

1 SOILS, SEC # • RESEARCH ARTICLE

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3 **Humic acid composition and humification processes in wetland soils of a Mediterranean**

4 **semiarid wetland**

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Código de campo cambiado

18 **Abstract**

19 | Purpose: The present study focuses on a compositional characterization of the [humic acid \(HA\)](#)
 20 | fraction of several wetland soils using solid-state ^{13}C NMR spectroscopy. The data were
 21 | analysed using the molecular mixing model (MMM), based on an empirical approach by
 22 | Nelson and Baldock. The compositional data from HAs [obtained with this model](#) were used to
 23 | obtain a wider assessment of the process of humification from comparison of total soil
 24 | wetland [organic matter](#)OM composition and HA composition

25 | Materials and methods: Twenty samples of humic acids (HAs) isolated from a Mediterranean
 26 | semiarid wetland ('Tablas de Daimiel', central Spain) ~~have been were~~ studied using [elementary](#)
 27 | [elemental](#) analysis and ~~CPMAS~~ (cross polarization magic angle spinning) ^{13}C -(CPMAS) ^{13}C
 28 | nuclear magnetic resonance (NMR) spectroscopy. The NMR data ~~have been were~~ analysed
 29 | with ~~a~~-the molecular mixing model (MMM) considering up to six generic components
 30 | (carbohydrate, protein, lignin, lipid, char, and 'carbonyl'). HAs are considered a conceptual
 31 | mixture of these model compon~~ents~~[sunds](#), and the MMM determines the proportions of the
 32 | characteristic biomolecules contributing to ~~the~~ HA composition.

33 | Results and discussion: The composition of the HAs ~~in question under study~~ depends on local
 34 | factors such as site vegetation and occurrence of fire. Correlations between the proportions of
 35 | the six generic components and further comparison with those determined for the ~~original~~
 36 | ~~entire~~, unfractionated OM [\(whole sample, WS\)](#), gave information on HA origin and
 37 | humification mechanisms. In particular, the ~~contents proportions~~ of char and carbohydrate (R^2
 38 | 0.637) and contents of lignin and protein (R^2 0.471) in the HAs were negatively correlated ($P <$
 39 | 0.05). Significant correlations (R^2 0.439) also existed for char contents in whole sample (WS)
 40 | compared to HA, and for carbohydrates in WS compared to HA (R^2 0.558). Char ~~contents~~
 41 | [proportion](#) grew in HA with respect to the WS, and ~~carbohydrates~~ dropped to a half on
 42 | average in HA compared to WS.

43 | Conclusions: Two different humification mechanisms could be identified for no-fire and fire
 44 | areas. In the former, HA-char was preserved selectively from char in the sample, whereas in
 45 | the latter, char was ~~mainly~~ newly formed [by fire effect from preexisting carbohydrates](#).

46

47 | **Keywords** CPMAS ^{13}C NMR spectroscopy • Humification mechanisms with and without fire •

48 | Molecular mixing method • Wetland soil humic acids

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51 1. Introduction

52 Humification is assumed to consist of widespread biochemical [and abiotic diagenetic](#) reactions
53 leading to recalcitrant, very complex macromolecular compounds (Aiken et al. 1985;
54 [Almendros 2008](#)). The resulting humic substances (HS) can originate from alteration of pre-
55 existing [biomacromolecules polymers](#) but also from abiotic or enzymatic condensation of
56 soluble biogenic precursors (Almendros 2008). Both processes result in the accumulation of
57 material which is more complex than the corresponding precursors and comparatively much
58 more resistant to microbial attack (Almendros 2008). However, it has also been pointed out,
59 that the recalcitrance of natural organic matter (OM), including HS, depends on association
60 with soil minerals, and may not be an intrinsic characteristic of the substrate (Kleber 2010). In
61 any case, humification can be seen as a key step within the wetland carbon cycle, strongly
62 contributing to the important role of wetlands as carbon sinks (Bridgham et al. 2006).

63 [The](#) HS are generally divided according to their solubility in aqueous solution (Hayes et al.
64 1989). The fraction only soluble under alkaline conditions is called the humic acid (HA) fraction.
65 HAs are polydisperse, heterogeneous substances. There are several conceptions concerning
66 their nature and composition: According to an early view, they can be seen as a system of
67 micelles of a polymeric nature, with high average molecular masses (20,000–50,000 Da; Aiken
68 et al. 1985). Some authors have considered that HAs do not exist in soil as a definite entity,
69 being mere mixtures of plant and microbial remains and their degradation products (Burdon
70 2001). As a matter of fact, specific chemical structures belonging to HS have not been
71 identified (Kelleher and Simpson 2006; Lehmann et al. 2008); an extended discussion on this
72 topic is given by Kleber and Johnson (2010). The polymeric character of HS is also a matter of
73 discussion: Some authors have introduced terminological insights which consider that HS are
74 supramolecular associations of self-assembling heterogeneous and relatively small molecules
75 (Piccolo 2001). This view is shared by others (Sutton and Sposito 2005), who consider that HS
76 would contain tightly bonded biomolecules or recognizable fragments of them.

77 The present study focuses on a compositional characterization of the HA fraction using solid-
78 state ^{13}C NMR spectroscopy. The data (from twenty samples of HAs) were analysed using the
79 molecular mixing model (MMM), based on an empirical approach by Nelson and Baldock
80 ~~(Nelson and Baldock (2005)~~ in an attempt to describe the molecular composition of natural
81 OM from quantitative data obtained from ^{13}C nuclear magnetic resonance (NMR)
82 spectroscopy. These authors showed that detrital OM, derived from different sources, can be
83 described in terms of six generic components (carbohydrate, protein, lignin, lipid, 'pure
84 carbonyl' and char). The contribution of each to the total OM can be inferred from the
85 respective ^{13}C NMR spectra by way of a linear combination of spectra of the six generic organic
86 components. The approach of Nelson and Baldock (2005) allows a more specific assignment of
87 the C-groups assigned from NMR to various compound classes. In particular, a better
88 discrimination between sources of aromatic C may help differentiate [pyrogenic C forms, i.e.,](#)
89 [char from other plant-derived](#) aromatic components. This improves the analysis of the origin of
90 HAs from different precursor macromolecular entities, which is especially appropriate for
91 wetland soils (in most cases peat or histosols) where selective preservation of [weakly](#)
92 [unaltered domains of biomass](#)-macromolecular [biomass](#) constituents is a classically recognized
93 factor in the C sequestration in these ecosystems.

94 The compositional data from HAs were used to obtain a wider assessment of the process of
95 humification ~~taking advantage of from~~ comparisons ~~between-of~~ total soil wetland OM
96 composition and HA composition. To our knowledge, [although](#) MMM has been applied to HAs
97 only ~~in the study by [ø](#)ee (Buurman et al. 2009),~~ [there is no previous research on MMM](#)
98 [applied to HAs from wetland soils.](#)
99 [and never to HAs from wetland soils.](#)

100

101 2. Materials and methods

102 2.1 Study site

103 The Tablas de Daimiel is a freshwater wetland in Central Spain on the River Guadiana drainage
104 basin (39° 08' N, 3° 43' W; Fig. 1). Its detailed description and environmental history were
105 recently reported ~~by Rodríguez Murillo et al., (2011) and by~~ Sánchez-Carrillo and Angeler
106 (2010) ~~and Rodríguez-Murillo et al., (2011).~~

107

108 2.2 Sample preparation and HA extraction

109 Samples (20) from the upper 20 to 30 cm of the wetland soils from Las Tablas de Daimiel,
110 corresponding to different local environments in the wetland, were collected in March 2007

111 (Fig. 1). ~~In the case of organic soils or sediments, sampling was carried out collecting 30 cm of~~
112 ~~the uppermost horizon. Only in sandy sediments with low content of organic C the material~~
113 ~~was collected in the organic horizon, i.e., 4–5 cm thickness topsoil layer.~~ The ~~samples~~ were
114 air-dried, ground and sieved to pass a 2 mm sieve. The HAs were extracted following a
115 standard procedure described by (Duchaufour and Jacquin, 1975). Briefly, the soil sample was
116 shaken with 0.1 M Na₄ P₂ O₇ for 5–6 h. After centrifugation at 4,000 rpm ~~for 5 min,~~ the
117 supernatant was separated from the ~~sedimentsolid pellet~~. The operation was repeated ~~3~~
118 and the supernatant ~~solutions~~ were combined. The solid residue was further extracted with
119 0.1 M NaOH by shaking the mixture for 3–4 h and centrifuging at 4,000 rpm for 5 min to
120 separate the supernatant ~~solution~~ from ~~a pellet with~~ the solid phase. The procedure was
121 repeated until the final extract showed a pale colour that did not change in successive
122 extractions (5–10~~x~~ depending on the concentration and extractability of organic C).

123 After acidification ~~with 6M HCl~~ of the extract ~~with 6M HCl to pH = 1.5,~~ the HAs were isolated
124 as a precipitate from the supernatant which formed the fulvic fraction, ~~after allowing to settle~~
125 ~~overnight~~. The HA fraction was re-dissolved in 0.5M NaOH, and centrifuged at 20,000 rpm for 5
126 min to remove clays and particulate OM. The HAs were precipitated again with HCl,
127 centrifuged, and dialyzed for purification ~~with distilled water under continuous agitation until~~

128 | [free of chlorides \(AgNO₃ test\), which took about 8 days.](#) The 20 soil samples resulted in only 18
129 | HAs extracts, since samples 2 and 4 yielded insignificant quantities of HAs.

130

131 | 2.3 Sample analysis

132 | Total C, H and N content (%) were determined with a Perkin-Elmer 2400 Series 2 CNHS/O
133 | elemental analyzer. [The](#) O content was obtained as the difference between 100% and the
134 | sum of measured C, N, and H proportions. [Based on previous analysis in Tablas de Daimiel](#)
135 | [wetland, as well as in the examination of the FTIR spectra of the HAs \(lack of intense signal in](#)
136 | [the 600-400 cm⁻¹ range\), we assume that ash content is ca. 1% or less, so it should not affect](#)
137 | [significantly to our results.](#)

138

139 | Solid state ¹³C NMR spectra were acquired with a Bruker DSX 200 (4.7 Tesla) operating at 50.29
140 | MHz for ¹³C, with HA samples contained in zirconium dioxide rotors of 7 mm diameter in a
141 | commercial double bearing probe. The magic angle spinning speed was 6.8 kHz. The recycle
142 | delay was 300 ms. Cross polarization contact time was 1 ms, and a ramped ¹H-pulse was
143 | applied to account for Hartmann-Hahn mismatches. Depending on sample, the number of
144 | free-induction decays was between 3000 and 20,000 and the line broadening ranged between
145 | 25 and 75 Hz. The chemical shifts were calibrated to tetramethylsilane (0 ppm) using glycine as
146 | external reference. For spectral interpretation the following ranges and assignments were
147 | [taken into account considered](#) (Wilson 1987; Wilson et al. 1988; Preston 1992; Preston et al.
148 | 1997; Huang et al. 1998): 0–46 ppm, alkyl (13, methyl; 21, acetate; 30, polymethylene), 46–110
149 | ppm, O/N-alkyl (56, methoxyl/α-amino; 73, glucopyranosyde-derived; 103, anomeric C in
150 | carbohydrate; 105, quaternary aromatic C in tannins); 110–160 ppm, aromatic/unsaturated
151 | [ca. 135: unsubstituted, ca. 145, heterosubstituted: guaiacyl (G) lignins/dihydroxyls of tannins;

152 ca. 153: ether-linked (syringyl (S) lignins)/tannins]; 160–200 ppm, carbonyl (172,
153 carboxyl/amide; 198, ketone/aldehyde).

154 Areas in nine spectral regions were measured with ImageJ software (<http://rsb.info.nih.gov/ij/>)
155 and normalized to 100.

156

157 2.4 Data treatment

158 The molecular mixing model (MMM) of Nelson and Baldock (2005) was applied to the
159 NMR data as described by Rodríguez-Murillo et al., (2011). The used ¹³C [spectral](#)-intensity
160 distribution among the seven chemical shift regions for the six components was obtained from
161 Baldock et al. (2004), who also supplied the appropriate elemental atomic H/C, N/C and O/C
162 ratios [for the six model components](#). Based on [these](#) data and the ¹³C [spectral](#) intensity
163 distribution determined from the ¹³C NMR spectra of our samples, HAs composition was
164 calculated in terms of the six generic biomolecules plus ‘pure carbonyl’ by solving a system of
165 equations for each sample using the Generalized Reduced Gradient nonlinear optimization
166 code in the Solver add-in of Microsoft Excel with two restrictions: (i) the sum of component
167 fractions was forced to be 100 and, (ii) the component fractions were forced to be equal to or
168 greater than zero.

169

170 3. Results

171 3.1 [Yield of HAs](#)-[yield](#) and elemental composition

172 [The](#) HA content and composition varied [considerably among](#)~~within~~ the different sampling
173 points (Table 1). From the Van Krevelen diagram (Van Krevelen 1950) shown ~~on~~-[in](#) Fig. 2, we
174 could distinguish up to three different [types-classes](#) of samples: predominantly aliphatic (high

175 H/C atomic ratio), predominantly aromatic, mature (low H/C), and highly oxidized (high O/C
176 atomic ratio).

177 The ^{13}C NMR CPMAS spectra of the HAs are depicted in Fig. 3a. The respective [spectral](#)
178 intensity distribution, as well as total aromaticity and aliphaticity, are given in Table 2. [Two](#)
179 [pairs of spectra \(WS and HA\), from two differentiated sampling points, are given in Fig. 3b.](#)

180 Clear [geographic](#) differences were evident between HAs, with the highly aliphatic HA
181 corresponding to the upper Tablas sector, with non-permanent flooding and charophyte
182 vegetation in open water areas whereas comparatively more aromatic HAs dominate in lower
183 Tablas, in which the permanent vegetation is dominated by vascular plants. Our data show an
184 increase in C/N of the HAs compared to the [whole sample-WS](#) (Rodríguez-Murillo et al. 2011)
185 in 16 of the 18 samples. Only for samples 16 and 19 was the C/N of the [whole sample-WS](#)
186 greater than that of the HA.

187

188 3.2 HA composition [of the HAs](#) inferred from the MMM

189 The HAs composition obtained from applying the MMM is shown in Fig. 4. We did not
190 introduce the experimental C/N values as a condition in our system of equations, as could be
191 done (Nelson and Baldock 2005), because in the MMM scheme, only peptides are significant
192 N-bearing compounds. This has indeed been confirmed in ^{15}N NMR spectroscopic studies
193 (Knicker 2000), despite the fact that in fire-affected soils, as many of our samples are, a
194 sizeable fraction of organic N could be present in heterocyclic compounds (Almendros et al.
195 2003; Knicker 2011), and we would overestimate the proteinaceous contribution assigning all
196 N to this fraction.

197 As ~~it~~ is the case in [WSwhole-soil](#) samples (Rodríguez- Murillo et al. 2011), the six organic
198 components of the HAs were widespread. Structural components as carbohydrate, protein,

199 and lipid dominate the HA composition across the wetland, and variable quantities of lignin
200 and char were also present in all the HAs.

201 The reliability of the MMM applied to HAs was assessed in two independent ways:

202 (i) Calculating errors in total spectrum area as the sum of absolute values of differences
203 between experimental (spectrum) areas and calculated (MMM) areas for each
204 chemical shift region (Nelson and Baldock 2005).

205 (ii) Calculating H/C and N/C ratios from the composition obtained with the MMM and
206 comparing the values with the corresponding H/C and N/C values from [elementary](#)
207 [elemental](#) analysis.

208 Mean error in total area [across HA samples](#) was 2.22 ± 0.75 , greater than that (0.93) found
209 in [whole samples-WS](#) (Rodríguez- Murillo et al. 2011; Fig. 5a). The spectral regions
210 reproduced most poorly were 95–110 ppm (C1 region of carbohydrate-like structures) and
211 145–165 ppm (N/O aromatic region). In the former, the mean error for calculated vs.
212 measured area was 32% and in the second 4.5%. In the remaining chemical shift regions,
213 differences between calculated and measured [spectral](#) intensities were lower. It was
214 estimated that a mean error “x” in determining intensity gives a double error (“2x”) in the
215 composition of the HA.

216 A strong correlation between MMM-calculated and experimental H/C ratio was obtained,
217 but with some bias; ~~;~~ calculated H/C values were bigger than experimental H/C ones, i.e.
218 real samples were more aromatic than MMM estimated composition (Fig. 5b). The
219 opposite happened with the N/C [ratio](#), the calculated values being generally lower than
220 the experimental ~~ones~~

221 , but with a less systematic trend in comparison with the H/C ratio case (Fig. 5c). Possible
 222 causes of the discrepancy between calculated and experimental ratios are discussed
 223 below in Section 4.1.

224

225 3.3. Relationship ~~between among relative proportions of~~ HA components and comparison
 226 between HAs composition and the ~~whole~~ OM composition of wetland ~~WS~~ soil samples

227 The most significant correlations ~~between among~~ HA components (Table 3) ~~was were~~ between
 228 char and carbohydrate (~~R^2 0.637 r -0.798~~), carbonyl and protein (~~R^2 0.610 r -0.781~~), lipid and
 229 lignin (~~R^2 0.502 r -0.709~~), lignin and protein (~~R^2 0.471 r 0.686~~), and carbohydrate and protein (~~R^2~~
 230 ~~0.463 r 0.680~~). Every significant relationship was negative, except in the case of carbohydrate-
 231 protein, carbohydrate-lipid, and carbonyl-char. In the ~~whole~~ wetland ~~soil samples~~ WS, the
 232 strongest correlation was lignin-protein, but the char-carbohydrate correlation was also
 233 noticeable (Rodríguez-Murillo et al. 2011).

234 The C/N ratio was positively related to lignin (~~R^2 0.622 r 0.789~~) and carbonyl (~~R^2 0.273 r 0.522~~),
 235 and negatively related to the other constituents (mainly to protein, ~~R^2 0.487 r -0.698~~). No
 236 significant relationship was found with char.

237 In Table 4, the HA composition is compared with the composition of the OM of the
 238 corresponding ~~whole~~ WS sample. The HAs differ from WSs mainly because of the higher
 239 aromaticity (relative area of 110–165 ppm region of ^{13}C NMR spectra; Fig. 6a). This increase in
 240 aromaticity ~~in HA compared to WS as a consequence of progressive humification~~ was fairly
 241 ~~regular-linear~~ (HA aromaticity = $0.912 \times (\text{WS aromaticity}) + 10.39$; ~~R^2 0.681 r 0.825~~, $P <$
 242 0.0001), ~~indicating that HA aromaticity is controlled to a great extent by WS aromaticity~~
 243 ~~indicating that WT and HA aromaticities are significantly related~~. In the data set of Mahieu et
 244 al. (1999) the mean increase in aromatic C content of HAs vs. WSs was 10 %, which is in line

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245 with our results. The total aromaticity depends strongly upon the sum of lignin content and
 246 char content (94% is explained by lignin + char, which were by far the most significant
 247 contributors).

248 The sum of lignin and char in HAs was strongly related to that of WSs ($HA = 0.879 \times WS + 15.39$,
 249 $R^2 = 0.570$, $r = 0.755$). Significant correlation also existed (Fig 6b) between WS and HA for char
 250 alone ($R^2 = 0.439$, $r = 0.663$, $P = 0.0027$) which increased from WS to HA, and for carbohydrates,
 251 which decreased sharply ~~in the HA compared to the WS for HA vs. WS~~ (Fig 6c). In the latter, the
 252 carbohydrate content of HA was ca.50% of that of WS (on average), with $R^2 = 0.558$, $r = 0.747$. For
 253 the HAs, the content of this compound class ~~depended—was strongly significantly related to on~~
 254 that of WS from the different sites. Lipid constituents in WS and HA were barely related (R^2
 255 0.278 , $r = 0.527$), and protein, lignin, and carbonyl showed non-significant relationships (at $P <$
 256 0.05) between their contribution to HA and to WS (protein, $R^2 = 0.161$, $r = 0.401$; lignin, $R^2 = 0.140$,
 257 $r = 0.374$; carbonyl, $r = 0.346$).

258

259 3.4 Influence of environmental factors in HA-WS component relationships

260 Humification is to a large extent influenced by environmental factors such as aerobic or semi-
 261 anaerobic conditions for plant detritus diagenesis. Wetlands are subjected to very ~~different~~
 262 ~~diverse~~ local physicochemical processes of variable intensity. In the ~~former~~ case ~~of~~ oxidizing
 263 environment, the OM tends towards a progressive depletion in carbohydrate, whereas in the
 264 ~~latter case of anaerobic conditions~~ the reducing environment leads to a selective increase of
 265 alkyl and lignin constituents (Filip and Alberts 1994). On the other hand, vegetation fires could
 266 produce severe thermal modification of existing OM, as well as the ~~differentiation-formation~~ of
 267 new carbon forms. During a fire, considerable rearrangement of C forms occurs and slightly
 268 more refractory and oxidation-resistant organic C forms with increased mean residence time,
 269 including black carbon, are formed (Schulze et al. 2000; Almendros et al. 2003).

270 We analyzed the effect of environmental conditions on humification (i.e. as reflected in the
271 composition of the HA fraction), by studying the relationship between HA and WS separately in
272 the different groups of sampling sites, which could be distinguished based on defined by
273 attending to two factors, viz. flooding regime and fire occurrence, by taking advantage of
274 information recorded from Tablas de Daimiel (Cirujano and Álvarez-Cobelas, personal
275 communication) and our own observations (Table 1).

276 Dividing our sample set into those groups (permanent/non-permanent flooded; fire-
277 affected/not fire-affected) and recalculating the correlations between the aromaticity, char
278 and carbohydrate contents for WS and HA (as the most significantly related variables in the
279 whole set of samples) clearly demonstrated the impact of environmental conditions. Further
280 differentiation between sampling sites with permanent and non-permanent flooding (irregular
281 or seasonal in Table 1), showed strong correlation between the aromaticity of HS and of WS
282 for the latter non-permanent flooding group (r 0.964, P = 0.0001), but only a weak relationship
283 for the former permanent flooding group (r 0.591, P = 0.072). If fire-affected and unaffected
284 areas were are differentiated, the aromaticity of HA and of WS wss are not related in fire areas
285 (r 0.533, P = 0.14), but shows a tight relationship for no fire areas (r 0.941, P = 0.0002). HA
286 aromaticity is higher and more constrained (30 - ca. 45%) in fire areas in comparison with no
287 fire areas (15 - ca. 40%).

288 No significant relationship (Fig. 7b) between char content of HA and of WS char was observed
289 for permanently flooded sites, as well as fire affected areas (r 0.055), but turned into being
290 fairly significant (Fig. 7a) for non-permanently flooded samples (r 0.964, P = 0.0008) and fire-
291 unaffected areas (r 0.940, P = 0.0002). Char content of HA and WS are significantly correlated
292 in non-permanently flooded samples (r 0.964, P = 0.0008) and fire-unaffected areas (r 0.940, P
293 = 0.0002) (Fig. 7a), but no significant relationship between char content of HA and of WS char
294 was observed for permanently flooded sites, as well as fire-affected areas (r 0.055) (Fig. 7b).

295 The relationship between carbohydrates in HA and WS was relatively strong and positive for

296 non-permanently flooded areas ($r = 0.821$, $P = 0.013$) (not shown) and fire-unaffected areas (r
297 0.733 , $P = 0.025$) (Fig. 8a), but not significant (Fig. 8b) for in the case of permanently flooded
298 areas and fire-affected areas ($r = 0.644$, $P = 0.061$) (not shown). ~~but relatively strong and~~
299 positive (Fig. 8a) for non-permanently flooded areas ($r = 0.821$, $P = 0.013$) and fire-unaffected
300 areas ($r = 0.733$, $P = 0.025$). Insignificant correlation was apparent for the other components
301 (lipid, protein and lignin) of WS and of HA.

302 ~~At the same time, data corresponding to subtraction between the amounts of the different~~
303 ~~constituents (Fig. 9, a and b) in WS and in HA (WS content minus HA content) in the case of~~
304 ~~aromaticity (HA aromaticity minus WS aromaticity), showed that there were significant~~
305 ~~relationships between the increase in aromaticity and the decrease in carbohydrate ($r = 0.680$, P~~
306 ~~$= 0.044$), and the increase in char ($r = 0.764$, $P = 0.017$) for fire-affected areas, and no significant~~
307 ~~relationship for the fire-unaffected ones. This confirms that aromaticity increase in fire-~~
308 ~~affected areas implies a parallel decrease in carbohydrate content (increased difference in~~
309 ~~carbohydrate content of HA vs. WS).~~

310

311 4 Discussion and conclusions

312 Previously (Rodríguez-Murillo et al. 2011), we studied the composition of OM in wetland soils
313 of Tablas de Daimiel. ~~Based on a combination of~~ Combining solid-state CPMAS ^{13}C NMR
314 spectroscopy with MMM (Nelson and Baldock 2005; Baldock et al. 2004), significant
315 differences were found in the composition of wetland soil OM derived from different areas.
316 The differences were ascribed mainly to the nature of the original detrital OM (non-vascular,
317 vascular, mixed and ~~bacterial~~ microbial). Additional perturbations such as fires, allochthonous
318 input and movement of sediment (resuspension) could be invoked to justify the presence of
319 lignin and perhaps of char in areas with non-vascular vegetation. Thus, Tablas de Daimiel
320 wetland can be considered as an interesting, mosaic-like ecosystem, with large variation in soil

321 OM characteristics caused by varying vegetation, environmental conditions and disturbances
322 in the different areas.

323 Humification processes are also likely to differ in local environments depending on the spatial
324 | variability of the microtopography and the ~~periodic or permanent duration of~~ flooding.
325 | Carbohydrates are the most labile macromolecular components, and tend to be depleted
326 | during the microbial and abiotic reworking of vegetal detritus. Proteins are more resistant, but
327 | are also depleted in part, with an effect of increasing the C/N ratio in the HAs ~~vs-versus~~ that of
328 | the whole sample. Progressive accumulation of aromatic structures in the course of HA
329 | formation is apparent in the sharp increase in lignin-type structures, as well as carbonyl and
330 | aromatic char. A Van Krevelen diagram offers some information on HA characteristics.
331 | Decreasing H/C ratio is frequently interpreted as progressive transformation of the OM
332 | towards a greater proportion of aromatic carbon, whereas higher H/C implies a molecular
333 | composition more similar to that of plant biomass. Besides, aliphatic samples have low C/N,
334 | and aromatic ~~ones~~ high C/N, and this can be related to the C/N of original OM (predominantly
335 | non-vascular in aliphatic samples and more vascular in mature samples) and/or different
336 | degree of transformation.

337 Fig. 2 illustrates the fact that in the Las Tablas ecosystem there are HAs of very different
338 nature: predominantly aliphatic HAs would correspond with WSs containing OM of aliphatic
339 | and alkyl character (non-vascular vegetation and microbial origin) ([Rodríguez-Murillo et al.](#)
340 | [2011](#)), indicating that the initial signature of detrital OM is kept through HA extraction. In
341 | particular, HA extraction preserves (although more weakly) the negative relationships between
342 | lignin and protein found in WSs, perhaps reflecting the original balance between vascular/non-
343 | vascular vegetation. The main ~~average~~ components of HAs, as calculated with MMM, were
344 | lipid > char > protein, whereas in whole sample the main components were protein > lipid >
345 | carbohydrate (Table 5). The relative content of carbohydrate is progressively depleted in the

346 course of humification, whereas, as expected, char and—to a lesser extent—lignin, increase in
347 concentration. Increase in lignin—comparing HA and WS—was not uniform in the samples,
348 and lignin, often considered the basis for HA structure in the case of peatland soils, was not
349 the main component identified in our HAs ~~-~~[applying the MMM](#).

350

351 4.1 Error assessment in the determination of composition from MMM

352 An obvious source of error in the determination of HA composition using the MMM could be
353 the occurrence of compounds other than the six generic constituents considered, ~~for instance,~~
354 tannins. ~~These are~~[These](#) polyphenols, present in higher plants (Kögel-Knabner 2002), ~~Tannins~~
355 display an intense NMR signal as two peaks in the 95–110 ppm region—which was the worst
356 reproduced signal with MMM—, due to their aromatic quaternary carbon (Preston et al.
357 1997). The 95–110 ppm region would then show considerable overlapping between anomeric
358 O-alkyl carbons in carbohydrates and aromatic quaternary carbon atoms in tannins and
359 dilignols. Although tannins ~~would not be~~[are usually far less](#) abundant ~~as~~[than](#) the other
360 MMM constituents, only 16.7% of carbohydrates, 8.6% of lignin and 6.3% of char peak in the
361 95–110 ppm region, ~~making it possible that which is compatible with a small proportion of~~
362 tannins ~~carbons could~~[make](#)ing a significant contribution to the signal in the 95–110 ppm
363 range. Incomplete agreement between experimental and simulated spectra (i.e. errors in total
364 spectrum area) might also be attributed to experimental errors in ¹³C-NMR spectra of HAs but,
365 considering the better agreement obtained in many cases using natural organic materials
366 (Nelson and Baldock 2005; Rodríguez-Murillo et al. 2011) instead of HAs, the occurrence in HAs
367 of specific chemical structures with special ¹³C-NMR signatures not considered in the MMM
368 treatment could be an important factor explaining the observed discrepancies. Unfortunately,
369 our data and their analysis can neither prove nor disprove the presence of such structures,
370 which should be quantitatively minor components.

371 Systematic discrepancies in experimental vs. calculated H/C (Fig. 5b) and N/C (Fig. 5c) ratios
372 were also probably due to the above considerations. ~~Problems of NMR in accurately detecting~~
373 ~~components with low proton content are also known (Knicker et al. 2005). If this were the case~~
374 ~~here, an underestimation of aromaticity would result. However, we observed neither an~~
375 ~~unusual lowering of the sensitivity for the sample nor problems during tuning the sample prior~~
376 ~~to measurement, which indicates that graphenic structures were present.~~ There was no
377 ~~apparent correlation~~ship of differences in experimental and calculated H/C (Fig. 5b) ~~and with~~
378 sample aromaticity or ~~sample~~ char content, although as H/C (~~real~~experimental) increased (less
379 ~~“low proton”~~ components), differences in H/C were smaller. The existence of a significant
380 proportion of N in heterocyclic structures could explain discrepancies in N/C ratio.

381

382 4.2 Humification mechanisms

383 In the areas permanently flooded in the past, fire has been a recent perturbation of Las Tablas,
384 coinciding with the first desiccation events (around 1960). Such fires, especially in peat zones,
385 have affected humus formation, as could be deduced from the different relationships of char
386 and carbohydrate content for fire and no-fire areas. At first sight, differences in humification
387 could be empirically ascribed to either fire action or flooding regime; the ~~existence-occurrence~~
388 of fire ~~seems to produce~~ in our system ~~seems to parallel the similar~~ effects ~~as of~~ the permanent
389 flooding in HA – WS component relationships but, as discussed below, fire effects could
390 explain these relationships, which is not the case for flooding. This coincidence between HA
391 and WS relationships in areas subjected to such different environmental influences (flooding
392 and fire) ~~is accidental and probably~~ arises from the fact that fires occur mainly in former
393 permanently flooded areas, after dessication. Fire consumes OM, but, at the same time,
394 increases aromaticity and the content of refractory substances, such as char, which ~~is-are~~ a
395 potential contributor to the formation of refractory long-term carbon sinks (Forbes et al.,

396 2006). This is due to selective enrichment of residual heat-resistant, strongly aromatic
397 components and also to endothermic neof ormation reactions involving carbohydrates, but
398 also amino acids and unsaturated alkyl chains (González-Pérez et al. 2004).

399 The concentration of char in HA was unrelated to the accumulation of WS char in fire areas,
400 but depended linearly on WS char in no fire areas (Fig. 7a, b). ~~If we plot the differences in~~
401 ~~carbohydrates and char between WS and HAs vs. the differences in aromaticity between HA~~
402 ~~and WS, we observe a linear relationship in fire affected areas and no significant relationship~~
403 ~~in the unaffected ones (Fig. 9a, b).~~

404 We can hypothesize that, in no-fire areas, HA 'char' comes mainly from char formerly
405 introduced into the sediment, as suggested by Haumaier and Zech (1995), and is selectively
406 preserved, whereas in fire-affected areas, char is ~~mainly~~ newly formed in situ as a
407 consequence of fire action. New formation of char in HAs in fire affected areas could explain
408 the loss of relationship between WS char and HA char in these areas. from preexisting
409 carbohydrates (González-Pérez 2004; Almendros et al. 2003). ~~The increase in aromaticity with~~
410 ~~humification in fire areas implies a parallel decrease in carbohydrate content (increased~~
411 ~~difference in carbohydrate of HA vs. WS; Fig. 9a), pointing to an accumulation of newly~~
412 ~~formed pyrogenic aromatic structures in HA from carbohydrate in the original sample via~~
413 ~~fire. In contrast, change in carbohydrate content in HA in fire unaffected areas with respect to~~
414 ~~WS does not show a relationship with change in char or aromatic content, as HA 'char' derives~~
415 ~~mainly from WS char (Fig. 9b).~~ Then, analysis of HA composition using MMM allows some
416 insight into humification mechanisms, comparing HA and whole sample composition: MMM
417 char ~~and carbohydrate~~ determination and constituent comparison between WS and HA allows
418 the detection of two contrasting HA-formation mechanisms, depending of on the occurrence
419 (or not) of fire.

420

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429

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510 **FIGURE CAPTIONS**511 **Fig. 1** Location of Tablas de Daimiel wetland and sampling points.512 **Fig. 2** Van Krevelen's diagram of [humic acids \(HAs\)](#) from the [sampling points of](#) Tablas de
513 Daimiel.514 **Fig. 3** ~~NMR spectra of the HAs.~~ [NMR spectra of the HAs. F: At least a fire has been recorded](#)
515 [in the area of the sampling point. NF: No fire recorded.](#)**Con formato:** Inglés (Reino Unido)516 b) Comparison between ¹³C NMR spectra of whole sample and its humic acids: the spectral
517 ranges for the main C-types are indicated.518 **Fig. 4** HA composition according to [the molecular mixing model \(MMM\)](#). The ~~HAs~~ are ordered
519 from lower aromaticity (bottom) to higher aromaticity (top). I, irregular; S, seasonal; P,
520 permanent. Flooding regime refers to that before 1973, year of the onset of large perturbation
521 in the hydrological regime of the Tablas. Fires are those recorded before sampling year (2007);
522 at sites 15, 17, and 17', the existence of fires in the past has been inferred from the occurrence
523 of charred material in the samples. "Y" ~~means that,~~ at least one fire has been recorded ~~and~~ ;
524 "N" ~~means~~ no fire recorded in the site.525 **Fig. 5** [a\)](#) Performance of MMM. Area error is the sum of absolute values of differences (%)
526 between experimental and calculated areas for each chemical shift region in each sample;**Con formato:** Espacio Después: 0 pto, No ajustar espacio entre texto latino y asiático, No ajustar espacio entre texto asiático y números527 [b\)](#) ~~Performance of MMM. Calculated vs experimental H/C ratios of the samples. The lower line~~
528 ~~represents the perfect concordance calculated-experimental;~~ c) Performance of MMM.**Con formato:** Inglés (Reino Unido)529 [Calculated vs experimental N/C ratios of the samples.](#)**Con formato:** Inglés (Reino Unido)530 ~~(a) Performance of MMM. Calculated vs. experimental H/C ratio for the samples; point-~~
531 ~~and dash line represents perfect agreement between calculated and experimental.~~532 ~~(b) Performance of MMM. Calculated vs. experimental N/C ratios for the samples; point-~~
533 ~~and dash line as in (b).~~

534 **Fig. 6** Comparison of components of HAs with components of OM-organic matter in whole
 535 samples (WS) (~~aromaticity, char and carbohydrate~~), according to MMM.

536 a) Comparison of aromaticities.

537 b) Comparison of char contents.

538 c) Comparison of carbohydrate contents.

539 **Fig. 7** Comparison of components of HAs with components of OM-organic matter in whole
 540 samples (WS), according to MMM. Effect of fire on char content change.

541 ~~(a) Comparison of char content in no fire areas.~~

542 ~~(b) Comparison of char content in fire areas.~~

543 **Fig. 8** Comparison of components of HAs with components of organic matter in whole samples
 544 (WS) according to MMM. Effects of fire on carbohydrate content change.

545 ~~**Fig. 8** Comparison of components of HAs with components of OM in whole samples (WS),~~
 546 ~~according to MMM. Effect of fire on carbohydrate content change. (a) Comparison of~~
 547 ~~carbohydrate content in fire areas. (b) Comparison of carbohydrate content in no fire areas.~~

548 ~~**Fig. 9** Differences in char content (in %) versus differences in carbohydrate (CH) content between WS~~
 549 ~~and HA . a) Fire areas. b) No fire areas~~ Differences in carbohydrate and char content (%) between
 550 WS and HA vs. aromaticity differences (%). (a) Fire areas. b) No fire areas

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567 [Table 1](#)568 [Concentration and elemental analysis of HAs from several wetland soils from Las Tablas de](#)569 [Daimiel and some environmental conditions. Atomic C/N, O/C and H/C ratios are shown \(Y, at](#)570 [least one fire recorded; N, means no fire recorded at the site\).](#)

571

Sample	<u>OC(g</u> <u>kg⁻¹)</u>	<u>C in HA^a</u>	<u>C(%)</u>	<u>H(%)</u>	<u>N(%)</u>	<u>O(%)</u>	<u>C/N</u>	<u>O/C</u>	<u>H/C</u>	<u>Flooding</u> <u>regime^b</u>	<u>Fire^b</u>
<u>1</u>	<u>149</u>	<u>11.9</u>	<u>50.6</u>	<u>5.11</u>	<u>4.15</u>	<u>40.2</u>	<u>12.2</u>	<u>0.60</u>	<u>1.21</u>	<u>I</u>	<u>N</u>
<u>3</u>	<u>49</u>	<u>1.52</u>	<u>52.7</u>	<u>4.79</u>	<u>4.62</u>	<u>37.9</u>	<u>11.4</u>	<u>0.54</u>	<u>1.09</u>	<u>S</u>	<u>N</u>
<u>5</u>	<u>105</u>	<u>10.2</u>	<u>51.7</u>	<u>5.13</u>	<u>3.46</u>	<u>39.8</u>	<u>14.9</u>	<u>0.58</u>	<u>1.19</u>	<u>I</u>	<u>N</u>
<u>6</u>	<u>58</u>	<u>2.80</u>	<u>51.7</u>	<u>5.80</u>	<u>4.63</u>	<u>37.9</u>	<u>11.2</u>	<u>0.55</u>	<u>1.35</u>	<u>S</u>	<u>N</u>

7	<u>53</u>	<u>43.5</u>	<u>52.8</u>	<u>4.07</u>	<u>2.96</u>	<u>40.1</u>	<u>17.8</u>	<u>0.57</u>	<u>0.92</u>	I	Y
8	<u>86</u>	<u>13.2</u>	<u>51.7</u>	<u>4.12</u>	<u>3.78</u>	<u>40.4</u>	<u>13.7</u>	<u>0.59</u>	<u>0.96</u>	S	Y
9	<u>365</u>	<u>8.85</u>	<u>52.9</u>	<u>4.06</u>	<u>2.49</u>	<u>40.5</u>	<u>21.3</u>	<u>0.57</u>	<u>0.92</u>	P	Y
10	<u>83</u>	<u>14.0</u>	<u>53.9</u>	<u>4.34</u>	<u>3.03</u>	<u>38.7</u>	<u>17.8</u>	<u>0.54</u>	<u>0.97</u>	P	Y
11	<u>148</u>	<u>8.81</u>	<u>53.3</u>	<u>4.44</u>	<u>2.82</u>	<u>39.4</u>	<u>18.9</u>	<u>0.55</u>	<u>1.00</u>	P	Y
12	<u>92</u>	<u>11.3</u>	<u>50.1</u>	<u>4.11</u>	<u>3.52</u>	<u>42.3</u>	<u>14.2</u>	<u>0.63</u>	<u>0.98</u>	P	Y
13	<u>88</u>	<u>8.13</u>	<u>51.5</u>	<u>4.90</u>	<u>4.49</u>	<u>39.1</u>	<u>11.5</u>	<u>0.57</u>	<u>1.14</u>	P	N
14	<u>133</u>	<u>14.2</u>	<u>51.2</u>	<u>3.84</u>	<u>3.10</u>	<u>41.9</u>	<u>16.5</u>	<u>0.61</u>	<u>0.90</u>	P	N
15	<u>200</u>	<u>5.73</u>	<u>51.4</u>	<u>4.34</u>	<u>3.90</u>	<u>40.3</u>	<u>13.2</u>	<u>0.59</u>	<u>1.01</u>	I	Y
16	<u>19</u>	<u>2.69</u>	<u>49.9</u>	<u>4.64</u>	<u>4.03</u>	<u>41.4</u>	<u>12.4</u>	<u>0.62</u>	<u>1.12</u>	P	N
17	<u>132</u>	<u>24.7</u>	<u>52.5</u>	<u>4.20</u>	<u>3.58</u>	<u>39.8</u>	<u>14.7</u>	<u>0.57</u>	<u>0.96</u>	P	Y
17'	<u>14</u>	<u>28.7</u>	<u>51.9</u>	<u>4.37</u>	<u>3.30</u>	<u>40.5</u>	<u>15.7</u>	<u>0.59</u>	<u>1.01</u>	P	Y
18	<u>37</u>	<u>8.40</u>	<u>50.1</u>	<u>3.93</u>	<u>3.93</u>	<u>42.0</u>	<u>12.8</u>	<u>0.63</u>	<u>0.94</u>	I	N
19	<u>63</u>	<u>11.9</u>	<u>50.8</u>	<u>4.29</u>	<u>4.18</u>	<u>40.7</u>	<u>12.3</u>	<u>0.60</u>	<u>1.01</u>	P	N

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573 ^a g C in the HA/g C in 100 g of total sample from which the HA was extracted;574 ^b I: irregular; S: seasonal; P: permanent. Flooding regime refers to that before 1973, year of the575 onset of large perturbation in the hydrological regime of the Tablas. Fires are those recorded576 before sampling year (2007); at sites 15, 17, and 17', the existence of fires in the past has been577 inferred from the occurrence of charred material in the samples.

578

579 **Table 2**580 ¹³C NMR areas from HAs from wetland soils of Las Tablas de Daimiel^a.

<u>Chemical shift range (ppm)</u>	<u>Alkyl</u>	<u>Methoxyl</u>	<u>C6</u>	<u>O-alkyl</u>	<u>C1</u>	<u>H-arom</u>	<u>O/N-arom</u>	<u>Carboxil</u>	<u>Carbonyl</u>	<u>Aliphaticity</u>	<u>Aromaticity</u>
	<u>0-45</u>	<u>46-58</u>	<u>59-65</u>	<u>66-94</u>	<u>95-110</u>	<u>111-146</u>	<u>147-167</u>	<u>168-188</u>	<u>189-200</u>	<u>111-167</u>	<u>0-110</u>
<u>Sample</u>											
<u>1</u>	<u>32.02</u>	<u>9.01</u>	<u>4.04</u>	<u>14.47</u>	<u>4.11</u>	<u>16.47</u>	<u>6.03</u>	<u>11.90</u>	<u>1.95</u>	<u>22.50</u>	<u>63.64</u>
<u>3</u>	<u>29.87</u>	<u>12.12</u>	<u>3.85</u>	<u>13.20</u>	<u>3.18</u>	<u>17.33</u>	<u>5.71</u>	<u>12.46</u>	<u>2.29</u>	<u>23.04</u>	<u>62.21</u>
<u>5</u>	<u>36.52</u>	<u>8.39</u>	<u>3.58</u>	<u>14.37</u>	<u>3.68</u>	<u>14.01</u>	<u>5.24</u>	<u>12.15</u>	<u>2.06</u>	<u>19.25</u>	<u>66.53</u>
<u>6</u>	<u>35.85</u>	<u>10.01</u>	<u>4.96</u>	<u>18.31</u>	<u>5.03</u>	<u>11.03</u>	<u>3.28</u>	<u>10.34</u>	<u>1.19</u>	<u>14.31</u>	<u>74.16</u>
<u>7</u>	<u>17.61</u>	<u>8.57</u>	<u>3.36</u>	<u>8.66</u>	<u>4.99</u>	<u>30.33</u>	<u>12.73</u>	<u>11.64</u>	<u>2.11</u>	<u>43.05</u>	<u>43.20</u>
<u>8</u>	<u>21.83</u>	<u>8.01</u>	<u>3.74</u>	<u>10.62</u>	<u>4.31</u>	<u>27.55</u>	<u>10.21</u>	<u>11.85</u>	<u>1.88</u>	<u>37.76</u>	<u>48.51</u>
<u>9</u>	<u>22.31</u>	<u>8.23</u>	<u>3.02</u>	<u>11.42</u>	<u>4.59</u>	<u>23.25</u>	<u>9.94</u>	<u>14.20</u>	<u>3.05</u>	<u>33.19</u>	<u>49.57</u>
<u>10</u>	<u>24.60</u>	<u>8.33</u>	<u>3.74</u>	<u>10.22</u>	<u>3.53</u>	<u>24.14</u>	<u>9.83</u>	<u>12.89</u>	<u>2.72</u>	<u>33.96</u>	<u>50.42</u>
<u>11</u>	<u>20.76</u>	<u>9.89</u>	<u>4.05</u>	<u>9.26</u>	<u>4.44</u>	<u>27.81</u>	<u>11.40</u>	<u>10.38</u>	<u>2.01</u>	<u>39.20</u>	<u>48.41</u>
<u>12</u>	<u>29.12</u>	<u>7.24</u>	<u>2.93</u>	<u>9.50</u>	<u>2.53</u>	<u>24.44</u>	<u>7.03</u>	<u>14.51</u>	<u>2.70</u>	<u>31.47</u>	<u>51.32</u>
<u>13</u>	<u>30.25</u>	<u>9.02</u>	<u>3.32</u>	<u>11.09</u>	<u>3.43</u>	<u>20.31</u>	<u>7.39</u>	<u>13.14</u>	<u>2.05</u>	<u>27.70</u>	<u>57.11</u>
<u>14</u>	<u>20.34</u>	<u>7.41</u>	<u>2.98</u>	<u>9.60</u>	<u>4.47</u>	<u>27.62</u>	<u>10.62</u>	<u>14.40</u>	<u>2.56</u>	<u>38.24</u>	<u>44.80</u>
<u>15</u>	<u>25.68</u>	<u>8.60</u>	<u>2.80</u>	<u>9.57</u>	<u>2.93</u>	<u>24.57</u>	<u>9.05</u>	<u>14.40</u>	<u>2.41</u>	<u>33.62</u>	<u>49.57</u>
<u>16</u>	<u>30.14</u>	<u>7.89</u>	<u>2.86</u>	<u>11.40</u>	<u>2.94</u>	<u>21.12</u>	<u>6.79</u>	<u>14.29</u>	<u>2.56</u>	<u>27.91</u>	<u>55.24</u>
<u>17</u>	<u>25.20</u>	<u>8.25</u>	<u>2.96</u>	<u>10.92</u>	<u>3.66</u>	<u>24.23</u>	<u>8.68</u>	<u>13.88</u>	<u>2.22</u>	<u>32.91</u>	<u>50.99</u>
<u>17'</u>	<u>27.65</u>	<u>8.13</u>	<u>3.20</u>	<u>9.64</u>	<u>2.96</u>	<u>22.75</u>	<u>8.47</u>	<u>14.50</u>	<u>2.71</u>	<u>31.22</u>	<u>51.58</u>
<u>18</u>	<u>23.32</u>	<u>7.39</u>	<u>3.10</u>	<u>8.86</u>	<u>2.82</u>	<u>28.94</u>	<u>9.90</u>	<u>13.47</u>	<u>2.20</u>	<u>38.84</u>	<u>45.50</u>
<u>19</u>	<u>29.32</u>	<u>8.41</u>	<u>3.74</u>	<u>12.10</u>	<u>2.91</u>	<u>19.66</u>	<u>6.71</u>	<u>14.72</u>	<u>2.44</u>	<u>26.37</u>	<u>56.47</u>

581

582 ^aAromaticity obtained from the NMR spectra strongly correlated with the elementary583 composition; H/C was strongly (negatively) related to HA aromaticity (R^2 0.794, $p < 0.0001$);584 the relationship between C/N and aromaticity was weak and positive (R^2 0.310, $p = 0.016$).

585

586 **Table 3**587 Pearson's linear correlation coefficients r for HA components obtained from MMM model and588 C/N ratio ^a [n.s. non-significant correlation ($P > 0.05$)].

<u>Carbohydrate</u>						
<u>0.681</u> <u>0.0019</u>	<u>Protein</u>					
<u>-0.563</u> <u>0.015</u>	<u>-0.687</u> <u>0.0017</u>	<u>Lignin</u>				
<u>0.649</u> <u>0.036</u>	<u>n.s.</u>	<u>-0.709</u> <u>0.001</u>	<u>Lipid</u>			
<u>-0.691</u> <u>0.0015</u>	<u>-0.781</u> <u>0.0001</u>	<u>n.s.</u>	<u>n.s.</u>	<u>Carbonyl</u>		
<u>-0.797</u> <u>0.0001</u>	<u>-0.502</u> <u>0.034</u>	<u>n.s.</u>	<u>-0.571</u> <u>0.013</u>	<u>0.528</u> <u>0.024</u>	<u>Char</u>	
<u>-0.547</u> <u>0.019</u>	<u>-0.698</u> <u>0.0013</u>	<u>0.789</u> <u><0.0001</u>	<u>-0.497</u> <u>0.036</u>	<u>0.523</u> <u>0.026</u>	<u>n.s.</u>	<u>C/N</u>

589

590 ^a P value of correlation in italics.

591

592

593 **Table 4**594 Pearson's linear correlation coefficients r for HA components and corresponding WS595 components obtained from the MMM model [n.s., non-significant correlation ($P > 0.05$)].^a

<u>Component</u>	<u>Carbohydrate</u>	<u>Protein</u>	<u>Lignin</u>	<u>Lipid</u>	<u>Carbonyl</u>	<u>Char</u>	<u>Lignin + char</u>
<i><u>r</u></i>	<u>0.747</u>	<u>0.401</u>	<u>0.374</u>	<u>0.527</u>	<u>0.346</u>	<u>0.663</u>	<u>0.755</u>
<i><u>p</u></i>	<u>0.0004</u>	<u>n.s.</u>	<u>n.s.</u>	<u>0.025</u>	<u>n.s.</u>	<u>0.0027</u>	<u>0.0003</u>

596

597 ^a P value of correlation in italics.

598

599 **Table 5**600 Average organic composition of whole samples and HAs (MMM results)

	<u>Whole</u>	
	<u>sample^a</u>	<u>Humic acid</u>
<u>Carbohydrate</u>	<u>19.9</u>	<u>10.7</u>
<u>Protein</u>	<u>24.8</u>	<u>20.7</u>
<u>Lignin</u>	<u>14.8</u>	<u>18.8</u>
<u>Lipid</u>	<u>24.6</u>	<u>22.8</u>
<u>Carbonyl</u>	<u>2.2</u>	<u>5.4</u>
<u>Char</u>	<u>13.7</u>	<u>21.7</u>

601 ^a Data from Rodríguez-Murillo et al.,(2011).

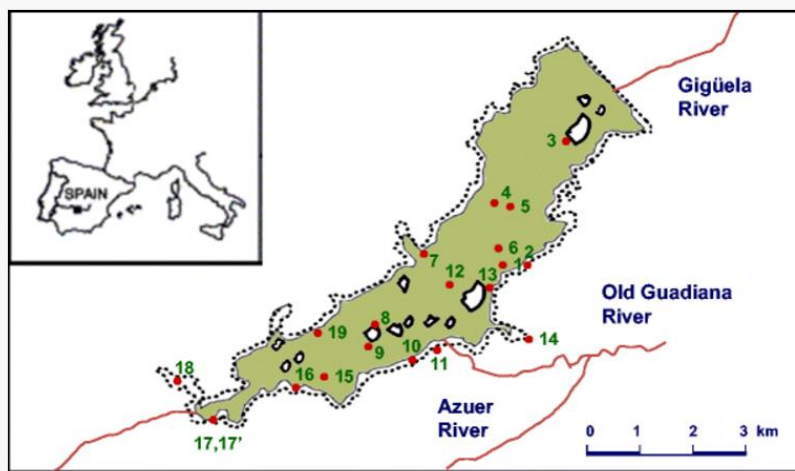


Fig. 1
Location of Tablas de Daimiel wetland and sampling points

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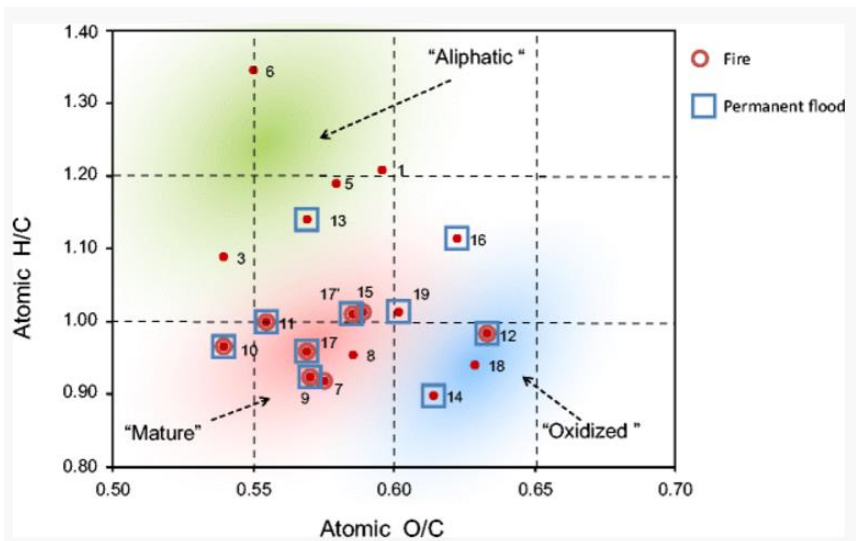


Fig. 2
Van Krevelen's diagram of humic acids (HAs) from the sampling points of Tablas de Daimiel

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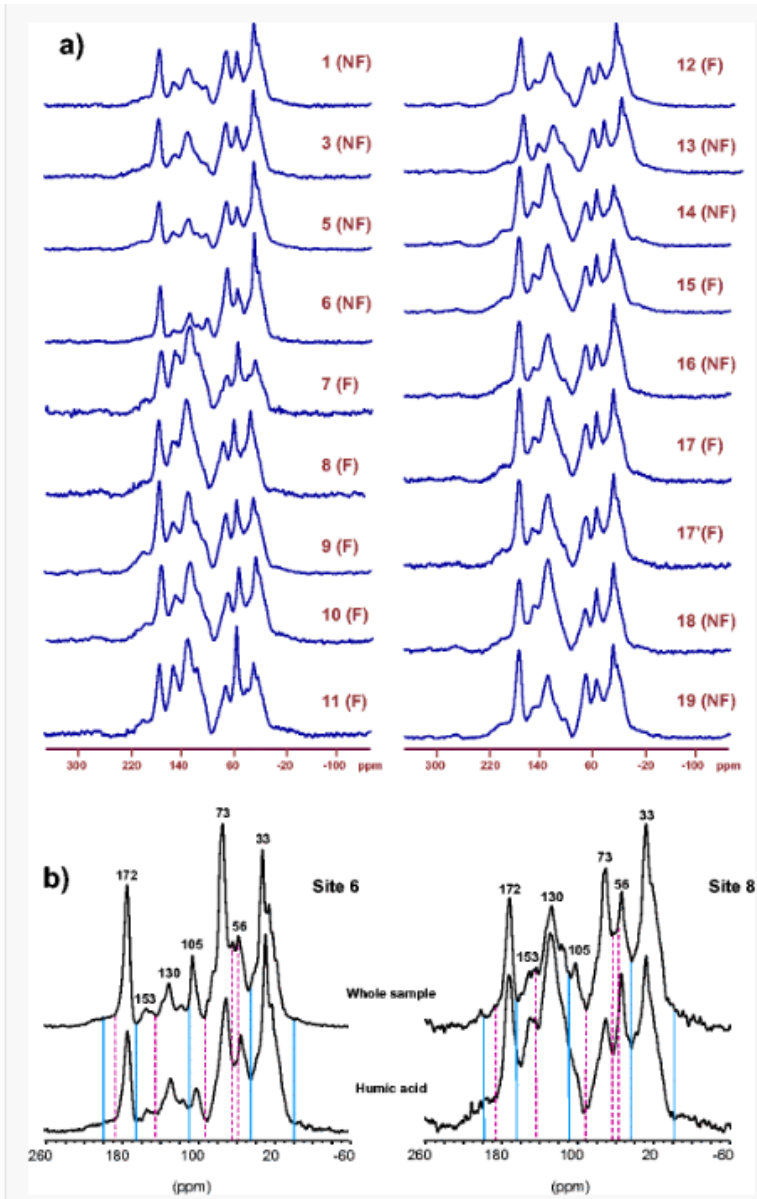


Fig. 3

a NMR spectra of the HAs. *F*: at least a fire has been recorded in the area of the sampling point. *NF* no fire recorded. **b** Comparison between ^{13}C NMR spectra of whole sample and its humic acids: the spectral ranges for the main C-types are indicated

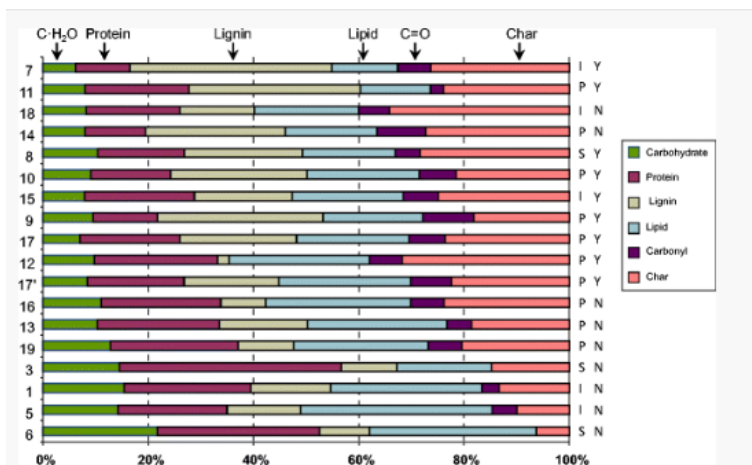


Fig. 4

HA composition according to molecular mixing model (MMM). HAs are ordered from lower aromaticity (*bottom*) to higher aromaticity (*top*). *I* irregular; *S* seasonal; *P* permanent. Flooding regime refers to that before 1973, year of the onset of large perturbation in the hydrological regime of the Tablas. Fires are those recorded before sampling year (2007); at sites 15, 17, and 17', the existence of fires in the past has been inferred from the occurrence of charred material in the samples. *Y* means that at least one fire has been recorded and *N* means no fire recorded in the site

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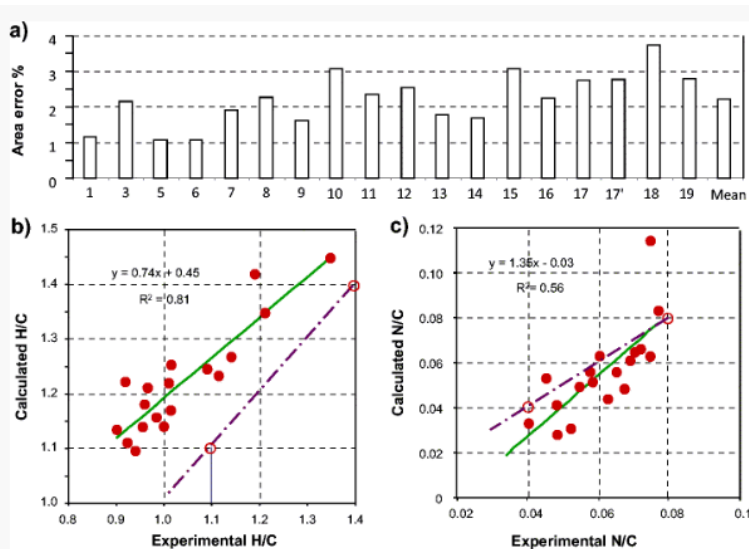


Fig. 5

a Performance of MMM. Area error is the sum of absolute values of differences (%) between experimental and calculated areas for each chemical shift region in each sample. b Performance of MMM. Calculated versus experimental H/C ratios of the samples. The *lower line* represents the perfect concordance calculated–experimental. c Performance of MMM. Calculated versus experimental N/C ratios of the samples

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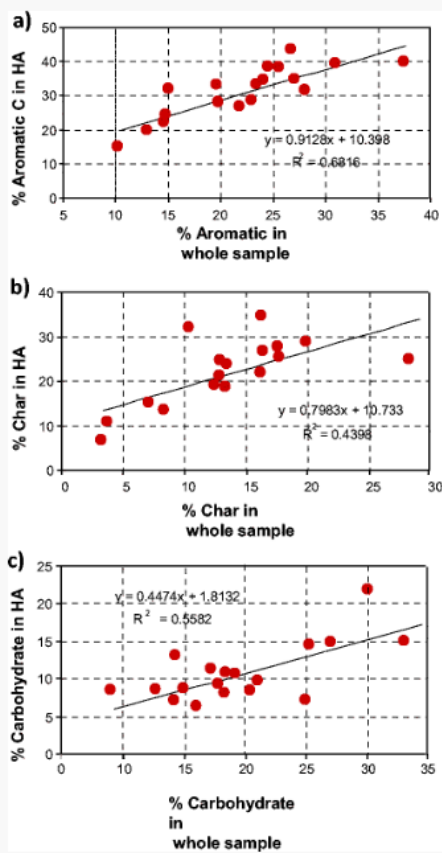


Fig. 6
Comparison of components of HAs with components of organic matter in whole samples (WS) according to MMM. **a** Comparison of aromaticities. **b** Comparison of char contents. **c** Comparison of carbohydrate contents

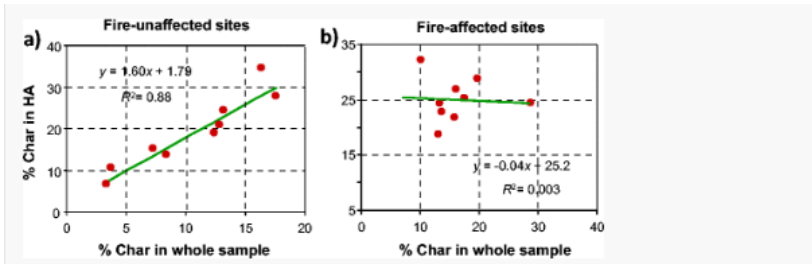


Fig. 7
Comparison of components of HAs with components of organic matter in whole samples (WS) according to MMM. Effects of fire on char content change. a Comparison of char content in no-fire areas. b Comparison of char content in fire areas

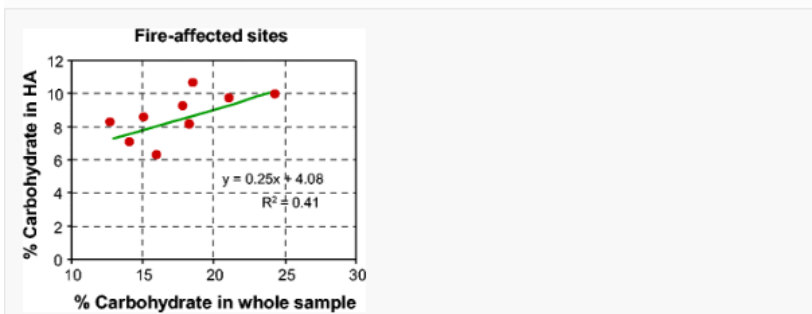


Fig. 8
Comparison of components of HAs with components of organic matter in whole samples (WS) according to MMM. Effects of fire on carbohydrate content change

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Con formato: Español (alfab. internacional)