



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 ($\mu=1.6\%$, $sd=0.9\%$) is
22 considerably smaller than the 95% uncertainty associated with the standard counting
23 procedure ($\mu=6.8\%$, $sd=2.7\%$). 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

32 **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, ZnCl₂, 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 ZnCl₂ 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 km²), with a surface area of 2.8 km² 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 10⁵ 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 km²)
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 10⁴ 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
112 10⁴ 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/cm³.
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:

$$144 \quad X\% = \frac{N_x}{TLP + N_x} \times 100$$

145

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 cm³.

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 ($p \leq 0.05$).

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

238 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 ($\mu=1.6\%$, $sd=0.9\%$) is considerably smaller than the 95% uncertainty associated with
253 the standard counting procedure ($\mu=6.8\%$, $sd=2.7\%$).

254 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 $<25 \mu\text{m}$ 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/cm³, 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/cm³ (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

389 when using the dense-media method. The health and safety risks of working with HF
390 are considerable; an HF approved lab is needed, HF-specific training and
391 supervision of the analyst, and suitable personal safety equipment and first aid
392 protocols. Initial per sample costs are typically much higher when using SPT (~£5, as
393 opposed to ~£0.18 for HF), although the used SPT can be recovered, filtered and
394 recycled. Other heavy liquids are much cheaper and offer similar performance at
395 costs comparable to HF; however, SPT (and LST) is a non-toxic liquid, and is
396 therefore preferable for routine use over Thoulet's liquid and ZnCl₂ (Munsterman and
397 Kerstholt, 1996). The removal of clays and breakdown products of alkali digestion
398 by conducting between six and twenty (in this case, sometimes more) water washes,
399 vastly improved the quality of the samples. Although removal of clays is most critical
400 for density separation (as highlighted by Nakagawa *et al.*, 1998), it is also beneficial
401 for maximising efficiency of the HF stage. It is therefore crucial to spend sufficient
402 time on this stage, depending on the clay content of the samples. Micro-sieving (e.g.
403 5µm mesh) as an alternative could be considered for further evaluation at this stage.
404 While these advantages and disadvantages may all be important in different
405 laboratory settings, the decisive factor when deciding which method to use is likely to
406 be the sediment type, with specifically either strongly minerogenic, or, very organic-
407 rich sediment standing to benefit particularly from the use of dense media.

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

417

418 **6. Conclusions**

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

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

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457

458 **8. References**

- 459 Allen JRM, Scourse JD, Hall AR, Coope GR. 2009. Palaeoenvironmental context of
460 the Late-glacial woolly mammoth (*Mammuthus primigenius*) discoveries at
461 Condover, Shropshire, UK. *Geological Journal* **44**: 414–446.
- 462 Amami B, Rhazi L, Chaibi M, Fauquette S, Ougougdal MA, Charif A, Ridaoui M,
463 Bouahim S, Grillas P, Muller SD. 2013. Late Quaternary history of a Mediterranean
464 temporary pool from western Morocco, based on sedimentological and palynological
465 evidence. *Palaeogeography, Palaeoclimatology, Palaeoecology* **392**: 281–292.
- 466 el Bait MN, Rhoujjati A, Eynaoud F, Benkaddour A, Dezileau L, Wainer K, Goslar T,
467 Khater C, Tabel J, Cheddadi R. 2014. An 18 000-year pollen and sedimentary record
468 from the cedar forests of the Middle Atlas, Morocco. *Journal of Quaternary Science*
469 **29**: 423–432.
- 470 Bell BA, Fletcher WJ. 2016. Modern surface pollen assemblages from the Middle
471 and High Atlas, Morocco: insights into pollen representation and transport. *Grana*: 1–
472 16.
- 473 Bennett K. 1992. PSIMPOLL: a quickBASIC program that generates PostScript page
474 description files of pollen diagrams. *INQUA Commission for the study of the*
475 *Holocene: working group on data handling methods newsletter* **8**: 11–12.
- 476 Björck S, Persson T, Kristersson I. 1978. Comparison of two concentration methods
477 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 charcoal concentration
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, Stuetgen 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, Stele 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

649 the sample. They were stirred and then centrifuged at 4500 rpm for 3
650 minutes, after which the supernatant was decanted. Working in a HF-
651 prepared fume cupboard with environmental scrubbers and wearing suitable
652 safety equipment (face mask, environmental hazard suit, rubber boots,
653 double nitrile gloves) 3 ml of HF was added, and the samples were stirred
654 careful with polypropylene stirring rods. They were then placed in a hot water
655 bath for 20 minutes, and the stirring rods were used to check for remaining
656 grittiness in the tube. The samples were heated until all silica had been
657 dissolved. The supernatant was decanted and 5ml 10% HCl was added to
658 the pellet and stirred. The tubes were placed back into the hot water bath for
659 15 minutes to remove any silicofluorides. They were centrifuged at 4500 rpm
660 for three minutes, decanted, and topped up with deionised water. This latter
661 step was repeated three times.

662 **b) Density separation using SPT.** A suitable volume of SPT (sodium
663 polytungstate) solution was prepared to a specific gravity between 1.88-1.91
664 g/cm³, allowing for 10 ml per sample. The software LSTCalc
665 (<http://www.polytungstate.co.uk/lstcalc.html>) was used to help determine the
666 correct volume of water to add to the starting volume of SPT of known specific
667 gravity. The samples were suspended in 5 ml 10% HCl to acidify the sample,
668 which aids with the density separation. They were mixed and centrifuged at
669 4500 rpm for three minutes, and the supernatant was decanted fully,
670 removing as much water as possible so as not to affect the density of the
671 SPT. 10 ml of the prepared SPT solution was added to each tube. The tubes
672 were capped and mixed using the vortex mixer for at least 20 seconds each.
673 The samples were centrifuged at 1800 rpm for 20 minutes. They were then
674 left overnight to further aid separation; this option allows for material in
675 suspension in the supernatant to float to the top, making it easier to see and
676 decant the floating organic material. The supernatant of each sample was
677 poured into a new centrifuge tube, topped up with deionised water to within 5
678 ml of the top of the tube (reducing the overall density to around 1.15-1.2
679 g/cm³), capped, and mixed by performing 8-10 inversions by hand. The
680 centrifuge tubes containing the supernatant were then centrifuged at 4500
681 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.

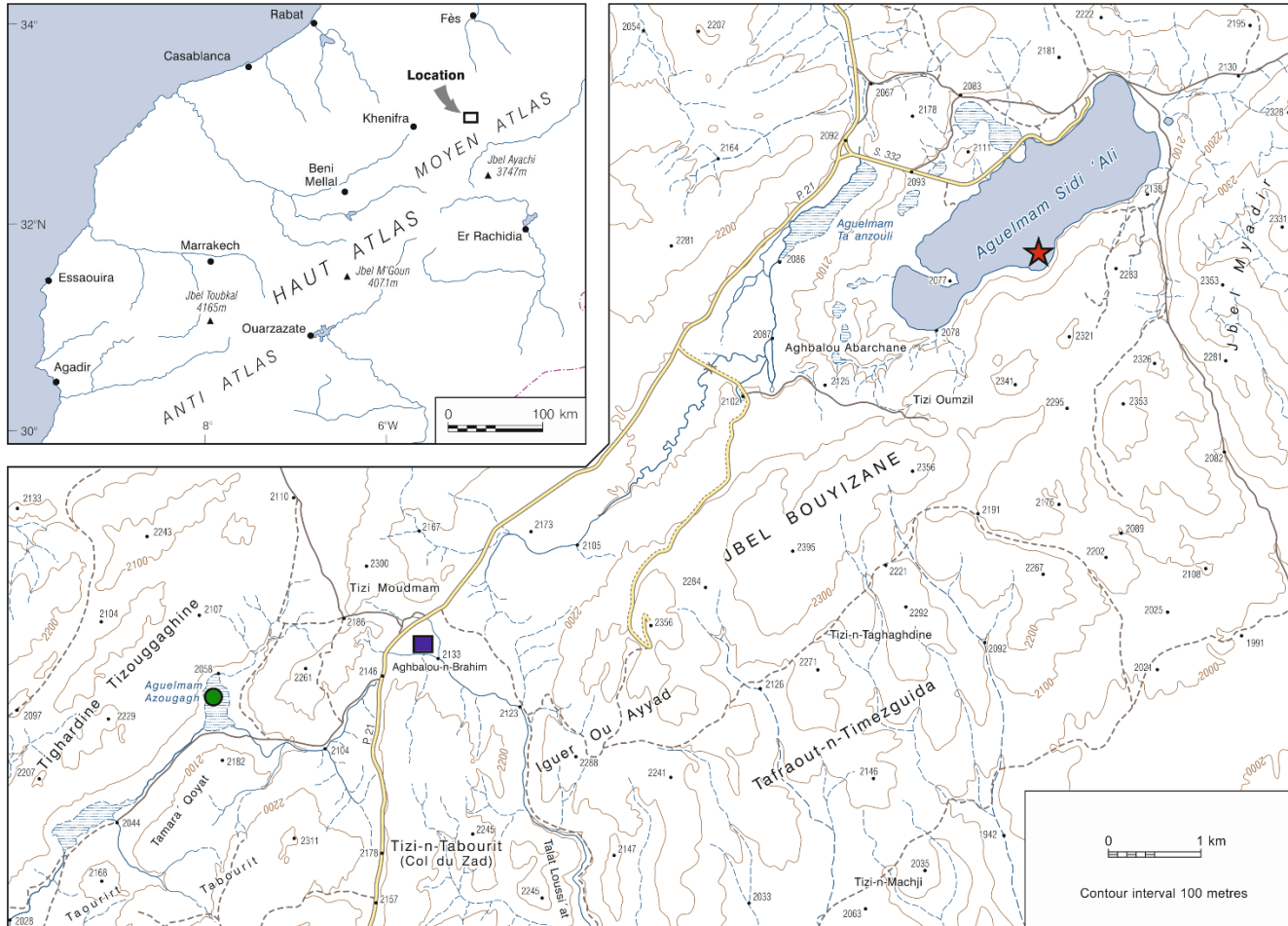
706 **9. Making slides.** Where necessary, additional silicone oil was added to the
707 samples, which were stirred with a microspatula. A small drop of the sample
708 was placed in the middle of the slide, and a glass cover slip was slowly
709 placed over the top. A seeker was used to apply gentle pressure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

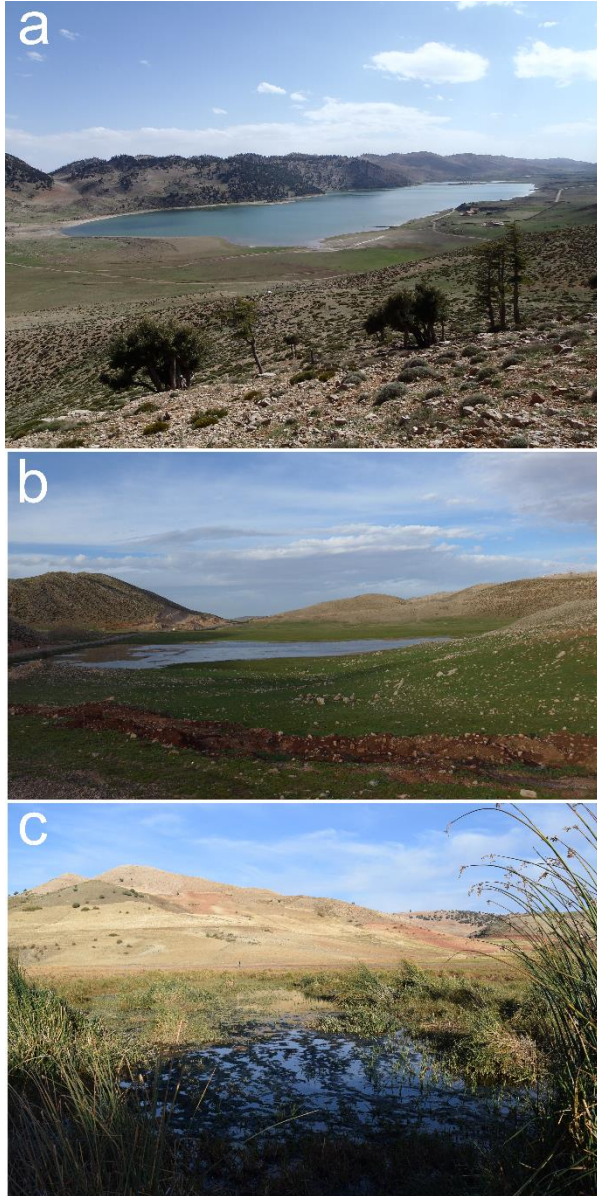
AA 1	HF	20.70	16.18	12.49	2.82	0.97	0.33	42.73	37.22	32.01	25.92	21.04	16.86	3.73	1.62	0.69
	SPT	24.00	19.22	15.20	1.82	0.33	0.06	38.34	32.90	27.88	28.50	23.45	19.06	4.63	2.28	1.11
AA 2	HF	15.79	11.67	8.51	6.03	3.33	1.82	42.26	36.67	31.41	22.02	17.33	13.47	3.84	1.78	0.71
	SPT	17.56	13.31	9.97	7.48	4.55	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
AA 7	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.



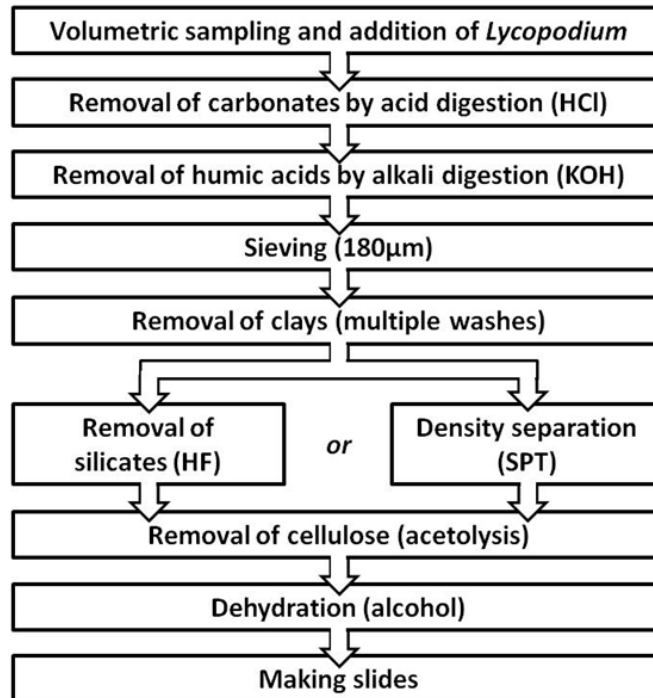
718 **Figure 2:** Photographs of the three sites: a) Lake Sidi Ali; b) Col du Zad; c)
719 Aguelmam Azougagh.

720



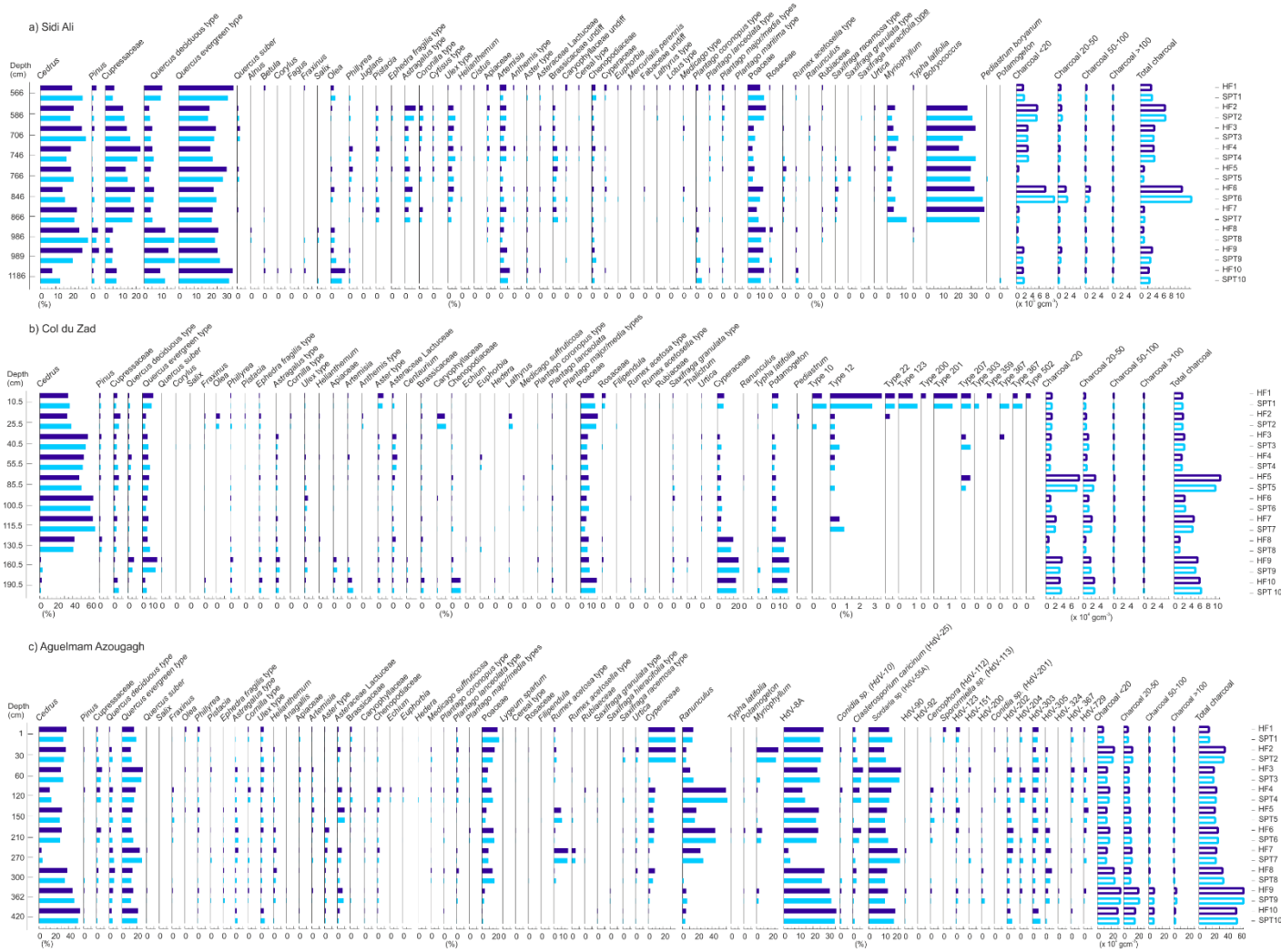
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722 **Figure 3:** A summary of the preparation protocol used for the pollen, NPP and
723 charcoal samples.



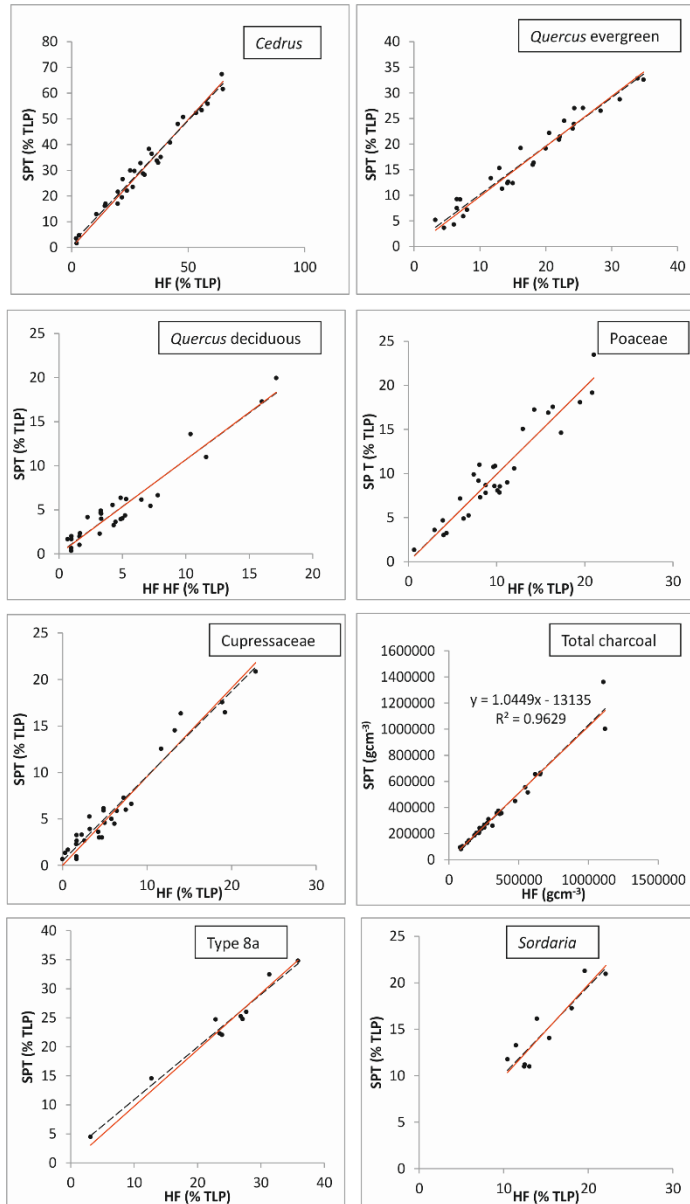
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725 **Figure 4:** Diagrams showing the pollen, fungal spore and charcoal counts for: a) Lake Sidi Ali, b) Col du Zad, and c) Aguelmam
 726 Azougagh.



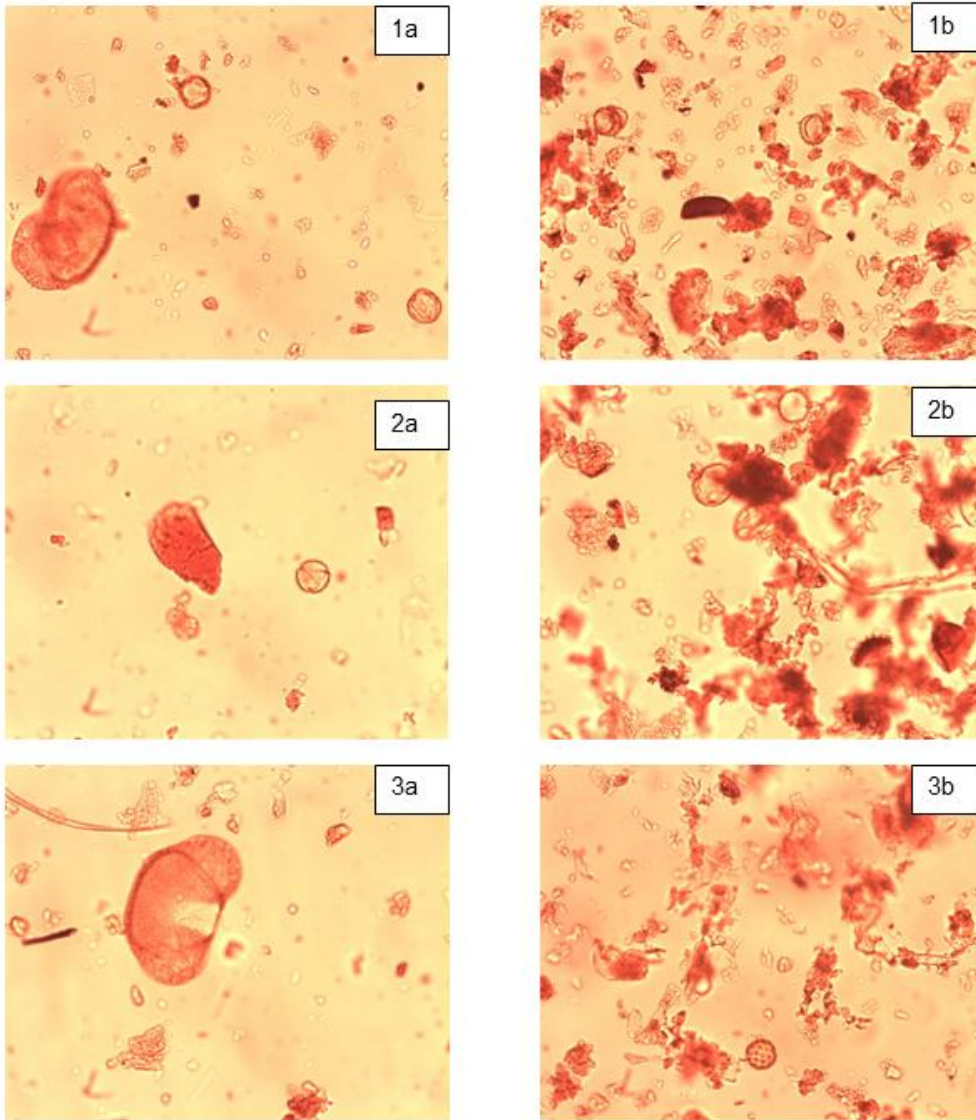
728 **Figure 5:** Linear regressions for the one-on-one statistics. The black dashed line
729 indicates the initial linear regression ($y= mx + c$); the solid red line indicates the linear
730 regression that was refit so that $y= mx$.

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732

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.



736