

## Moisture Effect on Soil Humus Characteristics in a Laboratory Incubation Experiment

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### Abstract

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A 180-day laboratory incubation experiment (30°C) was conducted to investigate the quantitative and qualitative characteristics of humic fractions in a Mollisol at different moisture conditions. The soil moisture contents were 30, 60, and 250% field water-holding capacity (WHC), which represented the low, middle, and high moisture levels, respectively. The results showed that the carbon contents of the total soil and corresponding humic fractions generally decreased with increasing soil moisture. A significant difference was observed between the 250% WHC and the two other moisture levels. By contrast, the carbon content of the water soluble fraction significantly increased with increasing soil moisture levels. The solid-state <sup>13</sup>C nuclear magnetic resonance (NMR) spectra showed that the alkyl C/O-alkyl C, aliphatic C/aromatic C, and hydrophobic C/hydrophilic C ratios were in the order of 250% WHC > 30% WHC ≈ 60% WHC, 30% WHC ≈ 60% WHC > 250% WHC and 250% WHC > 60% WHC ≈ 30% WHC for humic acid, and 250% WHC > 30% WHC ≈ 60% WHC, 60% WHC ≈ 250% WHC > 30% WHC and 30% WHC ≈ 250% WHC > 60% WHC for humin, respectively. These results indicated that a high moisture level was unfavourable for the carbon accumulation of the total soil and humic fractions, whereas it was favourable for the accumulation of water soluble carbon. Although soil moisture levels had a distinct effect on the chemical composition of humic acid and humin, the decomposition degree of the two humic substances components, as indicated by the alkyl C/O-alkyl C ratio, were both higher at a high moisture level than at a low moisture level. Therefore, the lower soil organic carbon content at a high moisture level than at a low moisture level can be ascribed to the higher water soluble carbon content and larger decomposition degree of humic acid and humin in the former. Our results are important for understanding the behaviour and mechanisms of humic substances at specific soil moisture conditions.

**Keywords:** humic acid; humin; humus composition; soil moisture; solid-state <sup>13</sup>C NMR

Soil organic matter is a major soil component. It has an important function in both soil fertility and the ecological environment (PAUL 2014). Approximately 60–70% of organic matter in soil is composed of humic substances (LOFFREDO & SENESI 2006). As the refractory organic carbon form of soil, humic substances play a vital role in the atmospheric CO<sub>2</sub> sequestration (SPACCINI *et al.* 2002). According to their solubility in aqueous solution at different pH values, humic

substances can be divided into three main fractions, namely humic acid, fulvic acid, and humin.

The formation of humic substances (i.e. humification) is primarily a microbially mediated process (ZECH *et al.* 1997). The humification process is generally accompanied by changes in the amount and chemical structure of humic substances (IKEYA *et al.* 2004). Therefore, the factors that control microbial activity, such as temperature, pH, and C/N ratio, determine

the quantity and quality of humic substances formed in soil. Among these factors, soil moisture is a major factor affecting microbial activity (BROCKETT *et al.* 2012). The optimum soil moisture content for microbial activity is found at intermediate levels (IQBAL *et al.* 2009), i.e. 50–70% of water-holding capacity (STRES *et al.* 2008). Previous studies mainly focused on the impact of soil moisture content on the mineralization process of soil organic matter (HOSSAIN & PUTEH 2013; CHEN *et al.* 2014; GUO *et al.* 2014; LU & XU 2014), however, its effects on the amount and chemical composition of humic substances remain unclear.

Mollisol, which accounts for about 7% of the world's ice-free land surface, is generally recognized as an inherently fertile and productive soil (LIU *et al.* 2012). Mollisol is characterized by high content of organic matter and humic substances with a higher aromatic structure compared with other soil types (GLASER & AMELUNG 2003). Moreover, a high aliphaticity has also been observed in both humic acid and humin in Mollisol (LI *et al.* 2015). Using sequential extraction with base + urea and DMSO + H<sub>2</sub>SO<sub>4</sub> solvent systems, SONG *et al.* (2011) showed that humin in Mollisol is largely made up of biological molecules from plants and microorganisms. Mollisol is mainly distributed in the northeast region of China. The region has been a key base for commodity grain production. However, long-term intensive cultivation practices have led to a remarkable decline in soil organic carbon in Mollisol over the last decades. Therefore, increasing carbon sequestration to sustaining soil productivity in the Mollisol region is important (LIU *et al.* 2010).

The present study attempts to provide a preliminary examination of the effects of different soil moisture contents on the quantitative and qualitative characteristics of humic fractions in a Mollisol of northeast China at controlled incubation conditions based on a combination of classical humus composition analysis and solid-state <sup>13</sup>C NMR spectroscopy.

## MATERIAL AND METHODS

**Soil samples.** Surface soil samples (0–20 cm) were collected in May 2013 from a maize (*Zea mays* L.) field located at the Lishu County (43°16'N, 124°26'E), Jilin Province, northeast China. The soil was classified as Mollisol (USDA Soil Taxonomy), Phaeozem (FAO Soil Taxonomy), or Isohumosol (Chinese Soil Taxonomy). The soil samples were air-dried, crushed, and passed through a 2 mm sieve after removing the visible plant residues. The physical and chemi-

cal properties of the soil were as follows: pH (H<sub>2</sub>O) 5.4, moisture content 52.1 g/kg, field water-holding capacity (WHC) 345.5 g/kg, total organic C (TOC) 11.1 g/kg, total N 1.11 g/kg, and C/N ratio 10.0.

**Incubation experiment.** Sieved soil samples (200 g) were weighed into 250 ml plastic beakers. Distilled water was added to bring the soil moisture contents to 30, 60, and 250% WHC, which represented the low, middle, and high (i.e. flooding condition with a 1 cm-deep water layer above the soil surface) moisture levels, respectively. The beakers were covered with plastic films with needle holes to maintain aerobic condition and placed in an incubator in a completely randomized design. The incubation experiment was conducted at 30°C for 180 days. During the incubation period, the soil moistures were held constant by periodically weighing and spraying distilled water as necessary. At the end of the incubation, three replicate soil samples from each treatment were removed, air-dried, and sieved through a 2 mm mesh sieve for subsequent analysis.

**Humus composition analysis.** Humus composition was analyzed according to the method described by ZHANG *et al.* (2010). Briefly, soil sample was first soaked in distilled water at 70°C for 60 min to isolate the water soluble fraction. The soil residue was then extracted using a mixture of 0.1 mol/l NaOH and 0.1 mol/l Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> at 70°C for 60 min. The dark brown alkaline supernatant solution, corresponding to the total humic extract, was acidified with 0.5 mol/l H<sub>2</sub>SO<sub>4</sub> solution to coagulate the humic acid fraction, with the fulvic acid fraction remaining in the supernatant solution. The solid residue after alkaline extraction was referred to as mineral-bound humin fraction. The carbon contents of water soluble fraction (WSFC), total humic extract (HEC), and humic acid fraction (HAC) were determined by dichromate oxidation method. The carbon contents of fulvic acid fraction (FAC) and mineral-bound humin fraction (MHUC) was calculated by subtracting the HAC from the HEC and subtracting the sum of WSFC and HEC from the TOC, respectively.

**Isolation of humic acid and humin.** Humic acid and humin were isolated at room temperature according to the procedure described previously (ZHANG *et al.* 2013). Briefly, 20 g of soil sample from each of the three replicates was first suspended in distilled water and 0.05 mol/l HCl solution to remove the poorly decomposed light fractions and carbonates, respectively. The soil residue was then extracted with 200 ml of 0.1 mol/l NaOH and 0.1 mol/l Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> for 48 h. The extraction procedure was repeated eight times until the supernatant was almost colourless.

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The humic acid fraction was precipitated by acidifying the alkaline supernatants to pH 1.0 using 6 mol/l HCl. After three cycles of dissolution in 0.1 mol/l NaOH and coagulation again in 6 mol/l HCl, the humic acid precipitate was demineralized with 0.5% HCl-HF solution. The alkaline-insoluble humin fraction was treated ten times with 10% HF-HCl solution to remove mineral impurity. The resulting humic acid and humin were dialyzed against distilled water, and then freeze-dried.

**Solid-state  $^{13}\text{C}$  NMR spectroscopic analysis of humic acid and humin isolated.** The solid-state  $^{13}\text{C}$  cross-polarization magic-angle-spinning and total-sideband-suppression (CPMAS TOSS) NMR spectra were recorded with a Bruker AVANCE III 400 WB spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) at 100.6 MHz. From 4000 to 10 000 scans with 2048 data points were collected over a spinning speed of 8 kHz, a spectral width of 50 kHz, an acquisition time of 20 ms, a recycle delay of 3 s, and a contact time of 2 ms. All the free induction decays were transformed by applying a zero filling of 16 384 and a line broadening of 80 Hz. Chemical shifts were referenced externally to the methylene resonance of adamantane standard at 38.4 ppm. The spectra were divided into four common chemical shift regions representing alkyl C (0–50 ppm), O-alkyl C (50–110 ppm), aromatic C (110–160 ppm), and carbonyl C (160–210 ppm). Moreover, the O-alkyl C was divided into regions of methoxyl C (50–60 ppm), carbohydrate C (60–95 ppm), and di-O-alkyl C (95–110 ppm); and aromatic C into regions of aryl C (110–145 ppm) and phenolic C (145–160 ppm). The relative intensity of each chemical shift region was obtained by integration using the MestReNova software package (Version 5.3.1).

**Statistical analysis.** The soil humus compositions among different moisture treatments were compared using one-way ANOVA followed by LSD test at  $P < 0.05$  (SPSS 16.0).

## RESULTS AND DISCUSSION

**Total organic carbon content in the soil.** The TOC content in the soil at different moisture treatments is shown in Table 1. The TOC content gradually decreased with the increase of soil moisture from 30 to 250% WHC. A significant difference was observed between the 250% WHC and the two other moisture treatments. The results suggested that a high moisture level was unfavourable to the organic carbon accumulation with respect to the low and middle moisture levels. Previous studies have also reported that organic carbon mineralization enhanced with increasing soil moisture from 20 up to 60–80% WHC (IQBAL *et al.* 2009; CHEN *et al.* 2014; GUO *et al.* 2014), which was consistent with our results. However, there is currently no agreement on the effects of flooding condition on soil organic carbon mineralization. Some studies have shown that flooding can promote organic carbon mineralization (SMITH 2005; LU & XU 2014), whereas other studies have found that flooding inhibited SOC mineralization (IQBAL *et al.* 2009; HOSSAIN & PUTEH 2013). As indicated above, the present study showed that flooding condition resulted in a higher SOC loss. The reason for this is explained in the following sections.

**Soil humus composition.** The carbon contents of humic fractions in soil at different moisture treatments are also shown in Table 1. The WSFC, which accounted for 0.92–1.80% of TOC, significantly increased with increasing moisture levels, which suggested that the accumulation of the water soluble fraction was more favourable at a high moisture level. Dissolved organic carbon is an easily available carbon source to soil microbial communities (STRAATHOF *et al.* 2014). Previous studies showed that the influence of flooding on soil organic carbon content depended on the equilibration between the

Table 1. Carbon contents of the total soil and corresponding humic fractions under different moisture treatments (in g/kg)

| Treatment<br>(% WHC) | TOC                      | WSFC                     | HEC                      | HAC                      | FAC                      | MHUC                     | HAC/FAC                  |
|----------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| 30                   | 9.77 ± 0.26 <sup>a</sup> | 0.09 ± 0.01 <sup>c</sup> | 3.99 ± 0.07 <sup>a</sup> | 1.71 ± 0.06 <sup>a</sup> | 2.28 ± 0.11 <sup>a</sup> | 5.68 ± 0.29 <sup>a</sup> | 0.75 ± 0.06 <sup>a</sup> |
| 60                   | 9.56 ± 0.19 <sup>a</sup> | 0.11 ± 0.00 <sup>b</sup> | 3.76 ± 0.03 <sup>b</sup> | 1.62 ± 0.05 <sup>a</sup> | 2.14 ± 0.02 <sup>a</sup> | 5.70 ± 0.19 <sup>a</sup> | 0.76 ± 0.03 <sup>a</sup> |
| 250                  | 8.61 ± 0.10 <sup>b</sup> | 0.16 ± 0.01 <sup>a</sup> | 3.37 ± 0.02 <sup>c</sup> | 1.40 ± 0.05 <sup>b</sup> | 1.97 ± 0.07 <sup>b</sup> | 5.08 ± 0.08 <sup>b</sup> | 0.71 ± 0.05 <sup>a</sup> |

WHC – field water-holding capacity; TOC – total organic carbon; WSFC – carbon content of water soluble fraction; HEC – carbon content of total humic extract; HAC – carbon content of humic acid fraction; FAC – carbon content of fulvic acid fraction; MHUC – carbon content of mineral-bound humin fraction; mean values ± standard deviation of three replicates are presented; values within the same column not followed by the same letter differ significantly ( $P < 0.05$ )

enhancement of dissolved organic carbon dissolution and the inhibition of microbial activity (HAO *et al.* 2011; LU & XU 2014). When the enhancement of dissolved organic carbon dissolution was larger than the inhibition of microbial activity, soil organic carbon content decreased. Although the microbial activity has not been determined, the higher WSFC content can reasonably explain the lower TOC content at flooding condition in the present study. By contrast, the HEC, HAC, FAC and MHUC, which accounted for 39.2–40.9%, 16.3–17.5%, 22.4–23.4%, and 58.2–59.6% of TOC, respectively, followed a pattern similar to TOC. The HAC, FAC, and MHUC also exhibited insignificant differences between the 30% and 60% WHC treatments. The results implied that a high moisture level was also unfavourable for the accumulation of humic fractions in soil.

Although no significant differences were observed among the three moisture treatments, the HAC/FAC ratio was lower at 250% WHC than at the other two moisture treatments. This implied that a high moisture level was favourable for the accumulation of fulvic acid fraction. TARDY *et al.* (1997) speculated that fulvic acid dominated in wet or humid climates and non-oxygenated condition based on the thermodynamic stability calculation, which was consistent with our results.

**Solid-state  $^{13}\text{C}$  NMR spectra of humic acid and humin.** The extraction yields of humic acid were 3.42, 3.33, and 2.63 g/kg, and those of humin were 11.8, 11.6, and 11.3 g/kg at 30, 60, and 250% WHC, respectively. The solid-state  $^{13}\text{C}$  CPMAS TOSS NMR spectra of humic acid and humin at different moisture treatments are presented in Figure 1, and the relative

intensities of different carbon functional groups are shown in Table 2. All spectra exhibited major signal peaks at approximately 25, 31, 33, 56, 72, 106, 129, 152, and 173 ppm. The peak assignments were reported in previous studies (ZHANG *et al.* 2009, 2011, 2013). The relative intensities of the carbon functional groups between humic acid and humin were different. Humic acid was predominated by the aromatic group, whereas humin was predominated by the O-alkyl group. However, in some soils, alkyl C dominated over O-alkyl C and aromatic C in humic acid (LUO *et al.* 2008; FERNANDES *et al.* 2010) and humin (DAI *et al.* 2001; CHEN *et al.* 2007). Moreover, NIEROP *et al.* (1999) reported that O-alkyl C dominated over alkyl C and aromatic C in humic acid. Therefore, further studies are needed to elucidate the possible reasons that lead to the different results. On average, compared with humic acid, humin had larger proportions of carbohydrate C and di-O-alkyl C, and smaller proportions of alkyl C, methoxyl C, aryl C, phenol C, and carbonyl C. The alkyl C/O-alkyl C and hydrophobic C/hydrophilic C ratios of humic acid were higher, whereas the aliphatic C/aromatic ratio was lower than those of humin. The larger values of alkyl C/O-alkyl C, aliphatic C/aromatic C, and hydrophobic C/hydrophilic C ratios generally indicated the higher degrees of alkylation, aliphaticity, and hydrophobicity of humic substances (ZHANG *et al.* 2013). Therefore, our results suggested that humic acid was more alkylated, more hydrophobic, and less aliphatic than humin. The higher aliphaticity for humin than for humic acid has also been observed in previous studies (SIMPSON *et al.* 2011).

The alkyl C for humic acid was in the order of 250% WHC > 60% WHC  $\approx$  30% WHC. The meth-

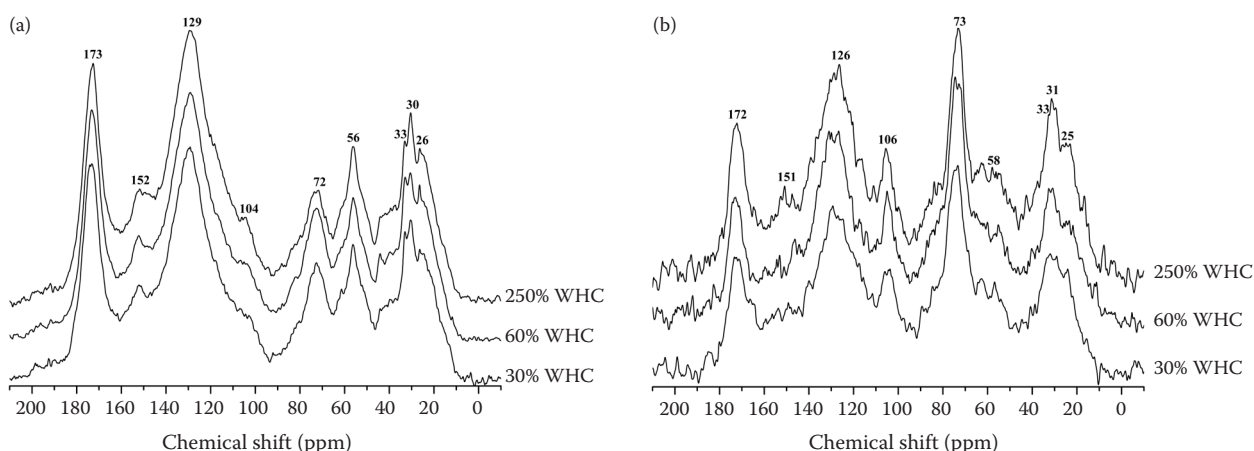


Figure 1. Solid-state  $^{13}\text{C}$  cross-polarization magic-angle-spinning and total-sideband-suppression (CPMAS TOSS) NMR spectra of soil humic acid (a) and humin (b) under different moisture treatments; WHC – field water-holding capacity



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Table 2. Relative carbon distribution (%) in different regions of chemical shift in  $^{13}\text{C}$  cross-polarization magic-angle-spinning and total-sideband-suppression (CPMAS TOSS) NMR spectra of soil humic acid and humin under different moisture treatments

| Treatment<br>(% WHC) | Chemical shift regions (ppm) |       |       |        |         |         |         | A/O-A | Alip/<br>Arom | HB/HI |
|----------------------|------------------------------|-------|-------|--------|---------|---------|---------|-------|---------------|-------|
|                      | 0–50                         | 50–60 | 60–95 | 95–110 | 110–145 | 145–160 | 160–200 |       |               |       |
| Humic acid           |                              |       |       |        |         |         |         |       |               |       |
| 30                   | 19.8                         | 9.72  | 11.5  | 4.77   | 29.9    | 6.71    | 17.7    | 0.76  | 1.25          | 1.29  |
| 60                   | 19.8                         | 10.2  | 10.9  | 5.68   | 29.7    | 7.47    | 16.2    | 0.74  | 1.25          | 1.32  |
| 250                  | 20.0                         | 9.69  | 10.3  | 4.43   | 31.2    | 7.88    | 16.4    | 0.82  | 1.14          | 1.45  |
| Average              | 19.9                         | 9.88  | 10.9  | 4.96   | 30.3    | 7.35    | 16.8    | 0.77  | 1.21          | 1.35  |
| Humin                |                              |       |       |        |         |         |         |       |               |       |
| 30                   | 17.0                         | 6.13  | 24.3  | 8.60   | 26.0    | 7.20    | 10.8    | 0.44  | 1.69          | 1.01  |
| 60                   | 18.2                         | 5.92  | 25.6  | 9.04   | 22.9    | 7.53    | 10.8    | 0.45  | 1.93          | 0.95  |
| 250                  | 20.4                         | 6.51  | 22.3  | 8.01   | 24.2    | 6.13    | 12.5    | 0.55  | 1.89          | 1.03  |
| Average              | 18.5                         | 6.19  | 24.1  | 8.55   | 24.4    | 6.96    | 11.3    | 0.48  | 1.83          | 0.99  |

WHC – field water-holding capacity; alkyl C/O-alkyl C =  $(0-50)/(50-110)$ ; aliphatic C/aromatic C =  $[(0-50) + (50-110)]/[(110-160) + (160-200)]$ ; hydrophobic C/hydrophilic C =  $[(0-50) + (110-160)]/[(50-110) + (160-200)]$

oxyl C, carbohydrate C, and di-O-alkyl C in the O-alkyl C region were in the order of 60% WHC > 30% WHC  $\approx$  250% WHC, 30% WHC > 60% WHC > 250% WHC, and 60% WHC > 30% WHC > 250% WHC, respectively. The aryl C and phenol C in the aromatic C region were in the order of 250% WHC > 30% WHC  $\approx$  60% WHC and 250% WHC > 60% WHC > 30% WHC, respectively. The carbonyl C was in the order of 30% WHC > 250% WHC  $\approx$  60% WHC. The above changes in relative intensity of the carbon functional groups resulted in the alkyl C/O-alkyl C ratio in the order of 250% WHC > 30% WHC  $\approx$  60% WHC, aliphatic C/aromatic C ratio in the order of 30% WHC  $\approx$  60% WHC > 250% WHC, and hydrophobic C/hydrophilic C ratio in the order of 250% WHC > 60% WHC  $\approx$  30% WHC. The results indicated that humic acid was more alkylated, more hydrophobic, and less aliphatic at a high moisture level than at the two other moisture levels.

The alkyl C for humin was in the order of 250% WHC > 60% WHC > 30% WHC. The methoxyl C in the O-alkyl C region was in the order of 250% WHC > 30% WHC > 60% WHC, and the carbohydrate C and di-O-alkyl C were in the order of 60% WHC > 30% WHC > 250% WHC. The aryl C and phenol C in the aromatic C region were in the order of 30% WHC > 250% WHC > 60% WHC and 60% WHC > 30% WHC > 250% WHC, respectively. The carbonyl C was in the order of 250% WHC > 30% WHC > 60% WHC. The above changes resulted in the alkyl C/O-alkyl C ratio in the order of 250% WHC > 30%

WHC  $\approx$  60% WHC, aliphatic C/aromatic ratio in the order of 60% WHC  $\approx$  250% WHC > 30% WHC, and hydrophobic C/hydrophilic C ratio in the order of 30% WHC  $\approx$  250% WHC > 60% WHC. These results indicated that humin was more alkylated and more aliphatic at a high moisture level than at a low moisture level, whereas the hydrophobicity degree was similar at the two moisture levels.

The above results showed that although soil moisture levels had a distinct effect on the chemical composition of humic acid and humin, the alkyl C/O-alkyl C ratios of the two humic substance fractions were all higher at a high moisture level than at a low soil moisture level. The O-alkyl C was generally considered as an easily bio-decomposable organic component. The greater value of the alkyl C/O-alkyl C ratio thereby indicates that soil organic matter was more decomposed (CHAVEZ-VERGARA *et al.* 2014; PANETTIERI *et al.* 2014). In our study, the larger alkyl C/O-alkyl C ratio of humic acid and humin at a high moisture level than at a low moisture level can explain their lower carbon contents in the former.

## CONCLUSION

This study has shown that the difference in soil moisture contents influenced the amounts and chemical composition of soil humic fractions. The increase in soil moisture was favourable for the accumulation of water soluble carbon within the range of the experimental moisture levels, whereas it resulted

in losses of humic fractions. Humic acid was more alkylated, less aliphatic, and more hydrophobic, whereas humin was more alkylated and more aliphatic at a high moisture level than at a low moisture level. The high water soluble carbon content and large decomposition degree of humic acid and humin can explain the low soil organic carbon content at a high moisture level against a low moisture level.

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