

Effects of elevated carbon dioxide and nitrogen fertilization on nitrate reductase activity in sweetgum and loblolly pine trees in two temperate forests

Susan M. Natali · Sergio A. Sañudo-Wilhelmy ·
Manuel T. Lerdau

Received: 15 March 2008 / Accepted: 2 July 2008 / Published online: 29 July 2008
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Abstract Nitrogen (N) availability is a major factor limiting plant production in many terrestrial ecosystems and is a key regulator of plant response to elevated CO₂. Plant N status is a function of both soil N availability and plant N uptake and assimilation capacity. As a rate-limiting step in nitrate assimilation, the reduction of nitrate is an important component of plant physiological response to elevated CO₂ and terrestrial carbon sequestration. We examine the effects of elevated CO₂ and N availability on the activity of nitrate reductase, the enzyme catalyzing the reduction of nitrate to nitrite, in two temperate forests—a closed canopy sweetgum (*Liquidambar styraciflua*) plantation in Tennessee (Oak Ridge National Laboratory (ORNL)) and a loblolly pine (*Pinus taeda*) stand in North Carolina

(Duke). Both CO₂ and N enrichment had species specific impacts on nitrate reductase activity (NaR). Elevated CO₂ and N fertilization decreased foliar NaR in *P. taeda*, but there were no treatment effects on *L. styraciflua* NaR at ORNL or Duke. NaR in 1-year *P. taeda* needles was significantly greater than in 0-year old needles across treatments. *P. taeda* NaR was negatively correlated with bio-available molybdenum concentrations in soils, suggesting that CO₂ and N-mediated changes in soil nutrient status may be altering soil-plant N-dynamics. The variation in response among species may reflect different strategies for acquiring N and suggests that elevated CO₂ may alter plant N dynamics through changes in NaR.

Keywords Free-air carbon dioxide enrichment (FACE) · *Liquidambar styraciflua* · Micronutrients · Molybdenum · NO₃⁻ assimilation · *Pinus taeda*

Responsible Editor: Herbert Johannes Kronzucker.

S. M. Natali (✉)
Department of Ecology and Evolution, 650 Life Sciences,
State University of New York at Stony Brook,
Stony Brook, NY 11794-5245, USA
e-mail: natalis@life.bio.sunysb.edu

S. A. Sañudo-Wilhelmy
Marine and Environmental Biology,
University of Southern California,
Los Angeles, CA 90089-0371, USA

M. T. Lerdau
Blandy Experimental Farm,
Department of Environmental Sciences,
University of Virginia,
Charlottesville, VA 22904, USA

Abbreviations

NaR nitrate reductase activity
FACE free-air carbon dioxide enrichment
SOM soil organic matter

Introduction

The ability of plants to acquire and assimilate nitrogen (N) is an important determinant of plant response to elevated CO₂ and of ecosystem carbon sequestration (Luo et al. 2004; Stitt and Krapp 1999).

The response of forests to increasing atmospheric CO₂ is of particular importance because forests comprise over one third of terrestrial ecosystems and account for 50–70% of terrestrial net primary production (NPP; Field et al. 1998; Melillo et al. 1993). Forest NPP, especially in northern and temperate regions, is commonly limited by the availability of inorganic N (Vitousek and Howarth 1991).

Plant N status is dependent upon soil N availability, as well as plant uptake and assimilation capacity. Plant species, including trees, vary in their ability to use nitrate (NO₃⁻) or ammonium (NH₄⁺) as their primary source of inorganic N (Templer and Dawson 2004). Although the energetic costs of NO₃⁻ utilization (~2.4 g glucose g⁻¹ protein produced) are higher than NH₄⁺ (~1.8 g glucose g⁻¹ protein (Zerihun et al. 1998)), NO₃⁻ is an important source of N for plants, fungi, and many species of bacteria in both managed (Haynes and Goh 1978) and natural systems (Stark and Hart 1997). Even in coniferous ecosystems where soil NO₃⁻ concentrations tend to be low, high rates of gross nitrification point to rapid turn-over of a small but ecologically important NO₃⁻ pool (Stark and Hart 1997). NO₃⁻ is particularly important as a plant N source because it is readily available to plants, and—in contrast to NH₄⁺—NO₃⁻ is mobile in negatively charged soils and is easily transported to the root surface by bulk flow.

Once taken up by plants, NO₃⁻ can be reduced in roots or in leaves. Both above and belowground assimilation processes may be altered by increasing concentrations of atmospheric CO₂. While a large number of recent studies show a decrease in foliar NaR activity with CO₂ enrichment (Bloom et al. 2002; Constable et al. 2001; Searles and Bloom 2003; Smart et al. 1998), CO₂ effects on NaR have been variable across species and studies. Elevated CO₂ has been shown to cause an increase (Geiger et al. 1998; Larios et al. 2001; Matt et al. 2001), decrease (Bloom et al. 2002; Hocking and Meyer 1991; Searles and Bloom 2003; Sicher 2001; Smart et al. 1998), or have no effect (Cousins and Bloom 2003) on foliar NaR activity. Variation in response may be attributed to soil N form and concentration (Geiger et al. 1999; Kruse et al. 2003; Matt et al. 2001; Yong et al. 2007), plant species/functional group (Cousins and Bloom 2003), mycorrhizal association (Constable et al. 2001) and altered diurnal rhythm of NaR activity (Geiger et al. 1998). Potential molecular and physiological

interactions between elevated CO₂ and N assimilation have been well-reviewed by Stitt and Krapp (1999).

One hypothesized mechanism of CO₂ effects on NO₃⁻ photoassimilation is competition for reductant. Under high light conditions, NO₃⁻ reduction in leaves may provide a sink for excess reductant not used in carbon assimilation (Guo et al. 2007). However, foliar NO₃⁻ reduction may compete for reductant with Calvin cycle reactions when light levels are lower (Huppe and Turpin 1994) or when carbon assimilation is increased under elevated CO₂ (Bloom et al. 1989; Bloom et al. 2002). Because of the potential competitive effect between NO₃⁻ and CO₂ reduction, foliar nitrate reductase activity (NaR) may decrease with CO₂ enrichment, and species that rely on NO₃⁻ may be competitively disadvantaged under elevated CO₂ unless they can increase NH₄⁺ acquisition or redirect NO₃⁻ assimilation to roots (Smart et al. 1998).

In addition to direct effects on plant NaR (i.e., competition for reductant), elevated CO₂ may also affect NaR through impacts on soil properties. For example, molybdenum (Mo) is a plant micronutrient that serves as a cofactor for nitrate reductase—the enzyme that catalyzes the reduction of NO₃⁻ to nitrite (NO₂⁻). When soil Mo is low, Mo can limit NaR and alter plant N assimilation capacity (Kaiser et al. 2005; Lang and Kaupenjohann 1999; Randall 1969; Stout and Meagher 1948). While total Mo in soils is often greater than plant demand, deficiency can occur in mildly acidic soils because Mo availability markedly decreases at pH of about 5.5 and lower (Marschner 1995; Stiefel 2002).

Soil Mo bio-availability may increase with CO₂ enrichment because soil organic matter (SOM) has been shown to increase under elevated CO₂ (Jastrow et al. 2005), and SOM is positively correlated with Mo bio-availability (Fontes and Coelho 2005). Alternatively, Mo bio-availability may decrease under elevated CO₂, because CO₂ enrichment has been shown to increase soil acidification (Andrews and Schlesinger 2001; Oh and Richter 2004) and the availability of soil Mo decreases with decreasing pH (Marschner 1995; Stiefel 2002).

Previous studies of CO₂ effects on NaR have been conducted on potted seedlings or in greenhouse/chamber experiments. While these experiments form a foundation for studying CO₂–NO₃⁻ dynamics, limitations of pot/chamber experiments for studying CO₂ effects on plant processes are well-recognized (Ainsworth and Long 2005). Constable et al. (2001) conducted a greenhouse study of CO₂ effects on N

uptake and assimilation by loblolly pine (*Pinus taeda*) and sweetgum (*Liquidambar styraciflua*) seedlings and found that CO₂ enrichment increased root and foliar NaR activity in *L. styraciflua* but had no effect on NaR activity in leaves or roots of *P. taeda*. In our study, we looked at CO₂ and N-fertilization effects on foliar NaR in adult *P. taeda* and *L. styraciflua* grown under natural conditions in long-term field experiments. We ask the question: how will elevated CO₂ and N fertilization affect NaR in these species in field conditions, where CO₂ can potentially have both direct effects on foliar NaR as well as indirect effects through CO₂-mediated changes in soil nutrient dynamics?

If foliar NaR is limited by reductant, we expect lower NaR in low light conditions (lower canopy leaves) across CO₂ treatments and a decrease in NaR with CO₂ enrichment. We expect that changes in soil properties (e.g. SOM and pH) with CO₂ enrichment will impact Mo bio-availability in soils. If foliar NaR is limited by Mo, then NaR will be positively correlated with bio-available soil Mo concentrations. We expect that fertilization with NH₄NO₃ will decrease NaR activity in *L. styraciflua* and *P. taeda* because both species have a greater capacity for uptake of NH₄⁺ than NO₃⁻ (Constable et al. 2001), and we expect preferential uptake of NH₄⁺ when both N forms are available because of NH₄⁺'s lower cost of assimilation.

We conducted this study in two temperate forest free-air carbon dioxide enrichment (FACE) sites—a loblolly pine forest in North Carolina (Duke) and a sweetgum plantation in Tennessee (Oak Ridge National Laboratory (ORNL))—which have been exposed to CO₂ enrichment since 1996 and 1998, respectively. An experimental N treatment was added to half of each CO₂ ring at Duke FACE in 2005; therefore, we examined N fertilization effects on NaR activity at Duke but not ORNL. We examined NaR in leaves from the dominant canopy tree at Duke FACE, *P. taeda*, and the dominant canopy tree at ORNL FACE, *L. styraciflua*. *L. styraciflua* was also sampled at Duke where it is a common understory tree.

Materials and methods

Site description

Duke FACE is a mixed evergreen-deciduous temperate forest dominated by loblolly pine (*P. taeda*),

located in the Blackwood Division of Duke Forest in Orange County, North Carolina (35°58' N, 79°05' W). The stand of loblolly pine, which was planted in 1983 at a spacing of 2.0×2.4 m, is located on low-fertility, acidic Hapludalf soils. The sub-canopy and understory are diverse, containing more than 50 species, but dominated by sweetgum (*L. styraciflua*). The FACE experiment began in August 1996 and is comprised of three ambient rings (~365 μmol mol⁻¹) and three elevated rings (~565 μmol mol⁻¹). The 30 m diameter experimental rings are arranged in a complete block design to account for topographic variation and potential fertility gradients. The CO₂ treatment is applied via a series of vertical pipes located around the perimeter of each ring. The pipes, which extend from the forest floor to the canopy, are equipped with blowers that deliver a controlled amount of CO₂-fumigated air to maintain ambient or elevated levels of CO₂ into the rings (Hendrey et al. 1999). An experimental N treatment, consisting of the addition of NH₄⁺NO₃⁻ (5.6 g N m⁻² year⁻¹), was added to one half of each ring in March and April 2005.

The deciduous forest site (ORNL) is a sweetgum (*L. styraciflua*) plantation located in the Oak Ridge National Environmental Research Park in Roane County, TN, USA (35°54' N, 84°20' W). The soil, which is classified as Aquic Hapludult, has a silty clay loam texture, is moderately well drained and is slightly acidic. The stand was planted with 1-year-old sweetgum seedlings in 1988 at a spacing of 1.2×2.3 m. The FACE apparatus (i.e., the CO₂ treatment as described above and detailed in Hendrey et al. 1999) is assembled in four of the five 25 m diameter experimental rings. There are three ambient (~393 μmol mol⁻¹) rings and two enriched (~549 μmol mol⁻¹) rings. CO₂ enrichment began in 1998 and continues during the growing season through the present time. The site description and experimental design have been thoroughly documented (Norby et al. 2001).

Field sampling

Soil samples and canopy leaves were collected at ORNL from 25–28 July 2005 and at Duke from 8–11 August 2005. A core sampler was used to collect two 2.5 cm (diameter) by 20 cm soil cores per ring (ORNL) or N-treatment within a ring (Duke) at each site (34 cores total). Butyrate plastic core liners, which were washed prior to use in 0.1 N HCl, were

used in the soil corer in order to maintain an intact core during extraction. Cores were divided into 5 cm depth increments and pooled within rings (within N treatment of each ring at Duke). Coarse and fine roots were removed from the soil cores and combined for elemental analysis, but because of the instability of the nitrate reductase enzyme, were not analyzed for enzyme activity.

Green leaves for elemental analysis were sampled from three canopy heights—low (10–12 m), mid (12–14 m) and upper (14–16 m)—from *L. styraciflua* at ORNL and Duke (lower and mid canopies only at Duke) and from *P. taeda* at Duke. The canopy at ORNL was accessed using a stationary hydraulic lift located near the center of each ring; at Duke the canopy was accessed by a central walk-up tower and by a mobile hydraulic lift. Both 0-year (needles that originated in 2005) and 1-year (needles that originated in 2004) needles were samples from *P. taeda*. At each canopy height three replicate samples were collected; each of these sub-samples consisted of approximately five leaves/20 needles from an individual tree. An additional set of replicate leaf samples for nitrate reductase analysis was collected from the lower and upper canopies (*L. styraciflua* at Duke were from lower canopy only). Petioles were removed from *L. styraciflua* prior to sample analysis. Nitrate reductase leaf samples were immediately placed in liquid N₂ upon collection and stored at –80°C until enzyme analysis was conducted.

Sample processing and chemical analyses

To remove surface deposits, leaves for elemental analysis were rinsed in milli-Q[®] ultrapure water, washed in 0.2N HCl and rinsed again three times in milli-Q water (Oliva and Raitio 2003). Roots were separated from the soil cores, washed in milli-Q water until visible soil deposits were removed and then washed in dilute acid as above. After removal of roots, soils were passed through a 2 mm screen, air-dried and homogenized using a mortar and pestle. Leaves and roots for elemental analysis were dried for 72 h at 60°C in a Fisher-Isotemp Oven and homogenized using a ball mill.

Soils for metal analysis were digested using repeated additions of concentrated nitric acid (HNO₃) and hydrogen peroxide (H₂O₂) with heating (EPA Method 3050B). Leaves were digested using repeated additions of HNO₃, followed by H₂O₂ and HCl (EPA Method

2003). Bio-availability of Mo in soils was determined by extraction of soil samples with ammonium oxalate solution at pH 6.0 (Liu et al. 1996). The ammonium oxalate extractable fraction of Mo is widely used as an indicator of Mo availability to plants and is the fraction used to set Mo deficiency thresholds. This extract contains the specifically sorbed, plus free and non-specifically bound, Mo—the more readily exchangeable forms of soil Mo (Lang and Kaupenjohann 1999). We assume that this plant available Mo is available to both plants and microbes and refer to the oxalate extractable fraction as bio-available Mo. We also analyzed total concentrations of two other plant micronutrients (Fe and Cu), two nonessential plant metals (Al and Pb), and bio-available Cu (Mehlich 1984) in soils to compare leaf NaR-soil metal relationships. While we were specifically interested in soil Mo effects on NaR, we looked at these other metals to determine if potential patterns detected were due to Mo effects per se or to more general changes in soils properties with CO₂ and N enrichment.

Mo and other metals (Al, Pb, Fe and Cu) were analyzed using a Thermo-Finnegan Element2 Inductively Coupled Plasma Mass Spectrometer, with apple leaf (NIST 1515) and San Joaquin soils (NIST 2709) as digestion standards, and river water (NIST 1643d) as an instrumental standard. Total nitrogen was analyzed in a CHN elemental analyzer, using atropine and apple leaf (NIST 1515) as standards. Soil samples for total N were pooled across soil depths. SOM was determined by the method of percent loss on ignition (8 h combustion at 400°C). Soil pH was determined in a 1:1 ratio of soil (grams) to water (milliliters) and 1:1 ratio of soil to 0.01 M CaCl₂. These two pH measures were highly correlated (R²=0.943, P<0.01) so only pH in water was used in the analyses.

Nitrate reductase assay

Frozen leaves were finely ground using liquid nitrogen and a chilled mortar and pestle. One gram of ground leaf material (wet weight) was extracted in four milliliters of extraction buffer (0.1 M KH₂PO₄, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 7.5, with 1% (w/v) bovine serum albumin, 2% (w/v) casein, 0.1% (w/v) cysteine, 5 mM dithiothreitol, 20 μM FAD, 25 μM leupeptin, 5 μM Na₂MoO₄, 0.5 mM phenylmethylsulfonyl fluoride, 1% (w/v) polyvinylpyrrolidone, 0.1% (v/v) Triton-X-100). The extract was filtered

through three layers of cheesecloth, and centrifuged at 4°C 10,000 rpm for 10 min. To determine nitrate reductase activity, 200 µl of the supernatant was added to 500 µl assay buffer (25 mM KH₂PO₄, 0.025 EDTA, pH 7.5) and 0.2 mM NADH, and incubated at 30°C for various times up to 5 min. The reaction was stopped by the addition of 50 µl of 1 M ZnAc and the solution centrifuged at 10,000 rpm for 5 min. The nitrite (NO₂⁻) content of 0.5 ml of the supernatant was determined by addition of 0.5 ml 1% (w/v) sulfanilamide in 3 N HCl and 0.5 ml 0.02% *N*-naphthylethylenediamine in d-I water. After 20 min incubation the absorption of the sample solution at 540 nm was compared to standards of known NO₂⁻ concentration. Each sample was run in triplicate and averaged. This procedure is the optimized assay (optimized by adjusting assay and extract buffer pH and reagent concentrations) for both species. Because we were interested in CO₂ and N effects—rather than species effects—on NaR (i.e., we did not make statistical comparisons between species), slight difference in optimal conditions for each species will not affect the conclusions of this study. Nitrate reductase activity is reported in units of NO₂⁻ produced per hour per gram leaf wet weight.

Statistical analyses

ORNL foliar data were analyzed using a partly-nested analysis of variance (ANOVA; SAS 9.0, SAS Institute, Cary, NC, USA) with CO₂ as the main plot factor, canopy height as the within plot factor, and ring (random) as the experimental unit for CO₂. We analyzed Duke *P. taeda* and *L. styraciflua* in separate ANOVAs so that we could include age and canopy structure into our *P. taeda* model. Duke *L. styraciflua* leaf samples were analyzed using a partly nested design with CO₂ as the main plot factor nested in block (random) and N-treatment as a within plot factor. The ANOVA on Duke *P. taeda* needles had two additional within plot factors—needle age and canopy height.

Root Mo and N, and soil N concentrations were analyzed using a mixed linear model ANOVA, with ring or block (random) as the unit of replication for CO₂ treatment, and N-treatment (at Duke) as a within plot factor. Total and bio-available Mo concentrations in soils were analyzed with a mixed linear model ANOVA to test for effects of CO₂, N (Duke), depth and interaction effects, with ring or block as the unit of replication for CO₂ effects.

To look at relationships among measured variables, a regression model was fit by method of ordinary least squares and Pearson correlation coefficients calculated to estimate the correlation between foliar NaR and soil and leaf variables. Data were pooled across canopy heights and soil depths for leaf-soil analyses. Based on these results, we used the main driver among soil variables of foliar NaR as a covariate in an analysis of covariance (ANCOVA) to test for indirect effects of elevated CO₂ (that is, CO₂-mediated changes in soil properties) on NaR.

All data were transformed when necessary to meet the assumptions of the statistical tests. Post-hoc comparison *p*-values were adjusted with Tukey's test to control the family-wise error rate. For the ANOVAs and ANCOVA with unequal treatment sample sizes, *dof* were estimated using Satterthwaite's approximation (Satterthwaite 1946). Because of the constraints on sample size of the FACE experiments and resulting low statistical power (Filion et al. 2000), effects were considered marginally significant for *P*<0.10 and significant for *P*<0.05 as in other FACE studies (e.g., Ellsworth et al. 2004, Jastrow et al. 2005). Errors presented in the text and tables are one standard error of the mean.

Results

Duke FACE: *P. taeda*

Nitrate reductase activity

There was a decrease in *P. taeda* foliar NaR with CO₂ enrichment (*F*=8.17, *P*=0.10) and a significant CO₂ × age effect (*F*=10.10, *P*=0.03). Foliar NaR decreased with CO₂ enrichment in 1-year needles (*t*=3.95, *P*=0.05) but not in 0-year-old needles (*t*=0.25, *P*=0.99), where NaR was three times lower than in 1-year needles across treatments (*F*=71.21, *P*<0.01; Fig. 1a).

There was also a decrease in NaR with N-fertilization (*F*=22.23, *P*=0.04) and a significant N × age interaction (*F*=8.54, *P*=0.02)—N-fertilization decreased NaR in 1-year needles (*t*=5.10, *P*<0.01) but not in 0-year needles (*t*=1.41, *P*=0.53). While we did not detect significant CO₂ × N (*F*=5.84, *P*=0.14) or CO₂ × N × age (*F*=2.03, *P*=0.20) interactions, CO₂ effects on NaR were greatest in 1-year needles in the N-control plots (Fig. 1a).

There was no detected canopy height effect on *P. taeda* NaR across treatments ($F=0.24$, $P=0.65$) and no $\text{CO}_2 \times$ canopy height interaction effects ($F=0.49$, $P=0.49$; Fig. 2a). There was, however, a significant $\text{N} \times$ canopy height interaction ($F=9.22$, $P=0.02$) because N-fertilization significantly decreased NaR in lower canopy ($t=4.99$, $P=0.01$) but not upper canopy needles ($t=1.27$, $P=0.61$; Fig. 2b).

Foliar Mo concentrations

There was no detected CO_2 ($F=4.50$, $P=0.17$) or N-fertilization effect ($F=0.03$, $P=0.88$) on *P. taeda* foliar Mo concentrations (Fig. 1b). As with NaR, Mo concentrations were significantly lower in 0-year needles compared to 1-year needles ($F=5.61$, $P=0.03$). There was a marginally significant effect of

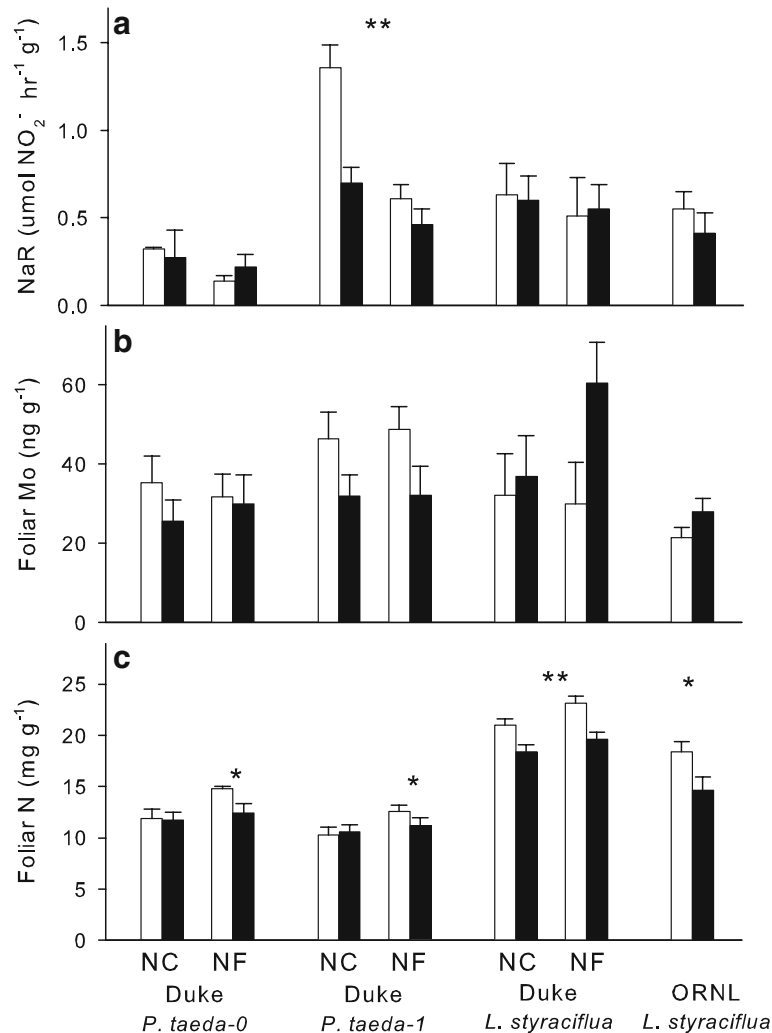


Fig. 1 Elevated CO_2 and N fertilization effects on foliar NaR, Mo and N in 0-year and 1-year *P. taeda* needles at Duke and *L. styraciflua* leaves at Duke and ORNL FACE. **a** CO_2 and N fertilization significantly decreased NaR ($p<0.05$) in 1-year old *P. taeda* needles but not in *L. styraciflua* leaves. **b** There were no significant CO_2 or N treatment effects on foliar Mo concentrations in either species. **c** In *P. taeda*, there was a significant CO_2 effect on foliar N concentrations in the N fertilized ($p<0.05$) but not in the N control treatments and a

significant effect of N fertilization on foliar N in the ambient CO_2 rings ($p<0.05$) but not in the elevated rings. *L. styraciflua* foliar N concentrations were significantly lower with CO_2 enrichment at both sites ($p<0.05$) and increased with N fertilization at Duke ($p<0.10$). Filled bars represent least squares means (\pm SE) from elevated rings and open bars represent ambient rings. NC = N control; NF=N fertilization. * denotes significant CO_2 effect ($p<0.05$); ** denotes significant CO_2 effect across N-treatments (no $\text{CO}_2 \times \text{N}$; $p<0.05$)

canopy height on *P. taeda* foliar Mo ($F=3.17$, $P=0.06$), with higher concentrations in lower canopy (41.3 ± 4.2 ng g⁻¹) than in upper canopy needles (30.4 ± 3.7 ng g⁻¹; $t=2.12$, $P=0.01$). Mid-canopy concentrations (33.3 ± 3.7 ng g⁻¹) were not significantly different from either the lower or upper canopies. There were no significant interaction effects on *P. taeda* foliar Mo ($P > 0.10$).

P. taeda foliar Mo was positively correlated with NaR across needle age classes ($R^2=0.22$, $P=0.03$). However, when age was included as a factor in the regression model (ANCOVA with age as a class variable), the relationship between foliar NaR and Mo was no longer significant ($P=0.13$), suggesting that differences in foliar NaR and Mo between age classes were, in part, driving the foliar NaR–Mo relationship.

Foliar N concentrations

While there was not a significant CO₂ main effect on *P. taeda* foliar N concentrations, there was a marginally significant CO₂ × age interaction effect ($F=3.54$, $P=0.07$; Fig. 1c). Foliar N concentrations in 0-year needles were lower with CO₂ enrichment, but there was no detected CO₂ effect on 1-year-old needles ($t=0.98$, $P=0.76$). Unlike NaR and foliar Mo, N concentrations were significantly higher in 0-year than 1-year needles across treatments ($F=59.52$, $P < 0.01$).

Concentrations of N in *P. taeda* needles were greater in the N-fertilized plots compared to control plots ($F=11.35$, $P=0.04$) in both needle age classes (N × age, $P > 0.10$), but there was a marginally significant CO₂ ×

N interaction ($F=3.76$, $P=0.06$); N fertilization significantly increased foliar N concentrations in the ambient CO₂ rings ($t=3.60$, $P < 0.01$) but not in the elevated CO₂ rings ($t=1.04$, $P=0.73$), and elevated CO₂ significantly decreased foliar N concentrations in the N fertilized plots ($t=2.78$, $P=0.05$) but not in the control plots ($t=0.07$, $P=0.99$; Fig. 1c). There was also a significant N × height interaction effect on *P. taeda* foliar N concentrations ($F=3.78$, $P=0.04$), with the greatest N fertilization effect in lower canopy needles (23% increase in foliar N; 10% increase in mid-canopy needles, 12% increase in upper canopy needles).

Foliar N concentrations were negatively correlated with *P. taeda* log NaR ($R^2=0.33$, $P < 0.01$). When age was included as a factor in the regression model (ANCOVA with age as a class variable) there still was a significant relationship between foliar NaR and N ($P < 0.01$); therefore, the relationship between NaR and N was not driven by differences between age classes.

Duke FACE: *L. styraciflua*

Nitrate reductase activity

There were no detected effects of CO₂ ($F=0.07$, $P=0.82$), N-fertilization ($F=0.54$, $P=0.54$), or N × CO₂ ($F=0.01$, $P=0.99$) on *L. styraciflua* NaR at Duke FACE (Fig. 1a).

Foliar Mo concentrations

There also were no significant effects of CO₂ treatment ($F=1.85$, $P=0.31$), N-fertilization ($F=1.45$; $P=0.29$) or N × CO₂ ($F=2.11$, $P=0.22$) on

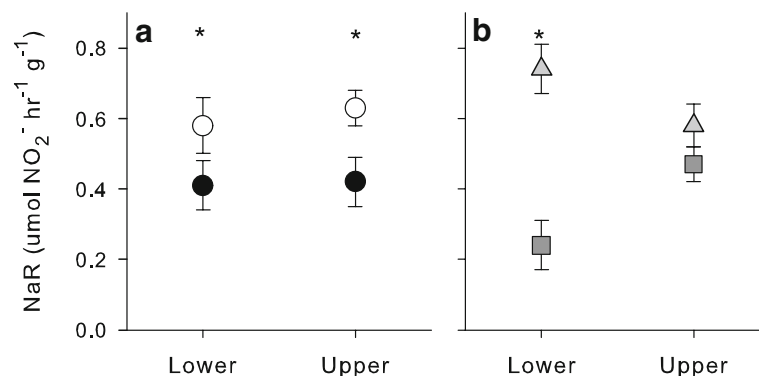


Fig. 2 NaR in 1-year *P. taeda* needles in the upper and lower canopies at Duke FACE. **a** There was no CO₂ × canopy height interaction effect on NaR **b** but there was a significant N fertilization × canopy height effect ($p < 0.05$). Symbols represent

least squares means (\pm SE) from **a** elevated (filled circles) and ambient CO₂ rings (open circles), and **b** N control (light grey triangles) and N fertilized sectors (dark grey squares). * denotes significant CO₂ or N fertilization effect ($p < 0.05$)

foliar Mo concentrations in *L. styraciflua* at Duke (Fig. 1b). There was not a significant correlation between *L. styraciflua* foliar Mo and NaR.

Foliar N concentrations

L. styraciflua foliar N concentrations decreased with CO₂ enrichment ($F=18.22$, $P=0.05$) and increased with N fertilization ($F=5.64$, $P=0.08$). There was no CO₂ × N interaction effect ($F=0.46$, $P=0.53$; Fig. 1c). There was not a significant correlation between *L. styraciflua* foliar N and NaR.

ORNL FACE: *L. styraciflua*

Nitrate reductase activity

As at Duke FACE, there was no effect of CO₂ on *L. styraciflua* NaR at ORNL ($F=0.87$, $P=0.37$, Fig. 1a), nor was there a detected effect of canopy height ($F=0.01$, $P=0.93$) or canopy height × CO₂ interaction ($F=2.24$, $P=0.16$).

Foliar Mo concentrations

At ORNL there was not a statistically significant effect of elevated CO₂ on *L. styraciflua* Mo concentrations ($F=2.06$, $P=0.19$); however, as at Duke, Mo concentrations were slightly higher in the elevated CO₂ rings than in the ambient CO₂ rings (Fig. 1b). There was no detected effect of canopy height on foliar Mo concentrations ($F=0.32$, $P=0.73$) at ORNL nor was there a significant CO₂ × height interaction ($F=1.76$, $P=0.23$). As at Duke, there was not a significant correlation between *L. styraciflua* foliar Mo and NaR.

Foliar N concentrations

At ORNL, *L. styraciflua* had lower foliar N ($F=5.30$, $P=0.08$) concentrations in the elevated CO₂ rings than in the ambient CO₂ rings (Fig. 1c). There was a marginally significant effect of height ($F=3.89$, $P=0.08$) on foliar N, with lower concentrations in the upper canopy (15.4 ± 1.7 mg g⁻¹) compared to the mid (17.1 ± 1.1 mg g⁻¹) and lower (17.2 ± 1.6 mg g⁻¹) canopies. There was no detected CO₂ × height interaction ($F=3.20$, $P=0.11$). N concentrations in ORNL *L. styraciflua* were slightly higher than generally found at this site (Norby and Iversen

2006), which may be due to petiole removal from leaves in this study. As at Duke, there was not a significant correlation between *L. styraciflua* foliar N and NaR.

Duke FACE: soil and roots

Mo and bio-available Mo concentrations

There were no significant effects of CO₂ ($F=0.22$, $P=0.66$), N fertilization ($F=0.92$, $P=0.39$), or CO₂ × N ($F=0.42$, $P=0.55$) on root Mo concentrations at Duke (Fig. 3a). There also were no significant effects of CO₂ enrichment ($F=1.21$, $P=0.39$) or N fertilization ($F=0.67$, $P=0.46$) on total soil Mo concentrations (Fig. 4a). There were marginally significant differences in soil Mo concentrations across depths (0–20 cm), with greater Mo concentrations in surface soils ($F=2.63$, $P=0.07$).

There were, however, significant CO₂ effects on bio-available Mo in soils. Mo bio-availabilities in the CO₂ enriched plots were greater in the lower soil depths (15–20 cm; CO₂ × depth: $F=2.39$, $P=0.09$), and there was a significant increase in bio-available Mo with N fertilization ($F=12.97$, $P=0.02$; Fig. 4b).

None of the other measured soil factors were correlated with bio-available Mo at Duke (total Mo: $R^2=0.01$, $P=0.56$; soil pH: $R^2=0.07$, $P=0.07$; log-SOM: $R^2=0.04$, $P=0.18$), and bio-available soil Mo was not correlated with foliar Mo concentrations in *L. styraciflua* ($R^2=0.30$, $P=0.30$) or *P. taeda* (1-year: $R^2=0.04$, $P=0.53$; *P. taeda* 0-year: $R^2=0.01$, $P=0.75$) at Duke FACE.

N concentrations

There were no significant CO₂ effects on total N concentrations in Duke FACE soils (0–20 cm) or roots. There was no detected N-fertilization effect on total N in soils, but root N concentrations were significantly greater ($F=9.01$, $P=0.04$) in the N fertilized plots compared to N control plots (Figs. 3b, 4c).

ORNL FACE: soil and root

Mo and bio-available Mo concentrations

At ORNL, root Mo concentrations were lower ($F=6.22$, $P=0.09$) in the elevated CO₂ rings compared to ambient

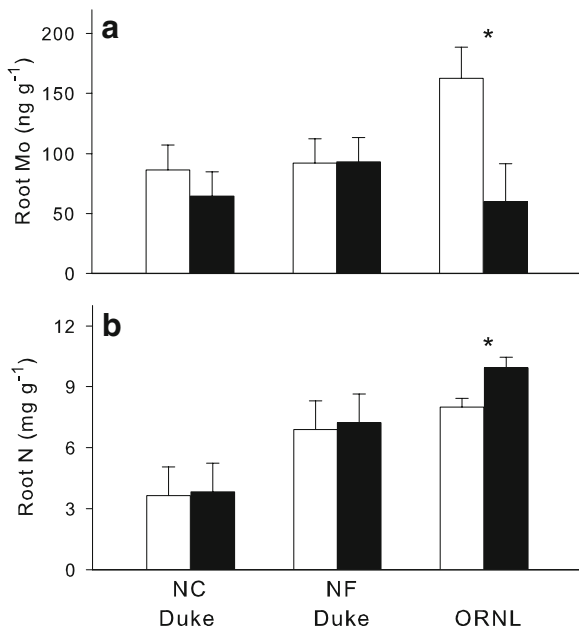


Fig. 3 Elevated CO₂ and N fertilization effects on root Mo and N concentrations at Duke and ORNL FACE. **a** Root Mo concentrations at Duke did not change with CO₂ or N enrichment, but there were significantly lower root Mo concentrations at ORNL in the CO₂ enriched rings ($p < 0.05$). **b** At Duke, there was no significant effect of elevated CO₂ on root N concentrations, but there were significantly greater root N concentrations with N fertilization. At ORNL, there was a significant increase in root N with CO₂ enrichment ($p < 0.05$). Filled bars represent means (\pm SE) from elevated rings and open bars from ambient rings. NC = N control; NF = N fertilization. * denotes significant CO₂ effect ($p < 0.05$)

CO₂ rings (Fig. 3a). However, there were no significant effects of CO₂ enrichment ($F = 2.40$, $P = 0.22$), soil depth ($F = 1.34$, $P = 0.32$), or CO₂ \times depth ($F = 0.91$, $P = 0.47$) on total soil Mo concentrations (Fig. 4a).

Bio-available soil Mo at ORNL was significantly greater in the elevated CO₂ rings compared to ambient rings ($F = 9.27$, $P = 0.01$), with increases in all soil depths but the 10–15 cm increment (CO₂ \times depth: $F = 2.90$, $P = 0.08$). Mo bio-availability decreased with depth from 0–20 cm in both CO₂ treatments at ORNL ($F = 8.92$, $P < 0.01$).

Bio-available Mo was positively correlated with log-SOM at ORNL ($R^2 = 0.32$, $P = 0.01$). No other measured soil factors were correlated with bio-available Mo (total Mo: $R^2 = 0.06$, $P = 0.31$; soil pH: $R^2 = 0.09$, $P = 0.21$), and bio-available soil Mo was not correlated with *L. styraciflua* foliar Mo at ORNL ($R^2 = 0.12$, $P = 0.30$).

N concentrations

ORNL root N ($F = 7.80$, $P = 0.07$; Fig. 3b) and soil N concentrations ($F = 6.96$, $P = 0.08$; Fig. 4c) were greater in the elevated CO₂ rings than in ambient rings. Because we collected bulk roots, these concentration differences may, in part, be driven by CO₂-mediated changes in root diameter.

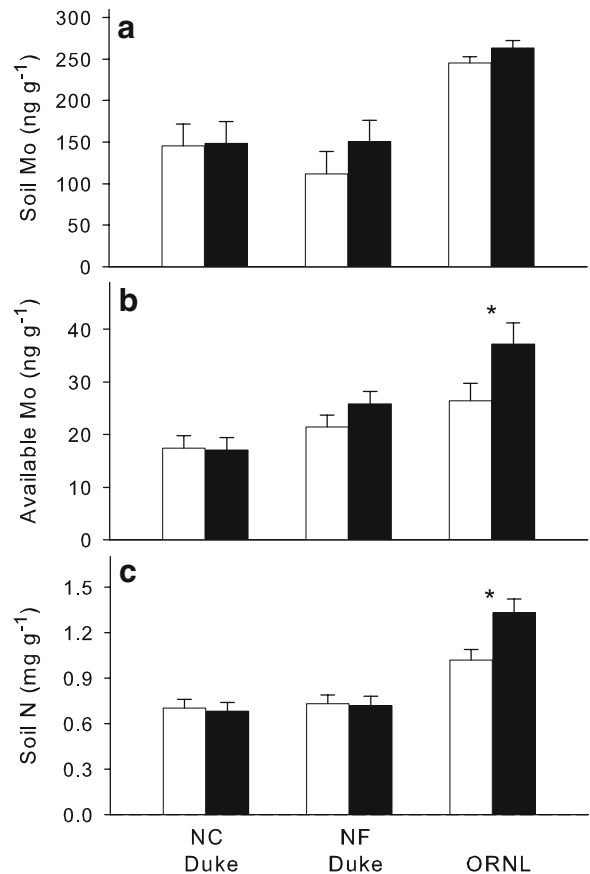


Fig. 4 Elevated CO₂ and N fertilization effects on soil (0–20 cm) Mo and N concentrations at Duke and ORNL FACE. **a** While there were no significant CO₂ or N fertilization effects on total soil Mo concentrations at either site, **b** at Duke, soil Mo bio-availability was greater in the CO₂ enriched plots in the lower soil depths (15–20 cm; $p < 0.10$) and there was a significant increase in bio-available Mo with N fertilization across depths ($p < 0.05$). Bio-available soil Mo at ORNL was also significantly greater with CO₂ enrichment ($p < 0.05$). **c** There were no detected effects of CO₂ or N fertilization on N concentrations in soils at Duke, but there were significantly greater soil N concentrations with CO₂ enrichment at ORNL. Filled bars represent least squares means (SE) from elevated rings and open bars from ambient rings. NC = N control; NF = N fertilization. * denotes significant CO₂ main effect ($p < 0.05$)

Effects of soil variables on NaR

To look at effects of soil variables on foliar NaR we focused on *P. taeda* 1-year needles because NaR in these leaves were the only ones affected by CO₂ and N treatments. We averaged NaR across canopy heights and soil variables across depths in the following analyses. There was a significant negative correlation between log NaR and bio-available soil Mo ($R^2=0.47$, $P=0.02$). The strength of this correlation increased markedly if a single outlier (CO₂-ambient, N-control sector) was removed from the analysis. When this sample was omitted, bio-available soil Mo now accounted for 86% ($P<0.01$) of the variation in foliar NaR. While we have no a priori reason to believe this NaR value is incorrect, its removal from the analysis does not alter the pattern of the relationship among these two variables. There is the potential for ring-level heterogeneity in soil N-dynamics (e.g., as referred to in Finzi et al. 2001), which could be driving the elevated levels of NaR in this ring.

None of the other soil metals or other soil variables measured were correlated with foliar NaR (Al: $R^2<0.01$, $P=0.91$; Pb: $R^2<0.01$, $P=0.96$; Fe: $R^2=0.04$, $P=0.55$; Cu: $R^2=0.03$, $P=0.60$; bio-available Cu: $R^2=0.19$, $P=0.17$; total Mo: $R^2=0.08$, $P=0.40$; N: $R^2<0.01$, $P=0.92$; pH: $R^2=0.05$, $P=0.53$; SOM: $R^2=0.01$, $P=0.76$).

To test the hypothesis that changes in soil properties caused by CO₂ and N fertilization are indirectly driving changes in foliar NaR, we used bio-available Mo as a covariate in an ANCOVA. This covariance test removes the variation in foliar NaR that is correlated with bio-available soil Mo. When bio-available Mo was included as a covariate in the statistical analysis, there was no significant difference in adjusted foliar NaR concentrations due to CO₂ ($F=3.18$, $P=0.33$) or N treatments ($F=1.93$, $P=0.40$) in 1-yr-old *P. taeda* needles, and there was no CO₂ × N interaction effect ($F=3.67$, $P=0.31$).

Discussion

The depression of *P. taeda* foliar NaR with CO₂ enrichment observed in this experiment (Fig. 1a) is consistent with a number of previous studies of CO₂ effects on NO₃⁻ assimilation (Bloom et al. 2002; Searles and Bloom 2003; Smart et al. 1998; Stitt and

Krapp 1999). One potential mechanism for this effect is increased competition for reductant between NO₃⁻ and carbon assimilation under elevated CO₂ (Bloom et al. 2002). CO₂ enrichment at both Duke and ORNL FACE sites has been shown to increase photosynthetic rates across canopy heights, despite large differences in light reaching upper and lower canopy leaves (Crous and Ellsworth 2004; Gunderson et al. 2002). Therefore, if competition for reductant between CO₂ and NO₃⁻ assimilation were limiting NaR, we would expect to see differences in NaR at different light levels—NaR should be lowest in low light conditions (lower canopy leaves) across CO₂ treatments and decrease with CO₂ enrichment.

In our study, however, there were no differences in NaR between the lower and upper canopies for either species nor was there a significant CO₂ × canopy height interaction (Fig. 2a), suggesting that observed CO₂ effects on NaR were not driven primarily by competition for reductant between carbon and NO₃⁻ assimilation. We should note, however, there is the potential for NaR activity to shift to other isoforms (our study focused on NADH:NaR) in response to environmental conditions or to overcome limitation by NADH (Kaiser et al. 2000). Therefore, further research is needed to rule out potential canopy level variation in the activity state of different NaR isoforms (NADH or NADPH).

The observed decrease in NaR in *P. taeda* may also have been driven by soil-mediated CO₂ effects, such as changes in nutrient status with CO₂ enrichment and N fertilization. While Mo is an essential nutrient for plant growth and for biological N transformations in plants and soils, little is known about Mo limitation thresholds and concentration ranges in nonagricultural species and systems. Foliar Mo concentrations in this study (Fig. 1b) were of similar magnitude to those found in Norway spruce needles in forests in southern Germany (20–250 ng g⁻¹; Lang and Kaupenjohann 1999) and slightly higher than those found in a leguminous vine in a Mo-limited scrub-oak community in Florida (10–20 ng g⁻¹; Hungate et al. 2004). Bio-available soil Mo concentrations at Duke and ORNL FACE (Fig. 4b) were slightly lower than values reported for German spruce forests (44–407 ng g⁻¹; Lang and Kaupenjohann 1999) and slightly lower than the deficiency range established for agricultural soils (100–200 ng g⁻¹; Sims and Evars 1997).

To test for indirect effects of elevated CO₂—that is, changes in soil nutrient status—on NaR we focused

on 1-year-old *P. taeda* needles because there was no effect of CO₂ enrichment or N fertilization on NaR in *L. styraciflua* leaves or 0-year *P. taeda* needles (Fig. 1a). We detected no CO₂ effects on total soil Mo or N concentrations at Duke FACE (Figs. 4a, c). Bio-available Mo, however, was greater with CO₂ enrichment in lower soil depths (15–20 cm) and greater across depths with N fertilization, and explained 47–86% of the variation in *P. taeda* NaR across CO₂ and N treatments. Soil Mo has been linked to limitation of N₂-fixation under elevated CO₂ in a scrub-oak community (Hungate et al. 2004), but Mo-limitation of NaR with CO₂ enrichment does not appear to be occurring at Duke FACE because bio-available Mo was *negatively* correlated with NaR. Although the direction of the correlation between bio-available Mo and foliar NaR was not as we expected, soil-mediated effects of CO₂ on NaR were further supported by results of the ANCOVA. When bio-available Mo was used a covariate in the analysis, there were no detected effects of elevated CO₂ or N-fertilization on adjusted NaR in *P. taeda*, suggesting that CO₂ and N-fertilization effects on *P. taeda* NaR were mediated by variation in bio-available Mo.

The negative relationship between bio-available Mo and foliar NaR appears contrary to expectations, as previous studies have demonstrated a positive correlation between NaR and soil Mo concentrations (Kaiser et al. 2005; Randall 1969; Stout and Meagher 1948). One possible explanation is that the bio-available Mo-leaf NaR correlation was driven by plant uptake of Mo from soils. Lang and Kaupenjohann (2000) suggest that Mo turnover in soils is governed by plant uptake and estimate the annual turnover of bio-available Mo (i.e., the oxalate extractable fraction) in soils to be about 30% in a Norway spruce forest. If plant uptake of Mo (for NaR) were driving soil Mo availabilities, we would expect a positive correlation between plant Mo and NaR and a negative correlation between plant and soil Mo. However, we found no relationship between Mo in leaves and soils. While *P. taeda* foliar Mo and NaR were correlated, this correlation was driven by differences between needle age classes. Alternatively, soil Mo-bioavailability may be driven by a complex interaction between plant and microbial Mo uptake as well as biological and chemical transformations in soils. Further research that incorporates soil Mo and N manipulations is needed to discern the mechanism of

the relationship between CO₂ and N treatments, bio-available Mo and leaf NaR.

CO₂ effects on NO₃⁻ and NH₄⁺ soil pools and plant uptake capacities may also be an important driver of NaR. While we did not measure NO₃⁻ and NH₄⁺ dynamics in this study, others have found no effect of elevated CO₂ on rates of N mineralization or nitrification at ORNL or Duke, and no effect on soil NO₃⁻ or NH₄⁺ pool sizes at Duke (Finzi et al. 2001; Sinsabaugh et al. 2003). CO₂ effects on NO₃⁻ and NH₄⁺ uptake by *P. taeda* have been variable across studies (BassiriRad 2000; BassiriRad et al. 1996a, b; Larigauderie et al. 1994). Larigauderie et al. (1994) found that elevated CO₂ increased *P. taeda* NO₃⁻ uptake when soil NO₃⁻ was high but decreased uptake under low NO₃⁻ conditions. Stitt and Krapp (1999) suggest one possible reason for reduced NO₃⁻ uptake with CO₂ enrichment—especially when NO₃⁻ concentrations are low—is that lower transpiration under elevated CO₂ may reduce bulk flow of soil water and water-soluble nutrients such as NO₃⁻ to roots. However, there have been no detected effect of elevated CO₂ on stomatal conductance or leaf-water relations in *P. taeda* at Duke (Ellsworth 1999) nor CO₂ effects on canopy water relations at Duke (Schafer et al. 2002).

Elevated CO₂ did have species-specific effects on NaR, but patterns were not as expected based on previous research on these species. Constable et al. (2001), who reported NaR activity of similar magnitude (0.1–0.6 μmol NO₂⁻ g⁻¹ h⁻¹) to levels found in our study, found an increase in *L. styraciflua* NaR with CO₂ enrichment, while we found no CO₂ effects on *L. styraciflua* NaR at Duke or ORNL (Fig. 1a). *P. taeda* NaR in 0-year needles did not change with CO₂ enrichment, as in Constable et al. (2001), but 1-year needle NaR decreased in our study (Fig. 1a). There are several possible reasons for the contrasting effect of elevated CO₂ on NaR in these two studies. In Constable et al. (2001), seedlings were grown in separate pots and were well-supplied with water and nutrients under relatively constant environmental conditions. In field conditions of the FACE sites, in addition to direct CO₂ treatment effects on NaR, there is potential for CO₂-mediated differences in soil water content, N concentrations and chemical forms, and competition among species for soil nutrients. Our study suggests that a change in soil Mo bio-availability, driven by CO₂ and N-fertilization, is

one important determinant of plant NaR. These system-level changes in soil properties can only be observed under longer time scales and larger spatial scales provided by the FACE experiments.

As with CO₂ treatment, N fertilization decreased foliar NaR in 1-year *P. taeda* needles but there was no change in *L. styraciflua*. While there were no detected differences in total soil N concentrations between the N-fertilized and control plots at Duke (Fig. 4c), total N concentrations in leaves and roots were greater with N fertilization (Figs. 1c, 3b). The negative correlation between *P. taeda* foliar NaR and leaf N concentrations may have been driven by a preferential uptake of NH₄⁺ with N-fertilization (fertilization treatment consisted of additions of NH₄⁺NO₃⁻), coupled with decreased uptake and assimilation of NO₃⁻. Previous studies have shown that the chemical form of N available to plants is an important regulator of NaR and also interacts with CO₂ effects on NaR (Geiger et al. 1999; Matt et al. 2001). Both species in our study have been shown to preferentially take up NH₄⁺ over NO₃⁻; Constable et al. (2001) determined that the proportion of N taken up as NO₃⁻ was about 30% in seedlings of both these species when both forms of inorganic N were available.

Instability of the nitrate reductase enzyme coupled with limitations of destructive harvest at the multi-user FACE experiments precluded us from measuring root NaR. We use caution in drawing conclusions about treatment effects on whole plant NO₃⁻ assimilation capacity, particularly since root NaR in both these species accounts for more than 50% of whole plant nitrate reduction (Constable et al. 2001). Root NaR may be enhanced by elevated CO₂ because the energy requirements for root NaR are met through respiration of root carbohydrates (Sechley et al. 1992), which tend to increase with CO₂ enrichment (BassiriRad et al. 1996b). Therefore, the observed decrease in *P. taeda* NaR may have been coupled to an increase in root NaR. However, if root Mo concentrations are correlated with NaR (as in leaves) then there does not appear to be an increase in root NaR with CO₂ enrichment because there were no CO₂ effects on root Mo concentrations at Duke and a decrease in root Mo concentrations at ORNL in the CO₂ enriched plots (Fig. 3a).

Although we did not measure leaf mass per unit area (LMA) in this study, differences in LMA with canopy height and CO₂ treatment at Duke and ORNL FACE have been reported in other studies. LMA in *L. styraciflua* at ORNL was two times greater in upper

canopy leaves than in lower canopy leaves (Norby and Iversen 2006), and LMA in *P. taeda* needles at Duke was about 1.5 times greater in the upper canopy than the lower canopy (Springer et al. 2005). Although we found no canopy effects on mass-based NaR, NaR on an area basis would therefore be greater in the upper canopy, as would be expected if NaR was limited by reductant. There was also a slight increase in *L. styraciflua* LMA with CO₂ enrichment at ORNL (Norby and Iversen 2006); while *L. styraciflua* mass-based NaR was not affected by CO₂ enrichment, area-based NaR would therefore be increased with CO₂ enrichment. There was no detected CO₂ effect on *P. taeda* LMA at Duke FACE in needles collected from 1999 through 2002 (Springer et al. 2005).

Our results demonstrate that elevated CO₂ is affecting plant NO₃⁻ dynamics and that soil nutrient status is an important component of plant response to CO₂ enrichment. As a required enzyme cofactor for several N transformation processes (e.g., N₂ fixation, nitrate assimilation, nitrification and denitrification) Mo is a key, yet often overlooked, player in biological N dynamics. Further research on diurnal and seasonal variation in NO₃⁻ assimilation and direct species manipulation experiments are needed to determine the potential role of CO₂-mediated changes in community and ecosystem dynamics on plant NaR. Research on multiple element interactions and indirect effects of micronutrients on C and N cycling will enhance our understanding of and ability to predict global change effects on plant and ecosystem processes. This study also highlights the value of field experiments—even for studying complex physiological processes; large-scale studies such as the FACE experiments allow observation of complex system-wide processes and indirect effects that may not be realized in a greenhouse or laboratory setting.

Acknowledgements We thank C. Iversen, R. Norby, R. Oren, and the staff at the FACE sites for field support, and R. Norby, S. Baines, and two anonymous reviewers for advice during manuscript preparation. This work was supported by the U. S. Department of Energy, Office of Science (BER), and fellowships from the National Science Foundation (S.M.N.) and Department of Energy (S.M.N.).

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