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Biochar Enhances Nitrous Oxide Reduction in Acidic but Not in Near-Neutral pH Soil

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Abstract: We quantified nitrous oxide (N_2O) fluxes and total denitrification ($N_2O + N_2$) in an acidic (Ferralsol) and a near-neutral pH soil (Cambisol) to determine whether biochar's alkalinization effect could be the mechanism inducing potential reductions in N_2O fluxes. In Ferralsol, decreases in N_2O emissions and in the N_2O to $N_2O + N_2$ ratio were observed in both biochar and lime treatments. In Cambisol, neither biochar nor lime decreased N_2O emissions, despite significantly increasing soil pH. The abundance and community structure of *nosZ* gene-bearing microorganisms indicated that gene abundances did not explain biochar effects, but a higher diversity of *nosZ* gene-bearing microorganisms correlated to lower total denitrification. Overall, our results suggest that biochar's potential to decrease N_2O emissions, through soil alkalinization, may be more effective in acidic soils.

Keywords: amendments; denitrification; qPCR; T-RFLP; Ferralsol; Cambisol

1. Introduction

Biochar is generally an alkaline charcoal-like material that can improve soil fertility [1] and potentially decrease the emissions of nitrous oxide (N_2O), a very potent greenhouse gas [2]. The latter is possibly related to soil alkalinization, as the activity of N_2O-reductase, the enzyme reducing N_2O into dinitrogen (N_2), is enhanced with soil pH increases [3,4]. Yet, some studies have observed soil pH increases without a consequent decrease in N_2O emissions [5,6]. Hence, this study aims to: (i) elucidate whether decreases in N_2O emissions following alkalinization are related to initial soil pH and (ii) to determine whether N_2O decreases are followed by changes in the abundance or composition of denitrifying bacteria. We hypothesize that in a soil with a low pH and buffer capacity (i.e., Ferralsol), the effect size of biochar on N_2O emissions will be larger than that in a near-neutral pH soil (i.e., Cambisol). Additionally, soil alkalinization in lime and biochar treatments will increase the abundance of *nosZ*-bearing bacteria and the reduction of N_2O to N_2.

2. Materials and Methods

To test our hypotheses, in a laboratory setting, a Swiss Stagnic Cambisol (pH_{H2O} 6.32, 3.0% total C) and a Brazilian Rhodic Ferralsol (pH_{H2O} 5.37, 2.0% total C) were submitted to three treatments: control (no amendments), biochar (10% *w/w*), and lime (3% *w/w* $CaCO_3$). The amount of $CaCO_3$ was based on the biochar's liming equivalency (30% $CaCO_3$ equivalent) [7]. Cambisol had a buffer capacity

of 120 mmol of H^+ or OH^- to change 1 pH unit/kg of soil, while Ferralsol had a buffer capacity of 25.54 mmol of H^+ or OH^- to change 1 pH unit/kg of soil. Biochar was produced from gasified walnut shells (900 °C, pH 9.7) [8]. Biochar was composed of 43% of particles larger than 2000 µm, 19% between 1000 and 2000 µm, 15% between 250 and 1000 µm, and 21% smaller than 250 µm. For pH stabilization, soil and amendments were incubated for 45 days at 24 °C maintaining soil moisture at 60% of water holding capacity. We measured denitrification potential according to [9], for which we prepared six jars (20 g soil each) per treatment and added a nutrient solution (300 µg glucose g^{-1} soil and 50 µg NO_3^- g^{-1} soil) reaching 60% of water holding capacity. Half of the jars received an acetylene (C_2H_2) enrichment (10% *v/v*) to inhibit N_2O reduction to N_2 allowing the estimation of total denitrification ($N_2O + N_2$), while the other half received a 10% *v/v* enrichment with argon and were used to quantify N_2O fluxes. We collected gas samples at 0, 3, 6, 9, and 12 h, and measured N_2O concentration using gas chromatography (Bruker Corporation, Karlsruhe, Germany). After the incubation, soil was collected for pH and microbial measurements. DNA was extracted using PowerLyzer Soil DNA Extraction kit, according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). Nitrite reductase (*nirK* and *nirS*) and N_2O-reductase (*nosZ*) gene abundances were quantified using real-time quantitative polymerase chain reaction (qPCR). For information about primers and PCR conditions see [5]. The diversity of the *nosZ* gene was assessed using the terminal restriction fragment length polymorphism (T-RFLP) method using the same conditions as in the qPCR, except that the forward primers were labeled with 6-carboxy-fluorescein (FAM). PCR products were digested by HhaI restriction enzyme (New England Biolabs, Inc., Ipswich, MA, USA) and fragments were analyzed by an ABI 3730XL sequencer (Applied Biosystems, Waltham, MA, USA) against an internal standard (GeneScanTM-500 LIZ®; Applied Biosystems, Waltham, MA, USA,). T-RF alignment was performed using the T-REX software [10] and analyzed for relative abundances of *nosZ* T-RFs and Shannon diversity index (H'). Analysis of variance was employed to determine the effects of amendments across both soil types with subsequent post hoc analyses (Tukey's honest significant differences test). Pearson correlation analysis was used to test the relationship between total denitrification and the *nosZ* T-RFs Shannon diversity index. All analyses were performed in R 3.2.1 [11].

3. Results

Over the incubation period (45 days), lime and biochar significantly increased soil pH compared to the unamended control (Table 1). In Cambisol, soil pH significantly increased from 6.32 in control to 7.99 and 8.05 in lime and biochar treatments, respectively. In Ferralsol, pH increased from 5.37 to 8.24 and 8.64 in lime and biochar treatments, respectively. Overall, total denitrification ($N_2O + N_2$) was two-fold larger in Cambisol than in Ferralsol (Figure 1A; $p = 0.003$). In both soil types, $N_2O + N_2$ was lower in biochar treatment than in unamended control and lime treatments. The production of N_2O was in general 4.8-fold larger in Cambisol than in Ferralsol (Figure 1B; $p < 0.0001$). In both soil types, N_2O production was lower in biochar than in unamended control (only significant for Ferralsol), while the lime treatment only decreased N_2O production in Ferralsol. The ratio of N_2O production over total denitrification ($N_2O/N_2O + N_2$) represents the proportion of N_2O that is converted into N_2. In Cambisol, none of the treatments altered $N_2O/N_2O + N_2$ ratios ($p > 0.05$). In Ferralsol, both lime and biochar decreased on average 93% of the $N_2O/N_2O + N_2$ ratio, compared to unamended control.

None of the treatments or soil types altered the abundance of *nirK* and *nirS* genes (Table 1). Abundance of *nosZ* was larger in Cambisol than in Ferralsol ($p = 0.007$), with no differences between the amendments within each soil type. The diversity of *nosZ*-bearing bacteria was represented by the Shannon H' index (Table 1, $p < 0.0001$). We also observed a negative correlation between total denitrification and the *nosZ* T-RFs Shannon diversity index (Pearson r = −0.69 $p = 0.0018$), with communities in Cambisol being less diverse than those in Ferralsol.

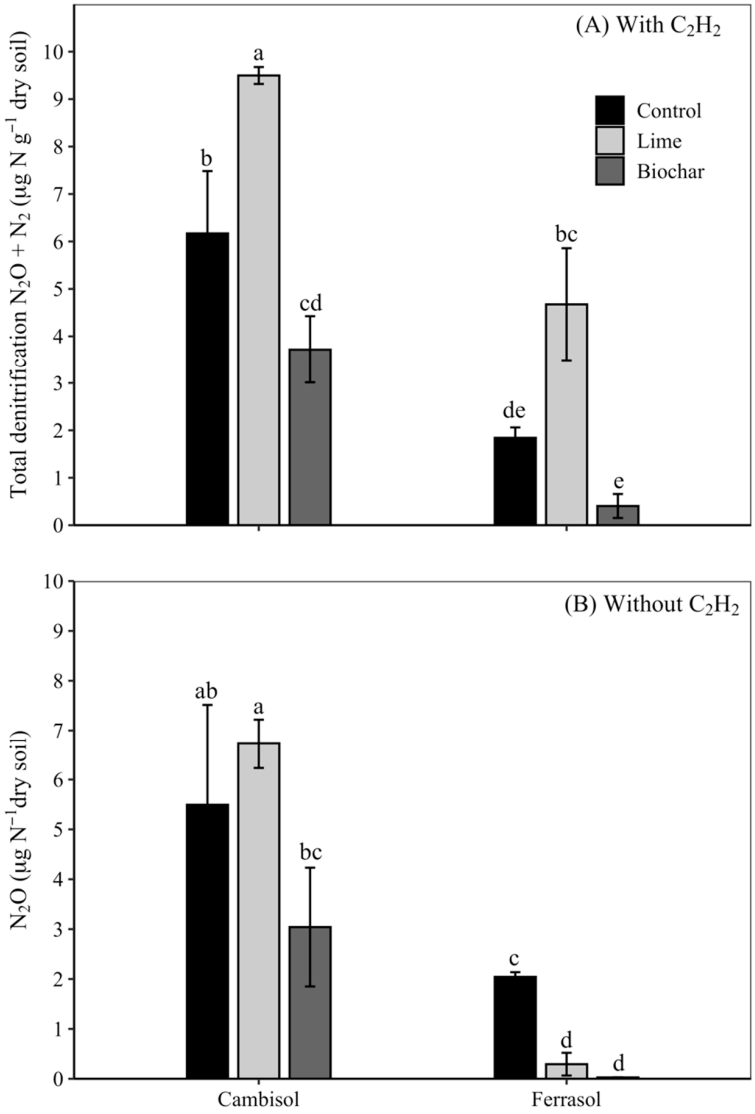

Figure 1. Total denitrification ($N_2O + N_2$) and cumulative nitrous oxide (N_2O) fluxes from a temperate Cambisol and a tropical Ferralsol after 12 h of incubation with (**A**) and without (**B**) acetylene (C_2H_2) enrichment. Error bars represent one standard error of the mean.

Table 1. Soil pH, proportion of N_2O to total denitrification ($N_2O/N_2O + N_2$), denitrifying gene abundances (*nirK, nirS*, and *nosZ*), and Shannon diversity index (H′) of *nosZ* terminal restriction fragment (T-RF) responses to biochar and lime amendments in a Cambisol and Ferralsol.

Soil	Amendment	Soil pH	$N_2O/N_2O + N_2$	*nirK*	*nirS*	*nosZ*	Shannon H′
				Log Gene Copies g^{-1} soil			
Cambisol	Control	6.32 c	0.88 a	7.79 a	6.89 a	7.15 ab	2.17 b
	Lime	7.99 b	0.71 a	7.99 a	7.10 a	6.93 abc	1.84 b
	Biochar	8.05 b	0.84 a	8.54 a	7.36 a	7.29 a	1.92 b
Ferralsol	Control	5.37 d	1.12 a	7.72 a	7.26 a	6.38 cd	2.58 a
	Lime	8.24 ab	0.06 b	8.11 a	7.22 a	6.27 cd	2.43 a
	Biochar	8.64 a	0.08 b	8.02 a	7.06 a	6.00 d	2.43 a

Means within a column followed by the same letter are not significantly different ($p > 0.05$).

4. Discussion

We expected that in biochar- and lime-amended soils, where pH is higher, N_2O emissions would decrease as a result of an enhanced proportion of N_2O that is completely denitrified to N_2 upon soil pH alkalinization. Despite increasing pH in both soils, this hypothesis was only confirmed in Ferralsol where N_2O emissions (Figure 1) and $N_2O/N_2O + N_2$ ratios (Table 1) in biochar and lime treatments were lower than those in the control. In Cambisol, lime and biochar amendments did not decrease N_2O emission or $N_2O/N_2O + N_2$ ratios. It is possible that pH increases in near-neutral pH soils do not further enhance N_2O-reductase activities. Accordingly, Thomsen et al. [12] studied denitrification across a range of pH (5.5–9.5) and showed that pH increases in the acidic range enhanced N_2O reduction to N_2 substantially more than pH increases in neutral or alkaline media. Soil aggregates may also explain the lack of biochar effects on N_2O emissions: Denitrifying activity has been hypothesized to occur mainly inside soil aggregates [13]; thus, denitrifiers would not be in direct contact with biochar located outside the aggregate assemblage. Given that Cambisol has 3% soil C content and higher cation exchange capacity than a weathered tropical soil, it is possible that it possesses larger proportion of soil aggregates than Ferralsol.

Biochar decreased total denitrification in both soils compared to the unamended control (Figure 1A). This effect was not observed in the lime treatment and, thus, does not seem to be related to soil alkalinization. The decreased total denitrification, without changing N_2O emissions in Cambisol, suggests that biochar may affect multiple steps of the denitrification pathway. For instance, biochar may decrease the availability of resources that support the first-steps of denitrification [14,15]. Labile C was suggested to adsorb to the biochar's surface [16–19], while microorganisms were suggested to immobilize N, which can be related to the ratio of available C and available N [2]. Based on our assessments, we could not identify the causes of a lower total denitrification; however, we suggest that future mechanistic studies investigate the interactions between biochar and the first segments of the denitrification pathway.

Denitrifying gene abundances did not predict biochar effects on total denitrification or N_2O emissions. However, a greater abundance of *nosZ* in Cambisol than in Ferralsol may have been related to higher pH levels in Cambisol than in Ferralsol. The significant differences between the two soil types on the *nosZ* T-RFs Shannon diversity index (Table 1), suggest that community structure, along with gene abundance, may have functional implications controlling total denitrification levels [20–22].

5. Conclusions

In conclusion, our findings confirm that biochar's alkalinization effect can promote a complete denitrification of N_2O, similar to that of lime treatment, but only in the acidic Ferralsol. Additionally, our results suggest that biochar can decrease N_2O production. In Cambisol, the effects of biochar were not as pronounced as those in Ferralsol, perhaps due to a higher native pH and potentially due to stronger aggregates shielding denitrifiers from biochar. Finally, our results suggest that larger total denitrification fluxes found in Cambisol may be related to a larger abundance and lower community diversity of *nosZ* gene-bearers, compared to that of the Ferralsol soil type.

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