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### **Paper:**

Mastrolonardo, G., Rumpel, C., Forte, C., Doerr, S. & Certini, G. (2015). Abundance and composition of free and aggregate-occluded carbohydrates and lignin in two forest soils as affected by wildfires of different severity.

*Geoderma*, 245-246, 40-51.

<http://dx.doi.org/10.1016/j.geoderma.2015.01.006>

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## **Abundance and composition of carbohydrates and lignin in two forest soils affected by wildfires of different severity**

5 Giovanni Mastrodonardo<sup>1,2</sup>, Cornelia Rumpel<sup>2</sup>, Claudia Forte<sup>3</sup>, Stefan H. Doerr<sup>4</sup>, Giacomo Certini<sup>1</sup>

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7 <sup>1</sup>*Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente (DISPAA), Università*  
8 *degli Studi di Firenze, Piazzale delle Cascine 28, 50144 Firenze, Italy.*

9 <sup>2</sup>*CNRS; nstitute of Ecology and Enviroment Paris (IEES, UMR Université Paris VI et XII – CNRS*  
10 *– IRD), Campus AgraParisTech, Thiverval–Grignon, France.*

11 <sup>3</sup>*Istituto di Chimica dei Composti OrganoMetallici (ICCOM), UOS Pisa, CNR, Pisa, Italy*

12 <sup>4</sup>*Geography Department, Swansea University, Singleton Park, Swansea SA28PP, UK.*

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14 Corresponding author G. Mastrodonardo, tel: +39 055 522 6509; fax: +39 055 522 6477

15 E-mail address: [giovanni.mastrodonardo@gmail.com](mailto:giovanni.mastrodonardo@gmail.com)

16

17 **Keywords:** wildfires; soil organic matter; non-cellulosic neutral sugars; lignin; soil density  
18 fractions; <sup>13</sup>C NMR.

19

### **1. Introduction**

21 Fire is a major ecological factor, affecting more land surface than any other natural disturbance

22 (Lavorel et al., 2007; Scott et al., 2014). Soils are affected by many direct and indirect

23 consequences of fire, which can alter the physical, mineralogical, chemical and biological

24 properties of soil, either temporarily or permanently (Bento-Gonçalves et al., 2012; Certini, 2005;

25 Neary et al., 1999). The organic component of soil is the one most affected by fire, in terms of both

26 content and composition (González-Pérez et al., 2004; Certini et al., 2011). The assessment of the

27 overall effect of fire on soil organic matter (SOM) is a complex task because burnt soils are  
28 generally a patchwork of areas affected to different degrees by burning. For example, besides the  
29 factors correlated with site, soil and vegetation features, fire effects on SOM depend on the degree  
30 and duration of heating, the availability of oxygen and the type of combustion (smouldering or  
31 flaming), which may lead to different SOM transformations (González-Pérez et al., 2004; Rumpel  
32 et al., 2007; Shakesby and Doerr, 2006). Most often fire causes a substantial reduction of the  
33 organic matter stock in the litter layer (Bento-Gonçalves et al., 2012; Certini et al., 2011; Nave et  
34 al., 2011). There are, however, contrasting results in the literature about quantitative changes in the  
35 mineral soil. For instance, meta-analyses by Johnson and Curtis (2001) and Nave et al. (2011), have  
36 contrasting outcomes, concluding that in the short-term the A horizon does experience, respectively,  
37 a significant fire-induced increase and decrease in C content. However, divergent results from  
38 different studies can arise from methodological differences, related to sampling strategy, including  
39 soil depth interval considered and time elapsed since fire, local conditions, as for example  
40 vegetation/climate type.

41 In terms of SOM quality, the reactions that take place during combustion, *e.g.* dehydration,  
42 dehydrogenation, volatilisation of nitrogenous compounds, decarboxylation, demethylation,  
43 demethoxylation, cyclisation and polycondensation (Hernández et al., 1997; Knicker, 2007), can  
44 substantially change the composition of the parent material. Charcoal formation is the most  
45 common outcome of wildfires and essentially represents the temperature and oxygen-depletion  
46 dependent transformation of the organic compounds into highly recalcitrant aromatic structures  
47 (Alexis et al., 2010). Once incorporated into the soil, charcoal may resist decomposition for  
48 centuries or even millennia, thereby sequestering carbon (Egli et al., 2012; Kuhlbusch and Crutzen,  
49 1995; Schmidt and Noack, 2000). Carbohydrates are believed to be among the most fire-affected  
50 components of SOM (Certini, 2005; González-Pérez et al., 2004; Knicker et al., 2006). On this  
51 basis, Martín et al. (2009) proposed the ratio of carbohydrates to total SOM as an index of fire  
52 impact on SOM quality. Such an index allows, in principle, also differentiating between low- and

53 high- soil burn severity wildfires. Lignin, which, after carbohydrates, is the second most abundant  
54 component of plant residues in terrestrial ecosystems, is rather resistant to fire and is totally  
55 oxidised only at 400–450 °C (DeBano, 1991; Kuo et al., 2008). Nevertheless, despite the high heat  
56 resistance of its backbone (Knicker, 2011; Sharma et al., 2004), lignin is affected by fire at much  
57 lower temperatures (200–250 °C) in terms of the distribution of phenols (Certini et al., 2011;  
58 Nocentini et al. 2010; Rumpel et al., 2007). Therefore, the composition of SOM regarding lignin  
59 and phenols has potential as an indicator of fire occurrence and severity. Quantifying post-fire  
60 lignin phenols and sugars in soil might be useful to evaluate the wider impact of fire on soil quality.  
61 In the mineral soil, the effects of fire are usually confined to the top few cm because of the low  
62 thermal conductivity of both minerals and pore spaces. Nonetheless, in several studies reporting on  
63 fire impacts, soil has been sampled to rather substantial depths, which is likely to have resulted in  
64 the dilution of the investigated fire effects.

65 In this study, we investigated the top 2.5 cm of mineral soil of two forests located in Italy and  
66 Australia, which have been affected by recent wildfires of moderate and extreme severity,  
67 respectively. The purpose of using two contrasting sites in terms of forest type and fire severity was  
68 to assess changes to SOM quality resulting directly from the fire and explore their implications. We  
69 focused particularly on the non-cellulosic neutral sugars, those digested by trifluoroacetic acid  
70 (TFA), and lignin-derived phenolic monomers, those released by cupric oxide (CuO) oxidation. We  
71 further analysed the distribution of density fractions in SOM and the composition of such fractions.  
72 The changes SOM experienced at the two sites were compared to relate them to fire severity,  
73 hypothesising common fire-related alterations in SOM, mainly driven by fire severity.

74

## 75 **2. Materials and Methods**

76

### 77 *2.1 Study sites*

78 The study sites are Orentano (hereafter called OR), 30 km east of Pisa, Tuscany, Central Italy, and  
79 Mount Gordon (hereafter called MG), near Marysville, in the Victoria State, south-east Australia  
80 (Fig. 1; Table 1).

81 Orentano (OR), 20 m a.s.l., has a mean annual precipitation of 893 mm and a mean annual  
82 temperature of 14.3 °C. The vegetation cover is a mixed forest of Downy oak (*Quercus pubescens*  
83 Willd) and Maritime pine (*Pinus pinaster* Aiton) with a rich understory of common fern (*Pteridium*  
84 *aquilinum* L.) and *Rubus spp.* Soils formed on sand and stony lacustrine deposits – where quartz is  
85 largely dominant and chlorite, illite, kaolinite, and goethite are accessory minerals – and are  
86 classified as Haplic Skeletic Acrisols according to the World Reference Base for Soil Resources  
87 (IUSS Working Group WRB, 2014). In July 2011, an area of 3.3 ha underwent a wildfire of  
88 moderate to high severity, based on the visual scale of litter and vegetation consumption proposed  
89 by Chafer et al. (2004). Most of the tree stems were still standing after the fire and were partly or  
90 totally scorched. The soil was covered entirely by charcoal and ash, with no or very little uncharred  
91 litter remaining. Soil sampling was carried out three days after the fire, on both the burnt area  
92 (coordinates WGS84: 43°47'22.82"N, 10°39'52.30"E) and an adjacent (50 meters away) unburnt  
93 area having the same characteristics of the burnt one prior to fire occurrence, thus used as control.

94 At Mount Gordon (MG) the sampling area is located 530 m a.s.l., where mean annual precipitation  
95 is 670 mm and mean annual temperature is 13 °C. The site was chosen because it represented an  
96 end-member in terms of fire severity. The sadly famous ‘Black Saturday’ fire, which involved also  
97 MG, in early February 2009, burned some 450,000 ha of eucalypt forest causing the loss of 173  
98 lives (Royal Commission, 2009). Average fire-line intensity is estimated to have exceeded 70,000–  
99 80,000 kW m<sup>-1</sup>, which is amongst the highest ever reported in Australia (Royal Commission, 2009).

100 Such an extreme intensity was promoted by particularly extreme weather conditions, such as wind  
101 speeds up to 100 km h<sup>-1</sup> and air temperatures even exceeding 45 °C. Fuel loads were very high,  
102 since the forest had not experienced a major fire since 1939 (fuel load, including the litter,  
103 amounted to 25–40 Mg ha<sup>-1</sup>), and fuel moisture was very low (3–4%) because of prolonged drought

104 conditions (McCaw et al., 2009). The sampling site (37°31'56.30"S, 145°43'17.14"E) is an  
105 *Eucalyptus spp.* mixed forest 3 km SW of Marysville on the road to Narbethong. The fire removed  
106 all ground fuel, green vegetation and woody stems <10 mm in diameter; accordingly, fire severity  
107 was classified as extreme, based on the classification of Chafer et al. (2004). A long unburnt site –  
108 last burned by wildfire in 1939 – was selected as control, approximately 3 km NW of Narbethong  
109 (37°32'54.10"S, 145°37'37.30"E). This site is 8.5 km away from the burnt site and as much as  
110 possible similar to the latter in terms of all environmental conditions, soil included. Soils of the area  
111 formed on sandy Devonian sediments – where quartz is largely prevailing and the clay fraction  
112 comprises vermiculite, illite, and kaolinite – and are classified as Dystric Humic Cambisols  
113 according to the World Reference Base for Soil Resources (IUSS Working Group WRB, 2014).  
114 Sampling was performed in April 2009, two months after the fire and following some light rainfall,  
115 but before the more intense precipitation of winter had caused significant ash removal via erosion.

116

## 117 2.2 *Soil sampling*

118 At both study sites, OR and MG, the sampling involved four parallel 20 m transects, laid out 5 m  
119 apart, at 5 m intervals. Twenty mineral soil samples were taken at each site down to 2.5 cm, after  
120 carefully removing the ash, charcoal, or any litter layer by a brush. In both burnt areas, ten samples  
121 of charcoal particles were randomly collected in 40 ×40 cm plots.

122

## 123 2.3 *Physico-chemical properties*

124 Soil pH was measured potentiometrically using deionised water to soil ratio of 5:1, while particle  
125 size analysis was performed according to the hydrometer method. Total C and N contents and stable  
126 carbon isotopic composition of the fine earth (the less than 2 mm soil fraction) and charcoal were  
127 measured by a Carlo Erba NA1500 elemental analyser coupled to an isotope ratio mass  
128 spectrometer (Micromass-Optima).  $\delta^{13}\text{C}$  isotope abundance was reported in per mil (‰) relative to  
129 the Pee Dee Belemnite standard (PDB).

130

131 *2.4 Density fractionation*

132 Density fractionation was carried out on two soil samples per site, both obtained combining equal  
133 aliquots of ten samples of fine earth. The procedure was based on the method of Golchin et al.  
134 (1994), modified according to Sohi et al. (2001) and Cerli et al. (2009). This procedure allows  
135 different fractions, related to the spatial arrangement and interactions of organic compounds with  
136 minerals, to be separated (Cerli et al., 2012). The method requires a sodium polytungstate (NaPT)  
137 solution adjusted at a specific density to isolate a *free light fraction* (f-LF). By addition of the same  
138 solution to the precipitated material, followed by ultrasonic dispersion to break down the  
139 aggregates, the *occluded light fraction* (o-LF) is separated from the *heavy fraction* (HF), the latter  
140 fraction mainly comprising minerals. We performed preliminary trials in order to determine the  
141 most suitable density cut off and sonication energy for the fractionation. We first used the density  
142 and sonication intensities most commonly found in the literature, *i.e.* 1.6 and 1.8 Mg m<sup>-3</sup> and 100  
143 and 300 J cm<sup>-3</sup>, respectively (Cerli et al., 2009, 2012; Golchin et al., 1997; Kiem and Kögel-  
144 Knabner, 2003), with the ultrasonic energy being calibrated calorimetrically according to Schmidt et  
145 al. (1999). Based on the criterion of the highest SOM concentration of the o-LF, *i.e.* the higher  
146 release of the o-LF with the smallest mineral “contamination” (data not shown), we selected 1.8 Mg  
147 m<sup>-3</sup> and 300 J cm<sup>-3</sup> for soils from both study areas. Hereafter the soil samples were fractionated  
148 according to the following procedure: 125 cm<sup>3</sup> of NaPT solution (density 1.8 Mg m<sup>-3</sup>, soil to  
149 solution ratio 1:5) were added to 25 g of soil, gently shaken and allowed to stand for one hour. After  
150 centrifugation at 6800 g for 20 min, the suspended material (f-LF <1.8 Mg m<sup>-3</sup>) was separated from  
151 the supernatant and filtered on a glass fibre filter (cut-off 0.7 µm) for washing away any residual  
152 NaPT. The precipitated material was ultrasonically dispersed in NaPT solution with the same  
153 density (1.8 Mg m<sup>-3</sup>, soil to solution ratio 1:5) by applying 300 J cm<sup>-3</sup> and allowed to stand for one  
154 hour. After centrifugation at 6800 g for 20 min, the o-LF <1.8 Mg m<sup>-3</sup> was recovered as described  
155 above. All three fractions obtained were repeatedly washed with deionised water until the electrical

156 conductivity of the supernatant was  $<50 \mu\text{S cm}^{-1}$ . They were then oven dried at  $50^\circ \text{C}$ , weighed and  
157 analysed for their C and N content. Apart from obtaining functionally more homogeneous fractions  
158 with a narrower range of properties compared to the bulk soil, this type of density fractionation  
159 allows free SOM, and SOM associated with minerals and physically protected into aggregates to be  
160 distinguished.

161

### 162 2.5 Lignin phenols determination

163 There is not yet any method able to reliably quantify the total lignin content in soil. Even the cupric  
164 oxide (CuO) oxidation, which is perhaps the most widely used method for this purpose, does not  
165 succeed in completely depolymerising lignin. It thus cannot be considered as a strictly quantitative  
166 method (Kögel, 1986). Nevertheless, CuO oxidation is able to release phenolic monomers and  
167 dimers from the end groups of the lignin macromolecules and, as such, it is a reliable indicator of  
168 lignin amount and composition in soil (Otto and Simpson, 2006; Spielvogel et al., 2007; Thevenot  
169 et al., 2010). On this basis, we submitted the bulk soil and density fractions to CuO oxidation  
170 according to the modified method proposed by Kögel and Bochter (1985) and Kögel-Knabner  
171 (1995). Briefly, 50-200 mg of sample (the higher the C concentration, the lower the quantity of  
172 sample), 250 mg CuO, 50 mg of glucose, 50 mg of  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  and  $15 \text{ cm}^3$  of 2 M NaOH  
173 were digested in a Teflon pot at  $172^\circ \text{C}$  under  $\text{N}_2$  atmosphere for 2 h. Afterwards, ethyl vanillin was  
174 added as an internal standard to assess the recovery of lignin products. The solution was adjusted to  
175 pH 1.8 – 2.2 and left overnight for humic acid precipitation. Thereafter, the lignin-derived phenols  
176 were purified by elution through a  $\text{C}_{18}$  column (International Sorbent Technology) and extracted  
177 from the column by adding  $5 \times 0.5 \text{ cm}^3$  ( $2.5 \text{ cm}^3$  in total) ethyl acetate. After evaporating ethyl  
178 acetate under  $\text{N}_2$  flow, the dried residue was dissolved in pyridine containing phenylacetic acid as  
179 internal quantification standard, and then derivatised by adding BSTFA [*N*, *O*-  
180 bis(trimethylsilyl)trifluoro-acetamide]. The silylated lignin monomers were separated using a HP  
181 6890 gas chromatograph (GC) equipped with a SGE BPX-5 column ( $65 \text{ m} \times 0.32 \text{ mm}$  internal

182 diameter, 0.25  $\mu\text{m}$  film thickness) and a flame ionization detector (FID). The GC oven temperature  
183 program was: 100  $^{\circ}\text{C}$  (2 min) to 172  $^{\circ}\text{C}$  at 8  $^{\circ}\text{C min}^{-1}$ , to 184  $^{\circ}\text{C}$  at 4  $^{\circ}\text{C min}^{-1}$ , to 300  $^{\circ}\text{C}$  at 10  $^{\circ}\text{C}$   
184  $\text{min}^{-1}$  and 310  $^{\circ}\text{C}$  for 5 min. Helium was used as carrier gas and samples were injected in split mode  
185 (1:10). CuO oxidation products are composed of vanillyl (V)-units (vanillin, acetovanillone, vanillic  
186 acid), syringyl (S)-units (syringaldehyde, acetosyringone, syringic acid), and cinnamyl (C)-units  
187 (ferulic and *p*-coumaric acids). The sum of V-, S- and C-type phenols (VSC) was used to estimate  
188 the total amount of lignin.  $\text{Ac}/\text{Al}_V$  and  $\text{Ac}/\text{Al}_S$  are the mass ratios of acid-to-aldehyde for vanillyl  
189 and syringyl units, respectively.  $\text{S}/\text{V}$  is the mass ratio of syringyl to vanillyl units and  $\text{C}/\text{V}$  is the  
190 mass ratio of cinnamyl to vanillyl units. These mass ratios are generally used to assess the state of  
191 degradation of lignin, since the  $\text{Ac}/\text{Al}$  ratios increase while  $\text{S}/\text{V}$  and  $\text{C}/\text{V}$  ratios decrease with  
192 increasing decomposition. Fire was shown to immediately produce a degraded lignin signature for  
193 pure organic matter, increasing the acid-to-aldehyde ratio of V- and S-type units (Nocentini et al.,  
194 2010; Rumpel et al., 2007), so partly mimicking the effect of microbial degradation.

195

## 196 2.6 *Non-cellulosic neutral sugars determination*

197 Sugar monomers were determined according to the method proposed by Amelung et al. (1996) as  
198 modified by Rumpel and Dignac (2006). The analysis was performed on both bulk soil and density  
199 fractions. Briefly, 200–500 mg of soil, depending on C concentration, were hydrolysed with 4 M  
200 trifluoroacetic acid (TFA) at 105  $^{\circ}\text{C}$  for 4 h. After the samples had cooled down, 0.5  $\text{cm}^3$  of  
201 myoinositol (concentration 2  $\text{mg l}^{-1}$ ) was added as internal standard. Thereafter, the hydrolysed  
202 samples were purified by filtration over glass fibre membrane (cut-off 1.2  $\mu\text{m}$ ) and dried using a  
203 rotary evaporator (58  $^{\circ}\text{C}$ ). Ethylenediaminetetraacetic acid (EDTA) was added, according to Eder et  
204 al. (2010), to keep iron in solution in a non-reactive form to avoid possible co-precipitation of  
205 dissolved organic carbon. Derivatisation of the samples was carried out in screw top test tubes.  
206 Aldoses were reduced to their corresponding alditols after addition of 1  $\text{cm}^3$   $\text{NaBH}_4$  dissolved in  
207 dimethyl sulfoxide (DMSO). Acetylation was carried out by adding 2  $\text{cm}^3$  acetic anhydride and 2

208 cm<sup>3</sup> glacial acetic acid, and using methylimidazole (2 cm<sup>3</sup>) as catalyst. The reaction was stopped  
209 after 10 min by 7 cm<sup>3</sup> ice-cold deionized water, which transformed acetic anhydride to acetic acid.  
210 The derivatised sugar monomers were extracted by liquid–liquid extraction with 1 cm<sup>3</sup>  
211 dichloromethane using a vortex mixer. After the phase separation, the darker organic lower phase  
212 was removed with a Pasteur pipette and transferred into a GC vial. The analyses were performed by  
213 a HP 6890 gas chromatograph (GC) equipped with a SGE BPX-70 column (60 m × 0.32 mm  
214 internal diameter, 0.25 μm film thickness) and a FID. The GC oven temperature program was:  
215 200 °C to 250 °C at 8° C min<sup>-1</sup> and 250 °C for 15 min. Helium was used as carrier gas and samples  
216 were injected in split mode (1:10).

217 The TFA digests the monosaccharides originated from plant-derived hemicelluloses and microbial  
218 products, while it is not able to digest crystalline cellulose (Guggenberger et al., 1994). Hence,  
219 hereafter, we will use the term sugars to indicate the non-cellulosic neutral polysaccharides. In  
220 particular, the sugar monomers detected by this method are: rhamnose, fucose, ribose, arabinose,  
221 xylose, mannose, galactose, glucose. Fructose is transformed into the same alditol as glucose during  
222 the reduction step (Rumpel and Dignac, 2006); however, the fructose content in soil is so low that  
223 its contribution can be neglected (Amelung et al., 1996). The concentration of individual sugar  
224 monomers was calculated based upon the internal standard myoinositol.

225 According to Oades (1984), the proportion of microorganism-derived sugars in relation to plant-  
226 derived sugars can be roughly estimated by means of the mass ratio of hexoses/pentoses sugars:  
227 (galactose + mannose)/(arabinose + xylose), hereafter called GM/AX. Low (<0.5) and high (>2)  
228 GM/AX ratios are peculiar of carbohydrates predominantly derived from plants and  
229 microorganisms, respectively (Oades, 1984).

230

### 231 2.7 *Solid-state <sup>13</sup>C NMR spectroscopy*

232 We analysed by solid-state <sup>13</sup>C cross polarisation with magic angle spinning (CP/MAS) nuclear  
233 magnetic resonance (NMR) spectroscopy the bulk soil and the density fractions, with the exception

234 of the heavy one (HF), which was too poor in C to provide reliable information (Mastrolonardo et  
235 al., 2013). The specimens subjected to NMR analysis were obtained combining equal aliquots of all  
236 the independent samples collected from each site. Before  $^{13}\text{C}$  NMR analysis, all samples were  
237 treated with 2% hydrofluoric acid, as described by Skjemstad et al. (1994), to remove paramagnetic  
238 minerals, which strongly reduce the signal-to-noise ratio of the spectra. NMR spectra were recorded  
239 on a Bruker AMX300-WB spectrometer, working at 300.13 MHz for proton and at 75.47 MHz for  
240 carbon-13, and equipped with a 4 mm CP/MAS probehead. The spectra were recorded with a  
241 contact time of 2 ms under proton decoupling conditions with a spinning rate of 8 kHz. The  $^1\text{H}$  90°  
242 pulse length was 3.4  $\mu\text{s}$ , the spin-lock field 72 kHz, and the recycle delay 4 s. From 4,000 to 40,000  
243 scans were acquired depending on the sample. The chemical shifts were referenced to  
244 tetramethylsilane (TMS) using adamantane as external standard.

245 The contribution of main C forms to total signal was determined by integration of corresponding  
246 chemical shift regions: 0 to 45 ppm (alkyl C), 45 to 110 ppm (*O*-alkyl C, subdivided in methoxyl/*N*-  
247 alkyl C, 45–60 ppm; *O*-alkyl C, 60–90 ppm; di-*O*-alkyl C, 90–110 ppm), 110 to 165 ppm (aryl C,  
248 subdivided in aromatic C–H and C–C, 110–140 ppm; *O* substituted C, 140–165 ppm), 165 to 185  
249 ppm (carboxyl C); no signals arising from aldehydes or ketones were observed in the 185 to 220  
250 ppm region.

251

## 252 2.8 *Statistics*

253 Data from burnt and unburnt soils were compared by two-tailed paired t-test at 95% confidence  
254 level (SigmaPlot 12.0). Where data did not show a normal distribution (Shapiro-Wilk test),  
255 Spearman rank correlation was used.

256

## 257 3. Results and Discussion

258

259 3.1 *Carbon and nitrogen in the bulk soil*

260 The measured pH values, all below 6, demonstrated that the two soils were carbonate-free, hence,  
261 that the measured C was entirely in organic forms. At OR, where fire severity was moderate to high,  
262 the concentrations of carbon and nitrogen, and consequently the C/N ratios, did not change  
263 significantly between the unburnt and burnt soil (Table 2). Perhaps, the fire-induced loss in SOM, if  
264 any, was counterbalanced by the input of organic residues, including charcoal, from aboveground  
265 biomass and litter. Doing a meta-analysis Johnson and Curtis (2001) found a significant increase in  
266 soil C in the A horizon of forest soils burnt less than 10 years earlier. They attributed such an  
267 increase, at least partly, to an accumulation on the ground of unburnt and charred residues. The  
268 incorporation of fresh charcoal into the mineral soil at OR is supported by the lower  $\delta^{13}\text{C}$  value of  
269 the burnt soil compared to the unburnt one (Table 2), since charcoal had lower  $\delta^{13}\text{C}$  than unburnt  
270 soil (-28.5 ‰ in charcoal versus -27.4 ‰ in unburnt soil).

271 At MG, where fire severity was extremely high, the burnt soil exhibited substantially lower C and N  
272 concentrations compared to the unburnt one: 88 and 164 g C kg<sup>-1</sup>, and 4.3 and 5.8 g N kg<sup>-1</sup>,  
273 respectively (Table 2). Such fire-induced losses are out of range when compared to data reported by  
274 Nave et al. (2011) in their meta-analysis. At MG, also the C/N ratio experienced a significant  
275 decrease, which might be explained by an accumulation of recalcitrant organic N-forms in the  
276 charred material (Almendros et al., 2003; González-Pérez et al., 2004; Mastrolonardo et al., 2014;  
277 Santin et al., 2008). The addition of any charred materials from the aboveground biomass at MG  
278 was evidently not sufficient to counterbalance the large loss of SOM. Nevertheless, it should be  
279 noted that the surface ash layer removed during sampling to expose the mineral soil was  
280 considerable (1.7 cm thick on average). It contained substantial amounts of C (62 g kg<sup>-1</sup>), mainly in  
281 the form of charcoal (Santin et al., 2012). Although the ash layer does usually not remain on  
282 hillslopes for a long time because of wind and water erosion (Bodi et al., 2014; Rumpel et al.,  
283 2009), it is conceivable that over time, at least some of the C retained in the ash layer would  
284 become incorporated into the mineral soil and hence increase the C content in the mineral soil.

285

## 286 3.2 Carbon and nitrogen in density fractions

287 The C and N concentrations of soil density fractions are given in Table 2. Light fractions, f- and o-  
288 LF, are generally assumed to comprise mainly plant debris and ancillary animal residues, charcoal  
289 and microorganisms colonising organic residues (Golchin et al., 1994; Wagai et al., 2009). Their  
290 main differences generally are in the particle size and location within the soil matrix: the f-LF is  
291 assumed to feature larger almost undecomposed organic materials, while the o-LF should comprise  
292 finer and more altered organic particles than the f-LF (Cerli et al., 2012; Golchin et al., 1994).

293 The C/N ratio of density fractions of the unburnt soil at both OR and MG supports the expectation  
294 that SOM ranges between the less degraded light fractions, having a higher C/N, to the comparably  
295 more decomposed heavy fraction showing a lower C/N. These considerations are consistent with  
296 the higher  $^{13}\delta$  C values for HF compared to light fractions, which support the hypothesis of a higher  
297 decomposition of the former fraction (Roscoe et al., 2001). In spite of low C and N concentrations,  
298 but due to its large relative mass (Table 2), HF stores one third of total SOC and almost half of total  
299 soil N (Fig. 2). Comparing density fractions from burnt and unburnt soils, it could be inferred if and  
300 how the aggregates were able to protect SOM from heating and if they collapsed because of fire. As  
301 quite recently reviewed by Mataix-Solera et al. (2011), the response of soil aggregates to heating by  
302 fire can be highly variable. Fire can oxidise organic binding agents in aggregates thereby causing  
303 their breakdown. Alternatively, a fast vaporisation of the water included in aggregates can lead to  
304 their destruction in a similar way as slacking does (Albalasmeh et al., 2013). However, under  
305 certain conditions, *i.e.* for wettable soils with SOM as main binding agent subject to low severity  
306 fires, aggregate stability may improve as a consequence of enhanced soil water repellency (Mataix-  
307 Solera and Doerr, 2004).

308 At OR, fire apparently caused an increase of C and N stock and concentration of f-LF (Table 2; Fig.  
309 2). This increase is probably due to the incorporation of some charred residues into the top  
310 centimetres of soil and the charring of part of SOM there present. The C stock in the o-LF was

311 slightly lower in the burnt soil compared to the unburnt one, although the C and N concentrations  
312 were actually higher (Fig 2; Table 2). This leads us to hypothesise that fire could have actually  
313 caused a partial disruption of aggregates; these latter released some high density mineral particles  
314 with no or little interaction with OM and some OM free particles, which may might have become  
315 part of the HF and the f-LF, respectively (Fig. 3). The so released SOM might also be more exposed  
316 to decomposition because more easily accessible by microorganisms and their enzymes. The same  
317 phenomenon, some fire-induced aggregate disruption, also occurred at MG, here resulting overall in  
318 an increase of the HF (Table 2). However, at MG the most affected fraction was the f-LF, that  
319 experienced a depletion in the C and N content and contribution (Table 2; Fig. 2).

320

### 321 3.3 Soil polysaccharides content

322 At OR, neutral sugars in the burnt and unburnt soils amounted to 7.4 and 8.2 g kg<sup>-1</sup>, respectively,  
323 which correspond to 76 and 97 g kg<sup>-1</sup> of SOC (Table 3), in the range of data reported by other  
324 authors for forest soils (*e.g.*, Guggenberger et al., 1994; Rumpel and Dignac, 2006). The apparent  
325 fire-induced decrease in neutral sugars was statistically significant if referred to total SOC, but not  
326 in absolute terms. The GM/AX ratio before fire occurrence was >2, indicating that sugars were  
327 synthesized mainly by microbial population (Guggenberger and Zech, 1994; Oades, 1984). Fire did  
328 not change this ratio indicating that, in principle, neither plant- nor microbial-derived sugars were  
329 preferentially affected by fire.

330 The SOC normalised sugar content slightly decreased due to fire in the f-LF, while in the other  
331 fractions, o-LF and HF, it increased (Table 3). A possible explanation for this result could be that  
332 sugars associated to minerals were relatively preserved compared to the other OM compounds. In  
333 fact, it is commonly reported that sugars of microbial origins contribute to the formation and  
334 stabilisation of soil aggregates, hence benefiting from physical protection. Moreover, they seem to  
335 be chemically stabilised by interaction with the mineral phase (Kiem and Kögel-Knabner, 2003;  
336 Martín et al., 2009; Rumpel et al., 2010).

337 At MG, the absolute concentration of non-cellulosic neutral polysaccharides in the bulk soil was  
338 substantially lower in the burnt area than in the unburnt one: 9 and 21 g kg<sup>-1</sup>, respectively. This  
339 apparent decrease, however, was not significant if sugar content was normalised to SOC (Table 3).  
340 This suggests that the polysaccharides present in the mineral soil were not preferentially affected by  
341 fire, despite being part of the most thermally labile SOM pool (De la Rosa et al., 2008). However, it  
342 must be pointed out that the method we used is not able to also detect cellulose C, which may have  
343 a different behaviour towards fire compared to non-cellulosic sugars. Like at OR, the GM/AX ratio  
344 at MG indicates approximately that carbohydrates were mainly originated from microorganisms and  
345 the ratio did not change after fire occurrence. Most of the sugars were stored in the f-LF and were  
346 apparently greatly lost because of the fire, in the former fraction as well as in the HF and o-LF,  
347 both in absolute terms (normalised to mass proportion of density fractions) and relative to SOC  
348 (Table 3).

349

#### 350 3.4 Soil lignin content

351 At OR, the yield of phenolic CuO oxidation products in the burnt soil was significantly lower than  
352 in the unburnt one, both in absolute terms and relative to SOC (Table 4). This suggests that lignin  
353 was somehow preferentially affected by fire, despite its assumed moderate recalcitrance to heating  
354 (Knicker et al., 2005). Fire apparently left the VSC content of SOC associated to HF almost  
355 unaltered, while it affected the VSC content of the light fractions, particularly o-LF, both in  
356 absolute value and relative to SOC. Therefore, occlusion into aggregates does not seem to guarantee  
357 lignin protection. Even, pyrolytic degradation of lignin polymers in aggregates could be favoured  
358 by inorganic catalysts, such as acidic clay minerals (Ohta and Venkatesan, 1992).

359 At MG, the absolute yield of phenolic CuO oxidation monomers was half in the burnt bulk soil  
360 compared to the unburnt one (Table 4), but such a difference was inconsistent if values were  
361 referred to SOC. As in the case of sugars, lignin monomers did not appear to be preferentially  
362 affected by fire compared to other SOM constituents. In the unburnt soil, lignin absolute content

363 was almost equally distributed among density fractions. Fire mainly affected the f-LF, causing large  
364 decrease in its VSC content. The o-LF showed the highest lignin contribution to SOC and the  
365 highest apparent lignin loss due to fire, while HF even shows a relative accumulation of lignin  
366 compounds.

367 At both study sites, none of the indicators describing the composition and degradation of lignin, *i.e.*  
368 acid-to-aldehyde ratios of V and S-type units, S-to-V and C-to-V ratios, changed significantly in  
369 response to fire (Table 4). Hence, it seems that fire affected unselectively all lignin units, which is  
370 in contrast to what has been reported by other authors, *i.e.* a higher thermal susceptibility of  
371 aldehydes in V and S phenols (Certini et al., 2011; Kuo et al., 2008; Nocentini et al., 2010; Ohta  
372 and Venkatesan, 1992; Rumpel et al., 2007).

373 Plotting the sugar vs. lignin contents from burnt soils at OR and MG, we found a fairly good linear  
374 correlation that did not occur in the unburnt soils (Fig. 4). This correlation probably depends on fire  
375 that, whatever its severity, would affect lignin and sugars leading to an overall decrease of both of  
376 them. Although based on a relatively small sample size here, this intriguing correlation deserves  
377 further investigation in future studies to ascertain its wider validity.

378

### 379 3.5 NMR analysis

380 The  $^{13}\text{C}$  CPMAS NMR spectra of bulk soil from burnt and unburnt areas at OR and MG are  
381 displayed in Fig. 5, while Table 5 shows the percent distribution of the total signal among seven  
382 chemical shift regions. The most evident difference between the burnt and unburnt soil specimens  
383 was the more intense signal of the former in the aromatic C region (110–160 ppm). This was clearly  
384 due to some input of charred material, whose signal is centred at ~130 ppm (Skjemstad et al., 1996).  
385 At OR, this enrichment was counterbalanced by a decrease in alkyl-C (0–45 ppm region) and a less  
386 substantial decrease in *O*-alkyl C (60–90 ppm). The two sharp peaks at around 72 and 104 ppm in  
387 the burnt soil revealed the persistence of substantial amounts of polysaccharides, possibly cellulose.  
388 Therefore, contrary to what is commonly found (*e.g.*, Certini et al., 2011; Knicker et al., 2005,

389 2006), here *O*-alkyl C does not seem to be the most fire-affected C form. At MG, in spite of the  
390 extremely high fire severity, spectra did not show major differences, apart from the evident  
391 enrichment in aromatic C in the burnt soil. Both burnt and unburnt soils were characterised by  
392 dominant signals in the alkyl C region, generally assigned to lipids and other aliphatic compounds,  
393 and in the *O*-alkyl C region, indicative for polysaccharides and amide C of proteins (Knicker and  
394 Lüdemann, 1995). The decrease in intensities of these signals, plausibly attributable to fire, is  
395 modest.

396 The NMR spectra of the light density fractions from OR and MG are shown in Fig. 6 and 7, while  
397 the signal distribution among the chemical shift regions of the spectra is in Table 5. The light  
398 fractions from the unburnt soils had similar patterns in the two sites, although the o-LF revealed  
399 more advanced stage of alteration than f-LF, as chiefly indicated by a lower *O*-alkyl C to alkyl C  
400 ratio (Baldock et al., 1992) and a much higher signal in the aryl C region (Golchin et al., 1994). In  
401 particular, the higher relative intensity observed in the aromatic region at ~150, 130 and 115 ppm  
402 for o-LF with respect to f-LF suggests higher lignin content (Golchin et al., 1994; Hatcher, 1987).  
403 The NMR analysis unravelled that in both soils the light fractions were to some extent affected by  
404 fire at both sites (Figs. 6 and 7). At OR, the f-LF from the burnt soil showed higher intensity of the  
405 peak at 130 ppm, and smaller signals in the alkyl (0–45 ppm), *O*-alkyl (60–90 ppm) and carboxyl C  
406 (160–185) regions compared to the counterpart from the unburnt soil, overall indicating charring  
407 processes. In the o-LF, the peak at 56 ppm (ascribable to lignin methoxyl carbon) and the signal at  
408 around 150 ppm (*O*-substituted phenolic carbon) decreased much in the burnt soil compared to the  
409 unburnt one, which suggests lignin decomposition. At MG, the most evident fire-induced change to  
410 light fractions of SOC was the increase of the peak at 130 ppm, while ancillary differences are the  
411 decrease of the peaks at about 150 and 53 (lignin), and the intensification of the peak at 174 ppm  
412 (carboxylic C). This latter was unexpected, because organic matter exposed to severe heating  
413 generally loses carboxyl C (Knicker et al., 2005).

414

#### 415 4. Conclusions

416 Our parallel investigation at Orentano and Mt. Gordon, two areas recently affected by wildfires of  
417 markedly different severity, showed that in both cases fire had a marked impact on composition of  
418 SOM from the uppermost mineral soil. This impact was partly independent of fire severity. At  
419 Orentano, Italy, where fire severity in the mixed oak-pine forest was moderate, soil did not  
420 experience any significant, loss of carbon. On the contrary, some charred material from the organic  
421 layer and the standing vegetation had joined the mineral soil. At Mt. Gordon, Australia, where the  
422 eucalypt forest had been burnt by an extremely severe fire, SOM experienced substantial loss not  
423 counterbalanced by the incorporation of charred materials, although it is likely that over time some  
424 of the OM still retained in the ash layer will be partly incorporated into the mineral soil.

425 Density fractionation enabled to examine three SOM pools with different characteristics and  
426 turnover time in soil, and to assess the fire impact on each of them. In spite of contrasting fire  
427 severity, at the two study sites we found similar fire impacts on the SOM assumed occluded in  
428 aggregates. Apparently, fire partly disrupted aggregates, causing release of SOM from this fraction.  
429 Such a SOM redistribution could imply substantial changes to C cycle. It is reasonable, for  
430 example, to assume higher availability of the released SOM to microorganisms. This outcome of  
431 fire should be taken into account, together with the significant immediate loss of SOM due to  
432 combustion and the increase in mean residence time of charred residues, when accounting for the  
433 fire impact on C balances.

434 In terms of SOM composition, at OR lignin was preferentially affected by fire compared to sugars,  
435 particularly in the light fraction occluded into aggregates, where presumably minerals offered  
436 different protection to different compounds. At MG, where lignin and sugars decreased a lot in  
437 response to fire occurrence, apparently none of the main C forms were preferentially affected by  
438 fire, either in the bulk SOM or the density fractions. Variables formerly proposed as reliable  
439 indicators of fire severity in soil, *i.e.* the sugar-to-total organic C ratio and phenols ratio in lignin,  
440 did not provide encouraging results in this study. Further studies are needed to elucidate the

441 complex impact of fire on SOM composition and to ultimately identify the chemical legacies that  
442 are most useful to reconstruct fire history.

443

444

#### 445 **Acknowledgments**

446 The work of the first author, G.M., was supported by a mobility fellowship provided by the  
447 European Science Foundation (Ref. number: 3690) under the framework of the MOLTER Program  
448 (MOLEcular structures as drivers and tracers of TERrestrial C fluxes). For site MG, sample  
449 collection and analysis were supported by a UK Natural Environment Research Council Urgency  
450 Grant (NE/F00131X/1). Patrick Lane, Petter Nyman, Richard Shakesby, Gary Sherdian and Hugh  
451 Smith supported sample collection in Australia. We are also indebted to Gianluca Borgogni, who  
452 kindly drew Figure 3.

453

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