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1 **Organo-mineral associations largely contribute to**
2 **the stabilization of century-old pyrogenic organic**
3 **matter in cropland soils.**

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15 **1. Abstract**

16 Understanding the processes underlying carbon (C) stability in soils is of utmost importance in the
17 context of climate change. In this setting, biochar is often studied for its persistence in soils and
18 reported to have positive impacts on soil fertility. Whilst recent research has mostly focused on the
19 short-term effects of biochar amendments to soil, a better understanding of its long-term persistence in
20 soils is needed.

21 Our study focuses on agricultural soils enriched in charcoal residues, produced in preindustrial kiln sites
22 *ca.* 220 years ago, as a proxy for aged biochar. Our aim is to better understand the processes governing
23 the long-term persistence of pyrogenic organic matter (PyOM) in cultivated soils. To achieve this, we
24 focus on i) the effect of PyOM on soil aggregation, ii) its distribution amongst soil fractions and iii) its
25 chemical and thermal properties. For this purpose, we combined a soil particle size-density fractionation
26 with elemental and thermal analyses on topsoil samples collected in charcoal enriched (CHAR) and
27 adjacent reference (REF) soils in a conventionally cropped field in Wallonia (Belgium).

28 The presence of charcoal in soils resulted in a 91 ± 34 % higher C content in CHAR soils than REF soils, of
29 which 84 to 94 % was PyOM and 6 to 16 % was additional non-PyOM. In CHAR soils, macroaggregation
30 was promoted at the expense of microaggregation ($\text{CHAR}_{\text{MACRO}} = 48.9 \pm 12.8$; $\text{CHAR}_{\text{MICRO}} = 25.5 \pm 8.3$ g
31 100 g^{-1} soil) whereas these were similar in REF soils ($\text{REF}_{\text{MACRO}} = 41.3 \pm 9.5$; $\text{REF}_{\text{MICRO}} = 36.0 \pm 8.7$ g 100 g^{-1}
32 soil). Elemental and thermal analyses revealed that PyOM did not only occur as free light fractions but
33 was also occluded in aggregates or sorbed onto mineral phases (56.4 ± 22.9 % of total PyOM) suggesting
34 PyOM may be further stabilized through organo-mineral associations. Century-old PyOM showed
35 increased H:C and O:C atomic ratios compared to recently pyrolyzed OM, particularly in occluded as
36 opposed to free light fractions, vouching for the functionalization of its surfaces. Furthermore,
37 regardless of high C contents in CHAR soils, similar C:N ratios between studied soils in mineral
38 dominated fractions suggested increased amounts of N-rich non-PyOM in charcoal rich soils. Our results
39 demonstrate that, over centuries in cultivated soils, PyOM plays an active role in aggregation patterns.
40 We conclude that century old PyOM is broken down from coarse to fine particles, and through
41 functionalization of its surface contributes to organo-mineral associations. Beyond its intrinsic chemical
42 recalcitrance, the long-term persistence of biochar is enhanced by occlusion and sorption processes.

43 **Keywords:**

44 Black carbon, size-density fractionation, thermal analysis, soil aggregation, aged biochar

45 2. Introduction

46 Globally, human land use and agriculture in particular have resulted in a substantial loss of soil organic C
47 (SOC) (Sanderman et al., 2017). As soil organic matter (OM) plays a crucial role in both the global C cycle
48 and in ensuring food security (Le Quéré et al., 2009; Chenu et al., 2019) implementing practices that
49 allow for SOC restoration and sequestration in cultivated soils offers a great potential to contribute to
50 the mitigation of anthropogenic greenhouse gas emissions, whilst improving soil agronomic and
51 ecological functions (Lal, 2004; Minasny et al., 2017; Chenu et al., 2019).

52 Amongst the various strategies allowing for SOC sequestration (i.e., a net removal of atmospheric CO₂),
53 the study of C forms displaying long residence times in soils such as biochar has received much
54 attention. Biochar is a form of pyrogenic organic matter (PyOM) whose purpose is to be amended to
55 soils for its conjoint potential in terms of long-term C storage (centennial or longer)(Forbes et al., 2006)
56 as well as soil fertility improvement (Lehmann et al., 2006). Meta-analyses of short-term (annual to
57 decadal) biochar impacts in agro-ecosystems report an overall increased plant productivity as explained
58 by the increase in soil water holding capacity, plant nutrient availability and liming effects (Jeffery et al.,
59 2011; Biederman and Harpole, 2013). Such changes are particularly marked in highly weathered, coarse
60 textured, tropical soils (Crane-Droesch et al., 2013). Biochar properties and their resulting agronomic
61 benefits depend on its organic precursor and the pyrolysis conditions (e.g., heating rates and maximum
62 temperatures) (Zimmerman et al., 2011; B. P. Singh et al., 2012; Baveye, 2014; Fang et al., 2015). Beyond
63 reported positive impacts on soil fertility, biochar is studied for its potential as a C sequestration tool
64 and related implications on the global C balance (Schmidt and Noack, 2000). The stability of biochar and
65 PyOM in general is attributed to its aromatic moieties which confer it recalcitrance against biotic or
66 abiotic oxidation (Hammes et al., 2008; Kuzyakov et al., 2014). The distribution and organization of these
67 aromatic moieties in charcoal fragments are characterized by the aromaticity and degree of aromatic
68 condensation of biochar (Wiedemeier et al., 2015) which will in turn determine its persistence in
69 ecosystems (Hammes et al., 2008; Kuzyakov et al., 2014). Despite this persistence in soils, biochar
70 properties evolve in soils over time through breakdown and functionalization (i.e., ageing) (Cheng et al.,
71 2006; Nguyen et al., 2008; Cheng and Lehmann, 2009; Hardy et al., 2017b). Recent studies have shown
72 that PyOM may decompose faster than initially predicted (Hamer et al., 2004; Hammes et al., 2008) and
73 that the persistence of PyOM in soils may have been overestimated particularly in arable settings (Singh
74 et al., 2012; Lehndorff et al., 2014; Lutfalla et al., 2017). The persistence of PyOM, as for other OM
75 forms, in soils is therefore strongly dependant on ecosystem properties rather than solely inherited
76 chemical recalcitrance (Schmidt et al., 2011; Lehmann and Kleber, 2015; Lehmann et al., 2020).
77 Therefore, spatial inaccessibility for decomposer organisms (i.e., occlusion in aggregates) and
78 interactions with mineral surfaces (i.e., adsorption) or metals (von Lützow et al., 2006; Kögel-Knabner et
79 al., 2008) need to be carefully investigated as stabilization processes for PyOM in soils particularly as
80 ageing renders it an active soil constituent.

81 While short-term biochar-induced changes in agro-ecosystems have been extensively characterized
82 (Jeffery et al., 2011; Biederman and Harpole, 2013; Crane-Droesch et al., 2013), insights into its long-
83 term effects are needed to verify its potential in climate-smart agriculture (Baveye, 2020). Worldwide
84 various field models have been addressed to better understand the long-term fate of biochar in soils.

85 Amongst these are the fertile Amazonian Dark Earth (Glaser and Birk, 2012), soils containing wildfire
86 derived PyOM (Schmidt and Noack, 2000; Bird et al., 2015), historical industrial sites (Cheng et al.,
87 2008b), crop residues burning or relic charcoal hearths sites (Borchard et al., 2014; Lehdorff et al.,
88 2014; Hernandez-Soriano et al., 2016b; Kerré et al., 2016; Hardy et al., 2017a; Hirsch et al., 2017;
89 Schneider et al., 2019). All these studies address aged PyOM however varying production environments
90 may result in a final product differing from that obtained through oxygen regulated pyrolysis as is done
91 today to produce biochar (Bird et al., 2015).

92 In southern Belgium, pre-industrial charcoal production kiln sites are easily spotted in agricultural fields
93 as “black patches” (Hardy and Dufey, 2015). The charcoal still found in these sites stands as left-overs
94 from the production kiln that has been incorporated in agro-ecosystems since their conversion from
95 woodlands *circa* 150-200 years ago (Hardy et al., 2017a). Due to similar oxygen-limited conditions in
96 kilns as those obtained in modern biochar production units, this setting is ideal for the study of century-
97 old biochar in temperate conventional cropping systems (Hardy et al., 2017a). Recent studies
98 investigating similar kiln sites in the region showed that black patches stored 60 to 80% more C than
99 adjacent reference soils in cropped systems, of which from 25% to 40% was non-pyrogenic organic
100 matter (non-PyOM)(Hernandez-Soriano et al., 2016a; Kerré et al., 2016; Hardy et al., 2017a). The total N
101 content as well as the C:N and C:P ratios are higher in kiln soils than reference soils (Hardy et al., 2017a).
102 In addition, kiln soils display greater cation exchange capacity (CEC), which was associated with larger
103 content of exchangeable Ca^{2+} and Mg^{2+} (Hardy et al., 2017a). Long-term ageing and continuous
104 cultivation have resulted in the increase in H:C and O:C ratios of charcoal particles in comparison to
105 freshly pyrolyzed OM (Hardy et al., 2017b). These modifications are associated with the oxidation of
106 charcoal particles and the creation of functional groups (mostly carboxyl, phenol or carbonyl) at the
107 edge of aromatic rings (Lehmann et al., 2005; Cheng et al., 2008a) which in turn promotes the
108 precipitation of 2:1 phyllosilicates and CaCO_3 on the surfaces of PyOM (Hardy et al., 2017b).

109 In the present study we aim to investigate the various associations between century-old biochar and soil
110 minerals in order to assess their contribution to PyOM persistence in soils. For this purpose, we aim to
111 separate SOC into pools displaying similar stabilization mechanisms (i.e., chemical recalcitrance, spatial
112 inaccessibility, interactions with mineral surfaces)(von Lützow et al., 2006, 2007). Numerous physical
113 (size, density) and chemical (oxidation, extraction) fractionation protocols have been developed to
114 isolate SOC pools of comparable stabilization mechanisms and turnover rates (von Lützow et al., 2007;
115 Poeplau et al., 2018). In soils, the most labile SOC pool, displaying turnover rates < 10 years, are
116 represented by free microbial biomass and the coarse light fraction (LF - density < 1.6-2 g cm⁻³) whereas
117 the SOC pools with the highest turnover rates comprise the oxidation-resistant SOC pool and the heavy
118 fraction after macroaggregate dispersion (HF - density > 2.8 g cm⁻³)(von Lützow et al., 2007; Virto et al.,
119 2010; Poeplau et al., 2018). In cultivated soils macroaggregates display rapid turnover as revealed by
120 their composition of a mix of young and older SOC originating from both labile organic matter, (i.e.,
121 plant roots, mucilage, exudates, fungal hyphae, microbial biomass)(Rillig, 2004; Rasse et al., 2005) and
122 more persistent physically protected and adsorbed organic compounds (Moni et al., 2010; Poeplau et
123 al., 2018). Their dispersion is therefore required to isolate SOC groups with similar turnover rates (Virto
124 et al., 2010; Poeplau et al., 2018). The few fractionation studies performed on soils enriched in aged
125 biochar showed that the century-old biochar particles were mostly present as free particulate organic

126 matter (i.e., non-associated with mineral particles)(Glaser et al., 2000; Herath et al., 2014; Kerré et al.,
127 2016; Grunwald et al., 2017). However PyOM has also been reported to increase aggregate formation
128 (Awad et al., 2013) and stability (Pituello et al., 2018) as well as to increase the abundance of non-PyOM
129 in the aggregate-protected silt and clay fraction (Liang et al., 2010; Hernandez-Soriano et al., 2016a;
130 Kerré et al., 2017) thus showing interactions with mineral phases.

131 Although the effects of PyOM in soils have been widely addressed, there is a lack of consensus
132 concerning the processes involved in its long-term persistence. Nonetheless it is known that ageing in
133 soils causes an oxidation of the charcoals surfaces and results in the formation of phenolic and
134 carboxylic functional groups along with a charging of the structures surface (Cheng et al., 2008a; Nguyen
135 et al., 2008; Cheng and Lehmann, 2009). As a result, physical protection through aggregation (Brodowski
136 et al., 2006) as well as an increased association with the mineral phases (Nguyen et al., 2008; Rodionov
137 et al., 2010; Hardy et al., 2017b) could play an important role in PyOM stabilization (Lehmann and
138 Kleber, 2015) beyond its chemical recalcitrance to oxidation.

139 In this study, we investigated the long-term persistence of PyOM in soils. To achieve this, soil samples
140 from five preindustrial charcoal kiln sites were compared to five adjacent reference soils in a
141 conventionally farmed Luvisol (Isnes, Belgium). These charcoal kilns are visible as black spots on bare
142 soils and procure an ideal setting for the study of aged biochar in conventionally cropped systems. This
143 site hence serves as a proxy to study the long-term effect of biochar in soils if it was to become a
144 common soil amendment in temperate soils of the region.

145 Our aim is to understand how and where PyOM persists in the soil matrix, as either free standing
146 particulate OM (POM) or protected through aggregate occlusion or mineral sorption. In particular, this
147 study aimed i) to quantify the effects of long-term PyOM accumulation on soil aggregation through a
148 size and density fractionation protocol, ii) to investigate the distribution of PyOM amongst the fractions
149 and iii) to determine the physico-chemical and thermal characteristics of organic matter in soil fractions.
150 Numerous analytical techniques exist to identify, characterize and quantify PyOM; physical density
151 separations, chemical oxidation, thermal analyses, optical or molecular marking techniques (Schmidt
152 and Noack, 2000; Brodowski et al., 2005; Hammes et al., 2007; Leifeld, 2007; Plante et al., 2009; Bird et
153 al., 2015; Kerré et al., 2016). Among these, thermal analyses and differential scanning calorimetry (DSC)
154 in particular, has been reported to be an efficient way to quantify and characterize number of samples
155 rapidly within the whole OM continuum, and was hence used to characterize PyOM forms (Leifeld,
156 2007; Plante et al., 2009).

157 We postulate that charcoal is found in all, light and heavy, soil fractions as a consequence of particle
158 physical breakdown and functionalization of its surfaces. Furthermore, we postulate that the elemental
159 composition of OM controls its thermal stability within the different fractions. Finally, we postulate that
160 pyrogenic C forms may be further stabilized as a result of organo-mineral associations which increases
161 its potential as a C sequestration tool.

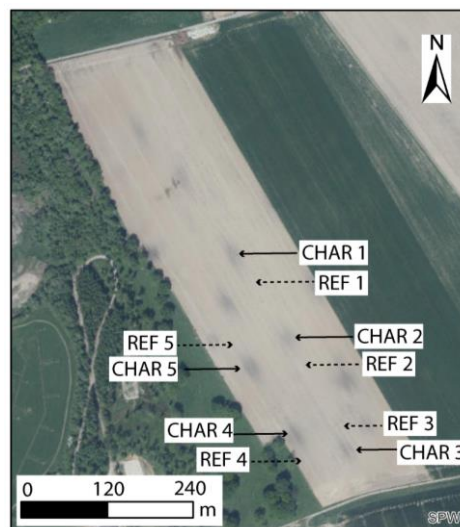
162 3. Materials and Methods

163 3.1. Study site

164 In cropped agroecosystems of the province of Namur (Belgium), century-old charcoal kiln sites are easily
165 spotted visually in the field or by aerial imagery as darker ellipses on the soil surface (Figure 1). The site
166 selected for this study is located in Isnes (50°31'N 4°44'E) and was converted from forest to cropland at
167 least 170 years ago as revealed by land occupation maps of Vandermaelen (1846-1854). Charcoal was
168 produced in kiln sites during the pre-industrial era to meet energy demands thus providing an estimated
169 age for the charcoal of interest of ca. 250 years (Hardy et al., 2017a). *Hardy et al. (2017b)* thoroughly
170 characterized kiln sites of the region and reported that charcoal mixtures are generally dominated by
171 deciduous hardwood such as oak (*Quercus* sp.), hornbeam (*C. betulus*), beech (*F. sylvatica*), hazel
172 (*Corylus avellana* L.) and birch (*Betula* sp.).

173 The soil of the studied site has a homogeneous silt loam texture throughout and is slightly sloped (Table
174 1). It is characterized as a Haplic Luvisol (WRB, 2014) developed from decarbonated quaternary loess.
175 The average annual precipitation is of 840 mm of rain and the mean annual temperature is 9.8°C. The
176 field follows a four-year crop rotation established approximately in 1985 and alternates between
177 chicory, winter wheat, beet root, potatoes and cover crops (mustard and phacelia).

178 # Figure 1: Aerial view of the study site.



179
180 *Figure 1 - Aerial view of the study site. Kiln sites are visible as black patches when the soil is bare. The image comes from the*
181 *“Service Public de Wallonie: Orthophotos 2009/2010”, arrows indicate the approximate location of the five soil sampling sites*
182 *per studied soil.*

183 3.2. Soil sampling and characterization

184 In this study, five field replicates of two soils, charcoal enriched (CHAR) and reference soils (REF) at least
185 40 m away from one another, were compared to study the effect of charcoal. Every soil sample collected
186 for each of the five replicates of CHAR and REF sites was bulked from five cores sampled in the topsoil
187 layer (0-30 cm) within a radius of 1 m. These samples were dried at 40 °C for 48h before further analysis.

188 A subsample of each soil was ground and sieved to 2 mm for baseline physico-chemical characterization.
189 Briefly, pH was determined in distilled water and KCl (1N) (ratio soil: solution 1:5, ISO 10390). The soil
190 particle-size distribution was determined by gravity sedimentation (NF X 31-107), C and N content were
191 quantified by near infra-red spectrometry after flash dry combustion (respectively ISO 10694; ISO
192 13878). We measured the cation exchange capacity (CEC) by percolation of 1 M ammonium acetate (pH
193 7) on soil columns according to the Metson procedure (Metson, 1956). The bioavailable major elements,
194 Ca, Mg, P and K were extracted using ammonium acetate-EDTA 1M (pH = 4.65; (Lakanen and Erviö,
195 1971)) and quantified by atomic absorption spectroscopy (Ca, Mg, K) or spectrophotometry (P).

196 3.3. Fractionation protocol

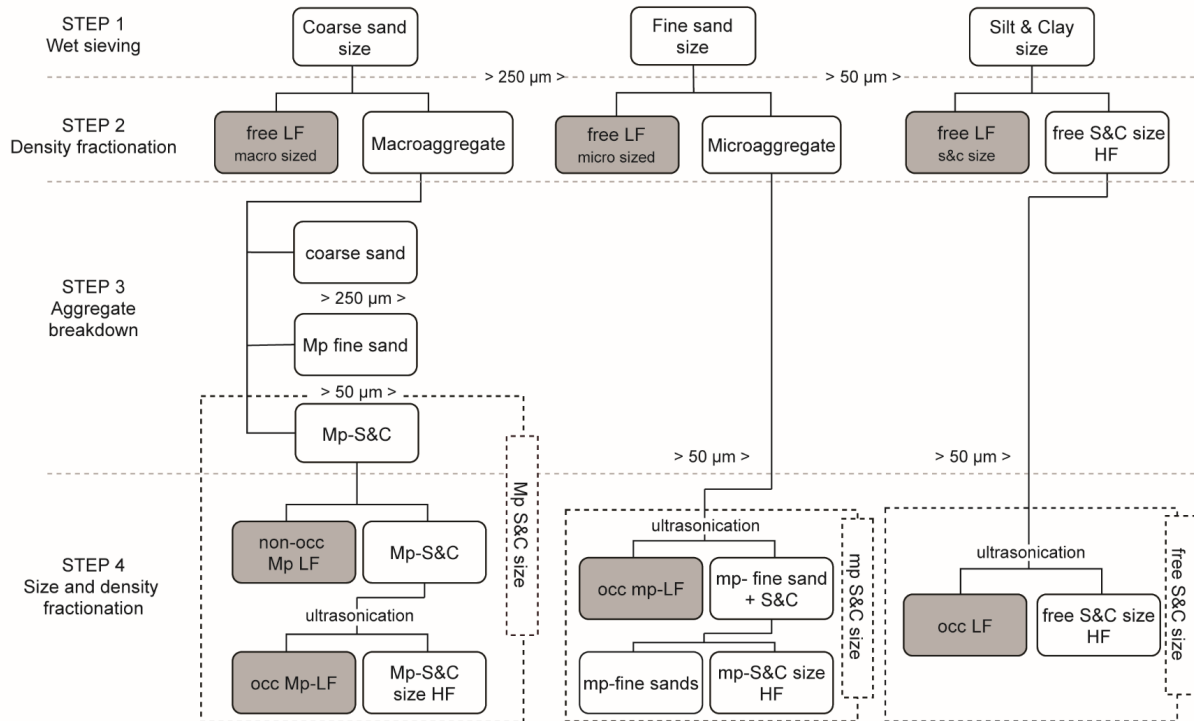
197 Soil samples from both studied soils followed a four step fractionation protocol (Figure 2), which was
198 adapted from the method described by *Chenu and Plante*, (2006), *Gulde et al.* (2008) and *Virto et al.*
199 (2010). In the first step, wet sieving fractionated the soil into four fractions according to size (>2000 µm,
200 coarse sand size: 2000-250 µm, fine sand size: 250-50 µm, silt and clay size (S&C) <50 µm). The
201 particulate material bigger than 2 mm was discarded. The second step used density to differentiate light
202 fractions (free LF, $d < 1.85 \text{ g cm}^{-3}$) from heavy fractions (HF: $d > 1.85 \text{ g cm}^{-3}$): Macroaggregate,
203 microaggregate, free silt and clay size heavy fractions (free S&C HF). Step 3 focused on breaking down
204 the macroaggregates (2000-250 µm) to isolate its components into three finer size class fractions
205 (coarse sand, macroaggregate protected fine sand, macroaggregate protected-S&C). Finally, step 4,
206 through a sequential size and density fractionation and dispersion of microaggregates, isolated non-
207 occluded and occluded S&C LF from S&C-size HF within each size class sizes from step 1.

208 We used the wording *free* for LF and HF after wet sieving, and *occluded* for LF obtained after ultrasonic
209 dispersion of aggregates. This fractionation protocol was applied on both studied soils, CHAR or REF, and
210 for all five replicates.

211 i. Step 1 – Size separation by wet sieving

212 We applied the wet sieving size separation protocol presented by *Six et al.* (1998). A soil sample of 80 g,
213 previously air-dried at 40°C for 48 h was submerged in deionized water for 5 min prior to deposition in a
214 water bath on a 2 mm sieve. The sieve was then moved up and down and tilted in a rotational
215 movement for 50 rotations in two minutes at room temperature. The sieve was then removed from the
216 water bath and its inner and outer walls were rinsed with deionized water. The reject (> 2 mm) was
217 collected by back-washing in a glass tray and air dried at 60°C. This wet sieving operation was repeated
218 firstly using a 250 µm sieve on the soil that went through the 2 mm sieve, then repeated a second time
219 on a 50 µm sieve to separate the two smallest fractions. All sieve rejects were back-washed in glass trays
220 after rinsing the sieve and dried at 60°C. This way four fractions per sample were differentiated as
221 follows: particulate (>2000 µm) > coarse sand size (2000-250 µm) > fine sand size (250-50 µm) > Silt &
222 Clay size materials (<50 µm) (Figure 2-step1).

Figure 2: Flow-chart of the four step fractionation protocol.



224

225 Figure 2 - Flow-chart of the four step fractionation protocol. S&C silt and clay; LF light fraction; HF heavy fraction; Mp
226 macroaggregate protected; mp microaggregate protected; occ. occluded; non-occ non-occluded. Dashed squares highlight the
227 difference between the three size classes

228 ii. Step 2 - Density fractionation

229 In this step we divided a 10 g subsample of the three size fractions smaller than 2 mm (coarse sand size
230 > fine sand size > S&C-size materials) obtained from step 1 into a light fraction (LF) and a heavy fraction
231 (HF) as described by Six et al. (1998). For this density separation subsamples were submerged in 30 mL
232 of sodium polytungstate (SPT, Na₆(H₂W₁₂O₄₀, - 1.85 g cm⁻³) in centrifuge tubes. These were then closed
233 and gently stroked 10 times to remove bubbles and without breaking aggregates. The centrifuge tubes
234 were then reopened and with an extra 20 ml of SPT its inner walls were rinsed to gather all the sample
235 in the solution. The samples were placed in a vacuum (-140 kPa) for 10 min, left to rest for 20 min and
236 centrifuged at 4700 g for 10 min. The supernatant was transferred on a filtering flask where a gentle
237 vacuum was applied (glass fiber filter Mechery-Nagel Germany, Φ = 0.45 µm). After having repeated the
238 centrifuge and the filtering thrice, the LF on the filter was thoroughly rinsed to remove the remaining
239 SPT under a light vacuum. The LF was then recovered in a glass tray by gently back washing the filter
240 with deionized water and then oven dried (60°C).

241 To recover the HF, 50 ml of deionized water was added to the pellet in a centrifuge tube and the tube
242 gently stroked until the pellet detached. An additional 20 ml of deionized water was used to clean the
243 tubes inner walls before centrifuging for 10 min at 4700 g. This rinsing was repeated thrice to rid the HF
244 of SPT before recovering the sample in a glass tray and oven drying at 60 °C for 24h. This step produces a

245 LF and a HF per size class obtained after step 1. These LF are referred to as free LF (macroaggregate,
246 microaggregate or S&C-size) and the HF as macroaggregates, microaggregates or free S&C-size (Figure 2-
247 step 2).

248 iii. Step 3: Macroaggregate breakdown

249 This step focused on the HF collected after density fractionation (step 2) of the macroaggregate soil
250 fraction separated from coarse sand size (2000-250 μm) material. The purpose of this step was to
251 separate this HF into: Coarse sands, macroaggregate protected fine sands and macroaggregate
252 protected S&C-size material. To achieve this, the sample was placed in a 250 μm sieve in contact with 50
253 glass beads in a water bath previously installed on a shaker plate. Deionized water was set to run
254 continuously on the sample while maintaining a 2 cm water front on top. The sample running with the
255 water through the first sieve was collected by means of a funnel and deposited onto a 50 μm sieve. This
256 breakdown procedure was set to last for 5 min on the shaker plate. After this step, deionized water was
257 used to thoroughly rinse the funnel into the 50 μm sieve. The 250 μm sieve was back-washed and oven
258 dried (60°C for 24 h) in a glass tray. Then the wet sieving procedure was repeated for the fraction
259 collected in the 50 μm sieve whereby the sieve was tilted and lifted up and down 50 times within two
260 minutes. Again after rinsing the sieve, the reject was back-washed and collected in a glass tray. Both
261 fractions (250-50 μm and <50 μm) were collected in glass trays and oven dried (24 h at 60°C).

262 iv. Step 4: Isolating occluded LF and HF

263 The first purpose of this step was to isolate the non-occluded (non-occ) S&C-size fraction (<50 μm)
264 contained in macroaggregates from the S&C-size HF (i.e., S&C aggregates or free minerals). For this step
265 an adapted density fractionation was undertaken. The sample was submerged in 30 ml SPT (1.85 g cm^{-3})
266 and gently shaken into suspension. Using an extra 20 ml of SPT the inner walls of the centrifuge tube
267 were rinsed before being centrifuged at 4700 g for 60 min (all material bigger than 0.7 μm sediments).
268 Using a micropipette, the topmost 15 ml were sampled and deposited on a glass fiber filter (0.4 μm)
269 without vacuum application (composes the non-occ LF). After each filtration the filter was rinsed
270 thoroughly to rid it of the SPT and then back-washed in a glass tray to collect the LF in order to avoid
271 filter clogging, this step was repeated thrice. After the third centrifuge, 40 ml of the supernatant were
272 collected and poured in the filter, rinsed thoroughly with distilled water through the filter. Finally, this
273 filter was back-washed to collect the LF in a glass tray.

274 Next, using SPT, the pellet of the centrifuge tube was shaken into suspension and then dispersed by
275 ultrasonication at 440 J ml^{-1} for 2 minutes (Soniprep 150 MSE) (Amelung and Zech, 1999) in order to
276 break down remaining S&C-sized aggregates thus freeing the occluded LF. This step was undertaken for,
277 macroaggregate protected S&C-size material, microaggregates HF and free S&C-sized material.
278 Following this dispersion, the LF was separated from the HF through a density fractionation (1.85 g cm^{-3})
279 to obtain three new fraction: occluded LF in macroaggregates (occ Mp-LF), occluded LF in
280 microaggregates (occ mp-LF) and occluded LF (occ LF).

281 Finally, to conclude step 4, a size separation through wet sieving on the HF obtained following the
282 ultrasonication of microaggregate HF only is undertaken as described previously to separate fine sands
283 from S&C size HF.

284 The density of SPT was controlled before each density fractionation step. Previous studies reported
285 solubilization of organic compounds with SPT use leading to a loss of C and a decrease the C recovery
286 after density fractionation (Chenu and Plante, 2006).

287 The elemental composition (C, H, N) was determined via dry combustion for all fractions (LF and HF).
288 The O concentration was determined on LF only through sample pyrolysis at 1000 °C. The C, H, N, O
289 concentrations were quantified using a Euro EA elemental analyzer (Hekatech, Germany). Prior to the
290 analysis, HF samples were acidified with HCl to remove residual inorganic C.

291 3.4. Differential Scanning Calorimetry (DSC)

292 We used thermal analysis to compare soil fractions isolated by the fractionation protocol to highlight the
293 presence of PyOM in fractions as well as its position in the OM continuum by comparing CHAR and REF
294 fractions. For this thermal analysis, three out of the five field replicates were selected for each studied
295 soil. For these repetitions, all fractions isolated by the fractionation protocol were analyzed. In addition,
296 three products were characterized as reference materials; hand-picked pyrolyzed OM (hand-picked
297 PyOM, n=3), hand-picked non pyrolyzed coarse OM (hand-picked non-PyOM, n=3) and freshly pyrolyzed
298 beech (*F. Sylvatica* –fresh PyOM, n=3). Hand-picked non-PyOM are crop residues hand-picked from REF
299 soils and hand-picked PyOM are charcoal fragments hand-picked from CHAR soils. The freshly pyrolyzed
300 beech was made in an industrial pyrolyzer (Greenpoch SA, Belgium). Finally, three bulk soils were also
301 used as comparison materials (n=3).

302 Samples were analyzed using a differential scanning calorimeter (Netzsch STA 449, Selb, Germany),
303 Agroscope, Zürich, Switzerland). Before sample analysis the sensor was calibrated for temperature and
304 sensitivity (i.e. reaction enthalpy) with six different standards (indium, tin, bismuth, zinc, aluminium and
305 silver).

306 Prior to scanning, samples were ground to powder. For HF between 15 and 25 mg of samples were
307 weighed and placed in open Al₂O₃ pans for analysis. For LF only 2 mg of samples were mixed to
308 approximately 18 mg Al₂O₃ powder, precise weights were recorded to enable a correction by the
309 dilution factor. Samples were analyzed under a constant flow of 50 ml min⁻¹ synthetic air from 50 °C to
310 700 °C at a heating rate of 20 °C min⁻¹. During data treatment a baseline correction was applied on
311 thermograms to account for increased heat capacity of samples. Resulting thermograms were drawn
312 between 200-600 °C to highlight the temperature range of interest as data below 200°C or above 600°C
313 were never used.

314 To characterize the material obtained we used values of peak height as the heat flow at a given
315 temperature (W g⁻¹), peak temperatures as the temperature at which main exotherms occur (°C), the
316 area under the curve as the total heat of reaction of a sample (J g⁻¹) delimited by the baseline, and the
317 thermogram 50% burn-off temperatures (°C) as the temperature where 50% of the total heat flow has
318 occurred. Finally ratios between areas or peak heights considered as thermally labile or stable were

319 used (Leifeld, 2007). In accordance with previous studies, a tipping temperature of ~380-390 °C was
320 used to differentiate the thermally labile or stable C forms (Dell'Abate et al., 2000; Lopez-Capel et al.,
321 2005).

322 Amongst various indicators available for the characterization of C forms using DSC, the sum of peak
323 heights of the exotherms ($R^2=0.78$ – p-value < 0.0001) and the total heat release ($R^2=0.91$ – p-value <
324 0.0001) displayed strong linear correlation with the C content of fractions.

325 3.5. PyOM and non-PyOM quantification in charcoal rich soils

326 In CHAR soils, the PyOM was quantified as the additional heat flow occurring in the thermally stable
327 region (>390 °C) as opposed to the heat flow in adjacent REF soils. This tipping temperature was
328 established based on our results and in line with previous findings (Dell'Abate et al., 2000; Lopez-Capel
329 et al., 2005). This additional heat flow over the sum of punctual heat flows (peak heights) of CHAR
330 samples multiplied by the samples C content corresponds to the PyOM content of CHAR soils (eq 1).

331 eq 1.

$$332 \quad PyOM = \frac{\sum CHAR_{st.peaks} - \sum REF_{st.peaks}}{\sum CHAR_{peaks}} * C_{CHAR}$$

333 Where **PyOM**; the PyOM content ($g\ kg^{-1}$), **st. peaks**; the heat flow of exotherms occurring above 390 °C
334 and **C_{CHAR}** the C content of CHAR samples.

335 Changes in non-PyOM contents, $\delta(\text{non-PyOM})$, were calculated as the ratio of differences in CHAR to
336 REF labile peaks height (< 390 °C) to the difference in sum of peaks between studied soils, the whole
337 multiplied by the difference of C content between CHAR and REF samples (eq 2.).

338 eq 2.

$$339 \quad \delta(\text{non PyOM}) = \frac{CHAR_{lab.peak} - REF_{lab.peak}}{\sum CHAR_{peaks} - \sum REF_{peaks}} * (C_{CHAR} - C_{REF})$$

340 Where **$\delta(\text{non-PyOM})$** ; the change in non-PyOM content ($g\ kg^{-1}$), **lab.peak**; the heat flow of
341 exotherms occurring below 390 °C, **C_{CHAR}** and **C_{REF}** the C content of CHAR and REF samples ($g\ kg^{-1}$).

342 3.6. Data analysis and signal treatment

343 All data analysis, DSC signal treatment and graph plotting were performed using R studio v. 3.4.2 (R Core
344 Team, 2017). Results of all parameters are presented as the means \pm standard deviation (SD).

345 Comparison between fractions of similar density and size class were undertaken by analysis of variances
346 (ANOVA) or by paired t-tests for soil baseline characteristics of bulk soils. Mean classification was
347 pursued through a least significant differences test (LSD-test) and indicated by different letters following
348 mean \pm SD. For the application of this test the normality of the distribution and homoscedasticity of
349 variances was presumed given the low number of repetitions ($n < 10$).

350 To ensure the validity of our soil fractionation results, the mass recovery and C content (mass by
351 concentration) were calculated at each fractionation step. These were obtained as the sum of fraction
352 masses and C content divided by that of sample used as input for the fractionation step.

353 4. Results

354 4.1. Soil characteristics

355 Beyond the presence of century-old charcoal kiln sites, the study site was selected for its homogeneity
 356 (REF soil) in terms of texture (sand = $7.5 \pm 0.4\%$, silt = $76.9 \pm 0.8\%$, clay = $15.5 \pm 0.7\%$), C concentration
 357 ($1.4 \pm 0.0\%$), CEC ($12.0 \pm 0.6 \text{ cmol}_c \text{ kg}^{-1}$) and pH (H₂O – 7.8 ± 0.2). On average, C concentrations in CHAR
 358 soils ($[1.9 - 3.3]\%$) were $91 \pm 34\%$ higher than in REF soils ($[1.3 - 1.4]\%$) (p-val. < 0.01). Similarly,
 359 organic N concentrations were $66 \pm 11\%$ higher (p-val. < 0.001) in CHAR ($[2.1 - 2.6] \text{ g kg}^{-1}$) than in REF
 360 soils ($[1.2 - 1.5]\%$). C, N concentrations as well as C:N ratios were more variable among replicates in the
 361 CHAR soils than in the REF soils. The two studied soils were of identical texture (silty loam) and had
 362 similar pH values. No significant differences in CEC were reported. Bioavailable Ca²⁺ concentrations were
 363 on average $26 \pm 15\%$ higher in CHAR soils (p-val. < 0.05) (Table 1).

364 *Table 1 – Main bulk soil physico-chemical parameters. Means \pm SD (n=5). When followed by * results are significantly different*
 365 *(p.val in text) according to a student paired T-test.*

	CHAR	REF
Sand (%)	7,7 \pm 0,3	7,5 \pm 0,4
Silt (%)	77,3 \pm 1,1	76,9 \pm 0,8
Clay (%)	15,0 \pm 1,0	15,5 \pm 0,7
C (%)	2,6 \pm 0,5 *	1,4 \pm 0,0
Norg (%)	2,3 \pm 0,2 *	1,4 \pm 0,1
C/N (-)	11,3 \pm 1,5	9,9 \pm 0,6
CEC (cmolc kg ⁻¹)	14,0 \pm 1,8	12,0 \pm 0,6
pH (H ₂ O)	7,7 \pm 0,2	7,8 \pm 0,2
pH (KCl)	7,0 \pm 0,3	7,1 \pm 0,2
P (mg 100g ⁻¹)	7,3 \pm 0,9	8,0 \pm 0,5
K (mg 100g ⁻¹)	21,4 \pm 2,9	18,2 \pm 1,1
Mg (mg 100g ⁻¹)	9,9 \pm 1,0	8,6 \pm 0,7
Ca (mg 100g ⁻¹)	314,4 \pm 39,1 *	251,6 \pm 31,7

366

367 # Table 1: Main bulk soil physico-chemical parameters.

368 4.2. Weight and Carbon distribution amongst fractions

369 In CHAR soils macroaggregates contributed significantly more (p-val. < 0.01) to total soil weight than
 370 microaggregates or free S&C fractions (CHAR_{MACRO} = 48.9 ± 12.8 , CHAR_{MICRO} = 25.5 ± 8.3 , CHAR_{Free S&C} =
 371 $25.6 \pm 4.6 \text{ g } 100 \text{ g}^{-1}$ soil). In reference soils, macroaggregate and microaggregate fractions were equal
 372 and accounted significantly more to total soil weight (p-val. < 0.01) than the free S&C fraction (REF_{MACRO} =
 373 41.3 ± 9.5 , REF_{MICRO} = 36.0 ± 8.7 , REF_{Free S&C} = $22.7 \pm 2.6 \text{ g } 100 \text{ g}^{-1}$ soil)(Figure 3).

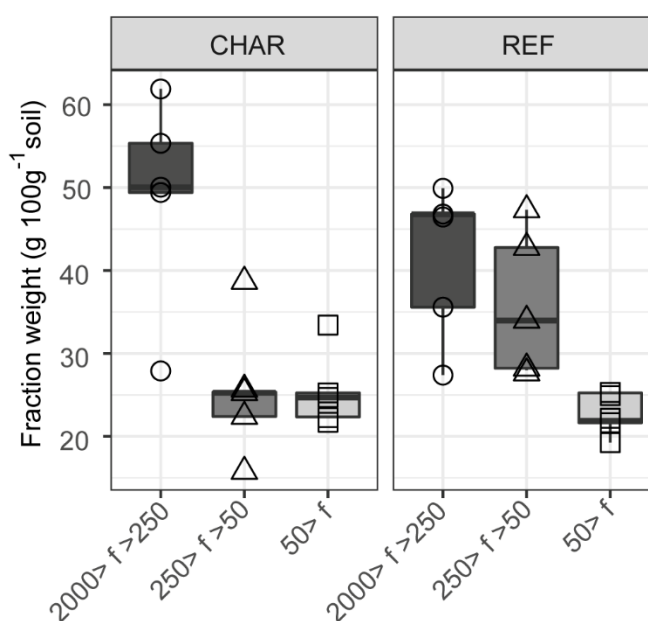
374 The weight recovery following the wet sieving procedure was of $99.4 \pm 0.3\%$ and $99.6 \pm 0.1\%$ for CHAR
 375 and REF respectively. For step 4 of our fractionation protocol, $93.6 \pm 4.8\%$ and $89.0 \pm 7.5\%$ of initial
 376 mass were recovered (CHAR and REF respectively). For this step the C content recovery was of $87.9 \pm$
 377 52.1% (CHAR) and $100.6 \pm 20.1\%$ (REF).

378 Macroaggregate-size free LF was the most abundant free LF in CHAR soils ($0.7 \pm 0.2\%$) and was also
 379 significantly more abundant (p-val. < 0.05) in CHAR soils than in REF soils (Table 2). Micro and S&C-size

380 free LF displayed similar contribution to the soil mass between the two studied soils (Table 2).
 381 Furthermore, free LF displayed systematically higher C contents (i.e., fraction C concentration multiplied
 382 by fraction mass) in CHAR soils than in REF soils (Table 2) due mainly to higher C concentrations (i.e.,
 383 mass of C per mass of the fraction). Macroaggregate size free LF of charcoal rich soils contributed the
 384 most to the C content compared to all other free LF studied soils for both treatments.

385 While no differences between studied soils were observed, occluded LF contributed more to the total
 386 soil weight than free LF (Table 2). In both studied soils, microaggregate-size fractions accounted the
 387 most to the total soil C content for both occluded LF and free LF. Microaggregate size occluded LF stored
 388 twice the amount of carbon than microaggregate-size free LF (Table 2).

389 # Figure 3: Size class distribution.



390
 391 *Figure 3 – Size class distribution. Weight distribution amongst fractions of different sizes following wet sieving of the studied*
 392 *soils. Points represent the 5 field replicates. Symbols and box shadings highlight different fraction sizes. Weight recoveries (mean*
 393 *± SD) are: CHAR= 99.4 ± 0.3 %, REF= 99.6 ± 0.1 %.*

394 The macroaggregate protected HF was significantly more abundant than microaggregate protected and
 395 free HF in charcoal rich soils. In reference soils, macroaggregate and microaggregate protected HF,
 396 which displayed the same contribution to the total soil mass, were more abundant than free HF (Table
 397 2). The C concentrations of HF ranged between 0.7 ± 0.2 to 0.8 ± 0.2 % in reference soils and from $0.9 \pm$
 398 0.1 to 1.0 ± 0.2 % in CHAR soils. In charcoal rich soils, macroaggregate protected HF (3.5 ± 1.2 g kg⁻¹ soil)
 399 contributed more to the total soil C content than microaggregate protected HF and free HF (2.2 ± 0.6 ,
 400 2.2 ± 0.3 g kg⁻¹ soil). In REF soils, macroaggregate and microaggregate protected HF accounted similarly
 401 to the total soil C content, which was a greater contribution than free HF (Table 2).

402 Summing all fractions regardless of size into either LF or HF showed that although HF accounted for
 403 most of the soil mass (CHAR=89.0 ± 2.2, REF= 86.5 ± 1.0 %), it merely accounted for 39.0 ± 25.0 % of the

404 recovered soil C in CHAR soils ($\text{CHAR}_{\text{HF}} = 7.9 \pm 1.4 \text{ g C kg}^{-1}$) and $48.0 \pm 23.0 \%$ in reference soils ($\text{REF}_{\text{HF}} =$
 405 $6.8 \pm 1.2 \text{ g C kg}^{-1}$). In charcoal rich soils, LF (free and occluded combined) comprised $12.2 \pm 3.1 \text{ g C kg}^{-1}$
 406 soil of which $6.8 \pm 3.0 \text{ g C kg}^{-1}$ soil occurred in occluded LF rather than free LF ($5.4 \pm 0.7 \text{ g C kg}^{-1}$ soil). In
 407 reference soils, LF combined comprised $7.5 \pm 1.5 \text{ g C kg}^{-1}$ soil, mainly as occluded LF ($\text{REF}_{\text{occ.LF}} = 4.7 \pm 1.3 \text{ g}$
 408 C kg^{-1} soil) rather than as free LF ($\text{REF}_{\text{free.LF}} = 2.8 \pm 0.7 \text{ g C kg}^{-1}$ soil). C concentrations of free LF increased
 409 with aggregate size and ranged between $25.6 \pm na$ and $41.9 \pm 7.4 \%$ for charcoal rich soils and between
 410 10.0 ± 2.9 and $36.7 \pm 10.1 \%$ in reference. Microaggregate occluded LF had higher C concentrations for
 411 both treatments ($\text{CHAR} = 31.1 \pm 5.4$, $\text{REF} = 30.3 \pm 4.2 \%$) than macroaggregate occluded LF ($\text{CHAR} = 26.1$
 412 ± 2.4 , $\text{REF} = 17.7 \pm 3.4 \%$) or S&C-size occluded LF ($\text{CHAR} = 24.4 \pm 4.2$, $\text{REF} = 21.3 \pm 2.4 \%$)(Table 2).

413 *Table 2 – Elemental composition of fractions. Mass, C & N concentration, C & N content, C:N ratio and PyOM content (mean \pm*
 414 *SD) distribution amongst fractions as a function of studied soils (CHAR, REF), size and density (LF, HF). Free LF, occluded (occ.) LF*
 415 *and HF were isolated from the three major aggregate size classes. Different letters indicate significant differences ($p \leq 0.05$)*
 416 *between studied soils as a function of fraction. “na” is indicated when not enough fraction mass was recovered for a complete*
 417 *set of analysis. Weight recovery (mean \pm SD) CHAR= $93.6 \pm 4.8 \%$, REF= $89.0 \pm 7.5 \%$. C content recovery CHAR = $87.9 \pm 52.1 \%$,*
 418 *REF = $100.6 \pm 20.1 \%$.*

frac.	soil	size (μm)	mass (g kg^{-1} soil)	C (%)	Ntot (%)	C (g kg^{-1} soil)	N (mg kg^{-1} soil)	C:N (-)	PyOM (g kg^{-1} soil)
free LF	CHAR	2000-250	6,7 \pm 2,0 A	41,9 \pm 7,4 A	15,3 \pm 2,8 A	2,7 \pm 0,4 A	98,4 \pm 17,7 A	27,5 \pm 3,0 A	1,1 \pm 0,7
		250-50	4,2 \pm 1,5 B	36,5 \pm 4,5 A	14,6 \pm 1,4 AB	1,7 \pm 0,5 B	67,2 \pm 18,4 B	25,0 \pm 1,5 A	0,3 \pm 0,1
		< 50	3,8 \pm 3,3 B	25,6 \pm na	11,8 \pm 2,8 B	1,0 \pm na	49,5 \pm 0,0 BCD	21,5 \pm na	0,1 \pm na
	REF	2000-250	3,9 \pm 0,7 B	36,7 \pm 10,1 A	14,8 \pm 1,2 AB	1,5 \pm 0,6 BC	58,2 \pm 10,3 BC	25,0 \pm 7,6 A	
		250-50	2,6 \pm 0,8 B	34,9 \pm 3,9 AB	15,5 \pm 2,9 A	1,0 \pm 0,3 CD	42,6 \pm 16,0 CD	22,8 \pm 2,5 A	
		< 50	3,6 \pm 1,8 B	10,0 \pm 2,9 C	6,5 \pm 2,2 C	0,3 \pm 0,2 D	22,3 \pm 12,0 D	15,7 \pm 1,8 B	
occ. LF	CHAR	2000-250	7,8 \pm 6,4 FG	26,1 \pm 2,4 FG	17,9 \pm 1,6 GH	2,1 \pm 1,8 FG	142,4 \pm 117,6 FG	14,6 \pm 0,8 FG	1,2 \pm na
		250-50	11,1 \pm 6,7 F	31,1 \pm 5,4 F	20,2 \pm 4,7 FG	3,8 \pm 2,3 F	247,1 \pm 149,6 FG	15,7 \pm 1,5 F	0,2 \pm 0,0
		< 50	4,2 \pm 0,9 FG	24,4 \pm 4,2 G	15,3 \pm 1,6 H	1,0 \pm 0,4 G	63,3 \pm 18,3 G	15,8 \pm 1,6 F	0,5 \pm 0,2
	REF	2000-250	5,9 \pm 2,4 FG	17,7 \pm 3,4 H	13,7 \pm 3,1 H	1,0 \pm 0,5 G	82,0 \pm 41,4 G	13,1 \pm 1,4 GH	
		250-50	9,1 \pm 1,9 FG	30,3 \pm 4,2 F	22,2 \pm 4,4 F	2,7 \pm 0,8 FG	200,2 \pm 60,3 FG	13,9 \pm 1,4 GH	
		< 50	4,1 \pm 3,5 G	21,3 \pm 2,4 GH	17,1 \pm 1,9 GH	0,9 \pm 1,0 G	70,1 \pm 72,9 G	12,4 \pm 0,7 H	
HF	CHAR	2000-250	414,2 \pm 127,6 J	0,9 \pm 0,2 JK	0,9 \pm 0,3 J	3,5 \pm 1,2 J	403,6 \pm 228,8 J	9,8 \pm 2,4 K	0,3 \pm 0,2
		250-50	221,6 \pm 70,4 L	1,0 \pm 0,2 J	0,8 \pm 0,2 JKL	2,2 \pm 0,6 KL	168,6 \pm 33,4 KL	13,1 \pm 3,2 JK	0,2 \pm 0,1
		< 50	252,1 \pm 56,1 JK	0,9 \pm 0,1 JK	0,7 \pm 0,2 JKL	2,2 \pm 0,3 KL	183,5 \pm 100,7 KL	13,9 \pm 3,3 J	0,4 \pm 0,1
	REF	2000-250	337,5 \pm 80,5 JK	0,8 \pm 0,2 K	0,9 \pm 0,4 JK	2,7 \pm 1,0 JK	306,6 \pm 168,9 JK	10,0 \pm 2,8 K	
		250-50	310,7 \pm 79,6 JKL	0,8 \pm 0,1 JK	0,6 \pm 0,1 KL	2,6 \pm 0,7 JK	178,3 \pm 43,5 KL	14,5 \pm 2,2 J	
		< 50	217,1 \pm 25,0 L	0,7 \pm 0,2 K	0,5 \pm 0,2 L	1,5 \pm 0,3 L	109,3 \pm 25,8 L	14,0 \pm 1,2 J	
hand-picked PyOM			37,8 \pm 7,9		5,4 \pm 1,7		76,6 \pm 27,4		
fresh PyOM			79,6 \pm 0,8		8,8 \pm 0,6		90,6 \pm 6,9		

419

420

Table 2: Elemental composition of fractions.

421

4.3. Differential Scanning Calorimetry

422

423

424

425

426

Three reference materials and two bulk soils were analyzed to allow for the discrimination of various forms of OM (Figure 4). Three distinct exotherms characterized hand-picked PyOM (top-Figure 4) with a major peak at $443 \pm 4 \text{ }^\circ\text{C}$. Fresh PyOM was characterized by a single exotherm ($485 \pm 2 \text{ }^\circ\text{C}$) and a higher punctual heat flow. Hand-picked non-PyOM (crop residues) were characterized by a strong first exotherm occurring at $350 \pm 2 \text{ }^\circ\text{C}$ and a second smaller at $430 \pm 1 \text{ }^\circ\text{C}$.

427

428

429

The total heat flow of CHAR bulk soils were on average $84 \pm 59 \%$ greater than REF bulk soils (Table 4). CHAR bulk soils were characterized by four exotherms occurring at temperatures similar to those occurring in hand-picked PyOM and hand-picked non-PyOM combined (top-Figure 4). Similarly,

430 exotherms of reference bulk soils occurred at similar temperatures to those which characterized hand-
431 picked non-PyOM.

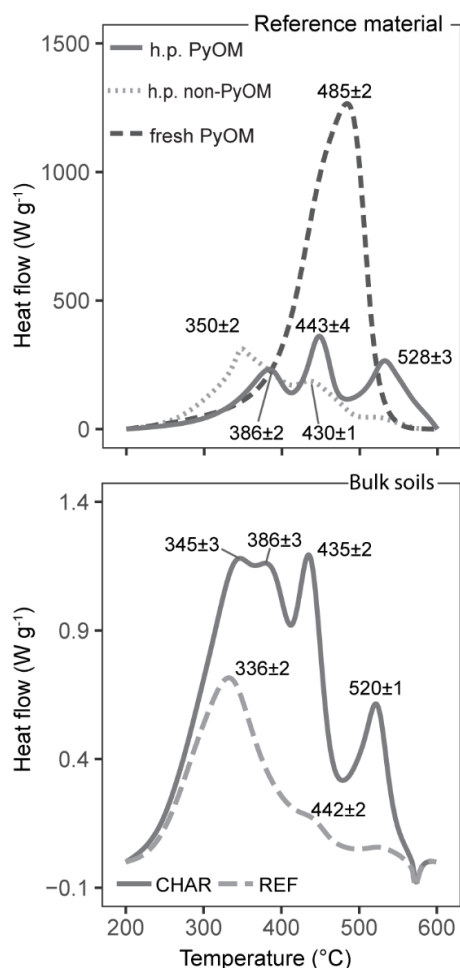
432 Figure 5 illustrates the thermograms of the three main types of fractions addressed in this study, the
433 free LF, occluded LF and HF (respectively row A, B, C - Figure 5) as a function of the aggregate size class
434 in which they occur (col. 1: macroaggregate, col. 2: microaggregate, col. 3: free S&C-size - Figure 5).

435 Thermograms of the free LF (Figure 5 - A) showed more pronounced exotherms occurring between 400-
436 500 °C for CHAR than REF fractions whereas thermograms of REF fractions tended to be more spread
437 out throughout the thermal continuum than CHAR fractions. Nonetheless, 50 % burn-off temperatures
438 were not significantly smaller in REF than CHAR fractions (p -val. > 0.05). Finally, a decrease in total heat
439 flow as a function of fraction size was reported whereby heat flows of S&C-size free LF were significantly
440 smaller (CHAR = $232 \pm 60 \text{ J g}^{-1}$, REF = $172 \pm 30 \text{ J g}^{-1}$) than microaggregate-size free LF (CHAR = $665 \pm 254 \text{ J g}^{-1}$,
441 REF = $753 \pm 160 \text{ J g}^{-1}$), and macroaggregate-size free LF (CHAR = $821 \pm 185 \text{ J g}^{-1}$, REF = $645 \pm 262 \text{ J g}^{-1}$)
442 regardless of the studied soil (p -val. < 0.01). However, total heat flows are equal between fractions when
443 normalized by their C content.

444 The occluded LF (Figure 5 - B) were characterized by two distinct exotherms visible as individual peaks or
445 marked shoulders occurring between 325-342 °C and 430-480 °C for both studied soils (Table 4). In
446 CHAR soils, the second exotherm (430-480 °C) had a higher relative importance as shown by the
447 increase of thermally stable:total peaks ratios by 42.6 % in macroaggregate occluded-LF, 44.3 % in
448 microaggregate occluded-LF and 50.0 % in S&C-size occluded-LF compared to REF soils (Table 4). No
449 differences in 50 % burn-off temperatures were noted between studied soils within a size class yet
450 macroaggregate protected occluded LF have significantly smaller 50 % burn-off temperatures than
451 microaggregate protected or S&C occluded LF (p -val. < 0.05) (Table 4).

452

Figure 4: DSC reference curves.



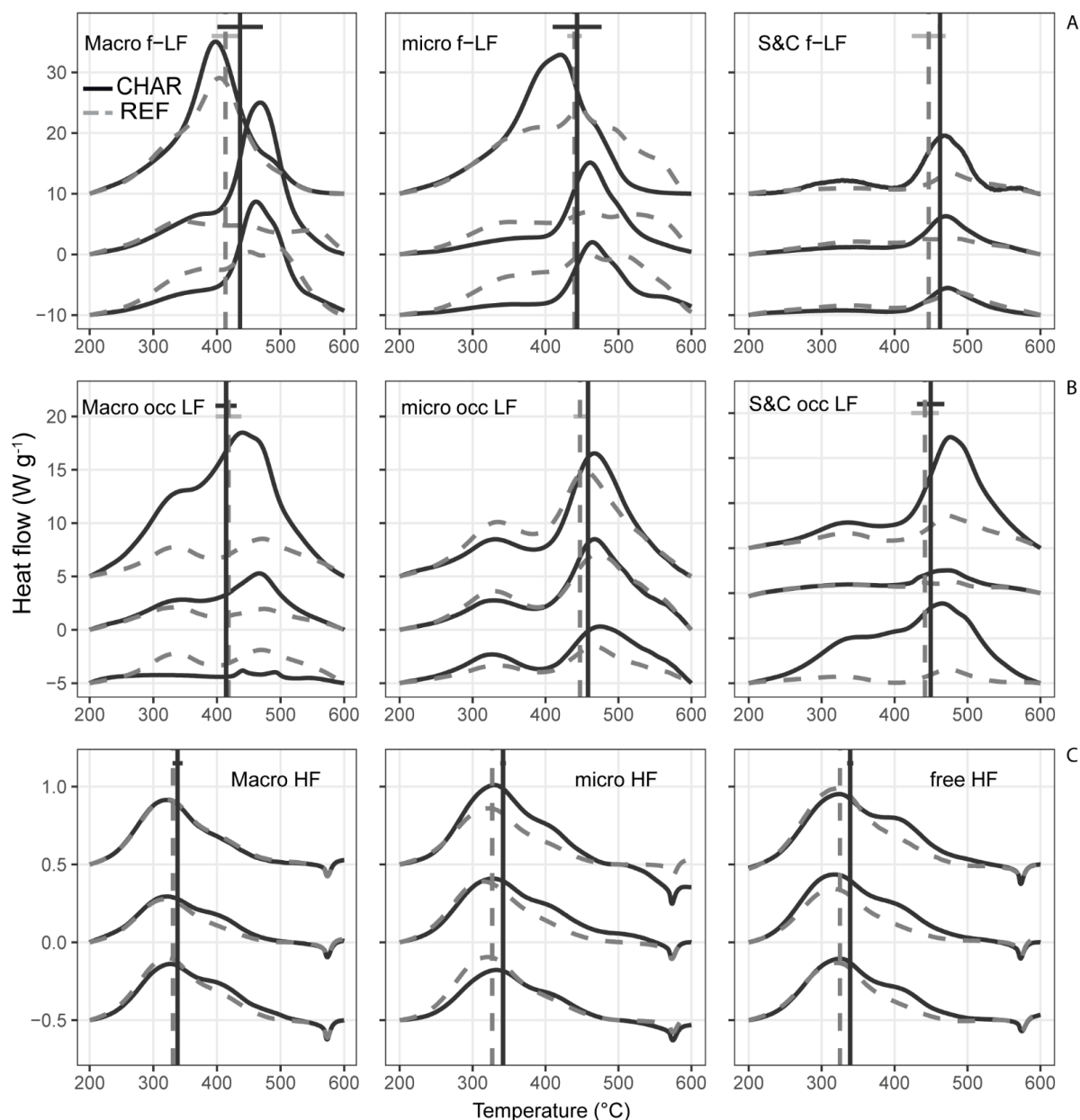
453

454 *Figure 4 – DSC reference curves. DSC thermograms of reference material for the characterization of C forms. Top panel:*
 455 *compares three types of OM forms, hand-picked (abbreviated h.p) non-PyOM, hand-picked PyOM and fresh PyOM. Bottom*
 456 *panel: compares CHAR and REF bulk soils.*

457 HF thermograms were characterized by a main exotherm occurring for both studied soils at ~ 323 °C.
 458 CHAR fractions were differentiated by a marked shoulder in the DSC curves occurring at ~ 405 °C.
 459 Thermograms of HF (Figure 5 C) were more systematic between repetitions as highlighted by the
 460 average heat flow. The average heat flow occurring in HF was of 187.1 ± 42.3 J g⁻¹ for CHAR soils and
 461 147.5 ± 31.5 J g⁻¹ for REF soils where the standard deviation represented respectively 22.5 and 21.3 % of
 462 the total heat flow. In occluded LF the average total heat flow was of 356.1 ± 159.0 J g⁻¹ for CHAR and
 463 242.8 ± 147.1 J g⁻¹ for REF soils with the standard deviation representing 44.6 and 60.6 % for CHAR and
 464 REF soils. In free LF, it represented 55.0 % for CHAR samples and 55.5 % in reference samples (CHAR_{free LF}
 465 $= 495.2 \pm 272.6$ J g⁻¹, REF_{free LF} $= 523.8 \pm 291.228$ J g⁻¹). For HF, 50 % burn-off temperatures were always
 466 significantly higher for charcoal rich than reference soils (p-val. < 0.01) in all size classes and notably
 467 smaller than in LFs (Table 4).

468 Finally, thermograms differed between hand-picked reference materials (Figure 4) and fractions isolated
 469 by the fractionation protocol (Figure 5). Indeed the first ($\sim 386 \pm 3$ °C) and last (~ 520 - 528 °C) exotherms
 470 of hand-picked PyOM (Figure 4) were not found in soil fraction thermograms (Figure 5).

471 # Figure 5: DSC signatures of soil fractions.

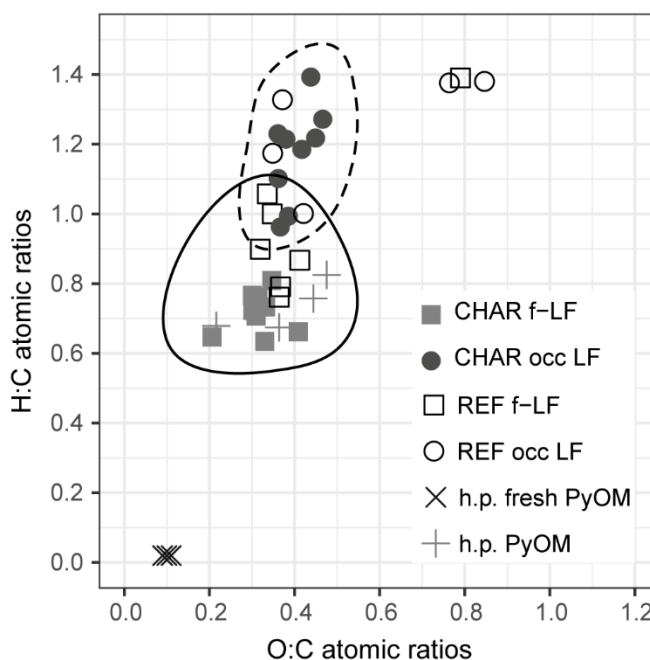


472
 473 *Figure 5 - DSC signatures of soil fractions. Graphs are presented as: free LF (A - top row) either macroaggregate-size (2000-250*
 474 *µm), microaggregate-size (250-50 µm) or silt and clay size (50 > µm). Middle (B) and bottom (C) rows show occluded LF or HF of*
 475 *silt and clay size, either protected in macroaggregates or microaggregates or as free material. In each graph the three replicas*
 476 *were represented, each one was plotted by pair with the adjacent samples of the opposite studied soil (CHAR/REF). Curves were*
 477 *vertically shifted to facilitate the comparison between replicas. Vertical lines show the mean (n=3) 50 % burn-off temperature*
 478 *per fraction (REF=dashed line, CHAR=solid line) and the horizontal line at the top of each graph represents the standard*
 479 *deviation (REF= light grey, CHAR= dark grey).*

480 4.4. Elemental analyses

481 Aged hand-picked PyOM displayed much higher H:C (0.70 ± 0.10) and O:C (0.40 ± 0.10) atomic ratios
482 than fresh PyOM (H:C = 0.02 ± 0.00 ; O:C = 0.10 ± 0.00) (Table 3). Differences in H:C and O:C ratios were
483 greater between soil fractions (free or occluded) than between CHAR and REF soils (Figure 6). Indeed,
484 free LF had lower ratios than occluded LF for both studied soils (Table 3). However, S&C-size free LF had
485 higher H:C and O:C ratios in REF soils than in CHAR soils. For occluded LF, differences in H:C ratios
486 between studied soils were not significant, and only O:C ratios of macroaggregate-size occluded LF were
487 greater in REF fractions than in CHAR fractions.

488 # Figure 6: Van Krevelen diagram of fractions.



489
490 *Figure 6 - Van Krevelen diagram of fractions. Results show H:C and O:C atomic ratios of LF isolated by the fractionation protocol.*
491 *Squares illustrate free-LF (f-LF, encircled by solid line) and circles illustrate occluded LF (occ-LF, encircled by dashed line). The*
492 *point color depends on studied soils, white= REF, grey= CHAR. Crosses show ratios for fresh PyOM or hand-picked PyOM.*

493 H:C atomic ratios increased from free LF, to occluded LF and HF for both studied soils (Table 3). For
494 microaggregate protected and free HF, H:C ratios were higher in REF (microaggregate protected HF: 3.22
495 ± 0.34 , free HF: 4.24 ± 0.39) than CHAR soils (microaggregate protected HF: 2.71 ± 0.15 ; free HF: $3.44 \pm$
496 0.43).

497 Inversely, C:N atomic ratios decreased from free LF ($[15.7 \pm 1.8 - 27.5 \pm 3.0]$), to occluded LF ($[12.4 \pm 0.7$
498 $- 15.8 \pm 1.6]$) and HF ($[9.8 \pm 2.4 - 14.5 \pm 2.2]$). In free LF, C:N ratios decreased as a function of size while
499 their values were similar in occluded LF of the three size classes. Furthermore, C:N ratios of HF were
500 lower in macroaggregate protected HF than in microaggregate protected and free HF materials (p-val. <
501 0.05), for both studied soils (Table 3).

502 Table 3 - H:C and O:C atomic ratios of soil fractions. Either free LF, occluded LF and HF as a function of treatment and of the size
 503 classes in which they were contained. Different letters indicate significant differences ($p \leq 0.05$) between studied soils as a
 504 function of fraction. "na" indicated that not enough fraction material was recovered for a complete set of analysis. No O:C ratios
 505 were measured for mineral dominated fractions.

frac.	soil	size (μm)	H:C (-)		O:C (-)	
free LF	CHAR	2000-250	0,69 \pm 0,11	C	0,30 \pm 0,10	A
		250-50	0,72 \pm 0,06	C	0,31 \pm 0,00	A
		< 50	0,67 \pm	na	0,41 \pm	na
	REF	2000-250	0,95 \pm 0,20	B	0,34 \pm 0,00	A
		250-50	0,75 \pm 0,13	C	0,41 \pm 0,00	A
		< 50	1,55 \pm 0,11	A	1,30 \pm 0,50	B
occ. LF	CHAR	2000-250	1,24 \pm 0,12	F	0,39 \pm 0,04	G
		250-50	1,19 \pm 0,20	FG	0,44 \pm 0,03	G
		< 50	1,01 \pm 0,12	G	0,38 \pm 0,01	G
	REF	2000-250	1,32 \pm 0,20	F	0,85 \pm 0,00	F
		250-50	1,31 \pm 0,12	F	0,37 \pm 0,00	G
		< 50	1,18 \pm 0,14	FG	0,39 \pm 0,05	G
HF	CHAR	2000-250	3,14 \pm 0,37	KL		
		250-50	2,71 \pm 0,15	L		
		< 50	3,44 \pm 0,43	KL		
	REF	2000-250	3,58 \pm 0,50	K		
		250-50	3,22 \pm 0,39	K		
		< 50	4,24 \pm 0,39	J		
	hand-picked PyOM		0,70 \pm 0,10		0,40 \pm 0,10	
	fresh PyOM		0,02 \pm 0,00		0,10 \pm 0,00	

506

507 **# Table 3: H:C and O:C atomic ratios of soil fractions.**

508 4.5. Combining analytical practices

509 Amongst the different fractions of both studied soils, H:C atomic ratios decreased (Table 3) alongside an
 510 increase in stable:total heat flow ratios (Table 4). HF (i.e., S&C minerals) were characterized by most of
 511 the heat flow occurring at low temperatures as opposed to LF. While H:C ratios were similar between
 512 studied soils, HF of CHAR soils displayed higher stable:total heat flows than HF of REF soils (CHAR = 24.0
 513 \pm 3.0, REF = 16.1 \pm 2.5 %). On average in CHAR soils, free LF showed lower H:C and higher stable:total
 514 ratios (H:C = 0.69 \pm 0.09; stable:total = 76.6 \pm 11.1 %) than for occluded LF (H:C = 1.14 \pm 0.16;
 515 stable:total = 68.2 \pm 7.8 %) whereas in REF soils, average H:C ratios for occluded fractions were lower
 516 than for free LF (occ LF= 1.27 \pm 0.15, free LF= 1.08 \pm 0.44) while stable:total ratios were similar (occ LF=
 517 63.2 \pm 4.8, free LF= 64.1 \pm 6.2 %)(Table 4)

518 4.6. PyOM and non-PyOM quantification in charcoal rich soils

519 Charcoal rich bulk soils contained between 5.8 and 11.9 g kg⁻¹ soil of PyOM. This amounts for between
 520 29.9 and 41.3 % of the total SOC in bulk CHAR soils. In free LF, the PyOM accounted between 13.7 and
 521 41.2 % of the total SOC in these fraction. Most of the PyOM was found as macroaggregate-size free LF
 522 and accounted for up to 1.1 ± 0.7 g kg⁻¹ soil (Table 2). Similar amounts of PyOM were found in occluded
 523 LF and accounted between 5.4 and 55.9 % of total SOC contained in occluded fractions. HF contained
 524 between 0.2 ± 0.1 and 0.4 ± 0.1 g kg⁻¹ soil of PyOM. This represented between 7.3 and 16.2 % of the SOC
 525 contained in HF and was highest for free HF (0.4 ± 0.1 g kg⁻¹ soil).

526 A higher heat of reaction occurring at thermally labile temperatures in CHAR soils than REF soils show
 527 that differences in C content did not solely occur as thermally stable OM but also as labile PyOM or non-
 528 PyOM. Bulk charcoal rich soils accounted 0.4 to 2.3 g C kg⁻¹ soil of additional thermally labile OM
 529 compared to reference soils. This represented between 5.8 and 16.2 % of the additional C content of
 530 CHAR soils. The combined heat flows of occluded LF were on average 38.7 % higher in CHAR than REF
 531 fractions (CHAR_{occ} = 9.8 ± 4.3 W g⁻¹, REF_{occ} = 7.1 ± 1.8 W g⁻¹) although non-significantly different due to
 532 the variability between CHAR repetitions. However, an increase in thermally labile peak height was not
 533 systematic between samples and hence not statistically significant for occluded LF (p-val. > 0.05) nor HF
 534 (p-val. > 0.05).

535 *Table 4 - DSC curve characteristics of soil fractions. 50 % burn-off T, peak T and heat flows of visible exotherms as well as ratios*
 536 *between labile:total and stable:total heat flow (mean ± SD) are represented followed by results of analysis of variance. For a*
 537 *given fraction (frac.) identical letters indicate equal means.*

frac.	soil	size (µm)	50% Burnoff T (°C)	exotherm 1		exotherm 2		Labile : Total Heat flow (%)	Stable : Total Heat flow (%)							
				peak T (°C)	Heat flow (W g ⁻¹)	peak T (°C)	Heat flow (W g ⁻¹)									
free LF	CHAR	2000-250	436,3 ± 35,9	AB			442,3 ± 38,6	A	22,9 ± 3,7	A	28,5 ± 15,2	BC	72,0 ± 14,9	AB		
		250-50	443,3 ± 33,5	AB			448,7 ± 24,0	A	16,7 ± 5,6	AB	24,2 ± 13,3	C	76,2 ± 13,0	A		
		< 50	462,3 ± 3,1	A			471,0 ± 2,0	A	6,8 ± 2,6	C	22,6 ± 1,0	C	77,5 ± 1,0	A		
	REF	2000-250	413,5 ± 21,9	B			442,3 ± 38,6	A	11,0 ± 7,3	BC	41,2 ± 7,5	B	59,3 ± 7,4	B		
		250-50	439,8 ± 9,9	AB			454,0 ± 6,2	A	10,5 ± 3,5	BC	32,4 ± 2,4	BC	67,9 ± 2,4	AB		
		< 50	446,8 ± 23,2	AB			483,0 ± 4,4	A	3,2 ± 0,6	C	34,0 ± 6,2	BC	66,2 ± 6,2	AB		
occ. LF	CHAR	2000-250	414,3 ± 16,6	G	335,0 ± 1,4	F	3,8 ± 3,7	F	449,7 ± 15,0	G	6,7 ± 6,3	F	40,8 ± 6,7	FG	59,5 ± 6,8	GH
		250-50	458,3 ± 2,0	F	328,7 ± 1,5	H	3,0 ± 0,4	F	469,7 ± 4,6	F	8,5 ± 3,1	F	26,6 ± 2,8	H	73,6 ± 2,8	F
		< 50	449,5 ± 18,8	F	342,0 ± 0,0	F	3,0 ± 2,1	F	472,3 ± 5,7	F	7,9 ± 5,0	F	28,7 ± 7,0	H	71,5 ± 6,9	F
	REF	2000-250	418,0 ± 20,2	G	335,0 ± 1,4	F	2,5 ± 0,4	F	471,7 ± 1,2	F	2,9 ± 0,8	F	42,8 ± 5,4	F	57,5 ± 5,4	H
		250-50	447,3 ± 8,8	F	332,0 ± 4,0	G	3,5 ± 1,7	F	464,3 ± 8,3	F	6,8 ± 3,2	F	31,7 ± 1,5	GH	68,5 ± 1,5	FG
		< 50	441,7 ± 18,9	FG	342,0 ± 0,0	F	1,1 ± 0,5	F	475,7 ± 4,0	F	2,1 ± 1,3	F	38,8 ± 6,2	FG	61,4 ± 6,2	GH
HF	CHAR	2000-250	338,2 ± 7,8	JK	324,0 ± 1,7	K	0,4 ± 0,1	J	405,0 ± 0,0		0,2 ± 0,0	JKL	76,9 ± 4,6	KL	23,5 ± 4,6	JK
		250-50	341,8 ± 3,8	J	329,7 ± 2,1	J	0,4 ± 0,1	J	405,0 ± 0,0		0,2 ± 0,0	JK	77,0 ± 4,2	KL	23,4 ± 4,2	JK
		< 50	339,2 ± 4,2	J	322,0 ± 4,4	K	0,4 ± 0,0	J	405,0 ± 0,0		0,2 ± 0,0	J	75,3 ± 2,2	L	25,1 ± 2,2	J
	REF	2000-250	330,8 ± 3,4	KL	322,3 ± 1,5	K	0,4 ± 0,1	J	405,0 ± 0,0		0,2 ± 0,0	KL	81,7 ± 2,7	JK	18,6 ± 2,7	KL
		250-50	327,0 ± 2,6	KL	319,0 ± 3,6	K	0,4 ± 0,0	J	405,0 ± 0,0		0,1 ± 0,0	L	85,3 ± 1,2	J	15,1 ± 1,2	L
		< 50	325,2 ± 2,6	KL	319,3 ± 3,8	K	0,4 ± 0,1	J	405,0 ± 0,0		0,1 ± 0,0	L	85,6 ± 1,6	J	14,7 ± 1,6	L

538

539

Table 4: DSC curve characteristics of soil fractions.

540 5. Discussion

541 5.1. Physico-chemical characteristics of charcoal rich soils

542 Our results show close to a twofold increase in C contents as a result of century-old PyOM accumulation
543 in cultivated soils as previously reported for similar sites (Hernandez-Soriano et al., 2016b; Kerré et al.,
544 2016; Hardy et al., 2017a). As both studied soils were sampled in the same field, they have received
545 similar amendments and field works in identical climatic conditions. We can therefore be confident such
546 an increase in C content is induced by the presence of PyOM and corresponds to increases in both PyOM
547 and non-PyOM contents in kiln soils. Accumulation of non-PyOM in kiln soils have been previously
548 shown to originate from a decreased mineralization rates of recent non-PyOM (Hernandez-Soriano et
549 al., 2016a; Kerré et al., 2016) and from the adsorption of dissolved organic compounds onto charcoal
550 particles (Hernandez-Soriano et al., 2016b; Kerré et al., 2017). A relative greater aboveground biomass
551 production on kiln soils (Heidarian Dehkordi et al., 2020) may also be responsible for a positive feedback
552 loop through an increase in non-PyOM inputs to the charcoal rich soils.

553 Previous studies on similar kiln sites in Wallonia reported significant increases in CEC related to the
554 presence of charcoal (Kerré et al., 2016; Hardy et al., 2017a). Our results show slight yet non-statistically
555 significant increases. Furthermore, higher CEC has been estimated for century-old PyOM fragments
556 (PyOM = 414 cmol_c kg⁻¹) compared to non-PyOM (non-PyOM = 213 cmol_c kg⁻¹) explained by an increase in
557 the amounts of functional groups with charcoal ageing and cultivation combined with the high specific
558 surface area of PyOM (Hardy et al., 2017a). Additionally, CHAR soils displayed greater bioavailable Ca²⁺
559 contents than adjacent REF soils as previously reported (Hardy et al., 2017a, 2017b). Increased Ca²⁺
560 contents in CHAR soils result from the high affinity between Ca²⁺ and carboxylate groups found on aged
561 PyOM thus potentially serving as a mediator for PyOM interaction with soil constituents (Kalinichev and
562 Kirkpatrick, 2007).

563 5.2. Element C:N, H:C and O:C ratios support charcoal-induced organo-mineral associations

564 The presence of PyOM in charcoal enriched soils resulted in a greater macroaggregate formation at the
565 expense of free microaggregates in comparison to reference soils. Macroaggregate turnover is known to
566 be relatively rapid particularly in cropped ecosystems where tillage damages soil structure (Six et al.,
567 2000; Chenu et al., 2019). In CHAR soils, where macroaggregates are more abundant than in REF soils, it
568 is likely that PyOM acts as an additional binding agent between soils constituents and microaggregates
569 (Six et al., 2000). Furthermore, a larger macroaggregate fraction in CHAR soils may also result from
570 higher aggregate stability (Pituello et al., 2018) caused by increased inter-particle cohesion (Sun and
571 Lu, 2014) or faster aggregate formation as a result of the specific surface area of PyOM. Century-old
572 PyOM therefore improves soil aggregation which is a key property for soil structure, essential in
573 maintaining soil fertility and which may also further increase the stability of OM through increased
574 physical disconnection with decomposers (Schmidt et al., 2011; Lehmann and Kleber, 2015).

575 Our results showed significant differences in O:C and H:C ratios between free and occluded LF for both
576 studied soils thereby showing that the position of PyOM and non-PyOM in the soil matrix and their
577 chemical composition are strongly linked. Lower O:C and H:C atomic ratios suggest a greater abundance
578 of large aromatic structures in free LF than in occluded LF whereas the latter may be enriched in either

579 PyOM with more functional entities such as carboxyl groups or additional non-PyOM (Brodowski et al.,
580 2005; Cheng et al., 2008a).

581 Long-term residence of PyOM in soils results in higher O:C and H:C atomic ratios (Cheng et al., 2008a;
582 Nguyen et al., 2008; Hardy et al., 2017b) compared to fresh PyOM (Hardy et al. 2017b). Ageing of PyOM
583 is known to occur mainly through oxidation of the particles and results in the development of phenol,
584 carboxyl and carbonyl functional groups on the PyOM aromatic structures (Cheng et al., 2008a).
585 Although free LF are highly weathered compared to fresh PyOM, their lower H:C and O:C ratios in
586 comparison to occluded LF suggests free LF may be less oxidized than occluded LF. In turn, this higher
587 functionalization of PyOM in occluded LF compared to free LF supports its increased reactivity towards
588 surrounding soil constituents. As all the PyOM in CHAR soils is of similar age range and as most of the
589 oxidation occurs rapidly following the addition of PyOM to the soil, it is expected that particles would
590 have faced similar oxidative agents (Nguyen et al., 2008). Therefore, differences in O:C and H:C ratios
591 between free and occluded LF may originate from products with varying initial properties. Indeed, slight
592 changes in pyrolysis conditions throughout the kiln during charcoal production may result in varying
593 degrees of aromaticity or aromatic condensation (B. P. Singh et al., 2012; Wiedemeier et al., 2015)
594 resulting in different degrees of hydroxylation and carboxylation (Lehmann et al., 2005) and in turn
595 leading to charcoal fragments of varying persistence. However, it may be that when first produced, the
596 PyOM was physically and chemically homogeneous in composition and that, with time and farming
597 practices, the PyOM fragments were broken down to different size classes which resulted in different
598 degrees of oxidation. In addition, higher H:C and O:C atomic ratios of occluded LF than free LF in
599 charcoal enriched soils may also originate from higher amounts of non-PyOM such as microbial related
600 biomass (Lehmann et al., 2011) or stabilized fresh non-PyOM residues (Hernandez-Soriano et al., 2016a).
601 In HF, differences in H:C and O:C ratios result either from mineral phases or changes in OM. However, as
602 texture and mineralogy are similar throughout the study site, differences in H:C and O:C ratios between
603 studied soils are mainly attributed to the presence of PyOM. Lower H:C ratios in HF of charcoal enriched
604 soils than in reference soils demonstrate the presence of PyOM (C-rich) associated with minerals
605 through S&C-size microaggregation or adsorption.

606 Similar C:N ratios between studied soils, despite generally higher C content in CHAR, indicate that larger
607 amounts of N occur in charcoal enriched soils than in adjacent REF soils. This may result from
608 significantly higher nitrate contents retained by the biochar (Hagemann et al., 2017) or N-rich organic
609 compounds derived from root and microbial activities (Brodowski et al., 2005; Meier et al., 2017).
610 Indeed, microbial-derived proteins and root exudates are known to strongly bind to minerals with
611 possibilities for their polar chains to serve as binding sites (Kleber et al., 2007; Vogel et al., 2014; Poirier
612 et al., 2018) and to interact with PyOM (Brodowski et al., 2005). In HF this is supported by C:N ratios
613 similar to those expected for microbial-derived OM sorbed onto mineral surfaces ranging [7-14]
614 (Aufdenkampe et al., 2001; Bell et al., 2014).

615 5.3. Thermal analysis confirms organo-mineral associations

616 The presence of charcoal in the bulk soil of kiln sites is distinguished from reference soils by three
617 distinct exotherms (Figure 4). These exotherms occur at higher temperatures than in reference
618 thermograms and correspond to peak temperatures characterizing hand-picked charcoal particles

619 (Hardy et al., 2017a). At first, fresh PyOM is characterized by a single sharp exotherm. With time, as the
620 PyOM particle oxidizes from its surface inwards (Brodowski et al., 2005; Cheng et al., 2008a) the
621 aromatic structures are broken down and functional groups appear (Plante et al., 2009). The occurrence
622 of these functional groups decreases the thermal stability of PyOM as shown by the occurrence of a new
623 exotherm at ~ 386 °C (Figure 4), which corresponds to the combustion of O-rich compounds (Hardy et al.,
624 2017b). Hardy et al. (2017b) suggested that Ca^{2+} acted as a thermal catalyst thereby resulting in a
625 decrease of thermal stability of functional groups visible in thermograms as a decrease in peak
626 temperatures towards more thermally labile OM. Interestingly, hand-picked PyOM is characterized by
627 the occurrence of a third peak (~ 528 °C) with higher thermal stability (Figure 4) than fresh PyOM as
628 previously reported by Hardy et al. (2017b). In this study authors showed an increase in this peak height
629 with time of cultivation thereby suggesting that amounts of highly thermally stable C increase relatively
630 to other, less stable forms which are preferentially altered or lost from the system. The third type of OM
631 used as a reference curve is hand-picked non-PyOM which is characterized by the occurrence of both
632 thermally labile (336 ± 2 °C) and thermally stable (442 ± 2 °C) OM (Figure 4). These peak temperatures
633 correspond to those of cellulose and lignin like molecules (Lopez-Capel et al., 2005). The occurrence of
634 these exotherms in both studied soils highlight that PyOM in soils can be determined as the additional
635 heat flow occurring at thermally stable temperatures in CHAR as opposed to REF soils.

636 In both studied soils, LF (i.e. occ. or free) are characterized by higher thermal stability than HF. Free LF in
637 CHAR soils are characterized by a punctual heat flow comparable to that of pristine fresh PyOM which
638 suggests little functionalization of PyOM particles in these fractions. Such a recalcitrance to oxidation for
639 CHAR free LF is explained by a greater aromaticity or degree of condensation of the PyOM (Wiedemeier
640 et al., 2015). Thermograms of REF free LF on the other hand show more diversity in the type of OM
641 composing the fraction as shown by a multitude of peaks. In agricultural soils, after biomass inputs, it
642 may be expected that free LF would be quickly deprived of small and energy rich forms of OM due to
643 rapid mineralization of thermally labile organic compounds more beneficial to decomposers (Leifeld,
644 2008; Rovira et al., 2008; Barré et al., 2016). As a result, following the preferential decomposition of
645 energy rich OM, free LF in CHAR soils are expected to be dominated by larger more persistent molecules
646 with higher binding energy and hence to be thermally more stable (Leinweber et al., 1992; Lopez-Capel
647 et al., 2005; De la Rosa et al., 2008). This is supported by higher 50 % burn-off temperatures in free LF
648 compared to other fractions (Leifeld, 2008)(Figure 5).

649 Occluded LF differ from free LF through the appearance of a second class of exotherms occurring around
650 ~ 330 °C (Figure 5). The occurrence of this thermally labile peak suggests an accumulation of energy-rich
651 OM resulting from physical inaccessibility of this OM to decomposers and organo-mineral associations
652 (Lehmann and Kleber, 2015). In occluded LF of REF soils significant heat flows occur at high thermal
653 stability. These exotherms coincide with peak temperatures attributed to PyOM (Figure 5) which
654 complicates a definite distinction between PyOM and non-PyOM (Leifeld, 2007). Such exotherms
655 highlight the importance of studying CHAR soils in comparison to thermograms of REF soils to determine
656 the PyOM pool as the additional heat flow occurring in CHAR soils. In CHAR occluded LF the presence of
657 PyOM is mainly underlined by a slightly higher 50 % burn-off temperatures (Figure 5) and higher
658 stable:total heat flow ratios (Table 4).

659 In HF the presence of an elbow occurring at $\sim 410^\circ\text{C}$ in CHAR and not in REF soils strongly suggests
660 the presence of PyOM tightly associated with minerals. Indeed, although these fractions are dominated
661 by thermally labile OM (Figure 5 - C - peaks $\sim 330^\circ\text{C}$), the sustained presence of PyOM in fractions
662 dominated by microaggregation and adsorption suggests that century-old charcoal displays strong
663 interactions with soil minerals (Chenu and Plante, 2006; Virto et al., 2010). The decrease in peak
664 temperatures of PyOM originates from the creation of phenol, carbonyl and carboxyl functionalities at
665 the surface of century-old charcoal particles (Hardy et al., 2017a). Our results support this assumption as
666 this peak value in HF corresponding to PyOM ($\sim 410^\circ\text{C}$) occurs between the two first peaks of hand-
667 picked PyOM ($p1 = 386 \pm 2^\circ\text{C}$, $p2 = 443 \pm 4^\circ\text{C}$). Furthermore, because functional groups are needed for
668 sorption to occur (Kleber et al., 2015) it is expected that PyOM particles tightly bound to mineral phases
669 have been strongly oxidized through either biotic or abiotic processes (Cheng et al., 2006). As
670 microaggregation and adsorption are the main stabilization mechanisms for OM in soils, it is expected
671 that beyond its chemical recalcitrance, PyOM is further stabilized by organo-mineral associations (von
672 Lützow et al., 2006; Dungait et al., 2012; Lehmann and Kleber, 2015) yet that this additional physical
673 protection is not discernable by thermal analyses (Plante et al., 2011).

674 5.4. A major part of PyOM is associated with mineral phases

675 DSC results show PyOM amounts for an important part of the total SOC [29.9 - 41.3 %] in bulk charcoal
676 rich soils. Due to the variability in chemical composition of PyOM no single method can correctly
677 quantify it (Lorenz and Lal, 2014; Kerré et al., 2016). Our methodology consisted in estimating the
678 amount of PyOM by comparing peak height ratios between CHAR and adjacent REF soil with respect to
679 differences in C content and is therefore not a direct quantification. Furthermore, our quantification
680 approach is developed on the assumption that no PyOM is found in REF soils. However, it may be
681 expected that small amounts of PyOM could be found in reference soils suggesting PyOM amounts are
682 slightly underestimated in charcoal rich soils. Using standard additions of PyOM to mineral soils free of
683 PyOM, Hardy et al., (2017a) reported that charcoal-C content in kiln sites amounts for 79.5 % of the
684 difference in C content between CHAR soils and REF soils. Using this relationship on our charcoal rich
685 soils we obtain an estimate of PyOM content ranging [25.3 – 39.2 %], slightly lower than our estimate
686 [29.9 - 41.3 %]. Using DSC on isolated soil fractions enabled an understanding of the distribution of
687 PyOM amongst soil fractions. Results show that after two centuries of residence in soils, PyOM is
688 located in all soil fractions and that, although a large amount occurred as free LF ($43.6 \pm 22.9\%$ of total
689 PyOM), the majority interacts with mineral phases ($56.4 \pm 22.9\%$ of total PyOM) through occlusion (29.6
690 $\pm 28.8\%$ of total PyOM) or sorption ($26.78 \pm 5.9\%$ of total PyOM) to mineral phases (Table 2).

691 Despite decades of incorporation in the soil matrix and sustained farming practices, results show that a
692 large amount of PyOM is still found as free LF in CHAR soils. This result illustrates the importance of
693 PyOM composition for its stability in soils and the role of charring against oxidative agents through its
694 polycondensed aromatic structure (Wiedemeier et al., 2015) and against decomposers by making it a
695 hardly decomposable form of OM (Rovira et al., 2008; Wang et al., 2016). Nonetheless, in the presence
696 of specific decomposers, PyOM may decompose faster than often reported (Hamer et al., 2004). Recent
697 studies show that the persistence of PyOM in soils may be overestimated (Hammes et al., 2008; N. Singh
698 et al., 2012; Lehndorff et al., 2014; Lutfalla et al., 2017). Nonetheless, given the high temperatures and

699 low oxygen contents found in kiln sites, it may be expected that the stability of the charcoal produced in
700 such environment would be higher than wildfire residues (Bird et al., 2015). In the studied CHAR soils,
701 the presence of PyOM amongst occluded LF and OM in the HF shows that its stability is not exclusively
702 explained by its chemical structure but further enhanced by a decreased accessibility for
703 microorganisms (Brodowski et al., 2006; Lützow et al., 2006). Charcoal-mineral associations likely
704 increase with PyOM ageing through increasing proportions of functional groups on the charcoal surfaces
705 as well as the occurrence of biotic or abiotic binding agents (Brodowski et al., 2006; Kögel-Knabner et al.,
706 2008; Kopittke et al., 2020). Finally, our results show increased amounts of non-PyOM in charcoal rich
707 soils which suggests PyOM may influence the stability of non-PyOM in soils by serving as a hotspot for its
708 sorption onto already existing organo-mineral clusters (Kopittke et al., 2020) or by creating
709 microaggregates in which it becomes physically inaccessible to decomposers.

710 **6. Conclusion**

711 Our work combined for the first time thermal and elemental analyses on soil fractions extracted from a
712 unique setting where PyOM from kiln sites was incorporated in conventionally cropped systems for over
713 two centuries.

714 The C content of CHAR soils was significantly higher than that of adjacent REF soils thus confirming the
715 potential of PyOM as a tool for C storage over centuries. Furthermore, our results show that such
716 increases in C content were not solely attributed to increases in PyOM content, but also to additional
717 non-PyOM accumulation in CHAR soils. The presence of PyOM in all soil fractions showed that after
718 centuries of incorporation in strongly anthropogenized environments PyOM still stands as free LF yet
719 also strongly associates with mineral phases. This association occurs through occlusion into aggregates
720 and sorption of PyOM onto mineral surfaces. Compared to fresh PyOM, century-old PyOM isolated from
721 charcoal rich soils showed higher H:C and O:C atomic ratios particularly in occluded LF vouching for a
722 functionalization of the PyOM. As such its reactivity with other soil constituents increases as suggested
723 by increased Ca^{2+} concentrations in CHAR soils. Furthermore, similar C:N ratios in CHAR and REF
724 fractions suggest increased amounts of N-rich organic products in charcoal rich soils which probably
725 serve as binding agents between PyOM and mineral phases. As a result of more binding sites, we
726 observed increased amounts of water stable macroaggregate in CHAR soils. The related improved soil
727 structure in charcoal rich soils is fundamental in maintaining fundamental ecosystems services such as
728 diminished erosion and enhanced plant primary production.

729 Our results highlight that beyond its chemical composition, PyOM is further stabilized by organo-mineral
730 associations and can not only serve as a C sequestration tool but also as a mean to improve the soil
731 quality of conventionally cropped agroecosystems in temperate Luvisols.

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