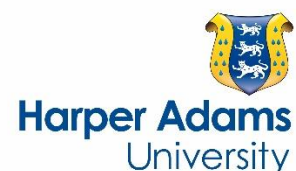


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DOI: 10.1007/s11104-016-3171-z



Jeffery, S., Memelink, I., Hodgson, E., Jones, S., van de Voorde, T.F.J., Bezemers, M., Mommer, L. and van Groenigen, J.W. 2017. Initial biochar effects on plant productivity derive from N fertilization. *Plant and Soil*.

13 January 2017

Number of text pages: 27

Number of Tables: 2

Number of Figures: 5

Short running title: Biochar increases plant productivity by N fertilization

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## Initial biochar effects on plant productivity derive from N fertilization

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Key words:

Pyrolysis; Organic amendment; Stable isotopes; C dynamics; N immobilisation; greenhouse gases

# 1 Abstract

2 *Background and Aim* Biochar application to soil is widely claimed to increase plant productivity.  
3 However, the underlying mechanisms are still not conclusively described. Here, we aim to elucidate  
4 these mechanisms using stable isotope probing.

5 *Methods* We conducted two experiments with uniquely double-labelled (<sup>15</sup>N and <sup>13</sup>C) biochar and its  
6 feedstock (residue), applied separately at 15 Mg ha<sup>-1</sup>. Both experiments contained three treatments:  
7 biochar amendment (Biochar), unpyrolysed residue amendment (Residue) and a no addition control  
8 (Control). Experiment I was a 119 day pot experiment seeded with *Lolium perenne*. Experiment II was  
9 a 71 day incubation experiment without plants in which CO<sub>2</sub> and N<sub>2</sub>O fluxes were measured.

10 *Results* Both Biochar and Residue significantly increased aboveground productivity compared to  
11 Control (140 % and 160 %, respectively). Initial N immobilisation was stimulated in Residue, whereas  
12 not in Biochar. <sup>13</sup>C-CO<sub>2</sub> analysis confirmed that biochar was significantly more recalcitrant than  
13 residue. <sup>15</sup>N analysis showed that 2 % and 0.3 % of grass N was derived from the amended material in  
14 Residue and Biochar, respectively.

15 *Conclusions* Our results suggest that biochar-induced yield increases derive from a combination of  
16 reduced N immobilization and a moderate N fertilization effect. Although in the short term biochar  
17 might offer benefits compared to residue incorporation, it is unlikely that biochar yield gains will be  
18 sustainable for the decades to centuries that biochar C can be expected to reside in soil.

19

## 20 Introduction

21 Interest in biochar has grown considerably since the term was first coined in 2000 (Karaosmanoğlu et  
22 al. 2000) and subsequently recognized as a soil conditioner (Lehmann et al. 2006). Biochar is  
23 produced through the heating of biomass (feedstock) to temperatures generally exceeding 350 °C, in  
24 low to zero oxygen environments (Shackley et al. 2013). It has been repeatedly demonstrated that  
25 biochar application to soil can bring benefits in terms of crop yield increases (Jeffery et al. 2011; Liu  
26 et al. 2013; Jeffery et al. 2015a). Other studies have shown that it can reduce greenhouse gas (GHG)  
27 emissions (Cayuela et al. 2014; Maestrini et al. 2014; Sagrilo et al. 2015), and increase carbon (C)  
28 storage in soils (Gurwick et al. 2013), thereby potentially mitigating climate change (Woolf et al.  
29 2010). However, negative effects have also been reported (Mukherjee and Lal 2014), including  
30 negative effects on crop yields (Singla et al. 2014; Nelissen et al. 2015).

31 Despite the growing body of research, the mechanisms behind observed effects following biochar  
32 application to soil remain poorly understood. This is largely due to a lack of appropriate experimental  
33 controls, as well as the systems-level research approach generally adopted (Jeffery et al. 2015b). A  
34 mechanistic understanding of biochar impacts is vital to allow effective predictions regarding biochar  
35 soil amendment and its consequences for soil-based ecosystem services including crop productivity.  
36 This will aid maximisation of the potential benefits of biochar application to soil while concurrently  
37 minimising trade-offs (Crombie et al. 2015; Jeffery et al. 2015b)

38 One potential mechanism underlying crop yield increases following biochar application is a  
39 fertilization effect. This has been shown for potassium (K) which is present in the ash component of  
40 biochar (Mia et al. 2014; Oram et al. 2014). Besides providing nutrients, biochar may also affect  
41 nutrient cycling and leaching of nutrients in indirect ways (Spokas et al. 2012; Clough et al. 2013).  
42 Two extensive reviews on the effects of biochar application to soil on N dynamics (Clough et al. 2013;  
43 Cayuela et al. 2014) suggest that one of the main mechanisms is adsorption leading to reduced N

44 leaching. This is particularly true for high temperature (>600 °C) biochars and for NO<sub>3</sub>. Conversely,  
45 NH<sub>4</sub><sup>+</sup> retention appears more dependent on the type of feedstock than on pyrolysis temperature  
46 (Karaosmanoğlu et al. 2000). Biochar has also been shown to interact with denitrification through its  
47 function as an electron shuttle during redox reactions (Cayuela et al. 2013).

48 Few studies have aimed to investigate the bioavailability of N from biochars beyond quantifying  
49 hydrolysable organic N (Clough et al. 2013). Meta-analyses have not found significant differences in  
50 yield effects with biochar applied alone or in combinations with fertilizers, either organic or inorganic  
51 (Jeffery et al. 2011; Liu et al. 2013). On an individual study level there is some evidence that biochar  
52 application to soil can decrease the apparent nitrogen recovery (ANR) of plants (Nelissen et al. 2015).  
53 The suggested mechanism for this effect was N immobilisation. However, this effect is dependent on  
54 pyrolysis conditions and type of feedstock, as they lead to different propensities for labile  
55 compounds to remain on the surface of biochar particles post production (Cornelissen et al. 2005).  
56 The application of stable isotope <sup>15</sup>N probing provides a means of quantifying the relative and  
57 absolute uptake of N from materials amended to soil (Bedard-Haughn et al. 2003), such as biochar,  
58 and so quantifying bioavailability of this key plant nutrient.

59 Besides interactions with mineral N, biochar application to soil has also been shown to interact with  
60 soil organic matter (SOM). These interactions include accelerated turnover of SOM (i.e. positive  
61 priming; Wardle et al. 1999), reduced turnover of SOM (i.e. negative priming; Zimmerman et al.  
62 2010) and no effect (Sagrilo et al. 2015). The application of <sup>13</sup>C labelled biochar can provide insights  
63 into the contribution of C pools to CO<sub>2</sub> fluxes as well as into immobilization / decomposition effects  
64 related to N availability (Boschker et al. 1998). Further, through combination with <sup>13</sup>C phospholipid  
65 fatty acid (PLFA) analysis, the main microbial groups able to utilise substrates can be identified,  
66 potentially providing insights into microbial-based mechanisms (Boschker et al. 1998).

67 Here, we utilise a double-labelled ( $^{13}\text{C}$  and  $^{15}\text{N}$ ) biochar and its feedstock to investigate the effects of  
68 biochar application to soil on N availability and relate C and GHG dynamics. Work conducted here  
69 was focussed on grasslands, which have been largely overlooked in biochar research despite  
70 suggestions that application to grassland will be required to maximise the GHG offsetting capabilities  
71 of biochar (Woolf et al. 2010). Through the use of unpyrolysed feedstock as a positive control we aim  
72 to elucidate biochar effects *per se*, i.e. those that are beyond what would have been observed with  
73 the application of the feedstock alone. To do so we will test the hypothesis that plant productivity  
74 increases following biochar application to soil derive from a fertility effect. If accepted, this suggests  
75 that yield effects may not last for as long as the residence time of C in soil, often estimated to be in  
76 the range of decades to centuries (Lehmann et al. 2006). Rather, they will last until available  
77 nutrients are utilised and become limiting locally once more.

78

## 79 **Materials and Methods**

80 This project was focussed on grasslands and so grassland species were used both for the feedstock  
81 and for the plants grown. *Plantago lanceolate* is a common plant in grasslands and is fast growing  
82 with broad leaves meaning it produces biomass relatively quickly. It was also applied in unpyrolysed  
83 form as a positive control (hereafter Residue). The feedstock was isotopically enriched with  $^{13}\text{C}$  and  
84  $^{15}\text{N}$  as described below. These materials were used in two experiments that used the same  
85 homogenised soil: a greenhouse experiment with *Lolium perenne* grown in pots (Experiment I) and  
86 an incubation experiment without plants in a climate controlled room to quantify GHG fluxes  
87 (Experiment II).

### 88 ***Isotopically labelling biomass***

89 Biomass (*Plantago lanceolata*) was grown in a growth chamber in a vermiculite substrate. Key  
90 characteristics can be found in Table 1. During the growth period it was pulse-labelled with  $^{13}\text{C}\text{-CO}_2$ ,  
91 following the method of Bromand et al. (2001). Biomass was labelled with  $^{15}\text{N}$  through fertilization  
92 with  $\text{Ca}(^{15}\text{NO}_3)_2$  added to a fertilizer solution applied to the vermiculite daily to achieve an  
93 enrichment approx. 58 % atom. Aboveground biomass of *Plantago lanceolata* was harvested twice,  
94 at pre-flowering stage. This was done to ensure only leaves were included and no stems or flowers,  
95 thereby reducing the heterogeneity of the feedstock. Harvests were performed by cutting plants  
96 back to approx. 2 cm above the surface of the vermiculite, after 5 weeks for the first harvest and 7  
97 weeks for the second. After harvest, biomass was oven-dried at 60 °C for 24 hours. The dried  
98 biomass of both harvests was ground to 2 mm, combined and mixed. A sub-sample of approximately  
99 70 % of the biomass was pyrolysed to produce biochar (pyrolysis under  $\text{N}_2$ , max temp 400 °C,  
100 residence time 30 min; Aberystwyth University, Wales). The remaining 30 % of the biomass was used  
101 for the Residue treatments described below. Analyses of biochar and residue from which it was  
102 produced were performed using a Pyris 1 thermogravimetric analyser (TGA) (Perkin–Elmer,  
103 Massachusetts, USA; Hodgson et al. 2011). In short, samples were pyrolysed under nitrogen at a flow  
104 rate of 20 mL min<sup>-1</sup> using the following temperature program: Heated from 40 to 105 °C at 10  
105 °C min<sup>-1</sup>; held at 105 °C for 10 min; heated from 105 to 905 °C at 10, 25, and 100 °C min<sup>-1</sup>; held at 905  
106 °C for 15 min; cooled from 905 to 105 °C at 25 °C min<sup>-1</sup>. A proximate analysis was performed on the  
107 TGA data to calculate the relative proportions volatiles, fixed carbon and ash (wt. %). Volatiles were  
108 calculated from mass loss occurring between 105 and 550 °C, fixed carbon from 550 °C to 900 °C and  
109 ash as the remaining material after heating. An elemental analysis was used for analysis of  $\text{H:C}_{\text{org}}$   
110 which is reported as a molar mass ratio.

111 For the C:N ratio,  $^{13}\text{C}$  and  $^{15}\text{N}$  content analysis, three replicates (2 mg) of both residue and biochar  
112 were placed into individual tin capsules and analysed using a PDZ Europa ANCA-GSL elemental  
113 analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd.; Cheshire,



114 UK) at The Stable Isotope Facility of UC Davis, USA. Key characteristics of both the residue and the  
115 biochar can be found in Table 1.

## 116 ***Soil***

117 Topsoil (top 10 cm) was collected from a nature restoration grassland area on the Veluwe, in  
118 Gelderland, Netherlands (52.059826N, 5.751354E) on 11<sup>th</sup> March 2014. The site is located on an ice  
119 pushed ridge formed during the Saalien Ice Age. The soil is characterised as a “holtpodzol” on coarse  
120 sand (gY30; Stiboka, 1975: map 40 W). The area was used as arable field until 1995 and had last been  
121 used to grow maize in 1995. Previous to that cropping had included cycles of sugar beet, potatoes  
122 and oats. Collected soil was sieved to pass 4 mm and thoroughly mixed to ensure homogenisation.  
123 After homogenisation the soil was split into two parts to be used for Experiments I and II. Soil  
124 characteristics were determined in Mia et al. (2014) and Oram et al. (2014). Further information on  
125 methods for soil analysis can be found in those studies. Key soil characteristics are presented in Table  
126 2.

## 127 ***Experiment I – Plant growth***

128 Soil for each treatment was amended with biochar and residue each at a rate equivalent to 15 t ha<sup>-1</sup>,  
129 incorporated into the top 10 cm of soil produced with five replicates. Soil was packed into 9.5 cm  
130 diameter 0.5 L polypropylene pots (505 g dry weight (dw) of soil, packed to a dry bulk density of 1.2 g  
131 cm<sup>-3</sup>). The control consisted of unamended soil packed to the same bulk density. A 1-cm deep layer  
132 consisting of 94 g of soil (i.e. without biochar or feedstock) was added to the surface of pots to  
133 function as a germination layer as biochar has previously been shown to occasionally inhibit  
134 germination. All treatments were replicated five times, totalling 15 pots, set up in a completely  
135 randomised design. The experiment was performed in a greenhouse (average 60 % relative humidity;  
136 average temperature 21 °C) of Wageningen University, The Netherlands.

137 In each pot, 10 wild type *Lolium perenne* (diploid) seeds were sown at a seeding rate equivalent to 27  
138 kg ha<sup>-1</sup>. Owing to reduced germination in some pots, pots were reseeded after a week in order to  
139 achieve 10 plants per pot.

140 After germination, pots were fertilized at rates equivalent to 30 kg P ha<sup>-1</sup> and 140 kg K ha<sup>-1</sup> (KH<sub>2</sub>PO<sub>4</sub>  
141 and K<sub>2</sub>SO<sub>4</sub>). Applications of fertilizer were spread over four days to minimise the risk of burning the  
142 seedlings. No N fertilizer was added to any of the pots. Water was added following fertilisation to  
143 bring all the pots to 60 % water-filled pore space (WFPS) and to ensure that the fertilizer moved  
144 deeper into the soil. The pots were then watered daily and maintained gravimetrically at 60 % WFPS.

145 Aboveground biomass was harvested at Day 35 by cutting the plants back to approximately 2 cm  
146 above the soil surface. The second, third and fourth harvest of biomass were respectively on Day 63,  
147 91 and 119. Biomass was oven dried at 60 °C for at least 48 h and weighed. Subsequently, all biomass  
148 from each pot was combined, ground and ball milled. A representative subsample (approx. 2 mg) of  
149 aboveground biomass was then isotopically analysed for <sup>13</sup>C and <sup>15</sup>N content as described below.  
150 Belowground biomass was collected by washing roots over a 2 mm sieve to remove soil particles.  
151 Roots were then oven-dried and weighed as described above.

## 152 ***Experiment II – Soil gas fluxes***

153 Experiment II consisted of the same three treatments as Experiment I but without plants. Pots  
154 (polypropylene 0.5 L – 6.6 cm diameter) were packed with 200 g soil dry weight (dw) to a dry bulk  
155 density of 1.2 g cm<sup>-3</sup>. All treatments were replicated 5 times, totalling 15 pots. The pots were placed  
156 on a table in a completely randomized design in a climate-controlled room at 20 °C and maintained  
157 at 60 % WFPS.

158 On days 1, 2, 5, 8, 16, 22, 29, 36, 43, 50, 57, 64, and 71, CO<sub>2</sub> and N<sub>2</sub>O gas samples were taken and  
159 fluxes were measured. This was done one hour after closing the pot with a lid containing two septa.

160 Two separate gas samples (7 ml each) were taken with a syringe and injected into pre-evacuated  
161 4.5ml borosilicate vials for analysis of  $^{13}\text{C}$ - $\text{CO}_2$  and  $^{15}\text{N}$ - $\text{N}_2\text{O}$  content. Soil gas fluxes were then  
162 quantified following a standard procedure with photoacoustic gas monitor (Brüel & Kjær, Monitor  
163 Type 1302; Nærum, Denmark; Velthof et al. 2002). Daily fluxes (ppm) were converted to  $\text{mg CO}_2\text{-C h}^{-1}$   
164  $\text{m}^{-2}$  and  $\mu\text{g N}_2\text{O-N h}^{-1} \text{m}^{-2}$  and to cumulative fluxes in  $\text{g CO}_2\text{-C m}^{-2}$  and  $\text{mg N}_2\text{O-N m}^{-2}$  assuming linearity  
165 of flux rate between each measurement day.

### 166 ***Stable Isotope analyses***

167 All isotope analyses were performed at The Stable Isotope Facility of University California, Davis. The  
168  $^{13}\text{C}$  content analyses were performed using a ThermoScientific PreCon-GasBench system interfaced  
169 to a ThermoScientific Delta V Plus isotope ratio mass spectrometer (ThermoScientific, Bremen,  
170 Germany). The  $^{15}\text{N}$  content analyses were performed using a ThermoFinnigan GasBench + PreCon  
171 trace gas concentration system interfaced to a ThermoScientific Delta V Plus isotope-ratio mass  
172 spectrometer (Bremen, Germany). Percentage C and N derived from the biochar and feedstock were  
173 calculated using  $^{13}\text{C}$  and  $^{15}\text{N}$  gas values and applying the equation of Bedard-Haughn et al. (2003).  
174 These percentage values were then used to calculate the  $\text{g CO}_2\text{-C m}^{-2}$  and  $\text{mg N}_2\text{O-N m}^{-2}$ , as a  
175 proportion of the total flux, derived from the Biochar and Residue.

176 On Day 71, the soil from each pot was sieved to pass 4 mm, homogenised by thorough mixing and  
177 split into sub-samples for analysis. A representative subsample (50 mg) of the soil was analysed ( $^{13}\text{C}$   
178 and  $^{15}\text{N}$  content) as described below. The pH and EC was determined after shaking each sample (5 g)  
179 for 1 h with demi-water (1:5 w/v).

### 180 ***Microbial biomass $^{15}\text{N}$***

181 Determination of microbial biomass N (MBN) was undertaken via an extension of the chloroform  
182 fumigation extraction (Vance et al. 1996). In short, soil (20 g) was shaken for 1 hour with 80 ml 0.5 M

183 KCl following 24 hours of fumigation. After shaking, extracts were filtered to pass 0.45  $\mu\text{m}$ . The  
184 difference in total soluble N content (TSN) between the fumigated and non-fumigated soil in the KCl  
185 extract was used to calculate the MBN. Microdiffusion was used to quantify the  $^{15}\text{N}$  content of MBN  
186 (Stark and Hart 1996) through the analysis of  $^{15}\text{N}$  that was obtained on the filter. Each glass  
187 microfiber filter used for the microdiffusion was put in tin capsules and analysed for  $^{15}\text{N}$  content as  
188 described above.

### 189 $^{13}\text{C}$ PLFA

190 The phospholipid fatty acid (PLFA) extraction and analysis as outlined by Bligh and Dyer (1959) and  
191 extended upon by Zelles (1999) was utilised to determine microbial community level phenotypes.  
192 Extractions were performed using 5-g aliquots of soil for each sample. Extracted PLFAs were analysed  
193 by gas chromatography using an HP 5 column on a G2070AA Chemstation, Model 6890N, gas  
194 chromatography appliance (Agilent Technologies, Santa Clara, California, USA). Peaks were compared  
195 with known retention times on the basis of a Supelco 26 peak standard to identify individual PLFAs  
196 (Sigma-Aldrich Ltd, Poole, Dorset, UK). The fungal:bacterial ratio was calculated using 18:2 $\omega$ 6 (fungal  
197 biomarker) divided by the summed % mol of biomarkers i15:0, ai15:0, 15:0, i16:0, 16:1 $\omega$ 7t, i17:0,  
198 ai17:0, 17:0, 18:1 $\omega$ 7 and cy19:0 as an expression of total bacterial abundance (Frostegård & Bååth,  
199 1996).  $\delta^{13}\text{C}$  values were measured on a Finnigan Delta-S gas chromatograph–isotope ratio monitoring  
200 mass spectrometer (GC-IRMS) as described in Boschker (2004). The increase in  $\delta^{13}\text{C}$  values of PLFAs  
201 in the treatments compared to the control indicates microbial uptake of labelled C from the  
202 amended material.

### 203 **Data analysis**

204 For statistical analyses, SPSS Statistics 19 (IBM) was used with the exception of Principal Component  
205 Analysis used to analyse PLFA profiles, which was performed using R Studio (version 0.99.903) with  
206 the Vegan package. The effects of the treatments (Biochar, Reside and Control) on cumulative fluxes

207 of CO<sub>2</sub> and N<sub>2</sub>O, the <sup>13</sup>C and <sup>15</sup>N content, pH and EC of the incubation soil, N content of the incubation  
208 soil and the microbial biomass N were compared using a one-way Analysis of Variance (ANOVA).  
209 Individual comparisons were performed using a Tukey's HSD *post hoc* test. The effects of the  
210 treatments on plant performance and <sup>13</sup>C and <sup>15</sup>N uptake by plants were compared using ANCOVA  
211 with the number of plants per pot included as a covariate. The treatment effects on daily CO<sub>2</sub> and  
212 N<sub>2</sub>O fluxes and the <sup>13</sup>C and <sup>15</sup>N content were tested with a repeated measures ANOVA.

## 213 **Results**

### 214 ***Experiment 1: Plant growth***

215 Despite the germination layer an average of 5.3 (S.E. 1.16), 5.2 (S.E. 1.2) and 8.4 (S.E. 0.68) plants  
216 germinated in Biochar, Residue and Control, respectively. Aboveground biomass production of *L.*  
217 *perenne* (dry weight) was significantly reduced by 89 % in the first harvest of Residue compared to  
218 Control (Fig. 1). However, biomass production in Residue was significantly higher than Control over  
219 the next three harvests. On average, biomass production was approx. 40 % and approx. 60 % higher  
220 than Control for Biochar and Residue, (P = 0.001 and P = 0.01, resp.). Belowground biomass increased  
221 significantly in Biochar by 115 % compared to Control; no significant difference was observed in  
222 belowground biomass production in Residue compared to Control. (Fig. 1). Differences in the  
223 shoot:root ratio between treatments were close to significant (P = 0.064).

224 Significant differences in N uptake were observed between Biochar and Residue (P < 0.001). Overall,  
225 more N was taken up by plants from the amended material in Residue than in Biochar (Fig. 2 a & b). At  
226 first harvest, significantly more N was taken up from the amended material in Biochar than in  
227 Residue in absolute terms (P < 0.001; Figure 2a). However, as a proportion of total N taken up over  
228 all harvests, significantly more plant N was derived from the amended material in Residue than in  
229 Biochar (P<0.001; Fig. 2b). After the first harvest, approximately 2 to 2.5 % of N taken up by plants in

230 Residue was derived from the amended material. For Biochar, this was less than 0.5 % for all harvests  
231 (Figure 2b). After four harvests, biochar N accounted for approx. 0.4 % of total plant N whereas for  
232 residue N this was approx. 2 %.

### 233 ***Experiment 2: Soil gas fluxes***

234 Cumulative N<sub>2</sub>O fluxes from Biochar did not vary significantly from Control throughout the timeframe  
235 of the experiment (P = 0.9; Fig. 3a). Residue resulted in a significantly greater release of N<sub>2</sub>O than  
236 either Control or Biochar up to Day 5 (P < 0.001; Figure 3a). The N<sub>2</sub>O flux from Residue was reduced  
237 greatly after this initial flush but increased again at Day 64 until the end of the experiment.

238 After 71 days, at the end of the incubation experiment, approximately 0.3 % of amended N had been  
239 lost as N<sub>2</sub>O from the amendment in Residue, compared to 0.05 % from Biochar (Fig. 3). N-loss from  
240 the amended material in Biochar as N<sub>2</sub>O was significantly lower than from Residue (P < 0.01)  
241 suggesting decreased availability of N from biochar than residue. However, N<sub>2</sub>O did not represent a  
242 significant source of N loss from either experimental treatment.

243 % %By Day 2, significantly higher cumulative CO<sub>2</sub> fluxes were measured in Residue compared to  
244 Biochar and Control (P = 0.001); this difference increased throughout the incubation period. There  
245 was no significant difference in total cumulative fluxes between Biochar and Control (P = 0.96; Fig.  
246 3b). Significantly more C was lost from Residue by Day 5 (Fig 3d; P = 0.008); by the end of the  
247 experiment approx. 20 % of the applied C was lost from Residue as CO<sub>2</sub> compared to approx. 2 % loss  
248 from Biochar (Fig. 3d). However, the rate of C loss from biochar reduced greatly after the initial flush.

249 By the end of the experiment, microbial biomass nitrogen (MBN) was four times higher in Residue  
250 than in Biochar or Control (P < 0.001; Fig. 4a). There was no significant difference in MBN between  
251 Biochar and Control (P = 0.78; Fig. 4a). Stable isotope analysis showed that for the Residue treatment

252 approximately 25 % of total MBN was derived from the amended material, while this was only  
253 approximately 0.3 % for the Biochar treatment (Fig. 4a).

254 The CO<sub>2</sub>-C derived from SOM did not differ significantly from Control in either treatment (P > 0.05;  
255 Fig. 4b). However, significantly more CO<sub>2</sub>-C was derived from the amended material in Residue than  
256 Biochar (56 % compared to 39 %; P<0.001; Fig. 4b). Further, significantly more C was mineralised  
257 from SOM in Residue than Biochar (Fig. 4b; P = 0.03) showing that both C pools had increased  
258 turnover in Residue.

259 PLFA profile analysis of the community level microbial phenotype showed strong discrimination  
260 between Residue compared to Biochar and Control where little discrimination was evident (Fig. 5a).  
261 Discrimination between treatments occurred mainly in PC1, which accounted for 86 % of variation.  
262 The PLFAs most responsible for the observed discrimination between treatments were C16:0  
263 (general biomarker for microbial biomass), C18:2 $\omega$ 6c and C18:1 $\omega$ 9c /2 $\omega$ 6t/3 $\omega$  (saprotrophic fungal  
264 biomarkers – note that with the methodology used it was not possible to discriminate between these  
265 PLFAs; Fig 5b). The bacterial: fungal ratios were significantly lower in Control (0.03) and Biochar  
266 (0.04) compared to Residue (0.3) (P < 0.01).

267 The stable isotope enrichment increased significantly from -30.4‰, -32.1‰ and -29.4‰ in Control to  
268 8.3‰, 5.5‰ and -0.5‰ in Biochar and 140‰, 87.8‰ and 154.4‰ in Residue for the PLFAs C16:0,  
269 C18:2 $\omega$ 6c and C18:1 $\omega$ 9c/2 $\omega$ 6t/3 $\omega$  respectively (Figure 5c; P < 0.01).

## 270 **Discussion**

271 The increased plant productivity following soil biochar amendment that we found in this study is in  
272 agreement with previous studies (Jeffery et al.2011; Spokas et al. 2012; Liu et al. 2013). However, the  
273 use of stable isotope probing allows us to investigate the mechanisms underlying this effect, rather  
274 than reporting results at the systems level. Previous biochar studies using soil from the same site

275 showed that micronutrients in the soil used were not limiting (Oram et al. 2014; van de Voorde et al.  
276 2014). In the present study, soils were fertilized with K and P to ensure that only N would be limiting  
277 (Table 2). Total plant production in Biochar and Residue was significantly higher than Control.  
278 Concurrently, stable isotope analysis demonstrated uptake of N from both Residue and Biochar.  
279 Therefore, the data support the hypothesis that observed differences in biomass production resulted  
280 from an N fertilisation from the amendment in the Biochar and Residue treatments. Plant N uptake  
281 from the amended material Residue was 7.6 times higher than in Biochar. Differences in biomass  
282 production were less apparent. Once N limitation has been alleviated, diminishing returns are  
283 expected from further increased N availability (Tillman et al. 2002). This effect was reflected in the  
284 biomass data.

285 Increased root growth was noted in Biochar compared to Residue and Control (Fig. 1). It has  
286 previously been reported that plants grown in biochar-amended soils can have increased  
287 “rhizosphere zones” compared to controls (Prendergast-Miller et al. 2014). During harvest, we noted  
288 that the rhizosphere contained more biochar particles than the bulk soil suggesting that roots may  
289 prefer soil containing biochar particles. This may have consequences beyond investigated effects in  
290 this experiment. For example, increased rooting may help alleviate the impact of drought as well as  
291 aiding nutrient acquisition beyond those included in the amended material. The trigger that led to  
292 increased root growth in the presence of biochar remains unclear and a necessary area for further  
293 research.

294 The same amendment application rate was used for Biochar and Residue. As they each contained  
295 very similar levels of N (Table 2), similar rates of N were applied to both treatments. However, the  
296 C:N of the applied materials differed significantly. It is not yet clear how the C:N stoichiometry  
297 interacts with soil processes as it is likely the quality of the C that is important rather than the  
298 quantity. In the Residue treatment more than four times as much N was taken up from the  
299 amendment than in the Biochar treatment showing enhanced ANR from the amended material in



300 Residue than Biochar. However, initial N immobilisation reduced ANR significantly at the first harvest  
301 in Residue. Such immobilisation was not observed in Biochar where ANR was shown to be highest at  
302 first harvest (Fig 2a and b) where it then decreased and remained at a consistent level thereafter.  
303 Biochar could have been expected to stimulate N immobilisation more than residue when  
304 considering only the C:N ratios alone. That this did not occur provides evidence that the C:N ratio of  
305 biochar is likely not an effective predictor as to whether that biochar will immobilise N when applied  
306 to soil.

307 There are few data on the availability of N from biochar (Clough et al. 2013; Cayuela et al. 2014).  
308 Studies that have investigated N dynamics following biochar application have typically focused on co-  
309 application of N fertilizer (Spokas et al. 2012; Zheng et al. 2012; Clough et al. 2013), N retention  
310 effects of biochar (Spokas et al. 2012; Zheng et al. 2012), or N<sub>2</sub>O flux effects (Zheng et al. 2012;  
311 Clough et al. 2013). However, no other study to date has quantitatively analysed the bioavailability of  
312 N from biochar itself. Our study shows that pyrolysis reduced availability of N for plant uptake in  
313 Biochar by >700 % compared to Residue, but that a significant proportion of N remained bioavailable  
314 (or mineralisable to available forms). Reduced N bioavailability led to decreased plant productivity in  
315 Biochar compared to Residue, while still being greater than Control. Plant biomass productivity was  
316 lowest in Control due to N limitation as it received no amendment (i.e. no input of N).

317 The significant decrease in plant biomass in Residue compared to Biochar and Control at the first  
318 harvest suggests N immobilisation. This was likely a consequence of the addition of the relatively  
319 large amounts of labile C added to this treatment (i.e. plant residue) as reflected in the CO<sub>2</sub> emission  
320 rate (Fig. 3). This is also reflected in the fact that very little N was taken up by plants from the  
321 amendment in Residue by the first harvest, and that microbial biomass N was significantly larger in  
322 the Residue treatment. Isotopic analysis showed that 0.3 % of MBN was derived from the  
323 amendment Biochar, compared to 22.5 % in Residue. This agrees with previous work that found little  
324 effect of biochar on MBN in contrast to wheat straw (Zhang et al. 2014). This was likely due to the

325 application of labile C (i.e. plant material) provided substrate that functioned as an energy source  
326 allowing microbes to scavenge for N from SOM through nitrogen-mining (Craine et al. 2007).

327 There was a significant increase in N<sub>2</sub>O production from Residue for the first 5 days of the incubation  
328 experiment indicative of increased microbial N cycling in this period. After this time, N<sub>2</sub>O fluxes  
329 decreased greatly suggesting that readily available N in the soil was immobilised in the microbial  
330 community, or denitrification increased due to depletion of O<sub>2</sub> within soil pores driven by respiration  
331 of labile C as. By the end of the incubation there was no significant difference in cumulative N<sub>2</sub>O  
332 emissions between treatments. This result contrasts with numerous studies which have reported a  
333 significant decrease (Cayuela et al. 2014; Case et al. 2015), or increase (Clough et al. 2010; Sánchez-  
334 García et al. 2014) in N<sub>2</sub>O fluxes following biochar application to soil. However, other studies have  
335 also reported no effect on N<sub>2</sub>O emissions (Suddick et al. 2013), or different effects from the same  
336 biochar applied to different soils (Yoo et al. 2012). These contrasting findings emphasise that  
337 generalisation of the effects of biochar should be taken with great care and that the results may  
338 depend greatly on characteristics of the biochar and soil used. It should be noted that we used a  
339 coarse soil (Table 2) and as such our results may differ from experiments that used a fine soils.

340 Utilisation of <sup>13</sup>C isotopes allowed proportional attribution of CO<sub>2</sub> flux to the different carbon pools in  
341 biochar and SOM (Boschker et al. 1998). <sup>13</sup>C analysis confirmed that a portion of emitted CO<sub>2</sub> was  
342 derived from biochar and that the biochar therefore contained a labile component. However, data  
343 presented here demonstrate that the C in the biochar was, on the whole, significantly more  
344 recalcitrant than the unpyrolysed feedstock with >3 % of amended C lost from Biochar over the  
345 course of the experiment compared to 19 % of amended C lost from Residue. We found no evidence  
346 of priming of SOM by addition of Biochar and Residue compared to the control. However,  
347 mineralisation rates of SOM differed between the Biochar and Residue treatments. This means that  
348 while no priming of SOM occurred compared to the control situation with no addition, differential  
349 interactions with SOM in terms of priming effects were observed following the application of Biochar

350 versus Residue. This was likely due to the increased microbial biomass (as suggested by the increased  
351 MBN in Residue; Fig. 4a) which is generally correlated with increased decomposition of soil organic  
352 matter (Balota et al. 2003; Lee et al. 2003). The decay constant (k) of SOM are usually imperfect  
353 representations of first order kinetics (Paul et al. 1996). As such linear extrapolation cannot be  
354 undertaken with confidence but the evidence suggests the residence time of the biochar C would be  
355 at least an order of magnitude greater than that of residue C.

356 Biochar application to soil has been shown to stimulate mycorrhizal fungi and their colonisation of  
357 plant roots (Warnock et al. 2007). We quantified microbial community effects through phenotypic  
358 fingerprinting using  $^{13}\text{C}$  PLFA. While PLFA discriminated between the microbial community in Residue  
359 compared to Biochar and Control, no strong discrimination between biochar and control was  
360 observed. The PLFA 16:1 $\omega$ 5 considered a biomarker for mycorrhizal fungi (Olsson 1995) did not vary  
361 significantly between treatments suggesting that, at least in our study, observed yield effects should  
362 not be attributed to increased mycorrhizal fungi as has been posited previously (Warnock et al.  
363 2007). However, the plant used in this experiment, *Lolium perenne*, forms a dense rooting system  
364 that may not be conducive to mycorrhizal colonisation. Further, the soil is relatively high in P (Table  
365 2), and was fertilised with soluble P, which tends to reduce mycorrhization. As such, different results  
366 may have been observed if different plants or different fertilisation regimes were used. The PLFA  
367 C16:0, considered a general microbial biomass marker (Bossio et al. 1998) and C18:1 $\omega$ 9c/2 $\omega$ 6t/3 $\omega$   
368 and C18:2 $\omega$ 6, all considered saprotrophic fungal biomarkers (Frostegård et al. 1996; von Rein et al.  
369 2016), contributed most to the discrimination observed between treatments. The  $\delta^{13}\text{C}$  of these PLFAs  
370 all increased significantly from Control to Biochar to Residue. This further confirms that some of the  
371 C in the biochar was labile and so available for microbial utilisation and incorporation into microbial  
372 cell membranes. However, considerably more C was incorporated into microbial cell membranes  
373 (and likely microbial cells in general) in Residue, as confirmed by the greatly increased  $\delta^{13}\text{C}$ . These  
374 data are again consistent with the fertilisation hypothesis. This evidence suggests that saprotrophic

375 fungal biomass increased in Biochar, and much more so in Residue, where it decomposed the  
376 amended material and mineralised organic N into plant available forms.

## 377 **Conclusions**

378 Our results demonstrate that the observed increases in plant productivity following biochar addition  
379 to soil were due to an N fertilisation effect. Stable isotope analysis using  $^{13}\text{C}$  confirmed that the C in  
380 biochar is considerably more recalcitrant than the feedstock from which it was produced.

381 Pyrolysis strongly reduced the bioavailability of N from the resulting biochar when compared to the  
382 initial feedstock. This means that nutrients in biochar are released slowly when compared to the  
383 initial feedstock. Further, due to the increased recalcitrance of the C in biochar, application of  
384 biochar to soil did not cause N immobilisation. Therefore, application of biochar rather than crop  
385 residues may circumvent the need of co-application of synthetic N fertilisers, which are sometimes  
386 applied to compensate for the effects of microbial N immobilisation. This study also highlights the  
387 need for rigorous controls in experiments to allow distinguishing fertilisation effects (short-term)  
388 from the “true” biochar effects, i.e. those effects associated with biochar C that will occur over the  
389 entire residence time of that C in the soil. For sustainable application of biochar it is vital to make  
390 informed decisions on where best to apply biochar, compost and/or green manures to maximise the  
391 potential benefits and minimise the negative impacts. Our results will help decisions makers such as  
392 farmers or policy makers to do so.

393 **Acknowledgements**

394 We gratefully acknowledge funding support from the Marie Curie Career Integration Grant (No.  
395 GA526/09/1762) and funding from the Emerging Science fund of the Production Ecology and  
396 Resource Conservation (PE&RC) graduate school of Wageningen UR, Netherlands. We are also  
397 grateful for the assistance of André Maassen for watering plants, and Jaap Nelemans and Willeke van  
398 Tintelen for technical assistance in the laboratory and Ana Prada for assistance with formatting.

399

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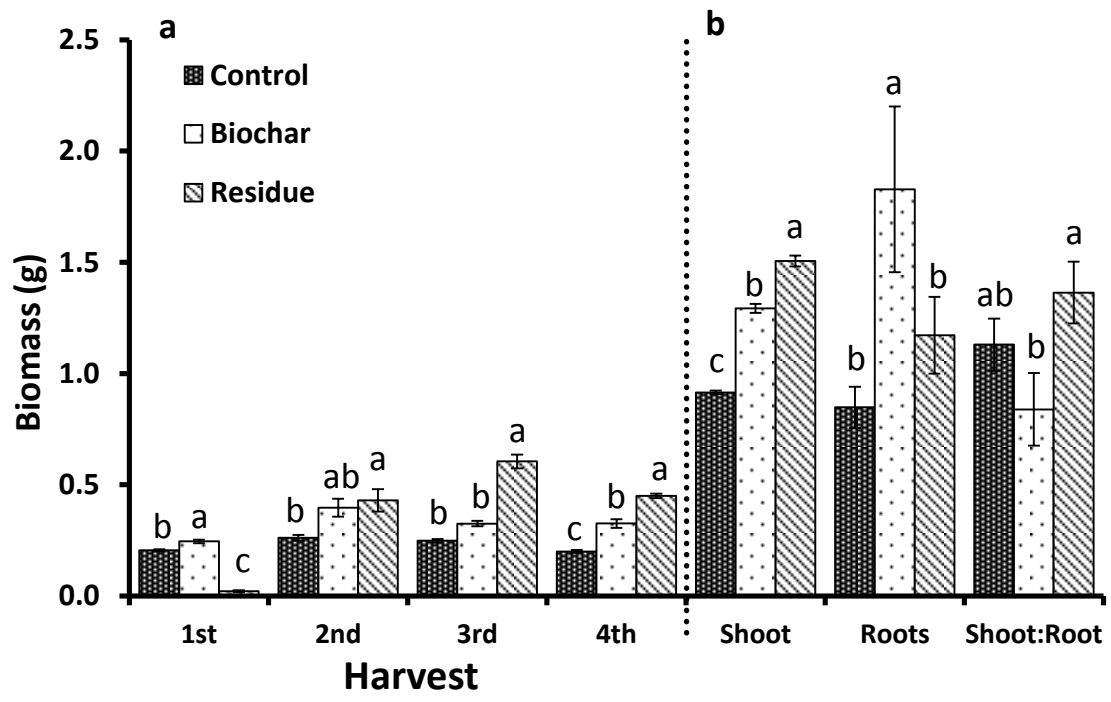
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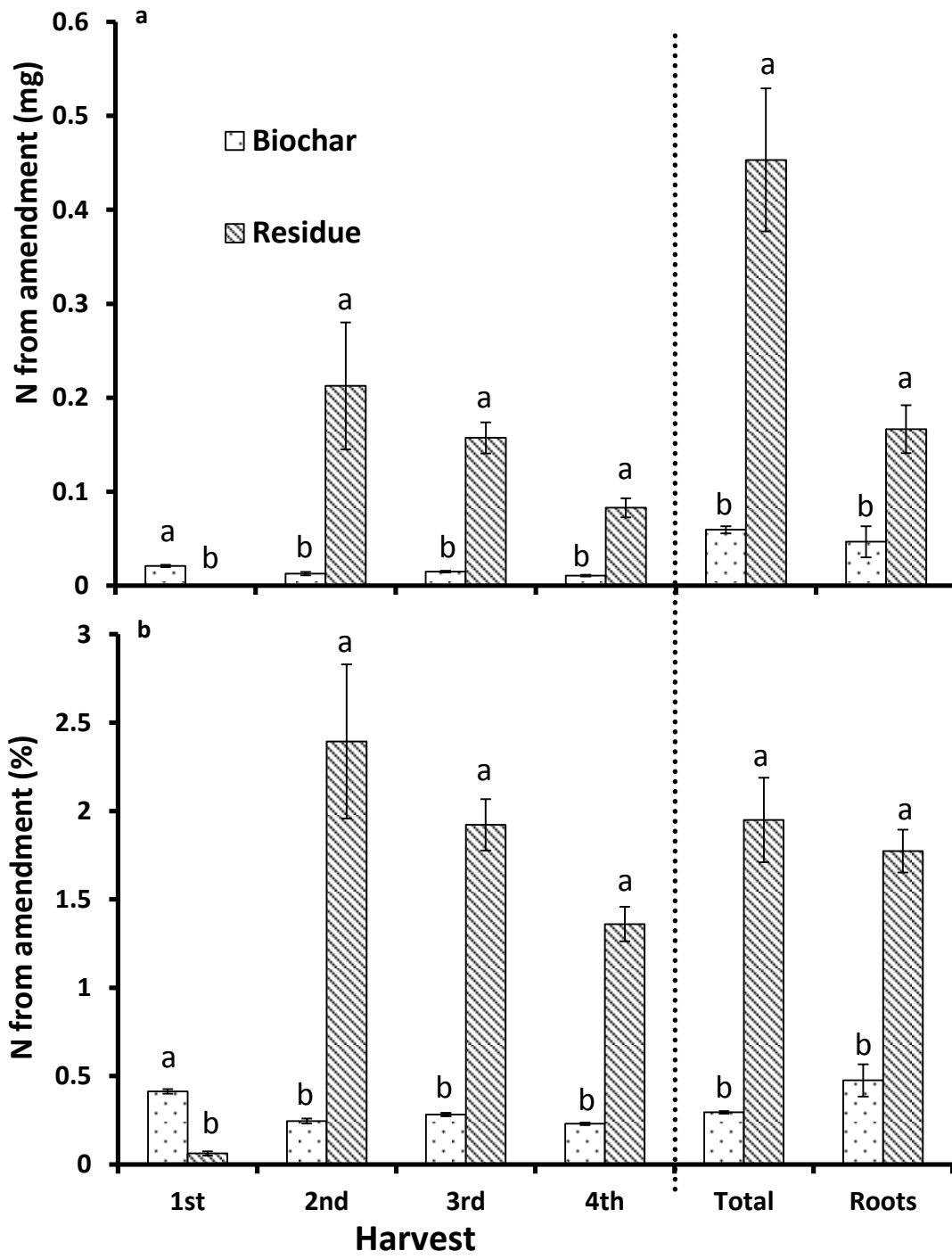
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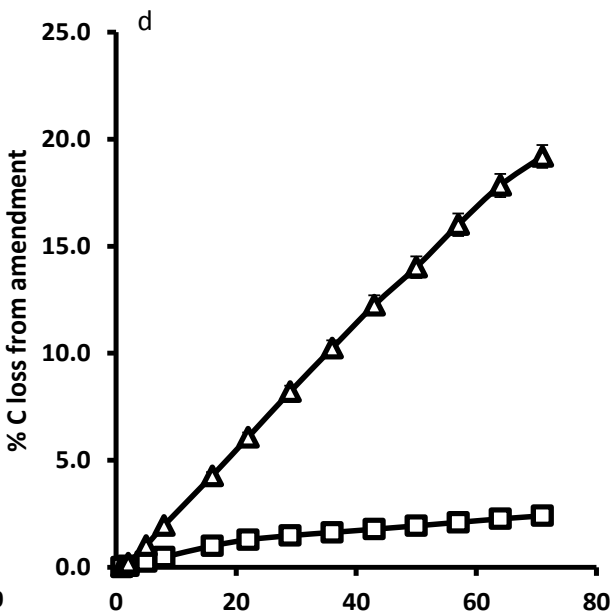
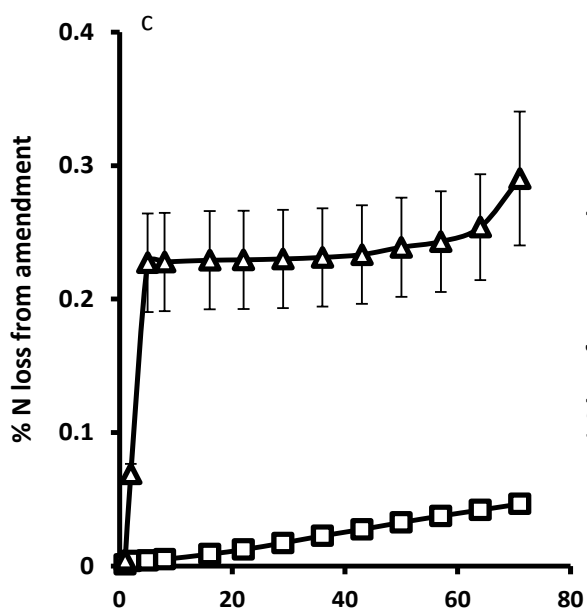
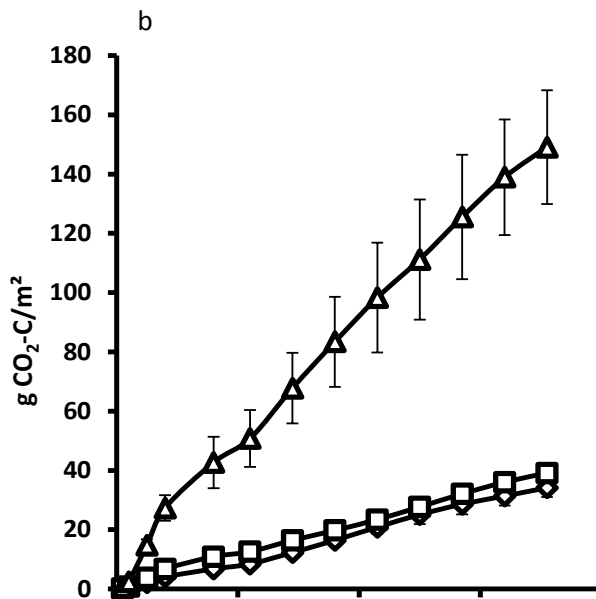
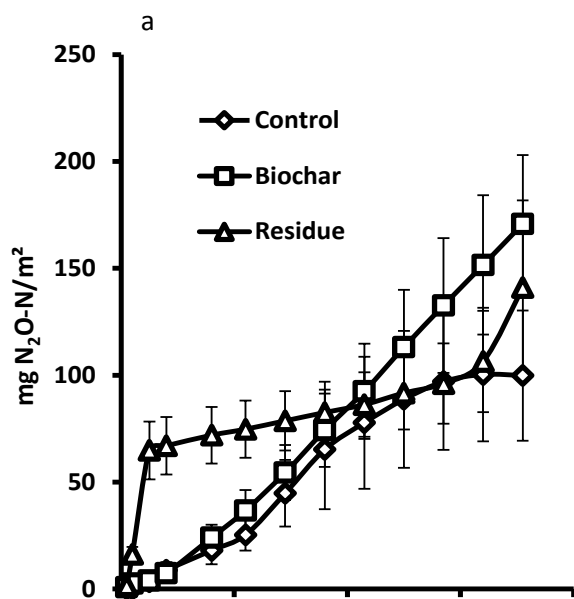
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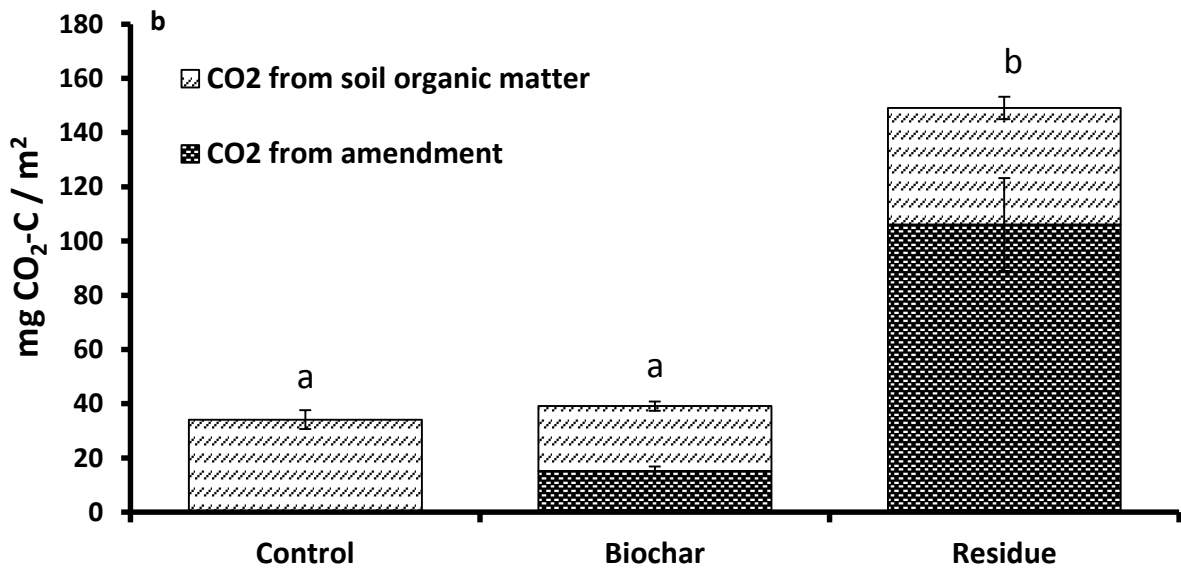
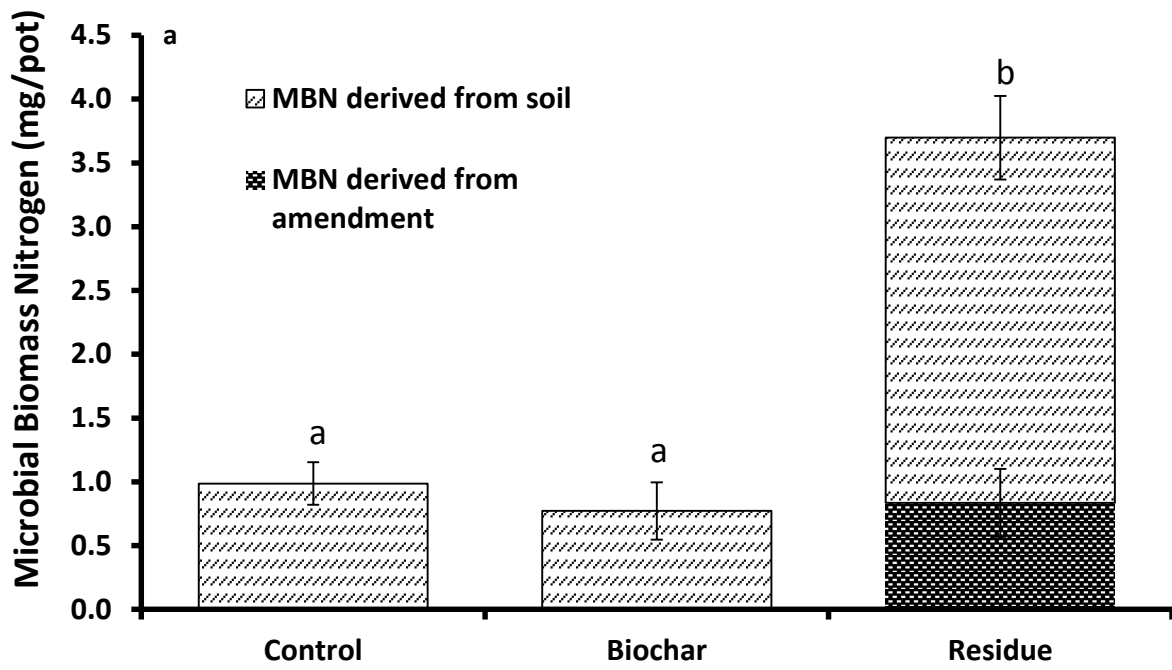
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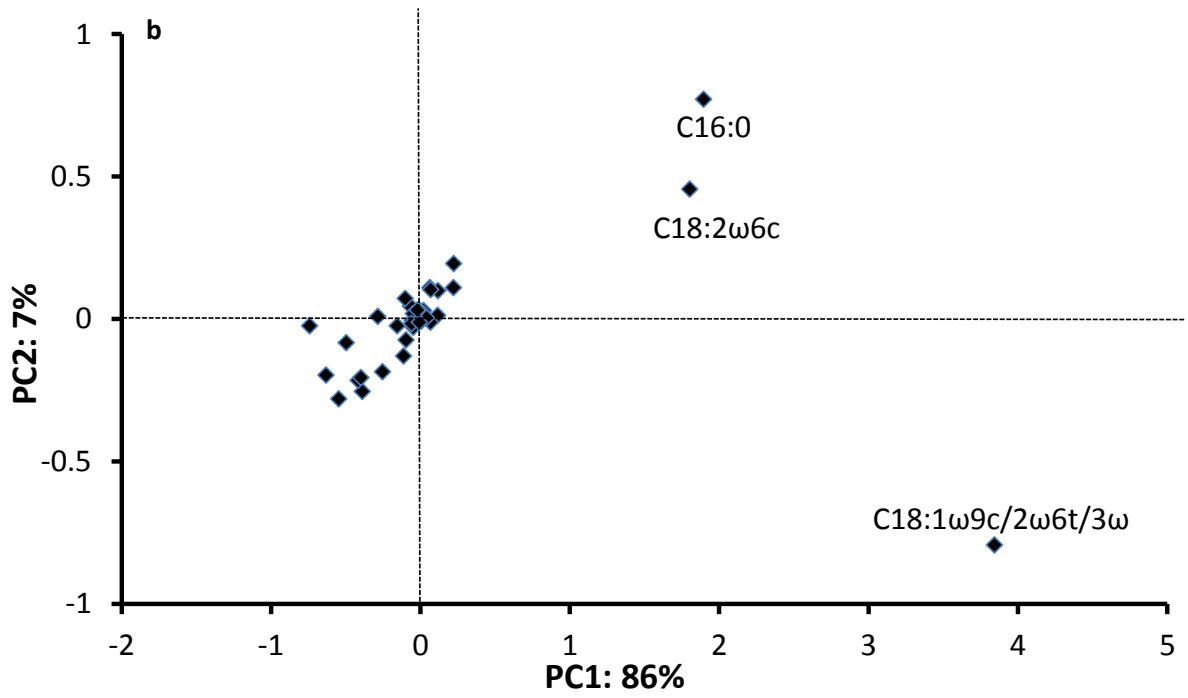
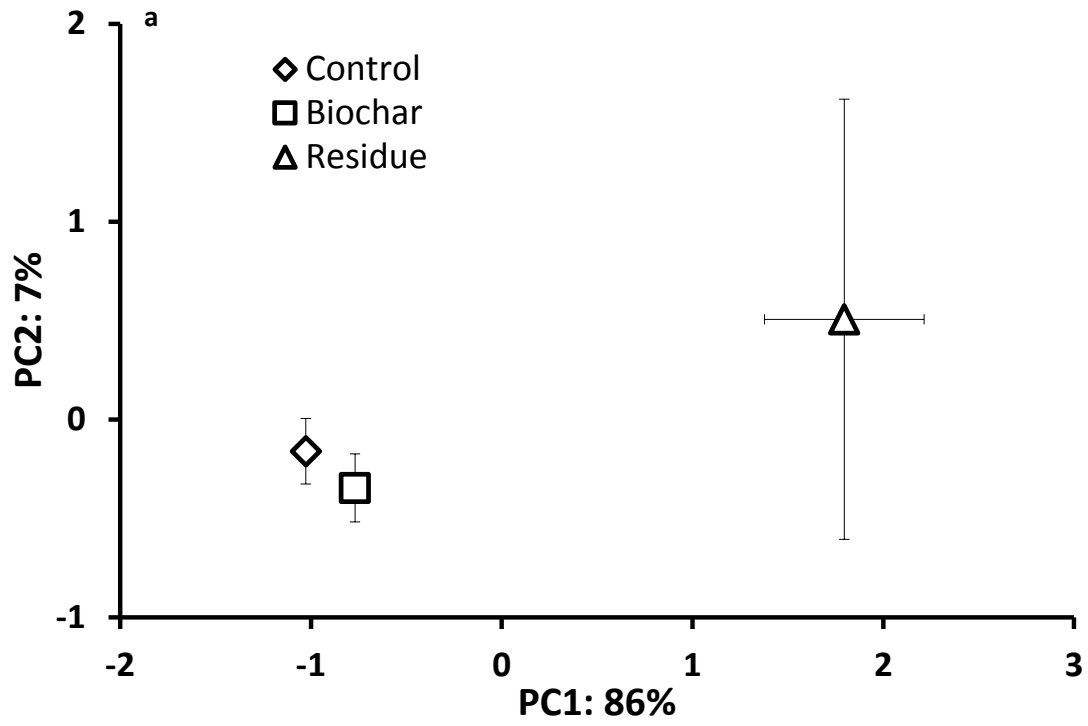




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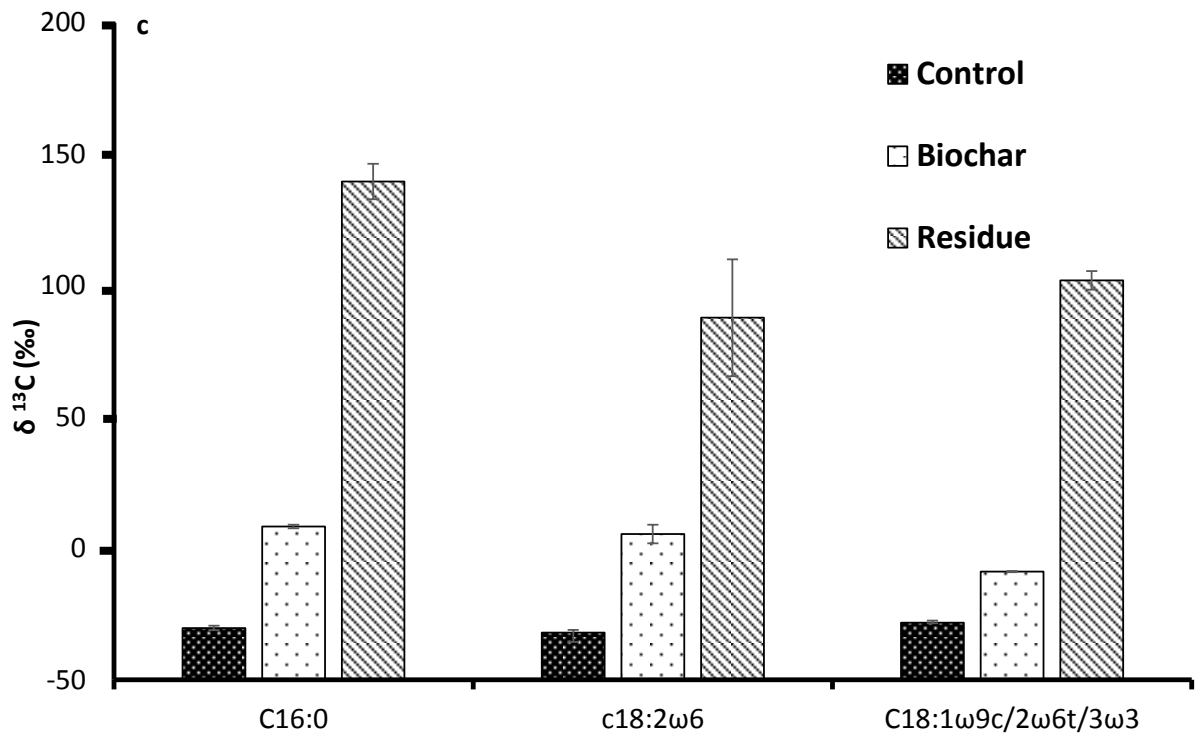


Figure 1a. *Lolium perenne* biomass (dry weight) produced from each treatment at each of the four harvests after 35, 63, 91 and 119 days; b. total aboveground biomass "Shoot" (i.e. from all harvests combined) and belowground biomass "Roots" (calculated as the average of the belowground biomass produced in each treatment), and the Shoot to Root Ratio after 119 days. Letters show significant differences within each harvest and for plant growth response characteristics. Columns show means, bars show  $\pm$ standard error (n=5).

Figure 2. (a) Total amount of N taken up from the amended material and (b) proportion of plant N derived from the amended material. Columns show means. Bars show  $\pm$ standard errors (n=5). N uptake from amendment was significantly different between treatments in all cases ( $P = 0.05$ ). Note that no material was amended to Control and so no data are reported for Control.

Figure 3. Cumulative  $N_2O$  (a) and  $CO_2$  (b) fluxes from microcosms in Exp. II over a 71 day incubation period and the percent loss of N (c) and C (d) from the amended material as determined by  $^{15}N$  or  $^{13}C$  analysis. Points show means. Bars show  $\pm$ standard errors (n=5, apart from Day 2 points where n=4). Note that no material was amended to Control and so no data are reported for Control in (c) or (d).

Figure 4. (a) Microbial biomass N (MBN) as determined by chloroform fumigation extraction with the contribution of each pool of N to microbial biomass N determined by stable isotope  $^{15}N$  71 days after application of amended material; (b) Cumulative  $CO_2$  emitted from each treatment, derived from each soil C pool using  $^{13}C$  isotope analysis over a 71 day incubation. Columns show means. Shaded columns show the mean contribution of each pool to the total. Bars show  $\pm$ standard errors (n=5).

Figure 5 (a) A principal component ordination plot of the first two principal components of PLFAs extracted from each sample. Points show mean coordinates of treatment replicates, bars show standard errors (n=5); (b) loading plot in which the PLFAs which contribute most to the discrimination between treatments are labelled; (c)  $\delta^{13}C$  profiles of the three PLFAs that contributed

most to the discrimination observed between treatments. Larger bars represent more uptake of applied enriched  $^{13}\text{C}$  material. Columns show means. Bars show  $\pm$ standard errors (n=5).

Table 1. Key Biochar and Feedstock characteristics

	Biochar	Residue
Fixed Carbon (%)	36.3	14.1
Volatiles (%)	32.9	71.4
Nitrogen (%)	2.7	2.3
Hydrogen (%)	3.4	NA
C:N ratio	22.0	31.9
Sulphur (%)	0.4	NA
H:C <sub>org</sub>	0.59	NA
Ash (%)	28.0	9.6
pH	9.2	5.4
<sup>13</sup> C enrichment	1.38 ±0.0002 at-%	1.40 ±0.003 at-%
<sup>15</sup> N enrichment	58.2 ±0.01 at-%	58.5 ±0.03 at-%

(All values provided on an oven dried (60°C) basis. pH was quantified in demineralised water 1:5 w/v)

Table 2: Key soil characteristics (based on soil dry weight)

Soil Texture	
Sand (%)	93.9
Silt (%)	4.3
Clay (%)	1.8
pH (CaCl <sub>2</sub> )	5.2
SOM (%)	4.6
N-content (mg kg <sup>-1</sup> )	
N-NH <sub>4</sub>	1.51 ±0.19
N-NO <sub>3</sub>	0.97 ±0.07
P-content (mg kg <sup>-1</sup> )	
P-PO <sub>4</sub>	3.96 ±0.17
K-content (mg kg <sup>-1</sup> )	16.49 ±0.92