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1 **Compositional Changes in the Hydrophobic acids fraction of**
2 **Drainage Water from Different Land Management Practices.**

3

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13 Byrne, C.M.P. et al., Compositional changes in the hydrophobic acids fraction of
14 drainage water from different land management practices, Water Research (2010),
15 doi: 10.1016/j.watres.2010.05.055

Abstract

16

17

18 Dissolved organic matter (DOM) can play a key role in many environmental
19 processes, including carbon cycling, nutrient transport and the fates of contaminants
20 and of agrochemicals. Hydrophobic acids (Ho) , the major components of the DOM,
21 were recovered from the drainage waters from a well drained (WDS) and a poorly
22 drained (PDS) Irish grassland soils in lysimeters and amended with N fertiliser (F)
23 and with bovine urine (U) and were studied using 1D and 2D solution state Nuclear
24 Magnetic Resonance (NMR) spectroscopy. The Diffusion Edited (DE) ¹H NMR
25 spectra indicated that the Ho consisted largely of larger molecules, or of molecules
26 that formed rigid aggregates, and the 1D and the 2D (Heteronuclear Multiple
27 Quantum Coherence – HMQC, the Total Correlation Spectroscopy –TOCSY, and the
28 Nuclear Overhauser Effect –NOESY) spectra indicated that the samples were
29 composed of lignin residues, carbohydrates, protein/peptides, and aliphatic
30 components derived from plant waxes/cuticular materials and from microbial lipids.
31 The F amendments increased the concentrations of Ho in the waters by 1.5 and 2.5
32 times those in the controls in the cases of WDS and PDS, respectively. The lignin-
33 derived components were increased by 50% and by 300% in the cases of the Ho from
34 the WDS and PDS, respectively. Applications of F + U decreased the losses of Ho,
35 (compared to the F amendments alone) and very significantly decreased those of the
36 lignin derived materials, indicating that enhanced microbial activity from U gave rise
37 to enhanced metabolism of the Ho components, and especially of lignin. In contrast
38 the less biodegradable aliphatic components containing cuticular materials increased
39 as the result of applications of F + U. This study helps our understanding of how
40 management practices influence the movement of C between terrestrial and aquatic
41 environments.

42

43 **Keywords**

44 Grassland; Dissolved organic matter; Hydrophobic acids; Drainage water; Fertiliser;
45 Urine; Solution state NMR

46

47 **1. Introduction**

48 Dissolved organic matter (DOM) is a complex, heterogeneous mixture found in all
49 natural waters, and it represents the largest fraction of mobile carbon (C) on earth. It
50 provides an intimate link between the terrestrial and aquatic environments (Lam et al.,
51 2007). Soil derived DOM can play a key role in many environmental processes,
52 including carbon cycling, nutrient transport and the fates of contaminants and of
53 agrochemicals (Qualls and Haines, 1991; Royer et al., 2007; Zsolnay, 2003). Despite
54 its obvious importance, the structural components of soil DOM and the variations of

55 these components with different land management practices, have not been well
56 resolved (Royer et al., 2007).

57 Temperate grassland ecosystems, which comprise 32% of the earth's natural
58 vegetation (Frank and Dugas, 2001), can be considered to have a significant role in
59 the uptake of atmospheric CO₂ and in balancing the global C budget (Batjes, 1998).
60 Grassland, the dominant ecosystem in Ireland, represents 90% of agricultural land and
61 56% of the total land area (Jaksic et al., 2006). Article 3.4 of the Kyoto protocol
62 (UNFCCC, 1998) makes provision for the use of soil C stock changes in grazing
63 lands to offset greenhouse gas (GHG) emissions and to facilitate the achievement of
64 emissions reduction targets (Byrne et al., 2007). On that basis, there is a need to better
65 understand the organic components leached from these carbon stocks under different
66 management practices.

67 Soils in long-term pasture are in a steady-state with regard to soil organic matter
68 (SOM) content. Carbon accumulation in grassland ecosystems occurs mostly below
69 ground and changes in soil organic C (SOC) stocks may result from changes in land
70 uses management (Soussana et al., 2004). Grassland C stocks represent at least 10%
71 of the global total, and some sources suggest up to 30% of that total (Scurlock and
72 Hall, 1998). The stocks of SOM result from the balance between inputs and outputs of
73 C. Inputs are primarily from leaf and root detritus. Outputs are dominated by the
74 efflux of carbon dioxide (CO₂) and of methane (CH₄) from the soil surface and by the
75 hydrologic leaching of dissolved and particulate C (Davidson and Janssens, 2006).
76 The pool of SOM is of particular interest because even small changes in flux rates into
77 or out of such a large pool could lead to the accumulation of significant quantities of
78 greenhouse gases (Billings and Ziegler, 2008).

79 Although land use and related management practices are known to affect the
80 amounts and compositions of SOM and soil properties, their influences on the
81 amounts and compositions of DOM have not been extensively studied (Chantigny,
82 2003). Various aspects of the effects of elevated nitrogen (N) deposition and of N
83 fertilization have been studied, yet little is known about their effects on DOM
84 turnover (Kalbitz et al., 2000). The same is true for organic amendments such as
85 urine. The OM in amendments is biodegradable and is generally readily transformed
86 by soil microbes. That may result in transient increases in the soil DOM (Chantigny,
87 2003). Amendment with slurry has been found to increase nitrogen (N)
88 immobilisation through increased microbial activity (Hoekstra, 2009). This may lead
89 to an increase in carbon mineralization and a decrease in DOM export. However, to
90 our knowledge, detailed studies have not been carried out on changes in DOM
91 compositions following mineral fertilization and organic amendments. Soil hydrology
92 is also likely to affect DOM dynamics. Differences have been found between DOM
93 fractions isolated from different drainage regimes (Hayes et al., 1997), and research
94 has shown that DOC exports were 33 Kg ha⁻¹ lower from drained than from
95 undrained plots (McTiernan et al., 2001).

96 In this study we characterise, in detail, the Ho from the DOM formed from two
97 soils, one well drained (WDS) and the second poorly drained (PDS), each amended
98 with fertiliser, and with fertiliser and urine. The emphasis is on the characterisation of
99 the components of the Ho released in the drainage water from these soils using
100 advanced Multidimensional nuclear magnetic resonance spectroscopy (NMR)
101 techniques that are widely used to study structures and interactions in environmental
102 chemistry (Simpson and Brown, 2005; Thrippleton and Keeler, 2003).

103

104 **2. Materials and Methods**

105 **2.1 Source of samples**

106 Intact soil monoliths lysimeters (0.8 m diameter by 1 m deep) were sampled
107 from a well drained (WDS) Brown Podzolic soil (Haplic Podzol (Anthric)) (FAO,
108 2007) and from a poorly drained (PDS) Gley (Luvic Stagnosol (Eutric, Siltic)) (FAO,
109 2007) were installed in 2004 in lysimeters in a pasture field at the Teagasc
110 Environmental Research Centre (ERC), Johnstown Castle, Wexford, Ireland. The
111 sand, silt, and clay contents of the soils are given in Table 1. The soils were collected
112 as undisturbed monoliths and installed according to an established protocol (Cameron
113 et al., 1992). Briefly this involved isolating a 1 by 1m soil column and then carefully,
114 reciprocally, pushing a 0.8 m HDPE pipe through the soil column. When the pipe
115 reached 1 m a cutting plate was hydraulically pushed beneath the lysimeter to cut it
116 from the soil beneath. To prevent edge flow liquid petrolatum was injected between
117 the soil and the HDPE pipe. The lysimeters were inverted and 5 cm of fine gravel
118 inserted at the base of the soil and a base plate with drainage outlet was welded to the
119 pipe. The completed lysimeters were installed in a field lysimeter facility under
120 natural rainfall and meteorological conditions. Each soil was sown with perennial
121 ryegrass (*Lolium perenne* L.). In order to replicate typical Irish grazed grassland
122 activities, some of the lysimeter soils were amended with fertiliser and some with
123 both fertiliser and bovine urine, and unamended soils served as controls as described
124 in Stark *et al.* (2007). With the exception of the controls, the lysimeter soils received
125 in 2004 and 2005, 291 kg N ha⁻¹ yr⁻¹ as fertiliser and 310 kg N ha⁻¹ yr⁻¹ as urine (Table
126 2). Treatments were applied in a randomised complete block design with 3 replicates
127 per treatment. Herbage was harvested regularly to correspond with a 28-day rotation
128 of livestock. A series of pipes transported the drainage water (DW) from each

129 lysimeter to storage vessels housed below ground level. Drainage water samples, 200
130 L from each treatment and control, were collected from the lysimeter facility between
131 June and December, 2005.

132 **2.2 Isolation of hydrophobic acids from drainage waters.**

133 The Ho were isolated from the drainage waters using previously described
134 procedures (Hayes et al., 2008; Malcolm and MacCarthy, 1992). Waters were filtered
135 under pressure (69 kPa) through 0.2 m Sartorius (Goettingen, Germany) cellulose
136 acetate membrane filters. The filtrates were adjusted to pH 2 (HCl) and applied to
137 XAD-8 resin [(poly)methylmethacrylate] (Rohm and Haas, Philadelphia). Two
138 column volumes of 0.01 M HCl were pumped through to ensure that the entire sample
139 had passed through the column. The resin was then desalted with distilled water until
140 effluent conductivities were $< 100 \mu\text{S cm}^{-1}$. Back elution was carried out using 0.1 M
141 NaOH and the centre cut eluates were H^+ exchanged (Amberlite IR-120, H^+ -form;
142 Rohm and Haas, Philadelphia), then freeze dried to give the XAD-8 hydrophobic (Ho)
143 acids.

144

145 **2.3 Solution State NMR Spectroscopy experimental details**

146 Samples (40 mg) were dissolved in 600 μL of deuterium oxide (D_2O) and
147 titrated to pH 12 using NaOD to ensure complete solubility. Additional samples (40
148 mg) were dissolved in 600 μL $\text{DMSO-}d_6$.

149 Samples were analysed using a Bruker Avance 500 MHz NMR spectrometer
150 equipped with a ^1H - ^{19}F - ^{15}N - ^{13}C 5 mm, quadruple resonance inverse probe with
151 actively shielded z-gradient (QXI). 1D solution state ^1H NMR spectra were obtained
152 with 128 scans, a recycle delay of 2 s, 16384 time domain points, and an acquisition
153 time of 0.79 s. Water suppression was achieved using PURGE (Simpson and Brown,

154 2005). Spectra were apodized through multiplication with an exponential decay
155 corresponding to 1 Hz line broadening, and a zero filling factor of 2. Diffusion-edited
156 (DE) spectra were obtained using a bipolar pulse longitudinal encode-encode
157 sequence. Scans (1600) were collected using a 2.5 ms, 49 gauss/cm, sine-shaped
158 gradient pulse, a diffusion time of 200 ms, 16384 time domain points, 0.82 s
159 acquisition time, and a sample temperature of 298 K.

160 Heteronuclear multiple quantum coherence (HMQC) spectra were obtained in
161 phase-sensitive mode using echo/anti-echo gradient selection and a $^1J \text{ } ^1\text{H}-^{13}\text{C}$ value of
162 145 Hz. Scans (512) were collected for each of the 128 increments in the F1
163 dimension. A total of 1048 data points were collected in F2, and a relaxation delay of
164 1 s was employed. The F2 dimension was multiplied by an exponential function
165 corresponding to a 10 Hz line broadening and a zero filling factor of 2. The F1
166 dimension was processed using a sine-squared function with a $\sqrt{2}$ phase shift and a
167 zero-filling factor of 2.

168 Total correlation spectroscopy (TOCSY) spectra were acquired in the phase-
169 sensitive mode, using time proportional phase incrimination (TPPI). TOCSY NMR
170 experiments were carried out using 512 scans with 128 time domain points in the F1
171 dimension and 1048 time domain points in the F2 dimension. A mixing time of 60 ms
172 was used with a relaxation delay of 1 s. Processing of both dimensions used a sine-
173 squared function with a $\sqrt{2}$ phase shift and a zero-filling factor of 2.

174 Nuclear Overhauser Effect Spectroscopy (NOESY) was obtained with the
175 elimination of zero-quantum interference (Thrippleton and Keeler, 2003). NOESY
176 NMR experiments were carried out using 256 scans with 128 time domain points in
177 the F1 dimension and 1048 time domain points in the F2 dimension. A mixing time of
178 250 ms was used with a relaxation delay of 1 s. Zero-quantum suppression was

179 achieved through the use of an adiabatic-pulse/gradient pair during the mixing time
180 (Thrippleton and Keeler, 2003). Both dimensions were processed using a sine-
181 squared function with a $\sqrt{2}$ phase shift and a zero-filling factor of 2.

182

183 **3. Results and discussion**

184 DOM in soil is composed of humic substances and a variety of specific identifiable
185 organic compounds, including carbohydrates and peptides. In this study the
186 hydrophobic acid fraction was isolated using an XAD-8 resin technique (Leenheer,
187 1981), and is the dominating constituent of bulk dissolved organic matter (DOM) in
188 soil solutions (Asakawa et al., 2006).

189

190 **3.1 Characterisation of the drainage water hydrophobic acids**

191 Two solvent systems were used for the NMR analysis of the Ho; D₂O/NaOD
192 and DMSO- *d*₆. D₂O or D₂O/NaOD systems are commonly used for studies of DOM
193 (Hertkorn et al., 2006; Kaiser et al., 2003; Kim et al., 2003; Lam et al., 2007;
194 Simpson, 2001; Smejkalova and Piccolo, 2008) and the D₂O/NaOD system in this
195 study enabled comparisons with previous studies. DOM samples in the protonated
196 form (achieved here through exchange with the IR-120 cation exchange resin) are
197 completely soluble in DMSO. DMSO is a dipolar aprotic solvent; hence signals from
198 exchangeable protons, for example, N-H, can be observed. Thus DMSO provides
199 excellent complimentary information for structural studies, especially for
200 protein/peptide components, and in many cases it provides spectra with better defined
201 resonances (Simpson, 2001). Our samples were completely soluble in both solvent
202 systems used. 1D and 2D NMR spectroscopy techniques were used to observe
203 compositional differences in the Ho components in the drainage waters.

204 Figure 1A shows the ^1H NMR spectrum in $\text{DMSO-}d_6$ for the Ho isolated from
205 the poorly drained soil (PDS) treated with fertiliser. Major structural components
206 present include aromatics, lignin (Lig), carbohydrates (Carb), proteins/peptides (P)
207 and aliphatic units. Figure 1B is the diffusion edited (DE) NMR spectrum of the same
208 sample. Signals from larger molecules or rigid molecular associations can be further
209 emphasised by the use of diffusion editing. Diffusion editing “spatially encodes”
210 molecules at the start and then “refocuses” these at the end of the experiment. Species
211 that diffuse and exhibit a high degree of motion during the experiment are not
212 refocused and are essentially gated from the final spectrum (Simpson et al., 2007b).
213 Thus the spectrum produced contains only signals from larger molecules or rigid
214 molecular associations. Because the majority of the signals remain after diffusion
215 editing, it can be considered that the components in the Ho are likely to be larger
216 molecules or very stable aggregates (Simpson, 2002). Main chain methylene signals at
217 ~1.3 ppm are consistent with aliphatic structures from plant-derived waxes/cuticles
218 (Deshmukh et al., 2003) that have previously been identified in humic extracts
219 (Kelleher and Simpson, 2006; Kelleher et al., 2006; Simpson et al., 2003), and to
220 contributions from microbial lipids (Simpson et al., 2007a). In this DE spectrum, the
221 CH_3 signal at ~ 0.8 ppm is likely to be mainly from methylated amino acid side chain
222 residues (Simpson et al., 2007a). This is further dealt with in discussion of Figure 1C.
223 There is considerable overlap in the 1D NMR resonances. However it has been
224 possible to confirm the suggested assignments by an array of 2D NMR experiments,
225 including HMQC, TOCSY, and NOESY. Applications of 2D NMR for studies of
226 natural organic matter (NOM), and interpretations of the data have been discussed
227 extensively in the literature (Cardoza et al., 2004; Simpson, 2001; Simpson et al.,
228 2001). Briefly, 2D NMR experiments provide increased spectral dispersion as well as

229 additional connectivity information allowing detailed assignments of the chemical
230 functionalities and structural components present (Lam et al., 2007). Figure 2A shows
231 the Heteronuclear Multiple Quantum Coherence (HSQC) spectrum for the Ho isolated
232 from the PDS that was treated with fertiliser. The HMQC experiment detects one
233 bond ^1H - ^{13}C connectivities in an organic structure (Simpson, 2001). When considered
234 together, the cross-peaks form a specific pattern that can be thought of as the
235 “molecular fingerprint” of a specific structure or class of structure (Kelleher and
236 Simpson, 2006). The HMQC NMR spectrum identifies a range of chemical
237 functionalities present (assignments and references are given in the Figure caption)
238 and suggests that the Ho are a mixture of predominately lignin, protein,
239 carbohydrates, and lipids/cuticular waxes (Deshmukh et al., 2005; Deshmukh et al.,
240 2003; Kelleher and Simpson, 2006; Lam et al., 2007; Simpson et al., 2007a; Simpson
241 et al., 2007b). This is further supported by the TOCSY (Fig. 2C) and NOESY (Fig.
242 2D) data. All these components have been assigned previously for NOM (Deshmukh
243 et al., 2003; Hertkorn et al., 2006; Kelleher and Simpson, 2006; Kelleher et al., 2006;
244 Lam et al., 2007; Simpson, 2001; Simpson et al., 2003; Simpson et al., 2007a;
245 Simpson et al., 2007b).

246 Signals due to *N*-acetyl and/or *O*-acetyl, previously seen in freshwater DOM
247 (Hertkorn et al., 2006; Lam et al., 2007) are evident in region 10 (Fig. 2A, 2B). Acetyl
248 groups (Lam et al., 2007), often found in peptidoglycan from microbial cell walls
249 (Simpson et al., 2007b) and in protein (Simpson et al., 2007a) could indicate
250 microbial inputs. The microbial contributions are most clearly evident from
251 comparisons between spectra for microbial biomass cultured from soil (Simpson et
252 al., 2007a) and those for the Ho in this study. Figures 1B and 1C compare the DE
253 spectrum of the Ho with that obtained for microbes cultured from a Canadian dark

254 grey Chernozem soil. The microbes on which Fig. 1C is based were isolated from a
255 different soil to that from which the Ho for Fig 1B was obtained. The microbes were
256 cultured in a minimal medium with glucose and acetate as carbon sources using a
257 "double spiking approach" (Simpson et al., 2007a). Previous studies have shown that
258 soil microbes give a relatively similar NMR spectrum, irrespective of the soil type
259 from which they are isolated (Simpson et al., 2007a), and the spectrum shown in
260 Figure 1C shows the extent to which the microbial contributions contribute to the Ho.

261 Comparison of the two spectra indicate that signals from microbial biomass,
262 mainly peaks labelled P, are clearly apparent in the NMR spectrum of the Ho.

263 Characteristic resonances seen for protein/peptide, namely amide (N-H),
264 phenylalanine (Phe), α -protons from amino acid side chains, and methylated side
265 chains are easily distinguishable in both the Ho acid and in the microbial biomass.

266 Furthermore, the region labelled "SC" in Figure 1C represents the side-chain
267 resonances from proteins and peptides. This region can generally be considered as a
268 "fingerprint" region representing the type of peptide/protein present (Simpson et al.,
269 2007a). The side-chain region in the Ho acid matches well with that of the microbes.

270 The similarities between the Ho spectrum and that of the microbes, highlights
271 the input of microbial biomass to the Ho isolated from the drainage waters.

272 Components from plant biomass, in addition to microbial inputs, are also in
273 evidence. There are clear indications for lignin-derived components. While these
274 signals are very clear in the HMQC and NOESY data (Figs 2A, 2D), they are still
275 apparent in the 1D spectra. Figure 1D displays the DE spectrum for a lignin standard
276 (organosolv lignin, Sigma Aldrich). The large resonance centered at ~3.7 ppm is
277 characteristic of the methoxyl of lignin. Comparison of the spectrum for Ho (1B) with
278 that of the Organosolv lignin (1D) clearly indicates that the apex of the central region

279 of the lignin peak (labelled Lig) in the Ho is from the methoxyl of lignin (Simpson et
280 al., 2007b). This is also confirmed by the intensity of the methoxyl signal in the
281 HMQC data (Figure 2A). Additionally, aromatic resonances from lignin at ~6.3-7
282 ppm (Lig), are evident in the Ho (Figure 1B) and these partially overlap with the
283 signal for aromatic residues in proteins/peptides. Thus it can be concluded that the Ho
284 is likely to be a mixture of soil derived plant and microbial materials that have
285 previously been identified in a range of NOM samples (Hertkorn et al., 2006;
286 Hertkorn et al., 2002; Kelleher and Simpson, 2006; Kelleher et al., 2006; Lam et al.,
287 2007; Simpson, 2001; Simpson, 2002; Simpson et al., 2001; Simpson et al., 2003;
288 Simpson et al., 2004; Simpson et al., 2007a; Simpson et al., 2007b).

289 **3.2 Investigation into the effects of the various treatment regimes**

290 Results have varied with regard to studies of the effects of N on OM
291 decomposition. Concentrations and fluxes of DOC from the forest floor remained
292 unchanged for field additions of N (Currie et al., 1996; McDowell et al., 1998)
293 whereas the DOC release rate was found to have decreased by 20% following N
294 fertilization of a forest soil (Cronan et al., 1992). N addition as urea resulted in the
295 increased release of water-soluble OC from a forest soil (Homann and Grigal, 1992).

296 Exports of hydrophobic acids in the drainage water from the well-drained and of
297 poorly-drained soils under different treatment applications are shown in Table 3. Both
298 of the control soils had similar exports of Ho acids in their DW. However, the
299 application of fertiliser gave rise to large increases. Exports of Ho were 1.5 times
300 greater from the WDS, and were almost 2.5 times greater from the PDS. This positive
301 correlation between N fertiliser application and total Ho exported in the cases of both
302 soils may have resulted from increased OM matter inputs arising from increase
303 grassland productivity.

304 This is proportional to N application (McTiernan et al., 2001), and leads to greater
305 returns of OM to the soil via leaf and root decay (Parsons et al., 1991). The additional
306 OM from the increased plant growth would be a potential source of the Ho that would
307 be transported from the plot by rainwater (McTiernan et al., 2001). In addition urea-
308 and ammonium-based fertilisers temporarily solubilise SOM and can, as the result of
309 an increase in soil pH, induce a marked increase in DOC content (Chantigny, 2003;
310 Myers and Thien, 1988). However, this effect has been found to be short-lived (Clay
311 et al., 1995).

312 The NMR spectra obtained for samples after dissolving in DMSO-*d*₆, shown in
313 Figure 4, are better resolved but contain the same major structural components seen in
314 D₂O (Figure 3). The contribution of peptides to the Ho is more evident in the DMSO-
315 *d*₆ spectra, as seen by the double “hump” at ~4-4.4 ppm (-protons) and by the large
316 amide and methyl resonances (Simpson et al., 2007b). This is most clear in the DE
317 spectra in DMSO (see Figure 5). The DE spectra are dominated by lignin and
318 microbial signatures indicating that these are the largest of the components in the
319 sample.

320 Regardless of solvent used, the NMR spectra indicate that there is an increase in
321 the lignin contribution to the Ho (Figures 3 and 4: A vs. B, D vs. E) as the result of
322 fertiliser applications. Absolute quantification from such complex 1D spectra is very
323 difficult, as discussed by Simpson et al. (2007b). However, relative quantification of
324 the methoxyl signal is possible from the 2D HMQC spectra. Absolute quantification
325 is not possible because the signal intensity in the HMQC employed in this study is
326 proportional to the one bond coupling constant ($^1J \text{ } ^1\text{H}-^{13}\text{C}$). The intensity of the
327 methoxyl signal with respect to the total intensity of all peaks in the HMQC (with the
328 exclusion of the DMSO peak) provides an estimation of the abundance of lignin in

329 each sample. This, in turn permits the relative increases/decreases in lignin contents in
330 the different samples to be estimated.

331 Semi-quantitative analysis indicates that, compared to the control, treatment of the
332 soil with fertiliser increased the lignin-derived components in the WD Ho by ca 50%.
333 An increase of 300% was found in the case of the PD Ho. The increases in lignin-
334 derived materials are likely to have resulted from the increased vegetative growth
335 arising from the fertiliser-N amendments.

336 Grazing can result in the deposition to soils of large quantities of urine-N (400 to
337 1200 Kg N ha⁻¹), and the effects of urine on changes in DOM compositions are not
338 well understood (Rooney et al., 2006). Ho was collected from lysimeter soils amended
339 with both fertiliser-N and urine-N. Applications of fertiliser plus urine (F+U) caused
340 less Ho losses than the treatment with fertiliser alone, (Table 3) but greater than from
341 the control. ¹H NMR spectra in both D₂O and DMSO-*d*₆ solvents show a significant
342 decrease in the lignin-derived signal in the Ho isolated from both F+U treated soils
343 (Figures 3 and 4: B vs. C, E vs. F). This correlates well with the semi-quantitative
344 analysis that suggested a decrease of 70 % (in comparison to the control) in the lignin-
345 derived OM signal for the WDS Ho as the result of treatment of the soil with F + U. A
346 decrease of 3% was found in the case of the PDS Ho as the result of a similar soil
347 treatment. It is probable that this decrease in C export in the drainage water from the
348 F+U treated soils resulted from increased microbial activity in the soil from the
349 addition of urine. Under the aerobic conditions that prevailed in the WDS F+U, the
350 lignin appears to have undergone greater oxidation. Soil respiration was found to be
351 higher from a soil treated with cow urine as the result of an immediate and significant
352 increase in microbial metabolic activity (Lovell and Jarvis, 1996). Urine contains only
353 small concentrations (0.01%) of soluble carbon (Kishan et al., 1989); however,

354 solubilisation of soil organic C has been shown to take place following urine
355 applications (Monaghan and Barraclough, 1993), and that soluble carbon could
356 provide substrate for increased microbial metabolism (Lovell and Jarvis, 1996). Soils
357 treated with varying concentrations of synthetic sheep urine had greater levels of
358 microbial activity than untreated soils (Rooney et al., 2006). Urine deposition has
359 been shown to alter substantially soil microbial communities, in terms of bacterial and
360 fungal counts and respiration rates (Williams et al., 2000). Differences in microbial
361 biomass activity between grassland types are related to differences in substrate
362 availability (Bardgett et al., 1998; Williams et al., 2000). A strong correlation between
363 N immobilization and C mineralization has been found (Barrett and Burke, 2000).
364 Rapid stabilization of N was facilitated by an active microbial community and the
365 availability of a readily mineralizable C substrate. It is likely that increased microbial
366 activity induced by the addition of urine promoted the decomposition of the lignin-
367 derived DOC (observed in the NMR spectra in this study) leading to the decrease in
368 the DOC concentration in the drainage water.

369 Conversely, cuticular coatings/leaf waxes are known to be highly recalcitrant
370 and to accumulate over time during the degradation of plants (Kelleher et al., 2006).
371 The relative contributions from aliphatic components compared to the lignin
372 components in the DE-NMR spectra for both PDS and WDS increased with
373 applications of F+U (Figure 5, C and F, see arrows). That would correspond to an
374 accumulation of aliphatic components in the Ho. Such may result from a decrease in
375 the more readily degradable fraction (i.e. lignin), resulting in higher concentrations of
376 the 'less digestible' cuticular fraction in the soil. Treatment of a grassland soil with
377 sheep urine was found to have led to an increase in the dead or decomposing root
378 mass from 2.2% in the untreated soil (control) to 6.3% in the urine treated soil (Shand

379 et al., 2002). They considered that part of the DOC in the soil solution from beneath
380 the urine patches came from roots damaged by the high concentrations of ammonia
381 (NH₃). That could explain the greater contribution of methylene units, possibly from
382 suberin in the root material, to the spectra of the Ho isolated from the DW of the soils
383 treated with F+U (Figure 5: C and F, see arrows). On the other hand, the signals
384 consistent with protein/microbial contributions are still dominant in the spectra. Such
385 would be expected as both the urea and N should stimulate microbial activity.

386 There are similarities in the Ho exported from the control soils. The various
387 treatment regimes, however, had greater effects on the PDS. As mentioned, the
388 application of fertiliser caused a greater increase in the exports of Ho from the PDS
389 (Table 3). That could arise, in the case of the poorly drained soil, from the decreased
390 aeration that would impede biological oxidation to carbon dioxide (CO₂) of the
391 increased organic matter (resulting from the application of fertiliser) (McTiernan et
392 al., 2001). On the other hand, rapid decomposition of organic materials may have
393 taken place in the WDS resulting in the removal of less DOM.

394 In contrast, the F+U application caused a decrease in the Ho from both the
395 PDS and from the WDS, in comparison to the application of fertiliser alone. That
396 could have arisen from increased microbial activity as a result of the urine additions,
397 leading to a greater metabolism of the SOM and leaving less material available to
398 contribute to the DOM.

399 In summary, the main effects of the varying treatment regimes on the Ho
400 composition from both soils are still not completely resolved. The contribution of
401 lignin components (peak labelled Lig or 6) increased with applications of fertiliser
402 and decreases with fertiliser plus urine addition. The most likely causes of the effects
403 is that the F+U applications lead to an increase in microbial activity causing microbial

404 utilisation of the more degradable lignin components. Irrespective of the causes of
405 these changes it appears that land management practices significantly alter the
406 composition of dissolved organic matter released into drainage water.

407

408 **3.3 Agricultural/Environmental Significance**

409

410 Results from the multidimensional solution-state NMR analysis, indicate that
411 the components of Ho in the drainage water of typical Irish grassland soils are
412 complex mixtures of both plant and microbial-derived materials. Strong contributions
413 from lignin and of peptides/proteins of microbial origins were evident in all spectra.

414 Treatment with fertiliser (F) resulted in an increase in the Ho export from both
415 the WDS and the PDS, and an increase in the lignin contribution to the compositions
416 of the Ho. This is thought to result directly from elevated OM inputs to the soil as the
417 result of increased dry matter production through fertilization. Enhanced microbial
418 activity is brought about by inputs of labile C (Lovell and Jarvis, 1996). Increased
419 microbial activity, stimulated by the addition of urine, could result in a degradation of
420 the increased OM input brought about by fertilization. That is reflected by a lower
421 lignin contribution to the Ho isolated from the fertiliser and urine treatment.

422 The drainage regime affected the responses of each soil to the treatments. The
423 decreased aeration in the PDS, compared to the WDS, resulted in a lesser
424 decomposition of the increased OM input in the Ho (McTiernan et al., 2001). In
425 contrast, the fertiliser plus urine application gave rise to a decrease in the Ho from the
426 PDS, compared to the treatment with fertiliser alone. A plausible explanation for this
427 might be that the urine may have been transported more slowly through the PDS

428 resulting in a higher level of microbial activity, increased decomposition, and a lower
429 export of Ho.

430 Growing concern about climate change has increased interest in the role of DOM
431 in the global carbon cycle (Kalbitz and Kaiser, 2008). This study provides further
432 information on the extent and the composition of the organic C lost from soils through
433 transport in drainage water from Irish grassland. Additions of plants with high lignin
434 content have been proposed as a means of building C stocks (Paustian et al., 1997) in
435 order to sequester C. Aromatic compounds from lignin are considered to be the most
436 stable components of DOM (Kalbitz and Kaiser, 2008). Our study indicates, however,
437 that the stimulation of microbial activity by the addition of urine decreases the
438 recalcitrance of the lignin components in the DOM.

439 Investigations of the compositions and the extents to which Ho is lost from soils,
440 as influenced by management practises and the processes involved, will help our
441 understanding of the movement of C between the terrestrial and aquatic environments.
442 Such information is important because it provides an insight into an area of the carbon

443 **4. Conclusions**

444 Hydrophobic acids (Ho) were isolated from drainage waters and characterised using
445 solution state NMR. The main conclusions from this study can be summarised as
446 follows:

- 447 1, Multidimensional solution-state NMR analysis indicates that the components of the
448 Ho from the drainage water of typical Irish grassland soils are complex mixtures of
449 both plant and microbial-derived materials;
- 450 2, Treatment with fertiliser (F) increased the Ho export from both well drained (WDS)
451 and poorly drained (PDS) soils, and increased the lignin contribution to the
452 compositions of the Ho. This possibly resulted from elevated OM inputs to the soil as

453 the result of increased dry matter production through fertilization. Application of a
454 fertiliser plus urine (F+U) mixture resulted in smaller losses of Ho and decreased the
455 lignin-derived signal. This is likely to be attributable to an increase in microbial
456 activity arising from the urine application;

457 3. The drainage regime affected the responses of each soil to the treatments.
458 Application of fertiliser caused a greater increase in the exports of Ho from the PDS.
459 That reflected the decreased aeration in the PDS, resulting in a lesser decomposition
460 of the increased OM input in the HO. The F+U application gave rise to a decrease in
461 the Ho from the PDS, compared to the treatment with fertiliser alone. The urine may
462 have been transported more slowly through the PDS resulting in a higher level of
463 microbial activity, increased decomposition, a lower export of Ho, and a lower lignin
464 contribution to the Ho.

465 4. Our study shows that the stimulation of microbial activity by the addition of urine
466 decreases the recalcitrance of the lignin components.

467

468 **Acknowledgement**

469 We thank the Teagasc, Ireland Walsh fellowship scheme, the Environmental
470 Protection Agency, Ireland, for funding this research, and the International Humic
471 Substance Society for a Training Bursary award to CMB for a research period in the
472 laboratory of AJS. AJS thanks NSERC (Discovery and Strategic Programs) and an
473 Early Researcher Award (Ontario Government) for providing support.

474 **Table 1:** Analyses of the well-drained and of poorly-drained soils.

Soil	Depth (cm)	Total C	Organic C	Total N	C\N ratio	% Sands	% Silts	% Clays
WD	0-10	3.22	3.18	0.3	10.7	45.2	20.4	12.0
	10-20	2.52	2.33	0.24	10.5	44.0	27.9	12.3
	20-30	1.42	1.43	0.14	10.1	48.6	28.0	12.4
	30-40	1.59	1.5	0.13	12.2	41.4	33.1	14.3
	40-50	1.19	1.12	0.08	14.9	40.0	42.5	9.2
	50-60	0.69	0.66	0.05	13.8	42.8	43.2	7.5
	60-70	0.17	0.14	0.02	8.5	46.6	35.7	6.5
	70-80	0.32	0.27	0.03	10.7	46.7	32.4	6.1
	80-90	0.22	0.19	0.02	11.0	50.2	36.4	5.9
	90-100	0.19	0.15	0.02	9.5	42.6	44.7	1.8
PD	0-10	4.36	4.23	0.35	12.5	24.8	35.0	25.2
	10-20	2.72	2.7	0.25	10.9	25.6	35.2	26.3
	20-30	0.83	0.77	0.09	9.2	27.2	34.7	30.0
	30-40	0.34	0.29	0.04	8.5	30.1	17.8	45.6
	40-50	0.25	0.22	0.03	8.3	31.0	34.6	28.7
	50-60	0.22	0.18	0.03	7.3	31.1	34.2	16.1
	60-70	0.14	0.11	0.03	4.7	34.6	34.5	25.3
	70-80	0.14	0.11	0.03	4.7	34.2	35.3	24.0
	80-90	0.12	0.09	0.03	4.0	35.8	34.4	23.9
	90-100	0.11	0.08	0.03	3.7	33.8	36.6	24.3

475

476 **Table 2:** Nutrient application rates to lysimeters.

Treatment	Nutrient application rates kg/ha				
	Inorganic Fertiliser		Cow Urine		
	Urea ¹	CAN ²	N	P	K
Control	0	0	0	0	0
Fertiliser only	58	233	0	0	0
Fertiliser & urine	58	233	310	0.8	465

1 Urea (46% N) manufactured by Goulding.

2 CAN- Calcium Ammonium Nitrate (27% N) manufactured by Goulding.

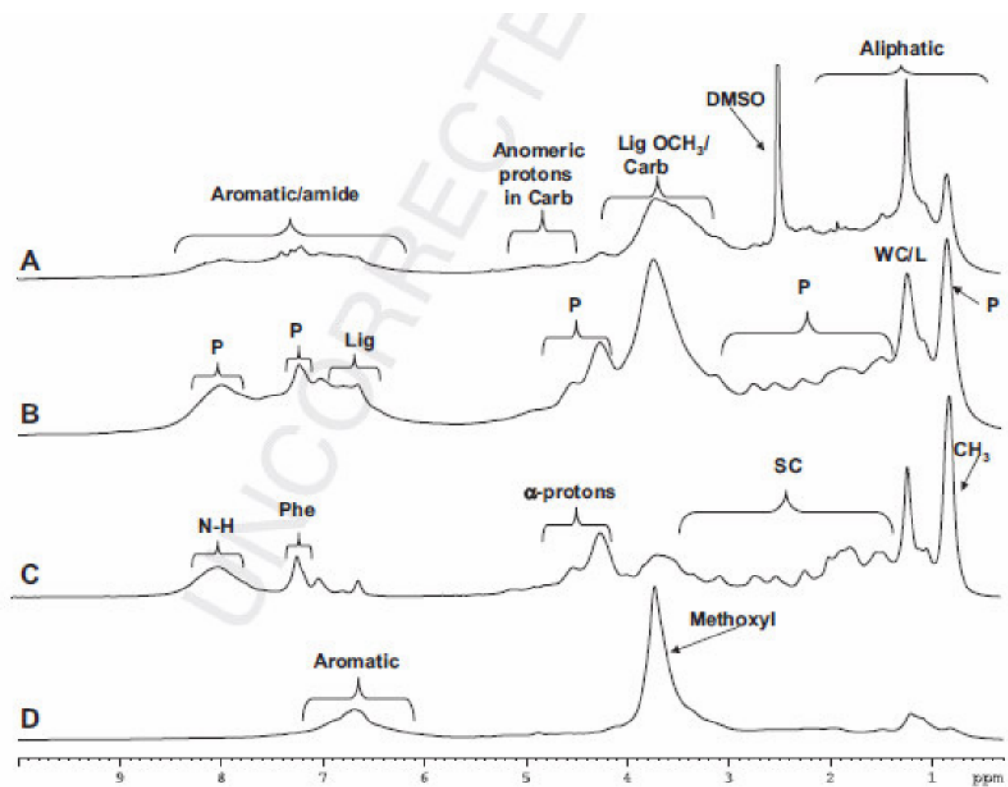
2004 At grass sowing all lysimeters received a basal application of NPK of 37, 37 and 74 kg/ha, respectively.

477

478 **Table 3:** Exports of hydrophobic acids (Ho) in the drainage water from the well-
479 drained and of poorly-drained soils under different treatment applications.

Treatment	Ho losses mg L⁻¹	
	Well drained soil (WDS)	Poorly drained soil (PDS)
Control	1.62	1.54
Fertiliser	2.42	3.78
Fertiliser & urine	2.25	1.87

480

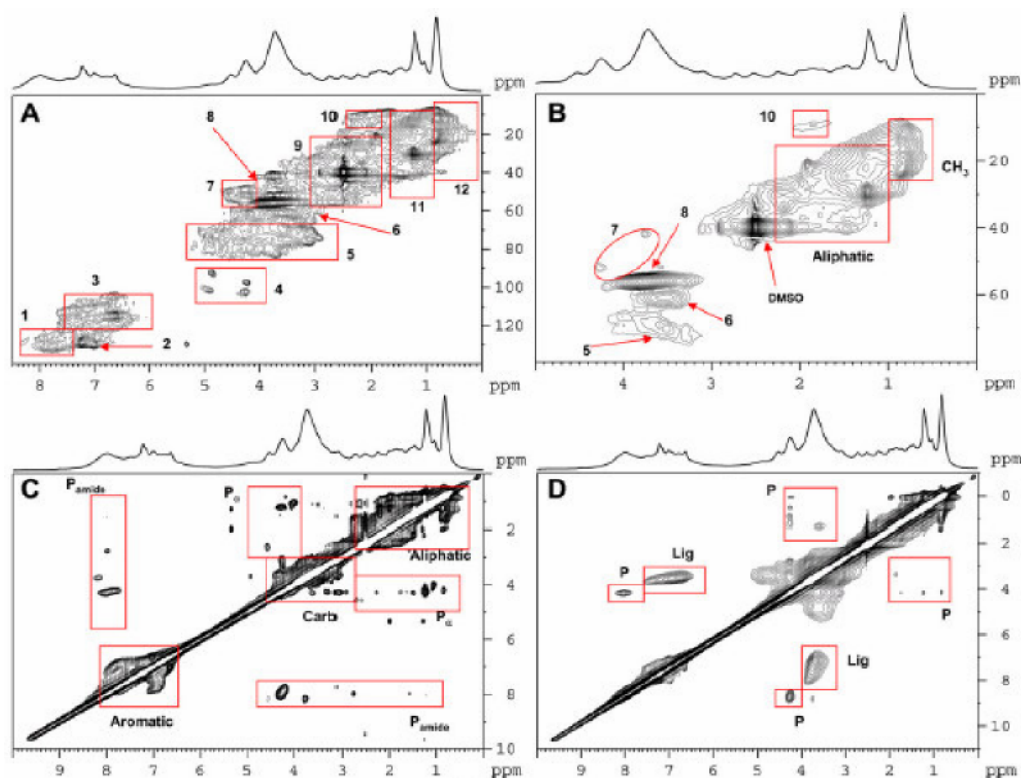


481
 482 **Figure 1.** (A), ^1H NMR spectrum in $\text{DMSO-}d_6$ for Ho isolated from the PDS treated
 483 with Fertiliser. (B), Diffusion edited ^1H NMR spectrum in $\text{DMSO-}d_6$ for the Ho. (C),
 484 Cultured soil microbes. (D), Organosolv Lignin. Assignments include lignin (Lig),

485 carbohydrates (Carb), protein/peptides (P), waxes, cuticles and lipids (WC/L),

486 protein/peptide side chains (SC), phenylalanine (Phe) and amide (N-H).

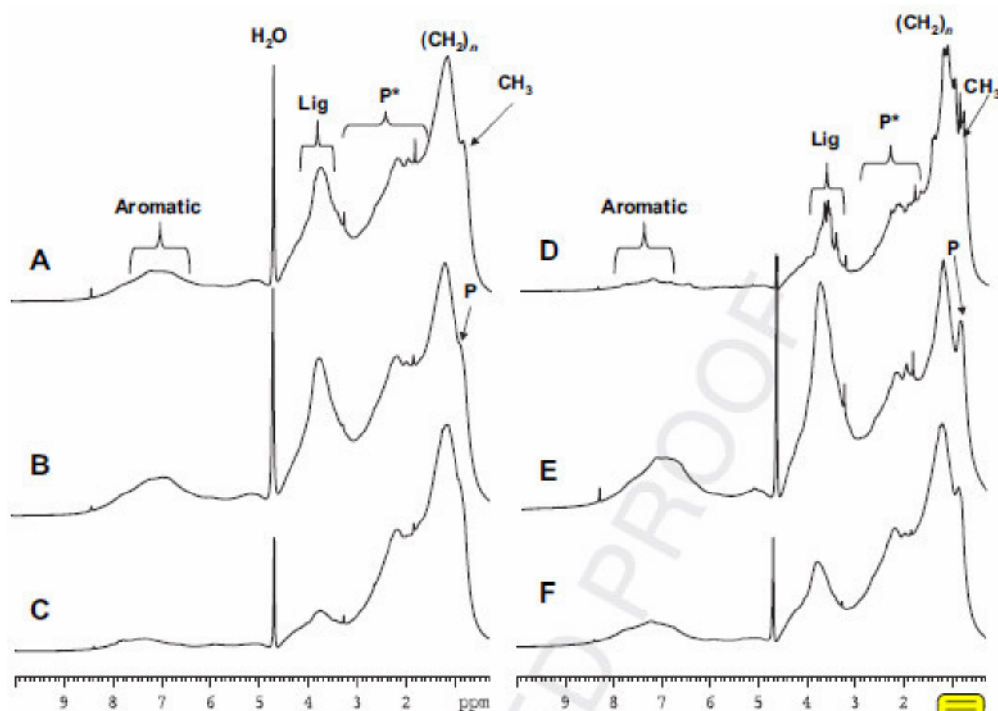
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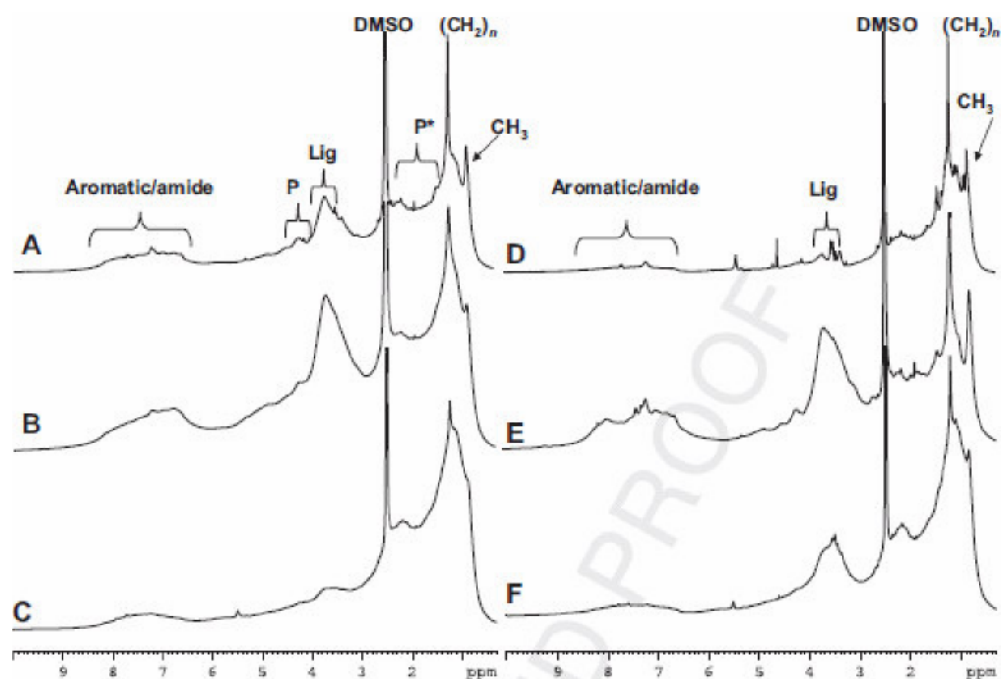
489 **Figure 2.** Various 2D NMR spectra of the Ho isolated from the PDS treated with
 490 Fertiliser. (A), HMQC Spectrum, main assignments can be summarized as 1, p-
 491 hydroxybenzoate aromatics in lignin (Kelleher and Simpson, 2006; Simpson et al.,
 492 2004); 2, phenylalanine in peptides (Kelleher and Simpson, 2006; Simpson et al.,
 493 2007a) ; 3, aromatic lignin units (Kelleher et al., 2006; Simpson et al., 2004); 4,
 494 anomeric protons in carbohydrates (Kelleher and Simpson, 2006; Lam et al., 2007); 5,
 495 methine in carbohydrates (Kelleher and Simpson, 2006; Lam et al., 2007); 6,
 496 methylene units in carbohydrates (Kelleher and Simpson, 2006; Lam et al., 2007); 7,
 497 -protons in peptides and proteins (Kelleher and Simpson, 2006; Simpson et al.,
 498 2007a; Simpson et al., 2007b); 8, methoxyl in lignin (Kelleher and Simpson, 2006;
 499 Simpson et al., 2003; Simpson et al., 2004); 9, aliphatic linkages including signals
 500 from various lipids and plant cuticles (Deshmukh et al., 2005; Deshmukh et al., 2003;
 501 Simpson et al., 2003; Simpson et al., 2007b), and side-chain protons in peptides
 502 (Kelleher and Simpson, 2006; Simpson et al., 2007a); 10, *N*-acetyl and/or *O*-acetyl

503 carbohydrates (Hertkorn et al., 2006; Lam et al., 2007); 11, methylene units in
504 aliphatic chains (Kelleher et al., 2006; Simpson et al., 2001; Simpson et al., 2003); 12,
505 methyl groups, a small contribution in this region will be from terminal CH₃ in lipids,
506 though the majority of signals are from peptides (Kelleher et al., 2006; Simpson et al.,
507 2003; Simpson et al., 2007a). (B), is an expanded region of the HMQC. The intense
508 lignin methoxyl signal is clearly evident in region 8. (C), is the TOCSY spectrum
509 which supports assignments made from the 1D and HMQC spectra. Key assignments:
510 aromatic couplings (Kelleher and Simpson, 2006; Simpson et al., 2004); P_{amide} =
511 amide-_{try} couplings in peptides (Kelleher and Simpson, 2006; Kingery et al., 2000;
512 Simpson et al., 2007a; Simpson et al., 2007b); P_a; -protons coupling to amino acid
513 side chains (Kelleher and Simpson, 2006; Kingery et al., 2000; Simpson et al., 2007a;
514 Simpson et al., 2007b); couplings in carbohydrates (Carb) and aliphatic couplings
515 (Deshmukh et al., 2005; Deshmukh et al., 2003; Kelleher et al., 2006; Simpson et al.,
516 2003). (D), is the NOESY spectrum that confirms the strong contribution of P,
517 peptides/proteins with cross-peaks from -protons in amino acid side chains. The
518 most important assignment is the through space interaction between aromatic rings
519 and methoxyl groups indicative of lignin (Lig) (Simpson, 2001).
520



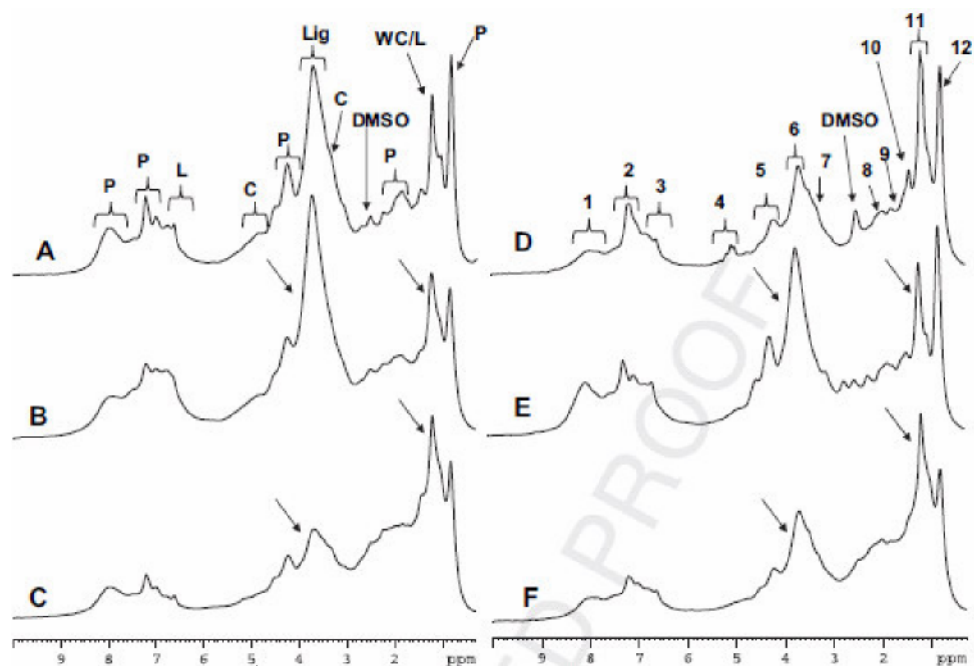
521

522 **Figure 3.** ¹H NMR spectra for Ho in D₂O, differing by soil and treatment. (A), WDS
 523 Control; (B), WDS Fertiliser; (C), WDS Fertiliser + Urine; (D), PDS Control; (E),
 524 PDS Fertiliser; and (F), PDS Fertiliser + Urine. Simple assignments for spectra
 525 indicate strong contributions from aromatic functionalities, from P, proteins/peptides;
 526 Lig, lignin; Carb, Carbohydrate; (CH₂)_n, aliphatic methylene units consistent with
 527 aliphatic structures from plant-derived waxes, cuticles and lipids, in addition to
 528 contributions from microbial lipids; (CH₃), could be due to methylated amino acid
 529 side residues plus contributions from terminal methyl groups from plant-derived
 530 residues. P* could contain contributions from other molecules such as Refractory
 531 carboxyl-rich alicyclic molecules (CRAM).
 532



533
 534 **Figure 4.** ^1H NMR spectra for Ho in DMSO- d_6 , differing by soil and treatment. (A),
 535 WDS Control; (B), WDS Fertiliser; (C), WDS Fertiliser + Urine; (D), PDS Control;
 536 (E), PDS Fertiliser; and (F), PDS Fertiliser + Urine. Assignments are the same as
 537 given in Figure 3.

538



539
 540 **Figure 5.** Diffusion edited ^1H NMR spectra for Ho in DMSO- d_6 , differing by soil

541 and treatment. (A), WDS Control; (B), WDS Fertiliser; (C), WDS Fertiliser + Urine;
542 (D), PDS Control; (E), PDS Fertiliser; and (F), PDS Fertiliser + Urine. Assignments
543 are the same as shown in Figure 3 in addition to WC/L, which refers to waxes, cutins
544 and/or lipids. More specific assignments shown for spectrum D refer to: 1, amide; 2,
545 phenylalanine; 3, aromatics in lignin; 4, anomeric protons in carbohydrates; 5, -
546 protons (peptides); 6, methoxyl (lignin); 7, carbohydrate protons; 8, methylene
547 adjacent to a carbonyl; 9, N-acetyl and/or O-acetyl group in peptidoglycan; 10,
548 aliphatic methylene units to an acid or ester; 11, aliphatic methylene; 12, CH₃.
549 Changes in the relative abundances of Lignin OCH₃ and aliphatic methylene are
550 highlighted by the arrows.

551

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