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1 **Radically different lignin composition in *Posidonia* species may link to differences in**
2 **organic carbon sequestration capacity**

3

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16 **Abstract**

17

18 There is considerable variability in the ability of seagrass ecosystems to sequester
19 organic carbon (C_{org}) in their sediments, which act as natural carbon sinks contributing to
20 climate change mitigation. In this work, we studied the chemistry of two *Posidonia* seagrass
21 species aiming to elucidate whether differences in chemical composition might explain
22 differences in their C_{org} sequestration capacity. Pyrolysis-GC-MS and Thermally assisted
23 Hydrolysis and Methylation (THM-GC-MS) showed a remarkable difference in phenolic
24 compound patterns between *P. oceanica* and *P. australis* bulk plants and individual organs
25 (leaves, sheaths, roots and rhizomes). The lignin of *P. australis* generates a series of *p*-
26 hydroxyphenyl (H), guaiacyl (G) and syringyl (S) products that are typical of herbaceous plants,
27 whereas *P. oceanica* is particularly rich in *p*-hydroxybenzoic acid (*p*BA) derivatives. The
28 structural characteristics of the lignins were further investigated by two-dimensional Nuclear
29 Magnetic Resonance (2D-NMR) and Derivatization Followed by Reductive Cleavage (DFRC),
30 focusing on sheath tissues. The analyses confirmed important differences in the lignin content
31 (19.8% in *P. australis* and 29.5% in *P. oceanica*) and composition between the two species;
32 intriguingly, the cell-walls of *P. oceanica* sheaths were highly enriched in *p*BA, a component
33 that was completely absent in *P. australis*. 2D-NMR and DFRC further revealed that *p*BA was
34 esterified to the lignin, acylating the γ -OH of the lignin side-chain. Interestingly, *P. oceanica*
35 lignin presented an extremely high degree of *p*-hydroxybenzoylation in both guaiacyl (73%) and
36 syringyl (61%) lignin units, the highest *p*-hydroxybenzoylation degree reported in plant lignins
37 to date. It is tempting to conclude that the higher soil C_{org} storage capacity of *P. oceanica*
38 ecosystems might be related to the higher abundance of *p*BA-rich lignin and its recalcitrant
39 nature.

40

41 **Keywords:** *Posidonia australis*, *Posidonia oceanica*, lignin, *p*-hydroxybenzoates, analytical
42 pyrolysis, 2D-NMR, DFRC, blue carbon

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43 1. Introduction

44

45 Coastal vegetated ecosystems – blue carbon ecosystems (tidal marshes, mangroves and
46 seagrasses) – occupy 0.2% of the ocean surface, but contribute 50% of carbon burial in marine
47 sediments (Duarte et al., 2005; Nellemann et al., 2009). Seagrass meadows of the genus
48 *Posidonia* rank amongst the most effective blue carbon sinks. More specifically, the organic
49 carbon (C_{org}) storage in *P. oceanica* and *P. australis* (ranging from 80 to 880 $Mg C_{org} ha^{-1}$;
50 Serrano et al., 2016a) is exceptional compared to other seagrass species (ranging from 6 to 120
51 $Mg C_{org} ha^{-1}$; Lavery et al., 2013; Campbell et al., 2015; Miyajima et al., 2015). Furthermore, *P.*
52 *oceanica* ($750 Mg C_{org} ha^{-1}$ and $84 g m^{-2} yr^{-1}$) has up to three-fold higher C_{org} stocks and up to
53 seven-fold higher C_{org} sequestration rates compared to *P. australis* ($250 Mg C_{org} ha^{-1}$ and $12 g$
54 $m^{-2} yr^{-1}$) (Serrano et al., 2016a). These differences have been attributed, among other reasons, to
55 the vertical rhizomal growth (i.e., orthotropic) of *P. oceanica*, producing a deep mat of plant
56 debris embedded within the sediment that can persist for millennia (Boudouresque et al., 1980;
57 Gobert et al., 2006; Mateo et al., 1997; Lo Iacono et al., 2008; Serrano et al., 2014).

58 Differences in the chemical composition of seagrass tissues can play a key role in the
59 accumulation and preservation of C_{org} in seagrass sediments (Klap et al., 2000; Torbatinejad et
60 al., 2007; Trevathan-Tackett et al., 2015, 2017). Nevertheless, molecular-scale studies on
61 seagrass composition have focused on free low molecular weight organic matter (OM), and
62 mostly in *P. oceanica* (Ncibi et al., 2009; Zapata and McMillan, 1979; Cuny et al., 1995). Little
63 attention has been given to the composition of the structural biopolymers, such as lignin, even
64 though degradation/preservation dynamics (“recalcitrance”) largely depends on that OM
65 fraction and the capacities of the microbial community to decompose such debris (Trevathan-
66 Tackett et al., 2017). It is well known that plant recalcitrance is caused, to a large extent, by the
67 presence of lignin, a highly complex aromatic cell-wall polymer typical of vascular plants that
68 plays crucial roles for plant growth and development, as well as for pathogen protection. The
69 lignin is formed by radical coupling of mainly three *p*-hydroxycinnamyl alcohols, namely, *p*-

70 coumaryl, coniferyl, and sinapyl alcohols, differing in their degree of methoxylation. These
71 monolignols give rise to the *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units,
72 respectively, producing a variety of structures and linkages within the lignin polymer
73 (Vanholme et al., 2010). The lignin content as well as the monomer composition widely varies
74 with taxon, cell type, and tissue (Harris, 2005). In general terms, gymnosperm lignins are
75 composed of G- units with minor amounts of H-units, woody and non-woody angiosperm
76 lignins are G-S lignins with variable S:G ratios, whereas grasses (monocots) contain all the
77 three units (Boerjan et al., 2003). Additionally, abundant research has indicated that lignins are
78 also produced from γ -acylated monolignol conjugates, with acetates, *p*-hydroxybenzoates, *p*-
79 coumarates, and ferulates (del Río et al., 2008; Martínez et al., 2008; Lu et al., 2015; Karlen et
80 al., 2016). Lignin is present in considerable amounts in both *Posidonia* species, especially in the
81 fiber-rich leaf sheaths (Kuo, 1978; Ncibi et al., 2009). A recent study using analytical pyrolysis
82 techniques (Kaal et al., 2016) showed that the roots, rhizomes and sheaths (not leaves) of *P.*
83 *oceanica* contain large amounts of phenolic material with a significance abundance of *p*-
84 hydroxybenzoic acids (*p*BA), but the lignin of *P. australis* has not been studied on the
85 molecular scale.

86 In the present study we describe the molecular composition of several *P. australis* and
87 *P. oceanica* plant organs, by conventional analytical pyrolysis (Py-GC-MS) and thermally
88 assisted hydrolysis and methylation (THM-GC-MS). Furthermore, we study the lignin
89 composition of sheaths in both *Posidonia* species, by state-of-the-art bidimensional Nuclear
90 Magnetic Resonance (2D-NMR) and Derivatization Followed by Reductive Cleavage (DFRC).
91 The ultimate objective is to provide the necessary framework for assessing differences in C_{org}
92 storage capacity based on differences in the recalcitrance of *Posidonia* tissues.

93

94 **2. Material and Methods**

95

96 *2.1 Sample materials and pre-treatment*

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97 Plants of *P. australis* were sampled in Shark Bay (Indian Ocean, Western Australia),
98 while plants of *P. oceanica* were sampled in Port Lligat (Spain). All materials were rinsed with
99 distilled water, assuring that epiphytes attached to the leaves and other allochthonous tissues
100 were removed, and subsequently oven-dried at 60 °C until constant weight. Subsamples of *P.*
101 *australis* were kept as “whole plants”, whereas others were subdivided into “leaves”, “leaf
102 sheaths” and “roots+rhizomes”. Subsamples of *P. oceanica* were obtained for “leaf sheaths”
103 only, which complements existing data for leaves, roots, rhizomes and three subfractions of
104 sheaths published by Kaal et al. (2016). This enabled comparisons between *P. australis* and *P.*
105 *oceanica*. All samples were ball milled to powder and then split for subsequent analyses.

106 For Py-GC-MS and THM-GC-MS, powder samples of whole plants of *P. australis*, the
107 dissected organs of *P. australis* and sheaths of *P. oceanica* were analyzed without any further
108 treatment. For 2D-NMR and DFRC, subsamples of *P. australis* and *P. oceanica* sheaths were
109 previously extracted with water (3 x 40 mL), 80% ethanol (3 x 40 mL), and finally with acetone
110 (2 x 40 mL), by sonicating in an ultrasonic bath for 30 min, centrifuging (8000 rpm, 25 min)
111 and eliminating the supernatant. The extractive-free samples were dried and then ball-milled for
112 2 h using a Retsch PM 100 planetary ball mill at 600 rpm. The lignin content of *P. australis* and
113 *P. oceanica* samples were determined as Klason lignin (Tappi, 2004).

114 For detailed NMR analyses, lignin-enriched cell wall preparations (cellulolytic enzyme
115 lignin; CEL) were obtained by enzymatically hydrolyzing the polysaccharides fraction with
116 Cellulysin (Calbiochem, San Diego, CA), a crude cellulase preparation from *Trichoderma*
117 *viride* that also contains hemicellulase activities. 200 mg of extracted ball-milled material were
118 suspended in 20 mM NaOAc buffer (30 mL, pH 5.0), in a 50 mL centrifuge tube, together with
119 8 mg of Cellulysin, and the reaction slurry was incubated at 30 °C for 48 h. The solids were
120 recovered by centrifugation (8000 rpm, 4 °C, 20 min), and the process was repeated twice with
121 fresh buffer and enzyme. The lignin-enriched preparation was finally recovered by filtration,
122 washed with distilled water and then lyophilized.

123

124 2.2 Analytical pyrolysis

125 Pyrolysis-GC-MS and THM-GC-MS were performed as described by Kaal et al. (2016).
126 Briefly, approximately 0.5 mg of plant materials were introduced into quartz tubes and
127 embedded in quartz wool, which were both pre-combusted at 900 °C using a muffle furnace.
128 The sample-containing quartz tubes were pyrolyzed using a Pyroprobe 5000 (CDS Analytical)
129 at 650 °C (setpoint temperature) for 10 s (heating rate 10 °C ms⁻¹). The pyrolysis interface,
130 isothermal at 325 °C, was connected to a 6890 gas chromatograph (Agilent Technologies)
131 equipped with a HP-5MS non-polar column (length 30 m; internal diameter 0.25 mm; film
132 thickness 0.25 µm). The oven temperature program was from 50 to 325 °C at 20 °C min⁻¹. The
133 mass selective detector (Agilent 5975B) operated in electron impact (EI) mode at 70 eV, with
134 the ion source at 230 °C and the quadrupole detector at 150 °C, and measuring fragments in the
135 *m/z* 50–500 range. The interfaces between the pyrolyzer, gas chromatograph and the mass
136 selective detector were at 325 °C. The same analytical conditions were used for THM-GC-MS,
137 but prior to the analysis a droplet of 25% (aq) tetramethyl ammonium hydroxide (TMAH,
138 Sigma-Aldrich) was added to the sample-containing quartz tubes and allowed to impregnate for
139 one hour (at room temperature).

140 The sheath materials of *P. oceanica* and *P. australis* were analyzed by both methods in
141 triplicate. For the sheaths, relative proportions of the products were calculated as the percentage
142 of the total quantified peak area (% TQPA), using the main fragment ions (*m/z*) of each product,
143 creating separate datasets for Py-GC-MS and THM-GC-MS, focusing on the phenolic
144 compounds (phenols, guaiacols, syringols). A one-way analysis of variance (ANOVA) was
145 applied to identify statistically significant differences in relative proportions of compounds,
146 groups of compounds and compound ratios, between the two *Posidonia* species. Replicates
147 were not obtained for the other samples (whole plant and different organs of *P. australis*) and
148 the interpretation of the chromatograms is based on visual inspection.

149

150 2.3 Two-dimensional Nuclear Magnetic Resonance (2D-NMR)

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151 For the 2D-NMR study, whole cell walls of *P. oceanica* and *P. australis* sheaths were
152 analyzed in two different ways: i) “*in situ*” (at the gel state) according to the method previously
153 described (Kim and Ralph, 2010), and ii) after acetylation, which provides NMR spectra with a
154 better resolution (Lu and Ralph, 2003). In the case of the samples without derivatization, ~85
155 mg of extracted and finely milled sheaths were swelled in 1 mL DMSO-*d*₆:pyridine-*d*₅ (4:1,
156 v/v), until forming a gel inside the NMR tube. For the NMR analysis of acetylated samples, 50
157 mg of ball-milled extractive-free samples were previously acetylated with *N*-methylimidazole
158 and acetic anhydride (Lu and Ralph, 2003), and were subsequently dissolved in chloroform-*d*.
159 In addition, *P. oceanica* lignin enriched preparation (CEL) was also acetylated and dissolved in
160 DMSO-*d*₆:pyridine-*d*₅ (4:1). Heteronuclear single quantum coherence experiments (2D-HSQC)
161 were acquired at 300 K on a Bruker AVANCE III 500 MHz spectrometer (Bruker, Karlsruhe,
162 Germany) equipped with a 5 mm TCI cryoprobe. The spectra were acquired using an adiabatic
163 pulse sequence (hsqcetgpsisp.2) and the following NMR parameters were set: spectral width
164 from 10 to 0 ppm in F2 (¹H) using 748 data points for an acquisition time (AQ) of 74.8 ms, an
165 interscan delay (D1) of 1 s, and from 200 to 0 ppm in F1 (¹³C) using 256 increments of 32
166 scans, for a total acquisition time of 2 h 34 min. The ¹J_{CH} used was 145 Hz. Spectra were
167 processed using Bruker’s Topspin 3.5 software and typical matched Gaussian apodization in F2
168 dimension (LB=-0.1 and GB=0.001) and squared cosine-bell apodization in F1 dimension
169 (LB=0.3 and GB=0.1) were applied. The residual peaks of DMSO (δ_C/δ_H 39.5/2.49) and CHCl₃
170 (δ_C/δ_H 77.0/7.26) were used as internal references. 2D-HSQC correlation signals were assigned
171 by literature comparison (Rencoret et al., 2013; Lu et al., 2015). The lignin compositions of *P.*
172 *oceanica* and *P. australis*, in terms of H-, G- and S-lignin units, and *p*BA were estimated based
173 on the volume integrals of the ¹H–¹³C correlation signals. The signals C₂–H₂ (from G units) and
174 C_{2,6}–H_{2,6} (from H and S lignin units, and from *p*BA) were used to estimate their abundances. It
175 is important to note that *p*BA quantitation relative to the lignin might be overestimated because
176 of the longer relaxation times of these end-units compared to the rapidly relaxing polymer.

177

178 2.4 Derivatization Followed by Reductive Cleavage (DFRC)

179 The DFRC degradation method was performed according to the protocol by Lu et al.
180 (2015). Fifty mg of extractive-free *P. australis* and *P. oceanica* whole plant samples were
181 treated with 4 mL of an acetyl bromide/acetic acid solution (1/4 v:v), and stirred at 50 °C for 3
182 h. The solvents were removed by rotary evaporation at reduced pressure. The products were
183 then suspended in 5 mL of dioxane/acetic acid/water, 5:4:1 (v/v/v) and 200 mg of powdered Zn
184 was added, and the mixture was stirred at room temperature for 24 h. After completion, the
185 reaction mixture was transferred into a separation funnel with dichloromethane (10 mL) and
186 saturated ammonium chloride (10 mL). The aqueous phase was adjusted to pH < 3 by adding
187 3% HCl(aq), the mixture vigorously mixed, and the organic layer separated. The water phase
188 was extracted twice more with 10 mL of dichloromethane. The combined dichloromethane
189 fractions were dried over anhydrous Na₂SO₄, and were evaporated on a rotary evaporator. The
190 residue was then acetylated with 5 mL of pyridine:acetic anhydride solution, 1:1 (v:v) for 24 h.
191 The acetylated lignin degradation products were collected after rotary evaporation of the
192 solvents and analyzed by GC/MS on a Saturn 4000 (Varian, Walnut Creek, CA) instrument
193 fitted with a medium-length high-temperature capillary column (DB5-HT, 15 m × 0.25 mm i.d.,
194 0.1 µm film thickness; from J&W Scientific). Helium was used as carrier gas at a rate of 2 mL
195 min⁻¹. The samples were injected with an autoinjector (Varian 8200), which was programmed
196 from 120 °C (0.1 min) to 340 °C at a rate of 200 °C min⁻¹ and held at the maximum temperature
197 until the end of the analysis. The oven temperature was programmed from 120 °C (1 min) to
198 340 °C (10 min) at a rate of 10 °C min⁻¹ and the transfer line temperature was set at 300 °C
199 during the analysis.

200

201

202 3. Results and discussion

203

204 3.1 Analytical pyrolysis of *Posidonia australis* organs

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205 The Py-GC-MS chromatograms of the whole plant sample of *P. australis* contain large
206 peaks for products of polysaccharides (acetic acid, 3/2-furaldehyde, 4-hydroxy-5,6-dihydro-
207 (2*H*)-pyran-2-one, 5-hydroxymethyl-2-dihydrofuraldehyde-3-one, 1,4:3,6-dianhydro- α -D-
208 glucose and levoglucosan), guaiacyl lignin (4-vinylguaiacol, C_{3:1}-guaiacols), syringyl lignin (4-
209 methylsyringol, 4-vinylsyringol, C_{3:1}-syringols), chlorophyll (phytadienes) and C₁₆-fatty acid, a
210 combination that is typical of a predominantly lignocellulose material (Fig. 1A). Among the
211 phenolic products, 4-vinylphenol and G and S products are dominant, typical of herbaceous
212 species in general (Hedges and Mann, 1979). The chromatograms of individual *P. australis*
213 organs show that the leaves (Fig. 1C) release, besides G and S moieties (from lignin) and
214 polysaccharides, relatively large amounts of fatty acids (probably occurring freely) and
215 phytadienes (from phytol in chlorophyll). Sheaths (Fig. 1E) have relatively large peaks of G and
216 S moieties, indicative of high lignin content. Roots and rhizomes (Fig. 1G) produce
217 predominantly G-type phenols and conspicuous peaks for catechol and methylcatechols, which
218 reflect the relatively large tannin content. The results obtained for *P. australis* whole plant and
219 individual organs are in concert with those of *P. oceanica* (Kaal et al., 2016), with the key
220 exception of the distribution of phenolic compounds.

221 These results are corroborated by the THM-GC-MS fingerprints of the same materials
222 (Fig. 1B-H). All samples were prolific in C₁₆-fatty acid methyl ester (FAME) and a
223 carbohydrate product with *m/z* 101, 111, 187 and 219 (Yeloff et al., 2008). None of the samples
224 from *P. australis* produced a significant peak for *p*B A methyl ester. The roots and rhizomes of
225 *P. australis* produce clear peaks for 1,3,5-trimethoxybenzene and 2,4,6-trimethoxytoluene,
226 which is in agreement with their interpretation as tannin markers (Nierop et al., 2005). The leaf
227 sample of *P. australis* produced a peak for 9,10-dimethoxy-C₁₆-FAME from cutin in leaf
228 cuticles (del Río and Hatcher, 1998) and all samples showed peaks for typical carbohydrate
229 products (e.g. methylated C₆-metasaccharinic acids) and G and S type phenols.

230

231 3.2 Analytical pyrolysis of *P. australis* and *P. oceanica* sheaths

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232 The sheaths showed the “cleanest” fingerprint of lignin-derived phenolic compounds
233 (least signal from tannin, polysaccharides, chlorophyll, cutin and fatty acids) for both *P.*
234 *australis* and *P. oceanica* (Fig. 1). Therefore, leaf sheaths were used for a further comparison of
235 lignin in *P. oceanica* and *P. australis* (triplicate analysis). The Py-GC-MS chromatograms show
236 that only *P. oceanica* (Fig. 1I) produces a dominant peak for phenol, whereas *P. australis* has a
237 more heterogeneous fingerprint of G phenols (4-vinylguaiacol, C_{3:1}-guaiacols) and S phenols (4-
238 vinylsyringol, C_{3:1}-syringols) (Fig. 1E). Markers of non-decarboxylated intact *p*BA (*m/z* 121
239 base ion) were found only in the chromatograms of *P. oceanica*. The ANOVA analysis of the
240 relative abundance of the phenolic products show that phenol and *p*BA compounds from Py-
241 GC-MS are significantly enriched in *P. oceanica* and all other compounds (including C₁- and
242 C₂-alkylphenols and most G and S compounds) are enriched in *P. australis* ($P < 0.001$; Table
243 1). Moreover, ratios that describe the phenolic composition such as *p*BA/total phenols
244 (*p*BA/Ph_t) –calculated as the sum of phenol and *p*-hydroxybenzoic acids divided by the sum of
245 the all phenols and methoxyphenols (Table 1)–, G/Ph_t and S/Ph_t, shows that these parameters
246 are significantly different between the two species (ANOVA, $P < 0.001$ for all ratios). This can
247 also be inferred for the THM-GC-MS fingerprints (compare Fig. 1F for *P. australis* and Fig. 1J
248 for *P. oceanica*; Table 2) albeit with lower degrees of significance ($P < 0.05$). These results
249 provide unambiguous evidence of a major difference in lignin composition of the sheaths of the
250 *Posidonia* species analyzed. Furthermore, the abundance of phenol (Py-GC-MS) and *p*BA
251 (THM-GC-MS) of *P. oceanica* is unrivalled by any other plant material. These compounds also
252 dominate millennial soil materials underneath *P. oceanica* meadows (Gadel and Bruchet, 1987;
253 Kaal et al., 2016), which is indicative of the good preservation of *p*BA-rich lignin. Hence, ratios
254 such as *p*BA/total phenols (*p*BA/Ph_t) can be used to estimate the contribution of *P. oceanica* in
255 blue carbon soils and sediments, likely enhancing the capability to discern between
256 autochthonous and allochthonous sources using stable carbon isotopes alone (Kennedy et al.,
257 2010)
258

259 3.3 2D-NMR analyses of sheaths of *P. oceanica* and *P. australis*

260 The Klason lignin contents of the extractive-free sheath materials were 19.8% and
261 29.5% (of dry weight sample) for *P. australis* and *P. oceanica*, respectively. This remarkably
262 high lignin content of sheath materials of *P. oceanica* was also reported by Ncibi et al. (2009).
263 A detailed analysis of the lignin composition in both species was performed by 2D-NMR.

264 The aromatic regions of the HSQC NMR spectra of the acetylated whole cell-wall from
265 *P. oceanica* and *P. australis* revealed notable differences between them (Fig. 2). The HSQC
266 spectrum of *P. australis* (Fig. 2A) shows correlation signals corresponding to the aromatic rings
267 of typical H-, G- and S-lignin units. The signal of syringyl units ($S_{2,6}$) is readily observed at
268 δ_C/δ_H 103.8/6.59, whereas the signals of guaiacyl units are detected at δ_C/δ_H 110.8/6.93,
269 118.6/6.86 and 122.1/6.99, and from H-lignin units at δ_C/δ_H 128.2/7.38, corresponding to
270 $C_{2,6}H_{2,6}$ ($C_{3,5}H_{3,5}$ is overlapped with $G_{5/6}$ signals). A semi-quantitative analysis, based on the
271 signals volume integrals, shows that the lignin from *P. australis* is enriched in G-lignin units
272 with an H:G:S composition of 7:57:36 (molar ratio). The S/G ratio obtained upon NMR (S/G of
273 0.6) is in good agreement with that obtained from Py-GC-MS (S/G of 0.5) (Table 1).

274 On the other hand, the aromatic region of the HSQC spectrum of *P. oceanica* (Fig. 2B)
275 shows the typical signals of H-, G- and S-lignin units, and two prominent signals at δ_C/δ_H
276 121.5/7.12 and 131.0/7.91, which correspond unequivocally to the $C_{3,5}H_{3,5}$ ($pBA_{3,5}$) and $C_{2,6}H_{2,6}$
277 ($pBA_{2,6}$) correlation signals of *pBA* (Leplé et al., 2007; Lu et al., 2015; Rencoret et al., 2013,
278 2018). The semi-quantitative analysis gave a H:G:S lignin composition of 3:81:16, but
279 accompanied with a *pBA* content of 102% (relative to total lignin defined as H+G+S = 100%).
280 2D-NMR data corroborated the pyrolysis results, and indicated that the lignin in *P. oceanica* is
281 more enriched in G-units than the lignin in *P. australis*, and that *pBAs* are the main components
282 in *P. oceanica*, whereas *pBA* is completely absent in *P. australis*. It is noted that the high
283 abundance of *pBA* might be exacerbated by the fact that *pBA* could be found as terminal group,
284 which are overestimated by 2D-HSQC (Lu et al., 2015).

285 In order to determine whether the *pBA* moieties in *P. oceanica* are linked to lignin or to
286 carbohydrates, a lignin-enriched cell wall preparation obtained after enzymatic hydrolysis of
287 carbohydrates (CEL) was also analyzed by 2D-HSQC and its spectrum was compared with that
288 of the whole cell walls (Fig. 3). The HSQC spectrum of the whole cell walls (Fig. 3A) displays
289 correlation signals of carbohydrates, especially from glucose (G1-G6), together with signals of
290 *pBA*. After the enzymatic hydrolysis and removal of the carbohydrates, the above mentioned
291 glucose signals strongly decreased, whereas signals from *pBA* remained unchanged (Fig. 3B).
292 This implies that the vast majority of the *pBA* units are not connected to the polysaccharides in
293 the cell-wall of *P. oceanica* sheath. Indeed, a comparison between the aromatic regions of the
294 spectra, after increasing their intensity (Fig. 3C and D), shows that the *pBA*/(H+G+S) ratio
295 remains essentially unaffected by the enzymatic hydrolysis of carbohydrates, which suggests
296 that *pBA* units in *P. oceanica* sheaths are bound to the lignin moiety. This is an important result
297 because if the *pBA* would have been located primarily in glycosidic linkages, as was
298 hypothesized by Kaal et al. (2016), degradation of the carbohydrates would release the *pBA*
299 units. The incorporation of the *pBA* in lignin structures implies a stronger (theoretical) inherent
300 chemical recalcitrance, especially under anoxic conditions (Arnosti et al., 1994; Traoré et al.,
301 2017).

302 Taking into account that the *pBA* can only be connected to lignin via ester (acting as
303 acylating group) or via ether (whose formation involves the 4-OH group) linkages, an
304 acetylation assay was performed to determine whether the 4-OH of *pBA* was free or etherified.
305 The 2D-HSQC of the non-acetylated and acetylated CEL preparation of *P. oceanica* clearly
306 shows the shift in the *pBA* signals (especially *pBA*_{3,5} that moves from δ_C/δ_H 115.0/6.84 to
307 121.8/7.21) after acetylation (Fig. 4), which indicates that the 4-OH groups of *pBA* in *P.*
308 *oceanica* occur freely and are not etherified. Consequently, the *pBA* moieties in *P. oceanica*
309 sheath are bound to lignin through ester linkages, acylating the γ -OH of the lignin side-chain,
310 and bearing a free phenolic (4-OH) group, as also occurs in the lignins of other
311 monocotyledonous plants (Rencoret et al., 2013, 2018; Lu et al., 2015; del Río et al., 2017).

312 Note that the positioning of *p*BA as terminal and pending substructures to G-dominated lignin is
313 similar to that of neolignans isolated from the rhizomes of *P. oceanica* (Bitam et al., 2012).

314

315 3.4 DFRC analysis

316 The incorporation of *p*BA into the lignin polymer of *P. oceanica* was further
317 conclusively demonstrated by using DFRC, a degradation method that cleaves β -ether linkages
318 (the most abundant linkages in the lignin polymer) but leaves esters unaltered (Lu et al., 2015).
319 This method has been recently employed to confirm that *p*BA are also found acylating the lignin
320 of some palm fruit endocarps (del Río et al., 2017; Rencoret et al., 2018). The chromatograms
321 of the DFRC degradation products of *P. oceanica* and *P. australis* sheaths are presented in Fig.
322 5. Both seagrasses released the *cis*- and *trans*-isomers of *p*-hydroxyphenyl (*cH*, *tH*), guaiacyl
323 (*cG*, *tG*), and syringyl (*cS*, *tS*) lignin monomers (as their acetylated derivatives) arising from
324 normal (γ -OH) units in lignin. The H:G:S lignin compositions obtained upon DFRC were of
325 6:54:40 for *P. australis* and 1:68:31 for *P. oceanica*, confirming the results obtained by Py-GC-
326 MS and 2D-NMR. More importantly, the chromatogram of *P. oceanica* was dominated by
327 prominent peaks of the *cis*- and *trans*- isomers of γ -*p*-hydroxybenzoylated G- (*cG-p*BA, *tG*-
328 *p*BA) and S-lignin units (*cS-p*BA, *tS-p*BA), which were identified by their mass spectra (Fig.
329 5C), and which were completely absent in *P. australis*. DFRC also indicated that *p*BA in *P.*
330 *oceanica* lignin extensively acylates both guaiacyl (73%) and syringyl (61%) lignin units. This
331 observation was crucial to confirm that *p*BA are linked to a lignin polymeric network of G and
332 S moieties, and that the vast majority of G and S units carry covalently bound *p*BA.

333 Lignin acylation with *p*BA has been reported in other monocotyledonous plants, such as
334 in palms (Rencoret et al., 2013, 2018; Lu et al., 2015; del Río et al., 2017; Karlen et al., 2017),
335 as well as in some eudicotyledonous plants like poplar, willow and aspen (Venverloo, 1971;
336 Landucci et al., 1992; Morreel et al., 2004). Interestingly, the pattern of *p*-hydroxybenzoylation
337 of *P. oceanica* widely differs from those observed in the lignins of other plants where *p*BA are
338 preferentially acylating the S-lignin units whereas G-units are barely *p*-hydroxybenzoylated (Lu

339 et al., 2015; del Río et al., 2017; Rencoret et al., 2018). On the other hand, the monolignol-*p*BA
340 ester conjugates in *P. oceanica* are responsible not only for the high extent of *p*-
341 hydroxybenzoylation of the lignin polymer but also for the production of γ -*p*-
342 hydroxybenzoylated neolignans (Bitam et al., 2012). Knowledge on the microbial or abiotic
343 decay of either lignin with an extraordinary *p*BA load or enigmatical neolignan is not available,
344 which implies that we can not elaborate on the link between the peculiar structure of polymeric
345 phenolic compounds in *P. oceanica* and its capacity as a C_{org} sink. Molecular assessment of
346 seagrass tissues before and after laboratory or field incubation assays might contribute to
347 establishing this link.

348

349 *3.5 Implications on C_{org} preservation dynamics*

350 Gobert et al. (2006) and Waycott and Les (2000) reported that there is a large genetic
351 difference between the Mediterranean-endemic *P. oceanica* and the Australian counterparts,
352 such as *P. australis*. Their habitat separation may have taken place as early as the Late Eocene,
353 associated with the disappearance of the Tethys Sea. Considering that the Australian seagrasses
354 have a phenolic composition that is similar to that of most angiosperms and in particular
355 herbaceous species, it remains to be assessed how (and why) the molecular skeleton of *P.*
356 *oceanica* had undergone a big evolutionary shift towards a lignin (or neolignan) complex
357 dominated by *p*BA-acylated G units.

358 The tissues of both *Posidonia* species studied are rich in lignin. Especially the leaf
359 sheaths are heavily lignified, which has been linked to the persistence of sheath materials after
360 plant death (Kuo, 1978; Torbatinejad et al., 2007). It is well-known that lignin has a large
361 preservation potential under anoxic conditions such as those of mat deposits in such seagrass
362 ecosystems (Burdige and Lerman, 2006), which might be related to the capacities of both
363 *Posidonia* species as C_{org} sinks. However, this effect of lignin composition on the preservation
364 of dead OM from *Posidonia*, and the relative importance of other biogeochemical factors (e.g.
365 Serrano et al., 2016b), is yet to be determined. The main chemical differences between *P.*

366 *oceanica* and *P. australis* are the higher lignin content and the high proportion of *pBA* in the
367 former and H, G and S moieties in the latter. It was conclusively demonstrated that the abundant
368 *pBA* units in *P. oceanica* form part of the lignin macromolecule, possibly transferring an
369 enhanced chemical recalcitrance to the cell-wall materials. These results are in agreement with
370 the description of *P. oceanica* as an outlier among seagrasses in terms of their biology, ecology
371 and C_{org} sink capacity (Walker et al., 1999; Fourqurean et al., 2012).

372 Even though the biogeochemical behavior of lignin in *Posidonia* seagrasses remains
373 largely unknown, detailed molecular characterization of macromolecular tissues seems to be a
374 promising approach to unravel the processes and pathways of C_{org} preservation, including
375 evaluating the magnitude and rate of greenhouse gas emissions with disturbance (Lovelock et
376 al., 2017).

377

378 **4. Conclusions**

379 Detailed analyses of the composition of *P. oceanica* and *P. australis* tissues, and
380 especially their lignin composition, showed that they are very different. The lignin of *P.*
381 *australis* presents a moderate enrichment of G-units (H:G:S molar ratio of 7:57:36) whereas the
382 lignin of *P. oceanica* is largely enriched in G-units (3:81:16) and is extensively acylated at the
383 γ -OH with *pBA*. *P. oceanica* lignin presented the highest *p*-hydroxybenzoylation degree (73%
384 of G- and 61% of S-lignin units are acylated with *pBA*) reported in plant lignins to date. It
385 seems likely that differences in C_{org} storage capacity between *P. oceanica* and *P. australis* are
386 partly explained by differences in the abundance of lignin and its molecular composition, which
387 may enhance the chemical recalcitrance of cell walls. Molecular characterization of more types
388 of seagrasses ecosystems (e.g. living plants and detritus embedded within soils) together with
389 incubation studies to assess the lability of different molecular structures in oxic and anoxic
390 conditions, are necessary to unravel the importance of phenolic composition for C_{org}
391 accumulation-storage-release dynamics.

392

393

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400

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553 **FIGURE CAPTIONS**

554

555 **Figure 1.** Pyrolysis-GC-MS (left) and THM-GC-MS (right) total ion current chromatograms of
556 whole plant *P. australis* (**A** and **B**) and its plant organs: leaf (**C** and **D**), leaf sheath (**E** and **F**)
557 and root+rhizome (**G** and **H**). The chromatograms of the sheath material of *P. oceanica* (**I** and
558 **J**) are also displayed. Symbols for Py-GC-MS: CAT= catechol, F16= C₁₆-fatty acid, G=
559 guaiacol, 4MG= 4-methylguaiacol, 4VG= 4-vinylguaiacol, C3G= *trans*-4-propenylguaiacol,
560 C3S= *trans*-4-propenylsyringol, P= phenol, Ps1= acetic acid, Ps2= 3/2-furaldehyde, Ps3= 5-
561 hydroxymethyl-2-dihydrofuraldehyde-3-one, Ps4= levoglucosan, Ph1= pristene, Ph2=
562 phytadiene 1, Ph3= phytadiene 2. Symbols for THM-GC-MS (for *p*BA, G and S derivatives, see
563 Table 1): F16= C₁₆-fatty acid methyl ester, Ps5-Ps8= unidentified carbohydrate products (Ps8=
564 *m/z* 101,111,187,219 compound, *see text*), T1= 1,3,5-trimethoxybenzene, T2= 2,4,6-
565 trimethoxytoluene.

566

567 **Figure 2.** 2D-HSQC NMR spectra of acetylated *P. australis* (**A**) and *P. oceanica* (**B**) sheath
568 whole cell walls. The main lignin structures identified are depicted at the bottom. *p*BAs are the
569 most abundant aromatic substructures in *P. oceanica*, whereas they are completely absent in *P.*
570 *australis*. The abundance of the different lignin units (H, G and S) are calculated from contour
571 volume-integrals, and *p*BA is estimated based on H+G+S=100%. It is important to note that
572 *p*BA are found as endgroup on the lignin polymer, presenting a higher mobility and therefore
573 their signal integration is over-represented in 2D-HSQC NMR outputs.

574

575 **Figure 3.** 2D HSQC spectra of acetylated *P. oceanica* sheath whole cell walls (**A**) and its
576 corresponding lignin enriched preparation, CEL (**B**), in DMSO-*d*₆:pyridine-*d*₅ (4:1,v:v). Note
577 that the intensities of the spectra **A** and **B** have been selected to clearly show the relative
578 proportion carbohydrates/*p*BA. If the intensity of the spectra are augmented, the correlation
579 signals of H-, G- and S-lignin units can also be observed (**C** and **D**).

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580

581 **Figure 4.** 2D-HSQC NMR spectra of *P. oceanica* EL preparation, before (A) and after (B)
582 acetylation, in DMSO-*d*₆:pyridine-*d*₅ (4:1,v:v). The shifts in the *p*BA correlation signals
583 (principally *p*BA_{3,5}) after acetylation demonstrate that *p*-hydroxybenzoate on lignin occurs in its
584 free phenolic form, susceptible to being acetylated, and are not etherified (in this case, the
585 acetylation of *p*BA group cannot take place and the *p*BA signals should therefore remain in
586 essentially the same place within the HSQC spectrum).

587

588 **Figure 5.** Reconstructed ion chromatograms (*m/z* 192+222+252+121, characteristic for the H,
589 G, S and *p*BA compounds) of the DFRC degradation products released from (A) *P. australis*
590 and (B) *P. oceanica*. *c*H, *t*H, *c*G, *t*G, *c*S, and *t*S represent the normal *cis*- and *trans-p*-coumaryl,
591 guaiacyl and syringyl monomers, respectively (as their acetyl derivatives). G-*p*BA and S-*p*BA
592 represent the coniferyl and sinapyl alcohol γ -acylated with *p*BA. (C) The structures of these
593 compounds and the mass spectra of G-*p*BA and S-*p*BA.

594 **Table 1.** Py-GC-MS products of the sheaths of *P. australis* and *P. oceanica*, and average
 595 relative proportions (%) with standard deviation (SD) of three replicates. ANOVA differences
 596 are indicated with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS $P > 0.05$) and the
 597 *Posidonia* species with higher concentration is indicated. *p*BA = *p*-hydroxybenzoic acid, Ph_t =
 598 total phenolic compounds, G = guaiacyl, S = syringyl, H = *p*-hydroxyphenyl.

599

	Main source	<i>P. australis</i>		<i>P. oceanica</i>		ANOVA (<i>P</i> value)
		(%)	SD	(%)	SD	
phenol		26.2	5.7	87.8	0.7	*** <i>P. oceanica</i>
4-hydroxybenzoic acid (<i>p</i> BA) methyl ester	<i>p</i> BA	0.0	0.0	1.7	0.2	*** <i>P. oceanica</i>
4-hydroxybenzoic acid (<i>p</i> BA)	<i>p</i> BA	0.0	0.0	1.0	0.1	*** <i>P. oceanica</i>
4-methoxybenzoic acid methyl ester	<i>p</i> BA	0.0	0.0	0.2	0.0	*** <i>P. oceanica</i>
2/3-methylphenol		4.2	0.2	1.6	0.2	*** <i>P. australis</i>
4-methylphenol		14.7	0.3	1.5	0.3	*** <i>P. australis</i>
C ₂ -alkylphenol		2.5	0.1	0.3	0.1	*** <i>P. australis</i>
C ₂ -alkylphenol		3.7	0.1	0.4	0.1	*** <i>P. australis</i>
C ₂ -alkylphenol		0.9	0.1	0.1	0.0	*** <i>P. australis</i>
4-vinylphenol	H	4.3	0.4	0.4	0.1	*** <i>P. australis</i>
guaiacol	G	5.9	0.7	0.8	0.1	*** <i>P. australis</i>
4-methylguaiacol	G	5.4	0.6	0.7	0.1	*** <i>P. australis</i>
4-ethylguaiacol	G	1.9	0.4	0.2	0.0	** <i>P. australis</i>
4-vinylguaiacol	G	10.6	1.4	1.4	0.3	*** <i>P. australis</i>
4-allylguaiacol	G	1.0	0.2	0.1	0.0	*** <i>P. australis</i>
<i>cis</i> -4-propenylguaiacol	G	0.8	0.1	0.1	0.0	*** <i>P. australis</i>
<i>trans</i> -4-propenylguaiacol	G	3.4	0.5	0.5	0.1	*** <i>P. australis</i>
syringol	S	1.8	0.4	0.2	0.0	** <i>P. australis</i>
4-methylsyringol	S	1.2	0.2	0.1	0.0	*** <i>P. australis</i>
4-ethylsyringol	S	0.6	0.1	0.0	0.0	*** <i>P. australis</i>
4-vinylsyringol	S	4.9	0.8	0.4	0.2	*** <i>P. australis</i>
4-allylsyringol	S	1.0	0.1	0.1	0.0	*** <i>P. australis</i>
<i>cis</i> -4-propenylsyringol	S	0.8	0.1	0.1	0.0	*** <i>P. australis</i>
<i>trans</i> -4-propenylsyringol	S	4.4	0.5	0.2	0.1	*** <i>P. australis</i>
<i>p</i> BA/Ph _t		0.4	0.1	0.9	0.0	*** <i>P. oceanica</i>
H/Ph _t		0.1	0.0	0.0	0.0	*** <i>P. australis</i>
G/Ph _t		0.4	0.0	0.0	0.0	*** <i>P. australis</i>
S/Ph _t		0.2	0.0	0.0	0.0	*** <i>P. australis</i>
S/G		0.5	0.0	0.3	0.1	** <i>P. australis</i>

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602 **Table 2.** THM-GC-MS products of the sheaths of *P. australis* and *P. oceanica*, and average
603 relative proportions (%) with standard deviation (SD) of three replicates. ANOVA differences
604 are indicated with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS $P > 0.05$) and the
605 *Posidonia* species with higher concentration is indicated. *p*BA = *p*-hydroxybenzoic acid, Ph_t =
606 total phenolic compounds, G = guaiacyl, S = syringyl, H = *p*-hydroxyphenyl.
607

	Main source	<i>P. australis</i>		<i>P. oceanica</i>		ANOVA (<i>P</i> value)
		(%)	SD	(%)	SD	
4-methoxybenzaldehyde (P4)		2.0	1.0	0.0	0.0	* <i>P. australis</i>
x-methoxybenzoic acid methyl ester (P6 isomer)		0.0	0.0	20.6	14.6	NS
4-methoxybenzoic acid methyl ester (P6)	<i>p</i> BA	3.2	1.5	71.8	16.9	** <i>P. oceanica</i>
3,4-dimethoxybenzaldehyde (G4)	G	7.0	3.7	0.4	0.2	* <i>P. australis</i>
3,4-dimethoxybenzoic acid methyl ester (G6)	G	6.5	3.4	0.8	0.6	* <i>P. australis</i>
3,4,5-trimethoxybenzaldehyde (S4)	S	4.1	1.1	0.1	0.1	** <i>P. australis</i>
2-methoxyethenyl-3,4-dimethoxybenzene (G7,G8)	G	18.8	6.9	3.2	1.5	* <i>P. australis</i>
3,4,5-trimethoxybenzoic acid methyl ester (S6)	S	0.6	0.2	0.1	0.0	* <i>P. australis</i>
<i>cis/trans</i> -1-(3,4,5-trimethoxybenzene)-2-methoxyethylene (S7,S8)	S	35.6	14.3	2.1	0,7	* <i>P. australis</i>
<i>threo/erythro</i> -1-(3,4-dimethoxybenzene)-1,2,3-trimethoxypropane (G14)	G	5.4	3.2	0.0	0,0	* <i>P. australis</i>
<i>threo/erythro</i> -1-(3,4-dimethoxybenzene)-1,2,3-trimethoxypropane (G15)	G	3.3	2.5	0.1	0,0	NS
<i>trans</i> -3-(3,4-dimethoxyphenyl)-3-propenoic acid methyl ester (G18)	G	7.1	2.5	0.7	0,7	* <i>P. australis</i>
<i>threo/erythro</i> -1-(3,4,5-trimethoxybenzene)-1,2,3-trimethoxypropane- (S14)	S	4.4	2.8	0.0	0,0	NS
<i>threo/erythro</i> -1-(3,4,5-trimethoxybenzene)-1,2,3-trimethoxypropane- (S15)	S	1.8	1.0	0.0	0,0	* <i>P. australis</i>
<i>p</i> BA/Ph _t		0.0	0.0	0.9	0,0	*** <i>P. oceanica</i>
H/Ph _t		0.0	0.0	0.0	0,0	* <i>P. australis</i>
G/Ph _t		0.5	0.1	0.1	0,0	*** <i>P. australis</i>
S/Ph _t		0.5	0.1	0.0	0,0	** <i>P. australis</i>
S/G		1.0	0.4	0.4	0,0	NS

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