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Long-term effect of biochar on the stabilization of recent carbon: soils with historical inputs of charcoal

Long-term biochar effects on carbon build-up

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Abstract

This study was set-up to identify the long-term effect of biochar on soil C sequestration of recent carbon inputs. Arable fields (n=5) were found in Belgium with charcoal enriched black spots (>50 m^2; n=14) dating > 150 years ago from historical charcoal production mound kilns. Topsoils from these "black spots" had a higher organic C concentration (3.6±0.9% OC) than adjacent soils outside these black spots (2.1±0.2 % OC). The soils had been cropped with maize for at least 12 years which provided a continuous input of C with a C isotope signature ($\delta^{13}C$) -13.1, distinct from the $\delta^{13}C$ of soil organic carbon (-27.4 ‰) and charcoal (-25.7 ‰) collected in the surrounding area. The isotope signatures in the soil revealed that maize derived C concentration was significantly higher in charcoal amended samples ("black spots") than in adjacent unamended ones (0.44% versus 0.31%; P=0.02). Topsoils were subsequently collected as a gradient across two "black spots" along with corresponding adjacent soils outside these black spots and soil respiration and physical soil fractionation was conducted. Total soil respiration (130 days) was unaffected by charcoal but the maize derived C respiration per unit maize derived OC in soil significantly decreased about half

($P<0.02$) with increasing charcoal derived C in soil. Maize derived C was proportionally more present in protected soil aggregates in the presence of charcoal. The lower specific mineralisation and increased C sequestration of recent C with charcoal is attributed to a combination of physical protection, C-saturation of microbial communities and, potentially, slightly higher annual primary production. Overall, this study provides evidence of the capacity of biochar to enhance C sequestration in soils through reduced C turnover on the long-term.

Introduction

Biochar production and application to soil is currently suggested as one of the economically feasible strategies for global carbon (C) sequestration (Spokas *et al.*, 2012). Charcoal production from biomass provides a sustainable source of energy, e.g. biofuels and syngas (synthesis gas) that are generated during the pyrolysis process (Manyà, 2012), it is a mean for disposal of organic wastes such as agricultural residues and it is a rational tool for combating the rise in atmospheric CO_2 (Sohi et al., 2009) and for sustainable agricultural production (McHenry, 2009). The C sequestration in soil through application of biochar is mostly attributed to its well-known persistence, its estimated lifetime ranging from few years to millennia (Gurwick *et al.*, 2013, Lehmann *et al.*, 2006) and it has a low sensitivity to positive priming effects upon addition of labile sources of C (Woolf & Lehmann, 2012). Biochar application can also indirectly increase C sequestration through enhancement of soil fertility (Criscuoli *et al.*, 2014), i.e. more annual biomass production. Soils enriched with biochar >800 years ago in the Brazilian Amazon Basin (*Terra Preta de Indio*) have a higher pH, higher content of stable organic matter (OM) and higher crop productivity compared to the surrounding low fertile soils (Lehmann & Joseph, 2012, Sohi *et al.*, 2010). Application of biochar has also been demonstrated to improve nutrient cycling on the short-term (Novak *et al.*, 2009) with enhancement of biomass production ranging 20–200% (Major *et al.*, 2010b).

The indirect effect of biochar on C-sequestration may also be related to lower C-turnover, i.e. lower mineralisation due to physical and chemical protection of other sources of organic carbon (OC) in soil. Biochar has generally a high porosity and surface area (Keiluweit *et al.*, 2010) and can potentially stabilize other sources of OC in soil through adsorption processes (Pignatello *et al.*, 2006), likely due to surface hydrophobic and hydrophilic interactions among biochar, organic compounds, and soil minerals (Kleber *et al.*, 2007). Oxidized charcoal residues contribute to increased cation exchange capacity (CEC) in soil (Mao *et al.*, 2012) and can promote OC stabilization through organo-mineral associations. Moreover, biochar can promote aggregation (Awad *et al.*, 2013, Brodowski *et al.*, 2006) and therefore the simultaneous stabilization of biochar particles with other sources of OC in microaggregates.

The assessment of indirect effects of biochar on C sequestration has generally been addressed by short-term studies of maximally a few years (Qayyum, 2012). Liang et al. (2010) reported that 3–8 % of plant residue applied to biochar-rich Amazonian soil was stabilized in the organo-mineral fraction (19–340% more than in adjacent biochar-poor soils) 532 days after residue application. Long-term data (> 10 years) are required to estimate the sustainability of biochar application to soil for C sequestration.

Recently, historical charcoal production kilns have been localized in Wallonia, south Belgium, which were in use during the 18th–19th centuries in beech and oak forests to supply charcoal required by the steel sector prior to the switch to coal (Hardy & Dufey, 2012). Aerial photography has identified "black spots" in arable land on former forest kilns (Atlas Ferraris, 1777) that extend over a few decametres, corresponding to residues from charcoal production in mound kilns >150 years ago. The C isotope signature ($\delta^{13}C$) of maize plants cultivated during last decade in agricultural fields on "black spots" is distinctly different (-13.1) from traditional crops and beech/oak biomass (-27.4). Thus, stable C isotope composition can be used to estimate the long-term (>10 years) C sequestration derived from maize crops (Balesdent *et al.*, 1987).

The objective of this study was to identify if long-term crop production on charcoal enriched soils leads to higher build-up of maize derived OC. The hypothesis is that the presence of aged charcoal in soil might reduce mineralization rates of maize derived C in soil while increasing crop residue stabilization in soil aggregates. Fourteen different kiln sites were identified as black spots in five arable maize crop fields. The soils in the black spots were collected together with soils adjacent to the black spots. In addition, soils were collected from gradients across two black spots for respiration studies and characterization of carbon in aggregates to identify mechanisms of biochar related C-sequestration in soil.

Material and methods.

Soil sampling and characterization

Soils were collected from 14 different kiln sites ("black spots") and the corresponding 14 adjacent sites outside those black spots from five arable fields in Wallonia, Belgium (Table

1). Fields 1-4 are located near Sivry-Rance and field 5 is located in Mettet. The “black spots” are a few decameters in size and correspond to charcoal production mound kilns dating from 18th-19th century. The soils were collected as 8 different subsamples in or out each spot and were thoroughly mixed to obtain 14 (in) and 14 (out) samples. All fields have a silt loam texture determined with laser Diffraction Particle Size (Beckman Coulter LS13 320) and have been dedicated to maize cultivation for at least 12 year. The samples were sieved (2 mm), oven dried and finely ground in a ball-mill before analysis of OC concentration and C isotopic signature.

In addition, two of the black spots were studied more in detail. Both samples are located in the same agricultural field (50°16’52’’ N, 4°44’6’’ E). On this field, maize has been continuously cultivated for 12 years. Composite soil samples were collected in 1 m^2 spots every 2 m along line transects across the two different “black spots”. For each transect, five composite samples were collected from the plough layer inside a “black spot” (B) and seven samples from adjacent soil outside (O) the “black spot”, these samples were within about 10 m of the edge. Soil respiration and DOC was determined for these 24 soil samples by two independent incubation experiments and we also conducted physical fractionation using a third set of subsamples. The soil samples were further analysed for pH (1/5 soil/CaCl2, 10 mM ratio) and CEC (cobalt hexamine trichloride).

Soil respiration and CO_2-C isotopic signature analysis

The 24 soil samples were sieved at 2 mm and moisture was adjusted to 20%. Triplicate subsamples of 20 g of each sample were placed in air-tight incubation jars (300 mL) equiped with three-way stopcocks to allow air sampling from the headspace. The jars were closed and incubated in the darkness at 25ºC for 125 days (transect 1) and 134 days (transect 2). Moisture content was corrected with MilliQ water as necessary to maintain moisture at 20 % (w/w). Three empty jars (blanks) were incubated in the same conditions.

The concentration of CO_2 in the headspace was determined by collecting air periodically with a 60 mL syringe and injection in a LI-COR CO_2 infrared gas analyser (LI-820). The gas stream was passed through a $Mg(ClO_4)_2$ (Sercon, UK) absorptive water trap to remove water vapour from the air sample. After each measurement, the jars were opened and soil moisture content was determined gravimetrically. Moisture content was corrected with MilliQ water as necessary to maintain moisture at 20 % (w/w). The jars were left open for 5–10 min to ensure equilibration with atmospheric CO_2. The CO_2-values measured (ppm) were corrected by subtraction of the blanks and the amount of C respired calculated using the ideal gas law. The detector was flushed with CO_2-free air between measurements using a CO_2 trap (Carbosorb, Sercon, UK) .

Parallel air samples from the jars headspace were collected at 7, 25 and 125 d and transferred to a glass vial with a rubber septum and the $\delta^{13}C$ of the CO_2 determined by gas chromatography IRMS (GC-IRMS) by means of a Gas bench II connected to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, Germany).

The CO_2-C isotopic signature ($\delta^{13}C$) was calculated according to Eqn. 1:

$$\delta^{13}C\ (‰) = \left(\frac{R_{sample}}{R_{standard}} - 1\right) * 1000 \qquad [1]$$

where R_{sample} is the $^{13}C/^{12}C$ ratio in the sample and $R_{standard}$ is the $^{13}C/^{12}C$ ratio of the Vienna Pee Dee Belemnite standard (Gonfiantini, 1984).

The $\delta^{13}C$ value of the CO_2 produced ($\delta^{13}C_{C\text{-}CO2}$) was determined after correcting for the contribution of atmospheric CO_2 with the isotopic mass balance equation:

$$\delta^{13}C_{C-CO_2} = \frac{\delta^{13}C_{sample} \cdot C_{sample} - \delta^{13}C_{blank} \cdot C_{blank}}{C_{sample} - C_{blank}} \qquad [2]$$

with $\delta^{13}C_{sample}$ and $\delta^{13}C_{blank}$ the measured isotopic ^{13}C ratio ($\delta^{13}C$) of the sample and the blank, and C_{sample} and C_{blank} the amounts of C-CO_2 (mg).

Average values obtained from three replicates were used for data analysis in the following sections.

Source appointment of carbon

The fraction of CO_2-C derived from the C4 pool (fresh or old maize residues) in soil (f_M) was calculated using a mixing model, which determines the relative contribution of each C pool to the mixture (Moore & Semmens, 2008):

$$f_M = \frac{\delta^{13}C_{C-CO_2} - \delta^{13}C_{SOM}}{\delta^{13}C_M - \delta^{13}C_{SOM}} \qquad [3]$$

with $\delta^{13}C_{SOM}$ the $\delta^{13}C$ values determined for soil organic matter (SOM) (-27.4 ‰) in soils under C3 crops (barley) that were sampled in the vicinity of the maize field nr.5 where the transect samples were collected (Table 2) and $\delta^{13}C_M$ (-11.0 ‰) the isotopic signature of CO_2-C_{maize}. That latter value was determined from the analysis of CO_2 respired from maize straw incubated with sterile sand and inocula obtained from soil by extraction with 0.1 M $MgCl_2$. Deviations in $\delta^{13}C$ from the values determined for the maize leaves $\delta^{13}C$ (-13.1 ‰) reflect a preferential microbial decomposition of specific compounds and structures of plant material

(Preston *et al.*, 2006). The source appointments for respiration were always derived from the cumulative soil respiration data in which a weighted average isotope signature was used, i.e. the $\delta^{13}C$ observed at different intervals (day 7, 25 and 125) multiplied with the cumulative CO_2 respired at the corresponding sampling interval. The fraction of CO_2-C derived from native (i.e. C3 based) soil organic matter (f_{SOM}) was calculated as 1-f_M. The contribution of the aged charcoal to CO_2-C was assumed to be negligible because this fraction of C is highly recalcitrant and unlikely to undergo mineralization.

The fractions of maize (f_M) and native SOM derived C (f_{SOM}) in the total soil OC outside the "black spots" were estimated according to a mixing model equivalent to Eqn. [3], using the bulk maize straw isotopic signature as $\delta^{13}C_M$ (-13.1 ‰) and the $\delta^{13}C_{SOM}$ (-27.4 ‰; for field 5). Fields 1-4 were located distinctly away from field 5 and grassland samples were locally taken for $^{13}C_{SOM}$ determination, yielding a slightly different value (-28.6 ‰). For soil samples from the "black spots", f_M, f_{SOM}, and charcoal derived C (f_C) cannot be directly determined since the three sources cannot be traced back with only two C-isotopes. Therefore, two different approaches were considered for the estimation of f_M, the maize derived C:

i) The average C isotope signature of charcoal ($\delta^{13}C_C$=-25.7±0.3 ‰; field 5) was determined in nine samples of charcoal collected at nearby ancient mound kilns located in forest land. For fields 1-4, individual charcoal particles were collected in the "black spots" yielding $\delta^{13}C_C$=-26.8 ±0.3 ‰. These values are relatively close to the isotope signature of native SOM as estimated from samples collected in a C3-field in the surrounding area ($\delta^{13}C_{SOM}$ =-27.4±0.3 ‰, field 5 and 28.6±0.7 ‰, fields 1-4), while both values are substantially different from that of the maize ($\delta^{13}C_M$=-13.1 ‰). Therefore, a $\delta^{13}C$ value for the pool of

SOM+charcoal ($\delta^{13}C_{SOM+C}$) can be estimated as the mean of both values, which yielded $\delta^{13}C_{SOM+C}$ =-26.6 ‰. (field 5) and $\delta^{13}C_{SOM+C}$ =-27.7‰ (fields 1-4). The choice for a mere average rather than a weighted average is justified as the samples inside the black spots contain about twice the amount of C determined outside the black spots and the bulk isotopic signature is closer to values determined for SOM and charcoal than to maize-derived OC, suggesting a similar share of SOM and charcoal in the soil C pool (Table 2). Accordingly, the estimated amount of maize derived C is a small contribution in soil, less than 12% of total SOC (see below). The fraction of maize (f_M) can then be estimated with a mixing model, i.e. ii)

$$f_M = \frac{\delta^{13}C_{sample} - \delta^{13}C_{SOM+C}}{\delta^{13}C_M - \delta^{13}C_{SOM+C}} \qquad [4]$$

With $\delta^{13}C_{sample}$ the isotope signature of the whole soil sample. The fraction of C derived from charcoal, f_C in the black spots (B) can be derived from relative differences in total OC between B and outer (O) samples, i.e.:

$$f_C = 1 - \frac{C_O}{C_B} \qquad [5]$$

and

$$f_{SOM} = 1 - f_M - f_C. \qquad [6]$$

with C_O the average OC concentration (%) for samples outside the local (corresponding)"black spot" and C_B the % OC of the sample inside the "black spot". This approach initially assumes no effect of charcoal on the C sequestration of the recent C input, however the approach (i) already predicts that the difference in percentage of maize derived C in soil between outside and inside the black spot is ≤8% of total C for all samples (Table 3), i.e. the differences in total OC between B and O is mainly attributed to charcoal.

iii) The fractions of maize derived C (f_M) can also be estimated according to the expression:

$$f_M = \frac{\delta^{13}C_{sample} - \delta^{13}C_{SOM}(1-f_C) - \delta^{13}C_C f_C}{\delta^{13}C_M - \delta^{13}C_{SOM}} \quad [7]$$

i.e. this Equation does not assume a 50/50 contribution of charcoal and SOM to the total soil SOM+charcoal C but distributes the partitioning according to the differences in total C between "black spots" samples and corresponding adjacent soils, again assuming that the main additional C in the "black spot" is due to charcoal only.

The first approach was used in the main text for reasons given in the results section. The statistical uncertainty of the f values, calculated according to approaches (i) or (ii), was estimated from the variance of mean δ^{13}C of the sources (Table 2) and of the mean C concentrations of the outer soil (Co) using standard statistical equations assuming independent parameters.

Mineralization kinetics

Cumulative specific C-mineralisation was fitted according to Brunner and Focht (1984):

$$P = S_0\left(1 - e^{-k_1 t - (k_2 t^2)/2}\right) + k_0 t \quad [8]$$

with P the mineralized per unit OC (g C 100 g^{-1} OC), S_0 the fraction of easily degradable C (mg CO_2-C 100 g^{-1} SOC), k_1 a first order mineralization rate constant (d^{-1}), k_2 an estimation of the microbial growth (d^{-2}) and k_0 a zero order constant that represents the rate of native SOM mineralized (% d^{-1}). The k_2 values corresponding to increase in microbial biomass were negligible and are not presented.

The use of cumulative respiration values normalized per unit soil C allows evaluating the C mineralization capacity or stability (Liang *et al.*, 2008). Model parameters were estimated from the best-fit model using SPSS v.17 software package. Curve fitting was performed using non-linear regression, using a Levenberg-Marquardt algorithm, which returns the best-fit parameters by minimizing the sum of squares of the residuals between measured and fitted values. Standard error of estimates (SEE) was used to determine the adequacy of the least squares line equation to fit each data set.

Carbon distribution in physical soil fractions

Thirteen samples (seven O and six B) selected from both transects were subjected to a physical fractionation procedure. Therefore, (20 g) of soil were sieved at 2 mm and oven-dried at 60 ºC, following isolation of macroaggregates, free microaggregates (f-mic) and silt+clay (f-sc) by wet sieving through a series of two sieves (250 and 53 µm mesh-size), according to Six et al. (2000). The macroaggregate fraction was further fractionated to isolate the particulate organic matter (POM) fraction (> 250 µm), microaggregates (i-mic) and silt and clay (i-sc) within the macroaggregates, according to the procedure described by Six et al. (2002). The fractions were collected in aluminium trays, oven-dried overnight (50°C) and the dry weight was recorded. Recoveries were calculated from the dry weights of the different fractions relative to the dry weight of the original soil sample.

Total organic C (OC) concentration, C isotopic signature and C/N ratios were determined for the bulk soil, soil fractions, maize straw and biochar by isotope ratio mass spectrometry (IRMS). For bulk soils, all the samples collected along each transect (seven O and 5 B) were analysed. Samples were bullet-grounded, weighted in silver capsules, acidified with 20 µL of

HCl 10% (w/w) to remove traces of inorganic C and oven-dried overnight at 50°C. Analysis where performed with a FlashEA 1112 HT elemental analyser coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, Germany). The fraction of C derived from maize was estimated according to Eqns [4].

Dissolved organic carbon

The concentration of dissolved organic carbon (DOC) was determined in soil solution isolated from 10 samples (O and B) selected from both transects at the end of the incubation experiment. The soil solution was isolated after soil incubation at 25°C (10 days) by centrifugation (10 min at 3000 g) using the 'double chamber' method (Bufflap & Allen, 1995) and immediately filtered through a 0.45-µm filter. The soil solutions were analysed for DOC using a TOC-analyser coupled to a Delta-V Advantage isotope ratio mass spectrometer.

Statistical analysis

The overall effect of charcoal (fixed factor) on the concentration of maize derived C (%CM) in soil was determined with the means values in the 14 black spots soils and corresponding values in adjacent soil and using kiln site as random factor (JMP 11.0). For the two transects, differences in soil properties between the charcoal amended and adjacent non-amended soils were tested using t-tests assuming equal variances (JMP 11.0). The uncertainty in source appointments of soil carbon or specific respiration was calculated for each sample as described above and is given in the figures and tables for illustration. That uncertainty was not used for testing the charcoal effects on these properties since sampling errors are assumed larger than the measurement (or calculation) errors. Further assessment of the variation in the percentage of maize derived OC along the two transects was conducted by log-transformation of CM values (%). This approach removes the heterogeneous variance due to the high variance associated to CM values for samples inside the black spots (B). Statistical analysis of the two transects was performed considering two separate data sets, one for each site (transect) and a block effect was included to capture possible systematic differences in the percentage of maize derived OC and specific maize C respiration between the areas to the left and to the right of the black spot since a significant block effect was found in the data. The sensitivity of these assumptions on the statistical significance of results is analysed below.

Results

Maize C build-up in charcoal amended soils

The concentration of maize C ranged 0.20-0.47% in soils outside the kiln sites and was somewhat lower in field 5 reflecting the shorter history of maize cropping (12 years; Table 1). The texture is similar for all five fields (see above) and other soil properties are also similar. The average concentration of maize C inside the black spots was significantly higher (P = 0.0169) than in adjacent soil. The mean maize derived C concentration (±standard deviation) was 0.44 ± 0.17% inside and 0.31 ± 0.11% outside the black spots (Table 1). In the following, results of source appointment of C is presented more in detail for transects of the two different black spots of field number 5.

Soil properties and estimated C-sources across two transects

The soil at the site is characterized as a silt loam Luvisol (International Union of Soil Sciences, 2006) with texture fractions according to the laser diffraction particle size analysis: 21/71/8 (sand/silt/clay) outside (=O) and 23/70/7 inside (=B) the “black spot”. The average top layer soil pH was 5.60±0.12 (10 mM $CaCl_2$), with no significant differences between outside and inside the "black spots". The CEC ranged from 13.9 (O) to 22.3 $cmol_c$ kg^{-1}(B) ($P<0.05$).

The soil total OC concentration, C/N ratio and $\delta^{13}C$ values of both transects are summarized in Table 2 and estimated sources of carbon given in Table 3.The average and total concentration of OC (%) in soil was circa 2-fold higher for samples collected inside the "black spot" (4.2±0.9) compared to samples outside (2.3±0.5). The average $\delta^{13}C$ values of total soil OC were -26.2±0.4 ‰ outside the "black spot" and -25.6±0.5 ‰ inside.. The uncertainty on the maize derived C concentration for each sample was calculated based on uncertainty in the $\delta^{13}C$ and %C values for either approach. The standard deviations for the fractions are about 37% of the mean (Table 3) and are typically lower than the standard deviation based on the sampling replicates. This means that the level of statistical significance for testing differences among parameters for samples inside and outside the "black spots" is not dominated by uncertainty in isotope signatures of the sources. The maize derived C concentration was significantly higher inside the "black spot" for both transects (Table 3) (P = 0.005 for transect 1, P = 0.02 for transect 2; both analyzed statistically as described above). Without accounting for block effects on C_M (only significant in transect 2), there was no significant charcoal effect (P>0.05) on maize derived C in transect 2. However, combining all data of transect 1&2 yielded an overall significant effect of charcoal on maize derived C (P<0.05 as in Table 1) and log-transformation of the data did not affect the conclusion of significance of charcoal effects on maize derived C (details not shown). In addition, a significant linear increase of maize derived C concentration with increasing charcoal derived C in soil was found when all data of the 14 kiln sites were combined (Fig. 1). The same trend analysed per transect yielded a significant increase of maize derived C concentration with increasing charcoal derived C in soil (Fig. S2; $P = 0.002$ for transect 1, $P = 0.01$ for transect 2 but $P>0.05$ for transect 2 when the block effect was not accounted for).

Soil respiration

The cumulative (g CO_2-C respired kg^{-1} soil) is summarized in Fig. 2. Respiration was unaffected by charcoal (average 0.39 ± 0.09 g CO_2-C kg^{-1} soil produced after 125–134 days), which suggests that microbial activity in soil might not be affected by the presence of charcoal. The cumulative respiration curve for the sample collected in the centre of the "black spot" in transect 1 (Fig. 1.1) yielded lower respiration compared to the other samples (0.12±0.09 g CO_2-C kg^{-1}) but also a lower maize derived C concentration (0.37±0.15 $\%C_M$).

The parameters values of the kinetic model [8] are summarized in Table 4. The k_2 values corresponding to increase in microbial biomass were negligible and have not been included. The estimated labile OC, as a fraction of the total SOC (S_0, mg CO_2-C 100 g^{-1} SOC) is lower inside the "black spot" (average 0.29±0.12 for transect 1, 0.34±0.02 for transect 2) compared to soil outside the "black spot" (average 0.63±0.22 for transect 1, 1.02±0.18 for transect 2). Since S_0 is expressed per unit total OC (including charcoal-C), this effect logically relates to the mixing of SOM with the recalcitrant charcoal. The mineralization constant k_1 was similar inside and outside the "black spots" (average for both transects 0.020±0.004 $days^{-1}$), in agreement with the respiration curves presented in Fig. 1. However, the zero order mineralization constant k_0 ($days^{-1}$) was significantly lower inside the "black spot" (average 0.007±0.002) compared to soil outside for transect 1 (average 0.003±0.002), but that might again be partially related to the recalcitrant charcoal that is part of the OC in the "black spots”. No significant difference in k_0 between inside and outside black spot samples were found for transect 2 (average 0.003±0.001).

The analysis of the C isotopic signature of the CO_2-C collected at 3, 25 and 125 days allowed the estimation of the ratio of maize derived C respired to soil maize derived C according to Eqns. [2]–[4]. The ratio of maize-C respired relative to total maize-C in soil was significantly different between O and B for both transects. This specific respiration was about 50% lower inside the “black spot” compared to soil outside for both transects (P=0.0178 for transect 1 and P=0.0120 for transect 2; Fig. 3).

The fraction of maize derived C in soils was also estimated by method (ii) (see method sections) and both approaches agreed strongly (Fig S1). In addition, method (i) was further examined with a sensitivity analysis assuming a range of charcoal/SOM contributions between 30/70 and 70/30. The results indicate that for any charcoal/SOM ratio above 40/60 the percentage of maize-derived carbon remains significantly higher in charcoal amended soil. Moreover, the linear increase of maize derived C concentration with increasing charcoal derived C (Figure 1) remains significant ($p<0.05$) for any charcoal/SOM ratio above 30/70.

Physical soil fractionation and dissolved organic carbon

The recoveries for the fractionation ranged 95.8–98.3% (mass) and 93–117% (OC). Table 5 summarizes the average distribution of maize among the size fractions outside and inside the “black spots” for the two transects. The carbon in the POM fraction, as % of total soil OC, was larger in charcoal amended (“black spots”) soil suggesting that free charcoal particles occur as POM. The fraction of maize-C (% of total maize C in soil) located in i-mic was significantly higher in charcoal enriched soil than in the unamended soil. Conversely, the fractions of maize C present in –silt+clay were significantly lower in charcoal enriched soil than in unamended soils. The latter was less pronounced when expressed as mg C/kg soil since that latter fraction is small. The silt and clay fractions contained lowest fractions of maize C, ranging 4–18%. Maize C, expressed per kg dry soil, was significantly higher in charcoal enriched soil than in unamended soil for the POM and i-mic fractions. The concentration of C in soil solution (DOC; mean values and standard deviation; n= 5 for O and B) was 23.0±2.4 mg L^{-1} outside the "black spot" and 24.2±3.0 inside. The average $\delta^{13}C$ values of DOC were -25.7±0.1 outside the "black spot" and -26.1±0.2 inside.

Discussion

Soils from the "black spots" have approximately twice the concentration of OC determined in adjacent soil after 150 years. This is most likely due to the occurrence of the recalcitrant pool of C-charcoal but, as indicated by the maize-C data, partially also due to a larger residual C from crop residues. The difference in maize derived OC between the amended ("black spots) and non-amended soils, i.e. 0.44-0.31%=0.13%, is equivalent to about 3-4 ton C ha^{-1} in the plough layer. Such is a relatively high additional C-sequestration after only about12 years of cultivation relative to estimations of C-sequestration potential in agricultural systems at global scale (Lal, 2011). The data of Table 3 suggest that the additional maize-C is compensated by lower native SOC in the soils with charcoal, i.e. suggesting that there is no net sequestration but likely a replacement of native SOC by maize-C. However, that result is a consequence of the source appointment method, i.e. Eqn. [5] that attributes all additional SOC in the charcoal amended soils ("black spots") to charcoal only, i.e. the concentrations of native SOC cannot be estimated with sufficient precision to accept or reject a replacement hypothesis. Borchard et al. (2014) have recently presented results that suggest long-term stabilization of SOC in the presence of aged charcoal at historical charcoal production sites comparable to the “black spots” selected for our study. However the authors did not analyse stable isotopes to discriminate the different sources of carbon in soil. We are currently testing other analytical tools to estimate charcoal-C (TGA and DSC methods); these methods combined with the isotope signature data confirm effects of charcoal on the maize derived C concentration (as in Fig.1; details not shown) but none of these are sufficiently precise to better quantify the native SOC concentration and test such replacement hypothesis.

Increased residue derived OC in charcoal amended soil (“black spots”) might result from the combination of lower mineralisation rate and larger annual primary production. Total respiration rates were not affected by the presence of charcoal (Fig. 1) despite somewhat higher concentrations of (more recent) maize derived C in the “black spot” samples. Thus, the respiration of maize- C relative to the maize-C for charcoal enriched soil was significantly lower in the “black spot” of both transects (Fig. 3) and may explain higher residual maize-C in the charcoal amended soils. Several mechanisms could be involved in the protective effect of charcoal on fresh C inputs. First, hydrophobic interactions of fresh C residue with charcoal may reduce mineralization.. Second, our physical fractionation data suggests that charcoal might promote physical protection of the maize C inside microaggregates (Table 5). Presumably, charcoal provides an ecological niche for soil microorganisms that is not yet well understood (Lehmann *et al.*, 2011), and that might influence chemical and biochemical transformation of carbon sources in soil. Interestingly, most of the charcoal C is located in the POM fraction, this being accompanied by significantly higher amounts of maize C in this fraction compared to non-charcoal amended soils. The differences in the fraction of maize C determined in the i-sc fraction for charcoal amended soils (“black spots”) and for unamended soils are not considered, because the corresponding amount of maize-C constitutes a negligible percentage of the total maize C budget (g maize C kg^{-1} soil).

The effect of charcoal on the respiration rate of labile inputs of C in soil has been addressed in several short-term experiments. Keith et al. (2011) reported negative priming effects of biochar in the mineralization of sugar cane residue applied to soil in combination with young biochar prepared at 450–550 °C during 120 days incubation. Zavalloni et al. (2011) reported a decrease in total C respired (84 days incubation) when wheat straw was added in combination with a commercial charcoal (500 °C) compared to respiration rates in soil added only with labile residue. In contrast, positive priming effects of biochar on the respiration of labile sources of OC have been also reported. Awad et al. (2013) and Qayyum (2012) described an increase in the mineralization of maize (80 days incubation) and wheat straw (1 year incubation) when applied in combination with a low pyrolysis biochar (180–250 °C). The study performed by Zimmerman et al (2011) has provided a more general insight and indicated that positive priming occurs generally at the early stages of charcoal in soil (approximately 90 days) and particularly for biochar prepared at low temperature (250–400 °C), while negative priming might prevail as charcoal ages in soil, particularly when hardwood is used as feedstock. Currently, the results from Liang et al. (2010) are the only research comparable with the presence of aged charcoal presented in our study. The authors examined the mineralization of sugar cane (C4 plant) added to charcoal-enriched, C3 plant-dominated *Terra Preta* soils during 532 days and reported similar total mineralization rates of sugar cane residue as in soils with no charcoal. It is possible that one pulse of C4 plant material added in the *Terra Preta* or adjacent soil samples is not sufficient to sensitively estimate differences in the build-up of residue derived C in soil compared to our data that result from 12 years of maize cultivation. Nevertheless, Liang et al.(2010) indicated that after

9 months, a greater ratio of sugar cane residue was located in the intra-aggregates and organo-mineral fractions, in agreement with the increase of maize derived C in i-mic reported in Table 5. The presence of charcoal in soil aggregates is consistent with results from Brodowski et al. (2006). The authors presented evidence that suggest the participation of charcoal as binding agent in aggregate formation. Recently, Awad et al. (2013) indicated that the presence of biochar in soil aggregates was accompanied by an increase in the incorporation of fresh residue in the aggregates. The presence of charcoal in microaggregates (41–43%) estimated from our δ^{13}C analysis supports the role of charcoal providing a new compartment for physical and chemical protection of C, this being a consequence of compartmentalization of biochemical activity, i.e. biochar might provide a suitable environment for microbial communities in the POM fraction (Lehmann *et al.*, 2011). Results from Liang et al (2010) for charcoal enriched soil also support a decrease in microbial activity accompanied by increased incorporation of maize-derived C into aggregates. Recent development of Nano secondary ion mass spectrometry (NanoSIMS) to study distribution of isotopes in soil particles might provide sufficient sensitivity to accurately discriminate distribution of different sources of carbon in biochar amended soil (Vogel *et al.*, 2014).

The long-term effects of charcoal on the primary production in well fertilised fields are unclear. In the fall of 2014, we performed paired sampling of maize plants (above ground biomass) inside and outside the “black spots” in 12 of the 14 of the kiln sites reported here. The above ground dry biomass was significantly larger by, on average, 10% on “black spots” compared to that on corresponding soil outside the spots (details not shown), reasons for this are yet to be determined. This suggests that the higher build-up of maize C in charcoal enriched soil can partly be attributed to higher crop yield, however results need to be confirmed over multiple growing seasons, taking into account yearly variations. It is well established that biochar or charcoal can enhance fertility and biomass production in *Terra Preta* soils in Amazonia (Lehmann *et al.*, 2006, Liang *et al.*, 2006), this being confirmed by numerous short-term studies (2 months – 4 years) (Major *et al.*, 2010b, Mao *et al.*, 2012, Novak *et al.*, 2009). However, impact on fertility might be more subtle for temperate soils (Jeffery *et al.*, 2011). For instance, similar pH values were determined for O and B soil samples whereas the liming effect of biochar are important in Anthrosols with high contents of aged charcoal (Liang *et al.*, 2006).

Taken together, this study provides the probably first evidence of the long-term potential of charcoal application to increase C retention and storage in agricultural soils. Charcoal provides a pool of C that is resistant to mineralization and that decreases the turnover rate of recent C due to better physical protection or C-saturation of microbial processes. In combination with small increased annual inputs of fresh C from crop residues, charcoal has the capability to enhance C sequestration in an indirect way.

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Figure legends

Fig. 1 The maize derived carbon in soil (soil air dry weigh basis, %) as affected by the concentration of charcoal; data of all 14 kiln sites, including the detailed gradient studies at field 5. The regression line is significant ($P<0.001$), the 95% confidence limit is shaded.

Fig. 2 Cumulative total soil respiration for samples collected outside (empty symbols) and inside (full symbols) the “black spots” for transects 1 (left) and 2 (right). Error bars denote standard deviations of the mean of three replicate subsamples. Difference in respiration between inside and outside samples are not significant ($P>0.05$).

Fig. 3 Total specific mineralization of maize derived OC, i.e. CO_2-C normalized per unit of total maize C in soil. Samples were collected at the end of a maize growing season in transects 1 (left, 120 days respiration) and 2 (right, 134 days respiration). The $\delta^{13}C$ of soil OC and respired C was used to infer the source of C in respiration. O = soil samples outside “black spots”, B = soil samples inside the “black spots”. Error bars are standard deviations derived by error propagation including the uncertainty of the isotope signatures of the sources and the variability of incubation replicates of the sample; n=3. Mean specific respiration for transect 1 is 6.8 g C 100 g C^{-1} (outside) and 2.8 g C 100 g C^{-} (inside), P=0.0178 and for transect 2 13.5 g C 100 g C^{-1} (outside) and 7.7 g C 100 g C^{-} (inside), P=0.0120.

Table 1. Soil properties and percentage of maize-derived carbon (C_M; means of the kiln sites per field ± standard deviation) in black spots (B) and adjacent soil (O) sampled from 14 charcoal kiln sites identified in five arable fields cropped by maize. The %C_M source was calculated with Eqn. [4]. Two transects were sampled across the two black spots of field nr. 5 and the results are presented in all other tables and figures. Soil pH and CEC values represent the background values, i.e. measured for the O samples.

Field	location	soil pH	CEC (cmol$_c$/kg)	year of maize cropping	Number of black spots		C_M (%)
1	Sivry-Rance	6.0	12.6	> 17	2	B	0.43 ± 0.04
						O	0.40 ± 0.04
2	Sivry-Rance	6.6	14.1	> 17	2	B	0.56 ± 0.07
						O	0.47 ± 0.03
3	Sivry-Rance	6.8	13.7	> 17	4	B	0.30 ± 0.06
						O	0.32 ± 0.01
4	Sivry-Rance	6.2	11.1	> 17	4	B	0.59 ± 0.19
						O	0.24 ± 0.11
5	Mettet	5.6	13.9	12	2	B	0.33 ± 0.18
						O	0.20 ± 0.04
					Average C_M (%)	**B**	0.44* ± 0.17
						O	0.31 ± 0.11

*significantly larger than O sample, P=0.02 for effects of ‘B’ with black spot as random effect.

Table 2. Concentration of OC (%, oven dried soil basis) and C isotope signature (‰, $\delta^{13}C$) in topsoil samples collected outside (O) and inside the “black spots” (B) over two transects in the agricultural field nr. 5, maize leaves from the same fields, soil samples collected in nearby fields under C3 crop (C3-soil) and charcoal pieces collected near ancient mound kilns in nearby forest. Means and standard deviations of n (indicated) independent samples.

		n	OC (%)	C/N	$\delta^{13}C$ (‰)
Transect 1	**O**	7	2.6 ± 0.3	7.2 ± 0.5	-26.2 ± 0.3
	B	5	4.5 ± 0.5	10.2 ± 0.6	-25.2 ± 0.4
Transect 2	**O**	7	2.0 ± 0.4	6.9 ± 0.5	-26.3 ± 0.5
	B	5	3.9 ± 1.1	10.0 ± 1.7	-25.9 ± 0.2
Maize		5	45 ± 3	39 ± 2	-13.1 ± 0.3
C3-soil		5	2.1 ± 0.2	10.5 ± 0.4	-27.4 ± 0.2
Charcoal		9	52 ± 9	214 ± 67	-25.7 ± 0.3

Table 3. Total organic carbon (OC oven dried soil basis) concentration in soil (OC_s) and concentrations of OC derived from charcoal (C_C), maize (C_M) and soil OM (C_{SOM}) in soil sampled in transects 1 and 2. O = soil samples outside "black spots", B = soil samples inside the "black spots" of field nr. 5. The C sources were identified using Eqns [4-6]. The statistical uncertainty (standard deviation) of C_C, C_M and C_{SOC} is calculated based on uncertainty of the mean $\delta^{13}C$ of the sources and mean %C of O samples; differences in mean C_M between B and O were tested with a t-test and including a block effect for the side of the spot (left=O1-O4, right= O5-O7), this block effect was significant in transect 2.

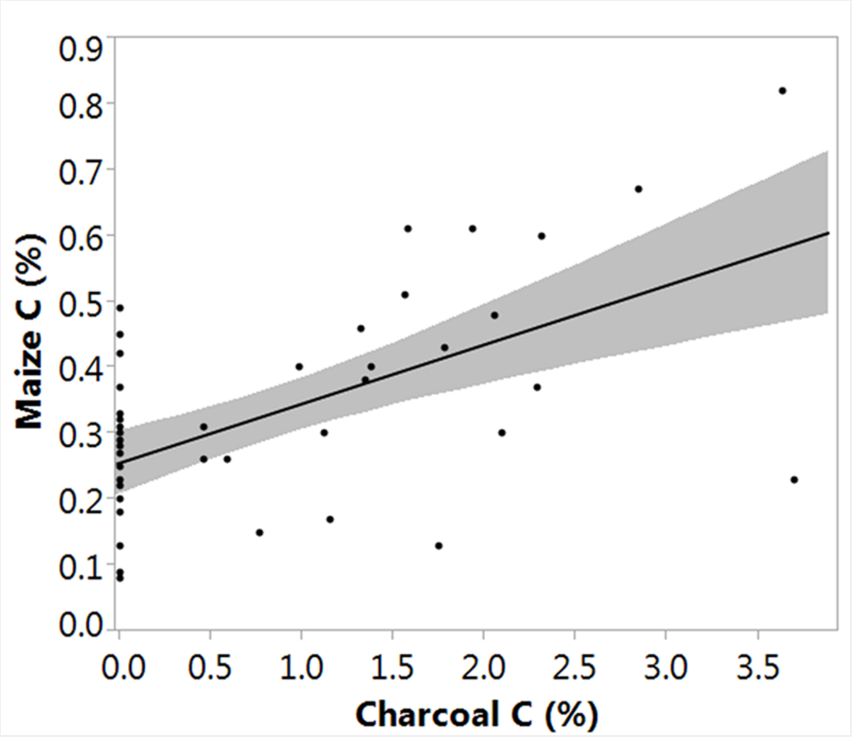
	Transect 1				**Transect 2**			
	OC_S	**C_C**	**C_M**	**C_{SOM}**	**OC_S**	**C_C**	**C_M**	**C_{SOM}**
	%				**%**			
O1	2.75		0.20 ± 0.06	2.55 ± 0.06	1.66		0.09 ± 0.04	1.57 ± 0.04
O2	2.56		0.13 ± 0.06	2.43 ± 0.06	1.71		0.18 ± 0.04	1.54 ± 0.04
O3	2.70		0.27 ± 0.06	2.44 ± 0.06	1.81		0.08 ± 0.04	1.74 ± 0.04
O4	3.00		0.29 ± 0.07	2.71 ± 0.07	2.18		0.09 ± 0.05	2.09 ± 0.05
B1	4.42	1.78 ± 0.28	0.43 ± 0.13	2.21 ± 0.31	2.81	0.76 ± 0.44	0.15 ± 0.08	1.90 ± 0.45
B2	4.94	2.31 ± 0.28	0.60 ± 0.15	2.03 ± 0.32	3.79	1.75 ± 0.44	0.13 ± 0.11	1.91 ± 0.46
B3	4.93	2.29 ± 0.28	0.37 ± 0.15	2.27 ± 0.32	5.74	3.70 ± 0.44	0.23 ± 0.17	1.81 ± 0.47
B4	4.57	1.93 ± 0.28	0.61 ± 0.14	2.02 ± 0.31	4.13	2.09 ± 0.44	0.30 ± 0.12	1.74 ± 0.46
B5	3.75	1.12 ± 0.28	0.30 ± 0.11	2.34 ± 0.30	3.20	1.15 ± 0.44	0.17 ± 0.09	1.87 ± 0.45
O5	2.47		0.22 ± 0.06	2.25 ± 0.06	2.36		0.23 ± 0.05	2.13 ± 0.05
O6	2.13		0.25 ± 0.05	1.88 ± 0.05	2.85		0.28 ± 0.06	2.57 ± 0.06
O7	2.82		0.27 ± 0.06	2.55 ± 0.06	1.74		0.23 ± 0.04	1.51 ± 0.04
Mean	O		0.23				0.17	
	B		0.46				0.20	
Significance			P = 0.0049				P = 0.0214	

Table 4. Parameters of the cumulative soil respiration model (Eqn. [8]) for soil samples from transect 1 and 2 of field nr. 5. S_0 = fraction of easily available C (mg CO_2-C 100 g^{-1} SOC), k_1 = first order mineralization rate constant for the easily available C pool (d^{-1}), k_0 = zero order mineralization rate constant (% d^{-1}). O = soil samples outside “black spots”, B = soil samples inside the “black spots”. The standard error of parameter values are given R^2 >0.98 for all fitted curves. The k_2 parameter has not been included.

		kinetic model $P = S_0\left(1\text{-}e^{-k_1t-(k_2t^2)/2}\right)+k_0t$		
Transect		**S_0**	**k_1**	**k_0**
(1)	**O1**	1.03 ± 0.14	0.019 ± 0.004	0.008 ± 0.001
	O2	0.80 ± 0.20	0.023 ± 0.013	0.006 ± 0.002
	O3	0.75 ± 0.16	0.024 ± 0.007	0.004 ± 0.001
	O4	0.52 ± 0.14	0.023 ± 0.016	0.005 ± 0.001
	B1	0.37 ± 0.08	0.020 ± 0.008	0.004 ± 0.001
	B2	0.35 ± 0.06	0.021 ± 0.008	0.004 ± 0.001
	B3	0.10 ± 0.02	0.034 ± 0.008	0.000 ± 0.000
	B4	0.23 ± 0.01	0.028 ± 0.006	0.003 ± 0.001
	B5	0.40 ± 0.18	0.016 ± 0.002	0.002 ± 0.001
	O5	0.60 ± 0.15	0.025 ± 0.005	0.006 ± 0.001
	O6	0.43 ± 0.08	0.020 ± 0.010	0.008 ± 0.001
	O7	0.47 ± 0.09	0.018 ± 0.008	0.008 ± 0.001
	O8	0.41 ± 0.06	0.012 ± 0.011	0.008 ± 0.001
(2)	**O1**	0.96 ± 0.20	0.025 ± 0.003	0.005 ± 0.001
	O4	0.79 ± 0.10	0.021 ± 0.002	0.004 ± 0.001
	B1	0.55 ± 0.04	0.026 ± 0.002	0.002 ± 0.000
	B3	0.46 ± 0.10	0.023 ± 0.002	0.003 ± 0.001
	B5	0.00 ± 0.04	0.016 ± 0.003	0.004 ± 0.000
	O6	1.19± 0.37	0.015 ± 0.002	0.002 ± 0.003
	O7	1.13 ± 0.35	0.015 ± 0.002	0.004 ± 0.003

Table 5. Physical fractions of soils outside (O) and inside (B) the "black spot" (n=7 for O, n=6 for B) of field nr. 5. Data are total OC distribution (as % of total soil concentration) and maize C distribution (as % of total soil concentration and as g maize C kg^{-1} soil) over the different fractions. The source appointments were calculated using Eqn. [4]. Fractions are particulate organic matter (POM), free microaggregages (f-mic), microaggregates inside macroaggregates (i-mic), free silt and clay (f-sc) and silt and clay inside macroaggregates (i-sc). The standard deviations of the maize C distribution (% of total maize C) are calculated based on the uncertainty of the isotope signatures of the sources and the variability between replicate samples, standard deviations of total OC distribution and maize C distribution (g maize C kg^{-1} soil) are only based on variability between replicate samples. Significances of pairwise difference between O and B are indicated (*).

	Total OC distribution		**Maize C distribution**			
	(% of total OC)		**(% of total maize C in soil)**		**(g maize C kg-1 soil)**	
	O	**B**	**O**	**B**	**O**	**B**
POM	11 ± 3	23 ± 6*	28 ± 8	38 ± 12	11.14 ± 1.36	17.43 ± 1.47*
f-mic	27 ± 7	31 ± 7	24 ± 7	25 ± 8	2.22 ± 1.07	3.06 ± 1.74
i-mic	41 ± 9	33 ± 3	34 ± 3	46 ± 7*	1.6 ± 0.67	4.28 ± 1.59*
f-sc	4.8 ± 0.8	5.7 ± 1.0	3.5 ± 2.3	3.5 ± 3.6	1.08 ± 0.67	1.11 ± 0.51
i-sc	16 ± 3	8.5 ± 1.4*	18 ± 12	5.8 ± 3.2*	1.52 ± 0.09	1.36 ± 0.42


Maize C (%)
Charcoal C (%)
0.0
0.1
0.2
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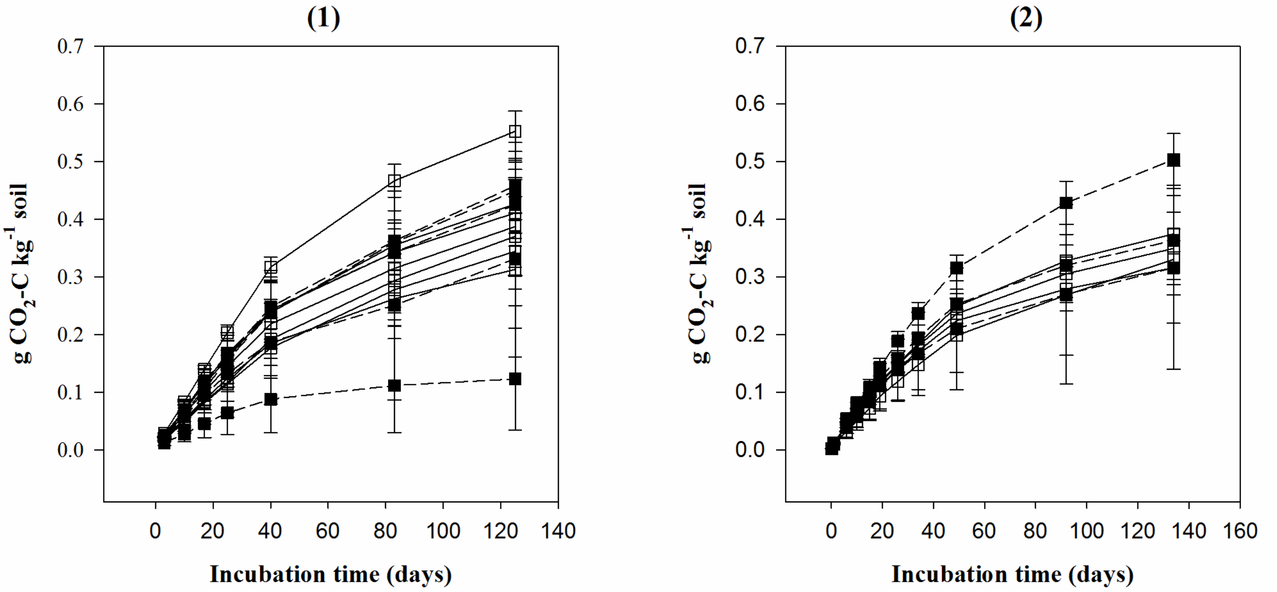
3.5


(1)
(2)
g CO_2-C kg^{-1} soil
Incubation time (days)
0.0
0.1
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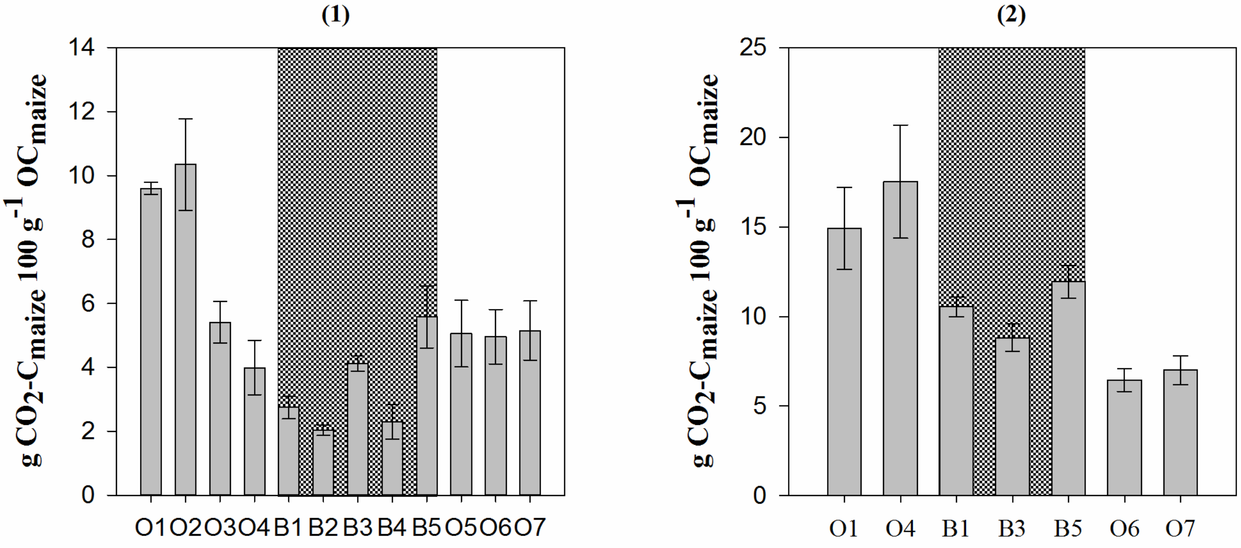
100
120
140
160


(1)
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g CO2-Cmaize 100 g-1 OCmaize
0
2
4
6
8
10
12
14
O1 O2 O3 O4 B1 B2 B3 B4 B5 O5 O6 O7
g CO2-Cmaize 100 g-1 OCmaize
0
5
10
15
20
25
O1 O4 B1 B3 B5 O6 O7