

# Quantifying the impact of chemicals on stable carbon and oxygen isotope values of raw pollen

CAROLINA MÜLLER,<sup>1,2\*</sup> JULIAN HENNIG,<sup>1</sup> FRANK RIEDEL<sup>1</sup> and GERHARD HELLE<sup>1,2</sup>

<sup>1</sup>Institute of Geological Sciences, Palaeontology, Freie Universität Berlin, Berlin, Germany

<sup>2</sup>GFZ German Research Centre for Geoscience, Section 4.3 Climate Dynamics and Landscape Evolution, Potsdam, Germany

Received 6 March 2020; Revised 22 January 2021; Accepted 4 February 2021

**ABSTRACT:** Purification protocols to extract pollen from lake sediments contain chemicals that alter the carbon and oxygen pollen-isotope values according to pollen characteristics and family affiliation. Modern (raw) pollen of broad-leaved (*Alnus glutinosa*, *Betula pendula*, *Carpinus betulus*, *Corylus avellana*, *Fagus sylvatica* and *Quercus robur*) and coniferous tree species (*Picea abies* and *Pinus sylvestris*) were treated with potassium hydroxide (KOH), hydrofluoric acid (HF), sodium hypochlorite (NaClO) and sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) to test the impact on  $\delta^{13}\text{C}_{\text{pollen}}$  and  $\delta^{18}\text{O}_{\text{pollen}}$  and assess the applicability in purification protocols. Pollen of broad-leaved and coniferous trees reacted differently to chemical exposure, but response patterns are generally alike. Alterations of  $\delta^{13}\text{C}_{\text{pollen}}$  values vary between +1.0‰ (*B. pendula*, NaClO-treatment) and –5.0‰ (*P. sylvestris*, H<sub>2</sub>SO<sub>4</sub>-treatment). The  $\delta^{13}\text{C}_{\text{pollen}}$  values of raw and chemically treated samples seem to be related after treatments with KOH, NaClO and HF, whereas the application of H<sub>2</sub>SO<sub>4</sub> led to inconsistent changes among species. The impact of chemicals on  $\delta^{18}\text{O}_{\text{pollen}}$  are more diverse and offsets range between +1.1‰ (*C. avellana*, NaClO-treatment) and –17.9‰ (*P. sylvestris*, H<sub>2</sub>SO<sub>4</sub>-treatment). In general, the use of isotope-altering chemicals in purification protocols should be brought to a minimum, but the application of KOH and NaClO seems mostly unproblematic before  $\delta^{13}\text{C}_{\text{pollen}}$  and  $\delta^{18}\text{O}_{\text{pollen}}$  analysis.

© 2021 The Authors. *Journal of Quaternary Science* Published by John Wiley & Sons Ltd.

**KEYWORDS:** chemical treatment; pollen; pollen purification protocol; stable carbon and oxygen isotopes

## Introduction

Modern (raw) pollen can function as reference material to interpret fossil pollen-isotope values. The application of certain chemicals on raw pollen imitates a fossilization process to enhance comparability, but chemical substances are also used to purify fossil pollen from organic and inorganic remains of a sample. It is already known that chemical application alters the pollen-isotope values (Amundson *et al.*, 1997), but the single effect of certain chemicals on pollen-isotope values of different species with variable pollen characteristics are unknown. Before the approach of pollen-isotope analysis can be applied in palaeoclimate studies, the impact of chemical substances from purification protocols on the  $\delta^{13}\text{C}_{\text{pollen}}$  and  $\delta^{18}\text{O}_{\text{pollen}}$  values needs to be thoroughly investigated.

Raw pollen differ from fossilized pollen, because decomposition of structurally weak pollen wall components and pollen wall coatings leaves only the sporopollenin layer of fossil pollen intact (Loader and Hemming, 2000). Sporopollenin, the main component of a pollen wall, is a highly resistant biopolymer approximately consisting of C<sub>90</sub>H<sub>150</sub>O<sub>33</sub> (Brooks and Shaw, 1978; Loader and Hemming, 2004; Fraser *et al.*, 2014; Li *et al.*, 2019; Mikhael *et al.*, 2019) and the proportion of sporopollenin within raw pollen of different species ranges between 55 and 85% (Nelson, 2012). Additional to sporopollenin, the outer wall of raw pollen contains lipids, proteins and in some cases pollenkitt, whereas the inner pollen wall is composed of pectin, cellulose and hemicellulose (Fan *et al.*, 2019). The structure and composition of a pollen grain wall

varies highly with species (e.g. Moore *et al.*, 1991; Stanley and Linskens, 2012) and therefore also carbon and oxygen isotope values of raw pollen have species-specific patterns and ranges (e.g. Amundson *et al.*, 1997; Jahren, 2004; Loader and Hemming, 2004; King *et al.*, 2012; Nelson, 2012; Schwarz, 2016; Müller *et al.*, 2020). Chemicals applied on raw pollen change the composition of the pollen wall coating substance-dependently, but the degree of contamination of the sporopollenin layer is unknown. To prevent contamination of the  $\delta^{13}\text{C}_{\text{pollen}}$  during sporopollenin extraction, Loader and Hemming (2000) tested an acid digestion method using sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) to remove all organic components from raw pollen except sporopollenin. Stable and species-specific offsets of the  $\delta^{13}\text{C}_{\text{pollen}}$  from *Pinus sylvestris* (–2.18‰), *Zea mays* (–1.81‰) and *Populus trichocarpa* (–3.55‰) were detected and all species yielded strong linear correlations between raw and chemically treated pollen-isotope values, which indicates a consistent impact of the chemicals on the pollen-isotope values and thus implies a replicable outcome ( $r^2 = 0.93$ ; Loader and Hemming, 2000). When Descolas-Gros and Schölzel (2007) applied H<sub>2</sub>SO<sub>4</sub> on raw pollen to investigate reflections of the C3 and C4 photosynthetic pathway in  $\delta^{13}\text{C}_{\text{pollen}}$ , they detected offsets of –1.18‰ (*Sorghum vulgare*) and –0.91‰ (*Platanus* sp.). Another test of the method using H<sub>2</sub>SO<sub>4</sub> to extract sporopollenin revealed an average offset of –1.5‰ between raw and treated  $\delta^{13}\text{C}_{\text{pollen}}$  of *Cedrus atlantica* (Bell *et al.*, 2017). In general, carbon pollen-isotope values are depleted after treatments including sulphuric acid, but the impact seems highly species-specific.

Chemicals are traditionally used to extract and purify pollen from lake sediments for palynological studies (Faegri *et al.*, 1989; Nelson *et al.*, 2006; Griener *et al.*, 2013). These are

\*Correspondence: Carolina Müller, as above.

E-mail: carolina.mueller@fu-berlin.de

mainly: hydrochloric acid (HCl) to remove inorganic carbon particles, potassium hydroxide (KOH) to digest humic acids, hydrofluoric acid (HF) to dissolve siliciclastic components and acetolysis [glacial acetic acid ( $\text{CH}_3\text{COOH}$ ) used together with acetic anhydride ( $\text{C}_4\text{H}_6\text{O}_3$ ) and  $\text{H}_2\text{SO}_4$ ] to remove organic remains and stain the pollen wall. However, traditional protocols were not designed for pollen extraction before stable isotope analysis and can thus lead to carbon-isotopic contamination of the pollen grain wall (Amundson *et al.*, 1997). In particular, a protocol containing acetolysis needs to be avoided due to its major impact on the  $\delta^{13}\text{C}$  values (Amundson *et al.*, 1997; Descolas-Gros and Schölzel, 2007). Nelson *et al.* (2006) established a protocol to purify pollen for isotope analysis containing HCl, KOH, HF, NaClO and  $\text{H}_2\text{SO}_4$ . Applied on raw pollen of grass and shrub species, it led to depletions ranging between  $-1.2$  and  $-3.7\%$  in the  $\delta^{13}\text{C}_{\text{pollen}}$  (Nelson *et al.*, 2006; Nelson, 2012). Griener *et al.* (2013) used this protocol on raw and fossil *Nothofagus* pollen and detected an average depletion of  $-1.7\%$  within the  $\delta^{13}\text{C}_{\text{pollen}}$  and Jahren (2004) suggested a treatment including sodium hydroxide (NaOH), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and HF to do both: simulate the effect of diagenesis avoiding acetolysis and  $\text{H}_2\text{SO}_4$  and additionally to be used for the isolation of isotopically unaltered pollen. So far, only the isotope-altering effect of protocols with a sequence of chemicals have been tested on raw pollen, and the individual impacts of most of these chemicals are unknown.

Most recently, Bell *et al.* (2019) successfully used a dense-media separation technique to extract fossil pollen from lake sediment avoiding  $\text{H}_2\text{SO}_4$  and other carbon-isotope-altering chemicals. However, most pollen samples from lake, bog or peat archives still require chemical purification because they contain various types of pollen, other organic matter and clastic debris which complicate the mandatory solubilization of pollen before extraction for isotope analysis. In general, applying fewer chemicals lessens isotope alterations, but if a sample requires chemical treatment (e.g. due to a high amount of humic acids or due to calcareous contents), it may in some cases still be beneficial to use substances with a strong but known impact on  $\delta^{13}\text{C}_{\text{pollen}}$  values to prepare the sample sufficiently for isotope analysis (Jahren, 2004). Therefore, it is important to be aware of the specific impact of certain chemicals to compose or adjust individual protocols with minimal but sufficient use of chemicals before each study.

There are no comparable studies on  $\delta^{18}\text{O}_{\text{pollen}}$  after chemical treatment procedures solely with potassium hydroxide (KOH), hydrofluoric acid (HF) and sodium hypochlorite (NaClO) and there is little suggestion regarding a method to avoid oxygen-isotope alteration of the pollen wall components available. A published technique including ethanol dilution to separate pollen from honey for oxygen pollen-isotope analysis

revealed a large but consistent impact on the  $\delta^{18}\text{O}_{\text{pollen}}$  values (Chesson *et al.*, 2013).

To advance the approach of fossil pollen-isotope analysis in palaeoclimate reconstructions, we test the particular effect of four chemical substances (KOH, NaClO, HF and  $\text{H}_2\text{SO}_4$ ) and a successive treatment including all chemicals on the  $\delta^{13}\text{C}_{\text{pollen}}$  and  $\delta^{18}\text{O}_{\text{pollen}}$  of raw pollen from eight different tree species. Our aims were to assess species-specific alterations of pollen-isotope values after exposure to the different chemicals and to promote the compilation of a treatment protocol minimizing the impact on fossil pollen isotopes.

## Materials and methods

### Sample location, collection and preparation

Individual pollen samples contain pollen of several inflorescences from individual trees. Modern pollen of three individuals from each species (*Alnus glutinosa*, *Betula pendula*, *Corylus avellana*, *Fagus sylvatica*, *Picea abies*, *Pinus sylvestris* and *Quercus robur*; Table 1) were collected in March and May 2016 within their respective flowering period in Parc naturel Forêt d'Anlier (Belgium: 49.7899°N, 5.6829°E; average elevation: 385 m a.s.l.). Mean annual temperature at this location is 9.1 °C (mean temperature of the coldest month:  $-0.1$  °C; mean temperature of the warmest month: 16.1 °C). Mean annual precipitation amounts to 1019 mm (based on the high-resolution gridded dataset CRU TS at www.cru.uea.ac.uk/data). Pollen of three individual trees of *Carpinus betulus* (Table 1) were collected in May 2016 at Nationalpark Steigerwald (Germany: 49.8616°N, 10.5241°E; average elevation: 390 m a.s.l.; Mean annual temperature: 9.4 °C; mean temperature of the coldest month:  $-1.2$  °C; mean temperature of the warmest month: 17.5 °C; Mean annual precipitation: 581 mm).

The samples were kept at 6 °C in a refrigerator during fieldwork and dried afterwards in a drying oven with a maximum temperature of 45 °C for 7 days. Dry samples were kept frozen at  $-16$  °C until further processing. The pollen were separated from the rest of the flower tissue through rinsing with deionized water and sieving with mesh sizes of 10–200  $\mu\text{m}$ . Afterwards, the pollen were transferred to safe lock tubes, frozen again at  $-16$  °C and freeze-dried until fully dehydrated. Dry pollen samples were kept frozen for preservation.

### Chemical treatment procedure

Pollen samples of 24 individual trees from eight species were treated separately with potassium hydroxide (KOH 10%; 20 min, ca. 70 °C water bath), sulphuric acid ( $\text{H}_2\text{SO}_4$

**Table 1.** Overview of species, taxonomic classification and pollen characteristics (Beug, 2004) of eight tree species examined in this study including their common names and family affiliation.

Family	Species	Common name	Size of pollen ( $\mu\text{m}$ )	Shape of pollen
Betulaceae	<i>Alnus glutinosa</i> (L.) Gaertn.	Black alder	24–34	Stephanoporatae, pentagonal
	<i>Betula pendula</i> Roth	Silver birch	26–32	Triporatae, triangular
	<i>Carpinus betulus</i> (L.)	European hornbeam	38–48	Stephanoporatae, roundish
	<i>Corylus avellana</i> (L.)	Common hazel	28–33	Triporatae, triangular
Fagaceae	<i>Fagus sylvatica</i> (L.)	European beech	28–39	Tricolporatae, roundish
	<i>Quercus robur</i> (L.)	Pedunculata oak	31–40	Tricolpatae, oval
Pinaceae	<i>Picea abies</i> (L.) H. Karst.	Norway spruce	110–148	Vesiculatae
	<i>Pinus sylvestris</i> (L.)	Scots pine	62–84	Vesiculatae

**Table 2.** Details of the chemical treatment procedure used to prepare pollen for stable carbon and oxygen isotope analysis including the common names and purity of the chemicals, the specifications of each treatment, the time for each treatment and the effect on the pollen sample.

Chemical formula	Common name	Purity	Type of treatment	Duration	Effect
KOH	Potassium hydroxide	10%	Water bath at 70 °C	20 min	Digestion of humic acids
H <sub>2</sub> SO <sub>4</sub>	Sulphuric acid	96%	Constantly on a shaker	5 h	Extraction of sporopollenin
HF	Hydrofluoric acid	38%	Resting at room temperature	24 h	Degeneration of clastic debris
NaClO	Sodium hypochlorite	3%	Gentle stirring at room temperature	90 s	Dissolution of small organic matter

96%; 5 h on a shaker) and sodium hypochlorite (NaClO 3%; 90 s; Table 2). The impact of hydrofluoric acid (HF 38%; 24 h) was tested on pollen samples of nine individual trees from three species (*C. avellana*, *P. abies* and *P. sylvestris*; Fig. 1). Repeated and thorough rinsing of the pollen samples with deionized water followed each chemical treatment. To test combined effects of the chemicals, as it occurs following a standard protocol for fossil pollen sample preparation, pollen samples of three individual trees from each of the eight species (in total 24 samples) underwent the chemical treatments with KOH, HF, H<sub>2</sub>SO<sub>4</sub> and NaClO successively in this order and with the same length as the single treatments (successive treatment is termed *all*; Table 2). All samples were visually inspected after each chemical treatment using a Meiji MT4300L microscope at magnifications of 400× and 600× to ensure intactness of the pollen wall. Additionally, the cellulose content of the pollen wall after H<sub>2</sub>SO<sub>4</sub> exposure and the successive treatment (*all*) was determined with a staining technique using Safranin and Fast Green.

### Stable isotope and statistical analysis

In total, 220 µg ± 10% of pollen material was weighed directly into silver capsules (0.02 mL; 3.3 × 4 mm) using a high-precision scale (Mettler Toledo AX 26 Delta Range) at the dendrochronological laboratory, section 4.3, GFZ Potsdam. The carbon and oxygen isotope values were determined using a DELTA V isotope ratio mass spectrometer (IRMS; Thermo Fisher Scientific, Bremen, Germany). Each sample was weighed and measured with three repetitions and vacuum dried for at least 12 h in a Thermo Scientific Heraeus VT 6060 P at 100 °C before stable isotope measurement. The pollen material was reduced to CO for simultaneous IRMS analysis of carbon and oxygen isotope ratios in a High Temperature Conversion Elemental Analyzer (TC/EA; 1400 °C; Thermo Fisher Scientific) coupled to the IRMS. Isotope ratios are expressed relative to VPDB for δ<sup>13</sup>C and VSMOW for δ<sup>18</sup>O. The isotope values were compared against laboratory-internal and international reference material (IAEA-CH3, IAEA-CH6, and IAEA 601 and 602). For a single-point normalization, two reference standards with widespread isotopic compositions were used (Paul *et al.*, 2007).

During the chemical treatment procedures, four samples treated with NaClO and one sample of the successive treatment (*all*) were dissolved and could not be measured isotopically. Each sample was measured three times and in total, and we conducted 296 measurements of chemically treated pollen and 72 measurements of untreated (referred to as raw) pollen of the same individuals (Supporting Information Table S1). Raw and chemically treated pollen have been plotted to visualize the relationship. All calculations and the graphic depiction were made using the programs R (R Core Team, 2017) and RStudio (RStudio Team, 2015). The impact of species and type of chemical applied during treatment of the samples on the mean and between raw and chemically treated pollen was assessed by two-way analysis of variance (ANOVA) in R v4.0.2 (R Core Team, 2017; Table S2).

## Results

### δ<sup>13</sup>C<sub>pollen</sub> after chemical treatment

Mean δ<sup>13</sup>C<sub>pollen</sub> values are mostly depleted after chemical treatment procedures compared to raw pollen-isotope values (Fig. 1; Table 3A). The depletion of mean δ<sup>13</sup>C<sub>pollen</sub> values after KOH exposure ranges from −0.3‰ (*F. sylvatica*) to −4.0‰ (*C. avellana*). The HF treatment results in a depletion ranging between −0.4‰ (*P. abies*) and −1.4‰ (*C. avellana*) and the impact of sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) on mean δ<sup>13</sup>C<sub>pollen</sub> values results in a depletion ranging from −1.2‰ (*F. sylvatica*) to −5.0‰ (*P. sylvestris*). A successive treatment with all chemicals (*all*) has the highest impact on δ<sup>13</sup>C<sub>pollen</sub> values compared to the untreated pollen material. The depletions range between −1.6‰ (*F. sylvatica*) and −6.4‰ (*P. sylvestris*).

Enriched mean δ<sup>13</sup>C<sub>pollen</sub> values occur only after NaClO treatment for *B. pendula* (+1.0‰) and *Carpinus betulus* (+0.8‰). Pollen-isotope values of other species are depleted after exposure to NaClO and range between −0.3‰ (*Pinus sylvestris*) and −1.4‰ (*Corylus avellana*). There was no effect on *Picea abies* pollen measurable for NaClO (Fig. 1; Table 3A).

### δ<sup>18</sup>O<sub>pollen</sub> after chemical treatment

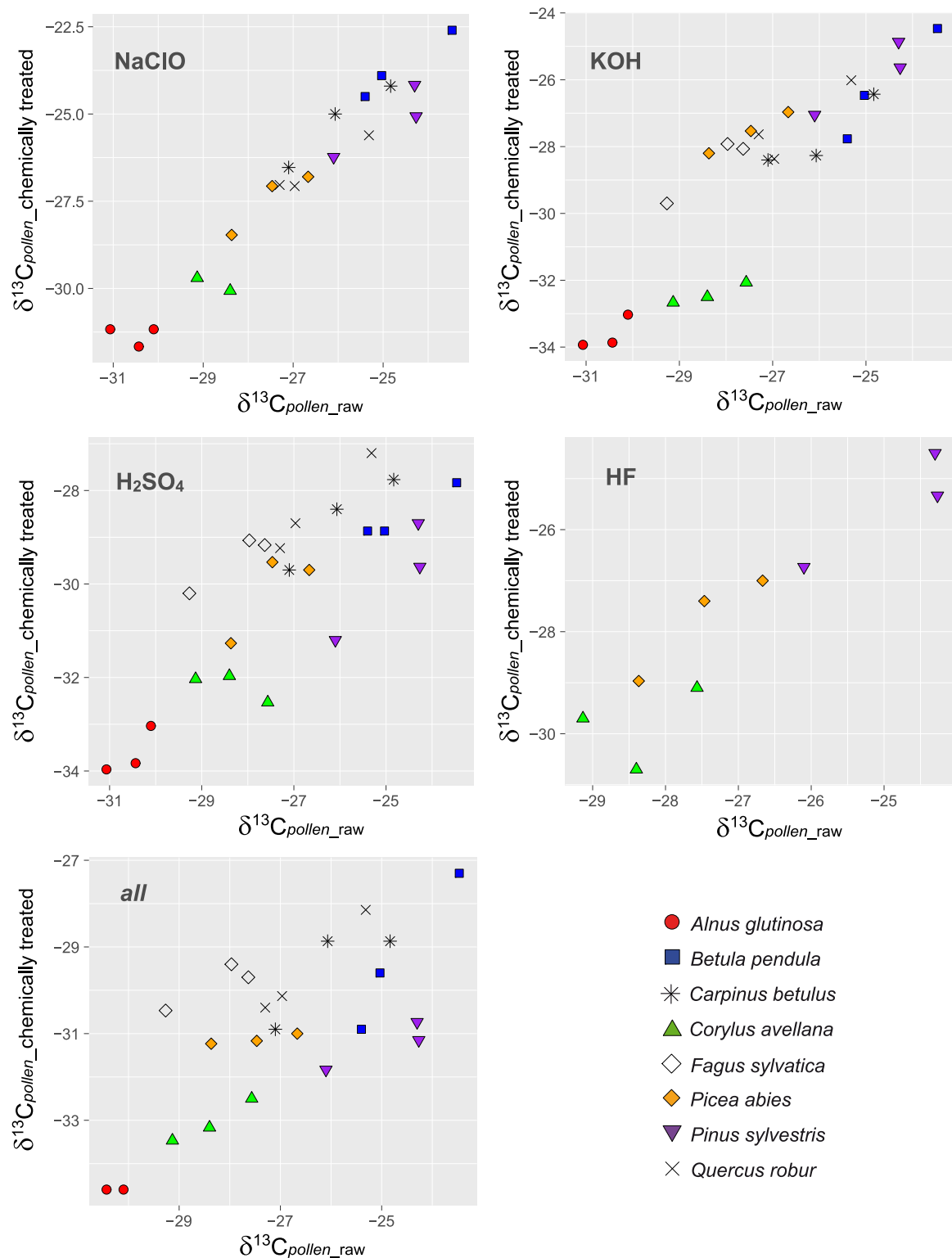
Mean δ<sup>18</sup>O<sub>pollen</sub> values of *B. pendula* (+0.1‰) and *Q. robur* (+1.0‰) are enriched after exposure to KOH, whereas values of all other species are depleted in a range of −0.3‰ (*Picea abies*) to −2.6‰ (*A. glutinosa*; Fig. 2; Table 3B). The HF treatment leads for *C. avellana* to enriched pollen-isotope values of +0.5‰, whereas the values of *P. abies* (−1.8‰) and *P. sylvestris* (−1.7‰) are depleted.

All broad-leaved species have enriched δ<sup>18</sup>O<sub>pollen</sub> values after the treatment with NaClO. The enrichment ranges between +1.1‰ (*C. avellana*) and +3.1‰ (*B. pendula*). NaClO had no measurable effect on *A. glutinosa* δ<sup>18</sup>O<sub>pollen</sub> values (Fig. 2; Table 3B). Coniferous δ<sup>18</sup>O<sub>pollen</sub> values (*P. abies*: −1.7‰; *P. sylvestris*: −0.5‰) are depleted as a result of the NaClO treatment.

The H<sub>2</sub>SO<sub>4</sub>-treatment has distinct effects on δ<sup>18</sup>O<sub>pollen</sub>. Mean pollen-isotope values of broad-leaved species are depleted in a range of −0.7‰ (*A. glutinosa*) to −5.8‰ (*C. betulus*), whereas coniferous δ<sup>18</sup>O<sub>pollen</sub> values are depleted by −9.7‰ (*P. abies*) and −17.9‰ (*P. sylvestris*), respectively. The depletion of mean δ<sup>18</sup>O<sub>pollen</sub> values after the successive treatment has a broad range between −3.7‰ (*A. glutinosa*) and −19.2‰ (*P. sylvestris*; Table 3B).

### Statistical analysis

Two-way ANOVA indicated significant impacts of species, type of chemical applied during treatment of the samples and their interaction term on the mean differences in and between raw and chemically treated pollen. However, due to the small number of samples, these results should be treated with caution (Supporting Information Table S2).



**Figure 1.** Scatter plots visualizing the relationship between raw and chemically treated  $\delta^{13}\text{C}_{\text{pollen}}$  values (expressed in ‰, relative to VPDB) of eight tree species (*Alnus glutinosa*, *Betula pendula*, *Carpinus betulus*, *Fagus sylvatica*, *Picea abies*, *Pinus sylvestris* and *Quercus robur*) after treatment with KOH (24 individuals, eight species), HF (nine individuals, three species),  $\text{H}_2\text{SO}_4$  (24 individuals, eight species) and the successive treatment *all* (23 individuals, eight species). [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)].

## Discussion

### $\delta^{13}\text{C}_{\text{pollen}}$ after chemical treatments

Chemical treatment alters the stable carbon isotope values of pollen and the amount of the deviation is species- and substance-dependent (Amundson *et al.*, 1997; Loader and Hemming, 2000; Jahren, 2004; Nelson *et al.*, 2006). However,  $\delta^{13}\text{C}_{\text{pollen}}$  values are mostly depleted compared to untreated

pollen material (Fig. 1; Table 3A). Even though the individual deviation between raw and treated pollen is species-specific for  $\delta^{13}\text{C}_{\text{pollen}}$ , the overall response patterns to each chemical reveal similarities among the species (Fig. 1), which have also been detected for other species (Loader and Hemming, 2000; Jahren, 2004). We assume that the species-specific pollen wall structure and coating as well as pollen shape and pollen size are factors that may determine the impact.

**Table 3.** Mean  $\delta^{13}\text{C}_{\text{pollen}}$  values (3A, expressed in ‰, relative to VPDB) and  $\delta^{18}\text{O}_{\text{pollen}}$  values (3B, expressed in ‰, relative to VSMOW) of the chemically untreated (raw) pollen of eight species and first standard deviation.  $\delta^{13}\text{C}_{\text{dif.}}$  and  $\delta^{18}\text{O}_{\text{dif.}}$  indicate depletion or enrichment of the isotope values after chemical treatment compared to the untreated (raw) pollen-isotope values and the first standard deviation. Chemicals used for treatment are KOH, HF, NaClO, H<sub>2</sub>SO<sub>4</sub> and *all* (successive treatment procedure following the protocol of four chemicals applied consecutively; see text). Dev. H<sub>2</sub>SO<sub>4</sub>/*all* refers to the difference between values after treatment solely with H<sub>2</sub>SO<sub>4</sub> and after the successive treatment (*all*).

(A)							
Plant species	$\delta^{13}\text{C}$ Raw	$\delta^{13}\text{C}_{\text{dif.}}$ KOH	$\delta^{13}\text{C}_{\text{dif.}}$ HF	$\delta^{13}\text{C}_{\text{dif.}}$ NaClO	$\delta^{13}\text{C}_{\text{dif.}}$ H <sub>2</sub> SO <sub>4</sub>	$\delta^{13}\text{C}_{\text{dif.}}$ <i>all</i>	Dev. $\delta^{13}\text{C}$ : H <sub>2</sub> SO <sub>4</sub> / <i>all</i>
<i>Alnus glutinosa</i>	-30.6 ± 0.3	-3.0 ± 1.4	-	-0.7 ± 0.2	-3.0 ± 0.7	-4.0 ± 0.4	1.0
<i>Betula pendula</i>	-24.6 ± 0.1	-1.6 ± 0.2	-	+1.0 ± 0.1	-3.9 ± 0.3	-4.6 ± 0.2	0.7
<i>Carpinus betulus</i>	-26.0 ± 0.1	-1.7 ± 0.3	-	+0.8 ± 0.2	-2.6 ± 0.3	-3.5 ± 0.3	0.9
<i>Corylus avellana</i>	-28.4 ± 0.3	-4.0 ± 0.2	-1.4 ± 0.3	-1.2 ± 0.1	-3.7 ± 0.7	-4.6 ± 0.1	0.9
<i>Fagus sylvatica</i>	-28.2 ± 0.1	-0.3 ± 0.2	-	-	-1.2 ± 0.5	-1.6 ± 0.1	0.4
<i>Quercus robur</i>	-26.1 ± 0.3	-1.1 ± 0.6	-	-0.4 ± 0.2	-2.3 ± 0.3	-3.4 ± 0.2	1.1
<i>Picea abies</i>	-27.4 ± 0.3	-0.1 ± 0.2	-0.4 ± 0.2	0.0 ± 0.2	-2.7 ± 0.3	-3.7 ± 0.3	1.0
<i>Pinus sylvestris</i>	-24.8 ± 0.3	-1.0 ± 0.2	-0.7 ± 0.2	-0.3 ± 0.1	-5.0 ± 0.3	-6.4 ± 0.2	1.4
(B)							
Plant species	$\delta^{18}\text{O}$ Raw	$\delta^{18}\text{O}_{\text{dif.}}$ KOH	$\delta^{18}\text{O}_{\text{dif.}}$ HF	$\delta^{18}\text{O}_{\text{dif.}}$ NaClO	$\delta^{18}\text{O}_{\text{dif.}}$ H <sub>2</sub> SO <sub>4</sub>	$\delta^{18}\text{O}_{\text{dif.}}$ <i>all</i>	Dev. $\delta^{18}\text{O}$ : H <sub>2</sub> SO <sub>4</sub> / <i>all</i>
<i>Alnus glutinosa</i>	21.7 ± 0.5	-2.6 ± 1.8	-	0.0 ± 0.5	-0.7 ± 0.6	-3.7 ± 0.8	3.0
<i>Betula pendula</i>	24.8 ± 0.2	+0.1 ± 0.2	-	+3.1 ± 0.1	-4.5 ± 0.4	-7.9 ± 0.2	3.4
<i>Carpinus betulus</i>	26.4 ± 0.2	-1.8 ± 0.3	-	+1.5 ± 0.1	-5.8 ± 0.1	-9.2 ± 0.2	3.4
<i>Corylus avellana</i>	21.5 ± 0.4	-0.9 ± 0.3	+0.5 ± 0.3	+1.1 ± 0.4	-0.8 ± 0.3	-5.5 ± 0.2	4.7
<i>Fagus sylvatica</i>	23.3 ± 0.2	-1.1 ± 0.2	-	-	-1.8 ± 0.1	-6.2 ± 0.4	4.4
<i>Quercus robur</i>	27.4 ± 1.0	+1.0 ± 0.7	-	+2.3 ± 0.5	-4.7 ± 0.3	-9.1 ± 0.3	4.4
<i>Picea abies</i>	24.5 ± 0.8	-0.3 ± 0.5	-1.8 ± 0.8	-1.7 ± 0.2	-9.7 ± 0.8	-10.6 ± 1.1	0.9
<i>Pinus sylvestris</i>	30.1 ± 0.5	-2.9 ± 0.2	-1.7 ± 0.5	-0.5 ± 0.2	-17.8 ± 0.5	-19.1 ± 0.6	1.3

### Impact of KOH, HF and NaClO on $\delta^{13}\text{C}_{\text{pollen}}$

Even though the mean depletion of carbon isotope values after KOH exposure varies between -0.3‰ (*F. sylvatica*) and -4.0‰ (*C. avellana*), species react in a similar fashion and a relationship between raw and treated  $\delta^{13}\text{C}_{\text{pollen}}$  values can be assumed (Fig. 1). Inter-specific differences in the  $\delta^{13}\text{C}_{\text{pollen}}$  offset after chemical treatment seem to be influenced by the shape and size of the pollen, which differ between the respective family affiliation (Tables 1 and 3A), as well as the differences within the species-specific amount of sporopollenin and pollen wall coating of raw pollen. In general, KOH has a greater impact on species of the family Betulaceae (*A. glutinosa*, *C. betulus*, *C. avellana* and *B. pendula*). Pollen values of Betulaceae species are on average -2.6‰ depleted, whereas the average difference of all other species (*F. sylvatica*, *P. abies*, *P. sylvestris* and *Q. robur*) is -0.5‰ (Fig. 1; Table 3A). Potassium hydroxide does not contain carbon and should thus not affect the  $\delta^{13}\text{C}$  of sporopollenin. However, it may alter the structurally weaker inner pollen wall (Fan *et al.*, 2019) and attack the coating of raw pollen from species of the family Betulaceae more severely compared to other species, because Betulaceae are known to contain more derivatives of humic acids (Moore *et al.*, 1991). Thus, already decomposed fossil pollen may react differently or significantly less to KOH exposure in a protocol applied to purify pollen for palaeoclimate analysis, but we did not test the direct impact of KOH on fossilized pollen.

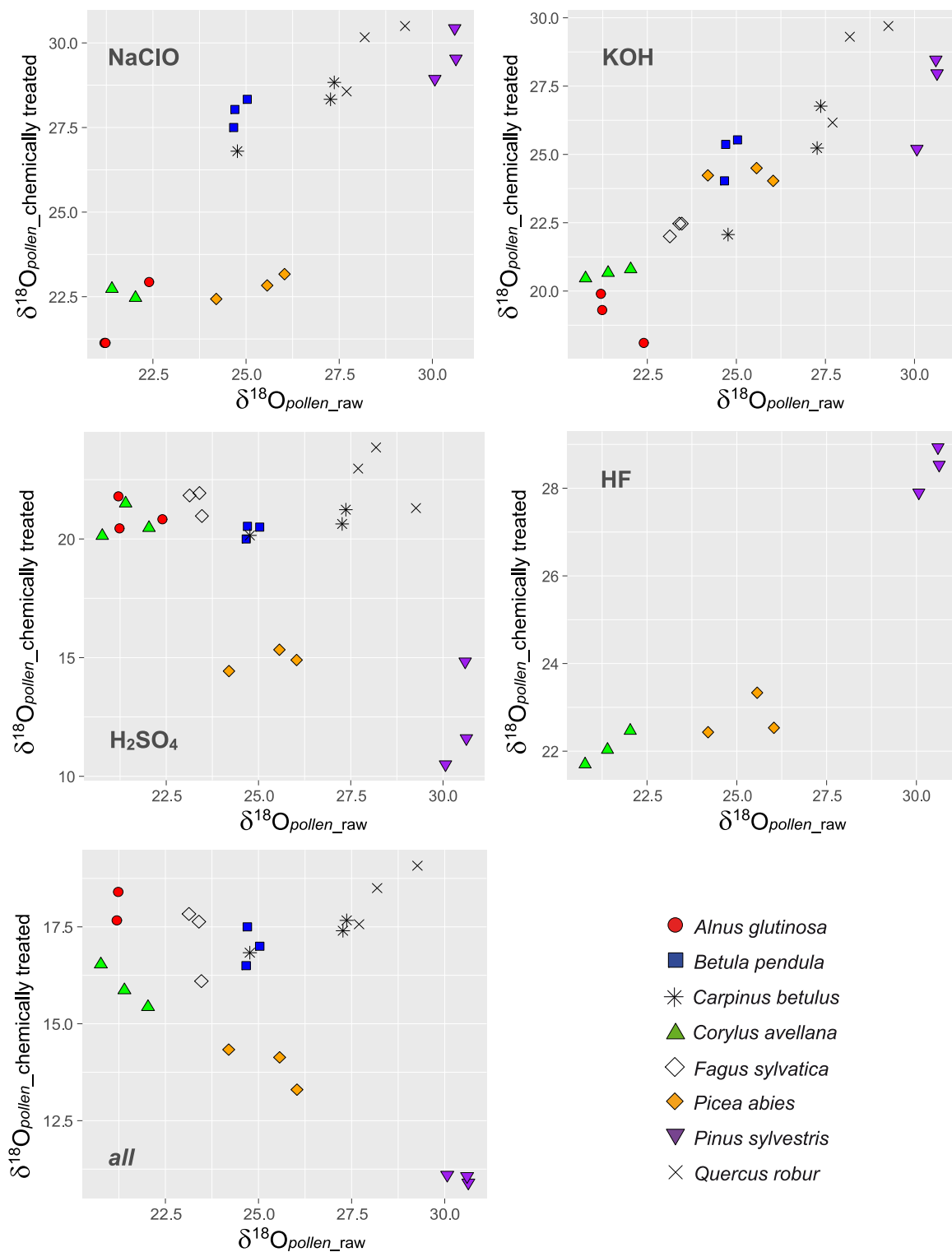
Hydrofluoric acid (HF) is widely used to extract pollen from lake sediments (Bennett and Willis, 2002). Even though it has only been tested in protocols containing several chemicals (Jahren, 2004; Nelson *et al.*, 2006), HF is suspected to have generally little influence on the organic structure of pollen (Jahren, 2004). The impact of HF alone on  $\delta^{13}\text{C}_{\text{pollen}}$  was smallest in comparison to other chemical substances applied in this study, ranging between -0.4‰ (*P. abies*) and -1.4‰

(*C. avellana*). Due to the small number of tested species, a relationship between raw and treated  $\delta^{13}\text{C}_{\text{pollen}}$  values can neither be confirmed nor denied (Fig. 1). The impact of NaClO on  $\delta^{13}\text{C}_{\text{pollen}}$  varies between +1.0‰ (*B. pendula*) and -1.4‰ (*C. avellana*) but the overall response is similar for most species and the carbon isotope values of raw and treated pollen seem linearly related (Fig. 1; Table 3A).

### Impact of H<sub>2</sub>SO<sub>4</sub> on $\delta^{13}\text{C}_{\text{pollen}}$

Average depletion of  $\delta^{13}\text{C}_{\text{pollen}}$  values after treatment with H<sub>2</sub>SO<sub>4</sub> is -3.2‰, but the isotopic shift varies highly among species and differ between pollen characteristics (Fig. 1; Tables 1 and 3A). A species-specific level of depletion for the  $\delta^{13}\text{C}_{\text{pollen}}$  values after H<sub>2</sub>SO<sub>4</sub> treatment has already been shown in other studies (Loader and Hemming, 2000; Nelson *et al.*, 2006; Descolas-Gros and Schölzel, 2007; Nelson, 2012).  $\delta^{13}\text{C}_{\text{pollen}}$  values of herbaceous plants and broad-leaved trees are on average -2.2‰ depleted, ranging between -0.15‰ (pollen of the family Asteraceae; Nelson, 2012) and -3.7‰ (grass pollen, Nelson *et al.*, 2006). That is in accordance with the depletion of isotope values of broad-leaved trees detected in this study, which averages -2.8‰ after H<sub>2</sub>SO<sub>4</sub> exposure (Table 3A).

In our study the impact of H<sub>2</sub>SO<sub>4</sub> on  $\delta^{13}\text{C}_{\text{pollen}}$  of the coniferous tree species *P. sylvestris* is distinct and the carbon isotope values are on average -5.0‰ depleted (Fig. 1; Table 3A). Loader and Hemming (2000) detected an average depletion of -2.18‰ for *P. sylvestris*  $\delta^{13}\text{C}_{\text{pollen}}$  after treatment with H<sub>2</sub>SO<sub>4</sub> and the comparison of chemically treated and raw pollen in their study gave a high correlation coefficient of  $r^2 = 0.93$ . A methodological reason for the different offsets between raw and chemically treated  $\delta^{13}\text{C}_{\text{pollen}}$  values of *P. sylvestris* among the studies might be the usage of a Whatman microcentrifuge tube with a mesh to extract the pollen from the sulphuric acid by Loader and Hemming (2000). In our



**Figure 2.** Scatter plots visualizing the relationship between raw and chemically treated  $\delta^{18}\text{O}_{\text{pollen}}$  values (expressed in ‰, relative to VSMOW) of eight tree species (*Alnus glutinosa*, *Betula pendula*, *Carpinus betulus*, *Fagus sylvatica*, *Picea abies*, *Pinus sylvestris* and *Quercus robur*) after treatment with KOH (24 individuals, eight species), HF (nine individuals, three species), NaClO (20 individuals, seven species),  $\text{H}_2\text{SO}_4$  (24 individuals, eight species) and the successive treatment *all* (23 individuals, eight species). [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.com)].

study, we slowly diluted the acid with deionized water until the velocity of the sulphuric acid was low enough to extract the pollen via centrifugation. The dilution had a warming effect on the liquid, whose impact on the  $\delta^{13}\text{C}_{\text{pollen}}$  values remain unknown. A similar method of slowly diluting the sulphuric acid was used by Bell *et al.* (2017), where a constant and low depletion of  $-1.5\text{‰}$  could be found for coniferous *Cedrus atlantica* pollen but no impact of a warming effect was reported.

Loader and Hemming (2000) tested the impact of the treatment time of  $\text{H}_2\text{SO}_4$  on *P. sylvestris* pollen and had most stable results after a treatment duration of 8 h. They suggested that the treatment time should not be  $<30$  min, otherwise cellulose residues remained on the pollen. However, a longer treatment resulted in partly degraded pollen grains (Loader and Hemming, 2000). Based on these findings, we settled on a treatment time of 5 h and additional constant shaking (Table 2). After  $\text{H}_2\text{SO}_4$  exposure, all samples were visually inspected and

no signs of decomposition were detectable. Also, cellulose residues on the pollen could not be detected after staining the pollen with Safranin and Fast Green (after Loader and Hemming, 2000). Therefore, we can exclude remaining residues on the pollen as causing the high offset of  $-5\%$ .

Descolas-Gros and Schölzel (2007) applied  $H_2SO_4$  for 8 h on pollen of different species and measured lower species-specific offsets between  $-0.91$  and  $-1.18\%$ . Bell *et al.* (2017) treated *Cedrus atlantica* pollen with  $H_2SO_4$  only for 45 min without additional stirring or heating, but verified the results of cellulose-free pollen grains using a staining technique (after Loader and Hemming, 2000), and Nelson *et al.* (2006) applied the sulphuric acid for 2 h on grass pollen grains, which resulted in constant offsets between raw and chemically treated pollen. All treatment durations entailed reliable results for different species. The reason for exceptionally high depletion of the *P. sylvestris*  $\delta^{13}C_{pollen}$  values after  $H_2SO_4$  exposure in this study requires further investigation.

Fossil pollen retrieved from lake sediments are at least partly decomposed and the remaining part of the pollen wall is mostly sporopollenin. Thus, the  $\delta^{13}C_{pollen}$  values of fossil pollen are believed to be isotopically closer to the values of raw pollen treated with sulphuric acid (Table 3A). However, to enhance the comparability of raw and fossil pollen, a treatment protocol imitating the diagenetic processes without using sulphuric acid may be beneficial (Jahren, 2004).

### Impact of successive chemical treatment (all) on $\delta^{13}C_{pollen}$

The successive treatment protocol imitates the chemical exposure that a fossil pollen sample might need to be fully cleared of other organic and inorganic debris. For all species, a successive application of KOH, HF, NaClO and  $H_2SO_4$  caused the highest depletion in  $\delta^{13}C_{pollen}$  (between  $-1.6\%$  for *F. sylvatica* and  $-6.4\%$  for *P. sylvestris*). Even though this protocol avoids carbon-contaminating chemicals, the overall mean depletion of chemically treated  $\delta^{13}C_{pollen}$  in comparison to raw  $\delta^{13}C_{pollen}$  is  $-4.0\%$  (Fig. 1).

In most cases, the successive treatment with four chemicals alters the samples only slightly more than sole treatment with  $H_2SO_4$  (Table 3A). The average additional depletion of  $\delta^{13}C_{pollen}$  for the successive treatment is  $0.9\%$  (range:  $0.4$ – $1.4\%$ ) and the deviation between  $\delta^{13}C_{pollen}$  values after applying the full protocol and the samples treated solely with sulphuric acid seems to be related to pollen morphology and family affiliation (Tables 1 and 3A). Thus, application of the full protocol may only in some cases be beneficial to purify samples for certain types of pollen designated for stable isotope analysis.

### $\delta^{18}O_{pollen}$ values after chemical treatment

Chemical treatment affects the  $\delta^{18}O_{pollen}$  of all species markedly (Fig. 2; Table 3B). The chemical treatment protocols to purify pollen from lake sediments were created to avoid chemical contamination of the  $\delta^{13}C_{pollen}$  and was not adjusted for the application to analyse stable oxygen pollen isotopes. Hence, the application of chemicals results in some cases in strongly depleted pollen-isotope values compared to the raw pollen isotopes (Fig. 2; Table 3B).

### Impact of KOH, HF and NaClO on $\delta^{18}O_{pollen}$

There are no comparable studies on  $\delta^{18}O_{pollen}$  after chemical treatment procedures solely with potassium hydroxide (KOH), hydrofluoric acid (HF) and sodium hypochlorite (NaClO)

available at present. Mean  $\delta^{18}O_{pollen}$  values of *B. pendula* ( $+0.1\%$ ) and *Q. robur* ( $+1.0\%$ ) are enriched after exposure to KOH, whereas the isotope values of all other species are depleted in a range of  $-0.3\%$  (*P. abies*) to  $-2.6\%$  (*A. glutinosa*; Fig. 2; Table 3B). Even though the impact of KOH on  $\delta^{18}O_{pollen}$  of different species is inconsistent, isotope values of raw and treated pollen seem to be related, especially when broad-leaved and coniferous species are looked at separately. Coniferous pollen have relatively thin pollen wall structures in relation to the grain size and thus may be more vulnerable to chemical treatment. In general, species-specific pollen membrane porosity needs to be considered when chemical substances are applied before stable isotope analysis (Loader and Hemming, 2000).

Treatment with hydrofluoric acid for the broad-leaved species *C. avellana* leads to enriched mean  $\delta^{18}O_{pollen}$  values of  $+0.5\%$ , whereas mean  $\delta^{18}O_{pollen}$  values of coniferous species are depleted, both in a similar fashion (*P. abies*:  $-1.8\%$ ; *P. sylvestris*:  $-1.7\%$ ; Table 3B). HF is the only non-oxygen-bearing chemical in this protocol, but nonetheless some diagenetic processes affecting the pollen wall occur during chemical exposure (Fig. 2).

Broad-leaved species have enriched  $\delta^{18}O_{pollen}$  values ranging between  $+1.1\%$  (*C. avellana*) and  $+3.1\%$  (*B. pendula*) following treatment with NaClO (Fig. 2; Table 3B), whereas the values of coniferous species are depleted as a result of NaClO exposure (*P. abies*:  $-1.7\%$ ; *P. sylvestris*:  $-0.5\%$ ; Table 3B). What exactly causes the different impact on pollen isotopes remains unknown.

### Impact of $H_2SO_4$ on $\delta^{18}O_{pollen}$

Raw and chemically treated mean  $\delta^{18}O_{pollen}$  values of coniferous species deviate strongly after  $H_2SO_4$  exposure (Fig. 2; Table 3B). In particular, *P. sylvestris* responses to  $H_2SO_4$  treatment are depleted ( $-17.9\%$ ) and also mean  $\delta^{18}O_{pollen}$  values of *P. abies* are highly depleted ( $-9.7\%$ ). Broad-leaved species exhibit a distinctly weaker response to  $H_2SO_4$  exposure. The average depletion of treated  $\delta^{18}O_{pollen}$  values compared to raw  $\delta^{18}O_{pollen}$  values of broad-leaved species is  $-3.1\%$ . The offset varies markedly between all broad-leaved species, and raw and treated  $\delta^{18}O_{pollen}$  values do not seem to be related (Fig. 2).

A strong response of coniferous pollen to chemical exposure may be caused by the size of the pollen grains and a relatively thin pollen wall, thus having a larger surface area on which chemicals can corrode the organic material. This applies in milder form also to *C. betulus* and *Q. robur* pollen (Fig. 2), which have the biggest pollen among the broad-leaved species examined in this study and a relatively thin pollen wall. However, the effect does not seem to be consistent: *A. glutinosa*, *B. pendula* and *C. avellana* are almost similar in size and shape (Beug, 2004), but the treatment affects *B. pendula* pollen more than *A. glutinosa* and *C. avellana* pollen (Fig. 2; Table 3B).

### Impact of successive chemical treatment (all) on $\delta^{18}O_{pollen}$

Depletion of mean  $\delta^{18}O_{pollen}$  values of broad-leaved species after successive treatment with all chemicals ranges between  $-3.7\%$  (*A. glutinosa*) and  $-9.1\%$  (*Q. robur*), whereas coniferous species pollen-isotopes are depleted by  $-10.6\%$  (*P. abies*) and  $-19.2\%$  (*P. sylvestris*, Table 3B). The successive treatment alters the  $\delta^{18}O_{pollen}$  values of broad-leaved trees on average  $3.9\%$  more than a treatment solely with  $H_2SO_4$ . However, the average additional depletion of coniferous species  $\delta^{18}O_{pollen}$  is only  $1.1\%$  (Table 3B). Thus, applying

the full protocol may have an assessable effect on some species, whereas others are severely altered. Applying chemicals before stable isotope analysis should generally be considered carefully, depending on the type of pollen and necessities based on the original fossil sample.

### $\delta^{13}\text{C}_{\text{pollen}}$ in relation to $\delta^{18}\text{O}_{\text{pollen}}$

Species-specific response patterns are generally alike, also when carbon and oxygen pollen-isotope patterns are compared (Figs 1 and 2). Even though the overall offset is higher for chemically treated  $\delta^{18}\text{O}_{\text{pollen}}$  values, the changes for each species among the different chemicals are almost similar. This may be caused by the species-specific pollen wall composition (proportion of sporopollenin) and shape of the pollen grain as well as the species-specific pollen wall coating (Table 1). The chemicals can alter the pollen isotopes only to a certain degree in relation to a species-specific pollen structure and vulnerability. Nevertheless, *A. glutinosa* and *C. avellana* show differing patterns for  $\delta^{18}\text{O}_{\text{pollen}}$  and  $\delta^{13}\text{C}_{\text{pollen}}$  after chemical treatment (Figs 1 and 2). This might be related to their plant-specific time of pollination from January to March, which is why these species have generally low baseline values of raw pollen isotopes (Müller *et al.*, 2020).

## Conclusions

Potassium hydroxide (KOH), hydrofluoric acid (HF) and sodium hypochlorite (NaClO) alter the  $\delta^{13}\text{C}_{\text{pollen}}$  and  $\delta^{18}\text{O}_{\text{pollen}}$  values of raw pollen species-specifically, according to their affiliation and pollen characteristics. The alterations are possibly caused by the removal of non-sporopollenin components of the pollen wall, whose composition and amount differ among species. However, the raw and chemically treated pollen isotope values seem to be related and thus the use of these chemicals to purify pollen samples before carbon and oxygen stable isotope analysis may generally be unproblematic. Care should be taken during the application of KOH on coniferous species before  $\delta^{18}\text{O}_{\text{pollen}}$  analysis and the application of HF on pollen of broad-leaved species.

The impact of sulphuric acid ( $\text{H}_2\text{SO}_4$ ) is highly species-specific and the offset between raw and chemically treated pollen-isotope values can be as high as 5.0‰ for  $\delta^{13}\text{C}_{\text{pollen}}$  and 17.9‰ for  $\delta^{18}\text{O}_{\text{pollen}}$  (both from *Pinus sylvestris*).  $\text{H}_2\text{SO}_4$ -treated and raw  $\delta^{13}\text{C}_{\text{pollen}}$  values of broad-leaved species seem to be related, whereas pollen of coniferous species react more strongly to  $\text{H}_2\text{SO}_4$  exposure and the impact seems unpredictable. Raw and  $\text{H}_2\text{SO}_4$ -treated  $\delta^{18}\text{O}_{\text{pollen}}$  values are not related for either broad-leaved species or coniferous species. Even though  $\text{H}_2\text{SO}_4$  is applied to extract sporopollenin while leaving it intact, stronger unknown alterations seem to occur.

In many cases, chemicals are needed to prepare fossil pollen samples before palaeoclimate investigations. Purification protocols for pollen should be adjusted due to the requirements of the source material, so the use of pollen-isotope-altering substances can be minimized. In general, protocols to purify fossil pollen samples and protocols to extract sporopollenin from modern pollen should avoid the use of sulphuric acid.

## Supporting information

Additional supporting information can be found in the online version of this article.

**Table S1.** Dataset of  $\delta^{13}\text{C}_{\text{pollen}}$  and  $\delta^{18}\text{O}_{\text{pollen}}$  measurements of raw and chemically treated pollen. Three individual trees of

the same species were treated with KOH, HF,  $\text{H}_2\text{SO}_4$ , NaClO and a treatment protocol (*all*) including all four chemical substances. The samples were measured with up to three repetitions.

**Table S2.** Results of a two-way analysis of variance (ANOVA) showing the differences in  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  between raw and chemically treated pollen among the plant species and type of chemical applied. The ANOVA indicated significant impacts of species, type of chemical and the interaction of both factors on the mean differences in  $\delta^{13}\text{C}$  and in  $\delta^{18}\text{O}$  between raw and chemically treated pollen (DF = degrees of freedom, Sum Sq = sum of squares, Mean Sq = mean square).

**Acknowledgements.** We are grateful to Maïke Glos (Freie Universität Berlin) who helped with sample preparation in the laboratory (Freie Universität Berlin) and Michèle Dinies (Deutsches Archäologisches Institut, Berlin) and Dr. Kai Hartmann (Freie Universität Berlin) for discussion, support and consultations concerning laboratory methods and statistical analysis. This work was financially supported by the Deutsche Forschungsgesellschaft (DFG) under grant no. RI 809/33-1. Open access funding enabled and organized by Projekt DEAL.

## Data availability statement

The data that support the findings of this study are available in the Supporting Information.

**Abbreviation.** IRMS, isotope ratio mass spectrometer.

## References

- Amundson R, Evett RR, Jahren AH *et al.* 1997. Stable carbon isotope composition of Poaceae pollen and its potential in paleovegetational reconstructions. *Review of Palaeobotany and Palynology* **99**: 17–24.
- Bell BA, Fletcher WJ, Cornelissen HL *et al.* 2019. Stable carbon isotope analysis on fossil *Cedrus* pollen shows summer aridification in Morocco during the last 5000 years. *Journal of Quaternary Science* **34**: 323–332.
- Bell BA, Fletcher WJ, Ryan P *et al.* 2017. Stable carbon isotope analysis of *Cedrus atlantica* pollen as an indicator of moisture availability. *Review of Palaeobotany and Palynology* **244**: 128–139.
- Bennett KD, Willis KJ. 2002. Pollen. In *Tracking Environmental Change Using Lake Sediments*. Springer: Dordrecht; 5–32.
- Beug HJ. 2004. *Leitfaden der Pollenbestimmung für Mitteleuropa und angrenzende Gebiete*. Gustav Fischer: Stuttgart.
- Brooks J, Shaw G. 1978. Sporopollenin: a review of its chemistry, palaeochemistry and geochemistry. *Grana* **17**: 91–97.
- Chesson LA, Tipple BJ, Erkkila BR *et al.* 2013. Hydrogen and oxygen stable isotope analysis of pollen collected from honey. *Grana* **52**: 305–315.
- Descolas-Gros C, Schölzel C. 2007. Stable isotope ratios of carbon and nitrogen in pollen grains in order to characterize plant functional groups and photosynthetic pathway types. *New Phytologist* **176**: 390–401.
- Faegri K, Kaland PE, Krzywinski K. 1989. *Textbook of Pollen Analysis*, 4th edn. John Wiley & Sons Ltd: Chichester.
- Fan TF, Potroz MG, Tan EL *et al.* 2019. Species-specific biodegradation of sporopollenin-based microcapsules. *Scientific Reports* **9**: 9626.
- Fraser WT, Watson JS, Sephton MA *et al.* 2014. Changes in spore chemistry and appearance with increasing maturity. *Review of Palaeobotany and Palynology* **201**: 41–46.
- Griener KW, Nelson DM, Warny S. 2013. Declining moisture availability on the Antarctic Peninsula during the Late Eocene. *Palaeogeography, Palaeoclimatology, Palaeoecology* **383–384**: 72–78.
- Jahren AH. 2004. The carbon stable isotope composition of pollen. *Review of Palaeobotany and Palynology* **132**: 291–313.
- King DC, Schubert BA, Jahren AH. 2012. Practical considerations for the use of pollen  $\delta^{13}\text{C}$  value as a paleoclimate indicator. *Rapid Communications in Mass Spectrometry* **26**: 2165–2172.



- Li FS, Phyto P, Jacobowitz J *et al.* 2019. The molecular structure of plant sporopollenin. *Nature Plants* **5**: 41–46.
- Loader NJ, Hemming DL. 2000. Preparation of pollen for stable carbon isotope analyses. *Chemical Geology* **165**: 339–344.
- Loader NJ, Hemming DL. 2004. The stable isotope analysis of pollen as an indicator of terrestrial palaeoenvironmental change: a review of progress and recent developments. *Quaternary Science Reviews* **23**: 893–900.
- Mikhael A, Jurcic K, Schneider C *et al.* 2019. *Demystifying and Unravelling the Factual Molecular Structure of the Biopolymer Sporopollenin*. Chemistry Department, Memorial University, St John's, Canada.
- Moore PD, Webb JA, Collinson ME. 1991. *Pollen Analysis*, 2nd edn. Blackwell Scientific Publications: Oxford.
- Müller C, Hethke M, Riedel F *et al.* 2020. Inter- and intra-tree variability of carbon and oxygen stable isotope ratios of modern pollen from nine European tree species collected in 2015 and 2016: exploring their potential for palaeoclimate studies. *PLoS ONE* **15**: e0234315.
- Nelson DM. 2012. Carbon isotopic composition of *Ambrosia* and *Artemisia* pollen: assessment of a C<sub>3</sub>-plant paleophysiological indicator. *New Phytologist* **195**: 787–793.
- Nelson DM, Hu FS, Michener RH. 2006. Stable-carbon isotope composition of Poaceae pollen: an assessment for reconstructing C<sub>3</sub> and C<sub>4</sub> grass abundance. *Holocene* **16**: 819–825.
- Paul D, Skrzypek G, Fórizs I. 2007. Normalization of measured stable isotopic compositions to isotope reference scales – a review. *Rapid Communications in Mass Spectrometry* **21**: 3006–3014.
- R Core Team. 2017. *R: A language and environment for statistical computing*. <https://www.r-project.org/>
- RStudio Team. *RStudio: integrated development for R*. RStudio, Inc.: Boston. <http://www.rstudio.com/>
- Schwarz DM. 2016. *A Stable isotope investigation of pollen from Pinery Provincial Park, Southwestern Ontario, Canada*. The University of Western Ontario. Electronic Thesis and Dissertation Repository; 4254.
- Stanley RG, Linskens HF. 2012. *Pollen: Biology Biochemistry Management*. Springer Science + Business Media: Berlin.