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Effects of the application of biochar and organic fertilizers as well as temperature on soil carbon and aggregate dynamics

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Witzenhausen, August 2016

(Dennis Grunwald)

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Die folgenden Publikationen sind Bestandteil der vorliegenden Arbeit:

Kapitel 4:

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Kapitel 6:

Grunwald, D., Kaiser, M., Piepho, H.-P., Koch, H.-J., Rauber, R., Ludwig, B.: Effects of biochar and slurry application and drying and rewetting on soil macro-aggregate formation in silty loam soils. In preparation for submission to Geoderma.

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Abkürzungsverzeichnis

B_{60} / B_{120}	60 / 120 g biochar kg^{-1} dry soil
C_{mic}	Microbial biomass carbon
Con	Control
C_{org}	Organic carbon
C_t	Total carbon
DRC	Drying-rewetting cycles
DRIFT	Diffuse reflectance infrared spectroscopy
fLF	Free light fraction
HF	Heavy fraction
OC	Organic carbon
oLF	Occluded light fraction
OM	Organic matter
SOC	Soil organic carbon
SOM	Soil organic matter
SPT	Sodium polytungstate
T_0-C	Ambient soil temperature without biochar
T_0-B	Ambient soil temperature with biochar
$T_{+2.5}$-C	Elevated soil temperature (+ 2.5 °C) without biochar
$T_{+2.5}$-B	Elevated soil temperature (+ 2.5 °C) with biochar
$S_{0.15}$ / $S_{0.3}$	0.15 / 0.3 g slurry-N kg^{-1} dry soil

1 Zusammenfassung

Die organische Bodensubstanz ist von Bedeutung für die Bodenfruchtbarkeit und stellt vor dem Hintergrund des Klimawandels einen wichtigen Kohlenstoffspeicher dar. Für den Schutz organischer Substanz und die langfristige Kohlenstoffspeicherung im Boden ist die Bildung von Bodenaggregaten ein bedeutender Prozess. Während der positive Einfluss von Pflanzenresten auf die Bildung von Makroaggregaten (> 250 µm) gezeigt werden konnte, ist der Einfluss organischer Dünger wie Rindermist oder Rindergülle auf die Bodenaggregatdynamik weniger bekannt.

Steigende Bodentemperaturen im Zuge des Klimawandels könnten darüber hinaus den Abbau organischer Bodensubstanz erhöhen, wohingegen die Einarbeitung von Pflanzenkohle in den Boden als eine Möglichkeit zur nachhaltigen Erhöhung der Kohlenstoffvorräte im Boden angesehen wird. Bisher sind allerdings sowohl die Effekte erhöhter Bodentemperaturen als auch der Einarbeitung von Pflanzenkohle auf die Bodenaggregatdynamik unklar.

Die Hauptziele dieser Arbeit sind daher, die Effekte von (i) Rindermist und Rindergülle, (ii) der Einarbeitung von Pflanzenkohle und (iii) verschiedenen Temperaturen auf Aggregat- und Kohlenstoffdynamik im Boden zu untersuchen. Hierzu wurden zwei Inkubationsversuche durchgeführt sowie ein Feldversuch beprobt und analysiert.

In beiden Inkubationsversuchen wurde Boden, der auf eine Größe von unter 250 µm zerkleinert wurde und somit keine Makroaggregate mehr beinhaltete, verwendet, um die Aggregatbildung während der Inkubation zu bestimmen. Im ersten Versuch wurde der Boden, neben einer Kontrolle ohne Zugaben, entweder mit Pflanzenkohle, Rindermist, Rindergülle oder einer Mischung aus Pflanzenkohle und Rindergülle gemischt und bei 5, 15 oder 25 °C für vier oder acht Wochen inkubiert. Neben dem Trockengewicht der gebildeten Makroaggregate und dem in den Aggregaten gebundenen Kohlenstoff wurden auch die CO_2-Emissionen sowie der mikrobiell gebundene Kohlenstoff bestimmt.

Im Rahmen der zweiten Studie wurden vier Varianten des Hohenheim Climate Change-Experiments in zwei Tiefen beprobt. Hierbei wurden die Faktoren Bodentemperatur (Umgebungstemperatur oder seit sechs Jahren um 2,5 °C erhöhte Temperatur) und die Einarbeitung von Pflanzenkohle (Kontrolle ohne Pflanzenkohle oder Einarbeitung von 30 t / ha *Miscanthus*-Kohle ein Jahr vor Beprobung) variiert. Die Proben wurden in einem kurzen Inkubationsversuch hinsichtlich der Basalrespiration und des mikrobiell gebundenen Kohlenstoffs untersucht. Darüber hinaus wurden die Kohlenstoffgehalte in Aggregat- und Dichtefraktionen bestimmt. Durch die Verwendung von *Miscanthus*-Kohle konnte zudem der Anteil des Kohlenstoffs aus der Pflanzenkohle in diesen Fraktionen durch ^{13}C-Isotopenmessungen quantifiziert werden.

In der dritten Studie wurde ein ergänzender Versuchsansatz zum ersten Experiment gewählt, allerdings mit dem Fokus auf der kombinierten Einarbeitung von Pflanzenkohle und Rindergülle. Hierbei wurden Bodenproben, die auf eine Größe von unter 250 µm zerkleinert wurden, mit Pflanzenkohle oder Gülle oder mit Mischungen der beiden Substrate in verschiedenen Verhältnissen bei 15 °C für 60 Tage inkubiert. Weiterhin wurden die Proben entweder bei einer konstanten Feuchte inkubiert oder während der Inkubationsdauer in drei Zyklen getrocknet und wiederbefeuchtet.

Die Ergebnisse der ersten Studie zeigen, dass der Effekt höherer Temperaturen auf die Abbauintensität der organischen Substanz am höchsten für die Variante mit Rindermist und am niedrigsten für die Varianten mit Pflanzenkohle ist. Im Vergleich mit niedrigeren Temperaturen resultierten höhere Temperaturen weiterhin in signifikant geringeren Trockengewichten der gebildeten Makroaggregate in der Kontrolle, der Variante mit Pflanzenkohle (beide nach vier Wochen) und der Variante mit Rindermist (nach vier und acht Wochen), was vermutlich mit der Metabolisierung der Aggregatkittstoffe zu erklären ist. Bei 15 °C wurde die höchsten Aggregatmengen für die Variante mit Gülle gefunden, was möglicherweise mit der flüssigen

Konsistenz der Gülle erklärt werden kann, durch die eine effiziente Verteilung der Gülle auf den Oberflächen von Mineralpartikel zu erfolgen scheint. Die Variante mit Mist hatte im Vergleich zur Kontrolle hingegen keinen Effekt auf die Aggregatmengen. Auch eine solitäre Einarbeitung von Pflanzenkohle hatte im Vergleich mit der Kontrolle keinen signifikanten Einfluss auf die Aggregatmengen, aber in Kombination mit der Gülle resultierte die Einarbeitung von Pflanzenkohle in kleineren Aggregatmengen als die Variante mit Gülle allein. Signifikante Korrelationen zwischen Aggregatmengen und CO_2-Emissionen oder dem mikrobiell gebundenen Kohlenstoff deuten weiterhin auf eine Aggregatbildung in Folge einer direkten Interaktion der eingemischten Substrate mit den Mineralpartikeln des Bodens und einen anschließenden Abbau der Substrate hin, bei einer parallel erfolgenden Produktion von mikrobiellen Aggregatkittstoffen.

In der zweiten Studie zeigten die Varianten mit Pflanzenkohle aus dem Feldversuch signifikant höhere Basalrespirationsraten als die Varianten ohne Pflanzenkohle, während die erhöhte Bodentemperatur in den meisten Fällen in einer Erhöhung des mikrobiell gebundenen Kohlenstoffs resultierte. Die mikrobielle Biomasse wies keinen Kohlenstoff aus der Pflanzenkohle auf, was darauf hindeutet, dass die Kohle nicht mineralisiert wurde. Die Einarbeitung von Pflanzenkohle hatte keine Auswirkungen auf die in Aggregaten gespeicherten Kohlenstoffvorräte, allerdings wurde Kohlenstoff aus der Pflanzenkohle in allen Fraktionen gefunden. Zudem erhöhte die Pflanzenkohleeinarbeitung die Kohlenstoffvorräte in der okkludierten leichten Fraktion (in Aggregaten gebundene Pflanzen- bzw. Pflanzenkohlereste), was auf eine Bildung von Bindungen zwischen der Kohle und der Mineralphase innerhalb eines Jahres nach Ausbringung hindeutet. Erhöhte Bodentemperaturen hatten hingegen keine Effekte auf die Kohlenstoffvorräte in den verschiedenen Fraktionen.

In der dritten Studie zeigten die Ergebnisse, dass die Einarbeitung von Pflanzenkohle, allein und in Kombination mit Gülle, zu geringeren Makroaggregatmengen im Vergleich zur Kontrolle oder der alleinigen Einarbeitung von Gülle führte. Jedoch waren die Kohlenstoffmengen in der

Makroaggregatfraktion in den Varianten mit Pflanzenkohle ähnlich oder sogar höher als in den Varianten ohne Pflanzenkohle, was auf einen Aggregateinschluss und eine weitere Stabilisierung der Kohle hindeutet. Trocknung und Wiederbefeuchtung resultierte generell in kleineren Mengen an Makroaggregaten und den entsprechend gebundenen Kohlenstoffvorräten. Dieser Effekt war am stärksten bei den Varianten mit Pflanzenkohle ausgeprägt, was auf eine höhere Anfälligkeit von Aggregaten, die Pflanzenkohle beinhalten, gegenüber einem Zerfall, der durch Trocknung und/oder Wiederbefeuchtung induziert wurde, hinweist.

Insgesamt deuten die Ergebnisse darauf hin, dass sich die Einarbeitung von Pflanzenkohle nachteilig auf die Bildung von Makroaggregaten auswirkt und unter Feldbedingungen nach einem Jahr keine Auswirkungen auf die Kohlenstoffvorräte in Aggregaten hat. Jedoch wurden Verbindungen zwischen Kohle und Mineralphase schon innerhalb kurzer Zeit nach Einarbeitung gefunden, was auf möglicherweise andere langfristige Effekte hindeutet. Selbst in diesen kurzen Zeitperioden von unter einem Jahr wurde die Pflanzenkohle durch die Okklusion in Aggregaten weiter stabilisiert, was den potenziellen Nutzen der Kohle als Mittel zur Kohlenstoffsequestrierung und damit Minderung des Klimawandels unterstreicht.

Die Verwendung organischer Dünger zur Steigerung der Makroaggregatbildung kann nur im Fall von Gülle unterstützt werden, welche in diesem Zusammenhang als sehr effektiv befunden wurde. Temperaturerhöhungen in 10 °C-Schritten wirkten sich aufgrund einer Metabolisierung der Aggregatkittstoffe nachteilig auf die Aggregatbildung aus. Jedoch hatte eine Erhöhung der Bodentemperatur im Feld um 2,5 °C nach sechs Jahren keine Auswirkungen auf Kohlenstoffspeicherung in Aggregat- oder Dichtefraktionen.

2 Summary

Soil organic matter is essential for soil fertility and constitutes a considerable carbon pool against the background of climate change. Soil aggregate formation is important for organic matter protection and long-term carbon storage in soil. While the positive effect of plant residue application on soil macro-aggregate (> 250 µm) formation is known, less is known about the effect of organic fertilizers like cattle manure and slurry on soil aggregate dynamics. Furthermore, rising soil temperatures due to climate change might increase organic matter decomposition, whereas the application of biochar to soil is seen as an option to sustainably increase soil organic matter stocks. However, the effects of elevated soil temperatures as well as biochar application on soil aggregate dynamics remain unclear. Thus, the main objectives of this thesis were to analyze the effects of (i) cattle manure and slurry application, (ii) biochar application and (iii) different temperature regimes on soil aggregate dynamics. For this, two incubation studies were performed and one field trial was sampled and analyzed.

In both incubation studies, soil that was crushed to a size smaller than 250 µm, i.e. without macro-aggregates, was used to determine aggregate formation during the incubation. In the first experiment, the soil was mixed with either biochar, cattle manure, cattle slurry or a combination of biochar and cattle slurry, besides a control. The mixtures were then incubated at 5, 15 or 25 °C for four or eight weeks. Besides macro-aggregate yields and associated carbon, CO_2 emissions during the incubation and microbial biomass C were measured.

For the second study, four treatments of the Hohenheim Climate Change experiment were sampled, including variations in the soil temperature (ambient or elevated (+ 2.5 °C) for six years prior to sampling) and biochar application to the soil (control without biochar or application of 30 t / ha *Miscanthus* biochar one year prior to sampling). The samples were analyzed for basal respiration and microbial biomass C in a short-term incubation and for carbon associated with

aggregate-size and density fractions. The use of *Miscanthus* biochar enabled the tracing of biochar-C by ^{13}C isotopic analyses.

In the third study, a complementary experimental setup to the first study was used, with the focus on the combined application of biochar and slurry. Soil crushed to a size < 250 µm was incubated either with an individual application of biochar or slurry or combined applications of biochar and slurry at different mixture ratios at 15 °C for 60 days. Furthermore, samples were either incubated at constant moisture levels or were subjected to three drying-rewetting cycles over the course of the incubation.

The results from the first study show that the effect of higher temperatures on the intensity of organic matter decomposition was highest for the manure and lowest for the biochar treatment. Higher temperatures also caused significantly lower macro-aggregate yields for the control and biochar (after four weeks) and the manure treatment (after four and eight weeks). This was probably due to a metabolization of the aggregate binding agents. At 15 °C, highest aggregate yields were found for the slurry application treatment. This is thought to be caused by the more liquid and mobile state of the slurry, leading to a more efficient spread across the mineral particles. The application of manure instead did not have an effect on aggregate yields compared to the control. Biochar application also did not cause significant differences in aggregate yield compared to the control and, when applied in combination with slurry, resulted in lower macro-aggregate yields than the individual application of slurry. Significant correlations found between aggregate yields and CO_2 emissions or microbial biomass C further suggest that the applied substrates directly interacted with mineral particles to form macro-aggregates after application, and were subsequently decomposed, which was accompanied by the production of microbially derived aggregate binding agents.

In the second study, the biochar-amended samples taken from the long-term field trial showed significantly higher basal respiration rates than the control, while elevated soil temperatures

mostly increased microbial biomass C. No biochar-C was found in the microbial biomass, indicating that the biochar was not mineralized. Aggregate-associated organic C was not affected by the application of biochar, however, biochar-C was found in all fractions and biochar application also significantly increased organic C associated with the occluded light fraction, indicating the formation of biochar-mineral interactions within one year of application. Elevated soil temperatures, however, had no effects on carbon stocks in the different fractions.

In the third study, the individual application of biochar and the combined application of biochar and slurry were found to result in lower macro-aggregate formation compared to the control and the individual application of slurry, respectively. However, organic C associated with the macro-aggregate fraction was found to be similar or higher in the biochar-amended compared to the control or slurry treatments, indicating an incorporation into the fraction and further stabilization of biochar-C. Drying and rewetting generally decreased macro-aggregate yields and associated C. This effect was especially pronounced for the biochar-amended samples, indicating a higher susceptibility of biochar-containing aggregates to disruption by either drying and/or rewetting.

In total, biochar was found to be detrimental to macro-aggregate formation and to have no effects on aggregate-associated C under field conditions after one year. However, biochar-mineral interactions were found within short periods of time after application, indicating possibly different long-term effects. Even within these short periods of only up to one year, biochar was further stabilized in soil by the occlusion in aggregates, underlining its potential use as a means for C sequestration and thus climate change mitigation. The use of organic fertilizers to increase macro-aggregate formation can only be encouraged in the case of slurry, which was found to be very efficient in this respect. Temperature increases in steps of 10 °C were found to be detrimental to aggregate formation due to a metabolization of the aggregate binding agents. However, a 2.5 °C temperature increase compared to ambient conditions in the field did not have any effects on carbon storage in aggregate or density fractions after six years.

3 General Introduction

Soil aggregate formation is of importance for many soil properties affecting soil productivity such as aeration, resistance against erosion and organic matter storage (Balesdent et al. 2000, Christensen 2001, Six et al. 2000). Major drivers of soil aggregation processes include soil microorganisms, plant debris and roots as well as climate (Six et al. 2004).

Soil aggregates can be subdivided into different size-classes with distinct ecological functions and differing turnover times. Macro-aggregates (> 250 µm) can be formed around organic particles (e.g. plant residues) rendered more reactive (i.e. whose surface contains a higher amount of reactive functional groups) by microbial decomposition. This process results in CO_2 emission as well as the production of microbial-derived aggregate binding agents (Six et al. 2004), binding together organic and mineral particles. Thus, macro-aggregates can also be an accumulation of micro-aggregates (53 – 250 µm) (Six et al. 1999).

Macro-aggregates offer immediate protection of organic matter from further decomposition but are more susceptible for physical disruption than micro-aggregates (von Lützow et al. 2007, Christensen 2001, Six et al. 2000), which are formed within macro-aggregates and released upon their disruption (Six et al. 1999). Organic matter stored in micro-aggregates was consequently found to have a higher turnover time than organic matter in macro-aggregates (von Lützow et al. 2007). Generally, macro-aggregate formation is thus important for the immediate, short-term protection of fresh organic matter, while micro-aggregates are responsible for long-term protection of organic matter in soil.

Several studies could show increasing macro-aggregate formation following the addition of crop residues to soil (e.g. Andruschkewitsch et al. 2014, Helfrich et al. 2008). However, the magnitude of this positive effect was found to differ for residues of different decomposability (Helfrich et al. 2008). While the effect of crop residues on macro-aggregate formation is known,

there is a lack of studies regarding the effect of fertilizers commonly used in organic agriculture such as slurry and manure.

The effect of organic amendments on soil aggregate formation might be also time-dependent. Positive effects on soil aggregation were found to last for a few weeks after residue application (e.g. Helfrich et al. 2008), while longer-term effects possibly rely on N availability, determining whether microbial decomposition following the initial period after application targets microbial-derived binding agents or undecomposed organic matter (Le Guillou et al. 2012). Thus, incubation periods spanning longer timeframes than the initial phase of organic amendment decomposition, usually reached after four weeks, are needed to gain further insight into aggregate formation mechanisms.

In recent years, the application of biochar to agricultural soils has been shown to be beneficial for many soil properties, including nutrient stocks and water holding capacity, as well as crop yield (e.g. Biederman and Harpole 2013). Besides these benefits, biochar is also considered as a means for C sequestration in the soil and thus climate change mitigation (Lehmann 2007). This is due to a high resistance against microbial decomposition, especially for biochars produced at high temperatures (Joseph et al. 2010, Ameloot et al. 2013) due to a high aromaticity and degree of aromatic condensation (McBeath et al. 2014).

Biochar contains reactive functional groups and may form biochar-mineral interactions, for example through adsorbed non-pyrolyzed organic matter (Lin et al. 2012). Interactions of this kind were found within months after biochar application to soil (Joseph et al. 2010, Lin et al. 2012). However, concerning the effect of biochar on soil aggregate yields, contrasting results were found, with several studies reporting positive (Awad et al. 2013, Sun & Lu 2014, Liu et al. 2014) and others no effects (Busscher et al. 2010, Herath et al. 2013, Lei et al. 2013, Awad et al. 2013, Sun & Lu 2014, Zhang et al. 2015). Important factors in determining the extent of a possible effect of biochar on soil aggregation seem to be soil and biochar characteristics, which

strongly differed among the studies, as well as the amount of biochar applied to soil, with larger effects found for larger amounts (Liu et al. 2014, Zhang et al. 2015) and biochar particle sizes, with larger particle sizes possibly limiting soil-biochar interactions (Herath et al. 2013, Zhang et al. 2015). The biochars applied might also differ in the amount of reactive functional groups and thus reactivity, controlling the extent of biochar-mineral interactions. Low biochar reactivity might be increased by the combined application with slurry, as slurry contains reactive compounds such as organic acids (Kirchmann and Lundvall 1993, Provenzano et al, 2014).

Contrastingly to the unclear effect on aggregate yields, however, increased organic C contents after biochar application to soil were found for several aggregate-size classes (Liu et al. 2014, Kaiser et al. 2014, Zhang et al. 2015). Hilscher & Knicker (2011) and Singh et al. (2014) also found up to 26 % of the total biochar applied to soil to be incorporated into the (aggregate-) occluded light or heavy fraction within a few months in incubation experiments. Field studies analyzing the effect of biochar on C associated with aggregate or density fractions under temperate conditions are however scarce.

Temperature influences microbial organic matter decomposition (Franzluebbers et al. 2001, Conant et al. 2011) and can affect the formation of organo-mineral complexes and soil aggregates (Davidson & Janssens 2006, Conant et al. 2011). However, detailed results on the effect of temperature on soil macro-aggregate formation are scarce, as in incubation experiments on this issue usually only one incubation temperature was used (De Gryze et al. 2005, Helfrich et al. 2008). Recent studies also found no effects of elevated soil temperatures on C stored in aggregate-size fractions in soils of (unclipped) grasslands (Cheng et al. 2011, Nie et al. 2014). Such studies are missing for agriculturally used soils so far.

Furthermore, changing moisture conditions in soil are known to influence soil aggregation (Denef et al. 2001a, b). However, although the application of biochar to soil might be of largest benefit in drought-affected regions as it is known to increase soil water holding capacity (Atkinson et al.

2010), studies on the effect of drying-rewetting cycles on aggregate formation in soils containing biochar are lacking so far.

Thus, the main objectives of this thesis were to analyze:

(i) the effects of the individual application of cattle manure (chapter 4) as well as the individual or combined application of cattle slurry and biochar (chapter 4 and 6) on soil macro-aggregate formation and associated carbon in incubation experiments,

(ii) the effect of biochar on carbon storage in aggregate-size and density fractions in a field experiment (chapter 5),

(iii) the effect of temperature on soil macro-aggregate formation and associated carbon in an incubation experiment (chapter 4) and carbon storage in aggregate-size and density fractions in a field experiment (chapter 5) and

(iv) the effect of drying and rewetting on soil macro-aggregate formation and associated carbon in an incubation experiment (chapter 6).

4 Effect of biochar and organic fertilizers on C mineralization and macro-aggregate dynamics under different incubation temperatures

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4.1 Abstract

Understanding the effects of soil amendments on organic matter dynamics is essential for the sustainable management of soil. Less is known about the effects of biochar in comparison to commonly used organic fertilizers on soil aggregation and organic matter dynamics. Here, we aimed to analyze the effect of biochar, slurry, and manure on carbon (C) mineralization and macro-aggregate formation. Soil material whose macro-aggregates (>250 µm) have been carefully crushed was mixed with biochar and/or slurry or manure and incubated at 5 °C, 15 °C, and 25 °C over a period of eight weeks. We determined and modelled the CO_2 emissions over the course of the incubation experiment and analyzed the contents of newly formed macro-aggregates, associated C and microbial biomass C after four and eight weeks. Temperature effects on the decomposition intensity were found to be highest for the manure and lowest for the biochar application. CO_2 fluxes across treatments and temperatures were mostly satisfactorily described using 1- or 2-pool-models. Macro-aggregate yields were smaller at higher temperatures in the control and biochar (after 4 weeks) as well as manure (after 4 and 8 weeks) treatments, presumably because of a metabolization of binding agents. At 15 °C, slurry addition resulted in significantly higher aggregate yields compared to all other treatments, which may be attributed to its more liquid state allowing an easier and more efficient contact with mineral particle surfaces. In combination with slurry, biochar resulted in lower aggregate yields than slurry alone at 15 °C. Manure application did not significantly change aggregate yields compared to the control. Correlations between aggregate yields and the microbial biomass or

CO_2 production point towards a direct interaction of the respective substrates with soil particles to form aggregates followed by decomposition of the amendments and production of microbially derived aggregate binding agents.

4.2 Introduction

Soil aggregates play an important role in organic matter (OM) dynamics and are a major factor in stabilizing the soil structure, which is important, for example, for the aeration of soils or their resistance against erosive forces (Balesdent et al., 2000, Christensen, 2001 and Six et al., 2000). According to Six et al. (2004), plant debris and roots, microorganisms, inorganic binding agents, and environmental variables such as climate and soil mineral characteristics are major drivers behind aggregation processes.

Macro-aggregates (>250 µm) can be formed around organic particles such as plant residues whose reactivity (i.e., amount of reactive functional groups on the residue surface) is increased by bacterial and fungal decomposition processes. Such processing by microorganisms leads to the emission of CO_2 and at the same time to the production of microbially derived binding agents rendering the residues into more reactive aggregation cores (Six et al., 2004). Macro-aggregates can further be an accumulation of micro-aggregates (53–250 µm), which in turn are formed within macro-aggregates and are released upon their disruption (Six et al., 1999). Micro-aggregates are supposed to be more stable against disruptive forces resulting from, for example, rain drops or tillage than macro-aggregates (Christensen, 2001 and Six et al., 2000). Consequently, the OM stored in micro-aggregates was found to have a higher turnover time (100–300 years) than that stored in macro-aggregates (15–50 years; von Lützow et al., 2007). Thus, macro-aggregates offer immediate, but rather weak protection from ongoing decomposition processes, while micro-aggregates contribute to the long-term storage of OM in soil.

The productivity especially of poorly structured agricultural soils could be improved by soil additives that promote the formation of aggregates. Several studies showed an increase in soil macro-aggregate formation due to the addition of crop residues (Andruschkewitsch et al., 2014, De Gryze et al., 2005, Helfrich et al., 2008 and Wagner et al., 2007). However, Helfrich et al. (2008) reported for roots and leaves from maize that differences in the decomposability between different types of residues can lead to variations in the magnitude of positive effects on macro-aggregate formation. Beside these uncertainties, respective data for the effects of other widely used organic soil additives such as slurry or manure on macro-aggregate dynamics are scarce.

Biochar addition to agricultural soils gained much recognition in the last decade because of the positive effects on crop yield and soil nutrient stocks among other parameters (e.g. Biederman and Harpole, 2013). Biochar is a C-rich solid material produced by combusting biomass in an oxygen-limited environment. Comparable to crop residues, biochar can be expected to promote aggregation processes because it contains reactive functional groups, which may engage in bonding interactions with suitable reaction partners (Joseph et al., 2010 and Kaiser et al., 2014). However, only few studies with contrasting results analyzed the effect of biochar on macro-aggregate dynamics. Sun and Lu (2014) and Lu et al. (2014) detected an increase in macro-aggregate formation and conservation after biochar addition to clayey soils due to physical soil improvements including an increased water holding capacity and reduced tensile strength. In contrast, Pronk et al. (2012) found negative effects of charcoal application on the amount of macro-aggregates >2 mm in an artificial soil within a one year incubation study. The contrasting effects might be a result of differences in the biochar reactivity (i.e., amount of reactive functional groups) that strongly depends on production conditions and feedstock (Keiluweit et al., 2010). The combined application of biochar and slurry might be a way to increase the biochar reactivity and, consequently, the ability to form macro-aggregates because slurry contains reactive compounds such as organic acids (Kirchmann and Lundvall, 1993 and Provenzano et al., 2014). To the best of our knowledge, the effects of biochar applied individually vs. applied in

combination with slurry on the dynamics of soil macro-aggregates and associated OM have not been analyzed.

Aggregation processes in soil are tightly linked to the activity of the decomposer community (Le Guillou et al., 2012). The addition of manure, slurry, or charcoal to soil might exert different effects on the activity of microorganisms because of differences in their composition (e.g., C/N ratio, amount of low molecular compounds) (Helfrich et al., 2008 and Le Guillou et al., 2012). An increase in microbial activity can lead to less aggregation due to the intensified decomposition of organic binding agents. On the other hand, enhanced microbial activity might also promote the formation of aggregates due to the production of microbially derived binding agents. The occurrence of such contrasting effects are supposedly time dependent because positive feedbacks on soil aggregation were observed to reach the maximum after a few days to weeks of residue addition while longer lasting effects seem to be less clear (e.g., Helfrich et al., 2008). According to a conceptual model from Le Guillou et al. (2012), longer termed effects (i.e. more than a few weeks) on aggregate dynamics highly depend on N availability governing the interaction between the production of microbial derived binding agents and their decomposition. Thus, an extended period of analysis exceeding the time needed to detect the previously reported short-term effects, usually observed within about four weeks, should reveal more insights in the longer lasting effects of OM addition on aggregate dynamics. Including different organic additives would also allow for analyzing the time dependency of interactive effects between (i) the type of organic amendment, (ii) the microbial activity as well as OM decomposition, and (iii) the formation of macro-aggregates and amount of associated OM.

Climatic effects on aggregation processes were mostly analyzed with respect to variations in soil moisture and connected wetting-drying-cycles (Bravo-Garza et al., 2009 and Wagner et al., 2007). Although temperature is known to influence microbially mediated decomposition processes (Franzluebbers et al., 2001), its influence on aggregation processes is unclear

because previous studies usually used one incubation temperature (De Gryze et al., 2005 and Helfrich et al., 2008). To elucidate the outlined gaps in knowledge in more detail, we performed microcosm experiments over a period of 8 weeks under 5 °C, 15 °C and 25 °C. In this study we aimed to analyze the effects of biochar, manure, and slurry applications under varying temperature on the formation of soil macro-aggregates and the amount of associated OM.

4.3 Material and Methods

4.3.1 Site description and soil sampling

Soil samples were taken in April 2013 from sites in Friemar (west of Erfurt) and Zschortau (north of Leipzig) in eastern Germany. The sites are part of long-term field experiments installed to analyze the impact of tillage intensity on soil properties and productivity in 1992/93 and 1997/98, respectively (Koch et al., 2009). The site in Friemar is a Haplic Phaeozem (3% sand, 68% silt, 29% clay) with a mean annual precipitation of 517 mm and a mean annual temperature of 7.8 °C, while the site in Zschortau is a Gleyic Luvisol (33% sand, 53% silt, 14% clay) with a mean annual precipitation of 512 mm and a mean annual temperature of 8.8 °C (Koch et al., 2009). The two contrasting soils were chosen as field replicates. Samples were taken from the conventionally tilled plots, managed with annual moldboard ploughing to the depth of 30 cm. More detailed site information are given in Koch et al. (2009). A composite soil sample was taken from each plot consisting of twelve individual core samples taken from 0 to 30 cm depth. The soil samples were sieved <5 mm.

4.3.2 Experimental setup: incubation experiment

The dry soil was carefully ground to pass a 250 µm sieve (Andruschkewitsch et al., 2014 and Helfrich et al., 2008) to ensure the complete dispersion of macro-aggregates. The amount of the remaining material on top of the 250 µm sieve was negligible and discarded in case of the clayey soil but was collected and mixed in equivalent parts into each of the <250 µm subsamples (see below) in case of the sandy soil.

The organic soil additives analyzed in this study were biochar, slurry and manure. We used commercially available biochar (Verora GmbH, Switzerland) manufactured from woodchips by combustion at 575 °C, containing 77.4% C and 1% N (C/N = 77.4), and produced according to the European biochar certificate (Schimmelpfennig and Glaser, 2012). The biochar was dried at 40 °C and ground to pass a 53 µm sieve. The analyzed slurry and manure were obtained from an organic cattle farm in Schwäbisch Hall (Southern Germany). The manure was cut into smaller pieces in order to allow a more homogenous application.

The sample size for the incubation was 150 g dry soil material, which was split up in two 75 g subsamples filled into 120 ml pots because of spatial restrictions. The biochar was added at a rate of 30 g per kg of dry soil (equivalent to 30 t ha^{-1}, 0 – 10 cm, assuming a dry bulk density of 1 t m^{-3}), which is in the range of commonly used rates in previous studies (e.g. Bruun et al., 2014, Sun and Lu, 2014 and Lu et al., 2014). Afterwards, the samples were shaken over night to assure a homogenous mixture of the biochar with the soil. For slurry and manure, the amounts added were adapted to the common field application rate of 150 kg N ha^{-1} corresponding to 0.3 g N per kg dry soil. Due to different C/N ratios, the slurry (C/N = 10.9) treatments received 3.28 g C kg^{-1} soil and the manure (C/N = 19.7) treatments 5.91 g C kg^{-1} soil. The dry soil or the soil/biochar mixture was thoroughly mixed with the slurry or the manure. All samples were brought up to 60 % of their water holding capacity. Thus, five treatments were analyzed: (i) control (no additives), (ii) biochar addition, (iii) biochar + slurry addition, (iv) slurry addition, and (v) manure addition. For all treatments three pseudo-replicates were incubated. Samples were incubated in incubation vessels containing water to prevent desiccation of the samples in climate chambers at three different temperatures: 5 °C, 15 °C or 25 °C. One subset of the samples was incubated for four weeks and a further one was incubated for eight weeks. The CO_2 evolution was monitored by continuous flushing of the headspace of each vessel with varying amounts of fresh air, depending on the expected and already measured CO_2 emissions, and subsequent chromatographic measurement of the CO_2 concentration in the exhaust air (Loftfield et al.,

1997). Depending on the total number of vessels connected to the gas chromatograph, one measurement took place at least every six hours. After finishing the incubation experiment, the two subsamples of 75 g each were combined and sieved to pass 10 mm. An aliquot of the material was then dried at 40 °C for aggregate fractionation, while a second aliquot was sieved to pass 2 mm and used for the determination of microbial C.

4.3.3 Laboratory analyses: separation of water-stable aggregates and determination of the bulk soil, aggregate, and microbial biomass C concentrations

Water-stable aggregates were separated according to a wet-sieving fractionation method first described by Cambardella and Elliott (1993) and modified by Jacobs et al. (2009). Thirty grams of the dried soil were placed on a 250 µm sieve and submerged in water for 10 min. Free organic particles floating on the water during the 10 min of submergence were skimmed off. Subsequently, the sieve was gently moved 50 times up and down to separate the fraction >250 µm. Thereafter, the sample material <250 µm was placed on a 53 µm sieve and treated accordingly to separate the fractions 250–53 µm and <53 µm. The fractions >250 µm (i.e., macro-aggregates) and 250–53 µm (i.e., micro-aggregates) were vacuum filtered (>0.45 µm) and dried at 40 °C for 48 h. The solid sample material <53 µm was precipitated with a 0.5 M $AlCl_3$ solution (2.5 ml l^{-1}). The supernatant was siphoned off and the precipitate was filtered and dried as described above.

The bulk soil samples and aggregate size fractions were ball-milled and analyzed for total C (C_t) by dry combustion using a CN elemental analyzer (Elementar Vario El, Heraeus, Hanau, Germany). As no carbonates were present in the soil, the C_t data correspond to organic C (C_{org}). C data for the aggregate-size fractions are given in percentage of the bulk soil C content. Microbial biomass C (C_{mic}) was determined by Chloroform-Fumigation-Extraction (Vance et al., 1987) on fresh samples directly after the incubation.

4.3.4 Statistical analyses

The data were tested for homoscedasticity using Bartlett's Test. The macro-aggregate dry yields, aggregate-associated as well as microbial biomass C concentrations after 4 and 8 weeks, and the cumulative CO_2 emission rates after 8 weeks were analyzed for significant differences ($p < 0.05$) with one-way analyses of variance for the effects of temperature and treatment. The residuals were checked for normal distribution graphically as well as with the Shapiro-Wilk test. In the case of non-normally distributed residuals, the data were log-transformed, which was done for the analysis of the treatment effect on cumulative CO_2 emission at 25 °C. In the case of non-homogeneity of variances, Welch analyses of variance (oneway.test, R Development Core Team, 2014) were used followed by pairwise t-tests with Bonferroni corrections without pooled standard deviations and without the assumption of equal variances. For correlation analyses, data were tested for normal distribution using the Shapiro-Wilk test. Pearson product-moment correlation coefficients (r) were calculated for macro-aggregate yields and microbial biomass C for each sampling date (n = 30). Spearman rank correlation coefficients (r_s) were calculated for macro-aggregate yields and CO_2 emission rates for each sampling date (n = 30) as the CO_2 data were not normally distributed. Only significant correlations ($p < 0.05$) are discussed.

The following equations were used to calculate non-linear models for the cumulative C emission rates (in mg CO_2-C kg^{-1} soil), which were calibrated for the 25 °C temperature treatment for the control (Eq. 1) and the slurry and manure treatments (Eq. 2):

$$Cumulative\ C\ mineralization\ = C_{max,\ con} * (1 - \exp(-a * k_{con} * t)) \quad \text{(Eq. 1)}$$

with $C_{max,con}$ being the maximum mineralisable content of C (1506.2 mg kg^{-1}, 11.2 % of the native C) at the experimental conditions, k_{con} being the rate constant for this pool ($2.6 * 10^{-3}$ day^{-1}) and t being the time.

$$Cumulative\ C\ mineralization$$

$$= C_{max,\ con} * (1 - \exp(-a * k_{con} * t)) + C_{max,\ substrate} * (1 - \exp(-a * k_{substrate} * t)) \quad \text{(Eq. 2)}$$

with $_{Cmax,substrate}$ being the maximum mineralisable content of C of the substrate at the experimental conditions (slurry: 1401.4 mg kg^{-1}, 42.7% of slurry C added, manure: 3160 mg kg^{-1}, 53.4% of manure C added) and $k_{substrate}$ being the rate constant for the substrate pool (slurry: 1.39 * 10^{-2} day^{-1}, manure: 1.41 * 10^{-2} day^{-1}).

In both equations the effect of temperature on the C mineralization was accounted for by defining a, the rate modifying factor for temperature as used in the RothC-26.3 model (Coleman and Jenkinson 1999) (Eq. (3)):

$$a = \frac{47.9}{1+exp^{(\frac{106}{T+18.3})}} \quad \text{(Eq. 3)}$$

with “T” the respective temperature in °C. The values for a are thus 3.81, 1.91 and 0.50 for 25, 15 and 5 °C, respectively.

Using the estimates from the calibration for the control, slurry and manure treatments, respectively, at 25 °C, CO_2 evolution was calculated for the 5 °C and 15 °C incubations of these treatments as well as all temperature variants of the treatments involving biochar, for which the parameter estimates of the control (biochar) or the slurry treatment (biochar + slurry) were used.

All statistical analyses were performed with R (R Development Core Team, 2014).

4.4 Results

4.4.1 CO_2 emissions and microbial biomass C

For all treatments, after eight weeks we detected an increase in the cumulative CO_2 emission rates with increasing incubation temperature (Fig. 4.1). Cumulative CO_2 emissions from the control and biochar treatments were similar and ranged from 77 to 529 mg C kg^{-1} soil across the incubation temperatures. Compared to these, the CO_2 emission rates from the slurry and biochar + slurry treatments were higher and ranged from 560 to 1662 mg C kg^{-1} soil, while the highest CO_2 emissions were detected for the manure treatments (513 – 3056 mg C kg^{-1} soil). The CO_2 fluxes were generally satisfactorily described (calibration of 25 °C for the control, slurry and manure treatment) or estimated (validation of 15 °C and 5 °C for all treatments and 25 °C for the biochar and biochar/slurry treatment) using one- or two-pool-models (Fig. 4.1). However, the model overestimated CO_2 fluxes for manure at 5 °C. The microbial biomass C concentration in the control and biochar treatments accounted for 84 – 176 mg C kg^{-1} soil after four weeks and for 101 – 165 mg C kg^{-1} soil after eight weeks (Fig. 4.2). In the slurry and manure treatments, the microbial biomass C concentration was between 192 and 468 mg C kg^{-1} soil after four weeks and between 267 and 497 mg C kg^{-1} soil after eight weeks.

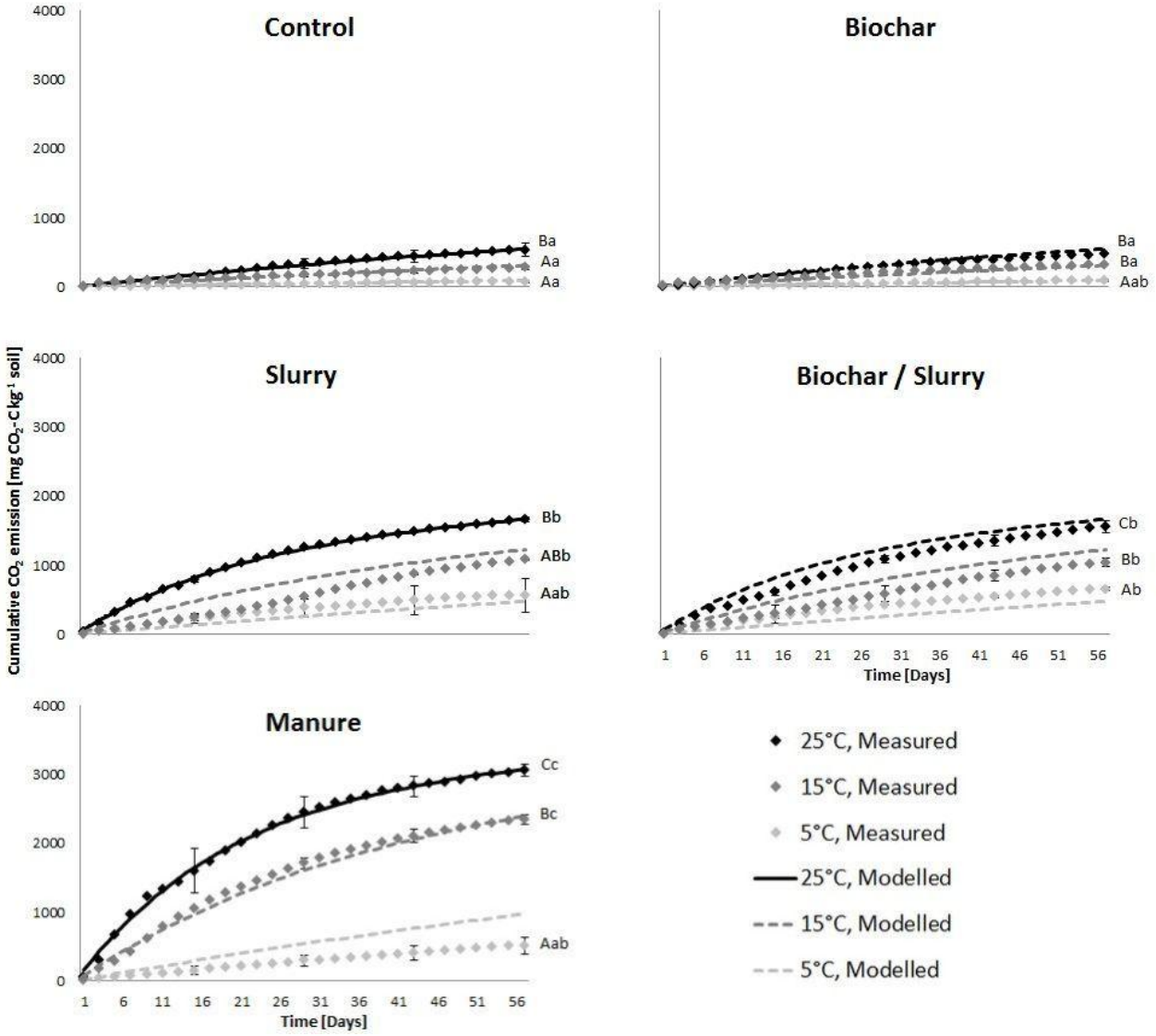

***Figure 4.1**: Measured and modelled cumulative CO_2 emissions for the different treatments over the eight weeks of incubation with standard deviations after 15, 29, 43 and 57 days as well as capital letters signifying significant differences between temperatures for the same treatment and lower-case letters signifying significant differences between treatments for the same temperature after 57 days, and model parameters calibrated for the 25°C variant of the control, slurry and manure treatments, respectively.*

4.4.2 Macro-aggregate yield and associated C

The concentration of macro-aggregates was mostly higher at lower than at higher temperatures, while the duration of the experiment (four or eight weeks) did not result in large differences. For 5 °C, 15 °C and 25 °C incubation temperatures, the macro-aggregate dry mass concentration ranged from 233 to 606 g kg^{-1} soil, from 91 to 637 g kg^{-1} soil, and from 100 to 383 g kg^{-1} soil (Fig. 4.2), respectively, with the highest value from the slurry or biochar + slurry treatment and the lowest from the biochar treatment.

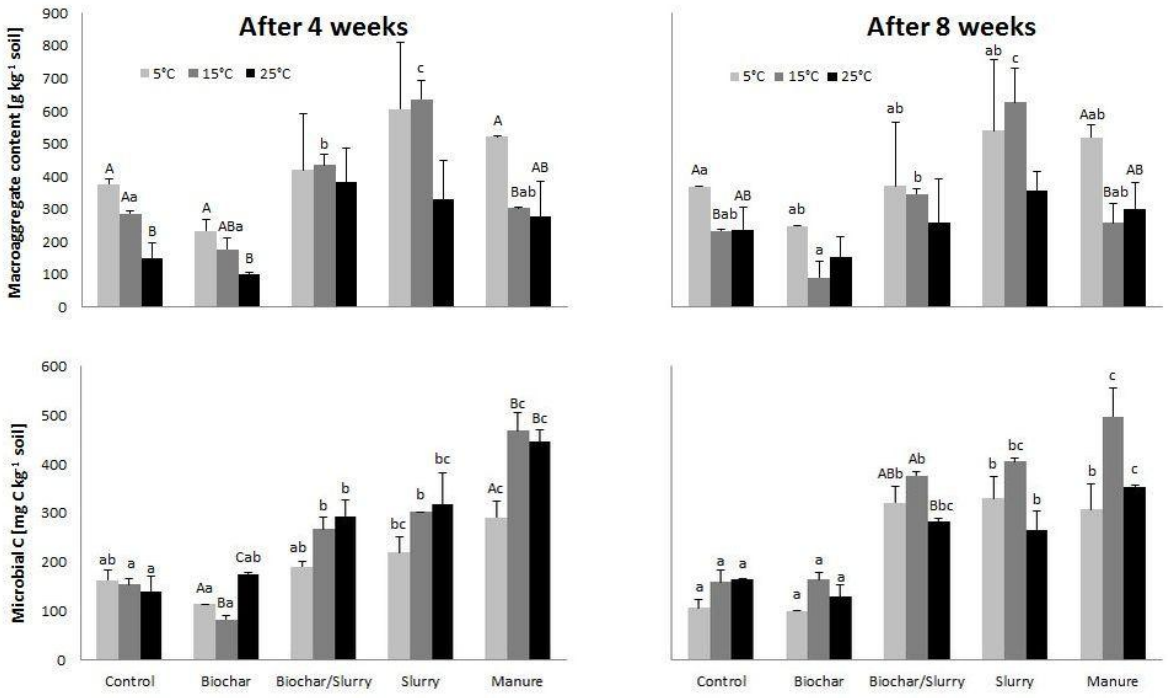

***Figure 4.2**: Macro-aggregate yields and concentrations of the microbial biomass C. Capital letters signify significant differences between temperatures for the same treatment and duration and lower-case letters signify significant differences between treatments for the same temperature and duration.*

The macro-aggregate associated C concentrations had a similar distribution among the treatments as the macro-aggregate dry mass concentrations. The macro-aggregates separated from the biochar treatments accounted for 5 – 22 % of bulk soil C (Table 4.1). Those separated

from the control and from the slurry or manure treatments accounted for 14 – 34 % and for 25 – 66 % of bulk soil C, respectively.

Table 4.1*: Organic C (OC) contents of the macro-aggregate fractions of the different treatments after incubation given in % of the bulk soil organic C (SOC) in the bulk soil, with standard deviations in brackets, capital letters signifying significant differences between incubation temperatures for the same treatment and duration and lower-case letters signifying significant differences between treatments for the same temperature and duration.*

		Temperature		
Duration	**Treatment**	**5°C**	**15°C**	**25°C**
		OC macro-aggregates/SOC (%)		
4 Weeks	**Control**	33.9 (2.5) A	23.0 (0.7) Bab	14.2 (1.4) Cab
	Biochar	20.0 (6.7)	12.6 (8.0) a	7.1 (3.4) a
	Biochar / Slurry	43.8 (15.3)	42.1 (0.7) bc	37.5 (7.8) b
	Slurry	65.6 (19.9)	56.5 (7.5) c	36.8 (8.4) b
	Manure	62.0 (7.0) Ab	33.0 (5.3) Bab	32.4 (5.2) Bb
8 Weeks	**Control**	32.8 (0.3)	19.6 (3.0) ab	21.2 (7.5)
	Biochar	22.1 (3.1)	4.8 (0.9) a	12.0 (10.1)
	Biochar / Slurry	38.3 (16.4)	33.2 (4.6) b	24.7 (10.1)
	Slurry	57.3 (18.4)	62.5 (11.7) c	38.3 (5.3)
	Manure	62.7 (5.5) A	33.0 (5.6) Bb	31.9 (3.7) B

4.5 Discussion

4.5.1 Carbon mineralization and microbial biomass

For each treatment, the CO_2 emission rates were significantly higher at 25 °C than at 5 °C, which indicates that the decomposition intensity generally increases with increasing temperature. The magnitude of this difference (CO_2 emission at 25 °C minus CO_2 emission at 5 °C) decreases in the sequence manure > slurry and biochar/slurry > biochar and control (differences are significant between the treatments separated by ">"). This suggests that the temperature effect on the decomposition intensity is substrate-specific and largest for manure while lowest for biochar. The overestimated CO_2 fluxes by the two-pool-model for manure at 5

°C probably result from this high temperature sensitivity of manure decomposition, leading to disproportionally higher CO_2 fluxes at 25 °C compared to 5 °C.

The amount of CO_2 cumulatively emitted within the first four weeks is in most cases larger than the amount emitted in weeks five to eight. This effect can be observed for all treatments incubated at 25 °C where the CO_2 cumulatively respired after the first four weeks accounted for 59 % (control) to 79 % (manure) of the total amount of respired CO_2 (cumulative after eight weeks). This effect is less pronounced for the CO_2 amounts emitted within the first four weeks at 15 °C and 5 °C incubation temperature. The emission rates account for 48 % (slurry) to 71 % (manure) of the total amount of the respired C at 15 °C and for 47 % (control) to 66 % (slurry) at 5 °C. Only for the treatments with organic soil additives at 25 °C incubation temperature, did the higher emissions rates measured within the first four weeks compared to those of weeks five to eight correspond with higher concentrations of the microbial biomass C measured after four rather than after eight weeks. For the samples incubated at 15 °C (except slurry) and at 5 °C (except control and biochar), the CO_2 emission rates were higher within the first four weeks but the microbial biomass C concentration was higher after eight weeks than after four weeks (3 % to 68 %). The data imply that the microbial biomass seems to become less active or less efficient over time in terms of decomposition processes and CO_2 production at incubation temperatures of 5 °C and 15 °C compared with 25 °C.

4.5.2 Macro-aggregate dynamics

The concentration of macro-aggregates and the macro-aggregate associated C in the control samples decreased with increasing temperature, showing significant differences between the samples incubated at 5 °C and 25 °C after four weeks. This inverse trend in comparison with the CO_2 emission rates suggests an accelerated decomposition of organic compounds at higher temperatures that are potentially involved in the formation of macro-aggregates at lower temperatures. Similar to the control treatments, the addition of 30 g biochar kg^{-1} soil resulted in

significantly lower macro-aggregate yields at 25 °C incubation temperature compared to 5 °C after four weeks with an opposite effect observed for the CO_2 emission rates. Independent from the incubation temperature and time, the concentrations of macro-aggregates and the macro-aggregate associated C (in % of the bulk soil C) detected for the biochar treatments were smaller than the respective values of all other treatments including the control. The lowest organic C recovery observed for the macro-aggregates of the biochar treatments imply a preferential accumulation of the applied biochar in aggregate or particle size fractions smaller than 250 µm. This is in line with studies from Brodowski et al. (2006) and Kaiser et al. (2014) and might have been promoted by size of the biochar used in this study being smaller than 53 µm.

The combined application of biochar and slurry resulted in a significantly increased macro-aggregate concentration compared to the control treatment after four weeks at 15 °C incubation temperature but a significantly decreased macro-aggregate concentration compared to the individual application of slurry at 15 °C. Similarly, Sun and Lu (2014) detected lower macro-aggregate concentrations in a clayey soil compared to the control after the application of biochar made from woodchips at a rate of 2 %. However, at a rate of 6 % larger concentrations compared to the control soil were found. These data suggest a strong effect of the biochar application rates on soil aggregate formation. The application rate of 3 % as used in our study might therefore be too low for a positive effect on the formation of macro-aggregates.

At 15 °C incubation temperature, the concentrations of macro-aggregates and the associated C (in % of the bulk soil C) were significantly higher for the samples of the slurry treatment compared to those of any other treatment (except for aggregate-associated C after four weeks compared to the biochar/slurry treatment) after four as well as eight weeks. This indicates the highest efficiency of slurry regarding macro-aggregate formation among the studied soil additives. This higher efficiency can be a result of the more mobile, liquid, and less dense state

of the slurry compared to biochar or manure, allowing it to overcome spatial disconnections more easily and to spread reactive slurry compounds more efficiently on surfaces of soil constituents, thus enhancing their ability to form aggregates.

The application of manure did not result in higher macro-aggregate yields or associated C (in % of the bulk soil C) compared to the control samples or any other treatment except biochar. Among the analyzed organic additives, only the manure application resulted in significantly higher macro-aggregate associated C (in % of the bulk soil C) at 5 °C than at 15 °C and 25 °C incubation temperatures, which was detectable after four as well after eight weeks. We assume the high temperature sensitivity of the decomposition intensity of manure as indicated by the CO_2 emission rates to be the driving force behind this observation. At 5 °C and low decomposition intensity, the manure seems to be involved in the formation of macro-aggregates but in the course of rising temperatures these binding agents are easily utilized by microorganisms. This might be a result of lower amounts of easily available carbon in the manure than in the slurry due to the straw content of the manure.

4.5.3 Conceptual considerations

The macro-aggregate formation after substrate addition can conceptually be subdivided into two pathways. The direct pathway would imply that the organic substrate (biochar, slurry, manure) enters the soil and immediately interacts with other soil compounds to form macro-aggregates. Furthermore, the organic additives serve as food for the microorganisms, leading to an increase in microbial biomass C and CO_2 emission rates. Considering all treatments, the significant positive correlation between the C_{mic} concentrations and the CO_2 emission rates after four weeks (r_s = 0.85) without detectable positive correlations between C_{mic} and the macro-aggregate parameters points towards the direct pathway in week one to four of the experiment. The larger amounts of bioavailable C derived from the biochar/slurry, slurry, and manure additions may

have led to larger microbial biomass and CO_2 evolution compared to the control and biochar treatments with no positive effect on the macro-aggregate formation.

The indirect pathway includes the usage of the organic substrate by microorganisms as an energy source, leading to an increase in the microbial biomass and CO_2 emission. Here, the microbial biomass, microbially derived residues such as hyphae or cell walls, and the microbially processed organic compounds act as the main macro-aggregate binding agents. The significant positive correlations between C_{mic} and CO_2 emissions (r_s = 0.78) and C_{mic} and the macro-aggregate yield (r = 0.42) as detected after eight weeks suggest a stronger effect of the microbial derived binding agents on aggregation processes in weeks five to eight. This is corroborated by the partly detected lower CO_2 emissions in weeks five to eight compared to weeks one to four that coincided with higher microbial biomass C concentrations measured after eight weeks.

4.6 Conclusions

In our study, we found biochar to have no significant effect on the formation of macro-aggregates except a negative one in combination with slurry at 15 °C. Slurry application was found to be most effective in aggregate formation at 15 °C, which presumably stems from its liquid form allowing it to spread its reactive compounds most efficiently. Manure application did not significantly increase aggregate yields compared to the control. Higher incubation temperatures resulted in mostly lower aggregate yields, hinting at an increased mineralization of aggregate binding agents. The temperature sensitivity of the decomposition intensity was satisfactorily understood through modelling and was found to be highest for manure and lowest for biochar. Correlations between aggregate yields and CO_2 flux or microbial C hint at a direct interaction of soil particles with the organic amendments and a subsequent decomposition of the substrates producing microbial products acting as aggregate binding agents.

5 Influence of elevated soil temperature and biochar application on organic matter associated with aggregate-size and density fractions in an arable soil

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5.1 Abstract

The effects of biochar amendments under elevated soil temperatures on the dynamics of soil organic matter are largely unknown. The objective of this study was to analyze the effect of biochar application and elevated soil temperature on the amount and composition of organic matter (OM) associated with soil fractions of different OM turnover rates. Samples were taken from four treatments of the Hohenheim Climate Change Experiment with the factors temperature (ambient or elevated by 2.5 °C, initiated in 2008) and biochar (control and 30 t / ha *Miscanthus* pyrolysis biochar, corresponding to approximately 12.4 to 13.9 g biochar kg^{-1} soil, applied in 2013) in two depths (0 - 5 and 5 - 15 cm) in August 2014. Microbial biomass C (C_{mic}) and basal respiration were analyzed within an incubation experiment. Aggregate-size fractions were separated by wet-sieving and the free light (fLF), occluded light (oLF), and heavy fractions were isolated by density fractionation. All fractions were analyzed for organic carbon (OC) and $\delta^{13}C$ and by infrared spectroscopy. C_{mic} was mostly significantly ($p \leq 0.05$) increased by elevated temperature in both depths and no biochar-C was found in the microbial biomass. Biochar

significantly increased basal respiration by 24 – 68 %. Biochar and elevated temperature had no significant effects on the OC associated with aggregate-size classes, although small amounts of biochar were incorporated into all fractions already after one year of application. Biochar application markedly increased the OC associated with fLF and oLF. The proportion of C=O groups of the aggregates > 250 µm and 250 – 53 µm and of the oLF fraction was significantly increased in the biochar treatments. The results suggest that already within one year, biochar-mineral interactions had occurred that had led to an aggregate occlusion of the applied biochar as revealed by the results for the oLF fractions. At least in the short-term, the effects of biochar on the amount and composition of OM associated with different aggregate-size and density fractions do not seem to be affected by elevated soil temperatures.

5.2 Introduction

Soil temperature is considered as a key factor for the microbial decomposition of organic matter (OM) (Conant et al., 2011). Due to climate change, soil temperatures will presumably increase in the future but the effects of elevated soil temperature on the microbial decomposition of OM in the long-term compared to the short-term are unclear (Conant et al., 2011; Davidson and Janssens, 2006). The decomposition of soil OM (SOM) is important for the nutrient cycling and productivity of agroecosystems whereas the accumulation and stabilization of OM against microbial decay is important not only for the function of soil to mitigate climate change by carbon (C) sequestration, but also for the sustainability of agricultural production, e.g. by improving the soil's water holding capacity and the energy and nutrient supply. The OM in temperate and aerated soils is mainly stabilized against microbial decomposition by different mechanisms such as the formation of organo-mineral complexes and aggregates, which might also be influenced by soil temperature (Conant et al., 2011; Davidson and Janssens, 2006).

Climatic and management effects on decomposition and stabilization processes can be analyzed by an experimental separation of the bulk SOM into discrete fractions with different

turnover times. The separation of different aggregate-size fractions by wet-sieving, for example, revealed an increase in the turnover time of the associated OM with decreasing aggregate size (Christensen, 2001; Tisdall and Oades, 1982; von Lützow et al., 2007). Using a fractionation scheme based on differences in the density of soil compartments, the light, unprotected and less stabilized plant residues can be separated from the heavier organo-mineral associations (Christensen, 2001; von Lützow et al., 2007). Recently, lack of effects of elevated soil temperature on organic C (OC) contents associated with aggregate or density fractions were reported for soils under unclipped grassland (Cheng et al., 2011; Nie et al., 2014). Such studies are scarce for temperate agricultural soils, which still have potential for C sequestration and may be strongly influenced by soil management (e.g., Freibauer et al., 2004).

Among soil management options, the application of biochar is considered to be an efficient means to sequester OC in the soil and, to a small extent, mitigate climate change (Lehmann, 2007), besides having other positive effects on soil properties such as nutrient retention or soil microbial biomass (e.g. Biederman and Harpole, 2013). Nevertheless, the effects of biochar on soil CO_2 fluxes vary strongly and depend on biochar and soil characteristics (Spokas and Reicosky, 2009). Especially biochars produced at high temperatures (i.e., > 500°C) are associated with higher resistance against microbial decomposition (Ameloot et al., 2013; Joseph et al., 2010), due to a high aromaticity and the degree of aromatic condensation (McBeath et al., 2014).

Additionally, biochar can be stabilized through occlusion in soil aggregates. Biochar-mineral interactions can evolve, for example, via adsorbed non-pyrolyzed OM (Lin et al., 2012) and were found to be formed already after less than a year after biochar application (Joseph et al., 2010; Lin et al., 2012). Several incubation studies reported an increase in aggregate stability through biochar application (Herath et al., 2013; Soinne et al., 2014; Sun and Lu, 2014), whereas field experiments showed contrasting results (Hardie et al., 2014; Liu et al., 2014; Zhang et al., 2015).

Relative increases in macro-aggregates compared to micro-aggregates from biochar application found by several studies depended on soil texture, biochar feedstock or application rate (Liu et al., 2014; Sun and Lu, 2014), while other authors found no or only temporal effects of biochar application on aggregate-size class distribution (Busscher et al., 2010; Lei and Zhang, 2013; Zhang et al., 2015). Possible explanations for the latter case were the biochar amounts applied being too low and the duration of the experiment being too short for significant effects. In contrast, an increase in the OC content of different aggregate-size classes was found by several authors (Kaiser et al., 2014; Liu et al., 2014; Zhang et al., 2015), indicating the incorporation of biochar and/or the increased incorporation of other OM into the respective fractions. The conflicting results suggest that although biochar promoted aggregate formation, the controlling factors behind this promotion are far from being understood. For a better understanding, field experiments are considered to be crucial (Ameloot et al., 2013; Herath et al., 2013; Soinne et al., 2014).

Besides aggregate-size fractions, incubation studies have shown that after several months biochar was found mainly in the free light fraction (fLF, i.e., undecomposed or partially decomposed, not aggregate-occluded plant residues) but up to 26% were also found in the occluded light (oLF) or remaining heavy fraction (HF, i.e., aggregate-occluded, strongly decomposed plant residues and mineral associated OM) (Hilscher and Knicker, 2011; Singh et al., 2014). However, field studies on the effects of biochar on density fractions and their composition are scarce.

In order to study individual and combined effects of biochar addition (30 t/ha, *Miscanthus*, 850 °C) and elevated soil temperature (2.5 °C above ambient) on SOM dynamics in agricultural soils, respective treatments from the long-term Hohenheim Climate Change (HoCC) Experiment were sampled. Besides basal respiration and microbial biomass C (C_{mic}), we analyzed the OC distribution among aggregate-size and density fractions. The composition of the different

fractions was examined via isotopic (C^{12}/C^{13}) and spectroscopic measurements. The application of biochar derived from a C_4-plant and the isotopic measurements enabled us to determine the amount of biochar-derived C in the microbial biomass and the aggregate as well as density fractions. Objectives were to analyze the effects of biochar application to soil and elevated soil temperatures on OC storage in aggregate and density fractions, the microbial biomass and basal respiration rates in a temperate agricultural soil.

5.3 Material and Methods

5.3.1 Study site, experimental setup, and soil sampling

The study site is an arable field at the experimental station Heidfeldhof of the University of Hohenheim (48° 42' 50'' N, 9° 11' 26'' E), with a mean annual temperature of 8.7 °C and a mean annual precipitation of 679 mm. The soil is a Stagnic Luvisol derived from loess with a silty loam texture (9 % sand, 68 % silt, 23 % clay) (Högy et al., 2013; Poll et al., 2013).

In July 2008, the Hohenheim Climate Change experiment was established, manipulating, among other parameters, the soil temperature by an increase of 2.5 °C. A split-plot design was used: Four complete blocks, each with one plot for the control and one plot with elevated temperature were established. Randomization within each block was not possible due to technical restrictions (treatments with roofs for rainfall manipulation required specific locations). For more detailed information on the site and experiment, see Poll et al. (2013). As a further treatment factor, slow pyrolysis biochar made from *Miscanthus* at 850 °C (Bamminger et al., under review) was manually incorporated into the upper 20 cm in August 2013 at the rate of 30 t / ha, corresponding to approximately 12.4 to 13.9 g biochar kg^{-1} soil, on a subplot in each plot, while another subplot remained untreated. The subplots with or without biochar addition were randomly assigned.

The experiment consisted of four treatments: ambient temperature with biochar (T_0-B) and ambient temperature without biochar (T_0-C) (C = control treatment) as well as elevated

temperature with biochar ($T_{+2.5}$-B) and elevated temperature without biochar ($T_{+2.5}$-C). Two undisturbed soil samples were taken from 0 - 5 cm (surface soil) and 5 - 15 cm (subsurface soil) soil depth from each of the four field replicates using 100 cm^3 cores (height 4 cm) in August 2014 after harvesting winter oilseed rape. Field moist soils were sieved < 10 mm and stored at 4°C, > three months.

5.3.2 Basal respiration and microbial biomass

Basal respiration was determined as described by Linsler et al. (2014). Briefly, two laboratory replicates of 20 g field-moist soil (< 2 mm) from each sample were adjusted to 60% water holding capacity and incubated in 1.5 l glass jars for two weeks at 20 °C. The jars also contained 10 ml of 1 M NaOH in a separate beaker, which was replaced after one week. The formed CO_2 was measured by the titration of the solution to pH 8.3 with 1 M HCl after the addition of 5 ml of 0.5 M $BaCl_2$ (Isermeyer, 1952). C_{mic} was determined by Chloroform-Fumigation-Extraction (Joergensen, 1996; Vance et al., 1987) directly after the incubation. Following Helfrich et al. (2008), 0.05 M instead of 0.5 M K_2SO_4 was used, as a large amount of salt would hamper ^{13}C-analyses (Potthoff et al., 2003).

5.3.3 Separation of aggregate-size and density fractions

Water-stable aggregates were separated according to a wet-sieving fractionation method described by Cambardella and Elliott (1993) and modified by Jacobs et al. (2009). Dried (48 h at 40 °C) soil (30 g) was placed on a 250 µm sieve and submerged in water for 10 minutes. Free, non-aggregate-occluded, organic particles (almost exclusively consisting of biochar) floating on the water were skimmed off and discarded. Subsequently, the sample was sieved by moving the sieve gently 3 cm vertically 50 times to separate the fraction > 250 µm. Especially the samples containing biochar still showed large amounts of free organic particles after the sieving, so the water used to wash the fraction > 250 µm from the sieve was decanted, refilled and decanted again until no free particles were visible anymore. Thereafter, the sample material < 250 µm was

placed on a 53 µm sieve and treated accordingly to separate the fractions 250 - 53 µm and < 53µm. The fraction 250 - 53 µm, especially for the biochar samples, still contained considerable amounts of free, non-aggregate-occluded, organic particles, which were removed with a syringe due to the small size of the particles and discarded. The fractions > 250 µm (i.e., macro-aggregates) and 250 - 53 µm (i.e., micro-aggregates) were vacuum filtered (> 0.45 µm) and dried at 40 °C for 48 h. The sample material < 53 µm was precipitated with a 0.5 M $AlCl_3$ solution (2.5 ml l^{-1}). The supernatant was siphoned off and the precipitate was filtered and dried as described above.

The density fractionation was performed as described by Jacobs et al. (2009), with modifications concerning the disaggregation of the soil, which was carried out via sonication (Branson Digital Sonifier, Branson Ultrasonics Corporation, Dietzenbach, Germany) to account for a reproducible input of disruptive energy (Kaiser and Berhe, 2014). Also, following Linsler et al. (2013), < 5 mm sieved soil was used instead of < 2 mm sieved soil in order to prevent aggregate disruption prior to the fractionation. Briefly, 7 g of field-moist soil was placed in a 60 ml centrifugation tube with 28 ml of a sodium polytungstate (SPT) solution. A density of 2.0 g cm^{-3} was chosen, based on a series of pre-tests aimed at recovering the maximum OC yield for the fLF and oLF. The tube was revolved five times by hand and left resting for 30 min. The samples were then centrifuged for 30 min at 4000 x g, after which the supernatant was vacuum-filtered (< 0.45 µm) and washed with 2 l of distilled water, to gain the fLF.

In order to separate the oLF, the soil pellet was dispersed by sonication at 300 J cm^{-3}, as this energy level was found to be most effective in a series of pre-tests. For sonication, samples were converted from the centrifugation tubes to glass beakers. During sonication, samples were cooled < 50 °C with ice surrounding the beaker. Afterwards, samples were converted back to the tubes and centrifuged, and the supernatant was filtered as described above following a 30 min resting period. The pellet remaining in the tube was regarded as HF. To clean the HF of

remaining SPT, the samples were placed in a beaker with 1.5 l of distilled water and precipitated with a 0.5 M $AlCl_3$ solution (2.5 ml l^{-1}) (John et al., 2005). The supernatant was siphoned off and the precipitate washed with an additional 0.5 l of distilled water. Subsequently, all fractions were dried at 40°C for at least 48 h and then weighed.

The C recovery rates in the density fractions ranged from 103 to 127 % (Tab. 1). Recovery rates above 100 % may partly be explained by the choice of the larger mesh size (5 mm) during the sample preparation for this analysis compared to the preparation of the bulk soil samples for C analysis (2 mm).

5.3.4 Carbon measurements and isotopic analyses

The bulk soil samples, aggregate-size fractions and the heavy fraction were ball-milled, while the light fractions were ground by hand in an agate mortar. Bulk soil and aggregate fraction samples were analyzed for total C (C_t) by dry combustion using a CN elemental analyzer (Elementar Vario El, Heraeus, Hanau, Germany), while C_t in the density fractions was analyzed during the isotope analysis (see below). No carbonates were found in the soil using the Scheibler method, thus C_t is considered to represent OC. The OC contents and the dry mass yields of the single fractions were multiplied to calculate the OC contents in g C kg^{-1} soil for each fraction.

In order to trace the biochar-C, $\delta^{13}C$ values in ‰ V-PDB were determined for the bulk soil samples, the aggregate and density fractions as well as for the K_2SO_4-extracts, used for the analysis of C_{mic}, at the Centre for Stable Isotope Research and Analysis in Göttingen, Germany. The fraction of biochar-derived C to the total C content was calculated as follows (Eq. 1) (Luo et al., 2011):

$$\textit{fraction of biochar-C} = (\delta^{13}C \text{ of sample} - \delta^{13}C \text{ of control}) / (\delta^{13}C \text{ of biochar} - \delta^{13}C \text{ of control}) \quad \text{(Eq.1)}$$

with “sample” being the biochar-containing sample in question and “control” the corresponding sample (same depth, same temperature treatment (ambient or elevated)) without biochar.

5.3.5 Infrared spectroscopic analyses

Shifts in OM composition were assessed using diffuse reflectance infrared spectroscopy (DRIFT) (Tensor 27, BRUKER, Ettlingen, Germany). Ball milled bulk soil samples and aggregate-size fractions were examined without any dilution. The density fractions (fLF and oLF: pestled, HF: ball milled) were mixed with KBr in a ratio of 15 mg sample to 220 mg KBr. The mixture was then homogenized in an agate mortar. The spectra were recorded by performing 200 scans in the range of wave numbers from 850 to 4000 cm^{-1} at a resolution of 4 cm^{-1} using an Easy Diff unit (Pike Technologies, Medison, USA). All spectra were baseline corrected using the same subroutine from the OPUS 7.2 software (Bruker, Ettlingen, Germany). Spectral information was parameterized following the method of Ellerbrock et al. (2005). The heights of the adsorption band maxima in the aliphatic region of the DRIFT spectrum (Capriel et al., 1995; Capriel, 1997) representing CH stretching at wavenumbers 2928 ± 20 cm^{-1} and 2856 ± 20 cm^{-1} (Band "A") were added up and related to the maximum height of the broad absorption band at 1625 ± 20 cm^{-1} (Band "B"). The latter signal has been taken in the past to represent C=O functional groups in SOM (Ellerbrock and Gerke, 2013; Kaiser et al., 2014). The magnitude of the absorption maxima in the 1625 ± 20 cm^{-1} region tends to vary with the (i) the abundance of ionizable carboxyl groups and the resulting cation exchange capacity of the OM in the sample, (ii) the polarity and hydrophilicity of the OM in the sample, and (iii) the abundance of proteinaceous material such as microbial debris. By relating the C=O region (B) to the aliphatic region (A) of the DRIFT spectrum, we thus obtain a numerical parameter "B/A" that allows for a comparison of sample spectra. The B/A ratio was then scaled with the OC concentrations (g OC kg^{-1} fraction) to derive a quantitative estimate of the functional group contribution to the concentration of total OM (Kaiser et al., 2008, 2014). This analysis was not feasible for the fLF, as the fraction yield was too small for most of the samples.

5.3.6 Statistical analyses

All statistical analyses were performed with R version 3.3.0 (R Core Team, 2016). Analyses of variances were calculated to study the effects of biochar application and soil temperature on (i) C_{mic}, (ii) basal respiration, (iii) carbon associated with aggregate-size classes, (iv) carbon associated with density fractions and (v) infrared-derived B/A ratio x OC content and hypotheses were as follows: (i) Biochar as well as elevated temperature increase C_{mic}, whereas (ii) basal respiration rates are expected to be unaffected by biochar or temperature after one or six years, respectively. (iii and iv) We expected to find biochar incorporation and thus increased OC contents in the macro-aggregates as well as in the free and occluded light fractions in the biochar treatments, as they usually show the fastest turnover among the analyzed fractions. Elevated temperature likely increases decomposition, but could both increase and decrease aggregate formation, thus we hypothesized no effects or a decrease in C content in the mentioned fractions. (v) We hypothesized an increase in the proportion of C=O groups in OM fractions affected by biochar or elevated soil temperature.

To test our hypotheses, surface and subsurface soils were analyzed separately by three-way analyses of variance with the factors temperature (ambient: T_0, increased: $T_{+2.5}$), biochar (no addition: C, biochar addition: B), block, and the interaction of temperature and biochar and by considering the error structure of the split plot design with the main plot error (error of the interaction of block by temperature, labeled as “residuals (Block x Temperature)”) and the remaining error (error of the subplots (interaction of block by temperature and biochar), labeled as “residuals (Within)”). Stepwise model reductions were carried out, eliminating first a non-significant interaction, then non-significant main effects (Crawley, 2013). Groups were checked for homoscedasticity by Levene’s test (package lawstat) and residuals of the final model were checked for homoscedasticity graphically and for normal distribution by the Shapiro-Wilk test as well as graphically.

In all cases, except for one, interactions were not significant and thus removed. For C_{mic} in the surface soil, the interaction between biochar and temperature was significant ($p \leq 0.05$). Comparisons of means for this case were carried out using a t test (package TukeyC) by considering the error structure of a split plot design.

The infrared-derived B/A-ratio multiplied by the OC content for the macro-aggregates (surface soil), for the micro-aggregates (both depths) and for the oLF (both depths) were Box-Cox-transformed using transformation parameters estimated by maximum likelihood.

For fLF-C in 0 - 5 and 5 - 15 cm one and five observations were missing as no C analysis was possible because of negligible fraction yields and no analyses of variance were carried out.

5.4 Results

5.4.1 C_{mic} and basal respiration

C_{mic} after 14 days of incubation ranged from 186 to 231 mg C_{mic} kg^{-1} soil (Fig. 5.1) with a significant interaction between biochar and temperature in the surface soil and significantly higher values due to elevated temperature in the subsurface soil (Tab. 5.2a). Subsequent t tests by considering the error structure of a split plot design for the surface soil showed significant ($p \leq 0.05$) increases in C_{mic} by elevated temperature ($p = 0.04$). Biochar application significantly increased C_{mic} at ambient temperature ($p = 0.03$), but not at elevated temperature. No biochar-derived C was found in the microbial biomass (data not shown).

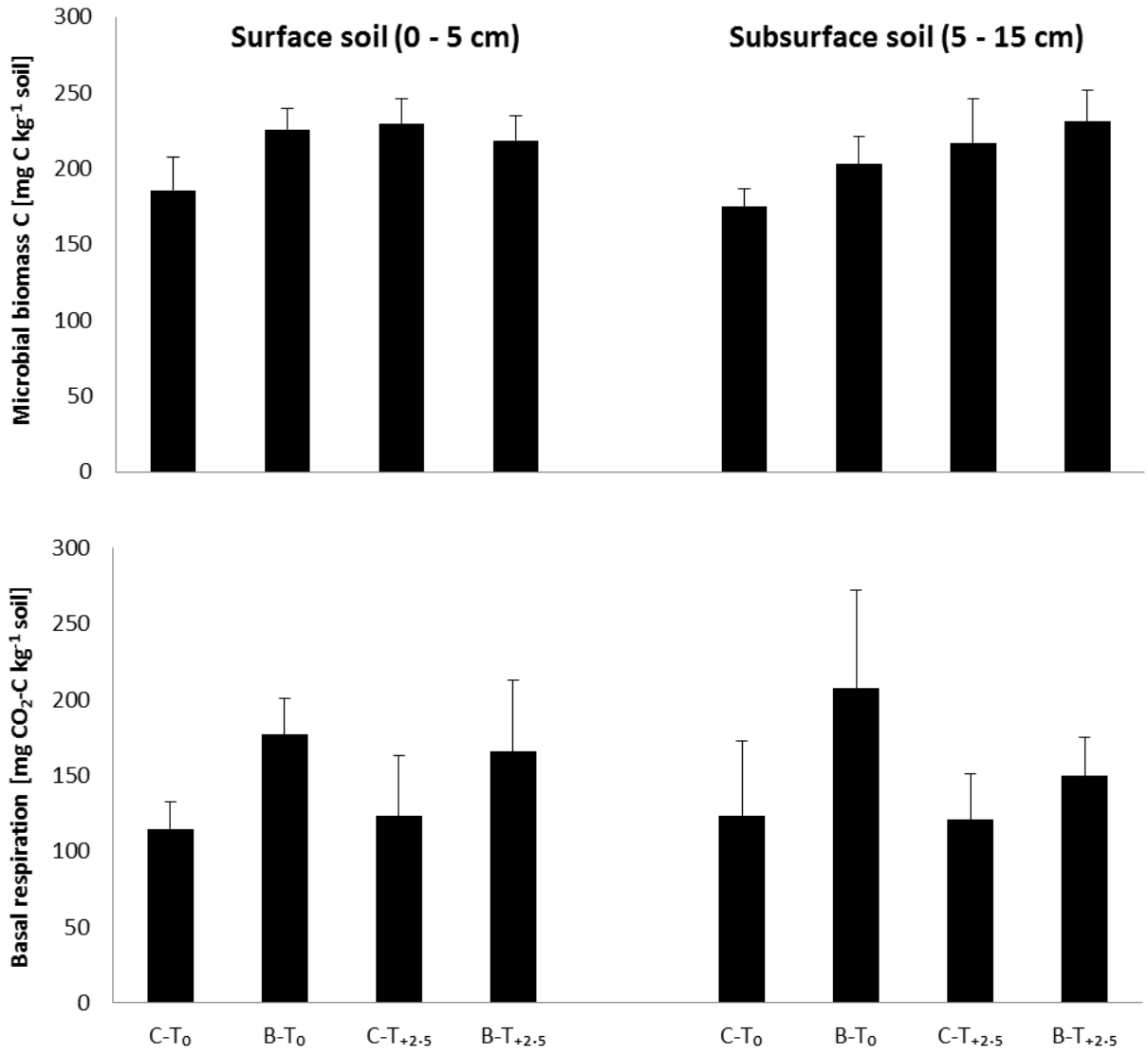

***Figure 5.1**: Cumulative CO_2 emissions over 14 days of incubation at 20°C and microbial biomass C following the incubation in mg C kg^{-1} soil for the treatments with control soil (C), soil with biochar amendment (B) at ambient temperature (T_0) and increased temperature ($T_{+2.5}$). Values shown are means of four field replicates with standard deviations.*

After 14 days, cumulative CO_2 emission rates ranged from 115 to 123 mg CO_2-C kg^{-1} soil in the control samples and from 150 to 207 mg CO_2-C kg^{-1} soil in the biochar samples (Fig. 5.1). In both soil depths, biochar significantly increased basal respiration rates, while elevated temperature had no effect (Tab. 5.2a).

5.4.2 Aggregate-associated C

The OC content associated with the macro-aggregate fraction ranged from 5.1 to 6.5 g C kg^{-1} soil (Fig. 5.2), while micro-aggregate-associated OC ranged from 3.7 to 4.8 g C kg^{-1} soil and OC associated with the silt and clay fraction ranged from 1.0 to 1.4 g C kg^{-1} soil. No effect of biochar or temperature was found for the macro-aggregate and micro-aggregate fractions (Tab. 5.2a). Between 7 and 12 % of the aggregate-associated OC in the respective samples was biochar-derived (Tab. 5.1), corresponding to 0.1 to 0.7 g C kg^{-1} soil.

***Table 5.1**: Biochar-derived C in the bulk soil, aggregate and density fractions in g C kg^{-1} soil and % of the total C in the respective fraction. Given are the means of four field replicates with standard deviations in brackets.*

	T_0, 0-5 cm		T_0, 5-15 cm		$T_{+2.5}$, 0-5 cm		$T_{+2.5}$, 5-15 cm	
	Biochar-derived C							
	[g kg^{-1}]	[%]	[g kg^{-1}]	[%]	[g kg^{-1}]	[%]	[g kg^{-1}]	[%]
Bulk soil	9.5 (1.7)	47 (3)	13 (10)	45 (21)	8.8 (3.0)	45 (11)	10 (3.0)	47 (8)
Aggregate-size classes								
> 250 µm	0.7 (0.2)	10 (3)	0.6 (0.3)	9 (5)	0.6 (0.3)	9 (4)	0.4 (0.2)	7 (3)
53-250 µm	0.3 (0.1)	9 (2)	0.4 (0.3)	10 (5)	0.3 (0.1)	7 (2)	0.3 (0.1)	7 (3)
< 53 µm	0.1 (0.0)	8 (1)	0.2 (0.1)	12 (7)	0.1 (0.0)	10 (2)	0.2 (0.0)	10 (3)
Density fractions								
fLF	10 (1.6)	99 (3)	11 (7.7)	98 (2)	9.4 (3.9)	91 (5)	9.7 (2.8)	95 (6)
oLF	1.7 (0.5)	36 (4)	1.6 (1.1)	36 (17)	1.4 (0.6)	31 (10)	1.3 (0.4)	30 (9)
HF	0.4 (0.1)	5 (2)	0.6 (0.5)	8 (7)	0.2 (0.1)	4 (2)	1.0 (0.3)	15 (6)
Recovery rate for biochar-derived C in the density fractions								
	127 %		103 %		125 %		120 %	

T_0 = ambient temperature; $T_{+2.5}$ = elevated temperature

> 250 µm / 53 – 250 µm / < 53 µm = aggregate-size fractions

fLF = free light fraction; oLF = occluded light fraction; HF = heavy fraction

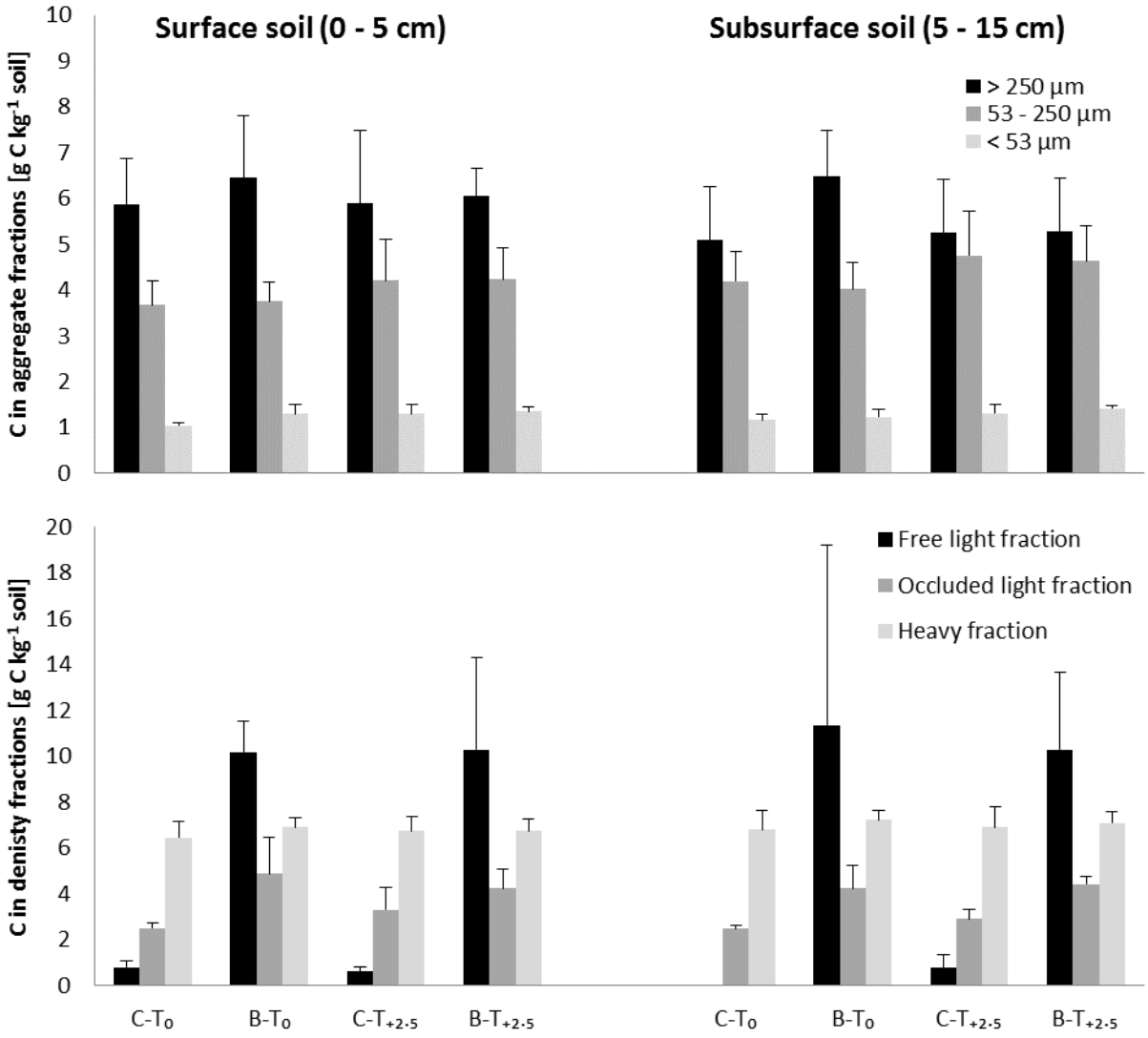

***Figure 5.2**: Organic carbon contents in the different aggregate-size and density fractions in g C kg^{-1} soil for the treatments with control soil (C), soil with biochar amendment (B) at ambient temperature (T_0) and increased temperature ($T_{+2.5}$). Values shown are means of four field replicates with standard deviations.*

5.4.3 Density fractionation

The OC content associated with the fLF ranged from 0.6 to 0.8 g C kg^{-1} soil for the control treatments and from 10.2 to 11.4 g C kg^{-1} (Fig. 5.2) soil for the biochar treatments. In 0 - 5 cm and under elevated soil temperature in 5 - 15 cm, biochar markedly increased OC contents. For the oLF, we detected contents between 2.5 and 3.3 g C kg^{-1} soil for the control and from 4.2 to 4.9 g C kg^{-1} soil for the biochar treatments. In both depths, biochar significantly increased the

OC contents, while elevated temperature had no effect (Tab. 5.2b). In the heavy fraction, between 6.4 and 7.2 g C kg^{-1} soil was found. In the biochar samples, the largest part of the fLF consisted of biochar (91 – 99 %, Tab. 5.1), while biochar-derived OC made up between 30 and 36 % of the oLF-C, corresponding to 1.3 to 1.7 g C kg^{-1} soil. In the remaining heavy fraction, 4 - 15 % of the associated OC was biochar-derived, corresponding to 0.2 to 1.0 g C kg^{-1} soil.

5.4.4 Infrared spectroscopy analyses

The B/A ratios scaled with the OC content of the respective sample were significantly higher for the biochar compared to the control treatments in the macro-aggregate fraction and the oLF in both depths and the micro-aggregate fraction in 0 - 5 cm. No effects were found for the temperature treatment (Tab. 5.2b).

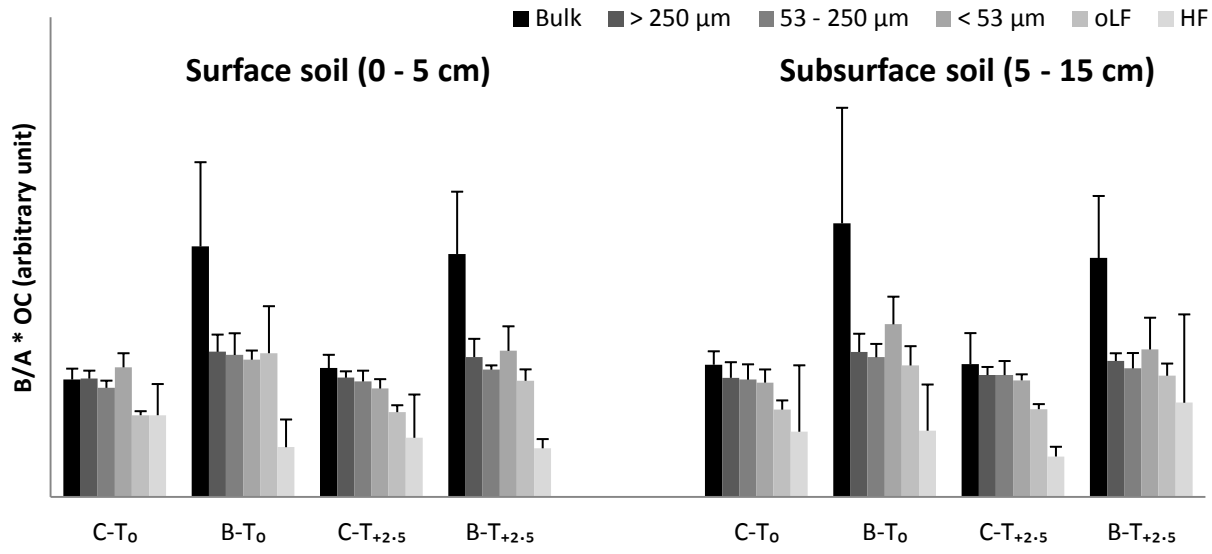

Figure 5.3*: Ratios of absorption band peak heights indicative for C-H (Band A) and C=O groups (Band B) as derived from DRIFT spectra for the different aggregate-size and density fractions and the bulk soil, multiplied by the organic C concentration of the respective fraction for the treatments with control soil (C), soil with biochar amendment (B) at ambient temperature (T_0) and increased temperature ($T_{+2.5}$). oLF = occluded light fraction and HF = heavy fraction. Values shown are means of four field replicates with standard deviations.*

Table 5.2a: Split plot ANOVAs for the effects of biochar, temperature, block and the interaction of biochar and temperature on different parameters in the surface and subsurface soil. The ANOVAs are the results of stepwise model simplifications.

Source	Degrees of freedom	Sum of squares	Mean square	F ratio	P	Source	Degrees of freedom	Sum of squares	Mean square	F ratio	P
	Surface soil						Subsurface soil				
	Microbial biomass C						Microbial biomass C				
Block	3	836	279			Block	3	687	229		
Temperature	1	1352	1352	13	0.04	Temperature	1	4846	4846	20	0.02
Residuals (Block x Temperature)	3	314	105			Residuals (Block x Temperature)	3	728	243		
Biochar	1	816	816	2.1	0.20	Residuals (Within)	8	5895	737		
Biochar x Temperature	1	2654	2654	6.8	0.04						
Residuals (Within)	6	2336	389								
	Basal respiration						Basal respiration				
Block	3	6749	2250			Block	3	7499	2500		
Residuals (Block x Temperature)	4	5749	1437			Residuals (Block x Temperature)	4	9176	2294		
Biochar	1	10909	10909	39	0.00	Biochar	1	12779	12779	6.2	0.04
Residuals (Within)	7	1955	279			Residuals (Within)	7	14538	2077		
	Organic C in the macro-aggregate fraction (> 250 µm)						Organic C in the macro-aggregate fraction (> 250 µm)				
	not significant						not significant				
	Organic C in the micro-aggregate fraction (250 – 53 µm)						Organic C in the micro-aggregate fraction (250 – 53µm)				
	not significant						not significant				

Table 5.2b: Split plot ANOVAs for the effects of biochar, temperature, block and the interaction of biochar and temperature on different parameters in the surface and subsurface soil. The ANOVAs are the results of stepwise model simplifications.

Source	Degrees of freedom	Sum of squares	Mean square	F ratio	P	Source	Degrees of freedom	Sum of squares	Mean square	F ratio	P
	Surface soil						Subsurface soil				
	Organic C associated with the occluded light fraction						Organic C associated with the occluded light fraction				
Block	3	0.95	0.32			Block	3	0.75	0.25		
Residuals (Block x Temperature)	4	3.4	0.84			Residuals (Block x Temperature)	4	1.0	0.25		
Biochar	1	11	11	7.1	0.03	Biochar	1	11	11	29	0.00
Residuals (Within)	7	11	1.5			Residuals (Within)	7	2.6	0.38		
	Box-Cox-transformed infrared-derived B/A ratio x org. C content (> 250 µm)						Infrared-derived B/A ratio x org. C content (> 250 µm)				
Block	3	$7.5 * 10^{-16}$	$2.5 * 10^{-16}$			Block	3	36827	12276		
Residuals (Block x Temperature)	4	$3.0 * 10^{-16}$	$7.5*10^{-17}$			Residuals (Block x Temperature)	4	33760	8440		
Biochar	1	$2.1 * 10^{-15}$	$2.1 * 10^{-15}$	18	0.00	Biochar	1	62527	62527	23	0.00
Residuals (Within)	7	$8.1 * 10^{-16}$	$1.2 * 10^{-16}$			Residuals (Within)	7	18725	2675		
	Box-Cox-transformed infrared-derived B/A ratio x org. C content (250 - 53 µm)						Box-Cox-transformed infrared-derived B/A ratio x org. C content (250 - 53 µm)				
Block	3	$1.8 * 10^{-12}$	$5.9 * 10^{-13}$				not significant				
Residuals (Block x Temperature)	4	$1.1 * 10^{-13}$	$2.7 * 10^{-14}$								
Biochar	1	$5.0 * 10^{-12}$	$5.0 * 10^{-12}$	13	0.01						
Residuals (Within)	7	$2.6 * 10^{-12}$	$3.8 * 10^{-13}$								
	Box-Cox-transformed infrared-derived B/A ratio x org. C content (occluded light fraction)						Box-Cox-transformed infrared-derived B/A ratio x org. C content (occluded light fraction)				
Block	3	$1.7 * 10^{-13}$	$5.7 * 10^{-14}$			Block	3	$1.1 * 10^{-13}$	$3.5 * 10^{-14}$		
Residuals (Block x Temperature)	4	$6.5 * 10^{-14}$	$1.6 * 10^{-14}$			Residuals (Block x Temperature)	4	$6.5 * 10^{-14}$	$1.6 * 10^{-14}$		
Biochar	1	$1.5 * 10^{-12}$	$1.5 * 10^{-12}$	116	0.00	Biochar	1	$1.1 * 10^{-12}$	$1.1 * 10^{-12}$	45	0.00
Residuals (Within)	7	$9.2 * 10^{-14}$	$1.3 * 10^{-14}$			Residuals (Within)	7	$1.7 * 10^{-13}$	$2.4 * 10^{-14}$		

5.5 Discussion

5.5.1 Effects of elevated soil temperature

Elevated soil temperature significantly increased C_{mic} compared to ambient temperature in the surface soil and independently of biochar in the subsurface soil. However, no higher basal respiration values were found for the elevated compared to the ambient temperature treatment, resulting in lower microbial biomass-specific respiration (0.54 – 0.76 for elevated temperature in comparison to 0.62 – 1.02 for ambient temperature, data not shown; Bradford et al., 2008; Luo et al., 2001). Possible reasons include a depletion in labile SOC during the six years of higher field temperatures (Knorr et al., 2005) and/or a microbial adaptation to the higher temperatures, resulting in lower microbial biomass-specific respiration at a given temperature than in the ambient treatment (Bradford et al., 2008).

Elevated temperature exerted no effect on macro-aggregate- and micro-aggregate-associated OC (Fig. 5.2; Tab. 5.1). Similarly, Cheng et al. (2011) and Nie et al. (2014) found no effects of elevated soil temperature on aggregate-associated OC in unclipped grassland soils. Also, no effects of elevated temperature were found for the isolated density fractions, confirming findings of Cheng et al. (2011) for density fractions isolated from aggregate-size fractions from unclipped grassland soil.

Furthermore, temperature did not affect OM composition in the different fractions or the bulk soil (Fig. 5.3). This lack of large effects of elevated temperature on OM retention in the different fractions might result from the ambivalent nature of temperature effects on organo-mineral interactions. While elevated soil temperatures favor desorption relative to adsorption for high-affinity organo-mineral interactions (e.g. van der Waals forces, hydrogen bonding; regulated by equilibrium thermodynamics), it is vice versa for low-affinity interactions (regulated by diffusion) (Conant et al., 2011). Thus, the net effect of higher temperatures on organo-mineral interactions can be minimal (Davidson and Janssens, 2006). Our findings and those of previous studies

(Cheng et al., 2011; Nie et al., 2014) suggest that an increased soil temperature will not result in an enhanced OM protection against microbial decomposition due to additional organo-mineral interactions and OM occlusion in aggregates. This is of relevance for an estimation of the fate of SOM under changing climatic conditions because the highest sensitivity for temperature change is given for the topsoil where usually also the highest OM contents can be found. However, since neither our nor other studies could detect a lasting increase in the basal respiration caused by higher soil temperatures, the overall effect of elevated soil temperature on the OM stored in soil remains unclear. For further elucidation, long-term studies combined with more detailed microbiological analyses are required.

5.5.2 Effects of the biochar application to soil

One year after its application, biochar had significantly increased C_{mic} for ambient temperature in the surface soil, while it had no significant effect at elevated temperature or in the subsurface soil (Tab. 5.2a). Former studies often found increases of C_{mic} after the application of biochar (Biederman and Harpole, 2013; Lehmann et al., 2011), with the biochar providing a habitat for microorganisms (Ameloot et al., 2013; Lehmann et al., 2011). The lack of effects of biochar application on C_{mic} at elevated soil temperature in this study is probably a result of the larger influence of higher temperatures on microbial growth (see above). Thus, the climatic effect seems to be more important than the management effect in this case.

Independent of depth and temperature, biochar significantly increased basal respiration. This is surprising, as previous studies found increases in soil respiration after biochar addition to be short-lived, i.e. not present one year after application (Lehmann et al., 2011). A possible reason for increased soil respiration may be a small mineralization of the biochar. However, as no biochar-C was found in the microbial biomass after the incubation, this seems to be unlikely in our study. Nevertheless, biochar is beneficial for parameters like water holding capacity and pH (data not shown) (e.g. Basso et al., 2013; Van Zwieten et al., 2010), which might have positively

influenced the microbial activity, especially during an incubation under optimal conditions (20 °C, 60 % water holding capacity).

Biochar exerted no significant effects on the macro- or micro-aggregate-associated OC. This is in contrast to results from Liu et al. (2014) and Zhang et al. (2015), who detected increased OC contents in all isolated aggregate-size fractions and in fractions larger than 1 mm, respectively. However, these studies were conducted under different climatic conditions and with soils of different textures. Nonetheless, in our study biochar-C was found to be incorporated into all aggregate fractions, making up of about 10 % of the total OC in each fraction (Tab. 5.1). This indicates that aggregation-promoting biochar-mineral interactions were already present after one year, confirming data from studies under different climate conditions (Joseph et al., 2010; Lin et al., 2012).

As expected, the biochar application led to an increase in the fLF-associated OC, as more than 80 % of total biochar-C was found in this fraction. Thus, the largest portion of the biochar amendment remained non-occluded in aggregates after one year, as similarly found under different environmental conditions (Hilscher and Knicker, 2011; Singh et al., 2014). Biochar significantly increased oLF-associated OC independently from depth and temperature (Fig. 5.2, Tab. 5.2), as reported before for intra-aggregate fractions (Kimetu and Lehmann, 2010; Qayyum et al., 2014). The additional OC in this fraction made up between 11 and 14 % of the total biochar-C present in the soil (Tab. 5.1), which is in the range found within previous studies (Hilscher and Knicker, 2011; Singh et al., 2014) and similar to the total biochar-C found in all aggregate fractions, accounting for 9 to 12 % of the bulk soil biochar-C. We assume that mainly the smaller biochar particles interacted with the soil compounds to form aggregates as it was hypothesized that coarser biochar particles might limit soil-biochar interactions (Herath et al., 2013; Zhang et al., 2015). However, the biochar particle size effect on the formation of soil aggregates needs further systematic evaluation. The remaining HF-associated OC remained

largely unchanged by the biochar amendment and mostly only small amounts of biochar-C were recovered in this fraction, as could have been expected after one year due to the relatively slow turnover of this fraction (von Lützow et al., 2007).

Concerning the infrared spectroscopic analyses, the significantly higher B/A*OC values in most fractions for the biochar compared to the control treatments (Fig. 5.3) indicate an increase in C=O groups in these samples. This increase could be derived from the biochar itself or from intensified microbial decomposition processes that can be concluded from higher basal respiration rates found for the biochar samples. The latter can be the precursor for further organo-mineral interactions and aggregate formation because of the increasing reactivity of the biochar caused by higher contents of C=O groups.

5.5.3 Interaction between elevated soil temperature and biochar

Biochar incorporation into aggregate-size and density fractions was very similar at ambient and elevated temperature (Tab. 5.1), indicating that temperature did not affect biochar allocation through, for example, increased decomposition, which could be expected given the higher temperature sensitivity of biochar compared to more easily decomposable, not thermally altered organic matter (Davidson and Janssens, 2006). However, this lack of interactions between biochar and elevated temperature is in line with Fang et al. (2014) who found that the temperature sensitivity of biochar can be lowered by organo-mineral interactions to levels comparable to native SOM pools, which were largely unaffected by elevated temperature in this study as well. We thus assume the suitability of biochar application for the long-term soil C sequestration to be amplified by interactions with minerals and occlusion in aggregates. Although no negative effect of elevated soil temperature on soil OM stabilization was found in this study, the impact of a changing climate on OM fractions of different turnover kinetics in soil remains unclear. The elucidation of the long-term mitigation potential of biochar for the

postulated accelerated OM decomposition due to rising soil temperatures remains a challenging task for further studies.

5.6 Conclusions

In this study, we found a 2.5 °C increase in soil temperature for six years to mostly increase C_{mic}. Elevated soil temperature had no effects on the OC stored in aggregate-size or density fractions. Therefore, in the short-term rising soil temperatures may have no or only minor effects on the decomposition and allocation of OM among fractions of different turnover rates. This lack of pronounced short-term effects might be beneficial for a balanced long-term adaption of agroecosystems to the expected increase in soil temperatures.

Biochar-C was found in each aggregate-size fraction already one year after the application of 30 t/ha, which had also led to a significant increase of the OC associated with the oLF fraction. Therefore, biochar-mineral interactions seem to be formed quickly and are probably not dependent on intensive long lasting microbial processing (i.e., increase of reactive-oxygen-containing functional groups). The results also suggest a fast additional stabilization of biochar, besides being less attractive for microorganisms than native OM, by interactions with soil minerals, which underscores the high potential of biochar for the long-term C sequestration in soil.

5.7 Acknowledgements

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6 Effects of biochar and slurry application and drying and rewetting on soil macro-aggregate formation in silty loam soils

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6.1 Abstract

Although the positive effect of organic soil additives on the formation of macro-aggregates (> 250 µm) is known, the impact of biochar in combination with other organic amendments remains unclear. The objectives were to analyze the effects of biochar and slurry on the formation of macro-aggregates under constant and under alternating moisture conditions. Silty loam soils were sampled from four arable German sites, the soil macro-aggregates were crushed, and each soil was incubated at 15 °C and at 60% of the water holding capacity for 60 days with the addition of biochar and slurry, each at two rates and individually as well as in combination. The soils were further subdivided into two groups that were incubated under conditions of constant soil moisture or three drying-rewetting cycles. Besides monitoring the CO_2 fluxes, samples were analyzed for microbial biomass C (C_{mic}), macro-aggregate yields and macro-aggregate-associated C and three-way analyses of variances (factors: management, moisture and block)

were calculated for these target variables, whereby C_{mic} had to be Box-Cox transformed and cumulative CO_2 emissions and macro-aggregate-C log-transformed. Application of biochar resulted in significantly ($p \leq 0.05$) lower macro-aggregate yields with or without slurry compared to the control or the individual slurry application, probably because of a reduced substrate availability following the adsorption of slurry by biochar particles, resulting in lower microbial binding agent production. In contrast, similar or higher C contents in the macro-aggregate fraction of the biochar-amended treatments as compared to the control or slurry treatment were found, indicating an incorporation of biochar into these fractions. This was especially strong for treatments with biochar and high amounts of slurry, thus the slurry possibly facilitated the formation of biochar-mineral interactions. Drying and rewetting decreased macro-aggregate yields and associated C. This effect was especially pronounced for the biochar-amended samples, indicating a higher susceptibility of aggregates formed by slurry-mediated biochar-mineral interactions compared to aggregates formed by microbial binding agents. Overall, more mechanistic insight into aggregate formation under changing moisture conditions in the presence of biochar is needed to clarify its beneficial role in agricultural soil management.

6.2 Introduction

The formation of aggregates at different scales is important for the soil productivity because of its positive effects on the infiltration capacity, aeration, and organic matter content, among others (Balesdent et al. 2000, Christensen 2001, Six et al. 2000). To elucidate the effects of aggregate characteristics on soil properties and functions, the aggregates are experimentally subdivided into different size-classes. Macro-aggregates (> 250 µm) are formed around organic particles that are rendered more reactive by decomposition processes, which offers immediate protection of fresh organic matter (OM) from further decay (Six et al. 2004). Micro-aggregates (53 - 250 µm) are considered to be more stable with higher turnover times of the associated OM than macro-aggregates (Christensen 2001, Six et al. 2000, von Lützow et al. 2007) and can be formed within macro-aggregates and be released upon their disruption (Six et al. 1999). In

general, aggregate-occluded OM can be stored in the soil for longer periods than non-occluded, free OM with an increasing turnover time by a decrease in aggregate size (von Lützow et al. 2007).

It has been shown that macro-aggregate formation increases after the application of crop residues (e.g., Andruschkewitsch et al. 2014, Helfrich et al. 2008). In contrast, the effects of common organic fertilizers such as slurry and manure on macro-aggregate formation are less understood (Grunwald et al. 2016). Beside organic fertilizers, the application of biochar to soil has been shown to be beneficial for crop yield, nutrient supply, and water holding capacity, among others (e.g., Biederman and Harpole 2013) but the effects of biochar on aggregate formation remain unclear. Previous studies found positive (Awad et al. 2013, Liu et al. 2014, Sun and Lu 2014) or no effects (Awad et al. 2013, Sun and Lu 2014, Zhang et al. 2015) of biochar on macro-aggregate yields. Factors that may affect the aggregate formation are soil and biochar characteristics as well as the amount of biochar applied. Zhang et al. (2015), for example, noted that the low amount of biochar (9 t/ha) might have prevented a positive effect on aggregate formation, while Liu et al. (2014) found strongest positive effects for the highest biochar amounts applied (up to 40 t/ha).

Furthermore, the reactivity of biochar (i.e., abundance of oxygen containing, reactive functional groups such as -COOH and -OH), which controls the extent of interactions between biochar and the soil mineral phase, strongly differs among feedstocks and production conditions (Keiluweit et al. 2010). Management-wise, the reactivity of biochar might be increased by the combined application with slurry. Slurry contains reactive compounds such as organic acids (Kirchmann and Lundvall 1993, Provenzano et al. 2014) that may spread efficiently on biochar surfaces due to the liquid state of the slurry. However, possible effects of combining different amounts of biochar and/or slurry on soil macro-aggregate formation remain unclear (Grunwald et al. 2016).

The application of biochar is postulated to be beneficial for agricultural production especially in areas affected by drought periods due to the increased soil water holding capacity (Atkinson et al. 2010). Although it is known that drying and rewetting can affect soil aggregation (Denef et al. 2001a, b), the effect of biochar on soil aggregate dynamics under drying-rewetting cycles is little understood.

In this study, we aimed to clarify the effects of the individual and combined application of biochar and slurry on macro-aggregate formation and associated organic C contents as affected by the soil moisture conditions in a range of silty loam soils from four sites. For this, we mixed soil material, where the existing macro-aggregates had been artificially crushed, with biochar and/or slurry and incubated the mixtures for 60 days at 15 °C under constant or alternating (three drying-rewetting cycles). To gain further understanding of the mechanisms involved, we also measured the CO_2 emissions and the microbial biomass C (C_{mic}).

6.3 Material and Methods

6.3.1 Site description and soil sampling

Soil samples were taken in June and July 2015 from the long-term field experiments Friemar (near Erfurt, running since 1992), Grombach (near Heilbronn, running since 1990), Garte-Süd and Hohes Feld (both near Göttingen, running since 1970 and 1967, respectively) in Germany. These experiments are used to analyze the effect of tillage intensity on soil properties and productivity. All samples were taken from the respective conventional tillage treatments. The site in Friemar is a Haplic Phaeozem, while the other three sites are Haplic Luvisols (Andruschkewitsch et al. 2013, Jacobs et al. 2009). All four soils have a silty loam texture (2 - 16 % sand, 65 - 78 % silt, 15 - 31 % clay) and were chosen to represent a variety in silty loam soils. Organic C contents ranged from 0.75 to 1.43 %, while N contents ranged from 0.08 to 0.14 %. More detailed site information is given in Andruschkewitsch et al. (2013) and Jacobs et al. (2009).

6.3.2 Setup of the incubation experiment

Each air dried soil was carefully ground to pass a 250 µm sieve (Andruschkewitsch et al. 2014, Helfrich et al. 2008) to ensure the complete dispersion of macro-aggregates. The amount of the remaining material on top of the 250 µm sieve was negligible and discarded.

As soil additives, we used biochar and cattle slurry. The added biochar was produced by combustion of woodchips at 575 °C according to the European biochar certificate (Schimmelpfennig and Glaser 2012) by Verora GmbH, Switzerland, and contained 77.4% C and 1% N. The biochar was dried at 40 °C and ground to a diameter size between 53 and 250 µm. The added cattle slurry originated from Braunschweig, Germany, contained 4.3 % C and had a C/N ratio of 14.

For each experiment, we used 120 g of dry soil material that was filled into 120 ml pots. Biochar was added at two different rates of 60 (B_{60}) and 120 g per kg dry soil (B_{120}), which is in the upper range of application rates used in previous studies (e.g. Jiang et al. 2016, Jones et al. 2011, Zimmerman et al. 2011). Slurry was added at two rates of 0.15 ($S_{0.15}$) and 0.3 g N per kg dry soil ($S_{0.3}$), corresponding to common annual field application rates of 75 and 150 kg N ha^{-1} and containing 2.0 and 4.1 g C per kg dry soil, respectively. When both additives were applied in combination, they were first mixed and then added to the soil. All samples were adjusted to 1.2 g cm^{-3} bulk density and to a water content of 60% of their water holding capacity.

In total we analyzed seven treatments: (i) control (no additives; Con), (ii) high level of biochar addition (B_{120}), (iii) high level of slurry addition ($S_{0.3}$), (iv) low amount of biochar and low amount of slurry addition ($B_{60}S_{0.15}$), (v) high amount of biochar and low amount of slurry addition ($B_{120}S_{0.15}$), (vi) low amount of biochar and high amount of slurry addition ($B_{60}S_{0.3}$) and (vii) high amount of biochar and high amount of slurry addition ($B_{120}S_{0.3}$).

Incubation experiments were carried out as described by Grunwald et al. (2016). Briefly, four field replications as represented by the four soils were incubated for each treatment. Samples were incubated in incubation vessels containing water at the bottom to avoid desiccation in climate chambers at 15°C for 60 days with or without three wetting/drying cycles as a further variant. Samples with drying-rewetting cycles (DRC) were dried at 40 °C for 48 hours for the first time after the first seven days, then rewetted (to 60 % of the water holding capacity) and incubated again. The two further dryings took place 18 days after each rewetting.

The CO_2 evolution during the incubation was monitored by continuous flushing of the headspace of each vessel with fresh air and subsequent chromatographic measurement of the CO_2 concentration in the exhaust air (Loftfield et al. 1997). After the incubation, the samples were sieved to pass a 10 mm sieve. An aliquot of the sample was dried at 40 °C for the aggregate fractionation procedure, while the remaining material was sieved to pass 2 mm to determine C_{mic}.

6.3.3 Determination of macro-aggregate yield, associated C and C_{mic}

Water-stable macro-aggregates were separated according to a wet-sieving fractionation method first described by Cambardella and Elliott (1993) and modified by Jacobs et al. (2009). Thirty grams of the dried soil were placed on a 250 µm sieve and submerged in water for 10 minutes. Free organic particles floating on the water during the 10 minutes of submergence were skimmed off. Subsequently, the sieve was gently moved 50 times up and down to separate the fraction >250 µm, which was vacuum filtered (>0.45 µm) and dried at 40 °C for 48 h. The macro-aggregate fraction and bulk soil samples were ball-milled and analyzed for total C concentrations, which correspond to organic C due to the absence of carbonates in the soil, using a CN elemental analyzer (Elementar Vario El, Heraeus, Hanau, Germany). Chloroform-Fumigation-Extraction was used to determine C_{mic} (Vance et al. 1987; Joergensen 1996) on

fresh samples directly after the incubation using a correction value of 0.45 to account for non-extractable C_{mic} (Wu et al. 1990).

6.3.4 Statistical Analyses

All statistical analyses were performed with R version 3.3.0 (R Core Team 2016). Analyses of variance were calculated to study the effects of biochar and/or slurry application as well as drying-rewetting cycles on (I) C_{mic}, (II) cumulative CO_2 fluxes, (III) macro-aggregate yields and (IV) carbon associated with the macro-aggregate fraction. We hypothesized that (i) slurry and biochar increase C_{mic} as well as cumulative CO_2 fluxes, because slurry is a source of organic material and biochar may improve soil physical conditions, while (ii) drying-rewetting cycles have a negative impact on these parameters due to the death of part of the microbial biomass following the drying. We further expected (iii) slurry and biochar to increase macro-aggregate yields, especially in combination and at higher application levels, for the same reasons as stated above for C_{mic} and CO_2 fluxes and, in the case of biochar and biochar-slurry mixtures, due to the formation of biochar-mineral interactions, and (iv) drying-rewetting cycles to negatively affect aggregate yields, due to aggregate disruption upon rewetting. We also expected (v) biochar to be incorporated into the macro-aggregate fraction and thus cause higher macro-aggregate-associated C levels, due to the formation of biochar-mineral interactions.

To test these hypotheses, each of the four parameters was analyzed by three-way analyses of variance with the factors management treatment (levels: Con, B_{120}, $S_{0.3}$, $B_{60}S_{0.15}$, $B_{120}S_{0.15}$, $B_{60}S_{0.3}$, $B_{120}S_{0.3}$), moisture treatment (levels: with or without DRC), block and the interaction of management and moisture treatment. Stepwise model reductions were carried out, eliminating first a non-significant interaction, then non-significant main effects (Crawley 2013). For all cases, interactions were not significant and thus removed.

Groups were checked for homoscedasticity by Levene's test (package lawstat) and residuals of the final model were checked for homoscedasticity graphically and for normal distribution by the Shapiro-Wilk test as well as graphically.

The data for cumulative CO_2 fluxes and carbon associated with the macro-aggregate fraction were log-transformed as no homoscedasticity was given. The data for C_{mic} were Box-Cox-transformed using a transformation parameter estimated by trial and error as this procedure yielded better results in terms of a normal distribution of the residues of the final model than the estimation of the parameter by maximum likelihood. For all analyses, a significant management treatment effect was found, so Tukey's HSD test was used to determine which treatments were different from each other.

6.4 Results

6.4.1 C_{mic} and CO_2 fluxes

The cumulative CO_2 emissions after 60 days ranged between 92 and 983 mg CO_2-C kg^{-1} soil (Tab. 6.1). The $S_{0.3}$ treatment had significantly higher emission rates than all other treatments except the $B_{120}S_{0.3}$ treatment (tested for Box-Cox-transformed data), while the B_{120} treatment showed significantly lower emissions than all other treatments. The treatments that have experienced drying-rewetting cycles (DRC) were found to emit significantly lower cumulative CO_2 amounts than the respective treatments under constant moisture conditions (Tab. 6.2).

Contents of C_{mic} ranged from 59 to 118 mg C kg^{-1} soil (Tab. 6.1). Except for the $B_{120}S_{0.15}$ treatment, all treatments with slurry significantly increased C_{mic} compared to the control or B_{120} treatment (tested for log-transformed data). Biochar or slurry amount had no further effect. The DRC treatments showed significantly lower contents of C_{mic} than the than treatments with constant moisture (Tab 6.2).

***Table 6.1**: Microbial biomass C (C_{mic}) and cumulative (Cum.) CO_2 fluxes for the different treatments with and without drying-rewetting cycles (DRC). Data shown are means of four replicates with standard deviations, letters show significant ($p < 0.05$) differences between management treatments, calculated for Box-Cox- and log-transformed data, respectively. The effect of drying-rewetting cycles was significant with $p = 0.00$ (C_{mic}) and $p = 0.00$ (Cum. CO_2 fluxes, calculated for the transformed data as well.*

	C_{mic} [mg C kg^{-1} soil]			Cum. CO_2 fluxes [mg C kg^{-1} soil]		
	Without DRC	**With DRC**	**Treatment effects**	**Without DRC**	**With DRC**	**Treatment effects**
Con	70 (18)	65 (16)	A	241 (77)	136 (80)	B
B_{120}	75 (11)	59 (15)	A	103 (19)	92 (48)	A
$S_{0.3}$	118 (17)	90 (14)	B	983 (334)	441 (171)	E
$B_{60}S_{0.15}$	114 (23)	93 (4)	B	462 (257)	246 (125)	CD
$B_{120}S_{0.15}$	89 (11)	80 (7)	AB	338 (172)	235 (125)	BC
$B_{60}S_{0.3}$	111 (22)	92 (15)	B	568 (284)	318 (186)	CD
$B_{120}S_{0.3}$	105 (10)	98 (19)	B	629 (331)	376 (160)	DE

Con: control, B_{120}: 120 g biochar per kg soil, S_3: 0.3 g slurry-N per kg soil; $B_{60}S_{0.15}$: 60 g biochar and 0.15 g slurry-N per kg soil; $B_{120}S_{0.15}$: 120g biochar and 0.15 g slurry-N per kg soil; $B_{60}S_{0.3}$: 60 g biochar and 0.3 g slurry-N per kg soil; $B_{120}S_{0.3}$: 120g biochar and 0.3 g slurry-N per kg soil

6.4.2 Macro-aggregate yields and associated C

Macro-aggregate yields ranged from 5.7 to 40.8 g fraction 100 g^{-1} soil (Fig. 6.1). For the $S_{0.3}$ treatment, significantly higher yields compared to all other treatments were found, while none of the other treatments significantly increased yields compared to the control. Yields for the B_{120} and the $B_{120}S_{0.15}$ treatments were significantly lower than for the control. Treatments with DRC were found to have significantly lower macro-aggregate yields than treatments without (Tab 6.2).

Macro-aggregate-associated C ranged from 2.0 to 24.1 g C kg^{-1} bulk soil (Fig. 6.1). The lowest values were found for the control and B_{120} treatments, while the highest values were found for the slurry-biochar mixtures with high slurry amounts ($B_{60}S_{0.3}$ and $B_{120}S_{0.3}$). The treatments that have experienced DRC showed significantly lower macro-aggregate associated organic C than the respective treatments under constant moisture conditions (tested for log-transformed data, Tab 6.2).

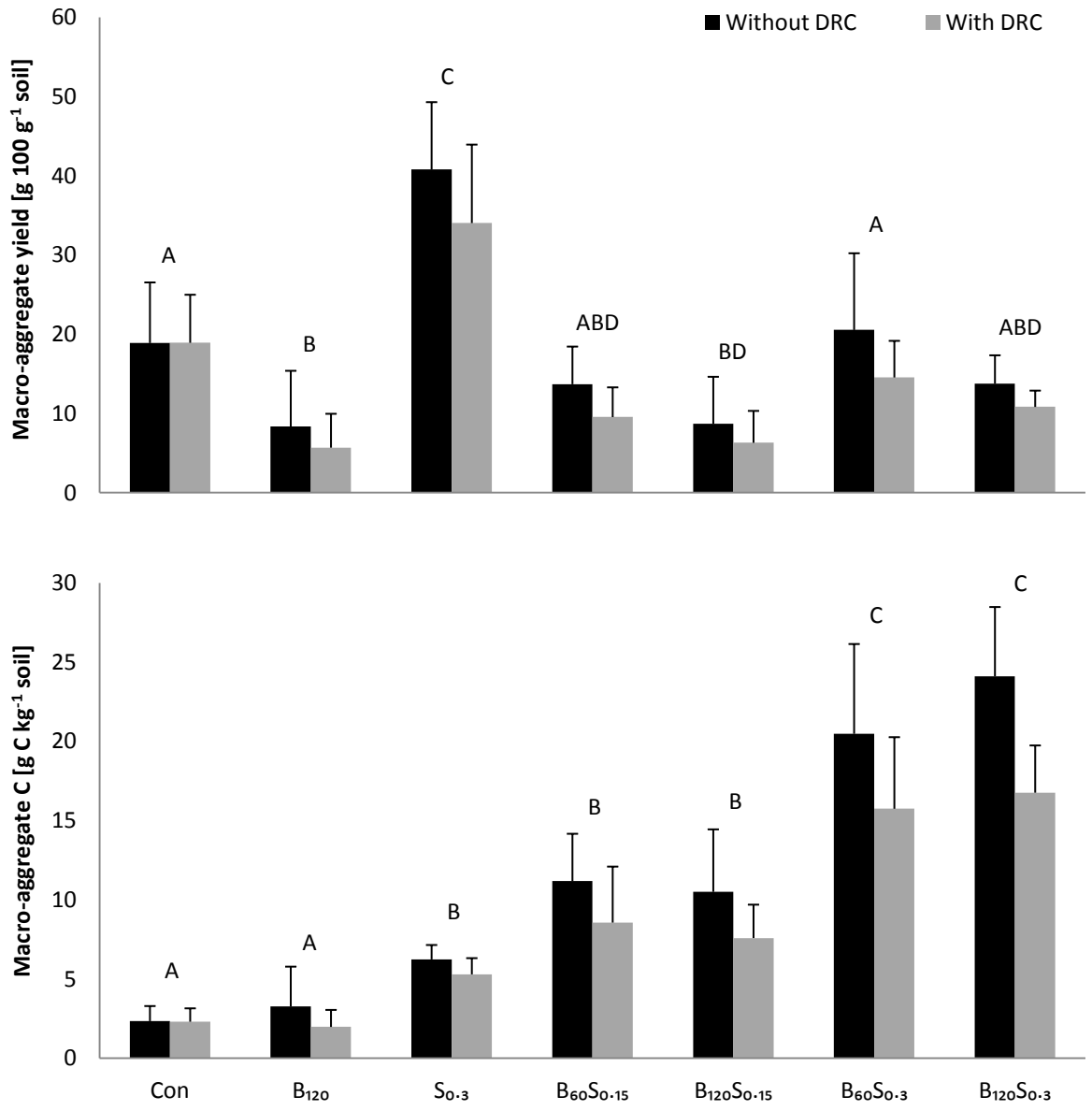

***Figure 6.1**: Macro-aggregate yields and associated C in g 100 g^{-1} soil and in g C kg^{-1}, respectively, for the different treatments with and without drying-rewetting cycles (DRC). Data shown are means of four replicates with standard deviations with letters showing significant ($p < 0.05$) differences between treatments (calculated for log-transformed data in the case of aggregate-associated C). The effect of DRC was significant with $p = 0.02$ (macro-aggregate yields) and $p = 0.01$ (log-transformed macro-aggregate C), respectively.*

***Table 6.2**: ANOVAs for the effects of management treatment, moisture treatment and block on different parameters. The ANOVAs are the results of stepwise model simplifications.*

Source	Degrees of freedom	Sum of squares	Mean square	F ratio	P
	Microbial biomass C (Box-Cox-transformed)				
Management	6	1.4	0.2	13	0.00
Moisture	1	0.3	0.3	16	0.00
Block	3	0.2	0.1	4.7	0.01
Residuals	45	0.8	0.02		
	Cumulative CO_2 fluxes (log-transformed)				
Management	6	3.8	0.6	37	0.00
Moisture	1	0.8	0.8	45	0.00
Block	3	1.4	0.5	28	0.00
Residuals	45	0.8	0.02		
	Macro-aggregate yields				
Management	6	5217	869	31	0.00
Moisture	1	165	165	6	0.02
Block	3	493	165	6	0.00
Residuals	45	1274	28		
	Macro-aggregate-associated C (log-transformed)				
Management	6	7.2	1.2	58	0.00
Moisture	1	0.2	0.2	8.3	0.01
Block	3	0.2	0.1	3.0	0.04
Residuals	45	0.9	0.02		

Management: control and 6 treatments with biochar and slurry individually and in combination

Moisture: with of without three drying/rewetting cycles

Block: four sites

6.5 Discussion

6.5.1 Effects of the organic amendments

6.5.1.1 Cumulative CO_2 emission rates and C_{mic}

As expected, slurry increased CO_2 fluxes and C_{mic} significantly (as tested for log- and Box-Cox-transformed data, respectively) compared to the control caused by the input of fresh organic matter, as found before (e.g., Andruschkewitsch et al. 2014). The CO_2 emissions were reduced in the biochar treatment compared to the control and in the biochar-slurry mixture treatments compared to slurry individually applied. This was found before for biochar-amended soils with and without further organic amendments (Jones et al. 2011, Mukherjee et al. 2016, Prayogo et al. 2014, Zimmerman et al. 2011) and is possibly caused by the adsorption of OM by the biochar, leaving a part of the OM inaccessible for microorganisms (Ameloot et al. 2013). This is probably more pronounced for biochar with high specific surface areas (Ameloot et al. 2013) as found for high temperature biochar (Keiluweit et al. 2010, Schimmelpfennig and Glaser 2012), which was used in this study.

The biochar and the biochar-slurry mixtures did not have an impact on C_{mic} compared to the control and the slurry treatment, respectively, despite the lower CO_2 emissions. A possible reason for this discrepancy might be the better C use efficiency of the microorganisms in the biochar-amended samples, caused by the co-location of microorganisms and adsorbed OM on the biochar surfaces and favoring microbial growth over respiration (Lehmann et al. 2011). This was also found by Jiang et al. (2016) with strongest positive effects of biochar on C use efficiency at 1 and 5 % of biochar addition rates compared to 10 and 20 %, which might explain the lack of differences between the different amounts of biochar applied in our study as a larger application rates seem not to lead to higher C use efficiency compared to a smaller ones.

6.5.1.2 Effects on macro-aggregate yields and associated C

Macro-aggregate yields and associated C in the S_3 treatment were largely increased (80 - 116 %) compared to the control. This could be either due to microbial processing of the slurry and thus the production of microbial binding agents or due to the slurry acting as binding agent itself. Possibly, both mechanisms took place, with one replacing the other over time. Macro-aggregate yields for the B_{120} treatment and the biochar-slurry mixtures were significantly lower than for the control (56 - 70 % less) and the slurry treatment (50 - 81 % less), respectively. This is probably caused by the above-mentioned overall decreased substrate availability through biochar OM adsorption resulting in reduced production of microbial binding agents. Also, significantly higher yields from the $B_{60}S_{0.3}$ treatment compared to the $B_{120}S_{0.15}$ treatment were found. This indicates that an increase in biochar and a decrease in slurry amount lead to lower aggregate yields, as presumably a larger proportion of the added slurry can be adsorbed by the biochar. It is also noteworthy that there was no significant difference between the $B_{60}S_{0.15}$ and $B_{120}S_{0.3}$ treatment despite the different amounts of slurry added. Apparently, when doubling both amendments from the rates used in the $B_{60}S_{0.15}$ treatment, a similar proportion of the available slurry can be adsorbed by the biochar resulting in a similar extent of aggregate formation.

The reduction of macro-aggregate formation is in contrast to former studies on aggregate yields following biochar application to soil, finding positive (Awad et al. 2013, Liu et al. 2014, Sun and Lu 2014) or no effects (Awad et al. 2013, Sun and Lu 2014, Zhang et al. 2015). Possible reasons for this discrepancy are differences between the studies in soil environmental conditions, as well as in the biochar feedstock, application rate, and particle size. For example, large application rates of biochar were found to be more beneficial for soil aggregate formation (Liu et al. 2014, Zhang et al. 2015) and large biochar particle sizes were hypothesized to be detrimental to aggregate formation (Herath et al. 2013, Zhang et al. 2015). The results of this study confirm the findings from Grunwald et al. (2016) where the same type of biochar was used at a rate of 3 % (instead of 6 or 12 % as in this study) and similar reductions in aggregate yields

were found without the addition of slurry. In combination with slurry, decreases in aggregate formation due to the addition of biochar were less pronounced (45 % less than slurry alone) than in this study, hinting at larger negative effects at higher biochar amendments, possibly because of an enhanced adsorption of slurry. Despite not being promotive for aggregation formation, the adsorption of slurry by biochar might be beneficial for nutrient retention. A following slow release of the nutrients over time should be beneficial especially in sustainable land management strategies.

Macro-aggregate associated C in the B_{120} treatment was not different from the control (Fig. 1) despite smaller aggregate yields. The biochar-slurry mixtures showed even higher macro-aggregate-associated C values than the $S_{0.3}$ treatment, which was significant (tested for log-transformed data) for $B_{60}S_{0.3}$ and $B_{120}S_{0.3}$, despite lower aggregate yields. This suggests biochar incorporation into the macro-aggregate fraction in these treatments. Concerning the biochar-slurry mixture, this is more pronounced at the high rate of slurry independent of biochar amount, as shown by significantly higher values for $B_{120}S_{0.3}$ and $B_{60}S_{0.3}$ in comparison to $B_{120}S_{0.15}$ and $B_{60}S_{0.15}$, respectively. The hypothesis by Lin et al. (2012) that biochar-mineral interactions evolved after the adsorption of slurry to the biochar surface, supported by Ca^{2+}-ions acting as a bridge, might serve as mechanistic explanations. Thus, the reactivity of the biochar surface could have been increased by the slurry and the amount of available Ca^{2+} mediating interactions at the biochar/slurry/soil interfaces. This would suggest an abiotic aggregate formation with the slurry itself acting as binding agent. Thus, larger amounts of slurry could result in the aggregation of larger numbers of individual biochar and/or mineral particles.

The longevity of aggregates formed through slurry adsorbed to the biochar surface seems questionable given the nature of slurry as highly decomposable substrate. However, the biochar in the aggregates could offer protection from microbial decomposition by rendering the adsorbed slurry less accessible. Similarly, Rogovska et al. (2011) hypothesized a possible physical

protection of manure-C by biochar, while Liang et al. (2010) found more rapid transition of added C in form of shredded sugar cane leaves into physically protected fractions in soils with biochar than in soils without, despite a lower microbial activity as shown through the metabolic quotient. Also, if the biochar-C is transferred to the more stable micro-aggregate fraction over time (Christensen 2001, Six et al. 2000), the resistance of biochar and adsorbed slurry against microbial decomposition could be further extended, improving the suitability for long-term C sequestration. However, previous studies found either increases in or no effects on aggregate stability after the application of biochar (e.g. Sun and Lu 2014, Zhang et al. 2015) with unknown effects of slurry addition.

6.5.2 Effects of drying-rewetting cycles

In contrast to other studies (summarized, for example, in Borken and Matzner 2009), we did not find a burst in CO_2 flux after rewetting the dried samples, but rather a period of several days in which CO_2 fluxes slowly increased up to the levels of the samples without DRC (data not shown). This led to significantly lower cumulative CO_2 emissions (tested for log-transformed data) for the DRC samples in comparison to the samples without DRC (11 to 55 %). Similar results were found by Muhr et al. (2008) for forest soils, who suggested that the death of a majority of microorganisms due to the drying is followed by a period of repopulation. Also, due to drying induced hydrophobicity, a certain period of time after rewetting might be needed until the previously moist soil domains are in contact with water again (Kaiser et al. 2015).

Contents of C_{mic} were also negatively affected by DRC, yet the reduction compared to the samples without DRC was lower than the reduction in CO_2 emission (7 to 24 %). As all sampled soils are located in rather humid regions, the native microbial community in the soils was probably less adapted to severe drying and rewetting cycles, thus the microbial abundance might not have recovered to full extent after drying and rewetting (Borken and Matzner 2009, Kaiser et al. 2015).

Macro-aggregate yields and associated C for the DRC samples were significantly lower (tested for log-transformed data in the case of aggregate-associated C) than for samples without DRC (17 to 32 % and 15 to 39 % lower values, respectively), except for the control, for which similar values were found in both treatments. As aggregate yields and associated C were reduced to a similar degree, the reduction in C contents (in g C in fraction kg^{-1} bulk soil) is likely a result of the reduction in macro-aggregate yields. Macro-aggregates are susceptible to slaking when dried and rewetted (Denef et al. 2001a, b), especially when rewetted fast (Kaiser et al. 2015). However, no flush of CO_2 derived from the mineralization of newly available OM due to aggregate disruption was found in this study. Also, Denef et al. (2001a, b) reported a decrease in aggregate disruption upon rewetting after the first two DRC, indicating that another process than aggregate slaking might be responsible for the decrease in aggregate formation under DRC. Also, reductions for the control (0 and 2 % for aggregate yields and associated C, respectively) and the $S_{0.3}$ treatment (17 and 15 %) were lower than reductions for the treatments with biochar (21 – 32 and 23 – 39 %), indicating a stronger effect on macro-aggregates containing biochar than formed otherwise.

The data of this study suggest that aggregates formed by the interaction of minerals, biochar, and undecomposed slurry, as hypothesized above, seem to be more susceptible to drying than aggregates formed by microbially produced aggregate binding agents. A reason for this might be a higher aggregate stability due to highly reactive extracellular polymeric substances that are produced as a response to drying at larger amounts in the biochar-free treatments that show a higher microbial activity than the treatments with biochar. The drying might also exert irreversible effects on the slurry at the surface of the biochar rendering it less reactive or less efficiently distributed thereby lowering its importance for the formation of aggregates. This also implies a weakened suitability of biochar to increase soil aggregation at least in areas with varying moisture conditions. Further mechanistic information of this process is necessary to understand the impact of biochar on soil aggregation under changing moisture conditions.

6.6 Conclusions

In our incubation study we found the application of slurry to increase the formation of macro-aggregates while biochar, individually and in combination with slurry in various mixture ratios, could not increase aggregate yields compared to the control. High amounts of biochar (120 g kg^{-1} soil) in combination with low rates (0.15 g N kg^{-1} soil) or no addition of slurry even decreased macro-aggregate yields compared to the control. Despite lower aggregate yields, the biochar and biochar-slurry mixtures resulted in similar or higher aggregate-associated C levels, indicating the incorporation of biochar into macro-aggregates. Due to the sorption characteristics of biochar, we assume the aggregate formation to be partially abiotic with direct interactions between biochar, (adsorbed) slurry, and the mineral phase of the soil. Therefore, in the presence of slurry, a prolonged period of microbial processing does not seem to be necessary to render the biochar suitable to form aggregates.

Drying and rewetting of the samples resulted in significantly lower aggregate yields especially for the biochar-slurry mixtures but aggregate slaking upon rewetting was found to be unlikely to serve as the main cause. The drying of undecomposed slurry as thought to be the most important macro-aggregate binding agent in these treatments might irreversibly disrupt large amounts of the macro-aggregates formed. More mechanistic insights into the effects of biochar, especially in combination with organic fertilizers, on aggregate formation with or without drying and rewetting is needed to be able to fully assess the role of biochar in agricultural soil management.

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7 General Conclusion

As a main finding of this work, biochar was found to be not beneficial for aggregate formation in two incubation experiments. Macro-aggregate yields were found to be significantly lower for combined slurry and biochar applications than for slurry applications without biochar, depending on the incubation temperature. A reason for this might be found in the strong absorption characteristics of biochar, effectively separating organic matter from the microbial biomass and thus preventing microbial processing essential for the production of aggregate binding agents.

In a one-year field trial, biochar did not have significant effects on aggregate-associated carbon. However, biochar-C was found in all analyzed aggregate fractions as well as the occluded light fraction, the C stocks of which were increased by biochar application, showing the formation of biochar-mineral interactions within one year in the field. Although not quantified, biochar-C was found to have been incorporated into newly-formed macro-aggregates in the second incubation study as well. This can possibly be traced to the adsorption of organic matter (i.e. slurry in the case of the incubation study) to the biochar surface, acting as aggregate binding agent. However, this effect was apparently not strong enough to yield similar or even higher macro-aggregate amounts compared to treatments without biochar.

Slurry was found to be more efficient in terms of aggregate formation than manure, which did not increase aggregate yields compared to the control. This is related to the liquid state of the slurry, allowing it to spread across the particle surfaces and act as a binding agent immediately. In the further course of the experiment, decomposition of the slurry and the subsequent production of microbial-derived aggregate binding agents can be assumed.

Higher incubation temperatures in steps of 10 °C were found to result in lower aggregate yields, which probably results from the mineralization of the aggregate binding agents after the pool of readily available organic matter is decomposed. This threshold appeared earlier at higher temperatures due to the larger microbial activity and thus caused lower aggregate yields in a

given time span. However, in a field trial with elevated soil temperatures (+ 2.5 °C) for six years prior to sampling, temperature had no effects on carbon associated with aggregate or density fractions. This might be related to the ambivalent nature of temperature influences on organo-mineral interactions, resulting in only minimal changes at higher temperatures.

Drying and rewetting caused lower aggregate yields in an incubation experiment. This effect was especially pronounced in treatments with biochar, indicating a larger susceptibility of macro-aggregates containing biochar particles. A possible reason for this might be that the drying of liquid, largely undecomposed organic matter acting as binding agent in these aggregates causes irreversible disruption.

The use of biochar in agricultural soils as a means to increase macro-aggregate formation cannot be encouraged by these results. However, the results presented here support the use of biochar as a means to increase carbon storage in soils as the relative high stability of biochar might be even increased by interactions with minerals and occlusion in aggregates. The apparently higher susceptibility of aggregates containing biochar to drying and rewetting, however, deserves further attention.

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