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A Comprehensive Method for Fractionating Soil Organic Matter Not Protected and Protected from Decomposition by Physical and Chemical Mechanisms

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A comprehensive method for fractionating soil organic matter into pools not protected and protected from decomposition by physical and chemical mechanisms

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Running title: Fractionation of soil organic matter

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Abbreviations: CPMAS, cross-polarization magic-angle spinning; NMR, nuclear magnetic resonance; SOM, soil organic matter.

Keywords: Aggregates; C stabilization; Nuclear magnetic resonance; Physical fractionation; Soil organic matter

Abstract

The objective of this work was to describe a method for isolating meaningful and measurable soil organic matter (SOM) pools that differ in the mechanisms by which they are protected from decomposition. The method is appropriate for soil C stabilization and sequestration studies and is different from previous ones in that it allows free SOM located between aggregates (unprotected C pool) and SOM occluded within both macroaggregates and microaggregates (C weakly and strongly protected by physical mechanisms, respectively) to be recovered separately, freed from the soil mineral matrix and the mineral-associated SOM pool (C pool protected by chemical mechanisms) and thus well suited to advanced chemical characterization by ^{13}C nuclear magnetic resonance. Briefly, free SOM is isolated by an initial density separation. Stable macroaggregates are broken up into stable microaggregates and intra-macroaggregate SOM, which is then separated by density. Finally, intra-microaggregate SOM is isolated from mineral-associated SOM by a third density separation after ultrasonic disruption. The SOM dissolved during the fractionation procedure is also recovered. Results obtained on soil samples with contrasting textures suggested that clay content induces a decrease of the proportion of free organic C and an increase of mineral-associated organic C content. Free SOM is characterized by a marked presence of undecayed organic materials and biologically labile substances, such as carbohydrates and proteins. In contrast, SOM occluded within aggregates, especially within microaggregates, represents a more decomposed fraction, relatively enriched in unsubstituted-aliphatic materials, most probably lipid biopolymers.

1 Introduction

Soil organic matter (SOM) comprises a wide variety of plant- and animal-derived components differently susceptible to degradation, having cycling rates from weeks to millennia [1]. According to widely accepted concepts, mechanisms of SOM stabilization and protection from decomposition include: (a) occlusion within soil aggregates (physical protection), which is due to the spatial inaccessibility for decomposers, limited microbial turnover due to soil microbiota protection from predation, reduced diffusion of enzymes, and limited O_2 diffusion; (b) intimate interaction with mineral particles (chemical protection), which leads to a reduced capacity of microorganisms to decompose bound substrates and to conformational changes of organic molecules which make them unavailable for soil enzymes; and (c) selective preservation and formation of SOM compounds with molecular structures more resistant to decomposition (biochemical protection) [2-4].

There is a wide body of evidence in the literature indicating that indicates that physical protection of SOM depends on the level of aggregation. In particular, C stability has shown to be much greater within microaggregates than within macroaggregates [5-7]. Based on these observations, Six et al. [3] developed a successful fractionation scheme for isolating meaningful and measurable pools of SOM that differ in cycling rates. This procedure isolates: (a) an unprotected C pool, which consists of free coarse particulate organic matter (i.e. > 250 μm) and represents the most labile material; (b) a physically protected C pool, consisting of the fine organic matter occluded within microaggregates; (c) a chemically-protected C pool, consisting of the C associated with the silt- and clay-sized fractions; and (d) a biochemically-protected C pool, i.e., the nonhydrolyzable organic fraction that represents the oldest and most resistant C pool in temperate soils. Briefly, coarse non-protected particulate organic matter, microaggregates, and silt- and clay-associated C are isolated by a method that

accomplishes a complete break up of macroaggregates without breaking up microaggregates, which are then separated by wet sieving. Fine unprotected particulate organic matter that is collected together with the microaggregates (i.e. 53-250 μm) is isolated by density flotation. Subsequently, microaggregates are dispersed and wet sieved to isolate microaggregate protected C versus silt and clay associated C. Finally, the silt- and clay-associated C fractions are hydrolyzed to differentiate the silt and clay protected C versus biochemically protected C.

Advanced molecular characterization of unprotected and physically protected SOM fractions is of intrinsic importance to analyze C transformation and stabilization processes in soils. Nuclear magnetic resonance (NMR) is among the most powerful analytical tools for investigating SOM structure and composition, because it can provide information on the abundance of specific functional groups and structural entities [8-10]. However, the presence of soil mineral components is problematic during NMR analysis [8, 11, 12], and has limited the application of this analytical tool to the study of the microaggregate SOM pool isolated by the method described by Six et al. [3].

Some authors have proposed physical fractionation procedures that are based on an initial density separation, which yields free light organic matter, and a second density separation after ultrasonic disruption, which gives light intra-aggregate organic matter freed from the soil mineral matrix [3-15]. In broad outline, NMR results obtained by these authors indicate that the proportion of O-alkyl C is lower and that of alkyl C higher in the occluded light fraction than in the free light fraction, suggesting that the occluded light fraction comprises more decomposed and transformed organic matter relative to the free. Despite the importance for a better understanding of the mechanisms of C storage in soils, however, these studies and the method described by Six et al. [3] do not distinguish SOM pools associated with different aggregate sizes.

The objective of this work was to describe a comprehensive method for isolating

functionally different SOM fractions mainly based on Six et al. [3] and Sohi et al. [14] that allows to recover separately free SOM located between aggregates (unprotected C pool) and SOM occluded within both macroaggregates and microaggregates (C pool weakly and strongly protected by physical mechanisms, respectively) freed from the soil mineral matrix and the mineral-associated SOM pool (C pool protected by chemical mechanisms) and thus well suited to advanced chemical characterization by NMR.

2 Experimental

2.1 Soil fractionation

The fractionation procedure, which is schematized in Fig. 1, uses a combination of density, aggregate fractionation, and sonication procedures to separate (a) free SOM between aggregates (unprotected C pool), (b) SOM occluded within macroaggregates (C pool weakly protected by physical mechanisms), (c) SOM occluded within microaggregates (C pool strongly protected by physical mechanisms), and (d) SOM associated with the mineral fractions (chemically-protected C pool); the SOM dissolved during the fractionation is also collected. In a first step, 150 mL of sodium polytungstate at a density of 1.85 g mL^{-1} is added to 20.00 g of 2-mm-sieved, air-dried soil in a 250-mL polycarbonate centrifuge bottle. The bottle is rotated at 1 revolution s^{-1} for 30 s in an overhead shaker and centrifuged at 13900 × g for 30 min. The floating light fraction (free particulate SOM) is separated from the heavy fraction by suction and filtration through a glass fiber filter (particle retention in liquid, 1.6 µm; GF/A, Whatman, UK). In a second step, macroaggregates in the heavy fraction are broken up into stable microaggregates by using a microaggregate isolator [6, 16]. In this step, the content of the centrifuge bottle (i.e., the heavy fraction) is gently transferred to the top of a 250-µm sieve, immersed in deionized water, and then shaken with 50 stainless steel beads (4 mm in diameter) at 150 strokes min^{-1} on a reciprocating shaker under a continuous,

steady deionized water flow (about 0.2 mL min^{-1}). Microaggregates and other soil components smaller than 250 µm are immediately flushed through the device and transferred to a beaker, thus preventing the fragmentation of the microaggregates freed. Shaking is stopped when water below the 250-µm sieve run clear and after visually checking that all macroaggregates are broken. In a third step, the fraction flushed through the 250-µm sieve mixed with the fraction collected over the sieve is oven-dried at 70 ºC and then gently transferred into a 250-mL polycarbonate centrifuge bottle together with the filtrate from the first step (sodium polytungstate solution). The bottle is rotated at 1 revolution s^{-1} for 30 s and centrifuged at 13900 × g for 30 min. The floating particles (intra-macroaggregate SOM) are separated from the heavy fraction by suction and filtration through a glass fiber filter. Finally, the heavy fraction is resuspended and dispersed in the sodium polytungstate solution from the third step by sonicating at an energy input of 1500 J g^{-1}. According to previous studies on a variety of soils, this ultrasonic dispersion energy is enough to maximize the breakdown of all aggregates [14] while not causing detachment of SOM from primary organomineral complexes and redistribution between fractions [17]. The floating particles (intra-microaggregate SOM) are recovered after centrifugation using the same procedure described above for free and intra-macroaggregate SOM fractions (i.e., suction and filtration through a glass fiber filter).

In the first step, sieves larger than 2 mm can be used to allow coarser aggregate sizes. As in the procedure described by Sohi et al. [14], the shaking and especially the centrifugation in this fractionation scheme may break up weak macroaggregares. The set of shaking and centrifugation conditions (especially in terms of volume, speed, and time) arguably define the intra-macroaggregate SOM proportion that is released during this step. For sake of simplicity, we used the terms "free SOM" and "intra-macroaggregate SOM" in this work, even though the terms "free SOM and SOM

occulded within weak macroaggregates" and "SOM occluded within strong macroaggregates", respectively, would be more accurate.

The residual heavy organomineral fraction recovered in the last step (mineral-associated SOM) can be separated into sand-, silt-, and clay-size fractions as in Sohi et al. [14]. Further, organomineral fractions can be hydrolyzed as in Six et al. [3] to differentiate mineral protected C versus biochemically protected C. It is noteworthy that, unlike in Six et al. [3], the method reported here allows recovering the dissolved C remaining in the fractionation medium and, also importantly, avoids the manual wet sieving procedure to separate the mineral-associated C from the microaggregate C pool, which is strongly dependent upon the operator's ability.

2.2 Soils

The protocol was tested using four replicates of two agricultural top soil samples (0-20 cm) with contrasting texture (Table 1). The fractions obtained were oven-dried at 70 ºC, weighed, and ground with a mortar. Free and intra-aggregate fractions were ground with the glass fiber filters to recover fine embedded particles [14]. For the NMR analysis, composite samples were prepared by mixing equal weights of the four corresponding replicates of each soil and soil fraction. Composite samples of the SOM dissolved during the fractionation procedure (i.e., SOM remaining in the sodium polytungstate solution) were also prepared by mixing equal volumes of the four replicates.

2.3 Analytical methods

Total C and N contents of soils and SOM fractions were determined using an Elementar Analysensysteme (Hanau, Germany) Vario MACRO CHNS elemental analyzer, inorganic C content was determined with a Shimadzu (Duisburg, Germany) TOC-5000A analyzer equipped with a SSM-5000 solid sample module, and total organic C content was computed as the difference between total C and inorganic C.

Total C, inorganic C, and total organic C remaining in the composite samples of sodium polytungstate solutions after fractionation (i.e., dissolved SOM pool) were determined in triplicate using a Shimadzu TOC 5000A analyzer.

The solid-state cross-polarization magic-angle spinning (CPMAS) ^{13}C NMR spectra of soils and soil fractions were obtained on a Bruker (Billerica, MA) Avance 400 MHz NMR spectrometer, operating at a frequency of 100.62 MHz. Approximately 200 mg of sample was packed into a zirconium rotor of 4-mm outer diameter with a Kel-F cap. The rotor spin rate was set at 5 kHz. Forty thousand scans were accumulated for each sample with a pulse delay of 0.5 s and a contact time of 3 ms. The NMR free induction decay signals were digitized and Fourier transformed after application of a line broadening of 80 Hz. The CPMAS ^{13}C-NMR spectra were then baseline corrected and integrated into the following chemical shift regions: 0-45 ppm, aliphatic C; 45-110 ppm, substituted-aliphatic C, including alcohols, amines, carbohydrates, ethers, methoxyl, and acetal C; 110-160 ppm, aromatic C; and 160-220 ppm, carboxyl and carbonyl C. The ^{13}C chemical shifts are reported relative to adamantane.

3 Results and discussion

3.1 Recoveries, C and N contents, and C/N ratio

Total organic C recovery in free, aggregate, and mineral-associated pools after fractionation is 94.5% for soil A and 91.6% for soil B (Table 2). The recovery rate increases to 102.3% and 101.8%, respectively, when the organic C dissolved during the fractionation procedure is taken into account (Table 2).

Of the total organic C pool, free organic C accounts for 28.8% in soil A and 37.6% in soil B, intra-macroaggragate organic C for 1.9% and 11.2%, intra-microaggregate organic C for 7.8% and 10.8%, and mineral-associated organic C for 56.0% and 31.9% (Table 2). The lower proportion of free organic C and the higher mineral-associated, or

chemical protected, organic C content in soil A than in soil B indicate a larger capacity for stabilization of organic materials, which may be directly related to the higher clay content. Chemical stabilization capacity is believed to be mainly governed by soil clay content because of the increase of specific surface area of mineral particles with decreasing particle size [19, 20]. Some data previously reported in the literature also show increases in physically protected organic C with increasing soil clay content [15], presumably due to the role of clays in aggregation and aggregate stability and the related indirect effect on enhancing C storage by occluding organic materials [20]. In the current study, however, the higher proportion of intra-aggregate, or physically protected, organic C is found in soil B, which has lower clay but higher total organic C content. Not only clays but also organic matter is well-known to play a paramount role on soil aggregation and aggregate stability [21]. Thus, the higher total organic C content of soil B could be offsetting the lower clay content for promoting aggregate formation.

Regardless of the soil origin, total N content increases in the order intra-macroaggregate SOM < intra-microaggregate SOM < free SOM < mineral-associated SOM (Table 2). Free SOM and intra-macroaggregate SOM exhibit the largest C/N ratios, whereas the smallest values are obtained for the mineral-associated SOM fraction (Table 2). Factors that may be involved in lowering of the C/N ratio include the microbial transformation of plant residue material into stable organic matter and, secondly, chemical fixation of NH_3 or amines by lignin-like substances [21]. The results obtained thus suggest that free and intra-macroaggregate SOM consists of less decomposed plant residues than the organic material occluded within microaggregates, which in turn represents a less decomposed fraction than the chemically protected SOM pool.

3.2 Molecular structure of soil organic matter pools

The CPMAS ^{13}C-NMR spectra of the soil samples and of the soil fractions are shown in Fig. 3, and the corresponding estimates of C distribution derived from the NMR integration are reported in Table 3. The CPMAS ^{13}C-NMR spectra of the two soils and the mineral-associated SOM fractions are featureless, showing very low signal-to-noise ratios (Fig. 3). These results may be related to the low C content and presence of paramagnetic species containing unpaired electrons (e.g., Fe^{3+}), which reduce the efficiency of signal acquisition [8,11]. On the contrary, the CPMAS ^{13}C-NMR spectra of the free and aggregate SOM fractions show well-resolved peaks, having much higher signal-to-noise ratios (Fig. 3).

The most intense peaks in the CPMAS ^{13}C-NMR spectra of the free and aggregate SOM fractions of the two soils examined are in the chemical shift regions between 45 and 90 ppm and between 10 to 45 ppm. The signals in the former region are primarily attributed to O-substituted alkyl C in carbohydrates and secondarily to methoxyl C and N-substituted alkyl C in proteins, whereas the signals in the latter region are due to unsubstituted-aliphatic C of methyl, methylene, and methine groups [8]. Other important signals are also observed in the chemical shift regions due to anomeric C (at about 105 ppm), unsubstituted and alkyl-substituted aromatic C (at about 125 ppm), aromatic C in phenolic groups, aromatic amines, and aromatic ethers (at about 155 ppm), carboxyl C (at about 175 ppm), and carbonyl C in ketonic groups (between 190 to 224 ppm) [8].

For both soils examined, with respect to the aggregate SOM fractions, the free SOM pool shows a smaller content of unsubstituted-aliphatic C and higher content of substituted-aliphatic C (Table 3). This most probably indicates greater presence of carbohydrates and proteinaceous materials arising from undecayed plant and microbial tissues. The free SOM fraction of soil A has smaller aromatic, carboxyl, and carbonyl C contents than the free SOM fraction of soil B (Table 3), probably reflecting the different

plant species from which these fractions mainly derive (wheat vs. vine). Compared to the macroaggregate SOM fractions, the microaggregate SOM fractions of both soils feature larger aliphatic C content and smaller aromatic, carboxyl, and carbonyl C contents.

It is noteworthy that, except for the free SOM, which mainly consists of undecayed plant material, the differences in chemical structure between the SOM pools are quite similar in the examined soils, which suggests that SOM decomposition and stabilization follow a major similar biogeochemical pattern. Biologically labile substances arising from plants and microorganisms, such as carbohydrates and proteins, are preferentially and gradually lost during SOM decomposition and incorporation into macro- and microaggregates, whereas unsubstituted-aliphatic materials, likely from lipid biopolymers such as cutin and suberin, are preserved and accumulated in physically protected fractions, especially in microaggregates.

3.3 Concluding remarks

The fractionation scheme described here, based on a combination of density, aggregate fractionation, and sonication procedures, has proved effective to separate unprotected, intra-macroaggregate, and intra-microaggregate SOM pools freed from soil mineral constituents and well suited for advanced chemical characterization. Soil properties, especially clay content, seem to affect the quantitative distribution of SOM in the isolated SOM pools, but not their major differences in chemical composition and structure, which suggests that SOM decomposition and stabilization follow a common biogeochemical pattern. In particular, free organic C content is lower in the soil with lower clay content, whereas mineral-associated organic C content is higher. Despite the soil properties, crop, and climatic conditions, free SOM between aggregates is characterized by a relatively more marked presence of undecayed organic materials and biologically labile substances, such as carbohydrates and proteins. In contrast, SOM

occluded within aggregates, especially within microaggregates, represents a more decomposed fraction, relatively enriched in unsubstituted-aliphatic materials, most probably lipid biopolymers. We expect that the application of this soil physical fractionation procedure to SOM research will prove especially useful for characterizing SOM structures and functions, as well as for developing a better understanding of the mechanisms of C stabilization and sequestration in soils under diverse management scenarios.

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LEGENDS

Figure 1. Fractionation scheme to isolate soil organic matter (SOM) pools unprotected and protected from decomposition by physical and chemical mechanisms. Briefly, free SOM is isolated by an initial density separation; in a second step, stable macroaggregates in the heavy fraction are broken up into stable microaggregates and intra-macroaggregate SOM, which is separated by density; finally, intra-microaggregate SOM is isolated from mineral-associated SOM by a third density separation after ultrasonic disruption.

Figure 2. Microaggregate isolator used to break up stable macroaggregates into stable microaggregates [16].

Figure 3. Solid-state cross-polarization magic-angle spinning ^{13}C nuclear magnetic resonance spectra of soil samples (A and B) before and after fractionation into free, intra-macroaggregate, intra-microaggregate, and mineral-associated soil organic matter (SOM) pools. SN is the signal-to-noise ratio.

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The authors have declared no conflict of interest.

Table 1. Designation, origin, crop, and major properties of the soil samples used in this work.

	Soil A	Soil B
Sampling site	Arganda del Rey, Madrid, Spain	Verín, Galicia, Spain
Crop	Wheat	Vine
WRB soil group [a]	Regosol	Regosol
Texture	Clay	Sandy loam
Sand (g kg^{-1})	423	530
Silt (g kg^{-1})	193	296
Clay (g kg^{-1})	385	173
pH (1:2 soil-to-water ratio)	8.5	5.0
Total organic C (g kg^{-1})	12.20	15.37
Total N (g kg^{-1})	1.25	1.22

[a] Classification according to the International Union of Soil Sciences Working Group on the World Reference Base for Soil Resources [18].

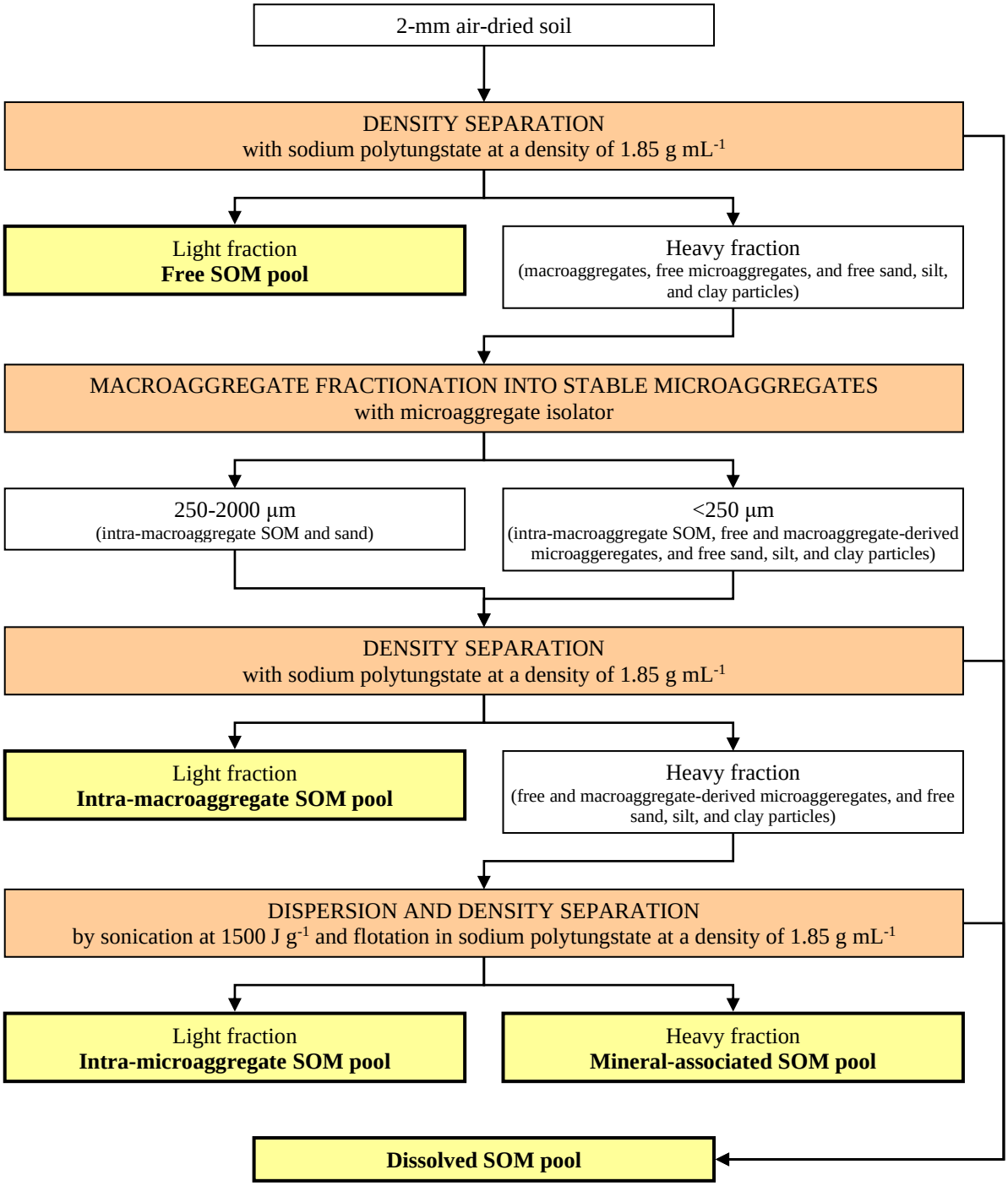
Table 2. Free, intra-macroaggregate, intra-microaggregate, and mineral-associated organic C and total N contents of soil samples (A and B) and the corresponding C/N ratios (± standard error, n = 4). Total organic C recovery in free, aggregate, and mineral-associated pools is 94.5% for soil A and 91.6% for soil B (102.3% and 101.8% when the organic C dissolved during the fractionation procedure recovered in the sodium polytungstate solution is taken into account).

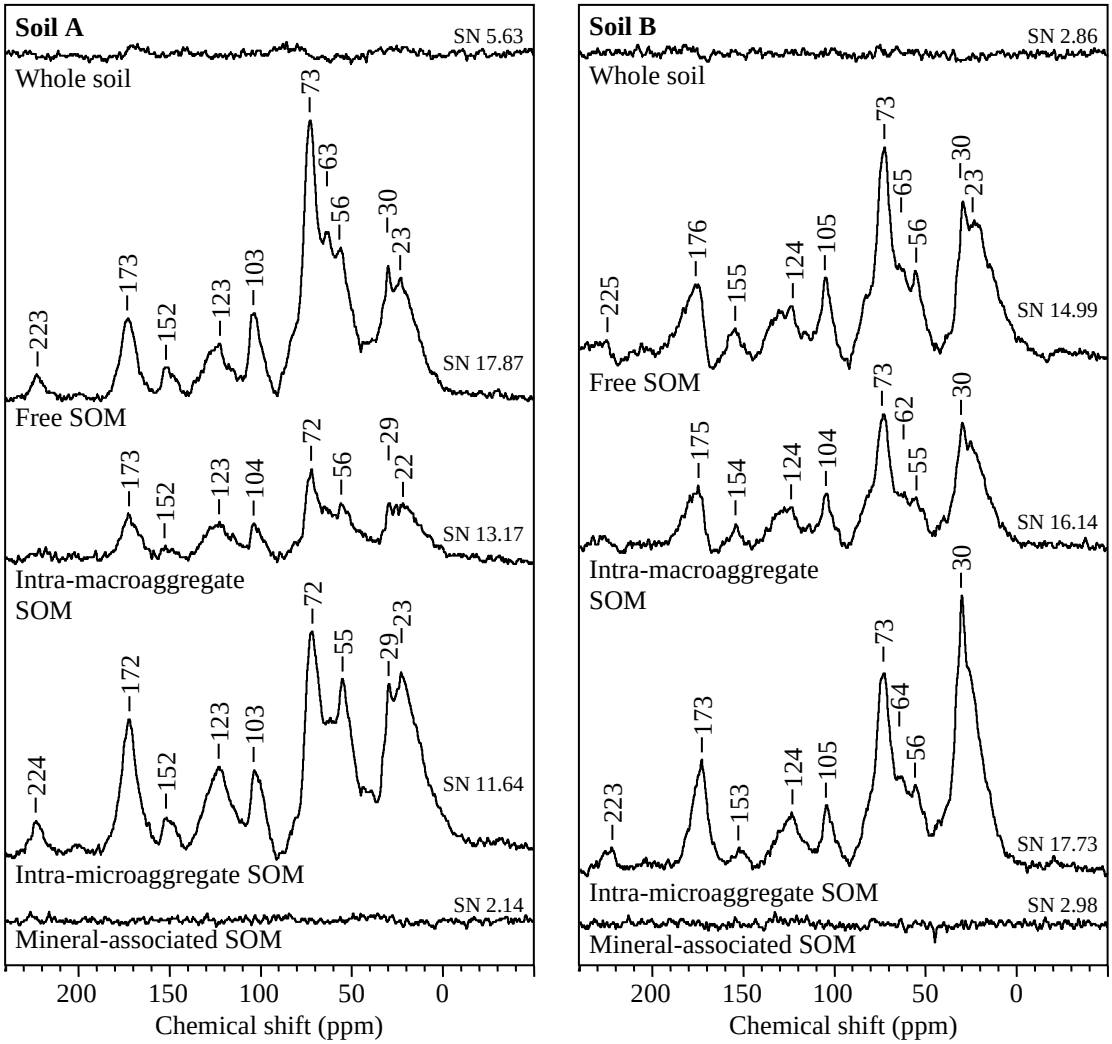
Soil organic matter (SOM) pool	Total organic C ($g\ kg^{-1}$)		Total N ($g\ kg^{-1}$)		C/N ratio	
	Soil A	Soil B	Soil A	Soil B	Soil A	Soil B
Free SOM	3.51 ± 0.21	5.78 ± 0.26	0.27 ± 0.01	0.34 ± 0.02	13.0 ± 0.2	17.0 ± 0.3
Intra-macroaggregate SOM	0.24 ± 0.02	1.73 ± 0.03	0.02 ± 0.00	0.10 ± 0.00	15.6 ± 0.8	17.5 ± 0.2
Intra-microaggregate SOM	0.96 ± 0.03	1.66 ± 0.10	0.08 ± 0.00	0.11 ± 0.01	12.6 ± 0.1	15.9 ± 0.8
Mineral associated SOM	6.83 ± 0.21	4.90 ± 0.29	1.15 ± 0.04	0.79 ± 0.04	5.9 ± 0.1	6.2 ± 0.4
Dissolved SOM [a]	0.94 ± 0.01	1.57 ± 0.01	-	-	-	-

[a] Soil organic matter dissolved during the fractionation procedure (± standard error, n = 3).

Table 3. Distribution of C in the free, intra-macroaggregate, and intra-microaggregate soil organic matter (SOM) pools isolated from two different soils (A and B) as estimated from CPMAS ^{13}C-NMR integration.

SOM pool	Unsubstituted-aliphatic C ($0 < \delta \leq 45$ ppm) (%)		Substituted-aliphatic C ($45 < \delta \leq 110$ ppm) (%)		Aromatic C ($110 < \delta \leq 160$ ppm) (%)		Carboxyl and carbonyl C ($160 < \delta \leq 220$ ppm) (%)	
	Soil A	Soil B	Soil A	Soil B	Soil A	Soil B	Soil A	Soil B
Free SOM	27.5	31.9	56.8	41.6	9.2	13.6	6.5	12.8
Intra-macroaggregate SOM	30.3	34.1	43.1	41.4	14.9	13.4	11.7	11.2
Intra-microaggregate SOM	34.8	40.8	42.1	39.7	12.4	9.0	10.6	10.5


2-mm air-dried soil
DENSITY SEPARATION
with sodium polytungstate at a density of 1.85 g mL-1
Light fraction
Free SOM pool
Heavy fraction
(macroaggregates, free microaggregates, and free sand, silt, and clay particles)
MACROAGGREGATE FRACTIONATION INTO STABLE MICROAGGREGATES
with microaggregate isolator
250-2000 µm
(intra-macroaggregate SOM and sand)
<250 µm
(intra-macroaggregate SOM, free and macroaggregate-derived microaggeregates, and free sand, silt, and clay particles)
DENSITY SEPARATION
with sodium polytungstate at a density of 1.85 g mL-1
Light fraction
Intra-macroaggregate SOM pool
Heavy fraction
(free and macroaggregate-derived microaggeregates, and free sand, silt, and clay particles)
DISPERSION AND DENSITY SEPARATION
by sonication at 1500 J g-1 and flotation in sodium polytungstate at a density of 1.85 g mL-1
Light fraction
Intra-microaggregate SOM pool
Heavy fraction
Mineral-associated SOM pool
Dissolved SOM pool


Soil A
SN 5.63
Whole soil
73
63
56
30
23
223
173
152
123
103
SN 17.87
Free SOM
173
152
123
104
72
56
29
22
SN 13.17
Intra-macroaggregate SOM
72
55
29
23
172
152
123
103
224
SN 11.64
Intra-microaggregate SOM
SN 2.14
Mineral-associated SOM
200
150
100
50
0
Chemical shift (ppm)
Soil B
SN 2.86
Whole soil
73
65
56
30
23
225
176
155
124
105
SN 14.99
Free SOM
73
62
55
30
175
154
124
104
SN 16.14
Intra-macroaggregate SOM
30
73
64
56
223
173
153
124
105
SN 17.73
Intra-microaggregate SOM
SN 2.98
Mineral-associated SOM
200
150
100
50
0
Chemical shift (ppm)