Specified Dosages of Biochar Application Not Impact Native Organic Carbon but Promote a Positive Effect on Native Humic Acid in Humicryepts Soil

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Abstract: Biochar is considered to have potential use in carbon (C) sequestration and has been widely used in soil amendment. Humic substances (HSs), assigned as the stable organic C, have obvious agronomic benefits. However, the response mechanisms of these carbonaceous substances to biochar are unclear in biochar-amended soils. In a two-year experiment, the δ13C technique was employed to trace the fate of the biochar-derived C in HSs and evaluate the effects of four treatments, including no biochar control (CK) and biochar addition at dosages of 6 t ha−1 (BC6), 12 t ha−1 (BC12), and 24 t ha−1 soil (BC24), on soil organic carbon (SOC) and HSs. Compared to CK, biochar application significantly improved total SOC contents and the C pool index, whereas the C labile index declined. Humic acid (HA) and humin were distinctly enhanced in bulk soil. Moreover, the alipheraticity was intensified in the chemical composition of HA. In particular, native HA contents substantially increased by 16.30–55.95%. Biochar-applied C of 4.08–6.43% was finitely involved in HA formation over the two years, which resulted in a genetic relationship between soil HA and biochar to some extent. The low dosages of biochar at 6 t ha−1, 12 t ha−1 and 24 t ha−1 did not obviously affect native SOC contents. Moreover, BC24 had less of an effect on HA formation compared to BC6 and BC12, but had the highest SOC. These results demonstrate that biochar application can improve SOC stocks, reduce C instability and promote HSs formation, and they suggest that determining and keeping an optimum dosage of biochar application can represent an effective strategy (i.e., not only sequestrate C, but also improve soil quality), which is beneficial to sustainability in the ecological environment and agriculture.

Keywords: biochar; humic substances; carbon sequestration; labile organic carbon; 13C isotope tracer

1. Introduction

The return of agricultural straw to the soil is still a predominant and effective practice for improving soil quality and recycling nutrients. It can increase carbon (C) input and nutrient element supply, decrease mineral fertilizer input, improve crop yield, and mitigate potential air pollution from in situ straw burning [1]. However, significant greenhouse gas emissions, especially CO2, can occur under circumstances where straw application increases the C inputs and C stocks [2–5], resulting in a higher global warming potential than conventional fertilization systems [6].

Biochar is defined as a carbon-rich solid product derived from agro-forestry biomass waste [7] such as energy crops (miscanthus, willow, etc.), forest waste (wood chip, sawdust,
etc.), agricultural waste (rape, sunflowers, crop straw, corn cobs, poultry litter, etc.) and sewage sludge [8–12]. It is produced through pyrolysis in an oxygen-limited environment and at relatively low temperatures (<700 °C) and is returned to farmland to promote soil quality and sequester C [7]. Due to abundant C and the highly aromatic C structure, biochar returning can increase soil organic carbon (SOC) stocks and mitigate greenhouse gas emissions [13], which begin to be considered as the ultimate C sink [14], and thus should be an effective and beneficial strategy for middle- and long-term soil management [15].

SOC pools possess the largest and most active C stock in terrestrial ecosystems, with a stock of 2400 Pg in the 2 m soil layer, which is 3.2 times as much as the atmosphere pool and 4.4 times that of the biotic pool [16]. Attributed to the enormous SOC pool, small changes in SOC stocks, particularly the unstable C pool, can greatly impact the concentration of carbon dioxide in the atmosphere and, consequently, global climate change [16,17]. Understanding the fundamental mechanisms of the storage and stabilization of SOC has thus garnered considerable attention. As for the stability mechanisms of SOC, the chemical protection provided by soil minerals through adsorbing C onto soil mineral surfaces, the physical protection of soil aggregates through occluding C into the aggregates, and biochemical protection through the biochemical transformation of C into products recalcitrant to microbial attack (e.g., humic substances (HSs)) may improve C stability and C sequestration [18]. Therefore, the classical humification theory supported by most scholars is one of the stability mechanisms of SOC [19–21]. HSs are traditionally treated as a proxy for soil organic matter [22] and their agronomic benefits are obvious. For instance, HSs favor the formation of soil aggregates, improve the retention of water and nutrients, enhance crop growth, and suppress crop disease [23]. These positive agronomic effects on soil quality are attributed to the functional groups of phenol and carboxylic acid owned by the HSs themselves [23]. HSs are heterogeneous complexes consisting of large macromolecules with functional groups formed by biochemical reactions [22]. HSs could be extracted from the soil according to their solubility with alkalis and acids and operationally categorized into “base-soluble/acid-insoluble humic acid (HA), acid/base soluble fulvic acid (FA), and insoluble humin (Hu)” [22]. As the major components of SOC, HSs account for more than 50% of SOC in soil [22]. Therefore, determining the responses of SOC and HSs to different dosages of biochar application can help us gain a deeper understanding of the mechanisms of SOC sequestration and agronomic effects in biochar-applied soil.

Biochar is generally considered the least bioavailable organic C due to its highly condensed aromatic structure [16]. In fact, biochar may be either more or less decomposed by biotic and abiotic factors [24–26]. Dissolved black carbon (black carbon is defined as a combustion product of biomass and fossil fuel, which includes biochar [14]) contributes to a substantial fraction (10.6%) of dissolved organic C fluxes in global rivers [27], suggesting its degradability as well. Biochar can be intensively oxidized under fresh organic substrate input [24,25] or abiotic factors such as temperature [28], which might constitute a mechanism of HSs formation and a potential source of highly aromatic constituents of HSs [29–31]. Biochar promotes the humification of soil organic matter [31] and the formation of HSs [32–35]. As a consequence, isotope tracer technology should be used to confirm whether the newly formed HSs are, in a way, derived from biochar and have a genetic relationship with biochar. This aspect is still unclear and suggests that another mechanism of SOC stabilization for biochar-applied soil might exist.

A two-year pot experiment was carried out using biochar from a C_4 source (maize straw) applied into the soil from a historical C_3 vegetation (soybean) using δ^{13}C, Fourier transform infrared (FTIR) spectroscopy, and elemental analysis technologies. The objectives of this study were to illuminate (i) the effects of biochar on SOC, labile C, HSs and native SOC contents; (ii) the influences of biochar on the formation and chemical composition of soil HA; and (iii) the genetic relationship between biochar and HA. We hypothesize that biochar application to the soil (i) improves the content and stability of SOC, (ii) promotes the formation and transformation of HSs, and (iii) causes the involvement of biochar-
derived C in HA formation, accordingly demonstrating the genetic relationship between biochar and soil HA.

2. Materials and Methods

2.1. Experimental Soil, Design, and Soil Sampling

A pot experiment was conducted in November 2015 by mixing different dosages of biochar derived from maize straw (Zea mays L., C₄ plant) into a soil historically planted with soybean (Glycine max (L.) Merr., C₃ plant) for more than 12 years. The difference in the natural abundance of ¹³C between the historical C₃ soil (δ¹³C of −24.59‰) and C₄ biochar (δ¹³C of −13.62‰) allowed us to trace the fate of the biochar-derived C in SOC [36,37].

Experimental soil: Applied soil in the pot experiment was gathered from the top soil layer (0–20 cm) in October 2015 in a national soybean breeding base set up in 2003 with continuous soybean monoculture, located in Heihe City, Heilongjiang province, China (50°15’ N, 127°27’ E). The soil has a clayey loam texture (45.07% sand, 36.63% silt, and 18.30% clay) and is classified as dark brown Alfisol soil (Chinese Soil Taxonomy) or Humicryperts, belonging to Inceptisols (USDA Soil Taxonomy), with a pH of 6.25. It contains an organic C of 22.33 g kg⁻¹, total nitrogen of 1.82 g kg⁻¹, total phosphorus of 0.33 g kg⁻¹, hydrolysable nitrogen of 338.33 mg kg⁻¹, and available phosphorus of 51.67 mg kg⁻¹ in the 0–20 cm soil layer. The soil was passed through a 5 mm sieve with stones and other impurities removed. Exactly 11 kg of sieved soil was put in a polyethylene bucket with the top diameter being 32 cm and the bottom diameter being 23 cm, with a height of 29 cm.

Experimental design and soil sampling: The pot experiment involved four treatments with three replicates (pots) per treatment, i.e., no biochar control (CK), and biochar addition at dosages of 6 t ha⁻¹ (BC6), 12 t ha⁻¹ (BC12), and 24 t ha⁻¹ soil (BC24), equivalent to application rates of 0%, 0.27%, 0.53%, and 1.07% (w/w), respectively. Biochar was applied to the above soil in a polyethylene bucket only once in early November 2015, and adopted dosages simulated the amounts of biochar applied into a 20 cm plough layer in farmland. Two maize seeds were sown per bucket in early May 2016 and 2017, respectively, and after two weeks one of the poor seedlings was uprooted. The aboveground parts of the plant and root of maize were removed in early October 2016 in order to mimic a growing season, and the experiment was terminated and soil sample was collected in October 2017. Three soil cores (5 cm diameter and 10 cm height) near the edge of each bucket were taken and then thoroughly mixed to form a single sample. Fresh soil samples were sieved to 5 mm and then air-dried for the analyses as required.

Annual fertilization rates were 0.1 g N kg⁻¹, 0.037 g P₂O₅ kg⁻¹, and 0.037 g K₂O kg⁻¹ soil, equivalent to 225 kg N ha⁻¹, 82.5 kg P₂O₅ ha⁻¹, and 82.5 kg K₂O ha⁻¹ soil. The batch buckets were placed in the natural environment of the teaching experimental field of Jilin Agricultural University in Jilin province, China (43°49′5″ N, 125°24′8″ E). Therein, the climate is temperate humid continental with an annual average temperature of 0 °C–6.7 °C, annual mean precipitation of 500–650 mm and a frost-free period of 135 days–140 days. Rainfall mainly occurs between July and September. The plant was watered every four days during growing season of maize when the weather was in drought.

2.2. Biochar Production and Properties

Biochar was produced from the maize straw by slow pyrolysis (elevated temperature at 100 °C every 3 h) with peak temperatures of 450 °C for 10 h in a vacuumized charring furnace with a subsequent N₂ environment. The biochar contained an organic C of 618.10 g kg⁻¹, total nitrogen of 7.80 g kg⁻¹, hydrogen of 31.64 g kg⁻¹, and oxygen and sulfur of 342.46 g kg⁻¹ (calculated by subtraction), determined by a VARIO EL-III Elemental Analyzer (Elementar Analysensysteme GmbH Inc., Frankfurt, Germany), and total phosphorus of 1.31 g kg⁻¹ and pH of 9.67. The biochar had a humic-like acid value of 0.31 g kg⁻¹.
2.3. Measurements of Labile Organic C

Labile organic C including microbial biomass C (MBC), easily oxidizable organic C (EOC), Water-soluble organic C (WSOC), and dissolved organic C (DOC) in bulk soil were extracted and measured as follows:

MBC was measured using the chloroform fumigation and extraction method [38], and EOC was determined by the KMnO$_4$ (333 mM) oxidation method [39]. WSOC and DOC were extracted with distilled water and K$_2$SO$_4$, respectively [40].

2.4. Extraction, Fractionation, and Purification of HSs

Generally, biochar includes an acid-precipitated fraction of higher molecular weight and aromaticity and an acid-soluble fraction of lower molecular weight and aromaticity [41,42], which were generated during pyrolysis. They are regarded as a humic-like acid and fulvic-like acid of biochar, respectively, because they show remarkable similarities to highly aromatic soil HA and relatively low aromatic FA in their spectroscopic properties and chemical compositions [33]. To distinguish humic-like C owned by the undecomposed biochar from soil HSs, soil HSs were extracted and fractionated after the density flotation of a heavy fluid. The free light fractions (fLFs) were removed from the soil samples firstly by NaI solution (d = 1.79 g cm$^{-3}$) and then the occluded light fractions (oLFs) protected within the soil structure were separated through the dispersion of the ultrasonic and flotation of the NaI solution. fLFs and oLFs were gathered. The residuals were the heavy fractions. Next, the heavy fractions were extracted using a solution of 0.1 M NaOH and 0.1 M Na$_4$P$_2$O$_7$. The alkaline supernatants were acidified to pH 1 with 0.5 M H$_2$SO$_4$ for precipitating the acid-insoluble HA from acid-soluble FA. The alkali-insoluble solid residuals were Hu.

For $\delta^{13}$C mass spectrometry and FTIR analysis, HA was extracted and purified by deashing according to the procedure recommended by the International Humic Substances Society. Briefly, the bulk soil samples were extracted with 1 M HCl at soil to liquid ratio of 1:1 and then adjusted to soil to liquid ratio of 1:10 with 0.1 M HCl. Solid residues were extracted three times by 0.1 M NaOH (soil: liquid = 1:10) under an N$_2$ atmosphere, and subsequently the dark-colored supernatant was acidified to pH 1 three times with 6 M HCl to flocculate the HA. The HA precipitate was redissolved using 0.1 M KOH until it contained 0.3 M K$^+$ with solid KCl addition. After acidizing with 6 M HCl again, mineral impurities were repeatedly removed from the HA precipitate using a mixture of 0.1 M HCl and 0.3 M HF (<1% in ash) three times. The HA precipitate was further dialyzed and freeze-dried afterward.

2.5. Organic C Measurement

The contents of total soil organic C in bulk soil were measured by a VARIO EL-III Elementary Analyzer (Elementar Analysensysteme GmbH Inc., Frankfurt, Germany), and Hu was in the heavy fractions as well. The contents of HA and FA (liquid) in the heavy fractions were determined using a TOC-V CPH analyzer (Shimadzu Company, Tokyo, Japan). The elementary composition (C, H, and N) of purified solid HA was determined using a VARIO EL-III Elemental Analyzer, and the O content was calculated by subtraction.

2.6. Calculation in C Management Index

The C Management Index (CMI) is an indication of the changes in the C dynamics of agriculture and ecosystem when a new practice is applied, which may assess the capacity of management systems in promoting soil fertility. The related calculation of CMI by Equations (1)–(4), from Blair et al. [39] and Dixit et al. [43], is as follows:

$$CL = \frac{EOC}{SOC - EOC}$$

(1)

$$CLI = \frac{CL_{tr}}{CL_{ck}}$$

(2)

$$CPI = \frac{SOC_{tr}}{SOC_{ck}}$$

(3)
\[ CMI = CPI \times CLI \times 100 \] (4)

Above, CL represents C lability; EOC is easily oxidizable organic C by KMnO₄ (333 mM); CLI denotes the C lability index; CL₁₀ and CLck are regarded as C lability for adding biochar and no biochar control (CK) treatments, respectively; CPI represents the C pool index; SOC₁₀ and SOCck are the total SOC contents for adding biochar and CK treatments, respectively; CMI represents the C management index.

2.7. δ¹³C Analysis

Biochar-derived C in the total SOC and HA was directly estimated from the δ¹³C (the ¹³C isotope signature) of the corresponding biochar application treatment (δ¹³C₁₀) using mixing Equations (5)–(7):

\[ f = (\delta^{13}C_{\text{tr}} - \delta^{13}C_{\text{ck}})/(\delta^{13}C_{\text{BC}} - \delta^{13}C_{\text{ck}}) \] (5)

with f (%) being the proportion of organic C derived from biochar in the sample, δ¹³C₁₀ and δ¹³Cck indicating the ¹³C isotope signature of the sample (bulk soil or HA) from biochar addition and CK treatments, respectively, δ¹³CBC representing the ¹³C isotope signature of the maize straw biochar itself with δ¹³C values of −13.62‰.

If the total SOC and HA contents are known, the amount of biochar-derived C (Biochar-C) in total SOC or total HA can be calculated by Equation (6), and native-C (i.e., native SOC or native HA), which excludes biochar-C through Equation (7):

\[ \text{Biochar-C} = tOC \times f \] (6)

\[ \text{Native-C} = tOC \times (1 - f) \] (7)

with tOC being the total SOC or total HA contents (g kg⁻¹).

The efficiency of transforming biochar-derived C applied to the soil into HA (EHA%) was calculated by Equation (8):

\[ EHA = \frac{\text{Biochar-C}_{\text{BC}}}{\text{BC}_{\text{tr}}} \times 100 \] (8)

with Biochar-C being the amount of biochar-derived C in total HA and BC₁₀ representing the initial C amount of biochar applied to the soil. Here, C amount of biochar applied to the soil in BC6, BC12 and BC24 were 1.65 g C kg⁻¹, 3.29 g C kg⁻¹ and 6.60 g C kg⁻¹ soil, respectively.

The δ¹³C values in the samples were determined by an Isoprime 100 Mass Spectrometer (Elementar Analysensysteme GmbH Inc., Frankfurt, Germany).

2.8. FTIR Spectra Analysis

The FTIR absorption spectra of purified solid HA were recorded on an AVATAR 360 FTIR spectrometer (NICOLET, ThermoFisher Scientific, Waltham, MA, USA), covering a scan range of wave numbers from 4000 to 400 cm⁻¹. Sixteen scans were averaged with a resolution 4 cm⁻¹, and pure potassium bromide (KBr) spectra were used as a background. The relative intensity of the absorption peaks from HA was calculated using Omnic Version 8 (NICOLET, ThermoFisher Scientific, Waltham, MA, USA).

2.9. Statistics

Statistical analyses were conducted using SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) with least significant difference (LSD) tests was applied to test for significance (\( p < 0.05 \)) among treatments. Redundancy analysis (RDA) was performed to assess the relationships between stable- and labile-organic C using CANOCO 5 (CANOCO, Microcomputer Power Inc., Ithaca, NY, USA).
3. Results

3.1. Total SOC, Labile Organic C and CMI

With regard to the labile organic C, according to Figure 1, the WSOC amounts (104.28 mg kg\(^{-1}\)–131.52 mg kg\(^{-1}\)) extracted by deionized water were observed to be similar to DOC (104.29 mg kg\(^{-1}\)–131.47 mg kg\(^{-1}\)) by K\(_2\)SO\(_4\), whereas the EOC amounts (0.80 g kg\(^{-1}\)–1.65g kg\(^{-1}\)) oxidized by K\(_2\)MnO\(_4\) were much greater than WSOC and DOC throughout all treatments. The smallest contents of WSOC and DOC were found in BC12 (\(p < 0.05\)). The EOC contents from BC6 and BC12 were observably lower than that of CK by 20.88% and 47.55% (\(p < 0.05\)), respectively, and were in the order BC24 (CK) > BC6 > BC12. The MBC contents in BC6, BC12 and BC24 were 6.17, 1.89, and 5.91 times higher than that measured in CK (\(p < 0.05\)) in CLI, with the sharpest decrease observed in BC12. For CMI, in addition to BC24 not differing significantly from CK, BC6 and BC12 declined significantly by 22.29% and 49.73% (\(p < 0.05\)).

![Figure 1. Contents of labile organic C (OC) including water-soluble organic C (WSOC), dissolved organic C (DOC), microbial biomass C (MBC), and easily oxidizable organic C (EOC) under different dosages of biochar application. CK, no biochar control; BC6, BC12, and BC24, biochar addition at a dosages of 6 t ha\(^{-1}\), 12 t ha\(^{-1}\), and 24 t ha\(^{-1}\), respectively. Different lower-case letters indicate significant differences (\(p < 0.05\)) among treatments.](image)

### Table 1. Carbon Management Index under different dosage biochar applications.

| Treatment | C Lability | C Lability Index | C Pool Index | C Management Index (%) |
|-----------|------------|-----------------|--------------|------------------------|
| CK        | 0.081 ± 0.007 a | 1.00 ± 0.00 a    | 1.00 ± 0.00 c | 100.00 ± 0.00 a        |
| BC6       | 0.053 ± 0.008 b | 0.66 ± 0.10 b    | 1.17 ± 0.07 b | 77.21 ± 12.39 b        |
| BC12      | 0.033 ± 0.006 c | 0.41 ± 0.08 c    | 1.23 ± 0.07 ab | 50.27 ± 10.90 c        |
| BC24      | 0.065 ± 0.007 b | 0.81 ± 0.09 b    | 1.33 ± 0.08 a | 106.96 ± 16.56 a       |

Notes: CK, no biochar control; BC6, BC12, and BC24, biochar addition at a dosages of 6 t ha\(^{-1}\), 12 t ha\(^{-1}\), and 24 t ha\(^{-1}\), respectively. Data are means ± SE (\(n = 3\)). Different lower-case letters indicate significant differences (\(p < 0.05\)) among treatments.
As for the total SOC contents in bulk soil, BC6, BC12, and BC24 treatments significantly increased them by 16.51%, 22.73%, and 32.39%, respectively (p < 0.05), compared with CK, and in the order BC24 > BC12 (BC6) > CK (Table 2).

### Table 2. Soil δ^{13}C values, the contents of total soil organic carbon (tSOC), biochar-derived C and native SOC.

| Treatment | tSOC  (g kg\(^{-1}\)) | Soil δ^{13}C (‰) | f (%) | 1− f (%) | Biochar-C  (g kg\(^{-1}\)) | Native SOC  (g kg\(^{-1}\)) |
|-----------|----------------|------------------|-------|-----------|----------------|------------------|
| CK        | 20.43 ± 1.03 c | −24.11 ± 0.17 d | /     | 100 a     | 20.43 ± 1.03 a |                   |
| BC6       | 23.80 ± 1.61 b | −23.42 ± 0.03 c | 6.54 ± 0.29 c | 93.46 ± 0.29 b | 1.55 ± 0.04 c | 22.25 ± 1.41 a |
| BC12      | 25.07 ± 1.24 b | −22.90 ± 0.13 b | 11.56 ± 1.22 b | 88.44 ± 1.22 c | 2.91 ± 0.46 b | 22.16 ± 0.94 a |
| BC24      | 27.05 ± 1.46 a | −21.60 ± 0.43 a | 23.89 ± 4.12 a | 76.11 ± 4.12 d | 6.42 ± 0.73 a | 20.63 ± 2.28 a |

Notes: CK, no biochar control; BC6, BC12, and BC24, biochar addition at a dosages of 6 t ha\(^{-1}\), 12 t ha\(^{-1}\), and 24 t ha\(^{-1}\), respectively. f, percentage of biochar-derived C of total SOC; 1− f, percentage of native SOC of total SOC; Biochar-C, the contents of biochar-derived C. Data are means ± SE (n = 3). Different lower-case letters indicate significant differences (p < 0.05) among treatments.

#### 3.2. HSs Distributed in Soil Heavy Fractions

HSs, including HA, FA, and Hu, were extracted from the heavy fractions of the soil. The contents of HA, FA and Hu accounted for 17.72–24.98%, 20.10–27.78% and 29.72–32.60% of total SOC (Figure 2), respectively. Noteworthy increments of 38.69% and 60.62% in HA contents were found in BC6 and BC12 treatment, for CK treatment, SOC was decomposed by 8.51% over two years. For biochar application, BC24 caused a decomposition rate of 7.63% of the native SOC, whereas BC6 and BC12 merely reduced native SOC by 0.37% and 0.74%, negligibly over two years.

**Figure 2.** Humic C contents from humic acid (HA), fulvic acid (FA), and humin (Hu) in soil heavy fractions and their percentage of total SOC under different dosages of biochar application. CK, no biochar control; BC6, BC12, and BC24, biochar addition at a dosages of 6 t ha\(^{-1}\), 12 t ha\(^{-1}\), and 24 t ha\(^{-1}\), respectively. Different lower-case letters indicate significant differences (p < 0.05) among treatments. Vertical bars denote standard deviation of the means (n = 3).

#### 3.3. δ^{13}C Analyses of SOC and HA

Compared with CK, the BC6, BC12 and BC24 treatments notably improved the soil δ^{13}C values by 2.85%, 5.03% and 10.39% in the order BC24 > BC12 > BC6 > CK (p < 0.05) (Table 2). The percentage of biochar-derived C of total SOC under biochar application ranged from 6.54% to 23.89% and was clearly enhanced with the increase in biochar dosage. It is noteworthy that BC6 and BC12 treatments caused increases of 8.90% and 8.49% of the native SOC contents, but with no significant increasing rates, the same as BC24 (Table 2). Moreover, relative to initial SOC content (22.33 g kg\(^{-1}\)) at the beginning of the present experiment, for CK treatment, SOC was decomposed by 8.51% over two years. For biochar application treatments, BC24 caused a decomposition rate of 7.63% of the native SOC, whereas BC6 and BC12 merely reduced native SOC by 0.37% and 0.74%, negligibly over two years.
As shown in Table 3, compared with CK, the δ^{13}C values of HA in BC6, BC12 and BC24 were significantly improved by 0.91%, 1.44% and 2.59%, respectively, and were in the order BC24 > BC12 (BC6) > CK (p < 0.05), suggesting the involvement of biochar in HA formation. Indeed, smaller percentages of biochar-derived C of HA were 1.96%, 3.13% and 5.61%, respectively, in the order BC24 > BC12 (BC6). Accordingly, the amounts of biochar-derived C of 0.11 g kg\(^{-1}\), 0.20 g kg\(^{-1}\) and 0.27 g kg\(^{-1}\) in HA were obtained in BC6, BC12 and BC24, with significant differences in the order of BC24 > BC12 > BC6 (p < 0.05). Based on the input C from the applied biochar, the biochar-applied C of 6.43%, 5.95% and 4.08% in BC6, BC12 and BC24 were transformed into HA, respectively, with the lowest BC24 in the two-year experiment. Notably, BC6, BC12 and BC24 significantly increased the amounts of native HA by 36.49%, 55.95% and 16.30% relative to CK (p < 0.05), respectively, which fully demonstrates the decrease in mineralization rate of HA or the formation of HA caused by biochar application. However, BC24 resulted in the smallest increasing rate of native HA relative to BC6 and BC12 treatments.

### Table 3. δ^{13}C values of humic acid (HA), the content of biochar-derived C in HA, and native HA content.

| Treatment | δ^{13}C of HA (%) | f (%) | 1–f (%) | Biochar-C (g kg\(^{-1}\)) | Native HA (g kg\(^{-1}\)) | EHA (%) | ΔRHA (%) |
|-----------|-------------------|-------|---------|---------------------------|---------------------------|--------|---------|
| CK        | −25.31 ± 0.11 c   | /     | 100 a   | 3.90 ± 0.31 d /           |                           |        |         |
| BC6       | −25.08 ± 0.08 b   | 1.96 ± 0.05 b | 98.04 ± 0.05 b | 0.11 ± 0.02 c | 5.30 ± 0.03 b | 6.43 ± 1.82 a | 36.49 ± 11.04 a |
| BC12      | −24.94 ± 0.12 b   | 3.13 ± 1.06 b | 96.88 ± 1.06 b | 0.20 ± 0.06 b | 6.06 ± 0.07 a | 5.95 ± 1.51 a | 55.95 ± 10.53 a |
| BC24      | −24.65 ± 0.05 a   | 5.61 ± 0.37 a | 94.39 ± 0.37 c | 0.27 ± 0.01 a | 4.52 ± 0.02 c | 4.08 ± 0.27 b | 16.30 ± 8.46 b |

Notes: CK, no biochar control; BC6, BC12, and BC24, biochar addition at dosages of 6 t ha\(^{-1}\), 12 t ha\(^{-1}\), and 24 t ha\(^{-1}\), respectively. f, percentage of biochar-C of total HA; 1–f, percentage of native HA of total HA. EHA, efficiency of transforming biochar-applied into HA; ΔRHA, increasing rates of native HA from biochar treatments relative to CK. Data are means ± SE (n = 3). Different lower-case letters indicate significant differences (p < 0.05) among treatments.

3.4. Elementary and FTIR Spectra Analyses on HA

In comparison to CK, biochar application had no effect on the contents of C, N, O and the O/C ratio of HA (Table 4), but significantly increased the H content and the H/C ratio by 3.94–5.73% and 4.95–5.69%, indicating a decline in the condensation degree of the HA.

### Table 4. Elementary composition of humic acid under different dosages of biochar application.

| Treatment | Element Content (g kg\(^{-1}\)) | Molar Ratio |
|-----------|-------------------------------|-------------|
|           | C    | H    | O    | N    | O/C | H/C |
| CK        | 571.10 ± 4.23 a | 42.94 ± 1.23 b | 355.23 ± 6.47 a | 30.76 ± 1.52 a | 0.47 ± 0.012 a | 0.90 ± 0.02 b |
| BC6       | 565.57 ± 1.24 a | 44.63 ± 0.64 ab | 357.23 ± 1.60 a | 32.60 ± 0.41 a | 0.47 ± 0.003 a | 0.95 ± 0.01 a |
| BC12      | 572.40 ± 1.22 a | 45.40 ± 1.37 a | 351.03 ± 2.26 a | 31.17 ± 1.90 a | 0.46 ± 0.002 a | 0.95 ± 0.03 a |
| BC24      | 569.37 ± 5.35 a | 45.25 ± 0.96 a | 354.53 ± 6.11 a | 30.85 ± 0.64 a | 0.47 ± 0.013 a | 0.95 ± 0.02 a |

Notes: CK, no biochar control; BC6, BC12, and BC24, biochar addition at dosages of 6 t ha\(^{-1}\), 12 t ha\(^{-1}\), and 24 t ha\(^{-1}\), respectively. Data are means ± SE (n = 3). Different lower-case letters indicate significant differences (p < 0.05) among treatments.

The FTIR spectra of HA are shown in Figure 3. Based on the literature [28,44,45], the main absorption peaks of HA included O–H stretching and hydrogen-bonded OH at a wavenumber of 3400 cm\(^{-1}\), asymmetric and symmetric C–H stretching vibrations of the CH\(_2\) group at 2920 cm\(^{-1}\) and 2850 cm\(^{-1}\), respectively, asymmetric C=O stretching of carboxyl at 1720 cm\(^{-1}\), aromatic C=C skeletal vibrations at 1620 cm\(^{-1}\), O–H deformation and C–O stretching of phenolic OH at 1400 cm\(^{-1}\), and stretching of aromatic C–O and phenolic OH at 1230 cm\(^{-1}\).
The relative intensities of these peaks from HA are presented in Table 5. Aliphatic hydrocarbon-C at 2920 cm\(^{-1}\) of 4.37–6.52% in the peak intensity, carboxyl-C at 1720 cm\(^{-1}\) of 16.15–20.15%, aromatic-C at 1620 cm\(^{-1}\) of 12.74–14.8%, and phenolic-C at 1400 and 1230 cm\(^{-1}\) of 34.30–40.02% were observed throughout all absorption bands. In comparison to CK, the BC6, BC12 and BC24 treatments markedly improved the aliphatic hydrocarbon-C by 42.79%, 48.74% and 49.20%, respectively, in the order BC24 (BC12) > BC6 > CK, with carboxyl-C observably increasing by 13.68–24.77% as well \((p < 0.05)\). Though aromatic-C under biochar application was significantly enhanced by 10.13–16.17% relative to CK, the ratio of I_{2920}/I_{1620}, reflecting the intensity of the ratio of aliphatic-C to aromatic-C, distinctly increased as well, suggesting improved aliphaticity in the HA structure by biochar application.

### Table 5. Relative intensity of the main absorption peaks of FTIR spectra of humic acid under different dosages of biochar application.

| Treatment | 3400 cm\(^{-1}\) | 2920 cm\(^{-1}\) | 2850 cm\(^{-1}\) | 1720 cm\(^{-1}\) | 1620 cm\(^{-1}\) | 1400 cm\(^{-1}\) | 1230 cm\(^{-1}\) | I_{2920}/I_{1720} | I_{2920}/I_{1620} |
|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| CK        | 3.21 ± 0.16 a   | 4.37 ± 0.16 a   | 0.73 ± 0.06 b   | 16.75 ± 0.20 a  | 12.74 ± 0.23 b  | 8.67 ± 0.71 b   | 25.62 ± 0.58 b  | 0.32 ± 0.00 b   | 0.40 ± 0.01 b   |
| BC6       | 3.65 ± 0.21 a   | 6.24 ± 0.09 b   | 0.65 ± 0.09 ab  | 18.36 ± 0.69 a  | 10.00 ± 0.55 a  | 10.49 ± 0.69 ab | 27.51 ± 0.71 ab | 0.36 ± 0.01 a   | 0.50 ± 0.02 a   |
| BC12      | 3.88 ± 0.37 a   | 6.50 ± 0.11 a   | 0.86 ± 0.06 ab  | 19.71 ± 0.75 a  | 14.36 ± 0.44 a  | 10.82 ± 0.43 ab | 29.23 ± 1.01 a  | 0.37 ± 0.02 a   | 0.51 ± 0.02 a   |
| BC24      | 4.01 ± 0.86 a   | 6.52 ± 0.04 a   | 1.02 ± 0.11 a   | 20.15 ± 0.95 a  | 14.00 ± 0.89 a  | 10.96 ± 0.97 a  | 28.62 ± 1.75 a  | 0.37 ± 0.02 a   | 0.50 ± 0.04 a   |

Note: I_{2920}/I_{1720} = (2920 + 2850)/1720; I_{2920}/I_{1620} = (2920 + 2850)/1620. CK, no biochar control; BC6, BC12, and BC24, biochar addition at a dosages of 6 t ha\(^{-1}\), 12 t ha\(^{-1}\), and 24 t ha\(^{-1}\), respectively. Data are means ± SE \((n = 3)\). Different lower-case letters indicate significant differences \((p < 0.05)\) among treatments.

#### 3.5. Redundancy Analyses of Stable- and Labile-Organic C

The full RDA model accounted for 61.67% of the total variation from the CK, BC6, BC12 and BC24 treatments, and the eigenvalues of axis 1 and axis 2 of the RDA biplot explained 50.94% and 9.52% of the total variation (Figure 4). RDA showed that the native SOC (nSOC), total HA (tHA), and native HA (nHA) were all significantly negatively correlated with labile organic C, including WSOC, DOC and EOC, but positively correlated with MBC, with a distinct difference between biochar application and CK treatment (Figure 4). Moreover, EOC, nHA and tHA had the longest vectors, suggesting that the change of EOC had a considerable effect on HA content due to biochar application.
Table 5. Relative intensity of the main absorption peaks of FTIR spectra of humic acid under different dosages of biochar

| Biochar Dosage | Relative Intensity (%) |
|---------------|------------------------|
|              | I2920/I1720            | I2920/I1620            |
| BC24         | 4.01 ± 0.89 a          | 1.02 ± 0.11 a         |
| BC12         | 3.88 ± 0.37 a          | 0.86 ± 0.06 ab        |
| BC6          | 3.63 ± 0.21 a          | 0.82 ± 0.09 ab        |
| CK           | 3.21 ± 0.16 a          | 0.73 ± 0.08 b         |

Letters indicate significant differences (p < 0.05) among treatments.

Note: $I_{2920}/I_{1720} = (2920 + 2850)/1720$; $I_{2920}/I_{1620} = (2920 + 2850)/1620$. CK, no biochar control; BC6, BC12, and BC24, biochar addition at a dosages of 6 t ha$^{-1}$, 12 t ha$^{-1}$, and 24 t ha$^{-1}$, respectively. tSOC, total soil organic C; nSOC, native soil organic C; tHA, total humic acid; nHA, native humic acid; Hu, humin; WSOC, water-soluble organic C; DOC, dissolved organic C; MBC, microbial biomass C; EOC, easily oxidizable organic C.

Figure 4. Redundancy analysis (RDA) of the correlations between labile organic C (including MBC, EOC, WSOC, and DOC) and SOC; humic C is based on the synthesis of CK, BC6, BC12, and BC24 treatments. CK, no biochar control; BC6, BC12, and BC24, biochar addition at a dosages of 6 t ha$^{-1}$, 12 t ha$^{-1}$, and 24 t ha$^{-1}$, respectively.

4. Discussion

4.1. Effects of Different Dosages of Biochar Application on the Total SOC and CMI

Compared to CK, different dosages of biochar application into the soil markedly increased the SOC contents (Table 2). This is mostly attributed to the natively recalcitrant properties of biochar due to its high aromaticity [7] and promotion of the physical protection of SOC by biochar application (e.g., adsorption or aggregate protection) [18,26,46,47]. Additionally, labile C was also possibly transformed into stable C in the present study (see Section 4.3), which can maintain SOC for a long time, and contribute to sequestrating C and mitigating climate change.

The SOC pools consist of labile C, slow C and passive C, based on their sensitivity to external factors and turnover rate [48]. Given the important functions of SOC in improving soil fertility, C sources and C sinks, its labile C pool is of great importance in evaluating soil fertility and the C pool balance between the C loss and C accumulation [39,49] due to labile C changes relatively quickly with alterations in soil management [43]. Therefore, CLI, CPI and CMI were established by Blair et al. [39] to assess the rate of change in soil C dynamics of agriculture and ecosystems when a new practice is introduced, and so were indicators estimating labile C pool and the capacity of management systems in promoting soil fertility. In the present study, a significant decline in CLI and an obvious improvement in CPI (Table 1) suggest that biochar application can weaken the instability of the soil C pools, which is responsible for C sequestration. Compared with BC6, BC12 and BC24, BC24 had the highest CMI, CLI and CPI due to the high rate of biochar application. This suggests that if biochar rate increases to a certain extent, a larger labile C input might have a positive impact on greenhouse gas emissions while soil fertility improves.
4.2. Effect of Different Dosages of Biochar Application on Native SOC

Though it appears that BC6 and BC12 treatments tend to have a negative priming effect due to improvements in the native SOC contents, the difference is nonsignificant relative to CK (Table 2). Therefore, BC6, BC12 and BC24 have no notable impact on the native SOC contents. Our present result is supported by a few studies [50, 51]. Most studies have documented that adding biochar to the soil reduces the mineralization of SOC, which has a negative priming effect on native SOC [37, 52–54]. However, biochar also causes positive priming of SOC [55, 56]. In addition, input of labile organic materials at a high rate has a negative [57] or a positive [25] effect on native SOC in biochar-amended soil. Nevertheless, a positive priming might occur in the short term with young biochar addition [58, 59], and a negative priming should take place over the long term, thereby sequestrating C [60]. So the effect of biochar on SOC mineralization is quite diverse. Factors such as experimental conditions and time scale, soil physicochemical properties (such as soil aggregates, organic C level, pH, and bulk density), the feedstock type, pyrolysis temperature of biochar, and application dose of biochar (see Section 4.4) probably result in the controversial conclusions discussed above. Thus, it can be seen biochar interacts with SOC in a complex way. Biochar addition may induce a positive priming effect on native SOC in the soils richer in organic C level [61]. Biochar application possibly impacts microbial functions and community structure through altering the physicochemical properties of the soil [61], but this largely depends on the biochar application dosages and biochar types. Due to its intrinsic recalcitrant character, biochar could not provide sufficient labile C or N substrates, and microorganisms may not be stimulated when the biochar application rate is not enough to affect the soil. Only when the application rates of biochar are high enough to significantly alter soil moisture retention capacity, pH conditions, and/or nutrient concentrations can the changes in microbial functions associated with metabolism of organic C mineralization occur [61]. Again, biochar application could promote physical protection of SOC by macro- and micro-aggregate formations via organo-mineral interactions [18, 53, 54] or direct adsorption to C [46] and improved HA and Hu (regarded as stable C) (Figure 2), which is beneficial to the stability and accumulation of SOC. Perhaps this offsets mineralized C resulting from microorganisms. Of course, the effects of different dosages of biochar application on native SOC need to be further researched based on the long-term field evidence.

4.3. Effects of Different Dosages of Biochar Application on Biochar-Derived HA and Native HA

A considerable increase of 22.82–60.62% in HA contents in the biochar-applied soil suggested that biochar application was beneficial to HA formation (Figure 2), which corroborates previous studies [31, 32, 34]. Biochar with abundant aromatic C is regarded as the source of highly aromatic structural components in soil HSs [14, 30, 31]. However, it needs to be clarified whether the newly formed HA is derived from biochar or not, and whether it has a genetic relationship with biochar or not. In the present study, the percentage of biochar-derived C of HA (1.96–5.61%) was smaller (Table 3), and only 4.08–6.43% of organic C from the biochar inputs were transformed into HA with the lowest BC24 over two years. According to the FTIR spectra of HA, additionally, a significant increase in aromatic-C at 1620 cm\(^{-1}\) under biochar application (Table 5) should be derived from the chemical precursors provided by the biochar and the promotion of aromatic polymer formation [14, 30, 31]. These results based on \(^{\delta}^{13}\)C and FTIR spectra analysis powerfully clarified the involvement of biochar in HA formation and the genetic relationship between soil HA and biochar. Although biochar is considered to be chemically stable, some studies have shown that its surface functional groups are gradually or rapidly oxidized with changes in chemical element composition and nonaromatic structure with its aging in the soil [62–65]. Oxidation and depolymerization of biochar might constitute a mechanism of HA formation [22, 29, 66, 67].

Interestingly, BC6, BC12 and BC24 significantly increased the contents of native HA by 36.49%, 55.95% and 16.30% in comparison to CK \((p < 0.05)\) (Table 3), respectively, which
suggests that biochar application indirectly promoted the formation of stable organic C. Generally, HA formation involves the transformation of biologically decomposable organic matter into a relatively stable HSs [68], which might be largely attributed to the effects of biochar on microorganisms. Biochar may provide a suitable habitat for microorganisms due to its abundant microporosity, large specific surface area, and strong sorptive capacity, resulting in microbial growth [68,69]. Moreover, active organic C in the soil and a few labile fractions from the biochar are the resources required for microbial activity [33]. Previous studies have suggested that biochar increases microbial activity, biomass C and N [70], and bacterial diversity indexes and richness [71]. It is possible, therefore, that labile organic C is decomposed and then partly transformed into HA by special microbial species in the soil [33]. In the present study, MBC increased dramatically, whereas other labile C, such as WSOC from BC12, and DOC and EOC from BC6 and BC12, distinctly decreased (Figure 1). Where is the destination of labile C in biochar-applied soil? First, leaching might induce the loss of labile C. The increase in pH due to biochar amendment results in a higher solubility and desorption of dissolved organic matter from mineral sites and is accordingly followed by the release and leaching of labile C from the soil [49,72,73]. Second, biochar application possibly stimulates the mineralization of labile organic C in soils [55], as biochar provides some unstable substrates and a suitable habitat for microorganisms. However, there is a distinct possibility that labile organic C could be microbially transformed into stable organic C, while microbial activity is concurrently promoted due to available substrates and a suitable habitat provided by the biochar. In fact, in the present study, a significant decline of EOC from both BC6 and BC12 (except BC24), concurrently, a dramatic improvement in MBC (Figure 1), followed by a high rate of increase in native HA in BC6, BC12 and BC24 from δ13C analysis (Table 3), seem to support this hypothesis. Moreover, H content in elementary composition of HA (Table 4), together with aliphatic hydrocarbon-C at 2920 cm\(^{-1}\) and 2850 cm\(^{-1}\) and carboxyl-C at 1720 cm\(^{-1}\) in FTIR (Table 5) were all significantly improved. They should originate from labile organic C in the soil. According to Zhang et al. [74], when easily soluble organic C is aggregated it can produce many aliphatic compounds. Therefore, the improvement in the aliphaticity in the HA structure likely demonstrates that biochar application potentially promotes the transformation of labile organic C into HA, which is supported by previous studies [1,8,33,75].

The RDA also showed that total HA and native HA were significantly negatively correlated with labile organic C, including WSOC, DOC and EOC, but were positively correlated with MBC, with a distinct difference between biochar application and CK (Figure 4). These results further confirmed that unstable C could be transformed into stable C under biochar application. Moreover, our results might also be explained by Huang et al. [1]. Huang et al. [1] suggested that biochar improved the linkage between rhizosphere bacteria and soil organic matter structure, resulting in a decrease in labile C with an increase in stable C. Orlova et al. [31] showed that increased HA could be attributed to the mineralization and humification of the more insoluble organic compounds and the humification of biochar itself, which coincides with our results.

4.4. Differences in the Effects of Three Dosages of Biochar Application on SOC and HA

In the present study, several variables did not show a linear response to the increasing dosage of biochar application. BC6 and, particularly, BC12 significantly reduced EOC (Figure 1) and improved biochar-derived HA and native HA (Table 3), further increased total HA (Figure 2), with the highest native or total HA detected under BC12, yet not BC24, which suggested that BC6 and BC12 might favor the formation of soil HA, thereby improving soil quality and the stabilization of SOC. Compared to BC6 and BC12, BC24 had the highest SOC, EOC, Hu, CLI, CPI and CMI, the mineralization rate of native SOC as well, but the lowest increasing rate in native HA. This indicates that while biochar addition rate was increased to twenty-four tons per hectare, SOC stocks and soil fertility were improved, but HA formation was possibly inhibited. Moreover, the effects of low/high rates of biochar addition on native SOC and soil HSs need to be further evaluated, because
they are closely related to soil biological properties. The application and doses of biochar impact microbial functions and associated metabolisms of organic C mineralization in the soil through altering the physicochemical properties of the soil [61]. The functions and community structure of microorganisms are only changed when the application dosage of biochar is high enough to distinctly alter soil bioavailable moisture dynamics, pH, and available C, N and P substrates [61], which may further affect soil C and N dynamics. High application dosages of biochar, for example, 50 t ha\(^{-1}\) vs. 0 t ha\(^{-1}\), 10 t ha\(^{-1}\) and 30 t ha\(^{-1}\) for Luo et al. [76] and 48 t ha\(^{-1}\) vs. 0 t ha\(^{-1}\) and 24 t ha\(^{-1}\) for Wang et al. [77], have greater potential to increase fungal than bacterial diversity due to the decrease in bacteria/fungi ratios, even though both bacteria and fungi in the abundance are significantly enhanced under biochar application. It is well-known that fungi are the dominant decomposers of recalcitrant C, which more beneficial to soil organic C degradation [77]. In addition, the abundance of nitrifying bacteria was raised and strong positive correlations were found in \(\text{Rhizobium} (r = 0.99)\) and \(\text{Azospirillum} \) abundance \((r = 0.70)\), with increased biochar dosages at 0%, 2.5%, 5% and 10% w/w; biochar loading of 10% even went so far as to cause an improvement in \(\text{methanotrophs}\) abundance, which would impact soil nitrogen cycle and then soil C dynamics [78]. Therefore, some studies documented no effect on native SOC under the application rates of 0.15% [50] or 1.8% w/w [51] of biochar, a negative priming effect under application rates of 1% w/w [53,54], 30 t ha\(^{-1}\) (appr. 1.3%–1.8% w/w) [37] and 2% w/w [52], and a positive priming effect under application rates of 30 t ha\(^{-1}\), 60 t ha\(^{-1}\), and 90 t ha\(^{-1}\) (equivalently 0.9%, 1.8%, 2.7% w/w) [55] and 4%, 7.3%, 13.5%, 25%, 38% w/w [56]. Orlova et al. [31] found that biochar application of 0.1% w/w did not affect the mineralization of soil organic C, but biochar of 1.0% w/w improved the mineralization rate by 15–18%. It is noteworthy that biochar triggered not only mineralization but also the formation of HA, exerting a negative priming effect on HA, which is similar to our study.

Biochar, regarded as “black gold” in the soil due to its agronomic [18,79] and environmental functions [80,81], has high production costs. If suitable dosages of biochar application can promote not only the stabilization of SOC but also the renewal of humic C to improve soil fertility and favor crop growth, it will be a win–win for ecological environment and agronomy, beneficial for the control of the costs of agricultural production as well.

5. Conclusions

In a two-year experiment, biochar application in soil improved the total SOC contents and C pool index and weakened the instability of the soil organic C. Biochar application significantly enhanced the contents of relatively stable HA and Hu and aliphaticity of HA as well. Though biochar application at the low dosages of 6 t ha\(^{-1}\), 12 t ha\(^{-1}\) and 24 t ha\(^{-1}\) had no distinct effect on native SOC, it did increase native HA substantially, indicating a positive effect on HA formation, with biochar exerting a positive influence on the transformation of labile C into stable C. Using \(\delta^{13}\text{C}\) and FTIR spectra techniques, biochar-derived C was found to be finitely involved in the formation of HA, resulting in a genetic relationship between soil HA and biochar to some extent. Comparing BC6, BC12 and BC24, however, the high dosage of biochar application (i.e., BC24) was less effective than BC6 and BC12 with respect to the positive effect on HA formation, though BC24 had the highest SOC contents and C management index. Therefore, future research is necessary to determine the optimum dosages of biochar application in order to not only sequestrate C in an ecological environment but also improve soil quality and crop yield in agronomy, and it is beneficial to control the costs of agricultural production as well based on the compelling long-term evidence.
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