Addition of activated switchgrass biochar to an aridic subsoil increases microbial nitrogen cycling gene abundances

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ABSTRACT

It has been demonstrated that soil amended with biochar, designed specifically for use as a soil conditioner, results in changes to the microbial populations that reside therein. These changes have been reflected in studies measuring variations in microbial activity, biomass, and community structure. Despite these studies, very few experiments have been performed examining microbial genes involved in nutrient cycling processes. Given the paucity of research in this area, we designed a 6 month study in a Portneuf subsoil treated with three levels (1%, 2%, and 10% w/w ratio) of a biochar pyrolyzed from switchgrass (*Panicum virgatum*) at 350 °C and steam activated at 800 °C to measure the abundances of five genes involved in N cycling. Gene abundances were measured using qPCR, with relative abundances of these genes calculated based on measurement of the 16S rRNA gene. At the end of the 6 month study, all measured genes showed significantly greater abundances in biochar amended treatments as compared to the control. In soil amended with 10% biochar, genes involved in nitrogen fixation (*nifH*), and denitrification (*nirS*), showed significantly increased relative abundances. Lastly, gene abundances and relative abundances correlated with soil characteristics, in particular NO_3-N, % N and % C. These results confirm that activated switchgrass-derived biochar, designed for use as a soil conditioner, has an impact on the treated soils microbial communities. We therefore suggest that future use of biochar as a soil management practice should take into account not only changes to the soil's physiochemical properties, but its biological properties as well.

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1. Introduction

Biochar, also referred to as black carbon or char, is the residual material after pyrolysis of organic feedstock with the intent for use as a soil amendment (Lehmann and Joseph, 2009). These carbon-stable, and plant nutrient enriched, materials have received considerable interest for use as a soil conditioner, can be tailored for specific soils and management practices (Ippolito et al., 2012), and can result in beneficial soil chemical and physical changes (Atkinson et al., 2010; Novak et al., 2009a, 2012). Though the potential impacts of biochar on soil have typically been focused on improvements in C sequestration and soil quality, they also have the potential for influencing the soil microbial communities which reside therein (Lehmann et al., 2011). While it has been demonstrated that a majority of biochar carbon (C) is resistant to microbial mineralization (Bruun et al., 2008; Smith et al., 2010; Zavalloni et al., 2011), it has been hypothesized that the soil's microbial community structure will change in response to the addition of a pool of recalcitrant biochar-C. This tenet is supported by Pietikainen et al. (2000) in a study examining microbial phospholipid fatty acid (PLFA) profiles. They reported that while microbial biomass did not vary between biochar treatments, microbial PLFA patterns were significantly altered. Using terminal restriction fragment length polymorphism (T-RFLP) analysis, Anderson et al. (2011) reported shifts in microbial community structure in the presence of a pine-derived (*Pinus radiata*) biochar. These authors also noted shifts in the relative abundances of organisms involved in soil nitrogen cycling; in particular, increases were reported in organisms shown to be involved in nitrogen fixation and denitrification, and decreases in organisms shown to be involved in nitrification (Anderson et al., 2011).

In addition to the report by Anderson et al. (2011), there have been a number of papers discussing the topic of N-cycling, in particular nitrous oxide emissions, and the effect of biochar on these processes (Clough and Condron, 2010). Ball et al. (2010) demonstrated the effects of charcoal, produced by wildfire in coniferous-dominated forests, on increased soil nitrification rates and nitrifier abundances. In a contrasting study of a soil treated with a wood-derived biochar, Clough et al. (2010) reported lower rates of nitrification. Nitrous oxide emissions decreased in soils amended

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with biochar in studies reported by Spokas et al. (2009) and Spokas and Reicosky (2009), while Rondon et al. (2007) reported increases in nitrogen fixation in soils receiving varying levels of biochar. However, despite these and similar reports, there have been no studies using soils amended with designed biochar, which have sought to quantify the abundance of genes involved in N-cycling.

Understanding the effect of designed biochars on soil microbiology may have a substantial practical impact on our ability to improve the productivity of eroded calcareous soils, whose lack of organic C inhibits microbial activity (Tarkalson et al., 1998). In this case, the switchgrass biochar employed is one developed specifically for its low pH, which potentially will help neutralize the target soil's alkaline character and further enhance microbial contributions.

Therefore, this study investigates the effect that switchgrass biochar has on genes involved in N-cycling of a calcareous subsoil. We further examined the effect that the physicochemical changes in those amended soils had on altering gene abundances. Our approach was to utilize quantitative Real-Time polymerase chain reaction (qPCR) analysis to measure the abundance of the following five genes involved in N-cycling: (i) *nifH*, which encodes the iron-containing subunit of nitrogenase, an enzyme which fixes dinitrogen (N_2) gas to ammonia (NH_3); (ii) *amoA*, which encodes the active site of ammonia monooxygenase, which oxidizes NH_3; (iii) *nirS* and *nirK*, which both encode nitrite reductases, which convert nitrite (NO_2) to nitric oxide (NO); and (iv) *nosZ*, which encodes a nitrous oxide reductase, responsible for degrading nitrous oxide (N_2O) to N_2.

2. Materials and methods

2.1. Biochar source and preparation

Full maturity switchgrass (*Panicum virgatum*) was collected from Clemson University Pee Dee Research and Education Center located in Darlington, SC. This material was dried at 40 °C and then hammer milled to 6 mm. Hammer milled switchgrass was pyrolyzed and activated using a Lindberg bench furnace equipped with a retort (Lindberg/MPH with retort, Riverside, MI). Details of this system and its controls can be found elsewhere (Cantrell and Martin, 2012). Approximately 1300 g of switchgrass (moisture content of 8.8%) at a time was pyrolyzed under N_2 gas and steam activated concurrently according to the schedule listed in Table 1. Steam activation involved injecting water at 5 ml min^{-1} using a peristaltic pump into the N_2 gas flow entering the heated retort. After retort cool down, samples were allowed to cool to room temperature overnight. Activated samples were not post-treated with an acid wash. Activated biochar recovery was the percentage weight ratio of activated biochar mass to feedstock mass. Surface area was measured in duplicate by N_2 adsorption isotherms at 77 K using a Nova 2000 surface area analyzer (Quantachrome Corp., Boynton Beach, FL). Specific surface areas were determined from adsorption isotherms using the Brunauer, Emmett, and Teller (BET) equation (Brunauer et al., 1938). Biochar pH was determined in deionized water at a 1% (w/v) ratio following shaking at 200 rpm for 24 h (Novak et al., 2009b). Biochar electrical conductivity (EC) was determined using a saturated paste extraction (Rhoades, 1996).

Table 2
Biochar and subsoil characteristics.

Property	Units	Biochar	Subsoil
Recovery	%$_{db}$	27.2 ± 0.2	ND[a]
BET surface area	m^2 g^{-1}	218.7 ± 39.9	ND
Micropore area	m^2 g^{-1}	126.4 ± 50.5	ND
Micropore volume	cm^3 g^{-1}	0.059 ± 0.024	ND
Ash	% (dry basis)	5.86 ± 0.01	ND
pH		5.8	7.6
EC	dS m^{-1}	0.70	0.77
NH$_4$-N	mg kg^{-1}	8.2	0.6
NO$_3$-N	mg kg^{-1}	2.6	18.1
Total C	%	88.0	3.53
Total N	%	0.68	0.08

[a] ND = not determined.

Biochar total C and N were determined by dry combustion (Nelson and Sommers, 1996) using a Flash EA 1112 CN Elemental Analyzer (CE Elantech, Inc., Lakewood, NJ), and biochar nitrate-nitrogen (NO$_3$-N) and ammonium-nitrogen (NH$_4$-N) were determined using a 2 M KCl extract (Mulvaney, 1996). Biochar physico-chemical characteristics are presented in Table 2.

2.2. Soil characterization

Subsoil was obtained from the edge of a field from a site located 1.7 km southwest of Kimberly, Idaho (42°31′N, 114°22′W) with a mean elevation and annual precipitation of 1190 m and 251 mm. Soil at the site was classified as Portneuf (coarse-silty, mixed, superactive, mesic Durinodic Xeric Haplocalcid) and is extensive in southern Idaho, occupying approximately 117,000 hectares (USDA-NRCS, 2011). The site was developed and utilized for eroded soil experiments as described by Robbins et al. (1997, 2000) and Lentz et al. (2011) whereby the topsoil (0–30 cm) was removed. The top 30 cm of exposed subsoil was collected, air-dried, and passed through a 2-mm sieve. Soil characteristics were measured using the same methods as for the biochar. Soil chemical characteristic data are presented in Table 2.

2.3. Soil-biochar incubation

The effect of switchgrass biochar to exposed subsoil was investigated during a 6 month incubation study. Treatments consisted of either 0, 1, 2, or 10% rates of biochar application to soil (w:w). Soil (300 g) and biochar mixtures were placed in square 8 cm^3 plastic pots lined with plastic to prevent leaching, placed in a growth chamber set at 22 °C and 30% humidity, and watered twice per week with reverse osmosis water to 80% of field capacity. Pots were destructively sampled at 1, 2, 3, 4, 5, and 6 month intervals; four replicates per treatment were utilized for each time step for a total of 96 pots. Prior to destructively sampling, pot weights were recorded and soil moisture content (by weight) was determined.

2.4. DNA extraction

Two DNA extractions – for a total of 48 – were performed for each soil treatment per month, based on odd (1 and 3) and even (2 and 4) numbering of quadruplicate soil replicates. A total of 4 g of

Table 1
Biochar preparation conditions.

Stages	Ramp (°C min^{-1})	Temperature (°C)	Hold time (min)	N_2 flow (L min^{-1})	Water flow (ml min^{-1})
Purge		200	30	15	–
Pyrolysis	2.5	350	120	15	–
Activation	5	800	180	1	5
Cool down	−2.5	100	–	1	–

Table 3
Primers used in this study.

Primers	Sequence (5′–3′)	Target	T_m[a]	Product length	Reaction T_m	Efficiency
amoA-1F	GGGGTTTCTACTGGTGGT	*amoA*	54.1 °C	491 bp	54 °C	1.92
amoAr NEW	CCCCTCBGSAAAVCCTTCTTC		58.8 °C			
cd3aF_nirS	GTSAACGTSAAGGARACSGG	*nirS*	57.1 °C	425 bp	55 °C	1.97
R3cd_nirS	GASTTCGGRTGSGTCTTGA		55.8 °C			
1F_nirK	GGMATGGTKCCSTGGCA	*nirK*	58.0 °C	516 bp	53 °C	1.92
nirK5R	GCCTCGATCAGRTTRTGG		52.8 °C			
nosZF	CGYTGTTCMTCGACAGCCAG	*nosZ*	58.6 °C	453 bp	55 °C	1.90
nosZ-1622R	CGSACCTTSTTGCCSTYGCG		63.1 °C			
PolF	TGCGATCCSAATGCBGACTC	*nifH*	55.9 °C	360 bp	55 °C	1.99
PolR	ATSGCCATCCTYTCRCCGGA		57.9 °C			
515F	TGCCAGCAGCCGCGGTAA	16S v4–v5 region	63.3 °C	412 bp	55 °C	1.99
927R	CTTGTGCGGGCCCCCGTCAATTC		65.1 °C			

[a] T_m, melting temperature.

soil were combined to form the composite samples and mixed thoroughly. From each composite soil sample, a total of 0.5 g of soil was used for microbial DNA extraction using a PowerLyzer PowerSoil DNA Isolation kit (MO Bio Laboratories, Inc., Carlsbad, CA), according to manufacturer specifications. DNA concentration and purity were determined by 260/280 nm and 260/230 nm measurements using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE).

2.5. Quantitative Real-Time polymerase chain reaction (qPCR) assays

All qPCR assays were run on a Lightcycler 480 Real Time PCR System (Roche Diagnostics, Indianapolis, IN). Primers used in these assays were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Table 3. Assays were carried out using SYBR GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA) in a total reaction volume of 25 μL as previously described (Ducey et al., 2011). Final reaction concentrations of reagents consisted of 1× SYBR GreenER qPCR SuperMix, 200 nM each of forward and reverse primers (Table 3), and 1 μL of a 1:100 dilution of DNA template. The qPCR conditions were conducted as follows: (i) an initial denaturation at 95 °C for 5 min; (ii) 50 cycles of denaturation at 95 °C for 30 s, the appropriate annealing temperature for 30 s (Table 3), and elongation at 72 °C for 30 s; (iii) melting curve analysis to confirm amplification product specificity. Fluorescent measurements were taken during the annealing phase of each cycle. All qPCR assays included negative controls without template, as well as reactions containing between 10^1 and 10^9 DNA copies to generate standard curves and calculate amplification efficiencies according to the equation: $E = 10^{[-1/\text{slope}]}$ (Pfaffl, 2001). DNA standards consisted of linearized plasmids carrying the appropriate target gene (Hou et al., 2010), which were sequenced to confirm their identity and primer binding site. Each assay was performed in triplicate, with triplicate measurements for each sample.

2.6. Statistics

Prior to statistical analysis, gene abundances were normalized to the amount of DNA collected per sample and $\log_{10}$ transformed. Gene abundances were likewise corrected for gene copy per organism: 1 copy per organism for *nifH*, *nirK*, *nirS*, and *nosZ* (Hall et al., 2008; Kandeler et al., 2006); 2 copies per organism for *amoA* (Chain et al., 2003); and 3.6 copies per organism for the 16S rRNA gene (Klappenbach et al., 2001). Relative abundances (from non-log transformed qPCR values) were calculated for each sample as a ratio of the abundance of each N-cycling gene to 16S rRNA gene abundance. Effects of biochar on gene abundances, relative abundances, and soil characteristics were analyzed using Duncan's multiple range test in SAS version 9.2 (SAS Institute, Cary, NC). Correlation

and regression analyses, using gene and relative abundance values from each DNA extraction ($n = 48$), was performed in SAS. Prior to statistical analysis for NH_4-N, non-detectable levels were adjusted to 0.002 mg kg^{-1} to account for the minimum detection limit of the assay. Month to month comparisons of soil characteristics were performed using Student's *t*-test in Excel 2010 (Microsoft Corporation, Redmond, WA) outfitted with the Analyse-it (Analyse-it Software, Ltd., Leeds, UK) plugin.

3. Results

3.1. Soil characteristics after biochar addition

The study was conducted with a Portneuf soil collected from the B horizon of the soil profile near the Kimberly, Idaho. This soil was amended with an activated switchgrass biochar such that soils contained 1%, 2%, or 10% (w/w) biochar. For the purposes of this conducted study, all use of the word biochar refers to this activated switchgrass biochar. Soils characteristics were compared both temporally (month 1 versus month 6) and based on the rate of biochar amendment; all values and statistical comparisons can be found in Table 4.

Over the course of the 6 month study, there were significant changes in several soil characteristics, some of which could be attributed to the biochar amendment. Soil electrical conductivity (EC) values increased over the course of the study; however the increase was less severe in the soils amended with biochar indicating that biochar may help retard soil salinity by potentially sequestering salts. Levels of NH_4-N were undetectable by the end of the study, however in the first month of the study soils receiving biochar showed a significant decrease. Temporally, NO_3-N levels increased in the control soil but trended downward in all three biochar amendments. Additionally, this reduction of NO_3-N had a linear relationship with increasing biochar application rate ($r^2 = 0.85$; $P < 0.0001$).

While a majority of soil characteristics saw temporal fluctuations within treatment, overall percentages between months 1 and 6 of soil nitrogen (% N) and carbon (% C) remained constant. Instead, % N and % C were primarily affected by rate of biochar addition. An increase in % N was seen with the 10% biochar amendment rate. For % C, increases were seen with 1%, 2%, and 10% biochar amendment rates.

3.2. Abundances of microbial genes involved in nitrogen cycling

Measurement of the 16S rRNA gene revealed an increase in gene abundance that coincided with biochar amendment rate (Fig. 1). A 6 month average of soils receiving biochar revealed 44%, 86%, and 136% increases of microbial 16S rRNA gene abundance over control soils (0% biochar addition) for 1%, 2%, and 10% biochar amendment

Table 4
Soil characteristics at months 1 and 6 following biochar addition.

	0% Biochar	1% Biochar	2% Biochar	10% Biochar
pH				
Month 1	8.7 (0.1)[†] a[‡]	8.6 (0.1) b	8.5 (0.1) b	8.4 (0.1) c
Month 6	7.8 (0.0) a	7.9 (0.1) a	7.8 (0.1) a	7.8 (0.1) a
	0.0001[§]	0.003	<0.0001	0.005
EC (dS m⁻¹)				
Month 1	0.15 (0.01) a	0.24 (0.05) b	0.26 (0.05) b	0.23 (0.03) b
Month 6	1.04 (0.03) a	0.83 (0.09) b	0.67 (0.02) c	0.50 (0.08) d
	<0.0001	0.003	0.0008	0.01
NH$_4$-N (mg kg⁻¹)				
Month 1	1.4 (0.2) a	1.2 (0.2) ab	0.8 (0.1) b	0.9 (0.5) b
Month 6	ND[¶] (0.0) a	ND (0.0) a	ND (0.0) a	ND (0.0) a
	0.0004	0.001	0.002	0.05
NO$_3$-N (mg kg⁻¹)				
Month 1	93.1 (7.4) ab	111.5 (36.5) a	73.2 (5.5) b	8.0 (2.1) c
Month 6	113.0 (14.4) a	73.5 (12.5) b	59.2 (5.1) b	0.7 (0.2) c
	0.04	ns	ns	0.01
% N				
Month 1	0.09 (0.00) a	0.09 (0.01) a	0.10 (0.00) a	0.13 (0.00) b
Month 6	0.09 (0.01) a	0.10 (0.00) a	0.10 (0.00) a	0.14 (0.01) b
	ns	ns	ns	ns
% C				
Month 1	2.94 (0.06) a	3.44 (0.03) b	3.78 (0.09) c	7.53 (0.14) d
Month 6	3.04 (0.04) a	3.51 (0.07) ab	3.98 (0.03) b	7.91 (0.81) c
	ns	ns	0.03	ns

[†] Mean, with standard deviation in parentheses ($n = 4$).
[‡] Lowercase letters denote significant differences between biochar treatments ($P < 0.05$, Duncan's multiple range test).
[§] Values denote statistical significance (P) between months (ns = not significant).
[¶] ND = not detected.

soils, respectively. After 1 month, 16S rRNA gene abundances were already significantly elevated in the 10% biochar amended soils, and remained so throughout the course of the study. The 1% and 2% biochar amended soils were significantly greater than the control soil after 1 month, however did not significantly separate from each other until month 2 of the study. The degree of separation between these two amended soil treatments continued to increase until at month 6 there was 48.8% more 16S rRNA gene copies per gram of soil in the 2% biochar amended soils (5.12×10^9) as compared to the 1% biochar amended soils (3.44×10^9).

Gene abundance of *nifH* followed a pattern very similar to the 16S rRNA gene. Analysis after 1 month of incubation revealed significant increases of *nifH* gene abundances for all biochar amended soils as compared to the control (Fig. 2A). Additionally, 10%
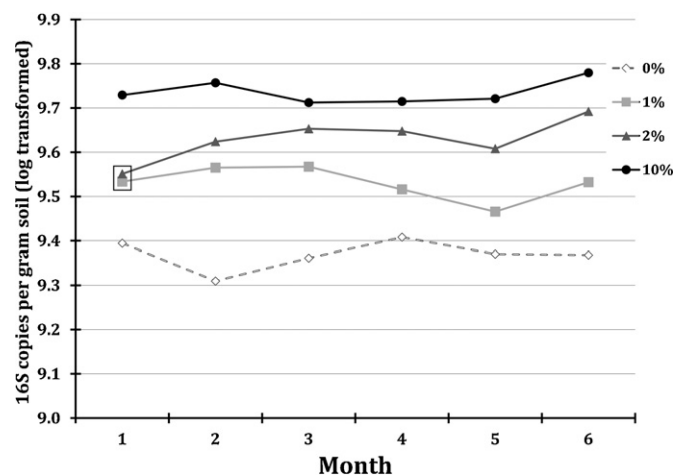

Fig. 1. Copies per gram of soil (log$_{10}$ transformed) of the bacterial 16S rRNA gene measured in soils incubated with switchgrass biochar. Biochar was added to soil on a w/w basis, and abundances were measured by quantitative polymerase chain reaction. Statistically similar measurements ($P > 0.05$) are boxed together.

biochar amended soils showed a significant increase from the other treatments. Like the 16S rRNA gene, the 1% and 2% biochar amended soils did not demonstrate a statistically significant difference until month 2 of the study. Overall, biochar amendment had a greater impact on *nifH* gene abundances than it did for the 16S rRNA gene, with 6 month average increases of 88%, 231% and 511% for 1%, 2%, and 10% biochar amendment, respectively. This disparity between treatments reached its peak at month 6, where 10% biochar amended soil had 793% more *nifH* gene copies than the control soil.

Examination of *amoA* gene abundances in response to biochar amendment shows a very different picture as compared to *nifH*. With the exception of the 2% biochar amended treatment, all other soil treatments demonstrated considerable fluctuation in *amoA* gene abundances (Fig. 2B). While the maximal fluctuation between gene abundances in 2% biochar amendment was 83% (between months 2 and 5), gene abundance fluctuated 653% (between months 2 and 6) for the control soil, 400% (between months 2 and 5) for 1% biochar amended soil, and 203% (between months 2 and 4) for the 10% biochar amended soil. The second month of the study saw gene abundances at their highest levels for all treatments before decreasing; only the 2% and 10% biochar amended soils approached their month 2 gene abundance levels by the end of the study. Six month averages for *amoA* copies per gram of soil were 1.00×10^7, 1.13×10^7, 1.58×10^7, and 1.40×10^7 for control, 1%, 2%, and 10% biochar-amendment, respectively.

Measurement of the genes involved in nitrite reduction revealed patterns more in line with the 16S rRNA and *nifH* genes, the exception being at month 1 where *nirS* gene abundances were significantly greater in the 1% biochar-amended soil than the 2% amended soil (Fig. 2C). For *nirS*, by the third month, the 1% and 2% biochar-amended soils remained significantly different, while all treatments achieved a significant difference from each other by the end of the study. At month 6, as compared to the control soil, 1%, 2% and 10% biochar-amended soils had 74%, 231%, and 475% more *nirS* gene copies per gram of soil. Gene abundances of *nirK* were roughly an order of magnitude lower than those of *nirS* (Fig. 2D). By the fourth month, all treatments were significantly different from each other, and by month 6 soils amended with 1%, 2%, and 10% biochar had gene copy increases of 152%, 336%, and 546% respectively, when compared to the control soil.

Similar to the *nirS* gene, *nosZ* gene abundances in 1% and 2% biochar-amended soils were flipped, though for the case of *nosZ*, this occurred over the first 5 months of the study as opposed to only the first month for *nirS* (Fig. 2E). By month 6 however, *nosZ* gene abundances were 24% higher in the 2% biochar-amended soil, as opposed to the 1% amended soil. Additionally, the 10% biochar-amended soil gene abundances remained higher than all other treatments throughout the course of the study – a pattern evident in all genes examined with the exception of *amoA* – with gene abundances (6 month average) 182%, 59%, and 83% greater than control, 1%, and 2% amended soils respectively.

3.3. Relative abundance of microbial nitrogen cycling genes

Relative abundance is presented as a percentage of the total microbial population that contains, in the case of this study, each of five N-cycling genes. This measure is useful as it allows for the assessment of a particular portion of the population's contribution to the biochemical function of the microbial community as a whole (Gordon and Giovannoni, 1996). Mean relative abundance values for the five N-cycling genes examined in this study are shown in Fig. 3, and month to month relative abundance values for each gene can be found in the Supplementary Material. For *nifH*, relative abundances increased with biochar amendment; 10% biochar-amended
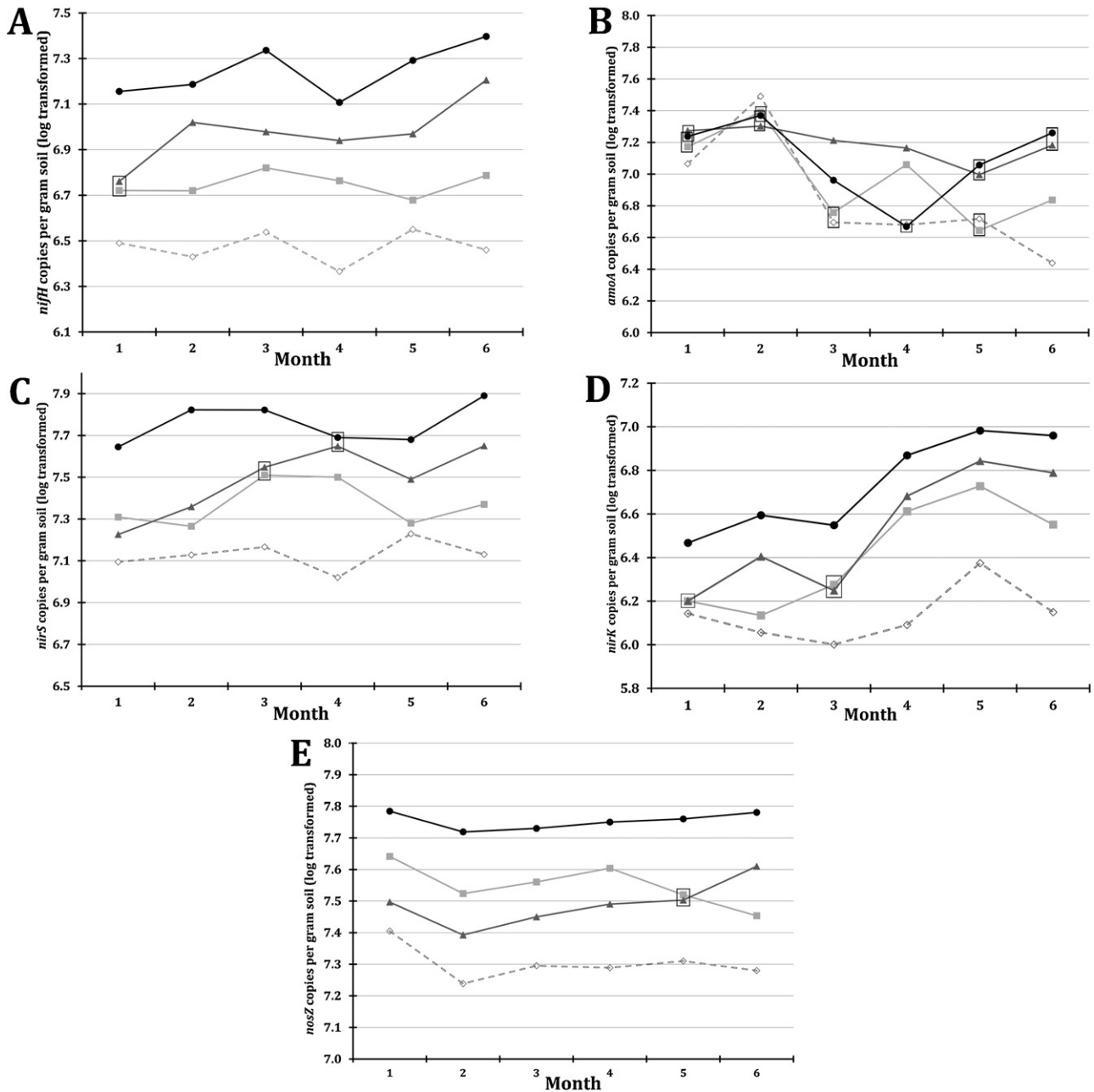
Fig. 2. Copies per gram of soil ($\log_{10}$ transformed) of the following genes: (A) *nifH*, involved in nitrogen fixation; (B) *amoA*, involved in ammonia oxidation; (C and D) *nirS* and *nirK* respectively, involved in the reduction of nitrate to nitric oxide; and (E) *nosZ*, involved in the reduction of nitrous oxide to dinitrogen. Genes were measured in soils incubated with switchgrass biochar. Biochar was added to soil on a w/w basis (0% ◇; 1% ■; 2% ▲; and 10% ●), and abundances were measured by quantitative polymerase chain reaction. Statistically similar measurements ($P > 0.05$) are boxed together.

soils displayed an almost three-fold increase over control soil, with 0.33% (as opposed to 0.12% for the control) of the total microbial population capable of fixing nitrogen. There was no statistical difference in relative abundances of *amoA*, although the percentage of the population capable of oxidizing NH_4 decreased by 44% (0.48% to 0.27%) between the control and 10% biochar amendment. Relative abundances for *nirS* trended upwards while the 10% biochar-amended soil grouped separately from the remainder of the treatments with 1.05% of the total soil microbial population carrying the gene responsible for reducing NO_2 to NO. Relative abundances of *nirK* showed no significant difference across treatments. For *nosZ*, both the 1% and 10% biochar-amended soils had 1.09% of the microbial population carrying the gene responsible for converting the greenhouse gas N_2O to N_2 (Fig. 3). When looking at

the relative abundances across months, while relative abundances trend in a manner similar to the gene abundance values, there is considerable more overlap between treatments (Supplementary Material). It should be noted that by month 6 however, the control soils had the lowest relative abundance values, while the 10% biochar-amended soils had the highest relative abundance values, in four of the five genes studied (Supplementary Material).

3.4. Correlations of gene abundances to soil characteristics

Correlation coefficients of gene and relative abundances with soil characteristics can be found in Table 5. Gene abundances of the 16S rRNA, *nifH*, *nirK*, *nirS* and *nosZ* genes all demonstrated a positive relationship with % N and % C, and a negative relationship
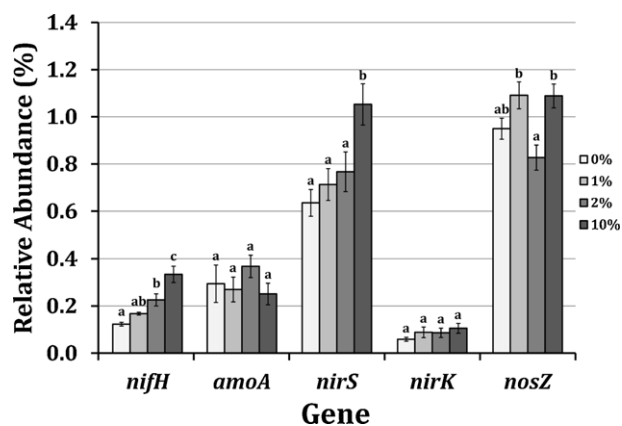
Fig. 3. Relative abundances of the five nitrogen cycling genes examined in this study. Means and standard errors of relative abundances reported for each biochar amendment over the course of 6 months. Groupings for each gene were calculated by Duncan's multiple range test. Different letters correlate to statistically distinct groups ($P < 0.05$).

with NO_3-N. With the exception of *nirK* and *nosZ*, these statistically significant relationships remained when comparing relative abundances to soil characteristics. Gene abundances of *nirK*, and relative abundances of both *nirK* and *nirS* correlated negatively with pH. Gene and relative abundances of *nosZ* correlated negatively with EC. The *amoA* gene did not significantly correlate with any soil characteristics, nor did NH_4-N with any of the genes targeted in this study.

4. Discussion

Analysis of the data revealed gene to gene variation in the patterns of the N-cycling genes monthly abundances as measured in this study. Irrespective of these variations and with the exception of *amoA*, by the first month and continuing throughout the study, the genes analyzed had significantly higher abundances in the biochar-amended soils as compared to the control treatment. Additionally, with *amoA* serving again as the lone exception, at month 6 these differences were statistically significant not only from the control but from each other, indicating that each treatment had a substantial effect on microbial gene abundances. Furthermore, these increased gene abundances coincided with the rate of biochar addition; soil receiving 10% biochar had the greatest increase, followed by 2%, and finally 1% biochar addition.

Table 5
Correlation of gene copy number and relative abundances to soil characteristics.

Gene	pH	EC	NH_4-N	NO_3-N	% N	% C
16S	ns	ns	ns	−0.89[****]	0.73[****]	0.78[****]
nifH	ns	ns	ns	−0.91[****]	0.78[****]	0.83[****]
amoA	ns	ns	ns	ns	ns	ns
nirS	ns	ns	ns	−0.88[****]	0.76[****]	0.81[****]
nirK	−0.42[*]	ns	ns	−0.71[****]	0.56[****]	0.64[***]
nosZ	ns	−0.55[**]	ns	−0.85[****]	0.77[****]	0.83[****]
Relative abundance						
nifH	ns	ns	ns	−0.85[****]	0.80[****]	0.82[****]
amoA	ns	ns	ns	ns	ns	Ns
nirS	−0.40[*]	ns	ns	−0.69[***]	0.68[***]	0.69[***]
nirK	−0.45[*]	ns	ns	ns	ns	Ns
nosZ	ns	−0.46[*]	ns	ns	ns	Ns

Statistical significance (*P*): ns (not significant).
[*] Statistical significance (*P*): 0.05.
[**] Statistical significance (*P*): 0.01.
[***] Statistical significance (*P*): 0.001.
[****] Statistical significance (*P*): <0.0001.

Correlation of gene abundances with soil characteristics revealed a number of relationships. The 16S rRNA gene, along with *nifH*, *nirS*, *nirK*, and *nosZ* all demonstrated a positive relationship with % N and % C and a negative relationship with NO_3-N. Correlation between microbial biomass and nitrogen availability has been documented previously, and indicates the importance of N in microbial metabolism (Kaiser et al., 1992). Furthermore, Strong et al. (1999) discuss the role of the soil matrix in N mineralization, hypothesizing that soil wetting cycles may result in the movement of N into microbial-laden pores. Concomitantly, biochar has been reported to increase moisture holding capacity (Chen et al., 2010; Glaser et al., 2002; Novak et al., 2012), which likewise held true for this study (data not shown). This increase in water storage, along with movement of N into areas of high microbial activity, may account for the strong correlation between microbial abundance and % N in this study. This hypothesis is seemingly further advanced by the negative relationships of the 16S rRNA gene, as well as *nifH*, *nirS*, *nirK*, and *nosZ* gene abundances to NO_3-N which, given the low levels of NH_4-N available throughout the course of this study, seem to indicate that, in biochar-amended soils, NO_3-N utilization would have been more pronounced (Burger and Jackson, 2003). Similarly, Lentz and Ippolito (2012) found a decrease in corn silage total N concentration and uptake, as compared to a control, when 0.5% biochar was applied to a Portneuf surface soil. The authors speculated that the reduction in plant N was related to an increase in microbial population growth and subsequently immobilization of N.

The positive relationship of the 16S rRNA gene to % C can be attributed to microbial heterotrophic metabolism (Zak et al., 1990). A similar relationship between *nifH*, *nirS*, *nirK*, and *nosZ* and % C can also be explained given the heterotrophic nature of the organisms which carry these genes (Knowles, 1982). Additionally, when factoring in the relationships with % N and NO_3-N, these results suggest that the addition of biochar increased microbial activity and growth. Increased gene abundances in biochar amended soils by the first month of this study are consistent with the results of Smith et al. (2010) that demonstrated a labile C pool rapidly consumed by microorganisms in the days immediately after biochar addition. These results are further supported by Pietikainen et al. (2000), who saw higher microbial growth rates in charcoal layers. Likewise, in a study designed to examine the effects of wildfire-produced charcoal on soil ecosystems, Kolb et al. (2009) demonstrated increasing microbial biomass and activity across several soil types. These increases corresponded to increasing biochar application rates, and occurred in all four soils that were examined. Biochar application rates were similar in the Kolb et al. study to those undertaken in our experiments, with a top application rate of 10% (w:w). We recognize that the 10% application rate is high, at approximately 100 tons ac^{-1}, however as we rise to the challenge of attaining sustainable rates of agricultural production to meet the required food resources for the world (Clark, 2009), it may require drastic measures to rehabilitate severely degraded soils. Therefore, while most instances of a 10% biochar application rate may be unwarranted, we would warn against completely dismissing such an approach.

The results of Kolb et al., combined with the data reported in this study, suggest that the effects of biochar amendment on microbial populations can be reasonably anticipated. It should also be recognized that in our study, these increased abundances persisted throughout the course of the 6 month study, indicating that these increased microbial populations are able to be supported by soils receiving biochar-amendment. Likewise, the responses of *nirS*- and *nosZ*-carrying organisms, which in the first month saw higher gene abundances in the 1% biochar amended soils than the 2%, rules out the possibility that these microorganisms originated in the biochar itself. Detailing the rapid growth of these populations

immediately following the addition of biochar will be the subject of future studies.

Such rapid growth however does not appear to have been mimicked by the *amoA*-carrying, chemolithoautotrophic, ammonia oxidizing bacterial (AOB) populations which rely predominantly on the oxidation of NH_4-N for energy production and growth (Arp et al., 2007). Despite the reliance of AOB populations on NH_4-N, there was no correlation between *amoA* and NH_4-N which indicates that additional factors, currently unknown, must have played a role in regulating *amoA* abundances. Abundances of *amoA* were seen to decrease after the second month, which is similar to results published by Anderson et al. (2011) that demonstrated decreases in the abundance of *Nitrosomonadaceae* in soils treated with ryegrass-derived biochar. It should be noted that control soils exhibited a similar pattern, indicating that the study conditions may have factored in the exhibited patterns of *amoA*. However by 6 months, while *amoA* abundances in the control soil continued to drop, *amoA* abundances in biochar-amended treatments began to increase; this may be indicative of a recovery in the NH_4-N oxidizing populations.

Interestingly, while gene abundances increased dramatically with biochar amendment, relative abundances had modest increases. In fact, only *nifH* and *nirS*, over the course of the 6 month study period, showed significant increases in relative abundance between control soil and the highest rate of biochar addition. However by month 6, four out of the five genes examined, with the exception of *nosZ*, showed trends where all three biochar treatments had higher relative abundances over the control. Given the month to month variability in these measurements however, further study needs to be conducted in order to determine the longer term trends of biochar amendment to microbial community structure and function. Such variability has been demonstrated in other studies that have looked at the effects of biochar application on soil microbial characteristics (Lehmann et al., 2011). In a field study by Castaldi et al. (2011), while they reported no negative impacts on amending soils with a wood-derived biochar, increases in microbial activity were only transient. If this proved to be the case in the longer-term for the biochar treatment examined in this study, physico-chemical benefits in the form of increased carbon storage, aggregate formation, and water holding capacity could still be realized (Novak et al., 2012).

Abundance measures, gene and relative, are worthy of consideration when looking at the soil biochemistry as they address two different issues. Gene abundance can be regarded as the total microbial biomass that contributes to a specific process (i.e., nitrogen fixation, nitrification, or denitrification), while relative abundance is the proportion of the microbial community that contributes to this process. In addition, these two values need not be mutually connected; as long as the abundance of a gene decreases or increases at a rate similar to the losses or gains in abundance that the microbial community experiences as a whole, that gene's relative abundance remains the same.

In the present study, the increased gene abundances indicate that – assuming conditions ideal for gene expression and subsequent enzymatic function – the amount of N cycled by these biochar-amended soils would also be increased. Furthermore, the consistency in the relative abundances of *amoA* and *nosZ* between treatments, indicate that the N cycled through these two steps would be proportionally the same. Increases in the relative abundances of *nifH* indicate that the use of biochar could have a beneficial impact on agricultural soil management by increasing the proportion of fixed nitrogen in those soils available for plants. When considering denitrification, the increase in relative abundance of *nirS* indicates increased nitrate reduction – once again assuming ideal conditions – in soils amended with 10% biochar, which could result in the loss of plant available N. Care should therefore be taken when considering such high rates of biochar application to land used for crop production, so that any shifts in microbial populations caused by biochar do not negatively impact plant growth. Statistically similar relative abundances of *nosZ* indicate that percentages of the greenhouse gas (GHG) nitrous oxide emanating from these soils should remain level, regardless of the rate of biochar amendment. Whereas reduced *nosZ* levels could have resulted in higher levels of GHG emissions. It should be noted however that these proportions do not take into account the environmental factors which regulate the expression of each gene, and may be controlled by biochar addition. Further study would need to be conducted to elucidate the role that biochar plays in controlling biogeochemical fluxes under various field conditions. Given the multitude of conditions that can be used to create biochar, all of which can result in changes to its physicochemical properties, these studies would ideally be tailored to address specific land management issues.

In order to study the efficacy of biochar in the management of erosion-degraded soils, we utilized an exposed B horizon soil; such exposures are a common phenomenon in the areas of Idaho from which this soil originated. The A horizon of soils in this area of Idaho are typically no more than 30 cm thick, and with a management history which usually involves furrow irrigation, are prone to erosion. The B horizon is highly calcareous ($\sim$25% free lime content; Robbins et al., 1997) with an alkaline pH; in our study the pH in the control started at 8.7. Our premise therefore was to apply a low pH biochar with the understanding that this would help reduce soil pH, reduce volatilization of ammonia, and increase micronutrient availability. Based on these preferred outcomes, we utilized switchgrass as the biochar feedstock. When created under conditions outlined in Table 1, this biochar is known for its low pH characteristics, as well as for its enhanced water storage capacity (Novak et al., 2012) and excellent biomass qualities (Monti et al., 2008).

In fact, while moisture storage and pH happen to be two of the most common soil factors targeted for alteration by biochar amendment (Clough and Condron, 2010; Novak et al., 2009a, 2012), they also happen to be two of the greatest factors for driving microbial diversity (Griffiths et al., 2011). Therefore, it is suggested that great care be taken when selecting a biochar for use as an amendment such that its use will improve not only the soils chemical and physical properties, but its biological properties as well. These biological properties, reflected in part by soil bacterial community structure and activity, will play a significant role in nutrient cycling processes which contribute to acceptable plant growth.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2013.01.006.

References

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

Arp, D.J., Chain, P.S.G., Klotz, M.G., 2007. The impact of genome analyses on our understanding of ammonia-oxidizing bacteria. Annu. Rev. Microbiol. 61, 503–528.

Atkinson, C.J., Fitzgerald, J.D., Hipps, N.A., 2010. Potential mechanisms for achieving agricultural benefits from biochar application to temperate soils: a review. Plant Soil 337, 1–18.

Ball, P.N., MacKenzie, M.D., DeLuca, T.H., Montana, W.E.H., 2010. Wildfire and charcoal enhance nitrification and ammonium-oxidizing bacterial abundance in dry montane forest soils. J. Environ. Qual. 39, 1243.

Brunauer, S., Emmett, P.H., Teller, E., 1938. Adsorption of gases in multimolecular layers. J. Am. Chem. Soc. 60, 309–319.

Bruun, S., Jensen, E.S., Jensen, L.S., 2008. Microbial mineralization and assimilation of black carbon: dependency on degree of thermal alteration. Org. Geochem. 39, 839–845.

Burger, M., Jackson, L.E., 2003. Microbial immobilization of ammonium and nitrate in relation to ammonification and nitrification rates in organic and conventional cropping systems. Soil Biol. Biochem. 35, 29–36.

Cantrell, K.B., Martin, J.H., 2012. Stochastic state–space temperature regulation of biochar production. Part ii: application to manure processing via pyrolysis. J. Sci. Food Agric. 92, 490–495.

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

Chain, P., Lamerdin, J., Larimer, F., Regala, W., Lao, V., Land, M., Hauser, L., Hooper, A., Klotz, M., Norton, J., Sayavedra-Soto, L., Arciero, D., Hommes, N., Whittaker, M., Arp, D., 2003. Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. J. Bacteriol. 185, 2759–2773.

Chen, Y., Shinogi, Y., Taira, M., 2010. Influence of biochar use on sugarcane growth, soil parameters, and groundwater quality. Aust. J. Soil Res. 48, 526–530.

Clark, M., 2009. Sustainable agriculture: feeding the world. Available at http://www.csiro.au/Portals/Multimedia/On-the-record/Sustainable-Agriculture-Feeding-the-World.aspx

Clough, T.J., Bertram, J.E., Ray, J.L., Condron, L.M., O'Callaghan, M., Sherlock, R.R., Wells, N.S., 2010. Unweathered wood biochar impact on nitrous oxide emissions from a bovine-urine-amended pasture soil. Soil Sci. Soc. Am. J. 74, 852–860.

Clough, T.J., Condron, L.M., 2010. Biochar and the nitrogen cycle: introduction. J. Environ. Qual. 39, 1218.

Ducey, T.F., Shriner, A.D., Hunt, P.G., 2011. Nitrification and denitrification gene abundances in swine wastewater anaerobic lagoons. J. Environ. Qual. 40, 610–619.

Glaser, B., Lehmann, J., Zech, W., 2002. Ameliorating physical and chemical properties of highly weathered soils in the tropics with charcoal – a review. Biol. Fert. Soils 35, 219–230.

Gordon, D.A., Giovannoni, S.J., 1996. Detection of stratified microbial populations related to *Chlorobium* and *Fibrobacter* species in the Atlantic and Pacific oceans. Appl. Environ. Microbiol. 62, 1171–1177.

Griffiths, R.I., Thomson, B.C., James, P., Bell, T., Bailey, M., Whiteley, A.S., 2011. The bacterial biogeography of British soils. Environ. Microbiol. 13, 1642–1654.

Hall, J.R., Mitchell, K.R., Jackson-Weaver, O., Kooser, A.S., Cron, B.R., Crossey, L.J., Takacs-Vesbach, C.D., 2008. Molecular characterization of the diversity and distribution of a thermal spring microbial community by using rRNA and metabolic genes. Appl. Environ. Microbiol. 74, 4910–4922.

Hou, Y.B., Zhang, H., Miranda, L., Lin, S.J., 2010. Serious overestimation in quantitative PCR by circular (supercoiled) plasmid standard: microalgal *pcna* as the model gene. PLoS ONE 5, 1–7.

Ippolito, J.A., Laird, D.A., Busscher, W.J., 2012. Environmental benefits of biochar. J. Environ. Qual. 41, 967–972.

Kaiser, E.A., Mueller, T., Joergensen, R.G., Insam, H., Heinemeyer, O., 1992. Evaluation of methods to estimate the soil microbial biomass and the relationship with soil texture and organic-matter. Soil Biol. Biochem. 24, 675–683.

Kandeler, E., Deiglmayr, K., Tscherko, D., Bru, D., Philippot, L., 2006. Abundance of *narG*, *nirS nirK*, and *nosZ* genes of denitrifying bacteria during primary succession of a glacier foreland. Appl. Environ. Microbiol. 72, 5957–5962.

Klappenbach, J.A., Saxman, P.R., Cole, J.R., Schmidt, T.M., 2001. Rrndb: the ribosomal rna operon copy number database. Nucleic Acids Res. 29, 181–184.

Knowles, R., 1982. Denitrification. Microbiol. Rev. 46, 43–70.

Kolb, S.E., Fermanich, K.J., Dornbush, M.E., 2009. Effect of charcoal quantity on microbial biomass and activity in temperate soils. Soil Sci. Soc. Am. J. 73, 1173–1181.

Lehmann, J., Joseph, S., 2009. Biochar for Environmental Management: Science and Technology, 1st ed. Earthscan, London, UK, p. 416.

Lehmann, J., Rillig, M.C., Thies, J., Masiello, C.A., Hockaday, W.C., Crowley, D., 2011. Biochar effects on soil biota – a review. Soil Biol. Biochem. 43, 1812–1836.

Lentz, R.D., Ippolito, J.A., 2012. Biochar and manure affect calcareous soil and corn silage nutrient concentrations and uptake. J. Environ. Qual. 41, 1033–1043.

Lentz, R.D., Lehrsch, G.A., Brown, B., Johnson-Maynard, J., Leytem, A.B., 2011. Dairy manure nitrogen availability in eroded and noneroded soil for sugarbeet followed by small grains. Agron. J. 103, 628–643.

Monti, A., Di Virgilio, N., Venturi, G., 2008. Mineral composition and ash content of six major energy crops. Biomass Bioenerg. 32, 216–223.

Mulvaney, R.L., 1996. Nitrogen – inorganic forms. In: Sparks, D.L. (Ed.), Methods of Soil Analysis, Part 3 – Chemical Methods. Soil Science Society of America, Madison, WI, pp. 1123–1184.

Nelson, D.W., Sommers, L.E., 1996. Total carbon, organic carbon, and organic matter. In: Sparks, D.L. (Ed.), Methods of Soil Analysis, Part 3 – Chemical Methods. Soil Science Society of America, Madison, WI, pp. 975–977.

Novak, J.M., Busscher, W.J., Laird, D.L., Ahmedna, M., Watts, D.W., Niandou, M.A.S., 2009a. Impact of biochar amendment on fertility of a southeastern coastal plain soil. Soil Sci. 174, 105–112.

Novak, J.M., Busscher, W.J., Watts, D.W., Amonette, J.E., Ippolito, J.A., Lima, I.M., Gaskin, J., Das, K.C., Steiner, C., Ahmedna, M., Rehrah, D., Schomberg, H., 2012. Biochars impact on soil-moisture storage in an ultisol and two aridisols. Soil Sci. 177, 310–320.

Novak, J.M., Lima, I., Xing, B., Gaskin, J.W., Steiner, C., Das, K., Ahmedna, M., Rehrah, D., Watts, D.W., Busscher, W.J., 2009b. Characterization of designer biochar produced at different temperatures and their effects on a loamy sand. Ann. Environ. Sci. 3, 2.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time rt-PCR. Nucleic Acids Res. 29, e45.

Pietikainen, J., Kiikkila, O., Fritze, H., 2000. Charcoal as a habitat for microbes and its effect on the microbial community of the underlying humus. Oikos 89, 231–242.

Rhoades, J.D., 1996. Electrical conductivity and total dissolved solids. In: Sparks, D.L. (Ed.), Methods of Soil Analysis, Part 3 – Chemical Methods. Soil Science Society of America, Madison, WI, pp. 417–435.

Robbins, C.W., Freeborn, L.L., Westermann, D.T., 2000. Organic phosphorus source effects on calcareous soil phosphorus and organic carbon. J. Environ. Qual. 29, 973–978.

Robbins, C.W., Mackey, B.E., Freeborn, L.L., 1997. Improving exposed subsoils with fertilizers and crop rotations. Soil Sci. Soc. Am. J. 61, 1221–1225.

Rondon, M.A., Lehmann, J., Ramirez, J., Hurtado, M., 2007. Biological nitrogen fixation by common beans (*Phaseolus vulgaris* l.) increases with bio-char additions. Biol. Fert. Soils 43, 699–708.

Smith, J.L., Collins, H.P., Bailey, V.L., 2010. The effect of young biochar on soil respiration. Soil Biol. Biochem. 42, 2345–2347.

Spokas, K.A., Koskinen, W.C., Baker, J.M., Reicosky, D.C., 2009. Impacts of woodchip biochar additions on greenhouse gas production and sorption/degradation of two herbicides in a Minnesota soil. Chemosphere 77, 574–581.

Spokas, K.A., Reicosky, D.C., 2009. Impacts of sixteen different biochars on soil greenhouse gas production. Ann. Environ. Sci. 3, 179–193.

Strong, D.T., Sale, P.W.G., Helyar, K.R., 1999. The influence of the soil matrix on nitrogen mineralisation and nitrification – iii. Predictive utility of traditional variables and process location within the pore system. Aust. J. Soil Res. 37, 137–149.

Tarkalson, D.D., Jolley, V.D., Robbins, C.W., Terry, R.E., 1998. Mycorrhizal colonization and nutrient uptake of dry bean in manure and compost manure treated subsoil and untreated topsoil and subsoil. J. Plant Nutr. 21, 1867–1878.

USDA-NRCS, 2011. Soil extent mapping tool. Available at http://www.cei.psu.edu/soiltool/semtool.html

Zak, D.R., Grigal, D.F., Gleeson, S., Tilman, D., 1990. Carbon and nitrogen cycling during old-field succession – constraints on plant and microbial biomass. Biogeochemistry 11, 111–129.

Zavalloni, C., Alberti, G., Biasiol, S., Delle Vedove, G., Fornasier, F., Liu, J., Peressotti, A., 2011. Microbial mineralization of biochar and wheat straw mixture in soil: a short-term study. Appl. Soil Ecol. 50, 45–51.