Biomolecular Research Reports
2022, 2
https://doi.org/10.24377/BRR602
Accepted: *October 2021*
Research Article

Changes in total and functional bacterial genera following biochar application to planted soil

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Biochar has the recognized potential to sequester carbon, facilitate contaminant amelioration and enhance agricultural crop yield. Different types of biochar have different impacts on ecosystems, and those that are produced locally, relative to where they will be used, are considered more sustainable. It is important, therefore, to determine how the locally produced biochars affect total and functional microbial communities, especially in agronomic contexts. In this study we tested the hypotheses that biochar augmentation would: (1) increase plant yield; and (2) differentially affect total and functional microbial community composition and structure in bulk vs. rhizosphere (*Trifolium pratense*) soils. Triplicate randomised seedling cells of a 5% (w/w) mixture of sandy clay loam soil (26% clay, 21% silt and 53% sand), with/without locally-produced mixed broadleaf forestry biochar, and with/without 0.1 g clover seeds, were sampled destructively at 2-week intervals for 8 weeks post clover germination. Microbial DNA of bulk and *T. pratense* rhizosphere soils were analysed with next-generation sequencing of the 16S rRNA gene. The results showed a statistically significant increase in plant biomass in response to biochar addition correlating to increased abundances of Armatimonadetes and Bacteroidetes specifically in the rhizosphere. Although no significant change in overall alpha diversity was observed, significant changes in abundance at the genus level were recorded particularly in the presence of biochar for a number of recognised nitrogen-fixing and plant growth-promoting bacteria, including those capable of indole acetic acid (IAA) production, plant disease suppression and degradation of toxic compounds. We conclude that although overall soil diversity may not be affected by biochar addition, key genera associated with soil health and nitrogen fixation, such as *Pseudoxanthomonas*, *Variovorax*, *Pseudonocardia*, *Devosia*, *Lysobacter* and *Hydrogenophaga*, increased and facilitated plant growth.

Key words: Biochar; Plant growth-promoting bacteria; Nitrogen-fixing; Next-generation sequencing; Rhizosphere

INTRODUCTION

Biochar – charcoal applied to land to sequester carbon and modify soil properties – has attracted considerable global research interest due to its unique physico-chemical properties and potential to address several contemporary challenges. In particular, multiple findings from laboratory, pilot and field studies of various time scales have shown that biochar has considerable potential to benefit plants through: protection against pathogens [1, 2]; and sorption of phytotoxic or growth-inhibitory chemical moieties [3], with subsequent increased yield of agricultural crops [4 - 7]). The proposed mechanisms for these benefits include: (i) increased water and fertilizer retention; (ii) mitigation of nitrogen loss by reduced ammonia (NH_3) volatilization; (iii) addition and/or retention of soil micronutrients such as trace elements; (iv) provision of substrate for functional microbial biofilm formation, including as an inoculum carrier; and (v) protection of functional microbial communities from predation and desiccation.

Neutral and negative impacts of biochar addition have also been documented [5, 6, 8]. Therefore, maintaining ecosystem functions remains central to the use of biochar. The maintenance of ecosystem services can be evidenced through microbial community dynamics by, for example, characterisation of total communities and analysis of unique functional glades such as plant growth-promoting bacteria (PGPB) in agricultural soils. Most reports on the latter example [9 - 15] have focused on inoculated PGPB instead of those indigenous to the soil being studied. Additionally, it is crucial to characterize the impacts of biochar on major biogeochemical cycles such as the nitrogen cycle, particularly in regard to the possibility that biochar augmentation can mitigate greenhouse gas emission from agronomic soil.

Robust proof of sustained agronomic ecosystem services following biochar augmentation can be achieved through use of complete study models that assess both plant yield and microbial profiles, in parallel. A

study by Ducey *et al*. (2013)[16] used non-planted laboratory-scale microcosms, greenhouse pot trials and field-scale designs to assess functional microbial community dynamics in biochar-supplemented soils and their controls. Subsequently, increased total bacterial diversity and shifts in metabolic potential were recorded in the rhizosphere of tomato (*Solanum lycopersicum* cv 1125; cv. Taiwan red cherry) and were associated with increased resistance to *Botrytis cinerea* [5] and *Ralstonia solanacearum* [2]. Also, increases in biological nitrogen fixation (BNF) were reported for soils planted with mash [17], soybean (*Glycine max* L.) [18] and common (*Phaseolus vulgaris* L.; [19]) bean in response to biochar addition. The same increase resulted for mixed pasture, with a dominance of *Trifolium repens*, due to amendment with aged biochar. Mia *et al.* (2018)[20] reported a similar trend for a pot study planted with a mixture of clover and grass (*Lolium perenne*). These positive findings were, however, based largely on biochemical and physiological analyses, including plant tissue characteristics, nodule content and mycorrhizae colonization, with no parallel microbial profiling.

The aim of our study was to adopt a holistic approach by investigating the impacts of biochar on plant yield, parallel to its effects on the bulk and rhizosphere bacterial communities of planted soil. Red clover (*Trifolium pratense*) was used because it is a well recognised model plant in agronomy and soil research, which will allow comparisons between our findings to similar investigations on biochar application to agricultural soil ecosystems. Studies of planted rhizosphere ecosystems have largely used straw-based biochar [14, 21 - 23] in temperate climates, and sometimes with additives such as urea [7]. The results presented in our study are derived from a mixed broadleaf biochar, on a U.K. soil, and thus contribute further to similar studies toward a knowledge base of how biochar affects ecosystem functions in planted (agricultural) soil. Uniquely, our research also monitored the occurrence of PGPB that were not added exogenously to the study soil. This will add to knowledge development on how the application of sustainable locally produced biochar maintains ecosystem services where enhanced crop yield is the target evidence. Biochar-augmented soils are sometimes planted with grasses, shrubs trees and other leguminous plants for improved bioremediation. Therefore, we also made fortuitous observations on shifts in functional microbial communities that have the capacity for contaminant degradation, or phytoremediation, within the rhizosphere.

In summary, this study explored two hypotheses: (1) addition of broadleaf biochar will enhance the yield of the red clover as a model crop; and (2) addition of broadleaf biochar will lead to an increase in abundance and diversity of the bulk and rhizosphere bacterial communities, however, bulk and rhizosphere soil may respond differently. To interrogate these hypotheses, we used next-generation sequencing to profile the structures and compositions of the bulk and rhizosphere soil bacterial community.

MATERIAL AND METHODS

Soil and biochar properties

Soil (20 kg) was dug from a secured site at Framwellgate Moor, County Durham, U.K. (Lat. 53.15° N, Long. 1.59° W) and stored in a sterilised 25-litre airtight bucket prior to sieving (ASTM - standard soil sieve No 10; 2 mm mesh) to ensure homogeneity. The soil was analyzed for % clay, % silt and % sand content (Forestry Commission, Surrey, U.K.). The physico-chemical characteristics of the soil and locally produced biochar (mixed broadleaf forestry pyrolysis – 500°C < T < 600°C; Yorkshire Charcoal Company) were also determined (Derwentside Environmental Testing Services Ltd, County Durham, U.K.) (Table 1; [24]).

Experimental design, sampling and DNA extraction

The experimental protocol consisted of seedling trays, maintained at room temperature (ca 25°C), with randomized triplicate treatments of: soil only (110 g fresh weight); soil + biochar (5% w/w = 50 g kg^{-1}); soil + clover (0.1 g *Trifolium pratense* seeds); and soil + biochar + clover (5% w/w biochar and 0.1 g *Trifolium pratense* seeds) where irrigation was maintained via capillary action with deionized water. Clover seeds were planted in study Week -2 and allowed to germinate for 2 weeks with germination recorded as study Week 0.

Triplicate tray cells were then sampled destructively to collect bulk and rhizosphere soil every two weeks up to Week 8. The plants were harvested and shaken to dislodge the bulk soil while the roots were washed subsequently in 5 ml sterile saline to collect the rhizosphere soil. The rinsates were centrifuged (10 000 x *g* for 10 minutes), the

Table 1. *Physico-chemical characteristics of the study soil and mixed broadleaf forestry-derived biochar (Orr et al. 2016; Data are triplicate analyses from the same soil or biochar sample).*

Parameter	Clay (%)	Silt (%)	Sand (%)	*Al	*Ca	*Mg	*K	*Na	*P	Electrical conductivity (uS cm^{-1})	Calorific value (MJ kg^{-1})	Total organic carbon (%)	Total S (%)	Nitrate aqueous extract as NO_3 (mg l^{-1})	pH
Soil	26	21	53	13	2.2	1.1	1.8	0.25	0.0012	140	2.5	4.1	0.03	4.6	6.3
Biochar	nd	nd	nd	7.6	26	2	1.8	0.34	0.057	1,400	16.6	10	0.03	26	9.6

** indicates (g kg^{-1})*

Table 2. *Average (n =3) plant height and biomass increases during the 8-week study (Data are presented with standard deviations; letters show Tukey's test; different letters show significant differences P<0.05).*

	Plant Biomass (g)		Plant Height (mm)	
	Biochar	No Biochar	Biochar	No Biochar
Week 0	1.149 ± 0.31 x	1.069 ± 0.32 x	44.67 ± 5.5 a	46.00 ± 9.2 a
Week 2	1.499 ± 0.85 x	1.011 ± 0.57 x	86.33 ± 12.3 bcde	80.33 ± 6.0 b
Week 4	1.395 ± 0.39 x	1.068 ± 0.28 x	105.67 ± 5.9 cde	89.00 ± 7.8 bc
Week 6	2.977 ± 0.48 xy	1.393 ± 0.54 x	108.33 ± 4.9 ce	83.33 ± 9.9 bd
Week 8	3.687 ± 1.81 y	2.663 ± 0.63 xy	102.33 ± 11.9 bcde	89.00 ± 6.1 be

supernatants discarded and 0.5 g of the pelleted soils used for DNA extraction (FastDNA™ SPIN Kit for Soil, MP Biomedicals) with the extracts stored at -80°C.

Microbiota analysis

Bacterial community DNA sequencing was made (NU-OMICs, Northumbria University, Newcastle Upon Tyne, U.K.) with a primer set targeting the V4 region of the 16S rRNA gene according to Kozich *et al.* (2013)[25]. The raw sequencing reads were processed in FASTQ format and analyzed with Mothur software package (version 1.36.1) (University of Michigan, U.S.A.). UCHIME was used to quality check and filter the FASTA formatted sequences for chimeras. These were aligned to the SILVA reference, and taxonomic identification of the reads was assessed by assigning sequences to operational taxonomic units (OTUs) with Ribosomal Database Project (RDP) classifier. PCR negative controls were run and sequenced in parallel to the experimental samples. The OTUs recorded in negative controls and samples were excluded from further analysis. Non-bacterial sequences (Archaea/Eukaryota/Unclassified) were discarded. OTUs less than 3% were classified as rare taxa with these and the unclassified OTUs omitted from the plots.

Data and statistical analyses

The Mothur [26] generated Taxonomy and Shared files with corresponding metadata were inputted into R version 3.5.0 [27]. Shapiro-Wilk and Bartlett tests were conducted in R to test for normality and homogeneity, respectively. Data were subsequently filtered in Microbiome Analyst [28] to remove low abundance features based on 20% prevalence in samples, and low variance features based on 10% inter-quantile range. A counts per million data normalisation was performed.

ANOVA tested for significance of biochar addition, sample week and presence of clover with Tukey test used to highlight differences between sampling weeks. Subsequently, the MarkerData Profiling (MDP) module of Microbiome Analyst [28] outlined the diversity measurements in relation to the different treatments. The MDP module used R phyloseq [29] and VEGAN package [30] analyses, and univariate analysis to test for significant effects. Specifically, alpha diversity measures of Chao, Shannon and Simpson diversity were calculated within Microbiome Analyst using the phyloseq package, with Kruskal Wallis highlighting significant differences. Bray-Curtis distances were plotted using PCoA showing clustering with and without biochar and significance measured by PERMANOVA. Significant differences in phyla and genera linked to biochar addition were determined by Kruskal-Wallis sum-rank tests, and subsequent linear discriminant analysis to determine the effective size of the different abundances.

RESULTS

Plant biomass

Since increased crop biomass is identified as a positive outcome of biochar application, clover seedlings were harvested bi-weekly for yield determination. Generally, both plant height and biomass increased during the study. In particular, the addition of biochar resulted in statistically significantly increased plant height ($P < 0.001$) and biomass ($P < 0.019$) (Table 2) with a final average plant height of 102.33 (±11.9) mm with biochar and 89.00 (± 6.1) mm for the control.

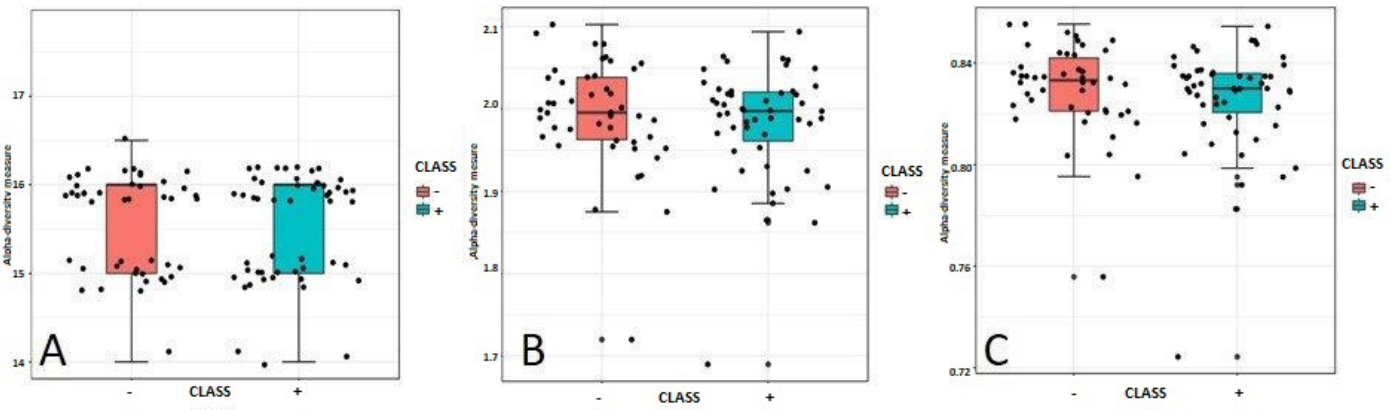

Figure 1. *Alpha diversity measurements with (+) and without (-) biochar. A = Chao1 (P = 0.522), B = Shannon diversity (P = 0.576), C = Simpson diversity (P = 0.283).*

Microbiota analysis

Many phyla have been associated with biochar addition with identities changing relative to biochar feedstock type and soil environment, although many observations have been contradictory [31, 32]. Therefore, the second hypothesis explored was that the addition of forestry biochar would differently affect the structure and composition of bulk and rhizosphere bacterial communities. No significant differences in alpha diversity resulted due to the presence or absence of biochar (Figure 1), presence or absence of plant, or between the rhizosphere and bulk soils (Figure 2). However, significant differences were recorded in the relative abundances of Bacteroidetes ($P = 0.005$), Armatimondates ($P<0.001$) and Planctomycetes ($P = 0.010$) when we assessed the impact of biochar addition on the rhizosphere communities in comparison with the bulk soil populations (Figure 2). Specifically, increases in Bacteroidetes and Armatimonadetes, and decreases in Planctomycetes, were recorded in the rhizosphere.

Beta diversity analysis with PCoA and PERMANOVA showed significant clustering of soils in the presence or absence of biochar (Figure 3), which were dependent on bacterial community shifts at the phylum level (Figure 4). Phyla that increased significantly in abundance with biochar addition were Gemmatimonadetes and Bacteroidetes, while Armatimonadetes and Verrucomicrobia recorded maximum abundances in its absence. Several significant changes in genera abundance were recorded in both the presence and absence of biochar (Table 3). Specifically, the genera with increased relative abundances in the presence of biochar are associated generally with plant growth-promoting ability, nitrogen fixation, pathogen predation, and toxic compound mineralization.

Impact on functional potential: Plant-growth promotion, pathogen suppression and pollutant degradation

The impact of biochar on ecosystem services has been investigated by measuring the changes in soil microbial community structure and how these affect plant growth, disease suppression and pollutant clean-up. Our study recorded increased abundances of *Hoeflea*, *Variovorax* and *Pseudoxanthomonas* that corresponded directly to clover biomass promotion, and *Lysobacter*, *Nocardiopsis* and *Pseudonocardia* that have been reported to suppress known plant pathogens. Also, *Pseudoxanthomonas, Lysobacter* and *Ohtaekwangia* have previously been correlated with contaminant degradation in the presence of biochar.

DISCUSSION

Plant yield and total bacterial community change

Measurements of total *T. pratense* plant height and weight showed statistically significant increases between the control and 5% (w/w) biochar-augmented soil (Table 2). These indicated that the mixed broadleaf forestry-derived biochar had positive effects on *T. pratense* yield with generally no noticeable negative effects on the study soil health, and no phytotoxic impacts on the clover.

The plant yield differences were not reflected by distinct shifts in total bacterial community structure and composition as overall alpha diversity was not affected (Fig. 1). Changes in abundance of Gemmatimonadetes, Bacteroidetes, Armatimonadetes and Verrucomicrobia were, however, observed (Fig. 4). Noyce *et al.* (2016)[33] recorded similar phyla-level changes to our study and suggested a pattern of biochar promoting Bacteroidetes and reducing Verrucomicrobia compared to surrounding soils. They proposed that these were, potentially, a result of oligotrophic bacteria such as Verrucomicrobia migrating towards the biochar particles, thus reducing their abundance in the char-free vicinities. Liao *et al*. (2019)[34] used a *Vicia faba* L. and *Zea mays* L. intercropping system with stable-isotope and reported increases in rhizosphere Bacteroidetes that assimilated plant-derived carbon. Further consistency has been recorded with increases in Gemmatimonadetes following biochar addition. For example, Jenkins *et al.* (2017) [32] monitored bacterial community changes at various European sites following supplementation with the same biochar and reported that, while changes at phyla level were variable with location, there were consistencies in the dynamics of Gemmatimonadetes. Gemmatimonadetes may be particularly sensitive to biochar addition possibly due to their correlation with moisture availability [35, 36]. In their critical analysis of the literature, Hagemann *et al.* (2016)[37] surmised that water retention and water-filled pore space, as determinants of soil moisture content, were central to microbial nitrogen cycling in soils in terms of transport (leaching) and oxygen concentration, respectively. Therefore, the level of impact of biochar augmentation on water transport, and oxygen availability and water activity in a specific soil, should affect the dominances of soil bacterial communities or phyla.

Given the metabolic diversity found within environmental phyla and the contradictory findings of previous studies, it is perhaps more valid to consider the bacterial genera associated with functional change between biochar-supplemented soils and their controls. In this study, we observed statistically significant increases in several Proteobacteria and Bacteroidetes genera typically associated with nitrogen fixation, plant growth promotion and organic toxin degradation (Table 3). Also, many Proteobacteria and Actinobacteria increased in numerical abundance with biochar addition. This aligns with the work of Zhu *et al.* (2019)[38] who reported that biochar addition increased heterotrophic microbial populations, and led to enriched metabolic pathways of biosynthesis, decomposition of secondary metabolites and polycyclic aromatic hydrocarbon (PAH). Many of the genera that correlated with biochar addition in our study were also found by other researchers [38, 39] to increase in the presence of biochar, where they were linked to increases in the capacity for nitrogen fixation.

Biochar impacts on the nitrogen-fixing community

Since the N-cycle is central to agriculture, the assessment of its dynamics and attendant economics

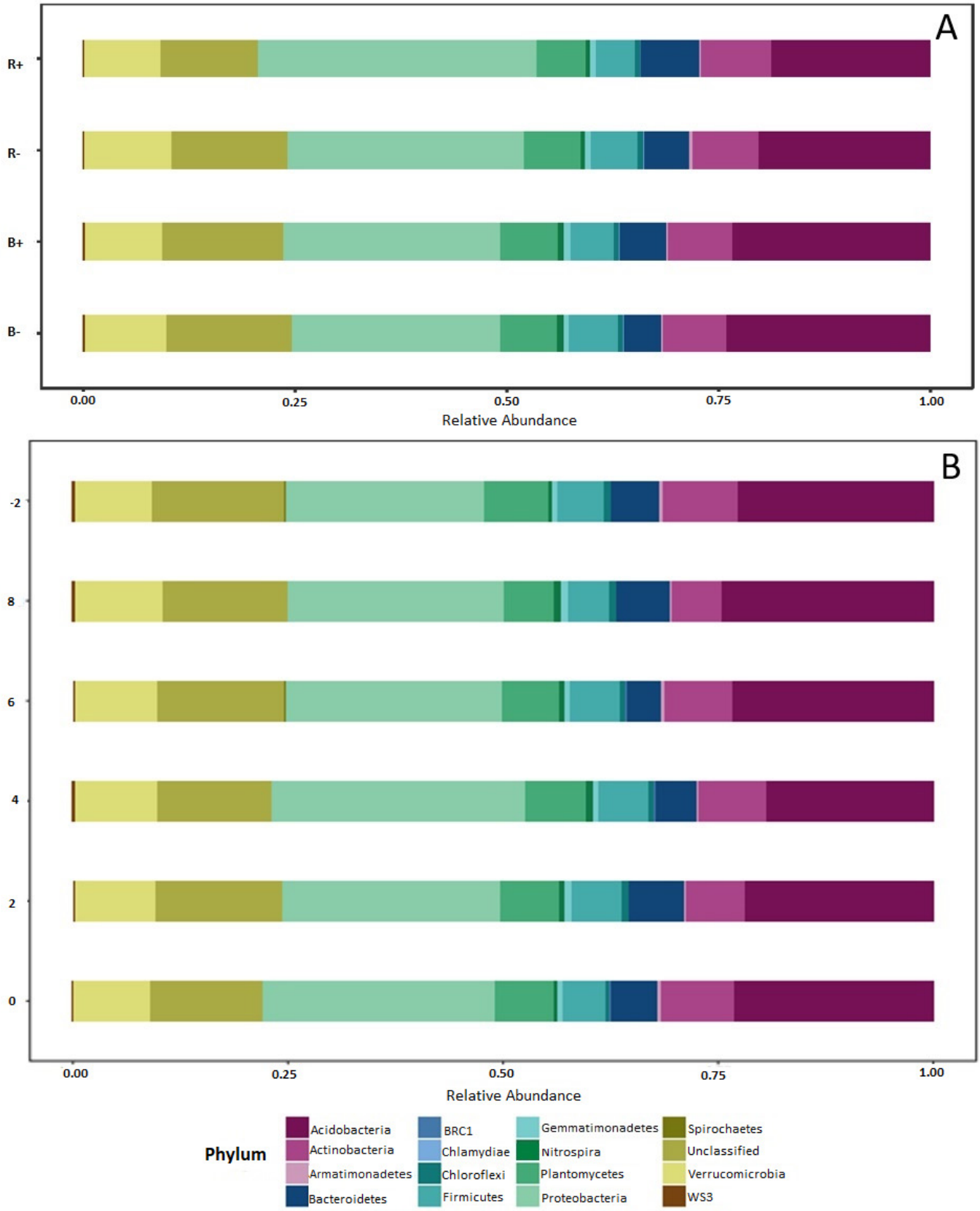

Figure 2. *Relative abundance of sequence data showing no significant difference in overall phyla throughout the weeks (-2 – 8) of the experiment for A: presence (+) or absence (-) of biochar in the rhizosphere (R); and B: bulk soil (B).*

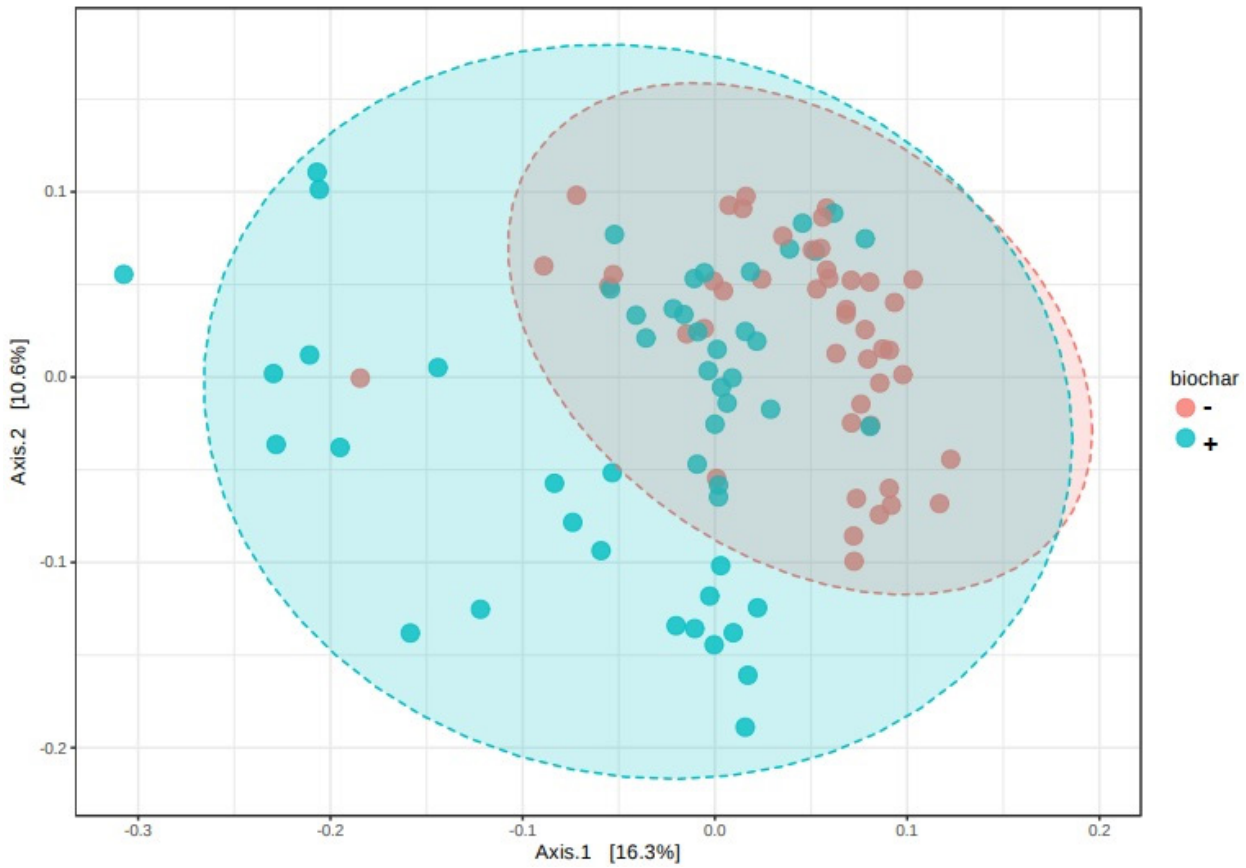

Figure 3. *Bray-Curtis distances plotted using PCoA showing clustering with and without biochar. PERMANOVA used to measure significance (P<0.001).*

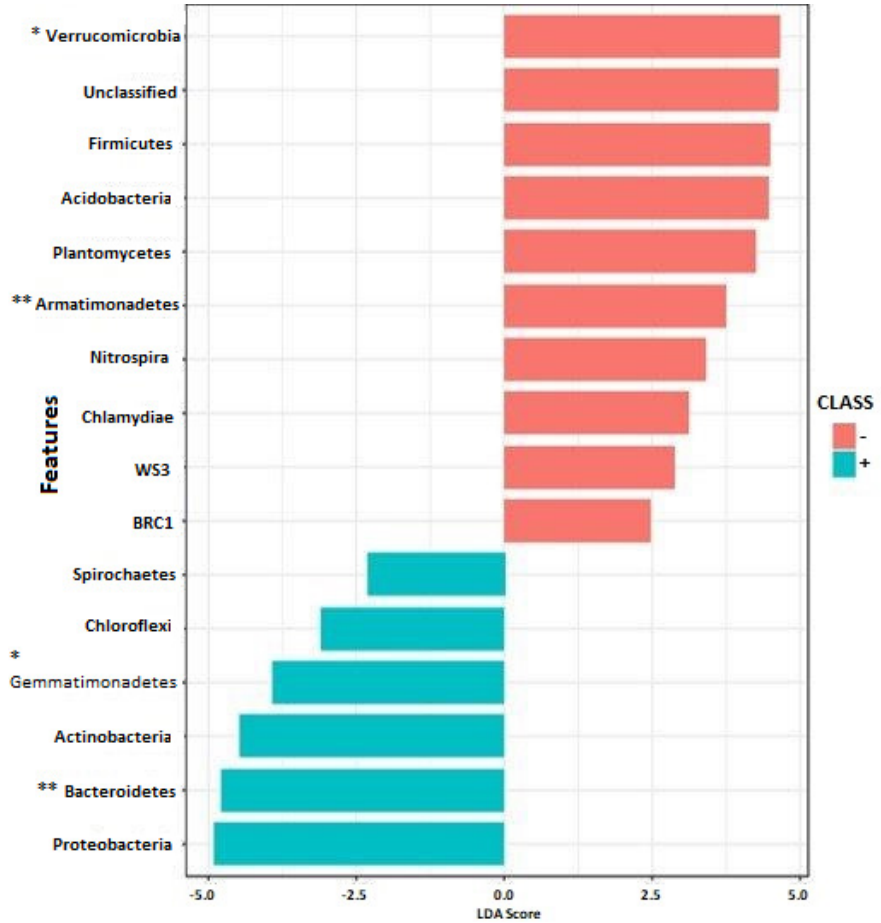

Figure 4. *Linear Discriminant Analysis Effect Size showing differences in abundant phyla with (+) and without (-) biochar. Significant differences are highlighted with an asterisks; * denotes P<0.01 and ** denotes P<0.001.*

Table 3. *Genera identified by Kruskal Wallis testing as being increased significantly in the presence or absence of biochar.*

Phylum	Taxa more abundant with biochar	P Value	Taxa more abundant without biochar	P Value
Alphaproteobacteria	Hoeflea*^ Brevundimonas Devosia*^ Sphingopyxis[1] Bosea*^ Mesorhizobium*^ Sphingobium[1] Novosphingobium[1]* Sphingomonas*[1]	8.54×10^{-10} 3.45×10^{-8} 8.72×10^{-6} 2.03×10^{-6} 6.90×10^{-4} 2.20×10^{-4} 5.00×10^{-4} 6.11×10^{-3} 2.27×10^{-3}	Rhizomicrobium[2] Rhodomicrobium*^	4.54×10^{-11} 2.33×10^{-3}
Betaproteobacteria	Hydrogenophaga* Variovorax^	4.10×10^{-8} 8.15×10^{-6}	Burkholderia[2] Cupriavidus[2]	3.21×10^{-9} 4.56×10^{-5}
Deltaproteobacteria	Nannocystis[3] Bacteriovorax[3] Enhygromyxa[3] Peredibacter[3]	9.01×10^{-4} 4.78×10^{-3} 7.77×10^{-3} 5.90×10^{-3}		
Gammaproteobacteria	Lysobacter^ Pseudoxanthomonas*^	3.51×10^{-8} 4.86×10^{-7}	Rudaea[2] Alkanindiges[1] Aquicella Dokdonella[1]	1.01×10^{-11} 9.49×10^{-3} 3.74×10^{-4} 1.42×10^{-2}
Bacteroidetes	Flavisolibacter^ Ohtaekwangia Emticicia Flavitalea Terrimonas[1] Fluviicola[1]	1.01×10^{-6} 1.83×10^{-6} 1.45×10^{-5} 3.31×10^{-5} 1.86×10^{-5} 1.39×10^{-4}	Mucilaginibacter[1]	3.26×10^{-7}
Actinobacteria	Nocardiopsis^ Nitriliruptor*[1] Pseudonocardia*^ Nocardioides[1]^	7.14×10^{-13} 3.36×10^{-9} 1.03×10^{-7} 1.31×10^{-2}		
Planctomycetes	Blastopirellula	1.14×10^{-5}		
Armatimonadetes			Chthonomonas	4.63×10^{-10}
Firmicutes	Gracilibacter	1.19×10^{-3}	Cohnella Thermoactinomyces	1.18×10^{-2} 1.21×10^{-2}
Gemmatimonadetes	Gemmatimonas	8.28×10^{-3}		
Chloroflexi			Bellilinea	1.95×10^{-3}
Verrucomicrobia			Verrucomicrobium	1.23×10^{-4}

**Nitrogen fixer, ^plant growth promoter, [1]degrades toxic compounds, [2]associated with poor soil health, [3]predators of Gram-negative bacteria.*

in biochar-supplemented soils remains a key research focus. Functionally, nitrogen fixation has been observed in a number of the genera sensitive to biochar addition including: *Brevundimonas* [40]; *Pseudoxanthomonas* [41, 42]; *Devosia* [1, 43]; *Lysobacter* [44]; and *Pseudonocardia* [45]). Previously, biochar augmentation enhanced nitrogen fixed within legume species both in terms of nitrogen accumulation [46], and increases in both the number of root nodules and nodule biomass [19]. Therefore, the increased abundance of *Mesorhizobium* and *Bradyrhizobium* such as *Bosea* could be indicative of increased clover nodulation in our study. Consequently, future investigations should consider other model plant species with no known capacity to either fix nitrogen or promote microbial communities involved in nitrogen fixation. This would provide a wider understanding and knowledge base on the tripartite relationship between biochar addition, microbial-based nitrogen fixation and enhanced crop yield.

Other genera that increased numerically and significantly in this study have been linked to other aspects of nitrogen cycling. Specific examples include: *Variovorax* that are capable of denitrification [47]; *Sphingopyxis* that are involved in nitrate respiration [48]; and *Flavisolibacter* that are capable of removing nitrogen from agricultural systems [49]. At the crop level, Wang *et al.* (2016)[50] observed increased apple yield following bioorganic fertilizer application and attributed this to enrichment of genera such as *Lysobacter* and *Ohtaekwangia*, which are linked to increases in organic matter and total nitrogen. Correlations with biochar and increased species capable of nitrification and denitrification has been reported in other systems such as composting with some authors suggesting that these processes are enhanced by biochar to a greater effect than nitrogen fixation [51, 52].

Quantitative PCR has been used previously to measure copy numbers of nitrogen-cycling genes in response to biochar. For example, Anderson *et al.* (2014)[53] and Liu *et al.* (2019)[54] conducted field studies with *Pinus radiata* and rice straw biochars and observed similar results to ours where overall alpha diversity remained unchanged. They also recorded changes in nitrogen-cycling gene copy numbers for denitrification and nitrogen fixation, which contrasted our findings where qPCR for *nifH* copy number suggested that significant increases in this gene were temporary (Data not shown) in response to the forestry broadleaf biochar. Future work on the current and similar biochars should combine qPCR and metatranscriptomic analyses for better understanding of how they impact the expression and dynamics of N-cycle genes within agronomic soils.

Increased abundance of plant growth-promoting and plant pathogen-suppressing bacteria following biochar addition

Several genera that increased in abundance have been identified as plant growth promoters. For example, *Brevundimonas* and *Pseudoxanthomonas* are capable of: nitrogen fixation; indole acetic acid (IAA), siderophore and ACC deaminase production; and P solubilization [40 - 42] and have, consequently, been used as PGPR to enhance crop growth/yield. Fox *et al.* (2014)[4] found increases of *Variovorax* and *Hydrogenophaga* species in soils supplemented with biochar. These bacterial genera have functional marker genes for S and P mobilization and, in particular, desulphination in crop- and grasslands [55]. Also, *Variovorax* spp have been identified as potential biopesticides and biofertilizers due to their ability to promote disease resistance, enhance plant stress tolerance, and improve nutrient availability and uptake [56].

It is possible, however, that other non-microbiological mechanisms accounted for the plant growth and yield enhancements recorded in our study. These could include increased abundance of mycorrhizal fungi and low concentrations/availability of potentially toxic elements in the biochar used, as proposed previously [3, 57, 58]. For example, Xia *et al*. (2020)[3] found that biochar led to decreases in aluminium toxicity as Al_2SiO_5 was complexed on the surface of biochar. The researchers proposed that the reduced toxicity and increased efficiency in the ability to use the available nitrogen led to increased growth of maize seedlings.

Other genera that showed statistically significant increases in the current work have been linked to biocontrol and the reduction of plant diseases. For example, *Pseudonocardia* have antibacterial [59] and antifungal [60] capabilities, with demonstrable siderophore production. *Lysobacter* and *Nocardiopsis* species also produce antifungal and antibacterial compounds, which can be used to suppress a key plant root pathogen, *Rhizoctonia solani* [61]. In studies of soils infected with *Ralstonia*, Wang *et al.* (2017)[62] found that healthy soils were often dominated by *Lysobacter* and *Pseudonocardia* while *Rhizomicrobium* and *Rudea*, whose abundances decreased with biochar addition in the current study, occurred, typically in unhealthy soils. These, together with the predatory behaviour demonstrated against Gram-negative bacteria by several Deltaproetobacteria genera, which were recorded in statistically significantly numbers following biochar application, may facilitate benefits in soil health.

Potential for degradation of organic compounds with biochar addition

Several researchers [22, 63 - 65] investigated the potential for biochar-enhanced bioremediation of organic and inorganic environmental contaminants in different soil-plant systems where specific focus was on profiling the rhizosphere microbial communities. Similar to these and other studies, we recorded shifts in functional genera such as *Pseudoxanthomonas, Lysobacter and Ohtaekwangia*, whose increases have previously been correlated with contaminant degradation in the presence of biochar. For example, Galitskaya *et al.* (2016)[66] recorded *Pseudoxanthomonas* increases in an oil-contaminated soil supplemented with biochar. Similarly, Ni *et al.* (2017)[67] reported that the addition of different biochar types to PAH contaminated soil led to increases in many genera including *Lysobacter*, *Ohtaekwangia* and

Pseudoxanthomonas, reductions in *Rhizomicrobium*, and resulted in significant remediation. Hou *et al.* (2015)[68] recorded statistically positive correlations between petroleum degradation and biosurfactant production with specific genera such as *Lysobacter*, *Pseudoxanthomonas*, *Planctomyces*, *Nocardioides*, *Hydrogenophaga* and *Ohtaekwangia*. Therefore, although not investigated, occurrences of these genera in our study could have reflected the remediating potential of the mixed broadleaf biochar together with its plant growth-promoting capability. Also, our findings confirmed key genera that can be targeted in future investigations, as exemplified by Sarma *et al*. (2019)[69] and Wang *et al*. (2019)[63], into the mechanisms for plant-microbiome enhanced rhizoremediation in biochar-augmented contaminated soils. For example, we recorded increases in *Sphingobium*, known toxic compound degraders and one of several genera that Li *et al*. (2020)[23] linked specifically to shifts in metabolite profiles within maize rhizosphere.

The soil used in this study had, to our knowledge, no recorded history of contamination. Therefore, the increases in microbial genera related to contaminant degradation raise a question on the properties of freshly prepared biochar and its impacts on soil health. This was outside the scope of our work, but has been investigated by several researchers where concentrations of volatile organic compounds are elevated in fresh compared to aged biochar.

CONCLUSIONS AND FURTHER WORK

To conclude, species within the genera identified in this work have been linked to mechanisms that are beneficial to plant growth including: nitrogen fixation; IAA production; siderophore production; pathogen number reduction; and toxic compound degradation. We propose, therefore, that the recorded increase in red clover yield resulted from a number of these and commend further studies to determine the likely underpinning mechanism(s). Overall, biochar did not change the genus-level community structure and composition of the bulk soil compared to the rhizosphere. The exceptions were Bacteroidetes – with known capacities for nitrogen fixation, phosphorus solubilization and reduction of iron and sulfur [34] – Armatimonadetes and Planctomycetes. Nonetheless, longer studies are required to confirm that addition of the locally-produced broadleaf biochar used in this report, and similar forestry waste-derived biochars, do not lead to deleterious changes to functional capacity, soil health and ecosystem services.

Ultimately, robust evidence is required to validate claims that biochar is an economically viable and value-added material where appropriate wastes can be used as feedstocks for the production of a sustainable and agriculturally important additive, whether added directly to soil or indirectly via manure and compost. Our results align with those from similar studies and show that biochar addition led to the enrichment of specific plant-growth promoting, nitrogen-fixing genera, and disease-suppressing members, with a subsequent increase in plant growth. This alignment highlights the significance of knowledge development that includes a wide range of feedstocks where we used broadleaf forestry-derived biochar instead of wheat straw-based, for example. Future work should investigate the likely biochar-stimulated rhizosphere mechanisms (shifts in SOM, pH, redox potential, net ion concentrations, plant N-uptake, plant-derived carbon sources), and how they impact bacterial community composition, as also demonstrated in several studies [18, 23, 34, 70, 71]. Also, transcriptomics within planted soils should be applied to measure the expression of functional genes, such as those involved in nitrogen fixation and cycling, to confirm the impact of forestry broadleaf biochar on nitrogen as a biogeochemical cycle that is important to sustainable agronomy.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

CHO and TKRS conceived and designed the study and drafted the manuscript; AN did all the sequencing and advised on data analysis; EAC analysed the sequencing data; SL and JJR did most of the laboratory work and were involved in study design; CHO, TKRS and EAC wrote sections of the manuscript. All authors contributed to manuscript revision, and read and approved the submitted version.

FUNDING

The authors acknowledge gratefully funding from the Teesside University's: Research Fund (Project140214); DfLD Students as Researchers Scheme; and Graduate Tutor Scheme.

ACKNOWLEDGMENTS

Grateful acknowledgements are made to Christopher Schroeter for robust contributions to experimental design, Jodie Harris for technical input and the Teesside University's Students as Researchers initiative which facilitated student contribution.

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