



INTER- AND INTRA-ANNUAL CARBON ISOTOPE FLUCTUATIONS IN *PINUS SYLVESTRIS* L. TREE RINGS WHOLE WOOD AND CELLULOSE IN NORTH-EASTERN LITHUANIA

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Abstract

In temperate regions trees typically exhibit growth sensitivity to climatic conditions during the growth season. Annual tree ring growth increments correlate with a variety of environmental factors. As an index of water use efficiency, $\delta^{13}\text{C}$ is a preferred proxy to allow accurate interpretation of environmental factors critical for the tree growth, including changes in climate patterns, variation in the ambient temperature and precipitation.

We assumed that isotopic differences within individual tree rings are likely to produce seasonal isotope fluctuations in the chronology that might be interpreted as response to environmental impacts. To verify the assumption, we measured $\delta^{13}\text{C}$ in annual tree rings of *Pinus Sylvestris* L. split into 13 intra-annual segments each and checked $\delta^{13}\text{C}$ correlations with temperature, precipitation, the number of sunshine hours and relative air humidity. For the investigation, we chose a site in north-eastern Lithuania, Zarasai, located in boreal latitude and remote from industrial pollution sources. The methodology of the research was based on high coherence of $\delta^{13}\text{C}$ chronologies measured in the whole wood and α -cellulose extracted by means of two different methods. The experiment produced strong $\delta^{13}\text{C}$ correlations with hydrometeorological parameters, especially in the earlywood formed in June.

Keywords

intra-annual $\delta^{13}\text{C}$ chronology, earlywood, latewood, α -cellulose, environmental factors, boreal forest

1. Introduction

Recent climate change have raised interest in boreal and temperate forest ecosystems functioning as a significant part of the terrestrial carbon sink that removes nearly 30% of anthropogenic carbon emissions each year (Le Quéré *et al.*, 2018, Romeiro *et al.*, 2022, Mirabel *et al.*, 2023) and encouraged research in the productivity, carbon fixation capacity, dynamics and sustainability of the ecosystem, dominated by coniferous trees in the north and transiting to a mix of hardwoods in the south. Being the largest pool of terrestrial biomass, boreal forest plays a vital role in global carbon dynamics. As the forest ecosystems are

subjected to simultaneous impacts by climatic changes and anthropogenic factors, a deeper understanding of how the environmental variables affect the carbon cycling in the ecosystem becomes critical. Along with the annual growth increment, research has to make a deeper insight into physiological processes taking place in the tree cell and internal and external effects on its growth (Remus *et al.*, 2018; Belmecheri and Lavergne 2020; Pukienė *et al.*, 2021).

As an ecosystem, boreal forests experience multiple abiotic and biotic risks, including low temperatures, windstorms, snow loading, drought, forest fires, insect and pathogen outbreaks (Jactel *et al.*, 2011; Seidl, *et al.*, 2014; Reyer *et al.*, 2014; Seidl *et al.*, 2017; Bentz *et al.*, 2019; Belmecheri and Lavergne 2020). While the atmospheric CO_2 elevations are likely to positively contribute to forest growth, changes in temperature and precipitation as well as shifts in seasonal onsets and length may adversely affect

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the photosynthetic rate of individual trees (Sensuła and Wilczyński, 2022). Researchers distinguish drought stress and the length of the growing season as the two key climate components limiting tree growth (Girardin and Tardif, 2005; Sensuła *et al.*, 2015; Sensuła and Wilczyński, 2018). Evergreen foliage allows conifers to take advantage of earlier springs and extended growing seasons, rendering drought stress the critical factor for the growth (Graumlich, 1993; Tardif and Bergeron, 1997; Girardin and Tardif, 2005). Bonan and Shugart (1989) also point out the role of the seasonal dynamics of atmospheric carbon dioxide.

Environmental effects on tree growth have been estimated by measuring various physical and biochemical parameters, including radial increment, cell number and wood density (Pritzkow *et al.*, 2014; Sensuła, 2016; Timofeeva *et al.*, 2017; Fonti *et al.*, 2018; Szejner *et al.*, 2018). Along with measurements of tree ring width and density, ratios of stable carbon isotope have been widely used to understand eco-physiological processes and environmental conditions governing tree growth (McCarroll and Loader, 2004; Gessler *et al.*, 2014). As an expression of photosynthetic carbon isotope fractionation $\Delta^{13}\text{C}$ and the ratio of CO_2 concentrations within the leaf mesophyll and the surrounding atmosphere (c_i/c_a), the tree ring carbon isotopes ratio ($\delta^{13}\text{C}$) is a proxy that allows disclosure of the relationship between the process of photosynthesis and the environment (Farquahar *et al.* 1989; McCarroll and Loader, 2004; Brooks *et al.*, 2019; Belmecheri and Lavergne, 2020). $\delta^{13}\text{C}$ values have been used to reconstruct drought impact on tree physiology in temperate climates (Lévesque *et al.*, 2014) and cloudiness in boreal environments (Young *et al.*, 2010). It also allows estimation of intra- and inter-annual rates of photosynthesis by means of internal water use efficiency (iWUE), the derivative expressing the ratio of carbon gain to water loss (Saurer *et al.*, 2004; Saurer *et al.*, 2014) with a further insight into environmental factors affecting the latter, such as air and soil pollution, fertilizing and other anthropogenic impact (Brooks and Mitchell, 2011; Sensuła *et al.*, 2015; Tomlinson, 2015; Barisevičiūtė *et al.*, 2017).

The meteorological parameters that could affect $\delta^{13}\text{C}$ values in tree-rings are those affecting the stomatal conductance and the photosynthetic rate. In arid environments, the stomatal conductance is the dominating factor regulating CO_2 intake along with evapotranspiration and a strong correlation of $\delta^{13}\text{C}$ with air humidity and precipitation is observed whereas in humid environments where moisture stress is absent the dominating factor is the photosynthetic rate and a high correlation with sunlight and temperature is observed (Barisevičiūtė *et al.*, 2017). This is clearly seen in iWUE, the ratio of photosynthetic rate (A) to stomatal conductance to water vapor (g_w), where the photosynthetic rate is directly dependent on the concentration of the atmospheric CO_2 and conductance to water vapour is limited by water pressure deficit. The increase in iWUE and decrease in $\delta^{13}\text{C}$ over the past century testifies the growing amount of anthropogenic CO_2 (Barisevičiūtė *et al.*, 2017;

Belmecheri and Lavergne, 2020; Sensuła and Wilczyński, 2022).

Measurements of stable carbon isotope ratios can be done in different wood components, including whole wood, holocellulose, hemicellulose, α -cellulose, lignin (Belmecheri *et al.*, 2022) and glucose (Sensuła, 2016). The α -cellulose is often preferred to other components as it is most stable and least prone to disintegration under environmental effects while hemicellulose, for example, undergoes isotopic exchange in its carbonyl bonds. Because of the complexity of lignification, measurements of lignin's isotopic composition may be difficult to interpret despite its high resistance to environmental degradation. Several studies estimate the climate signal recorded in whole wood as highly coherent with that measured in α -cellulose (Leavitt and Danzer, 1993; Borella *et al.*, 1998; Leuenberger *et al.*, 1998; Barbour *et al.*, 2001; Loader *et al.*, 2003; Gori *et al.*, 2013, McCarroll and Loader, 2004; Ferrero and Voltas, 2005; Cullen and Grierson, 2006; Sidorova *et al.*, 2008; Szymczak *et al.*, 2011; Sensuła and Pazdur, 2013; Bégin *et al.*, 2015; Mischel *et al.*, 2015; Weigt *et al.*, 2015; Andreu-Hayles *et al.*, 2019; Shestakova *et al.*, 2019). Hence, whole wood remains second mostly preferred substance for isotope rate measurements in view of shorter processing and reduced cost.

Stable carbon isotope composition in tree rings have also been used to trace intra- and inter-annual climate variability and anthropogenic pressure in the Baltic region (Pawelczyk *et al.*, 2004; Pazdur *et al.*, 2007; Sensuła, 2015; Barisevičiūtė *et al.*, 2017; Pukienė *et al.*, 2021). The present study is an attempt to obtain additional data on seasonal isotope fluctuations in the tree ring wood and associate the latter with the environmental factors. One of the objectives of the study is to compare $\delta^{13}\text{C}$ sequences obtained from whole wood and α -cellulose of *Pinus sylvestris* L. tree rings by means of two different techniques described in Loader *et al.*, 1997 and Brendel *et al.*, 2000. Another objective of the study is to recover annual and seasonal $\delta^{13}\text{C}$ variations and identify correlations between the $\delta^{13}\text{C}$ values and atmospheric temperature, sunshine duration, precipitations and relative air humidity as the key hydroclimatic factors. To reach this objective, we measured seasonal $\delta^{13}\text{C}$ values in a 13-year tree ring chronology of *P. sylvestris*. Measurements were done in tree disks cut from pine trees growing in north-eastern Lithuania, Zarasai region.

2. Study site

The site (55.729195N / 26.179808E) is located in north-eastern Lithuania on the Baltic upland (Fig. 1). The location is in close proximity to Aukštaitija National Park with no industries in vicinity. The closest industrial city Daugavpils, Latvia, is 35 km away from the site and the closest road Utena – Daugavpils is at the distance of 2 km. The territory is dominated by luvisols. The landscape contains loamy plateaus and hills overgrown by mixed pine and

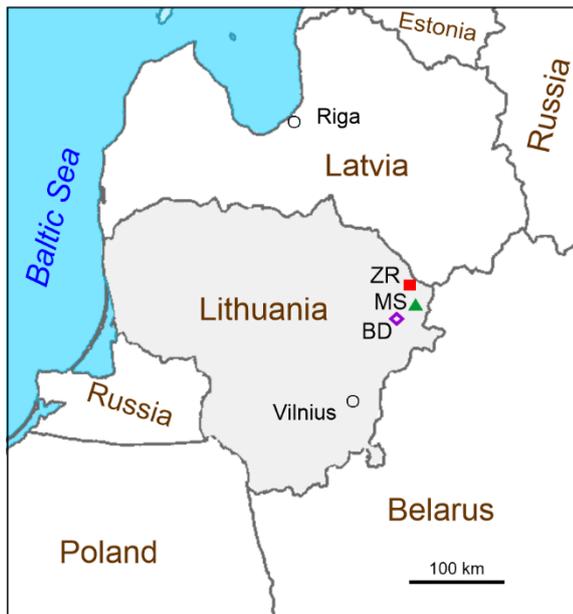


Fig. 1. Location of the study site (ZR), Dukštas meteorological station (MS) and permanent study site for long-term band dendrometer measurements (BD).

spruce forests with scattered lowland meadows, bogs and lakes. The location belongs to the continental climatic zone with an expressed seasonal climatic change. The mean temperature ranges from -5°C in January to 17°C in July. The mean annual precipitation is 600–650 mm. In winter, the site is covered by a 20–25 cm snowpack for 100 days on average. Also, the site is in vicinity of a meteorological station in Dukštas ($55.521202\text{N} / 26.323686\text{E}$) and the site in Vaišnoriškės ($55.426062\text{N} / 26.025975\text{E}$) where long-term measurements of tree growth rate are taken with a band dendrometer, allowing estimations of meteorological parameters and the wood growth dynamics of the sample tree.

3. Material and methods

Two Scots pine trees growing on formerly cultivated land at the edge of a forest were picked for sampling in order to obtain a “clean” signal. In order to trace the coherence between the climate signal measured in whole wood and cellulose in our region and verify the choice of whole wood samples instead of cellulose, we selected 15 tree rings, cut from a disk of a 90 year old tree (*Pinus sylvestris* L, tree ZR-11-1) growing in the study site, corresponding to years with distinct meteorological gradients and performed several cellulose extraction experiments from tree rings attributed to years 1960–1964, 1979–1983, and 1991–1995. Further investigation of inter- and intra-annual carbon isotope fluctuations was carried out on a disk cut from a ~14 year old *Pinus sylvestris* L (tree ZR-22-2). We examined in detail the 13 seasonal cycles of $\delta^{13}\text{C}$ (2010–2022),

dividing each annual tree ring into 13 segments of the same size, which were studied both whole wood and cellulose extracted from homogenized wood.

3.1. Dendrochronological analysis

Tree-ring analysis of the selected trees was performed following standard dendrochronological methodologies (Fritts, 1976; Eckstein, 1983; Eckstein, 1987). Tree-ring width was measured using a semi-automatic tree-ring measurement stage to an accuracy of 0.01 mm. The obtained data was processed with the Dendro software package (I. Tyers, unpublished). Annual ring width (RW) was measured in the case of the α -cellulose extraction experiments, but earlywood (EW) and latewood (LW) width was measured separately for studying intra-annual $\delta^{13}\text{C}$ fluctuations. However, wood formation rate is not a linear process and changeable throughout the growing season (Leavitt and Lone, 1991; Helle and Schleser, 2004; Seo et al., 2011; Vitas, 2011; Cuny et al., 2015; Matveev et al., 2020). We roughly estimated timing of each tree-ring subdivision (segment) based on average intra-annual wood formation rates in *Pinus sylvestris* obtained from a long-term series of band dendrometer measurements in a permanent study site at Vaišnoriškės located 35 km apart (Pukiene et al., 2021), the closest location with dendrometric data to the site under investigation (Fig. 1).

We developed an intra-annual growth model based on the band dendrometer (BD) data obtained at the Vaišnoriškės site. The BD site is located at 55.426062N latitude and 26.025975E longitude, with an elevation of c. 160 m a.s.l.. The soil is podzolic, and the parent material is sand with gravel and pebble admixtures. Groundwater table is deeper than 5 m. The site is in the same Aukštaitija National Park and subject to similar environmental conditions, in terms of annual range of air temperature and precipitation, implying a coherent wood growth dynamics. As the BD site is in vicinity of the site where sample tree disks were collected for isotopic analysis, characterized by similar growth conditions and comparable meteorological parameters and the obtained growth curve is comparable with preferred sigmoid models (Rossi et al., 2003; Rossi et al., 2006), we assumed the model suitable to identify approximate dates of the onset and the end of the growth of each intra-annual sample segment subject to $\delta^{13}\text{C}$ analysis.

3.2. Wood treatment methods and $\delta^{13}\text{C}$ analysis

The wood treatment methods tested for this study on homogenised by milling (with stone or metal beads placed into 2 ml “Eppendorf” tubes for 4–10 min. in “Retsch MM2” mill) wood shavings to powder were based on two somewhat different approaches described in Loader et al., 1997 and Brendel et al., 2000. The wood powder itself was also tested for $\delta^{13}\text{C}$ and compared to that of α -cellulose isolated by the mentioned methods.

In the first method (Loader et al., 1997), when processing one sample to produce α -cellulose, we used 200 mg of material after resin extraction. The produced

α -cellulose was dried by lyophilisation for at least 6 hr. The α -cellulose yield was $31.6\% \pm 1.8\%$ (\pm SD, standard deviation), and elemental analysis done by (FlashEA1112) of cellulose isolated from pine wood samples estimated the relative carbon content to be *ca.* $43.2\% \pm 1.2\%$.

The second method (the so-called Brendel-modified method, Brendel *et al.*, 2000) is useful for the rapid isolation of micro-samples of highly purified cellulose for isotope analysis of 10–100 mg of secondary (*Pinus sylvestris* L.) plant cell wall material (Brendel *et al.*, 2000). The method details follow the chemistry of Crampton and Maynard (1938) and have been modified (Brendel *et al.*, 2000; Evans and Schrag, 2004) to adjust for small sample sizes.

After processing the samples, the α -cellulose was freeze-dried for at least 6 hr. The α -cellulose yield was $29.9\% \pm 7.5\%$ (\pm SD), and elemental analysis of cellulose samples isolated from pine wood estimated the relative carbon content to be *ca.* $42.3\% \pm 1.4\%$.

For $\delta^{13}\text{C}$ analysis of each sample, up to 1 mg of purified α -cellulose, whole wood or other wood components was weighed into a tin cup and processed using an isotope ratio mass spectrometer (IRMS) at Center for Physical Sciences and Technology and using a laser absorption spectrometry (LAS), which was recently installed in our laboratory at Nature Research Centre. In IRMS measurements, the prepared samples were combusted with the elemental analyser (FlashEA1112) connected to the IRMS (Thermo Finnigan Delta Plus Advantage). The LAS consisted of a combustion module (Costech Analytical Technologies, Inc.) connected via a Picarro Liaison Interface A0301 to a laser-based Picarro Cavity Ring-Down Spectrometer G2121-i. The international standards (IAEA-600, IAEA CO-8 and SRM 4990C) were used for the system calibration and control. Repeated analysis of homogeneous material gave a standard deviation of $\delta^{13}\text{C}$ values less than 0.1‰ and 0.2‰ with IRMS and LAS, respectively.

Since carbon isotope discrimination is expressed as

$$\Delta^{13}\text{C} = \frac{\delta^{13}\text{C}_a - \delta^{13}\text{C}_p}{1 + \delta^{13}\text{C}_p}, \quad (3.1)$$

where $\delta^{13}\text{C}_a$ and $\delta^{13}\text{C}_p$ are carbon isotope ratio of CO_2 in the atmosphere and the plant correspondingly, the obtained delta values can be used to evaluate iWUE.

Faquhar *et al.* (1982) described the dependence of $\Delta^{13}\text{C}$ on the ratio of intercellular to atmospheric CO_2 concentrations c_i/c_a as

$$\Delta^{13}\text{C} \cong a + (b - a) \frac{c_i}{c_a}, \quad (3.2)$$

where a ($= 4.4\%$) is the discrimination against $^{13}\text{CO}_2$ during CO_2 diffusion through stomata, and b ($= 27\%$) is the discrimination during enzymatic carbon fixation. Thus, $\frac{c_i}{c_a}$ is linearly related to $\Delta^{13}\text{C}$ and can be calculated as

$$\frac{c_i}{c_a} = \frac{\Delta^{13}\text{C} - a}{b - a}, \quad (3.3)$$

and then used to calculate iWUE, the ratio of the photosynthetic rate (A) to stomata conductance to water vapour:

$$iWUE = \frac{A}{g} = \frac{c_a - c_i}{1.6} = c_a \left(1 - \frac{c_i}{c_a}\right) / 1.6 \quad (3.4)$$

3.3. Influence of environmental conditions on intra-annual $\delta^{13}\text{C}$ fluctuations

To select years with distinct meteorological gradients and to investigate climatic forcing of intra-annual fluctuations in $\delta^{13}\text{C}$ values., we used meteorological data from the closest Dūkštas meteorological station (Dūkštas MS, 55.521202N / 26.323686E). Monthly mean air temperature, relative humidity, the number of sunshine hours and precipitation totals were used as primary data. We used monthly meteorological parameters for the periods matching the estimated time of each wood segment formation. Where the growth period of the segment extended over the monthly boundaries, the monthly temperature and relative humidity values were averaged, and precipitation and sunshine values were summed proportionally. The correlation between $\delta^{13}\text{C}$ values in each segment of tree rings and the matching meteorological parameters was established using Pearson correlation coefficients. As the actual dates of the onset and termination of wood formation in individual tree-ring segment could only be identified approximately, we also calculated correlations between $\delta^{13}\text{C}$ values in each of the segment with the means of monthly meteorological parameters during the year.

4. Results

4.1. Wood treatment methods and $\delta^{13}\text{C}$ analysis

The comparison of different wood treatment methods was performed with the samples cut from stem discs of two sample trees – tree ZR-11-1 and tree ZR-22-2. From tree ZR-11-1 (~90 year old), we selected 15 tree rings corresponding to years with distinct meteorological gradients and performed several cellulose extraction experiments on annual tree rings attributed to years 1960–1964, 1979–1983, and 1991–1995. The set of 15 samples was subjected to resin extraction by toluene-methanol in a Soxhlet apparatus. Subsamples of whole wood of the same set were treated using the method of Loader *et al.* (1997) and a modified method of Brendel *et al.* (2000) to isolate α -cellulose.

The mean of carbon isotope ratios of whole wood ($\delta^{13}\text{C}_{\text{ww}}$) from the 15 rings was -26.1% ($\text{SD}=0.3\%$). $\delta^{13}\text{C}_{\text{ww}}$ displayed somewhat smaller range than carbon isotope ratios of α -cellulose ($\delta^{13}\text{C}_{\text{cell}}$) extracted by both methods: -24.8% ($\text{SD}=0.5\%$) according to Loader *et al.* (1997) and -24.9% ($\text{SD}=0.5\%$) according to Brendel *et al.* (2000). $\delta^{13}\text{C}_{\text{ww}}$ were on average 1.3‰ ($\text{SD}=0.3\%$) and 1.2‰ ($\text{SD}=0.3\%$) isotopically lighter than the corresponding $\delta^{13}\text{C}_{\text{cell}}$ according to Loader *et al.* (1997) and Brendel *et al.* (2000), respectively. $\delta^{13}\text{C}_{\text{ww}}$ and $\delta^{13}\text{C}_{\text{cell}}$ showed the same Pearson correlation coefficient ($r = 0.81$;

$p < 0.05$; $n = 15$) independently of the α -cellulose extraction method. The variations of $\delta^{13}\text{C}$ in the studied components of wood corresponding to 1991–1995 and compared to Panemunė data from ISONET project (Treydte *et al.*, 2007; Frank *et al.*, 2015) are listed in **Table 1** and graphically shown in **Fig. 2** and **3**.

The $\delta^{13}\text{C}$ values of α -cellulose isolated by two methods (tree ZR-11-1) and corrected for changes in atmospheric CO_2 isotope ratio compared to the ISONET project data (Panemunė tree), obtained from α -cellulose extracted according to Jayme-Wise (JW-alpha) chemical approach (Jayme, 1942; Wise *et al.*, 1946; Green, 1963), combusted

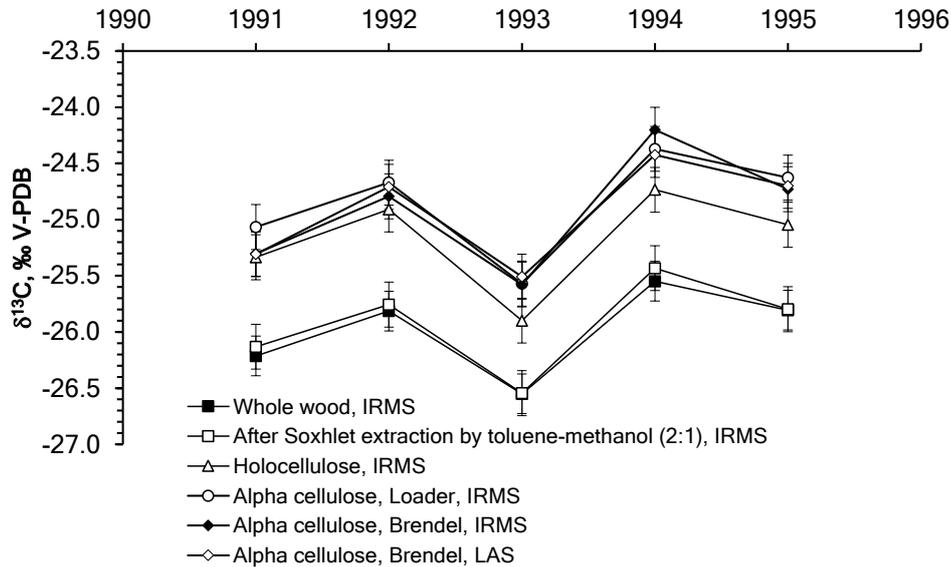


Fig. 2. The $\delta^{13}\text{C}$ values from whole wood and α -cellulose (tree ZR-11-1) isolated by two methods (Loader *et al.*, 1997; Brendel *et al.*, 2000) and measured by IRMS and LAS. Data not corrected for changes in the isotopic ratio of atmospheric CO_2 . Data for holocellulose and whole wood after extraction by toluene-methanol are provided for comparison as well. Uncertainty of each result is not shown, but after 2–3 repetitions it is $< 0.2\%$.

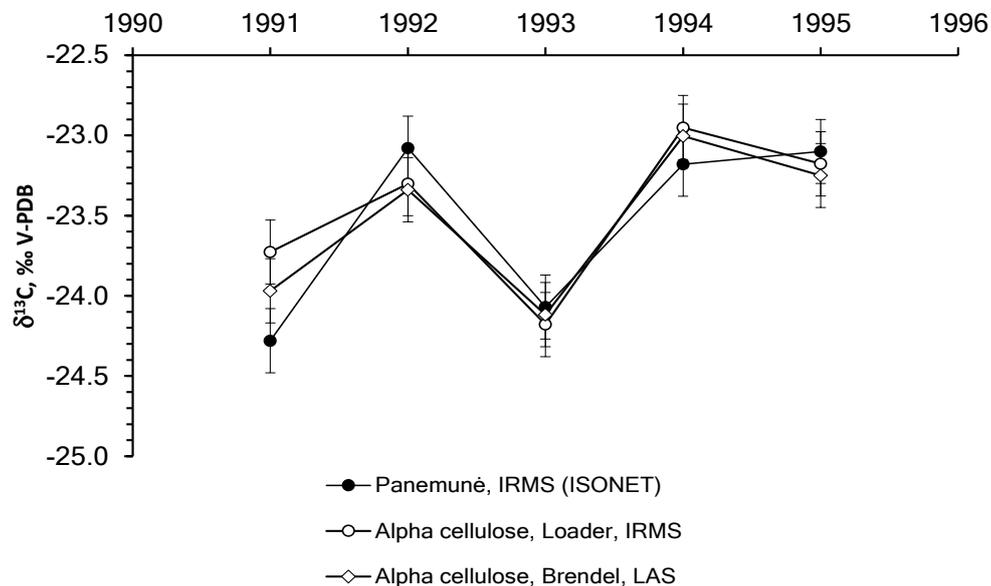


Fig. 3. The $\delta^{13}\text{C}$ values from α -cellulose (tree ZR-11-1) isolated by two methods (Loader *et al.*, 1997; Brendel *et al.*, 2000) and measured by IRMS and LAS. Data corrected for changes in the isotopic ratio of the atmospheric CO_2 . Panemunė tree data from ISONET project are provided for comparison. Uncertainty of each result after 2–3 repetitions is $< 0.2\%$.

Table 1. The $\delta^{13}\text{C}$ values from whole wood and α -cellulose (tree ZR-11-1) isolated by two methods (Loader *et al.*, 1997; Brendel *et al.*, 2000) and measured by IRMS and LAS.

Year	Panemuné, IRMS (ISONET), corrected	Alpha cellulose, Loader, IRMS, corrected	Alpha cellulose, Brendel, LAS, corrected	Whole wood, IRMS, not corrected	After Sxhlet, IRMS, not corrected	Holocellulose, IRMS, not corrected	Alpha cellulose, Loader, IRMS, not corrected	Alpha cellulose, Brendel, IRMS, not corrected	Alpha cellulose, Brendel, LAS, not corrected
1995	-23.1	-23.2	-23.3	-25.8	-25.8	-25.0	-24.6	-24.7	-24.7
1994	-23.2	-23.0	-22.8	-25.5	-25.4	-24.7	-24.4	-24.2	-24.4
1993	-24.1	-24.2	-24.2	-26.6	-26.5	-25.9	-25.6	-25.6	-25.5
1992	-23.1	-23.3	-23.4	-25.8	-25.8	-24.9	-24.7	-24.8	-24.7
1991	-24.3	-23.7	-24.0	-26.2	-26.1	-25.3	-25.1	-25.3	-25.3

at temperature of 1020°C and measured by means of standard IRMS system coupled to Elemental Analyzers, showed the same correlation ($r = 0.71$; $p < 0.05$; $n = 15$), despite the fact that the distance between these two sampling sites is rather long (190 km).

4.2. Intra-annual $\delta^{13}\text{C}$ analysis

From tree ZR-22-2 (~14 years old), 13 seasonal cycles (2010–2022) of $\delta^{13}\text{C}$, consisting of 13 series of samples each, were selected to compare inter- and intra-annual $\delta^{13}\text{C}$ fluctuations in whole wood and α -cellulose of *Pinus sylvestris* L. The 13 subsections are described in Table 2. Depending on the width of a tree ring, the thickness of these segments varies from 0.49 to 0.87 mm in different years, but is constant in the same year. Widths of individual tree-rings and their subdivisions are given in Table 2. The set of 169 samples was treated using a modified method of Brendel *et al.* (2000) to isolate α -cellulose without resin extraction in a Soxhlet apparatus.

The mean $\delta^{13}\text{C}_{\text{ww}}$ value obtained for intra-annual wood segments for 2010–2022 was -26.0‰ ($\text{SD} = 1.0\text{‰}$) with significant intra-annual fluctuations in $\delta^{13}\text{C}$ values in some years exceeding 3‰ (Fig. 4). Moreover, a significant difference between $\delta^{13}\text{C}_{\text{ww}}$ values for earlywood segments (mean = -25.8‰ , $\text{SD} = 1.0\text{‰}$) and latewood segments (mean = -26.5‰ , $\text{SD} = 0.9\text{‰}$) was detected.

In this case, cellulose was extracted according to Brendel *et al.* (2000); however, for some intra-annual wood segments, the amount of material was insufficient to accurately measure $\delta^{13}\text{C}_{\text{cell}}$. This limits the ability to determine a reliable difference between $\delta^{13}\text{C}_{\text{cell}}$ and $\delta^{13}\text{C}_{\text{ww}}$, which, based on the data available right now, is tentatively $0.6 \pm 0.3\text{‰}$. The high variability of this difference depends on values reaching 1.0–1.2‰ at some periods of the growing season and on values approaching 0‰ at other periods. These periods vary in different years and no distinct regularities were detected, though differences between $\delta^{13}\text{C}_{\text{cell}}$ and $\delta^{13}\text{C}_{\text{ww}}$ values tend to be smallest in the last (No 13) segment of a ring, in the latewood section, which is formed in the second half of August – beginning of September (see below). Although the variability of the difference between $\delta^{13}\text{C}_{\text{cell}}$ and $\delta^{13}\text{C}_{\text{ww}}$ is irregular, the $\delta^{13}\text{C}_{\text{ww}}$ and $\delta^{13}\text{C}_{\text{cell}}$ data for intra-annual wood segments for 2010–2019 showed a

Table 2. Widths of tree-rings and tree-ring subdivisions of tree ZR-22-2, used for intra-annual $\delta^{13}\text{C}$ analysis.

Year	EW width (mm)	LW width (mm)	Ring width (mm)	Segment length (mm)
2010	6.62	1.56	8.18	0.63
2011	4.84	2.5	7.34	0.56
2012	4.06	4.52	8.58	0.66
2013	4.69	3.19	7.88	0.61
2014	8.33	2.95	11.28	0.87
2015	7.18	2.57	9.75	0.75
2016	7.21	2.15	9.36	0.72
2017	7.25	1.39	8.64	0.66
2018	8.46	2.25	10.71	0.82
2019	7.44	1.94	9.38	0.72
2020	6.39	2.43	8.82	0.68
2021	5.32	1.99	7.31	0.56
2022	4.8	1.57	6.37	0.49

strong correlation with a Pearson correlation coefficient of 0.97 ($r = 0.95$; $n = 123$; $p < 0.05$).

The $\delta^{13}\text{C}$ values measured in whole wood were eventually converted into iWUE to produce a sequence presented in Fig. 5 below. As the actual values of CO_2 concentration and $\delta^{13}\text{C}_{\text{atm}}$ corresponding to specific years of tree ring formation were used in the calculations, the obtained variation accounts both for the rising CO_2 and $^{12}\text{CO}_2$ concentrations in the atmosphere. The variation exposes peaks in the middle of most of the growing seasons, coinciding with the pattern of the inter-annual $\delta^{13}\text{C}$ variation, and a trend of a gradual increase of about 20 $\mu\text{mol/mol}$ over the decade in question.

4.3. Intra-annual $\delta^{13}\text{C}$ fluctuations and environmental forcing

The intra-annual isotopic analysis was done for the sample pine tree ZR-22-2. For the intra-annual $\delta^{13}\text{C}$ variability analysis each annual tree ring was divided into 13 equal segments. For the purpose of dating of individual segments, we developed a polynomial growth model based on band dendrometer measurements taken in Vaišnoriskės. The band dendrometer data allowed identification of specific dates in DOY (day of the year) when the accumulative growth attains 1/13th of the tree-ring for each tree-ring.

The obtained data set was then approximated to yield a polynomial growth curve (Fig. 6). Based on the established growth model, we estimated average time periods of each segment formation. The range of the duration of the formation of a 1/13th of the segment of an annual ring is from 6 days (in June) to 23 days (in August – September). The estimated timing of all segment formation is presented in Table 2.

Depending on the estimated segment formation time we determined corresponding meteorological parameters. Mean monthly values of air temperature (T) and relative air humidity (RH), and monthly sum of precipitation (P) and sunshine duration (S) were used as climatic variables

for segments formed in the corresponding months. Estimates of the climatic variables for segments the growth of which was overlapping adjacent months had to be weighted separately. The weighting coefficients correspond to the proportion of the segment growth duration during the particular month. Weighting coefficients for monthly meteorological parameters (mean temperature, relative humidity, sum precipitation, the number of sunshine hours) are presented in Table 3.

We used $\delta^{13}\text{C}_{\text{ww}}$ values of the ring segments and the corresponding weighted meteorological parameters to reveal possible correlations (Fig. 7, Fig. 9). The $\delta^{13}\text{C}_{\text{ww}}$ values exhibit predominantly significant negative correlations

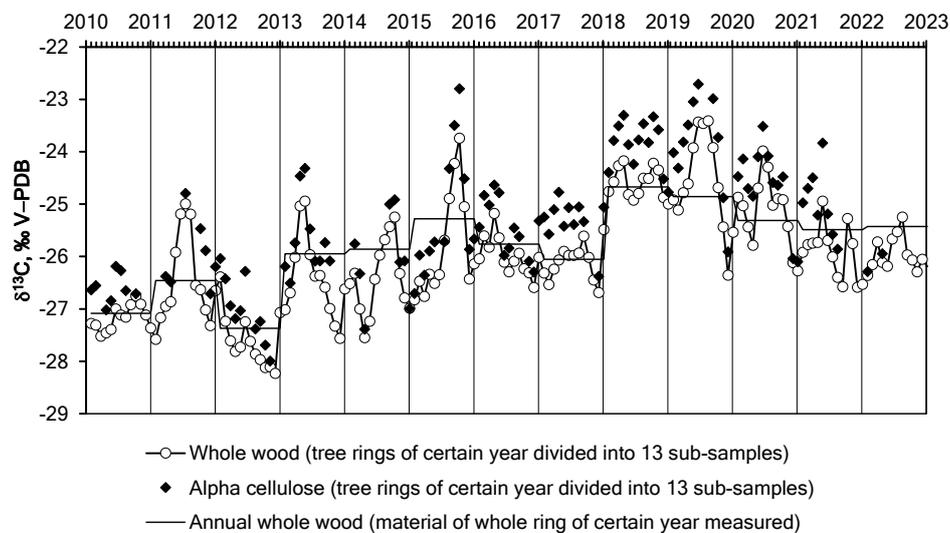


Fig. 4. The $\delta^{13}\text{C}$ values from whole wood and α -cellulose (tree ZR-22-2, intra-annual segments representing a 1/13 part of a tree ring) isolated according to Brendel et al. (2000) and measured by Picarro. Data not corrected for changes in the isotopic ratio of the atmospheric CO_2 . Vertical lines indicate the position of the ring boundary. Uncertainty of each result is not shown, but after 2-3 repetitions it is $<0.2\%$.

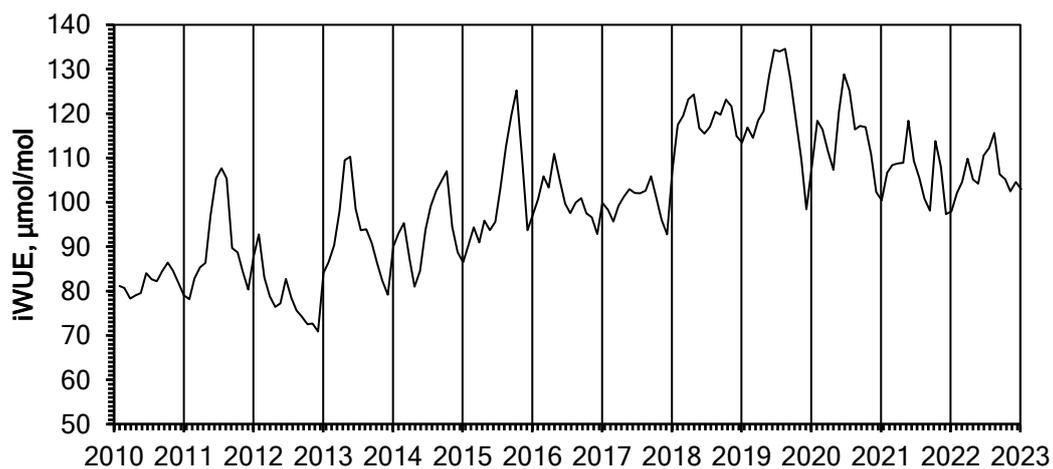


Fig. 5. Variability of iWUE in the study site (district of Zarasai, Lithuania).

Table 3. Estimated growth time of each wood segment and the corresponding meteorological parameter (*T* for temperature, *P* for precipitation, *S* for the number of sunshine hours, *RH* for relative humidity) used as a climatic variable in $\delta^{13}\text{C}$ correlation analysis. The subscripts indicate the corresponding months. Weighted monthly data is used where the estimated period extended over a month boundary.

Segment No.	Estimated growth duration in days	Estimated growth period		Corresponding meteorological parameter			
		In DOY	In calendar days	Temperature	Precipitation	Number of sunshine hours	Relative humidity
1	12	122–133	2–13 May	T_5	P_5	S_5	RH_5
2	8	134–141	14–21 May	T_5	P_5	S_5	RH_5
3	7	142–148	22–28 May	T_5	P_5	S_5	RH_5
4	7	149–155	29 May–4 June	$0.42 \cdot T_5 + 0.58 \cdot T_6$	$0.42 \cdot P_5 + 0.58 \cdot P_6$	$0.42 \cdot S_5 + 0.58 \cdot S_6$	$0.42 \cdot RH_5 + 0.58 \cdot RH_6$
5	6	156–161	5–10 June	T_6	P_6	S_6	RH_6
6	6	162–167	11–16 June	T_6	P_6	S_6	RH_6
7	6	168–173	17–22 June	T_6	P_6	S_6	RH_6
8	8	174–181	23–30 June	T_6	P_6	S_6	RH_6
9	9	182–190	1–9 July	T_7	P_7	S_7	RH_7
10	11	191–201	10–20 July	T_7	P_7	S_7	RH_7
11	12	202–213	21 July–1 August	$0.92 \cdot T_7 + 0.08 \cdot T_8$	$0.92 \cdot P_7 + 0.08 \cdot P_8$	$0.92 \cdot S_7 + 0.08 \cdot S_8$	$0.92 \cdot RH_7 + 0.08 \cdot RH_8$

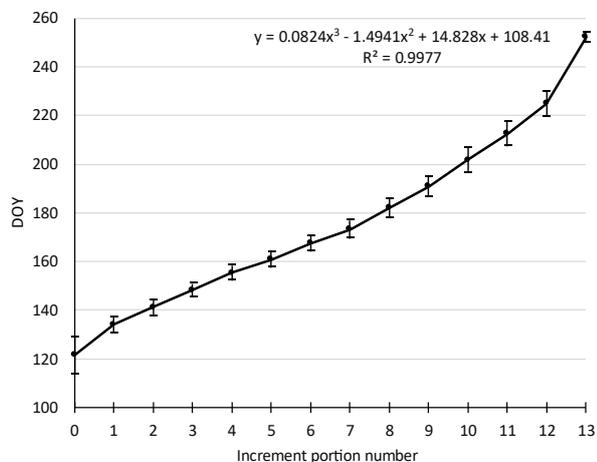


Fig. 6. Model of time required to form equal wood increment portions throughout the growing season, based on 40 year length band dendrometer measurements of 24 Scots pine trees (Pukienė et al., 2021). DOY indicates the average day of the year when the accumulative growth attains a subsequent 1/13th of the tree-ring (numbers on horizontal axis). The bars represent 95% confidence intervals.

($r_{\min} = -0.66$; $n = 13$; $p < 0.05$) with precipitation and positive ($r_{\max} = 0.77$; $n = 13$; $p < 0.05$) with temperatures in June (segments 4, 5, 6, 7, and 8). To the contrary, correlations with May temperature and precipitation are low. Interestingly, $\delta^{13}\text{C}_{\text{ww}}$ values show a significant negative correlation with temperature at the beginning of July. An insignificant but still negative correlation persists throughout July. Because of the alternating reaction to temperatures, the correlation between the annual $\delta^{13}\text{C}_{\text{ww}}$ and the mean seasonal temperature is close to zero. Steady negative correlations with precipitation result in a significant negative correlation ($r = -0.61$; $n = 13$; $p < 0.05$) between the mean annual $\delta^{13}\text{C}_{\text{ww}}$ and the sum precipitation during the growth season.

Fig. 7 shows that correlations are only significant for segments 4, 5, 6, 7 which were formed in June. The best $\delta^{13}\text{C}$ correlations, both with temperature and precipitation, were identified in segment 5, i.e. in the first half of June. The correlation can also be clearly seen in the yearly fluctuation of the $\delta^{13}\text{C}$ of the 5th segment, presented in Fig. 8.

$\delta^{13}\text{C}$ correlations with sunshine hours are positive for segments 1 to 8, being significant only for segments 4 to 8, representing June, with the highest value ($r = 0.8$) of segment 4 (Fig. 9). In segments 9 to 11, the correlation shifts to negative and regains in segment 12, i.e. in August. The strongest $\delta^{13}\text{C}$ correlations with sunshine are observed almost in the same interval as the best correlations with air temperature. Unlike in case of correlations with air temperature, a significant correlation may be observed for the mean annual $\delta^{13}\text{C}_{\text{ww}}$ value as well. Correlations with relative humidity are negative with the exception of segment 9. However significant values are observed only for segments 4, 5, 8 and the mean annual $\delta^{13}\text{C}_{\text{ww}}$ value.

Correlations between average values of $\delta^{13}\text{C}$ measured in the 13 sample segments and the mean monthly temperatures are given in Fig. 7. Significant positive correlations ($r > 0.506878$, $n = 13$, $p < 0.05$) are observed mostly with June temperatures and in the middle of the vegetation period (segments 3–7). Also, a strong correlation with October temperature may be seen in the first half of the growth season (segments 1–7). Segments 6–8 also strongly correlate with January temperatures. Surprisingly, correlation with July temperature turns to negative, being significant ($r = -0.50702$, $n = 13$, $p < 0.05$), however, only in segments 8 and 9, i.e. closer to latewood formation. A strong correlation with mean temperature values is observed in all segments.

Correlations with precipitation are shown in Fig. 7. Most of the correlations are negative here. Significant correlations with April precipitation are observed in the middle of the vegetation period (segments 6 and 7) and with June precipitation (segments 8–10). Strong correlations with sunshine hours (Fig. 9) are observed with spring

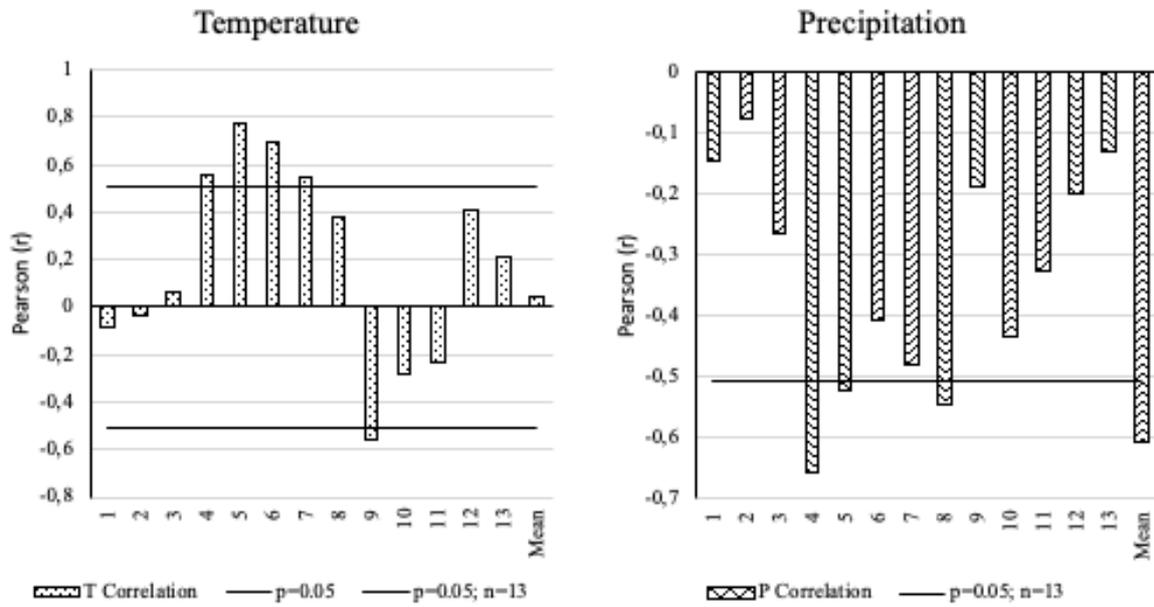


Fig. 7. Pearson correlation coefficients between intra-annual $\delta^{13}C_{wv}$ values and corresponding meteorological parameters. Horizontal lines indicate the significance threshold ($p=0.05$). X axis labels segment number, while 'Mean' indicates correlation between the mean annual $\delta^{13}C_{wv}$ value and the mean value of a meteorological parameter during the growth season.

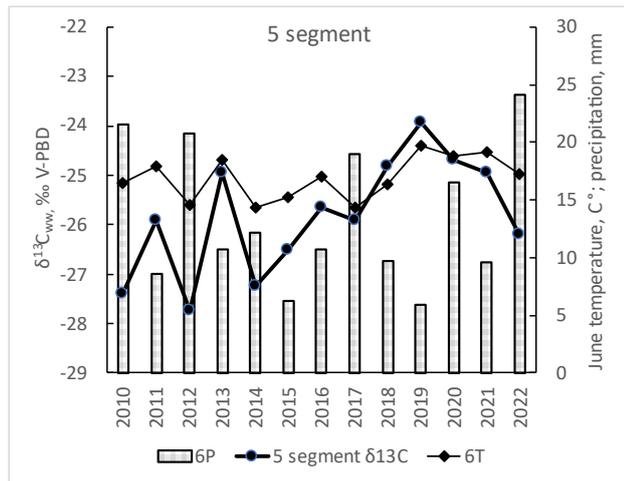


Fig. 8. Fluctuation of $\delta^{13}C$ values in segment 5 of the annual ring and the corresponding meteorological parameters – June mean temperature (6T) and sum precipitation (6P).

summer and autumn data mostly in the middle segments. The strongest correlations are with June data and in segments of the tree rings (segment 3–7). Surprisingly, correlation with July sunshine hours turns out to be negative, being strongest in the initial tree-ring segments. As for correlation with relative humidity, significant values are observed only with June data nearly in all segments and with April data in segments 6–8 (Fig. 9).

5. Discussion

Measurements of $\delta^{13}C$ values in whole wood and α -cellulose show nearly identical patterns with a maximal difference of 1.3‰, the α -cellulose being enriched compared to the whole wood. Differences in $\delta^{13}C$ values may arise from differential fractionation during the complex processes of carbon uptake, assimilation and allocation within different tissues of the tree. Many studies report strong coherence between $\delta^{13}C$ chronologies within whole wood and its components with the α -cellulose being about 2‰ lighter (Loader *et al.*, 2003), suggesting that the same climatic signal may be obtained independently of the substance. Studies also point to asynchronous development and degradation of the components (Suberkropp and Klug, 1976, Spiker and Hatcher, 1987, Benner *et al.*, 1991, Schleser *et al.*, 1999) manifesting in a certain lag between trends measured in the whole wood and α -cellulose. The lag may be preconditioned by allocation of newly assimilated carbon with a different isotopic signature to different carbon pools within the tree (Loader *et al.*, 2003). However, such offset is usually visible in long chronologies and miniscule in juvenile trends (Wilson and Grinsted, 1977).

Carbon in the latewood part of rings tends to be 'lighter' compared to the earlywood part exhibiting higher carbon isotope discrimination during latewood formation. These periods vary in different years with no distinct regularities, though differences between $\delta^{13}C_{cell}$ and $\delta^{13}C_{wv}$ values tend to be smallest in the last (No 13) segment of a ring, *i.e.* in the latewood section, which is formed in the second half of August – beginning of September. Irregular variability

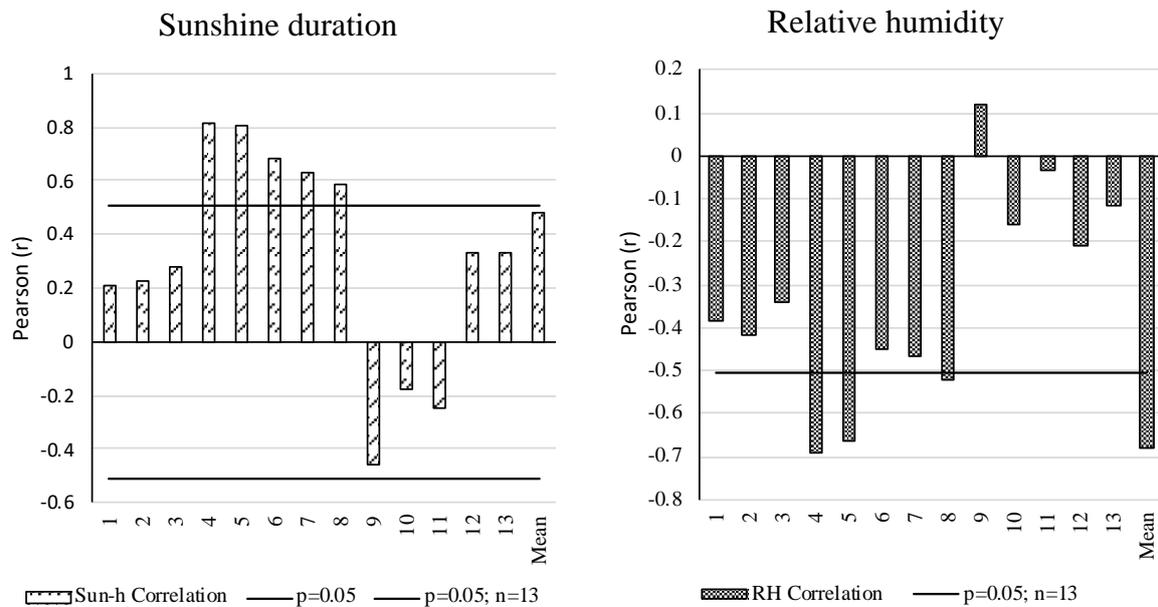


Fig. 9. Correlation coefficients of $\delta^{13}\text{C}_{\text{ww}}$ values in intra-annual wood segments with monthly sunshine hours and relative air humidity. Horizontal lines indicate significance threshold ($p=0.05$). X axis labels segment number, while 'Mean' indicates correlation between the mean annual $\delta^{13}\text{C}_{\text{ww}}$ value and the mean value of a meteorological parameter during the growth season.

of the difference between $\delta^{13}\text{C}_{\text{cell}}$ and $\delta^{13}\text{C}_{\text{ww}}$, depending mainly on varying cellulose to lignin ratios, was also observed in intra-annual $\delta^{13}\text{C}$ patterns of broadleaf *Fagus sylvatica* rings (Helle and Schleser, 2004), Douglas fir and ponderosa pine (Leavitt and Lone, 1991). Although this point is not yet clear, the $\delta^{13}\text{C}_{\text{ww}}$ and $\delta^{13}\text{C}_{\text{cell}}$ data for intra-annual wood segments for 2010–2019 showed a strong correlation with a Pearson correlation coefficient of 0.97 ($r=0.95$; $n=123$; $p<0.05$). This implies that whole wood samples could be used instead of cellulose samples to achieve the same result considering basic patterns of climate variability.

Most of the analyzed tree rings show a gradual increase in $\delta^{13}\text{C}$ values at the beginning of the growing season and a decrease toward the end. A slight increase in the end of the latewood is also visible in most of the tree rings. The obtained variability of $\delta^{13}\text{C}$ values provides certain insights into environmental and physiological factors affecting tree growth in the investigated site. The increase at the onset of the growing season suggests an increase in the photosynthesis rate, favourable growing conditions and a growing deficit of accessible carbon sources. The increase coincides with the onset of temperature growth in spring and activation of metabolic processes within the cell requiring large amounts of assimilates. As the spring soil provides sufficient moisture for intensive transpiration, the positive correlation between $\delta^{13}\text{C}$ and temperature is in line with photosynthesis activity during the first half of the growing season (Pukienė *et al.*, 2021). Peaking, normally occurring in June–July, may be shifted or moderated depending on summer droughts and unusual temperature

variations. The subsequent drop or plateau in the pattern occurs towards the period of latewood formation and reflects the response to changes in both the temperature and physiological processes within the tree. The shift may be due to changing lignin content in the wood composition or various physiological processes. The increase in $\delta^{13}\text{C}$ values at the end of the growing season suggests a response to limited water availability or changes in carbon fixation patterns in the latewood. It may also indicate incomplete lignification of cell walls at the end of the season (Piermattei *et al.*, 2015). Vaganov *et al.* (2009) report a pattern with a slow increase of $\delta^{13}\text{C}$ in earlywood peaking in autumn followed by a sharp decrease in latewood, specifically substantial in wide rings and reaching even lower values at the end of the growth season than those registered at the onset of the season. The study also reports a variation of 0.8–1.6‰ in intra-annual variation of $\delta^{13}\text{C}$ depending on the season. As a possible reason for the increase in $\delta^{13}\text{C}$ in autumn, the reserve formation of ^{13}C -enriched compounds, resulting in heavy early season wood, is suggested. Cuny *et al.* (2015) identify a 12 to 22 day and 6 to 50 day lag between cell enlargement and cell wall thickening in earlywood and latewood respectively. Such a lag shows the non-linear accumulation of carbon in earlywood and latewood and may subsequently manifest in the changes in the isotopic composition of the tissue. The studies also report 2–3‰ annual variation of $\delta^{13}\text{C}$ values, which is comparable with the results of our study.

The calculated seasonal iWUE values, not surprisingly, reiterate the pattern of $\delta^{13}\text{C}_{\text{ww}}$ variation during the season, peaking mostly in summer just before the onset of

latewood formation. Since by definition *i*WUE is the ratio of the photosynthetic rate to stomatal conductance to water vapour, the growth in the *i*WUE may be explained by either the growth of the intensity of photosynthesis or reduction of the stomatal conductance, regulating the intensity of transpiration and the ratio of CO₂ concentrations in the atmosphere and the leaf mesophyll. At the leaf level, such growth implies an increased carbon uptake at the same intensity of water evaporation, *i.e.* the same stomatal aperture (Lavergne *et al.*, 2019). As the δ¹³C_{ww} shows stronger correlations with temperature and sunshine than precipitation and relative humidity in June, the intensity of photosynthesis seems to be the prevailing factor until the summer solstice followed by the subsequent drop in the latewood presumably caused by the gradual decrease of sunshine necessary for carbon fixation by the plant. Here the correlations with temperature and sunshine duration shift from positive to negative additionally contributing to the

concurrent effects of precipitation and humidity. The latter however are not statistically significant; hence cannot be fully relied upon in the interpretation.

Correlations between δ¹³C values in individual segments and monthly temperature values, specifically strong in the first half of the tree ring formation process (Fig. 10), indicate temperature being a limiting factor regulating the plant carbon fixation in June, when photosynthesis is most intense due to extensive photoperiod. The growing correlation values may be explained by physiological processes in the tree, *i.e.* the growing water conductivity of the enlarging tracheid. Strong correlations with October temperatures may be explained by the choice of the lighter carbon in processes of lignification and photosynthate storage. Negative correlations with July temperatures, manifesting in the latewood segments may indicate stomatal closure triggered by dry July environments or metabolic processes in the latewood as significant correlations are observed in

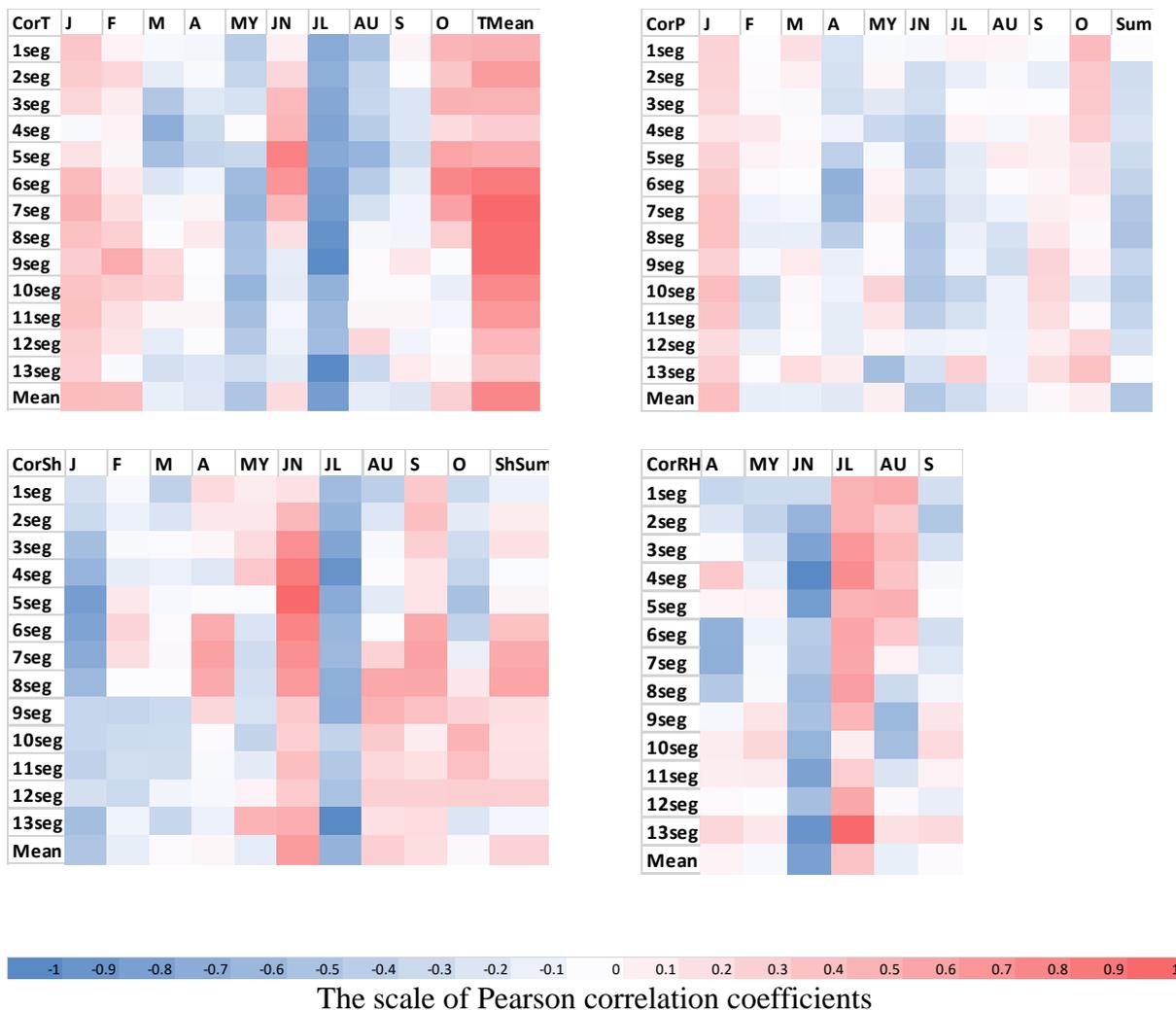


Fig. 10. Correlations between δ¹³C values in individual segments of the tree ring with monthly and annual mean temperatures (T), precipitation (P), the number of sunshine hours (Sh) and relative humidity. *p*=0.05, *n*=13. Statistically significant values range from -0.506878 to 0.506878.

segments closer to the latewood. Surprisingly, January temperatures seem to be also important. This implies existence of certain photosynthetic and metabolic processes. However, they are difficult to explain within the framework of the present study as significant correlations appear only in the middle of the tree ring. A similar picture may be observed in correlations of monthly sunshine hours, testifying in favour of the greater importance of the photosynthetic processes.

Correlations with precipitation and relative humidity (Fig. 10) are, as expected, mostly negative with significant values in April and June. This corroborates the importance of moisture accumulated before the onset of the growth season and availability of water critical for photosynthesis and maintaining turgor necessary for cell swelling and enlargement during the period of most intensive and extensive carbon fixation processes.

The observed decadal increase of about 20 $\mu\text{mol/mol}$ in the $i\text{WUE}$ value is comparable with the growth reported by several studies as plant adaptation to climate changes (Barisevičiūtė *et al.*, 2017; Belmecheri and Lavergne, 2020; Sensuła and Wilczyński, 2022). The growth is preconditioned by the rising CO_2 concentration in the atmosphere and by the increase in atmospheric $^{12}\text{CO}_2$ concentration resulting from fossil fuel combustion (Lavergne *et al.*, 2019; Sensuła and Wilczyński, 2022). Mean temperature of June, positively linked with $\delta^{13}\text{C}$ values, has an increasing trend from 2010 to 2019 too. Also, the increase may be partly preconditioned by the juvenile effects as the samples were collected from initial decades of wood formation. Hence, the absolute value of the increase cannot be accurately interpreted.

The negative $\delta^{13}\text{C}_{\text{ww}}$ correlations with precipitation and positive with temperatures in June are typical in boreal latitudes and have been mentioned in numerous studies (Traydte *et al.*, 2007). Such reaction is in agreement with the general model of isotope discrimination by Farquhar *et al.* (1982, 1989a, 1989b), which relates it to the partial pressure of leaf internal vs. external CO_2 depending on stomatal conductance and photosynthesis rate. Loader *et al.* (2003) also distinguish July and August as the months when $\delta^{13}\text{C}$ values are mostly affected by the climatic factors. $\delta^{13}\text{C}$ correlation with temperature changes from positive to negative in July and August. The shift is difficult to explain and may be attributable to certain changes in metabolic processes, e.g. cell wall lignification; however,

most of the correlations are not statistically significant, suggesting the need for additional investigation. However, the predominating positive correlation of $\delta^{13}\text{C}_{\text{ww}}$ in ring segments with monthly temperatures and negative with monthly precipitation result in a statistically significant positive correlation of the mean $\delta^{13}\text{C}_{\text{ww}}$ value in the tree ring with the mean annual temperature and a negative correlation with the annual precipitation sum.

6. Conclusions

$\delta^{13}\text{C}$ values retrieved from different wood components demonstrate that whole wood can be successfully used instead of extracted cellulose for analysis of tree growth response to environmental and hydroclimatic factors. The use of whole wood for isotopic analysis substantially reduces the time required for sampling and thus allows processing of larger amounts of samples in expectation to obtain high accuracy estimates. However, it should be noted that the choice of the substance for experiments is primarily predetermined by the specific signal to be tracked in the course of the research.

The obtained $\delta^{13}\text{C}$ interannual variation pattern reiterates in most of the rings with different absolute, maximum and minimum values. There is a statistically significant difference in earlywood and latewood. However, correlations with hydroclimatic indices are only strong enough in the initial stage of the ring formation. This suggests that earlywood $\delta^{13}\text{C}$ is more suitable for interpretation of the growth response to climatic variations as the signal may be mitigated by the lower values in the latewood where other factors become dominating. This also suggests the potential of latewood in the analysis of complex physiological processes, such as assimilation, translocation and storage of assimilated carbohydrates. The inter-annual pattern discloses environmental factors with good correlations only in certain months.

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