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RESEARCH ARTICLE

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Biochar amendment suppresses N_2O emissions but has no impact on ^{15}N site preference in an anaerobic soil

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Rationale: Biochar amendments often decrease N_2O gas production from soil, but the mechanisms and magnitudes are still not well characterized since N_2O can be produced via several different microbial pathways. We evaluated the influence of biochar amendment on N_2O emissions and N_2O isotopic composition, including ^{15}N site preference (SP) under anaerobic conditions.

Methods: An agricultural soil was incubated with differing levels of biochar. Incubations were conducted under anaerobic conditions for 10 days with and without acetylene, which inhibits N_2O reduction to N_2. The N_2O concentrations were measured every 2 days, the SPs were determined after 5 days of incubation, and the inorganic nitrogen concentrations were measured after the incubation.

Results: The SP values with acetylene were consistent with N_2O production by bacterial denitrification and those without acetylene were consistent with bacterial denitrification that included N_2O reduction to N_2. There was no effect of biochar on N_2O production in the presence of acetylene between day 3 and day 10. However, in the absence of acetylene, soils incubated with 4% biochar produced less N_2O than soils with no biochar addition. Different amounts of biochar amendment did not change the SP values.

Conclusions: Our study used N_2O emission rates and SP values to understand biochar amendment mechanisms and demonstrated that biochar amendment reduces N_2O emissions by stimulating the last step of denitrification. It also suggested a possible shift in N_2O-reducing microbial taxa in 4% biochar samples.

1 | INTRODUCTION

Nitrous oxide (N_2O) is a greenhouse gas that has 298 times the global warming potential of carbon dioxide[1,2] and has increased from a pre-industrial level of 270 ppb to 330 ppb in recent years.[3] N_2O is also one of the most important stratospheric ozone-depleting compounds.[4,5] The main source of N_2O from terrestrial ecosystems is agricultural soils.[1,6] A variety of options for reducing N_2O production from agricultural soils are being explored and evaluated, but mitigation efforts remain hampered by an incomplete understanding of the microbial pathways that produce N_2O, as well as those that reduce N_2O to N_2. It is therefore essential to identify sources of N_2O production from agricultural soils and the underlying mechanisms driving variation in its flux to the atmosphere.

The primary microbial processes that produce N_2O in the soil environment are nitrification, nitrifier denitrification, and denitrification.[7-12] During nitrification, nitrifying bacteria can oxidize one of the intermediates, hydroxylamine, releasing N_2O as a byproduct. Nitrifier denitrification can occur under anaerobic conditions when ammonia oxidizers successively reduce nitrite (NO_2^-) to nitric oxide (NO), nitrous oxide (N_2O), and molecular nitrogen (N_2). Denitrification occurs under anaerobic conditions when heterotrophic denitrifying organisms (bacteria and fungi) sequentially reduce nitrate (NO_3^-) to NO_2^-, NO, N_2O, and then

finally N_2, where N_2O is produced as an intermediate or the final product. It is expected that most N_2O emitted from agricultural soils is produced through denitrification.[13] Other sources of N_2O formation include chemodenitrification[14-17] and other biotic processes such as archaeal nitrification and co-denitrification.[18,19] It is difficult to estimate N_2O fluxes from each potential microbial process since the pathways are complex and vary spatially and temporally in different soil types and under different environmental conditions, depending on soil nitrogen availability, pH, temperature, soil water content, oxygen concentration, organic matter content, and redox condition.[12,20]

A powerful tool to identify N_2O production pathway is ^{15}N site preference (SP) of N_2O.[12,21-28] The linear N_2O molecule exhibits non-random intramolecular distributions of ^{15}N (isotopomers) that have been related to microbial production pathways (see Toyoda et al[12] for currently understood mechanisms). The SP is the difference in relative abundances of ^{15}N in the central (α) and in the terminal (β) N atoms, quantified as $\delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$.[29,30] The SP values of N_2O produced by hydroxylamine oxidation by ammonia-oxidizing bacteria (nitrification) and fungal denitrification are ~33‰ (16–37‰[20,23,31-36]) whereas N_2O produced through denitrification and nitrifer denitrification yields SP values near 0‰.[20,34,35,37,38] Note that SP values are independent of the mineral or organic (precursors).[34]

While early SP results generated significant enthusiasm and several papers have been published pursuing SP as a tool for probing sources of N_2O production in soils, no international standards exist for SP and some questions have arisen about consistency across laboratories in the reported SP values. In 2014, an interlaboratory comparison of N_2O isotopomers was performed. Three gases (T, REF1, and REF2) were provided to 11 laboratories and the bulk stable N and O isotope ratios ($\delta^{15}N^{bulk}$ and $\delta^{18}O$ values, respectively) and SP values were compared.[39] The $\delta^{15}N^{bulk}$, $\delta^{18}O$, and SP values measured in some laboratories were quite different from values measured by Tokyo Institute of Technology (Tokyo, Japan) – source of the current *de facto* gas standards.

An amendment of biochar to soils generally reduces N_2O emission from soils.[40-47] However, increases in N_2O production also have been observed in some cases.[48-51] These discrepancies appear to be related to biochar types and other differences in experimental conditions, such as a shift in the availability of organic carbon, available soil NO_3^- concentration, soil moisture, soil pH, soil porosity, or microbial community composition,[13,41,47,52-57] but the mechanisms and magnitudes still remain unclear. Cayuela et al[13] have reported that biochar stimulates the last step of denitrification (N_2O reduction to N_2) and proposed that biochar works as an electron shuttle, facilitating the transfer of electrons to soil-denitrifying microorganisms. However, Ameloot[40] observed no decrease in the $N_2O/(N_2 + N_2O)$ ratio and concluded that biochar did not induce N_2O reduction. Thus, there is a significant need to better understand the relationships between biochar addition and net N_2O production in soils.

The purpose of this study was to evaluate the influence of biochar amendment on emissions and SP values of N_2O from agricultural soil under anaerobic conditions. To accomplish this, we incubated agricultural soil with different amounts of biochar amendment under anaerobic conditions and with and without acetylene (an inhibitor of N_2O reduction to N_2), and measured emission rates and SP values of N_2O produced by the incubated soils. We hypothesized that (i) SP values of N_2O and concentrations of nitrate and nitrite in soil would demonstrate that bacterial denitrification is the major N_2O production process; (ii) differences in N_2O emissions from soils incubated with and without acetylene would show that biochar affects the last step of denitrification; and (iii) the different N_2O emissions from soils with and without acetylene would yield slightly different SP values.

2 | EXPERIMENTAL

2.1 | Samples

Soil samples were collected from a grassland at the Texas A&M AgriLife Research & Extension Center at Overton, TX, USA. The field plot has been used for perennial forages for 20 years. The top 0–20 cm of a Lilbert loamy fine sand soil (N = 0.22%, C = 2.34%, soil pH = 5.8) (Order: Ultisols) was collected from one location using a spade, transferred to the laboratory in sealed polyethylene bags, and stored at 4°C for one week prior to initiation of incubation. Before starting incubation, the soil was passed through a 4 mm mesh size sieve to remove large roots. The biochar used for this experiment was created at Baylor University (Waco, TX, USA) by heating pine (species unknown) at 455–485°C for 100 min, and then passed through a 0.75 mm mesh sieve (N = 0.12%, C = 81.64%; pH = 6.9). The biochar was then mixed thoroughly with the soil to create four aliquots: 0% biochar (no amendment), 1% biochar, 2% biochar, and 4% biochar (w/w).

2.2 | Incubation

After mixing and homogenizing the soil and biochar, each aliquot was subdivided (~125 g dry weight) among 8 glass jars (1 L). Soil moisture was elevated to approximately 50% water-filled pore space (WFPS) based on soil bulk density. The jars were left at room temperature (20°C) in the dark for 5 days to stabilize microbial activity. After the 5-day aerobic pre-incubation, these 32 jars were then sealed and the headspace was made anaerobic by repeated evacuation and filling with ultrahigh-purity helium gas (>99.999%). An NH_4NO_3 solution was injected via a syringe, adding 10 μg of nitrogen to soil per gram and bringing the soil moisture to 70% WFPS. In addition, 10% (v/v) acetylene (C_2H_2) was injected into four jars of each aliquot (sample IDs with "+"). In summary, the following treatments were tested: 0% biochar (no amendment) without acetylene (C0−), 1% biochar without acetylene (C1−), 2% biochar without acetylene (C2−), 4% biochar without acetylene (C4−), 0% biochar (no amendment) with acetylene (C0+), 1% (w/w) biochar with acetylene (C1+), 2% (w/w) biochar with acetylene (C2+), 4% (w/w) biochar with acetylene (C4+). This yielded four replicates per treatment.

All jars were placed in an incubator (20°C) in the dark for 10 days. The headspace gas was sampled using a 10-mL syringe on day 1 (24 h after incubation started), and days 3, 5, 7, and 10. The air pressure in each jar was kept constant by adding ultrahigh-purity helium to replace the volume of collected sample gases. The headspace was flushed and filled with new helium (and C_2H_2 for "+" samples) after sampling on days 3 and 7 in order to maintain the acetylene concentrations (10%) and prevent potential O_2 contamination into the headspace by manual

gas sampling. All the collected gas samples were kept in 10-mL crimped glass containers with 13 mm polyisobutylene snap-on stoppers (Wheaton-DWK Life Sciences, Millville, NJ, USA) until analysis.

In addition to these 32 jars, soil sterilized by an autoclave (Tuttnauer, Hauppauge, NY, USA) was tested without biochar amendment to evaluate any abiotic contributions to N_2O formation via chemodenitrification (reduction of NO_x^- by ferrous iron, Fe^{2+}). The sterilized soil was distributed to eight jars: four jars with acetylene and four jars without acetylene. They were incubated under the same conditions and sampled in the same way as the other 32 jars. After 10 days of incubation, no N_2O emissions were detected from these sterilized soil samples, and so we presumed that there was no production of abiotically derived N_2O.

2.3 | Measurements of N_2O concentrations

The N_2O concentrations on days 1, 3, 5, 7, and 10 were measured by gas chromatography (GC) using a Master GC (Dani Instruments SpA, Milan, Italy) equipped with an electron capture detector. Gas samples (5 mL) were injected in a 0.25 μL sample loop connected with a capillary column (Restek, Bellefonte, PA, USA; Rt®-Q-Bond, 30 m length, 0.53 mm diameter). The column oven temperature was kept initially at 30°C for 4 min and then increased at 60°C min^{-1} to 70°C. Helium was used as the carrier gas at 0.27 atm constant pressure. The results were calibrated using 0.3 and 3.1 ppm N_2O in N_2 balance gas purchased from Airgas (College Station, TX, USA). The reproducibility error was below 2%.

The emission rates were calculated by dividing the measured N_2O concentrations by the dry soil weights in each jar and the incubation time. Cumulative N_2O emissions were calculated by adding the measured N_2O concentrations of each sampling day, and then dividing those values by the soil weights in each jar.

2.4 | Isotope analysis of N_2O

Nitrous oxide samples from day 5 were analyzed for the N and O isotope ratios ($\delta^{15}N^{bulk}$ and $\delta^{18}O$ values, respectively) and SP using a modified Precon/Gasbench II sample introduction system coupled to a Delta V Advantage isotope ratio mass spectrometer (all from Thermo Fisher Scientific, West Palm Beach, FL, USA) in the Stable Isotopes for Biosphere Science Laboratory (Department of Ecosystem Science and Management, Texas A&M University, College Station, TX, USA; http://sibs.tamu.edu). Precon modifications and the determination of a rearrangement factor are described in the supporting information.

Reference gas (99.9% N_2O) and our working standard gas (30 ppm N_2O in nitrogen balance gas) were purchased from Airgas and their isotope ratios were determined at Tokyo Institute of Technology (Tokyo Tech, Tokyo, Japan). The $\delta^{15}N^{bulk}$, $\delta^{18}O$, $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$, and SP values of the working standard gas from Tokyo Tech are 1.83 ± 0.05‰, 39.68 ± 0.33‰, 1.25 ± 0.61‰, 2.40 ± 0.62‰, and −1.16‰, respectively.

Each sample gas and the working standard gas were manually injected into the modified Precon system through an 11 mm Thermolite® septum (Restek) using a gastight syringe (VICI Precision Sampling, Baton Rouge, LA, USA). Injection volumes for N_2O sample gases were calculated in advance based on the N_2O concentrations measured by GC, and were between 0.08 and 5 mL. The volume of injected working standard (30 ppm N_2O) was 2.4 mL, producing approximately 4000 mV of m/z 44 and *ca* 1300 mV of m/z 30. The Delta V Advantage mass spectrometer is equipped with three collectors so the gas was analyzed twice to obtain SP values: once to measure m/z 44, 45, and 46 of the N_2O^+ molecular ion, and a second time to measure m/z 30 and 31 of the NO^+ fragment ion. The duration of each analysis was 35 min, and the working standards were analyzed before and after sample analyses each day. A blank (ultrapure helium injection) was also analyzed after changing the septa of the Precon injection ports. For gas samples containing acetylene, 1 mL of 0.3 M potassium permanganate ($KMnO_4$) was added into the crimped vials 24 h prior to the isotope analysis in order to oxidize NO and acetylene.

The SP values were calculated using the following equation:[30]

$$SP = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta} \quad (1)$$

The $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values ($^{15}N/^{14}N$ ratios at the center and end sites of the nitrogen atoms, respectively) were calculated by solving the following equations:[30]

$$^{45}R = {}^{15}R^{\alpha} + {}^{15}R^{\beta} + {}^{17}R \quad (2)$$

$$^{46}R = {}^{18}R + \left({}^{15}R^{\alpha} + {}^{15}R^{\beta}\right){}^{17}R + {}^{15}R^{\alpha}{}^{15}R^{\beta} \quad (3)$$

$$^{31}R = {}^{15}R^{\alpha} + {}^{17}R \quad (4)$$

$$^{17}R = A\left({}^{18}R\right)^{\gamma} \quad (5)$$

where $A = 0.00937035$ and $\gamma = 0.516$.[58] Values of ^{45}R, ^{46}R, and ^{31}R were obtained by measurements of m/z 45/44, 46/44, and 31/30, respectively. The isotope ratios are reported in units of per mil (‰) using delta notation (δ):

$$\delta^{15}N = \left(\frac{^{15}R_{smp}}{^{15}R_{std}}\right) - 1 \quad (6)$$

where subscipts "smp" and "std" represent the isotope ratios of the sample and the standard, respectively. The standards are atmospheric N_2 for nitrogen and Vienna Standard Mean Ocean Water (VSMOW) for oxygen. The measurement precision was typically better than 0.15‰ for $\delta^{15}N^{bulk}$ values, 0.35‰ for $\delta^{18}O$ values, and 0.80‰ for $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values.

We compared our calibrations with those of other laboratories by analyzing three additional reference gases. "REF1" and "REF2" were provided by Laboratory for Air Pollution & Environment Technology, Empa Materials Science and Technology (Dübendorf, Switzerland), and "AK2" was provided by the Institute of Climate-Smart Agriculture, Johann Heinrich von Thünen Institute (Braunschweig, Germany). We compare our results with those of Tokyo Tech ($\delta^{15}N^{bulk}$ = 6.24‰, $\delta^{18}O$ = 35.16‰, SP = 18.92‰ for REF1, $\delta^{15}N^{bulk}$ = −3.66‰, $\delta^{18}O$ = 32.73‰, SP = 18.42‰ for REF2, and $\delta^{15}N^{bulk}$ = −1.19‰, $\delta^{18}O$ = 40.03‰, and SP = −1.84‰ for AK2) and other published values. We were unfortunately unable to analyze the "T gas" that was also discussed in Mohn et al[39] due to a cylinder leak.

2.5 | Inorganic nitrogen analysis of soil

Inorganic N concentrations ($NH_3 + NH_4^+$ and $NO_2^- + NO_3^-$) were determined at the end of the 10-day incubation. Soil inorganic N was extracted from 10 g of each soil sample using 30 mL of 2 M KCl immediately after incubation was terminated. The mixture of the soil and KCl solution was shaken for 1 h, filtered using pre-leached (2 M KCl) #40 ashless filter paper (Whatman, Maidstone, UK), and the extracts were stored in a freezer until analysis. The concentrations of $[NH_3 + NH_4^+]$-N and $[NO_2^- + NO_3^-]$-N were determined colorimetrically using an AQ2+ Discrete Chemistry Analyzer (SEAL Analytical Ltd, Southhampton, UK). Color development for determination of NH_4^+ was based on indophenol-blue chemistry,[59] and the determination of $NO_2^- + NO_3^-$ was based on cadmium reduction and subsequent diazotization.[60] The inorganic N concentrations of the soil before incubation were not measured.

2.6 | Statistical analysis

One-way ANOVA in conjunction with Tukey's *post hoc* comparisons and Pearson's correlation was performed using JMP®[61] to determine significant differences between samples. A value of $p < 0.05$ was used to indicate statistical significance.

Five samples with extremely low N_2O emission rates or extremely high inorganic nitrogen concentrations, probably due to leaks or inadvertent excessive N addition, are excluded from further discussions.

3 | RESULTS

3.1 | Accuracy and precision of N_2O isotope measurement

Our empirically determined rearrangement factor was 0.101 (Figure S1, supporting information), which is comparable with those of other laboratories (0.08 to 0.117[29,30,62-64]).

The $\delta^{15}N^{bulk}$, $\delta^{18}O$, and SP values obtained for the AK2 standard were − 1.17 ± 0.16‰, 40.05 ± 0.42‰, and −2.93 ± 0.43‰ ($n = 13$). These differ by 0.03‰, 0.02‰, and − 1.09‰, respectively, from the values determined by Tokyo Tech. The $\delta^{15}N^{bulk}$, $\delta^{18}O$, and SP values obtained for the REF1 gas were 6.24 ± 0.06‰, 35.85 ± 0.17‰, and 17.77 ± 1.04‰, respectively ($n = 8$), and those of REF2 were −3.61 ± 0.08‰, 34.13 ± 0.18‰, and 18.00 ± 0.27‰, respectively ($n = 6$). These $\delta^{15}N^{bulk}$, $\delta^{18}O$, and SP values differ by 0.00‰, 0.69‰, and −1.15‰ (REF1) and 0.05‰, 1.40‰, and −0.42‰ (REF2) from values measured by Tokyo Tech, respectively (see Figure 1 for comparison with all published values).

3.2 | N_2O emissions

Soil N_2O flux measurements were made on days 1, 3, 5, 7, and 10. The N_2O emission rates were highest on day 1 (0.92 to 2.32 μg-N g^{-1} day^{-1}) across all treatments, and then decreased gradually to lowest values on day 10 (0 to 0.08 μg-N g^{-1} day^{-1}) (Figure 2; Table S1, supporting information).

Biochar significantly reduced N_2O emission rates on day 1 in the absence of acetylene and irrespective of biochar amounts (1.31 ± 0.19, 1.26 ± 0.26, and 1.44 ± 0.08 μg-N g^{-1} day^{-1}, for 1%, 2%, and 4%, respectively), compared with 2.12 ± 0.18 μg-N g^{-1} day^{-1} for non-amended soil ($p < 0.01$). A similar pattern was observed on day 1 in samples with acetylene (2.21 ± 0.16, 1.44 ± 0.24, 1.71 ± 0.06, and 1.67 ± 0.13 μg-N g^{-1} day^{-1}, for 0%, 1%, 2%, and 4% biochar amounts, respectively) ($p < 0.001$).

The response of N_2O emission rates to the treatments after 5 days of incubation was different. First, emission rates in the presence of acetylene were 5–26 times higher than those without acetylene. Second, biochar had no effect on emissions in the presence of acetylene (1.29 ± 0.08, 1.41 ± 0.17, 1.18 ± 0.01, and 1.42 ± 0.27 μg-N g^{-1} day^{-1}, for 0%, 1%, 2%, and 4% biochar amounts, respectively), whereas the samples with no biochar produced four times as much N_2O as those with 4% biochar, in the absence of acetylene (0.22 ± 0.03 μg-N g^{-1} day^{-1} for 0% biochar amounts, compared with

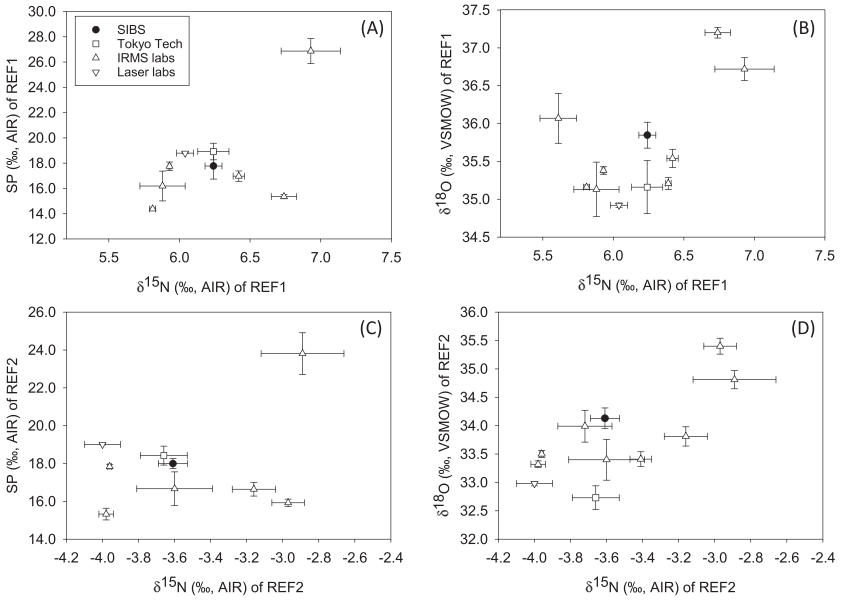

FIGURE 1 Comparison of isotope ratios of REF1 (A, B) and REF2 (C, D) gases measured by Stable Isotopes for Biosphere Science Laboratory (this study) (closed circle), Tokyo Tech (open square), laboratories using isotope ratio mass spectrometry (IRMS) (open upward triangle), and laboratories using laser-based spectrometers (open downward triangle)[39]

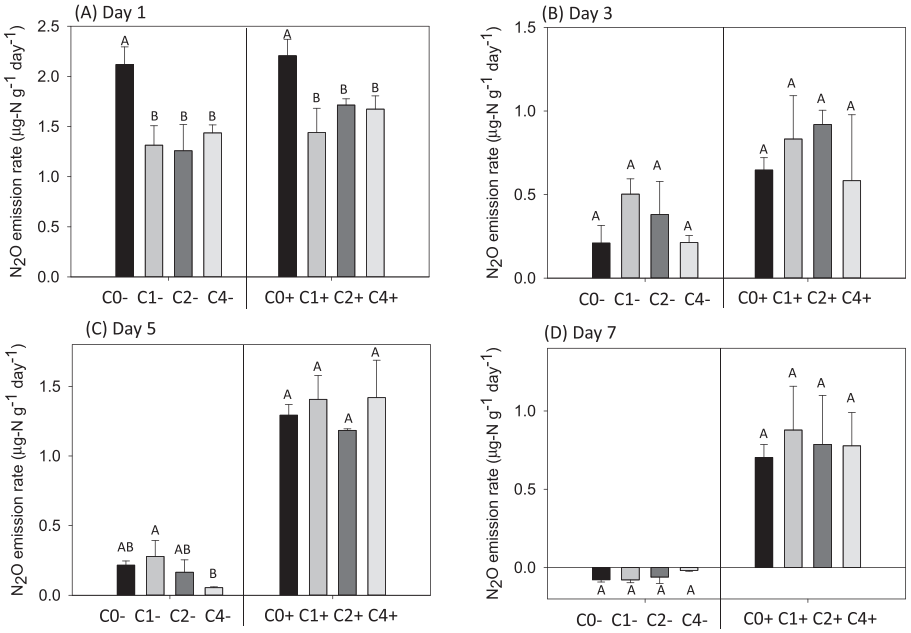

FIGURE 2 N_2O emission rates of incubation days 1 (A), 3 (B), 5 (C), and 7 (D) from samples incubated without (C−) and with acetylene (C+). Significant differences are indicated with different letters (ANOVA followed by Tukey HSD, $\alpha = 0.05$). Note scales vary for each day

0.05 ± 0.01 µg-N g^{-1} day^{-1} for 4% biochar amounts) (Figure 2C). When acetylene was not added, the cumulative N_2O production declined with increasing biochar content for all incubation times (Figure 3; Table S2, supporting information).

3.3 | Isotope ratios of N_2O

N_2O from samples with and without acetylene (irrespective of biochar treatment) showed clear differences in their SP values after 5 days of incubation (Figure 4). Average SP values of day 5 samples with acetylene (−3.26 ± 1.98‰) were significantly lower than those without acetylene (13.74 ± 1.96‰; $p < 0.0001$). Although biochar affected soil N_2O production in the absence of acetylene, there was no apparent effect on the SP (Table 1).

The $\delta^{15}N^{bulk}$ values of N_2O showed no difference with and without acetylene (irrespective of biochar treatment): 24.88 ± 8.83‰ for day 5 C− samples and 27.80 ± 7.70‰ for day 5 C+ samples (Table 1). In contrast, the $\delta^{18}O$ values of N_2O produced in the absence of acetylene (60.30 ± 4.73‰) were significantly greater than those with acetylene (30.31 ± 3.09‰) ($p < 0.0001$).

3.4 | Ammonium, nitrate, nitrite of post-incubation soils

The $[NH_3 + NH_4^+]$-N concentrations in soil after 10 days of incubation were 6.88 ± 4.27 and 7.20 ± 4.12 µg N g^{-1} for the samples without and with acetylene, respectively (Table 2). The $[NO_2^- + NO_3^-]$-N concentrations were low for all samples (0.38 ± 0.11 µg N g^{-1}; Table 2). Note that the inorganic N concentrations of the soil before incubation were not measured, so changes in those concentrations before and after incubations are not shown.

4 | DISCUSSION

4.1 | Pathways of N_2O production during incubations

Under complete anaerobic condition (0% oxygen), N_2O production from soil with 50% water holding capacity (WHC) is contributed only by heterotrophic denitrification.[65] Our incubations were anaerobic with 70% WHC and, assuming that it is 0% oxygen, the dominant microbial process generating N_2O should be denitrification:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (7)$$

Enhanced N_2O production after 5 days of incubation in the presence of acetylene was clearly consistent with acetylene blocking the last step of denitrification. The SP values after 5 days of incubation with acetylene are close to 0‰ and are similar to those of N_2O produced by denitrifying organisms. Reported SP values of N_2O that is instantaneously produced by denitrification and is not affected by partial reduction are −0.5 ± 0.6‰, −0.5 ± 1.9‰, and −5.1 ± 1.8‰ for $NO_3^- \rightarrow N_2O$[20,34] and −5.9 ± 2.1‰ for $NO \rightarrow N_2O$.[35] On the other hand, when N_2O is produced by fungi, the SP values are considerably higher (20–40‰[32,36]). If the oxygen concentration of the headspace was >0.5%, it is possible that approximately half of N_2O production is by denitrification and the other half by nitrifier denitrification.[65] The SP values of N_2O produced by nitrifier denitrification (SP = −0.8 ± 5.8‰ for *Nitrosomonas europaea* and SP = 0.1 ± 1.7‰ for *Nitrosospira multiformis*;[34,63] SP = −10.7 ± 2.9‰ for *Nitrosomonas marina* C-113a)[23] overlap with those produced by denitrification, and it is not possible to differentiate them. Our headspace was evacuated repeatedly and flushed with ultrahigh-purity helium gas (>99.999%) on days 3 and 7, so it is reasonable to assume that the headspace contained 0% oxygen. Hence the N_2O emitted during our incubation

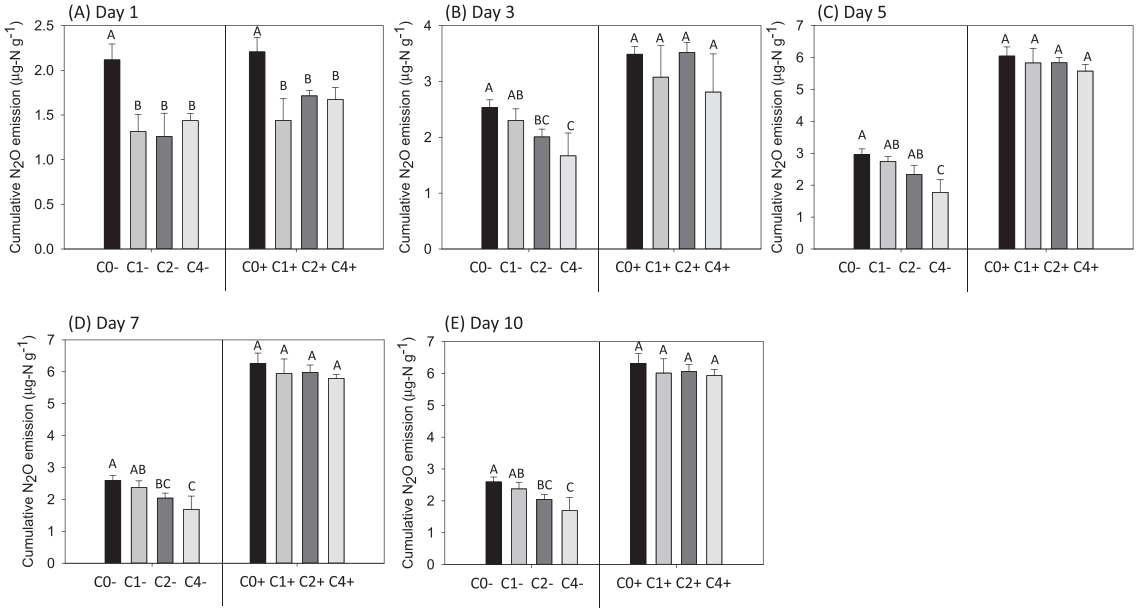

FIGURE 3 Cumulative N_2O emissions after incubation days 1 (A), 3 (B), 5 (C), 7 (D), and 10 (E) without (C−) and with acetylene (C+). Significant differences are indicated with different letters (ANOVA followed by Tukey HSD, $\alpha = 0.05$, $p < 0.05$). Note scales vary for each day

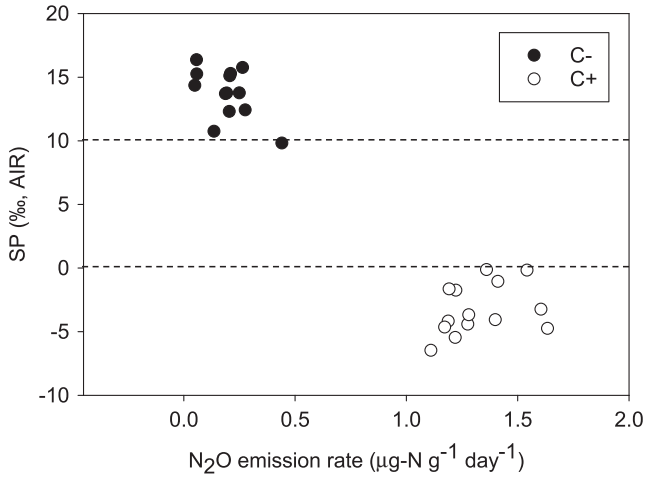

FIGURE 4 N_2O emission rates and SP of N_2O without (C−) and with acetylene (C+) after 5 days of incubation

should be dominated by bacterial denitrification. The decreasing concentrations of NO_3^- and NO_2^- measured in both C− and C+ soil samples after 10 days of incubation also support the hypothesis that NO_3^- and NO_2^- were used to produce N_2O.

The only difference between the samples incubated with and without acetylene should be that N_2O reduction to N_2 is blocked, and thus the process to produce N_2O in the samples without acetylene should have also been heterotrophic denitrification. The SP values of the day 5 samples without acetylene are 10 to 16‰. These values must result from a mixture of N_2O produced from/via NO_3^- and NO_2^- (presumably from the same processes and at the same rates in both acetylene treatments) and residual N_2O after reduction to N_2.

During N_2O reduction to N_2, the ^{14}N–O bond is easier to break than that of ^{15}N–O, causing an increase in the ^{15}N at the central N of the residual N_2O as that pool is reduced.[12] Therefore, the partial consumption of N_2O during the reduction process increases the SP values of residual N_2O. The difference in the isotope ratios between the product and unconsumed reactant is apparent fractionation. The apparent fractionation factor (ε) for the N_2O reduction process can be estimated using a Rayleigh distillation approach:[66-68]

$$\delta_R \cong \delta_P + \varepsilon \times \ln f \quad (8)$$

where δ_R is the isotope ratio of residual N_2O measured in the absence of acetylene, δ_P is the isotope ratio of produced N_2O (unconsumed N_2O) measured in the presence of acetylene where no N_2O reduction process occurred, and f is the product ratio of the reduction process $N_2O/(N_2O + N_2)$ calculated based on comparison of acetylated and non-acetylated samples.[68] The N_2O emitted from the samples in the presence of acetylene represents the total denitrification ($N_2O + N_2$). The calculations using Equation 8 show that the apparent fractionation factor for the N_2O reduction in our soil samples without biochar amendment is −10.4 ± 1.5‰. A study by Ostrom et al showed apparent SP fractionation factors for N_2O reduction using pure microbial cultures of −6.8‰ (*Paracoccus denitrificans*) and −5‰ (*Pseudomobas stuzeri*).[38] Averages of the apparent SP fractionation factors calculated based on soil samples incubated after removing all oxidized inorganic nitrogen and adding N_2O gas in the headspace are −7.9 to −3.6‰ (actual range: −7.9 ± 2.8‰ to −3.6 ± 1.6‰)[67] and −4.5 to −2.9‰ (actual range: −6.1 to −2.1‰).[69] Averages of the apparent SP fractionation factors calculated from soils incubated under 70–80% WFPS are −8 to −2‰ (actual range: −9.4 ± 4.0‰ to −1.3 ± 0.8‰)[68] and − 8.6 to −6.7‰.[70] In these two studies, a contribution of nitrifier denitrification to N_2O production was assumed negligible because their anoxic incubation conditions with high soil moisture are favored by denitrification.[70] Our value is slightly lower than those from previous studies but comparable.

TABLE 1 $\delta^{15}N$ and $\delta^{18}O$ values of N_2O sampled on day 5 of incubation without (C−) and with acetylene (C+). Significant differences in $\delta^{18}O$ and SP between different treatments are indicated with different superscript letters (ANOVA followed by Tukey HSD, $\alpha = 0.05$, $p < 0.0001$)

Day	Biochar (%, w/w)	Presence of acetylene	ID	$\delta^{15}N$ (‰, AIR)	$\delta^{18}O$ (‰, VSMOW)	$\delta^{15}N_\alpha$ (‰, AIR)	$\delta^{15}N_\beta$ (‰, AIR)	SP (‰, AIR)
Day 5	0	Without acetylene	C0−	25.45 ± 1.89	61.65 ± 1.36^{A}	32.56 ± 1.73	18.35 ± 2.10	14.21 ± 0.78^{A}
	1	Without acetylene	C1−	22.89 ± 5.57	59.62 ± 3.11^{AB}	29.80 ± 6.09	16.00 ± 5.10	12.81 ± 1.43^{A}
	2	Without acetylene	C2−	24.35 ± 7.37	59.43 ± 2.80^{AB}	31.26 ± 7.35	17.46 ± 7.64	13.80 ± 2.71^{A}
	4	Without acetylene	C4−	37.53 ± 3.66	66.72 ± 0.74^{B}	44.94 ± 3.35	30.13 ± 3.98	14.80 ± 0.63^{A}
	0	With acetylene	C0+	29.06 ± 2.32	30.89 ± 0.66^{C}	26.85 ± 2.15	31.26 ± 2.53	-4.41 ± 0.77^{B}
	1	With acetylene	C1+	26.25 ± 6.29	28.21 ± 3.36^{C}	25.28 ± 7.26	27.20 ± 5.33	-1.92 ± 2.00^{B}
	2	With acetylene	C2+	34.01 ± 1.25	33.41 ± 0.65^{C}	32.27 ± 0.62	35.76 ± 2.01	-3.49 ± 1.61^{B}
	4	With acetylene	C4+	21.96 ± 14.11	29.23 ± 4.30^{C}	20.31 ± 12.77	23.60 ± 15.49	-3.29 ± 3.15^{B}

TABLE 2 Concentrations of inorganic nitrogen after 10 days of incubation without (C−) and with acetylene (C+)

Day	Biochar (%, w/w)	Presence of acetylene	ID	$NH_3 + NH_4^+$ (μg-N g^{-1})	$NO_2^- + NO_3^-$ (μg-N g^{-1})
Day 10	0	Without acetylene	C0−	9.24 ± 1.33^{A}	0.49 ± 0.05^{A}
	1	Without acetylene	C1−	9.92 ± 4.60^{A}	0.33 ± 0.16^{A}
	2	Without acetylene	C2−	4.63 ± 2.66^{A}	0.48 ± 0.04^{A}
	4	Without acetylene	C4−	1.72 ± 1.94^{A}	0.29 ± 0.12^{A}
	0	With acetylene	C0+	11.04 ± 3.77^{A}	0.46 ± 0.04^{A}
	1	With acetylene	C1+	7.31 ± 0.73^{A}	0.28 ± 0.08^{B}
	2	With acetylene	C2+	18.91 ± 6.69^{A}	0.39 ± 0.00^{AB}
	4	With acetylene	C4+	3.20 ± 0.44^{A}	0.32 ± 0.08^{AB}

4.2 | Changes in N_2O emission rates with biochar amendment

Emission rates of N_2O produced on day 5 with 0–4% biochar in the presence of acetylene are not significantly different from each other (1.33 ± 0.11 μg-N g^{-1} day^{-1}), suggesting that biochar did not affect N_2O production. However, in the absence of acetylene, the soils incubated with 4% biochar produced only a quarter of the N_2O produced by soils with no biochar addition (Figure 2C). Cumulative N_2O emissions on days 3, 5, 7, and 10 of incubation in the presence of acetylene are also not significantly different from each other, where as those on days 3, 5, 7, and 10 in the absence of acetylene showed significantly lower emissions from samples with 4% biochar than from samples with no biochar amendment (Figures 3B–3E). The ratios of $N_2O/(N_2O + N_2)$ calculated based on comparison of acetylated and non-acetylated samples showed a decrease from 0.17 (0% biochar) to 0.04 (4% biochar) (Table 3). These results suggest that biochar enhances the last step of denitrification (N_2O reduction to N_2). Our results are consistent with those of a previous study which used a ^{15}N gas-flux method to measure $N_2O/(N_2 + N_2O)$ and proposed that the biochar works as an "electron shuttle," promoting the transfer of electrons to denitrifying bacteria in soil.[13] Ameloot et al,[40] however, did not observe a decrease in the $N_2O/(N_2 + N_2O)$ ratio using the acetylene inhabitation incubation method in their soil samples where biochar was added 7 months prior to the incubation experiments. They concluded that biochar did not facilitate the reduction of N_2O to N_2 because of degraded available C for denitrification. In our study, biochar enhanced the reduction of N_2O to N_2 in the soil samples probably because labile carbon was available from biochar that was added only 5 days prior to the incubation experiments and also because the agricultural soils generally contain high organic carbon (C = 2.34%, 3.15%, 3.85%, and 5.34% for our soil samples with 0%, 1%, 2%, and 4% biochar after 10 days of incubation, respectively).

After 5 days of incubation without acetylene, we observed no difference in SP values between different amounts of biochar (Table 1). This was a surprising result since, to a first approximation, the SP values of residual N_2O are expected to increase because the $^{14}N–O$ bond is easier to break than that of $^{15}N–O$, causing an increase in ^{15}N at the central N of the residual N_2O as that pool is reduced.[12] In this experiment, a higher reduction of N_2O to N_2 was observed for soils with 4% biochar than those with no addition (Figure 3C), which should increase SP values of N_2O produced by these biochar-amended soils. We did not observe such an increase. In our soil samples, the apparent fractionation factors calculated using

TABLE 3 Ratios of $N_2O/(N_2 + N_2O)$ on day 5 and apparent fractionation factors calculated using Equation 8

Biochar (%, w/w)	ID	$N_2O/(N_2 + N_2O)$	Apparent fractionation factor (‰)
0	C0	0.17 ± 0.03	-10.41 ± 1.46^{A}
1	C1	0.20 ± 0.11	-9.10 ± 1.89^{AB}
2	C2	0.14 ± 0.06	-8.78 ± 1.96^{AB}
4	C4	0.04 ± 0.00	-5.54 ± 0.96^{B}

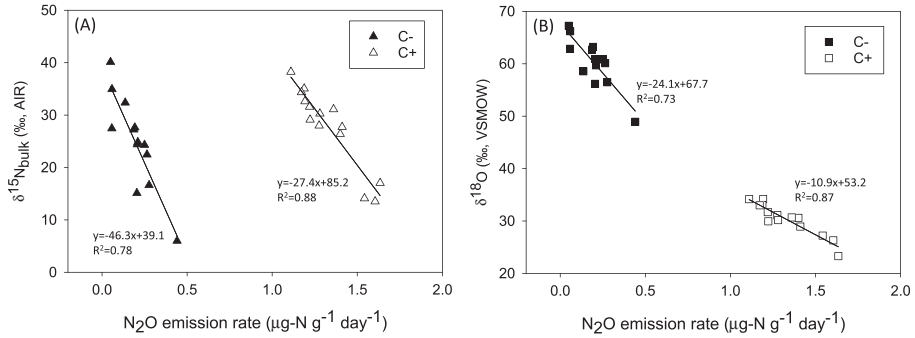

FIGURE 5 N_2O emission rates and $\delta^{15}N$ values (A) and $\delta^{18}O$ values (B) of N_2O without (C−) and with acetylene (C+) after 5 days of incubation

Equation 8 for the samples with 4% biochar are −5.54 ± 0.96‰, whereas those for the samples with 0% biochar are −10.41 ± 1.46‰ (Table 3). It is possible that a shift in microbial community with biochar amendment occurred and different bacteria have different fractionation factors. Harter et al[54,71] found that biochar amendment significantly altered N_2O-reducing microbial taxa (bacterial genes *nosZ* encoding for N_2O reducing enzyme) and were probably responsible for N_2O suppression in soil. Malghani et al[47] also showed higher abundance of the denitrifying gene *nosZ* in biochar treatment than in the control, possibly due to the high availability of labile carbon. We speculate that the activities of N_2O-reducing microbial taxa in the samples with 4% biochar might have been modified by the biochar addition and they have different fractionation factors from the original microbial taxa for the processes of N_2O reduction, causing the unchanging SP values.

Future research should include microbial DNA sequence analyses in an effort to identify the microbes that may be involved, and incubation experiments should be performed with higher amounts of biochar amendment to produce more prominent differences in the SP values and N_2O emission rates. In addition, better constraints on SP values for the N_2O reduction to N_2 are needed since, to the best of our knowledge, there is only one pure culture study that reported the fractionation factors of SP for N_2O reduction (−6.8‰ for *Paracoccus denitrificans* and −5.0‰ for *Pseudomonas stuzeri*[38]).

The pattern of N_2O emission rates from 0 to 4% biochar samples with acetylene on day 1 is similar to that without acetylene on day 1 (24 h after the start of incubation; Figure 2A). This observation suggests that the microbial N_2O reduction to N_2 had not started, at least to any significant degree, 24 h after incubation started. It is worth noting further that in day 1 samples, the N_2O emission rates with biochar (1–4%) are significantly lower than with 0% biochar ($p < 0.005$). This suggests that the N_2O production process was slowed by the presence of biochar during the first 24 h of the incubation.

4.3 | The $\delta^{15}N^{bulk}$ and $\delta^{18}O$ values

We observed negative correlations between the $\delta^{15}N$ of bulk N_2O ($\delta^{15}N^{bulk}$) and N_2O emission rate irrespective of acetylene treatment (Figure 5A). During N_2O production by bacterial denitrification, substrates with ^{14}N tend to react faster than those with ^{15}N, leading to declines in $\delta^{15}N^{bulk}$, with fractionation factors reported by previous studies ranging from −37 to −10‰.[12,20,34,73,74] The negative correlation between the $\delta^{15}N^{bulk}$ values and N_2O emission rates in samples with acetylene is consistent with N_2O production driving lower $\delta^{15}N^{bulk}$ values. In the process of reduction of N_2O to N_2, on the other hand, the $\delta^{15}N^{bulk}$ value of the residual N_2O would be expected to increase (fractionation factors from −26 to −4‰).[12,38,74,75] The $\delta^{15}N^{bulk}$ values from samples without acetylene (24.9 ± 8.8‰) were not significantly different from those with acetylene (27.8 ± 7.7‰), showing that N_2O reduction to N_2 had a negligible impact on the $\delta^{15}N^{bulk}$ values in this experiment. It is worth noting that all $\delta^{15}N^{bulk}$ values are considerably higher than those reported in previous denitrification studies.[25,28,38,69,72] During our incubation, the headspace of all samples was flushed and filled with new helium after sampling on day 3 and day 7 in order to maintain the acetylene concentrations. It is likely that the N_2O production process left ^{15}N-rich NO_3^- and NO_2^- in the soil, which became a source of new N_2O after flushing the headspace, and is also consistent with the near-complete consumption of available NO_3^- during the incubation. Note that the $\delta^{15}N^{bulk}$ values of the precursors do not influence the SP values of N_2O.[34]

There were also negative correlations between the $\delta^{18}O$ values and N_2O emission rates (Figure 5B). N_2O production by denitrification should drive ^{18}O enrichment of the produced N_2O because the N–^{16}O bond is easier to break during the elimination of O in NO_3^- to produce N_2O (fractionation factors from 4 to 40‰).[12,20,79] However, there is also an exchange with oxygen of ambient H_2O during denitrification, which may vary from 10 to 100%.[76-78] The observed negative correlation in the presence of acetylene could have been the result of accelerated O exchange by specific enzyme and microbial activities (e.g., Nir and Nor[76]), along with the expected enrichment associated with denitrification. In the absence of acetylene, all $\delta^{18}O$ values were higher, consistent with additional enrichment caused by N_2O reduction (fractionation factors range from −42 to −11‰ for the process of $N_2O \rightarrow N_2$).[12,38,75]

5 | CONCLUSIONS

Our study demonstrated the influence of different amounts of biochar amendment on emissions and SP values of N_2O from anaerobic agricultural soil. The emission rates, cumulative emissions, and SP values of N_2O collected from soil with different amounts of biochar

amendment and incubated with and without acetylene showed that (1) bacterial denitrification was the major process that produced N_2O; (2) biochar induced the last step of denitrification (N_2O reduction to N_2); (3) SP values were constant despite the great magnitude of N_2O reduction in samples with 4% biochar, possibly because N_2O-reducing microbial taxa were altered by biochar amendment; (4) N_2O production processes were slowed by the presence of biochar, at least in the first 24 h of incubation; and (5) high $\delta^{15}N$ and $\delta^{18}O$ values of N_2O reflected ^{15}N and ^{18}O enrichment by both N_2O production and reduction and O exchange in the absence of the acetylene inhibitor. These results facilitate understanding of relationships between biochar addition and net N_2O production in soils and the mechanism of biochar amendment reducing N_2O production.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supporting information

Biochar amendment suppresses N_2O emissions but has no impact on ^{15}N site preference in an anaerobic soil

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Method

Isotope analysis of N_2O

A rearrangement factor was determined using 98% $^{15}N^{14}NO$ and $^{14}N^{15}NO$ labeled gases purchased from the Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA). The rearrangement factor accounts for partial scrambling of ^{15}N between the α and β position in the ion source[1-3]. The labeled gases were mixed with the 99.9% N_2O reference gas in our laboratory and the R45 (ratios of *m/z* 45 and *m/z* 44) of N_2O and the R31 (ratios of *m/z* 31 and *m/z* 30) of the fragment ion NO were measured using dual inlet mode of the Delta V Advantage isotope ratio mass spectrometer. The results are shown in Figure S1.

To optimize our isotopic measurements, the following modifications were made to our Precon trace gas pre-concentrator: (1) an injection port was built using Swagelok stainless tube fittings and valves and installed on the Precon for manual injection of sample gases through a septum; (2) the furnace oven and a liquid nitrogen trap that were built-in for CH_4 analysis were removed; (3) seals and fittings containing fluorocarbons were removed or replaced (e.g., Viton O-rings)[4-6]; (4) the water/carbon dioxide trap was replaced with a larger trap that is more leak-tight and facilitates the replacement of sorbent chemicals; (5) the "gas transfer" time from the injection port to the first liquid nitrogen trap was extended to 400 sec; (6) the Valco 4-port valve was set back to "load" during the isotope measurements after transferring the sample gas from the first to the second liquid nitrogen traps; (7) 300 sec pause was added before the isotopic measurements for better stability of background. The mass spectrometer was set to optimize the sensitivity, linearity, and stability, using an electron energy of 85eV.

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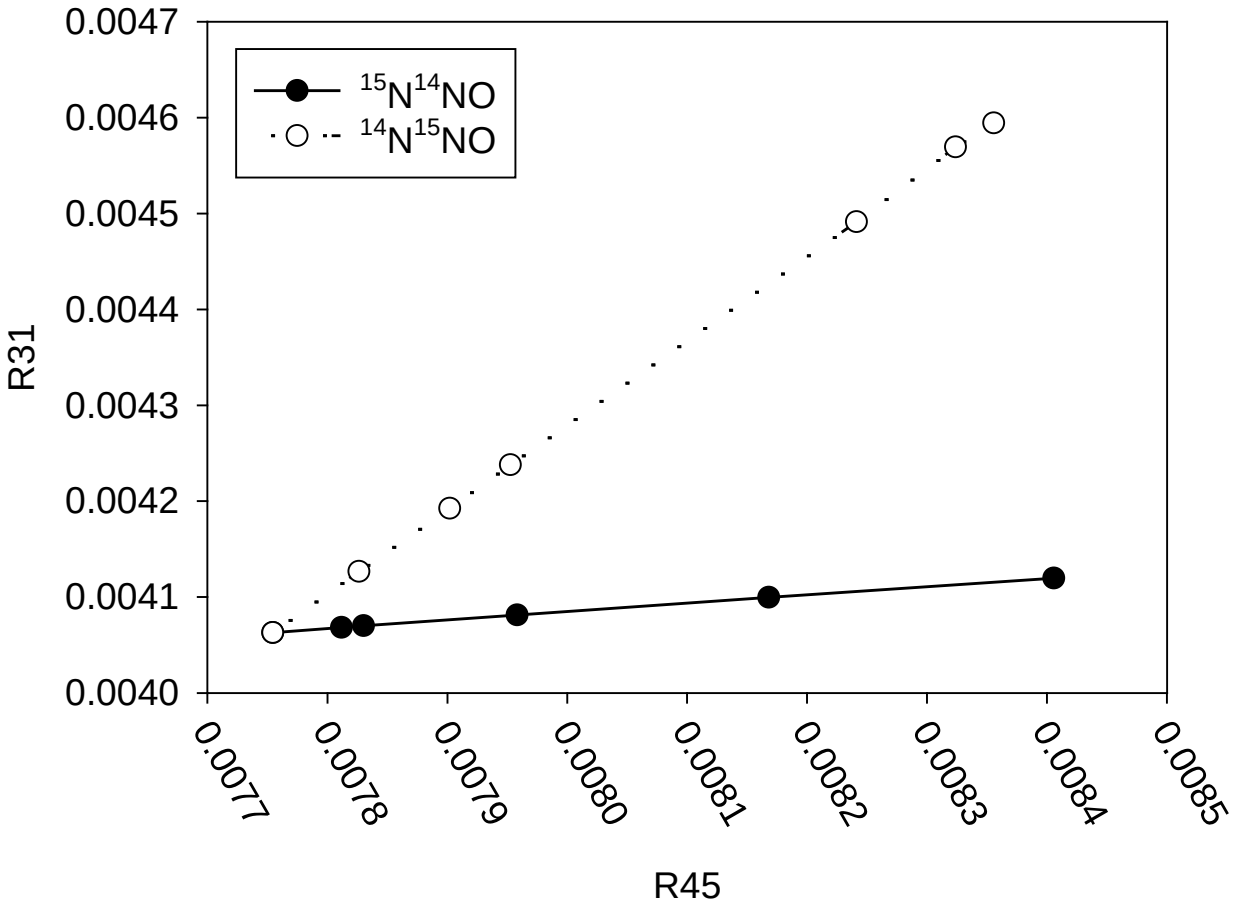

Figure S1

Relationships between R45 and R31 of gas mixtures of 99.9% N_2O reference gas and 98% $^{15}N^{14}NO$ and $^{14}N^{15}NO$ labeled gases used to determine a rearrangement factor.

Table S1

N_2O emission rates on Days 1, 3, 5, 7, and 10.

Biochar (%w/w)	Presence of acetylene	ID	Day 1 (μg-N g^{-1} day^{-1})	Day 3 (μg-N g^{-1} day^{-1})	Day 5 (μg-N g^{-1} day^{-1})	Day 7 (μg-N g^{-1} day^{-1})	Day 10 (μg-N g^{-1} day^{-1})
0%	Without acetylene	C0-	2.12 ± 0.18	0.21 ± 0.11	0.22 ± 0.03	-0.08 ± 0.01	0.00 ± 0.00
1%	Without acetylene	C1-	1.31 ± 0.19	0.50 ± 0.09	0.28 ± 0.11	-0.08 ± 0.02	0.00 ± 0.00
2%	Without acetylene	C2-	1.26 ± 0.26	0.38 ± 0.2	0.17 ± 0.09	-0.06 ± 0.04	0.00 ± 0.00
4%	Without acetylene	C4-	1.44 ± 0.08	0.21 ± 0.04	0.05 ± 0.01	-0.02 ± 0.01	0.00 ± 0.00
0%	With acetylene	C0+	2.21 ± 0.16	0.65 ± 0.07	1.29 ± 0.08	0.70 ± 0.08	0.02 ± 0.01
1%	With acetylene	C1+	1.44 ± 0.24	0.83 ± 0.26	1.41 ± 0.17	0.88 ± 0.28	0.03 ± 0.02
2%	With acetylene	C2+	1.71 ± 0.06	0.92 ± 0.09	1.18 ± 0.01	0.79 ± 0.31	0.03 ± 0.03
4%	With acetylene	C4+	1.67 ± 0.13	0.58 ± 0.39	1.42 ± 0.27	0.78 ± 0.21	0.05 ± 0.03

Table S2

Cumulative N_2O emissions on Days 1, 3, 5, 7, and 10.

Biochar (%w/w)	Presence of acetylene	ID	Day 1 (μg-N g^{-1})	Day 3 (μg-N g^{-1})	Day 5 (μg-N g^{-1})	Day 7 (μg-N g^{-1})	Day 10 (μg-N g^{-1})
0%	Without acetylene	C0-	2.12 ± 0.18	2.53 ± 0.14	2.96 ± 0.17	2.60 ± 0.15	2.60 ± 0.15
1%	Without acetylene	C1-	1.31 ± 0.19	2.30 ± 0.21	2.74 ± 0.15	2.37 ± 0.21	2.37 ± 0.21
2%	Without acetylene	C2-	1.26 ± 0.26	2.01 ± 0.14	2.33 ± 0.29	2.04 ± 0.16	2.04 ± 0.15
4%	Without acetylene	C4-	1.44 ± 0.08	1.67 ± 0.41	1.77 ± 0.40	1.69 ± 0.41	1.69 ± 0.41
0%	With acetylene	C0+	2.21 ± 0.16	3.49 ± 0.14	6.05 ± 0.29	6.26 ± 0.32	6.32 ± 0.31
1%	With acetylene	C1+	1.44 ± 0.24	3.08 ± 0.56	5.83 ± 0.45	5.94 ± 0.46	6.01 ± 0.45
2%	With acetylene	C2+	1.71 ± 0.06	3.52 ± 0.18	5.84 ± 0.16	5.98 ± 0.23	6.07 ± 0.21
4%	With acetylene	C4+	1.67 ± 0.13	2.81 ± 0.68	5.58 ± 0.20	5.79 ± 0.12	5.93 ± 0.20