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

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ABSTRACT

N_2O is a major greenhouse gas and the majority of anthropogenic N_2O emissions originate from agriculturally managed soils. Therefore, developing N_2O 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_2O reducers has been proposed as possible mechanisms for N_2O mitigation. An experiment was undertaken to investigate whether changes in the community composition of N_2O 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 –II* across a vegetation period of *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_2O emissions and emphasizes the possibility to shape the functioning of the N_2O reducing microbial community through the addition of biochar at a field scale.

1. Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas that also catalyzes stratospheric ozone depletion (Ravishankara et al., 2009). Atmospheric N_2O 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_2O yr^{-1}, developing effective mitigation strategies is a key challenge for the agricultural sector (Butterbach-Bahl et al., 2013). As N_2O is a long lived greenhouse gas with an atmospheric half life time of 114 years, elevated atmospheric N_2O concentrations will be a long term issue (IPCC, 2013). N_2O 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_2O producing processes (Davidson, 2009). Denitrification describes the stepwise reduction of nitrate (NO_3^-) to molecular nitrogen (N_2) and occurs under oxygen limited conditions in water saturated soils, especially after fertilization which increases the bioavailability of nitrogen compounds (Davidson, 2009). N_2O is produced as an obligatory intermediate in the denitrification process. The last step in denitrification, the reduction of N_2O to N_2, presents the only known biological sink for N_2O (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_2O 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_2O reducers might be an important controlling factor for N_2O emissions (Jones et al., 2014; Philippot et al., 2011). Around one third of denitrifiers lack the genetic capability to reduce N_2O and therefore act as a source for N_2O (Philippot et al., 2011). On the other hand, some N_2O reducers lack

the genes to produce N_2O from NO_3^- and thus might act as an N_2O sink by reducing exogenous N_2O. This especially applies to the recently described *nosZ*-II bearing bacteria since $\sim 50\%$ of the so far known *nosZ*-II bearing bacteria lack the genetic capability to produce N_2O (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 N_2O reducers and affect the $N_2O/(N_2O + N_2)$ ratio (Domeignoz-Horta et al., 2015). Identifying and developing agricultural practices that promote complete denitrification through stimulation of exclusive N_2O reducers might be one way to reduce N_2O 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 physicochemical 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 physicochemical 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 N_2O emission since decreased N_2O 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 N_2O mitigation through biochar addition (Clough et al., 2013), but the key mechanism causing decreased N_2O emissions is still poorly understood (Cayuela et al., 2013, 2014; Clough et al., 2013). Since there is evidence that a high soil pH fosters N_2O 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 N_2O emissions under controlled incubation conditions (Obia et al., 2015; van Zwieten et al., 2010; Zheng et al., 2012). Furthermore, incubation experiments showed that reduced N_2O 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 N_2O reducing bacteria. In a short term incubation experiment biochar addition resulted in a reduction of N_2O emissions (Harter et al., 2014), which could be linked to community shifts in *nosZ* and *nosZ-II* bearing bacteria, favoring bacteria specialized in N_2O reduction (Harter et al., 2016b). Community shifts might have been induced by entrapment of N_2O on biochar surfaces (Harter et al., 2016a). However, other underlying mechanisms driving the reduction of N_2O emissions after biochar addition cannot be excluded, including an abiotic N_2O reduction on biochar surfaces (Quin et al., 2015) or enhancement of N_2O reduction induced by pH (Clough et al., 2013; Obia et al., 2015). Although a recent field study observed a reduction of N_2O emissions three years after biochar amendment (Hagemann et al., 2016b), the effect of biochar amendment on N_2O 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 N_2O 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 N_2O 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, 437m 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 harrowed 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*, N-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 (NH_4NO_3) at a rate of 80 kg N ha^{-1}. The harvesting took place on 13th of October. In order to assess community structure of N_2O reducers, the first set of soil samples from each micro-plot was taken after "harrowing" at 31st of March. An N_2O 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 N_2O and CO_2 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 $\sim 80\%$ softwood and $\sim 20\%$ 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

Table 1
Basic biochar and soil properties as described in Hüppi et al. (2015).

parameter	biochar	soil
pH	9.8	6.3
C/N mass ratio	94.0	9.0
C_{org} (%)	62.1	2.62
N_{total} (%)	0.66	0.29
BET surface ($m^2\ g^{-1}$)	148.0	–
Ash content (%)	20.0	–
O/C molar ratio	0.11	–
H/C molar ratio	0.33	–
Bulk density ($g\ cm^{-3}$)	–	1.3

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 N_2 adsorption after 12 h of vacuum degassing with a NOVA2e from Quantachrome Instruments (Odelzhausen 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 NH_4^+ and NO_3^- concentrations, 20g of moist soil were mixed with 100 ml of 0.01 M $CaCl_2$ 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.

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 9.1×10^9 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 (Thonar 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 ul⁻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 ~10^8 to ~10^2 gene copy numbers 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 assessing 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 (Thonar 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 the ENA Sequence Read Archive (SRA) under the accession number PRJEB19689.

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 N_2O reducers (Anderson, 2001; Jari Oksanen et al., 2009). In a first step, the sampling date and treatment factors and its

Table 2
R^2 and P-value for experimental factors and geochemical parameters explaining the variance in dissimilarity matrices (Bray Curtis) of *nosZ* and *nosZ*-II bearing bacterial communities as analyzed by Permanova.

	nosZ		*nosZ*-II	
	R^2	P-value	R^2	P-value
Experimental factors				
Sampling date	0.26	< 0.001 ***	0.12	0.013 *
Treatment	0.06	*0.101 ns*	0.20	< 0.001 ***
Plot	0.34	< 0.001 ***	0.42	< 0.001 ***
Sampling date x Treatment	0.09	*0.258 ns*	0.04	*0.638 ns*
Geochemical parameters				
NO_3^-	0.17	0.003 **	0.04	0.338 *ns*
NH_4^+	0.10	0.013 *	0.01	0.672 *ns*
Soil pH	0.13	0.006 **	0.02	0.651 *ns*
N_2O	0.04	0.323 *ns*	0.04	0.263 *ns*
CO_2	0.04	0.224 *ns*	0.02	0.445 *ns*

Level of significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, *ns* not significant.

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 multipatt 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. N₂O 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 ± 16.5, 353 ± 31.7 and 359 ± 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 $1.84 \times 10^8 \pm 3.17 \times 10^7$ 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
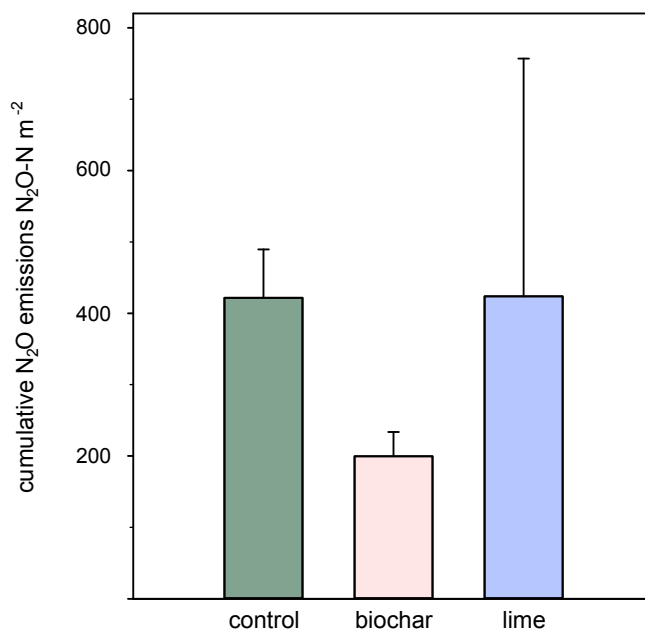

Fig. 1. Cumulated N_2O emissions in control, biochar and lime treatment for the whole vegetation period (data from Hüppi et al. (2015)).

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 $7.06 \times 10^8 \pm 1.14 \times 10^7$ 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 permanova 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 harvesting compared to harrowing and fertilization. The abundance of *Thiobacillus 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 harrowing and
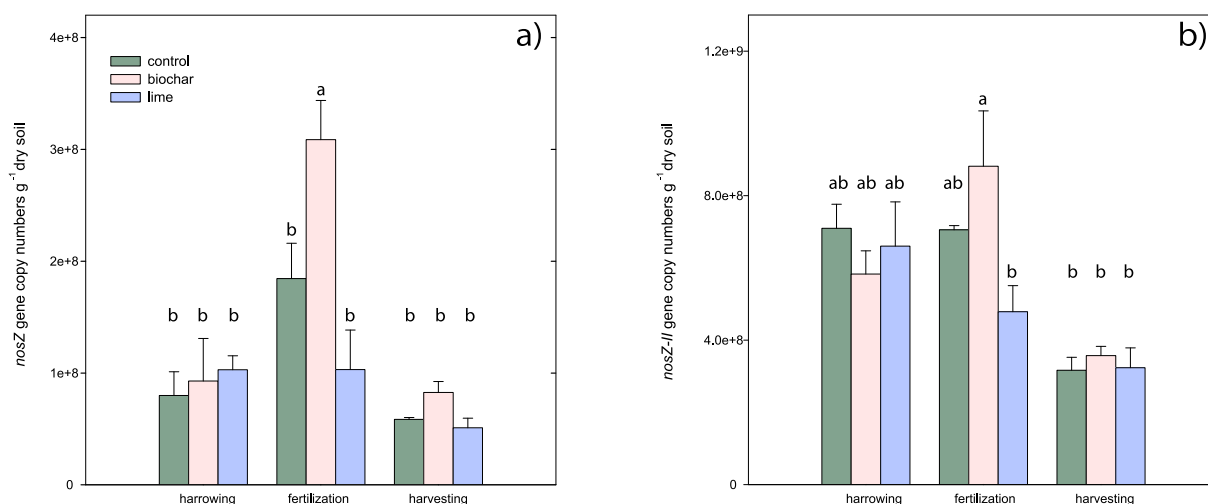
Fig. 2. Gene copy numbers per g dry soil of the functional marker gene *nosZ* (a) and *nosZ*-II (b) after harrowing, fertilization and harvesting of *Zea mays*. Treatments with the same letter were not significantly different at P < 0.05.
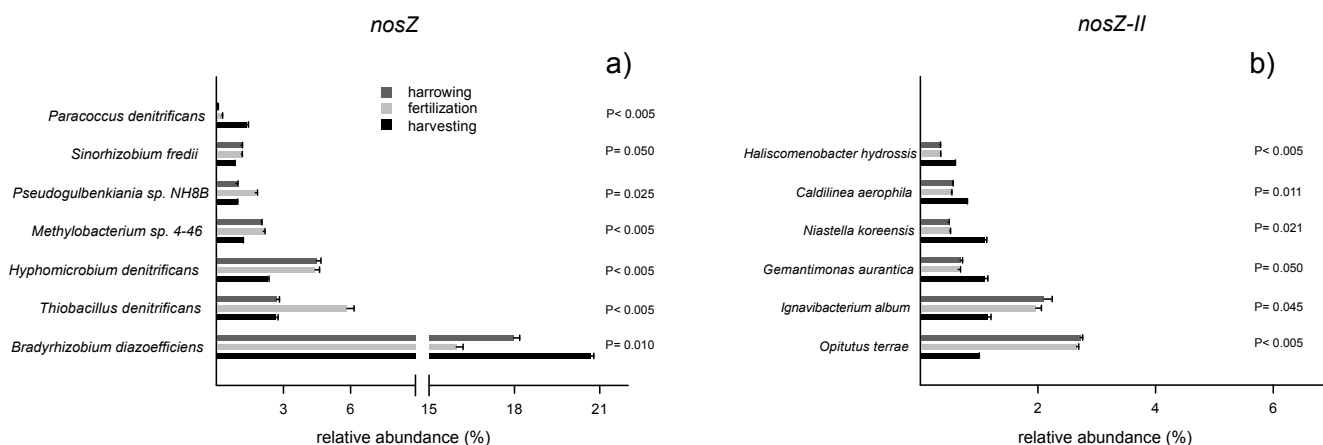

Fig. 3. Relative abundance and P-values of indicator species of the soil sampling dates "harrowing", "fertilization" and "harvesting" across all treatments. Taxa were identified using the multipatt function in the indicspecies package of R. Only taxa with a mean relative share above 0.5% are displayed.

fertilization, while the opposite effect was observed for *Niastella koreensis, Haliscomenobacter hydrossis, Gemantimonas aurantica* and *Caldinella 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 *Anaeromyxobater* 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_2O emission by 52% compared to the control. This conforms very well with a recent meta-analysis that showed biochar addition lowers N_2O 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_2O emissions and there are other field studies lacking significant reductions in N_2O 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_2O emissions by influencing abundance and/or activity of N_2O 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_2O fluxes and abundances of *nosZ* bearing bacteria in a field experiment could not observe any influence of biochar amendment, either on N_2O emissions nor on the size of the N_2O 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_2O reducers (Harter et al., 2014; Xu et al., 2014). In the study of Dicke et al. (2015) generally low N_2O emissions together with low water contents favoring nitrification as a
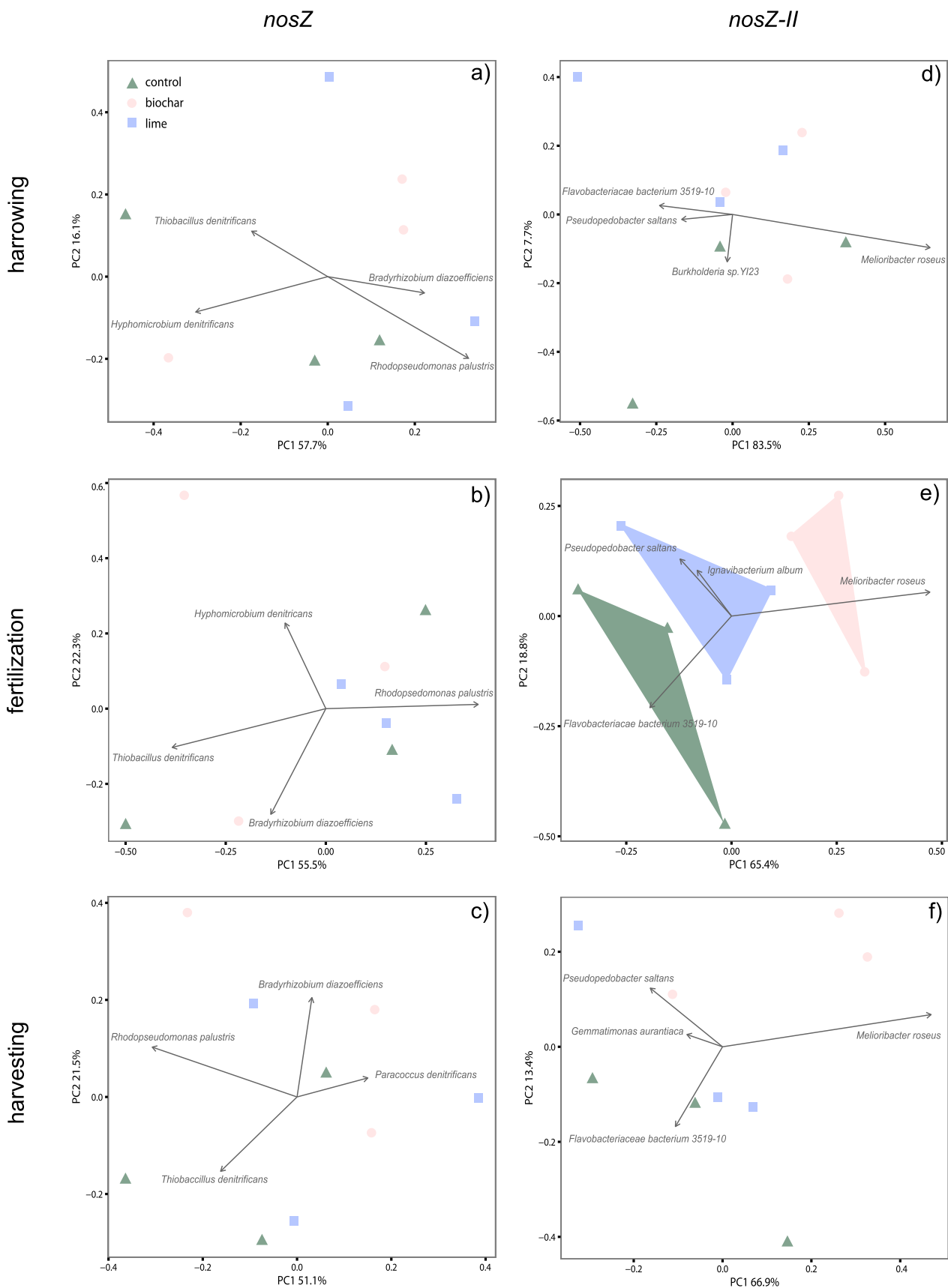
Fig. 4. Principal coordinate analysis (PCoA) plot based on the genetic diversity of the functional marker genes *nosZ* (a)-c)) and *nosZ*-II (d)-f)) after harrowing (a) and d)), fertilization (b) and e)) and harvesting (c) and f)) of *Zea mays* in the control, biochar and lime treatment. The PCoA was 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.
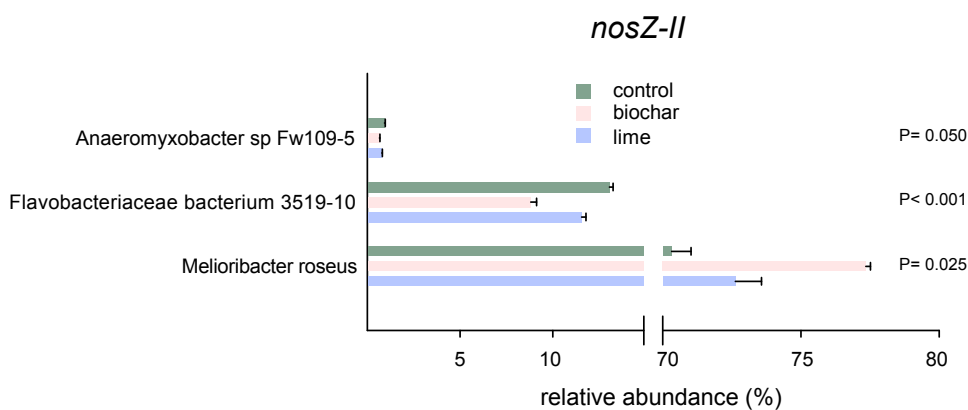
Fig. 5. Relative abundance and P-values of indicator species in the control, biochar and lime treatments at fertilization. Taxa were identified using the multipatt function in the indicspecies package of R. Only taxa with a mean relative share above 0.5% are displayed.

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 N$_2$O reduction (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. *NH8B,* 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 (Delamuta 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 *Anaeromyxobacter* 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 N_2O in soils due to biochar addition seem one possible mechanism which could explain promotion of specialized N_2O 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 N_2O 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 N_2O reducers and N_2O emission (Quilliam et al., 2012). To resolve this open question, long term field experiments investigating the impact of biochar on N_2O emission over multiple years are needed. However, we could confirm that biochar is a promising option, which can assist in decreasing N_2O emissions through manipulation of the N_2O reducing community under field conditions.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2018.01.018.

References

Anderson, C.R., Condron, L.M., Clough, T.J., Fiers, M., Stewart, A., Hill, R.A., Sherlock, R.R., 2011. Biochar induced soil microbial community change: implications for biogeochemical cycling of carbon, nitrogen and phosphorus. Pedobiologia 54, 309–320. https://doi.org/10.1016/j.pedobi.2011.07.005.

Anderson, C.R., Hamonts, K., Clough, T.J., Condron, L.M., 2014. Biochar does not affect soil N-transformations or microbial community structure under ruminant urine patches but does alter relative proportions of nitrogen cycling bacteria. Agriculture, Ecosystems & Environment 191, 63–72. https://doi.org/10.1016/j.agee.2014.02.021.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26, 32–46. https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x.

Atkinson, C.J., Fitzgerald, J.D., Hipps, N.A., 2010. Potential mechanisms for achieving agricultural benefits from biochar application to temperate soils: a review. Plant and Soil 337, 1–18. https://doi.org/10.1007/s11104-010-0464-5.

Baggs, E., Smales, C., Bateman, E., 2010. Changing pH shifts the microbial sourceas well as the magnitude of N_2O emission from soil. Biology and Fertility of Soils 46, 793–805. https://doi.org/10.1007/s00374-010-0484-6.

Bent, E., Németh, D., Wagner-Riddle, C., Dunfield, K., 2016. Residue management leading to higher field-scale N_2O flux is associated with different soil bacterial nitrifier and denitrifier gene community structures. Applied Soil Ecology 108, 288–299. https://doi.org/10.1016/j.apsoil.2016.09.008.

Buchfink, B., Xie, C., Huson, D.H., 2015. Fast and sensitive protein alignment using DIAMOND. Nature Methods 12, 59–60. https://doi.org/10.1038/nmeth.3176.

Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Zechmeister-Boltenstern, S., 2013. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? Philosophical Transactions of the Royal Society 368, 20130122. https://doi.org/10.1098/rstb.2013.0122.

Castaldi, S., Riondino, M., Baronti, S., Esposito, F.R., Marzaioli, R., Rutigliano, F.A., Vaccari, F.P., Miglietta, F., 2011. Impact of biochar application to a Mediterranean wheat crop on soil microbial activity and greenhouse gas fluxes. Chemosphere 85, 1464–1471. https://doi.org/10.1016/j.chemosphere.2011.08.031.

Cayuela, M.L., Sánchez-Monedero, M.A., Roig, A., Hanley, K., Enders, A., Lehmann, J., 2013. Biochar and denitrification in soils: when, how much and why does biochar reduce N_2O emissions? Scientific Reports 3, 1732. https://doi.org/10.1038/srep01732.

Cayuela, M.L., van Zwieten, L., Singh, B.P., Jeffery, S., Roig, A., Sánchez-Monedero, M.A., 2014. Biochar's role in mitigating soil nitrous oxide emissions: a review and meta-analysis. Agriculture, Ecosystems & Environment 191, 5–16. https://doi.org/10.1016/j.agee.2013.10.009.

Chen, J., Liu, X., Li, L., Zheng, J., Qu, J., Zheng, J., Zhang, X., Pan, G., 2015. Consistent increase in abundance and diversity but variable change in community composition of bacteria in topsoil of rice paddy under short term biochar treatment across three sites from South China. Applied Soil Ecology 91, 68–79. https://doi.org/10.1016/j.apsoil.2015.02.012.

Chen, J., Liu, X., Zheng, J., Zhang, B., Lu, H., Chi, Z., Pan, G., Li, L., Zheng, J., Zhang, X., Wang, J., Yu, X., 2013. Biochar soil amendment increased bacterial but decreased fungal gene abundance with shifts in community structure in a slightly acid rice paddy from Southwest China. Applied Soil Ecology 71, 33–44. https://doi.org/10.1016/j.apsoil.2013.05.003.

Clough, T., Condron, L., Kammann, C., Müller, C., 2013. A review of biochar and soil nitrogen dynamics. Agronomy 3, 275–293. https://doi.org/10.3390/agronomy3020275.

Cornelissen, G., Rutherford, D.W., Arp, H.P.H., Dörsch, P., Kelly, C.N., Rostad, C.E., 2013. Sorption of pure N_2O to biochars and other organic and inorganic materials under anhydrous conditions. Environmental Science & Technology 47, 7704–7712. https://doi.org/10.1021/es400676q.

Cuhel, J., Simek, M., Laughlin, R.J., Bru, D., Cheneby, D., Watson, C.J., Philippot, L., 2010. Insights into the effect of soil pH on N_2O and N_2 emissions and denitrifier community size and activity. Applied and Environmental Microbiology 76, 1870–1878. https://doi.org/10.1128/Aem.02484-09.

Davidson, E.A., 2009. The contribution of manure and fertilizer nitrogen to atmospheric nitrous oxide since 1860. Nature Geoscience 2, 659–662. https://doi.org/10.1038/ngeo608.

De Cáceres, M., Legendre, P., Wiser, S.K., Brotons, L., 2012. Using species combinations in indicator value analyses. Methods in Ecology and Evolution 3, 973–982. https://doi.org/10.1111/j.2041-210X.2012.00246.x.

Delamuta, J.R.M., Ribeiro, R.A., Ormeño-Orrillo, E., Melo, I.S., Martínez-Romero, E., Hungria, M., 2013. Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens sp. nov*. International Journal of Systematic and Evolutionary Microbiology 63, 3342–3351. https://doi.org/http://dx.doi.org/10.1099/ijs.0.049130-0.

Dicke, C., Andert, J., Ammon, C., Kern, J., Meyer-Aurich, A., Kaupenjohann, M., 2015. Effects of different biochars and digestate on N_2O fluxes under field conditions. The Science of the Total Environment 524–525, 310–318. https://doi.org/10.1016/j.scitotenv.2015.04.005.

Domeignoz-Horta, L.A., Putz, M., Spor, A., Bru, D., Breuil, M.C., Hallin, S., Philippot, L., 2016. Non-denitrifying nitrous oxide-reducing bacteria - an effective N_2O sink in soil. Soil Biology and Biochemistry 103, 376–379. https://doi.org/10.1016/j.soilbio.2016.09.010.

Domeignoz-Horta, L.A., Spor, A., Bru, D., Breuil, M.-C., Bizouard, F., Léonard, J., Philippot, L., 2015. The diversity of the N_2O reducers matters for the N_2O: N2 denitrification end-product ratio across an annual and a perennial cropping system. Frontiers in Microbiology 6, 971. https://doi.org/10.3389/fmicb.2015.00971.

Dufrêne, M., Legendre, P., 1997. Species assemblages and indicator species: the need for a flexible asymetrical approach. Ecological Monographs 67, 345–366. https://doi.org/10.1890/0012-9615(1997)067[0345:SAAIST]2.0.CO;2.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200. https://doi.org/10.1093/bioinformatics/btr381.

Gower, J.C., 1966. Some distance properties of latent root and vector methods used in multivariate analysis. Biometrika 53, 325–338. https://doi.org/10.2307/2333639.

Graf, D.R.H., Jones, C.M., Hallin, S., 2014. Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for N_2O emissions. PLoS One 9, e114118. https://doi.org/10.1371/journal.pone.0114118.

Griffiths, R.I., Whiteley As Fau - O'Donnell, A.G., O'Donnell Ag Fau - Bailey, M.J., Bailey, M.J., 2010. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Applied and Environmental Microbiology 66 (12), 5488–5491.

Hagemann, N., Harter, J., Behrens, S., 2016a. Elucidating the impacts of biochar applications on nitrogen cycling microbial communities. In: Ralebitso-Senior, T.K., Orr, C.H. (Eds.), Biochar Application: Essential Soil Microbial Ecology. Elsevier, New York, pp. 163–198. chap. 7. https://dx.doi.org/10.1016/B978-0-12-803433-0.00007-2.

Hagemann, N., Harter, J., Kaldamukova, R., Guzman-Bustamante, I., Ruser, R., Graeff, S., Kappler, A., Behrens, S., 2016b. Does soil aging affect the N_2O mitigation potential of biochar? A combined microcosm and field study. GCB Bioenergy 953–964. https://doi.org/10.1111/gcbb.12390.

Harter, J., Guzman-Bustamante, I., Kuehfuss, S., Ruser, R., Well, R., Spott, O., Kappler, A., Behrens, S., 2016a. Gas entrapment and microbial N_2O reduction reduce N_2O emissions from a biochar-amended sandy clay loam soil. Scientific Reports 6, 39574. https://doi.org/10.1038/srep39574.

Harter, J., Krause, H.-M., Schuettler, S., Ruser, R., Fromme, M., Scholten, T., Kappler, A., Behrens, S., 2014. Linking N_2O emissions from biochar-amended soil to the structure and function of the N-cycling microbial community. The ISME Journal 8, 660–674. https://doi.org/10.1038/ismej.2013.160.

Harter, J., Weigold, P., El-Hadidi, M., Huson, D.H., Kappler, A., Behrens, S., 2016b. Soil biochar amendment shapes the composition of N_2O-reducing microbial communities. The Science of the Total Environment 562, 379–390. https://doi.org/10.1016/j.scitotenv.2016.03.220.

Hüppi, R., Felber, R., Neftel, A., Six, J., Leifeld, J., 2015. Effect of biochar and liming on

soil nitrous oxide emissions from a temperate maize cropping system. Soils 1, 707–717. https://doi.org/10.5194/soil-1-707-2015.

Huson, D., Auch, A., Qi, J., Schuster, S., 2007. MEGAN analysis of metagenomic data. Genome Research 17, 377–386. https://doi.org/10.1101/gr.5969107.

Huson, D.H., Beier, S., Flade, I., Górska, A., El-Hadidi, M., Mitra, S., Ruscheweyh, H.-J., Tappu, R., 2016. MEGAN community edition - interactive exploration and analysis of large-scale microbiome sequencing data. PLoS Computational Biology 12, e1004957. https://doi.org/10.1371/journal.pcbi.1004957.

IPCC, 2013. In: Stocker, T.F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M. (Eds.), The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge.

Ishii, S., Tago, K., Nishizawa, T., Oshima, K., Hattori, M., Senoo, K., 2011. Complete Genome sequence of the denitrifying and N(2)O-Reducing bacterium Pseudogulbenkiania sp. Strain NH8B. Journal of Bacteriology 193, 6395–6396. https://doi.org/10.1128/JB.06127-11.

IUSS Working Group WRB, 2015. World Reference Base for Soil Resources 2014, Update 2015. International Soil Classification System for Naming Soils and Creating Legends for Soil Maps. FAO, Rome.

Jari Oksanen, F., Blanchet, G., Kindt, R., Legendre, P., O'Hara, R.G., Simpson, G., Solymos, P., Henry, Wagner, H., 2009. Vegan: Community Ecology Package. R Package Version 1.16-0. http://CRAN.R-project.org/package=vegan.

Jones, C.M., Spor, A., Brennan, F.P., Breuil, M.-C., Bru, D., Lemanceau, P., Griffiths, B., Hallin, S., Philippot, L., 2014. Recently identified microbial guild mediates soil N$_2$O sink capacity. Nature Climate Change 4, 801–805. https://doi.org/10.1038/nclimate2301.

Kadnikov, V.V., Mardanov, A.V., Podosokorskaya, O.A., Gavrilov, S.N., Kublanov, I.V., Beletsky, A.V., Bonch-Osmolovskaya, E.A., Ravin, N.V., 2013. Genomic analysis of melioribacter roseus, facultatively anaerobic organotrophic bacterium representing a novel deep lineage within bacteriodetes/chlorobi group. PLoS One 8, e53047. https://doi.org/10.1371/journal.pone.0053047.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., Tanabe, M., 2016. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Research 44, D457–D462. https://doi.org/10.1093/nar/gkv1070.

Kappler, A., Wuestner, M.L., Ruecker, A., Harter, J., Halama, M., Behrens, S., 2014. Biochar as an electron shuttle between bacteria and Fe(III) minerals. Environmental Science and Technology Letters 1, 339–344. https://doi.org/10.1021/ez5002209.

Kolton, M., Harel, Y.M., Pasternak, Z., Graber, E.R., Elad, Y., Cytryn, E., 2011. Impact of biochar application to soil on the root-associated bacterial community structure of fully developed greenhouse pepper plants. Applied and Environmental Microbiology 77, 4924–4930. https://doi.org/10.1128/aem.00148-11.

Lam, P., Kuypers, M.M.M., 2011. Microbial nitrogen cycling processes in oxygen minimum zones. Annual Review of Materials Science 3, 317–345. https://doi.org/10.1146/annurev-marine-120709-142814.

Lehmann, J., Joseph, S., 2009. Biochar for environmental management: an introduction. In: Lehmann, J., Joseph, S. (Eds.), Biochar for Environmental Management, Science and Technology. Earthscan, London, pp. 1–12.

Liu, B., Frostegard, A., Bakken, L.R., 2014. Impaired reduction of N$_2$O to N$_2$ in acid soils is due to a posttranscriptional interference with the expression of nosZ. MBio 5 01383–01314. https://doi.org/10.1128/mBio.01383-14.

Liu, Q., Liu, B., Zhang, Y., Lin, Z., Zhu, T., Sun, R., Wang, X., Ma, J., Bei, Q., Liu, G., Lin, X., Xie, Z., 2017. Can biochar alleviate soil compaction stress on wheat growth and mitigate soil N$_2$O emissions? Soil Biology and Biochemistry 104, 8–17. https://doi.org/10.1016/j.soilbio.2016.10.006.

Mandal, S., Sarkar, B., Bolan, N., Novak, J., Ok, Y.S., Van Zwieten, L., Singh, B.P., Kirkham, M.B., Choppala, G., Spokas, K., Naidu, R., 2016. Designing advanced biochar products for maximizing greenhouse gas mitigation potential. Critical Reviews in Environmental Science and Technology 46, 1–35. https://doi.org/10.1080/10643389.2016.1239975.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.Journal 17 (1), 10–12. https://doi.org/10.14806/ej.17.1.200.

Obia, A., Cornelissen, G., Mulder, J., Dörsch, P., 2015. Effect of soil pH increase by biochar on NO, N$_2$O and N$_2$ production during denitrification in acid soils. PLoS One 10, e0138781. https://doi.org/10.1371/journal.pone.0138781.

Philippot, L., Andert, J., Jones, C.M., Bru, D., Hallin, S., 2011. Importance of denitrifiers lacking the genes encoding the nitrous oxide reductase for N$_2$O emissions from soil. Global Change Biology 17, 1497–1504. https://doi.org/10.1111/j.1365-2486.2010.02334.x.

Philippot, L., Hallin, S., Schloter, M., 2007. Ecology of denitrifying prokaryotes in agricultural soil. Advances in Agronomy 96, 249–305. https://doi.org/10.1016/S0065-2113(07) 96003–96004.

Quilliam, R.S., Marsden, K.A., Gertler, C., Rousk, J., DeLuca, T.H., Jones, D.L., 2012. Nutrient dynamics, microbial growth and weed emergence in biochar amended soil

are influenced by time since application and reapplication rate. Agriculture, Ecosystems & Environment 158, 192–199. https://doi.org/10.1016/j.agee.2012.06.011.

Quin, P., Joseph, S., Husson, O., Donne, S., Mitchell, D., Munroe, P., Phelan, D., Cowie, A., Van Zwieten, L., 2015. Lowering N$_2$O emissions from soils using eucalypt biochar: the importance of redox reactions. Scientific Reports 5, 16773. https://doi.org/10.1038/srep16773.

Ramakers, C., Ruijter, J.M., Deprez, R.H.L., Moorman, A.F.M., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neuroscience Letters 339, 62–66. https://doi.org/10.1016/S0304-3940(02)01423-4.

Ravishankara, A.R., Daniel, J.S., Portmann, R.W., 2009. Nitrous oxide (N$_2$O): the dominant ozone-depleting substance emitted in the 21st century. Science 326, 123–125. https://doi.org/10.1126/science.1176985.

Sanford, R.A., Wagner, D.D., Wu, Q., Chee-Sanford, J.C., Thomas, S.H., Cruz-García, C., Rodríguez, G., Massol-Deyá, A., Krishnani, K.K., Ritalahti, K.M., Nissen, S., Konstantinidis, K.T., Löffler, F.E., 2012. Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proceedings of the National Academy of Sciences 109, 19709–19714. https://doi.org/10.1073/pnas.1211238109.

Scheer, C., Grace, P., Rowlings, D., Kimber, S., Van Zwieten, L., 2011. Effect of biochar amendment on the soil-atmosphere exchange of greenhouse gases from an intensive subtropical pasture in northern New South Wales, Australia. Plant and Soil 345, 47–58. https://doi.org/10.1007/s11104-011-0759-1.

Shao, M.F., Zhang, T., Fang, H.H., 2010. Sulfur-driven autotrophic denitrification: diversity, biochemistry, and engineering applications. Applied Microbiology and Biotechnology 88, 1027–1042. https://doi.org/10.1007/s00253-010-2847-1.

Singh, B.P., Hatton, B.J., Balwant, S., Cowie, A.L., Kathuria, A., 2010. Influence of biochars on nitrous oxide emission and nitrogen leaching from two contrasting soils. Journal of Environmental Quality 39, 1224–1235. https://doi.org/10.2134/jeq2009.0138.

Sohi, S.P., Krull, E., Lopez-Capel, E., Bol, R., 2010. A review of biochar and its use and function in soil. Advances in Agronomy 105, 47–82. https://doi.org/10.1016/S0065-2113(10)05002-9.

Thomson, A.J., Giannopoulos, G., Pretty, J., Baggs, E.M., Richardson, D.J., 2012. Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. Philosophical Transactions of the Royal Society 367, 1157–1168. https://doi.org/10.1098/rstb.2011.0415.

Thonar, C., Erb, A., Jansa, J., 2012. Real-time PCR to quantify composition of arbuscular mycorrhizal fungal communities—marker design, verification, calibration and field validation. Molecular Ecology Resources 12, 219–232. https://doi.org/10.1111/j.1755-0998.2011.03086.x.

van Zwieten, L., Kimber, S., Morris, S., Downie, A., Berger, E., Rust, J., Scheer, C., 2010. Influence of biochars on flux of N$_2$O and CO$_2$ from Ferrosol. Australian Journal of Soil Research 48, 555–568. https://doi.org/10.1071/Sr10004.

van Zwieten, L., Singh, B.P., Kimber, S.W.L., Murphy, D.V., Macdonald, L.M., Rust, J., Morris, S., 2014. An incubation study investigating the mechanisms that impact N$_2$O flux from soil following biochar application. Agriculture, Ecosystems & Environment 191, 53–63. https://doi.org/10.1016/j.agee.2014.02.030.

Verheijen, F., Jeffery, S., Bastos, A., van der Velde, M., Diafas, I., 2009. Biochar Application to Soils - a Critical Scientific Review of Effects on Soil Properties, Processes and Functions. EUR 24099 EN. Office for the Official Publications of the European Communities, Luxembourg.

Verhoeven, E., Pereira, E., Decock, C., Suddick, E., Angst, T., Six, J., 2017. Toward a better assessment of biochar–nitrous oxide mitigation potential at the field scale. Journal of Environmental Quality 46, 237–246. https://doi.org/10.2134/jeq2016.10.0396.

Verhoeven, E., Six, J., 2014. Biochar does not mitigate field-scale N$_2$O emissions in a Northern California vineyard: an assessment across two years. Agriculture, Ecosystems & Environment 191, 27–38. https://doi.org/10.1016/j.agee.2014.03.008.

WMO, 2016. Greenhouse Gas Bulletin. World Meterological Organization. http://www.wmo.int/gaw/.

Woolf, D., Amonette, J.E., Street-Perrott, F.A., Lehmann, J., Joseph, S., 2010. Sustainable biochar to mitigate global climate change. Nature Communications 1, 1–9. https://doi.org/10.1038/ncomms1053.

Xu, H.-J., Wang, X.-H., Li, H., Yao, H.-Y., Su, J.-Q., Zhu, Y.-G., 2014. Biochar impacts soil microbial community composition and nitrogen cycling in an acidic soil planted with rape. Environmental Science & Technology 48, 9391–9399. https://doi.org/10.1021/es5021058.

Zheng, J., Stewart, C.E., Cotrufo, M.F., 2012. Biochar and nitrogen fertilizer alters soil nitrogen dynamics and greenhouse gas fluxes from two temperate soils. Journal of Environmental Quality 41, 1361–1370. https://doi.org/10.2134/jeq2012.0019.

Zumft, W.G., Kroneck, P.M.H., 2006. Respiratory transformation of nitrous oxide (N$_2$O) to dinitrogen by bacteria and archaea. Advances in Microbial Physiology 52, 107–227. https://doi.org/10.1016/S0065-2911(06)52003-X.