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Physical, chemical, and biochemical mechanisms of soil organic matter stabilization under conservation tillage systems: A central role for microbes and microbial by-products in C sequestration

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ABSTRACT

Conservation tillage practices that entail no or reduced soil disturbance are known to help preserve or accumulate soil organic matter (OM). However, the underlying mechanisms especially at the molecular level are not well understood. In this study soil samples from 25-year-old experimental plots continuously cropped with barley (*Hordeum vulgare* L.) under no-tillage (NT) and chisel tillage (CT) were subjected to a new physical fractionation method to isolate dissolved OM, mineral-free particulate OM located outside aggregates (physically and chemically unprotected), OM occluded within both macroaggregates and microaggregates (weakly and strongly protected by physical mechanisms, respectively), and OM in intimate association with minerals (protected by chemical mechanisms). The whole soils and OM fractions were analyzed for organic C and N content and by modern nuclear magnetic resonance (NMR) techniques. The soil under NT stored 16% more organic C and 5% more N than the soil under CT. Compared to CT, NT increased free organic C content by 7%, intra-macroaggregate organic C content by 20%, intra-microaggregate organic C content by 63%, and mineral-associated organic C content by 16% and decreased dissolved organic C content by 11%. The mineral-associated OM pool accounted for 65% of the difference in total organic C content between NT and CT, whereas the intra-microaggregate OM only explained 18%, intra-macroaggregate OM 14%, and free OM 11%. The NMR experiments revealed that the free and intra-aggregate OM fractions were dominated by crop-derived materials at different stages of decomposition, whereas the mineral-associated OM pool was predominately of microbial origin. Overall, our results indicate that microbes and microbial by-products associated with mineral surfaces and likely physically protected by entrapment within very small microaggregates constitute the most important pool of OM stabilization and C sequestration in soils under NT. Most probably the slower macroaggregate turnover in NT relative to CT boosts not only the formation of microaggregates and thereby the physical protection of crop-derived particulate OM, but more importantly the interaction between mineral particles and microbial material that results in the formation of very stable organo-mineral complexes.

1. Introduction

Preserving the organic matter (OM) content in agricultural soils is of paramount importance, due to its central role in determining soil properties that strongly affect crop production and the quality of the wider environment (Lal, 2009; Powlson et al., 2012). Fresh OM is stabilized in soil by biochemical processes including the formation and selective preservation of molecules, structural rearrangements, and molecular associations more resistant to decomposition (Piccolo, 2001; Six et al., 2002a; Schnitzer and Monreal, 2011). However, the persistence of soil OM is known to depend not only on its molecular structure alone but also on the combined action of physical and chemical protection mechanisms (Six et al., 2002a; Von Lützow et al., 2006; Schmidt et al., 2011). Physical protection mechanisms refer to the occlusion of soil OM within aggregates, which forms a physical barrier that limits the accessibility of decomposers and enzymes to the organic substrates and the diffusion of O_2; physical protection depends on the level of aggregation and has been shown to be much greater within microaggregates than within macroaggregates (Pulleman and Marinissen, 2004). Chemical stabilization mechanisms refer to the intimate association of OM with mineral particles, which

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reduces the degrading capacity of decomposers and enzymes (Six et al., 2002a).

It has been widely documented that conservation practices that entail no or reduced soil disturbance help maintain and build up surface soil OM, in contrast to intensive tillage (Paustian et al., 2000; West and Post, 2002; Lal, 2004; Six et al., 2004). Soil aggregate dynamics are known to be central to the stabilization of OM. According to the widely accepted description of aggregate dynamics proposed by Golchin et al. (1994), fresh plant material entering the soil is colonized by microorganisms and encrusted by primary particles through the binding action of microbial agents (e.g., mucilage and polysaccharides), thus forming stable macroaggregates. With time, the fresh plant material within macroaggregates is selectively decomposed leaving the more chemically recalcitrant plant structural materials, which are coated with microbial metabolites and mineral particles to form stable microaggregates. On the basis of this description, Six et al. (1998, 1999, 2000) developed a conceptual model that links soil aggregate turnover and OM dynamics to explain differences in OM stabilization and C sequestration under different tillage systems. According to this model, soil tillage increases macroaggregate turnover and thereby inhibits the formation of microaggregates within macroaggregates in which particulate OM is stabilized in the long term.

Much of the most valuable research on soil OM dynamics as affected by tillage has relied on physical fractionation methods (e.g., dry and wet sieving, aggregate dispersion, and density separation) followed by C and N analysis to assess the quantity of OM physically and chemically protected in different aggregate classes and organo-mineral complexes (Jastrow, 1996; Six et al., 1998, 1999, 2000; Denef et al., 2004; Bayer et al., 2006). However, additional molecular-level information is required to better understand the composition and chemical structure of these OM pools. The objective of this study was to investigate comparatively the effects of long-term no-tillage and chisel plow on the quantity and molecular structure of soil OM dissolved and located outside aggregates (unprotected), occluded within both macroaggregates and microaggregates (weakly and strongly protected by physical mechanisms, respectively), and in intimate association with soil minerals (protected by chemical mechanisms), in order to provide a better understanding of the mechanisms of OM stabilization and gain an insight into the conservation tillage options for C sequestration in soils. To achieve this objective, soil samples were fractionated by the procedure recently developed by Plaza et al. (2012). This physical fractionation method enables isolation of the targeted OM pools, which are measurable and meaningful inasmuch as they are directly connected to the aforementioned conceptual mechanisms of soil OM preservation (Fig. 1). The whole soils and their OM fractions were analyzed by a range of modern nuclear magnetic resonances (NMR) techniques, including solid-state cross polarization magic angle spinning (CP-MAS), solution-state, and high resolution (HR)-MAS NMR spectroscopy. Such techniques are among the most, if not the most, powerful analytical tools for investigating soil OM structure and composition at the molecular level (Kögel-Knabner, 1997, 2000; Simpson and Simpson, 2009; Simpson et al., 2011) and have been proven capable to provide valuable information in recent studies of degradation and transformation of plant residues and microbial biomass (Kelleher et al., 2006; Spence et al., 2011). In particular, solid-state ^{13}C CP-MAS NMR provides an excellent overview as to the total C distribution in a sample, while solution-state ^{1}H provides the highest resolution possible as to the H distribution in soluble materials. Nowadays, also the use of HR-MAS NMR spectroscopy is possible, which allows the acquisition of very high resolution spectra of swellable, partially-soluble, and soluble organic materials. It also allows the potential application of a number of advanced experiments also available in solution-state NMR, such as diffusion edited (DE) ^{1}H and $^{1}H-^{13}C$ heteronuclear single quantum coherence (HSQC) NMR.

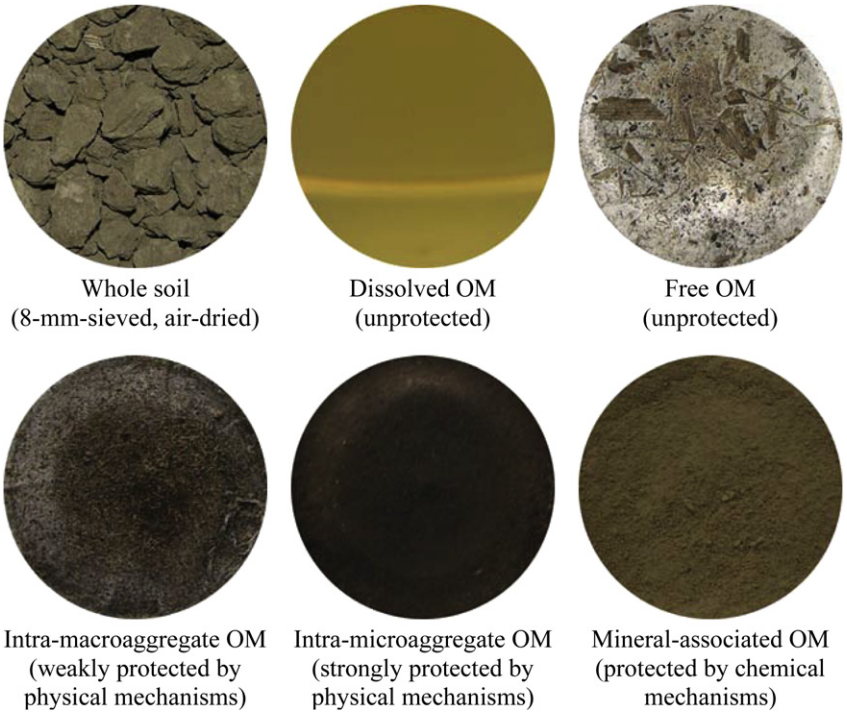

Fig. 1. Dissolved organic matter (OM), mineral-free particulate OM located outside aggregates (not protected from decomposition by physical and chemical mechanisms), mineral-free OM occluded within both macroaggregates and microaggregates (weakly and strongly protected by physical mechanisms, respectively), and OM in intimate association with soil minerals (protected by chemical mechanisms) isolated by the physical fractionation method of Plaza et al. (2012) from an 8-mm-sieved, air-dried soil.

Specifically, DE ^{1}H NMR selectively attenuates signals from water, solvents, and small molecules undergoing little self-diffusion and relatively enhances those from large molecules and aggregates. ^{1}H–^{13}C HR-MAS HSQC is a multidimensional technique (one axis for ^{1}H and the other for ^{13}C chemical shift) that provides additional spectral dispersion and one-bond connectivity information which facilitates assignments of the chemical species in complex matrices.

2. Materials and methods

2.1. Field experiment and soil sampling

The field experiment used in this study was established in 1986 on a site that had been under long-term intensive tillage at the research farm "La Higueruela" of the Spanish National Research Council (CSIC), located near Santa Olalla, Toledo, Spain (40°3′ N, 4°25′ W; 440 m above sea level). The site has a Mediterranean climate, with moist cool winters and warm dry summers. The mean annual temperature is 15 °C, annual rainfall averages 450 mm, and potential evapotranspiration is 800 mm. The soil is a Typic Haploxeralf (Soil Survey Staff, 2010). The 0- to 20-cm horizon is sandy loam with 58% sand, 24% silt, and 18% clay; the content of non-crystalline minerals is 6%; non-crystalline Al content is 343 mg kg^{-1}, non-crystalline Fe content is 157 mg kg^{-1}, non-crystalline Si content is 2892 mg kg^{-1}, and non-crystalline Mn content is 23 mg kg^{-1}; feldspars and quartz are the most abundant minerals of the <2 mm fraction (approximately 47% and 40%, respectively); and the mineralogical composition of the clay (<2 μm) fraction is approximately 57% illite, 19% smectite, 10% quartz, 7% kaolinite, and 7% feldspars.

The experiment had a completely randomized design with three replicates. Each plot measured 7 by 20 m. The treatments were no-tillage (NT), which entailed no soil disturbance other than seed planting, and chisel tillage (CT), which consisted in the use of a chisel plow to a depth of 20 cm in the early autumn. All plots were under continuous winter barley (*Hordeum vulgare* L.) planted with a drill with double-disc openers for NT and a conventional hoe drill for CT.

Soil samples were collected after barley harvest in the summer of 2011, 25 years after the beginning of the experiment. Each soil sample consisted of a composite of 15 cores taken randomly from each plot with a probe to 20-cm depth. The samples were air-dried, gently crushed, and passed through a 2-mm sieve prior to OM fractionation. For 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 by combining the three corresponding replicates.

2.2. Soil organic matter fractionation

Soil OM was separated into dissolved, free, intra-macroaggregate, intra-microaggregate, and mineral-associated OM using the procedure described by Plaza et al. (2012) with slight modifications. Briefly, 20 g of 2-mm sieved, air-dried soil was placed into a 100-mL centrifuge tube, and 80 mL of sodium polytungstate (SPT) at a density of 1.85 g mL^{-1} was added. The centrifuge tube was rotated at 1 revolution s^{-1} for 30 s in an overhead shaker to allow free OM outside aggregates (and inside unstable aggregates) to float. After centrifugation at 2500 × *g* for 30 min, the floating light fraction (free OM) was separated from the heavy fraction by suction and filtration through a glass fiber filter (GF/A, Whatman, UK) and washed thoroughly with deionized water. The heavy fraction in the centrifuge tube was transferred to the top of a 250-μm sieve, immersed in deionized water, and shaken with 50 stainless steel beads (4-mm diameter) at 150 strokes min^{-1} on a reciprocating shaker under a continuous, steady deionized water flow of about 0.2 L min^{-1}, using the device designed by Six et al. (2000, 2002b) to break up stable macroaggregates. Microaggregates and other soil components <250 μm flushed through the sieve were transferred to a beaker. Shaking was stopped when water below the 250-μm sieve ran clear and after visually checking that all macroaggregates were broken. The fraction flushed through the 250-μm sieve and the fraction collected over the sieve were oven-dried at 70 °C and then recombined and gently transferred into a 100-mL polycarbonate centrifuge tube together with the filtrate from the first step (SPT solution). The tube was rotated at 1 revolution s^{-1} for 30 s and centrifuged at 2500 × *g* for 45 min. The floating light particles (intra-macroaggregate OM) were separated from the heavy fraction by suction and filtration through a glass fiber filter and washed thoroughly with deionized water. Finally, the heavy fraction was resuspended and dispersed in the SPT solution by sonication at an energy input of 1500 J g^{-1}. The floating light fraction (intra-microaggregate OM) was separated from the heavy fraction (mineral-associated OM) by centrifugation at 2500 × *g* for 60 min, suction, and filtration through a glass fiber filter, and washed thoroughly with deionized water. The fractionation procedure was repeated three times to obtain enough material for analysis.

The free, intra-macroaggregate, intra-microaggregate, and mineral-associated OM fractions recovered after fractionation were oven-dried at 70 °C, weighed, and ground with a ball mill prior to analysis. For C and N analysis, the free and intra-aggregate OM fractions were ground with the glass fiber filters, whereas for NMR analysis they were completely washed off the glass fiber filters before drying and grinding. An aliquot of the SPT solution after fractionation, which contained the dissolved OM fraction, was freeze-dried and ground with a mortar. For NMR analysis, composite samples of all OM fractions were prepared by combining the three corresponding replicates.

2.3. C and N analysis

Whole soil samples and solid OM fractions were analyzed for organic C and total N content by dry combustion using a Thermo Flash 2000 NC Soil Analyzer. Prior to analysis, carbonates were removed from the whole soil samples and mineral-associated OM fractions by acid fumigation (Harris et al., 2001). Organic C remaining in the SPT solution after fractionation (i.e., dissolved OM pool) was determined using a Shimadzu TOC 5000A analyzer. The SPT powder used to prepare the solution for density separation contained no C but some N (0.665 g kg^{-1}). Because some SPT inevitably remained in the heavy fraction after the last separation, the N content in the mineral-associated OM fraction and the dissolved OM pool had to be estimated. In particular, the N content in the dissolved OM fraction was assumed to be equal to the water-soluble total N content determined on extracts obtained at a soil-to-water ratio of 1:4 (equivalent to the ratio used for soil OM fractionation) by the 2,6-dimethylphenol method after digestion with peroxodisulphate. The N content in the mineral-associated OM fraction was then estimated by subtracting the N content in the free and intra-aggregate OM pools plus the water-soluble N content from the total N content in the whole soil.

2.4. Nuclear magnetic resonance analysis

All NMR spectra were acquired on a Bruker Avance III 500 MHz NMR spectrometer at 298 K if not otherwise stated. Prior to analysis, the whole soil samples and mineral-associated OM fractions were repeatedly treated with 10% HF to remove paramagnetic compounds and minerals and enhance the signal-to-noise ratio (Schmidt et al., 1997).

Solid-state ^{13}C CP-MAS NMR spectra were recorded with a 4-mm H-X MAS probe at room temperature, using a MAS frequency

of 13,000 Hz, a recycle delay of 1 s, a ramp-CP contact time of 1 ms, and between 2112 and 225,280 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 calibrated using the carboxyl signal of glycine as an external standard (176.03 ppm).

Solution-state ^{1}H and DE ^{1}H NMR spectra of the OM dissolved during the fractionation procedure were acquired with a 5-mm QXI probe fitted with an actively shielded Z gradient. Two g of freeze-dried sample was dissolved in 0.8 mL of D_2O and transferred into 5-mm NMR tubes. For ^{1}H NMR spectra, a total of 1024 scans and 4096 time domain points were recorded. DE ^{1}H NMR spectra were acquired with a bipolar pulse longitudinal encode–decode sequence using a 2.5 ms encoding–decoding gradients at 50 G cm^{-1}, a diffusion time of 200 ms, 4096 time domain points, and 1024 scans. Both solution-state ^{1}H and DE ^{1}H NMR spectra were processed with a zero-filling factor of 2 and a line broadening of 1 Hz. Spectra were externally calibrated to the methyl group of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) (0.00 ppm).

^{1}H, DE ^{1}H, and ^{1}H–^{13}C HSQC HR-MAS NMR spectra were collected 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. For these analyses, ~35 mg of sample was placed in a zirconium rotor, mixed with 65 μL of deuterated dimethyl sulfoxide (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 bipolar pulse longitudinal encode–decode sequence using a 1.25 ms, 33.3 G 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 and an exponential function corresponding to 1 Hz line broadening in the transformed spectrum and calibrated using the solvent residual peak at 2.50 ppm. The ^{1}H–^{13}C HSQC HR-MAS NMR spectra were obtained in phase-sensitive mode using echo/anti-echo gradient selection and a 1 J ^{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 90°.

3. Results

3.1. C, N, and C/N ratio

The soil under NT stores 16% more organic C than the soil under CT (Table 1), the difference being statistically significant ($P < 0.05$). The organic C recovery in dissolved, free, intra-macroaggregate, intra-microaggregate, and mineral-associated OM pools after separation is 97.5% for NT and 99.6% for CT (Table 1), indicating that the C losses due to the fractionation procedure can be considered negligible. The mineral-associated OM fraction accounts for the greatest proportion of the total organic C in both NT and CT soils (55.7% and 54.4%, respectively), followed by the free (20.0% and 21.3%), intra-macroaggregate (10.3% and 9.8%), dissolved (8.3% and 10.6%), and intra-microaggregate (5.7% and 3.9%) fractions. Compared to the soil under CT, the soil under NT contains 7% more free organic C, 20% more intra-macroaggregate organic C, 63% more intra-microaggregate organic C, 16% more mineral-associated organic C, and 11% less dissolved organic C. Although the greatest relative organic C increase is found in the intra-microaggregate OM fraction, it is the mineral-associated OM pool that explains most of the difference in total organic C content between the NT and CT soils by far. In particular, up to 65% of the absolute difference in the total organic C content was explained by the mineral-associated OM, whereas only 18% were explained by the intra-microaggregate OM, 14% by the intra-macroaggregate OM, and 11% by the free OM.

The total N content of the soil under NT is slightly greater (5%) than that of the soil under CT, but the difference is not statistically significant ($P > 0.05$). For both NT and CT, approximately 74% of the total N is estimated to be associated with the mineral fraction. The C/N ratio of the soil under NT is slightly larger than that of the soil under CT (Table 1). For both NT and CT, the C/N ratio decreases from 25–29 to 7.3–6.5 in the order dissolved > free > macro-aggregate > micro-aggregate > mineral-associated OM (Table 1). With the exception of the dissolved and free OM, the C/N ratios of the OM fractions in the soil under NT are higher than those in the soil under CT, the differences being more pronounced in the intra-aggregate OM fractions.

3.2. Nuclear magnetic resonance spectra

Fig. 2 shows the solid state ^{13}C CP-MAS NMR spectra of barley straw, the whole soils, and their corresponding OM fractions, together with ^{13}C chemical shift range assignments according to Simpson and Simpson (2009), Simpson et al. (2011), and references therein. Table 2 lists the integrated values of the unsubstituted alkyl, substituted alkyl, anomeric, aromatic, and carboxyl and carbonyl C regions. The ^{13}C CP-MAS NMR spectrum of barley straw is dominated by signals in the 60–65, 70–80, and 90–110 ppm regions, which are attributed to carbohydrates. Compared to the spectrum of barley straw, the spectra of the two whole soils feature more intense signals in the 0–50 ppm region due to terminal methyl C, methylene C in aliphatic rings, and methylene C in alkyl chains, at 55 ppm due to methoxyl C, at 130 ppm due to alkyl-substituted aromatic C, and at 170 ppm due carboxyl and amidic

Table 1
Organic C and N in no-tillage (NT) and chisel tilled (CT) soils and in the free, intra-macroaggregate, intra-microaggregate, mineral-associated, and dissolved organic matter (OM) fractions (mean ± standard error, $n = 3$).

Pool	Organic C (g kg^{-1}) [a]		N (g kg^{-1}) [a]		C/N ratio	
	NT	CT	NT	CT	NT	CT
Whole soil	8.11 ± 0.21	6.99 ± 0.31	0.82 ± 0.04	0.79 ± 0.02	9.9 ± 0.2	8.9 ± 0.2
Free OM	1.58 ± 0.27	1.48 ± 0.20	0.09 ± 0.01	0.08 ± 0.01	16.7 ± 0.4	17.8 ± 0.2
Intra-macroaggregate OM	0.81 ± 0.10	0.68 ± 0.05	0.06 ± 0.01	0.06 ± 0.00	14.8 ± 0.6	11.9 ± 0.9
Intra-microaggregate OM	0.45 ± 0.04	0.27 ± 0.07	0.04 ± 0.00	0.03 ± 0.00	10.5 ± 0.5	7.9 ± 1.0
Mineral-associated OM	4.41 ± 0.25	3.79 ± 0.26	0.61 ± 0.04[b]	0.59 ± 0.01[b]	7.3 ± 0.2	6.5 ± 0.4
Dissolved OM	0.66 ± 0.06	0.74 ± 0.01	0.03 ± 0.00[c]	0.03 ± 0.00[c]	25.2 ± 0.8	28.8 ± 3.2

[a] On whole soil basis.
[b] Estimated by subtracting N content in free and intra-aggregate OM pools, plus water-soluble N content, from total N content in whole soil.
[c] Water-soluble N.

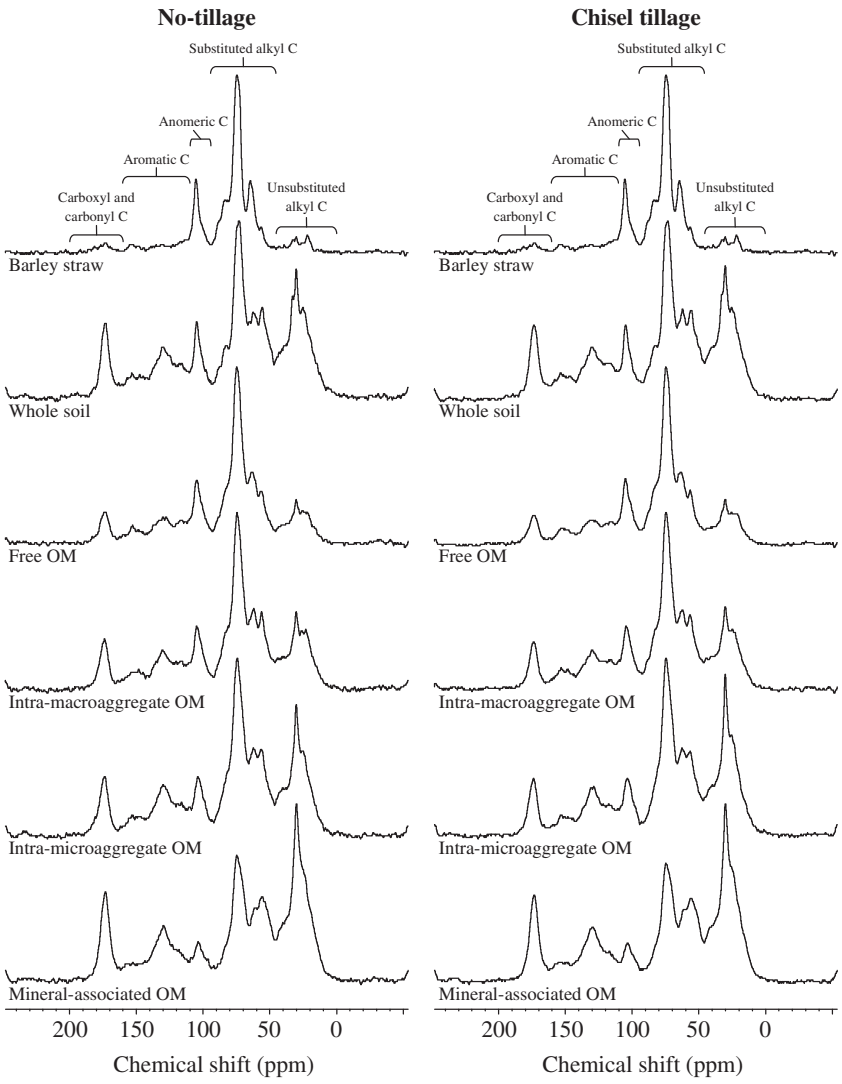

Fig. 2. ^{13}C CP-MAS NMR spectra of barley straw, soils under chisel tillage and no-tillage management for 25 years, and their respective organic matter (OM) fractions.

C, and less intense signals at 105 ppm due to anomeric C and ring C at 70 ppm due to carbohydrates. For both NT and CT, the intensity of the signals attributed to carbohydrates decreases in the order free > macro-aggregate > micro-aggregate > mineral-associated OM, along with an increase in the intensity of the resonances attributed to unsubstituted alkyl, methoxyl, alkyl-substituted aromatic, carboxyl, and amidic C.

The ^{1}H NMR and DE ^{1}H NMR spectra of the samples examined are shown in Figs. 3 and 4, respectively, together with structural assignments according to Simpson et al. (2011) and references therein. Free and intra-aggregate OM fractions contain traces of SPT as indicated by the resonance at around 6.7 ppm in the ^{1}H NMR and DE ^{1}H NMR spectra, although they were washed thoroughly with deionized water. This resonance does not occur in the spectra of the

Table 2
Distribution of C structures in barley straw, soils under chisel tillage and no-tillage management for 25 years, and their respective organic matter (OM) fractions as estimated from ^{13}C CP-MAS 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) (%)
Barley straw	7.5	65.8	13.7	9.3	3.7
No-tillage					
Whole soil	26.4	41.5	7.2	16.6	8.3
Free OM	14.3	54.1	10.4	15.2	5.9
Intra-macroaggregate OM	20.6	48.4	8.4	15.8	6.8
Intra-microaggregate OM	24.8	44.2	7.0	16.6	7.4
Mineral-associated OM	33.0	35.7	4.8	16.2	10.4
Chisel tillage					
Whole soil	26.2	40.9	7.0	17.3	8.7
Free OM	14.4	54.1	10.4	15.2	5.9
Intra-macroaggregate OM	20.6	47.8	8.5	16.5	6.6
Intra-microaggregate OM	28.4	42.8	6.8	15.4	6.6
Mineral-associated OM	33.1	34.7	4.8	16.9	10.6

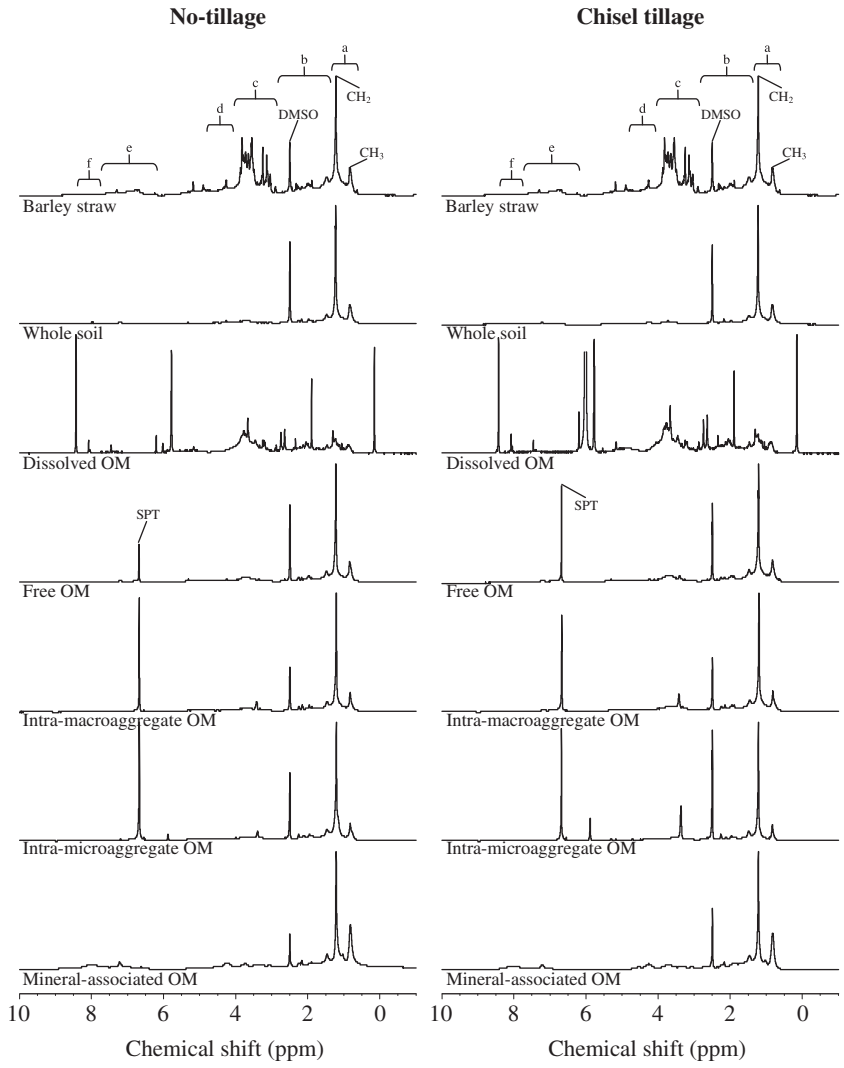

Fig. 3. ^{1}H HR-MAS NMR spectra of barley straw, soils under chisel tillage and no-tillage management for 25 years, and their respective free, intra-macroaggregate, intra-microaggregate, and mineral-associated organic matter (OM) pools, and solution-state ^{1}H NMR spectra of the dissolved OM fractions. General assignments of chemical shift ranges 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 (4.1–4.8 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.

mineral-associated OM because this fraction was repeatedly treated with HF.

The ^{1}H HR-MAS NMR spectrum of barley straw shows relatively strong and sharp signals at 0.6 and 1.3 ppm due to terminal methyl and methylene groups, slightly less intense sharp resonances in the 2.9–4.1 ppm region due to *O*-alkyl mainly from carbohydrates and lignin, and relatively weak signals in the 6.2–7.8 region due to aromatic moieties. The resonances between 6.2 and 7.8 ppm probably arise mainly from lignin and are observed in all samples except the mineral-associated OM fractions, which are dominated by a signature from protein aromatic groups in this region (Simpson et al., 2011). After diffusion editing, the intensity of the resonances attributed to terminal methyl and methylene groups decreases slightly, which indicates the presence of small aliphatic structures. These are common in soil and have been attributed to aliphatic acids and alcohols present in both plant and microbial biomass (Simpson et al., 2011).

The ^{1}H NMR and DE ^{1}H NMR spectra of the whole soil and its OM fractions under NT management are very similar to the corresponding spectra of the CT soil and its OM fractions. In contrast, considerable differences do exist between the various OM pools. In particular, the solution-state ^{1}H NMR spectra of dissolved OM exhibits a number of sharp signals attributable to the presence of a high variety of relatively small compounds not observed in the DE spectra. The DE spectra are dominated by strong signals in the 2.9–4.1 ppm which are consistent with relatively large carbohydrates. The lack of aromatic signals indicates there is very little lignin in this fraction. In contrast, the ^{1}H HR-MAS NMR spectra of the free and intra-aggregate OM pools are dominated by strong sharp resonances from aliphatic chains at 0.8 and 1.2 ppm. The relative intensity of these signals is attenuated after diffusion editing, indicating most to be relatively mobile species; the signal that remains could be lipids restricted in membranes (for example inside microbial cells) or larger molecular weight cuticular type materials derived from plants (Simpson et al., 2007a). In addition there is a strong contribution in the 2.9–4.1 ppm region attributable to relatively large carbohydrates overlapping with methoxyl signal from lignin. In the DE spectra, the intensity of the signals of carbohydrates and lignin decreases in the order free > intra-macroaggregate > intra-microaggregate OM, concomitant with an increase of the resonances from aliphatic compounds.

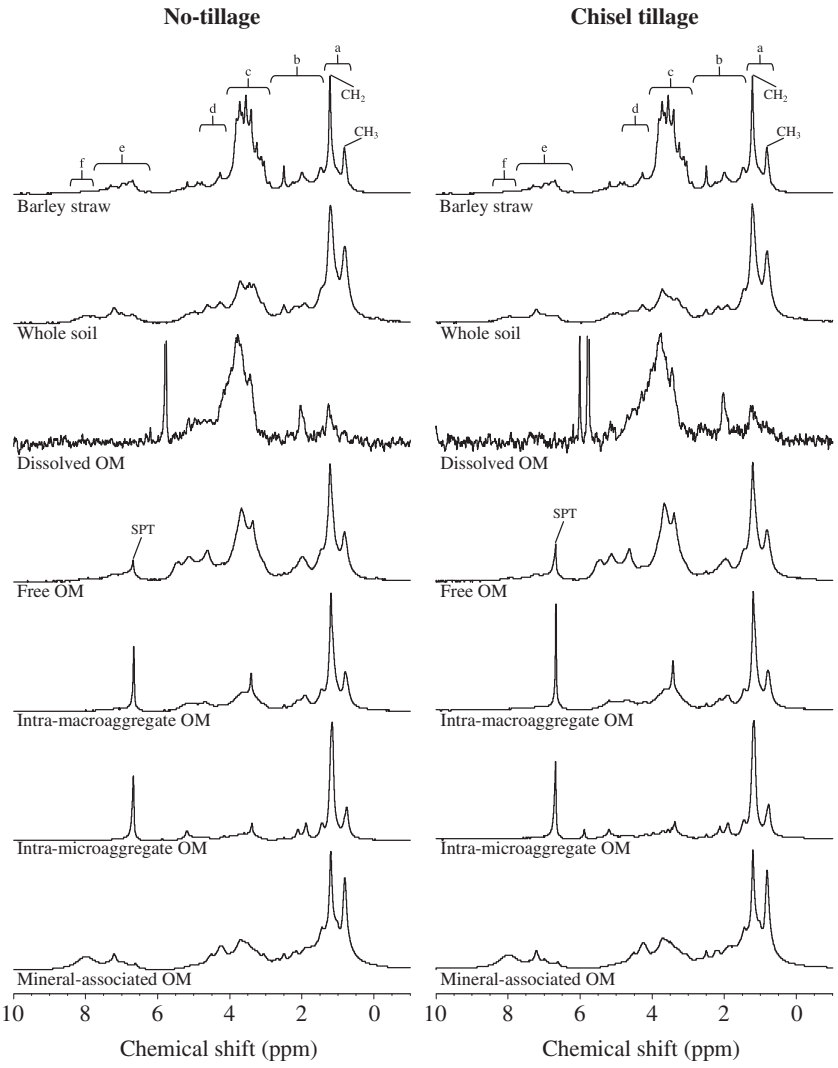

Fig. 4. Diffusion edited (DE) ^{1}H HR-MAS NMR spectra of barley straw, soils under chisel tillage and no-tillage management for 25 years, and their respective free, intra-macroaggregate, intra-microaggregate, and mineral-associated organic matter (OM) pools, and solution-state DE ^{1}H NMR spectra of the dissolved OM fractions. General assignments of chemical shift ranges 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 (4.1–4.8 ppm); (e) aromatic, from lignin and peptides (6.2–7.8 ppm); and (f) amide in peptides (7.8–8.4 ppm). SPT, sodium polytungstate.

The ^{1}H HR-MAS NMR and DE ^{1}H HR-MAS NMR spectra of the mineral-associated OM fractions differ radically from those of the free and intra-aggregate OM. In particular, the similarity between the ^{1}H NMR and DE ^{1}H NMR spectra is stronger for the mineral-associated OM than for the other OM fractions, which suggests a more marked presence of relatively large molecules and/or compounds in rigid domains. In addition, the intensity of the peak at 1.2 ppm due to methylene groups relative to that at 0.8 ppm due to terminal methyl groups in the ^{1}H HR-MAS NMR spectra and especially in the DE ^{1}H HR-MAS NMR spectra of the mineral-associated OM fractions is much smaller than in the spectra of the other OM pools. This strongly suggests a significant contribution from methyl rich amino acids rather than methylene-containing compounds, such as cutin, suberin, and other plant-derived lipid biopolymers (Simpson et al., 2007b). Further, unlike the spectra of the free and intra-aggregate OM pools, the spectra of the mineral-associated OM pool exhibit signals at 4.1–4.8 ppm and 7.8–8.4 ppm that have been shown to be attributed to α-H and amides in proteins, at 6.6 ppm and 7.0 ppm attributable to tyrosine, and at 7.2 ppm consistent with the presence of phenylalanine (Simpson et al., 2007b). It is noteworthy that the ^{1}H HR-MAS NMR and DE ^{1}H HR-MAS NMR spectra of the mineral-associated OM pools, especially the 2.5–9 ppm region, are very similar to those of albumin (Fig. 5), a pure protein, and also match very well those of soil microbial cultures reported in other studies (Simpson et al., 2007b). As pointed out by Simpson et al. (2007b), a significant contribution of crop-derived protein structures to these signals is unlikely considering the low content in plants and high degradability in a soil environment. Indeed near identical NMR profiles as seen here have been thoroughly assigned to microbial biomass in both whole soils (Simpson et al., 2007b) and humin fractions (Simpson et al., 2007a). Thus, overall, these clearly indicate that mineral-associated OM is predominately of microbial origin.

The ^{1}H–^{13}C HSQC HR-MAS NMR spectra of barley straw, free OM, and mineral-associated OM (Fig. 6) confirm the information provided by the 1D NMR spectra. In particular, the HSQC spectrum of barley straw is dominated by resonances due to carbohydrates and lignin, accompanied by strong signals from aliphatic structures with high contributions from methylene groups. The HSQC spectrum of free OM exhibits strong signals attributed to plant-derived aliphatic structures and weak resonances due to carbohydrates and lignin. Finally, compared to the HSQC spectra of barley straw and

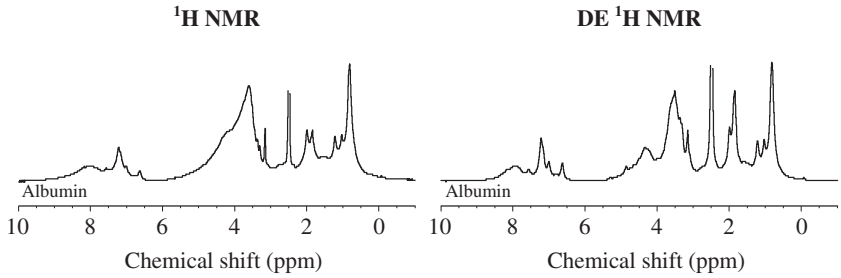

Fig. 5. ^{1}H HR-MAS NMR spectra and diffusion edited (DE) ^{1}H HR-MAS NMR spectra of albumin.

free OM, the HSQC spectrum of mineral-associated OM shows much more intense signals attributed to terminal methyl groups relative to those due to methylene, as well as resonances from α-H/C in proteins/peptides and aromatic amino acids (e.g., tyrosine and phenylalanine) consistent with microbial protein (Simpson et al., 2007b).

4. Discussion

In addition to this work, numerous studies report greater storage of organic C and N in soils under NT than in tilled soils, especially as the period of the comparisons and the difference in tillage intensity increase (e.g., Six et al., 2004; Paustian et al., 2000; Denef et al., 2004; Wang et al., 2008; Babujia et al., 2010). Using a global database of 67 long-term agricultural experiments, West and Post (2002) estimated that a change from conventional tillage to NT can sequester an average of 0.57 ± 0.14 g C m^{-2} $year^{-1}$ (excluding wheat-fallow systems). However, some studies have recently raised concerns about the actual effectiveness of conversion to NT, since possible changes in root growth and translocation of surface OM into soil depths below the plow layer have been largely ignored (Baker et al., 2007; Lal, 2009; Luo et al., 2010). For example, Dolan et al. (2006) found that the 0–20-cm layer of soils under NT had more than 30% higher soil organic C contents than moldboard plow and chisel plow tillage treatments after 23 years; however, no differences were found when the sampling depth was extended to 45 cm.

In agreement with Von Lützow et al. (2008), the potential for OM stabilization and C sequestration in agricultural soils is site- and horizon-specific. Von Lützow et al. (2008) also suggested that tillage decreases the contribution of aggregation to OM stabilization and increases the relative importance of organo-mineral interactions, due to the input of easily biodegradable crop-derived materials and chemical conditions that stimulate microbial activity. Our results, on the other hand, show that the relative distribution of OM in dissolved, free, intra-macroaggregate, intra-microaggregate, and mineral-associated pools in the CT soil is very similar to that in the NT soil.

The much greater content of organic C occluded within microaggregates in the soil under NT compared to CT reported in this study is consistent with the conceptual model developed by Six et al. (1998, 1999, 2000), which postulates that NT enhances microaggregate formation within macroaggregates, and consequently physical protection of fine particulate OM, due to a reduced macroaggregate turnover. However, it is the mineral-associated OM pool that explains to the greatest extent the difference in the total organic C content found between NT and CT. On the basis of these results, we argue that a reduced aggregate turnover, as in NT relative to CT, boosts not only the formation of microaggregates and, therefore, the physical protection of particulate OM, but more importantly the interaction between soil mineral particles and OM that results in the formation of stable organo-mineral complexes. Moreover, we suggest that microaggregates contribute to the long-term stabilization of OM in NT systems by providing physical protection not only to mineral-free OM but also to chemically-protected OM adsorbed on mineral surfaces.

Previous studies also suggest that C sequestration in soils under NT is enhanced in the mineral-associated OM fraction (Jastrow, 1996; Six et al., 2000; Denef et al., 2004), whereas others report no or little differences (Bayer et al., 2006; Álvaro-Fuentes et al., 2009). For example, in three soils characterized by different mineralogy, Denef et al. (2004) found that more than 90% of the total difference in soil organic C between NT and conventional tillage systems was explained by the difference in microaggregate-associated C, and more than 75% of microaggregate-associated C was mainly as mineral-associated C. In contrast, Bayer et al. (2006) reported that total and particulate organic C increased in soils under NT compared to conventional tillage, but mineral-associated organic C was not affected. The fractionation methods used in all these studies generally involve a first size separation step followed by a density separation to obtain free particulate OM fractions; particulate OM fractions occluded within the different aggregates are usually obtained subsequently by chemical or physical dispersion and sieving. The OM obtained in the fine fractions after sieving (usually <53 μm) are all assumed to be mineral associated, but such may not be the case. Recent research indicates that a substantial amount of OM not bound to minerals (i.e., not chemically protected) can be physically entrapped in silt-sized microaggregates (Virto et al., 2008, 2010). As previously suggested (Virto et al., 2010), the assumption that the OM pool in this fraction is homogeneous in terms of turnover rate and mechanisms by which it is protected can lead to important misinterpretations.

In our study, the mineral-associated OM fraction is isolated from mineral-free OM by density after full dispersion of aggregates by sonication, which reduces the uncertainty of previous works to support the hypothesis that long-term OM stabilization and C sequestration in undisturbed soils compared to tilled soils occur mainly in the mineral-associated OM pool. A study by Chenu and Plante (2006) strongly suggests that this fraction would include OM bound to soil minerals forming very small (clay-sized) microaggregates resistant to the ultrasonic dispersion treatment. These very small microaggregates are believed to constitute major sites of OM stabilization, not only chemically by adsorption but also physically by entrapment (Chenu and Plante, 2006). Transmission electron microscopy images of very small microaggregates showed clay particles surrounding plant cell wall debris, bacteria, and amorphous OM (Chenu and Plante, 2006), albeit the exact contribution of each one of these OM forms to soil OM was not determined.

The C/N ratios measured in our study suggest that, compared to CT, the OM accumulated in the soil under NT, especially in the physically-protected fractions, is relatively less decomposed. The C/

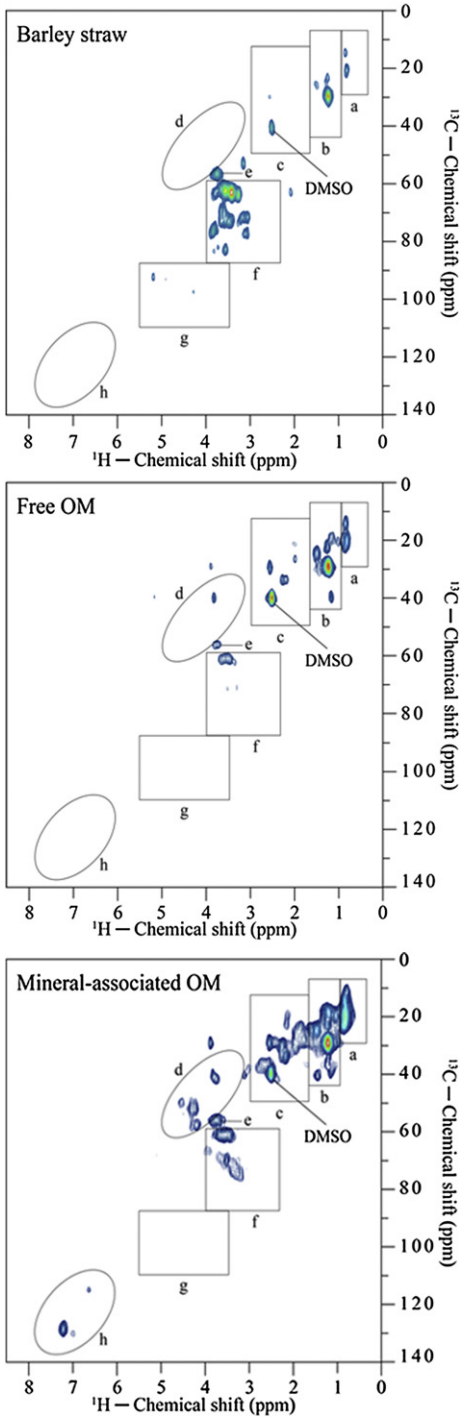

Fig. 6. ^{1}H–^{13}C HSQC HR-MAS NMR spectra of barley straw and free and mineral-associated organic matter (OM) pools isolated from the soil under no-tillage management for 25 years. General assignments of chemical shift regions are as follows: (a) CH_3 from lipids and/or peptides; (b) CH_2 in lipids; (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; (f) C–H bonds in carbohydrates; (g) anomeric H (carbohydrates); and (h) aromatic structures, mainly from aromatic amino acids. DMSO, dimethyl sulfoxide.

N ratios also suggest that the presence of undecayed plant tissues decreases and microbial biomass increases in the order free > macro-aggregate > micro-aggregate > mineral-associated OM. These interpretations are based on numerous studies documenting that the C/N ratio of fresh plant material entering the soil is usually much higher than those of soil OM and microbial biomass (~10–12 and ~6–7, respectively) and tends to decrease with increasing decomposition (Stevenson, 1994; Schlesinger, 1997; Simpson et al., 2007b).

The results obtained by NMR confirm and strongly complement the information provided by the C/N ratio and are consistent with the model of aggregate dynamics and microaggregate formation within macroaggregates described by Golchin et al. (1994). In particular, the NMR spectra of free and intra-aggregate OM fractions are dominated by identifiable signals from plant residues at different stages of decomposition. Free OM is relatively rich in fresh plant materials and labile compounds such as carbohydrates, whereas the intra-macroaggregate OM and especially the intra-microaggregate OM contains plant debris that have been selectively degraded and enriched in unsubstituted-aliphatic compounds with high methylene content, likely from recalcitrant biopolymers such as cutin and suberin. Previous ^{13}C NMR studies also show that the proportion of *O*-alkyl relative to unsubstituted alkyl C is lower in occluded OM pools or in fractions with higher density, compared to free light OM fractions or fractions with lower density, which in turn have structures consistent with the fresh plant tissues from which they originate (Golchin et al., 1994; Baldock and Skjemstad, 2000; Sohi et al., 2001; Helfrich et al., 2006; Clemente et al., 2011).

In their model, Golchin et al. (1994) suggested that most of the mineral-associated OM in soils is of microbial origin. Our study strongly supports this statement not only by the C/N ratio but also by NMR spectroscopy. In addition, for the first time we provide evidence by NMR that the OM accumulated in the mineral-associated fraction in soils under NT is predominately of microbial origin. In support of this, numerous studies in the literature document that NT increases the stocks of soil microbial biomass C and N (Balota et al., 2004; Franchini et al., 2007; Wang et al., 2008; Babujia et al., 2010). Recent work has found the contribution of microbial biomass to soil OM to be much more important than previously thought (Simpson et al., 2007b; Miltner et al., 2012). Further, it has been also shown that bacteria "build" hutches on clay surfaces to protect themselves in soil (Lünsdorf et al., 2000). Under NT, such structures will be less perturbed leading to accumulation of more stable and abundant microbe–mineral complexes.

On the basis of this study, we argue that a reduced aggregate turnover due to less soil disturbance, as in NT relative to CT, enhances the formation of stable organo-mineral complexes between mineral particles and microbial materials. It is likely that at least in part microbes play an active role in anchoring themselves to clay surfaces through the secretion of biofilms that mix with various inorganics to produce "hutches" (Lünsdorf et al., 2000). Once anchored to clay it is likely that the microbes can only access readily available components close to the mineral surfaces. Eventually, over time, labile compounds may disappear and microbes die or pass to a dormant state, entrapped and physically disconnected from electron acceptors, energy sources, and predators. Microbes and microbial by-products adsorbed on mineral surfaces and physically protected by entrapment within very small micro-aggregates appear to constitute an important pool of OM stabilization and C sequestration in soils under NT. This microbial OM pool has probably been underestimated when compared to crop-derived OM physically protected within microaggregates. The stabilization mechanism of soil OM described here is probably valid not only for conservation tillage systems but for any agricultural practice able to reduce soil disturbance and aggregation turnover or increase C inputs into the soil. It will be important for future studies to determine the ratio of necromas vs. viable cells at mineral surfaces. This will be key in determining whether NT leads to the accumulation of cellular materials due to reduced disruption and

mixing or whether NT promotes the accumulation of viable microbial species which could have important implications for sustainable agriculture as well as C sequestration.

5. Conclusions

Compared to CT, 25 years of NT resulted in 16% more organic C and 5% more N in soil. By applying a comprehensive fractionation procedure and advanced NMR techniques, we provided evidence that organo-mineral complexes formed by intimate interaction between microbial-derived inputs and mineral particles during the degradation of plant-derived materials constitute the most important pool of long-term OM stabilization and C sequestration in NT systems, at least in the OM-depleted agricultural soils examined here. Physical protection within small microaggregates of live, dormant, and decaying microorganisms adsorbed on mineral surfaces may be an important mechanism of soil OM preservation. However, further research is needed to determine quantitatively the precise microbial contribution to both total soil OM and the functionally different OM fractions (dissolved, free, intra-macroaggregate, intra-microaggregate, and mineral-associated OM), as well as the contribution of live, dormant, and decaying microbial cells to total soil microbial biomass. Future work should be also focused on the whole soil profile rather than on the surface layer only, in order to achieve a more complete understanding of soil OM dynamics under conservation tillage systems and a better evaluation of soil management options to sequester C.

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