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δ^2H, δ^{13}C and δ^{18}O from whole wood, α-cellulose and lignin methoxyl groups in *Pinus sylvestris*: a multi-parameter approach

Maria Mischel[a,b*], Jan Esper[a], Frank Keppler[c], Markus Greule[c] and Willy Werner[b]

aDepartment of Geography, Johannes Gutenberg University, Mainz, Germany; bDepartment of Geobotany, University of Trier, Trier, Germany; cInstitute of Earth Sciences, University of Heidelberg, Heidelberg, Germany

Dedicated to Professor Dr Hanns-Ludwig Schmidt on the occasion of his 85th birthday

Novel tree ring parameters – δ^{13}C and δ^2H from methoxyl groups – have been developed to reconstruct palaeoclimate. Tests with δ^{13}C and δ^{18}O derived from whole wood and cellulose samples, however, indicated differences in the isotopic composition and climate signal, depending on the extracted wood component. We assess this signal dependency by analysing (i) δ^{13}C and δ^{18}O from whole wood and cellulose and (ii) δ^{13}C and δ^2H from methoxyl groups, using *Pinus sylvestris* L. growing near Altenkirchen (Germany). Results indicate significant correlations among the time series derived from whole wood, cellulose, and lignin methoxyl groups. Compared with the whole wood samples, δ^{13}C from methoxyl groups showed a different and overall lower response to climate parameters. On the other hand, δ^2H from methoxyl groups showed high correlations with temperature and was also correlated with ring width, indicating its potential as a temperature proxy. Isotope time series with the highest correlation with climatic parameter were: (i) whole wood and cellulose δ^{13}C with growing season precipitation and summer temperature; (ii) methoxyl groups with spring precipitation; (iii) whole wood and cellulose δ^{18}O correlates with annual evapotranspiration and water balance; and (iv) methoxyl group δ^2H with spring temperatures. These findings reveal that multiple climate elements can be reconstructed from different wood components and that whole wood proxies perform comparably to cellulose time series.

Keywords: carbon-13; climate; dendroclimatology; hydrogen-2; isotope ecology; oxygen-18; pine trees; tree rings; wood components

1. Introduction

Annual growth rings of trees can serve as high-resolution archives for climate and ecological information. Tree ring parameters (stable isotopes, ring width or density) from temperate regions often reflect several climate parameters (e.g. precipitation amount, temperature, global radiation and potential evapotranspiration) [1]. In these regions, tree growth is not limited by only one single climate element (e.g. temperature or precipitation) and, therefore, single tree ring parameters can present correlations with climatic parameters that are not significant. Here, interpreting correlations between multiple tree ring parameters and climate is helpful in identifying climate and ecological drivers [2–8].

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*Corresponding author. Email: stantsma@uni-mainz.de

Wilson and Grinsted [9] proved that different wood components (α-cellulose, hemicellulose, lignin, resins and tannins) contain distinct isotopic compositions. α-cellulose from annual growth rings is preferentially analysed, as it is an immobile wood component in contrast to resins and tannins [10]. However, the time-consuming extraction steps are a major disadvantage. Different extraction protocols have been developed to reduce laboratory work and time, for example, the Jayme-Wise method [11], diglyme-HCl method [12], and variants of the Brendel protocol [13–15]. Whereas some studies have shown that whole wood can be used for $\delta^{13}C$ and $\delta^{18}O$ analysis on recent wood material [16–19], other papers demonstrated a loss of signal strength in $\delta^{13}C$ and $\delta^{18}O$ if cellulose is not extracted [20–24].

Recently, it was suggested that stable hydrogen isotope values of lignin methoxyl groups in tree rings might also be used to derive climate signals and could potentially be applied as a palaeoclimate proxy [25,26]. Lignin methoxyl groups are considered to be stable, that is, the hydrogen atoms of the methoxyl moiety do not exchange with those of plant water during ongoing metabolic reactions in the plant. Thus, the initial δ^2H value of the methoxyl groups of lignin in woody tissue at formation is retained throughout the lifetime of the tree and in preserved tissue. The methoxyl content of lignin in wood is usually determined by the Zeisel method [27] – the reaction between methyl ethers and hydrogen iodide (HI) to form methyl iodide (CH_3I). Exploiting the reaction for the measurement of δ^2H values of lignin methoxyl groups ensures that during the entire analytical procedure, the isotope signal is preserved since no isotopic exchange occurs between the methyl groups and HI and no isotopic fractionation in the course of CH_3I formation is observed [28–30]. For the general principles of stable isotope fractionation of hydrogen, carbon and oxygen in plants, the reader is referred to the literature [31–35 and references therein].

The aims of this study are to:

(1) quantify the variability and offset of the wood components (whole wood, cellulose and lignin methoxyl groups),
(2) evaluate the correlation between individual trees,
(3) assess the climate signals in ring width, carbon, oxygen and hydrogen stable isotopes.

2. Material and methods

2.1. *Study site*

Sixteen *Pinus sylvestris* trees were sampled in a monitoring area located near Altenkirchen (50°39′ N, 7°38′ E), Germany (Figure 1(a)). The sampling site consists mainly of Scots pine trees (*P. sylvestris*), beech (*Fagus sylvatica*) and oak (*Quercus petraea*). It contains mainly mature trees with ages from 120 to 125 years.

2.2. *Sample preparation*

Stem discs were cut out at breast height and used for ring width analysis with the software package WinDendro density 2006 (Regent Instruments®). Each stem disc (Figure 1(c)) was polished to ease ring identification. Ring width measurements were performed on 4–6 radii, omitting parts of the disc showing anatomical irregularities and hazel growth. Ring width time series were cross-dated among all trees considering the Gleichläufigkeit and Skeleton plot technique. Gleichläufigkeit is a measure of the year-to-year agreement between the individual trees [1].

Four trees were selected for isotopic analysis. Four to eight radii including the rings from 1989 to 2009 were cut out, and the whole annual growth rings (latewood and earlywood) were separated by manual dissection with a scalpel under a magnifier. The samples were then ground
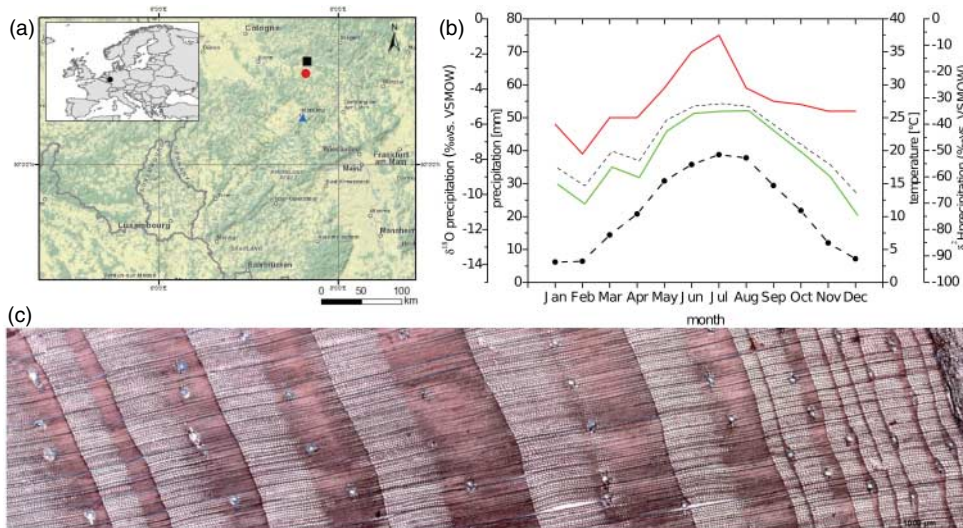
Figure 1. (a) Location of study site Altenkirchen (red), climate station Hilgenroth (black) and GNIP station Koblenz (blue). (b) Monthly mean air temperature (circle), monthly mean precipitation amount (red), monthly mean δ^2H (dashed) and monthly mean $\delta^{18}O$ (green) in precipitation of the time period from 1989–2009. (c) Thin section of one tree sample (Colour online).

in steal grinding jars for about 2–3 min using a mixer mill (Retsch®). The ground sample amount varied from 50 to 300 mg.

Samples of whole wood were treated using a modified Brendel method [14] to isolate α-cellulose from annual growth rings. Samples (2.2–3.0 mg) of whole wood were prepared in 2 ml polypropylene tubes. A volume of 240 μl acetic acid (80 %) and 24 μl nitric acid (9 %) were added to the samples. The closed tubes were inserted into a heating block and heated in an oven for 30 min at 118 °C. Afterwards, the samples were cooled for approximately 5 min at room temperature, and 800 μl ethanol (99.8 %) was added. The samples were then vortexed, centrifuged for 15 min at 13,000 rpm, and the supernatant removed. A volume of 600 μl of bi-distilled water was added to eliminate excess of unreacted nitric acid, and the samples were shaken and centrifuged for another 12 min. The next step was to pipette 300 μl ethanol (99.8 %) to the samples, to centrifuge these for 7 min at 13,000 rpm, and to remove the supernatant. Finally, 300 μl acetone was added, the samples were centrifuged again for 2 min (13,000 rpm), and supernatant was removed. Samples were sealed with Parafilm© and dried at 50 °C (5–30 min). The samples were then placed in a refrigerator for at least 30 min and lyophilised for 1–5 h. Cellulose samples (250–300 μg) were packed in tin capsules for $\delta^{13}C$ analysis and in silver capsules for $\delta^{18}O$ analysis.

Hydrogen and carbon stable isotopes of methoxyl groups were analysed according to a method recently presented by Greule et al. [29,30], based on the Zeisel method [27]. Without any pretreatment, 0.5 ml hydroiodic acid (55–58 %) was added to wood samples (2 mg for carbon and 10 mg for hydrogen isotopic analysis) in a sealed vial (1.5 ml) and heated for 30 min at 130 °C. Afterwards, the samples were left to equilibrate for at least 30 min and an aliquot (10–90 μl) of the gaseous reaction product methyl iodide (CH_3I) was transferred to the gas chromatography–combustion (for $\delta^{13}C$)/thermal conversion (for δ^2H)–isotope ratio mass spectrometry (GC-C/TC-IRMS) system using an autosampler.

Whole wood measurements were based on milled samples, dried at 60 °C for 24 h, and packed (170–200 μg) in tin capsules for $\delta^{13}C$ and silver capsules for $\delta^{18}O$ measurement (IVA Analysentechnik, Meerbusch, Germany).

2.3. *Stable isotope analysis*

2.3.1. *TC/EA-IRMS analysis of cellulose and whole wood samples*

Samples for δ^{13}C analysis were stored in a desiccator until combustion with an elemental analyser (Flash EA 1112, ThermoFinnigan, Bremen, Germany). Samples for δ^{18}O analysis were stored in an evacuated desiccator to prevent adsorption with water vapour from the atmosphere until analysis and pyrolysed in a high-temperature conversion/elemental analyser (TC/EA ThermoFinnigan). The TC/EA was coupled to a continuous-flow isotope ratio mass spectrometer (Delta V Advantage, ThermoFinnigan). Carbon isotopes were measured with a standard deviation of ± 0.1 for the periodic quality standard (barley), and oxygen isotopes were measured with a standard deviation of ± 0.3 for the periodic quality standards (benzoic acid (IAEA-601, Austria), cellulose (Sigma Aldrich, Germany) and sucrose (Roth, Germany) after drift correction.

2.3.2. *GC-C/TC-IRMS analysis of lignin methoxyl groups*

Hydrogen and carbon isotope signatures of lignin methoxyl groups were measured as CH_3I released upon treatment of wood samples with HI as described in Section 3.2. δ^{13}C and δ^{2}H isotope ratios were determined using a GC-C/TC-IRMS system consisting of an HP 6890N gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with an A200S autosampler (CTC Analytics, Zwingen, Switzerland), coupled to a Delta^{PLUS}XL isotope ratio mass spectrometer (ThermoQuest Finnigan, Bremen, Germany) via an oxidation reactor (δ^{13}C) [ceramic tube (Al_2O_3), length 320 mm, 0.5 mm i.d., with Cu/Ni/Pt wires inside (activated by oxygen), reactor temperature 960 °C] or via a pyrolysis reactor (δ^{2}H) [ceramic tube (Al_2O_3), length 320 mm, 0.5 mm i.d., reactor temperature 1450 °C] and a GC Combustion III interface (ThermoQuest Finnigan, Bremen, Germany). The gas chromatograph was fitted with a ZB-5ms capillary column (Phenomenex, Torrance, CA, USA) (30 m × 0.25 mm i.d., d_f 1.0 mm).

The GC conditions for δ^{13}C analysis were: split injection (split ratio 10:1), injector temperature 200 °C; initial oven temperature at 30 °C for 3.8 min, ramp at 30 °C/min to 100 °C. Helium was used as the carrier gas and the flow rate was 1.8 ml/min. For δ^{2}H analysis, the following GC conditions were employed: split injection (split ratio 4:1), injector temperature 200 °C; initial oven temperature at 30 °C for 7 min, ramp at 40 °C/min to 120 °C with a constant helium carrier gas flow of 0.6 ml/min. All δ^{13}C and δ^{2}H values were normalised relative to V-PDB or V-SMOW using a CH_3I standard. The δ^{13}C value of CH_3I was calibrated against international reference substances (IAEA-CH-6, IAEA-CH-7, NBS-22) using an offline elemental analyser (EA)/IRMS system (Iso-Analytical Ltd., Sandbach, UK). The calibrated δ^{13}C value in ‰ *vs*. V-PDB for CH_3I was -69.27 ± 0.05‰ ($n = 15$, 1 s).

The δ^{2}H value of CH_3I was also calibrated against international reference substances (IA–R002, IAEA-CH-7, NBS-22) using the same EA-IRMS system described before. The calibrated δ^{2}H value in ‰ *vs*. V-SMOW was -179.0 ± 2.9‰ ($n = 15$, 1 s). The reference material was measured after every fourth sample injection. As this procedure represents solely a 1-point calibration, it has to be pointed out that the δ^{2}H and δ^{13}C values might be affected by an additional error ('scale compression'). Unfortunately, CH_3I working standards with distinct isotopic signatures spanning the full range of measured δ^{2}H and δ^{13}C values were not available for this study to eliminate or minimise such an error. The authors are aware that international comparability of stable isotope abundance measurements ideally requires a 2-scale anchor calibration with accepted isotope abundance values as recommended by the IUPAC guidelines [36].

Throughout this paper, the conventional delta notation, which expresses the isotopic composition of a material relative to a standard, is used: δ^{2}H and δ^{18}O relative to V-SMOW, and values

δ^{13}C relative to V-PDB are defined as

$$\delta = (R_{\text{sample}}/R_{\text{standard}}) - 1, \tag{1}$$

where R is the ratio between the heavier isotope and the lighter one for the sample and the standard, respectively [36]. Values of δ in the text are quoted as per mil (‰).

2.4. *Climate and GNIP data*

Climate data for the monitoring plot in Altenkirchen were provided by the DWD (Deutscher Wetterdienst, station 'Hilgenroth') on a monthly basis including mean, minimum and maximum temperatures, precipitation and relative humidity. A modified climate diagram using these data (1989–2005) indicates that average precipitation exceeds 39 mm in any month, and temperatures reach a minimum in January (3.1 °C) and a maximum in July (19.4 °C).

A subprogram of the assembly tool 'evaporation' of the soil water model SIMPEL was used to calculate the water balance (Precip-pET) available at http://www.hydrology.uni-kiel.de/forschung/projekte/simpel. Climate data on a daily basis as driving input were provided from a monitoring plot of the Research Institute for Forest Ecology and Forestry near Koblenz. The Penman–Montheith equation was used to estimate pET [37].

Precipitation isotope data of the station in Koblenz were downloaded from the Global Network of Isotopes in Precipitation (GNIP, Vienna) database at http://isohis.iaea.org. At this station, precipitation δ^2H and δ^{18}O data are available from 1989 to 2005.

2.5. *Atmospheric carbon dioxide correction*

Since industrialisation, increasing combustion of fossil fuels has led to an increase in carbon dioxide concentration and to a decrease of δ^{13}C in the atmosphere. Correction for the industrial δ^{13}C decline due to fossil fuel emission was conducted using the equation:

$$\delta^{13}C_{\text{cor}} = \delta^{13}C_P - (\delta^{13}C_{\text{Atm}} + 6.4‰), \tag{2}$$

where $\delta^{13}C_{\text{cor}}$ is the isotopic value of the corrected δ^{13}C, $\delta^{13}C_P$ is the isotopic value of the plant, $\delta^{13}C_{\text{Atm}}$ is the isotopic value of the atmosphere. $\delta^{13}C_{\text{Atm}}$ data until 2003 were used from McCarroll and Loader [35], and thereafter linearly extrapolated until 2009.

2.6. *Statistical analysis*

Isotopic and ring width time series were compared with climatic parameters (monthly temperature, precipitation, relative humidity, vapour pressure deficit, potential evapotranspiration, Precip-pET, δ^{18}O and δ^2H in precipitation) to assess the climatic sensitivity, using Pearson correlations. Lag-1 autocorrelation (AC) was considered to evaluate the dependency of growth and δ^{13}C on previous years' photoassimilates, and, for δ^{18}O, on ground instead of soil water. We used sensitivity as a measure of inter-annual variability [38] and mean standardised sensitivity (MSS) as a measure of the variance of whole time series, defined as:

$$S = \frac{\sum_{i=1}^{n-1} |x_{i+1} - x_i|}{n-1}. \tag{3}$$

The expressed population signal (EPS) was used to estimate the minimum number of trees needed to capture a representative signal from stable isotopes and ring widths [35,39]:

$$\text{EPS} = \frac{(n \times r_{\text{mean}})}{(n \times r_{\text{mean}}) + (1 - r_{\text{mean}})}, \tag{4}$$

where n is the number of trees and r_{mean} is the mean inter-tree correlation. MSS and EPS were calculated for the different isotopic and ring width time series.

3. Results

3.1. *Tree ring parameters of individual trees*

The δ^{13}C range of the same year between different individuals of *P. sylvestris* was 0.22–0.96‰ for cellulose and 0.46–2.15‰ for methoxyl groups. δ^2H$_{meth}$ of the same year ranged from 6.4 to 26‰, and the δ^{18}O range was 0.45–2.94‰ for whole wood and 0.32–2.27‰ for cellulose. Ring width ranged from 0.41 to 1.58 mm (Figure 2).

First, second and third order AC, MSS and EPS results are given in Table 1. All tree ring parameters showed non-significant AC ($p > .05$). MSS was highest for δ^2H$_{meth}$ and lowest for δ^{13}C$_{meth}$ and δ^{18}O$_{whole}$. EPS indicated that four trees are acceptable to capture a representative

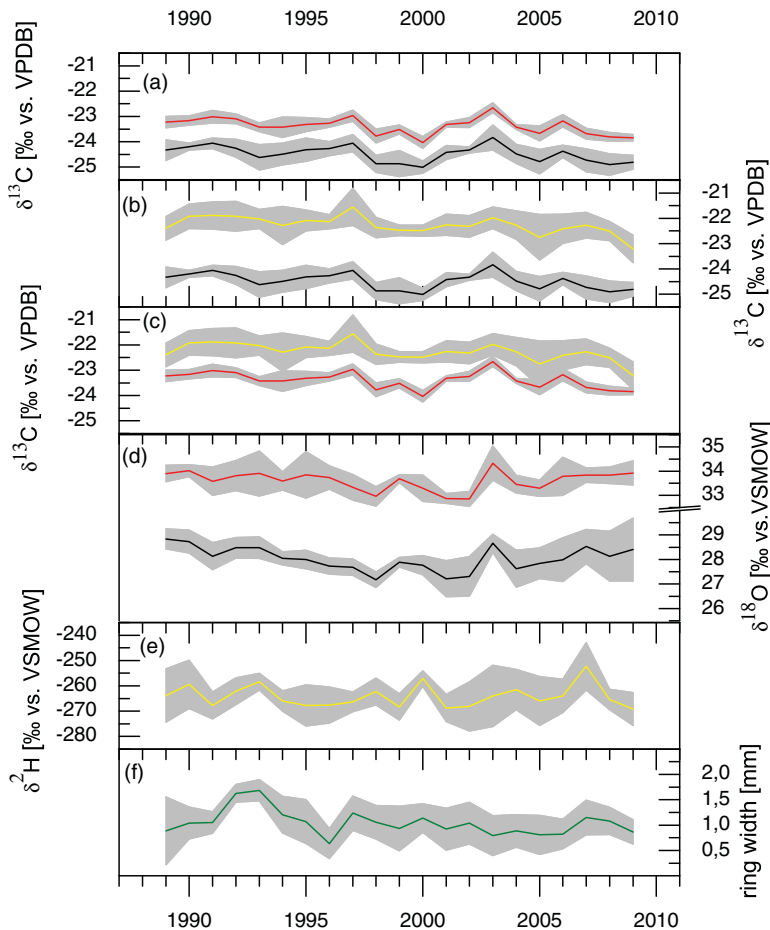

Figure 2. Time series of arithmetic mean of ring width, carbon, oxygen and hydrogen isotopes ($n = 21$). Arithmetic mean of (a) δ^{13}C from cellulose (red) and δ^{13}C from whole wood (black), (b) δ^{13}C from methoxyl groups (yellow) and δ^{13}C from whole wood (black), (c) δ^{13}C from cellulose (red) and δ^{13}C from methoxyl groups (yellow), (d) δ^{18}O from cellulose (red) and δ^{18}O from whole wood (black), (e) δ^2H from methoxyl groups (yellow) and (f) ring width (green). Standard error (2xSE) is shown as grey area (Colour online).

Table 1. First, second and third order autocorrelation (AC), mean standardised sensitivity (MSS) and expressed population signal (EPS).

	AC1	AC2	AC3	MSS	EPS
$\delta^{13}C_{ww}$	0.39	− 0.04	− 0.33	0.86	0.88
$\delta^{13}C_{cell}$	0.21	0.06	− 0.30	0.93	0.91
$\delta^{13}C_{meth}$	0.29	0.04	0.03	0.79	0.77
$\delta^{2}H_{meth}$	− 0.16	− 0.27	0.10	1.22	0.75
$\delta^{18}O_{ww}$	0.36	0.22	0.32	0.79	0.74
$\delta^{18}O_{cell}$	0.18	− 0.09	0.27	0.90	0.61
RW	0.38	0.09	− 0.14	0.86	0.77

Abbreviations: ww – whole wood, cell – cellulose, meth – methoxyl group, RW – ring width.

site isotope signal for $\delta^{13}C_{cell}$ and $\delta^{13}C_{whole}$, but not for $\delta^{13}C_{meth}$, $\delta^{2}H_{meth}$, $\delta^{18}O_{whole}$, $\delta^{18}O_{cell}$ and ring width. EPS of $\delta^{18}O_{cell}$ was the lowest among all measured parameters.

3.2. *Stable isotopes from different wood components*

3.2.1. *$\delta^{18}O$ of whole wood and α-cellulose*

The mean time series of $\delta^{18}O_{whole}$ presented a larger range (27.99 to 28.84‰; $\bar{x} = 28.03$‰; SD = 0.49‰) than $\delta^{18}O_{cell}$ (32.73 to 34.33‰; $\bar{x} = 33.61$‰; SD = 0.39‰). $\delta^{18}O_{whole}$ were on average 5.58‰ (SD = 0.23‰) lower than $\delta^{18}O_{cell}$. Pearson correlation coefficients between whole wood and cellulose indicated high positive correlations ($r = 0.89$; $p < .001$) (Table 2, Figures 2(d) and 3).

3.2.2. *$\delta^{13}C$ of whole wood, α-cellulose and lignin methoxyl groups*

The mean time series of $\delta^{13}C$ from whole wood ranged from − 25.01 to − 23.83‰ ($\bar{x} = -24.48$‰; SD = 0.32‰). $\delta^{13}C_{cell}$ displayed a greater range than whole wood (− 24.03 to − 22.66‰; $\bar{x} = -23.38$‰; SD = 0.33‰), and $\delta^{13}C_{meth}$ showed the largest range (− 23.21 to − 21.55‰; $\bar{x} = -22.26$‰; SD = 0.35‰). $\delta^{13}C_{whole}$ were on average 1.09‰ (SD = 0.09‰) isotopically lighter than the corresponding $\delta^{13}C_{cell}$ and 2.23‰ (SD = 0.25‰) lighter than corresponding $\delta^{13}C_{meth}$. $\delta^{13}C_{whole}$ and $\delta^{13}C_{cell}$ showed a high correlation ($r = 0.96$, $p < .001$), but it was lower than between $\delta^{13}C_{whole}$ and $\delta^{13}C_{meth}$ ($r = 0.72$, $p < .001$). $\delta^{13}C_{meth}$ and $\delta^{13}C_{cell}$ showed the same correlation ($r = 0.72$, $p < .001$) (Figures 2(a)–(c) and 3, Table 2).

Table 2. Pearson correlations among all measured tree ring parameters.

	$\delta^{13}C_{ww}$	$\delta^{13}C_{cell}$	$\delta^{13}C_{meth}$	$\delta^{2}H_{meth}$	$\delta^{18}O_{ww}$	$\delta^{18}O_{cell}$
$\delta^{13}C_{cell}$	0.96**					
$\delta^{13}C_{meth}$	0.72**	0.72**				
$\delta^{2}H_{meth}$	− 0.23	− 0.24	0.16			
$\delta^{18}O_{ww}$	0.23	0.24	0.07	0.36*		
$\delta^{18}O_{cell}$	0.26	0.29	0.08	0.21	0.89**	
RW	− 0.06	− 0.03	0.40*	0.41*	0.21	0.03

Note: Significant values are marked with asterisks ($p < .1$*; $p < .001$**). Abbreviations: ww – whole wood, cell – cellulose, meth – methoxyl group, RW – ring width.
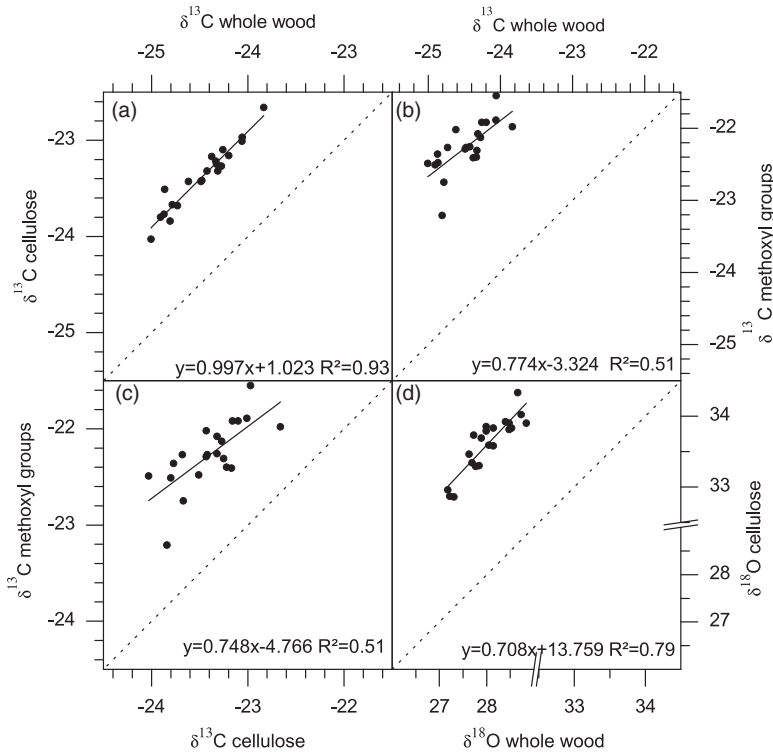
Figure 3. Records of δ^{13}C whole wood *vs.* corresponding δ^{13}C from (a) cellulose and (b) methoxyl groups, respectively, and (c) records of δ^{13}C cellulose *vs.* corresponding δ^{13}C from methoxyl groups. (d) Records of δ^{18}O from whole wood *vs.* corresponding δ^{18}O from cellulose. The regression is shown as black line.

3.2.3. *Stable isotopes and ring width*

Pearson correlations between different isotopic parameters and ring width are presented in Table 2. $\delta^{18}O_{whole}$, $\delta^{18}O_{cell}$, $\delta^{13}C_{whole}$ and $\delta^{13}C_{cell}$ demonstrated insignificant correlations with ring width ($p > .1$). Pearson correlation between ring width and $\delta^{13}C_{meth}$ was higher than
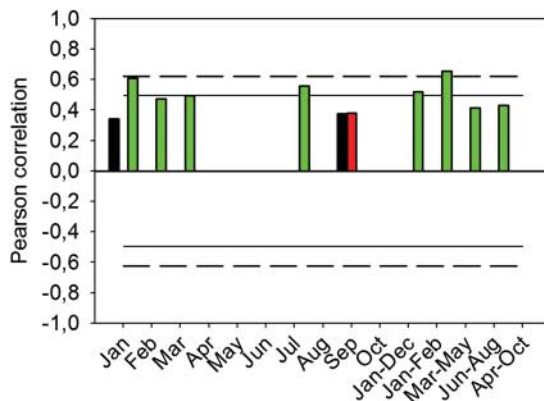

Figure 4. Pearson correlations between δ^{18}O whole wood (black bar), δ^{18}O cellulose (red bar) and δ^2H methoxyl groups (green bar) in tree ring and weighted isotopes in precipitation for years 1989–2005. Significance level $p < .05$ is shown as continuous line and $p < .01$ as dashed line (Colour online).
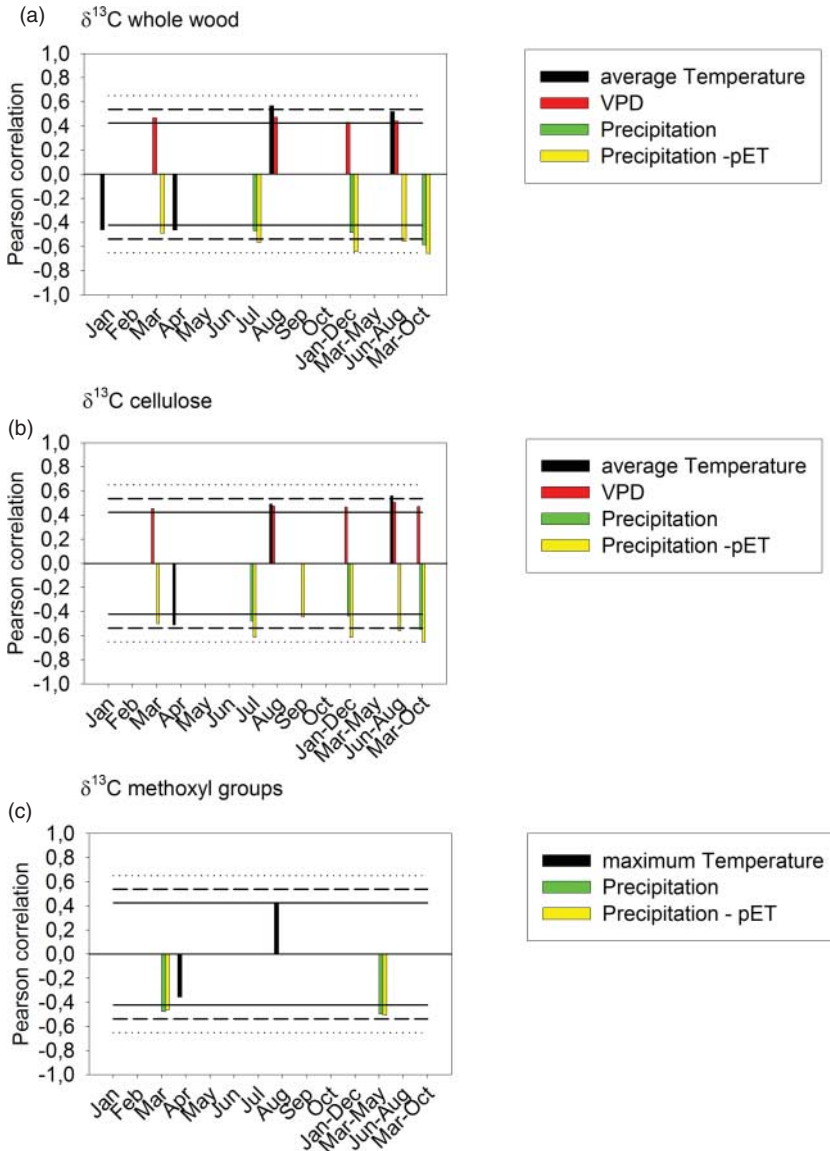
Figure 5. Pearson correlation between (a) $\delta^{13}C$ whole wood, (b) $\delta^{13}C$ cellulose or (c) $\delta^{13}C$ methoxyl groups and climate data for years 1989–2009. Selected climate data are monthly temperature (black bar), monthly sum of precipitation (green bar), vapour pressure deficit VPD (red bar) and Precip-pET (yellow bar). Significance level $p < .05$ is shown as continuous line, $p < .01$ as dashed line and $p < .001$ as dotted line (Colour online).

between ring width and $\delta^{13}C_{whole}$ and $\delta^{13}C_{cell}$, respectively. δ^2H_{meth} showed moderate positive correlations with ring width ($r = 0.41$; $p < .1$) (Table 2).

3.3. *Environmental signals in isotopic records*

3.3.1. *Environmental signals in hydrogen and oxygen isotopes from tree rings and isotopic composition of precipitation*

Hydrogen and oxygen isotopic data in precipitation were taken from the climate station in Koblenz, Germany (GNIP station) from 1989 to 2005 (Figure 1(b)). δ^2H_{meth} showed highest

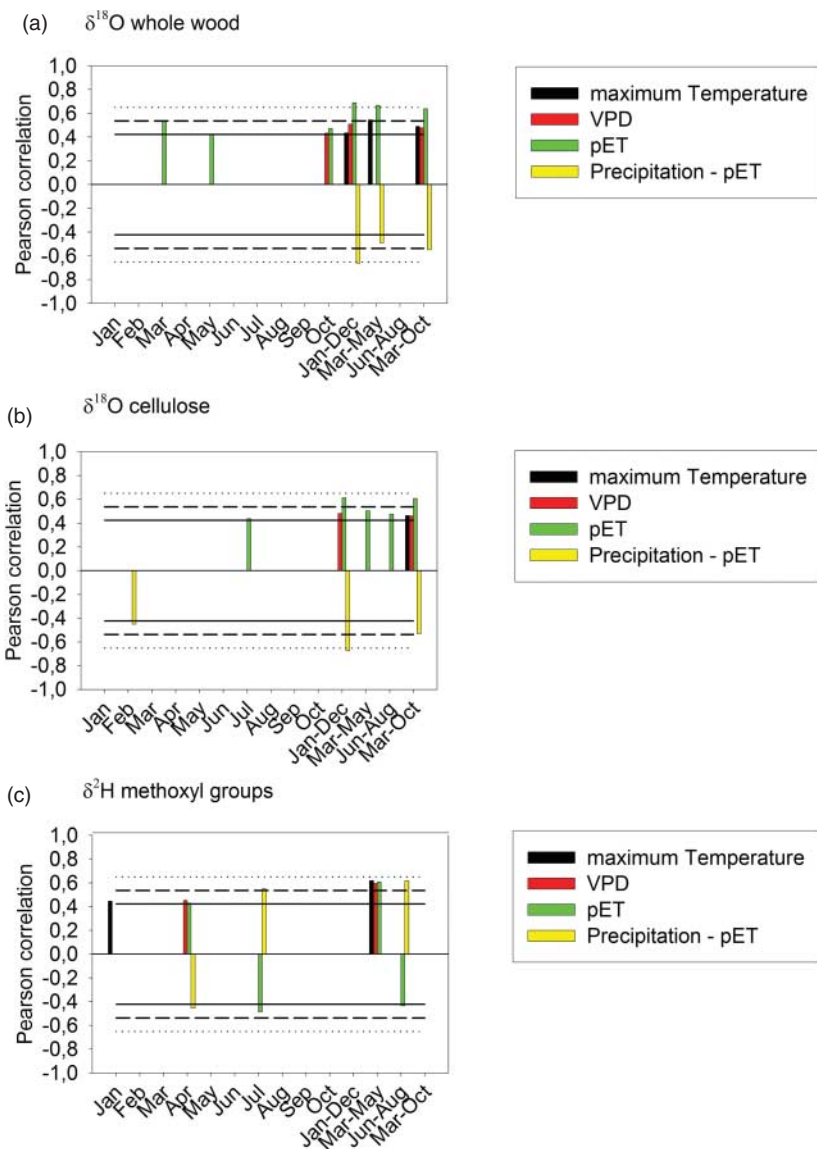Figure 6. Pearson correlation between (a) δ^{18}O whole wood, (b) δ^{18}O cellulose, (c) δ^2H methoxyl groups and climate data for years 1989–2009. Selected climate data are monthly temperature (black bar), vapour pressure deficit VPD (red bar), potential evapotranspiration pET (green bar) and Precip-pET (yellow bar). Significance level $p < .05$ is shown as continuous line, $p < .01$ as dashed line and $p < .001$ as dotted line (Colour online).

positive correlations with January to February hydrogen isotopes in precipitation. September oxygen isotopes in precipitation showed the highest positive correlations with δ^{18}O$_{whole}$ and δ^{18}O$_{cell}$ (Figure 4).

3.3.2. Climate data and δ^{13}C records from whole wood, cellulose and methoxyl groups

δ^{13}C values from tree rings were significantly correlated with temperature, vapour pressure deficit, precipitation and Precip-pET. δ^{13}C$_{whole}$ was significantly correlated with August average temperature ($r = 0.57$; $p < .01$), March to October precipitation ($r = -0.59$; $p < .01$), March

and August vapour pressure deficit ($r = 0.47$; $p < .05$) and March to October Precip-pET ($r = -0.66$; $p < .001$) (Figure 5(a)).

$\delta^{13}C_{cell}$ was significantly correlated with June to August average temperature ($r = 0.56$; $p < .01$), March to October precipitation ($r = -0.55$; $p < .01$), June to August vapour pressure deficit ($r = 0.51$; $p < .05$) and March to October Precip-pET ($r = -0.66$; $p < .001$) (Figure 5(b)).

$\delta^{13}C_{meth}$ showed significant correlations with August maximum temperature ($r = 0.42$; $p < .05$), March to May precipitation ($r = -0.50$; $p < .05$) and March to May Precip-pET ($r = -0.51$; $p < .05$) (Figure 5(c)).

3.3.3. *Climate data and δ^2H from methoxyl groups*

δ^2H_{meth} records showed significant correlations with maximum temperature of March to May ($r = -0.62$; $p < .01$), VPD of March to May ($r = 0.60$; $p < .01$), potential evapotranspiration of March to May ($r = 0.61$; $p < .01$) and Precip-pET of April ($r = -0.45$; $p < .05$) (Figure 6(c)).

3.3.4. *Climate data and $\delta^{18}O$ from whole wood and cellulose*

$\delta^{18}O_{whole}$ showed significant correlations with maximum temperature of March to May ($r = 0.55$; $p < .01$), VPD of January to December ($r = 0.51$; $p < .05$), pET of January to December ($r = 0.69$; $p < .001$) and Precip-pET of January to December ($r = -0.66$; $p < .001$) (Figure 6(a)).

Also, $\delta^{18}O_{cell}$ revealed significant correlations with maximum temperature of March to October ($r = 0.46$; $p < .05$), pET of January to December ($r = 0.61$; $p < .01$) pET of March to October ($r = 0.61$; $p < .01$) and Precip-pET of January to December ($r = -0.68$; $p < .001$) (Figure 6(b)).

4. Discussion

4.1. *Tree ring parameters of individual trees*

The coherence of inter-tree variability is an important measure which is helpful in deciding the number of trees necessary to capture a site-specific isotope signal. Inter-tree variance typically ranges from 1 to 3‰ for carbon isotopes, 1–4‰ for oxygen isotopes, and 5–30‰ for hydrogen isotopes [39]. Our findings differ in parts from Leavitt [39]. We found smaller inter-tree variability in $\delta^{13}C_{whole}$, $\delta^{13}C_{cell}$ and $\delta^{13}C_{meth}$, compared with the 2–3‰ $\delta^{13}C$ range among five *Pinus edulis* trees [40]. Ramesh et al. [2] report a similar range from 0.5 to 4‰.

The variability of $\delta^{18}O_{cell}$ and $\delta^{18}O_{whole}$ in our study is at the lower end, and agrees with $\delta^{18}O$ values of *F. sylvatica* with a range of 0.8‰ [41]. Treydte et al. [42] reported a 2–4‰ range for *Juniperus excelsa*.

For the first time, variability between trees of the same site is reported for $\delta^{13}C$ and δ^2H methoxyl groups. Methoxyl groups show a greater range of $\delta^{13}C$ in tree rings compared with $\delta^{13}C_{whole}$ and $\delta^{13}C_{cell}$. δ^2H_{meth} varies from 6.4 to 26‰ in tree rings of the same year, and the variability is similar to the range of δ^2H from nitrocellulose reported by Ramesh et al. [2] and Savard et al. [43].

EPS values higher than 0.85 are considered to be adequate to capture a representative site signal. With an EPS = 0.91, the sample size of four trees for $\delta^{13}C_{cell}$ can be considered adequate

and, as a consequence, the number of trees might be reduced to a sample size of three trees. Four trees are still adequate for $\delta^{13}C_{whole}$, but the number of trees would need to increase to seven for $\delta^{13}C_{meth}$. The study on $\delta^{13}C$ isotopes from Gagen et al. [4] indicated four *Pinus uncinata* trees were sufficient (EPS = 0.90). The study on $\delta^{13}C$ isotopes from McCarroll and Pawellek [44] showed two to five *P. sylvestris* to be sufficient for sites in Northern Finland (EPS = 0.90–0.95). Two to six *Quercus robur* were found sufficient to produce representative $\delta^{13}C$ chronologies in Southern Finland [45].

EPS of δ^2H_{meth} was substantially lower (0.75) indicating that a minimum of eight trees would be needed to achieve values exceeding 0.85. Similarly, the number of trees would need to be increased to eight trees for $\delta^{18}O_{whole}$. $\delta^{18}O_{cell}$ achieved the lowest EPS indicating that 14 trees would be needed. In contrast, three to four trees were found sufficient to obtain representative $\delta^{18}O$ chronologies in Pakistan [42].

Furthermore, moderate AC was present in $\delta^{13}C_{whole}$. According to Kagawa et al. [46] working with *Larix gmelinii*, earlywood formation in conifers depends on the photoassimilates formed during the end of the previous growing season (end of summer and autumn) and on the current photoassimilate (current spring). However, latewood formation depends predominantly on the photoassimilate formed during the current growing season (summer and autumn).

AC was not observed for δ^2H_{meth}, indicating that ground water from previous growing seasons was not used for the synthesis of methoxyl groups. Moderate, insignificant AC was found in $\delta^{18}O_{whole}$ potentially indicating an uptake of depleted ground water that is stored over several growing periods in the deeper soil layer [47].

Standardisation prior to calculation of MSS made the different parameters comparable and showed which parameter contains the largest year-to-year variability. MSS of $\delta^{13}C_{cell}$ exceeded the values of $\delta^{13}C_{whole}$ and $\delta^{13}C_{meth}$ (Table 1). Lowest MSS was found in $\delta^{13}C_{meth}$ and $\delta^{18}O_{whole}$, and highest in δ^2H_{meth}, revealing this parameter to be most sensitive. Due to the MSS and the non-existent AC, δ^2H_{meth} is the most suitable climate proxy followed by $\delta^{18}O_{cell}$.

4.2. *Isotope time series from whole wood, α-cellulose and methoxyl groups*

Wilson and Grinsted [9] and Brugnoli and Farquhar [48] reported that varying wood components show differences in the isotopic ratio and demonstrated that cellulose and lignin reflect distinct climate signals. Several studies examined whether whole wood contains the same climate signal as cellulose or lignin and whether cellulose extraction is necessary. Leuenberger et al. [49], Barbour et al. [17], Loader et al. [18] (*Q. robur*), Verheyden et al. [19] (*Rhizophora mucronata*), and Taylor et al. [50] (*Pseudotsuga menziesii*) found that whole wood contains the same climate signal as cellulose and that extraction of cellulose is unnecessary. In contrast, Borella et al. [16], Schleser et al. [51] (*Q. robur, F. sylvatica, P. sylvestris* and *Sequoiadendron giganteum*), Battipaglia et al. [21] (*F. sylvatica* and *Acer pseudoplatanus*), Sidorova et al. [22–24] (*Larix cajanderi and L. gmelinii*) all reported a significant difference between whole wood and cellulose, indicating that cellulose extraction is necessary for carbon and oxygen isotope analysis.

Here, we compared carbon and oxygen isotopes from whole wood and cellulose, as well as carbon isotopes from methoxyl groups with whole wood and cellulose. Our findings suggest that cellulose extraction may not be mandatory for carbon and oxygen isotope analysis from *P. sylvestris* in Altenkirchen. The results between $\delta^{13}C_{whole}$ and climate data are similar to those observed between $\delta^{13}C_{cell}$ and climate data (Figure 5(a) and 5(b)), but different for $\delta^{13}C_{meth}$ and climate data (Figure 5(c)). Furthermore, we found differences and lower correlations between $\delta^{13}C_{whole}$ and $\delta^{13}C_{meth}$ than between $\delta^{13}C_{whole}$ and $\delta^{13}C_{cell}$ (Table 2, Figure 3), indicating that methoxyl groups contain a different climate signal. The correlation between $\delta^{13}C_{cell}$ and $\delta^{13}C_{meth}$ was identical to the correlation between $\delta^{13}C_{whole}$ and $\delta^{13}C_{meth}$ (Table 2, Figure 3).

Table 3. Comparison of correlation coefficient and mean differences of different wood compounds.

Reference	Species	Correlation coefficient between $\delta^{13}C_{whole}$ and $\delta^{13}C_{cell}$	Mean difference between $\delta^{13}C_{whole}$ and $\delta^{13}C_{cell}$	Correlation coefficient between $\delta^{18}O_{whole}$ and $\delta^{18}O_{cell}$	Mean difference between $\delta^{18}O_{whole}$ and $\delta^{18}O_{cell}$
[17]	Pine and oak species			$r = 0.89$	3.9 ‰
[21]	*A. pseudoplatanus; F. sylvatica*				4.7 ‰
[20]	*Q. robur*	$r = 0.90$		$r = 0.81$	
[52]	cypress pine	$r = 0.64$		$r = 0.70$	7.60 ‰
[26]	*Picea abies*		1.4 ‰		3.3–4.0 ‰
[53]	*Picea glauca*				5.0 ‰
[22]	*L. cajanderi*	$r = 0.92$	1.84 ‰	$r = 0.63$	3.51 ‰
[23]	*L. gmelinii*	$r = 0.75$		$r = 0.61$	
[24]	*L. gmelinii*	$r = 0.84$ and $r = 0.87$	1.64 ‰	$r = 0.58$ and $r = 0.45$	3.46 ‰
[19]	*R. mucronata*	$r = 0.98$	0.97 ‰ (SD = 0.03)		
[54]	*Pinus pinaster; Pinus radiata*	$r = 0.96$	0.87 ‰		

Correlations between $\delta^{13}C_{whole}$ and $\delta^{13}C_{cell}$ from *P. sylvestris* L. are high ($r = 0.96$) and comparable to other studies (Table 3). $\delta^{13}C_{meth}$ showed lower correlations with $\delta^{13}C_{whole}$ ($r = 0.72$) and $\delta^{13}C_{cell}$ ($r = 0.72$). The mean difference between $\delta^{13}C_{whole}$ and $\delta^{13}C_{cell}$ was 1.09 ‰ (SD = 0.09 ‰) and was smaller compared with other studies (Table 3, Figure 2(a)).

The extent of the difference between whole wood and cellulose mainly depends on the relative concentration of other wood components, for example, lignin and resins. Lignin is on average 3 ‰ lighter than cellulose, but does not bias the correlation between whole wood and cellulose [18,19]. Resins of whole wood move radially within the stem [55]. The linear regression model (Figure 3) demonstrates a slope close to one between $\delta^{13}C_{whole}$ and $\delta^{13}C_{cell}$, revealing that whole wood samples could be used instead of cellulose samples to achieve the same result.

Correlations between $\delta^{18}O_{whole}$ and $\delta^{18}O_{cell}$ ($r = 0.89$) are similar to those reported in other studies (Table 3). The mean difference between $\delta^{18}O_{whole}$ and $\delta^{18}O_{cell}$ in this study was 5.58 ‰, comparable to other studies (Figure 2(d)).

4.3. *Climate signals in carbon, hydrogen and oxygen isotope time series*

Highest correlations were found between $\delta^{13}C_{whole}$ as well as $\delta^{13}C_{cell}$ and climate data. $\delta^{13}C_{whole}$ showed high correlations with March to October Precip-pET and August temperature. $\delta^{13}C_{cell}$ showed similar correlations with the June to August temperatures and March to October Precip-pET. Highest correlations were found between $\delta^{13}C_{meth}$ and March to May precipitation ($r = -0.50$) and Precip-pET reaching $r = -0.51$, respectively.

Several other studies reported similar positive correlations between $\delta^{13}C$ time series and temperature data [6,8,56]. Positive correlations between $\delta^{13}C$ and temperature data are reported from dry environments [22,57], whereas negative correlations between $\delta^{13}C$ and precipitation indicate a sensitivity to moisture conditions related to this parameter [4,58]. Saurer et al. [8] found that trees growing in dry sites correlate better with climate data than trees growing in moist sites. The lower correlations between $\delta^{13}C_{meth}$ and March to May temperature might indicate moist soil conditions during the beginning of the growing period. Based on these results, we assume that $\delta^{13}C_{whole}$ and $\delta^{13}C_{cell}$ are more sensitive to climate parameters (precipitation and Precip-pET) of the whole growing season (March to October) than $\delta^{13}C_{meth}$. However, $\delta^{13}C_{meth}$

appears to be more sensitive to climate conditions during the beginning of the growing season (March to May).

$\delta^{13}C_{meth}$, δ^2H_{meth} and $\delta^{18}O_{whole}$ were observed to be positively correlated with ring width (Table 1). Positive correlations between ring width and $\delta^{13}C$ values appear characteristic for moist sites [57,58], whereas negative correlations are indicative for dry sites [38]. $\delta^{13}C_{meth}$ showed higher positive correlations and appears more sensitive in humid sites.

In the case of δ^2H_{meth}, we found high correlations with maximum temperature, VPD and pET at the beginning of the growing season. The highest correlations were found between $\delta^{18}O_{whole}$ and pET or Precip-pET from January to December. $\delta^{18}O_{cell}$ showed similar correlations with January to December pET and Precip-pET. A possible explanation can be the use of melt water from snow or frozen soil water in the early growing season.

$\delta^{18}O$ and δ^2H in tree rings are most likely related to the isotopic composition of the source water [32]. The mean time series of $\delta^{18}O_{whole}$ and $\delta^{18}O_{cell}$ showed insignificant correlations with $\delta^{18}O$ in precipitation, suggesting that the trees used a water source different from precipitation, for example, deeper ground water with a different isotopic composition. In agreement with Rebetez et al. [7], we found higher correlations between $\delta^{18}O$ as well as δ^2H and maximum temperature, compared with mean and minimum temperature.

5. Conclusion

Our findings show that $\delta^{13}C_{whole}$ and $\delta^{18}O_{whole}$ are good climate proxies, similar to proxies derived from cellulose samples. The high correlation between whole wood and cellulose $\delta^{13}C$ and $\delta^{18}O$ time series in *P. sylvestris* offers the possibility to use whole wood instead of cellulose. This is in agreement with work by Leuenberger et al. [49], Barbour et al. [17], Loader et al. [18], Verheyden et al. [19], Taylor et al. [50] and Gori et al. [26]. The results of the correlations can perhaps not be applied to sub-fossil *P. sylvestris* wood, as the degradation in fossil wood results in an increased percentage of lignin [18]. Furthermore, the wood samples used here did not include heartwood. The utilisation of heartwood in longer time series can be problematic, as it contains a higher percentage of extractives [18,19].

δ^2H_{meth} showed high correlations with maximum temperatures and is also correlated with ring width (highest correlation among all tree ring parameters), indicating its potential as a temperature proxy. $\delta^{13}C_{meth}$ showed generally lower correlations with climate parameters, except for the period from March to May. Correlations between climate parameters and $\delta^{13}C_{whole}$ or $\delta^{13}C_{cell}$ are equally strong. $\delta^{18}O_{whole}$ showed similar correlations with climate as $\delta^{18}O_{cell}$.

A low AC and high standardised mean sensitivity of δ^2H_{meth} and $\delta^{18}O_{cell}$ showed the applicability for ecophysiological and palaeoclimatic studies. Ring width, $\delta^{13}C_{whole}$, $\delta^{13}C_{meth}$ and $\delta^{18}O_{whole}$ all contain moderate, though insignificant, ACs, indicating the influence of stored photoassimilates from the previous year.

In summary, our findings reveal that multiple climate elements can be reconstructed from different wood components and that stable carbon and oxygen values of whole wood proxies perform comparably to cellulose time series. In addition, we would like to highlight that δ^2H measurements of methoxyl groups might open new possibilities for climate reconstructions in tree ring research.

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