Soil biochar amendment shapes the composition of N₂O-reducing microbial communities

Johannes Harter a, Pascal Weigold a, Mohamed El-Hadidi b, Daniel H. Huson b, Andreas Kappler a, Sebastian Behrens a,b,c,d,e

HIGHLIGHTS
• Biochar promoted anaerobic, alkalinity-adapted, and polymer-degrading microbial taxa.
• Biochar fostered the development of distinct N₂O-reducing microbial taxa.
• Taxonomic shifts among N₂O-reducing microbes might explain lower N₂O emissions.

GRAPHICAL ABSTRACT

ABSTRACT
Soil biochar amendment has been described as a promising tool to improve soil quality, sequester carbon, and mitigate nitrous oxide (N₂O) emissions. N₂O is a potent greenhouse gas. The main sources of N₂O in soils are microbially-mediated nitrogen transformation processes such as nitrification and denitrification. While previous studies have focused on the link between N₂O emission mitigation and the abundance and activity of N₂O-reducing microorganisms in biochar-amended soils, the impact of biochar on the taxonomic composition of the nosZ gene carrying soil microbial community has not been subject of systematic study to date. We used 454 pyrosequencing in order to study the microbial diversity in biochar-amended and biochar-free soil microcosms. We sequenced bacterial 16S rRNA gene amplicons as well as fragments of common (typical) nosZ genes and the recently described 'atypical' nosZ genes. Our results showed that biochar

Keywords:
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Biochar
Nitrous oxide emission
nosZ genes
Soil microbial community
454 amplicon pyrosequencing
amendment can affect the relative abundance and taxonomic composition of N$_2$O-reducing functional microbial traits in soil. Thus these findings broaden our knowledge on the impact of biochar on soil microbial community composition and nitrogen cycling.

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1. Introduction

Biochar is a carbon-rich solid produced by thermal decomposition of organic material under low oxygen conditions. Biochar has recently gained a lot of attention as a soil additive because of its soil quality enhancing properties (Atkinson et al., 2010; Lehmann et al., 2011). Biochar production and soil amendment has been discussed as one way to address environmental issues related to current agricultural practices and mankind’s use of fossil energy sources, such as problems associated with the excessive use of synthetic fertilizers and greenhouse gas emission from combustion processes (Atkinson et al., 2010; Clough and Condron, 2010; Sohi, 2012). For example, it has been shown that the physicochemical properties and reactivity of biochar in soil can help decrease nutrient leaching and greenhouse gas emission. Biochar’s physicochemical properties vary widely with the type of feedstock and pyrolysis condition but the majority of biochars share common characteristics, such as a high content of aromatic carbon structures, an elevated pH, and a large surface area (Atkinson et al., 2010; Singh et al., 2010).

Despite variations in physicochemical properties among different biochars, several studies showed that soil biochar amendment does reduce soil nitrous oxide (N$_2$O) emissions and leads to shifts in the soil microbial community composition (Cayuela et al., 2014; Khodadad et al., 2011; Kuzyakov et al., 2014). However, if and how these are linked has not yet been intensively studied.

N$_2$O is a potent greenhouse gas with an atmospheric lifetime of 114 years and an almost 300-fold greater global warming potential compared to CO$_2$ (Thomson et al., 2012). Microbially-mediated nitrogen transformation reactions in soils represent world’s largest sources of atmospheric N$_2$O (Thomson et al., 2012). In soils, denitrification represents one of the major N$_2$O producing pathways. N$_2$O is an obligate intermediate of microbial denitrification, which occurs frequently in oxygen-limited soil horizons and anoxic microsites, especially in the presence of high amounts of nitrogen fertilizer (Braker and Conrad, 2011; Pfäb et al., 2011; Philippot et al., 2009). Denitrification describes the stepwise reduction of nitrate (NO$_3^-$) to dinitrogen gas (N$_2$) and is performed by many facultative and strict anaerobic chemoorganotrophic bacteria of which many belong to the phylum Proteobacteria (Braker and Conrad, 2011; Philippot et al., 2007). The enzymes catalyzing these stepwise reduction reactions are encoded by the functional genes narG and napA (nitrate reductases), nirK and nirS (nitrite reductases), and nosZ (nitrous oxide reductase) (Philippot et al., 2007; Richardson et al., 2009). Due to the fact that some denitrifiers lack a functional nosZ gene and the high oxygen and pH sensitivity of N$_2$O reductases, denitrification is often incomplete and N$_2$O is released and not reduced to N$_2$ (Bakken et al., 2012; Mckenney et al., 1994; Philippot et al., 2011). Besides denitrification, other microbial processes also contribute to the formation of N$_2$O (e.g. nitrifier denitrification, nitrification) under certain environmental conditions. However, the only known biological sink for N$_2$O is the reduction of N$_2$O to N$_2$ by N$_2$O-reducing microorganisms (Thomson et al., 2012).

Many “classical” denitrifiers belonging to the Proteobacteria contain a typical nosZ gene and synthesize Z-type nitrous oxide reductases (Philippot et al., 2007; Zumft and Körner, 2007). Recently the existence of a second clade of N$_2$O reducers comprising microorganisms from several different phyla has been demonstrated. These organisms possess a phylogenetically distinct atypical nosZ gene (Jones et al., 2013; Philippot et al., 2007; Sanford et al., 2012; Zumft and Körner, 2007). About half of the atypical nosZ gene containing microorganisms do not carry the functional genes encoding nitrate, nitrite and nitric oxide reductases and are thus only capable of reducing N$_2$O to N$_2$ (Jones et al., 2013; Orellana et al., 2014). Interestingly, atypical nosZ gene carrying N$_2$O reducers outnumber typical nosZ gene carrying microorganisms in many environments suggesting that the ratio of typical to atypical nosZ gene carrying microbial populations might impact soil N$_2$O emissions (Jones et al., 2014; Orellana et al., 2014).

To date, most studies on the effect of soil biochar amendment on the microbial community composition used molecular fingerprinting techniques such as PLFA (Gomez et al., 2014), DGGE (Chen et al., 2013), or T-RFLP (Anderson et al., 2011) to analyze community shifts. Other studies relying on next generation sequencing approaches mainly targeted the bacterial 16S rRNA gene to describe the microbial community composition (Chen et al., 2015; Kolton et al., 2011). According to these studies, soil biochar amendment increases the ratio of bacteria to fungi, the proportion of gram-negative over gram-positive bacteria, and the relative abundance of bacterial taxa capable of degrading aromatic hydrocarbons and transforming (in)organic nitrogen species (Anderson et al., 2011; Chen et al., 2013; Chen et al., 2015; Gomez et al., 2014; Kolton et al., 2011). Biochar-induced taxonomic shifts among nitrogen-transforming functional groups of microorganisms, especially microbial taxa capable of nitrous oxide reduction, might directly affect soil N$_2$O release, because microbial nitrous oxide reduction is the main biotic sink of atmospheric N$_2$O (Thomson et al., 2012).

It is known from several studies that soil biochar amendment can change the abundance and activity of denitrifiers, especially of N$_2$O-reducing microorganisms possessing a typical nosZ gene (Ducey et al., 2013; Harter et al., 2014; Van Zwieten et al., 2014). An increased abundance and activity of typical nosZ gene containing N$_2$O reducers is thought to be one of the reasons for the frequently observed decreased N$_2$O emissions from biochar-amended soils (Harter et al., 2014; Van Zwieten et al., 2014). However, the impact of biochar on the taxonomic composition of typical and atypical nosZ containing N$_2$O-reducing microbial communities has not been studied in detail yet. Since it has been shown that individual N$_2$O-reducing taxa can differ considerably in N$_2$O reduction activity, taxonomic shifts among typical and atypical nosZ gene carrying N$_2$O-reducing microbial communities might affect N$_2$O reduction rates and net soil N$_2$O release (Cavigelli and Robertson, 2001; Tago et al., 2011).

In order to investigate biochar-induced shifts in the taxonomic composition and structure of the soil bacterial community, as well as shifts among the typical and atypical nosZ gene carrying microbial communities, we performed 454 amplicon pyrosequencing on soil samples collected from a previously conducted soil microcosm experiment described in detail in Harter et al. (2014). The main objectives of this study were to i) describe biochar-induced taxonomic shifts among the soil bacterial community, to ii) investigate how soil biochar amendment can affect the composition of the typical and atypical nosZ gene carrying microbial communities, and to iii) evaluate if changes in the functional community composition might help to explain the observed reduction in N$_2$O emissions after biochar amendment in the soil microcosm experiment performed by Harter et al. (2014).

2. Material and methods

The soil samples analyzed in this study were collected from a microcosm experiment previously described by Harter et al. (2014). We briefly describe the experimental setup and sample collection below. A detailed description of the experimental setup and the geochemical...
2.2. Experimental setup

Soil microcosms were set up in 500 ml DURAN® wide neck glass bottles (Schott AG, Mainz, Germany). Control microcosms consisted of soil and biochar physicochemical properties are summarized in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Soil</th>
<th>Biochar</th>
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<tbody>
<tr>
<td>Sand [%]</td>
<td>44.94</td>
<td>n.d.</td>
</tr>
<tr>
<td>Silt [%]</td>
<td>35.37</td>
<td>n.d.</td>
</tr>
<tr>
<td>Clay [%]</td>
<td>19.69</td>
<td>n.d.</td>
</tr>
<tr>
<td>pH (H2O)</td>
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<td>9.8</td>
</tr>
<tr>
<td>Cao [%]</td>
<td>1.87</td>
<td>51.90</td>
</tr>
<tr>
<td>Corg [%]</td>
<td>0.91</td>
<td>48.87</td>
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<tr>
<td>Nreal [%]</td>
<td>0.17</td>
<td>0.59</td>
</tr>
<tr>
<td>S [%]</td>
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<td>88</td>
</tr>
<tr>
<td>C/N</td>
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<td>n.d.</td>
</tr>
<tr>
<td>Particle density (g cm^{-3})</td>
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<tr>
<td>Ash content [%]</td>
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<td>45.7</td>
</tr>
<tr>
<td>CEC [mmol, kg^{-1}]</td>
<td>n.d.</td>
<td>103.4</td>
</tr>
<tr>
<td>EC (mS m^{-1})</td>
<td>n.d.</td>
<td>33.7</td>
</tr>
<tr>
<td>Total surface area [m^2 g^{-1}]</td>
<td>n.d.</td>
<td>303</td>
</tr>
</tbody>
</table>

n.d. not determined.

2.3. Nucleic acid extraction and quantitative PCR of typical and atypical nosZ genes

Total DNA was extracted from 0.25 g of thawed soil from each soil sub-sample using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the instructions given by the manufacturer. In total DNA was extracted from 16 control/biochar samples.

One extraction from each of the duplicate soil sub-samples collected from the 8 microcosms. DNA concentration and quality were determined spectrophotometrically (NanoDrop™ 1000, Thermo Scientific, Waltham, MA, USA), fluorometrically (Qubit® 2.0 Fluorometer, Life Technologies, Carlsbad, CA, USA), and by agarose gel electrophoresis. DNA extraction efficiencies varied only slightly between different soil samples (mean DNA yield 4.0 ± 0.6 µg g^{-1} dry soil, n = 8) and did not show any biochar-related bias.

Quantification of typical and atypical nosZ genes was carried out by quantitative PCR (qPCR) using the iCycler IQ™ Real-Time PCR Detection System and the IQ™ 5 Optical System software (Bio-Rad laboratories, Hercules, CA, USA). Typical nosZ genes were quantified with the nosZ22F (5′-CGCRAAGGCAAAGGTSMSSG-3′) and nosZ22R (5′-CAKRTGCGAKGCGTGGCAGAA-3′) primer pair (Heny et al., 2006). For atypical nosZ genes nosZ-II-F (5′-CTTGGCCCACTKACAYAC-3′) and nosZ-II-R (5′-CCGIARGCATAATCGTTRC-3′) were used (Jones et al., 2013). qPCRs were setup in 96 well plates with a reaction volume of 10 µl. Reaction mixtures for typical nosZ genes consisted of 1× Advanced™ Universal SYBR® Green Supermix (Bio-Rad laboratories, Hercules, CA, USA), nosZ22F and nosZ22R (250 nM each) and 1.5–2.5 ng template DNA. Atypical nosZ genes were quantified in a reaction mixture consisting of IQ™ SYBR® Green Supermix (Bio-Rad laboratories, Hercules, CA, USA), nosZ-II-F and nosZ-II-R (1 µM each), and 1.5–2.5 ng template DNA. As standards, dilution series with plasmids containing the different nosZ genes (typical nosZ gene of Sinorhizobium meliloti 1021; atypical nosZ gene of Gemmatimonas aurantiaca T-27) were used. Thermal cycling conditions for typical nosZ genes consisted of an initial denaturing step of 98 °C for 2 min, followed by 40 cycles of 98 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. For atypical nosZ genes thermal cycling conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, and a final step of 80 °C for 30 s at which SYBR Green fluorescence was measured. In all qPCRs, standards, samples, and negative controls were analyzed in triplicates and amplicon specificity was confirmed by performing melt curve analyses and 2% agarose gels. Amplification efficiencies were 72% for typical and 85% for atypical nosZ genes with R² values >0.99 for both assays. The presence of PCR inhibitors in the soil DNA extracts was tested by comparing a standard DNA dilution series with a dilution series of standard DNA to which DNase-treated soil DNA extracts were added. No PCR inhibition was detected when the soil DNA extracts were diluted 1:10 prior to qPCR.

2.4. 454 pyrosequencing of bacterial 16S rRNA genes, typical nosZ genes and atypical nosZ genes

For 454 pyrosequencing DNA extracts from each soil sub-sample were used for amplification of bacterial 16S rRNA genes, typical nosZ genes, and atypical nosZ genes. The bacterial 16S rRNA genes were amplified using primer 27F (5′-AGAGTTTGATCMTGCTAGCAG-3′) (Lane, 1991) and 534R (5′-ATTACCGCGGCTGTGCTAC-3′) (Li et al., 2007) targeting the V1–V3 region of the 16S rRNA gene. For typical nosZ genes primer nosZ-F-1181 (5′-CCTGTCCITCAGACAYAC-3′) and nosZ-R-1880 (5′-ATGTCAGKICGRTGGCAGAA-3′) (Rich et al., 2003) were used. Atypical nosZ genes were amplified with the primers nosZ-II-F and nosZ-II-R developed by Jones et al. (2013). The primers 534R, nosZ-R-1880, and nosZ-II-F contained Roche’s 454 pyrosequencing barcodes and adaptor A, while primers 27F, nosZ-F-1181, and nosZ-II-R contained adaptor B. PCR reactions for each of the DNA samples were performed in duplicates with the
FastStart High Fidelity PCR system (Roche Diagnostics, Rotkreuz, Switzerland) using the thermal protocols and reaction mixtures described in Table S2 in the supplementary data file. Quality of the amplified DNA was confirmed on an Experion® automated electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA). Prior to sequencing, the PCR products were quantified using the Quant-it™ PicoGreen® dsDNA assay kit (Life Technologies, Carlsbad, CA, USA) with a Quantifluor® ST fluorometer (Promega, Fitchburg, WI, USA) and replicates were pooled in equimolar amounts. 454 pyrosequencing was performed on a Roche GS Junior Sequencer (454 Life Sciences, Branford, CT, USA) according to the manufacturer’s instructions for amplicon sequencing. Due to technical problems it was impossible to generate typical nosZ gene amplicons from samples taken at day 0 from the control microcosms.

2.5. Sequence analysis

Quality control, alignment, and classification of the sequencing data were performed using the software package MOTHUR, version 1.33.3 (Schloss et al., 2009) according to Weigold et al. (2016). Pyrosequencing noise and chimeras were removed with the in MOTHUR implemented algorithms PyroNoise (Quince et al., 2009) and UCHIME (Edgar et al., 2011) as described previously (Schloss et al., 2011). Sequences shorter than 200 bp and sequences with homopolymers longer than 8 bp were removed from the dataset. The remaining sequences were aligned against the non-redundant (NR) SILVA SSU Ref database (v.119) (Puesse et al., 2007) and preclustered with MOTHURs implementation of the single linkage algorithm by Huse et al. (2010). A distance matrix was created and sequences were assigned to operational taxonomic units (OTUs) on the species level at 1.3% genetic distance (Yarza et al., 2014) using the average neighbor algorithm (Schloss and Westcott, 2009). Rarefaction curves, diversity indices (Shannon diversity, Simpson diversity), richness (Chao1, ACE), and coverage estimators (Good’s coverage) were calculated based on 1.3% genetic distance using MOTHURs implementation of DOTUR (Schloss and Handelsman, 2005). Prior to the calculation of diversity indices, richness and coverage estimators random subsampling was performed to normalize the dataset to the sample with the lowest number of reads.

nosZ (typical and atypical) gene quality processing was performed using MOTHUR implementations of PyroNoise (Quince et al., 2009), SeqNoise (Quince et al., 2011), and UChime (Edgar et al., 2011) and the criteria stated above for trimming and homopolymer removal. To make sure that typical and atypical nosZ primers were target specific, all sequences were mapped against specific typical (nosZ SEED alignment from fungenes v7.8.1, Fish et al. (2013)) and atypical (sequences from Jones et al. (2013) and Sanford et al. (2012)) nosZ databases. Mapping was performed using DIAMOND (v0.7.9) (Buchfink et al., 2015) in blastx mode with a 70% sequence identity cutoff. For each sequence, only one hit with the highest bit score (− k set to 1) was considered. Sequences not matching the corresponding databases were considered as unclassified. Afterwards typical nosZ gene sequences matching the typical nosZ database and atypical nosZ gene sequences matching the atypical nosZ database were mapped against the RefSeq protein database (release 66, accessed on 14.07.2014) for taxonomic classification. Mapping against RefSeq was also performed with DIAMOND (v0.7.9) in blastx mode with a 70% sequence identity cutoff and an e-value cutoff of 10−10. For each sequence, only matches within 0% of the highest bit score were considered (− top set to 0). For classification of sequences with more than one RefSeq database entry with the same score the Lowest Common Ancestor (LCA) algorithm of MEGAN (MEGAN v5.8.0, Huson et al. (2011)) was applied at default parameters. The LCA algorithm assigns species-specific sequences to specific taxa. Sequences that are conserved among different species (e.g. as consequence of horizontal gene transfer) will only be assigned to taxa of higher rank (Huson et al., 2007). Nonetheless, since it is very difficult to directly prove that a given nosZ gene appears in a specific microbial taxa, whenever we mention a specific species name in the results and discussion we refer to bacteria that contain a nosZ gene closely related to the nosZ gene of the respective species.

2.6. Statistical analysis

Significant differences among typical and atypical nosZ gene copy numbers (Fig. 1) were statistically evaluated by applying t-tests using SAS 9.2. (SAS Institute, Cary, NC, USA). Statistically significant differences among the relative sequence abundance of bacterial 16S rRNA, typical nosZ and atypical nosZ genes were determined using STAMP (v2.0.8) (Parks et al., 2014). The PCA plot (Fig. 2) was generated based on relative sequence abundances on the genus level using the default settings in STAMP. For the extended error bar plots (Figs. 4, 5 and 6) Fisher’s exact test was used with the Newcombe-Wilson confidence interval method and Storey’s FDR multiple test correction (alpha < 0.05). In order to exclude minor abundance differences, only species differing by at least 1% in relative sequence abundance (bacterial 16S rRNA genes, typical nosZ genes and atypical nosZ genes) and by at least a factor of 2 between control and biochar microcosms were considered (bacterial 16S rRNA genes).

![Fig. 1. Typical (A) and atypical (B) nosZ gene copy numbers per g dry soil over time (day 0 and day 15) in control (white) and biochar (black) microcosms. Data points and error bars represent means and standard errors, respectively. Asterisks indicate significant differences (p-value < 0.05).](image-url)
that 93.2% of typical and 0% of atypical sequence identity cutoff against the species, respectively.

645–

12,414), 2695 (day 0: 4466; day 15: 1518 over time (Fig. 1B). Even though average typical and atypical numbers ranged from 1.0 × 10⁷ to 2.6 × 10⁷ copies per g dry soil and decreased over time (Fig. 1B). Even though average typical and atypical nosZ gene copy numbers were higher in biochar microcosms at each time point of sampling (Fig. 1A,B) significant differences (p = 0.046) were only determined for typical nosZ gene copy numbers at day 15 (Fig. 1A). All other incubation time and biochar-related differences were not statistically significant.

3.3. Alpha diversity of the general bacterial community

In order to estimate the alpha diversity of the different bacterial 16S rRNA gene sequence libraries we performed rarefaction analyses and calculated diversity indices (Simpson, Shannon), richness estimators (Chao1, ACE), and Good's nonparametric coverage estimator.

Rarefaction curves did not reach plateaus for any sample indicating that sampling did not fully cover the total estimated diversity (Fig. S1). Even though the number of OTUs per sampled reads was higher during initial sampling (control day 0 and biochar day 0) rarefaction curves constantly increased in all samples. Rarefaction curve slopes were higher for samples taken at the beginning of the experiment compared to samples from day 15. In agreement with the rarefaction analyses, richness estimators (Chao1 and ACE) indicated a higher average OTU richness in samples from day 0 compared to samples taken after 15 days (Table S3). As observed for the OTU richness, bacterial OTU diversity (Simpson and Shannon diversity indices) was also higher in samples taken at the initial time point of sampling compared to samples from day 15 (Table S3). OTU coverage, however, was with an average value of 87.2% after 15 days slightly higher than at the beginning of the experiment, when on average only 81.5% of the expected OTUs were covered (Table S3). The results from the alpha diversity calculations indicated that the bacterial diversity (Simpson and Shannon richness) and Chao1, ACE and rarefaction curves decreased while the OTU coverage increased over time. A biochar effect was not observed.

3.4. Taxonomic composition and distribution of the bacterial community

In order to visualize general differences in the composition of the bacterial communities (16S rRNA gene sequencing) among the different samples we performed a principal component analysis (PCA) and analyzed the contribution of different phyla to each community. The PCA was performed for control and biochar microcosms at the two time points of sampling (0 and 15 days). The first two principal components (PC1 and PC2) explained 99.2% of the variance. The taxonomic composition and distribution of the bacterial community in control and biochar microcosms was very similar at initial sampling (day 0), which was directly after biochar and fertilizer application (Fig. 2). Over time, however, the taxonomic composition and community structure changed and thus control and biochar samples from day 15 clustered very distinct from samples taken at day 0 and from each other.

On the phylum level nine different phyla with a relative sequence abundance of at least 1% were identified (Fig. 3). Remaining sequences could either not be classified (unclassified) or were below the 1% relative sequence abundance threshold (Other) we applied. At day 0, control and biochar microcosms showed a very similar pattern. The three most abundant phyla in both samples were Proteobacteria (control: 29.4%, biochar: 31.0%), Acidobacteria (control: 18.2%, biochar: 17.7%), and Actinobacteria (control: 14.8%, biochar: 14.5%). After 15 days of incubation the most abundant phyla were Proteobacteria (control: 36.1%, biochar: 48.9%), Firmicutes (control: 36.5%, biochar: 28.5%), and Bacteroidetes (control: 5.6%, biochar: 4.8%). The most prominent abundance changes over time were observed for the Firmicutes. Their relative sequence abundance increased from 1.1–1.2% at day 0 to 28.5–36.5% after 15 days. Over the same period Acidobacteria decreased from 17.7–18.2% to 14.5–14.8% at day 0 to 4.1–5.3% and 2.4–4.1% at day 15, respectively. Biochar related changes were much lower but still observable. Proteobacteria had a 12.8% higher (control: 36.1%,
biochar: 48.9%) and Firmicutes an 8% lower (control: 36.5%, biochar: 28.5%) relative sequence abundance in biochar compared to control microcosms at day 15.

Initially (day 0) different genera of the bacterial community were distributed very evenly and no genus exceeded a relative sequence abundance of 4%. Over time, however, distributions became uneven and the following five genera reached relative sequence abundances far above 4% at day 15: Bacillus (up to 17.4%), Pseudomonas (up to 10.6%), Geobacter (up to 8.8%), Simplicispira (up to 5.9%), Azospira (up to 5.2%). All of these genera had a remarkably low initial abundance of below 0.2% at day 0 and thus their fraction of the total bacterial community increased over time.

No genera with significantly different relative sequence abundances in control and biochar microcosms were identified at day 0. However, as shown in Fig. 4 significant differences (q-values from 1.65 × 10^{-10} to <1.0 × 10^{-15}) between control and biochar microcosms were detected for six genera after 15 days of incubation. The genera Pseudomonas (control: 1.2%, biochar: 10.6%), Ensifer (control: 0.4%, biochar: 2.0%), and Geobacter (control: 1.2%, biochar: 2.4%) had significantly higher relative sequence abundances in the biochar-amended microcosms. The relative sequence abundances of Bacillus (control: 17.4%, biochar: 5.5%), Azospira (control: 5.2%, biochar: 0.5%), and Simplicispira (control: 5.9%, biochar: 1.6%) were higher in the control microcosms.

3.5. Classification of typical nosZ genes

The typical nosZ gene carrying microbial community was initially fairly evenly structured with five major species accounting for 71.9% of all typical nosZ sequences (Polydomus guilum: 17.2%, Bradyrhizobium diazoefficiens: 16.8%, Ensifer meliloti: 14.9%, Ensifer fredii: 11.7%, Oligotropha carboxidovorans: 7.7%). After 15 days of incubation only two species (Azoarcus sp. KH32C and Pseudomonas stutzeri) made up 80.3% and 77.9% of the typical nosZ gene sequences in control and biochar microcosms, respectively. While the fraction of Azoarcus sp. KH32C and Pseudomonas stutzeri increased substantially over time, the total cumulative abundance of the five initially abundant species dropped from 71.9% to 4.4% of the total typical nosZ carrying microbial community.

At day 15 significant differences (q-values from 0.048 to <1.0 × 10^{-15}) between control and biochar microcosms were detected for three species (Fig. 5). Azoarcus sp. KH32C had a significantly higher relative sequence abundance in control (67.7%) compared to biochar (47.6%) microcosms. Relative sequence abundances of Pseudomonas stutzeri and Ensifer mellotii were significantly higher in biochar (Pseudomonas stutzeri: 30.3%, Ensifer mellotii: 2.7%) compared to the control (Pseudomonas stutzeri: 13.6%, Ensifer mellotii: 1.3%) samples.

3.6. Classification of atypical nosZ genes

Among the atypical nosZ gene carrying microbial community we identified five species with initial relative sequence abundances above 2%: Runella siliquoformis (up to 29.5%), Pedobacter saltans (up to 26.7%), Belliella baltica (up to 14.6%), Melioribacter roseus (up to 9.5%), and Niastella koreensis (up to 3.7%). In contrast to our findings for the typical nosZ gene containing microbial community the relative sequence abundance of these species did not change considerably over time and their relative sequence abundances remained above 2% also after 15 days of incubation. In addition Flavobacteriaceae bacterium 3519–10 and Dechloromonas aromatica which had a relative sequence abundance below 2% at day 0 reached values of up to 13.1% and 4.7% relative sequence abundance at day 15, respectively.

Statistical analyses revealed that at day 0 no species had a significantly different relative atypical nosZ gene sequence abundance in control and biochar microcosms. However, after 15 days of incubation significant differences (q-values from 6.01 × 10^{-10} to <1.0 × 10^{-15}) between control and biochar microcosms have been identified for five species (Fig. 6). At day 15, Flavobacteriaceae bacterium 3519–10 (control: 13.1%, biochar: 8.0%), Belliella baltica (control: 9.0%, biochar: 4.5%), Dechloromonas aromatica (control: 4.7%, biochar: 0.9%) and Niastella koreensis (control: 5.7%, biochar: 3.1%) showed significantly higher relative sequence abundances in the control microcosms. Pedobacter saltans (control: 26.7%, biochar: 50.9%) had a significantly higher relative sequence abundance in biochar microcosms.

4. Discussion

Similar to other studies, Harter et al. (2014) showed that biochar addition to soil can significantly decrease soil N₂O emissions by altering the abundances and activity of denitrification marker genes (Ducey et al., 2013; Van Zwieten et al., 2014).

The goal of the present study was to investigate the impact of soil biochar amendment on the taxonomic composition and structure of the general bacterial community and the microbial community carrying
typical and atypical nosZ genes. In the following discussion we will compare the sequence data obtained in this study to the geochemical properties of the soil, gas emission rates as well as the gene and transcript content of the different functional marker genes described in Harter et al. (2014) because both studies are based on samples from the same soil microcosm experiment.

Fig. 4. Relative sequence abundances of bacterial genera significantly different in biochar and control microcosms. Panel A illustrates relative sequence abundance and difference in relative sequence abundance (95% confidence intervals based on Newcombe-Wilson) of all genera, which are according to Fisher’s exact test (multiple test correction: Storey’s FDR), significantly different (q-value \(< 0.05\)) in biochar (black) and control (white) microcosms at day 15. Effect size filters “difference between proportions” and “ratio of proportions” were set to 1 and 2, respectively. Panel B shows relative sequence abundances in biochar (black) and control microcosms (white) over time (day 0 and day 15) for the significantly different genera shown in panel A.
In recent years many studies investigating biochar related changes in the microbial community have been published. Most of them showed that soil biochar amendment significantly alters the composition and diversity of the microbial community. Several studies revealed an increase in specific taxa known to have specific ecological functions, life styles or physiological characteristics (Anderson et al., 2011; 2014; Chen et al., 2013; Khodadad et al., 2011; Kolton et al., 2011; Nielsen et al., 2014; Xu et al., 2014). In this study we observed biochar-induced taxonomic shifts within the general bacterial community and identified six genera with significantly different relative sequence abundances in control and biochar microcosms after 15 days of incubation (Bacillus, Pauicmonas, Geobacter, Simplicispira, Azospira, Ensifer).

In agreement with Chen et al. (2013), who observed a biochar related increase in anaerobic bacteria, we recorded a significantly higher relative sequence abundance of Geobacter in our biochar microcosms, a genus known to be strictly anaerobic (Lovley et al., 1993). This confirms the findings of Chen et al. (2015) who documented a strong increase of Geobacter in biochar-amended rice paddy soil. Apart from that we observed that Ensifer (formerly Sinorhizobium), a genus very well adapted to high pH and a broad range of toxins (such as heavy metals) and the polymer degrader containing genus Pauicmonas increased in abundance in the presence of biochar (Braaz et al., 2002; Elboutahiri et al., 2010; Jendrossek, 2001). This supports the findings of Kolton et al. (2011) who detected a biochar-induced increase of chitin- and aromatic carbon-degrading microorganisms. Graber et al. (2010) identified a variety of different organic compounds including hydroxy, acetoxo, and alkanoic acids in biochar extracts which might serve as growth substrates for Pauicmonas. Interestingly, all three genera that were significantly more abundant in biochar microcosms (Geobacter, Ensifer, Pauicmonas) comprise strains described as strict or facultative anaerobes capable of denitrification or fixing molecular nitrogen (Jendrossek, 2001; Kashima and Regan, 2015; Torres et al., 2014). These attributes, however, also apply to the three genera with significantly higher relative sequence abundance in the control microcosms. The genera Bacillus, Azospira, and Simplicispira all comprise strains capable of denitrification and nitrogen fixation (Grabovich et al., 2006; Reinhold-Hurek and Hurek, 2000; Verbaendert et al.,

![Graph A](imageA.png)

**Fig. 5.** Relative sequence abundances of typical nosZ carrying N2O-reducing microbial species significantly different in biochar and control microcosms. Panel A illustrates relative sequence abundance and difference in relative sequence abundance (95% confidence intervals based on Newcombe-Wilson) of all species, which are according to Fisher’s exact test (multiple test correction: Storey’s FDR), significantly different (q-value < 0.05) in biochar (black) and control (white) microcosms at day 15. The effect size filter “difference between proportions” was set to 1. Panel B shows relative sequence abundances in biochar (black) and control microcosms (white) over time (day 0 and day 15) for the significantly different species shown in panel A.

were expected to be equal. Therefore we believe it is reasonable to assume that control and biochar microcosms also contained similar typical nosZ gene communities at the start of the experiment although we failed to amplify typical nosZ genes from the control microcosms at day 0.

The shift from rather diverse but evenly distributed initial microbial communities to functionally more specialized and uneven communities after 15 days of incubation can therefore be ascribed to the experimental setup and conditions during incubation. Highest N2O emission fluxes occur when geochemical conditions are favorable for denitrification (e.g. after fertilization, heavy rainfall) (Pfab et al., 2011; Walter et al., 2014). Accordingly, we set up microcosms with high water content and added organic carbon and mineral nitrogen as fertilizer. In agreement with Chen et al. (2013), who observed a biochar related increase in anaerobic bacteria, we recorded a significantly higher relative sequence abundance of Geobacter in our biochar microcosms, a genus known to be strictly anaerobic (Lovley et al., 1993). This confirms the findings of Chen et al. (2015) who documented a strong increase of Geobacter in biochar-amended rice paddy soil. Apart from that we observed that Ensifer (formerly Sinorhizobium), a genus very well adapted to high pH and a broad range of toxins (such as heavy metals) and the polymer degrader containing genus Pauicmonas increased in abundance in the presence of biochar (Braaz et al., 2002; Elboutahiri et al., 2010; Jendrossek, 2001). This supports the findings of Kolton et al. (2011) who detected a biochar-induced increase of chitin- and aromatic carbon-degrading microorganisms. Graber et al. (2010) identified a variety of different organic compounds including hydroxy, acetoxo, and alkanoic acids in biochar extracts which might serve as growth substrates for Pauicmonas. Interestingly, all three genera that were significantly more abundant in biochar microcosms (Geobacter, Ensifer, Pauicmonas) comprise strains described as strict or facultative anaerobes capable of denitrification or fixing molecular nitrogen (Jendrossek, 2001; Kashima and Regan, 2015; Torres et al., 2014). These attributes, however, also apply to the three genera with significantly higher relative sequence abundance in the control microcosms. The genera Bacillus, Azospira, and Simplicispira all comprise strains capable of denitrification and nitrogen fixation (Grabovich et al., 2006; Reinhold-Hurek and Hurek, 2000; Verbaendert et al.,

![Graph B](imageB.png)
Thus the taxonomic shifts we observed in the general bacterial community do not confirm the findings of Anderson et al. (2011), Xu et al. (2014), and Chen et al. (2015) who concluded that biochar promoted the growth of nitrogen cycling microorganisms. Based on our findings we conclude that soil biochar amendment induces shifts in the taxonomic composition and structure of the soil microbial community. However, functional interpretation of 16S rRNA gene data should be done with great care since the 16S rRNA gene is not a good marker for microbial physiology.

Khodadad et al. (2011) suggested a wide range of environmental factors that could potentially affect the microbial community composition in biochar-amended soils. They assumed that consumable
4.2. Impact of biochar on the N₂O-reducing microbial community

The higher abundance of Geobacter in our biochar microcosms might be promoted by the formation of water saturated anoxic microsites on char particles (Harter et al., 2014; Van Zwieten et al., 2009). The slight pH increase and potential toxin content (Singh et al., 2010) might help explain the higher abundance of species belonging to the genus Ensifer while refractory organic carbon compounds of the biochar (Graber et al., 2010) could be responsible for the observed increase in abundance of Pausimonas-related species. Further experiments relying on more quantitative techniques to assess the abundance of individual taxa as a function of varying environmental parameters will show if our conclusions can be confirmed.

4.2. Impact of biochar on the N₂O-reducing microbial community

In agreement with Harter et al. (2014) and other studies, the present study showed that, based on qPCR data, soil biochar addition increased typical nosZ gene copy numbers after 15 days of incubation (Ducey et al., 2013; Van Zwieten et al., 2014; Xu et al., 2014). As discussed before variation in nosZ gene copy numbers might be due to biochar-induced changes in pH, the formation of anoxic microsites or biochar's ability to act as electron donor and electron shuttle (Cayuela et al., 2013; Harter et al., 2014; Kappler et al., 2014; Xu et al., 2014). In contrast to our results for typical nosZ, atypical nosZ gene copy numbers were not affected by biochar addition. Nonetheless our qPCR results confirmed the findings of Jones et al. (2013) and Orellana et al. (2014) who demonstrated that microbes containing atypical nosZ were overall more abundant than typical nosZ carrying microbes in soils.

Apart from the effect of biochar on the abundance of typical nosZ genes the presence of biochar also changed the taxonomic composition of the typical and atypical nosZ gene carrying microbial community. The microbial community with typical nosZ genes mainly comprises species belonging to the phylum Proteobacteria (Jones et al., 2013; Philippot et al., 2007; Sanford et al., 2012; Zumft and Körner, 2007). In contrast to atypical nosZ-type N₂O reducers most of these microorganisms are capable of complete denitrification and thus also contain functional genes encoding nitrate (narG, napA), nitrite (nirK, nirS), and nitric oxide (norB) reductases (Orellana et al., 2014). After 15 days of incubation we identified three species differing significantly in relative sequence abundance in comparison of biochar and control microcosms. Pseudomonas stutzeri and Ensifer melloti had significantly higher relative sequence abundances in the biochar microcosms and Azorarcus sp. KH32C was more abundant in the control microcosms. Pseudomonas stutzeri is a facultative anaerobe often considered as denitrification model organism. Pseudomonas stutzeri is capable of complete denitrification, carries all necessary denitrification genes, and has been shown to degrade a broad variety of aliphatic and aromatic hydrocarbons (Dados et al., 2015; Lalucat et al., 2006; Lors et al., 2010; Moscoso et al., 2015). Ensifer melloti has been described to be tolerant with respect to many environmental stress factors such as elevated alkalinity (Elbouthahir et al., 2010). Azorarcus sp. KH32C contains the complete denitrification gene set and gene clusters encoding nitrogen fixation (Nishizawa et al., 2012).

All three species with significantly different relative sequence abundances in control and biochar microcosms (Pseudomonas stutzeri, Ensifer melloti, Azorarcus sp. KH32C) are known denitrifiers commonly found in soils (Palmer et al., 2012; Philippot et al., 2007). The observed taxonomic shifts among the typical nosZ gene carrying microbial community in the biochar-amended microcosms might be associated with an increased availability of aromatic carbon compounds (higher abundance of Pseudomonas stutzeri) and the slightly elevated pH as consequence of biochar addition (higher abundance of Ensifer melloti) (Elbouthahir et al., 2010; Graber et al., 2010; Lalucat et al., 2006; Singh et al., 2010). As described in Harter et al. (2014) soil pH values were initially close to neutral in all microcosms and increased slightly during incubation. In the control microcosms, the pH increased from 7.2 to 7.9 and in the biochar-containing microcosms from pH 7.5 to 8.2. In accordance with other studies the pH was higher in biochar-containing microcosms (Ameloot et al., 2013; Van Zwieten et al., 2014). Although all microcosms had a neutral to slightly alkaline pH, the fact that the pH in biochar microcosms was on average 0.3 pH units higher than in the control microcosms might have promoted the growth and activity of specific microbial taxa adapted to higher pH values such as Ensifer melloti (Elbouthahir et al., 2010).

In contrast to typical nosZ genes atypical nosZ genes have been found in many different bacterial and archaeal phyla (Jones et al., 2013; Sanford et al., 2012). As shown by Jones et al. (2013) and Sanford et al. (2012) typical and atypical nitrous oxide reductases are transported via the cytoplasmic membrane by different secretion pathways. While typical nosZ genes possess a Tat signal peptide motif, atypical nosZ genes mainly encode a Sec signal recognition motif (Jones et al., 2013; Sanford et al., 2012). In this study we identified one species with an atypical nosZ gene with significantly higher relative sequence abundance in biochar microcosms and four species that were significantly more abundant in control microcosms after 15 days of incubation. Pedobacter saltans, a common gram-negative soil bacterium found in many different soils across the world, had a significantly higher relative sequence abundance in the biochar microcosms (Fulltorpe et al., 2008; Janssen, 2006; Steyn et al., 1998). Flavobacteriaceae bacterium 3519–10, Belliella baltica, Dechloromonas aromatica, and Niastella koreensis had significantly higher relative sequence abundances in the control microcosms. In addition to nosZ Belliella baltica, Dechloromonas aromatica, and Flavobacteriaceae bacterium 3519–10 also contain other denitrification genes such as narG/napA, nirK/nirS and norB which provide them with the genetic potential to additionally perform the denitrification steps prior to N₂O reduction (Jones et al., 2014; Markowitz et al., 2014; Sanford et al., 2012). Interestingly, Niastella koreensis and Pedobacter saltans only carry an atypical nosZ gene, are therefore restricted to N₂O reduction and rely on the availability of N₂O produced by other species (Jones et al., 2014; Markowitz et al., 2014; Sanford et al., 2012). The species we identified to be significantly different with respect to their relative sequence abundance in control and biochar microcosms have previously been described as atypical nosZ carrying microorganisms. However, the lack of information about their ecophysiological requirements and life styles makes it difficult to speculate about their occurrence or absence from biochar-amended soil microcosms (Jones et al., 2013, 2014; Orellana et al., 2014; Sanford et al., 2012).

4.3. Biochar, N₂O emissions and the diversity of typical and atypical nosZ genes

Harter et al. (2014) showed that decreased N₂O emissions from biochar-amended soil microcosms were linked to a higher abundance and activity of microorganisms carrying a typical nosZ gene. Here we show that soil biochar addition not only affects copy numbers of typical nosZ genes and transcripts but also the diversity of typical and atypical nosZ genes. Tago et al. (2011) have shown that under similar growth conditions even closely related species and genera can differ significantly with respect to denitrification activity and gaseous end product ratio (N₂O to N₂). In addition Cavugeli and Robertson (2001) showed that N₂O reductases of individual denitrifier isolates differed considerably regarding oxygen sensitivity. These findings suggest that taxonomic
shifts within the typical and atypical nosZ carrying N₂O-reducing microbrial community can have a strong impact on N₂O release. According to Jones et al. (2014) about 47% of atypical and only 17% of typical nosZ-containing microorganisms lack either of the nir genes (nirS/nirK) and thus depend on the production of N₂O by other microorganisms. Furthermore Jones et al. (2014) showed that soils with a high N₂O sink capacity also exhibited a high abundance of atypical nosZ carrying microorganisms lacking nitrite reductase genes (nirS/nirK). In this study we have shown that Pseudomonas stutzeri, a typical nosZ-type gene containing species that is known to perform complete denitrification with negligible N₂O release (Carlson and Ingraham, 1983; Lalucat et al., 2006), had a significantly higher relative sequence abundance in the presence of biochar. Among the atypical nosZ gene carrying microbial community Pedobacter saltans was significantly more abundant in biochar microcosms. Pedobacter saltans does not carry other known nitrate, nitrite or nitric oxide reductase genes and therefore seems to be restricted to N₂O reduction (Jones et al., 2014; Markowitz et al., 2014).

Based on these results we conclude that the decreased N₂O emissions from the biochar-amended soil microcosms described by Harter et al. (2014) might be explained by i) an increase in relative sequence abundance of typical nosZ containing taxa efficient in N₂O reduction such as Pseudomonas stutzeri and ii) an increased relative sequence abundance of atypical nosZ gene carrying microorganisms that lack any of the other denitrification genes (nitrate, nitrite and nitric oxide reductases) such as Pedobacter saltans. N₂O for reduction by these only atypical nosZ gene containing strains could be produced by denitrifiers lacking any nosZ gene (Phillippot et al., 2011). Future experiments should target the active N₂O-reducing microbial community based on typical and atypical nosZ mRNA transcript sequencing and quantification to evaluate if the shifts in the nosZ gene community composition described here are also reflected in the organisms transcription profiles.

5. Conclusion

Our study demonstrated that biochar addition to fertilized, water-saturated soil microcosms induced taxonomic shifts within the microbial community including the functional group of N₂O-reducing microorganisms carrying either a typical or atypical nitrous oxide reductase. Since individual microbial taxa can vary considerably with respect to N₂O reduction activities, biochar-induced changes to the community composition of typical and atypical N₂O reducers might provide an additional explanation for the repeatedly reported N₂O mitigation potential of various biochars. Our results suggest that biochar addition can increase the relative sequence abundance of complete denitrifiers such as Pseudomona

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.03.220.

References


