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Effects of elevated concentrations of atmospheric CO_2 and tropospheric O_3 on leaf litter production and chemistry in trembling aspen and paper birch communities†

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Summary Human activities are increasing the concentrations of atmospheric carbon dioxide ($[CO_2]$) and tropospheric ozone ($[O_3]$), potentially leading to changes in the quantity and chemical quality of leaf litter inputs to forest soils. Because the quality and quantity of labile and recalcitrant carbon (C) compounds influence forest productivity through changes in soil organic matter content, characterizing changes in leaf litter in response to environmental change is critical to understanding the effects of global change on forests. We assessed the independent and combined effects of elevated $[CO_2]$ and elevated $[O_3]$ on foliar litter production and chemistry in aspen (*Populus tremuloides* Michx.) and birch–(*Betula papyrifera* Marsh.) aspen communities at the Aspen free-air CO_2 enrichment (FACE) experiment in Rhinelander, WI. Litter was analyzed for concentrations of C, nitrogen (N), soluble sugars, lipids, lignin, cellulose, hemicellulose and C-based defensive compounds (soluble phenolics and condensed tannins). Concentrations of these chemical compounds in naturally senesced litter were similar in aspen and birch–aspen communities among treatments, except for N, the C:N ratio and lipids. Elevated $[CO_2]$ significantly increased C:N (+8.7%), lowered mean litter N concentration (–10.7%) but had no effect on the concentrations of soluble sugars, soluble phenolics and condensed tannins. Elevated $[CO_2]$ significantly increased litter biomass production (+33.3%), resulting in significant increases in fluxes of N, soluble sugars, soluble phenolics and condensed tannins to the soil. Elevated $[O_3]$ significantly increased litter concentrations of soluble sugars (+78.1%), soluble phenolics (+53.1%) and condensed tannins (+77.2%). There were no significant effects of elevated $[CO_2]$ or elevated $[O_3]$ on the concentrations of individual C structural carbohydrates (cellulose, hemicellulose and lignin). Elevated $[CO_2]$ significantly increased cellulose (+37.4%) input to soil, whereas elevated $[O_3]$ significantly reduced hemicellulose and lignin inputs to soil (–22.3 and –31.5%, respectively). The small changes in litter chemistry in response to elevated $[CO_2]$ and tropospheric $[O_3]$ that we observed, combined with changes in litter biomass production, could significantly alter the inputs of N, soluble sugars, condensed tannins, soluble phenolics, cellulose and lignin to forest soils in the future.

Keywords: cellulose, chemical fluxes, C:N ratio, condensed tannins, FACE, foliar litter, global change, lignin, soluble phenolics, soluble sugars.

Introduction

Atmospheric carbon dioxide concentration ($[CO_2]$) has increased from pre-industrial values of 280 ppm to current values of about 367 ppm (IPCC 2001). Background tropospheric ozone concentrations ($[O_3]$) have doubled in the past 100 years (Andersen 2003, Karnosky et al. 2003). These increases are projected to continue well into the future, likely impacting above- and belowground plant production and the chemical composition of senesced plant material (Zak et al. 2000, Andersen 2003, Giardina et al. 2005). Northern mid-latitude forests appear to be an important sink for atmospheric CO_2 (IPCC 2001). Litter inputs determine substrate availability for soil microbial metabolism, thereby controlling soil organic carbon (C) formation and nutrient release rates, which could influence the C-sink strength of forest ecosystems.

The effects of elevated $[CO_2]$ and elevated $[O_3]$ on the quantity and timing of nutrient release to plants and on soil C formation rates are influenced by the combined change in litter quality and quantity. Loya et al. (2003) showed that, in northern forests in the presence of elevated $[CO_2]$, exposure to elevated $[O_3]$ results in lower rates of accumulation of acid-insoluble soil C compared with the rates at ambient $[O_3]$, in part because of lower production rates (Karnosky et al. 2003). Johnson et al. (2004) observed that elevated $[CO_2]$ significantly reduced mean nitrogen (N) concentration ([N]) of sweetgum litter, but the increase in litterfall biomass offset this reduction and so N flux to soil was unaffected. The effects of elevated $[CO_2]$ and elevated $[O_3]$ on plant physiology and growth have been studied extensively, but most studies have examined short-term responses of seedlings during chamber fumigation. The response of mature trees to environmental change will

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likely differ from that of seedlings because growth rates, tree size, leaf-level properties and canopy environment change dramatically as trees age. Consequently, extrapolating short-term results from studies of seedlings to mature trees could be misleading (Saxe et al. 1998, Samuelson and Kelly 2001, Karnosky et al. 2003).

In this study, we used free-air CO_2 enrichment (FACE) technology to examine leaf litter production and biochemical input to soil in response to elevated [CO_2] and [O_3] treatments. We collected litter from aspen and birch–aspen communities exposed to 6 years of free-air CO_2 and O_3 enrichment. Aspen and birch are early successional species common to northern forests. They are relatively fast growing, and competition is high when these species occupy the same site (King et al. 2001*b*). Therefore, both aspen and birch growth may increase relative to production of C-based secondary compounds when C availability is relatively high and soil resources are relatively low (Herms and Mattson 1992).

We used the growth-differentiation balance hypothesis (GDBH) (Lorio 1986, Herms and Mattson 1992) as the conceptual framework to develop hypotheses about litter chemistry responses to elevated [CO_2] and [O_3]. The GDBH posits that environmental factors that increase the net balance of plant C sources relative to growth sinks will increase the allocation of photosynthate to the production of C-based secondary compounds. Elevated [CO_2] increased stand production at Aspen FACE (Isebrands et al. 2001, Percy et al. 2002). Based on this finding we predicted that, under conditions of elevated [CO_2], the synthesis of secondary metabolites is disproportionately stimulated because carbohydrate accumulation exceeds the requirements for plant growth. Further, we predicted that litter [N] will decrease because of the dilution effect of high C assimilation. As a phytotoxin, elevated [O_3] damages Rubisco activity and decreases C assimilation rates (Reich and Amundson 1985, Friend et al. 1992). Accordingly, we predicted that elevated [O_3] will reduce secondary compound concentrations as a result of reduced carbohydrate availability. We also reasoned that litter [N] will increase because of the relatively lower photosynthate production in elevated [O_3]. We predicted that the combined effects of elevated [CO_2] and elevated [O_3] on tissue chemistry would cancel each other, because enhanced photosynthesis in response to elevated [CO_2] would be offset by the toxic effects of elevated [O_3].

Materials and methods

Field site

The study was conducted at the Aspen FACE experiment near Rhinelander, Wisconsin (45°40.5′ N, 89°37.5′ E). The Aspen FACE experiment, established in 1997, is the first open-air facility to study the response of intact forest vegetation to both elevated [CO_2] and elevated [O_3]. The experiment is divided into three blocks from north to south. Within each block, two treatments (CO_2 and O_3), each at two concentrations (ambient and elevated), are randomly assigned to four plots, resulting in four treatment combinations: control (ambient [CO_2] + ambient [O_3]), elevated [CO_2] (elevated [CO_2] + ambient [O_3]), elevated [O_3] (ambient [CO_2] + elevated [O_3]), and elevated [CO_2] + elevated [O_3]. In 1997, the eastern half of each plot was planted with five genotypes of O_3-sensitive and O_3-tolerant trembling aspen clones (*Populus tremuloides* Michx.) at a 1 × 1-m spacing. The other half of each plot was divided into two quarters. One quarter was planted with sugar maple (*Acer saccharum* Marsh.) seedlings and cuttings of a single clone of aspen (Clone 216) and the other quarter was planted with birch (*Betula papyrifera* Marsh.) seedlings and the same single clone of aspen. Fumigation began in May 1998 and was conducted only during the daylight hours of the growing season. The elevated [CO_2] treatment was 560 μmol mol^{-1}, about 200 μmol mol^{-1} above ambient. The elevated [O_3] was about 1.5× ambient, or about 50–60 nmol mol^{-1} on cloudy days and 90–100 nmol mol^{-1} on sunny days. At the time of litter collection for this study, the stature of the maple trees was small, and so the results for the aspen–maple community are not presented. A more complete description of the Aspen FACE project is provided by Dickson et al. (2000).

Litter sample collection

Naturally senesced leaf litter was collected in 43-cm-diameter plastic baskets. The litter collection baskets were evenly distributed along a concentric circle at one-half diameter of the plots. Twelve baskets were placed in the aspen subplots, and six baskets were placed in the birch–aspen subplots before leaf out. Foliar litter samples for biomass estimation were collected every 2 weeks from June to October 2003 from eight of the traps in the aspen subplots and four of the traps in the birch–aspen subplots. After removing twigs, understory litter and other coarse woody material, aspen and birch leaf litter were composited by community type within each plot for each collection date. Litter from each collection date was dried to constant mass at 60 °C and pooled to determine biomass production.

Tissue for all chemical analyses was collected from a subset of litter traps in each of the subplots on the same schedule as the biomass collections. Litter from each collection date was pooled across collection dates and freeze-dried, after which two 2-g samples per plot were ground in liquid N and stored at –20 °C for future chemical analysis. For each birch–aspen subplot, leaf litter was composited according to the mass ratio of total annual aspen leaf litter to total annual birch leaf litter for that subplot.

Litter chemistry

Two replicate samples per plot were analyzed for tissue chemistry. Total C and total N were measured with a Carlo Erba NA1500 Series II elemental analyzer (Beverly, MA).

Soluble sugars and lipids About 25 mg of sample was extracted three times (3×) with 2 ml of methanol:chloroform:water (12:5:3, v/v). Sample extracts were then mixed with 2 ml of water in 4-ml vials and stored overnight at 4 °C. A 200-μl aliquot was then taken from the top of the extract in each vial, diluted with 800 μl of water and treated with 1 ml of 5% phenol and 5 ml of concentrated H_2SO_4 for 30 min. Light absorbance (490 nm) of each sample was measured with a Beckman DU-

640 spectrophotometer (Fullerton, CA). A standard curve was prepared with glucose (Dubois et al. 1956, Tissue and Wright 1995). After evaporating the chloroform from the bottom fraction of the extract in each vial, lipids were determined as the mass of the residue (Poorter and Villar 1997).

Soluble phenolics Concentrations of soluble phenolics were determined by the Folin-Ciocalteu method. About 50 mg of ground sample was extracted (1×) with 1.5 ml of 70% acetone at 25 °C, followed (3×) with 1 ml of 70% acetone. After diluting 1:5 with 70% acetone, a 50-µl aliquot was reacted with 0.475 ml of 0.25 N Folin-Ciocalteu reagent and 0.475 ml of 1 M Na_2CO_3 for 1 h. Absorbance of the solution was measured at 724 nm, and compared with a standard curve prepared with catechin (Booker et al. 1996).

Condensed tannins Concentrations of condensed tannins were measured by the acid butanol method of Porter et al. (1986). About 100 mg of ground sample was extracted (×5) with 1 ml of ice-cold 70% acetone containing 10 mM ascorbic acid (AA) at 4 °C for 30 min. After centrifugation, the supernatants from the five extractions were pooled by sample. After diluting the sample with 350 µl of AA, a 150-µl aliquot was reacted with 3.0 ml of 19:1 (v/v) *N*-butanol:concentrated HCl and 100 µl of 0.02 g ml^{-1} $FeNH_4(SO_4)_2 \cdot 12H_2O$ in 2 N HCl, and incubated in a water bath (at 100 °C) for 50 min. Absorbance of the solution was measured at 550 nm.

A condensed tannins standard curve was prepared according to a method adapted from Czochanska et al. (1980) and Booker (2000) from purified condensed tannins extracted from green aspen and birch leaves, which were collected from mature trees in Houghton, Michigan. Freeze-dried leaf tissues were ground in liquid N, and 5 g quantities were extracted (×2) with 40 ml of ethanol (100%) containing 0.1% (w/v) AA (EAA). After centrifugation, the residue was re-extracted (×3) with 50 ml of EAA and re-centrifuged. Acetone was evaporated from the supernatant in a rotary evaporator, and the remaining aqueous solution was extracted (×2) with 50 ml of diethyl ether and then ethyl acetate (×3). After evaporating the residual ethyl acetate, the aqueous fraction was freeze-dried. The crude freeze-dried product was dissolved in 25 ml of degassed 50% (v/v) methanol, and applied to a column of Sephadex LH-20. The column was washed with 1.9 l of 50% methanol and eluted with 70 ml of 50% acetone. After evaporating the acetone, the aqueous solution was freeze-dried to isolate the purified tannins. Purity was verified by UV absorbance. The UV spectrum of the purified pro-anthocyanidin standards in water had maxima at 210 and 280 nm, with a shoulder at 236 nm, and the mean UV absorbance value in 70% acetone ($E_{1\%550}$) was 420. These values are similar to published values (Czochanska et al. 1980, Porter et al. 1986, Booker 2000).

Hemicellulose The pellets resulting from the condensed tannins analyses were air-dried and then extracted with 2 ml of 10% KOH at 30 °C for 24 h. The extracts were mixed with 20 ml of ice-cold absolute ethanol containing 4 M acetic acid, maintained at –20 °C for 24 h and then centrifuged at 4500 rpm for 10 min. After washing (×2) with 2 ml of absolute ethanol, the precipitate was oven dried at 65 °C. Hemicellulose was determined as the dry mass of the precipitate (Dickson 1979).

Lignin and cellulose Lignin concentrations of senescent leaf samples were measured by the method of Booker et al. (1996). About 50 mg of ground sample was extracted (×3) with 1 ml of 50% methanol, rinsed (×2) with 0.8 ml of methanol (53%):chloroform (26%):water (21%), rinsed (×2) with 0.8 ml of liquefied phenol (51%):acetic acid (25%):water (24%), and then washed (×5) with 1.0 ml of ethanol. The extractive-free cell wall material was oven dried at 70 °C, treated with 3.75 ml of 5% H_2SO_4 at 100 °C for 1 h, and then centrifuged at 4500 rpm for 10 min. The resulting pellet was resuspended and washed (×2) with 2 ml of hot water (×2), 2 ml of 95% ethanol, and 2 ml of acetone (×2). After oven drying at 70 °C, the residue was mixed with 1 ml of 72% H_2SO_4, incubated for 2 h at 20 °C, and then diluted with 28 ml of water. After further incubation for 2 h in boiling water, the solution was filtered through a fine mesh glass filter of known dry mass, and the filter plus residue dried at 70 °C. Lignin was determined as residue dry mass. Cellulose concentrations were estimated by subtracting hemicellulose and lignin mass from the extractive-free cell wall materials.

Chemical fluxes and nutrient-use efficiency

Chemical flux to the forest floor was calculated as: Litter chemical flux = chemical concentration of litter (g g^{-1}) × litter production (g m^{-2} $year^{-1}$). Ecosystem N-use efficiency (NUE) was calculated according to Finzi et al. (2001) as: NUE = litter biomass (g m^{-2} $year^{-1}$)/[N] of litter (g m^{-2} $year^{-1}$).

Literature survey

To compare our results with published studies that examined plant chemistry responses to elevated [CO_2] and elevated [O_3], we summarized the published results obtained by searching the ISI Web of Sciences database. Published data included in our analysis were screened based on three criteria: (1) the ambient [CO_2] treatment was lower than 400 ppm and the elevated [CO_2] treatment was between 550 to 800 ppm; (2) the ambient [O_3] treatment was lower than 40 ppb and the elevated [O_3] treatment was between 55 to 100 ppb; and (3) [N], soluble sugar, soluble phenolics and condensed tannins were reported on a mass basis. Four exposure systems (open top chambers (OTC), phytotron, FACE and natural CO_2 springs) and 13 tree species were included in our comparison (Table 1). We did not conduct a formal meta-analysis, but instead, for each study, we calculated a response ratio (after Hedges et al. 1999) to quantify mean responses to elevated [CO_2] or elevated [O_3]. The response ratio was calculated as: Response ratio = chemical concentration in elevated treatment/chemical concentration in ambient air.

Data for all species and treatments reported in the published papers were included in our comparisons. For example, a study reporting chemistry of two species in a three-way factorial design (two concentrations of CO_2, two concentrations of O_3, and two rates of N supply) generated a total $2 \times 2 \times 2 \times 2 = 16$ data points. We analyzed and present the mean of all replicates for a specific treatment combination within a study.

Table 1. Sources of published observations of effects of elevated carbon dioxide concentration ($[CO_2]$) or elevated ozone concentration ($[O_3]$), or both, on litter chemistry. Abbreviations: OTC = open top chambers; FACE = free-air CO_2 enrichment; and CS = natural CO_2 spring enrichment.

Reference	Exposure system	Species	Plant part
Booker (2000)	OTC	*Gossypium hirsutum* L.	Live foliage
Booker and Maier (2001)	OTC	*Pinus tadeda* L.	Live foliage
Booker and Miller (1998)	OTC	*Glycine max* (L.) Merr.	Live foliage
Booker et al. (2005)	OTC	*Gossypium hirsutum*	Litter
Chapman (2004)	FACE	*Populus tremuloides*	Live fine roots
Chapman (2004)	FACE	*Betula papyrifera*	Live fine roots
Chapman (2004)	FACE	*Acer saccharum* Marsh.	Live fine roots
Friend et al. (1992)	OTC	*Pinus taeda*	Live foliage, stems and roots
Gebauer et al. (1998)	OTC	*Pinus taeda*	Live foliage
Heyworth et al. (1998)	OTC	*Pinus sylvestris* L.	Live foliage
Holton et al. (2003)	FACE	*Populus tremuloides*	Live foliage
Kainulainen et al. (1998)	OTC	*Pinus sylvestris*	Live foliage
King et al. (2001*a*)	OTC	*Acer saccharum*	Live foliage and foliar litter
King et al. (2001*c*)	OTC	*Populus tremuloides*	Foliar litter
Kull et al. (1996)	OTC	*Populus tremuloides*	Live foliage
Lindroth et al. (2001)	FACE	*Populus tremuloides*	Live foliage
Lindroth et al. (2001)	FACE	*Betula papyrifera*	Live foliage
Parsons et al. (2004)	FACE	*Betula papyrifera*	Foliar litter
Peñuelas et al. (2002)	CS	*Myrtus communis* L.	Foliar litter
Peñuelas et al. (2002)	CS	*Erica arborea* L.	Foliar litter
Peñuelas et al. (2002)	CS	*Juniperus communis* L.	Foliar litter
Pfirrmann et al. (1996)	Phytotron	*Picea abies* (L.) Karst.	Live fine roots and foliage
Sallas et al. (2001)	OTC	*Pinus sylvestris*	Live foliage
Scherzer et al. (1998)	OTC	*Liriodendron tulipifera* L.	Live foliage
Scherzer et al. (1998)	OTC	*Pinus strobus* L.	Live foliage
Utriainen et al. (2000)	OTC	*Pinus sylvestris*	Live foliage

Statistical analysis

For data from the Aspen FACE experiment, chemical concentrations were derived from the mean of two samples per subplot. Across the experiment, inspection of residuals and normal probability plots indicated that data were normally distributed and had equal variances. Treatment effects on litter biomass and chemistry were analyzed with a fixed-effects model in an analysis of variance (ANOVA) for a randomized complete block design, where community type is treated as a split-plot factor. We used the PROC GLM procedure in SAS (Cary, NC) and the following model adapted from King et al. (2001*b*) to analyze the results:

$$Y_{ijkl} = \mu + \rho_i + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \gamma_{ijk} + \chi_l + (\alpha\chi)_{jl} + (\beta\chi)_{kl} + (\alpha\beta\chi)_{jkl} + \zeta_{ijkl} \quad (1)$$

where Y_{ijkl} is the mean response of block i (ρ_i, $i = 3$), $[CO_2]$ j (α_j, $j = 2$), $[O_3]$ k (β_k, $k = 2$), and community type l (χ_l, $l = 2$). Fixed effects include block (ρ_i), CO_2 (α_j), O_3 (β_k) and their interaction terms $CO_2 \times O_3$ ($(\alpha\beta)_{jk}$), $CO_2 \times$ Community ($(\alpha\chi)_{jl}$), $O_3 \times$ Community ($(\beta\chi)_{kl}$) and $CO_2 \times O_3 \times$ Community ($(\alpha\beta\chi)_{jkl}$). Here, γ is the random component associated with whole units and ζ is the random component associated with the split-plot effect. In this analysis, block is considered a fixed effect because of a gradient in soil fertility from south to north across the site (Dickson et al. 2000, King et al. 2001*b*). Treatment effects were considered significant if $P \leq 0.05$.

For our literature survey, mean response ratios for each treatment and species combination within a study were calculated from the data of individual selected studies. Treatment effects were considered significant if the 95% confidence interval of the mean response ratio did not overlap with 1 (Koricheva et al. 1998).

Results

Litter biomass

Leaf litter biomass ranged from 151.9 to 333.4 g m^{-2} across plots (Table 2). The main effects of both elevated $[CO_2]$ and elevated $[O_3]$ were significant. Elevated $[CO_2]$ increased litter biomass production by 31.3% for the aspen community and 37.6% for the birch–aspen community relative to control plots. Elevated $[O_3]$ reduced litter biomass production by 20.5% for the aspen community and 14.2% for the birch–aspen community compared with control plots. The $CO_2 \times O_3$ interaction was not statistically significant.

Litter chemistry

Leaf litter [N] ranged from 10.8 to 16.8 mg g^{-1} across plots (Table 2). Averaged across O_3 treatments and species, elevated $[CO_2]$ significantly reduced mean litter [N] by 10.5% relative

Table 2. Means ± SE ($n = 3$) and P values of biochemical constituents of aspen and birch–aspen litter produced in the experimental treatments at the Aspen FACE experiment, Rhinelander, WI. Units: biomass = g m^{-2}; nitrogen (N), carbon (C), soluble sugars (SS), soluble phenolics (SP), condensed tannins (CT), lipids, hemicellulose (HC), cellulose and lignin = mg g^{-1}. Abbreviation: Com = community.

	Biomass	N	C	C:N	SS	SP	CT	Lipids	HC	Cellulose	Lignin
Aspen											
Control	230.0 (4.6)	15.3 (1.9)	512.9 (4.3)	34.0 (4.4)	15.5 (2.1)	18.7 (2.3)	21.1 (6.1)	56.6 (6.4)	154.7 (26.1)	462.0 (43.8)	286.3 (49.0)
+CO_2	302.0 (36.8)	13.1 (1.2)	502.1 (5.9)	38.5 (3.2)	22.3 (7.7)	21.8 (9.7)	17.5 (5.3)	60.3 (23.7)	155.0 (20.9)	342.3 (78.5)	392.1 (108.1)
+O_3	182.1 (26.7)	14.3 (1.3)	516.5 (6.0)	36.3 (3.3)	26.2 (9.3)	27.5 (2.4)	37.7 (8.2)	54.6 (7.3)	170.9 (65.0)	361.1 (83.9)	359.6 (73.1)
+CO_2+O_3	246.3 (7.3)	13.7 (0.1)	503.0 (6.1)	36.8 (0.1)	32.4 (13.2)	31.4 (3.2)	33.9 (6.0)	52.2 (3.2)	132.1 (8.0)	527.6 (42.7)	232.4 (28.2)
Birch–aspen											
Control	209.8 (28.5)	15.3 (1.1)	510.6 (8.3)	33.6 (2.7)	11.9 (5.0)	19.6 (7.0)	17.2 (4.8)	73.7 (33.0)	164.0 (40.5)	407.0 (32.9)	323.9 (75.0)
+CO_2	288.7 (42.0)	12.5 (0.5)	501.2 (9.6)	40.2 (2.0)	20.2 (5.3)	20.4 (5.3)	28.0 (3.5)	70.9 (18.8)	168.9 (21.4)	375.7 (31.0)	343.8 (40.9)
+O_3	186.7 (7.7)	12.5 (0.5)	516.4 (7.4)	41.3 (1.5)	26.4 (8.8)	29.1 (3.4)	33.3 (4.8)	68.0 (14.4)	168.8 (27.0)	400.4 (61.1)	307.2 (73.3)
+CO_2+O_3	241.1 (13.0)	11.9 (1.0)	502.3 (3.4)	42.4 (3.3)	39.4 (19.7)	35.1 (7.8)	43.5 (13.1)	56.2 (16.8)	147.9 (29.9)	483.3 (122.8)	238.1 (126.0)
Source (P values)											
CO_2	**0.001**	**0.012**	**0.003**	**0.022**	0.088	0.185	0.276	0.404	0.407	0.509	0.684
O_3	**0.004**	0.073	0.294	**0.043**	**0.017**	**0.004**	**0.001**	0.082	0.722	0.234	0.254
$CO_2 \times O_3$	0.441	0.076	0.481	0.059	0.823	0.539	0.949	0.343	0.329	**0.029**	0.099
Com	0.439	**0.030**	0.731	**0.013**	0.935	0.533	0.253	**0.042**	0.310	0.778	0.568
$CO_2 \times$ Com	0.946	0.704	0.959	0.483	0.648	0.996	**0.017**	0.424	0.523	0.960	0.78
$O_3 \times$ Com	0.453	0.107	0.825	**0.040**	0.475	0.458	0.901	0.597	0.790	0.862	0.719
$CO_2 \times O_3 \times$ Com	0.702	0.715	0.864	0.722	0.765	0.581	0.961	0.881	0.707	0.097	0.173

to control litter values. Litter C concentrations, ranging from 490.8 to 523.8 mg g^{-1}, were reduced by elevated [CO_2], but differed little across species. Averaged across CO_2 and O_3 treatments, mean litter [N] was higher for aspen (14.1 mg g^{-1}) than for birch (13.0 mg g^{-1}). Thus, elevated [CO_2] alone and elevated [O_3] alone increased C:N by 16.3 and 14.9%, respectively. Across treatments, aspen litter had a lower C:N ratio than litter from the aspen–birch community (Table 2).

Concentrations of soluble sugars in leaf litter ranged from 6.2 to 54.4 mg g^{-1}. Across species and CO_2 treatments, elevated [O_3] significantly increased mean soluble sugar concentration by 78.1%, with the highest concentrations in litter in the elevated [CO_2] + elevated [O_3] treatment (161.8% higher than control plots). Concentrations of soluble phenolics ranged from 11.9 to 43.7 mg g^{-1}. Elevated [O_3] significantly increased mean concentration of soluble phenolics by 53.1% across treatments. The highest concentrations of soluble phenolics were observed in litter in the elevated [CO_2] + elevated [O_3] treatment (Table 2). Concentrations of condensed tannins ranged from 11.4 to 56.2 mg g^{-1} (Table 2), and increased in response to elevated [O_3] (77.2% across treatments). A significant $CO_2 \times$ Community interaction was associated with the differential effects of elevated [CO_2] on condensed tannins in aspen litter and the combined birch–aspen litter. Specifically, elevated [CO_2] decreased the mean concentration of condensed tannins by 17.1% for aspen communities, but increased the mean concentration by 62.8% for the birch–aspen community compared with control values. Mean concentration of lipids was not significantly altered by elevated [CO_2] or elevated [O_3], though values ranged from 3.7 to 109.5 mg g^{-1}. Across treatments, birch–aspen litter (67.2 mg g^{-1}) had significantly higher lipid concentrations than litter from the aspen community (55.9 mg g^{-1}) (Table 2).

Concentrations of hemicellulose ranged from 117.0 to 223.1 mg g^{-1} and were not consistently affected significantly by the CO_2 and O_3 treatments or by species. Cellulose ranged from 252.5 to 576.5 mg g^{-1}, with a significant interaction of $CO_2 \times O_3$ (Table 2). Averaged across species, elevated [CO_2] in ambient [O_3] reduced mean cellulose concentration by 17.4%, but elevated [CO_2] + elevated [O_3] increased mean cellulose concentration by 32.7%. Lignin concentrations of senesced leaf tissue ranged from 155.1 to 512.3 mg g^{-1} but no significant treatment effects were observed.

Litter chemical flux

Carbon fluxes from foliar litter to the forest floor were significantly altered by elevated [CO_2] and by elevated [O_3] (Table 3), with elevated [CO_2] increasing C flux by 32.7% relative to control plots and elevated [O_3] reducing C flux by 15.4% (Figure 1A). Greater litter production in the elevated [CO_2] plots offset lower [N] such that N flux to the forest floor increased by 12.5% in elevated [CO_2] compared with control plots. As a result of decreased litter production in elevated [O_3], N flux was 26.5% lower than in control plots. Fluxes of C and N in elevated [CO_2] and elevated [O_3] were similar to those in control plots (Figure 1B). Mean NUE in the birch–aspen community was significantly higher than in the aspen

Table 3. The *P* values ($n = 3$) for chemical constituent inputs to soil through leaf litter produced in the experimental treatments at the Aspen FACE project. Abbreviations: C = carbon; N = nitrogen; SP = soluble phenolics; SS = soluble sugars; CT = condensed tannins; HC = hemicellulose; Cell = cellulose; and Com = community.

Source	C	N	SP	SS	CT	Lipids	HC	Cell	Lignin
CO_2	0.007	0.005	0.030	0.008	0.005	0.105	0.059	0.020	0.115
O_3	0.005	0.008	0.019	0.049	0.007	0.041	0.035	0.684	0.038
$CO_2 \times O_3$	0.425	0.406	0.342	0.668	0.650	0.437	0.210	0.074	0.065
Com	0.451	0.124	0.880	0.972	0.297	0.136	0.496	0.346	0.444
$CO_2 \times$ Com	0.951	0.734	0.974	0.713	0.056	0.687	0.636	0.838	0.742
$O_3 \times$ Com	0.465	0.925	0.328	0.386	0.995	0.715	0.801	0.672	0.729
$CO_2 \times O_3 \times$ Com	0.697	0.844	0.744	0.811	0.685	0.757	0.887	0.038	0.396

community. Across O_3 treatments and species, relatively low [N] and high litter production rates in elevated [CO_2] significantly increased mean NUE by 11.3% (Figure 2).

The elevated [CO_2] and elevated [O_3] treatments had large effects on stand-level fluxes of most leaf litter constituents. Fluxes of soluble sugars, soluble phenolics and condensed tannins to the forest floor were all significantly altered by elevated [CO_2] and elevated [O_3] (Table 3), with the highest fluxes observed in the elevated [CO_2] + elevated [O_3] treatment. In elevated [CO_2] + elevated [O_3], fluxes of soluble sugars, soluble phenolics and condensed tannins increased by 197.0, 94.3 and 122.2%, respectively, compared with those in control plots (Table 3, Figures 1G–1I). There was a significant effect of elevated [O_3] on fluxes of lipids and hemicellulose (Table 3). Averaged across CO_2 treatments and species, elevated [O_3] significantly reduced lipid input to the forest floor by 28.4%, and reduced hemicellulose inputs by 22.4% (Figures 1C and 1F). Fumigation with elevated [CO_2] significantly increased cellu-

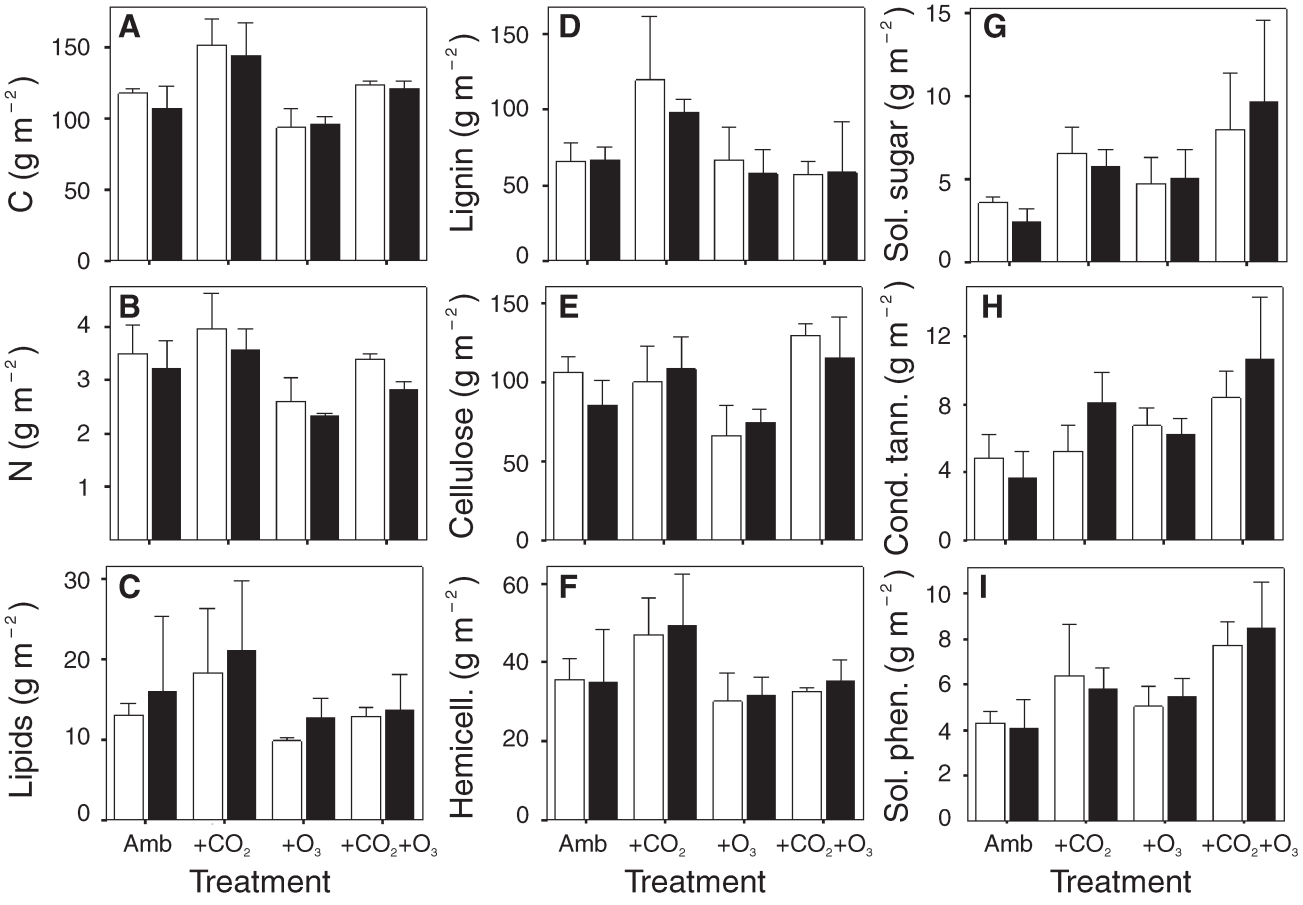

Figure 1. Chemical fluxes from foliar litterfall to the forest floor of aspen (open bars) and birch–aspen litter (filled bars) produced in the experimental treatments at the Aspen FACE experiment, Rhinelander, WI. Values are means ± SE ($n = 3$). Abbreviations: amb = ambient; C = carbon; hemicell. = hemicellulose; N = nitrogen; sol. phen. = soluble phenolics; cond. tann. = condensed tannins; and sol. sugar = soluble sugar.

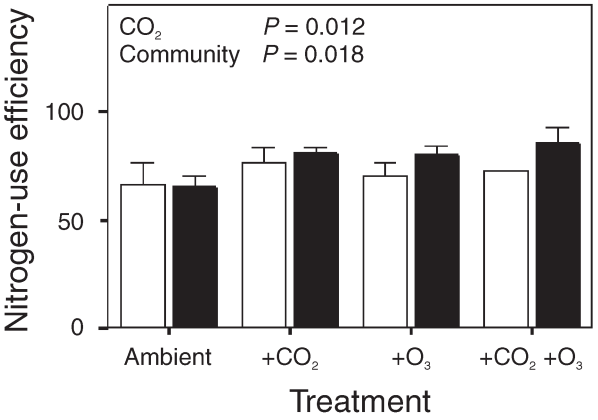

Figure 2. Nitrogen-use efficiency of aspen (open bars) and birch–aspen (filled bars) communities in the experimental treatments at the Aspen FACE project. Values are means ± SE (n = 3).

lose input to the soil, but a significant interaction of $CO_2 \times O_3$ × community (Table 3, Figure 1E) indicated that elevated $[CO_2]$ increased cellulose input only in the presence of elevated $[O_3]$ and only in the aspen community, whereas elevated $[CO_2]$ increased cellulose input in ambient and elevated $[O_3]$ in the birch–aspen community. Mean lignin flux to the forest floor was significantly reduced by 6.2% in elevated $[O_3]$ compared with control plots (Figure 1D). A near significant $CO_2 \times O_3$ interaction for lignin flux (P = 0.065) indicated that the effects of elevated $[CO_2]$ and elevated $[O_3]$ on lignin flux were nonadditive. In ambient $[CO_2]$ plots, elevated $[O_3]$ reduced mean lignin flux by 6.3% compared with control plots, whereas in the elevated $[CO_2]$ plots, elevated $[O_3]$ reduced the lignin flux by 46.9% compared with the elevated $[CO_2]$ + ambient $[O_3]$ plots.

Literature survey

Across three forest FACE sites (Duke, Oak Ridge and Rhinelander), response ratios for litter [N] in elevated $[CO_2]$ ranged from 0.70 to 1.05 and averaged 0.89 across sites. The ANOVA indicated that, compared with litter N in ambient $[CO_2]$ plots, elevated $[CO_2]$ significantly reduced litter [N] across the three FACE sites by 12.8% (P = 0.026). Although the Aspen FACE site had significantly higher litter [N] than the other two sites (P = 0.017), there was no $CO_2 \times$ site interaction for litter [N] (P = 0.976), indicating that the effect of elevated $[CO_2]$ was significant across sites. The response ratios for sugars, tannins and phenolics in elevated $[CO_2]$ were significantly different from 1, indicating that elevated $[CO_2]$ can increase the concentrations of these constituents (Figure 3).

In contrast to elevated $[CO_2]$, elevated $[O_3]$ had no significant effects on litter [N] (Figure 4). Elevated $[O_3]$ alone also had no significant effects on the concentrations of sugars, tannins and phenolics. However, the elevated $[CO_2]$ + elevated $[O_3]$ treatment significantly increased the concentrations of sugars and tannins (but not that of phenolics) relative to elevated $[CO_2]$ alone (Figure 3).

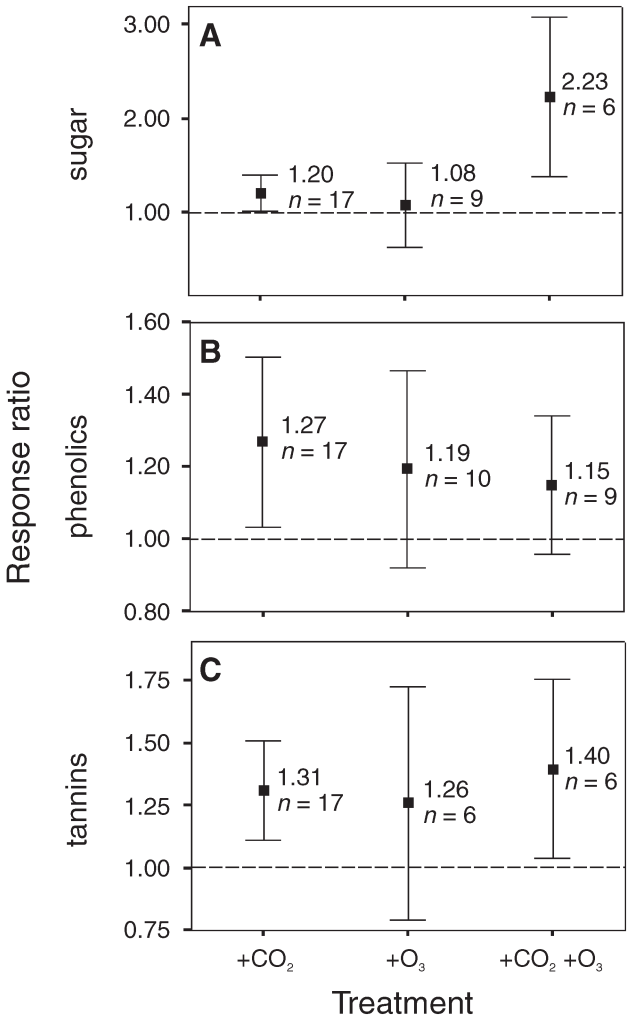

Figure 3. Response ratios for concentrations of sugars (A), phenolics (B) and condensed tannins (C) to elevated carbon dioxide or ozone concentration ($[CO_2]$ or $[O_3]$), or both. The response ratio in elevated $[CO_2]$ = chemical concentration in elevated $[CO_2]$/chemical concentration in ambient $[CO_2]$; the response ratio in elevated $[O_3]$ = chemical concentration in elevated $[O_3]$/chemical concentration in ambient atmosphere; and the response ratio of elevated $[CO_2]$ + elevated $[O_3]$ = chemical concentration in elevated $[CO_2]$ + $[O_3]$/chemical concentration in ambient atmosphere. Square symbols denote means; bars denote the 95% confidence intervals (CI); and n indicates the number of observations. The dashed line corresponds to a response ratio of 1. The effect is considered significant if the 95% CI does not overlap 1. Sources of data: Friend et al. 1992, Booker and Miller 1998, Gebauer et al. 1998, Heyworth et al. 1998, Kainulainen et al. 1998, Booker and Maier 2001, King et al. 2001*a*, 2001*c*, Lindroth et al. 2001, Sallas et al. 2001, Peñuelas et al. 2002, Holton et al. 2003, Chapman 2004, Parsons et al. 2004 and Booker et al. 2005.

Discussion

We predicted that increased C availability in elevated $[CO_2]$ promotes the accumulation of total nonstructural carbohydrates (TNC). As plant growth becomes limited by factors other than photosynthate supply (e.g., mineral nutrients), an increasing proportion of photosynthetic production becomes available for the synthesis of secondary compounds (Herms

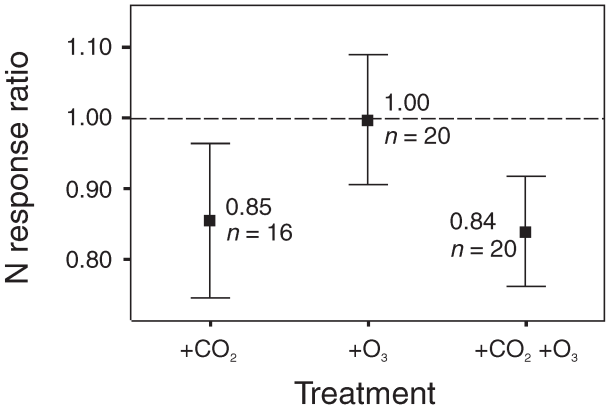

Figure 4. Response ratios of leaf litter nitrogen (N) concentration ([N]) to elevated carbon dioxide or ozone concentration ([CO_2] or [O_3]). The response ratio in elevated [CO_2] = leaf litter [N] in elevated [CO_2]/leaf litter [N] in ambient atmosphere; the response ratio in elevated [O_3] = leaf litter [N] in elevated [O_3]/leaf litter [N] in ambient atmosphere; and the response ratio in elevated [CO_2] + elevated [O_3] = leaf litter [N] in elevated [CO_2] + [O_3]/leaf litter [N] in ambient atmosphere. Square symbols denote means; bars denote the 95% confidence intervals (CI); and *n* indicates the number of observations. The dashed line corresponds to a response ratio of 1. The effect is considered significant if the 95% CI does not overlap 1. Sources of data: Kull et al. 1996, Pfirrmann et al. 1996, Scherzer et al. 1998, Booker 2000, Utriainen et al. 2000, Lindroth et al. 2001, Parsons et al. 2004 and Booker et al. 2005.

and Mattson 1992). We also hypothesized that the decline in photosynthesis in elevated [O_3] (Anderson 2003) reduces the availability of photosynthate for C-based defensive compounds. We found that elevated [CO_2] significantly increased litter production rates, but had minor effects on concentrations of secondary compounds. Elevated [O_3] significantly reduced biomass production but increased concentrations of C-based defensive compounds. These findings do not support our GDBH-based predictions, perhaps because the GDBH considers the secondary metabolism of the whole plants, whereas we examined only the properties of leaf litter (Herms and Mattson 1992, Stamp 2004). Further, the response of plants to altered resource availability may be more complex than could be addressed by our experimental design.

Litter production

Elevated [CO_2] generally stimulates photosynthesis and net primary production in short-term fumigations (Saxe et al. 1998). However, this initial stimulation may become suppressed by excess carbohydrate or because nutrient supply is reduced as nutrients are increasingly immobilized by larger quantities of reduced quality litter (Luo and Reynolds 1999, Long et al. 2004). In the Aspen FACE experiment, elevated-[CO_2]-related stimulation of biomass production has persisted throughout 6 years of free air fumigation, with no indication of reduced responsiveness (King et al. 2005). In line with findings for total stand biomass (King et al. 2005), we found that litter production in the elevated [CO_2] + elevated [O_3] treatment was only slightly greater than that in control plots, indicating that the positive effects of elevated [CO_2] were largely offset by the negative effects of elevated [O_3]. The small stimulation of litter production by elevated [CO_2] in the presence of elevated [O_3] could result from two mechanisms: elevated [CO_2] reduced O_3 uptake by reducing leaf stomatal conductance (Andersen 2003); or elevated [CO_2] stimulated biomass productivity by increasing assimilation rates and decreasing photorespiration (Booker et al. 1997).

Litter nitrogen

Nitrogen limitations to plant growth predominate in northern forests, and much emphasis has been placed on understanding how elevated [CO_2] affects litter [N]. O'Neill and Norby (1996) suggested that most of the observed decline in litter [N] in response to elevated [CO_2] is a result of pot size limitations on root growth. However, in a meta-analysis of naturally senesced leaves grown in the field, Norby et al. (2001) found that litter N was significantly reduced by 7.1% in response to elevated [CO_2]. In our Aspen FACE experiment, elevated [CO_2] significantly lowered mean [N] by 10.8% for the aspen community and by 12.2% for the birch–aspen community. Our findings corroborate previous studies showing significantly decreased mean [N] in response to elevated [CO_2] for sweetgum litter at Oak Ridge FACE (Johnson et al. 2004) and for paper birch litter in an earlier study at Aspen FACE (Parsons et al. 2004). A small but insignificant decline in mean litter [N] was also observed in the elevated [CO_2] treatment at the Duke FACE experiment (Finzi and Schlesinger 2002). Our data survey indicates that the mean response ratio for litter N across three FACE sites (0.89) is lower than the mean of 0.94 reported by Norby et al. (2001), indicating a general reduction in litter N of 12.8% compared with the 7.1% reduction reported by Norby et al. (2001).

In contrast to the many studies on the effects of elevated [CO_2] on litter N, there has been little research on how elevated [O_3] influences litter [N]. Booker (2000) reported that elevated [O_3] increased foliar [N] in cotton, whereas Scherzer et al. (1998) found that elevated [O_3] had no significant effect on foliar [N] of yellow-poplar or white pine. In the earlier study at the Aspen FACE, Parsons et al. (2004) reported that elevated [O_3] had no effect on paper birch litter [N], but Lindroth et al. (2001) found that elevated [O_3] significantly reduced aspen foliar [N]. In our study, elevated [O_3] appeared to reduce mean litter [N] by 6.6% ($P = 0.073$), and significantly increased the litter C:N ratio ($P = 0.043$). Our literature survey suggests that elevated [O_3] has no consistent effect on leaf [N] (Figure 4), which is in line with the meta-analysis of Koricheva et al. (1998).

Soluble sugars, condensed tannins and soluble phenolics

In our study, concentrations of soluble sugars, condensed tannins and soluble phenolics generally increased in elevated [CO_2], but these changes were not statistically significant. Low replication is common in FACE experiments, and the low power of our test may explain why differences were not detected. Our literature survey indicates that elevated [CO_2] significantly increases concentrations of soluble sugars, condensed tannins and soluble phenolics (Figure 3), which is consistent with the findings of Peñuelas and Estiarte (1998).

We predicted that the phytotoxic effect of elevated [O_3] would lower concentrations of secondary compounds as a result of a reduction in carbohydrate supply. However, we found that elevated [O_3] significantly increased leaf litter concentrations of soluble sugars, soluble phenolics and condensed tannins, indicating that elevated [O_3] may trigger a biochemical defense or damage response that elevates synthesis of secondary compounds despite lower carbohydrate availability. The significantly higher concentration of soluble sugars could be a result of impaired phloem loading (Andersen 2003), which could also increase C supply within the leaf. Observed changes in concentrations of soluble phenolics and condensed tannins are consistent with previous studies (Friend and Tomlinson 1992, Booker and Miller 1998, Wustman et al. 2001) reporting that elevated [O_3] can alter C partitioning to defense processes by stimulating the phenylpropanoid pathway, which results in increased production of phenolic compounds. In contrast, several studies have reported that elevated [O_3] has no impact on concentrations of tannins or phenolics. For example, Parsons et al. (2004) found no significant effect of elevated [O_3] on the concentration of condensed tannins in birch litter. Our literature survey also suggests that elevated [O_3] has no significant effects on the concentrations of soluble sugars, phenolics and tannins (Figure 3). We found that elevated [CO_2] + elevated [O_3] resulted in the highest concentrations of soluble sugars and soluble phenolics (Table 2). These findings corroborate data from previous studies showing that the combined effects of elevated [CO_2] and elevated [O_3] can increase concentrations of soluble sugars and condensed tannins (Figure 3). Overall, these findings do not support our hypothesis that the effects of elevated [CO_2] and elevated [O_3] on litter chemistry cancel each other, and indicate that leaf-level responses to multiple factors are more complex than can be predicted from responses identified in single factor studies.

Lignin

In our Aspen FACE experiment, mean lignin concentration in ambient conditions was 28.6% in aspen litter and 32.4% in birch litter. These values are higher than have been reported in other FACE experiments (Norby et al. 2001, Finzi and Schlesinger 2002) and in earlier studies at the Aspen FACE facility (Parsons et al. 2004), but differences may be methodological. We ground whole leaves, including petioles, for biochemical analysis, so more lignified portions of the leaf may have been included than in other studies. Mean lignin concentration of our aspen litter was higher than the 21.6% reported for aspen stem wood (Hu et al. 1999), in agreement with findings that the lignin concentration of birch leaf litter (33.0%) can exceed that of birch wood (21.7%; Berg and McClaugherty 2003).

Overall, the relative responses of leaf litter lignin to the elevated [CO_2] and elevated [O_3] treatments were comparable with previous studies. In the Aspen FACE experiment, mean lignin concentration was higher in elevated [CO_2] than in ambient conditions, but this was not statistically significant, which is consistent with findings at the Duke FACE facility (Finzi and Schlesinger 2002) and in an earlier study at the Aspen FACE facility (Parsons et al. 2004). Again, the limited statistical power of individual FACE experiments may obscure real but small effects of elevated [CO_2] on tissue chemistry. Across experiments, Norby et al. (2001) showed a significant increase (6.5%) in litter lignin concentration in elevated [CO_2].

Chemical fluxes

Strain and Bazzaz (1983) hypothesized that elevated [CO_2] could trigger a negative feedback between biomass production and nutrient availability if a decrease in nutrient concentrations of litter slows decomposition rates, nutrient release rates, and nutrient supply to growing plants. In our Aspen FACE experiment, elevated [CO_2] reduced mean litter [N] and increased stand-level NUE by 12.5% compared with control plots ($P = 0.05$), but also increased the flux of litterfall N to the forest floor by 13.9%. Given lower [N], this increase probably did not result from reduced translocation rates in response to elevated [CO_2]. More likely, increased N flux is associated with enhanced N uptake from soil (Finzi et al. 2001) or perhaps enhanced N translocation from wood. Evidence for the former includes the finding in the Aspen FACE facility that N transferred from inorganic pools to organic pools is greater in elevated [CO_2] than in ambient [CO_2] (Holmes et al. 2003), which indicates that trees may absorb more N from soil to support increased growth. Johnson et al. (2004) also found that elevated [CO_2] resulted in an increased uptake of N in sweetgum. This increased uptake capacity may relate to higher total belowground C allocation in elevated [CO_2] (King et al. 2001*b*, Giardina et al. 2005).

Elevated [CO_2] had no significant effects on concentrations of soluble sugars, soluble phenolics, condensed tannins or cellulose, but significantly elevated litter production rates resulting in higher fluxes of these compounds to soil. From an ecosystem perspective, increased fluxes of soluble sugars and cellulose to the forest floor provide labile C sources to soil microbial communities, in part explaining the higher soil respiration rates observed in plots fumigated with elevated [CO_2] (King et al. 2001*b*, 2004). Higher concentrations of cellobiohydrolase, an extracellular microbial enzyme that degrades cellulose, have been observed in soil in elevated [CO_2] (Larson et al. 2002, Phillips et al. 2002).

Elevated [O_3] significantly increased litter C:N in our Aspen FACE experiment, and the combination of lower [N] and lower litter production rates reduced N fluxes to the forest floor. Further, the reduced N flux resulting from elevated [O_3] was associated with increased returns of tannins and phenolics. These findings lead us to conclude that regional increases in tropospheric [O_3] will retard litter decomposition rates and N cycling in northern hardwood forests. This conclusion is consistent with the finding of Holmes et al. (2003) that elevated [O_3] reduces gross N mineralization rates and microbial biomass N.

We found a near significant $CO_2 \times O_3$ interaction for leaf litter lignin flux to the forest floor ($P = 0.065$). To examine this potential interaction, we conducted a paired *t* test analysis and showed that, under ambient [O_3] conditions, lignin flux was significantly higher in elevated [CO_2] than in ambient [CO_2] ($P = 0.037$). However, under elevated [O_3] conditions, the effect of elevated [CO_2] was not significant, indicating that ele-

vated [O_3] may offset elevated-[CO_2]-related increases in lignin input to soil. This result is consistent with the finding that elevated [O_3] could reduce soil C formation under elevated [CO_2] conditions (Loya et al. 2004). Further, elevated [CO_2] and elevated [O_3] treatments can impact the soil microorganisms that control litter decomposition (Larson et al. 2002, Phillips et al. 2002, Loranger et al. 2004). Additional studies are needed to elucidate these multi-level controls on C and N cycling and retention in forests exposed to changing atmospheric conditions.

In conclusion, elevated [CO_2] significantly increased litter production, whereas elevated [O_3] decreased it, and in plots exposed simultaneously to both trace gases, the positive effects of elevated [CO_2] were largely eliminated by elevated [O_3]. Increases in soluble sugars and soluble phenolics caused by elevated [O_3] were most pronounced in the presence of elevated [CO_2]. Overall, elevated [CO_2] had little effect on the concentrations of soluble sugars, soluble phenolics, condensed tannins and cellulose, but reduced [N] in litter. Because of higher litter production rates, however, fluxes of these constituents all increased significantly in the elevated [CO_2] treatment. Similarly, elevated [O_3] had little impact on litter N and lignin concentrations, but reduced litter production led to significantly lower N and lignin fluxes to the forest floor. Our results indicate that the small changes in litter chemistry caused by elevated [CO_2] or elevated [O_3], combined with changes in litter production rates, will significantly alter stand-level chemical inputs to soil.

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