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Contamination risk of stable isotope samples during milling

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RATIONALE: Isotope analysis of wood is an important tool in dendrochronology and ecophysiology. Prior to mass spectrometry analysis, wood must be homogenized, and a convenient method involves a ball mill capable of milling samples directly in sample tubes. However, sample-tube plastic can contaminate wood during milling, which could lead to biological misinterpretations.
METHODS: We tested possible contamination of whole wood and cellulose samples during ball-mill homogenization for carbon and oxygen isotope measurements. We used a multi-factorial design with two/three steel milling balls, two sample amounts (10 mg, 40 mg), and two milling times (5 min, 10 min). We further analyzed abrasion by milling empty tubes, and measured the isotope ratios of pure contaminants.
RESULTS: A strong risk exists for carbon isotope bias through plastic contamination: the $\delta^{13}C$ value of polypropylene deviated from the control by −6.77‰. Small fibers from PTFE filter bags used during cellulose extraction also present a risk as the $\delta^{13}C$ value of this plastic deviated by −5.02‰. Low sample amounts (10 mg) showed highest contamination due to increased abrasion during milling (−1.34‰), which is further concentrated by cellulose extraction (−3.38‰). Oxygen isotope measurements were unaffected.
CONCLUSIONS: A ball mill can be used to homogenize samples within test tubes prior to oxygen isotope analysis, but not prior to carbon or radiocarbon isotope analysis. There is still a need for a fast, simple and contamination-free sample preparation procedure. Copyright © 2016 John Wiley & Sons, Ltd.

The stable carbon and oxygen isotope signatures ($\delta^{13}C$ and $\delta^{18}O$ values) of tree-rings are parameters useful in dendroclimatology, dendroecology, ecophysiology and biology since they are datable to specific calendar years and are sensitive to environmental variation. They are increasingly being used as proxies for past climate changes,[1–5] to understand tree response to drought[6,7] and thinning,[8,9] to explain genetic differentiation in water use efficiency,[10,11] and in interpreting forest response under changing atmospheric conditions and climates.[12,13] These important analyses are based on the fact that environmental conditions influence stomatal control and photosynthetic activity, which in turn affects isotope ratios in synthesized macromolecules later archived in the tree-rings. Such relationships are built on well-established understanding of carbon isotope fractionation processes[14–16] and a more recent understanding of oxygen isotope fractionation processes in plants.[17–23]

All methods of processing wood samples for isotope analysis require homogenization prior to mass spectrometry analysis.[24,25] There is no ideal homogenization method, however. For example, ultra-centrifugal mills are commonly used for tree-ring stable isotope research,[1,7,26] but samples must be laboriously cut into fine pieces with a scalpel before milling to avoid burning and unintentional fractionation. Material collection and cleaning of the mill are both cumbersome and time consuming processes, and may result in high loss of sample material[25]. Ultrasonic homogenization is an alternative process, but it only works for small cellulose samples (<10 mg).[25,27] This procedure involves placing the extracted cellulose into a sample tube with deionized water, and homogenizing it with ultrasonic waves.[25] This alternative method requires freeze-drying, however, and is therefore not necessarily faster. Other methods exist but are usually limited (e.g. grinding with a Wig-L-bug mill,[28–30] a microtome,[31–33] or small drill bits[8,34,35]) or require very specialized and often expensive equipment that is only appropriate for some applications (e.g. UV-laser ablation,[32] cryo-mill,[25] and cellulose cross-sections[36,37]).

Because of these limitations, a ball mill fitted with a sample-tube holder is one of the most convenient methods of homogenizing wood for isotope analysis. The use of a ball mill is faster than using an ultra-centrifugal mill because larger wood pieces can be milled, and, hence, less time is required to cut wood into small pieces with a scalpel. Since the wood can remain in the original sample tube, there is no loss of material and time can be saved: steel milling balls are simply added to each tube, and multiple samples can be placed in a tube holder and milled simultaneously. The resulting homogenized material is then ready for cellulose extraction[38,39] or for mass spectrometric analysis of whole wood (resins and extractives may have to be removed first[2,30,38]). After a recent study found no evidence of isotope fractionation due to heating from

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friction of wood samples during ball milling, the ball mill was explicitly recommended for tree-ring isotope research.[40]

The ball-mill configuration became a matter of concern, however, after unpublished isotope data showed atypical patterns: radiocarbon measurements of the same samples indicated the presence of 'dead carbon'. These atypical results might have stemmed from sample-tube abrasion during milling, which could taint the sample with plastic. Since most standard sample tubes are made of polypropylene (PP) plastic derived from fossil fuels, they have specific isotope signatures. Fossil fuels represent the remains of prehistoric plants with low $\delta^{13}C$ values,[41,42] although the exact $\delta^{13}C$ signatures of fossil fuel types can vary.[43] If the inside of a plastic sample tube is ground during ball milling and this does indeed cause plastic contamination, the isotope signature could be altered. This could lead to biological or climatological misinterpretations. A thorough literature review produced no information on the isotope signatures of polypropylene sample tubes.

Here, we evaluate the suitability of a common ball-mill design for continued use as a fast and contamination-free method of homogenizing wood for isotope measurements. First, we assess the potential for plastic contamination to alter isotope values by measuring the $\delta^{13}C$ and $\delta^{18}O$ values of polypropylene-based sample tubes. Second, we observe possible sample-tube abrasion under different milling configurations. Third, we quantify any loss of polypropylene plastic during cellulose extraction, and test the remaining isotope contamination on both cellulose and whole wood. Finally, we assess alternative homogenization methods.

EXPERIMENTAL

Tree-ring selection and separation

For this study, we chose a large stem disk (~80 cm in diameter) of a Norway spruce (*Picea abies* L.H. Karst.) tree that was free of any damage and had a large ring (~6 mm). Taking samples from the same ring was important because a constant isotope signature was required to ensure that any variation in isotope values would be based on procedure alone. Because the stem disk was so large and the ring was so wide, enough material for all treatments could be taken from within ~4 cm, thereby reducing the potential of isotope value variation along the same ring.[44] The stem disk was sawed into a section about 1 cm thick for easier cutting, and the surface was cleaned with a scalpel. The wide ring was separated and finely cut into small pieces using a scalpel under a stereomicroscope (Leica Wild M3B, Wetzlar, Germany).

Experimental design

To determine the $\delta^{13}C$ and $\delta^{18}O$ values of the pure contaminants, we analyzed eight samples of polypropylene plastic cut from sample tubes: four underwent the full laboratory procedure for cellulose extraction and four were weighed directly into tin capsules for analysis by mass spectrometry. Each sample was from a new sample tube, but all sample tubes were from the same batch of standard 2.0-mL laboratory polypropylene sample tubes (Rotilabo-safety reaction tubes, Item No.: NA16.1, Carl Roth GmbH, Karlsruhe, Germany). As part of a thorough study, we also evaluated possible contamination through fibers of the PTFE filter bags often used for cellulose extraction: samples of polytetrafluoroethylene plastic (PTFE) were prepared by cutting pieces from a F57 filter bag (Ankom Technology, Macedon, NY, USA). While different extraction methods can be used,[38,39] a common technique involves placing the samples inside filter bags made of PTFE, then exposing the samples to NaOH and $NaClO_2$ (sodium chlorite) to remove extractives and lignin.[39] However, PTFE fibers can occasionally become embedded in the sample and may go unnoticed before mass spectrometry. Similar to polypropylene, this could cause isotope contamination because PTFE is also made from fossil fuels. The chemical structures of polypropylene $[(C_3H_6)_n]$ and PTFE $[(C_2F_4)_n]$ contain no oxygen atoms, so only $\delta^{13}C$ values would be expected to be affected by plastic contamination. We nevertheless measured the $\delta^{18}O$ values of a small subset to verify that no unexpected source of fractionation existed.

To test polypropylene contamination of wood due to ball milling, we used a multi-factorial design involving two milling times (5 min and 10 min), two different numbers of balls (2 or 3 balls), two sample amounts (10 mg and 40 mg) and two measurement types (whole wood and cellulose). These categories were chosen because they represent a range of common applications of the ball mill for homogenizing wood for stable isotope analysis. Each category contains four samples (Tables 1 and 2). The samples were carefully weighed to a precision of 0.01 mg using an analytical balance (model

Table 1. Mean $\delta^{18}O$ values and standard errors for polytetrafluoroethylene plastic (PTFE), polypropylene plastic, and wood that underwent different ball-milling treatments

Material	Mill	Weight (mg)	No. balls	Time (min)	Mean $\delta^{18}O$ (‰)	Mean $\delta^{18}O$ offset (‰)	SE	N	adj. *p*-value
Polypropylene	–	–	–	–	0	–	–	4	
Polytetrafluroethylene	–	–	–	–	0	–	–	4	
Cellulose*	CM	40	–	–	29.17	–	0.19	4	–
Cellulose	BM	10	2	5	29.02	−0.15	0.17	4	0.865
Cellulose	BM	40	2	5	29.08	−0.09	0.27	4	0.951

The ball mill is denoted by BM, the ultra-centrifugal mill is represented by CM. Sample weights (*Weight*) are in milligrams (mg), and the milling time (*Time*) is in min. The control is marked with a star, and the offsets to this control are provided (*Mean $\delta^{18}O$ Offset*). Standard error is denoted by *SE*, while the number of samples is denoted by *N*. The difference of each group from its respective control can be evaluated with the *p*-value adjusted with a Bonferroni correction to compensate for experiment-wise alpha inflation (*adj. p-value*).

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Table 2. Mean $\delta^{13}C$ values and standard errors for polytetrafluoroethylene plastic (PTFE), polypropylene plastic, and wood that underwent different ball-milling treatments

Material	Mill	Weight (mg)	No. balls	Time (min)	Mean $\delta^{13}C$ (‰)	Mean $\delta^{13}C$ offset (‰)	SE	N	adj. *p*-value
Polypropylene	–	–	–	–	−30.61	−6.77	0.01	4	–
Polytetrafluoroethylene	–	–	–	–	−28.87	−5.02	0.02	4	–
Whole wood*	CM	40	–	–	−23.85	–	0.01	4	–
Whole wood	BM	10	2	10	−24.29	−0.44	0.12	4	0.024
Whole wood	BM	10	2	5	−24.03	−0.18	0.10	4	0.828
Whole wood	BM	10	3	10	−25.19	−1.34	0.05	4	<0.001
Whole wood	BM	10	3	5	−24.30	−0.45	0.16	4	0.020
Whole wood	BM	40	2	10	−23.86	−0.01	0.05	4	>0.999
Whole wood	BM	40	2	5	−23.90	−0.05	0.02	4	>0.999
Whole wood	BM	40	3	10	−24.01	−0.16	0.09	4	0.914
Whole wood	BM	40	3	5	−23.87	−0.02	0.02	4	>0.999
Cellulose*	CM	40	–	–	−22.77	–	0.05	4	–
Cellulose	BM	10	2	10	−24.09	−1.33	0.43	4	0.013
Cellulose	BM	10	2	5	−23.06	−0.30	0.21	4	0.992
Cellulose	BM	10	3	10	−26.14	−3.38	0.10	4	<0.001
Cellulose	BM	10	3	5	−23.76	−0.99	0.41	4	0.122
Cellulose	BM	40	2	10	−23.03	−0.27	0.21	4	0.996
Cellulose	BM	40	2	5	−22.64	−0.13	0.05	4	>0.999
Cellulose	BM	40	3	10	−22.98	−0.21	0.21	4	>0.999
Cellulose	BM	40	3	5	−22.78	−0.01	0.07	4	>0.999

The ball mill is denoted by BM, the ultra-centrifugal mill is represented by CM. Sample weights (*Weight*) are in milligrams (mg), and the milling time (*Time*) is in minutes. The control is marked with a star, and the offsets to this control are provided (*Mean $\delta^{13}C$ Offset*). Standard error is denoted by *SE*, while the number of samples is denoted by *N*. The difference of each group from its respective control can be evaluated with the *p*-value adjusted with a Bonferroni correction to compensate for experiment-wise alpha inflation (*adj. p-value*).

XS105, Mettler Toledo, Greifensee, Switzerland) before being placed into 2.0-mL polypropylene sample tubes. We initially compared the sample tubes by Carl Roth with Eppendorf sample tubes, both of which are made of polypropylene plastic, but a visual assessment found no apparent difference. We therefore continued with the Carl Roth sample tubes that are standard in our laboratory. The ball mill used was the 'Mixer Mill' by Retsch (MM 200, Retsch GmbH, Haan, Germany).

To test the base level of abrasion of each treatment on the sample tube itself, we ran empty sample tubes under two time treatments (5 min and 10 min) with differing numbers of milling balls (2 or 3 balls). The abrasion from these sample tubes was detected visually and a binary label of 'opaque' (abrasion present) or 'clear' (abrasion absent) was assigned (Fig. 1). For the control, four 40-mg samples were milled with an ultra-centrifugal mill (ZM 2000, Retsch GmbH, Germany) with a 0.25-mm mesh size to ensure homogeneity.[2,24,45] This mill is a suitable control because it is all-metal and therefore free from any potential plastic contamination. All samples were then separated into cellulose and whole wood samples, and samples for both $\delta^{13}C$ and $\delta^{18}O$ measurements were packed for mass spectrometric analysis.

We also conducted a follow-up experiment to test if the addition of liquid to the sample tube prior to ball-milling could reduce sample tube abrasion without inhibiting sample homogenization. We determined the suitability of distilled water and 96% ethanol. We initially observed that sample tubes milled with 1 mL of liquid remained 'optically clear', but subsequent runs produced very poor homogenization results. Continued re-milling was not considered because it could increase abrasion and plastic contamination. Follow-up mass spectrometry on a subset of treatments with the highest contamination potential indicated that some samples were significantly different from the control (data not shown). We therefore did not pursue this option further and cannot recommend its use without further investigation.

Cellulose extraction

Half of the samples were whole wood (Table 2), which were packed directly into tin capsules after milling. The other half underwent the cellulose-extraction procedure after milling. The latter samples were packed into PTFE filter bags (F57, Ankom Technology) and cellulose was extracted by applying a modified Jayme-Wise Holocellulose Isolation method.[38,39] For a 10-mg sample, this typically involves two washes with a 5% NaOH solution for 2 h at 60°C followed by a wash with 7% $NaClO_2$ for 30 h at 60°C. We assumed the reaction to be a linear process, and initially scaled the chemical exposure time according to sample weight. We found, however, that the percentage of the sample that was extracted varied substantially after completing an initial round of cellulose extraction. This probably arose from a high variability in particle size after milling. In a normal study design, a researcher would probably mill such a heterogeneous sample again to ensure particle uniformity and equal cellulose extraction rates.[24] Since we were testing the effect of milling time, we chose not to re-mill any such samples. To allow us to nevertheless compare the cellulose from such heterogeneously

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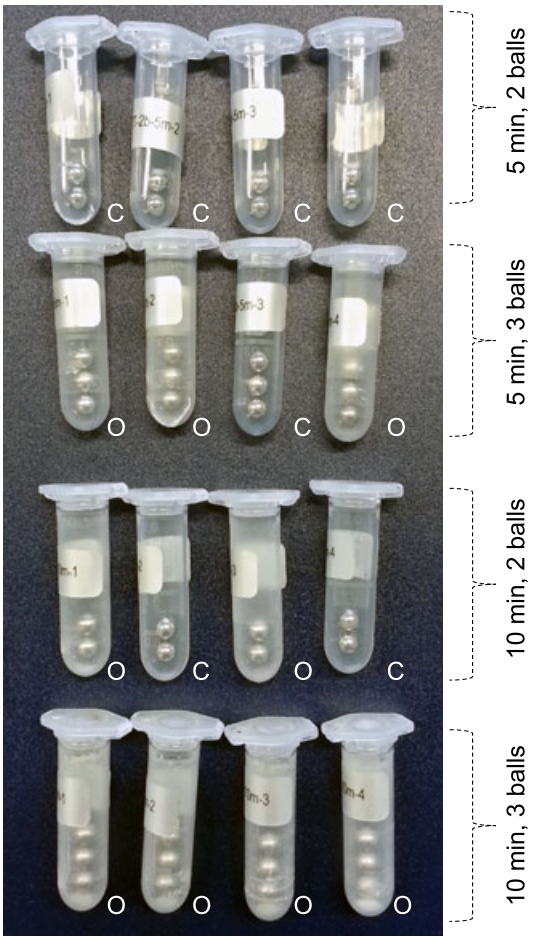

Figure 1. The results of testing the base level of abrasion during milling. Empty sample tubes were milled with two time treatments and two numbers of steel milling balls. A white letter to the bottom-right of each tube shows the assigned level of abrasion, a binary response variable. Abrasion is clearly visible in test tubes that appear opaque, denoted by 'O'. Tubes that are clear indicate no abrasion, labelled with a 'C'. The sample tubes that were milled for 10 min using 3 balls showed the highest abrasion because they were all opaque. In contrast, the tubes milled for 5 min with only 2 balls were all clear, showing the least abrasion.

milled samples, we attempted to ensure a consistent rate of extraction by processing the samples until at least only 45% of the original sample mass remained. As a further precaution, the final extraction rate was accounted for in the model.

Measurements of isotope ratios

For $\delta^{13}C$ measurements, the samples were combusted to CO_2 at 1080°C using a EURO EA elemental analyzer (EuroVector, Milan, Italy). For $\delta^{18}O$ measurements, the samples were pyrolyzed to CO at 1400°C using a high-temperature oxygen analyzer (HEKAtech, Wegberg, Germany). The stable isotope ratios were determined by isotope ratio mass spectrometry (Delta V Advantage Mass Spectrometer, Thermo Scientific, Bremen, Germany). All isotope measurements were conducted at the WSL Central Laboratory (Birmensdorf, Switzerland) at a precision of ±0.02‰ for $\delta^{13}C$ values and ±0.3‰ for $\delta^{18}O$ values. The $\delta^{13}C$ values were referenced to Vienna Pee Dee Belemnite (VPDB) and the $\delta^{18}O$ values to Vienna Standard Mean Ocean Water (VSMOW) according to the following formula: $R = (R_{sample}/R_{standard} - 1)$, where R is the ratio of the heavy to light isotope.[2,15]

Statistical analysis

All analyses were conducted in the R statistical programming environment.[46] To test differences in abrasion among the empty sample tubes after milling, we used binary logistic regression. Statistical analysis of the multi-factorial design relies on a simple linear model analysis of variance (ANOVA) implemented with the *lm* function in the base package in R. This analysis was first completed to compare different amounts of losses among treatments after cellulose extraction, using the percentage remaining as a response variable after logit transformation[47] using the *logit* function in the *car* package. We then analyzed cellulose and whole wood separately to account for slightly different predictor variables. Both models use $\delta^{13}C$ values as the response variable, and the predictor variables include amount (10 mg or 40 mg), the number of steel milling balls (2 or 3), and the milling time (5 min or 10 min). The percentage of the sample's mass after cellulose extraction was included in the cellulose model as a covariate. In the whole wood model, the percentage of carbon was included as a covariate. A similar model was run on a smaller subset with $\delta^{18}O$ values as the response variable. To calculate the proportional contribution of the three main treatments (amount, number of balls and milling time) to the total explained variance, we estimated the partial eta-squared (η_p^2) using the *etaSquared* function from the *lsr* package on the ANOVA outputs. Post-hoc tests were completed with the *TukeyHSD* function, which uses Tukey's Honest Significant Difference to account for experiment-wide alpha inflation.

RESULTS

Sample-tube abrasion and isotope signatures of potential contaminants

The link to polypropylene comes from the abrasion on the inside of the sample tubes during milling. Results from testing the base level of abrasion can be seen in Fig. 1. This figure can be used to evaluate the level of abrasion present: the appearance of empty sample tubes after milling with different numbers of balls (2 or 3 balls) for different durations (5 min and 10 min) is assigned a binary label of abrasion. Those tubes assigned a label of 'clear' indicate little to no abrasion, while those assigned a label of 'opaque' suggest high abrasion (Fig. 1). The visual results together with the results of binary logistic regression show that there are significant differences among groups ($p = 0.008$). The sample tubes from the 3-ball–10-min category showed the most abrasion, while the sample tubes from the 2-ball–5-min category showed the least.

Effect of cellulose extraction on the amount of material

Cellulose extraction did not reduce the amount of polypropylene plastic in samples (Fig. 2 and Supplementary Table S1, Supporting Information). The yield after cellulose extraction of the wood samples depended on the type of

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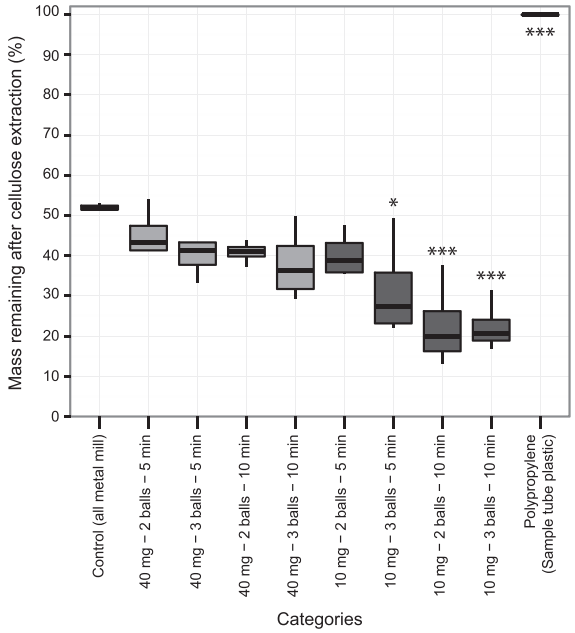

Figure 2. Boxplots of the percentage of sample mass remaining after the first round of cellulose extraction (they were later extracted until <45% of the sample mass remained). The inter-quartile range of 40-mg samples is shown in light grey while the inter-quartile range of 10-mg samples is shown in dark grey. Each category represents four samples. Stars represent categories that are significantly different from the control (***$p < 0.001$, *$p < 0.05$).

mill and the amount of material that was milled (Fig. 2 and Supplementary Table S1). The control samples, which were milled with the all-metal, ultra-centrifugal mill, showed a high remaining amount with low variability between samples. Similarly, samples that were milled in the ball mill with more material (40 mg) had more material remaining and less variability in its amount after cellulose extraction. The samples ball-milled with less material (10 mg) showed a significantly higher loss of material after cellulose extraction, combined with higher variability. The samples that were ball-milled for 10 min showed the least amount remaining after cellulose extraction. In fact, the only three categories that were significantly different in their amount from the control were 10-mg–2-ball–10-min ($p < 0.001$), 10-mg–3-ball–10-min ($p < 0.001$) and 10-mg–3-ball–5-min ($p = 0.029$).

Carbon and oxygen isotope signatures in contaminants, milled wood and cellulose

We found no oxygen atoms in either polypropylene or PTFE (Table 1). However, both plastics contain carbon atoms, and our analyses showed distinct $\delta^{13}C$ signatures (Table 2). For polypropylene, the mean $\delta^{13}C$ values were −30.6‰. For PTFE, the mean $\delta^{13}C$ values were −28.9‰. These values are −6.8‰ and −5.0‰ lower than the mean $\delta^{13}C$ value of our wood control sample (−23.8‰), respectively.

The $\delta^{13}C$ values were similar among the control samples and the large (40 mg) samples milled with the ball mill (Figs. 3(a) and 3(b), Table 2). Post-hoc tests further confirmed no statistical difference between the 40-mg samples and the control for both cellulose and whole wood. In comparison, the small samples (10 mg) milled with the ball mill showed generally more deviation from the control and more variability in their $\delta^{13}C$ values (Figs. 3(a) and 3(b), Table 2). Both cellulose and whole wood samples milled for longer (10 min) and with less material (10 mg) showed significantly lower $\delta^{13}C$ values than the control samples (Figs. 3(a) and 3(b), Table 2). The number of balls, time and material all significantly contributed to the model (Tables 3 and 4). In the cellulose model, the yield (% remaining) also contributed significantly to the model (Table 4). Using $\delta^{13}C$ values as the response variable in both cellulose and whole wood models, the effect size for the sample amount (10 mg or 40 mg) was found to be of higher relevance ($\eta_p^2 = 0.505$ in both) than the effect size for the number of steel milling balls (2 or 3 balls), which was weaker in both cellulose and whole wood models ($\eta_p^2 = 0.204$ and 0.263, respectively). The effect of milling time (5 min or 10 min) was also weaker in both cellulose and whole wood models ($\eta_p^2 = 0.341$ and 0.252, respectively).

A comparison of the $\delta^{13}C$ values of whole wood and cellulose indicated a relatively consistent offset (Fig. 3(c)): the $\delta^{13}C$ values in cellulose were less negative than those in whole wood. This offset was most prominent and consistent in samples that were milled with more material (40 mg), where they deviated on average by −1.053 ‰ (Table 2). These values fall within the range of previously published work on wood-cellulose comparisons of conifers.[48,49] Interestingly, the offset became reduced in the samples milled with less material (10 mg) and even reversed in the treatment with the highest abrasion: the 10-mg–3-ball–10-min category showed cellulose being even more lower $\delta^{13}C$ values than whole wood (Fig. 3(c)). In the 10 mg samples, the mean offset was only −0.190‰ because individual offsets were not consistent in direction (Table 2).

DISCUSSION

Sample-tube abrasion and heterogeneity of particle size in milled wood

Our milling test with empty sample tubes showed that abrasion can occur. We also demonstrated that the intensity of abrasion increases when using 3 instead of 2 balls and when extending the milling time from 5 to 10 minutes. This is the first evidence that there is a risk of contamination of wood samples with plastic during the ball-mill procedure.

During cellulose extraction, samples of similar mass unexpectedly showed different amounts of loss. This serendipitously provided a means to estimate variations in yield created under our milling configurations. Here, we confirm the importance of particle size on the yield and rate of cellulose extraction.[24] The reduction in yield can result from both higher exposure (smaller particles have higher surface area) and direct loss through the PTFE filter bag used for cellulose extraction (porosity of 25 μm). The control samples milled with the ultra-centrifugal mill showed the highest remaining amount and least variability. This suggests that the ultra-centrifugal mill produces particles that are relatively large compared with those produced with the ball mill, but uniform.

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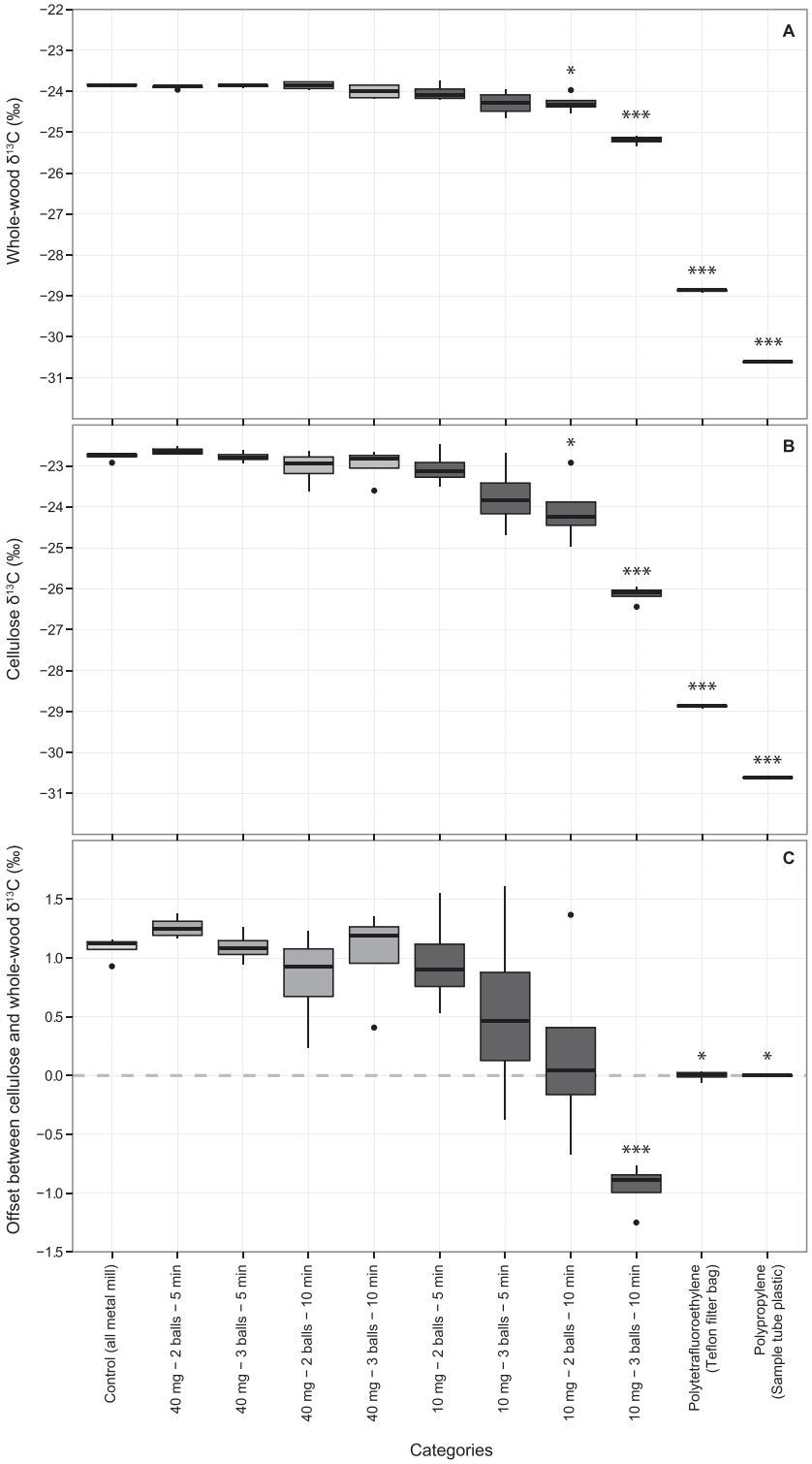

Figure 3. Boxplots of the distribution of $\delta^{13}C$ values of treatments, represented as ‰ deviations from VPDB. (A) $\delta^{13}C$ values from whole wood samples in each treatment category. (B) $\delta^{13}C$ values from cellulose samples in each treatment category. (C) $\delta^{13}C$ value offset between whole wood and cellulose in each treatment category. The inter-quartile range of the control samples, which were milled with an all-metal mill, is displayed in light grey. The inter-quartile range of samples milled with 40 mg is shown in darker grey. The inter-quartile range of 10-mg samples is shown in the darkest shade of grey. Each category represents four samples. Stars represent categories that are significantly different from the control (***$p < 0.001$, *$p < 0.05$).

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Table 3. Analysis of variance from the cellulose model

Source of variation	SS	MS	df	F-value	*p*-value
Mill	2.23	2.24	1	27.03	<0.001
Balls	4.00	4.00	1	48.26	<0.001
Time	8.01	8.01	1	96.71	<0.001
Amount	15.81	15.81	1	190.84	<0.001
Percentage remaining	5.07	5.07	1	61.24	<0.001
Interaction (Balls:Time:Amount)	8.29	2.07	4	25.01	<0.001
Residuals	2.15	0.08	26		

Reported values include the sum of squares (*SS*), the mean square (*MS*), degrees of freedom (*df*), the calculated test statistic (*F-value*) and the *p*-value (*p-value*). Residuals refer to variation that is not explained by the model.

Table 4. Analysis of variance for the whole wood model

Source of variation	SS	MS	df	F-value	*p*-value
Mill	0.38	0.39	1	14.00	<0.001
Balls	0.82	0.83	1	29.86	<0.001
Time	0.77	0.78	1	28.03	<0.001
Amount	2.35	2.35	1	85.16	<0.001
Carbon amount	0.06	0.06	1	2.15	0.155
Interaction (Balls:Time:Amount)	1.53	0.38	4	13.80	<0.001
Residuals	0.72	0.028	26		

The fixed effects are shown, and the amount of carbon present in the sample was specified as a random effect. Reported values include the sum of squares (*SS*), the mean square (*MS*), degrees of freedom (*df*), the calculated test statistic (*F-value*) and the *p*-value (*p-value*). Residuals refer to variation that is not explained by the model.

Contamination concentration due to cellulose extraction

Since the yield of plastic after cellulose extraction remained the same, plastic is not lost during extraction, in contrast to the non-cellulose components of wood which are lost. The concern, therefore, is that cellulose extraction can exacerbate the effects of plastic contamination by increasing the plastic-to-sample ratio. Since both plastics had more negative $\delta^{13}C$ values than the cellulose from the control samples used in this study, we would expect that any increase in the plastic-to-sample ratio would result in more negative $\delta^{13}C$ values than usual. This effect was not seen in the large (40 mg) samples because the well-known offset in $\delta^{13}C$ values of cellulose and whole wood[24] remained consistent among treatments. However, the plastic-contamination effect was visible in the samples milled with less material (10 mg). These samples showed a decreased whole-wood to cellulose offset, and even a reversal of the offset occurred in the category with the highest abrasion (10-mg–3-balls–10-min). This trend change provides evidence that smaller samples do incur plastic contamination and that the plastic-to-sample ratio increases after cellulose extraction.

Recommendations

A ball mill equipped with holders to mill wood samples in polypropylene sample tubes may be suitable for $\delta^{18}O$ analysis: no oxygen atoms are present in plastic, and, hence, we found no evidence that $\delta^{18}O$ values would be affected by plastic contamination. We also did not detect any evidence of fractionation from heating, which is in line with a previous experiment.[40]

Using a ball mill is not advisable for milling wood prior to $\delta^{13}C$ radiocarbon measurement. Changes in isotope values due to plastic contamination were most concerning for smaller amounts (10 mg). An increase in the plastic-to-sample ratio during cellulose extraction further contributes to erroneously low $\delta^{13}C$ values in these smaller samples. We cannot recommend the ball mill for milling larger sample amounts, either. Our results showed that $\delta^{13}C$ values in larger sample amounts (40 mg) in both whole wood and cellulose did not deviate significantly from the control, but there is potential for contamination if the samples are re-milled since the abrasion in sample tubes increases with milling time. In a follow-up test, milling very large sample amounts (40–90 mg) for 10 min within their test tubes was often ineffective, and re-milling for another 10 min did not improve the homogenization. However, with each successive milling treatment, the probability of sample-tube abrasion and isotope contamination increases, even if the risk had been initially low. We found similarly poor homogenization when milling samples with liquid. Accordingly, we cannot recommend milling with a ball mill, in direct disagreement with the recommendations made by a previous study.[40]

Possible alternatives

Several modifications to the current ball-mill configuration can reduce or eliminate the potential for plastic contamination. For example, the wood samples could be transferred into inert

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milling containers made of materials such as ceramic, metal, or possibly more abrasion-resistant PTFE sample tubes. Using these types of containers is, however, also a slow procedure since usually only two inert containers can be run simultaneously, compared with milling up to 16 sample tubes at once. Time-savings are also lost because the sample must be transferred into and out of the container and the milling containers must be carefully cleaned between samples. Nevertheless, the ball mill fitted with inert containers remains one of the most simple and least problematic (albeit slow) options for homogenizing wood prior to isotope measurements (Table 5).

The all-metal ultra-centrifugal mill remains a good option for homogenization, but it is time-consuming, and often much of the sample is lost (Table 5). Sample loss is primarily due to a loose fit of the mechanical mill pieces and the fact that it was designed for much larger sample volumes. We therefore tested a smaller version of the milling pieces specially designed by the manufacturer for smaller samples, described as a 'small-sample-converter-kit'. However, this small-sample-converter-kit did not solve the problem of sample loss because it also had loose-fitting parts. Such loss of sample is especially problematic when there is little sample material to begin with (<10 mg) and when cellulose extraction is done later.

If researchers choose to work with cellulose, they would be advised to use the ultrasonic homogenization method[25] (Table 5). This method is, however, also limited in that it can only homogenize cellulose samples of less than 10 mg. These cellulose samples must be soaked in 1 mL of deionized water for this process to work, and care must be taken to minimize and standardize the treatment length. Over-heating can otherwise occur, potentially increasing the exchange of oxygen atoms between the sample and water.[37] Ultrasonic homogenization of small samples of whole wood is not an option: We found no change after applying a full-power, 2-min ultrasonic treatment on two water-logged whole wood samples. Much more energy is apparently needed to break the lignin molecules than the cellulose chains.

In conclusion, there is a clear need for further development of simple, fast, effective and contamination-free methodologies to homogenize wood prior to isotope analysis. This would further facilitate tree-ring isotope research aimed at answering important biological, ecophysiological, and dendrochronological questions.

Table 5. Options for homogenizing wood and cellulose prior to stable- and radio-carbon isotope analyses

	Sample category				
	Cellulose		Whole wood		
Homogenization options	10 mg	40 mg	10 mg	40 mg	Notes
Ball mill, samples milled in plastic containers	–	–	–	–	• The ball mill cannot be recommended for homogenizing wood prior to carbon isotope analyses, but may for only oxygen isotope analyses. • While milling samples in water or ethanol may help reduce abrasion in some cases, it also reduces the efficacy of homogenization. It cannot be recommended as an alternative solution.
Ball mill, samples milled in inert containers	✔	✔	✔	✔	• All check-marks are black, because there is no risk of plastic contamination while using ceramic or metal containers. • This is a time-consuming option since samples must be moved from sample-tubes into the inert containers (usually max. of 2/run), the powder collected and returned into the sample tubes, and the containers must be cleaned. • Unlike with the ultra-centrifugal mill, wood does not need to be cut as finely prior to milling.
Ultrasonic homogenizer	✔	–	–	–	• Using the ultrasonic homogenizer may be easiest for small cellulose samples (<10 mg). • The ultrasonic homogenizer did not work on water-logged whole wood samples.
Ultra-centrifugal mill	✔	✔	✔	✔	• Although the milling is almost instantaneous, the process of collecting the powder across the mill surfaces and returning it to the sample tubes is slow. Cleaning all surfaces between samples takes approx. 7–10 min. • The check-marks for the small samples are grey because much sample is lost.

As seen in the *Notes* column, there is a trade-off between the speed of the procedure and the potential for plastic contamination.

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