavy fraction has a density greater than 1.65 g cm^{-3}, and sinks in a solution of that density, whereas the light fraction is low density and floats in the same solution (Sollins et al. 1999). In the heavy fraction, C is bound to mineral soil, physically protected from decomposers, whereas light soil C is presumed to be freely available to microbial decomposers. The history, advantages, and limitations of the density fraction method are reviewed in detail by Crow et al. (2007). The relevant limitation of the method for this study is that C quality varies considerably within the light fraction: some C molecules are easily-degradable simple sugars and starches, and some are large and unruly lignin-related compounds with complex phenolic structures, which have low density, yet are not rapidly metabolized by microbial decomposers. We expect N addition to decrease decomposition of the humic materials within the light fraction. If the decomposition of the lignin-related compounds in the light fraction slows, the size of the light fraction pool should increase over time, yet the density fractionation method will only yield information about the physical stability of the soil C pools and not the chemical nature of the C. Nonetheless, density fractionation gives us a more detailed view of two soil C pools, and we used the method to test for N effects on soil C fractions whose differences could be masked by the larger and even more heterogeneous bulk soil pool.

We conducted a soil decomposition experiment to estimate the magnitude of the response of soil C to N addition in northeastern forest soils, and to elucidate microbial mechanisms underlying that decomposition response. We measured the consequence of long-term N addition on four soil C pools: light fraction, heavy

fraction, microbial biomass and dissolved organic carbon (DOC). We also measured extracellular enzyme activity to discern the effect of long-term N addition on microbial decomposition of soil C.

METHODS

Study Sites

We collected forest soils from six long-term N addition experiments at five sites spanning the northeastern United States (Figure 1.1): Fernow Forest, West Virginia; Cary Institute, New York; Harvard Forest, Massachusetts (Hardwood and Pine); Mt. Ascutney, Vermont, and Bear Brook, Maine (Table 1.1). Each of these experiments was established to evaluate the effects of acid rain and/or chronic N additions on forests. The sites were established in the late 1980's to mid 1990's, and vary in ambient N deposition rates, addition rates and type of N fertilizer applied (Table 1.1). To date, very little information has been published on soil CO_2 fluxes or stocks across these six experiments. With the exception of Harvard Forest, where soil respiration decreased (Bowden et al. 2004, Micks et al. 2004) but soil %C had not changed detectably after 15 years of N addition (Magill et al. 2004), no other sites have published N effects on soil C stocks.

Figure 1.1: Study Sites

Table 1.1 Characteristics of field sites. Field site abbreviations are FF = Fernow Forest, CI = Cary Institute, HH = Harvard Forest Hardwood, HP = Harvard Forest Pine, AS = Mt. Ascutney, and BB = Bear Brook.

Field Site	Latitude, Longitude	Soil	Forest Type	Fertilized Area	Fertilizer Type	Fertilizer Rate ($kg\ ha^{-1}\ year^{-1}$)	Ambient Deposition ($kg\ ha^{-1}\ year^{-1}$)	Start Year	Soil Collection Date	References
FF	39°03'N, 29°49'W	Typic Dystrochrept	Mixed Hardwood	Watershed[a]	$(NH_4)_2SO_4$	36	18	1989	7 June 2007	1, 2
CI	41°50'N, 73°45'W	Lithic Dystrochrept	Oak	Plot	NH_4NO_3	50[b]	10	1996	21 June 2007	3
HH	42°30'N, 72°10'W	Typic Dystrochrepts	Mixed Hardwood	Plot	NH_4NO_3	50	8	1988	21 Oct 2008	4
HP	42°30'N, 72°10'W	Typic Dystrochrepts	Red Pine	Plot	NH_4NO_3	50	8	1988	22 Oct 2008	4
AS	45°26'N, 72°27'W	Typic Haplorthods	Spruce-Fir	Plot	NH_4Cl	31.4	5.4	1988	3 July 2007	5, 6
BB	44°52'N, 68°96'W	Typic & Aquic Haplorthods	Mixed Hardwood	Watershed	$(NH_4)_2SO_4$	25.2	4.5	1989	5 July 2007	7

References:

1. Gilliam et al. 1994
2. Gilliam et. al. 1995
3. Wallace et al. 2007
4. Aber et al. 1993
5. McNulty and Aber, 1993
6. McNulty et al. 1996
7. Norton et al. 1994

Notes:

a. We sampled WS# 13 (Ambient) and WS #3 (Nitrogen Fertilized).

b. For the first 3 years, the nitrogen fertilizer rate was 100, and has been 50 from 2000 to present.

Soil Sampling

Soils were collected in May – August 2007, and October 2008. Forest floor samples were collected to the depth of the forest floor – mineral horizon boundary with a tulip bulb corer (7 cm diameter). We visually assessed the forest floor – mineral horizon boundary to separate the two types of soil. Separating mineral soil and forest floor is notoriously subjective (Federer 1982). We made the following distinctions to separate our soils: Forest floor material at the boundary with mineral soil was dark brown to black in color, and was dense with roots. Mineral soil was generally orange to pale yellow in color. At Bear Brook and Mt. Ascutney, the forest floor – mineral horizon boundary was the E horizon. For each treatment (N-fertilized, Ambient unfertilized), we made three soil composites, each consisting of five forest floor cores collected from points distributed around the plot or watershed, depending on the scale of the field experiment. After removing each forest floor sample, we collected 0 - 15 cm of mineral soil with a 3.5 cm corer to make three mineral soil composites each containing five mineral soil cores. Sampling differed slightly for the Harvard Forest Hardwood and Pine sites, where we received three 0 - 10 cm mineral soil composites per plot collected in conjunction with the 20th anniversary soil sampling for the Harvard Forest chronic N addition study. Each composite was comprised of two soil cores collected with an engine-driven, diamond-bit corer, internal diameter 9.5 cm. Using the tulip bulb corer, we collected five forest floor cores for each of the three forest floor composites, selected from the immediate vicinity of the mineral soil sampling points. All soils were stored at 4 ^{o}C (Table 1.2) until the incubation experiment began.

Table 1.2: Soil incubated per microlysimeter, and incubation timing.

Site	Horizon	Soil Incubated (g dry mass per microlysimeter)	4°C Storage Duration Between Collection and Sieving (Days)	20°C Storage Time Between Sieving and Adjustment to 30% Moisture (Weeks)
FF	Forest Floor	50	1	4
	Mineral	50	1	4
CI	Forest Floor	25	1	8
	Mineral	50	1	8
HH	Forest Floor	25	4	1
	Mineral	50	2	1
HP	Forest Floor	25	8	1
	Mineral	50	2	1
AS	Forest Floor	15	14	12
	Mineral	50	14	12
BB	Forest Floor	25	7	16
	Mineral	50	7	16

Incubation Experiment

We conducted an incubation experiment to measure the effects of long-term N addition on heterotrophic respiration. All soils were sieved to 4 mm. Several recent studies report no effect of sieving to 2 mm on C mineralization (Thomson et al. 2010, Oorts et al. 2006, Kristensen et al. 2003), though sieving soils to less than 2 mm stimulated soil C turnover (Oorts et al. 2006). Soils in this study were sived to 4 mm, quite a bit larger than the 2 mm threshold for stimulating C mineralization. Sieved soil was weighed into microlysimeters (Nadelhoffer et al. 1999) constructed from modified Millipore 150 mL Stericup Steirle Vacuuum Filter Units (Millipore, Billerica, MA, USA). Cups were modified by removing the standard filter provided with the cup, and replacing the filter with a Whatman GF/F glass fiber filter (Whatman plc, Kent, UK), sealed to the filter cup using a ring of silicone caulk. Mineral soil incubations contained 50 g dry weight equivalent of sieved mineral soil per microlysimeter. Soil bulk density determined how much forest floor material could fit in the microlysimeters, resulting in 15-50 g dry weight sieved forest floor in each incubation cup (Table 1.2). Each cup was capped with polyethylene film secured by a rubber band, which allowed oxygen exchange while maintaining soil moisture.

Field-moist soils were stored in the microlysimeters at room temperature (20^oC) for 1 up to 16 weeks due to processing limitations (Table 1.2), at which time we adjusted soil moisture to 30% of dry mass and allowed the soils to equilibrate at room temperature for an additional two weeks before we made the first CO_2 measurement. On review, CO_2 measurements indicated that initiation more than 8 weeks after sieving meant missing the exponential decline of CO_2 respiration at the start of the incubation. We excluded Mt. Ascutney and Bear Brook from the calculation of cumulative CO_2 respired in the first week of incubation, but present their one year basal CO_2 respiration results. We set up two identical sets of soil

incubations at the beginning of the experiment, "one-week" and "one-year," each containing three forest floor composites and three mineral soil composites from each treatment from each site. We measured CO_2 emissions of all soils for one week, then harvested all "one-week" soils to destructively measure microbial biomass, enzyme assays, and density fractionation, as described below. The "one-year" soils continued to incubate for an additional 51 weeks, over which time we maintained 30% soil moisture. Nine times per year, following each CO_2 measurement, we added 100 mL of a low-concentration nutrient solution containing all essential micronutrients with the exclusion of N, following Nadelhoffer (1990). We froze the resulting extract solutions; however, we only present results from the first extract of the background samples here. The remaining sieved soil not allocated to a mycrolysimeter was used to measure soil moisture, pH, and total C and N, as described below.

Carbon Dioxide Production

We measured soil CO_2 flux on incubating soils nine times over the course of the year-long soil incubation (Figure 1.3) using a Li-Cor 6200 infrared gas analyzer (Li-Cor Inc., Lincoln, NE, USA). We removed the polyethylene film from each microlysimeter, and sealed each sample into a CO_2-tight chamber fashioned from a 2.6 L LOCK&LOCK food storage container (Seoul, Korea) for the duration of each CO_2 measurement. All measurements were made at 20 ^{o}C, and each CO_2 measurement consisted of the average of three CO_2 fluxes. For each flux, the Li-Cor monitored change in CO_2 concentration for 90 seconds, and integrated the resulting CO_2 curve to calculate the rate of CO_2 evolved over time. We calculated cumulative CO_2 respired over the first week of the soil incubation by calculating the average daily respiration rate from three measurements during the first week, and multiplying that rate by 7. We scaled the estimate from our soil cup measurements to estimate the cumulative

CO_2 respired per square-meter of forest area using soil bulk density measurements for each site.

Microbial Biomass

We measured microbial biomass using the chloroform fumigation/extraction method (Brookes et al. 1985, Davidson et al. 1989). We placed two replicate soil samples from each "one-week" microlysimeter incubation (5 g dry weigh equivalent for organic soils, and 10 g dry weight equivalent for mineral soils) in a 60 mL centrifuge tube and 25 mL glass beaker, respectively. Soils in tubes were immediately extracted with 45 mL of 0.5 M K_2SO_4. The beakers containing soil were fumigated with chloroform in a vacuum desiccator for 4 days. We transferred the fumigated soils to centrifuge tubes, and extracted those soils with 0.5 M K_2SO_4. We filtered all extracts through ashed Whatman GF/F filter paper, dried the K_2SO_4 salt extracts at 55^{o}C for a minimum of 48 hours, ground the salts to a powder, and submitted the salts for C and N analysis by a Europa Scientific ANCA-GS- 2020 (PDZ Europa, Cheshire, UK) at the University of California, Davis Stable Isotope Facility. Microbial biomass C and N was calculated as the difference between extractable C or N in chloroform fumigated versus unfumigated samples (Paul et al. 1999), with correction factors of $K_{EC} = 0.45$ (Beck et al. 1997) and $K_{EN} = 0.54$ (Brooks et al. 1985) for C and N, respectively. We express microbial biomass as microgram C or N per gram soil C.

Extracellular Enzymes

We measured two lignin-degrading enzymes, phenol oxidase and peroxidase, and two cellulolytic enzymes, b-1,4-glucosidase and cellobiohydrolase, following Allison and Jastrow (2006). We made a slurry beginning with 3 g of forest floor material harvested at the end of the one week incubations. We added 30 mL 50 mM

pH 5 acetate buffer, and stirred the suspension for a minimum of 3 minutes. Then, while stirring, we pipetted 0.75 mL subsamples into 2 mL centrifuge tubes. Substrates were added to the soil-buffer slurries to target each enzyme (see details below), and all enzymes were incubated at 20 ^{o}C on the shaker-table, mixing the slurry-substrate solutions throughout the incubation. At the conclusion of the incubation, we centrifuged all tubes to separate soil from supernatant solution, then pipetted 0.26 mL supernatant from each tube into a well of a 95-well plate, and ended the reactions by adding 0.013 mL 1 M NaOH to each well to denature enzymes and develop color. We analyzed the plates on a Thermo Scientific Varioskan Flash spectral scanning multimode plate reader (Thermo Fisher Scientific, Waltham, MA, USA). For the phenol oxidase and peroxidase assays, we used 5 mM solution of the substrate L-DOPA. We incubated the assays for 1.5 hours and read the results at 450 nm. For peroxidase we also added plus 0.075 mL 0.3% H_2O_2 to the incubating slurry. The for the b-1,4-glucosidase assay, we used 5 mM pNP- b-1,4-glucopyranoside substrate. We incubated the assay for 1 hour and read the results at 405 nm. For the cellobiohydrolase assay, we used a 2 mM concentration of the substrate pNP-cellobioside, incubated the assay for 4 hours, and analyzed the results at 405 nm. We express enzyme activities as micromole substrate consumed per gram soil C per hour.

Density Fractions

We measured light fraction and heavy fraction soil carbon using a standard density fractionation method (Sollins et al. 1999). We weighed 5 g of dried "one-week" soil into 60 mL centrifuge tubes. We added 36 mL of sodium polytungstate (SPT) solution, adjusted to a density of 1.65 g cm^{-3}. We placed the tubes containing the soil and SPT on a shaker table. Two hours later, we removed the tubes from the shaker table, swirled the tubes by hand to remove any soil adhering to the sides or cap

of the tube, and then centrifuged the tubes at 7,000 rpm for 12 minutes. We allowed the tubes to sit for 12 to 24 hours following centrifuging to allow for additional particle settling, which resulted in a more transparent SPT solution, making viewing the separation between the light and heavy fractions more obvious. We aspirated the light fraction using a flask and vacuum pump, and rinsed and collected the separate light and heavy fractions on ashed 7 mm diameter Whatman GF/F filter paper. We rinsed the fractions with a minimum of 250 mL deionized water. We transferred the fractions from the filter paper into foil drying tins, and dried the fractions at 55 ^{o}C for a minimum of 4 days. We weighed all of the dried fractions, and ground each fraction to a homogenized fine powder for analysis.

Soil C and N Analysis, DOC, DON, and pH

Total C and N were measured on dried, ground bulk soil samples, light fractions and heavy fractions using an Elementar vario EL elemental analyzer to (Elementar vario MACRO CHNS, Hanau, Germany). DOC and DON were extracted on the seventh day after initiating CO_2 measurements by adding 100 mL of a low-concentration nutrient solution containing all essential micronutrients with the exclusion of N, following Nadelhoffer (1990) to the incubating soils in microlysimeter cups. The soil and solution equilibrated for a minimum of 30 minutes, and the solution was extracted from the soils using vacuum filtration (Nadelhoffer 1990). Soils were extracted with the micronutrient solution following each CO_2 measurement; however, we only present the day seven extract data here. We froze the soil extracts 125 mL Nalgene HDPE bottles (Thermo Fisher Scientific, Waltham, MA, USA) at -20 ^{o}C for up to 16 months until we thawed the extracts and measured DOC and total dissolved N (TDN) using a Shimadzu TOC-VCPN with TNM-1 analyzer (Kyoto, Japan). Inorganic N was measured at the Analytical Services Center in the

School of Forest Resources at the University of Washington on an Auto-Analyzer (OI Analytical, College Station, TX, USA), and DON was estimated by difference as TDN – DIN. Soil pH was measured using an Accumet Basic AB15 pH meter (Fisher Scientific, Waltham, MA, USA), by making a 1:10 soil : deionized water slurry and letting the soils equilibrate in the water for 30 minutes before measurement (Robertson et al. 1999).

Statistical Analysis

We tested the effects of N addition on soil CO_2 flux, microbial biomass (C, N and C:N), soil enzyme activities, DOC production, soil CN (%N, %C, C:N), and pH within sites using two-way ANOVA on log-transformed data, with site as a block, and with a Student's t post-hoc test to view comparisons within the site x treatment interaction. To constrain sample size to an amount of soil that was possible to process during this experiment, we used a sample size of n=3 per treatment. More replicates would have given us more power in our analysis, but exceeded our capacity to make time-sensitive biological measurements. Thus, we present results for both $P < 0.05$ and $P < 0.10$ for some comparisons. All models were fit using JMP version 7.0 (SAS Institute, Cary, North Carolina, USA).

RESULTS

Soil Respiration

During the first week of the soil incubation, N-fertilized forest floor soils had significantly lower rates of respiration than ambient soils at all four sites with complete CO_2 measurements ($P = 0.0001$): Fernow Forest (-12%), Cary Institute (-36

%), Harvard Forest Hardwood (-23%), and Harvard Pine (-43%) (Figure 1.2). However, by the conclusion of one year, the N addition effect in suppressing soil respiration had disappeared; the suppression lasted from nearly 100 days in Fernow Forest, Cary Institute and Harvard Forest Hardwood forest floor samples to more than 300 days for Harvard Forest Pine forest floor samples (Figure 1.3). There was no N addition treatment effect on respiration from mineral soils at any time point, nor was there a statistically significant difference in cumulative C respired between N-fertilized and ambient soils over one year for either forest floor and mineral soils (Table 1.3).

Microbial Biomass

Over all sites, forest floor microbial biomass C at the start of the incubations decreased with N addition ($P = 0.011$). At individual experiments, the decrease in microbial biomass was statistically significant ($P < 0.05$) at Harvard Forest Hardwood (40% decrease), Harvard Forest Pine (60% decrease), and Mt. Ascutney (44% decrease). Bear Brook, the site whose incubation started with the longest equilibration time (16 weeks), had low microbial biomass C in both treatments and no difference between them (Figure 1.4a). We saw similar trends in forest floor microbial biomass N as for C. On average, N addition reduced microbial biomass N in the forest floor relative to ambient plots ($P = 0.005$); however, again, the decrease in microbial biomass N at individual sites was only statistically significant ($P < 0.05$) at Harvard Forest Hardwood (40% decrease), Harvard Forest Pine (92% decrease), and Mt. Ascutney (60% decrease). Bear Brook had low microbial biomass N and no significant effect of addition on microbial biomass N between treatments (Figure 1.4b).

CO_2 respired during the first week of incubation was correlated with microbial

biomass for ambient forest floor for Fernow Forest, Cary Institute, Harvard Hardwood, and Harvard Pine. However, for N fertilized forest floor there was no apparent relationship between microbial biomass and soil respiration (Figure 1.5).

Extracellular Enzymes

In the forest floor, there were no consistent significant treatment effects of long-term N addition on C-degrading enzyme activities across sites, though several significant within-site effects were observed. The cellulytic enzyme b-1,4-glucosidase significantly declined in activity at Harvard Forest Pine (28% decrease, $P < 0.05$) (Figure 1.6a). N addition induced an increase in activity for the cellulytic enzyme cellobiohydrolase at Harvard Forest Hardwood (62% increase, $P < 0.10$), and a decline in activity at Harvard Forest Pine (38% decrease, $P < 0.10$) (Figure 1.6b). The concentration of the lignin-degrading enzyme phenol oxidase decreased at three sites, Fernow Forest (29% decrease), Harvard Forest Hardwood (100% decrease), and Harvard Forest Pine (100% decrease) (all $P < 0.10$) (Figure 1.6c), and N addition also induced a decline in the lignin-degrading enzyme peroxidase in the forest floor at Harvard Forest Pine (19% decrease, $P < 0.10$) (Figure 1.6d).

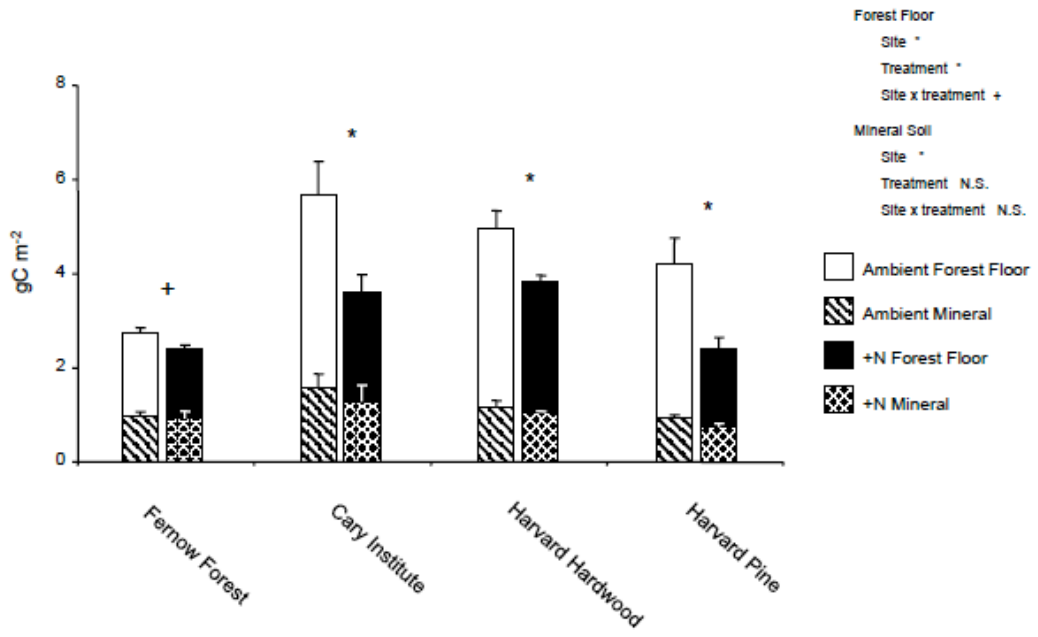

Figure 1.2: Estimate of one week cumulative CO_2 for four northeastern forest sites. * Denotes treatment pairs with a significant difference of $p < 0.05$, and + signifies $p < 0.10$.

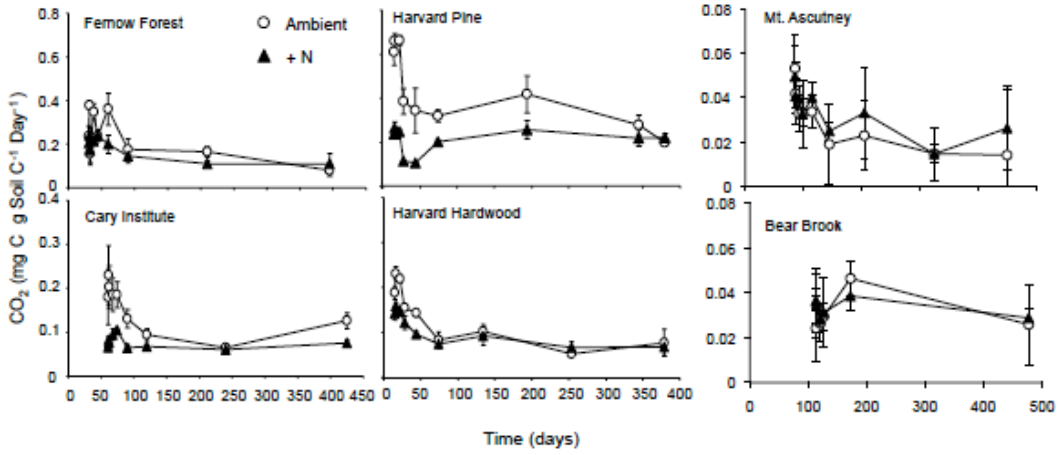

Figure 1.3: CO_2 measured over one year of soil incubation. Scale of X-axis reflects time since soil was sieved.

Table 1.3: Cumulative respiration for the 1 year soil incubation, as well as estimated soil carbon. Standard error is in parentheses

		Respiration ($g\ C\ m^{-2}\ year^{-1}$)		Soil Carbon ($kg\ m^{-2}$)	
Field Site	Horizon	Ambient	+ N	Ambient	+ N
FF	Forest Floor	50 (9)	57 (8)	1.5 (0.1)	1.9 (0.1)
	Mineral	22 (3)	33 (7)	**10.1 (0.9)**	**6.5 (1.5)**
CI	Forest Floor	101 (6)	8 (10)	1.8 (0.2)	1.9 (0.4)
	Mineral	54 (6)	51 (8)	7.1 (1.2)	11.3 (1.7)
HH	Forest Floor	80 (9)	79 (6)	2.3 (0.4)	2.7 (0.4)
	Mineral	37 (3)	40 (5)	4.4 (0.2)	4.6 (1.0)
HP	Forest Floor	81 (7)	65 (7)	2.4 (0.4)	3.0 (0.3)
	Mineral	31 (1)	31 (1)	9.8 (0.9)	8.8 (0.4)
AS	Forest Floor			5.3 (0.7)	4.9 (0.5)
	Mineral			6.3 (0.5)	8.6 (0.9)
BB	Forest Floor			4.4 (1.1)	3.8 (0.8)
	Mineral			8.6 (1.2)	11.5 (2.8)

%C, %N, and C:N of Soils and Density Fractions

The long-term N addition treatment did not have an effect on %C or %N across sites, in either the forest floor and mineral soil. However, within sites, forest floor C:N ratio increased significantly at the Harvard Forest Pine, while it decreased significantly at Mt. Ascutney ($P < 0.05$, Table 1.4). Mineral soil C:N ratio increased in response to addition at the Harvard Forest Pine ($P < 0.05$, Table 1.4).

N addition did not affect % light fraction at any site or in either horizon, nor was there a significant N addition treatment effect on % heavy fraction for forest floor or mineral soil. Within sites, there was a significant decline in % heavy fraction with N addition in mineral soil at Cary Institute (Table 1.5).

There were significant N addition treatment effects on forest floor heavy %C, heavy %N, and heavy C:N, all of which decreased with N addition, as well as mineral soil light %N and heavy %N, both of which increased with N addition. There were no other significant treatment effects of N addition on the density fractions (Table 1.5).

DOC, DON, and pH

We did not observe an N addition treatment effect on water-extractable DOC from the forest floor or the mineral soil, though extractable DOC differed significantly across sites (forest floor $P < 0.0001$, mineral soil $P = 0.0002$) (Table 1.4). Forest floor pH declined in N-fertilized soils at two sites (Fernow Forest and Harvard Forest Pine), and mineral soil pH declined at Harvard Forest pine in the N fertilized soil compared to the ambient soil. pH differed significantly across sites (forest floor and mineral soil $P < 0.0001$) (Table 1.4).

In the mineral soil, DON increased significantly with N addition across sites (P

= 0.004), but there was no significant treatment effect in the forest floor across sites. DON increased significantly in the forest floor at Cary Institute (43 %) and mineral soil (299 %) as well as in the mineral soil at Mt. Ascutney (131 %). DOC:DON declined significantly in Harvard Forest hardwood mineral soil and Harvard Forest pine forest floor. There was no overall site or treatment effect on DOC:DON in forest floor, though DOC:DON differed among sites in the mineral soil (P = 0.003) (Table 1.4).

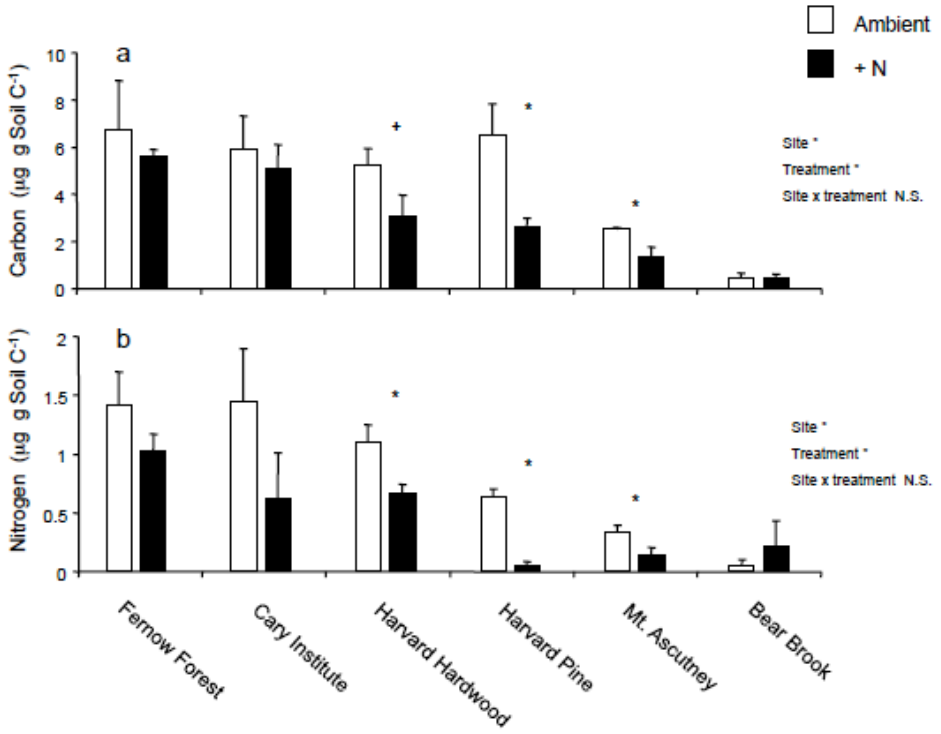

Figure 1.4: Forest floor microbial biomass carbon (a) and nitrogen (b). * Denotes treatment pairs with a significant difference of $p < 0.05$, and + signifies $p < 0.10$.

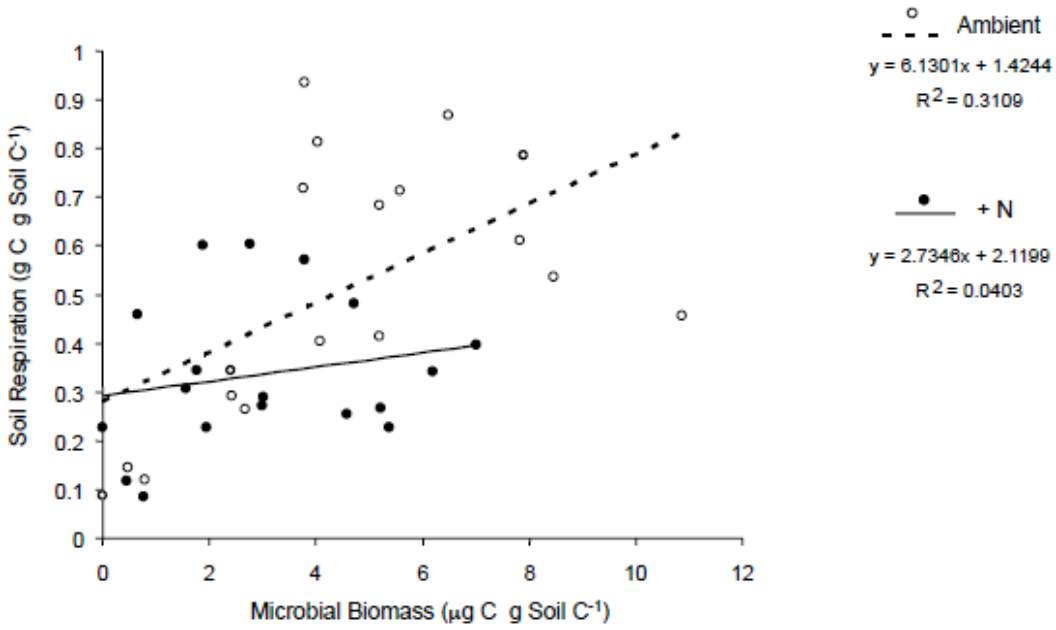

Figure 1.5: Microbial biomass and soil respiration are more strongly correlated for ambient soils than for N fertilized soils.

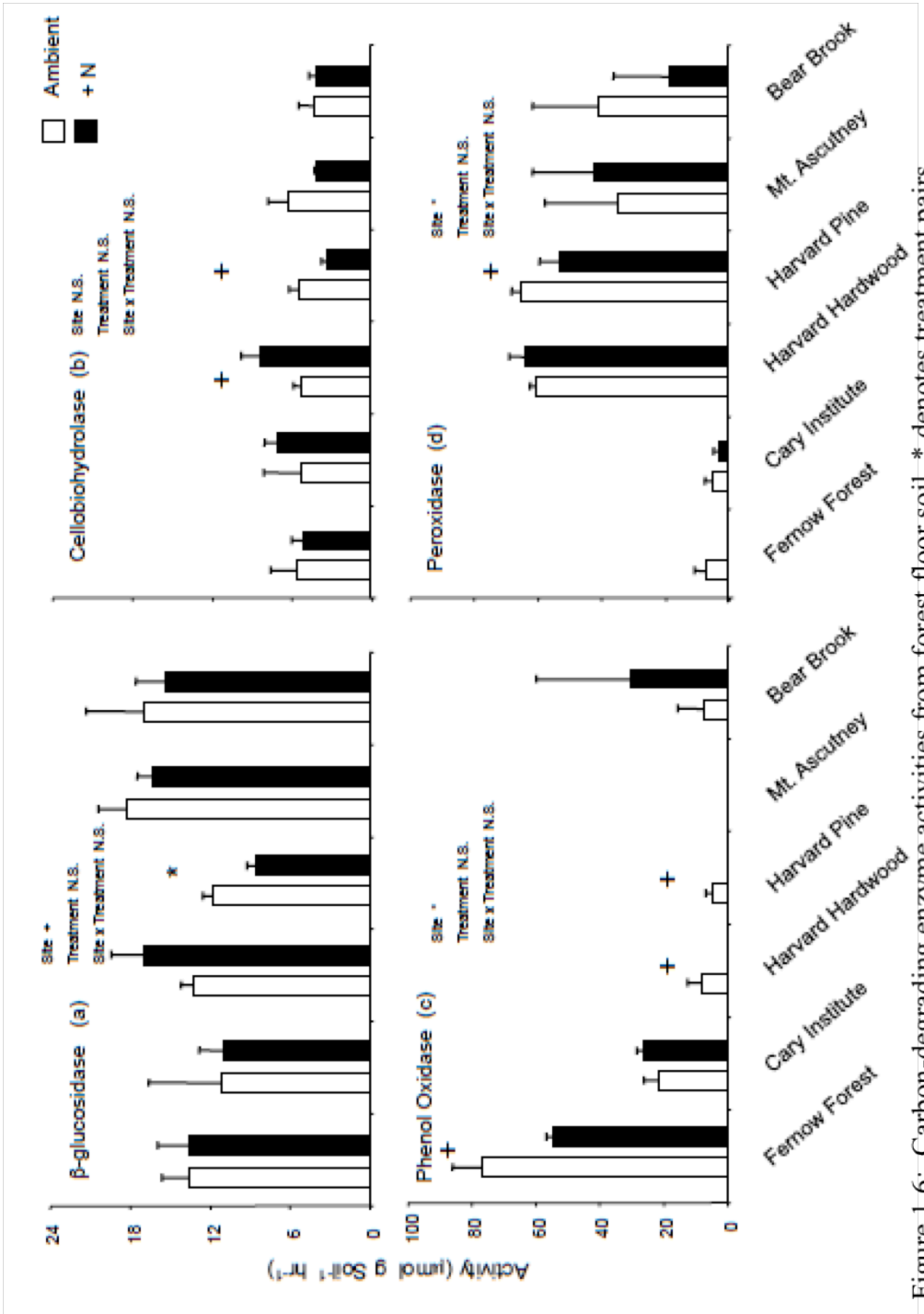

Figure 1.6: Carbon-degrading enzyme activities from forest floor soil. * denotes treatment pairs

Table 1.4: Soil characteristics for all sites and forest floor and mineral horizons.

		%C		%N		C:N		pH		DOC (mg g soil C^{-1})		DON (mg g soil C^{-1})		DOC:DON	
Field Site	Horizon	Ambient	+ N	Ambient	+ N	Ambient	+ N	Ambient	+ N	Ambient	+ N	Ambient	+ N	Ambient	+ N
FF	Forest Floor	7.5	9.8	0.4	0.6	16.6	16.3	4.1	3.8	4.0	3.1	2.1	1.6	1.9	2.0
	Mineral	2.1	2.7	0.1	0.2	15.0	15.6	4.2	4.1	2.0	2.4	2.9	2.6	0.7	0.9
CI	Forest Floor	20.2	23.4	1.2	1.3	17.5	18.4	4.5	4.4	13.2	13.3	**2.1**	**3.0**	6.4	5.1
	Mineral	6.2	6.7	0.3	0.4	17.8	18.0	4.1	4.1	2.2	2.7	**0.9**	**3.6**	4.2	0.7
HH	Forest Floor	17.8	18.2	0.7	0.8	24.8	23.4	4.0	3.9	5.2	5.7	0.2	0.2	29.6	50.0
	Mineral	4.5	4.7	0.2	0.2	22.1	21.3	**4.4**	**4.1**	3.0	2.9	0.1	0.2	**59.2**	**19.4**
HP	Forest Floor	17.0	19.9	0.7	0.8	**22.6**	**26.1**	**4.1**	**3.6**	8.0	6.4	0.4	0.7	**23.3**	**9.8**
	Mineral	4.6	5.0	0.2	0.2	19.7	24.0	4.1	4.0	3.1	4.2	0.2	0.3	33.4	16.6
AS	Forest Floor	49.7	39.9	1.7	1.5	29.7	26.3	3.6	3.9	10.1	9.3	0.9	0.8	12.2	10.8
	Mineral	6.2	4.7	0.2	0.2	27.1	26.2	3.5	3.6	12.9	10.5	**1.4**	**3.2**	9.3	3.8
BB	Forest Floor	39.9	35.2	1.7	1.7	23.1	20.9	4.0	4.0	8.5	10.8	2.1	2.0	4.3	12.2
	Mineral	4.8	5.6	0.2	0.3	21.9	19.8	4.2	4.2	4.8	5.3	**3.5**	**4.7**	1.3	1.1

Table 1.5: Density fractions from all sites, forest floor and mineral horizons.

		% Light		% Heavy		Light %C		Light %N		Light C:N		Heavy %C		Heavy %N		Heavy C:N	
Field Site	Horizon	Ambient	+ N	Ambient	+ N	Ambient	+ N	Ambient	+ N	Ambient	+ N	Ambient	+ N	Ambient	+ N	Ambient	+ N
FF	Forest Floor	6.9	12.4	93.1	87.6	**22.0**	**27.6**	3.1	4.1	7.3	10.5	5.6	5.6	0.4	0.4	16.9	15.0
	Mineral	1.4	1.7	98.6	98.3	20.7	20.7	**2.8**	**4.3**	7.6	4.8	1.7	2.3	0.1	0.2	12.1	13.1
CI	Forest Floor	41.9	58.3	58.1	41.7	27.9	28.6	3.9	4.6	7.8	6.2	**10.3**	**7.6**	**0.6**	**1.3**	**17.9**	**5.9**
	Mineral	6.8	11.4	**93.2**	**88.6**	24.1	21.1	2.7	2.1	9.0	14.3	4.6	4.6	**0.3**	**1.0**	**16.2**	**4.6**
HH	Forest Floor	37.9	40.3	62.1	59.7	38.3	36.3	4.9	5.1	7.8	7.1	5.6	5.9	0.9	1.0	6.1	6.1
	Mineral	3.6	4.1	96.4	95.9	34.3	31.7	**2.0**	**3.2**	**22.4**	**9.8**	3.4	3.6	0.6	0.6	5.7	5.7
HP	Forest Floor	36.1	42.8	63.9	57.2	34.1	37.8	4.8	4.8	7.1	7.8	6.7	5.3	**1.2**	**0.8**	5.6	6.2
	Mineral	4.6	6.4	95.4	93.6	25.4	23.9	2.8	1.8	9.6	13.2	3.4	3.2	0.7	0.5	5.2	5.9
AS	Forest Floor	96.6	82.3	3.4	17.7	37.4	38.1	4.1	4.6	9.2	8.3	**10.0**	**3.2**	**1.4**	**0.5**	7.0	6.1
	Mineral	11.9	9.5	88.1	90.5	35.4	35.5	**2.4**	**3.7**	14.9	9.6	2.0	2.4	0.4	0.4	5.3	6.1
BB	Forest Floor	83.9	79.6	16.1	20.4	**34.9**	**29.0**	4.7	4.3	7.6	6.8	11.1	9.6	**1.7**	**1.2**	6.8	8.5
	Mineral	4.3	5.6	95.7	94.4	30.2	29.0	3.5	3.7	8.6	7.9	3.8	4.1	0.7	0.8	5.7	5.3

	Forest Floor % Light	Forest Floor % Heavy	Forest Floor Light %C	Forest Floor Light %N	Forest Floor Light C:N	Forest Floor Heavy %C	Forest Floor Heavy %N	Forest Floor Heavy C:N
Site	*	*	*	N.S.	N.S.	*	*	*
Treatment	N.S.	N.S.	N.S.	N.S.	N.S.	*	*	*
Site x Treatment	N.S.	N.S.	*	N.S.	N.S.	*	*	*

	Mineral % Light	Mineral % Heavy	Mineral Light %C	Mineral Light %N	Mineral Light C:N	Mineral Heavy %C	Mineral Heavy %N	Mineral Heavy C:N
Site	*	*	*	*	N.S.	*	*	*
Treatment	N.S.	N.S.	N.S.	*	N.S.	N.S.	*	N.S.
Site x Treatment	N.S.	N.S.	N.S.	*	N.S.	N.S.	*	N.S.

DISCUSSION

The objective of this study was to examine the effects of long-term N addition on soil C pools, fluxes, and corresponding microbial processes. Though we saw few significant changes in light or heavy soil C fractions, we did see a significant decrease in CO_2 produced by N fertilized soils during the several weeks of the year-long incubation, as well as declines in microbial biomass and extracellular enzyme activity, all indicating that microbial activities were diminished by long-term N addition.

The lack of a discernible N addition treatment effect on proportions of light and heavy soil C could be a consequence of how we defined and measured C pools. The physical fractionation method we used may not correspond with microbial use of soil C. Also, if N contributes to the chemical stabilization to the heavy and/or light C pools, the total C accumulation over 11 – 20 years of N addition may yet be too small to detect with our sample size. Spatial heterogeneity of soil C stocks can make differences between sites difficult to detect. Our enzyme assay results suggest that within the light pool, decomposition of chemically recalcitrant compounds like lignin has decreased with N addition; perhaps the light fraction is slowly accumulating C via inhibition of lignin decomposition over time. On the other hand, if N addition first accelerated turnover of starches and sugars before stabilizing lignin, the lack of change in light C could indicate a balance between increased turnover of cellulose in the past and decreased turnover of lignin in the present with N addition. Also, if decomposition slows by roughly the same amount in both the light and heavy fractions, the relative proportions of each fraction would remain the same, masking the decrease in decomposition. Density fractionation only explores one of many C stabilization mechanisms (Crow et al. 2007); methods for digging into the vast array of mechanisms of soil C stability are actively being developed and refined, yielding valuable information about the complexity of soil C (Sollins et al. 2007). To

accurately assess the sizes and composition of these soil C pools independent of microbial activity, and to understand the interactions between physical and chemical mechanisms of C stabilization would require a larger sample size, and fractionation techniques designed to assess the chemical structure of soil C (Paul et al. 2006, Grandy et al. 2008). Alternatively, an proxy for chemical fractionation could be to use C isotopes to separate soils by age, because soil age class corresponds with soil chemical properties (Hagedorn et al. 2003).

We observed an average reduction of CO_2 production by 28% in N fertilized soil during the first week of incubation measurements. Assuming that our measurements during the first week of incubation are most similar to field conditions and that the N effect is be maintained in the field over time at each site, that value is consistent with the calculation made by Janssens et al. (2010). They found heterotrophic respiration decreased by 15% with N addition in a meta-analysis of 37 studies.

Though N addition decreased soil respiration in the first week at the four field experiments for which we conducted detailed 1-week measurements of heterotrophic respiration, we could not detect an increase in the soil carbon pools. Thus, our data do not support our hypothesis that N addition is resulting in soil C accumulation at these sites, or at least, not one we could detect. However, in contrast to our results, Frey (Unpublished Data) has documented a significant soil carbon accumulation with N addition in Harvard Forest Hardwood and Harvard Forest Pine soils. Frey's analysis used a larger sample size, and a different estimate of soil bulk density. Estimates of N-induced soil carbon sequestration for temperate forests converge on the range of 5 to 30 kg C per kg N annually (Högberg 2007, de Vries et al. 2006, Sutton et al. 2008, Janssens et al. 2010, Goodale et al. Submitted). Correctly calculating the potential for soil and vegetation to accumulate C with added N is an important topic for continued

investigation as measurements and models improve. Moreover, ecosystem models require reliable measurements of microbial processes to accurately predict the response of ecosystem C to N addition.

Our CO_2 measurements indicate that the effects of N addition may indeed differ with soil carbon quality. N significantly inhibited respiration for the first week of soil incubations at Fernow Forest, Cary Institute, Harvard Hardwood, and Harvard Pine; however, respiration occurred at the same rate in both fertilized and unfertilized soil by the end of the one year incubation. We assume that C respired at the start of a year-long incubation is, by definition, more respirable and therefore higher quality or more accessible than the C respired near the end. Soils in the N addition treatment plots had received N fertilizer for 11 – 20 years; if N addition had stimulated the decomposition of the most easily decomposed substrates, the remaining soil C pool in the N fertilized plots at the time these samples were collected should be harder to decompose than the available C in the ambient plots, as we observed here. The microbial communities in N fertilized soils at the start of this experiment had lower CO_2 production and lower chloroform-labile biomass than the microbes in the ambient soil, supporting the explanation that a long-term effect of N addition could have been to reduce labile C substrates. By the end of one year, when the higher quality soil carbon had presumably been metabolized, both ambient and N fertilized soils were likely limited by the availability of degradable substrates; at that time, C limitation superseded the N addition treatment effect. We did not observe any enhanced decomposition with N addition, most likely because we were measuring CO_2 on soils that had received N fertilizer for 11 – 20 years; in past work at Harvard Forest, stimulation of CO_2 with N addition was observed when N was added to previously unfertilized soils, but not in long-term N fertilized soils (Bowden et al. 2004). After long-term N addition, that highest quality C would have likely already been

metabolized.

Forest floor microbial biomass declined significantly with N addition, and N addition also reduced the correlation between microbial biomass and soil CO_2 across these sites. Decreasing the correspondence between CO_2 efflux and microbial biomass with N addition makes predicting changes in decomposition with N addition more challenging.

Long-term N addition reduced oxidative enzyme activity, indicating that N addition could be slowing the turnover of complex phenolic compounds such as lignin. Taken together with the N-induced decline in microbial biomass and CO_2, we infer that N addition is decreasing microbial abundance and activity, thereby slowing decomposition rates in forest floor humus. Additionally, N may be stabilizing soil C, making C less available to decomposers, and in turn decreasing microbial biomass.

Our results are broadly consistent with past work at some of these same field sites. Inhibition of soil respiration by long-term N addition has been measured at Harvard Hardwood and Harvard Pine (Bowden et al. 2004, Micks et al. 2004), along with a decrease in oxidative enzyme activity (Frey et al. 2004). Decreased microbial biomass in response to N addition has been observed at the Harvard Forest (Compton et al. 2004, Frey et al. 2004, Wallenstein et al. 2006), Mt. Ascutney (Wallenstein et al. 2006), and Bear Brook (Wallenstein et al. 2006).

Soil C composition plays a role in the response of soil C decomposition to N addition, as summarized by the hypothesis that N addition stimulates labile C decomposition and inhibits recalcitrant C decomposition (Fog 1988, Berg and Matzner 1997, Neff et al. 2002, Hagedorn et al. 2003, Knorr et al. 2005, Janssens et al. 2010). Our soil respiration data show inhibition of decomposition with added N. One possible explanation could be that these soils have large recalcitrant C pools which were stabilized by added N.

Additional factors aside from soil C quality may be important in determining the effects of N addition on soil C decomposition. Fog (1988) and Berg and Matzner (1997) proposed that N can directly stabilize soil organic matter. They hypothesize that N can be abiotically incorporated into phenolic C molecules, forming even more stable molecules which are resistant to decomposition by microbial enzymes. However, empirical evidence for this hypothesis is scarce (Janssens et al. 2010). Our experiment neither confirms nor refutes that hypothesis. Another possible mechanism for a decrease in decomposition with N addition is referred to as a decreased priming effect (reviewed by Janssens et al. 2010). Under ambient conditions, tree roots appear to prime the nearby decomposers by exuding easily metabolized C from an extensive system of fine roots and mycorrhizae, priming rhizosphere microbes with energy to decompose complex organic matter. During decomposition, the microbes release essential nutrients from the organic matter and make them available to the plants. With N addition, plants generally decrease fine root biomass and decrease exudate production, which should decrease priming of decomposition (Phillips and Fahey 2007). We removed roots from our soils by sieving, so our experiment did not test the rhizosphere priming hypothesis

We measured a decrease in pH with N addition in two soils (Harvard Forest hardwood mineral and Harvard Forest pine forest floor). Though decreasing pH is positively correlated with decreasing DOC export (Evans et al. 2008), neither N addition nor pH was associated with any change in DOC production in these soils.

The forest soil response to N addition is variable over space and time; for most of the parameters we measured, site differences exceeded N treatment effects, and the decrease in CO_2 with N addition we observed at the start of the 1-year soil incubation was no longer discernable by the end of the year. Anthropogenic change may increase the variability of the soil C response to N addition, making responses across sites even

more difficult to discern and predict. Enhancing estimates of the magnitude and mechanisms of the soil C response to N addition will require large sample sizes and advanced microbial techniques to precisely characterize soil C pools and microbially-mediated C transformations.

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CHAPTER 2

The response of soil carbon decomposition to short-term and long-term nitrogen fertilization

ABSTRACT

Soils contain a vast reservoir of carbon (C); anthropogenic nitrogen (N) deposition has the potential to alter microbial decomposition of the soil C pool. The goal of our study was to understand the microbial activities driving the response of decomposition to N fertilization in northeastern forest soils. We measured the transient soil respiration to detect the response of soil microbial activity to a pulse of N fertilization applied in the lab (response over one week). We also measured the effects of long-term field N addition on soil respiration in soils which have received N addition for up to 20 years (response measured over one year). We collected fertilized and unfertilized (ambient) soils from 6 long-term N addition field studies, and added a standard amount of N to all soils in the lab. We incubated the soils for one year, and monitored decomposition rate by measuring carbon dioxide (CO_2) production throughout the incubation. At the conclusion of the incubation, we measured microbial biomass and extracellular enzyme activity for four enzymes: the cellulose-degrading enzymes b-glucosidase and cellobiohydrolase and the lignin-degrading enzymes phenol oxidase and peroxidase. Here we present detailed soil respiration results from two of the six sites, Harvard Forest Hardwood and Harvard Forest Pine, and carbon-degrading enzyme activity from all six sites. At Harvard Forest Hardwood, we observed a brief pulse of CO_2 corresponding with an increase in cellobiohydrolase activity following the lab N fertilization of previously unfertilized soil. We also saw a decline in phenol oxidase activity, suggesting an inhibition of

lignin decomposition. In Harvard Forest Pine forest floor, long-term N fertilization decreased b-glucosidase and peroxidase activity, and both field and lab N fertilization decreased phenol oxidase activity, and while we saw a dramatic inhibition of CO_2 release from the field N addition, we did not see any change in CO_2 release from the lab N fertilization. We did not see changes in microbial activity in response to a pulse of lab N fertilization for soils from any of the other sites we studied. Our results provide evidence to support the hypothesis that N fertilization initially stimulates decomposition of easily-degraded carbon compounds and over time inhibits decomposition of harder to degrade, more complex carbon substrates.

Keywords:
decomposition, carbon cycle, nitrogen fertilization, soil respiration, extracellular enzyme, microbial biomass

INTRODUCTION

Soils contain a larger carbon (C) reservoir than the combined amount of C in plants and the atmosphere; decomposition, mediated by micro-organisms, is the primary means by which C leaves the soil pool and returns to the atmosphere as carbon dioxide (CO_2) (Schlesinger and Andrews 2000; Denman et al. 2007). Microbial decomposers have a large cumulative impact on CO_2 flux from land to atmosphere, and are subject to various types of anthropogenic change. One such change is nitrogen (N) deposition. Humans currently generate more reactive N than all natural terrestrial processes combined (Vitousek et al. 1997). Much of this reactive N is emitted to the atmosphere and redeposits on downwind ecosystems (e.g., Galloway et al. 2008, Dentener et al. 2006), and within forests, is predominantly

retained in soils (Nadelhoffer et al. 1999). A range of N addition experiments have been used to simulate the effects of N deposition on soil processes (reviewed by Janssens et al. 2010). Though N addition can alter microbial activity (Treseder 2008), we are yet unable to reliably predict or mechanistically understand how microbial decomposers respond to added N. Two issues of particular concern are: 1) The time-scale of the response; decomposition may initially be stimulated by N addition but later inhibited, and 2) The microbial activities mediating the responses at different time scales.

In the field, a new N addition can stimulate a transient pulse of soil respiration (Bowden et al. 2004). But the long-term effect of added N is generally to reduce rates of soil CO_2 respired in temperate and boreal forests (Bowden et al. 2004, Micks et al. 2004, Treseder 2008, Janssens et al. 2010). Microbial biomass also generally declines in forests with N addition (Compton et al. 2004, Frey et al. 2004, Wallenstein et al. 2006, Treseder 2008). However, some studies find microbial biomass unresponsive to N addition (Wallenstein et al. 2006, Allison et al. 2009.

Assays measuring the activity of the extracellular enzymes produced by microbes to decompose soil organic matter can provide more detailed information about the mechanisms underlying decomposition. In general, phenol oxidase, an oxidative enzyme which degrades lignin, is less abundant in N-fertilized soils than in unfertilized soils (Carreiro et al. 2000, Saiya-Cork et al. 2002, DeForest et al. 2004, Frey et al. 2004, Waldrop and Firestone 2004, Waldrop et al. 2004, Waldrop et al. 2004, Sinsabaugh 2010). Inhibition of phenol oxidase production by N is believed to result in suppression of lignin decomposition, and a slowing of the turnover of more complex, resistant C substrates (Carreiro et al. 2000, Sinsabaugh 2010). However, sometimes the relationships among oxidative enzymes, N addition, and decomposition are more complicated. In one study, a decrease in phenol oxidase levels corresponded

with an increase in turnover of old, presumably recalcitrant C (Waldrop and Firestone 2004). Phenol oxidase is sometimes unresponsive to N (Carreiro et al. 2000, Vitousek et al. 2002, Allison and Vitousek 2004, Waldrop et al. 2004, Allison et al. 2009, Keeler et al. 2009, Allison et al. 2010), and is occasionally stimulated by N addition (Carreiro et al. 2000, Saiya-Cork et al. 2002, Waldrop et al. 2004), and peroxidase, another lignin-degrading oxidative enzyme is frequently stimulated by N fertilization, or both stimulated and inhibited for different samples within the same study (Saiya-Cork et al. 2002, Waldrop and Firestone 2004, Waldrop et al. 2004, Waldrop et al. 2004, Keeler et al. 2009). Thus, the standard interpretation that oxidative enzymes decrease with N fertilization and decrease decomposition of lignin-based C compounds appears to be generally supported, though exceptions emphasize the complexity of the soil ecosystem.

In contrast to phenol oxidase, the cellulose-degrading enzymes b-glucosidase and cellobiohydrolase are commonly stimulated by N fertilization (Carreiro et al. 2000, Saiya-Cork et al. 2002, Allison and Vitousek 2004, Waldrop et al. 2004, Allison et al. 2009, Keeler et al. 2009, Allison et al. 2010), though sometimes they are unresponsive to N (Allison and Vitousek 2004, DeForest et al. 2004, Frey et al. 2004, Waldrop and Firestone 2004, Waldrop et al. 2004). Stimulation of cellulose-degrading enzymes by N fertilization is generally interpreted as a process that will increase the turnover of fast-cycling, easily decomposed, simple-structure C molecules (Carreiro et al. 2000).

We can apply enzyme assays to discern mechanisms mediating changes in the short and long-term turnover of soil C pools as a consequence of N fertilization. Here we propose a conceptual model for N effects on decomposition rates for different C pools within the soil, with four response phases (Figure 2.1): Phase (1) Transient (ie: short-term) stimulation of decomposition rates in a labile C pool; phase (2) No effect,

owing to a balance between stimulation of decomposition rates in labile C pools and inhibition of decomposition rates in slower cycling pools, also a transient effect; phase (3) Suppression of decomposition rates in slower cycling C pools, and phase (4) No effect, because C limitation of decomposition supersedes any N fertilization effect, and decomposition rates are very low, regardless of the N status of the soil. These responses are all functions of the available soil C pool and time. Soils may show all four responses to N fertilization over time, or some subset of responses depending on the soil C pools present at the time of N fertilization and measurement. Microbial activities mediate the decomposition response in each soil C pool; the transient stimulation phase (1) is presumably mediated by an increase in cellulase activity. The lack of effect noted in phase (2) would occur through a balance between an increase in cellulase activity and a decrease in oxidative enzyme activity. Suppression in phase (3) could result from a decrease in oxidative enzyme activity, and no effect in phase (4) may be an indication of microbial C limitation. Mechanistically understanding how N additions affect the decomposition of discrete soil pools will facilitate developing accurate models and predictions of the responses of soil C pools to N fertilization and chronic N deposition.

We assessed the response of microbial decomposers to N addition; the goal of our experiment was to test the conceptual model described above (Figure 2.1), and to link observed changes in N effects on decomposition rate with underlying microbial activities. Hereafter, we use the term "ambient" to refer to forest floor samples which have not previously received a specified N addition treatment. We added a pulse of N fertilizer to both ambient and N-fertilized forest floor samples from six long-term field N addition experiments in northeastern US forests, to observe subsequent changes in enzyme activity and microbial biomass. We present additional measurements from only two of the six sites because delays in respiration measurements at four of six sites

resulted in a prolonged equilibration; the measurement period ultimately probably did not capture transient responses we intended to measure to test the short-term response of forest floor C to a pulse of N fertilization: We measured the short-term (one week) and long-term (one year) response of CO_2 production to the lab N addition on forest floor from the Hardwood and Pine stands in the Chronic Nitrogen Amendment Study at Harvard Forest, where the soils had been fertilized for 20 years at the time of our sampling. We collected forest floor from both ambient and N-fertilized plots to contrast the effects of long-term N addition with the short-term effects of a uniform pulse of N. We tested the hypotheses that N addition stimulates CO_2 production of previously unfertilized forest floor for a transient period shortly following the N addition, while the long-term consequence of N addition is to decrease CO_2 production. We predicted that N addition would stimulate b-glucosidase and cellobiohydrolase activity only in field ambient forest floor material receiving the short-term lab N addition because only those samples would have a readily metabolized labile C pool; labile C would likely already have been depleted in forest floor from long-term N addition experiments. We expected both the short-term lab N addition and long-term field N treatment would decrease phenol oxidase and peroxidase activity, because N fertilization may stabilize lignin-based C compounds (Fog et al. 1988, Berg and Matzner 1997).

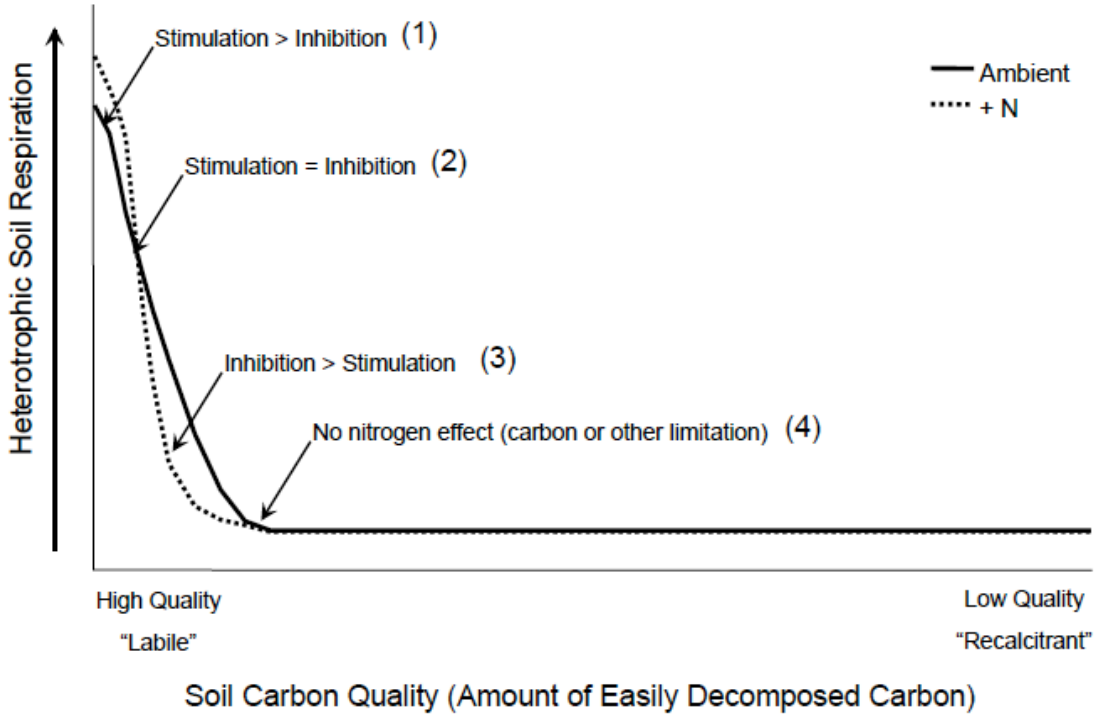

Figure 2.1 Conceptual model for soil carbon quality as a factor determining changes in decomposition rate with nitrogen fertilization.

METHODS

Study Sites

We collected forest floor from six long-term N fertilization studies at five sites across the Northeastern U.S.: the Harvard Forest, in Petersham, Massachusetts (studies in Pine and Hardwood stands); Fernow Experimental Forest, West Virginia; the Cary Institute of Ecosystem Studies in Millbrook, NY; Mt. Ascutney, Vermont; and at the Bear Brook Watershed Manipulation Experiment, Maine. The Harvard Forest Chronic N Addition Experiment began in 1988 (42°32'N, 72°10'W), with the establishment of "low N" (+ 50 kg N ha^{-1} yr^{-1}) and "high N" (+ 150 kg N ha^{-1} yr^{-1}) plots in both a red pine plantation ("Pine") and an adjacent mixed hardwood ("Hardwood") stand (Aber et al. 1993). The N fertilizer is applied as dissolved NH_4NO_3 in six equal applications every four weeks from May to September, below the canopy via a backpack sprayer. The red pine plantation was planted in 1926, and was 82 years old in 2008, when we collected forest floor samples. The hardwood stand is dominated by black and red oak and includes black birch, red maple, American beech and black cherry. The soils are stony- to sandy-loam Inceptisols (Magill et al. 2004), and wet + dry N deposition is about 8 kg ha^{-1} yr^{-1} (Ollinger et al. 1993). We collected forest floor on October 21 and 22, 2008 from ambient (unfertilized control) plots, and "low N" plots. We collected forest floor samples from Harvard Forest in conjunction with the 20th anniversary soil sampling for the Harvard Forest chronic N addition study in October 2008.

We also collected forest floor from four additional sites to provide a cross-site comparison of enzyme activity and microbial biomass. Fernow Forest in West Virginia is a mixed hardwood forest with a watershed-scale $(NH_4)_2SO_4$ fertilization experiment (+35 kg N ha^{-1} yr^{-1}) which began in 1989. We collected forest floor from Fernow Forest watershed 13 (ambient) and watershed 3 (N fertilized) on June 7, 2007.

Cary Institute in New York is an oak forest with a plot-scale NH_4NO_3 addition experiment (+100 kg N ha^{-1} yr^{-1} for initial three years, +50 kg N ha^{-1} yr^{-1} from 2000 to present) which began in 1996. We collected forest floor from Cary Institute on June 21, 2007. Mt. Ascutney in Vermont is a spruce-fir forest with a plot-scale NH_4Cl (+31.4 kg N ha^{-1} yr^{-1}) addition experiment which began in 1988. We collected forest floor from Mt. Ascutney on July 3, 2007. Bear Brook is a mixed hardwood forest with a watershed-scale $(NH_4)_2SO_4$ addition experiment (+25 kg N ha^{-1} yr^{-1}) which began in 1989. We collected forest floor from Bear Brook on July 5, 2007.

Forest Floor Sampling

We brushed away loose litter (Oi horizon), and collected forest floor material (Oe + Oa) to the depth of the mineral horizon boundary, using a 7 cm diameter tulip bulb corer. For each plot or catchment, we collected three composite samples each consisting of five forest floor cores. We stored the forest floor samples at 4^{o}C for 24 hours to two weeks, at which time we sieved the forest floor to 4 mm.

Lab Fertilization and Incubation Experiment

We constructed microlysimeters (Nadelhoffer et al. 1999) from modified Millipore 150 mL Stericup Steirle Vacuum Filter Units (Millipore, Billerica, MA, USA). We replaced the cups' pre-installed filters with a Whatman GF/F glass fiber filters (Whatman plc, Kent, UK), which we sealed to the cups using a ring of silicone caulk. We incubated 25 g dry weight equivalent of forest floor material from Harvard Forest Hardwood and Pine, Cary Institute, and Bear Brook. We incubated 50 g dry weigh equivalent from Fernow Forest and 15 g from Mt. Ascutney. For each site, this was the amount of forest floor which filled the capacity of the cups.

In the lab, we split each sieved forest floor composite into two equal portions

to conduct a lab fertilization experiment. Prior to beginning the fertilization and incubation, we adjusted moisture to 30%. We allowed forest floor samples to equilibrate for two weeks following the rewetting disturbance before beginning CO_2 measurements. Within 12 hours following the initial CO_2 measurement, we added N fertilizer to half of the samples using 0.25 mg N per g dry sample, added as $(NH_4)_2SO_4$ dissolved in 12 mL of deionized water. Each paired forest floor sample received 12 mL of deionized water.

We incubated three replicate composites for each treatment in a fully factorial design (n = 3) for each of four treatments (also see Figure 2.2): field ambient / lab ambient, field ambient / lab +N, field +N / lab ambient, and field +N / lab +N. Forest floor samples equilibrated in incubation cups at field moisture and 20^{o}C for a variable amount of time between sieving and adjusting soil moisture. Harvard Forest samples equilibrated for 1 week between sieving and moisture adjustment. The other sites all equilibrated for more than 4 weeks. Because of the long lag between sieving and moisture adjustment for the respiration measurement, measurements from those samples likely did not capture the transient dynamics of C response to a pulse of added N.

For the Harvard Forest, we report forest floor CO_2 production measured immediately before adding the N fertilizer, 24 hours after fertilization, and 7, 14, 30, 60, 120, 240, and 365 days after fertilization, using a Li-Cor 6200 infrared gas analyzer (Li-Cor Inc., Lincoln, NE, USA). We placed the microlysimeter containing the incubating forest floor samples into a CO_2-tight chamber fashioned from a 2.6L LOCK&LOCK food storage container (Seoul, Korea) for the duration of each CO_2 measurement. Each CO_2 measurement consisted of the average of 3 measured CO_2 fluxes; each flux integrated CO_2 produced over 90 seconds.

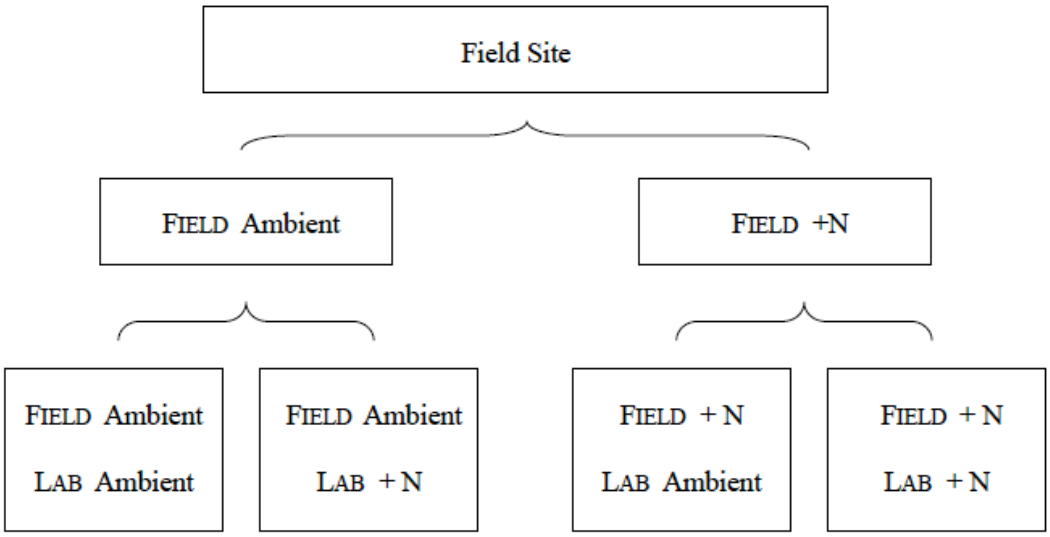

Figure 2.2 Schematic of treatments.

Extracellular Enzymes

For all six studies, we measured the activity of four carbon-degrading enzymes at the conclusion of the incubation experiment, one year after the lab fertilization: the lignolytic enzymes phenol oxidase and peroxidase, and the cellulolytic enzymes b-1,4-glucosidase and cellobiohydrolase. We followed the procedure described by Allison and Jastrow (2006). First, we made a slurry using 3 g forest floor material and 30 mL 50 mM pH 5 acetate buffer and stirred the suspension for a minimum of 3 minutes. Then, while stirring, we pipetted 0.75 mL subsamples into 2 mL centrifuge tubes. We mixed the forest floor-buffer slurries with specific substrates to target each enzyme, and incubated all enzymes at 25 ^{o}C on a shaker-table for the duration of each incubation (times listed below). We then centrifuged all tubes to separate forest floor from supernatant solution, pipetted 0.26 mL supernatant from each tube into a well of a 95-well plate, and ended the cellulytic assays by adding 0.013 mL 1 M NaOH to each well to denature enzymes and develop color. We analyzed the plates on a Thermo Scientific Varioskan Flash spectral scanning multimode plate reader (Thermo Fisher Scientific, Waltham, MA, USA). For the phenol oxidase assay, we used 5 mM of the substrate L-DOPA. We incubated the assay for 1.5 hours, and analyzed the samples at 450 nm. For the peroxidase assay, we used 5 mM of the substrate L-DOPA plus 0.075 mL 0.3 % H_2O_2. We incubated the assay for 1.5 hours, and analyzed the samples at 450 nm. For the b-1,4-glucosidase assay, we used 5 mM of the substrate pNP- b-1,4-glucopyranoside. We incubated the assay for 1 hour and analyzed the samples at 405 nm. For the cellobiohydrolase assay, we used 2 mM of the substrate pNP-cellobioside. We incubated the assay for 4 hours and analyzed the samples at 405 nm. We express enzyme activities as micromole substrate consumed per gram forest floor C per hour.

Microbial Biomass

We measured microbial biomass at the conclusion of the experiment for all six studies using the chloroform fumigation/extraction method (Brookes et al. 1985, Davidson et al. 1989). We collected two replicate samples from each mycrolysimeter. Each contained 5 g dry weight equivalent. One sample of each pair was extracted immediately in a 60 mL centrifuge tube with 45 mL of 0.5 M K_2SO_4. We placed the second sample of each pair in a 25 mL glass beaker, and fumigated the forest floor samples with 20 mL ethanol-free chloroform in a vacuum dessicator. After 4 days, we extracted the fumigated forest floor samples using the same procedure as for the immediately extracted forest floor samples. All extracts were filtered through ashed Whatman GF/F glass fiber filters. We dried the K_2SO_4 salt extracts at 55 ^oC for a minimum of 48 hours to remove all water, ground the salts to a powder, and submitted the salts for C and N analysis on a Carlo Erba NC2500 elemental analyzer (CE Elantech, Inc., Lakewood, New Jersey, USA) at the Cornell Stable Isotope Lab. We calculated microbial biomass C and N as the difference between extractable C or N in chloroform fumigated versus unfumigated samples (Paul et al. 1999), with correction factors of $K_{EC} = 0.45$ (Beck et al. 1997) and $K_{EN} = 0.54$ (Brooks et al. 1985) for C and N, respectively.

Statistical Analysis

We tested the effects of field- and lab-N fertilization on forest floor CO_2 flux, microbial biomass, forest floor enzyme activities within each site and among all treatments on log-transformed data using ANOVA, with site (Hardwood or Pine) as a block, at $P < 0.05$. All models were fit using JMP version 7.0 (SAS Institute, Cary, North Carolina, USA).

RESULTS

Nitrogen additions had both short and long-term effects on CO_2 release from forest floor samples from the Harvard Forest. The lab N fertilization stimulated CO_2 release during the first week of incubation for the Harvard Forest Hardwood forest floors, for both the previously unfertilized samples (+17%) and for those that had received long-term N additions (+14%). The stimulation of CO_2 with lab N fertilization was transient, and disappeared within a week (Figure 2.2a). Laboratory N fertilization did not induce any change in CO_2 release from the Pine forest floors from either the ambient or the field fertilized plots (Figure 2.2b). In contrast, independent of any N additions in the lab, 20 years' worth of field N additions led to significantly reduced CO_2 release from forest floor samples from the Hardwood stand for one month of incubation, and from the Pine stand for two months (Figure 2.3).

Though all incubating forest floor samples had small but detectible CO_2 fluxes after one year of incubation as well as measurable enzyme activity, we were unable to detect chloroform-extractable dissolved organic C, which we used to measure microbial biomass, because many of our samples fell below the detection limit of the instrument (47 mg C). However, microbial biomass N samples were within the detection limit (11 mg N). Microbial biomass N significantly increased in both forest types in the lab N-fertilized forest floor samples which had not previously been fertilized in the field (Table 2.1).

Parallel to the short-term CO_2 responses, the Harvard Forest Hardwood forest floors had a significant increase in cellobiohydrolase activity with lab N fertilization, for both the field ambient and field N-fertilized forest floor samples. However, 20 years of field N addition did not suppress cellobiohydrolase activity (Figure 2.4b). A similar, though not statistically significant pattern was observed for b-glucosidase, with a slight elevation in activity with lab N fertilization for both the field ambient and

field N-fertilized forest floor samples (Figure 2.4a). Lab N fertilization also decreased phenol oxidase activity in previously unfertilized forest floor samples, and phenol oxidase was suppressed in both lab-and field N treatments (Figure 2.4c). Neither lab N fertilization nor field N addition altered peroxidase activity in Hardwood forest floors (Figure 2.4a,d).

Harvard Forest Pine forest floor samples had a significant decrease in b-glucosidase activity in response to the long-term N addition in the field, but lab N fertilization had no effect (Figure 2.5a). Cellobiohydrolase activity in Pine forest floor samples was not altered by either lab- or field N addition treatment (Figure 2.5b). However, both lab and field N additions suppressed phenol oxidase activity in the previously unfertilized Pine forest floors (Figure 2.5c). Peroxidase activity was only reduced by long-term N addition, and did not change with lab N fertilization (Figure 2.5d).

While the lab N addition induced a short-term pulse of CO_2 and a concomitant increase in cellobiohydrolase activity and decrease in phenol oxidase activity at the Harvard Hardwood site, no other sites showed this suite of responses (Table 2.1). A few significant effects were observed at the various sites: At Fernow Forest, peroxidase activity declined with field N addition. At Mt. Ascutney and Bear Brook, b-glucosidase activity increased with lab N fertilization in previously unfertilized forest floor samples, and at Mt. Ascutney, peroxidase activity increased with field N addition. Aside from those effects, the microbial and enzymatic dynamics we measured were largely unresponsive to lab N fertilization (Table 2.1).

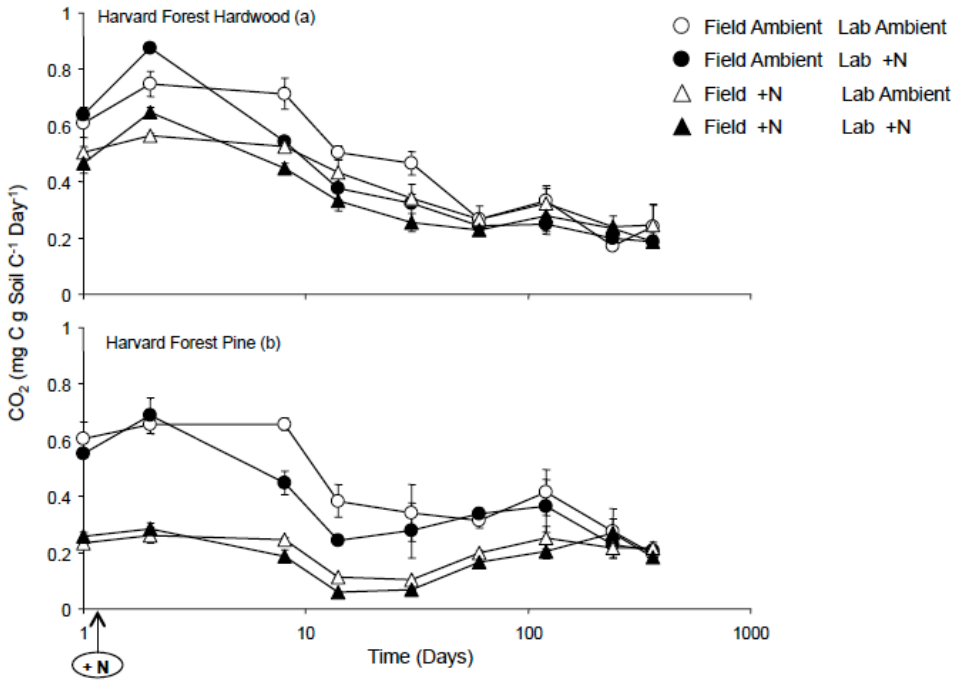

Figure 2.3 Harvard Forest Hardwood (a) and Pine (b) soil respiration for one year. Arrow notes addition of N fertilizer to "Lab +N" soils, after background CO_2 measurement.

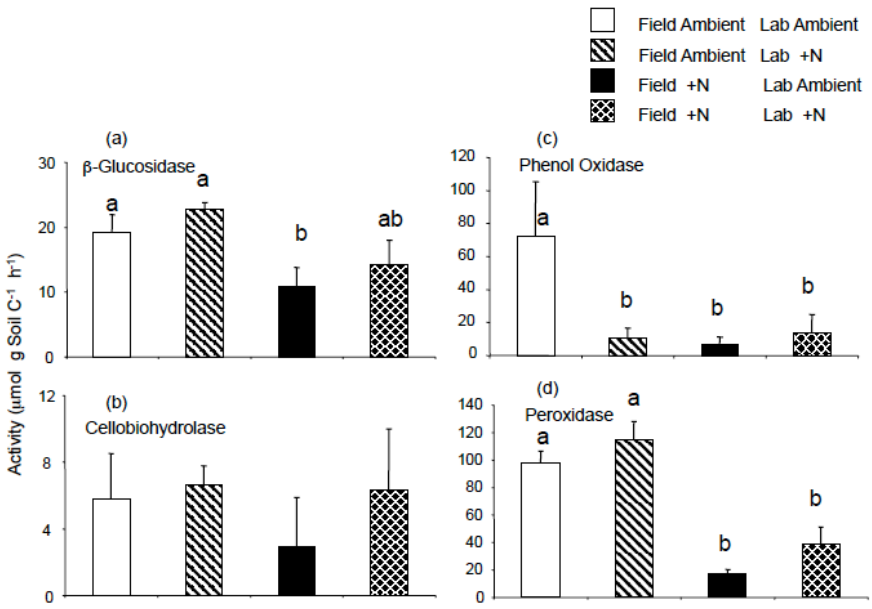

Figure 2.4 Harvard Forest Hardwood forest floor enzymes at one year. Lab +N stimulated cellobiohydrolase activity and both field +N and lab +N inhibited phenol oxidase activity.

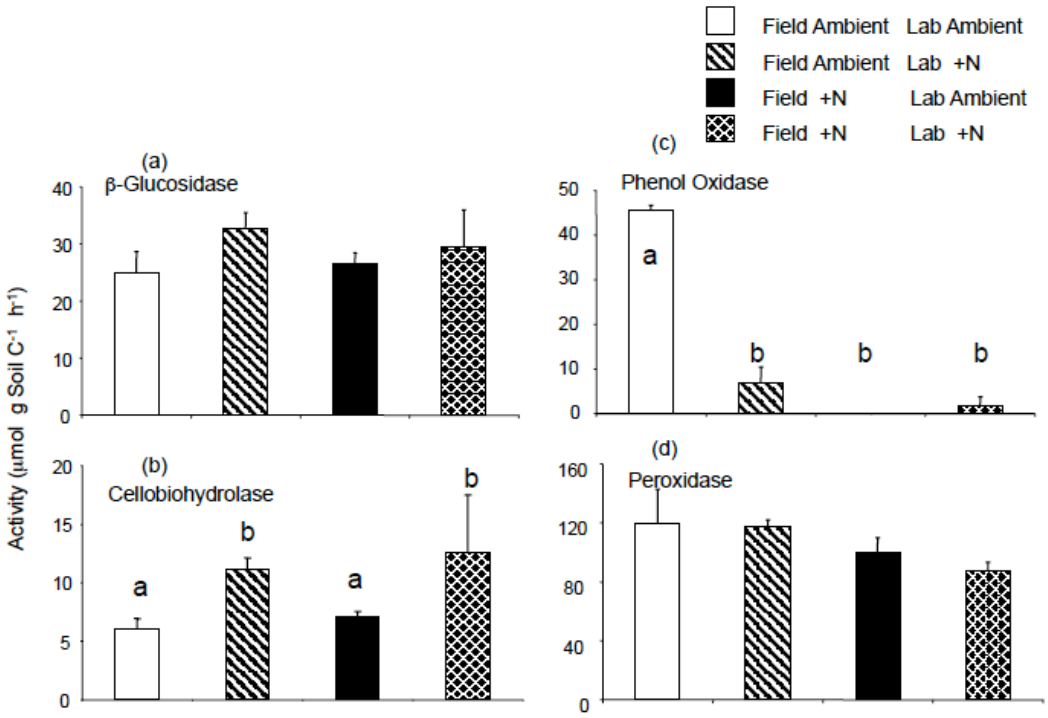

Figure 2.5 Harvard Forest Pine forest floor enzymes at one year. Field +N inhibited b-glucosidase and peroxidase activity, and both field +N and lab +N inhibited phenol oxidase activity.

Table 2.1 Effects of lab N fertilization and field fertilization treatments on six forest floor soils. + means increase with lab N fertilization, - means decrease with lab N fertilization, N.S. means not significant. CO_2 for Mt. Ascutney and Bear Brook excluded.

Site	Comparison: Field Ambient v.	CO_2	Microbial Biomass	β-Glucosidase	Cellobiohydrolase	Phenol Oxidase	Peroxidase
Harvard Hardwood	Lab + N	+	N.S.	N.S.	+	-	N.S.
	Field + N	-	N.S.	N.S.	N.S.	-	-
Harvard Pine	Lab + N	N.S.	N.S.	N.S.	N.S.	-	N.S.
	Field + N	-	N.S.	-	N.S.	-	-
Fernow Forest, WV	Lab + N	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Field + N	-	N.S.	N.S.	N.S.	N.S.	-
Cary Institute, NY	Lab + N	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Field + N	-	N.S.	N.S.	N.S.	N.S.	N.S.
Mt. Ascutney, VT	Lab + N		N.S.	+	N.S.	N.S.	N.S.
	Field + N		N.S.	N.S.	N.S.	N.S.	+
Bear Brook, ME	Lab + N		N.S.	+	N.S.	N.S.	N.S.
	Field + N		N.S.	N.S.	N.S.	N.S.	N.S.

DISCUSSION

Our objective in this study was to test the hypotheses that the short-term effect of N fertilization is to stimulate a pulse of CO_2 production through stimulation of decomposition of labile C, and over time, the dominant consequence of added N is to inhibit CO_2 production through inhibition of decomposition of recalcitrant C. Our expectation that the lab N fertilization treatment would quickly and briefly stimulate decomposition was supported by our results from the Harvard Forest Hardwood forest floor, in that we observed a pulse of CO_2 when N fertilizer was added to those samples in the lab. Both ambient and field-N fertilized samples showed this response to the lab N fertilization treatment. In the Hardwood forest floor, we observed three distinct phases of response to N fertilization, which may correspond to three soil C pools: (a) A small labile pool, the decomposition of which is stimulated by short-term N fertilization, (b) a larger slower cycling pool, the decomposition of which is inhibited by N fertilization, and (c) a large refractory C pool which is unchanged by N fertilization. These three soil carbon pools and responses to N fertilization correspond with the conceptual model described above. We measured the stimulation phase on a timescale of days (phase 1), an inhibition phase on a timescale of months (phase 3), and a no effect phase at the annual or longer timescale (phase 4).

The short-term pulse of CO_2 efflux following N fertilization in Harvard Forest Hardwood forest floor samples was detectable in both forest floor with no history of added N (field ambient) and samples which had received 20 years of N addition in the field. We were surprised to observe a CO_2 response to added lab N in the field N fertilized forest floor because we expected long-term N addition would have already stimulated the decomposition of the most labile C pool. Our results indicate that long-term N addition decreases decomposition; less CO_2 was respired in field N fertilized soils than in field ambient forest floor samples at the start of the incubation, but the N

fertilized soils could still contain a small amount of labile C prone to short-term loss with addition of new N, particularly in fall when leaf-fall may be providing a small labile C pool to these soils. This labile C pool is small; compared to forest floor samples which did not receive lab N addition, per area of forest soil, only an additional 1.4 mg C m^{-2} was respired by field ambient samples and an additional 1 mg C m^{-2} was lost from field N fertilized forest floor. By the end of the experiment, regardless of lab treatment, field ambient forest floor had respired 80 g C m^{-2} and field N fertilized forest floor had respired 79 g C m^{-2}, much exceeding the short term stimulation of CO_2 respired with added N.

In Harvard Forest Pine soils, we saw evidence for the two slower C pools, but not the more labile C pool. The two pools correspond with the inhibition phase (3) and long-term no effect phase (4) in our conceptual model. Although we did not see a pulse of CO_2 production with the lab N addition, Schimel and Weintraub (2003) caution that N fertilization may cause microbial decomposers to increase efficiency, such that they could increase decomposition without increasing CO_2 respired. Yet N fertilization did not increase C-degrading enzyme activity in these forest floor samples, providing no evidence that N fertilization increased decomposition when CO_2 efflux declined.

The origin of the labile C which was rapidly decomposed in Hardwood forest floor samples following the pulse of N fertilization likely enters the soil through a combination of root exudation and fresh leaf litter. The soils in this study were collected in late October, during leaf fall, and readily metabolized C may have been more abundant in the Hardwood forest floor as a result. In contrast, Pine forest floor had no pool of readily metabolized C mobilized by a pulse of N fertilization. As a conifer stand, the Pine site does not does not experience as strong a seasonal pulse of labile C from litter as does Hardwood. The differences in species composition

between Hardwood and Pine sites may be particularly relevant to soil C dynamics during leaf fall in the Hardwood stand.

Our Harvard Forest CO_2 production results are consistent with past work at that site. That is, N fertilization stimulated Hardwood but not Pine forest floor respiration during the first year of the Chronic N field experiment (Bowden et al. 2004). When Micks et al. (2004) initiated a new field N fertilization experiment on nearby unfertilized soils, they also observed a transient increase in CO_2 respired during one measurement of Hardwood soils. Most Hardwood soil measurements showed no stimulation of CO_2 production, and N fertilization did not stimulate CO_2 production at any time in the Pine soils (Micks et al. 2004). At the field study, by its year 2, the "low N" fertilized Pine soils showed less soil respiration than ambient soils, though there was no treatment effect for Hardwood soils (Bowden et al. 2004). By year 13, the "low N" fertilized Pine soils again showed a decrease in soil respiration relative to ambient soils, and though there was still no "low N" treatment effect on Hardwood soil respiration, the Hardwood "high N" treatment decreased soil respiration relative to both ambient and "low N" soils (Bowden et al. 2004), indicating that cumulative N addition can ultimately decrease CO_2 release. Our year 20 results are consistent with the increasing inhibition of CO_2 release with N addition measured by Bowden et al (2004). Both Micks et al. (2004) and Bowden et al. (2004) made field measurements of soil respiration, which includes CO2 produced by both microbial decomposition of detrital material and by root growth. In year 13, Bowden et al. (2004) also measured CO_2 production in a root-free incubation, and found that for Harvard Forest Pine soils both total soil respiration and heterotrophic respiration decreased: total soil respiration measured in the low-N field plots declined by about 40% and heterotrophic respiration of root-free low-N soil measured in a lab incubation declined by about 50%. For comparison, here in our lab incubation, we added a pulse

of new N to forest floor samples which had never been fertilized before, as well as to the 20 year field fertilized samples. We focus here on forest floor heterotrophic respiration, while Bowden et al. (2004) incubated only the mineral soil. However following Bowden et al. (2004), we would expect field measurements to show the same qualitative treatment responses.

A goal of this work was to link microbial activities to changes in decomposition rate measured by CO_2 release. Concurrent with a pulse of CO_2 released following our lab N fertilization, we expected to observe increases in b-glucosidase and cellobiohydrolase activity, signifying a stimulation of the decomposition of simple C compounds. We also expected that long-term N fertilization would decrease CO_2 production and phenol oxidase and peroxidase activity, evidence of an inhibition of the decomposition of lignin-based complex C substrates. Our Harvard Hardwood results support the hypothesis that N fertilization stimulates the decomposition of easily-degradable high quality C compounds and inhibits the decomposition of harder to decompose more complex C substrates like lignin (Fog 1988, Berg and Matzner 1997), and illustrate that multiple enzymatic pathways contribute to regulating the rate of soil decomposition. The net effect of N fertilization on decomposition rates depends on the available substrate quality, and the responses of the different suites of enzymes which degrade the specific substrates. In the case of Harvard Harwood, presumably at first, N fertilization stimulated decomposition of cellulose-based C, yielding a small transient net increase in CO_2 release. However once those cellulose-based substrates had been decomposed, inhibition of decomposition in the larger remaining lignin-based C pool dominated the decomposition response.

Measurements of C-degrading enzyme activities can aid a mechanistic understanding of changes in decomposition processes with N fertilization in Harvard

Forest Pine forest floor as well. Here, both lab N fertilization and field N addition decreased phenol oxidase activity, while long-term field N addition alone decreased peroxidase activity and b-glucosidase activity. Those decreases in C-degrading enzyme activity correspond with the decrease in CO_2 respired with N fertilization, suggesting that both short term N fertilization and long term N addition slowed decomposition of lignin-based C. Long-term N addition also appears to slow decomposition of cellulose-based C, whereas the short-term pulse of N did not change either cellulase enzyme activity in Harvard Pine forest floor.

Our lab N fertilization treatment did not stimulate CO_2 release at Fernow Forest and Cary Institute, though any labile C in these samples may well have been lost during prolonged equilibration time at these sites, prior to the start of their N additions. The observed pulse of CO_2 efflux in Harvard Forest Hardwood forest floor samples was so transient that if an analogous carbon pool with the potential to be stimulated by N fertilization existed in forest floor from Fernow Forest or Cary Institute, that C was likely to have been respired during the equilibration period, prior to our initial measurements or the N fertilization itself. In addition, we collected forest floor samples from these deciduous forests in June, as opposed to the Harvard Forest Hardwood forest floor samples which were collected in October and possibly included the labile C from fresh litter. At the longer timescale of months, we did observe an inhibition of respiration due to the long-term field N addition treatment at Fernow Forest and Cary Institute, showing that despite the prolonged equilibration period, decomposers were still active and inhibited by N addition in these forest floor samples. The extended equilibration times of these samples likely prevented detection of effects on a C pool with a turnover time of days to weeks but fully allowed detection of effects on soil C pools with turnover times of months or more. In Fernow Forest soils, a decline in peroxidase activity with the long-term field N addition

treatment suggests that a decrease in decomposition of lignin-based C compounds could contribute to the decrease in CO_2 respired. We observed no changes in enzyme activity at the end of the year-long incubation at Cary Institute.

We used enzyme assays and soil respiration measurements to understand the microbial activities driving decomposition responses to changes in N availability. We measured enzyme activity at the conclusion of the year-long soil incubation experiment, which we interpret to be a reflection of microbial enzyme activity during the course of the incubation. Enzymes have been shown to stabilize in soils; Allison et al. (2006) showed that at the end a 21 day incubation, both phenol oxidase and b-glucosidase activity maintained steady rates of activity in soil. Also, enzymes can remain active independent of the fate of the organism which produced the enzyme (Allison 2006). The most severe limitation of these methods is that we are comparing a metric of immediate microbial activity, respiration, with an estimate of the maximum capacity of the extracellular enzymes to degrade model substrates. Current enzyme assay techniques tell us much about enzymatic capacity and maximum potential enzymatic rates, but unfortunately very little about the actual rates of the processes in soil (Wallenstein and Weintraub 2008). A great leap forward in our capacity to understand microbial activities driving biogeochemical processes and to predict how those processes will change in the future will be to develop methods for accurately measuring *in situ* enzyme activity (Wallenstein and Weintraub 2008), so we can monitor enzymes in the environment and precisely document the responses of the enzymes to change.

We found evidence at Harvard Forest Hardwood to support the hypothesis that N fertilization briefly stimulates decomposition of a very small pool of labile C and inhibits decomposition of the more abundant and recalcitrant constituents of soil organic matter, and to support our conceptual model (Figure 2.1) that N fertilization

can simultaneously interact with different soil C pools, changing those soil C pools and thus the overall soil decomposition response over time. C-degrading enzyme activity explained changes in CO_2 respired for both Harvard Forest Hardwood and Pine forest floor.

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CHAPTER 3

Effects of long-term nitrogen addition on nitrogen retention in four soil nitrogen pools from six northeastern forest soils: A ^{15}N tracer approach

ABSTRACT

Nitrogen deposition to northeastern forests has increased since industrialization. Soils represent the largest sink for added nitrogen in most forest ecosystems, yet few studies have followed the added nitrogen into discrete soil pools. Here we present results from a multi-site ^{15}N tracer soil incubation experiment to study the fate of soil nitrogen retention and loss with long-term nitrogen addition. We collected forest floor and surface mineral soils from six long-term N addition experiments in the northeastern U.S. We added a ^{15}N – ammonium sulfate ($(NH_4)_2SO_4$) tracer in the lab to all soils, and measured its recovery in bulk soil, microbial biomass, and light and heavy density fractions after a 1-year incubation. We found that long-term nitrogen addition decreased recovery of the ^{15}N tracer in mineral soil for all pools (bulk soil, heavy fraction, light fraction and microbial biomass). There was no N addition treatment effect on ^{15}N tracer recovery in the forest floor. In both the mineral soil and the forest floor, ^{15}N recovery was in proportion with the size of each fraction; recovery was higher in larger fractions. In mineral soil, most ^{15}N was recovered in the heavy fraction, whereas in the forest floor, most ^{15}N was recovered in the light fraction. Microbial biomass generally had the lowest ^{15}N recovery of the N pools; only 0.2 to 5% of N was recovered in microbial biomass. Within the light fraction, % ^{15}N recovery was positively associated with light fraction %C for both ambient and N-fertilized soils; neither the heavy fraction nor bulk soil showed a

significant relationship between % ^{15}N recovery and %C of the respective pool. The forest floor had higher percent recovery of ^{15}N across sites and higher organic matter content than mineral soil, though organic matter alone did not explain differences in recovery across sites. These results indicate that stabilized organic matter associated with the mineral fraction provides a substantial sink for added N, a sink that can be reduced by long-term N addition. In contrast, N sinks associated with free organic matter dominate in the forest floor, and were not reduced by 11 – 20 years' worth of N addition.

Keywords:
nitrogen addition, ^{15}N tracer, nitrogen retention, density fractions, microbial biomass

INTRODUCTION

Nitrogen (N) deposition to northeastern U.S. forest ecosystems has increased 4 to 15-fold since industrialization (Dentener et al. 2006, Elliott et al. 2007, Galloway et al. 2008). N deposition to forested ecosystems has caused concern about long-term negative consequences including tree mortality and a decreased capacity of forests to retain reactive N, leading to water pollution (Aber et al. 1998) and has also led to an ongoing debate over whether N enhances carbon (C) sequestration in forest biomass and/or soils (Nadelhoffer et al. 1999, Magnani et al. 2007, LeBauer and Treseder 2008, Sutton et al. 2008, Thomas et al. 2010, Goodale et al. Submitted). The ecosystem consequences of atmospheric N deposition depend in large part on its fate: how much of this N deposition reaches plants, how much leaches to drainage waters, and how much is retained in soil pools of various turnover times.

Whole-ecosystem ^{15}N tracer studies have yielded much useful information about the fate of added N in forests. Small amounts of inorganic N, highly enriched in ^{15}N, have been applied to forests, typically as a solution sprayed on the forest floor, and the added ^{15}N is traced into different ecosystem pools over various timescales. These studies show that most ^{15}N added to forests is retained in soils one to seven years later (Seely and Lajtha 1997, Tietema et al. 1998, Nadelhoffer et al. 1999, Perakis and Hedin 2001, Kaye et al. 2002, Nadelhoffer et al. 2004, Templer et al. 2005, Morier et al. 2010; but see Zak et al. 2004).

Of the whole-ecosystem ^{15}N tracer studies, a subset has examined the effect of long-term N addition on N retention in soils. The dominant finding of the N addition studies is that N amended soils retain less ^{15}N than unfertilized soils (Nadelhoffer et al. 1995, Tietema et al. 1998, Nadelhoffer et al. 2004, Templer et al. 2005), indicating that N addition decreases the capacity of soils to retain additional N by saturating some component of the soil N pool (Nadelhoffer et al. 2004). Few studies have followed the added ^{15}N into discrete soil N pools, and no studies of which we are aware have looked at the effect of N addition on ^{15}N retention in multiple soil N pools using a ^{15}N tracer approach. Understanding why soil N retention decreases with N addition requires delving into specific soil N pools.

Of the ^{15}N retained in the bulk soil pool, the ^{15}N could be incorporated into microbial biomass, could be retained in soil organic matter (SOM), or could rest in the exchange complexes of clay lattices within mineral soil (Vitousek et al. 1979, Johnson et al. 2000). Studies pairing short term (hours to days) and long-term (one year or more) ^{15}N recovery measurements demonstrate that microbial immobilization is an important short-term fate of added ^{15}N. Much of this N is maintained in the microbial biomass for days to a week or so, at which time it is mineralized, and may be lost, retained in SOM, re-incorperated into microbial biomass, or taken up by plants (Seely

and Lajtha 1997, Perakis and Hedin 2001, Zak et al. 2004). Though microbial biomass is not an important pool for long-term N retention, ^{15}N retained in long-term, stable soil pools often shows signs of microbial processing (Morier et al. 2008, Sollins et al. 2009), indicating that microbial processing plays an important role in N stabilization, even without retaining the N directly.

Though biotic mechanisms of N retention appear to be dominant in ambient soils, ^{15}N studies also reveal a significant role for a variety of abiotic mechanisms of N immobilization (Schimel and Firestone 1989, Johnson et al. 2000, Dail et al. 2001, Perakis and Hedin 2001, Davidson et al. 2003, Moritsuka et al. 2004, Fricks et al. 2009). Ammonium (NH_4^+) can be retained in sterilized soils, indicating an abiotic N retention, though the exact mechanism remains elusive (Johnson et al. 2000, Morier et al. 2008). Increasing N availability has been demonstrated to increase the importance of abiotic relative to biotic ^{15}N-NH_4^+ retention, even while decreasing overall soil N retention, because biotic N retention decreases while abiotic retention remains constant (Johnson et al. 2000). Rapid abiotic assimilation of ^{15}N-nitrate (NO_3^-) into sterilized soils implies an active abiotic mechanism (Dail et al. 2001, Davidson et al. 2003). The positive association between abiotic NO_3^- retention and dissolved organic C (DOC) content in soils suggests a role for organic matter in abiotic NO_3^- retention. One hypothesis suggests DOC reduces iron; the reduced iron in return reduces nitrate to nitrite, and the nitrite readily binds with DOC (Davidson et al. 2003). A detailed understanding of how biotic and abiotic factors ultimately interact to stabilize N in soils is still lacking, but work to measure these mechanisms is consistent with the conclusions of Sollins et al. (2009), that microbial processing of retained N is probably the dominant mechanism in most soils relative to abiotic chemical associations between N and soil clays or minerals (Johnson et al. 2000, Morier et al. 2008).

Chronic N additions to soils have consequences for both N retention and

interactions with the soil C pool. If microbial processing is critical to the soil N retention processes, microbial decomposers could be C limited in their ability to process added N (Kaye et al. 2002a). In this case, recovery of a pulse of ^{15}N tracer should be reduced by N addition both in microbial biomass as well as in other soil pools, because adding N will enhance C limitation of the microbial community. A second reason ^{15}N recovery may decline in microbial biomass with N addition is that N addition commonly causes soil microbial biomass to decline (Treseder 2008).

To determine the effect of N addition on soil N retention, we added a ^{15}N tracer to soils from six northeastern forested sites with long-term N addition experiments, and measured ^{15}N recovery in bulk soil, microbial biomass, and two operationally defined soil fractions: heavy fraction and light fraction. These two fractions are distinguished by flotation in a solution of defined density (here, 1.65 g cm^{-3}): the heavy fraction sinks, whereas the light fraction floats (Sollins et al. 1999). This method distinguishes between physically stabilized heavy SOM and an SOM pool not bound to a mineral fraction. While the light SOM is not physically stabilized, and is therefore presumed to be freely available to decomposers, other stabilization mechanisms such as chemical stabilization can occur in the light fraction (Gregorich et al. 2000), and are not accounted for by the density fractionation method. SOM associated with the heavy fraction tends to be older than light fraction material (Gaudinski et al. 2000). Here, density fraction allows us to see whether ^{15}N is predominantly recovered in the free SOM fraction or in mineral fractions within the bulk soil pool. This is particularly useful for tracing the fate of ^{15}N into SOM fractions that are small components of the SOM pool, where changes in ^{15}N retention in those SOM fractions could not be distinguished from the response of the bulk soil pool.

Past work has shown that long-term N addition decreases the capacity of soils to retain N (Nadelhoffer et al. 1995, Tietema et al. 1998, Nadelhoffer et al. 2004, Templer et al. 2005). Therefore, we expected that bulk soils from long-term N addition studies will retain less of our ^{15}N tracer than unfertilized soils. We anticipated that N addition would decrease recovery of ^{15}N in microbial biomass. We expected the majority of the added ^{15}N to be retained in the light SOM fraction and ^{15}N retention in the light fraction would decline with N addition, consistent with past work showing strong N retention in organic soil horizons, which still decrease N retention with N addition (Nadelhoffer et al. 2004). Further, we expected ambient mineral soil to retain less ^{15}N than forest floor. Last, we expect mineral soil ^{15}N retention to decline with N addition, as has been observed previously at Harvard Forest (Nadelhoffer et al. 2004).

METHODS

Study Sites

We collected soils from six long-term N addition experiments at five sites in the northeastern United States: Fernow Forest, West Virginia, Cary Institute, New York, Harvard Forest, Massachusetts, Mount Ascutney, Vermont, and Bear Brook, Maine. Each of these experiments was established to evaluate the effects of acid rain and/or chronic N additions on forests. The experiments were initiated in the late 1980's to mid 1990's, and vary in ambient N deposition rates, addition rates and type of N fertilizer applied.

Fernow Forest in West Virginia is a mixed hardwood forest, with a watershed-scale $(NH_4)_2SO_4$ addition experiment adding 36 kg N ha^{-1} yr^{-1} since 1989 (Gilliam et al. 1994, Gilliam et al. 1995, DeWalle et al. 2006). We collected soils from Fernow

Forest watershed 13 (ambient) and watershed 3 (N fertilized). The study at the Cary Institute in Millbrook, New York is in a mixed oak forest with a plot-scale NH_4NO_3 addition experiment which began in 1996. The initial N addition rate was 100 kg N ha^{-1} y^{-1}, but was reduced to 50 kg N ha^{-1} yr^{-1} in 2000 (Wallace et al. 2007). We studied two sites at the Harvard Forest Chronic N Addition Experiment, which began in 1988 at the Harvard Forest Long-Term Ecological Research (LTER) site, in Petersham, Massachusetts. The Harvard Forest Hardwood site is a mixed hardwood stand dominated by black oak, and the Harvard Forest Pine site is a red pine plantation. Both sites receive 50 kg ha^{-1} yr^{-1} of N fertilizer ("low N" plots), as NH_4NO_3 Aber (Aber et al. 1993, Magill et al. 2004). This study did not include soils from Harvard Forest plots receiving "high N" additions of 150 kg N ha^{-1} yr^{-1}. Mt. Ascutney in Vermont is a spruce-fir forest that began in 1988 with plot-scale addition experiments using several rates of NH_4Cl and $NaNO_3$ addition (McNulty and Aber 1993, McNulty et al. 1996). We collected soils from the 32 kg N ha^{-1} y^{-1} NH_4Cl experiment. The Bear Brook experiment in Maine consists of a mixed hardwood forest with a watershed-scale $(NH_4)_2SO_4$ addition experiment at 25 kg N ha^{-1} yr^{-1} since 1989 (Norton et al. 1994).

Soil Sampling and Preparation

We collected soils from Fernow Forest, Cary Institute, Mt. Ascutney, and Bear Brook in May – July 2007. We collected forest floor samples using a 7 cm diameter tulip bulb corer to extract forest floor material to the depth of the forest floor – mineral horizon boundary. To separate the two types of soil, we visually assessed the forest floor – mineral horizon boundary. Separating mineral soil and forest floor is notoriously subjective (Federer 1982). We made the following distinctions to separate our soils: Forest floor material at the boundary with mineral soil was dark brown to

black in color, lacked visible mineral grains, and was dense with roots. Mineral soil was generally orange to pale yellow in color. At Bear Brook and Mt. Ascutney, the forest floor – mineral horizon boundary was at a pale E horizon. For each treatment (N-fertilized, Ambient unfertilized), we made three forest floor composites, each comprised of five cores collected from points distributed around the plot or watershed, depending on the scale of the field experiment. At each sampling point where we had removed a forest floor sample, we used a 3.5 cm diameter corer to collect the top 15 cm of mineral soil, making three mineral soil composites of five soil cores.

For the Harvard Forest Pine and Hardwood sites, in October 2009, we received three mineral soil composites containing 0 – 10 cm soil that were collected in conjunction with 20th anniversary soil sampling for the Harvard Forest chronic N addition study. Each composite was comprised of two 9.5 cm diameter soil cores which were collected with an engine-driven, diamond-bit corer. As for the other sites, we used the tulip bulb corer to collect three forest floor composites each consisting of five cores; however, we collected these forest floor samples from the close vicinity of the mineral soil sampling points, rather than from directly above the mineral soil sampling locations.

We stored the soils at 4 ^{o}C for 24 hours to three weeks, at which time we sieved all soils to 4 mm and placed the soils into microlysimeter incubation cups (Nadelhoffer 1990). The cups were constructed from modified Millipore 150 mL Stericup Steirle Vacuuum Filter Units (Millipore, Billerica, MA, USA). We modified the cups by replacing the pre-installed filters with Whatman GF/F glass fiber filters (Whatman plc, Kent, UK), sealed using a ring of silicone caulk. We incubated 25 g dry weight equivalent of sieved forest floor material from Harvard Forest Hardwood and Pine, Cary Institute, and Bear Brook, 50 g dry weigh equivalent from Fernow Forest, and 15 g from Mt. Ascutney. These values reflect the amount of forest floor

from each site needed to fill the cups to capacity. Mineral soil incubations contained 50 g dry weight equivalent of sieved mineral soil per microlysimeter.

Both forest floor and mineral soil were sieved to 4 mm, and measurements reported here pertain to the material that passed the sieve. Soils were refrigerated prior to sieving and at held at room temperature thereafter. All soils were adjusted to 30% moisture after sieving, then allowed to equilibrate at room temperature for two weeks prior to addition of the ^{15}N tracer. However, processing constraints induced variable lags between collection and sieving and between sieving and moisture adjustment; For Harvard Forest Hardwood and Pine soils, we sieved soils within a week of collection and adjusted soil moisture one week after sieving. For Fernow Forest, we sieved the soils within a day of collection and adjusted moisture after four weeks. We followed a similar procedure for soils from Cary Institute, Mt. Ascutney, and Bear Brook though the time between sieving and adjusting soil moisture was 8 weeks, 12 weeks, and 16 weeks respectively (Table 3.1).

Table 3.1 Amount of soil incubated, amount of ^{15}N added, and incubation timing. FF = Fernow Forest, CI = Cary Institute, HH = Harvard Forest Hardwood, HP = Harvard Forest Pine, AS = Mt. Ascutney, and BB = Bear Brook

Site	Horizon	Soil Incubated (g dry mass per microlysimeter)	^{15}N Added (µg)	4°C Storage Duration Between Collection and Sieving (Days)	20°C Storage Time Between Sieving and Adjustment to 30% Moisture (Weeks)
FF	Forest Floor	50	116	1	4
	Mineral	50	116	1	4
CI	Forest Floor	25	58	1	8
	Mineral	50	116	1	8
HH	Forest Floor	25	58	4	1
	Mineral	50	116	2	1
HP	Forest Floor	25	58	8	1
	Mineral	50	116	2	1
AS	Forest Floor	15	35	14	12
	Mineral	50	116	14	12
BB	Forest Floor	25	58	7	16
	Mineral	50	116	7	16

^{15}N Tracer

We applied a 99 atom% ^{15}N-$(NH_4)_2SO_4$ tracer to the soils at a rate of 10 mg ^{15}N per g dry soil in each incubation cup, and we incubated the soils for one year. Over the course of the year we monitored soil moisture, and extracted the soils on 7, 14, 30, 60, 120, 240, and 365 days after addition with 100 mL of a low-concentration nutrient solution containing all essential micronutrients except N, following Nadelhoffer (1990). At the conclusion of the year-long incubations, we determined the fate of the ^{15}N tracer by measuring ^{15}N of bulk soil, light fraction, heavy fraction, and microbial biomass, as described below. All samples were analyzed for ^{15}N and %N by the Cornell Stable Isotope Lab (Ithaca, NY), using a Finnigan MAT Delta Plus isotope ratio mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) plumbed to a Carlo Erba NC2500 elemental analyzer (CE Elantech, Inc., Lakewood, NJ, USA) for elemental and isotopic composition of solid samples.

We calculated ^{15}N recovery following Nadelhoffer and Fry (1994):

$$^{15}N_{RECOVERED} = \frac{N_{POOL}\,(ATOM\ \%\ ^{15}N_{POOL} - ATOM\ \%\ ^{15}N_{REF})}{(ATOM\ \%\ ^{15}N_{TRACER} - ATOM\ \%\ ^{15}N_{REF})}$$

We used the equation above to calculate the $^{15}N_{RECOVERED}$ (mg ^{15}N soil cup^{-1}), where N_{POOL} equals the total mass of soil in the incubation cup multiplied by its %N (mg N soil cup^{-1}). We calculated ATOM % $^{15}N_{REF}$ from background samples, which were subsamples our composites from both treatments (ambient, N-fertilized) that were processed identically to our other incubation cups but did not receive our lab ^{15}N tracer. We analyzed background bulk soil, light fraction, heavy fraction, and microbial biomass from all sites and both treatments (fertilized and control soils). We report ^{15}N recovery as % of total ^{15}N added ("percent recovery").

Bulk Soil

At the conclusion of the one year incubation experiment, we destructively harvested the incubating soil for measurements including microbial biomass and density fractions, as described below. To measure ^{15}N recovery in the bulk soil pool, we dried the remaining soil not allocated the other measurements (about 20% of initial dry weight) at 55^{o}C for a minimum of 48 hours and ground the soils to a fine powder for ^{15}N analysis.

Microbial Biomass

We measured microbial biomass using the chloroform fumigation/extraction method (Brookes et al. 1985, Davidson et al. 1989). We collected two subsamples from each incubation cup, of 5 g dry weight equivalent for forest floor samples and 10 g dry weight equivalent for mineral soils. We placed one subsample from each pair in a 60 mL centrifuge tube and immediately extracted soils with 45 mL of 0.5 M K_2SO_4. We placed the second subsample in a 25 mL glass beaker and fumigated with chloroform in a vacuum desiccator for four days, at which time we transferred the fumigated soils to centrifuge tubes, and extracted those soils with 0.5 M K_2SO_4. A shaker table agitated all tubes containing soil and K_2SO_4 for one hour. After homogenization, we swirled the tubes by hand to remove any soil adhering to the sides or cap of the tube, and then centrifuged the tubes at 7,000 rpm for 12 minutes to separate soil from the extract solution, which accelerated filtering. We filtered all extracts through ashed Whatman GF/F glass fiber filters. We dried the K_2SO_4 salt extracts at 55 ^{o}C for a minimum of 48 hours, then ground the salts to a powder. We calculated microbial biomass N and ^{15}N as the difference between extractable N or ^{15}N in chloroform fumigated versus unfumigated samples (Paul et al. 1999), with the correction factor $K_{EN} = 0.54$ (Brookes et al. 1985).

Density Fractions

We separated soils into light and heavy components using density fractionation (Sollins et al. 1999). We weighed 5 g of dried forest floor or mineral soil into 60 mL centrifuge tubes and added 36 mL of sodium polytungstate (SPT) solution that had been adjusted to a density of 1.65 g cm^{-3}. A shaker table agitated the soil-SPT slurry for two hours. After homogenization, we swirled the tubes by hand to remove any soil adhering to the sides or cap of the tube, and then centrifuged the tubes at 7,000 rpm for 12 minutes. Following centrifuging, the tubes sat for 12 to 24 hours, during which time suspended particles either settled to the bottom or rose to surface, which resulted in a more transparent SPT solution and made the separation between the light and heavy fractions obvious. We aspirated the light fraction using a flask and vacuum pump, and collected the separate light and heavy fractions on ashed 7 mm diameter Whatman GF/F filters. We rinsed the material with a minimum of 250 mL deionized water, and transferred the fractions from the filter paper into foil drying tins. We dried the fractions at 55 ^{o}C for a minimum of four days, weighed all of the dried fractions, and ground each fraction to a homogenized fine powder for isotope analysis.

pH

Soil pH was measured using an Accumet Basic AB15 pH meter (Fisher Scientific, Waltham, MA, USA), by making a 1:10 soil : deionized water slurry and letting the soils equilibrate in the water for 30 minutes before measurement (Robertson et al. 1999).

Statistical Analysis

We tested the effects of N addition on ^{15}N recovery in bulk soil, heavy fraction, light fraction, and microbial biomass on log-transformed data using two-way

ANOVA, with the terms site and treatment, as well as the site by treatment interaction. We used a Student's-t post-hoc text to identify significant treatment differences within site. All models were fit using JMP version 7.0 (SAS Institute, Cary, North Carolina, USA).

RESULTS

Bulk Soil

In mineral soil, ^{15}N tracer recovery was significantly less in N fertilized soils than ambient ($P = 0.016$). However, in the forest floor, there was no significant effect of addition on ^{15}N recovery ($P = 0.90$, Figure 3.1). Across sites, percent recovery ranged from 10% to 51% in the mineral soil, and from 14% to 82% in the forest floor. For both mineral soil and forest floor, Harvard Forest hardwood had the highest recovery. Forest floor recovery was lowest in Fernow Forest soils, and mineral soil recovery was lowest at both Fernow Forest and Bear Brook.

Soil N Pools: Distribution of Recovered ^{15}N

The forest floor consisted largely of light fraction material, and the majority of the recovered ^{15}N was in the light fraction (Figure 3.2a). Fernow Forest was an exception, as its forest floor had a very small light fraction and the majority of recovered ^{15}N was in the heavy fraction.

In the mineral soil, the majority of the recovered ^{15}N was in the heavy fraction, which was by far the largest fraction by mass in the mineral soil (Figure 3.2b). In mineral soil, recovery in light fraction and microbial biomass were often similar in magnitude (0.3 - 15%), with microbial biomass recovery sometimes exceeding light fraction recovery (Fernow Forest, Harvard Forest Pine).

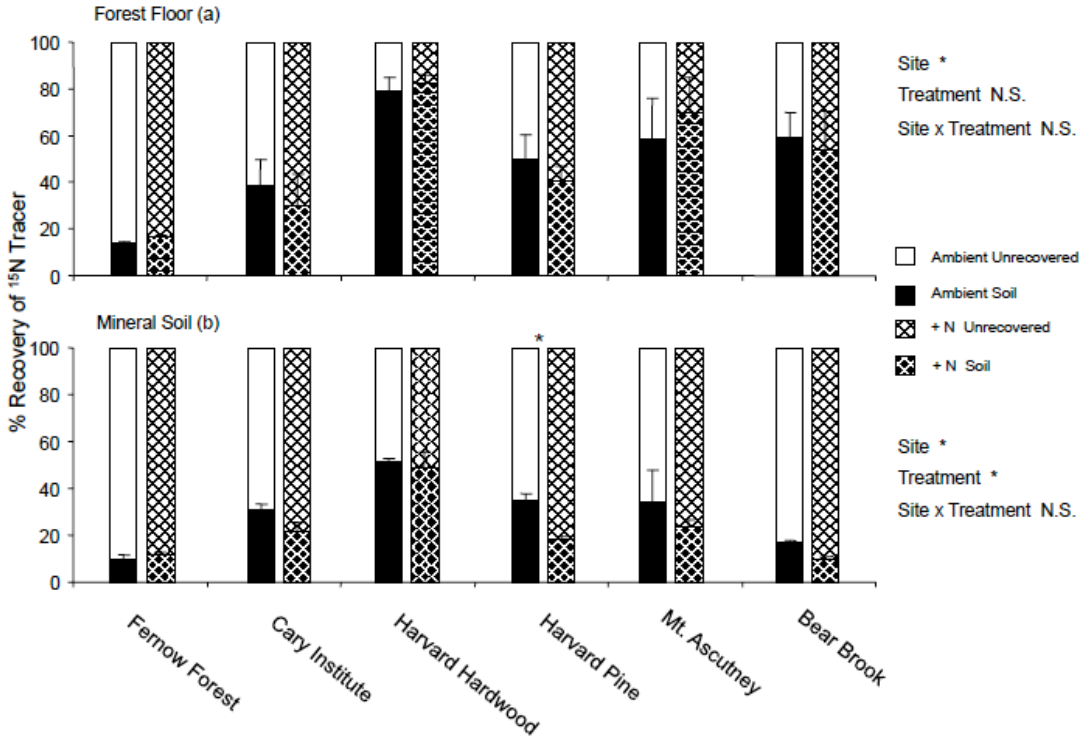

Figure 3.1 Bulk soil ^{15}N % Recovery. * Denotes statistically significant comparisons ($p < 0.05$).

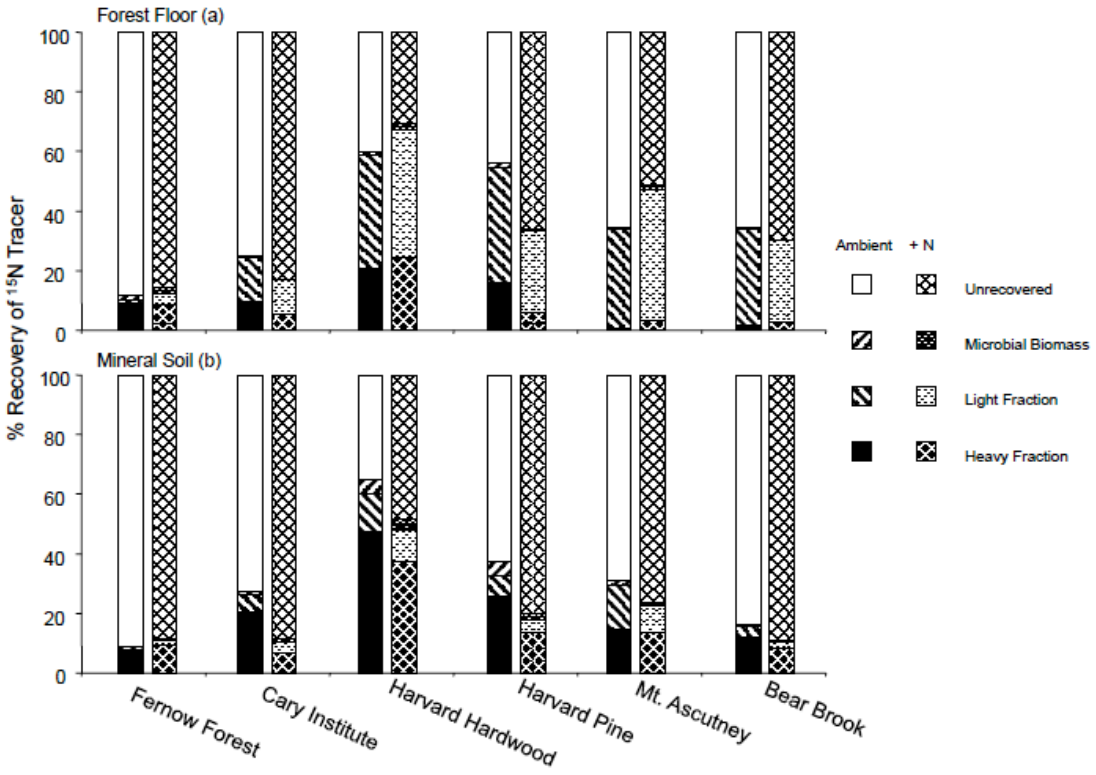

Figure 3.2 Recovery of ^{15}N tracer in three soil pools: light, heavy, and microbial biomass.

The ^{15}N recovery in the light fraction in the forest floor and in the heavy fraction in the mineral soil were both proportional to the size and N content of each fraction within that horizon. That is, ^{15}N recovery reflected soil N composition in the forest floor and mineral soil (Figure 3.3a and b).

In the light fraction, across sites in both the forest floor and the mineral soil, there was a strong positive relationship between % ^{15}N recovery and %C (Figure 3.4a and c). This relationship between recovery and %C was not present for the heavy fraction (Figure 3.4b and d) or for bulk soil. Soil pH was not significantly correlated with ^{15}N recovery.

N addition reduced ^{15}N recovery in all of the mineral soil N pools that we measured. Within each site, both ambient and N fertilized soils contained the same relative proportions of each N pool in mineral soil (Figure 3.5b).

Soil N Pools: Cumulative Recovery Compared to Bulk Soil

The cumulative sum of ^{15}N recovered in the light fraction, the heavy fraction, and microbial biomass ranged from 61 to 106% recovery relative to bulk soil, and averaged 87%. The lowest recovery was found in Bear Brook forest floor, while the highest recovery soil was in Bear Brook mineral soil (Table 3.2). Artifacts of the methods could be responsible for low recovery as well as over 100% recovery compared to bulk soil. Low recovery in the sum of fractions compared to bulk soil could be because some soil N is extractable with SPT (Crow et al. 2007). We did not measure ^{15}N recovery in the SPT soluble fraction to account for this loss. On the other hand, some cumulative recoveries exceeded 100% of that measured in the bulk soil. The primary reason for this is likely due to variability in the measurements of the three soil pools. Moreover, we may have effectively counted a subset of the microbial

biomass twice in our sum of the fractions. Though we have lost some of extractable N with the SPT, intact microbes should not pass through the filter used to collect the density fractions, and therefore would be included with the density fraction. Since we do not know the partitioning of the microbial biomass within the density fractions, it is difficult to correct the data for this addition, which could be no larger than the amount of microbial biomass in the whole soil sample which, while small on average (3%), reached as much as 9% of added ^{15}N. Microbial biomass was calculated with a correction factor from the literature of $K_N = 0.54$ to account for the limited efficiency of the extraction. This correction factor is an estimate, and may not necessarily represent the extraction efficiencies for all of the soils in this study. The combination of variability in the measurements of the three pools plus a correction factor which may not be perfectly suited to each particular site could have contributed to on-average recoveries slightly greater than 100%.

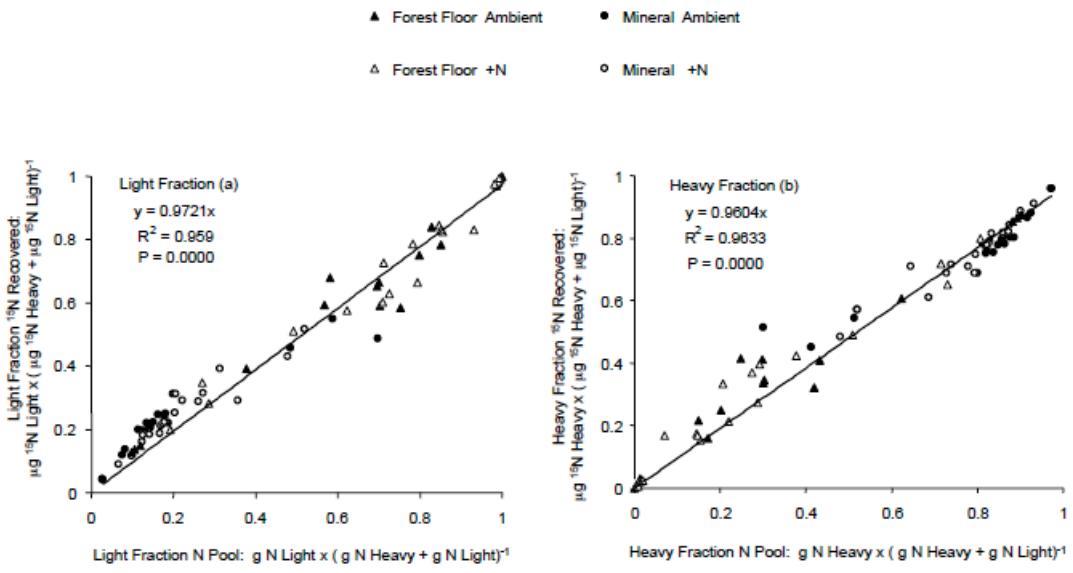

Figure 3.3 ^{15}N tracer recovery in light and heavy fractions, proportional to the total N pool of each fraction.

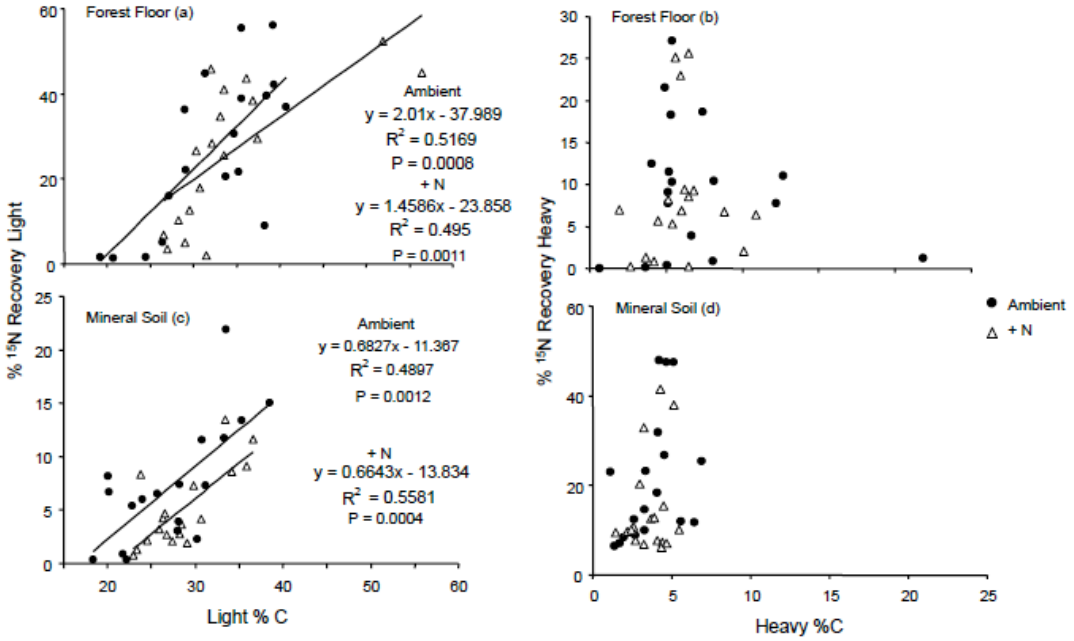

Figure 3.4 % ^{15}N recovery in light or heavy fractions versus light or heavy fraction %C in both forest floor and mineral soils.

Density Fractions

In the mineral soil, long-term N addition decreased ^{15}N recovery significantly in both the heavy and light fractions when considered across all six studies ($P = 0.0001$ and $P = 0.015$ respectively, Figure 3.6b). Within sites, addition reduced ^{15}N recovery in the mineral soil light fraction at one site (39% at Mt. Ascutney), and in the heavy fraction at three sites: Cary Institute (66%), Harvard Forest Hardwood (21%), and Harvard Forest Pine (47%).

In the forest floor, there was no overall effect of addition across sites on ^{15}N recovery in either the light or heavy fraction (Figure 3.6a). Within sites, however, for Harvard Forest Pine, the forest floor heavy fraction showed a significant 63% decrease in ^{15}N recovery with N addition.

Microbial Biomass

Long-term N addition reduced recovery of ^{15}N in microbial biomass in the mineral soil ($P = 0.016$, Figure 3.7b), but not in the forest floor (Figure 3.7a). In both the forest floor and the mineral soil, Harvard Forest Hardwood and Harvard Forest Pine had the largest recovery in microbial biomass, with both sites recovering 4% in the forest floor and 9% in unfertilized mineral soil. The lowest recovery in microbial biomass was at Bear Brook, where recovery was 0.4% for forest floor and 0.3% for mineral soil. Across sites, a larger fraction of the added ^{15}N was recovered in microbial biomass in the mineral soil (4%) than in microbial biomass in the forest floor (2%).

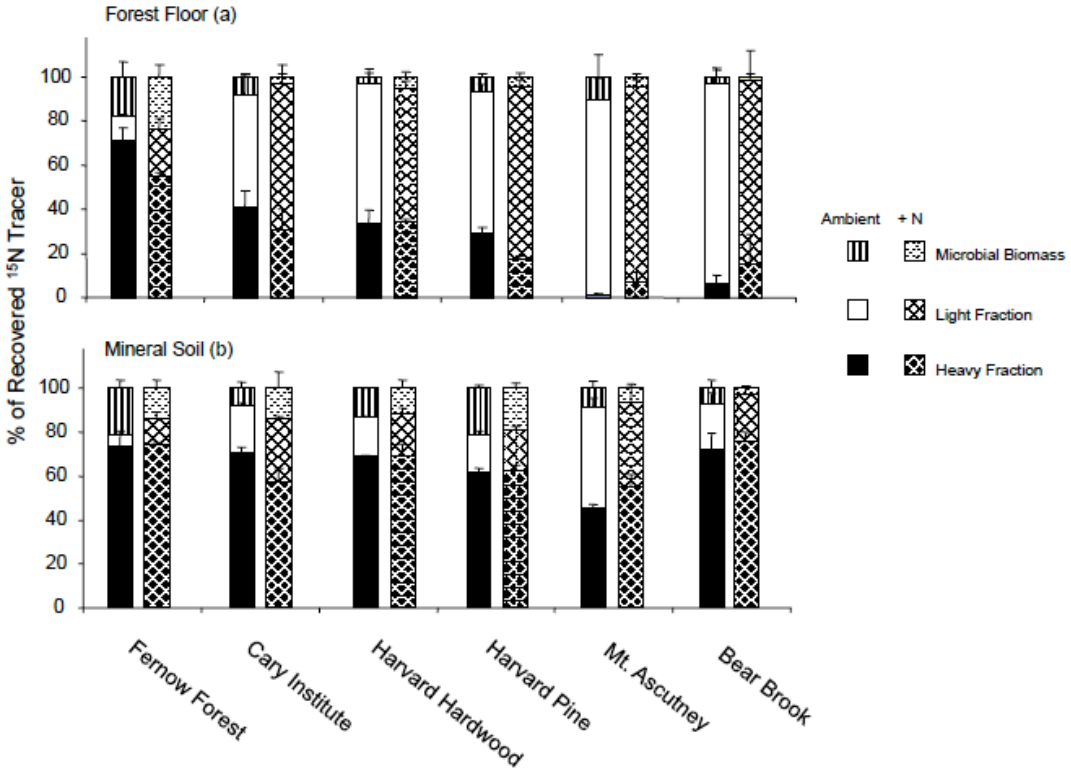

Figure 3.5 Of the total ^{15}N recovered, % distribution of ^{15}N tracer in three soil pools: light, heavy, and microbial biomass

Table 3.2 Sum of heavy fraction, light fraction, and microbial biomass ^{15}N recovery expressed as a % of bulk soil ^{15}N recovery.

Site	Treatment	% Recovery of Fractions Relative to Bulk Soil (SE)
Fernow Forest	Forest Floor	85 (4)
	Mineral	96 (7)
Cary Institute	Forest Floor	67 (7)
	Mineral	72 (10)
Harvard Hardwood	Forest Floor	81 (6)
	Mineral	107 (5)
Harvard Pine	Forest Floor	104 (19)
	Mineral	106 (5)
Mt. Ascutney	Forest Floor	69 (12)
	Mineral	101 (5)
Bear Brook	Forest Floor	61 (9)
	Mineral	101 (3)

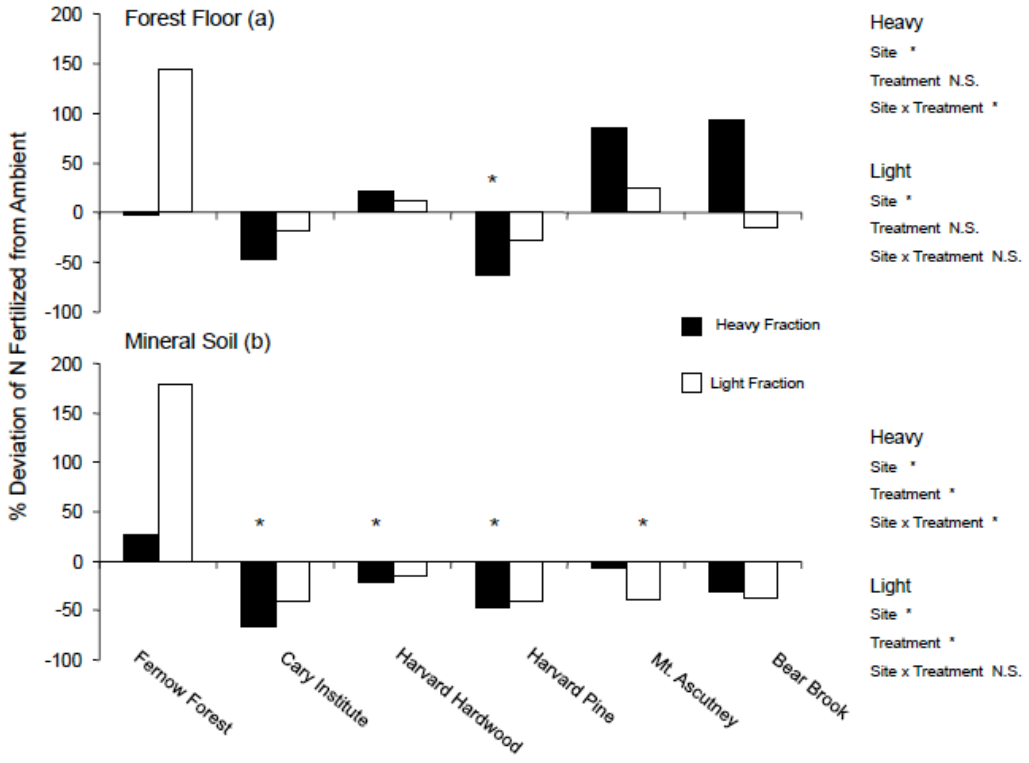

Figure 3.6 The effect of N fertilizer on ^{15}N recovery, calculated as % difference in ^{15}N recovery between ambient and N-fertilized soils. * Denotes significant N fertilizer treatment effect for marked fractions ($p < 0.05$).

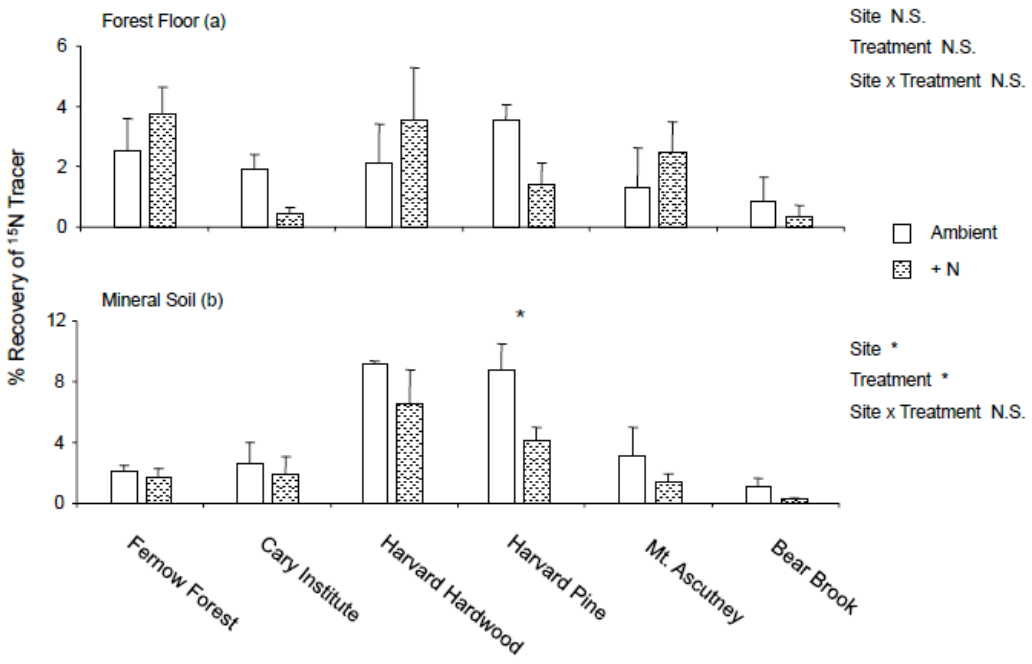

Figure 3.7 Microbial biomass ^{15}N % recovery. * Denotes significant comparisons ($p < 0.05$).

DISCUSSION

Our objective was to examine the effect of long-term N addition on ^{15}N retention in soils by measuring the fate of a ^{15}N tracer in four soil N pools: bulk soil, microbial biomass, heavy fraction and light fraction, at six long-term N addition experiments. We found three main results: (1) in the mineral soil, most added ^{15}N was retained in the heavy fraction, whereas in the forest floor, most ^{15}N was retained in the light fraction, in rough proportion to the distribution of these SOM fractions within these soil horizons; (2) ^{15}N retention increased with SOM content, and (3) N addition reduced ^{15}N retention in microbial biomass, heavy fraction, light fraction, and bulk soil in the mineral horizon.

Because our methods are insufficient for distinguishing between abiotic and microbial mechanisms underlying soil N retention and loss, our inferences about mechanisms of N incorporation into these various soil fractions are speculative and inferential. We saw only a small amount of ^{15}N retention in microbial biomass. Although microbial immobilization may be an important early fate of ^{15}N added to forest soils (Seely and Lajtha 1997, Perakis and Hedin 2001, Zak et al. 2004), N retained in soils is ultimately stabilized through associations with SOM or the mineral fraction. These associations may form following microbial processing of N, or without N passing through a microbial pathway at all (Johnson et al. 2000, Kaye et al. 2002, Sollins et al. 2009, Morier et al. 2010). Moreover, N addition status may influence which N retention mechanisms are dominant in a particular soil. For example, Johnson et al (2000) observed that as soils proceed towards N saturation, biotic N retention declines, increasing the relative importance of abiotic N retention. The $^{15}NH_4^+$ retained in bulk ambient soil in this study was most likely initially assimilated by microbial biomass, and was released either as NO_3^- or organic N, which

then became either physically or chemically associated with soil organic matter. In N-fertilized soils, abiotic immobilization may have played a more important role in directly immobilizing the $^{15}NH_4^+$, if N fertilization decreased biotic N retention (Johnson et al. 2000).

In the mineral soil, N does not appear to be retained primarily in association with the SOM pool: there was no relationship between bulk soil ^{15}N retention and bulk soil %C, nor between heavy fraction ^{15}N retention and heavy fraction %C. In the mineral soils, ^{15}N recovery was largest in the heavy fraction, and low in the more C-rich light fraction, indicating that a large amount of new N can be retained in association with a relatively old, stable SOM pool. Similarly, Hagedorn et al. (2005) observed that fine clay mineral soils can retain added ^{15}N more strongly than the associated C-rich fraction. The N retained in the clay fraction appeared fairly stable over time (Hagedorn et al. 2005), implying that ^{15}N recovered in the mineral fraction in this study may be stable over time as well, if similar mechanisms are retaining the N.

The distribution of ^{15}N recovery between the density fractions was highly correlated with the distribution of the N pool between the fractions; as the total N pool of a fraction increased, so did the ^{15}N recovery in that fraction. Though we are not able to discern the mechanisms underlying ^{15}N retention in the heavy fraction, understanding the contributions of direct and indirect microbial processing and abiotic N retention is critical for accurately predicting the capacity of soils to retain N with chronic N additions.

In the forest floor, the majority of the ^{15}N tracer was recovered in the C-rich light fraction, and N addition did not decrease ^{15}N retention in that pool. One possible mechanism is the capacity of SOM to form stabilizing interactions with N (Sollins et al. 2009). Other mechanisms could include abiotic condensation reactions through

which N directly associates with SOM and microbial uptake and re-exudation of nitrogen in enzymes which then associate with organic matter. ^{15}N NMR work indicates that the stable N associated with SOM is most commonly found in amides, presumably as a result of microbial processing of NH_4^+ (Morier et al. 2008). The relatively high recovery of the ^{15}N tracer in the C-rich forest floor compared to the mineral soil suggests that SOM plays an important role in N retention. Additionally, across sites, light fraction %C explained variation in ^{15}N retention in the light fraction, and the two showed a strong positive correlation in both forest floor and mineral soil. This suggests that when retained in a C-rich fraction, N appears to primarily associate with the SOM. Separating soils into light and heavy fractions revealed the positive association between light fraction ^{15}N retention and light fraction %C which would otherwise have been masked within the bulk soil pool.

N addition reduced ^{15}N retention in all four of the mineral soil N pools we measured, and the relative reductions in ^{15}N retention for each pool were approximately equivalent, such that the N fertilized soils had the same relative ^{15}N distribution as ambient soils. However, in the forest floor, there was no significant effect of N addition on ^{15}N retention. The most striking difference between the mineral soils and the forest floor is SOM amount and composition, which we suspect played a role in ^{15}N retention in the low density fraction material from both horizons. Our density fraction analysis results suggest that N is retained by different mechanisms in SOM-rich versus SOM-poor soils, and that SOM-rich soils are able to retain more N overall. The higher capacity to retain N buffers a soil against N loss with added N.

The N addition-induced decrease in ^{15}N retention in microbial biomass, heavy fraction, and bulk soil in the mineral horizon is consistent with our hypothesis. The decrease matches plot-scale results from Harvard Forest Hardwood and Pine

(Nadelhoffer et al. 2004), Bear Brook (Nadelhoffer et al. 1995), and in a cross-site experiment in Europe (Tietema et al. 1998), with the exception that those studies found reduced ^{15}N recovery in N fertilized organic soil as well as mineral soils. In contrast, we did not detect a treatment effect in the forest floor, though we expected to measure a decline in light fraction ^{15}N retention based on past work by others. One difference between our results and past work is likely methodological; we added ^{15}N directly to each incubating forest floor and mineral soil sample in the lab, whereas in the other studies, the ^{15}N is applied to the surface of the forest floor in the field. For the forest floor, applying the ^{15}N to sieved soil and subjecting the soils to leaching events led to less ^{15}N retention than is observed in the field studies, and may have contributed to the lack of a treatment effect of added N. For mineral soils, in the field studies those soils only access the ^{15}N which is not first retained in the forest floor, decreasing interaction between mineral soil and the ^{15}N tracer. Also consistent with our hypothesis and past work was our finding that ^{15}N retention was greater in forest floor than in mineral soil, and that retention in mineral soil declined with N addition. These observations are consistent with N saturation (Nadelhoffer et al. 2004), begging the question of whether biotic N retention in these soils is decreasing, increasing the relative importance of abiotic N retention (Johnson et al. 2000), and if so, what the implications are for SOM stabilization.

We measured a positive association between light fraction C content and ^{15}N retention. Considered together with higher ^{15}N retention in the relatively SOM-rich forest floor compared to the mineral soil, both observations indicate that a higher SOM content increases the capacity of a soil to retain ^{15}N. Further, mineral soils were more susceptible to decreased ^{15}N retention with N addition than were forest floor soils, so SOM may buffer soils against increasing N leaching with N deposition. N stabilized within the SOM pool appears to have a slower turnover time than the C in

the same SOM pool (Hagdorn et al. 2005), yet the turnover time of the SOM fraction is an important factor contributing to the duration over which N will be retained in a soil fraction. Forest floor soils and the light SOM fraction have higher turnover times (< 100 years) compared to the heavy fraction (> 130 years) (Gaudinski et al. 2000). Thus, though more ^{15}N is retained in association with light fraction SOM, ^{15}N retained in association with the heavy fraction in the mineral soil is likely be more stable over time.

Our results showed that long-term N addition decreased ^{15}N retention in mineral soil N pools in six northeastern experiments. Multiple lines of evidence suggest SOM increases the capacity of the forest floor to retain ^{15}N. The bulk of the retained ^{15}N in mineral soil was recovered in association with the relatively old, mineral-associated heavy SOM fraction. Though the light SOM fraction has a much higher turnover time for C than does the heavy SOM fraction, we do not know whether those turnover times also apply to the added N in each fraction. If they do apply, light SOM provides a greater capacity to absorb new N, but mineral-associated SOM may be a more durable long-term stable sink for added N.

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