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Effects of vegetation and seasonality on bacterial communities in Amazonian dark earth and adjacent soils

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Amazonian Dark Earth (ADE) in the Brazilian Amazon is the main evidence left by pre-Columbian indigenous populations indicating that infertile soils can be transformed into highly fertile ground. Changes in vegetation cover and seasonality are likely to influence microbial communities; however, little is known about these effects on ADE. Therefore, this study compared the effects of two land use systems in ADE and adjacent soil (ADJ) during the rainy and dry seasons using biochemical and molecular tools. Bacterial community function was determined by community level physiological profile (CLPP), bacterial community structure by terminal restriction length polymorphism (T-RFLP), and bacterial community composition by pyrosequencing of the V4 16S rRNA gene region. Our results show that the community structure is highly affected by vegetation, in both, ADE and ADJ soils. Regarding community function, Average Well Color Development (from Biolog substrates) were higher in ADE than ADJ during the rainy season and kept the same pattern of substrate utilization during the dry season and finally, community composition showed to be influenced even at the level of family, mostly by soil type rather than vegetation. Collectively, our study provides insights into processes affecting the bacterial community assemblages in both, ADE and adjacent soils.

Key words: Amazonian soils, vegetation type, seasonality, soil bacteria.

INTRODUCTION

Most of the upland Amazon rainforest is located on heavily weathered and nutrient-poor soils. Their productivity depends on vegetation diversity and also relies on the efficient recycling of organic matter (Sanchez et al., 1982). Slash-and-burn agriculture is a typical smallholder land use system in the Amazon region. The release of nutrient-rich ashes leads to an increase in soil pH and cation contents of the surface soil layer, consequently providing new nutrient input (Hölscher et al., 1997). However, after continuous use for

cropping, there is a gradual decrease in soil fertility (Sanchez et al., 1982); another factor is nutrient losses due to the burn, harvest, and leaching during the process of slash-and-burn agriculture (Hölscher et al., 1997).

Concerning the same region, the existence of scattered patches of fertile black soils know as Amazonian Dark Earth (ADE) (locally called *Terra Preta de Índio*) is the main evidence left by pre-Columbian indigenous populations indicating that poor soil can be transformed into highly fertile ground. Analyses of this anthropogenic soil have shown that they present high levels of stable organic matter and chemical nutrients, such as carbon, phosphorous, calcium and manganese (Lehmann et al., 2003). Moreover, the anthropic horizon of ADE shows high resilience to soil management and remarkable soil physical qualities, such as good soil aggregation and high porosity in comparison to the surrounding soils (Teixeira and Martins, 2003). It is believed that these elements were added to the soils through human depositional activity and prehistoric semi-intensive or intensive agriculture (Denevan, 1996). For these reasons, anthropogenic ADE is frequently cultivated by traditional smallholders for subsistence farming.

In spite of the unique properties of ADE, little is known about the effects of modern agricultural practices, current land use, and seasonality effects on these anthrosols. Furthermore, different types of aboveground vegetation are known to influence soil bacterial communities (Mitchell et al., 2010; Chaparro et al., 2012). There is also growing concern that current climate change may cause a large “dieback”or degradation of Amazonian rainforest with a higher probability of intensified dry seasons (Malhi et al., 2009). This, in turn, will influence soil microbial communities which mostly regulate ecosystem processes (Neher, 1999). Few studies have characterized the bacterial community composition and distribution in different ADE sites (O´Neill et al., 2009; Grossman et al., 2010; Navarrete et al., 2010). Recently, using the DNA pyrosequencing technology, Taketani et al. (2013) observed that vegetation cover had an effect over the bacterial community structure independent of soil type and in the same sites of the present study.

Therefore, it is important to further assess ADE microbial communities to identify possible shifts in these communities that may influence soil fertility and quality. One way to assess changes in soil function is the use of Biolog ecoplates to generate a community-level physiological profile (CLPP) of mixed aerobic heterotrophic bacteria (Garland and Mills, 1991). Despite the methodological implications of BIOLOG ecoplates, the method was successful used to detect differences in microbial communities in soil such as Arctic tundra soils (Campbell et al., 2010) and wetlands under different land management regimes (Doutorelo et al., 2010). The molecular toolbox [group-specific-PCR; Denaturing Gradient Gel Electrophoresis (DGGE); Terminal Restriction Fragment Length Polymorphism (T-RFLP)] has also been successfully used to describe changes in microbial community structure in tropical forest soils (Jesus et al., 2009) and agricultural soils (Enwall et al., 2007) and DNA pyrosequencing technology has proven to be a powerful tool for rapid and sensitive investigations into complex microbial communities.

Here we investigated the bacterial community function, structure and composition at finer taxonomic level in ADE (Hortic Anthrosol) and the adjacent soil (Haplic Acrisol, ADJ) under different vegetation types and seasons at the Caldeirão Experimental Research Station in the Brazilian Central Amazon. This study combined CLPP, T-RFLP and pyrosequencing technology to test the hypothesis that aboveground plant diversity and seasonal effects might differentially influence the ADE and ADJ inhabiting bacterial communities. In addition, we provide correlational insights relating the relative abundance of bacterial families and genera in these soils to the differences between the soil chemical properties detected among sites.

MATERIALS AND METHODS

Study sites and soil sampling

The studied sites were located in the Caldeirão Experimental Research Station of Embrapa Amazônia Ocidental in Iranduba County in the Brazilian Central Amazon (03°26’00”S, 60°23’00”W). Four different sites were chosen based on the presence of pre-historic anthropic soil horizons (Hortic Anthrosols) referred to as ADE, along with the adjacent soils without an anthropic horizon (Haplic Acrisol, ADJ) according to the World Reference Base for Soil Resources (FAO, 1998). At both sites, the vegetation cover types were a 35-year-old secondary forest (SF) and a 5-year-old manioc (*Manihot esculenta*) plantation. The soil samples were collected during the rainy season (January 2009) with mean monthly rainfall of approximately 400 mm, and the dry season (August 2009) with mean monthly rainfall of approximately 30 mm (http://clima1.cptec.inpe.br/~rclima1/monitoramento_brasil.shtml).
At each site, the sample plot was determined by choosing a random point and from this reference point, three points 5 m apart were chosen for the collection of intact soil cores 5 cm in diameter and 15 cm in length. Soil samples were collected using sterile techniques and transported (< 24 h) in an isolated box on dry ice for DNA extraction and on ice packs for physiological and microbial biomass measurements at CENA in Piracicaba (SP, Brazil). Total microbial biomass measurement was performed at Embrapa Soybean (Londrina, Brazil), and chemical analysis at Embrapa Amazônia Ocidental in Manaus, Brazil.

Determination of soil chemical properties

Soil samples were analyzed in triplicate for pH (H_2O, 1:1), soil

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extractable Al, Ca, and Mg (1 M KCl), soil extractable P and K (double acid solution of 0.025 M sulfuric acid and 0.05 M hydrochloric acid Mehlich 1), soil C (Walkely-Black method) and effective cation exchange capacity (sum of all base cations plus exchangeable Al and H). For more details on the methods used for such measurements (Embrapa, 1998). The soil moisture was determined after drying the samples overnight at 105°C.

Soil microbial biomass carbon (MBC) was estimated following the fumigation-extraction method (Vance et al., 1987) and soil microbial biomass nitrogen (MBN) was assessed by the method of Brookes et al. (1985), both slightly modified by Hungria et al. (2009). For both measurements, triplicates were used from each site (n = 9). MBC measurements were based on the difference between organic C extracted with 0.5 M K_2SO_4 (Bartlett and Ross, 1988) from chloroform fumigated and unfumigated soil samples (Vance et al., 1987), using a correction factor of 0.41 as recommended for tropical soils (Feigl et al., 1995). MBN was determined by the difference between extractable N in fumigated and unfumigated samples using a correction factor of 0.54 (Brookes et al., 1985).

Biolog functional analysis

Microbial community level physiological profiles (CLPP) were assessed using Biolog Ecoplates® (Biolog, Hayward, CA, USA) which contained three replicate wells of 31 carbon sources and a water blank (Insam, 1997). Measurements were performed for each soil sample collected from the three points of each site with three replicates per carbon substrate (n = 9). Inoculation density was previously estimated by counting colony forming units on nutrient agar medium at 25°C for 48 h. Each soil suspension was inoculated into Biolog Ecoplates (120 µL per well) which were incubated at 28°C and were read after 12 h, then every 24 h for seven days using an ELISA microplate reader at 590 nm. The generated Biolog ecoplate data were transformed by dividing the raw values by the respective average well color development (AWCD) values (Garland and Mills, 1991). The corrected values were used to evaluate average heterotrophic metabolism and to estimate kinetic parameters as proposed by Lindström et al. (1998): $AWCD = K / [1 + e^{-r(t-s)}]$, where K (asymptote) is the maximum degree of color development, R (degradation rate) is the exponential rate of AWCD change (h^{-1}), t is the time of following inoculation of the plates (h), and S is the time when the mid-point of the exponential portion of the curve (that is when Y=K/2) has been reached (h).

DNA extraction, T-RFLP and 454-pyrosequencing

Soil DNA was extracted in triplicate for each sample using the MoBio PowerSoil DNA extraction kit according to the manufacturer's instructions (MoBio Laboratories, USA). The purity and quantity of the extracted DNA were determined by UV-spectrophotometry at 260 and 280 nm (NanoDrop® ND-1000 UV/vis-spectrophotometer, Peqlab Biotechnologie GmbH, Erlangen, Germany). The obtained DNAs were further stored at −20°C.

T-RFLP analysis was performed with the primer set 27F-FAM-labeled (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), used to amplify the near-full 16S rRNA gene (Lane, 1991). Each PCR amplification was performed in triplicate (n = 9) in 25 µL reactions containing 2.5 µL 10x reaction buffer (Invitrogen, Carlsbad, CA), 3 mM $MgCl_2$, 0.2 mM of each dNTP (Eppendorf, Germany), 0.1 mM BSA (New England Biolabs, USA), 0.25 mM forward-labeled primer 27F, 0.25 mM reverse primer 1492R, 1 U of Platinum Taq DNA Polymerase (Invitrogen, USA), and 2 ng of template DNA. Cycling conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 59°C for 45 s, 72°C for 1 min, and a final extension step of 72°C for 15 min. Obtained products were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA) and digested at 37°C for 3 h with the endonuclease MspI (Invitrogen, USA). DNA was precipitated using isopropanol (Sambrook and Russell, 2001) and resuspended in 9.8 µL of deionized formamide and 0.2 µL of GeneScan-500 ROX internal size standard (Applied Biosystems, USA), then denatured at 94°C for 5 min. Terminal Restriction Fragments (TRFs) were analyzed using an ABI PRISM 3100 genetic sequencer (Applied Biosystems, USA).

Partial bacterial 16S rRNA gene sequences were amplified for pyrosequencing using the following primers to target the V4 region (fragment length of 270-300 bp) of the 16S rRNA gene at corresponding *Escherichia coli* positions 563 and 802: primers 563F and 802R (Sul et al., 2011) containing the Roche 454 pyrosequecing adaptors and barcodes of 8 bp (attached to the forward primers). Each PCR reaction mixture contained 1x reaction buffer, 1.8 mM $MgCl_2$, 0.2 mM of each dNTP, 10 mg mL^{-1} of BSA, 0.2 µM of each primer, 1 U of FastStart high-fidelity PCR system enzyme blend (Roche Applied Sciences, IN, USA), and 4 ng of DNA template. Cycling conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 45 s, and 72°C for 1 min, and a final extension step of 72°C for 4 min. PCR products were separated by agarose gel electrophoresis and the products with the expected size (ca. 270-300 bp) were excised and purified using the Qiagen gel extraction kit (Qiagen, CA, USA), followed by a second purification with the Qiagen PCR purification kit (Qiagen, CA, USA). Sequencing was performed on the GS FLX sequencer (454 Life Sciences, CT, USA) at the Michigan State University Research Technology Support Facility. The dominant phyla and class composition of the bacterial communities from the same sites of this study was previously reported (Taketani et al., 2013). Here, we incorporated such dataset to gain insights into a deepest taxonomical resolution of such effects.

Soil chemical properties, microbial biomass, and Biolog data analysis

Variance analyses of soil chemical properties were tested separately for land use and season by ANOVA. Results showing significant overall changes were subjected to Tukey's post-hoc test with significance set at $P < 0.005$. The kinetic parameters were submitted to analysis of variance (ANOVA) and differing pairs were identified with post hoc Tukey test ($P < 0.05$). These results were also correlated with soil chemical properties and microbial biomass using the Spearman correlation coefficient. Statistical analyses were carried out using STATISTICA version 10 (StatSoft, USA).

T-RFLP data analysis

T-RFLP data were analyzed using Peak Scanner software v1.0 (Applied Biosystems). TRFs smaller than 50 bp and larger than 800 bp were excluded from the analysis. True peaks were determined using T-REX online software according to Abdo et al. (2006) (http://trex.biohpc.org, last updated on 2010/03/01). TRF sizes were rounded to the nearest integer and peak heights were relativized to account for uncontrolled differences in the quantity of DNA between samples (Culman et al., 2009). Normalized peak heights were used to calculate the relative abundance of TRFs. Statistical analysis of T-RFLP data were performed on square-root transformed data to obtain homogeneity of variances. Multivariate analysis of the T-RFLP fingerprints from all sites was performed using multidimensional scaling (MDS) based on Bray-Curtis similarity matrices. Permutational ANOVA (PERMANOVA) was used to verify significant differences between samples from all sites and seasons (Anderson, 2001). The influence of soil properties on the bacterial community structure was assessed using BEST analysis (BIOENV procedure), which selects the soil properties that may

Table 1. Selected soil properties of Amazonian Dark Earth and adjacent soil (Haplic Acrisol) under secondary forest and manioc plantation during the rainy and dry seasons.

Soil properties	Amazonian Dark Earth				Adjacent Soil			
	Secondary forest		Manioc plantation		Secondary forest		Manioc plantation	
	Rainy	Dry	Rainy	Dry	Rainy	Dry	Rainy	Dry
pH	5.40[a]	5.25a	5.46a	5.33a	3.63b	3.67b	3.68b	3.73b
Soil C (g kg^{-1})	32.35a	28.17a	28.44a	26.47a	30.47aA	18.68aB	17.62b	16.15b
P (mg dm^{-3})	140aA	83aB	174a	205a	9b	4b	6b	4b
Ca ($cmol_c$ dm^{-3})	9.05aA	3.93aB	8.68aA	3.93aB	0.89b	0.24b	0.16b	0.11b
Mg ($cmol_c$ dm^{-3})	1.43aA	0.86aB	1.53a	1.11a	0.31b	0.11b	0.10b	0.06b
Al ($cmol_c$ dm^{-3})	0.01a	0.03a	0.01a	0.02a	2.06bA	1.52bB	1.72b	1.49b
CEC† ($cmol_c$ dm^{-3})	10.64aA	4.90aB	10.37aA	5.13aB	3.42b	1.95b	2.07b	1.74b
MBC (mg kg^{-1})	656.97aA	431.00aB	372.53b	346.43ab	378.53b	452.93a	248.33c	232.73b
MBN (mg kg^{-1})	51.93a	63.47a	20.90bA	15.33bB	19.37bA	51.33aB	12.83b	13.00b
SMC (%)	41.8aA	23.1aB	24.0bA	16.2bB	40.0aA	12.7bcB	37.7aA	12.1cB

[a]Abbreviations: CEC, cation exchange capacity; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; SMC, soil moisture content. [b]Means separately for eADJ season within a column followed by the same lower case letter are not significantly different ($P < 0.05$, Tukey post hoc).[c]Significant differences between seasons are followed by different upper case letter ($P < 0.05$, Tukey pos hoc).

explain biotic patterns (Clarke, 1993). All multivariate statistical analyses aforementioned were performed with PRIMER 6 software and the PERMANOVA add-on (Clarke and Gorley, 2006; Anderson et al., 2008).

Pyrosequencing data analysis

The resulting sequence reads were screened to remove sequences that contained any errors in the forward primer and barcode regions, ambiguities, and sequences shorter than 150 bp using the RDP Pyrosequencing Initial Process Tool (Cole et al., 2009). Chimeric sequences were identified by the Chimera Check program in the RDP pipeline (http://www.rdp.cmc.msu.edu). Quality trimmed sequences were aligned using the RDP pyrosequencing function *Aligner* and clustered with default parameters of the RDP function *Clustering*. The resulting aligments were manually checked and corrected if necessary. The resulting clusters were used to construct rarefaction curves at a dissimilarity value of 3% and were subsequently phylogenetically classified using the RDP Classifier (Wang et al., 2007). Distance matrices were constructed using the *dist.seqs* function and LIBSHUFF comparisons were made between the four studied sites using MOTHUR software (Schloss et al., 2009).

RESULTS

Soil properties and microbial biomass

The results of the different soil properties measured in ADE and ADJ under secondary forest (SF) and manioc plantation (M) during the rainy and dry seasons are presented in Table 1. Soil chemical properties of ADE-SF were chemically similar to ADE-M during both seasons. ADJ-SF and ADJ-M chemical properties were also very similar with the exception of soil organic carbon (SOC), which was significantly higher in ADJ-SF. ADE showed higher soil pH independent of vegetation comparatively to ADJ. As expected, in contrast to ADJ, ADE showed higher CEC, Ca, Mg, and P, indicating the high fertility of these anthropic soil horizons. Particularly, ADE had significant higher exchangeable bases (Ca, Mg) at both sites compared to ADJ. Decreases in the Ca content were observed during the dry season in ADE-SF (57%) and ADE-CP sites (55%). Similarly, there was a significant decrease (40%) in the Mg content, but this was only observed in ADE-SF. Seasonal changes in CEC were also observed in ADE for both sites with a significant decrease during the dry season. For ADJ, seasonal changes influenced only the contents of SOC and SOM under SF.

Microbial biomass carbon (MBC) was higher in ADE-SF compared to ADE-M, which presented similar MBC values as ADJ-SF during the rainy season. ADJ-M showed a decrease in MBC and MBN values for both seasons. Furthermore,

seasonality affected MBC in ADE-SF with a 34% decrease along with a 27% reduction in MBN for ADE during the dry season. For ADJ-SF, there was a significant increase in MBC and MBN from the rainy to the dry season. On the other hand, ADJ-M presented a significant decrease in MBC contents from the rainy season to the dry season. Soil moisture content decreased by 45-68% from the rainy to the dry season.

Bacterial community function

Average Well Color Development (AWCD) data represented by the average utilization intensity of 31 carbon substrates (during the evaluation period) are shown in Figure 1. The AWCD of plates inoculated with all studied soil samples increased rapidly after 30 h in both seasons, with the exception of ACH-SF-Rainy. In the rainy season, AWCD varied among the different soil types with higher overall AWCD values in ADE compared to the ACH soils. Differences due to vegetation type were observed only for the ACH soil samples with the lowest activity in ACH-SF. Nevertheless, there were no changes in AWCD for all soils in the dry season. Microbial utilization patterns of specific substrate groups are presented in Figure 2. Differences in microbial utilization patterns were observed only during the rainy season. The microbial utilization of carbohydrates was higher in ADJ-SF during the rainy season. Furthermore, the ADJ-SF presented lower microbial utilization of carboxylic and acetic acids, amino acids and amines when compared to the other sites.

Bacterial community structure

T-RFLP data analysis by multidimensional scaling (MDS) showed clearly differences between community structures in ADE and ADJ, and distinct clusters were formed according to vegetation type and sampling period (Figure 3). These results were further statistically confirmed by PERMANOVA, showing a significant effect of both, vegetation (SF and CP) and seasonality (rainy-R; dry-D) ($P = 0.002$). The BIO-ENV routine was used to determine which set of variables (environmental and microbial biomass) mostly explained the biological patterns observed in the T-RFLP analysis. The results indicate Al, Ca, P, pH, and SMC ($Rho = 0.911$; $P < 0.01$) as major drivers of community structure in the rainy season. For the dry season, Al, MBN and pH ($Rho = 0.877$; $P < 0.01$) were the major variables explaining the observed distribution.

Bacterial community composition

The pyrosequencing-based analysis of the V4 region of 16S rRNA was previously used to assess the bacterial community of ADE and ADJ (Taketani et al. 2013). It was shown that the most abundant phyla in all sites were Actinobacteria, Acidobacteria, Verrucomicrobia and Proteobacteria, represented by approximately 70% of the total number of sequences. However, at the class level, community composition showed differences between ADE and ADJ and, also, an effect of vegetation type was observed. In this sense we here use the same dataset to investigate these effects at a deepest taxonomic level.

Classification of sequences at the family and genus levels showed differences in their relative abundances according to the soil and vegetation type (Tables 2 and 3). The ADE soil was dominated by Gaiellaceae, Gemmataceae and Syntrophobacteraceae. In the ADJ soil, Acidobacteriaceae, Acetobacteraceae, Alicyclobacillaceae, Burkholderiaceae, Caulobacteraceae, Conexibacteraceae, Sinobacteraceae, Solibacteraceae and Xanthomonadaceae were the most abundant. Relative abundance of Hyphomicrobiaceae was higher in both soils under secondary forest. Moreover, higher bacterial family abundance in both soils under manioc plantation included Gemmataceae, Thermogemmatisporaceae and Oxalobacteraceae. At the genus level, the most dominant genera were *Alicyclobacillus*, *Bradyrhizobium*, *Candidatus solibacter* and *Rhodoplanes*. Among the most abundant genera under secondary forest were *Burkholderia* and *Rhodoplanes*. The genera *Luteibacter* and *Salinispora* were only observed in the ADJ soils. The relative abundance of bacterial families and genera lower than 1% also confirmed differences between ADE and ADJ soils (Tables S1 and S2).

We analyzed the relationship between bacterial family relative abundance and soil properties using Spearman correlation (Table 4). Most of the selected bacterial families were negatively correlated with soil properties typically found in higher amounts in ADE soils, indicating that ADJ soil properties may favor the higher abundance of these bacterial groups. Gaiellaceae, Gemmataceae and Syntrophobacteriaceae presented positive correlation with ADE soil properties and negative correlation with Al. In specific, the relative abundance of Gaiellaceae showed strong positive correlation with Ca, Mg and CEC, while the abundance of Syntrophobacteriaceae was positively correlated with P.

DISCUSSION

Temporal variability in soil properties

Losses of SOC and SOM by the conversion of native forest to agricultural use in the Brazilian Amazon are well known (Fearnside and Barbosa, 1998). This is in agreement with the results obtained in the ADJ soil samples, which showed a significant decrease in SOC and SOM after the conversion of secondary forest (SF) to a manioc plantation (M). However, SOC and SOM in

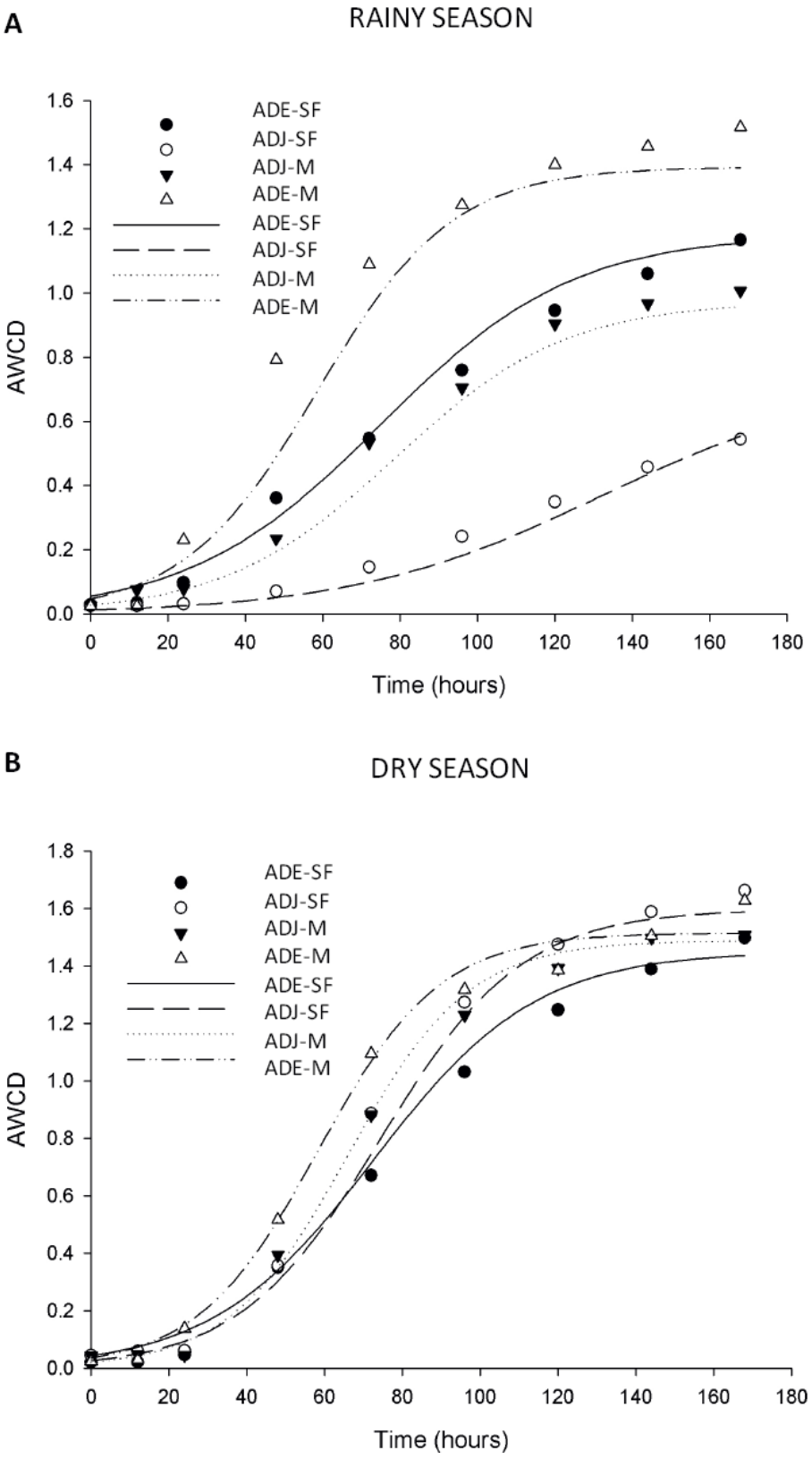

Figure 1. Average well color development (AWCD) of community-level physiological profiles (CLPP) of Amazonian Dark Earth (ADE) and adjacent soil (ADJ) under secondary forest (SF) and manioc plantation (CP) during the rainy (A) and dry (B) seasons. The lines represent the fitted equations and the dots represent the means of eADJ treatment (*n*=3).

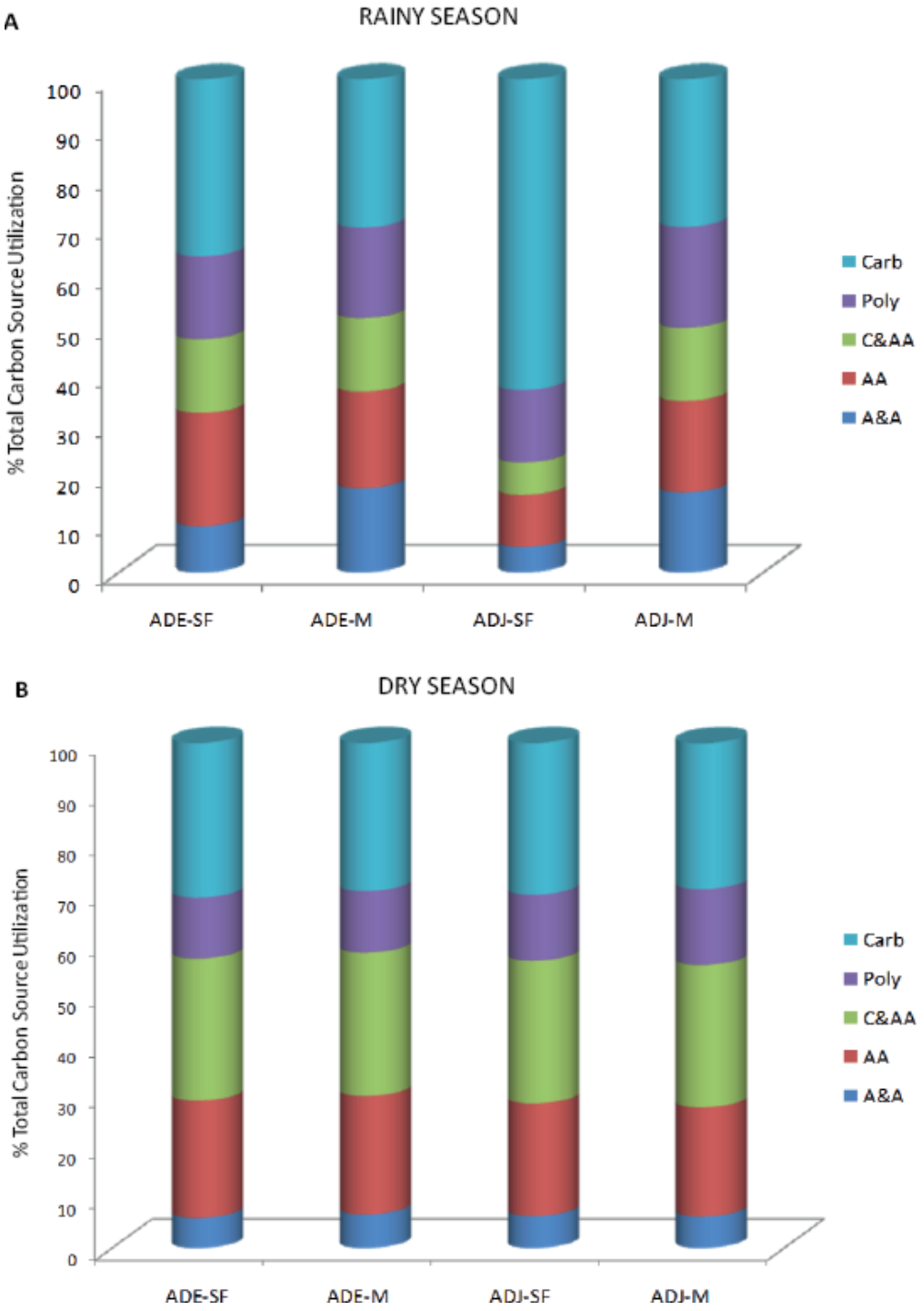

Figure 2. Percent of total carbon source utilization in ADE and ADJ soil samples collected in the rainy season (January 2009) and dry season (August 2009) under secondary forest (SF) and manioc plantation (M) for the different carbon substrate groups: carbohydrates (Carb), polymers (Poly), carboxylic and acetic acids (C & AA), amino acids (AA) and amines and amides (A & A).

ADE samples were not influenced by vegetation type, confirming findings that SOM in ADE is highly stable, even under agricultural use (Woods and McCann, 1999). The large amounts of biochar found in ADE soils are thought to improve and maintain soil fertility by stabilizing organic C in soil and increasing soil C sequestration (Zavalloni et al., 2011).

Soil MBC was significantly higher in ADE. Surprisingly,

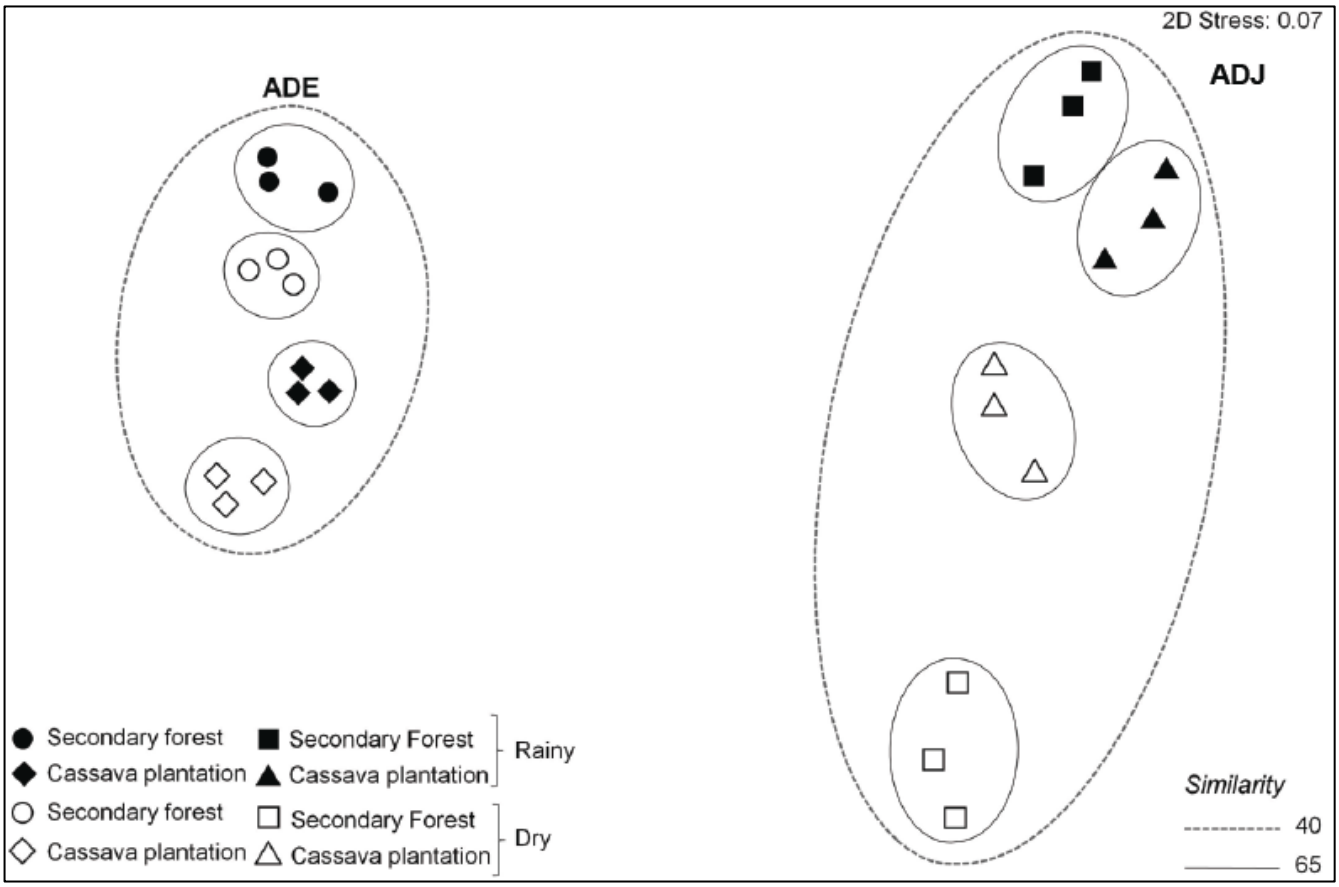

Figure 3. Multidimensional scaling (MDS) ordination based on Bray Curtis similarity analysis of T-RFLP data (square root transformed) of bacterial communities from Amazonian Dark Earth (ADE) and adjacent soil (ADJ) under secondary forest and manioc plantation during the rainy and dry seasons.

Table 2. Percentage of detected bacterial family greater than 1% for Amazonian Dark Earth and adjacent soil under secondary forest and manioc plantation.

Bacterial family	Amazonian Dark Earth (ADE)		Acrisol (ADJ)	
	Secondary forest (SF)	Manioc plantation (M)	Secondary forest (SF)	Manioc plantation (M)
Acidobacteria				
Acidobacteriaceae	0.1	0.1	5.0	6.1
Solibacteraceae	1.1	2.1	5.0	5.5
Actinobacteria				
Conexibacteraceae	-	0.2	1.1	1.4
Gaiellaceae	3.6	2.5	0.3	0.3
Micrococcaceae	0.1	-	0.3	2.6
Alphaproteobacteria				
Acetobacteraceae	0.5	0.4	0.7	1.0
Bradyrhizobiaceae	4.4	4.7	7.8	5.5
Caulobacteraceae	0.5	0.5	3.7	1.9
Hyphomicrobiaceae	14.9	10.9	15.7	8.5
Betaproteobacteria				
Burkholderiaceae	0.7	-	5.0	2.1
Oxalobacteraceae	-	0.2	-	3.7
Chloroflexi				

Table 2. Contd

Thermogemmatisporaceae	0.3	1.8	1.9	4.2
Deltaproteobacteria				
Syntrophobacteraceae	4.1	7.8	0.5	0.4
Firmicutes				
Alicyclobacillaceae	1.4	1.5	5.0	3.1
Bacillaceae	0.2	0.3	0.6	1.0
Gammaproteobacteria				
Sinobacteraceae	0.5	0.2	3.9	4.6
Xanthomonodaceae	0.4	0.4	3.2	1.5
Planctomycetes				
Gemmataceae	1.8	2.4	0.3	0.9

Table 3. Percentage of detected bacterial genera greater than 1% for Amazonian Dark Earth and adjacent soil under secondary forest and manioc plantation.

Bacterial genus	Amazonian Dark Earth (ADE)		Acrisol (ADJ)	
	Secondary forest (SF)	Manioc plantation (M)	Secondary forest (SF)	Manioc plantation (M)
Acidobacteria				
Candidatus solibacter	1.1	2.1	5.0	5.5
Edaphobacter	0.1	-	0.2	1.7
Firmicutes				
Alicyclobacillus	1.4	1.4	1.9	2.1
Bacillus	0.2	0.3	0.6	1.0
Alphaproteobacteria				
Bradyrhizobium	3.8	4.3	7.3	5.4
Pedomicrobium	2.1	1.4	0.1	0.2
Rhodoplanes	12.1	8.9	15.3	8.3
Betaproteobacteria				
Burkholderia	0.7	-	1.6	0.4
Salinispora	-	-	3.4	1.7
Gammaproteobacteria				
Luteibacter	-	-	2.8	0.4

MBC in ADE-M was not significantly different from ADJ-SF. This suggests that the presence of biochar in ADE soils may enhance MBC (Steiner et al., 2008; Liang et al., 2010). However, there was a clear decline in MBC due to the change in vegetation type for both ADE and ADJ during the rainy season and only for ADJ during the dry season. Such declines in MBC occurring according to the vegetation have been shown in tropical soils of the Central Amazon (Luizão et al., 1992). Seasonal variation in MBC was only observed in ADE-SF (Table 1) with higher values during the rainy season. Cleveland et al. (2004) have reported that high MBC in the rainy season may be controlled by precipitation, which transports the leached organic carbon accumulated in the dry season, thus increasing MBC. However, this effect could not be observed in ADJ-SF, indicating that MBC in ADE-SF acts as a sink during the rainy season; this may be due to high amounts of biochar in ADE combined to plant litter and debris accumulation during the dry season. MBN was strongly affected by land use for both soil types. Low MBN at manioc plantation sites is an indication of enhanced N supply to the plant, and mineral nitrogen is likely to be limited to the MBN. Seasonal variations in MBN were observed at the ADE-M and ADJ-SF sites. August showed very low monthly precipitation (~30 mm), which is less than the average of 58 mm for this month (http://www.bdclima.cnpm.embrapa.br/resultados/index.php). Furthermore, January 2009 reported one of the largest rainfall anomalies in Central Amazonia, between 25 and 50% above normal (Marengo, 2010). This could explain the decline in MBN during the rainy season due to elevated soil moisture (Tiemann and Billings, 2011). Interestingly, ADJ-SF showed no indication of N-mineralization during the rainy season. The presence

Table 4. Spearman rank correlation between selected bacterial family and soil properties.

Soil properties	Acido.	Alicy.	Brady.	Burkh.	Caulo.	Gaiella.	Gemma.	Hypho.	Sino.	Soli.	Syntro.	Thermo.
pH	-0.731*	-0.779**	-0.779**	-0.771**	-0.779**	0.7409*	0.755*		-0.779**	-0.779**	0.826**	-0779**
SOC (g kg^{-1})								0.643*				
SOM (g kg^{-1})								0.640*				
P (mg dm^{-3})	-0.835**			-0.747**		0.765**	0.826**		-0.898***	-0.706*	0.934***	-0.707*
Ca ($cmol_c$ dm^{-3})	-0.764**	-0.640*	-0.643*		-0.635*	0.934***	0.691*		-0.738**	-0.833**		
Mg ($cmol_c$ dm^{-3})	-0.763**	-0.643*	-0.642*		-0.633*	0.934***	0.690*		-0.730**	-0.830**		
Al (g kg^{-1})		0.913*	0.901**	0.919**	0.924**	-0.804**	-0.913***		0.710*	0.710*	-0.736**	0.710*
CEC (g kg^{-1})	-0.763**	-0.643*	-0.632*		-0.633*	0.934***			-0.735**	-0.833**	0.858**	
MBC (g kg^{-1})	-0.812**		-0.634*		-0.630*					-0.929***		-0.929***
MBN (g kg^{-1})	-0.760**					0.682*				-0.881**		-0.881**

[a] *P < 0.05, **P < 0.01, ***P < 0.001. [b] SOC, soil organic carbon; SOM, soil organic matter; CEC, cation exchange capacity; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; Acido., Acidobacteraceae; Alicy., Alicyclobacillaceae; Brady., Bradyrhizobiaceae; Burk., Burkholderiaceae; Caulo., Caulobacteraceae; Gaiella., Gaiellaceae; Gemma., Gemmataceae; Hypho., Hyphomicrobiaceae; Sino., Sinobacteraceae; Soli., Solibacteraceae; Syntro., Syntrophobacteriaceae; Thermo., Thermogemmatisporaceae.

of biochar in ADE is probably the main cause of N immobilized in the MBN because no significant changes in SOC and SOM were observed between ADE-SF and ADJ-SF. Steiner et al. (2008) have suggested that N immobilization in biochar amended soils is a desirable phenomenon in soils under heavy rainfall conditions. Furthermore, it is more likely that ADE-SF soils have higher availability of organic C compounds and higher rates of microbial activity, which might trigger N immobilization (Barret and Burke, 2000).

Community functioning as revealed by Biolog

The results of soil function (measured by Biolog substrates) indicate that seasonality has an influence on the metabolism of soil heterotrophic microorganisms (Figures 1 and 2). The patterns of bacterial carbon utilization show that vegetation type and seasonality affected more ADJ than ADE, (Figure 1).

High level of soil moisture observed during the rainy season might have affected the ACH microorganisms (Table 1). Dunn et al. (1985) also observed that physiologically active microorganisms were more sensitive to moist soil rather than dried ones. In addition, cycles of drying and rewetting has been shown affecting the respiration rates in soils, as being significant lower than observed in non-stressed soils (Fierer and Schimel, 2002). Interestingly, ADE bacterial carbon utilization was not influenced by either vegetation and time (Figures 1 and 2), which shows an important feature of ADE soils as usually belowground microbial activity, are very sensitive to soil moisture (Li et al., 2005; Feng et al., 2009). It is also remarkable to state that, despite it is known that substrate utilization is dependent on the initial cell density of the soil inoculums, which can therefore bias subsequent analysis of utilized substrate patterns (Garland, 1996), Biolog plates used here were read after no color development had occurred. Therefore observed differences reliably reflect the ability of a subset of the bacterial community to utilize the Biolog substrates.

Effects of vegetation type and seasonality on bacterial community structure

The bacterial community structure varied with seasonality, with differences observed between the rainy and dry seasons for both, soil and vegetation types, indicating community structure in these soils to be affected by both, moisture and temperature variations (Gordon et al., 2008; Bárcenas-Moreno et al., 2009). MDS demonstrated that seasonality and vegetation affected both soils (Figure 2). It seems that the bacterial communities in ADJ were more sensitive to seasonality, suggesting that ADE communities might be more resistant to such temporal stress. Here, resistant is defined as the ability to withstand a perturbation or stress (McNaughton, 1994). Cruz-Martínez et al. (2009) have indicated that soil microbial communities may be more robust to changes in climate than associated

aboveground macroorganisms. Furthermore, land use appeared to have a stronger effect on structuring the bacterial community in ADJ during the dry season. Perhaps the heavy rainfall in January 2009 (Marengo, 2010) imposed severe stress on the structure of the bacterial communities, diminishing the effect of vegetation. The ADE bacterial community structure appeared to be more affected by vegetation type than seasonality. In agreement with these results, studies in Amazonian tropical soils have shown changes in bacterial community structure according to land use (Jesus et al., 2009; Navarrete et al., 2010). Contrary to these results in other anthropic ADE, Grossman et al. (2010) were not able to detect changes in ADE under different vegetation, which may be explained by the sampling strategy of one single soil horizon or the age of the secondary forest studied.

In addition, BEST analysis in ADE showed correlation with soil P together with MBC and MBN (data not shown). Kuramae et al. (2011) found that P was the major predictor shaping microbial communities in a series of neutral pH fields (pH = 7.0-7.5). Furthermore, Habekost et al. (2008) detected distinct seasonal changes in the microbial community structure; these changes were thought to be driven by the availability and quality of organic resources, which are likely to influence microbial biomass. Interestingly, BEST analysis for ADJ also included MBN as one of the properties shaping the structure of these communities, together with Al, which is known to shape bacterial communities in Amazonian soils (Jesus et al., 2009; Navarrete et al., 2010). Such findings are of great importance for soil management practices, as microbial biomass may act as a sink or source of available N to plants (Friedel et al., 2001).

Effects of vegetation cover and soil type on bacterial community composition

As reported in a previous paper (Taketani et al., 2013), soil type have a stronger selective effect on the class composition of bacterial community, which outpaces the effects imposed by the vegetation. In the present study, the analysis at lower taxonomic level (the family or genus) also demonstrated a stronger effect due to soil type. The most abundant sequences at the family level in ADE soil originated from Gaiellaceae, Gemmataceae and Syntrophobacteraceae. For example, Gaillaceae is a novel family within the class Actinobacteria and what is known is that members of this family are strictly aerobic and chemoorganotrophic (Albuquerque et al., 2011). The chemoorganotrophic bacteria are capable of growing on accumulated organic matter from dead cells and trapped debris which could explain their high abundance in ADE soils, especially under SF. Furthermore, ADJ soils showed higher abundance in nine different groups of family comprising the phyla Acidobacteria, Actinobacteria, Firmicutes and Proteobacteria. Of these, Acidobacteriaceae and Acetobacteriaceae are typical bacteria of acidic environments, in accordance with the low pH of most Amazonian soils and with the highest acidobacterial abundances found in environments with the lowest pH (Fierer et al., 2007; Lauber et al., 2008).

In addition, we also accessed the influence of vegetation cover on the bacterial community composition independently of the soil type. It is well known that microbial communities are not only influenced by soil properties but that plant species also shape the structure and composition of these communities (Berg and Smalla, 2009; Buée et al., 2009; Ladygina and Hedlund, 2010). Interestingly, it was possible to observe the imposed effect of vegetation type on bacterial groups independent of the contrasting soil characteristics of ADE and ADJ.

The bacterial composition of some families and genera smaller than 1% were exclusively detected in ADE soils (Tables S1 and S2). Interestingly, some of these bacterial members are known to play an important role in the carbon and nitrogen cycles. Beijerinckiaceae is a family known to harbor methanotrophs (Dedysh et al., 2000) and seemed to prefer their growth on media of pH 5 (Folman et al., 2008), which is within the pH range of ADE soils. Nitrospiraceae (nitrifying bacteria) was also only observed in ADE and it may indicate that anthropogenic biochar stimulated the presence of bacterial members from this family (Chen et al., 2013). Another particular family detected in ADE was Rhodobiaceae (photoheterotrophic *α-Proteobacteria*) that require carbon under anoxic conditions in light. ADE contains high amounts of anthropogenic biochar and is full of pieces (sherds) of unfired pottery that could increase water-holding capacity and create anoxic microenvironments suitable for bacteria able to grow under these conditions.

Various studies have shown that soil properties influenced microbial communities (Lauber et al., 2008; Singh et al., 2009; Kuramae et al., 2012). In this study, we found that the relative abundance of bacterial families was strongly affected by the differences between the soil properties of ADE and ADJ. One of the main drivers of change in the abundance of the selected bacterial families was soil pH, which is well known to affect soil bacterial communities (Lauber et al., 2009; Singh et al., 2009; Nacke et al., 2011). The strong correlation between Gaiellaceae, Gemmataceae and Syntrophobacteriaceae with soil P also appeared to favor an increase in the abundance of these bacterial groups. This strong correlation with soil P has been previously observed in an old growth forest (DeForest and Scott, 2010), as well as in soils under different land use types (Kuramae et al., 2012).

Conclusion

Concluding, we demonstrated that vegetation cover and seasonality influence the bacterial communities of ADE

and their adjacent soil (Haplic Acrisol, ADJ). The microbial community structure differed in both soils and a higher number of T-RFs were observed in ADE. Average Well Color Development (from Biolog substrates) was higher in ADE than ADJ during the rainy season and kept the same pattern of substrate utilization during the dry season. Considering these results, ADE functional microbial activity was less affected by seasonality. The presence of biochar in ADE likely suggests a buffer effect protecting the system against environmental changes. However, this assumption needs to be further tested with other methods and higher number of samples. Bacterial community composition at deepest taxonomic resolution showed that some groups were in higher abundance or only present in ADE. Taken all together, these results show that ADE maintains important bacterial groups and active bacterial communities. These findings provide insights into microbial community composition, structure and functionality in ADE and their ADJ locations, highlighted by the assessment of how temporal changes in the local environmental conditions and land use types underpin changes in community dynamics.

Conflict of interests

The authors did not declare any conflict of interest.

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Table S1. Percentage of selected bacterial family smaller than 1% for Amazonian Dark Earth and adjacent soil under secondary forest and manioc plantation.

Bacterial family	Amazonian Dark Earth (ADE)		Acrisol (ADJ)	
	Secondary Forest (SF)	Manioc plantation (M)	Secondary Forest (SF)	Manioc plantation (M)
Actinobacteria				
Actinospicaceae	-	-	0.4	0.3
Micromonosporaceae	0.1	0.1	-	-
Nocardioidaceae	0.9	0.3	-	0.2
Patulibacteraceae	0.2	0.1	-	-
Solirubrobacteraceae	0.1	0.1	-	-
Streptomycetaceae	0.3	0.2	0.3	0.1
Thermomonosporaceae	-	0.2	-	0.1
Alphaproteobacteria				
Beijerinckiaceae	0.5	0.2	-	-
Methylocystaceae	0.2	0.1	0.7	0.3
Phyllobacteriaceae	0.1	0.1	-	-
Rhodobiaceae	0.9	0.3	-	-
Xanthobacteraceae	0.2	0.2	-	-
Armatimonadetes				
Chthonomonadaceae	-	0.2	0.1	0.3
Bacteroidetes				
Chitinophagaceae	0.5	0.4	0.5	0.2
Flavobacteriaceae	-	0.1	-	0.1
Firmicutes				
Clostridiaceae	0.4	0.3	0.4	0.2
Paenibacillaceae	0.8	0.2	0.8	0.5
Ruminococcaceae	-	-	0.1	0.1
Sporolactobacillaceae	-	-	0.1	0.1
Thermoactinomycetaceae	0.1	0.1	-	-
Turicibacteraceae	-	-	0.1	0.3
Nitrospirae				
Nitrospiraceae	0.1	0.2	-	-
Planctomycetes				
Isosphaeraceae	0.1	0.1	0.7	0.3
Pirellulaceae	0.5	0.7	0.1	-

Table S2. Percentage of selected bacterial genera smaller than 1% for Amazonian Dark Earth and adjacent soil under secondary forest and manioc plantation.

Bacterial genus	Amazonian Dark Earth (ADE)		Acrisol (ADJ)	
	Secondary Forest (SF)	Manioc plantation (M)	Secondary Forest (SF)	Manioc plantation (M)
Alphaprotebacteria				
Balneimonas	0.3	0.2	-	-
Devosia	0.1	-	0.1	-
Hyphomicrobium	0.2	0.2	0.1	-
Labrys	0.2	0.1	-	-
Rhizobium	0.3	-	-	-
Sphingomonas	0.1	-	-	-
Phenylobacterium	0.1	0.4	0.7	0.3
Acidobacteria				
Acidobacterium	-	-	0.1	-

Table S2. Contd.

Actinobacteria				
Microbacterium	0.1	-	-	-
Nocardioides	0.6	0.1	-	-
Sinomonas	-	-	0.3	0.1
Streptomyces	0.1	0.1	-	-
Terracoccus	-	-	-	0.2
Armatimonadetes				
Chthonomonas	-	0.2	0.1	0.3
Deltaproteobacteria				
Syntrophobacter	-	0.1	-	-
Firmicutes				
Brevibacillus	0.1	-	-	-
Lactobacillus	-	-	0.1	-
Paenibacillus	0.6	0.1	0.7	0.3
Pullulanibacillus	-	-	0.1	0.1
Thermosinus	-	-	0.1	-
Gammaproteobacteria				
Acinetobacter	0.2	-	-	-
Aquicella	0.7	0.2	0.6	0.2
Cupriavidus	-	0.1	-	-
Erwinia	0.3	-	-	-
Lysobacter	0.2	-	-	-
Rhodanobacter	-	-	-	0.6
Stenotrophomonas	0.1	-	-	-
Thermomonas	0.1	-	-	-
Nitrospirae				
Nitrospira	0.1	-	-	-
Planctomycetes				
Gemmata	0.1	0.2	-	-
Verrucomicrobia				
Opitutus	0.1	-	-	-
Pedosphaera	-	-	-	0.1