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ORIGINAL ARTICLE

Effects of long-term elevated CO_2 treatment on the inner and outer bark chemistry of sweetgum (*Liquidambar styraciflua* L.) trees

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Abstract

Key message **Long-term exposure of sweetgum trees to elevated atmospheric CO_2 concentrations significantly shifted inner bark (phloem) and outer bark (rhytidome) chemical compositions, having implications for both defense and nutrient cycling.**

Abstract Changes in plant tissue chemistry due to increasing atmospheric carbon dioxide (CO_2) concentrations have direct implications for tissue resistance to abiotic and biotic stress while living, and soil nutrient cycling when senesced as litter. Although the effects of elevated CO_2 concentrations on tree foliar chemistry are well documented, the effects on tree bark chemistry are largely unknown. The objective of this study was to determine the effects of a long-term elevated CO_2 treatment on the contents of individual elements, extractives, ash, lignin, and polysaccharide sugars of sweetgum (*Liquidambar styraciflua* L.) bark. Trees were harvested from sweetgum plots equipped with the Free-Air CO_2 Enrichment (FACE) apparatus, receiving either elevated or ambient CO_2 treatments over a 12-year period. Whole bark sections were partitioned into inner bark (phloem) and outer bark (rhytidome) samples before analysis. Principal component analysis, coupled with either Fourier transform infrared spectroscopy or pyrolysis–gas chromatography–mass spectrometry data, was also used to screen for differences. Elevated CO_2 reduced the N content (0.42 vs. 0.35 %) and increased the C:N ratio (109 vs. 136 %) of the outer bark. For the inner bark, elevated CO_2 increased the Mn content (470 vs. 815 mg kg^{-1}), total extractives (13.0 vs. 15.6 %), and residual ash content (8.1 vs. 10.8 %) as compared to ambient CO_2; differences were also observed for some hemicellulosic sugars, but not lignin. Shifts in bark chemistry can affect the success of herbivores and pathogens in living trees, and as litter, bark can affect the biogeochemical cycling of nutrients within the forest floor. Results demonstrate that increasing atmospheric CO_2 concentrations have the potential to impact the chemistry of temperate, deciduous tree bark such as sweetgum.

Keywords Ash · Climate change · Extractives · Lignin · Phloem · Rhytidome

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Introduction

The global atmospheric concentration of carbon dioxide (CO_2) has risen by 35 % over pre-industrial levels and is expected to continue to rise through the end of the century (IPCC 2007). For plants that only use the Calvin cycle (C_3 plants), photosynthesis is still limited by current atmospheric CO_2 concentrations (Norby et al. 2001; Körner 2006; Natali

et al. 2009). Exposure to elevated CO_2 concentrations generally increases plant photosynthetic carbon fixation rates, leading to increases in net primary production (Gielen and Ceulemans 2001; Ainsworth and Long 2005; Norby et al. 2005) if other resources such as soil nutrients are not limiting (Norby et al. 2010). In addition to carbon fixation, plants extract micro- and macronutrients from the soil for assimilation into a wide spectrum of simple and complex chemical compounds, with some of these ending up in phloem (inner bark) tissues that transport sugars and other compounds from the source leaves to various plant sinks, or into periderm tissues that protect the plant against abiotic and biotic stressors (Franceschi et al. 2005). Plant litter from abscised tissues, or whole-plant mortality, returns those elements to the soil where litter decomposition rates are dependent on structure and chemical compositions (Talbot and Treseder 2012; Van Nevel et al. 2014). Thus, any chemical shifts in the inner bark (phloem) or outer bark (rhytidome) caused by elevated CO_2 concentrations would have impacts on tree vascular function, sensitivity of living trees to herbivores and pathogens, and biogeochemical cycling of nutrients within the forest floor.

Decreased foliar levels of the macronutrient nitrogen are common under elevated CO_2 concentrations as a result of greater photosynthetic N-use efficiency (Saxe et al. 1998; Peterson et al. 1999; Calfapietra et al. 2007). The impact of elevated CO_2 concentrations on other plant nutrients, including micronutrients, is not well understood (Loladze 2002) and may be complicated by increases or decreases of specific elements based on their particular functionality in metabolic or structural processes (Natali et al. 2009). To date, numerous studies have compared wood (xylem) chemistry (Kaakinen et al. 2004; Luo et al. 2008; Kostiainen et al. 2008, 2009; Kim et al. 2015) and growth/anatomy (Yazaki et al. 2005; Kilpeläinen et al. 2007; Overdieck et al. 2007; Kostiainen et al. 2008, 2009; Watanabe et al. 2010) under ambient or elevated CO_2 treatments. In the wood chemistry studies, the macronutrient N was not affected by elevated CO_2; wood micronutrient concentrations were not determined. Extractive contents (e.g., nonstructural polysaccharides, fatty acids) increased for birch wood (Kostiainen et al. 2008) from elevated CO_2, whereas no effects were observed for aspen, sweetgum, or spruce woods (Kaakinen et al. 2004; Kostiainen et al. 2008, 2009; Kim et al. 2015). Quantitative analyses of the cell wall polymers (i.e., cellulose, lignin) in wood indicated no significant differences for trees from elevated CO_2 (Kaakinen et al. 2004; Luo et al. 2008; Kostiainen et al. 2008, 2009; Kim et al. 2015), the exception being lower hemicellulose concentrations observed for birch wood (Kaakinen et al. 2004). Among the very few studies investigating the effects of elevated CO_2 on bark, total bark tissue thicknesses were unchanged in *Picea abies* trees (Kostiainen et al. 2009), but increased in *Abies faxoniana* seedlings (Qiao et al. 2008); consistent effects of elevated CO_2 on nonstructural carbohydrates were not observed in *Populus nigra* trees (Luo et al. 2006).

Prior studies at the Oak Ridge National Laboratory sweetgum Free-Air CO_2 Enrichment (FACE) site have indicated that elevated CO_2 concentrations initially increased net primary productivity and stem biomass (Norby et al. 2005), but the effect declined through time due to soil resource limitations (Garten et al. 2011) that reduced nitrogen availability for photosynthesis (Warren et al. 2015) and reduced net primary productivity (Norby et al. 2010). Results also indicated that shifts in internal carbon allocation led to increased root biomass and distribution (Norby et al. 2004; Iversen 2010), soil nutrient extraction (Garten et al. 2011), and plant nutrient sequestration (Norby et al. 2010)—including increases in base cation (Ca, Mg) concentrations in the wood (Kim et al. 2015). There was also substantially reduced transpiration (Warren et al. 2011) indicating less movement of soil water and mass flow of dissolved nutrients into the roots.

The effects of elevated CO_2 treatment on sweetgum tree bark have not previously been investigated and bark tissues from other species have received very little attention. The lack of data is disconcerting, since the bark serves essential biological and ecological functions of the living tree. The essentially dead outer bark serves as a barrier, sealing moisture within, and providing passive protection against damaging agents (e.g., fungi, insects) and events (e.g., fire). Active protection is afforded by the living inner bark through responses to wounding and pathogen invasion; of course, arguably the most critical function of the inner bark is the translocation of the products of photosynthesis. As a tree grows, the outermost zones of the inner bark are converted into the innermost layers (i.e., periderms) of the persistent outer bark, such that the extant tissue represents development over many years. Long-term treatments, such as in FACE experiments, are necessary to analyze for any impacts of elevated CO_2 on the outer bark, analogous to wood by its cumulative deposition; however, unlike wood, the individual periderms comprising the outer bark are neither concentric, specifically formed annually, nor are they retained for the life of the tree (Eberhardt 2013).

In this study, we leveraged a long-term (12-year) FACE field experiment in sweetgum trees to explore the effects of elevated CO_2 concentrations on bark chemical characteristics. Since foliar nitrogen content, carbon allocation patterns, and wood cation concentrations were all strongly affected by elevated CO_2 concentrations, we hypothesized that shifts in nutrient concentrations in bark would occur. Phenylpropanoid metabolism appears to be related to nitrogen metabolism with the recycling of nitrogen from phenylalanine affording the production of phenolic

extractives and lignin (van Heerden et al. 1996). With increased partitioning to the phenylpropanoid pathway under elevated CO_2 concentrations (Mattson et al. 2005), we hypothesized increased C allocation to the production of extractives and lignin. Age-related changes in outer bark extractives chemistry, such as secondary reactions in tannins (Matthews et al. 1997), are not directly linked to active metabolism; therefore, we hypothesized the effects of the elevated CO_2 treatment on extractives contents to be greater in the inner bark than in the outer bark. To test these hypotheses, we measured elemental concentrations of micro- and macronutrients, residual ash (a measure of the total concentration of inorganic constituents), yields of extractives recovered by sequential solvent extractions, acid-insoluble lignin, and polysaccharide sugars. Principal component analysis (PCA) was applied to Fourier transform infrared (FTIR) spectroscopy and pyrolysis–gas chromatography–mass spectrometry (pyrolysis–GC–MS) data as a rapid analysis technique to screen for compositional differences. Herein, this study represents the first-ever characterization of the inner bark apart from the outer bark for trees subjected to a long-term treatment with an elevated CO_2 concentration.

Materials and methods

Free-air CO_2 enrichment

The FACE experiment was conducted in a sweetgum (*Liquidambar styraciflua* L.) plantation located in the Oak Ridge National Laboratory Research Park, Roane County, Tennessee, USA. The study site coordinates were 35°54′N, 84°20′W. The soil was classified as Aquic Hapludult, with a silty clay loam texture with moderate drainage; soil pH and nutrient concentrations were reported by Johnson et al. (2004). The stand was planted in 1988 (1.2 m × 2.3 m spacing) with 1-year-old seedlings. Five 25-m-diameter treatment rings were sited in a staggered arrangement with four of the treatment rings encircled with FACE apparatus (radial blower, plenum, vent pipes). Beginning in 1998, two of the treatment rings released a targeted ~550 ppm CO_2 during the daytime through the growing season of April through October. The remaining three treatment rings, two of these having FACE apparatus, experienced ambient CO_2 (~393 ppm) concentrations. Fumigation with CO_2 ceased in late September 2009.

Bark collection and processing

Ten trees, each assigned a number for identification, were harvested in July 2009. During the harvest, 40-cm stem sections were taken at 60 cm above the ground level. Sweetgum bark has deep furrows by the age of 25 years (Gilman and Watson 1993) and thickness measurements change with diurnal patterns of tree water status and phloem (inner bark) loading (Mencuccini et al. 2013), as well as relative humidity of the surrounding air (Gall et al. 2002). While we did not make detailed measurements in the 22-year-old stand, whole bark thicknesses estimated in situ ranged from approximately 0.6 to 1.3 cm as a function of tree diameter from 7 to 22 cm, independent of the CO_2 treatments; dried inner bark thicknesses ranged from 2 to 4 mm. Six of the trees were from the two treatment plots that received CO_2 enriched air. The remaining four trees were from two control plots with FACE apparatus that received ambient CO_2 air. No trees were selected from the single ambient CO_2 plot without apparatus. Stem sections were transported on ice to the laboratory and placed in a walk-in freezer for storage until analysis.

Partitioning the inner bark from the outer bark was deemed necessary in the current study given results from other bark studies showing differences between them in extractive content and PCA data (So and Eberhardt 2006; Eberhardt 2012). Tree sections were briefly thawed to allow nearly intact strips of whole bark (i.e., inner and outer bark still together) to be peeled from the wood at the cambium around the circumference, keeping bark samples separated by both tree number and quadrants for cardinal direction. Subsequent processing of whole bark sections, from northern and southern directions, separated the inner bark from the outer bark by careful peeling facilitated by the use of a woodworking knife. Samples from the northern and southern orientations were not pooled, but analyzed separately, and the resultant data averaged for each tree. Small amounts of inner bark still adhering to the outer bark samples were gently removed by scraping with the blade; likewise, any outer bark still adhering to the inner bark samples was removed. The outer surface of the outer bark samples was also gently scraped with the blade to remove adhering lichen/moss. Partial removal of the outermost layer(s) of the outer bark was unavoidable. Partitioned bark samples were freeze dried before grinding in a Wiley mill equipped with a 2-mm mesh screen; heat generation during grinding was negligible. Moisture contents, determined by drying subsamples at 100 °C, were used to account for any reuptake of moisture during grinding/handling/storage. All quantitative data are reported on the basis of dry mass.

Ash and elemental analyses

Ash content is a determination of the percentage of inorganic elements (as oxides and salts) on the basis of the mass of dry woody tissue. The ash contents of unextracted bark samples were determined by combustion in a muffle furnace (525 °C, 6 h). Carbon, hydrogen, and nitrogen

concentrations were determined using a Perkin Elmer 2400 Series II CHNS/O analyzer. Inorganic elements were analyzed (Lindsey et al. 2013) by inductively coupled plasma-optical emission spectroscopy (ICP-OES) using a Perkin Elmer Optima 7300 DV spectrometer. Samples (0.5 g) were suspended in a mixture of 8 mL HNO_3 (trace metal grade, 67–70 %), 3 mL H_2O_2 (35 %), and 0.2 mL HF (48 %) followed by digestion at 210 °C (20 min) using an Anton Parr Multiwave 3000 microwave digester. After digestion, 4 % (w/w) boric acid (1 mL) was added and diluted with deionized water to make a total volume of 50 mL. The cooled solutions were filtered (0.45 µm, Teflon filter) before determination of inorganic elements.

Extractive analysis

Extractives represent isolates of mostly organic compounds present in plant tissues that are soluble in a particular organic or aqueous solvent. Sequential extractions with solvents of increasing polarity are routinely used with bark tissues (McGinnis and Parikh 1975; Labosky 1979; Eberhardt 2012), because they afford a progressive and exhaustive removal of a broad spectrum of extractive compounds. Processed (i.e., dried, milled) inner and outer bark samples (ca. 7 g) were sequentially extracted (6 h) using Soxhlet apparatus with reagent-grade organic solvents of increasing polarity (hexane, acetone, 95 % ethanol) followed by deionized water. Organic solvent extracts were concentrated by rotary evaporation. The resultant oils were transferred to glass vials using small amounts of solvent for washing. The extracts were then evaporated further under a stream of nitrogen to obtain residues that were dried in vacuo over a desiccant (Drierite) before weighing. Aqueous extracts, likely comprising water-soluble non-structural carbohydrates (Raessler et al. 2010), extractives, and inorganics, were freeze dried and weighed. The extractive-free inner bark and outer bark tissues were dried in an oven (100 °C) before weighing.

Furthermore, we also had access to small inner bark samples (1-cm-diameter punches) collected from the base of the stem from five trees per plot in early June 2009. For these samples, after freeze drying, we assessed only the total ethanol (71 %) extractable phenolics based on the nonspecific Folin–Ciocalteu assay (Ainsworth and Gillespie 2007). Samples were analyzed in duplicate and considered valid if within 5 % of one another; results were reported as gallic acid equivalents (GAE ± SD).

Lignin and hydrolyzed polysaccharide sugar analyses

Extractive-free inner and outer bark samples, to be used for acid-insoluble lignin (Klason lignin) and hydrolyzed polysaccharide sugar analyses, were ground further to pass a 1-mm screen. Dried subsamples (100 mg) were hydrolyzed in 72 % (w/w) sulfuric acid (1 h, 30 °C) and diluted to 4 % (w/w) sulfuric acid with distilled water. Fucose was added as an internal standard, and subsamples were further hydrolyzed (1 h, 120 °C). After cooling, an aliquot of the hydrolysate was analyzed for polysaccharide sugars using the method of Davis (1998); lignin content was determined as described by Effland (1977) except that the acid-insoluble lignin was filtered using a glass fiber filter (Whatman 934-AH) in a Gooch crucible.

Fourier transform infrared spectroscopy

Extractive-free inner bark samples and the extractive solids (oils, powders) were scanned (650–4000 cm^{-1} spectral range, 8 cm^{-1} spectral resolution, 32 scans per spectrum) using a Thermo Nicolet Nexus Model 670 FTIR spectrometer equipped with a Golden Gate MKII Single Reflection ATR accessory. The ATR pressure anvil was applied to the bark samples and extractive powders to ensure sufficient contact with the diamond window. Spectra used for PCA included those from three independently scanned subsamples.

Pyrolysis–gas chromatography–mass spectrometry

Inner bark samples (200 µg), without extraction, were weighed into stainless steel cups and pyrolyzed (12 s, 450 °C) using a Frontier EGA/PY-3030 D pyrolyzer; each sample was analyzed five times. Separations of the pyrolysis vapors were carried out on a Perkin Elmer Clarus 680 gas chromatograph with an Elite 17 MS (cross bond 50 % phenyl- 50 % methyl polysiloxane) capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness). The split ratio was 80:1 with helium as the carrier gas (1 mL/min); the oven temperature for the gas chromatograph was held at 50 °C for 4 min and then ramped to 270 °C (5 °C/min) requiring a total analysis time of 48 min. Peaks representing individual pyrolysis degradation products were identified using a Perkin Elmer Clarus SQ 8 GC mass spectrometer.

Statistical analysis

This experiment was analyzed as a completely random design with two replicates of the main effects of elevated CO_2 treatment on the chemical analysis data. After averaging with PROC SUMMARY in SAS® 9.3, a one-way analysis of variance was conducted on plot means using PROC GLM in SAS/STAT® 9.3. Due to the small sample size, a determination of nonsignificance was made for all tests with a $P_\alpha > 0.1$. The control data corresponded to only the two ambient CO_2 plots with the FACE apparatus.

Multivariate analyses of the inner bark FTIR spectroscopy and pyrolysis–GC–MS data were performed using the CAMO Unscrambler (version 8.0) software. Multiplicative scatter correction was applied to remove the systematic variations in the spectral data unrelated to the properties studied. Principal component analysis (PCA) was performed on the spectral data to observe differences and groupings between the sample sets. Pyrolysis–GC–MS chromatograms were analyzed in an analogous manner; the chromatogram peak, assigned to pyrolysis-derived CO_2, was removed from the data to increase the sensitivity of the PCA.

Results

Ash and elemental analyses

Comparison of the ash content (Table 1) between the elevated and ambient CO_2 bark samples showed a significantly higher (10.8 vs. 8.1 %, $P_\alpha = 0.062$) value for the inner bark, but not the outer bark ($P_\alpha = 0.336$). Coinciding with the higher ash content for the elevated CO_2 inner bark was a significantly lower concentration of carbon (35.9 vs. 40.1 %, $P_\alpha = 0.064$); values for the outer bark were not different ($P_\alpha = 0.856$). A similar trend was shown for the hydrogen content (4.4 vs. 5.1 %, $P_\alpha = 0.092$) in the elevated CO_2 inner bark. While nitrogen concentrations were not different for the inner bark, they were lower for the outer bark of elevated CO_2 trees ($P_\alpha = 0.016$); this coincided with a higher C/N ratio (136 vs. 109, $P_\alpha = 0.046$) for the outer bark. Analysis of the inner bark samples following solvent extraction gave no significant differences ($P_\alpha > 0.1$) in either carbon or hydrogen concentrations (data not shown). Analysis of the inner bark by ICP-OES (Table 2) showed mostly no differences between elevated and ambient CO_2 samples, the exception being higher Mn content (815 vs. 470 ppm, $P_\alpha = 0.040$) for the elevated CO_2 treatment.

Inner and outer bark extractives

Sequential extractions of the inner and outer bark samples afforded increasing extractive values as the polarity of the solvent increased from hexane to water (Table 3). The hexane extractive contents for the elevated CO_2

Table 1 Effect of elevated CO_2 on ash, carbon, hydrogen, and nitrogen contents of sweetgum inner and outer bark tissues

	Ash (%)	Carbon (%)	Hydrogen (%)	Nitrogen (%)	C/N ratio
Inner bark					
Ambient CO_2	8.1 ± 0.1	40.1 ± 0.3	5.1 ± 0.2	0.32 ± 0.01	127 ± 2.5
Elevated CO_2	10.8 ± 1.0	35.9 ± 1.5	4.4 ± 0.3	0.24 ± 0.04	150 ± 20
P_α value	0.062	0.064	0.092	0.149	0.255
Outer bark					
Ambient CO_2	7.1 ± 0.2	46.2 ± 0.2	5.2 ± 0.2	0.42 ± 0.00	109 ± 1.0
Elevated CO_2	7.9 ± 0.8	46.1 ± 0.5	5.2 ± 0.1	0.35 ± 0.01	136 ± 8.4
P_α value	0.336	0.856	0.542	0.016	0.046

Data are the mean percentages of dry matter ± SD, the exception being the C/N ratio which is without units

Table 2 Effect of elevated CO_2 on elemental concentrations of sweetgum inner bark tissue

Element	Ambient CO_2 (mg kg^{-1})	Elevated CO_2 (mg kg^{-1})	P_α value
Na	74.6 ± 18.2	96.7 ± 24.6	0.415
Mg	1130 ± 74.7	1180 ± 21.8	0.458
Al	71.2 ± 14.4	66.3 ± 11.3	0.742
Si	1860 ± 292	2000 ± 114	0.579
P	457 ± 29.5	434 ± 6.16	0.381
S	914 ± 111	1090 ± 5.59	0.148
K	2760 ± 142	2670 ± 56.5	0.497
Ca	36,500 ± 5650	44,900 ± 228	0.169
Mn	470 ± 79.2	815 ± 62.9	0.040
Fe	18.4 ± 10.3	31.0 ± 28.6	0.618
Zn	25.5 ± 1.91	19.8 ± 7.91	0.430
Ba	223 ± 99.0	256 ± 40.1	0.706

Data shown are the mg kg^{-1} concentrations of dry matter ± SD

Table 3 Effect of elevated CO_2 on extractive contents of sweetgum inner and outer bark tissues

	Extraction solvents				Total extractives (%)
	Hexane (%)	Acetone (%)	Ethanol (%)	Water (%)	
Inner bark					
Ambient CO_2	0.38 ± 0.01	1.27 ± 0.01	4.65 ± 0.40	6.71 ± 0.42	13.01 ± 0.02
Elevated CO_2	0.37 ± 0.03	1.59 ± 0.05	5.89 ± 0.62	7.75 ± 0.32	15.60 ± 1.01
P_α value	0.824	0.010	0.141	0.108	0.068
Outer bark					
Ambient CO_2	1.92 ± 0.03	3.00 ± 0.54	2.42 ± 0.24	4.01 ± 0.77	11.4 ± 0.50
Elevated CO_2	1.92 ± 0.20	2.00 ± 0.40	2.93 ± 0.15	4.91 ± 0.81	11.8 ± 1.56
P_α value	0.986	0.172	0.129	0.759	0.373

Data are the mean percentages of dry matter ± SD

(0.37 %) and ambient CO_2 (0.38 %) inner bark were the lowest among the four solvents used and showed no significant difference ($P_\alpha = 0.824$). A higher acetone extractive content was observed in the elevated CO_2 inner bark (1.59 vs. 1.27 %, $P_\alpha = 0.010$). Although the contents of ethanol (5.89 vs. 4.65 %) and water-soluble extractives (7.75 vs. 6.71 %) for the elevated CO_2 inner bark seemed to follow a similar trend, they did not meet the criterion for statistical significance ($P_\alpha < 0.1$). The total extractive content for the elevated CO_2 inner bark was also higher than that for the ambient CO_2 inner bark (15.60 vs. 13.01 %, $P_\alpha = 0.068$). Results for the outer bark showed no significant difference ($P_\alpha > 0.1$) for elevated and ambient CO_2 extractive content with any solvent. The ethanol-soluble total phenolic content of the inner bark punches collected in June was similar for elevated (87.8 ± 0.7 mg g^{-1} GAE) and ambient (86.8 ± 0.6 mg g^{-1} GAE) CO_2 treatments ($P_\alpha = 0.37$).

FTIR spectroscopy of inner bark extracts

Screening of the ambient CO_2 inner bark extracts by infrared spectroscopy gave spectra (Fig. 1) with strong bands at 2800–3000 cm^{-1} (C-H stretch) and 1700–1750 cm^{-1} (C = O stretch) for the hexane and acetone extracts (Fig. 1a, b), consistent with the chemical functionalities of waxes and fatty acids. Bands assigned to hydroxyl (O–H stretch, 3100–3600 cm^{-1}) and aromatic (ca. 1600 cm^{-1}, C=C stretch) functionalities were readily apparent in the spectra of the extracts isolated with the three most polar solvents, acetone, ethanol, and water (Fig. 1b–d); likely, compounds include highly polar extractives such as hydrolysable tannins and their precursors. Visual inspection of the FTIR spectrum from each individual elevated CO_2 inner bark extract showed no obvious differences from the corresponding ambient CO_2 spectrum; thus, only the ambient CO_2 spectra are shown in Fig. 1.

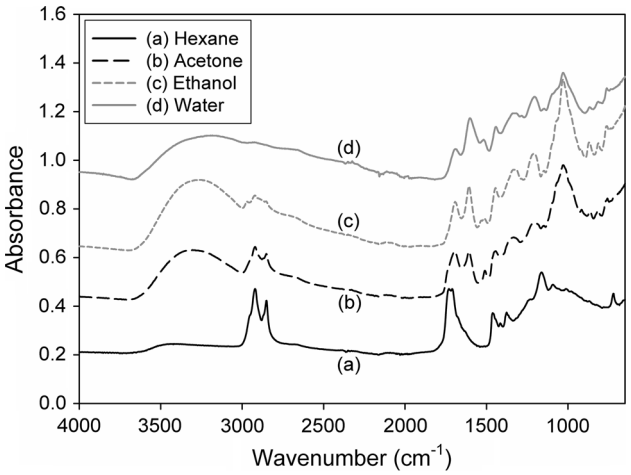

Fig. 1 FTIR spectra of extracts from ambient CO_2 sweetgum inner bark. Strong bands at 2800–3000 cm^{-1} and 1700–1750 cm^{-1} in hexane (*a*) and acetone (*b*) extracts are consistent with waxes and fatty acids; bands assigned to hydroxyl (3100-3600 cm^{-1}) and aromatic (ca. 1600 cm^{-1}) functionalities were apparent in the more polar acetone (*b*), ethanol (*c*), and water (*d*) extracts

Lignin and hydrolyzed polysaccharide sugar analyses

Analyses of the extractive-free inner and outer bark tissues showed no difference in lignin content as determined by the acid-insoluble residue following acid hydrolysis (Table 4). There was also no difference in the total yield of the five polysaccharide sugars found in plant cell walls. An increase in galactose (1.23 vs. 1.31 %, $P_\alpha = 0.025$) was offset by a decrease in xylose (8.88 vs. 7.22 %, $P_\alpha = 0.068$) for the elevated CO_2 inner bark. The polysaccharide sugars in the outer bark were nearly equivalent, the exception being a subtle decrease in arabinose (2.57 vs. 2.41 %, $P_\alpha = 0.017$).

Table 4 Effect of elevated CO_2 on lignin, and polysaccharide sugar analyses of extractive-free sweetgum inner and outer bark tissues

	Acid-insoluble lignin (%)	Polysaccharide sugars					
		Arabinose (%)	Galactose (%)	Glucose (%)	Xylose (%)	Mannose (%)	Total (%)
Inner bark							
Ambient CO_2	17.38 ± 2.85	2.86 ± 0.34	1.23 ± 0.08	33.18 ± 2.24	8.88 ± 1.06	1.20 ± 0.10	47.35 ± 3.06
Elevated CO_2	15.39 ± 3.24	3.19 ± 0.46	1.31 ± 0.11	29.50 ± 2.36	7.22 ± 1.24	1.18 ± 0.12	42.39 ± 3.25
P_α value	0.240	0.172	0.025	0.125	0.068	0.682	0.141
Outer bark							
Ambient CO_2	40.48 ± 1.36	2.57 ± 0.20	1.35 ± 0.46	17.47 ± 1.55	5.61 ± 0.81	1.23 ± 0.11	27.98 ± 2.18
Elevated CO_2	40.70 ± 1.99	2.41 ± 0.15	1.11 ± 0.11	17.66 ± 0.72	5.69 ± 0.49	1.28 ± 0.08	28.15 ± 1.22
P_α value	0.635	0.017	0.441	0.692	0.571	0.554	0.744

Data are the mean percentages of extractive-free dry matter ± SD

Principal component analysis

An initial PCA was performed on the spectra from all the inner bark extracts and showed four separate groupings, representing the four extract types, in the scores plot (not shown). Individual PCA plots for inner bark extracts gave overlapping points with no distinguishable grouping of elevated CO_2 samples separated from ambient CO_2 samples (Fig. 2). Likewise, comparisons of inner bark samples after extraction showed no obvious groupings indicative of spectral differences (plots not shown).

Major peaks in the pyrolysis–GC–MS chromatograms included typical biomass pyrolysis compounds such as aliphatic acids (e.g., acetic, propanoic), furfural, cyclic aliphatics (e.g., 4-acetyl-1-methycyclohexene), and phenolics (e.g., phenol, 2-methoxyphenol); peak assignments are shown in Table 5. PCA of the pyrolysis–GC–MS data from elevated and ambient CO_2 inner bark exhibited some group separation, with the elevated CO_2 data points showing a broader distribution than those for the ambient CO_2 data points (Fig. 3). Loadings plot for PC2 showed elevated CO_2 inner bark to have lower levels of levoglucosan and sucrose (peaks below baseline) and higher levels of aromatic fragments (peaks above baseline), typically derived from lignin (Fig. 4; Table 5).

Discussion

Key findings in the present study include that elevated CO_2 treatment increased ash content, Mn content, and total extractive content of the inner bark. For the outer bark, elevated CO_2 reduced the nitrogen content and increased the C:N ratio. Differences were observed for some hemicellulosic sugars, but not lignin. Principal component analysis coupled with FTIR spectroscopy did not show compositional differences in either individual extracts or cell wall polymers, the latter present in the extractive-free inner bark residues. Findings are compared with prior observations for wood from trees receiving elevated CO_2 treatments. Ramifications of such shifts in bark chemistry remain to be determined.

Ash and elemental analyses

Ash content is a ubiquitous proximate analysis applied to woody tissues (Toscano et al. 2013; Duca et al. 2014) providing a measure of the percentage of inorganic constituents on a dry-weight basis. Yet, in only one study to date was ash content compared between wood samples from a FACE study, showing elevated CO_2 wood with a significantly higher (+20 %) ash content (Kim et al. 2015). Here, we report the first ash content data for bark which shows a significantly higher (+33 %) value attributable to an elevated CO_2 treatment (Table 1). The biological and ecological consequences of such a dramatic increase are not known. Even a small increase in ash content almost always has a negative impact on the utilization of woody biomass, because ash is associated with problems in wood processing, ranging from tool wear during lumber production, deposits formed during pulp and paper processing operations, ash sintering during combustion and, of more recent concern, catalytic effects during biofuel conversions/syntheses (Porankiewicz et al. 2006; Lestander et al. 2012; López-González et al. 2014).

The relatively high proportion of ash in the inner bark, and its higher value with the elevated CO_2 treatment, undoubtedly contributed to the lower content of carbon and hydrogen (Table 1). Hydrogen content, commonly determined along with carbon content in biofuel studies, has hitherto not been reported for characterization of wood from FACE studies. Thus, it appears that an elevated CO_2 treatment impacted not only the carbon content of the inner bark, but also the corresponding hydrogen content. The

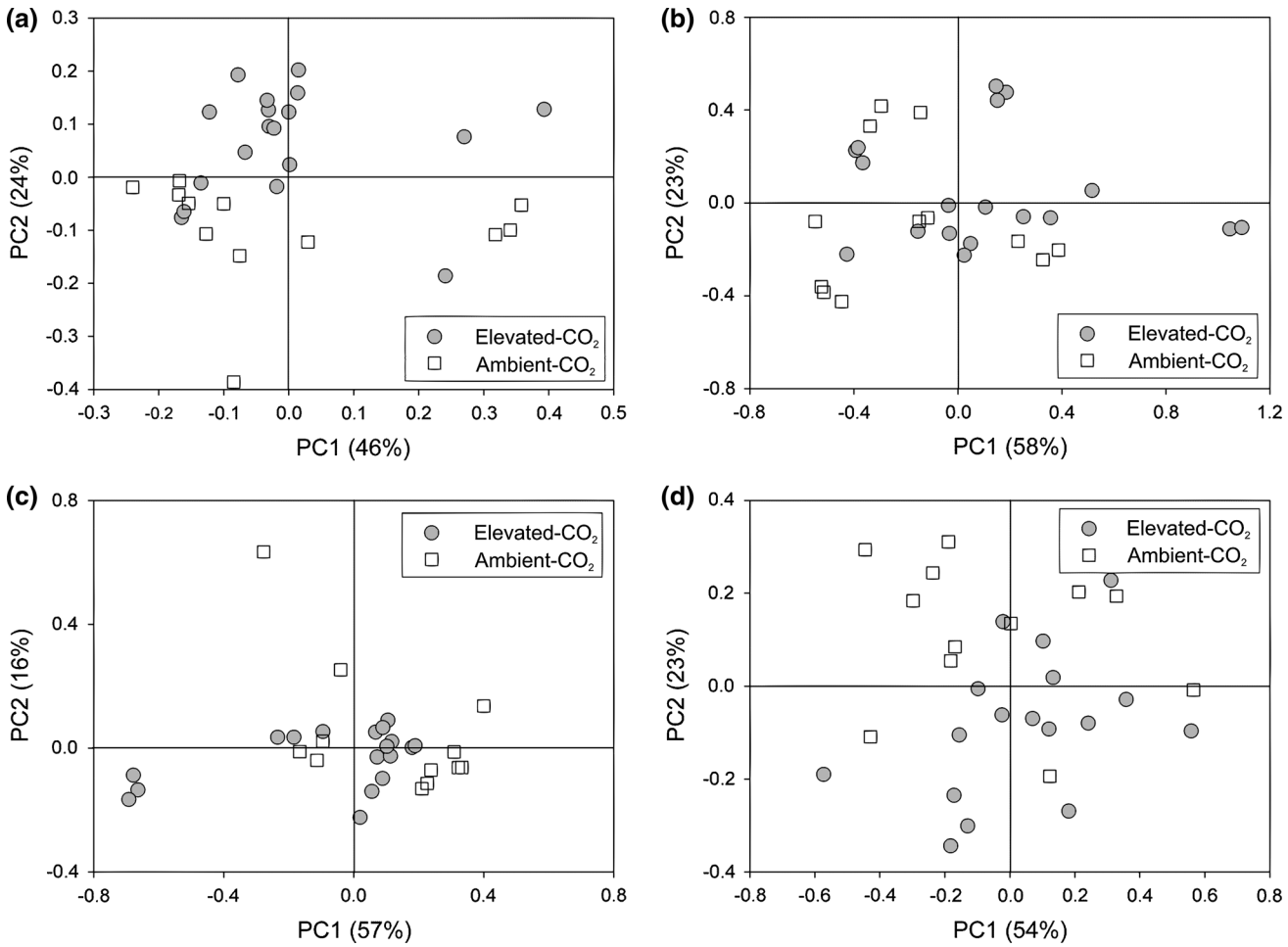

Fig. 2 PCA score plots of elevated and ambient CO_2 sweetgum inner bark hexane (**a**), acetone (**b**), ethanol (**c**), and water (**d**) extracts. The lack of grouping of ambient CO_2 extracts apart from elevated CO_2 extract suggests that no compositional differences were imparted by the elevated CO_2 treatment

Table 5 Peak assignments for selected compounds in pyrolysis–GC–MS loadings plot for PC2 (Fig. 4)

Signal	RT (min)	Compound	*m/z*	Assigned source
A	2.059	Acetic acid	60	Polysaccharides
B	8.627	Furfural	96	Polysaccharides
C	11.718	2-hydroxy-2-cyclopenten-1-one	98	Polysaccharides
D	16.500	2-methoxy-phenol	124	Lignin
E	21.372	Sucrose	57, 73	Polysaccharides
F	23.078	2-methoxy-4-vinylphenol	150	Lignin
G	26.514	*trans*-isoeugenol	164	Lignin
H	30.416	Levoglucosan	162	Polysaccharides

Assignments based on best mass spectrometry library match where *m/z* represents the predominant spectral ion(s)

RT retention time

caveat here is that we are also reporting an impact on both the extractives and ash contents of the inner bark. The carbon and hydrogen values were still lower even after adjusting the values to an ash-free basis. Conducting the carbon and hydrogen analyses on extractive-free inner bark gave essentially identical values between the elevated and ambient CO_2 samples (data not shown). Thus, we can attribute the differences in the inner bark carbon and

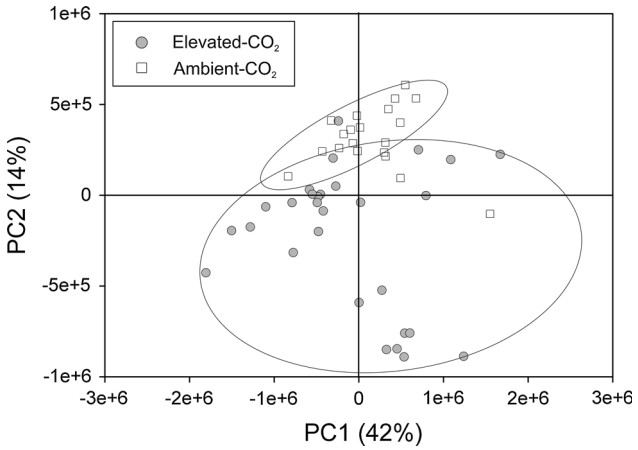

Fig. 3 PCA scores plot of pyrolysis–GC–MS data from elevated and ambient CO_2 sweetgum inner bark. Weak grouping of ambient CO_2 from elevated CO_2 inner bark suggests that some compositional differences were imparted by the elevated CO_2 treatment

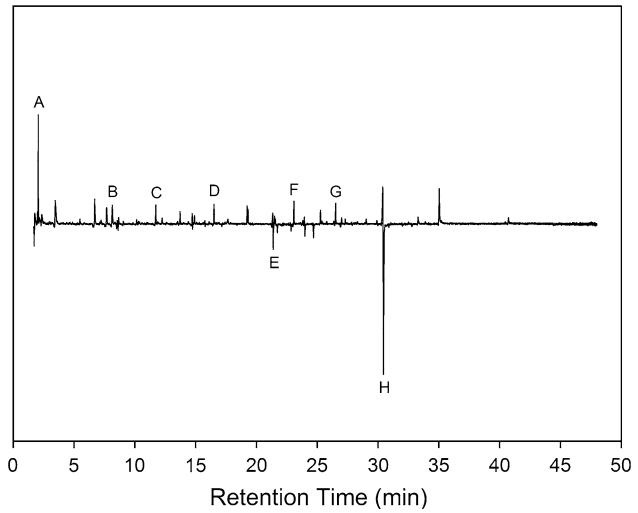

Fig. 4 Loadings plot for PC2 of pyrolysis–GC–MS data from elevated and ambient CO_2 sweetgum inner bark; see Table 5 for selected peak assignments

hydrogen contents to differences in the contents of the extractives; in the case of the aqueous extract, that would include water-soluble inorganics accompanying the nonstructural carbohydrates.

Curiously, nitrogen content determinations showed a difference for the outer bark as opposed to the inner bark. The difference in nitrogen content afforded a difference in the corresponding C/N ratio. Analyses of nitrogen contents and C/N ratios in wood from FACE studies have generally not shown an impact of an elevated CO_2 treatment (Kostiainen et al. 2008, 2009), the exception being results obtained for aspen (Kaakinen et al. 2004). It should be noted that while every effort was taken to remove relatively nitrogen-rich lichens and moss (Boltersdorf et al. 2014) from the outer bark by gentle scraping, a minor presence of these nonwoody plants (or their rooting structures) remains a possibility.

Inorganic analysis of inner bark samples showed the Mn content to be higher for the elevated CO_2 treatment (Table 2). It is interesting to note that analysis of trace metals in the sweetgum foliage also found increased Mn content, as well as Mo and Zn for the elevated CO_2 treatment (Natali et al. 2009). The same study reported a consistent increase in Mn (+28 %) across species and sites, and that of the ten elements sampled, Mn was the most responsive element (Natali et al. 2009). It was postulated that the increased foliar Mn concentration resulted from the increased photosynthesis in response to CO_2 enrichment, leading to an increased plant demand for Mn, which would increase inner bark Mn concentrations. Root uptake of limiting nutrients, such as N or Mn, would need to be increased under elevated CO_2. Results from the sweetgum site report greater fine root development and deeper rooting systems under elevated CO_2 treatments (Norby et al. 2004; Iversen 2010), which could potentially boost uptake of key resources despite lower transpiration rates (Warren et al. 2011) that reduce water uptake. Such observations could also explain the higher ash and Mn levels, with greater uptake of nutrient elements at the same or deeper soil levels as a result of differences in root system size and distribution.

Inner and outer bark extractives

Sequential extractions of sweetgum inner and outer bark samples showed increasing extract yields with increasing solvent polarity (Table 3), paralleling that reported for the sequential extraction of sweetgum whole bark (Choong and Fogg 1976). Compared to the inner bark, the outer bark had a higher proportion of nonpolar (hexane- and acetone-soluble) extractives (e.g., fatty acids, phenolics) and a lower proportion of polar (ethanol- and water-soluble) extractives (e.g., nonstructural carbohydrates). Intuitively, this reflects the hydrophilic nature of the inner bark and the hydrophobic nature of the outer bark. Detailed characterizations of individual extractive compounds were outside the scope of the current study. Simply for the purpose of confirmation, FTIR spectroscopy of the extracts showed that the nonpolar extractives spectra were consistent with fatty acids and waxes present in sweetgum resins (Hillis 1962); likewise, the polar extractive spectra were consistent with the hydrolysable tannin compounds isolated during extractions of sweetgum bark and wood (Choong and Fogg 1976; Spencer and Choong 1977).

Based on our results, we report that rising atmospheric CO_2 concentrations have the potential to increase the extractive content of sweetgum inner bark. Results for the

outer bark showed no differences for any of the extractive contents. The outer bark is derived from the inner bark by the processes of obliteration and periderm formation. These individual periderms comprising the outer bark are neither concentric nor specifically formed annually; thus unlike wood, it is not possible to assign the year a periderm was formed. Sensitivity may have been lost for the outer bark given that the elevated CO_2 treatment did not commence until 10 years after planting. Unless much of the oldest outer bark formed during the first 10 years of growth was sloughed off from weathering, the outer bark collected at the time of harvesting would not fully represent that for an elevated CO_2 treatment. It is likely that if whole bark had been used in the current study, any impact of the elevated CO_2 treatment on the extractives content would have been obscured.

The minimal deterioration of wood while remaining in the living tree allows for assessment of the cumulative impacts of elevated CO_2 treatments over many years; however, it should be noted that wood properties and chemistry do change with time as the living sapwood is continually transformed into dead heartwood. Studies to date on the extractives contents from wood have given mixed results when comparing elevated and ambient CO_2 samples. Whereas there was no difference for aspen (Kaakinen et al. 2004, Kostiainen et al. 2008) and spruce (Kostiainen et al. 2009), there was a higher extractive content for birch (Kostiainen et al. 2008) with elevated CO_2 treatment. In all the above-mentioned studies with wood, extractive contents were determined by extraction with acetone alone. Kostiainen et al. (2009) did extract soluble sugars from spruce wood with 80 % ethanol in water; however, there was no difference between the elevated and ambient CO_2 treatments. Our ethanol extraction of total inner bark phenolics gave essentially identical values (87.8 vs. 86.8 mg g^{-1} GAE) between elevated and ambient CO_2 treatments, but those reported for sweetgum wood (91 vs. 108 mg g^{-1} GAE) were significantly higher with elevated CO_2 treatment (Kim et al. 2015). The difference in phenolics between multi-year wood tissue and new inner bark tissue may be attributable to the progressive loss of a CO_2 photosynthetic response due to low N content (Warren et al. 2015), whereby the wood can accumulate the CO_2 responses through time, but the inner bark primarily reflects current conditions.

Lignin and hydrolyzed polysaccharide sugar analyses

The absence of significant differences in lignin content for both the inner bark and the outer bark, between elevated and ambient CO_2 treatments, parallels prior determinations for wood (Kaakinen et al. 2004; Luo et al. 2008; Kostiainen et al. 2008, 2009 ; Kim et al. 2015). The caveat herein is that the lignin contents we report must be interpreted only as relative values, knowing that the acid-insoluble (Klason) method can give seemingly inflated values for lignin content when applied to bark (Eberhardt 2012). Treatments of bark with alkaline solutions have been used to remove interfering non-lignin phenolics (McGinnis and Parikh 1975, Labosky 1979); however, the values for lignin content may still be higher than those obtained by other methods (Eberhardt 2012), thus providing no advantage to the present study. Efforts to remove purported interfering compounds from non-wood tissues may also lead to losses in lignin and/or those cell wall polymers on which the total composition is based (Hatfield and Fukushima 2005). Extracted inner bark samples exhibited low nitrogen content (0.29 ± 0.056 %) indicating that any interference from proteins was minimal (Eberhardt et al. 2010). If any increased partitioning to the phenylpropanoid pathway under elevated CO_2 (Mattson et al. 2005) had occurred as hypothesized, it did not appear to significantly impact lignin or any other acid-insoluble phenolics found in bark tissues.

Likewise, to provide the fewest analytical artifacts, the polysaccharide sugars were quantified with no attempt to partition the cellulose from the hemicelluloses, the former contributing exclusively to the glucose content. The absence of significant differences in total sugars and glucose contents for both the inner bark and the outer bark, between elevated and ambient CO_2 treatments, again parallels prior determinations for the wood (Kaakinen et al. 2004; Luo et al. 2008; Kostiainen et al. 2008, 2009; Kim et al. 2015). In those studies, cellulose was determined by a colorimetric assay of the hydrolysate of the residue remaining after hemicellulose dissolution (Anttonen et al. 2002); hemicelluloses were determined by an indirect method. Kaakinen et al. (2004) provided the only instance where a hemicellulose determination gave a lower value for elevated CO_2 wood. Herein, significant differences in the inner bark values for galactose and xylose also provide evidence for changes not only to hemicellulose content, but also to composition.

Principal component analysis

Focusing on the inner bark, PCA coupled with either FTIR spectroscopy or pyrolysis–GC–MS data was performed as screening tool for detecting compositional differences imparted by the elevated CO_2 treatment. The complexity of the bark, and the corresponding FTIR spectrum, does not permit the assignment of single bands to single components (Chen et al. 2010); however, FTIR spectroscopy coupled with PCA can be used to detect subtle spectral differences imparted by a treatment. It should be noted that the

sensitivity of the technique was sufficient to detect differences in beech wood only differing in its site of collection (Rana et al. 2008). In this current study, PCA coupled with FTIR spectroscopy was able to distinguish between the inner and outer bark, and also between the extracted and unextracted bark (data plots not shown). Similarly, this technique was able to distinguish between the extracts, but the analysis of the individual extracts (Fig. 2) did not afford readily distinguishable elevated CO_2 groupings. Although the quantity of acetone-soluble materials differs between elevated and ambient CO_2 samples, there is no evidence to suggest that there are compositional differences. Analysis of the extractive-free inner bark samples also showed no discernible grouping, suggesting the lack of substantial impacts of CO_2 enrichment on the cell wall polymers, consistent with prior reports for sweetgum (Kim et al. 2015) and other species (Kaakinen et al. 2004; Luo et al. 2006; Kostiainen et al. 2009) of wood.

Inner bark samples were then subjected to pyrolysis–GC–MS in an attempt to increase the sensitivity of the PCA. This technique involves the fragmentation and detection of discrete chemical compounds, albeit degradation products. Pyrolysis–GC–MS coupled with PCA has been sufficiently sensitive to discriminate between genetically modified poplar clones (Meier et al. 2005). Given the rapid and intense pyrolysis conditions (12 s, 450 °C), the degradation of the semi-volatile extractive components was expected to coincide with the degradation of the cell wall polymers. PCA revealed a broadly scattered group of data points, corresponding to the elevated CO_2 inner bark samples, overlapping with a relatively tight group of data points, corresponding to the ambient CO_2 samples (Fig. 3). Although the technique appeared to detect a difference between the elevated and ambient CO_2 inner bark samples, the traditional wet chemical methods (e.g., extraction) were not supplanted. Remaining to be determined for the PCA of the pyrolysis-GC–MS data is the cause for the apparent greater variability in the data from the elevated CO_2 inner bark samples.

Conclusions

Collection, partitioning, and analysis of sweetgum bark from the Oak Ridge FACE experiment in this study provide the first demonstration of chemical changes in bark resulting from an elevated CO_2 treatment, particularly the inner (phloem) and outer (rhytidome) bark components. The higher extractive content, higher ash content, and corresponding changes to elemental composition (e.g., carbon and hydrogen) have the potential to impact the utility of this forest resource, particularly in the increasingly important biofuels sector. Since the outer bark and inner bark serve essential biological and ecological functions on the living tree, such compositional changes have the potential to impact forest health. We attribute the higher Mn content in the inner bark to increased plant demand for this nutrient to satisfy increased levels of photosynthesis from CO_2 enrichment. Our limited success thus far with PCA-based screening tools necessitated the reliance on time-consuming traditional chemical methods to demonstrate bark compositional changes imparted by the elevated CO_2 treatment.

Author contribution statement TLE coordinated the study, interpreted the data, and wrote the manuscript with technical and editorial contributions from all other authors. NL, CS, and KK contributed to data collection, analysis, and interpretation. KGR coordinated bark processing and the collection of analytical data. DJL coordinated the statistical analyses. JMW provided access to the study site and made significant contributions toward the preparation of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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