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Composition and structure of humic substances in long-term fertilization experimental soils of southern China

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Humic substances are extremely important attributes of soil quality but the impacts of long-term fertilization on their composition and structure remain controversial and are still not fully understood. A study, employing various methods was conducted to elucidate the influence of long-term fertilization practices on the composition and structure of humic substances. Soil samples were collected from eight treatments at the Red Soil Experimental Station of the Chinese Academy of Agricultural Sciences. Data were subjected to analysis of variance (ANOVA), using the MSTAT-C software package. In general, the Fourier transform-infrared (FT-IR) spectra discriminated bands between humic fractions. The humic acid (HA) from the NPK plus manure (NPKM) and manure alone (M) treatments showed higher values of the O/R ratios (1.24 and 1.18, respectively) as compared to that from the 1.5 NPKM and fallow (H) treatments, which decreased these ratios (0.66 and 0.85, respectively). Except for NPKM plus wheat-soybean-sweet potato between the lines (NPKMR), the O/R ratios of the fulvic acid (FA) did not differ among the treatments. However, the humin (HM) from H and the control (CK) displayed the highest value of O/R ratios (about 3.23 for both). Humic substances from organic treatments showed more aliphatic nature, whereas more condensed-alkali soluble humic substances were formed in the unfertilized and mineral treatments. Among fractions, the HA formed more phenolic or aromatic structures than did the FA or HM. In addition, the C, H, N and S contents of humic substances increased significantly, whereas their oxygen content decreased.

Key words: Long-term fertilization, soil quality, humic substances, Fourier transform-infrared (FT-IR) spectroscopy, elemental composition.

INTRODUCTION

Concerns regarding progressive soil degradation and the long-term sustainability of current agricultural practices have led to the study and assessment of soil quality (Haynes, 2008). Humic substances (HS) are the major components of soil organic matter (SOM) and extremely important attributes of soil quality since they profoundly influence the physical, chemical and biological properties and processes of soils. Humic substances are source of

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energy and nutrients for soil biota. They are also charge carriers and play vital roles in complexing organic compounds and adsorbents in controlling the toxicity, transport and fate of a variety of pollutants. Humic substances are involved in soil formation. They affect aggregate stability, trafficability, water retention and hydraulic properties. As a result, soil organic matter content and quality are now recognized as key factors in the evaluation of the sustainability of soil management practices.

Agricultural management practices, namely tillage, crop rotation and fertilization are known to affect SOM strongly with respect to the amount and composition at shorter and longer time scales. By evaluating the chemical changes of SOM in a sandy clay loam in North-east Viterbo city of Central Italy, Marinari et al. (2007) found that the humic-C content in the upper soil layer was 55% greater under organic than conventional management. They also noticed significantly higher humification degree and less condensed humic substances under the organic management. After 11 years of different tillage operations in Chinese Mollisols, Liu et al. (2005) reported higher levels of soil organic C and N in the upper soil layer. Ding et al. (2002) studied the changes of SOM quantity under conventional and conservation tillage in a Norfolk loamy sand soil of South Carolina, USA. They found that the aliphatic-C content of HA declined with the increase of soil depth under both systems, whereas the reverse was true for the aromatic- C content. Their results further revealed that HA contained more recalcitrant functional groups in conventional than in conservation tillage system. Recently, Liu et al. (2006) reported that the inclusion of legume crops in a long-term rotation trial of Pellic and Chromic vertisols in Australia resulted in an increase of liable carbon concentrations compared with continuous wheat or a long fallow period. They also observed that during a 9-year crop rotation experiment in the Chinese Mollisol, the soil organic carbon in the treatments of the wheat-sweet clover and wheat-soybean with addition of pig manure or wheat straw was significantly (22%) greater than that of the commonly used wheat-soybean alone. In a Pullman soil of Texas in USA, Acosta-Martinez et al. (2004) found that organic C, microbial biomass C, N and the enzymes activities were higher in perennial pasture compared with continuous cotton.

Although humic substances represent the most microbially recalcitrant and stable reservoir of organic carbon in soil, the application of organic matter can influence their amount and structural characteristics (Dou et al., 2008). These authors reported for example, that maize stalk and wheat straw applications increased the E_4/E_6 ratio and the content of carboxylic and aliphatic carbon in humic substances from a Dark brown soil of Heihe City, China. In a fluvi-calcaric cambisol of the long-term rotation experiment at Legnaro in Italy, Nardi et al. (2004) demonstrated that farmyard fertilizations improved the production of humus with a high degree of polycondensation, whereas the absence of organic fertilizer inputs led to a higher percentage of non-complex and light-weight humus.

Das and Nand (2006) studied the nature of humic substances under long-term manuring and fertilization in a rice-wheat-cowpea sequence on a mollisol at Pantnagar (India). They found that in general, in terms of quantity, the fulvic acid fraction dominated about 2 to 5 times over humic acid fraction, possibly due to biochemical transformations of open-chain compounds of plant residues into soil humus. They further noticed that regarding the elemental composition, the humic acid showed comparatively wider C/H and C/N but narrower O/H than fulvic acid isolated from the same treatment due to the stronger aromatization of humic acid in comparison with fulvic acid. According to these authors, the absorbance of humic substances measured at 465 (E_4) and 665 (E_6) revealed that humic acid absorbed more light than fulvic acid and this was attributed to accelerated mobility of electrons over unsaturated structures conjugated with humic substance nuclei.

During the past few decades, there has been much research on HS, but their chemical structure and composition remain controversial and not fully understood (Baldock et al., 1992; Dong et al., 2006). Previous long-term studies mostly focused on temporal changes of the amount of SOM (Kaiser et al., 2007) and changes induced by anthropogenic factors such as long-term fertilization practices on humic substances are still being researched. There is a crucial need in a more fundamental understanding of HS functions in the stabilization of soil aggregates, soil formation and quality, the binding of anthropogenic organic chemicals, and the sequestration of C from atmospheric CO_2 among other functions. To our knowledge most studies conducted on these issues have dealt predominantly with the HA fraction. However, the characterization and evaluation of long-term fertilization effects on the composition and structure of all the three fractions of humic substances, using integrative approaches are still scarce. We hence hypothesized that: (1) a better understanding of the changes occurring in HA, FA and HM fractions might give additional and comprehensive information on the effects of fertilisation practices and (2) reliable and complementary techniques are an essential precondition for more accurate interpretations of these structural changes in HS. The objectives of the present study were (1) to elucidate the influence of different long-term fertilization practices on the composition and structure of the isolated HAs, FAs and Humin fractions; (2) to discriminate between HAs, FAs and Humin fractions based on their characteristics that are influenced by the long-term fertilization conditions; and (3) to compare nondegradative spectroscopic (FT-IR) data with those produced by the elemental analyses to evaluate the suitability and specificity of individual results.

Table 1. Basic data of the long-term fertilization experimental soils (0-20 cm) (2008).

Treatment	Available nutrients(mg.kg^{-1})				Total Nutrients (g.kg^{-1})			Al (%)		Fe (%)		C$_{org}$ (g.kg^1)	pH	Moisture (%)
	NO$_3^-$	NH$_4^+$	P	K	N	P	K	DCB	PYRO	DCB	PYRO			
CK	7.07[f]	3.82[f]	3.41[g]	81.5[g]	0.86[e]	0.52[g]	12.6[e]	9.06[b]	3.10[b]	13.6[b]	4.73[b]	0.48h	4.91	10.8[g]
NPK	12.9[e]	9.96[d]	10.3[e]	138.7[e]	1.55[d]	1.16[f]	14.2[d]	8.41[c]	2.85[c]	12.6[c]	4.27[c]	1.20[g]	5.11[f]	11.8[e]
NPKM	19.6[a]	13.6	17.9[a]	254.8[a]	2.98[a]	2.82[a]	17.7[a]	6.51[e]	2.06[e]	9.75[e]	3.10[e]	2.65[a]	5.87[c]	12.9[c]
1.5NPKM	14.6[d]	10.2	13.1[c]	179.4[c]	2.08[c]	1.81[d]	14.9[c]	6.16[f]	1.88[f]	9.24[f]	2.82[f]	1.65[e]	5.58[d]	12.2[d]
NPKMR	16.2[c]	11.1[c]	15.3[b]	157.6[d]	1.16[e]	2.09[c]	14.1[d]	7.58[d]	2.47[d]	11.4[d]	3.71[d]	2.06[c]	5.32[e]	11.5[f]
NPKS	17.0[b]	12.8	7.06[f]	205.6[b]	1.69[d]	2.47[b]	16.4[b]	4.65h	0.88h	6.97h	1.33h	1.89[d]	4.34h	13.1[b]
M	14.2[d]	8.18[e]	11.3[d]	121.2[f]	1.86[cd]	1.52[e]	14.8[c]	9.53[a]	3.62[a]	14.3[a]	5.53[a]	1.40[f]	6.33	11.5[f]
H	7.14[f]	10.0	3.44[g]	65.7[h]	2.54[b]	0.93[f]	16.6[b]	5.64[g]	1.56[g]	8.46[g]	2.34[g]	2.38[b]	6.75[a]	14.2[a]

DCB, Dithionate-citrate bicarbonate; Pyro, pyrophosphate; C$_{org}$, organic carbon in soil; Different letters in the same column indicate a significant difference at P<0.05 (ANOVA).

MATERIALS AND METHODS

Experimental site and design

A long-term fertilization experiment with double cropping wheat and corn was begun in September 1990 at the Red Soil Experimental Station (RSES) of the Chinese Academy of Agricultural Sciences (CAAS), Southern Hunan Province (26.8°N, 111.9°E, 120 m altitude). The soil was originated from the Quaternary Red Clay and classified as Ultisols. Under average climate conditions, the area receives 1290 mm of annual precipitation, about 70 to 80% of which occurs from April to October. The mean annual temperature, annual evaporation; annual frost-free days and sunshine hours are 18°C, 1470 mm, 300 day and 1610 h, respectively. Prior to the experiment, the field had been under wheat-corn rotation for several years.

Based on the organic matter contents, eight plots (27 m × 10 m - 270 m^2) with different fertilization treatments were chosen for this study, that is, (1) CK, (2) NPK, (3) NPKM, (4) NPKMR, (5) 1.5 NPKM, (6) NPKS, (7) M and (8) H. The N, P and K fertilizers for corn and wheat were provided as urea at 456 and 195 kg N hm^{-2} ; P as single superphosphate at 699 and 300 kg P hm^{-2}, and K as KCl at 140 and 60 kg K hm^{-2} , respectively. Chemical fertilizer N was yearly applied as top dressing (40%) and base dressing (60%); chemical P and K fertilizers and manure were fertilized only as base dressings. Thirty percent of total N was used as chemical N and 70% as manure for the NPKM treatment; the same amount of chemical N was applied for other treatments. Thirty percent (30%) of the total amount of individual fertilizer applied each year was used for wheat and 70% was used for corn.

Soil sampling and analyses

Soil samples from the top red soil (0-20 cm) were collected in September 2008 at nine randomly selected points in each plot and mixed to form a bulk sample. Samples were sieved (<2 mm) and kept in polyethylene bags at 4°C. The basic data of soils were determined conventionally and summarized in Table 1. In brief, total organic carbon was determined by the method of Walkely and Black (1934) which consisted to oxidize the sample with potassium dichromate and sulphuric acid. The excess potassium dichromate was titrated against ferrous ammonium sulphate. Soil total nutrients were determined in the Kjeldahl acid digest using FIASTAR 5000 (total N and P) and flame photometer (for total K). The available N, P and K were measured by FIASTAR 5000 after their extraction with 2 M KCl, 0.5 M NaHCO$_3$ (pH 8.5) and 1 M NH$_4$OAC (pH 7), respectively (Page et al. 1982). The determination of dithionite extractable iron and aluminum was performed by the procedure of Mehra and Jackson (1960). The principle of the method consisted to heat the sample in a complexing buffer of sodium citrate/bicarbonate to which solid sodium dithionite was added as a reducing agent. Iron and aluminium were then measured in the extract by AAS. For the determination of the pyrophosphate extractable Al and Fe, 44.6 g of Na$_4$P$_2$O$_7$.10H$_2$O (0.1 M) were dissolved in distilled water and brought to 1 l and the pH was adjusted to 10.0. One gram soil was placed into a 250 ml polyethylene tube (with screw stopper) and 100 ml of 0.1 M sodium pyrophosphate reagent were added and agitated on a rotary shaker for 16 h at ambient temperature (20°C). The mixture was centrifuged at 20,000 g. The supernatant was retained in test tubes and two blanks (with only reagents) and two reference samples in each series were also performed. The concentrations of Al and Fe were measured by atomic absorption spectrometry with standards diluted in the extraction matrix.

Extractions and separation of humic fractions

Method "Schnitzer (1982)" was used with some modifications to maximize the extraction of humic compounds and to minimize their degradation. Soil humic substances were extracted with a solution containing 0.1 M NaOH and 0.1 M Na$_4$P$_2$O$_7$ (60 ml per 15 g of soil) with permanent shaking for 18 h at 25°C and the suspension was centrifuged at 9000 x g for 15 min. The supernatant was carefully decanted into a clean centrifuge bottle and retained for separation into humic acid (HA) and fulvic acid (FA) fractions. The residue (humin fraction) was mixed with 50 ml of deionized water, sonicated for 10 min and the suspension was centrifuged. The supernatant was removed and discarded, while the residue was dried and transferred into polyethylene bags for storage and analysis.

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Table 2. Some characteristics of humic fractions in long-term fertilization experimental soils (0-20cm) (2008).

Treatment	Elemental composition (%)														
	Humic acids (HA)					Fulvic acids (FA)					Humin				
	C	H	N	S	O	C	H	N	S	O	C	H	N	S	O
CK	40.7[g]	0.60[h]	0.90[h]	0.20[g]	57.6[a]	34.6[h]	0.40[g]	0.70[g]	0.60[e]	63.7[a]	41.0[h]	0.90	1.20[g]	0.70[f]	56.2[a]
NPK	42.6[fg]	1.30[g]	1.60[g]	1.00[f]	53.5[b]	39.4[g]	1.00[fg]	1.30[fg]	1.70[d]	56.6[b]	43.7[g]	1.70[f]	2.30[f]	1.20[f]	51.1[b]
NPKM	53.5[ab]	5.60[b]	6.40[b]	1.70[ef]	32.8[g]	51.0[b]	3.70[bc]	5.00[b]	2.30[d]	38.0[e]	54.2[b]	6.40[b]	6.90[a]	1.90[e]	30.6[g]
1.5NPKM	48.6[cd]	3.20[d]	4.60[d]	2.60[cd]	40.7[e]	44.9[d]	2.10[de]	3.60[c]	3.73[c]	45.6[d]	49.5[d]	3.90[d]	5.20[c]	2.50[d]	38.9[e]
NPKMR	56.3[a]	7.40[a]	8.20[a]	3.40[b]	25.0[h]	53.2[a]	4.80[a]	6.20[a]	4.50[bc]	31.3[f]	56.9[a]	8.20[a]	7.40[a]	3.60[c]	23.9[h]
NPKS	51.4[bc]	4.20[c]	5.30[c]	4.80[a]	34.4[f]	48.7[c]	3.00[cd]	3.10[cd]	5.90[a]	39.3[e]	52.0[c]	5.00[c]	6.00[b]	5.10[a]	31.9[f]
M	44.2[ef]	2.60[e]	2.60[f]	2.00[de]	48.6[c]	41.3[f]	1.50[ef]	2.00[ef]	4.00[c]	52.2[c]	45.3[f]	3.40[de]	3.50[e]	3.00[d]	44.8[c]
H	46.4[de]	2.00[f]	3.40[e]	3.00[bc]	45.2[d]	42.7[e]	4.20[ab]	2.60[de]	5.20[ab]	45.3[d]	48.1[e]	2.70[e]	4.20[d]	4.20[b]	41.5[d]

Different letters in the same column indicate a significant difference at P<0.05.

Using a dropping tube and a pH-meter, 6 M HCl was added to the decanted supernatant in the centrifuge bottle until a pH of 1.5 was attained. To separate the humic fractions, the dark brown to black precipitated portion and that of yellowish in color dissolved in the solution were centrifuged and the supernatant (FA) was removed and retained in 50-ml centrifuge bottle for storage and analysis. 50 ml of 0.1 M NaOH were added to the centrifuge bottle containing the precipitate (HA) to redissolve it. The HA was then transferred into 50-ml centrifuge bottle for storage and analysis. In a further step, the obtained HA and FA were freeze-dried and the dried samples were stored. The extractions were carried out three times for each sample.

Fourier-transform infrared spectroscopy

FT-IR spectra of humic fraction samples were collected with a Nicolet 380 spectrophotometer, in the 400 to 4000 cm^{-1} range with a 4 cm^{-1} resolution and 32 scans per sample. Pellets were prepared by pressing a mixture of 0.8 mg of sample and 80 mg of spectroscopy grade KBr.

Elemental analysis

Elemental analysis of humic fractions was determined by using CHNS analyzer (Perkin Elmer EA 2400). Oxygen was calculated by difference:

O%= 100−(C+H+N+S)%.

Statistical analysis

All results for basic soil properties and the other parameters were expressed on an oven-dried soil weight basis (105°C, 24 h). The data were subjected to analysis of variance (ANOVA), using the MSTAT-C software package (Michigan, USA). The means and standard deviations for triplicates were calculated. Significant differences of means for all treatments were judged by least significant difference (LSD) multiple-comparison tests and expressed at P<0.05 and/or P<0.01.

RESULTS AND DISCUSSION

Elemental and atomic ratios analyses of humic fractions

Elemental analysis of humic substances is often used to establish their nature and origin (Baddi et al., 2004). The contents of C, H, N and S among fertilization treatments significantly increased in all humic fractions as compared to the control (CK) (Table 2). In contrast, the oxygen content decreased in these fractions. C, H, N and S concentrations of humic fractions reached maximum values in 1.5 NPKM followed by NPKM, while their minimum values were recorded in CK followed by NPK. Among the treatments, the mean carbon content was higher in HA fraction (47.96%) than that in the FA fraction (44.47%) but remained lower if compared to the mean C content in the HM fraction (48.83%). The mean content of H and N in the treatments revealed a similar behavior, though to a lesser extent. Conversely, the mean content of S was higher in the FA fraction (3.49%) than that in the HA (2.34%) and HM (2.77%) fractions. These results showed that the carbon, hydrogen, and sulphur contents of humic fractions were relatively higher in the organic treatments (1.5 NPKM, NPKM, NPKMR, NPKS, M and H), although their C/N ratios were low as compared to the unfertilized (CK) and mineral (NPK) treatments. For example the C/N ratios of the mentioned organic treatments for the HA fraction were 8.01, 9.75, 12.3, 11.3, 19.9 and 15.9, respectively while that of the control and the NPK treatments recorded higher values (52.8 and 31.1,

Table 3. Atomic ratios of humic substances in long-term fertilization experimental soils (0-20 cm).

Treatments	Humic acids			Fulvic acids			Humin		
	H/C	O/C	C/N	H/C	O/C	C/N	H/C	O/C	C/N
CK	0.18	1.06	52.7	0.14	1.38	5.77	0.26	1.03	39.9
NPK	0.37	0.94	31.1	0.30	1.08	35.4	0.47	0.88	22.2
NPKM	1.26	0.46	9.75	0.87	0.56	11.9	1.42	0.42	9.16
NPKMR	0.79	0.63	12.3	0.56	0.76	14.5	0.94	0.59	11.1
1.5 NPKM	1.58	0.33	8.01	1.08	0.44	10.0	1.73	0.31	8.97
NPKS	0.98	0.50	11.3	0.74	0.60	18.3	1.15	0.46	10.1
M	0.70	0.82	19.8	0.43	0.95	24.1	0.90	0.74	15.1
H	0.52	0.73	15.9	1.18	0.80	19.2	0.67	0.65	13.3

respectively). This decreasing trend of the values of the C/N ratios in organic treatments as compared to the mineral ones was also true for the FA and humin fractions. However, for the FA fraction, the control recorded the lowest value of the C/N ratio (5.77), whereas its highest value was observed in the NPK treatment (35.4). The low value of the C/N ratio in organic treatments could probably be attributed to the high levels of products arising from protein decomposition and hence may indicate a greater level of humification. According to Baddi et al. (2004), high values of nitrogen and sulphur are indicative of high contents of non-humified biomolecules (polysaccharides and polypeptides) in the material. A high nitrogen values could also be attributed to the incomplete hydrolysis of proteinaceous constituents.

The decomposition of the organic material in soil tends to lead to the formation of phenolic structures deriving from lignin. These structures are hardly decomposed with respect to sugars and proteins (Rosa et al., 2005). Therefore, the C/H, C/N, and C/O atomic ratios have been used as important indicators of the aromaticity and level of organic material decomposition. The highest H/C ratios of HA and humin fractions were observed in 1.5 NPKM, followed by NPKM, whereas that of FA was recorded in H, followed by 1.5 NPKM. The lowest H/C ratios of all the three fractions were found in CK, followed by NPK (Table 3). According to the literature (Stevenson, 1994), higher C/H, C/O, and C/N atomic ratios are associated with higher degrees of humification due to decreased acid, carbohydrate, and amino acid/protein content.

For the different humic fractions, our results generally indicate that the H/C ratios in organic treatments were higher than those observed in the unfertilized and mineral plots. This suggests that the humic fractions from organic treatments may be more aliphatic than those from the unfertilized and mineral ones, which should contain more aromatic carbon. In contrast to our findings, Dou et al. (2008) have reported that the C/H ratio decreased after organic matter application and consequently reduced the degree of condensation. The values of the O/C atomic ratio appeared higher in CK and lower in 1.5 NPKM for all

humic fractions. While the C/N atomic ratio of the HA and humin fractions displayed a similar trend, that of the FA fraction showed higher values in NPK and lower ones in CK. Moreover, the mean values of the C/N ratio for the different humic fractions did not follow the changes in C and N content in these fractions. These values were relatively higher in the HA fraction (20.12) than those in FA (17.40) and HM (16.23) fractions, suggesting the formation of more phenolic or aromatic structure in HA fraction as compared to that in FA and HM fractions.

The distribution of carbon in different humic fractions (Table 4) revealed that the mean bulk of it was present in humin (48.83%), followed by HA (47.96%) and FA (44.47%) fractions in all treatments. The HA/FA ratio showed a predominance of HA in all treatments. This may be attributed to the supply of raw humus through manure, straw and the incorporated detritus of fallow causing incomplete decomposition of humic acids. Despite the lower expected biological activity in CK and NPK, it is interesting to observe that the HA/FA ratios of these treatments displayed relatively higher values. This probably indicates an encouragement for the formation of condensed alkali-soluble humic substances with greater stability in CK and NPK. The reasons for such a controversy should be further investigated because they are still unclear.

FT-IR spectra

Based on values from FT-IR spectroscopy (Appendix1), the spectra for humic acid, fulvic acid and humin from each treatment of the long-term fertilization soils were presented in Figure 1. In general, the FT-IR spectra showed similar bands in all treatments but different bands occurred between the humic fractions (HA, FA and HU). The broad intense bands at 3400 and 3700 cm^{-1} are due to the stretching vibration of bonded and non-bonded hydroxyl groups (Solomon et al., 2007; Meccozi et al., 2009). Small contributions of the bands at 2280 and 2360 cm^{-1} come from, NH_2^+, $CO-NH_2$, CO-NH-, S-H, and P-H (Tatzber et al., 2007). According to Mecozzi et al. (2009),

Table 4. Total organic carbon content and its distribution in humic fractions from long-term fertilization experimental soils (0-20 cm)*.

Treatment	TOC (%)	TN(%)	C/N ratio	HAC (%)	FAC (%)	HUC (%)	HAC/FAC ratio
CK	0.48	0.09	5.58	40.7	34.6	41.0	1.18
NPK	1.20	0.15	7.74	42.6	39.4	43.7	1.08
NPKM	2.65	0.30	8.89	53.5	51.0	54.2	1.05
NPKMR	1.65	0.21	7.93	48.6	44.9	49.5	1.08
1.5 NPKM	2.06	0.12	17.8	56.3	53.2	56.9	1.06
NPKS	1.89	0.17	11.2	51.4	48.7	52.0	1.05
M	1.40	0.19	7.53	44.2	41.3	45.3	1.07
H	2.38	0.25	9.37	46.4	42.7	48.1	1.09
Mean	1.71	0.18	9.50	47.9	44.5	48.8	1.08

*TOC, Total organic carbon; TN, total nitrogen; HAC, FAC and HUC are the contents of elemental carbon in humic acid, fulvic acid and humin fractions, respectively.
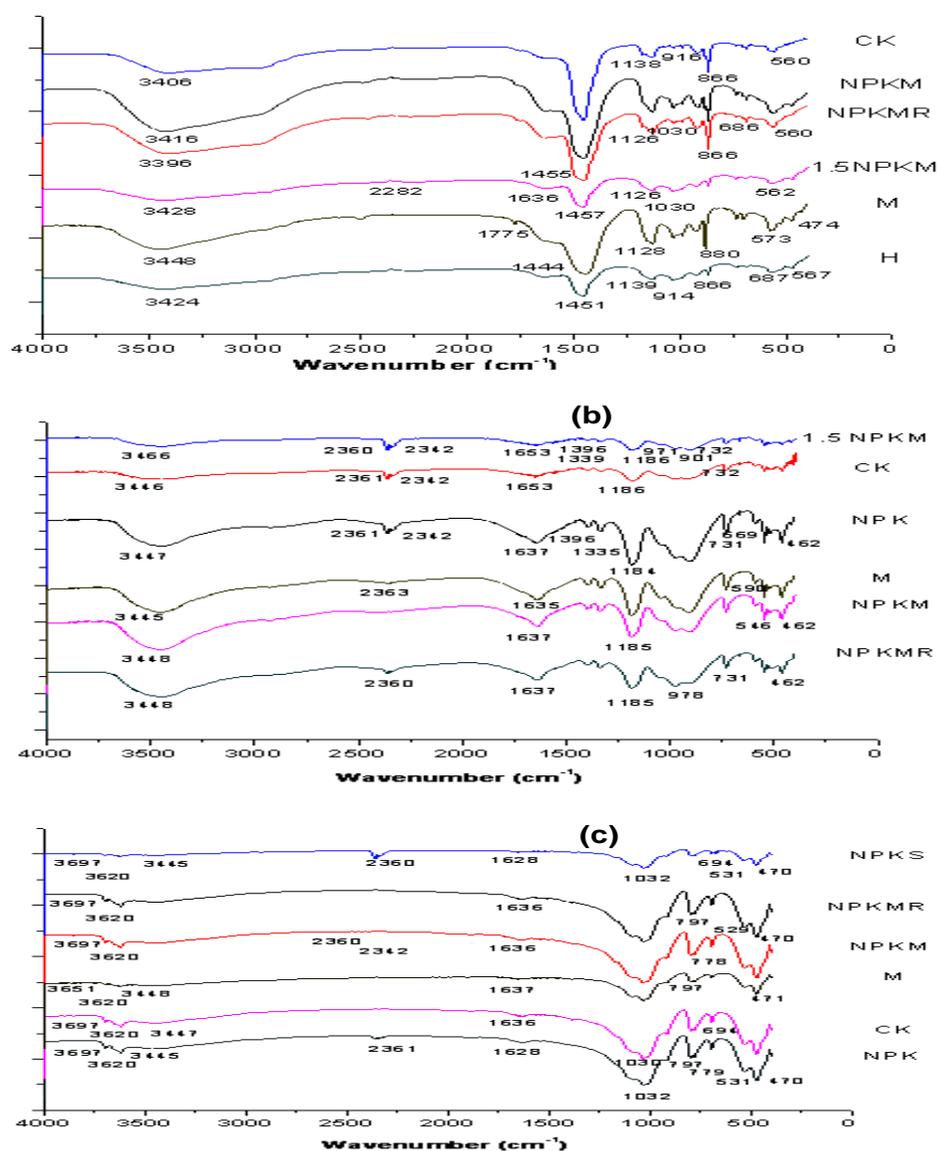

Figure 1. FT-IR spectra of humic substances in long-term fertilization soils (0-20 cm): humic acid (a), Fulvic acid (b), and Humin (c).

Table 5. The O/R ratios of humic substances from FT-IR spectra in the long-term fertilization soils (0-20 cm)*.

Treatments	HA[a]	FA[b]	HM[c]
CK	1.01	0.48	3.22
NPK	1.02	0.46	1.74
NPKM	1.24	0.46	2.28
NPKMR	1.04	0.54	0.91
1.5 NPKM	0.66	0.40	2.21
NPKS	1.01	0.46	0.92
M	1.18	0.46	2.21
H	0.85	0.46	3.23

*O/R ratios are the intensities of oxygen-containing functional groups vs. aliphatic and aromatic groups; HA is humic acid; FA is fulvic acid; and HM stands for humin. [a]HA O/R ratio was generated from HA FT-IR spectrum and calculated at (3406 +1138)/(1454 + 916 +866+ 686+ 560); [b]FA O/R ratio was generated from FA FT-IR spectrum and calculated as: (3446+1186)/(2361+1653+1397+1338+974+732+592+545+463); [c]HM O/R ratio was generated from HM FT-IR spectrum and calculated at (3697+3620+3447+1636)/(1030+797+694+530+470).

the band at 1775 cm-1 originates from the C=O stretch of ester fatty acids. The broad band at 1630 cm^{-1} can be assigned to the aromatic C=C vibrations, symmetric stretching of COO$^-$ groups, and H-bonded C=O of conjugated ketones (Mao et al., 2008). The bands at 1440 to 1457 cm^{-1} can be attributed to the CH and NH (amide II) bending motions, molecule skeleton vibrations and CO-bond vibration (Antil et al., 2005). These bands can also be due to the carbonate, C-O and C=C stretches as well as to the aliphatic C-H stretches (Piccolo et al., 1992, Richard et al., 2007, Hardie et al., 2009). Evidence for the presence of COOH groups was indicated by the peaks at 1120 to 1150 cm^{-1} which were attributed to the C-O stretches. Sulfone functional groups and C-O-C polysaccharides can also be associated with these peaks (Mao et al., 2008, Mecozzi et al., 2009). Si-O vibrations of clay minerals were assigned at around 1030 cm^{-1}(Artz et al., 2008, D'Orazio and Senesi, 2009). The bands at 900 to 460 cm^{-1} were indicative of inorganic materials, such as clay and quartz minerals (Ding et al., 2002; Filip and Demnerova, 2007).

By comparing the spectra among fractions, strong alkyl halide stretches (C-F at1000-1400 cm^{-1}; C-CL at 600-800 cm^{-1}; C-Br at 500-600 cm^{-1}; and amine N-H stretch at 3300-3500 cm^{-1}) were common to all the three fractions. The humin fraction was distinguished by the presence of a strong and sharp free OH stretch (3620-3700 cm^{-1}) whereas, a strong carbonyl C=O stretch (1670-1820 cm^{-1}) appeared in the HA fraction only. A C-N stretch (1080-1360 cm^{-1}) was present in both the HA and FA fractions but the humin fraction lacked this type of vibration.

It was a common practice to draw conclusions on factors affecting SOM composition by spectral peak positions and intensity changes. However, this method does not often provide the kind of detailed information

required to ascertain fertilization effects on SOM.

Therefore, detailed insight on the reactivity of HA, FA and HM was provided by calculation of O/R ratios, which were the intensities of oxygen-containing functional groups vs. aliphatic and aromatic groups (Ding et al., 2006). The reactivity of SOM was related with organic oxygen because it was present in all major functional groups, e.g., carboxyl, phenolic, hydroxyl, alcohol, and carbonyl and could be associated with SOM binding characteristics. The impact of fertilization on spectral composition was summarized in the O/R ratio (Table 5).

With different fertilization applications, the HA fraction isolated from the NPKM treatment had the highest O/R ratio (1.24) followed by the manure alone treatment (1.18), while that from 1.5 NPKM and H exhibited the lowest values (0.66 and 0.85, respectively). The relatively high O/R ratios in the NPKM and M treatments may indicate that HA was chemically and biologically more active in these treatments if compared to the unfertilized and other plots. This higher biological activity is probably related to the intrinsic accumulation of fatty acids in animal manure, corroborated in our study by the presence of a pronounced carbonyl C=O stretch at 1775 cm^{-1}. In addition, the cellular residues from increased soil microbial growth that is often associated with animal manure application may also explain the observed high biological activity of HA in the NPKM and M treatments. It is interesting to note that 1.5 NPKM and H treatments significantly decreased the O/R ratio of the HA, whereas no impact was observed for NPK, NPKS and NPKMR as compared to CK. This phenomenon is difficult to explain. However, either an inhibition of microbial activity or a formation of more recalcitrant materials is supposed to be occurred in 1.5 NPKM and H. It can also be speculated that the type, quality and quantity of the incorporated amendments are key factors in the potential structural changes in the HA fraction. In this regard, tannins and lignins could be formed as main contributors of aromatic and phenolic structures in these treatments. Such a structure can be linked with the E_4/E_5 ratio which is independent of the concentration of humic substances but varies with source and origin of incorporated residues.

The O/R ratios of the FA fraction did not differ among the treatments. Interestingly the HM fraction isolated from H and CK demonstrated high but similar values of O/R ratios (3.23 and 3.22, respectively), whereas that from NPKMR and NPKS had the lowest but also very close values of this ratio (0.91 and 0.92, respectively). NPKM, 1.5 NPKM and M treatments displayed little differences in the O/R ratio although these values remained higher than that for the NPK treatment. This probably indicated that the intrinsic accumulation of fatty acids in animal manure and the cellular residues from increased soil microbial growth were key factors for the high chemical and biological activities in organic treatments if compared to the control or mineral fertilizers alone. Among the humic

substances, our results showed higher O/R ratios in the HM fraction followed by those in the HA fraction, whereas lower values were recorded in the FA fraction. It can be deduced that the degree of complexity of the humic fraction controls its biological activity.

Conclusion

The results of this study demonstrated that organic fertilizers, namely 1.5 NPKM, NPKM, NPKMR, NPKS, M and H were the best treatments in increasing the elemental composition of the humic substances and in decreasing the C/N ratios which may indicate their greater level of humification as compared to the control or the mineral fertilizers. In addition these results were corroborated by the FT-IR analysis through the O/R ratios which revealed higher chemical and biological activities in the aforementioned organic treatments in comparison to the control or the mineral fertilizers. However, the type, quantity and quality of the incorporated amendments have to be considered for fertilization. The elemental analysis, the atomic and O/R ratios as well as the spectral characterization are important indicators to evaluating the impacts of fertilization on the structure and composition of humic substances. However, the in-depth understanding of the functional and structural features of soil humic substances appeals to more cross-site collaborative experiments and more specific techniques such as ^{13}C-NMR and fluorescence resonance. Foreseeing the future in this area can also be achieved through approach of coupling long-term experiments and modeling.

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APPENDIX

Appendix 1. FT-IR spectral values of humic substances in different long-term fertilization treatments (2008).

CK	NPK	NPKM	NPKMR	1.5NPKM	NPKS	M	H
			Humic acids (HA)				
3406	3452	3416	3396	3428	3399	3448	3424
1454	1452	1452	1455	1457	1451	1444	1451
1138	1137	1127	1126	1126	1140	1128	1139
916	914	915	917	920	913	926	914
866	866	866	866	867	866	-	866
686	686	686	686	-	686	-	687
560	560	559	560	562	562	573	567
-	-	1030	1030	1030	-	1030	-
-	-	880	-	-	-	880	880
-	-	-	-	2282	-	-	-
-	-	-	-	1636	-	-	-
-	-	-	-	735	-	735	-
-	-	-	-	474	-	474	-
-	-	-	-	-	-	1775	-
-	-	-	-	-	-	989	-
-	-	-	-	-	-	702	-
			Fulvic acids (FA)				
CK	NPK	NPKM	NPKMR	1.5NPKM	NPKS	M	H
3446	3447	3448	3448	3466	3448	3445	3458
2361	2361	-	2360	2360	2365	2363	2362
1653	1637	1637	1637	1653	1637	1635	1636
1397	1396	1397	1397	1396	1396	1395	1396
1338	1335	1335	1336	1339	1334	1335	1335
1186	1184	1185	1185	1186	1185	1185	1185
974	-	974	978	971	-	-	-
732	731	731	731	732	731	731	731
592	591	591	591	592	591	590	591
545	545	546	545	544	546	546	545
463	-	462	462	463	463	462	462
-	522	522	521	521	523	523	522
-	907	909	-	901	908	909	907
-	-	-	-	669	-	-	-
			Humin				
3697	3697	3697	3697	-	3697	-	3697
3620	3620	3620	3620	3620	3620	3620	3620
3447	3445	-	-	3433	-	3448	3446
1636	1628	1636	1636	1636	1654	1637	1636
1030	1032	1032	1032	1032	1031	1032	1032
797	797	797	797	797	796	797	797
694	694	694	694	694	694	694	694
530	531	528	529	529	531	530	529
470	470	471	470	470	470	471	470
-	2361	2360	-	-	2361	-	-
-	779	778	-	-	-	-	-
-	-	2342	-	-	2342	-	-
-	-	-	-	-	669	-	-