Does soil aging affect the N\textsubscript{2}O mitigation potential of biochar? A combined microcosm and field study

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Abstract

The application of biochar as a soil amendment to improve soil fertility has been suggested as a tool to reduce soil-borne CO\textsubscript{2} and non-CO\textsubscript{2} greenhouse gas emissions, especially nitrous oxide (N\textsubscript{2}O). Both laboratory and field trials have demonstrated N\textsubscript{2}O emission reduction by biochar amendment, but the long-term effect (>1 year) has been questioned. Here, we present results of a combined microcosm and field study using a powdered beech wood biochar from slow pyrolysis. The field experiment showed that both CO\textsubscript{2} and N\textsubscript{2}O emissions were still effectively reduced by biochar in the third year after application. However, biochar did not influence the biomass yield of sunflower for biogas production (Helianthus annuus L.). Biochar reduced bulk density and increased soil aeration and thus reduced the water-filled pore space (WFPS) in the field, but was also able to suppress N\textsubscript{2}O emission in the microcosms experiment conducted at constant WFPS. For both experiments, biochar had limited impact on soil mineral nitrogen speciation, but it reduced the accumulation of nitrite in the microcosms. Extraction of soil DNA and quantification of functional marker genes by quantitative polymerase chain reaction showed that biochar did not alter the abundance of nitrogen-transforming bacteria and archaea in both field and microcosm experiments. In contradiction to previous experiments, this study demonstrates the long-term N\textsubscript{2}O emission suppression potential of a wood biochar and thus highlights its overall climate change mitigation potential. While a detailed understanding of the underlying mechanisms requires further research, we provide evidence for a range of biochar-induced changes to the soil environment and their change with time that might explain the often observed N\textsubscript{2}O emission suppression.

Keywords: biomass yield, carbon dioxide, charcoal, nirK, nirS, nitrogen cycling, nosZ, quantitative polymerase chain reaction, sunflower

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Introduction

Biochar is defined as charcoal that is not used in thermal application, but as a beneficial soil amendment (European Biochar Certificate – EBC, 2012). It is produced by pyrolysis, that is, the heating of biomass in the partial or total absence of oxygen, and was already used as an agronomic input in the 19th and 20th century (Allen, 1847; Tryon, 1948; Lehmann & Joseph, 2009). Biochar can reduce nutrient leaching (Zheng \textit{et al.}, 2013; Laird & Rogovska, 2015), can increase soil water retention (Laird \textit{et al.}, 2010; Kammann \textit{et al.}, 2011; Karhu \textit{et al.}, 2011; Obia \textit{et al.}, 2016), and can promote crop growth (Jeffery \textit{et al.}, 2015). Due to its long-term stability in soil, biochar is discussed as a potential carbon sink for climate change mitigation (Lehmann \textit{et al.}, 2015; Wang \textit{et al.}, 2015). Biochar has also been shown to mitigate non-CO\textsubscript{2} greenhouse gas (GHG) emissions from soil (Van Zwieten \textit{et al.}, 2015).

Soil-borne N\textsubscript{2}O emissions

\textit{N}_2\textit{O} is both a potent GHG with a global warming potential of 298 (IPCC, 2007) and a precursor of the ozone-depleting nitrogen oxides (Crutzen, 1970). Due to
intensive N fertilization, agriculture is the most important anthropogenic source of \(\text{N}_2\text{O}\) and the key driver of the human disturbance of the biogeochemical flow of nitrogen (Rockström et al., 2009; Steffen et al., 2015).

Soil-borne \(\text{N}_2\text{O}\) emissions are predominantly produced by two microbially mediated processes, namely nitrification and denitrification (Braker & Conrad, 2011). Nitrification is the aerobic oxidation of ammonia to nitrate (\(\text{NO}_3^-\)) via nitrite (\(\text{NO}_2^-\)). \(\text{N}_2\text{O}\) can be produced as a nonobligate intermediate by chemical oxidation of hydroxylamine (NH\(_2\)OH) at rates \(10^3\)–\(10^6\) times lower than that of nitrite formation. Denitrification is the anaerobic reduction in \(\text{NO}_3^-\) or \(\text{NO}_2^-\) to dinitrogen (\(\text{N}_2\)) via NO and \(\text{N}_2\text{O}\). It is considered as a major pathway for N losses from agroecosystems through the production and release of \(\text{N}_2\text{O}\) and \(\text{N}_2\) at varying ratios (Braker & Conrad, 2011), which are mostly controlled by soil pH, with higher pH resulting in lower \(\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)\) ratios (Bergaust et al., 2010). The relative contribution of nitrification and denitrification to total N cycling and soil \(\text{N}_2\text{O}\) emissions is to a large extent determined by the soil water-filled pore space (WFPS; Davidson, 1991). Bateman & Baggs (2005) showed that denitrification is the dominant source of \(\text{N}_2\text{O}\) at WFPS of 70% or higher as water reduces the availability of oxygen due to a limited diffusion. A 60% WFPS is the optimum for nitrification as the transport of substrate becomes a limiting factor under drier conditions. Further microbial nitrogen-transforming metabolisms can form \(\text{N}_2\text{O}\) as a by-product, but are typically not considered a relevant source of \(\text{N}_2\text{O}\) in agroecosystems. These include nitrifier denitrification, dissimilatory nitrate reduction to ammonium (DNRA), or anaerobic ammonia oxidation (anammox) (Hagemann et al., 2016).

**\(\text{N}_2\text{O}\) mitigation potential of biochar**

The suppression of soil-borne \(\text{N}_2\text{O}\) emissions can be a major component of the overall climate change mitigation potential of biochar. It is one of the most intensively studied biogeochemical effects of biochar in soil and was first described by Yanai et al. (2007). In their meta-analysis, Cayuela et al. (2014, 2015) analyzed a total of 56 field and laboratory studies with respect to the impact of biochar on soil \(\text{N}_2\text{O}\) emissions. They found an overall emission reduction of 49 ± 5%. However, it has rarely been investigated whether decreases in \(\text{N}_2\text{O}\) emissions co-occur with yield increases. Additionally, aside from legislative and economic questions, there are still two major challenges hindering the large application of biochar for climate change mitigation.

**First,** there is limited knowledge on the persistence of the \(\text{N}_2\text{O}\) emission suppressing effect. Felber et al. (2014) reported a decreasing capacity of biochar to reduce \(\text{N}_2\text{O}\) emissions in their combined field and microcosm study, already 1 year after amendment. Spokas (2013) showed that wood and macadamia shell biochars lost their initial ability to suppress soil \(\text{N}_2\text{O}\) emissions in a laboratory experiment with biochar that was recovered after aging for 3 years in a field trial.

**Second,** despite the fact that many different mechanisms have been suggested in the literature (e.g., Harter et al., 2014; Obia et al., 2015; Quin et al., 2015), an integrative explanation for the \(\text{N}_2\text{O}\) emission suppression by biochar is still missing. Considering the precautionary principle, a mechanistic understanding is necessary to avoid negative impacts of biochar amendment. It is important to note that increased \(\text{N}_2\text{O}\) emissions after biochar amendment have been reported and depended on both soil and biochar characteristics (Spokas & Reicoscoky, 2009; Cayuela et al., 2014).

The combination of field and laboratory experiments constitutes a promising approach to address these questions. So far, to the best of our knowledge, this approach has only been followed by Felber et al. (2014) and Schimmelpfennig et al. (2014).

**Objectives**

The goal of this study was to assess the impact of soil aging on the \(\text{N}_2\text{O}\) mitigation potential of a beech wood biochar produced by slow pyrolysis. We quantified \(\text{N}_2\text{O}\) emissions and mineral N speciation in both plant-free soil microcosms amended with fresh biochar as well as in a field site cropped with sunflower (Helianthus annuus L.) under conventional management in the third year after biochar amendment. Both experiments included fertilized controls without biochar. While the field experiment provided quantitative data on the \(\text{N}_2\text{O}\) emission reduction in field-aged biochar over several months, the microcosm experiment allowed mechanistic insights into immediate effects of biochar on soil chemistry and microbiology.

**Materials and methods**

**Study location, soil, and biochar**

The biochar field trial was established on the experimental field ‘Goldener Acker’ at University of Hohenheim, Germany (48°42.5′N, 9°12.5′E, 400 m a.s.l). The soil was characterized as a Haplic Luvisol (WRB, 2006). The site has an annual precipitation of 698 mm and a mean annual temperature of 8.8 °C. We determined trace gas fluxes on eight plots (plot size: 30 m\(^2\)) of a randomized block experiment. In 2010, four of the plots of the ‘biochar treatment’ received 180 kg of charcoal dust (<300 μm, equivalent to 60 Mg ha\(^{-1}\)) that was produced from beech wood (Fagus sylvatica L.) using the Degussa/Reichert process, a slow pyrolysis conducted at 400 °C (proFagus...
demand of the sunflowers during the growth season, as the total amount of nitrogen was applied in one dose. A herbicide was applied on May 9, 2012 (Bandur SC600; Bayer CropScience, Langenfeld, Germany, 4 L ha\(^{-1}\)), to suppress weeds.

**Field experiment sampling**

Water-filled pore space was determined in triplicates in each plot. Therefore, three 100-cm\(^3\) soil samples were collected with stainless steel cylinders in 5–15 cm depth of the top soil. Gravimetric water content \(u\) was determined after drying at 105 °C for 24 h. Porosity \(\varphi\) and WFPS were calculated as suggested by Yanai et al. (2007) and Hagemann et al. (2016):

\[
WFPS = \frac{u \times \rho_{\text{bulk}}}{\rho_{H_2O} \times \varphi},
\]

\[
\varphi = 1 - \frac{\rho_{\text{bulk}}}{\rho_{\text{particle}}},
\]

With \(\rho_{\text{bulk}} = m_{\text{dry}}/100\text{ cm}^3\) and \(u = m_{\text{moist}} - m_{\text{dry}}/m_{\text{dry}}, m_{\text{moist}}\) is the mass of fresh soil in 100 cm\(^2\) volume, \(m_{\text{dry}}\) is the mass of the dry soil in this volume, and the density of water is \(\rho_{H_2O} = 1\text{ g cm}^{-3}\). The particle density of the mineral soil was assumed as \(\rho_{\text{particle}} = 2.65\text{ g cm}^{-3}\).

For geochemical analysis, four soil cores with a diameter of 2.5 cm were taken from the A\(_2\) horizon (0–30 cm depth) of each plot and pooled. For DNA extraction, 12 subsamples from the upper 10 cm of the top soil from three plots were pooled and stored at − 20 °C. Each replicate plot was analyzed separately.

Soil for the microcosms was sampled next to the research plots on May 12, 2012. Soil was sieved < 2 mm and stored at 10 °C in closed plastic containers to avoid drying.

Sunflower biomass from 9 m\(^2\) in each plot was harvested on October 15, 2012, with a combine harvester. Fresh matter yield was determined, and subsamples from each plot were dried at 65 °C to calculate the plant dry matter (dm) content.

**Microcosm experiment**

Microcosms were set up as described by Harter et al. (2014). In brief, 204.2 g wet soil (180 g dm, control treatment) and 194.0 g wet soil (171 g dm) + 9 g dry proFagus biochar (5% w/w) were incubated in 0.5-L Schott bottles. Bulk density was 1.03 g cm\(^{-3}\) for the control and 1.07 g cm\(^{-3}\) for the biochar treatment. WFPS was adjusted to 60% by adding deionized (DI) water with dissolved nutrients. Each microcosm received 360 mg molasses as a carbon source, 49 mg N as NH\(_4\)NO\(_3\), and 27 mg P and 34 mg K as KH\(_2\)PO\(_4\) (p.a. grade; Merck, Darmstadt, Germany). Treatments were set up in triplicates. In total, 36 sacrificial microcosms were set up and incubated under daylight conditions at 28 °C. Soil sampling was conducted after GHG sampling, at the following time-points: right after setup (day 0) and after 1, 3, 5, 7 and 9 days. Bottles were open to ambient atmosphere during incubation. Soil sampling was performed by transferring all soil from the Schott bottle into a clean container for manual homogenization with a spatula. Subsamples for geochemical analysis and DNA extraction were taken from this container.

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**Table 1** Soil and biochar characteristics

| Parameter | Biochar | Soil |
|-----------|---------|------|
| BET ssa (m\(^2\) g\(^{-1}\)) | 65.47 |  |
| Ash (%) | 2.6 |  |
| H (%) | 2.98 |  |
| C\(_{\text{tot}}\) (%) | 87.7 |  |
| C\(_{\text{org}}\) (%) | 87.7 | 1.8 |
| N\(_{\text{tot}}\) (%) | 0.6 | 0.21 |
| O (%) | 6.1 |  |
| S (%) | 0.05 |  |
| pH (CaCl\(_2\)) | 8.4 | 7.1 |
| Pb (mg kg\(^{-1}\)) | b.d. | 29 |
| Cd | b.d. |  |
| Cu (mg kg\(^{-1}\)) | 5 | 116 |
| Ni (mg kg\(^{-1}\)) | 2 | 42 |
| Hg | b.d. |  |
| Zn (mg kg\(^{-1}\)) | 13 | 98 |
| Cr (mg kg\(^{-1}\)) | b.d. | 42 |
| B (mg kg\(^{-1}\)) | 13 |  |
| Mn (mg kg\(^{-1}\)) | 620 | 900 |
| P (mg kg\(^{-1}\)) | 370 | 1100 |
| Mg (mg kg\(^{-1}\)) | 1100 | 9700 |
| Ca (mg kg\(^{-1}\)) | 6300 | 34 000 |
| K (mg kg\(^{-1}\)) | 3300 | 16 000 |
| Na (mg kg\(^{-1}\)) | 140 | 790 |
| Fe (mg kg\(^{-1}\)) | 270 | 27 000 |
| Si (mg kg\(^{-1}\)) | 1200 | 330 000 |
| S (mg kg\(^{-1}\)) | 300 | 700 |
| PAH (16 EPA) (mg kg\(^{-1}\)) | 18.5 |  |
| Naphthalene (mg kg\(^{-1}\)) | 13.0 |  |
| Phenanthrene (mg kg\(^{-1}\)) | 3.1 |  |

B.d., below detection limit. Bold numbers indicate exceedance of EBC threshold for ‘basic’ biochar (EBC, 2012).
Greenhouse gas measurements

Greenhouse gas emissions were quantified using the closed chamber method (Hutchinson & Livingston, 1993) once a week. We used dark round chambers with an inner diameter of 30 cm. The chambers were equipped with a vent and a gas sampling port. The cylinders had a volume of 10 L and were described in detail by Flessa et al. (1995). Base frames were inserted into the upper 10 cm of the soil between the plant rows. They were installed upon planting and were only removed once for a soil management measure. During gas sampling, four gas samples were taken from the chambers’ atmosphere periodically (total enrichment of at least 30 min) with a double-sided cannula and evacuated gas vials (<10 mbar, V = 22.4 mL) through a septum on the top of the chambers.

Microcosms were closed with butyl-rubber stoppers, and four gas aliquots (25 mL) were sampled using a gas tight syringe (1000 Series, Gastight®; Hamilton, Reno, NV, USA) within 3 h of enrichment using the same type of vial described above. Resulting overpressure in the vials was released by connecting the vial to a pipe ending in a water bath until no more bubbles were visible in the water, that is, atmospheric pressure was reached. Low pressure in chambers and microcosms was compensated with N2 from a gasbag (PLASTIGAS®; Linde AG, Pul- lach, Germany, Harter et al., 2014). Concentrations of N2O and CO2 were quantified using a gas chromatograph (GC; 5890 Series 2; Hewlett Packard, Palo Alto, CA, USA) equipped with a 63Ni electron capture detector and an autosampler (HS40; PerkinElmer, Waltham, MA, USA). The GC configuration with a backflush system for water vapor was described in more detail by Löffeld et al. (1997). Resulting data from the microcosms were corrected for the dilution during sampling according to the following equation:

\[ c_{cor} = c_{meas}k^d \]

with

\[ k = \left(1 + \frac{V_s}{V_h}\right). \]

\( c_{meas} \) represents the concentration measured from nth vial, \( c_{cor} \) the corrected concentration, \( V_h \) is the headspace volume of the headspace of the microcosm (400 mL), and \( V_s \) (25 mL) is the volume extracted on sampling. Subsequently, fluxes were calculated based on the linear regression of the increase in the respective trace gases during the enrichment according to Ruser et al. (1998).

Geochemical analysis

Dissolved organic carbon (DOC), nitrite, nitrate, and ammonium were extracted with 0.5 M K2SO4. Samples were shaken for 1 h in snap cap vials at 130 rpm on HS901 horizontal shaker (IKA®, Staufen, Germany). Soil suspension was percolated through an ashless cellulose filter (Whatman, Maidstone, UK) and filtered through a 0.45-µm syringe filter (Millex-HA; Merck Millipore, Billerica, MA, USA). DOC was extracted 1 : 9 (5 g soil dm equivalent and 40 mL extractant). The analysis was conducted using a HighTOC analyzer (Elementar Analysensysteme, Hanau, Germany). Inorganic nitrogen species were extracted 1 : 5. For the microcosm study, ammonium and nitrate were quantified photometrically in a plate reader as described below. Nitrite was quantified using a test kit according to the manufacturer’s instruction (Spectroquant® Nitrite Test No 1.14776; Merck). For the field study, ammonium, nitrite, and nitrate were quantified in a continuous-flow analyzer system (Seal Analytical, Norderstedt, Germany). The pH was determined in a 1 : 5 soil suspension in DI water after horizontal shaking for 1 h at 130 rpm. Measurements were performed in the deposition solution after 1 and 24 h (DIN ISO 10390).

Quantification of ammonium and nitrate in a plate reader

For the microcosms study, both ammonium and nitrate were quantified photometrically in a FlashScan 550 plate reader (Jena Analytics, Jena, Germany) after using a test kit (ammonium kit Cat. No 1.14752, nitrate kit Cat. No 1.09713, all Merck) with an adapted protocol. For both essays, samples and reagents were directly mixed in the wells of a 96-well plate and volumes were adjusted to a final volume of 200 µL.

For ammonium, 15 µL of solution NH4–1 and 50 µL of an aqueous solution of reagent NH4–2 (1 ‘spoon’ provided with the kit on 2 mL water) were added to 125 µL of sample in a polystyrene plate. After 5 min, 10 µL of an aqueous solution of reagent NH4–3 (4 drops on 2 mL water, vortex prior to application) was added. The measurement was conducted after 5 min of incubation in the dark. The absorption in the range of 656–695 nm was evaluated and calibrated using an NH4Cl standard (0.16–10.0 mg L−1 NH4+). A plate with 200 µL water in each well was measured as a reference for the FlashScan Software.

A 96-well quartz plate was used for nitrate. Thirty microliters of sample was added to 140 µL of solution NO3–1. After addition of 30 µL NO3–2, the plate was covered and incubated for 30 min in the dark at 60 °C and measured afterward. The absorption in the range of 336–345 nm was evaluated. Measurements were calibrated using a KNO3 standard (200.0–6.3 mg NO3–); air measurement was set as reference. If necessary, nitrate was removed from nitrate samples prior to the measurement using sulfamic acid (reduction in nitrite to molecular nitrogen).

DNA extraction and quantitative PCR

We extracted soil microbial DNA and quantified process-specific functional marker genes as indicators for the capability (genetic potential) of the microbial community to express this genes, synthesize the encoded enzymes, and to ultimately perform a certain enzymatic nitrogen transformation reaction. Differences in abundance of functional marker genes can be interpreted as higher or lower ability of the soil microbial community to perform specific enzymatic transformation reactions as result of cellular growth or decay of different functional groups (‘guilds’) of microorganisms.

Homogenized soil from the field and from the microcosms was stored at ~20 °C prior to extraction. Soil DNA was extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the
were performed on ice. DNA was eluted in solution 6 (10 mM Tris pH 8). DNA concentrations were checked using NanoDrop™ 1000 (NanoDrop products, Wilmington, DE, USA). Extraction yields ranged from 20.5 to 61.2 ng µL⁻¹ with a 260–280 nm ratio of 1.67–1.94.

The abundance of the functional marker genes including amoA (bacteria and archaea), nirK, nirS, and nosZ was assessed by quantitative polymerase chain reaction (qPCR) using protocols, primers, and plasmid standards as described by Harter et al. (2014). qPCR was performed in analytical triplicates using the iCycler iQ Real-Time PCR Detection System and the IQ5 OPTICAL SYSTEM software, version 2.0 (Bio-Rad, Hercules, CA, USA). Standard plasmid concentrations were quantified fluorometrically using Qubit 2.0 (Invitrogen, Carlsbad, CA, USA). Gene copy numbers were obtained from the IQ5 software and are determined based on the threshold cycle (CT).

Statistics

Data evaluation and statistical analysis were conducted in ORIGIN PRO 8 (OriginLab, Northampton, MA, USA). We calculated cumulative gas emissions by integration and compared treatments using the paired t-test.

Results

Geochemical data field experiment

Geochemical data from the field experiment are summarized in Fig. 1 along with data on precipitation and soil temperature. During the entire growing season, biochar-amended plots exhibited a lower WFPS (41 ± 5% to 60 ± 11% WFPS) than the control plots (48 ± 5% to 72 ± 6% WFPS). Gravimetric water content did not vary considerably between treatments, but biochar significantly reduced the bulk density of the soil from 1.40 ± 0.06 to 1.25 ± 0.13 kg L⁻¹ (P = 0.001). Soil pH was 7.1 ± 0.1 for both biochar and control treatments.

N₂O emission rates from the nonbiochar-amended control plots showed three peaks of up to 0.39 ± 0.20 mg N₂O–N m⁻² h⁻¹ (June 5) in the period following fertilization to the beginning of June. These peaks were less pronounced in the biochar plots. After June 19, emissions from both treatments did not show considerable differences. In summary, cumulative emissions from the biochar treatment were with 63% significantly lower compared to the biochar-free control treatment with 1.0 ± 0.4 kg N₂O–N ha⁻¹ vs. 2.7 ± 0.5 kg N₂O–N ha⁻¹, respectively (P = 0.009).

Concentrations of both nitrate and ammonium increased after fertilization (April 25) and decreased over the growing season. For nitrate, there were no differences between the treatments. The decrease in ammonia concentrations was faster in the biochar treatment, because concentrations in June were lower than in the control treatment (4.5 ± 0.8 vs. 9.1 ± 2.9 mg NH₄⁺–N kg⁻¹). Nitrite was below detection limit at all sampling time-points.

Concentrations of DOC hardly changed over time and only showed minor differences between treatments (higher concentration in the biochar treatments in June). CO₂ emissions showed a peak on May 15 that was higher for the control treatment (1.0 ± 0.6 g CO₂–C m⁻² h⁻¹) than for the biochar treatment (0.4 ± 0.2 g CO₂–C m⁻² h⁻¹). The CO₂ emission peak coincided with the N₂O emission peak. At the other two N₂O emission peaks, only minor CO₂ emission peaks could be identified. In total, CO₂ emissions from the biochar treatments were significantly lower (43%) than from the control treatments (7.7 ± 1.6 Mg CO₂–C ha⁻¹ vs. 4.4 ± 1.1 Mg CO₂–C ha⁻¹, P = 0.025).

Geochemical data microcosm experiment

The microcosm experiment was conducted at constant temperature (28 °C) and constant WFPS (60%). Biochar increased the soil pH in the microcosms from 6.5 ± 0.0 to 6.7 ± 0.0. Other geochemical data are summarized in Fig. 2. N₂O emissions peaked on day 0 (the day when microcosms were setup and sampled for the first time) for both control and biochar treatments. On this day, gas enrichment started 3 h after fertilization. Emissions rates from the control treatment were significantly higher than the emissions from the biochar treatment (2.7 ± 0.4 vs. 0.6 ± 0.1 mg N₂O–N m⁻² h⁻¹, P = 0.007). The emission rate of the control treatment dropped by 57% within the first 24 h. Compared to the biochar-free control, N₂O emission of the biochar treatment was lower or equal at all sampling events. Cumulative N₂O emissions were significantly reduced by 55% by biochar amendment. (0.37 ± 0.25 kg N₂O–N ha⁻¹ vs. 0.83 ± 0.18 kg N₂O–N ha⁻¹, P = 0.048).

Nitrite concentrations peaked on day 0 with a 62% lower concentration in the biochar microcosms compared to the control. After day 3, nitrite concentrations were approximately 1 µg NO₂⁻–N kg⁻¹ for both treatments. The concentration of nitrate increased continuously, while the concentration of ammonium decreased. There was no considerable difference between the treatments for the concentrations of both of these two N species.

Emissions of CO₂ peaked on day 0 for both treatments with a higher emission from the control (313 ± 19 mg CO₂–C m⁻² h⁻¹) than from the biochar treatment (248 ± 26 mg CO₂–C m⁻² h⁻¹, P = 0.113). Cumulative CO₂ emissions were 30% lower in the biochar treatment; however, this reduction was not
significant by conventional criteria (140 ± 28 Mg CO₂–C ha⁻¹ vs. 97 ± 22 Mg CO₂–C ha⁻¹,  P = 0.272), too. Highest DOC concentrations were quantified on day 0 with 0.23 ± 0.04 mg DOC g⁻¹ for the control treatment and 0.20 ± 0.06 mg DOC g⁻¹ for the biochar treatment. DOC concentrations dropped within the first 24 h by 36% for the control and by 29% for the biochar treatment and remained constant thereafter.

Abundance of nitrogen-transforming microorganisms

The abundance of nitrogen-transforming microorganisms was estimated by quantifying gene copy numbers of key functional genes involved in nitrogen transformation reactions by quantitative PCR. Gene copy numbers of nosZ (encoding for a N₂O reduc-tase), nirS, and nirK (encoding for two different
nitrite reductases) and amoA (encoding for an ammonia oxidase) of both ammonia-oxidizing bacteria and archaea were quantified for both the field (Fig. 3) and the microcosm study (Fig. 4). For both, the microcosm and field study, we found no temporal changes in gene abundances and no differences between the biochar and control treatments. Only on day 5 of the microcosm study, a lower abundance of nirS + nirK was detected for the biochar treatment compared to the control (Fig. 4). In general, the copy numbers of functional marker genes for denitrification (nirK, nirS, and nosZ) were one order of magnitude higher than those for nitrification (amoA).

Sunflower yield
In the third growing season after biochar amendment, total biomass yield of H. annuus did not differ between the nonamended control treatment (13.1 ± 0.5 Mg ha⁻¹, average ± standard deviation, n = 4) and the biochar treatment (13.1 ± 0.4 Mg ha⁻¹).
Discussion

In this study, we quantified the impact of biochar in an agricultural field experiment on N$_2$O emissions, mineral nitrogen speciation, and crop yield in the third year after initial soil biochar amendment at a rate of 60 t ha$^{-1}$. N$_2$O emissions and nitrogen speciation were also quantified in soil microcosm experiments that were performed under controlled laboratory conditions using nonweathered/pristine biochar. For both field and laboratory experiments, the abundance of key functional marker genes of microbial nitrification (amoA) and denitrification (nirK, nirS, and nosZ) was quantified using qPCR.

Biochar effectively reduced N$_2$O emissions in the third year in the field, especially during three major emission events of N$_2$O between April and June. It is remarkable that the N$_2$O emission mitigation of biochar was still that pronounced in the third year after biochar amendment. Previous studies have questioned that the N$_2$O emission suppression would persist for longer time periods (Spokas, 2013; Felber et al., 2014). However, sunflower biomass yield was unaffected by the presence of biochar. So the biochar used in this study did not provide a direct economic benefit to farmer in our intensively managed field experiment applying inorganic fertilizer and a nitrification inhibitor.

Source of N$_2$O in microcosm and field soil experiments

Quantitative PCR revealed a higher genetic potential of the soil microbial community for denitrification compared to nitrification, while according to the WFPS, field and soil microcosms soil conditions should both favor nitrification (Bateman & Baggs, 2005). Ruser et al. (2006) showed that more than half of the N$_2$O emission from a silty and loamy soil was a result of nitrate reduction even under very dry conditions of 40% WFPS. Denitrification can be a relevant process even under oxic conditions due to microscale anoxia within individual soil aggregates (Sexstone et al., 1985; Bateman & Baggs, 2005; Russow et al., 2009). Therefore, it seems reasonable to assume that both aerobic and anaerobic processes of N$_2$O production can occur on a microscale within a soil aggregate. In our microcosm experiment, soil was sieved to 2 mm, that is, there were no larger soil aggregates. But water was added from the top and its infiltration might not have been homogenous.

Overall, the decrease in NH$_4^+$ and increase in NO$_3^-$ suggested that nitrification contributed to N$_2$O production. However, N$_2$O emission rates declined from day 0 to day 3 and remained constant at low levels thereafter. In contrast, NH$_4^+$ concentrations declined constantly until day 5. Thus, the rapid decline of the emission rates was not caused by NH$_4^+$ limitation. However, DOC concentrations declined rapidly within the same time period as N$_2$O and CO$_2$ emissions decrease, suggesting a correlation of heterotrophic microbial activity with N$_2$O emissions. This might suggest a direct or indirect coupling of nitrification and denitrification via nitrifier denitrification contributing to the initial N$_2$O emission.

Long-term effect of biochar on reduction in greenhouse gas emission

The suggested mechanisms of soil N$_2$O emission mitigation by biochar amendment are manifold and vary with soil and biochar type and experimental conditions. Here, we considered the impacts of biochar on soil pH, WFPS, substrate limitation, and the abundance of nitrogen-transforming microorganisms.

Soil pH was proposed to control the ratio of N$_2$O/ (N$_2$O + N$_2$) emissions derived from denitrification with higher pH resulting in lower ratios (Bergaust et al., 2010). The increase in soil pH found in the microcosms after biochar amendment was not observed in the third year of the field experiment, where control and biochar plots had the same pH. This was expected, as also Spokas (2013) and Heitkötter & Marschner (2015) found lower pH of biochar after aging in soil. Thus, long-term N$_2$O emission suppression cannot solely be explained by pH effects. This is in contrast to results reported by...
Van Zwieten et al. (2014) and Obia et al. (2015). These studies found N₂O emission reduction in acidic/acid soils after biochar addition, while the soil from the ‘Goldener Acker’ used in this study had a circumneutral pH. Also Hippi et al. (2015) did not find evidence for pH induced N₂O emission suppression by biochar in a temperate soil. However, they could not fully reject this hypothesis, too.

Water-filled pore space governs the overall contribution of denitrification to soil microbial N transformations (Davidson, 1991). While microcosms were set up with a constant WFPS of 60% for both treatments, biochar reduced the bulk density and by that reduced the WFPS in the field. This is contrary to the findings of Felber et al. (2014), who reported a slight increase in WFPS in a pasture field after amendment with greenwaste biochar. However, they used biochar that was not milled. In our study, the powdered biochar did not influence the gravimetric water content, but decreased soil bulk density. Interestingly, biochar increased the bulk density in the microcosm experiment due to its small particle size <250 μm. Thus, the effect of reduced bulk density seems to have evolved over time by improved soil aggregation induced by biochar in the field. This effect was also shown by Quin et al. (2014) using X-ray μ-CT and Baiamonte et al. (2014) using the high-energy moisture characteristic technique. However, both studies used freshly prepared soil biochar mixtures. Unfortunately, we only started to measure GHG emissions, soil bulk density, and WFPS in the third year of the field experiment. Obia et al. (2016) performed a rainfall simulation experiment with disturbed soil from a field trial. They also found improved soil aggregation after biochar amendment. Reduced WFPS results in increased soil aeration, which was previously suggested as one mechanism for N₂O emissions suppression by biochar (Van Zwieten et al., 2010). Improved aeration might reduce denitrification activity (Van Zwieten et al., 2010). However, Ameloot et al. (2016) found the same rate of biochar-induced N₂O emission reduction for both undisturbed and disturbed (sieved, grounded) soil cores from a field experiment. Thus, increased soil aggregation or other indirect physical controls could not explain the observed emission reduction as it was the case for our short-term microcosm experiment, too. Additionally, Case et al. (2012) found that improved aeration could explain biochar-induced N₂O emission suppression in a sandy loam soil only to a limit extend, still it could contribute to N₂O mitigation in the field experiment.

Substrate limitation, that is, a reduced availability of NO₂⁻, NH₄⁺, and DOC, was suggested as another mechanism (Singh et al., 2010; Van Zwieten et al., 2010; Taghizadeh-Toosi et al., 2011; Harter et al., 2014). In our field experiment, the availability of NH₄⁺ was reduced. NH₄⁺ sorbed to the biochar might be only partly extractable with 2 M KCl extraction due to slow release of both anionic and cationic ions form biochar (Kammann et al., 2015). Additionally, it needs to be considered that ammonium was added to the field as ENTEC®, which contains DMPP to avoid or retard nitrification (Zerulla et al., 2001). Biochar might sorb DMPP, reduce its effect, and thus ammonium might have been nitrified more rapidly. NH₄⁺ is the substrate for both nitrification and nitrifier denitrification, and its limitation might partially contribute to the observed emissions suppression in the field experiment.

Biochar amendment can alter the soil microbial community as a result of aforementioned changes in soil geochemistry and by providing a new habitat for microbes (Quilliam et al., 2013). Biochar has also been shown to enhance microbial redox reactions by serving as direct source or sink for electrons (Klüpfel et al., 2014; Saquing et al., 2016) or by facilitating electron shuttling (Kappler et al., 2014). However, the abundance of nitrogen metabolizing bacteria did not alter with time nor between treatments in both the field and microcosm experiment. This is in line with Van Zwieten et al. (2014) who found changes in the abundance of amoA and nosZ just in one of four soils after biochar amendment. One needs to keep in mind that the measured abundance of functional genes just provides information on the genetic potential of the microbial community. Still, biochar could increase the activity of the functional genes as shown in other studies (Harter et al., 2014; Xu et al., 2014) that quantified transcript copy numbers following soil RNA extraction.

Accumulation of nitrite

In the microcosm experiment, biochar decreased the accumulation of NO₃⁻ on day 0 and 1. This is of interest, as already Yanai et al. (2007) claimed that further studies on NO₃⁻ accumulation in soil are necessary to understand the mechanisms of biochar-induced N₂O emissions suppression. Also, Russow et al. (2009) described the importance of NO₃⁻ for both nitrification and denitrification. Also, Müller et al. (2006) and Rütting et al. (2008) described the contribution (10% and 6% of total NO₃⁻, respectively) of heterotrophic nitrification to the NO₃⁻ pool in different soils. Heterotrophic nitrification produces NO₂⁻ from organic N independent of NH₄⁺ and NO₃⁻ pools. However, the study by Rütting et al. (2008) also highlights the functional link between heterotrophic nitrification and DNRA as well as between nitritation and subsequent NO₂⁻ reduction. Harter et al. (2014) found a decrease in NO₂⁻ accumulation parallel to N₂O emission mitigation, but their study
was conducted at 90% WFPS. Clough et al. (2010) found higher peak concentrations of NO\textsubscript{2} and higher N\textsubscript{2}O emissions in soil + biochar + urine microcosms if compared to just soil + urine microcosms. Sánchez-García et al. (2014) added Na\textsuperscript{15}NO\textsubscript{2} as a N source to one of their incubations and showed that biochar was not able to reduce N\textsubscript{2}O emissions from the NaNO\textsubscript{2} treatment. If biochar does not influence N\textsubscript{2}O production from NO\textsubscript{2}, then one potential hypothesis for the explanation of the observed N\textsubscript{2}O emission reduction in this study might be lower NO\textsubscript{2} concentrations in the biochar treatments. However, our study just quantified concentrations, but no fluxes based on, for example, \textsuperscript{15}N stable isotope data. Thus, it is not possible to differentiate if biochar reduced the production of nitrite or increased nitrite consumption. The latter explanation would refute the hypothesis of biochar-induced suppression of nitrite formation. Besides microbially mediated biochar effects, nitrite can be also immobilized abiotically by biochar. Fitzhugh et al. (2003) showed the abiotic immobilization of nitrite by soil organic matter. Additionally, NO\textsubscript{2} was below the detection limit for both treatments in the field experiment. Still, NO\textsubscript{3} could play a role in denitrification hotspots within single soil aggregates. This would not be detectable at a bulk level. Unfortunately, most studies on biochar and N\textsubscript{2}O emissions do not report NO\textsubscript{2} data, or they just quantify ‘NO\textsubscript{2} + NO\textsubscript{3}’ (Cayuela et al., 2010) or ‘mineral N’ (Schouten et al., 2012).

**Sunflower yield in the field experiment**

Biochar did not alter the yield of sunflower biomass for biogas production in this study. In their meta-analysis, Jeffery et al. (2015) calculated a grand mean of 18% yield increase by biochar across 60 studies. Considering the more specific characteristics for the field trial in the present study according to the classifications of Jeffery et al. (2015) (biochar application rate of 51–60 t ha\textsuperscript{-1}, ~19% yield increase; wood biochar, ~28% yield increase; initial pH of soil in the range of 7.1–7.5, ~20% yield increase but not significant at the 5% level), a yield increase could have been expected. However, Paneque et al. (2016) observed an increase in sunflower yield after application only with one of four biochars tested in a Mediterranean soil. Our results suggest that biochar is not able to further increase biomass yields in an optimized system, that is, an ecosystem in which plant growth is obviously not limited by nutrients, etc. The Haplic Luvisol at our study site ‘Goldener Acker’ is a very fertile loamy Loess soil. It was fertilized according to optimized standard practices for sunflower. This included a N input of 120 kg N ha\textsuperscript{-1}. Thus, there might have been no limitation to plant growth that could be improved by biochar. However, Graber et al. (2010) described the improvement of pepper plant growth by biochar in a coco fiber and tuff growth medium even ‘under optimal fertigation conditions’. In the present study, we focused on total biomass yield and did not find an effect of biochar, while we did not assess more specific parameters, as, for example, sunflower seed yield or quality. Additionally, Graber et al. (2010) described benefits for green pepper, but did not find significant effects of biochar on tomato yield. Biochar-induced yield increase beyond physiological optima seems to be restricted to certain plant species and could not be confirmed for sunflower in this study.

**Limitations and open questions on mechanisms**

This combined microcosm and field study showed that a wood biochar from slow pyrolysis (400 °C) effectively reduced the N\textsubscript{2}O emissions in both freshly amended soil microcosms and under field conditions in the third year after amendment. However, for a complete assessment of the mitigation potential of biochar, year-round field measurements are necessary.

It is important to note that the microcosm experiments were normalized to total dm of soil and soil+biochar mixture. Thus, the biochar treatments contained 5% less soil and with that 5% less soil microbes and 5% less soil organic matter and organic nitrogen as potential substrates for N\textsubscript{2}O producing metabolisms. However, these minor differences between the control and biochar microcosms were within the measurement error for N\textsubscript{2}O quantification.

The mechanism of biochar-induced N\textsubscript{2}O emission suppression requires further research. Biochar did not alter the abundance of functional marker genes of neither nitrification nor denitrification and hardly affected soil geochemistry beyond the reduction in N\textsubscript{2}O emissions. Biochar reduced the accumulation of NO\textsubscript{2} at the beginning of the microcosm experiment, which needs further investigation to understand underlying mechanisms of nitrite production and reduction in the presence of biochar. In the field, the reduced bulk density and WFPS in the biochar plots might be related to the observed N\textsubscript{2}O emission mitigation. This emphasizes that biochar most likely exerts multiple influences on the pedosphere resulting in N\textsubscript{2}O emission reduction. These effects might vary over time with respect to their overall contribution to N\textsubscript{2}O mitigation.

Biochar might have an immediate effect on (bio)chemical N transformations in both field and laboratory-based microcosm experiments by altering nitrogen availability, soil pH, and soil aeration (due to particle size) as soon as fresh biochar is mixed into soil. However, while nitrogen immobilization and liming capacity of biochar decrease with time (e.g. lower pH of aged
biochar as described by Spokas, 2013), biochar particles also break into smaller and smaller fractions due to physical interaction with water (Spokas et al., 2014). Aging of biochar in soil can induce enhanced soil aggregation and with that affect soil aeration. Additionally, biochar forms organo-mineral complexes and the amount oxidized functional groups on the biochar surface increases (Lin et al., 2012; Pignatello et al., 2015). While all these different processes might happen more or less simultaneously as biochar ages in soil, they all might contribute to the long-term effects of biochar on soil N2O emission in the field. In order to further elucidate the consequences of the ‘evolution of biochar properties in soil’ (Pignatello et al., 2015) on soil-borne N2O emissions, more studies should be performed that evaluate the effect of manually picked, ‘aged’ biochar particles from soil of long-term field trials for use in laboratory microcosm studies as previously done by Spokas (2013). However, this depends on initial biochar particles sizes and was not possible in this study because biochar particles were <300 μm.

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