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Degradation of *Miscanthus × giganteus* biochar, hydrochar and feedstock under the influence of disturbance events

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Abstract

Little is known about the degradation and environmental impacts of carbon (C) amendments such as hydrochar and biochar in soil under the influence of disturbance events such as wetting, freeze-thaw cycles, manual stirring, and glucose additions. Thus, we assessed the degradation and greenhouse gas (GHG) emissions of Miscanthus x giganteus biochar (from pyrolysis), hydrochar (from steam and water hydrothermal carbonization, HTCs and HTCw), the uncarbonized feedstock material in a sandy and a loamy soil, compared to a control, with four replicates per treatment. The C amendments were mixed with soil at a rate of ~20 t/ha wt% and incubated at 30°C over the period of 441 days. Over the whole incubation period, the soil mixtures were exposed consecutively to different disturbance events, with the intention to simulate a worst-case scenario for C degradation and GHG emissions. The degradation kinetics were quantified by source partitioning of the headspace \(^{13}\)C-CO\(_2\) and the application of an isotope two-component mixing model. Additionally, microbial biomass and composition were quantified and characterized at the end of the experimental period by chloroform fumigation extraction and phospholipid fatty acid analysis. The molecular composition and structural properties of the C amendments obtained by elemental analysis and NMR spectroscopy proved to be suitable indicators for the CO\(_2\) emissions which followed the sequence feedstock > HTCs > HTCw > biochar over the experimental duration. The addition of glucose triggered a short-lived, temporary co-mineralization of the otherwise recalcitrant materials HTCw and biochar. Among all C amendments, biochar proved most recalcitrant against decomposition and disturbance in both soils with a calculated recovery rate of 95-99% of the initially added biochar-C. Additionally, biochar amendment led to a decreased decomposition of soil organic C, especially in sandy soil.
Keywords: biochar, hydrochar, degradation, greenhouse gases, SOC mineralization, *Miscanthus × giganteus*

1 Introduction

A range of biomass carbonization techniques have been developed over the last few decades to cater for different feedstock qualities and end-use applications (Meyer et al., 2011). Water hydrothermal carbonization (HTCw) for example is well suited to process wet feedstock material, being energy sufficient, and yielding a material similar to brown coal or peat, i.e. a possible peat substitute (Mumme et al., 2011; Cao et al., 2013). Another HTC production process, which is even more energy conserving, is when water vapor is used instead of liquid water and is labeled as vapo-thermal or steam carbonization (HTCs) (Funke et al., 2013a). Pyrolysis is better-suited for drier feedstocks. Pyrolysis for energy and charcoal generation has been likely practiced for thousands of years (Antal and Grønli, 2003). Inspired by the discovery of anthropogenic dark earths, as well as natural black soils, the charcoal-like remains of pyrolysis are tested for their suitability for soil C sequestration and soil improvement (biochar) (Chang, 1996; Reina et al., 1998; Ponomarenko and Anderson, 2001; Nurul Islam et al., 2005; Marris, 2006).

The carbonization of plant material increases the half-life when incubated with soil to about 1.6 - 3.4 years on average for hydrochar (Steinbeiss et al., 2009), 6.75 years (Qayyum et al., 2012), 24.6-34.2 (Bai et al., 2013) and 60 years (Budai et al., 2016) for biochar, compared to the respective uncarbonized reference material. As a consequence, the different carbonization processes, especially hydrothermal carbonization and pyrolysis processes, may be suitable indicators for the properties and recalcitrance of C amendments against degradation (Lehmann et al., 2009; Spokas, 2010; Schimmelpfennig and Glaser, 2012; Singh et al., 2012).
The measurement of CO₂ emissions from soil is a well-established method to quantify the degradation of soil carbon. Many studies report CO₂ emission peaks after biochar and particularly hydrochar application to soil, which has been related to a labile C pool, e.g. volatile compounds or carbonates, functional groups or a biochar/hydrochar induced pH shift, providing suitable conditions for microorganisms (Zimmerman, 2010; Jones et al., 2011; Keith et al., 2011; Wang et al., 2011; Eibisch et al., 2013; Farrell et al., 2013; Malghani et al., 2013). Subsequent biochar mineralization, without any disturbance events, levels out to very low rates, indicating that biochar is a rather recalcitrant, stable C-pool (Kuzyakov et al., 2014). However, biochars’ recalcitrant behavior may be reduced by the addition of labile C substrates, such as glucose or straw, indicating its susceptibility to co-metabolization (Hamer et al., 2004; Kuzyakov et al., 2009). Hydrochar typically continues to mineralize after the initial degradation period following soil addition (Kammann et al., 2012; Malghani et al., 2013; Bamminger et al., 2014). Additionally, hydrochar mostly induced positive priming effects on SOC (Steinbeiss et al., 2009; Bamminger et al., 2014), though also a negative priming on SOC, after an initial period of positive priming of three months, has been reported (Malghani et al., 2015). Biochar was found to both increase and decrease SOC mineralization (positive and negative priming of SOC), depending on soil properties such as texture and SOC content, SOC structure, and microbial community composition or the biochar feedstock (Zimmerman, 2010; Keith et al., 2011; Dempster et al., 2012; Cely et al., 2014).

Concerning non-CO₂ GHG emissions, it has been shown that HTCw and HTCs, as well as uncarbonized feedstock, can lead to both higher and lower soil N₂O emissions compared to unamended controls in incubations and field studies (Kammann et al., 2012; Schimmelpfennig et al., 2014; Malghani et al., 2013). Both feedstock and HTCs increased the methane oxidation potential of the soil. However, if the soil was flooded or fertilized, the methane sink temporarily
Biochar reduced \( \text{N}_2\text{O} \) emissions on average by 54\% (Cayuela et al., 2014) and decreased methane emissions from soils (Dong et al., 2013, Shen et al., 2014, Van Zwieten et al., 2009, Feng et al., 2012, Barbosa de Sousa et al., 2014). Conversely, in connection with urea fertilization, biochar can also lead to increased soil \( \text{CH}_4 \) emissions (Zhang et al., 2010). Thus, the mechanisms and processes of the involvement of C-amendments in GHG emissions from soil are considerably influenced by soil properties (SOC-content, texture, pH and nutrient status), management (fertilization) and the type of C amendment (feedstock and production process conditions, C/N ratio etc.).

Despite quite complex models on the stability of C amendments in soil found in literature, the influence of disturbance/extreme weather events on the stability of C-amendments and associated GHG fluxes is still barely investigated. Events such as high temperatures and heavy precipitation as well as more pronounced freeze-thaw events due to a reduced snow cover are likely to increase in the future (Field, 2012; Reichstein, 2012).

To the authors’ awareness, the present study is the first to investigate the effects of a sequence of various physicochemical disturbance events and glucose addition on the degradation of the soil and C amendments, associated greenhouse gas emissions, and priming effects. The overall aim of the study was to create a “worst-case scenario” for the recalcitrance of the C-amendments. This worst-case scenario included a wetting event (simulating heavy precipitation), frost-thaw cycles, disruption of soil by stirring and glucose addition. Additionally, to stimulate microbial activity, the incubation temperature was set to 30°C.

All disturbance events were applied in sequence, since the repeated application of single extreme events can lead to an intensification of single effects, as has been shown for frequent frost-thaw cycles (Larsen et al., 2002, Chen et al., 1995). The experiment was completed by the examination of the response of soil microorganisms to the different C amendments.
2 Material and methods

2.1 Material

Topsoil samples (0-10 cm) were taken from two contrasting temperate soils; a grassland soil (Haplic Stagnosol, WRB 2007) with a texture of 25% sand, 47% silt and 28% clay, TOC of 2.23%, 0.2% nitrogen (N), a pH of 6.88±0.02, a bulk density of 1 g cm$^{-3}$, a $\delta^{13}$C value of -26.5‰, and an agricultural Cambisol (HLUG, 2014), with a texture of 85% sand, 10% silt and 5% clay, TOC of 0.63%, 0.04% N, a pH of 5.52±0.05, a bulk density of 1.6 g cm$^{-3}$, and a $\delta^{13}$C value of -27‰. The field collected soils were sieved through a 10 mm sieve to remove large roots, rhizomes and stones.

All C amendments (also referred to as “Miscanthus-C”) were produced from chopped Miscanthus × giganteus chaff, including untreated (= feedstock, $\delta^{13}$C -12.38‰), hydrothermally steam or water treated chaff (designated as hydrochar steam = HTCs, $\delta^{13}$C -12.41‰, and hydrochar water = HTCw, $\delta^{13}$C -14.32‰, respectively), or pyrolyzed chaff (biochar, $\delta^{13}$C -14.51‰). The field grown, senescent Miscanthus was harvested in winter 2009 when all aboveground plant material had died off. Hydrothermal carbonization (steam) was carried out by keeping the feedstock in a water vapor atmosphere for 2 hours at a temperature of 200±3 °C under a pressure of 1.6 MPa (Revatec, Geeste, Germany, at that time Hydrocarb GmbH, Ohmes, Germany). For water-based HTC, the Miscanthus straw was processed at 240 °C and 3.1-3.8 MPa for 8 h in distilled water using an 18.75 liter Parr series 4555 pressure reactor (Parr Instruments, Moline, IL, USA). The reactor contents were heated by an external heater operated by a Parr controller. The heating rate was set to 2 K min$^{-1}$. The reactor content was not stirred. After the process, the heater was switched off and the reactor was cooled overnight to about 50°C. Subsequently, the HTC slurry
was removed from the reactor, filtered by a wired mesh and the resulting solids were dried for 48 h at 60 °C.

Biochar was produced using a pyrolysis unit with a continuous flow reactor at 550-600 °C and a mean residence time of the material in the reactor of 15 min (Pyreg GmbH, Bingen, Germany). All C amendments were ground to < 10 mm before the experimental setup (SM 300, Retsch GmbH, Haan, Germany), to allow for the comparison of GHG emissions with an appending field experiment (Schimmelpfennig et al., 2015).

2.2 Methods

2.2.1 Experimental setup

The experiment was set up at the end of May 2012 and kept until mid-August 2013 (441 days). Seven grams of the C amendments were mixed with 350 g sandy/loamy soil at the rate of 1.96 wt% (equivalent to 29.2 or 19.6 t amendment per hectare as calculated for an application depth of 10 cm for sandy or loamy soil, respectively), placed into glass incubation jars (1100 ml, Weck®, Germany) and kept, with the lid ajar to allow air exchange, inside a drying closet at 30°C to stimulate microbial activity (n = 7 replicates per treatment). Four replicates were used for weekly measurements of the GHG fluxes of CO₂, N₂O and CH₄. The other three replicates were used to perform elemental analysis, to measure pH values at the beginning and the end of the experiment, and for substrate-induced respiration (SIR) measurement after eight months. The water content of the samples was set to 60% of the maximum water holding capacity (WHC) and adjusted weekly. The WHC of each mixture was previously determined as described by Schimmelpfennig et al. (2014).
The soil mixtures were fertilized with dissolved NH$_4$NO$_3$ five times during the experimental period, equivalent to 50 µg nitrogen (N) per g soil mix applied each time, to stimulate microbial activity and trigger N$_2$O emissions.

We forced the degradation of the C amendments by several disturbances throughout the experiment, which are termed “sections” in the following. The first four months (days 0-131) of the incubation are considered a period of labile Miscanthus-C degradation; with a constant WHC of 60% and no further disturbances (Section 1). On day 132, we increased the water content of the soil mixtures to 100% WHC, with a subsequent drying phase decreasing the WHC to 50% (Section 2, days 132-232). From day 233 onward, four freeze/thaw cycles were performed, where the jars were repeatedly put to -20° C overnight and taken out to thaw and to perform gas flux measurements (Section 3, days 232-302). On day 303, a plowing event was simulated, by stirring the soil mixtures with a plastic spoon (Section 4, days 303-353). After 12 months of incubation, a priming experiment was carried out to test the effects of a labile C source (glucose) on the mineralization of the C amendments. 80 mg glucose powder g$^{-1}$ soil mix was added as a labile C source to each jar (Section 5, days 354-399).

From a wetting event, simulating heavy precipitation, we expected high N$_2$O emission outbursts due to increased N-losses via nitrification and denitrification and a short flush of CO$_2$ (Borken and Matzner, 2009). Likewise, we expected that frost-thaw events lead to an increase in N$_2$O and CO$_2$ emissions with decreasing soil temperature (Koponen and Martikainen, 2004; Goldberg et al., 2010). By the disruption of soil, we aimed at simulating more frequent soil cultivation due to a longer vegetation period in the future, leading to a substantial increase in CO$_2$ emissions (Willems et al., 2011). By applying an incubation temperature of 30°C, we aimed at intensifying microbial activity, leading to an increase in soil and Miscanthus-C degradation as well as higher
N$_2$O emissions (Li et al., 2015). Other than for CO$_2$ and N$_2$O, we expected rather small effects of the disturbance events on methane emissions (Priemé and Christensen, 2001).

2.2.2 Analytical procedures

Soil pH (H$_2$O) was measured using a pH meter (ratio 2.5:1 soil/water) (InoLab; WTW, Weilheim, Germany). Elemental analyses including C, N, S and H of the raw materials and the soil mixtures were conducted using an elemental analyzer (CHNS Macro/VarioMax Elemental Analyzer, Elementar Analysensysteme GmbH, Hanau, Germany). The oxygen content (in %) was determined as difference between 100 and the sum of the other measured elements plus ash, see Table 1.

Table 1

| Structural analysis of all C amendments was carried out by high-resolution $^{13}$C solid-state NMR spectroscopy (cross polarization-magic angle spinning) on a Unity INOVA 400 spectrometer (Varian Inc. USA). The spectra were Fourier transformed and base lines were corrected and integrated with the spectrometer's software package VNMRJ 2.2D. For quantification of selected structural elements, all spectra were divided in four sections and their signal intensities were normalized to a total intensity of 100. Section boundaries were 0-50 ppm (aliphatic C), 50-60 ppm (-O-CH$_3$ = lignin), 60-110 ppm (cellulose), 110-220 ppm (aromatic C). GHG fluxes were determined on a weekly basis following the method described by Hutchinson and Livingston (1993) adapted to jar measurements (Kammann et al., 2008; Kammann et al., 2009). Briefly, after jar closure, 50 ml gas samples were taken with syringes three times in equal time steps over 0.5 – 2 hours (depending on the incubation temperature). Gas samples were
analyzed within 24 hours on a gas chromatograph equipped with a flame ionization detector (for CH₄) and an electron capture detector (for N₂O and CO₂; set-up according to Loftfield et al., 1997). The GHG fluxes were calculated by linear regression, considering the ideal gas law as well as average air pressure and temperature during the sampling period (Kammann et al., 2009). The overall GHG budget of the C amendment effects was expressed as CO₂ equivalents (CO₂eq), calculated from the CO₂, N₂O and CH₄ fluxes using the respective 100-year global warming potentials (GWPs) as given in the IPCC 4AR (GWP N₂O = 298 and GWP CH₄ = 25) (IPCC, 2007).

At the end of Section 1 (day 115) and subsequent glucose addition (day 368, in Section 5), gas samples were taken with syringes and transferred into evacuated 12 ml vials (Labco, UK) and measured against the IAEA standards CH6, 305 and 310, Vienna, Austria to analyze the isotopic composition of CO₂ (Microgas IRMS, Isoprime Ltd., Wytenshaw, UK).

The ¹³C/¹²C ratios of the soil and the C amendments were determined using an elemental analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS, Eurovector, Thermo Fisher Delta V Advantage, Thermo Fisher Scientific GmbH, Dreieich, Germany).

2.2.3 TOC change and isotope mass balance calculation

The TOC contents of the soil mixtures during the first 365 of the experiment as compared to day 368 after glucose addition were determined by subtracting the cumulated CO₂-C losses from the initial C-amounts within the soil mixtures. The relative C losses of the soil mixtures and their respective shares (SOC or Miscanthus-C) were calculated by using the isotopic signature of the headspace CO₂, sampled at day 115 for the period prior to the priming experiment, and the signature of the gas sampled after glucose addition (day 368) for the period during the priming experiment, compared to a control (Hamer et al., 2004), and source partitioning by Keeling plots.
(Pataki et al., 2003). The loss of Miscanthus-C was estimated by determining the relative C-share in CO$_2$ using equation 1:

$$^{13}\text{C}\text{O}_2(\%) = \frac{\delta_{\text{mix}} - \delta_{\text{soil}}}{\delta_{\text{Miscanthus}} - \delta_{\text{soil}}} \times 100$$

(1)

where $^{13}\text{C}\text{O}_2$ is the percentage of Miscanthus-C in the respired CO$_2$, $\delta_{\text{mix}}$ is the isotopic ratio of the soil mix (soil+Miscanthus (+glucose)), $\delta_{\text{soil}}$ is the isotopic ratio of the pure soil (+glucose) and $\delta_{\text{Miscanthus}}$ the isotopic ratio of the specific Miscanthus, (charred or uncharred), naturally enriched in $^{13}\text{C}$ (Amelung et al., 2008; Luo et al., 2011). The calculations are based on the following assumptions: (1) The integrated $^{13}\text{C}$ signature for the soil-plus-glucose CO$_2$ would be identical for the control and the Miscanthus-amended soils. Additional glucose mineralization, possibly induced by the presence of C-amendments was not taken into consideration. (2) The respective signatures of CO$_2$-C originating from the amendment or soil are similar to that of their sources. (3) The calculated ratio of the measurement on day 115 can be extrapolated to all CO$_2$ measurement time points in the period before glucose addition, and that the ratio in the beginning of the priming experiment applies to all subsequent measurements of the priming section. From the calculated cumulative Miscanthus-C loss, the daily Miscanthus-C loss was determined according to the five Sections, and the influence of glucose addition on Miscanthus-C mineralization was quantified.

The mean residence time MRT = (k*365)$^{-1}$ and half-life = MRT*ln(2) of the C-amendments were estimated using a single first-order exponential decay model (Lehmann et al., 2009), since our data did not comply with the requirements for a double exponential model such as found for constant experimental conditions and an experimental duration of several years (Kuzyakov et al., 2009).
2.2.4 Extractable organic C, microbial biomass and community composition

At the end of the experiment (day 441), the soil mixtures were analyzed for their extractable organic C (EOC) and soil microbial biomass C ($C_{mic}$) by the chloroform-fumigation-extraction (CFE) method (Vance et al., 1987). In brief, 10 g of the chloroform fumigated soil subsample (24 h at 22°C) were extracted with 40 ml 0.5 M $K_2SO_4$ on a horizontal shaker for 30 min at 250 rpm and centrifuged for 30 min at 4400 g. Another 10 g subsample remained non-fumigated but was extracted similar to the fumigated sample. C and N in supernatants of fumigated and non-fumigated samples were measured on a Multi N/C 2100S TOC/TN-analyzer (Analytik Jena, Jena, Germany). $C_{mic}$ was calculated by subtracting the extractable C values of the non-fumigated from that of the fumigated samples. For calculation of $C_{mic}$, the conversion factor $k_{EC}$ 0.45 (Joergensen, 1996) was used. EOC was calculated from C concentration in supernatants of the non-fumigated samples.

Phospholipid fatty acids (PLFA) of the soil mixtures were extracted following the method of Frostegård et al. (1993). A Bligh and Dyer solution (chloroform, methanol, citrate buffer, pH=4, 1:2:0.8;v/v/v) was used to extract glycol-, neutral- and phospholipid fatty acids and the fractions were separated by silica acid columns (0.5 g silicic acid, 3 ml: Varian Medical Systems, Palo Alto, California). PLFAs were classified according to Frostegård & Bååth (1996), Zelles (1999) and Kaiser et al. (2010), whereby the branched fatty acids i15:0, a15:0, i16:0 and i17:0 were summed as from Gram-positive bacteria, the cy17:0 and cy19:0 as from Gram-negative bacteria, the biomarker 16:1$ω_7$ served as additional fatty acid for identification of total bacteria and 18:2$ω_6,9c$ for total fungal PLFA. Total PLFA ($PLFA_{mic}$) is the sum of bacterial and fungal PLFA. To exclude CO$_2$ flux data from the proliferation period following glucose addition, only the extrapolated and cumulated fluxes from day 400 onward were chosen for the correlation with the microbial activity and the microbial parameters.
2.3 Statistics and calculations

Statistics were carried out using SigmaPlot 11.0 (Systat Software Inc., San José, California, USA), IBM SPSS Statistics Version 20 (IBM Corporation, Armonk, New York, USA) and Xact 8.03 (SciLab, Saint Yrieix, France). The cumulated CO₂, N₂O, CH₄ and CO₂eq fluxes were integrated over the experiment duration using linear interpolation between measurement dates. Differences in CO₂eq over the incubation period were determined by a repeated measurement ANOVA and post-hoc tests (Tukey HSD) and factoring in the different time intervals between the gas measurements. Overall treatment effects, Miscanthus-C losses in g per day within the five time sections, and differences between the single sections within one treatment were tested by one-way ANOVAs followed by LSD or Holm-Sidak post-hoc tests, or by ANOVA on Ranks followed by SNK or Tukey HSD post-hoc tests. Differences in the Miscanthus-C degradation rates as affected by soil type or treatments were determined by one-way ANOVAs on ranks followed by Tukey HSD post-hoc tests.

CO₂ and N₂O flux sums, percent SOC, Miscanthus-C and TOC (SOC+Miscanthus-C, excluding glucose-C) losses, Cₘic, EOC, pH, WHC and MRT were tested for significant effects of the soil type or treatment by two-way ANOVAs, followed by Holm-Sidak post-hoc tests (CI=95%). CH₄ flux sums were tested by a two-way ANOVA on ranks, due to lack of normal distribution or heterogeneity of variances, followed by a Tukey HSD post-hoc test (CI=95%; Table S.1). Correlations of the cumulated CO₂ and N₂O-N fluxes were tested by Spearman Rank Order Correlation, since the data were not normally distributed. Correlations between microbial parameters and pH and CO₂ fluxes of the end period of the experiment (days 400-440) were performed using Pearson Correlation. Correlations of the ratio of initial TOC [g] / remaining TOC [g] (g Cₚ/g Cₚrem) and the characterization parameters H/C-O/C ratio, cellulose and aromatics content of the Miscanthus samples were calculated using Michaelis-Menten equations.
3 Results

3.1 pH and WHC

Feedstock initially increased the pH value of sandy soil (pH 5.52) by half a unit, as did biochar, in both the sandy and loamy soil (Table S.2). At the end of the experiment, pH values had decreased significantly compared to initial pH values, especially in the loamy soil (on average from 5.33 to 4.62). While the pH values of the sandy control soil remained similar to initial values, all C amended soils exhibited a pH drop of 0.5 units. Initial treatment differences in loamy soil disappeared during the experimental period. The WHC\textsubscript{max} was increased by all C amendments by 20% compared to the control in both soils, with the exception of biochar in the loamy soil (Figure S.1).

3.2 \(^{12}C\text{O}_2\), \(^{15}N\text{O}_2\) and CH\textsubscript{4} fluxes

The cumulated \(^{12}C\text{O}_2\) emissions during the five sections (days 0-399) ranged from 3.7 to 32.8 g \(^{12}C\text{O}_2\) kg\(^{-1}\) soil mix (Figure 1 a-c). Emissions from the feedstock treatment were higher in the sandy soil (32.8 g \(^{12}C\text{O}_2\) kg\(^{-1}\) soil mix), compared to the loamy soil (25.5 g \(^{12}C\text{O}_2\) kg\(^{-1}\) soil mix, p < 0.001) (Figure 1 a-c). \(^{12}C\text{O}_2\) emissions from the control/HTCs/HTCw treatments were not different between the two soil types. Emissions from the biochar treatments were 3.7 and 6.5 g kg\(^{-1}\) soil and lower in sandy compared to loamy soil, respectively (p < 0.05). The cumulative degradation pattern was similar in both soils and followed the carbonization degree of the material (feedstock > HTCs > HTCw > biochar). Cumulative \(^{15}N\text{O}_2\) emissions during the five sections (days 0-399) ranged between 0.95-14.6 mg \(^{15}N\text{O}_2\)-N kg\(^{-1}\) soil mix. Emissions from the feedstock treatment were higher from loamy compared to sandy soil (p < 0.05), whereas no soil-treatment interactions were found for the other treatments. Nitrous oxide fluxes from feedstock/HTCs/HTCw treatments were higher than those from the control and biochar treatments in both soils (p < 0.001), with highest
fluxes from feedstock amended loamy soil (Figure 1 d-f). Emissions from the biochar treatments were not different compared to emissions from the control in both soils.

Cumulated methane fluxes during the five sections (days 0-399) from the sandy soil were negligible and emissions ranged from -0.02 to +72.1 CH$_4$ µg kg$^{-1}$ soil mix. Methane fluxes in the loamy soil were dominated by methane oxidation ranging from -72 to -303 µg CH$_4$ kg$^{-1}$. Here, soil methane oxidation capacity was improved by all C amendments, inversely to their carbonization degree, with highest methane oxidation rates in feedstock-amended soil (Figure 1 g-i).

Figure 1

The cumulated N$_2$O emissions correlated significantly with the cumulated CO$_2$ emissions in both soils ($r = 0.832$ in sandy soil, $r = 0.901$ in loamy soil, $p<0.01$) (Figure S.2).

3.3 Shares of the single GHGs in total CO$_2$-equivalent emissions

Carbon dioxide made up for 85.6% and 87.6% and N$_2$O for 14.4% and 12.5% of the total CO$_2$eq in the sandy and loamy soil, respectively, with large treatment differences. Methane fluxes corresponded to 0.02% (sandy soil) and -0.14% (loamy soil) of total CO$_2$eq, with only the feedstock treatment in loamy soil leading to a higher share in CO$_2$eq compared to the control ($p < 0.05$, Table S.3, Figure S.3).

The N$_2$O emissions during the disturbance events were very variable. In particular, water logging in Section 2 led to N$_2$O emission peaks, which strongly contributed to the CO$_2$eq sum during the wetting-drying cycle. As a consequence, the CO$_2$eq fluxes rather than the sole CO$_2$ fluxes during
the single Sections were used for an overall evaluation of the effects of the C amendments under the influence of disturbance events on the GHG budget of the soils.

3.4 GHG (CO₂eq) emissions during disturbance and priming events

Most of the CO₂eq (mean 58.4% in sandy soil and 54.3% in loamy soil) were lost during the period of labile C degradation (Section 1) in the first four months, enhanced by two fertilization events (Figures 2 A and B). The WHC increase to 100% (Start of Section 2) led to a renewed emission outburst in both soils, amounting to 20.4 and 19.1% of the total cumulated CO₂eq emissions. Thereafter, neither the third, fourth and fifth fertilization event, nor the freeze-thaw cycles (Section 3) led to larger GHG emission outbursts, contributing only 2.4 and 3.8% to the total cumulated CO₂eq emissions in sandy and loamy soil, respectively. Plowing (Section 4) and glucose addition (priming, Section 5) stimulated emissions from all treatments in both soils, contributing 10.4/11.9% (Section 4) and 8.6/11.0% (Section 5) to total CO₂eq emissions in the sandy/loamy soils, respectively.

Total CO₂eq emissions from both control soils were similar in Sections 1, 2 and 4 (labile C degradation, wet-dry and plowing). During Section 3 (freeze-thaw), the loamy control soil showed larger emissions than the sandy soil, while it was vice versa during Section 5 (priming) (Figures 2 A and B; Table 2). The non- or low-carbonized C amendments led to significantly higher GHG emission sums in most of the Sections (1, 2 and 4: feedstock > HTCs > HTCw), compared to the control and biochar treatments. During Section 3 (freeze-thaw), the sandy control soil emitted the lowest CO₂eq sum, followed by biochar/HTCs/HTCw while feedstock treatments exhibited the highest emissions. In the loamy soil, during freeze-thaw events, emissions from the HTCw treatment were as low as from the control, followed by biochar < HTCs/feedstock treatments. Subsequent to glucose addition (Section 5, priming), in sandy soil,
the control and HTCw treatments showed the highest CO$_2$eq emissions, followed by the feedstock and HTCs/biochar treatments. In loamy soil, the CO$_2$eq emissions in Section 5 were not different between treatments (Figures 2 A, B, Table 2).

Figures 2 A and B

The C amendments interacted differently with the two soils during the five sections (Table 2). None of the C amendments showed a distinctive degradation pattern according to the soil type except biochar, with higher CO$_2$eq emissions from the loamy compared to the sandy soil throughout all sections, except Section 1 (Table 2).

Table 2

3.5 Miscanthus-C losses during the disturbance events and priming

The fractions of Miscanthus-C and SOC in total CO$_2$ emissions were different for the period before and during the priming experiment, revealing an altered degradation behavior (Table S.4). During Section 1 (days 0-131), the ratio of Miscanthus-C/SOC in total emissions followed the carbonization degree with highest ratios for feedstock and HTCs, followed by HTCw and biochar. The source partitions of SOC- and Miscanthus-C in total CO$_2$ emissions in Section 5 were different, as compared to the experimental period before. While the relative fractions of HTCw- and biochar-C in total CO$_2$ emissions were higher, the relative fractions of feedstock and HTCs in total CO$_2$ emissions were lower (Table S.4).
The mean daily *Miscanthus*-C loss in the five sections was lowest in Section 3 (freeze-thaw) \( p = < 0.05 \), compared to all other sections, with no significant differences among the other Sections. Testing the losses of the single treatments for each soil showed very different daily C losses of the C amendments (Table 3). Mean daily C losses were highest in the initial phase of the experiment (Section 1, labile C) for feedstock and HTCs in both soils. Losses of HTCw were slightly elevated in the beginning (Section 1), decreased in the following Sections and were triggered again in Section 5 (priming) by glucose addition, similar in the two soils. Consequently, initial and final HTCw degradation rates (Sections 1 and 5) were higher than during the disturbance events (Sections 2-4: wet-dry, freeze-thaw and plowing). Biochar degradation in both sandy and loamy soil was slightly increased in the beginning, compared to Sections 2-4, and was increased by glucose addition, resulting in highest mean *Miscanthus*-C losses in Section 5 (Table 3). Expressed as percentages of the total *Miscanthus*-C loss, 1.3, 3.3, 16.0 and 52.2% and 3.2, 5.3, 16.4 and 25.1% were lost in Section 5 from the feedstock, HTCs, HTCw and biochar treatments in sandy and loamy soil.

Table 3

3.6 Carbon balance and degradation rate

The remaining C contents (SOC, *Miscanthus*-C and TOC) of the soil mixtures after 13 months of incubation (days 0-399) are given as percent of the initial C (=100%, \( t_0 \)) in Figure 3. The SOC losses from sandy soil were higher throughout all treatments. In sandy soil, SOC loss of the feedstock and HTCs treatments was similar to that of the control, whereas HTCw increased SOC losses. In loamy soil, SOC loss of the feedstock treatment was higher compared to the control soil, whereas no significantly different SOC loss was observed for the other treatments, except
for biochar. Biochar amendment led to significantly lower SOC losses in both soils, compared to the respective controls (Figure 3 A).

\textit{Miscanthus}-C loss followed the carbonization degree in sandy soil: feedstock > HTCs > HTCw > biochar. Also in loamy soil, feedstock/HTCs degradation was higher than that of HTCw/biochar. Feedstock degradation was higher in sandy soil compared to loamy soil, whereas it was vice versa for biochar with higher degradation rates in loamy than sandy soil (Figure 3 B, Figures 4 A and B). Comparing initial and end \textit{Miscanthus}-C amounts of the single treatments revealed that all C amendments except biochar lost significant amounts of \textit{Miscanthus}-C over the incubation period in both soils. Practically all C was lost from feedstock in sandy soil (97.65 ± 4.69%), whereas it was more stable in loamy soil (43.3 ± 9.0% C loss) (Table S.1: significant soil × treatment interactions). Degradation of HTCs and HTCw was not different between soil types with mean C loss of 42.9% from HTCs and 10.6% from HTCw. Biochar-C loss was insignificant in both soils with 98.5 and 96.3% of the initial C remaining at the end of the experiment in sandy and loamy soil, respectively.

TOC (SOC+\textit{Miscanthus}-C) loss from the treatments control, feedstock, HTCs and HTCw was higher in sandy soil, compared to loamy soil, whereas TOC loss from biochar treatments was low and not soil dependent. TOC loss was highest from the feedstock treatments in both soils, followed by HTCs>control>HTCw>biochar treatments in sandy soil and by HTCs/HTCw/control>biochar in loamy soil (Figure 3 C).

Figure 3

Total C build-up by C amendments was more pronounced in the sandy soil. There was a negative carbon build-up (decrease in TOC) due to feedstock amendment, and a significant linear increase
in C build-up, depending on the carbonization degree of the substrates, with C gains of initial C for HTCs, HTCw and biochar, respectively. In loamy soil, the C build-up was less pronounced (Table 4).

Table 4

All structural properties (elemental ratios H/C and O/C, cellulose and the aromatic component from the NMR measurements) were in line with the ratio of initial TOC / remaining TOC (C_{in}/C_{rem}), independent of the soil type (p<0.001). In sandy soil, the correlations of C_{in}/C_{rem} were highest for cellulose and H/C ratios (r^2_{adj} = 0.992 and 0.993, respectively); in loamy soil, best fits were obtained with O/C ratios (r^2_{adj} = 0.972) (Figures S.4 A and B).

3.7 MRT and half-life

The single exponential decay model fitted the data with R^2 values ranging from 0.92 to 0.95. Both MRT and half-life of the C amendments depended linearly on their carbonization degree and on soil type, with the highest MRT of around 90 years for biochar in sandy soil (Table 4). In loamy soil, biochar MRT and half-life was significantly lower than in sandy soil (27 years), nevertheless it was still significantly higher than the MRT and half-life of all other C amendments (2-12 years). MRT and half-life of feedstock and HTCw was significantly higher in loamy soil compared to sandy soil (2 vs 0.3 years and 12 vs 10 years, respectively), whereas MRT and half-life of HTCs material were similar in both soils (≈ 2 years).

3.8 C_{mic} and EOC with appending CO_{2} emissions

Both mean C_{mic} and EOC were higher in loamy soil (C_{mic} = 266.8 µg g^{-1} dw, EOC = 200.2 µg g^{-1} dw) compared to sandy soil (C_{mic} = 149.9 µg g^{-1} dw, EOC = 119.8 µg g^{-1} dw, p<0.001),
throughout all treatments except HTCw with similar values in both soils. HTCs led to highest EOC and Cmic amounts in loamy soil, whereas HTCw exhibited highest Cmic and EOC in sandy soil (Figure 5 A and B). Both Cmic and EOC correlated positively with the cumulated CO2 fluxes of the days 400-440 of the experiment. (0.681, p<0.001 and r=0.815, p<0.001) and final pH values (r = 0.67, p<0.001 and r= 0.68 p<0.001). The CO2 fluxes (days 400-440) were different for the factors soil and treatment with significant interactions for all treatments except the control treatment, where fluxes were similar in sandy and loamy soil. All other treatments exhibited higher fluxes in the loamy soil. The CO2 flux patterns were slightly altered compared to the experimental period before glucose addition, with a shift from feedstock mineralization towards HTCs and/or HTCw mineralization; fluxes from biochar treatment were reduced to levels similar to the time period before glucose addition, in both soils (Figure 5 C).

3.9 PLFAs

All bacterial (gram-positive and gram-negative) and fungal PLFAs were more abundant in loamy soil (total mean 22.4 nmol g⁻¹ soil mix dry weight, HTCs > control/feedstock/HTCw/biochar), compared to sandy soil (7.7 nmol g⁻¹ soil mix dry weight, no differences between treatments) (Figure 6). The C amendments had significant effects on most PLFA groups, with significant interactions regarding the soil type. In the loamy soil, HTCs caused a significantly larger abundance of gram-positive bacteria compared to all other treatments. The abundance of gram-negative bacteria followed the sequence control/biochar ≤ HTCs/HTCw ≤ feedstock in sandy soil and control/feedstock/HTCw/biochar < HTCs in loamy soil. The abundance of fungal biomass was different according to the treatments in the sequence HTCs ≤ biochar ≤ feedstock/HTCw ≤ control in the sandy soil and control/biochar ≤ feedstock/HTCw < HTCs in the loamy soil.
In the loamy soil, fungi, gram-positive and gram-negative bacteria correlated well with EOC contents \( (r=0.778, \ p<0.001; \ r=0.710, \ p<0.001; \ r=0.827, \ p<0.001) \). In sandy soil, only gram-positive bacteria correlated weekly with EOC \( (r=0.535, \ p<0.05) \). Fungi, gram positive and gram negative bacteria as well as the total PLFA groups correlated with the cumulated CO\(_2\) flux (days 400-440) \( (r = 0.657, \ p <0.001; \ r = 0.627, \ p <0.001; \ r = 0.645, \ p <0.001; \ r = 0.725, \ p <0.001) \).

4 Discussion

4.1 C-degradation

Laboratory incubation studies under constant conditions may underestimate the degradability of carbonized plant material (Ventura et al., 2014). Hence, in this study, a series of alternating conditions including N and labile-C addition were applied to accelerate degradation under high soil temperatures (except freeze-thaw cycles). The results suggest that especially C from feedstock and steam-carbonized HTCs was readily available to microorganisms, as indicated by high amounts of cellulose and large elemental ratios H/C and O/C (Siu and Reese, 1953; Eibisch et al., 2013). Water-carbonized HTCw was less degradable than HTCs and feedstock, but still significantly more degradable than biochar. Interestingly, HTCw triggered the degradation of native SOC rather than being mineralized itself, especially in sandy soil, as indicated by the isotope data shown in Figure 3. This behavior was also found by others and might origin from extra nutrients in HTCw available to microorganisms, thus increasing microbial mineralization of SOC (Steinbeiss et al., 2009; Bamminger et al., 2014). Nevertheless, the relative TOC loss was reduced by HTCw in sandy soil and was similar to the control in loamy soil, indicating, together with the relative C build-up at the end of the experiment, its suitability for C accumulation especially in sandy soils (Table 4, Figure 3). Biochar reduced the SOC- and TOC-losses from
both soils, though the reduction was more distinct in sandy soil, pointing to a negative priming effect. Negative priming, i.e. reduced SOC degradation following biochar amendment to soil has been observed before, mostly as medium- to long-term effect (5-16 months) (Jones et al., 2011; Zimmerman et al., 2011; Maestrini et al., 2014), whereas in the short-term (18-90 days), biochar amendment often increased the mineralization of SOC (Zimmerman et al., 2011; Maestrini et al., 2014). Nevertheless, in our experiment, biochar did neither in the short- nor the long term increase the degradation of SOC independent of the soil type, nor was mineralized in significant amounts itself, which was also found by Cross and Sohi (2011). Consequently, the TOC contents in the sandy and loamy soils after biochar application remained significantly increased compared to all other C amendments, regardless of the alternating experimental conditions applied to foster decomposition.

Biochar was less degraded in sandy soil, compared to loamy soil. The low initial SOC content of the sandy soil could imply that C-saturation had not occurred at the beginning of the experiment. Thus, a small but considerable C storage capacity, emanating from the clay content (5%) could have facilitated biochar stabilization by the formation of clay-biochar complexes (Baiamonte et al., 2014). Additionally, the increase of the soils’ WHC due to biochar amendment compared to the control could have facilitated the formation of soil aggregates in the biochar amended sandy soil (Piccolo et al., 1996; Glaser et al., 2002; Dugan et al., 2010), even though such effects are reported to occur only in the long term and with biochar application rates > 20t/ha (Borchard et al., 2014; Tammeorg et al., 2014). Moreover, these properties could apply to all C amendments in sandy soil and may thus not fully explain the stabilization of organic material by the biochar in sandy soil. Hence, sorption of (dissolved) organic material onto the biochar surface or encapsulation into biochar pores, limiting the substrate available to microorganisms, may better explain the negative priming effects observed here (Kasozi et al., 2010; Zimmerman et al., 2011;
Barnes et al., 2014; Lu et al., 2014). Chemical as well as structural properties of carbon(ized) substrates such as the elemental ratios of H/C and O/C and/or the amount of aromatic C compounds have long been identified as indicators for recalcitrance against degradation (Krull et al., 2009; Lee et al., 2010; Nguyen et al., 2010; Spokas, 2010; Schimmelpfennig and Glaser, 2012; Singh et al., 2012), together with the ash content (Bai et al., 2013). This was confirmed by our experiment for the continuum of all four different C amendments, as the ratios of initial/remaining TOC correlated well with all structural properties (Figures S.4 A and B). However, as the C amendments, especially feedstock and biochar, interacted differently with the two soils, it seems obvious that soil characteristics such as diverse microbial populations in the different soils influenced the C degradation patterns as well, as was reported in other studies (Nguyen et al., 2014). Also, our observation that feedstock material was less degraded in loamy soil compared to sandy soil is confirmed by other studies (Zhang et al., 2014). Most likely, the lower C degradation rate in loamy soil can be attributed to the higher content of soil aggregates, rendering the amendments more recalcitrant against microbial decomposition as compared to sandy soil.

Besides abiotic factors, negative priming of SOC observed with biochar was also attributed to biotic factors such as pH induced changes of the microbial population or inhibition of microbial metabolism by potentially toxic compounds released from biochar (Cross and Sohi, 2011; Jones et al., 2011; Zimmerman et al., 2011). Inhibitory effects e.g. by toxic components seem unlikely here, since the biochar did not contain toxic substances such as dioxins or polycyclic aromatic hydrocarbons when analyzed (unpublished results). Furthermore, substrate induced respiration measurements using subsamples of this experiment eight months after initiation showed that microbial biomass was increased but not decreased by all C amendments, compared to the control. Microbial biomass followed the reverse degradation order in the sandy soil (feedstock
>HTCs> HTCw > biochar > control) and the order HTCs > feedstock > biochar > HTCw > control in the loamy soil (Eckhardt, 2013). Nevertheless, negative priming caused by a decrease of the microbial and enzymatic activity has been found by others, possibly due to sorption of quorum sensing molecules onto biochar, rendering such molecules less available to soil microorganisms (Chintala et al., 2014).

The slightly acidic pH of the sandy soil was increased significantly by biochar amendment at the start of the experiment and thus could have improved the milieu for microbial metabolism (Farrell et al., 2013); however, the observed decrease in C mineralization here counteracts this assumption.

4.2 Kinetics, C loss and gain

The MRT and half-life of the C materials could clearly be linked to their carbonization grade. Thereby, the structural properties such as cellulose content or the elemental ratios of H/C and O/C were suitable predictors for the C losses during the experimental degradation period over one year in both soils.

The amount of C lost from the different C amendments during the experiment (Table 4) is slightly higher than data reported from other authors. Bai et al. (2013) used the same C-amendments (except HTCw), mixed them with a range of soils and reported C-losses of 32-37% from feedstock (here: 43-98 %), 27-30% C-loss from HTCs (here: 41-45 %) and 0-3% C-loss from biochar (here: 2-5 %) over a period of 200 days. The differences are easily explained by a longer experimental duration, a higher incubation temperature and the alternating, diverse degradation-promoting conditions that were applied in this study. Nguyen et al. (2014) incubated a biochar produced at 450°C from switchgrass together with five different soils at 25°C and 60% WHC. Their reported C losses, in the range of 2.1-4.1%, are in line with the results obtained here.
Fang et al. (2014) incubated mixtures of woody (*Eucalyptus salinga* Sm.) biochar produced at 550°C, with a range of soils over 12 months at 20-40°C and reported biochar-C losses of 0.30-0.42% and 0.97-1.16% at 20 and 40°C respectively. Here, lower values may be explained by the difference in feedstock; woody biochars are considered more stable than biochar from more brittle plant material such as *Miscanthus* chaff, due to a higher amount of fused aromatic C-structures and lower ash (SiO₂) and mineral contents (Kloss et al., 2012; Singh et al., 2012; Zhao et al., 2013).

The calculated MRT and half-lives for biochar in loamy soil in this experiment are rather short compared to the MRT of 550°C biochar reported in other studies (90-10¹⁴ years) (Keith et al., 2011; Gajić et al., 2012). This might be attributable to the disturbance events plus N and glucose additions during the experiment, since the fluctuations in the C-degradation rates following these events were mostly very high and consisted of several degradation boosts. Thus, the influence of disturbance events on the degradation of C amendments might have been underestimated in modeling of their stability so far. Nonetheless, our general result, the prolongation of the half-life of uncarbonized *Miscanthus × giganteus* straw by hydrothermal carbonization and pyrolysis are in good agreement with the results of Bai et al. (2013) and Quayyum et al. (2012), although none of these studies used a continuum of four carbonization types or disturbance events, fertilization and priming. Thus, the results confirm the suitability of hydrochar (especially HTCw) and more so biochar to increase the soils’ TOC content and to sequester C even under exposure to degradation-promoting conditions.

### 4.3 N₂O emissions in relation to the carbonization grade

All C amendments except biochar significantly increased the cumulative N₂O emissions over the course of the incubation study compared to the control. Biochar reduced the N₂O emissions by
26.5 and 8.5 % in sandy and loamy soil compared to the control however the reductions were not significant. The largest N\textsubscript{2}O emission increase was observed with the straw-like uncarbonized Miscanthus feedstock amended to loamy soil, while the increase was less in the sandy soil (although highest among all C amendments). The same effect – increased N\textsubscript{2}O emissions with uncarbonized compared to pyrolyzed wheat straw – was observed by Cheng et al. (2012) in a field study on Chernozem soil. Wolf et al. (2010) observed that grazing, i.e. the removal of grassy litter, reduced the N\textsubscript{2}O emissions of Mongolian steppe significantly, and that grassy litter decomposition may be an underestimated source of N\textsubscript{2}O formation in natural or semi-natural grasslands. The latter, as well as our findings, are in line with earlier observations in the grassland field site (where the loamy soil for the incubation had been taken): Schimmelpfennig et al. (2014) observed significantly increased N\textsubscript{2}O emissions in the second year after top-dressing of Miscanthus straw. No increase was found when the same amount of straw-C was applied as biochar. Rather, N\textsubscript{2}O emissions tended to be reduced during a freeze-thaw period (Schimmelpfennig et al., 2014). The reduction in N\textsubscript{2}O emissions by (woody) biochar amendment is a common finding confirmed by meta-analysis (Cayuela et al., 2014; Van Zwieten et al., 2015).

For hydrochar, less information is available. While Malghani et al. (2013) observed N\textsubscript{2}O emission reductions following hydrochar application to non-fertilized soils, Kammann et al. (2012) reported that soil N\textsubscript{2}O emissions were greatly stimulated in an incubation study after mineral-N fertilizer had been added to loamy soil amended with two different hydrochars. In the above-mentioned grassland field study, Schimmelpfennig et al. (2014) found no changes in the N\textsubscript{2}O emissions due to hydrochar application. However, this study indicates that hydrochar amendments still stimulated N\textsubscript{2}O emissions significantly compared to the unamended control, but that the increase was reduced (more so with increasing HTC carbonization grade) and retarded in time and/or dependent on N-fertilization compared to the uncarbonized feedstock.
In our study, differences in the water/oxygen content of the soils were likely eliminated by adjusting the WHC (Case et al., 2012). However, continuous O$_2$ consumption during the mineralization of the more labile C compounds may have reduced the redox potential of the soil mixtures, triggering the use of NO$_3^-$ as electron donor for denitrification (Miller et al., 2008). Especially in the SOC richer loamy soil, a higher amount of anaerobic microsites may have, together with the higher pH, promoted denitrifying bacteria (Firestone, 1982; Herold et al., 2012). Moreover, fungal co-denitrification often dominates N$_2$O emissions in temperate grassland soil (Laughlin and Stevens, 2002). A larger fungal biomass fraction was indeed confirmed for the loamy-soil HTCs and HTCw treatments at the end of the incubation study (p<0.001), whereas the fungi in the feedstock treatment were only slightly increased. Here, the microbial community composition likely had changed over the course of the experimental period. The easily accessible feedstock C may have been depleted so that the fungal biomass was reduced again when finally measured. Summarizing the evidence obtained here and earlier (Schimmelpfennig et al., 2014), we have evidence that N$_2$O production by denitrifying fungi may have been responsible for the higher N$_2$O emissions with uncarbonized or weaker carbonized materials (Laughlin and Stevens, 2002; Oehl et al., 2010), and that this effect was more pronounced in the loamy than sandy soil. Additionally, biochar but not hydrochar application offers the possibility to reduce N$_2$O emissions from soils compared to the application of decomposable straw materials. The most interesting open question is if the combination of labile straw material with biochar would result in lower-than-straw-alone N$_2$O emissions.

4.4 Predicting N$_2$O emissions: correlation of CO$_2$ and N$_2$O

A high amount of cumulative CO$_2$ emissions (i.e. mineralizable C) correlated with high cumulative N$_2$O emissions. Vice versa, the lack of an additional organic C source (control) as
well as a C amendment with little mineralizable C (biochar) resulted in low N₂O emissions. This behavior only became evident because of the carbonization gradient used in our experiment and due to the varied incubation conditions over 13 months. Other char-application studies where CO₂ and N₂O emissions were monitored simultaneously did not report a correlation; however, the fluxes were not cumulated for comparison (Van Zwieten et al., 2010). As to hydrochar, such a relation was found earlier, with high mean CO₂/N₂O fluxes from beet/bark hydrochar applied to a loamy soil, assumedly due to stimulation of microbial activity (Kammann et al., 2012). Our results suggest that using the CO₂ emissions after litter amendment for predicting cumulative N₂O emissions may be a promising approach that deserves further study.

4.5 Methane fluxes

The methane fluxes in the experiment were negligible in the sandy soil, whereas methane oxidation was improved by all C amendments in loamy soil, inversely to their carbonization degree (strongest with feedstock, lowest by biochar). Karhu et al. (2011) also observed a doubling of the CH₄ uptake in grassland soil when biochar was ploughed into the top soil; the authors attributed this to improved soil aeration. This can largely be excluded for this study since the WHC was adjusted to equal values and the most labile substrates, where more O₂ must have been consumed by decomposition, showed the strongest stimulation in CH₄ oxidation. The capacity of soil to take up methane is generally a function of land use, with tilled agricultural soil exhibiting lower oxidation potentials than no-tilled, temperate soils (Mosier 1997). The methane oxidation capacity of soils was found to correlate with the N status of the soil, in a way that high amounts of available N limited the activity of methanotrophs in non-wetland soils (Chan and Parkin, 2001; Aronson and Helliker, 2010). Thus, interactions of the denitrifying and the methanotrophic/methanogenic microorganisms may have been underestimated so far. More
mechanistic studies are necessary to identify the causes for the observed increase in the CH$_4$
consumption following C amendment.

4.6 Disturbance events

Initial CO$_2$ outbursts following the admixture of biochar, hydrochar and feedstock material such
as straw to soil have been widely reported (Zimmerman, 2010; Qayyum et al., 2012; Eibisch et
al., 2013; Fang et al., 2014), and have been confirmed by our experiment for feedstock and both
hydrochars. In contrast to most reported studies, CO$_2$ emissions from biochar amended soil in our
experiment were at none of the measured time points higher than emissions from the control soil.
Rather, they were mostly lower than the control, especially in the sandy soil, (except during
freeze-thaw events). Such contrasting results can likely be explained by the different properties of
the biochars used here compared to other studies (low temperature biochar $\leq$ 375°C (Bruun et al.,
2008), or a biochar with smaller particle sizes (Jones et al., 2011). Also, our biochar has been
stored 12 months in a closed but not air-tight box before use, thus some production prone, easily
degradable volatile compounds might have outgassed over the storage time.

By applying N-fertilizer, we aimed at lowering the C/N ratio of the soil to facilitate C degradation
and trigger CO$_2$ and/or N$_2$O emissions (Chantigny et al., 1999). This occurred for the first two
fertilization events and led to a CO$_2$ emissions peak in the feedstock and HTC treatments,
indicating the presence of labile, biologically available C compounds. None of the later
fertilization events triggered further peak emissions from any of the treatments, suggesting that
most of the labile compounds had been degraded. Only the disturbance of the soil structure by
simulation of plowing led to renewed C and N degradation of feedstock and hydrochar in both
soils, assumedly due to destruction of soil aggregates, improving the accessibility of C
compounds, and provision of oxygen (West and Marland, 2002). Similar results have been
reported from an experiment with two hydrochars and four biochars mixed with a Luvisol, where simulated plowing led to high CO₂ and N₂O emissions from the hydrochar soil mixtures, compared to the control (Kammann et al., 2012).

Water logging of N-rich soil promotes denitrifying enzyme gene expression in bacteria using NO₃⁻ as electron acceptor. The increase of the WHC to 100%, simulating heavy rainfall, led to an immediate short-term increase of CO₂ and N₂O emissions from all treatments, though emissions from biochar treatments were still lower than, or equal to, the control soil. Also, Kammann et al. (2012) reported high N₂O emissions of biochar amended, N-fertilized and nearly water-logged soil (80% WHC).

Freeze-thaw cycles had unexpectedly a rather small though stimulating effect on the GHG emissions. This might be explained by the experimental setup, i.e. the disturbed soil structure and removal of vegetation due to sieving of the soil. During freeze-thaw events, the die-off of fine roots and disruption of soil aggregates largely contribute to CO₂ and N₂O emissions (Logsdail and Webber, 1959). Thus, intact soil cores could have led to more distinct effects of frost-thaw cycles. Moreover, a different sequence of the disturbance events in the experiment, e.g. freeze-thaw cycles at an earlier time point might have resulted in greater effects on the degradation of the C amendments. However, freeze-thaw cycles led to significantly higher although small amounts of CO₂ emissions from biochar amended soil compared to the control soil, indicating some small effects of freeze-thaw events on biochar degradation. This can possibly be explained by the disruption of small biochar-soil aggregates, generating particles susceptible to microbial attack (Eastman, 2011). In another experiment, biochar significantly reduced both ammonium and nitrate leaching and N₂O emissions from vegetated soil cores subjected to freeze-thaw events, assumedly due to N-retention (Kettunen and Saarnio, 2013). The same tendency was
observed in data from a grassland field experiment, indicating that this interaction requires further attention (Schimmelpfennig et al., 2014).

4.7 Priming experiment

Glucose addition led to positive priming of *Miscanthus*-C, indicating that *Miscanthus*-C was susceptible to co-metabolism during glucose mineralization, especially since N was not a limiting factor (Kuzyakov et al., 2000). The relative fraction of positive priming (although low in absolute terms) was most prominent for the higher-temperature C-amendments such as HTCw and biochar, especially for biochar in sandy soil, where the biochar may have been less protected within soil aggregates compared to loamy soil (Hamer et al., 2004). Glucose addition increased mean biochar mineralization by the factor 6.5, compared to the average daily flux of all other sections (Table 4), as was found also by (Kuzyakov et al., 2009), incubating Haplic Luvisol with ryegrass char. This could indicate that possibly similar biochar compounds may generally be prone to co-metabolization.

Glucose addition had little priming effects on the degradation of feedstock and HTCs, likely due to the fact that these materials had been largely mineralized already (Table 3). Thus, labile-C priming (e.g. root exudates) and freeze-thaw cycles deserve further study with regard to their impact on long-term biochar stability.

4.8 Microbial biomass and composition

Microbial biomass and composition were analyzed at the end of the experimental period, corresponding to the end of the proliferation period following glucose amendment (Stotzky, 1965). Therefore, the results reflect the microbial population and related microbial data at the experimental period when the glucose effect had declined. EOC, Cmic and all PLFAmic groups were higher in the loamy soil compared to sandy soil. This is likely due to the loamy texture,
supporting aggregate formation and habitats for microorganisms (Monreal and Kodama, 1997). Additionally, disturbance of the soil structure and aggregates during glucose admixture likely triggered the availability of aggregate protected SOC and EOC to microorganisms (Rovira and Greacen, 1957). The considerable drop of the pH of the soil mixtures, especially in the loamy soil (about one unit) confirms the higher biological activity in loamy soil, leaving protons as metabolite from oxidation.

The increase in the microbial biomass and activity following EOC provision in soil, especially for fungi, is well known (Burford and Bremner, 1975). Interestingly, only HTCs increased the total microbial biomass in loamy soil significantly, coinciding with high EOC values in this treatment (Figure 5 a and b). An increase in C_mic due to hydrochar amendment (maize silage, application rate 4-8 g kg^{-1}) compared to the arable control soil was also found by Bamminger et al. (2014), even in the same order of magnitude (doubling of C_mic compared to the control). Concentrations of nutrients plus depolymerization of C structures during the hydrothermal production process, increasing the availability of both nutrients and C to microorganisms are likely the reasons for the preference of microorganisms for HTCs, as compared to all other materials including the uncarbonized feedstock (Funke et al., 2013a; Funke et al., 2013b).

C_{mic}, PLFA and especially EOC in biochar amended sandy soil were low, leading consequently to low CO_{2} emissions (Figure 5 c). A lower fungal and total biomass in the biochar- compared to the control soil indicates on the one hand higher persistence of microbial biomass in the control soil after glucose addition, on the other hand that biochar decreased the availability of organic material to microorganisms, possibly by sorption mechanisms or blocking of pores, also inducing the negative priming effect found earlier in the experiment. In loamy soil, biochar had no such effect; if EOC sorption occurred, the high background C values, as compared to the sandy soil, may have overlaid the influence of sorption. Since the incubation temperature was very high, the
microbial community might have adapted and changed during the experimental period. The results might therefore not be comparable to field studies, in-situ experiments and incubation experiments under temperature regimes simulating the current climate.

4.9 Remarks on the experimental setup

One limitation of our experimental setup was a limited number of CO₂ isotope measurements. The extrapolation of one isotope measurement to the whole experimental period until glucose addition might have disregarded any changes in the Miscanthus-C fraction during the different sections. However, results from Luo et al. (2011) and Nguyen et al. (2014) suggest that most of the source variations in the CO₂ occur during the early degradation period of 22-30 days. Thus, the time point of our ¹³CO₂ measurement at the end of Section 1 represents the rather moderate C-degradation period when the labile fraction already had been mineralized. As a consequence, our data more likely underestimate than overestimate the Miscanthus-C fraction in total CO₂ emissions.

Another limitation of the experimental setup is the interdependence of the degradation events, as they were applied consecutively instead of as parallel treatments. The sequential application of the degradation events may have overlaid the effect of one single event. However, the repeated exposition to degradation promoting events may also lead to intensification of the processes leading to C-loss, triggering positive feedbacks and legacy effects. Thus, our experiment creates a worst-case scenario for the degradation of the C-amendments. Less extreme experimental conditions might have resulted in a higher stability of the C-amendments.
5 Conclusion

In this study, the intrinsic recalcitrance of the C amendments was the most important factor determining degradation. However, the experimental setup i.e. the use of sequential disturbance events could have blurred the influence of one single disturbance event. Further studies that focus more closely on the influence of specific disturbance events such as frost-thaw cycles or wet-dry cycles on GHG emissions and C degradation are necessary to provide a clearer picture. Highest degradation rates were found for feedstock, and lowest for biochar. On average, CO$_2$ accounted for 86.5% of the cumulative GHG equivalents, highlighting the importance of the stability of C amendments in soil with respect to GHG mitigation and C sequestration. The cumulated CO$_2$-emissions correlated with the cumulated N$_2$O-emissions, an effect that is worth to be studied further, to be able to better predict the environmental consequences of biochar/HTC application to soil. C was lost in significant amounts from all C amendments except biochar over the experimental period in both soils. The CO$_2$ emissions from biochar amended soil were, throughout all degradation events, never higher than emissions from the control soil. Rather, they were mostly lower than the control, especially in the sandy soil (except during freeze-thaw events). Additionally, biochar induced a negative priming of SOC, whereas HTCw and feedstock led to a positive priming effect in sandy or loamy soil. The mechanisms and processes leading to negative priming of SOC following biochar amendment require further research. Also, as incubations only allow for first insights, verification of the degradation behavior of C amendments under the influence of degradation promoting disturbance events in field experiments, including vegetation and its root-C delivery as well as its below-ground microbiome are necessary.
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Tables

Table 1: Elemental composition of the carbon amendments, means ± SD (n=3). Oxygen values were determined by difference (100-the sum of the other elements and ash). All values refer to the dry weight. Letters depict significant treatment differences (One way ANOVA, Holm-Sidak Post-hoc test P<0.05).

Table 2: Mean cumulated GHG (CO₂eq) fluxes, means (n=4) ± SD during the different sections [g CO₂eq kg-1 soil mix]. Letters indicate significant differences between the treatments in each section across both soil types (One-way ANOVA and Holm-Sidak Post-hoc tests, CI=95%).
Table 3: Mean daily char-C-loss [mg d\(^{-1}\)] during the five sections, means ± SD

Table 4: Remains of initial char-C, TOC build-up on day 399 of the experiment in %, mean residence time (MRT) and half-life [years] of the carbon amendments in the two different soils (mean ± SD). Letters depict significant differences between the treatments (ANOVA on Ranks, followed by SNK-post-hoc-test, CI = 95%).

Table S.1: Statistical results of the two way ANOVA with the factors soil, treatment and soil × treatment effects (CI = 95%); MRT, mean residence time; WHC, water holding capacity.

Table S.2: pH values of the soil-substrate mixes, at initiation and end of the experiment (n=3 ± SD). Letters indicate significant treatment differences at the different time points (ANOVA+Holm-Sidak post-hoc test (CI = 95%)

Table S.3: Greenhouse gas emissions (CO\(_2\), N\(_2\)O, CH\(_4\)) over the incubation period, expressed in GHG equivalents with their respective shares in percent

Table S.4: Shares of char-C in total CO\(_2\) fluxes [%] on the 21\(^{st}\) of September 2012 (end of Section 1) and the 31\(^{st}\) of May 2013 (Start of Section 5), as determined by Keeling Plots and an isotope mixing model

**Figure captions**

Figure 1: Cumulated mean CO\(_2\), N\(_2\)O and CH\(_4\) fluxes over time (left, a, d, g; and right, c, f, i) and total GHG flux sums at the end of the experiment (middle, b, e, h). Error bars indicate
standard deviation (n=4); letters mark significant differences between treatments (one way ANOVA and Holm-Sidak post-hoc test (CO₂, N₂O), or ANOVA on Ranks and Tukey post-hoc test (CH₄), CI = 95%).

Figure 2: Total CO₂eq fluxes [g CO₂ kg⁻¹ soil mix, left Y-axis] of the five treatments over the incubation period (days 0-399) with mean and standard deviation. The white area marks the WHC [in %, right Y-axis]; differently patterned areas mark the five Sections of the disturbance events. A: sandy soil, B: loamy soil

Figure 3: Percental SOC, char-C and TOC-remains (A, B and C) at the end of the incubation period (day 399, n=4), means ± SD. Letters depict significant treatment differences, as determined by one way ANOVAs and Holm-Sidak or LSD Post-hoc tests or an ANOVA on Ranks with a SNK Post-hoc test (CI = 95%).

Figure 4: Percental char-C-loss over the incubation period, separated according to the different sections. Letters depict significant differences among the treatments (Kruskal-Wallis-ANOVA on Ranks and Tukey test, CI=95%). A=sandy soil, B=loamy soil.

Figure 5: Mean and standard deviation of Cmic, EOC [µg g⁻¹ soil mix] and cumulated CO₂ fluxes [mg g⁻¹ soil mix] at the final period of the experiment in the two different soils. Letters depict significant treatment differences (Two way ANOVA and Holm-Sidak post-hoc test, CI = 95%).

Figure 6: Total mean PLFA concentration [nmol g⁻¹] ± SD and their gram⁺, gram⁻ and fungal shares given in grey shades. The white lowercase letters indicate significant treatment differences.
in gram\(^+\) bacteria concentrations, black lowercase letters mark treatment differences in gram- bacteria concentrations, Greek letters show differences between fungal PLFA concentrations and uppercase letters give differences in total PLFA concentrations.

Figure S.1: Initial maximum water holding capacity [%] of the soil-substrate mixtures. Letters depict significant differences between treatments in sandy (small letters) and loamy (big letters) soil (two-way ANOVA with Holm-Sidak Post-hoc test CI = 95%).

Figure S.2: Pearson correlation of cumulated CO\(_2\) and N\(_2\)O fluxes [g kg\(^{-1}\) soil mix].

Figure S.3: Shares of the single GHGs in total CO\(_2\)eq fluxes [g CO\(_2\) kg\(^{-1}\) soil mix] of the two soils. Latin letters and asterisks mark differences according to the single GHGs (one way ANOVA followed by Tukey HSD post-hoc tests, CI = 95%), Greek letters mark significant differences of the total cumulated CO\(_2\)eq emissions. Note different scales.

Figure S.4 A and B: Non-linear regressions of the elemental composition and structural properties of the different carbon amendments with C initial/C remaining ratios. A=sandy soil, B=loamy soil.