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Long-term global change effects on forest biogeochemistry in the north-central United States

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Abstract

Human activities have substantially altered the composition of the atmosphere and many of these changes directly affect the biogeochemistry of forest ecosystems. Because of the geography of industrialization, these impacts are particularly acute in northern temperate forests. Unfortunately, most studies examining the effects of altered atmospheric composition on forest ecosystems may not be accurate predictors of the long-term impacts on mature forests because these studies used immature trees and were short in duration. Here, I use measurements from two large long-term collaborative experiments to examine the impacts of altered atmospheric composition on forest biogeochemistry in the north-central United States.

At the Rhinelander free-air carbon dioxide (CO_2) enrichment experiment in Wisconsin, I examined the independent and interactive effects of increased concentrations of atmospheric CO_2 and tropospheric ozone (O_3) on leaf production and soil carbon (C) storage in three forest communities. To estimate leaf production, litter traps were used to collect fallen leaves from 2002 to 2008 (years five through eleven of the experiment). In addition to leaf production (g m^{-2}), these collections were used to assess leaf area (m^2 m^{-2}), leaf litter nitrogen (N) concentration (mg g^{-1}), and the leaf N content (g N m^{-2}). On average, the factorial elevated CO_2 effect ($+CO_2$ and $+CO_2+O_3$) stimulated leaf production by 36% and the factorial elevated O_3 effect ($+O_3$ and $+CO_2+O_3$) decreased leaf production by 18%. Similar effects were observed for leaf area. However, the relative effects of the individual treatments were highly dynamic. From 2002 to 2008, the positive effect of the elevated CO_2 treatment ($+CO_2$) on leaf production relative to the

ambient treatment decreased from +52% to +25%, while the negative effect of the elevated O_3 treatment ($+O_3$) relative to ambient changed from -5% to -18%. The CO_2 and O_3 treatments did not have significant overall effects on litter N concentrations. Consequently, the leaf litter N content (g m^{-2}) was increased 30% by the elevated CO_2 treatments and decreased 16% by the elevated O_3 treatments. To estimate changes in soil C pools, the top 20 cm of the mineral soil was sampled seven times between 1998 and 2008. Despite an increase in the input of leaf and root litter by elevated CO_2 and a decrease in litter inputs by elevated O_3, there were no significant effects of CO_2 and O_3 on soil C storage for the overall experiment. However, within the forest community containing only aspen (*Populus tremuloides*), there was significantly less soil C (-17.4 Mg ha^{-1}) beneath forests receiving the elevated CO_2 treatments ($+CO_2$ and $+CO_2+O_3$) in the 2008 samples. In addition, I was able to use the unique ^{13}C signature of fumigation CO_2 to trace the input of new C into the soil in the elevated CO_2 treatments ($+CO_2$ and $+CO_2+O_3$). Initially, soils from the $+CO_2+O_3$ treatment had less new C than soils from the $+CO_2$ treatment, but this difference gradually disappeared. This gradual disappearance matched trends in fine root production. Combining the leaf production study with the soil C study, these results suggest that the rate of soil C cycling accelerated under elevated CO_2 and declined under elevated O_3 because changes in soil C accumulation did not match changes in litter production.

The other long-term experiment tests the influence of atmospheric deposition on four mature northern hardwood forests spread across 500 km in northern Michigan. These four forests sit along a north to south gradient, with warmer temperatures and higher

inputs of both acid deposition and N deposition at the southern end of the gradient. These sites were established in 1987 to examine the impacts of atmospheric deposition along this gradient, but a parallel experiment was established at the same four sites to simulate potential increases in N deposition. I utilized both aspects of this experimental design, using the existing deposition gradient to examine the ongoing effects of atmospheric deposition and using the N addition experiment to test the long-term influence of added N on leaf-level photosynthesis. Since these sites were established in 1987, there have been major changes in federal emissions regulations. These new regulations greatly restricted emissions of acid deposition precursors, but did not attempt to control overall N deposition. In the time since this policy was enacted, there have been remarkable changes in the impacts of acid deposition and N deposition on the biogeochemistry of these four forests. Using data only from the plots receiving ambient deposition, I found that there have been decreases in leaf sulfur, calcium, and aluminum concentrations over the past two decades. Acid deposition usually increases concentrations of these elements in soil solution, so the observed changes in leaf chemistry signal a waning influence of this pollutant. In comparison, leaf $\delta^{15}N$ and soil lysimeter data show that persistent ambient N deposition has caused widespread increases in both the availability of inorganic nitrogen and soil nitrate leaching. The declining influence of acid deposition shows that environmental policy can quickly and broadly influence forest biogeochemistry. Although there are large amounts of nitrate being leached from these forests as a result of ambient N deposition, the parallel N addition experiment at these same sites resulted in increased aboveground growth. Because of the key role of N in photosynthesis, conceptual models often attribute growth increases from increased N availability to

higher photosynthesis. However, increases in leaf-level photosynthesis have not often been observed in long-term N addition experiments. We tested the effects of 14 years of N additions on photosynthesis in two ways: by making instantaneous measurement from both canopy towers and excised branches, and by analyzing leaf tissue for $\delta^{13}C$ and $\delta^{18}O$, isotopes integrate changes in photosynthesis through time. Trees receiving N additions had higher foliar N concentrations, but there were no differences in instantaneous measurements of photosynthesis from canopy towers or excised branches. Further, there were no significant changes in $\delta^{13}C$ and $\delta^{18}O$ in either current foliage or leaf litter collected annually throughout the N addition experiment (1994-2007). Together, these data suggest that increases in photosynthesis are not responsible for the higher rates of aboveground growth.

Together, these experiments show that changes in atmospheric composition expected to occur in the next century will alter the functioning of forest ecosystems in the north-central United States. However, predictions from short-term experiments did not often match the results observed in these long-term projects. Alternately, the recovery of forests in the north-central United States from acid deposition suggests that forest biogeochemistry can respond positively if pollution reductions are prioritized by policy makers.

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Chapter One: Background and Introduction

The impacts of human population growth and mounting industrialization on the biosphere are so great that most ecosystems are now directly or indirectly dominated by human activities (Vitousek et al. 1997). Humans have dramatically changed global land cover, redistributed plant and animal species around the world, altered the climate, and modified major biogeochemical cycles (Vitousek et al. 1997). Although these changes are the subject of extensive research, scientists still struggle to understand how these processes will individually and interactively affect the biosphere.

Human-induced changes in atmospheric composition are having an obvious influence on the climate, and the effect of climate change on terrestrial ecosystems has received considerable attention (e.g. Nabuurs et al. 2007). However, in addition to altering the climate, many of the changes in atmospheric composition have a direct effect on terrestrial ecosystems. In particular, it has become clear over the last several decades that humans broadly influence forest biogeochemistry by increasing the concentrations of a number of key compounds in the atmosphere (Aber et al. 1989, Likens et al. 1996, Ainsworth and Long 2004, Wittig et al. 2009).

The most widely distributed of these changes in atmospheric composition is the increase in atmospheric carbon dioxide (CO_2). In the atmosphere, CO_2 is relatively stable and has a long turnover time (Seinfeld and Pandis 1998). Consequently, emissions lead to global rather than local increases in CO_2 concentrations and CO_2-impacts. Much of the effort dedicated to understanding the effects of elevated CO_2 on the biosphere has focused on

crop species and trees, the former because of global food production and nutrition concerns, while the latter because forests and woodlands dominate terrestrial productivity (Field et al. 1998). Plant scientists have long hypothesized that increases in atmospheric CO_2 concentrations will increase plant growth (Ainsworth and Long 2004). This is because CO_2 provides the carbon (C) substrate for photosynthesis in a reaction (for C_3 plants) that follows Michaelis-Menten kinetics (Lambers et al. 1998): higher concentrations of CO_2 lead to greater C assimilation. In this way, it is expected that increases in atmospheric CO_2 will create a "CO_2 fertilization" effect that stimulates both plant productivity and ecosystem C storage. The stimulation of ecosystem C storage is projected to capture some of the CO_2 emitted by human activities and slow the overall growth in atmospheric CO_2. However, there is considerable uncertainty in the magnitude of this effect (Nabuurs et al. 2007) because the raw stimulation of photosynthesis will be modified by interactions with plant physiology, forest biology, environmental variables (water, nutrients, temperature, etc.), and other global change factors (N deposition, tropospheric O_3, land use).

In contrast to the global distribution of elevated CO_2, several of the other important changes in atmospheric composition have more localized effects. The compounds involved in acid deposition, N deposition, and tropospheric O_3 pollution have a shorter residence time in the atmosphere than CO_2. Consequently, these compounds and their ecological effects tend to be concentrated in regions near emissions sources (Seinfeld and Pandis 1998). The first of these atmospheric pollutants to be widely linked to forest biogeochemistry and ecosystem function was acid deposition (Driscoll et al. 2001). In

the United States, increases in acid deposition resulted primarily from increases in the emission of sulfur dioxide (SO_2) and oxidized N (NO_x) due to the combustion of fossil fuels (Driscoll et al. 2001). These compounds react in the atmosphere to form H_2SO_4 and HNO_3, which are subsequently added to the landscape through wet deposition (acid rain) or dry deposition. The addition of these acidic compounds to forests alters biogeochemical cycling by increasing the movement of calcium (Ca), magnesium (Mg), and aluminum (Al) into the soil solution (Ruess and Johnson 1986). Once in the soil solution, these elements can be removed from the soil through leaching or taken up by plants or microbes. The loss of Ca and Mg from soils through this process has been a major concern in regions where availability of these essential plant nutrients is low (Driscoll et al. 2001), while increases in soil solution Al are alarming because Al can be toxic to plants (Cronan and Grigal 1995). Partly in response to these impacts on forest biogeochemistry, the U.S. Congress passed the 1990 Clean Air Act Amendments (Waxman 1991). This legislation greatly restricted emissions of SO_2 and led to reductions in acid deposition across the eastern and central U.S (Stoddard et al. 1999). However, acid deposition still far exceeds pre-industrial levels and the speed and magnitude of the response to recent changes in acid deposition remains unclear (Likens et al. 1996, Driscoll et al. 2001).

Although acid deposition acts indirectly to influence forest growth by changing the availability of other elements, N added through atmospheric deposition directly affects forest growth. Most forests are limited in their growth by N (LeBauer and Tresseder 2008), and the addition of N through atmospheric deposition is thought to fertilize forests

on a regional scale (Magnani et al. 2007). Nitrogen deposition results from emissions of oxidized N (NO_x), reduced N (NH_y) and organic N (Holland et al. 2005). Over the past century, there has been an extraordinary increase in the global production of these forms of reactive N as a result of fossil fuel combustion, fertilizer use and production, animal husbandry, and the cultivation of N-fixing plants (Galloway et al. 2008). The causes of increased N deposition are clear, but the consequences of N deposition on temperate forest ecosystems are complex (Aber et al. 1998). In particular, the magnitude of the N fertilization effect on plant growth and ecosystem C storage is the subject of an ongoing debate (Nadelhoffer et al. 1999, Magnani et al. 2007). Furthermore, N deposition can also reduce biodiversity and water quality (Vitousek et al. 2003). The 1990 Clean Air Act Amendments regulated NO_x emissions, but these reductions were relatively weak (Aber et al. 2003) and lowering total N deposition was not a goal of the legislation. Consequently, total N deposition has remained relatively unchanged in the U.S. over the past two decades (Watmough et al. 2005) and the complexity of forest biogeochemistry makes it difficult to assess the effects of these chronic N additions (Aber et al. 2003).

One of the reasons that NO_x emissions have been a focus of regulation in the U.S. is that NO_x plays a key and often limiting role in the formation of tropospheric O_3 (Seinfeld and Pandis 1998). Tropospheric O_3 negatively effects human respiratory health and is phyto-toxic to a wide range of plants (Wittig et al. 2009). Increases in tropospheric O_3 are likely to reduce forest C storage (Karnosky et al. 2005), but like atmospheric CO_2, O_3 effects are modified by interactions with plant physiology, forest biology, environmental variation (water, N, temperature, etc), and other global change factors (N deposition,

atmospheric CO_2). Furthermore, there is almost no experimental data on the long-term (+ 5 years) effects of O_3 on forest productivity, making it even more difficult to predict the magnitude of its effects on forest growth.

In the studies that follow, my collaborators and I explore the impacts of atmospheric CO_2, acid deposition, N deposition, and tropospheric O_3 on forests in the north-central U.S. These studies were conducted within two large collaborative experiments that are each over a decade in length. In northern Wisconsin, free-air CO_2 enrichment (FACE) technology has been used to expose forests to increased concentrations of atmospheric CO_2 and tropospheric O_3 since 1998. These trace gases were applied to three planted forest communities in order to address the knowledge gaps around both competitive interactions under elevated CO_2 and long-term exposure to elevated O_3. This project is the only long-term forest experiment examining both the independent and interactive effects of CO_2 and O_3 and the only forest FACE experiment designed to examine how competitive interactions among species and genotypes will modify ecosystem responses to these trace gases. Within the context of the greater FACE experiment, I examined changes in soil carbon storage over the first eleven years of the project (chapter two) and leaf production during years five through eleven of the project (chapter three).

The other collaborative experiment was started in 1987 to examine the impacts of atmospheric deposition. To do this, research plots were established in four closely matched mature northern hardwood forests located along a 500 km atmospheric deposition gradient in Michigan. In addition, a parallel N deposition experiment was

established in 1994 at each of the sites in order to more mechanistically understand the effects of N deposition. This project is one of the longest running ecosystem experiments of its kind in the world and has provided a number of important insights into interactions between the C and N cycles. At the Michigan gradient experiment, I examined the influence of ambient atmospheric acid and nitrogen deposition on forest biogeochemistry over the first two decades of this project (chapter four) and the long-term effects of the experimental N additions on leaf-level photosynthesis (chapter five).

Literature Cited:

Aber, J. et al. 1998. Nitrogen saturation in temperate forest ecosystems. Bioscience 48: 921-934.

Aber, J.A. et al. 2003. Is nitrogen deposition altering the nitrogen status of northeastern forests? Bioscience 53: 375-389.

Ainsworth E.A., Long S.P. 2005. What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New Phytologist 165: 351-372.

Cronan, C.S., Grigal D.F. 1995. Use of calcium/aluminum ratios as indicators of stress in forest ecosystems. Journal of Environmental Quality 24: 209-226.

Dentener F., et al. 2006. The global atmospheric environment for the next generation. Environmental Science and Technology 40: 3586-3594.

Driscoll, C.T., et al. 2001. Acidic deposition in the northeastern United States: sources and inputs, ecosystem effects, and management strategies. Bioscience 51: 180-198.

Field C.B., Behrenfeld M.J., Randerson J.T., Falkowski P. 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281: 237-240.

Galloway, J.N., et al. 2008. Transformations of the nitrogen cycle: recent trends, questions, and potential solutions. Science 320: 889-892.

Holland, E.A., Braswell, B.H., Sulzman, J., Lamarque, J.-F. 2005. Nitrogen deposition onto the United States and Western Europe: Synthesis of observations and models. Ecological Applications 15: 38-57.

Lambers, H., Chapin, F.S., T.L. Pons. 1998. Plant physiological ecology. Springer-Verlag, New York. 540 p.

LeBauer, D.S., Treseder, K.K. 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. Ecology 89: 371-379.

Likens, G.E., Driscoll, C.T. & Buso, D.C. 1996. Long-term effects of acid rain: response and recovery of a forest ecosystem. Science 272: 244-246.

Magnani F., et al. 2007. The human footprint in the carbon cycle of temperate and boreal forests. Nature 447: 848-852.

Nabuurs G.J., et al. 2007. Forestry. In: Metz B, Davidson OR, Bosch PR, Dave R, Meyer LA eds. Climate Change 2007: Mitigation. Contribution of Working Group III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge, UK and New York, NY, USA: Cambridge University Press, 543-584.

Nadelhoffer, K.J., et al. 1999. Nitrogen deposition makes a minor contribution to carbon sequestration in temperate forests. Nature 398: 145-148.

Reuss, J.O., Johnson D.W. 1986. *Acid deposition and the acidification of soils and waters*. Springer-Verlag, New York. 119 p.

Stoddard, J.L. *et al.* 1999. Regional trends in aquatic recovery from acidification in North America and Europe. Nature 401: 575-578.

Vitousek, P.M., Mooney, H.A., Lubchenco, J., Melillo, J.M. 1997. Human domination of Earth's ecosystems. Science 277: 494-499.

Watmough, S.A., et al. 2005. Sulphate, nitrogen, and base cation budgets at 21 forested catchments in Canada, the United States and Europe. Environmental Monitoring and Assessment 109: 1-36.

Waxman, H.A. 1991. Overview and critique: an overview of the Clean Air Act Amendments of 1990. Environmental Law 21: 1721-1816.

Wittig, VE, Ainsworth EA, Naidu SL, Karnosky DF, Long SP. 2009. Quantifying the impact of current and future tropospheric ozone on tree biomass, growth, physiology and biochemistry: a quantitative meta-analysis. Global Change Biology 15: 396-424.

Chapter Two: Species-Specific Responses to Atmospheric Carbon Dioxide and Tropospheric Ozone Mediate Changes in Soil Carbon*

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Abstract

We repeatedly sampled the surface mineral soil (0-20 cm depth) in three northern temperate forest communities over an 11-year experimental fumigation in order to understand the effects of elevated CO_2 and/or elevated O_3 on soil C. After 11 years, there was no significant main effect of CO_2 or O_3 on soil C. However, within the community containing only aspen (*Populus tremuloides* Michx.), elevated CO_2 caused a significant decrease in soil C content. Together with observations of increased litter inputs, this result strongly suggests accelerated decomposition under elevated CO_2. In addition, an initial reduction in the formation of new (fumigation-derived) soil C by O_3 under elevated CO_2 proved to be only a temporary effect, mirroring trends in fine root biomass. Our results contradict predictions of increased soil C under elevated CO_2 and decreased soil C under elevated O_3 and should be considered in models simulating the effects of Earth's altered atmosphere.

Introduction

One of the uncertainties in modeling the Earth's future climate is predicting the rate at which terrestrial ecosystems will sequester carbon (C) as the composition of the atmosphere changes (IPCC 2007). It is expected that higher concentrations of atmospheric carbon dioxide (CO_2) will lead to an increase in C sequestration by stimulating plant growth and net primary productivity (NPP; Ainsworth & Long 2005). This stimulation of C sequestration includes enhanced belowground growth (de Graaff *et al.* 2006) and an expected enhancement of C storage in the soil. These predictions have largely held in the forest free-air elevated CO_2 experiments (e.g. Jastrow *et al.* 2005;

Lichter *et al.* 2005; Hoosbeek *et al.* 2006) and a meta-analysis suggests that the increase in soil C outweighs the CO_2 stimulation of plant productivity (Luo *et al.* 2006). However, in many regions, the future atmosphere is also expected to contain increased concentrations of phyto-toxic ozone (O_3; Dentener *et al.* 2006), which could reduce soil C storage via reductions in plant growth and belowground C allocation (Grantz *et al.* 2006). There are few multi-year studies of O_3 effects on soil C. Some recent models suggest that the largest changes in terrestrial C storage from O_3 exposure occur through reductions in soil C (Ren *et al.* 2007; Sitch *et al.* 2007).

In addition to the direct physiological effects, exposure to increased levels of CO_2 or O_3 can alter competitive interactions and change dominance hierarchies (Poorter & Navas 2003; Kubiske *et al.* 2007; Zak *et al.* 2007). Physiological differences among the genotypes or species within a community mean that these modified communities can exhibit productivity responses to altered atmospheric composition that are either stronger or weaker than those found in the original community (Bradley & Pregitzer 2007).

Very few studies have examined long-term belowground responses of forest ecosystems to CO_2 and O_3 (Kasurinen *et al.* 2004). The Rhinelander FACE (free-air CO_2 enrichment) experiment is currently the only FACE experiment that examines the responses of forest communities exposed to elevated CO_2 ($+CO_2$), elevated O_3 ($+O_3$), and both elevated CO_2 and elevated O_3 ($+CO_2+O_3$). One early finding of the Rhinelander FACE experiment was that the pool of new soil C in the $+CO_2+O_3$ treatment was significantly lower than that under $+CO_2$ alone (Loya *et al.* 2003). A significant $CO_2 \times O_3 \times$ time interaction affecting

belowground processes (e.g., soil respiration, fine root production) has occurred at both Rhinelander FACE (Pregitzer *et al.* 2008) and in a multi-year chamber experiment (Kasurinen *et al.* 2004). However, there is no research to-date on whether such a CO_2 × O_3 × time interaction also affects soil C dynamics. Consequently, it was unknown whether the initial reduction in new soil C content by O_3 under elevated CO_2 at Rhinelander FACE would persist in the long-term. Effects of the treatments on other factors that can influence soil C cycling such as NPP (King *et al*. 2005), community composition (Kubiske *et al*. 2007), and decomposition (Chapman *et al*. 2005; Parsons *et al*. 2008) also suggested a need to reexamine soil C pools.

Our predictions for surface mineral soil C were that (a) soil C pools would be greater under elevated CO_2 than under ambient CO_2 and that (b) elevated O_3 would decrease soil C pools relative to ambient O_3, regardless of the CO_2 fumigation level. In addition, we expected that (c) elevated CO_2 ($+CO_2$) would create a larger pool of new soil C than elevated CO_2 and elevated O_3 combined ($+CO_2+O_3$). At Rhinelander FACE, the treatment gases have largely had the predicted effects on leaf litter production. However, the shifts in species composition (Kubiske *et a*l. 2007) and the transient response of fine roots and soil respiration (Pregitzer *et al*. 2008) in the two elevated O_3 treatments ($+CO_2$, $+CO_2+O_3$) may have influenced mineral soil C through time. The first objective of this report was to determine if changes in surface mineral soil C were consistent with the initially predicted treatment effects. The second objective was to understand how these dynamics have influenced the proportion of new soil C, i.e., C fixed during the 11 years of fumigation.

Materials and Methods

The FACE experiment in Rhinelander, Wisconsin, USA (45º 40.5' N, 89º 37.5' W, 490 m.a.s.l.) consists of twelve 30-m diameter rings, arranged in three randomized complete blocks (Dickson *et al.* 2000). Treatments consisted of factorial CO_2 and O_3 fumigation, composed of ambient and elevated levels of each trace gas; these treatments were randomly assigned within each block. Fumigation began in 1998 and occurred during the daylight hours of the growing season. Annual concentrations during fumigation are approximately 50-55 nL L^{-1} for elevated O_3 and 520-525 μL L^{-1} for elevated CO_2 (Kubiske *et al*. 2007). The source CO_2 used to create the elevated CO_2 treatment was fossil-fuel derived and highly depleted in ^{13}C (-43.7 ‰ ± 0.2; Pregitzer *et al*. 2006). Soils at the site are Alfic Haplorthods (Pandus series) with a sandy loam Ap horizon overlaying a sandy clay loam Bt horizon. Historical use of the site was predominantly agricultural, but the site was planted with trees in 1972. These trees were cut prior to establishment of the experiment; stumps were removed and soils were disked. More detailed description of the soils, experimental design, fumigation technique, and fumigation performance can be found in Karnosky *et al*. (2005).

Small trees (< 25 cm tall) initiated from potted stock were planted in the rings during July 1997. Half of each ring was planted at 1-m × 1-m intervals with five different aspen (*Populus tremuloides* Michx.) genotypes (Karnosky *et al*. 2005). The remaining two quarters of each FACE ring were mixed communities planted with either paper birch

(*Betula papyrifera* Marsh) or sugar maple (*Acer saccharum* Marsh) at equal densities with a single aspen genotype at 1-m × 1-m spacing.

Sampling

Sampling involved the collection of three distinct ecosystem components: surface mineral soil for the determination of both total C and new C, and fine roots and leaf litter to use as components of the ^{13}C model to determine new C. Soil samples were collected in 1997, 1999, 2001, and annually from 2003 to 2008. In 1999, 2001, 2003-2006, and 2008, five soil cores (4.8 cm diameter) were collected from each community within each FACE ring (15 cores per ring). In 2007, three cores were collected from each community in each ring. After collection, the cores were composited by community. All of the soil samples were removed from the Ap horizon.

In 1997, the soil was sampled to depth of 30 cm; in all other years sampling was to a depth of 20 cm. Sampling in 1997 pre-dated the soil disking and the planting of the three community types, so there is no differentiation by community type in the data presented for that year. As a result of these differences and the difference in sampling depth, we do not directly compare soil C content and concentrations in 1997 with samples collected after the experiment was initiated. However, we did use the 1997 data as starting points in the ^{13}C mixing model.

From 2003 to 2008, the soil samples were sieved and hand sorted to remove roots and coarse fragments (organic material, rocks). Roots were subsequently sorted by size class

and herbaceous and dead roots were removed. The soil was then placed in a 65 °C oven for at least 48 h, and later ground for analysis. The sorted roots were rinsed and placed in a 65 °C oven for at least 48 h. The root samples were then ground for analysis. Processing in 1999 and 2001 differed from sampling in the other years. In 1999, roots were extracted from the soil by elutriation of the entire sample (see King *et al*. 2001). This elutriation technique precluded analysis of the soil. Conversely, although the roots were removed from the soil samples in 2001, the roots were not analyzed for $\delta^{13}C$. Roots were not sampled in aspen-maple community in 1999.

Bulk density was measured in all years that soil analyses were conducted except 2001, 2003, and 2008. In order to calculate the C pool sizes in 2001 and 2003, we used the bulk density values for 2004. In 2008, we used bulk density values for 2007. We are confident in these substitutions as bulk density changed little through time (see results). Each bulk density measurement was corrected for the presence of coarse fragments.

From 2002-2008, four to twelve litter traps (0.15 m^2) were used to collect leaf litter from each of the three community types in each ring (Liu *et al.* 2005). Leaf litter was collected every two weeks during the period of active leaf senescence (late August through early November), and approximately monthly during the rest of the growing season. After collection, the leaf litter samples were sorted by species. These samples were then ground and used to create a biomass-weighted composite annual sample for analysis.

Stable isotope analysis for the soil, roots, and leaf litter was conducted using a Costech (Valencia, CA) Elemental Combustion System 4010 connected to a Thermo (Waltham, MA) Finnigan ConfloIII Interface and Delta^{plus} Continuous Flow-Stable Isotope Ratio Mass Spectrometer located at the Michigan Technological University Forest Ecology Analytical Laboratory. Samples were measured against a CO_2 reference gas calibrated with IAEA reference materials (International Atomic Energy Agency, Vienna, Austria). The standard deviation of measurements of a laboratory standard was 0.1‰ for $\delta^{13}C$.

Calculation of new soil carbon

To examine the input of recently fixed C into the soil under elevated CO_2, we used methods similar to those in earlier reports on soil C formation and soil respiration at Rhinelander FACE (Loya *et al*. 2003; Pregitzer *et al*. 2006). Briefly, we used the depletion in $\delta^{13}C$ caused by the use of fossil-fuel derived CO_2 for fumigation as a tracer in the treatment combinations receiving elevated CO_2 ($+CO_2$ and $+CO_2+O_3$). The proportional contribution (*f*) of soil C derived from C fixed during the fumigation was calculated with the following equation:

(1) $$f = (\delta_t - \delta_o)/(\delta_i - \delta_o)$$

where: δ_t is the current $\delta^{13}C$ value for soil C, δ_o is the $\delta^{13}C$ value for soil C at the start of the experiment, and δ_i is the $\delta^{13}C$ value for C inputs from roots and leaves. $\delta^{13}C_i$ values were calculated by averaging root and leaf $\delta^{13}C$ values from growing seasons prior to the calculation year. Like the earlier reports from Rhinelander FACE (Loya *et al*. 2003; Pregitzer *et al*. 2006), we assumed equivalent inputs from fine roots (<1 mm in diameter)

and leaf litter to calculate δ_i. Unlike those reports, we did not assume equal leaf litter inputs from both species in the mixed species communities. Over time, competition has shifted the proportional contribution of each species to the total aboveground biomass (Kubiske *et al*. 2007). Instead, we adjusted the overall leaf litter $\delta^{13}C$ value for each community according to the proportion of each species found in the leaf litter traps for that community in that year. Root biomass within a community is not differentiated by species. Roots and leaf litter were not sampled during every growing season. The only sampling of roots prior to 2003 occurred in 1999; the small size of the trees made leaf litter traps impractical prior to 2002. In years in which only one pool was sampled, we estimated the value of the missing input (leaves in 1999, roots in 2002) based on the average difference in $\delta^{13}C$ between roots and leaves in each individual ring across all years. For the 2001 calculation of new soil C, we assumed that 1999 $\delta^{13}C$ values (2002 values for the aspen-maple community) were representative of litter inputs prior to 2001. The mass of new C formed was computed by multiplying the fraction of new C by the size of the C pool. In addition, we calculated the mass of old soil C by subtracting the pool of new C from the total pool of C.

Statistical analysis

The statistical model was a randomized complete block with a split-plot design; the analyses were conducted using the SAS statistical package (Version 9.1.3, SAS Institute, Cary, NC). Block was a fixed effect because of a known gradient in aspen productivity across the site. These analyses used type III sums of squares within PROC GLM with post-hoc LSMEANS Tukey's adjusted for multiple comparisons. Statistical analyses of

fumigation effects closely followed those detailed in King *et al*. (2001) for this experiment, including specification of correct error terms for *F*-Tests using test statements within SAS. In addition, we tested trends through time with logarithmic, exponential, and linear regression models; the best fit among these models is reported. We used an $\alpha = 0.05$ to determine statistical significance. Given the difficulty of observing changes to soil bulk C pools (Hungate *et al*. 1996a), results of $0.05 \leq P < 0.1$ were considered to be of marginal significance (Jastrow *et al*. 2005).

Results

Prior to the initiation of our experiment, bulk density was greater under elevated CO_2 and elevated O_3 (Table 1). Post-hoc tests found that the $+CO_2+O_3$ treatment had a greater bulk density than the ambient ($P = 0.033$) and $+O_3$ treatments ($P = 0.093$). In subsequent years, there were no significant fumigation effects on bulk density. Bulk density differed between individual years (Table 2 & Table S1 in Supporting Information), but there were no significant trends over time in bulk density between 2004 and 2007 ($R^2 < 0.1$, $P > 0.5$). Before the start of fumigation, there were no significant differences in soil C concentrations (Table 1). Subsequently, overall concentrations increased linearly from 13.4 (SE: ± 0.8) mg g^{-1} in 2001 to 16.0 (± 0.6) mg g^{-1} in 2008 ($R^2 = 0.751$, $P = 0.012$). Likewise, soil C content did not significantly differ among treatments prior to fumigation (Table 1), and overall increased linearly from 33.5 (± 2.3) Mg ha^{-1} in 2001 to 40.1 (± 1.6) Mg ha^{-1} in 2008 ($R^2 = 0.707$, $P = 0.017$; Table S1).

After 11 years of fumigation, the effects on soil C content were negative for $+CO_2$ (-2.2 Mg ha^{-1}) and $+CO_2+O_3$ (-6.1 Mg ha^{-1}) relative to ambient, but positive for $+O_3$ (+1.4 Mg ha^{-1}); these effects varied by community. For instance, the overall CO_2 effect ($+CO_2$ and $+CO_2+O_3$ pooled vs. ambient and $+O_3$ pooled) on soil C content in 2008 was large and negative in the aspen-only community (-17.4 Mg ha^{-1}), but small and positive in the aspen-maple (+1.7 Mg ha^{-1}) and aspen-birch (+1.0 Mg ha^{-1}) communities. Likewise, the overall O_3 effect was negative in the aspen-only (-2.3 Mg ha^{-1}) and the aspen-maple (-4.0 Mg ha^{-1}) communities, but positive in the aspen-birch community (+2.6 Mg ha^{-1}). Consequently, there were no significant main effects of the fumigation treatments on soil C content ($P > 0.35$; Table 2). However, there were marginally significant interactive effects on soil C content (community × CO_2, community × CO_2 × time, $P < 0.1$, Table 2). Post-hoc tests revealed that the effect of CO_2 on soil C content was most significant in the aspen-only community ($P = 0.077$), particularly in 2008 ($P = 0.003$, Figure 1). When the aspen-only community data were analyzed separately to explore the effects in this community, both CO_2 and CO_2 × time significantly affected soil C content ($P = 0.042$ & $P = 0.046$, respectively). This CO_2 effect was not a loss of soil C per se, but a lack of accrual over the last several measurements. The significant interaction between CO_2 and time occurred because there was a linear increase in soil C content from 2001 to 2008 in the aspen-only community under ambient CO_2 ($R^2 = 0.784$, $P = 0.005$), but no clear trend of accumulation over time under elevated CO_2 ($R^2 = 0.026$, $P = 0.732$; Figure 1).

Surface mineral soil $\delta^{13}C$ values prior to fumigation were not significantly different between the $+CO_2$ and $+CO_2+O_3$ treatments (Table 1). The percent new C (Figure 2) and

the pool of new C formed in the treatments fumigated with elevated CO_2 ($+CO_2$, $+CO_2+O_3$) grew exponentially between 2001 and 2008 ($R^2 > 0.97$, $P < 0.001$ overall). Over the course of the experiment, the average annual increases in new C content for the $+CO_2$ and $+CO_2+O_3$ treatments were 1.45 (± 0.23) Mg ha^{-1} yr^{-1} and 1.27 (± 0.09) Mg ha^{-1} yr^{-1}, respectively. These values are approximately 3% of the overall C pool.

The proportion of new C added to the soil over the course of the study differed by community (Table 2, Figure 3), wherein the percent of new C in the soil was significantly lower in the aspen-maple community than in the aspen-only community ($P = 0.021$). New soil C content (Mg C ha^{-1}) did not differ among communities or treatments (Tables 2, S2). However, old soil C content did differ among communities (Table 2, Figure 3); the aspen-only community had less old C over the course of the study than either the aspen-maple ($P = 0.028$) or aspen-birch communities ($P = 0.074$). These community differences were not initially present (2001), but developed over time (community × time: $P = 0.025$). The aspen-only community lost old C at a rate of 0.83 (± 0.24) Mg ha^{-1} yr^{-1} between 2001 and 2008 (linear: $R^2 = 0.57$, $P = 0.050$), whereas the aspen-birch and aspen-maple communities lost 0.46 (± 0.20) Mg ha^{-1} yr^{-1} and 0.43 (± 0.33) Mg ha^{-1} yr^{-1} of old C and did not show significant trends in the loss of old C through time ($P > 0.4$)

When first measured (2001), fumigation with O_3 reduced the proportion of new C by almost a quarter ($+CO_2$: 12.2 ± 0.6%, $+CO_2+O_3$: 9.4 ± 1.3%) and new C content by almost a third ($+CO_2$: 4.5 ± 0.8 Mg ha^{-1}, $+CO_2+O_3$: 3.1 ± 0.5 Mg ha^{-1}), although these differences were not significant ($P = 0.798$ & $P = 0.704$, respectively; Figure 2, Table

S2). In subsequent years, the amount of new soil C in the $+CO_2$ and $+CO_2+O_3$ treatments converged. Averaged over the last four years of the study, $+CO_2+O_3$ reduced the proportion of new C by only 8% ($+CO_2$: 25.2 ± 2.4%, $+CO_2+O_3$: 22.2 ± 1.4%) and new C content over this period by only 14% ($+CO_2$: 9.7 ± 0.5 Mg ha^{-1}, $+CO_2+O_3$: 8.3 ± 0.6 Mg ha^{-1}). Overall, there was no significant effect of O_3 on the percentage of new C formed under elevated CO_2. Furthermore, there were no significant interactions among O_3, community, and time (Table 2). The amount of old soil C present was also not significantly affected by O_3 or the interactions of O_3 with community and/or time (Table 2).

Discussion

Elevated CO_2 effects on soil C content

Meta-analyses of elevated CO_2 experiments (Jastrow *et al.* 2005; Luo *et al.* 2006) and results from other forest FACE studies have shown that elevated CO_2 tends to increase the rate of soil C storage (Jastrow *et al.* 2005; Hoosbeek *et al.* 2006), although not always significantly (Lichter *et al.* 2005). We predicted that elevated CO_2 ($+CO_2$, $+CO_2+O_3$) would raise surface mineral soil C content, but there was not a significant increase in soil C content in any of the three forest communities and the overall CO_2 effect on soil C content was slightly negative. Our results are interesting in the context of our overall experiment, where elevated CO_2 has caused clear increases in litter inputs to the soil. For instance, although CO_2 had a large negative effect on soil C content in the aspen-only community (Figure 1), this community exhibited a CO_2 stimulation of 25% for leaf litter mass (Pregitzer & Talhelm *unpublished*) and 40% for fine root mass in 2005 (Pregitzer *et*

al. 2008). This implies that the decomposition of organic matter has accelerated under elevated CO_2. This response is consistent with the greater rates of soil N cycling that we have previously observed (Holmes *et al*. 2006), as well as with the higher activity of extracellular enzymes mediating cellulose and chitin degradation (Larson *et al*. 2002).

It has been proposed that elevated CO_2 will change litter chemistry and alter decomposition (Norby *et al*. 2001). Decomposition studies at Rhinelander FACE found that litter decay rates over two years decreased (Parsons *et al.* 2008) or did not change (Chapman *et al*. 2005) under elevated CO_2, but extrapolations to determine longer-term effects were inconclusive (Parsons *et al*. 2008). Similarly, a meta-analysis found no significant effect of elevated CO_2 on decomposition, but cautioned that long-term effects needed further examination (Norby *et al*. 2001). Together, these results suggest that changes in the initial stages of litter decomposition are not a likely cause of the lack of soil C accumulation in our study.

Several studies have revealed that increases in C inputs to the soil, particularly greater root growth, can enhance the decomposition of existing soil organic matter (Fontaine *et al.* 2004; Hoosbeek *et al*. 2004; Trueman & Gonzalez-Meler 2005; Carney *et al.* 2007; Dijkstra & Cheng 2007). The results of these studies may be due to a "priming" effect, the stimulation of microbial activity and decomposition in response to new inputs of organic matter (Fontaine *et al*. 2004). Recent modeling results suggest that a greater loss of old soil C under elevated CO_2 may negate gains in new C (Niklaus & Fallon, 2006). Consistent with this idea, two forest experiments have observed lower total soil C under

elevated CO_2 that resulted from greater losses of older soil C (Hoosbeek *et al.* 2004, Langley *et al.* 2009). Although the largest loss of old soil C under elevated CO_2 ($+CO_2$ & $+CO_2+O_3$) in our experiment occurred in the aspen-only community (Figure 3), we cannot conclude that the loss of old C caused the significant CO_2 effect because we do not know the fate of old C under ambient CO_2.

Effect of O_3 on soil C formation

The initial (2001) assessment of soil C formation under elevated CO_2 revealed that elevated O_3 reduced new C content in the aspen-only and aspen-birch communities (Loya *et al*. 2003). It is now clear that this was a transient effect (Figure 2). The most recent estimates of NPP found that the phyto-toxic effects of O_3 on NPP were apparent under both ambient and elevated CO_2 (King *et al.* 2005), so it was surprising that concurrent fumigation with O_3 has not reduced the proportion of recently fixed C in the soil under elevated CO_2 or had a meaningful effect on the overall surface mineral soil C pool.

The early results of reduced soil C formation matched observations on allocation to fine roots (<1 mm diameter), which were initially suppressed by fumigation with O_3 (King *et al*. 2001). However, since that time, the effect of O_3 on fine root biomass has reversed, wherein fine root biomass is now greatest in the $+CO_2+O_3$ treatment (Pregitzer *et al*. 2008). This fine root response is likely why the earlier gap in new soil C between the $+CO_2+O_3$ and $+CO_2$ treatments narrowed in later years. Over the course of the experiment, dominance by more O_3-tolerant plant genotypes and species has increased under elevated O_3 (Zak *et al*. 2007). This change toward a less O_3-responsive plant

community and an increase in the proportional allocation of C to roots under elevated O_3 (Pregitzer *et al*. 2008) are likely responsible for the increased root biomass under elevated O_3.

Soil C turnover

Our mixing model for determining new soil C assumed a 50/50 mix of roots and leaves as inputs, an assumption which may not be accurate (Hobbie *et al.* 2004). However, the average difference between leaves and roots in $\delta^{13}C$ was small (<0.5‰). Consequently, altering the mixing model to 75% roots/25% leaves or 25% roots/75% leaves only changed the proportion of new C in 2008 from 30.2% to 30.3% or 30.0%. Furthermore, these changes in the mixing model had no meaningful statistical consequences (Table S3).

The trends in accrual of new C from 2001 and 2008 suggest that the pool of new C has not yet reached equilibrium (Figure 2). Most models of soil C cycling include pools of C with both long and short turnover times, with pools that turnover quickly containing large portions of the total soil C (Trumbore 2000; Jenkinson & Coleman 2008). This explains the apparent contrast between our study, in which roughly one-third of the soil C pool is new C after 11 years, and the many studies using ^{14}C that estimate the average age of soil C to range from 200 to 1300 years (Trumbore 2000). The results from Rhinelander FACE also contrast with a pasture FACE study where the amount of new C began to plateau at approximately 25% of the soil C pool during the middle of 10 years of CO_2 fumigation (van Kessel *et al*. 2006). The pasture FACE study examined the surface 10 cm of soil,

whereas our study encompassed a greater depth (20 cm); earlier work from the pasture FACE experiment found new soil C accruing more slowly at 10-25 cm (van Kessel *et al.* 2000) and the differences in sampling depth may explain why the accrual of new C at Rhinelander FACE has been relatively slower to saturate.

The Rhinelander FACE study is similar to other elevated CO_2 studies in the size of the recent C flux into the soil C pool. Most elevated CO_2 studies fumigated with ^{13}C depleted CO_2 have found that annual inputs of new C are 2 - 5% of the overall soil C pool (Leavitt *et al*. 1994; Leavitt *et al*. 2001; Hagedorn *et al.* 2001; Xie *et al.* 2005; Lichter *et al.* 2005; van Kessel *et al*. 2006). Comparatively, the mass-based flux of new C into the total soil pool appears to be more variable, ranging from less than 0.5 Mg ha^{-1} yr^{-1} (Leavitt *et al*. 2001) to more than 6 Mg ha^{-1} yr^{-1} (Van Kessel *et al*. 2000). However, a number of studies have documented rates that are similar to our study, approximately 1.0 to 1.5 Mg C ha^{-1} yr^{-1} (Hagedorn *et al*. 2001; Xie *et al*. 2005; Lichter *et al*. 2005; van Kessel *et al*. 2006). This is similar to the range of average annual inputs to the soil in several ecosystems based on bomb ^{14}C methodology (0.8 - 1.4 Mg C ha^{-1} yr^{-1}; Trumbore 2000).

Plant community effects

In this study, soil C cycling has differed among communities, with a lower proportion of new soil C in the aspen-maple community (Figure 3), and a steady decrease in old soil C over time and a negative CO_2 effect on total C found solely in the aspen-only community (Figure 1). The presence of community differences might be expected because species-specific effects on soil properties are commonly observed (Binkley & Giardina 1998) and

because species-level controls on the cycling of soil organic matter under elevated CO_2 have been noted in the past (Hungate *et al.* 1996b; Dijkstra *et al.* 2004). Some species effects on soil properties are driven by differences in litter production (Binkley & Giardina 1998). The amounts of new and old C in the aspen-only and aspen-maple communities (Figure 3) are congruent with this idea because these communities have the highest and lowest NPP (King *et al.* 2005) and fine root biomass (Pregitzer *et al*. 2008) under elevated CO_2. At least under elevated CO_2, NPP appears to be a key factor in creating the community-level differences in soil C cycling. However, the aspen-only and aspen-birch communities differ by only a few percent in both NPP and leaf litter production under elevated CO_2 (King *et al.* 2005; Pregitzer & Talhelm *unpublished*), but these communities have shown very different responses to CO_2 in terms of soil C content (Figure 1). These results suggest that factors other than NPP have created the observed differences in soil C content under elevated CO_2.

Tissue chemistry responses to elevated CO_2 that vary among communities may also be responsible for the observed differences in community responses. For instance, elevated CO_2 has increased the concentration of condensed tannins in the aspen-birch community but decreased the concentration of condensed tannins in the aspen-only community (Liu *et al.* 2005). Condensed tannins are relatively recalcitrant compounds, which can slow microbial transformations of N (Maie *et al.* 2003) and tannin concentrations could be influencing decomposition. However, it is beyond the scope of our study to determine whether such a change is influencing soil C pools. Rather, our point is that species and genotypes respond to global change agents such as elevated CO_2 in individualistic ways

(Bradley & Pregitzer 2007). The results from our study demonstrate how these species-specific responses make it challenging to predict the extent to which elevated CO_2 and O_3 will influence the biogeochemical cycling of C. Other than time, the interaction between community type and CO_2 was the most significant factor driving change in soil C at Rhinelander FACE (Figure 1).

Implications

The Rhinelander FACE experiment has generated several unexpected results: lower soil C under elevated CO_2 in the aspen-only community and the transient effect of $+CO_2+O_3$ on soil C formation, soil respiration, and fine root biomass (Pregitzer *et al.* 2008). That each of these unexpected responses developed only after years of fumigation demonstrates the necessity of long-term experiments for the understanding of global change ecology. Across the study, community differences controlled the accrual of total C, inputs of new C, and the loss of old C over time. These community-specific responses mean that care must be made in selecting species for forestry-based C sequestration programs. Overall, our results do not match most conceptualizations of how soil organic matter dynamics will be altered by changing concentrations of CO_2 (McMurtie *et al.* 2000; Luo *et al*. 2006) and O_3 (Ren *et al.* 2007; Sitch *et al.* 2007). Particularly for O_3, the effects on soil C appear to have been overstated. The composition of the dominant plant community, competitive interactions, and positive feedbacks on microbial communities appear to play the most important role in controlling the pool of surface mineral soil C through time.

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References

Ainsworth, E.A. & Long, S.P. (2005). What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *New Phytol.,* 165, 351-372.

Binkley, D. & Giardina, C, (1998). Why do tree species affect soils? The Warp and Woof of tree-soil interactions. *Biogeochem.,* 42,89-106.

Bradley, K.L. & Pregitzer, K.S. (2007). Ecosystem assembly and terrestrial carbon balance under elevated CO_2. *Trends Ecol. Evol*., 22, 538-547.

Carney, K.M., Hungate, B.A., Drake, B.G. & Megonigal, J.P. (2007). Altered soil microbial community at elevated CO_2 leads to loss of soil carbon. *Proc. Natl. Acad. Sci.,* 104, 4990-4995.

Chapman, J.A., King, J.S., Pregitzer, K.S. & Zak, D.R. (2005). Effects of elevated concentrations of atmospheric CO_2 and tropopsheric O_3 on decomposition of fine roots. *Tree Physiol*., 25, 1501-1510.

de Graaf, M.-A., van Groenigen, K.-J., Six, J., Hungate, B. & van Kessel, C. (2006). Interactions between plant growth and soil nutrient cycling under elevated CO_2: a meta-analysis. *Glob. Change Biol.,* 12, 2077-2091.

Dentener, F., Stevenson, D., Ellingsen, K., van Noije, T., Schultz, M., Amann, M., Atherton, C., Bell, N., Bergmann, D., Bey, I., Bouwman, L., Butler, T., Cofala, J., Collins, B., Drevet, J., Doherty, R., Eickhout, B., Eskes, H., Fiore, A., Gauss, M., Hauglustaine, D., Horowitz, L., Isaksen, I. S. A., Josse, B., Lawrence, M., Krol, M., Lamarque, J. F., Montanaro, V., Müller, J. F., Peuch, V. H., Pitari, G., Pyle, J., Rast, S., Rodriguez, J., Sanderson, M., Savage, N. H., Shindell, D., Strahan, S., Szopa, S., Sudo, K., Van Dingenen, R., Wild, O. & Zeng, G. (2006). The global atmospheric environment for the next generation. *Environ. Sci. Technol*., 40, 3586-3594.

Dijkstra, F.A. & Cheng, W. (2007). Interactions between soil and tree roots accelerate long-term soil carbon decomposition. *Ecol. Lett.,* 10, 1046-1053.

Dijkstra, F.A., Hobbie, S.E., Knops, J.M.H. & Reich, P.B. (2004). Nitrogen deposition and plant species interact to influence soil carbon stabilization. *Ecol. Lett.,* 7, 1192-1198.

Fontaine, S., Bardoux, G., Abbadie, L. & Mariotti, A. (2004). Carbon input to soil may decrease soil carbon content. *Ecol. Lett.,* 7, 314-320.

Grantz, D.A., Gunn, S. & Vu, H.-B. (2006). O_3 impacts on plant development: a meta-analysis of root/shoot allocation and growth. *Plant Cell Environ*., 29, 1193-1209.

Hagedorn, F., Maurer, S., Egli, P., Blaser, P., Bucher, J.B. & Siegwolf, R. (2001). Carbon sequestration in forest soils: effects of soil type, atmospheric CO_2 enrichment, and N deposition. *Euro. J. Soil Sci.,* 52, 619-628.

Hobbie, E.A., Johnson, M.G., Rygiewicz, P.T., Tingey, D.T., & Olszyk D.M. (2004). Isotopic estimates of new carbon inputs into litter and soils in a four-year climate change experiment with Douglas-fir. *Plant Soil,* 259, 331-343.

Holmes, W.E., Zak, D.R., Pregitzer, K.S. & King, J.S. (2006). Elevated CO_2 and O_3 alter soil nitrogen transformations beneath trembling aspen, paper birch, and sugar maple. *Ecosystems*, 9, 1354-1363.

Hoosbeek, M.R., Li, Y. & Scarascia-Mugnozza, G.E. (2006). Free atmospheric CO_2 enrichment (FACE) increased labile and total carbon in the mineral soil of a short rotation poplar plantation. *Plant Soil,* 281, 247-254.

Hoosbeek, M.R., Lukac, M., van Dam, D., Godbold, D. L., Velthorst, E.J., Biondi, F.A., Peressotti, A., Cotrufo, M.F., de Angelis, P. & Scarascia-Mugnozza, G. (2004). More new carbon in the mineral soil of a poplar plantation under Free Air Carbon Enrichment (POPFACE): Cause of increased priming effect? *Glob. Biogeochem. Cycles,* 18, GB1040.

Hungate, B.A., Canadell, J., and Chapin, F.S. (1996b). Plant Species Mediate Changes in Soil Microbial N in Response to Elevated CO_2. *Ecology,* 77, 2505-2515.

Hungate, B.A., Jackson, R.B., Field, C.B. & Chapin, F.S., III. (1996a). Detecting changes in soil carbon in CO_2 enrichment experiments. *Plant Soil* 187, 135-145.

IPCC. (2007). *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* [Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor M. & Miller H.L. (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 996 pp.

Jastrow, J.D., Miller, R. M., Matamala, R., Norby, R.J., Boutton, T.W., Rice, C.W. & Owensby, C.E. (2005). Elevated atmospheric carbon dioxide increases soil carbon. *Glob. Change Biol.*, 11, 2057-2064.

Jenkinson, D.S. & Coleman, K. (2008). The turnover of organic carbon in subsoils. Part 2. Modelling carbon turnover. *Euro. J. Soil Sci*., 59, 400-413.

Karnosky, D.F., Pregitzer, K.S., Zak, D.R., Kubiske, M.E., Hendrey, G.R., Weinstein, D., Nosal, M. & Percy, K.E. (2005). Scaling ozone responses of forest trees to the ecosystem level in a changing climate. *Plant Cell Environ*., 28, 961-981.

Kasurinen, A., Kokko-Gonzales, P., Riikonen, J., Vapaavouri, E. & Holopainen, T. (2004). Soil CO2 efflux of two silver birch clones exposed to elevated CO2 and O3 levels during three growing seasons. *Global Change Biol.,* 10, 1654-1665.

King, J.S., Kubiske, M.E., Pregitzer, K.S., Hendrey, G.R., McDonald, E.P., Giardina, C.P., Quinn, V.S. & Karnosky, D.F. (2005). Tropospheric O_3 compromises net primary production in young stands of trembling aspen, paper birch and sugar maple in response to elevated atmospheric CO_2. *New Phytol*., 168, 623-636.

King, J.S., Pregitzer, K.S., Zak, D.R., Sober, J., Isebrands, J.G., Dickson, R.E., Hendrey, G.R. & Karnosky, D.F. (2001). Fine-root biomass and fluxes of soil carbon in young stands of paper birch and trembling aspen as affect by elevated atmospheric CO_2 and tropospheric O_3. *Oecologia*, 128, 237-250.

Kubiske, M.E., Quinn, V.S., Marquardt, P.E. & Karnosky, D.F. (2007). Effects of elevated atmospheic CO_2 and/or O_3 on intra-and interspecific competitive ability of aspen. *Plant Biol*., 9, 342-355.

Langley, J.A., McKinley, D.C., Wolf, A.A., Hungate, B.A., Drake, B.G., & Megonigal, J.P. (2009). Priming depletes soil carbon and releases nitrogen in a scrub-oak ecosystem exposed to elevated CO_2. *Soil Biol. Biochem*., 41. 54-60.

Larson, J.L., Zak, D.R. & Sinsabaugh, R.L. (2002). Microbial activity beneath temperate trees growing under elevated CO_2 and O_3. *Soil Sci. Soc. Am. J.*, 66, 1848-1856.

Leavitt, S.W., Pendall, E., Paul, E. A., Brooks, T., Kimball, B. A., Pinter Jr., P. J., Johnson, H. B. Matthias, A., Wall, G. W. & LaMorte, R. L. (2001). Stable-carbon isotopes and soil organic carbon in wheat under CO_2 enrichment. *New Phytol*., 150, 305-314.

Leavitt, S.W., Paul, E.A., Kimball, B.A., Hendrey, G.R., Mauney, J.R., Rauschkolb, R., Rogers, H., Lewin, K.F., Nagy, J., Pinter, Jr., P.J. & Johnson, H.B. (1994). Carbon isotope dynamics of free-air CO_2-enriched cotton and soils. *Agric. For. Meteorol*., 70, 81-101.

Lichter, J., Barron, S.H., Bevacqua, C.E., Finzi, A.C., Irving, K.F, Stemmler, E.A. & Schlesinger, W.H. (2005). Soil carbon sequestration and turnover in a pine forest after six years of atmospheric CO_2 enrichment. *Ecology*, 86, 1835-1847.

Liu, L., King, J.S. & Giardina, C.P. (2005). Effects of elevated atmospheric CO_2 and tropospheric O_3 on leaf litter production and chemistry in trembling aspen and paper birch communities. *Tree Phys.,* 25, 1511-1522.

Loya, W.M., Pregitzer, K.S., Karberg, N.J., King, J.S. & Giardina, C.P. (2003). Reduction of soil carbon formation by tropospheric ozone under increased carbon dioxide levels. *Nature,* 425, 705-707.

Luo, Y., Hui, D. & Zhang, D. (2006). Elevated CO_2 stimulates net accumulations of carbon and nitrogen in land ecosystems: a meta-analysis. *Ecology,* 87, 53-63.

Maie, N., Behrens, A., Knicker, H. & Kögel-Knabner, I. (2003). Changes in the structure and protein binding ability of condensed tannins during decomposition of fresh needles and leaves. *Soil Biol. Biochem*., 35, 577-589.

McMurtrie, R.E., Dewar, R.C., Medlyn, B.E., & Jeffreys, M.P. (2000). Effects of elevated [CO_2] on forest growth and carbon storage: a modelling analysis of the consequences of changes in litter quality/quantity and root exudation. *Plant Soil,* 224, 135-152.

Niklaus, P.A. & Falloon, P. (2006). Estimating soil carbon sequestration under elevated CO_2 by combining carbon isotope labelling with soil carbon cycle modelling. *Global Change Biol*. 12, 1909-1921.

Norby, R.J., Cotrufo, M.F., Ineson, P., O'Neill, E.G. & Canadell, J.G. (2001). Elevated CO_2, litter chemistry, and decomposition: a synthesis. *Oecologia*, 127, 153-165.

Parsons, W.F.J., Bockheim, J.G. & Lindroth, R.L. (2008). Independent, interactive, and species-specific responses of leaf litter decomposition to elevated CO_2 and O_3 in a northern hardwood forest. *Ecosystems*, 11, 505-519.

Poorter, H. & Navas, M.-L. (2003). Plant growth and competition at elevated CO_2: on winners, losers and functional groups. *New Phytol*., 157, 175–198.

Pregitzer, K., Burton, A., King, J. & Zak, D. (2008). Soil respiration, root biomass, and root turnover following long-term exposure of northern forests to elevated atmospheric CO_2 and tropospheric O_3. *New Phytol.,* 180, 153-161.

Pregitzer, K., Loya, W., Kubiske, M. & Zak, D. (2006). Soil respiration in northern forests exposed to elevated carbon dioxide and ozone. *Oecologia*, 148, 503-516.

Ren, W., Tian, H., Chen, G., Liu, M., Zhang, C., Chappelka, A.H., & Pan, S. (2007). Influence of ozone pollution and climate variability on net primary productivity and carbon storage in China's grassland ecosystems from 1961 to 2000. *Environ. Poll.,* 149, 327-335.

Sitch, S., Cox, P.M., Collins, W.J., & Huntingford, C. (2007). Indirect radiative forcing of climate change through ozone effects on the land-carbon sink. *Nature,* 448, 791-794.

Trueman, R.J. & Gonzalez-Meler, M.A. (2005). Accelerated belowground C cycling in a managed agriforest ecosystem exposed to elevated carbon dioxide concentrations. *Glob. Change Biol*., 11, 1258-1271.

Trumbore, S. (2000). Age of soil organic matter and soil respiration: radiocarbon constraints on belowground C dynamics. *Ecol. Appl*., 10, 399-411.

van Kessel, C., Boots, B., de Graaff, M.-A., Harris, D., Blum, H. & Six, J. (2006). Total soil C and N sequestration in a grassland following 10 years of free air CO_2 enrichment. *Glob. Change Biol*., 12, 2187-2199.

van Kessel, C., Nitschelm, J., Horwarth, W.R., Harris, D., Walley, F., Lüscher, A. & Hartwig, U. (2000). Carbon-13 input and turn-over in a pasture soil exposed to long-term elevated atmospheric CO_2. *Glob. Change Biol.*, 6, 123-135.

Xie, Z., Cadisch, G., Edwards, G., Baggs, E.M. & Blum, H. (2005). Carbon dynamics in a temperate grassland soil after 9 years exposure to elevated CO_2 (Swiss FACE). *Soil Biol. Biochem*., 37, 1387-1395.

Zak, D.R., Holmes, W.E., & Pregitzer, K.S. (2007). Atmospheric CO_2 and O_3 alter the flow of ^{15}N in developing forest ecosystems. *Ecology,* 88, 2630-2639.

SUPPORTING INFORMATION

The following Supporting Information is available for this article:

Table S1 *Bulk density, C concentration, and pool size by year, treatment, and community*

Table S2 *^{13}C mixing model inputs and old and new C pools*

Table 1: Pre-treatment (1997) soil C traits (means ± SE) and ANOVA *P*-values. CO_2 is not a factor in the analyses of $\delta^{13}C$ because only elevated CO_2 plots ($+CO_2$, $+CO_2+O_3$) were later fumigated with $\delta^{13}C$ depleted CO_2. Community is not a factor in this analysis because this sampling pre-dated the establishment of the model communities and did not distinguish between soils in the various parts of the rings. Effects with $P < 0.1$ are in bold; effects with $P < 0.05$ are in bold and italics.

		Bulk Density ($g\ cm^{-3}$)	**Carbon ($mg\ g^{-1}$)**	**Carbon ($Mg\ ha^{-1}$)**	**Soil $\delta^{13}C$**
Treatment	Ambient	1.2 ± 0.0	15.4 ± 1.5	37.2 ± 4.1	--
	$+CO_2$	1.3 ± 0.1	15.2 ± 0.5	39.5 ± 0.9	-26.4 ± 0.0
	$+O_3$	1.3 ± 0.0	15.0 ± 1.5	37.8 ± 4.0	--
	$+CO_2 +O_3$	1.4 ± 0.0	13.0 ± 1.1	37.2 ± 3.6	-26.1 ± 0.2
Source	CO_2	***0.018***	0.453	0.831	--
	O_3	**0.071**	0.381	0.835	0.251
	CO_2 x O_3	0.394	0.550	0.736	--

Table 2: ANOVA *P*-values for the differences in overall soil C traits, isotopically new C, and old soil C. CO_2 is not a factor in the analyses of new and old C because only elevated CO_2 plots ($+CO_2$, $+CO_2+O_3$) were fumigated with $\delta^{13}C$ depleted CO_2. Effects with $P < 0.1$ are in bold; effects with $P < 0.05$ are in bold and italics.

Source	Carbon (mg g^{-1})	Bulk Density	Carbon (Mg ha^{-1})	New Carbon (%)	New Carbon (Mg ha^{-1})	Old Carbon (Mg ha^{-1})
CO_2	0.406	0.894	0.365	--	--	--
O_3	0.365	0.502	0.413	0.548	0.148	0.645
$CO_2 \times O_3$	0.660	0.710	0.452	--	--	--
Community	0.896	0.457	0.956	***0.024***	0.262	***0.027***
$CO_2 \times$ community	0.174	**0.083**	**0.095**	--	--	--
$O_3 \times$ community	0.293	0.995	0.390	0.967	0.812	0.201
$CO_2 \times O_3 \times$ community	0.823	0.577	0.704	--	--	--
Time	***<.001***	***0.001***	***<.001***	***<.001***	***<.001***	0.247
$CO_2 \times$ time	0.348	0.616	0.343	--	--	--
$O_3 \times$ time	0.965	0.283	0.990	0.821	0.686	0.979
$CO_2 \times O_3 \times$ time	0.993	0.268	0.983	--	--	--
Community × time	0.628	0.565	0.784	0.101	0.441	***0.025***
$CO_2 \times$ community × time	0.106	0.383	**0.061**	--	--	--
$O_3 \times$ community × time	0.487	0.559	0.507	0.923	0.941	0.582
$CO_2 \times O_3 \times$ community × time	0.846	0.275	0.936	--	--	--

Figure 1: Mean surface mineral soil C (0 to 20 cm) for the three community types under ambient CO_2 (ambient and $+O_3$) and elevated CO_2 ($+CO_2$ and $+CO_2+O_3$). Error bars are ± 1 SE. Reported *P*-values are from post-hoc Tukey's tests. Asterisks denote the significant CO_2 effect in 2008 in the aspen-only community ($P = 0.003$).

Figure 2: The annual proportion of new surface mineral soil C (fixed since 1998; 0 to 20 cm) for the three community types in the plots fumigated with elevated CO_2 and either ambient ($+CO_2$) or elevated O_3 ($+CO_2+O_3$). Error bars are ± 1 SE. Ozone effects were not statistically significant.

Figure 3: The (a) proportion of new surface mineral soil C (fixed since 1998; 0 to 20 cm) and (b) the pool of old soil C in the plots fumigated with elevated CO_2 ($+CO_2$ and $+CO_2$ $+O_3$) for each the three community types averaged through time (2001-2008) and across treatments ($+CO_2$ and $+CO_2+O_3$). Different letters denote significant differences among the communities ($P < 0.05$).

Figure 1:

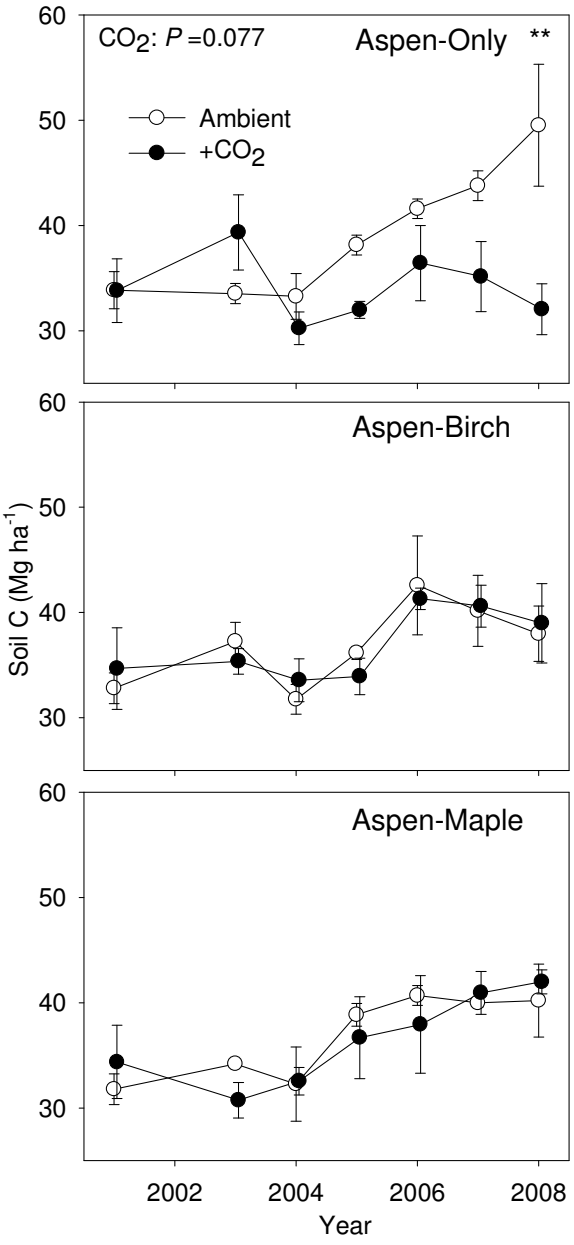

CO2: P =0.077
Aspen-Only **
Ambient
+CO2
Aspen-Birch
Aspen-Maple
Soil C (Mg ha-1)
Year
60
50
40
30
2002
2004
2006
2008

Figure 2

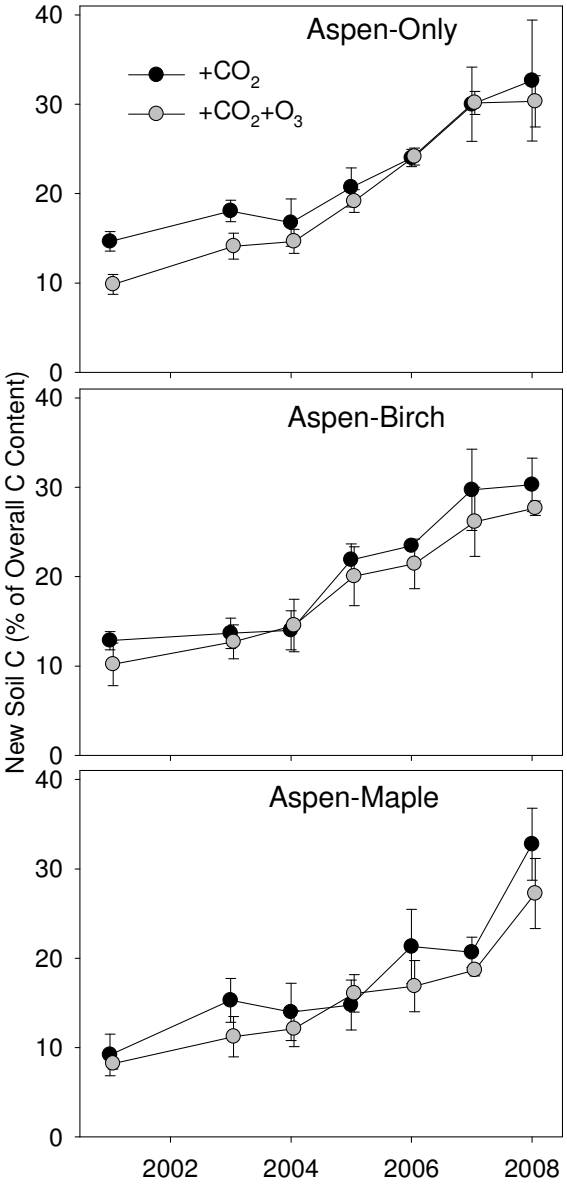
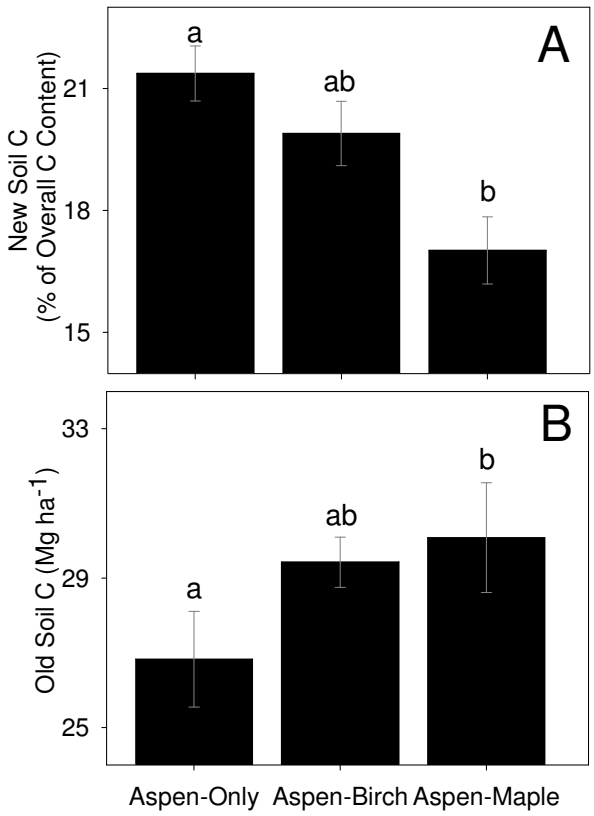

Aspen-Only
+CO2
+CO2+O3
Aspen-Birch
Aspen-Maple
New Soil C (% of Overall C Content)
0
10
20
30
40
2002
2004
2006
2008

Figure 3


A
a
ab
b
New Soil C
(% of Overall C Content)
21
18
15
B
a
ab
b
Old Soil C (Mg ha-1)
33
29
25
Aspen-Only
Aspen-Birch
Aspen-Maple

Supplementary Table 1: Mean soil bulk density, C concentration, and C pool size (± SE) in each of the three forest communities for each treatment. Means with different letters are significantly different ($P < 0.05$). In 1997, the top 30 cm of mineral soil were sampled. In 2001-2008, the top 20 cm of mineral soil were sampled.

Bulk Density ($g\ cm^{-3}$)	**Aspen-Only**			
	Ambient	**+CO_2**	**+O_3**	**+CO_2 +O_3**
1997	1.2 ± 0.0[a]	1.3 ± 0.1[ab]	1.3 ± 0.0[a]	1.4 ± 0.0[b]
2004	1.3 ± 0.0	1.3 ± 0.0	1.2 ± 0.0	1.3 ± 0.0
2005	1.2 ± 0.1	1.2 ± 0.0	1.3 ± 0.1	1.2 ± 0.0
2006	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.0	1.3 ± 0.0
2007	1.2 ± 0.0	1.2 ± 0.1	1.3 ± 0.0	1.2 ± 0.1
Carbon ($mg\ g^{-1}$)				
1997	15.4 ± 1.5	15.2 ± 0.5	15.0 ± 1.5	13.0 ± 1.1
2001	13.9 ± 0.6	13.9 ± 2.1	12.9 ± 0.4	12.6 ± 0.4
2003	14.2 ± 1.3	17.5 ± 0.3	12.4 ± 1.6	13.5 ± 2.0
2004	12.6 ± 0.8	12.8 ± 0.6	13.8 ± 0.6	11.0 ± 0.3
2005	15.7 ± 0.3	14.3 ± 2.0	15.5 ± 0.6	12.3 ± 1.6
2006	16.3 ± 1.6	15.4 ± 2.0	15.7 ± 2.1	13.2 ± 0.7
2007	18.0 ± 0.7	15.7 ± 1.3	16.3 ± 1.0	14.0 ± 1.8
2008	20.8 ± 4.1	13.8 ± 1.1	17.8 ± 1.8	13.4 ± 1.6
Carbon ($Mg\ ha^{-1}$)				
1997	55.8 ± 6.1	59.2 ± 1.4	56.6 ± 6.0	55.9 ± 5.4
2001	35.8 ± 2.4	35.6 ± 6.7	31.9 ± 1.2	32.0 ± 0.6
2003	36.3 ± 2.3	44.4 ± 1.9	30.8 ± 4.2	34.3 ± 5.3
2004	32.4 ± 2.8	32.4 ± 2.5	34.2 ± 1.5	28.0 ± 0.7
2005	37.4 ± 1.4	35.1 ± 5.3	38.9 ± 3.1	28.9 ± 4.7
2006	42.4 ± 2.7	38.4 ± 6.4	40.7 ± 4.5	34.4 ± 1.9
2007	43.7 ± 1.7	37.6 ± 4.7	43.8 ± 2.2	32.7 ± 4.7
2008	51.2 ± 11.3	32.7 ± 0.8	47.8 ± 5.2	31.4 ± 4.8

Supplementary Table 1 (cont.)

Bulk Density ($g\ cm^{-3}$)	**Aspen- Birch**			
	Ambient	**+CO_2**	**+O_3**	**+CO_2 +O_3**
1997	1.2 ± 0.0[a]	1.3 ± 0.1[ab]	1.3 ± 0.0[a]	1.4 ± 0.0[b]
2004	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	1.2 ± 0.0
2005	1.2 ± 0.0	1.3 ± 0.1	1.2 ± 0.0	1.2 ± 0.0
2006	1.3 ± 0.0	1.3 ± 0.1	1.3 ± 0.0	1.4 ± 0.0
2007	1.3 ± 0.1	1.2 ± 0.1	1.4 ± 0.0	1.3 ± 0.0
Carbon ($mg\ g^{-1}$)				
1997	15.4 ± 1.5	15.2 ± 0.5	15.0 ± 1.5	13.0 ± 1.1
2001	12.8 ± 1.0	14.7 ± 1.8	12.9 ± 0.1	12.8 ± 1.1
2003	13.2 ± 0.9	12.6 ± 0.4	16.0 ± 1.9	15.6 ± 1.1
2004	13.5 ± 0.6	13.3 ± 1.0	11.5 ± 0.9	13.4 ± 1.6
2005	15.1 ± 1.4	13.8 ± 1.4	15.5 ± 1.5	14.0 ± 1.1
2006	17.1 ± 4.6	16.5 ± 1.2	15.9 ± 2.1	14.5 ± 1.1
2007	16.1 ± 2.7	15.8 ± 1.3	14.6 ± 1.1	16.6 ± 0.5
2008	13.7 ± 0.6	16.0 ± 2.3	15.3 ± 2.0	14.9 ± 2.9
Carbon ($Mg\ ha^{-1}$)				
1997	55.8 ± 6.1	59.2 ± 1.4	56.6 ± 6.0	55.9 ± 5.4
2001	32.6 ± 2.8	38.2 ± 5.0	33.1 ± 0.3	31.2 ± 3.0
2003	33.5 ± 1.8	32.7 ± 1.0	41.1 ± 5.1	38.0 ± 2.5
2004	34.1 ± 0.8	34.6 ± 2.9	29.4 ± 2.3	32.6 ± 3.5
2005	36.5 ± 2.9	34.4 ± 2.1	35.8 ± 2.6	33.5 ± 1.6
2006	44.3 ± 10.8	42.0 ± 1.7	40.8 ± 5.5	40.6 ± 3.8
2007	41.3 ± 8.4	38.3 ± 6.3	39.1 ± 1.8	42.9 ± 2.3
2008	34.8 ± 2.6	39.5 ± 9.8	41.1 ± 6.5	38.5 ± 2.7

Supplementary Table 1 (cont.)

Bulk Density ($g\ cm^{-3}$)	Aspen-Maple			
	Ambient	**+CO_2**	**+O_3**	**+CO_2 +O_3**
1997	1.2 ± 0.0[a]	1.3 ± 0.1[ab]	1.3 ± 0.0[a]	1.4 ± 0.0[b]
2004	1.2 ± 0.1	1.3 ± 0.0	1.3 ± 0.1	1.2 ± 0.1
2005	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.3 ± 0.0
2006	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	1.4 ± 0.1
2007	1.2 ± 0.1	1.3 ± 0.0	1.2 ± 0.1	1.3 ± 0.1
Carbon ($mg\ g^{-1}$)				
1997	15.4 ± 1.5	15.2 ± 0.5	15.0 ± 1.5	13.0 ± 1.1
2001	12.6 ± 1.2	14.2 ± 2.0	13.7 ± 0.8	13.7 ± 0.8
2003	14.4 ± 0.9	12.8 ± 0.7	13.8 ± 0.7	12.1 ± 0.6
2004	13.4 ± 0.4	13.1 ± 1.0	13.3 ± 2.0	13.3 ± 0.6
2005	16.9 ± 1.4	16.3 ± 2.2	15.1 ± 2.1	13.3 ± 2.2
2006	15.5 ± 1.8	14.7 ± 2.3	15.4 ± 1.8	13.8 ± 0.5
2007	16.9 ± 1.1	17.2 ± 1.4	16.5 ± 0.7	15.0 ± 0.8
2008	16.5 ± 0.5	18.0 ± 0.3	17.1 ± 3.1	14.9 ± 1.0
Carbon ($Mg\ ha^{-1}$)				
1997	55.8 ± 6.1	59.2 ± 1.4	56.6 ± 6.0	55.9 ± 5.4
2001	29.1 ± 2.3	35.4 ± 5.4	34.5 ± 1.1	33.3 ± 1.6
2003	33.3 ± 2.5	31.9 ± 2.3	35.1 ± 3.0	29.6 ± 2.1
2004	31.4 ± 3.8	32.7 ± 2.4	33.2 ± 3.9	32.4 ± 0.4
2005	42.1 ± 4.0	39.6 ± 5.6	35.6 ± 6.0	33.8 ± 6.4
2006	40.2 ± 3.8	38.5 ± 6.2	41.2 ± 4.3	37.4 ± 3.3
2007	40.1 ± 0.7	44.5 ± 3.1	39.8 ± 1.1	37.4 ± 1.1
2008	39.5 ± 2.2	46.7 ± 1.9	40.9 ± 5.5	37.2 ± 3.4

Supplementary Table 2: (a) $\delta^{13}C$ values (± SE) for the soil, leaf litter, and fine roots used in calculating (b) the amount of recently fixed soil C and the amount of "old" C that existed prior to fumigation. Soil samples in 1997 were not differentiated by community and $\delta^{13}C$ values for this year in the two treatments were -26.1 ± 0.2 ($+CO_2$) and -26.4 ± 0.0 ($+CO_2+O_3$; not significantly different: $P = 0.251$). No root sampling occurred in the aspen-maple community in 1999.

Supplementary Table 2a

		Soil		Litter		Fine Roots	
		$+CO_2$	**$+CO_2$ $+O_3$**	**$+CO_2$**	**$+CO_2$ $+O_3$**	**$+CO_2$**	**$+CO_2$ $+O_3$**
Aspen-Only	1999			-38.9 ± 0.4*	-40.4 ± 0.8*	-39.1 ± 0.8	-39.1 ± 0.5
	2001	-28.2 ± 0.1	-27.4 ± 0.1				
	2002			-43.2 ± 0.7	-42.6 ± 0.3	-43.3 ± 0.5*	-41.4 ± 0.5*
	2003	-29.0 ± 0.2	-28.1 ± 0.1	-43.6 ± 0.4	-43.7 ± 0.8	-41.2 ± 0.3	-40.4 ± 0.2
	2004	-28.8 ± 0.4	-28.2 ± 0.1	-41.0 ± 0.8	-41.7 ± 0.6	-41.9 ± 0.1	-40.9 ± 0.4
	2005	-29.4 ± 0.3	-28.9 ± 0.1	-40.4 ± 1.6	-42.8 ± 0.3	-41.2 ± 0.4	-40.5 ± 0.3
	2006	-29.9 ± 0.2	-29.7 ± 0.2	-40.1 ± 0.4	-40.5 ± 0.2	-40.4 ± 0.5	-40.5 ± 0.6
	2007	-30.8 ± 0.6	-30.5 ± 0.2	-38.8 ± 0.5	-38.8 ± 0.3	-40.0 ± 0.1	-38.9 ± 0.3
	2008	-31.1 ± 1.0	-30.5 ± 0.4	-37.7 ± 0.6	-38.4 ± 0.2	-38.9 ± 0.6	-38.4 ± 0.2
Aspen-Birch	1999			-42.6 ± 1.3*	-43.2 ± 1.0*	-42.0 ± 1.6	-42.2 ± 0.9
	2001	-28.4 ± 0.1	-27.7 ± 0.1				
	2002			-44.4 ± 0.7	-45.2 ± 0.6	-43.9 ± 0.5*	-44.2 ± 0.5*
	2003	-28.6 ± 0.1	-28.3 ± 0.1	-43.3 ± 0.7	-44.8 ± 0.6	-42.1 ± 0.6	-42.4 ± 0.5
	2004	-28.7 ± 0.4	-28.6 ± 0.3	-42.1 ± 0.9	-44.3 ± 1.0	-42.4 ± 0.3	-43.2 ± 0.4
	2005	-30.0 ± 0.3	-29.5 ± 0.3	-43.1 ± 0.9	-44.7 ± 0.6	-41.0 ± 0.6	-42.8 ± 0.7
	2006	-30.2 ± 0.3	-29.8 ± 0.3	-41.7 ± 0.5	-43.3 ± 0.5	-41.1 ± 0.7	-42.8 ± 0.7
	2007	-31.2 ± 0.7	-30.6 ± 0.4	-40.9 ± 1.3	-40.6 ± 0.3	-41.5 ± 0.7	-41.4 ± 0.5
	2008	-31.2 ± 0.5	-30.8 ± 0.3	-41.5 ± 1.0	-41.5 ± 0.7	-41.3 ± 0.6	-40.9 ± 0.1
Aspen-Maple	1999						
	2001	-28.0 ± 0.5	-27.6 ± 0.1				
	2002			-43.6 ± 1.1	-45.1 ± 0.1	-44.0 ± 1.1*	-45.9 ± 0.3*
	2003	-29.0 ± 0.4	-28.2 ± 0.4	-43.7 ± 1.1	-44.8 ± 0.7	-41.1 ± 1.1	-43.1 ± 0.4
	2004	-28.8 ± 0.6	-28.4 ± 0.3	-39.9 ± 0.8	-42.5 ± 0.8	-41.4 ± 1.1	-42.9 ± 0.4
	2005	-28.8 ± 0.4	-29.0 ± 0.5	-42.2 ± 0.6	-43.4 ± 0.3	-40.7 ± 0.9	-42.6 ± 0.5
	2006	-29.9 ± 0.5	-29.2 ± 0.4	-40.8 ± 0.9	-42.0 ± 0.6	-40.7 ± 0.9	-42.6 ± 0.0
	2007	-29.7 ± 0.4	-29.4 ± 0.1	-39.4 ± 1.4	-38.5 ± 0.2	-40.4 ± 1.1	-40.7 ± 0.4
	2008	-31.6 ± 0.8	-30.9 ± 0.6	-40.4 ± 1.2	-40.0 ± 0.4	-41.0 ± 1.3	-40.3 ± 0.2

*Not sampled. Values calculated from applying the average difference between root and leaves to values for either roots or leaves sampled that year.

Supplementary Table 2b

		New C (Mg ha^{-1})		Old C (Mg ha^{-1})	
		+CO_2	**+CO_2 +O_3**	**+CO_2**	**+CO_2 +O_3**
Aspen-Only	1999				
	2001	5.3 ± 1.1	3.1 ± 0.3	30.3 ± 5.3	28.9 ± 0.9
	2002				
	2003	8.0 ± 0.8	5.0 ± 1.3	36.3 ± 1.2	29.3 ± 4.0
	2004	5.3 ± 0.5	4.1 ± 0.5	27.1 ± 2.9	23.9 ± 0.3
	2005	7.3 ± 1.4	5.4 ± 0.6	27.8 ± 4.2	23.5 ± 4.1
	2006	9.1 ± 1.3	8.3 ± 0.5	29.3 ± 5.2	26.1 ± 1.5
	2007	9.8 ± 1.4	9.9 ± 1.6	22.9 ± 1.4	22.8 ± 3.2
	2008	11.8 ± 1.9	9.8 ± 2.2	25.8 ± 5.7	21.6 ± 2.7
Aspen-Birch	1999				
	2001	5.3 ± 1.4	3.0 ± 0.8	33.2 ± 3.9	27.9 ± 2.1
	2002				
	2003	4.4 ± 0.5	4.7 ± 0.4	28.3 ± 1.4	33.2 ± 2.9
	2004	4.7 ± 0.4	4.6 ± 0.9	29.9 ± 3.2	27.9 ± 3.4
	2005	7.5 ± 0.6	6.7 ± 1.2	26.9 ± 1.9	26.8 ± 1.4
	2006	9.9 ± 0.7	8.5 ± 0.6	32.1 ± 1.1	32.1 ± 4.0
	2007	12.0 ± 3.7	11.0 ± 1.2	27.4 ± 6.9	31.8 ± 3.0
	2008	11.5 ± 2.0	10.6 ± 1.1	26.8 ± 4.8	27.8 ± 1.7
Aspen-Maple	1999				
	2001	3.0 ± 0.4	2.8 ± 0.3	32.4 ± 5.6	30.6 ± 1.3
	2002				
	2003	4.8 ± 0.6	3.3 ± 0.7	27.1 ± 2.6	26.3 ± 2.0
	2004	4.4 ± 0.8	3.9 ± 0.7	28.3 ± 3.1	28.5 ± 0.3
	2005	5.6 ± 0.7	5.7 ± 1.8	34.0 ± 5.9	28.1 ± 4.6
	2006	7.9 ± 0.8	6.5 ± 1.7	30.6 ± 6.1	30.9 ± 1.6
	2007	9.7 ± 0.8	7.0 ± 0.2	37.1 ± 1.8	30.4 ± 1.0
	2008	14.4 ± 1.2	10.4 ± 2.4	30.1 ± 3.8	26.8 ± 1.3

Supplementary Table 3: ANOVA *P*-values for the differences in isotopically new C and old soil C based on the results of three alternate mixing models for determining new soil C: (a) 50% roots, 50% leaf litter, (b) 25% roots, 75% leaf litter, and (c) 75% roots, 25% leaf litter. CO_2 is not a factor in the analyses of new and old C because only elevated CO_2 plots were fumigated with $\delta^{13}C$ depleted CO_2. Effects with $P < 0.1$ are in bold; effects with $P < 0.05$ are in bold and italics.

(a) 50% Roots

Source	New Carbon (%)	New Carbon (Mg ha^{-1})	Old Carbon (Mg ha^{-1})
O_3	0.548	0.148	0.645
Community	***0.024***	0.262	***0.027***
O_3 x community	0.967	0.812	0.201
Time	***<.001***	***<.001***	0.247
O_3 x time	0.821	0.686	0.979
Community x time	0.101	0.441	***0.025***
O_3 x community x time	0.923	0.941	0.582

(b) 25% Roots

Source	New Carbon (%)	New Carbon (Mg ha^{-1})	Old Carbon (Mg ha^{-1})
O_3	0.527	0.149	0.656
Community	***0.028***	0.289	***0.029***
O_3 x community	0.974	0.781	0.214
Time	***<.001***	***<.001***	0.251
O_3 x time	0.825	0.679	0.978
Community x time	0.107	0.431	***0.026***
O_3 x community x time	0.940	0.949	0.582

(c) 75% Roots

Source	New Carbon (%)	New Carbon (Mg ha^{-1})	Old Carbon (Mg ha^{-1})
O_3	0.573	0.148	0.633
Community	***0.021***	0.232	***0.024***
O_3 x community	0.914	0.831	0.187
Time	***<.001***	***<.001***	0.243
O_3 x time	0.811	0.689	0.981
Community x time	0.102	0.454	***0.023***
O_3 x community x time	0.907	0.931	0.584

Chapter Three: Long-Term Leaf Production Response to Elevated Atmospheric Carbon Dioxide and Tropospheric Ozone*

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Abstract

We measured leaf production, leaf area, and leaf litter nitrogen (N) from 2002 to 2008 in three young northern temperate forest communities exposed to elevated CO_2 and/or elevated O_3 since 1998. Leaf production in these communities exhibited a strong positive response to elevated CO_2 and a negative response to elevated O_3 early in their development, but it was unclear if these responses would be maintained as stands aged. On average, the factorial effect of elevated CO_2 ($+CO_2$ and $+CO_2+O_3$ versus ambient and $+O_3$) increased leaf production by 36% and the factorial O_3 effect decreased leaf production by 13%; effects were similar for leaf area. While there was no overall $CO_2 \times O_3$ interaction, the effects of the $+CO_2$ and $+O_3$ treatments on leaf production relative to ambient changed dramatically between 2002 and 2008: $+CO_2$ treatment stimulation dropped from +52% to +25%, while the $+O_3$ treatment effect changed from -5% to -18%. When growing competitively with maple (*Acer saccharum* Marsh.), aspen (*Populus tremuloides* Michx.) made a greater proportional contribution to community leaf production in the elevated CO_2 treatment ($+CO_2$) than under ambient conditions. Overall, neither CO_2 nor O_3 affected leaf litter N concentrations. Consequently, the leaf litter N content (g m^{-2}) was increased 30% by the elevated CO_2 treatments and decreased 16% by the elevated O_3 treatments. There is little evidence that N became progressively more limiting under elevated CO_2. Overall, these findings support the idea that stand development processes are important modifiers of CO_2 and O_3 effects on plant productivity.

Keywords: carbon dioxide, competition, leaf area, long-term, nitrogen cycling, northern temperate forests, ozone, stand age

Introduction

The industrialization of human activities has significantly increased the concentrations of atmospheric carbon dioxide (CO_2) and tropospheric ozone (O_3), with still greater increases in both gases predicted to occur over the next century (Dentener and others 2006, IPCC 2007). Both of these changes are having strong ecological impacts: CO_2 has been widely observed to stimulate plant productivity (Ainsworth and Long, 2004), while O_3 is phyto-toxic to a range of plant species (Chappelka and Samuelson 1998, Wittig and others 2009). With increasing concentrations of these gases in the coming century, the direct and interactive effects of these gases will exert greater influences in the composition, structure and functioning of natural and managed ecosystems.

Much of the effort dedicated to understanding the effects of these trace gases on the biosphere has focused on crop species and trees, the former because of global food production and nutrition concerns, while the latter because forests and woodlands dominate terrestrial productivity (Field and others 1998). Despite the large role of forests in the global carbon (C) cycle, there is still considerable uncertainly in how changes in atmospheric composition will affect the magnitude and even the direction of forest C storage (Nabuurs and others 2007). Although this uncertainty hinders efforts to develop forest-based C mitigation programs (Nabuurs and others 2007), relatively few experiments have examined the long-term response of forest ecosystems to elevated CO_2 and/or O_3. Globally, only a small number of experiments study the response of forests using the free-air CO_2 enrichment (FACE) technology that minimizes changes to other

environmental factors while allowing for in-situ exposure of large trees to increased concentrations of trace gases (Hendrey and others 1999).

Results from forest FACE experiments have emphasized the importance of leaf properties, including physiology, chemistry, anatomy, and production, in determining the response of forests to altered atmospheric composition (McCarthy and others 2006, Finzi and others 2007). These leaf traits are directly affected by CO_2, but are also affected by factors such as stand development (Gielen and others 2003), nitrogen (N) availability (McCarthy and others 2006), and species identity (Cotrufo and others 2005), all of which express important interactions with elevated CO_2. While it appears that at least some of these factors play roles in determining the forest response to elevated O_3 (Chappelka and Samuelson 1998), the number of experiments that have exposed forest systems to both elevated O_3 and elevated CO_2 is too few to draw similar conclusions (Wittig and others 2009).

Changes in atmospheric chemistry are also likely to modify competitive interactions among species and genotypes (McDonald and others 2002, Poorter and Navas 2003). The potential for wide differences in physiology among species means that shifts in the relative abundance of species caused by changes in competitive interactions can have larger effects on ecosystem properties like litter chemistry than the direct effects of elevated CO_2 or other global change factors (Bradley and Pregitzer 2007). Clearly, the forest C balance response to elevated CO_2 depends not only on direct changes in plant traits such as leaf area and N concentration, but also on how elevated CO_2 will change

community composition. However, insight into these interactions from FACE experiments is limited because most forest FACE experiments were established around single-species plantations.

Although there is considerable evidence that CO_2 and O_3 will each influence the physiology, productivity, and composition of forests, it is less clear how these influences change as stands mature (Körner 2006). Resource availability decreases as stands age, with light and soil resources such as water and N becoming more limiting as spaces both above and belowground become fully exploited (Ryan and others 1997). These other factors could limit the responsiveness of plants to elevated CO_2, or allow production in stands hampered by exposure to elevated O_3 to catch up to production in newly resource-limited forests. In particular, it has been hypothesized that as forests accumulate biomass, N will become scarce and progressively limit the stimulation of growth by elevated CO_2 (Luo and others 2004). Unfortunately, few studies have followed young forests long enough to examine whether early changes in productivity from CO_2 and O_3 will persist as forests mature (Körner 2006)

The Rhinelander FACE experiment was developed to address the knowledge gaps around both competitive interactions under elevated CO_2 and long-term exposure to elevated O_3 (Dickson and others 2000). To date, it is the only long-term forest experiment examining both the independent and interactive effects of CO_2 and O_3 and the only forest FACE experiment designed to examine how competitive interactions among species and genotypes will modify ecosystem responses to these trace gases. In addition, the

development of these stands from densely planted seedlings over the 11-year duration of the experiment provides the opportunity for unique insight into how the response of forests to CO_2 and O_3 will change as stands age.

Many of the important properties of the forest canopies at Rhinelander FACE have been examined at some point during this experiment. The most recent of these studies have shown that leaf litter production (g m^{-2}) and leaf litter N content (g N m^{-2}) increased under elevated CO_2 and decreased under elevated O_3 (Liu and others 2007). In comparison, canopy N concentrations (mg g^{-1}) were unaffected by the treatments (Zak and others 2007). However, previous examinations of leaf dynamics at Rhinelander FACE have been limited to short-term observations or to a subset of the species and communities included in the experiment. The objective of this study was to use annual leaf litter collections to comprehensively examine long-term leaf dynamics at Rhinelander FACE from 2002 until the last full growing season (2008) before the experiment was harvested. We sought to use these data to assess the response of both individual species and communities to elevated O_3 and CO_2 and identify if the relative influences of O_3 and CO_2 changed as these stands aged over this seven-year period. To do this, we focused on five variables: leaf production (L_{mass}, g m^{-2}), litter leaf area (L_{area}, m^2 m^{-2}), leaf litter N concentration (N_{conc}, mg g^{-1}), leaf litter N content (N_{mass}, g N m^{-2}), and species canopy dominance (as measured by proportion of L_{mass} in mixed communities, %). Inter-annual variability in plant production at Rhinelander FACE has previously been described (Kubiske and others 2006), so here we specifically conducted our statistical analyses to test for changes in trends through time.

We made several predictions about how leaf litter traits would respond to the fumigation treatments. First, we expected that because canopy development was thought to be nearly or fully complete in two of the three communities in 2003 (Norby and others 2005), leaf production and leaf area would maintain their responses to the fumigation treatments (+45% in 2003 L_{mass} for factorial elevated CO_2, -23% for factorial elevated O_3; King and others 2005) over the seven year sampling period. However, we did expect that the response to CO_2 and O_3 would vary by species and communities because previous results had observed these effects for net primary productivity (King and others 2005) and stem growth (Kubiske and others 2007). Lastly, we predicted that there would be no significant effect of the fumigation treatments on N concentrations, but that the overall N content of leaf litter would increase in response to changes in leaf production.

Materials and Methods

The FACE experiment in Rhinelander, Wisconsin, USA (45º 40.5' N, 89º 37.5' W, 490 m.a.s.l.) consists of twelve 30-m diameter rings, arranged in three randomized complete blocks (Dickson and others 2000). Treatments consisted of factorial CO_2 and O_3 fumigation, composed of ambient and elevated levels of each trace gas; these treatments were randomly assigned within each block. Fumigation began in 1998 and occurred during the daylight hours of the growing season (bud burst to leaf off). Average annual concentrations during fumigation are approximately 50-55 nL L^{-1} for elevated O_3 and 520-525 μL L^{-1} for elevated CO_2 (Kubiske and others 2007). Soils at the site are Alfic Haplorthods (Pandus series) with a sandy loam Ap horizon overlaying a sandy clay loam

Bt horizon. More detailed descriptions of the experimental design, fumigation technique, and fumigation performance can be found in Dickson and others (2000) and Karnosky and others (2005). Annual data on length of the fumigation season, average CO_2 and O_3 concentrations, and meteorological variables during this study are provided in the appendix.

Small trees (< 25 cm tall) initiated from potted stock were planted in the rings during July 1997. Half of each ring was planted at 1-m × 1-m intervals with five different aspen (*Populus tremuloides* Michx.) genotypes representing a range of responsiveness to elevated O_3 or elevated CO_2 (Dickson and others 2000). The remaining two quarters of each FACE ring were mixed communities planted with either paper birch (*Betula papyrifera* Marsh.) or sugar maple (*Acer saccharum* Marsh.) at equal densities with a single aspen genotype at 1-m × 1-m spacing.

In 2002 and 2003, four litter traps (0.15 m^2) were used to collect leaf litter from the aspen-maple and aspen-birch communities in each ring, with eight litter traps in the aspen-only community of each ring (Liu and others 2007). Starting in 2004, the number of traps in the aspen-only community was increased to twelve and the number of traps in the aspen-birch community was increased to six. Leaf litter was collected bi-weekly during the period of active leaf senescence (late August through early November), and approximately monthly during the rest of the growing season. After collection, the leaf litter samples were sorted by species. A subsample (10 - 15 leaves) from the litter collected in each community in each ring during September and October was analyzed

for leaf area using the LI-3100 Leaf Area Meter (Li-Cor Biosciences, Lincoln, NE, USA). Both the overall sample and the subsample were then oven dried to a constant mass and weighed. Specific leaf area (SLA, $cm^2\ g^{-1}$) of the litter was determined for the subsample (data not shown) and then applied to the mass of the overall sample to determine litter leaf area. Although September and October collections captured the large majority of leaf litter in every year, collections not sub-sampled for leaf area analysis were assigned SLA values from the nearest date when this sub-sampling had occurred. In addition, leaf area measurements were not made on samples collected from the aspen-maple community in 2003 and 2004. For these years, we used the average of SLA values in 2002 and 2005. Annual L_{area} ($m^2\ m^{-2}$) was the sum of L_{area} collected over the entire growing season.

The samples collected from throughout the growing season were ground and used to create a biomass-weighted composite annual sample for N analysis using a Costech (Valencia, CA) Elemental Combustion System 4010. Leaf N_{mass} ($g\ N\ m^{-2}$) was calculated by multiplying annual L_{mass} ($g\ m^{-2}$) by the N_{conc} ($mg\ g^{-1}$) of the annual sample. All of the measurements were completed at the species-level within each community type. Except in the case of N_{conc}, community-level data in the aspen-birch and aspen-maple communities were simply the sum of the results for the individual species within that community. Community N_{conc} for the aspen-birch and aspen-maple communities was calculated by dividing the combined N_{mass} of the two species within each community by the combined L_{mass} of those two species. For the aspen-birch and aspen-maple

communities, we calculated the canopy dominance of aspen in terms of its proportion of the total community L_{mass}.

Statistical analysis

The statistical model was a randomized complete block with a split-plot design; the analyses were conducted using the SAS statistical package (Version 9.1.3, SAS Institute, Cary, NC). These tests used type III sums of squares within a repeated measures analysis of variance (Proc Mixed) and post-hoc least squared means (LSMeans) adjusted for multiple comparisons. Block was considered a random effect (Parsons and others 2008, Riikonen and others 2008). Because we were more interested in trends through time rather than annual variability, we used year as a continuous rather than categorical variable. With year as a continuous variable, significant year effects reflect trends through time and year × treatment interactions test the hypothesis that the slope of these trends differs among treatments (Littell and others 1996). Starting with the highest level interaction terms, weak ($P \geq 0.1$) interactions between year and fumigation or species treatments were iteratively removed from the model until only main effects (CO_2, O_3, community, time, and interactions between the first of these three) and significant time interactions remained (Littell and others 1996). When there were significant date × treatment interactions, we also examined changes in the relative strength of the treatment effects (e.g., +CO_2/ambient) through time using linear regression (Proc Reg; after Norby and Iversen 2006). We conducted the overall analyses using community-level data, but we also conducted separate tests for each community individually and explore these results when there were significant community effects in the overall results. Furthermore,

data were analyzed for each community component (species) individually to provide mechanistic detail (community and species results provided in the Appendix). Data for the proportional dominance (in terms of percent of total community L_{mass}) of aspen in the aspen-birch and aspen-maple communities were arcsine transformed prior to statistical analysis to meet the assumption of normality. We used an alpha = 0.05 to determine statistical significance, but we report *P* values 0.05 < 0.10 because nearly significant differences in means for a study with necessarily low replication may have biological significance (e.g, Johnson and others 2004, Norby and Iversen 2006). Data reported throughout are means ± standard error.

Results

Experiment-wide

At the community-level, elevated CO_2 ($+CO_2$ and $+CO_2+O_3$) increased both leaf production (L_{mass}) (+36%, $P < 0.001$) and litter leaf area (L_{area}) (+31%, $P = 0.001$), while elevated O_3 ($+O_3$ and $+CO_2+O_3$) decreased L_{mass} (-13%, $P = 0.018$; Table 1) and L_{area} (-18%, $P = 0.004$). There were no significant fumigation effects or interactions for leaf litter N concentration (N_{conc}) and the effects of CO_2 and O_3 on leaf litter N mass (N_{mass}) were similar to those for L_{mass} (Table 1). L_{area} gradually increased during the study period (0.12 ± 0.03 m^2 m^{-2} yr^{-1}, $P < 0.001$), but L_{mass}, N_{mass}, and N_{conc} did not consistently change through time ($P > 0.15$).

In addition to the overall effects of CO_2 and O_3 on L_{mass}, L_{area}, and N_{mass}, each of these three community-level measures had significant $CO_2 \times O_3 \times$ year, year × community,

CO_2 × O_3 × community interactions (Table 1 and Table 2). The two significant interactions with year denote differences in trends through time. For L_{mass}, L_{area}, and N_{mass}, the relative differences between the +CO_2 treatment and both the ambient and +CO_2+O_3 treatments (+CO_2/ambient and +CO_2/+CO_2+O_3) diminished through time ($r^2 \geq 0.578$, $P \leq 0.048$), while the relative differences between the +O_3 treatment and both the ambient and +CO_2+O_3 treatments grew through time ($r^2 \geq 0.481$, $P \leq 0.084$ except for +CO_2+O_3/ambient for N_{mass}: $r^2 = 0.425$, $P = 0.113$). However, the overall effects of CO_2 and O_3 did not change significantly through time ($P > 0.2$). The CO_2 × O_3 × community and year × community interactions for L_{mass}, L_{area}, and N_{mass} are explored below in the sub-sections for each community.

Aspen was a much higher proportion of total community L_{mass} in the aspen-maple (84.0 ± 1.4% of leaf biomass) community than in the aspen-birch community (39.6 ± 1.6%). Trends through time varied by community ($P = 0.001$), with the proportion of aspen L_{mass} increasing in the aspen-birch community (1.3 ± 0.7% yr^{-1}, $P = 0.061$) and decreasing in the aspen-maple community (2.1 ± 0.7% yr^{-1}, $P = 0.003$). Overall, elevated CO_2 increased the proportion of aspen in the mixed-species communities from 57.4 ± 2.6% to 66.2 ± 3.0% ($P = 0.062$). However, CO_2 effects were only significant when comparing the +CO_2 treatment in the aspen-maple community with the ambient ($P = 0.033$) treatment in this community (CO_2 × O_3 × community: $P = 0.001$).

Aspen-Only Community

In the aspen-only community, there were gradual increases through time in L_{mass} (5.9 ± 2.8 g m^{-2} yr^{-1}; $P = 0.004$; Fig. 1), L_{area} (0.14 ± 0.04 m^2 m^{-2} yr^{-1}, $P < 0.001$; Fig. 2), and N_{mass} (0.06 ± 0.03 g N m^{-2} yr^{-1}, $P = 0.001$). These trends did not vary by fumigation treatment, except that N_{mass} did not increase through time in the $+CO_2$ treatment ($P = 0.846$; $CO_2 \times O_3 \times$ year: $P = 0.081$). Despite this interaction, the relative effects of the treatments on N_{mass} did not significantly vary through time ($P > 0.13$).

On average, L_{mass}, L_{area}, and N_{mass} were increased by elevated CO_2 (+59.0 g m^{-2}, +0.6 m^2 m^{-2}, and +0.55 g N m^{-2} respectively, all $P \leq 0.001$) and decreased by elevated O_3 (-35 g m^{-2}, -0.8 m^2 m^{-2}, and -0.52 g N m^{-2} respectively, all $P \leq 0.001$).

Aspen-Birch Community

In the aspen-birch community, only L_{area} showed an overall increase with time (0.27 ± 0.06 m^2 m^{-2}, $P < 0.001$). L_{mass}, L_{area}, and N_{mass} were all increased by elevated CO_2 (+70.4 g m^{-2}, +1.06 m^2 m^{-2}, and +0.51 g N m^{-2} respectively, all $P \leq 0.018$) and decreased by elevated O_3 (-29.3 g m^{-2}, -0.51 m^2 m^{-2}, and -0.32 g N m^{-2} respectively, all $P \leq 0.038$), however, there were strong $CO_2 \times O_3 \times$ year interactions for each ($P \leq 0.061$). The stimulatory effects of the $+CO_2$ treatment relative to both the ambient and $+CO_2+O_3$ treatments gradually declined for L_{mass}, L_{area}, and N_{mass} ($r^2 \geq 0.599$, $P \leq 0.041$). The negative effects of the $+O_3$ treatment relative to the ambient and $+CO_2+O_3$ treatments grew gradually stronger for L_{mass} and N_{mass} ($r^2 \geq 0.485$, $P \leq 0.082$), but not for L_{area} ($r^2 \leq 0.305$, $P \geq 0.199$).

These community-level results aggregate changes occurring at the level of individual species. At this level, aspen had overall increases through time in L_{mass} (3.7 ± 0.9 g m^{-2} yr^{-1}; $P = 0.002$), L_{area} (0.08 ± 0.02 m^2 m^{-2} yr^{-1}, $P < 0.001$), and N_{mass} (0.04 ± 0.01 g N m^{-2} yr^{-1}, $P = 0.001$). Year was also a strong influence on L_{mass}, L_{area}, and N_{mass} in birch ($P \leq 0.076$), but there were also significant $CO_2 \times O_3 \times$ year interactions for these traits in birch ($P \leq 0.02$). Over time, birch L_{mass} and N_{mass} decreased in the $+CO_2$ treatment (-11.3 ± 5.9 g m^{-2} yr^{-1} and 0.13 ± 0.06 g N m^{-2} yr^{-1}, $P \leq 0.051$) and birch L_{mass} decreased in the $+O_3$ treatment (-7.4 ± 3.7 g m^{-2} yr^{-1}, $P = 0.060$). Birch L_{mass} and N_{mass} increased with time in the $+CO_2+O_3$ treatment (4.5 ± 1.9 g m^{-2} yr^{-1} and 0.04 ± 0.03 g N m^{-2} yr^{-1}, $P \leq 0.097$). For L_{mass}, L_{area}, and N_{mass}, the difference between the $+CO_2$ treatment and both the ambient and $+CO_2+O_3$ treatments declined with time ($r^2 \geq 0.472$, $P \leq 0.088$) while the difference between the $+O_3$ treatment and both the ambient and $+CO_2+O_3$ treatments increased with time ($r^2 \geq 0.645$, $P \leq 0.030$, except $+O_3$/ambient for N_{mass} where $r^2 = 0.290$, $P = 0.212$).

For birch, L_{mass}, L_{area}, and N_{mass} were higher under elevated CO_2 (+36.4 g m^{-2}, +0.62 m^2 m^{-2}, and +0.29 g N m^{-2} respectively) and lower under elevated O_3 (-8.0 g m^{-2}, -0.16 m^2 m^{-2}, and +0.02 g N m^{-2} respectively). Of these, only the CO_2 effect on L_{area} approached significance ($P = 0.064$, all others: $P \geq 0.147$). For aspen, L_{mass}, L_{area}, and N_{mass} were also higher under elevated CO_2 (+34.0 g m^{-2}, +0.44 m^2 m^{-2}, and +0.26 g N m^{-2} respectively) and lower under elevated O_3 (-21.3 g m^{-2}, -0.35 m^2 m^{-2}, and -0.31 g N m^{-2} respectively). Of these, only the CO_2 effect on L_{mass} approached significance ($P = 0.090$, all others: $P \geq 0.104$).

Neither CO_2 nor O_3 affected N_{conc} at the community level (P = 0.100 and P = 0.471, respectively). But in aspen, the ambient treatment had 1.9 mg g^{-1} less N than the +CO_2 (P = 0.025) and +CO_2+O_3 (P = 0.027) treatments and 1.4 mg g^{-1} less N than the +O_3 treatment (P = 0.086, $CO_2 \times O_3$: P = 0.074). There were no significant effects in birch (P > 0.15).

In addition to changes in the proportional contribution of aspen to community L_{mass} described within the overall effects, there was also $CO_2 \times O_3 \times$ year interaction within the aspen-birch community (P = 0.013). Here, the relative effects of the +CO_2 and +O_3 treatments on the proportional contribution of aspen declined over time relative to both the ambient and +CO_2+O_3 treatments ($r^2 \geq 0.544$, $P \leq 0.04$).

Aspen-Maple Community

For the aspen-maple community, there were no significant overall trends with time or interactions between year and the fumigation treatments for L_{mass}, L_{area}, and N_{mass} ($P \geq$ 0.249). Community L_{mass}, L_{area}, and N_{mass} were significantly increased by elevated CO_2 (+53.2 g m^{-2}, +0.59 m^2 m^{-2}, and +0.52 g N m^{-2} respectively, all $P \leq 0.047$) and lower under elevated O_3 (-20.3 g m^{-2}, -0.42 m^2 m^{-2}, and -0.25 g N m^{-2} respectively). However, for O_3 only the difference in L_{area} approached significance (P = 0.097, all others: $P \geq$ 0.258).

At the species level, there were significant decreases over time in aspen L_{mass} (7.6 ± 2. 5 g m^{-2}, $P = 0.003$) and L_{area} (0.06 ± 0.03 g N m^{-2} yr^{-1}, $P = 0.015$) and there were increases in maple L_{mass} (1.5 ± 0.9 g m^{-2} yr^{-1}; $P = 0.090$), L_{area} (0.07 ± 0.03 m^2 m^{-2} yr^{-1}, $P = 0.012$), and N_{mass} (0.02 ± 0.01 g N m^{-2} yr^{-1}, $P = 0.089$).

The overall effects of CO_2 and of O_3 were unique in maple: elevated O_3 slightly increased L_{mass} (+4.2 g m^{-2}, $P = 0.363$), L_{area} (+0.06 m^2 m^{-2}, $P = 0.707$), and N_{mass} (0.03 g N m^{-2}, $P = 0.553$), while elevated CO_2 decreased L_{mass} (-15.1 g m^{-2}, $P = 0.109$), L_{area} (-0.34 m^2 m^{-2}, $P = 0.066$), and N_{mass} (-0.11 g N m^{-2}, $P = 0.079$). For aspen, L_{mass}, L_{area}, and N_{mass} were increased by elevated CO_2 (+68.2 g m^{-2}, +0.93 m^2 m^{-2}, and +0.63 g N m^{-2} respectively, all $P \leq 0.012$) and decreased under elevated O_3 (-24.5 g m^{-2}, -0.48 m^2 m^{-2}, and -0.27 g N m^{-2} respectively), but the O_3 effect of were not significant ($P \geq 0.102$) There were $CO_2 \times O_3 \times$ year interactions for maple L_{mass} ($P = 0.069$) and N_{mass} ($P = 0.068$). These interactions denote a steady decrease in the difference between the $+O_3$ treatment and the ambient treatment in L_{mass} and N_{mass} ($r^2 \geq 0.491$, $P \leq 0.079$) and a decreasing difference between the $+O_3$ treatment and the $+CO_2+O_3$ treatment in L_{area} ($r^2 = 0.459$, $P = 0.094$) and N_{mass} ($r^2 = 0.666$, $P = 0.025$).

Community (Fig. 3) and maple N_{conc} were not significantly affected by either CO_2 or O_3 ($P > 0.40$), but elevated CO_2 decreased N_{conc} in aspen by 0.9 mg g^{-1} ($P = 0.012$).

Discussion

Changes in CO_2 and O_3 Effects Through Time

Over the seven years of this study (years five through eleven of the experiment), there were clear changes in the effects of CO_2 and O_3 on canopy production (Table 2). Most dramatically, the average stimulation of L_{mass} by elevated CO_2 compared to current ambient conditions ($+CO_2$/ambient) dropped by more than half between 2002 and 2008 (Fig. 1, Table 2). This type of transient response to $+CO_2$ has been predicted and observed in several experiments (Norby and others 1999, Körner, 2006). It appears that the initial stimulation of growth by elevated CO_2 allows forests to more quickly reach the point in stand development where environmental factors (light, water, nutrients, etc.) limit canopy production and that eventually, trees growing under ambient conditions reach a similar set of limitations (Körner 2006). In coppiced *Populus* forests (Gielen and others 2003), a stimulatory effect of elevated CO_2 on leaf area was only observed until stands reached canopy closure and light and competitive interactions limited leaf area. A similar response appears to have occurred in our study, where the declining $+CO_2$ effect was most apparent in the aspen-birch community. In 2002, the $+CO_2$ treatment in this community had an L_{area} of 4.1 ± 0.2 m^2 m^{-2}, while the ambient treatment had an L_{area} of only 2.9 ± 0.2 m^2 m^{-2}. By 2008, both treatments had L_{area} values of just over 4.0 m^2 m^{-2} (4.3 ± 0.5 for $+CO_2$, 4.1 ± 0.2 for ambient). We did not observe this response in the aspen-only or aspen-maple communities, but the aspen-only community continued to add both L_{area} and L_{mass} and in the aspen-maple community L_{area} was considerably lower than that in the aspen-birch community (Fig. 2). Overall, the results from our long-term survey of leaf production are evidence of a declining $+CO_2$ stimulation relative to ambient at Rhinelander FACE, a result first observed in measurements of stem growth (Kubiske and others 2006).

Although the declining CO_2 stimulation observed here is supported both by theory (Körner 2006) and observations from similar experiments (e.g. Gielen and others 2003), much less is known about the long-term response of forests to elevated O_3. Earlier work at Rhinelander FACE found that the negative effect of the $+O_3$ treatment on the relative rate of tree growth was dissipating over time (Kubiske and others 2006). We found evidence to support this earlier work in the aspen-only and aspen-maple communities, where there is no evidence that the $+O_3$ treatment added or lost L_{area} or L_{mass} at a different rate than the ambient treatment (no significant $O_3 \times$ year or $CO_2 \times O_3 \times$ year effects). However, there was an increasing difference between the $+O_3$ treatment and the ambient treatment in L_{mass} in the aspen-birch community and to a lesser extent the overall experiment (Fig. 1, Table 2). Several other multi-year experiments have found increasing O_3 effects similar to those observed in the aspen-birch community (Oksanen 2003; Ottoson and others 2003; Volk and others 2006). In these cases, current year O_3 effects may be compounding due to reduction in the pool of plant resources available to initiate shoot growth the next growing season (e.g., C reserves, bud size; Oksanen and Saleem 1999, Oksanen 2003). At Rhinelander FACE, the size of aspen buds and the starch content of birch buds have both been found to be lower in the $+O_3$ treatment (Riikonen and others 2008). If this effect is creating the growth response in the aspen-birch community, it is unclear why aspen in the other two communities are not showing similar trends.

There were also transient effects when the $+CO_2$ and $+O_3$ treatments were compared to the interaction ($+CO_2+O_3$) treatment: the O_3 effect gradually decreased under elevated CO_2 ($+CO_2+O_3/+CO_2$) and CO_2 effect under elevated O_3 gradually increased ($+CO_2+O_3/+O_3$, Table 2). There are few other multi-year experiments that have exposed trees to $+CO_2+O_3$, but a chamber experiment exposing *Liriodendron tulipifera* (L.) seedlings to five years of $+O_3$ and $+CO_2+O_3$ found increasingly greater plant growth over time under $+CO_2+O_3$ compared to $+O_3$ (Rebbeck and Scherzer 2002). In our experiment, the ambient and $+CO_2+O_3$ treatments often showed similar responses for leaf production and litter leaf area (Figs. 1, 2, and 4). This result is unsurprising because experiments have often shown CO_2 and O_3 to have compensating effects on plant growth and leaf-level photosynthesis (Mulchi and others 1992, Volin and others 1998, Grams and others 1999, Gaucher and others 2003). It is clear from the Rhinelander FACE experiment that these compensating effects are not only temporary, but can be long-lasting.

Effects of CO_2 and O_3 on Species Dominance

Elevated CO_2 did not affect leaf production equally in all species. In this study and in a previous analysis of tree growth and survivorship (Kubiske and others 2007), the strongest effects of CO_2 on species dominance were found in the aspen-maple community. Here, elevated CO_2 produced a large increase in the proportional contribution of aspen to community L_{mass}. Of the three species included in the experiment, aspen and birch exhibit high relative growth rates and indeterminate shoot growth, while maple grows slowly and is highly determinate. Indeterminate growth

would be a likely advantage in an elevated CO_2 environment because leaves produced later in the season would multiply the stimulatory effects on photosynthesis. In general, it appears that species with greater relative growth rates show stronger responses to elevated CO_2 (Poorter and Navas 2003). Although O_3 did not significantly affect the proportional contribution of aspen to L_{mass} in the aspen-maple community, maple was the only species to show a positive leaf production response to elevated O_3 (Fig. 5). This species of maple (*Acer saccharum*) has been found to be relatively insensitive to elevated O_3 exposure (Noble and others 1992, Rebbeck and Loats 1997) and it appears likely that this trait provides maple with a competitive advantage under higher levels of O_3 that allowed it to benefit from the reduction in growth of the relatively O_3-sensitive aspen (Karnosky and others 2005).

Earlier in the development of the forests at Rhinelander FACE, it was noted that the $+CO_2$ treatment affected birch more favorably than aspen in both stem volume and mortality (Kubiske and others 2007). Birch also appeared competitively favored by $+O_3$ in this early analysis (Kubiske and others 2007). However, these effects disappeared during the 2002-2008 period of leaf litter collection. Here, the proportional contribution of aspen to community L_{mass} increased in all treatments (Fig. 4), but the declines in birch L_{mass} in the $+CO_2$ and $+O_3$ treatments meant that increases in the contribution of aspen were stronger in these treatments. Although the contrasting trends between the earlier analysis and this study may reflect differences in the way dominance was measured (contribution to L_{mass} versus stem volume), it is likely that the current trends reflect

changes in how competition between these species is altered by the treatment gases over time and as the canopies developed.

Canopy Nitrogen Cycling

As predicted, CO_2 and O_3 effects on N_{conc} were not significant overall. However, elevated CO_2 did reduce N_{conc} for aspen in the mixed-species communities. This result is unsurprising given similarly mixed findings in previous measurements of N concentrations at Rhinelander FACE for green foliage (Kopper and others 2001, Takeuchi and others 2001, Kopper and Lindroth 2003, Zak and others 2007) and leaf litter (Liu and others 2005, 2007, Parsons and others 2004, 2008). More consistent at Rhinelander FACE is the finding that the total mass of N in leaves and total N return to soil via leaf litter has been increased by elevated CO_2 and decreased by elevated O_3, observed in this and previous studies (Liu and others 2007; Zak and others 2007). A similar increase in leaf litter N mass under elevated CO_2 was observed at Duke Forest FACE (Finzi and others 2001). In contrast, other forest FACE studies have found a decrease in the N_{mass} (Cotrufo and others 2005) or no significant change in N_{mass} or the pool of green leaf N (Körner and others 2005, Norby and Iversen 2006).

A stimulation of N uptake by elevated CO_2 at Rhinelander FACE has also been observed for roots, stems, and branches (Zak and others 2007). This increase in N uptake rather than an increase in N-use efficiency appears to be supporting higher NPP under elevated CO_2 at Rhinelander FACE and two other forest FACE sites (Finzi and others 2007). In addition to the increases in N uptake, the increase in N mineralization (Holmes and others

2006), the greater activity of cellulose degrading extracellular enzymes (Larson and others 2002), and the lack of soil C accumulation (Talhelm and others 2009) are strong evidence that the additional leaf litter (Fig. 1), root litter (Pregitzer and others 2008) and total belowground carbon flux (Giardina and others 2005) under elevated CO_2 is quickly processed by the microbial community. Thus, rather than creating the negative feedbacks on plant N supply that would be consistent with an ecosystem experiencing progressive N limitation (Luo and others 2004), the rate of N cycling has increased under elevated CO_2 (Zak and others 2007). In comparison, elevated O_3 has had effects that suggest a slowed N cycle: decreased leaf production (Fig. 1) with no corresponding reduction in soil C (Talhelm and others 2009), lower plant N uptake (Zak and others 2007), and reduced N mineralization (Holmes and others 2006).

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References

Ainsworth EA, Long SP. 2005. What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New Phytologist 165: 351-372.

Bradley KL, Pregitzer KS. 2007. Ecosystem assembly and terrestrial carbon balance under elevated CO_2. Trends in Ecology and Evolution 22**:** 538-547.

Chappelka AH, Samuelson LJ. 1998. Ambient ozone effects on forest trees of the eastern United States: a review. New Phytologist 139: 91-108.

Cotrufo MF, De Angelis P, Polle A. 2005. Leaf litter production and decomposition in a poplar short-rotation coppice exposed to free air CO_2 enrichment (POPFACE). Global Change Biology 11: 971-982.

Dentener F, Stevenson D, Ellingsen K, van Noije T, Schultz M, Amann M, Atherton C, Bell N, Bergmann D, Bey I, *et al*. (2006). The global atmospheric environment for the next generation. Environmental Science and Technology 40: 3586-3594.

Dickson RE, Lewin KF, Isebrands JG, Coleman MD, Heilman WE, Riemenschneider DE, Sober J, Host GE, Zak DR, Hendrey GR, *et al.* 2000. Forest atmosphere carbon transfer storage-II (FACTS II) - The aspen free-air CO_2 and O_3 enrichment (FACE)

project in an overview. USDA Forest Service North Central Research Station. General Technical Report NC-214. 68 pp.

Field CB, Behrenfeld MJ, Randerson JT, Falkowski P. 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281: 237-240.

Finzi AC, Allen AS, DeLucia EH, Ellsworth DS, Schlesinger WH. 2001. Forest litter production, chemistry, and decomposition following two years of free-air CO_2 enrichment. Ecology 82: 470-484.

Finzi AC, Norby RJ, Calfapietra C, Gallet-Budynek A, Gielen B, Holmes WE, Hoosbeek MR, Iversen CM, Jackson RB, Kubiske ME, *et al*. Increases in nitrogen uptake rather than nitrogen-use efficiency support higher rates of temperate forest productivity under elevated CO_2. 2007. Proceedings of the National Academy of Science 104: 14014-14019.

Gaucher C, Costanzo N, Afif D, Maufette Y, Chevrier N, Dizengremel P. 2003. The impact of elevated ozone and carbon dioxide on young *Acer saccharum* seedlings. Physiologia Plantarum 117: 392-402.

Giardina CP, Coleman MD, Binkley D, Hancock JE, King JS, Lilleskov EA, Loya WM, Pregitzer KS, Ryan MG, Trettin CC. 2005. The response of belowground carbon allocation in forests to global change. In: Binkley D, Menyailo O, eds. Tree species

effects on soils: implications for global change. Dordrecht, the Netherlands: NATO Science Series, Kluwer Academic Publishers, 119-154.

Gielen B, Liberloo M, Bogaert J, Calfapietra C, DeAngelis P, Miglietta F, Scarascia-Mugnozza G, Ceulemans R. 2003. Three years of free-air CO_2 enrichment (POPFACE) only slight affect profiles of light and leaf characteristics in closed canopies of *Populus*. Global Change Biology 9: 1022-1037.

Grams TEE, Anegg S, Häberle K-H, Langebartels C, Matyssek R. 1999. Interactions of chronic exposure to elevated CO_2 and O_3 levels in the photosynthetic light and dark reactions of European beech (*Fagus sylvatica*). New Phytologist 144: 95-107.

Hendrey GR, Ellsworth DS, Lewin KF, Nagy J. 1999. A free-air enrichment system for exposing tall forest vegetation to elevated atmospheric CO_2. Global Change Biology 5: 293-300.

Holmes WE, Zak DR, Pregitzer KS, King JS. 2006. Elevated CO_2 and O_3 alter soil nitrogen transformations beneath trembling aspen, paper birch, and sugar maple. Ecosystems 9: 1354-1363.

IPCC. 2007. Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL, eds. Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on

Climate Change. Cambridge, UK and New York, NY, USA: Cambridge University Press 996 pp.

Johnson DW, Cheng W, Joslin JD, Norby RJ, Edwards NT, Todd Jr. DE. 2004. Effects of elevated CO_2 on nutrient cycling in a sweetgum plantation. Biogeochemistry 69: 379-403.

Karnosky DF, Pregitzer KS, Zak DR, Kubiske ME, Hendrey GR, Weinstein D, Nosal M, Percy KE. 2005. Scaling ozone responses of forest trees to the ecosystem level in a changing climate. Plant Cell and Environment 28: 961-981.

King JS, Kubiske ME, Pregitzer KS, Hendrey GR, McDonald EP, Giardina CP, Quinn VS, Karnosky DF. 2005. Tropospheric O_3 compromises net primary production in young stands of trembling aspen, paper birch and sugar maple in response to elevated atmospheric CO_2. New Phytologist 168: 623-636.

Kopper BJ, Lindroth RL, Nordheim EV. 2001. CO_2 and O_3 effects on paper birch (Betulaceae: *Betula papyrifera* Marsh.) phytochemistry and white-marked tussock moth (Lymantriidae: *Orgyia leucostigma* J.E. Sm.) performance. Environmental Entomology 30: 1119-1126.

Kopper BJ, Lindroth RL. 2003b. Effects of elevated carbon dioxide and ozone on the phytochemistry of aspen and performance of an herbivore. Oecologia 134: 95-103.

Körner C, Asshoff R, Bignucolo O, Hättenschwiler S, Keel SG, Peláez-Riedl S, Pepin S, Siegwolf RTW, Zotz G. 2005. Carbon flux and growth in mature deciduous forest trees exposed to elevated CO_2. Science 309: 1360-1362.

Körner C. 2006. Plant CO_2 responses: an issue of definition, time and resource supply. New Phytologist 172: 393-411.

Kubiske ME, Quinn VS, Heilman WE, McDonald EP, Marquardt PE, Teclaw RM, Friend AL, Karnosky DF. 2006. Interannual climatic version mediates elevated CO_2 and O_3 effects on forest growth. Global Change Biology 12: 1054-1068.

Kubiske ME, Quinn VS, Marquardt PE, Karnosky DF. 2007. Effects of elevated atmospheric CO_2 and/or O_3 on intra-and interspecific competitive ability of aspen. Plant Biology 9: 342-355.

Larson JL, Zak DR, Sinsabaugh RL. 2002. Microbial activity beneath temperate trees growing under elevated CO_2 and O_3. Soil Science Society of America Journal 66: 1848-1856.

Liu L, King JS, Giardina CP. 2005. Effects of elevated atmospheric CO_2 and tropospheric O_3 on leaf litter production and chemistry in trembling aspen and paper birch communities. Tree Physiology 25: 1511-1522.

Liu L, King JS, Giardina CP. 2007. Effects of elevated atmospheric CO_2 and tropospheric O_3 on nutrient dynamics: decomposition of leaf litter in trembling aspen and paper birch communities. Plant and Soil 299: 65-82.

Luo Y, Su B, Currie WS, Dukes JS, Finzi A, Hartwig U, Hungate B, McMurtrie RE, Oren R, Parton WJ. 2004. Progressive nitrogen limitation of ecosystem responses to rising atmospheric carbon dioxide. Bioscience 54: 731-740.

McCarthy HR, Oren R, Finzi, AC, Johnsen KH. 2006. Canopy leaf area constrains [CO_2]-induced enhancement of productivity and partitioning among aboveground carbon pools. *Proceedings of the National Academy of Science* **103**: 19356-19361.

McDonald EP, Kruger EL, Riemenschneider DE, Isebrands JG. 2002. Competitive status influences tree-growth responses to elevated CO_2 and O_3 in aggrading aspen stands. Functional Ecology 16: 792-801

Mulchi, CL, Slaughter L, Saleem M, Lee EH, Pausch R, Rowland R. 1992. Growth and physiological characteristics of soybean in open-top chambers in response to ozone and increased atmospheric CO_2. Agriculture, Ecosystems and Environment 38: 107-118.

Nabuurs GJ, Masera O, Andrasko K, Benitez-Ponce P, Boer R, Dutschke M, Elsiddig E, Ford-Robertson J, Frumhoff P, Karjalainen T, *et al.* 2007. Forestry. In: Metz B, Davidson

OR, Bosch PR, Dave R, Meyer LA eds. Climate Change 2007: Mitigation. Contribution of Working Group III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge, UK and New York, NY, USA: Cambridge University Press, 543-584.

Noble R, Jensen KF, Ruff BS, Loats K. 1992. Response of *Acer saccharum* seedlings to elevated carbon dioxide and ozone. Ohio Journal of Science **92**: 60-62.

Norby, RJ, Wullschleger SD, Gunderson CA, Johnson, DW, Ceulemans R. 1999. Tree responses to rising CO_2 in field experiments: implications for the future forest. Plant, Cell and Environment 22: 683-714.

Norby RJ, DeLucia EH, Gielen B, Calfapietra C, Giardina CP, King JS, Ledford J, McCarthy HR, Moore, DJP, Ceulemans R, *et al*. 2005. Forest response to elevated CO_2 is conserved across a broad range of productivity. Proceedings of the National Academy of Science 102: 18052-18056.

Norby RJ, Iversen CM. 2006. Nitrogen uptake, distribution, turnover, and efficiency of use in a CO_2-enriched sweetgum forest. Ecology 87: 5-14.

Nowak RS, Ellsworth DS, Smith, SD. 2004. Functional responses of plants to elevated atmospheric CO_2 – do photosynthetic and productivity data from FACE experiments support early predictions? New Phytologist 162: 253-280.

Oksanen E, Saleem A. 1999. Ozone exposure results in various carry-over effects and prolonged reduction in biomass in birch (*Betula pendula* Roth). Plant, Cell and Environment 22: 1401-1411.

Oksanen E. 2003. Responses of selected birch (*Betula pendula* Roth) clones to ozone change over time. Plant, Cell and Environment 26: 875-886.

Ottosson S, Wallin G, Skärby L, Karlsson PE, Medin EL, Räntfors M, Pleijel H, Selldén G. 2003. Four years of ozone exposure at high or low phosphorus reduced biomass in Norway spruce. Trees 17: 299–307.

Parsons WFJ, Lindroth RL, Bockheim JG. 2004. Decomposition of *Betula papyrifera* leaf litter under the independent and interactive effects of elevated CO_2 and O_3. Global Change Biology 10: 1666-1677.

Parsons WFJ, Bockheim JG, Lindroth RL. 2008. Independent, interactive, and species-specific responses of leaf litter decomposition to elevated CO_2 and O_3 in a northern hardwood forest. Ecosystems 11: 505-519.

Poorter H, Navas M-L. 2003. Plant growth and competition at elevated CO_2: on winners, losers and functional groups. New Phytologist 157: 175–198.

Pregitzer K, Burton A, King J, Zak D. 2008. Soil respiration, root biomass, and root turnover following long-term exposure of northern forests to elevated atmospheric CO_2 and tropospheric O_3. New Phytologist 180: 153-161.

Rebbeck J, Loats KV. 1997. Ozone effects on seedling sugar maple (*Acer saccharum*) and yellow-poplar (*Liriodendron tuliperfera*): gas exchange. Canadian Journal of Forest Research 27: 1595-1605.

Rebbeck J, Scherzer AJ. 2002. Growth responses of yellow-poplar (*Liriodendron tuliperfera* L.) exposed to 5 years of O_3 alone or combined with elevated CO_2. Plant, Cell and Environment 25: 1527-1537.

Riikonen J, Kets K, Darbah J, Oksanen E, Sober A, Vapaavuori E, Kubiske ME, Nelson N, Karnosky DF. 2008. Carbon gain and bud physiology in *Populus tremuloides* and *Betula papyrifera* under long-term exposure to elevated concentrations of CO_2 and O_3. Tree Physiology 28: 243-254.

Talhelm AF, Pregitzer KS, Zak DR. 2009. Species-specific responses to atmospheric carbon dioxide and tropospheric ozone mediate changes in soil carbon. Ecology Letters, 12: 1219-1228.

Takeuchi Y, Kubiske ME, Isebrands JG, Pregitzer KS, Hendrey G, Karnosky DF. 2001. Photosynthesis, light and nitrogen relationships in a young deciduous forest canopy under open-air CO_2 enrichment. Plant, Cell and Environment 24: 1257-1268.

Taylor G, Tallis MJ, Giardina CP, Percy KE, Miglietta F, Gupta PS, Gioli B, Calfapietra C, Gielen B, Kubiske ME, *et al.* 2008. Future atmospheric CO_2 leads to delayed autumnal senescence. Global Change Biology 14: 264-275.

Uddling J, Teclaw RM, Kubiske ME, Pregitzer KS, Ellsworth DS. 2008. Sap flux in pure aspen and mixed aspen-birch forests exposed to elevated concentrations of carbon dioxide and ozone. Tree Physiology 28: 1231-1243.

Volk M, Bungener P, Contat F, Montani M, Fuhrer J. 2006. Grassland yield declined by a quarter in 5 years of free-air ozone fumigation. Global Change Biology 12: 74-83.

Volin JC, Reich PB, Givnish TJ. 1998. Elevated carbon dioxide ameliorates the effects of ozone on photosynthesis and growth: species respond similarly regardless of photosynthetic pathway or plant functional group. New Phytologist 138: 315-325.

Wittig, VE, Ainsworth EA, Naidu SL, Karnosky DF, Long SP. 2009. Quantifying the impact of current and future tropospheric ozone on tree biomass, growth, physiology and biochemistry: a quantitative meta-analysis. Global Change Biology 15: 396-424.

Zak DR, Holmes WE, Pregitzer KS. 2007. Atmospheric CO_2 and O_3 alter the flow of ^{15}N in developing forest ecosystems. Ecology 88: 2630-2639.

Table Legends

Table 1: Abbreviations: L_{mass}: leaf production, L_{area}: litter leaf area, N_{conc}: leaf nitrogen concentration, N_{mass}: leaf N mass. Effects with $P < 0.05$ are in bold. Weak interactions ($P > 0.1$) between year and species or CO_2/O_3 removed from the analysis (see methods) denoted with "--".

Table 2: Overall CO_2 and O_3 effects defined from factorial treatments, e.g., ($+CO_2$ and $+CO_2+O_3$)/(Ambient and $+O_3$) . Effects with $P < 0.05$ are in bold.

Table 1. Experiment-wide ANOVA *P*-values from leaf litter collections.

Source	L_{mass} ($g\ m^{-2}$)	L_{area} ($m^2\ m^{-2}$)	Leaf N_{conc} ($mg\ g^{-1}$)	Leaf N_{mass} ($g\ N\ m^{-2}$)	Aspen Dominance (% of L_{mass})
CO_2	**<0.001**	**0.001**	0.132	**0.003**	0.062
O_3	**0.018**	**0.004**	0.176	**0.012**	0.375
$CO_2 \times O_3$	0.154	0.179	0.192	0.238	0.845
Community	**0.001**	**0.046**	**<0.001**	0.106	**<0.001**
$CO_2 \times$ community	0.313	**0.025**	0.487	0.967	**<0.001**
$O_3 \times$ community	0.433	0.127	0.617	0.104	0.424
$CO_2 \times O_3 \times$ community	**0.011**	**0.005**	0.191	**0.001**	**<0.001**
Year	0.691	**<0.001**	0.169	0.670	0.420
$CO_2 \times$ year	0.599	0.969	--	0.643	--
$O_3 \times$ year	0.444	0.650	--	0.255	--
$CO_2 \times O_3 \times$ year	**0.028**	**0.017**	--	**0.005**	--
Community × year	**0.001**	**0.012**	--	**0.002**	**0.001**
$CO_2 \times$ community × year	--	--	--	--	--
$O_3 \times$ community × year	--	--	--	--	--
$CO_2 \times O_3 \times$ community × year	--	--	--	--	--

Table 2. Relative treatment effects on overall leaf production through time.

Contrast	2002 Effect	2008 Effect	Annual Change	R^2	*P*
$+CO_2$/Ambient	+52.4 %	+24.9%	-4.0 ± 1.3%	0.654	**0.028**
$+O_3$/Ambient	-5.5%	-18.5%	-1.8 ± 0.8%	0.481	0.084
$+CO_2+O_3$/Ambient	+23.0%	+15.4%	-0.1 ± 1.5%	0.001	0.956
$+CO_2+O_3/+CO_2$	-19.3%	-7.6%	2.5 ± 0.7%	0.834	**0.004**
$+CO_2+O_3/+O_3$	+30.2	+41.6%	2.8 ± 0.5%	0.649	**0.029**
Overall CO_2 Effect	+41.6	+32.4	-0.9 ± 0.9%	0.149	0.392
Overall O_3 Effect	-13.8%	-12.5%	0.7 ± 0.5%	0.268	0.234

Figure 1: Annual leaf production (g m^{-2}) in the three community types for the ambient (filled circles, filled bar), +CO_2 (empty circles, empty bar), +O_3 (solid triangles, filled hatched bar), and +CO_2+O_3 (empty triangle, empty hatched bar). Bar graphs are means over the entire collection period. Error bars are ± 1 SE. Reported ANOVA *P*-values are from analyses specific to each community. Letters denote significant differences in pair-wise comparisons ($P < 0.05$) among the treatments within a community.

Figure 2: Annual litter leaf area (m^2 m^{-2}) in the three community types for each fumigation treatment. Symbols and bars as in figure 1. Reported ANOVA *P*-values are from analyses specific to each community. Letters denote significant differences in pair-wise comparisons ($P < 0.05$) among the treatments within a community.

Figure 3: Mean (a) leaf litter nitrogen concentration and (b) leaf litter nitrogen mass for each treatment in the three forest communities across all years. Symbols and bars as in figure 1. Reported *P*-values are from the overall experiment-level ANOVA. Letters denote significant differences in pair-wise comparisons ($P < 0.05$) among the treatments within a community.

Figure 4: Annual leaf production (g m^{-2}) of the individual components (species) in the mixed species communities (aspen-birch and aspen-maple) for each fumigation treatment. Symbols and bars as in figure 1. Reported ANOVA *P*-values are from analyses specific to each community. Note the difference in scale for maple.

Figure 1

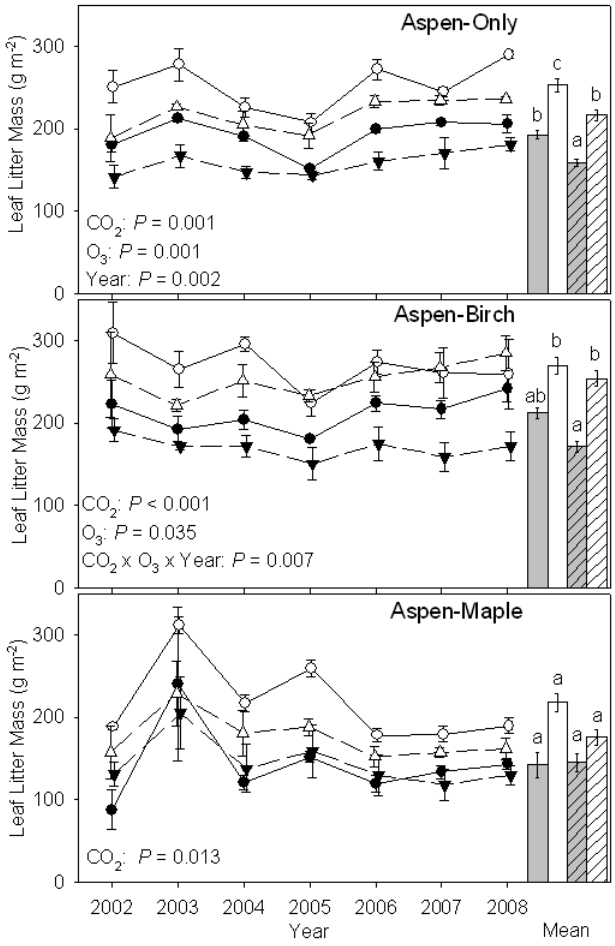

Aspen-Only
Leaf Litter Mass (g m-2)
CO2: P = 0.001
O3: P = 0.001
Year: P = 0.002
Aspen-Birch
CO2: P < 0.001
O3: P = 0.035
CO2 x O3 x Year: P = 0.007
Aspen-Maple
CO2: P = 0.013
2002
2003
2004
2005
2006
2007
2008
Year
Mean

Figure 2

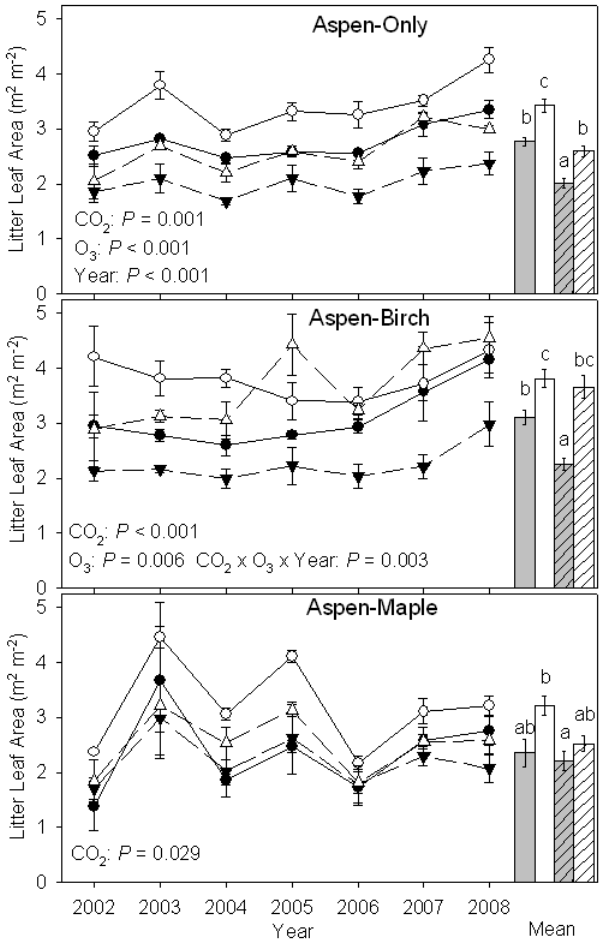

Aspen-Only
Litter Leaf Area (m² m⁻²)
CO2: P = 0.001
O3: P < 0.001
Year: P < 0.001
Aspen-Birch
CO2: P < 0.001
O3: P = 0.006 CO2 x O3 x Year: P = 0.003
Aspen-Maple
CO2: P = 0.029
2002 2003 2004 2005 2006 2007 2008
Year
Mean

Figure 3

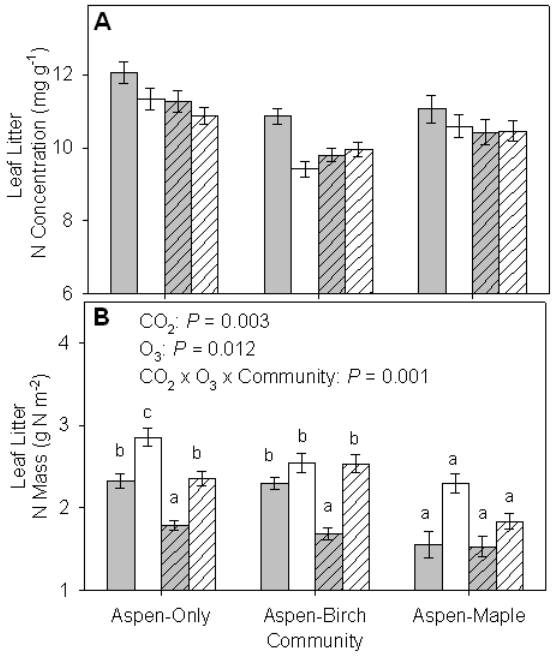
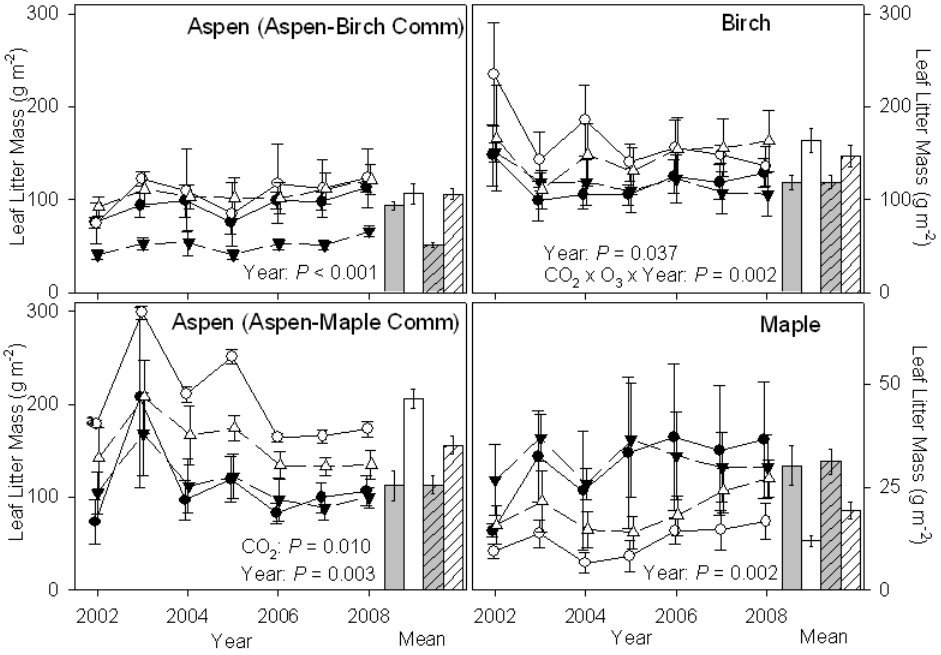

A
Leaf Litter
N Concentration (mg g-1)
12
10
8
6
B
CO2: P = 0.003
O3: P = 0.012
CO2 x O3 x Community: P = 0.001
Leaf Litter
N Mass (g N m-2)
4
3
2
1
b
c
a
b
b
b
a
b
a
a
a
a
Aspen-Only
Aspen-Birch
Aspen-Maple
Community

Figure 4


Aspen (Aspen-Birch Comm)
Year: P < 0.001
Birch
Year: P = 0.037
CO2 x O3 x Year: P = 0.002
Aspen (Aspen-Maple Comm)
CO2: P = 0.010
Year: P = 0.003
Maple
Year: P = 0.002
Leaf Litter Mass (g m-2)
Year
Mean
2002
2004
2006
2008

Appendix Table 1: Average fumigation season concentrations of CO_2 and O_3. Means calculated from: Jones, W.S. and D.F. Karnosky. 2009. FACTS II (Rhinelander, Wisconsin) FACE CO_2 Data. Carbon Dioxide Information Analysis Center, U.S. Department of Energy, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

Year	Fumigation Season Length (Days)	Ambient CO_2 Conc. (ppmv)	Elevated CO_2 Conc. (ppmv)	Ambient O_3 Conc. (ppbv)	Elevated O_3 Conc. (ppbv)
2002	137	358.9	542.3	32.6	48.6
2003	144	364.2	539.8	38.5	51.0
2004	150	371.2	514.7	34.4	41.9
2005	143	371.2	526.1	37.6	49.0
2006	141	382.0	535.8	38.5	44.1
2007	126	385.9	515.1	36.5	42.5
2008	140	382.0	539.7	36.5	42.0

Appendix Table 2: Average fumigation season meteorological information. Air temperature and soil water data are from the measurements taken in four FACE rings (see Kubiske *et al.* 2006). Precipitation data are from the US National Oceanic and Atmospheric Administration Rhinelander weather station (ID # 477113). Ratio of actual to potential evapotranspiration calculated from Thornthwaite and Mather (1955). Evapotranspiration data are from May through October, all other data are from the first to the last day of fumigation each season.

Year	Air Temp. (°C)	Volumetric Soil Water at 5 cm (θ_v)	Volumetric Soil Water at 50 cm (θ_v)	Daily Precip. (mm)	Actual/Potential Evapotranspiration
2002	17.4	0.27	0.42	4.3	0.92
2003	16.3	0.23	0.30	2.1	0.62
2004	14.9	0.17	0.33	2.8	0.77
2005	17.0	0.14	0.27	2.5	0.62
2006	17.9	0.14	0.32	2.7	0.67
2007	18.4	0.12	0.11	2.4	0.72
2008	15.1	0.10	0.08	2.4	0.68

Reference: Thornthwaite CR, Mather JR. 1955. Instructions and Tables for Computing Potential Evapotranspiration and the Water Balance. Centerton, NJ: Drexel Institute of Technology, Laboratory of Climatology, Publications in Climatology 10. 311 p.

Appendix Table 3: Community and community component (species) level ANOVA *P*-values for the differences in leaf production (L_{mass}), litter leaf area (L_{area}), leaf nitrogen concentration (N_{conc}), leaf N mass (N_{mass}), and the proportion of aspen in community leaf production of mixed species communities. Effects with $P < 0.05$ are in bold. Weak interactions ($P > 0.1$) between year and species or CO_2/O_3 removed from the analysis denoted with "--".

Aspen-Only Commuity) Source	L_{mass} ($g\ m^{-2}$)	L_{area} ($m^2\ m^{-2}$)	Leaf N_{conc} ($mg\ g^{-1}$)	Leaf N_{mass} ($g\ N\ m^{-2}$)
CO_2	**<0.001**	**0.001**	0.125	**0.001**
O_3	**0.001**	**<0.001**	0.095	**<0.001**
$CO_2 \times O_3$	0.856	0.735	0.625	0.735
Year	**0.002**	**<0.001**	0.192	**<0.001**
$CO_2 \times$ year	--	--	--	--
$O_3 \times$ year	--	--	--	--
$CO_2 \times O_3 \times$ year	--	--	--	--

Appendix Table 3 (continued)

Aspen-Birch Commuity Source	L_{mass} (g m^{-2})	L_{area} (m^2 m^{-2})	Leaf N_{conc} (mg g^{-1})	Leaf N_{mass} (g N m^{-2})	Aspen Dominance (% of L_{mass})
CO_2	**<0.001**	**<0.001**	0.100	**0.018**	0.564
O_3	**0.035**	**0.006**	0.471	**0.038**	0.686
$CO_2 \times O_3$	0.299	0.403	0.052	0.799	0.142
Year	0.850	**<0.001**	0.449	0.555	**0.001**
$CO_2 \times$ year	0.796	0.801	--	0.756	0.430
$O_3 \times$ year	0.461	0.163	--	0.365	0.628
$CO_2 \times O_3 \times$ year	**0.007**	**0.003**	--	0.061	**0.013**

Aspen (Aspen-Birch Commuity) Source	L_{mass} (g m^{-2})	L_{area} (m^2 m^{-2})	Leaf N_{conc} (mg g^{-1})	Leaf N_{mass} (g N m^{-2})
CO_2	0.090	0.104	**0.012**	0.231
O_3	0.261	0.182	0.089	0.163
$CO_2 \times O_3$	0.266	0.256	0.074	0.152
Year	**<0.001**	**<0.001**	0.807	**0.001**
$CO_2 \times$ year	--	--	--	--
$O_3 \times$ year	--	--	--	--
$CO_2 \times O_3 \times$ year	--	--	--	--

Birch (Aspen-Birch Commuity) Source	L_{mass} (g m^{-2})	L_{area} (m^2 m^{-2})	Leaf N_{conc} (mg g^{-1})	Leaf N_{mass} (g N m^{-2})
CO_2	0.147	0.064	0.426	0.241
O_3	0.434	0.190	0.460	0.509
$CO_2 \times O_3$	0.166	0.148	0.179	0.308
Year	**0.037**	**0.009**	0.754	0.076
$CO_2 \times$ year	0.657	0.635	--	0.897
$O_3 \times$ year	0.171	**0.030**	--	0.148
$CO_2 \times O_3 \times$ year	**0.002**	**<0.001**	--	**0.020**

Appendix Table 3 (continued)

Aspen-Maple Commuity Source	L_{mass} (g m^{-2})	L_{area} (m^2 m^{-2})	Leaf N_{conc} (mg g^{-1})	Leaf N_{mass} (g N m^{-2})	Aspen Dominance (% of L_{mass})
CO_2	**0.013**	**0.029**	0.632	**0.047**	**0.025**
O_3	0.258	0.097	0.424	0.301	0.399
$CO_2 \times O_3$	0.221	0.251	0.587	0.363	0.674
Year	0.352	0.239	0.640	0.280	**<0.001**
$CO_2 \times$ year	--	--	--	--	--
$O_3 \times$ year	--	--	--	--	--
$CO_2 \times O_3 \times$ year	--	--	--	--	--

Aspen (Aspen-Maple Commuity) Source	L_{mass} (g m^{-2})	L_{area} (m^2 m^{-2})	Leaf N_{conc} (mg g^{-1})	Leaf N_{mass} (g N m^{-2})
CO_2	**0.010**	**0.007**	**0.012**	**0.033**
O_3	0.258	0.102	0.246	0.293
$CO_2 \times O_3$	0.242	0.228	0.120	0.368
Year	**0.003**	0.129	0.247	**0.015**
$CO_2 \times$ year	--	--	--	--
$O_3 \times$ year	--	--	--	--
$CO_2 \times O_3 \times$ year	--	--	--	--

Maple (Aspen-Maple Commuity) Source	L_{mass} (g m^{-2})	L_{area} (m^2 m^{-2})	Leaf N_{conc} (mg g^{-1})	Leaf N_{mass} (g N m^{-2})
CO_2	0.109	0.066	0.654	0.079
O_3	0.363	0.707	0.510	0.553
$CO_2 \times O_3$	0.843	0.699	0.321	0.526
Year	**0.002**	**<0.001**	0.062	0.178
$CO_2 \times$ year	0.844	--	--	0.506
$O_3 \times$ year	0.167	--	--	0.668
$CO_2 \times O_3 \times$ year	0.069	--	--	0.068

Chapter Four: The Changing Influence of Atmospheric Deposition on Biogeochemistry in Northern Forests*

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Abstract

During the 20th century, growing industrialization created air pollution that increased the deposition of both acid and nitrogen to terrestrial ecosystems. In more recent decades, it became clear that these pollutants altered the biogeochemistry of temperate forests across large parts of the northern hemisphere[1, 2]. In the United States, these impacts were among the primary motives for national air pollution legislation enacted in 1990 (ref. 3) that targeted and successfully reduced acid deposition, but did not appreciably lower nitrogen deposition[1, 4]. Here, we demonstrate that since this legislation was enacted, there have been remarkable changes in the impacts of these two pollutants on forests in the north-central United States. In these forests, decreases in leaf sulfur, calcium, and aluminum concentrations signal a waning influence of acid deposition, while persistent nitrogen deposition has caused widespread increases in both the availability of inorganic nitrogen and soil nitrate leaching. This change in N availability could simultaneously increase forest growth[2, 5, 6] and reduce species diversity[6, 7], while increased nitrate leaching may reduce water quality[6]. This study demonstrates the tight linkage between atmospheric pollution and forest biogeochemistry and is clear evidence that environmental policy decisions can directly affect forest ecosystem function at broad scales.

Main text

In terrestrial ecosystems, external inputs from mineral weathering, atmospheric deposition, and biological fixation are important supplies of many elements[8]. In large areas of the industrialized world, the influence of atmospheric inputs greatly increased during the 20th century as air pollution added numerous compounds to terrestrial

ecosystems[1, 2, 9]. In particular, several decades of research made it clear that acid (H^+) deposition (acid rain) and nitrogen (N) deposition have altered the biogeochemistry of northern temperate forests[1, 2]. In the United States (U.S.), these impacts on forest biogeochemistry formed part of the impetus for the 1990 Clean Air Act Amendments (CAAA) that targeted reductions in acid deposition pre-cursors and set modest goals to cut the emissions of some N compounds[1, 3, 4]. However, although there have been subsequent changes in both emissions and atmospheric deposition, the size and complexity of most biogeochemical cycles makes it difficult to understand how forest biogeochemistry has changed[10] since this legislation was enacted. Here, we quantify two decades of change in four forests located along a 500 km air pollution gradient in the north-central U.S. by examining the cycling of four elements: sulfur (S), calcium (Ca), aluminum (Al), and N.

Much of the acid deposition in the U.S. results from sulfur dioxide (SO_2) emissions, which react to form sulfuric acid (H_2SO_4) in the atmosphere[11]. In most forests, deposition of H_2SO_4 increases soil concentrations of hydrogen ions (H^+) and sulfate (SO_4^{2-}), speeding the movement of Ca and Al from soil particles into the soil solution[10]. Once in the soil solution, Ca and Al can be removed from the soil through leaching or taken up by plants. The loss of Ca from soils through this process has been a major concern in regions where availability of this plant nutrient is low[12], whereas increases in soil solution Al are alarming because Al can be toxic to plants[13].

Following passage of the 1990 CAAA, SO_2 emissions dropped[14]. Consequently, both acid and SO_4^{2-} deposition have declined at monitoring stations in the study region (Figs 1 & 2a). These changes in atmospheric deposition have broadly improved water chemistry[9], but there is yet little evidence of similar regional recoveries in plant and soil chemistry[15, 16]. To assess changes in terrestrial cycling of S, Ca, and Al, we examined the chemistry of freshly fallen leaves collected annually since 1987. Foliar concentrations of Ca and Al correlate with those in soil solution[13] and concentrations of Ca and Al are maintained in fallen leaves at our sites because these elements are not actively removed during leaf senescence[17]. In this region, the S concentration of fallen leaves correlates with SO_4^{2-} deposition[17]. Since 1987, there have been significant declines in foliar Ca and Al concentrations study-wide (Fig. 2b) and at each of the study sites (Supplementary Table 1). Similarly, leaf S concentrations have declined significantly study-wide (Fig. 2a) and at three of the four sites (Supplementary Table 1), with steeper reductions at the more polluted southern sites. These three changes are clear evidence that the influence of acid deposition on forest biogeochemistry has declined following reductions in SO_2 emissions.

Unlike acid deposition, reducing total N deposition was not a focus of the 1990 CAAA[3] and N deposition remained relatively constant in this region during the study period (Fig. 1). Estimates of N deposition at the study sites range from 6.8 kg N ha^{-1} yr^{-1} in the north to 11.8 kg N ha^{-1} yr^{-1} in the south[18], spanning much of the range in N deposition observed in the eastern U.S., and lower than N deposition in much of Europe[19]. Forest growth is typically limited by the availability of N (ref. 20), but N availability only increases if the rate of ecosystem N sequestration is outpaced by either external inputs or the

mineralization of organic N in the soil. Consequently, N deposition may increase forest growth[5]. However, increases in N supply can also have negative consequences for water quality, plant species composition, and greenhouse gas emissions[6, 7, 21]. Despite the importance of N, long-term trends in terrestrial N availability are not well understood even in intensively-studied areas such as the eastern U.S.[4, 10]. This stems from the influence of other factors on N cycling (atmospheric CO_2, climate change, land use)[10] and from the fact that most long-term measurements have focused on stream water N concentrations that are influenced by both terrestrial N cycling and in-stream N processing[22].

The forests at the study sites are mature (~100 yrs old), with leaf area and basal area measurements that are near maximum for this forest type[18]. Consequently, the amount of N needed to meet growth demands is small: root[23] and leaf biomass (Supplementary Fig. 1) have not changed consistently during the study and average N demand from wood production ranges from 3.2 ± 0.1 (SE) kg N ha^{-1} yr^{-1} in the north to 6.6 ± 0.3 kg N ha^{-1} yr^{-1} in the south (Supplementary Table 1). Because N demand is relatively small, we estimate that between 1987 and 2008, atmospheric inputs of N exceeded sequestration in wood by a cumulative 70 to 120 kg N ha^{-1}.

During this same period, a trend of increasing inorganic N availability has become obvious in measurements of soil solution N. In the soil, nitrate (NO_3^-) is easily leached when NO_3^- production outpaces biotic uptake[24]. The forests at these study sites are dominated by sugar maple (*Acer saccharum* Marsh.; >75% of basal area), a species often

associated with high rates of nitrification[24], low affinity for NO_3^- uptake[24, 25], and significant soil NO_3^- leaching[24]. Our measurements of soil solution N are consistent with this and on average, the flux of dissolved inorganic N (DIN) through the soil is 94.9 ± 0.3 % NO_3^--N. Over the last two decades, the flux of DIN from these forests has increased at all sites ($P < 0.001$; Fig. 3). Nitrate loss increased at all four sites, but only significantly ($P < 0.04$) at the three receiving the greatest levels of N deposition. When dissolved organic N (measured since 1994) is added to inorganic N, leaching losses are clearly having an effect on ecosystem N balance: the average amount N lost from the most southerly site increased from an average of 1.2 ± 0.3 kg N ha^{-1} (10% of N dep.) in 1995 to 5.3 ± 0.2 kg N ha^{-1} (45% of N dep.) in 2006 (Supplementary Fig. 2).

The changes in soil solution N are corroborated by measurements of $\delta^{15}N$ in fallen leaves. $\delta^{15}N$ is often measured in both active[26] and fallen leaves[27] as an indicator of N cycling. In the northern hardwood forests of eastern North America, increases in foliar $\delta^{15}N$ are positively correlated to several metrics of N availability such as plant and soil C:N ratios, but are most tightly linked to increases in nitrification[26]. At our sites, there was no clear trend in $\delta^{15}N$ at the most northerly site, but there were significant increases in $\delta^{15}N$ at the same three sites where increases in NO_3^- leaching were observed ($P \leq 0.05$; Fig. 3). We consider the consistent results between two independent measurements ($\delta^{15}N$, soil DIN) to be convincing evidence that N availability has increased. Further evidence of altered N cycling comes from the significant increases in the N concentrations of fallen

leaves observed during the study period at two of the three most southerly sites (Supplementary Fig. 1; $P \leq 0.005$).

We attribute these trends to N deposition because the greatest changes in N cycling have occurred at the sites receiving the greatest N deposition, with relatively minor changes at the "cleanest" site. However, as noted earlier, other factors can also influence N cycling. In particular, a warming climate could increase soil temperatures and the mineralization of soil N. Average growing season (May-October) soil temperatures have significantly increased at some sites (Supplementary Fig. 2); however these trends do not geographically fit the observed changes in N cycling. Thus, it does not appear that temperature increases are the primary cause of the changes in N cycling at these sites.

The large losses of N at these sites fit the most widely accepted definition for ecosystem N saturation[2]. In that context, the observed N losses would signal that these forests are no longer N limited. However, we know from a parallel long-term N addition experiment at these sites that there is additional capacity for plant N uptake, soil N retention, and ecosystem C storage at these sites[18, 28]. This is consistent with the observation of increased tree growth from N deposition in this region[19]. It appears that in these forests, large N losses occur prior to the saturation of biotic uptake and that the chronology of N saturation[2] may have to be revised.

Our ecosystem-level responses directly parallel patterns in atmospheric deposition in this region, demonstrating tight coupling between the atmosphere and biosphere. Emissions

regulations reduced the impacts of acid deposition on forest biogeochemistry, but the lack of a similar effort to reduce N deposition[4] has substantially altered terrestrial N cycling. This study shows the clear link between environmental policy and ecosystem function, but future policy decisions intended to affect N deposition will have to weigh increased forest growth[5] against reduced water quality, reduced species diversity, and increased emissions of powerful greenhouse gases[5, 20, 21].

Methods Summary

The four sites are northern hardwood forests (three 30 m × 30 m plots per site) and span gradients of atmospheric deposition and temperature from central lower to western upper Michigan[15] (Supplementary Table 1 & Supplementary Fig. 3). Soils are sandy (Kalkaska series, Typic haplorthod) with low cation exchange capacity (2.6-3.8 $cmol^{+}\ kg^{-1}$), but high percent base saturation (70-96%). The methodologies for determination of wood growth[16], plant N (ref. 28), leaf biomass[16], and soil solution N (ref. 29) have been detailed elsewhere. Leaf litter fall sampling used eight (1987-2003) or four (2003-2007) 0.5 m^2 traps per plot, collected periodically throughout the growing season[16]. We analyzed fallen sugar maple leaves for S in 1987-1991, 1994-1996, 1998, 1999, 2001, 2003, and 2005 and Ca and Al in these same years except 1992 was analyzed instead of 1987. $\delta^{15}N$ and N concentration were measured in these leaves annually (1988-2007). Average annual demand for N in wood growth (1988-2004) was calculated from wood growth[16] and wood N concentrations[28]. Soil solution measurements (1989-1990, 1992-2006) used four tension lysimeters per plot installed at 75 cm depth. Soil solution was collected biweekly during the spring and fall; water rarely moves through these soils in the summer.

Estimates of water flux for each sampling period were calculated using the water balance method[30]. Overwinter water flux was assigned the concentration of the first spring collection. Growing season soil temperature (1988-2005) was measured at 15 cm depth using thermistors read every 30 minutes and averaged every three hours. Data for wet atmospheric deposition are from the three National Atmospheric Deposition Program monitoring sites nearest to the study sites. Statistical analyses used a repeated measures analysis of variance for leaf litter and soil solution data (PROC Mixed) and regression for deposition and soil temperature data, both conducted in SAS Version 9.1.3.

References

1. Driscoll, C.T., *et al.* Acidic deposition in the northeastern United States: sources and inputs, ecosystem effects, and management strategies. *Bioscience* **51**, 180-198 (2001).

2. Aber, J. *et al.* Nitrogen saturation in temperate forest ecosystems. *Bioscience* **48**, 921-934 (1998).

3. Waxman, H.A. Overview and critique: an overview of the Clean Air Act Amendments of 1990. *Environ. Law* **21**, 1721-1816 (1991).

4. Aber, J.A. *et al*. Is nitrogen deposition altering the nitrogen status of northeastern forests? *Bioscience* **53**, 375-389 (2003).

5. Thomas, R.Q., Canham, C.D., Weathers, K.C. & Goodale, C.L. Increased tree carbon storage in response to nitrogen deposition in the US. *Nature Geosci.* **3**, 13-17 (2010).

6. Vitousek, P.M., Mooney, H.A., Lubchenco, J. & Melillo, J.M. Human domination of Earth's ecosystems. *Science* **277**, 494-499 (1997).

7. Emmett, B.A. Nitrogen saturation of terrestrial ecosystems: some recent findings and their implications for our conceptual framework. *Water Air Soil Pollu.: Focus* **7**, 99-109 (2007).

8. Chapin, F.S., Matson, P.A. & Mooney H.A. *Principles of Terrestrial Ecosystem Ecology*. (Springer-Verlag, New York, 2002). 436 p.

9. Stoddard, J.L. *et al.* Regional trends in aquatic recovery from acidification in North America and Europe. *Nature* **401**, 575-578 (1999).

10. McLauchlan, K.K., Craine, J.M., Oswald, W.W., Leavitt, P.R. & Likens, G.E. Changes in nitrogen cycling during the past century in a northern hardwood forest. *Proc. Natl. Aca. Sci. USA* 104, 7466-7470 (2007).

11. Likens, G.E., Driscoll, C.T. & Buso, D.C. Long-term effects of acid rain: response and recovery of a forest ecosystem. *Science* **272**, 244-246 (1996).

12. Reuss, J.O. & Johnson D.W. *Acid deposition and the acidification of soils and waters*. (Springer-Verlag, New York, 1986). 119 p.

13. Cronan, C.S. & Grigal D.F. Use of calcium/aluminum ratios as indicators of stress in forest ecosystems. *J. Environ. Qual.* **24**, 209-226 (1995).

14. Schmalensee, R., Joskow, P.L., Ellerman, A.D., Montero, J.P. & Bailey, E.M. An interim evaluation of sulfur dioxide emissions trading. *J. Econ. Perspect.* **12**, 53-68 (1998).

15. Miller, D.E. & Watmough S.A. Soil acidification and foliar nutrient status of Ontario's deciduous forest in 1986 and 2005. *Environ. Pollut.* **157**, 664-672 (2009).

16. Warby, R.A.F., Johnson, C.E. & Driscoll, C.T. Continuing acidification of organic soils across the northeastern USA: 1984-2001. *Soil Sci. Soc. Am. J.* **73**, 274-284 (2009).

17. Burton, A.J., Pregitzer, K.S. & MacDonald N.W. Foliar nutrients in sugar maple forests along a regional pollution-climate gradient. *Soil Sci. Soc. Am. J.* **57**, 1619-1628 (1993).

18. Pregitzer, K.S., Burton, A.J., Zak, D.R. & Talhelm A.F. Simulated chronic nitrogen deposition increases carbon storage in Northern Temperate forests. *Glob. Change Biol.* **14**, 142-153 (2008).

19. Holland, E.A., Braswell, B.H., Sulzman, J. & Lamarque, J.-F. Nitrogen deposition onto the United States and Western Europe: Synthesis of observations and models. *Ecol. Appl.* **15**, 38-57 (2005).

20. LeBauer, D.S. & Treseder, K.K. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* **89**, 371-379 (2008).

21. Hall, S.J. & Matson, P.A. Nitrogen oxide emissions after nitrogen additions in tropical forests. *Nature* **400**, 152-155 (1999).

22. Bernhardt, E.S. *et al.* Can't see the forest for the stream? In-stream processing and terrestrial nitrogen exports. *Bioscience* **55**, 219-230 (2005).

23. Burton, A.J., Pregitzer, K.S., Crawford, J.N., Zogg, G.P. & Zak, D.R. *Glob. Change Biol.*, **10**, 1080-1091 (2004).

24. Lovett, G.M. & Mitchell M.J. Sugar maple and nitrogen cycling in the forests of eastern North America. *Front. Ecol. Environ.* **2**, 81-88 (2004).

25. Eddy, W.C., Zak, D.R., Holmes, W.E. & Pregitzer, K.S. Chronic atmospheric NO_3^- deposition does not induce NO_3^- use by *Acer saccharum* Marsh. *Ecosystems*, **11**, 469-477 (2008).

26. Pardo, L.H. *et al.* Regional assessment of N saturation using foliar and root $\delta^{15}N$. *Biogeochem.* **80**, 143-171 (2006).

27. Pardo, L.H., Hemond, H.F., Montoya, J.P. & Pett-Ridge J. Natural abundance ^{15}N in soil and litter across a nitrate-output gradient in New Hampshire. *Forest Ecol Manag.* **251**, 217-230 (2007).

28. Zak, D.R., Holmes, W.E., Burton, A.J., Pregitzer, K.S. & Talhelm, A.F. Simulated atmospheric NO_3^- deposition increases soil organic matter by slowing decomposition. *Ecol. Appl.* **18**, 2016-2027 (2008).

29. Pregitzer, K.S., Zak, D.R., Burton, A.J., Ashby, J.A. & MacDonald, N.W. Chronic nitrate additions dramatically increase the export of carbon and nitrogen from northern hardwood ecosystems. *Biogeochem.* **68**, 179-197 (2004).

30. Thornthwaite C.W. & Mather J.R. Instructions and tables for computing potential evapotranspiration and the water balance. *Publ. Climatol.* **10**, 185–311 (1957).

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Author Contributions K.S.P. and A.J.B designed the study. K.S.P., A.J.B., D.R.Z., and A.F.T. were responsible for sample collection and analysis. A.F.T. and A.J.B. analyzed the data and A.F.T. wrote the manuscript with K.S.P., D.R.Z., and A.J.B.

Figure Legends

Figure 1: Mean annual H^+ (triangles) and N (circles) wet deposition from the three atmospheric deposition monitoring sites nearest to the four study locations.

Figure 2: (a) Mean annual SO_4^{2-} wet deposition (triangles) from the three atmospheric deposition monitoring sites nearest to the four study locations and annual study-wide mean leaf S concentrations (circles). (b) Annual study-wide mean leaf Al concentrations (triangles) and annual study-wide mean leaf Ca concentrations (circles). Error bars are ± 1 SE (n = 12). Declines with time were significant for both Al ($P < 0.02$) and Ca: ($P = 0.03$).

Figure 3: Spring and fall soil solution dissolved inorganic nitrogen (circles, dashed trend lines) and annual leaf $\delta^{15}N$ (triangles, solid trend lines) at each of the four study sites ranging from north (lowest deposition, site A) to south (highest deposition, site D). Trend lines shown for time trends with $P < 0.05$.

Figure 1

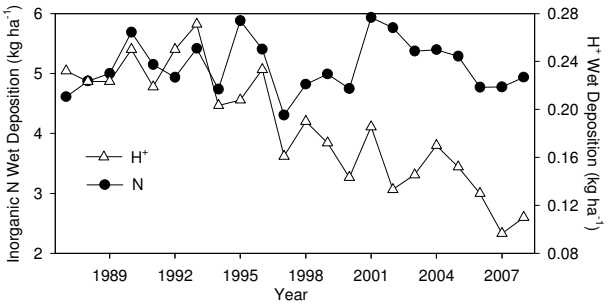

Inorganic N Wet Deposition (kg ha-1)
H+ Wet Deposition (kg ha-1)
Year
H+
N
6
5
4
3
2
0.28
0.24
0.20
0.16
0.12
0.08
1989
1992
1995
1998
2001
2004
2007

Figure 2

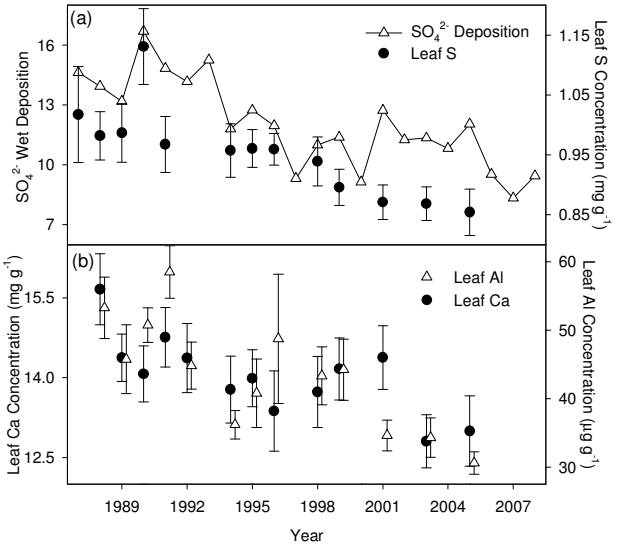
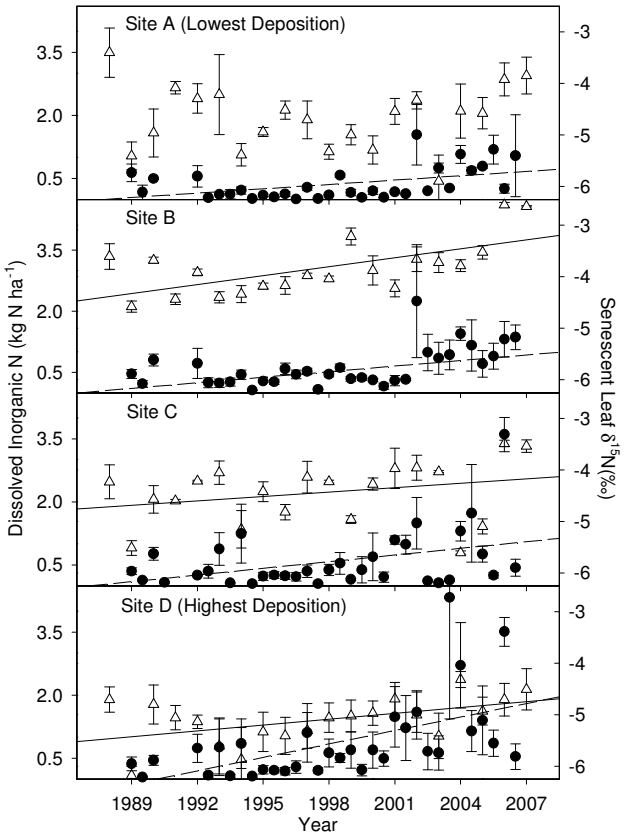

(a)
SO_4^{2-} Deposition
Leaf S
SO_4^{2-} Wet Deposition
Leaf S Concentration (mg g^{-1})
16
13
10
7
1.15
1.05
0.95
0.85
(b)
Leaf Al
Leaf Ca
Leaf Ca Concentration (mg g^{-1})
Leaf Al Concentration (µg g^{-1})
15.5
14.0
12.5
60
50
40
30
1989
1992
1995
1998
2001
2004
2007
Year

Figure 3


Site A (Lowest Deposition)
Site B
Site C
Site D (Highest Deposition)
Dissolved Inorganic N (kg N ha-1)
Senescent Leaf δ15N(‰)
Year
1989
1992
1995
1998
2001
2004
2007
0.5
2.0
3.5
-3
-4
-5
-6

Methods

Site Characteristics. The four study sites span a gradient of atmospheric deposition and temperature from central lower to western upper Michigan[15] (Supplementary Table 1 and Supplementary Fig. 3). Sites are northern hardwood forests (98 - 104 yrs old, basal area: 34.7 ± 0.8 m^2 ha^{-1}) dominated by sugar maple (*Acer saccharum* Marsh.)[16]. Soils are sandy (Kalkaska series, Typic haplorthod) and pH values range from 4.4 to 4.7 (top 10 cm of mineral soil). The soil cation exchange capacity is low (2.6-3.8 $cmol^+$ kg^{-1}), but percent base saturation is high (70-96%, measured 2004). Here we report data from plots (three 30 m × 30 m plots per site) that have never been experimentally manipulated.

Leaf Chemistry. The methodologies used at these sites for determination of wood growth, plant N uptake, leaf biomass, and soil solution N are described briefly below but have been described in detail in other recent publications[16, 28, 29]. Leaf litter fall sampling used eight (1987-2003) or four (2003-2007) 0.5 m^2 traps per plot, collected monthly from April through September and biweekly in October and November[16]. Leaf litter was sorted by species and a biomass-weighted annual sample was ground for elemental analysis. We analyzed the sugar maple leaf litter for S in 1987-1991, 1994-1996, 1998, 1999, 2001, 2003, and 2005 and Ca and Al in all of these same years except 1987 was replaced by 1992. Early analyses (1987-1992) were conducted using HNO_3-$HClO_4$ digestion, with DC-Ar plasma atomic emission spectrometry (AES) for Ca and Al and automated turbidity for S (ref. 15). For samples collected after 1992, analysis for Ca and Al was conducted at the Cornell Nutrient Analysis Laboratory with dry-ashing followed by inductively coupled plasma (ICP)-AES. Post-1992 samples were analyzed for S at the

University of Wisconsin Soil and Plant Analysis Lab using an HNO_3-$HClO_4$ digestion followed by ICP-AES. We reanalyzed 10 samples from the early period using the post-1992 techniques to test for analytical differences. A paired t-test determined that Al and S concentrations were higher using the earlier techniques ($P \leq 0.001$) and data from this period were adjusted downward proportionally. These same leaf samples were analyzed annually (1988-2007) for ^{15}N and N concentration at Michigan Technological University with an elemental analyzer (Costech 4010, Costech Analytical Technologies, Inc., Valencia, CA) coupled to a continuous-flow isotope ratio mass spectrometer (Delta^{PLUS}, Finnigan MAT, Bremen, Germany). Samples were measured against a N_2 reference gas calibrated with IAEA reference materials (International Atomic Energy Agency, Vienna, Austria). The standard deviation of measurements of a laboratory standard was 0.5‰ for $\delta^{15}N$.

Wood Growth. Wood growth was determined by combining annual measures of stem diameter and periodic measurements of tree height (every 5–6 years) with species-specific biomass equations[14]. Wood N concentration was determined on samples removed from stems at breast height with a 2.5 cm diameter hole saw in August 2004 (ref. 28). Average annual demand for N in wood growth (1988-2004) was calculated from wood growth and wood N concentration.

Soil Solution N. Soil solution measurements (1989-1990, 1992-2006) used four porous ceramic cup tension lysimeters per plot installed at a 75 cm depth (Model 1900 Soil Water Samplers, Soilmoisture Equipment Corp., Goleta, CA). Lysimeters were allowed

to recover from installation disturbance (fall 1987) for one year before sampling. Soil solutions were collected biweekly during the spring (April to early June) and fall (late August to early November). No vacuum was applied during the summer or winter. Water does not move below the rooting zone of these soils in the summer.

Through 2001, each sample was analyzed for NO_3-N, NH_4-N and persulfate-N by colorimetric analysis on an O.I. Analytical Flow Solution 3000 analyzer (O.I. Analytical, College Station, TX). Beginning in 2002, total dissolved N was analyzed using an Antek 9000VN chemiluminescence detector (Antek Instrument, Houston, TX) for total nitrogen in liquids. Dissolved organic N (DON) concentrations were determined as the difference between persulfate-N or total dissolved N and NO_3-N plus NH_4-N. Estimates of water flux for each sampling period were calculated using the water balance method based on meteorological data collected at each site[30]. These were multiplied by soil solution N concentrations for the same period and summed to determine soil solution fluxes. Water flux for the overwinter period was assigned the concentration of the first collection in the spring.

Soil Temperature. At each site, soil temperature was measured at 15 cm depth using thermistors (Model ES-060-SW, Data Loggers Inc., Logan, UT) read every 30 minutes by Omnidata EasyLoggers (Models 824 & 925, Data Loggers Inc., Logan, UT) and averaged every three hours. Measurements started in fall of 1988, but were only made during the growing season (May through October) prior to fall of 1994.

Atmospheric Deposition. Data for sulfate, acid, and N wet deposition are from the three National Atmospheric Deposition Program monitoring sites nearest to the study sites: Wellston (~70 km N of site D, ~24 km S of site C), Douglas Lake (~15 km E of site B), and Chassell (~40 km NE of site A).

Statistical Analysis. Statistical analyses used a repeated measures analysis of variance for leaf litter and soil solution data (PROC MIXED, restricted maximum likelihood) and regression for deposition and soil temperature data, both conducted in SAS (SAS Version 9.1.3, SAS Institute, Cary, NC).

Supporting Material

Table S1: Site locations and site-level time trends in wet deposition (SO_4^{2-} H^+, N), and leaf litter concentrations of sulfur (S), aluminum (Al), and calcium (Ca). Standard errors of intercept and slope estimates are given in parentheses. Wet deposition data are from the three NADP monitoring locations nearest to the study sites (one location is nearest to both sites C and D).

		Less Polluted ← Site → More Polluted			
		A	B	C	D
Location	Latitude (N)	46°52’	45°33’	44°23’	43°40’
	Longitude (W)	88°53’	84°51’	85°50’	86°09’
Wet N	Intercept (kg ha^{-1})	3.45 (0.29)	5.07 (0.30)	6.68 (0.32)	6.68 (0.32)
Deposition	Trend (kg ha^{-1} yr^{-1})	0.03 (0.02)	0.00 (0.02)	-0.01 (0.03)	-0.01 (0.03)
	P	0.211	0.855	0.709	0.709
Wood	Growth (kg ha^{-1} yr^{-1})	4626.5 (149.8)	5221.3 (174.7)	6288.9 (191.4)	6725.2 (282.9)
	N conc. (mg g^{-1})	0.70 (0.05)	0.99 (0.07)	0.90 (0.03)	0.97 (0.05)
	N uptake (kg ha^{-1} yr^{-1})	3.2 (0.1)	5.2 (0.2)	5.6 (0.2)	6.6 (0.3)
Total N dep*	(kg ha^{-1} yr^{-1})	6.8	9.1	11.7	11.8
Wet SO_4^{2-}	Intercept (kg ha^{-1})	9.52 (0.74)	15.20 (0.70)	19.92 (1.03)	19.92 (1.03)
Deposition	Trend (kg ha^{-1} yr^{-1})	-0.14 (0.06)	-0.28 (0.06)	-0.38 (0.08)	-0.38 (0.08)
	P	**0.025**	**<.001**	**<.001**	**<.001**
Wet H^+	Intercept (kg ha^{-1})	0.146 (0.009)	0.263 (0.011)	0.358 (0.019)	0.358 (0.019)
Deposition	Trend (kg ha^{-1} yr^{-1})	-0.003 (0.001)	-0.006 (0.001)	-0.010 (0.002)	-0.010 (0.002)
	P	**<.001**	**<.001**	**<.001**	**<.001**
Leaf S	Intercept (mg g^{-1})	0.818 (0.020)	0.926 (0.020)	1.14 (0.027)	1.30 (0.040)
	Trend (mg g^{-1} yr^{-1})	-0.005 (0.002)	-0.003 (0.002)	-0.013 (0.002)	-0.021 (0.002)
	P	**0.017**	0.173	**<0.001**	**<0.001**
Leaf Al	Intercept (μg g^{-1})	51.0 (4.0)	41.2 (1.8)	49.4 (2.5)	72.3 (6.7)
	Trend (μg g^{-1} yr^{-1})	-1.4 (0.4)	-1.0 (0.2)	-0.8 (0.2)	-1.5 (0.5)
	P	**0.001**	**<0.001**	**<0.001**	**0.005**
Leaf Ca	Intercept (mg g^{-1})	13.54 (0.51)	17.44 (0.82)	13.68 (0.95)	14.91 (0.62)
	Trend (mg g^{-1} yr^{-1})	-0.14 (0.03)	-0.13 (0.04)	-0.07 (0.03)	-0.07 (0.03)
	P	**0.008**	**<0.001**	**0.014**	**0.040**

*Wet + dry inorganic N (NH_4^+, NO_3^-); from MacDonald, N.W. *et al.* Ion leaching in forest ecosystems along a Great Lakes air pollution gradient. *J. Environ. Qual*. 21:614-623 (1992).

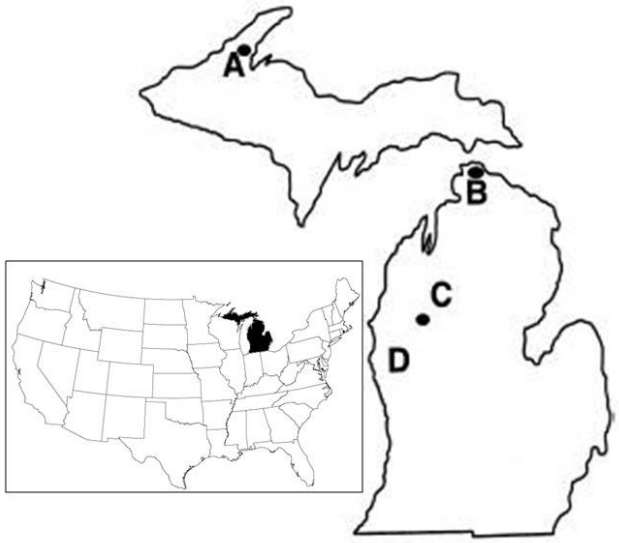
Supplementary Figure Legends:

Fig S1: Study site locations (letters) and atmospheric deposition monitoring site locations (black dots) in the state of Michigan, in the north-central United States.

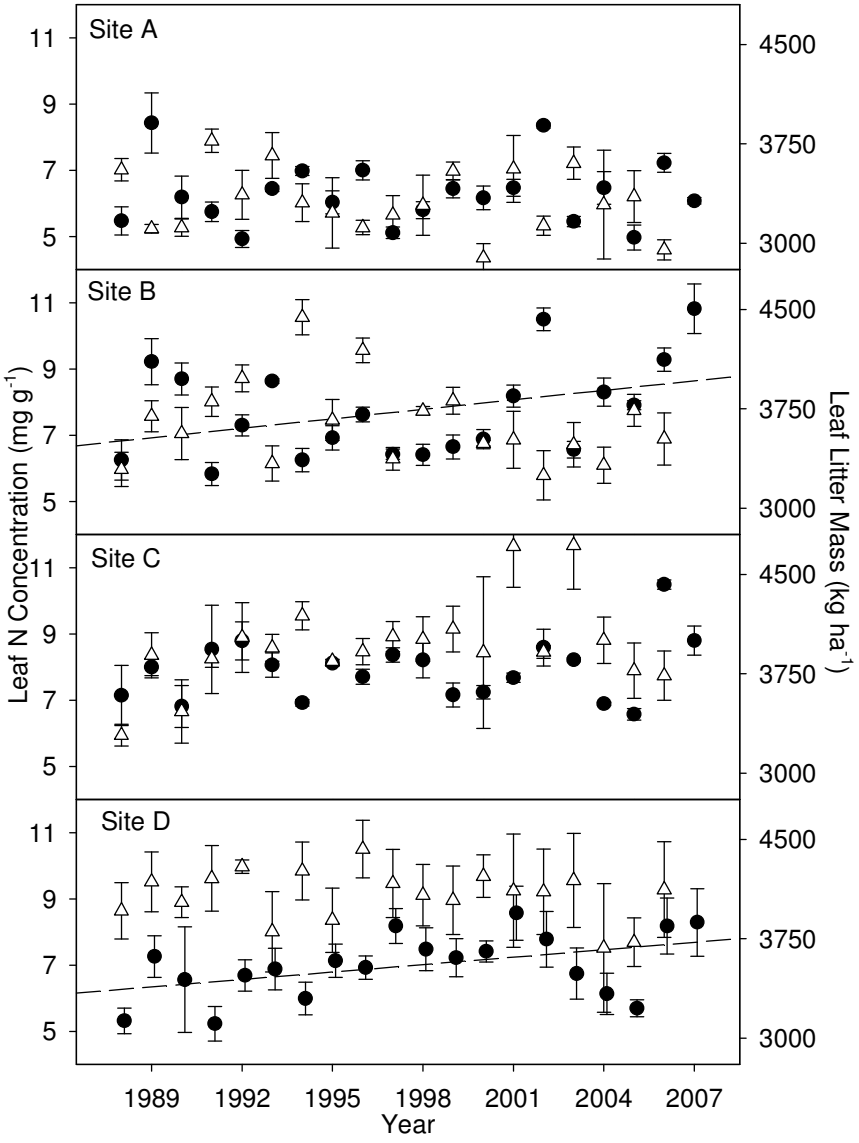
Fig S2: Annual N concentration in fallen leaves (circles) and annual leaf biomass from litter fall traps (triangles) at each of the four study sites ranging from north (lowest deposition, site A) to south (highest deposition, site D) from 1988-2007. Time trends for leaf N with $P < 0.05$ shown as dashed lines.

Fig S3: Spring and fall soil solution total (inorganic and organic) dissolved nitrogen (circles, dashed trend lines) and growing season (April-October) soil temperature (triangles, solid trend lines). Trend lines shown for time trends with $P < 0.05$. Total dissolved N measured from 1994-2006 and soil temperature measured from 1988-2005.

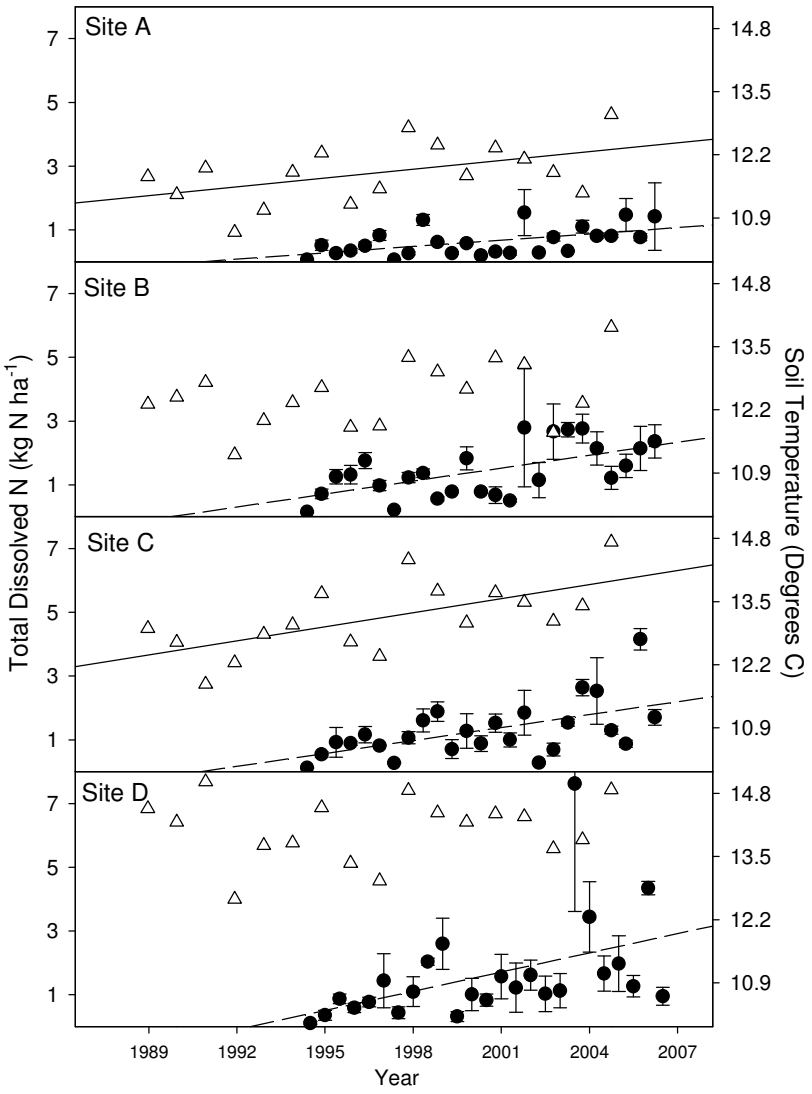
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Chapter Five: Chronic Nitrogen Additions Do Not Increase Leaf-Level Photosynthesis in Mature Sugar Maple Forests*

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*Formatted for submission to *Ecological Applications*

Abstract

Predicted growth in the anthropogenic production of reactive nitrogen (N) and subsequent N emissions makes it important to understand how atmospheric N deposition impacts the global carbon (C) cycle. Nitrogen deposition is thought to increase forest C sequestration by enhancing leaf-level photosynthesis, but this has been infrequently observed in long-term studies. We tested the effect of long-term (14 years) N additions (30 kg N ha^{-1} yr^{-1} as $NaNO_3$) on photosynthesis in sugar maple (*Acer saccharum*) growing in four mature northern hardwood forests in the north-central United States using instantaneous measurements and a dual isotope (^{13}C and ^{18}O) approach that integrates changes in photosynthesis through time. Forests receiving NO_3^- additions in this study have shown increased aboveground growth, but have not added additional leaf area or leaf biomass. We hypothesized that the increase in growth from the NO_3^- additions was the result of increases in foliar N and leaf-level photosynthesis. Trees receiving NO_3^- additions did have significantly more foliar N. However, there was no difference between the ambient and $+NO_3^-$ treatments in two seasons (2006-2007) of instantaneous measurements of photosynthesis from either canopy towers or excised branches. Furthermore, NO_3^- additions did not create significant differences in $\delta^{13}C$ and $\delta^{18}O$ in either current foliage or leaf litter collected annually throughout the study (1993-2007). Combined, these data suggest that NO_3^- additions have not stimulated photosynthesis. However, photosynthetic nitrogen use efficiency (μmol CO_2 s^{-1} g^{-1} N) was significantly decreased (-13%) by NO_3^- additions. Unlike other studies of photosynthesis in N saturated forests, we cannot attribute the lack of a stimulation of photosynthesis to nutrient limitations at these sites. Rather than increases in C

assimilation, the observed increases in aboveground growth at our study sites may be due to shifts in C allocation.

Introduction

Around the world, human activities have greatly increased the availability of reactive nitrogen (N) (Galloway et al. 2008). Much of this anthropogenic N is moved into the atmosphere where it is transported away from emissions sources and later added to ecosystems through atmospheric deposition (Galloway et al. 2008). Forecasts for the next century predict that as agricultural and industrial activities intensify around the world, terrestrial ecosystems could experience even greater loads of atmospheric N deposition (Dentener et al. 2006). Consequently, it is important to understand the effects of N deposition in order to predict how terrestrial ecosystems will function in the future.

The widespread limitation of forest growth by N (LeBauer and Tresseder 2008) suggests that in many areas N deposition may increase forest productivity. For temperate forests in particular, increases in N deposition have led to many predictions (e.g. Aber et al. 1998, Ollinger et al. 2002) and observations of increased aboveground productivity and carbon (C) storage in forests (Nadelhoffer et al. 1999, Magnani et al. 2007, Pregitzer et al. 2008, Thomas et al. 2010). Studies of N deposition have widely reported increases in foliar N concentration (e.g, Hutchinson et al. 1998, Baron et al. 2000, Bauer et al. 2004, Elvir et al. 2005, Boggs et al. 2005), and because of the key role of N in photosynthesis (Evans 1989), many empirical and conceptual models of the effects of N deposition on

aboveground productivity explicitly include increases in photosynthesis (Aber et al. 1989, Aber et al. 1997, Sievering et al. 2000, de Vries et al. 2006, Hyvönen et al. 2007).

However, in some cases, the supply of N can exceed the ability of ecosystems to use and sequester N, a condition termed N saturation (Aber et al. 1989). In N saturated ecosystems, the leaching of base cations is suspected to create nutrient limitations that limit plant C gain and reduce plant growth (Aber et al. 1998). In the poorly buffered soils of the northeastern United States, there are several ecosystems where long-term saturating N additions have not increased leaf-level photosynthesis and where other nutrients (particularly Ca and Mg) are thought to be limiting photosynthesis and growth (Schaberg et al. 1997, Bauer et al. 2004, Elvir et al. 2006). In fact, there is only limited evidence (Elvir et al. 2006) that higher leaf N concentrations are associated with increased leaf-level photosynthesis in N saturated forests. Where photosynthesis has not been stimulated, several other effects on leaf physiology and morphology have been observed: changes in leaf size (Schaberg et al. 1997, Bauer et al. 2004), increases in foliar respiration (Schaberg et al. 1997), and decreases in photosynthetic nitrogen-use efficiency (NUE, μmol CO_2 g^{-1} N s^{-1}; Schaberg et al. 1997, Bauer et al. 2004). However, it is unclear if similar responses to N saturated conditions would be observed in more nutrient-rich systems.

In this context, we sought to examine changes in photosynthesis induced by long-term N additions at the Michigan Gradient Study. At each of the four temperate forests sites included in this study (Figure 1), the large increases in the leaching of inorganic N from

the soil are evidence that experimental N additions have induced N saturation (Pregitzer et al. 2004). However, the observed increases in aboveground productivity (Pregitzer et al. 2008) indicate the N additions have altered plant C balance. Foliar N concentrations have increased (Zak et al. 2008), but there have not been significant changes in leaf (Pregitzer et al. 2008) or root production (Burton et al. 2004). Unlike many forests in the northeastern United States, the soils at these sites are rich in base cations. This makes increases in leaf-level C gain a plausible source of the additional aboveground C despite the large leaching losses of N. In addition, the one observation of increased leaf-level photosynthesis (Elvir et al. 2006) in an N-saturated forest (Jefts et al. 2004) was in the same species (*Acer saccharum* Marsh.) that dominates these sites.

To examine the current effects of long-term N additions on photosynthesis, we made two sets of instantaneous measurements: one set from two canopy towers located at one site with measurements repeated throughout the growing season and another set on excised branches at each of the four sites with measurements during three discrete periods. In addition to examining the effects under current conditions, we also sought to examine changes in photosynthesis retrospectively throughout the duration of the study. This retrospective analysis allowed us to examine whether effects of long-term N additions on photosynthesis had changed over time as these sites became more N saturated.

To evaluate changes through time, we used a dual isotope ($\delta^{18}O$ and $\delta^{13}C$) assessment of leaf gas exchange (Scheidegger et al. 2000). Leaf $\delta^{18}O$ reflects changes in transpiration and stomatal conductance because as organic compounds are created and transported

within the leaf, O atoms in these molecules are exchanged and take on the $\delta^{18}O$ of leaf water. Air temperature, vapor pressure deficit, and water source all influence leaf water $\delta^{18}O$ (Barbour 2007). However, because the trees within a study site are likely to use the same water sources and experience the same environmental conditions, changes in stomatal conductance that affect the rate of transpiration should be the only difference between N addition and ambient plots.

For $\delta^{13}C$, the relative abundance of ^{13}C in C_3 plant tissue is positively related to the ratio of the partial pressure of CO_2 in the chloroplasts (C_c) to the partial pressure of CO_2 in the atmosphere (C_a) surrounding the leaf during C fixation (Seibt et al. 2008). Although there are other influences on the $\delta^{13}C$ of plant tissue (Seibt et al. 2008), a simple model can predict the $\delta^{13}C$ of plant tissue ($\delta^{13}C_{tissue}$) with good accuracy:

$$\delta^{13}C_{tissue} = \delta^{13}C_{atmosphere} - \mathbf{a} - (\mathbf{b} - \mathbf{a})C_c/C_a$$

where $\delta^{13}C_{atmosphere}$ is the $\delta^{13}C$ of atmospheric CO_2 surrounding the leaf, **a** (about -4.4‰) is the C isotope fractionation that occurs through diffusion, and **b** (about -27‰) is the kinetic fractionation by the enzyme Rubisco in photosynthesis (Farquhar et al. 1989).

Previous studies often assumed that the partial pressure of CO_2 in the intercellular space (C_i) was equal to C_c because internal (or mesophyll) conductance to CO_2 was non-limiting (Niinemets et al. 2009). With this assumption, variation in plant $\delta^{13}C$ was often described as a function of C_i, with changes in both C_i and $\delta^{13}C$ dependent on the balance between the draw-down of CO_2 from photosynthesis and the influx of CO_2 through stomatal conductance (Seibt et al. 2008). Simultaneous measurement of plant $\delta^{18}O$ made

it possible to attribute the changes in C_i and $\delta^{13}C$ to variation in either photosynthesis or stomatal conductance (Scheidegger et al. 2000).

Now, it is clear that internal conductance can have a large effect on C_c and alter tissue $\delta^{13}C$ (Seibt et al. 2008). Internal conductance varies with environmental factors such as drought (Niinemets et al. 2009), but as with $\delta^{18}O$, these factors should not differ between plots at the same site. However, internal conductance also varies with leaf structural properties such as specific leaf area (SLA, cm^2/g) that can be affected by nutrient availability (Poorter et al. 2009; Niinemets et al. 2009). Thinner/less dense leaves increase the internal conductance of CO_2 to chloroplasts (Niinemets et al. 2009) and this relationship is thought to be one reason why there is a negative correlation between leaf $\delta^{13}C$ and SLA (Körner et al. 1991, Araus et al. 1997, Hanba et al. 1999, Hultine and Marshall 2000, Takahashi and Miyajima 2008, but see: Monclus et al. 2005). Given the link between SLA and nutrient availability, we accounted for differences in SLA when interpreting changes in $\delta^{13}C$.

We used the dual isotope model to examine changes in the effect of chronic N additions on photosynthesis through time by analyzing $\delta^{13}C$ and $\delta^{18}O$ in leaves collected annually over a 15-year period. We tested the robustness of the dual isotope model by comparing changes in $\delta^{13}C$ and $\delta^{18}O$ of green leaves and leaf litter to gas exchange measurements made in the same years (Scheidegger et al. 2000) and by analyzing the influence of SLA on $\delta^{13}C$.

Based on previous observations of increased aboveground growth and higher foliar N (Pregitzer et al. 2008, Zak et al. 2008), we hypothesized that the plants receiving chronic N additions would have higher leaf-level rates of light-saturated photosynthesis in instantaneous measurements. Furthermore, we hypothesized that increase would consistently be observed in stable isotope measurements of photosynthesis in previous years.

Methods

The four sites of the Michigan Gradient Study span 500 km across northern lower and western upper Michigan, in the north-central United States (Fig. 1). These sites occur across south to north gradients of climate and N deposition and encompass most of the northern hardwoods biome in the Great Lakes region (Braun 1950, Pregitzer et al. 2004). The forests in this study are characteristic of much of the region: second growth stands that originated shortly after the beginning of the 20^{th} century (Pregitzer et al. 2004). Soils are sandy (Kalkaska series, Typic haplorthod) and pH values range from 4.4 to 4.7 (top 10 cm of mineral soil). The soil cation exchange capacity is low (2.6-3.8 $cmol^{+}/kg^{-1}$), but percent base saturation is high (70%-96%, measured 2004).

Since 1994, three of the six 900 m^2 plots located at each site have received experimental additions of 30 kg N ha^{-1} yr^{-1} spread evenly across the growing season in six increments in the form of $NaNO_3$ pellets. In comparison, ambient N deposition ranges from 6.8 kg N

ha^{-1} yr^{-1} at the most northerly site to 11.8 kg N ha^{-1} yr^{-1} at the most southerly site, with NO_3^- being the dominant form of N deposition (MacDonald et al. 1992). Although the experimental additions are much greater than ambient deposition, the rates of total N addition are similar to those occurring in some areas of the United States and Europe (Holland et al. 2005).

Tower Based Measurements

The canopy was accessed using two scaffolding towers at Site B (Fig. 1), one each in a NO_3^- addition plot and an ambient plot. This site is in the geographic middle of the four study sites and because of ^{15}N tracer experiments conducted at the site (Zogg et al. 2000, Zak et al. 2008), it is the best characterized. Each canopy tower was approximately 25 m in height and provided access to three (ambient) or four ($+NO_3^-$) mature trees.

Measurements were conducted on leaves in the top 2.5 - 3.5 m of the canopy using a Li-Cor 6400 photosynthesis system (Li-Cor Inc., Lincoln, NE) during the 2006 and 2007 growing seasons. Measurement of light-saturated rates of photosynthesis were conducted at a saturating light intensity of 1600 μmol m^{-2} s^{-1} maintained by a red-blue LED light source, a chamber temperature of ~ 25° C, at near ambient humidity, and with a reference CO_2 concentration of 390 μl l^{-1}. Leaves were placed in the measurement cuvette and after at least two minutes, stable gas exchange values were recorded every 15 seconds for at least one minute. During 2006, measurements of light saturated photosynthesis started during the end of July and were made subsequently every three weeks until leaf fall in

early October. During 2007, measurements of light-saturated photosynthesis started shortly after leaf-out in mid-May and continued until late August. The frequency of these measurements varied during 2007 from every three weeks during the beginning and end of the growing season to every ten days or less during the peak of the growing season.

In addition to measurements of light-saturated photosynthesis, we measured the response of light-saturated photosynthesis to changes in the intercellular (C_i) partial pressure of CO_2 (referred to as A-C_i curves) in order to assess the biochemical controls on leaf-level gas exchange and determine whether additional foliar N is influencing these controls. For these measurements, we followed the protocols suggested by Long and Bernacchi (2003). Briefly, photosynthesis was measured at 390 μL CO_2/L until stable. Then, the cuvette CO_2 level was decreased in four steps to 100 μL/L. After measurement at 100 μL/L, the CO_2 level was returned to 390 μL/L for several minutes before being increased in seven steps to 2000 μL/L. Measurement at each step was completed as soon as the reference CO_2 level stabilized. These A-C_i curves were conducted over a two-week period in mid- to late July 2007.

As with previous descriptions of ^{13}C discrimination, it had been assumed for A-C_i measurements that C_i equaled C_c (Sharkey et al. 2007). However, it is now understood that the biochemical limitations to photosynthesis should be calculated from C_c. We did not directly estimate C_c during our measurements, but instead used the publicly available A-C_i curve fitting utility created by Sharkey et al. (2007) that calculates C_c from estimates of internal conductance and uses these values of C_c to determine the rate of

dark respiration (R_d), the maximum velocity of Rubisco for carboxylation (V_{cmax}), and the maximum rate of electron transport/RuBP regeneration (J_{max}). Curves were fit for each leaf individually.

All photosynthesis measurements were conducted on eight to twelve individual leaves per tree and these measurements were averaged to create a single tree-level value. In addition to gas exchange measurements, leaf samples (8-15 leaves) were collected every three to four weeks for analysis of changes in specific leaf area, N concentration, and $\delta^{13}C$ and $\delta^{18}O$ values (see below).

Excised Branch Measurements

Gas exchange measurements were conducted at all four study sites during three separate periods (August 2006, June 2007, and August 2007). During these periods, branches and intact foliage were gently removed from the upper canopy of dominant and co-dominant sugar maples (> 15 cm diameter at breast height (dbh)) with a shotgun firing steel shot. In order to ensure an overall sample that accurately represented a broad range of canopy trees, sampling during 2007 was stratified into two levels based on stem diameter (co-dominant: 15 ≤ 25 cm dbh; dominant: > 25 cm dbh), with equal representation from both strata. During the first sampling (August 2006) three to six measurements were taken per plot. Sampling intensity increased in 2007, with ten to twelve samples per plot in June and eight to ten samples per plot in August. Following removal from the tree, the branches were quickly re-cut under water and one leaf from each excised branch was placed in the gas exchange cuvette under a saturating light intensity (photon flux density:

1600 μmol m^{-2} s^{-1}) for seven minutes. This methodology has been used in similar studies (including with sugar maple; Elvir et al. 2006) and tests suggest that gas exchange measurements do not significantly differ between intact and excised foliage for 15 minutes following excision (Gower et al. 1993). After gas exchange measurements were completed, all of the leaves from each sampled branch were stored on ice and preserved for analysis of specific leaf area, N concentration, $\delta^{13}C$, and $\delta^{18}O$ values (see below).

Leaf N, $\delta^{13}C$, and $\delta^{18}O$

We applied the dual isotope technique to freshly fallen sugar maple leaves collected over a 15-year period as well as fresh foliage collected from the towers and from excised branches. Fallen leaves were collected during one year prior to application of the NO_3^- treatment (1993) and in each year until the last year of instantaneous gas exchange measurements (1994-2007). Sampling of fallen leaves (litter) used eight (1993-2003) or four (2003-2007) 0.5 m^2 litter traps per plot, collected monthly from April through September and biweekly in October and November (Pregitzer et al. 2008). These collections were used to create a biomass-weighted annual sample, from which a subsample was ground and analyzed. Two duplicate samples were analyzed per plot in each year for N concentration, $\delta^{13}C$, and $\delta^{18}O$.

All fresh foliage samples were frozen until they were analyzed for leaf area using the Li-Cor 3100 leaf area meter. Each sample was then oven dried, weighed, and ground for analysis. For foliage collected from the towers, two duplicate samples from each tree were analyzed for N concentration, $\delta^{13}C$, and $\delta^{18}O$. For leaf samples from excised

branches, the number of samples and the analytical procedure varied by year. For samples taken in 2006, foliage was analyzed from each tree sampled. In 2007, two duplicate leaf samples were analyzed for $\delta^{13}C$ from material bulked by date, site, plot, and size class. However, each tree sampled during 2007 was individually measured for foliar N concentration. This allowed us to match gas exchange and leaf area measurements to leaf N. In this case, these individual measurements were then averaged to create plot- and size class- level values. For both tower and excised branch measurements, gas-exchange measurements and leaf tissue analysis were combined to create tree-level estimates of photosynthesis per unit of leaf mass (nmol CO_2 s^{-1} g^{-1}) and photosynthetic nitrogen use efficiency (NUE; μmol CO_2 s^{-1} g^{-1} N).

Samples analyzed for both leaf $\delta^{13}C$ and N concentration were analyzed at the Michigan Technological University Forest Ecology Analytical Laboratory with an elemental combustion system (Costech 4010, Costech Analytical Technologies, Inc., Valencia, CA) coupled to a continuous-flow isotope ratio mass spectrometer (Delta^{PLUS}, Thermofinnigan MAT, Bremen, Germany). Samples were measured against CO_2 reference gas calibrated with IAEA reference materials (International Atomic Energy Agency, Vienna, Austria). The standard deviation of repeated measurements of a laboratory standard was 0.10‰ for $\delta^{13}C$. Measurements of $\delta^{18}O$ were conducted at the Washington State University Stable Isotope Core Lab using a pyrolysis elemental analyzer (TC/EA, Thermofinnigan MAT, Bremen, Germany) coupled to a continuous-flow isotope ratio mass spectrometer (Delta^{PLUS}, Thermofinnigan MAT, Bremen, Germany). Samples were measured against internal standards calibrated with IAEA reference materials. The standard deviation of

repeated measurements of a laboratory standard was less than 0.4 ‰ for $\delta^{18}O$. Tree-level measurements of leaf N concentrations from the 2007 excised branch sampling occurred at the University of Nevada, Reno Forest Ecology Laboratory using an elemental combustion system (Costech 4010).

Statistical Analyses

Statistical analyses used a mixed model in SAS (Proc Mixed, Version 9.1.3, SAS Institute, Cary, NC) to conduct analysis of variance tests (repeated measures where appropriate). Post-hoc pair-wise contrasts of treatment effects were adjusted for multiple comparisons using the Šidák correction. As in other studies of canopy physiology (Ryan et al. 2004, Keel et al. 2007), analyses of tower data were conducted using each tree as a replicate. All other analyses, including data from excised branches, were conducted using plot-level data. Analyses of data generated from the 2007 excised branch samples from co-dominant and dominant trees tested size class effects as a split-plot factor. To see if the effects of NO_3^- on leaf litter $\delta^{13}C$ and $\delta^{18}O$ changed through the course of the study, we modified the mixed model to use year as a continuous rather than a discrete variable to create an analysis of covariance (Littell et al. 1996). Relationships between SLA and $\delta^{13}C$ were tested using regression (Proc Reg, SAS). For litter, $\delta^{13}C$ values were converted to $\Delta^{13}C$ and adjusted for the Suess effect (Feng 1999) before regression with SLA. Differences in the SLA - $\delta^{13}C$ (or $\Delta^{13}C$) relationship between the $+NO_3^-$ and ambient plots were tested as an analysis of covariance (Proc Mixed, SAS).

Results

Tower Measurements

Overall, leaves collected from trees accessed by the $+NO_3^-$ tower had an N_{mass} that was 12% greater than leaves collected from trees accessed by the ambient tower (P = 0.015, Table 1). Leaf N per unit leaf area (N_{area}, g N/m^2) was on average 11% greater in $+NO_3^-$ trees, but N_{area} was actually 10 to 14% lower in $+NO_3^-$ trees at the end of 2006 growing season during leaf senescence and the beginning of the 2007 growing season while the leaves were still developing (NO_3^- × date: P = 0.037, Table 1). Average leaf area, average leaf mass, and specific leaf area did not significantly differ between ambient and $+NO_3^-$ trees (Table 1).

Over the 13 periods during which instantaneous gas exchange measurements were taken, NO_3^- additions stimulated photosynthesis per unit leaf area (A_{area}, μmol CO_2 m^{-2} s^{-1}) by an average of only 1.4% (P = 0.750, Table 2) and only significantly increased once (28 July 2006, P = 0.018). Photosynthesis per unit leaf mass (A_{mass}, nmol CO_2 g^{-1} s^{-1}) was only 2% greater in $+NO_3^-$ trees (P = 0.771, Table 2). Photosynthetic nitrogen use efficiency (NUE, μmol CO_2 g^{-1} N s^{-1}) was somewhat lower in the $+NO_3^-$ trees than in the ambient trees (Table 2), but not significantly different (P = 0.122). Stimulation of stomatal conductance (mol H_2O m^{-2} s^{-1}) was larger (15%) than that of photosynthesis, though only marginally significant (P = 0.076, Table 2). The most significant NO_3^- effect from the tower-based instantaneous measurements was an increase in the calculated intercellular CO_2 concentration (C_i, +5.7%, P = 0.047, Table 2), but the increase was only significant on one individual date (28 June 2007, P = 0.019).

Analysis of A-C_i curves (Table 2) found that trees exposed to chronic NO_3^- additions had higher apparent Rubisco activity ($V_{cmax\text{-}area}$, μmol CO_2 m^{-2} s^{-1}) increased dark respiration (R_d), but these changes were only marginally significant (P = 0.061 and P = 0.092, respectively). There were small changes in the ratio of photosynthesis (at 390 μmol CO_2/mol) and the rate of electron transport for RuBP regeneration ($J_{max\text{-}area}$, μmol CO_2 m^{-2} s^{-1}), but these differences were not significant (P = 0.599 and P = 0.246, respectively). Differences between $+NO_3^-$ and ambient trees in, R_d, V_{cmax}, and J_{max} were greater on a mass basis than when compared per unit N (Table 2).

Leaf $\delta^{18}O$ values were lower in $+NO_3^-$ tower foliage (P < 0.001, Table 1) and consistent with differences in instantaneous measurements of stomatal conductance. However, the NO_3^- effect on $\delta^{18}O$ varied by date (P < 0.001). Leaf samples collected from the $+NO_3^-$ tower had significantly lower $\delta^{13}C$ values (P = 0.023, Table 1), consistent with $+NO_3^-$ trees showing greater stomatal conductance and similar rates of photosynthesis.

Excised Branch Measurements

Across sites and measurements periods, $+NO_3^-$ plots had significantly greater N_{mass} and N_{area} (P < 0.001 for both, Table 3). The strength of the NO_3^- effect varied by site (P = 0.038 for N_{mass} and P = 0.002 for N_{area}) and was the strongest at site A (both P < 0.001). N_{mass} and N_{area} were always greater in the $+NO_3^-$ plots at the other sites, but these differences were not always significant (P = 0.161 to P = 0.039). The $+NO_3^-$ treatment slightly increased average leaf area (1.3 cm^2/leaf), but this effect varied by site (P = 0.030; Table 3) and the overall difference was not significant. Average leaf mass

increased by 0.02 g/leaf in $+NO_3^-$ plots ($P = 0.027$), but this effect also varied by site ($P < 0.001$) and leaf mass was actually significantly lower at site B ($P = 0.016$, Table 3). The effects of NO_3^- on specific leaf area (SLA) were small (-3% overall), varied by site, and were not significant (Table 3).

Overall, trees in the $+NO_3^-$ plots had slightly greater A_{area} during each of the three sampling periods (+1.3% to +7%, +3.5% overall), but neither the overall NO_3^- effect ($P = 0.407$; Table 4*)* nor the NO_3^- by time interaction were significant ($P = 0.853$). In contrast, the effects of NO_3^- on A_{mass} were not consistent across sampling periods (-4.3% to +5.9%, +0.1% overall). Across all dates and sites, photosynthetic NUE was greater in ambient plots (6.0 ± 0.3 μmol CO_2 s^{-1} g^{-1} N) than in $+NO_3^-$ plots (5.2 ± 0.1 μmol CO_2 s^{-1} g^{-1} N; $P = 0.013$; Table 4). The effects of NO_3^- were not significant for stomatal conductance (+1.8%; $P = 0.752$; Table 4) or C_i (-1.3%, $P = 0.466$, Table 4).

On average, excised branch foliage from $+NO_3^-$ plots was 0.3‰ more depleted in ^{18}O than foliage from ambient plots, but this difference was not significant ($P = 0.123$; Fig 2b). For $\delta^{13}C$, foliage from $+NO_3^-$ plots was on average 0.1‰ more enriched in ^{13}C than foliage from the ambient plots, but this difference was also not significant ($P = 0.273$; Fig. 2b). Site-level differences between ambient and $+NO_3^-$ in $\delta^{13}C$ matched differences in SLA ($n = 4$, $r^2 = 0.900$, $P = 0.051$), but had a stronger relationship with differences in log-transformed SLA ($r^2 = 0.938$, $P = 0.032$). Across all sites, plot-level variation in $\delta^{13}C$ was well explained by variation in SLA ($n = 72$, $r^2 = 0.373$, $P < 0.001$) and log-transformed SLA ($r^2 = 0.375$, $P < 0.001$, Supplementary Figure 1). There were no

significant differences in the slope or intercepts of this relationship in response to NO_3^- additions ($P > 0.6$).

In the 2007 sampling of dominant and co-dominant trees, there were no significant interactions between size class and NO_3^- for any measured gas exchange or leaf tissue trait.

Leaf Litter $\delta^{13}C$ and $\delta^{18}O$

Comparing leaf litter to summer foliage collected in the same year, leaf litter was more depleted in $\delta^{18}O$ and $\delta^{13}C$ (0.7‰ and 0.1‰, respectively) than summer foliage. These differences varied strongly for both isotopes according to which period the summer foliage was collected in ($P \leq 0.001$). There was no significant NO_3^- effect ($P > 0.5$ for $\delta^{18}O$ and $\delta^{13}C$) or significant NO_3^- interaction (all $P > 0.19$) for differences between summer foliage and litter. Overall, NO_3^- effects were roughly equivalent: -0.3‰ vs. -0.2‰ for $\delta^{18}O$ and 0.1‰ vs. 0.0‰ for $\delta^{13}C$ in green leaves and litter from 2006-2007, respectively.

In the year prior to the initiation of the NO_3^- additions (1993), leaf litter collected in the ambient plots averaged 23.5 ± 0.2‰ and -28.2 ± 0.1‰ while leaf litter collected in $+NO_3^-$ plots averaged 23.9 ± 0.3‰ and -28.2 ± 0.1‰ for $\delta^{18}O$ (NO_3^-: $P = 0.054$) and $\delta^{13}C$ (NO_3^- : $P = 0.735$), respectively. Averaged across 1994-2007 (Fig 2), site-level differences in both $\delta^{13}C$ and $\delta^{18}O$ between $+NO_3^-$ plots and ambient plots for leaf litter were similar to

the differences observed for excised foliage: slight depletion in $\delta^{18}O$ at all sites (-0.1‰), depletion in $\delta^{13}C$ at site B (-0.4‰) and enrichment in $\delta^{13}C$ at sites A, C, and D (+0.1 – +0.3‰). Although the NO_3^- effects on $\delta^{13}C$ and $\delta^{18}O$ were largely consistent from year to year within each site (Supplementary Fig. 3 and 4), the effects were not significant overall ($P = 0.593$ and $P = 0.261$ for $\delta^{13}C$ and $\delta^{18}O$, respectively) or when interacting with site ($P = 0.093$ and $P = 0.963$) or year ($P = 0.397$ and $P = 0.848$). However, there was a significant NO_3^- by site by year interaction ($P = 0.002$) for $\delta^{13}C$ because of a significant NO_3^- effect at site B in 1999 ($P = 0.005$). There were no significant trends in size of the NO_3^- effects through time for $\delta^{13}C$ or $\delta^{18}O$ ($P > 0.4$).

As with excised branches, there was a strong relationship between plot-level variation in $\Delta^{13}C$ and log-transformed SLA ($n = 336$, $r^2 = 0.140$, $P < 0.001$); there was not a significant difference in the SLA - $\Delta^{13}C$ relationship between ambient and $+NO_3^-$ litter ($P > 0.5$).

Discussion

In other studies, trees in N saturated forests have shown increases in average leaf area and mass (Schaberg et al. 1997, Bauer et al. 2004). In our excised branch samples, NO_3^- additions significantly affected both average leaf area and average leaf mass (Table 3), but the nature of the NO_3^- effect varied by site and was positive (sites A and C), negative (B), or nearly neutral (D). As in these other studies (Schaberg et al. 1997, Bauer et al.

2004), the changes in leaf area and mass occurred in parallel and there was not a significant NO_3^- effect on specific leaf area (Table 3).

The changes in average leaf area and mass observed in this study seemed counterintuitive given that earlier assessments (Pregitzer et al. 2008) based on leaf litter collections had not found NO_3^- effects on total leaf mass (g/m^2) or leaf area index (m^2/m^2) at these sites. However, changes in average leaf area (cm^2/leaf) and mass (g/leaf) had not been assessed in the leaf litter data. Analysis of the leaf litter data from 2007 (A.J. Burton, unpublished) found changes in average leaf area and mass for sugar maple that paralleled the site-level NO_3^- effects we observed in excised branches, but were not significant ($P > 0.3$). In the leaf litter data, NO_3^- trends in average leaf area and mass for sugar maple were counteracted by opposite changes in leaf number (leaves/m^2). Applying these leaf counts to the changes in the average leaf mass and average leaf area of green leaves observed in the excised branch samples negates any potential NO_3^- effects on total leaf biomass (P = 0.390) and leaf area index (P = 0.876). Therefore, if the observed increases in aboveground growth in the $+NO_3^-$ plots (Pregitzer et al. 2008) were from additional C assimilation, these gains would have to come from increases in leaf-level photosynthesis.

Based on foliar N concentration alone, increases in leaf-level photosynthesis were plausible. In samples collected from both the canopy towers (Table 1) and from excised branches (Table 3), leaves in plots receiving NO_3^- additions had higher concentrations of N on both a mass (mg/g^1) and area basis (g/m^2). This is a response that has been consistently observed in other forests receiving chronic N additions (Hutchinson et al.

1998, Baron et al. 2000, Bauer et al. 2004, Elvir et al. 2005, Boggs et al. 2005). The effects of the NO_3^- additions were most pronounced at the site (A) receiving the least ambient N deposition, but leaf N was greater in plots receiving NO_3^- additions at all sites (Table 3). Many models suggest that this boost in foliar N should increase photosynthesis and forest growth (e.g., Aber et al. 1989, Aber et al. 1997, Hyvönen et al. 2007).

However, there is little evidence that NO_3^- additions stimulated photosynthesis. Although there were some modest differences in A-C_i measurements describing leaf biochemistry (Table 2), no significant overall change in photosynthesis was observed using instantaneous measurements. In the tower-based measurements of photosynthesis, there was one measurement date when the trees exposed to NO_3^- additions had significantly greater A_{area}, but there was little difference between NO_3^- and ambient trees across all measurements (Table 1). Similarly, there were no significant NO_3^- effects on photosynthesis on a leaf mass or area basis in excised branch measurements (Table 4), with A_{mass} differing only by 0.1% between treatments.

Likewise, there is little evidence from the integrated dual isotope measures of photosynthesis that NO_3^- additions stimulated photosynthesis. There were significant NO_3^- effects on both $\delta^{13}C$ and $\delta^{18}O$ in foliage collected from the canopy towers (Table 1), but these changes do not suggest increased photosynthesis. Instead, the decreases in both $\delta^{18}O$ and $\delta^{13}C$ respectively imply increased stomatal conductance (Barbour 2007) and higher chloroplast CO_2 concentrations (C_c) (Seibt et al. 2008), both consistent with instantaneous measurements (Table 1). There were no significant overall NO_3^- effects on

$\delta^{18}O$ or $\delta^{13}C$ in leaves from excised branch samples or litter samples (Fig. 2), and there was little variation in the size of the NO_3^- effects across the duration of the study (Supplementary Figs. 3 and 4). There were small differences in $\delta^{13}C$, with sites A, C, and D tending to be enriched and site B tending to be depleted (Fig. 2). However, the tight relationship between $\delta^{13}C$ and SLA noted earlier (and in Supplementary Fig. 1) appears to explain these trends. Thus, there is no evidence from stable isotope measurements that NO_3^- additions increased photosynthesis.

Across sites, across time, and irrespective of measurement technique, we did not find conclusive evidence that photosynthesis was stimulated in our N saturated forests. Similar results have been reported elsewhere (Schaberg et al. 1997, Bauer et al. 2004, Elvir et al. 2006) and have been attributed to nutrient deficiencies created by N saturation (Schaberg et al. 1997, Elvir et al. 2006). However, there is no evidence that nutrient imbalances were responsible for our findings. Base saturation in surface soils is high (70-96%) and preliminary analyses suggest that despite large leaching losses of N, there have not been consistent effects of the NO_3^- additions on foliar concentrations of Ca, K, Mg, Na, or P (K. Fedak and D.R. Zak, unpublished data). The failure to observe increased photosynthesis at our sites suggests that although decreases in foliar nutrients and the lack of an N stimulation of photosynthesis may be coincident in other studies, the relationship between the two may not always be causal.

We observed that NO_3^- additions decreased photosynthetic NUE (Table 4) and similar decreases have been repeatedly observed in N saturated forests (Schaberg et al. 1997,

Bauer et al 2004). In plants exposed to high levels of soil N, surplus foliar N can be stored in leaves as free amino acids (Bauer et al. 2004), soluble non-Rubisco proteins (Bauer et al. 2004), or as Rubisco (Warren et al. 2004). In cases where Rubisco is stored in leaves, much of the additional Rubisco appears to be physiologically inactive (Cheng and Fuchigami 2000, Warren et al. 2000, Warren et al. 2003). This would mean that much of the additional N found in the foliage of N saturated forests does not play an active role in C uptake, consistent with our finding of significantly lower photosynthetic NUE.

In our forests, aboveground production has increased in response to NO_3^- additions (Pregitzer et al. 2008). This has occurred without apparent gains in C assimilation because no differences in leaf area index (Pregitzer et al. 2008) or in leaf-level photosynthesis have been observed. We suspect an alternate hypothesis, with increases in aboveground productivity resulting from a shift in plant C allocation. There is no evidence that C allocation has shifted away from root production or root respiration (Burton et al. 2004). However, mycorrhizae and their associated fungi require large amounts of plant C (Hobbie 2006) and these associations can decline in ecosystems exposed to chronic N deposition (Egerton-Warburton and Allen 2000, Lilleskov et al. 2002, van Diepen et al. 2007). Although we are unaware of any studies that have linked N deposition-related growth increases to shifts in C allocation away from mycorrhizae, there have been significant decreases in the abundance of arbuscular mycorrhizae in response to N additions at our study sites (van Diepen et al. 2007). Although mycorrhizal

fungi appear to account for a significant portion of autotrophic respiration in these forests (van Diepen 2008), annual C allocation to mycorrhizae has not been quantified.

Conclusion

Although chronic N additions increased both foliar N and aboveground growth, there is little evidence that these changes are directly linked because we did not observe increases in leaf-level photosynthesis in either instantaneous measurements or with $\delta^{13}C$ and $\delta^{18}O$ measurements. Instead, PNUE has decreased and the additional foliar N may be largely inactive. Increases in aboveground growth in response to the N additions may instead be due to a shift in plant C allocation, possibly away from mycorrhizal fungi. If this result is confirmed, it would be a novel observation and provide a new mechanism for increases in aboveground growth. Furthermore, there is no evidence that the availability of other nutrients prevented gains in photosynthesis in response to NO_3^- additions. Overall, our findings suggest models that assume N deposition increases growth through gains in leaf-level photosynthesis may not be mechanistically accurate.

Literature Cited

Aber, J.D., W. McDowell, K. Nadelhoffer, A. Magill, G. Bernston, M. Kamakea, S. McNulty, W. Currie, L. Rustad, and I. Fernandez. 1998. Nitrogen saturation in temperate forest ecosystems. Bioscience 48: 921-934.

Aber, J.D., K.J. Nadelhoffer, P. Steudler, and J.M. Melillo. 1989. Nitrogen saturation in northern forest ecosystems. Bioscience 39: 378-387.

Aber, J.D., S.V. Ollinger, and C.T. Driscoll. 1997. Modeling nitrogen saturation in forests ecosystems in response to land use and atmospheric deposition. Ecological Modelling 101: 61-78.

Araus, J.L., T. Amaro, Y. Zuhair, and M.M. Nachit. 1997. Effect of leaf structure and water status on carbon isotope discrimination in field-grown durum wheat. Plant, Cell and Environment 20: 1484-1494.

Barbour, M.M. 2007. Stable oxygen isotope composition of plant tissue: a review. Functional Plant Biology 34: 83-94.

Baron, J.S., H.M. Rueth, A.M. Wolfe, K.R. Nydick, E.J. Allstott, J.T. Minear, and B. Moraska, 2000. Ecosystem responses to nitrogen deposition in the Colorado Front Range. Ecosystems 3: 352-368.

Bauer, G.A., F.A. Bazzaz, R. Minocha, S. Long, A.Magill, J. Aber, and G.M. Berntson. 2004. Effects of chronic N additions on tissue chemistry, photosynthetic capacity, and carbon sequestration potential of a red pine (*Pinus resinosa* Ait.) stand in the NE United States. Forest Ecology and Management. 196: 173-186.

Boggs, J.L., S.G. McNulty, M.J. Gavanni, and J.M.Myers. 2005. Tree growth, foliar chemistry, and nitrogen cycling across a nitrogen deposition gradient in southern Appalachian deciduous forest. Canadian Journal of Forest Research 35: 1901-1913.

Braun, E.L. 1950. *Deciduous Forests of Eastern North America*. Macmillan, New York. 596 p.

Burton, A.J., K.S. Pregitzer, J.N. Crawford, G.P. Zogg, and D.R. Zak. 2004. Simulated chronic NO_3^- deposition reduces soil respiration in northern hardwood forests. Global Change Biology 10: 1080-1091.

Cheng, L., and L.H. Fuchigami. 2000. Rubisco activation state decreases with increasing nitrogen content in apple leaves. Journal of Experimental Botany 51: 1687-1694.

Dentener, F. et al. 2006. The global atmospheric environment for the next generation. Environmental Science and Technology 40: 3586-3594.

de Vries, W., G.J. Reinds, P. Gundersen, and H. Sterba. 2006. The impact of nitrogen deposition on carbon sequestration in European forests and forest soils. Global Change Biology 12: 1151-1173.

Egerton-Warburton, L.M., and E.B. Allen. 2000. Shifts in arbuscular mycorrhizal communities along an anthropogenic nitrogen deposition gradient. Ecological Applications 10: 484-496.

Elvir, J.A., L. Rustad, G.B. Wiersma, I.J. Fernandez, A.S. White, and G.J. White,. 2005. Eleven-year response of foliar chemistry to chronic nitrogen and sulfur additions at the Bear Brook Watershed in Maine. Canadian Journal of Forest Research. 35: 1402-1410.

Evans, J.R. 1989. Photosynthesis and nitrogen relationships in leaves of C3 plants. Oecologia 78: 9-19.

Farquhar, G.D., J.R. Ehleringer, and K.T. Hubick. 1989. Carbon isotope discrimination and photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology 40: 503-557.

Galloway, J.N., A.R. Townsend, J.W. Erisman, M. Bekunda, Z. Cai, J.R. Freney, L.A. Martinelli, S.P. Seitzinger, and M.A. Sutton. 2008. Transformations of the nitrogen cycle: recent trends, questions, and potential solutions. Science 320: 889-892.

Gower, S., P. Reich, and Y. Son, 1993. Canopy dynamics and aboveground production of five tree species with different leaf longevities. Tree Physiology 12: 327-345.

Hanba, Y.T., S.-I., Miyazawa, and I. Terashima. 1999. The influence of leaf thickness on the CO_2 transfer conductance and leaf stable carbon isotope ratio for some evergreen tree species in Japanese warm-temperate forests. Functional Ecology 13: 632-639.

Hobbie, E.A. 2006. Carbon allocation to ectomycorrhizal fungi correlates with belowground allocation in culture studies. Ecology 87: 563-569.

Holland, E.A., B.H. Braswell, J. Sulzman, and J.-F. Lamarque. 2005 Nitrogen deposition onto the United States and Western Europe: Synthesis of observations and models. Ecological Applications 15: 38-57.

Hultine, K.R., and J.D. Marshall. 2000. Altitude trends in conifer leaf morphology and stable carbon isotope discrimination. Oecologia 123: 32-40.

Hutchinson, T.C., S.A. Watmough, E. P. S. Sager, and J.D. Karagatzides. 1998. Effects of excess nitrogen deposition and soil acidification on sugar maple (*Acer saccharum*) in Ontario Canada: an experimental study. Canadian Journal of Forest Research 28: 299-310.

Hyvönen, R., et al. 2007. The likely impact of elevated [CO_2], nitrogen deposition, increased temperature and management on carbon sequestration in temperate and boreal forest ecosystems: a literature review. New Phytologist 173: 463-480.

Jefts, S., I.J. Fernandez, L.E. Rustad, and D.B. Dail. 2004. Decadal responses in soil N dynamics at Bear Brook Watershed in Maine, USA. Forest Ecology and Management 189: 189-205.

Keel, S.G., S. Pepin, S. Louzinger, and C. Körner. 2007. Stomatal conductance in mature deciduous forest trees exposed to elevated CO_2. Trees 21: 151-159.

Körner, C., G.D. Farquhar, and S.C. Wong, 1991. Carbon isotope discrimination by plants follows latitudinal and altitudinal trends. Oecologia 88: 30-40.

LeBauer, D.S. and K.K. Treseder. 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. Ecology 89: 371-379.

Lilleskov, E.A., T.J. Fahey, T.R. Horton, and G.M. Lovett. 2002. Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. Ecology 83: 104-115.

Littell, R.C., G.C. Milliken, W.W. Stroup, and R.D. Wolfinger. 1996. SAS System for Mixed Models. SAS Institute Inc., Cary, NC. 633 p.

Long, S.P. and C.J. Bernacchi. 2003. Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. Journal of Experimental Botany 54: 2393-2401.

MacDonald, N.W., A.J. Burton, H.O. Liechty, J.A. Witter, K.S. Pregitzer, G.D. Mroz, and D.D. Richter, 1992. Ion leaching in forest ecosystems along a Great Lakes air pollution gradient. Journal of Environmental Quality. 21: 614-623.

Magnani F, et al. 2007 The human footprint in the carbon cycle of temperate and boreal forests. Nature 447: 848-852.

Monclus, R., et al. 2005. Productivity, leaf traits, and carbon isotope discrimination in 29 *Populus deltoids x P. nigra* clones. New Phytologist 167: 53-62.

Nadelhoffer, K.J., B.A. Emmett, P. Gundersen, O.J. Kjonaas, C.J. Koopmans, P. Schleppi, A. Tietema, and R.F. Wright. 1999. Nitrogen deposition makes a minor contribution to carbon sequestration in temperate forests. Nature 398: 145-148.

Niinemets, U, A. Diaz-Espejo, J. Flexas, J. Galmes, and C.R. Warren. 2009. Role of mesophyll conductance in constraining potential photosynthetic productivity in the field. Journal of Experimental Botany 60: 2249-2270.

Ollinger, S.V., J.D. Aber, P.B. Reich, and R.J. Freuder. 2002. Interactive effects of nitrogen deposition, tropospheric ozone, elevated CO_2 and land use history on the carbon dynamics of northern hardwoods forests. Global Change Biology 8: 545-562.

Pregitzer, K.S., A.J. Burton , D.R. Zak, and A.F. Talhelm 2008. Simulated chronic nitrogen deposition increases carbon storage in Northern Temperate forests. Global Change Biology 14: 142-153.

Pregitzer, K.S., D.R. Zak, A.J. Burton, J.A. Ashby, and N.W. MacDonald. 2004. Chronic nitrate additions dramatically increase the export of carbon and nitrogen from northern hardwood ecosystems. Biogeochemistry 68: 179-197.

Ryan, M.G., D. Binkley, J.H. Fownes, C.P. Giardina, and R.S. Senock. 2004. An experimental test of the causes of forest growth decline with stand age. Ecological Monographs 74: 393-414.

Schaberg, P.G., T.D. Perkins, and S.G. McNulty. 1997. Effects of chronic low-level N additions on foliar elemental concentrations, morphology, and gas exchange of mature montane red spruce. Canadian Journal of Forest Research 27: 1622-1629.

Scheidegger, Y., M. Saurer, M. Bahn, and R. Siegwolf. 2000. Linking oxygen and carbon isotopes with stomatal conductance and photosynthetic capacity: a conceptual model. Oecologia 125: 350-357.

Seibt, U., A. Rajabi, H. Griffiths, and J.A. Berry. 2008. Carbon isotopes and water-use efficiency: sense and sensitivity. Oecologia 155: 441-454.

Sharkey, T.D., C.J. Bernacchi, G.D. Farquhar, and E.L. Singsaas. 2007. Fitting photosynthetic carbon dioxide response curves for C_3 leaves. Plant, Cell and Environment 30: 1035-1040.

Sievering, H., I. Fernandez, J. Lee, J. Hom, and L. Rustad. 2004. Forest canopy uptake of atmospheric nitrogen deposition at eastern U.S. conifer sites: Carbon storage implications? Global Biogeochemical Cycles 14: 1153-1159.

Takahashi, K., and Y. Miyajima. 2008. Relationships between leaf life span, leaf mass per area, and leaf nitrogen cause different altitudinal changes in leaf $\delta^{13}C$ between deciduous and evergreen species. Botany 86: 1233-1241.

Thomas, R.Q., C.D. Canham, K.C. Weathers, and C.L. Goodale. 2010. Increased tree carbon storage in response to nitrogen deposition in the US. Nature Geoscience 3: 13-17.

van Diepen, L.T.A., E.A. Lilleskov, K.S. Pregitzer, and R.M. Miller. 2007. Decline of arbuscular mycorrhizal fungi in northern hardwood forests exposed to chronic nitrogen amendments. New Phytologist 176: 175-183.

van Diepen, L.T.A. 2008. The role and diversity of arbuscular mycorrhizal fungi in *Acer saccharum* dominated forest ecosystems under natural and N-amended conditions. PhD Dissertation. Michigan Technological University, Houghton, Michigan, USA.

Warren, C.R., M.A. Adams, and Z. Chen, 2000. Is photosynthesis related to concentrations of nitrogen and Rubisco in leaves of Australian native plants? Australian Journal of Plant Physiology 27:407-416.

Warren, C.R., E. Dreyer, and M.A. Adams. 2003. Photosynthesis-Rubisco relationships in foliage of *Pinus sylvestris* in response to nitrogen supply and the proposed role of Rubisco and amino acids as nitrogen stores. Trees 17: 359-366.

Zak, D.R., W.E. Holmes, A.J. Burton, K.S. Pregitzer, and A.F. Talhelm. 2008. Simulated atmospheric NO_3^- deposition increases soil organic matter by slowing decomposition. Ecological Applications 18: 2016-2027.

Zogg, G.P., D.R. Zak, K.S. Pregitzer, and A.J. Burton, 2000. Microbial immobilization and the retention of anthropogenic nitrate in northern hardwoods forests. Ecology 81:1858-1866.

Table 1: Gas exchange and leaf traits from tower based instantaneous measurements

		Means		*P* values	
	Sample Dates	Ambient	+NO_3	NO_3^- Effect	Date × NO_3^-
A_{area} (μmol CO_2 m^{-2} s^{-1})	13	6.5 (0.2)	6.6 (0.2)	0.750	**0.005**
Conductance (mol H_2O m^{-2} s^{-1})	13	0.072 (0.003)	0.083 (0.002)	0.076	0.143
C_i (μmol CO_2/mol)	13	214.8 (3.4)	227.1 (3.0)	**0.047**	**0.037**
Foliar N_{mass} (mg/g)	10	16.3 (0.4)	18.3 (0.4)	**0.015**	0.068
Leaf Area (cm^2)	10	35.7 (4.1)	39.8 (3.5)	0.394	0.896
Leaf Mass (g)	10	0.36 (0.04)	0.36 (0.03)	0.955	0.115
SLA (cm^2/g)	10	108.9 (3.6)	113.1 (3.2)	0.410	0.106
A_{mass} (nmol CO_2 s^{-1} g^{-1})	10	66.0 (3.5)	67.4 (3.2)	0.771	0.233
N_{area} (g N/m^2)	10	1.52 (0.07)	1.68 (0.06)	0.140	**0.037**
NUE (μmol CO_2 s^{-1} g^{-1} N)	10	4.01 (0.21)	3.60 (0.19)	0.122	0.216
$\delta^{18}O$ (‰)	10	26.8 (0.1)	25.9 (0.1)	**<0.001**	**<0.001**
$\delta^{13}C$ (‰)	10	-25.7 (0.3)	-27.0 (0.3)	**0.023**	0.496

Statistical results with $P < 0.05$ are in bold. In addition to the results provided, date was a highly significant factor for each variable $P < 0.001$.

Table 2: Tower based A-Ci measurements and mean leaf traits during these measurements.

	Means		*P* values NO_3^- Effect
	Ambient	$+NO_3^-$	
$V_{cmax\text{-}area}$ (μmol CO_2 m^{-2} s^{-1})	90.7 (4.7)	105.7 (4.1)	0.061
J_{max} (μmol CO_2 m^{-2} s^{-1})	124.0 (5.6)	133.8 (4.9)	0.246
$R_{d\text{-}area}$ (μmol CO_2 m^{-2} s^{-1})	4.6 (0.6)	6.2 (0.5)	0.092
A_{area} (μmol CO_2 m^{-2} s^{-1})	6.8 (0.6)	7.9 (0.5)	0.219
Photo/R_d	1.5 (0.2)	1.3 (0.2)	0.599
SLA (cm^2/g)	101.7 (5.6)	101.4 (4.9)	0.972
$V_{cmax\text{-}mass}$ (nmol CO_2 g^{-1} s^{-1})	920.5 (54.7)	1068.9 (47.4)	0.096
$J_{max\text{-}mass}$ (nmol CO_2 g^{-1} s^{-1})	1.26 (0.08)	1.35 (0.07)	0.412
$R_{d\text{-}mass}$ (nmol CO_2 g^{-1} s^{-1})	46.5 (7.0)	63.0 (6.0)	0.135
Foliar N_{area} (g N/m^2)	1.7 (0.1)	1.9 (0.1)	0.108
$V_{cmax\text{-}N}$ (μmol CO_2 g^{-1} N s^{-1})	55.0 (2.3)	56.8 (2.0)	0.573
$J_{max\text{-}N}$ (μmol CO_2 g^{-1} N s^{-1})	75.2 (3.9)	72.0 (3.3)	0.560
$R_{d\text{-}N}$ (μmol CO_2 g^{-1} N s^{-1})	2.8 (0.4)	3.3 (0.3)	0.300

Data are means (SE). n = 3 for ambient, n = 4 for $+NO_3^-$

Table 3: Leaf traits from excised branch samples at each study site.

Site	Treatment	Foliar N_{mass} (mg/g)	Leaf Area (cm^2)	Leaf Mass (g)	SLA (cm^2/g)	Foliar N_{area} (g/m^2)
A	Ambient	14.3 (0.4)	33.5 (1.3)	0.22 (0.01)	155.8 (3.3)	0.9 (0.0)
	$+NO_3^-$	18.4 (0.5)*	37.1 (1.4)	0.27 (0.01)*	145.6 (5.1)	1.3 (0.1)*
B	Ambient	16.3 (0.5)	31.3 (1.2)	0.24 (0.01)	139.6 (6.2)	1.2 (0.0)
	$+NO_3^-$	18.1 (0.5)*	27.0 (1.7)	0.19 (0.01)*	143.2 (4.3)	1.3 (0.0)
C	Ambient	18.2 (0.4)	31.4 (2.0)	0.19 (0.01)	176.9 (5.7)	1.1 (0.0)
	$+NO_3^-$	19.9 (0.4)	37.7 (2.1)	0.24 (0.02)*	162.3 (6.1)	1.3 (0.1)*
D	Ambient	17.9 (0.6)	33.8 (1.3)	0.19 (0.01)	184.7 (4.4)	1.0 (0.0)
	$+NO_3^-$	19.5 (0.4)	33.4 (1.8)	0.20 (0.02)	186.3 (10.9)	1.1 (0.1)
P values						
Site		**< 0.001**	**0.008**	**0.001**	**< 0.001**	**< 0.001**
NO_3^-		**< 0.001**	0.297	**0.027**	0.215	**< 0.001**
Site × NO_3^-		**0.038**	**0.030**	**< 0.001**	0.291	**0.002**
Date		**< 0.001**	0.202	0.269	**< 0.001**	0.098
Site × Date		0.089	0.259	0.146	**0.042**	**0.016**
Date × NO_3^-		0.703	0.212	0.098	0.761	0.701
Site × Date × NO_3^-		0.827	0.582	0.173	0.302	0.435

Statistical results with $P < 0.05$ are in bold. Data are means (SE). $n = 3$. Site-level differences between ambient and $+NO_3^-$ with $P < 0.05$ are represented by asterisk (*).

Table 4: Gas exchange measurements from excised branch samples at each study site.

Site	Treatment	A_{area} (μmol CO_2 m^{-2} s^{-1})	A_{mass} (nmol CO_2 g^{-1} s^{-1})	NUE (μmol CO_2 g^{-1} N s^{-1})	Cond (mol H_2O m^{-2} s^{-1})	C_i (μmol CO_2/mol)
A	Ambient	6.7 (0.3)	100.3 (4.1)	7.1 (0.3)	0.09 (0.01)	237.6 (15.6)
	+NO_3^-	7.0 (0.3)	100.9 (2.6)	5.5 (0.3)	0.11 (0.02)	236.9 (13.9)
B	Ambient	5.9 (0.5)	82.7 (9.2)	5.0 (0.4)	0.11 (0.01)	268.4 (4.4)
	+NO_3^-	5.9 (0.5)	84.0 (7.5)	4.7 (0.4)	0.12 (0.02)	270.8 (6.5)
C	Ambient	6.7 (0.5)	114.8 (11.3)	6.4 (0.6)	0.13 (0.01)	283.5 (8.6)
	+NO_3^-	6.5 (0.4)	102.8 (5.9)	5.2 (0.3)	0.12 (0.01)	280.6 (9.4)
D	Ambient	5.3 (0.3)	92.9 (5.6)	5.3 (0.3)	0.12 (0.01)	283.1 (8.5)
	+NO_3^-	6.0 (0.2)	103.6 (4.4)	5.3 (0.2)	0.11 (0.01)	270.4 (10.3)
P values						
Site		**0.009**	**0.008**	**0.014**	0.068	**< 0.001**
NO_3^-		0.407	0.978	**0.013**	0.752	0.466
Site × NO_3^-		0.630	0.387	0.204	0.664	0.684
Date		**0.003**	**0.002**	0.255	**<.001**	**< 0.001**
Site × Date		0.059	0.173	**0.026**	**0.008**	**< 0.001**
Date × NO_3^-		0.853	0.637	0.743	0.397	0.082
Site × Date × NO_3^-		0.943	0.915	0.762	0.306	0.233

Statistical results with $P < 0.05$ are in bold. Data are means (SE). $n = 3$.

Figure Captions

Figure 1. Site locations of the Michigan N Gradient study.

Figure 2. Mean leaf $\delta^{13}C$ and $\delta^{18}O$ values for (A) leaves collected from excised branches in 2006 and 2007 or (B) from leaf litter collected from 1994-2007. Empty circles represent plots receiving ambient atmospheric deposition and filled circles represent plots receiving chronic NO_3^- additions. Dashed ovals group the ambient and $+NO_3$ plots from each site, with adjacent site labels (A-D) as in figure 1. Error bars are ± 1 SE.

Figure 1

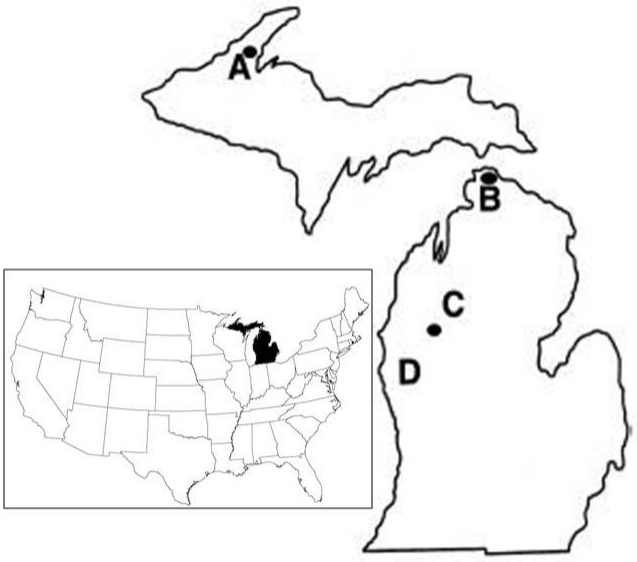
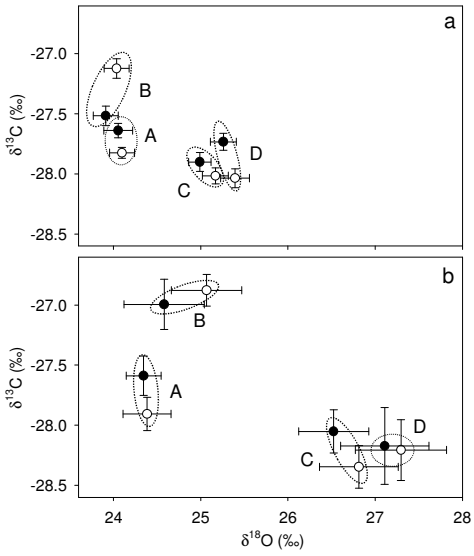


Figure 2



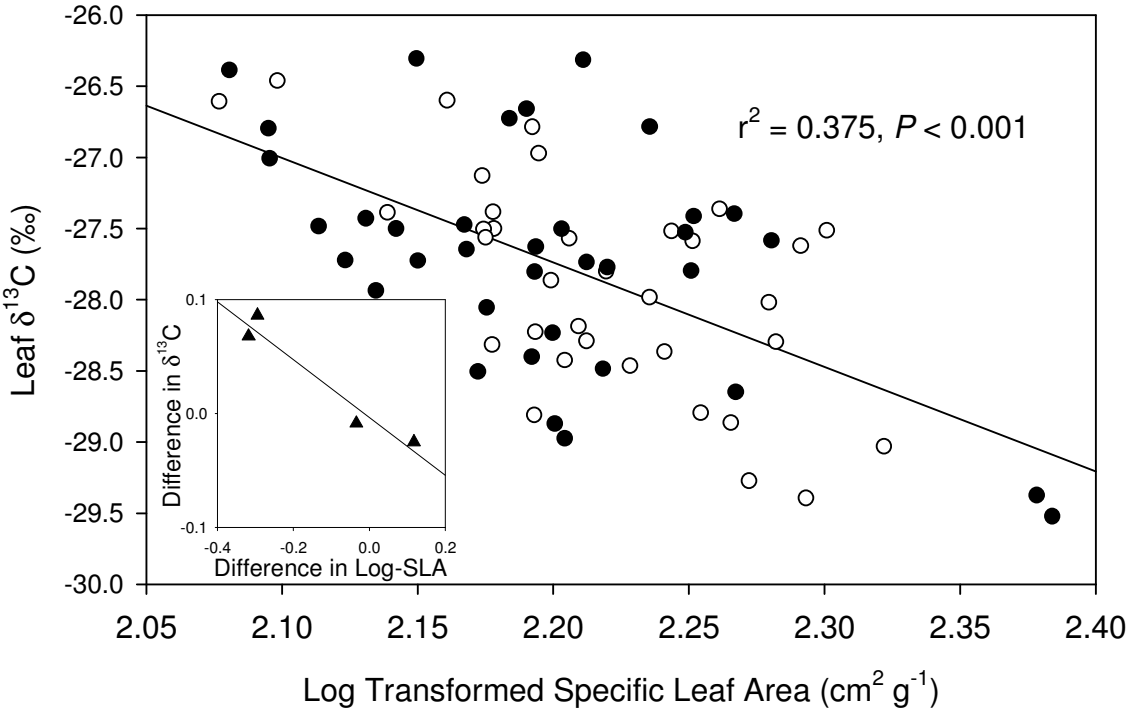
Supplementary Figure Captions

Supplementary Figure 1: Relationship between leaf $\delta^{13}C$ and log-transformed specific leaf area (SLA) for excised branch samples for each plot during the three sampling periods (n = 72). Empty circles represent ambient plots, filled circles represent $+NO_3^-$ plots. The fitted regression line represents the overall relationship between SLA and $\delta^{13}C$ because this relationship did not significantly differ between ambient and $+NO_3^-$ plots. Inset is the relationship between the average difference among ambient and $+NO_3^-$ plots at each site (n = 4) in $\delta^{13}C$ and the difference between these plots in log-transformed SLA. For the inset, $r^2 = 0.938$ and $P = 0.032$.

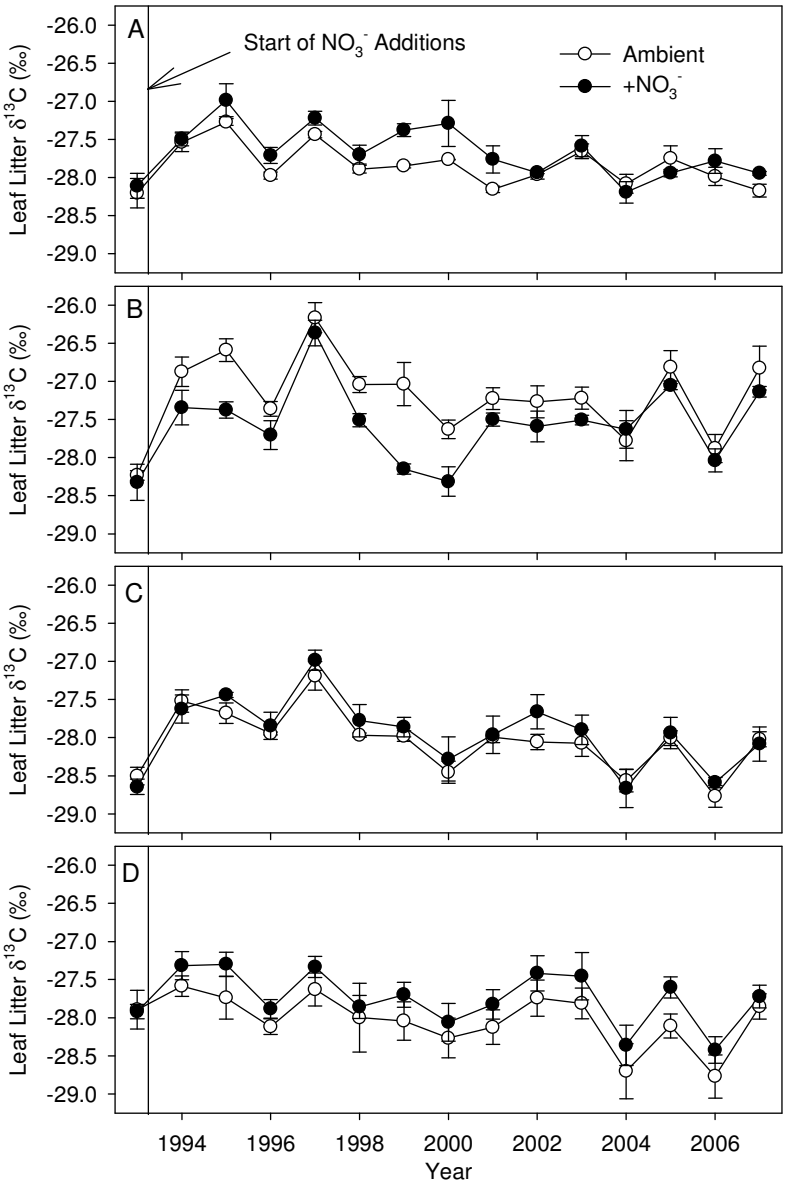
Supplementary Figure 2: Annual mean leaf litter $\delta^{13}C$ for ambient (empty circles) and $+NO_3^-$ (filled circles) at each of the four sites (A-D) including one year (1993) prior to NO_3^- additions. Site designations as in Fig. 1 of the main text. Error bars are ± 1 SE.

Supplementary Figure 3: Annual mean leaf litter $\delta^{18}O$ for ambient (empty circles) and $+NO_3^-$ (filled circles) at each of the four sites (A-D) including one year (1993) prior to NO_3^- additions. Site designations as in Fig. 1 of the main text. Error bars are ± 1 SE.

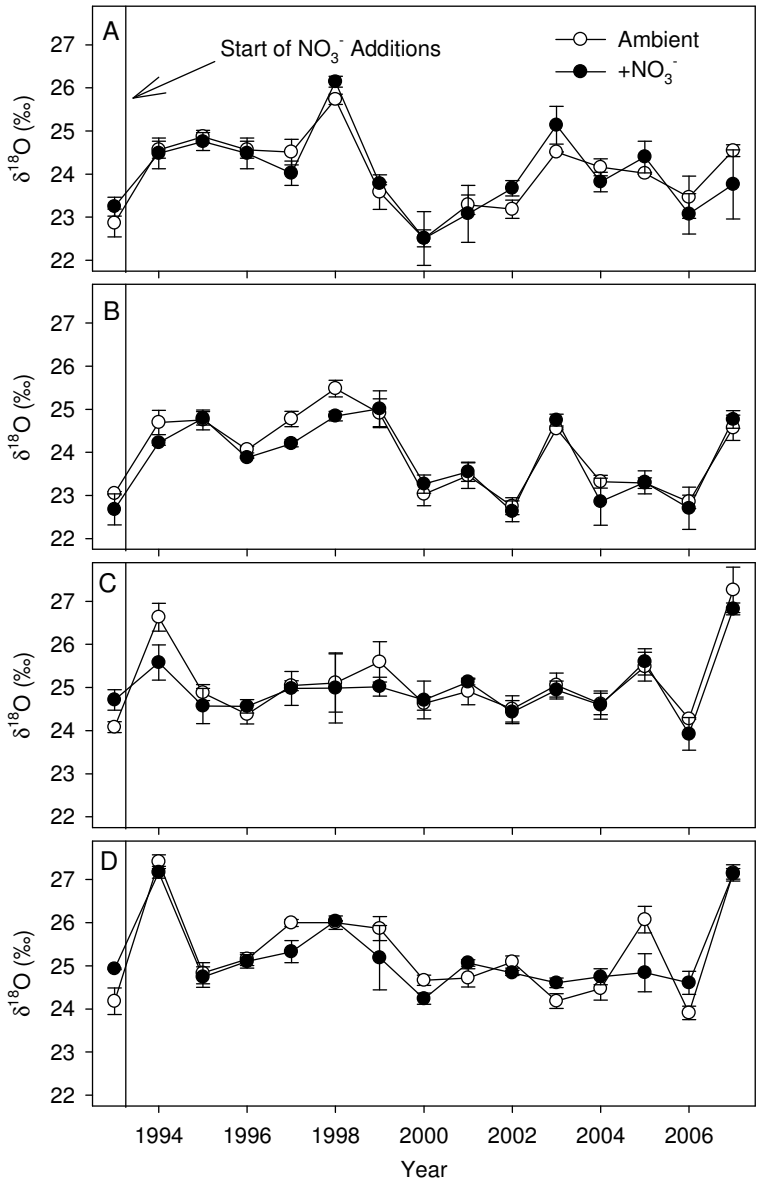
Supplementary Figure 1



Supplementary Fig. 2



Supplementary Figure 3



Chapter Six: Conclusions and Recommendations

As is often the case in ecological research, each of the four studies described here has obvious limitations that restrict its insight. In chapter two, soil C sampling occurred only in the top 20 cm of the mineral soil. This part of the soil typically contains the highest concentrations of C and receives the greatest inputs of plant C, but changes in the top 20 cm are not necessarily predictive of differences deeper in the soil profile. A more exhaustive sampling of the soil profile was conducted in 2009 and the resulting data will assess the treatment effects on soil C storage to one meter in depth. In chapter three, I attempted to use leaf litter collections to assess annual leaf production and leaf area. However, in neither case is an estimate derived from leaf litter collections a completely accurate estimate. In particular, the effects of herbivory are unaccounted for in these data and likely had a meaningful influence on our estimates of leaf production in some years (C.P. Giardina, personal communication). Similarly, the annual estimate of leaf area derived from litter collections is not a completely accurate assessment of leaf area index. Factors such as indeterminate growth, herbivory, premature senescence, and weather dynamically affect leaf area throughout the growing season.

Although there were clear trends in leaf litter calcium, aluminum, sulfur concentrations in the Michigan gradient study (chapter four), these changes are only proxies for effects of acid deposition on soil solution chemistry. A more direct measure of soil solution chemistry from lysimeter samples would have been preferable for this analysis, but relevant measurements have been made infrequently over the past two decades. Likewise, measurements of green leaf N concentrations and $\delta^{15}N$ values would have been

preferable when assessing the effects of ambient N deposition in this experiment. This is because the N chemistry of green leaves more frequently measured in other studies, meaning that our results would have been more comparable to other experiments. Finally, the assessment of leaf-level photosynthesis (chapter five) is handicapped because sampling only occurred periodically. In particular, sampling in the spring during leaf development and in the fall during senescence was limited. The additional leaf N could have played a more active role in photosynthesis during the physiologically dynamic periods of the growing season.

Despite these limitations, each study demonstrates the value of long-term ecological research experiments. At the Rhinelander FACE experiment, the multi-year trend of decreasing soil carbon in the aspen-only community did not become statistically significant until the eleventh year of the experiment (chapter two). Similarly, the declining stimulation of leaf production by the elevated carbon dioxide (CO_2) treatment and the increasingly negative effect of the elevated ozone (O_3) treatment (chapter three) would not have been evident if the experiment had been carried out for less than a decade. In the Michigan gradient experiment, neither the decreases in leaf calcium, sulfur, and aluminum nor the increase in soil nitrate leaching would have been apparent had these measurements been concluded in the year 2000, thirteen years after the study was established (chapter four). Increases in leaf-level photosynthesis in response to nitrogen (N) additions (chapter five) would not have been hypothesized had there not been an increase in aboveground growth, a change that did not become significant until the late 1990s. Although some of these results could have been predicted (such as

increased tree growth with N additions), other effects were not suggested by the results of previous experiments (such as the decrease in soil carbon (C) under elevated CO_2). The CO_2 effects on soil C, the O_3 effects on soil C, and the effects of N additions on photosynthesis were all poorly predicted by existing models. Overall, these results suggest the information we now have regarding the impacts of global change on forest ecosystem function is insufficient. Clearly, long-term research creates valuable opportunities for insight that would otherwise not be possible. Funding agencies should balance the development of new experiments with the maintenance of long-term research projects so that the scientific community and society at large can continue to benefit from the insights provided by both types of research.

In addition to highlighting the value of long-term research, the results from these studies suggest that society cannot necessarily expect forest ecosystems to absorb and buffer anthropogenic changes in the environment. At the Rhinelander FACE experiment, the stimulatory effect of the elevated CO_2 treatment on leaf production had declined significantly by the eleventh year of the study and no additional C had been sequestered in the soil. Together, these results challenge the idea that the growth in atmospheric CO_2 will be buffered by large increases in forest C storage. In the Michigan gradient study, the capacity of the forests to absorb current levels of atmospheric N deposition is clearly limited. Future additions of atmospheric N may further alter the biogeochemistry of these forests. However, the observed decrease in the influence of acid deposition on forest biogeochemistry is positive sign. That such a change occurred so quickly in response to a

change in environmental policy suggests that forests may be resilient if and when changes are made to reduce atmospheric CO_2, tropospheric O_3, and N deposition.