

Biochemical composition is not the main factor influencing variability in carbon isotope composition of tree rings

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Summary From June to December, we determined the effects of variations in biochemical composition on $\delta^{13}\text{C}$ of tree rings of 2-year-old oaks (*Quercus petraea* (Matt.) Liebl.) growing under semi-natural conditions, and the dependence of these effects of water stress during the growth season. Percent abundance, carbon concentration and $\delta^{13}\text{C}$ were measured in holocellulose, lignin, extractive-free wood, starch and a water-soluble fraction. Relative concentrations of lignin and holocellulose in the extractive-free wood varied little during the season or in response to water stress, indicating that these compounds could not quantitatively explain the variations in whole-wood $\delta^{13}\text{C}$. Among all sampled tree rings, the relative concentration of each structural compound (holocellulose and lignin) accounted for less than 5% of the $\delta^{13}\text{C}$ variability in whole wood. Variations in holocellulose and extractive-free wood $\delta^{13}\text{C}$ between tree rings were almost identical ($r > 0.95$), whereas variations in lignin $\delta^{13}\text{C}$ were less well correlated to these compounds. Whole-wood $\delta^{13}\text{C}$ had a slightly altered isotopic signal compared with that of the structural compounds because of the presence of the extractive component. These results showed that variations in lignin $\delta^{13}\text{C}$ and lignin concentration have little influence on extractive-free wood $\delta^{13}\text{C}$ and whole-wood $\delta^{13}\text{C}$. Rather, holocellulose influences $\delta^{13}\text{C}$ the most. Thus, we confirmed that, for climatic reconstruction from tree rings, removal of extractives by soxhlet is generally sufficient and sometimes unnecessary. Our findings also indicate that, in the case of rapid and severe water stress, the structural component did not accurately record the associated increase in $\delta^{13}\text{C}$ because of dilution with previously formed organic matter and cessation of trunk growth. The effect of drought on carbon isotope ratios was more pronounced in the extractive compounds, making them good water stress indicators but only on a scale of days to months.

Keywords: growth, holocellulose, lignin, *Quercus petraea*, stable carbon isotope, water stress.

Introduction

Tree rings record the quantity and isotopic ratio of the carbon assimilated by trees during each growth season (McCarroll and Loader 2004). Therefore, measuring stable carbon isotope ratios in tree rings provides a useful tool for reconstructing past environmental conditions. Stable carbon isotopes can capture environmental information beyond that available from ring width and density measurements, and some works have suggested that the carbon isotope signal is more robust than these conventional growth proxies (McCarroll et al. 2003, Gagen et al. 2004, Kirilyanov et al. 2008).

Numerous studies have shown correlations between carbon isotope composition of wood components and climatic variables as well as environmental characteristics such as soil water availability, precipitation, vapor pressure deficit, temperature and solar irradiance (Leavitt 1992, Dupouey et al. 1993, Saurer et al. 1997, Edwards et al. 2000, McCarroll and Pawellek 2001, Barbour et al. 2002, McCarroll et al. 2004, Gagen et al. 2007). These relationships are mechanistically explained at the leaf scale by the effect of climate on variations in water-use efficiency (WUE; the ratio between CO_2 assimilation and stomatal conductance). According to the model of Farquhar et al. (1982), the carbon isotope composition of assimilates is negatively correlated to the ratio of intercellular and atmospheric concentration of CO_2 (C_i/C_a) and therefore positively to the WUE. It is usually assumed that WUE information derived from the carbon isotope composition of leaves is transferable to tree-rings. However, stable carbon isotope

composition of wood integrates many other physiological processes that differ in their responses to varying environmental conditions. For example, the WUE information recorded in newly formed assimilates could be altered by use, mixing with carbon reserves (Damesin and Lelarge 2003), post-photosynthetic fractionations during the transport of assimilates, biosynthesis of wood components, respiration (Panek and Waring 1997, Duranceau et al. 1999, Damesin and Lelarge 2003, Tcherkez et al. 2003, Badeck et al. 2005) or varying wood biochemical composition within (Walcroft et al. 1997, Helle and Schleser 2004) and between tree rings (Farmer 1979, Poole and van Bergen 2006).

Whole wood is composed of several compounds that differ in relative concentrations and in carbon isotope composition (Gleixner et al. 1993, 1998, Badeck et al. 2005). Pectin, hemicellulose, cellulose, starch and soluble sugars are typically enriched in ^{13}C compared with lignins and lipids (Brugnoli et al. 1988, Damesin and Lelarge 2003). Therefore, $\delta^{13}\text{C}$ in whole wood reflects the relative proportions of the different compounds (Wilson and Grinstead 1977).

The structural components of wood, which comprise the major part of its bulk composition (70–90%), are mainly holocellulose and lignin. Lignin represents 17–31% of bulk matter (Benner et al. 1987) and is $3.2 \pm 0.3\text{‰}$ depleted in ^{13}C compared with whole-plant material (reviewed by Badeck et al. 2005, including tree species). Holocellulose makes up 55–77% (Benner et al. 1987) and is $1.3 \pm 0.08\text{‰}$ enriched compared with whole-plant material (reviewed by Badeck et al. 2005, including tree species). Variability in the relative concentrations of lignin and holocellulose could explain differences in $\delta^{13}\text{C}$ between rings or within a ring (Helle and Schleser 2004). For example, high ^{13}C depletion in a tree ring could be correlated with high lignin concentration.

To avoid the consequence of varying biochemical composition, many studies on isotope ratios in tree rings focus on the cellulose fraction, which is the largest and least mobile component of wood. Some recent studies, however, showed high correlations between $\delta^{13}\text{C}$ in whole wood, cellulose and lignin (Barbour et al. 2002, Loader et al. 2003, Verheyden et al. 2005), between $\delta^{13}\text{C}$ in whole wood, cellulose and extractive-free wood (Harlow et al. 2006) and between $\delta^{13}\text{C}$ in holocellulose and whole-leaf material (Rundgren et al. 2003).

Our main objective was to quantify the part of $\delta^{13}\text{C}$ variability of tree-ring bulk matter that may be explained by the relative concentrations of wood components and, in particular, structural compounds. Holocellulose can be subdivided into α -cellulose and hemicellulose fractions. Generally, hemicellulose contributes 10–20% of the overall ligno-cellulose complex (Poole and van Bergen 2006). Because available data show only a 0.39‰ difference in $\delta^{13}\text{C}$ between hemicellulose and α -cellulose (Boettger et al. 2007), these compounds were not studied separately. The relative concentrations and the $\delta^{13}\text{C}$ of whole wood, extractive-free wood, lignin, holocellulose, starch and a water-soluble fraction were determined in the current-year tree rings of young sessile oaks (*Quercus petraea* (Matt.) Liebl.) grown in controlled conditions during the period of wood growth (from June to August) and maturation

(from September to December). Because the biochemical composition of wood is known to vary within tree rings, we hypothesized that the $\delta^{13}\text{C}$ of bulk matter formed during the growth of the current-year tree ring changes in relation to the relative concentrations of holocellulose and lignin. Water stress occurring during wood growth is generally linked to an increase in wood $\delta^{13}\text{C}$ (Leavitt 1992, Dupouey et al. 1993, Barbour et al. 2002) and it is assumed that this increase is associated with ^{13}C -enrichment of substrates for wood growth during drought. However, a decrease in the concentrations of wood components with low $\delta^{13}\text{C}$, in particular lignin, could also be hypothesized.

Materials and methods

Experimental design

Experiments were conducted on 2-year-old sessile oaks (*Quercus petraea*) during the year 2006 at the University of Paris XI (48°50' N, 02°10' E, 65 m elevation). Trees were planted in December 2005 in 90-l pots containing a 1:1 (v/v) sand:compost mixture without the addition of fertilizer. All pots were watered once per week until the experiments began. Thereafter, the 23 trees that served as well-watered controls were watered once per week during the experiments. Six trees were subjected to water stress from May 29 to August 16 by withholding irrigation and excluding rainfall. The severity of water stress was assessed weekly by measuring predawn leaf water potential (Maunoury et al. 2007). Predawn leaf water potential ranged between -0.2 and -0.4 MPa during June, was minimal in July with a value of -1.3 MPa and increased to -0.2 MPa after watering resumed. Bud burst occurred from April 10 to May 10. Trunk radial growth started in early May and was completed by mid-August. For trees in the drought treatment, trunk growth ceased at the beginning of the drought, resulting in a mean stem diameter increment that was 24.9% less than the control value at the end of the study.

Wood sampling

The well-watered control trees were sampled twice during the growth season (June 28 ($n = 5$) and August 9 ($n = 5$)), and three times from September to December (September 28 ($n = 3$), October 26 ($n = 5$) and December 7 ($n = 5$)). Water-stressed trees were sampled twice (August 9 ($n = 3$) at the end of trunk growth and in December 7 ($n = 3$)). At each sampling, trunk slices were collected at 30-cm height and stored at -20°C until lyophilized. The current-year ring was isolated from the trunk slice with a razor blade. Samples were ground to a fine powder before extractions and carbon isotope analysis.

Carbohydrate extraction and determination of starch concentration

Starch and water-soluble fractions were extracted according to Duranceau et al. (1999). Fifty mg of powdered wood was put in a 2-ml Eppendorf tube with 1 ml of distilled water at 4°C . After centrifugation (5 min, 5°C , 12,000 g), the pellet was retained for starch purification, the supernatant was

heated at 100 °C for 3 min to denature proteins and recentrifuged. The resulting supernatant was filtered through a 0.45- μm filter (HV, Nihon Millipore Kogyo K.K, Japan) and the filtrate saved as the water-soluble fraction.

Starch was purified from the pellet by solubilization with 1 ml of 6 M HCl (Damesin and Lelarge 2003). After a 1-h incubation at 5 °C, the hydrolyzate was centrifuged (20 min, 5 °C, 12,000 g), the supernatant retained and the pellet re-extracted with HCl. The supernatants were combined in a 50-ml tube and methanol was added to a final concentration of 80%. After incubation overnight at 5 °C, the extract was centrifuged and the starch pellet was lyophilized and weighed.

Removal of extractives

Extractives in wood, which comprise a variety of compounds including lipids, soluble carbohydrates and amino acids (Benner et al. 1987), were removed from whole-wood samples in three steps. Cartridges filled with 1 mg of sample were placed in a soxhlet apparatus with 2:1 (v/v) toluene:ethanol for 24 h. The solvent was then replaced by ethanol for another 24 h and finally by water for 48 h. The resulting extractive-free wood samples were dried in an extractor hood and weighed to determine the extraction yield.

Holocellulose extraction

Holocellulose was extracted as described by Leavitt and Danzer (1993). Fifty mg of extractive-free powder was placed in a 47-mm diameter glass microfiber filter (X100 MB Mitex 10 μm 47 mm) tied with a Teflon string. Pouches were weighed and placed in a 1.5-l Erlenmeyer flask containing 700 ml of deionized water, 7 g of sodium chlorite (NaClO_2) and 1 ml of glacial acetic acid and incubated overnight at 70 °C. The next day, three successive additions of the same quantities of reagents were made at 2-h intervals. Heating was stopped 3 h after the last addition. Pouches were rinsed in an ultrasonic bath (Bransonic 52, 112 W) eight times over a 30-min period, dried for two days at 70 °C and weighed. Holocellulose was then removed from the glass fiber filters.

Lignin extraction and determination of concentration

The lignin fraction was isolated by the Klason method from 300 mg of extractive-free powder, according to the standard procedure (Dence 1992). Each sample was added to 3 ml of 72% (v/v) H_2SO_4 , incubated for 2 h at ambient temperature, and diluted into 40 ml of deionized water by refluxing for 3 h. Acid-insoluble lignin was collected by filtration, washed with deionized water and dried overnight at 70 °C. Acid-insoluble lignin concentration referred to as Klason lignin concentration was determined as a percentage of the extractive-free wood mass and reported as the mean of two independent determinations on the same sample. The absorbance at 205 nm of the soluble product of the hydrolytic reaction was also measured and the acid-soluble lignin concentration was calculated (Dence 1992). The reproducibility of lignin concentration determinations was within 0.19%.

Carbon concentration and carbon isotope analysis

Carbon concentration and carbon isotope composition were determined in whole wood, extractive-free wood, lignin, holocellulose, starch and water-soluble fractions. To determine carbon concentration, 1 mg of a sample was enclosed in a 3.3 \times 5-mm tin capsule (Säntis analytical) and combusted and measured in an elementary analyzer (Model NA-1500, Carlo Erba, Milan, Italy). Carbon concentrations are expressed in %. The mean standard deviation of 35 duplicates was 1.10%.

Carbon isotope compositions were determined with a stable ratio mass spectrometer (VG Optima; Fison, Villeurbanne, France, precision $\pm 0.2\text{‰}$) with a continuous-flow inlet system and the results expressed according to the conventional δ (‰) notation:

$$\delta^{13}\text{C} = \frac{R_s - R_{\text{PDB}}}{R_{\text{PDB}}} 1000 \quad (1)$$

where R_s and R_{PDB} are the molar abundance ratios of the carbon isotopes, $^{13}\text{C}/^{12}\text{C}$, in the sample and the Pee Dee Belemnite (VPDB) standard (Coplen 1995), respectively. The mean standard deviation for $\delta^{13}\text{C}$ in 35 duplicate samples was 0.16‰.

The global $\delta^{13}\text{C}$ of wood compounds extracted with the Soxhlet apparatus (total extractive $\delta^{13}\text{C}$) was estimated from their concentration in whole wood and the $\delta^{13}\text{C}$ of whole wood and extractive-free wood.

Checking the reliability of the measurements on wood structural components with mixing models

To check the reliability of the measurements, the measured $\delta^{13}\text{C}$ and carbon concentrations in extractive-free wood samples were compared by two mixing models taking into account the $\delta^{13}\text{C}$ values of lignin and holocellulose, their relative proportions in the extractive-free wood and their carbon concentrations:

$$C_{\text{EF}} = [H]C_{\text{H}} + [L]C_{\text{L}} \quad (2)$$

$$\delta^{13}\text{C}_{\text{EF}} = \frac{\delta^{13}\text{C}_{\text{H}}[H]C_{\text{H}} + \delta^{13}\text{C}_{\text{L}}[L]C_{\text{L}}}{C_{\text{EF}}} \quad (3)$$

where C_{EF} , C_{H} and C_{L} are the carbon concentrations of extractive-free wood, holocellulose and lignin, $[H]$ and $[L]$ are the holocellulose and lignin concentrations in extractive-free wood and $\delta^{13}\text{C}_{\text{EF}}$, $\delta^{13}\text{C}_{\text{H}}$ and $\delta^{13}\text{C}_{\text{L}}$ are the carbon isotope compositions of extractive-free wood, holocellulose and lignin fraction, respectively.

A good reconstruction of C_{EF} and $\delta^{13}\text{C}_{\text{EF}}$ with these mixing models is effective only if (1) $\delta^{13}\text{C}$, carbon concentrations and relative concentrations of structural wood components are accurately measured, and if (2) the $\delta^{13}\text{C}$ in these wood compounds is not modified by the different extraction processes. The extractions of holocellulose and lignin from extractive-free wood were independent. Consequently, if there is some fractionation during these processes, it is unlikely that compensation occurs between them.

Because the holocellulose extraction yield was low, holocellulose concentration in extractive-free wood was calculated as the complement of lignin concentration in extractive-free wood. Moreover, the lignin concentration used in the models could be determined either as the Klason lignin only or as the sum of the Klason and acid-soluble lignins. Therefore, we compared mixing models that considered the acid-soluble lignin as being similar to holocellulose or to Klason lignin in terms of $\delta^{13}\text{C}$ and carbon concentration. Modeling accuracy was determined as the coefficient of determination (r^2), the root mean square error (RMSE) and the mean error (Bias) between predicted and measured values.

Statistical analysis

When sample sizes were sufficient ($n = 23$) and the data were normally distributed according to the Kolmogorov-Smirnov test, differences in $\delta^{13}\text{C}$ among compounds were analyzed by paired t -tests. When sample sizes were small, nonparametric tests were used: the Mann-Whitney U-test for comparison between water-stressed and control trees and the Kruskal-Wallis analysis of variance (ANOVA) for seasonal variation. The significance level was set at 5%. The relationships between $\delta^{13}\text{C}$ of the different compounds were assessed by Pearson correlations to obtain values comparable with those in the literature (Loader et al. 2003, Verheyden et al. 2005, Harlow et al. 2006). When the Pearson correlations were superior to 0.95 and significant ($P < 0.05$), the slopes of the relationships were compared with a slope of 1 by bivariate least squares (BLS) regression analysis that takes into account the analytical uncertainties on both axes for estimation of the slope (Verheyden et al. 2005). Significance tests for the slopes were based on the 95% confidence interval.

Results

Wood component contents, carbon content and $\delta^{13}\text{C}$

In all tree rings sampled from well-watered trees, the structural component (holocellulose + lignin) amounted to $82.9 \pm 2.5\%$ of the bulk matter (Table 1). The yield of extracted holocellulose from extractive-free wood was $58.6 \pm 7.6\%$, which is close to the yields of about 50% reported in the literature (Leavitt and Danzer 1993, Cullen and Macfarlane 2005, Harlow et al. 2006). Overall total lignin concentration in the structural component was $17.6 \pm 1.3\%$, with a Klason lignin concentration of $15.3 \pm 1.8\%$ and an acid-soluble lignin concentration of $2.2 \pm 0.7\%$. Among extractives, water-soluble and starch fractions amounted to $0.8 \pm 0.2\%$ and $5.9 \pm 2.2\%$ of whole wood, respectively. The sum of the starch and water-soluble fraction concentrations amounted to only $60 \pm 30\%$ of the total extractive concentration measured following Soxhlet extraction.

Carbon concentrations in lignin and holocellulose were $57.7 \pm 1.4\%$ and $41.4 \pm 1.6\%$, respectively, which is consistent with lignin containing 50% more carbon than cellulose (Benner et al. 1987). Carbon concentrations in whole wood and the extractive-free fraction were intermediate between those of cel-

lulose and lignin with values of 43.0 ± 1.1 and $44.7 \pm 0.5\%$, respectively.

Ranges of whole-wood, extractive-free wood, holocellulose and lignin $\delta^{13}\text{C}$ values were 2.7, 2.4, 2.9 and 3.1‰, respectively. The $\delta^{13}\text{C}$ of each compound studied differed significantly except between whole wood and extractive-free wood, between total extractives and all the other compounds and between holocellulose and the water-soluble fraction (Table 1). Extractive-free wood and whole wood were isotopically depleted compared with holocellulose by 0.9 ± 0.1 and $1.0 \pm 0.1\%$, respectively, and enriched compared with Klason lignin by 3.1 ± 0.2 and $3.0 \pm 0.2\%$, respectively. The difference in $\delta^{13}\text{C}$ between holocellulose and lignin was on average $4.0 \pm 0.2\%$. Starch $\delta^{13}\text{C}$ was higher than that of the water-soluble fraction, structural components and total wood.

Correlations between $\delta^{13}\text{C}$ values in wood components

The $\delta^{13}\text{C}$ values of all wood components analyzed in the 29 sampled tree rings were strongly correlated (Table 2). The highest correlation was found between holocellulose and extractive-free wood (Pearson correlations: $r > 0.95$). The slope of the bivariate least squares (BLS) regression was not significantly different from 1 (Slope: 0.87; 95% confidence interval: 0.71–1.03). The lowest correlation was between whole-wood and lignin $\delta^{13}\text{C}$. No significant correlation was found between estimated $\delta^{13}\text{C}$ of total extractives and $\delta^{13}\text{C}$ of any of the other compounds studied.

Checking the reliability of the measurements on wood structural components with mixing models

The $\delta^{13}\text{C}$ values of extractive-free wood were accurately predicted using $\delta^{13}\text{C}$ values of lignin and holocellulose, relative concentrations and respective carbon concentrations (Figure 1). Considering acid-soluble lignin $\delta^{13}\text{C}$ equal to Klason lignin $\delta^{13}\text{C}$ did not change the correlation between measured and predicted $\delta^{13}\text{C}$ but halved the root mean square error (RMSE) from 0.04 to 0.02‰. Moreover, when holocellulose

Table 1. Extraction yield, carbon concentration and $\delta^{13}\text{C}$ values for components of all sampled current-year tree-rings of well-watered trees. Total extractives $\delta^{13}\text{C}$ was calculated from the other data. Values are means \pm SD of 23 individuals. Abbreviation: nd = not determined. Within the right column, different letters indicate a significant difference based on the paired t -test at the 5% threshold.

Wood component	Extractive yield (% of dry matter)	Carbon concentration (%)	$\delta^{13}\text{C}$ (‰)
Whole wood	100	43.0 ± 1.1	-25.1 ± 0.6^a
Extractive free wood	82.9 ± 2.5	44.7 ± 0.5	-25.0 ± 0.6^a
Holocellulose	50.4 ± 9.1	41.4 ± 1.6	-24.1 ± 0.7^b
Klason lignin	12.4 ± 1.6	57.7 ± 1.4	-28.1 ± 0.7^c
Acid-soluble lignin	2.0 ± 0.6	nd	nd
Total extractives	17.1 ± 2.5	nd	$-26.3 \pm 2.1^{a,b,c,d}$
Starch	5.9 ± 2.2	nd	-22.8 ± 0.7^d
Water-soluble fraction	0.8 ± 0.2	nd	-23.7 ± 0.5^b

Table 2. Summary of correlations for $\delta^{13}\text{C}$ values for all of the wood components analyzed. All individuals were used for calculations (*, $P < 0.05$; t -test).

	Total wood	Extractive-free wood	Holocellulose	Klason lignin	Total extractives	Starch	Water-soluble fraction
Total wood	1	0.90*	0.92*	0.81*	0.25	0.93*	0.94*
Extractive-free wood		1	0.97*	0.90*	0.05	0.94*	0.82*
Holocellulose			1	0.89*	0.08	0.92*	0.85*
Klason lignin				1	0.06	0.89*	0.84*
Total extractives					1	0.05	0.35
Starch						1	0.89*
Water-soluble fraction							1

$\delta^{13}\text{C}$ was substituted for acid-soluble lignin $\delta^{13}\text{C}$, predicted values were always higher than measured values with a mean error of 0.12‰. When Klason lignin $\delta^{13}\text{C}$ was used, the bias was largely removed.

The carbon concentration in extractive-free wood was not well predicted by the mixing models (r^2 of 0.03 and 0.09 for both models). However, by considering acid-soluble lignin carbon concentration equal to Klason lignin concentration, the prediction was improved, with an RMSE of 1.4% versus 1.9% if acid-soluble lignin carbon concentration was considered similar to that of holocellulose. The prediction bias was also reduced with a value of -0.3% versus -0.6% .

Structural wood compounds: $\delta^{13}\text{C}$ and seasonal variations

Seasonal variations in $\delta^{13}\text{C}$ were 0.5, 0.6, 1.1 and 0.5‰ in whole wood, holocellulose, lignin and extractive-free wood, respectively. The seasonal $\delta^{13}\text{C}$ pattern was similar among holocellulose, lignin and extractive-free wood (Figure 2). The first sampling in July had the highest $\delta^{13}\text{C}$ value in all wood compounds measured; however, there was no significant temporal effect on $\delta^{13}\text{C}$ (Kruskal-Wallis ANOVA). The variability in $\delta^{13}\text{C}$ among individual trees was of the same order or larger than the temporal variability in $\delta^{13}\text{C}$ among the structural compounds studied. For example, $\delta^{13}\text{C}$ of the rings sam-

pled in December ranged between -26.6 and -24.3% , and total lignin, Klason lignin and acid-soluble lignin concentrations were not significantly different between sampling dates (Kruskal-Wallis ANOVA).

Labile wood compounds: $\delta^{13}\text{C}$ and seasonal variations

The $\delta^{13}\text{C}$ in the water-soluble fraction and starch decreased from June to December by 0.5 and 1.3‰, respectively. The main decrease in starch $\delta^{13}\text{C}$ occurred between June and August (0.9‰). Because the seasonal pattern of whole-wood $\delta^{13}\text{C}$ differed slightly from that of the structural compounds (Figure 3), extractive compounds had a minor effect on whole-wood $\delta^{13}\text{C}$.

The concentration of wood extractives was constant from June to December except at the end of September when it dropped to a minimum mean value of $13.8 \pm 1.5\%$ of bulk matter. This minimum corresponded to the maximum starch concentration ($13.0 \pm 1.4\%$) and to the minimum water-soluble fraction concentration ($1.3 \pm 0.3\%$). The decrease in the concentration of the water-soluble fraction could not explain quantitatively the decrease in the concentration of total extractives. Starch concentration increased markedly from June to September and then decreased to a minimum of $7.7 \pm 1.0\%$ in December. The seasonal pattern in the concentration of the water-soluble fraction content was the inverse of the starch pattern.

Water stress and variations in $\delta^{13}\text{C}$ of wood components

Whole wood, extractives and structural compounds of current-year tree rings were generally enriched in ^{13}C in water-stressed trees compared with well-watered trees for all sampling dates (Tables 3 and 4), but no change in lignin concentration was detected. Among the structural compounds, water stress caused nonsignificant increases in $\delta^{13}\text{C}$ of 0.6‰ in whole wood, extractive-free wood, holocellulose and lignin in rings sampled in August and 0.7–0.9‰ in rings sampled in December (Mann-Whitney U-test).

The enrichment in ^{13}C in response to drought was significant in extractive compounds and higher than in structural compounds (Table 4). Starch $\delta^{13}\text{C}$ increased about 1.9 and 2.5‰ in response to drought in the tree rings sampled in August and December, respectively. In trees in the drought treatment, the water-soluble fraction was enriched by 2.5‰ in Au-

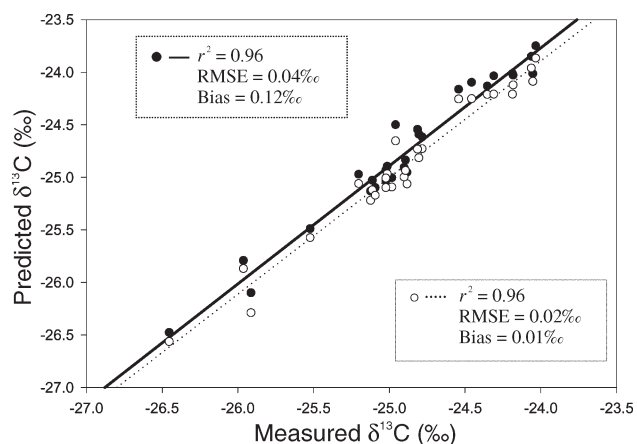


Figure 1. Comparison between measured and predicted $\delta^{13}\text{C}$ in extractive-free wood when acid-soluble lignin $\delta^{13}\text{C}$ was considered equal to holocellulose $\delta^{13}\text{C}$ (●) or to Klason lignin $\delta^{13}\text{C}$ (○).

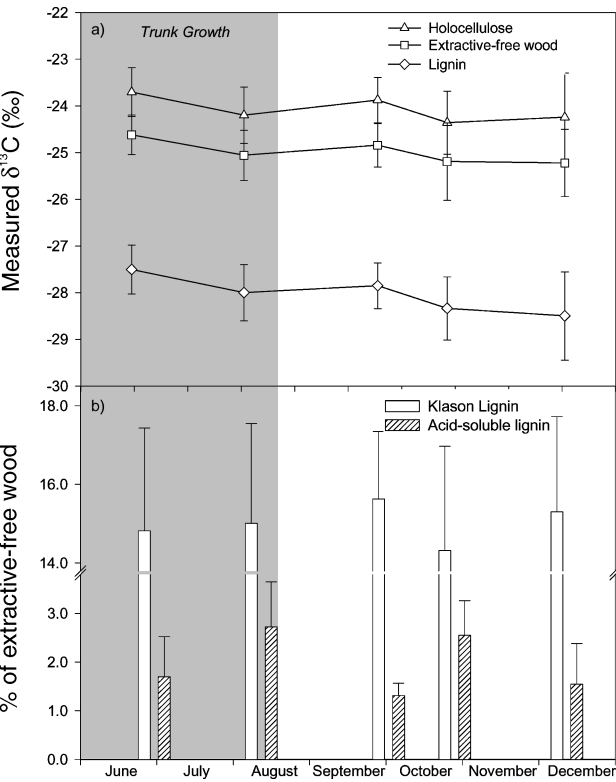


Figure 2. Seasonal variations in well-watered trees of (a) $\delta^{13}\text{C}$ of structural compounds and (b) Klason lignin and acid-soluble lignin concentrations in extractive-free wood.

gust and 1.5‰ in December, and the decrease in $\delta^{13}\text{C}$ between August and December was significant (Mann-Whitney U-test). In water-stressed trees, the $\delta^{13}\text{C}$ values in starch and in water-soluble fraction in August were similar, whereas starch was more enriched in ^{13}C compared with the water-soluble fraction in the well-watered trees. Drought had no significant effects on the concentrations of starch and the water-soluble fraction on any sampling date.

Discussion

Reliability of the measurements

In all sampled tree rings, measured lignin concentrations were slightly lower than values reported for mature trees (Benner et

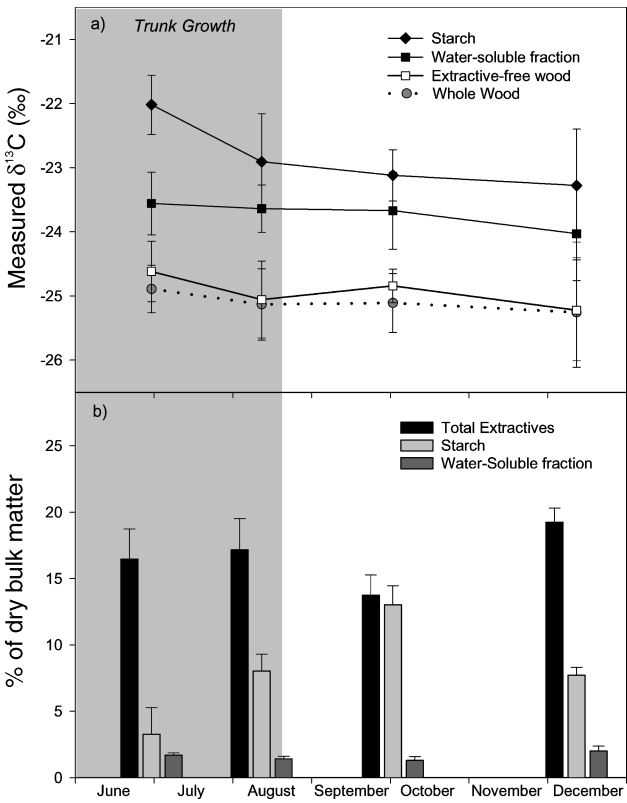


Figure 3. Seasonal variations in current-year tree rings of well-watered trees of (a) $\delta^{13}\text{C}$ of whole wood, starch, water-soluble fraction and extractive-free wood and (b) concentrations of extractives, starch and water-soluble fraction in whole wood.

al. 1987, Dence 1992). Our experiments were conducted on seedlings that contain juvenile wood with low density, thin-walled cells, which may differ in lignin to cellulose ratios from wood of mature trees.

The $\delta^{13}\text{C}$ values of all compounds analyzed corresponded well to reported values for C_3 plant material ranging from -21.4‰ for starch to -29.6‰ for lignin, and the observed differences between compounds were consistent with previous studies. The $0.9 \pm 0.1\text{‰}$ offset between extractive-free $\delta^{13}\text{C}$ and holocellulose $\delta^{13}\text{C}$ was similar to the $1.1 \pm 0.3\text{‰}$ value observed by Harlow et al. (2006) in a broad set of 44 gymnosperm and angiosperm species and slightly lower than the 0.99 to 1.24‰ values obtained by Borella et al. (1998) for α -cellu-

Table 3. Concentrations and $\delta^{13}\text{C}$ values of structural wood components in tree rings of water-stressed ($n = 3$ by date) and well-watered control trees ($n = 5$ by date) sampled in August and December. Values are means \pm SD.

Sampling date	Treatment	Total wood $\delta^{13}\text{C}$ (‰)	Extractive-free wood $\delta^{13}\text{C}$ (‰)	Holocellulose $\delta^{13}\text{C}$ (‰)	Lignin $\delta^{13}\text{C}$ (‰)	Total lignin concentration in extractive-free wood (%)
August 9	Control	-25.2 ± 0.5	-25.1 ± 0.5	-24.2 ± 0.6	-28.0 ± 0.6	17.5 ± 1.8
	Drought	-24.6 ± 0.4	-24.5 ± 0.5	-23.6 ± 0.5	-27.4 ± 0.5	17.1 ± 1.5
December 7	Control	-25.3 ± 0.8	-25.2 ± 0.7	-24.2 ± 0.9	-28.5 ± 0.7	17.7 ± 0.7
	Drought	-24.5 ± 0.4	-24.4 ± 0.5	-23.5 ± 0.6	-27.4 ± 0.6	18.3 ± 1.2

Table 4. Concentrations and $\delta^{13}\text{C}$ values of total extractives, starch and a water-soluble fraction in current-year tree rings of water-stressed ($n = 3$ by date) and well-watered control trees ($n = 5$ by date) sampled in August and December. Values are means \pm SD. Different letters between control and drought treatments indicate a significant difference at the 5% level (Mann-Whitney U-test).

Sampling date	Treatment	Total extractives $\delta^{13}\text{C}$ (‰)	Starch $\delta^{13}\text{C}$ (‰)	Water-soluble fraction $\delta^{13}\text{C}$ (‰)	Starch concentration (%)	Water-soluble fraction concentration (%)
August 9	Control	-25.8 ± 0.1 a	-22.9 ± 0.7 a	-23.6 ± 0.4 a	8.0 ± 1.3 a	1.4 ± 0.2 a
	Drought	-24.8 ± 0.01 b	-21.0 ± 0.7 b	-21.1 ± 0.9 b	5.9 ± 2.2 a	1.5 ± 0.2 a
December 7	Control	-26.2 ± 0.8 a	-23.3 ± 0.9 a	-24.0 ± 0.7 a	7.7 ± 0.6 a	2.0 ± 0.4 a
	Drought	-24.2 ± 2.0 a	-20.8 ± 1.0 b	-22.5 ± 0.7 b	8.2 ± 1.5 a	1.9 ± 0.4 a

lose and extractive-free wood. The offset between Klason lignin $\delta^{13}\text{C}$ and holocellulose $\delta^{13}\text{C}$ was 4‰, which is larger than the value of 3‰ measured in ring series of *Quercus petraea* (Loader et al. 2003) and within rings of *Pinus radiata* D. Don (Wilson and Grinstead 1977). Whole-wood $\delta^{13}\text{C}$ was lower than extractive-free wood $\delta^{13}\text{C}$ because of the presence of ^{13}C -depleted extractive compounds compared with structural compounds. However, starch and water-soluble fractions were ^{13}C enriched compared with extractive-free wood. Total extractives contained other ^{13}C -depleted compounds compared with whole wood including lipids, proteins and tannins. In our study, starch had the highest $\delta^{13}\text{C}$ values of the compounds analyzed. Enrichment in ^{13}C of starch compared with soluble sugars has been reported previously (Brugnoli et al. 1988).

Based on our finding that $\delta^{13}\text{C}$ in extractive-free wood could be precisely reconstructed with mixing models, particularly when acid-soluble lignin was considered equal to Klason lignin in terms of $\delta^{13}\text{C}$ and carbon concentration, we made three inferences. (1) Acid-soluble lignin is similar to Klason lignin in $\delta^{13}\text{C}$ value and carbon concentration. (2) Structural compounds that were not characterized as lignin can be assumed to be holocellulose. Thus, holocellulose extraction yields underestimated real holocellulose concentration by about 20%. Excessive processing during holocellulose extraction could explain these yields (Harlow et al. 2006). (3) Holocellulose and lignin $\delta^{13}\text{C}$ were accurately estimated and little or no fractionation occurred during extraction of these structural compounds from extractive-free wood.

The mixing models failed to predict carbon concentration in extractive-free wood accurately. This could have been the result of the 1.1% mean standard error associated with the analysis, which is comparable to the 0.6–1.5% variability measured between tree rings for the different structural compounds.

High correlations between structural wood compounds $\delta^{13}\text{C}$

Extractive-free wood and holocellulose globally showed similar isotopic patterns that were highly correlated ($r \geq 0.95$), as reported by Harlow et al. (2006). The correlation between lignin and cellulose $\delta^{13}\text{C}$ was lower than published values (Wilson and Grinstead 1977, Loader et al. 2003). Extractive-free wood $\delta^{13}\text{C}$ could be accurately reconstructed by taking into account the relative concentration of lignin. However,

the mixing model improved the Pearson correlation by only 1% ($r = 0.98$) compared with the correlation between holocellulose $\delta^{13}\text{C}$ and extractive-free $\delta^{13}\text{C}$ ($r = 0.97$), indicating that the isotopic signal was hardly altered by the different proportions of lignin in wood or by differences between the $\delta^{13}\text{C}$ patterns of holocellulose and lignin.

Correlations were lower between the different structural compounds and whole wood because the carbon isotope composition of the extractives had lower correlations with the other compounds. Starch $\delta^{13}\text{C}$ was more closely correlated to the $\delta^{13}\text{C}$ of structural compounds than the water-soluble fraction $\delta^{13}\text{C}$, perhaps because starch has a lower turnover rate than the water-soluble fraction and therefore integrates an isotopic signal over a longer period of time. The observed high correlations between the $\delta^{13}\text{C}$ values of structural and labile compounds could be related to the fact that these compounds were constructed in the same year under similar environmental conditions. In contrast, starch and water-soluble fraction $\delta^{13}\text{C}$ values of older tree rings were not significantly correlated to the $\delta^{13}\text{C}$ of these compounds in the current-year tree ring (data not shown).

Lignin concentration and extractive-free $\delta^{13}\text{C}$ variations in the current-year tree ring: during the season

Seasonal variations in the $\delta^{13}\text{C}$ values of all structural compounds were low and not significant. Furthermore, we were able to demonstrate that the relative concentrations of the different structural compounds could not explain the small temporal variations in extractive-free wood $\delta^{13}\text{C}$ and, therefore, in whole-wood $\delta^{13}\text{C}$. Lignin concentration in extractive-free wood did not differ significantly between sampling dates and did not explain the quantitative variations in extractive-free wood $\delta^{13}\text{C}$. For example, $\delta^{13}\text{C}$ values of extractive-free wood were maximal at the June sampling, and extractive-free wood $\delta^{13}\text{C}$ decreased by 0.4‰ between June and August. During this period, the mean increase in lignin concentration of 0.9% could explain a decrease in $\delta^{13}\text{C}$ of 0.04‰ only on the basis of a difference of 4‰ between holocellulose and lignin $\delta^{13}\text{C}$. Moreover, the similarity in the $\delta^{13}\text{C}$ seasonal patterns of extractive-free wood, lignin and holocellulose suggest that variation in $\delta^{13}\text{C}$ of their common substrates was the main explaining factor. In tree rings, early wood in angiosperms is assumed to be built with carbohydrate reserves from past years

(Helle and Schleser 2004), which are mainly starch in oaks (Barbaroux and Bréda 2002), whereas the construction of late wood is based on newly formed assimilates that are usually depleted in ^{13}C compared with starch. In our study, low temporal variations suggest that the difference in $\delta^{13}\text{C}$ between early wood and late wood was small, suggesting that the early wood was built with a mix of reserves and current-year assimilates.

Seasonal variations in whole-wood $\delta^{13}\text{C}$ and extractive-free wood $\delta^{13}\text{C}$ were similar. The large seasonal variations in the concentrations of starch and the water-soluble fraction had little effect on whole-wood $\delta^{13}\text{C}$ compared with extractive-free wood $\delta^{13}\text{C}$.

Lignin concentration and extractive-free $\delta^{13}\text{C}$ variations in the current-year tree ring: effect of water stress

The drought treatment increased $\delta^{13}\text{C}$ in all compounds analyzed just after the end of the growth season and at the end of the year. This increase was around 0.6–0.9‰ and similar for lignin, holocellulose and extractive-free wood, whereas the increase was larger (1.5–2.5‰) for the extractive fraction. The increase in $\delta^{13}\text{C}$ in response to drought was not entirely incorporated in the structural component because trunk growth stopped at the beginning of the drought and the isotopic signal was diluted by previously formed organic matter. For these reasons, the extractive compounds were better indicators of water stress than the structural components, but for a short time only because of their high turnover rates. For example, the water-soluble fraction $\delta^{13}\text{C}$ of the water-stressed trees showed a 1.4‰ decrease from August to December and was less ^{13}C enriched compared with control values in December than in August.

Variation in $\delta^{13}\text{C}$ in the structural component could not be accounted for by changes in the relative concentration of lignin in extractive-free wood, rather changes in the substrate $\delta^{13}\text{C}$ were the dominant factor. The increase in $\delta^{13}\text{C}$ in response to drought could be explained by the use of ^{13}C -enriched substrates for growth, which could be related to a reduced stomatal conductance in water-stressed trees resulting in a higher WUE (Farquhar et al. 1982, Bréda et al. 2006) or to the use of ^{13}C -enriched starch (Maunoury et al. 2007), as is indicated by the similar $\delta^{13}\text{C}$ values in starch and the water-soluble fraction when trunk growth ceased.

High variability in $\delta^{13}\text{C}$ among trees grown in similar conditions

The variability in $\delta^{13}\text{C}$ between individuals was high for each sampling date and could have hidden the seasonal variations (Figures 2 and 3). Because the ranges in $\delta^{13}\text{C}$ and lignin concentration in the extractive-free wood samples were 2.8‰ and 3.3%, respectively, the variation in relative lignin concentration could explain at the most 5% of the $\delta^{13}\text{C}$ variability assuming a difference of 4‰ between lignin $\delta^{13}\text{C}$ and holocellulose $\delta^{13}\text{C}$. High tree to tree variability was previously reported in wood $\delta^{13}\text{C}$ studies at the same site (Leavitt and Long 1986, Chevillat et al. 2005). Chevillat et al. (2005) ob-

served a mean intra-specific variability of 2.6‰ in stem xylem $\delta^{13}\text{C}$ of various deciduous and evergreen species and this variability is known to occur even with a single component. Variations among individuals within a site amounted to 2–3‰ in the cellulose of tree rings of *Pinus edulis* Engelm. (Leavitt and Long 1984) and to 1.5–2.8‰ in the cellulose of *Pinus pinaster* (Soland) (Nguyen-Queyrens et al. 1998, Porté and Loustau 2001). These ranges of variation in $\delta^{13}\text{C}$ are comparable with those in our study for whole wood and structural compounds.

Inter-tree variability could be explained by both environmental and genetic components (Porté and Loustau 2001, McCarroll and Loader 2004). For example, inter-tree variability could be linked to different hydraulic properties exerting different constraints on leaf gas exchange. Ponton et al. (2001) found a significant relationship between early wood vessel surface area and ^{13}C isotopic discrimination in *Quercus robur* L. and *Q. petraea*.

In conclusion, the Klason lignin and holocellulose methods of extraction were validated for $\delta^{13}\text{C}$ analysis and the results strongly suggested that the acid-soluble lignin was similar to the Klason lignin in terms of $\delta^{13}\text{C}$ and carbon concentration. In current-year tree rings, the relative concentrations of lignin and holocellulose in the extractive-free wood showed no variation during the season or in response to water stress. Therefore the variations in whole-wood $\delta^{13}\text{C}$ during the growth season and in response to water-stress were not quantitatively explained by the relative concentration of either structural compound; however, they could be accounted for by variations in the $\delta^{13}\text{C}$ of the substrates of the structural compounds. Large inter-tree variability was observed, even though the trees were similar in age, provenance and growth conditions, that could not be explained by the relative concentration of lignin, which accounted for less than 5% of the $\delta^{13}\text{C}$ variability in extractive-free wood and therefore in whole wood.

Variations in holocellulose and extractive-free wood $\delta^{13}\text{C}$ between tree rings were practically identical although lignin $\delta^{13}\text{C}$ was less well correlated. Whole-wood $\delta^{13}\text{C}$ exhibited a slightly altered isotopic signal compared with the $\delta^{13}\text{C}$ of the structural compounds because of the presence of the extractive component. These results confirmed that, for climatic reconstruction from tree rings, removal of extractives by Soxhlet is generally sufficient (Harlow et al. 2006) and sometimes unnecessary. Our experiments were made on seedlings, so the applicability of our findings to mature trees is unknown. However, our findings corroborate previous studies showing high correlations between $\delta^{13}\text{C}$ in whole wood, cellulose and lignin of mature trees (Barbour et al. 2002, Loader et al. 2003, Verheyden et al. 2005).

The structural component did not accurately record the increase in $\delta^{13}\text{C}$ associated with a severe water stress because of dilution of the isotopic signal with previously formed organic matter and the early cessation of trunk growth. Although drought effects on carbon isotope ratios were more pronounced in the extractive compounds than in the other compounds analyzed, the high turnover rates of the extractive compounds limits their usefulness as water stress indicators to time scales of days to months.

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