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Soil biochar amendment shapes the composition of N_2O-reducing microbial communities

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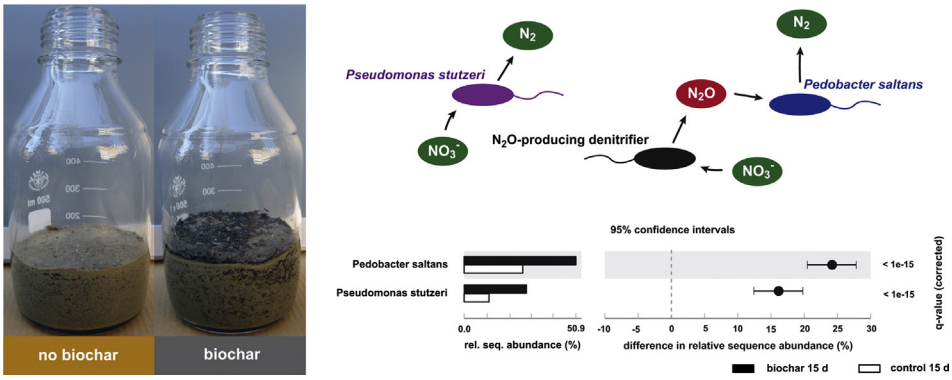
HIGHLIGHTS

- Biochar promoted anaerobic, alkalinity-adapted, and polymer-degrading microbial taxa.
- Biochar fostered the development of distinct N_2O-reducing microbial taxa.
- Taxonomic shifts among N_2O-reducing microbes might explain lower N_2O emissions.

GRAPHICAL ABSTRACT



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ABSTRACT

Soil biochar amendment has been described as a promising tool to improve soil quality, sequester carbon, and mitigate nitrous oxide (N_2O) emissions. N_2O is a potent greenhouse gas. The main sources of N_2O in soils are microbially-mediated nitrogen transformation processes such as nitrification and denitrification. While previous studies have focused on the link between N_2O emission mitigation and the abundance and activity of N_2O-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. The aim was to describe biochar-induced shifts in general bacterial community diversity and taxonomic variations among the *nosZ* gene containing N_2O-reducing microbial communities. While soil biochar amendment significantly altered the 16S rRNA gene-based community composition and structure, it also led to the development of distinct functional traits capable of N_2O reduction containing typical and atypical *nosZ* genes related to *nosZ* genes found in *Pseudomonas stutzeri* and *Pedobacter saltans*, respectively. Our results showed that biochar

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amendment can affect the relative abundance and taxonomic composition of N_2O-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.

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 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_2O) 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_2O 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_2O (Thomson et al., 2012). In soils, denitrification represents one of the major N_2O producing pathways. N_2O 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; Pfab 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), *norB* (nitric oxide reductase), 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_2O reductases, denitrification is often incomplete and N_2O 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_2O (e.g. nitrifier denitrification, nitrification) under certain environmental conditions. However, the only known biological sink for N_2O is the reduction of N_2O to N_2 by N_2O-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_2O 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_2O to N_2 (Jones et al., 2014; Orellana et al., 2014). Interestingly, atypical *nosZ* gene carrying N_2O 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_2O 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_2O release, because microbial nitrous oxide reduction is the main biotic sink of atmospheric N_2O (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_2O-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_2O reducers is thought to be one of the reasons for the frequently observed decreased N_2O 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_2O-reducing microbial communities has not been studied in detail yet. Since it has been shown that individual N_2O-reducing taxa can differ considerably in N_2O reduction activity, taxonomic shifts among typical and atypical *nosZ* gene carrying N_2O-reducing microbial communities might affect N_2O reduction rates and net soil N_2O 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 if 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_2O 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

data collected during the microcosm experiment (including N_2O emissions) is described in Harter et al. (2014).

2.1. Soil sampling and biochar production

Soil for the soil microcosms was collected at a vineyard of the Ithaka Institute for Carbon Intelligence located in Ayent (Switzerland). The soil is characterized as loamy sand and was collected from the top 10 cm. Biochar was produced by Swiss Biochar Sàrl (Belmont-sur-Lausanne, Switzerland) from green waste via slow pyrolysis (700 °C). Soil and biochar physicochemical properties are summarized in Table 1.

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 the field moist equivalent of 180 g dry soil. Biochar microcosms contained the field moist equivalent of 162 g dry soil and 18 g dry biochar, which corresponded to a biochar content of 10% (w/w). Both, control microcosms and biochar microcosms were set up in duplicates. In total 8 microcosms were set up. 4 microcosms (2 control and 2 biochar microcosms) were prepared for sampling at day 0 and 4 other microcosms (2 control and 2 biochar microcosms) were set up for sampling after 15 days of incubation. After preparation all soil microcosms were homogenized with a spatula and then carefully compacted by tapping the microcosms on a soft surface. In order to create denitrification favoring conditions corresponding to situations in the field when highest N_2O emission fluxes occur (e.g. after fertilization, heavy rainfall) soil microcosms were fertilized and a high constant water content was established. At beginning of the experiment carbon (555 mg kg^{-1} as molasses), nitrogen (250 mg kg^{-1} as NH_4NO_3), phosphorus, and potassium (150 and 188 mg kg^{-1} as KH_2PO_4) were added to all soil microcosms with a nutrient solution (refer to Table S1 in the supplementary data file for more details about molasses). After adding the fertilizer solution the water content of the soil microcosms was adjusted to a water filled pore space (WFPS) of 95% using deionized water. Soil microcosms were incubated open to ambient atmosphere at 28 °C in a daylight incubator. During incubation WFPS was held constant by periodically replenishing the evaporated water. Soil samples for DNA extraction were taken by destructively sampling both control and biochar microcosms after an incubation period of 0 and 15 days. After sampling, soil from the control microcosms and from the biochar microcosms were homogenized thoroughly. From the homogenized soil of each microcosm 2 soil sub-samples were transferred into centrifuge tubes and directly frozen at −20 °C.

Table 1
Physicochemical properties of the soil (calcaric leptosol) and the biochar used in this study. From Harter et al. (2014).

Parameter	Soil	Biochar
Sand [%]	44.94	n.d.
Silt [%]	35.37	n.d.
Clay [%]	19.69	n.d.
pH (H_2O)	8.4	9.8
C_{tot} [%]	1.87	51.90
C_{org} [%]	0.91	48.87
N_{total} [%]	0.17	0.59
S [%]	0.04	0.15
C:N	11	88
Particle density [g cm^{-3}]	n.d.	2.0
Ash content [%]	n.d.	45.7
CEC [$mmol_c$ kg^{-1}]	n.d.	103.4
EC [mS m^{-1}]	n.d.	33.7
Total surface area [m^2 g^{-1}]	n.d.	303

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 nosZ2F (5′-CGCRACGGCAASAAGGTSMSSG-3′) and nosZ2R (5′-CAKRTGCAKSGCRTGGCAGAA-3′) primer pair (Henry et al., 2006). For atypical *nosZ* genes nosZ-II-F (5′-CTIGGICCIYTKCAYAC-3′) and nosZ-II-R (5′-GCIGARCARAAITCBGTRC-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 SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad laboratories, Hercules, CA, USA), nosZ2F and nosZ2R (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 R^2 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′-AGAGTTTGATCMTGGCTCAG-3′) (Lane, 1991) and 534R (5′-ATTACCGCGGCTGCTGGC-3′) (Liu et al., 2007) targeting the V1–V3 region of the 16S rRNA gene. For typical *nosZ* genes primer nosZ-F-1181 (5′-CGCTGTTCITCGACAGYCAG-3′) and nosZ-R-1880 (5′-ATGTGCAKIGCRTGGCAGAA-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) (Pruesse 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, 2011). Sequences were classified using the Naïve Bayesian Classifier (Wang et al., 2007) and the SILVA reference taxonomy (v. 119). 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 MOTHURs 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).

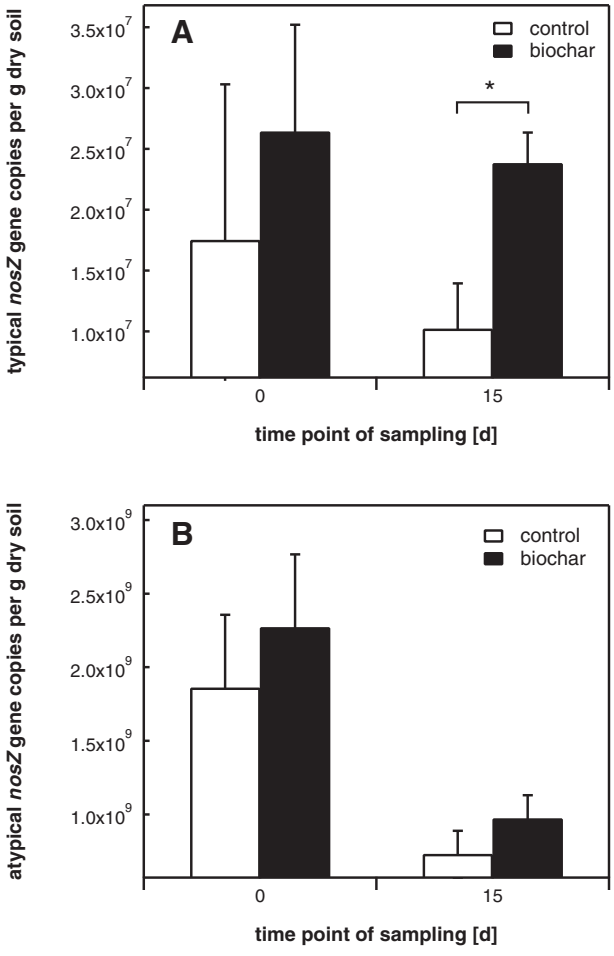

Fig. 1. Typical (A) and atypical (B) *nosZ* gene copy numbers per g dry soil over time (day 0 and day 15) in control (white) and biochar (black) microcosms. Data points and error bars represent means and standard errors, respectively. Asterisks indicate significant differences (*p*-value < 0.05).

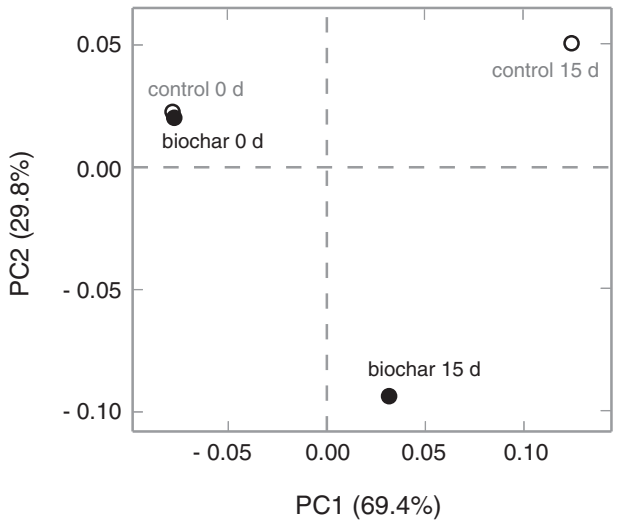

Fig. 2. PCA plot illustrating the taxonomic composition and distribution based on relative sequence abundances of genera among the total bacterial community (bacterial 16S rRNA gene sequencing) in control and biochar microcosms at different time points of sampling. Black circles indicate biochar microcosms. White circles indicate control microcosms.

2.7. Accession numbers

Pyrosequencing reads have been deposited in the ENA Sequence Read Archive (SRA) under accession number PRJEB9850.

3. Results

3.1. Typical and atypical nosZ gene copy numbers

Quantitative polymerase chain reactions (qPCR) targeting typical and atypical *nosZ* genes revealed time- and biochar-related variations among the different samples (Fig. 1). Average typical *nosZ* gene copy numbers ranged from 1.0×10^7 to 2.6×10^7 copies per g dry soil and did not vary much over time (Fig. 1A). Atypical *nosZ* gene copy numbers ranged from 7.3×10^8 to 2.3×10^9 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.

3.2. Sequencing statistics and nosZ primer specificity

Bacterial 16S rRNA, typical *nosZ* and atypical *nosZ* gene sequencing yielded 94,492, 9537, and 8081 raw sequence reads with average read lengths of 421, 479, and 352 bp, respectively. After quality processing with MOTHUR 47,486 high quality bacterial 16S rRNA gene sequences remained. For typical and atypical *nosZ* genes quality processing resulted in 8086 and 3889 high quality reads, respectively. On average bacterial 16S rRNA gene, typical *nosZ* gene and atypical *nosZ* gene sequencing resulted in 11,872 (day 0: 11,343–13,153; day 15: 10,576–12,414), 2695 (day 0: 4466; day 15: 1518–2102), and 972 (day 0: 645–719; day 15: 1231–1294) high quality sequence reads per sample, respectively.

Mapping of quality processed *nosZ* gene sequences with a 70% sequence identity cutoff against the specific typical *nosZ* database revealed that 93.2% of typical and 0% of atypical *nosZ* gene sequences could be matched to database entries. When quality processed *nosZ* gene sequences were mapped against the specific atypical *nosZ* database 0% of typical and 92.4% of atypical *nosZ* gene sequences matched database entries with a sequence identity >70%. According to this sequence filtering step 93.2 and 92.4% of typical and atypical *nosZ* gene sequences could be identified as "true" typical or atypical *nosZ* gene sequences, respectively. This proves that both primer sets amplified their specific *nosZ* gene type with high specificity and that our sequence analysis approach clearly distinguished both gene types.

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) and richness (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 Acido- and Actinobacteria decreased from 17.7–18.2% and 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%,

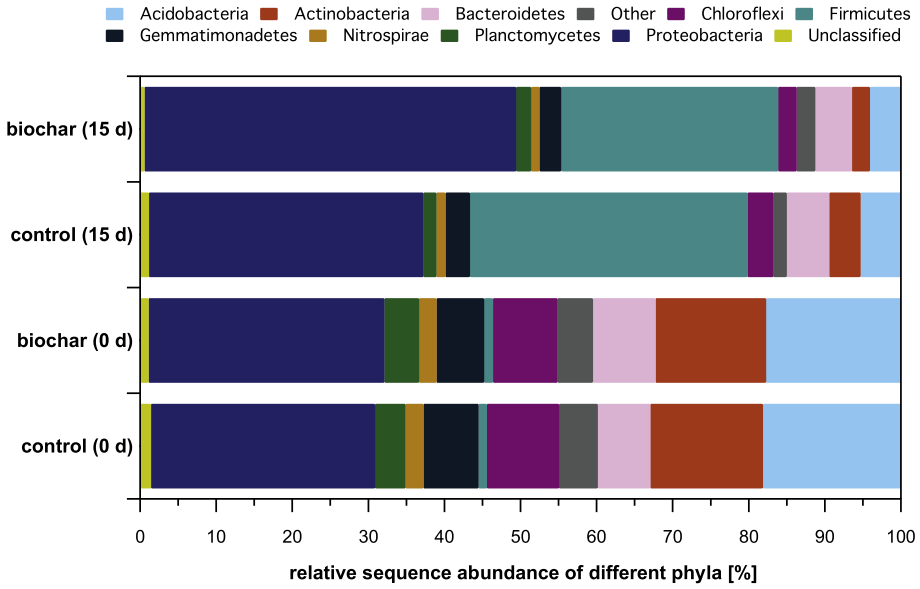

Fig. 3. Relative sequence abundance of different phyla in biochar and control microcosms after 0 and 15 days. Other represents all phyla with relative sequence abundances below 1%.

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%), *Paucimonas* (up to 10.6%), *Gracilibacter* (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 0.25% 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 $<1.0 \times 10^{-15}$) between control and biochar microcosms were detected for six genera after 15 days of incubation. The genera *Paucimonas* (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 (*Polymorphum gilvum*: 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 $<1.0 \times 10^{-15}$) 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 meliloti* were significantly higher in biochar (*Pseudomonas stutzeri*: 30.3%, *Ensifer meliloti*: 2.7%) compared to the control (*Pseudomonas stutzeri*: 13.6%, *Ensifer meliloti*: 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 slithyformis* (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^{-3} to $<1.0 \times 10^{-15}$) 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 N_2O 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

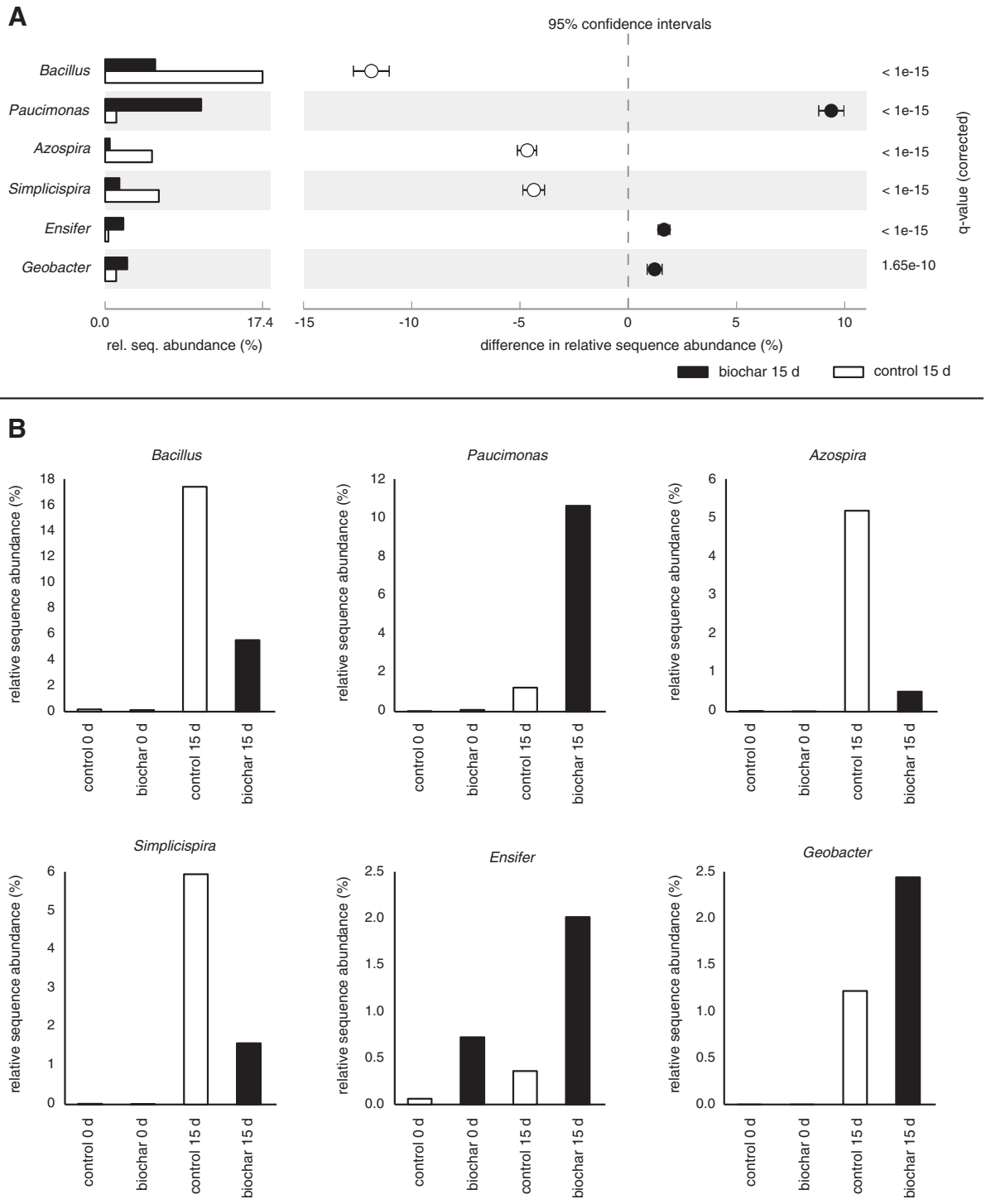

Fig. 4. Relative sequence abundances of bacterial genera significantly different in biochar and control microcosms. Panel A illustrates relative sequence abundance and difference in relative sequence abundance (95% confidence intervals based on Newcombe-Wilson) of all genera, which are according to Fisher's 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.

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.

Amplicon sequencing of the general bacterial community and the atypical *nosZ* gene-containing microbial community revealed very small differences between biochar and control microcosms at the beginning of the experiment (day 0). Samples were taken from freshly established microcosms directly after fertilizer application and since all microcosms contained the same soil their microbial communities

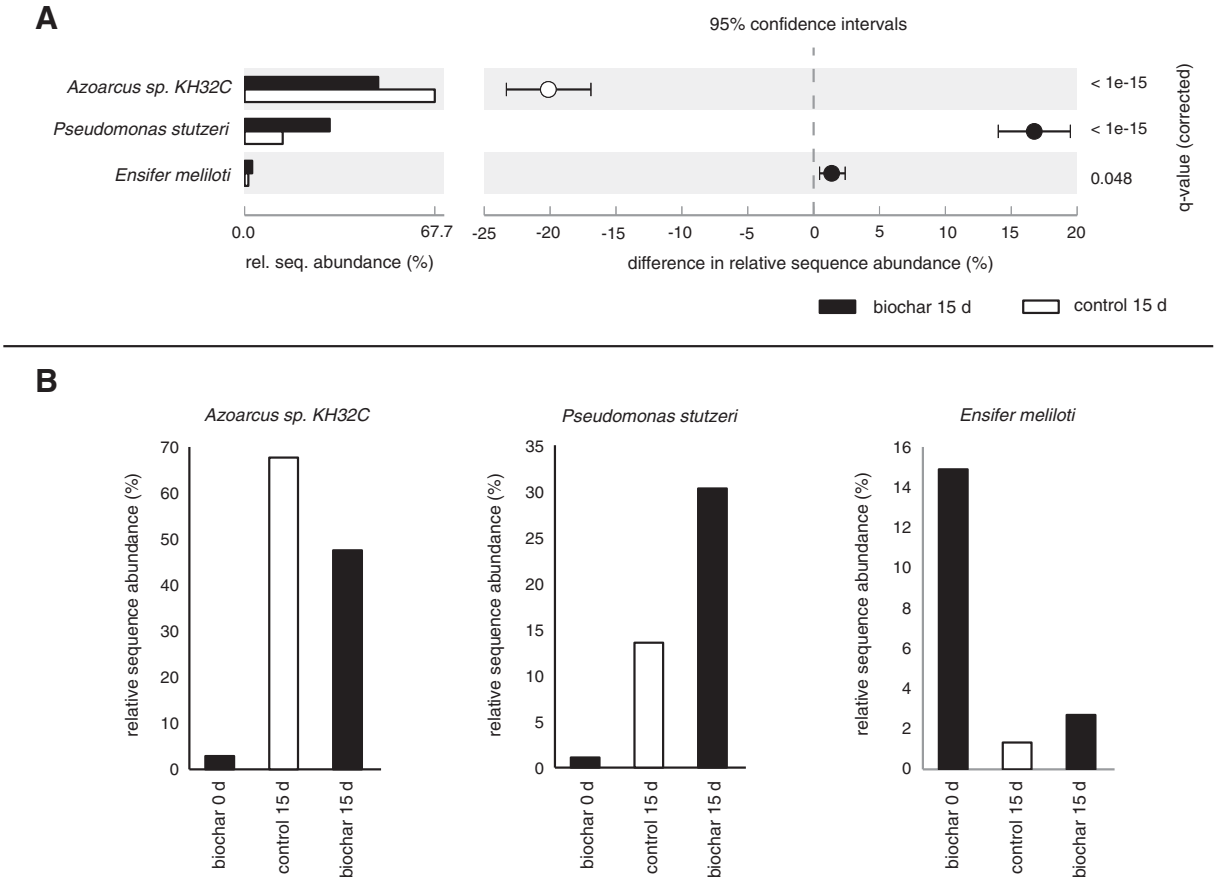

Fig. 5. Relative sequence abundances of typical *nosZ* carrying N_2O-reducing microbial species significantly different in biochar and control microcosms. Panel A illustrates relative sequence abundance and difference in relative sequence abundance (95% confidence intervals based on Newcombe-Wilson) 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 N_2O 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 the findings of other studies we observed a shift in the microbial community composition towards taxa adapted to lower oxygen partial pressures that are capable of using constituents of the applied fertilizer as growth substrates (Chen et al., 2010; Drenovsky et al., 2004; Zhong et al., 2009).

4.1. Impact of biochar on the general bacterial community

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*, *Paucimonas*, *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 *Paucimonas* 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, acetoxy, and alkanoic acids in biochar extracts which might serve as growth substrates for *Paucimonas*. Interestingly, all three genera that were significantly more abundant in biochar microcosms (*Geobacter*, *Ensifer*, *Paucimonas*) 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.,

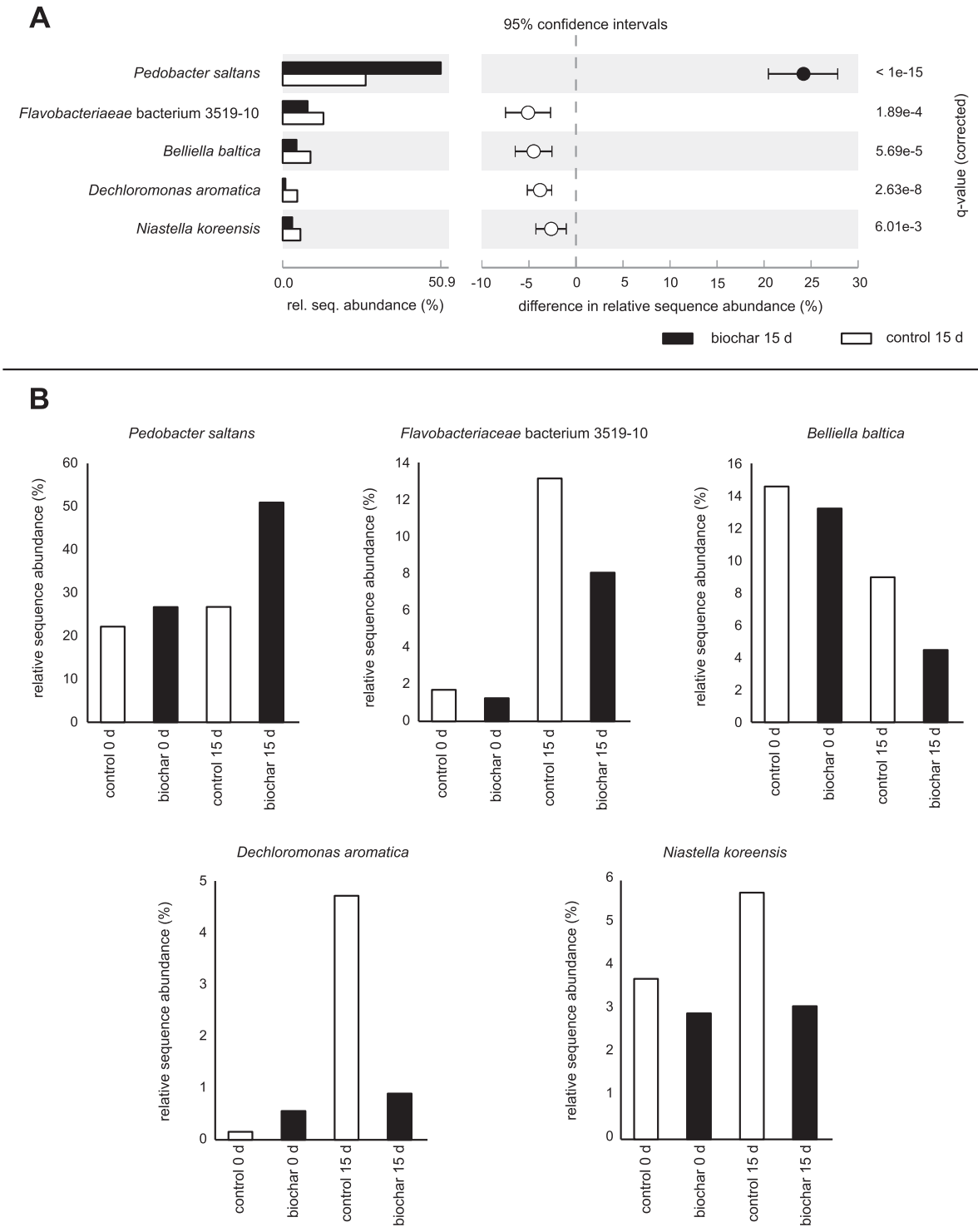

Fig. 6. Relative sequence abundances of atypical *nosZ* carrying N_2O-reducing microbial species significantly different in biochar and control microcosms. Panel A illustrates relative sequence abundance and difference in relative sequence abundance (95% confidence intervals based on Newcombe-Wilson) 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.

2011). 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

refractory carbon or other nutrients supplied with the biochar could affect microbial community compositions. They also suggest that the biochar could be toxic for certain taxa and that biochar-induced changes in pH and soil water availability might promote changes in soil microbial community diversity (Khodadad et al., 2011). Even though the role and individual importance of these variables for microbial community composition and functionality in biochar-amended soils are highly speculative it is likely that some of these variables were also responsible for the observed taxonomic shifts among the bacterial community in our study.

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 *Paucimonas*-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 N_2O-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 N_2O 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 meliloti* had significantly higher relative sequence abundances in the biochar microcosms and *Azoarcus* 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 meliloti* has been described to be tolerant with respect to many environmental stress factors such as elevated alkalinity (Elboutahiri et al., 2010). *Azoarcus* sp. KH32C contains the complete dentrification 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 meliloti*, *Azoarcus* 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 meliloti*) (Elboutahiri 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 microcosm might have promoted the growth and activity of specific microbial taxa adapted to higher pH values such as *Ensifer meliloti* (Elboutahiri 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 two 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 (Fulthorpe 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 N_2O 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 N_2O reduction and rely on the availability of N_2O 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, N_2O emissions and the diversity of typical and atypical nosZ genes

Harter et al. (2014) showed that decreased N_2O 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 (N_2O to N_2). In addition Cavigelli and Robertson (2001) showed that N_2O reductases of individual denitrifier isolates differed considerably regarding oxygen sensitivity. These findings suggest that taxonomic

shifts within the typical and atypical *nosZ* carrying N_2O-reducing microbial community can have a strong impact on N_2O 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 N_2O by other microorganisms. Furthermore Jones et al. (2014) showed that soils with a high N_2O 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 N_2O 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 N_2O reduction (Jones et al., 2014; Markowitz et al., 2014).

Based on these results we conclude that the decreased N_2O 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 N_2O 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*. N_2O for reduction by these only atypical *nosZ* gene containing strains could be produced by denitrifiers lacking any *nosZ* gene (Philippot et al., 2011). Future experiments should target the active N_2O-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 N_2O-reducing microorganisms carrying either a typical or atypical nitrous oxide reductase. Since individual microbial taxa can vary considerably with respect to N_2O reduction activities, biochar-induced changes to the community composition of typical and atypical N_2O reducers might provide an additional explanation for the repeatedly reported N_2O mitigation potential of various biochars. Our results suggest that biochar addition can increase the relative sequence abundance of complete denitrifiers such as *Pseudomonas stutzeri* and atypical N_2O reducers restricted to N_2O reduction such as *Pedobacter saltans*. Although biological explanations for lower N_2O emissions from biochar-amended soils seem likely we cannot exclude that abiotic mechanisms such as sorption of N_2O onto biochar particles or chemodenitrification also contributed to the decreased N_2O emissions (Cornelissen et al., 2013; Lin et al., 2014). To what extent abiotic and biological mechanisms other than the community composition of typical and atypical N_2O reducers contributed to the decreased N_2O emissions requires further study. It also has to be taken into account that the findings presented here are based on a plant-free, fertilized soil microcosm experiment carried out at a constant water filled pore space. Further research including long-term field trials with different soils and biochars is needed to broaden our understanding of the impact of biochar on the nitrogen cycling microbial community and soil N_2O emissions.

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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.

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