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Ecosystem type effects on the stabilization of organic matter in soils: combining size fractionation with sequential chemical extractions

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

24 The long-term stabilization of organic matter in mineral soils is widely believed to be mainly 25 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 26 27 according to land use. Here we study organo-mineral interactions in soils under different land 28 uses (coniferous and broadleaved forest, grasslands, and technosols), widely differing in their 29 soil organic matter (SOM) content, by an approach involving two consecutive steps: (i) a size 30 fractionation by wet sieving after sonication, to isolate the particulate organic matter (POM, 31  $>20 \,\mu\text{m}$ ) from the organo-mineral complex (OMC,  $<20 \,\mu\text{m}$ ), and (ii) a characterization of the 32 OMC by sequential extractions with different chemicals, each one disrupting a specific kind 33 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 34 35 affected the total amount of SOM, but barely its distribution between POM and OMC. The 36 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 37 38 less dominant than expected: the organic matter stabilized by carbonate coating (5-6% of 39 total OC in the OMC) or by iron oxyhydroxydes (3-6%). By far, the main pool of the OMC 40 was the final residue remaining after all extractions (34-48% of total OC in the OMC). 41 Because this residue remains unextractable with NaOH after removing all the active mineral 42 components, such unextractability can be due only to its own chemical characteristics. Future 43 research should aim at its in-depth characterization and help elucidating its ecological 44 functions.

46 Introduction

Soils represent the largest terrestrial organic carbon (OC) reservoir (Jobbágy and Jackson, 2000; Schmidt et al., 2011), and a major challenge for soil scientists is to clarify the dynamics of soil carbon in the context of climate change (Davidson et al., 2000; Amundson et al., 2015; Doetterl et al., 2018). Modelling primary drivers and mechanisms of OC stability requires a clear understanding of the mechanisms responsible for soil organic matter (SOM) physicalchemical features, its build-up, its location within the mineral matrix, and its long-term 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; Amelung et al., 1998; Christensen, 2001; Plaza et al., 2013). The size fractions (sand, silt and 60 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 Lützow et al., 2006; Basile-Doelsch et al., 2007). 66

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 clear that the distribution of SOM among the several size fractions affects its stability; as shown in laboratory incubation experiments, the higher the proportion of total SOM in the finest fractions (particularly the  $<20 \ \mu\text{m}$ : fine silt and clay), the higher its overall stability (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 N<sub>2</sub>) 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;

Schnitzer and Schuppli 1989), and the sequence NaTB - NaPP - NaOH was proposed for 94 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 material in SOM stocks formation and persistence have shown to be crucial (e.g., 114 115 Guggenberger et al., 1994, 1995; Leifeld and Kögel-Knabner, 2005; Clemente et al., 2011; Catoni et al., 2016), and thus we used a set of soil samples under a variety of land uses and 116 117 over a variety of geological substrates.

118

#### 119 **2.** Materials and methods

120 *2.1. Site selection and soil samples collection* 

Four land uses were compared in this study: coniferous forests (CF), broadleaved forests (BF), grassland soils (GL) and technosols (TS), all of them located in the Marche region (Italy). Here, the term 'land use' must be meant in a broad sense, because technosols 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 between 810 and 1570 mm. More details about these sites are reported elsewhere (Giannetta 134 135 et al., 2018).

Five different sites for each land use were sampled between May and June 2016. At each site, depending on site surface and homogeneity, 3-10 topsoil samples (generally, 0-25 cm depth, although in some places soil thickness was just 10 cm) were collected randomly from several points separated 50 to 100 cm apart using a soil auger, and mixed to obtain a composite sample. In these samples, soil texture (sand, silt and clay contents) was obtained by the pipette method; pH in H<sub>2</sub>O, at a soil-to-water ratio of 1:2.5; electrical conductivity (EC) in water extracts at proportion soil-to-water 1:5; OC by dry combustion, after
elimination of carbonates by acid fumigation; total N by dry combustion; and carbonate
content by the calcimeter method. Soil properties averaged by ecosystem type are reported in
Table 2.

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## 2.2. Physical fractionation of SOM

Size fractionation was performed by wet sieving, splitting soil particles into four
different size fractions: coarse sand (CSa: 2000-200 μm), fine sand (FSa: 200-50 μm), coarse
silt (CSi: 50-20 μm), and fine silt plus clay (FSCL: <20 μm) (Lopez-Sangil and Rovira,</li>
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  $\mu$ m: 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 sedimentation of the solid particles. The overlying water was siphoned off and discarded. The 162 163 sediment was recovered by centrifugation (15 min at 2500 g), transferred to a pre-weighed flask and dried at 60 °C to constant weight. 164

165	While in the coarse fractions (>20 $\mu$ m) organic matter occurs in the form of dark
166	particles, clearly distinguishable from mineral ones, below the 20 $\mu$ m threshold both
167	components, organic and mineral, are no longer visually distinguishable. Following Lopez-
168	Sangil and Rovira (2013), SOM in fractions >20 $\mu$ m was taken as particulate organic matter
169	(POM), whereas the $<\!20 \ \mu m$ 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 <sub>2</sub> SO <sub>4</sub> ), to extract water-soluble compounds;
177	2) 0.1 M NaTB (Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> ), 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 (Na <sub>4</sub> P <sub>2</sub> O <sub>7</sub> ), 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 <sub>2</sub> SO <sub>4</sub> ) at 5 °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 (Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub> ), followed by 0.1 M
186	NaOH, to extract compounds stabilized by free Fe <sup>3+</sup> forms, previously made soluble through
187	their reduction to Fe <sup>2+</sup> ;

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

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

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

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# 2.4. Organic C and total N analyses

196Total C and N contents of SOM fractions obtained after physical fractionation were197determined by dry combustion using a Thermo Flash 2000 NC Analyzer. Before analysis, an198aliquot of each sample was ground with a ball mill. For OC determination, the aliquots were199subjected 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 (H<sub>2</sub>SO<sub>4</sub>) 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 ( $H_2SO_4$ , 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 Sommers, 1996), and total N in a ThermoQuest CHN analyzer, by evaporation of an aliquot 208 209 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.

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

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#### 218 2.5. Statistical analysis

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

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 order without intercept (i.e.,  $Y = b_1X + b_2X^2$ ) were fitted using SigmaPlot v.10. Figure 2 228 229 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 apparently independent from the amount of OC accumulated in the soil (curve 3). 234

#### **3. Results**

#### 237 *3.1. Carbon and nitrogen in bulk soils*

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

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# 245 *3.2. Carbon and nitrogen in the size fractions*

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

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

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

The OC and N contents of the fractions and those of the whole soil samples are positively and significantly correlated (Fig. 4). The significance of these correlations is higher the smaller the size of the fraction, and is comparatively higher for N than for OC (Fig. 4). This also suggests that the differences in OC and N content in the fractions merely reflect the richness in OC and N in the whole soil sample, rather than a specific effect of the land use.
Even though the relationships were often curvilinear rather than strictly linear, all them
clearly match in concept the curve 1 in Fig. 2; no signs of OC nor N saturation were detected
for any size fraction.

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

The recovery of OC in the SOF fractions (sum of OC in all fractions, as % of the initial OC in the OMC) was on average  $89.2 \pm 11.6$  %. The HF hydrolysate, which was not analyzed for OC, partly explains this loss; however, C losses may also be attributed to the repeated NaOH extractions, which may result in decarboxylations in spite of being all carried out under N<sub>2</sub>.

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

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

When translated to percentages over the total OC in the OMC (Table 5), some of the differences between land uses disappear, while significant differences are still found for most SOF fractions. Thus, in GL the OC associated to mineral matter through weak bonds (NaTB fraction) is abundant, while the soluble fraction ( $K_2SO_4$  extract) and especially the fraction directly associated to clays through very strong bonds (HF-NaOH) are scarce. We note that in GL such a low relative prevalence is due to the abundance of the other fractions; the absolute amount of OC in this fraction is actually similar to that of other land uses (Table 5). Moreover, another specific land-use effect is the relevance of the first NaOH extract in TS, 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<sub>2</sub>SO<sub>4</sub> 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.

For four SOC fractions (NaTB, NaPP, NaOH, and H<sub>2</sub>SO<sub>4</sub>-NaOH), the OC content consistently increases with the total OC in the OMC. For NaTB-extracted, NaPP-extracted, and NaOH SOM fractions the relationships are strong and best described by growing curves, meaning that the relative prevalence of these fractions increase with the total OC in the OMC. In contrast, for the H<sub>2</sub>SO<sub>4</sub>-NaOH fraction data dispersion is high, likely because the accumulation of OC in this fraction depends on the amount of carbonates in fine silt and clays, thus making OC in the fraction and in the OMC less directly related.

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

The recovery of N in the SOF fractions (i.e., the sum of the total N in all SOF fractions) was  $66.5 \pm 11.1$  % of the total initial N in the OMC.

311 Humin accounts 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 the sequence NaTB - NaPP - NaOH, the amount of OC extracted was roughly similar or 313 314 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.

As observed for OC, the amount of N in each SOF fraction relates to the total amount of N in the OMC. The relationships are somewhat different for each SOF fraction (Fig. 6). In fact, for the first four fractions, the relations are more significant (p<0.01), whereas for the H<sub>2</sub>SO<sub>4</sub>-NaOH, the dithionite-NaOH and the HF-NaOH fractions a greater data dispersion was observed. It is also noteworthy the extremely low amount of N in the HF-NaOH fraction, apparently independent from the total N in the OMC.

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

#### **4. Discussion**

#### 333 *4.1. Methodological constraints*

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

In the size fractionation, the ultrasonic treatment plus the extensive water leaching during sieving are obvious reasons for both OC and N losses, for they imply that soluble or easily solubilizable compounds are extracted massively. Nitrogen-rich compounds, including amino acids, peptides, proteins, alkaloids, nucleotides, are more likely to be part of this labile pool, whereas macromolecules such as lignin, suberin, cutins, lipid compounds, rich in C and poor in N, remain largely insoluble and are likely to resist much better the ultrasonic plus 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 addition to these underestimations, the repeated NaOH extractions are likely to affect the 348 characteristics of the obtained fractions. In theory, at the pH values attained with a NaOH 349 350 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 classical N fractionation methods (e.g., Pansu and Gautheyrou, 2006), the N of amino sugars 352 353 is obtained precisely by steam distillation after deamination by an alkaline reagent as phosphate-borate buffer at pH 11.2. The SOF method does not include boiling with NaOH, 354 355 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).

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# 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 µm (POM) is higher in forest- and grassland soils than in technosols in absolute amounts, but not in relative terms (i.e., as % of the total organic matter). As 372 373 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 OC, whereas the organo-mineral complex is depleted. We must stress that, except for CF, the 375 effects of land use were significant on the total amount of SOM but not on the distribution of 376 377 organic matter among fractions.

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

litter a low decomposition rate and microbial use efficiency (Kooch et al., 2012; Cotrufo et
al., 2013), thus leading to a relatively higher OC accumulation in coarse POM fractions
(Castellano et al., 2015). The OC/N ratios of all size fractions are higher for CF that for the
other ecosystems (Table 3), consistently with previous reports (Guggenberger et al., 1994),
and also with the relatively low decomposition degree of SOM in coniferous forests
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 Zaccone 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 complex, observed in our dataset (Table 4), specific processes must also be accounted for. As 414 415 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; Zaccone 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 al., 1996; Nielsen et al., 2006; Sollins et al., 2006) and through the formation of aggregate-425 426 protected particulate and non-particulate organic matter (e.g., Yoo and Wander, 2008), with most of this organic N (>80%) being in the form of amides, peptides and proteins of
microbial origin (Simpson et al., 2007a). By contrast, the high OC/N ratio found in the POM
fractions suggests a relatively high occurrence of slightly-to-partially decomposed plant
materials in these fractions (Rasmussen et al., 2005).

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### 4.3. Organic matter in the organo-mineral complex

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434 4.3.1. Humin
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By far, the last residue (humin *stricto sensu*, in our fractionation) is the most important pool: in CF, it approaches 50% of the total OC in the organo-mineral complex. Slightly lower values, always <35%, have been obtained by Lopez-Sangil and Rovira (2013), but in their study the humin was still very abundant. This pool remains insoluble after the long set of treatments and extractions, and in particular after the substantial destruction of the mineral matrix by HF; therefore, its strong insolubility is not due to any association with the mineral matter, but to its own biochemical composition.

442 In this regard, Simpson et al. (2007b) found that peptides, aliphatic species, carbohydrates, peptidoglycan, and lignin, as macromolecules or aggregate species, are major 443 444 contributors of humin. They also found that microbial organic residues may account for 445 ~45% of the humin fraction in some soils (Simpson et al., 2007a). However, we must stress 446 that the existing literature about humin could be of little use, because the humin is by 447 definition the residue remaining after alkaline extractions, and thus its meaning depend 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.

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# 464 *4.3.2. Labile fractions*

465 Labile fractions include the extracts obtained with K<sub>2</sub>SO<sub>4</sub>, Na-tetraborate (Na-TB) 466 and Na-pyrophosphate (Na-PP). Strictly the K<sub>2</sub>SO<sub>4</sub> extract is of minor importance in its own, but these three fractions altogether represent the organic matter bound to the mineral surfaces 467 468 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 the OMC. 473

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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.

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# 4.3.3. Organic matter occluded by carbonates

In spite of the presence of carbonates in many of our soils (Table 2), the proportion of OC extracted after the cold acid attack (H<sub>2</sub>SO<sub>4</sub>-NaOH fraction) is low. This is in agreement with the work of Lopez-Sangil and Rovira (2013), in which two calcareous soils and two granite soils were investigated by the SOF method. In that work, the organic matter stabilized by occlusion within carbonate coatings accounted overall for <5% of the total OC. Most 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 are a huge source of calcium ions (Ca<sup>2+</sup>), which should act as the true and powerful stabilizer 496 497 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 pyrophosphate (Na-PP: step 3) (Bruckert, 1979). A dominant role of Ca bonds in SOM 502 503 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 total OC in the OMC, in acidic soils it accounted for 20-30 %. 508

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#### 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 unexpected result in the framework of current paradigms that give to Fe and Al 513 514 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 may have reduced the role of other chemical agents, including Fe oxyhydroxydes. 516 517 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 OMC. These figures are much lower than those reported by Gonzalez-Prieto et al. (1989), 519 520 also on acidic soils, who found that the OC extracted after dithionite treatment accounted for up to 30% of the total OC in the soil. Thus, soil type (e.g., lithology) may be of great 521 importance. The two acidic soils studied by Lopez-Sangil and Rovira (2013) were over 522

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.

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# 529 **5.** Conclusions

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

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

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

In contrast, other fractions of the OMC seem of minor importance. Thus, carbonate coatings and Fe oxyhydroxydes seem to play a minor role in the stabilization of organic matter in the OMC, at least in the conditions characterizing the studied sites. Whether this result may be extrapolated, or rather it is a very particular characteristic of our soils, should
be verified in future work. Finally, the organic matter associated to mineral surfaces by very
strong bonds, breakable only after HF treatment, seems always present in low amounts.

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

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

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Figure 2. Three types of behaviour expected for OC in a given fraction, obtained either in the 738 size fractionation or in the SOF fractionation. Curve 1: the amount of OC in the fraction 739 740 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 (or in the OMC). The same behaviours may be expected for the relationships between N in 744 745 the fraction and N in the soil (or in the OMC).

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

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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  $Y = b_1 X + b_2 X$  $b_2X^2$ , in which  $b_2$  may be zero. There is no  $b_0$  value, and therefore the regression lines pass 753 754 through the origin of coordinates (0,0). The signification of correlations is given: (\*) at p =0.05; (\*\*) at p = 0.01; (\*\*\*) at p = 0.001. 755

Figure 5. Relationships between total organic carbon (OC) in the organo-mineral complex and OC in each SOF fraction. Data have been fitted to polynomial equations of grade 1 or 2 (quadratic), 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 regression lines pass through the origin of coordinates (0,0). The signification of correlations is given: (\*) at p = 0.05; (\*\*) at p = 0.01; (\*\*\*) at p = 0.001.

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Figure 6. Relationships between total nitrogen (N) in the organo-mineral complex and N in each SOF fraction. Data have been fitted to polynomial equations of grade 1 or 2 (quadratic), 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 regression lines pass through the origin of coordinates (0,0). The signification of correlations is given: (\*) at p = 0.05; (\*\*) at p = 0.01; (\*\*\*) at p = 0.001.