# Carbon Stable Isotope Analyses of Mosses—Comparisons of Bulk Organic Matter and Extracted Nitrocellulose

Grzegorz Skrzypek,[a,b] Adam Kałużny,[b] Mariusz O. Jędrysek[b]

[a] Department of Earth and Environmental Science, The University of Texas at San Antonio, San Antonio, Texas, USA

[b] Laboratory of Isotope Geology and Geoecology, Institute of Geological Sciences, The University of Wrocław, Wrocław, Poland

The commonly used technique for determination of plant stable carbon isotope composition is analysis of $CO_2$ liberated during combustion of chemically extracted nitrocellulose or $\alpha$-cellulose. The $\delta^{13}C$ of cellulose is usually accepted as a more reliable record of growth environment conditions compared with bulk plant material analysis. Unfortunately, cellulose extraction techniques are time-consuming, and usually require toxic chemicals such as toluene, chloroform, benzene, methanol, concentrated acids, etc. We tested the possibility of replacing nitrocellulose analysis with bulk organic analysis. *Sphagnum* and *Polytrichum* mosses collected along a vertical transect (altitudes 500 to 1400 m), provided material for analysis in the wide range of $\delta^{13}C$: −32.66‰ and −26.20‰ for bulk organic matter and −24.11‰ and −31.86‰ for nitrocellulose. The correlation for $\delta^{13}C$ value of extracted cellulose and $\delta^{13}C$ values of bulk organic matter were very good (>0.95). Our results suggested that $\delta^{13}C$ analyses can be performed on bulk plant material instead of cellulose, without significant loss of information, at least for *Polytrichum* and *Sphagnum* mosses. Moreover, we confirmed that the extraction process of nitrocellulose did not cause any significant isotopic fractionation. (J Am Soc Mass Spectrom 2007, 18, 1453–1458) © 2007 American Society for Mass Spectrometry

The isotopic composition of organic matter ($\delta^{13}C$, $\delta^{18}O$, and $\delta D$) has a broad range of applications, including paleoclimatic and paleoenvironmental reconstruction. Cellulose is one of the most abundant and resistant organic compounds present in plants. It is, therefore, a compound commonly used for paleoenvironmental reconstruction [1, 2]. Its use as a paleoclimatic proxy comes from results of earlier studies finding a direct correlation between the isotopic composition of carbon, hydrogen, and oxygen cellulose and mean annual temperature [1, 3, 4]. However, other factors including partial pressure and $\delta^{13}C$ value of atmospheric $CO_2$, as well as relative humidity and amount of precipitation, have also been found to influence the carbon isotope composition of cellulose (e.g., [5–8]). Although most studies assessing the relationship between cellulose isotope data and environmental conditions have been performed on tree rings, studies based on material such as mosses, lichens, and liverworts are uncommon. Despite its great potential as a climatic archive, the usefulness of peat as a paleoclimate proxy is still uncertain. In addition to sedges, mosses are a major component of peat in bogs and fens which may be used as a register of past climates.

Address reprint requests to Dr. G. Skrzypek, Department of Earth and Environmental Science, The University of Texas at San Antonio, One UTSA Circle, San Antonio, 78249 Texas, USA. E-mail: buki@ing.uni.wroc.pl

Unlike vascular plants, mosses employ a simpler physiological water-use strategy because they lack stomata and vascular tissue. In *Sphagnum* mosses, the photosynthetic cell is surrounded by so-called hyaline cells, which form significant water reservoirs. Due to these attributes, mosses cannot regulate their carbon uptake, which differentiates them from vascular plants [9]. Nonvascular plants can be very useful for stable isotope environmental studies because of this simplification in the physiology, e.g., the important fractionation for carbon isotope composition of vascular plants, due to the so-called "stomata effect", does not exist and neither does isotopic fractionation due, to wood formation [9].

Most theories about the decay of plant matter were developed from observations in soil science rather than from peat studies [10]. Early diagenetic processes decompose labile plant material rapidly in aerobic soil environments with adequate water supplies. Oxidative and hydrolytic biodegradation of this dead plant material by microorganisms (mainly fungi and bacteria) is believed to be a primary source of humic substances [10, 11]. Plant cellulose is probably the most isotopically stable chemical compound, even under conditions of partial decomposition [1, 12–14]. Nevertheless, the primary isotopic plant composition can also be well preserved in bulk organic matter, especially in acid bog or fen conditions [14]. Indeed, cellulose extracted from


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plant and sediment is commonly used as the most reliable register of $\delta^{13}C$, $\delta^{18}O$, and $\delta D$ isotope signals formed during photosynthesis.

In general, plant organic matter mainly consists of cellulose (15%–50%), hemicellulose (10%–40%), lignin (5%–30%), proteins (2%–15%), and lipids [15]. Dry *Sphagnum* organic matter mainly consists of cellulose (~43%), including three major types: $\alpha$-cellulose (~20%), $\beta$-cellulose (~7%) and $\gamma$-cellulose (~15%) [16].

The question of whether stable isotopes can be analyzed on whole wood instead of cellulose has been indirectly addressed by many authors (e.g., [17–19], as well as studied in detail by Harlow et al. [20]. The question, however, was not considered in the case of mosses. Only Menot and Burns [21] have done the very first comparative studies based on 24 samples of various moss species. The generally postulated necessity of extracting cellulose is discussed in this paper, and it seems not to be required (at least for mosses).

Laboratories all over the world have developed various modifications of cellulose extraction methods, but all these methods were based on the very first studies by Green [22]. In principle, two major varieties of these techniques are widely used: the method of $\alpha$-cellulose extraction, the most common (e.g., [23]), and the cellulose extraction method with nitration (e.g., [1, 24]). The cellulose nitration method is also used for hydrogen stable isotope analysis, and gives very high reproducibility. This process, however, is more complicated and time-consuming.

All $\alpha$-cellulose techniques have been based on multiple chemical treatments with [22]: acetic acid and sodium chlorite at 70 °C, sodium hydroxide (17%), with addition of HCl (10%) and acetic acid (10%). However, each laboratory did not use a Soxhlet apparatus for extractive removal. Even when it was used, not all the same organic solvents were applied. Among recently published papers, the most commonly used organic solvents were (1) 2:1 benzene-methanol mixture (24 h) and acetone (24 h) by Zanazzi and Mora [25] modified after Wolfe et al. [26]; (2) no organic solvents, Pazdur et al. [27] after Loader et al. [28]; (3) 2:1 toluene-ethanol for 48 h, and ethanol for another 48 h by Harlow et al. [20] after Borella et al. [19]; (4) 1:1 toluene-ethanol 14 h or 8 to 10 h by Menot and Burns [21] after Brenninkmeijer [29]; (5) multiple ethanol (99%) washing and acetone by Brendel et al. [30]; (6) 2:1 chloroform-ethanol by Menot and Burns [21] after Price et al. [31] and Wise and D'Addieco [32].

Borella et al. [23] recommended the last listed procedure (6) for mosses. A general evaluation and comparison of some of these listed techniques was done by Sheu and Chiu [33] and Borella et al. [23]; nevertheless, the difference between bulk material and extracted cellulose was studied for wood only and cellulose nitration has not been studied.

All techniques of cellulose extraction are time-consuming and require toxic chemicals; therefore an alternative is desired. If possible, whole plant analysis could be used, provided there is no significant loss of information. The purpose of this research was to compare the carbon stable isotope composition of whole plant and extracted cellulose from selected moss samples. Our aim was to calibrate the $\delta^{13}C$ bulk and $\delta^{13}C$ nitrocellulose relation. The environmental causes of presented $\delta^{13}C$ value variation for mosses were a subject of other publications [2, 34]. The stable isotopic analyses were performed at the Laboratory for Stable Isotope Geology and Geoecology, the University of Wrocław.

## Experimental Methods and Materials

Samples of two moss species, *Sphagnum girgensohnii* Russow and *Polytrichum commune* Hedw., were collected from northern hillsides in the Karkonosze Mountains, (vertical transect 739–1393 m) and Izerskie Mountains (500–1100 m) SW Poland during two field seasons (July and October 2004). Among 18 sampling points, one point located at Hala Izerska was selected for extended studies [34], where besides *Sphagnum girgensohnii* and *Polytrichum commune*, the five other most common bog plant species, *Carex enchinata*, *Molinia caerulea*, *Eriophoroum vaginatum*, *Sphagnum fallax*, *Sphagnum majus*, and *Sphagnum papillosum* were collected.

The whole live plant samples (about 2 to 3 g dry matter each) were stored in the freezer (−20 °C) after collection. The proper botanical field identification of plant species was confirmed in the laboratory based on morphological features. After washing with distilled water, each sample was divided in two parts. Part one, intended for bulk organic matter preparation, was dried under vacuum and then milled to a fine powder. Part two was used for chemical extraction of cellulose.

The cellulose was extracted from the moss and vascular plant samples following the modified techniques developed by Epstein et al. [1] based on previous studies [16]. The preparation consisted of three major steps: (1) multiple chemical treatments, (2) nitrification, (3) dissolution and precipitation of nitrocellulose. The samples were vacuum-dried after each step.

During the first step, lipids were removed by washing with a 1:1 benzene-methanol mixture for 24 h. Subsequently, samples were washed with distilled water and mixed with acetone for 24 h. Then they were rinsed with water and HCl (4%) and again washed with distilled water and boiled (1 h). Lignin was removed by treating the samples with acetic acid and sodium chlorite at 70 °C, which was repeated five times in 45 min intervals. Samples were then well rinsed with distilled water again. Hemicellulose was removed by treating samples with 17% solution of sodium hydroxide for 1 h. After rinsing with distilled water, the samples were treated with 10% solution of acetic acid for 10 min. Samples were rinsed with water, placed in desiccators and vacuum-dried. This first step is very similar to the technique described for moss $\delta^{18}O$ analyzed by Zanazzi and Mora [25] developed after Green [22] and Wolfe et al. [26].

**Table 1.** Summary of regression estimates. The model using $\delta^{13}C$ values of bulk organic carbon (x) to predict $\delta^{13}C$ of nitrocellulose (y). In all cases the null hypothesis was that, slope (a) equals to one and intercept (b) equals zero. The relationship was described with a simple linear model y = ax + b

| | Location/ sampling period | n | a (±st. error) | *P* a ≠ 1 | b (±st. error) | P b ≠ 0 | $R^2$ | RMSE |
|---|---|---|---|---|---|---|---|---|
| Polytrichum | Izery/1 | 10 | 0.97 ± 0.07 | 0.67 | 0.06 ± 1.82 | 0.97 | 0.96 | 0.14 |
| | Izery/2 | 11 | 1.05 ± 0.06 | 0.42 | 2.91 ± 1.74 | 0.12 | 0.97 | 0.14 |
| | Karkonosze/1 | 9 | 1.02 ± 0.07 | 0.78 | 2.23 ± 2.08 | 0.31 | 0.96 | 0.28 |
| | Karkonosze/2 | 8 | 1.12 ± 0.07 | 0.13 | 5.27 ± 2.00 | 0.03* | 0.98 | 0.30 |
| | All above | 38 | 1.04 ± 0.03 | 0.19 | 2.84 ± 0.95 | 0.01* | 0.96 | 0.24 |
| Sphagnum | Izery/1 | 10 | 1.05 ± 0.08 | 0.55 | 2.05 ± 2.16 | 0.37 | 0.96 | 0.24 |
| | Izery/2 | 11 | 1.11 ± 0.09 | 0.25 | 4.20 ± 2.52 | 0.13 | 0.95 | 0.28 |
| | Karkonosze/1 | 9 | 0.97 ± 0.04 | 0.47 | -0.26 ± 1.22 | 0.84 | 0.99 | 0.24 |
| | Karkonosze/2 | 9 | 1.01 ± 0.08 | 0.90 | 0.73 ± 2.34 | 0.76 | 0.97 | 0.28 |
| | All above | 39 | 1.02 ± 0.04 | 0.62 | 1.18 ± 1.04 | 0.26 | 0.96 | 0.30 |
| All plants | Hala Izerska/1 | 7 | 1.31 ± 0.09 | 0.01* | 8.96 ± 2.48 | 0.01* | 0.97 | 0.17 |
| | Hala Izerska/2 | 8 | 1.17 ± 0.09 | 0.10 | 5.80 ± 2.47 | 0.05* | 0.96 | 0.26 |
| | All above | 15 | 1.18 ± 0.12 | 0.16 | 5.73 ± 3.14 | 0.09 | 0.89 | 0.31 |

Sampling periods: (1) July 2004, (2) October 2004. In total 3 outliers from 39 points for Sphagnum and 3 from 38 points for Polytrichum are abandoned for root mean square error (RMSE) calculations. *P*-values are calculated for two-tailed *t*-test, evaluated at $\alpha = 0.05$, statistically significant tests are flagged with an asterisk (*).

During the second step, dry samples where nitrated using nitric acid ($HNO_3$ 100%, Merck catalog no. 1.00455) at 5 °C for 48 h, according to the modified procedure of Epstein et al. [1] based on Goring and Timmell [35] and Alexander and Mitchell [36]. The nitrated samples were washed with distilled water and mixed with methanol for 6 h and again washed with distilled water, and then vacuum-dried.

The nitrated product still might have contained traces of nitrated lignin and hemicellulose besides nitrocellulose. During the third step of preparation, essentially pure nitrocellulose was extracted by dissolving in acetone [35]. After centrifugation and decantation, the nitrocellulose was precipitated from the solution by quickly adding distilled water. The white pure fluffy nitrocellulose was well rinsed with distilled water and dried under vacuum.

As was shown by Timmell [37], the nitration procedure extracts the same yields for cellulose as does the sodium chlorination technique, e.g., [16]. The experiments, which were done by Epstein et al. [1] and Feng et al. [12] confirm that the advantage of the nitration technique is that the exchangeable OH hydrogen [38] in cellulose is replaced by nitrates during the esterifications, leaving the cellulose carbon-bound hydrogen unaffected. Therefore, the $\alpha$-cellulose extraction technique, although suitable for $\delta^{18}O$, is not proper for $\delta D$ analysis due to the presence of exchangeable hydrogen.

All stable isotope analyses were carried out using an off-line preparation system technique used for maximum precision. About 3 to 5 mg of pure nitrocellulose or bulk plant samples were combusted with CuO wire in a sealed quartz tube, under vacuum at 900 °C [14]. The $CO_2$ gas produced was cryogenically purified off-line (liquid nitrogen and dry ice + ethanol mixture) and introduced into an isotope ratio mass spectrometer (IRMS; Finnigan-Mat Delta E/dual inlet, Bremen, Germany) for stable carbon isotope ratio analysis. The $\delta^{13}C$ values were normalized against NBS22 and USGS24 international standards (distributed by the International Atomic Energy Agency, Vienna) and then reported relative to the Vienna Pee Dee Belemnite (VPDB) scale with precision ± 0.05‰. The $\delta^{13}C_{VPDB}$ value is defined as the relative difference, in parts per thousand (‰), between the isotope ratio of the sample and the VPDB standard.

## Results and Discussion

The stable carbon isotopic composition ($\delta^{13}C_{VPDB}$) of mosses collected along selected transects varied due to different growth period temperatures observed at different altitudes [2, 34]. The selection of such sampling points provided desirable material characterized by significantly different isotopic composition. The $\delta^{13}C$ values for *Sphagnum* varied from −32.66‰ to −26.20‰ (bulk organic matter) and from −31.86‰ to −25.03‰ (nitrocellulose). A similar range was observed for *Polytrichum*, from −32.18‰ to −26.22‰ (bulk organic matter) and from −30.51‰ to −24.11‰ (nitrocellulose). The correlations between pairs of $\delta^{13}C$ values for nitrocellulose and for bulk organic matter (for each moss sample) were analyzed in four scopes: two sampling periods (1st period in July or 2nd period October) and two locations (Karkonosze or Izerskie Mountains.).

A very high coefficient of determination ($R^2$) was observed for each pair $\delta^{13}C_{bulk}$-$\delta^{13}C_{nitrocellulose}$. The $R^2$-factors for Karkonosze equal: 0.96, 0.98, 0.99, and 0.97 for *Polytrichum* (July), *Polytrichum* (October), *Sphagnum* (July), *Sphagnum* (October), respectively (Table 1). Only one sample, which did not match the trend, was excluded. Similarly, very high $R^2$-factors were observed for Izerskie: 0.96, 0.97, 0.96, and 0.95 for *Polytrichum* (July), *Polytrichum* (October), *Sphagnum* (July), *Sphagnum* (October), respectively (also, only one sample was excluded). The overall $R^2$ factor for both places and

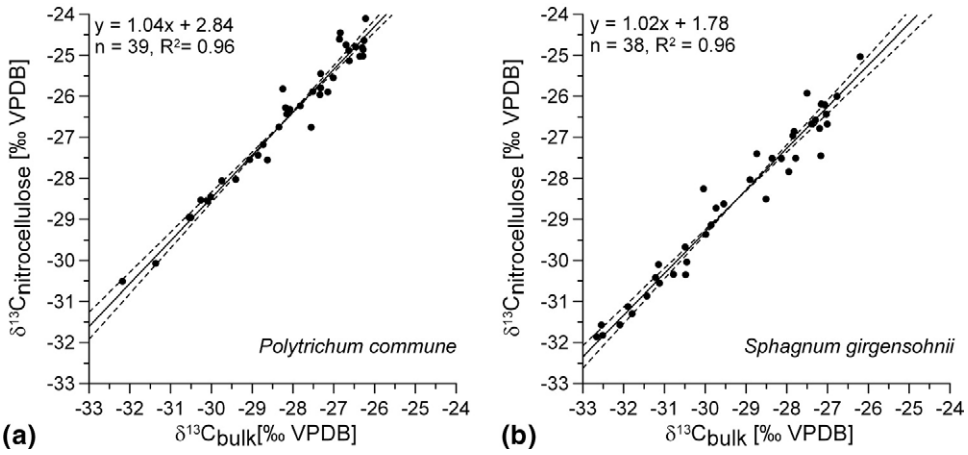


**Figure 1.** Representative $\delta^{13}C$-values in bulk plant material versus $\delta^{13}C$-values in nitrocellulose from *Polytrichum commune* (**a**) and *Sphagnum girgensohnii* (**b**). Summary for both regions Izerskie and Karkonosze Mountains and both sampling periods (July and October). The solid line represents the model and the dashed lines represent 95% confidence intervals of the model (reduced major axis regression).

sampling periods was equal to 0.96 for all *Polytrichum* samples and was the same for all *Sphagnum* samples (Figure 1, Table 1).

The regression model (summarized in Table 1) using $\delta^{13}C_{bulk}$ to predict $\delta^{13}C_{nitrocellulose}$ provided best fit (reduced major axis regression). Besides the high correlations ($R^2$), the calculated slopes for the linear regression lines for both moss species, locations and periods were significantly similar to unity (*P*-values from 0.13 to 0.90, two-tailed *t*-test), and observed differences were smaller then the highest standard errors for slope (<0.09). The overall slope for all *Polytrichum* samples was 1.04 ($n = 38$, $P = 0.19$) and for *Sphagnum* 1.02 ($n = 39$, $P = 0.62$). The intercepts were significantly similar to zero for *Sphagnum* (all cases) and for *Polytrichum* samples collected in Izerskie (July and October) and Karkonosze (July), *P*-values from 0.12 to 0.97. The intercept for *Polytrichum* (October) was different from zero ($P = 0.03$). The overall intercept for *Polytrichum* was 2.84 (±0.95, $P = 0.01$), and for *Sphagnum* 1.18 (±1.04, $P = 0.26$) (Table 1). No significant differences were observed between groups of mosses collected during different seasons (*P*-values range from 0.3 to 0.8, *t*-unpaired test). It was evident from the data presented in Figure 1 that, in general, the average $\delta^{13}C$ value of nitrocellulose was about 1.6‰ more positive versus bulk organic matter for *Polytrichum* and 0.7‰ for *Sphagnum*. The standard deviations for these calculated offsets were 0.40 ($n = 38$) and 0.41 ($n = 39$), respectively. This rule seems to be valid for the entire analyzed $\delta^{13}C$ range, however prediction of $\delta^{13}C_{nitrocellulose}$ from $\delta^{13}C_{bulk}$ may introduce an uncertainty. The root mean square error (RMSE) for all analyzed pairs of $\delta^{13}C$ values (bulk– cellulose) varies from 0.14‰ to 0.30‰. The introduced uncertainty (RMSE) is within the typically accepted precision for the EA technique (elemental analyzer in continuous flow) which is about ±0.15‰ (1 $\sigma$). Despite these differences and introduced uncertainty, the relative variation of the $\delta^{13}C$ trend (e.g., between locations) can be observed for bulk organic matter as well as for the extracted nitrocellulose (e.g., *Sphagnum* in the Izerskie Mountains, (Figure 2).

These results are in general agreement with the previous preliminary studies published by Menot and Burns [21], where they also observed a high correlation ($R^2 = 0.87$) and slope (about 0.9), but a different intercept (−0.85) for $\alpha$-cellulose from 24 unclassified moss species.

In addition, all the most common bog plant species were collected at Hala Izerska. These include (1) mosses: *Sphagnum girgensohnii*, *Polytrichum commune*, *Sphagnum fallax*, *Sphagnum majus*, *Sphagnum papillosum*, and (2) vascular plants: *Carex enchinata*, *Molinia caerulea*, *Eriophoroum vaginatum* (all these species are C3 photosynthesis). A high correlation ($R^2 = 0.97$ and 0.96) was also found for samples of various species of *Polytrichum*

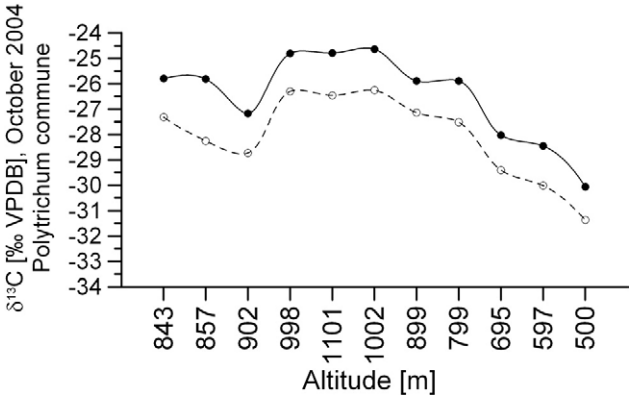


**Figure 2.** Example of $\delta^{13}C$-values variations in vertical transect from Izerskie Mountains. Bulk plant material $\delta^{13}C$-values (dashed line) versus $\delta^{13}C$-values in nitrocellulose (solid line).

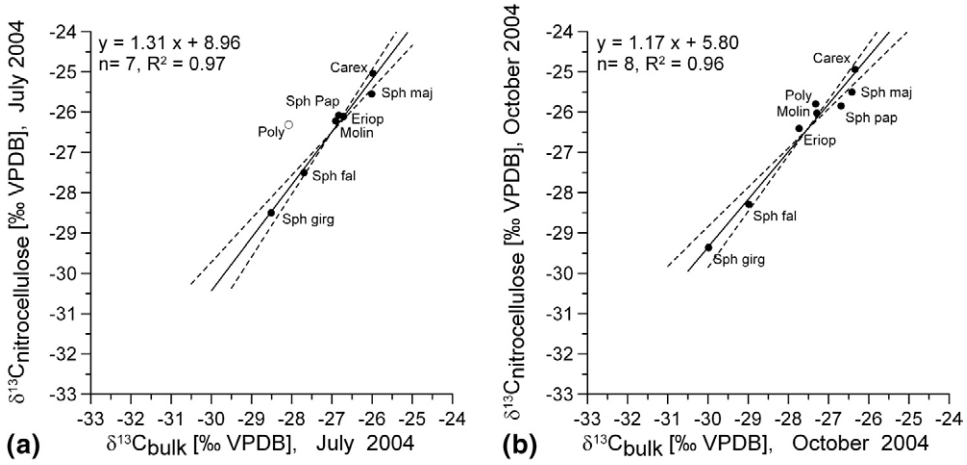


**Figure 3.** Representative $\delta^{13}C$-values in bulk plant material versus $\delta^{13}C$-values in nitrocellulose from selected plants, collected in July (**a**) and October (**b**) 2004 at Hala Izerska bog, SW Poland. Plants: *Sphagnum girgensohnii* (Sph girg), *Polytrichum commune* (Poly), *Carex enchinata* (Carex), *Molinia caerulea* (Molin), *Eriophoroum vaginatum* (Eriop), *Sphagnum fallax* (Sph fal), *Sphagnum majus* (Sph maj), *Sphagnum papillosum* (Sph pap). At plot A, one sample (Poly) excluded (open symbol). The solid line represents the model and the dashed lines represent 95% confidence intervals of the model (reduced major axis regression).

and *Sphagnum*. It was not surprising that $\delta^{13}C$ for all moss species fit this linear regression because they have very similar physiology and grow in only slightly different conditions. No significant differences were observed for groups of plant samples collected during different seasons (*P*-values from 0.48 to 0.82). On other hand, it is very interesting that $\delta^{13}C$ of vascular plants such as sedges, grasses, and cotton-grass followed the same regression line (Figure 3). However, a wide confidence interval for slope and interval for intercept were observed for mosses (Figures 1 and 3). The slope is significantly different from unity for samples collected in July ($P < 0.01$) but not significantly different for samples collected in October ($P = 0.10$). However, the relative difference between slopes (1.31 for July and 1.17 for October) was within the range of two standard error ±0.09 (Table 1).

We may assume that the isotope fractionation during the metabolic process of cellulose formation during plant growth in each analyzed plant species was affected by the same controlling factor, probably mainly by temperature [2, 34]. Because of that, the same differences between $\delta^{13}C$ values for bulk organic matter and $\delta^{13}C$ of nitrocellulose could be observed for all collected species, despite completely different types of plants (average 0.83‰). Previously, Menot and Burns [21] stated that it was not proper to consider vascular and nonvascular plants together because vascular species contain varying amounts of cellulose and noncellulose organic fractions. Regardless of this conclusion, we suggest that considering the vascular plants together with nonvascular plants in this case could be relevant. The influence of the temperature on the difference between carbon isotopic composition of nitrocellulose and bulk organic matter could be more crucial then physiological differences between analyzed species [2, 34].

## Conclusions

The correlation factors between pairs of $\delta^{13}C$ values: extracted nitrocellulose–bulk organic matter were very high for all analyzed plants ($R^2$ factors were ≥0.95). These results suggested that the extraction process of nitrocellulose did not cause any crucial isotopic fractionation; the slope of linear regression equals 1.02 (*Sphagnum*) and 1.04 (*Polytrichum*). The $\delta^{13}C$ values of extracted nitrocellulose and bulk plant organic matter were not equal. A more or less consistent difference between $\delta^{13}C$ for extracted nitrocellulose and bulk organic matter of 0.7‰ for *Sphagnum* and 1.6 ‰ for *Polytrichum* was observed. These observations support the conclusion that $\delta^{13}C$ analysis can be performed on bulk plant material instead of cellulose, without significant loss of information, at least for *Polytrichum* and *Sphagnum* mosses. This will reduce cost and time of analysis as well as the use of toxic chemicals.

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