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Effects of Elevated Carbon Dioxide on Trace Metal Cycling in Forests

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Abstract of the Dissertation

Effects of Elevated Carbon Dioxide on Trace Metal Cycling in Forests

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Changes in plant growth and function associated with increased atmospheric carbon dioxide (CO_2) can affect element cycling and ecosystem processes. My dissertation examines CO_2 effects on trace metals in plants and soils because of the massive impact plant processes have on metal cycling and the importance of metals as both contaminants and micronutrients.

I examined CO_2 effects on a suite of metal micronutrients and contaminants in forest trees and soils at two free-air CO_2 enrichment (FACE) sites—a loblolly pine forest in North Carolina and a sweetgum plantation in Tennessee—and an open-top chamber experiment in a scrub oak community in Florida. I found that CO_2-mediated changes in soil properties affected the storage of metals in soils. There was a general decline in foliar metal concentrations with CO_2 enrichment; however, CO_2 effects on foliar metals were species and element specific.

I also focused on CO_2 effects on the cycling of mercury (Hg) because Hg is an ecosystem contaminant whose movement through the environment is mediated by biological activity. Hg concentrations in soils increased with CO_2 enrichment at both FACE sites, but there were no direct CO_2 effects on litterfall or throughfall Hg deposition.

To better understand the link between plant essential metals, elevated CO_2 and plant function, I focused on CO_2 effects on nitrate reductase activity (NaR) and the NaR cofactor metal, molybdenum (Mo). As a rate-limiting step in nitrate assimilation, the reduction of nitrate is an important component of plant physiological response to elevated CO_2 and terrestrial carbon sequestration. I found that both CO_2 and N enrichment had species-specific impacts on NaR and that NaR was negatively correlated with bio-available soil Mo across treatments, suggesting that CO_2 and N-mediated changes in soil nutrient status/plant function are altering soil Mo dynamics.

This dissertation demonstrates that increased atmospheric CO_2 has the potential to affect the biological cycling, storage, and stoichiometry of trace metals in terrestrial systems. These changes in metal dynamics may have important implications for plant and ecosystem processes, human nutrition, and the movement of contaminants through the biosphere.

To Clancy, Ciati and Marc

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Chapter 1: Trace metal stoichiometry: human perturbation and elemental imbalance in terrestrial systems

1. Introduction

For centuries, scientists have recognized the requirement of nutrients for plant growth and survival and have been addressing the role of macronutrient acquisition in both managed and unmanaged terrestrial systems (Chapin 1980; Rubio et al. 2003; van der Ploeg et al. 1999). Resource acquisition models have formed the bases of theories of plant competition and coexistence (Tilman 1985; Tilman et al. 1997), diversity and distribution (Anderson et al. 2004; Chesson 2000), anti-herbivore defense (Bryant et al. 1983), and allocation strategies (Gleeson and Tilman 1992). Many studies have highlighted the importance, not only of single nutrient dynamics, but also of nutrient ratios in mediating ecological processes (Sterner and Elser 2002). Much of the ecological focus on element dynamics, however, has been centered on carbon and macronutrients (e.g., Knecht and Goransson 2004; Vanarendonk and Poorter 1994; Willby et al. 2001).

While trace metals are important as both micronutrients and ecosystem contaminants, little attention has been paid to the ecology of trace metals. One reason for the lack of attention to trace metals in ecological literature is that, until recently, metal supply may often have been in excess of plant demand and metal contamination was rare. Anthropogenic activities, however, are changing both metal supply levels and plant demands. For example, human inputs of nitrogen (N) may be alleviating historical N limitation in plants and creating limitation by metal micronutrients in natural ecosystems, as has been found in many agricultural systems. Changes in plant physiology and growth with CO_2 fertilization may also change plant demand for metal micronutrients and alter the biological role of plants in the cycling of metal contaminants.

Through both direct (e.g., industrial runoff and agricultural practices) and indirect (e.g., increased atmospheric CO_2 and soil acidification) processes, human activities are

altering total soil metal content, biological availability, as well as trace metal limitation and toxicity thresholds in plants. These changes in metal dynamics may have important implications for ecological interactions, human nutrition, and the movement of contaminants through the biosphere.

In the following sections, I will: *i.* review the role of trace metals in plant processes; *ii.* examine the role and mechanisms of trace metal interactions in alleviating or exacerbating element limitation and toxicity thresholds; *iii.* discuss our current knowledge of trace metals in unmanaged systems; and *iv.* address the potential impacts of human alteration of element cycles and the global energy balance on trace metal dynamics.

2. Trace metals overview

2.1. Geological trace metal

In geological terms, a trace metal is a metal that occurs in only minor amounts (< 1.0 mg kg^{-1}) in water or sediment [e.g., arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn); Berndt et al. 1998]. Plants can affect the cycling of metals—even those that are not used by plants—through uptake from soils, redistribution via litterfall, and changes in root exudates and rhizosphere chemistry. In the case of Hg, plant canopies serve as an important conduit for the transfer of this contaminant from the atmosphere to soils (Grigal 2003; St Louis et al. 2001). At high concentrations trace metals can limit plant productivity by interfering with physiological processes and uptake of biologically essential nutrients (Barcelo and Poschenrieder 1990; Clijsters and Vanassche 1985; Vanassche and Clijsters 1990). In this review, I will not focus on toxicity effects of single geological trace metals on plant dynamics because this research has already been well-summarized (Clemens 2001; Foy et al. 1978). In discussing non-essential trace metals, I will focus on element interactions and human alteration of metal stoichiometries.

2.2. Biological trace metals

Some elements that are geological trace metals are also essential plant nutrients. In biological terms a trace element is an element required in minor amounts that has specific and essential metabolic functions (Marschner 1995). For an element to be considered essential it must be required by an organism to complete its life cycle and be non-replaceable (Arnon and Stout 1939). Six of the eight known plant micronutrients are metals [copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), and zinc (Zn)]. All of the metal micronutrients are transition metals. Some of the properties of transition metals as a group that make them well-suited to their biological role as micronutrients include multiple oxidation states (with the exception of Zn) and tendency to form strong complexes with biological ligands (Clarkson and Hanson 1980).

Some metal micronutrients have high soil concentrations (e.g., Fe), but it is important to note that total abundance in soils does not necessarily reflect availability. While plant concentrations of trace metals are generally lower than those found in soils, trace metal limitation may occur because plants do not have access to the total soil pool due to metal adsorption and formation of insoluble organic and inorganic complexes (Guerinot and Yi 1994; Gupta and Lipsett 1981; Sims and Evarsi 1997). The physicochemical properties that make metals biologically important can also restrict their uptake under certain soil conditions. For example, Fe frequently is limiting even when total soil pools are high because Fe forms insoluble ferric oxides, which are not biologically available (Guerinot and Yi 1994).

2.3. Variation in soil metals across soil types

Metal concentrations and bioavailabilities vary with parent material and soil age. While metal distribution in soils can be highly heterogeneous, there are some general patterns of soil metal distribution over large geographic areas driven by parent material, soil age, and pedogenic processes (Holmgren et al. 1993). For example, serpentine soils are characterized by high concentrations of heavy metals (e.g., Ni, Cr, and Co) because of high metal content of the ultramafic parent material (Gasser and Dahlgren 1994; Gasser et al., 1995). Young soils, such as andisols, derived from volcanic material are characterized by high fertility and high metal content. Variation in volcanic source

material, however, can have a greater impact on soil metal content than soil age (Burt 2003). Low metal availabilities are common in older/weathered soils, such as oxisols and ultisols, common in tropical and subtropical regions; both of these tropical soil types are generally characterized by low organic matter contents, low pH values and high levels of Fe oxides (Rieuwerts 2007, USDA 1999). Although Fe oxides bind metals in soils, many tropical soils have low overall metal content because of low organic matter, low cation exchange capacity, and low pH (Rieuwerts 2007).

3. Functional role of trace metals in plants

As common components of enzymes, trace metals are key regulators of metabolism and growth and play a central role in the acquisition of macronutrients and in the assimilation of carbon. Micronutrients in plants function as constituents of enzymes, regulators of enzyme activity, and mediators of numerous essential redox reactions (Marschner 1995). Examples of some of the functions of plant metal micronutrients can be found in the Table 1 and in the text that follows. In the following sections, I provide a brief overview of the biological role of each metal micronutrient in plants, as well as soil and environmental conditions that may lead to limitation or toxicity thresholds under natural ecological conditions (i.e., not under conditions of direct anthropogenic inputs). A more extensive overview can be found in Marschner (1995) and in reviews by Clarkson and Hanson (1980) and Welch (1995).

3.1. Iron

Fe serves a redox function in a wide-scope of physiological processes in plants including photosynthesis (ferredoxin), nitrogen assimilation (nitrate reductase), and respiration (cytochrome; Clarkson and Hanson 1980). The characteristics of Fe that support its broad use in biological processes are its ability to transition between redox states II and III, and to complex with a diverse group of ligands (Hell and Stephan 2003). These characteristics, however, also restrict the availability of Fe in soils. Fe (II) is the form of Fe absorbed by plants. In well-aerated alkaline soils, however, Fe (II) is oxidized

to the insoluble Fe (III); Fe in aerated soils can be orders of magnitude lower than plant requirement (Hell and Stephan 2003).

When Fe bio-availability is low, plant Fe uptake is supported by deficiency-induced chelation and reduction systems (Hell and Stephan 2003). Plants can acquire Fe (III) after reduction to Fe (II), primarily through up-regulation of ferric reductase, which is induced by low Fe availability but is inhibited in high pH soils (Graham and Stangoulis 2003). Under low Fe, plants in the family Graminaceae take up Fe (III) after chelation with phytosiderophores, which are not inhibited by high pH (Graham and Stangoulis 2003). These Fe uptake strategies have been well-documented and reviewed (Lindsay and Schwab 1982; Marschner and Romheld 1994; Romheld 1987). Fe toxicity tends to occur in soils that are water-logged, particularly those with moderate to high acidities, because of reduction of insoluble Fe(III) oxides to Fe(II) in reducing environments (Favre et al. 2002; Lucassen et al. 2000).

3.2. Copper

Cu has two oxidation states—Cu (I) and Cu (II)—and serves a redox function in several oxidase enzymes. Cu is a component of superoxide dismutase, an enzyme important for detoxification of superoxide radicals and in protection of plants from ozone damage (Pitcher and Zilinskas 1996). Cu is also a component of plastocyanin—an electron donor to Photosystem I (Clarkson and Hanson 1980). Further information on the role of Cu in plants can be found in Gladstones et al. (1975), Maksymiec (1997), and Sommer (1931).

Most Cu in soils is not available for plant uptake because it is bound to organic matter, to metal oxides, or trapped in soil minerals (Bolan and Duraisamy 2003; do Nascimento et al. 2007). The divalent form of Cu is the form readily absorbed by plants (Grusak et al. 1999). Bioavailability of Cu is low in calcareous soils (Grusak et al. 1999), and increases with decreasing pH (Chaignon et al. 2003; Gupta and Aten 1993).

3.3. Manganese

Mn also plays a central role in photosynthesis—Mn is a required component of the oxygen evolving complex (OEC) of Photosystem II and is required for PEP carboxykinase activation in C_4 plants (Henriques 2003). Mn serves a redox role in a range of enzymes (e.g., Mn superoxide dismutase, Mn peroxidase, Mn catalase). As with other transition metals, the biological role of Mn is governed by its chemical properties, primarily, its ability to transition between oxidation states II, III and IV (Yachandra et al. 1996).

Mn availability is often limited in calcareous soils (Grusak et al. 1999). Like Fe and Cu, the biologically active form (and form that is absorbed by plants) of Mn is the divalent form. Mn availability increases in reducing atmospheres, such as waterlogged anaerobic soils and soils with low redox potentials (Jones and Ethering 1970; Tao et al. 2007). Mn toxicity, which is more common in tropical soils, is tied to soil pH because Mn availability increases with soil acidity (Ohki 1976; Ohki 1985; Vadez et al. 2000). Throughout the US, Mn deficiency is more common, especially in high pH soils (Graham et al. 1995; Saberi et al. 1999). Mn deficient plants have lower leaf photosynthetic capacity, primarily due to decreased number of PS II units per unit leaf area (Henriques 2003). Mn-deficiency can also limit N_2-fixation because Mn is involved in ureide degradation (Todd et al. 2006; Vadez et al. 2000).

3.4. Molybdenum

There are only four known Mo-dependent enzymes in plants: nitrate reductase, xanthine dehydrogenase, aldehyde oxidase, and sulfite oxidase (Kaiser et al. 2005; Mendel and Hansch 2002). Mo is also a required cofactor of nitrogenase, the bacterial enzyme that catalyzes the N_2-fixation reaction (Mendel and Hansch 2002). Soil Mo can have oxidation state II through VI, but only Mo VI is soluble and available to plants (Mendel and Hansch 2002). Plants absorb dissolved Mo primarily as the molybdate ion, MoO_4^{-2}.

Mo soil availability is dependent upon soil pH, soil metal oxide content, organic matter content, and soil drainage (Kaiser et al. 2005). Unlike other metal micronutrients, Mo availability decreases in acidic soils (<5.5) due to increased adsorption onto metal

oxides (Kaiser et al. 2005; Stiefel 2002). Although plant requirement for Mo is quite low, Mo deficiency occurs across a range of species and plant functional types (Gupta 1997). Mo-deficiency is widespread in tropical legumes (Schwenke and Kerridge 2000) because of soil acidity and high Mo requirements for nitrogenase. Because Mo limitation affects nitrate reductase, Mo-deficient plants can exhibit symptoms of N-deficiency (Mendel and Hansch 2002).

3.5. Nickel

The only known role of Ni in plants is the activation of urease. Urea is an important N metabolite in plants and important for within plant N transport; as such, Ni deficient plants are often metabolically N-deficient (Bai et al. 2006; Gerendas and Sattelmacher 1997; Marschner 1995). Ni has been recognized as an essential plant nutrient for less than a decade (Bai et al. 2006), and Ni deficiency has only been reported in a few species. Patterns of Ni availability in soils are similar to other metal micronutrients (with the exception of Mo). Soil pH is the most important factor determining the bioavailability of Ni in soils; Ni availability decreases with increasing pH because increased binding of Ni to soil organic and inorganic matter (Weng et al. 2003; Weng et al. 2004).

3.6. Zinc

Zn is a component of a diverse group of enzymes, which include carbonic anhydrase, superoxide dismutase, RNA and DNA polymerase, and several dehydrogenases, to name a few (Clarkson and Hanson 1980). Unlike other metal micronutrients, Zn does not transition between oxidation states and does not serve a redox role; rather, Zn serves a catalytic and structural role (Berg and Shi 1996). The main property of Zn that makes it a ubiquitous micronutrient in plants (and across eukaryotic taxa) is its strong tendency to form stable tetrahedral bonds and lack of redox activity (Berg and Shi 1996; Cakmak 2000; Clarkson and Hanson 1980).

Zn deficiency is common in plants worldwide, especially in arid and semi-arid regions where alkaline soils are common (Cakmak 2000; Guerinot and Eide 1999; Rashid

and Ryan 2004). Zn deficiency is more prevalent in calcareous soils, alkaline soils, and soils with low organic matter (Rashid and Ryan 2004). Most soil Zn is not available because it is bound to organic matter and to metal oxides or held in soil minerals (Grusak et al. 1999).

4. Interactions among trace metals

While soil properties may govern metal micronutrient bioavailabilities, plant populations and species vary in their requirement for metal micronutrients and tolerance of metal contaminants. Biological availability of metals may also be affected by elemental interactions in soils and plants, and interactions among trace metals can be important determinants of plant limitation/toxicity thresholds. Competitive uptake between two metals can increase deficiency symptoms or alleviate toxicity based on concentrations of the interacting metals in soils and plants.

Antagonistic or synergistic interactions between elements may occur as a result of ion interactions in soils, at the plant-soil interface, in plant transport systems, and through physiological interference. Chapin (1980) recognized the vital role of element interactions in resource acquisition in noting that the rate of absorption of one nutrient frequently depends upon the concentration of another. This dependence results from the use of one element to obtain another, competitive inhibition for ion absorption sites, or maintenance of cation-anion balance.

In the following sections I present a few examples of trace metal interactions (or interactions of macronutrients with trace elements) as they pertain to element deficiency or toxicity. This is not meant to be a complete review of trace element function in plant metabolism, element uptake and assimilation, or overview of all possible synergistic and antagonistic element interactions (see Foy et al. 1978, Marschner 1995, Kabata-Pendias 2000). The element interactions discussed are provided to exemplify the scope of stoichiometric effects, the role of trace metal stoichiometry in plant metabolism, growth, and survival, and potential impacts of human induced stoichiometric imbalance.

4.1. Induced deficiency

The ratio of elements in soils and plants can affect plant growth and survival through stoichiometrically induced element-deficiency. Induced deficiency may occur due to dilution of the deficient nutrient through growth enhancement (Loneragan et al. 1979; Neilsen and Hogue 1986) or through one of several interference mechanisms. Interference can take place at the soil-root interface (Loneragan et al. 1979; Marschner 1995; Marschner and Schropp 1977) or can affect within plant element mobility (Hill et al. 1978).

Induced deficiency can also occur as a result of altered plant physiology, morphology, and phenology (Hill et al. 1978; Marschner 1995). For example, N fertilization can aggravate Cu deficiency (Dias and Oliveira 1996) because as plant N-levels increase, a greater proportion of total Cu is complexed to amino acids and proteins. High N supply also causes a delayed senescence of leaves and thus a delay in Cu translocation (Hill et al. 1978). Both of these mechanisms essentially decrease the amount of available Cu for new growth. Because of the effect of N on senescence and translocation, N concentrations may also affect other elements whose availability for new growth is dependent upon within plant element recycling.

Phosphorus (P) induced Zn deficiency is common in agricultural systems with low Zn soils (Marschner 1995). P induced Zn deficiency has been attributed to growth-enhanced dilution effects (Loneragan et al. 1979), within plant physiological interactions (Cakmak and Marschner 1986), and uptake interference (Marschner and Schropp 1977). High P content in soils can inhibit Zn uptake by decreasing soil Zn solubility (Marschner and Schropp 1977) and by decreasing root growth and mycorrhizal infection (Marschner 1995).

Ionic competition is likely to occur between elements that share similar chemical and physical properties. Cellular ionic uptake competition between Zn and Fe, and Zn and magnesium (Mg), due to similarity in size and properties may result in induced Mg or Fe deficiency when Zn levels are high (Boardman and McGuire 1990; Marschner 1995). Induced deficiency of Mg when Mn levels are high can also occur as a result of structural and chemical similarities, and because Mg has low binding strength and can be competitively displace by Mn (Heenan and Campbell 1981). Ionic competition is also

important for the bioavailability of Mo, which is often limited by competition between MoO_4^{2-} and sulfate (SO_4^{2-}) ions (Guyette et al. 1989; Marschner 1995; Zimmer and Mendel 1999).

4.2. Induced tolerance

When elements interact in such a way that one element interferes with uptake of another, there is the possibility for both an induced deficiency effect, as discussed above, or an induced tolerance effect, depending upon elemental concentrations and plant requirements. This is the case for the interactions between SO_4^{2-} and MoO_4^{2-}, in which competitive uptake of these two divalent anions can occur. At low Mo levels, SO_4^{2-} can induce deficiency; while at high soil Mo levels, SO_4^{2-} may depress Mo uptake, thereby increasing the toxicity threshold of soil Mo supplies (Pasricha et al. 1977). The effect of Mg-Mn interactions on plant growth also may be beneficial or detrimental dependent upon element concentrations. At low Mg levels, Mn can induce deficiency symptoms but by the same mechanism can decrease uptake of Mg at otherwise toxic levels (Heenan and Campbell 1981).

Silicon (Si) has a number of beneficial effects on plant growth, especially in the alleviation of plant stress (Marschner et al. 1990). One well-documented beneficial role of Si is the alleviation of Mn and Al toxicity through external (soil) and internal (plant) processes (Barcelo et al. 1993; Horst and Marschner 1978; Ma 2004). S increases the toxicity threshold for Mn by affecting a more even distribution of Mn within the leaf and by decreasing transport of Mn from shoots to roots (Horst and Marschner 1978).

Cd is a widespread contaminant and is a toxic heavy metal for many organisms. Competitive uptake between Zn and Cd has been well-documented, as has Zn induced Cd tolerance (Grant et al. 1998; Hart et al. 2002; Puschenreiter and Horak 2003). The antagonistic effect of Zn on Cd uptake can be attributed to a common transport system at the root cell plasma membrane (Hart et al. 2002) and also to within plant transport interference (Cakmak 2000; Koleli et al. 2004). Koleli et al. (2004) suggest that Zn alleviates Cd toxicity by interfering with Cd uptake and transport, but also by improving plant defense against Cd-induced oxidative stress.

4.3. Induced toxicity

While there are fewer examples of accentuated toxicity, stoichiometrically-mediated toxicity may occur as a result of synergistic interactions among elements. In a study of metal uptake by rice, it was shown that upward transport of heavy metals in rice was significantly increased when multiple metals were present in soil (Zhou et al. 2003).

Fe deficiency increases root uptake of Cu and other metals from contaminated soils because Fe-deficiency induced chelation and reduction uptake mechanisms may facilitate uptake of other divalent cations such as Cd, Zn and Mn (Chaignon et al. 2002; Cohen et al. 1998; Cornu et al. 2007; Graham and Stangoulis 2003).

While much of the work on trace element interactions and deficiency/toxicity has thus far been focused on agricultural species, mechanisms and effects are a function of basic plant physiology, and therefore, can be applied to species in both managed and unmanaged systems. Differences in toxicity and deficiency thresholds among species may play a pivotal role determining species distribution and ecological dynamics.

5. Natural plant communities and ecological implications

Plants vary in their uptake capacity, requirement for, and tolerance of trace metals (Brown et al. 1996; Freitas et al. 2004; Woodbury et al. 1999). These differences among plant populations and species have been the focus of strategies of phytoremediation of toxic trace metals (Meagher 2000; Salt et al. 1998) and strategies of biofortification for increasing micronutrient content of food crops (Bouis 2002; DellaPenna 1999). These differences are also likely to be important mediators of ecological dynamics in non-managed systems.

The ecological role of metals in plant communities has been well-recognized in serpentine systems. In the United States, serpentine soils are most common in California, but they can be found throughout the United States and are distributed worldwide. Serpentine systems are characterized by low calcium to magnesium ratios in soils, elevated levels of soil metals (Fe, Ni, Cr, Co), and low plant nutrient concentrations

(Brady et al. 2005). These unique soil characteristics lead to low plant productivity, high rates of endemism, and distinct plant morphologies (Brady et al. 2005; Whittaker 1954). Metal hyperaccumulation (particularly Ni) has been reported in numerous serpentine plants (Iturralde 2001; Reeves et al. 1999), and a high metal tolerance level is one potential mechanism supporting the high number of endemic species found on serpentine soils (Brady et al. 2005).

Trace metal dynamics may be particularly important in the preservation of rare and endemic species because soil metals can be highly heterogeneous, and micronutrient limitation/toxicity thresholds are often location and genotype specific (Rashid and Ryan 2004). Habitat characterization and predictive distribution models for rare and endemic species, however, rarely consider soil trace metal supply. Soil availabilities of three micronutrients—Fe, boron (B, a nonmetal micronutrient), and Cu—have been shown to be significant predictors of rare-plant distribution in southern Appalachian rock outcrops (Wiser et al. 1998). Bowker et al. (2006) also found that the occurrence of lichen-moss soil crusts (which, like rare vascular plants, are patchily distributed on soil microsites) was correlated with soil metal concentrations (Zn, Mn, K and Mg).

Spatiotemporal patterns in soil metal resources were also found to be an important factor mediating occurrence and spread of an invasive exotic species (Miller et al. 2006). Winter growth of the invasive exotic grass, *Bromus tectorum*, was found to be nutrient limited, and growth was positively correlated with soil Mn (and negatively correlated with P). This study was conducted on calcareous soils, where Mn limitation is common because of sorption reactions of Mn with carbonates (Grusak et al. 1999; Miller et al. 2006). Therefore, a competitive advantage in Mn uptake may support the growth and spread of *B. tectorum* in low Mn soils.

Trace element interactions would be most likely to be significant and noticeable in systems in which interacting elements are at or near an imbalanced (in terms of plant nutrition) level. Van der Welle et al. (2007) looked at biogeochemical interactions between Fe and SO_4^{2-} and the effect of element interactions on interspecific competition between two freshwater macrophytes—*Stratiotes aloides* and *Elodea nuttallii*. In waterlogged soils, SO_4^{2-} is reduced to sulphide (S^{2-}). High levels of S^{2-} can be highly

toxic to plants. S^{2-} also binds to Fe, which may cause Fe deficiency in aquatic plants (van der Welle et al. 2006). Van der Welle et al. (2007) found that growth of *S. aloides* was directly regulated by Fe-SO_4^{2-} interactions and indirectly regulated by the effects of Fe and SO_4^{2-} on the competitive strength of *E. nuttallii*.

Lucassen et al. (2006) also found that Fe plays an important ecological role in wetland and riverine species. However, in this study Fe toxicity—which is more common in waterlogged soils—was affecting species distribution. Lucassen et al. (2000) first reported Fe-toxicity in a natural system—in flote grass (*Glyceria fluitans*) growing in water-logged soils—and Lucassen et al. (2006) reported that Fe was among the most important factors that explained variation in species distribution of *Alnus glutinosa* (black alder).

Endo and ectomycorrhizal associations can be important determinants of heavy metal resistance in plants (Bradley et al. 1982; Galli et al. 1994; Gildon and Tinker 1983; Krupa and Kozdroj 2007; Newsham et al. 1995). Bradley et al. (1982) found lower concentrations of metal contaminants in shoots of plants with ericoid mycorrhizal associations, but higher concentrations in roots compared to non-mycorrhizal plants. While the mechanisms of mycorrhizal-mediated resistance to heavy metals are still uncertain (Jentschke and Goldbold 2000), reduced translocation of metals from roots to shoots in mycorrhizal-associated plants has been found in a number of studies (Bradley et al. 1982, Krupa and Kozdroj 2007). Increased atmospheric CO_2 and temperature both have been shown to enhance mycorrhizal-fungal abundance (Clemmensen et al. 2006; Fujimura et al. 2008; Olsrud et al. 2004), and thus, may alter plant-metal toxicity thresholds.

Natural variation in soil metal concentrations and species level differences in toxicity and limitation thresholds suggest a role for trace metal stoichiometries in ecological interactions and in the structuring of natural plant communities. Human alteration of trace element stoichiometries may alter species distributions and ecological interactions through element limitation/toxicity effects and interactions between elements, as discussed above.

6. Human Impacts

6.1. Direct effects

Anthropogenic activities have had both direct and indirect effects on local, regional, and global metal stoichiometries. Through activities such as mining, coal combustion, and waste disposal, humans have moved metals from deep ore deposits in the lithosphere to biologically available pools (Han et al. 2002; Nriagu and Pacyna 1988). The problem of metal pollution is exacerbated by the fact that unlike many organic pollutants, metals accumulate once they enter the soil system (Han et al. 2002).

Atmospheric circulation patterns and deposition processes have facilitated global transport and transformation of metals from high activity areas to regions distant from sources of metal extraction and redistribution (Steinnes and Friedland 2006; Vukmirovic et al. 2004). Data from Vostok ice cores and from peat cores show a pattern of human-mediated metal redistribution that has been occurring for over 2000 years. Pb used more than two millennia ago by Roman and Greek civilizations had been transported and distributed on a global scale (Hong et al. 1994), and anthropogenic inputs of arsenic (As), antimony (Sb), and Pb have been recorded in peat cores dating to over 2000 years before present (Shotyk et al. 1996). Metal inputs associated with the Industrial Revolution have been observed in pristine areas for a number of elements including Cd, Cu, and Zn (van de Velde et al. 2000). These are just a few of many examples of documented impact of human perturbation on local, regional, and global trace metal distributions.

6.2. Indirect effects

Indirect effects of global warming, hydroclimatic changes, and alteration of physical and chemical properties of soils can also affect the distribution of trace metals in soils and organisms. As mentioned in Section 3, two of the most important soil factors regulating the bioavailability of metal micronutrients are soil organic matter and pH. These two factors are also key regulators of bioavailability of metal contaminants in soils (Crowder 1991; McLaughlin et al. 2000; Oborn et al. 1995). In the following sections I will focus on global change effects on these two soil properties—pH and SOM. I will also discuss the interactions between global change and biological processes on plant

metal stoichiometry. I will focus on effects of elevated CO_2 on trace metals in plants and soils, because CO_2 effects on many plant and ecosystem processes have been well-documented, yet little is known about how these changes may affect trace metal stoichiometry.

6.2.1. Soil pH

Soil pH is one of the main factors influencing the solubility and availability of soil metals (see references in Section 3). The effects of human emissions of sulfur and nitrogen oxides—and their subsequent chemical conversion to nitric and sulfuric acids—on forest soils and ecosystem processes are well-recognized. 'Acid rain' can increase soil acidity, mobilize Al, and leach macronutrient cations such as Ca, K, and Mg (Johnson et al. 1981; Likens et al. 1996). Increased soil acidity can also increase the solubility, availability and toxicity of soil metals such as Pb, Zn, Cu, Cd, Mn, and V (Hutchinson and Whitby 1977; Singh and Agrawal 2008).

Unlike other metal micronutrients, Mo availability in soils decreases with decreasing pH. Lang and Kaupenjohann (1999) measured Mo in acidic forest soils and found lowest extractable Mo associated with low pH values. Plass (1983; in Lang and Kaupenjohann 1999) hypothesized that leaching of NO_3^- from acidic forests soils is not a sign of N saturation, but rather of Mo deficiency. Deposition of sulfur compounds may be exacerbating this problem by lowering pH and by increasing ionic competition between MoO_4^{2-} and SO_4^{2-} (Lang and Kaupenjohann 1999).

Other global change factors may also affect soil acidity. Increased microbial and root respiration under elevated CO_2 may increase carbonic acid in soil water (Oh and Richter 2004). In column leaching experiments, Oh and Richter (2004) found that CO_2-mediated increases in carbonic acid could increase soil acidity across soil types and also may solubilize Al in weathered soils. Further research and syntheses of existing data are needed to determine CO_2 effects on soil acidity, as well as effects on soil metal mobility.

6.2.2. Soil organic matter

Soil organic matter (SOM) is also a key regulator of soil metal mobility and bioavailability. Increased global temperatures, particularly in high latitudes, can increase decomposition and nutrient availability, and mobilize a significant pool of soil carbon (Dutta et al. 2006; Mack et al. 2004; Nowinski et al. 2008; Zimov et al. 2006). Increased fire frequency, associated with higher global temperatures, can also alter both above and below ground carbon storage (Mack et al. 2004; Treseder et al. 2004).

Elevated CO_2 can affect carbon inputs to soils through increased litterfall, fine root production, and increased root exudates. Changes in litter quality with CO_2 enrichment (higher C: N, higher lignin) may limit decomposition and lead to increased soil carbon storage under elevated levels of atmospheric CO_2 (Cotrufo et al. 1994; Parsons et al. 2004). Norby et al. (2001), however, found that while litter chemistry was altered by elevated CO_2, effects on decomposition were not consistent across systems and studies. Jastrow et al. (2005) found an increase in C storage in temperate ecosystems with elevated CO_2 exposure, and increased root production in grassland and forests exposed to elevated CO_2. Elevated CO_2 may also affect soil carbon through increased rhizodeposition (Cardon 1996; Paterson et al. 1997). Cheng and Johnson (1998) found a 60% increase in soluble carbon in the rhizosphere of wheat plants grown at high CO_2. Organic acids exuded by plants into the rhizosphere can act as metal complexing agents (Mucha et al. 2005); therefore, changes in the quality and quantity of rhizodeposits with CO_2 fertilization may affect metal uptake and bioavailability. Based on what we know about metals in soils, and global change effects on soil properties, it can be expected that metal biogeochemistry will be affected by these indirect effects on soil properties.

6.2.3. Elevated CO_2

CO_2 effects on both soil and plant functional dynamics have been well-studied. Direct measurements of metals in leaves and soils, however, are limited. While soil properties are key mediators of plant metal bioavailabilities, metal uptake at the root surface and metal transport within plants can also be affected by plant requirement and assimilation capacities, which may be altered by increased CO_2.

Loladze (2002) provides some evidence of an overall decline of the essential elements: carbon ratio in plant foliage grown in elevated CO_2. He suggests this change is driven by growth dilution effects and reduced bulk flow of soil water with CO_2 enrichment. Foliar element concentrations, however, may also be affected by soil metal concentrations and availabilities, plant requirement, physicochemical properties of metals, and elemental interactions, as discussed above. Natali et al. (in prep., Chapter 2) found that CO_2 effects on soil metals varied across sites (a deciduous forest, pine forest, and scrub oak community). There was a trend of decreased foliar metal concentrations for most metals; however, some metals micronutrients, such as Mn, showed increasing trends with CO_2 enrichment. There was some indication of biological regulation of foliar metals; foliar concentrations of essential metals (as a group) decreased with CO_2 enrichment less than nonessential metals in some, but not all, species studied. This species level variation, which may have been driven by differences in uptake capability, rhizosphere chemistry, and/or metal requirement, highlights the potential role of metals in ecological dynamics. Because CO_2-mediated changes in foliar metal stoichiometry vary across species, shifts in interspecific competition for metal micronutrients may occur.

Changes in soil properties (pH and SOM) can have significant effects on storage and mobility of metals. This is particularly true for Hg, whose cycling is tightly linked to biological activity. Natali et al. (in review, Chapter 3) found that soil Hg concentrations were almost 30% greater under elevated atmospheric CO_2 in two temperate forests. There were no direct CO_2 effects, however, on litterfall, throughfall, or stemflow Hg inputs. Soil Hg was correlated with percent soil organic matter (SOM), suggesting that CO_2-mediated changes in SOM have influenced soil Hg concentrations. Through its impacts on SOM, elevated atmospheric CO_2 may affect the Hg storage capacity of soils and modulate the movement of Hg through the biosphere. At an open-top chamber CO_2 experiment in a scrub-oak community with low SOM, soil Hg concentrations were also correlated with SOM; however, elevated CO_2 caused a decrease in soil Hg concentrations at this site (Natali et al., in prep. Chapter 2).

Physiological changes associated with CO_2 enrichment may also affect concentrations of essential trace metals in leaves and soil. Mo is an essential metal-cofactor in nitrate reductase, the enzyme that catalyzes the reduction of nitrate to nitrite. As the rate-limiting step in nitrate assimilation, the reduction of nitrate is an important component of plant physiological response to elevated CO_2 and terrestrial carbon sequestration. Natali et al. (in review, Chapter 4) examined the effects of elevated CO_2 and N availability on the activity of nitrate reductase in two temperate forests —a closed canopy sweetgum plantation in Tennessee and a loblolly pine stand in North Carolina. Both CO_2 and N enrichment had species specific impacts on nitrate reductase activity (NaR). Elevated CO_2 and N fertilization decreased foliar NaR in loblolly pine, but there were no treatment effects on sweetgum NaR at either site. Loblolly pine NaR was positively correlated with foliar Mo and negatively correlated with bio-available soil Mo, suggesting that CO_2 and N-mediated changes in plant function are altering soil-plant Mo-dynamics. These results demonstrate that metal micronutrient status is an important component of plant response to CO_2 enrichment. As a required enzyme cofactor for several N transformation reactions (e.g., N_2 fixation, nitrate assimilation, nitrification and denitrification) Mo is a key, yet often overlooked, player in biological N dynamics.

7. Conclusion

While trace metals are important resources for metabolism and growth, and can be present in both limiting and toxic levels, very little focus has been paid to trace metals in unmanaged terrestrial systems. However, trace metals are key mediators of ecological processes. Direct and indirect effects of global change factors on metal distributions and availabilities may have increased trace metal limitation and toxicity in terrestrial systems. The lack of attention paid to trace metals in natural systems may be a result not only of the relatively recent change in trace metal dynamics (with human industrial activities), but also to past limitations in analytical techniques. Recent advances in analytical chemistry techniques (e.g., inductively coupled plasma mass spectrometer, ICPMS) allow precise quantification of trace levels of elements in soils, water, plants, and other organisms. These techniques provide a new opportunity to further address ecological

concepts and applied management questions. Knowledge of the role of trace metal interactions is essential to understanding basic biological and ecological processes and potential impacts of anthropogenic alteration of trace metal inventories and fluxes.

Table 1. Examples of the roles of metal micronutrients in plant function (from Marschner 1995).

	Oxidation state	Enzymes	Function
Mo	IV, V, VI	Nitrate reductase, sulfite oxidase, xanthine oxidase/dehydrogenase	N assimilation
Cu	I, II	Plastocyanin, tyrosinase, cytochrome oxidase, superoxide dismutase (SOD)	PSI, superoxide radical detoxification
Mn	II, III, IV	Mn-SOD; Mn-protein in PSII	cofactor for 35+ enzymes, PSII
Fe	II, III	Cytochromes, catalase, peroxidase, ferredoxin, nitrate reductase	lignin, chlorophyll, protein synthesis, photorespiration, N assimilation, PS I
Zn	II	Alcohol dehydrogenase, phospholipase, carbonic anhydrase, RNA polymerase	Protein and carbohydrate synthesis, membrane integrity
Ni	I, II, III	Urease	N metabolism

Chapter 2: Effects of elevated carbon dioxide on trace metals in leaves and soil

Introduction

Human industrial activities over the past 200 years have led to a 35% increase in atmospheric CO_2 concentrations, and concentrations are expected to continue to rise through the end of the century (IPCC 2007). In addition to its role as a greenhouse gas, CO_2 has an essential biological role as the building block of plant biomass. Under current atmospheric CO_2 concentrations, photosynthesis is CO_2 limited (in C_3 plants); therefore, as atmospheric CO_2 concentrations increase, plant photosynthetic rates, carbon assimilation and dry matter production may also increase (Ainsworth and Long 2005; Curtis and Wang 1998; Ellsworth et al. 2004).

Elevated atmospheric CO_2 not only affects the total amount of plant biomass produced, but also the chemical make-up of plant tissue (e.g., Cotrufo et al. 1998; Roth and Lindroth 1995; Taub et al. 2008). Ainsworth and Long (2005) found an 80% increase in foliar starch (area-based) with CO_2 enrichment across a number of studies. Curtis and Wang (1998) found an increase in mass-based foliar starch in woody plants, with significantly greater effects in angiosperms than in gymnosperms. Numerous studies have also found an overall decrease in the concentrations of nitrogen (N) in plants grown in elevated CO_2 (Ainsworth and Long 2005, Cotrufo et al. 1998); in a meta-analysis of 75 published studies, Cotrufo et al. (1998) found that N concentrations in aboveground tissue declined by an average of 14% in plants grown in high CO_2. While results are more variable for phosphorus (P), there is also a general trend of decreased P concentrations in plant tissue with CO_2 enrichment (Gifford et al. 2000; Loladze 2002).

Loladze (2002) suggests that there will be a decrease in concentrations of all soil-derived nutrients in plants because the increase in atmospheric CO_2 is not matched by an increase in soil derived elements. Based on stoichiometric theory, Loladze argues that high CO_2, as a rule, will lead to an element imbalance in plants. While data on micronutrients are less abundant, Loladze (2002) summarized several studies of

herbaceous and woody plants, and found an overall decline in elemental concentrations of macro and micronutrients in plants exposed to elevated CO_2. However, there was variation among studies, among elements (e.g. there was no overall change in Fe or Mn in foliage), and plant parts (concentrations in grains decreased more than green leaves). Loladze suggests this overall decline in nutrient concentrations may be attributed to reduced mass flow of soil waters due to reduced transpiration, and increased carbohydrate accumulation in a higher CO_2 environment ('dilution' effect). He concludes that this stoichiometric imbalance (i.e., increase in C: nutrient ratio) may exacerbate human micronutrient malnutrition, as plants produce more biomass, but become less nutritious.

While plant stoichiometry is unequivocally less constrained than that of organisms at higher trophic levels (Sterner and Elser 2002), plant nutrient uptake is not a completely passive process. For example, increased production of fine roots with CO_2 enrichment (Norby et al. 2004; Pritchard et al. 2008) may allow plants to match increased C assimilation with increased uptake of soil-derived elements. Observed decreases in N concentrations with CO_2 enrichment may be attributed not only to dilution effects, but also to biochemical adjustments in a high CO_2 environment. Therefore, the extent that a specific element concentration decreases with CO_2 enrichment may also be a function of plant nutrient requirement and uptake capacity.

In this study we focus on CO_2 effects on trace metal concentrations. Trace metals are important both as micronutrients and as environmental contaminants. As a group, metals are characterized by low ionization energies, variable oxidation states, and the ability to form complex ions—properties that make metals well-suited to their predominant functional role in plants as enzyme cofactors (Marschner 1995). Metal solubility in soils (and availability to organisms) is strongly influenced by soil pH and soil organic matter (SOM). The sorption capacity of most metals increases with increasing pH (Marschner 1995), and organic matter provides a large surface area and negative charge—both of which support the binding of metals (McBride et al. 2004). Observed and predicted changes in pH and SOM with CO_2 enrichment (Andrews and Schlesinger 2001; Jastrow et al. 2005; Oh and Richter 2004) may therefore affect soil

metal availability and plant uptake and assimilation. Plant stoichiometry (and metal stoichiometry, in particular) may therefore also be mediated by CO_2-changes in soil properties.

Six of the eight known plant micronutrients are metals [copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni) and zinc (Zn)], and they are involved in a wide scope of physiological processes, including photosynthesis, respiration, and N assimilation. As is the case with foliar N, plant response to CO_2 enrichment may change the requirement for, and stoichiometry of, trace metals that are functionally important for plant metabolism. For example, as photosynthetic activity shifts from being CO_2-limited under ambient CO_2 to electron transport limited under elevated CO_2 (Farquhar and von Caemmerer 1982), plant nutrient requirements may change with these changing biochemical activities. Plant requirements for trace metals involved in electron transport, such as Fe, Cu and Mn, may increase under elevated CO_2 to allow for continued photosynthetic stimulation.

We also examine nonessential trace metals [aluminum (Al), cobalt (Co), lead (Pb), mercury (Hg) and vanadium (V)]—that is, metals taken up by plants at low levels, but not required for plant metabolism. Many of these elements are important contaminants whose movement through the biosphere is modulated by plant activities. By analyzing leaves for both essential and nonessential metals, we will seek to determine if changes in metal concentrations with CO_2 enrichment are due purely to non-regulatory processes (i.e., reduced uptake or growth dilution), or if there is a detectable level of biological regulation of trace metal concentrations in plants exposed to elevated CO_2.

We expect that elevated CO_2 will decrease foliar metal concentrations, *but* essential metals as a group will decrease less than nonessential metals because of biological regulation. To test this hypothesis, we measured concentrations of metals in plants and soils at two free air CO_2 enrichment (FACE) sites—a loblolly pine forest in North Carolina (Duke) and a sweetgum plantation in Tennessee (ORNL)—and an open-top chamber (OTC) experiment in a scrub oak community in Florida (SERC). By looking across sites that vary in climate, species composition, and soil chemistry we hope to

identify CO_2 effects that are common across systems and those that are unique to a particular species or site.

Materials and methods

Site descriptions

Duke FACE

The free-air carbon dioxide enrichment experiment at Duke Forest is located on a mixed evergreen-deciduous temperate forest dominated by loblolly pine (*Pinus taeda)* and is 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 m × 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 (*Liquidambar styraciflua*). The FACE experiment began in August 1996 and consists of three 30 m diameter ambient CO_2 rings (~382 μmol mol^{-1}) and three 30 m diameter elevated CO_2 rings (~582 μmol mol^{-1}) that are arranged in a complete block design to account for topographic variation and potential fertility gradients. The CO_2 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 regulated blowers that deliver a controlled amount of CO_2-fumigated air to maintain ambient or elevated levels of CO_2 into the rings (Hendrey et al. 1999). The site, experimental design and FACE technology have been well-described (Finzi et al. 2001; Hendrey et al. 1999). A summary of all three sites can be found in Table 1.

ORNL FACE

The deciduous forest site (ORNL) is a sweetgum (*L. styraciflua*) plantation located in the Oak Ridge National Environmental Research Park in Roane County, Tennessee (35°54'N, 84°20'W). Soils at the site, classified as Aquic Hapludult, have a silty clay loam texture, are moderately well drained and slightly acidic. The stand was planted with one-year-old sweetgum seedlings in 1988 at a spacing of 1.2 m × 2.3 m. There are three ambient CO_2 (~ 393 μmol mol^{-1}) rings and two enriched CO_2 (~ 549 μmol mol^{-1}) rings,

which are each 25 m in diameter. The FACE apparatus (Hendrey et al. 1999) is assembled in both elevated CO_2 rings, and in two of the three ambient CO_2 rings. CO_2 enrichment began in 1998 and continues during the growing season through the present time. The site and experimental design are described in Norby et al. (2001).

SERC OTC

The Smithsonian Environmental Research Center (SERC) site is an open-top chamber (OTC) CO_2 enrichment experiment located in a scrub-oak ecosystem on Merritt Island Wildlife Refuge, Cape Canaveral, Florida (28°38'N, 80°42'W). Soils are sandy, well-drained Pomello and Poala sand with low nutrient content and low pH (Schmalzer and Hinkle 1992). The experiment is comprised of 16 octagonal open top chambers (3.6m diameter, 3.7m height), half of which are exposed to current ambient CO_2 and half to elevated CO_2 (ambient + 350 ppmv). CO_2 treatment has been in progress since May 1996. The plots are arranged in a randomized block design; blocks were established based on vegetation composition. Aboveground biomass is dominated by *Quercus myrtifolia* (76%), *Q. geminata,* and *Q. chapmanii*. Other species present include *Galactia elliottii*, *Serenoa repens* and *Vaccinium myrsinites* (Dijkstra et al. 2002). Complete description of the open-top chamber system can be found in Stiling et al. (1999), and further site description can be found in Hymus et al. (2003).

Field sampling

Soils

Soil samples were collected at ORNL from 25-28 July 2005, at Duke from 8-11 August 2005, and at SERC from 29 to 31 August 2006. A core sampler was used to collect two 2.5 cm diameter by 20 cm deep soil cores per ring at each FACE site; three cores were collected per chamber at SERC. Acid-washed butyrate plastic core liners 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 ring/chamber. In all of our analyses, 'ring' or 'chamber' is the unit of replication for CO_2 treatment, so pooling of cores within a ring has no consequence in testing for CO_2 or depth effects.

Leaves

Canopy leaves were collected at ORNL from 25-28 July 2005, at Duke from 8-11 August 2005, and at SERC from 25-29 August 2006. Green leaves were sampled from three canopy heights—low (10-12m), mid (12-14m) and upper (14-16m)—from *L. styraciflua* at ORNL and Duke (lower and mid canopies only, pooled for analysis), and from *P. taeda* at Duke (all canopy heights). 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.* In each canopy height three replicate samples were collected. At SERC three replicate samples were collected from the ground at approximately two meters height. For all leaves collected, a sample consisted of approximately 5 leaves/20 needles from an individual tree.

Sample analysis

After removal of roots, soils were passed through a two mm screen and air-dried. Leaves were dried at 60^{o}C in a Fisher Isotemp oven and homogenized using a ball mill (using acid-washed polypropylene tubes and glass grinding balls). Soils for metal analysis (Al, Co, Pb, Hg, V, Cu, Fe, Mn, Mo, Ni, Zn) were digested using repeated additions of concentrated nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) with heating (EPA Method 3050B); leaves were digested using repeated additions of HNO_3, followed by H_2O_2 and HCl (EPA Method 200.3).

Samples were analyzed for metal concentrations using a Thermo-Finnegan Element2 Inductively Coupled Plasma Mass Spectrometer (ICP-MS), with apple leaf (NIST 1515) and San Joaquin soils (NIST 2709) as digestion standards, and river water (NIST 1643d) as an instrumental standard. Percent soil organic matter (SOM) was determined by the method of percent loss on ignition (eight hours combustion at 400^{o}C). Soil pH was determined in a 1:1 ratio of soil (g) to water (ml) and 1:1 ratio of soil to

0.01M CaCl. These two pH measures were highly correlated ($R^2 = 0.94$, $P<0.01$); only pH in water was used in our analyses.

Statistical analyses

Principal components analysis (PCA; JMP, SAS Institute, Cary, NC) was used to look at correlations among metals in soils and in leaves and to reduce the number of variables for further analysis. Variables were transformed to reduce the influence of outliers and to improve the linear association between variables (Quinn and Keough 2002). For all PCAs we used a correlation matrix (variables standardized to zero mean and unit variance) because the variables (metal concentrations, pH, SOM) had different measurement units, and we were not concerned with differences in variances among variables. To obtain a more simple structure for the components (so that coefficients in each component are close to 0 or 1), we applied a varimax (orthogonal) rotation to the eigenvectors (Quinn and Keough 2002).

To test for CO_2 effects on soil metals, the reduced soil components were used as variables in an analysis of variance (ANOVA, SAS 9.0, SAS Institute, Cary, NC). At each site, soils were analyzed in a nested design with CO_2 as the main plot factor, depth as a within plot factor and ring or chamber (random) as the experimental unit for CO_2. Because the components are independent of each other, we used Hochberg's method (Hochberg 1988) for p-value adjustment to control family-wise error rate.

As we were specifically interested in CO_2-mediated pH and SOM effects on soil metals, we ran separate ANOVAs for these two variables. We were interested in common trends across sites, so for the pH and SOM ANOVAs we included site in our model as a main plot factor (rather than run separate tests at each site, as was done on the components). Ring/chamber was the experimental unit for testing for CO_2 and site effects. We could not include block (Duke and SERC) into this model; however, block was included in the ANOVAs conducted on soil components at each site.

Reduced leaf components (PCA) were not used as ANOVA variables because leaf metals were less correlated than soils, and variables did not load strongly onto few components, as with soils. Foliar data were analyzed using multivariate analysis of

variance (MANOVA); when MANOVA was significant, treatment effects on each metal were tested with ANOVA, and ANOVA p-values were adjusted to control family-wise error rate. ORNL foliar data were analyzed using a partly-nested design with CO_2 as the main plot factor, canopy height as the within plot factor, and ring (random) as the experimental unit for CO_2. 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 with CO_2 as the main plot factor, nested in block (random). The ANOVA on Duke *P. taeda* needles had two within plot factors—needle age and canopy height. SERC leaf data were analyzed as a nested design with chamber (random) as the unit of replication for CO_2 treatment—the main plot factor—and species as a within plot factor. For the ANOVAs with unequal treatment sample sizes, degrees of freedom were estimated using Satterthwaite's approximation (Satterthwaite 1946).

CO_2 effects on foliar elemental concentrations were tested with an *a priori* one-tailed hypothesis, based on expectations that growth dilution with CO_2 enrichment will decrease foliar elemental concentrations (H_0: $[metal]_{elevated} \geq [metal]_{ambient}$). CO_2 effects on soil variables, and all other factors and interactions were tested with two-tailed tests.

When CO_2 interactions were significant, we conducted one-tailed planned comparisons to look at CO_2 effects within a species or canopy height. To compensate for the increased chance of making Type II errors, due to the large number of variables in this study (and p-value adjustment for family-wise error rate), we conducted *a priori* comparisons based on pre-adjusted p-values. For example if 'CO_2 × canopy height' had a p-value of 0.05 prior to adjustment, we looked at CO_2 effects within each height, even if the interaction p-value was not significant after adjustment. We have noted this in the results text and tables.

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 and OTC studies (e.g., Carney et al. 2007; Ellsworth et al. 2004; Jastrow et al. 2005). Errors presented in the text and tables are one standard error of the mean.

To compare overall changes in essential and nonessential metal concentrations in leaves, we computed range standardized means for each metal. Range standardization was achieved by dividing each observation by the maximum value for each metal within a site. We then averaged and compared (graphically) range standardized means for all essential metals and all nonessential metals in leaves in ambient and elevated CO_2 treatments for each species.

To look at relationships among variables, a regression model was fit by the method of ordinary least squares and Pearson correlation coefficients calculated to estimate the correlations between variables. We used analysis of covariance (ANCOVA) to compare CO_2 effects on slopes/intercepts of regression lines fit between essential (Fe) and nonessential (V) foliar metal concentrations. If growth dilution is the primary mechanism driving CO_2 effects on foliar concentrations, we expect no CO_2 effect on regression slopes/intercepts between essential and nonessential metal pairs.

Results

Soil

Duke

Soil variables (principal components analysis, PCA) at Duke FACE were highly correlated; the first four components explained 93% of the variation in the data (Table 2). Most variables (Al, V, Fe, Cu and Mo) loaded strongly (≥0.7) onto component 1; Hg and log SOM correlated with component 2; Co, Mn and Ni correlated with component 3; and pH with component 4. Pb had moderate correlations (0.4-0.6) with the first three components (Table 2).

Because there were strong correlations among soil variables, we used the derived PCA variables in an ANOVA to test for CO_2 effects on soil metals, pH, and SOM (Supp. Table 1). CO_2 effects on soil metals were primarily concentrated in surface (0-5 cm) soils (Table 3, Figure 1a). There was a significant CO_2 × depth interaction effect for component 1, with a significant CO_2 effect ($p<0.05$) in the surface soil layer (0-5 cm). While Al, V, Fe, Cu, Mo had strongest loadings on component 1, all metals with the exception of Hg were positively correlated with component 1 (Table 2), and there was a

general trend of increased concentrations for all metals in upper soil depths (0-5 cm) at Duke FACE (Figure 1a).

There was also a significant CO_2 × depth interaction for component 4 (pH; Supp. Table 1), with significant CO_2 effects ($p<0.05$) at all soil levels except the 0-5 cm increment ($p<0.05$). Average pH in 0-5 cm soils was 5.08 ± 0.17 in ambient and 4.99 ± 0.17 in elevated rings. In 5-20 cm soils, average pH was 5.49 ± 0.05 in ambient and 5.27 ± 0.06 in elevated rings.

There was a significant depth effect for component 2 ($F = 22.55$, $P < 0.01$), which primarily represents soil Hg and SOM (Table 2). While there was no CO_2 effect on component 2, there was a significant effect of CO_2 on both soil Hg and SOM when tested by ANOVA ($p < 0.05$).

ORNL

Soil variables at ORNL were also highly correlated, and the first four components explained 89% of the variation in the data (Table 4). Al, V, Fe, Cu (same metal suite as at Duke) and Ni loaded strongly (≥0.7) onto component 1, with moderate loadings (0.4-0.6) from Mo and Zn. SOM and pH correlated with component 2; Hg and pH with component 3; and Co with component 4, with moderate loadings from Pb and Mn (Table 4).

There was a general trend of increased soil metals with CO_2 enrichment at ORNL, but the increase—which was mainly in surface soils (0-5 cm)—was less than that at Duke FACE (Table 3, Figure 1a). There was a significant CO_2 effect on component 3, which had strong loadings from Hg and pH, and moderate loadings from Pb, Mo, and Zn (Supp. Table 2). Metals associated with component 3 increased with CO_2 enrichment, and there was a decrease in pH (Table 3, Figure 1a).

SERC

Soil variables were also highly correlated at SERC. The first four components explained 87% of the variation in the data. Al, Co, V, Fe, and Mo loaded strongly (≥0.7)

onto component 1; SOM, Ph and Hg onto component 2; Co and Zn onto component 3; and Mn onto component 4 (Table 5).

Concentrations of soil metals at SERC were lower than at either FACE site; with the exception of Hg, concentrations were one to three orders of magnitude lower at SERC than at ORNL and Duke (Table 3). In contrast to ORNL and Duke, there was a general decline in metal concentrations with CO_2 enrichment at SERC (Table 3, Figures 1a); however, there were no significant CO_2 effects on any of the components (Supp. Table 3).

Patterns across sites

At all three sites there were significant correlations ($P < 0.05$) in soils between SOM and Hg (Figure 2) and between Al, V, Fe and Mo (Supp. Table 4). We looked at CO_2 effects on the slope/intercept of the relationship between V and Fe because these two metals were also correlated in leaves of all species. There were no statistically significant CO_2 effects on the slopes or intercepts of V-Fe correlations at Duke, ORNL or SERC, and there were no CO_2 effects on the ratio of Fe:V in soils (Supp. Table 5).

CO_2 effects on SOM and pH were significant across all sites (SOM: $F = 3.69$, $P = 0.07$; pH: $F = 4.12$, $P = 0.06$; Supp. Table 6), and there were no significant $CO_2 \times$ site interactions. Mean SOM increased with CO_2 enrichment and mean pH decreased with CO_2 enrichment (Table 3, Figure 1a).

Leaves

Duke *P. taeda*

There was a significant effect of elevated CO_2 on trace metal concentrations in *P. taeda* leaves at Duke (MANOVA; Wilk's lambda = 0.47, $P < 0.001$; Figures 1b and 3, Supp. Table 7). There was a significant difference in overall metal concentrations between 0-year and 1-year needles (Wilk's lambda = 0.14, $P < 0.001$, Supp. Table 7), as well as significant needle age effects on all individual metals (ANOVA, $p<0.1$), with the exceptions of Al and Mo (Supp. Table 8).

There was a significant age × CO_2 (Wilk's lambda = 0.55, P = 0.002) and age × canopy height (Wilk's lambda = 0.37, P < 0.001) MANOVA interaction. Canopy height (Wilk's lambda = 0.69, P = 0.67) and canopy height × CO_2 (Wilk's lambda = 0.57, P = 0.19) were not significant, but there was a significant canopy height × age × CO_2 interaction effect (Wilk's lambda = 0.36, P < 0.001). There was a significant decline ($p<0.1$) in foliar metal concentrations with CO_2 enrichment in a particular needle age-class/canopy height (i.e., CO_2 ×canopy ht × age interaction) for the following metals: Co, V, Cu, Fe, Mn, Ni, Zn (Figure 6, Supp. Table 9).

P. taeda foliar metals were less tightly correlated than metal concentrations in Duke soils (Supp. Table 10); the first four components explained 78% of the variation in Duke *P. taeda* leaf metal data. V, Fe, Zn and Mn loaded strongly (≥0.7) onto component 1, with moderate loadings from Al, Co and Mo; Ni and Cu loaded onto component 2; Pb onto component 3, and Mo onto component 4.

As in soils, there was a significant correlation between foliar Fe and V across (R^2 = 0.78, P < 0.001) and within CO_2 treatments (ambient: R^2 = 0.80, P <0.001; elevated: R^2 = 0.81, P < 0.001) for 0 and 1-yr needles combined. There was a significant CO_2 effect on the intercept of the relationship between leaf Fe and V for both needles ages combined and for 1-yr needles (Supp. Table 11). We did not test for intercept effects on 0-yr needles because regression slopes were heterogeneous between CO_2 treatments. Because V has no known biological role in terrestrial plants, we expected foliar V concentrations to decrease more than Fe concentrations in the elevated CO_2 plots, resulting in an increase in Fe:V. As expected, there was an increase in the Fe:V ratio across needle ages (ambient 1.07 ± 0.12, elevated 1.18 ± 0.04 μg Fe/ng V; Supp. Table 11).

Duke *L. styraciflua*

There was a significant effect of elevated CO_2 on trace metal concentrations in *L. styraciflua* leaves at Duke (MANOVA; Wilk's lambda = 0.05, P < 0.001). While no individual metal had significantly lower leaf concentrations under elevated CO_2 (ANOVA $p>0.10$; Supp. Table 12), there was a general trend of decreased foliar metal

concentrations with CO_2 enrichment for most nonessential metals and some essential metals (Figures 1b and 4, Supp. Table 7).

The first four principal components explained 82% of the variation in *L. styraciflua* leaf metal concentrations. Co, Mo, Zn loaded strongly (≥0.7) onto component 1; Ni onto component 2; Hg and Mn onto 3; and V and Fe onto component 4 (Supp. Table 13).

There was a significant correlation between foliar Fe (log) and V (log) across (R^2 = 0.27, P = 0.01) and within CO_2 treatments (ambient: R^2 = 0.88, P <0.01; elevated: R^2 = 0.43, P = 0.01). We did not test for intercept differences between the ambient and elevated CO_2 treatments because slopes were heterogeneous between treatments (Supp. Table 11). Mean foliar Fe: V was lower in the elevated than in ambient CO_2 plots (ambient 0.67 ± 0.05, elevated 0.49 ± 0.09 μg Fe/ng V).

ORNL *L. styraciflua*

There was a significant effect of elevated CO_2 on trace metal concentrations in leaves at ORNL (MANOVA; Wilk's lambda = 0.23, P<0.01; Figures 1b and 4, Supp. Table 7) but no individual metal had significantly lower leaf concentrations across all canopy heights in elevated CO_2 (ANOVA p>0.10; Supp. Table 14). However, there was a significant difference in metal concentrations among canopy heights (Wilk's lambda = 0.23, P <0.01), and significant CO_2 × canopy height interactions (Wilk's lambda = 0.36, P = 0.09).

There were significant CO_2 × height interaction effects for several metals (ANOVA, Supp. Table 14). Because of the large number of elements analyzed, many of these effects were not significant (p>0.1) after adjusting p-values to control family-wise error rate. To balance the risk of Type II errors, we ran subsequent ANOVAs on all CO_2 × height interactions whose p-values were significant prior to adjustment, to look at CO_2 effects within canopy heights.

Al, Co, Pb, V, Fe and Ni all had significantly lower upper canopy foliar concentrations with CO_2 enrichment (p<0.05; Figure 7, Supp. Table 15). While Mo had a significant CO_2 × height interaction effect, Mo concentrations tended to increase with CO_2 enrichment.

Leaf variables at ORNL were less correlated than soil variables. We extracted four components from 11 trace metal variables. The first four components explained only 69% of the variation in the data. No single component had more than two variables with strong loadings (≥0.7; Supp. Table 16).

There was a significant correlation between foliar Fe and V both across ($R^2 = 0.60$, $P < 0.01$) and within CO_2 treatments (ambient: $R^2 = 0.56$, $P < 0.01$; elevated: $R^2 = 0.85$, $P < 0.01$). There was a significant CO_2 effect on the intercept of the Fe-V regression (Supp. Table 11), and foliar Fe:V decreased at ORNL with CO_2 enrichment (ambient 1.39 ± 0.13, elevated 1.19 ± 0.19 μg Fe/ ng V).

SERC

There was a significant effect of elevated CO_2 on trace metal concentrations in leaves at SERC (MANOVA; Wilk's lambda = 0.24, $P<0.001$; Figures 1c and 5, Supp. Table 7). There were significant differences across species (Wilk's lambda = 0.042, $P < 0.001$) for most metals, and significant CO_2 × species interactions (Wilk's lambda = 0.26, $P = 0.10$). There were significant decreases in Al concentrations in *Q. geminata* and *Q. myrtifolia*, and significant decreases in Ni concentrations in *Q. chapmanii* (ANOVA, Supp. Tables 17 and 18).

Principal component analyses were conducted separately on each of the three species at SERC (Supp. Tables 19-21). The first four components explained 82% of the variation in the data in *Q. chapmanii*, 80% in *Q. geminata*, and 74% in *Q. myrtifolia.*

There was a significant correlation between foliar Fe and V in leaves of all species (*Q. chapmanii*: $R^2 = 0.68$, $P < 0.01$; *Q. geminata*: $R^2 = 0.74$, $P < 0.01$; *Q. myrtifolia*: $R^2 = 0.40$, $P < 0.01$). There was no effect of elevated CO_2 on the slope or intercept of the Fe-V regression for any of these species (Supp. Table 11).

Discussion

Soils

CO_2 effects on soil metals were greatest in surface soils (0-5cm), where there was an overall increase in metal concentrations at Duke, a slight increase at ORNL and a

decrease in metal concentrations at SERC (Figure 1a). CO_2 effects on SOM also varied across sites. Changes in percent SOM with CO_2 enrichment were greatest at Duke (18% increase), followed by ORNL (7% increase), with limited effect at SERC (3% increase). Soil organic matter is a key factor governing the sorption of trace metals onto soils (Linde et al. 2007) and may be mediating CO_2 effects on soil metal concentrations. Sorption of metals onto organic matter can exceed mineral sorption 6 to 13 times (for Cu, Cd, Zn; Lair et al. 2007); therefore, increased SOM may increase the metal-binding capacity of soils. Soil organic matter is effective at retaining metals in soils because it contains a large number of functional groups, and has a high cation exchange capacity (Bradl 2004). While elevated CO_2 has been shown to increase soil carbon across a range of studies (Jastrow et al. 2005), Carney et al. (2007) found decreased carbon in SERC soils with CO_2 enrichment. Changes in soil metal concentrations, therefore, may vary across sites that have different soil carbon responses to elevated CO_2.

The link between CO_2-mediated changes in SOM and soil metals is demonstrated by patterns of soil Hg. Across sites, SOM was positively correlated with soil Hg concentrations (Figure 2). While soil Hg concentrations were 20% higher at Duke and 34% higher at ORNL with CO_2 enrichment, soil Hg concentrations were 36% lower with CO_2 enrichment at SERC. Greater concentrations of soil Hg at ORNL and Duke were not driven by increased Hg litter or throughfall inputs (Natali et al., in review); based on the correlations between soil Hg and SOM, changes in soil Hg concentrations appear to be driven by CO_2-mediated changes in percent SOM. These results are in agreement with other studies that have found positive correlations between SOM and soil Hg. Across a range of peatland and forest soils in Europe and United States, Grigal (2003) found an average change in soil Hg of 0.22 µg per g SOM. While soil Hg concentrations in our study were an order of magnitude lower than those in Grigal (2003), the relationship between Hg and SOM was similar (Duke: 0.41 µg Hg g^- SOM; ORNL: 0.32 µg Hg g^- SOM; SERC: 0.20 µg Hg g^- SOM).

Another important driver of metal distributions in soils is pH. Elevated CO_2 may increase soil acidity through increased inputs of carbonic acid from root and microbial respiration (Oh and Richter 2004). Trace metal mobility in soils is tightly linked to

dissolved organic matter (DOM; Grybos et al. 2007; Linde et al. 2007), which tends to increase with increasing pH (You 1999). Metal losses from soils associated with DOM mobility, would, therefore, tend to be lower at lower pH levels. However, in addition to the effects of acidity on organic matter dissolution, increased acidity tends to decrease adsorption of metals onto organic matter and metal oxides (Bradl 2004). Decreasing pH alone, therefore, would tend to increase soil metal mobility, but pH will have interactive effects with soil organic matter. While we found decreases in soil pH in the CO_2 enriched plots at all sites (Figure 1a, Supp. Table 6), effects of pH changes on metal binding capacity in soils may be more pronounced in soils with low organic carbon content, such as those found at SERC.

Concentrations of most metals in SERC soils were one to three orders of magnitude lower than concentrations at Duke and ORNL; the low metal binding capacity of these sandy soils may be exacerbated by elevated CO_2. Hungate et al. (2004) suggest that decreased soil Mo-availability at SERC may have led to a decline in N_2-fixation by the leguminous vine *Galactia elliottii* under elevated CO_2. While we found no significant CO_2 effects on soil components (PCA) at SERC, potential decreases in essential metal concentrations (suggested by a general decline in all SERC soil metals, Figure 1a) at this low-fertility site may be an important regulator of ecosystem response to elevated CO_2.

While SOM and pH are two of the most important factors governing metal mobility in soils (Bradl 2004; Kalbitz and Wennrich 1998), other factors, such as mineral weathering rates and metal binding to Fe and Mn oxyhydroxides, are also important. Andrews and Schlesinger (2001) found that elevated CO_2 increased rates of mineral weathering, which may be an important process determining metal concentrations in soils (Starr et al 2002). It is likely that CO_2 effects on soil metals are driven by a combined effect of CO_2-mediated changes in SOM, pH, and other soil physico-chemical factors at each site.

Leaves

In all tree species in this study, elevated CO_2 significantly altered foliar trace metal stoichiometry (MANOVA, $p<0.05$; Figures 1b-c). CO_2-mediated changes in soil metals,

however, do not appear to be driving changes in foliar metal concentrations. Despite site-specific patterns of CO_2 effects on soil metals, patterns in leaves were more variable, and correlations among metals less constrained than in soils. While there was a general increase in soil metal concentrations at Duke and ORNL, foliar concentrations of several metals declined with CO_2 enrichment at all three sites, consistent with a growth dilution and/or reduced transpiration effect (Figures 1b-1c). It is expected that elevated CO_2 may lead to a general decline in elemental concentrations in plants if the increase in carbon uptake and assimilation with CO_2 enrichment (and increase in nonstructural carbohydrates) is not matched by increased uptake of soil derived elements (Loladze 2002).

Based on principles of growth dilution, we would expect the decrease in elemental concentrations with CO_2 enrichment to be greatest in the upper canopy compared to the lower canopy, because CO_2 effects on leaf mass per unit area (LMA) and leaf density are often more pronounced in upper canopy leaves. LMA and density in upper canopy leaves of *L. styraciflua* at ORNL increased in the CO_2 enriched plots (Norby and Iversen 2006), as did foliar content of nonstructural carbohydrates (Sholtis et al. 2004). In agreement with the growth dilution hypothesis, we found significantly lower concentrations of Al, Co, Pb, V, Fe, and Ni in upper canopy *L. styraciflua* leaves at ORNL (Figure 7). At Duke, there were CO_2 × canopy height effects for six metals (V, Cu, Fe, Mn, Ni, Zn), but patterns were more variable. Of the nonessential foliar metals at Duke, V had significantly lower concentrations with CO_2 enrichment in the upper canopy (0-yr needles), and Co concentrations in 0-yr needles were lower in all canopy heights. Of the essential metals at Duke, only Cu and Ni (1-yr) had decreased concentrations with CO_2 enrichment in upper canopy leaves (Figure 6). The expected CO_2 × canopy height effect at Duke may have been less pronounced because there were no detected CO_2 effects on Duke FACE *P. taeda* LMA (1-yr needles; Rogers and Ellsworth 2002; Springer et al. 2005).

We hypothesized that the decrease in foliar metals with CO_2 enrichment would be greater for nonessential metals than for essential metals. To test this hypothesis we looked at CO_2 effects on the correlation between foliar concentrations of a nonessential-

essential element pair. We used the correlation between Fe and V, because Fe and V were correlated in both soils and leaves at all three sites. We expected foliar concentrations of the essential metal, Fe, to decrease less than the non-essential metal, V, with CO_2 enrichment. There were no CO_2 effects on Fe-V correlations in soils at any of the sites. There were significant CO_2 effects on the intercept of the regression between foliar V and Fe for *P. taeda* at Duke and *L. styraciflua* at ORNL, but there were no CO_2 effects on any of the *Quercus* species at SERC. Consistent with the biological regulation hypothesis, there was an increase in *P. taeda* foliar Fe: V in the CO_2 enriched treatments. However, this pattern did not hold up across species—foliar Fe: V decreased in *L. styraciflua* at both ORNL and at Duke. The reasons for these interspecific differences are unclear, but they are likely due to interactions between plant Fe requirement, specific uptake kinetics, and changes in soil properties and microbial activities with CO_2 enrichment. Loladze (2002) notes that the magnitude of the potential CO_2-mediated effects of reduced bulk flow on element stoichiometry will vary by element because of differences in specific element diffusion and uptake kinetics. Our results suggest that species-level variation in elemental uptake is also an important factor in CO_2-metal uptake dynamics.

There does seem to be some evidence for biological regulation of essential metals with CO_2 enrichment when all essential and nonessential metals are looked at as a group. At ORNL, we found statistically significant decreases in foliar metal concentrations with CO_2 enrichment for four (Al, Co, Pb, V) of the five nonessential metals (either across canopies or in a specific canopy height), but in only two (Fe and Ni) of the six essential metals. At SERC, there were significant CO_2 effects (prior to p-value adjustment) in one or more of the *Quercus* species for four (Al, Co, Hg, V) of the five nonessential metals, but for only two (Cu and Ni) of the six essential metals. While at Duke, there were no significant decreases in foliar metals with CO_2 enrichment in *L. styraciflua*, and in *P. taeda* CO_2 effects were variable.

Foliar concentrations of nonessential metals as a group decreased with CO_2 enrichment, while essential metals often were not changed or increased in the elevated CO_2 plots (Figure 8), suggesting that there is some level of biological control of metal

stoichiometry. This pattern was most evident in the CO_2 effects on essential versus nonessential metals in the leaves of *L. styraciflua* at both Duke and ORNL (Figure 8). In the studies of CO_2 effects on elemental concentrations in plant tissue, reviewed by Loladze (2002), decreases in foliar concentrations were consistently greater in grains than in foliage, which may also reflect biological requirement in leaves for metals used in metabolic processes.

While many metals did decrease with CO_2 enrichment, consistent with the 'dilution' effect, in some cases there was no change and even an increase in foliar metal concentrations with CO_2 enrichment. For example, Mn concentrations in leaves tended to increase with CO_2 enrichment in all species, except *P. taeda*, at all three sites (Figures 1b-1c, 3-5). Mn concentrations in 1-yr *P. taeda* were, however, higher in the upper canopy elevated leaves compared to upper canopy ambient (Figure 6). This trend of increased foliar Mn with CO_2 enrichment may be a function of changes in plant requirement for Mn and soil chemistry under high CO_2. Mn serves a redox role in a number of plant enzymes, most notable in the oxygen evolving complex of photosystem II (Yachandra et al. 1996), and so increased photosynthetic activity with CO_2 enrichment may increase plant demand for Mn. Increased soil acidity (Oh and Richter 2004) and root exudation with CO_2 enrichment (Vanveen et al. 1991) would also increase Mn availability to plants (Sims 1986). An increased demand for Mn in plants grown in low nutrient soils, such as SERC, may limit plant photosynthetic and growth responses to elevated CO_2.

Conclusion

This study examined trace metal stoichiometry in plants because of the massive impact that plant processes have on element cycling and the importance of metals as both contaminants and micronutrients. Decreased foliar concentrations of essential metals in food crops can exacerbate human micronutrient malnutrition (Loladze 2002) and may be a factor contributing to increased rates of herbivory on plants grown in high CO_2. As reported by Loladze (2002), we found a general trend of decreased foliar metals with CO_2 enrichment, especially for nonessential plant metals. However, CO_2 effects on foliar

metal stoichiometry were species and element-specific. While not a focus of this study, CO_2 effects on foliar stoichiometry may also be modulated by plant photosynthetic pathway (C_3 v. C_4) because carbohydrate accumulation with CO_2 enrichment often does not occur (or there is a smaller effect) in C_4 plants (Ainsworth and Long 2005; Ghannoum et al. 2000; Ward et al. 1999). To alleviate potential CO_2 effects on human micronutrient malnutrition, human agricultural practices may need to increase dependence on C_4 crop species (e.g., corn and sorghum) and take advantage of species and population level variation in stoichiometric response of plants to CO_2 enrichment.

Forest ecosystems play a key role in the transport and storage of metal contaminants. Biogeochemical processes that affect the storage of metals in forest soils are important to the transport of metal contaminants to downstream systems (Aastrup 1995, Scott 2001). Changes in soil storage capacity and plant uptake of metals with CO_2 enrichment will have important implications for the movement of contaminants through natural and managed ecosystems. By altering plant growth, morphology, physiology and biochemistry, increased atmospheric CO_2 may affect the biological cycling, storage, and stoichiometry of trace metals in terrestrial and freshwater systems.

Tables

Table 1. Summary of ORNL, Duke and SERC field sites.

	ORNL	**Duke**	**SERC**
Location	Roane county, TN	Orange county, NC	Brevard county, FL
Lat-longitude	35°54'N,84°20'W	35 °58'N, 79 °05'W	28 °38'N, 80 °42'W
Mean annual T	14.2° C	15.5° C	21.8 ° C
Annual precipitation	1390mm	1118 mm	1341 mm
Experiment started	1998	1996	1996
CO_2 treatment	A: ~393µmol mol^{-1} E: ~544µmol mol^{-1}	A: ~382µmol mol^{-1} E: ~582µmol mol^{-1}	A: ~380 µmol mol^{-1} E: ~730 µmol mol^{-1}
Plot size	25 m diameter	30 m diameter	3.6 m diameter
Number plots	3 ambient 2 elevated	3 ambient 3 elevated	8 ambient 8 elevated
Dominant species	*L. styraciflua*	*P. taeda*	*Quercus* sp.

Table 2. Eigenvalues and loadings (correlations) of Duke soil variables onto rotated components. Variables that load strongly (≥0.7) are in bold.

Component	Eigenvalue	% variance
1	7.55	58.07
2	2.90	22.28
3	5.14	39.54
4	2.69	20.67

Variable	Comp. 1	Comp. 2	Comp. 3	Comp. 4
Log SOM	-0.112	**0.895**	-0.031	-0.084
pH	0.094	-0.643	0.100	**0.711**
Al	**0.800**	-0.030	0.529	0.052
Log Co	0.593	-0.223	**0.707**	-0.025
Pb	0.557	0.454	0.535	0.330
Hg	-0.181	**0.957**	0.079	-0.043
V	**0.914**	-0.169	0.314	0.141
Cu	**0.866**	-0.136	0.332	-0.233
Fe	**0.903**	-0.173	0.336	0.178
Mn	0.348	-0.084	**0.883**	-0.007
Mo	**0.912**	-0.029	0.332	0.157
Ni	0.464	0.181	**0.829**	0.114
Zn	0.463	0.425	0.606	0.300

Table 3. Means (± s.e.) of Duke, ORNL and SERC soil metal concentrations, percent SOM and pH in 0-20 cm soil depth.

	CO_2	Al (mg g^{-1})	Co (µg g^{-1})	Pb (µg g^{-1})	Hg (ng g^{-1})	V(µg g^{-1})	SOM (%)	pH
Duke	A	14.50 ± 1.97	27.53 ± 6.44	17.88 ± 1.71	16.5 ± 1.0	203.91 ± 21.83	3.81 ± 0.18	5.41 ± 0.09
	E	15.48 ± 2.78	42.31 ± 12.74	18.38 ± 3.01	19.0 ± 0.4	210.71 ± 47.37	4.48 ± 0.15	5.20 ± 0.09
ORNL	A	17.68 ± 1.08	11.38 ± 0.50	17.11 ± 0.95	23.1 ± 1.4	25.40 ± 1.37	3.85 ± 0.28	4.85 ± 0.10
	E	17.47 ± 0.07	11.72 ± 0.11	19.13 ± 0.29	31.0 ± 2.3	25.05 ± 0.00	4.12 ± 0.24	4.72 ± 0.06
SERC	A	0.11 ± 0.04	0.02 ± 0.001	0.97 ± 0.10	4.9 ± 0.3	0.39 ± 0.03	1.62 ± 0.11	4.97 ± 0.06
	E	0.11 ± 0.02	0.01 ± 0.001	0.89 ± 0.05	2.9 ± 0.5	0.37 ± 0.02	1.67 ± 0.20	4.78 ± 0.10

	CO_2	Cu (µg g^{-1})	Fe (mg g^{-1})	Mn(µg g^{-1})	Mo(µg g^{-1})	Ni(µg g^{-1})	Zn(µg g^{-1})
Duke	A	16.15 ± 1.94	47.84 ± 6.32	1667.88 ± 468.17	145.85 ± 25.71	4.43 ± 1.04	26.28 ± 5.35
	E	16.77 ± 2.25	48.12 ± 11.13	1716.41 ± 323.91	148.34 ± 42.05	4.97 ± 0.85	23.69 ± 5.25
ORNL	A	10.26 ± 0.24	15.10 ± 0.64	1124.66 ± 146.79	244.93 ± 8.90	15.50 ± 1.05	58.53 ± 2.79
	E	9.55 ± 0.67	15.49 ± 0.37	1145.73 ± 146.69	263.13 ± 3.38	15.43 ± 1.36	62.23 ± 4.92
SERC	A	0.18 ± 0.03	0.11 ± 0.02	2.85 ± 0.18	25.50 ± 2.41	0.12 ± 0.01	2.52 ± 0.60
	E	0.17 ± 0.02	0.10 ± 0.01	2.68 ± 0.27	22.73 ± 1.21	0.11 ± 0.01	1.93 ± 0.37

Table 4. Eigenvalues and loadings (correlations) of ORNL soil variables onto rotated components. Variables that load strongly (≥0.7) are in bold.

Component	Eigenvalue	% variance
1	4.92	37.86
2	2.49	19.19
3	2.10	16.12
4	2.08	16.04

Variable	Comp. 1	Comp. 2	Comp. 3	Comp. 4
SOM	0.248	**0.842**	0.370	0.006
pH	-0.009	**0.907**	**-0.807**	-0.090
Al	**-0.916**	-0.116	-0.038	-0.241
Co	-0.440	0.032	0.149	**-0.819**
Pb	-0.107	0.113	0.670	-0.686
Hg	0.176	0.221	**0.921**	-0.171
V	**-0.928**	-0.121	0.031	-0.169
Cu	**-0.907**	0.110	-0.173	0.039
Fe	**-0.874**	-0.167	0.127	-0.371
Mn	-0.216	0.641	0.124	-0.667
Mo	-0.610	-0.321	0.609	-0.021
Ni	**-0.778**	0.361	0.117	-0.400
Zn	-0.559	0.433	0.429	-0.274

Table 5. Eigenvalues and loadings (correlations) of SERC soil variables onto rotated components. Variables that load strongly (≥0.7) are in bold.

Component	Eigenvalue	% variance
1	4.21	32.35
2	3.14	24.15
3	2.80	21.53
4	1.21	9.29

Variable	Comp. 1	Comp. 2	Comp. 3	Comp. 4
Log SOM	0.279	**-0.852**	0.338	0.147
pH	-0.307	**0.847**	-0.134	-0.148
Log Al	**0.859**	-0.338	0.106	0.277
Co	**0.739**	-0.275	0.277	-0.278
Pb	0.559	-0.497	0.537	0.277
Hg	0.266	**-0.735**	0.400	0.154
V	**0.832**	-0.355	0.361	-0.050
Cu	0.206	-0.234	**0.857**	0.188
Log Fe	**0.944**	-0.185	0.141	-0.120
Mn	0.124	0.287	-0.066	**-0.924**
Mo	**0.730**	-0.171	0.478	-0.060
Ni	0.353	-0.563	0.584	0.189
Log Zn	0.260	-0.268	**0.819**	-0.094

Figures

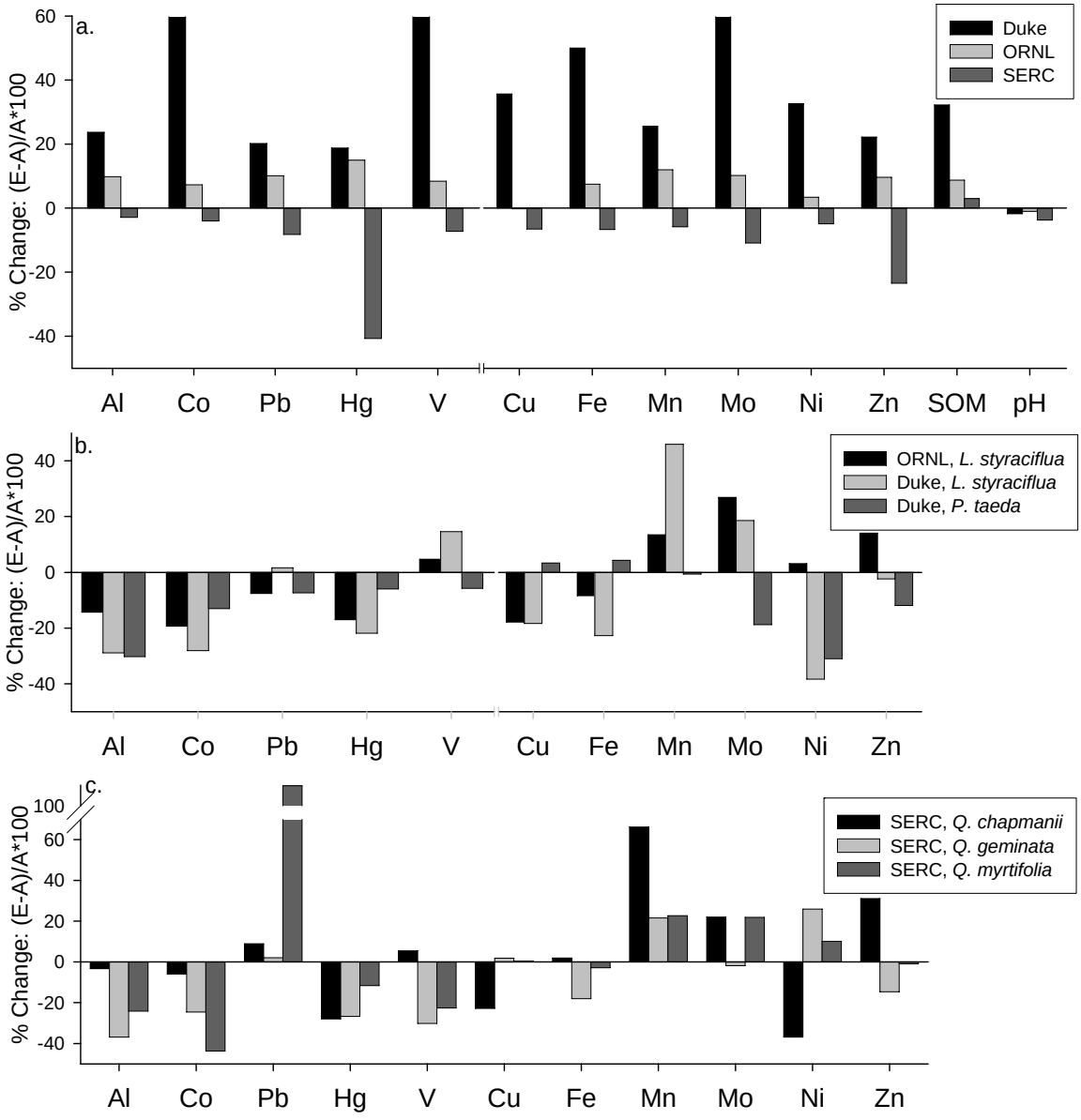

Figure 1. CO_2 effects on soil and leaf metal concentrations at Duke and ORNL FACE, and SERC OTC experiments. a. Percent change in metal concentrations, pH and SOM in surface soils (0-5 cm) from elevated plots relative to soils from ambient plots. b. Percent change in foliar metal concentrations in *P. taeda* at Duke, and *L. styraciflua* at ONRL and Duke. *P. taeda* concentrations are averages for 0-yr and 1-yr needles. All species are averaged across canopy heights. c. Percent change in foliar metal concentrations for *Q. chapmanii, Q. geminata* and *Q. myrtifolia* at SERC.

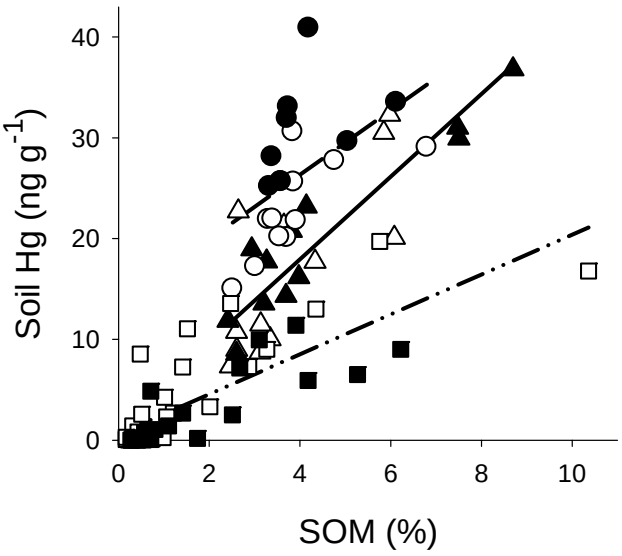

Figure 2. Relationship between soil Hg concentrations and percent soil organic matter (SOM). Soil Hg was positively correlated with percent SOM at Duke (triangles) and ORNL (circles) FACE sites, and SERC (squares) open-top chamber CO_2 experiment. Soil Hg concentrations increased with CO_2 enrichment at ORNL and Duke, and decreased with CO_2 enrichment at SERC. Filled symbols represent elevated rings and open, ambient rings.

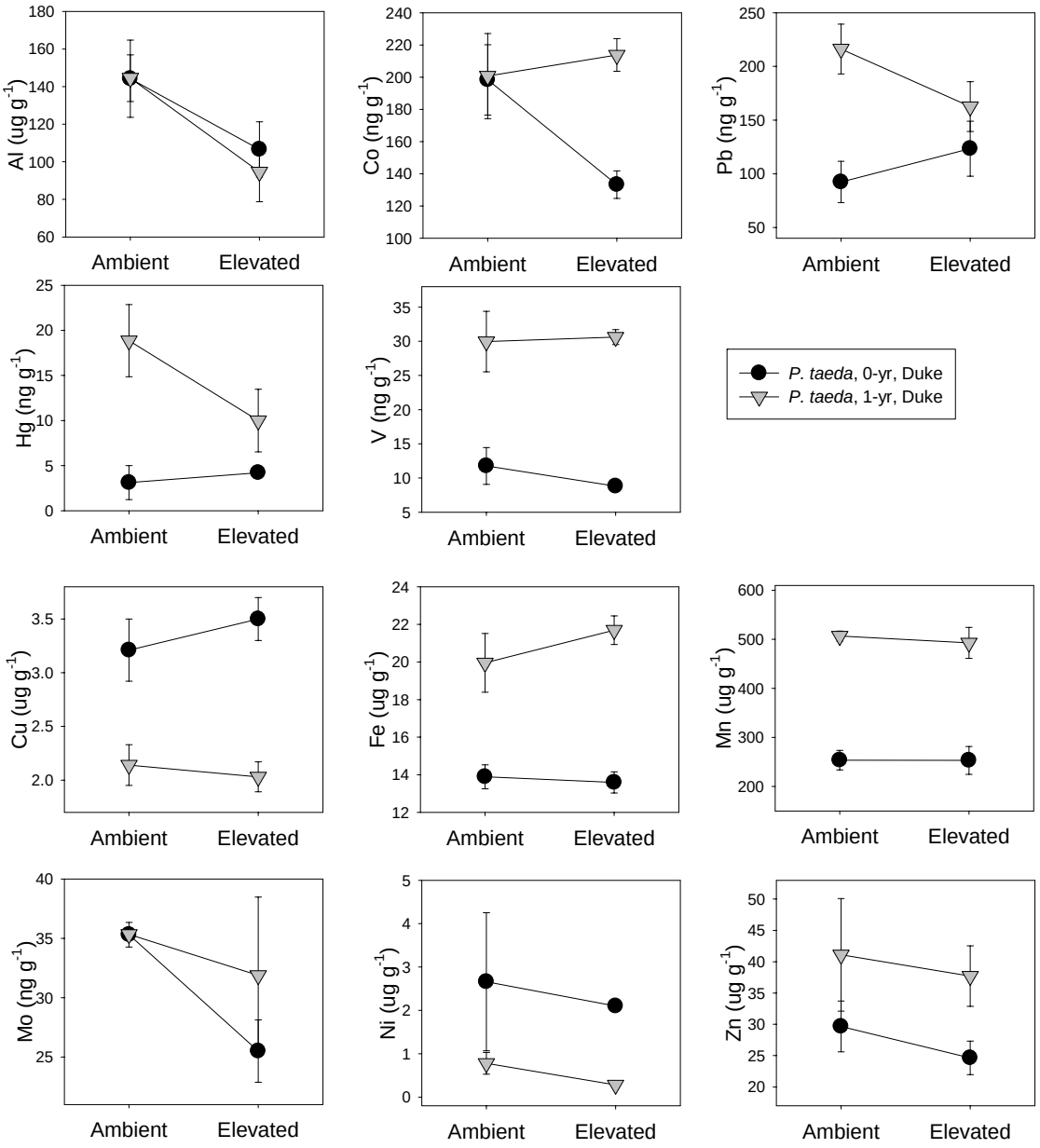

Figure 3. *P. taeda* foliar metal concentrations at Duke FACE. There was a significant effect of elevated CO_2, age, and CO_2 × age on *P. taeda* foliar metals (MANOVA, $p<0.05$). Symbols represent mean (± s.e.) foliar metal concentrations in 0-year (black circles) and 1-year (grey triangles) needles.

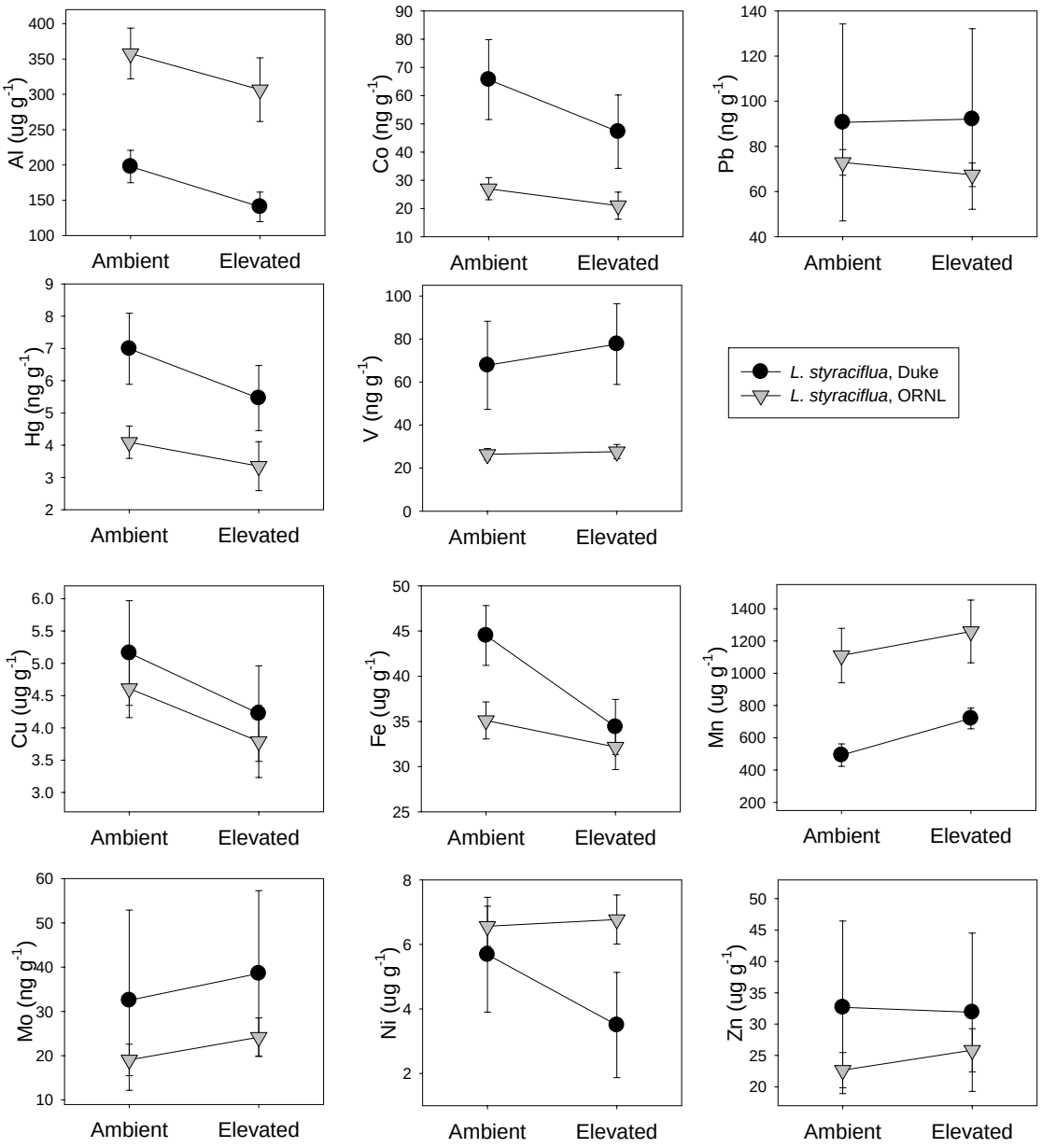

Figure 4. *L. styraciflua* foliar metal concentrations at Duke and ORNL FACE. There was a significant effect of elevated CO_2 on *L. styraciflua* foliar metals at both sites (MANOVA, $p<0.05$). Symbols represent mean (± s.e.) metal concentrations in *L. styraciflua* leaves at Duke (black circles) and ORNL (grey triangles).

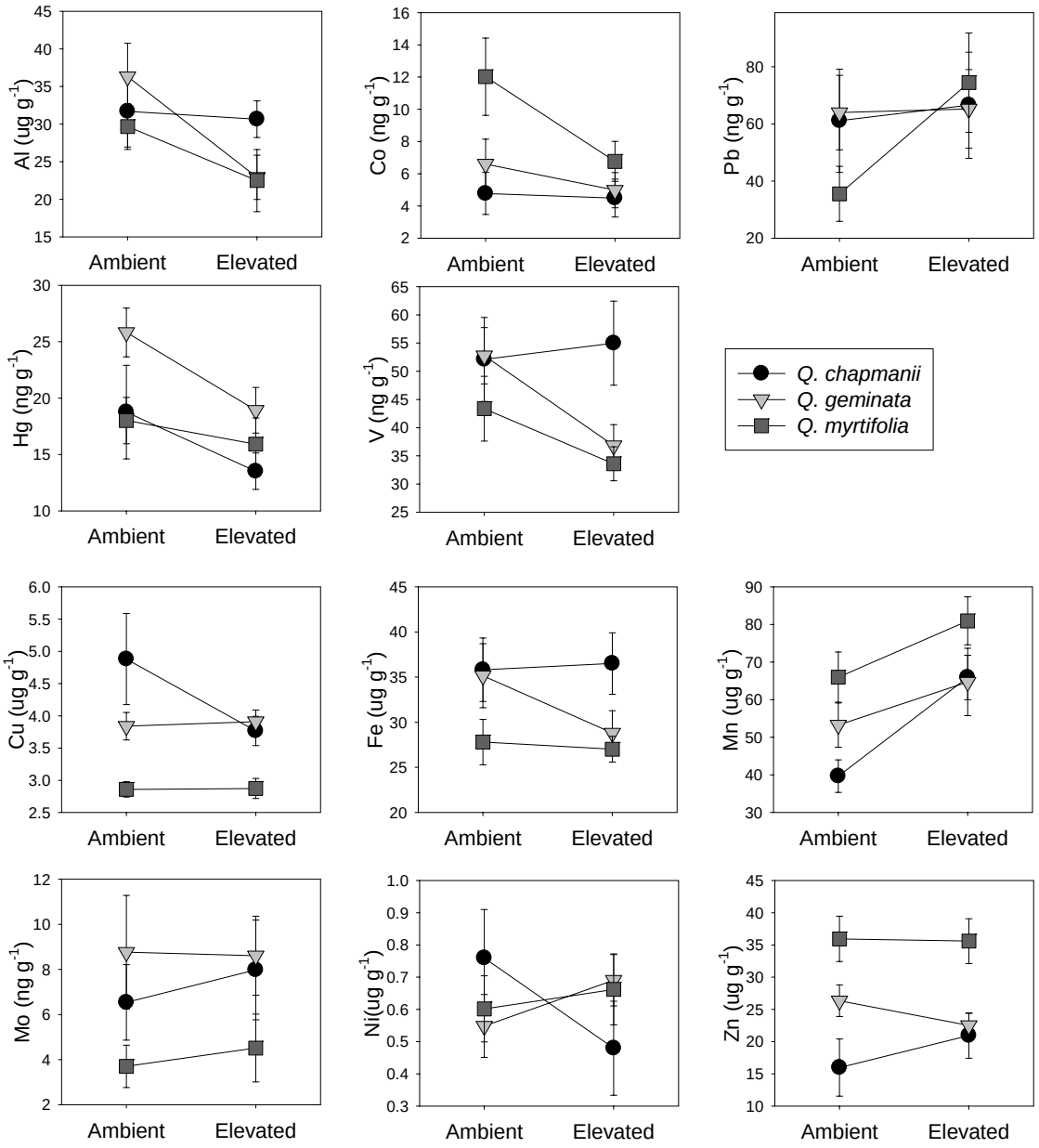

Figure 5. Foliar metal concentrations in *Quercus* sp. ORNL. There was a significant effect of elevated CO_2 (MANOVA, $p<0.05$) on foliar metal concentrations in *Q. chapmanii* (black circles), *Q. geminata* (light grey triangles), and *Q. myrtifolia* (dark grey squares).

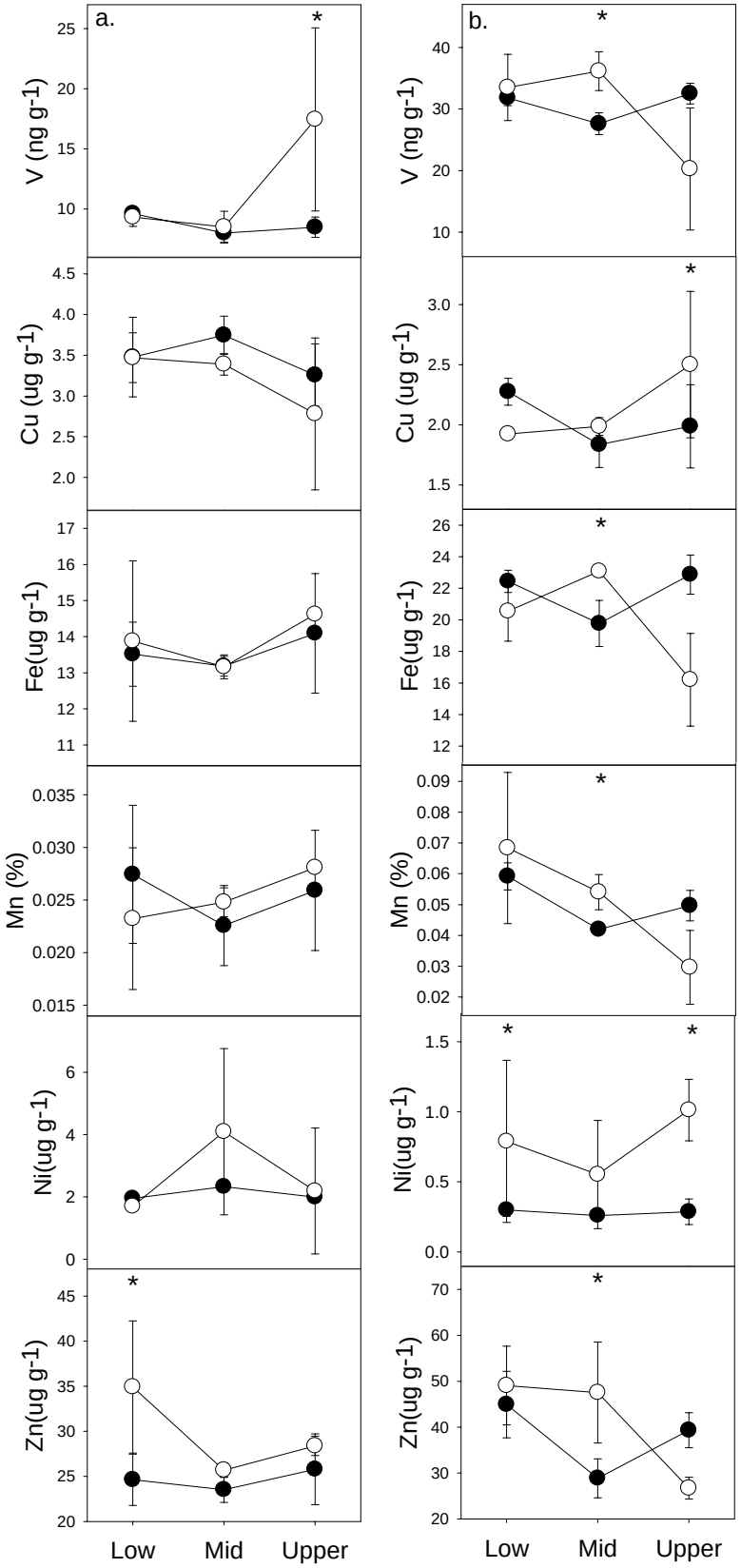

Figure 6. CO_2 effects on foliar metals in 0-yr (column a) and 1-yr (column b) *P. taeda* needles at Duke FACE. Only metals with significant CO_2 × canopy interaction effects are shown (low = 10-12 m; mid = 12-14 m; upper = 14-16 m canopy leaves). Significant decreases ($p<0.1$) with CO_2 enrichment are marked with *. Filled symbols are elevated; open are ambient.

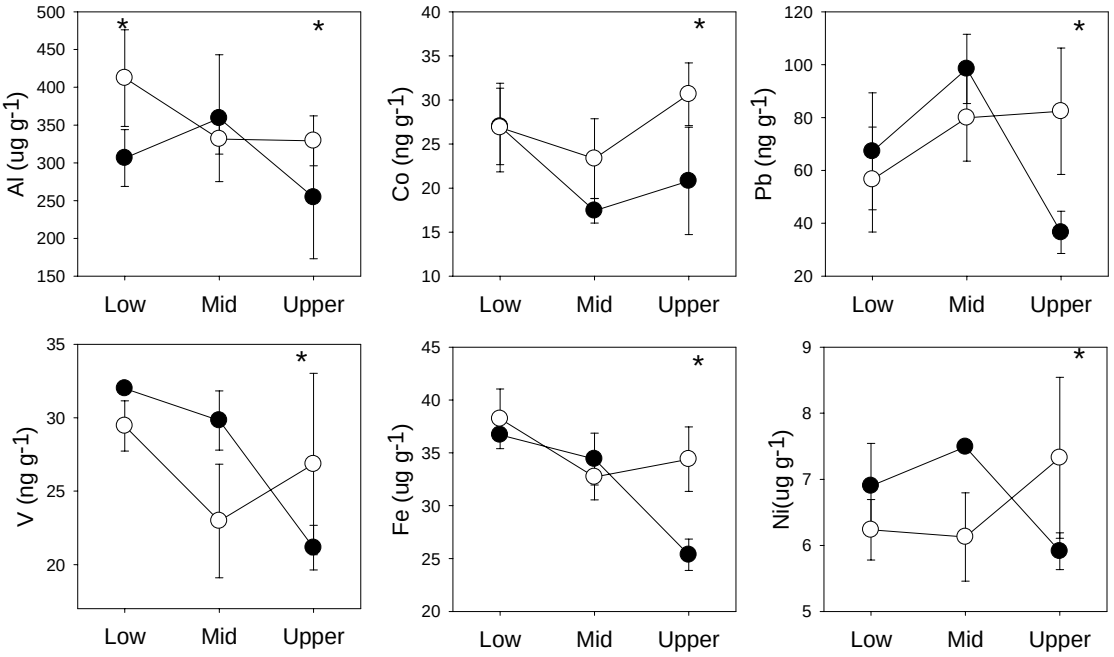

Figure 7. CO_2 effects on foliar metals at ORNL. Only metals with significant CO_2 × canopy interaction effects are shown. Significant decreases ($p<0.1$) with CO_2 enrichment are marked with *. Filled symbols are elevated; open are ambient.

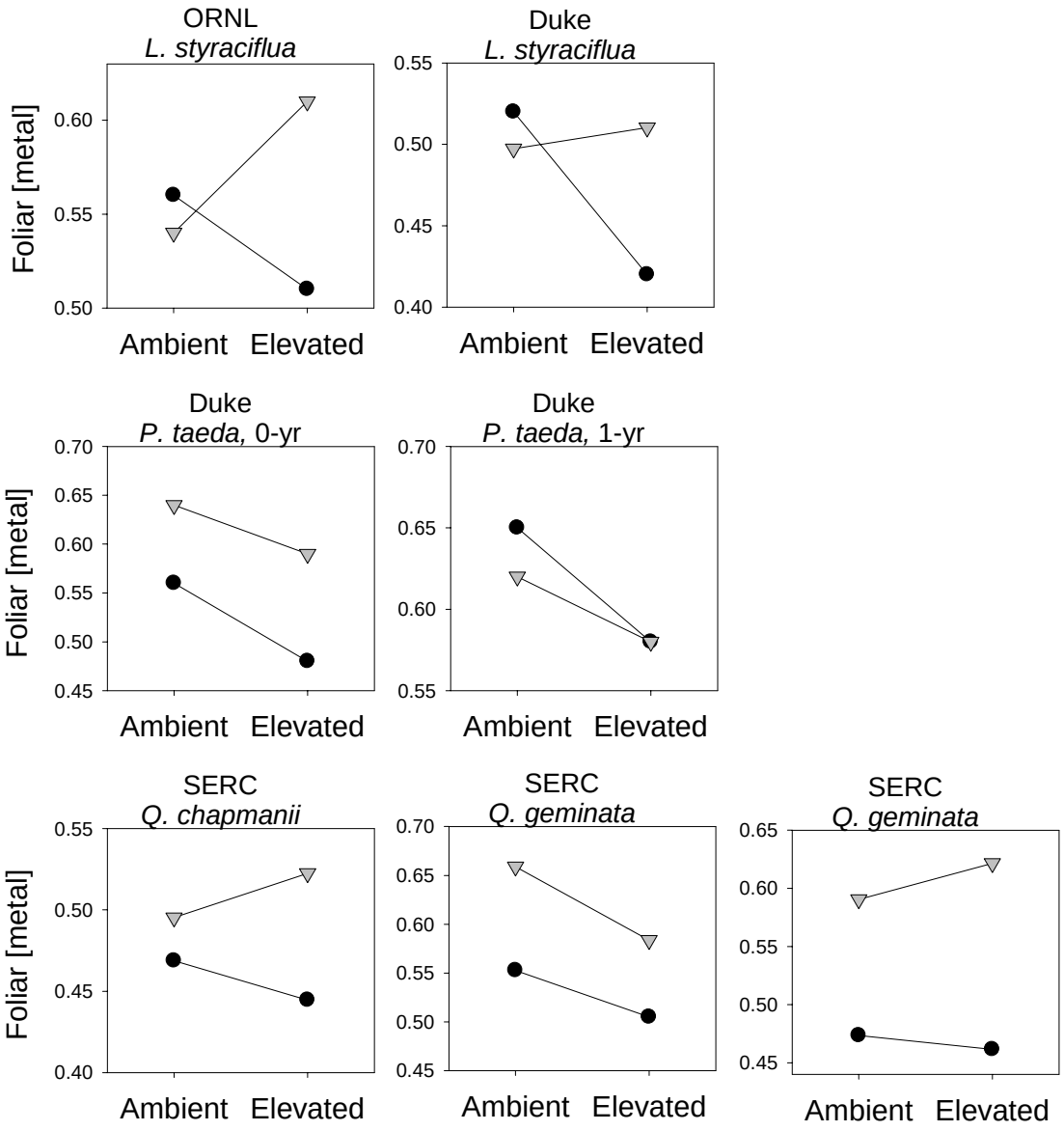

Figure 8. CO_2 effects on essential and nonessential metal concentrations in leaves at Duke, ORNL and SERC. Nonessential metals (black squares) are averaged range standardized mean concentrations of foliar Al, Co, Pb, Hg, and V. Essential metals (grey triangles) are averaged range standardized mean concentrations of foliar Fe, Cu, Mn, Mo, Ni, and Zn.

Supplemental Tables

Supp. Table 1. Summary of CO_2 and depth effects on soil components (Table 2) from Duke FACE. P-values have been adjusted to control family-wise error rate. Significant effects ($p<0.1$) in bold.

Effect	Comp. 1		Comp. 2		Comp. 3		Comp. 4	
	F	*P*	F	*P*	F	*P*	F	*P*
CO_2	0.01	0.93	0.60	0.93	0.30	0.93	2.7	0.68
Depth	2.30	0.25	22.55	**<0.01**	1.54	0.25	34.5	**<0.01**
CO_2 × depth	4.15	**0.09**	1.59	0.48	0.59	0.63	13.74	**<0.01**
Block	15.53	**<0.01**	0.96	**<0.01**	12.54	**<0.01**	56.87	**<0.01**

Supp. Table 2. Summary of CO_2 and depth effects on soil components (Table 4) from ORNL FACE. P-values have been adjusted to control family-wise error rate. Significant effects ($p<0.1$) in bold.

Effect	Comp. 1		Comp. 2		Comp. 3		Comp. 4	
	F	*P*	F	*P*	F	*P*	F	*P*
CO_2	0.23	0.66	0.43	0.66	24.68	**0.06**	0.44	0.66
Depth	0.25	0.86	12.51	**<0.01**	12.55	**<0.01**	1.81	0.43
CO_2 × depth	0.80	0.99	0.22	0.99	2.28	0.59	0.04	0.99
Ring	1.18	0.37	6.10	**0.06**	2.35	0.28	2.36	0.28

Supp. Table 3. Summary of CO_2 and depth effects on soil components (Table 5) from SERC CO_2 OTC experiment. P-values have been adjusted to control family-wise error rate. Significant effects ($p<0.1$) in bold.

Effect	Comp. 1		Comp. 2		Comp. 3		Comp. 4	
	F	*P*	F	*P*	F	*P*	F	*P*
CO_2	0.21	0.79	0.76	0.79	0.45	0.79	0.08	0.79
Depth	**5.12**	**0.01**	**28.14**	**<0.01**	**5.30**	**0.01**	1.04	0.39
CO_2 × depth	0.02	0.99	0.04	0.99	0.77	0.99	0.58	0.99
Block	**2.90**	**0.04**	1.15	0.36	1.84	0.20	2.18	0.15

Supp. Table 4. Correlations (R^2 values) between soil Al, V, Fe and Mo concentrations at Duke, ORNL and SERC. All correlations were significant at $p<0.005$.

	Duke	ORNL	SERC
Al-V	0.79	0.96	0.73[b]
Al-Fe	0.84	0.80	0.84
Al-Mo	0.79	0.40[a]	0.44[b]
Fe-V	0.98	0.70	0.84[a]
Fe-Mo	0.95	0.33[a]	0.59[c]
Mo-V	0.94	0.50[a]	0.70[a]

[a] both variables logged; [b] log Al; [c] log Fe

Supp. Table 5. ANCOVA test of CO_2 effects on soil Fe-V regression slopes and intercepts, and mean Fe (ppm): V (ppb) (± s.e.).

		Slope		Intercept		
		F	P	F	P	Fe:V
Duke	A	0.81	0.38	1.60	0.22	0.23 ± 0.01
	E					0.23 ± 0.01
ORNL	A	0.88	0.36	2.85	0.16	0.60 ± 0.01
	E					0.62 ± 0.01
SERC	A	0.23	0.63	0.12	0.73	0.26 ± 0.02
	E					0.26 ± 0.02

Supp. Table 6. CO_2, depth and site (Duke, ORNL, SERC) effects on soil pH and SOM.

	pH		SOM	
	F	P	F	P
CO_2	**4.12**	**.06**	**3.69**	**0.07**
Site (S)	**13.36**	**<0.01**	**113.10**	**<0.01**
Depth (D)	**9.18**	**<0.01**	**88.11**	**<0.01**
CO_2 × S	0.06	0.94	1.25	0.31
CO_2 × D	0.41	0.74	1.00	0.39
S ×D	**19.97**	**<0.01**	**2.97**	**0.01**
CO_2 × S ×D	0.06	0.99	0.86	0.53

Supp Table 7. Means (± s.e.) for Duke, ORNL and SERC leaf metal concentrations.

		CO_2	Al (μg g^{-1})	Co (ng g^{-1})	Pb (ng g^{-1})	Hg (ng g^{-1})	V (ng g^{-1})
Duke	*P. taeda*, 0	A	144.2 ± 20.6	198.3 ± 21.8	92.4 ± 19.3	3.1 ± 1.9	11.8 ± 2.7
		E	106.6 ± 14.7	133.3 ± 8.6	123.4 ± 25.6	4.2 ± 0.5	8.8 ± 0.4
	P. taeda, 1	A	144.5 ± 12.4	200.6 ± 26.5	216.2 ± 23.2	18.9 ± 4.1	30.0 ± 4.4
		E	94.6 ± 15.8	213.7 ± 10.1	162.5 ± 23.2	10.1 ± 3.5	30.6 ± 1.1
	L. styraciflua	A	197.7 ± 22.9	65.6 ± 14.2	90.6 ± 43.6	7.0 ± 2	67.8 ± 20.5
		E	140.6 ± 21.0	47.2 ± 13.0	92.1 ± 40.0	5.5 ± 1.0	77.7 ± 18.8
ORNL	*L. styraciflua*	A	357.7 ± 36.4	27.0 ± 3.9	72.9 ± 5.7	4.1 ± 0.5	26.4 ± 2.7
		E	306.6 ± 44.6	21.7 ± 4.8	67.4 ± 5.2	3.4 ± 0.6	27.7 ± 3.3
SERC	*Q. chapmanii*	A	31.7 ± 4.8	4.8 ± 1.3	61.1 ± 18.1	18.8 ± 4.2	52.1 ± 7.4
		E	30.6 ± 2.4	4.5 ± 1.2	66.6 ± 18.6	13.5 ± 1.6	55.0 ± 7.5
	Q. geminata	A	36.3 ± 4.4	6.6 ± 1.5	64.0 ± 13.0	25.8 ± 2.2	52.8 ± 5.0
		E	22.9 ± 2.9	5.0 ± 1.1	65.3 ± 13.8	18.9 ± 2.0	36.8 ± 3.7
	Q. myrtifolia	A	29.7 ± 3.0	12.0 ± 2.4	35.5 ± 9.6	18.0 ± 2.1	43.4 ± 5.7
		E	22.5 ± 4.1	6.8 ± 1.2	74.5 ± 17.4	15.9 ± 2.3	33.6 ± 3.0

		CO_2	Cu (μg g^{-1})	Fe (μg g^{-1})	Mn (μg g^{-1})	Mo (ng g^{-1})	Ni (μg g^{-1})	Zn (μg g^{-1})
Duke	*P. taeda*, 0	A	3.2 ± 0.3	13.9 ± 0.6	253.6 ± 19.9	35.3 ± 1.0	2.7 ± 1.6	29.7 ± 4.1
		E	3.5 ± 0.2	13.6 ± 0.6	253.0 ± 28.4	25.5 ± 2.6	2.1 ± 0.1	24.7 ± 2.7
	P. taeda, 1	A	2.1 ± 0.2	20.0 ± 1.6	506.7 ± 10.2	35.3 ± 0.1	0.8 ± 0.3	41.1 ± 9.0
		E	2.0 ± 0.1	21.7 ± 0.8	502.5 ± 31.6	31.9 ± 6.6	0.3 ± 0.1	37.7 ± 4.8
	L. styraciflua	A	5.2 ± 0.8	44.5 ± 3.3	492.8 ± 69.5	32.5 ± 20.4	5.7 ± 1.8	32.7 ± 13.8
		E	4.2 ± 0.7	34.4 ± 3.0	719.8 ± 63.7	38.6 ± 18.7	3.5 ± 1.6	31.9 ± 12.6
ORNL	*L. styraciflua*	A	4.6 ± 0.5	35.1 ± 2.1	1109.8 ± 168.4	19.1 ± 3.55	6.6 ± 0.6	22.7 ± 2.8
		E	3.8 ± 0.6	32.2 ± 2.5	1258.9 ± 194.8	24.2 ± 4.35	6.8 ± 0.8	25.8 ±3.4
SERC	*Q. chapmanii*	A	4.9 ± 0.7	35.8 ±3.5	39.66 ± 4.32	6.5 ± 1.7	0.8 ± 0.2	16.0 ± 4.4
		E	3.8 ± 0.2	36.5 ± 3.4	65.89 ± 5.90	8.0 ± 2.2	0.5 ± 0.2	20.9 ± 3.5
	Q. geminata	A	3.8 ± 0.2	35.2 ± 3.6	53.25 ± 5.87	8.76 ± 2.52	0.6 ± 0.1	26.4 ± 2.5
		E	3.9 ± 0.2	28.8 ± 2.5	64.71 ± 8.96	8.61 ± 1.75	0.7 ± 0.1	22.5 ± 1.9
	Q. myrtifolia	A	2.9 ± 0.1	27.8 ± 2.5	65.99 ± 6.69	3.70 ± 0.94	0.6 ± 0.1	35.9 ± 3.5
		E	2.9 ± 0.2	27.0 ± 1.4	80.96 ± 6.39	4.52 ± 1.50	0.7 ± 0.1	35.6 ± 3.5

Supp Table 8. F and P values for Duke *P. taeda* leaf ANOVA. All p-values have been adjusted to control family-wise error rate; effects that were significant ($p<0.1$) before adjusting p-values are in bold.

	Al		Co		Pb		Hg		V	
	F	P	F	P	F	P	F	P	F	P
CO_2	1.16	0.44	0.69	0.44	0.03	0.60	7.84	0.44	0.69	0.44
Ht	0.10	0.90	0.50	0.86	1.68	0.55	0.24	0.29	0.46	0.86
Age	0.25	0.66	**13.14**	**<0.01**	**5.04**	**0.04**	**32.14**	**0.01**	**192.8**	**<0.01**
CO_2 × Ht	1.38	0.47	0.61	0.76	1.13	0.52	2.70	0.47	1.41	0.48
CO_2 × Age	2.16	0.33	**8.75**	**0.03**	0.77	0.45	0.61	0.77	**7.05**	**0.04**
Ht × Age	0.93	0.44	1.29	0.39	**3.12**	**0.09**	0.31	0.74	**13.87**	**<0.01**
CO_2 × A × H	0.34	0.78	0.47	0.77	0.91	0.56	1.87	0.25	**18.99**	**<0.01**
Block	15.22	<0.01	6.14	<0.01	3.23	0.05	1.27	0.33	1.04	0.38

	Cu		Fe		Mn		Mo		Ni		Zn	
	F	P	F	P	F	P	F	P	F	P	F	P
CO_2	0.05	0.64	0.34	0.70	0.002	0.66	1.73	0.44	0.51	0.44	0.43	0.44
Ht	0.28	0.86	0.39	0.86	2.68	0.30	0.29	0.86	0.25	0.86	3.83	0.30
Age	**46.33**	**<0.01**	**85.36**	**<0.01**	**52.48**	**<0.01**	0.20	0.66	**108.7**	**<0.01**	**24.23**	**<0.01**
CO_2 × Ht	0.10	0.92	**4.38**	**0.19**	**3.28**	**0.28**	1.40	0.47	0.08	0.92	**2.51**	**0.13**
CO_2 × Age	**4.92**	**0.08**	1.35	0.45	0.82	0.45	0.53	0.47	**15.32**	**<0.01**	1.08	0.45
Ht × Age	**5.22**	**0.02**	**3.48**	**0.08**	**5.13**	**0.02**	1.16	0.39	**12.11**	**<0.01**	**2.59**	**0.13**
CO_2 × A × H	**2.73**	**0.15**	**5.09**	**0.03**	**3.53**	**0.11**	0.11	0.90	**9.82**	**<0.01**	**2.33**	**<0.01**
Block	5.80	<0.01	2.03	0.17	6.27	<0.01	1.84	0.18	11.08	<0.01	7.18	<0.01

Supp. Table 9. Probability values for Duke *P. taeda* leaf ANOVA interactions (CO_2 × Age × Ht). Co had a significant CO_2 × age effect, but not a significant three-way interaction, so canopy p-values not shown. Tests of CO_2 effects were one-tailed test; therefore, significant effects represent decreased foliar metal concentrations in elevated CO_2 rings.

	Co	V	Cu	Fe	Mn	Ni	Zn
0-yr	**<0.01**	**0.01**	0.97	-	-	0.16	-
Low		0.54	0.64	0.54	0.68	0.39	**0.07**
Mid		0.38	0.78	0.40	0.18	0.16	0.16
Upper		**<0.01**	0.98	0.37	0.23	0.77	0.18
1-yr	0.72	0.87	0.14	-	-	**<0.01**	-
Low		0.23	0.72	0.23	0.43	**0.09**	0.37
Mid		**0.02**	0.32	**0.02**	**0.04**	0.13	**<0.01**
Upper		1.00	**0.02**	1.00	1.00	**<0.01**	0.96

Supp. Table 10. Eigenvalues and loadings (correlations) of Duke *P. taeda* variables onto rotated components. Variables that load strongly (≥0.7) are in bold.

Component	Eigenvalue	% variance
1	3.42	31.09
2	2.67	24.18
3	1.28	11.61
4	1.25	11.38

Variable	Comp. 1	Comp. 2	Comp. 3	Comp. 4
Log Al	-0.551	0.545	0.174	-0.357
Log Co	-0.538	-0.285	0.390	0.225
Log Pb	-0.132	-0.181	**-0.846**	0.244
Log Hg	-0.125	-0.107	0.608	0.309
Log V	**-0.700**	-0.642	0.049	0.175
Log Cu	0.103	**0.894**	0.044	0.217
Log Fe	**-0.820**	-0.340	-0.009	0.231
Log Mn	**-0.879**	-0.179	-0.005	-0.130
Log Mo	-0.146	0.064	0.042	**0.850**
Log Ni	0.239	**0.932**	-0.049	-0.083
Log Zn	**-0.881**	0.094	-0.037	0.204

Supp. Table 11. ANCOVA test of CO_2 effects on leaf V-Fe regression slopes and intercepts, and mean Fe (ppm): V (ppb) (± s.e.) at Duke, ORNL and SERC. If slopes were heterogeneous across CO_2 treatments, no intercept test was conducted.

	Slope		Intercept		Fe:V	
	F	P	F	P	Ambient	Elevated
Duke, *P. taeda*	2.42	0.12	**9.69**	**<0.01**	1.07 ± 0.12	1.18 ± 0.04
Duke, *P. taeda*, 0-yr	6.54	0.02	--	--	1.42 ± 0.33	1.64 ± 0.07
Duke, *P. taeda*, 1-yr	2.25	0.14	**4.74**	**0.04**	0.74 ± 0.08	0.72 ± 0.03
Duke, *L. styraciflua*	6.28	0.02	--	--	0.67 ± 0.05	0.49 ± 0.09
ORNL, *L. styraciflua*	2.03	0.16	**16.42**	**<0.01**	1.39 ± 0.13	1.19 ± 0.19
SERC, *Q. chapmanii*	0.35	0.57	0.51	0.49	0.77 ± 0.07	0.67 ± 0.04
SERC, *Q. geminata*	0.34	0.57	0.21	0.61	0.69 ± 0.04	0.80 ± 0.05
SERC, *Q. myrtifolia*	0.04	0.84	0.75	0.40	0.70 ± 0.06	0.85 ± 0.10

Supp. Table 12. F and P values for Duke *L. styraciflua* leaf ANOVA. All p-values have been adjusted to control family-wise error rate; effects that were significant ($p<0.1$) before adjusting p-values are in bold.

	Al		Co		Pb		Hg		V	
	F	P	F	P	F	P	F	P	F	P
CO_2	3.38	0.42	1.06	0.42	0.05	0.65	0.43	0.42	0.06	0.59
Ring	**2.56**	**0.11**	**6.13**	**0.01**	**5.00**	**0.02**	0.29	0.33	**4.23**	**0.03**

	Cu		Fe		Mn		Mo		Ni		Zn	
	F	P	F	P	F	P	F	P	F	P	F	P
CO_2	0.75	0.42	5.05	0.42	4.95	0.95	0.05	0.65	0.69	0.42	0.08	0.63
Ring	**6.22**	**<0.01**	0.93	0.44	**2.60**	**0.11**	**6.61**	**<0.01**	**23.63**	**<0.01**	**26.02**	**<0.01**

Supp. Table 13. Eigenvalues and loadings (correlations) of Duke *L. styraciflua* variables onto rotated components. Variables that load strongly (≥0.7) are in bold.

Component	Eigenvalue	% variance
1	3.72	33.79
2	1.93	17.57
3	1.64	14.92
4	1.70	15.50

Variable	Comp. 1	Comp. 2	Comp. 3	Comp. 4
Al	-0.653	-0.480	-0.229	0.122
Co	**-0.946**	-0.086	-0.020	0.149
Pb	-0.632	0.563	0.172	0.073
Log Hg	0.068	0.119	**-0.888**	0.037
Log V	0.049	0.383	0.220	**0.838**
Log Cu	-0.613	-0.499	0.300	-0.334
Fe	-0.479	-0.307	0.032	**0.772**
Mn	-0.103	0.171	**0.763**	0.351
Log Mo	**-0.729**	0.076	0.166	0.343
Log Ni	-0.018	**-0.916**	0.045	-0.076
Zn	**-0.917**	0.046	0.138	-0.061

Supp. Table 14. F and P values for ORNL *L. styraciflua* leaf ANOVA. All p-values have been adjusted to control family-wise error rate; effects that were significant (p<0.1) before adjusting p-values are in bold.

	Al		Co		Pb		Hg		V	
	F	P	F	P	F	P	F	P	F	P
CO_2	0.79	0.46	0.70	0.46	0.13	0.46	0.45	0.28	0.08	0.46
Ht	2.89	0.19	**4.73**	**0.07**	1.75	0.32	0.51	0.63	**4.24**	**0.07**
CO_2×H	**2.76**	**0.16**	**2.40**	**0.16**	**1.98**	**0.16**	0.19	0.82	**3.7**	**0.11**
Ring	**5.94**	**<0.01**	**10.74**	**<0.01**	1.15	0.37	0.98	0.41	**4.93**	**0.01**

	Cu		Fe		Mn		Mo		Ni		Zn	
	F	P	F	P	F	P	F	P	F	P	F	P
CO_2	1.32	0.46	0.96	0.46	0.23	0.46	0.83	0.46	0.04	0.46	0.51	0.46
Ht	1.60	0.32	**8.77**	**<0.01**	2.59	0.20	0.57	0.68	0.13	0.88	1.55	0.32
CO_2×H	0.33	0.79	**4.97**	**0.11**	2.20	0.17	**2.64**	**0.16**	**4.07**	**0.11**	0.59	0.67
Ring	**5.77**	**<0.01**	**3.84**	**0.02**	**22.27**	**<0.01**	**3.15**	**0.05**	**5.58**	**<0.01**	**4.36**	**0.02**

Supp. Table 15. Probability values for ORNL *L. styraciflua* leaf ANOVA, CO_2 × canopy height interactions. Tests of CO_2 effects were one-tailed tests; significant effects represent decreased foliar metal concentrations in elevated CO_2 rings.

	Al	Co	Pb	V	Fe	Mo	Ni
Low	**<0.01**	0.52	0.66	0.78	0.35	0.48	0.82
Mid	0.74	0.13	0.69	0.97	0.97	0.99	0.97
Upper	**0.04**	**<0.01**	**0.04**	**0.05**	**<0.01**	0.52	**0.03**

Supp. Table 16. Eigenvalues and loadings (correlations) of ORNL *L. styraciflua* leaf variables onto rotated components. Variables that load strongly (≥0.7) are in bold.

Component	Eigenvalue	% variance
1	1.68	15.24
2	1.62	14.74
3	2.00	18.16
4	2.24	20.39

Variable	Comp. 1	Comp. 2	Comp. 3	Comp. 4
Al	0.221	-0.039	0.072	**0.790**
Co	0.060	0.159	-0.326	**0.758**
Log Pb	-0.026	0.074	0.422	0.426
Log Hg	0.365	0.797	-0.016	0.217
V	**0.933**	-0.004	0.045	0.060
Cu	0.304	-0.324	-0.210	0.077
Fe	**0.826**	-0.098	-0.251	0.331
Log Mn	0.242	-0.570	-0.290	0.625
Log Mo	-0.113	0.094	**-0.742**	0.246
Log Ni	0.389	-0.081	**-0.746**	-0.017
Zn	0.434	**-0.745**	0.175	0.012

Supp. Table 17. F and P values for SERC leaf ANOVA. All p-values have been adjusted to control family-wise error rate; effects that were significant ($p<0.1$) before adjusting p-values are in bold.

	Al		Co		Pb		Hg		V	
	F	P	F	P	F	P	F	P	F	P
CO_2	**2.42**	**0.06**	**2.77**	**0.15**	1.52	0.97	**3.06**	**0.15**	**2.82**	**0.15**
Species	2.41	0.11	**4.05**	**0.04**	0.28	0.75	**4.22**	**0.03**	**4.21**	**0.03**
CO_2×S	**2.52**	**0.46**	0.52	0.73	1.25	0.55	0.15	0.89	1.69	0.46
Block	**2.18**	**0.15**	1.43	0.33	**2.54**	**0.11**	1.20	0.39	1.51	0.31

	Cu		Fe		Mn		Mo		Ni		Zn	
	F	P	F	P	F	P	F	P	F	P	F	P
CO_2	**1.62**	**0.22**	1.00	0.29	9.45	0.99	0.75	0.73	0.23	0.45	0.19	0.43
Species	**16.85**	**<0.01**	**5.65**	**0.02**	**4.66**	**0.03**	**9.26**	**<0.01**	0.52	0.66	**15.44**	**<0.01**
CO_2×S	1.74	0.46	1.02	0.59	0.62	0.73	0.11	0.89	**3.93**	**0.33**	1.80	0.46
Block	1.31	0.36	1.71	0.24	0.86	0.66	**5.70**	**<0.01**	**2.03**	**0.17**	0.70	0.75

Supp. Table 18. P values for SERC leaf ANOVA, CO_2 × Species. Tests of CO_2 effects were one-tailed tests; significant effects represent decreased foliar concentrations in elevated CO_2.

	Al	Ni
Q. chapmanii	0.61	**<0.01**
Q. geminata	**<0.01**	0.89
Q. myrtifolia	**0.03**	0.66

Supp. Table 19. Eigenvalues and loadings (correlations) for SERC Q. *chapmanii* leaf variables onto rotated components. Variables that load strongly (≥0.7) are in bold.

Component	Eigenvalue	% variance
1	3.33	30.30
2	2.27	20.60
3	1.88	17.11
4	1.54	14.00

	Comp. 1	Comp. 2	Comp. 3	Comp. 4
Al	**0.926**	-0.097	0.130	0.147
Co	-0.246	**0.881**	0.153	-0.216
Pb	0.194	**0.917**	-0.035	-0.008
Hg	**0.827**	0.005	0.363	0.083
V	**0.846**	-0.073	-0.325	-0.258
Log Cu	-0.575	0.380	0.514	-0.432
Fe	**0.755**	0.015	-0.149	-0.509
Mn	0.110	0.255	**-0.819**	0.279
Mo	0.089	0.078	0.680	0.091
Log Ni	0.072	0.177	0.082	**-0.928**
Zn	-0.229	0.622	-0.421	-0.081

Supp Table 20. Eigenvalues and loadings (correlations) for SERC Q. *geminata* leaf variables onto rotated components. Variables that load strongly (≥0.7) are in bold.

Component	Eigenvalue	% variance
1	3.70	33.66
2	1.68	15.31
3	2.26	20.54
4	1.20	10.91

	Comp. 1	Comp. 2	Comp. 3	Comp. 4
Al	**-0.899**	-0.144	0.240	0.122
Co	-0.200	-0.138	-0.008	**0.885**
Pb	-0.304	-0.027	**0.759**	0.091
Hg	**-0.811**	-0.281	0.144	0.261
V	**-0.898**	0.014	0.195	0.131
Log Cu	-0.178	0.292	**0.793**	0.062
Fe	**-0.785**	0.201	0.464	0.084
Mn	0.087	**0.812**	-0.118	-0.158
Mo	-0.171	-0.113	**0.741**	-0.465
Log Ni	-0.110	**0.807**	0.392	0.035
Zn	**-0.776**	0.341	0.081	-0.233

Supp Table 21. Eigenvalues and loadings (correlations) for Q. *myrtifolia* leaf variables onto rotated components. Variables that load strongly (≥0.7) are in bold.

Component	Eigenvalue	% variance
1	2.91	26.43
2	2.15	19.50
3	1.82	16.58
4	1.30	11.85

	Comp. 1	Comp. 2	Comp. 3	Comp. 4
Al	-0.473	0.178	-0.426	0.384
Co	0.048	**0.823**	-0.108	-0.074
Pb	**-0.853**	0.061	-0.069	0.309
Hg	-0.328	-0.251	**-0.748**	-0.234
V	0.181	0.592	-0.598	0.028
Log Cu	-0.588	0.635	0.191	-0.129
Fe	0.072	0.329	**-0.755**	0.013
Mn	-0.152	0.024	0.129	**0.911**
Mo	**-0.847**	-0.016	-0.234	0.202
Log Ni	**-0.844**	-0.073	0.108	-0.107
Zn	-0.095	**0.708**	-0.138	0.318

Chapter 3: Increased mercury in forest soils under elevated carbon dioxide

Introduction

Mercury (Hg) is a persistent contaminant in terrestrial and freshwater systems, where it may enter into foodwebs, affecting wildlife and human health (U.S. EPA 1997a). Since the onset of the industrial revolution, anthropogenic Hg deposition has increased three to five times pre-industrial rates (Lamborg et al. 2002). Particulate and gaseous Hg emitted via coal combustion and other industrial activities (such as waste incineration, manufacturing and smelting) can be deposited locally or remain in the atmosphere for a year or more (U.S. EPA 1997b), making Hg important as a local, regional and global contaminant. Vegetation plays a critical role in the transfer of atmospheric Hg to the biosphere—Hg from the atmosphere is deposited onto leaf surfaces or taken up by stomata and subsequently transferred to soils in litterfall and throughfall (Rea et al. 1996; St Louis et al. 2001; Figure 1). Deposition of Hg under forest canopies can be three to four times greater than deposition to bare soils (Grigal 2003; St Louis et al. 2001) and almost all the Hg in forest canopies is derived from the atmosphere (Ericksen et al. 2003). Because of the critical role of vegetation in the transfer of Hg from air to surface, processes that affect plant structure and function can significantly alter Hg inputs to soils. Soil-bound Hg can become a large source of Hg to aquatic systems, where Hg is an important ecosystem contaminant.

One important factor that is affecting terrestrial ecosystem structure and function is the rise in atmospheric carbon dioxide (CO_2) concentrations. CO_2 concentrations are increasing at an unprecedented rate, primarily due to anthropogenic combustion of fossil fuels (IPCC 2007). Because of the strong biological component that governs Hg cycling, CO_2-induced changes in plant and soil properties may affect both the fluxes and storage of Hg in terrestrial systems. Although some of the effects of elevated CO_2 on plants and soils have been well-studied, the impacts on Hg cycling are less known. For example,

while it is known that CO_2 enrichment may affect soil acidity and organic matter (Andrews and Schlesinger 2001; Jastrow et al. 2005; Oh and Richter 2004) and that these soil properties can affect Hg adsorption (Schuster 1991; Yin et al. 1996), it is still unclear how increasing atmospheric CO_2 may affect Hg in soils. Elevated CO_2 may also affect Hg inputs to soils through changes in leaf litter biomass—an increase in litterfall biomass with CO_2 enrichment (Finzi et al. 2001; Norby et al. 2001) may increase Hg uptake and deposition to the forest floor. CO_2-mediated changes in leaf area and leaf tissue chemistry (Lichter et al. 2000) may also affect Hg deposition in litterfall, throughfall and stemflow (Figure 1).

Understanding the factors that may alter the cycling of Hg through soils is critical because soil Hg represents a major portion of the total Hg pool; surface soils store an estimated 95% of the 200,000 tons of Hg mobilized since the 1890's (EPMAP 2004). As such, terrestrial systems, and forests in particular, have been recognized as major components in the cycling of Hg between the atmosphere and biosphere (Lindberg et al. 1998). Interestingly, Hg concentrations in most forest soils tend to be low; it is the large volume of forest soils globally that leads to this large amount of storage (Fitzgerald and Lamborg 2003). As Hg leaves soils though leaching it can enter vadose and ground water flow paths and move into aquatic systems, where it becomes an important contaminant through methylation and biomagnification processes (Schroeder and Munthe 1998). A challenge facing the scientific community is to understand how rising global CO_2 concentrations may affect this large Hg pool.

To study the potential effects of increased atmospheric CO_2 on Hg cycling in forests we examined plants and soils from two free-air carbon dioxide enrichment (FACE) experiments—a loblolly pine forest in North Carolina (Duke) and a sweetgum plantation in Tennessee (ORNL)—which have been exposed to CO_2 enrichment (ambient + 200 ppmv) since 1996 and 1998, respectively. In 2005 we sampled leaves and litter from the dominant canopy tree at Duke FACE, loblolly pine (*Pinus taeda*), and the dominant canopy tree at ORNL FACE, sweetgum (*Liquidambar styraciflua*). *L. styraciflua* was also sampled at Duke where is it a common understory tree. In 2007 we focused sampling on Hg deposition at ORNL in litterfall, stemflow and throughfall. CO_2 effects

on plants and certain ecosystem processes at these sites have been well-documented (e.g., Andrews and Schlesinger 2001; Finzi et al. 2001; Norby and Iversen 2006; Norby et al. 2001), but the effects of elevated CO_2 on Hg have not been examined.

Materials and methods

Site descriptions

Duke forest FACE site

Duke FACE is a mixed evergreen-deciduous temperate forest dominated by loblolly pine (*Pinus 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 m × 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 (*Liquidambar styraciflua*). The FACE experiment began in August 1996 and is comprised of three 30 m diameter ambient rings (~382 μmol mol^{-1}) and three 30 m diameter elevated rings (~582 μmol mol^{-1}). An experimental N-fertilization treatment ($NH_4^+NO_3^-$ at a rate of 11.2 g N m^{-2} yr^{-1}) was added to one half of each ring in April 2005. The experimental rings are arranged in a complete block design to account for topographic variation and potential fertility gradients. The CO_2 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 regulated blowers that deliver a controlled amount of CO_2-fumigated air to maintain ambient or elevated levels of CO_2 into the rings (Hendrey et al. 1999). Descriptions of the site, experimental design and FACE technology have been well-documented (Finzi et al. 2001; Hendrey et al. 1999).

Oak Ridge FACE site

The deciduous forest site (ORNL) is a sweetgum (*L. styraciflua*) plantation located in the Oak Ridge National Environmental Research Park in Roane County, Tennessee (35°54'N, 84o20’W). The soil at the site, classified as Aquic Hapludult, has a silty clay loam texture, is moderately well drained and is slightly acidic. The stand was planted

with one-year-old sweetgum seedlings in 1988 at a spacing of 1.2 m × 2.3 m. The FACE apparatus 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. CO_2 enrichment began in 1998 and continues during the growing season through the present time. The site description and experimental design are well documented in Norby et al. (2001).

Field sampling

Soils

Soil samples were collected at ORNL from 25-28 July 2005 and at Duke from 8-11 August 2005. A core sampler (AMS with slide hammer) 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). Acid-washed butyrate plastic core liners were used in the soil corer in order to maintain an intact core during extraction. We used cores from the N-fertilized sectors of the Duke FACE rings to increase our sub-sampling size. Cores were divided into 5 cm depth increments and pooled within rings (within N treatment of each ring at Duke). In all of our analyses, 'ring' is the unit of replication for CO_2 treatment, so pooling of cores within a ring has no consequence in testing for CO_2 or depth effects.

We obtained pre-treatment soil samples (that is, samples collected prior to initiation of FACE in 1996 at Duke and 1998 at ORNL) from archives at each site. The number of pre-treatment soil sub-samples (cores within a ring) varies between sites and among rings within a site because these samples were not collected for this experiment, but rather were provided for our analysis from the FACE soil archives. Pre-treatment samples consisted of 16-18 pooled randomly sampled cores per ring (0-7.5 and 7.5-15 cm) at Duke, and two to six randomly collected cores per ring (0-5 and 0-15 cm) at ORNL. Archived soils were stored in a sealed container at room temperature; ambient and elevated soil samples from each site were stored under identical conditions.

Leaves

Canopy leaves were collected at ORNL from 25-28 July 2005 and at Duke from 8-11 August 2005. Green leaves were sampled from three canopy heights—low (10-12m), mid (12-14m) and upper (14-16m)—from *L. styraciflua* at ORNL and Duke (lower and mid canopies only, pooled for analysis) and from *P. taeda* at Duke (all canopy heights). The canopy at ORNL was accessed using a stationary hydraulic lift located near the center of each ring. At Duke the canopy as 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.* In each canopy height three replicate samples were collected. For all leaves collected, a sample consisted of approximately 5 leaves/20 needles from an individual tree.

In October 2005, freshly fallen leaf litter was collected from the forest floor at ORNL (15-20 leaves per ring). Senescent leaves at Duke were collected from three to five trees per species per ring via the central walk-up tower (*P. taeda*) or by gently shaking trees (*L. styraciflua*) and collecting leaves as they fell to the forest floor.

In 2007, leaves were collected from ORNL throughout the growing season on the following dates: 9 June, 5 July, 5 August, 3 September, and 11 November (senescent leaves). Because the lifts in several of the rings were not in operation during the collection periods, green leaves were collected from 3-5 trees at a height of approximately 8-10 m using a pole pruner and senescent leaves were collected by shaking 3-5 trees in each plot. By both methods leaves were collected as they fell, but prior to reaching the forest floor. All leaves were collected using particle-free gloves into double-bagged polyethylene bags.

Leaf litter biomass was determined from litter basket collections (Finzi et al. 2001; Norby et al. 2001). At Duke, litterfall was collected into twelve 0.16 m^2 baskets per plot. Litterfall was collected once per month between January and August and twice per month between September and December. At ORNL, leaf litter was collected into seven randomly placed 0.19 m^2 baskets per plot, and collected biweekly to monthly. Further details of litter collection methods can be found in Finzi et al. (2001) and Norby et al. (2001).

Litter Hg deposition was calculated as the product of litter Hg concentration and litterfall biomass. Duke leaf litter was comprised of *P. taeda* plus a number of broadleaf species. We use litter Hg concentrations from *L. styraciflua,* the most common broadleaf tree at Duke FACE, to represent all broadleaf litter Hg concentrations. While we expect inter-specific variation in litter Hg concentrations, *L. styraciflua* dominates broadleaf biomass, so effects of this variation on total Hg litter deposition will be minimal. Duke leaf litter concentrations presented in the text are a weighted average of these two species, based on percent leaf litter biomass of pines and broadleaf trees.

Throughfall

Six bulk throughfall collectors for Hg analysis were placed in each of the five experimental rings at ORNL in 2007. Collectors were placed in the field on 9 June and collections were made on the following dates: 4 July, 22 July, 5 August, 3 September, and 14 September.

Throughfall collectors were modeled after Iverfeldt (1991; Figure 2). Collectors consisted of a 100 mm i.d. clean borosilicate glass funnel (washing protocol described below) fitted with an inverted watch glass to filter large debris. Acid washed C-flex tubing was used to secure 0.5 m length acid washed Teflon® tubing (3/16" i.d.) to the funnel. A loop was formed in the Teflon® tubing to allow retention of a small amount of sample water, which created an airlock over the sample container. The tubing was attached to a clean 1 L borosilicate bottle (wrapped in foil) via a Teflon® compression fitting that was screwed into the bottle cap. Each bottle cap was also fit with a LDPE screw with a small cut made in the threads to allow for pressure release.

At the start of each collection period, 5 ml of tested high-purity 10% HCl was added to each collection bottle as a preservative. Prior to removing sample-filled collection bottles, 10 ml of milli-Q® ultrapure water was pipetted through the collection apparatus to wash out remaining sample from the filter and tubing. The sample bottle was removed and fitted with a Teflon® lined polyethylene lid for transport to the lab. The funnel, tubing and sampling lid were rinsed with 50 ml milli-Q® water and a clean sampling bottle was fitted to the collector. We used milli-Q® water for washing/rinsing

in the field, instead of dilute acid, in order to protect the integrity of the FACE experiment. Two bulk samplers, of like design, were set up outside of the experimental rings in an open area to determine rainfall Hg inputs. Particle free gloves were worn for all sample collection and clean-hands/dirty-hands procedures were used for collection and handling of samples. Throughfall from the Hg collectors was used for Hg analysis and to determine total throughfall volume.

Three dissolved organic carbon (DOC) throughfall collectors were placed in each ring, following the same collection schedule as for the Hg collectors. Collectors consisted of a 72 mm i.d. glass funnel placed directly into a 250 ml borosilicate collection bottle. Collectors were housed in PVC piping covered with fiberglass screening. When placed in the field, 2.5 ml of 10% HCl was added to each bottle. Funnels were rinsed with 50 ml milli-Q water between each collection period. Bottles were removed, capped with a Teflon® lined cap and a clean bottle was placed in the field.

Stemflow

Three stemflow collectors were placed in each of the five experimental rings at ORNL FACE on 22 July 2007. Stemflow, which is the flow of precipitation water that runs down tree branches and trunks, was collected from on 5 August, 3 September and 14 September. Stemflow collector design was based on Kolka et al. (1999). Each collector consisted of 9.6 mm i.d. Teflon tubing snugly wrapped around each tree and attached with plastic wire clips. Along the length of the tubing a wide slit was cut to create a "gutter" for collecting stemflow and the slit tubing flowed into borosilicate collection vesicles. Large particles were filtered using a plug of acid-washed glass fiber. The stemflow collectors were designed to capture an unbiased sample of stemflow but not total stemflow.

Hg quality assurance/quality control

All sample handling and analysis was conducted using rigorously tested cleaning and analytical procedures. All reagents, water and equipment were routinely analyzed.

Clean-room quality polyvinyl gloves and clean hands/dirty hands techniques were used for handling of all Hg samples and equipment.

Prior to use, borosilicate glassware (new or used for low Hg samples) was soaked in 1N HCl for 24 hours, rinsed and heated to 500^{o}C for 6-8 hours. C-flex tubing and polyethylene caps were soaked in 1 N HCl for two weeks, and Teflon tubing and fittings were leached in 6N HCl (reagent-grade) at 70-80^{o}C for 48 hours, placed in 1N HCl (high-grade) for two weeks, rinsed with milli-Q® water and dried in a class 100 unit and double bagged.

For aqueous samples, one field blank was collected for every ten samples (three throughfall and two stemflow blanks per collection period) by passing milli-Q® through the sample collector and treating the blank exactly as samples. Every tenth field sample was split into two bottles for duplicate analysis.

Sample analysis

Soils and leaves

After removal of roots, soils were passed through a two mm screen and air-dried. All soils for Hg analysis were digested using repeated additions of concentrated nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) with heating (EPA Method 3050B). Green and senescent leaves from 2005 were digested using repeated additions of HNO_3, followed by H_2O_2 and HCl (EPA Method 200.3). Leaves were dried at 60^{o}C in a Fisher Isotemp oven and homogenized using a ball mill (using acid-washed polypropylene tubes and glass grinding balls).

Hg and other metals (Al, Fe, Mn) in 2005 leaf and soil samples were analyzed using a Thermo-Finnegan Element2 Inductively Coupled Plasma Mass Spectrometer (ICP-MS), with apple leaf (NIST 1515) and San Joaquin soils (NIST 2709) as digestion standards, and river water (NIST 1643d) as an instrumental standard. Digestion recoveries for Hg in NIST 1515 were 98% (coefficient of variation, CV = 3.2%) and in NIST 2709 were 90% (CV = 5.5%). Instrument detection limits for Hg were 5 ng L^{-1}; method detection limits for Hg were 0.06 µg L^{-1} (soils) and 0.02 µg L^{-1} (leaves). All samples were run in duplicate and averaged.

Green and senescent leaves from 2007 were analyzed using a Milestone^{TM} Direct Mercury Analyzer 80 (Milestone Inc., Monroe CT; EPA Method 7473), which determines total Hg by thermal decomposition, amalgamation, and atomic absorption spectrometry. NIST 1515 and 2709 were used to confirm instrument calibration. Mean % recovery on NIST 1515 was 100 % (CV = 1.4%) and the method detection limit was 2.4 μg kg^{-1}. All samples were run in duplicate and averaged, and every tenth sample was run in triplicate. Standards were analyzed after every tenth sample to check for instrument drift.

Percent soil organic matter (SOM) was determined by the method of percent loss on ignition (eight hours combustion at 400^{o}C). Soil pH was determined in a 1:1 ratio of soil (g) to water (ml) and 1:1 ratio of soil to 0.01M CaCl. These two pH measures were highly correlated (R^2 = 0.94, P<0.01); only pH in water was used in our analyses.

Throughfall and stemflow

Within 24 hours of collection, aqueous samples were preserved in 1% BrCl and stored at 4^{o}C. Throughfall, rainwater and stemflow samples for total Hg analysis were shipped to Studio Geochimica (Seattle, WA) and analyzed by cold vapour atomic fluorescence spectrometry (CVAFS) using modified EPA Method 1631. NIST 1641d was used as a standard reference material with an average percent recovery of 99% (COV = 7.5%). Every tenth sample was run in duplicate for quality control. Average method detection limit was 0.06 ng L^{-1}.

Throughfall samples for DOC determination were filtered through a 0.45 μm filter and stored at 4^{o}C in 2% HCl until analysis. Samples were analyzed at the University of Georgia Stable Isotope/Soil Biology Laboratory using a Shimadzu TOC-5000A Total Organic Carbon Analyzer.

Estimated mercury inputs

Mercury inputs to forest soils in the United States (U.S.) were calculated based on an estimated U.S. Hg deposition rate of 87 Mg yr^{-1} (U.S. EPA 1997b). The total land area of the U.S. is nearly 2.3 billion acres and total forested land is 749 million acres, or

approximately 30% of total U.S. land area (Lubowski et al. 2006). The flux of Hg to forested areas is estimated to be four times non-forested areas (Grigal 2003). Using these values, we calculated deposition to U.S. forests of 57 Mg yr^{-1} and inputs to non-forested soils of 30 Mg yr^{-1}.

Statistical analyses

ORNL 2005 and pre-treatment soil data were analyzed with a mixed linear model analysis of variance (ANOVA; SAS 9.0) using a partly-nested design with CO_2 as the main plot factor, soil depth as the within plot factor, and ring (random) as the experimental unit for CO_2. Duke 2005 soil samples were analyzed using a partly nested design with CO_2 as the main plot factor, and N-treatment and soil depth as within plot factors. The blocked design structure of Duke FACE was removed from our statistical model because there was no block effect on soil Hg concentration ($P = 0.98$). We included samples from the N-addition sector in our soil analysis to increase our sub-sample size (and estimation of the mean). There was no effect of N-fertilization on Hg concentrations in soils ($P = 0.330$) nor was there a $CO_2 \times N$ interaction effect ($P = 0.555$), and removal of the N-fertilized soils from our analysis did not change our statistical results. We, therefore, limit our results and discussion to CO_2 effects on soil Hg.

Multiple regression analysis was used and Pearson correlation coefficients calculated to estimate the relationship between soil Hg and SOM, pH, Al, Mn, and Fe. Based on this analysis, we used the two main drivers of soil Hg concentration—SOM and pH—as covariates in an analysis of covariance (ANCOVA).

ORNL 2005 leaf data were analyzed using a partly-nested design with CO_2 as the main plot factor, canopy height as the within plot factor, and ring (random) as the experimental unit for CO_2. 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 CO_2 as the main plot factor nested in block (random). The ANOVA on Duke *P. taeda* needles had two additional within plot factors—needle age and canopy height. Litterfall Hg

deposition (both sites) was analyzed using a single factor ANOVA, with ring or block (random) as the unit of replication for CO_2.

2007 ORNL samples were analyzed using a repeated measures ANOVA to test for CO_2 effects on Hg concentrations in leaves, throughfall volume, DOC and Hg concentrations in throughfall, throughfall Hg and DOC deposition and stemflow Hg concentrations. Mauchley's W test statistic was used to test for sphericity, and when conditions of sphericity were not met, degrees of freedom of the F statistic were adjusted using the ε estimator (Huynh and Feldt 1976). A single factor ANOVA was used to test for CO_2 effects on the summed litterfall and throughfall deposition for the 2007 sampling period. A regression model was fit by method of ordinary least squares and Pearson correlation coefficients calculated to estimate the correlation between rainfall and throughfall volumes, and Hg and DOC throughfall deposition.

For the ANOVAs and ANCOVA with unequal treatment sample sizes, degrees of freedom 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), we used a probability level of 0.1 for the ANOVAs and ANCOVA, as in other FACE studies (e.g., Jastrow et al. 2005). All data were log-transformed when necessary to meet the assumptions of the statistical tests. Errors presented in the text and tables are one standard error of the mean.

Results

Soil Hg

There were significantly greater soil Hg concentrations in the elevated CO_2 rings at both Duke and ORNL FACE after nine and seven years of CO_2 enrichment, respectively (Duke: $F = 7.97$, $P = 0.05$; ORNL: $F = 9.87$, $P = 0.05$; Figure 3). Hg concentrations were 20% greater at Duke and 34% greater at ORNL in the top 20 cm of soils from elevated CO_2 plots compared to ambient plots (Duke ambient: 16.8 ± 0.9 ng Hg g^{-1} soil, elevated: 20.2 ± 0.7 ng Hg g^{-1} soil; ORNL ambient: 23.1 ± 1.6 ng Hg g^{-1}soil, elevated: 31.0 ± 1.9 ng Hg g^{-1} soil). This CO_2 effect occurred across soil depths from surface to 20 cm ($CO_2 \times$ depth, Duke: $F = 0.62$, $P = 0.61$; ORNL: $F = 1.33$, $P = 0.32$).

There were no pre-FACE treatment differences in soil Hg concentrations at either site (Duke: F = 0.94, $P = 0.39$; F = 0.01, ORNL: $P = 0.95$); therefore, observed differences in soil Hg occurred after CO_2 treatment was initiated and were not due to pre-existing soil concentrations.

One possible cause of the observed changes in soil Hg concentrations may be CO_2-mediated effects on the Hg-trapping efficiency of these soils. Soil Hg adsorption is affected by soil properties, such as percent SOM, that may be altered by elevated CO_2. At both Duke and ORNL, there was a significant relationship between Hg and percent SOM (Duke $R^2 = 0.81$, $P < 0.01$; ORNL $R^2 = 0.34$, $P < 0.01$; Figure 4a), and there was a significant relationship between Hg and pH at Duke ($R^2 = 0.33$, $P < 0.01$). There was not a significant soil Hg-pH relationship at ORNL, but soil Hg concentrations and soil pH were correlated across both sites ($R^2 = 0.33$, $P < 0.01$; Figure 4b). SOM and pH explained 76% of the variation in soil Hg across sites and CO_2 treatments (multiple regression, $R^2 = 0.76$, $P<0.01$). Therefore, changes in percent SOM at Duke (ambient: 4.0 ± 0.4%; elevated: 4.5 ± 0.1%; F = 16.54, $P = 0.02$) and ORNL (ambient: 3.9 ± 0.5; elevated: 4.1 ± 0.4; F = 0.74, $P = 0.45$), and acidity at Duke (ambient: 5.36 ± 0.04; elevated: 5.33 ± 0.1; F = 0.18, $P = 0.69$) and ORNL (ambient: 4.85 ± 0.18; elevated: 4.72 ± 0.08; F = 0.84, $P = 0.43$) under elevated CO_2, even when not statistically significant in and of themselves, may be affecting soil Hg concentrations.

To test the hypothesis that CO_2-mediated effects on soil properties are driving changes in soil Hg concentrations, we used percent SOM and pH as covariates in our statistical model. This covariance test removes the variation in soil Hg that is correlated with SOM and pH. When SOM and pH were included as covariates at Duke, there were no differences in adjusted Hg concentrations between CO_2 treatments (analysis of covariance, ANCOVA: F = 1.52, $P = 0.29$), suggesting that CO_2 effects on Duke soil Hg are mediated by SOM and pH. However, SOM alone as a covariate provided similar results (F = 1.53, $P = 0.28$). While pH does account for some of the variation in soil Hg at Duke, it appears that SOM is the main driver of CO_2 effects on soil Hg. At ORNL, we used SOM alone as a covariate since there was no detected pH-soil Hg relationship. The effect of elevated CO_2 on SOM-adjusted soil Hg was still significant (F = 16.32, $P =$

0.09), suggesting that there are other CO_2 effects, independent of SOM, influencing Hg cycling at ORNL.

Green leaf Hg concentrations

L. styraciflua green leaf Hg concentrations in 2005 were lower, but not significantly different under elevated CO_2 treatment relative to ambient, at both Duke ($F = 0.43$, $P = 0.55$) and ORNL FACE ($F = 0.45$, $P = 0.55$; Figure 5a). *P. taeda* needle Hg concentrations were also lower with CO_2 enrichment, but not significantly different ($F = 7.84$, $P = 0.11$). *P. taeda* foliar Hg concentrations were significantly lower in 0-yr than in 1-yr needles ($F = 32.14$, $P < 0.01$; Figure 5a). There was no needle age × CO_2 interaction ($F = 0.61$, $P = 0.44$).

There were no detected effects of canopy height on *L. styraciflua* leaves at ORNL in 2005 ($F = 0.51$, $P = 0.62$). At Duke FACE there was a significant CO_2 × canopy interaction effect ($F = 2.70$, $P = 0.07$) on *P. taeda* foliar Hg concentrations. Foliar Hg concentrations were lower under elevated CO_2 relative to ambient CO_2 in the lower and mid canopy for 1-yr needles but not in the upper canopy (Figure 6). There were no canopy height effects on 0-yr needles (Figure 6).

In 2007 we collected *L. styraciflua* leaves at ORNL throughout the growing season. There was a significant increase in foliar Hg with time ($F = 180.97$, $P < 0.01$) but no significant difference between CO_2 treatments ($F = 1.65$, $P = 0.29$) nor a time × CO_2 interaction ($F = 0.26$, $P < 0.90$). Hg concentrations in the elevated CO_2 plots were slightly lower that in the ambient plots across all measurement periods (Figure 5b).

Litterfall Hg concentrations and deposition

Concentrations of Hg in 2005 litterfall were lower in the elevated CO_2 rings at both Duke ($F = 9.27$, $P = 0.09$) and ORNL (non-significant; $F = 3.37$ $P = 0.16$; Table 1). There was an increase in 2005 litterfall biomass under elevated CO_2 at both sites (Duke: $F = 77.85$, $P = 0.01$; ORNL: $F = 5.89$, $P = 0.09$; Table 1) but there was no significant effect of elevated CO_2 on the total litterfall Hg deposition at either site (Duke: $F = 1.04$, $P = 0.42$; ORNL: $F = 2.08$, $P = 0.25$, Table 1). Litterfall Hg deposition was greater at Duke

than at ORNL (Table 1) because leaf litter Hg concentrations in the most common tree at Duke, *P. taeda*, were greater by a factor of eight than concentrations in the predominant species at ORNL, *L. styraciflua.* This difference in Hg concentrations may be due, in part, to differences in leaf life span between these two species; *P. taeda* needle longevity is 18 months while *L. styraciflua* leaf longevity is less than six months.

We collected litterfall at ORNL in 2007 and found similar results to 2005. Litterfall biomass was greater under elevated CO_2 than ambient ($F = 33.74$, $P = 0.01$; Table 1) but there was no CO_2 effect on litterfall Hg deposition ($F = 0.01$, $P = 0.97$) because Hg concentrations in senescent leaves were (non-significantly) lower with CO_2 enrichment ($F = 0.37$, $P = 0.59$; Figure 5b, Table 1). Litterfall Hg concentrations were five times greater in 2007 compared to 2005 values. This may be due to differences in rainfall between the two years and differences in sampling methods and collection dates. In October 2005 we collected freshly fallen leaves off the forest floor, while in November 2007 we collected senescent leaves from the canopy by shaking individual trees.

Throughfall and stemflow

Throughfall and rainfall amounts at ORNL from June through September 2007 were significantly correlated ($R^2 = 0.97$, $P < 0.01$; Figure 7a), with an average canopy interception of 10% across sampling periods. Throughfall volume varied across sampling dates ($F = 0.01$, $P = 0.97$) but was not affected by CO_2 treatment ($F = 0.01$, $P = 0.97$). The total amount of throughfall during the 2007 collection period also did not differ between the ambient and elevated treatments ($F = 0.01$, $P = 0.97$; Table 1).

There was a significant effect of sampling date on throughfall Hg concentrations ($F = 136.41$, $P < 0.01$) but no CO_2 effect ($F = 0.12$, $P = 0.75$; Figure 8a). Throughfall Hg deposition also varied across sampling periods ($F = 88.68$, $P < 0.01$) but was not affected by CO_2 treatment ($F = 0.16$, $P = 0.71$; Figure 8b). There was no CO_2 effect on throughfall Hg deposition summed across all collection dates ($F = 0.05$, $P = 0.84$; Table3).

There was a significant effect of sampling time ($F = 7.59$, $P < 0.01$) and CO_2 treatment on DOC concentrations in throughfall ($F = 13.49$, $P = 0.03$; Figure 8c), with

lower DOC concentrations in the elevated rings during all but one sampling period. CO_2 × time was not statistically significant (F = .91, P = 0.49). DOC throughfall deposition varied across sampling periods (F = 10.33, $P < 0.01$) but was not affected by CO_2 treatment (F = 1.76, P = 0.28; Figure 8d). Total DOC throughfall inputs summed across all sampling periods also did not differ between the ambient (0.86 ± 0.04 g m^{-2}) and elevated rings (0.78 ± 0.05 g m^{-2}; F = 1.76, P = 0.28). Average DOC concentrations in throughfall (4.8 ± 0.4 mg L^{-1}) were slightly more than 2.5 times concentrations in rainwater (1.8 ± 0.2 mg L^{-1}).

There was a positive correlation between throughfall Hg deposition and DOC deposition (R^2 = 0.14, P = 0.06). The strength of this correlation markedly increased (R^2 = 0.60, $P < 0.01$) with the removal of the final collection period, when Hg inputs were low relative to DOC inputs (Figure 7b).

Stemflow Hg concentrations differed among sampling periods (F = 5.08, P = 0.07) but again there was no CO_2 effect (F = 1.12, P = 0.37). Across all sampling periods and both CO_2 treatments, Hg concentrations in stemflow (54.27 ± 3.17 ng L^{-1}) were three times greater than concentrations in throughfall (18.37 ± 1.18 ng L^{-1}) and 5.5 times greater than concentration in rainwater (9.75 ± 0.58 ng L^{-1}). While we did not measure total stemflow amount, based on previous studies (Kolka et al. 1999) we expect stemflow inputs to this stand to be about 2-3% of open precipitation, which was 240 mm during the 2007 sampling period (Riggs et al. 2007). Estimated stemflow Hg deposition would therefore be about 0.25-0.39 μg m^{-2} sampling $season^{-1}$ or 10% of throughfall Hg deposition.

Discussion

CO_2 effects on soil Hg

The observed CO_2 effect on soil Hg concentrations—which were 20% greater with CO_2 enrichment at Duke and 34% greater at ORNL (Figure 3)—was likely driven by increased soil retention rather than increased litter, throughfall or stemflow Hg deposition. Our soil pH and SOM data support the hypothesis that CO_2-mediated changes in these two factors [note that changes in these factors have been found in other elevated CO_2

experiments (Andrews and Schlesinger 2001; Jastrow et al. 2005; Oh and Richter 2004)] underlie this higher retention. Our hypothesis regarding the Hg-trapping efficiency of soils under high CO_2 is consistent with previous studies of the impact of SOM and pH on soil Hg adsorption (Schuster 1991; Yin et al. 1996). For example, maximum Hg adsorption in soils has been shown to occur at low pH (range of 3-5) when SOM is present (Schuster 1991; Yin et al. 1996); SOM is thus a key determinant of Hg soil-binding capacity (Schuster 1991; Yin et al. 1996). In addition, soil pH affects the physical fractionation of SOM (dissolved versus adsorbed), which ultimately determines the fate of soil Hg (Schuster 1991; Yin et al. 1996). We found that soil Hg concentrations were significantly correlated with SOM at both sites, and with pH at Duke and across sites (Figure 4). The results of our ANCOVA, particularly at Duke, further support the hypothesis that CO_2-mediated changes in SOM are driving changes in soil Hg concentrations.

While the soils used in this study are from relatively uncontaminated forests, we expect increasing atmospheric CO_2 to affect soil Hg at a range of concentrations and soil types due to the strong relationship between CO_2 and SOM and the relationship between SOM and Hg (Grigal 2003; Schuster 1991). Changes on a global-scale that can affect soil Hg, even at low Hg-levels, will have a large overall effect on global Hg fluxes and pools. For example, in the United States, the estimated amount of Hg stored in the forest floor is 1350 Mg (Grigal 2003) and the deposition rate to forests is approximately 57 Mg yr^{-1} (details of this deposition estimate can be found in the Methods section). The increase in Hg storage under elevated CO_2 found in this study (27% over 8 years, or 3.4% yr^{-1}) suggests that greater Hg storage in forests under elevated CO_2 is on the same order of magnitude as the annual Hg deposition of 57 Mg to these forests and thus may have strong impacts on the dynamics of Hg in terrestrial ecosystems.

While these results are congruent with the retention hypothesis, other soil factors that may be affected by atmospheric CO_2 concentrations could also be at play. For example, in addition to the above-described pH and SOM impacts, Hg retention can vary with incident solar radiation, soil temperature, and soil moisture (Carpi and Lindberg 1998; Gustin and Stamenkovic 2005). The CO_2 enrichment treatment itself is unlikely to

be a source of added Hg to soils in the elevated rings because green leaf, litter and throughfall Hg concentrations in the elevated rings were all lower or similar to concentrations in the ambient-CO_2 rings.

CO_2 effects on leaf and litter Hg concentrations

There was a trend of decreased leaf and litter Hg concentrations in the elevated CO_2 rings in both species and sites. This decrease may have been caused by growth dilution effects (that is, increased carbohydrate accumulation/leaf density; Loladze 2002). If leaf density changes are an important driver of foliar Hg concentrations, we would expect to see concentration differences in the upper canopy because changes in leaf density with CO_2 enrichment are generally more pronounced in upper canopy leaves (Norby and Iversen 2006). However, there were no canopy effects on foliar Hg concentrations in *L. styraciflua* at ORNL and none in the upper canopy in *P. taeda* (Figure 6).

One potential source of Hg to canopy leaves is volatile emissions from soils. Elevated CO_2 may alter soil Hg fluxes through a CO_2-mediated decrease in forest floor light levels or increased adsorption of soil Hg to soil organic matter, both of which have been shown to lower volatile Hg fluxes (Carpi and Lindberg 1998; Gabriel and Williamson 2004; Gustin and Stamenkovic 2005). If soil Hg emissions were an important source of Hg to leaves in these forests then we would expect Hg concentrations in the lower canopy to reflect this source. Hg concentrations in *P. taeda* lower canopy leaves were higher relative to upper canopy in the ambient but not in the elevated plots, suggesting that soil fluxes may be greater under ambient CO_2 conditions than elevated CO_2 at Duke FACE. At ORNL, however, we found no canopy effect on foliar Hg concentrations.

Decreased stomatal conductance with CO_2 enrichment has also been shown to affect foliar Hg uptake (Millhollen et al. 2006); however, the detected patterns of foliar Hg concentrations in these forests were not congruent with CO_2-mediated changes in conductance (Ellsworth 1999; Wullschleger et al. 2002). Beyond CO_2 effects, our leaf concentration data support several other Hg studies, which report greater foliar Hg concentrations in evergreen versus broadleaf deciduous trees (Rasmussen et al. 1991;

Figure 5a), increased Hg during the growing season (Rasmussen 1995; Rea et al. 2002; Figure 5b), and increased Hg concentrations with needle age ((Fleck et al. 1999; Rasmussen 1995; Figure 6).

CO_2 effects on Hg deposition

The forest canopy provides a means of capturing atmospheric Hg and depositing it to soils, primarily through litterfall. While there was an increase in litterfall biomass under elevated CO_2, there was no change in litterfall Hg deposition because litter Hg concentrations decreased with CO_2 enrichment (Table 1). Potential changes in leaf area index (LAI, leaf area per unit ground area) with increased CO_2 may be a more important factor affecting the uptake of Hg by the forest canopy than changes in biomass. However, at ORNL, elevated CO_2 has had no effect on LAI (Norby et al. 2003; Norby et al. 2001) and reported effects at Duke have been small and variable (DeLucia et al. 2002; Lichter et al. 2000).

While litterfall Hg deposition may represent the greatest flux of Hg to forest soils (Lichter et al. 2000; St Louis et al. 2001), throughfall Hg deposition also is an important source of soil Hg (Iverfeldt 1991; Munthe et al. 1995; Rea et al. 2001; Rea et al. 2002). Throughfall, which is the flow of precipitation water through the forest canopy, can be affected by canopy architecture, leaf area and rainfall intensity (Lovett et al. 1996; Whelan and Anderson 1996). Lichter et al. (2000) found that elevated CO_2 increased throughfall volume and caused a significant increase in DOC throughfall deposition, attributed to increased soluble carbon in foliage grown in elevated CO_2. Because of the strong relationship between Hg and DOC (Kolka et al. 1999), we hypothesized that throughfall Hg may also increase with elevated CO_2. As in Kolka et al. (1999) we found a significant relationship between throughfall DOC and throughfall Hg (Figure 7b). While there was a significant CO_2 effect on DOC throughfall concentrations, concentrations were lower with CO_2 enrichment during most sampling periods (Figure 8c). There was no effect of elevated CO_2 on throughfall DOC or Hg deposition throughout the 2007 sampling period at ORNL FACE (Figures 8b and 8d). Stemflow Hg deposition also was not affected by elevated CO_2.

Our litter, throughfall and stemflow deposition data allow us to reject the hypothesis that higher soil Hg concentrations in elevated CO_2 soils were driven by increased deposition. These data support the hypothesis that CO_2-mediated changes in soil properties are increasing the Hg storage capacity of forest soils. One challenge of conducting research at large-scale multi-user facilities such as the FACE sites is the restrictions on sample size. The fact that a CO_2 effect on soil Hg was detected, in spite of this limitation, suggests that the observed response is robust, but future studies at other FACE would strengthen these conclusions.

Conclusions

These results highlight the wide-reaching effects of elevated CO_2—well beyond the traditional focus on carbon, water and nutrient cycles. An interesting challenge for further research lies in the differences observed between the two sites. While CO_2-mediated changes in percent SOM were linked to changes in soil Hg concentrations at Duke and ORNL, there seems to be an additional CO_2 effect on soil Hg at ORNL, which is not driven by deposition. As the two sites differ in soil properties, dominant plant species and soil Hg concentrations, it is not surprising that there is variation in the CO_2 effect on soil Hg. This study is a first-step in looking at CO_2 effects on Hg cycling in terrestrial ecosystems. A crucial area for future research includes global change effect on Hg speciation and methylation processes.

We expect that other global change factors that affect ecosystem carbon pools (*e.g.*, changes in temperature and tropospheric ozone concentration) may also affect terrestrial Hg fluxes. Increasing atmospheric CO_2 concentrations have the potential to lead to significant changes in the terrestrial component of the Hg cycle and these changes are likely to translate to shifts in the global biogeochemistry of Hg. These results suggest that rising atmospheric levels of CO_2 may be changing the terrestrial biogeochemistry of Hg by altering the Hg storage capacity of soils. On a broader scale, it is clear that the effects of elevated CO_2 extend beyond the carbon, water and nutrient cycles of ecosystems.

Tables

Table 1 Mean (±SE) annual litterfall (LF) biomass, litterfall Hg concentrations and annual litterfall Hg deposition at Duke (2005) and ORNL (2005 and 2007); mean throughfall (TF) volume and throughfall Hg deposition at ORNL during the 2007 sampling period, which ran from 9 June through 14 September 2007. Ambient and elevated refer to CO_2 concentrations.

	LF biomass ($g\ m^{-2}\ yr^{-1}$)	LF Hg concentration ($ng\ g^{-1}$)	LF Hg deposition ($\mu g\ m^{-2}\ yr^{-1}$)	TF amount (mm)	TF Hg deposition ($\mu g\ m^{-2}$)
2005					
Duke Ambient	611.7 ± 48.2	23.9 ± 1.0	14.6 ± 1.4		
Duke Elevated	783.6 ±61.4	20.2 ± 0.7	15.9 ± 1.8		
ORNL Ambient	497.6 ± 16.0	5.0 ± 0.9	2.5 ± 0.5		
ORNL Elevated	554.7 ± 15.5	2.6 ± 0.9	1.4 ± 0.5		
2007					
ORNL Ambient	447.7 ± 4.2	20.1 ±1.5	9.0 ± 0.6	21.6 ± 0.3	3.9 ± 0.1
ORNL Elevated	479.4 ± 0.6	18.8 ± 1.1	9.0 ± 0.6	21.7 ± 0.3	4.0 ± 0.1

Figures

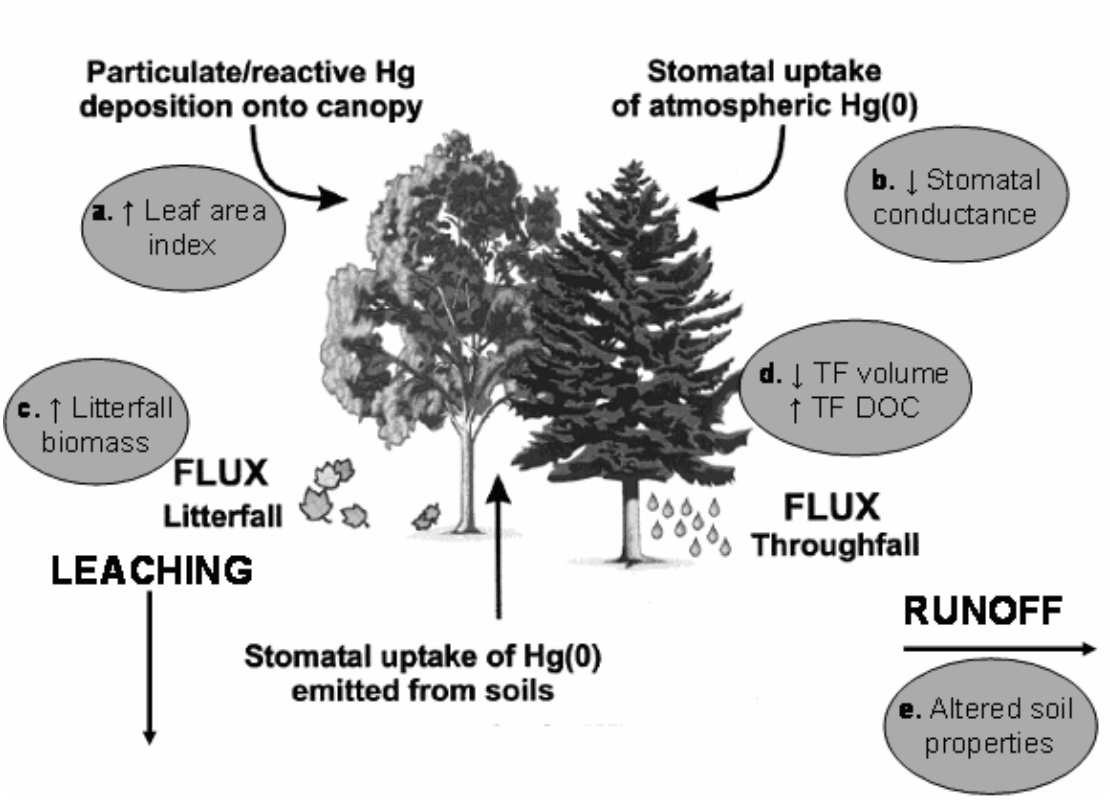

Figure 1. **Potential effects of elevated CO_2 on Hg cycling in forests.** CO_2 mediated changes in canopy structure (a) and function (b) may affect the amount of Hg deposited to foliage from the atmosphere. Fluxes from the canopy to the forest floor may also be affected by CO_2- mediated changes in litterfall biomass (c) and throughfall chemistry/volume (d). Changes in soil properties (e), such as pH and soil organic matter concentrations, can affect Hg storage in soils, and Hg lost via runoff, leaching and gaseous emissions. Potential CO_2 effects on forest structure and function are circled, and further described in the text. Figure adapted from St. Louis et al. (2001).

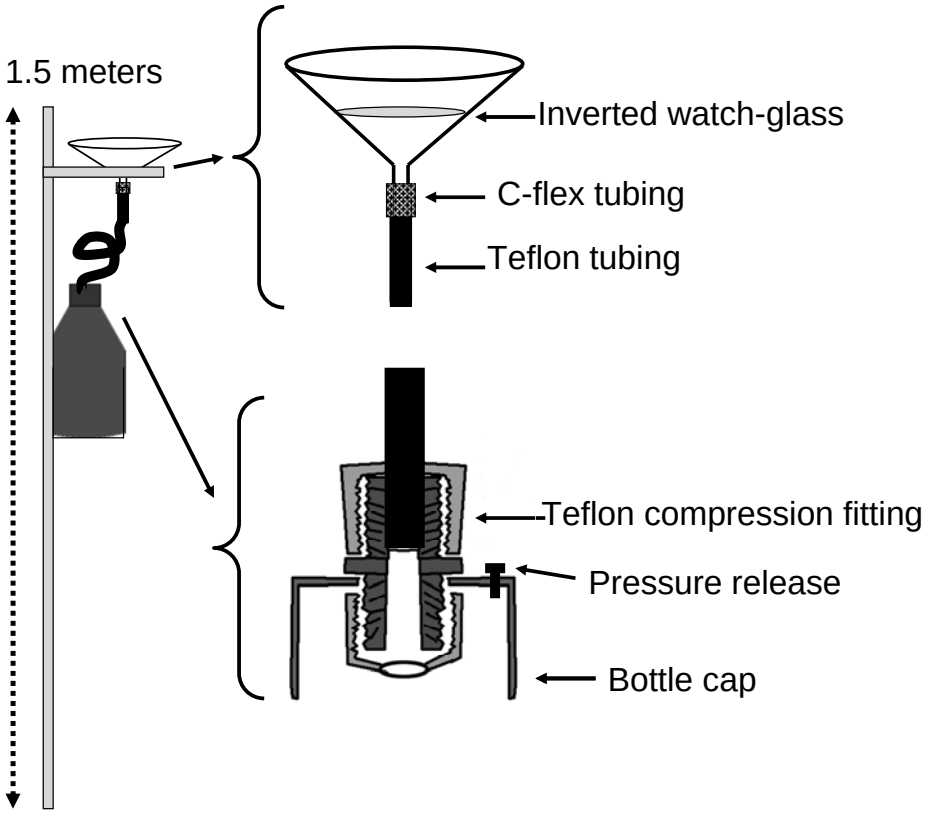

Figure 2. Bulk throughfall collectors used at ORNL FACE in 2007. Six Hg throughfall collectors were set up in each of the five experimental CO_2 rings. Samples were collected five times throughout the 2007 growing season.

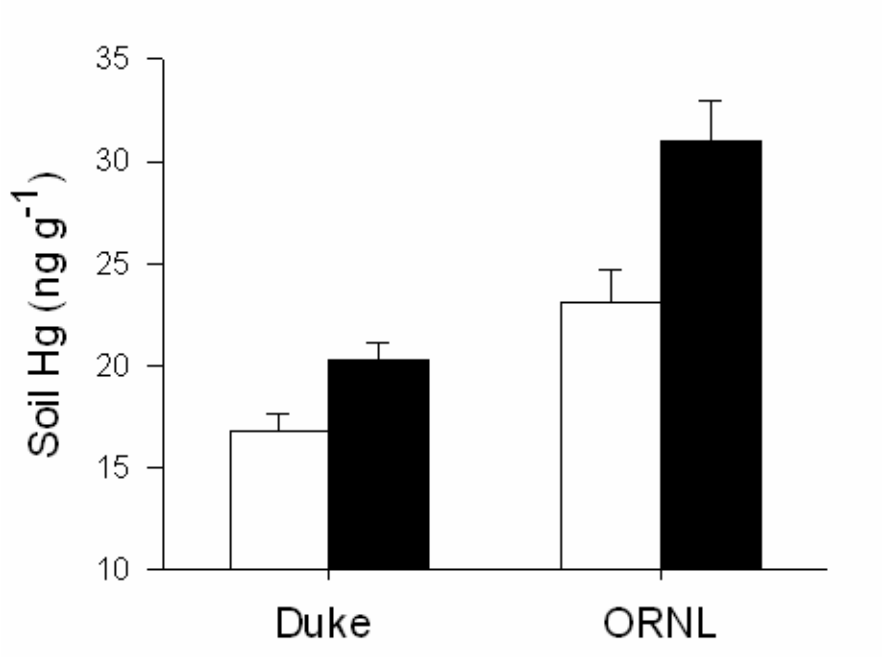

Figure 3. Soil Hg concentrations in the top 20 cm of soils were significantly greater ($p<0.1$) in the elevated CO_2 plots at both Duke and ORNL FACE. Points represent least squares means (± SE). Elevated CO_2 plots are represented by filled bars and ambient plots by open bars.

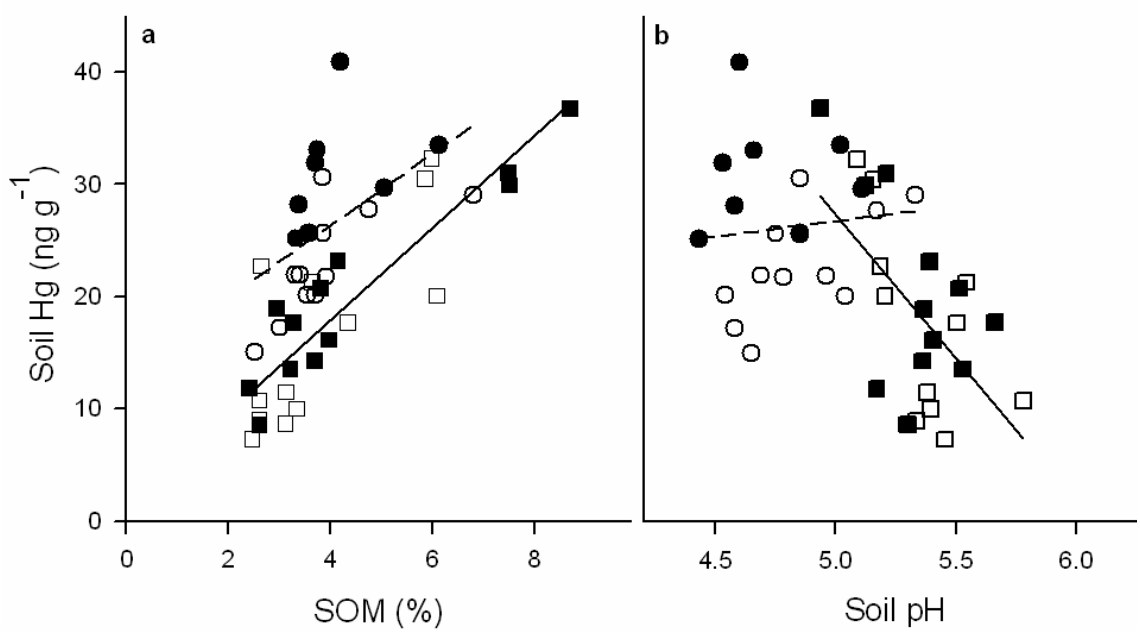

Figure 4 Hg concentrations in soils (0-20 cm) were correlated with **a.)** percent SOM at Duke and ORNL, and **b.)** pH at Duke ($P<0.1$). Dashed lines and circles represent ORNL; solid lines and squares represent Duke. Open symbols represent ambient CO_2 samples and closed, elevated. Points represent five cm depth increments within each ring.

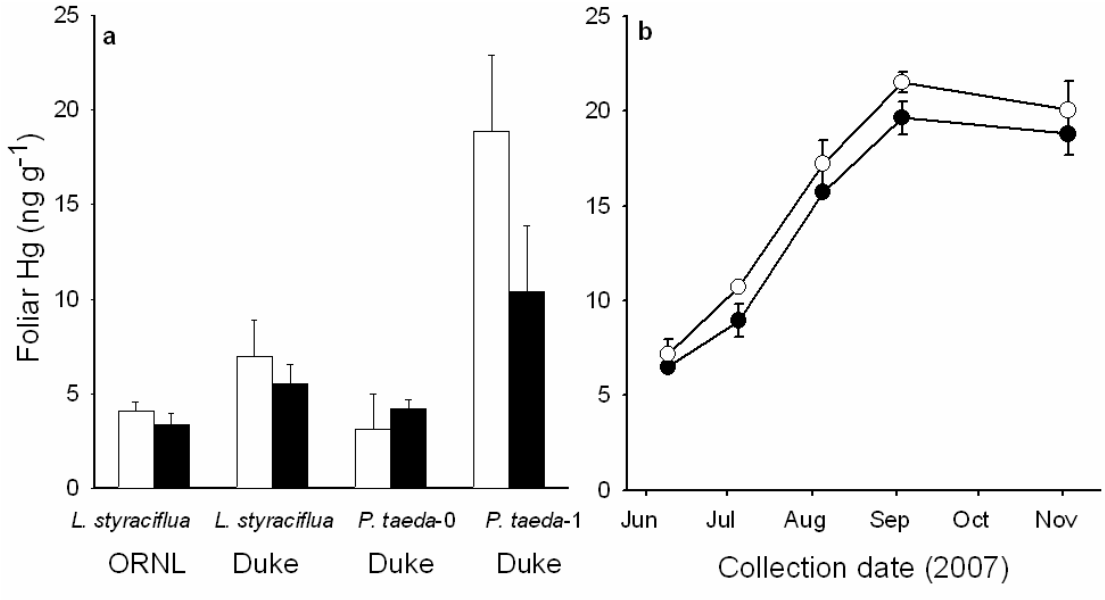

Figure 5 a. Least squares mean (± SE) Hg concentrations in 2005 green leaves from *L. styraciflua* at ORNL and Duke, and 0-yr and 1-yr *P. taeda* needles at Duke averaged across canopy heights. **b.** Hg concentrations in *L. styraciflua* green and senescent (last sampling date) leaves collected throughout the 2007 growing season at ORNL. Open symbols represent ambient CO_2 and closed, elevated.

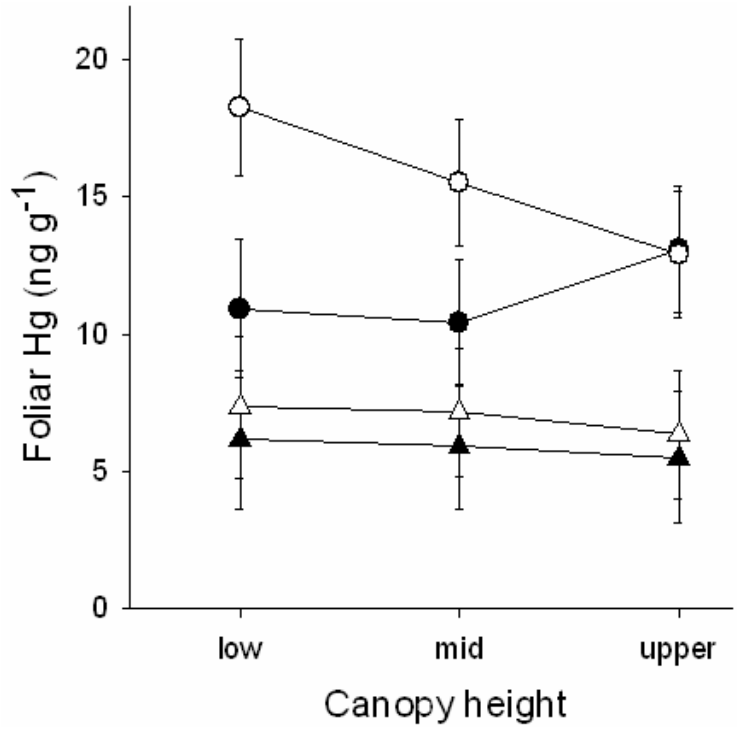

Figure 6 There was a significant CO2 × canopy height interaction effect (P<0.1) on Hg concentrations in *P. taeda* 1-year needles (circles) but not 0-year needles (triangles) across low (10-12 m), mid (12-14 m) and upper (14-16 m) canopy heights. Elevated CO_2 rings are represented by filled symbols and ambient rings by open symbols (least squares means ± SE).

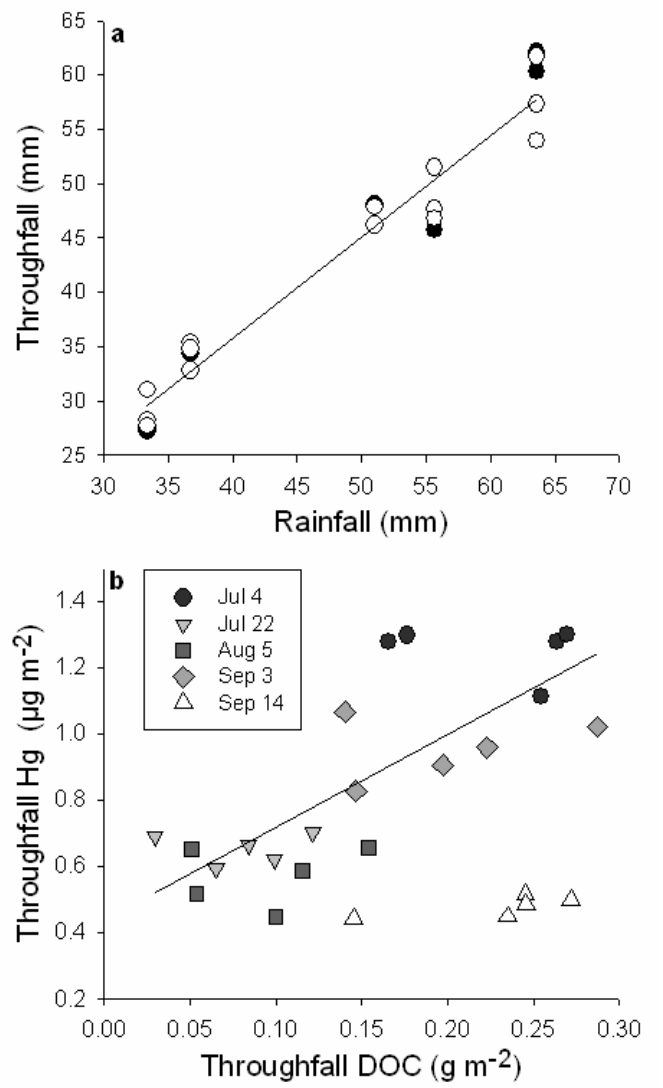

Figure 7 a. There was a significant relationship between rainfall and throughfall volume at ORNL FACE from June through September 2007 ($p<0.1$). Symbols represent throughfall volume in each experimental ring (elevated CO_2, filled symbols; ambient CO_2, open symbols) and rainfall volumes during each of the five collection periods. Rainfall data from Riggs et al. (2007). **b.** There was also a significant relationship between DOC and Hg throughfall deposition ($p<0.1$). Regression line does not include final sampling period (3-14 September). Dates in the legend are collection dates and symbols represent total deposition per ring during each collection period. The first collection began on 9 June 2007.

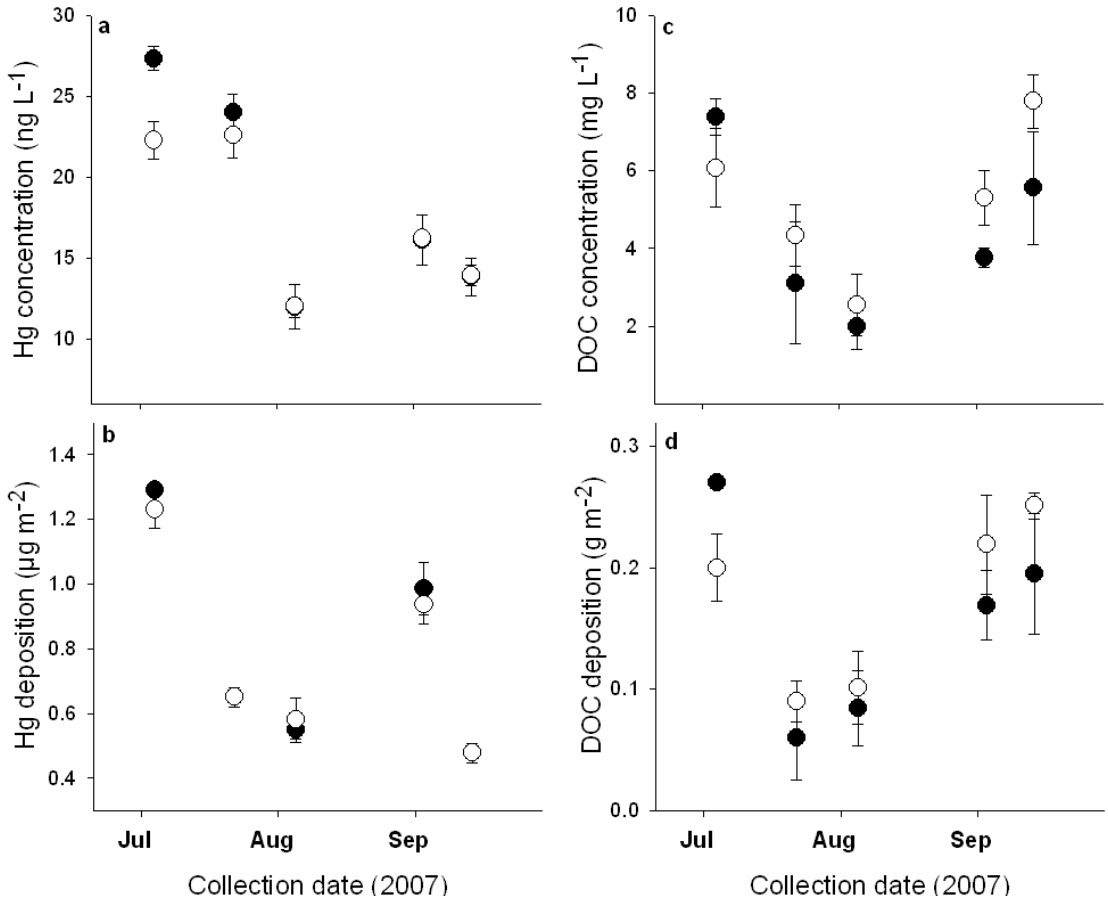

Figure 8 There was a significant effect of sampling date (P<0.1) on throughfall **a.** Hg concentrations, **b.** Hg deposition, **c.** DOC concentrations and **d.** DOC deposition. The only significant effect of CO_2 treatment was on DOC concentrations (p<0.1). Sample collection began on 9 June and was collected on the following dates in 2007 [rainfall totals during each collection period (Riggs et al. 2007) in parentheses]: 4 July (51.0 mm), 22 July (33.4 mm), 5 August (55.7 mm), 3 September (63.6), 14 September (36.7 mm).

Chapter 4: Effects of elevated carbon dioxide and nitrogen fertilization on nitrate reductase activity in sweetgum and loblolly pine trees in two temperate forests

Introduction

The ability of plants to acquire and assimilate nitrogen (N) is an important determinant of plant response to elevated CO_2 and of ecosystem carbon sequestration (Hungate et al. 2003; Luo et al. 2004). The response of forests to increasing atmospheric CO_2 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 (NO_3^-) or ammonium (NH_4^+) as their primary source of inorganic N (Templer and Dawson 2004). Although the energetic costs of NO_3^- utilization (~2.4 g glucose g^{-1} protein produced) are higher than NH_4^+ (~1.8 g glucose g^{-1} protein; Zerihun et al. 1998), NO_3^- 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 systems with relatively low levels of soil NO_3^-, the spatial and temporal heterogeneity of inorganic N (Garbin et al. 2006; Jackson and Caldwell 1993), the potential for species-level partitioning of N chemical forms (McKane et al. 2002; Miller and Bowman 2002; Reynolds et al. 1997), and the accessibility of soil NO_3^- (due to its high solubility and mobility), make NO_3^- an important component of plant and ecosystem N dynamics.

Once taken up by plants, NO_3^- can be reduced in roots or in leaves. Both above and belowground assimilation processes may be altered by increasing concentrations of atmospheric CO_2. Under high light conditions, NO_3^- reduction in leaves can provide a sink for excess NADH not used in carbon assimilation (Guo et al. 2007). However, foliar NO_3^- reduction may compete for reductant with Calvin cycle reactions when light levels

are low (Huppe and Turpin 1994) or when carbon assimilation is increased under elevated CO_2 (Bloom et al. 1989; Bloom et al. 2002). Because of this direct competitive effect between NO_3^- reduction and carbon assimilation, foliar nitrate reductase activity (NaR) may decrease with CO_2 enrichment, and species that rely on NO_3^- may be competitively disadvantaged under elevated CO_2 unless they can increase NH_4^+ acquisition (Smart et al. 1998).

In addition to this direct effect on plant NaR (i.e., competition for reductant), elevated CO_2 may also affect NaR through impacts on soil properties. Molybdenum (Mo) is a plant micronutrient that serves as a cofactor for nitrate reductase—the enzyme that catalyzes the reduction of NO_3^- to nitrite (NO_2^-). 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 CO_2 enrichment because soil organic matter (SOM) has been shown to increase under elevated CO_2 (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 CO_2 because CO_2 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).

While a large number of recent studies show a decrease in foliar NaR activity with CO_2 enrichment (Bloom et al. 2002; Constable et al. 2001; Searles and Bloom 2003; Smart et al. 1998), CO_2 effects on NaR have been variable across species and studies. For example, in a greenhouse study of loblolly pine (*Pinus taeda*) and sweetgum (*Liquidambar styraciflua*) seedlings, Constable et al. (2001) found that CO_2 enrichment increased root and foliar NaR activity in *L. styraciflua*, but had no effect on NaR activity in leaves or roots of *P. taeda*. It is uncertain, however, how elevated CO_2 will affect NaR in field conditions, where CO_2 can potentially have both direct effects on foliar NaR via

competition for reductant, as well as indirect effects through CO_2-mediated changes in soil nutrient dynamics.

In this study, we looked at CO_2 effects on foliar NaR in *P. taeda* and *L. styraciflua* in long-term field experiments. We tested the following hypotheses about the effects of elevated CO_2 and N-fertilization on foliar NaR in forest trees:

i.) Elevated CO_2 may *directly* limit NaR via competition for reductant between NO_3^- assimilation and photosynthesis. If foliar NaR is limited by reductant, we expect lower NaR in low light conditions (lower canopy leaves) across CO_2 treatments, and a decrease in NaR with CO_2 enrichment, particularly in the upper canopy.

ii.) Elevated CO_2 may *indirectly* affect NaR through soil-mediated effects. We expect that changes in soil properties (e.g. SOM and pH) with CO_2 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.

iii.) Fertilization with NH_4NO_3 will decrease NaR activity in *L. styraciflua* and *P. taeda* because both species have a greater capacity for uptake of NH_4^+ than NO_3^- (Constable et al. 2001), and we expect preferential uptake of NH_4^+ when both N forms are available.

We tested these hypotheses 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 (ORNL)—which have been exposed to CO_2 enrichment (ambient + 200 ppmv) since 1996 and 1998, respectively. An experimental N treatment was added to half of each CO_2 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.

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 m × 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 (~382 ppmv) and three elevated rings (~582 ppmv). The 30 m diameter experimental rings are arranged in a complete block design to account for topographic variation and potential fertility gradients. The CO_2 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 CO_2-fumigated air to maintain ambient or elevated levels of CO_2 into the rings (Hendrey et al. 1999). An experimental N treatment—which consisted of the addition of $NH_4^+NO_3^-$ (5.6 g N m^{-2} yr^{-1})—was added to soil in 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, Tennessee (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 one-year-old sweetgum seedlings in 1988 at a spacing of 1.2 m × 2.3 m. The FACE apparatus (i.e., the CO_2 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 ppmv) rings and two enriched (~ 549 ppmv) rings. CO_2 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 were sampled for elemental analysis from three canopy heights—low (10-12m), mid (12-14m) and upper (14-16m)—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.* In each canopy height three replicate samples were collected; each of these replicate sub-samples consisted of approximately 5 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 place in liquid N_2 upon collection and stored at -80^{o}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 0.2*N* 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 hours at 60^{o} 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 (HNO_3) and hydrogen peroxide (H_2O_2) with heating (EPA Method 3050B). Leaves were digested using repeated additions of HNO_3, followed by H_2O_2 and HCl (EPA Method 200.3). 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 CO_2 and N enrichment.

Mo and other metals (Al, Pb, Fe and Cu) were analyzed using a Thermo-Finnegan Element2 Inductively Coupled Plasma Mass Spectrometer (ICP-MS), 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. Percent soil organic matter (SOM) was determined by the method of percent loss on ignition (8 hours combustion at 400^{o}C). Soil pH was determined in a 1:1 ratio of soil (g) to water (ml) and 1:1 ratio of soil to 0.01M CaCl. These two pH measures were highly correlated ($R^2 = 0.943$, $P<0.01$) 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 KH_2PO_4, 1 mM EDTA, pH 7.5, with 1% (w/v) BSA, 2% (w/v) casein, 0.1% (w/v) cysteine, 5 mM dithiothreitol, 20 μM FAD, 25 μM leupeptin, 5 μM Na_2MoO_4, 0.5 mM phenylmethylsulfonyl fluoride, 1% (w/v) polyvinylpolypyrrolidone, 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 ten minutes. To determine nitrate reductase activity, 200 μl of the supernatant was added to 500 μl assay buffer (25 mM KH_2PO_4, 0.025 EDTA, pH 7.5) and 0.2 mM NADH, and incubated at 30°C for various times up to five minutes. The reaction was stopped by the addition of 50 μl of 1 M ZnAc and the solution centrifuged at 10 000 rpm for five minutes. The nitrite (NO_2^-) 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 minute incubation the absorption of the sample solution at 540 nm was compared to standards of known NO_2^- 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. Nitrate reductase activity is reported in units of NO_2^- 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) with CO_2 as the main plot factor, canopy height as the within plot factor, and ring (random) as the experimental unit for CO_2. By 'canopy height', we refer to the position of the sampled leaf in the forest canopy. 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 CO_2 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 CO_2 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 CO_2, N (Duke), depth and interaction effects, with ring or block as the unit of replication for CO_2 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 comparisons. 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 CO_2 (that is, CO_2-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, degrees of freedom 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

Nitrate reductase activity in leaves

At Duke FACE there was a significant $CO_2 \times$ age interaction effect ($F = 10.10$, $P = 0.03$) on *P. taeda* NaR. In 1-yr needles, NaR was significantly lower in the elevated plots compared to ambient ($t = 3.95$, $P = 0.05$; Figure 1a) but there was no CO_2 effect in 0-yr-old needles ($t = 0.25$, $P = 0.99$) where NaR was low under both CO_2 treatments (Figure 2a; Tables 1-2). There was also a significant N × age interaction ($F = 8.54$, $P = 0.02$),

again driven by N-fertilization effects on NaR in 1-yr needles ($t = 5.10$, $P < 0.01$; Figure 1a), but not in 0-yr needles ($t = 1.41$, $P = 0.53$) where NaR was low across both N-treatments (Figure 2b, Tables 1-2). NaR was three times greater in 1-yr needles than 0-yr needles across treatments at Duke FACE ($F = 71.21$, $P < 0.01$; Tables 1-2).

Canopy height effects on *P. taeda* NaR were not significant across treatments ($F = 0.24$, $P = 0.65$) and there was no detected CO_2 × height interaction ($F = 0.49$, $P = 0.49$; Figure 3a). There was, however, a significant N × canopy height interaction ($F = 9.22$, $P = 0.02$) because N-fertilization significantly decreased NaR in the lower canopy ($t = 4.99$, $P = 0.01$) but not in the upper canopy ($t = 1.27$, $P = 0.61$; Figure 3b).

There was no effect of CO_2 on *L. styraciflua* NaR at either Duke ($F = 0.07$, $P = 0.82$) or ORNL ($F = 0.87$, $P = 0.37$, Figure 1a, Tables 1-2). There also was no effect of N-fertilization ($F = 0.54$, $P = 0.54$) or N × CO_2 interactions ($F = 0.01$, $P = 0.99$) on *L. styraciflua* NaR at Duke. At ORNL, there was no detected effect of canopy height ($F = 0.01$, $P = 0.93$) or canopy height × CO_2 interaction ($F = 2.24$, $P = 0.16$).

Mo concentrations in leaves

P. taeda Mo concentrations in 1-yr needles (Figure 1b) and 0-yr needles in the elevated CO_2 rings were lower but not significantly different from concentrations in the ambient rings ($F = 4.50$, $P = 0.17$). However, when all non-significant interactions terms were removed from the ANOVA model, there was a marginally significant decline in foliar Mo with CO_2 enrichment in *P. taeda* leaves ($F = 8.63$, $P = 0.10$). Like NaR, Mo concentrations were significantly lower in 0-yr needles compared to 1-yr needles ($F = 5.61$, $P = 0.03$; Tables 1-2).

There was a marginally significant effect of canopy height on *P. taeda* foliar Mo concentrations ($F = 3.17$, $P = 0.06$), with higher concentrations in the lower canopy (41.3 ± 4.2 ng g^{-1}) than in the upper canopy (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.

Foliar Mo concentrations in *L. styraciflua* at Duke were not significantly different between ambient and elevated CO_2 treatments ($F = 1.85$, $P = 0.31$; Figure 1b, Tables 1-

2). There was no detectable effect of N-fertilization on foliar Mo concentrations in either species at Duke FACE (Figure 1b, Tables 1-2).

At ORNL there was not a statistically significant effect of elevated CO_2 on foliar Mo concentrations ($F = 2.06$, $P = 0.19$) in *L. styraciflua;* however, the trend was similar to the response of this species at Duke, with slightly higher concentrations in the elevated CO_2 rings than in the ambient CO_2 rings (Figure 1b, Tables 1-2). Like NaR, there was no detectable effect of canopy height on foliar Mo concentrations ($F = 0.32$, $P = 0.73$) at ORNL nor was there a significant $CO_2 \times$ height interaction ($F = 1.76$, $P = 0.23$).

N concentrations in leaves

Foliar N concentrations were significantly higher in 0-yr than 1-yr needles ($F = 59.52$, $P < 0.01$; Tables 1-2). There was a marginally significant $CO_2 \times$ age interaction effect on *P. taeda* foliar N concentrations ($F = 3.54$, $P = 0.07$). Foliar N concentrations in 0-yr needles were lower in the elevated rings than in the ambient rings ($t = 2.45$, $P = 0.09$), but there was no detected CO_2 effect (across N fertilization treatments) on foliar N in 1-yr-old needles ($t = 0.98$, $P = 0.76$; Figure 1c). There was, however, a marginally significant $CO_2 \times$ N interaction effect on *P. taeda* foliar N concentrations ($F = 3.76$, $P = 0.06$). CO_2 decreased foliar N concentrations in both needle age classes in the N fertilized plots ($t = 2.78$, $P = 0.05$) but not in the control plots ($t = 0.07$, $P = 0.99$; Table 1-2). *P. taeda* foliar N concentrations increased with N fertilization in the ambient CO_2 rings ($t = 3.60$, $P < 0.01$) but not in the elevated CO_2 rings ($t = 1.04$, $P = 0.73$).

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 on foliar N concentrations in lower canopy needles (23% increase in foliar N; 10% increase in mid-canopy needles, 12% increase in upper canopy needles).

In *L. styraciflua* at Duke, foliar N concentrations decreased with CO_2 enrichment ($F = 18.22$, $P = 0.05$) and increased with N fertilization ($F = 5.64$, $P = 0.08$). There was no $CO_2 \times$ N interaction effect ($F = 0.46$, $P = 0.53$; Figure 1c, Tables 1-2).

At ORNL, *L. styraciflua* had lower foliar N ($F = 5.30$, $P = 0.08$) concentrations in the elevated CO_2 rings than in the ambient CO_2 rings (Figure 1c, Table 1-2). 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 CO_2 × 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**.**

Mo concentrations in roots

There were no significant effects of CO_2 (F = 0.22, P = 0.66), N fertilization (F = 0.92, P = 0.39), or CO_2 × N (F = 0.42, P = 0.55) on root Mo concentrations at Duke (Figure 4a, Tables 1-2). Root Mo concentrations at ORNL were lower (F = 6.22, P = 0.09) in the elevated CO_2 rings compared to ambient CO_2 rings (Figure 4a, Tables 1-2).

N concentrations in roots

At Duke, there was no CO_2 effect on root N concentrations, but root N was significantly greater (F = 9.01, P = 0.04) in the N fertilized plots compared to N control plots (Figure 4b, Tables 1-2). ORNL root N concentrations were greater (F = 7.80, P = 0.07) in the elevated CO_2 rings compared to ambient rings (Figure 4b, Tables 1-2).

Mo concentrations in soils

At Duke, there were no significant effects of CO_2 enrichment (F = 1.21, P = 0.39) or N fertilization (F = 0.67, P = 0.46) on total soil Mo concentrations (Figure 5a, Tables 1-2). 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). At ORNL there were no significant effects of CO_2 enrichment (F = 2.40, P = 0.22), soil depth (F = 1.34, P = 0.32), or CO_2 × depth (F = 0.91, P = 0.47) on total soil Mo concentrations (Figure 5a, Tables 1-2).

There were, however, significant CO_2 effects on bio-available Mo at both sites. At Duke, there were greater concentrations of bio-available Mo in the CO_2 enriched plots in the lower soil depths (15-20 cm; CO_2 × depth: F = 2.39, P = 0.09) and there was a

significant increase in bio-available Mo with N fertilization ($F = 12.97$, $P = 0.02$; Figure 5b, Tables 1, 2). Bio-available soil Mo at ORNL was significantly greater in the elevated CO_2 rings compared to ambient rings ($F = 9.27$, $P = 0.01$), with increases in all soil depths but the 10-15 cm increment ($CO_2 \times$ depth: $F = 2.90$, $P = 0.08$). Concentrations of bio-available Mo decreased with depth from 0-20 cm in both CO_2 treatments at ORNL ($F = 8.92$, $P < 0.01$).

At ORNL, bio-available Mo was positively correlated with log-percent soil organic matter (SOM; $R^2 = 0.32$, $P = 0.01$), which, like bio-available Mo, also decreased with soil depth. No 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: $R^2 = 0.04$, $P = 0.18$) or ORNL (total Mo: $R^2 = 0.06$, $P = 0.31$; soil pH: $R^2 = 0.09$, $P = 0.21$). Bio-available soil Mo was not correlated with foliar Mo at ORNL ($R^2 = 0.12$, $P = 0.30$) or Duke (*L. styraciflua*: $R^2 = 0.30$, $P = 0.30$; *P. taeda* 1-yr: $R^2 = 0.04$, $P = 0.53$; *P. taeda* 0-yr: $R^2 = 0.01$, $P = 0.75$).

N concentrations in soils

At Duke, there were no significant effects of elevated CO_2 or N-fertilization on total N concentrations in soils (0-20cm). At ORNL, soil N concentrations (0-20cm) were greater ($F = 6.96$, $P = 0.08$) in the elevated CO_2 rings than in ambient rings (Figure 5c, Tables 1, 2).

Effects of leaf and soil variables on foliar NaR

There were no significant correlations between NaR and foliar concentrations of Mo or N for *L. styraciflua* at either site. *P. taeda* log NaR was negatively correlated with foliar N concentrations ($R^2 = 0.33$, $P < 0.01$; Figure 6a) and positively correlated with foliar Mo concentrations ($R^2 = 0.22$, $P = 0.03$; Figure 6b) across both needle ages. 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 ($P < 0.01$). The relationship between foliar NaR and Mo, however, was no longer significant (P =

0.13), suggesting that differences in foliar NaR and Mo between age classes are driving the foliar NaR-Mo relationship.

To look at effects of soil variables on foliar NaR we focused on *P. taeda* 1-yr needles because NaR in these leaves were the only ones affected by CO_2 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 ($R^2 = 0.47$, $P = 0.02$; Figure 7). The strength of this correlation increased markedly if a single outlier (CO_2-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 overall trend of the relationship among these two variables. There is strong 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 CO_2 and N fertilization are indirectly driving changes in foliar NaR, we used bio-available Mo as a covariate in an analysis of covariance (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 CO_2 ($F = 3.18$, $P = 0.33$) 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 CO_2 enrichment observed in this experiment (Figure 1a) is consistent with a number of previous studies of CO_2 effects on

NO_3^- 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 NO_3^- and carbon assimilation under elevated CO_2 (Bloom et al. 2002). If reductant 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 CO_2 treatments, and decrease with CO_2 enrichment, particularly in the upper canopies. In our study, however, there were no differences in NaR between the lower and upper canopies, nor was there a significant CO_2 × canopy height interaction (Figure 3a), suggesting that observed CO_2 effects on NaR were not driven primarily by competition for reductant between carbon and NO_3^- assimilation, but rather may have been driven by soil-mediated CO_2 effects, such as Mo bio-availability.

Little is known about Mo limitation thresholds and concentration ranges in nonagricultural species and systems. Foliar Mo concentrations in this study (Figure 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 that 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 (Figure 5b) were slightly lower than values reported for Germen 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 Evarsi 1997).

To test for indirect effects of elevated CO_2—that is, changes in soil nutrient status—on NaR, we focused on 1-year-old *P. taeda* needles because there was no effect of CO_2 enrichment or N fertilization on NaR in *L. styraciflua* leaves or 0-yr *P. taeda* needles (Figures 1a, 2). Low NaR in 0-yr needles across treatments (Figure 2) may reflect the source of N allocated to new growth—foliar N in 0-yr needles may be derived from N recycled within the plant, rather than newly fixed N. Because soil N supplies are often lower than plant demand, N recycling within plants is an important component of plant N nutrition, and many studies have found that N retranslocation to new growth is an important mechanism to enhance N supply (Proe et al. 2000, Weatherall et al. 2006)

We detected no CO_2 effects on total soil Mo or N concentrations at Duke FACE (Figures 5a, 5c). However, bio-available Mo—which was greater with CO_2 enrichment in lower soil depths (15-20 cm) and greater across depths with N fertilization—explained 47-86% of the variation in *P. taeda* NaR across CO_2 and N treatments. Soil Mo has been linked to limitation of N_2-fixation under elevated CO_2 in a scrub-oak community (Hungate et al. 2004), but Mo-limitation of NaR with CO_2 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, results of the ANCOVA further support a link between foliar NaR and soil Mo. When bio-available Mo was used a covariate in the analysis, there were no detected effects of elevated CO_2 or N-fertilization on adjusted NaR (i.e., adjusted for bio-available Mo in the ANCOVA) in *P. taeda*, suggesting that CO_2 and N-fertilization effects on *P. taeda* NaR were tied to 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 for the negative correlation observed between bio-available Mo and leaf NaR 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 they 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. 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 is needed to discern the mechanism of the relationship between CO_2 and N treatments, bio-available Mo and leaf NaR.

CO_2 effects on NO_3^- and NH_4^+ soil pools and plant uptake capacities may also be an important driver of NaR. While we did not measure NO_3^- and NH_4^+ dynamics in this study, others have found no effect of elevated CO_2 on rates of N mineralization or nitrification at ORNL or Duke, and no effect on soil NO_3^- or NH_4^+ pool sizes at Duke (Finzi et al. 2001; Sinsabaugh et al. 2003). CO_2 effects on NO_3^- and NH_4^+ uptake by *P.*

taeda have been variable across studies (BassiriRad 2000; BassiriRad et al. 1996a; BassiriRad et al. 1996b; Larigauderie et al. 1994). Larigauderie et al. (1994) found that elevated CO_2 increased *P. taeda* NO_3^- uptake when soil NO_3- was high but decreased uptake under low NO_3^- conditions. Stitt and Krapp (1999) suggest one possible reason for reduced NO_3^- uptake with CO_2 enrichment—especially when NO_3^- concentrations are low—is that lower transpiration under elevated CO_2 may reduce bulk flow of soil water and water-soluble nutrients such as NO_3^- to roots. However, there have been no detected effect of elevated CO_2 on stomatal conductance or leaf-water relations in *P. taeda* at Duke (Ellsworth 1999) nor CO_2 effects on canopy water relations at Duke (Schafer et al. 2001). Based on these studies and on our soil Mo results, it appears that the plant-soil Mo dynamic is the main driver of CO_2 effects on leaf NaR.

Elevated CO_2 did have species-specific effects on NaR, but patterns were not as expected based on previous research on theses species. Constable et al. (2001), who reported NaR activity of similar magnitude (0.1-0.6 μmol NO_2^- g^{-1} h^{-1}) to levels found in our study, found an increase in *L. styraciflua* NaR with CO_2 enrichment, while we found no CO_2 effects on *L. styraciflua* NaR at Duke or ORNL (Figure 1a). *P. taeda* NaR in 0-year needles did not change with CO_2 enrichment, as in Constable et al. (2001), but 1-year needle NaR decreased in our study (Figure 2a). There are several possible reasons for the contrasting effect of elevated CO_2 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 CO_2 treatment effects on NaR, there is potential for CO_2-mediated differences in soil water content, N concentrations and chemical forms, and competition among species for soil nutrients. In addition, our study suggests that changes in soil Mo bio-availability, driven by CO_2 and N-fertilization are important determinants 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 CO_2 treatment, N fertilization effects on NaR also varied between species. N fertilization decreased foliar NaR in 1-yr *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 (Figure 5c), total N concentrations in leaves and roots were greater with N fertilization (Figures 1c, 4b). The negative correlation between *P. taeda* foliar NaR and leaf N concentrations may have been driven by a preferential uptake of NH_4^+ with N-fertilization (fertilization treatment consisted of additions of $NH_4^+NO_3^-$), coupled with decreased uptake and assimilation of NO_3^-. Previous studies have shown that the chemical form of N available to plants is an important regulator of NaR and also interacts with CO_2 effects on NaR (Geiger et al. 1999; Matt et al. 2001). Both species in our study have been shown to preferentially take up NH_4^+ over NO_3^-; Constable et al. (2001) determined that the proportion of N taken up as NO_3^- was about 30% in seedlings of both these species when both forms of inorganic N were available. Under field conditions, however, N uptake will be affected not only by root uptake kinetics but also by inter-specific competitive interactions for inorganic soil N.

Instability of the nitrate reductase enzyme coupled with logistics of extracting roots from trees under field conditions precluded us from measuring root NaR. We use caution in drawing conclusions about treatment effects on whole plant NO_3^- 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 CO_2, because the energy requirements for root NaR are met through respiration of root carbohydrates (Sechley et al. 1992), which tend to increase with CO_2 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 CO_2 enrichment because there were no CO_2 effects on root Mo concentrations at Duke and a decrease in root Mo concentrations at ORNL in the CO_2 enriched plots (Figure 4a).

Although we did not measure leaf mass per unit area (LMA) in this study, differences in LMA with canopy height and CO_2 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; when normalized to area, the pattern of NaR in *P. taeda* leaves across canopy heights does suggest competition for reductant between carbon fixation and assimilatory NO_3^- reduction. There was also a slight increase in *L. styraciflua* LMA with CO_2 enrichment at ORNL (Norby and Iversen 2006); while *L. styraciflua* mass-based NaR was not affected by CO_2 enrichment, area-based NaR would therefore be increased with CO_2 enrichment. There was no detected CO_2 effect on *P. taeda* LMA at Duke FACE in needles collected from 1999 through 2002 (Springer et al. 2005).

As a required enzyme cofactor for several N transformation processes (*e.g.,* N_2 fixation, nitrate assimilation, nitrification and denitrification) Mo is a key, yet often overlooked, player in biological N dynamics. Further 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.

Table 1. Summary of N and CO_2 effects on foliar NaR, and N and Mo concentrations in leaves, roots and soils at ORNL and Duke FACE sites. Values are least squares means (and standard errors). A = ambient CO_2; E = elevated CO_2; NC = N-control; NF = N-fertilized.

		Mo (ng g^{-1})		N (mg g^{-1})		NaR (NO_2^- hr^{-1} g^{-1})	
	N-treatment	A	E	A	E	A	E
Leaves: Duke							
P. taeda, Yr-0	N-C	35.3 (1.0)	25.5 (2.6)	11.9 (0.9)	11.7 (0.8)	0.32 (0.01)	0.27 (0.16)
	N-F	31.8 (6.1)	29.9 (3.9)	14.8 (0.2)	12.4 (0.9)	0.14 (0.03)	0.22 (0.07)
P. taeda, Yr-1	N-C	46.5 (11.0)	31.9 (6.8)	10.3 (0.1)	10.6 (0.8)	1.36 (0.13)	0.70 (0.04)
	N-F	48.7 (4.3)	32.1 (3.7)	12.5 (0.5)	11.2 (0.6)	0.61 (0.01)	0.46 (0.19)
L. styraciflua	N-C	32.2 (8.4)	36.9 (21.9)	21.0 (0.4)	18.4 (0.5)	0.63 (0.18)	0.60 (0.14)
	N-F	30.0 (9.6)	60.5 (6.7)	23.2 (0.1)	19.6 (0.1)	0.51 (0.22)	0.55 (0.14)
Leaves: ORNL							
L. styraciflua		21.4 (3.7)	28.1 (1.0)	18.4 (1.0)	14.7 (1.3)	0.55 (0.11)	0.41 (0.12)
Roots							
Duke	N-C	86.6 (18.3)	64.4 (11.9)	3.6 (1.9)	3.8 (0.8)	-	-
	N-F	92.1 (17.0)	93.0 (30.0)	6.9 (1.3)	7.2 (1.3)	-	-
ORNL		162.6 (31.6)	59.7 (8.5)	8.0 (0.5)	9.9 (0.4)	-	-
Soil							
Duke	N-C	145.9 (25.7)	148.3 (42.1)	0.7 (0.02)	0.7 (0.01)	-	-
	N-F	112.1 (5.7)	150.4 (16.3)	0.7 (0.01)	0.7 (0.01)	-	-
ORNL		244.9 (7.49)	263.1 (9.1)	1.0 (0.1)	1.3 (0.01)	-	-

Table 2. Probability values for CO_2, N and needle-age effects on foliar NaR, and Mo and N concentrations in leaves, roots and soils. Significant values ($p \leq 0.10$) in bold.

		Mo	N	NaR
Leaves				
Duke	CO_2	0.31	**0.05**	0.82
L. styraciflua	N	0.30	**0.08**	0.54
	$CO_2 \times N$	0.22	0.53	0.99
Duke	CO_2	0.17	0.204	**0.10**
P. taeda[*]	N	0.88	**0.04**	**0.04**
	Age	**0.03**	**<0.01**	**<0.01**
	$CO_2 \times N$	0.78	**0.06**	0.14
	$CO_2 \times$ Age	0.22	**0.07**	**0.03**
	N × Age	0.92	0.48	**0.02**
	$CO_2 \times N \times$ age	0.53	0.54	0.20
ORNL	CO_2	0.19	**0.08**	0.37
Roots				
Duke	CO_2	0.66	0.89	-
	N	0.39	**0.04**	-
	$CO_2 \times N$	0.55	0.95	-
ORNL	CO_2	**0.09**	**0.07**	-
Soil				
Duke	CO_2	0.39	0.65	-
	N	0.46	0.60	-
	$CO_2 \times N$	0.41	0.90	-
ORNL	CO_2	0.22	**0.08**	-

* Full models for leaves at ORNL and Duke *P. taeda* analysis included height and height interaction terms.

Figures

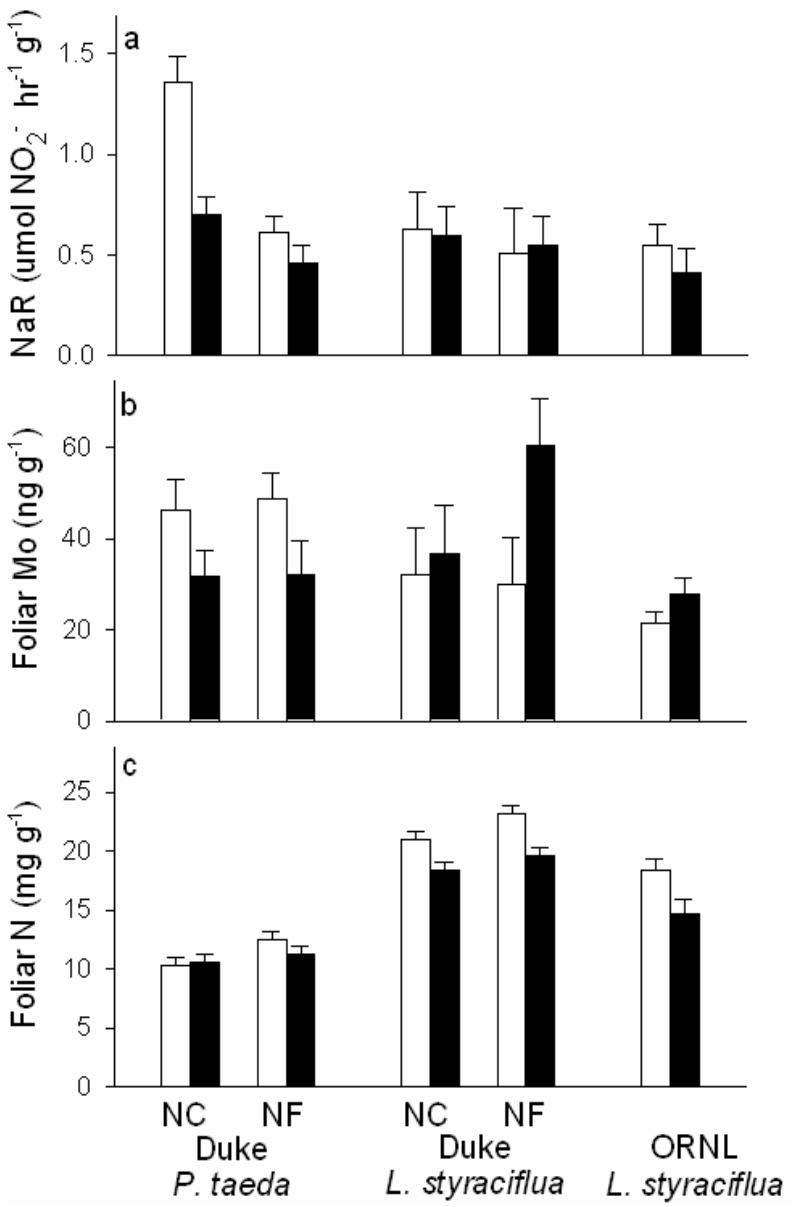

Figure 1. Elevated CO_2 and N fertilization effects on foliar NaR, Mo and N in 1-yr *P. taeda* needles at Duke and *L. styraciflua* leaves at Duke and ORNL FACE.
a.) CO_2 and N fertilization significantly decreased NaR ($p<0.05$) in 1-yr old *P. taeda* needles but not in *L. styraciflua* leaves. b.) There were no significant CO_2 or N treatment effects on foliar Mo concentrations in either species. c.) There was a significant $CO_2 \times N$ interaction effect on foliar N in *P. taeda* ($p<0.05$). *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 (±SE) from elevated rings and open bars represent ambient rings. NC= N control; NF = N fertilization.

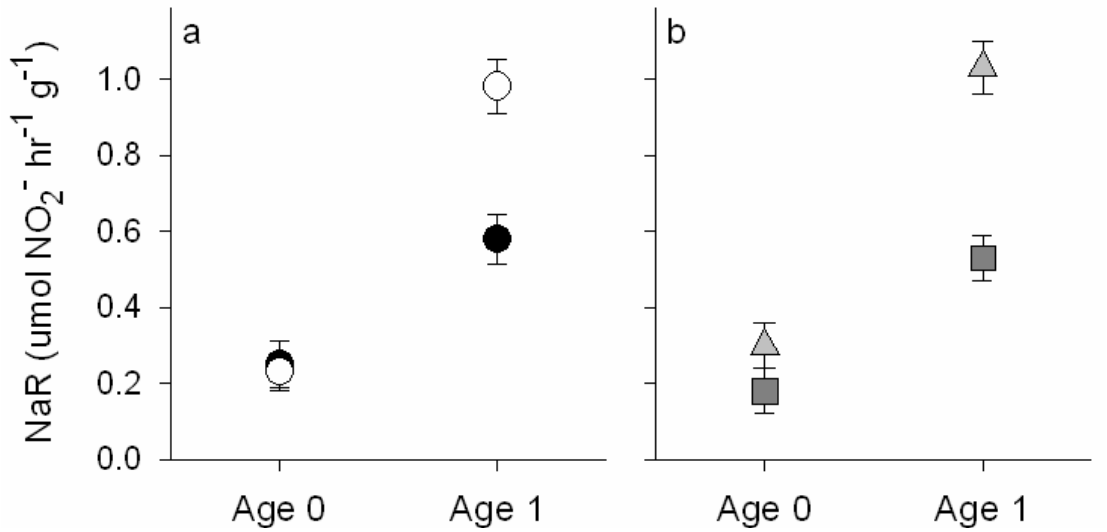

Figure 2. NaR in 0-yr and 1-yr *P. taeda* needles at Duke FACE. a.) There was a significant effect of (a.) elevated CO_2 and (b.) N fertilization on NaR in 1-yr *P. taeda* needles ($p < 0.05$) but not in 0-yr-old needles. Symbols represent least squares means (± SE) from a.) elevated (filled circles) and ambient CO_2 rings (open circles), and b.) N control (light grey triangles) and N fertilized sectors (dark grey squares).

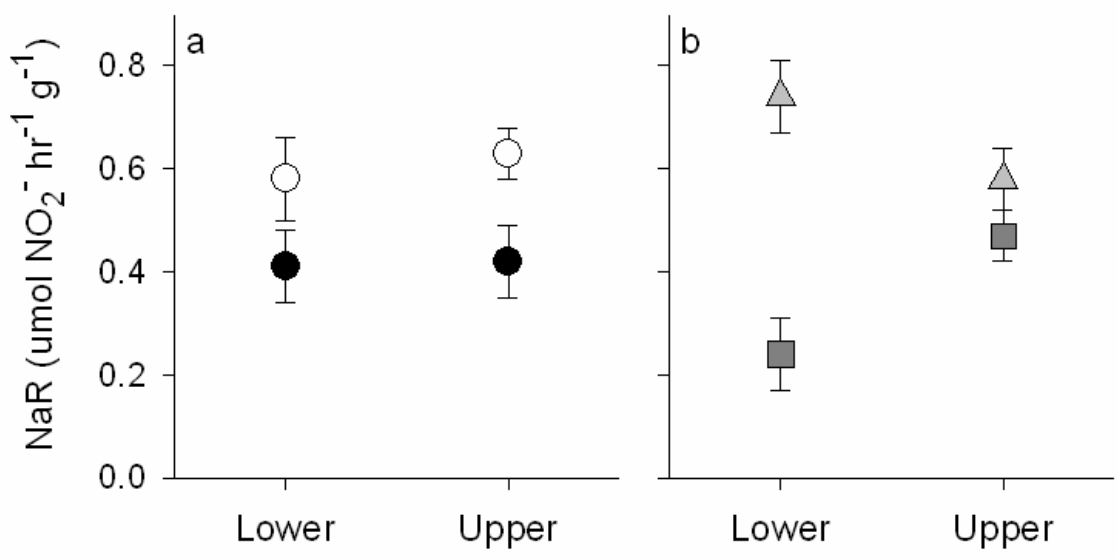

Figure 3. NaR in 1-yr *P. taeda* needles in the upper and lower canopies at Duke FACE. a.) There was no CO_2 × canopy height interaction effect on NaR b.) but there was a significant N fertilization × canopy height effect ($p < 0.05$). Symbols represent least squares means (± SE) from a.) elevated (filled circles) and ambient CO_2 rings (open circles), and b.) N control (light grey triangles) and N fertilized sectors (dark grey squares).

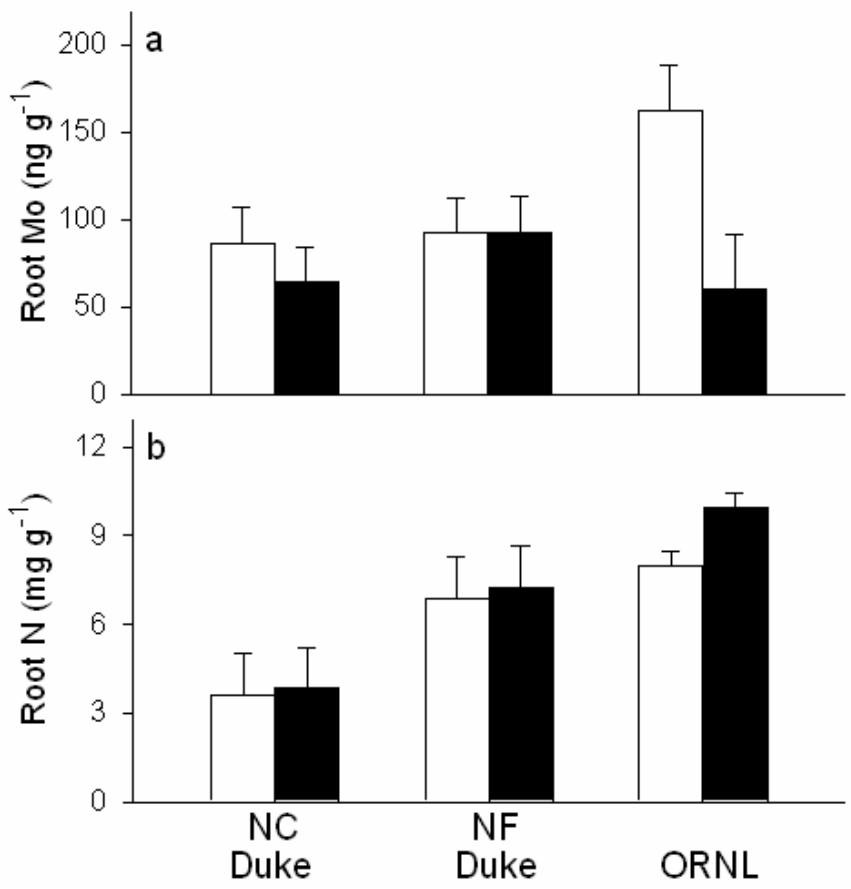

Figure 4. Elevated CO_2 and N fertilization effects on root Mo and N concentrations at Duke and ORNL FACE. a.) Root Mo concentrations at Duke did not change with CO_2 or N enrichment, but there were significantly lower root Mo concentrations at ORNL in the CO_2 enriched rings ($p < 0.05$). b.) At Duke, there was no significant effect of elevated CO_2 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 CO_2 enrichment. Filled bars represent means (± SE) from elevated rings and open bars from ambient rings. NC= N control; NF = N fertilization.

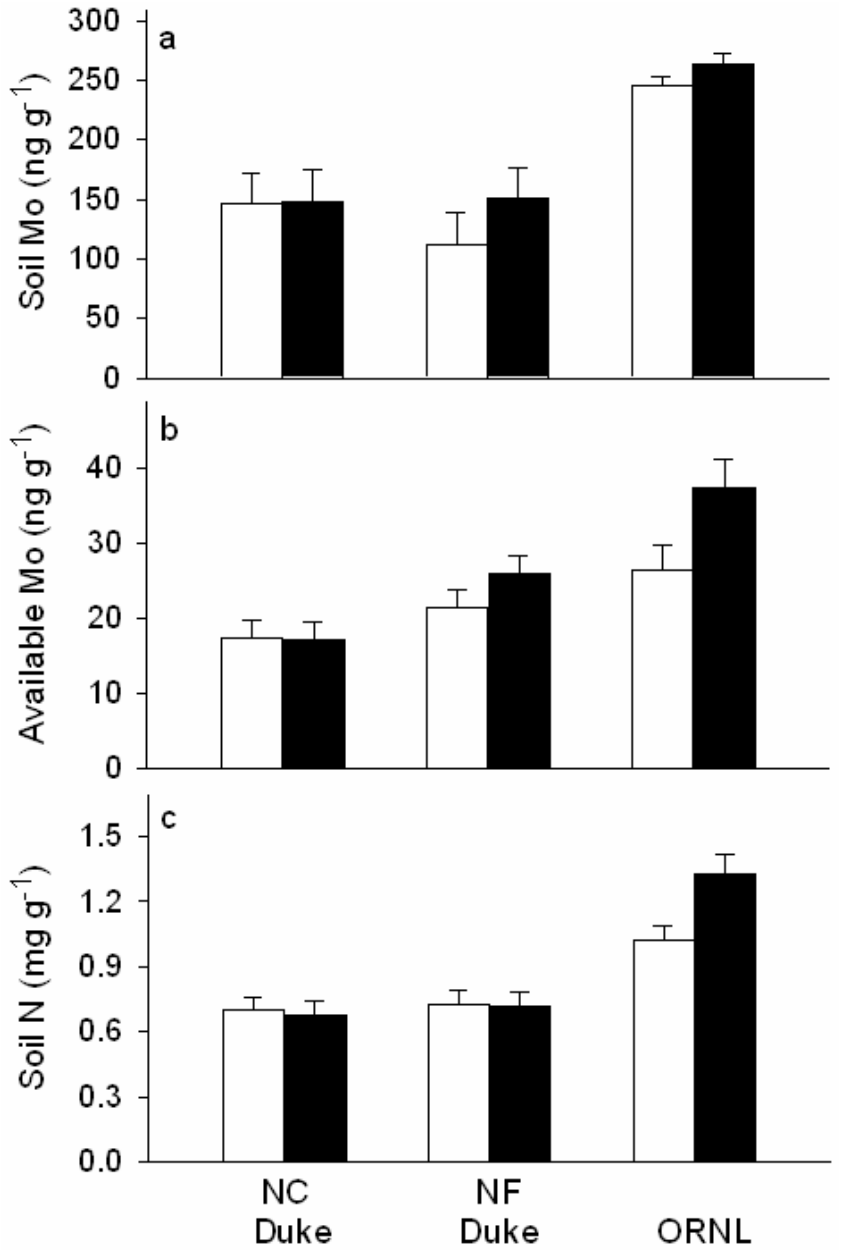

Figure 5. Elevated CO_2 and N fertilization effects on soil (0-20cm) Mo and N concentrations at Duke and ORNL FACE. a.) There were no significant CO_2 or N fertilization effects on total soil Mo concentrations at either site; b.) however, at Duke, soil Mo bio-availability was greater in the CO_2 enriched 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 CO_2 enrichment ($p < 0.05$). c.) There were no detected effects of CO_2 or N fertilization on N concentrations in soils at Duke, but there were significantly greater soil N concentrations with CO_2 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.

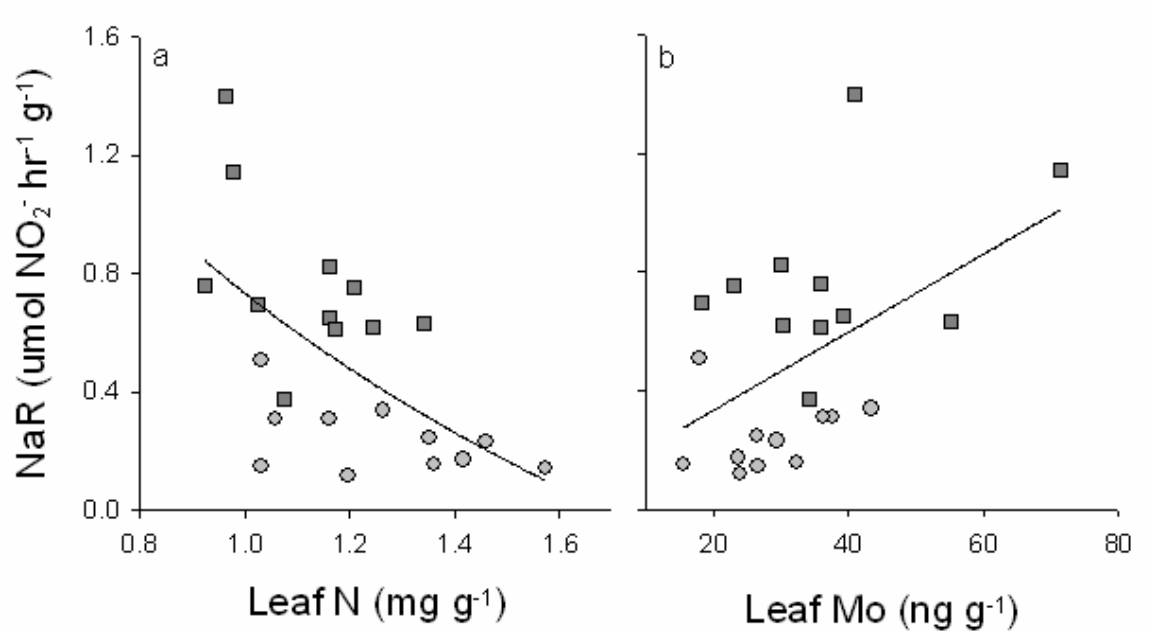

Figure 6. Relationship between foliar NaR and foliar N and Mo concentrations in 0 and 1-yr *P. taeda* needles. a. Foliar NaR was negatively correlated with foliar N concentrations ($R^2 = 0.33$, $P < 0.01$) b. and positively correlated with foliar Mo concentrations ($R^2 = 0.22$, $P = 0.03$) across needle age classes. Points represent least squares means (SE) from N treatment sectors in each experimental ring. Values were averages across canopies heights. Light grey circles represent 0-yr needles and dark grey squares represent 1-yr needles.

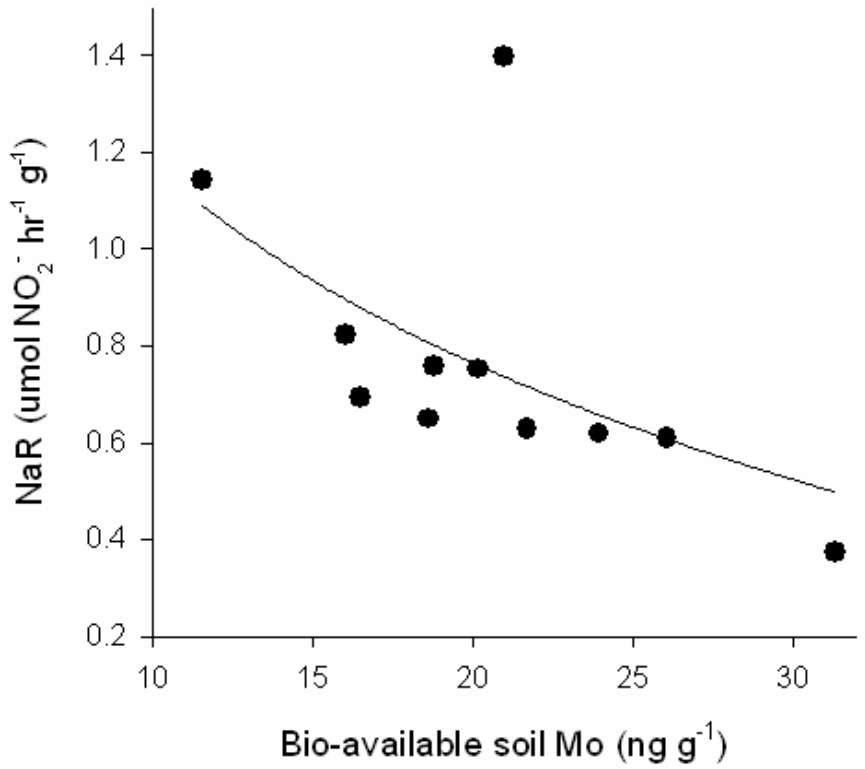

Figure 7. Relationship between foliar NaR in 1-yr *P. taeda* needles and soil Mo bioavailability at Duke FACE. There was a significant negative correlation between log NaR and bio-available soil Mo ($R^2 = 0.47$, $P < 0.05$). The strength of this correlation increased markedly with the outlier removed ($R^2 = 0.86$, $P < 0.01$). Points represent least squares means (SE) from N treatment sectors in each experimental ring. Leaf NaR values were averaged across canopies, and soil values were averaged across depths.

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