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Biochar increases soil N_2O emissions produced by nitrification-mediated pathways

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In spite of the numerous studies reporting a decrease in soil nitrous oxide (N_2O) emissions after biochar amendment, there is still a lack of understanding of the processes involved. Hence the subject remains controversial, with a number of studies showing no changes or even an increase in N_2O emissions after biochar soil application. Unraveling the exact causes of these changes, and in which circumstances biochar decreases or increases emissions, is vital to developing and applying successful mitigation strategies. With this objective, we studied two soils [Haplic Phaeozem (HP) and Haplic Calcisol (HC)], which showed opposed responses to biochar amendment. Under the same experimental conditions, the addition of biochar to soil HP decreased N_2O emissions by 76%; whereas it increased emissions by 54% in soil HC. We combined microcosm experiments adding different nitrogen fertilizers, stable isotope techniques and the use of a nitrification inhibitor (dicyciandiamide) with the aim of improving our understanding of the mechanisms involved in the formation of N_2O in these two soils. Evidence suggests that denitrification is the main pathway leading to N_2O emissions in soil HP, and ammonia oxidation and nitrifier-denitrification being the major processes generating N_2O in soil HC. Biochar systematically stimulated nitrification in soil HC, which was probably the cause of the increased N_2O emissions. Here we demonstrate that the effectiveness of using biochar for reducing N_2O emissions from a particular soil is linked to its dominant N_2O formation pathway.

Keywords: nitrous oxide, charcoal, nitrification, DCD, codenitrification, nitrogen fertilizers

INTRODUCTION

Biochar, a carbonaceous material produced during the pyrolysis of biomass, has been found to decrease N_2O emissions from soils (Spokas and Reikosky, 2009; Cayuela et al., 2010; Van Zwieten et al., 2010). A recent meta-analysis of 30 papers (published from 2007 to 2013) revealed a statistically significant reduction of 54% in N_2O emissions when soils were amended with biochar (Cayuela et al., 2014). However, a substantial number of studies contradict this result, they reporting no difference or even an increase in soil N_2O emissions after biochar application (Clough et al., 2010; Saarnio et al., 2013; Suddick and Six, 2013). A remarkable finding was that the same biochar could lead to opposite effects (increasing or decreasing N_2O emissions) depending on the soil to which the biochar was applied (Yoo and Kang, 2012; Malghani et al., 2013).

Soils are a major source of N_2O, which is a potent greenhouse gas and contributor to ozone layer destruction. N_2O is produced during several soil processes and its release to the atmosphere is almost entirely controlled by microbial activities. Current knowledge suggests five N_2O-genic soil microbial sources (Baggs, 2011; Spott et al., 2011). These are the nitrate or nitrite reducing processes of denitrification and dissimilatory nitrate reduction to ammonium (DNRA), and ammonia oxidation (the first step in nitrification, facilitated by ammonia oxidizing bacteria). Nitrifier denitrification, the ability of ammonia oxidizing bacteria to denitrify, is often also seen as a separate process. Finally, codenitrification has also been identified as a relevant N_2O formation pathway in soils (Spott et al., 2011). Understanding the mechanisms of the interactions of biochar with soil N_2O formation pathways represents a difficult challenge. No evidence has been reported that would serve to unambiguously define the cause for the observed variations (increase or decline) in soil N_2O fluxes. This is due to the extremely complex set of reactions leading to N_2O formation and consumption in soils and also to the fact that the number of studies which analyze how biochar influences specific N_2O formation pathways is still very limited.

In a recent study using the ^{15}N gas flux method, Cayuela et al. (2013) observed a consistent decrease in the N_2O/N_2 ratio after biochar amendment in 15 agricultural soils, pointing to denitrification as the N_2O formation pathway that biochar might be altering. According to this, biochar would enhance the last step of denitrification (i.e., the reduction of N_2O–N_2). Subsequently, Harter et al. (2014) found that soil biochar amendment increased the relative gene and transcript copy numbers of the nosZ-encoded bacterial N_2O reductase, a result which could explain the previous mechanistic findings. Nevertheless, Cayuela et al. (2013) also found contrasting results for the flux of total denitrified N ($N_2O + N_2$), which was significantly reduced in the majority of soils (10 out of 15), but highly amplified in others. No conclusive explanation was found for this paradoxical finding.

In this study we aimed to look more closely at the reasons for these contrasting results. Our hypothesis was that, besides denitrification, other microbial processes (e.g., nitrifier-denitrification, dissimilatory nitrate reduction to ammonia, codenitrification) could have led to N_2O and N_2 formation in these soils, mechanisms that had not been addressed in previous studies. Hence, we studied two soils that, under identical experimental conditions, showed opposite responses to biochar amendment, i.e., whereas biochar addition decreased N_2O emissions in one soil, it increased emissions in the other. The main objective was to investigate by ^{15}N gas measurements and the use of nitrification inhibitors, the main pathways leading to N_2O formation in these two soils, with the aim of understanding why biochar might be influencing N_2O emissions differently.

MATERIALS AND METHODS

SOILS AND BIOCHAR SELECTED FOR THE EXPERIMENTS

Two agricultural soils were selected for the experiments (**Table 1**). Soil HP was used as a reference soil, since it had been previously used in numerous studies that proved that denitrification was the major process responsible for N_2O emissions (Čuhel et al., 2010). Soil HC was selected from a series of agricultural soils because it was the only one where (under identical optimal denitrifying conditions) the addition of greenwaste biochar increased N_2O emissions. The soils were sampled from a depth of 0–0.25 m, air-dried and sieved (<2 mm).

We used a biochar produced by continuous slow pyrolysis of greenwaste at 550°C provided by Pacific Pyrolysis Pty. Ltd. (Australia) (**Table 1**). Herbaceous and woody biochars have been found to be the most promising for mitigating N_2O emissions from soil (Cayuela et al., 2014). Therefore, this biochar was selected for its mitigation potential and as a representative standard biochar commonly used in other studies. The biochar was ground to a particle size <1 mm before soil application.

Table 1 | Physical and chemical characteristics of soil and biochar samples used in the experiments.

	Soil HP	Soil HC	Biochar
Management	Pasture	Olive orchard (organic farm)	–
Location	48°52′ N, 14°13′ E	38°23′ N 1°22′ W	–
Cassification (WRB)	Haplic phaeozem	Haplic calcisol	–
Texture	Loamy sand	Sandy loam	–
Sand (%)	78	57	–
Clay (%)	6	16	–
Volatile matter (%)	–	–	26.8
Ash (%)	–	–	7.0
$H:C_{org}$	–	–	0.534
pH (in water, 1:20 w:w 25°C)	6.89	8.01	7.87
EC ($\mu S\ cm^{-1}$)	140	518	166
Ca CO_3 (%)	–	30	–
TOC ($g\ kg^{-1}$)	11.6	16.8	701.7
Total N ($g\ kg^{-1}$)	2.0	2.4	2.7
DC ($mg\ kg^{-1}$)	439.5	694.0	285.1
DOC ($mg\ kg^{-1}$)	315.7	356.9	113.2
DN ($mg\ kg^{-1}$)	34.7	74.0	8.6
DON ($mg\ kg^{-1}$)	10.2	35.9	7.1
NH_4^+- N ($mg\ kg^{-1}$)	19.3	5.0	1.3
NO_2^- -N ($mg\ kg^{-1}$)	<0.2	16.2	<0.2
NO_3^- -N ($mg\ kg^{-1}$)	5.3	16.9	<0.2

TOC, total organic carbon; DN, dissolved nitrogen; DON, dissolved organic nitrogen; DC, dissolved carbon; DOC, dissolved organic carbon.

MICROCOSMS EXPERIMENTS

The incubation experiments were performed in 250 ml polypropylene jars at optimum conditions for denitrification: 25°C and moisture content of 90% water filled pore space (WFPS). The control treatments consisted of 100 g dry soil and the biochar treatments of 98 g dry soil and 2 g biochar (2% w:w). The biochar was thoroughly mixed with the dry soil to obtain a completely homogeneous mixture. Subsequently deionized water (or a solution containing the appropriate concentration of N fertilizer) was added to reach 90% WFPS (and the required N concentration in the fertilized treatments). The jars were incubated aerobically, covered with a polyethylene sheet that allows gas exchange but minimizes evaporation. Moisture was gravimetrically adjusted every other day with the addition of deionised water for each individual jar. The experiments were laid out as randomized block designs with four replicates per treatment.

Experiment 1. Impact of biochar on soil N_2O emissions and mineral N after the addition of different N fertilizers

A set of 48 jars [2 soils (HP/HC) × 2 management treatments (biochar/control) × 3 fertilization treatments (no fertilizer/KNO_3/$CO(NH_2)_2$) × 4 replicates] was set up for the first experiment. The fertilizers were homogeneously distributed in the soil at a rate of 200 kg N Ha^{-1} (corresponding to 55 mg N kg^{-1} based on a plough layer of 25 cm). N_2O samples were taken twice a day during the first 2 days decreasing subsequently to daily measurements, then every other day, then three times per week, etc. (see **Figure 1**). At the end of the incubation (14 days) mineral N (NH_4^+, NO_3^-, and NO_2^-) was extracted and determined in all jars.

Experiment 2. Isotopic composition of N_2O and N_2 emitted after application of labeled ^{15}N fertilizers

The following ^{15}N-tracer experiments were performed:

(i) Soil HP + $^{15}NO_3^-$, vs. soil HP + $^{15}NO_3^-$ + biochar,
(ii) Soil HC + $^{15}NO_3^-$ vs. soil HC + $^{15}NO_3^-$ + biochar,
(iii) Soil HC + $CO(^{15}NH_2)_2$ vs. Soil HC + $CO(^{15}NH_2)_2$ + biochar

Moisture was adjusted to 90% WFPS in each jar by adding the required volume of a solution containing $K^{15}NO_3$ or $CO(^{15}NH_2)_2$ (>99% ^{15}N enrichment) at the appropriate concentration to obtain 90% WFPS and exactly 5.5 mg of ^{15}N-per jar. Rewetting the soils in this way guaranteed a homogenous ^{15}N pool. Gas samples for isotopic analysis were taken daily during the

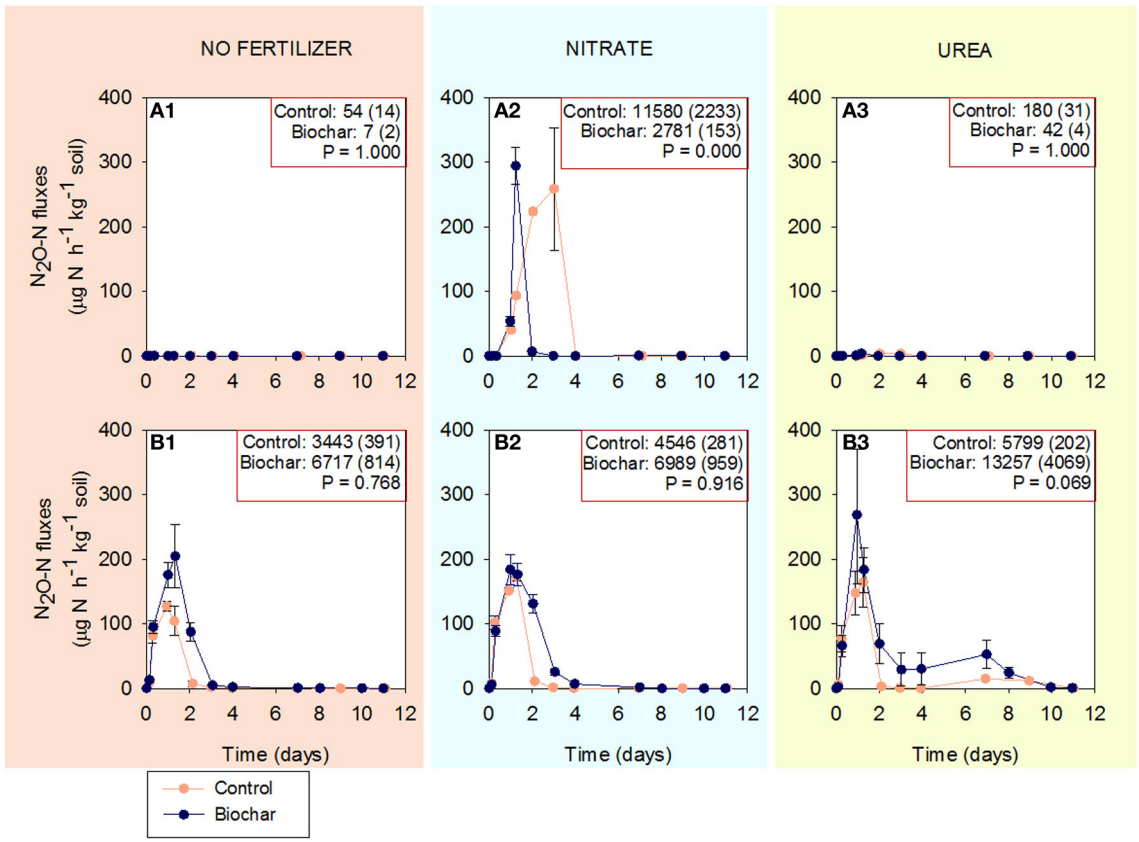

FIGURE 1 | Fluxes of N_2O in soils HP and HC in control and biochar treatments (mean ± *SE*; *n* = 4). (A1–A3) correspond to soil HP unfertilized, fertilized with KNO_3 and fertilized with $CO(NH_2)_2$, respectively. **(B1–B3)** correspond to soil HC unfertilized, fertilized with KNO_3 and fertilized with $CO(NH_2)_2$, respectively. Cumulative N_2O emissions (μg N-N_2O kg^{-1} soil) and standard errors by the end of the incubation are reported in the right-above box for each treatment.

first 3 days and on day 10. For each treatment, two gas samples were collected using a 12-ml syringe and needle: one immediately after the screw cap was fitted to the jar ($t = 0$) and the second after 60 min ($t = 60$). The gas samples were transferred to 12-ml vials (Labco) previously purged with He and evacuated. Selected samples (a total of 192 samples) were analyzed for the isotope ratios of N_2 [29/28 (29R) and 30/28 (30R)] and N_2O [45/44 (45R) and 46/44 (46R)] by automated isotope ratio mass spectroscopy [ThermoFinnigan GasBench and PreCon trace gas concentration system interfaced to a ThermoScientific Delta V Plus isotope-ratio mass spectrometer (Bremen, Germany)].

Experiment 3. N_2O emissions, mineral N, and N_2O isotopic composition after addition of NO_2^- in soil HC

Experiments 1 and 2 were reproduced in soil HC with a different source of nitrogen: $NaNO_2$ was added to a set of 8 jars [4 replicates × 2 management treatments (biochar/control)] and homogeneously distributed in the soil at a rate of 200 kg N Ha^{-1}. N_2O and final concentrations of mineral N were determined as for Experiment 1 (see **Figure 5**).

Subsequently, the following ^{15}N tracer experiment was performed: Soil HC +$Na^{15}NO_2$ vs. Soil HC + $Na^{15}NO^2$ + biochar (as for Experiment 2).

Moisture was adjusted to 90% WFPS in each jar by adding the required volume of a solution containing $NaNO_2$ (>98% ^{15}N enrichment) at the appropriate concentration to obtain 90% WFPS and exactly 5.5 mg of ^{15}N-per jar. Gas samples for isotopic analysis were taken daily during the first 3 days and on the 10th day of incubation in the same way as in Experiment 2. A total of 64 gas samples [2 management treatments (biochar/control) × 4 replicates × 4 days (1/2/3/10) × 2 times per day ($t = 0/t = 60$)] were analyzed.

Experiment 4. N_2O emissions and mineral N after addition of dicyandiamide to soil HC

The nitrification inhibitor dicyandiamide (DCD) was applied in combination with N fertilizers in soil HC. DCD inhibits the first stage of nitrification, the oxidation of NH_4^+ to NH_2OH, by rendering the enzyme ammonia monooxygenase (AMO) ineffective. It is not a bactericide, and does not affect other heterotrophs responsible of the soil biological activity (Zacherl and Amberger, 1990).

A set of 24 jars [2 management treatments (biochar/control) × 3 fertilization treatments (no fertilizer/KNO_3/$CO(NH_2)_2$) × 4 replicates] was set up for the experiment. DCD was applied at a rate of 30 mg kg^{-1} soil to ensure its persistence over the entire

incubation period (Rajbanshi et al., 1992). The fertilizers were homogeneously distributed in the soil at the same rate as in the previous experiments (200 mg N Ha^{-1}) in the solution including the DCD. N_2O samples were taken following the same intervals as in Experiment 1. Mineral N (NH_4^+, NO_3^-, and NO_2^-) was also extracted and determined in all jars at the end of the incubation period.

N_2O SAMPLING AND MEASUREMENTS

For N_2O sampling each unit was sealed with gas-tight polypropylene screw caps for an accumulation period of 60 min. The headspace gas was then sampled directly with a membrane air pump (Optimal 250, Schego, Offenbach am Main, Germany), attached to a gas chromatograph (VARIAN CP-4900 Micro-GC, Palo Alto, CA, USA) (Mondini et al., 2010).

N_2O fluxes were calculated assuming a linear increase during the accumulation (closed) period, an approach which was verified prior to the experiments. Cumulative N_2O was calculated assuming linear changes in fluxes between adjacent measurement points (Velthof et al., 2003).

CHEMICAL-PHYSICAL ANALYSES OF BIOCHAR AND SOILS

Biochar

Proximate analysis was conducted using ASTM D1762-84 Chemical Analysis of Wood Charcoal. Total N and C were analyzed by automatic elemental analysis (FlashEA 1112 Series, Thermo scientific, Madrid, Spain). Water soluble C and N were determined in 1:10 (w/v) water extracts using a Photometer Nanocolor 500 D MACHEREY-NAGEL. Electrical conductivity (EC) and pH were determined in a 1:10 (w/v) water-soluble extract. NH_4^+ was extracted with 2.0 M KCl at 1:10 (w/v) and determined by a colorimetric method based on Berthelot's reaction. NO_3^- and NO_2^- were extracted with water at 1:10 (w/v) and determined by ion chromatography (HPLC, model 861, Metrohm AG, Herisau, Switzerland).

Soil

Soil texture was determined using the pipette method according to Kettler et al. (2001). Soils were extracted by shaking four replicates of moist soil (1/10, w/v dry weight basis) with 2.0 M KCl (for NH_4^+) or water (for NO_3^- and NO_2^-) for 2 h. Extracts were centrifuged (2509 G) and filtered (0.45 μm) before analysis. NH_4^+ was determined by a colorimetric method based on Berthelot's reaction. NO_3^- and NO_2^- were determined by ion chromatography (HPLC, model 861, Metrohm AG, Herisau, Switzerland).

^{15}N CALCULATIONS

The ^{15}N atomic fraction in N_2O was calculated from the 45/44 and 46/44 ratios of N_2O. The ^{15}N gas-flux method (Mulvaney and Boast, 1986; Stevens et al., 1993; Stevens and Laughlin, 2001) was used to quantify N_2O and N_2 emissions from denitrification in soil HP. The molar fraction of ^{15}N-NO_3^- ($^{15}X_N$) in the soil pool was calculated from Δ45R and Δ46R according to Stevens and Laughlin (2001). The flux of N_2 and N_2O was then calculated by the equations given by Mulvaney and Boast (1986). The presence of hybrid nitrous oxide ($^{45}N_2O$) co-metabolically introduced into the reaction pathway of denitrification was tested by the model developed by Spott and Florian Stange (2011). This model considers two different N sources, where each source generates non-hybrid N_2O ($^{46}N_2O$ and $^{44}N_2O$) and, simultaneously, both N sources can be combined to form hybrid N_2O ($^{45}N_2O$). According to this model, the contribution of each pathway to the total N_2O formation can be calculated from the mass distribution of the released N_2O and the ^{15}N mole fraction of the labeled N source (Spott and Florian Stange, 2011).

STATISTICAL ANALYSIS

Univariate analysis of variance was used to investigate the significant differences in N_2O emissions and mineral N concentrations between biochar and control treatments with IBM SPSS Statistics 21, Sommers, USA.

RESULTS

EXPERIMENT 1. CUMULATIVE N_2O EMISSIONS AND MINERAL N IN SOILS A AND B

Soil HP emitted N_2O when NO_3^- was added but not in the absence of fertilizer or after the addition of urea. In this soil, biochar significantly reduced N_2O emissions, by an average of 76% (**Figures 1A1–A3**).

Soil HC emitted N_2O in all treatments: without N fertilization, after the addition of NO_3^- and urea. In this soil, biochar consistently increased total cumulative N_2O emissions and the average increase was larger in the non-fertilized (95%) and urea (129%) treatments than in the NO_3^- treatment (54%), (**Figures 1B1–B3**).

Comparing treatments without biochar, the addition of NO_3^- increased total N_2O emissions in soil HP (from 54 to 11580 μg N_2O-N kg^{-1} soil), whereas it increased N_2O emissions slightly in soil HC (from 3443 to 4546 μg N_2O-N kg^{-1} soil). The addition of urea had no impact on soil HP, and increased emissions in soil HC (from 3443 to 5799 μg N_2O-N kg^{-1} soil).

Figure 2 shows NH_4^+-N concentration in soils HP and HC at the end of the experiment. The original concentration of NH_4^+ in soil HP was 19.3 mg N kg^{-1} soil. After 14 days of incubation, soil HP underwent a significant increase in NH_4^+ content for all fertilization treatments (74.5–110.4 mg N kg^{-1} soil). The highest increase was observed when soil HP was fertilized with urea. Biochar addition did not have a significant impact on the final NH_4^+ concentration in this soil.

Soil HC similarly increased its NH_4^+ concentration throughout the incubation (initial concentration: 2.8 mg kg^{-1} soil), excluding the KNO_3 treatment. In this soil biochar significantly decreased the amount of NH_4^+ by the end of the incubation for the non-fertilized soil. Biochar also decreased mean NH_4^+ concentration in the urea treatment, although not significantly due to the high variability in the biochar samples.

Figure 3 shows ($NO_3^- + NO_2^-$)-N concentrations in soils HP and HC. The concentrations of ($NO_3^- + NO_2^-$)-N in soil HP were very low (<2.0 mg kg^{-1}) for all fertilization treatments and biochar did not have a significant impact. However, NO_2^- was detected in biochar amended soils and not in the control. Soil HC had low ($NO_3^- + NO_2^-$)-N concentrations when no fertilizer was added or after the addition of urea. In contrast, 33.3 mg of NO_3^--N kg^{-1} were found in the KNO_3 treatment irrespective of the biochar addition.

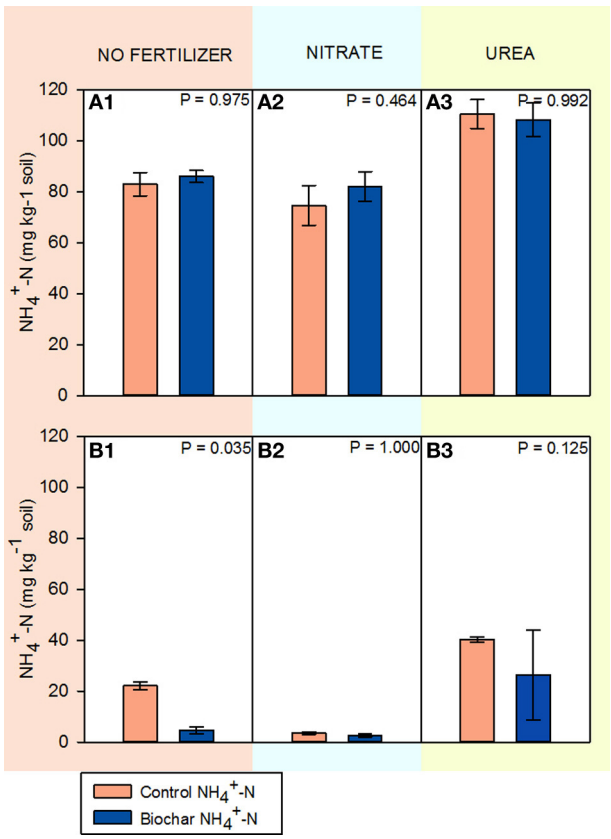

FIGURE 2 | NH_4^+-N concentrations in soils HP and HC after 14 days of incubation (mean ± *SE*; *n* = 4). (A1–A3) correspond to soil HP unfertilized, fertilized with KNO_3 and fertilized with $CO(NH_2)_2$ respectively. **(B1–B3)** correspond to soil HC unfertilized, fertilized with KNO_3 and fertilized with $CO(NH_2)_2$ respectively.

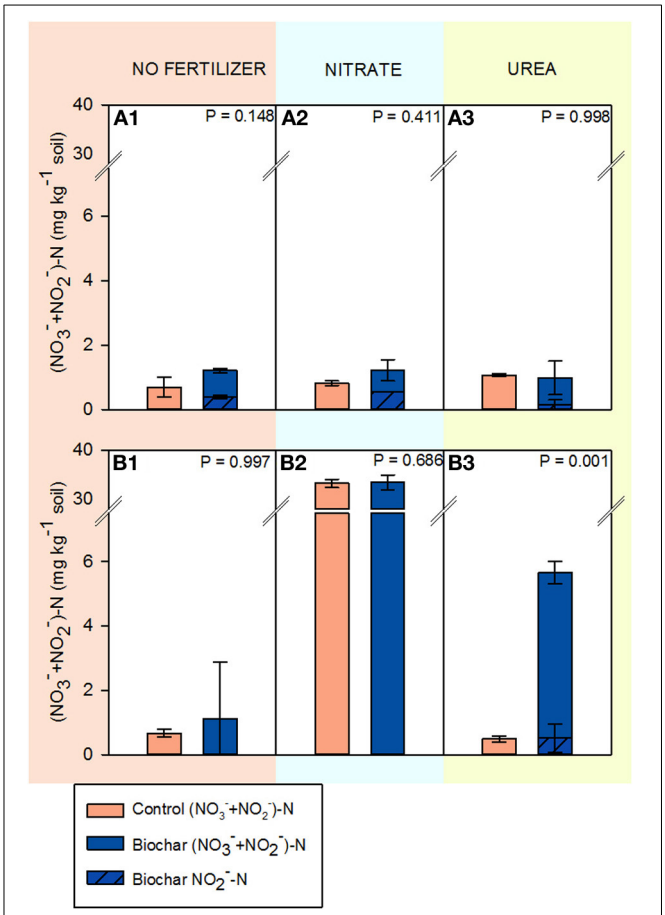

FIGURE 3 | (NO_3^- + NO_2^-)-N concentrations in soils HP and HC after 14 days of incubation (mean ± *SE*; *n* = 4). (A1–A3) correspond to soil HP unfertilized, fertilized with KNO_3 and fertilized with $CO(NH_2)_2$, respectively. **(B1–B3)** correspond to soil HC unfertilized, fertilized with KNO_3 and fertilized with $CO(NH_2)_2$, respectively.

EXPERIMENT 2. ISOTOPIC COMPOSITION OF N_2O EMITTED FROM SOILS A AND B

Figure 4 shows the ^{15}N atomic fraction in N_2O emitted from soils HP and HC in Experiment 2. When $^{15}NO_3^-$ was added, the initial ^{15}N atomic fraction in N_2O emitted from soil HP was 0.74, decreasing gradually to reach 0.04 at day 10 (**Figure 4A**). In contrast, the ^{15}N isotopic composition in soil HC followed totally different dynamics: the initial ^{15}N atomic fraction in N_2O was only 0.18; it increased slightly to 0.33 by day three, and reached a final value of 0.10 by day 10 (**Figure 4B1**). Biochar altered the isotopic composition of N_2O emitted in both soils.

When urea was added, soil HP did not emit N_2O (**Figure 1A3**). In soil HC (even when emissions were high) the initial ^{15}N atomic fraction in N_2O was zero (**Figure 4B2**), it successively increased, but always remained beneath 0.15. The biochar and control treatments showed identical ^{15}N-N_2O concentration dynamics.

Table 2 shows the molar fraction of ^{15}N-NO_3^- and the ratio $N_2O/(N_2+N_2O)$ calculated by the ^{15}N gas flux method (Mulvaney and Boast, 1986) and the contribution of codenitrification to N_2O formation according to Spott and Florian Stange (2011) in soil HP. The ratio $N_2O/(N_2+N_2O)$ was very high during the first 3 days, which demonstrates that most N was lost as N_2O. Biochar decreased the N_2O/N_2 ratio, particularly at day three (the peak of emissions in the control soil). The contribution of codenitrification was zero (see C in **Table 2**). This method of calculation could not be applied to soil HC, since other mechanisms than denitrification were operating in this soil and we could not calculate the enrichment of the source [$^{15}NO_3^-$ in soil ($^{15}X_N$)] (Mulvaney and Boast, 1986). Nonetheless, we found a high proportion of N_2O with a hybrid bond ($^{45}N_2O$) in soil HC.

EXPERIMENT 3. N_2O EMISSIONS, ^{15}N ISOTOPIC COMPOSITION AND MINERAL N AFTER FERTILIZATION OF SOIL HC WITH NO_2^-

Addition of NO_2^- to soil HC produced the highest N_2O emissions peak monitored in this soil (**Figure 5B1**); fourfold higher than that of the non-fertilized soil (**Figure 1B1**). Under these conditions, the biochar amendment did not modify cumulative N_2O emissions.

The ^{15}N atomic fraction in N_2O (**Figure 5B2**) followed a different pattern than with $^{15}NO_3^-$ (Experiment 2; **Figure 4B1**). The initial ^{15}N atomic fraction in the N_2O emitted was 0.30,

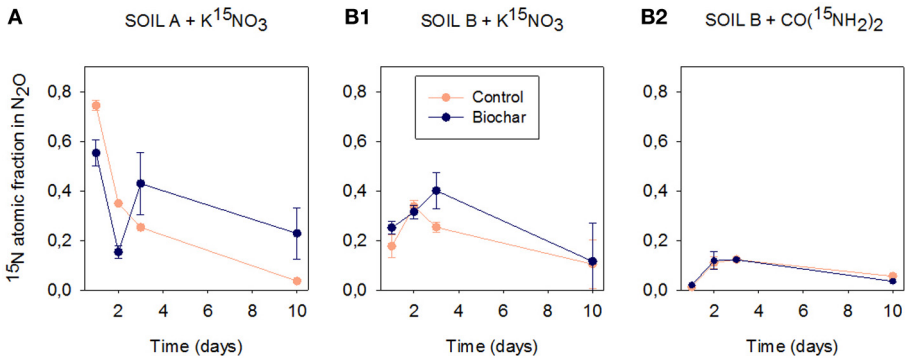

FIGURE 4 | ^{15}N atomic fraction in N_2O emitted from soils HP and HC in control and biochar treatments after 1, 2, 3, and 10 days of incubation (mean ± *SE*, *n* = 4). **(A)** corresponds to soil HP fertilized with $K^{15}NO3$ (>99% enrichment). **(B1)** and **(B2)** correspond to soil HC amended with $K^{15}NO_3$ and $CO(^{15}NH_2)_2$ respectively (both at >99% enrichment).

Table 2 | Means and standard deviations (*n* = 4) of $^{15}X_N$, the ratio $N_2O/(N_2+N_2O)$ and the three fractions (A, B, C) of hybrid and non-hybrid N_2O (Spott and Florian Stange, 2011) in soil HP.

Parameter	Treatment	Time (days)			
		1	2	3	10
$^{15}X_N$	Control	0.98 (0.00)	0.99 (0.00)	0.99 (0.00)	0.84 (0.01)
(molar fraction of ^{15}N-NO_3^- in soil, calculated by the ^{15}N gas flux method)	Biochar	0.99 (0.00)	0.99 (0.00)	0.92 (0.06)	0.91 (0.09)
$N_2O/(N_2+N_2O)$	Control	1.01 (0.12)	0.99 (0.01)	0.99 (0.00)	0.14 (0.27)
(calculated by the ^{15}N gas flux method)	Biochar	0.93 (0.05)	0.89 (0.08)	0.04 (0.05)	0.05 (0.08)
A	Control	0.19 (0.05)	0.02 (0.02)	0.00 (0.00)	0.95 (0.00)
(fraction of non-hybrid N_2O from the unlabeled source)	Biochar	0.03 (0.03)	0.01 (0.01)	0.48 (0.28)	0.75 (0.22)
B	Control	0.81 (0.05)	0.98 (0.02)	1.00 (0.00)	0.05 (0.00)
(fraction of non-hybrid N_2O from the labeled source)	Biochar	0.99 (0.03)	1.00 (0.01)	0.49 (0.31)	0.24 (0.22)
C	Control	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
(fraction of hybrid N_2O formed by a 1:1 linkage of labeled and unlabeled sources)	Biochar	−0.02 (0.00)	−0.02 (0.00)	0.04 (0.03)	0.01 (0.00)

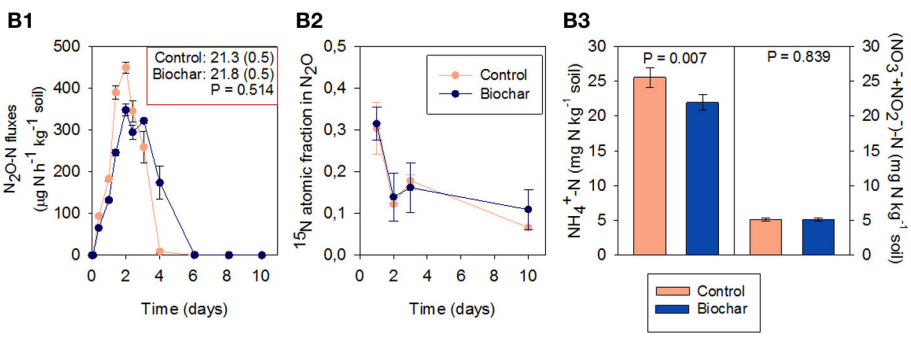

FIGURE 5 | Fluxes of N_2O (B1) ^{15}N atomic fraction in N_2O at days 1, 2, 3, and 10 (B2) and NH_4^+-N and NO_3^--N concentrations after 14 days of incubation (B3) in soil HC (mean ± *SE*, *n* = 4). Soil HC had been fertilized with $Na^{15}NO_2$ (>98% enrichment). Cumulative N_2O emission (mg N-N_2O kg^{-1} soil) and standard error by the end of the incubation is reported in the right-above box in **B1**.

decreasing gradually to reach 0.06 at day 10 (**Figure 5B2**). Biochar did not significantly modify this pattern.

The biochar amended soil had a significantly lower concentration of NH_4^+ at the end of the incubation (**Figure 5B3**). The concentration of NO_3^- was low (below 5 mg kg^{-1} soil) and not affected by biochar addition.

EXPERIMENT 4. IMPACT OF THE NITRIFICATION INHIBITOR DICYCIANDIAMIDE (DCD) ON N_2O EMISSIONS AND MINERAL N CONCENTRATION IN SOIL HC

N_2O emissions almost ceased when DCD was added to soil HC (**Figure 6**). The highest emissions were observed when the soil was fertilized with NO_3^- (**Figure 6B2**), but still represented less

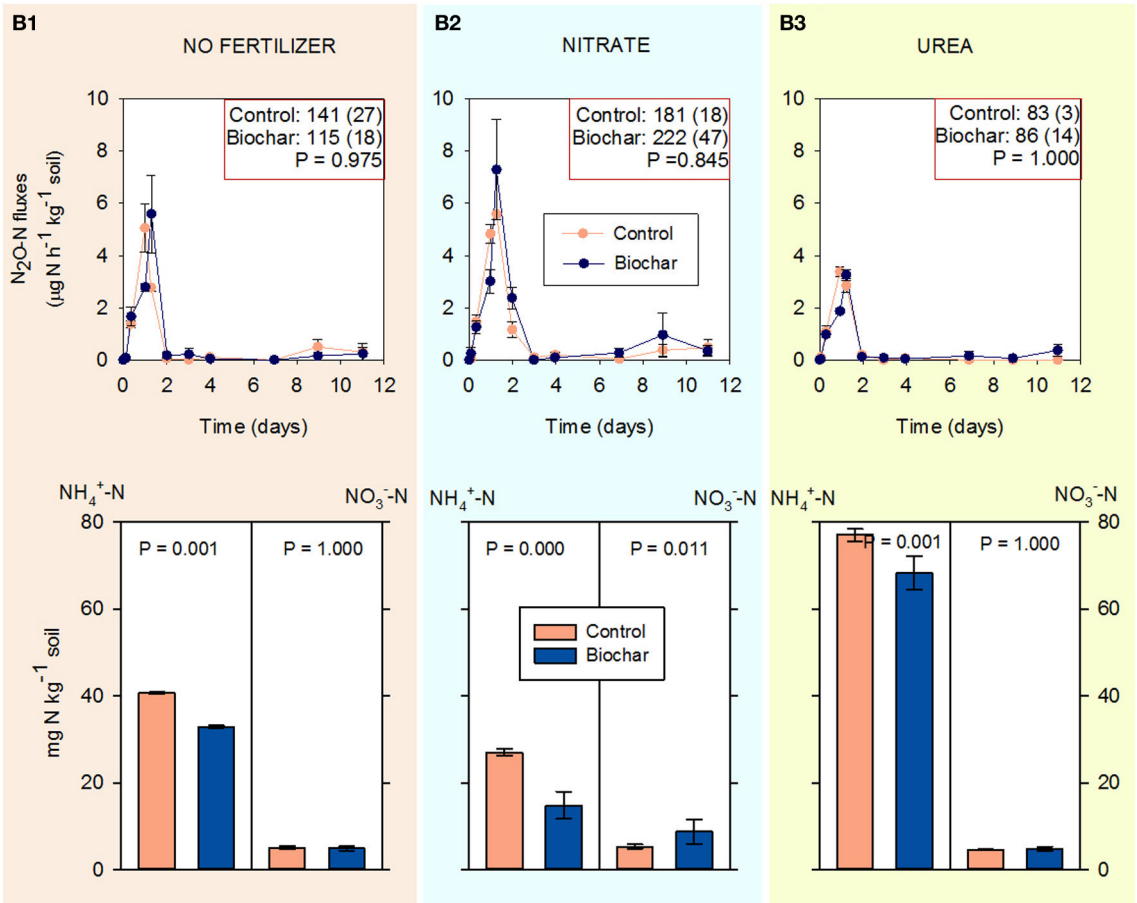

FIGURE 6 | Fluxes of N_2O (above) and NH_4^+-N and NO_3^--N concentrations during 14 days of incubation (below) in soil HC containing DCD (30 mg kg^{-1} soil) in control and biochar treatments (mean ± *SE*, $n = 4$). (B1–B3) correspond to unfertilized soil, fertilized with KNO_3 and fertilized with $CO(NH_2)_2$ respectively. Cumulative N_2O emissions (μg N-N_2O kg^{-1} soil) and standard errors by the end of the incubation are reported in the right-above box for each treatment.

than 0.4% of the added N (compared to 12.7% without DCD (**Figure 1B2**).

The highest NH_4^+ concentrations were found in the soil amended with urea, followed by the non-fertilized soil and the soil amended with KNO_3. Biochar (compared to the control) systematically decreased the concentration of NH_4^+ by the end of the incubation for all treatments (non-fertilized soil, KNO_3, and urea). NO_3^- concentration was lower than the original in soil (16.9 mg NO_3^--N kg^{-1} soil).

DISCUSSION

PRE-DOMINANT N_2O FORMATION PATHWAYS IN SOIL HP AND HC

Nitrous oxide emissions patterns and their response to the addition of different N fertilizers were different in soils HP and HC, which clearly reflected the different N_2O production pathways involved.

Figure 7 illustrates the main pathways for N_2O formation in soil. Ammonia oxidation takes place in two steps: first NH_3 is oxidized to NH_2OH, which is then oxidized to NO_2^-. N_2O may be directly released as a by-product of ammonia oxidation (nitrifier-nitrification) (Hooper and Terry, 1979) or it can be produced through a denitrification pathway where NO_2^- is reduced to N_2O (nitrifier-denitrification) (Kool et al., 2011). The ability to denitrify is a widespread, if not ubiquitous, attribute in ammonia oxidizers (Shaw et al., 2006). Classically, denitrification (from NO_3^-) has been considered the main N_2O formation pathway in soils. However, other pathways that have been systematically overlooked in soil studies could play a more important role than originally estimated (Baggs, 2011; Spott et al., 2011). This is the case for codenitrification, which is potentially a widespread pathway of microbial N transformation in terrestrial environments (Spott et al., 2011) and dissimilatory nitrate reduction to ammonia (DNRA) (Giles et al., 2012). Although our knowledge of microbial N transformation in soil has evolved significantly over the last decades, recent findings show that, even today, our understanding of N_2O formation and consumption in soil is still very limited (Sanford et al., 2012; Long et al., 2013).

In the nearly water-saturated soil conditions used in our experiments (90% WFPS), N_2O production is expected to be dominated by denitrification of NO_3^-. This was the case in soil HP, where emissions were clearly controlled by the conventional denitrification pathway. This can be deduced from the following

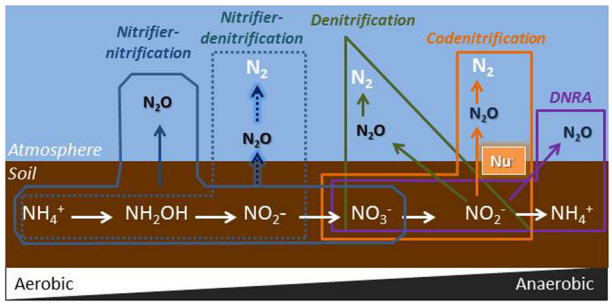

FIGURE 7 | Microbial sources of N_2O during transformations of mineral nitrogen in soil. Nu^-: nuclophile (e.g., R-NH_2, NH_4^+, amino acids or other organic N compounds). During codenitrification, nitrous acid reacts with a nucleophile in soil through nitrosation reactions forming a hybrid N-N bond (Spott et al., 2011); DNRA, dissimilatory nitrate reduction to ammonium.

facts: (i) This soil only emitted N_2O after the addition of NO_3^- (**Figure 1A2**); (ii) the ^{15}N atomic fraction of the N_2O emitted at day one was 0.74 (**Figure 4A**), which shows that N_2O was primarily produced from the added $^{15}NO_3^-$. The ^{15}N atomic fraction decreased over time, showing the depletion of the labeled source; (iii) given the limited nitrification activity detected in this soil, addition of NO_3^- did not increase the final NH_4^+ concentration (with respect to the non-fertilized soil), which suggests that DNRA was not a relevant pathway, and (iv) applying the equations developed by Spott and Florian Stange (2011), codenitrification was found to be null (**Table 2**).

As previously found in other soils under analogous optimal denitrifying conditions (Cayuela et al., 2013), biochar significantly decreased total N_2O emissions in this soil.

In soil HC, the weak response of N_2O emissions to NO_3^- addition pointed out to a low contribution of denitrification or DNRA in this soil. Given that the original NO_3^- concentration in the soil was 16.9 mg N kg^{-1} at a natural abundance of 0.364% ^{15}N, and that we added 55 mg N kg^{-1} of $^{15}NO_3^-$ (>99% enrichment), the ^{15}N-NO_3^- enrichment in the soil at the beginning of the incubation was 75.8%. Yet, the ^{15}N atomic fraction in the N_2O emitted at day one (**Figure 4B1**) was only 0.18, which demonstrates that some N_2O originated from denitrification, but also that NO_3^- was not the only source of N_2O. Moreover, the low C:N ratio of this soil and the NH_4^+ concentration at the end of the incubation in the KNO_3 treatment (**Figure 2B2**) indicates that DNRA was not a major N_2O formation route in this soil (Giles et al., 2012). Instead, we hypothesize that N_2O formation in soil HC was mainly the result of nitrification-mediated processes. The results supporting this hypothesis can be summarized: (i) The addition of extra NO_3^- did not increase N_2O emissions in this soil, whereas the addition of extra urea did; (ii) the ^{15}N atomic fraction of the N_2O emitted at day one was 17.7% (**Figure 4B1**), which shows that N_2O was not pre-dominantly formed from the added $^{15}NO_3^-$. (iii) The concentration of dissolved organic N in this soil was very high (35.9 mg N kg^{-1} soil), which can explain the low contribution of the labeled urea to the emitted $^{15}N_2O$ (**Figure 4B2**). However, significant hybrid N_2O ($^{45}N_2O$) was produced (data not shown) and we cannot discard the contribution of codenitrification to N_2O formation in soil HC.

To better understand which processes (within nitrification-mediated pathways) biochar might be modifying we performed Experiments 3 and 4.

IMPACT OF BIOCHAR IN N_2O BY NITRIFICATION-MEDIATED PATHWAYS

In Experiment 3 the addition of NO_2^- to soil HC showed that, under high moisture conditions, this soil was able to rapidly reduce NO_2^- to N_2O, which was emitted in large quantities (38% of added NO_2^--N). It is very unlikely that the N_2O emitted was just the product of the chemical decomposition of NO_2^- (chemodenitrification), since this process, largely controlled by soil pH, only occurs in neutral and acidic soils (Bremner, 1997). Instead, NO_2^- was most probably used as electron acceptor for microbial respiration (nitrifier-denitrification). The high N_2O production in Experiment 3 (21.3 mg N kg^{-1} compared to 3.4 mg N kg^{-1} in Experiment 1) may be related to enhanced nitrifier-denitrification for detoxifying NO_2^- (Jung et al., 2014).

The subsequent tracer experiment with application of $^{15}NO_2^-$, demonstrated that significant nitrite reduction to N_2O occurs (the N_2O originating from the added $^{15}NO_2^-$ at day one was 31.5%, see **Figure 5B2**), but also that it could not be the only process leading to N_2O emissions. This experiment demonstrated that biochar was not increasing N_2O emissions through the nitrifier-denitrification pathway, since N_2O emissions in the biochar and control treatments were not statistically different.

In our final experiment (Experiment 4), the high NH_4^+ and low NO_3^- concentrations by the end of the experiment demonstrate the effectiveness of the DCD treatment to inhibit ammonia oxidation, which correlated with a large decrease in N_2O emissions for all treatments. We assumed that DCD did not inhibit other possible N_2O formation pathways. Although the impacts of DCD on other aspects of microbial N transformation in soil are largely unknown, Bremner and Yeomans (1986) demonstrated that DCD does not inhibit N_2O and N_2 emissions by denitrification when applied at similar rates to those used in this study. More recently, Wakelin et al. (2013) also demonstrated in a field study that the application of DCD had a minor impact on denitrifying bacteria activity (*nir*S).

Addition of biochar significantly and consistently decreased the NH_4^+ concentration in soil HC. These results reinforce our conclusion that the production of N_2O in soil HC must be the consequence of nitrification processes (nitrifier-nitrification and associated nitrifier-denitrification). It seems that biochar does not promote the denitrification from NO_2^- (as was deduced from Experiment 3), but it does promote the oxidation of ammonia and concomitantly the formation of N_2O through nitrifier-nitrification. Clearly, if biochar raises the production of NO_2^- in soil, it will intrinsically enhance its denitrification (nitrifier-denitrification) when the soil is under low oxygen conditions (as in our experiments).

Our results are in agreement with recent findings by Prommer et al. (2014), who showed that biochar promotes soil ammonia-oxidizer populations and accelerates gross nitrification rates in a calcareous arable soil. The importance of nitrifier-nitrification

and nitrifier-denitrification for N_2O production in calcareous soils has been recently documented by Huang et al. (2014), who demonstrated that these processes accounted for 35–53% and 44–58% of total N_2O emissions, respectively.

Here we present preliminary evidence that explains how biochar might affect N_2O emissions differently depending on the N_2O formation pathway operating in the soil. When denitrification was the main N_2O formation pathway (soil HP), biochar was found to decrease the $N_2O/(N_2 + N_2O)$ ratio (**Table 2**), which is in agreement with previous findings (Cayuela et al., 2013). Recent studies have reported that biochar promotes an increase in the abundance of nitrous oxide reductase (nosZ) in soil (Harter et al., 2014), an enzyme that enhances the reduction of N_2O to N_2 (the last step in denitrification). In contrast, when N_2O was produced by nitrification (soil HC), biochar addition might have increased emissions by promoting gross nitrification. To our knowledge, there are not published studies explicitly relating to biochar and nitrification-N_2O production.

Another question that arises from this study is: why these two soils under identical experimental conditions follow different N_2O formation pathways, which we hypothesize might be linked to different soil microbial communities. In conclusion, predicting which N_2O formation pathway pre-dominates in a certain kind of soil will be necessary for guaranteeing the success of biochar as a N_2O mitigation strategy.

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