

## RESEARCH ARTICLE

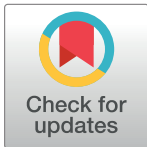
# Influence of crop rotation, tillage and fertilization on chemical and spectroscopic characteristics of humic acids

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## Abstract

The changes in soil organic matter composition induced by anthropogenic factors is a topic of great interest for the soil scientists. The objective of this work was to identify possible structural changes in humic molecules caused by a 2-year rotation of durum wheat with faba bean, lasted for a decade, and conducted with different agricultural practices in a Mediterranean soil. Humic acids (HA) were extracted at three depths (0–30, 30–60 and 60–90 cm) from a Mediterranean soil subjected to different tillage (no tillage, minimum tillage and conventional tillage), crops (faba bean and wheat), and fertilization. The changes in HA quality were assessed by several chemical (ash, yield and elemental analysis) and spectroscopic techniques (solid-state <sup>13</sup>C nuclear magnetic resonance, Fourier transform infrared and fluorescence). The results suggest that the different agronomic practices strongly affected the quality of HA. Smaller but more aromatic molecules were observed with depth, while the fertilization induced the formation of simpler and less aromatic molecules due to the enhanced decomposition processes. Under no tillage, more stable humic molecules were observed due to the less soil aeration, while under conventional tillage larger and more aromatic molecules were obtained. Compared to wheat, more aromatic and more oxidized but less complex molecules were observed after faba bean crop. The inorganic fertilization accelerates the decomposition of organic substances rather than their stabilization. At the end of each crop cycle, humic matter of different quality was isolated and this confirms the importance of the rotation practice to guarantee a diversification of the soil organic matter with time. Finally, no tillage induces the formation of more stable humic matter.

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## Introduction

Soil is considered the most important compartment in the carbon cycle. It behaves as a CO<sub>2</sub>-producing system because of the respiration and mineralization of organic matter (OM). In

contrast, soils are systems that reduce CO<sub>2</sub> emissions by means of the fixation and immobilization of organic carbon (OC) in relatively stable forms difficult to decompose, especially through humification processes [1]. Humic substances are supramolecular associations of several heterogeneous and relatively small molecules deriving from the degradation and decomposition of biological material. Humic acids (HA) are components of humic substances that appear to be held together mainly by van der Waals interactions, hydrogen bonds, hydrophobic interactions and metal-bridged intermolecular electrostatic bonds [2]. Since HA in themselves are not biologically refractory, they survive in soil due to the strong interactions with minerals, and if the molecules complexed have hydrophobic domains exposed to the soil solution, they can act as condensation nuclei for the supramolecular association. The HA nearest the mineral surfaces are protected from the microbial community, while the external ones would be susceptible to desorption and to microbial attack [3]. Therefore, the larger the hydrophobicity of the biomolecules (long-chain alkyl or aromatic compounds), the slower is their degradation in the soil solution [2, 4].

Stable humic molecules are subjected to lower rate of mineralization and, therefore, the corresponding soils release less CO<sub>2</sub> [5]. The same humic molecules sequester further carbon in the soil, reducing the negative consequences of climate change in terms of CO<sub>2</sub> production [6]. Humic acids improve soil health and quality through the amelioration of its physical, biological, and chemical properties [7]. In particular, they constitute an important source of nutrients, promote a good soil structure improving the water retention [8–10], and have the capacity to chemically and physically interact with other components in the environment [11–13].

Most agricultural soils in the Mediterranean area contain small amounts of OM, which is a limiting factor for crops growth and production. Different agronomic practices, such as tillage, inorganic fertilization and crop rotation, influence the biological, chemical and physical properties of soil that in turn, [determine changes](#) in the quality of OM from which they originate [14–18]. For example, Moussadek et al. demonstrated that the no tillage, without removing the crop residues from the field, increased the soil organic matter content compared to the conventional tillage [7]. Zhang et al. found HA less complex and less aromatic after the application of the inorganic fertilizers [19]. In a perspective of sustainable agriculture, especially in the Mediterranean area, it is necessary to know the effects of different agronomic practices on the quantity and quality of the soil OM.

A detailed molecular characterization of HA appears, thus, as an essential requirement for evaluating their stability and quality and understanding the role of the different agronomic practices in agricultural and environmental processes. Non-destructive spectroscopic methods such as Fourier transform infrared spectroscopy (FT IR) and <sup>13</sup>C cross-polarization magic-angle-spinning nuclear magnetic resonance (<sup>13</sup>C-CPMAS-NMR) have been widely used to identify the content and distribution of organic molecules in a wide range of solid organic matrices [20–22]. Fluorescence spectroscopy is another sensitive, non-destructive technique for the evaluation of molecular and quantitative aspects of the structural and functional chemistry of humic substances. The intrinsic fluorescence of soil organic components contains information related to structural components, functional groups, conformation and heterogeneity [23].

Little has been published on the changes in the structure and element composition of HA as affected by tillage, fertilization and crop rotation. The objective of this work is to identify possible structural changes caused by soil management in HA from a Mediterranean soil under different agronomic practices making use of spectroscopic techniques. The results provided could suggest the best agronomic practices in order to conserve the soil organic matter with a view to agronomic and environmental sustainability.

## Materials and methods

### 2.1 Humic acids

The trial was conducted at the experimental station of the University of Bari located at Polignano, latitude 40° 10' 20" North and longitude 16° 39' 04" East. The experiments included a 2-year rainfed rotation of faba bean (*Vicia faba* Pers. var. equina) cv PROTHABAT 69 with durum wheat (*Triticum turgidum* L. var durum) cv IRIDE, in a split-plot block design with three replications per treatment.

Three different tillage were compared for each crop: (i) conventional tillage (CT, 35 cm deep moldboard plowing in late August and 15 cm deep disk harrowing in November); (ii) minimum tillage (MT, 20 cm deep subsoiling in late August and 15 cm deep disk harrowing in November); and (iii) no tillage (NT).

Regardless the tillage, each new wheat crop received 90 kg N ha<sup>-1</sup> and each new faba bean crop received 30 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup>. Control plots without fertilization were prepared too. Details of the fertilization are reported elsewhere [24].

After a decade of the aforementioned management, soil was sampled from all plots of the faba bean—wheat rotation 2015–2016 at three different depths (0–30, 30–60 and 60–90 cm), after the harvest of each crop. The humic acids (HA) were isolated from: i) NT and no fertilization; ii) NT and crops fertilization; iii) MT and no fertilization; (iv) MT and crops fertilization; (v) CT and no fertilization; (vi) CT and crops fertilization, according to a slightly modified procedure by Swift [25] and reported by Zaccone et al [26].

### 2.2 Chemical characterization of humic acids

Moisture and ash contents were measured by heating the HA for one night at 105°C and for 5 h at 550°C, respectively. The elemental composition was determined by a CHNS Elemental Analyser Flash 2000 (Thermo Scientific) calibrated by a BBOT [2,5-bis-(5-tert-butyl-benzoxazol-2-yl)-thiophene] standard (ThermoQuest Italia s.p.a.) [27]. Oxygen was calculated by difference: O g kg<sup>-1</sup> = 1000 – (C+H+N+S) g kg<sup>-1</sup>. Data obtained were corrected for moisture and ash contents.

### 2.3 Spectroscopic characterization of humic acids

**2.3.1 The E<sub>4</sub>/E<sub>6</sub> ratio.** The E<sub>4</sub>/E<sub>6</sub> ratio was calculated as the ratio of the absorbances at 465 and 665 nm measured by a Spectrophotometer Perkin Elmer model Lambda 15 UV–Vis on solutions of 3.0 mg of each HA dissolved in 10 ml of 0.05 M NaHCO<sub>3</sub>.

**2.3.2 The Fourier transform infrared spectroscopy.** The Fourier transform infrared (FT IR) spectra were recorded in the range 4000–400 cm<sup>-1</sup> on pellets obtained by pressing under reduced pressure a mixture of 1 mg of HA and 400 mg of dried KBr, spectrometry grade, using a Nicolet Nexus FT IR spectrophotometer and a Nicolet Omnic 6.0 software. Spectra were acquired at 2 cm<sup>-1</sup> resolution, and 64 scans min<sup>-1</sup> were averaged to reduce noise.

**2.3.3 Fluorescence spectroscopy.** Fluorescence spectra were obtained on aqueous solutions of HA samples at a concentration of 100 mg l<sup>-1</sup> after overnight equilibration at RT and adjustment to pH 8 with 0.05 M NaOH [28]. Spectra were recorded using a Perkin Elmer LS 55 luminescence spectrophotometer equipped with the WinLab 4.00.02 software (Perkin-Elmer, Inc., 2001, Norwalk, CT) for data processing. Total luminescence spectra, in the form of excitation–emission matrices (EEMs, contour maps), were recorded over the emission wavelength range from 300 to 600 nm by increasing sequentially by 5 nm step the excitation wavelength from 250 to 500 nm. A scan speed of 1200 nm min<sup>-1</sup> was selected for both

monochromators. The EEM plots were generated as contour maps from spectral data by using Surfer 8.0 software (Golden Software, Inc., 2002, Golden, CO).

The humification index (HIX) was calculated according to Ohno [29]. This index is expressed as the ratio between the area in the upper quarter (435–480 nm) and the sum of the area in the lower quarter (300–345 nm) and in the upper quarter of the emission spectra of HA measured at an excitation wavelength fixed at 254 nm.

**2.3.4 NMR Spectroscopy.** The  $^{13}\text{C}$ -CPMAS-NMR experiments were performed on a Bruker AMX 400 operating at 100.625 MHz on the carbon-13. The rotor spin rate was set at 4500 Hz. A recycle time of 2 s and an acquisition time of 13 ms were used. All experiments were conducted with Variable Contact Time (VCT) pulse sequence in order to find the Optimum Contact Time (OCT) for each sample and minimize errors on evaluation of peak areas [30]. The OCT ranged between 0.8 and 1.0 ms. A 50-Hz line broadening was used, and side bands of the carboxyl-C signal were subtracted from the 110–140 ppm region by automatic integration after spectra acquisition [22].

## 2.4 Statistical analysis

All analyses performed on HA were conducted in triplicate. Data were analysed using the four-way ANOVA and Tukey's test. All statistical analyses were executed using the R software version 3.2.3.

## Results and discussion

### 3.1 Chemical properties of HA

Table 1 shows the effect of the variables depth, tillage, fertilization, and crop (year) on chemical properties of HA. Nitrogen and H content were slightly higher under NT management with respect to the other treatments, in accordance to results by Szajdak et al. [31]. Humic acids incorporate ammonium-N either abiotically, in form of amide-N, or microbiologically, in the form of free or ionized  $\text{NH}_2$ -groups in amino acids and sugars, and  $\text{NH}_4^+$  [32]. The enhanced activity of nitrifiers in more aerated MT and CT plots resulted in a possible major leaching and lower incorporation of N in the corresponding HA.

No significant difference was observed along the profile and between the two years considered, while the percentage of N was expectedly larger in fertilized soils. The behaviour of N influenced the C/N atomic ratio that was significantly lower in NT and fertilized plots. The atomic ratios H/C and O/C, considered indicators of origin and structural changes of humic substances, did not change significantly in all treatments due to the same origin of HA [33–34]. Further, the smaller O/H ratio of the HA isolated from NT would suggest the occurrence of humic molecules with relatively low polarity and high hydrophobicity [35].

The organic matter content decreased with depth [24], therefore the corresponding HA yield resembled the same trend. With respect to the tillage, the HA yield was slightly larger under CT, in contrast with the results of Moussadek et al. [7] that removed the aboveground crop residues only from the CT plots and not from all treatments, as in present experiments. Finally, fertilization and crop did not influence significantly the HA yield.

### 3.2 UV-Vis properties of HA

The effects of depth, tillage, fertilization and crop (year) on UV-Vis properties of HA are reported in Table 2. Significant differences were observed between  $E_4/E_6$  ratios in relation to the tillage. In particular, the  $E_4/E_6$  ratio of HA from CT was smaller than those of the other treatments, suggesting the presence of larger humic molecules. In fact, Chen et al.

**Table 1. Analysis of variance and mean values of the chemical parameters of HA, subdivided by depth, tillage, fertilization and year.**

	Ash	N	C	H	S	O	C/N	H/C	O/C	O/H	HA yield	
	%	g kg <sup>-1</sup>										g kg <sup>-1</sup>
Depth	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***
Tillage	n.s.	**	n.s.	*	n.s.	n.s.	*	n.s.	n.s.	*	*	*
Fertilization	n.s.	*	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.
Crop (year)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
<b>Depth</b>												
0–30	5.6 a (0.34)	47.4 a (0.83)	568.2 a (2.37)	50.1 a (0.42)	19.6 a (3.87)	314.6 a (4.74)	14.0 a (0.30)	1.06 a (0.01)	0.42 a (0.01)	0.39 a (0.01)	2.30 c (0.12)	
30–60	6.2 a (0.40)	46.5 a (1.53)	577.5 a (3.98)	51.6 a (1.09)	12.1 a (2.28)	312.1 a (5.50)	14.6 a (0.50)	1.07 a (0.02)	0.41 a (0.01)	0.38 a (0.01)	1.36 b (0.17)	
60–90	6.2 a (0.35)	47.7 a (1.27)	574.4 a (6.61)	52.5 a (0.78)	23.4 a (6.27)	301.9 a (6.43)	14.2 a (0.52)	1.10 a (0.02)	0.40 a (0.01)	0.36 a (0.01)	0.30 a (0.07)	
<b>Tillage</b>												
NT	6.1 a (0.42)	49.8 b (1.08)	576.0 a (4.85)	53.1 b (1.07)	21.2 a (6.57)	299.7 a (7.25)	13.6 a (0.32)	1.11 a (0.02)	0.39 a (0.01)	0.35 a (0.01)	1.24 a (0.30)	
MT	5.6 a (0.36)	46.8 ab (0.98)	565.1 a (3.21)	50.2 a (0.63)	21.2 a (2.90)	316.6 a (2.85)	14. ab (0.38)	1.07 a (0.02)	0.42 a (0.01)	0.39 b (0.01)	1.19 a (0.27)	
CT	6.2 a (0.31)	45.0 a (1.23)	579.0 a (5.07)	50.9 ab (0.54)	12.7 a (3.10)	312.3 a (5.25)	15.1 b (0.51)	1.06 a (0.01)	0.41 a (0.01)	0.38 ab (0.01)	1.52 b (0.25)	
<b>Fertilization</b>												
No	6.2 a (0.30)	45.7 a (1.21)	576.6 a (3.71)	51.0 a (0.76)	15.3 a (2.34)	311.2 a (3.96)	14.9 b (0.45)	1.06 a (0.02)	0.41 a (0.01)	0.38 a (0.01)	1.31 a (0.20)	
Yes	5.8 a (0.30)	48.7 b (0.55)	570.1 a (3.85)	51.8 a (0.60)	21.4 a (4.66)	307.9 a (5.30)	13.7 a (0.15)	1.09 a (0.01)	0.41 a (0.01)	0.37 a (0.01)	1.33 a (0.24)	
<b>Crop (year)</b>												
Faba bean (2015)	5.9 a (0.29)	48.0 a (1.08)	574.2 a (4.39)	51.5 a (0.85)	14.2 a (4.41)	312.1 a (6.02)	14.1 a (0.38)	1.08 a (0.01)	0.41 a (0.01)	0.38 a (0.01)	1.44 a (0.23)	
Wheat (2016)	6.1 a (0.31)	46.4 a (0.89)	572.5 a (3.24)	51.3 a (0.49)	22.6 a (2.58)	307.1 a (2.67)	14.5 a (0.34)	1.08 a (0.01)	0.40 a (0.01)	0.37 a (0.005)	1.20 a (0.21)	

The values in each column followed by a different letter are significantly different according to Tukey’s test.

\* Significant at the  $P \leq 0.05$

\*\* Significant at the  $P \leq 0.01$ ;

\*\*\* Significant at the  $P \leq 0.001$ ;

n.s.: not significant. The standard errors are reported in parentheses.

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demonstrated that the  $E_4/E_6$  ratio is governed by the molecular size, where higher values of  $E_4/E_6$  ratio are associated with smaller in size humic molecules, whereas lower  $E_4/E_6$  ratio indicates the contrary [36]. In addition, the  $E_4/E_6$  ratio slightly increased along the profile, implying the occurrence of smaller or lower in size humic molecules isolated from the deeper horizons, and those results are in accordance to the ones by Marinari et al. [37]. Finally, this ratio was slightly larger in soils after fertilization possibly due to the increased microbial activity that accelerated the decomposition of the organic matter forming humic molecules of smaller size.

### 3.3 FT IR spectroscopy

The FT IR spectra of the HA samples isolated in the year 2015 and 2016 under different agromonomic management systems are reported in Figs 1 and 2 and showed common absorption bands with some differences in their relative intensity. The most important peaks were: (i)  $3400\text{ cm}^{-1}$  associated to OH stretching of OH groups; (ii)  $2925\text{ cm}^{-1}$  due to aliphatic C-H stretching; (iii)  $1715\text{ cm}^{-1}$  attributed to C = O stretching of COOH and ketones; (iv)  $1650\text{ cm}^{-1}$  associated to structural vibrations of aromatic C = C or COO<sup>-</sup> stretching; (v) approximately  $1460\text{ cm}^{-1}$  attributed to asymmetric bending of C-H groups; (vi) approximately  $1384\text{ cm}^{-1}$  due to asymmetric stretching of COO<sup>-</sup> groups, bending of C-H groups, symmetric vibrations of C-NO<sub>2</sub> groups, vibration of X-N = O groups and asymmetrical stretching of COO<sup>-</sup> groups and; (vii)  $1240\text{ cm}^{-1}$  attributable to C-O stretching and OH bending of COOH groups.

In order to quantify the relative changes in FT IR spectra, the intensity ratios of FT IR peaks  $2920+2850\text{ cm}^{-1}$  (aliphatic structures)/ $1620-1630\text{ cm}^{-1}$  (aromatic structures), and  $2920+2850$

**Table 2. Analysis of variance and mean values of the spectroscopic parameters of HA subdivided by depth, tillage, fertilization, and crop.**

	E <sub>4</sub> /E <sub>6</sub>	(2920+2850)/1720	(2920+2850)/1620	Fluorophore A	Fluorophore B	HIX
Depth	*	n.s.	*	**	**	**
Tillage	**	n.s.	*	*	*	*
Fertilization	*	n.s.	n.s.	n.s.	n.s.	**
Crop (Year)	n.s.	***	***	*	**	***
Depth						
0–30	6.12 a (0.08)	1.86 a (0.09)	1.64 b (0.10)	113.0 a (4.16)	108.1 a (5.98)	96.3 a (22.47)
30–60	6.19 a (0.12)	1.81 a (0.08)	1.63 b (0.09)	123.3 a (6.35)	117.3 a (6.79)	141.7 b (31.47)
60–90	6.50 b (0.18)	1.77 a (0.09)	1.57 a (0.09)	165.7 b (5.82)	158.0 b (4.04)	122.3 ab (19.35)
Tillage						
NT	6.39 b (0.11)	1.76 a (0.08)	1.56 a (0.10)	135.3 ab (8.66)	127.3 a (8.33)	128.1 b (24.39)
MT	6.44 b (0.16)	1.83 a (0.09)	1.62 b (0.09)	142.8 b (10.02)	133.7 b (9.50)	116.9 a (24.08)
CT	5.98 a (0.09)	1.85 a (0.10)	1.66 b (0.09)	123.8 a (6.45)	122.3 a (7.82)	115.2 a (27.94)
Fertilization						
No	6.14 a (0.14)	1.81 a (0.07)	1.60 a (0.08)	134.1 a (7.25)	129.3 a (6.77)	138.0 b (26.51)
Yes	6.40 b (0.07)	1.82 a (0.07)	1.62 a (0.08)	133.9 a (6.94)	126.3 a (7.18)	102.2 a (10.28)
Crop (Year)						
Faba bean (2015)	6.26 a (0.09)	1.53 a (0.02)	1.31 a (0.01)	120.9 a (6.08)	114.2 a (6.60)	71.4 a (7.81)
Wheat (2016)	6.28 a (0.13)	2.10 b (0.03)	1.91 b (0.03)	147.1 b (6.58)	141.4 b (5.69)	168.8 b (22.49)

The values in each column followed by a different letter are significantly different according to Tukey's test. n.s.: not significant;

\* Significant at the  $P \leq 0.05$ ;

\*\* Significant at the  $P \leq 0.01$ ;

\*\*\* Significant at the  $P \leq 0.001$ . The standard errors are reported in parentheses.

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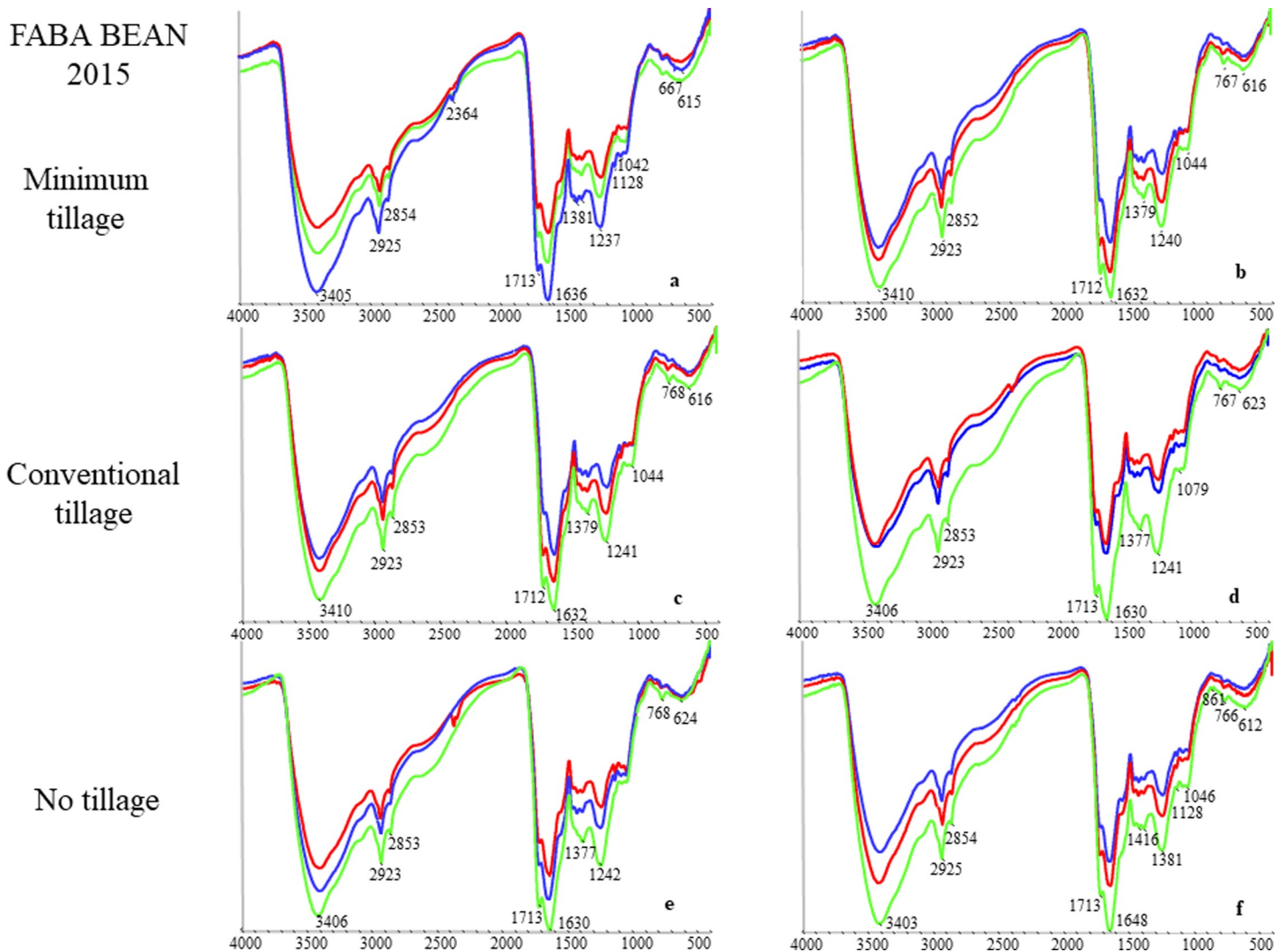
$\text{cm}^{-1}/1720 \text{ cm}^{-1}$  (oxidized structures) of HA were compared according to Zhang et al. (Table 2) [19]. Results suggested a decrease of aliphatic moieties of HA isolated after faba bean, from the NT plots and, generally speaking, with depth.

### 3.4 Fluorescence spectroscopy

The total luminescence spectra of HA samples isolated in the year 2015 and 2016, respectively, are shown in Figs 3 and 4, while in Table 2 are reported the corresponding fluorescence intensity (FI) values of the main fluorophores. The fluorescence investigation revealed the presence of two fluorophores in each sample (A and B), located at an EEWP of about 390ex/480em and 450ex/510em, respectively, ascribed to extensively conjugated quinones and phenols with an elevated polycondensation degree [28]. Miiikki et al. associated the FI with the age of HA [38]. In our study, the higher FI observed in the HA isolated after wheat cultivation suggested a more recent formation of the same compared to those found after faba bean cultivation. In addition, the aforementioned higher FI could be related to the presence of substituent groups with strong fluorescence intensity (eg. hydroxys and amidogens) [28]. No evident difference was observed regarding the fertilization, while the FI increasing with depth could be reasonable ascribed to the modification of substituents on the molecule that emitted fluorescence. The lower FI showed in HA under CT could be reasonably ascribed to the higher aromaticity of these HA, as previous confirmed by the (2920+2850)/1620 ratio of the FT IR spectra.

In accord with the results obtained by Tadini et al. [39], the HIX was smallest in the surface horizon (Table 2) possibly due to the presence of fresh debris in the upper layers whose decomposition resulted in the percolation of humic material of low molecular size and high





**Fig 1.** FT-IR spectra of HA samples isolated from faba bean soils, year 2015. a: MT unfertilized; b: MT fertilized; c: CT unfertilized; d: CT fertilized; e: NT unfertilized; f: NT fertilized. Sampling depth: Blu spectra, 0–30 cm; Red spectra, 30–60 cm; Green spectra, 60–90 cm. X-axis: wavenumbers ( $\text{cm}^{-1}$ ); Y-axis: transmittance (%).

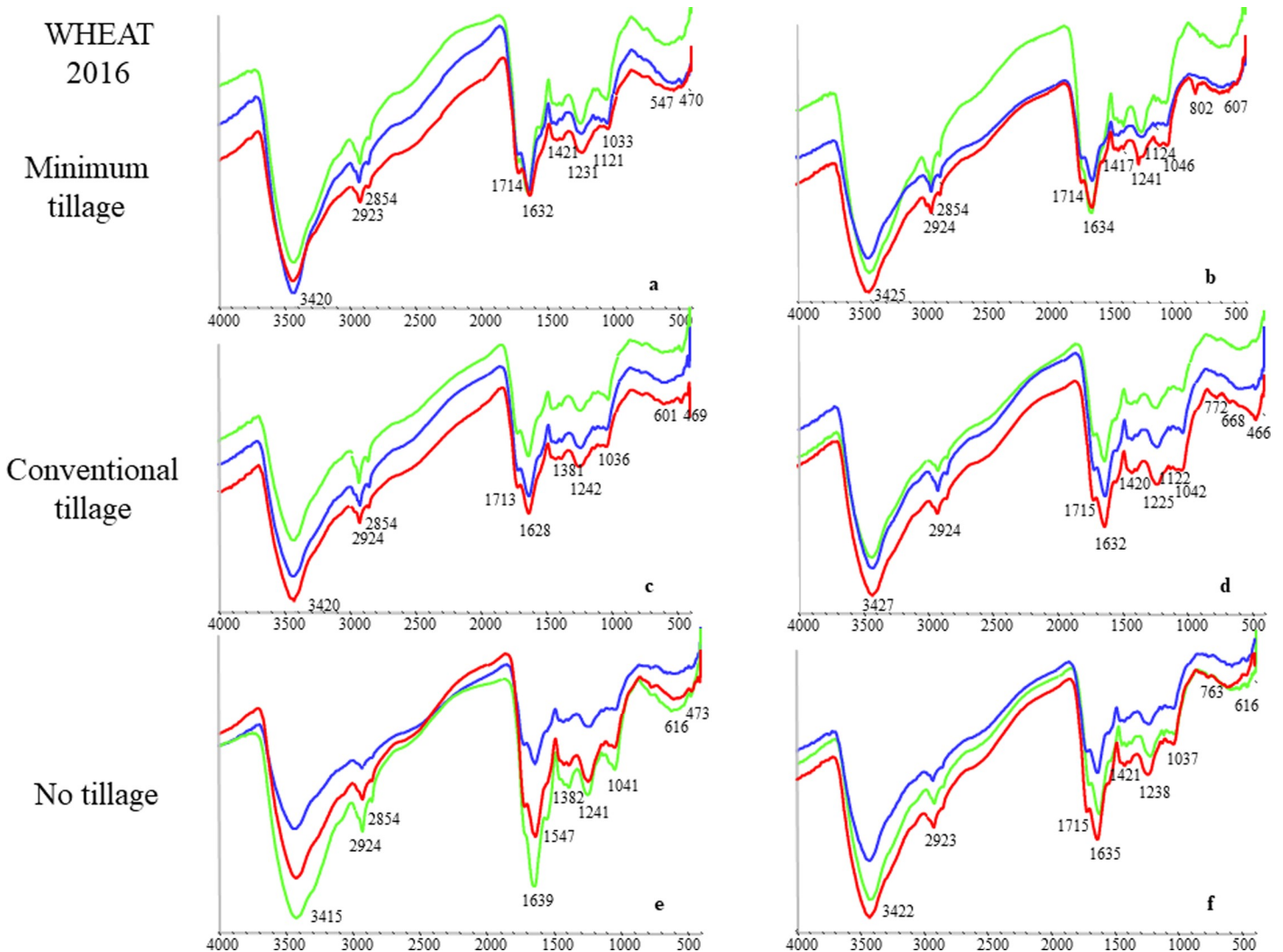
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solubility in deeper layers, as reported by Bardy et al. too [40]. This result was in agreement also with Segnini et al. too, who found lower values of humification index in the first layer of soils under different tillage managements, and with the  $E_4/E_6$  ratio that suggested the presence of smaller molecules in the deeper layers [41].

The HIX of fertilized soils was smaller than that of the control plots, suggesting an enhanced microbial activity in the former plots that resulted in an increased OM decomposition and in a release of low molecular size humic matter of. Similarly, the different materials produced by the two crops had certainly influenced the HIX with time. In fact, after the faba bean cultivation, a smaller HIX value was found, reasonably attributable to the more suitable C/N ratio of those residues for the microbial community.

### 3.5 $^{13}\text{C}$ -CPMAS-NMR spectroscopy

The NMR spectra of HA samples are shown in Fig 5, while the corresponding peak assignment and the semi-quantitative results are reported in Table 3. The peaks within the chemical shift



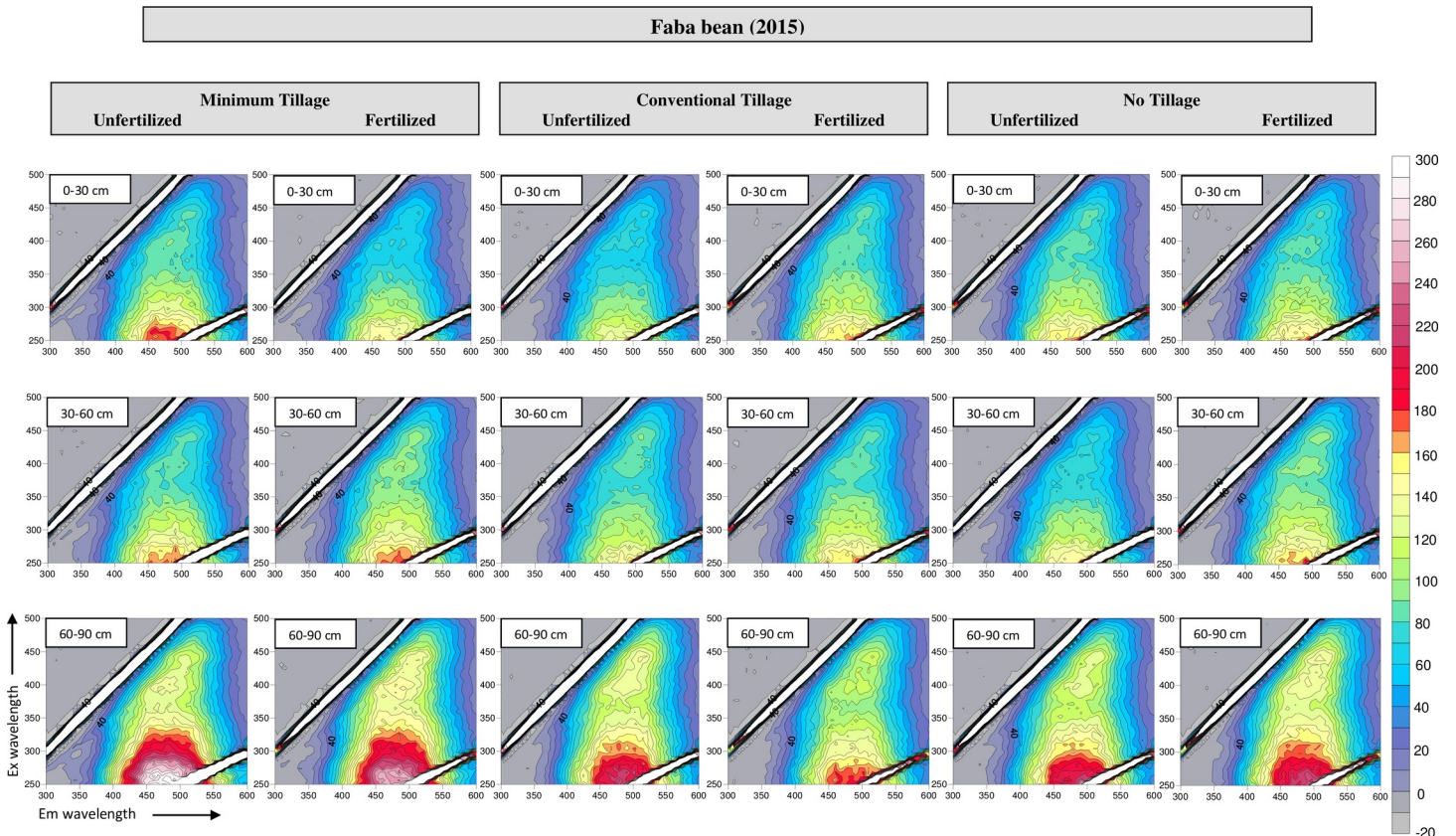
**Fig 2. FT-IR spectra of HA samples isolated from wheat soils, year 2016.** a: MT unfertilized; b: MT fertilized; c: CT unfertilized; d: CT fertilized; e: NT unfertilized; f: NT fertilized. Sampling depth: Blu spectra, 0–30 cm; Red spectra, 30–60 cm; Green spectra, 60–90 cm. X-axis: wavenumbers ( $\text{cm}^{-1}$ ); Y-axis: transmittance (%).

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region 0–45 ppm have been assigned to alkyl chains, while those between 45 and 60 ppm are usually associated with the resonance of lignin methoxy groups or C-N in amino acids [42]. Moreover, signals in the region 60–110 ppm have been attributed to O-Alkyl-containing molecules, mainly carbohydrates, whereas peaks within the 110–160 ppm interval arose from the resonance of aromatic and phenolic carbons, such those in lignin, tannins or resins [43–45]. Finally, signals within the 160–210 ppm range were assigned to the carboxyl and amide functional groups [43].

Along the profile of soils under NT (Fig 5, spectra A, B and C; Table 3), an increase of alkyl- and aromatic/phenolic-related signals in HA can be noted, while the content of methoxyl-C, carbohydrates and carboxyl moieties significantly decreased. An increased amount of aromatic molecules, together with a concomitant decrease of O-Alkyl compounds, may indicate a larger stability of HA with soil depth, due to a larger protection of SOM from microbial degradation [45]. Such result is also supported by the significantly larger Alkyl-C to O-Alkyl-C ratio found





**Fig 3. Excitation/Emission matrices of HA samples isolated from faba bean soils, year 2015.**

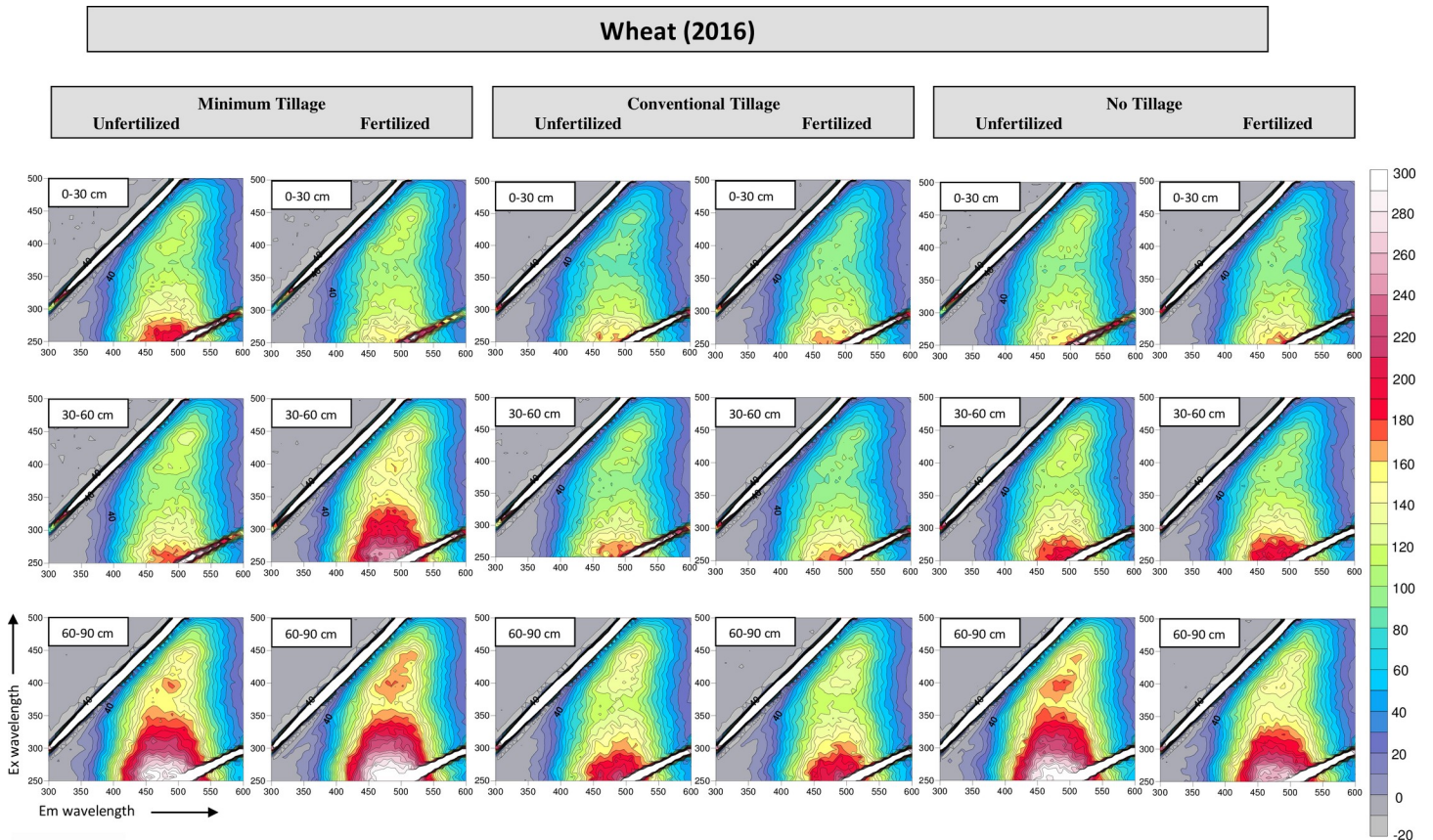
<https://doi.org/10.1371/journal.pone.0219099.g003>

at increasing depths (Table 3). Such parameter is commonly employed as an index for the degradation degree of SOM [46].

The HA isolated from fertilized soils under CT (Fig 5, spectrum D; Table 3) showed a larger relative content of alkyl-C (0–45 ppm) and a reduction of the amount of *O*-Alkyl and aromatic moieties with respect to the HA isolated from unfertilized soils under CT (Fig 5, spectrum E; Table 3). These results agree with those reported by Drosos and Piccolo [18], who found an increased content of alkyl C and a decreased content of aromatic C in HA due to the application of an inorganic fertilizer. According to Galantini and Rosell [47], the fertilization promotes an increase of aliphatic groups in respect to aromatic moieties, probably due to the large inputs in fertilized than in *non*-fertilized soils, thus increasing the amount of less transformed organic compounds, which were bound to HA. Such results are well in line with the  $E_4/E_6$  ratio and the HIX showed before.

Humic acids isolated from soils under MT after faba bean (2015; Fig 5, spectrum F and Table 3) contained a significantly larger relative content of alkyl- and *O*-Alkyl-C than HA isolated from soils under MT after wheat (2016; Fig 5, spectrum G and Table 3). Conversely, the relative amount of aromatic/phenolic moieties significantly increased after wheat cultivation (Table 3). A reduced content of Alkyl-C has been associated with an oxidation of SOM, thus suggesting the occurrence of SOM degradation processes [48].

Finally, the comparison among the three tillage systems studied (Fig 5, spectra G, H and I; Table 3) showed a larger relative content of Alkyl-, methoxy- and *O*-Alkyl-C and a lower relative amount of aromatic and phenolic moieties in HA from NT compared with that from CT.



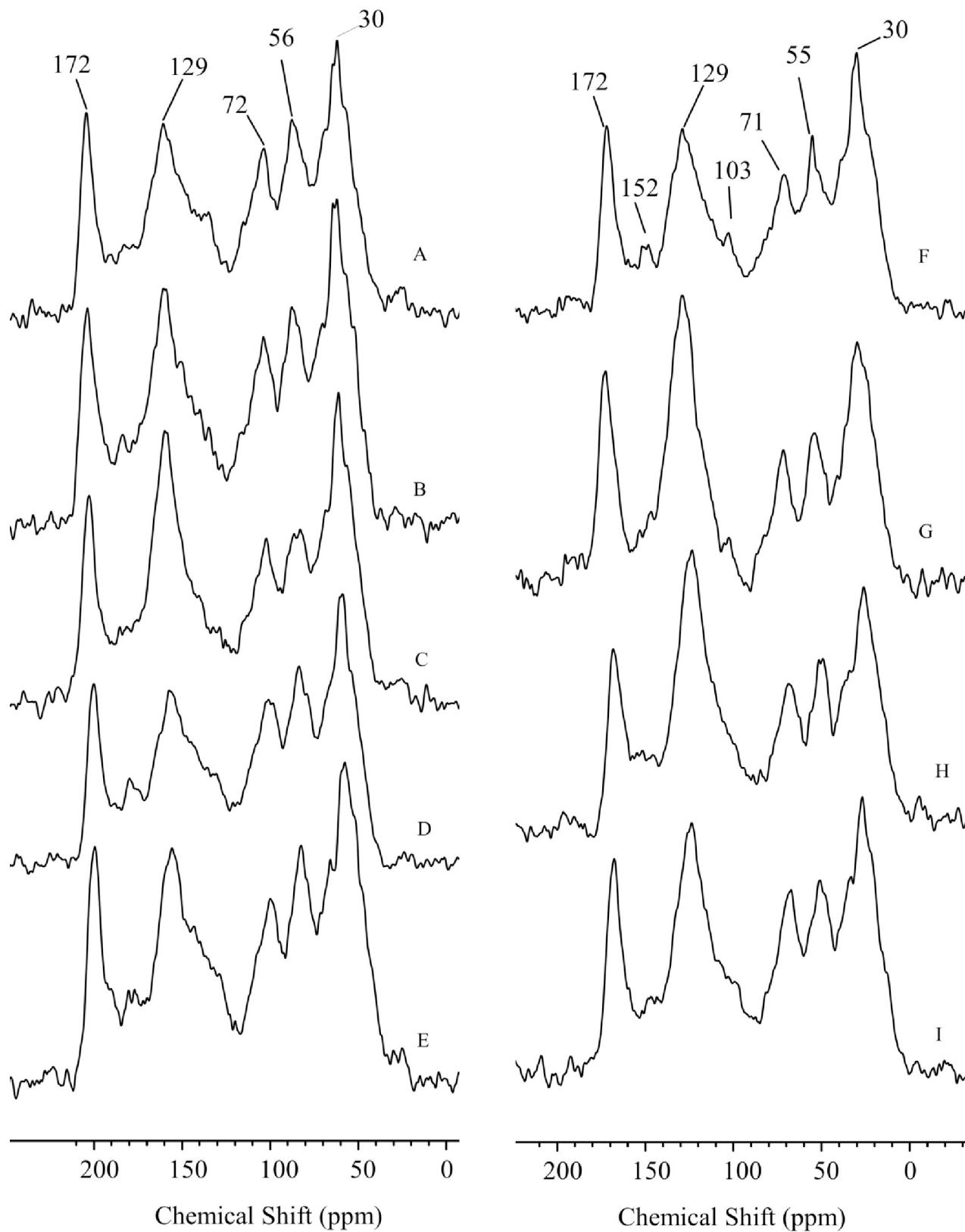
**Fig 4.** Excitation/Emission matrices of HA samples isolated from wheat soils, year 2016.

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Conversely, the content of such functional groups in HA from MT soils was either intermediate or comparable to that found for HA from CT, except for the content of carboxyl groups, which was significantly larger in HA from MT soil than in those from CT soils (Table 3). The general trend observed here is in line with previous reports where large amount of *O*-Alkyl groups in NT were related to a lesser degradation of carbohydrates [49]. This finding, together with the smaller relative content of both carboxyl moieties and Alkyl-C and a larger Alkyl/*O*-Alkyl ratio for soils under conservational tillage practices (Table 3), suggested a lower SOM degradation for these soils [48]. Indeed, the larger stability found for the HA under NT may be due to the fact that, under this tillage system, soils were less disturbed, and the growth of SOM-decomposing microbes was limited, thus inhibiting the rate of SOM degradation [45].

## Conclusions

The present study reports the effects of different soil managements on quality and quantity of HA when the aboveground crop residues have been removed from all treatments. The NT apparently induced the formation of HA more stable that can contribute to the soil fertility for a longer time. In contrast, CT induced the formation of a slightly greater quantity of HA with more aromatic and larger structure. The inorganic fertilization, favoring a more intense OM decomposition, induced the formation of simpler and more aliphatic humic molecules that should persist less time in the soil. Humic acids extracted at the end of the legume crop showed



**Fig 5.**  $^{13}\text{C}$  CP MAS NMR spectra of humic acids isolated from various soil samples. A. NT 2015 0–30 cm; B. NT 2015 30–60 cm; C. NT 2015 60–90 cm; D. CT fertilized 2015 0–30 cm; E. CT 2015 0–30 cm; F. MT 2015 30–60 cm; G. MT 2016 30–60 cm; H. CT 2016 30–60 cm; I. NT 2016 30–60 cm.

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**Table 3. Relative carbon distribution (%) for different chemical shift regions (ppm) in  $^{13}\text{C}$ -CPMAS-NMR spectra (Fig 5) of HA extracted from soils managed with different tillage systems: No Tillage (NT), Conventional Tillage (CT) and Minimum Tillage (MT). The HA samples have been extracted at different depths: 0–30, 30–60 or 60–90 cm.**

Attribution	Chemical shift range (ppm)	NT 0–30 cm 2015	NT 30–60 cm 2015	NT 60–90 cm 2015	CTF 0–30 cm 2015	CT 0–30 cm 2015	MT 30–60 cm 2015	MT 30–60 cm 2016	CT 30–60 cm 2016	NT 30–60 cm 2016
Alkyl-C	45–0	24.0 a (0.17)	28.3 b (0.52)	27.8 b (0.58)	29.0 b (0.38)	27.1 a (0.24)	26.8 b (0.14)	23.9 a, A (0.07)	23.1 A (0.32)	26.7 B (0.24)
Methoxyl-C	64–45	13.0 b (0.51)	12.7 ab (0.44)	11.7 a (0.31)	13.7 a (0.32)	13.3 a (0.29)	12.6 a (0.32)	11.1 a, A (0.09)	11.3 A (0.03)	12.3 B (0.22)
O-Alkyl	105–64	18.8 b (0.32)	17.1 ab (0.23)	16.3 a (0.21)	17.5 a (0.20)	18.4 a (0.22)	17.6 b (0.09)	16.3 a, A (0.19)	17.5 A (0.12)	18.1 A (0.10)
Aromatic-C	145–105	24.0 a (0.40)	23.5 a (0.40)	27.2 b (0.47)	22.3 a (0.31)	23.9 b (0.23)	24.1 a (0.09)	30.4 b, B (0.15)	30.5 B (0.23)	25.9 A (0.19)
Quaternary aromatic C	160–145	5.4 a (0.10)	5.0 a (0.21)	4.9 a (0.21)	5.1 a (0.23)	5.0 a (0.20)	4.9 a (0.12)	4.9 a, A (0.23)	5.8 B (0.24)	4.7 A (0.17)
Carboxyl-C, Amide-C	210–160	14.7 b (0.32)	13.4 ab (0.25)	12.0 a (0.40)	12.4 a (0.29)	12.3 a (0.25)	14.0 a (0.35)	13.5 a, B (0.37)	11.9 A (0.03)	12.3 A (0.12)
Alkyl/O-Alkyl		1.3 a (0.02)	1.7 b (0.01)	1.7 b (0.05)	1.7 b (0.03)	1.5 a (0.03)	1.5 a (0.002)	1.5 a, A (0.02)	1.3 A (0.02)	1.5 A (0.01)

All the measurements refer to HA isolated from non-fertilized soils, except for CTF (CT fertilized).

Different letters in a row indicate significant differences according to the Tukey's range post hoc test ( $p < 0.05$ ). A One-Way ANOVA was carried out to compare the means of HA from NT soils (2015) at increasing depth, and another one for comparing HA from different tillage systems (MT, CT and NT 2016, uppercase letters). The HA from CTF and CT, as well as those from MT soils after faba bean (2015) and wheat cultivation (2016), were compared by using the t-test ( $p < 0.05$ ). The standard errors are reported in parentheses.

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more aromatic and oxidized character but less complex structure with respect to the ones isolated after the wheat crop. Finally, smaller but more aromatic HA were observed with depth.

Future investigations should interest the impact of the variables studied (tillage, fertilization, crop and depth) on HA when the aboveground crop residues return to soil. In that case, one can predict a major yield of HA, that influence positively the soil fertility, but the effects on the HA quality could differ from the ones of the present study.

## Supporting information

**S1 Dataset. Results of chemical and spectroscopic analyses.**  
(XLSX)

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## References

1. Andr n O, K tterer T. ICBM: the introductory carbon balance model for exploration of soil carbon balances. *Ecol Appl*. 1997; 7(4):1126–1236. <http://jordek10.eom.slu.se/olle/ICBM.html>
2. Piccolo A, Spaccini R, Drosos M, Vinci G, Cozzolino V. The Molecular Composition of Humus Carbon: Recalcitrance and Reactivity in Soils. In: Garcia C, Nannipieri P, Hernandez T (Eds.), *The Future of Soil Carbon*. Academic Press; 2018, pp 87–124.
3. Sposito G. *The chemistry of soils*. Oxford University Press: New York; 2008.
4. Drosos M, Leenheer JA, Avgeropoulos A, Deligiannakis Y. H-binding of size- and polarity-fractionated soil and lignite humic acids after removal of metal and ash components. *Environ Sci Pollut R* 2014; 21: 3963–3971. <https://doi.org/10.1007/s11356-013-2302-9>
5. Tan KH. *Humic matter in soil and the environment: Principles and controversies*. 2nd ed. Boca Raton: CRC Press; 2014.
6. Zepp RG, Sonntag C. *The role of non-living organic matter in the earth's carbon cycle*. Wiley, Chichester; 1995.
7. Moussadek R, Mrabet R, Dahan R, Zouahri A, ElMourid M, Van Ranst E. Tillage system affects soil organic carbon storage and quality in central Morocco. *App Environ Soil Sci*. 2014; 2014: 1–8. <http://dx.doi.org/10.1155/2014/654796>
8. Singer MJ, Le Bissonnais Y. Importance of surface sealing in the erosion of some soils from a Mediterranean climate. *Geomorphology* 1998; 24: 79–85. [https://doi.org/10.1016/S0169-555X\(97\)00102-5](https://doi.org/10.1016/S0169-555X(97)00102-5)
9. Stevenson FJ. *Humus Chemistry: Genesis, Composition, Reactions*; Wiley-Interscience: New York; 1994.
10. Bronick CJ, Lal R. Soil structure and management: a review. *Geoderma* 2005; 124: 3–22. <https://doi.org/10.1016/j.geoderma.2004.03.005>
11. Baran ikova G, Senesi N, Brunetti G. Chemical and spectroscopic characterization of humic acids isolated from different Slovak soil types. *Geoderma* 1997; 78: 251–266. [https://doi.org/10.1016/S1001-0742\(08\)62224-X](https://doi.org/10.1016/S1001-0742(08)62224-X)
12. Xing B, Liu JD, Liu XB, Han XZ. Extraction and characterization of humic acid and humin fractions from a black soil in China. *Pedosphere* 2005; 15:1–8.
13. Chefetz B, Deshmukh AP, Hatcher PG. Pyrene sorption by natural organic matter. *Environ Sci Technol* 2000; 34(14): 2925–2930. <https://doi.org/10.1021/es9912877>
14. Aziz I, Mahmood T, Raut Y, Lewis W, Islam R, Weil RR. Active organic matter as a simple measure of field soil quality. In: *ASA International Meetings*, Pittsburg, PA; 2009.
15. Derpsch R, Friedrich T, Kassam K, Hongwen L. Current status of adoption of no-till farming in the world and some of its main benefits. *Int J Agr Biol Eng* 2010; 3(1): 1–25. <https://doi.org/10.3965/j.issn.1934-6344.2010.01.0-0>
16. Celik I, Barut ZB, Ortas I, Gok M, Demirbas A, Tulun Y, et al. Impacts of different tillage practices on some soil microbiological properties and crop yield under semi-arid Mediterranean conditions. *Int J Plant Prod* 2011; 5 (3): 237–254. <https://doi.org/10.22069/ijpp.2012.736>
17. Ding X, Zhang B, Zhang X, Yang X, Zhang X. Effects of tillage and crop rotation on soil microbial residues in a rainfed agroecosystem of northeast China. *Soil Till Res* 2011; 114: 43–49. <https://doi.org/10.1016/j.still.2011.03.008>
18. Drosos M, Piccolo A. The molecular dynamics of soil humus as a function of tillage. *Land Degrad Dev* 2018; 29: 1792–1805. <https://doi.org/10.1002/ldr.2989>
19. Zhang J, Wang J, An T, Wei D, Chi F, Zhou B. Effects of long-term fertilization on soil humic acid composition and structure in Black Soil. *Plos One* 2017; 1–14. <https://doi.org/10.1371/journal.pone.0186918>



20. Lguirati A, Baddi GA, Mousadik AE, Gilard V, Revel JC, Hafidi M. Analysis of humic acids from aerated and non-aerated urban landfill composts. *Int Biodeter Biodegr* 2005; 56: 8–16. <https://doi.org/10.1016/j.ibiod.2005.03.004>
21. Adani F, Ricca G, Tambone F, Genevini P. Isolation of the stable fraction (the core) of humic acids. *Chemosphere* 2006; 65: 1300–1307. <https://doi.org/10.1016/j.chemosphere.2006.04.032> PMID: 16735055
22. Spaccini R, Piccolo A. Molecular characterization of compost at increasing stages of maturity. 1. Chemical fractionation and infrared spectroscopy. *J Agricult Food Chem* 2007; 55: 2293–2302. <https://doi.org/10.1021/jf0625398> PMID: 17298079
23. D'Orazio V, Traversa A, Senesi N. Forest soil organic carbon dynamics as affected by plant species and their corresponding litters: a fluorescence spectroscopy approach. *Plant Soil* 2014; 374: 473–484. <https://doi.org/10.1007/s11104-013-1897-4>
24. De Mastro F, Brunetti G, Traversa A, Coccozza C. Effect of crop rotation, fertilisation and tillage on main soil properties and its water extractable organic matter. *Soil Res* 2019. <https://doi.org/10.1071/SR18297>
25. Swift RS. Organic Matter Characterization. In: Sparks DL, Page AL, Helmke PA, Loeppert RH, Soltanpour PN, Tabatabai MA, Johnston CT, Sumner ME (Eds.), *Method of soil analysis: part 3, Chemical methods*. SSSA Book Series No. 5. ASA and SSSA, Madison, WI; 1996, pp 1011–1069.
26. Zaccone C, Santoro A, Coccozza C, Terzano R, Shoty W, Miano TM. Comparison of Hg concentrations in ombrotrophic peat and corresponding humic acids, and implications for the use of bogs as archives of atmospheric Hg deposition. *Geoderma* 2009; 148: 399–404. <https://doi.org/10.1016/j.geoderma.2008.11.017>
27. Schnitzer M., Organic matter characterization, in: Page AL, Miller RH, Keeney DR, editors. *Methods of soil analysis, Part 2, Chemical and microbiological properties*. Second edition. Agronomy Monograph no 9. Madison: American Society of Agronomy, Soil Science Society of America; 1982. p. 581–594.
28. Senesi N, Miano TM, Provenzano MR, Brunetti G. Characterization, differentiation, and classification of humic substances by fluorescence spectroscopy. *Soil Sci* 1991; 152: 259–271. DOI: 00010694-199110000-00004
29. Ohno T. Fluorescence inner-filtering correction for determining the humification index of dissolved organic matter. *Environ Sci Technol* 2002; 36: 742–746. <https://doi.org/10.1021/es0155276> PMID: 11878392
30. Conte P, Piccolo A, van Lagen B, Buurman P, de Jager PA. Quantitative aspects of solid phase <sup>13</sup>C-NMR spectra of humic substances from soils of volcanic systems. *Geoderma* 1997; 80: 327–338. [https://doi.org/10.1016/S0016-7061\(97\)00058-X](https://doi.org/10.1016/S0016-7061(97)00058-X)
31. Szajdak L, Jezierski A, Cabrera ML. Impact of conventional and no-tillage management on soil amino acids, stable and transient radicals and properties of humic and fulvic acids. *Org Geochem* 2003; 34: 693–700. [https://doi.org/10.1016/S0146-6380\(03\)00024-X](https://doi.org/10.1016/S0146-6380(03)00024-X)
32. Dong LH, Yuan HL. Nitrogen Incorporation into Lignite Humic Acids during Microbial Degradation. *Geomicrobiol J* 2009; 26: 484–490. <https://doi.org/10.1080/01490450903061085>
33. Rice JA, MacCarthy P. Statistical evaluation of the elemental composition of humic substances. *Org Geochem* 1991; 17: 635–648. [https://doi.org/10.1016/0146-6380\(91\)90006-6](https://doi.org/10.1016/0146-6380(91)90006-6)
34. Kumada K. *Chemistry of soil organic matter*. Japan Scientific Societies Press, Tokyo; 1987.
35. Traversa A, D'Orazio V, Mezzapesa GN, Bonifacio E, Farrag K, Senesi N, et al. Chemical and spectroscopic characteristics of humic acids and dissolved organic matter along two Alfisol profiles. *Chemosphere* 2014; 111: 84–194. <https://doi.org/10.1016/j.chemosphere.2014.03.063>
36. Chen Y, Senesi N, Schnitzer M. Information provided on humic substances by E<sub>4</sub>/E<sub>6</sub> ratios. *Soil Sci Soc Am J* 1977; 41: 352–358. <https://doi.org/10.2136/sssaj1977.03615995004100020037x>
37. Marinari S, Dell'Abate MT, Brunetti G, Dazzi C. Differences of stabilized organic carbon fractions and microbiological activity along Mediterranean Vertisols and Alfisols profiles. *Geoderma* 2010; 156: 379–388. <https://doi.org/10.1016/j.geoderma.2010.03.007>
38. Miikki V, Senesi N, Hanninen K. Characterization of humic materials formed by composting of domestic and industrial biowastes. *Chemosphere* 1997; 34: 1639–1655. [https://doi.org/10.1016/S0045-6535\(97\)00021-0](https://doi.org/10.1016/S0045-6535(97)00021-0)
39. Tadani AM, Nicolodelli G, Senesi GS, Ishida DA, Montes CR, Lucas Y, et al. Soil organic matter in podzol horizons of the Amazon region: Humification, recalcitrance, and dating. *Sci Total Environ* 2018; 613–614: 160–167. <https://doi.org/10.1016/j.scitotenv.2017.09.068> PMID: 28915453
40. Bardy M, Fritsch E, Derenne S, Allard T, Nascimento NR, Bueno GT. Micro-morphology and spectroscopic characteristics of organic matter in waterlogged podzols of the upper Amazon basin. *Geoderma* 2008; 145: 222–230. <https://doi.org/10.1016/j.geoderma.2008.03.008>

41. Segnini A, Carvalho JLN, Bolonhezi D, Milori DMBP, Lopes da Silva WT, Simões ML, et al. Carbon stock and humification index of organic matter affected by sugarcane straw and soil management. *Sci Agric* 2013; 70: 321–326. <http://dx.doi.org/10.1590/S0103-90162013000500006>
42. Savy D, Cozzolino V, Vinci G, Nebbioso A, Piccolo A. Water-Soluble Lignins from Different Bioenergy Crops Stimulate the Early Development of Maize (*Zea mays*, L.). *Molecules* 2015; 20: 19958–19970. <https://doi.org/10.3390/molecules201119671> PMID: 26556330
43. Savy D, Mazzei P, Drosos M, Cozzolino V, Lama L, Piccolo A. Molecular characterization of extracts from biorefinery wastes and evaluation of their plant biostimulation. *ACS Sustain Chem Eng* 2017; 5: 9023–9031. <https://doi.org/10.1021/acssuschemeng.7b01928>
44. Dick DP, Nunes Gonçalves C, Dalmolin RSD, Knicker H, Klamt E, Kögel-Knabner I, et al. Characteristics of soil organic matter of different Brazilian Ferralsols under native vegetation as a function of soil depth. *Geoderma* 2005; 124(3–4): 319–333. <https://doi.org/10.1016/j.geoderma.2004.05.008>
45. Spaccini R, Piccolo A. Effects of field managements for soil organic matter stabilization on water-stable aggregate distribution and aggregate stability in three agricultural soils. *J Geochem Explor* 2013; 129: 45–51. <https://doi.org/10.1016/j.gexplo.2012.10.004>
46. Baldock JA, Preston CM. Chemistry of carbon decomposition processes in forests as revealed by solid-state carbon-13 nuclear magnetic resonance. In: Kelly JM, McFee WW (Eds.), *Carbon Forms and Functions in Forest Soils*. Soil Science Society of America, Madison, WI; 1995, pp 89–117.
47. Galantini J, Rosell R. Long-term fertilization effects on soil organic matter quality and dynamics under different production systems in semiarid soils. *Soil Till Res* 2006; 87: 72–79. <https://doi.org/10.1016/j.still.2005.02.032>
48. Panettieri M, Knicker H, Berns AE, Muillo JM, Majedón E. Moldboard plowing effects on soil aggregation and soil organic matter quality assessed by <sup>13</sup>C CPMAS NMR and biochemical analyses. *Agr Ecosyst Environ* 2013; 177: 48–57. <https://doi.org/10.1016/j.agee.2013.05.025>
49. Ding G, Novak JM, Amarasiriwardena D, Hunt PG, Xing B. Soil Organic Matter Characteristics as Affected by Tillage Management. *Soil Sci Soc Am J* 2002; 66: 421–429. <https://doi.org/10.2136/sssaj2002.4210>