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Sequential chemical extractions of the mineral-associated soil organic matter: An integrated approach for the fractionation of organo-mineral complexes

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ABSTRACT

Long-term stabilisation of soil organic matter (SOM) largely depends on its interaction with the active mineral components of soils. SOM may become associated with the mineral active surfaces through a wide variety of linkages, with different strength. Thus, fractionation procedures capable of assessing the strength through which mineral-associated SOM is stabilised can be very useful. This paper presents a soil organo-mineral fractionation method (henceforth, SOF) that essentially resumes the work of classical pedologists, who aimed to quantify the different modes through which organic compounds are bound to the mineral matrix using sequential extractions with chemical reagents (0.1 M sodium tetraborate, 0.1 M sodium pyrophosphate, 0.1 M sodium hydroxide, 0.1 M sodium hydroxide after sodium dithionite pre-treatment, and 0.1 M sodium hydroxide after hydrofluoric acid pretreatment). We added a previous extraction with 0.5 M potassium sulfate to remove soluble organic compounds, and a weak acid attack with 0.33 M sulfuric acid to destroy possible SOM-occluding carbonate films, which are often assumed to contribute to SOM stability in calcareous soils. The proposed sequence is applied only to the organo-mineral complexes (<20 μm), after the removal of the particulate organic matter (POM) by ultrasonic dispersion and wet sieving.

We tested the SOF method on four contrasting soils: two Haplic Calcisols (under crop and forest) and two Humic Cambisols (under forest and pasture), with organic C (OC) contents ranging from 1.8 to 3.4% and pH from 3.9 to 8.0. Our results showed that the mineral-associated SOM represents the largest SOM fraction (67–72% of the total organic C content), and that a substantial part of it is weakly associated with the mineral matrix, as it can be extracted by sodium tetraborate or sodium pyrophosphate. While the sodium tetraborate extract was the main fraction in acid soils, the sodium pyrophosphate extract was the main fraction in calcareous soils, thus highlighting the role of Ca in SOM stability. In contrast, our results suggest a small role of carbonate precipitation in the stabilisation of SOM < 20 μm. The sodium hydroxide extractions after both the sodium dithionite and HF treatments released little SOM in the studied soils, but the remaining (insoluble) residue accounted for 15–30% of total OC, and deserves further study. The SOF method can be a valuable tool for splitting mineral-associated SOM into different fractions regarding their proneness for extraction, and its thoroughness may prove most useful for comparative studies about SOM stabilisation.

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1. Introduction

The study of soil organic matter (SOM) dynamics through fractionation procedures has been practised for at least one century (Vaughan and Ord, 1985). Fractionation involves splitting SOM among several fractions, whose relative proportion provides us with information about SOM stability, which results in its long-term persistence. Ideally, these fractions should account for SOM functional pools, reflecting the soil C cycle to some extent.

Chemical fractionation methods based on the solubility of SOM in specific solvents (usually alkalis, mainly sodium hydroxide) dominated SOM studies until the 1980s (Stevenson, 1982). These methods aimed to isolate the so-called humic substances, a stabilised yet chemically active SOM fraction. Humic substances were further divided into humic (soluble in alkaline solutions, insoluble in acid) and fulvic (soluble at all pHs) acids. Radiocarbon dating studies showed that these fractions actually represent distinct stabilisation pools: humic acids are usually more stable, resistant to

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biodegradation and much older than fulvic acids (Guillet, 1979). Although several refinements of this fractionation were proposed over the years (for instance, Schnitzer and Schuppli, 1989), the basic concept still remains.

In recent decades, the fractionation methods based on physical properties (density, particle-size, or a combination of both) have become the dominant ones in SOM studies. These methods, which primarily aim to isolate and further study soil organo-mineral complexes (Bruckert, 1979a), became increasingly popular in the last decades of XXth century (Christensen, 1992). Today's predominant physical fractionation procedures result from evidence that the obtained fractions are often good predictors of SOM stability and decomposition (Janzen et al., 1992; Rovira et al., 2010; Zimmerman et al., 2012, among many others).

Some studies have evidenced the relevance of organo-mineral interactions for SOM stability (Kaiser and Guggenberger, 2000; Kalbitz et al., 2005; Kögel-Knabner et al., 2008). A recent review (Schmidt et al., 2011) stressed that while chemical recalcitrance explains SOM persistence during the first decades, in the long-term – centuries or millenia – SOM stabilisation depends on its interaction with the active components of the mineral matrix. Thus, any fractionation method designed to understand SOM stabilisation in a given soil should start by quantifying the SOM attached to active mineral surfaces. Density- or particle-size fractionation methods are appropriate for this (Christensen, 1992). The problem, however, is that physical fractionation alone cannot resolve the exact nature of the bond between organic and active mineral components. The generic term 'associated with mineral matrix' involves many different and contrasting organo-mineral interactions, each one with its own properties: hydrogen bonds, weak electrostatic unions with clay particles, precipitation by cations, association with Fe and Al oxides and hydroxides, occlusion by fine carbonate coatings, etc. Neither density- nor size fractionation methods are useful for a complete and absolute quantification of the relevance of the different stabilisation modes.

An alternative way is to use specific chemical reagents capable of counteracting a particular type of binding, thus solubilising most, if not all, of the SOM stabilised by such an interaction. These chemicals are already known, some of them for many years. Here we emphasize old literature, precisely to stress this fact. Sodium tetraborate has been shown to displace the organic substances attached to mineral surfaces by Van der Waals and weak electrostatic forces (Bruckert, 1970, 1979b; Greenland, 1971). Sodium pyrophosphate is a very useful chelating agent to solubilise organic compounds precipitated by metallic elements (Bremner et al., 1946; McKeague, 1967; Kononova and Bel'chikova, 1970). Sodium dithionite is a known reductive agent of iron oxyhydroxides, enabling the extraction of their bound SOM (Gonzalez-Prieto et al., 1989). Traditional extraction with sodium hydroxide must also be considered given its powerful extractive properties.

The return to fractionation methods based on chemical extractions should not consist of merely repeating old recipes, but in designing new, sound fractionation protocols in which several extractants are arranged in a rational and well-ordered sequence. Indeed, attempts have been made to do just this. The sodium tetraborate–sodium pyrophosphate–sodium hydroxide sequence was recommended by French researchers (Chouliaras et al., 1975; Duchaufour 1976; Bruckert, 1979a; Bruckert and Kilbertus, 1980), and applied sometimes by spanish researchers (Dorado et al., 1979). Schnitzer and Schuppli (1989) applied the sodium pyrophosphate–sodium hydroxide sequence to particle-size fractions after extracting lipids with organic solvents. After noting that the sodium hydroxide-insoluble fraction often accounted for most of the SOM, Duchaufour (1976) suggested pursuing fractionation by applying further treatments, including reduction with sodium dithionite, hydrofluoric attack or density fractionation. Actually the protocol of Dabin (1976) already applied these ideas. On these grounds, Almendros et al. (1979) developed a quite exhaustive fractionation scheme, involving the previous isolation of free (= light) organic matter by density fractionation with organic solvents, and the further treatment of the organo-mineral (= dense) fraction with a sequence of extractants: sodium pyrophosphate, sodium hydroxide, hydrochloric acid (in calcareous soils), sodium dithionite and hydrofluoric acid. Sodium tetraborate was not included in the sequence. Gonzalez-Prieto et al. (1989) put together the previous sequences in a single and long one: after removing the light fraction by density fractionation, they carried out extractions with sodium tetraborate, sodium pyrophosphate, sodium hydroxide, sodium dithionite, and hydrofluoric acid. The protocol by Gonzalez-Prieto et al. (1989), though complex and time-consuming, represents in our view the right point of departure to perform a complete description of the mineral-associated SOM.

In this study, we propose a sequential, integrated fractionation procedure designed to quantify different organo-mineral fractions according to the strength of their bond. Rather than studying a single, specific interaction type, the soil organo-mineral fractionation (SOF) described herein aims to achieve a compelling insight into how – and to what extent – the SOM of a given soil sample is stabilised by its mineral components.

2. Materials and methods

2.1. Overview of the SOF procedure and reagents

Broadly, our proposal involves a two-step procedure. The soil sample was first subjected to physical fractionation by size fractionation after ultrasonic dispersion, in order to separate the particulate organic matter (POM > 20 μm) from the organo-mineral complexes. Whereas POM is assumed to account for the free organic debris, not associated with the mineral components, the organo-mineral fraction (<20 μm) was subjected to a sequential set of extracting solutions within the SOF procedure (Fig. 1) by using the following reagents:

1) Potassium sulfate, to extract the water-soluble organic compounds present (or potentially present) in the soil solution (Jones and Willett, 2006), thus easily available for microbes (Casals et al., 2009, 2011).
2) Sodium tetraborate buffer at pH 9.7 ($Na_2B_4O_7$), to extract mainly those organic molecules adsorbed to mineral surfaces by weak unions. Sodium tetraborate displaces, through anionic exchange or ionisation of the functional groups, those organic compounds weakly adsorbed to the mineral matrix by means of electrostatic or Van der Waals interactions, such as hydrogen or cationic bridges with clay particles (Greenland, 1971, Bruckert, 1979b). These chemical bonds are characteristic of the SOM recently adsorbed by the mineral matrix (Bruckert, 1979b, Evans and Wilson, 1985; Lemaître et al., 1995), less stabilised and more prone to be detached from its adsorption site during soil disturbances or microbial attack.
3) Sodium pyrophosphate buffer at pH 9.8 ($Na_4P_2O_7$), a well-known chelating reagent, to extract organic substances precipitated by metallic cations (Ca, Mg, Fe, Al). Multivalent cations can form insoluble complexes with organic molecules, inducing their coagulation (Oste et al., 2002). Sodium tetraborate does not extract these complexes (Bruckert, 1979a; Evans and Wilson, 1985). Sodium pyrophosphate extraction is

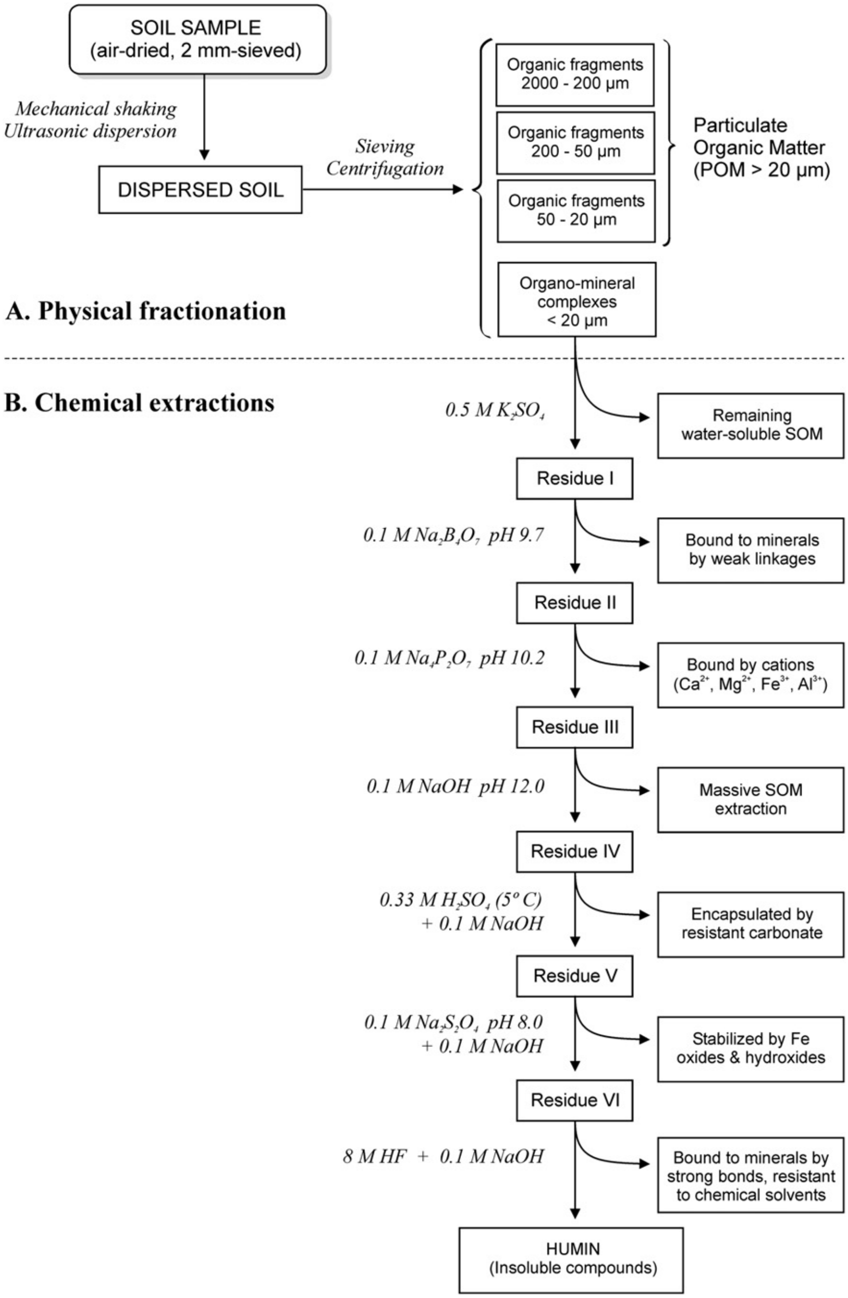

Fig. 1. Scheme of the methodological steps for (A) the physical fractionation, which collects the different fractions of the particulate organic matter (POM > 20 μm); and (B) the chemical extractions (applied only to the organo-mineral complexes, < 20 μm).

quite specific because it does not affect amorphous or crystalline Fe and Al oxyhydroxides (McKeague, 1967).

4) Sodium hydroxide (NaOH), for massive SOM extraction. Its high alkalinity (pH ~ 12) enables the disruption of strong linkages between SOM and active mineral surfaces.
5) Cold weak sulfuric acid (H_2SO_4), to destroy possible carbonate coatings physically occluding SOM in calcareous soils (Olk et al., 1995; Mahieu et al., 2002); plus further extraction with sodium hydroxide.
6) Sodium dithionite ($Na_2S_2O_4$), a well-known reagent for reducing Fe in both amorphous and crystalline oxides and hydroxides (Mehra and Jackson, 1960; Schulten and Leinweber 1995; Kumar et al. 2004; Rasse et al., 2006), by solubilising the organic compounds stabilised by them, plus further extraction with sodium hydroxide.
7) Hydrofluoric acid (HF), to solubilise the fraction associated with clay by bonds strong enough to resist all previous treatments, plus further extraction with sodium hydroxide.

The alkaline extractants in steps 2 to 4 are not expected to be perfectly specific as to their effects. Thus sodium tetraborate mainly extracts weakly adsorbed organic matter, but the solubilisation of small amounts of complexes of organic matter with metals by sodium tetraborate is possible, if the ratio organic matter/cation is high (see Discussion). Further, sodium pyrophosphate is meant to mainly extract organic matter precipitated through complexes with metals and especially calcium, but the solubilisation of some amounts of strongly adsorbed organic compounds can not be discarded, owing to its relatively high pH.

2.2. Soils used

The SOF procedure was tested on four soils with different parent materials and land uses (Table 1). Two of them ("Cropland (calcareous)" and "Forest (marl)") were calcareous soils under Mediterranean conditions, from Catalonia region; the other two ("Forest (granite)" and "Pasture (granite)") were acidic soils developed over the same granite bedrock, under humid-temperate climate, from Galicia region. All the soils were collected between November 2010 and April 2011, and were air-dried, thoroughly homogenised and passed through a 2-mm sieve prior to their analysis. Analyses of soil organic carbon (OC) and total nitrogen (TN) contents, as well as pH, soil texture and inorganic C, were performed from bulk soil samples (Table 2).

2.3. Physical fractionation

Duplicate subsamples (15 g each) were weighed into 50 mL falcon-type vials, which were filled to 3/4 of their volume with two glass marbles and deionised water. The vials were then subjected to smooth vertical agitation (20 rpm) for 60 min. The glass marbles were then removed and the soil–water suspension was transferred into 60 mL polypropylene vials for ultrasonic dispersion of the soil particles. The sonication was performed for 10 min at 60% of the total amplitude (Branson 450 Sonifier), with the sonifier horn inserted 1.5 cm into the soil–water mix. The vials were surrounded by ice-cold water during sonication, in order to counteract associated warming. The soil–water suspension was then wet-sieved, splitting particles into three different size fractions: 2000–200, 200–50 and 50–20 μm diameter (Fig. 1). The particles passing through the 20-μm sieve were brought to ca. 1 liter volume and left stand refrigerated for 2 days, after addition of 3 mL of saturated aluminum potassium sulfate ($AlKSO_4$) solution as flocculant. After sedimentation, most of the water was carefully discarded by siphonation, and the lowest part of the water column (containing the whole organo-mineral fraction) was transferred into 250 mL polypropylene vials and centrifuged for 15 min at 2500 g (Hettich Rotina 420 R).

The fraction <20 μm was taken as the organo-mineral complex, and was the one subjected to the SOF procedure, explained below. The organic matter in the fractions >20 μm was mostly composed of dark organic particles, and taken overall as particulate organic matter (POM).

Table 1
Description of the soils used for this study. All sites were located in Spain.

	Cropland (calcareous)	Forest (marl)	Forest (granite)	Pasture (granite)
Location	Vilabertran	Cardona	Lugo	Lugo
Coordinates	42°16′51″N 02°58′37″E	41°56′14″N 01°42′44″E	43°02′56″N 07°31′26″W	43°02′56″N 07°31′26″W
Altitude (m a.s.l.)	19	460	480	480
Slope	Negligible	Negligible	Negligible	Negligible
Substrate	Limestone terrace	Reddish marl	Granite	Granite
Taxonomy (IUSS WRB, 2006)	Haplic Calcisol	Haplic Calcisol	Humic Cambisol	Humic Cambisol
Land use	*Zea mays* field	*Pinus nigra* stand	*Pinus radiata* stand	Grassland
Temperature, annual mean	15.3 °C	13.2 °C	11.5 °C	11.5 °C
Precipitation, annual mean	607 mm	630 mm	1083 mm	1083 mm
Sampled depth	0–20 cm	1–5 cm	0–10 cm	0–10 cm

Table 2
Main physico-chemical characteristics of the four soil types studied. Values are means of $n = 3$ except for texture ($n = 1$). OC: total organic carbon. TN: total nitrogen.

	Cropland (calcareous)	Forest (marl)	Forest (granite)	Pasture (granite)
OC (g kg^{-1})	17.5	34.4	27.0	31.7
TN (g kg^{-1})	2.1	2.8	2.1	2.7
C:N ratio	8.3	12.3	12.8	11.7
Carbonate (g kg^{-1})[a]	375	129	<0.5	<0.5
pH in H_2O (1:5)	8.0	7.7	3.9	4.9
Texture (%)[b]	41–28–31	48–28–24	57–25–17	55–27–18

[a] Soil carbonate content is expressed as the percentage in $CaCO_3$-equivalent.

[b] Texture is expressed as the relative content of sand (2000 to 50 μm)–silt (50–2 μm)–clay (<2 μm) in the fine earth (<2 mm).

2.4. Chemical extractions (SOF procedure)

Pellets (containing the soil particles <20 μm) were subjected to a battery of successive extractions (Fig. 1). Each one consisted basically of redissolving pellets with the selected extractant (usually 65 mL), adjusting pH when required. The 250 mL vials were then shaken until resuspension of soil particles and placed on a horizontal shaker (160 rpm) for 16 h (overnight). Vials were then centrifuged (3300 g for 15 min) and the supernatant transferred into a new set of 250 mL vials. This cycle was normally repeated at least twice, though with only 1 h of shaking instead of overnight. Supernatants were combined and brought to an exact volume (usually 200 mL, depending on the number of extractions), and finally stored under refrigeration until further analysis.

Seven types of extractions were performed in the following sequence. Pellets were:

1) extracted twice with 50 mL 0.5 M potassium sulfate;
2) extracted 3 times with 65 mL 0.1 M sodium tetraborate, with pH adjusted to 9.7;
3) extracted 3 times with 65 mL 0.1 M sodium pyrophosphate, pH adjusted to 10.2;
4) extracted 3 times with 65 mL 0.1 M sodium hydroxide;
5) treated once with 0.33 M sulfuric acid at a cold temperature (5 °C), and then extracted 3 times with 65 mL 0.1 M sodium hydroxide;
6) treated once with 20 mL 0.1 M sodium dithionite with pH adjusted to 8, and then extracted 3 times with 65 mL 0.1 M sodium hydroxide;
7) finally, treated twice with 20 mL 8 M hydrofluoric acid, and then extracted twice with 50 mL 0.1 M sodium hydroxide.

The remaining pellet (strongly insoluble SOM < 20 μm, resistant to the whole SOF procedure) was considered as humin (Fig. 1).

The rationale behind adjusting the pH (using drops of 0.1 M sodium hydroxide) is to favour the solubilisation of the extracted organic compounds through the ionisation of their functional groups (e.g. carboxylic and phenolic groups). In the last three set of extractions (from 5 to 7), sulfuric, sodium dithionite and hydrofluoric acid solutions were shaken vigorously for about 16 h (overnight) in a horizontal shaker at 160 rpm, for resuspension of pellets. The sulfuric acid solution should be added at cold temperature and its molarity must be low, in order to minimise collateral hydrolysis of the organic substances. Thus, the amount of sulfuric acid solution must be calculated according to the amount of inorganic C present in soil samples (e.g., in "cropland (calcareous)" subsamples, 120 mL 0.33 M solution were added for the presence of 40 mmoles $CaCO_3$ maximum). In extraction 6), the added sodium dithionite solution was cleaned twice with 65 mL deionised water, followed by 1 h of shaking and centrifugation; deionised water joined sodium dithionite solution and thus was analysed in conjunction. Sodium dithionite solutions are unstable and may undergo rapid self-decomposition (see Varadachari et al., 2006), so it is highly recommended to use only fresh solutions. In extraction 7), both hydrofluoric acid extractions were shaken for 15 h in a horizontal shaker, and then centrifuged and poured off into a residual basin with sodium hydroxide pellets in excess. After the second hydrofluoric acid extraction, the pellet was cleaned twice with 65 mL of deionised water (1 h shaking + centrifugation), which was similarly treated in order to neutralize this dangerous reagent.

2.5. *OC and TN within SOF fractions*

All the extracts were analysed for total organic carbon (OC) and nitrogen (TN) contents. Organic carbon was measured by colorimetry at 600 nm following acid dichromate oxidation (Nelson and Sommers, 1996), in which up to 5 mL of the extract were mixed with 0.17 M $K_2Cr_2O_7$ and conc. sulfuric acid, and digested in a block heater for 30 min at 155 °C (Soon and Abboud, 1991). Total nitrogen was measured by drying an aliquot into tin capsules (evaporation over hot plates at 60 °C). Total nitrogen in capsules was then analysed by combustion oxidation (ThermoScientific Flash2000). Both sodium dithionite and sulfuric acid solutions were similarly analysed; for the sodium dithionite solution, however, it was not possible to measure its OC content by colorimetry (reduced dithionite would interfere with dichromate oxidation), so in this case, OC was measured together with TN in the combustion oxidizer. This step was also performed for all the non-alkaline extracts, which confirmed that the data from dichromate and combustion oxidations matched. The hydrofluoric acid solution was not analysed but discarded after total neutralisation with sodium hydroxide. Insoluble humin was also analysed for OC (acid dichromate oxidation with subsamples of 200 mg) and TN (combustion oxidizer).

2.6. *Ca, Mg, Fe and Al contents within SOF fractions*

The amount of soil cations in all the extracted organo-mineral fractions was analysed by the inductively coupled plasma technique (ICP-OES). Briefly, 25 mL of each fraction were digested at 95 °C with 1 mL 65% nitric acid and 3 mL of 30% hydrogen peroxide until a totally clean, colourless solution was obtained, which was taken as a sign of total oxidation of SOM within the extracts. The concentration of the metallic cations within each SOF extract was finally standardised for the amount of organo-mineral fraction isolated for each soil.

This analysis was not performed in the extracts obtained after hydrofluoric acid treatment, because this step (step 7 in our protocol) destroys most, if not all, the mineral component of the organo-mineral fraction.

2.7. *Humic substances fractionation*

Aliquots of the extracts (either 50 or 100 mL, depending on the OC concentration) were transferred to polypropylene centrifuge flasks and their pH were adjusted down to 1 with 2.5 M sulfuric acid. Extracts were left to stand for at least 1 h and were then centrifuged (2500 g, 7 min). The supernatant was transferred to an equivalent set of vials. This cycle was repeated once. The pellet (humic acids: HA) was redissolved with 0.1 M sodium hydroxide, the pH adjusted to about 7.5 with diluted sulfuric acid, and the obtained HA solution stored refrigerated for the analysis. The supernatant was passed through a 140 × 20 mm column containing Amberlite™ XAD7 (Rohm & Haas) at a rate of 3.5 mL min^{-1}: the fulvic acid (FA) fraction was retained within the column, whereas the remaining organic compounds (non-humified fraction) went through and were discarded. The FA fraction was eluted with 0.1 M sodium hydroxide (Aiken, 1985).

The OC content of the HA and FA fractions was analysed by colorimetric dichromate oxidation (see above). The degree of humification of the extracts was quantified by the HS/OC ratio (where the amount of OC in humic substances [HS] is the sum of those of humic and fulvic acids).

2.8. *Statistical analysis*

Two subsamples of each soil type ($n = 2$) were used for the physical and chemical fractionations described herein. For all the OC and TN analyses, two methodological blanks were performed and subtracted from the sample values. When values from samples were not statistically different from blanks, they were considered as ‘not detected’. All the values and figures are given as mean ± standard deviation (SD). Differences between means were analysed by one-way ANOVA, by applying the post-hoc Tukey test. Pearson’s correlation was used to assess the relation between the chemical nature of the different SOF fractions of two given soil types. All the statistical analyses were performed using SPSS for Windows, version 15.0.

3. Results

3.1. *Overall C and N pools*

Fig. 2 summarizes the distribution of SOM among the three main pools generated during the fractionation procedure: POM, organo-mineral complexes, and lost. The POM fraction (>20 μm) accounted overall for 18–28% of the total OC content, and 10–14% of TN. This results in higher C:N ratios in the POM fraction (Table 5, $P < 0.001$). We detected differences between samples in the % of OC recovered within the POM fraction ($P < 0.05$), but not in the % of TN.

The organo-mineral complexes were the dominant SOM pool in all studied soils. The amount of OC in the soil organo-mineral complexes was roughly twice that of the POM (>20 μm) fraction. In the case of TN, this difference was even greater: 2-fold for the calcareous soils, and 3–4 times greater for the granite soils.

During the whole analytical procedure, 3–11% of the initial OC and 8–32% of the TN were lost, likely during wet sieving in physical fractionation, in which soil samples were extensively washed with deionised water.

3.2. *Chemical extractions*

In the granite soils, the chemical extractions recovered around 50% of the initial OC and 60% of the initial TN contents. For both calcareous soils, these percentages were lower ($P < 0.05$); however, they still represented around 35–45% of both the initial soil OC and TN contents (Tables 3 and 4). The C:N ratios of the mineral-associated SOM were mostly within the range 9–11 (Table 5), except for the ‘marl forest’, which showed a higher C:N ratio (16; $P < 0.001$). All these ratios were significantly lower than those for POM.

In calcareous soils, sodium pyrophosphate was the main extracting reagent (15% of total soil OC), while in the granite soils it was the sodium tetraborate (22–30% of the total soil OC and 28–38% of TN). Overall, sodium tetraborate, sodium pyrophosphate and sodium hydroxide extracted the largest proportion of OC (Fig. 3), although sodium hydroxide extraction after sodium dithionite pretreatment was also notorious (>5% of the total OC in most soils).

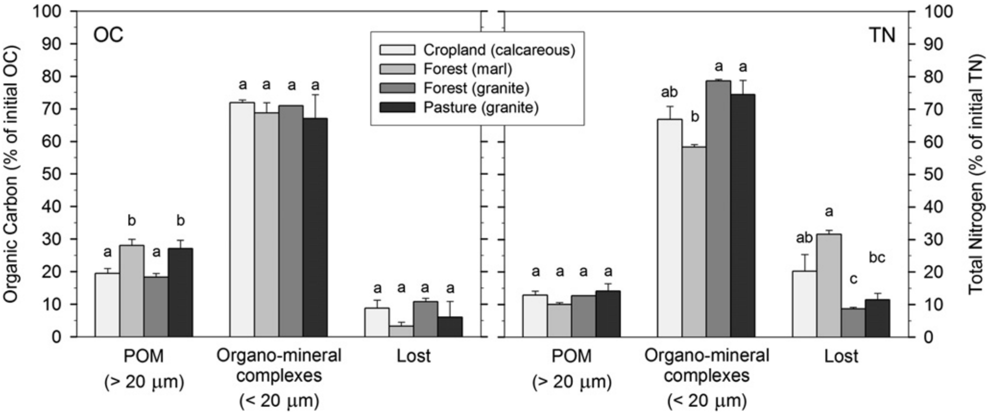

Fig. 2. Percentage (mean + SD) of the initial soil organic carbon (OC) and total nitrogen (TN) in the three main pools obtained in the fractionation: POM (>20 μm), organo-mineral complexes (<20 μm) and lost during fractionation ($n = 2$). For a given element (OC or N) and a given fraction, different letters denote the statistical significance between the means of the different soils ($n = 2$; Tukey test, $P < 0.05$).

In contrast, potassium sulfate, sulfuric acid and HF treatments were less relevant, as they extracted less than 5% of the total OC in all soil types. The sulfuric acid attack and the subsequent regular extraction with sodium hydroxide isolated only small percentages of OC (1–2.5%) in the calcareous samples. Similar percentages were extracted in the granite samples, though only by the sodium hydroxide solution, in contrast to the calcareous soils in which organics were directly extracted by the sulfuric acid solution (Table 3). For TN contents, percentages broadly followed the same pattern as for OC throughout the different SOF extractions.

The insoluble organic residue after SOF extractions (humin) accounted for a considerable part of the total SOM (18–32% of the initial OC content, and 14–30% of the TN, Fig. 3). While in the calcareous soils humin showed C:N ratios similar to the bulk soil, in the granite soils humin was proportionally depleted in nitrogen content, as shown by their high C:N ratios (Table 5).

3.3. Metallic cation contents in the organo-mineral fractions

The distribution of the measured cations (Ca, Mg, Fe, Al) throughout the organo-mineral fractions was clearly influenced by the soil chemistry (Fig. 4). The pattern of metallic cation distribution showed a higher presence of Ca or Fe cations in sodium tetraborate extracts from calcareous or granite samples respectively. The large proportion of sodium pyrophosphate-extracted Ca in calcareous soils stood out, particularly compared with the granite samples (Fig. 4), but without entailing significantly lower Fe/Al contents in those extracts. Sodium dithionite reagent (6a) collected large amounts of iron species but very little aluminum. Leaving aside the potassium sulfate extraction (expected to remove exchangeable cations that do not necessarily contribute to SOM stabilisation), Ca contents increased dramatically in calcareous soil extractions after the sulfuric acid treatment (5a), which evolved soil carbonates and resulted in the formation of considerable amounts of gypsum precipitate as a by-product.

3.4. Chemical nature of the organo-mineral fractions

Humus fractionation revealed a lower degree of humification of the hydrofluoric acid-extracted fraction in comparison to the majority of the other fractions, as denoted by their lower HS:TOC ratios (Fig. 5). This was observed in all the soils. Likewise, the humification ratios of the sulfuric acid/sodium hydroxide treatment showed large differences depending on soil type (Fig. 5), although we must stress that humic substances fractionation was performed with either the sulfuric (calcareous soils) or the subsequent sodium hydroxide solution (granite soils), depending on where the organic C solubilised (Table 3). Significantly, these results revealed correlations between the C:N and humification ratios when comparing soils with similar characteristics. Overall, the humification ratios of

Table 3
Mean ± SD (in mg per 15.0 g of initial soil sample) of organic carbon (OC) recovered from soil subsamples during the physical fractionation (POM > 20 μm), the chemical extractions (SOF procedure) and the resultant insoluble residue (humin).

Step		Cropland (calcareous)	Forest (marl)	Forest (granite)	Pasture (granite)
Total soil sample, initial		262.6 ± 1.4	515.6 ± 2.4	406.0 ± 0.6	474.4 ± 0.4
POM (<20 μm)		50.9 ± 4.2	144.4 ± 9.8	74.3 ± 4.5	128.6 ± 12.3
SOF step (<20 μm)					
1) Potassium sulphate		2.5 ± 0.2	3.1 ± 0.2	4.5 ± 0.8	5.0 ± 0.0
2) Sodium tetraborate		18.2 ± 2.2	45.1 ± 1.8	120.7 ± 0.0	105.0 ± 4.0
3) Sodium pyrophosphate		39.7 ± 0.7	78.4 ± 2.5	22.0 ± 0.4	25.1 ± 5.4
4) Sodium hydroxide		27.0 ± 0.2	54.1 ± 4.3	37.2 ± 1.5	51.8 ± 6.9
5) Sulfuric acid + sodium hydroxide	H_2SO_4 attack	6.6 ± 0.0	4.9 ± 0.5	Not detected[a]	
	NaOH extraction	Not detected		8.2 ± 0.8	10.7 ± 1.4
6) Sodium dithionite + sodium hydroxide	$Na_2S_2O_4$ reduction	5.9 ± 0.1	9.4 ± 0.2	2.4 ± 0.4	2.4 ± 0.2
	NaOH extraction	0.7 ± 0.2	34.6 ± 0.4	19.1 ± 0.9	26.8 ± 5.2
7) Hydrofluoric acid + sodium hydroxide	HF attack	Not analysed[b]			
	NaOH extraction	2.5 ± 0.8	2.4 ± 0.6	1.5 ± 0.2	5.8 ± 0.3
Humin		85.4 ± 1.1	122.4 ± 7.7	72.1 ± 0.3	85.1 ± 14.4

[a] Below detection threshold: OC content in these extracts did not statistically differ from methodological blanks.
[b] The hydrofluoric acid solution was discarded and not analysed.

Table 4
Mean ± SD (in mg per 15.0 g of initial soil sample) of the total nitrogen (TN) recovered from soil subsamples during the physical fractionation (POM > 20 μm), the chemical extractions (SOF procedure) and the resultant insoluble residue (humin).

Step		Cropland (calcareous)	Forest (marl)	Forest (granite)	Pasture (granite)
Total soil sample, initial		31.82 ± 0.17	42.05 ± 0.19	31.84 ± 0.05	40.58 ± 0.03
POM (<20 μm)		4.10 ± 0.38	4.21 ± 0.21	4.04 ± 0.00	5.71 ± 0.94
SOF step (<20 μm)					
1) Potassium sulphate		0.54 ± 0.26	0.28 ± 0.03	0.69 ± 0.30	1.37 ± 0.07
2) Sodium tetraborate		2.93 ± 0.37	3.35 ± 0.37	11.86 ± 0.09	11.68 ± 0.05
3) Sodium pyrophosphate		3.99 ± 0.03	3.56 ± 0.32	2.36 ± 0.10	2.71 ± 0.21
4) Sodium hydroxide		2.24 ± 0.12	2.95 ± 0.10	3.21 ± 0.63	4.36 ± 0.30
5) Sulfuric acid + sodium hydroxide	H_2SO_4 attack	1.09 ± 0.01	1.54 ± 1.09	Not analysed[a]	
	NaOH extraction	Not analysed[a]		0.77 ± 0.11	0.78 ± 0.16
6) Sodium dithionite + sodium hydroxide	$Na_2S_2O_4$ reduction	0.64 ± 0.07	0.63 ± 0.22	0.32 ± 0.03	0.41 ± 0.10
	NaOH extraction	0.06 ± 0.03	2.46 ± 0.03	1.43 ± 0.00	2.06 ± 0.29
7) Hydrofluoric acid + sodium hydroxide	HF attack	Not analysed[b]			
	NaOH extraction	0.23 ± 0.10	0.17 ± 0.06	N.D.[c]	0.84 ± 0.28
Humin		9.56 ± 0.87	9.60 ± 0.65	4.42 ± 0.06	5.93 ± 0.74

[a] The extracts with undetected OC content (see Table 3) were not analysed.
[b] The hydrofluoric acid solution was discarded and not analysed.
[c] Not detected. Nitrogen below the detection limit.

the several organo-mineral fractions were significantly correlated in soils with similar texture and pH, but not in contrasting soil types (Table 6).

4. Discussion

4.1. Precedents of our approach

The protocol we propose relies on previous works of many researchers. Our aim was to essentially put all the steps in a logical sequence to offer an integrative approach to SOM-mineral interactions. The exhaustive protocols developed by Dabin (1976) and Almendros et al. (1979) aimed to a complete description of the status of SOM relative to the way it is stabilized by the active mineral component of the soil. Our closest precedent is the work of Gonzalez-Prieto et al. (1989), which applied most of the chemical extractions described herein after isolating the mineral-associated SOM by densimetry with a mixture of ethanol and bromoform. Our approach differs from theirs in three main aspects: (i) the initial extraction of water-soluble organic compounds with 0.5 M potassium sulfate (Jones and Willett, 2006), thus avoiding an overestimation of sodium tetraborate-extracted SOM; (ii) the addition of a step aimed to destroy possible carbonate coatings in calcareous soils (Almendros et al., 1979; Olk et al., 1995); and (iii) the replacement of previous density with size fractionation, by which organic debris are isolated as POM > 20 μm (Rovira et al., 2010). While size fractionation entails only extensive water washing, density fractionation involves chemicals that may alter SOM features. For instance, a mixture of ethanol and bromoform will extract part of the lipidic component of SOM. Sodium polytungstate has been shown to negatively affect SOM decomposition under laboratory conditions (Magid et al., 1996).

4.2. Extraction in the potassium sulfate–sodium tetraborate–sodium pyrophosphate–sodium hydroxide sequence

Owing to the previous washing with water during size fractionation, the potassium sulfate extract is not a measure of water-soluble organic matter; instead it is mainly a way of removing any residual SOM not (or extremely weakly) bound to active mineral surfaces, thus avoiding an overestimation of the next fraction, the sodium tetraborate extract.

A relevant result of our extractions is that a major proportion of SOM in the organo-mineral complexes may be extracted by sodium tetraborate and/or sodium pyrophosphate, i.e., before the massive extraction with sodium hydroxide. Even though very few references can be found in the literature that can be compared with our results, in general, studies with extraction procedures similar to

Table 5
Mean ± SD of C:N ratio of the fractions recovered from soil subsamples during the physical fractionation (POM > 20 μm), the chemical extractions (SOF procedure) and the resultant insoluble residue (humin).

Step		Cropland (calcareous)	Forest (marl)	Forest (granite)	Pasture (granite)
Total soil sample, initial		8.3	12.3	12.8	11.7
POM (<20 μm)		12.4 ± 0.1	34.4 ± 4.5	18.5 ± 1.1	22.6 ± 1.6
SOF step (<20 μm)					
1) Potassium sulphate		5.1 ± 2.0	11.1 ± 0.7	7.6 ± 4.5	3.7 ± 0.2
2) Sodium tetraborate		6.2 ± 0.1	13.5 ± 1.0	10.2 ± 0.1	9.0 ± 0.3
3) Sodium pyrophosphate		10.0 ± 0.3	22.1 ± 1.3	9.4 ± 0.3	9.2 ± 1.3
4) Sodium hydroxide		12.1 ± 0.6	18.4 ± 2.1	11.9 ± 2.8	11.8 ± 0.8
5) Sulfuric acid + sodium hydroxide	H_2SO_4 attack	6.1 ± 0.0	4.1 ± 2.6	Not analysed[a]	
	NaOH extraction	Not analysed[a]		10.8 ± 0.5	13.7 ± 1.0
6) Sodium dithionite + sodium hydroxide	$Na_2S_2O_4$ reduction	9.3 ± 1.2	15.9 ± 5.8	7.7 ± 1.9	6.0 ± 1.0
	NaOH extraction	12.0 ± 1.1	14.1 ± 0.3	13.4 ± 0.6	13.0 ± 0.7
7) Hydrofluoric acid + sodium hydroxide	HF attack	Not analysed[b]			
	NaOH extraction	10.9 ± 1.4	14.5 ± 1.5	N.D.[c]	7.2 ± 2.0
Humin		9.0 ± 0.7	12.8 ± 0.1	16.3 ± 0.2	14.3 ± 0.6

[a] Extracts in which OC was not detectable were not analysed for N.
[b] The hydrofluoric acid solution was discarded and not analysed.
[c] Not detected: OC was still quantifiable but N was below the detection limit.

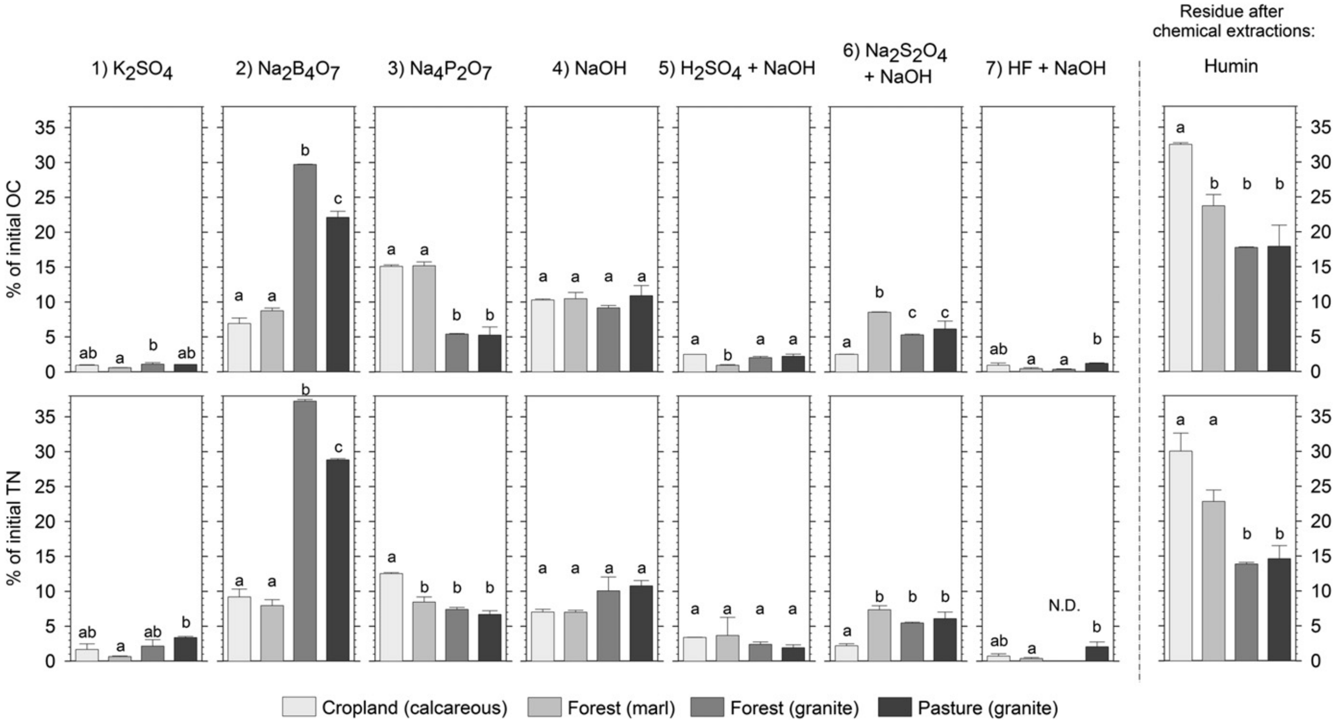

Fig. 3. Percentage (mean + SD) of the initial soil organic carbon (OC) and total nitrogen (TN) recovered by the sequence of SOF chemical extractions, and within the insoluble residue after SOF (humin). "ND" means "not detected" as values did not statistically differ from methodological blanks. Different letters denote the statistical significance between the means of the different soils ($n = 2$; Tukey test, $P < 0.05$).

ours also suggested that a substantial fraction of mineral-associated SOM is often bound through weak (i.e., sodium tetraborate- and sodium pyrophosphate-extractable) bonds to inorganic components. Thus, Gonzalez-Prieto et al. (1989) observed on Dystric Cambisol samples that the buffered sodium tetraborate extract was the dominant organo-mineral fraction in acid soil samples (pH = 4.7), accounting for 24% of total OC, with sodium pyrophosphate and sodium hydroxide extracts obtaining lower percentages (15 and 13%, respectively). Chouliaras et al. (1975) noted that sodium tetraborate and sodium pyrophosphate extracted more organic matter from a forest rendzina than sodium hydroxide did. Dorado et al. (1979) observed that the amount of carbon extracted by each solvent (either sodium tetraborate, sodium pyrophosphate or sodium hydroxide) depended on the size of the fraction: whereas for particles > 5 μm the dominant fraction was the sodium pyrophosphate extract, for particles < 5 μm it was the sodium tetraborate extract.

Free multivalent cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} or Al^{3+} can stabilise SOM by i) bridging negatively charged SOM (anionic groups) with negatively charged clay surfaces (phyllosilicates), thus reducing SOM accessibility to decomposers (Oades, 1988; Clough and Skjemstad, 2000; Hobbie et al., 2007) or ii) through direct precipitation by forming insoluble complexes after reducing the negative charge of the dissolved organic molecules (Oste et al., 2002). Whereas the former (weaker) cation-bridging interaction can be solubilised by previous sodium tetraborate extraction, the latter is extracted by chelating agents, such as sodium pyrophosphate. Both stabilisation modes can be of particular relevance in soils with basic (7–8.5) or very acidic pH (<5), like those studied herein, in which free Ca or Fe/Al hydroxypolycations contents can be high (Oades, 1988). In general, this supports the fact that both sodium tetraborate and sodium pyrophosphate are the main chemical reagents to extract SOM-mineral complexes in these soils, which specifically suggests a role for calcium (Fig. 4) as being mainly responsible for the higher ability shown by calcium-rich soils to stabilise SOM (Oades, 1988; Muneer and Oades, 1989).

4.3. The role of soil carbonates in SOM stabilisation

The idea of applying an alkaline extraction before and after mild acid treatment to extract SOM occluded by pedogenic carbonate coatings was taken from the work of Dan Olk and collaborators (Olk et al., 1995; Mahieu et al. 2002). The main difference with our approach is that the soil subsamples were previously extracted with Na-pyrophosphate. This previous step allows the differentiation between the stabilising effect of Ca^{2+} ions and that from occluding soil carbonates *stricto sensu*, which was not possible in Olk et al. (1995). In that study, the percentage of SOM extracted after the acid treatment was substantial. In our work, sodium pyrophosphate led to significant amounts of SOM, whereas sulfuric acid treatment and the subsequent sodium hydroxide treatment extracted low amounts of organic matter, about 1–2.5% of total OC, within the same range as the acid granite samples (Fig. 3), thus casting a doubt as to whether these minor OC fractions actually come from carbonate-occluded SOM or from additional non-specific sodium hydroxide-extracted compounds. Whatever the case, our results suggest a minor role for carbonates in the stabilisation of SOM < 20 μm, at least in the two calcareous soils studied here. At first glance this disagrees with studies affirming that soil carbonates form coatings, which cover either plant debris -roots- or soil aggregates (Müller and Védy, 1978; Alonso et al., 2004; Gocke et al., 2011). Their research objectives differed from ours. Specifically, Gocke et al. (2011) studied encrustations of pedogenic carbonate which helped protect roots. By densimetric fractionation, Müller and Védy (1978) extracted considerable amounts of organic debris after carbonate destruction. In our protocol, organic debris was isolated in the POM fraction (>20 μm). Thus, the question here is whether pedogenic carbonates are relevant in stabilising fine SOM (<20 μm). Apparently, the previous sodium

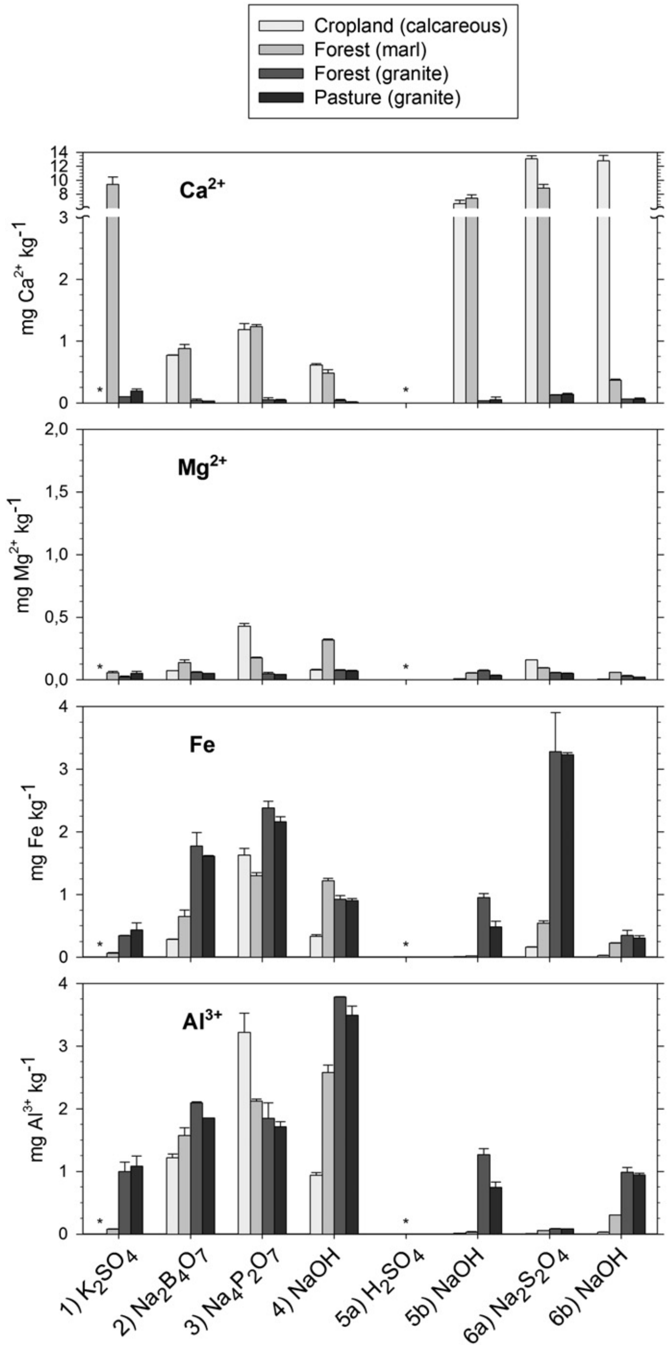

Fig. 4. Mean + SD (in μg cation · g organo-mineral fraction^{-1}) of the calcium, magnesium, iron and aluminium contents within the fractions (<20 μm) collected by the SOF chemical extractants ($n = 2$). Asterisks refer to those extractions in which not enough sample was available to perform the analysis (potassium sulfate and the sulfuric acid attack). The analysis was not performed on extraction 7) as the mineral matrix was severely destroyed by HF treatment.

pyrophosphate extraction is largely redundant with the mild acid attack. Nevertheless, our protocol should be applied to a wider range of calcareous soils before deciding to delete the H_2SO_4/NaOH extraction. Only two calcareous soil samples have been included in this study: our results could be an exception rather than the rule.

4.4. SOM stabilisation by Fe/Al oxides and hydroxides

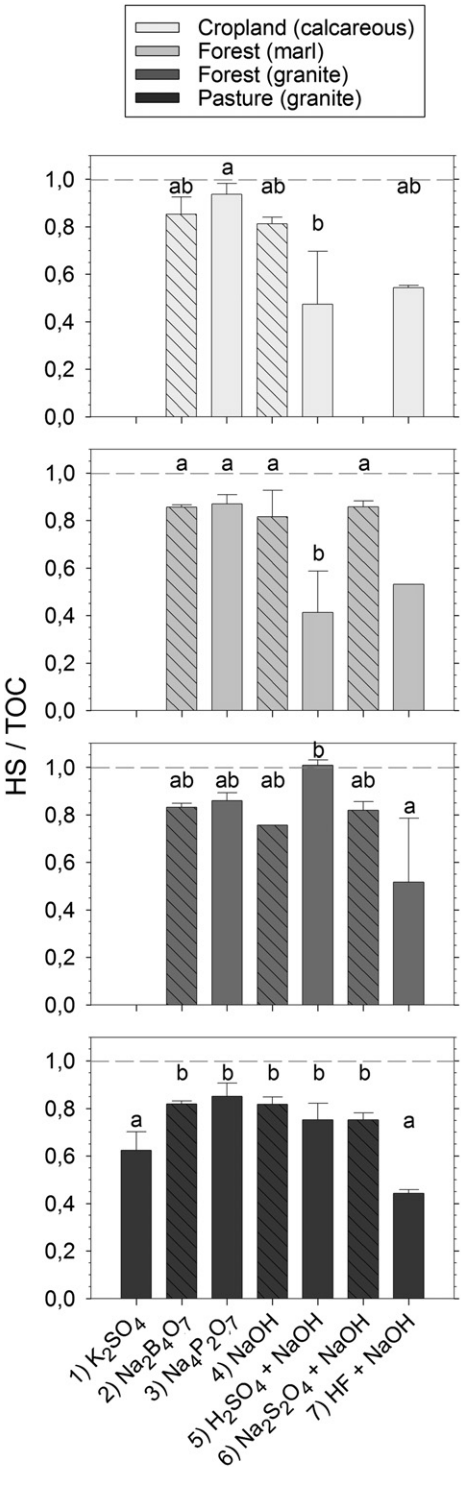

Fig. 5. Mean (+SD) ratios obtained from the humic substances (HS) fractionation. (HS:TOC ratio). Different letters denote statistical significance between the different soil organo-mineral fractions ($n = 2$; Tukey test, $P < 0.05$). Some ratios are not represented (mainly from the "cropland (calcareous)"), as OC concentration in the extracts was too small to reliably perform the HS fractionation.

The role of iron and aluminum oxyhydroxides in SOM stabilisation has been repeatedly addressed (e.g., Kaiser and Guggenberger, 2000; Kögel-Knabner et al., 2008). Although their relevance is well-accepted, it is noteworthy that most available evidence relies on indirect data such as correlations (Kaiser et al., 1996; Mikutta et al., 2006; Eusterhues et al., 2005; Rasse et al., 2006), or on chemical extractions performed with sodium dithionite, oxalate or HF

Table 6
Correlation (expressed as Pearson's coefficient) between the C:N or HS:TOC ratios of two given soils, obtained from their seven organo-mineral fractions. Sample number *n* was between 10 and 14, as the HS fractionation values were not available for all SOF fractions. Asterisks denote statistical significance ($P < 0.05$); NS means "not significant" ($P > 0.05$; actually they were $P > 0.1$ in all cases).

		Cropland (calcareous)	Forest (marl)	Forest (granite)
Forest (marl)	C:N	0.650*		
	HS:TOC	0.742*		
Forest (granite)	C:N	NS	NS	
	HS:TOC	NS	NS	
Pasture (granite)	C:N	NS	NS	0.619*
	HS:TOC	0.649*	NS	0.652*

reagents, but without previous exhaustive extractions of mineral-associated SOM not bound to oxyhydroxides (Eusterhues et al., 2003; Kiem and Kögel-Knabner, 2003; Mikutta et al., 2006). In our soils, we failed to extract large amounts of SOM upon sodium dithionite treatment and with subsequent sodium hydroxide extraction. The highest sodium dithionite-extracted OC content (9%) was found in the forest over marl (Fig. 3). Surprisingly, this percentage does not correspond to high sodium dithionite-extractable Fe values: 5-fold higher values are found in both granite soils (Fig. 4) where, in contrast, sodium dithionite treatment obtains significantly less OC than in marl soils (Table 3).

Sodium dithionite is usually applied in conjunction with an organic acid, either citrate and bicarbonate (DCB method: Mehra and Jackson, 1960), or oxalate and carbonate (Varadachari et al., 2006). Nevertheless, if the intention is to quantify SOM associated with oxides, and not the oxides themselves, then this addition should be discarded. Extractions with sodium dithionite are often performed at temperatures between 40 °C and 90 °C (Varadachari et al., 2006), to ensure a solubilisation of iron oxyhydroxides as complete as possible. But the combination of hot temperature plus highly reducing environment could result in generation of artifacts and distortions in the main characteristics of the extracted SOM. These are the reasons why in our procedure the extractions were performed at room temperature, and an organic acid was not included in the extracting mixture. Therefore, we cannot exclude the possibility of an incomplete dissolution of the Fe oxyhydroxides.

As to the relevance of the sodium dithionite-extracted organic matter, contrasting results can be found in the literature. In a forest rendzina, Chouliaras et al. (1975) obtained small amounts of OC in association with sodium dithionite-extractable Fe oxides, but Gonzalez-Prieto et al. (1989) found that this fraction may account for up to 30% of OC in the horizon. Kaiser et al. (1996) failed to establish a relation between sodium dithionite (DCB)-extractable Fe and soil OC in a range of forest soils. Mikutta et al. (2006) reported positive correlations between mineral-associated SOM and the content in crystalline Fe oxides (which are preferentially extracted by sodium dithionite). However, Rasse et al. (2006) found no correlations at all between SOC and DCB-extracted Fe/Al contents at different depths in agricultural loamy soils. Thus the low relevance of the sodium dithionite-extracted organic matter in the four soils we studied is not an isolated result.

4.5. *Hydrofluoric acid extraction and insoluble humin*

The hydrofluoric acid (plus sodium hydroxide) treatment recovered small amounts of OC (≤1%) in the four soils studied. In contrast, a marked amount of SOM remains insoluble after hydrofluoric acid treatment, particularly in calcareous soils. In a set of 12 subsurface forest acid soil horizons, Mikutta et al. (2006) obtained slightly lower amounts of hydrofluoric acid-insoluble humin (12 ± 1%) than in our granite soils after treating samples with 20 mL 5 M hydrofluoric acid four times.

In classic books (e.g., Duchaufour, 1977), this fraction is called 'Insolubilisation humin', and it was thought to be composed of highly processed, amorphous SOM (Müller and Védy, 1978). This view is challenged by recent evidences showing that even the most recalcitrant fractions of humin are mainly composed of molecules of plant or microbial origin, protected by hydrophobic interactions and sorption to clays, not necessarily generated by long and complex decomposition processes (e.g., Song et al., 2011). This residual fraction is clearly of great importance in soils and should be a matter of further research by applying up-to-date analytical tools.

4.6. *Advantages and limitations of the method*

One limitation of the proposed fractionation lies in the fact that some of the chemical extractants behave to some extent as non-specific: they solubilise organo-mineral fractions not by the type of minerals attached to them, but by the chemical strength of their bond. This is the case of sodium tetraborate with the Fe/Al oxyhydroxides entangling SOM with clay particles through hydroxypolycation bridges (Oades, 1988), which is reflected by the amounts of sodium tetraborate-extracted Fe and Al (Fig. 4). Similarly, this may be applied to the massive sodium hydroxide extraction. Application of sulfuric acid may also entail the hydrolysis of soil humic substances, even if the amount of acid applied is exactly that needed to remove carbonates. Last, but not least, the length of our method results from the fact that several extractions with the same reagent may be needed to solubilise all (or as much as possible of) the targeted organo-mineral fractions. The number of extractions within each step must be adapted to ensure that no significant amounts of SOM-mineral complexes are left unsolubilised; the lack of colour of the last extract is an obvious indicator for this. Three consecutive extractions should likely suffice in most cases, but obviously OM-rich soil samples could need more extractions at any step.

In spite of these drawbacks, the SOF procedure presented a high degree of thoroughness in the quantitative assessment of the different organo-mineral fractions, and it may be very useful for obtaining conclusive results when performing comparative studies. The rationale behind this protocol aims to establish a compelling sequence of the organo-mineral fractions according to the strength of their interactions. This could give soil researchers a valuable tool for splitting the mineral-associated SOM into different functional pools regarding their ease of extraction or possible availability for microbial decomposition and leaching.

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