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Testing the performance of sodium polytungstate and lithium heteropolytungstate as non-toxic dense media for pollen extraction from lake and peat sediment samples

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ABSTRACT

Pollen analysis is one of the most important methods to reconstruct past climate change and to understand prehistoric and early historic human-environment interactions. Every study based on fossil pollen assemblages from sedimentary archives starts with the preparation of collected sample material. The most widely employed protocols to concentrate pollen involve the use of several chemicals including hydrofluoric acid (HF), which is extremely hazardous to human health. As an alternative to HF, we have tested the reliability of dense media separation using two non-toxic substances, sodium polytungstate (SPT) and lithium heteropolytungstate (LST). Our test, which is based on five different samples representing different palaeoenvironmental archives partly revealed statistical disagreement between HF-treated samples and those treated by SPT and LST. In most cases, the observed differences in taxa proportions of the SPT and LST samples are unidirectional. In general, they do not appear to be pollen-taxa-specific but sample-specific thus probably linked to properties associated with the respective study material. However, sample comparison indicates that SPT-based dense media separation produces pollen concentrates that are statistically more comparable to those obtained by protocols based on HF-treatment. Discrepancies between both methods were also recognised for pollen concentrations and generally support the sample-specific character of dense media separation performance as suggested by the pollen proportion comparison. To verify the observed significant differences in pollen proportions and concentrations and to understand the factors that control them, further studies based on a larger number of test samples are required. In addition, we evaluated the effect of ultrasonic-aided fine sieving to bi-saccate pollen types. Our results indicate that this commonly used method to remove clays may lead to fragmentation of bi-saccate pollen into corpora and sacci, thus making identification more complicated. Although more time-consuming, we recommend to use less destructive differential centrifugation as an alternative, if indicated by preliminary tests.

1. Introduction

Pollen is the most important proxy in Quaternary palaeoenvironmental studies (Birks and Birks, 1980). Pollen-derived information about past environmental and climate changes may also help to understand prehistoric and early historic cultural developments (Berglund, 2003). Moreover, fossil pollen assemblages are crucial to reconstruct the history of human impact on vegetation and past cultural landscapes (Gaillard, 2013). Every analysis of fossil pollen assemblages begins with the preparation of the collected sediment sample material, which is essential to allow identification and counting of the contained pollen grains. The most frequently used methods to concentrate pollen from Quaternary deposits like lake and peat sediments involves the digestion of minerogenic and biogenic components using a set of different chemicals including weak to strong acids and bases (e.g. Fægri and Iversen, 1989). These chemical compounds have different properties regarding their hazard to human health.

By far the greatest health risk is linked to hydrofluoric acid (HF), that is commonly utilised at high concentrations (>20%) to digest siliceous matter. This inorganic chemical is one of the most dangerous acids known. At concentrations >40%, skin contact with

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small amounts of HF can cause a corrosive burn, which immediately results in severe tissue destruction (McKee et al., 2014). Moreover, HF is highly lipophilic and quickly penetrates deep into tissue. Here, the dissociated and highly reactive fluoride ions bind cellular enzymes as well as calcium and magnesium cations leading to the production of insoluble salts liquefaction necrosis, decalcification and bone destruction, which has devastating effects on cellular metabolism (Özcan et al., 2012). Similar serious damage to the human organism may result from HF inhalation. This acute systemic toxicity, which even arises at minor quantities of several millilitres and concentrations <50%, may cause fatal injuries to the affected person, if treatment is inadequate or not immediately provided (Özcan et al., 2012). Fatal accidents in connection with HF have been reported from palynological laboratories (Muriale et al., 1996) and other fields of application (Takase et al., 2004). In different parts of the world, the reduction of risks, which arise from the use of harmful substances, has received growing attention over the last decades. Today, in many countries (e.g. Australia, EU member states, South Korea, USA), the substitution of hazardous chemical compounds, if possible, is explicitly required by law.

An alternative to the digestion of siliceous matter by HF treatment is the application of dense media or heavy liquid separation. This approach takes advantage of the differences in density of pollen exines and silicates. Non-toxic dense media like SPT (sodium polytungstate) and LST (lithium heteropolytungstate) became available over the last three decades and have been applied in different palynological studies (e.g. Dodson and Lu, 2000; Zabenskie and Gajewski, 2007). However, thus far, there has been little work on evaluating the applicability of these chemical compounds in the concentration of pollen from sediment samples (Caffrey and Horn, 2013; Campbell et al., 2016; Zabenskie and Gajewski, 2007).

Here we test the applicability of dense media separation as a substitute for the digestion of siliceous matter by HF using the non-toxic dense media SPT and LST. Furthermore, we compare the use of differential centrifugation as an alternative to the widely used ultrasonic fine sieving method for removing small-sized particles (especially clays and organic residue). The methodological analyses focus on a set of five sediment samples extracted from different palaeoenvironmental archives representing different ages and environmental conditions.

2. Dense media separation for extracting pollen from sediments

This approach takes advantage of the higher density of silicates, ranging between ca. 2.6 and 4.3 cm^3 (Wicander and Monroe, 2006), in comparison to the exines of pollen grains. There is no agreement about the exine density. Determined values given in the literature vary between ca. 1.4–1.5 g/cm^3 (Flenley, 1971; Regnéll and Everitt, 1996) and ca. 1.4–1.7 g/cm^3 (Fægri and Iversen, 1989). Juvigné (1973) suggests even higher values of ca. 1.9 and 2.1 g/cm^3 for specimens from the last glacial period and the Paleogene, respectively, which implies that exine densities may increase over time. To our knowledge, the earliest documented attempt to use dense media separation was made by Grichuk (1937). As heavy medium, he used Thoulet's solution, which is a mixture of mercury iodide (Hg_2I_2), potassium iodide (KI), and water, thus highly hazardous to the human health. During the following decades, palynologists have employed additional compounds, which also exhibit different degrees of toxicity. Among the most commonly used are bromoform ($CHBr_3$), a solution of bromoform and acetone (C_3H_6O) or ethanol (C_2H_6O), zinc bromide ($ZnBr_2$), zinc chloride ($ZnCl_2$), caesium chloride (CsCl), or a solution of KI and cadmium iodide (CdI_2). Since the 1980s, different water soluble, non-toxic salts like sodium polytungstate (also known as sodium metatungstate, SPT; Kamps et al., 1985), lithium metatungstate (LMT, Duyvesteyn et al., 1993), and lithium heteropolytungstate (LST; Patrick and Patrick, 1997) are available as alternatives to traditionally used harmful substances. These non-toxic compounds have been successfully employed for concentrating pollen for radiocarbon dating (e.g. Howarth et al., 2013; Proske et al., 2015; Vandergoes and Prior, 2003) and for microscopic palynological analysis (e.g. Eisele et al., 1994; Moss et al., 2005; Munsterman and Kerstholt, 1996; Zabenskie and Gajewski, 2007). Especially when dealing with strongly minerogenic sediments where standard pollen preparation protocols including HF treatment fail to concentrate a sufficient amount of palynomorphs, dense media separation has proven to be useful (Allen et al., 2009). This has been also confirmed for SPT, which has been recently used by Fletcher and Hughes (2017) to prepare minerogenic-rich sediments from the High Atlas for palynological analysis. Disregarding the employed heavy liquid, a range of densities has been chosen in previous approaches, including 2.4 (Simes and Wrenn, 1998), 2.35 (Lentfer and Boyd, 2000), 2.3 (Frey, 1951), 2.2 (Waterbolk, 1954), 2.1 (Munsterman and Kerstholt, 1996), 2.0 (Moss et al., 2005; Proske et al., 2015), 1.95 (Zabenskie and Gajewski, 2007), 1.88 (Nakagawa et al., 1998), and 1.85 (Nakagawa et al., 2013) g/cm^3.

In contrast to concentrating pollen for radiocarbon dating, the application of dense media separation in pollen analytical studies usually requires that the pollen composition in the prepared samples reflect that of the raw (unprepared) sediment samples thus actual pollen composition. Although it has been noted that not the entire pollen fraction of the raw sample is recovered by dense media separation procedures (e.g. Nakagawa et al., 1998), there are only few available studies, which aim at evaluating the quality of dense-media-treated pollen samples. Nakagawa et al. (1998) have focused on assessing the comparability of the pollen fraction of organic-rich samples prepared by the HF-based method and a dense media (KI-CdI_2 solution) separation method. Caffrey and Horn (2013) attempted to test LST applying different settings for isolating pollen from lacustrine sediments, but failed due to the extremely low pollen concentration in the analysed samples. Thus far, there are two successful efforts reported (Campbell et al., 2016; Zabenskie and Gajewski, 2007), which positively evaluated the performance of SPT-based dense media separation versus preparation protocols using HF (Fægri and Iversen, 1989) by paired sample analysis.

3. Study material

For this study, we have compiled a set of five samples (TS I–V), each of which originate from sedimentary successions recovered from individual palaeoenvironmental archives including four lacustrine depositional systems and one peat bog situated in different parts of Eurasia (Table 1). All five samples represent different ages (i.e. taphonomic stages), spanning from the early Eemian to the late Holocene, and regional vegetation distributions including densely forested to high-alpine steppe environments. Each of the samples TS I–V was homogenised and divided into three identical subsamples a–c (Table 2) for lab preparation following different methods that are outlined in the subsequent section.

4. Methods

The samples of each subset were prepared according to two different protocols (Table S1) in the pollen lab of the Paleontology

Table 1
Summary of the five sediment samples (TS I–V) originating from different palaeoenvironmental archives used for comparing different pollen preparation methods (Table 2).

Sample ID (original)	Sample ID (this study)	Environmental archive (name; type; location)	Sediment type	Age (cal ka BP)	Reference
OKN-2 54–56 cm	TS Ia–c	Okunevoe; peat bog; Southern Siberia, Russia (51°27′N, 104°51′E; 500 m a.s.l.)	Compact brown peat	late Holocene	unpublished
KTK10-2-6-1 82–83 cm	TS IIa–c	Lake Kotokel; lake; Southern Siberia, Russia (52°47′N, 108°07′E; 458 m a.s.l.)	Compact homogenous silty clay	23.182	(Müller et al., 2014)
TMD 269–281 cm	TS IIIa–c	Tso Moriri; lake; NW Transhimalaya, India, (32°56′N, 78°19′E; 4522 m a.s.l.)	Laminated calcareous silty clay	10.1–10.6	(Leipe et al., 2014)
JW PZ4	TS IVa–c	Eemian basin Jänschwalde; lake; Brandenburg state, Germany (51°50′N, 14°32′E; 60 m a.s.l.)	Calcareous organic-rich silt	126–115	(Strahl, 2016)
RK12-P1-1 74–76 cm	TS Va–c	Lake Kushu; lake; Rebun Island, Japan (45°26′N, 141°02′E; 5 m a.s.l.)	Organic-rich clay	0.61	(Müller et al., 2016)

Table 2
Summary of the 15 subsamples (TS Ia–Vc) used for comparison of three different pollen preparation methods (see Table S1 for details on the preparation protocols).

Sample ID (original)	Sample ID (this study)	Weight/ volume	Preparation method
OKN-2 54–56 cm	TS Ia	1 cm^3	Conventional (HF)
	TS Ib	1 cm^3	Dense-media separation (LST)
	TS Ic	1 cm^3	Dense-media separation (SPT)
KTK10-2-6-1 82–83 cm	TS IIa	1 cm^3	Conventional (HF)
	TS IIb	1 cm^3	Dense-media separation (LST)
	TS IIc	1 cm^3	Dense-media separation (SPT)
TMD 269–281 cm	TS IIIa	2.3 cm^3	Conventional (HF)
	TS IIIb	2.3 cm^3	Dense-media separation (LST)
	TS IIIc	2.3 cm^3	Dense-media separation (SPT)
JW PZ4	TS IVa	0.2 g (dry)	Conventional (HF)
	TS IVb	0.2 g (dry)	Dense-media separation (LST)
	TS IVc	0.2 g (dry)	Dense-media separation (SPT)
RK12-P1-1 74–76 cm	TS Va	0.5 g (wet)	Conventional (HF)
	TS Vb	0.5 g (wet)	Dense-media separation (LST)
	TS Vc	0.5 g (wet)	Dense-media separation (SPT)

Section of the Freie Universität Berlin. The first preparation protocol, which is commonly applied in palynological studies, includes treatment of samples with hydrochloric acid (HCl, 10%), potassium hydroxide (KOH, 10%), HF (40%), and acetolysis (9 parts $C_4H_6O_3$ + 1 part H_2SO_4, 95%) in order to digest carbonates, humic acids, siliceous matter, and cellulose, respectively (e.g. Fægri and Iversen, 1989). Before samples were mounted in glycerol, they had been washed through a 7-µm nylon mesh aided by an ultrasonic water bath to remove clays and other small non-pollen particles. The other two preparation procedures also followed this protocol except for the HF and fine sieving steps.

In the second protocol the HF treatment was substituted by dense media separation using LST for samples TS I–Vb and SPT for samples TS I–Vc at 2.1 g/cm^3. This density represents approximately the average of the range of densities applied in previous studies (see chapter 2 for details) and has been commonly used to isolate the pollen fraction from other, non-pollen sedimentary sample material. SPT ($Na_6H_2W_{12}O_{40}$) was obtained from TC-Tungsten Compounds GmbH (Grub am Forst, Germany) and LST was purchased as LST Fastfloat (chemical formula is unpublished) from Central Chemical Consulting Pty Ltd (Malaga, Australia) via Polytungstates Europe (Chippenham, United Kingdom). Applying up to 7 ml SPT and LST per sample, which is sufficient when working with 15 ml centrifuge tubes, corresponds to initial per-sample costs of ca. EUR3.00/USD3.50 and EUR2.60/USD3.00, respectively. Compared to the use of HF, which amounts to ca. EUR/USD0.20 per sample (Campbell et al., 2016), the expenses for using SPT/LST are higher, but may be reduced by recycling. However, how often recycling of SPT/LST can be successfully carried out depends on the chemical composition of the treated sediment samples (S. Kamps pers. comm.). Regarding the recycling process, LST offers two advantages over SPT. After filtration, LST can be boiled to evaporate water for re-adjusting the density, whereas SPT becomes thermally instable at >60 °C (Patrick and Patrick, 1997) making the recycling step more time-consuming. Although, there are cases reported where boiling of SPT apparently did not affect its quality (M. Dinies pers. comm.). Another, although minor, difference between the two dense media is the viscosity, which is ca. 4.6 cP and ca. 3.6 cP at 25 °C for SPT and LST, respectively (Central Chemical Consulting, 2016).

Instead of ultrasonic sieving, we performed differential centrifugation (e.g. Barss and Williams, 1973) after treatment with KOH in order to evaluate the effect on the preservation of bi-saccate pollen types that are usually more susceptible to fragmentation than other more compact built types. Fragmented bi-saccate pollen are represented by sacci counts. To allow for comparison, we calculated percentages for counts of complete and fragmented (sacci counts divided by 2) grains based on the total sum of the respective bi-saccate taxon.

To increase the reproducibility of the pollen counts (Birks and Birks, 1980), we counted more than 600 terrestrial pollen grains per sample. For the calculation of pollen percentages, the total sum of terrestrial pollen taxa identified in each spectrum was taken as 100%. The comparison is based on the 95% confidence limits ($\pm 2\sigma$) of the true proportion of the pollen taxa calculated according to Mosimann (1965). For each of the five samples (TS I–V), a pollen taxon was considered in the comparison, if its 95% confidence interval indicated a contribution of $\geq$5% to the overall pollen assemblage of the sample treated by the HF-based method. Pollen taxon proportions of a dense-media-treated sample and the corresponding sample treated by the HF procedure are considered statistically comparable, if both confidence intervals, at least partly, overlap. Hereafter, the samples treated by the SPT- and LST-based dense media separation, and the HF-based method are simply referred to as SPT, LST, and HF samples, respectively.

Estimation of pollen concentration is a standard measure in palynological studies and may hold information about disturbances in the vegetation cover, changes in sedimentation rates within palaeoenvironmental archives, or may be used for indirect dating of peat sections (Middeldorp, 1982). To check whether the density separation techniques have an influence on the pollen concentration estimates, we determined this measure for each sample. Therefore, we added a known quantity of exotic *Lycopodium clavatum* marker spores (tablet batch no. 483216, Department of Geology, Lund University) to each sample prior to lab preparation and applied the formula given in Stockmarr (1971). A detailed step-by-step description of the preparation protocols is outlined in Table S1.

5. Results

Regarding all five HF samples (TS I–Va), 21 pollen taxa proportions contribute ≥ 5% to the respective pollen spectrum, thus were considered for comparison. The results indicate that the pollen taxa proportions of the dense-media-treated samples partly differ from those of the HF samples (Fig. 1). Of the 21 comparisons, 17 (ca. 80%) and 12 (ca. 57%) proportions of the SPT and LST samples, respectively, are in statistical agreement with the HF sample. The cumulative differences in taxa proportions are 75% for the SPT and 117% for the LST samples. In the following paragraphs, we outline and compare the results of the different preparation methods for TS I–V regarding (i) the relative contributions of all relevant pollen taxa (Fig. 1), (ii) the proportion of complete and fragmented (sacci) grains of bi-saccate pollen taxa (Fig. 2), and (iii) the calculated pollen concentrations (Fig. 3). We also checked the supernatant (clay fraction) collected during the differential centrifugation step 4b (Table S1) of samples TS I–Vb for pollen content. We detected negligible amounts of pollen, which are not further outlined in the following sections. Moreover, minor non-uniform differences among the respective subsamples were obtained in terms of pollen taxa diversity (number of identified pollen taxa per subsample). For a complete overview of the pollen counting results, the pollen taxa diversity, the taxa proportions including the 95% confidence limits of the taxa considered relevant for the comparison, and pollen concentration estimates, the reader

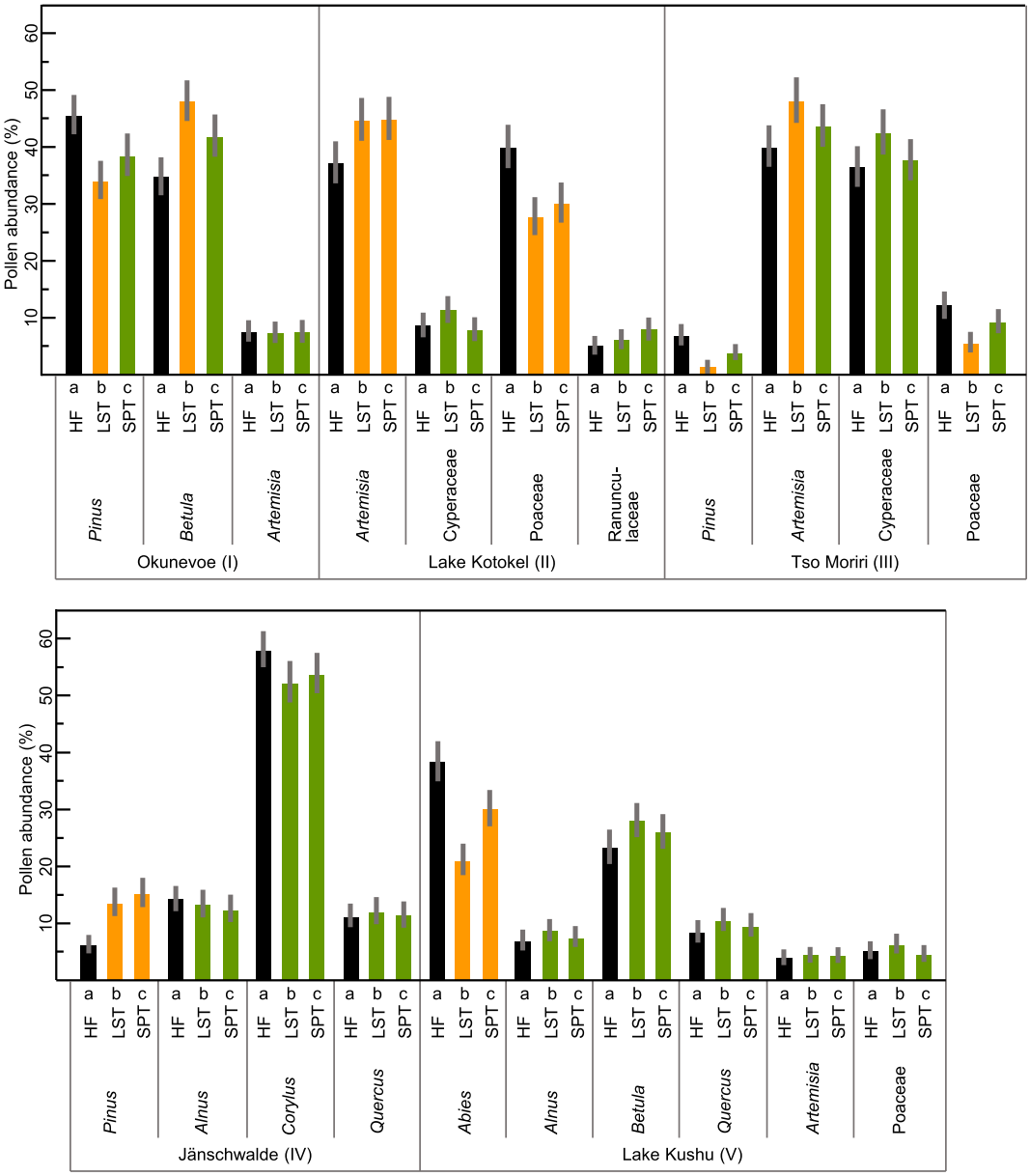

Fig. 1. Pollen analysis results for the five test sediment samples (TS I–V; see Table 1 for details) prepared applying (a) a HF-based protocol and dense media separation using (b) LST and (c) SPT (see Table S1 for details on the employed lab preparation methods). Only taxa, which were included in the comparison (i.e. comprise ≥5% of the total terrestrial pollen sum) are presented. Pollen percentages are based on the total sum of identified terrestrial pollen grains (see Table S2 for complete pollen count results). Narrow grey bars indicated 95% confidence intervals for the true pollen proportion (Mosimann, 1965). Pollen proportions of the dense-media-treated samples (b and c) that are statistically comparable and incomparable are highlighted in green and orange, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

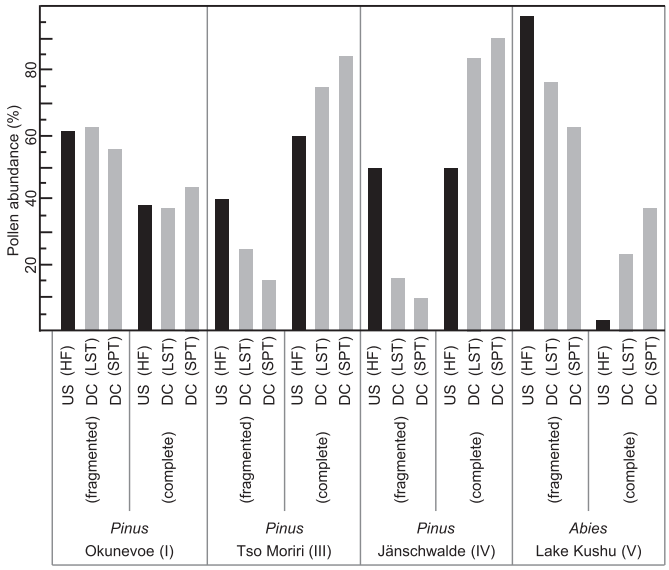

Fig. 2. Proportions of the fragmented (sacci) and complete grains of the bi-saccate pollen taxa (*Pinus* and *Abies*) contained in TS I, III, IV, and V (see Table 1 for details) of the analysed samples. Calculation of the proportions is based on the sum of the respective bi-saccate pollen taxon. Numbers of sacci are halved to allow comparison with the amounts of complete grains. Ultrasonic sieving and differential centrifugation are abbreviated by US and DC, respectively.

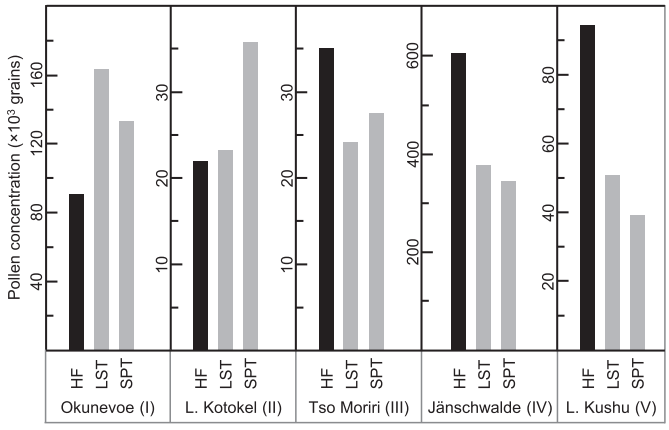

Fig. 3. Pollen concentrations for the five sediment samples (TS I–V; see Table 1 for details) prepared applying a HF-based method and dense media separation using LST and SPT (see Table S1 for details on the employed lab preparation methods).

is referred to the supplementary material (Table S2) of this article.

TS I contains three taxa (*Pinus*, *Betula*, *Artemisia*), which individually comprise more than 5% of the pollen spectrum of the HF-treated subsample. For *Pinus* and *Betula*, the proportions of the LST and SPT samples are, respectively, lower and higher than the HF-treated sample. While the proportions of the SPT sample are in agreement with those of the HF sample, they are statistically different in the LST sample. For *Artemisia,* the percentages of all three samples are well in accordance with each other. In the HF sample treated by ultra-sonic sieving the fragmented and complete *Pinus* pollen comprise ca. 60% and 40%, respectively. Very similar results were derived for fragmented and complete grains for the samples treated by LST (ca. 62% and 38%, respectively) and SPT (ca. 56% and 44%, respectively). The pollen concentration in the HF-treated sample is about 91,000 grains. The concentrations are 1.8 and 1.5 times higher in the LST (ca. 160,000 grains) and SPT (ca. 133,000 grains) samples, respectively.

In TS II there are four pollen taxa (*Artemisia*, Cyperaceae, Poaceae, Ranunculaceae) comprising $\geq 5\%$ of the pollen sum. In the dense-media-prepared samples, *Artemisia* frequencies are similarly higher than the one obtained for the HF sample. Both are outside the 95% confidence interval of the HF sample. Similar results are also derived for Poaceae, although in this case the percentages of the LST and SPT samples are statistically significantly lower than in the HF sample. The Cyperaceae and Ranunculaceae percentage ranges calculated for the LST and SPT samples are within the confidence interval of the HF sample. The values of the estimated pollen concentration in the HF (ca. 22,000 grains) and LST (ca. 23,000 grains) samples are similar. In contrast, a greater difference, corresponding to a factor of 1.6, was detected for the SPT (ca. 36,000 grains) sample.

For TS III, we compared four different pollen taxa (*Pinus*, *Artemisia*, Cyperaceae, Poaceae). For the LST sample, the confidence intervals of the true pollen proportion for *Pinus*, *Artemisia*, and Poaceae are outside those of the HF sample. While for *Pinus* and Poaceae the concentrations are significantly lower, they are higher for *Artemisia* with regard to the HF sample. Only the relative contributions of Cyperaceae of all three samples are statistically comparable. On the other hand, all taxa percentage ranges for the SPT sample are in agreement with the HF sample. Regarding the preservation of the bi-saccate *Pinus* pollen, it appears that the proportion of complete grains is higher in the LST (ca. 75%) and SPT (ca. 85%) samples treated by differential centrifugation compared to the ultrasonic-treated HF sample (ca. 60%). The pollen grain concentration estimates for the LST (ca. 24,000) and SPT (ca. 27,000) samples are lower by about 31% and 23%, respectively, than for the HF sample (ca. 35,000).

Pinus, *Alnus*, *Corylus*, and *Quercus* were considered for comparison in TS IV. The 2σ confidence intervals of the relative frequency of *Pinus* pollen in the LST (ca. 11–16%) and SPT (ca. 13–18%) samples are similar to each other, but significantly deviate from that of the HF sample (ca. 5–8%). While the *Pinus* frequencies in the dense-media-treated samples are higher, they are lower for *Corylus*, which represents the major pollen contributor in TS IV. However, for both samples the derived error ranges indicate statistical agreement with the HF sample. A higher degree of comparability is indicated for the proportions of *Alnus* and *Quercus* for both SPT and LST subsamples. The amount of intact versus fragmented bi-saccate *Pinus* pollen grains is clearly higher in the samples that did not undergo ultrasonic sieving. The proportion of complete *Pinus* grains is 84% in the LST and 90% in the SPT sample. In the HF sample, the percentages of complete and incomplete specimens are balanced. A substantial difference is also evident for the pollen concentration estimates. With about 1,210,000 grains, the determined HF sample concentration is almost twice as high as in the LST (ca. 750,000 grains) and SPT (ca. 690,000 grains) samples.

Six terrestrial pollen taxa (*Abies*, *Alnus*, *Betula*, *Quercus*, *Artemisia*, Poaceae) were considered for comparison for TS V. The greatest pollen contributor is *Abies* reaching about 40% (ca. 35–42%) in the HF sample. By comparison, the proportions of this taxon are lower in the samples that underwent dense media separation. In the LST sample, the relative contribution of *Abies* (ca. 18–24%) is about half of that registered in the HF sample. Although being much closer to that of the HF sample, the *Abies* percentage range of the SPT sample (ca. 27–33%) is also statistically incomparable. While the proportions of *Abies* do not conform to the one of the HF-treated sample, they statistically match for the remaining taxa. Of all subsamples, the HF one contains the lowest relative amount (ca. 3%) of intact bi-saccate pollen (i.e. *Abies*) grains. The proportion of intact grains of this taxon is higher in the LST (ca. 23%) and SPT (ca. 37%) samples. Regarding the absolute pollen

concentration, the situation compares with TS IV. While the estimate for the HF sample suggests a total amount of about 94,000 grains, it is about half (ca. 51,000) in the LST sample and even lower (ca. 39,000) in the SPT sample.

6. Interpretation and discussion

Dense media separation has been suggested a useful method providing several advantages in concentrating pollen from sediment samples. On the one hand, it appears especially beneficial when dealing with minerogenic-rich material deposited under arid conditions (Allen et al., 2009) or organic-rich samples as derived from peat bogs (Nakagawa et al., 1998). On the other hand, by using non-toxic substances like SPT and LST, dense media separation allows for significant health risk reduction by substituting HF treatment that is being frequently used in preparation protocols to digest siliceous matter. However, whether dense-media separation using SPT or LST produces statistically comparable pollen assemblages, thus can be straightforwardly employed instead of HF in pollen sample preparation, has not been satisfactorily verified by numerical tests.

In the present study, the comparison of relative taxa abundances between dense-media-prepared (using LST/SPT) and HF-treated samples partly revealed statistical differences (Fig. 1). This is in contrast to results of previous studies concerned with testing the reliability of dense media separation as an alternative to HF treatment that has been widely used in sample preparation protocols for pollen analysis. Without presenting further details, Zabenskie and Gajewski (2007) claim that comparison of replicate samples of lacustrine deposits from northern Canada prepared by a HF-based protocol and with heavy liquid separation using SPT "…*showed no statistical difference in the pollen counts* …". Campbell et al. (2016) provide detailed numerical results about their test that is based on 30 sediment sample pairs from three different sites in the Moroccan Atlas Mountains. Their results indicate that the percentages of all identified pollen taxa in the SPT-treated sample set are statistically comparable with those prepared using a HF-based method largely following the protocol outlined in Fægri and Iversen (1989). Using another, toxic heavy liquid (KI-CdI_2 solution) Nakagawa et al. (1998) compared percentage values for 14 different taxa from 11 samples (i.e. 151 data pairs). In six cases, the percentages differ by more than 5% between the dense media samples and HF-treated samples. Confidence limits of the true proportion of pollen taxa were not determined in this study. Based on their results, the authors concluded that both methods yield comparable pollen taxa proportions and suggest dense media separation as preferable over the traditional (HF-based) method.

Our results reveal a more complex picture reflecting partly non-systematic differences between the HF-prepared samples and those treated by dense media separation that do not allow for straightforward interpretation and discussion. In general, it appears that, compared to the LST samples, the derived pollen proportions of the SPT samples are more similar to those of the HF-treated samples. The cumulated differences in pollen percentages of all 21 data comparisons are lower for SPT (ca. 75%) than for LST (ca. 117%) resulting in a higher rate of statistical agreement for the compared SPT (ca. 80%) versus LST (ca. 57%) samples. Regarding the LST samples there is statistical disagreement for all five samples (TS I–V) for at least one of the relevant pollen taxa. For the SPT samples, on the other hand, our results indicate statistical conformity for TS I and III. In this regard, our findings do not contradict with the statistical correspondence between pollen proportions of the SPT-treated and HF-treated samples in the study by Campbell et al. (2016). Moreover, the here obtained results indicate that the proportional composition of pollen residues obtained by SPT-based dense media separation is "more comparable" to HF-treated samples in comparison to samples treated by LST. However, this is yet to be verified by further examinations based on a higher, statistically significant number of sample pairs.

In most cases, the differences in the LST and SPT sample percentages revealed in the present study have the same direction suggesting that the overall effect of the dense media separation on the pollen contained in a particular sample is generally comparable. This is well reflected, for example, in TS II where the Poaceae percentages are similarly lower in favour of *Artemisia* percentages that are, in turn, similarly higher in both LST and SPT samples. Looking at proportions of particular pollen taxa, there is no clear evidence for a systematic offset between the HF and dense-media-treated samples. While the results for TS I, III, and V suggest that proportions of bi-saccate coniferous pollen types are generally lower in the LST and SPT samples, the *Pinus* percentages of TS IV imply the opposite. For *Artemisia* the SPT and LST percentages are comparatively high for TS II and III, but very close to the HF sample values for TS I and V. Our test results provide no indication for a pollen-taxa-specific behaviour that generally occurs in dense media separation. They rather imply that the observed differences in pollen proportions are sample-specific. At this point, it has to be noted that the properties (e.g. chemical sample composition, sample age, pollen taphonomy, pollen taxa etc.), which may play a role on the performance of the dense media separation procedure remain speculative and that further studies are needed for clarification. However, as inferred above, dense media separation may produce pollen concentrates that are statistically comparable to HF-treated samples depending on the properties of the sample material (including pollen and non-pollen components) and the chemical compound used as dense medium.

We have employed the marker grain method based on *Lycopodium clavatum* spores (Stockmarr, 1971), which still appears to be the most widely used approach for estimating pollen concentration. More recently, Kitaba and Nakagawa (2017) have introduced an alternative for the calculation of microfossil concentrations using black ceramic spheres. Although the usability of these marker grain has yet to be confirmed in future applications, tests show that the presented ceramic spheres are advantageous over the frequently used *Lycopodium* spores when applied on pollen-poor samples in combination with dense media separation (Kitaba and Nakagawa, 2017).

In this study, we obtained non-systematic differences between the HF-treated and the dense-media-treated samples for the pollen concentration (Fig. 3). While the calculated values in the SPT and LST samples are comparatively higher in case of TS I and II, they are lower in case of TS III–V. A common feature of both SPT and LST samples in this regard is the offset direction (either higher or lower) compared to the value of the HF-treated sample. Except for TS II, the pollen concentrations of the SPT and LST samples appear to be generally similar. This suggests that the dense media separation acts specifically on the *Lycopodium clavatum* marker spores, on the one hand, and fossil pollen, on the other hand, and that this process is likely comparable for both SPT and LST. For TS I and II it appears that, in relation to fossil pollen, more marker spores remained in the sink fraction. By contrast, a relatively greater proportion of fossil pollen versus marker spores remained in the sink fractions of the SPT and LST samples of TS III, IV, and V. However, if we assume that the revealed discrepancies in concentration are systematic in relation to an entire sample set originating from a single sedimentary environment (i.e. palaeoenvironmental archive), there would be no problem with interpreting this measure when using SPT- or LST-based dense media separation as part of the preparation protocol. Why the marker spores and the fossil pollen fraction react differently to the dense media separation and why this

difference does not follow a common trend with regard to the HF sample pollen concentration cannot be verified based on the results of the current study. Potential variations in absolute amount of *Lycopodium* spores per tablet (Berglund and Persson, 2004; Stockmarr, 1971) can be excluded as possible cause for the detected discrepancies in concentration. Since the differences in concentration show non-uniform trends, they can also not be attributed to density differences between the acetolysed modern *Lycopodium* spores and the treated fossil pollen. Given the contrary results for TS I and V that were both deposited during the late Holocene, sample (pollen) ages also do not appear to control the concentration differences between HF and SPT/LST samples. Instead, the obtained pollen concentration data appear to support a sample-specific effect of the dense media separation as indicated by the results for the pollen proportions (Fig. 1).

We also aimed at comparing the impact of two different methods commonly used to eliminate fine-grained minerogenic material on bi-saccate pollen grain preservation. The results obtained for the four bi-saccate pollen-bearing samples (Fig. 2) allow a more straightforward interpretation. For TS III, IV, and V, the proportion of complete bi-saccate pollen (i.e. *Pinus* or *Abies*) are higher in SPT and LST samples, which were both treated by differential centrifugation. The difference is especially large for TS IV and V. For TS IV the relative amount of intact grains ranges between 90% (SPT sample) and 84% (LST sample) versus 50% in the HF sample, which underwent ultrasonic-aided fine sieving. In the HF-treated sample of TS V preserved *Abies* pollen amounts to 3%. This value is multiple times higher in the differentially centrifuged SPT (37%) and LST (23%) samples. This suggests that in these three samples (TS III, IV, and V) many of the contained bi-saccates broke during fine sieving under the physical force of the ultrasonic water bath. Broken bi-saccate pollen grains, which are usually represented by sacci and corpora, are a common feature in fossil pollen assemblages. Being more fragile than other, more compact built pollen types, they are more often subjected to fragmentation under unfavourable depositional conditions or preparational treatments, which may complicate reliable identification in palynological analyses. An exception seems to be TS I where the complete/fragmented *Pinus* pollen ratio is comparable among all three subsamples. This may be explained by the very short time of ultrasonic treatment of the HF sample due to the low amount of fine mineral particles contained in this peat bog sediment. Our results suggest that differential centrifugation should be preferably used over fine sieving aided by an ultrasonic bath. Although the former method is more labour-intensive and time-consuming, especially when the sample material contains large quantities of fine-grained particles, it facilitates faster and more reliable pollen counting. However, depending on the resilience of the contained bi-saccate pollen, this might not apply to all study materials. Whether differential centrifugation is beneficial should be verified for each study material under investigation based on a set of test samples.

What factors control the described differences in pollen proportions and concentrations between the HF and SPT/LST samples, on the one hand, and the SPT and LST performances, on the other hand, cannot be clarified within the scope of the current test. It appears that sample-specific properties may play a role and influence the quality of the dense media separation. The presence of pyrite (FeS_2) may be a potential problem that could explain discrepancies between HF and dense-media-treated samples. This sulphide mineral has a high density of about 5.0 g/cm^3 (Kennedy, 2015) and is formed under reducing conditions by bacteria-driven decomposition of organic matter. It may build up within pollen grains and may consequently lead to the accumulation of affected grains in the sink fraction as previously observed by Barss and Williams (1973). Nevertheless, such problem can be excluded for the present test, as we did not observe any pyrite particles in the analysed HF samples. In addition, we do not assume that the minor differences in viscosity between SPT and LST may explain the differences between the relative pollen compositions of the dense-media-treated samples. Likewise, sample-specific ages do not appear to influence the detected pollen proportional dissimilarities.

Indications for phenomena similar to the here described inconsistencies may be found in previous studies aimed at testing the capability of dense media separation for pollen sample preparation. In their study, Nakagawa et al. (1998) identified few cases where the percentages of the dense-media-treated sample differed from those of the HF sample by more than 5%. When taking into account the high counts of mostly more than 2000 pollen grains per sample, these cases, and perhaps even more, might be regarded as inconsistent in a statistical sense when confidence intervals of individual pollen taxa are considered. In addition, Munsterman and Kerstholt (1996) report differences in the performance of SPT versus bromoform to concentrate palynomorphs from Lower Cretaceous deposits. They observed a much greater amount of smooth-walled trilete spores in samples that were treated with bromoform. On the other hand, coniferous pollen including *Classopollis* and bi-saccate types appear at higher proportions in the SPT-treated samples. Together with these findings, our study indicates that samples prepared by dense-media separation may not always lead to pollen taxa percentages statistically comparable with HF-based methods or other dense media. Whether these potential differences require a critical evaluation or may be accepted as systematic, however, depends on the objectives of the particular palynological analysis. Nevertheless, to understand what factors control the different results requires further investigations.

7. Conclusions

We examined the performances of SPT and LST versus HF treatment and recorded partly significant differences in relative abundances of main pollen taxa. These differences seem to be non-systematic with regard to particular pollen taxa/types. Although there seems to be taxa-specific trends in both SPT and LST samples regarding a particular sample. This may suggest that the performance quality (i.e. statistical comparability) of pollen concentrates obtained by SPT/LST-based dense media separation is linked to the specific sample under analysis. Differences in both preparation methods (dense media separation and HF treatment) are also evident for calculated pollen concentration per sample. However, if we assume that these differences are systematic within the same sample set, this parameter may be confidently used to detect relative changes in pollen concentration over time.

A positive effect on bi-saccate pollen taxa is determined for differential centrifugation as an alternative to ultrasonic-aided fine sieving with generally higher proportions of intact pollen grains that may substantially improve and accelerate taxa identification. However, like the use of non-toxic recyclable dense media versus HF treatment, differential centrifugation is more labour-intensive than ultrasonic sieving. Consequently, it is worthwhile evaluating the physical resilience of bi-saccate pollen contained in the sample material to be analysed.

Due to the small number of analysed samples, it has to be noted that the current study provides valuable information on the performance of dense media separation versus more frequently used HF-based methods, though drawing statistically robust inferences requires a more comprehensive set of test samples. To further elucidate the here indicated potential differences in pollen concentrates obtained by the different preparation methods calls for additional examination. Future tests may benefit from considering different sample properties like chemical composition and their

impact on dense media separation performance.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.quaint.2018.01.029 and in the Open Access information system PANGAEA at https://doi.org/10.1594/PANGAEA.885267.

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