Biochar affects community composition of nitrous oxide reducers in a field experiment

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\begin{abstract}
NO\textsubscript{2} is a major greenhouse gas and the majority of anthropogenic N\textsubscript{2}O emissions originate from agriculturally managed soils. Therefore, developing N\textsubscript{2}O mitigation strategies is a key challenge for the agricultural sector and biochar soil treatment is one reported option. Biochar's capacity to increase soil pH and to foster activity of specialized N\textsubscript{2}O reducers has been proposed as possible mechanisms for N\textsubscript{2}O mitigation. An experiment was undertaken to investigate whether changes in the community composition of N\textsubscript{2}O reducers was observed under field conditions after biochar application. The study objective was to assess the abundance and taxonomic composition of the functional marker genes nosZ and nosZ\textsubscript{II} across a vegetation period of \textit{Zea mays} L. after biochar or lime addition compared to an untreated control. After fertilization, biochar amendment resulted in a significant increase of nosZ gene copy numbers compared to the control and the lime treatment. Simultaneously a shift in community composition of nosZ-II bearing bacteria was observed in the biochar treatment that went beyond the sole liming effect. This study broadens our understanding of the functional impact of biochar on N\textsubscript{2}O emissions and emphasizes the possibility to shape the functioning of the N\textsubscript{2}O reducing microbial community through the addition of biochar at a field scale.
\end{abstract}

\section{1. Introduction}

Nitrous oxide (N\textsubscript{2}O) is a potent greenhouse gas and also catalyzes stratospheric ozone depletion (Ravishankara et al., 2009). Atmospheric N\textsubscript{2}O concentrations are constantly rising and reached 328 ppb in 2016 (121\% of preindustrial levels) mainly due to anthropogenic intervention in the nitrogen cycle (Davidson, 2009; WMO, 2016). Since agriculturally managed soils emit 4.3–5.8 Tg N\textsubscript{2}O yr\textsuperscript{-1}, developing effective mitigation strategies is a key challenge for the agricultural sector (Butterbach-Bahl et al., 2013). As N\textsubscript{2}O is a long lived greenhouse gas with an atmospheric half life time of 114 years, elevated atmospheric N\textsubscript{2}O concentrations will be a long term issue (IPCC, 2013). N\textsubscript{2}O in soils is mainly produced by microbial mediated nitrogen transformations and the process of denitrification is widely recognized as one of the most important N\textsubscript{2}O producing processes (Davidson, 2009). Denitrification describes the stepwise reduction of nitrate (NO\textsubscript{3}\textsuperscript{-}) to molecular nitrogen (N\textsubscript{2}) and occurs under oxygen limited conditions in water saturated soils, especially after fertilization which increases the bioavailability of nitrogen compounds (Davidson, 2009). N\textsubscript{2}O is produced as an obligatory intermediate in the denitrification process. The last step in denitrification, the reduction of N\textsubscript{2}O to N\textsubscript{2}, presents the only known biological sink for N\textsubscript{2}O (Thomson et al., 2012). The enzyme performing this last reduction step is encoded by the functional gene nosZ (Philippot et al., 2007). The functionality of this enzyme is highly sensitive to oxygen and low soil pH (Liu et al., 2014; Zumft and Kroneck, 2006). Therefore the last step in denitrification is often impaired and high amounts of N\textsubscript{2}O is produced.

Since denitrification is a modular process and not all microbes involved possess the whole set of denitrification genes (Graf et al., 2014), the community composition of denitrifiers and especially N\textsubscript{2}O reducers might be an important controlling factor for N\textsubscript{2}O emissions (Jones et al., 2014; Philippot et al., 2011). Around one third of denitrifiers lack the genetic capability to reduce N\textsubscript{2}O and therefore act as a source for N\textsubscript{2}O (Philippot et al., 2011). On the other hand, some N\textsubscript{2}O reducers lack
the genes to produce N₂O from NO₃⁻ and thus might act as an N₂O sink by reducing exogenous N₂O. This especially applies to the recently described nosZ-II bearing bacteria since ~50% of the so far known nosZ-II bearing bacteria lack the genetic capability to produce N₂O (Graf et al., 2014; Jones et al., 2014; Sanford et al., 2012). Recently it was shown that some agricultural practices can change the diversity of N₂O reducers and affect the N₂O/(N₂O + N₂) ratio (Domeignoz-Horta et al., 2015). Identifying and developing agricultural practices that promote complete denitrification through stimulation of exclusive N₂O reducers might be one way to reduce N₂O emissions and a step towards climate smart agriculture.

Biochar is a carbon-rich product of incomplete combustion that is added to the soil to improve soil quality and enhance carbon sequestration (Verheijen et al., 2009). Although the physiochemical properties of biochar largely depend on the feedstock and production temperature, the majority of biochars share some common characteristics such as an alkaline pH, high surface area and stable aromatic carbon structures (Atkinson et al., 2010; Mandal et al., 2016). Due to these physiochemical properties, the addition of biochar to agriculturally managed soils is discussed as a management tool to address environmental issues associated with current agricultural practice, such as nitrogen leaching and long term stabilization of organic carbon (Lehmann and Joseph, 2009; Mandal et al., 2016; Singh et al., 2010; Sohi et al., 2010; Woolf et al., 2010). Biochar amendment is also discussed as a management option to mitigate N₂O emission since decreased N₂O emissions were reported from a range of field and incubation experiments (Cayuela et al., 2013; Harter et al., 2014; Singh et al., 2010; van Zwieten et al., 2014). Parameters such as the biochar feedstock, soil texture and the chemical form of N fertilizer were found to influence the extent of N₂O mitigation through biochar addition (Clough et al., 2013), but the key mechanism causing decreased N₂O emissions is still poorly understood (Cayuela et al., 2013, 2014; Clough et al., 2013). Since there is evidence that a high soil pH fosters N₂O reduction (Baggs et al., 2010; Cuhel et al., 2010), some authors suggest an increased pH due to biochar addition to be the main driver in lowering soil derived N₂O emissions under controlled incubation conditions (Obia et al., 2015; van Zwieten et al., 2010; Zheng et al., 2012). Furthermore, incubation experiments showed that reduced N₂O emission after biochar addition was accompanied by an increased activity of nosZ bearing denitrifiers and a shift in denitrifier community composition (Anderson et al., 2014; Harter et al., 2014, 2016b; van Zwieten et al., 2014). Molecular fingerprinting techniques revealed that biochar addition can affect the microbial community composition (Anderson et al., 2011; Chen et al., 2013). Furthermore, functional communities involved in nitrogen transformation and degradation of complex organic structures can be affected by the addition of biochar (Chen et al., 2015; Kolton et al., 2011). While the potential of biochar to shape the general microbial community composition seems to be evident, to our knowledge there is only one study that investigated the impact of biochar on the community composition of N₂O reducing bacteria. In a short term incubation experiment biochar addition resulted in a reduction of N₂O emissions (Harter et al., 2014), which could be linked to community shifts in nosZ and nosZ-II bearing bacteria, favoring bacteria specialized in N₂O reduction (Harter et al., 2016b). Community shifts might have been induced by entrapment of N₂O on biochar surfaces (Harter et al., 2016a). However, other underlying mechanisms driving the reduction of N₂O emissions after biochar addition cannot be excluded, including an abiotic N₂O reduction on biochar surfaces (Quin et al., 2015) or enhancement of N₂O reduction induced by pH (Clough et al., 2013; Obia et al., 2015). Although a recent field study observed a reduction of N₂O emissions three years after biochar amendment (Hagemann et al., 2016b), the effect of biochar amendment on N₂O emissions under field conditions generally seems less pronounced compared to incubation experiments (Cayuela et al., 2014; Verhoeven et al., 2017). Therefore we aimed to investigate whether biochar alters abundance or community composition of N₂O reducers under field conditions compared to a control and a limed treatment. For this purpose we assessed the abundance and composition of the functional marker genes nosZ and nosZ-II in a biochar field trial across a vegetation period accompanied to a long term N₂O measuring campaign (Hüppi et al., 2015). By including a limed control treatment we aimed at assessing the liming effect of the biochar independently.

2. Material and methods

2.1. Sampling site

The experiment was established on arable land at the Agroscope research station in Zürich (Switzerland, 47.427°N, 8.522°E, 437 m a.s.l.) on a Eutric Mollic Gleysol (IUSS Working Group WRB, 2015) in January 2014. The field trial is located in a temperate climate with mean annual temperature of 9.4 °C and mean annual precipitation of 1054 mm (Climate data from 1981-2010, Meteoswiss). The soil texture was classified as clay loam with a particle size distribution of 37% sand, 27% silt and 36% clay. Before the establishment of the experiment the field was under conventional agricultural management. The year before the start of the experiment Zea mays L. was cultivated and after harvesting the field was ploughed in autumn 2013. Thereafter, no cover or catch crop was sown until the start of the vegetation period in 2014. Further details on the field trial can be reviewed elsewhere (Hüppi et al., 2015).

2.2. Experimental setup

Each treatment was replicated three times in experimental plots sized 3 × 2 m in a randomized complete block design with 1 m buffer zone between the plots. Soil sampling was undertaken using micro-plots of 30 × 30 cm. In order to investigate the liming effect of biochar an additional treatment was established by adding 5 t ha⁻¹ of limestone. The field plots were sown on 31st of March and the upper layer of soil (15 cm) was thus thoroughly mixed. For the first and third N fertilization of Zea mays L. fertilizer (LONZA-Ammonsalpeter; 27.5% N) was applied at a rate of 40 kg N ha⁻¹ on May, 26th and July 16th. The second N fertilization was carried out on June 16th using Ammonium Nitrate (NH₄NO₃) at a rate of 80 kg N ha⁻¹. The harvesting took place on 13th of October. In order to assess community structure of N₂O reducers, the first set of soil samples from each micro-plot was taken after “harrowing” at 31st of March. An N₂O emission event occurred 9 days after the second fertilization, post-rainfall. Therefore, the second sampling date labeled as “fertilization” refers to soil sampling at the 25th of June 2014 (Supplementary Fig. S1). The third soil sampling was performed at the end of the cropping period after “harvesting” on 13th of October. For the soil sampling from each microplot, 10 soil cores up to 10 cm were taken and immediately homogenized for DNA analysis, which was immediately frozen in liquid nitrogen and stored at −80 °C until further processing. Greenhouse gas measurement with an automated closed chamber system started from the month after biochar application in mid-February and continued until harvesting of the Zea mays in October 2014. Details of N₂O and CO₂ quantification as well as pH adjustment in the lime and biochar treatments are described elsewhere (Hüppi et al., 2015).

2.3. Geochemical analysis of soil and biochar

The biochar was produced by slow pyrolysis in a Pyreg reactor (Pyreg GmbH, Dörth, Germany) with a peak temperature of 650 °C and an average residence time of 25 min. The feedstock was green waste obtained from tree pruning and was made up of ~80% softwood and ~20% hardwood. The pH of the soil and biochar was assessed in water at a ratio of 1:2.5 w/v using a PH100 ExStick pH meter (Extech Instruments Corp., Nashua, NH, USA). Elemental composition (C, N and H) of soil and biochar was measured by dry combustion of milled
subsamples in an elemental analyzer equipped with GC-TCD (Euro EA, HEKATECH GmbH, Wegberg, Germany). The same analyzer was used to measure oxygen content of the biochar after pyrolysis at 1000°C. The surface area of the biochar was quantified by N₂ adsorption after 12 h of vacuum degassing with a NOVA2e from Quantachrome Instruments (Odellhausen Germany). The biochar ash content was determined after combustion at 800°C for 2h with addition of oxygen. The bulk density of soil was measured at a depth of 3–8 cm with 3 steel cores (100 cm³) in each plot. For determination of soil NH₄⁺ and NO₃⁻ concentrations, 20g of moist soil were mixed with 100 ml of 0.01 M CaCl₂ solution for 30 min. The suspension was filtered and subsequently analyzed by segmented flow injection analysis (SKALAR SANplus, Skalar Analytical B.V., Breda, Netherlands). Soil texture was characterized by a combination of sieving and sedimentation analysis following DIN ISO 11277.

Basic properties of the soil and the biochar are listed in Table 1 and Supplementary Table S1.

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<tr>
<td>Bulk density (g cm⁻³)</td>
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### 2.4. Molecular biological methods

#### 2.4.1. DNA extraction and qPCR

DNA was extracted via phenol chloroform extraction from 0.5 g of soil sample (Griffiths et al., 2010). Before bead beating 9.1 × 10⁹ copies of a linearized plasmid (pJET1.2, CloneJET PCR Cloning Kit, Thermo Scientific, Waltham, MA) carrying a fragment of cassava mosaic virus (APA9, gene accession Nr. AJ427910) were added to the soil samples in order to assess DNA recovery rates for each sample (Thoner et al., 2012). DNA yield was assessed fluorometrically with Qubit 2.0 (Qubit dsDNA HS Assay Kit and Qubit RNA HS Assay Kit, Invitrogen, Carlsbad, CA, USA) directly after extraction and ranged from 21.7 to 60 ng µl⁻¹. DNA extracts were further purified using OneStep™ PCR Inhibitor Removal Kit (D6030, Zymo Research, Irvine, USA). Quantitative PCR of functional genes was performed using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA) and a Rotor-Gene-Q (QIAGEN, Venlo, Netherlands). Each 10 µl reaction volume included 1 ng of template DNA. Primers and thermal protocols used for functional gene quantification are listed in Supplementary Tables S2 and S3. Standard curves were constructed by running a serial dilution of a plasmid bearing a copy of the respective gene with concentrations ranging from 10⁸ to 10¹⁰ gene copies per reaction. Specifications of vector plasmids and host genes are given in Supplementary Table S4. Concentrations of standards were measured fluorometrically with Qubit 2.0 (Qubit dsDNA HS Assay Kit, Invitrogen, Carlsbad, CA). Each reaction was performed in analytical duplicates and was repeated if Ct values differed by more than 0.5. Efficiencies of qPCRs were 86–90% for the APA9 gene fragment, 79–84% for nosZ and 77–78% for nosZ-II assays. R² and y-intercepts of qPCR analysis can be reviewed in Supplementary Table S5. Specificity of the amplification was checked by melt curve and agarose gel analysis. Raw data was analyzed via LinReg PCR by examining enzyme kinetics for each reaction individually (Ramakers et al., 2003). In addition to normalization of functional gene abundances per soil dry weight (g), DNA extraction efficiencies obtained by APA9 quantification were used to correct functional gene copy numbers (Thoner et al., 2012).

#### 2.4.2. Illumina amplicon sequencing

A total of 27 DNA extracts (3 sampling dates, 3 treatments and 3 replicates) were employed for Illumina sequencing using a two-step PCR procedure. In the first step the products of three independent PCRs were pooled and purified using DNA Clean & Concentrator™ (D4033, Zymo Research, Irvine, USA). Primer, master mixes and thermal cycling conditions for nosZ and nosZ-II are shown in Supplementary Table S6. The size of the purified products was verified using agarose gel electrophoresis. Subsequent library preparation and sequencing were performed at Genome Quebec Innovation Center (Montreal, Canada) according to the amplicon sequencing guidelines given by Illumina (San Diego, CA, USA). Sequencing was performed on an Illumina MiSeq™ sequencing system using the 2 × 250 bp providing MiSeq Reagent Kit v2 (Illumina, San Diego, CA, USA). Primer sequences of the obtained raw sequences were removed using Cutadapt v1.9 (Martin, 2011). Given the length of the fragments of ca. 700bp reads could not be merged and forward reads were used for further processing due to higher phred scores. Reads were quality filtered using USEARCH fastq_filter function with a maximum error threshold of 1. Chimeric sequences were removed using the uchime_denovo algorithm implemented in USEARCH (Edgar et al., 2011). Quality filtering yielded a mean of 57,206 ± 9594 and 27,473 ± 6997 high quality reads per sample for nosZ and nosZ-II genes, respectively. No treatment specific bias could be detected. Quality filtered sequences for nosZ and nosZ-II were translated to protein sequences and mapped against the NCBI Reference Sequence protein database (RefSeq) using DIAMOND (Buchfink et al., 2015) in blastx mode with a minimum protein sequence identity cutoff of 70% and an e-value cutoff of 10⁻¹⁰. 98.7 ± 0.4% and 97.9 ± 0.7% of the reads matched nosZ and nosZ-II types of nitrous oxide reductase genes in the KEGG database (Kanehisa et al., 2016), respectively. Matched sequences were used for further analysis using the weighted Lowest Common Ancestor (LCA) algorithm implemented in MEGAN6 Ultimate Edition (Huson et al., 2016). The LCA analysis parameters “Top percent” and “Min Support” were set to 0.5% and 15. This implies that all hits within the top 0.5% of the best bit score are used for further analysis and a minimum of 15 reads is needed for each taxa to be considered. Given the basic principle of the LCA algorithm, sequences that are conserved among different species were assigned to taxa of a higher rank (Huson et al., 2007). On average 50.527 ± 8.515 and 22.346 ± 5.771 nosZ and nosZ-II sequences were assigned on a species level and used for further statistical analysis. It should be noted that a direct proof that a given sequence is associated with a specific microbial taxon is difficult to obtain, therefore caution should be observed when specific species names are mentioned. When we refer to specific names, we infer microbes that contain nosZ and nosZ-II genes that are closely related to the respective gene of the named species. The raw sequences have been deposited in theENA Sequence Read Archive (SRA) under the accession number PRJE19689.

#### 2.5. Statistical analysis

All statistical analyses were performed using R version 3.3.1. Differences in abundance of functional marker genes nosZ and nosZ-II were assessed by performing an ANOVA with a Post Hoc Tukey test using sampling date and treatment as factor. Furthermore, a linear regression between functional gene abundances and geochemical parameters across all sampling dates was conducted. Permutational multivariate analysis of variance (Permanova) was performed using the “vegan” package in order to assess significant effect of experimental factors and geochemical parameters on community composition of nosZ and nosZ-II N₂O reducers (Anderson, 2001; Jari Oksanen et al., 2009). In a first step, the sampling date and treatment factors and its
interaction were assessed. The factor plot, which was nested in the factor sampling date, was included in this analysis as a measure for spatial heterogeneity (Table 2). In a second step the same procedure was employed to test significant treatment effects at each sampling date. All Permanova analyses were performed with $10^4$ permutations. Community composition of nosZ and nosZ-II gene sequences was visualized in vegan by performing an Analysis of Principal Coordinates (PCoA) using Bray Curtis dissimilarities (Gower, 1966). Indicator species associated with one or two specific sampling dates were assessed using the multipart function of the “indicspecies” package with the group-site association function “Indval,g” and a maximum of 2 treatments combinations allowed (De Cáceres et al., 2012; Dufrêne and Legendre, 1997) (Fig. 3). Analysis of indicator species also includes the calculation of P-values as a measure for statistical significance. The same analysis was used to assess indicator species associated with one or two treatments at specific sampling dates.

3. Results

3.1. $N_2O$ emissions and basic soil parameters

As described in detail in Hüppi et al. (2015), liming treatment and biochar addition resulted in an increase of soil pH by 0.4 pH units on average. The pH fluctuated across the vegetation period but was lowest in the control treatment throughout the vegetation period. Soil pH in the control treatment was 6.3 on average. Bulk density and soil mineral N content did not statistically differ between treatments throughout the experimental period. Cumulative $N_2O$ emissions across the vegetation period were $170 \pm 16.5, 353 \pm 31.7$ and $359 \pm 164$ mg $N_2O-N$ m$^{-2}$ for biochar, control and lime treatments, respectively (Hüppi et al., 2015). Although biochar amendment reduced $N_2O$ emissions by 52% compared to the control (Fig. 1), due to high variability of $N_2O$ emissions in the lime treatment no significant treatment effect across all three treatments could be reported (Hüppi et al., 2015).

3.2. Community size of nosZ and nosZ-II bearing bacteria

NosZ gene abundances did not show any significant effect of treatment at the beginning of the vegetation period after harrowing. One week after fertilization nosZ gene abundances in the control and especially in the biochar treatment increased compared to harrowing. After fertilization, significantly higher nosZ abundances in the biochar treatment were observed compared to the lime and the control treatments (biochar: $3.09 \times 10^8 \pm 3.51 \times 10^7$, control $1.84 \times 10^7 \pm 3.17 \times 10^7$ and lime $1.03 \times 10^8 \pm 3.54 \times 10^7$) (Fig. 2). At harvest, nosZ gene abundances in all treatments declined almost below initial levels and no treatment effects could be detected (Fig. 2). Furthermore, abundances of the nosZ-II gene did not show any treatment effect after harrowing (Fig. 2b). For the nosZ-II genes, significantly increased gene copy numbers in the biochar treatment compared to the lime treatment were found after fertilization. However, nosZ-II gene copy numbers did not differ significantly between the control and biochar treatment (biochar: $8.82 \times 10^8 \pm 1.53 \times 10^8$, control $7.66 \times 10^8 \pm 1.14 \times 10^7$ and lime $4.79 \times 10^8 \pm 7.21 \times 10^7$). After fertilization, significantly increased nosZ and nosZ-II gene copy numbers were observed. In all treatments lowest nosZ-II gene copy numbers had been observed after harvesting without any significant effect in treatments (Fig. 2b). Across all sampling dates, nosZ gene abundance showed a significant correlation with $NO_3^{-}$, $NH_4^+$ and $N_2O$ emissions. The size of the nosZ-II bearing community correlated with $NO_3^{-}$ concentrations (Supplementary Table S7).

3.3. Community composition of nosZ and nosZ-II bearing bacteria

Statistical analysis via permmanova showed a significant effect of sampling date on community composition of nosZ and nosZ-II bearing bacteria (Table 2). The factor “plot” introduced significant variance in community composition of nosZ and nosZ-II bearing $N_2O$ reducers. (Table 2). Treatment (lime and biochar amendment) was a significant factor explaining changes in nosZII abundance but not the abundance of nosZ. (Table 2). Although the R$^2$ values are low, community composition of nosZ bearing bacteria had been significantly affected by $NO_3^{-}$ and $NH_4^+$ concentrations as well as soil pH. For nosZ-II bearing bacteria no geochemical parameter showed a significant effect on community structure (Table 2 and Supplementary Table S8).

Taxa indicative for one or two sampling dates with a relative share above 0.5% are displayed in Fig. 3. For the nosZ bearing bacteria, relative abundance of Paracoccus denitrificans increased while Sinorhizobium fredii, Methylobacterium sp. 4–46 and Hyphomicrobium denitrificans decreased at harvest compared to harrowing and fertilization. The abundance of Thioacutatius denitrificans and Pseudogulbenkiania sp. NH8B was significantly higher after fertilization compared to harrowing and harvesting. The abundance of Bradyrhizobium diazoefficiens decreased from harrowing to fertilization, but recovered again and showed highest relative abundance at harvesting (Fig. 3a). For nosZ-II the relative share of Opitutus terrae and Ignavibacterium album was significantly decreased at harvesting compared to harvesting and
fertilization, while the opposite effect was observed for *Niastella kor-eensis*, *Haliscomenobacter hydrossis*, *Gemantimonas aurantica* and *Caldilinea aerophila* (Fig. 3b).

Assessing the treatment effect on community composition of *nosZ* and *nosZ*-II bearing bacteria at specific sampling dates using PCoA revealed no clear clustering of *nosZ* bearing bacteria throughout the vegetation period (Fig. 4). The same was true for the *nosZ*-II gene sequences after harrowing. After fertilization, *nosZ*-II bearing bacteria showed treatment specific clusters and analysis of variance via permanova revealed a significant treatment effect ($P = 0.04$) (Fig. 4). At harvesting a similar clustering was observed but lacked statistical significance. At fertilization and harvesting, the samples of the lime treatment clustered in between the control and biochar treatment (Fig. 4e and f).

At fertilization, when a significant treatment effect was observed, *nosZ*-II sequences assigned to *Anaeromyxobacter* sp. Fw109-5 and *Flavobacteriaceae bacterium* 3519-10 were indicative for the control and lime treatment. On the contrary, a significant increase of the *nosZ*-II gene affiliated with *Melioribacter roseus* was detected in the biochar treatment (Fig. 5).

**4. Discussion**

Biochar addition decreased N$_2$O emission by 52% compared to the control. This conforms very well with a recent meta-analysis that showed biochar addition lowers N$_2$O emissions by an average of 54% across 30 studies (Cayuela et al., 2014). However, it should not be neglected that field data generally showed a less pronounced impact on N$_2$O emissions and there are other field studies lacking significant reductions in N$_2$O emissions after biochar addition (Scheer et al., 2011; Verhoeven and Six, 2014). Nevertheless, there is a growing body of studies that show biochar’s potential to decrease N$_2$O emissions by influencing abundance and/or activity of N$_2$O reducing bacteria (Harter et al., 2014; Liu et al., 2017; van Zwieten et al., 2014; Xu et al., 2014). One of the few studies investigating N$_2$O fluxes and abundances of *nosZ* bearing bacteria in a field experiment could not observe any influence of biochar amendment, either on N$_2$O emissions nor on the size of the N$_2$O reducing microbial community across a whole vegetation period (Dicke et al., 2015). In our case, biochar amendment increased abundance of *nosZ* and partly *nosZ*-II bearing bacteria after fertilization (Fig. 1). After fertilization the highest NO$_3^-$ concentration throughout the vegetation period were observed, accounting for 9.19 ± 1.66, 9.38 ± 3.69 and 11.65 ± 1.24 mg NO$_3$-N kg$^{-1}$ in the biochar control and lime treatment respectively. Additionally a heavy rain event increased water content of the soil and further stimulated denitrifying conditions (Hüppi et al., 2015). In line with other incubation experiments denitrifying conditions were a prerequisite for biochar induced changes on activity and abundance of N$_2$O reducers (Harter et al., 2014; Xu et al., 2014). In the study of Dicke et al. (2015) generally low N$_2$O emissions together with low water contents favoring nitrification as a
Fig. 4. Principal coordinate analysis (PCoA) plot based on the genetic diversity of the functional marker genes nosZ (a-c) and nosZ-II (d-f) after harrowing (a) and d), fertilization (b) and e) and harvesting (c) and f) of Zea mays in the control, biochar and lime treatment. The PCoA was calculated using a Bray-Curtis dissimilarity matrix based on the relative share of assigned gene sequences in the respective soil samples. Biplots show the four most abundant gene sequences affecting ordination and the assigned taxa. Data points of a treatment were connected by lines when permanova showed a significant treatment effect.
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major N$_2$O producing pathway might have hampered differentiation of N$_2$O emissions and nosZ gene abundances in between treatments.

NosZ bearing bacteria are more likely to possess a complete set of denitrification genes compared to nosZ-II bearing N$_2$O reducers (Graf et al., 2014). The greater growth of nosZ compared to nosZ-II bearing N$_2$O reducers as a response to fertilization might thus be linked to the more widespread genetic capability to process NO$_3^-$ and other intermediate products of denitrification in this functional guild (Fig. 2). In fact, N$_2$O reducing bacteria performing all denitrification steps will gain more energy compared to N$_2$O reducers which rely on external N$_2$O supply (Lam and Kuypers, 2011). This is further strengthened by the fact that nosZ gene abundance across all sampling dates significantly correlated with NO$_3^-$ ($R^2 = 0.38$) and NH$_4^+$ concentrations ($R^2 = 0.30$) as well as N$_2$O emissions ($R^2 = 0.17$), while nosZ-II gene abundances could only be related to NO$_3^-$ concentrations ($R^2 = 0.21$) (Supplementary Table S8). Biochar amendment especially increased the abundance of nosZ bearing bacteria which further indicates enhanced denitrification after N addition in this treatment as in other studies (Castaldi et al., 2011; Xu et al., 2014). Since biochar was shown to enhance electron shuttling (Kappler et al., 2014), it seems reasonable to assume that in this treatment, nosZ bearing bacteria involved in all denitrification steps outcompete nosZ-II bearing bacteria, which are more likely to be genetically restricted to N$_2$O reduction.

Apart from abundance and activity of the N$_2$O reducing microbial community, community structure was reported to affect community differentiation (Domeignoz-Horta et al., 2015). There are strong indications that some agricultural practice have the potential to shape diversity of N$_2$O reducers as was shown for residue management in a long term field trial (Bent et al., 2016). The diversity of the nosZ-II bearing bacteria was especially shown to have a strong influence on denitrification end products and determine the N$_2$O sink capacity of soils (Jones et al., 2014). Furthermore, inoculation with the nosZ-II bearing and non-denitrifying strain Dyadobacter fermentans was shown to significantly reduce N$_2$O emissions in 4 out of 11 soils in an incubation experiment (Domeignoz-Horta et al., 2016). There is growing evidence that managing the community structure of N$_2$O reducers might be a useful tool in mitigating soil derived N$_2$O emissions. In our study, however, no statistical relationship between community structure of nosZ or nosZ-II bearing bacteria with N$_2$O emissions could be detected (Table 2). It is possible that the high variability of N$_2$O emissions, especially in the lime treatment (Hüppi et al., 2015), and/or increased retention time of N$_2$O in the biochar treatment might have impeded statistical significance.

In our case the date of sampling affected community structure of nosZ and nosZ-II bearing bacteria to a greater extend compared to the experimental treatments (Fig. 3). This seems reasonable considering variations in N-availability, soil temperature and water contents in between sampling dates (Hüppi et al., 2015). In line with this, indicator species associated with the fertilization treatment, like Thiobacillus denitrificans and Pseudogulbenkiania sp. NHBB, are known for their capability to process large amounts of NO$_3^-$ (Ishii et al., 2011; Shao et al., 2010). However, the slow growing and effective N fixing Bradyrhizobium diazoefficiens was associated with sampling dates at harvest and harrowing when N-availability was low (Delamut et al., 2013).

The fact that the only significant treatment effect was observed after fertilization might be linked to a high supply of N, leading to an increased formation of N$_2$O (Supplementary Fig. S1). In line with our study, Anderson et al. (2014) found seasonal changes to be the major driver of the general bacterial community composition in a biochar field trial. The authors reported that the addition of biochar did not have any significant impact on bacterial community composition. Still, nosZ abundance increased through the course of the experiment (Anderson et al., 2014).

A significant treatment effect on the community structure of nosZ-II bearing bacteria was observed after fertilization. The indicator species Melioribacter roseus, which was only associated with the biochar treatment, lacks the complete set of denitrification genes and might thus act as a sink for N$_2$O (Kadnikov et al., 2013). In contrast Flavobacteriaceae bacterium 3519-10 and Anaeromycobacter sp. Fw 109-5, which were associated with the lime and control treatments, also possess other genes involved in denitrification (Graf et al., 2014). This observation is in agreement with a recent hypothesis which suggests promotion of complete denitrifiers and N$_2$O reducers, relying on external N$_2$O to be a major driver for N$_2$O mitigation after biochar addition (Hagemann et al., 2016a), Harter et al. (2016b) observed this effect in an incubation study and similarly to our experiment, relative abundance of Flavobacteriaceae bacterium 3519-10 was significantly decreased in the biochar treatment. Yet, for Pseudopedobacter saltans which was the most abundant nosZ-II bearing bacteria and associated with the biochar treatment in the study of Harter et al. (2016b) we could not detect any treatment effect. Given the fact that our experiment was conducted under field conditions, it seems reasonable that the effect was less pronounced and partly superimposed by spatial heterogeneity. Nevertheless, we found indications for the same functional pattern as per Harter et al. (2016b), as Melioribacter roseus and Pseudopedobacter saltans both rely on external N$_2$O from the environment as they lack other denitrification genes. Furthermore, the increased abundance of N$_2$O reducers lacking preceding denitrification genes due to biochar addition was a community specific effect (Supplementary Fig. S2). Response of different taxa to biochar addition seems attributable to the soil inherent differences in community composition of nosZ-II bearing bacteria. Since indicator species were either associated with the lime and control treatment or the biochar treatment, it seems unlikely that changes in community composition can be attributable to the liming effect or biochar only. Since the lime treatment at fertilization was clustered between the control and biochar treatment, other mechanisms seem to contribute to differentiation of community composition of nosZ-II bearing bacteria. Biochars potential to retain N$_2$O had been
demonstrated in different experiments (Cornelissen et al., 2013; Harter et al., 2016a; Quin et al., 2015), and increased retention time of N₂O in soils due to biochar addition seem one possible mechanism which could explain promotion of specialized N₂O reducers (Harter et al., 2016a).

In conclusion we could show that the amendment of biochar induces a shift of community composition of nosZ-II bearing bacteria under field conditions and alters the relative abundance of specialized N₂O reducers. Biochar amendment affected community composition of nosZ-II bearing bacteria beyond the sole effect of liming. Since differentiation of nosZ-II bearing bacteria in between treatments was not significant after harvesting it is still unclear whether this effect would hold true for subsequent vegetation periods. The fading effect on nosZ-II community composition at harvesting might be due to decreased N turnover. However, the transient nature of the biochar might also have decreased the functional impact on N₂O reducers and N₂O emission (Quilliam et al., 2012). To resolve this open question, long term field experiments investigating the impact of biochar on N₂O emission over multiple years are needed. However, we could confirm that biochar is a promising option, which can assist in decreasing N₂O emissions through manipulation of the N₂O reducing community under field conditions.

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Appendix A. Supplementary data

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