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A comparison of pollen extraction methods: confirmation of dense-media separation as a reliable method of pollen preparation

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Running head

Dense-media separation as a reliable method of pollen preparation

Abstract

Palynology is a crucial proxy for understanding Quaternary environmental change. A range of laboratory preparation techniques has been developed to deal with the extraction of pollen in different sedimentary contexts. Here, we present a comparison of the conventional hydrofluoric acid (HF) method and the dense-media separation method using sodium polytungstate (SPT). We examine pollen, non-pollen palynomorphs (NPPs) and microcharcoal by undertaking parallel preparation and counting of thirty paired samples from three sites in the Middle Atlas Mountains, Morocco, with contrasting environmental and sedimentary conditions. Differences between microfossil counts are assessed visually and statistically, using ANOVA and linear regression. We observe that the typical offset between counts for the five most common pollen and NPPs produced by the two methods ($\mu=1.6\%$, $sd=0.9\%$) is considerably smaller than the 95% uncertainty associated with the standard counting procedure ($\mu=6.8\%$, $sd=2.7\%$). There is no statistically significant difference associated with the preparation methods, and no significant deviation from a linear relationship between the results obtained by the two methods. There are advantages and disadvantages to both methods in terms of preparation time and visual characteristics. This study confirms that results for pollen, NPPs and microcharcoal obtained by either preparation method can be directly compared.

Key words: pollen extraction; dense-media separation; hydrofluoric acid; non-pollen palynomorphs; microcharcoal

1. Introduction

Pollen analysis is a commonly used method for reconstructing past environmental change, and is used widely in Quaternary research (Seppä and Bennett, 2003). Part of the challenge of extracting pollen and other organic microfossils from sediment is separating organic material from siliceous mineral matter of similar size to pollen (silts). The method that is most commonly used involves the dissolution of siliceous material using hydrofluoric acid (HF) (Faegri *et al.*, 2000). An alternative approach is the use of dense-media to separate pollen from the rest of the sediment matrix, such as Thoulet's liquid, ZnCl₂, sodium polytungstate (SPT) and lithium heteropolytungstate (LST) (Wood *et al.*, 1996; Nakagawa *et al.*, 1998; van Geel *et al.*, 2011; Caffrey and Horn, 2013). The dense-media separation method is typically used to extract pollen grains from clay-rich or silty sediments, where it may be used to extract very small levels of pollen from highly minerogenic sediments, often where the use of HF has failed to yield substantial quantities of pollen (e.g. Allen *et al.*, 2009; Caffrey and Horn, 2013; Fletcher and Hughes, in press). Dense-media separation can also be used for pollen extraction in more organic-rich material (Nakagawa *et al.*, 1998). Organic-rich samples are often abundant in pollen; however, isolating pollen can be problematic as a result of the difficulty of digesting and removing small organic fragments (Nakagawa *et al.*, 1998). There is evidence to suggest that dense-media separation is highly efficient when preparing organic material for pollen analysis, and can often produce material for analysis that is easier to count than samples that undergo the conventional method using HF (Phipps and Playford, 1984; Forster and Flenley, 1993; Nakagawa *et al.*, 1998; Allen *et al.*, 2009). More recently, dense-media separation has also been adopted as a method in palaeoecology for extraction of non-pollen palynomorphs (NPPs) and microcharcoal analyses (e.g. Turner *et al.*, 2008; van Geel *et al.*, 2011).

However, there are few studies that compare the influence of this key stage in the pollen preparation method – that is, the influence of using HF vs. dense-media

separation. A pioneering study (Björck *et al.*, 1978) compared the results of pollen analysis via the HF and ZnCl₂ separation methods on clay-rich lake sediments from south-eastern Sweden, and reported good correspondence between the results obtained by the two methods. Nakagawa *et al.* (1998) examined a set of eleven samples from a range of depositional contexts, and observed compatible results for HF and dense-medium preparations. To date, however, the papers that compare the two methods focus solely on pollen analysis, and do not provide comparative records for either NPPs or microcharcoal (e.g. Nakagawa *et al.*, 1998; Caffrey and Horn, 2013). In addition, comparisons so far have used fairly small numbers of paired samples, and there is little statistical support for the findings. Overall, robust evaluation of key methodological stages in microfossil preparation remains relatively rare (cf. Wolfe, 1997; Riding and Kyffin-Hughes, 2004; Mertens *et al.*, 2009), but is critical for understanding the extent to which results from different studies can be considered compatible. This paper aims to test whether dense-media separation using SPT is a reliable method of pollen preparation for both organic and minerogenic sediments. It provides a comparison of thirty paired samples prepared in parallel for both HF and dense-media separation, analysing pollen, NPPs and microcharcoal in the residues.

2. Research sites

The three research sites that are used in this investigation are located in the Middle Atlas Mountains, Morocco. The sites lie within 10 km of each other, close to the transition between the Mediterranean and Saharan climatic zones (Figure 1). The regional climate is semi-arid, and is influenced by the sub-tropical high-pressure belt during the summer, and the Atlantic westerly circulation in the winter (Lamb *et al.*, 1999). The sites are located in carbonate bedrock areas and occur in depressions related to karstic and tectonic processes. Table 1 summarises the key characteristics of each site.

INSERT FIGURE 1 HERE.

The first site is Lake Sidi Ali (Figure 2a). Sitting at 2080 m.a.s.l., Sidi Ali is a permanent, closed waterbody and one of the largest and highest lakes in the Middle

Atlas Mountains. It is located at 33° 03' N, 05° 00' W, within a small closed catchment (~14 km²), with a surface area of 2.8 km² and a water depth of up to 40m. A 19.56 metre core was extracted using a UWITEC piston corer with a 2m core chamber at the deepest part of the lake (full method described in Zielhofer *et al.*, in review). The sediment at Lake Sid Ali is classified as organic silt, averaging approximately 12% total organic carbon (TOC), and is rich in pollen, with a typical concentration of 2.5 x 10⁵ grains cm⁻³.

INSERT FIGURE 2 HERE.

Col du Zad (33° 01' 44.8" N, 05° 04' 7.9" W, 2148 m a.s.l) is a small (0.25 km²) waterlogged depression fed by groundwater springs containing a shallow, seasonal pond (Figure 2b). A 2.2 m core was collected from the site using a Russian corer. The sedimentary infill is predominantly minerogenic silts and clay, with organic matter content determined by loss-on-ignition typically 14% (i.e. an estimated ~6-9% TOC following Veres, 2012). The pollen concentrations are an order of magnitude lower than at Sidi Ali, typically 7.0 x 10⁴ grains cm⁻³.

The third site is a shallow lake with a surrounding marshy area (Figure 2c). Aguelmam Azougagh lies a couple of kilometres south west of Col du Zad (33° 01' 23.2" N, 05° 05' 29" W, 2058 m a.s.l). A 4.4 m core from the marsh was collected using a combination of Russian corer and gouge auger. The sediment is predominantly minerogenic, but has a moderate organic content throughout the core, averaging 13% (~5-9% TOC), reaching > 30% (i.e. an estimated ~12-20% TOC maximum). Typical pollen concentrations are slightly higher than at Col du Zad (9.8 x 10⁴ grains cm⁻³).

These three sites present contrasting environmental conditions, with sediment from a deep lake, a shallow, marshy lake, and a seasonally waterlogged bog. In addition, the three cores contain a range of sediment types, and as a result we have tested our hypotheses on sediments that are organic rich, highly minerogenic, and less minerogenic clays.

3. Methodology

3.1. Pollen preparation

Ten pairs of samples were taken from ten different depths in each core, or thirty pairs in total. A known quantity of exotic marker spores (*Lycopodium*) was added in tablet form, following a standard approach to allow for estimation of the absolute concentration of palynomorphs (Stockmarr, 1971). Samples were prepared using both the HF method and the dense-media separation method. All samples were prepared in parallel until stage 6 (Figure 3), when half were treated with hydrofluoric acid and the other half from the same depths underwent pollen extraction using the dense-media separation method, with SPT prepared to a density of 1.88-1.91 g/cm³. The preparation was identical and continued in parallel for the remaining few steps (Figure 3). The detailed preparation protocol is provided in Appendix 1.

INSERT FIGURE 3 HERE

3.2. Pollen, NPP and charcoal analysis

Full pollen counts were conducted for each of the twenty samples at each site. This involved counting a minimum of 300 terrestrial grains, along with any aquatic grains, spores and NPPs. Pollen taxa were identified primarily using Moore *et al.* (1991) and Reille (1999), and percentages were calculated against the total land pollen. The taxonomy of NPPs includes a mixture of taxa, some of which are of known biological affinity at the species, genus or family level, while others are known only from microfossils. The taxa identified here are listed as ‘type’ following the Hugo de Vries (HdV) Laboratory numbering, and were identified based on the descriptions outlined in various studies, including: van Geel, 1972, 1978; Pals *et al.*, 1980; Kuhry, 1985; van Geel *et al.*, 2003; van Geel and Aptroot, 2006. The abundances of NPPs, aquatics and spores are expressed as a percentage, with the individual spore count included in the pollen sum:

$$X\% = \frac{N_x}{TLP + N_x} \times 100$$

Where X% is the percentage of the individual NPP taxon, TLP is total land pollen counted and N_x is the number of NPPs of that taxon counted (Moore *et al.*, 1991).

Microscopic charcoal analysis was conducted using the same slides as the pollen analysis. A sum of 200 charcoal particles and *Lycopodium* spores was counted in each sample, to reach an accurate estimate of particles for the entire sample, with less than 5% error (Finsinger and Tinner, 2005). These were split into four size classes: < 20 µm, 20-50 µm, 50-100 µm, >100 µm, measured using a calibrated graticule. Charcoal concentrations are expressed in number of fragments per cm³.

3.3. Statistical analysis

The statistical approach includes three stages: (i) comparison of differences in abundances between the pairs against 95% confidence limits on the counts, (ii) ANOVA to test for the influence of method on the pollen and NPP abundances, and (iii) a series of linear regressions for individual microfossil taxa/types, including pollen and NPP abundances, charcoal concentrations and *Lycopodium* counts. The rationale for stage (i) is to evaluate the amplitude of the difference in the counts for main taxa produced by the two methods against counting uncertainty associated with the standard counting method. Stage (ii) tests for the influence of preparation method on the most common pollen and NPP taxon abundances. Stage (iii) models the relationship for individual, significant taxa between the counts produced by each method to test for departure from a predictable linear relationship. Our working hypothesis is there will be no significant difference between the pollen, fungal spore and charcoal counts produced from the paired samples prepared using two different methods: dense-media separation, with SPT, and the conventional method, with HF. All relationships were tested at the 95 % level ($p \leq 0.05$).

3.3.1. 95% confidence limits

When conducting pollen counts, it is understood that any count only provides an estimate of the true values of the population of the sample (Maher, 1972; Faegri *et al.*, 2000). Analysis of confidence limits was performed on the top five most common pollen taxa in terms of average abundance per sample (*Cedrus*, Cupressaceae, *Quercus* evergreen type, *Quercus* deciduous type, Poaceae). These were calculated using the method of nomograms as presented in (Maher, 1972), and implemented in the software Psimpoll (Bennett, 1992).

3.3.2. ANOVA (analysis of variance)

An analysis of variance (ANOVA) following a general linear model (GLM) approach (Rutherford, 2001) was employed to determine the statistical significance of the influence of two factors (method and taxa), and their interaction (method*taxa), on pollen and NPP abundance for the major taxa. The ANOVAs were implemented in the software package SPSS. There are two levels for method (HF, SPT) and five levels for pollen taxa (*Cedrus*, Cupressaceae, *Quercus* evergreen type, *Quercus* deciduous type, Poaceae). The test was repeated for NPPs with the same two factors (method, taxa) with five levels for the most common taxa (Type 8a, *Sordaria*, Type 25, Type 202, Type 303). These two tests were performed separately to independently assess the influence of preparation method on pollen and NPPs, respectively. In each case, the influence of method on the overall dataset of common taxa is of primary interest. Also of interest is the interaction between the factors (method*taxa), which will highlight whether the method factor (i.e. the two preparation methods) has a different influence on specific levels of the second factor (taxa), i.e. a specific influence on the abundances of any of the individual pollen taxa. The significance of the results is evaluated with respect to the p value and ω^2 , a measure of the effect size (Rutherford, 2001).

3.3.3. Assessment of one-on-one relationship by linear regression

An assessment of the relationship between the two methods was then conducted using a linear regression approach, adapted from the assessment carried out for geochemical data obtained by different measurement techniques (assessing accuracy of field portable X-ray fluorescence measurements), by Kilbride *et al.* (2006) and Shuttleworth *et al.* (2014). Linear regression was performed on all pollen and NPP taxa occurring at least once at > 5% abundance, as well as on total microcharcoal concentrations and *Lycopodium* counts. Rare taxa are excluded because the abundance of zero values makes them unsuitable for this analysis. The analyses are summarised in Figure 4.

For each taxon (pollen and NPP), least-squares linear regression is undertaken, where:

208 $Y = mx + c + \varepsilon$

209 Y is the percentage abundance from the SPT preparation (SPT%), x is the
 210 percentage abundance from the paired HF preparation (HF%), c is the y-intercept of
 211 the regression line, m is the slope and ε is the error term. Following regression, the
 212 distribution of residuals was checked for normality, and a classification procedure
 213 followed relating to the nature of the relationship between SPT% and HF%. In this
 214 case, where $R^2 > 0.85$ and $c = 0$ and $m = 1$ (within 5% confidence level), then the
 215 relationship $y = x$ is accepted and the two datasets are considered statistically
 216 similar, i.e. displaying a definitive, linear, one-on-one relationship. Where R^2 is
 217 between 0.7 and 0.85, or R^2 is > 0.85 and either $c = 0$ and $m = 1$ is not accepted at
 218 the 5% level, then either the relationship $y = mx + c$ or $y = mx$ is accepted,
 219 respectively, such that the two datasets are statistically different in terms of either the
 220 slope or the intercept, i.e. displaying a linear but not strictly one-on-one relationship
 221 (quantitative data). Where $R^2 < 0.7$, the relationship between SPT% and HF% is
 222 weakly linear and the data are statistically different (qualitative data).

223

224 **4. Results**

225 *4.1. Comparison of the total pollen counts, fungi and charcoal*

226 The microfossil data from each core is presented in Figures 4a, b and c. Visual
 227 comparison of the HF and SPT results indicates that abundances are very similar for
 228 the pairs of samples prepared using the two techniques at all three sites. Overall, the
 229 differences in abundances are small, both for common and rare taxa, with a
 230 maximum difference in any single sample of 5% (*Cedrus*). While the counts are not
 231 identical, a perfect agreement would not be expected – even performing two counts
 232 from the exact same sample would not yield identical results. There does not appear
 233 to be a consistent offset (i.e. consistent over- or under-representation of particular
 234 taxa), 24.6% of pairs show higher values following SPT treatment while 24.1% of
 235 pairs show higher values following HF. We can see that this is also the case when
 236 looking at the NPP abundances and the charcoal concentration.

INSERT FIGURE 4 HERE

4.2. 95% confidence intervals

The 95% confidence intervals of the five most common pollen taxa were calculated for each of the sixty counts (*Cedrus*, *Quercus* evergreen type, *Quercus* deciduous type, Poaceae, Cupressaceae). For every single sample, the HF count falls within the confidence interval of the SPT count, and vice versa (Appendix 2). The average difference between the upper and lower limits of the 95% confidence intervals were calculated, and these were as follows: *Cedrus* 9.6%, *Quercus* evergreen type 8.1%, *Quercus* deciduous type 4.7%, Poaceae 6.6%, Cupressaceae 5.1%. These values clearly indicate that there is a fairly wide uncertainty associated with the standard counting procedure (300 pollen grain main sum), and that the confidence intervals are greater than the absolute differences associated with the two preparation method (averages: *Cedrus* 2.6%, *Quercus* evergreen type 1.7%, *Quercus* deciduous type 1.1%, Poaceae 1.5%, Cupressaceae 1.1%). Overall, the typical offset between counts for the five most common pollen and NPPs produced by the two methods ($\mu=1.6\%$, $sd=0.9\%$) is considerably smaller than the 95% uncertainty associated with the standard counting procedure ($\mu=6.8\%$, $sd=2.7\%$).

4.3. ANOVA

The results of the ANOVA for major pollen taxa and NPPs are given in Table 2 and Table 3, respectively. For both tests, there is no significant influence of the method factor on the pollen or NPP abundances (95% confidence level). As would be expected, there are significant effects of the second factor (taxa) on pollen/NPP abundances, as some of the taxa are simply more common than others. Importantly, however, no statistically significant interaction between the two factors (method*taxa) is observed, i.e. no patterns of difference between taxa can be related to the influence of method. For pollen (NPPs), the ω^2 indicates that 31% (26%) of the variation between the samples can be associated with the taxa; the other 69% (74%) is unaccounted for by the test. This large unaccounted proportion of the variance reflects inter-sample differences related to palaeoecological changes at the sites.

Effectively, the method and the interaction of method and taxa are responsible for none of the variation in abundances of major pollen and NPP types.

INSERT TABLES 2 AND 3 HERE

4.4. Linear regression

Table 4 summarises the results of the linear regression analysis, and Figure 5 displays examples of the linear regression models for several taxa. The R^2 values are all above 0.85. This shows that the strength of linear association between the two methods is very high. The quality level shows that in all but one case, we can accept that $y = x$ within the 5% confidence level, i.e. that both methods of pollen preparation yield statistically similar results. Values for one pollen taxon, Caryophyllaceae, conform strongly to a linear relationship ($R^2 = 0.981$) but the one-on-one line falls narrowly outside the 5% confidence limits of the best fit model, with slightly higher values in the SPT preparations. However, average abundance of this taxon is low (0.7%) and therefore the model will be sensitive to small differences in raw counts. Overall, the regression analysis highlights how closely the values for pollen and NPP abundances, and microcharcoal concentrations, conform to a one-to-one, linear relationship, underlining a negligible influence of HF vs SPT treatment during the preparation stages. It is also important to note that counts for the exotic marker spore, *Lycopodium*, strongly conform to the highest category of definitive linear relationship (Table 4).

INSERT TABLE 2 AND FIGURE 5 HERE

5. Discussion

So, is the use of the dense-media separation a reliable method for preparing samples for pollen, fungal spore and charcoal analysis? The results of all analyses here clearly indicate that there is no significant difference between the results produced by both methods. We have compared microfossils with a range of different morphologies: charcoal, NPPs, and a variety of pollen types, including conifer taxa (e.g. *Cedrus*), which have substantial air bladders (sacci) that might be anticipated to

influence the density of the grain. *Cedrus* is a particularly crucial taxon in Middle Atlas Holocene pollen records, as the nature of its glacial and Holocene biogeographical history is of keen regional interest (Lamb and van der Kaars, 1995; Cheddadi *et al.*, 1998; Lamb *et al.*, 1999). Despite having the largest differences in the results presented, the differences between the parallel *Cedrus* abundances are still very small, and are seen to be insignificant (Figure 5); the results indicate that the use of the dense-media separation method has no impact on these samples and the results that are produced. Our findings confirm previous conclusions drawn by Björck *et al.* (1978) and Nakagawa *et al.* (1998), that pollen abundances are not altered when samples undergo dense-media separation, and furthermore extend this finding to other microfossil components of the samples (microcharcoal and NPPs). The comparable results obtained for the *Lycopodium* marker spores are also important because they confirm that concentration values calculated on the basis of the ratio of pollen or NPPs to *Lycopodium* will not be significantly affected by the choice of HF or dense media separation at the preparation stage.

The visual and statistical results show that there is no bias towards larger or smaller pollen grains when using the dense-media method – this would be highlighted clearly, for example, in significant deviation in slope or intercept in the linear models, which we do not observe. We analysed a range of sizes and morphologies, including *Cedrus* – a large, bisaccate pollen grain, typically 60-80 µm longest dimension – and *Quercus* evergreen – typically much smaller, with <25 µm longest dimension. The variation that does occur appears essentially random – that is, there is not typically more of one taxon occurring in samples prepared using SPT rather than HF (or vice versa). Overall, statistically similar abundances are obtained from both methods, confirming that the dense-media separation is a reliable method of preparation.

The charcoal results are also of particular interest. Figures 4a, b and c show that there is very little difference between not only the total charcoal concentrations, but also the concentrations of the different size categories. This indicates that, while some of the charcoal may become fragmented during the pollen preparation process, the same level of fragmentation occurs regardless of the method used.

Where fragmentation is of critical concern, other preparation protocols aiming to reduce the handling of the sample should be considered (e.g. Rhodes, 1998; Turner *et al.*, 2008). Nevertheless, our findings suggest that dense-media separation does not preclude comparison of size class results as compared to samples prepared with the HF technique.

The results of these visual and statistical analyses therefore show that records produced using either preparation technique (i.e. dense-media separation or HF), can be directly compared. This finding is crucial, for example, for the comparison of Late Quaternary Moroccan vegetation records. Previous researchers have used both techniques; the majority of these Moroccan vegetation studies prepared pollen samples using HF (Lamb *et al.*, 1989, 1991, 1999; Lamb and van der Kaars, 1995; McGregor *et al.*, 2009; Rhoujjati *et al.*, 2010; Amami *et al.*, 2013; Muller *et al.*, 2014), however several recent studies prepared samples using dense-media separation protocols (Zapata *et al.*, 2013; el Bait *et al.*, 2014; Bell and Fletcher, 2016; Fletcher and Hughes, in press). In a region that is receiving an increasing amount of interest, particularly regarding Holocene environmental change, the knowledge that these records are directly comparable enables us to begin to accurately study biogeographical changes across the region, and how the nature and timing of environmental and vegetation shifts varied at a regional scale.

Despite the statistical similarity of the microfossil content, visual characteristics of the residues are notably different between the HF and SPT approaches. As noted by Nakagawa *et al.* (1998), pollen samples prepared using the dense-media separation method are substantially cleaner and more efficient for counting. When reviewing the palaeoenvironmental context of Late-glacial woolly mammoth discoveries, Allen *et al.* (2009) had to re-prepare their samples for pollen analysis using the dense-media separation method after insufficient results were achieved using the conventional method (using HF). They too found the dense-media separation provided much more efficient material for counting. Figure 6 shows photographs of pollen slides used in this investigation. We draw similar conclusions to those of Nakagawa *et al.* (1998) and Allen *et al.* (2009). The dense-media separation samples generated much cleaner slides; the majority of material was in fact pollen grains, NPPs and charcoal, and there was a much smaller amount of excess organic debris on the slides. As a

result of these cleaner, clearer slides, the NPP and pollen grains were much easier to identify, and measuring the size of the charcoal grains was more accurate. In addition, the slides were much quicker to count, as there was less detrital material, meaning that a greater number of slides could be counted in the same period of time. Similar advantages of SPT have been reported for microinvertebrate preparation techniques (Mitchell and Heckert, 2010).

INSERT FIGURE 6 HERE

An interesting point to note is that there were high levels of other organic matter in the HF samples, but much less in the dense-media separation samples. Both sets of samples were subjected to the same preparation, other than stage 6. HF treatment removes siliceous material, while the removal of humic material from the samples occurs at stage 3, using potassium hydroxide (Faegri *et al.*, 2000). This suggests that during dense-media separation, some dense organic fragments that have not already been destroyed sink to the bottom of the centrifuge tube, and are not transferred along with the pollen grains after dense-media separation has been carried out. Microscopic observation of the heavy residue confirms that there is organic material in the dense fraction settling at 1.88-1.91 g/cm³, including compact, angular unidentifiable organic fragments, as well as rare pollen grains. Also, some very low density organic matter may be further separated from the main pollen residue at the end of the density separation, when the density is lowered to around 1.15-1.2 g/cm³ (typically the lowest achievable density without resorting to partitioning the sample across multiple centrifuge tubes). Overall, while the exact reasons are not fully known, the density separation procedure appears to concentrate microfossils with respect to other organic detritus. This concentration effect is capitalised upon, for example, in the preparation of pollen concentrates for radiocarbon dating (Vandergoes and Prior, 2003).

In addition to the clarity of the slides, we also evaluated a range of other advantages and disadvantages of each method. Typically, the HF method has a quicker preparation time than the dense-media separation method when pre-preparation time is taken into account (i.e. preparation of the solution to appropriate density), and time for the samples to settle (not strictly required but makes the decantation easier; Appendix 1). However, due to the cleaner slides, counting and analysis is reduced

when using the dense-media method. The health and safety risks of working with HF are considerable; an HF approved lab is needed, HF-specific training and supervision of the analyst, and suitable personal safety equipment and first aid protocols. Initial per sample costs are typically much higher when using SPT (~£5, as opposed to ~£0.18 for HF), although the used SPT can be recovered, filtered and recycled. Other heavy liquids are much cheaper and offer similar performance at costs comparable to HF; however, SPT (and LST) is a non-toxic liquid, and is therefore preferable for routine use over Thoulet's liquid and ZnCl_2 (Munsterman and Kerstholt, 1996). The removal of clays and breakdown products of alkali digestion by conducting between six and twenty (in this case, sometimes more) water washes, vastly improved the quality of the samples. Although removal of clays is most critical for density separation (as highlighted by Nakagawa *et al.*, 1998), it is also beneficial for maximising efficiency of the HF stage. It is therefore crucial to spend sufficient time on this stage, depending on the clay content of the samples. Micro-sieving (e.g. 5µm mesh) as an alternative could be considered for further evaluation at this stage. While these advantages and disadvantages may all be important in different laboratory settings, the decisive factor when deciding which method to use is likely to be the sediment type, with specifically either strongly minerogenic, or, very organic-rich sediment standing to benefit particularly from the use of dense media.

The results of this investigation support an additional degree of flexibility for researchers in terms of choice of preparation procedure. The dense-media separation method may be applied to specific stratigraphic sections within a sediment core, for example a very minerogenic layer, where pollen extraction may prove difficult with HF. Interpretation of the results in this case will need to be approached with caution, as other taphonomic factors (e.g. changes in transport vectors and pollen source areas, pollen preservation, etc.) may influence the nature of the microfossil assemblages; however, the application of dense-media separation does not introduce taphonomic bias in the results.

6. Conclusions

The aim of this investigation was to test if dense-media separation is an accurate method for microfossil (pollen, NPPs, microcharcoal) preparation, and then compare its efficiency to the standard preparation method. Our results indicate that the dense-media separation does not introduce any bias in the microfossil counts as compared with HF preparation. Offsets between the comparison counts are typically small ($\mu=1.6\%$, $sd=0.9\%$), and considerably less than the 95% confidence intervals associated with a main counting sum of 300 terrestrial pollen. The ANOVAs indicate that no significant difference between the samples is attributed to the preparation method. The linear regression models further highlight that there is a strong one-on-one linear relationship between results from the two methods. For all common taxa (occurring at least once at an abundance $>5\%$) except one relatively rare taxon (Caryophyllaceae), it can be accepted that $y = x$, that is that the two methods yield statistically similar results. There is no evidence in this investigation to suggest that SPT has any effect on the composition of microfossil assemblages within the samples. The results also showed that the use of dense-media separation was effective across a range of depositional contexts in the Middle Atlas, Morocco, including a deep, permanent lake, a seasonally waterlogged pond, and a marsh. While we chose to test multiple samples from across the depth range of each of the three sites, future methodological tests in palynology could use the standard sample method suggested by Nakagawa *et al.* (2013), thus allowing for similarly rigorous statistical evaluation with replication of results while minimising loss of core material.

In terms of wider advantages and disadvantages, dense-media separation preparation may slightly increase the overall preparation time and laboratory costs due to allowing the samples to settle, and because the SPT medium is expensive (but can be recycled). However, the health and safety risks are greatly reduced compared with HF. In addition, the dense-media separation method leads to cleaner slides that maximise counting efficiency. In summary, dense-media separation is as effective a preparation method as the conventional method, using HF, and the methods produce results that are directly comparable.

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8. References

- Allen JRM, Scourse JD, Hall AR, Coope GR. 2009. Palaeoenvironmental context of the Late-glacial woolly mammoth (*Mammuthus primigenius*) discoveries at Condover, Shropshire, UK. *Geological Journal* **44**: 414–446.
- Amami B, Rhazi L, Chaibi M, Fauquette S, Ougougdal MA, Charif A, Ridaoui M, Bouahim S, Grillas P, Muller SD. 2013. Late Quaternary history of a Mediterranean temporary pool from western Morocco, based on sedimentological and palynological evidence. *Palaeogeography, Palaeoclimatology, Palaeoecology* **392**: 281–292.
- el Bait MN, Rhoujjati A, Eynaud F, Benkaddour A, Dezileau L, Wainer K, Goslar T, Khater C, Tabel J, Cheddadi R. 2014. An 18 000-year pollen and sedimentary record from the cedar forests of the Middle Atlas, Morocco. *Journal of Quaternary Science* **29**: 423–432.
- Bell BA, Fletcher WJ. 2016. Modern surface pollen assemblages from the Middle and High Atlas, Morocco: insights into pollen representation and transport. *Grana*: 1–16.
- Bennett K. 1992. PSIMPOLL: a quickBASIC program that generates PostScript page description files of pollen diagrams. *INQUA Commission for the study of the Holocene: working group on data handling methods newsletter* **8**: 11–12.
- Björck S, Persson T, Kristersson I. 1978. Comparison of two concentration methods for pollen in minerogenic sediments. *Geologiska Föreningen i Stockholm*

- 478 *Förhandlingar* **100**: 107–111.
- 479 Caffrey MA, Horn SP. 2013. The use of lithium heteropolytungstate in the heavy
480 liquid separation of samples which are sparse in pollen. *Palynology* **37**: 143–150.
- 481 Faegri K, Krzywinski K, Kaland PE. 2000. *Textbook of Pollen Analysis*. Blackburn.
- 482 Finsinger W, Tinner W. 2005. Minimum count sums for charcoalconcentration
483 estimates in pollen slides: accuracy and potential errors. *The Holocene* **15**: 293–297.
- 484 Fletcher WJ, Hughes, PD. in press. Anthropogenic triggers for Late Holocene soil
485 erosion in the JebelToubkal, High Atlas, Morocco. *Catena*.
486 doi:10.1016/j.catena.2016.03.025
- 487 Forster M, Flenley JR. 1993. Pollen purification and fractionation by equilibrium
488 density gradient centrifugation. *Palynology* **17**: 137–155.
- 489 Kilbride C, Poole J, Hutchings TR. 2006. A comparison of Cu, Pb, As, Cd, Zn, Fe, Ni
490 and Mn determined by acid extraction/ICP–OES and ex situ field portable X-ray
491 fluorescence analyses. *Environmental Pollution* **143**: 16–23.
- 492 Kuhry P. 1985. Transgression of a raised bog across a coversand ridge originally
493 covered with an oak—lime forest. *Review of Palaeobotany and Palynology* **44**: 303–
494 353.
- 495 Lamb H, Roberts N, Leng M, Barker P, Benkaddour A, van der Kaars S. 1999. Lake
496 evolution in a semi-arid montane environment: response to catchment change and
497 hydroclimatic variation. *Journal of Paleolimnology* **21**: 325–343.
- 498 Lamb H., Eicher U, Switsur VR. 1989. An 18,000-year record of vegetation, lake-
499 level and climatic change from Tigalmamine, Middle Atlas, Morocco. *Journal of*
500 *Biogeography* **16**: 65–74.
- 501 Lamb HF, van der Kaars S. 1995. Vegetational response to Holocene climatic
502 change: pollen and palaeolimnological data from the Middle Atlas, Morocco. *The*
503 *Holocene* **5**: 400–408.
- 504 Lamb HF, Damblon F, Maxted RW. 1991. Human impact on the vegetation of the

505 Middle Atlas, Morocco, during the last 5000 years. *Journal of biogeography* **18**: 519–
506 532.

507 Maher Jr L. 1972. Nomograms for computing 0.95 confidence limits of pollen data.
508 *Review of Palaeobotany and Palynology* **13**: 85–93.

509 McGregor H V., Dupont L, Stuut J-BW, Kuhlmann H. 2009. Vegetation change,
510 goats, and religion: a 2000-year history of land use in southern Morocco. *Quaternary*
511 *Science Reviews* **28**: 1434–1448.

512 Mertens KN, Verhoeven K, Verleye T, Louwye S, Amorim A, Ribeiro S, Deaf AS,
513 Harding IC, De Schepper S, González C, et al. 2009. Determining the absolute
514 abundance of dinoflagellate cysts in recent marine sediments: The Lycopodium
515 marker-grain method put to the test. *Review of Palaeobotany and Palynology* **157**:
516 238–252.

517 Mitchell J, Heckert A. 2010. The setup, use and efficacy of sodium polytungstate
518 separation methodology with respect to microvertebrate remains. *Journal of*
519 *Paleontological Techniques* **7**: 1–12.

520 Moore PD, Webb JA, Collinson ME. 1991. *Pollen Analysis* (Blackwell, Ed). London.

521 Muller SD, Rhazi L, Andrieux B, Bottollier-Curtet M, Fauquette S, Saber E-R, Rifai N,
522 Daoud-Bouattour A. 2014. Vegetation history of the western Rif mountains (NW
523 Morocco): origin, late-Holocene dynamics and human impact. *Vegetation History and*
524 *Archaeobotany*.

525 Munsterman, D Kerstholt, S. 1996. Sodium polytungstate, a new non-toxic
526 alternative to bromoform in heavy liquid separation. *Review of Palaeobotany and*
527 *Palynology* **91**(1): 417-422.

528 Nakagawa T, Brugiapaglia E, Digerfeldt G, Reille M, Beaulieu J-L De, Yasuda Y.
529 1998. Dense-media separation as a more efficient pollen extraction method for use
530 with organic sediment/deposit samples: comparison with the conventional method.
531 *Boreas* **27**: 15–24.

532 Nakagawa T, Kitagawa, H, Payne, R, Tarasov, P, Demske, D. 2013. A standard

533 sample method for controlling microfossil data precision: A proposal for higher data
534 quality and greater opportunities for collaboration. *Quaternary International* **290**: 239-
535 244.

536 Pals J., Van Geel B, Delfos A. 1980. Paleoecological studies in the Klokkeweel bog
537 near hoogkarspel (prov. of Noord-Holland). *Review of Palaeobotany and Palynology*
538 **30**: 371–418.

539 Phipps D, Playford G. 1984. Laboratory techniques for extraction of palynomorphs
540 from sediments. *Papers of the Department of Geology, University of Queensland* **11**:
541 1–23.

542 Reille M. 1999. *Pollen et Spores d'Europe et d'Afrique du nord*. Laboratoire de
543 Botanique Historique et Palynologie: Marseille.

544 Rhodes AN. 1998. A method for the preparation and quantification of microscopic
545 charcoal from terrestrial and lacustrine sediment cores. *The Holocene* **8**: 113–117.

546 Rhoujjati A, Cheddadi R, Taïeb M, Baali A, Ortu E. 2010. Environmental changes
547 over the past c. 29,000 years in the Middle Atlas (Morocco): A record from Lake
548 Ifrah. *Journal of Arid Environments* **74**: 737–745.

549 Riding JB, Kyffin-Hughes JE. 2004. A review of the laboratory preparation on
550 palynomorphs with a description of an effective non-acid technique. *Revista*
551 *Brasileira de Paleontologia* **7**: 13–44.

552 Rutherford A. 2001. *Introducing ANOVA and ANCOVA: a GLM Approach*. Sage,
553 London.

554 Seppä H, Bennett KD. 2003. Quaternary pollen analysis: recent progress in
555 palaeoecology and palaeoclimatology. *Progress in Physical Geography* **27**: 548–
556 579.

557 Shuttleworth EL, Evans MG, Hutchinson SM, Rothwell JJ. 2014. Assessment of
558 Lead Contamination in Peatlands Using Field Portable XRF. *Water, Air, & Soil*
559 *Pollution* **225**: 1844.

560 Stockmarr J. 1971. Tablets with spores used in absolute pollen analysis. *Pollen et*

- 561 *Spores* **13**:615-621.
- 562 Turner R, Kelly A, Roberts N. 2008. A critical assessment and experimental
563 comparison of microscopic charcoal extraction methods. In *Charcoal from the past:
564 cultural and palaeoenvironmental implications. Proceedings of the Third International
565 Meeting of Anthracology*, Fiorentino G, , Magri D (eds). BAR International Series,
566 Archaeopress, Oxford, UK: Cavallino (Lecce).
- 567 van Geel B. 1972. Palynology of a section from the raised peat bog 'Wietmarshcer
568 Moor', with special reference to fungal remains. *Acta Botanica Neerlandica* **21**: 261–
569 284.
- 570 van Geel B. 1978. A palaeoecological study of holocene peat bog sections in
571 Germany and The Netherlands, based on the analysis of pollen, spores and macro-
572 and microscopic remains of fungi, algae, cormophytes and animals. *Review of
573 Palaeobotany and Palynology* **25**: 1–120.
- 574 van Geel B, Aptroot A. 2006. Fossil ascomycetes in Quaternary deposits. *Nova
575 Hedwigia* **82**: 313–329.
- 576 van Geel B, Buurman J, Brinkkemper O, Schelvis J, Aptroot A, van Reenen G,
577 Hakbijl T. 2003. Environmental reconstruction of a Roman Period settlement site in
578 Uitgeest (The Netherlands), with special reference to coprophilous fungi. *Journal of
579 Archaeological Science* **30**: 873–883.
- 580 van Geel B, Gelorini V, Lyaruu A, Aptroot A, Ruchina S, Marchant R, Damste JSS,
581 Verschuren D. 2011. Diversity and ecology of tropical African fungal spores from a
582 25,000-year palaeoenvironmental record in southeastern Kenya. *Review of
583 Palaeobotany and Palynology* **164**: 174–190.
- 584 Vandergoes MJ, Prior CA. 2003. AMS dating of pollen concentrates-a
585 methodological study of late Quaternary sediments from south Westland, New
586 Zealand. *Radiocarbon*, **45**(3): 479-492.
- 587 Veres D. 2012. A Comparative Study Between Loss on Ignition and Total Carbon
588 Analysis on Mineralogenic Sediments. *Studia UBB, Geologia* **47**: 171–182.

- 589 Wolfe AP. 1997. On diatom concentrations in lake sediments: results from an inter-
590 laboratory comparison and other tests performed on a uniform sample. *Journal of*
591 *Paleolimnology* **18**: 261–268.
- 592 Wood GD, Gabriel AM, Lawson JC. 1996. Palynological techniques—processing and
593 microscopy. In *Palynology: Principles and Applications 1*, Jansonius J, , McGregor
594 DC (eds). American Association of Stratigraphic Palynologists Foundation; 29–50.
- 595 Zapata L, Lopez-Saez JA, Ruiz-Alonso M, Linstadter J, Perez-Jorda G, Morales J,
596 Kehl M, Pena-Chocarro L. 2013. Holocene environmental change and human impact
597 in NE Morocco: Palaeobotanical evidence from Ifri Oudadane. *The Holocene* **23**:
598 1286–1296.
- 599 Zielhofer C, Fletcher WJ, Mischke S, De Batist M, Campbell JFE, Joannin S,
600 Tjallingii R, El Hamouti N, Junginger A, Steele A, Bussmann J, Schneider B, Lauer T,
601 Spitzer K, Brachert T, Mikdad A. in revision. Synchronous Atlantic cooling and
602 Western Mediterranean winter 589 rain minima during the last 12,000 years.
603 *Quaternary Science Reviews*.

604 *Table 1: Characteristics of the study sites.*

605

Site	Site description	Location	Altitude (m.a.s.l.)	Basin size (km ²)	Catchment area (km ²)	Estimated TOC (%)	Average pollen concentration (grains cm ⁻³)
Sidi Ali	Permanent lake	33° 03' N, 05° 00' W	2080	2.8	14	12	2.5 x 10 ⁵
Col du Zad	Waterlogged depression	33° 01' 44.8" N, 05° 04' 7.9" W	2148	0.25	1	6-9*	7.0 x 10 ⁴
Aguelmam Azougagh	Shallow lake and marsh	33° 01' 23.2" N, 05° 05' 29" W,	2058	0.2	1.2	5-9*	9.8 x 10 ⁴

606 **estimated at 0.4-0.7 x LOI (see Veres, 2012)*

607

608

609 *Table 2: ANOVA results for pollen.*

610

Source	p value	ω^2
Method	0.981	0.00
Taxa	<0.001	0.31
Method*taxa	1.000	0.00
Total		0.31

611

612 *Table 3: ANOVA results for NPPs.*

613

Source	<i>p</i> value	ω^2
Method	0.939	0.00
Taxa	<0.001	0.26
Method*taxa	1.000	0.00
Total		0.26

614

615

616 *Table 4: Linear regression statistics for major pollen and NPP taxa, and*
617 *microcharcoal concentration*
618

Taxa	Type	Average %	Residuals normal?	R ²	c	Accept c = 0?	m	Accept m = 1?	Quality level
<i>Cedrus</i>	Pollen	27.8	Yes	0.973	1.452	Yes	0.957	Yes	Definitive (y = x)
<i>Botryococcus</i>	Pollen	19.1	Yes	0.999	-0.068	Yes	0.995	Yes	Definitive (y = x)
<i>Quercus evergreen</i>	Pollen	17.1	Yes	0.957	0.686	Yes	0.955	Yes	Definitive (y = x)
Poaceae	Pollen	10.1	Yes	0.901	0.033	Yes	0.988	Yes	Definitive (y = x)
<i>Ranunculus</i>	Pollen	9.4	Yes	0.957	1.016	Yes	1.002	Yes	Definitive (y = x)
Cyperaceae	Pollen	7.0	Yes	0.989	-0.137	Yes	0.999	Yes	Definitive (y = x)
Cupressaceae	Pollen	6.3	Yes	0.952	0.498	Yes	0.912	Yes	Definitive (y = x)
<i>Quercus deciduous</i>	Pollen	5.0	Yes	0.930	0.062	Yes	1.062	Yes	Definitive (y = x)
<i>Potamogeton</i>	Pollen	4.3	Yes	0.986	0.007	Yes	0.982	Yes	Definitive (y = x)
<i>Myriophyllum</i>	Pollen	3.8	Yes	0.946	0.276	Yes	0.892	Yes	Definitive (y = x)
Asteraceae Lactuceae	Pollen	2.3	Yes	0.898	0.0663	Yes	0.893	Yes	Definitive (y = x)
<i>Ulex</i> type	Pollen	2.2	Yes	0.851	0.328	Yes	0.961	Yes	Definitive (y = x)
<i>Artemisia</i>	Pollen	1.9	Yes	0.854	0.264	Yes	0.872	Yes	Definitive (y = x)
<i>Astragalus danicus</i>	Pollen	1.8	Yes	0.889	1.075	Yes	0.889	Yes	Definitive (y = x)
Chenopodiaceae	Pollen	1.7	Yes	0.870	0.180	Yes	0.915	Yes	Definitive (y = x)
<i>Rumex acetosa</i>	Pollen	1.5	Yes	0.974	0.989	Yes	0.078	Yes	Definitive (y = x)
Asteraceae aster	Pollen	1.3	Yes	0.940	0.077	Yes	0.8665	Yes	Definitive (y = x)
<i>Olea</i>	Pollen	0.9	Yes	0.974	1.452	Yes	0.957	Yes	Definitive (y = x)
<i>Caryophyllaceae</i>	Pollen	0.7	Yes	0.981	-0.137	Yes	1.155	No	Quantitative (y = mx)
<i>Rumex acetosella</i>	Pollen	0.4	Yes	0.879	0.023	Yes	0.898	Yes	Definitive (y = x)
Type 8a	NPP	23.3	Yes	0.972	1.182	Yes	0.906	Yes	Definitive (y = x)
Sordaria	NPP	14.8	Yes	0.904	1.173	Yes	0.931	Yes	Definitive (y = x)
Type 25	NPP	3.7	Yes	0.923	0.372	Yes	0.886	Yes	Definitive (y = x)
Type 303	NPP	3.0	Yes	0.892	0.6252	Yes	0.810	Yes	Definitive (y = x)
Type 202	NPP	2.3	Yes	0.938	0.552	Yes	0.8573	Yes	Definitive (y = x)
Charcoal	Charcoal	N/A	Yes	0.963	-13135	Yes	1.045	Yes	Definitive (y = x)
<i>Lycopodium</i>	<i>Lycopodium</i>	N/A	Yes	0.982	-7.580	Yes	1.056	Yes	Definitive (y = x)

619 APPENDIX 1. Pollen preparation protocol, including HF and dense media options.
620 Please refer to Figure 3 for an overview.

- 621 1. **Volumetric sampling and the addition of *Lycopodium*.** Plastic syringes
622 with a 1 cm³ volume were used to measure out each individual sample. The
623 samples were transferred to large (50 mL) centrifuge tubes and a single
624 *Lycopodium* tablet was added to each sample. The addition of the
625 *Lycopodium*, an exotic marker grain, to a known volume of sediment enables
626 the absolute abundance of pollen grains to be determined (Stockmarr, 1971).
- 627 2. **Removal of carbonates by acid digestion (HCl).** 10 ml of 10% v/v
628 Hydrochloric acid (HCl) was slowly added to each sample, and stirred using a
629 glass stirring rod. The samples were placed in the waterbath at 90°C for 20
630 minutes. They were then centrifuged in a Heraeus Megafuge 16 at 4500 rpm
631 for three minutes. After the supernatant had been decanted they were mixed
632 with deionised water and centrifuged again.
- 633 3. **Removal of humic acids by alkali digestion (KOH).** 10ml 10% w/v KOH
634 was added to the samples, which were then placed in the water bath at 90°C
635 for 10 minutes. The samples were then centrifuged at 4500 rpm for three
636 minutes, and the supernatant was decanted.
- 637 4. **Removal of coarse plant debris.** Approximately 5 ml of water was added to
638 each sample. The samples were then agitated into suspension using the
639 vortex mixer, and washed through 180 micron sieve mesh down a funnel into
640 a clean 50 mL centrifuge tube. Deionised water was added to balance the
641 samples, and then they were centrifuged at 4500 rpm for three minutes and
642 the supernatant was decanted.
- 643 5. **Removal of clays and breakdown of products of alkali digestion.** The
644 samples were re-suspended in water using the vortex mixture and then
645 centrifuged at 2200 rpm for three minutes. The supernatant was decanted.
646 This step was repeated up to 20 times, until the supernatant was clear.
- 647 6. a) **Removal of silicates using HF.** The samples were suspended in 5 ml of
648 10% HCl, to ensure the sample is entirely free from carbonates and to acidify

the sample. They were stirred and then centrifuged at 4500 rpm for 3 minutes, after which the supernatant was decanted. Working in a HF-prepared fume cupboard with environmental scrubbers and wearing suitable safety equipment (face mask, environmental hazard suit, rubber boots, double nitrile gloves) 3 ml of HF was added, and the samples were stirred carefully with polypropylene stirring rods. They were then placed in a hot water bath for 20 minutes, and the stirring rods were used to check for remaining grittiness in the tube. The samples were heated until all silica had been dissolved. The supernatant was decanted and 5ml 10% HCl was added to the pellet and stirred. The tubes were placed back into the hot water bath for 15 minutes to remove any silicofluorides. They were centrifuged at 4500 rpm for three minutes, decanted, and topped up with deionised water. This latter step was repeated three times.

b) **Density separation using SPT.** A suitable volume of SPT (sodium polytungstate) solution was prepared to a specific gravity between 1.88-1.91 g/cm³, allowing for 10 ml per sample. The software LSTCalc (<http://www.polytungstate.co.uk/lstcalc.html>) was used to help determine the correct volume of water to add to the starting volume of SPT of known specific gravity. The samples were suspended in 5 ml 10% HCl to acidify the sample, which aids with the density separation. They were mixed and centrifuged at 4500 rpm for three minutes, and the supernatant was decanted fully, removing as much water as possible so as not to affect the density of the SPT. 10 ml of the prepared SPT solution was added to each tube. The tubes were capped and mixed using the vortex mixer for at least 20 seconds each. The samples were centrifuged at 1800 rpm for 20 minutes. They were then left overnight to further aid separation; this option allows for material in suspension in the supernatant to float to the top, making it easier to see and decant the floating organic material. The supernatant of each sample was poured into a new centrifuge tube, topped up with deionised water to within 5 ml of the top of the tube (reducing the overall density to around 1.15-1.2 g/cm³), capped, and mixed by performing 8-10 inversions by hand. The centrifuge tubes containing the supernatant were then centrifuged at 4500 rpm for three minutes. The supernatant was decanted into a wash bottle for

later recycling of SPT. The samples were topped up with deionised water and centrifuged and decanted again twice.

7. **Removal of cellulose by acetolysis.** The acetolysis mixture was prepared by mixing nine parts of acetic anhydride with one part concentrated sulphuric acid. The samples were suspended in 5 ml of glacial acetic acid, to dehydrate the sample; they were centrifuged at 4500 rpm for three minutes, and the supernatant was decanted. 5 ml of the acetolysis mixture was added to each tube. The samples were then placed in the water bath at 90°C for two minutes. They were centrifuged at 4500 rpm for two minutes and decanted. 5 ml of glacial acetic was added; the samples were stirred, centrifuged at 4500 rpm for three minutes, and decanted. They were suspended in 5ml 10% KOH to neutralise the acid, stirred, centrifuged at 4500 rpm for three minutes and then decanted. Finally they were suspended in 5 ml of deionised water, centrifuged at 4500 rpm for three minutes, and the supernatant was decanted.

8. **Alcoholic dehydration.** After suspending the samples in 5 ml of ethanol, they were mixed and centrifuged for three minutes at 4500 rpm and then decanted. They were then suspended in 1 ml of tert-butyl alcohol (TBA) and centrifuged at 4500 rpm, and then decanted. 1 ml TBA was added to each sample; they were mixed, and then transferred to a glass vial with a pasteur pipette. The samples were centrifuged at 4500 rpm for three minutes, and then decanted. Approximately 0.5 ml of silicone oil was added to the samples, and the vials were topped with cotton wool. The samples were left for two days to allow the remaining TBA to evaporate.

9. **Making slides.** Where necessary, additional silicone oil was added to the samples, which were stirred with a microspatula. A small drop of the sample was placed in the middle of the slide, and a glass cover slip was slowly placed over the top. A seeker was used to apply gentle pressure to the slip, allowing the sample to spread out. The slip was sealed with clear nail varnish, and labelled.

Appendix 2: 95% confidence intervals for the five most common taxa

Sample		% Quercus evergreen			% Quercus deciduous			% Cedrus			% Poaceae			% Cupressaceae		
		Upper 95%	Mid point	Lower 95%	Upper 95%	Mid point	Lower 95%	Upper 95%	Mid point	Lower 95%	Upper 95%	Mid point	Lower 95%	Upper 95%	Mid point	Lower 95%
SA 1	HF	40.55	35.06	29.95	15.76	11.69	8.56	27.04	22.08	17.81	11.71	8.12	5.56	8.99	5.81	3.70
	SPT	38.04	32.56	27.51	15.00	10.96	7.91	31.84	26.58	21.91	15.00	10.96	7.91	8.06	4.98	3.04
SA 2	HF	24.89	20.00	15.87	6.03	3.33	1.82	26.67	21.67	17.38	16.17	12.00	8.80	15.79	11.67	8.51
	SPT	23.95	19.14	15.11	6.79	3.96	2.28	24.30	19.47	15.41	14.53	10.56	7.58	16.75	12.54	9.27
SA 3	HF	29.19	24.10	19.66	8.30	5.21	3.23	32.27	27.04	22.37	6.71	3.91	2.25	18.34	14.01	10.57
	SPT	28.09	23.00	18.60	7.27	4.33	2.55	35.07	29.67	24.78	7.68	4.67	2.80	20.94	16.33	12.58
SA 4	HF	25.44	20.53	16.36	8.43	5.30	3.29	24.73	19.87	15.76	5.57	2.98	1.58	27.91	22.85	18.47
	SPT	27.12	22.15	17.86	9.46	6.19	4.00	21.54	16.94	13.16	6.30	3.58	2.01	25.74	20.85	16.68
SA 5	HF	36.67	31.23	26.26	7.25	4.32	2.54	24.81	19.93	15.81	7.25	4.32	2.54	17.59	13.29	9.91
	SPT	33.98	28.71	23.96	5.84	3.23	1.76	26.53	21.61	17.39	5.84	3.23	1.76	18.87	14.52	11.03
SA 6	HF	27.81	22.80	18.46	9.85	6.51	4.26	18.69	14.33	10.85	13.98	10.10	7.21	24.00	19.22	15.20
	SPT	29.60	24.52	20.06	9.37	6.13	3.96	20.63	16.13	12.45	11.63	8.06	5.52	20.98	16.45	12.74
SA 7	HF	27.12	22.15	17.86	7.11	4.23	2.49	28.85	23.78	19.36	9.08	5.86	3.74	23.65	18.89	14.91
	SPT	26.35	21.43	17.21	8.66	5.52	3.47	27.04	22.08	17.81	10.58	7.14	4.76	22.17	17.53	13.69
SA 8	HF	29.37	24.38	19.99	21.70	17.19	13.45	30.35	25.31	20.86	15.18	11.25	8.24	7.57	4.67	2.85
	SPT	29.05	23.92	19.45	24.81	19.93	15.81	35.30	29.90	25.01	12.74	8.97	6.24	5.58	2.99	1.58
SA 9	HF	30.90	25.67	21.05	20.57	16.00	12.29	31.59	26.33	21.67	14.29	10.33	7.38	8.08	5.00	3.05
	SPT	31.38	26.27	21.72	24.35	19.62	15.62	27.72	22.78	18.50	11.05	7.59	5.16	7.51	4.56	2.74
SA 10	HF	39.45	34.07	29.07	14.26	10.41	7.51	14.61	10.73	7.78	14.26	10.41	7.51	10.62	7.23	4.87
	SPT	38.16	32.81	27.87	17.77	13.56	10.23	17.08	12.93	9.68	12.11	8.52	5.92	10.65	7.26	4.88
CZ 1	HF	17.70	13.38	9.98	3.85	1.67	0.72	40.00	34.45	29.29	21.01	16.39	12.62	7.30	4.35	2.56
	SPT	15.32	11.26	8.17	4.71	2.32	1.13	41.99	36.42	31.20	22.24	17.55	13.67	5.57	2.98	1.58
CZ 2	HF	7.51	4.56	2.74	3.76	1.63	0.70	38.67	33.22	28.19	25.74	20.85	16.68	11.74	8.14	5.58
	SPT	6.38	3.63	2.04	2.87	0.99	0.34	43.87	38.28	32.99	23.95	19.14	15.11	9.97	6.60	4.31
CZ 3	HF	9.82	6.49	4.24	3.74	1.62	0.70	63.81	58.44	52.87	12.45	8.77	6.09	5.87	3.25	1.77
	SPT	10.96	7.47	5.03	4.18	1.95	0.90	61.28	55.84	50.26	11.33	7.79	5.29	6.69	3.90	2.24
CZ 4	HF	10.38	7.01	4.67	7.34	4.46	2.67	58.94	53.50	47.98	11.49	7.96	5.45	5.76	3.28	1.74
	SPT	12.91	9.15	6.41	6.32	3.59	2.02	57.82	52.29	46.70	12.91	9.15	6.41	8.32	5.23	3.24
CZ 5	HF	11.60	8.04	5.50	5.82	3.22	1.76	53.45	47.91	42.42	13.44	9.65	6.84	9.72	6.43	4.20
	SPT	10.58	7.14	4.76	4.62	2.27	1.11	56.19	50.65	45.09	14.67	10.71	7.73	9.05	5.84	3.73
CZ 6	HF	9.38	6.06	3.87	2.42	0.67	0.18	70.19	64.98	59.40	10.96	7.41	4.94	3.88	1.68	0.72
	SPT	7.18	4.28	2.52	3.79	1.64	0.70	66.81	61.51	55.93	13.74	9.87	7.00	5.11	2.63	1.34
CZ 7	HF	10.99	7.49	5.04	2.83	0.98	0.33	69.64	64.50	58.99	10.23	6.84	4.52	7.11	4.23	2.49
	SPT	9.11	5.88	3.75	3.77	1.63	0.70	72.33	67.32	61.88	8.32	5.23	3.24	6.32	3.59	2.02
CZ 8	HF	9.79	6.47	4.23	2.83	0.98	0.33	47.93	42.35	36.95	13.52	9.71	6.89	4.63	2.28	1.11
	SPT	12.99	9.21	6.45	4.24	1.97	0.91	46.40	40.79	35.41	12.24	8.55	5.90	5.95	3.29	1.80
CZ 9	HF	22.81	18.09	14.17	10.68	7.21	4.81	4.22	1.97	0.90	13.74	9.87	7.00	3.78	1.64	0.70
	SPT	20.38	15.92	12.29	8.50	5.41	3.41	6.16	3.50	1.97	14.75	10.83	7.85	2.77	0.96	0.33
CZ 10	HF	5.87	3.25	1.77	2.82	0.97	0.33	4.62	2.27	1.11	24.27	19.48	15.44	7.88	4.87	2.97
	SPT	8.22	5.16	3.20	2.32	0.65	0.18	3.72	1.61	0.69	22.73	18.06	14.18	9.37	6.13	3.96

AA 1	HF	20.70	16.18	12.49	2.82	0.97	0.33	42.73	37.22	32.01	25.92	21.04	16.86	3.73	1.62	0.69
	SPT	24.00	19.22	15.20	1.82	0.33	0.06	38.34	32.90	27.88	28.50	23.45	19.06	4.63	2.28	1.11
AA 2	HF	15.79	11.67	8.51	6.03	3.33	1.82	42.26	36.67	31.41	22.02	17.33	13.47	3.84	1.78	0.71
	SPT	17.56	13.31	9.97	7.48	4.55	2.73	39.22	33.77	28.71	18.99	14.61	11.10	5.87	3.25	1.77
AA 3	HF	33.62	28.34	23.59	2.83	0.98	0.33	34.98	29.64	24.81	11.74	8.14	5.58	10.99	7.49	5.04
	SPT	31.74	26.49	21.83	4.27	1.99	0.91	38.26	32.78	27.73	10.78	7.28	4.86	9.22	5.96	3.80
AA 4	HF	22.87	18.18	14.28	7.88	4.87	2.97	18.99	14.61	11.10	17.20	12.99	9.68	7.88	4.87	2.97
	SPT	20.90	16.34	12.62	6.73	3.92	2.26	21.60	16.99	13.20	19.47	15.03	11.46	9.11	5.88	3.75
AA 5	HF	18.57	14.19	10.71	5.97	3.30	1.80	36.78	31.35	26.39	9.59	6.27	4.05	1.85	0.33	0.06
	SPT	16.48	12.34	9.12	7.88	4.87	2.97	33.52	28.25	23.51	7.88	4.87	2.97	2.34	0.65	0.18
AA 6	HF	17.15	12.94	9.65	4.60	2.27	1.10	36.10	30.74	25.86	20.35	15.86	12.21	9.40	6.15	3.97
	SPT	19.68	15.29	11.73	6.95	4.14	2.44	33.90	28.66	23.94	21.42	16.88	13.14	7.34	4.46	2.67
AA7	HF	29.44	24.35	19.89	7.88	4.87	2.97	5.87	3.25	1.77	12.45	8.77	6.09	2.34	0.75	0.18
	SPT	32.29	27.00	22.29	9.68	6.33	4.09	7.68	4.67	2.80	12.40	8.67	5.98	3.84	1.67	0.71
AA 8	HF	20.68	14.29	9.63	13.13	7.79	4.51	46.19	38.31	31.01	20.68	14.29	9.63	6.49	2.60	1.01
	SPT	18.82	12.58	8.21	11.76	6.62	3.64	43.00	35.10	27.94	24.03	17.22	12.03	6.61	2.65	1.03
AA 9	HF	19.48	15.00	11.40	8.08	5.00	3.05	51.32	45.67	40.12	6.86	4.00	2.30	3.84	1.67	0.71
	SPT	16.54	12.33	9.08	6.86	4.00	2.30	53.64	48.00	42.41	5.60	3.00	1.59	2.40	0.77	0.18
AA 10	HF	27.03	22.04	17.74	5.95	3.29	1.80	61.39	55.92	50.30	2.37	0.66	0.18	1.84	0.33	0.06
	SPT	25.80	20.86	16.66	7.63	4.64	2.78	58.86	53.31	47.68	3.36	1.32	0.52	3.36	1.32	0.52

715 **Figure 1:** The location of the three sites: Sidi Ali, Col du Zad and Aguelmam Azougagh (AA), indicated by the star, the square and
716 the circle, respectively.

718 **Figure 2:** Photographs of the three sites: a) Lake Sidi Ali; b) Col du Zad; c)
719 Aguelmam Azougagh.

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722 **Figure 3:** A summary of the preparation protocol used for the pollen, NPP and
723 charcoal samples.

724

725 **Figure 4:** Diagrams showing the pollen, fungal spore and charcoal counts for: a) Lake Sidi Ali, b) Col du Zad, and c) Aguelmam
726 Azougagh.

Figure 5: Linear regressions for the one-on-one statistics. The black dashed line indicates the initial linear regression ($y = mx + c$); the solid red line indicates the linear regression that was refit so that $y = mx$.

733 **Figure 6:** Images of the slides prepared for all three sites, using the two separate
734 preparation methods. 1a - Sidi Ali, SPT; 1b - Sidi Ali, HF; 2a - Col du Zad, SPT; 2b -
735 Col du Zad, HF; 3a - Aguelmam Azougagh, SPT; 3b - Aguelmam Azougagh, HF.