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1 Biochar suppresses N₂O emissions while 2 maintaining N availability in a sandy 3 loam soil

4 Running title: Biochar, soil N₂O suppression and N availability

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11

12 **Keywords**

13 Biochar, Nitrous oxide, Immobilisation, Denitrification, Mineralisation, Nitrification, Ammonium,

14 Nitrate, FLUAZ

15 **Abstract**

16 Nitrous oxide (N₂O) from agricultural soil is a significant source of greenhouse gas emissions.

17 Biochar amendment can contribute to climate change mitigation by suppressing emissions of N₂O
18 from soil, although the mechanisms underlying this effect are poorly understood. We investigated the
19 effect of biochar on soil N₂O emissions and N cycling processes by quantifying soil N
20 immobilisation, denitrification, nitrification and mineralisation rates using ¹⁵N pool dilution
21 techniques and the FLUAZ numerical calculation model. We then examined whether biochar
22 amendment affected N₂O emissions and the availability and transformations of N in soils.

23 Our results show that biochar suppressed cumulative soil N₂O production by 91 % in near-saturated,
24 fertilised soils. Cumulative denitrification was reduced by 37 %, which accounted for 85 - 95 % of
25 soil N₂O emissions. We also found that physical/chemical and biological ammonium (NH₄⁺)
26 immobilisation increased with biochar amendment but that nitrate (NO₃⁻) immobilisation decreased.
27 We concluded that this immobilisation was insignificant compared to total soil inorganic N content. In
28 contrast, soil N mineralisation significantly increased by 269 % and nitrification by 34 % in biochar-
29 amended soil.

30 These findings demonstrate that biochar amendment did not limit inorganic N availability to nitrifiers
31 and denitrifiers, therefore limitations in soil NH₄⁺ and NO₃⁻ supply cannot explain the suppression of
32 N₂O emissions. These results support the concept that biochar application to soil could significantly
33 mitigate agricultural N₂O emissions through altering N transformations, and underpin efforts to
34 develop climate-friendly agricultural management techniques.

35 1 Introduction

36 Nitrous oxide is a significant greenhouse gas (GHG) that has a global warming potential 298 times
37 that of carbon dioxide (CO₂) over a 100-year time period and is responsible for approximately 6 % of
38 total anthropogenic radiative forcing (Davidson, 2009). Agricultural land contributes approximately
39 60 % to global anthropogenic N₂O emissions; new agricultural practices are therefore needed to
40 minimise soil N₂O emissions and mitigate the effects of climate change (Reay et al., 2012; Smith et
41 al., 2007).

42 Biochar amendment to soil has been proposed as a method to increase soil C storage and suppress soil
43 N₂O emissions on a global scale (Woolf et al., 2010). Biochar consists of biomass heated in an O₂-
44 limited environment (typically to between 350 and 600 °C) that can be subsequently applied as a soil
45 amendment (Sohi et al., 2010). Laboratory incubations and several short-term field studies have
46 shown that biochar amendment can suppress soil N₂O emissions (Clough et al., 2013; Taghizadeh-
47 Toosi et al., 2011; Zhang et al., 2012). However, more extensive studies are needed to conclude with
48 certainty whether biochar addition has a consistent and long-term effect on soil N₂O emissions (Jones
49 et al., 2012; Spokas, 2012).

50 Denitrification, nitrification and nitrifier-denitrification are the three main processes that produce N₂O
51 in agricultural soils (Butterbach-Bahl et al., 2013; Kool et al., 2011). Denitrification is the primary
52 source, which also produces nitric oxide (NO) and dinitrogen (N₂) from nitrite (NO₂⁻) and nitrate
53 (NO₃⁻), whilst nitrification comprises the oxidation of ammonium (NH₄⁺) to NO₂⁻ and NO₃⁻. The rates
54 of denitrification and the relative proportions of N₂O, NO and N₂ produced by this process depend on
55 complex interactions between soil physico-chemical properties and climatic factors such as soil
56 temperature, pH, moisture status, and the availability of oxygen (O₂), nitrogen (N) and labile carbon
57 (C) (Gillam et al., 2008; Saggar et al., 2013; Šimek et al., 2002). The ratio of N₂O: N₂ produced via
58 denitrification decreases with increasing soil pH, labile C availability, soil water-filled pore space
59 (WFPS) and decreasing soil NO₃⁻ concentrations (Senbayram et al., 2012). Conditions that favour

60 nitrification include high soil NH_4^+ concentrations, high soil temperature and aerobic conditions
61 (greatest at a moderate WFPS, ~ 60 %) (Norton and Stark, 2011).

62 The mechanisms to explain how biochar amendment influences soil N_2O emissions are uncertain
63 (Spokas et al., 2012). Biochar affects soil aeration by increasing soil water holding capacity (WHC)
64 and decreasing soil bulk density (BD), conditions under which denitrifier activity is typically lower
65 (Basso et al., 2012; Karhu et al., 2011). However, we recently demonstrated that biochar-induced
66 suppression of soil N_2O emissions in soil subjected to wetting/drying cycles was not due to increased
67 soil aeration (Case et al., 2012).

68 One alternative mechanism for biochar N_2O suppression is a restriction in the availability of inorganic
69 N to soil nitrifiers and denitrifiers via immobilisation in biochar-amended soil (Bruun et al., 2012;
70 Case et al., 2012; Nelissen et al., 2014). Inorganic N availability may be affected by changes in the
71 rates of N mineralisation or nitrification. Increased gross mineralisation rates following biochar
72 addition have been attributed to stimulated mineralisation of native soil organic matter (Nelissen et
73 al., 2012), whilst increased nitrification rates have been attributed to greater soil pH in a biochar-
74 amended arable soil (Nelissen et al., 2012) and the uptake of inhibitive phenolic compounds by
75 biochar in a forest soil (DeLuca et al., 2006). However, research in this area is limited; the effect of
76 biochar amendment on the net availability of inorganic N to soil nitrifiers and denitrifiers and the
77 subsequent effect on soil N_2O emissions is poorly understood (Clough et al., 2013). This represents a
78 significant knowledge gap in determining the potential for biochar to contribute to climate change
79 mitigation. To address this knowledge gap, we analysed those soil N cycling processes that control
80 substrate availability for N_2O production (i.e. denitrification, nitrification, immobilisation and
81 mineralisation) in fertilised, near-saturated soil amended with biochar. Our aim was to identify
82 whether biochar affects the availability and transformations of N in arable soils underlying soil N_2O
83 emissions.

84 **2 Materials and methods**

85 **2.1 Biochar and field site description**

86 The field site near Lincoln, Lincolnshire, UK was cultivated with an arable rotation of three years of
87 wheat (*Triticum aestivum*) followed by one year of oilseed rape (*Brassica Napus*). The field received
88 a total of 140 kg N ha⁻¹ yr⁻¹ as ammonium nitrate (NH₄NO₃) divided into three separate applications.
89 The soil association of the field the samples were taken from was Beccles 1, which was a fine loam
90 over clay. The bedrock was a Charnmouth mudstone formation. The soil was a sandy loam (57 %
91 sand, 32 % silt and 10 % clay) with a bulk density (BD) of 1.39 g cm⁻³. The biochar (also used in a
92 previous study, Case et al. (2012)) was derived from a slow-pyrolysis batch process, heated first to
93 180 °C to release volatile gas, then to 400 °C for the next 24 hours, using the thinnings of hardwood
94 trees as feedstock (ash, oak and cherry, Bodfari Charcoal, UK). The biochar had a total C content of
95 72.3 %, a total N content of 0.71 %, low extractable inorganic N concentrations (< 1.0 and 1.3 mg N
96 kg⁻¹ of NH₄⁺ and NO₃⁻ respectively), and a pH of 9.25. For more biochar properties refer to the
97 supplementary information of Case et al. (2012).

98 A four-treatment factorial experiment using ¹⁵N pool dilution was designed to investigate the effects
99 of biochar amendment on N transformations in arable soil. Soil was collected from the field in
100 January 2012 (during which time winter wheat was growing), sieved to < 4 mm then covered and
101 stored at 4 °C. Biochar (< 2 mm) was mixed with soil at a rate of 2 % d. wt. soil (equivalent to 28 t ha⁻¹)
102 ¹). One week later, 100 g d. wt. soil was put into plastic containers (H 17.4 cm, D 11.6 cm, V = 1.7 l)
103 to 10 mm depth (bulk density, BD = 0.91 ± 0.02 g cm⁻³) and pre-incubated in the dark at 16 °C for
104 seven days to allow for any initial flush of soil CO₂ emissions (Reichstein et al., 2000; Reicosky,
105 1997). Mineral fertiliser in de-ionised water solution was added to the soil at a rate of 100 mg N kg⁻¹
106 (d. wt. soil, equivalent to 110 kg N ha⁻¹) in the form of ¹⁵NH₄NO₃ or NH₄¹⁵NO₃ (10 atom % ¹⁵N
107 enrichment, Sigma-Aldrich, USA), adjusting the soil to 90 % WFPS to create favourable conditions
108 for denitrification, and also N₂O production (Weier et al., 1993). Pre-tests had demonstrated that soil

109 CO₂ emissions were linear, and O₂ concentrations adequate over at least four days of enclosure, so the
110 containers were sealed for the duration of the incubation to enable a mass balance to be calculated.

111 At four time points after ¹⁵N addition (30 mins, 1, 2 and 4 days), four replicates of each treatment
112 were destructively sampled for total C and N content, soil pH, gravimetric moisture content (GMC),
113 extractable soil NH₄⁺ and ¹⁵NH₄⁺, NO₃⁻ and ¹⁵NO₃⁻, and organic N and ¹⁵N concentrations (methods in
114 Section 3.3). The first sampling time point was chosen as 30 minutes after ¹⁵N addition, when it was
115 assumed that the chemical or physical immobilisation of N was completed, and any further N
116 immobilisation came exclusively from biological processes (Mary et al., 1998).

117 At seven time points following ¹⁵N addition (0, 0.5, 1, 1.5, 2, 3 and 4 days), 10 ml gas samples were
118 taken from the soil container headspace for N₂O and CO₂ analysis using a gas-tight syringe and
119 injected into evacuated 3 ml vials (Labco, USA). For ¹⁵N₂O analysis, 80 ml headspace samples were
120 injected into evacuated 60 ml glass serum bottles (Wheaton Science Products, USA). After gas
121 samples were removed, laboratory air of equivalent volume (N₂O and CO₂ concentration analysed)
122 was injected into the enclosed sample headspace. This dilution of laboratory air was taken into
123 account in the final calculations of GHG emissions.

124 **2.2 Gas sampling and N₂O source separation**

125 Headspace gas samples were analysed for N₂O and CO₂ concentrations using the same Gas
126 Chromatograph system (PerkinElmer Autosystem XL, PerkinElmer, USA) described in Case et al.
127 (2014) and calibrated against certified standards (Air Products, UK).

128 For ¹⁵N₂O analysis, ~ 4 ml of the 80 ml sample was injected into a TraceGas Preconcentrator coupled
129 to an isotope ratio mass spectrometer (IRMS, Isoprime Ltd, UK) whereupon the sample was directed
130 through a series of chemical traps to remove H₂O and CO₂. The N₂O was cryogenically trapped under
131 liquid N. The waste was flushed out, and then the N₂O was further cryofocused in a second liquid N
132 trap prior to being introduced onto a 25 m x 0.32 mm Poraplot Q column (Chrompack column,
133 Varian, UK). The column separated N₂O from any residual CO₂, and both entered the IRMS via an
134 open split. The retention time between the first eluting CO₂ (< 2^{E-10} amplitude) and second eluting

135 N₂O peak typically fell in the range between 60 - 70 seconds to avoid isobaric interference of the CO₂
136 with the calculated ¹⁵N. The N₂O was directed towards the triple collectors of the IRMS where *m/z* 44,
137 *m/z* 45 and *m/z* 46 mass ions were measured. Mass/charge ratios for the *m/z* 44, *m/z* 45 and *m/z* 46 NO
138 were then recorded for each sample and delta values for both ¹⁵N were calculated with respect to N₂O
139 reference gas (BOC Industrial Gases, UK).

140 The experimental design allowed us to differentiate the source of N₂O emissions from nitrification +
141 nitrifier-denitrification and denitrification. The proportions of soil N₂O emissions attributed to the two
142 processes were calculated using Equation 1, based on data from the analysis of the ¹⁵NO₃⁻ labelled soil
143 treatment (Mathieu et al., 2006). Outputs greater than 100% and lower than 0% were rounded to the
144 nearest boundary.

$$d = \frac{(a_m - a_n)}{(a_d - a_n)} \text{ with } a_d \neq a_n \quad (1)$$

145 Where 'd' is the proportion of N₂O emissions from denitrification in a time period, 'a_m' is the average
146 % ¹⁵N atom enrichment of the N₂O mixture during the time period, 'a_n' is the average % ¹⁵N
147 enrichment of the nitrification pool (NH₄⁺) during the time period and 'a_d' is the average % ¹⁵N
148 enrichment of the denitrification pool (NO₃⁻) during the time period.

149 **2.3 Analysis of soil properties and soil N isotopic composition**

150 Extractable inorganic NH₄⁺ and NO₃⁻ concentrations were determined using 5 g d. wt. equivalent of
151 wet soil and 50 ml of 0.8 M potassium chloride (KCl, 6 %). The samples were shaken for 1 hour, and
152 then filtered through Whatman no. 44 filter paper disks (Whatman, USA). Extracts were analysed on
153 a Seal AQ2 analyser (Bran and Luebbe, UK) using discrete colorimetric procedures (Maynard and
154 Kalra, 1993).

155 Extractable inorganic ¹⁵N concentrations (¹⁵NH₄⁺ and ¹⁵NO₃⁻) were analysed following the acidified
156 disk method (Khan et al., 1998). First, inorganic N was extracted from soil, using 2 M KCl and the
157 same method as that described for inorganic N extraction above. Then, 20 ml of the extract was
158 placed in air-tight 500 ml glass jars (Kilner, USA). For ¹⁵NH₄⁺ concentrations, 0.2 g of magnesium

159 oxide (MgO) was added. For $^{15}\text{NH}_4^+ + ^{15}\text{NO}_3^-$ concentrations, 1 ml of 0.2 M sulfamic acid was added
160 to decompose NO_2^- , followed by 0.2 g of MgO and 0.2 g Devarda's alloy. Whatman no. 41 filter
161 paper disks (Whatman, USA) were suspended above the solution with added 5 μl of 2.5 M potassium
162 hydrogen sulphate solution. The jars were sealed and placed in a 30 °C environment for at least 72
163 hours to enable near 100 % adsorption of the extractant N. The filter disks were then dried at 40 °C
164 for 24 hours.

165 Three-quarters of each of the two filter papers were weighed together and sealed in a single tin
166 capsule (Elemental Microanalysis Ltd, UK). The samples were combusted using an automated
167 NA1500 elemental analyser (Carlo Erba, Italy) coupled to an IRMS (Dennis Leigh Technology, UK).
168 Atom % abundances of $^{15}\text{NO}_3^-$ were calculated from the ($^{15}\text{NH}_4^+ + ^{15}\text{NO}_3^-$) atom % abundance and
169 respective inorganic N concentrations using the method described in Khan et al., (1998).

170 Organic ^{15}N contents were used as an analogue for microbial biomass and were assumed to have an
171 atom % ^{15}N excess of 0.0025 % (Mary et al., 1998). First, 3 g of soil was oven dried at 80 °C for 24
172 hours, and then the dried soil was mixed with 10 ml of 1 M KCl in a 12 ml polystyrene test tube and
173 mechanically shaken for 15 minutes. The tube was then centrifuged for 15 minutes at 3,000 rpm and
174 subsequently the KCl was removed and replaced (Recous et al., 1998). This process was repeated four
175 times. Afterwards, the soil was dried at 80 °C for 24 hours. 50 mg of dried soil was sealed in a tin
176 capsule and analysed in the same fashion as described for the analysis of the acidified disks above.

177 The total C and N contents of dried, ground soil samples (0.1 g, < 1 mm) were analysed using a Tru-
178 spec total CN analyser (Leco Corp., USA) (Sollins et al., 1999). Gravimetric moisture content, soil pH
179 (soil: H_2O , 1: 2.5), particle density, BD and WFPS analyses were conducted according to standard
180 methods (Blake, 1965; Emmett et al., 2008; Ohlinger, 1995a, 1995b).

181 **2.4 Estimating soil N transformations with and without biochar**

182 To assess whether biochar amendment affected the availability and transformations of soil N
183 underlying N_2O production, we quantified mineralisation, immobilisation, nitrification and
184 denitrification rates using the FLUAZ numerical N-cycling model (Mary et al., 1998). The model

185 consists of two parts (Mary et al., 1998). First, a numerical model that solves differential equations
186 from the N and ^{15}N mass equations based on a 4th order Runge-Kutta algorithm with a variable time
187 step. Second, a non-linear fitting program to calculate N rates based on Marquardt's algorithm
188 (Marquardt, 1963).

189 Inorganic N, organic N and respective ^{15}N concentrations were input into the FLUAZ model and
190 analysed using a paired treatment design. The final model fitted mineralisation ('m + s',
191 mineralisation of soil organic N and biochar-derived N to NH_4^+), nitrification ('n', the conversion of
192 NH_4^+ to NO_3^-), immobilisation of NH_4^+ and NO_3^- ('ia' and 'in', the sum of NH_4^+ and NO_3^- taken up by
193 the organic N pool) and denitrification rates ('kd', the sum of conversion of NO_3^- to N_2O , NO or N_2),
194 over three time periods following ^{15}N addition (30 minutes - 1 day, 1 - 2 days, 2 - 4 days).

195 For the FLUAZ model analysis we made several assumptions. As the incubation only lasted for four
196 days, and the temperature was maintained at 16 °C it was assumed that remineralisation of
197 immobilised N ('r') was negligible (Murphy et al., 2003). It was also assumed that the conversion of
198 plant residue N directly into microbial biomass ('j', N humification) and ammonia volatilisation were
199 negligible (Mary et al., 1998; Whitehead and Raistrick, 1990).

200 **2.5 Statistical analysis**

201 Student's t tests were used to test for significant differences in soil N_2O and CO_2 emissions, inorganic
202 N contents, total C, N and pH between un-amended and amended soil. For all statistical analyses the
203 software package R was used (version 3.0.2, The R Project, 2013).

204 **3 Results**

205 Cumulative soil N₂O emissions after four days were suppressed by 91 % with biochar amendment,
206 from 0.61 ± 0.20 to 0.05 ± 0.02 mg N₂O-N kg⁻¹ for un-amended and amended soils respectively (two-
207 sample t-test, $p < 0.05$, $t = 2.5$, $df = 13$, Fig. 1a). Soil CO₂ production was 56 compared to 32 mg CO₂-
208 C kg⁻¹ in amended and un-amended soil respectively over the same time period, equivalent to a 75%
209 increase (two-sample t-test, $p < 0.001$, $t = 4.7$, $df = 13$, Fig. 1b).

210 Using ¹⁵N analysis of N₂O emissions (Fig. 1c, d), and soil NH₄⁺ and NO₃⁻ concentrations (Fig. 1e, f),
211 soil N₂O emissions were source partitioned over the four-day incubation period. Nitrification +
212 nitrifier denitrification produced 40% and 33% of N₂O emissions in amended and un-amended soils
213 respectively from day 0 to 2 (Fig. 1e, f). Between day 2 and 4, all soil N₂O emissions were produced
214 via denitrification in both treatments. Considering the entire four-day incubation, 95% of un-amended
215 soil N₂O emissions came from denitrification, compared to 85% in amended soil (Fig. 1e, f).

216 To test whether transformations of soil N were affected by biochar amendment we analysed the
217 concentrations and isotope ratios of inorganic and organic N and input these data into the FLUAZ
218 model. Soil NH₄⁺ concentrations decreased over time whilst soil NO₃⁻ concentrations increased over
219 time in both un-amended and amended soils (Fig. 2a, c, Fig. 3a, c). Soil NH₄⁺ concentrations
220 decreased at a similar rate in all treatments (Fig. 2a, c, Fig. 3a, c). Soil NO₃⁻ concentrations were
221 initially lower in biochar-amended soil (88.7 ± 2.1 vs 77.2 ± 2.6 NO₃⁻ N mg kg⁻¹, $p < 0.01$ for un-
222 amended and amended soil respectively), but during the four-day period increased more rapidly (28.9
223 ± 13.8 vs 69.1 ± 8.9 NO₃⁻ N mg kg⁻¹, $p < 0.05$ for un-amended and amended soil respectively, Fig. 2a,
224 c, Fig. 3a, c). Soil ¹⁵NH₄⁺ enrichment decreased more rapidly in amended soils (Fig. 2b), but there was
225 no difference in ¹⁵NO₃⁻ enrichment between the treatments (Fig. 2d, 3d). Initial soil organic N content
226 was $2,162 \pm 46$ mg N kg⁻¹; organic ¹⁵N enrichment did not vary significantly between un-amended
227 and amended soil over the course of the incubation (Fig. 2f, 3f).

228 The FLUAZ model outputs generally fitted well to analysed soil inorganic N and ¹⁵N concentrations,
229 resulting in a mean-weighted error of 0.8 for the un-amended and 1.3 for the amended soil models.

230 Total N recovery was calculated from inorganic, organic N and respective ^{15}N concentrations in the
231 soil. Total N recovery for the ^{15}N -labelled NO_3^- treatments remained close to 100 % throughout the
232 incubation, whereas it was lower for the ^{15}N -labelled NH_4^+ treatments (typically above 80 %, but
233 attained a minimum of 62 % on day 4 in the amended treatment, Fig. 2e, 3e).

234 Cumulative mineralisation, nitrification, denitrification and immobilisation of N over 4 days were
235 estimated by using the FLUAZ model. Cumulative denitrification after four days was 37 % lower in
236 amended than in un-amended soil (0.17 and 0.27 mg N kg⁻¹ respectively, Table 1). Mineralisation of
237 N and nitrification were greater in amended compared to un-amended soil. Cumulative mineralisation
238 was 55.0 in amended soil compared to 14.9 mg N kg⁻¹ in un-amended soil (269 % greater), and
239 cumulative nitrification was increased by 34%, from 75.6 to 101.1 mg N kg⁻¹ (Table 1).

240 The magnitude of initially immobilised N (within 30 minutes of ^{15}N addition) was similar in biochar-
241 amended (5.7 mg N kg⁻¹) and un-amended (5.5 mg N kg⁻¹) soils (Table 1). Biological NH_4^+
242 immobilisation over the subsequent four days was 50 % greater in amended compared with un-
243 amended soil according to the FLUAZ outputs (17.6 and 11.9 mg N kg⁻¹, respectively, Table 1). Soil
244 NO_3^- immobilisation only increased between day 0 and 2 (Table 1). After two days, NO_3^-
245 immobilisation was 17 % lower in amended than un-amended soil (7.8 compared to 9.4 mg N kg⁻¹,
246 Table 1).

247 Biochar amendment significantly altered soil physico-chemical properties. Soil pH increased from
248 6.31 ± 0.03 to 6.62 ± 0.03 in amended soil ($p < 0.001$, Table 2). Total soil C content was also greater
249 in amended treatments (3.71 ± 0.19 compared to 1.99 ± 0.01 mg C kg⁻¹, $p < 0.001$, Table 2), while
250 total N contents were similar. The soil C: N ratio increased with biochar amendment ($p < 0.001$, Table
251 2).

4 Discussion

Suppression of soil N₂O emissions following biochar application has been demonstrated in a number of short-term studies. Here, we optimised experimental conditions to favour for denitrification and also high soil N₂O emissions and observed a significant (91 %) suppression of those emissions with biochar amendment, consistent with suppressions of 50 - 80 % reported in studies using other soil and biochar combinations (Ameloot et al., 2013; Cayuela et al., 2013; Nelissen et al., 2012). The proportions of N₂O emissions derived from nitrification + nitrifier denitrification and denitrification (calculated by source partitioning) were similar in un-amended and amended soils; 95 % of emissions came from denitrification in un-amended soil compared with 85 % in amended soil over four days (Fig. 1b). This is consistent with results from a near-saturated, agricultural soil (not amended with biochar), where 85 % of N₂O emissions were attributed to denitrification (Mathieu et al., 2006).

Our findings indicated that denitrification was the dominant source of N₂O emissions and that N₂O emissions from both denitrification and nitrification were suppressed by biochar addition. The suppression of soil N₂O emissions from denitrification may have been due to reduced denitrifier activity or increased complete denitrification (i.e. increased conversion of N₂O to N₂). To examine this, we estimated denitrifier activity with the FLUAZ model and found that denitrification was 37 % lower with biochar amendment (Table 1). Lower overall denitrifier activity could feasibly be due to a lower supply of substrate (i.e. NO₃⁻) for denitrifying organisms. We observed that initial concentrations of NO₃⁻ in soil were lower than in un-amended soil, but they increased at a more rapid rate than in un-amended soil, and were not significantly different on day 4 (Fig. 2a, c, Fig. 3a, c).

Therefore it was unlikely that NO₃⁻ substrate limitation could explain the suppression of denitrification activity in this study. To confirm this, we considered the processes that controlled N transformations of inorganic N in the soil, including N mineralisation, nitrification and immobilisation.

Biochar addition increased gross N mineralisation by 269 %, and an additional 40 mg N kg⁻¹ soil was mineralised in biochar-amended soil over four days (FLUAZ, Table 1). Mineralised N could be derived from the biochar itself; recent studies have suggested that organic N derived from biochar

279 may be mineralised in a matter of weeks (de la Rosa and Knicker, 2011; Hilscher and Knicker, 2011).
280 The biochar addition rate used in this study added 142 mg N kg⁻¹ soil in organic form, some of which
281 may have been mineralised during the incubation. Alternatively, the addition of labile C as fresh
282 biochar to this relatively low C agricultural soil may have stimulated soil microbial activity, priming
283 the mineralisation of native soil C and the release of bound N (Luo et al., 2011; Nelissen et al., 2012).
284 We could not discern the source of mineralised N (biochar or native soil organic matter) using this
285 experimental design. This could be investigated using ¹⁵N-labelled biochar to differentiate between
286 biochar and SOM-derived mineralised N.

287 Cumulative nitrification was also increased with biochar (34 %), with nitrification rates greater than
288 20 mg N kg⁻¹ per day in biochar-amended soil (Table 1). An increase in nitrification with biochar
289 addition is consistent with previous biochar studies, although the magnitude of effect has been
290 observed to vary with N addition rate (Nelissen et al., 2012; Prommer et al., 2014). For example,
291 nitrification rates between 1 and 9 mg N kg⁻¹ were observed following the addition of < 5 mg N kg⁻¹
292 of inorganic N (Nelissen et al., 2012; Prommer et al., 2014). Possible explanations for this increase in
293 nitrification include increased soil pH or increased soil NH₄⁺ concentrations as a result of biochar
294 amendment (Mørkved et al., 2007; Norton and Stark, 2011). Soil pH, which was greater than 6.3 in
295 this study, has been found to have little effect on nitrification rates above pH 5 (Mørkved et al., 2007),
296 and so does not explain the increased nitrification observed here. Furthermore, we did not directly
297 observe an increase in NH₄⁺ concentrations in soil with biochar, although there was a more rapid
298 increase in NO₃⁻ concentrations (Fig. 2c, 3c). Assuming that the NH₄⁺ provided by freshly mineralised
299 organic N was rapidly nitrified, we suggest that biochar amendment did increase soil NH₄⁺ availability
300 (Fig. 2c, 3c).

301 Examining N immobilisation more closely, we found that initial chemical or physical N
302 immobilisation was minimal following biochar addition (Table 1). Furthermore, biological N
303 immobilisation in un-amended and amended soil was also small relative to the magnitude of
304 mineralisation and nitrification; equivalent to less than 8 % of the initial soil NH₄⁺-N content (Table 1,
305 Fig. 1a, 2a). This magnitude of N immobilisation was insignificant compared to total inorganic N

306 availability in soil and therefore was not sufficient to explain the 91 % suppression of soil N₂O
307 emissions. This corroborates findings from a similar study in which hardwood biochar suppressed soil
308 N₂O emissions in excess N conditions (Cayuela et al., 2013). The rates of biological NH₄⁺
309 immobilisation reported here (2 - 9 mg N kg⁻¹ d⁻¹) were greater than those from a comparative study
310 of maize biochar (~ 2 mg N kg⁻¹ d⁻¹), potentially due to greater N addition rates in this study (< 3 mg
311 N kg⁻¹ compared to 100 mg N kg⁻¹) (Nelissen et al., 2012). We also observed a small decrease in NO₃⁻
312 immobilisation in biochar-amended soil (- 1.6 mg N kg⁻¹), possibly as a result of decreased anion
313 exchange capacity and increased soil pH (Nelissen et al., 2012).

314 Increased mineralisation, nitrification, insignificant increases in N immobilisation, and similar final
315 NO₃⁻ concentrations are indicative of similar N substrate availability to soil nitrifiers and denitrifiers
316 in biochar-amended compared to un-amended soil. Despite similar N availability, soil N₂O emissions
317 were significantly decreased. We therefore concluded that the processes underlying N supply (from
318 mineralisation, nitrification and immobilisation) did not explain the suppression of soil N₂O emissions
319 in biochar-amended soil, or reduced denitrification rates. Alternative hypotheses to explain reduced
320 denitrification rates include: pH increase (Šimek et al., 2002); the capacity of biochar to act as an
321 electron sink for NO₃⁻, therefore competing with soil denitrifiers (Cayuela et al., 2013); or the
322 presence of inhibitory compounds in biochar (Quilliam et al., 2012; Spokas et al., 2011, 2010;
323 Taghizadeh-Toosi et al., 2011). The first two hypotheses were not supported by evidence from this
324 study: the increase in soil pH was relatively small (0.3), and the biochar did not contain significant
325 amounts of magnesium (0.24 %) or iron (0.32 %) compared to other biochars that could act as
326 electron acceptors (from supplementary information of Case et al. (2012)). We would therefore suggest
327 that the presence of inhibitory compounds in biochar and their effects on denitrification should be the
328 focus of further research.

329 As discussed above, suppression of N₂O emissions could result from reduced rates of denitrification,
330 however it could alternatively result from a difference in the proportions of N₂O, N₂ and NO produced
331 through denitrification, (e.g. a reduction in the N₂O: N₂ ratio) (Baggs, 2011). This was demonstrated
332 in a recent study which showed that biochar consistently reduced the N₂O: N₂ ratio promoting the last

333 step of denitrification (Cayuela et al., 2013). We did not analyse N_2 emissions as a part of this study
334 and so could not confirm this finding. The observed increase in soil pH may, however, have either
335 directly decreased the proportion of N_2O : N_2 emitted from soil, or enabled the biochar to act as an
336 'electron shuttle' increasing the transfer of electrons to denitrifying bacteria (Cayuela et al., 2013). On
337 addition, the incorporation of biochar into the soil introduces fresh labile C which may have increased
338 the conversion of N_2O to N_2 , by increasing the availability of C electron acceptors for denitrifying
339 organisms (Azam et al., 2002; Morley and Baggs, 2010; Saggar et al., 2013; Senbayram et al., 2012).
340 In this study we observed a 75 % increase in soil CO_2 emissions with biochar amendment (Fig. 1b),
341 equivalent to 0.35 % of the biochar C added to the soil (assuming that biochar emission did not prime
342 the mineralisation of soil C), indicating that a significant proportion of labile C was present in the
343 biochar. This provided evidence in support of this mechanism but was not conclusive.

344 Taken together, the evidence presented in this study indicates that the supply of inorganic N, and
345 particularly NO_3^- , to N_2O -producing organisms was not a limiting factor constraining soil N_2O
346 emissions in biochar-amended soil. Future research should focus on the potential of inhibitive
347 substances and labile C in biochar to alter the N_2O : N_2 ratio from denitrification.

348 **5 Conclusions**

349 Biochar amendment has been observed to suppress soil N₂O emissions; this characteristic could be of
350 great value in efforts to reduce agricultural greenhouse gas emissions and therefore mitigate
351 anthropogenic climate change. However, it is not known how and under which environmental
352 conditions biochar consistently suppresses soil N₂O emissions.

353 In this study, several soil N transformation processes were affected following the addition of biochar
354 to a sandy loam soil, including increased mineralisation and nitrification, slightly increased
355 immobilisation and decreased denitrification. Nitrate-supplying transformation rates were increased or
356 un-affected by biochar amendment, so we concluded that the suppression of soil N₂O emissions was
357 not due to limitations of inorganic N availability in the soil caused by biochar-induced inorganic N
358 immobilisation.

359 This investigation into N transformations in soil following addition of biochar adds to the body of
360 knowledge regarding the efficient utilisation of biochar in agriculture with minimal environmental
361 impact. The findings suggest that adding biochar to agricultural soil with mineral fertilisers can
362 suppress N₂O emissions without suppressing the activity of soil biota involved in N transformation
363 processes such as mineralisation or nitrification. Finally, they support the concept that biochar
364 application to agriculture could significantly mitigate agricultural N₂O emissions.

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372

373 **Table 1.** The effect of biochar amendment on soil N cycling processes in soils treated with ¹⁵N-
 374 labelled NH₄NO₃ and wetted to 90% WFPS for 4 days from N addition. Nitrogen transformations
 375 were estimated from the FLUAZ model, described in Section 3.4.

Nitrogen cycling process	Day	Cumulative production (mg N kg ⁻¹)		
		0-1	0-2	0-4
Mineralisation	Un-amended	-8.2	9.3	14.9
	Amended	9.8	25.2	55
Nitrification	Un-amended	17.2	34.8	75.6
	Amended	23.4	54.9	101.1
NH ₄ ⁺ immobilisation	Un-amended	9.2	9.7	11.9
	Amended	6.7	6.9	17.6
NO ₃ ⁻ immobilisation	Un-amended	3.1	9.4	9.4
	Amended	6.8	7.8	7.8
Denitrification	Un-amended	0.06	0.12	0.27
	Amended	0.00	0.15	0.17

376

377

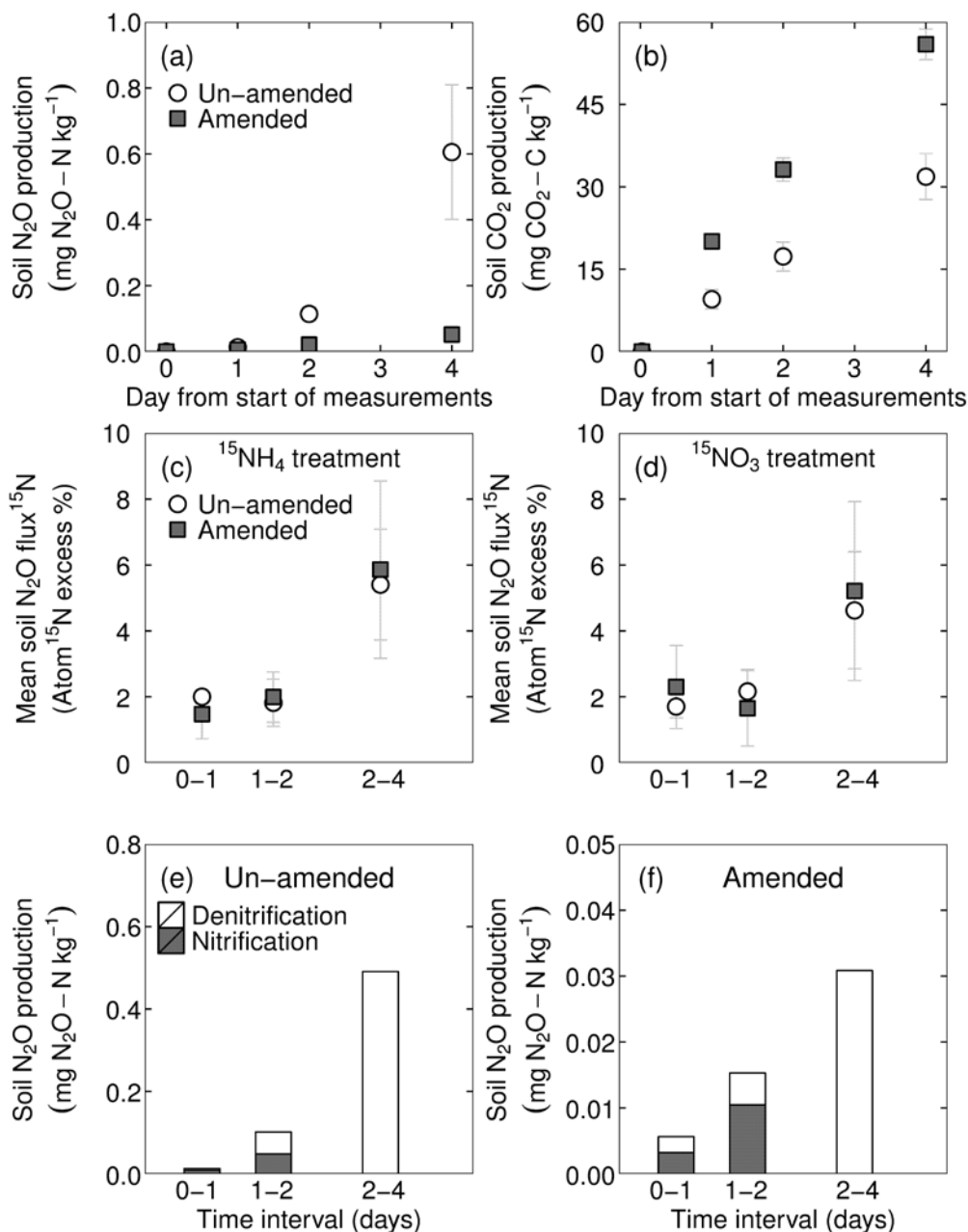
378 **Table 2.** The effect of biochar amendment on physico-chemical properties of soil, treated with ¹⁵N-
 379 labelled NH₄NO₃ and wetted to 90% WFPS. Values represent mean (± standard error) of analyses
 380 from four time points following addition: 30 minutes, 1 day, 2 days and 4 days. Asterisks indicate
 381 significant difference between adjacent un-amended and amended soils: *** = p < 0.001.

Biochar amendment	Total C (%)	Total N (%)	CN ratio	pH
Un-amended	1.99 (0.03)	0.26 (0.001)	7.94 (0.41)	6.31 (0.03)
Amended	3.71 (0.19) ***	0.27 (0.001)	13.90 (1.29) ***	6.62 (0.03) ***

382

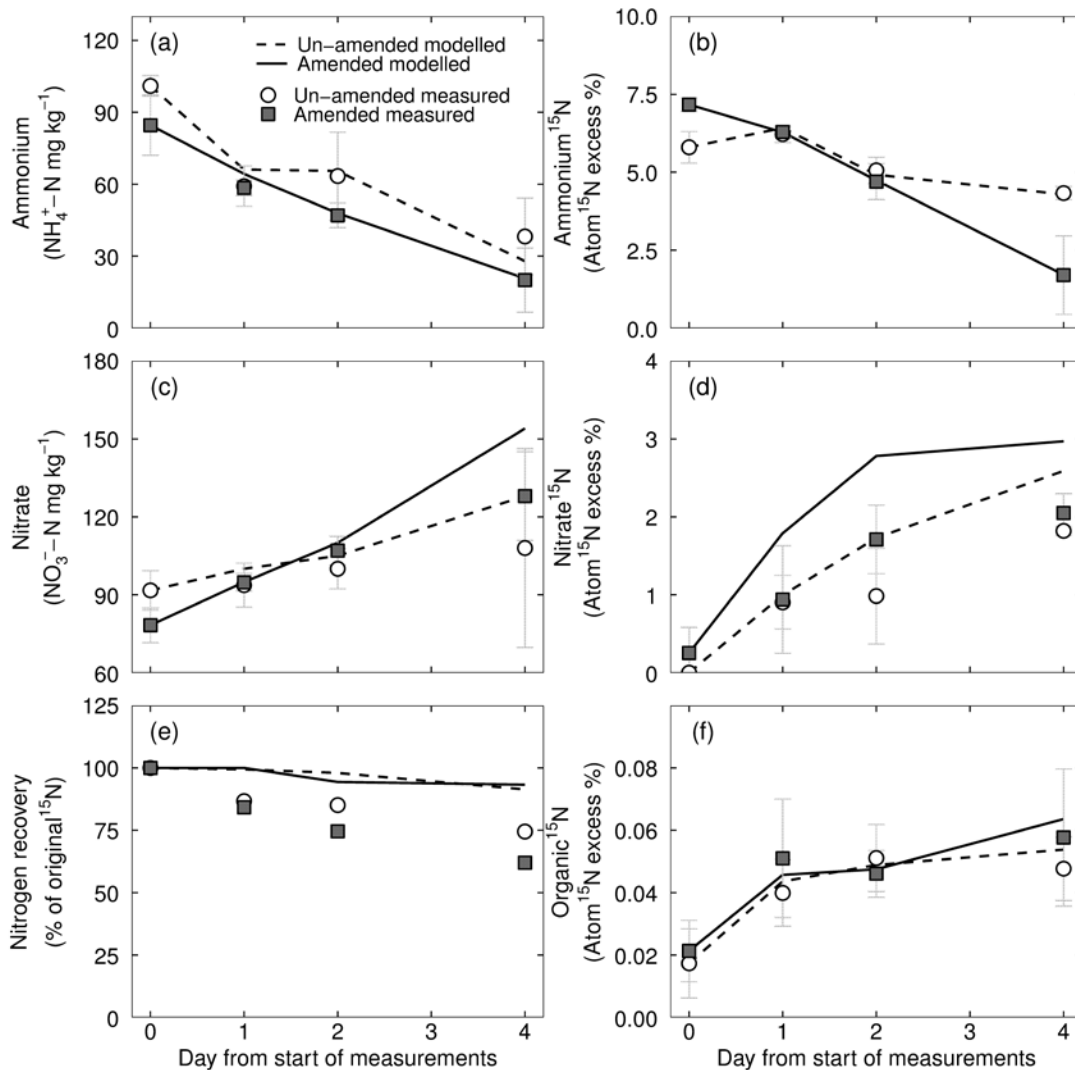
383

384 **Fig. 1.** The effect of biochar amendment on (a) cumulative soil N₂O production, (b) cumulative soil
 385 CO₂ production, mean soil N₂O ¹⁵N flux in un-amended and biochar-amended soils treated with (c) ¹⁵N-
 386 labelled NH₄⁺ or (d) ¹⁵N-labelled NO₃⁻, and the source partitioning of soil N₂O emissions attributed to
 387 denitrification and nitrification + nitrifier denitrification in (e) un-amended and (f) biochar-amended
 388 soils treated with ¹⁵N-labelled NO₃⁻. Data points for graphs a) – d) represent mean ± standard error.



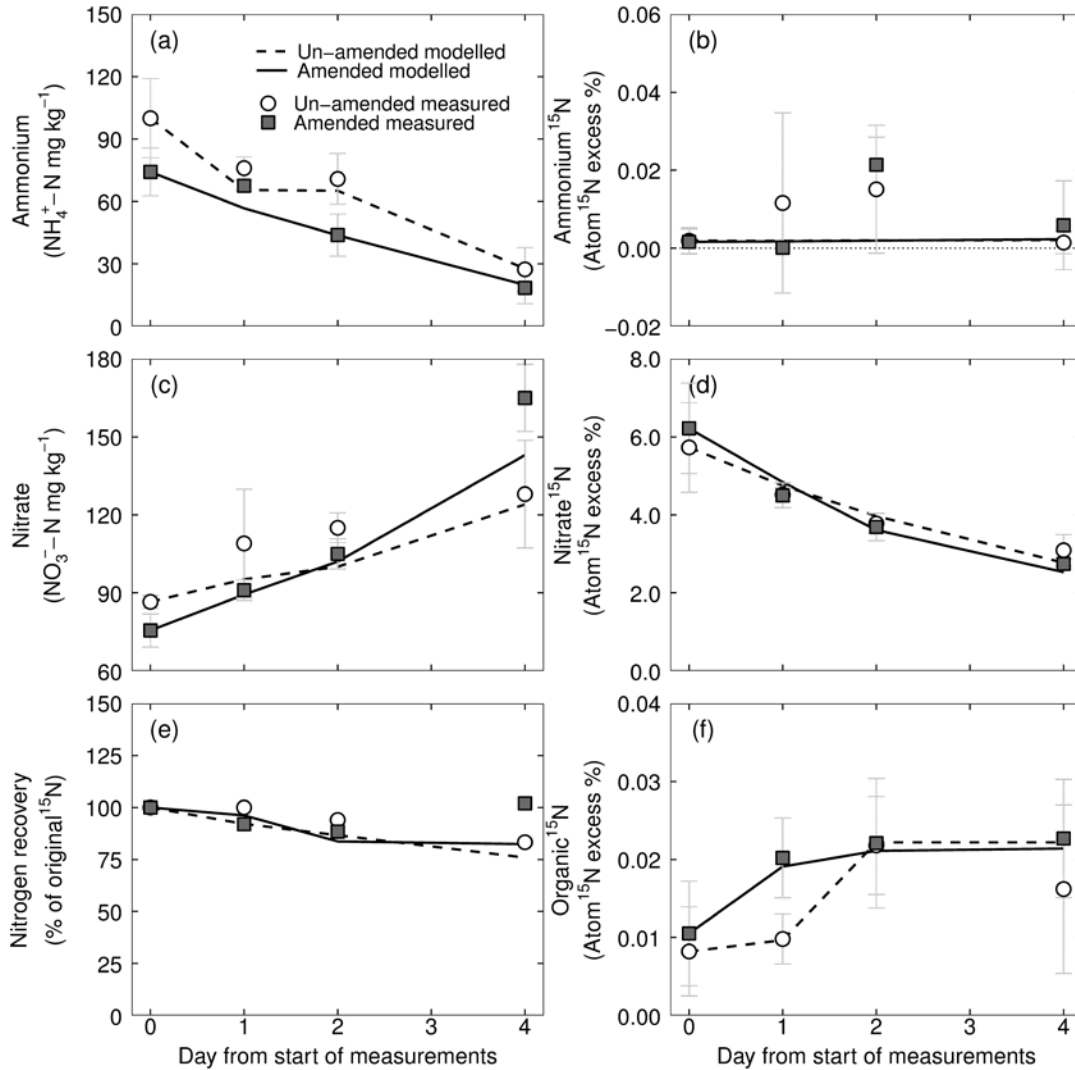
389

390 **Fig. 2.** The effect of biochar amendment on soil inorganic-N concentrations and ^{15}N atom abundance.
 391 in soils labelled with $^{15}\text{NH}_4^+$. Soil properties presented are: (a) soil extractable NH_4^+ concentration; (b)
 392 soil NH_4^+ atom ^{15}N % excess; (c) soil extractable NO_3^- concentration; (d) soil NO_3^- atom ^{15}N %
 393 excess; (e) % N recovery of ^{15}N measured at t_0 ; and (f) soil organic N atom ^{15}N % excess. Points
 394 indicate the mean of directly measured values \pm standard error ($n = 4$), whereas lines indicate
 395 simulated values from FLUAZ model analysis.



396
 397

398 **Fig. 3.** The effect of biochar amendment on soil inorganic-N concentrations and ^{15}N atom abundance.
 399 in soils labelled with $^{15}\text{NO}_3^-$. Soil properties presented are: (a) soil extractable NH_4^+ concentration;
 400 soil NH_4^+ atom ^{15}N % excess; (c) soil extractable NO_3^- concentration; (d) soil NO_3^- atom ^{15}N %
 401 excess; (e) % N recovery of ^{15}N measured at t_0 ; and (f) soil organic N atom ^{15}N % excess. Points
 402 indicate the mean of directly measured values \pm standard error ($n = 4$), whereas lines indicate
 403 simulated values from FLUAZ model analysis.



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