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Wood biochar increases nitrogen retention in field settings mainly through abiotic processes

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ABSTRACT

Nitrogen (N) is an essential element associated with crop yield and its availability is largely controlled by microbially-mediated processes. The abundance of microbial functional genes (MFG) involved in N transformations can be influenced by agricultural practices and soil amendments. Biochar may alter microbial functional gene abundances through changing soil properties, thereby affecting N cycling and its availability to crops. The objective of this study was to assess the effects of wood biochar application on N retention and MFG under field settings. This was achieved by characterising soil labile N and their stable isotope compositions and by quantifying the gene abundance of *nifH* (nitrogen fixation), *narG* (nitrate reduction), *nirS*, *nirK* (nitrite reduction), *nosZ* (nitrous oxide reduction), and bacterial and archeal *amoA* (ammonia oxidation). A wood-based biochar was applied to a macadamia orchard soil at rates of 10 t ha^{-1} (B10) and 30 t ha^{-1} (B30). The soil was sampled after 6 and 12 months. The abundance of *narG* in both B10 and B30 was lower than that of control at both sampling months. Canonical Correspondence Analysis showed that soil variables (including dissolved organic C, NO_3^-–N and NH_4^+–N) and sampling time influenced MFG, but biochar did not directly impact on MFG. Twelve months after biochar application, NH_4^+–N concentrations had significantly decreased in both B10 (4.74 μg g^{-1}) and B30 (5.49 μg g^{-1}) compared to C10 (13.9 μg g^{-1}) and C30 (17.9 μg g^{-1}), whereas NO_3^-–N concentrations increased significantly in B30 (24.7 μg g^{-1}) compared to B10 (12.7 μg g^{-1}) and control plots (6.18 μg g^{-1} and 7.97 μg g^{-1} in C10 and C30 respectively). At month 12, significant $\delta^{15}N$ of NO_3^-–N depletion observed in B30 may have been caused by a marked increase in NO_3^-–N availability and retention in those plots. Hence, it is probable that the N retention in high rate biochar plots was mediated primarily by abiotic factors.

1. Introduction

Reliance on inorganic fertiliser is increasing globally to meet the needs of a growing population for food production (Lal, 2004; Bouwman et al., 2013). However, inorganic nitrogen (N) inputs do not always ensure high yields as plant-available N can be lowered through leaching, sorption and volatilisation (Jaynes et al., 2001). Furthermore, the movement of N into ground water and the atmosphere can have negative environmental impacts (Thorburn et al., 2011). Therefore, N-use efficiency needs to be maximised in cropping systems (Bramley and Roth, 2002; Manlay et al., 2007). Previous studies have shown that adding organic matter residues to the soil improves soil N retention (Blumfield and Xu, 2003; Bai et al., 2014; Reverchon et al., 2015) and provides labile carbon (C) sources to the soil microorganisms involved in N transformations (Steiner et al., 2008). Biochar is a C-rich product of the pyrolysis of different feedstocks such as crop residues, wood chips, poultry litter or manure (Lehmann and Joseph, 2009; Bai et al., 2015; Xu et al., 2015) and is used as a soil amendment to increase soil

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quality (Lehmann, 2007). Biochar affects N cycling through different mechanisms including sorption of NO_3^-, NH_3, NH_4^+ and organic-N as well as through changes in microbial processes and activities (Van Zwieten et al., 2010a, 2010b; Cayuela et al., 2014; Van Zwieten et al., 2014). However, these processes may reduce N availability to plants (Deenik et al., 2010). Biochar also alters cation and anion exchange capacity in the soil, which further influences N retention (Clough et al., 2013; Slavich et al., 2013). Feedstock, production temperature, residence time at maximum temperature and post biochar treatments may influence the retention of NO_3^-–N and NH_4^+–N (Clough and Condron, 2010; Mukherjee et al., 2011; Ippolito et al., 2012; Reverchon et al., 2014). However, recent evidence has shown that the N adsorbed by biochar can eventually become available to plants (Taghizadeh-Toosi et al., 2012a, 2012b).

The mechanisms through which biochar influences N availability and thus plant productivity remain largely unclear, although they seem to be principally mediated by microbial processes (Güereña et al., 2013; Anderson et al., 2014). The effects of biochar on microbially-mediated processes such as nitrification, denitrification and N fixation have been previously investigated (Rondon et al., 2007; Spokas and Reicosky, 2009; Van Zwieten et al., 2014, 2015). However, the influence of biochar on microbial functional genes (MFG) involved in N-cycling is still poorly understood. Recently, Ducey et al. (2013) and Harter et al. (2014) reported that biochar enhanced the abundance of MFG involved in N fixation, nitrification and denitrification, while Van Zwieten et al. (2014) suggested biochar increased the abundance of *nosZ* and hence reduced the emissions of N_2O, most likely through an increase in soil pH by 1–1.3 units. However, not all biochars increase soil pH and the effect of biochar on soil pH depends on biochar production temperature and ash content of feedstock (Mukherjee et al., 2011; Slavich et al., 2013; Zhao et al., 2013). These studies were undertaken in the laboratory and there is therefore a need to investigate how biochar amendment influences the abundance of MFG under field conditions.

Soil N isotope composition ($\delta^{15}N$) is a reliable indicator of N cycling (Hietz et al., 2011; Bai et al., 2012; Ibell et al., 2013; Wang et al., 2014). When N losses occur, soils will usually become enriched in $\delta^{15}N$ (Nadelhoffer and Fry, 1994). This is because microbial processes involved in N transformations discriminate against the heavier isotope, resulting in the ^{15}N enrichment of the substrates (Criss and Criss, 1999). For example, when NH_4^+ is transformed into NO_3^-, $^{14}NH_4^+$ is preferably used which results in the enrichment of $^{15}NH_4^+$ retention in the soil (Choi and Ro, 2003). Soil N cycling can be investigated with $\delta^{15}N$ in soil–plant–biochar systems (Reverchon et al., 2014, 2015; Bai et al., 2015). In the current study, we additionally determined soil $\delta^{15}N$ of NH_4^+–N and $\delta^{15}N$ of NO_3^-–N in order to better understand the mechanisms underlying N cycling in the presence of biochar.

Biochar is usually mixed and incorporated into soil, however, macadamia develops surface feeding roots and it is not possible to incorporate biochar into soil by ploughing. Therefore, biochar was applied to the surface. The results of this experiment are valuable to different systems (e.g. agroforestry, orchards and no-till cropping systems) where it is not possible to incorporate biochar into the soil by tilling. We examined the effect of wood-based biochar on soil N cycling within the first year following biochar application in a macadamia orchard in subtropical Australia, through its influence on MFG and $\delta^{15}N$ of N species. The main objectives were to: (a) assess the effects of biochar on soil labile N dynamics, including inorganic N, $\delta^{15}N$ of NH_4^+–N and NO_3^-–N, and dissolved organic N; and (b) determine the relationships between the abundances of MFG involved in N cycling and soil chemical properties.

2. Materials and methods

2.1. Biochar characterisation

Biochar (Black Earth, Kurwongbah) was produced from pine wood chips (*Pinus* spp.) in a slow pyrolysis unit at highest treatment temperature (HTT) of 550 °C and residence time of ca 45 min. Biochar properties are summarised in Table 1.

2.2. Site description and experimental design

The experimental site was established at Beerwah in south-east Queensland, Australia (26°50′14.16″S 152°56′49.96″E), in 2012. This area is subtropical with most precipitation in summer (December–February) (Fig. 1). The soil classified as a Kurosol with an acidic pH of 5.0. Soil properties are shown in Table 2. The orchard was planted with macadamia (*Macadamia integrifolia* Maiden & Betche: Proteaceae, *variety 741*) in 2003. The experimental site was set up with a randomised complete block design with six replicates per treatment. Twenty-four plots (4 m × 4 m) were established under 24 macadamia trees, with tree at the centre. The tree spacing was 4 m × 9 m. To prevent any contamination, at each row, there was a spacing of three trees between each plot and therefore plots in each row were 12 m apart.

Biochar was surface applied nine years after macadamia planting at two rates of 10 dry t ha^{-1} (B10) and 30 dry t ha^{-1} (B30). Although biochar is often incorporated into soil using rotary tillage or ploughing, it was not possible in this orchard setting (e.g. macadamia) where soil disturbance severely damages the established root system. Before application, biochar was mixed with the soil at the ratio of 1:1.5 (w/w; dry weight) to minimise wind and rain erosion. Soil was provided from the same farm and the properties of the soil did not differ from soil collected under trees (Table 2). Each plot was divided into 16 sub-plots (1 m × 1 m) and soil and biochar were mixed for 5 min for each sub-plot and the prepared mixture was then applied homogeneously onto each sub-plot. In B10 and B30 plots, the depth of the mixture added were 1 cm and 3 cm respectively. The B10 and B30 plots received 16 kg and 48 kg dry weight biochar respectively. The control plots received the same amount of soil with no biochar, namely 10 t ha^{-1} (C10) and 30 t ha^{-1} (C30). The whole orchard was fertilised on a monthly

Table 1
Biochar characteristics and available nutrients.

pH	DI 1:25	8.21
H:C molar ratio		0.33
Ash	%	34.2
Total C	%	50.8
$\delta^{13}C$	‰	−25.1
NH_4^+–N	μg g^{-1}	14.3
NO_3^-–N	μg g^{-1}	3.82
Total N	%	0.13
$\delta^{15}N$	‰	7.71
CEC	cmol(+) kg^{-1}	44.3
Al	mg kg^{-1}	2.67
Ca	Wt%	0.77
K	Wt%	0.11
Mg	Wt%	0.031
Na	mg kg^{-1}	71.3
P	mg kg^{-1}	102.0
S	mg kg^{-1}	30.9
Zn	mg kg^{-1}	9.70
B	mg kg^{-1}	1.15
Cu	mg kg^{-1}	0.61
Fe	mg kg^{-1}	597
Mn	mg kg^{-1}	40.8

DI: Deionised water.

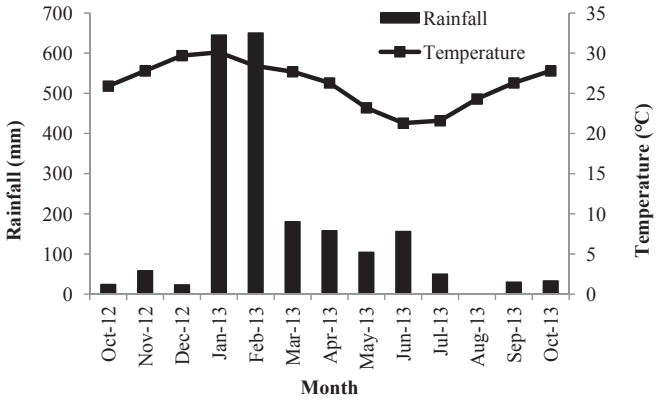

Fig. 1. Mean monthly rainfall and mean monthly maximum daily temperature at the experimental site, data extracted from Bureau of Methodology on-line database.

basis (total rate equivalent to 120 kg N ha^{-1} yr^{-1}). Due to the fact that the orchard had been managed similarly nine years prior to the experiment and during the experiment, the isotopic signature of the fertiliser has been well incorporated within the soil. Weeds were controlled mechanically but herbicide (glyphosate at label rate) was applied once a year before harvesting because the mechanical control was not possible when the kernels were on the orchard floor.

2.3. Soil sampling and chemical analyses

Soil sampling was carried out immediately before biochar application, and at 6 and 12 months following biochar application in March and October 2013. Soil samples were collected at 8 points chosen randomly within a 50 cm radius from the base of the tree stem using an auger (20 mm internal diameter) at depth of 0–5 cm at the end of a fertilisation cycle (i.e. before new fertiliser application). Soil samples collected at the same depth under the same tree were bulked and mixed to provide one homogenised sample per depth for each plot. Soil samples were sieved (2 mm) when field moist in the laboratory and stored at 4 °C before analysis. A sub-sample was stored at −20 °C immediately after sieving for subsequent molecular analyses. All extractions were obtained within 5 days following soil sampling.

A sub-sample of each soil sample was oven dried (50 °C) to a constant weight and ground to a fine powder (Rocklabs™ ring grinder). Approximately 20 mg were transferred into 8 mm × 5 mm tin capsules for total N (TN), $\delta^{13}C$ and $\delta^{15}N$ analysis using an isotope ratio mass spectrometer (GV Isoprime, Manchester, UK).

Soil NH_4^+–N and NO_3^-–N were extracted after agitation for 60 min with 2 M KCl followed by a 10-min centrifugation (3220 *g*) and then filtered through a Whatman 42 filter paper. Soil NH_4^+–N and NO_3^-–N concentrations were determined using a SmartChem 200, Discrete Chemistry Analyser (DCA). Total inorganic N (TIN) was the sum of NH_4^+–N and NO_3^-–N. The same 2 M KCl extractions were used to determine dissolved organic C (DOC) and total soluble N (TSN) using a Shimadzu TOC-$V_{CSH/CSN}$ TOC/N analyser. Dissolved organic N was calculated as the difference between TSN and TIN. Soil $\delta^{15}N$ of NH_4^+–N and $\delta^{15}N$ of NO_3^-–N were determined using a micro-diffusion technique as described in Stark and Hart (1996).

Table 2
Soil background information.

	Orchard soil	Soil used to mix with biochar
pH_{DI}	5.01	4.95
NH_4^+–N (μg g^{-1})	32.05	28.8
NO_3^-–N (μg g^{-1})	1.86	2.02
TN (%)	0.112	0.125
$\delta^{15}N$ (‰)	3.27	3.49

DI: Deionised water.

2.4. DNA extraction and quantification of functional gene abundance

DNA was extracted from 0.3 g soil using the MoBio Powersoil DNA isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions, with the final elution step in deionised water. Extractions were carried out no more than two weeks after sampling. The total amount of DNA was quantified using a NanoDrop spectrophotometer (Thermoscientific) and diluted in deionised water (1:10).

Quantitative PCR (qPCR) was performed immediately after DNA extractions to assess the abundance of the following genes: 16S rRNA gene for total bacteria, *amoA* gene for archaeal ammonia oxidizers (AOA) and bacterial ammonia oxidizers (AOB), *narG* (nitrate reductase gene), *nifH* (N fixation gene), *nirK* and *nirS* (nitrite reductase genes), and *nosZ* (nitrous oxide reductase gene). All qPCR reactions were conducted in duplicate using an Eppendorf Mastercycler ep realplex real-time PCR system (Eppendorf, Hamburg, Germany) and standard curves obtained using 10-fold serial dilutions of plasmid DNA containing the cloned genes of interest and spanning seven orders of magnitude. Specific primer combinations and qPCR conditions are listed in Table S1. Single qPCR reactions were prepared in a total volume of 20 μl including 10 μl SYBR green PCR Master Mix (Takara SYBR Premix Ex Taq (Perfect Real Time)), 0.4 μl of forward and reverse primers (10 μM) and approximately 5 ng of DNA. Melting curves and agarose gel running of PCR products were used at the end of each quantitative real-time PCR to check amplification specificity and purity of negative controls. The PCR efficiency ranged between 88 and 100%. The presence of PCR inhibitors in DNA extracted from soil was estimated by a 1:10 soil DNA dilution; no inhibition was detected.

The same sets of standards were used to quantify gene abundance at month 6 and month 12, except for 16S and *nifH*. Because qPCR was performed at different times for samples from month 6 and month 12 samplings, we calibrated the measured cycle threshold (C_t) values of standards quantification by placing the threshold lines at the same level for each same gene. All gene data were/are expressed in number of gene copies ng^{-1} DNA as recommended in several studies (Bru et al., 2007; Cŭhel et al., 2010; Rachid et al., 2013).

2.5. Statistical analysis

A repeated measures ANOVA was performed to detect significant difference among treatments, sampling time and their interaction followed by Tukey test where significant differences were detected to compare treatments and sampling month. All data were tested for normality using Shapiro–Wilk normality test and for homogeneity of variance using Levene's test. The $\delta^{15}N$ of NH_4^+–N and all gene data were transformed using Ln and Log_{10} transformation respectively. Pearson correlations were used to investigate relationships between soil chemical variables and functional gene abundances. SPSS 21 software was used for all above statistical analyses. Canonical Correspondence Analysis (CCA) was performed using XLSTAT 2015 to assess the relationships between abundance of functional genes and soil properties, using data from months 6 and 12.

3. Results

3.1. Soil characteristics and labile fractions

Soil pH and EC were significantly influenced by both treatment and sampling time. The interactions between treatment and sampling time were not significant (Table 3). Soil pH was higher in B10 and B30 compared to C10 and C30 at months 6 and 12 following biochar application (Table 4). Soil EC in B10 was significantly lower than that of C10, at 12 months after biochar amendment (Table 4).

Soil TN was not influenced by treatment nor by sampling time. The interactions between treatment and sampling time were not significant (Table 3). The C:N ratio was significantly higher in both B10 and B30 compared to C10 and C30 at 6 and 12 months (Table 4). Both soil NH_4^+–N and NO_3^-–N were significantly affected by biochar application and sampling time, and the interactions between biochar application and sampling time for NH_4^+–N and NO_3^-–N were significant (Table 3). Soil NH_4^+–N concentration was not influenced by biochar application at month 6. However, at month 12, soils receiving biochar treatments had significantly lower NH_4^+–N concentration than the control soils (Fig 2a). Soil NH_4^+–N at month 12 was significantly lower than those of month 6 in all treatments. No significant differences in soil NO_3^-–N were observed among treatments at month 6 (Fig 2b). In contrast, soil NO_3^-–N concentration was significantly greater in B30 plots compared to B10, C10 and C30 plots, 12 months after biochar application (Fig. 2b).

Differences in $\delta^{15}N$–NH_4^+–N were significant with respect to biochar application and sampling time but interactions between biochar application and sampling time were not significant (Table 3). The $\delta^{15}N$ of NH_4^+–N was significantly enriched in B10 and B30 compared with that of C10 and C30, 12 months following biochar application (Fig. 2c). The $\delta^{15}N$ of NO_3^-–N was influenced by biochar application but not by sampling time (Table 3). The $\delta^{15}N$ of NO_3^-–N in B30 was significantly lower than that of B10, C10 and C30 at month 12 (Fig. 2d). Soil total $\delta^{15}N$ was not significantly influenced by biochar application but sampling time and interactions between biochar application and sampling time were significant (Table 3, Fig. 2e). Soil DOC and DON were influenced by sampling time and there was no significant influence of biochar application (Table 3).

3.2. Abundance of functional genes involved in N cycle

Both biochar and sampling time independently affected the abundance of some MFG (Table 5). The lowest gene abundance of the *amoA* gene of AOA was found in B30 plots 12 months following biochar application. Adding biochar also decreased the abundance of *narG* at both sampling times (Table 6).

Differences in MFG abundances were also observed between sampling times (Table 6). The AOA (B10), AOB (B10 and B30) and *nirS* genes (B10 and B30) were higher 12 months after biochar application than after 6 months. The abundance of the 16S and *nirS* genes also increased in the control plots after 12 months. In contrast, abundances of *nifH* (B10) and *nosZ* (B10 and B30) decreased between months 6 and 12 after biochar application (Table 6). The denitrification genes *narG* and *nirK* were not influenced by sampling time.

Pearson correlations showed that relationships between soil variables and abundances of MFG involved in N cycling varied depending upon sampling time (Table 7). Six months after biochar application, significant correlations were found between soil $\delta^{15}N$ and $\delta^{15}N$ of NH_4^+–N and abundances of the *amoA* gene of AOB. The $\delta^{15}N$ of NH_4^+–N was also negatively correlated with *nosZ*. These relationships were not significant at month 12. At month 6, bacterial abundance as measured by the abundance of the 16S gene was positively correlated with soil EC. Soil TN was strongly and positively correlated with the abundance of *nirS*, while soil DOC correlated significantly and negatively with the denitrification genes *nirS* and *nosZ*. The abundance of the *amoA* gene of AOB was negatively correlated with soil pH, which was the only relationship still valid at month 12. Twelve months after biochar application, soil pH, EC and NH_4^+–N were significantly correlated with the abundance of AOB while AOA was negatively correlated with soil NO_3^-–N. The abundance of *narG* was significantly correlated with soil pH, NO_3^-–N, NH_4^+–N, and DON. Soil DON was also significantly correlated with the denitrification genes *nirS*, *nirK* and *nosZ*. No significant correlations were found between the abundance of *nifH* and the measured soil parameters at either 6 or 12 months.

A CCA analysis was performed to determine the soil variables that best explained the patterns in MFG abundances. The soil parameters which were included in the CCA analysis were selected on the basis of the strength of their correlations with MFG abundances when both month 6 and month 12 data were pooled. The selected soil variables were pH, EC, DOC, DON, NO_3^-–N and NH_4^+–N. The CCA biplot confirmed that sampling time influenced the abundance of

Table 4
Soil pH, electrical conductivity (EC), total N (TN), C:N ratio, dissolved organic C (DOC) and dissolved organic N (DON) at months 6 and 12 following biochar application in a macadamia orchard, in biochar (B10 and B30) and control (C10 and C30) plots. Means followed with different letters show a significant difference at $P < 0.05$ at each sampling time. Values in bold cases represent significant differences between sampling time ($P < 0.05$). Mean standard errors are presented in the brackets.

	pH		EC ($\mu S\ cm^{-1}$)	
	Month 6	Month 12	Month 6	Month 12
B10	**5.00 (0.09)a**	**5.38 (0.1)a**	**122 (5.3)**	**243 (10)b**
B30	5.21 (0.05)a	5.37 (0.06)a	**114 (9.1)**	**265 (11)ab**
C10	**4.66 (0.07)b**	**5.02 (0.1)b**	**135 (9.0)**	**286 (10)a**
C30	4.93 (0.08)ab	4.94 (0.1)b	**143 (5.04)**	**272 (20)ab**
	TN (%)		C:N ratio	
	Month 6	Month 12	Month 6	Month 12
B10	0.14 (0.008)	0.15 (0.008)	18.6 (0.4)b	18.3 (1.1)b
B30	0.15 (0.007)	0.14 (0.009)	20.1 (0.6)a	21.6 (2.4)a
C10	0.14 (0.006)	0.15 (0.007)	17.1 (0.5)c	16.8 (2.7)c
C30	0.14 (0.004)	0.14 (0.004)	16.5 (0.4)c	16.0 (1.8)c
	DOC ($\mu g\ g^{-1}$)		DON ($\mu g\ g^{-1}$)	
	Month 6	Month 12	Month 6	Month 12
B10	**41.1 (4.5)**	**149 (6.1)**	12.5 (4.2)	18.5 (1.3)
B30	**39.4 (2.6)**	**136 (7.0)**	**12.0 (2.0)**	**20.4 (2.6)**
C10	**40.6 (2.5)**	**169 (8.0)**	**13.1 (2.8)**	**28.9 (2.7)**
C30	**40.4 (2.6)**	**162 (14)**	**9.16 (2.7)**	**23.7 (2.6)**

Table 3
P values from a repeated measures ANOVA for biochar application (B), sampling time (ST) and their interactions (B × ST) following biochar application in a macadamia orchard.

	df	pH	EC	TN	C:N ratio	NH_4^+–N	NO_3^-–N	$\delta^{15}N$–NH_4^+–N	$\delta^{15}N$–NO_3^-–N	$\delta^{15}N$	DOC	DON
B	3	***P* < 0.0001**	**0.04**	0.92	***P* < 0.0001**	**0.007**	**0.002**	**0.055**	**0.045**	**0.085**	0.083	0.12
ST	1	**0.003**	***P* < 0.0001**	0.44	0.64	***P* < 0.0001**	***P* < 0.0001**	**0.013**	0.09	***P* < 0.0001**	***P* < 0.0001**	***P* < 0.0001**
B × ST	3	0.21	0.46	0.48	0.002	**0.006**	***P* < 0.0001**	0.22	**0.002**	***P* < 0.0001**	0.16	0.36

Bold cases are significant.

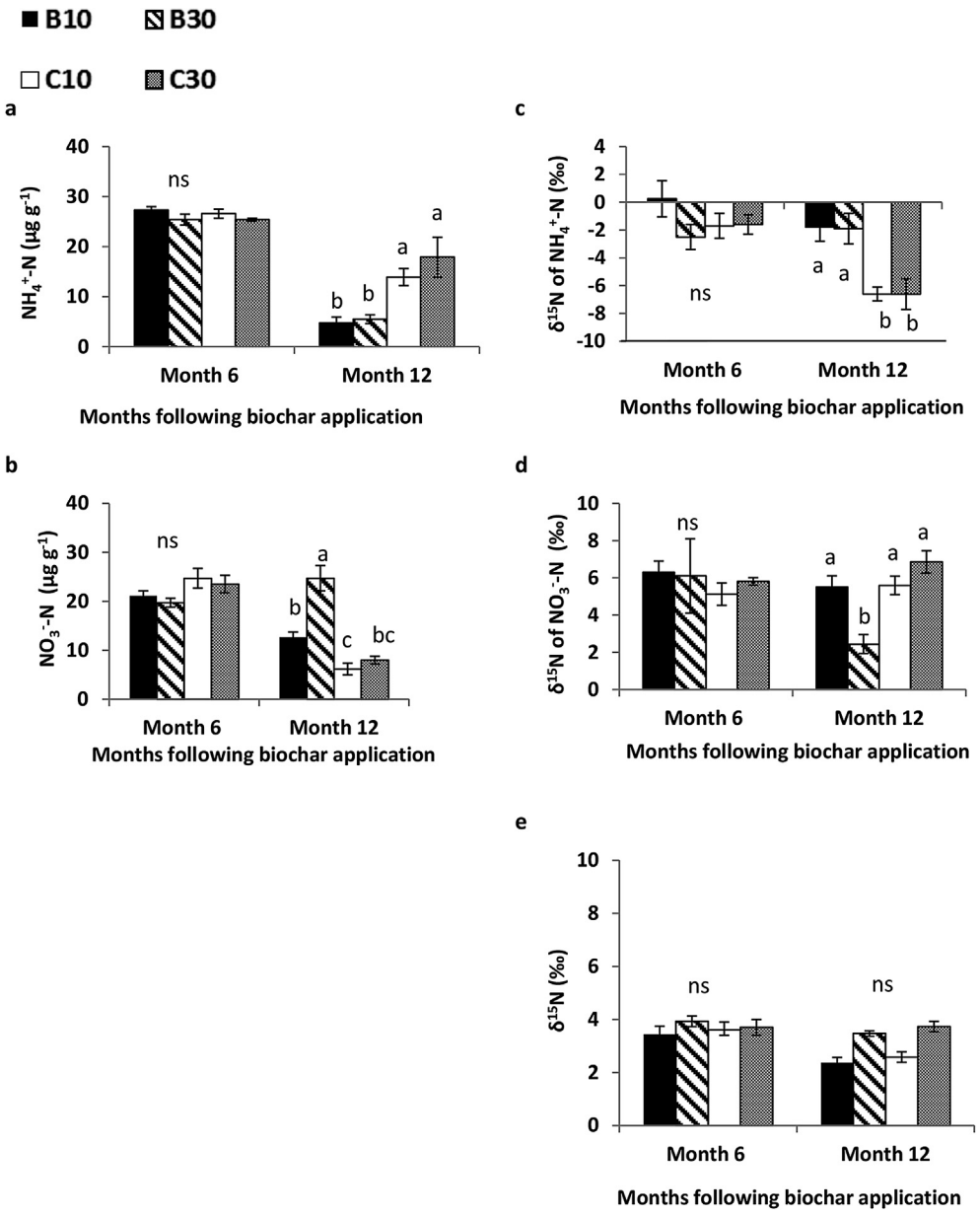

Fig. 2. Soil NH_4^+–N (**a**), NO_3^-–N (**b**), δ^{15}N of NH_4^+–N (**c**), δ^{15}N of NO_3^-–N (**d**) and soil total δ^{15}N (**e**) among treatments at months 6 and 12 following biochar application. Treatments were B10 (black columns), B30 (hatch columns), C10 (white columns) and C30 (grey columns) plots. Different letters show significant differences at $P < 0.05$ and ns presents no significant difference ($P > 0.05$).

MFG as month 6 and month 12 data were clearly segregated, month 6 data being clustered at the left of the "y" axis and month 12 data on its right but no segregations among treatments were found (Fig. 3). The first and second axes of the CCA biplot explained 88.2% and 11.4% of the gene abundance variance, respectively. Soil DOC, EC and NH_4^+–N were the constraining variables with the highest scores for the "x" axis (0.929, 0.898 and −0.792 respectively) while soil DON was the variable with the highest score for the "y" axis (0.660). The abundance of *nirS* was associated with larger soil DOC while *nifH* was related to larger NH_4^+–N concentrations. The

Table 5
P values of microbial functional gene abundance from a repeated measures ANOVA for biochar application (B), sampling time (ST) and their interactions (B × ST) following biochar application in a macadamia orchard.

	df	*16S*	AOA	AOB	*narG*	*nifH*	*nirK*	*nirS*	*nosZ*
B	3	0.317	**0.037**	0.497	**0.036**	0.062	0.094	0.489	0.146
ST	1	**0.001**	**0.033**	***P* < 0.0001**	0.732	***P* < 0.0001**	0.693	***P* < 0.0001**	***P* < 0.0001**
B × ST	3	0.305	0.496	0.308	0.620	0.501	0.379	0.203	0.418

Bold cases are significant.

Table 6
Abundances of N-cycling genes (number of gene copies ng^{-1} DNA) at months 6 and 12 following biochar application in a macadamia orchard, in biochar (B10 and B30) and control (C10 and C30) plots. Mean standard errors are presented in the brackets. Lower case letters indicate significant differences between treatments at P < 0.05 at each sampling time. Values in bold indicate significant differences between sampling times ($P < 0.05$).

	16S		AOA	
	Month 6	Month 12	Month 6	Month 12
B10	2.31×10^8 (2.8×10^7)	2.91×10^8 (2.5×10^7)	**3.92×10^5 (4.8×10^4)**	**6.15×10^5 (4.4×10^4)a**
B30	2.22×10^8 (5.4×10^7)	3.58×10^8 (3.2×10^7)	3.60×10^5 (3.5×10^4)	4.10×10^5 (7.0×10^4)b
C10	**2.08×10^8 (1.3×10^7)**	**4.83×10^8 (1.3×10^8)**	4.92×10^5 (8.1×10^4)	7.17×10^5 (1.3×10^4)a
C30	**2.61×10^8 (2.5×10^7)**	**4.32×10^8 (3.5×10^7)**	5.36×10^5 (6.6×10^4)	5.55×10^5 (4.6×10^4)ab
	AOB		*nifH*	
	Month 6	Month 12	Month 6	Month 12
B10	**5.74×10^5 (1.2×10^5)**	**8.52×10^5 (1.5×10^5)**	**5.48×10^6 (6.1×10^5)**	**3.92×10^6 (3.1×10^5)**
B30	**5.11×10^5 (7.9×10^4)**	**8.66×10^5 (8.4×10^4)**	4.24×10^6 (2.7×10^5)	3.80×10^6 (2.4×10^5)
C10	6.45×10^5 (1.2×10^5)	9.18×10^5 (1.2×10^5)	**5.74×10^6 (5.0×10^5)**	**4.01×10^6 (3.3×10^5)**
C30	**5.65×10^5 (7.1×10^4)**	**1.24×10^6 (1.1×10^5)**	**6.01×10^6 (4.4×10^5)**	**4.14×10^6 (3.6×10^5)**
	narG		*nirK*	
	Month 6	Month 12	Month 6	Month 12
B10	8.34×10^7 (8.34×10^6)b	8.07×10^7 (4.66×10^6)b	2.52×10^5 (4.9×10^4)	1.98×10^5 (3.2×10^4)
B30	7.98×10^7 (3.84×10^6)b	8.21×10^7 (5.97×10^6)b	2.52×10^5 (4.0×10^4)	3.67×10^5 (7.0×10^4)
C10	1.07×10^8 (1.50×10^7)a	9.56×10^7 (3.54×10^6)ab	2.74×10^5 (4.8×10^4)	3.38×10^5 (6.7×10^4)
C30	8.79×10^7 (6.98×10^6)ab	1.00×10^8 (4.94×10^6)a	4.26×10^5 (1.0×10^5)	3.32×10^5 (2.5×10^4)
	nirS		*nosZ*	
	Month 6	Month 12	Month 6	
B10	**2.10×10^6 (4.4×10^5)**	**2.48×10^7 (2.2×10^6)**	**2.54×10^4 (5.0×10^3)**	**1.01×10^4 (2.0×10^3)**
B30	**1.81×10^6 (1.1×10^5)**	**3.43×10^7 (4.0×10^6)**	**2.58×10^4 (3.2×10^3)**	**1.81×10^4 (4.8×10^3)**
C10	**1.91×10^6 (1.9×10^5)**	**3.53×10^7 (2.6×10^6)**	**3.04×10^4 (2.6×10^4)**	**1.62×10^4 (2.0×10^4)**
C30	**2.04×10^6 (2.2×10^5)**	**2.67×10^7 (9.0×10^5)**	**2.87×10^4 (2.7×10^4)**	**1.30×10^4 (1.0×10^4)**

Table 7
Pearson coefficients for correlation between the abundance of functional genes involved in N cycling and soil characteristics, 6 months and 12 months following biochar application. Significant coefficients are presented in bold (* significant at $P < 0.05$; ** significant at $P < 0.01$).

	16S		AOA		AOB		*nifH*	
	Month 6	Month 12	Month 6	Month 12	Month 6	Month 12	Month 6	Month 12
pH	−0.047	−0.192	0.047	−0.016	**−0.416***	**−0.539****	−0.357	−0.203
EC	**0.474***	0.187	0.084	0.129	−0.133	**0.427***	0.298	−0.030
TN	0.372	0.005	−0.006	−0.063	0.088	−0.274	0.115	−0.154
NH_4^+−N	−0.087	0.195	0.080	0.127	−0.083	**0.469***	0.122	−0.008
NO_3^-−N	0.199	−0.315	−0.307	**−0.489***	−0.301	−0.096	−0.045	−0.242
DOC	0.342	−0.103	−0.146	0.215	−0.378	0.051	−0.382	−0.051
DON	−0.047	0.010	−0.010	−0.01	0.235	0.276	0.063	−0.066
$\delta^{15}N$	−0.134	0.048	−0.058	−0.169	**−0.442***	0.113	−0.350	−0.084
$\delta^{15}N$–NH_4^+−N	0.145	−0.153	−0.177	−0.223	**−0.557****	−0.210	−0.344	−0.091
$\delta^{15}N$–NO_3^-−N	0.053	0.247	0.039	0.305	−0.136	0.265	−0.163	0.097
	narG		*nirK*		*nirS*		*nosZ*	
	Month 6	Month 12	Month 6	Month 12	Month 6	Month 12	Month 6	Month 12
pH	−0.253	**−0.476***	0.118	−0.144	0.305	0.009	−0.088	0.084
EC	0.234	0.380	0.204	0.130	0.165	0.111	0.292	−0.035
TN	0.317	0.072	0.271	0.188	**0.544****	0.202	0.393	0.314
NH_4^+−N	0.303	**0.414***	0.069	0.221	0.390	0.005	−0.034	0.027
NO_3^-−N	0.259	**−0.483***	−0.055	0.053	−0.030	−0.076	−0.022	−0.061
DOC	−0.208	0.215	−0.337	0.272	**−0.406***	0.152	**−0.410***	0.218
DON	0.360	**0.430***	0.026	**0.528****	0.080	**0.444***	0.183	**0.469***
$\delta^{15}N$	−0.288	0.225	−0.210	0.349	−0.324	0.065	−0.308	0.249
$\delta^{15}N$–NH_4^+−N	−0.217	−0.139	−0.294	0.024	−0.206	0.068	**−0.565****	0.083
$\delta^{15}N$–NO_3^-−N	−0.122	0.267	0.145	−0.016	0.126	−0.019	−0.094	0.048

denitrification genes *narG*, *nirK* and *nosZ* were distributed on the upper left side of the biplot and were associated with larger NO_3^-−N concentrations. However, this was not the case for *nirS* abundance.

4. Discussion

Our results indicate that the concentrations of both NH_4^+−N and NO_3^-−N were influenced by biochar application 12 months after application. At twelve months, biochar influenced soil inorganic N availability with a decrease in NH_4^+−N concentration in both B10 and B30 and with an increase in NO_3^-−N concentration in B30 in comparison to control plots. Decreases in soil NH_4^+−N and increases in soil NO_3^-−N have been observed in other biochar studies under controlled laboratory-based conditions (Ding et al., 2010; Van Zwieten et al., 2010a) and our work extends these findings to the present field settings. The changes in soil NH_4^+−N and NO_3^-−N

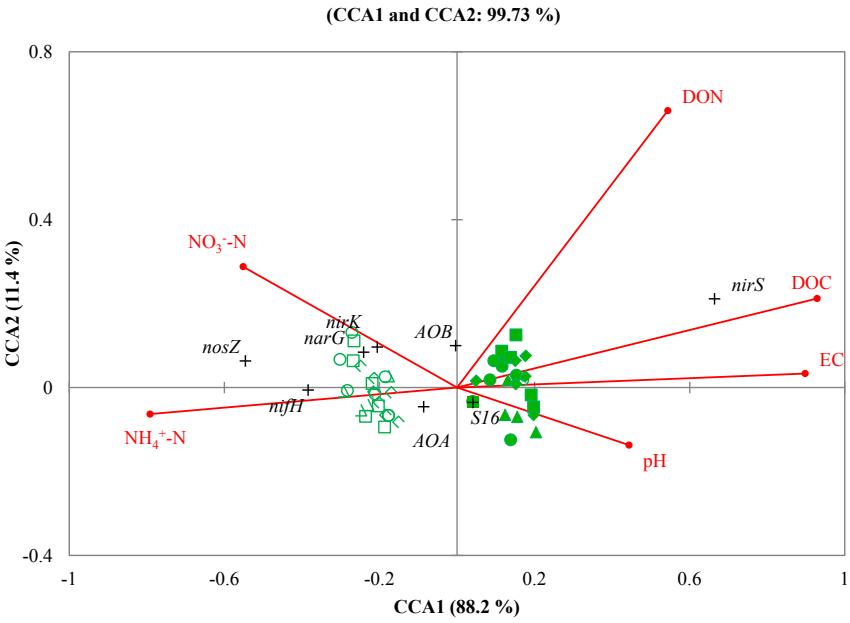

Fig. 3. Canonical Correspondence Analysis (CCA) biplot representing the relationships between abundance of functional genes and soil properties, using the months 6 and 12 data. Vectors represent the soil characteristics from 0 to 5 cm (EC: electrical conductivity; DOC: dissolved organic C; DON: dissolved organic N). Axes 1 and 2 represent 88.2% and 11.4% of the variation in the data, respectively. Open green: Month 6; Close green: Month 12; Circles: C10 plots; Rectangles: B10 plots; Diamonds: C30 plots; Triangles: B30 plots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentrations when biochar is applied may be caused by a number of abiotic and biotic factors (Clough and Condron, 2010). Biochar presents negatively charged functional groups on its surface leading to adsorption of positively charged NH_4^+–N (Novak et al., 2010; Singh et al., 2010). In the present study, TN did not differ between control and biochar plots but NH_4^+–N decreased in biochar applied soils, suggesting immobilisation of NH_4^+–N by biochar. Lack of biochar effects on the MFG data confirms that immobilisation by biochar is likely to be the principal responsible mechanism for the observed lowering in soil NH_4^+–N concentration (Fig 2 and Table 6).

There was a significant depletion of $\delta^{15}N$ of NO_3^-–N in B30 compared to B10, C10 and C30 at month 12. Both leaching and denitrification can result in enriched soil ^{15}N signals (Nadelhoffer and Fry, 1994; Choi and Ro, 2003) but nitrification may result in depleted soil ^{15}N signals due to increased NO_3^-–N availability (Choi and Ro, 2003). A reduction in nitrification or increased denitrification rates would decrease soil NO_3^-–N concentrations, leading to $\delta^{15}N$ enrichment of NO_3^-–N (Hogberg, 1997; Pörtl et al., 2007). In our experiments, biochar only decreased the abundance of *narG*. Decreased *narG* could increase NO_3^-–N but NO_3^-–N only increased in B30. Thus, although part of increased NO_3^-–N might be related to decreased abundance of *narG*, the decreased abundance of AOA in B30 could have offset the effects of decreased *narG*. Therefore, the abundance of MFG could not explain the depleted $\delta^{15}N$ of NO_3^-–N and increased NO_3^-–N concentration in B30. Although we did not assess NO_3^-–N leaching, based on soil $\delta^{15}N$ of NO_3^-–N, NO_3^-–N losses were significantly reduced in high rate biochar plots (B30) compared to low rate biochar and control plots. If leaching occurs, $\delta^{15}N$ of NO_3^-–N would be enriched due to the loss of lighter N from the system, as explained by Hogberg (1997). Therefore, in the absence of evidence for nitrification enhancement by biochar, decreases in leaching could explain the larger soil NO_3^-–N concentrations in B30 plots. We recorded higher soil NO_3^-–N concentrations in B30 and depleted foliar $\delta^{15}N$ in both B10 and B30 plots at months 15, 18 and 24 following biochar application (data not presented).

The abundances of only AOA and *narG* were influenced by biochar application. Previous laboratory-based experiments have shown that biochar can alter functional gene abundance (Ducey et al., 2013; Harter et al., 2014), and our research confirms these findings under field conditions but only for AOA and *narG*. The AOA abundance was negatively influenced by high rates of biochar application, but not when 10 t ha^{-1} biochar were applied. Biochar has been shown to increase nitrification rates (Prommer et al., 2014; Ulyett et al., 2014; Case et al., 2015) and the community size of ammonia oxidisers (Taketani and Tsai, 2010; Song et al., 2014), most likely through an increase in soil pH that creates more favourable conditions for soil nitrifying microorganisms (Nelissen et al., 2012). In the present study, although biochar did induce an increase in soil pH, the soil remained acidic (pH 5.0 and 5.4) even after biochar amendment, which may explain the lack of increase in AOA and AOB abundances.

Biochar also decreased the abundance of *narG*, but did not influence the abundance of other denitrification genes. Reductions of denitrification rates following biochar amendment have been reported by previous studies (Nelissen et al., 2012; Cayuela et al., 2013; Case et al., 2015). The biochar used in this study was produced at 550 °C and had a relatively low molar H/C ratio, which could constitute an insufficient source of bioavailable C to sustain denitrifying communities, as recently reported by Van Zwieten et al. (2014). In our experiment there was a high association between *nirS* and DOC as shown in Fig. 3 but biochar did not affect either DOC concentrations nor *nirS* abundance. In addition, Anderson et al. (2014) suggested that biochar may influence the relative proportion of nitrifiers and denitrifiers in the microbial population rather than their absolute gene abundance. This may also explain the absence of a biochar effect on denitrification genes in B30 plots.

Soil pH has been shown to be significantly and positively correlated with nitrifier and denitrifier gene abundance (Lindsay et al., 2010; Shen et al., 2014), and is a reliable determinant of soil bacterial community structure (Lauber et al., 2009; Rousk et al.,

2010). However, in our study, soil pH was not correlated with MFG abundance, with the exception of AOB at both sampling times and with *narG* at month 12. Our results contrast with previous findings that AOA abundance was higher than AOB in acidic soils (Nicol et al., 2008; Moin et al., 2009; Wessén et al., 2011). The lack of overlap between the explanatory variables to the variation in the abundance of the *amoA* gene of the AOA and AOB communities had been previously reported (Bru et al., 2011; Wessén et al., 2011). These endorse the recommendation made by Wessén and Hallin (2011) who proposed the combined quantification of AOB and AOA communities as bio-indicators for soil monitoring and land management planning (Wessén and Hallin, 2011).

Our experiment indicated that MFG abundances were more strongly influenced by environmental factors than by biochar application, as shown by CCA. The soil collection was undertaken at the beginning and end of growing season in the Southern hemisphere and soil moisture was higher at month 6 compared to the 12 month sampling time (data not presented). Therefore, the segregation of gene data between sampling time may be due to the seasonal differences. Seasonal effects may further influence soil microbial communities and soil–plant–microbe–biochar interactions by affecting plant growth and productivity, and hence the amount and composition of root exudates (Anderson et al., 2014).

It should be noted that changes in MFG abundance may not necessarily suggest changes in microbial activity and function (Nannipieri et al., 2003; Levy-Booth et al., 2014), which is supported by the lack of strong correlations between the measured soil variables and the gene data. However, several studies showed changes in MFG abundance that were associated with N transformations (Van Zwieten et al., 2014; Xu et al., 2014). In an incubation study, Van Zwieten et al. (2014) found that N_2O emission decreased at the same time that *nosZ* abundances increased. The authors suggested this could be due to the liming effect of biochar increasing pH of acidic soils. It has also been shown that N_2O could be further transformed to N_2 due to increased *nosZ* abundances leading to decreased N_2O emission after biochar application (Xu et al., 2014). N_2O emissions have been coupled to *nosZ* abundances as reported by Németh et al. (2014). Therefore, although the presence of functional genes may not necessarily explain microbial functional capacity, functional gene abundances still constitute a proxy that may partly explain the N transformations in soil.

5. Conclusions

The present study investigated changes in soil properties and MFG abundances associated with the N cycle following biochar amendment in a field experiment. After 12 months, biochar lowered soil NH_4^+–N concentrations at rates of 10 and 30 t ha^{-1} and increased NO_3^-–N concentrations at the higher biochar rate compared to control plots and amendment at 10 t ha^{-1}. We conclude that biochar enhanced N retention in soil through increased immobilisation and decreased leaching, as evidenced by isotopic data. Biochar addition also affected some MFG involved in N transformations, causing a general decrease in AOA and *narG* abundances independent of biochar application rates. The general lack of significant effects of biochar on the abundance of MFG may be associated with the absence of the effect of biochar on soil labile C (DOC) and of the insignificant liming value of the biochar used. Therefore, the N retention observed in this study may have been dominantly driven by abiotic factors rather than microbially meditated processes. Soil disturbance is an issue and in sustainable agriculture, the no till method is highly recommended. In these situations and if it is a no till practice then biochar needs to be applied to the surface and therefore our results have implications for any system where tilling is not possible.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.08.007.

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