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Density fractions versus size separates: does physical fractionation isolate functional soil compartments?

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Abstract

Physical fractionation is a widely used methodology to study soil organic matter (SOM) dynamics, but concerns have been raised that the available fractionation methods do not well describe functional SOM pools. We also examine the question whether physical fractionation techniques isolate ecologically meaningful, functionally relevant soil compartments. In this study we explore whether the kind of information that aggregate density fractionation (ADF) and particle size-density fractionation (PSDF) yield on soil OM dynamics is method-specific, similar, or complimentary. We do so by following the incorporation of a ^{15}N label into mineral soils of two European beech forests a decade after its application as ^{15}N labelled litter.

Both density and size-based fractionation methods suggested that OM became increasingly associated with the mineral phase as decomposition progressed, within aggregates and onto mineral surfaces. Our results suggest that physical fractionation methods do isolate ecologically relevant functional soil subunits. However, scientists investigating specific aspects of OM dynamics are pointed towards ADF when adsorption and aggregation processes are of interest, whereas PSDF is the superior tool to research the fate of particulate organic matter (POM).

Some methodological caveats were observed mainly for the PSDF procedure, the most important one being that fine fractions isolated after sonication can not be linked to any defined decomposition pathway or stabilisation process. This also implies that historical assumptions about the “adsorbed” state of carbon associated with fine fractions need to be re-evaluated. Finally, this work demonstrates that establishing a comprehensive picture of whole soil OM dynamics requires a combination of both methodologies and we offer a suggestion for an efficient combination of the density and size-based approaches.

1 Introduction

Mineral soil is a complex mixture of mineral and organic materials attached to each other by a variety of mechanisms. Soil process research has addressed the resulting mechanistic and structural complexity through the development of a large number of physical fractionation protocols (Balesdent et al., 1991; Christensen, 1992; Golchin et al., 1994b; Six et al., 2000b). These methods are based on the premise that the association of soil particles and their spatial arrangement play a key role in soil organic matter (SOM) dynamics, because bioaccessibility and bioavailability are prerequisites for decomposition (Balesdent, 1996; Gregorich et al., 2006; von Lützow et al., 2007). Protocols involve various degrees of soil dispersion, followed by density and/or size separation to isolate pools of SOM based on their size and degree of organo-mineral interaction (Torn et al., 2009). They intend to isolate SOM pools meaningful at different time-scales, but their relevance remains poorly explored. Here, an exhaustive state of the art of the subject was drawn up.

1.1 Soil dispersion

Several procedures generating different levels of soil disaggregation ranging from moderate to strong are currently used. Moderate dispersion treatments include: various types of shaking with or without glass beads, mild sonication, slacking, disruption with a jet of water, blade mixing, and wet sieving (e.g. Billings et al., 2005; Huygens et al., 2005; Kong et al., 2005; Shang and Tiessen, 2000; Six et al., 2002). Strong dispersion treatments include chemical dispersion with sodium hexametaphosphate and high-energy sonication treatments (e.g. Lehman et al., 2001; Sohi et al., 2001). Depending on whether a strong or a moderate dispersion treatment is being used we will refer to a *particle* or an *aggregate fractionation* procedure, respectively. *Particle fractionation* theoretically aims at isolating non-aggregated particles but appears biased when considering small-size fractions resistant to disaggregation (Chenu and Plante, 2006). On the contrary, *aggregate fractionation* does not yield only aggregates but rather a mixture

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of aggregated and non-aggregated particles. It is therefore important to realise that the terms *particles* and *aggregates,* traditionally used in many previous studies, are often misleading. With this realisation in mind, the philosophy behind each procedure can be expressed as follows:

- *Particle fractionations* are based on the idea that equivalent soil particles are the seat of equivalent OM dynamic controlling processes. This type of procedure puts principally the accent on adsorption mechanisms.
- *Aggregate fractionation* procedures are based on the assumption that soil structure is a major control on SOM turnover through physical protection. The emphasis of aggregate fractionations is on the isolation of ecologically meaningful subunits of the soil structure.

1.2 Separation into fractions

The two major separation principles are based on the physical categories “size” and “density”, both pertaining to individual particles and aggregated structures. Size separation mostly relies on wet sieving for soil subunits coarser than 20 µm, and sedimentation for finer soil subunits. The sedimentation method is based on Stokes’ law, but the conditions for its validity, which are a spherical shape of particles/aggregates and homogenous particle/aggregate density, are never realised in soil systems. The consequence is an inherent, yet unknown degree of experimental error which effectively turns the sedimentation approach into some kind of density-size separation method as opposed to a physically rigorous size separation.

Density separation is usually performed through floatation or swirling decantation procedures. Floatation can be performed on water to isolate intact plant remnants with a density $> 1\,\mathrm{g\,cm^{-3}}$, or in dense liquids, such as Ludox (i.e. colloidal silica), NaI or Sodium Polytungstate (SPT) to separate aggregates or particles composed of minerals that vary in density. SPT-solutions are the most common separation liquids, because SPT is non-toxic and allows to create solutions as dense as $2.8\,\mathrm{g\,cm^{-3}}$. However, SPT is known to solubilise a certain proportion of C which may redistribute across fractions or become lost with the supernatant (Chenu and Plante 2006; Crow et al., 2007; Six et al., 1999b; Virto et al., 2008). The swirling decantation procedure takes place in water and is used to separate mostly organic from mostly mineral soil subunits. This last method combines floatation and sedimentation and therefore is different from the water floatation methods presented above (see Sect. 2.2.1 for a brief description of the method).

Over the last three decades, depending on the functional soil compartments tracked, various procedures of density/size separations involving one or several dispersion steps, have been performed. A quick overview of the main types of fractionation procedures is given below (see also Fig. 1). In an attempt at organising information for the readers convenience, terms in bold characters and followed by an asterisk are further defined in Table 1.

1.3 A comprehensive inventory of fractionation procedures

- *Particle density fractionation (PDF)* has been carried out to separate free OM and different types of mineral fractions. The idea behind this approach was that (i) the association or the absence of associations with the mineral phase control OM dynamics (e.g. Rumpel et al., 2000; Swanston et al., 2002; Whalen et al., 2000), and that (ii) the mineralogy of the minerals within the size fractions is distinct and of variable effect on the stability of the adsorbed SOM. Recent examples include the study of mineral-specific associations with organic matter and mineral-specific bonding mechanisms (e.g. Basile-Doelsch et al., 2007, 2009).

- *Particle size fractionation (PSF)* (e.g. Amelung et al., 1999; Jimenez et al., 2011; Rumpel et al., 2000; Sa et al., 2001; Schmidt et al., 1999b; Solomon et al., 2000, 2002) was based on the assumption that particle sizes reflect the state of decomposition of associated SOM as decay induces fragmentation. Yet, coarse fractions that include both relatively fresh plant debris (particulate organic matter, POM),

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as well as more processed and protected OM bound to coarse mineral grains were too heterogeneous to correspond closely to the functional pools used by SOM turnover models. As further described below, this problem was on occasion addressed by combining the particle-size and the particle-density fractionation procedures in what we designate a *particle size-density fractionation (PSDF)* scheme.

- *Particle size-density fractionation (PSDF)* In addition to the size separation, a density separation which removed POM of low density was performed on coarse fractions. This density separation step has been performed using both H_2O as a separation liquid (e.g. Balesdent, 1996; Balesdent et al., 1998, 1991; Derrien et al., 2006) and flotation in dense liquids (e.g Kapkiyai et al., 1999; Sohi et al., 2001).

- *Aggregate density fractionation (ADF)* (e.g. Arnarson and Keil, 2001; Billings, 2006; Bock and Mayer, 2000; Castanha et al., 2008; Crow et al., 2007; Echeverria et al., 2004) was initially developed to isolate *true aggregate fractions** of intermediate density from fully dispersed particles that could be either dense/mineral or light/organic. *True aggregate fractions** were also separated by density in an attempt to separate aggregates of different properties that control soil microbial activity, such as the ratio between organic matter and mineral phase or the importance of aggregates trapped porosity (e.g. Arnarson and Keil, 2001; Hatton et al., 2012). Sollins et al. (2006) also performed a sequential ADF but aimed at isolating particles and not aggregates.

- *Aggregate size fractionation (ASF)* (e.g. Bayer et al., 2000; Billings, 2006; Jimenez et al., 2011; John et al., 2005; Moni et al., 2010; Ranjard et al., 2000) relies on the idea that OC acts as a glue in aggregates and that aggregate size is directly related to the state of decomposition of the organic carbon. Yet, ASF fractionation does not enable to isolate *true aggregate fractions* but rather a mixture of POM, fully dispersed particles and aggregates which renders a robust interpretation difficult. Several studies proposed ways to correct results in a way that would allow to estimate the *true aggregate* characteristics (e.g. Balabane and Plante, 2004; Moni et al., 2010).

- *Aggregate size-density fractionation (ASDF)* (e.g. Cayet and Lichtfouse, 2001; Lehmann et al., 2001; Magid et al., 2010; Nelson et al., 1994; Römkens et al., 1999; Shang and Tiessen, 2000) relies on the same ideas as the *ASF* but a density separation is performed together with the size separation to ensure that only *true aggregates* are separated by size. Yet these fractionation schemes are often incomplete since only a few studies seek to cleanse the *true aggregate fraction* from POM and fully dispersed mineral particle simultaneously.

- *Single vs Multiple step fractionation procedures* Because the above mentioned fractionation procedures all involve just a single dispersion step, we call them *single step fractionation procedures**. Yet, *single step fractionation procedures** were also successively combined to give rise to *multiple step fractionation procedures** (Fig. 1). Those involve the successive redispersion/separation of aggregate fractions obtained from a *single step fractionation procedure** and were used to investigate the internal architecture of soil aggregates (e.g. Baisden et al., 2002; Golchin et al., 1994a, 1995a, b; Huygens et al., 2005; John et al., 2005; Kong et al., 2005; Paul et al., 2008; Six et al., 1998, 2000a; Swanston et al., 2005; Tan et al., 2007; Virto et al., 2008). Multiple step procedures are largely inspired from the aggregate hierarchy concept (Oades, 1984; Oades and Waters, 1991; Tisdall and Oades, 1982), which posits that large, fast cycling aggregates are made of small slow cycling aggregates and that this aggregate organisation controls SOM dynamics in soil. *Multiple step fractionation procedures** are the most informative fractionation procedures available to date, at the cost of being extremely time consuming and prone to generate propagated measurement errors.

1.4 Objectives of the study

With all this variety of fractionation protocols, and the scepticism prevailing with regard to the general usefulness of the fractionation approach (von Lützow et al., 2007), the fundamental question emerges which soil functions or soil process regimes are best represented by a given physical fraction scheme.

This study aims at evaluating the specificity and relevance of the information provided by size and density fractionations for the research question of nitrogen transfer from the litter layer into the underlying mineral soil after a decade of litter decay. By checking the progress of a ^{15}N label through physical fractions we can estimate to which extent a given physical fraction was involved in the turnover of organic matter in general and organic N in particular. We test the hypothesis that physical fractions may allow the observer to identify functional subunits of the soil fabric and the associated process dynamics of OM turnover.

The fractionation procedures selected for this study include an aggregate density fractionation (ADF), a fairly new procedure that has shown promise as a means to isolate ecologically meaningful aggregate structures (Sollins et al., 2006), while traditional particle size-density fractionation (PSDF) was selected because it can be considered as one of the most detailed and widely established fractionation procedures. The fractions obtained were characterised by a suite of analytical techniques with emphasis on parameters that would be informative of the intensity of N turnover.

Our strategy to draw inference involved two steps. First, principle component analysis (PCA) was used to reduce the set of organic matter related data (including C and N contents, C/N ratio, δ^{13}C) to two independent variables or principal components (PC) that account for the majority of the data variability. The second step consisted of resolving the plane defined by the two principal components into contour maps of ^{15}N label incorporation among physical fractions from both fractionation procedures. By doing this, dynamics of litter derived N transformation can be visualized as trajectories in the PCA plane.

2 Materials and methods

2.1 Experimental design

A detailed description of soil sites and sampling procedures was given by Hatton et al. (2012). Briefly, soils were collected from long-term field experiments located at Ebrach (Germany, 49°52′ N, 10°27′ E) and Fougères (France, 48°23′ N, 1°8′ W) (Table 2). Both sites represent managed beech forests (*Fagus sylvatica* L.). According to the FAO classification (IUSS Working Group WRB, 2006), Ebrach is an acidic dystric Cambisol with a sandy loam texture while Fougères keyed out as acidic glossalbic Cambisol with a silty loam texture.

At both sites, the label was applied as a single pulse of highly ^{15}N-enriched beech litter. The litters were obtained by foliar application of urea to ten year old beech trees in another forest (Zeller et al., 1998). In February 1996 at Ebrach and in February 2000 at Fougères, undecomposed litters were removed and replaced by the labeled beech leaves in an amount equal to the respective mean annual leaf litter input and covered with a 2-cm mesh nylon net (Zeller et al., 2001).

Twelve years (November 2007) and eight years (January 2008) after tracer application, labeled and control soils were collected at Ebrach and Fougères, respectively. The A-horizon was collected in triplicate and sieved to pass 2 mm. Observable roots were removed. Replicates were stored at +4 °C. Soil moisture was measured at 105 °C. The first 2.5 cm were investigated in this study as most of the litter-derived ^{15}N was concentrated here (Hatton et al., 2012).

2.2 Description of fractionation protocols

We compared two *single step fractionation procedures** that differ in the number of fractions generated, in the intensity of the dispersion step and in the type of separation used. Particle size-density fractionation (PSDF) uses high-energy ultrasonic dispersion, wet sieving, sedimentation and density separation in water to separate different size classes of organic and mineral fractions, while aggregate density fractionation (ADF) employs a low-energy dispersion step and uses a salt solution diluted to various densities to separate out fractions of varying density.

2.2.1 Particle size-density fractionation (*PSDF*)

We followed a method originally proposed by Balesdent et al. (1991) and sequentially isolated the following size fractions: > 2000, 2000–630, 630–200, 200–63, 63–20, 20–6, 6–2, 2–0.2, 0.2–0.035, and $< 0.035\,\mu$m (see Fig. 2). The procedure entails the suspension of aggregates $> 63\,\mu$m without breaking POM using mechanical shaking with glass beads (Balesdent et al., 1998) followed by the complete dispersion of $< 63\,\mu$m micro-aggregates using ultrasound. Briefly, deionised water (360 ml) was added to ~50 g of air-dried bulk soil, and shaken overnight with 20 glass beads (diameter = 5 mm). The fractions $> 63\,\mu$m were recovered by wet sieving, whereas the fraction $< 63\,\mu$m was sonified using conditions calibrated to obtain a clay-sized fraction equivalent in proportion to that achieved during standard particle size analyses (Balesdent et al., 1998; Schmidt et al., 1999a). The input of energy delivered by the ultrasonic probe to the soil suspension (soil mass (g) to water volume (ml) ratio of 1:10) was fixed at 320 J ml^{-1} delivered over a 20 min period of time. An ice bath was used to limit temperature increase and to avoid reduction of cavitation during sonication (Roscoe et al., 2000). The 63–20 µm fraction was then recovered by wet sieving, whereas all fractions $< 20\,\mu$m were separated by sequential sedimentation performed either under normal gravity for fractions $> 2\,\mu$m or under increased gravity for fractions $< 2\,\mu$m. Assuming an average particle density of 2.44 g cm^{-3}, sedimentation times were determined according to Stokes' law under normal gravity, and according to an adapted version under centrifugation (Poppe et al., 1988). Fractions coarser than 6 µm were further separated by density in water into a dense fraction mostly mineral and a lighter fraction mostly organic, using a method similar to gold panning and sometimes referred to as the swirling decantation method (e.g. Shang and Tiessen, 2000). Repeatedly, soil fractions immersed in water were gently swirled in a beaker to achieve preferential resuspension of light organic particles as opposed to denser particles. Subsequently, the upper part of the beaker containing POM was poured away into another beaker. The full PSDF-procedure yields a total of 16 fractions: 6 coarse fractions denser than water, 6 coarse fractions lighter than previous fractions and 4 fine fractions (Fig. 2). Coarse dense fractions were oven dried at 105 °C, whereas coarse light and fine fractions were freeze-dried.

2.2.2 Aggregate density fractionation (ADF)

Aggregate density fractionation was performed as described by Sollins et al. (2006) to isolate different soil fractions by flotation on sodium polytungstate solutions (SPT) of varying density. Seven density fractions were isolated: < 1.65; 1.65–1.85; 1.85–2.0; 2.0–2.2; 2.2–2.4; 2.4–2.65 and $> 2.65\,\mathrm{g\,cm^{-3}}$. For more details about the procedure, see Hatton et al. (2012) or Fig. 2.

2.3 Characterization of physical fractions

The seven aggregate fractions generated by the ADF procedure are labeled A1–A7 in Figs. 3 and 4.

The PSDF procedure generates 16 fractions falling in three categories which we designate as follows:

(a) fractions $> 6\,\mu\mathrm{m}$ and with a mineral content that renders their density greater than that of water are labeled "coarse dense" (designated P7–P12 in Figs. 3 and 4),

(b) fractions $> 6\,\mu\mathrm{m}$ whose mineral content is low enough to allow them to float on water are labeled "coarse light" (labeled P1–P6 in Figs. 3 and 4),

(c) fractions $< 6\,\mu\mathrm{m}$ are labeled as "fine" (P13–P16 in Fig. 3 and 4).

2.3.1 Physical appearance

All fractions were examined with a stereomicroscope. For PSDF, fractions were directly observed after drying, whereas fractions recovered by ADF were observed after dispersion in water. Aliquots were immersed in Petri dishes (ø: 4 cm) filled with deionised water. Petri dishes were gently hand shaken to check for the presence of different density phases. Visual description was performed under a range of magnifications starting from 6× to 50×. Samples were checked for: recognizable organic debris, black carbon, aggregates, non-aggregated mineral particles (including oxides and concretions).

2.3.2 Organic matter

Total C, total N, δ^{15}N and δ^{13}C were determined in triplicate using a 20–20 coupled continuous flow elemental analyzer–isotope ratio mass spectrometer (EA-IRMS; PDZ Europa Ltd., Crewe, Cheshire, England). The degree of microbial processing was determined using δ^{13}C, δ^{15}N and C:N values as proxies (Baisden et al., 2002).

2.3.3 ^{15}N Tracer

In bulk soil and isolated fractions, the ^{15}N tracer enrichment (excess ^{15}N; E^{15}N(%)) was quantified as a proportion of total N as follows:

$$\mathrm{E}^{15}\mathrm{N}(\%) = \mathrm{A}^{15}\mathrm{N}(\%)_{\mathrm{labelled\_plot}} - \mathrm{A}^{15}\mathrm{N}(\%)_{\mathrm{control\_plot}} \qquad (1)$$

where $\mathrm{A}^{15}\mathrm{N}(\%)_{\mathrm{labelled\_plot}}$ and $\mathrm{A}^{15}\mathrm{N}(\%)_{\mathrm{control\_plot}}$ are the abundances of the ^{15}N isotope, expressed in percent of total N, in labelled and reference plots.

2.3.4 Statistics

Total C, total N, δ^{15}N and δ^{13}C data are presented as means of three replicates with their standard deviations.

2.3.5 Principal component analysis

Fractionation procedures were compared through standardized principal component analysis (PCA). The procedure reduces the overall variability in the data from the fractions by derivation of a small number of linear combinations of the original variables, called the principal components. The results of a PCA are usually discussed in terms of component scores and loadings (Shaw, 2003). Scores represent the coordinates of fractions in the new space defined by the principal components, while loadings represent the correlation between the principal components and the original variables. To perform the PCA, we combined the following original variables: C, N, C/N ratio, $A^{13}C$ (%) measured on all the fractions isolated from Ebrach and Fougères, including both labelled and control treatments. Since we did not want to separate our fractions on the basis of the ^{15}N labeling, $A^{15}N$ (%) was excluded from multivariate analyses. This way of representation constitutes a convenient way of assessing the level of similarity between fractions at a glance. Fractions whose positions are close together in the PCA plane share overall characteristics without being necessarily equivalent, i.e. an aggregate fraction can display the same carbon content as a fraction encompassing a mixture of fully dispersed mineral particles and POM.

Visualization of the label incorporation within fractions was performed by applying a contour map representing the excess of ^{15}N ($E^{15}N$) in the plane defined by the main PCs behind related scatter plots. Interpolated contour maps were obtained by kriging using a default linear variogram (slope = 1, nugget effects = 0) using the software "Surfer", version 7.02. Here the goal was to provide an easy way to visualize a three dimensional data set.

3 Results

3.1 Recoveries

Mass losses in the course of the fractionation process were characterised by calculating mass budgets as well as C and N recovery rates. For both fractionation procedures mass losses never exceeded 2 %. Carbon and nitrogen recovery rates averaged 91.4 ± 2.6 % for ADF and 91.7 ± 7.2 % for PSDF and were similar to recovery values presented by other studies (Balabane and Plante, 2004; Balesdent et al., 1998; Schmidt et al., 2000; Schmidt and Kogel-Knabner, 2002; Schöning and Kögel-Knabner, 2006).

3.2 Physical appearance of isolated fractions

Microscopic observations of separates from Fougères and Ebrach are summarized in Table 3.

For ADF, the fraction $< 1.65\,\mathrm{g\,cm^{-3}}$ was composed of free plant debris with minor encrustations of mineral grains. Fractions from 1.65 to $2.4\,\mathrm{g\,cm^{-3}}$ mostly consisted of aggregates whose content of non-aggregated mineral particles (determined after mild grinding and resuspension in water) increased with increasing density, from 10 % to 60 % of observed items.

Traces of charcoal (i.e. about 3 % of items) were identified in every fraction below a density of $2.4\,\mathrm{g\,cm^{-3}}$. Fractions $> 2.4\,\mathrm{g\,cm^{-3}}$ were mostly composed of non-aggregated mineral particles. Brown to red colored oxides and concretions were nearly exclusively observed in fractions $> 2.65\,\mathrm{g\,cm^{-3}}$ and accounted for about 10 % of the material there.

For the PSDF, coarse dense fractions were almost exclusively composed of non-aggregated mineral particles, whereas coarse light fractions were almost exclusively composed of POM. The purity of these mineral and organic fractions slightly decreased with decreasing particle size, which illustrates that it became increasingly difficult to separate organic and mineral phases by density in water as particle size approached colloidal dimensions. Oxide concretions were observed in varying proportions in coarse dense fractions. Representing in mass up to < 1 % and nearly 100 % of the > 2000 µm fractions at Fougère and Ebrach, respectively, their abundance decreased quickly with decreasing fraction size. A few particles of charcoal were identified in all coarse fractions (i.e. > 6 µm). Visual description of fine fractions was beyond the resolution of the stereomicroscope, yet it was still possible to see that the fine fractions were composed of two phases, a black one and white one with black fractions likely corresponding to low density materials and white ones to minerals.

3.3 Organic matter

For both fractionation procedures, fractions isolated from labeled and control soils had comparable C and N concentrations and C/N ratio and similar δ^{13}C values (Fig. 3).

3.4 Dry mass, total C, N

Dry mass peaked in fraction 2.4–2.65 g cm^{-3} recovered from ADF with about 58 % of total soil mass in this fraction at Fougères and 83 % at Ebrach. These values were equivalent to the mass percentage of the coarse dense fractions isolated by PSDF in both soils. Mass proportions in other fractions isolated by ADF or PSDF ranged from 1 and 10 % of dry weight. Soil mass distributions among fractions were similar between labeled and control soils (Fig. 3).

For both Ebrach and Fougères samples, C and N content of ADF fractions dropped by an order of magnitude from the lightest fraction to the two densest fractions. Some PSDF fractions exhibited some similarities with ADF fractions. Coarse dense fractions were as depleted in C and N as the ADF fractions denser than 2.4 g cm^{-3}. Coarse light fractions had on average the same C and N concentrations as the lightest ADF fractions. Their C content slightly decreased with decreasing particle size with the exception of the 63–20 µm fractions that reached a local maximum, while their N contents slightly peaked in the 63–20 µm fractions. Within fine fractions, C and N increased with decreasing particle size.

3.5 Indicators for microbial processing: C/N ratio, natural abundance in ^{13}C and ^{15}N

Within ADF fractions, C/N ratios decreased and natural abundance of ^{13}C and ^{15}N increased with increasing density from plant-like to microbe-like values (in Fig. 3; C:N ratios from 21 ± 1 to 11 ± 3; δ^{13}C from −28.0 ± 0.3 ‰ to −25.8 ± 1.7 ‰ and natural δ^{15}N from −3.4 ± 0.6 ‰ to −0.1 ± 3.2 ‰). Within the organic rich fractions generated by PSDF, i.e. coarse light and fine fractions, the same trend was observed with decreasing particle size, (C/N ratios from 28 ± 4 to 9.4 ± 4, δ^{13}C from −29.1 ± 0.3 ‰ to −26.9 ± 0.5 ‰ and natural δ^{15}N from −3.3 ± 0.8 ‰ to −1.4 ± 0.9 ‰). In the coarse dense fractions generated by PSDF, no clear trend could be observed when size decreased: C/N ratios ranged between 9.5 and 22, δ^{13}C between −29 ‰ and −25 ‰, and natural δ^{15}N between −7 ‰ and 0 ‰.

3.6 Principal component analysis

3.6.1 Gradients within the PCA plane

The two first principal components (PC) accounted for 88 % of the total variance in the samples (Fig. 4 and Table 4, PC1 66 %, PC2: 22 %). With respect to component loadings (Table 4), two gradients set at ∼45° from the PC1 and PC2 were identified (Fig. 4a). The first gradient was characterised by a decreasing C/N ratio and increasing δ^{13}C, and represented the degree of OM microbial processing from a plant-like to a microbe-like pool of OM. The second gradient follows increasing levels of C and N content and corresponds to the gradient of OM content within fractions.

3.6.2 Plot of the isolated fractions in the PCA plane

Coordinates of the isolated fractions in the plane defined by the two first components PC1 and PC2 were similar for both control and the label treatments. Therefore only fractions isolated from labeled plots are displayed in Fig. 4 (Fougères on panel b and Ebrach on panel c). A schematic interpretation of fraction distribution in the PCA plane is given in Fig. 5a.

In the PCA plane, fractions are separated along the two previously identified gradients according to their OM characteristics. Their geometric arrangement can be interpreted as indicating their proximity to either a plant or a microbe-like state of OM and between a more organic-poor and a more organic rich state. These four different states can be represented as the four sides of a parallelogram delimiting a space where all possible OM combinations may be observed (Fig. 5a). Consequently, fractions located within this space are characterized by intermediary carbon content and must be interpreted as *composite fractions** (defined as fractions neither purely organic nor purely mineral made of heterogeneous elements that may be aggregated or not).

3.7 ^{15}N Tracer distribution in soil fractions a decade after litter application

The ^{15}N tracer was applied as a pulse of labelled litter on the top of the forest humus layer. Its release into the first centimetres of mineral soil took several years as shown by Zeller and Dambrine (2011). After a decade, it was mainly located in the *two lightest ADF* fractions or in the *coarse light fractions isolated by PSDF*, revealing that these fractions acted as recipient of litter residues at the decadal-timescale (Fig. 4), and how N cycling differed across fractions.

4 Discussion

4.1 Process dynamics as inferred from the combination of fractionation procedures

The numerous trajectories and trends revealed by the principal component analysis are explained in Fig. 6 in a step by step fashion.

Fractions isolated from the ADF spread only along PC1 crossing the composite domain (see Figs. 4, 5a) from the pole of unaltered fresh OM to the pole of adsorbed microbial derived OM. This indicated that with increasing density the level of microbial processing increased and the OM content decreased, and confirms the aggregated nature of mid-density fractions (Fig. 5).

Fractions isolated from the PSDF followed a fundamentally different pattern and were discriminated according to both principal components. Figures 3 and 4 show that the three groups of PSDF fractions (i.e. coarse-dense, coarse-light and fine-fractions) were well separated.

Coarse light fractions (upper right hand corner, Figs. 4, 5a) were characterised by high positive scores on PC1 and PC2 that decreased with decreasing fraction size. Fractions $> 63\,\mu m$ (P1, P2, P3 and P4) are on the gradient of increasing degree of OM microbial processing when they get finer. The application of ultrasound dispersion to particles less than $63\,\mu m$ induced an increase of OM content from P4 to P5 (see also Fig. 6 step 4). This process may have removed mineral matter loosely attached to organic 63–20 µm particles or may have redistributed OM from finer fractions, although several authors demonstrated ultrasonic dispersion has minor effects on OM redistribution within fine fractions (Morra et al., 1991; Oorts et al., 2005; Schmidt et al., 1999a; Yang et al., 2009). The next finer coarse light fractions (20–6 µm, P6) show less OM and more mineral matter. This increase of mineral matter from P5 to P6 was also evident from visual observation and is likely to result from an imperfect separation of organic matter from mineral particles during suspension in water (see also Fig. 6 step 3). In theory, had the dispersion and the separation of organic and mineral phases been ideal, coarse light fractions should have been restricted to the pole of pure OM and evolved from a plant like to a more microbe like signature without intruding the composite domain.

Coarse dense fractions (upper left hand corner, Figs. 4, 5a) were characterised by high negative scores on PC1 and high positive scores on PC2. They were almost exclusively composed by non-aggregated mineral particles, had very low OM content and differed mainly from each other by the degree to which OM has undergone microbial processing, which explains why they were aligned parallel to the plant-microbe gradient on the adsorbed OM pole (Figs. 4, 5a). However, decreasing particle size was not equivocally related to increasing degree of microbial alteration. This could in theory result from a lack of analytical sensitivity at low OM contents, but visual observations also suggested that some black carbon particle may have interfered with the detection of consistent change related to decreasing particle size. There have been reports of high density for black carbon particles (up to $2.4\,\mathrm{g\,cm^{-3}}$ according to Glaser et al., 2000), which would clearly make it impossible to segregate dense black carbon from mineral matter of similar density. Moreover, the imperfection of the density separation in water might be responsible for a contamination of the mineral phase by organic materials, negligible in terms of overall mass but significant in terms of OM content (see also Fig. 6 step 8).

Fine fractions (Aligned with PC2 in the centre, Figs. 4, 5a) were parallel with the PC2 axis and are characterised by PC2 scores that decreased with decreasing size of particles, indicating that OM content and the level of microbial processing increased with decreasing particle size (from P13 going to P16). The fact that fine fractions are located in the composite domain (see also Fig. 6 upper part) confirms that they combine all small scale organic or mineral debris that are generated by the ultra-sonic dispersion. Increasing OM content with decreasing particle size may be explained by (i) a lower efficiency of ultrasonic dispersion to detach OM patches from small clay particles than from larger silt particles as suggested by Chenu and Plante (2006), (ii) the accumulation of colloidal OM released by the previous dispersion of coarser aggregates or (iii) may result from variations in settling velocities as a result of small variations in density. The unavoidable violation of the conditions for the application of Stokes law (spherical shape, uniform density) will have introduced an error and will have enhanced the organic content of fine fractions as the speed of sedimentation of light organic-rich particles is slower than the one of heavy mineral grains, for a given diameter (see also Fig. 5 step 2). Although the (P16) fraction < 0.035 µm was relatively depleted in mineral matter, its carbon content suggests that it might not be completely mineral free. Therefore P16 might not qualify as dissolved organic matter (DOM).

4.2 ^{15}N tracer distribution and evaluation of the turnover of processes captured by physical fractions

The superimposition of the ^{15}N excess map to the PCA plane provides several insights in the contrasted N cycling processes captured by individual fractions (Fig. 4).

Of particular interest are the coarse light PSDF fractions from P1 to P4 that displayed contrasting levels of ^{15}N incorporation. As they plot on the pole of pure OM, they keyed out as a functionally homogeneous group especially relevant to capture such processes that are directly related to decomposer activity and can be considered as controlling N cycling, with no influence from the mineral phases. Thus the maximum of ^{15}N tracer was located within the 630–200 µm fraction (P3) at Fougères but within the 200–63 µm fraction (P4) at Ebrach. This indicates that, independent from the time elapsed since tracer application; the mesofauna fragmentation and microbial transformation of the labelled litter was more advanced at Ebrach than at Fougères.

Contrastingly, *mineral-rich fractions* that are the coarse dense PSDF fractions and the ADF fractions above 1.85 g cm^{-3}, incorporated very little tracer-N a decade after its application. This shows that they correspond to reservoirs of older organic N in forest soil. They either cycled nitrogen very slowly, or did not come into contact with the label on a decadal time scale, possibly because the label was preferentially retained in the OM rich fractions. A distinction must be done between these mineral-rich fractions isolated either by size or density regarding their relevance to N cycling processes though. Coarse dense PSDF fractions consisting of individual grains coated with adsorbed OM exhibited no real difference in term of ^{15}N tracer incorporation. This might be due to a level of tracer close to the detection limit of the instrument, or to the fact that size separation of coarse dense fractions was neither able nor expected to separate defined mineralogical fractions that would have influenced the nitrogen dynamic. More interestingly, the (low) level of tracer incorporation in ADF fractions progressively decreased as density increased. These fractions thus appear to be particularly promising as tools for the investigation of time-dependent N sequestration mechanisms (see Hatton et al., 2012 for example).

The absence of contrast in tracer enrichment in the finest fractions isolated by PSDF probably resulted from the incomplete dispersion and separation procedure. This confirmed the methodological artefacts already revealed by the location of the fraction in the PCA plane and clearly demonstrated that fine particle size fractions can not be treated as functional soil compartments regarding N and OM cycling. They constitute a heterogeneous mixture of debris and leftovers of both mineral and organic sorts and can not be expected to exist as an identifiable fraction in natural soil.

4.3 Derivation of functional soil compartments (FSC's) among physical fractions

These observations on OM characteristics and N cycling can be synthesized to identify *functional soil compartments (FSC)**, which we define as groups of fractions for which organic matter transformation and decomposition processes are controlled by fundamentally different process regimes (Table 5). In other words, FSC's* represent an extra level of organisation, encompassing physical fractions in which the majority of constitutive elements (i.e. particles or aggregates) undergo the same combination of decomposition processes.

We identified three groups of physical fractions that could be regarded as FSC*. They are listed below and on Figs. 4 and 5.

1. Free particulate organic matter (POM), where litter derived-N resides for about one decade in forest ecosystems (A1, P1–6).
2. Non-aggregated mineral particles with adsorbed organic matter, location of N cycling processes occurring on timescale longer than decades (A6–A7, P7–P12).
3. Aggregates, also determining N dynamics on pluridecadal time scales in soil (A2–A5).

4.4 PSDF versus ADF procedures: recommendations

In addition to our goal of investigating the general usefulness of soil fractionation for the isolation of ecologically relevant soil subunits, we examined how the process of soil fractionation can be economised such that only meaningful fractions are being retrieved. Below we offer some considerations to help with the choice of fractionation procedure.

As revealed previously, both ADF and PSDF successfully isolate the functional soil compartment POM and non-aggregated mineral particles coated with OM. To help with the decision for any of the two, we recommend favouring the fractionation procedure that better mimics the natural processes. PDSF is better suited than ADF to investigate the dynamic of POM, as POM sizes are directly related to the natural fragmentation that occurs with the decomposition process. In the case of mineral particles associated with adsorbed carbon, ADF isolated fewer of these fractions (i.e. A6 and A7) than PSDF (i.e. P7–P12). Yet, since the mineralogy and the subsequent adsorption capacity of a particle are more directly linked to its density than to its size, the ADF must be considered to be more appropriate for the investigation of the dynamics of adsorbed OM than the PSDF. Finally, only ADF enables the study of aggregates.

As a conclusion, the two fractionation methods can not be considered alternatives; they must be seen as complementary. However, we recognized 4 major methodological issues of the PSDF procedure which need to be considered when data are interpreted:

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1. Density separation in water as performed in PSDF is not designed to achieve strict separation of the mineral and organic phases and therefore generates slight impurities. Coarse dense and coarse light fractions were slightly polluted with residual POM and black carbon in one case, as well as mineral grains in the other case. In the case of coarse light fractions the OC pollution was negligible, though (see also Fig. 6 steps 3 and 8).

2. There is an absence of good separation within the fine fractions, which are obviously composed of phases with contrasting density in contradiction with the sedimentation separation methods which require a unique density for all particles (Stokes' Law) (see also Fig. 6 step 5).

3. Separating coarse dense fractions by size was not very informative.

4. Ultrasonic dispersion at the energy level chosen for this work (i.e. 320 $\mathrm{J\,ml^{-1}}$ delivered over a 20 min period of time) does not achieve complete dispersion at submicron scales, and was responsible for slight redistributions in the finest coarse light fractions (i.e. P5, P6) (see also Fig. 6 step 4).

In response to this situation, we propose a PSDF fractionation protocol that reduces the creation of ecologically irrelevant fractions. To do so, we recommend separating the mineral from the organic phases directly after the dispersion procedures, prior to size separations by wet sieving and sedimentation. To avoid interference of dense organic phases (some forms of charcoal) we suggest to adjust the density of the separating liquid to 2.4 $\mathrm{g\,cm^{-3}}$ (using a solution of sodium polytungstate) to exclude all residual POM as well as a maximum of black carbon particles from the mineral phase. Finally, we propose to abandon the size separation of the mineral fraction ($d > 2.4\,\mathrm{g\,cm^{-3}}$). In oxide rich soils, these fractions could eventually be pooled and further separated by density at 2.65 $\mathrm{g\,cm^{-3}}$ to obtain reactive and less reactive mineral fractions (Fig. 7). Ultrasonication, although unable to achieve complete dispersion at the submicron scale is still extremely efficient at larger size scales and should be kept for that reason. Finally, the size separation of light fraction < 20 µm would be performed after density exclusion of dense fractions to limit the effects of varying particle density during sedimentation. The characteristics of the fractions recovered with this new procedure are displayed in Fig. 5b.

Most contemporary PSDF procedures opt for a separation of the organic phase at ∼1.6–1.8 g cm^{-3} after various degrees of dispersion (e.g. Bruun et al., 2008; Magid et al., 2010; Paul et al., 2008; Shang and Tiessen, 2000; Sohi et al., 2001). In theory this standard method allows the separation of the organic from the mineral phase in most soils, however if applied in soils that contain substantial amounts of black carbon particles and pedogenic oxides, it will not be possible to isolate confounding effects of charcoal when interpreting the fractionation data.

All these issues are resolved with the proposed fractionation procedure (Fig. 7) while keeping the advantages of the old methods.

5 Conclusions

1. We set out to test whether physical fractionation would allow us to identify functional subunits of the soil fabric and their associated process dynamics. We demonstrated how physical fractions can be separated into well-defined functional categories (Table 5) and we showed that both fractionation procedures brought complementary information about SOM dynamics. PSDF fractionation was more adapted to the investigation of the natural fragmentation of POM at decadal time scale whereas ADF was more adapted to the study of adsorption and aggregations at pluridecadal time scale. This suggests that SOM dynamics can not be fully understood when using a unique single step fractionation procedure.

2. We evidenced a few defects in the PSDF procedure that might be wide spread among similar procedures, the most important one being that fine fractions isolated after sonication can not be linked to any defined decomposition pathway or

stabilisation process. An improved PSDF fractionation procedure was proposed to address most of the methodological issues observed.

3. Our work demonstrated that it is fundamentally possible to use physical fractionation for the purpose of isolating organic matter of progressing decomposition stage from soils. This applies to organic matter associated with minerals as well as to particulate organic matter. Figure 7 illustrates how this purpose can be achieved through the application of an optimised, combined fractionation scheme.

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Table 1. Definitions.

"True" aggregate fraction:	Fraction that contains only aggregates, opposed to non-fully dispersed fractions including a mixture of aggregates, POM and fully dispersed mineral particles, which are sometimes referred as aggregates fraction.
Single step fractionation procedure:	Fractionation procedure that involves a single dispersion step followed by a separation step that could include a size, a density or a size-density separation.
Multiple step fractionation procedure:	Fractionation procedure that involves the re-dispersion of fractions obtained from a single step fractionation procedure. The redispersion can only occur on aggregate fractions.
Composite fractions:	Soil fractions made of heterogeneous elements aggregated or not. These heterogeneous elements can be fully dispersed mineral particles, POM, oxide concretions, black carbon.
Functional soil Compartment (FSC):	Represents an extra level of organisation gathering groups of physical fractions in which the majority of constitutive elements (i.e. particles or aggregates) undergo the same combination of decomposition processes.

Table 2. Basic soil properties of the 0–2.5 horizon.

C	N	pH_{H_2O}	$CEC_e^\dagger$	Base saturation†	Years after the ^{15}N pulse	Remaining ^{15}N tracer	Texture (%)		
($mg\,g^{-1}$)	($mg\,g^{-1}$)		($mmol\,kg^{-1}$)	(%)	(yr)	(%)	Sand	Silt	Clay
Ebrach:dystric Cambisol									
45.9	2.0	3.9	87.8	34.5	12	11	76.2	19.0	4.8
Fougères: glossalbic Cambisol									
79.5	4.7	3.8	87	26	8	15	2.5	85.5	12.0

Data from Hatton et al. (2011).

† Values from depth 0–5 cm.

Table 3. Visual description of fractions.

Fractions Names	Ebrach: dystric Cambisol Mineral phase Quartz, Feldspar	 Concretions/ oxides	 Organic phase POM	 Aggregates	 Black carbon	Fougères: glossalbic Cambisol Mineral phase Quartz, Feldspar	 Concretions/ oxides	 Organic phase POM	 Aggregates	 Black carbon
Particle Size Density Fractionation (PSDF)										
Coarse dense										
> 2000 µm		*100 %*				*99 %*	+++			+
2000–630 µm	*50 %*	*50 %*			+	*99 %*	++			+
630–200 µm	*99 %*		+		+	*99 %*	++	+		+
200–63 µm	*99 %*		+		+	*99 %*	+	+		+
63–20 µm	*99 %*		++		+	*99 %*		++		+
20–6 µm	*99 %*		++		+	*99 %*		++		+
Coarse light										
> 2000 µm			99 %		+			99 %		+
2000–630 µm			99 %		+			99 %		+
630–200 µm	+		99 %		+	+		99 %		+
200–63 µm	+		99 %		+	+		99 %		+
63–20 µm	++		99 %		+	++		99 %		+
20–6 µm	++		99 %		+	++		99 %		+
Fine fractions <6 µm	**Stereomicroscope insufficient resolution 2 distinct phases: a dark light organic and a dense clear mineral**					**Stereomicroscope insufficient resolution 2 distinct phases: a dark light organic and a dense clear mineral**				
Aggregates Density Fractionation (ADF)										
Density Fractions										
< 1.65 g cm^{-3}	+		99 %		+	+		99 %		+
1.65–1.85 g cm^{-3}	**10 %**		**+++**	**89 %**	**+**	**10 %**		**+++**	**89 %**	**+**
1.85–2.0 g cm^{-3}	**16 %**		**++**	**83 %**	**+**	**16 %**		**++**	**83 %**	**+**
2.0–2.2 g cm^{-3}	**26 %**		**+**	**73 %**	**+**	**21 %**		**+**	**78 %**	**+**
2.2–2.4 g cm^{-3}	**40 %**		**+**	**59 %**	**+**	**35 %**		**+**	**64 %**	**+**
2.4–2.65 g cm^{-3}	99 %	+	+			99 %	+	+		
> 2.65 g cm^{-3}	80 %	20 %	+			80 %	20 %	+		

+, ++, +++ The fraction element is present as trace of increasing importance.
Fractions susceptible to be composite in term of OM.
Fractions composite* in term of OM.

Table 4. Eigenvalues and percentage of explained variance for the fraction datasets on OM quality.

	PC1	PC 2
Eigenvalues	2.645	0.893
Variance (%)	66.13	22.32
Correlation coefficients (loading) between the original reduced data and the two first components		
	PC1	PC2
C	0.948	−0.265
N	0.787	−0.599
C/N ratio	0.770	0.465
A^{13}C	−0.731	−0.497

PC: principal component.

Table 5. Identification of functional soil compartment* (FSC's) among physical fractions.

Soil subunit	Represented by	FSC?	Associated processes and their *timescale*	Remarks
Particulate organic matter (POM)	PSDF: (P1 to P6) and ADF: (A1)	Yes	Progressive processing by mesofauna, provides adhesive nuclei for aggregate formation *Year to decade*	POM fractions isolated by PSDF represent a decomposition gradient that can be seen as representing the litter – soil organic matter transition. ADF with only one fraction can not characterize the dynamics in this cohort. Changes in this fraction are independent from interactions with minerals.
Aggregated stuctures	ADF: (A2 to A5)	Yes	Physical isolation of substrate from decomposition actors and factors within defined micro-environments (i.e. microbes, O_2 and H_2O supply, etc.) *Several decades*	ADF is effective at isolating functional micro-structures. The biogeochemical stability of individual aggregates, the proportion of mineral materials and the microbial characteristics increase with increasing aggregate density.
Coarse mineral grain coated with OM	PSDF: (P7 to P12) and ADF: (A6 to A7)	Yes	All processes that are controlled by surface chemistry, including adsorption, electron transfer, catalytic effects. Involved dissolved organic matter generated in any stage of the dynamics *Several decades*	Organic matter associated with the mineral grains isolated by ADF had a greater microbial character than such as was isolated by PSDF.
Residuals	PSDF: (P13 to P16)	No	Does not represent specific soil process	These fractions represent a mixture of mineral and organic materials that (1) were incompletely dispersed, that (2) could not be separated by density in water and that (3) could not be properly separated by size using the single density assumption of Stoke's law. These materials were not necessarily joined or even co-localized in soil.

PSDF = Particle size density fractionation, ADF = Aggregate density fractionation.

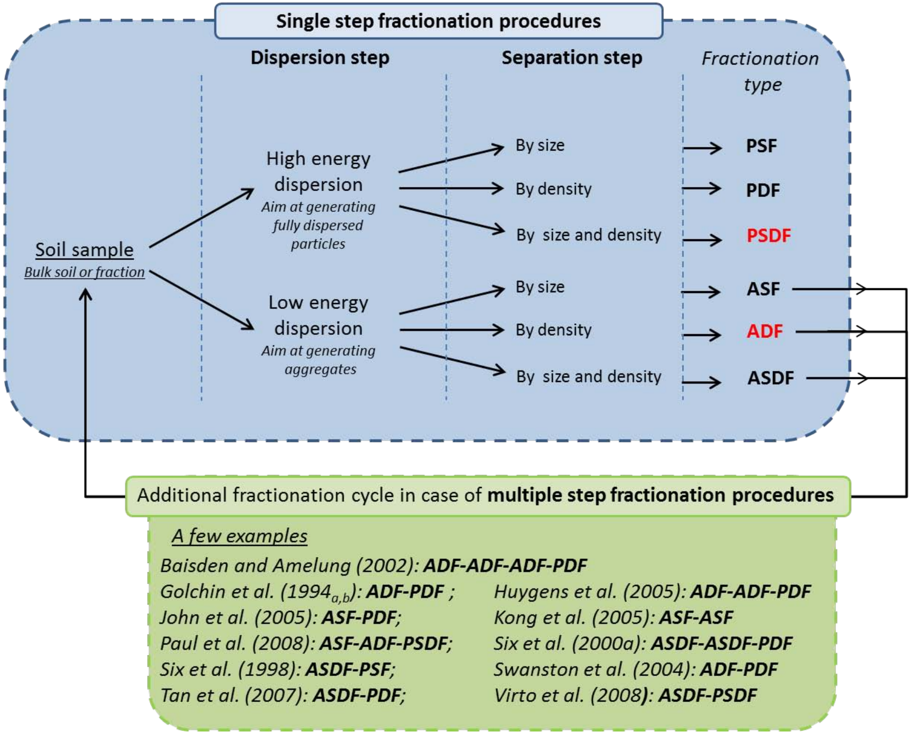

Fig. 1. Flow chart to illustrate methodological differences between soil fractionation procedures. PDF = Particle density fractionation; PSF = Particle size fractionation; ADF = aggregate density fractionation; ASF = aggregate size fractionation; PSDF = Particle size density fractionation and ASDF = aggregate size density fractionation. ADF and PSDF are highlighted in red because they were chosen for this study.

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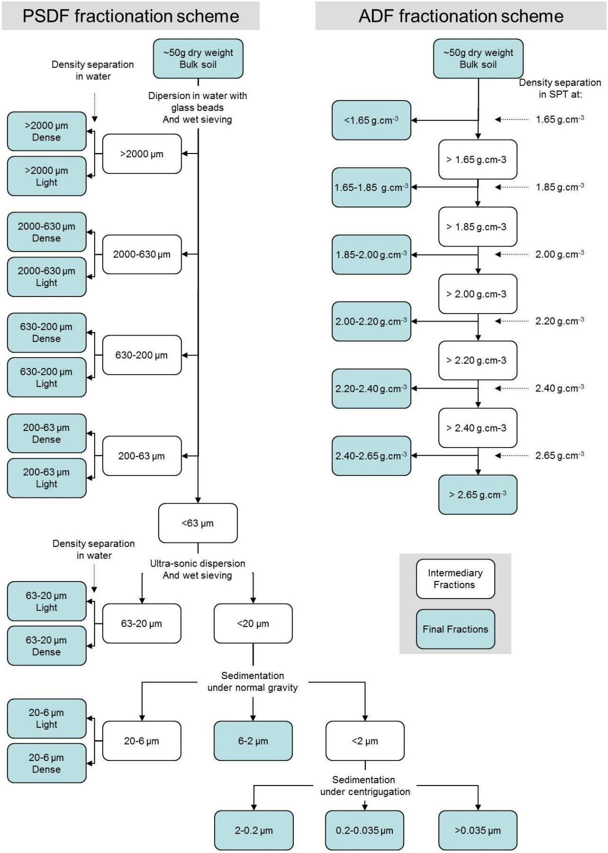

Fig. 2. PSDF and ADF fractionation schemes.

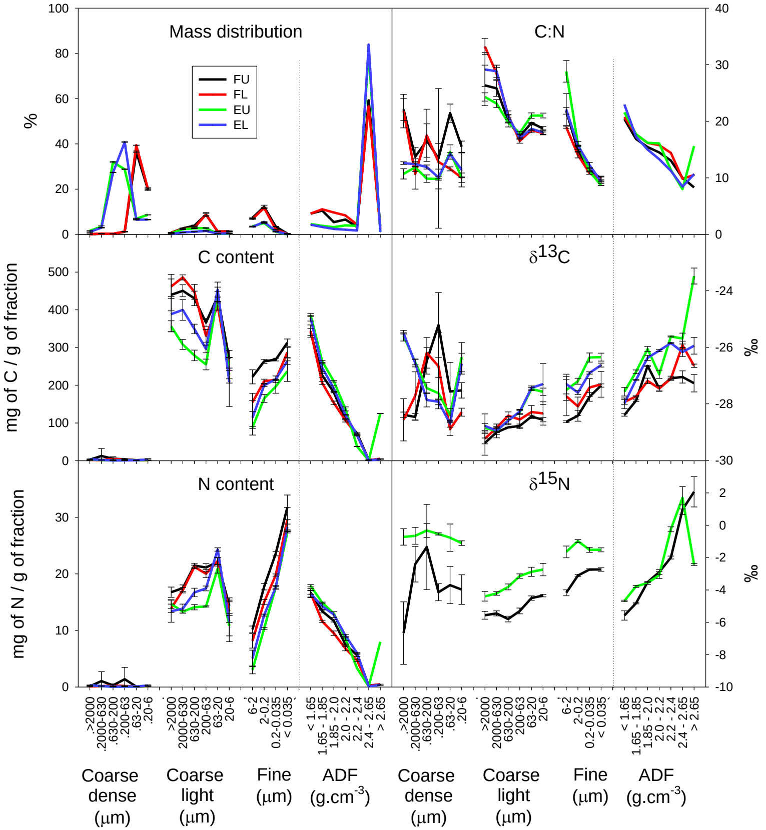

Fig. 3. Chemical characteristics of PSDF and ADF fractions isolated from Ebrach and Fougères labelled and unlabelled soils. (Mass distribution, C and N contents, C/N ratio, as well as, δ^{13}C and δ^{15}N natural abundances.) Need to include separation line between PSF and AF mass distribution.

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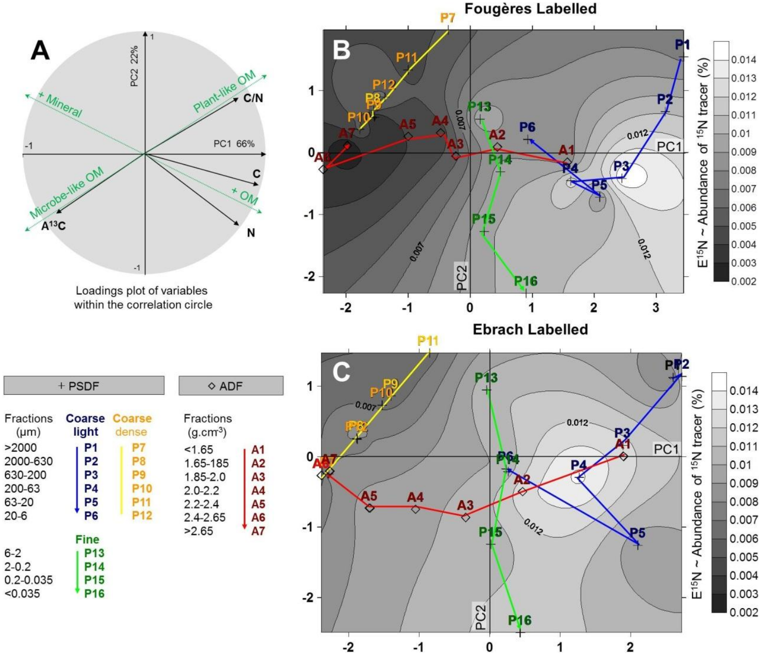

Fig. 4. Principal component analyses (PCA). PCA was performed for both sites independently on fractions isolated from labelled and unlabelled soil samples. Fractions were discriminated as a function of their C and N content, C/N ratio and the abundance of ^{13}C, A^{13}C. **(A)** Correlation circle between the new components and the original variables. **(B)** and **(C)** Score plots for labelled soil fractions only. Visualization of ^{15}N tracer abundance (see Eq. 1) within fractions was performed by applying the relevant contour map as a background image. Interpolated contour maps were obtained by kriging using a default linear variogram (slope = 1, nuggets effects = 0) using the software ″Surfer v. 7.02). Fractions are grouped for similarity based on their level of affiliation with one of four clearly discernible trajectories (indicated by line style) within the PCA plane. A synthetized representation of the PCA results as well as its visual interpretation is given in Figs. 5 and 6.

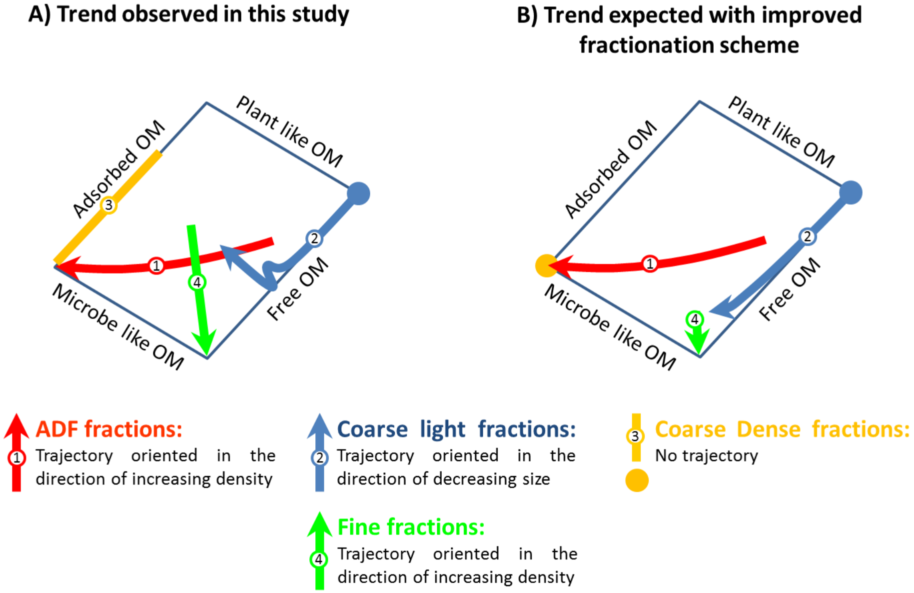

Fig. 5. Schematic representation of **(A)** the PCA results and **(B)** the change that could be expected in the PCA plan after application of the improved PSDF fractionation procedure suggested for future work in Fig. 6.

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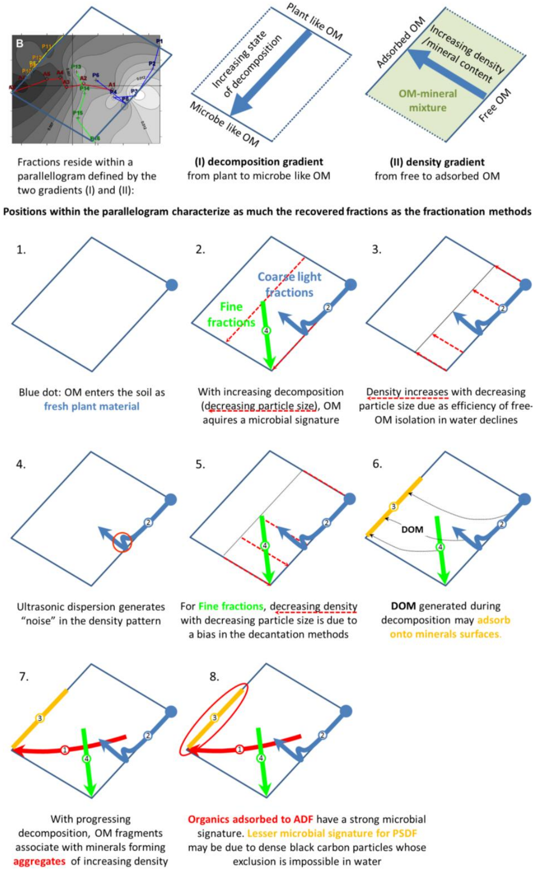

Fractions reside within a parallellogram defined by the two gradients (I) and (II):

(I) decomposition gradient from plant to microbe like OM

(II) density gradient from free to adsorbed OM

Positions within the parallelogram characterize as much the recovered fractions as the fractionation methods

Blue dot: OM enters the soil as fresh plant material

With increasing decomposition (decreasing particle size), OM aquires a microbial signature

Density increases with decreasing particle size due as efficiency of free-OM isolation in water declines

Ultrasonic dispersion generates "noise" in the density pattern

For Fine fractions, decreasing density with decreasing particle size is due to a bias in the decantation methods

DOM generated during decomposition may adsorb onto minerals surfaces.

With progressing decomposition, OM fragments associate with minerals forming **aggregates** of increasing density

Organics adsorbed to ADF have a strong microbial signature. Lesser microbial signature for PSDF may be due to dense black carbon particles whose exclusion is impossible in water

Fig. 6. Interpretative schemes of the PCA analysis presented Fig. 4. Meanings of arrows are describes in Fig. 5. The green zone in the upper right scheme represents a zone where fractions must be composite* (i.e. made of heteroclite elements aggregated or not). Thin dashed red arrows highlight the component of OM processing that is being discussed.

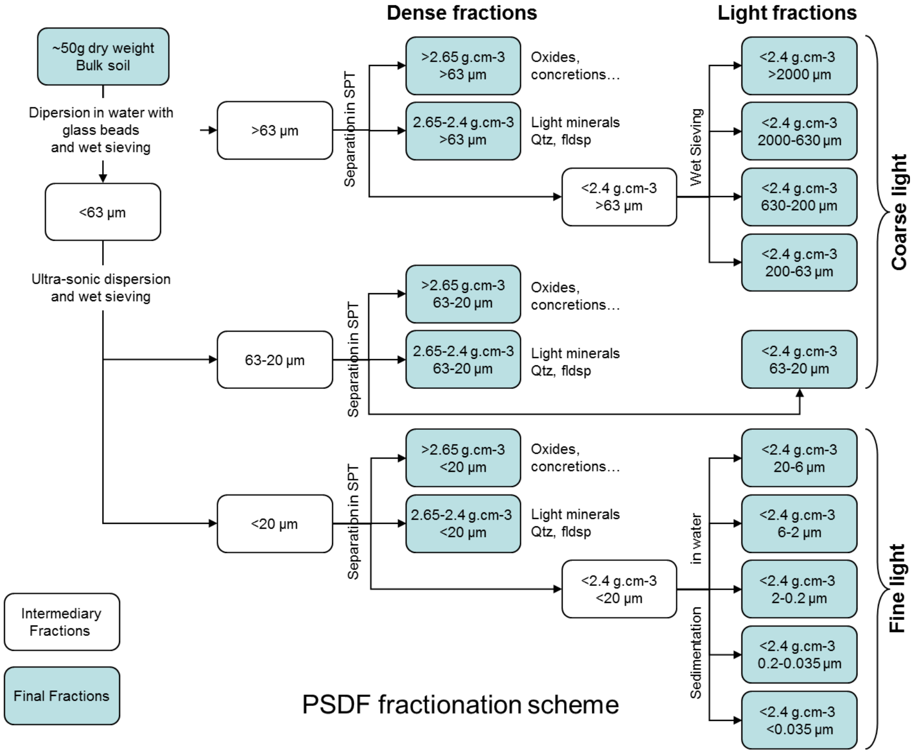

Fig. 7. Suggestion for the improvement of the PSDF fractionation scheme.