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

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1 **A comparison of pollen extraction methods: confirmation of dense-media**

2 **separation as a reliable method of pollen preparation**

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8 **Running head**

9 Dense-media separation as a reliable method of pollen preparation

10 **Abstract**

11 Palynology is a crucial proxy for understanding Quaternary environmental change. A

12 range of laboratory preparation techniques has been developed to deal with the

13 extraction of pollen in different sedimentary contexts. Here, we present a comparison

14 of the conventional hydrofluoric acid (HF) method and the dense-media separation

15 method using sodium polytungstate (SPT). We examine pollen, non-pollen

16 palynomorphs (NPPs) and microcharcoal by undertaking parallel preparation and

17 counting of thirty paired samples from three sites in the Middle Atlas Mountains,

18 Morocco, with contrasting environmental and sedimentary conditions. Differences

19 between microfossil counts are assessed visually and statistically, using ANOVA and

20 linear regression. We observe that the typical offset between counts for the five most

21 common pollen and NPPs produced by the two methods (μ=1.6%, sd=0.9%) is

22 considerably smaller than the 95% uncertainty associated with the standard counting

23 procedure (μ=6.8%, sd=2.7%). There is no statistically significant difference

24 associated with the preparation methods, and no significant deviation from a linear

25 relationship between the results obtained by the two methods. There are advantages

26 and disadvantages to both methods in terms of preparation time and visual

27 characteristics. This study confirms that results for pollen, NPPs and microcharcoal

28 obtained by either preparation method can be directly compared.

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

31

1. Introduction

33 Pollen analysis is a commonly used method for reconstructing past environmental
34 change, and is used widely in Quaternary research (Seppä and Bennett, 2003). Part
35 of the challenge of extracting pollen and other organic microfossils from sediment is
36 separating organic material from siliceous mineral matter of similar size to pollen
37 (silts). The method that is most commonly used involves the dissolution of siliceous
38 material using hydrofluoric acid (HF) (Faegri *et al.*, 2000). An alternative approach is
39 the use of dense-media to separate pollen from the rest of the sediment matrix, such
40 as Thoulet's liquid, $ZnCl_2$, sodium polytungstate (SPT) and lithium
41 heteropolytungstate (LST) (Wood *et al.*, 1996; Nakagawa *et al.*, 1998; van Geel *et
42 al.*, 2011; Caffrey and Horn, 2013). The dense-media separation method is typically
43 used to extract pollen grains from clay-rich or silty sediments, where it may be used
44 to extract very small levels of pollen from highly minerogenic sediments, often where
45 the use of HF has failed to yield substantial quantities of pollen (e.g. Allen *et al.*,
46 2009; Caffrey and Horn, 2013; Fletcher and Hughes, in press). Dense-media
47 separation can also be used for pollen extraction in more organic-rich material
48 (Nakagawa *et al.*, 1998). Organic-rich samples are often abundant in pollen;
49 however, isolating pollen can be problematic as a result of the difficulty of digesting
50 and removing small organic fragments (Nakagawa *et al.*, 1998). There is evidence to
51 suggest that dense-media separation is highly efficient when preparing organic
52 material for pollen analysis, and can often produce material for analysis that is easier
53 to count than samples that undergo the conventional method using HF (Phipps and
54 Playford, 1984; Forster and Flenley, 1993; Nakagawa *et al.*, 1998; Allen *et al.*, 2009).
55 More recently, dense-media separation has also been adopted as a method in
56 palaeoecology for extraction of non-pollen palynomorphs (NPPs) and microcharcoal
57 analyses (e.g. Turner *et al.*, 2008; van Geel *et al.*, 2011).

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

60 separation. A pioneering study (Björck *et al.*, 1978) compared the results of pollen

61 analysis via the HF and ZnCl$_2$ separation methods on clay-rich lake sediments from

62 south-eastern Sweden, and reported good correspondence between the results

63 obtained by the two methods. Nakagawa *et al.* (1998) examined a set of eleven

64 samples from a range of depositional contexts, and observed compatible results for

65 HF and dense-medium preparations. To date, however, the papers that compare the

66 two methods focus solely on pollen analysis, and do not provide comparative records

67 for either NPPs or microcharcoal (e.g. Nakagawa *et al.*, 1998; Caffrey and Horn,

68 2013). In addition, comparisons so far have used fairly small numbers of paired

69 samples, and there is little statistical support for the findings. Overall, robust

70 evaluation of key methodological stages in microfossil preparation remains relatively

71 rare (cf. Wolfe, 1997; Riding and Kyffin-Hughes, 2004; Mertens *et al.*, 2009), but is

72 critical for understanding the extent to which results from different studies can be

73 considered compatible. This paper aims to test whether dense-media separation

74 using SPT is a reliable method of pollen preparation for both organic and

75 minerogenic sediments. It provides a comparison of thirty paired samples prepared

76 in parallel for both HF and dense-media separation, analysing pollen, NPPs and

77 microcharcoal in the residues.

78 **2.   Research sites**

79 The three research sites that are used in this investigation are located in the Middle

80 Atlas Mountains, Morocco. The sites lie within 10 km of each other, close to the

81 transition between the Mediterranean and Saharan climatic zones (Figure 1). The

82 regional climate is semi-arid, and is influenced by the sub-tropical high-pressure belt

83 during the summer, and the Atlantic westerly circulation in the winter (Lamb *et al.*,

84 1999). The sites are located in carbonate bedrock areas and occur in depressions

85 related to karstic and tectonic processes. Table 1 summarises the key characteristics

86 of each site.

87 INSERT FIGURE 1 HERE.

88 The first site is Lake Sidi Ali (Figure 2a). Sitting at 2080 m.a.s.l., Sidi Ali is a

89 permanent, closed waterbody and one of the largest and highest lakes in the Middle

90   Atlas Mountains. It is located at 33° 03' N, 05° 00' W, within a small closed

91   catchment (~14 km^2), with a surface area of 2.8 km^2 and a water depth of up to 40m.

92   A 19.56 metre core was extracted using a UWITEC piston corer with a 2m core

93   chamber at the deepest part of the lake (full method described in Zielhofer *et al.*, in

94   review). The sediment at Lake Sid Ali is classified as organic silt, averaging

95   approximately 12% total organic carbon (TOC), and is rich in pollen, with a typical

96   concentration of 2.5 x 10^5 grains cm^{-3}.

97   INSERT FIGURE 2 HERE.

98   Col du Zad (33° 01' 44.8" N, 05° 04' 7.9" W, 2148 m a.s.l) is a small (0.25 km^2)

99   waterlogged depression fed by groundwater springs containing a shallow, seasonal

100 pond (Figure 2b). A 2.2 m core was collected from the site using a Russian corer.

101 The sedimentary infill is predominantly minerogenic silts and clay, with organic

102 matter content determined by loss-on-ignition typically 14% (i.e. an estimated ~6-9%

103 TOC following Veres, 2012). The pollen concentrations are an order of magnitude

104 lower than at Sidi Ali, typically 7.0 x 10^4 grains cm^{-3}.

105 The third site is a shallow lake with a surrounding marshy area (Figure 2c).

106 Aguelmam Azougagh lies a couple of kilometres south west of Col du Zad (33° 01'

107 23.2" N, 05° 05' 29" W, 2058 m a.s.l). A 4.4 m core from the marsh was collected

108 using a combination of Russian corer and gouge auger. The sediment is

109 predominantly minerogenic, but has a moderate organic content throughout the core,

110 averaging 13% (~5-9% TOC), reaching > 30% (i.e. an estimated ~12-20% TOC

111 maximum). Typical pollen concentrations are slightly higher than at Col du Zad (9.8 x

112 10^4 grains cm^{-3}).

113 These three sites present contrasting environmental conditions, with sediment from a

114 deep lake, a shallow, marshy lake, and a seasonally waterlogged bog. In addition,

115 the three cores contain a range of sediment types, and as a result we have tested

116 our hypotheses on sediments that are organic rich, highly minerogenic, and less

117 minerogenic clays.

118 **3.   Methodology**

119 *3.1.   Pollen preparation*

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

130 INSERT FIGURE 3 HERE

131 *3.2. Pollen, NPP and charcoal analysis*

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

144 $$X_\% = \frac{N_X}{TLP + N_X} \times 100$$

146 Where $X_\%$ is the percentage of the individual NPP taxon, TLP is total land pollen
147 counted and N_X is the number of NPPs of that taxon counted (Moore *et al.*, 1991).

148 Microscopic charcoal analysis was conducted using the same slides as the pollen
149 analysis. A sum of 200 charcoal particles and *Lycopodium* spores was counted in
150 each sample, to reach an accurate estimate of particles for the entire sample, with
151 less than 5% error (Finsinger and Tinner, 2005). These were split into four size
152 classes: < 20 µm, 20-50 µm, 50-100 µm, >100 µm, measured using a calibrated
153 graticule. Charcoal concentrations are expressed in number of fragments per cm^3.

154 *3.3. Statistical analysis*

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

170 *3.3.1. 95% confidence limits*

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

178 *3.3.2. ANOVA (analysis of variance)*

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

196 *3.3.3. Assessment of one-on-one relationship by linear regression*

197 An assessment of the relationship between the two methods was then conducted
198 using a linear regression approach, adapted from the assessment carried out for
199 geochemical data obtained by different measurement techniques (assessing
200 accuracy of field portable X-ray fluorescence measurements), by Kilbride *et al.*
201 (2006) and Shuttleworth *et al.* (2014). Linear regression was performed on all pollen
202 and NPP taxa occurring at least once at > 5% abundance, as well as on total
203 microcharcoal concentrations and *Lycopodium* counts. Rare taxa are excluded
204 because the abundance of zero values makes them unsuitable for this analysis. The
205 analyses are summarised in Figure 4.

206 For each taxon (pollen and NPP), least-squares linear regression is undertaken,
207 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.

237 INSERT FIGURE 4 HERE

4.2.   95% confidence intervals

239 The 95% confidence intervals of the five most common pollen taxa were calculated
240 for each of the sixty counts (*Cedrus, Quercus* evergreen type, *Quercus* deciduous
241 type, Poaceae, Cupressaceae). For every single sample, the HF count falls within
242 the confidence interval of the SPT count, and vice versa (Appendix 2). The average
243 difference between the upper and lower limits of the 95% confidence intervals were
244 calculated, and these were as follows: *Cedrus* 9.6%, *Quercus* evergreen type 8.1%,
245 *Quercus* deciduous type 4.7%, Poaceae 6.6%, Cupressaceae 5.1%. These values
246 clearly indicate that there is a fairly wide uncertainty associated with the standard
247 counting procedure (300 pollen grain main sum), and that the confidence intervals
248 are greater than the absolute differences associated with the two preparation method
249 (averages: *Cedrus* 2.6%, *Quercus* evergreen type 1.7%, *Quercus* deciduous type
250 1.1%, Poaceae 1.5%, Cupressaceae 1.1%). Overall, the typical offset between
251 counts for the five most common pollen and NPPs produced by the two methods
252 (μ=1.6%, sd=0.9%) is considerably smaller than the 95% uncertainty associated with

253 the standard counting procedure (μ=6.8%, sd=2.7%).

4.3.   ANOVA

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

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

268 INSERT TABLES 2 AND 3 HERE

269 *4.4. Linear regression*

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

286 INSERT TABLE 2 AND FIGURE 5 HERE

287

288 **5.   Discussion**

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

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

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

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

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

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

344 Despite the statistical similarity of the microfossil content, visual characteristics of the
345 residues are notably different between the HF and SPT approaches. As noted by
346 Nakagawa *et al.* (1998), pollen samples prepared using the dense-media separation
347 method are substantially cleaner and more efficient for counting. When reviewing the
348 palaeoenvironmental context of Late-glacial woolly mammoth discoveries, Allen *et al.*
349 (2009) had to re-prepare their samples for pollen analysis using the dense-media
350 separation method after insufficient results were achieved using the conventional
351 method (using HF). They too found the dense-media separation provided much more
352 efficient material for counting. Figure 6 shows photographs of pollen slides used in
353 this investigation. We draw similar conclusions to those of Nakagawa *et al.* (1998)
354 and Allen *et al.* (2009). The dense-media separation samples generated much
355 cleaner slides; the majority of material was in fact pollen grains, NPPs and charcoal,
356 and there was a much smaller amount of excess organic debris on the slides. As a

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

363   INSERT FIGURE 6 HERE

364   An interesting point to note is that there were high levels of other organic matter in
365   the HF samples, but much less in the dense-media separation samples. Both sets of
366   samples were subjected to the same preparation, other than stage 6. HF treatment
367   removes siliceous material, while the removal of humic material from the samples
368   occurs at stage 3, using potassium hydroxide (Faegri *et al.,* 2000). This suggests
369   that during dense-media separation, some dense organic fragments that have not
370   already been destroyed sink to the bottom of the centrifuge tube, and are not
371   transferred along with the pollen grains after dense-media separation has been
372   carried out. Microscopic observation of the heavy residue confirms that there is
373   organic material in the dense fraction settling at 1.88-1.91 g/cm^3, including compact,
374   angular unidentifiable organic fragments, as well as rare pollen grains. Also, some
375   very low density organic matter may be further separated from the main pollen
376   residue at the end of the density separation, when the density is lowered to around
377   1.15-1.2 g/cm^3 (typically the lowest achievable density without resorting to
378   partitioning the sample across multiple centrifuge tubes). Overall, while the exact
379   reasons are not fully known, the density separation procedure appears to
380   concentrate microfossils with respect to other organic detritus. This concentration
381   effect is capitalised upon, for example, in the preparation of pollen concentrates for
382   radiocarbon dating (Vandergoes and Prior, 2003).

383   In addition to the clarity of the slides, we also evaluated a range of other advantages
384   and disadvantages of each method. Typically, the HF method has a quicker
385   preparation time than the dense-media separation method when pre-preparation
386   time is taken into account (i.e. preparation of the solution to appropriate density), and
387   time for the samples to settle (not strictly required but makes the decantation easier;
388   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 (μ=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.

7.   Acknowledgements

457

8.   References

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

614

615

Taxa	Type	Average %	Residuals normal?	R^2	c	Accept c = 0?	m	Accept m = 1?	Quality level
Cedrus	Pollen	27.8	Yes	0.973	1.452	Yes	0.957	Yes	Definitive (y = x)
Botryococcus	Pollen	19.1	Yes	0.999	-0.068	Yes	0.995	Yes	Definitive (y = x)
Quercus evergreen	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)
Ranunculus	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)
Quercus deciduous	Pollen	5.0	Yes	0.930	0.062	Yes	1.062	Yes	Definitive (y = x)
Potamogeton	Pollen	4.3	Yes	0.986	0.007	Yes	0.982	Yes	Definitive (y = x)
Myriophyllum	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)
Ulex type	Pollen	2.2	Yes	0.851	0.328	Yes	0.961	Yes	Definitive (y = x)
Artemisia	Pollen	1.9	Yes	0.854	0.264	Yes	0.872	Yes	Definitive (y = x)
Astragalus danicus	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)
Rumex acetosa	Pollen	1.5	Yes	0.974	0.989	Yes	0.078	Yes	Definitive (y = x)
Ateraceae *aster*	Pollen	1.3	Yes	0.940	0.077	Yes	0.8665	Yes	Definitive (y = x)
Olea	Pollen	0.9	Yes	0.974	1.452	Yes	0.957	Yes	Definitive (y = x)
Caryophyllaceae	Pollen	0.7	Yes	0.981	-0.137	Yes	1.155	No	Quantitative (y = mx)
Rumex acetosella	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)
Lycopodium	*Lycopodium*	N/A	Yes	0.982	-7.580	Yes	1.056	Yes	Definitive (y = x)

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

1. **Volumetric sampling and the addition of *Lycopodium.*** Plastic syringes with a 1 cm^3 volume were used to measure out each individual sample. The samples were transferred to large (50 mL) centrifuge tubes and a single *Lycopodium* tablet was added to each sample. The addition of the *Lycopodium*, an exotic marker grain, to a known volume of sediment enables the absolute abundance of pollen grains to be determined (Stockmarr, 1971).

2. **Removal of carbonates by acid digestion (HCl).** 10 ml of 10% v/v Hydrochloric acid (HCl) was slowly added to each sample, and stirred using a glass stirring rod. The samples were placed in the waterbath at 90°C for 20 minutes. They were then centrifuged in a Heraeus Megafuge 16 at 4500 rpm for three minutes. After the supernatant had been decanted they were mixed with deionised water and centrifuged again.

3. **Removal of humic acids by alkali digestion (KOH).** 10ml 10% w/v KOH was added to the samples, which were then placed in the water bath at 90°C for 10 minutes. The samples were then centrifuged at 4500 rpm for three minutes, and the supernatant was decanted.

4. **Removal of coarse plant debris.** Approximately 5 ml of water was added to each sample. The samples were then agitated into suspension using the vortex mixer, and washed through 180 micron sieve mesh down a funnel into a clean 50 mL centrifuge tube. Deionised water was added to balance the samples, and then they were centrifuged at 4500 rpm for three minutes and the supernatant was decanted.

5. **Removal of clays and breakdown of products of alkali digestion.** The samples were re-suspended in water using the vortex mixture and then centrifuged at 2200 rpm for three minutes. The supernatant was decanted. This step was repeated up to 20 times, until the supernatant was clear.

6. a) **Removal of silicates using HF.** The samples were suspended in 5 ml of 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 careful 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/cm^3, 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/cm^3), 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

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

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

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

706 **9. Making slides.** Where necessary, additional silicone oil was added to the
707 samples, which were stirred with a microspatula. A small drop of the sample
708 was placed in the middle of the slide, and a glass cover slip was slowly
709 placed over the top. A seeker was used to apply gentle pressure to the slip,
710 allowing the sample to spread out. The slip was sealed with clear nail
711 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

714

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
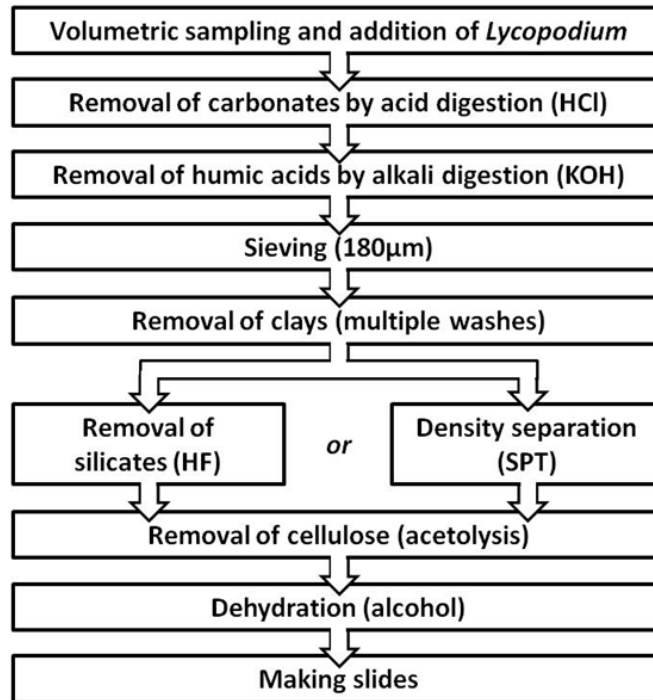
716 the circle, respectively.

717
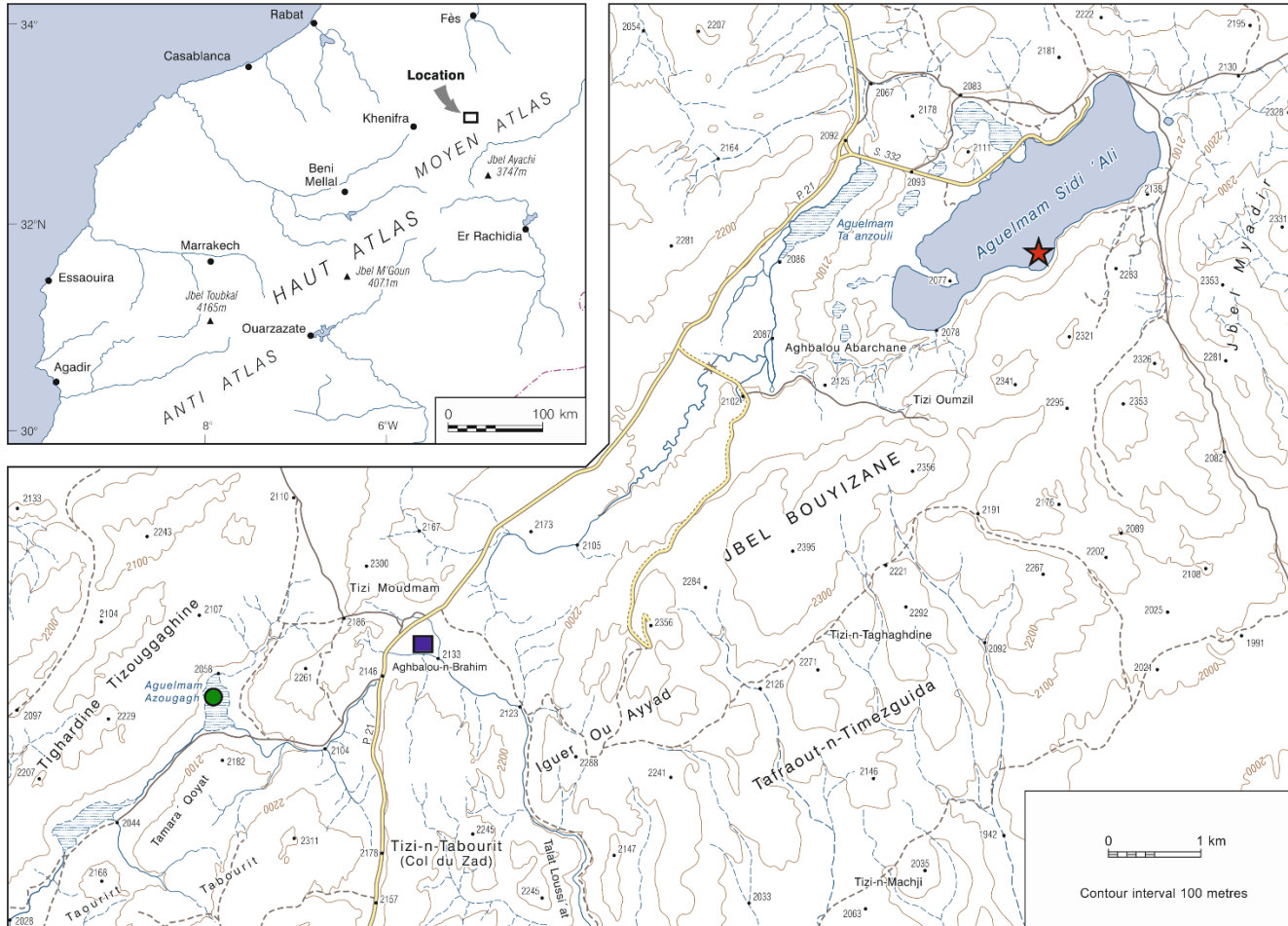
718   **Figure 2**: Photographs of the three sites: a) Lake Sidi Ali; b) Col du Zad; c)
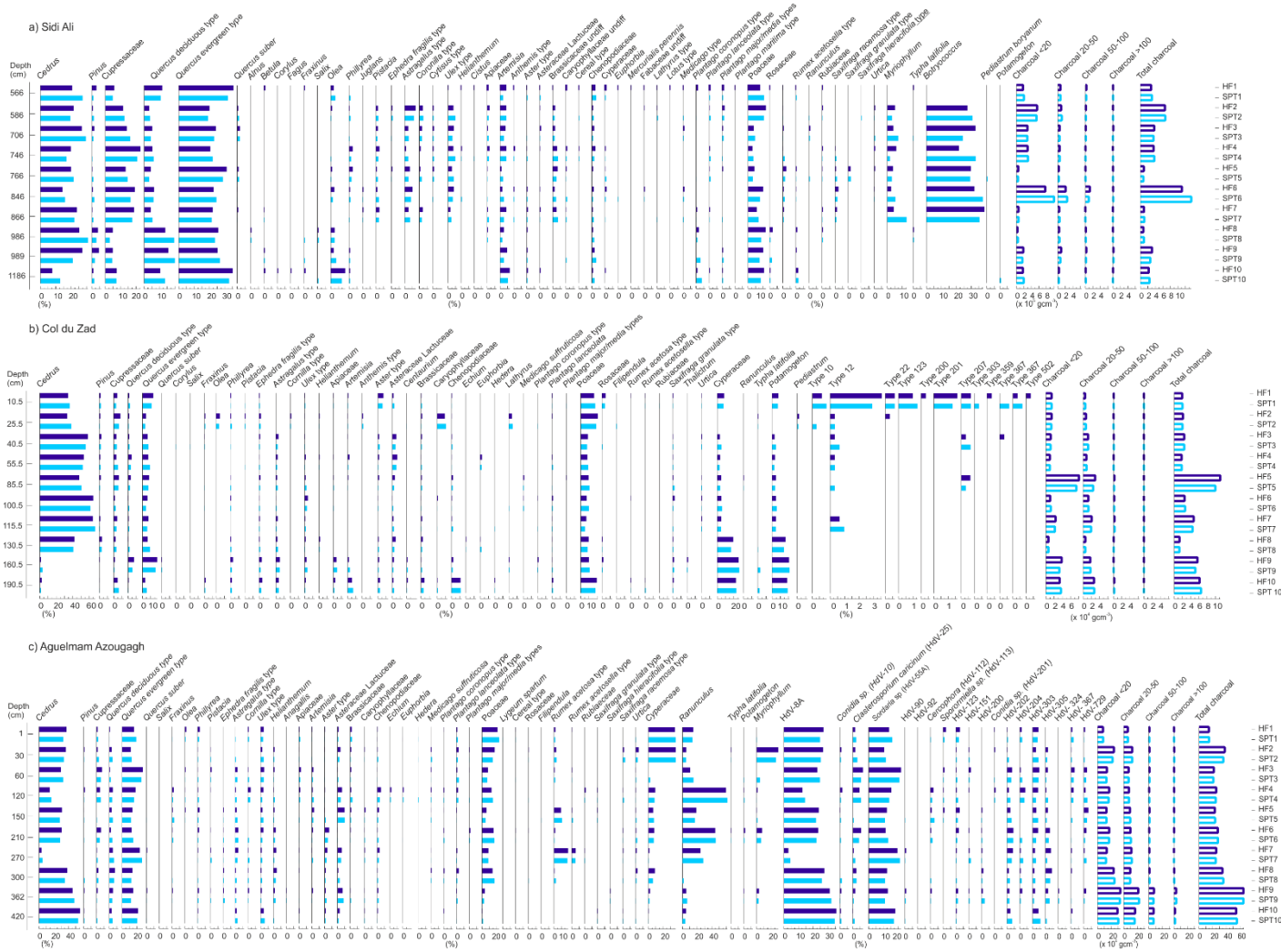719   Aguelmam Azougagh.

720
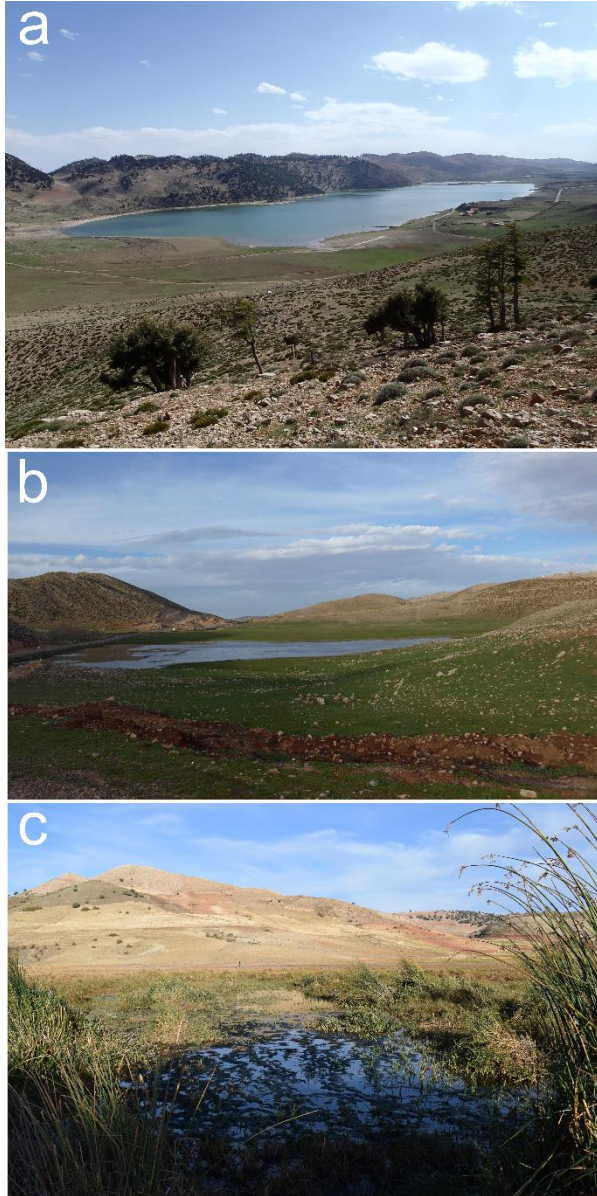
721

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

724

Figure 4: Diagrams showing the pollen, fungal spore and charcoal counts for: a) Lake Sidi Ali, b) Col du Zad, and c) Aguelmam Azougagh.
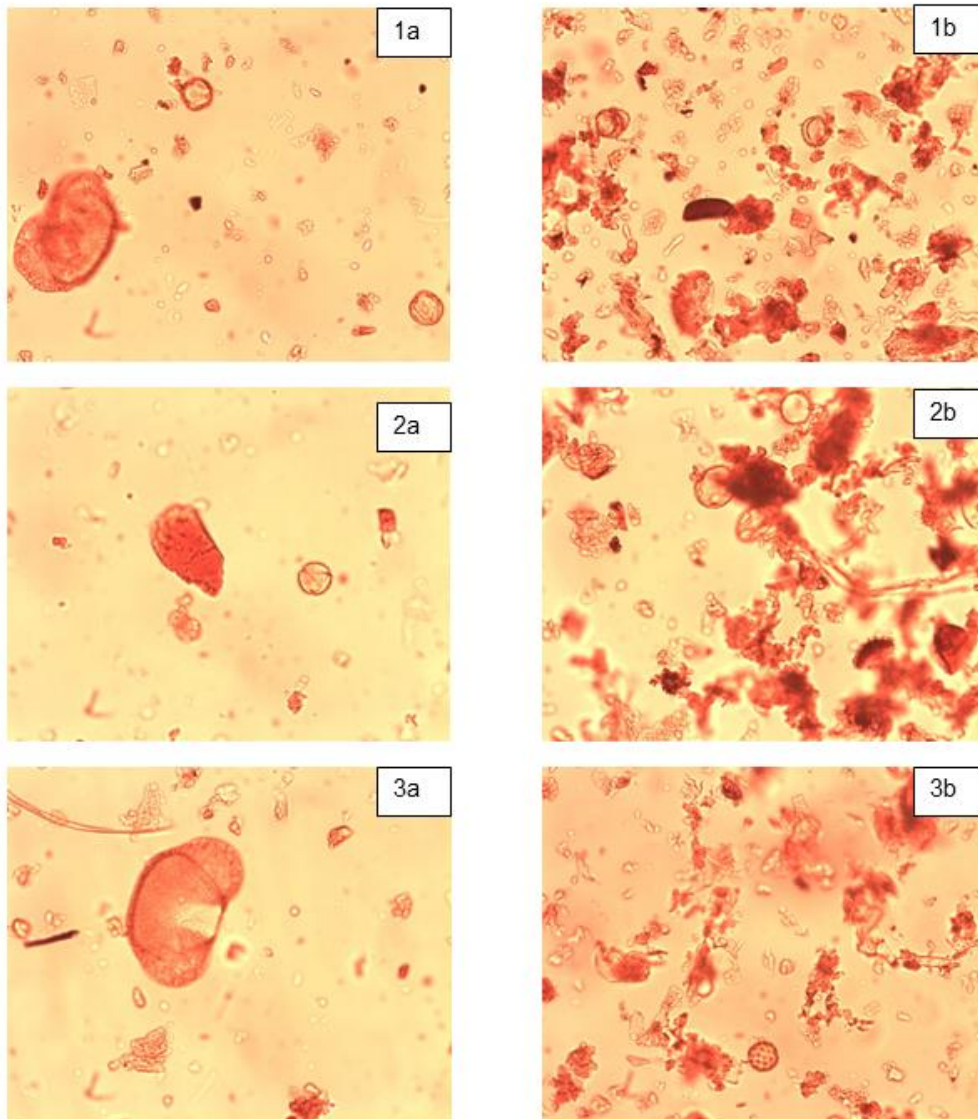
728 **Figure 5**: Linear regressions for the one-on-one statistics. The black dashed line
729 indicates the initial linear regression (y= mx + c); the solid red line indicates the linear
730 regression that was refit so that y= mx.

731

732
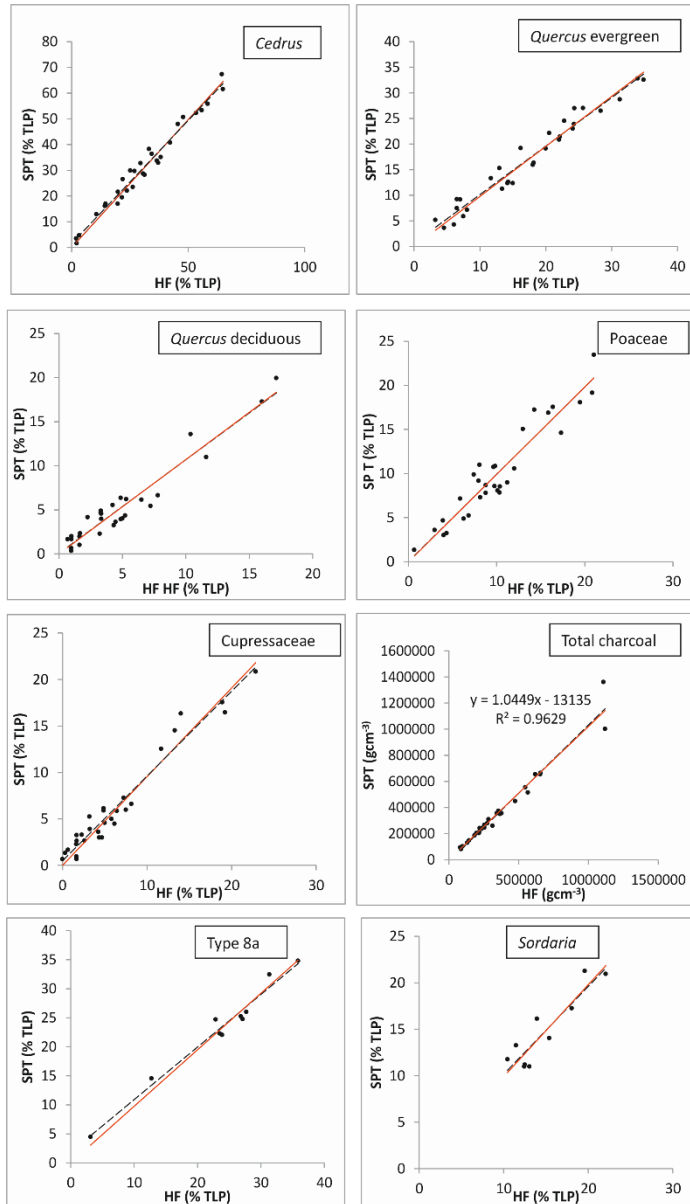
Figure 6: Images of the slides prepared for all three sites, using the two separate preparation methods. 1a - Sidi Ali, SPT; 1b - Sidi Ali, HF; 2a - Col du Zad, SPT; 2b - Col du Zad, HF; 3a - Aguelmam Azougagh, SPT; 3b - Aguelmam Azougagh, HF.