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# Biochar alters the soil microbiome and soil function: results of next-generation amplicon sequencing across Europe

JOSEPH R. JENKINS<sup>1</sup>, MAUD VIGER<sup>1</sup>, ELIZABETH C. ARNOLD<sup>1</sup>, ZOE M. HARRIS<sup>1</sup>, MAURIZIO VENTURA<sup>2</sup>, FRANCO MIGLIETTA<sup>3,4</sup>, CYRIL GIRARDIN<sup>5</sup>, RICHARD J. EDWARDS<sup>1,6</sup>, CORNELIA RUMPEL<sup>5,7</sup>, FLAVIO FORNASIER<sup>8</sup>, COSTANZA ZAVALLONI<sup>9</sup>, GIUSTINO TONON<sup>2</sup>, GIORGIO ALBERTI<sup>3,9</sup> and GAIL TAYLOR<sup>1</sup>

<sup>1</sup>Centre for Biological Sciences, University of Southampton, Southampton SO17 1BJ, UK, <sup>2</sup>Faculty of Science and Technology, Free University of Bolzano/Bozen, Piazza Università 1, 39100 Bolzano, Italy, <sup>3</sup>MountFor Project Centre, European Forest Institute, Via E. Mach 1, 38010 San Michele a/Adige, Trento, Italy, <sup>4</sup>FoxLab, Forest & Wood Science, Fondazione E.Mach, Via E.Mach 1, 38010 San Michele a/Adige, Trento, Italy, <sup>5</sup>Ecosys, UMR INRA-AgroParisTECH, Bâtiment EGER, Aile B, 78820 Thiverval-Grignon, France, <sup>6</sup>School of Biotechnology and Biomolecular Sciences, University of New South Wales, Kensington, Sydney, NSW 2052 Australia, <sup>7</sup>IEES, UMR 7618, CNRS-UPMC-UPEC-INRA-IRD, Bâtiment EGER, Aile B, 78820 Thiverval-Grignon, France, <sup>8</sup>Agricultural Research Council (CRA), Research Centre for the Soil-Plant System, via Treste 23, 34170 Gorizia, Italy, <sup>9</sup>Department of Agricultural and Environmental Sciences, University of Udine, Via delle Scienze 206, 33100 Udine, Italy

## Abstract

Wide-scale application of biochar to soil has been suggested as a mechanism to offset increases in CO<sub>2</sub> emissions through the long-term sequestration of a carbon rich and inert substance to the soil, but the implications of this for soil diversity and function remain to be determined. Biochar is capable of inducing changes in soil bacterial communities, but the exact impacts of its application are poorly understood. Using three European sites [UK SRC, short rotation coppice, French grassland (FR) and Italian SRF, short rotation forestry (IT)] treated with identical biochar applications, we undertook 16S and ITS amplicon DNA sequencing. In addition, we carried out assessments of community change over time and N and P mobilization in the UK. Significant changes in bacterial and community structure occurred due to treatment, although the nature of the changes varied by site. STAMP differential abundance analysis showed enrichment of *Gemmatimonadete* and *Acidobacteria* in UK biochar plots 1 year after application, whilst control plots exhibited enriched *Gemmataceae*, *Isosphaeraceae* and *Koribacteraceae*. Increased mobility of ammonium and phosphates was also detected after 1 year, coupled with a shift from acid to alkaline phosphomonoesterase activity, which may suggest an ecological and functional shift towards a more copiotrophic ecology. Italy also exhibited enrichments, in both the *Proteobacteria* (driven by an increase in the order *Rhizobiales*) and the *Gemmatimonadetes*. No significant change in the abundance of individual taxa was noted in FR, although a small significant change in unweighted UNIFRAC occurred, indicating variation in the identities of taxa present due to treatment. Fungal  $\beta$  diversity was affected by treatment in IT and FR, but was unaffected in UK samples. The effects of time and site were greater than that of biochar application in UK samples. Overall, this report gives a tantalizing view of the soil microbiome at several sites across Europe and suggests that although application of biochar has significant effects on microbial communities, these may be small compared with the highly variable soil microbiome that is found in different soils and changes with time.

**Keywords:** 16SrDNA, Acidobacteria, Alphaproteobacteria Bacteria, amplicon, biochar, fungi, ITS, metabarcoding, microbiome, Proteobacteria, Rhizobiales, soil

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## Introduction

Soil contains thousands of bacterial and fungal taxa of which the majority remain uncharacterized and their effects on soil function are yet to be elucidated. Whilst

Correspondence: Gail Taylor, tel. + 44 0 238 059 2335, fax + 44 0 238 059 4469, e-mail: g.taylor@soton.ac.uk

we have some understanding of the factors which drive microbial diversity (Fierer *et al.*, 2012; Serna-Chavez *et al.*, 2013), we know relatively little about community changes in soils below the level of phylum. Although the driving factor in bacterial community diversity has been determined to be pH (Fierer & Jackson, 2006), we still have limited knowledge on the impacts of ecosystems manipulation experiments in these communities.

There has been much discussion regarding the use of biochar (pyrolysed biomass) as both a soil conditioner and a method for carbon sequestration (Lehmann *et al.*, 2006; Major, 2010; Mao *et al.*, 2012). Addition of biochar to soil has also been shown to increase plant growth (Baronti *et al.*, 2010; Vaccari *et al.*, 2011; Jones *et al.*, 2012; Viger *et al.*, 2015), possibly related to altered abiotic characteristics including increased pH, cation exchange capacity (CEC) and improved soil water content (Verheijen *et al.*, 2010; Jeffery *et al.*, 2011; Jones *et al.*, 2012). The soil physico-chemical changes induced by biochar addition may also play a pivotal role in determining soil bacterial biodiversity because pH influences the biogeographical distribution of bacteria (Fierer & Jackson, 2006). Shifts in microbial communities may result from a wide range of biochar-mediated interactions, including variations in microbial signalling (through sorption of the molecules themselves; Masiello *et al.*, 2013), increased transfer of electrons, resulting in augmentation of biological processes (Cayuela *et al.*, 2013), shifts in microbial N cycling (Harter *et al.*, 2014) and decreased abundance of fungi relative to bacteria (which could utilize biochar substrates for growth; Gomez *et al.*, 2014). The increased fertility associated with biochar amendment could be linked to these changes in the microbiome. For example, addition of biochar has been found to increase the abundance of bacteria and archaea oxidizing ammonia to nitrates and nitrites (Prommer *et al.*, 2014), increase *Bradyrhizobiaceae* and *Hyphomicrobiaceae* populations in short-term pot experiment on ryegrass (Anderson *et al.*, 2011) and increased nitrification (*amoA*, *amoB*), nitrogen fixing (*nifH*) and nitrite reduction (*nirS*, *nirK* and *nosZ*) gene abundances (Ducey *et al.*, 2013). A 126-day pot experiment studying the effects of biochar application on S and P mobilizing bacteria in *Lolium perenne* indicated increased abundance of *Rhizobacteria* associated with the mineralization of S and P in nutrient limited soils (Fox *et al.*, 2014). However, previous studies have been undertaken over short time scales and in microcosm experiments, thus their relevance to long-term field impacts remains unknown.

Observed effects of biochar on edaphic microbial processes are often conflicting. Different studies have observed increases in soil respiration (Kolb *et al.*, 2009; Zavalloni *et al.*, 2011; Belyaeva & Haynes, 2011; Castaldi *et al.*, 2011; Quilliam *et al.*, 2012; Ventura *et al.*, 2014);

although decreases (Dempster *et al.*, 2011; Paz-Ferreiro *et al.*, 2011; Carlsson *et al.*, 2012) or no change have also been observed (Galvez *et al.*, 2012; Bamminger *et al.*, 2014).

Microbial biomass is also altered following biochar application, with increases (Kolb *et al.*, 2009; Belyaeva & Haynes, 2011; Paz-Ferreiro *et al.*, 2011), decreases (Dempster *et al.*, 2011) and no change (Castaldi *et al.*, 2011; Galvez *et al.*, 2012; Bamminger *et al.*, 2014; Ventura *et al.*, 2014) all reported. Again, these are primarily short-term pot experiments, enabling accurate measurement of microbial biomass, with the trade-off of limited validity in terms of effects of treatment *in situ*. Therefore, it appears that a range of microbial responses to biochar application can occur, depending on the biochar (its feedstock, nutrient content and pyrolysis temperature), the initial edaphic conditions (pH, soil organic matter (SOM), soil moisture, bulk density and aeration), land use and management regimes, vegetation types and the microbial community. Effects of biochar treatment have also been noted on microbial community structures, with decreases in *Betaproteobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Planctomycetes* noted (Kolton *et al.*, 2011; Ding *et al.*, 2013; Hu *et al.*, 2014), as have (sometimes contradictory) increases in *Bradyrhizobiaceae*, *Hyphomicrobiaceae*, *Actinomycetes*, *Chloroflexi*, *Nitrospiraceae*, *Proteobacteria*, *Trichoderma*, *Pseudomonas*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Gemmatimonadetes* (Graber *et al.*, 2010; Anderson *et al.*, 2011; Khodadad *et al.*, 2011; Kolton *et al.*, 2011; Ding *et al.*, 2013; Chen *et al.*, 2014; Hu *et al.*, 2014). These studies suggest that biochar-mediated bacterial shifts have the potential to change the mineralization of nutrients in the soil (Kolton *et al.*, 2011), or impact on biocontrol, plant growth promotion and organic compound degradation (Graber *et al.*, 2010). Few studies have determined the impact of biochar on fungal abundance and diversity, but these communities have also displayed a range of responses, including fluctuations in arbuscular mycorrhizal fungi (AMF) colonization and abundance (Warnock *et al.* 2010; Elmer & Pignatello 2011), decreased diversity (Hu *et al.*, 2014), increased fungal growth (Sun *et al.* 2013), decreased fungal growth (Quilliam *et al.* 2012) and decreases in the abundance of fungi (Ameloot *et al.* 2014). Studies of fungi have indicated a decline in alpha ( $\alpha$ ) diversity due to the inability of fungal taxa to adapt to rapid variation in the soil environment (Hu *et al.*, 2014) and shifts in community composition (Chen *et al.*, 2013). Increased abundance of *Trichoderma* and *Paecilomyces* in biochar samples has also been noted (Hu *et al.*, 2014), known to improve soils and promote plant growth.

Using the 16S rRNA subunit gene and the ribosomal internal transcribed spacer region (ITS), surveys of the relative abundance of bacterial and fungal operational taxonomic units (OTUs) within a sample can be undertaken without the need for culturing. These methods

have the added advantage of detecting changes even in unidentified species such that we can now begin to unravel complex ecological processes with the aid of molecular approaches. For example, 16S amplicon surveys comparing existing agricultural practises with low and high applications of biochar enhanced with chicken manure and rock phosphate indicated significant differences between high biochar and control bacterial diversity. This was due to decreased abundance of the *Bacteroidete* families *Flavobacteriaceae* and *Saprospiraceae*, the *Planctomycete* genus *Planctomyces*, the *Alphaproteobacteria* families *Hyphomonadaceae* and *Rhodobacteraceae* and two *Verrucomicrobia* genera, *Rubritalea* and *Roseibacillus* (Nielsen *et al.*, 2014). However, this utilized enriched biochars at a single field site, and so whilst representative of the changes under those conditions, it is likely variation in response will occur in taxa treated with nonenriched biochars. Furthermore, to date, next-generation sequencing (NGS) ITS amplicon surveys have not been used to study shifts in fungal abundance after biochar application in a field trial.

It therefore remains unclear how biochar application will effect bacterial and fungal populations within the soil. Furthermore, it is uncertain whether the disparate findings of previous studies are due to differences in the biochar used, the nature of biodiversity assessment, differences in environments/communities studied or some combination of these factors. In this study, we applied 16S rRNA and ITS short read amplicon sequencing to assess detailed taxonomic changes in both bacterial and fungal microbiomes as a result of field-scale treatment using a standardized biochar, applied at three contrasting sites across Europe and attempted to link our findings in the UK to assessment of soil chemistry using measures of enzymatic activity and nutrient leachate. It was hypothesized that in time series data for the UK, a short-term increase in copiotrophic taxa would occur, as labile portions of biochar become available as microbial substrates. In addition, increases in the proportion of *Actinobacteria* and *Acidobacteria* were expected across all sites, as these have been associated with carbon cycling and the decomposition of complex carbon molecules (Lehmann *et al.*, 2011; Nielsen *et al.*, 2014).

## Materials and methods

### Biochar characterization

Biochar was produced by Advanced Gasification Technologies (AGT s.r.l., Cremona, Italy), using *Zea mays* feedstock in a fixed bed, open core, down draft gasifier as previously described (Ventura *et al.*, 2015). Detailed chemical characterization of biochar produced by the gasification process can be found in Wiedner *et al.* (2013) (Table 1).

### Field sites

Three field sites (Fig. 1) were established across Europe. The sites were part of the EuroChar project ([www.eurochar.eu](http://www.eurochar.eu)) and were located in West Sussex (UK), Prato Sesia (IT) and Lusignan (FR) (Table 2).

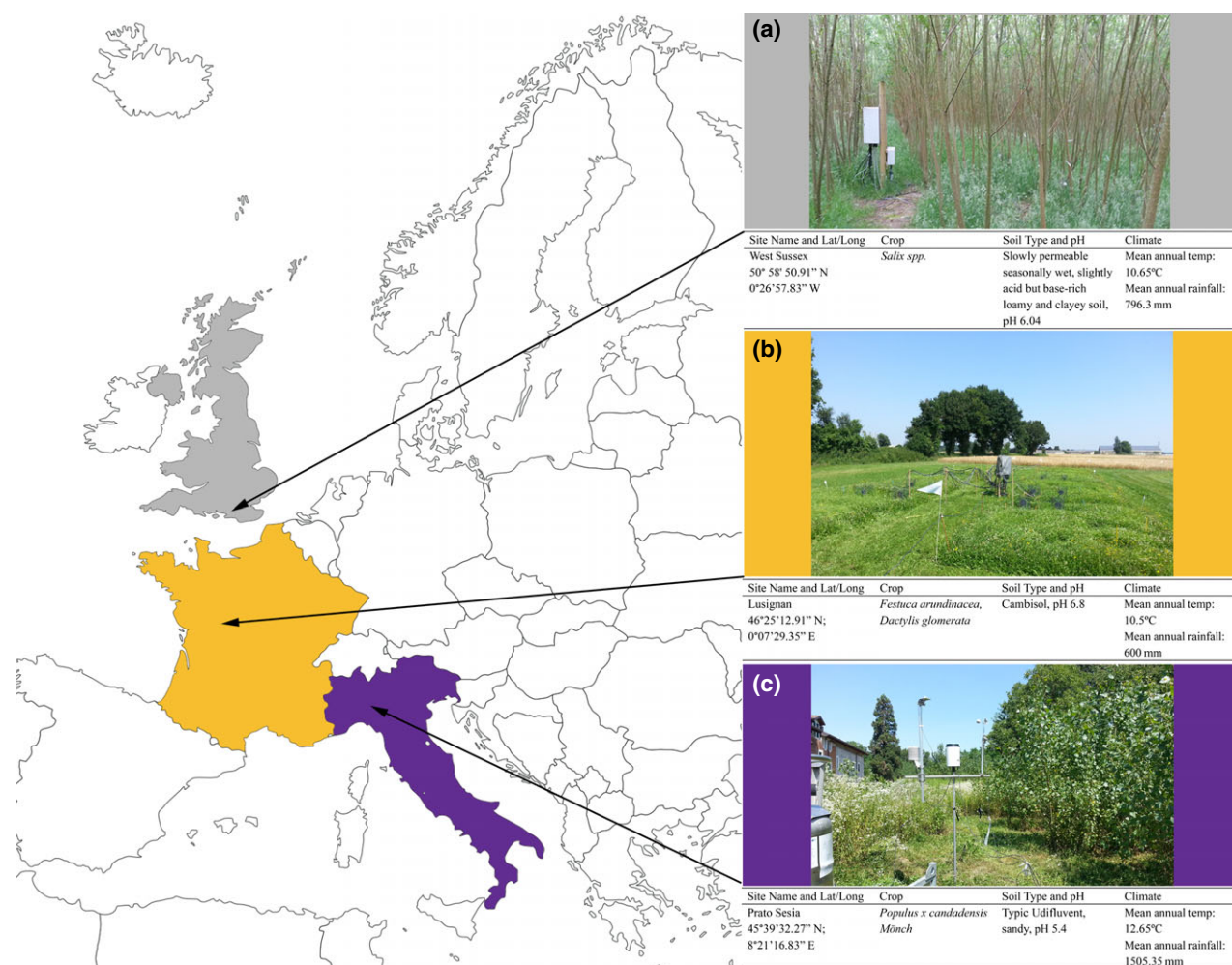
At all sites, biochar was added to the soil as previously described (Ventura *et al.*, 2015). A single biochar application of 30 t ha<sup>-1</sup> (65 kg of biochar fresh weight, equivalent to 5.5 kg m<sup>-2</sup> dry weight, 45% water content) was applied at each site in June 2012 to a depth of 15 cm. In the UK, this application was carried out using hand tools to minimize damage to the pre-established *Salix* crop, whilst a rotary hoe was used for application in IT and FR. Biochar was shipped to each site in sealed plastic bags within weeks of its production, in order to maintain its sterility. Biochar was not sterilized after production, as this would not be representative of real-world application scenarios. Treated and control plots were arranged in a completely randomized design, with four replicates per treatment. Plots were 4.3 × 2.75 m, 5 × 9 m and 5 × 4 m at UK, IT and FR sites, respectively. Difference in plot size reflected the different cropping methods applied at each site. However, only three replicates were sampled for microbial community analysis.

### Sampling

The microbial community was assessed at each site 1 year after biochar application (July 2013). An additional intensive time series experiment was carried out at the UK site, with samples collected pretreatment during March 2012, 1 month after biochar amendment during July 2012 and 1 year after biochar application in July 2013. At all sites, biochar-treated plots were referred to as B, whilst control was denoted C. A total of 130 soil samples were collected from biochar amended plots and control plots using a systematic sampling design, with 30 samples from FR, IT, UK 1 month and UK 1 year (5 samples × 3 replicates × 2 treatments). Ten samples were collected prior to biochar addition from UK pretreatment. Samples were collected at a 1.5 m radius from the centre of each plot. Considerable effort was maintained throughout sampling to ensure clean, uncontaminated samples, including use of gloves during

**Table 1** Physico-chemical properties of AGT biochar applied in UK, FR and IT

Parameter	Value	Units
Bulk Density	0.65	g cm <sup>-3</sup>
pH (H <sub>2</sub> O)	11.6	—
Salinity	758	mS m <sup>-1</sup>
H	2.3	%
H/C	0.5	—
C	56.1	%
N	1.35	%
C:N	42.9	—
Ca	38.1	g kg <sup>-1</sup>
K	32.3	g kg <sup>-1</sup>
P	8.56	g kg <sup>-1</sup>
S	1.32	g kg <sup>-1</sup>



**Fig. 1** Details of each of the three field sites sampled (a) West Sussex UK (Grey), (b) Lusignan FR (Orange) and (c) Prato Sesia IT (Purple). Tables include mean annual temperature and rainfall, crop species, site coordinates and soil data.

**Table 2** Site properties for UK, IT and FR

Location and site name	Mean annual temperature and rainfall	Soil type	pH	Altitude	Crop
West Sussex (UK) 50°58'38"N; 0°27'33"W	10 °C 742.3 mm	Permeable, seasonally wet, clay and loam	6.04	33 m a.s.l.	<i>Salix</i> sp. SRC
Prato Sesia, Novara (IT) 45°39'32.27"N; 8°21'16.83"E	12 °C 1200 mm	Sandy	5.4	279 m a.s.l.	<i>Populus x canadensis</i> Mönch, clone 'Oudenberg', SRF
Lusignan (FR) 46°25'12.91"N; 0°07'29.35"E	10.5 °C 600 mm	Loamy cambisol	6.8	153 m a.s.l.	<i>Festuca arundinacea</i> and <i>Dactylis glomerata</i> grassland

sample collection and decontamination of equipment prior to and during sampling. Collection was carried out using a sterilized stainless steel soil corer (15 cm × 2.5 cm). Once collected, soil samples were passed through sterilized stainless steel sieves (mesh size 2 mm) and homogenized. For DNA

extraction, a 50-ml sterile falcon tube was then filled with a homogenized portion of the sieved sample, prior to freezing in liquid nitrogen. Samples were transported back to the laboratory at −80 °C by cryoshipper. Between the sampling at each site, previously collected samples were stored at −80 °C.

### Extraction protocol

DNA extraction used MoBio Powersoil Extraction kits (MO BIO Laboratories, Carlsbad, CA, USA). Briefly, 0.5 g (increased from the recommended 0.25 g, as a result of experimental testing of methods to increase DNA yield) of homogenized frozen soil was placed into a PowerSoil Bead Tube, before following manufacturer's specifications. DNA quality and concentration were assessed using a NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA) ensuring all samples had a minimum 260/280 ratio of 1.8. Extracted samples were stored at  $-80^{\circ}\text{C}$  until all extractions were complete and ready for transport to LGC Genomics (Berlin, Germany).

### Amplification and sequencing

Isolated DNA from each sample was amplified using the bacterial 16S rRNA gene primers 341F (5'-TCC TAC GGG NGG CWG CAG-3') and 785R (5'-GAC TAC HVG GGT ATC TAA KCC-3') (Klindworth *et al.*, 2013) and the fungal primers fITS7 (5'-TGTGARTCATCGAATCTTTG-3') and ITS4 (5'-TTCCTCC GCTTATTGATATGC-3') (Ihrmark *et al.*, 2012). In the case of the 16S region, these primers were chosen as they provide approximately 470 bp of sequence and are suitable for a wide range of bacterial taxa, amplifying the hypervariable V3-V4 region and have the additional ability to detect a small range of archaea. The ITS primers were chosen as they amplify the ITS2 region and include a portion of the 5.8S region. This primer pair has been shown to increase the diversity of fungi identified, whilst decreasing misrepresentation in communities. Each sample was tagged with an individual eight nucleotide barcode to allow demultiplexing of pooled sequences into their original samples. Tagged samples were randomly pooled prior to library construction, to ensure a mix of treatments in each sequencing lane (Carlsen *et al.*, 2012). Amplification was carried out using 15 pmol of each forward and reverse primer, added to 20  $\mu\text{l}$  of MyTaq buffer, including 1.5 units of MyTaq DNA polymerase and 2  $\mu\text{l}$  of BiostabII PCR Enhancer. Thirty cycles of PCR were undertaken for 2 min at  $96^{\circ}\text{C}$ , followed by  $96^{\circ}\text{C}$  for 15 s,  $50^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 60 s, and gel electrophoresis was utilized to assess concentration. Finally, approximately 20 ng of PCR product was pooled prior to purification using preparative gel electrophoresis. Purified amplicon, barcode and primer complexes were sequenced on an Illumina MiSeq, using V3 reagent chemistry, producing  $2 \times 300$ -bp paired-end reads. Reads were demultiplexed and separated by their sample-specific barcodes. These steps were undertaken at LGC Genomics (GmbH), Berlin, Germany.

### Sequence Analysis Pipeline

**16S pipeline.** For each site, paired-end reads were first quality controlled and combined using PandaSeq (Masella *et al.*, 2012). PandaSeq combines paired-end reads through areas of overlapping sequence, converting  $2 \times 300$  bp reads into a single read of approximately 500 bp in length and clips adapters and primers from each read. Using .fastq input, PandaSeq is able to determine the quality score of each base, and in cases where

reads disagree on a basecall, the programme utilizes the base with the highest quality score. Combined reads were then renamed and preprocessed using BESPOKE software (SeqSuite, <http://bioware.soton.ac.uk>), which renamed each read and ensured names were compatible with QIIME. Formatted files were run through the Quantitative Insights into Microbial Ecology (QIIME v1.8) pipeline (Caporaso *et al.*, 2010b). Unless otherwise stated, named python scripts are from the QIIME package. Reads were clustered into OTUs using the pick\_denovo\_otus.py workflow, clustering all reads at 97% identity using UCLUST (Edgar, 2010), prior to alignment using PyNAST (Caporaso *et al.*, 2010a). Classification of sequences was undertaken using the RDP Classifier (Wang *et al.*, 2007), trained by the GREENGENES 13.5 database (DeSantis *et al.*, 2006). Phylogenetic trees were produced using the make\_phylogeny.py command using FastTree2 (Price *et al.*, 2010). Filtering of errant sequences was undertaken through use of filter\_otus\_from\_otu\_table.py, removing singletons from the data set, before sorting samples by treatment utilizing sort\_otu\_table.py. Taxonomic summaries were generated using the summarize\_taxa\_through\_plots.py script, generating bar charts showing the raw relative abundance output of the pipeline, and mean values by treatment.

**ITS pipeline.** Initial QC and read combination of ITS reads were carried out identically to the methods outlined in the 16S pipeline above. OTUs were picked using the open reference pipeline which clusters reads against a reference sequence database (in this case, the UNITE ITS database 12.11; Kõljalg *et al.*, 2013). Reads failing to match any within the database were grouped with the closest matching cluster.

### Nutrient leaching, pH, respiration and soil enzymatic activities

To coincide with the time series amplicon data at the UK site, further detailed analyses were undertaken to link soil microbiome to functional attributes of the soil. pH was measured for each sample, using a 1 : 5 water soil:water dilution (weight:volume) method in deionized water. Samples were agitated and left to equilibrate for 1 h before measurement using a Jenway 3510 pH meter. Total and heterotrophic soil respirations were monitored on site as part of a previous work during 2012–2013 (Ventura *et al.*, 2015). These used an automatic soil respiration (SR) system to collect soil respiration data from control and biochar plots every 4 h. Furthermore, heterotrophic and total respirations were measured in each plot through the installation of two SR chambers per plot, one unconstrained chamber measuring total SR and another surrounded by a root exclusion cylinder, measuring heterotrophic SR only (for further detailed methodology see Delle Vedove *et al.*, 2007; Ventura *et al.*, 2014). Samples were checked for curvature and rejected if the relationship between cumulative flux and time was concave, or if a difference of  $<3$  ppm was detected between initial and final flux measurements (Ventura *et al.*, 2015). If this occurred, data was gapfilled using a model based on soil temperature and moisture content (Qi & Xu, 2001; Delle Vedove *et al.*, 2007). Mean daily flux was calculated during the period of the time series sampling (19 June 2012–18 June 2013) for each treatment

group: total biochar, heterotrophic (root excluded) biochar, total control and heterotrophic (root excluded) control.

During the same time period, resin lysimeters were installed in biochar and control plots to assess concentrations of ammonium ( $\text{NH}_4^+$ ), phosphates ( $\text{PO}_4^{3-}$ ) and nitrate ( $\text{NO}_3^-$ ) present in leachate after treatment. Lysimeters were positioned to capture leachate from within the row and from between adjacent rows. Lysimeters consisted of a mixed ion-exchange resin (16.2 g, Amberlite MB-150, Sigma-Aldrich, Dorset, UK) held within PVC pipe sections with a height of 3 cm and a diameter of 5 cm. To prevent direct contact with soil, a section of glass beads (2 mm diam.) was placed at either end of the resin and held in place using 125  $\mu\text{m}$  nylon mesh (Scubla s.n.c., Remanzacco, UD, Italy; Ventura *et al.*, 2013). Installation of lysimeters was carried out on the 10 July 2012. Three lysimeters were buried vertically at a depth of 20 cm in each plot. These were collected during July 2013, approximately 1 year after their placement. Once collected, lysimeters were opened in the laboratory prior to washing of resin with 100 mL of 2M KCl solution within 500 mL Erlenmeyer flasks. These were then shaken at 100 rpm for 1 h using an orbital shaker before filtration (Whatman no. 42 filters).  $\text{NO}_3^-$  and  $\text{NH}_4^+$  concentrations were detected in the washing solution through a continuous flow automatic analyser (AxFlow AA3, Bran+Luebbe, Norderstedt, Germany). Ammonium was detected using a combination of salicylate and dichloro-isocyanuric acid (ISO 11732:2005), whilst sulphanilamide-NEDD [N-(1-Naphthyl)ethylenediamine] was used for nitrate (ISO 13395:2006) (Ventura *et al.*, 2013).  $\text{PO}_4^{3-}$  analysis of extracts was carried out using an inductively coupled plasma optical emission spectrometer (ICP-OES, Spectro Arcos, Ametek, Germany).

During the campaign to collect soil material for sequencing, additional samples were collected for soil enzymatic activity (EA) analysis. Samples were collected using the same method as the amplicon samples, prior to analysis. dsDNA was extracted from soil samples following the procedure from Fornasier *et al.* (2014). Briefly, DNA was extracted with a 0.12 M, pH 8  $\text{Na}_2\text{HPO}_4$  buffer using bead beating; dsDNA was quantified in a crude (not purified) extract using the PicoGreen reagent. Soil EAs quantified were as follows: aryl-sulfatase,  $\beta$ -glucosidase, acid and alkaline phosphatase, phosphodiesterase, esterase and leucine aminopeptidase. EA substrates were determined after treating soil subsamples with an extraction/desorption procedure (Fornasier & Margon, 2007). Extracts were obtained using 400 mg of soil and 1.2 mL of extractant (3% lysozyme, Cowie *et al.*, 2013) in 2 mL Eppendorf tubes containing 0.4 mL of 1 mm diameter ceramic beads and 0.4 mL of 100 micron glass beads. Tubes were shaken for 3 min at 30 strokes  $\text{s}^{-1}$  using a Retsch 400 beating mill then centrifuged at 15 000 g for 3 min. Aliquots of supernatants were dispensed in 384-well microplates with appropriate buffer to determine EA using fluorescent 4-methylumbelliferyl substrates.

## Statistical methods

To understand the impacts of biochar on the number of taxa present in each samples,  $\alpha$  diversity was calculated

at the level of OTU. Due to difficulties in aligning the ITS sequences, nonphylogenetic measures (Chao1 or Bray–Curtis distance) were used to analyse  $\alpha$  and beta ( $\beta$ ) diversity of fungal samples. Each sample was randomly subsampled to 90% of the smallest sample at each site, to ensure that each sample was directly comparable. Reported values represent the mean for each rarefied metric per treatment at each site (Table S1). These rarefied values represent a normalized mean for the samples at each site. Species richness was measured through use of  $\alpha$  diversity metrics (observed species: OBS, Chao1 and phylogenetic diversity: PD), indicating whether a change in the number of different OTUs between treatments occurred. Significance of differences between sites and treatments used two-sample *t*-tests adjusted with Monte Carlo methods (using QIIME's compare\_alpha\_diversity.py script).

$\beta$  diversity, the similarity between the identities of taxa and their abundances by treatment, was assessed through pairwise UNIFRAC distances (Lozupone & Knight, 2005) prior to plotting using EMPeror (Vázquez-Baeza *et al.*, 2013) or bespoke R scripts. Again, a single rarefaction of 90% of the smallest sample in each site was used to normalize samples. Unweighted UNIFRAC methods determine whether the identities of taxa within communities change, whilst weighted UNIFRAC represents the identities of the taxa, and their relative abundances. Using both metrics, we determined whether community structure varied due to changes in taxonomic abundance or shifts in the identities of taxa present due to treatment. To statistically assess the differences between  $\alpha$  diversities, a nonparametric two-sample *t*-test was utilized using the compare\_alpha\_diversity.py script within QIIME.  $\beta$  diversities at the level of OTU in treated and control samples were analysed through use of principle coordinate analysis (PCoA), prior to ADONIS statistical testing for significance (999 permutations) (Oksanen *et al.*, 2016). To compensate for the multiple ADONIS tests carried out (four unweighted UNIFRAC tests for 16S – UK 1 month, UK 1 year, FR and IT, 4 weighted UNIFRAC tests for 16S and 4 ITS tests of Bray–Curtis distances = 12 in total), a Benjamini–Hochberg correction was applied to an FDR of 0.05 (Benjamini & Hochberg, 1995). As a result, each *q*-score which passed the threshold set for significance represents only a 5% chance of a false positive.

Finally, differential abundance testing of each taxonomic level (from phylum to genus) was carried out using STAMP (Parks & Beiko, 2010), using two-sided Whites nonparametric *t*-tests (White *et al.*, 2009), with Benjamini–Hochberg FDR correction for multiple testing (Benjamini & Hochberg, 1995). These analyses were carried out to compare the differences between treatments at each site and to compare temporal differences within

treatments in UK time series samples. Statistically significant results were filtered to include only OTUs which had >5 sequences, where the difference between proportions was >0.5% or the ratio of proportions >2. A  $q$ -value of 0.05 was used, representative of a 95% confidence that a significant result is not a false discovery.

In addition, to detect the differences between the initial community compositions of each site, a further comparison of the control samples from each site at 1 year was undertaken using the same methods described above.

To assess correlation between taxonomic and pH distance matrices, Mantel tests were conducted for each site using QIIME (compare\_distance\_matrices.py) to determine whether there was a significant correlation between biochar induced pH change and community structure. Additionally, a two-way analysis of variance (ANOVA) looking at the effect of site (IT, FR and UK) and treatment (biochar and control) on soil pH was conducted in R.

For respiration measurements, ANOVA was carried out using SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA: IBM Corp) using a between subjects design, factoring the treatment (biochar vs. control), the partitioning of respiration (total vs. heterotrophic) and the interaction between the two factors.

To test for differences in the leachate in the UK, a two-way ANOVA was used for each chemical ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$  and  $\text{PO}_4^{3-}$ ) extracted from the lysimeters. Homogeneity of variance was checked using Levene's test. When homogeneity of variants was not respected, Mood's median test was carried out to identify divergences between treatments. Normality of data was not checked, due to the relatively low number of replicates. Treatment (control vs. biochar) and position of lysimeter (outside left, within row, outside right) were independent variables. STATGRAPHICS software (Statpoint Inc., Warrenton, VA, USA) was used for statistical analysis.

Finally, to detect changes taking place in enzymatic activities, ANOVA for dsDNA and EAs was performed in SPSS (IBM SPSS Statistics for Windows, Version 22.0: IBM Corp) using a one-way ANOVA. ANOVAs were performed including soil treatment (biochar vs. control) as fixed factor.

## Results

### Sequence data

A total of 2 453 023 reads were produced, of which 299 593 ( $\mu = 19972.867$ ,  $\sigma = 3859.847$ ) were from UK pretreatment, 502 318 ( $\mu = 16743.933$ ,  $\sigma = 5414.146$ ) were from UK samples 1 month after treatment, and 237 707 ( $\mu = 7923.567$ ,  $\sigma = 3832.652$ ) were from the UK

1 year after treatment. FR samples contained 581 030 reads ( $\mu = 19367.667$ ,  $\sigma = 7074.381$ ), whilst IT contained a total of 832 375 reads ( $\mu = 27745.833$ ,  $\sigma = 10757.089$ ). After rarefaction of data (to 90% of the reads from the smallest sample), downstream analysis used 7619 (UK 1 month), 1827 (UK 1 year) and 3354 (FR) 2940 (IT) reads, respectively.

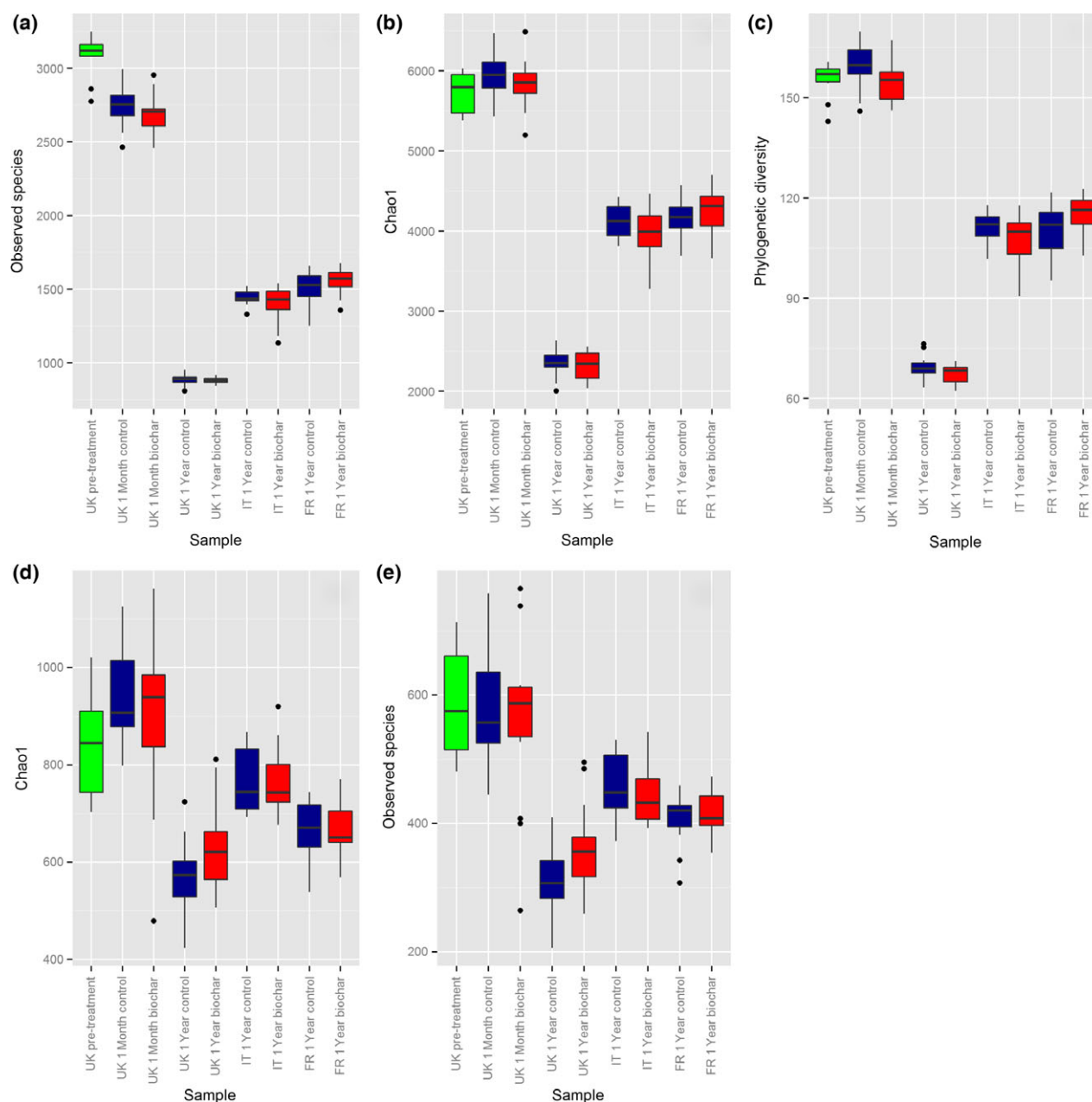
### Bacterial community structure and diversity

*The effect of site on initial community structure.* Comparison of the sites prior to treatment showed significant differences in community structure.  $\alpha$  diversity analysis revealed significant differences between UK and continental Europe for bacterial richness regardless of the metric used. UK samples displayed lower richness than the continental samples, although both FR and IT communities had similar  $\alpha$  diversities (Fig. 2).

$\beta$  diversity of the sites revealed significant differences in bacterial community structure and abundance. Results of both weighted UNIFRAC PCoA revealed significant clustering of samples (Fig. 3a) by site (ADONIS  $R^2 = 0.16$ ,  $P = 0.001$ ). Therefore, we conclude that there were substantial differences between the bacterial taxa present, and their abundances within the communities present at each of the three sites.

$\alpha$  diversity analysis of fungal communities revealed no significant differences in fungal richness by site. However, fungal  $\beta$  diversity analysis by site revealed a similar pattern to that observed in the bacterial samples (Fig. 3b), in that distinct clusters formed based on the site of origin of each sample (ADONIS:  $R^2 = 0.56$ ,  $P = 0.001$ ). Therefore, it appears that there are significant differences in the taxa present and in their relative proportions at each site.

*The effect of biochar treatment on community structure.* Biochar treatments had no significant effect detected for any of the metrics used to assess bacterial  $\alpha$  diversity at any of the sites 1 year after treatment (Table S1). The impact of biochar on bacterial  $\beta$  diversity was significant but differed depending on site. PCoA for biochar samples collected after 1 year showed a significant difference in weighted (ADONIS:  $R^2 = 0.12$ ,  $q = 0.004$ ) and unweighted (ADONIS:  $R^2 = 0.06$ ,  $q = 0.004$ ) UNIFRAC distances between UK control and biochar samples (Fig. 4a). IT results showed significant clustering by treatment on weighted (ADONIS:  $R^2 = 0.08$ ,  $q = 0.013$ ) and unweighted (ADONIS:  $R^2 = 0.06$ ,  $q = 0.004$ ) UNIFRAC distance (Fig. 4b). Finally, results for FR showed no significant clustering by treatment in the weighted UNIFRAC analysis (Fig. 4c) although unweighted (ADONIS:  $R^2 = 0.04$ ,  $q = 0.004$ ) UNIFRAC showed significant differences



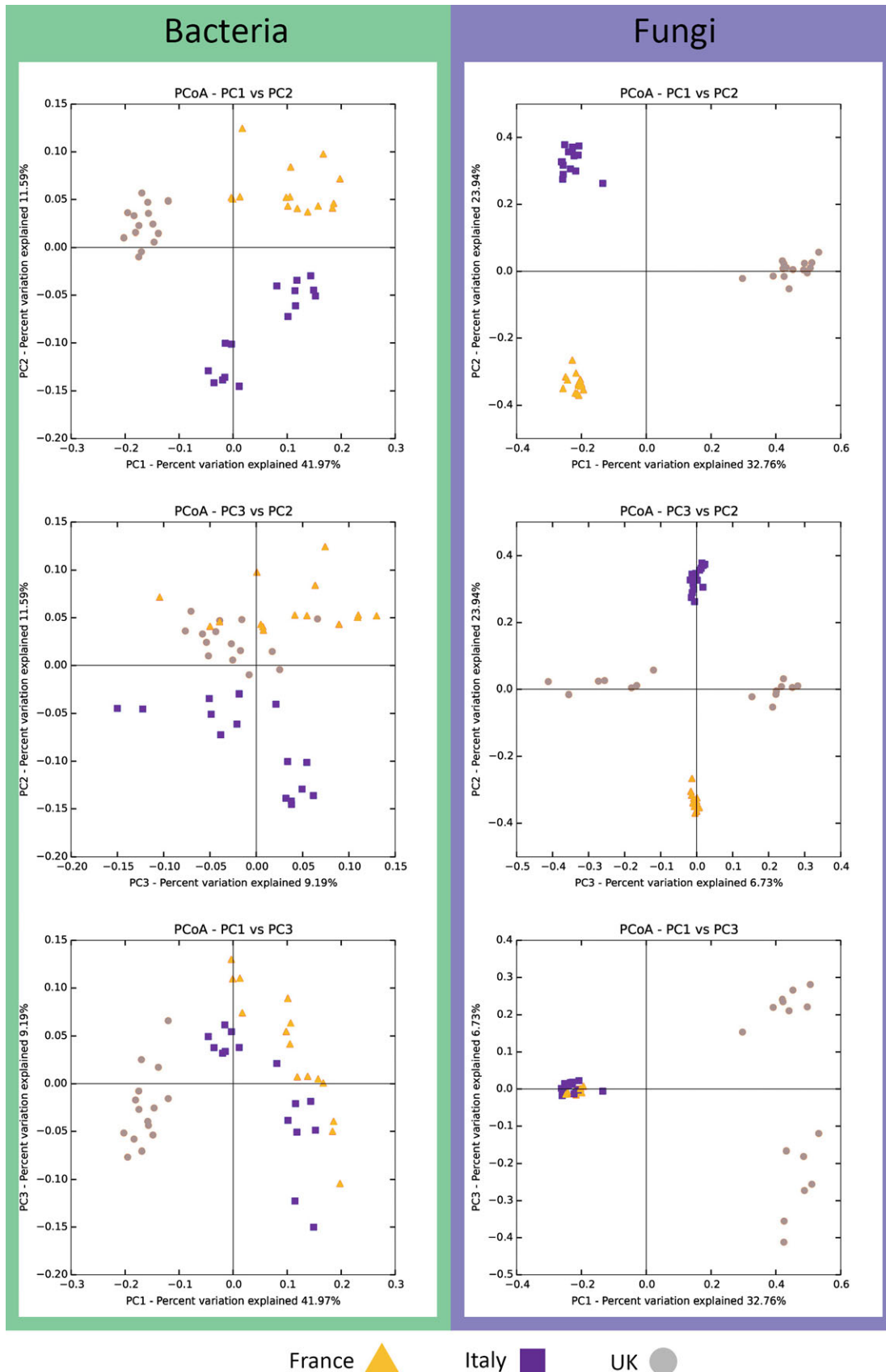
**Fig. 2**  $\alpha$  diversity metrics showing (a) Observed species, (b) Chao1 and (c) Phylogenetic diversity metric data for UK pretreatment, UK after 1 month, UK after 1 year, IT and FR (IT and FR are labelled to indicate that samples were taken 1 year after treatment). ITS analysis of Observed species and Chao1 is shown in (d) and (e). Each plot shows average values for biochar in red, control in blue and pretreatment in green.

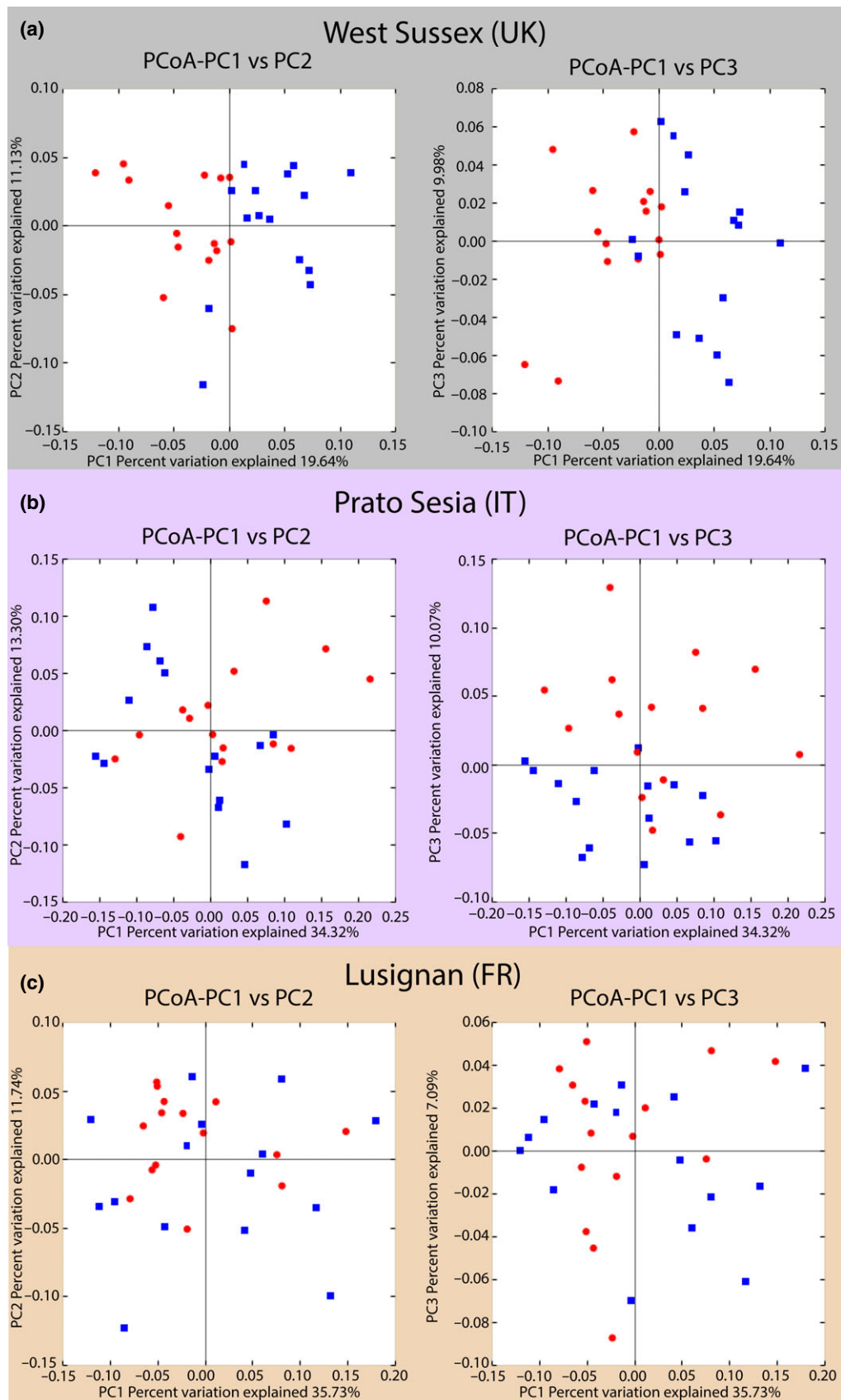
between treatments, indicating a shift in OTUs present between treatment types (Fig. S1).

Mantel tests comparing bacterial unweighted UNIFRAC  $\beta$  diversity with pH distance matrices

revealed a significant linear positive correlation (Mantel:  $R = 0.15$ ,  $P = 0.001$ ; Fig. S2). This reflects an increasing diversity of taxa as pH increased, as a consequence of the biochar treatment.

**Fig. 3** Principle coordinate analysis of bacterial (left hand column) and fungal (right hand column) OTU weighted UNIFRAC distances for control samples at UK (grey circles), IT (purple squares) and FR (yellow triangles) sites. Samples are from the summer of 2013, 1 year after biochar application at each site.





**Fig. 4** Principle coordinate analysis of bacterial OTU weighted UNIFRAC distances for (a)UK (grey), (b) IT (purple) and (c) FR (orange) sites. Weighted UNIFRAC is adjusted for both the number of shared species between samples and the abundance of those species. Biochar-treated samples are shown in red, whilst control samples are displayed in blue. Samples are from the summer of 2013, 1 year after biochar application at each site.

Fungal  $\alpha$  diversity showed a significant difference between treatments for the Chao1 metric 1 year after treatment. OBS displayed a similar, albeit nonsignificant increase. No significant shift in fungal richness was noted in either FR or IT.

Fungal samples from UK biochar and control samples collected 1 year after treatment showed no significant difference in fungal  $\beta$  diversity, with no distinct clustering occurring due to treatment (Fig. 5a) whilst fungal results from IT indicated a significant difference between biochar and control plots (ADONIS:  $R^2 = 0.09$ ,  $q = 0.008$ ; Fig. 5b).

There was also a significant effect of biochar on fungal  $\beta$  diversity for the FR samples (ADONIS:  $R^2 = 0.05$ ,  $q = 0.021$ ; Fig. 5c). Mantel tests of pH vs. fungal  $\beta$  diversity revealed a similar pattern to that observed in the bacterial data sets. A significant positive correlation was detected between the Bray–Curtis  $\beta$  diversity and pH (Mantel:  $R = 0.158$ ,  $P = 0.001$ ).

#### *The effect of time of sampling and treatment in samples from the UK site*

In common with the 1-year timepoint, no effect of treatment was detected on bacterial  $\alpha$  diversity in UK 1 month samples.

Temporal shift in bacterial  $\alpha$  diversity for the UK site between the 1-month and 1-year samples showed significant differences between treatments, with biochar samples collected 1 month after treatment having significantly higher richness than biochar samples collected after 1 year. Similarly, control samples collected after 1 month had a higher richness compared with control samples collected after 1 year. Therefore, there was no significant difference between treatments at either timepoint. Thus, it appears that time of sampling had a greater impact on bacterial  $\alpha$  diversity than the biochar treatment. A similar pattern was also detected in the results of the time of sampling for fungal  $\alpha$  diversity results.

Time series data for the UK bacterial  $\beta$  diversity showed weighted UNIFRAC for UK one-month samples displayed no significant difference by treatment. However, unweighted UNIFRAC for the same time period comparing treated and untreated plots showed a significant difference (unweighted UNIFRAC ADONIS:  $R^2 = 0.05$ ,  $q = 0.04$ ). The lack of difference in weighted UNIFRAC indicates no significant shift in abundance of the taxa present, whilst the shift in unweighted UNIFRAC suggests a shift in the identity of OTUs between

treatments (for exact ADONIS values,  $P$ -values and  $q$ -scores, see Table S2).

However, a significant difference was detected in fungal community due to treatment at 1 month (ADONIS:  $R^2 = 0.06$ ,  $q = 0.017$ ), which was not reflected in the 1-year data set.

STAMP analysis of differential abundance comparing UK 1-month and UK 1-year biochar samples revealed an enrichment of *Actinobacteria* in the 1-year samples (7%), whilst one-month biochar samples exhibited an enrichment in *Acidobacteria* and *Proteobacteria* (approximately 4% and 3%, respectively; Fig. S3). The majority of the *Proteobacteria* enrichment in biochar samples at 1 month were due to a 3% increase in an unidentified genus within the candidate order *iii1-15*, a member of the *Betaproteobacteria*. The *Actinobacteria* increase noted in the 1-year biochar sample was due to a large increase in the class *Thermoleophilia* and a range of small shifts in other genera.

Comparison of control samples collected during 1 month and 1 year at the UK site revealed significantly increase *Actinobacteria* in the UK one-month samples (10%) due to a 4.5% enrichment in the order *Actinomycetales*. Control samples for UK 1 year showed elevated abundance of the *Acidobacteria* (6%) due to an increase in the *Solibacterales* (4%) (Fig. S3). Whilst temporal variation occurred in the abundances of taxa in both conditions, biochar may additionally modulate the taxa present as temporal changes were not uniform.

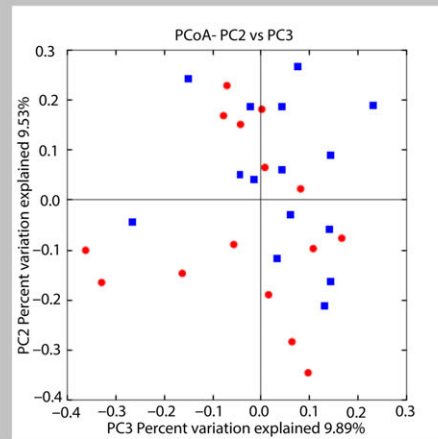
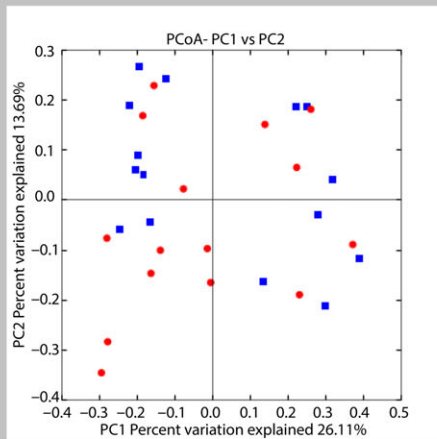
When run collectively, the weighted UNIFRAC distance for UK samples showed a distinct effect of the date of sample collection on the population present, with all samples collected during 2012 (both UK pre-treatment and 1 month) clustering closely together (Fig. 6). However, samples collected 1 year after biochar treatment dislocated to the right of the axis and showed differentiation in clustering into control and biochar-treated samples. This indicated that whilst there was short-term change in the species present in biochar samples, this is the beginning of a gradual shift in communities with time since biochar application.

#### *What are the differences in OTU abundance due to site?*

Sites differed in their dominant bacterial phyla. UK samples collected after 1 year were dominated by *Proteobacteria* and *Acidobacteria*, whilst IT was dominated by *Actinobacteria*, *Proteobacteria* and *Acidobacteria*. Proportions of phyla in communities from FR were similar to

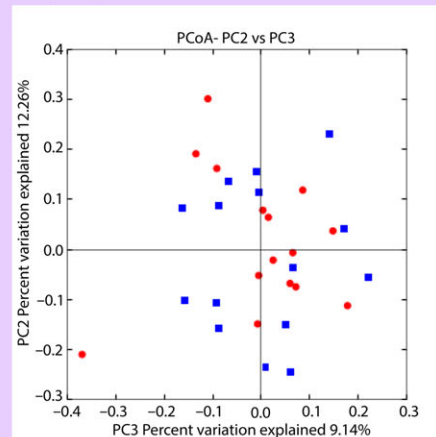
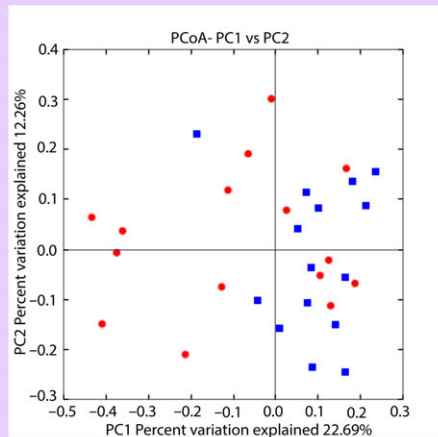
(a)

## West Sussex (UK)



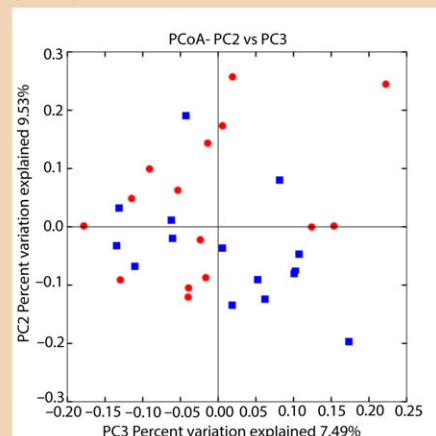
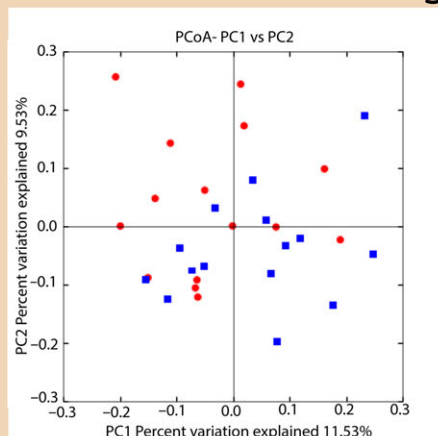
(b)

## Prato Sesia (IT)



(c)

## Lusignan (FR)



**Fig. 5** Principle coordinate analysis of Bray–Curtis distances for (a) UK (grey), (b) IT (purple) and (c) FR (orange) sites showing difference in fungal diversity. Biochar samples are displayed in red, whilst control samples are shown in blue.

those present in IT (Fig. 7a). Results of fungal analysis also showed variation in community composition by site (Fig. 7b). UK samples collected after 1 year were dominated by *Basidiomycota*, unidentified fungi and *Ascomycota* (35–37%, 42–42.5% and 15–16%, respectively). IT samples were prevalently *Basidiomycota*, with similar proportions of *Ascomycota*, whilst FR samples consisted of *Ascomycota* and unidentified fungi, with small numbers of *Basidiomycota*. It should be noted that the phyla of *Glomeromycota* and *Zygomycota* were only detected in FR.

#### What changes in differential OTU abundance occur due to treatment?

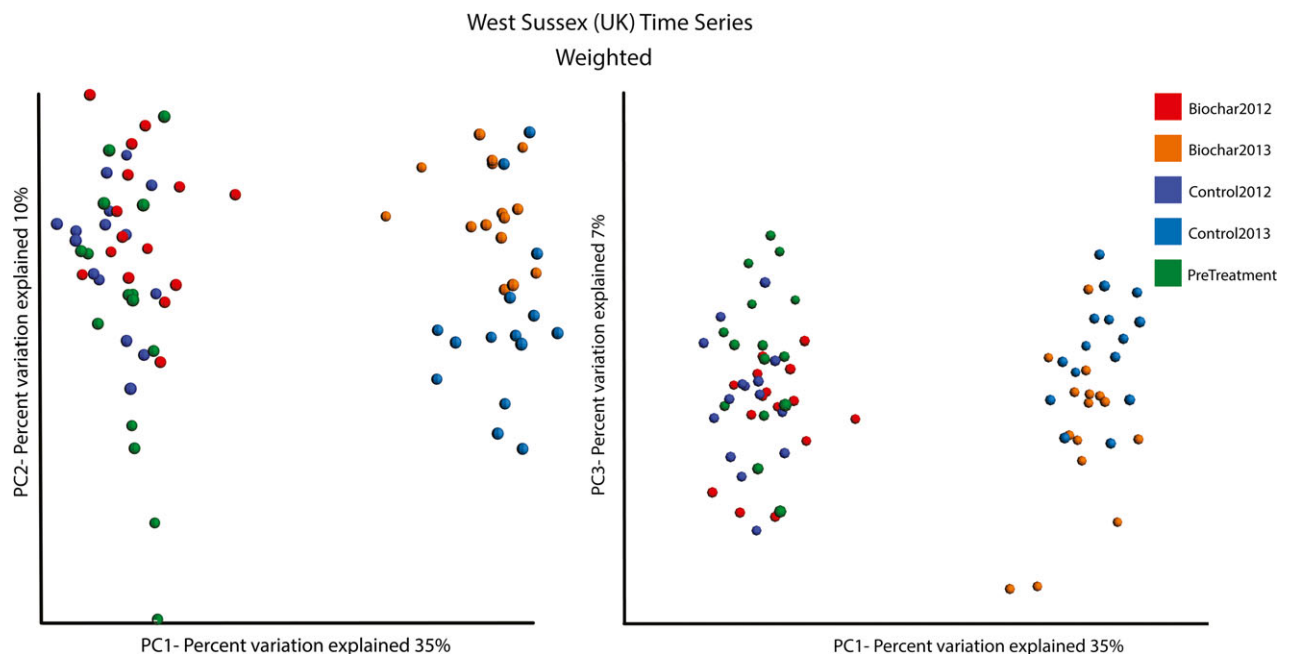
Results from the time series collected in the UK indicate a different community structure after treatment. UK pretreatment samples were dominated by *Proteobacteria*, *Actinobacteria* and *Acidobacteria* (Table S3). UK samples collected after 1 month were still dominated by *Proteobacteria*, *Actinobacteria* and *Acidobacteria* although both the latter phyla had declined when compared with the UK pretreated abundances. STAMP analysis of treatment revealed significant decreases in the *Gemmataceae* and the *Koribacteraceae*

in biochar samples (1.14% and 1.21%, respectively; Fig. 8). A similar trend was also detected in IT, although no significant difference was detected in FR.

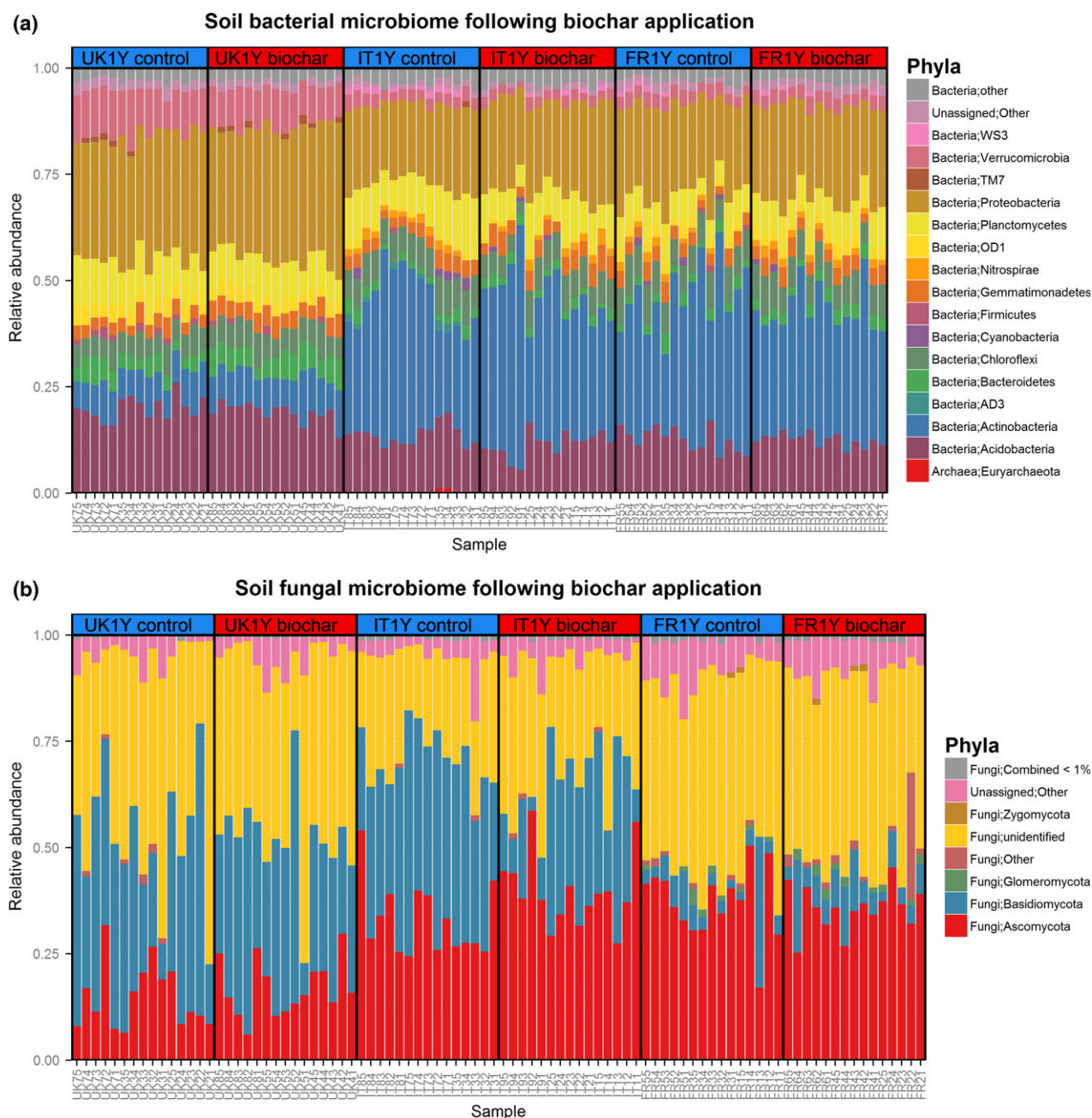
#### Soil function assessed from respiration, chemistry and enzyme activity

pH data revealed a significant difference between control and biochar samples ( $F_{(1,81)} = 10.72$ ,  $P = .002$ ) with biochar-treated samples having a pH between 0.3 and 1.2 pH higher than controls (Table 3). No interaction between site and treatment was identified ( $P = 0.653$ , ns). Data for soil respiration, extended from that previously published (Ventura *et al.*, 2015), showed seasonally increased respiration in control and treated plots during the summer, as a result of elevated soil temperature (Fig. 9a). The presence of roots significantly increased CO<sub>2</sub> flux, regardless of treatment ( $P = 0.08$ , total  $\mu = 2.04$ ,  $\sigma = 0.36$ , heterotrophic  $\mu = 1.34$ ,  $\sigma = 0.39$ ). No other main effects of interactions were identified (in all cases  $P > 0.15$ ).

Results from lysimeters indicated that biochar significantly increased the leachate of NH<sub>4</sub><sup>+</sup> ( $\mu = 4.30$  kg N-NH<sub>4</sub> ha<sup>-1</sup>,  $\sigma = 1.83$ ) and P-PO<sub>4</sub><sup>3-</sup> ( $\mu = 2.77$  kg



**Fig. 6** Principle coordinate analysis of weighted UNIFRAC distances for all West Sussex (UK) time series samples. Pretreated (green); Biochar 2012 (red); Control 2012 (dark blue); Biochar 2013 (orange) and Control 2013 (light blue). Division along PC1 is a result of time since treatment, with all samples collected in 2012 appearing on the left of the axis, and samples collected in 2013 on the right.



**Fig. 7** Taxonomic breakdown of proportional change in (a) bacteria and (b) fungi. Relative abundance of each of the core phyla (above 1%) is represented. Blocks at the top of the figure indicate the treatment type associated with each 'block' of replicated samples. Red represents biochar-treated samples, whilst blue indicates a control treatment. Labels within each block indicate the sample site, UK, IT or FR. All samples shown are from samples collected 1 year after biochar application.

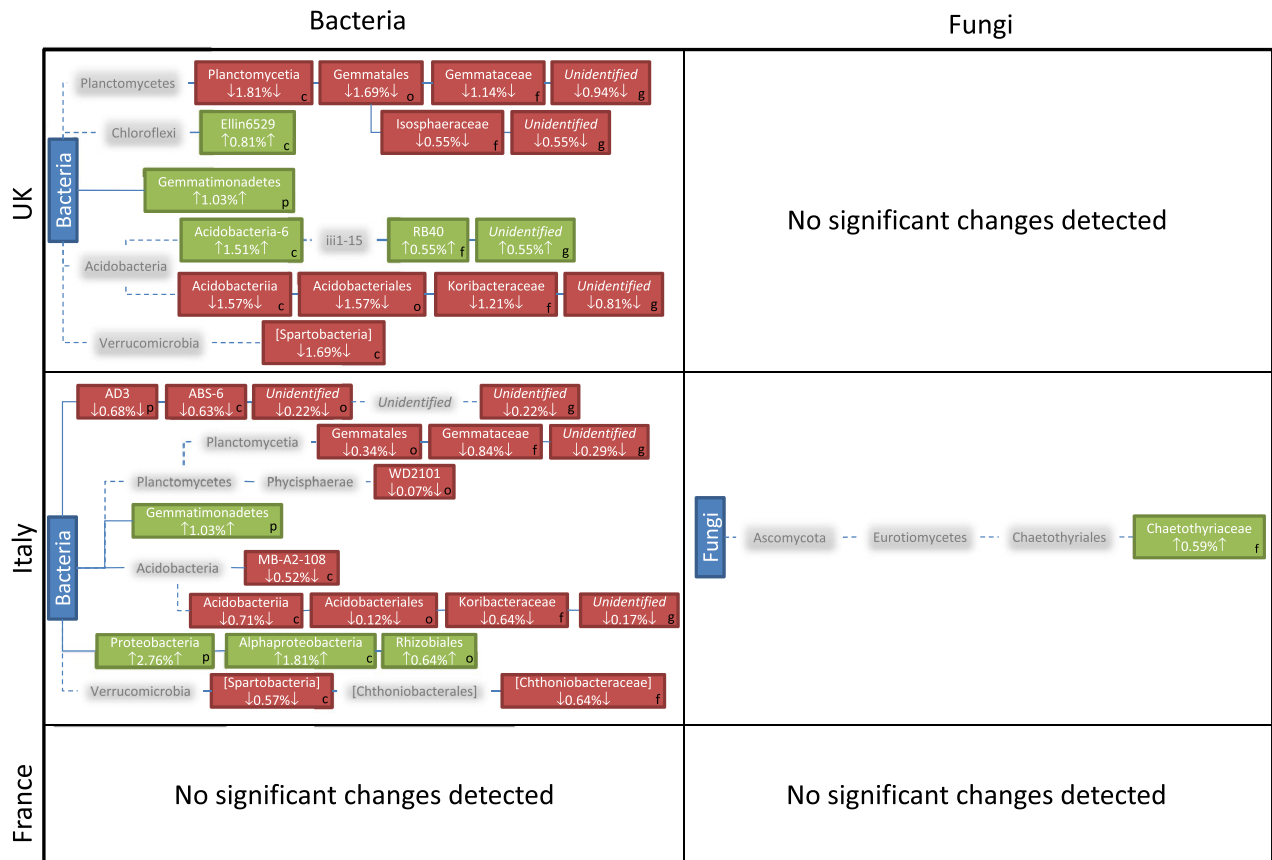
$\text{P-PO}_4 \text{ ha}^{-1}$ ,  $\sigma = 1.26$ ) when compared to control ( $\mu = 2.79 \text{ kg N-NH}_4 \text{ ha}^{-1}$ ,  $\sigma = 1.35$ ;  $\mu = 1.43 \text{ kg P-PO}_4 \text{ ha}^{-1}$ ,  $\sigma = 0.97$ , respectively). No other main effects or interactions were identified (in all cases  $P > 0.18$ ) (Fig. 9b).

EA analysis showed a significant decrease in acid phosphomonoesterase ( $P = 0.017$ ) and increased alkaline phosphomonoesterase activity ( $P < 0.001$ ) in biochar-treated plots (Fig. 9c).

## Discussion

### *Biochar causes significant changes in the structure of microbial communities*

We present the first study using NGS to survey taxonomic and community shifts in bacteria and fungi in a range of biochar-treated field sites across Europe. By



**Fig. 8** Significant differences in taxa from STAMP differential abundance testing. Taxa in red declined in biochar samples, whilst taxa in green were increased.

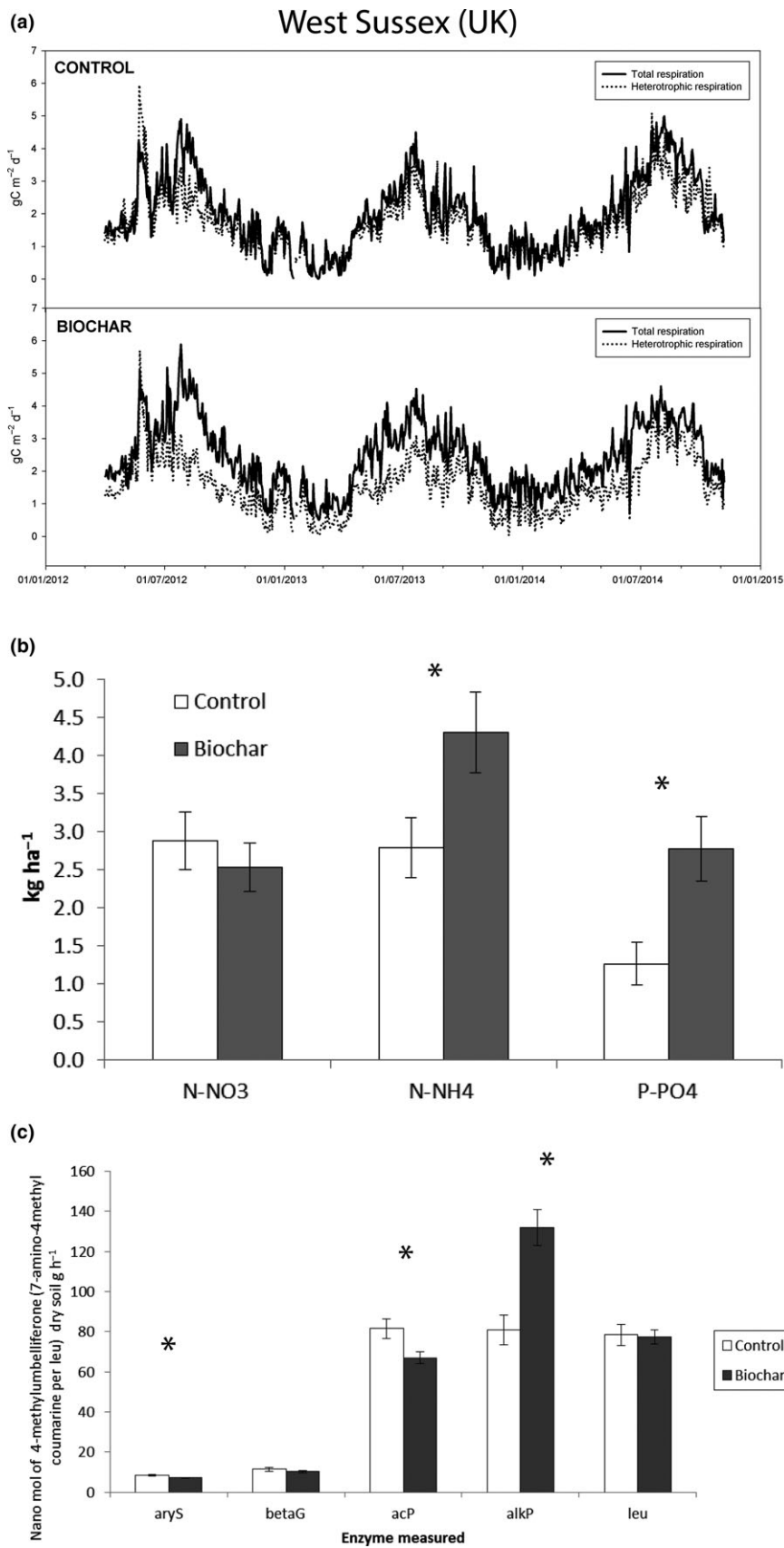
**Table 3** Mean pH values for biochar and control plots at each site

	Biochar	Control
UK 1 month	7.369	6.216
UK 1 year	7.380	6.230
France	7.075	6.754
Italy	7.245	6.624

applying consistent methods and biochar to several soils, we measured microbial community response to detect whether similar modifications in community structure occurred in contrasting locations and environments. We found that whilst some phyla (such as the *Gemmatimonadetes*) showed similar responses at multiple sites, many taxa showed variation in response. In addition, we identified small but significant changes in bacterial and fungal community diversity and composition following biochar application, consistent with previous findings in microcosms, using target specific markers (Khodadad *et al.*, 2011; Kolton *et al.*, 2011; Hu *et al.*, 2014; Xu *et al.*, 2014). However, we note that whilst biochar influenced community structure at all sites in some

way (be it through shifts in bacterial or fungal beta diversity), a wide range of responses were noted and site differences and changes with time were much larger than the impacts of biochar on the soil microbiome. Bacterial community change in response to biochar appears to result from several small changes in abundance across phyla in the UK with the UK time series revealing temporal shifts in the bacterial community after 1 year of treatment that were not present after 1 month. For fungi, the inverse was apparent, with community difference revealed after 1 month, but not after 1 year. These shifts across site and time suggest that diverse responses to biochar remain likely and extend previous research by providing greater resolution of biochar impact on bacterial and fungal diversity across location and time. In addition, whilst a community change due to biochar was noted at each site, the UK time series experiment indicated that temporal variation in the soil community diversity was often greater than that of the treatment.

No change in the number of bacterial taxa present was noted, regardless of treatment. This contrasts with data obtained in laboratory experiments in which



**Fig. 9** (a) Mean change in total soil respiration (solid line) and heterotrophic respiration (dotted line) over 2012–2014 monitoring period at UK, for both biochar and control treatments; (b) Total amounts of N-NO<sub>3</sub>, N-NH<sub>4</sub> and P-PO<sub>4</sub> collected by resin lysimeters installed at UK during summer of 2012 and collected during Summer 2013; (c) shift in soil enzymatic activities at UK after biochar treatment. \**P* < 0.05.

biochar significantly increased taxonomic diversity (Hu *et al.*, 2014; Xu *et al.*, 2014) and questions the relevance of such studies. However, both of these soils were of very low pH (4.5 and 3.7, respectively). Neutral pH soils are known to support greater diversity of taxa than acid soils (Fierer & Jackson, 2006). Thus, the potential for liming effects of biochar in such studies may drive the increased diversity noted. In our study, pH at each site was significantly increased by biochar application after 1 year, although as pH's were not as acid as those in the aforementioned studies, this may explain why no significant change in  $\alpha$  diversity occurred. However, it should be noted that other unquantified differences between sites such as soil moisture, vegetation cover, interaction with plant rhizospheres and temperature may also have influenced bacterial richness. UK time series data showed a significant decline in bacterial richness with time. As this occurred in both treatment and control plots, we suggest that this may be through variations in annual conditions such as meteorological events, soil moisture content and root exudates (Cruz-Martínez *et al.*, 2009), although to confirm this increased sampling frequency would be required.

Differences in bacterial  $\beta$  diversity were detected between treatment and control at each site. In the UK and IT,  $\beta$  diversity results suggest a change in both bacteria and fungi present and their abundances. Application of biochar represents an influx of nutrients, as the labile portion can be rapidly mineralized (Kolb *et al.*, 2009; Farrell *et al.*, 2013). It is possible that the shifts in community structure detected in the  $\beta$  diversity analysis may be a result of edaphic change (such as the significant increase in soil pH) associated with biochar. No significant effect was detected in FR bacterial data, with the exception of unweighted  $\beta$  diversity. We can therefore conclude that whilst changes in the identities of bacterial taxa present in FR occurred in response to biochar application, there was no linked change in abundance. Thus, FR bacterial communities had differences in rare taxa, although abundances of extant taxa were not significantly changed.

Fungal diversity also exhibited shifts in UK one-month samples when comparing biochar with controls. This may suggest a short-term shift in fungal richness due to biochar application, potentially as a result of sudden changes in edaphic variables, which fungi may struggle to rapidly adapt to (Lehmann *et al.*, 2011). However, a significant change in fungal community was also noted in IT and FR. FR samples were driven

by multiple small shifts in abundance. Therefore, although microbial communities responded at all sites, the nature of the response varied, probably due to interactions between the biochar and the range of initial soil conditions, vegetation types and extant communities.

#### *Biochar application impacts on different taxa at each site, but selection against oligotrophs occurs*

As the rate and production method of biochar applied at each site was identical, it is interesting to note the range of responses. Previous incubation and pot experiments with biochar have found a range of changes in community structure. For example, 454 sequencing of root associated bacteria in a pot study of pepper plants revealed an increased abundance of *Bacteroidetes* and particularly the genus *Flavobacterium* in biochar samples (Kolton *et al.*, 2011). A similar pot study using 454 sequencing combined with TRFLP detected significant increases in *Bradyrhizobiaceae* and *Hyphomicrobiaceae*, both associated with N cycling (Anderson *et al.*, 2011). Whilst a further DGGE pot study of *Lolium perenne*, treated with biochar, revealed significant shifts in total community structure as a result of biochar related pH changes (Fox *et al.*, 2014). This indicates the potential of biochar to cause shifts in biodiversity, but the nature of these changes differs with environment. Our analysis revealed significant shifts in the structure of communities and that this was correlated with pH change at all sites. Given the significant increase in soil pH due to biochar treatment that we detected, it is probable that the two responses are linked. By undertaking STAMP analysis, we were able to determine which taxonomic groups revealed the largest shifts in differential abundance, thus contributing to the shifts in  $\beta$  diversity detected.

STAMP analysis revealed significant enrichment of *Acidobacteria* in control samples 1 year after application, implying biochar amendment led to a decline in their abundance. We also found an enrichment of *Acidobacteria* in IT control samples. *Acidobacteria* dominate oligotrophic, low pH soils; therefore, it is likely that the decline in *Acidobacteria* was a response to biochar-treated soil that was more neutral and copiotrophic (Verastegui *et al.*, 2014). Previous studies have suggested that this class is often unaffected by the presence of charcoal in terra preta soils (Grossman *et al.*, 2010; Taketani *et al.*, 2013), although our results appear to contradict this. However, terra preta soils often contain ancient sources

of pyrolysed carbon, and as such, the effect occurs on a short term rather than decadal timescale. Soil microbiome studies of Central Park have previously noted the driving effect of pH gradients and also noted its particular effect on *Acidobacteria* proportional abundance (Ramirez *et al.*, 2014). Therefore, the influx of high pH biochar into neutral or slightly acidic soils at our sites may have further lead to their decline in proportional abundance.

STAMP analysis showed significantly elevated abundance of *Alphaproteobacteria* in IT biochar samples, a class known to be able to metabolize arabinose, a component of vegetable derived hemicelluloses and bacterial membranes, possibly indicating an increased role for this taxa in decomposition (Verastegui *et al.*, 2014). Approximately 1.25% of this increase was in the order *Rhizobiales*, a taxa associated with nitrogen fixation, plant growth promotion and increase SOM turnover (Spain *et al.*, 2009; Bruto *et al.*, 2014; Tkacz & Poole, 2015).

We revealed a single change in differential abundance of fungal taxa in the IT site, an increase in the pathogenic fungus *Chaetothyriaceae* in IT biochar samples (0.6%). Although the increase is relatively small, it should be considered that this is representative of a threefold increase in this taxon after biochar application. These are known leaf mould forming fungi (Chomnunti *et al.*, 2012), and as such biochar application either increases their abundance within the soil directly, or alternatively may be elevated due to an increase in their rate of infection on fallen leaves.

#### *Annual and site effects may be greater than that of treatment*

Our time series data revealed significant divergence between bacterial communities by treatments over time. This suggests that the year of sampling may have a greater influence than the treatment. PLFA-based methods suggested that bacterial diversity is strongly related to mean annual precipitation, indicating that biogeographical variables can trigger change in soil microbial communities (de Vries *et al.*, 2012). Seasonal shifts in community have been associated with temperature, although variation in functional groups remained low (Delmont *et al.*, 2012). However, biochar appeared to cause grouping along PC2, although only in samples collected after 1 year. This indicates a change in the effect of biochar on communities over time, possibly as recalcitrant portions of the biochar became available (Watzinger *et al.*, 2014) following physical weathering (Naisse *et al.*, 2015). This gradual change in the nutrient profile of the soil again may suggest selection in favour of copiotrophic organisms, as faster growing bacteria are able to outcompete more specialized oligotrophs. The shift from

acid to alkaline phosphomonoesterase further supports this theory (Caldwell, 2005). Furthermore, differences in  $\alpha$  diversity between UK samples collected 1 month and 1 year after treatment showed an impact of time on overall diversity. It is also possible that the presence of biochar in the soil may slow some naturally occurring annual variations. For example, we noted that an unidentified genus of *Ascomycota* was detected in increased proportions in control samples 1 month after treatment, which no longer occurred after 1 year. A similar effect was not noted in biochar plots during the same time period. This may reflect a natural succession event occurring within untreated soils, which was retarded by the application of biochar. Similarly, a multitude of genera (see Fig. 8) experienced annual shifts in one treatment, not detected in the other. This suggests that combinations of seasonal variations with biochar could further explain the variations observed. For example, biochar is known to increase pore space and decrease bulk density in soils, which in turn can lead to increased soil water content (Baronti *et al.*, 2014) which could result in a selection pressure for bacterial communities (Manzoni *et al.*, 2012). Many bacterial taxa are almost ubiquitous, being able to adapt to survive across a range of environments due to their ability to partake in horizontal gene transfer. Furthermore, those bacteria which have larger genomes often have greater capacity for surviving in unsuited environments as they have a larger range of processes available (Barberán *et al.*, 2014). This may result in difficulties resolving the limiting factors in bacterial temporal niche and biogeography taxonomically, as organisms of the same taxa may have varying functional attributes depending on annual or seasonal conditions (Van Der Heijden *et al.*, 2008).

The UK fungal time series displayed the inverse of the UK bacterial time series for  $\beta$  diversity, with a significant effect in UK samples collected after 1 month, but no change in UK samples after 1 year. Drought experiments investigating heathland fungal diversity have noted a greater effect of seasonality than the treatment itself, with a gradual decline in diversity in the summer months. Such summer declines may be as a result of declines in SOM reaching the soil for decomposition and subsequently limitations to the niche creation within the soil environment (Toberman *et al.*, 2008).

We detected significant differences in the  $\beta$  diversity of both bacteria and fungi depending on site in control samples. This represents the substantial variation in the communities present and will be influenced by edaphic variables, vegetation types and the abiotic variables of each geographical location. However, we note that at all sites there was a significant correlation between pH and fungal and bacterial diversity, corresponding with

previous research indicating that soil pH is one of the main drivers of microbial biogeography (Fierer *et al.*, 2012).

Our results show short-term shifts in fungi, followed by longer term shifts in bacteria contrary to expectations. Due to their ability to rapidly acclimatize to nutrient influxes, bacteria would be expected to respond to biochar more quickly. It is possible that this rapid change noted in fungal communities may be a response to the disturbance of biochar application, although it is interesting to note that differences occur by treatment, as both biochar and control plots were disturbed equally. Taken together with the significant increase in *Ascomycota* in UK 1-year control compared with at 1 month, and the absence of the same effect in biochar-treated plots, we suggest that biochar may negatively influence the recovery of these taxa after disturbance effects.

#### *Biochar impacts on microbially mediated biogeochemical cycles*

Environmental measurements in the UK time series suggest no change in respiration due to biochar over the year. Previous work has indicated that biochar application either increases microbially mediated respiration (Belyaeva & Haynes, 2011; Castaldi *et al.*, 2011) or can in some cases retard it (Dempster *et al.*, 2011; Carlsson *et al.*, 2012). It is possible that this is a result of differences in methodology, given that respiration rates are often measured through use of incubation experiments (Kolb *et al.*, 2009; Belyaeva & Haynes, 2011; Zavalloni *et al.*, 2011). Whilst a valuable and accurate method, incubation experiments in the laboratory cannot capture natural seasonal and temporal variation in microbial communities, making it difficult to determine how applicable their results are to biochar in the field. Additionally, many previous studies (both those showing increases and decreases in respiration) are carried out over short timescales, of months, or even weeks. The respiration data collected for this study encompassed a full year, indicating that in long-term *in situ* environments, bacterial respiration may be unaffected by biochar. Biochar application is known to have transient effects as available C portions are rapidly utilized by bacterial growth, but no long-term effects on growth rates are noted (Rousk *et al.*, 2013). Taken together with the limited degradation and high stability of the biochar at the site (Ventura *et al.*, 2015), it seems unlikely that biochar provides a substantial long-term additional source of C for microbial activity. However, we cannot determine whether microbial activity may switch to metabolizing biochar C in future. Previous work at the same site found that biochar degraded more rapidly in the presence of roots, although the rate of SOM

degradation decreased, indicating that biochar may protect extant SOM from mineralization (Ventura *et al.*, 2015).

Elevated alkaline phosphomonoesterase and a decline in acid phosphomonoesterase suggest a shift in response to the increased soil pH detected in biochar samples. The increased presence of alkaline phosphomonoesterase is associated with a shift in acidophilic bacterial taxa to those better adapted to neutral or alkaline environments. Taken together with the data from the lysimeters, we can see increased available P within the soil, indicating an impact of biochar on phosphate cycling. Given that phosphate is often a limiting nutrient, its increased availability will have implications for plant and microbial growth and as a consequence, nutrient cycling. Biochar has been previously suggested to augment bacterial phosphate mobilization (Fox *et al.*, 2014), whilst AMF have been shown to liberate phosphorus from biochar, making them available to plant roots (Hammer *et al.*, 2014). Alternatively, these changes may be due to influx of P within the biochar itself. Whilst the bioavailability of the P within the biochar is unknown, a portion is likely to have remained present within the ash fraction of the biochar, and as such, this may explain the increased leaching noted. Leachate of ammonium was significantly higher in biochar plots, although no change in leucine aminopeptidase activity was observed. This may suggest an increase in the rate of ammonification by soil communities in response to biochar, or a decline in the rate of nitrification, leading to a build-up of ammonium within the soil, in agreement with previous studies (DeLuca *et al.*, 2006; Anderson *et al.*, 2011). However, we detected no large increases in abundances of taxa associated with these roles, and so in the case of ammonium, as well as for phosphorus, the increased leaching could be due to the N-NH<sub>4</sub> content of biochar itself.

In conclusion, we have shown that NGS DNA metabarcoding is a powerful technique that can be used to detect changes in the soil microbiome – between different sites and with time. In contrast to earlier studies using microcosms, metabarcoding of soil following field exposure to biochar revealed changes occurring consistently in the proportional abundance in the microbiome revealing that the application of biochar may change soil ecology, with the potential for subsequent shifts in soil function. Although the nature of the shift often varied depending on soil, climate and crop conditions, there were clear indications of a move in soil function towards a more nutrient rich and higher pH environment, with increases and decreases in *Proteobacteria* and *Acidobacteria*, respectively, in biochar and increased availability of P and N from chemical analyses. Although shifts in individual taxa were relatively small, overall community structures showed sizable shifts.

Given that soil fungal and bacterial communities provide essential biogeochemical cycling and a range of ecosystem services, variation of this type in response to biochar application may have implications for soil function above that associated with carbon mitigation. These are not only dependent upon the type of biochar applied, but on the soil environment subject to its application. These interacting effects on the soil microbiome should be investigated further prior to wide-scale application of this treatment.

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## Data accessibility

- 1 Raw DNA sequences will be submitted to Dryad.
- 2 Results of QIIME pipeline will also be submitted to Dryad, in the form of .biom files containing taxa ID's and frequency counts.

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## Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1.** PCoA of UNIFRAC distances for (a) weighted and unweighted UNIFRAC in FR and (b) weighted and unweighted UNIFRAC in UK 1 month (UK1M) samples.

**Figure S2.** Mantel Correlogram showing the significance of soil pH in bacterial (a) and fungal (b) composition for all sites and treatments.

**Figure S3.** UK time series showing shifts in differential abundance from 1 month to 1 year.

**Table S1.** Results of *t*-tests for difference in (a) 16S  $\alpha$  diversity (i.e. bacterial diversity), (b) ITS  $\alpha$  diversity (i.e. fungal diversity) between treatment (B, biochar, or C, control) at each of three sites.

**Table S2.** Results of ADONIS tests for weighted and unweighted 16S beta diversity, and Bray-Curtis ITS beta diversity, comparing the effects of treatment or site for UK1M, UK1Y, IT and FR.

**Table S3.** Mean proportional abundances of phyla present in UK control, biochar and pre-treated plots in UK1M and UK1Y samples.