

Carbon Dioxide Effects on Heterotrophic Dinitrogen Fixation in a Temperate Pine Forest

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Increased net primary productivity (NPP) under elevated atmospheric CO₂ requires additional N inputs to sustain C sequestration. We hypothesized that heterotrophic N₂ fixation would be stimulated by enhanced litter production under elevated CO₂, thus augmenting N availability to plants. To test if N₂ fixation is limited by organic substrates alone or in combination with nutrients required for the nitrogenase enzyme, we measured nitrogenase activity (acetylene reduction) in laboratory incubations with water, nutrient, and O₂ manipulations. Response of N₂ fixation to water, glucose, P, Fe, or Mo was measured under aerobic and anaerobic conditions in forest floor and mineral soil samples from the Duke Forest, NC. Potential nitrogenase activity in forest floor and mineral soil from the Duke Forest Free Air CO₂ Enrichment (FACE) site were measured to determine if elevated CO₂ enhances N₂ fixation. In homogenized slurries with glucose additions, nitrogenase activity was 2 and 400 times greater than controls in organic and mineral soils, respectively ($P < 0.01$). In laboratory studies, water additions increased N₂ fixation 25-fold in intact soil cores ($P < 0.01$). Additions of nutrients alone or in combination with C and water did not consistently stimulate N₂ fixation in intact soil cores. We detected no CO₂ effect on potential nitrogenase activity in Duke FACE soil. Since heterotrophic N₂ fixation is not stimulated in temperate pine forests under elevated CO₂, additional N assimilation by trees will require increased acquisition of endogenous N, such as increased nutrient use efficiency or enhanced root exploration of the soil.

Abbreviations: FACE, free air carbon dioxide enrichment; NPP, net primary production.

The rapid increase of atmospheric CO₂ during the past century has focused attention on C sequestration in forests as a means of mitigating this anthropogenic change in atmospheric chemistry. Free Air CO₂ Enrichment research has demonstrated that increased atmospheric CO₂ enhances NPP in forest ecosystems. Additional N is required to sustain enhanced NPP and promote C sequestration for the long term (Hungate et al., 2003), but the source of this additional N supply has not been identified. One potential mechanism of increasing plant-available N is through increased heterotrophic N₂ fixation in the soil.

Energy availability (i.e., organic substrates) most frequently limits N₂ fixation. If the availability of organic substrates limits heterotrophic N₂ fixation rates, a greater supply of C derived from higher NPP under elevated atmospheric CO₂ could stimulate asymbiotic N₂ fixation. Increased photosynthesis would augment above- and belowground litter production as well as enhancing root exudation of labile substrates (Zak et al., 1993; Norby, 1994; Finzi et al., 2001). More C would be available to stimulate heterotrophic activity, potentially subsidizing the energetic demands for heterotrophic N₂ fixation. Increased substrate availability under elevated CO₂ could enhance N₂ fixation to sustain increased NPP and to sequester atmospheric CO₂ (Gifford et al., 1996).

Under elevated CO₂, enhanced primary production can stimulate symbiotic N₂ fixation (Thomas et al., 1991; Hartwig et al., 2000; Luscher et al., 2000; Hungate et al., 2004). Heterotrophic N₂ fixation has been shown to increase under elevated CO₂ in soil environments with low redox potential (Dakora and Drake, 2000; Cheng et al., 2001; Hoque et al., 2001). No published studies to date have evaluated the effects of elevated CO₂ on heterotrophic N₂ fixation in well-oxygenated forest soils, which are the primary focus for terrestrial C sequestration.

In addition to energy requirements, it is possible that heterotrophic N₂ fixation is limited by nutrients or O₂. Biological N₂ reduction requires Fe, Mo, and P. Iron and Mo are required for the nitrogenase enzyme, responsible for biological N₂ reduction. Phosphorus has been linked to the activation of the gene for nitrogenase synthesis in bacteria (Stock et al., 1990). In addition, O₂ in the soil can limit N₂ fixation rates, since O₂ reacts with the Fe component of the nitrogenase enzyme, rendering the enzyme permanently inactive (Stacey et al., 1992). Soil water limits diffusion of O₂ in soils, and strongly influences asymbiotic N₂ fixation in woody debris in forest ecosystems (Wei and Kimmins, 1998; Hicks et al., 2003).

Our objectives were to determine (i) if substrate availability limits heterotrophic N₂ fixation in Duke Forest soils and (ii) if increased NPP and C inputs could enhance heterotrophic N₂ fixation. To address the hypothesis that energy limits heterotrophic N₂ fixation in Duke Forest soils, we conducted laboratory manipulations to examine factors controlling N₂ fixation, including C and nutrient supply, O₂ in the soil pore space, and water availability. Our second hypothesis was that greater plant growth and litter input under elevated atmospheric CO₂ stimulates heterotrophic N₂ fixation. To test this

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hypothesis, we estimated rates of heterotrophic nitrogenase activity in intact forest floor and mineral soil samples from the Duke Forest FACE site on five dates during 3 yr.

MATERIALS AND METHODS

Study Site

Soils were sampled from the Duke Forest FACE site, Orange County, North Carolina, to compare rates of asymbiotic N_2 fixation under conditions of ambient and elevated atmospheric CO_2 . The Duke FACE experiment maintains three 30-m-diameter treatment plots (ambient $CO_2 + 200 \mu L L^{-1}$) and three similar control plots (ambient $CO_2 \sim 370 \mu L L^{-1} CO_2$) in a loblolly pine (*Pinus taeda* L.) forest planted in 1983. Pine comprises 98% of the basal area (DeLucia et al., 1999) with the canopy reaching 20 m. The site has deep and highly weathered soils, developed from igneous parent materials and classified as Ultic Hapludalfs of the Enon series, with a pH of 5.75. The CO_2 treatment was initiated on 27 Aug. 1996. For further details of the experiment, see Hendrey et al. (1999). No known symbiotic N_2 fixers inhabit the Duke Forest FACE site. Although several members of the genus *Cercis* can fix N_2 , nitrogenase activity in the *Cercis canadensis* L. present at the site has not been observed (Bryan et al., 1996).

Rates of Heterotrophic Dinitrogen Fixation in Duke Forest Soil

To quantify free-living N_2 fixation rates, forest floor samples (100 cm^2) and mineral soil cores (5 cm diameter by 10 cm deep) were collected on five dates (September 2000, May and August 2001, September 2002, and January 2003). One forest floor and one mineral soil sample (0–10 cm) were taken from each of four sectors in each FACE plot. Each mineral soil sample was collected directly below the corresponding forest floor sample. Samples were placed on ice in the field, returned to the laboratory, and immediately incubated for measurements of N_2 fixation. Nitrogenase activity was measured on intact soil cores and forest floor samples using the acetylene reduction assay at room temperature (Hardy et al., 1968; Hendrickson, 1990). Gas-tight incubation chambers (1-L mason jars) were fitted with rubber septa and sealed. Jars containing field samples were injected with 30 mL of air, and the volume of the headspace was measured using a pressure transducer. Jars were equilibrated to atmospheric pressure, and 10% of the headspace was removed and replaced with acetylene (C_2H_2) generated from the reaction of CaC_2 and water.

Samples were incubated at field moisture for 3 h, after which a 2-mL gas sample was withdrawn and analyzed for C_2H_4 concentration on a Varian gas chromatograph (Model 3700, Varian Inc., Palo Alto, CA) using a flame ionization detector and a Poropak N column with He as the carrier gas. Two samples from each FACE plot were incubated without C_2H_2 to determine background levels of net C_2H_4 production. Empty chambers incubated with 10% C_2H_2 were used to determine background C_2H_4 generated by C_2H_2 production (blank correction). Ethylene produced during acetylene generation (average [mean \pm SE] $0.03 \pm 0.0009 \mu mol L^{-1}$) was equivalent to 5% of the sample ethylene production. Gravimetric soil moisture was measured after the incubation and acetylene-reduction rates were calculated on a dry-weight basis.

Laboratory Manipulations of Duke Forest Soil

We used laboratory incubations with C, water, and nutrient additions under aerobic and anaerobic conditions to test for limitations to N_2 fixation in this forest. Incubations were performed on intact soil cores (0–10 cm) collected adjacent to FACE plots in the Duke Forest in

November 2001. Four samples were collected for each treatment, including controls, and four additional cores were collected to determine gravimetric soil moisture content. Nutrient additions included C as glucose, Fe as FeDTPA, Mo as MoO_3 , and P as KH_2PO_4 . We chose C additions ($460 mg C kg^{-1}$ soil) based on the annual increment of detrital C between high CO_2 and ambient plots ($60 g C m^{-2}$; Schlesinger and Lichter 2001). Similarly, we added P at 20% of the annual litterfall input to the soil or $50.4 mg P m^{-2}$ ($0.388 mg P kg^{-1}$ soil; Finzi et al., 2001). We added micronutrients (Mo and Fe) based on the molar ratio of each micronutrient to P in Hoagland's solution (viz. for each mole of P, we added 0.1 mol Mo kg^{-1} soil and 22 mol Fe kg^{-1} soil).

Water-addition experiments were designed to test for water limitation by increasing soil water content. Four samples were collected to determine gravimetric soil moisture content under field conditions. The dry soil mass was calculated for each sample, using the average soil moisture content from four harvested cores (7%). From the calculated dry mass of each sample, we derived a new wet mass with 30% soil moisture content. Water was added to increase soil water from ambient to 30% gravimetric soil moisture content. The nutrient addition experiment on intact cores was repeated under anaerobic conditions to determine if O_2 availability was inhibiting nitrogenase activity. Anaerobic chambers were sealed, vacuumed, and filled with He three times before acetylene reduction assays were conducted.

The nutrient, C, or water solution was injected at five points in the core to ensure its even distribution throughout the soil. For each injection point, a syringe with a 15-cm-long, 20-gauge needle was inserted 10 cm into the core and then 1 mL of nutrient or C solution was injected as the needle was slowly withdrawn. Controls received 5 mL of water delivered at five injection points. These were designed to control for the effects of delivering nutrient solutions in water. Water treatments received enough water to bring each sample to 30% soil moisture content. Four replicate samples of the control, nutrient, and C treatments were incubated at 7% gravimetric soil moisture content (field conditions) and four water-treatment replicates were incubated at 30% gravimetric soil moisture content for 48 h and measured for C_2H_4 production.

To reduce the variability inherent in intact soil cores, we repeated these experiments with sieved soils to allow an even distribution of water and nutrients. Nutrient-addition incubations were performed on 200 g of sieved (2 mm) mineral soil collected from the Duke Forest in November 2001. Nutrients were added in a dissolved nutrient solution (as above) and mixed into the soil. Acetylene reduction assays were conducted on 3-h incubations of field-moist soils (23% soil moisture on this date). Next, this experiment was repeated under anaerobic conditions to determine if O_2 availability was inhibiting nitrogenase activity. Jars were sealed, vacuumed, and filled with He three times before anaerobic acetylene reduction assays were conducted. Four replicate samples were incubated for each treatment.

In a final experiment, forest floor and mineral soil samples (0–10 cm) were collected adjacent to FACE plots in the Duke Forest and homogenized. This experiment was designed to compare the N_2 fixation response in organic and mineral horizons with supplemental water. Sixty grams of forest floor or 200 g of field-moist soil was placed in each incubation chamber, and nutrient solution was distributed in enough water to bring samples to 30% soil moisture, creating a slurry. Four replicate samples were incubated for each treatment. Acetylene reduction was measured after 48 h.

Calculations and Statistics

Ethylene concentrations ($\mu mol L^{-1}$) were multiplied by headspace volume to estimate the volume of C_2H_4 generated, which was then cor-

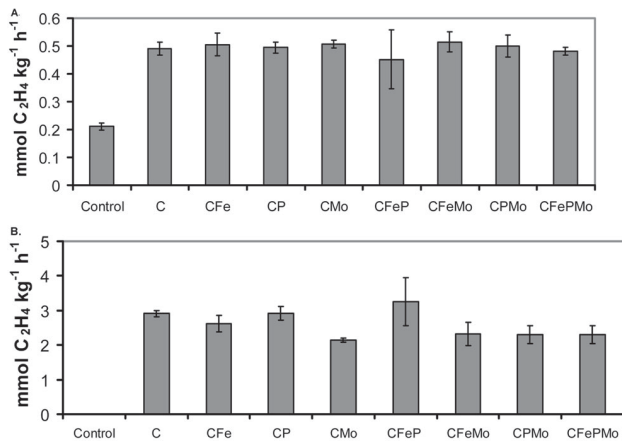


Fig. 1. Acetylene reduction assays in (A) homogenized forest floor and (B) mineral soil with nutrient additions (30% gravimetric soil moisture content). Bars represent average \pm SE N_2 fixation rates. Treatments consisted of combinations of P, Mo, Fe, and glucose (C).

rected for natural C_2H_4 production and C_2H_4 generation during acetylene production. Soil water potential was calculated as $\psi = \psi_s \theta^{-b}$ (converted to MPa), where ψ is the soil water potential (cm H_2O), ψ_s is the saturated soil water potential, θ is the average soil moisture ($m^3 m^{-3}$), and b is an empirical parameter (Clapp and Hornberger, 1978). Soil moisture was based on field measurements and all other parameters were from published results from the Duke FACE site (Schäfer et al., 2002).

We conducted a repeated measures ANOVA to determine the effect of CO_2 , soil moisture, soil horizon, and sample date on N_2 fixation activity. Linear regression was used to evaluate the relationship between soil moisture and nitrogenase activity. To capture a wide range of soil moisture conditions, we used all data from potential field measurements to determine the response of N_2 fixation to soil moisture. Student's t -test was used to compare soil moisture in mineral and forest floor samples. Nutrient addition results were compared using a one-way ANOVA. For significant treatment effects, the ANOVA was followed by post hoc multiple comparisons analysis. All statistics were computed using Splus 6.1 (Insightful Corp., Reinach, Switzerland).

RESULTS

Nutrient Additions

The addition of water was the most important factor controlling N_2 fixation activity in laboratory incubations of Duke Forest soil. For intact mineral soil cores taken outside

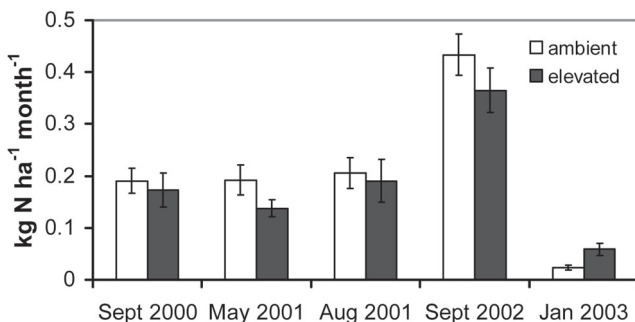


Fig. 2. Average (\pm SE) monthly rates of total N_2 fixation (forest floor + mineral soil horizons). Open bars represent ambient conditions, closed bars represent elevated CO_2 ($+200 \mu L L^{-1}$).

the FACE plots and incubated at field moisture (7% gravimetric soil moisture content), C and nutrient additions had no significant effect on N_2 fixation. When soil moisture was enhanced to 30%, N_2 fixation rates increased 25-fold from 3.7 to 92.3 $mmol C_2H_4 kg^{-1} h^{-1}$ ($P < 0.001$). When the nitrogenase enzyme was protected under anaerobic conditions N_2 fixation rates significantly increased relative to aerobic rates for all treatments ($P < 0.0001$). Mean \pm SE N_2 fixation rates were 47 ± 8 and 107 ± 8 $nmol kg^{-1} h^{-1}$ for aerobic and anaerobic conditions, averaged across all nutrient treatments. Aerobic nutrient additions had no significant effect on N_2 fixation, with the exception of stimulation by Fe with Mo ($P = 0.02$). Under anaerobic conditions, N_2 fixation was stimulated, relative to controls with no nutrient additions, in soils with C in combination with P and Mo (C + P + Mo, $P < 0.01$) and with Fe and Mo (C + Fe + Mo, $P = 0.04$). Overall laboratory incubations of intact cores demonstrated a consistent increase in N_2 fixation when O_2 in the soil pore space was reduced by water additions or anaerobic incubations.

To reduce between-core variability and water limitation, labile C and nutrient additions were added to homogenized samples at 30% soil moisture. Relative to controls, labile C additions significantly increased N_2 fixation activity in both forest floor ($P < 0.01$) and mineral horizons ($P < 0.01$; Fig. 1). Nutrient additions caused no additional stimulation to N_2 fixation relative to C additions alone. Carbon additions in the presence or absence of nutrients consistently doubled N_2 fixation rates in the organic horizon relative to controls. In the mineral horizon, C stimulation of N_2 fixation was >400 times greater than controls, while nutrient additions in combination with C provided no additional stimulation of N_2 fixation (Fig. 1).

Potential Rates under Ambient and Elevated Carbon Dioxide

Atmospheric CO_2 treatment had no effect on average potential rates of N_2 fixation in forest floor or mineral soil. Over five collections and several years, nitrogenase activity was variable, with no significant CO_2 treatment effects on any date (Fig. 2). Averaged across CO_2 treatment, fixation rates in the forest floor were 17 times greater than in the mineral soil ($P < 0.01$). Potential N_2 fixation rates in forest floor and mineral soil samples were positively related to gravimetric soil water content ($P < 0.01$; Fig. 3), wherein soil water

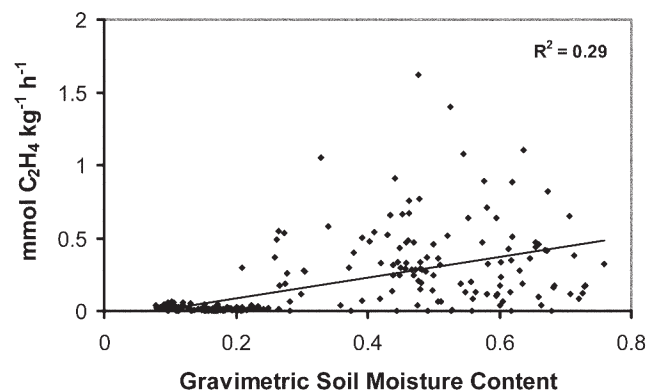


Fig. 3. Measurements of potential heterotrophic N_2 fixation rates as a function of gravimetric soil moisture content in forest floor and mineral soil horizons.

accounted for 29% of the variability in N₂ fixation rates. The forest floor was significantly wetter than the mineral soil ($P < 0.01$), with average soil moisture contents of 50 and 15% for forest floor and mineral soil samples, respectively.

DISCUSSION

We hypothesized that heterotrophic N₂ fixation would be stimulated by increased substrate availability provided by plant growth under elevated atmospheric CO₂, offering an additional source of N to sustain increased NPP. Carbon dioxide enrichment has increased NPP at the Duke FACE site, requiring an additional 6.7 kg N ha⁻¹ yr⁻¹ of N from the soil to sustain this increase in plant growth (Table 3 in Finzi et al., 2002). Understanding the factors controlling free-living N₂ fixation is necessary for predicting N availability required to support increased NPP under future climate scenarios.

Despite increases in forest floor mass and substrate availability at the Duke FACE site (Lichter et al., 2005), the rates of N₂ fixation were not stimulated under elevated CO₂. Several mechanisms could be responsible for this observation, one of which might be water limitation. In laboratory manipulations of intact soil cores, water additions increased N₂ fixation 25-fold. In addition to limiting O₂ in the soil pore space, liberation of labile C may be contributing to the strong response to water additions. The combined benefit of labile C and increased water availability was demonstrated by slurry incubations (Fig. 1). Contrary to measurements on intact cores, C additions in slurry incubations produced a much greater N₂ fixation response in the mineral soil than in the forest floor (Fig. 1). Slurry conditions increased the potential for reduced microsites that harbor N₂ fixation, increased the distribution of glucose, and potentially released additional labile C. The strong positive effects of water and glucose addition on N₂ fixation suggest that soil moisture is limiting the nitrogenase response, despite increased substrate availability under elevated CO₂.

Nitrogen fixation rates in intact FACE samples were most influenced by soil moisture. Nitrogenase activity was minimal below 30% soil moisture ($\psi = -0.03$ MPa), suggesting a moisture threshold constraining N₂ fixation in this forest. These results are consistent with field studies from the Pacific Northwest, where moisture content is the most important factor controlling heterotrophic N₂ fixation activity in woody debris (Wei et al., 2003) and a threshold of 50% soil moisture was reported for free-living N₂ fixation (Hicks et al., 2003).

No studies to date demonstrate a CO₂ stimulation of heterotrophic N₂ fixation in well-aerated soil; however, N₂ fixation is stimulated under elevated CO₂ in rice fields and wetland sediments (Dakora and Drake, 2000; Cheng et al., 2001; Hoque et al., 2001). In these environments, water is not limiting and the nitrogenase enzyme is adequately protected from O₂. Our results are consistent with a FACE experiment in the Mojave Desert, where no CO₂ stimulation was detected in heterotrophic N₂ fixation activity in soils or their cryptobiotic crusts (Billings et al., 2003). Our measurements reveal that soil moisture has the greatest effect on N₂ fixation in laboratory manipulations of both homogenized and intact Duke FACE soils.

With the addition of water, nitrogenase activity in Duke Forest soil showed a strong response to substrate additions

(Fig. 1). These results are consistent with previous studies demonstrating substrate limitation to microbial respiration in both organic and mineral soils of the Duke Forest (Allen and Schlesinger, 2004). Although N₂ fixation is stimulated by labile C under laboratory manipulations, the increase in C inputs to soil under elevated CO₂ is apparently insufficient to increase rates of N₂ fixation in the field experiment. Our experiments only examined the effects of labile C inputs. Because N₂ fixation is energetically expensive, it is unlikely that more complex forms of C will stimulate N₂ fixation. In addition to water limitation, competition for organic substrates among members of the heterotrophic soil community may be limiting the response of N₂ fixers under elevated CO₂.

Quantifying the response of heterotrophic N₂ fixation is necessary to understand the ecological impact of rising atmospheric CO₂ on ecosystem N cycling. Without substantial increases, available N will be insufficient to support model projections of C sequestration (Hungate et al., 2003). Based on our results, asymbiotic N₂ fixation in forest ecosystems will not support enhanced growth of plants under elevated CO₂. Heterotrophic N₂ fixation does not increase the contribution to the N economy of the forest under elevated CO₂. These results indicate that sustaining long-term C sequestration on N-poor pine forests will require plants to increase root exploration of soil or increase nutrient-use efficiency to enhance biomass production.

Our experiments demonstrate that water is the factor most limiting to N₂ fixation in the Duke FACE experiments. It seems unlikely that increased substrate availability due to plant growth at high CO₂ will stimulate fixation in upland soils without concurrent increases in soil moisture. If future changes in precipitation patterns result in increased soil moisture, asymbiotic N₂ fixation rates in aerobic soils may increase, altering N availability.

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