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

Beatrice Giannetta <sup>a</sup>, César Plaza <sup>b</sup>, Claudio Zaccone <sup>c\*</sup>, Costantino Vischetti <sup>a</sup>, Pere Rovira <sup>d</sup>

<sup>a</sup> Department of Agricultural, Food and Environmental Sciences, Polytechnic University of  
Marche, via Brecce Bianche 10, 60131 Ancona, Italy

<sup>b</sup> Instituto de Ciencias Agrarias, Consejo Superior de Investigaciones Científicas, Serrano 115  
bis, 28006 Madrid, Spain

<sup>c</sup> Department of the Sciences of Agriculture, Food and Environment, University of Foggia,  
via Napoli 25, 71122 Foggia, Italy

<sup>d</sup> Forest Science and Technology Centre of Catalonia, Carretera de St Llorenç de Morunys,  
km 2, 25280 Solsona, Spain

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\* Corresponding author.

E-mail address: [claudio.zaccone@unifg.it](mailto:claudio.zaccone@unifg.it)



## 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,  $>20\ \mu\text{m}$ ) from the organo-mineral complex (OMC,  $<20\ \mu\text{m}$ ), 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.

## 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) physical-chemical features, its build-up, its location within the mineral matrix, and its long-term persistence across different soils and ecosystem types.

The physical protection of SOM and its association to active minerals (fine silt and clays, free forms of iron and aluminium, carbonates) are widely recognized as the main driver for its long-term persistence in mineral soils (Kaiser and Guggenberger, 2000; Kögel-Knabner et al., 2008; Schmidt et al., 2011; Lehmann and Kleber, 2015). Physical fractionations based on particle-size, density or a combination of both have been widely used 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 clay-sized, all of them containing both mineral particles and organic matter) represent the primary organo-mineral units; the secondary structures are the aggregates built from the former (Christensen, 2001). SOM turnover strongly depends on the size fraction: fast in coarse fractions (Balesdent, 1996), and much slower in fine fractions (fine silt and clay-sized), for which the longest turnover times have been suggested (von Lützow et al., 2006; Basile-Doelsch et al., 2007).

The diversity of rationale, goals and mechanistic theories about SOM formation, stabilization and turnover has led to the application of different fractionation protocols, which 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  $<20\text{ }\mu\text{m}$ : fine silt and clay), the higher its overall stability (Rovira et al., 2010).

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

The 'ideal' chemical extractant for SOM should perform a complete, massive, non-selective SOM extraction, without any generation of chemical artefacts (Stevenson, 1994). Of course, such an extractant does not exist. To date, sodium hydroxide (NaOH) extraction under inert atmosphere (Ar or N<sub>2</sub>) is the closest available proxy for such a chemical extractant (Stevenson, 1994). Well on the contrary, 'non-ideal', non-massive, selective extractants are of particular interest for our work. Sodium tetraborate (NaTB henceforth) or sodium pyrophosphate (NaPP) have been used as mild extractors to obtain specific SOM 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 SOM fractionation in the organo-mineral complex (Bruckert and Kilbertus, 1980). González-Prieto et al. (1989) described an extended version of that method, which involved a further extraction with NaOH after reduction of Fe oxides with dithionite, and another after destruction of silicates with hydrofluoric acid (HF). The soil organo-mineral fractionation (SOF) method (Lopez-Sangil and Rovira, 2013) still introduced two steps, a preliminary extraction with a neutral saline solution of potassium sulfate to extract soluble or easily solubilizable compounds, and a mild attack with acid to destroy carbonate coatings, which is essential for carbonate-rich soils, common in the Mediterranean basin. In such a way, a quite extensive characterization of the organo-mineral complex can be achieved. It is to note that, in our context, 'characterization' must be meant not in the sense of an in-depth study of its chemical structure – which has been argued to change during alkaline extraction (Kleber and Johnson, 2010) – but in the sense of establishing the quantitative relevance of each kind of physico-chemical bond between organic matter and the mineral surfaces.

The aim of this paper was to attain a comprehensive view of SOM stabilization in representative Mediterranean soils. To do so, we applied the two-step approach described above: (a) first, a size fractionation, to isolate and quantify the organo-mineral complex, plus (b) the detailed fractionation of organic matter in the organo-mineral complex using the sequential extraction method of Lopez-Sangil and Rovira (2013). To attain such a 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., 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 over a variety of geological substrates.

## 2. Materials and methods

### 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.

The coniferous forests were *Pinus nigra* woodlands occurring between 350 and 1400 m a.s.l. The broadleaved forests were Orno-Ostryetum associations with *Quercus pubescens* growing between 320 and 1300 m a.s.l. Grassland soils were under meadows and pastures covered by meso- to xerophilous species (e.g., *Bromus erectus*) and *Medicago sativa*, located between 890 and 1700 m a.s.l. Finally, technosols were all placed below 300 m a.s.l., and included (i) two open-pit mines, filled and then reclaimed, one with *Helianthus annuus* since 2010, the other with *Triticum* sp. since 2000; (ii) two dumps, reclaimed in 2000 and in 2011; and (iii) a site of disposal of compost and other non-hazardous waste materials. Mean annual 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 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.

## 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  $\mu\text{m}$ ), fine sand (FSa: 200-50  $\mu\text{m}$ ), coarse silt (CSi: 50-20  $\mu\text{m}$ ), and fine silt plus clay (FSCL: <20  $\mu\text{m}$ ) (Lopez-Sangil and Rovira, 2013).

To do that, soil samples (15 g), air-dried and sieved at 2 mm, were put into 50 mL vials, which were filled with deionized water to 3/4 of their volume. Samples were then subjected to rotatory agitation (20 rpm) for 60 minutes, and to further ultrasonic dispersion for 10 minutes (100W output) using a Branson 45 sonifier. The derived suspensions were immediately wet-sieved through a set of three sieves (200  $\mu\text{m}$ , 50  $\mu\text{m}$  and 20  $\mu\text{m}$  mesh), thus splitting particles into four different size fractions. The fractions retained by sieves were directly recovered from them, quantitatively transferred to pre-weighted flasks and dried at 60 °C to constant weight. The particles passing through the last sieve (<20  $\mu\text{m}$ : FSCL) were brought to about 1 L with water. Two mL of saturated solution of potassium alum were 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 sediment was recovered by centrifugation (15 min at 2500 g), transferred to a pre-weighted flask and dried at 60 °C to constant weight.

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

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

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

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

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.

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.

#### *2.4. Organic C and total N analyses*

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

Steps 5-7 involved two parts. First, the extract obtained with the pre-treatment (sulfuric acid, dithionite, hydrofluoric acid); next, that obtained with 0.1 M NaOH. While the 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 not be analyzed for total N, and in step 7 the first extract (HF) was discarded and thus could not be analyzed neither for OC nor for total N. The reason was in both cases that the presence of a solvent (H<sub>2</sub>SO<sub>4</sub>, HF) is strongly harmful for elemental analyzers. Thus, extracted OC in step 7, and extracted N in steps 5 and 7, are underestimated. Leaving aside these exceptions, 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 of each extract into tin capsules over a hot plate at 60 °C. In the first extract of step 6 (dithionite) OC was quantified in the CHN analyzer, because of the interference of dithionite, 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.

## 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  $p \leq 0.05$ .

In order to understand how the fractions become replenished as the soil accumulates OC or N, we studied by linear regression the relationships between (i) the OC and N content of each size fraction, *versus* that of the whole soil sample, and (ii) the OC and N content of 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 shows the main kinds of dynamics we expect to find in such analysis: the amount of OC in a given fraction is expected to increase together with the total OC in the soil sample, either without any apparent limit (curve 1), either showing clear signs of an apparent upper limit (saturation of the fraction: curve 2), whose detection will be one of the focus of our work. In 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).

### 3. Results

#### 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).

#### 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.

### 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.

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

For four SOC fractions (NaTB, NaPP, NaOH, and  $H_2SO_4$ -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  $H_2SO_4$ -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.

Humin accounts for >60 % of the OMC total N, and thus its dominance is higher than 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 tended to increase; in contrast, the amount of extracted N clearly tends to decrease, being lowest in the first NaOH extract. The NaOH extract contains always <10% of the total N on average, and in most cases <5%. Thus, while this fraction is one of the most important for OC, it is not for N. Another difference is that the extract obtained after dithionite treatment is more relevant for N than for OC; in soils under grasslands and broadleaved forests, it 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  $\text{H}_2\text{SO}_4$ -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  $\text{K}_2\text{SO}_4$  extract, it is not observed for  $\text{K}_2\text{SO}_4$ -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

### 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.

For the SOF method, the reasons for the low recovery of N compounds are different. In the SOF method, all extractions are done quantitatively, without any losses of material. On the other hand, we were not able to analyse some extracts for OC, N or both (as explained in 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 characteristics of the obtained fractions. In theory, at the pH values attained with a NaOH solution (13 or more) the amino groups do not change their ionization status; therefore, amino 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 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, but it includes a NaOH extraction overnight repeated four times (steps 4, 5, 6 and 7), and

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

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

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

Our results do not perfectly match this view. We found that the amount of organic matter in fractions  $>20\ \mu\text{m}$  (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 shown in Table 4, the distribution of organic matter between the four size fractions is similar in TS, GL and BF. Only in CF soils, the coarsest POM fraction (2000-200  $\mu\text{m}$ ) is enriched in OC, whereas the organo-mineral complex is depleted. We must stress that, except for CF, the effects of land use were significant on the total amount of SOM but not on the distribution of 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 than 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).

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

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

Of particular interest is the distribution of N among the size fractions. The decrease in the OC/N ratio is an indicator of humification, well described in classical books (Duchaufour, 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 shown in a previous work by Rovira and Vallejo (2002), the split of plant-derived debris between N-poor in the coarse fractions and N-rich in the fine fractions occurs from the very beginning of the mixing of plant debris with a mineral, organic matter-depleted soil. Clay surfaces, together with active mineral components such as Fe and Al oxyhydroxydes, establish bonds with soluble organic matter, including nitrogenous compounds such as amino acids, amino sugars, peptides or proteins, nucleic acids, partly plant-derived but also from microbial origin (Ladd et al., 1996; Knicker, 2004; Sollins et al., 2006; Nannipieri and Paul, 2009; Paul, 2016; Zacccone et al., 2018). The large capacity of clays for stabilizing proteins has been known for many years (Pinck et al., 1954; Harter and Stotzky, 1971; Kleber et al., 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-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).

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

#### *4.3.1. Humin*

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.

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

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

#### *4.3.2. Labile fractions*

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

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

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

#### *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 ( $\text{H}_2\text{SO}_4$ -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.

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

calcareous soils Ca is likely to play the dominant role.

Calcium ions generate bonds between organic matter and clay surfaces: these bonds 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 stabilization, then, should be evidenced by the importance of the Na-PP extract (step 3), which in our dataset accounts for 10-15 % of the total OC in OMC (Table 5). In agreement with this view, Lopez-Sangil and Rovira (2013) observed that in calcareous soils the Na-PP extract accounted for 15% of the total OC in the OMC, whereas in acidic soils it was just 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 %.

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

The amounts of OC and N recovered in step 6 (dithionite reduction, plus NaOH 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 oxyhydroxydes a pivotal role in the long-term stabilization of SOM (Mikutta et al., 2006; 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. Nevertheless, Lopez-Sangil and Rovira (2013) observed that also for soils over granite this 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), 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 importance. The two acidic soils studied by Lopez-Sangil and Rovira (2013) were over



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

## 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  $\mu\text{m}$  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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## Figure captions

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

**Figure 2.** Three types of behaviour expected for OC in a given fraction, obtained either in the size fractionation or in the SOF fractionation. Curve 1: the amount of OC in the fraction increases as total OC in the soil (or in the OMC), without any apparent limit. Curve 2: same as before, but there is an uppermost limit, at least apparent: while total OC in the soil (or in the OMC) may increase, the amount of OC in the fraction does not increase anymore. Curve 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 the fraction and N in the soil (or in the OMC).

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

**Figure 4.** Relationships between total organic carbon (OC) in the whole soil and OC in each size fraction, and between total nitrogen (N) in the whole soil and N in the size 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  $p = 0.001$ .

**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  $p = 0.001$ .

**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  $p = 0.001$ .