Unraveling the long-term stabilization mechanisms of organic materials in soils by physical fractionation and NMR spectroscopy

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Abstract

The fundamental mechanisms whereby organic inputs stabilize in soil are poorly resolved, which limits our current capacity to predict the dynamics of soil organic matter (OM) turnover and its influence on soil quality and functioning. Here we fractionated soil OM from long-term experimental field plots either unamended or amended with two organic materials of different quality (i.e., solid cattle manure and crop residues) for 44 years into five measurable and meaningful pools directly related to conceptual preservation mechanisms: dissolved OM, mineral-free particulate OM located outside aggregates (unprotected from decomposition), OM occluded within both macroaggregates and microaggregates (weakly and strongly protected by physical mechanisms, respectively), and OM intimately associated with soil mineral particles (protected by chemical mechanisms). Compared to the unamended soil, the application of cattle manure and crop residues increased total organic C content by 35 and 10%, respectively. Most of these increases (up to 60 and 72% for cattle manure and crop residues, respectively) were explained by the mineral-associated OM pool, followed by the intra-microaggreggate OM fraction. In general, the distribution and dynamics of N content paralleled those of C content. As determined by a range of modern nuclear magnetic resonances (NMR) techniques, including ^{13}C cross polarization magic angle spinning (MAS), ^{1}H high resolution (HR)-MAS, and ^{1}H-^{13}C heteronuclear single quantum coherence HR-MAS NMR, the mineral-associated OM fraction was found to be predominately of microbial origin, unlike free and intra-aggregate OM pools, which were dominated by plant structures at different stages of decomposition. As a whole, our results indicate that the main mechanism by which organic inputs are stabilized and OM accrues in soils is not the physical and chemical protection of undecayed or partially degraded organic structures, but the adsorption on mineral surfaces of microbial biomass and microbial by-products resulting from microbial growth, transformation, and degradation processes. It is possible that organic amendments increase more than previously thought the microbial populations of the soil, which live, thrive, and die in close association with the mineral surfaces. This mechanism appears to be enhanced with the addition of stable organic materials.

Keywords: Crop residues; Cattle manure; NMR spectroscopy; Organic amendments;

Organomineral complexes; Physical fractionation; Soil aggregates; Soil organic matter.

1. Introduction

The addition of organic amendments, such as animal manures and crop residues, is universally known to improve soil fertility (Watson et al., 2002; Diacono and Montemurro, 2011). This practice has the benefit of providing plant nutrients and especially organic matter (OM), which is essential for soil quality and functioning (Johnston et al., 2009). Besides improving soil physical, chemical, and biological conditions, increasing the levels of soil OM through organic material additions has the potential to contribute to climate change mitigation by sequestering C from the atmosphere, especially if applied to degraded arable lands depleted in soil OM, albeit this potential depends on the alternative fate of the organic material (Powlson et al., 2011).

Understanding the underlying processes of soil OM stabilization is pivotal for agricultural and environmental considerations. Leaving aside climatic and environmental factors that govern soil OM persistence (Schmidt et al., 2011), the fundamental mechanisms that protect organic compounds entering the soil against decomposition include (a) selective preservation and synthesis of resistant molecular structures, arrangements, and associations (biochemical mechanisms), (b) physical disconnection from decomposers, enzymes, O_2, etc. by occlusion within aggregates (physical mechanisms), and (c) chemical inaccessibility by intimate association (sorption) with mineral surfaces (chemical mechanisms) (Piccolo, 2001; Six et al., 2002a; Lützow et al., 2006; Schnitzer and Monreal, 2011).

Chemical extraction and fractionation of soil OM to quantify and characterize nonhumic and humic constituents has been by far the dominant approach to investigate transformation and stabilization processes of soil OM in organically-amended soils

(Francioso et al., 2000; Senesi and Plaza, 2007; Senesi et al., 2007). More recently, driven by growing evidence that the stability of soil OM does not depend on its molecular structure alone (Schmidt et al., 2011), physical fractionation techniques have been increasingly used to capture the influence of the soil mineral matrix on soil OM dynamics (Olk and Gregorich, 2006). However, physically-fractionated OM pools still need to be better resolved especially at the molecular level to achieve a more complete understanding of the biochemical, physical, and chemical mechanisms that together control the dynamics of soil OM turnover. Nuclear magnetic resonance (NMR) spectroscopy is arguably one of the most powerful tools to unravel the highly complex structural composition of soil OM (Simpson et al., 2011). In particular, advanced solid-state cross polarization magic angle spinning (CP-MAS) and high resolution (HR)-MAS NMR methods can be used complementarily to extract valuable information. While solid-state CP-MAS NMR spectroscopy provides semi-quantitative information on the types and distribution of C present in soil OM, HR-MAS NMR provides increased spectral resolution and structural information as to the soluble and swellable OM components.

The main objective of this research was to obtain new molecular-level information about the mechanisms and processes that control the stabilization of OM in agricultural soils amended with organic materials. To achieve this objective, soil OM from long-term experimental field plots unamended and amended with solid cattle manure and crop residues were fractionated by a recent physical methodology into five pools meaningfully related to the abovementioned conceptual protection mechanisms: dissolved OM, mineral-free particulate OM located outside aggregates (unprotected), OM occluded within both macroaggregates and microaggregates (weakly and strongly protected by physical mechanisms, respectively), and OM intimately associated with soil mineral particles (protected by chemical mechanisms). The whole soils and OM fractions were analyzed for organic C and N content, and the molecular composition was investigated using CP-MAS and HR-MAS NMR methods.

2. Materials and methods

2.1. Long-term field experiment and soil samples

The long-term field plot experiment used in this work started in 1966 at the research and outreach farm of the Agricultural Faculty of the University of Bologna, located in Cadriano, northern Italy (44º33’ N, 11º24’ E; 31 m above sea level). The climate is humid subtropical with an average annual precipitation of 714 mm and an average annual temperature of 13.0 °C. The soil is a Haplic Calcisol (IUSS Working Group WRB, 2006). The topsoil (0-20 cm) is sandy clay loam (560 g kg^{-1} sand, 160 g kg^{-1} silt, and 280 g kg^{-1} clay). In 1966, bulk density was 1.16 g mL^{-3}; pH, 6.9; total organic C content, 7.7 g kg^{-1}; total N content, 1.1 g kg^{-1}; and C/N ratio, 7.0.

The experiment was laid out in a randomized split-plot design with three replicates, several types of organic amendments on the main plots, and different rates of mineral N fertilizer at the split subplot level. Each subplot was 3 m wide and 11 m long, and all of them were in a corn (*Zea mays* L.)-winter wheat (*Triticum aestivum* L.) rotation. For this study, we selected three treatments with no mineral N application: a control without any amendment, amendment with cattle manure, and amendment with crop residues (wheat straw and corn stalk alternately). The organic amendments were applied every year in early October at rates of either 7.5 t ha^{-1} after corn crops or 6.0 t ha^{-1} after wheat crops, and immediately buried by moldboard plowing. Average organic C content of the cattle manure, wheat straw, and corn stalk applied was 331, 328, and 326 g kg^{-1}, average total N content was 26.8, 4.7, and 7.0 g kg^{-1}, and average C/N ratio was 12.4, 69.8, and 46.6, respectively. Further experimental details and data were reported in Triberti et al. (2008).

Soil samples were taken from each plot after wheat harvest in 2010. Ten soil cores were taken per plot with a probe to a depth of 20 cm and composited. The samples were air-dried, gently crushed, and passed through a 2-mm sieve prior to OM fractionation. For organic C and N analysis, a representative aliquot of each soil sample was ground with a ball mill. For NMR analysis, composite samples of ground soils were prepared for each treatment and repeatedly treated with 10% HF to improve the signal-to-noise ratio (Schmidt et al., 1997).

2.2. Soil organic matter fractionation

Soil OM fractionation into dissolved, free, intra-macroaggregate, intra-microaggregate, and mineral-associated OM was accomplished using the method of Plaza et al. (2012a) modified by Plaza et al. (2013). Briefly, 80 mL of sodium polytungstate (SPT) solution at a density of 1.85 g mL^{-1} was added to 20 g of soil and shaken on an overhead shaker (1 revolution s^{-1}, 30 s). After centrifugation (2500 × g, 30 min), the floating light fraction (free OM) was separated from the heavy fraction by suction and filtration through a glass fiber filter and washed thoroughly with deionized water. The heavy fraction was transferred to a device designed by Six et al. (2000, 2002b) to break up macroaggregates into stable microaggregates (< 250 μm), oven-dried at 70 ºC, and gently transferred into a centrifuge tube together with the filtrate from the first step (SPT solution). The tube was rotated (1 revolution s^{-1}, 30 s) and centrifuged (2500 × g, 45 min). The floating light particles (intra-macroaggregate OM) were separated from the heavy fraction by suction and filtration and washed with deionized water. Finally, the heavy fraction was dispersed in the SPT solution by sonication (1500 J g^{-1}) and the floating light fraction (intra-microaggregate OM) was separated from the heavy fraction (mineral-associated OM) by centrifugation (2500 × g, 60 min), suction, and filtration and washed with deionized water.

The free, intra-macroaggregate, intra-microaggregate, and mineral-associated OM fractions recovered after fractionation were oven-dried at 70 ºC, weighed, and ground with a ball mill. Free and intra-aggregate fractions were ground with the glass fiber filters. For NMR analysis, composite samples of OM fractions were prepared by mixing equal amounts of the replicates. To improve the signal-to-noise ratio, the mineral-associated OM fractions were treated with 10% HF (Schmidt et al., 1997).

2.3. Organic C and N analysis

Organic C and total N content of the whole soil samples and solid OM fractions were determined by dry combustion using a Thermo Flash 2000 NC Soil Analyzer. Dissolved organic C in the SPT solution after fractionation (dissolved OM fraction) was determined using a Shimadzu TOC 5000A analyzer. We detected no C in the SPT powder used to prepare the solution for density separations, but relatively high levels of N (0.665 g kg^{-1}). Since some N inevitably remained in the mineral-associated OM pool after the fractionation procedure, the N content in the mineral-associated pool was calculated together with dissolved OM pool by subtracting the measured free and aggregated N content from the total N content of the whole soil.

2.4. Nuclear magnetic resonance analysis

Solid-state ^{13}C CP-MAS NMR spectra were recorded at room temperature with a 4-mm H-X MAS probe on a Bruker Avance III 500 MHz NMR spectrometer, using a MAS frequency of 13000 Hz, a recycle delay of 1 s, a ramp-CP contact time of 1 ms, and between 1344 and 81920 scans. The free induction decay signal was digitized, zero filled twice, multiplied by an exponential function corresponding to 50 Hz line broadening in the final transformed spectrum. Spectra were externally calibrated to the carboxyl group of glycine (176.03 ppm).

^{1}H, DE ^{1}H, and ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) HR-MAS NMR spectra were collected at 298 K on a Bruker Avance III 500 MHz NMR spectrometer with a 4-mm ^{2}H-^{1}H-^{13}C-^{15}N HR-MAS probe with actively shielded Z gradient at a spinning speed of 6666 Hz. Approximately 35 mg of sample was placed in a zirconium rotor, mixed with 65 μL of DMSO-d_6, and sealed using a Kel-F insert and a Kel-F rotor cap. The ^{1}H HR-MAS NMR spectra were acquired with 1024 scans, a 2 s delay between pulses, a sweep width of 20 ppm, and 4096 time domain points; PURGE was employed for water suppression (Simpson and Brown, 2005). The DE ^{1}H HR-MAS NMR spectra were collected with a BPPLED sequence using a 1.25 ms, 33.3 gauss cm^{-1} sine-shaped gradient pulse, a diffusion time of 60 ms, 4096 time domain points, and 1024 scans. Both ^{1}H and DE ^{1}H HR-MAS NMR spectra were processed with a zero-filling factor of 2, exponential functions corresponding to 1 and 10 Hz line broadening, respectively, and calibrated using the solvent residual peak at 2.50 ppm. The ^{1}H-^{13}C HSQC spectra were obtained in phase-sensitive mode using echo/anti-echo gradient selection and a 1J ^{1}H-^{13}C value of 145 Hz. From 896 to 2560 scans and 1024 data points were collected for each of the 256 increments in the F1 dimension, employing a relaxation delay of 0.5 s. The F2 dimension was processed with an exponential function corresponding to 15 Hz line broadening in the transformed spectrum and F1 was processed using a sine-squared function with phase shift of 90º.

2.5. Statistical analysis

The significance of the effects of the treatments on total, free, intra-macroaggregate, intra-microaggregate, mineral-associated, and dissolved organic C and N contents and C/N ratios was determined by one-way ANOVA using IBM SPSS (version 20). Comparisons of means were performed using the Bonferroni test at the 0.05 level. Pearson's correlation coefficient *r* was used to measure the effect size of SM compared to UN, CR compared to UN, and SM compared to CR. Effects with *r* values of 0.10, 0.30, and 0.50 were assessed as small, medium, and large effects, respectively.

3. Results

3.1. Content and distribution of organic C and N

With respect to the unamended soil, organic C and N content increases by 35 and 31%, respectively, in the soil amended with cattle manure and by 10 and 7% in the soil amended with crop residues (Table 1). Total organic C recovery after soil OM fractionation into dissolved, free, intra-macroaggregate, intra-microaggregate, and mineral-associated OM pools is 99.6% for the unamended soil, 97.7% for the soil amended with cattle manure, and 98.3% for the soil amended with crop residues (Table 1), indicating negligible C losses during the separation procedure. For the three soils examined, approximately 70% of the total organic C is found in the mineral-associated OM; similarly, the mineral-associated and dissolved OM pools together store most (approximately 90%) of the total N and exhibit the lowest C/N ratios (Table 1).

Compared to the unamended soil, the application of cattle manure increases dissolved, free, intra-macroaggregate, intra-microaggregate, and mineral-associated organic C content by 22, 35, 59, 65, and 28%, respectively, whereas crop residues only increases dissolved, intra-microaggregate, and mineral-associated organic C content by 14, 22, and 9%, respectively (Table 1). For both amended soils, the mineral-associated OM fraction explains most of the increase in total organic C content (60 and 72% for manure and crop residues, respectively), followed by the intra-microaggreggate OM pool (17 and 20% for SM and CR, respectively). Consistently with this, the mineral-associated and dissolved OM pools together accounts for most (71 and 79% for SM and CR, respectively) of the increase in total N content, followed by the intra-microaggregate OM pool (15 and 21% for SM and CR, respectively) (Table 1).

3.2. Nuclear magnetic resonance spectra

The solid-state ^{13}C CP-MAS NMR spectra of cattle manure, wheat straw, corn stalk, the unamended and amended soils, and their respective OM fractions exhibit common signals of different intensity (Fig. 1), which results in varying distributions of main C structures (Table 2). In particular, compared with the spectra of the whole soils, the spectra of the organic materials, especially the crop residues, show relatively stronger signals arising from C in carbohydrates at 60-65, 70-80, and 95-110 ppm and less intense resonances due to terminal methyl C, methylene C in aliphatic rings, and methylene C in alkyl chains between 0 and 45 ppm, methoxyl C at 56 ppm, alkyl substituted aromatic C at 130 ppm, O or N substituted aromatic C at 150 ppm, and carboxyl and amidic C at 170 ppm. The intensity of the resonances due to unsubstituted alkyl, methoxyl, alkyl-substituted aromatic, carboxyl, and amidic C is higher for the intra-aggregate OM (especially for the intra-microaggregate OM) than for the free OM, whereas the intensity of the signals attributed to carbohydrates is lower. Compared with the free and intra-aggregate OM fractions, the mineral-associated OM pools show stronger resonances derived from unsubstituted alkyl, carboxyl, and amidic C and weaker signals attributed to carbohydrates. In general, the spectra of the unamended soil and its OM fractions closely match the corresponding spectra of the amended soils and their OM fractions, except for the slightly higher intensity of the signals due to carbohydrates in the spectra of the intra-aggregate OM fractions of the amended soils, especially in the spectrum of the macroaggregate OM fraction of the soil amended with crop residues, and the slightly higher intensity of the resonances due to methoxyl C in the spectrum of the intra-microaggregate OM fraction of the manured soil.

The ^{1}H HR-MAS NMR spectra of the cattle manure and crop residues show (a) two sharp resonances due to terminal methyl and methylene groups between 0.6 and 1.3 ppm; (b) weak signals due to O- and N-substituted aliphatic structures in the 1.3-2.9 ppm region, which are more intense for the crop residues; (c) signals due to O-alkyl structures mainly from carbohydrates and lignin methoxyl in the 2.9-4.1 ppm region, which are more intense for the crop residues than for the cattle manure; (d) overlapping weak resonances in the 4.1-5.2 ppm region generally attributed to a number of moieties, including α-H from peptides (4.1-4.8 ppm) and anomeric H in carbohydrates, which are only observable for the crop residues; and (e) signals from aromatic structures, in the

6.2-7.8 ppm region, which are only abundant for the crop residues and likely result from lignin in these samples (Fig. 2).

The ^{1}H HR-MAS NMR spectra of the whole soils and the free and intra-aggregate OM fractions are very similar to each other, featuring a very intense and sharp signal at 1.2 ppm due to methylene groups accompanied by a weak resonance at 0.8 ppm due to terminal methyl groups, which is generally attributed to plant-derived lipid biopolymers, such as waxes, cutin, and suberin (Fig. 2). On the other hand, the ^{1}H HR-MAS NMR spectra of the mineral-associated OM pools show stronger resonances from terminal methyl groups, indicative of methyl rich amino acids instead of polymethylene structures from plant-derived lipid biopolymers, and also exhibit weak signals in the 4.1-4.8, 6.2-7.8, and 7.8-8.4 ppm regions, indicative of α-H, aromatic, and amide structures from proteins, such as albumin (Simpson et al., 2007).

Diffusion edited NMR is useful for suppressing signals from smaller molecules exhibiting self-diffusion and accentuating signals from macromolecular components (Simpson et al., 2011). The selective attenuation of signals from small molecules in the DE ^{1}H HR-MAS NMR spectra, in comparison with the corresponding ^{1}H HR-MAS NMR spectra, is mainly observed in the resonances from terminal methyl and methylene groups (Fig. 3), indicating the presence of relatively small aliphatic structures. The observed attenuation is concomitant with an enhancement of resonances arising from large or rigid molecules and aggregates, especially in the 2.9-4.1 ppm region due to large carbohydrates and lignin. The effects of diffusion editing are considerably less evident for the cattle manure and crop residues than for the whole soils, suggesting a less presence of organic degradation products in the organic amendments. Diffusion editing also highlights substantial differences in lipid, peptide, and carbohydrates composition between the OM fractions examined, especially between the free and aggregate OM and the mineral-associated OM pools. In particular, the DE ^{1}H HR-MAS NMR spectra of the intra-aggregate and especially free OM fractions show weaker resonances from terminal methyl groups at 1.2 ppm and relatively stronger resonances due to methylene groups at 0.8 ppm, consistent with plant-derived lipid biopolymers, as well as stronger signals from carbohydrates and lignin in the 2.9-4.1 ppm region; in contrast, the DE ^{1}H HR-MAS NMR spectra of the mineral-associated OM fractions closely resemble that of albumin (Fig. 4), showing resolved signals at 4.1-4.8 and 7.8-8.4 ppm, at 6.6 and 7.0 ppm, and at 7.2 ppm that have been shown to be attributable to α-H and amides from proteins, tyrosine, and phenylalanine, respectively (Simpson et al., 2007). These NMR features are very similar to those found for soil microbial cultures (Fig. 4; Simpson et al., 2007), which strongly suggest that mineral-associated OM is predominately of microbial origin.

The ^{1}H-^{13}C HSQC HR-MAS NMR spectrum of wheat straw exhibits a very strong cross-signal at 3.7 ppm in the ^{1}H dimension and 56 ppm in the ^{13}C dimension due to aromatic methoxyl in lignin, strong cross-resonances at 2.9-4.1 ppm and 60-80 ppm due to carbohydrates, a strong cross-resonance at 1.2 ppm and 29 ppm due to methylene groups, and other relatively weaker cross-resonances at 6.0-8.0 ppm and 100-140 ppm due to aliphatic rings and at 0.5-1.0 ppm and 10-25 ppm due to terminal methyl groups in aliphatic chains (Fig. 5). The HSQC spectrum of free OM (Fig. 5) is characterized by intense cross-resonances due to methylene groups and weak signals due to terminal methyl groups, carbohydrates, and lignin, consistent with plant structures (Kelleher et al., 2006). In contrast, the HSQC spectrum of mineral-associated OM (Fig. 5) shows cross-resonances in the aliphatic region with strong signals from terminal methyl groups, cross-resonances due to α-H/C in proteins/peptides (at about 4.0-5.0 ppm in the ^{1}H dimension and 40-60 ppm in the ^{13}C dimension), and cross-resonances in the aromatic region that have been shown to be attributed to aromatic amino acids, such as tyrosine and phenylalanine, and consistent with microbial biomass structures (Spence et al., 2011). The multidimensional NMR examination by HSQC thus corroborates the microbial origin of the mineral-associated OM suggested by the 1D NMR results.

4. Discussion

Greater organic C and N content in soils amended with organic materials, including crop residues and especially animal manures, with respect to unamended soils are widely reported in the literature (Smith et al., 1997; Lemke et al., 2010; Powlson et al., 2012). The greater storage of OM with cattle manure than with crop residues in this long-term experiment has been previously attributed to the more marked presence of recalcitrant organic compounds, such as humic and humic-like substances, in consequence of more extended decomposition and humification processes (Triberti et al., 2008; Plaza et al., 2012b). In the current study, NMR results, such as the higher unsubstituted alkyl C relative to substituted alkyl C resonances observed for cattle manure, are also indicative of more extended OM degradation (Clemente et al., 2011). In addition to the higher recalcitrance of the added OM, the greater OM content might also be partially attributed to the likely higher inputs of root and stubble biomass in the cattle manured plots, as suggested by the generally better wheat and corn yields obtained throughout the experiment (Triberti et al., 2008). In particular, average corn grain and stalk yields were 6.8 and 6.8 t ha^{-1} for UN, 9.6 and 8.4 t ha^{-1} for SM, and 7.0 and 7.0 t ha^{-1} for CR; and average wheat grain and straw yields were 3.2 and 2.1 t ha^{-1} for UN, 4.0 and 5.5 t ha^{-1} for SM, and 3.4 and 2.3 t ha^{-1} for CR.

The much higher mineral-associated organic C and N content compared with the dissolved, free, and intra-aggregate organic C and N content in the three soils examined indicates that the mineral-associated OM pool has higher storage capacity and is less susceptible to mineralization. It has been widely documented that cultivation practices that entail strong soil disturbance, such as the annual moldboard plowing used in our study, stimulate the microbial decomposition of unprotected and physically-protected soil OM by increasing soil oxygenation, increasing macroaggregate turnover, and inhibiting the formation of microaggregates within macroaggregates in which particulate OM is stabilized in the long term (Six et al., 1998, 1999, 2000; Balesdent et al., 2000; Zotarelli et al., 2007; Mishra et al., 2010). In this respect, earlier works by Guggenberger et al. (1995) suggested that microbial products bound to clay surfaces are more stable against mineralization induced by cultivation than plant residues in aggregates.

The increase in free and intra-aggregate organic C and N content measured in the cattle manured soil is consistent with other studies showing that animal manure applications increase the particulate OM pool and promote aggregate formation (Whalen and Chang, 2002; Jiao et al., 2006; Yan et al., 2012). The considerably less effect of crop residues on these fractions may be attributed to the higher degradability of the added OM. In agreement with Sun et al. (1995), relatively less stable organic materials, such as crop residues relative to animal manures, may cause a greater but less lasting increase in aggregation. Nevertheless, our results clearly indicate that it is the mineral-associated pool and not the intra-aggregate fractions that stores by far most of the organic C and N added with both cattle manure and crop residues. However, aggregates, especially microaggregates, may provide physical protection not only to mineral-free dissolved and particulate OM, but also to the chemically-protected mineral-associated OM pool. On the basis of our results, we suggest that this joint physical-chemical mechanism of OM stabilization is enhanced in amended soils, especially in soils treated with organic materials relatively richer in compounds with molecular structures and/or assemblies more resistant to decomposition.

In agreement with previous ^{13}C CP-MAS NMR studies on similar systems (Golchin et al., 1994; Sohi et al., 2001; Poirier et al., 2005; Helfrich et al., 2006), our results indicate that the free and intra-aggregate OM fractions both in the soil amended with crop residues and in the manured soil consist mainly of plant material at different stages of decomposition. In passing from the free to the intra-macroaggregate and to the intra-microaggregate pools, the presence of unsubstituted-aliphatic compounds relatively rich in methylene, probably from lipid biopolymers such as waxes, cutin, and suberin, increases while the content of carbohydrates decreases, indicating a greater degree of decomposition. Many studies have also shown that mineral-associated OM fractions, especially, fine mineral fractions, have narrow C/N ratios and are generally depleted in lignin and plant-derived components and enriched in microbial C (Golchin et al., 1994; Guggenberger et al., 1994, 1995; Kögel-Knabner et al., 2008). To our knowledge, however, our results provide the first direct evidence obtained by advanced NMR spectroscopy that microorganisms and microbial by-products intimately associated with minerals constitute the most important pool in which organic amendments are stored in soils in the long term. According to earlier works by Golchin et al. (1994), who also suggested a microbial origin for this fraction mainly on the basis of the low C/N ratio and absence of lignin, organic materials entering the soil are colonized by microorganisms, encrusted by mineral particles in macroaggregates, decomposed into fragments of more resistant structures, coated by mineral particles to form stable microaggregates, eventually incorporated into microbial biomass and metabolites, and associated with soil minerals. Our study suggests that it is also possible that the amendments increase the microbial populations of the soil, which live, thrive and die in close association with the mineral surfaces (Fig. 6). This mechanism, which our results suggest to be enhanced with the addition of stable organic materials, is strongly consistent with the increased soil microbial biomass contents in organically-amended soils reported in many studies (García-Gil et al., 2000; Kallenbach and Grandy, 2011; Liang et al., 2011; Jost et al., 2012), with recent findings suggesting that the contribution of microbes and microbial residues to soil OM is higher than previously thought (Simpson et al., 2007; Miltner et al., 2011), and also with Jenkinson's (1977) classical description of the soil microbial biomass as "the eye of the needle through which all natural organic materials that enters the soil must pass".

5. Conclusions

Our results show that long-term soil incorporation of crop residues and especially solid cattle manure increases soil organic C and N content due to the addition of OM with these materials and the higher crop yields in amended soils. The most important pools in which the organic inputs stabilize in the soil are, primarily, the mineral-associated OM fraction and, secondarily, the intra-microaggregate OM pool. The key mechanism by which OM is stabilized in amended soils appears to be the adsorption of microbial biomass and microbial by-products on mineral surfaces during the microbial transformation and degradation of added fresh organic materials, rather than the physical and chemical protection of undecayed or partially degraded structures. Organic amendments may also increase soil microbial populations, which live, thrive, and die in close association with mineral surfaces. The quality of the organic input appears to affect the amount but not the composition of the OM stabilized in amended soils in the long term. Further investigations are still required to ascertain whether or not these findings are also valid for organic amendments of different origin and nature and under different environmental conditions. Since soil microorganisms and microbial by-products may contribute substantially to soil OM, the specific protection mechanisms of microbial cells and microbial metabolites from decomposition also merit further research, as well as the molecular composition of soil OM as affected by the soil microbial community structure.

Acknowledgements

This work was partly supported by the Spanish Ministry of Science and Innovation (research grant AGL2009-09124). C. Plaza thanks the Spanish National Research Council (CSIC) for the travel grant (PA1003114) which made possible his visit to the University of Toronto. Andre Simpson thanks the NSERC Discovery and Strategic Programs for providing support.

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Figure captions

Fig. 1. ^{13}C CP-MAS NMR spectra of cattle manure, wheat straw, corn stalk, soils unamended and amended with cattle manure and crop residues, and their respective organic matter (OM) fractions. ^{13}C chemical shift range assignments according to Simpson and Simpson (2009), Simpson et al. (2011), and references therein.

Fig. 2. ^{1}H HR-MAS NMR spectra of cattle manure, wheat straw, corn stalk, soils unamended and amended with cattle manure and crop residues, and their respective organic matter (OM) pools. General assignments of chemical shift ranges (Simpson et al., 2011, and references therein) are as follows: (a) CH_3 and CH_2 (0.6-1.3 ppm); (b) CH_3 and CH_2 near O and N (1.3-2.9 ppm); (c) O-alkyl, mainly from carbohydrates and lignin (2.9-4.1 ppm); (d) α-H from peptides, anomeric H in carbohydrates, H in esters, and double bonds (4.1-5.2 ppm); (e) aromatic, from lignin and peptides (6.2-7.8 ppm); and (f) amide in peptides (7.8-8.4 ppm). DMSO, dimethyl sulfoxide; SPT, sodium polytungstate.

Fig. 3. Diffusion edited (DE) ^{1}H HR-MAS NMR spectra of cattle manure, wheat straw, corn stalk, soils unamended and amended with cattle manure and crop residues, and their respective organic matter (OM) pools. General assignments of chemical shift ranges (Simpson et al., 2011, and references therein) are as follows: (a) CH_3 and CH_2 (0.6-1.3 ppm); (b) CH_3 and CH_2 near O and N (1.3-2.9 ppm); (c) O-alkyl, mainly from carbohydrates and lignin (2.9-4.1 ppm); (d) α-H from peptides, anomeric H in carbohydrates, H in esters, and double bonds (4.1-5.2 ppm); (e) aromatic, from lignin and peptides (6.2-7.8 ppm); and (f) amide in peptides (7.8-8.4 ppm).

Fig. 4. Diffusion edited ^{1}H HR-MAS NMR spectra of bobine serum albumin protein and microorganisms cultured from soil. General assignments of chemical shift ranges (Simpson et al., 2011, and references therein) are as follows: (a) amino acid side chains

(0-4 ppm);; (b) α-H from peptides (4.1-5 ppm), (c) aromatic amino acid signals (6.2-7.8 ppm); and (d) amide in peptides (7.8-8.4 ppm).

Fig. 5. ^{1}H-^{13}C HSQC HR-MAS NMR spectra of wheat straw and free and mineral-associated organic matter (OM) pools isolated from the unamended soil. General assignments of chemical shift regions (Simpson et al., 2011, and references therein) are as follows: (a) CH_3 from lipids and/or peptides; (b) CH_2 in lipids; (c) C-H bonds in various aliphatic structures including fatty acids and amino acids; (d) α-H/C in peptides; (e) CH_2 in carbohydrates and aromatic methoxyl (derived predominately from lignin); (f) C-H bonds in carbohydrates; and (g) aromatic structures. DMSO, dimethyl sulfoxide.

Fig. 6. Schematic representation of long-term stabilization mechanisms of organic materials in soils.

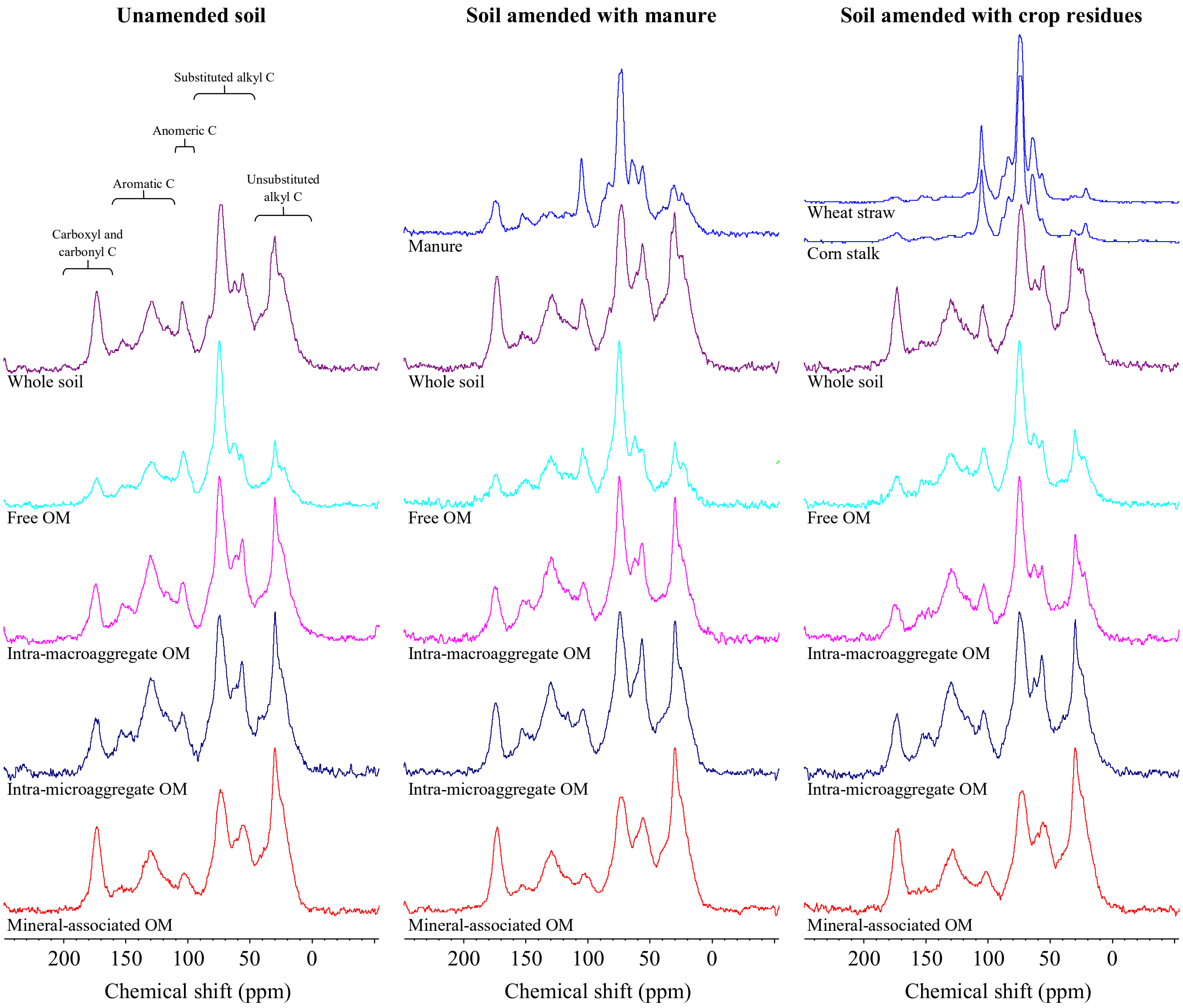

Unamended soil
Soil amended with manure
Soil amended with crop residues
Substituted alkyl C
Anomeric C
Aromatic C
Unsubstituted alkyl C
Carboxyl and carbonyl C
Manure
Wheat straw
Corn stalk
Whole soil
Free OM
Intra-macroaggregate OM
Intra-microaggregate OM
Mineral-associated OM
200
150
100
50
0
Chemical shift (ppm)

Fig. 1

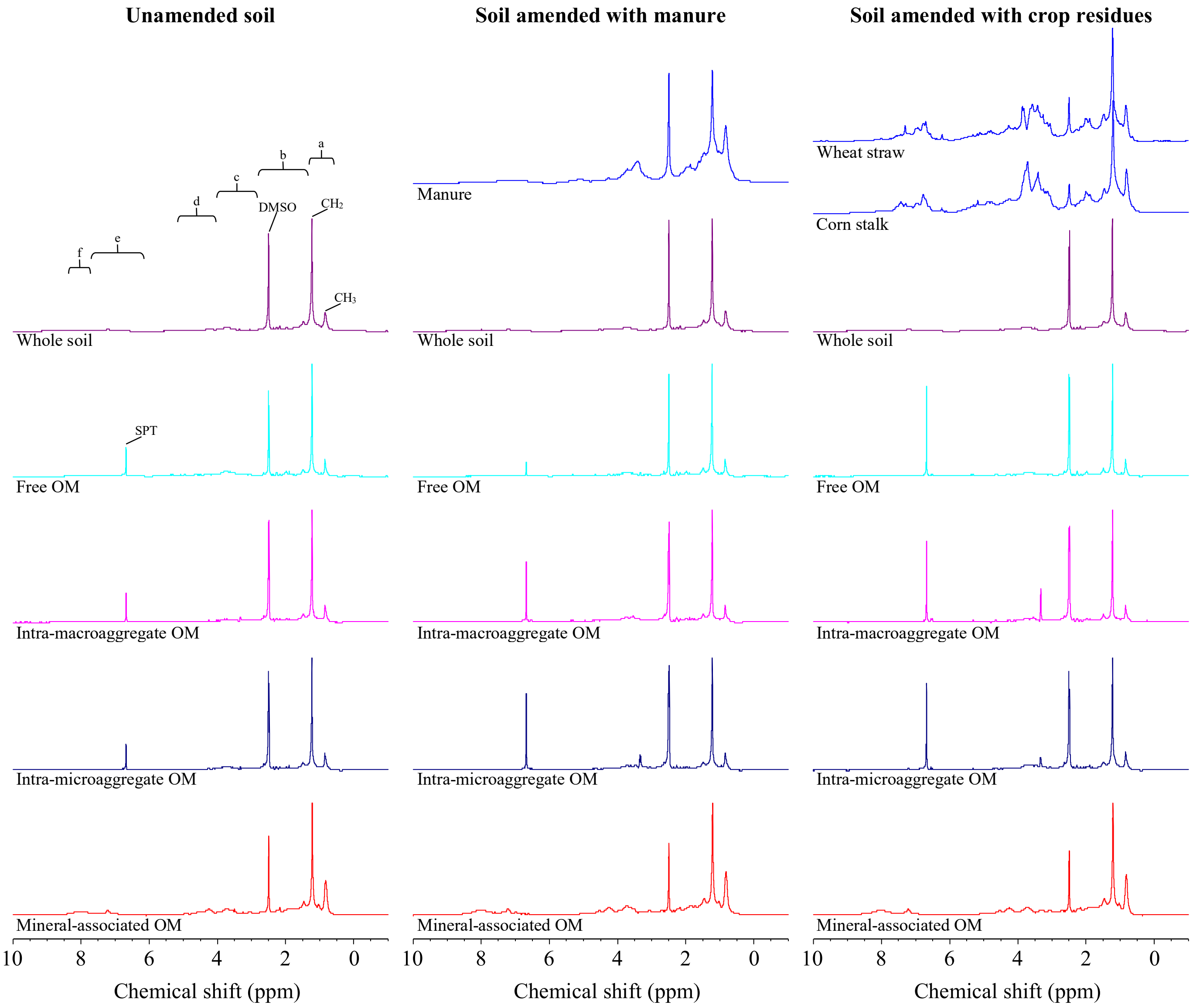

Unamended soil
Soil amended with manure
Soil amended with crop residues
a
b
c
d
e
f
DMSO
CH_2
CH_3
SPT
Manure
Wheat straw
Corn stalk
Whole soil
Free OM
Intra-macroaggregate OM
Intra-microaggregate OM
Mineral-associated OM
10
8
6
4
2
0
Chemical shift (ppm)

Fig. 2

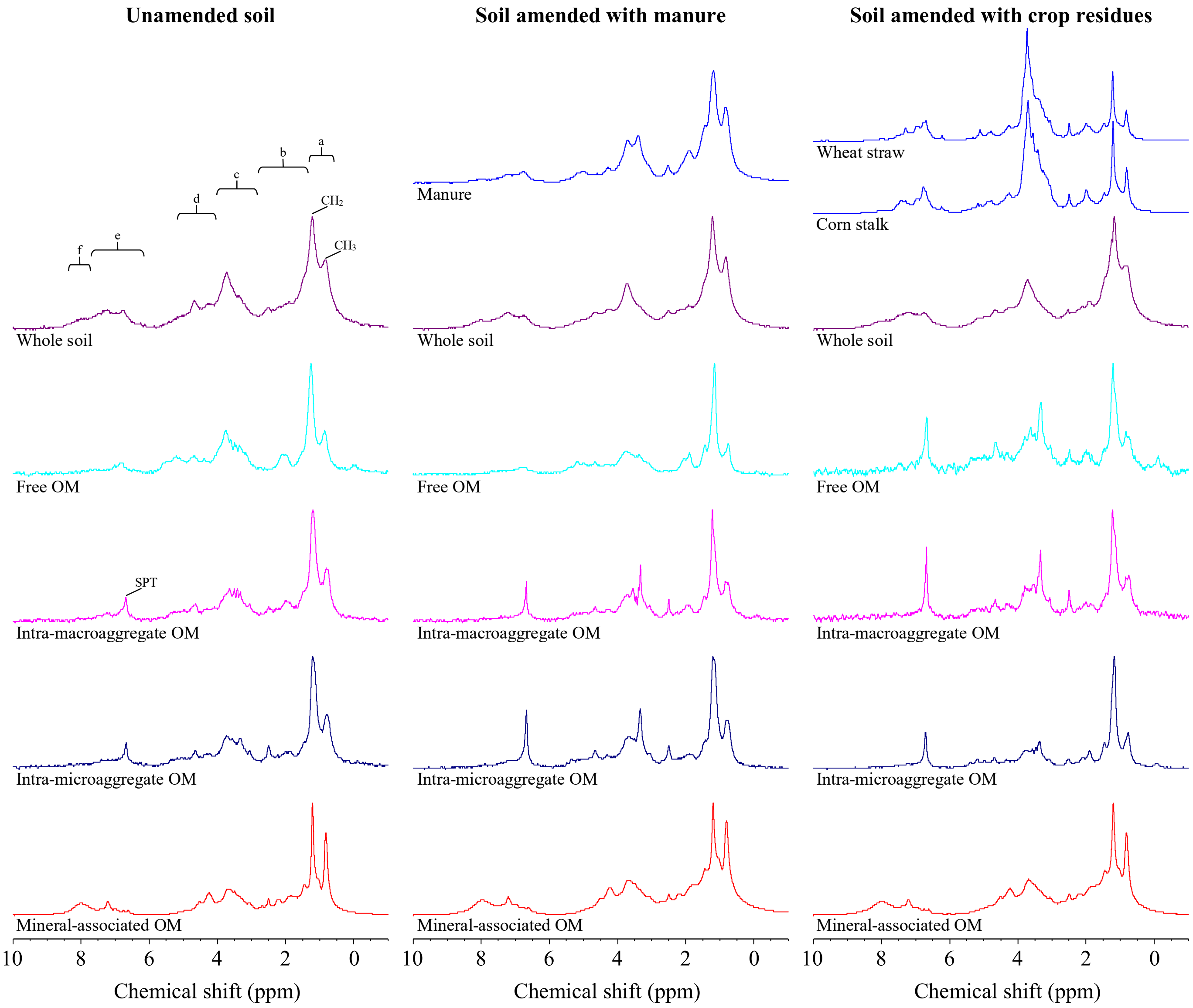

Unamended soil
Soil amended with manure
Soil amended with crop residues
a
b
c
d
e
f
CH_2
CH_3
SPT
Manure
Wheat straw
Corn stalk
Whole soil
Free OM
Intra-macroaggregate OM
Intra-microaggregate OM
Mineral-associated OM
10
8
6
4
2
0
Chemical shift (ppm)

Fig. 3

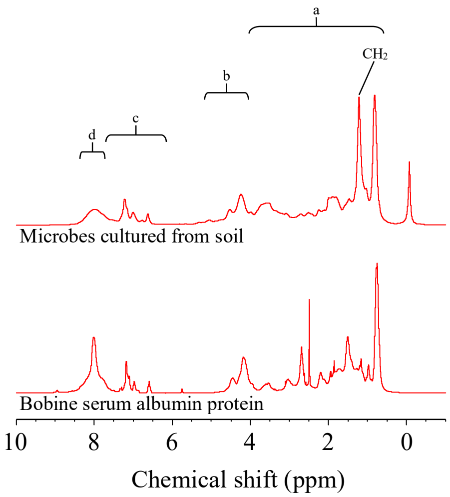
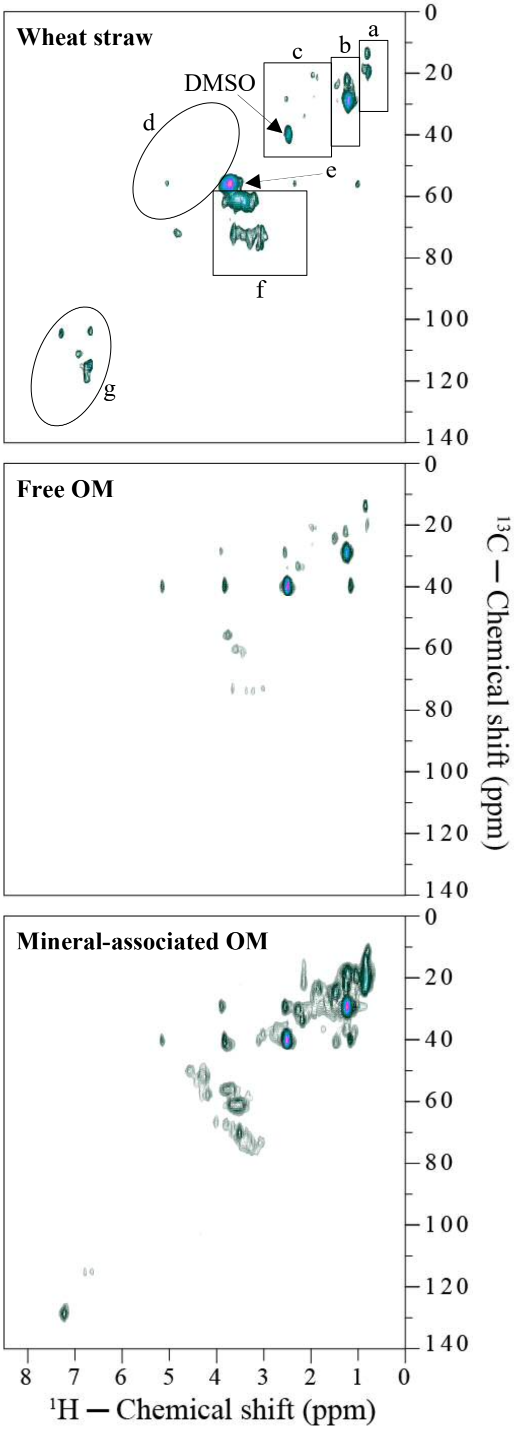

a
CH2
b
c
d
Microbes cultured from soil
Bobine serum albumin protein
10
8
6
4
2
0
Chemical shift (ppm)

Fig. 4



Wheat straw
a
b
c
DMSO
d
e
f
g
Free OM
Mineral-associated OM
0
20
40
60
80
100
120
140
^{13}C — Chemical shift (ppm)
8
7
6
5
4
3
2
1
0
^{1}H — Chemical shift (ppm)

Fig. 5

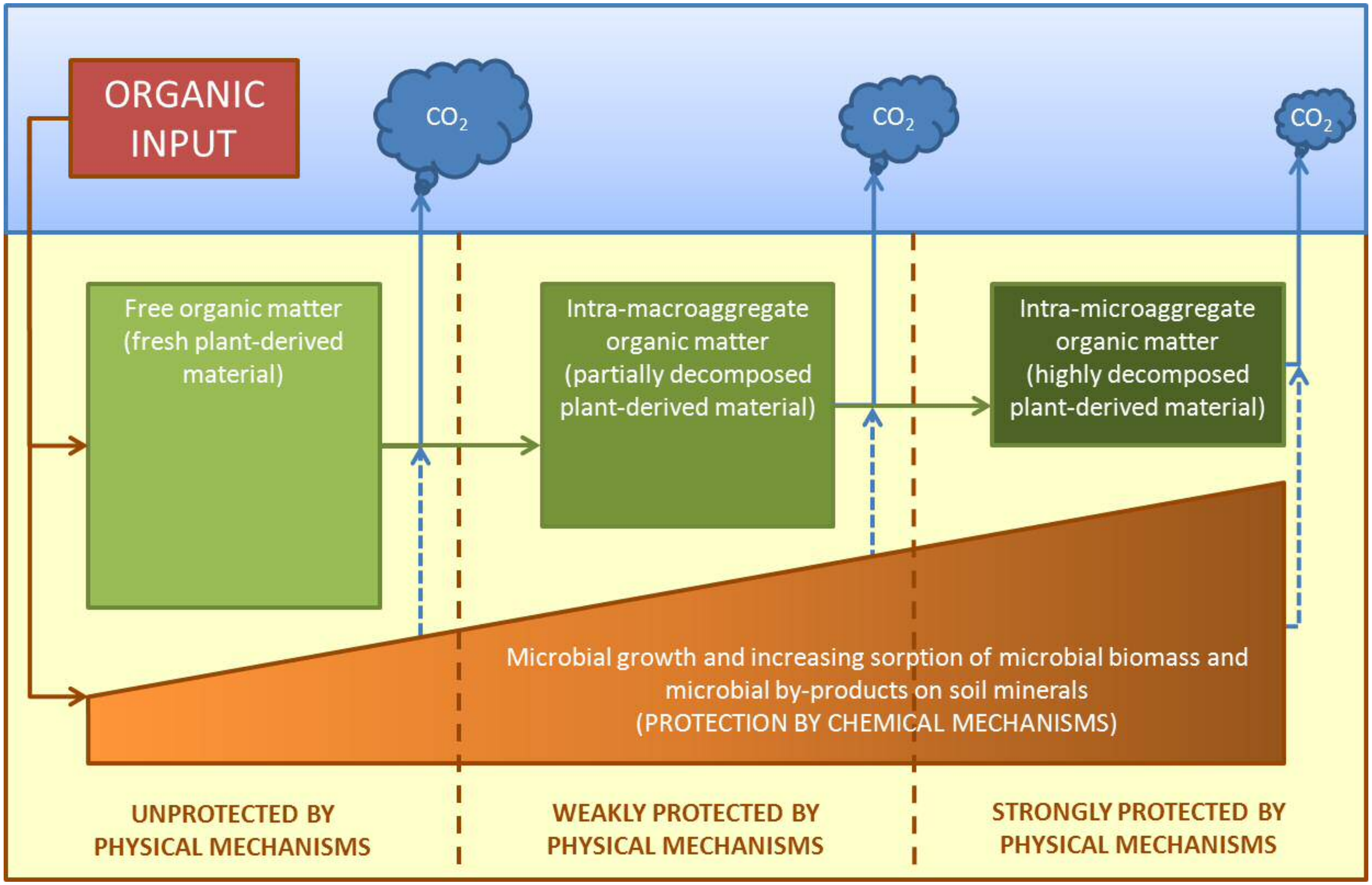

ORGANIC INPUT
CO2
CO2
CO2
Free organic matter (fresh plant-derived material)
Intra-macroaggregate organic matter (partially decomposed plant-derived material)
Intra-microaggregate organic matter (highly decomposed plant-derived material)
Microbial growth and increasing sorption of microbial biomass and microbial by-products on soil minerals (PROTECTION BY CHEMICAL MECHANISMS)
UNPROTECTED BY PHYSICAL MECHANISMS
WEAKLY PROTECTED BY PHYSICAL MECHANISMS
STRONGLY PROTECTED BY PHYSICAL MECHANISMS

Fig. 6

Table 1

Content of total, free, intra-macroaggregate, intra-microaggregate, mineral-associated, and dissolved organic C (mean ± standard error, $n = 3$) in soils unamended (UN) and amended with solid cattle manure (SM) and crop residues (CR), p-values, and effect sizes

Organic matter (OM) fraction	Organic C (g kg^{-1})			p-value	Effect size, r		
	UN	SM	CR		UN vs. SM	UN vs. CR	CM vs. CR
Total OM	6.46 ± 0.38 b	8.71 ± 0.50 a	7.15 ± 0.40 ab	0.025	0.84 (Large)	0.42 (Medium)	0.73 (Large)
Free OM	0.42 ± 0.09 a	0.57 ± 0.03 a	0.39 ± 0.02 a	0.139	0.60 (Large)	0.16 (Small)	0.67 (Large)
Intra-macroaggregate OM	0.27 ± 0.01 b	0.43 ± 0.03 a	0.26 ± 0.02 b	0.002	0.91 (Large)	0.19 (Small)	0.92 (Large)
Intra-microaggregate OM	0.55 ± 0.14 a	0.91 ± 0.12 a	0.67 ± 0.05 a	0.141	0.69 (Large)	0.30 (Medium)	0.53 (Large)
Mineral-associated OM	4.51 ± 0.22 a	5.75 ± 0.46 a	4.93 ± 0.36 a	0.120	0.71 (Large)	0.32 (Small)	0.55 (Large)
Dissolved organic OM	0.68 ± 0.07 a	0.84 ± 0.02 a	0.78 ± 0.06 a	0.225	0.62 (Large)	0.43 (Medium)	0.30 (Small)

Within OM fractions, means followed by the same letter are not significantly different according to the Bonferroni test at the 0.05 level.

Table 2

Content of total, free, intra-macroaggregate, intra-microaggregate, and mineral-associated + dissolved N (mean ± standard error, *n* = 3) in soils unamended (UN) and amended with solid cattle manure (SM) and crop residues (CR), *p*-values, and effect sizes

Organic matter (OM) fraction	N (g kg^{-1})			*p*-value	Effect size, *r*		
	UN	SM	CR		UN vs. SM	UN vs. CR	CM vs. CR
Total OM	0.82 ± 0.01 b	1.07 ± 0.05 a	0.87 ± 0.06 ab	0.019	0.84 (Large)	0.32 (Medium)	0.72 (Large)
Free OM	0.02 ± 0.00 b	0.04 ± 0.00 a	0.03 ± 0.00 ab	0.005	0.91 (Large)	0.68 (Large)	0.78 (Large)
Intra-macroaggregate OM	0.02 ± 0.00 b	0.04 ± 0.01 a	0.01 ± 0.00 ab	0.019	0.70 (Large)	0.55 (Large)	0.85 (Large)
Intra-microaggregate OM	0.03 ± 0.01 b	0.06 ± 0.01 a	0.04 ± 0.00 ab	0.016	0.86 (Large)	0.46 (Medium)	0.76 (Large)
Mineral-associated + dissolved OM	0.75 ± 0.00 a	0.92 ± 0.06 a	0.79 ± 0.06 a	0.076	0.74 (Large)	0.26 (Small)	0.65 (Large)

Within OM fractions, means followed by the same letter are not significantly different according to the Bonferroni test at the 0.05 level.

Table 3

C/N ratio of total, free, intra-macroaggregate, intra-microaggregate, and mineral-associated + dissolved organic matter (OM) pools (mean ± standard error, *n* = 3) in soils unamended (UN) and amended with solid cattle manure (SM) and crop residues (CR), *p*-values, and effect sizes

OM fraction	N (g kg^{-1})			*p*-value	Effect size, *r*		
	UN	SM	CR		UN vs. SM	UN vs. CR	CM vs. CR
Total OM	7.9 ± 0.4 a	8.2 ± 0.2 a	8.2 ± 0.1 a	0.621	0.31 (Medium)	0.36 (Medium)	0.06
Free OM	23.8 ± 1.2 a	14.3 ± 1.0 b	14.4 ± 0.3 b	0.000	0.95 (Large)	0.95 (Large)	0.02
Intra-macroaggregate OM	12.0 ± 0.8 a	12.5 ± 2.3 a	18.6 ± 1.2 a	0.041	0.10 (Small)	0.78 (Large)	0.75 (Large)
Intra-microaggregate OM	20.2 ± 1.8 a	14.2 ± 0.2 b	17.4 ± 0.3 ab	0.019	0.85 (Large)	0.61 (Large)	0.66 (Large)
Mineral-associated + dissolved OM	6.9 ± 0.4 a	7.1 ± 0.2 a	7.2 ± 0.0 a	0.722	0.21 (Small)	0.32 (Medium)	0.11 (Small)

Within OM fractions, means followed by the same letter are not significantly different according to the Bonferroni test at the 0.05 level.

Table 4
Distribution of C structures in cattle manure, wheat straw, corn stalk, soils unamended and amended with cattle manure and crop residues, and their respective organic matter (OM) fractions, as estimated from ^{13}C CPMAS NMR integration

	Unsubstituted alkyl C ($0 < \delta \leq 45$ ppm) (%)	Substituted alkyl C ($45 < \delta \leq 95$ ppm) (%)	Anomeric C ($95 < \delta \leq 110$ ppm) (%)	Aromatic C ($110 < \delta \leq 160$ ppm) (%)	Carboxyl and carbonyl C ($160 < \delta \leq 200$ ppm) (%)
Cattle manure	18.4	51.9	9.4	14.1	6.1
Wheat straw	5.8	69.1	14.2	8.6	2.4
Corn stalk	8.0	67.4	13.7	7.4	3.5
Unamended soil					
Whole soil	26.9	38.5	6.9	19.3	8.4
Free OM	17.2	45.4	9.4	21.8	6.3
Intra-macroaggregate OM	25.3	36.9	6.3	24.0	7.4
Intra-microaggregate OM	25.2	35.6	6.3	25.9	6.9
Mineral-associated OM	30.6	35.3	5.0	18.2	10.8
Soil amended with cattle manure					
Whole soil	27.9	37.5	6.4	19.2	9.0
Free OM	16.3	45.3	9.0	22.5	7.0
Intra-macroaggregate OM	23.9	37.1	6.8	25.0	7.1
Intra-microaggregate OM	23.7	37.6	6.7	24.3	7.7
Mineral-associated OM	30.9	35.5	5.1	18.2	10.3
Soil amended with crop residues					
Whole soil	27.3	39.3	6.2	19.1	8.1
Free OM	18.5	43.8	8.9	22.8	6.0
Intra-macroaggregate OM	22.2	40.1	7.3	24.7	5.7
Intra-microaggregate OM	24.7	37.5	6.6	24.8	6.5
Mineral-associated SOM	32.0	35.3	5.0	17.4	10.2

Highlights

- We examined changes in soil organic matter fractions from long term amended plots
- Organic matter fractions were analyzed by a range of modern NMR techniques
- Manure and crop residues increase total organic C content by 35 and 10%
- Organic matter accrues on mineral surfaces as microbial biomass and by-products