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(Article begins on next page)

1 **Land use effects on the stabilization of organic matter in soils: combining size**
2 **fractionation with sequential chemical extractions**

3

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22

Abstract

The long-term stabilization of organic matter in mineral soils is widely believed to be mainly driven by its interaction with the mineral matrix rather than by its recalcitrance. This interaction involves several processes, and the relative importance of each one may vary according to land use. Here we study organo-mineral interactions in soils under different land uses (coniferous and broadleaved forest, grasslands, and technosols), widely differing in their soil organic matter (SOM) content, by an approach involving two consecutive steps: (i) a size fractionation by wet sieving after sonication, to isolate the particulate organic matter (POM, >20 μm) from the organo-mineral complex (OMC, <20 μm), and (ii) a characterization of the OMC by sequential extractions with different chemicals, each one disrupting a specific kind of bond between SOM and active mineral surfaces. The OMC accounted for most of the SOM, i.e., >60% of total organic C (TOC) and >75% of total N. Differences in land uses affected the total amount of SOM, but barely its distribution between POM and OMC. The sequential extractions showed that a substantial amount of the organic matter in the OMC (20-30%) was weakly bound to minerals. Other components of the OMC, in contrast, were less dominant than expected: the organic matter stabilized by carbonate coating (5-6% of total OC in the OMC) or by iron oxyhydroxydes (3-6%). By far, the main pool of the OMC was the final residue remaining after all extractions (34-48% of total OC in the OMC). Because this residue remains unextractable with NaOH after removing all the active mineral components, such unextractability can be due only to its own chemical characteristics. Future research should aim at its in-depth characterization and help elucidating its ecological functions.

45

46 **Introduction**

47 Soils represent the largest terrestrial organic carbon (OC) reservoir (Jobbágy and Jackson,
48 2000; Schmidt et al., 2011), and a major challenge for soil scientists is to clarify the dynamics
49 of soil carbon in the context of climate change (Davidson et al., 2000; Amundson et al., 2015;
50 Doetterl et al., 2018). Modelling primary drivers and mechanisms of OC stability requires a
51 clear understanding of the mechanisms responsible for soil organic matter (SOM) physical-
52 chemical features, its build-up, its location within the mineral matrix, and its long-term
53 persistence across different soils and ecosystem types.

54 The physical protection of SOM and its association to active minerals (fine silt and
55 clays, free forms of iron and aluminium, carbonates) are widely recognized as the main driver
56 for its long-term persistence in mineral soils (Kaiser and Guggenberger, 2000; Kögel-
57 Knabner et al., 2008; Schmidt et al., 2011; Lehmann and Kleber, 2015). Physical
58 fractionations based on particle-size, density or a combination of both have been widely used
59 to isolate functional SOM pools (e.g., Cambardella and Elliott, 1993; Golchin et al., 1994;
60 Amelung et al., 1998; Christensen, 2001; Plaza et al., 2013). The size fractions (sand, silt and
61 clay-sized, all of them containing both mineral particles and organic matter) represent the
62 primary organo-mineral units; the secondary structures are the aggregates built from the
63 former (Christensen, 2001). SOM turnover strongly depends on the size fraction: fast in
64 coarse fractions (Balesdent, 1996), and much slower in fine fractions (fine silt and clay-
65 sized), for which the longest turnover times have been suggested (von Lützwow et al., 2006;
66 Basile-Doelsch et al., 2007).

67 The diversity of rationale, goals and mechanistic theories about SOM formation,
68 stabilization and turnover has led to the application of different fractionation protocols, which
69 have in turn hampered comparisons among studies (Poeplau et al., 2018). However, it seems

70 clear that the distribution of SOM among the several size fractions affects its stability; as
71 shown in laboratory incubation experiments, the higher the proportion of total SOM in the
72 finest fractions (particularly the <20 μm : fine silt and clay), the higher its overall stability
73 (Rovira et al., 2010).

74 The size fractionation of SOM seems, therefore, a first and obvious step to study its
75 stabilization. However, an in-depth characterization of SOM in the finest fractions must
76 follow, because these fractions usually account for most SOM, particularly in soil layers
77 below 15-cm depth (Rovira et al., 2010). It is in the finest fractions where chemical
78 interactions between SOM and active mineral surfaces mostly occur. These interactions are
79 of several kinds: slight electrostatic adsorptions, association to clay surfaces through metal
80 bonding, strong adsorptions to fine silt and especially clay surfaces, or association to iron
81 (Fe) and aluminium (Al) free forms (Kögel-Knabner et al., 2008). In order to pursue an
82 understanding of SOM stabilization, we need to go beyond the simple fact of its association
83 to mineral particles (i.e., generation of the organo-mineral complex), and investigate the
84 relative importance of each kind of association between the organic and the mineral
85 component of such a complex.

86 The 'ideal' chemical extractant for SOM should perform a complete, massive, non-
87 selective SOM extraction, without any generation of chemical artefacts (Stevenson, 1994). Of
88 course, such an extractant does not exist. To date, sodium hydroxide (NaOH) extraction
89 under inert atmosphere (Ar or N₂) is the closest available proxy for such a chemical
90 extractant (Stevenson, 1994). Well on the contrary, 'non-ideal', non-massive, selective
91 extractants are of particular interest for our work. Sodium tetraborate (NaTB henceforth) or
92 sodium pyrophosphate (NaPP) have been used as mild extractors to obtain specific SOM
93 fractions, associated to silt and clay surfaces by mild bonds or metal bridges (Bruckert, 1979;

94 Schnitzer and Schuppli 1989), and the sequence NaTB - NaPP - NaOH was proposed for
95 SOM fractionation in the organo-mineral complex (Bruckert and Kilbertus, 1980). González-
96 Prieto et al. (1989) described an extended version of that method, which involved a further
97 extraction with NaOH after reduction of Fe oxides with dithionite, and another after
98 destruction of silicates with hydrofluoric acid (HF). The soil organo-mineral fractionation
99 (SOF) method (Lopez-Sangil and Rovira, 2013) still introduced two steps, a preliminary
100 extraction with a neutral saline solution of potassium sulfate to extract soluble or easily
101 solubilizable compounds, and a mild attack with acid to destroy carbonate coatings, which is
102 essential for carbonate-rich soils, common in the Mediterranean basin. In such a way, a quite
103 extensive characterization of the organo-mineral complex can be achieved. It is to note that,
104 in our context, 'characterization' must be meant not in the sense of an in-depth study of its
105 chemical structure – which has been argued to change during alkaline extraction (Kleber and
106 Johnson, 2010) – but in the sense of establishing the quantitative relevance of each kind of
107 physico-chemical bond between organic matter and the mineral surfaces.

108 The aim of this paper was to attain a comprehensive view of SOM stabilization in
109 representative Mediterranean soils. To do so, we applied the two-step approach described
110 above: (a) first, a size fractionation, to isolate and quantify the organo-mineral complex, plus
111 (b) the detailed fractionation of organic matter in the organo-mineral complex using the
112 sequential extraction method of Lopez-Sangil and Rovira (2013). To attain such a
113 comprehensive view, we took into account that the roles of vegetation, litter inputs and parent
114 material in SOM stocks formation and persistence have shown to be crucial (e.g.,
115 Guggenberger et al., 1994, 1995; Leifeld and Kögel-Knabner, 2005; Clemente et al., 2011;
116 Catoni et al., 2016), and thus we used a set of soil samples under a variety of land uses and
117 over a variety of geological substrates.

118

119 **2. Materials and methods**

120 *2.1. Site selection and soil samples collection*

121 Four land uses were compared in this study: coniferous forests (CF), broadleaved
122 forests (BF), grassland soils (GL) and technosols (TS), all of them located in the Marche
123 region (Italy). Here, the term 'land use' must be meant in a broad sense, because technosols
124 are a soil type, rather than a definite land use.

125 The coniferous forests were *Pinus nigra* woodlands occurring between 350 and 1400
126 m a.s.l. The broadleaved forests were Orno-Ostryetum associations with *Quercus pubescens*
127 growing between 320 and 1300 m a.s.l. Grassland soils were under meadows and pastures
128 covered by meso- to xerophilous species (e.g., *Bromus erectus*) and *Medicago sativa*, located
129 between 890 and 1700 m a.s.l. Finally, technosols were all placed below 300 m a.s.l., and
130 included (i) two open-pit mines, filled and then reclaimed, one with *Helianthus annuus* since
131 2010, the other with *Triticum* sp. since 2000; (ii) two dumps, reclaimed in 2000 and in 2011;
132 and (iii) a site of disposal of compost and other non-hazardous waste materials. Mean annual
133 temperature of sampled sites ranged from 7.3 and 15.0 °C, and mean annual precipitation
134 between 810 and 1570 mm. More details about these sites are reported elsewhere (Giannetta
135 et al., 2018).

136 Five different sites for each land use were sampled between May and June 2016. At
137 each site, depending on site surface and homogeneity, 3-10 topsoil samples (generally, 0-25
138 cm depth, although in some places soil thickness was just 10 cm) were collected randomly
139 from several points separated 50 to 100 cm apart using a soil auger, and mixed to obtain a
140 composite sample. In these samples, soil texture (sand, silt and clay contents) was obtained
141 by the pipette method; pH in H₂O, at a soil-to-water ratio of 1:2.5; electrical conductivity

142 (EC) in water extracts at proportion soil-to-water 1:5; OC by dry combustion, after
143 elimination of carbonates by acid fumigation; total N by dry combustion; and carbonate
144 content by the calcimeter method. Soil properties averaged by ecosystem type are reported in
145 Table 2.

146

147 *2.2. Physical fractionation of SOM*

148 Size fractionation was performed by wet sieving, splitting soil particles into four
149 different size fractions: coarse sand (CSa: 2000-200 μm), fine sand (FSa: 200-50 μm), coarse
150 silt (CSi: 50-20 μm), and fine silt plus clay (FSCL: <20 μm) (Lopez-Sangil and Rovira,
151 2013).

152 To do that, soil samples (15 g), air-dried and sieved at 2 mm, were put into 50 mL
153 vials, which were filled with deionized water to 3/4 of their volume. Samples were then
154 subjected to rotatory agitation (20 rpm) for 60 minutes, and to further ultrasonic dispersion
155 for 10 minutes (100W output) using a Branson 45 sonifier. The derived suspensions were
156 immediately wet-sieved through a set of three sieves (200 μm , 50 μm and 20 μm mesh), thus
157 splitting particles into four different size fractions. The fractions retained by sieves were
158 directly recovered from them, quantitatively transferred to pre-weighted flasks and dried at
159 60 °C to constant weight. The particles passing through the last sieve (<20 μm : FSCL) were
160 brought to about 1 L with water. Two mL of saturated solution of potassium alum were
161 added, and the suspension was left to stand refrigerated for 2 days, until complete
162 sedimentation of the solid particles. The overlying water was siphoned off and discarded. The
163 sediment was recovered by centrifugation (15 min at 2500 g), transferred to a pre-weighted
164 flask and dried at 60 °C to constant weight.

165 While in the coarse fractions ($>20\ \mu\text{m}$) organic matter occurs in the form of dark
166 particles, clearly distinguishable from mineral ones, below the $20\ \mu\text{m}$ threshold both
167 components, organic and mineral, are no longer visually distinguishable. Following Lopez-
168 Sangil and Rovira (2013), SOM in fractions $>20\ \mu\text{m}$ was taken as particulate organic matter
169 (POM), whereas the $<20\ \mu\text{m}$ fraction was taken as the organo-mineral complex (OMC). The
170 OMC was subjected to the SOF fractionation procedure, as described below.

171
172 *2.3. Chemical fractionation of the organo-mineral complex: SOF procedure*

173 The method has been thoroughly described by Lopez-Sangil and Rovira (2013), and
174 thus only the essential details are given here (Fig. 1). Briefly, the OMC was submitted to the
175 following sequence of extractions:

- 176 1) 0.5 M potassium sulfate (K_2SO_4), to extract water-soluble compounds;
- 177 2) 0.1 M NaTB ($\text{Na}_2\text{B}_4\text{O}_7$), at pH 9.7, to extract compounds adsorbed to mineral
178 surfaces by Van der Waals- or weak electrostatic bonds;
- 179 3) 0.1 M NaPP ($\text{Na}_4\text{P}_2\text{O}_7$), at pH 9.8, to extract compounds precipitated or stabilized
180 by coordination complexes with metals (Ca, Mg, Fe, Al);
- 181 4) 0.1 M NaOH, at pH >12 , to extract compounds strongly associated to mineral
182 surfaces;
- 183 5) mild attack with 0.33 M sulfuric acid (H_2SO_4) at $5\ ^\circ\text{C}$, followed by 0.1 M NaOH, to
184 extract compounds occluded by carbonate coatings;
- 185 6) reduction with 20 mL 0.1 M sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), followed by 0.1 M
186 NaOH, to extract compounds stabilized by free Fe^{3+} forms, previously made soluble through
187 their reduction to Fe^{2+} ;

188 7) attack with cold 8 M hydrofluoric acid (HF), followed by 0.1 M NaOH, to extract
189 compounds associated to clays by strong bonds, resistant to all the previous steps.

190 The remaining pellet after all previous steps was taken as humin.

191 All SOF extracts were brought up to a fixed volume, and stored at 4 °C until OC and
192 N analyses. The remaining residue (humin) was dried at 60 °C, weighed, and stored at room
193 temperature until OC and total N analyses.

194 195 *2.4. Organic C and total N analyses*

196 Total C and N contents of SOM fractions obtained after physical fractionation were
197 determined by dry combustion using a Thermo Flash 2000 NC Analyzer. Before analysis, an
198 aliquot of each sample was ground with a ball mill. For OC determination, the aliquots were
199 subjected to acid fumigation before the analysis to remove carbonates (Harris et al., 2001).

200 Steps 5-7 involved two parts. First, the extract obtained with the pre-treatment
201 (sulfuric acid, dithionite, hydrofluoric acid); next, that obtained with 0.1 M NaOH. While the
202 second one was analyzed for OC and N in all cases, in step 5 the first extract (H₂SO₄) could
203 not be analyzed for total N, and in step 7 the first extract (HF) was discarded and thus could
204 not be analyzed neither for OC nor for total N. The reason was in both cases that the presence
205 of a solvent (H₂SO₄, HF) is strongly harmful for elemental analyzers. Thus, extracted OC in
206 step 7, and extracted N in steps 5 and 7, are underestimated. Leaving aside these exceptions,
207 in the SOF extracts OC was quantified colorimetrically by dichromate oxidation (Nelson and
208 Sommers, 1996), and total N in a ThermoQuest CHN analyzer, by evaporation of an aliquot
209 of each extract into tin capsules over a hot plate at 60 °C. In the first extract of step 6
210 (dithionite) OC was quantified in the CHN analyzer, because of the interference of dithionite,
211 a strong reductor, with the dichromate oxidation.

212 In each of the steps 5-7 two consecutive extracts were obtained, and the analyses of
213 OC and N were performed for each one separately. Nevertheless, here we consider both
214 extracts altogether, for it is the sum of both extracts what covers the whole organic matter
215 that becomes soluble after the blocking obstacle (carbonates, oxyhydroxydes, the clay
216 herself) has been removed by the pre-treatment.

217

218 2.5. Statistical analysis

219 All studied variables (i.e., the amounts of OC and N in the whole soils, in the size
220 fractions, and in the obtained SOF fractions) were compared by one-way ANOVA, taking
221 land use as a factor. For such analyses, data were log-transformed. Analyses were done with
222 SPSS version 19. Within a given ANOVA, differences between the several land uses were
223 tested by the Duncan's test. Effects were deemed as significant when $p \leq 0.05$.

224 In order to understand how the fractions become replenished as the soil accumulates
225 OC or N, we studied by linear regression the relationships between (i) the OC and N content
226 of each size fraction, *versus* that of the whole soil sample, and (ii) the OC and N content of
227 each SOF fraction, *versus* that of the whole OMC. Polynomial equations of either 1st or 2nd
228 order without intercept (i.e., $Y = b_1X + b_2X^2$) were fitted using SigmaPlot v.10. Figure 2
229 shows the main kinds of dynamics we expect to find in such analysis: the amount of OC in a
230 given fraction is expected to increase together with the total OC in the soil sample, either
231 without any apparent limit (curve 1), either showing clear signs of an apparent upper limit
232 (saturation of the fraction: curve 2), whose detection will be one of the focus of our work. In
233 addition to these two main curves, we may found that the amount of OC in the fraction is
234 apparently independent from the amount of OC accumulated in the soil (curve 3).

235

236 3. Results

237 3.1. Carbon and nitrogen in bulk soils

238 The OC and total N contents of the studied soils span a broad range (Table 2). Briefly,
239 GL have the highest average contents of OC (8.6%) and total N (0.9%), and TS the lowest
240 (1.3 and 0.2%, respectively). Both OC and N average contents rank as GL > CF > BF > TS,
241 thus mirroring the link between OC and N dynamics, also evidenced by the linear
242 relationship between OC and N contents (Fig. 3). Specific land use effects are also visible in
243 the OC/N ratios, which rank as CF > BF > GL > TS (Table 2).

244

245 3.2. Carbon and nitrogen in the size fractions

246 The recovery of OC in the size fractions (i.e., the sum of all fractions) averaged
247 around 96.2 ± 16.2 % of total OC in the whole sample, and that of N around 79.7 ± 8.8 %.

248 Both OC and N contents in each fraction are highest in GL soils (for all fractions),
249 whereas they tend to be lowest in TS, except for the coarsest fraction (2000-200 μm) (Table
250 3). Overall, OC and N contents follow the trend GL > CF > BF > TS.

251 The OMC accounts for 63 (in CF) to 77% (in GL) of total OC, and for 80% of total N
252 (Table 4). Whereas in all coarse fractions (>20 μm : POM) the total OC content is higher than
253 in the <20 μm fraction (OMC), the opposite is found for total N. The OMC is by far the main
254 sink for N-rich compounds; this role is the underlying reason for their OC/N ratios, the lowest
255 among all (Table 3).

256 The OC and N contents of the fractions and those of the whole soil samples are
257 positively and significantly correlated (Fig. 4). The significance of these correlations is higher
258 the smaller the size of the fraction, and is comparatively higher for N than for OC (Fig. 4).
259 This also suggests that the differences in OC and N content in the fractions merely reflect the

260 richness in OC and N in the whole soil sample, rather than a specific effect of the land use.
261 Even though the relationships were often curvilinear rather than strictly linear, all them
262 clearly match in concept the curve 1 in Fig. 2; no signs of OC nor N saturation were detected
263 for any size fraction.

264

265 *3.3. Organic C in the organo-mineral complex: SOF fractionation*

266 The recovery of OC in the SOF fractions (sum of OC in all fractions, as % of the
267 initial OC in the OMC) was on average 89.2 ± 11.6 %. The HF hydrolysate, which was not
268 analyzed for OC, partly explains this loss; however, C losses may also be attributed to the
269 repeated NaOH extractions, which may result in decarboxylations in spite of being all carried
270 out under N₂.

271 Table 5 summarizes OC amounts in each SOF fraction, both in absolute terms (mg
272 OC per g of OMC) and in relative terms (% of the total recovered OC). As expected, the
273 absolute amounts reflect the SOM richness of each land use: for each fraction, the amount of
274 OC follows the order $GL > CF \sim BF > TS$. The differences between land uses are significant
275 in all cases except one, the HF-NaOH fraction, in which the absolute amount of OC in HF-
276 NaOH fraction is notably homogeneous among the four land uses (1-2 mg OC per g OMC).

277 For all land uses, the highest OC amounts are found in the humin, which accounts for
278 34 to 48% of total OC in the OMC. Thus, in all cases a substantial part of SOM in the OMC
279 remains insoluble even after clay minerals, bond-making metals, carbonates, Fe and Al
280 oxides (precipitating factors) have been removed.

281 When translated to percentages over the total OC in the OMC (Table 5), some of the
282 differences between land uses disappear, while significant differences are still found for most
283 SOF fractions. Thus, in GL the OC associated to mineral matter through weak bonds (NaTB

284 fraction) is abundant, while the soluble fraction (K_2SO_4 extract) and especially the fraction
285 directly associated to clays through very strong bonds (HF-NaOH) are scarce. We note that in
286 GL such a low relative prevalence is due to the abundance of the other fractions; the absolute
287 amount of OC in this fraction is actually similar to that of other land uses (Table 5).
288 Moreover, another specific land-use effect is the relevance of the first NaOH extract in TS,
289 about 4% of total OC, thus much lower than that of other land uses.

290 Values in Table 5 are affected by the total richness of the soils in OC. The amount of
291 OC in most SOF fractions increases with the total OC in the OMC, but the increase is not
292 homogeneous in all fractions (Fig. 5). Two fractions, the K_2SO_4 extract and the humin, seem
293 to have an upper limit. For the K_2SO_4 extract, at about 2 mg OC per g OMC; for the humin,
294 at about 28-30 mg OC per g OMC, even though the later seem highly variable, and could be
295 related to land use, since most points at the saturation zone are from GL. In two fractions
296 (Dithionite-NaOH and HF-NaOH), the relationship between OC in the fraction and total OC
297 in the OMC seems to match curve 3 in Fig. 2; that is, the amount of OC does not seem related
298 to that of the whole OMC. In the HF-NaOH fraction the OC remains consistently low for all
299 samples, always below 3 mg OC per gram OMC.

300 For four SOC fractions (NaTB, NaPP, NaOH, and H_2SO_4 -NaOH), the OC content
301 consistently increases with the total OC in the OMC. For NaTB-extracted, NaPP-extracted,
302 and NaOH SOM fractions the relationships are strong and best described by growing curves,
303 meaning that the relative prevalence of these fractions increase with the total OC in the OMC.
304 In contrast, for the H_2SO_4 -NaOH fraction data dispersion is high, likely because the
305 accumulation of OC in this fraction depends on the amount of carbonates in fine silt and
306 clays, thus making OC in the fraction and in the OMC less directly related.

307

308 *3.4. Nitrogen in the organo-mineral complex*

309 The recovery of N in the SOF fractions (i.e., the sum of the total N in all SOF
310 fractions) was 66.5 ± 11.1 % of the total initial N in the OMC.

311 Humins account for >60 % of the OMC total N, and thus its dominance is higher than
312 for OC. The N extractable after HF treatment is minimal, i.e., <1% of total N on average. In
313 the sequence NaTB – NaPP – NaOH, the amount of OC extracted was roughly similar or
314 tended to increase; in contrast, the amount of extracted N clearly tends to decrease, being
315 lowest in the first NaOH extract. The NaOH extract contains always <10% of the total N on
316 average, and in most cases <5%. Thus, while this fraction is one of the most important for
317 OC, it is not for N. Another difference is that the extract obtained after dithionite treatment is
318 more relevant for N than for OC; in soils under grasslands and broadleaved forests, it
319 accounts for about 10% of total N.

320 As observed for OC, the amount of N in each SOF fraction relates to the total amount
321 of N in the OMC. The relationships are somewhat different for each SOF fraction (Fig. 6). In
322 fact, for the first four fractions, the relations are more significant ($p < 0.01$), whereas for the
323 H_2SO_4 -NaOH, the dithionite-NaOH and the HF-NaOH fractions a greater data dispersion was
324 observed. It is also noteworthy the extremely low amount of N in the HF-NaOH fraction,
325 apparently independent from the total N in the OMC.

326 Moreover, while an uppermost limit was clearly observed for OC in the K_2SO_4
327 extract, it is not observed for K_2SO_4 -extractable N. For N in the OMC, an uppermost limit
328 was observed only in one case, the humin, at about 2.5 mg N per g OMC. The same comment
329 given for humin OC can be applied here: this limit, that must be taken with caution, could be
330 due to land use, since most points in the saturation area are from GL.

331

332 **4. Discussion**

333 *4.1. Methodological constraints*

334 The recovery of N after both sieving and SOF fractionation was lower than that of
335 OC. Therefore, the results for N, both in size fractions and in SOF extracts, are prone to
336 larger biases than those for OC.

337 In the size fractionation, the ultrasonic treatment plus the extensive water leaching
338 during sieving are obvious reasons for both OC and N losses, for they imply that soluble or
339 easily solubilizable compounds are extracted massively. Nitrogen-rich compounds, including
340 amino acids, peptides, proteins, alkaloids, nucleotides, are more likely to be part of this labile
341 pool, whereas macromolecules such as lignin, suberin, cutins, lipid compounds, rich in C and
342 poor in N, remain largely insoluble and are likely to resist much better the ultrasonic plus
343 sieving treatment.

344 For the SOF method, the reasons for the low recovery of N compounds are different.
345 In the SOF method, all extractions are done quantitatively, without any losses of material. On
346 the other hand, we were not able to analyse some extracts for OC, N or both (as explained in
347 2.4), and thus their OC or N could not be accounted for in the recovery calculations. In
348 addition to these underestimations, the repeated NaOH extractions are likely to affect the
349 characteristics of the obtained fractions. In theory, at the pH values attained with a NaOH
350 solution (13 or more) the amino groups do not change their ionization status; therefore, amino
351 groups should be unresponsive to the alkaline treatment (Kleber and Johnson, 2010). But in
352 classical N fractionation methods (e.g., Pansu and Gautheyrou, 2006), the N of amino sugars
353 is obtained precisely by steam distillation after deamination by an alkaline reagent as
354 phosphate-borate buffer at pH 11.2. The SOF method does not include boiling with NaOH,
355 but it includes a NaOH extraction overnight repeated four times (steps 4, 5, 6 and 7), and

356 therefore deamination events cannot be discarded. This may explain why the relatively
357 amount of N recovered in the first NaOH extract was low (Table 6), whereas the OC extract
358 presented one of the major fractions (Table 5).

359

360 *4.2. Distribution of organic matter among the size fractions: land use effects*

361 A main result of our size fractionation work (summarized in Table 4) is the clear
362 dominance of the OMC in all land uses. This agrees with the results obtained by Giannetta et
363 al. (2018) using the same soils but with a different fractionation approach (Plaza et al., 2013).
364 It has been often claimed that the organic matter in coarse fractions (particulate organic
365 matter: POM) is more sensitive to differences in ecosystem types and land use changes
366 (Poeplau and Don, 2013). Since the organic matter accrual in large size fractions is mainly
367 determined by organic inputs and not by texture (Hassink, 1997), a change in land use from a
368 low C-input ecosystem to a high C-input one should logically affect mainly the coarse SOM
369 fractions.

370 Our results do not perfectly match this view. We found that the amount of organic
371 matter in fractions $>20\ \mu\text{m}$ (POM) is higher in forest- and grassland soils than in technosols
372 in absolute amounts, but not in relative terms (i.e., as % of the total organic matter). As
373 shown in Table 4, the distribution of organic matter between the four size fractions is similar
374 in TS, GL and BF. Only in CF soils, the coarsest POM fraction (2000-200 μm) is enriched in
375 OC, whereas the organo-mineral complex is depleted. We must stress that, except for CF, the
376 effects of land use were significant on the total amount of SOM but not on the distribution of
377 organic matter among fractions.

378 In our work, the CF land use become thus an easily distinguishable group. Coniferous
379 needles are rich in recalcitrant compounds (lignin, suberin, lipidic polymers) which confer to

380 litter a low decomposition rate and microbial use efficiency (Kooch et al., 2012; Cotrufo et
381 al., 2013), thus leading to a relatively higher OC accumulation in coarse POM fractions
382 (Castellano et al., 2015). The OC/N ratios of all size fractions are higher for CF than for the
383 other ecosystems (Table 3), consistently with previous reports (Guggenberger et al., 1994),
384 and also with the relatively low decomposition degree of SOM in coniferous forests
385 compared to other ecosystems (Castellano et al., 2015).

386 The results obtained for TS are noteworthy, in the sense that their behaviour is
387 surprisingly similar to that of highly mature ecosystems. In the current paradigm about the
388 filling of the several fractions, the accumulation of organic matter in soils involves its transfer
389 between fractions, in which fresh inputs predominantly accumulate in the coarsest fraction
390 (coarse sand size), pass both fine sand and coarse silt pools during the degradation
391 continuum, and eventually accrue in the organo-mineral complex (Baldock et al., 1992).
392 According to this paradigm, we should expect substantial differences between young and
393 poorly evolved soils (such as technosols) and more complex, SOM-rich and evolved systems,
394 such as soils under mature forests. Instead, in our TS, the distribution of OC and N between
395 particulate organic matter and the organo-mineral complex does not differ from that of GL or
396 BF (Table 4). Moreno-Barriga et al. (2017) studied the evolution of OC and N pools
397 following the addition of different materials to mine tailings, and concluded that easily-
398 available organic compounds triggered microbial growth during the first days of incubation
399 and enhanced the formation of aggregates, which in turn stabilize coarse debris. This
400 phenomenon, which could occur in most technosols, mimics early stages of pedogenesis
401 (Giannetta et al., 2018) and could make the SOM in technosols similar to that of much more
402 evolved soil systems.

403 Coarse fractions of SOM are mainly similar to plant litter (Guggenberger et al., 1994)
404 and are mainly plant debris in large pieces, not associated with minerals (Ladd et al., 1996).
405 In contrast, fine fractions, in particular clay, accumulate more evolved materials, depleted in
406 lignin and plant-derived components, and enriched in products of microbial origin (Paul and
407 Clark, 1989; Golchin et al., 1994; Kögel-Knabner et al., 2008; Plaza et al., 2013, 2016;
408 Zacccone et al., 2018). The decrease in OC/N ratios from coarse to fine fractions thus reflects
409 a change from a chemistry dominated by plant materials to the chemistry of microbial
410 biomass and metabolites.

411 Of particular interest is the distribution of N among the size fractions. The decrease in
412 the OC/N ratio is an indicator of humification, well described in classical books (Duchaufour,
413 1977); but in addition to this explanation for the low OC/N ratio of the organo-mineral
414 complex, observed in our dataset (Table 4), specific processes must also be accounted for. As
415 shown in a previous work by Rovira and Vallejo (2002), the split of plant-derived debris
416 between N-poor in the coarse fractions and N-rich in the fine fractions occurs from the very
417 beginning of the mixing of plant debris with a mineral, organic matter-depleted soil. Clay
418 surfaces, together with active mineral components such as Fe and Al oxyhydroxydes,
419 establish bonds with soluble organic matter, including nitrogenous compounds such as amino
420 acids, amino sugars, peptides or proteins, nucleic acids, partly plant-derived but also from
421 microbial origin (Ladd et al., 1996; Knicker, 2004; Sollins et al., 2006; Nannipieri and Paul,
422 2009; Paul, 2016; Zacccone et al., 2018). The large capacity of clays for stabilizing proteins
423 has been known for many years (Pinck et al., 1954; Harter and Stotzky, 1971; Kleber et al.,
424 2007). The effect of clays may be either through a direct stabilization of organic N (Ladd et
425 al., 1996; Nielsen et al., 2006; Sollins et al., 2006) and through the formation of aggregate-
426 protected particulate and non-particulate organic matter (e.g., Yoo and Wander, 2008), with

427 most of this organic N (>80%) being in the form of amides, peptides and proteins of
428 microbial origin (Simpson et al., 2007a). By contrast, the high OC/N ratio found in the POM
429 fractions suggests a relatively high occurrence of slightly-to-partially decomposed plant
430 materials in these fractions (Rasmussen et al., 2005).

431

432 *4.3. Organic matter in the organo-mineral complex*

433

434 *4.3.1. Humins*

435 By far, the last residue (humins *stricto sensu*, in our fractionation) is the most
436 important pool: in CF, it approaches 50% of the total OC in the organo-mineral complex.
437 Slightly lower values, always <35%, have been obtained by Lopez-Sangil and Rovira (2013),
438 but in their study the humins were still very abundant. This pool remains insoluble after the
439 long set of treatments and extractions, and in particular after the substantial destruction of the
440 mineral matrix by HF; therefore, its strong insolubility is not due to any association with the
441 mineral matter, but to its own biochemical composition.

442 In this regard, Simpson et al. (2007b) found that peptides, aliphatic species,
443 carbohydrates, peptidoglycan, and lignin, as macromolecules or aggregate species, are major
444 contributors of humins. They also found that microbial organic residues may account for
445 ~45% of the humins fraction in some soils (Simpson et al., 2007a). However, we must stress
446 that the existing literature about humins could be of little use, because the humins is by
447 definition the residue remaining after alkaline extractions, and thus its meaning depends on
448 how exhaustive the previous alkaline extractions have been. The solubility of a molecule in
449 NaOH is linked to the presence of R-OH groups (in particular carboxyls: -COOH) which
450 dissociate in water with the help of alkali in the solution. A very low proportion COOH/total

451 OC is likely to result in poor or null extractabilities of the compound, particularly if its
452 molecular weight is high. Because the decomposition plus humification process is, partly at
453 least, a process of chemical oxidation, an increased extractability of organic matter is
454 indicative of aerobic decomposition (Kleber and Johnson, 2010). Thus, the unextractability of
455 humin may precisely suggest poor biochemical evolution: it could be made essentially of
456 relatively fresh plant- or microbial-derived materials. However, it is not possible to discard
457 the opposite, that is, the presence in this fraction of organic materials that have suffered a
458 biochemical evolution resulting in the formation of nonpolar structures (e.g., poly-condensed
459 aromatics without substitution) not extractable with alkali. Because of the dominant status of
460 this fraction in the whole SOM, a priority research topic should be a detailed chemical
461 characterization of its composition. Without such an in-depth characterization, its ecological
462 function and meaning can only be supposed.

463

464 *4.3.2. Labile fractions*

465 Labile fractions include the extracts obtained with K_2SO_4 , Na-tetraborate (Na-TB)
466 and Na-pyrophosphate (Na-PP). Strictly the K_2SO_4 extract is of minor importance in its own,
467 but these three fractions altogether represent the organic matter bound to the mineral surfaces
468 by relatively mild bonds, breakable by smooth solvents at pH values substantially lower than
469 those of a standard NaOH solution (pH 13; Kleber and Johnson, 2010). In all land uses, the
470 sum of these three extracts account for more OC than that extracted by NaOH, thus showing
471 that a substantial portion of organic matter in the organo-mineral complex is rather loosely
472 associated to the mineral surfaces: between 21% (in BF) and 31% (in GL) of the total OC in
473 the OMC.

474 This result is in agreement with the results of Lopez-Sangil and Rovira (2013), at least

475 for acid soils. In his review on organo-mineral complexes, Bruckert (1979) already observed
476 that tetraborate often extracted more OC than NaOH. In forest and grassland soils, tetraborate
477 extracts on average less OC than NaOH (Table 4), but González-Prieto et al. (1989) obtained
478 similar results for a dystric cambisol and two eutric fluvisols, in an estuarine zone of Galicia
479 (NW Spain). The crucial question is whether the organic matter bound to the minerals by
480 these mild bonds is really labile and, consequently, be released from the OMC more easily
481 than OC pools retained by stronger bonds (breakable by NaOH, or by harder treatments).
482 This is a matter deserving further detailed research.

483

484 *4.3.3. Organic matter occluded by carbonates*

485 In spite of the presence of carbonates in many of our soils (Table 2), the proportion of
486 OC extracted after the cold acid attack (H_2SO_4 -NaOH fraction) is low. This is in agreement
487 with the work of Lopez-Sangil and Rovira (2013), in which two calcareous soils and two
488 granite soils were investigated by the SOF method. In that work, the organic matter stabilized
489 by occlusion within carbonate coatings accounted overall for <5% of the total OC. Most
490 interestingly, this percentage was not higher in calcareous soils than in acid (granite) soils.

491 These data suggest that in the studied soils carbonate coatings do not play a
492 substantial role in protecting organic compounds, in contrast with previous claims (Alonso et
493 al., 2004; Gocke et al., 2011). Lopez-Sangil and Rovira (2013) explained such an apparent
494 contradiction by proposing that, without denying the presence of carbonate coatings that may
495 protect some organic compounds, the main role of carbonates is actually indirect: carbonates
496 are a huge source of calcium ions (Ca^{2+}), which should act as the true and powerful stabilizer
497 of organic matter. While Fe or Al are regarded as the main responsible cations for SOM
498 stabilization, at least in acid soils (Mikutta et al. 2006; Kögel-Knabner et al. 2008), in

499 calcareous soils Ca is likely to play the dominant role.

500 Calcium ions generate bonds between organic matter and clay surfaces: these bonds
501 resist the action of sodium tetraborate (Na-TB: step 2), but they are disrupted by sodium
502 pyrophosphate (Na-PP: step 3) (Bruckert, 1979). A dominant role of Ca bonds in SOM
503 stabilization, then, should be evidenced by the importance of the Na-PP extract (step 3),
504 which in our dataset accounts for 10-15 % of the total OC in OMC (Table 5). In agreement
505 with this view, Lopez-Sangil and Rovira (2013) observed that in calcareous soils the Na-PP
506 extract accounted for 15% of the total OC in the OMC, whereas in acidic soils it was just
507 about 5%; in contrast, while in calcareous soils the Na-TB extract accounted for <10 % of the
508 total OC in the OMC, in acidic soils it accounted for 20-30 %.

509

510 *4.3.4. Organic matter stabilized by its association to Fe oxyhydroxydes*

511 The amounts of OC and N recovered in step 6 (dithionite reduction, plus NaOH
512 extraction) are low: on average, <5 % of the total OC in the OMC. This may seem an
513 unexpected result in the framework of current paradigms that give to Fe and Al
514 oxyhydroxydes a pivotal role in the long-term stabilization of SOM (Mikutta et al., 2006;
515 Kögel-Knabner et al., 2008). The probable dominant role of Ca as the main stabilizer cation
516 may have reduced the role of other chemical agents, including Fe oxyhydroxydes.
517 Nevertheless, Lopez-Sangil and Rovira (2013) observed that also for soils over granite this
518 fraction (step 6) accounted for a relatively low amount of OC, just 5-6 % of total OC in the
519 OMC. These figures are much lower than those reported by Gonzalez-Prieto et al. (1989),
520 also on acidic soils, who found that the OC extracted after dithionite treatment accounted for
521 up to 30% of the total OC in the soil. Thus, soil type (e.g., lithology) may be of great
522 importance. The two acidic soils studied by Lopez-Sangil and Rovira (2013) were over

523 granite; possibly their result would have been different working on soils developed on other
524 acidic substrates, such as basalts, slates or gneiss, prone to generate soils chemically more
525 active. The future application of the SOF protocol to this kind of acidic soils could add new
526 and relevant data about the quantitative importance of iron oxyhydroxydes for SOM
527 stabilization.

528

529 **5. Conclusions**

530 We provide evidence that size fractionation coupled with an extensive set of chemical
531 extractions allows obtaining new insights about SOM sequestration in mineral soils
532 characterized by different land use. The distribution of OC and N among the obtained size
533 fractions is little affected by land use; the only significant effects of land use on the
534 distribution of SOM among fractions occurs in CF. Interestingly, TS behaviour is similar to
535 that of highly mature ecosystems.

536 Irrespective of land use, the OMC (< 20 μm fraction) accounts for most of total OC
537 and N in the studied soils. Most of the SOM (60-75 %) is physically protected. The
538 differences between land uses as to the proportion of OC and N in the OMC are low: thus, the
539 mere quantification of OMC may be a too rough measure of SOM stability.

540 In the studied soils, a substantial part of the organic matter in the OMC (20-30 %) is
541 bound loosely through electrostatic, van der Waals-, or metallic bonds. The NaOH extract,
542 involving organic matter bound to clays by relatively strong bonds (breakable only at pH 13),
543 accounts also for a notorious part of the organic matter in the OMC (10-15 % of the OC).

544 In contrast, other fractions of the OMC seem of minor importance. Thus, carbonate
545 coatings and Fe oxyhydroxydes seem to play a minor role in the stabilization of organic
546 matter in the OMC, at least in the conditions characterizing the studied sites. Whether this

547 result may be extrapolated, or rather it is a very particular characteristic of our soils, should
548 be verified in future work. Finally, the organic matter associated to mineral surfaces by very
549 strong bonds, breakable only after HF treatment, seems always present in low amounts.

550 For all land uses, the main fraction of OMC is humin, i.e., the last, insoluble residue
551 which remains unextractable with alkali after all the active mineral components causing
552 precipitation have been removed. The precise ecological meaning of such fraction could only
553 be understood after an in-depth chemical characterisation, which should be the next step of
554 our work.

555

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560

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733 **Figure captions**

734

735 **Figure 1.** Flow diagram of the SOF method. Taken from Lopez-Sangil and Rovira (2013),
736 with permission.

737

738 **Figure 2.** Three types of behaviour expected for OC in a given fraction, obtained either in the
739 size fractionation or in the SOF fractionation. Curve 1: the amount of OC in the fraction
740 increases as total OC in the soil (or in the OMC), without any apparent limit. Curve 2: same
741 as before, but there is an uppermost limit, at least apparent: while total OC in the soil (or in
742 the OMC) may increase, the amount of OC in the fraction does not increase anymore. Curve
743 3: no apparent relationship between the amount of OC in the fraction and total OC in the soil
744 (or in the OMC). The same behaviours may be expected for the relationships between N in
745 the fraction and N in the soil (or in the OMC).

746

747 **Figure 3.** Linear correlation between organic carbon (OC) and total nitrogen (N) in the
748 studied soils.

749

750 **Figure 4.** Relationships between total organic carbon (OC) in the whole soil and OC in each
751 size fraction, and between total nitrogen (N) in the whole soil and N in the size fraction. Data
752 have been fitted to polynomial equations of grade 1 or 2 (quadratic), of the type $Y = b_1X +$
753 b_2X^2 , in which b_2 may be zero. There is no b_0 value, and therefore the regression lines pass
754 through the origin of coordinates (0,0). The signification of correlations is given: (*) at $p =$
755 0.05; (**) at $p = 0.01$; (***) at $p = 0.001$.

756

757 **Figure 5.** Relationships between total organic carbon (OC) in the organo-mineral complex
758 and OC in each SOF fraction. Data have been fitted to polynomial equations of grade 1 or 2
759 (quadratic), of the type $Y = b_1X + b_2X^2$, in which b_2 may be zero. There is no b_0 value, and
760 therefore the regression lines pass through the origin of coordinates (0,0). The signification of
761 correlations is given: (*) at $p = 0.05$; (**) at $p = 0.01$; (***) at $p = 0.001$.

762

763 **Figure 6.** Relationships between total nitrogen (N) in the organo-mineral complex and N in
764 each SOF fraction. Data have been fitted to polynomial equations of grade 1 or 2 (quadratic),
765 of the type $Y = b_1X + b_2X^2$, in which b_2 may be zero. There is no b_0 value, and therefore the
766 regression lines pass through the origin of coordinates (0,0). The signification of correlations
767 is given: (*) at $p = 0.05$; (**) at $p = 0.01$; (***) at $p = 0.001$.

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