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Successional and seasonal variations in soil and litter microbial community structure and function during tropical postagricultural forest regeneration: a multiyear study

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Abstract

Soil microorganisms regulate fundamental biochemical processes in plant litter decomposition and soil organic matter (SOM) transformations. Understanding how microbial communities respond to changes in vegetation is critical for improving predictions of how land-cover change affects belowground carbon storage and nutrient availability. We measured intra- and interannual variability in soil and forest litter microbial community composition and activity via phospholipid fatty acid analysis (PLFA) and extracellular enzyme activity across a well-replicated, long-term chronosequence of secondary forests growing on abandoned pastures in the wet subtropical forest life zone of Puerto Rico. Microbial community PLFA structure differed between young secondary forests and older secondary and primary forests, following successional shifts in tree species composition. These successional patterns held across seasons, but the microbial groups driving these patterns differed over time. Microbial community composition from the forest litter differed greatly from those in the soil, but did not show the same successional trends. Extracellular enzyme activity did not differ with forest succession, but varied by season with greater rates of potential activity in the dry seasons. We found few robust significant relationships among microbial community parameters and soil pH, moisture, carbon, and nitrogen concentrations. Observed inter- and intrannual variability in microbial community structure and activity reveal the importance of a multiple, temporal sampling strategy when investigating microbial community dynamics with land-use change. Successional control over microbial composition with forest recovery suggests strong links between above and belowground communities.

Keywords: extracellular enzymes, forest succession, land-use change, litter, microbial communities, PLFA-FAME, soil, tropics

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Introduction

Land-use change is an important source of carbon (C) emissions and is the primary cause of biodiversity loss in tropical and subtropical regions (Grace *et al*., 2014; Malhi *et al*., 2014). While deforestation is the most studied land-use transition in the tropics, forest regeneration or reforestation now characterizes many sites on formerly cultivated or cleared lands (Grau *et al*., 2004; Wright, 2005; Meiyappan & Jain, 2012). In the Caribbean, reforestation explained 70% of forest cover change from 2001 to 2010, with an estimated 5771 km^2 net increase (Aide *et al*., 2013). On the island of Puerto Rico, forest cover increased from 13% in the 1940s to ~ 53% in 2009 due to widespread agricultural abandonment following changes in regional social and economic policies (Brandeis & Turner, 2013). Postagricultural forests can sequester C in aboveground biomass and in soil organic matter (SOM) (Johnson *et al*., 2000; Silver *et al*., 2000; Brandeis & Turner, 2013), as well as provide habitat for biodiversity (Chazdon *et al*., 2009). The geographic expansion of secondary forests across the tropics provides the opportunity to examine how land-use change affects soil biogeochemistry and forest recovery, important research priorities for understanding human effects on the global C cycle (US DOE, 2012).

Shifts in plant species composition during postagricultural succession in the tropics, and their consequence on ecosystem processes, are well-documented (Guariguata & Ostertag, 2001; Lugo & Helmer, 2004; Norden *et al*., 2009). Fewer studies have followed changes in microbial community composition during reforestation, especially in tropical ecosystems, despite the important role of microorganisms in biogeochemical cycling (but see Hedlund, 2002; Macdonald *et al*., 2009; Zhang *et al*., 2005). Soil microbes can respond to shifts in leaf litter and root inputs with changes in plant

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communities (Bardgett *et al.*, 2005; Kardol *et al.*, 2006). Changes in microbial community structure, physiology and function are hypothesized to alter ecosystem processes, such as plant litter decomposition, SOM transformations, and nutrient availability (Wardle *et al.*, 2002; Schimel *et al.*, 2007; McGuire & Treseder, 2010). As these processes can influence forest growth, understanding the response of soil microorganisms to land-use change is important for predicting successional trajectories in ecosystem C. Our overall understanding of how shifts in microbial community structure affect important biogeochemical processes and ecosystem function is poor, especially in highly-weathered tropical soils (Leff *et al.*, 2012; Whitaker *et al.*, 2014). Predictions of microbial community dynamics in species-rich tropical ecosystems can be further complicated by high resource and habitat heterogeneity (Townsend *et al.*, 2008).

In addition to spatial variability in soil resources and microenvironmental conditions, temporal heterogeneity may confound detection of effects of land cover, management or experimental treatments on microbial communities. For example, in a multifactorial global change experiment, increased nitrogen (N) addition altered the abundance of gram-negative (Gm-) and gram-positive (Gm+) bacteria in some years, but not others (Gutknecht *et al.*, 2012). Similarly, responses to elevated CO_2 and warming varied from year to year. Yearly shifts in microbial structure altered the significance of interactive effects of elevated CO_2, warming and/or increased N. Seasonal variations in temperature, moisture, and litter inputs or root exudates within a year and from year to year may also influence microbial community biomass and composition (Wardle, 1998; Bouskill *et al.*, 2012). An increased understanding of microbial dynamics at seasonal and yearly time scales can improve model predictions of biogeochemical process responses to increased climate variability and changing resource availability (Allison *et al.*, 2010; Treseder *et al.*, 2011; Wieder *et al.*, 2013).

Here we used a well-replicated, long-term successional chronosequence to evaluate the effects of post-agricultural forest regeneration on microbial community composition and enzyme activity in the subtropical wet forest life zone of Puerto Rico. Replicated successional chronosequences provide the opportunity to investigate temporal changes in community composition and ecosystem processes, thus serving as a model system for investigating interactions between plant and soil community dynamics. This study asked: (1) How does microbial community composition respond to changes in land cover? (2) What are the effects of seasonal variability in precipitation and successional changes in plant species on microbial communities?, and (3) What is the effect of potential changes in community composition on microbial activity?

We hypothesized that forest regeneration on former pastures would result in changes in microbial community composition, especially early during succession and that this would be driven by observed changes in plant inputs and shifts in SOM pools (Marín-Spiotta *et al.*, 2008; Ostertag *et al.*, 2008). We predicted that land cover effects on microbial communities would persist despite seasonal and yearly differences in precipitation. We expected that changes in seasonal rainfall and community composition would result in changes in microbial function as measured by the activity of extracellular enzymes, and that enzyme activity would be greatest in the wet season. Most studies comparing microbial communities under different land covers report data collected at one fixed time point. Our study incorporated multiple sampling time periods to assess temporal variability and evaluate whether potential differences in composition and activity were related to decadal-scale changes in vegetation during succession rather than more short-term fluctuations in resource availability.

Materials and methods

Field site description

This study takes advantage of previously established chronosequence sites (Marín-Spiotta *et al.*, 2007) in the subtropical wet forest life zone of Puerto Rico. The chronosequence consists of replicated actively grazed pastures, secondary forests growing on pastures abandoned ~ 20, 30, 40, 70, and 90-years before the time of this study, and reference primary forest sites that have not been under pasture or agricultural use. All sites are located on private land, 580–700 m above sea level and within ~ 5 km of each other in the Sierra de Cayey in southeastern Puerto Rico (18°01′ N, 66°05′ W). Mean air temperature between 1971–2000 was recorded as 21.5 °C (Southeast Regional Climate Center http://sercc.com/climateinfo/historical/historical_pr.html) with little seasonal variation (Daly *et al.*, 2003). Mean annual precipitation was ~ 2184 mm, with monthly mean precipitation varying from ~ 310 mm in the wet season (May–October) to 54 mm during the dry season (November–April) (Fig. 1) during the years of sampling (2010–2012) (Jajome Alto climate station, data from the Caribbean Atmospheric Research Center, http://atmos.uprm.edu/). Soils at all sites are characterized as very-fine, kaolinitic, isothermic Humic Hapludox in the *Los Guineos* soil series (Soil Survey Staff, 2006). The pasture sites are dominated by *Axonopus compressus, Panicum laxum,* and *Sporobolus jacquemontii*. Early successional secondary forests, late successional forests, and primary forests have

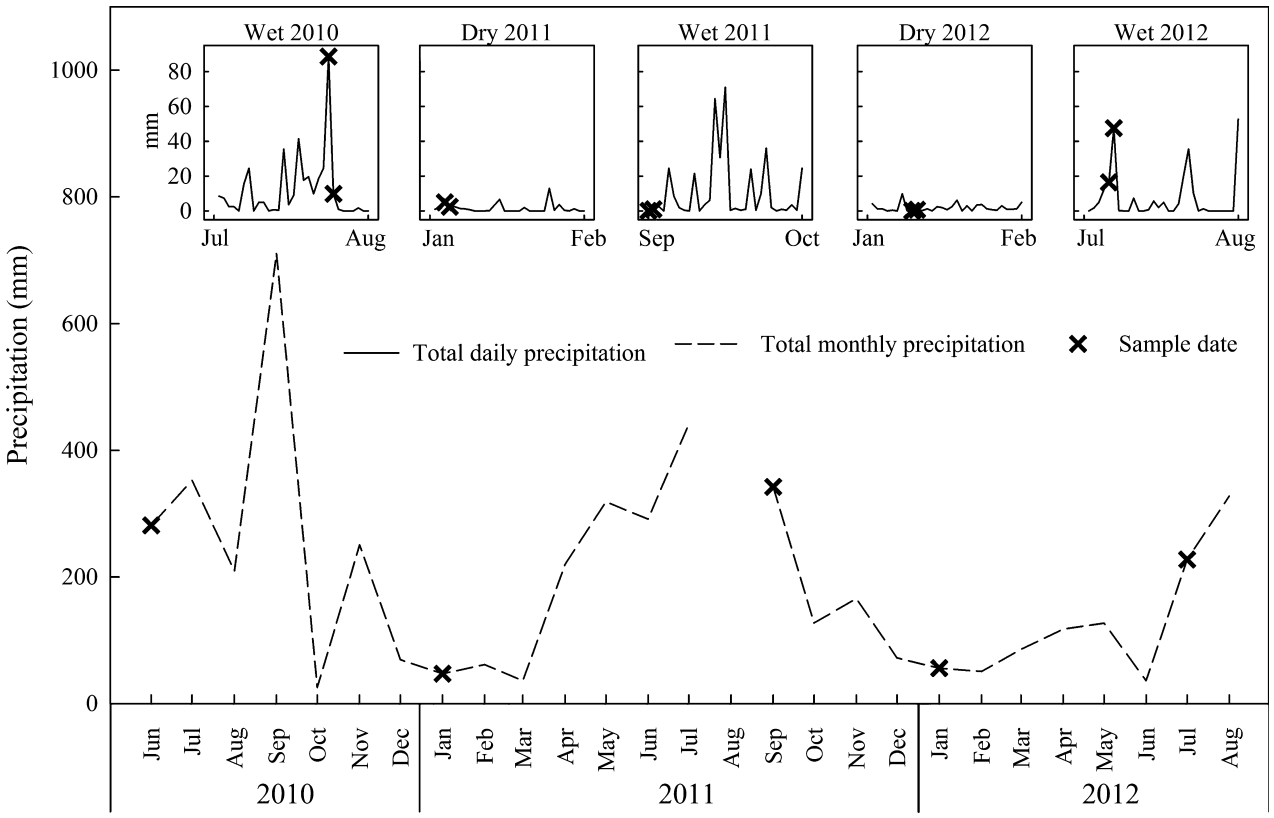

Fig. 1 Total (a) daily and (b) monthly precipitation for the Jajome Alto climate station in southeastern Puerto Rico, located ~ 8 km from the tropical forest successional chronosequence field sites for years 2010–2012. Data acquired from the Caribbean Atmospheric Research Center (http://atmos.uprm.edu/).

distinct tree communities (described in Marín-Spiotta *et al*., 2007; Souther, 2014). Young secondary forests are dominated by *Tabebuia heterophylla* and *Syzygium jambos* stems, whereas older forests show large abundance of *Dacryodes excelsa*, *Sloanea berteriana*, and the Sierra palm *Prestoea acuminata* var. *montana*.

Sampling and experimental design

For each of the land cover types, we sampled three replicate sites, with the exception of the 40 year-old secondary forests, where one of the sites was deforested since establishment of the chronosequence; for a total of 17 forested sites and three pastures. Litter layer and soil samples were collected in the forest sites. The pasture sites were sampled for mineral soil only due to the absence of a litter layer. Sampling occurred biannually, during both the wet and dry season, from July 2010 to July 2012 for a total of five sampling time points to account for any potential effects of season and interannual variability on microbial community profiles.

At each of the forest sites, standing litter was collected from three 20 cm^2 quadrats located at random directions and distances from a center pole, which had been previously established and marked (Ostertag *et al*., 2008). Forest litter was comprised of various components of leaf litter, including freshly fallen leaves, fine twigs, and coarse organic materials. Mineral soil was collected using a 1.7 cm diameter soil core to a 20 cm depth. In the forests, this soil was collected immediately underneath the sampled litter layer. At each of the three quadrats, several soil cores (five to eight) were collected and composited to incorporate spatial heterogeneity at the field scale, for a total of three replicate soil samples per site. Following collection, litter and soil were stored in coolers and shipped to the University of Wisconsin-Madison. Soils were subsampled and processed separately for enzyme analysis, microbial phospholipid fatty acid analysis (PLFA) and other physical and chemical analyses. Subsamples of litter and soil for PLFA analysis were frozen at −20 °C within 24–36 h of shipping and then freeze-dried for later analysis. Subsamples intended for enzyme analysis were stored at 4 °C and processed within 1–5 days upon arrival in the lab.

Soil and litter chemical analyses

Field moisture content, pH, total C and N concentrations (%) were determined for both mineral soils and the litter layer. We report soil moisture and pH for all collection dates.

Given the lack of significant differences in soil and litter C and N concentrations between years, we report data only from the dry (January) and wet (August) season of 2011. Moisture content was determined gravimetrically on 10 g of soil and 5 g of litter oven dried at 105 °C or 60 °C, respectively, for 48 h or until no change in mass was observed. Water (%) by mass was calculated as [(wet mass − dry mass)/dry mass] × 100 (Klute, 1986). Soil and litter pH was measured on air dried and ground samples using a Sartorius PP-20 professional pH reader in a 1 : 1 (by volume) 1 M KCl slurry (Sparks *et al*., 1996).

Total C and N concentrations were determined on air-dried, ground soil and oven-dried (60 °C) litter using a Flash 2000 NC Analyzer (Thermo Scientific, Wilmington, Delaware) at University of Wisconsin-Madison. Soil samples were ground to fine powder using a SPEX Sample Prep Mixer/Mill (Metuchen, NJ, USA) and litter samples were ground through a size 60 mesh screen on a Thomas Wiley Mini-Mill (Swedesboro, NJ, USA). All samples for elemental analysis were run in duplicate with replicate error < 10% using aspartic acid as calibration and internal standards. Total % C represents organic carbon concentration due to the absence of inorganic C. Litter and soil C-to-N ratios were calculated as molar ratios (Cleveland & Liptzin, 2007).

Microbial community composition

Microbial community composition was measured using a hybrid phospholipid fatty acid (PLFA) and fatty acid methyl ester (FAME) analysis protocol, simplified as PLFA (Vestal & White, 1989; Smithwick *et al*., 2005). Whereas PLFA does not provide taxonomic information at a species level, it does provide gross functional group information, which has been shown to be sensitive to ecologically-relevant treatments such as changes in plant cover, land management or nutrient availability and reveal both short- and long-term compositional shifts due to environmental changes (Ruess & Chamberlain, 2010; Frostegård *et al*., 2011; Wixon & Balser, 2013). Briefly, PLFAs were extracted from freeze-dried and homogenized soil (3 g) or litter (0.25 g) using a specific ratio (1 : 2 : 0.9) of chloroform, methanol, and a phosphorus-buffer. After isolating and concentrating the extracted PLFAs, they were then saponified, methylated, transferred to an organic phase and then washed with a basic NaOH solution. Stock standards (9 : 0, 19 : 0) of known concentrations (7.08 μg ml^{-1} for 19 : 0 and 9 μg ml^{-1} for 9 : 0) were then added to each sample. Samples were run on a Hewlett-Packard 6890 Gas Chromatograph equipped with a flame ionization detector and an Ultra 2 capillary column (Agilent Technologies Inc., Santa Clara, CA, USA). Peaks were identified using bacterial fatty acid standards and peak identification software (MIDI Inc, Newark, DE, USA). Peak areas were converted to μmol PLFA g $soil^{-1}$ (absolute abundance) using internal standard peaks (9 : 0, 19 : 0). Microbial PLFA biomass was calculated as the sum of all peaks (as μmol PLFA g^{-1} soil) identified < 20.5 C atoms long (Vestal & White, 1989; Zelles, 1999) and is strongly correlated with other measurements of microbial biomass (Frostegård & Bååth, 1996).

Individual phospholipid fatty acids used as indicator PLFAs, or groups of lipid biomarkers used in calculations of PLFA biomass, guilds and ratios are detailed in Table 1. Specific PLFA biomarkers representing indicator species of broad taxonomic groups such as fungi and gram-positive (Gm+), gram-negative (Gm−), methanotrophic, and anaerobic bacteria, were used to describe microbial community composition in soils (Gutknecht *et al*., 2012; Smith *et al*., 2014). In plant litter samples, multiple PLFAs, or guilds, were used to characterize microbial composition. For example, Gm+ bacteria were represented by all iso and anteiso lipids with estimated chain lengths between 14 and 17-C long for litter samples, whereas only 15 : 0 iso was used to represent Gm+ bacteria in soil samples (Balser & Firestone, 2005; Kaur *et al*., 2005). The presence of plant waxes can make the use of individual indicator PLFAs inaccurate (Zelles, 1997; Ruess & Chamberlain, 2010; Frostegård *et al*., 2011).

Microbial community functional activity

Microbial community functional activity was measured as potential extracellular enzyme activity on soils and litter samples collected from January 2011 through July 2012. Key enzymes involved in nutrient cycling processes, such as *β*-glucosidase, α-glucosidase, cellobiohydrolase, and xylosidase (involved in decomposition of cellulose and hemi-cellulose compounds), N-acetylglucosaminidase (catalyzes the decomposition of chitin and N polymers stored in SOM) and acid phosphatase (used for microbial phosphorous acquisition) were measured using a modified fluorescent-linked substrate (4-methylumbelliferone, MUB) microplate protocol (Tate, 1995; Sinsabaugh *et al*., 1999; German *et al*., 2011) optimized for *in situ* temperature and pH conditions (German *et al*., 2011). The homogenate was prepared using 1–2 g fresh soil or 0.5 g fresh litter in 100 ml sodium acetate buffer (pH 5). Enzyme substrates (200 μM) were dispensed in 50 μl aliquots into 200 μl soil homogenate and incubated for one hour (*β*-glucosidase, N-acetylglucosaminidase, phosphatase) or 3 h (α-glucosidase, cellobiohydrolase, xylosidase) at room temperature (which is similar to *in situ* soil temperatures). Following incubation, 10 μl of 1M NaOH was added to stop the reaction and then the plates were read (~ 4 min following the NaOH addition) on a Beckman-Coulter DTX880 fluorescent microplate reader (Beckman-Coulter, Fullerton, CA, USA). Potential enzyme activities were measured as μmol enzyme g^{-1} soil h^{-1} using the following equation (modified from German *et al*., 2011):

$$\text{Total Activity } (\mu\text{mol g}^{-1}\text{ soil h}^{-1}) = (\text{net F}/\varepsilon) \times (\text{h}^{-1}\text{ incubation}) \times (\text{assay volume} \times \mu\text{l}^{-1}\text{ homogenate volume}) \times (\text{total buffer volume} \times \text{g}^{-1}\text{ dry sample weight})$$

where: net Fluorescence (F) = (average substrate value - homogenate control) - (substrate control- plate blank) and ε = (slope of MUB in presence of homogenate/assay volume). For the potential enzyme activities reported for soils, values were also normalized to soil C concentrations (μmol g^{-1} C h^{-1}).

Table 1 Microbial PLFA biomarkers and metrics used

Community Category	Community Metric	PLFA Biomarker	References*
PLFA Biomass		Sum named and unnamed PLFAs	(Tunlid *et al*., 1985, Zelles, 1999)
Total Fungal PLFAs		16:1 ω5c 18:1 ω9c, 18:2 ω6,9c	
Total Bacterial PLFAs		15:0iso, 15:0anteiso, 16:1ω7c, 17:0anteiso, 17:0iso, 17:0cyclo, 18:1ω5c, 18:1ω7c, 19:0cyclo	(Zelles, 1997, Zelles, 1999)
F:B ratio		18:1 ω9c, 18:2 ω6,9c/total bacterial PLFAs	(Bardgett *et al*., 1996, Kaur *et al*., 2005)
Indicator PLFAs†	Gram-positive bacteria	15:0 iso	(Kaur *et al*., 2005, Zelles, 1997, Zelles, 1999)
	Actinobacteria	16:10 methyl	(Ratledge & Wilkinson, 1988)
	Gram-negative bacteria	16:1 ω7c	(Zelles 1999, Ratledge & Wilkinson 1988)
	Arbuscular mycorrhizal fungi	16:1 ω5c	(Olsson, 1999, Olsson *et al*., 1995)
	Saprotrophic fungi or ectomycorrhizal fungi§	18:1 ω9c	(Bardgett *et al*., 1996, Frostegård *et al*., 2011)
	Methanotrophic bacteria	18:1 ω7c	(Sundh *et al*., 2000)
	Saprotrophic fungi	18:2 ω6,9c	(Frostegård & Bååth, 1996, Joergensen & Wichern, 2008, Kaiser *et al*., 2010)
	Anaerobic, gram-negative bacteria	19:0 cyclo	(Vestal & White, 1989)
Community Guilds‡	Gram-positive bacteria	14:0iso, 15:0anteiso, 15:0iso, 16:0iso, 16:0anteiso, 17:0iso, 17:0anteiso	(Balser & Firestone, 2005, Mentzer *et al*., 2006, Waldrop & Firestone, 2006, Williamson & Wardle, 2007)
	Actinobacteria	16:0 10methyl, 17:0 10methyl, 18:0 10methyl, 19:0 10methyl	
	Gram-negative bacteria	16:1ω7c, 16:1ω9c, 17:1ω8c, 18:1ω5c	
	Total bacteria	16:0 10methyl, 17:0 10methyl, 18:0 10methyl, 19:0 10methyl, 14:0iso, 15:0anteiso, 15:0iso, 16:0iso, 16:0anteiso, 17:0iso, 17:0anteiso, 16:1ω7c, 16:1ω9c, 17:1ω8c, 18:1ω5c	
	Total fungi	18:1ω9c, 18:2ω6,9c	
Community Structure	Principal Component Analysis of PLFAs	Soil: Indicator Lipids	(Chaer *et al*., 2009, Frostegård *et al*., 2011, Mentzer *et al*., 2006, Ushio *et al*., 2008)
		Forest Litter: all named and unnamed PLFAs	
		Combined: all named and unnamed PLFAs	

*Selective list of all publications supporting use of specific PLFA metrics and/or biomarkers.
†Used solely for soil samples and not for forest litter samples.
‡Used for community composition and structure of forest litter layer analysis.
§Biomarker has been described as identifying both ectomycorrhizal and saprotrophic fungi (Bardgett et al., 1996, Frostegård et al., 2011). We were unable to detect if 18:1ω9c was ectomycorrhizal or saprotrophic in our analyses.

Statistical analysis

Statistical analyses were performed using JMP Pro Version 11 (SAS Inst. Inc., Cary, NC, USA). A mixed model nested analysis of variance (ANOVA), random effects standard least square model was used to test for effects of season and land cover type on soil (C and N concentrations, C:N ratios, pH and moisture) and microbiological (extracellular enzyme activities, PLFA biomass, indicator lipids or guilds, fungal-to-bacterial ratio, etc.) variables, with site replicate and collection year as random factors. Restricted maximum likelihood (REML) models, a maximum likelihood based-estimation for linear mixed models, were run on site means and weighted by within site replicates to account

for uneven replication (Patterson & Thompson, 1971). Site means of soil C concentration were also used in the calculation of extracellular enzyme activities. Untransformed relative abundance values (%) of indicator PLFAs or PLFA guilds were used in the mixed-models. Enzyme activities and other physical or chemical data were log transformed to satisfy assumptions of normality. Propagated standard error (SE) was calculated to account for within and among site variability for reported means in the text and in tables. Relationships are reported as significant at $P < 0.05$, unless otherwise indicated.

Principal Component Analysis (PCA) was performed on the arcsine-square root transformed, relative abundance of PLFA biomarkers (Table 1) (Ramette, 2007). Standard error calculations for PCA figures were pooled for fixed treatment effects. Only indicator PLFAs (see Table 1) were used in PCAs of soil communities, whereas all PLFA biomarkers (< 20.5 C chain length) were analyzed for litter communities. Ordination analyses for soil samples using all lipids, rather than select indicator lipids, yielded similar results with significant differences in microbial structure with season and land cover type. Therefore, we chose to focus on the biomarkers, or indicator lipids, that explained most of these differences (Smith *et al.*, 2014). Of the identified microbial lipids used in the ordination analysis of litter communities, lipids with a known C chain length but an unknown molecular structure were labeled 'unnamed' and followed by the number of C atoms contained in the estimated chain length.

Results

Soil microbial community structure

Overall soil microbial community PLFA structure represented by select indicator lipids varied by season and land cover (Fig. 2a,b). Values along both axes of the principal components analysis (PC1 and PC2) differed by season (wet and dry) for each year when analyzing all years and seasons combined ($P < 0.0001$; data not shown). Therefore, data for the wet and dry season were analyzed separately to determine differences in indicator PLFAs explaining the majority of the variation within each season (Fig. 2a,b). For samples collected in the wet season, the PLFA biomarker for anaerobic Gm- bacteria (19 : 0 cyclo) explained 90% of the variation along PC1 (Table 2a, Fig. 2a). For the dry season samples, the PLFA biomarker indicating Gm+ bacteria (15:0 iso) described 92% of variation along PC1 (Table 2a, Fig. 2b). The PLFA biomarkers for fungi (18:1ω9c) described the majority of variation along the second principal component for both the wet and dry season (80% and 78%, respectively).

Land cover type had a significant effect on microbial PLFA community composition (Fig. 2a,b). The soil microbial communities from pastures and early secondary forests, aged 20, 30 and 40-years old, differed from those in the older forests (70 and 90-year old secondary and primary forests) along both axes ($P < 0.01$). The clustering of sites into successional stages was consistent when analyzing all community PLFA data together (data collected biannually from July 2010 until July 2012) or individually by season and collection date.

Individual PLFA indicator lipids differed in their response to land cover and season (Table 3a, Fig. 3). Total PLFA and bacterial PLFA biomass (nmols PLFAs g^{-1} soil) and the relative abundance of indicator lipids for Gm-, anaerobic Gm- bacteria and methanotrophic bacteria in soil showed seasonal, but no land cover

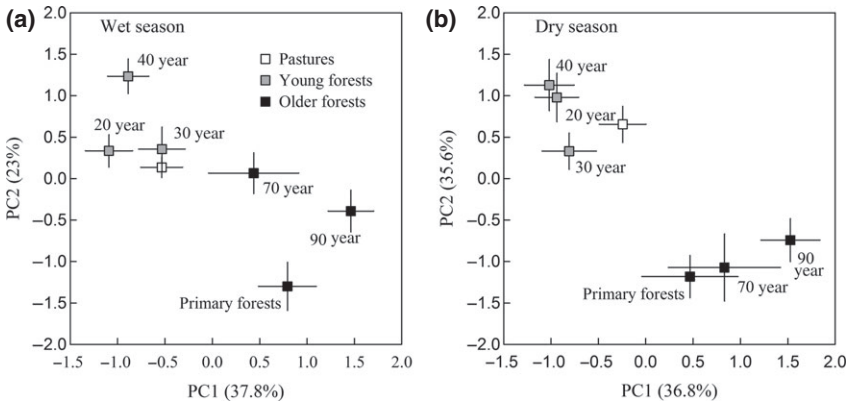

Fig. 2 Principal component analysis of soil microbial community structure using indicator PLFAs. (a) Points represent mean community structure for the wet seasons (across 3 years) averaged by site means, with three replicate sites per land cover type, except in the 40 year secondary forests where there are only two sites; (b) Points represent mean community structure for the dry seasons (across 2 years) averaged by site means, with three sites per land cover type, except in the 40-year secondary forests where there are only two sites. Error bars represent one standard error from the mean.

Table 2 Correlation coefficients, or loading factors, of microbial PLFA biomarkers with the first and second axis (PC1 and PC2, respectively) of principal components analyses of (a) soil and (b) forest litter samples corresponding to Figs 2 and 5 respectively

	Wet Season		Dry Season	
Principal Component	PLFA	Correlation coefficient (%)	PLFA	Correlation coefficient (%)
(a) Soil				
PC1	19:0 cyclo	90	15:0 iso	92
	15:0 iso	89	19:0 cyclo	88
	16:10 methyl	85	16:10 methyl	82
PC2	18:1ω9c	80	18:1ω9c	78
	16:1ω5c	71	18:2ω6,9c	70
(b) Forest litter layer				
PC1	Unnamed ECL 13.5–14.49	85	Unnamed ECL 11.5–12.49	80
	12:0 3OH	77	Unnamed ECL 12.5–13.49	76
	Unnamed ECL 11.5–12.49	74	16:0	75
PC2	16:0 iso	72	16:0 iso	75
	15:0 anteiso	63	19:0 cyclo	71

ECL = estimated chain length.

effects (Table 3a). Seasonal differences were not consistent between wet and dry seasons from year to year (Table S2). Many indicator PLFAs were least abundant during the dry season in 2012, which was in general a hot and dry year. The relative abundance of biomarkers for fungi (18:1ω9c and 18:2ω6,9c) and anaerobic, Gm- bacteria (19:0 cyclo) showed significant effects of season and land cover (Table 3a). The greatest differences in the relative abundance of indicator PLFAs occurred between the early secondary forests and the older forests (both late secondary and primary forests) (Fig. 3, Table S2). The arbuscular mycorrhizal fungi biomarker (16:1ω5c) and the saprotrophic or ectomycorrhizal biomarker (18:1ω9c) were more abundant in the early secondary forest soils compared to the older forest soils, whereas Gm+ bacteria (15:0 iso) and the indicator PLFA for anaerobic Gm- bacteria (19:0 cyclo) were less abundant in the early secondary forests compared to the late secondary and primary forests (Fig. 3).

There was a significant interaction between season and land cover for total fungal PLFAs, the fungal-to-bacterial ratio, Gm+ bacteria (15:0 iso) and for arbuscular mycorrhizal fungi (AMF, 16:1ω5c). Overall, the differences in indicator PLFA abundance between the early successional forests (secondary forests 20, 30 and 40 years old) and late successional forests (70- and 90-year old secondary and primary forests) were greater in some seasons than others, but similar successional patterns endured throughout the study period (Fig. 3a–e). For example, the relative abundance of Gm+ bacteria was significantly greater in the late successional forests than in the early successional forests during the wet season in 2010, dry season of 2011, the wet season of 2012 and averaged across all seasons, but only marginally so ($P < 0.10$) during the wet season in 2011 (Table S2).

Soil microbial enzyme activity

The potential activity of most extracellular enzymes showed a significant effect of season, but not land cover, with the exception of NAGase, which showed an effect of both (Table 3a). There were no significant interactions between land cover and season for any of the soil enzymes studied. Mean potential enzyme activities across all land cover types were highest during the dry season in 2011 and lowest during the wet season of 2012. Total enzyme activity (sum of all enzyme activities) showed strong seasonal variability, with greater activities measured in the dry seasons (Fig. 4). The mean potential activity of all enzymes during the dry season was 16 330 $\pm$ 145 and 11 788 $\pm$ 124 μmol g^{-1} soil h^{-1} in 2011 and 2012, respectively, whereas it was 8050 $\pm$ 77 and 6234 $\pm$ 62 μmol g^{-1} soil h^{-1} in 2011 and 2012, respectively (Fig. 4). High variability in the potential activity of extracellular enzymes in soils within and among site replicates resulted in no significant differences across the pasture to forest regeneration chronosequence (data not shown). Specific enzyme activity normalized by soil organic C concentrations (Table 4) also did not follow any consistent pattern with land cover or with forest age.

Across all sites and collection dates, mean phosphatase activity was 12–500 times greater (averaging ~ 6000–16 000 μmol g^{-1} soil h^{-1}, or 10 000–500 000 μmol g^{-1} C h^{-1}) than all other enzymes measured

Table 3 Statistical *P*-values for the fixed effects of season and land cover from a restricted maximum likelihood model for (a) soil and (b) forest litter samples for PLFA biomass and other community indicators. *P*-values in bold are considered significant (*P*-value < 0.05)

		Effects (*P*-value)					
		(a) Soil			(b) Forest litter layer		
Response variables		Season (year)*	Land cover	Season (year)* × Land cover	Season (year)*	Forest age	Season* × Forest age
PLFA biomass†	Total PLFA	**< 0.0001**	0.3045	0.0548	**< 0.0001**	0.0631	0.5965
	Fungal PLFA	**< 0.0001**	**0.0451**	**0.0175**	**0.0011**	0.5382	0.2563
	Bacterial PLFA	**< 0.0001**	0.8712	0.0522	**< 0.0001**	**0.0418**	0.2942
F:B		**0.0008**	**0.0394**	**0.0062**	**0.0017**	0.1855	**0.0258**
PLFA	Gm+	**< 0.0001**	**0.0051**	**0.0057**	**0.0182**	0.2697	0.1575
community‡	Actinobacteria	**< 0.0001**	0.0754	**0.0232**	**< 0.0001**	0.2973	**0.0497**
	Gm-	**0.0245**	0.0995	0.2704	0.7465	**0.0429**	0.7441
	Fungi				**< 0.0001**	0.1777	0.1919
	AMF	**< 0.0001**	**0.0272**	**0.003**			
	SF or ECTO	**0.0352**	**0.0007**	0.2959			
	SF	**0.0088**	**0.0126**	0.087			
	Methanotrophic	**0.001**	0.5079	0.8435			
	Gm - anaerobic	**< 0.0001**	**0.0061**	0.0618			
Enzyme	Beta	**< 0.0001**	0.3748	0.9033	0.5076	0.7076	**0.0175**
activities§	NAG	**< 0.0001**	**0.0304**	0.9184	0.2169	0.2267	**0.0274**
	Phos	**< 0.0001**	0.1174	0.9822	**0.0007**	0.0892	0.2688
	Alpha	**< 0.0001**	0.216	0.5071	0.0535	0.2014	0.5626
	CBH	**0.0011**	0.2438	0.8954	0.3322	0.4262	0.5972
	Xylo	**< 0.0001**	0.1357	0.7121	0.4976	**0.0183**	0.4137

P-values for enzyme activities for both soil and forest litter performed on log-transformed data.

*The effect of season is nested within year. Data covers two seasons, wet and dry, for years 2010–2012 with the exception of 2010 where there is only data from the dry season.

†Models performed on absolute abundance (nmols g^{-1} soil) for PLFA biomass values.

‡Models performed on relative abundance (%) for PLFA community indicators and guilds. Guilds for forest litter communities were not calculated for different groups of fungi, methanotrophic or Gm anaerobic bacteria as there were only one biomarker associated with each taxonomic classification known (see Table 1 for PLFA structures).

§Enzyme Activities; Beta = *β*-glucosidase, NAG = N-acetylglucosaminidase, Phos = acid phosphatase, Alpha = *α*-glucosidase, CBH = cellobiohydrolase, Xylo = xylosidase.

(Table 4). NAGase, *β*-glucosidase, and xylosidase averaged between 300–1700 μmol g^{-1} soil h^{-1} (5000–50 000 μmol g^{-1} C h^{-1}), while *α*-glucosidase and cellobiohydrolase had the lowest activities (~ 20–300 μmol g^{-1} soil h^{-1}, or 500–10 000 μmol g^{-1} C h^{-1}) (Table 4).

Litter layer microbial community structure

Litter layer microbial community structure varied with season and forest successional stage (no litter was collected from the pastures) (Fig. 5a,b). Similar to the soil community, the PC scores for both axes (PC1 and PC2) varied by season ($P < 0.01$) and therefore, were analyzed separately for ordination analyses. Forest age only varied ($P < 0.01$) along PC1 in both the wet (Fig. 5a) and dry (Fig. 5b) seasons. This pattern was mostly explained by differences between the 70 year-old secondary forests and 40 year-old secondary forests for both seasons. In both seasons, unnamed PLFAs with > 11-C chain length explained the majority of variation along the first principal component; 85% and 80% for the wet and dry seasons, respectively (Table 2b). A biomarker generally representing Gm+ bacteria, 16:0iso (Zelles, 1999), was most strongly correlated with PC2; 72% and 75% during the wet and dry season, respectively.

Similar to microbial indicators for the soil community, PLFA biomass (nmols PLFAs g^{-1} litter) and the abundance of select PLFA guilds of the litter layer community varied by season within each year (Table 3b). Differences were neither consistent among microbial guilds nor between wet and dry seasons. For example, total PLFA biomass was highest during the wet season in 2011 and dry season in 2012 and lowest in the dry season of 2011 and wet season of 2010 (see Table S3).

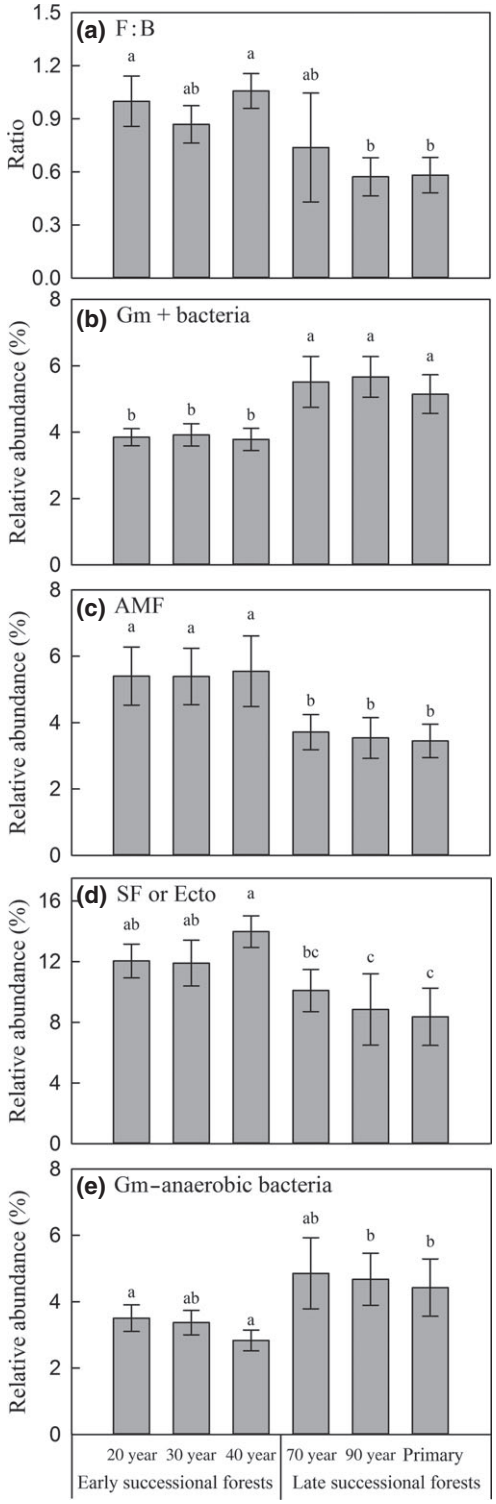

Fig. 3 Relative abundance of soil indicator PLFAs with a significant effect of land cover type (from Table 3a) for the early successional (20, 30, and 40-year old secondary forests) and late successional (70, 90-year old secondary and primary forests) for (a) the fungal-to-bacterial ratio (F:B), (b) Gm+ bacteria (15:0iso), (c) arbuscular mycorrhizal fungi, AMF (16:1ω7c), (d) saprotrophic or ecotmycorrhizal fungi (18:1ω9c), and (e) anaerobic, Gm- bacteria (19:0 cyclo). Bars represent mean relative percent for both wet and dry seasons from 2010 to 2012 averaged by site means, with three sites per land cover type, except in the 40-year secondary forests where there are only two sites. Error bars represent one standard error from the mean.

Total fungal PLFAs were highest during the wet season of 2010 and dry seasons in 2011 and 2012, and lowest during the wet season in 2011 (Table S3). There was a significant interaction between season and forest age on the fungal-to-bacterial ratio and the relative abundance of actinobacteria.

Forest age had a significant effect on the total bacterial PLFAs and the relative abundance of Gm− bacteria (Table 3b). Bacterial PLFA biomass was greatest in the 70-year old secondary forests and smallest in the 20-year old secondary forests. In general, the relative abundance of Gm− bacteria (sum of 16:1ω7c, 16:1ω9c, 17:1ω8c, 18:1ω5c) was greater in the late secondary (70 and 90-years old) and primary forests compared to the early secondary forests (20, 30, and 40-years old) (Table S3).

Litter layer microbial enzyme activity

Potential activity for most enzymes measured in the litter layer did not differ by season, sampling years or forest ages (Table 3b), likely due to high variability among replicate field sites within each class of forest ages (Table S4). There was a significant interaction of forest age and season for the activities of β-glucosidase and NAGase. This was mainly due, in part to differences between the activity of the primary forests in dry season of 2011 and 40-year old secondary forests in the wet season of 2011. Phosphatase activity differed by season and year, with greater activities in the wet season than the dry season in 2012 (Table S4). The activity of xylosidase varied by forest age but not season, with the 40-year secondary forests having greater potential activities compared to the primary, 20 and 70-year old secondary forests.

Environmental controls on microbial parameters

There were few to no significant relationships among soil microbial community composition, extracellular enzyme activity and soil properties (C, N, C:N, moisture, pH). Many of the soil variables measured did not differ by land cover type (Table S1). Average soil values across all sites were 0.26 ± 0.01% N, 3.36 ± 0.15% C, and C:N of 15.18 ± 0.27. In contrast, there was a signifi-

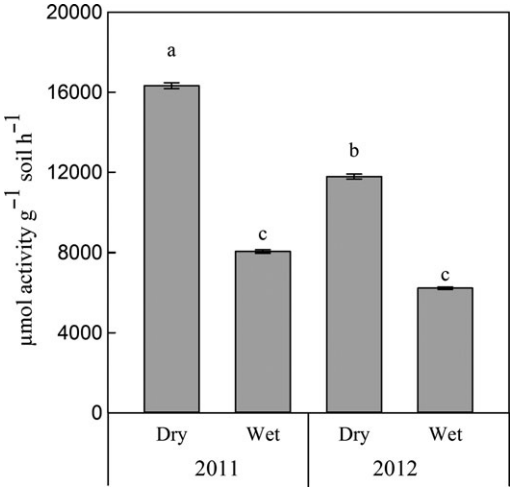

Fig. 4 Total soil extracellular enzyme activity, or sum of all enzymes measured, averaged across all land cover types per year and season. Error bas represent one standard error from the mean. Letters not shared across columns represent significantly different means via Tukey's mean comparison test.

cant effect of land cover on soil pH and soil moisture, although the differences did not predictably track forest recovery over the seasons and years (data not shown). For example, soil pH only differed between the 30 year-old secondary forests and the primary forests and 70-year old forests. Overall, soil moisture was greatest in the pastures (80.3% ± 5.2) and lowest in the 20-year old secondary forests (40.9% ± 3.2). Average soil moisture across all land cover types was greater in the wet seasons (67.5% ± 2.9) than in the dry seasons (55.8% ± 3.2). Despite seasonal or land cover effects on soil microbial properties and soil moisture, there were few to no significant correlations between soil PLFA absolute abundance or the relative abundance of indicator PLFAs and soil extracellular enzyme activities and pH, moisture, elemental concentrations or ratios. Significant linear relationships between PLFA variables or enzyme activities with soil physical and/or chemical properties were weak (with r^2 values at or below 0.10). For example, the specific activity of β-glucosidase and NAGase (normalized to g soil C) were positively correlated with soil pH ($P = 0.019$, 0.018 respectively), and negatively correlated with soil moisture ($P = 0.028$ and 0.010, respectively). The strongest relationship (i.e., largest r^2 value = 0.11) was between total PLFA biomass and soil C concentration ($P = 0.0032$).

In contrast with soil values, litter % N and C:N differed by forest age but without any discernable linear trend with time since pasture abandonment and reforestation (See Table S1). Season had a significant effect on litter C:N and % C, with a greater % C and C:N in the wet season than in the dry season. Litter layer properties, such as pH, % C, and % N did not explain observed patterns in microbial community composition. The activities of individual enzymes were highly correlated with each other. In particular, NAGase activity was positively correlated with β-glucosidase ($r^2 = 0.85$), α-glucosidase ($r^2 = 0.71$), and phosphatase ($r^2 = 0.69$).

Microbial community structure and composition in the litter layer and soil communities differed greatly (Table S2 and S3). Microbial PLFA biomass and the absolute abundance of important guilds, such as Gm+ bacteria, fungi, and actinobacteria, were 2–5 times greater in the litter layer than in the soil. The absolute abundance (nmols PLFA g soil^{-1}) and the relative abundance of the fungal biomarkers 18:1ω9c and 18:2ω6,9c were greater in the litter than in the soil ($P < 0.0001$, data not shown).

Discussion

Microbial communities follow successional trends with reforestation

Belowground microbial community structure showed strong successional patterns along a chronosequence of secondary forests growing on former pastures in the wet subtropical forest life zone of Puerto Rico. These successional trends were consistent between the wet and dry seasons and from year to year, even given inter- and intra-annual variability in the specific lipids and guilds responsible for differences among land cover types. The observed clustering of soil microbial community structure into early and late successional groups paralleled successional stages in forest tree species composition at the same sites (Marín-Spiotta *et al*., 2007). Ordination analyses of tree species during an earlier survey showed differences in composition between early successional forests (then 10, 20, and 30-year old forests), secondary forests (60 and 80-year forests), and primary forests. These results show strong relationships between above and belowground community composition during forest regrowth.

The long-term nature of our chronosequence allowed us to examine the effects of a change in plant cover with forest regrowth on the soil and forest litter microbial community during almost a century of forest succession. The importance of different drivers of change on microbial communities is debated in the literature (Cleveland *et al*., 2003; Paterson, 2003; Hooper *et al*., 2005). Microbial communities have been shown to respond to changes in plant diversity and composition (Carney & Matson, 2006), whereas other studies link changes in microbial composition to changes in litterfall production (Zak *et al*., 2003). As aboveground plant

Table 4 Mean and propagated standard error of soil extracellular enzyme activities normalized by total soil C (μmol g^{-1} C h^{-1}) for all land cover types in both the wet and dry season from 2011 to 2012. Propagated error is calculated as the square root of the sum of errors of sample reps and sites to take site error into account

				Soil enzymes activities (μ mol g^{-1} C h^{-1})					
Year	Season	Land cover	Forest age (years)	Beta	NAG	Phos	Alpha	CBH	Xylo
2011	Dry	Pasture		40 399 ± 18 508	24 250 ± 9120	207 163 ± 117 979	1839 ± 667	6395 ± 2484	25 437 ± 7089
			20	74 575 ± 34 814	73 697 ± 65 689	399 237 ± 208 662	3105 ± 1278	12 559 ± 5679	59 534 ± 22315
			30	81 882 ± 68 668	175 011 ± 165 307	498 884 ± 422 397	3203 ± 1502	16 409 ± 10 786	45 439 ± 30 906
		Secondary forest	40	49 986 ± 8257	48 809 ± 15 145	300 059 ± 146 501	1903 ± 702	9541 ± 3521	32 303 ± 13 336
			70	52 045 ± 31 545	34 741 ± 17 201	551 320 ± 356 011	2311 ± 711	9648 ± 5465	37 281 ± 19 413
			90	38 805 ± 16 021	26 635 ± 12 420	272 418 ± 120 426	1692 ± 1375	8487 ± 2898	26 423 ± 3545
	Wet	Primary forest pasture		25 968 ± 10 367	29 864 ± 11 750	279 813 ± 218 383	1308 ± 417	2760 ± 967	9987 ± 3391
				14 295 ± 9072	14 962 ± 8636	124 363 ± 62 538	1613 ± 590	5282 ± 4350	19 073 ± 12 058
			20	13 423 ± 5674	34 790 ± 35 034	221 689 ± 75 018	1659 ± 566	8103 ± 5966	22 945 ± 7320
			30	14 823 ± 5708	35 694 ± 17 175	222 744 ± 46 474	1433 ± 547	7192 ± 3091	17 345 ± 7020
		Secondary forest	40	15 471 ± 3802	20 860 ± 7077	155 403 ± 38 093	1363 ± 545	6284 ± 2646	18 477 ± 4582
			70	15 388 ± 7173	21 673 ± 12 556	279 126 ± 101 755	2513 ± 1921	9504 ± 10 157	16 244 ± 8212
			90	13 263 ± 5620	13 810 ± 6173	205 437 ± 41 238	1681 ± 747	6843 ± 5289	19 753 ± 11 713
2012	Dry	Primary forest pasture		4516 ± 2599	8925 ± 4475	145 789 ± 48 511	748 ± 253	1527 ± 658	7345 ± 4045
				30 535 ± 15 220	24 730 ± 11 917	268 497 ± 121 638	2379 ± 1703	5798 ± 1669	20 135 ± 4899
			20	16 769 ± 7998	18 149 ± 8161	202 549 ± 131 709	893 ± 225	3124 ± 1567	12 479 ± 3323
			30	17 072 ± 7769	40 367 ± 28 931	259 595 ± 89 655	730 ± 262	2929 ± 2114	6757 ± 4315
		Secondary forest	40	36 798 ± 18 570	36 833 ± 14 863	294 499 ± 135 420	927 ± 489	4000 ± 2495	13 292 ± 8565
			70	20 232 ± 13 043	22 112 ± 11 448	409 127 ± 171 368	1166 ± 525	3695 ± 2238	11 350 ± 6573
			90	16 988 ± 6279	19 495 ± 6756	285 112 ± 116 248	1000 ± 256	4168 ± 1672	14 804 ± 3098
	Wet	Primary forest pasture		14 744 ± 8674	26 058 ± 22 715	363 170 ± 198 988	884 ± 356	1498 ± 748	6020 ± 2575
				11 763 ± 3348	8430 ± 2415	123 082 ± 37 097	802 ± 296	2839 ± 1081	16 316 ± 4341
			20	9010 ± 2818	6896 ± 3601	10 0989 ± 26 098	458 ± 129	1742 ± 519	11 540 ± 2282
			30	10 083 ± 7349	13 883 ± 8602	138 943 ± 40 582	440 ± 203	2234 ± 655	7930 ± 3149
		Secondary forest	40	15 299 ± 3627	11 274 ± 3213	144 813 ± 40 678	596 ± 184	4116 ± 1431	15 345 ± 6935
			70	13 209 ± 3638	12 994 ± 7440	233 386 ± 70 976	975 ± 335	3221 ± 1404	15 556 ± 4076
			90	11 432 ± 2376	9752 ± 2716	187 512 ± 25 183	655 ± 222	3202 ± 873	13 266 ± 4378
		Primary forest		8718 ± 4208	10 104 ± 4879	155 830 ± 65 753	535 ± 139	1569 ± 929	5748 ± 1577

Column Headings: Enzyme Activities; Beta = β-glucosidase, NAG = N-acetylglucosaminidase, Phos = acid phosphatase, Alpha = α-glucosidase, CBH = cellobiohydrolase, Xylo = xylosidase.

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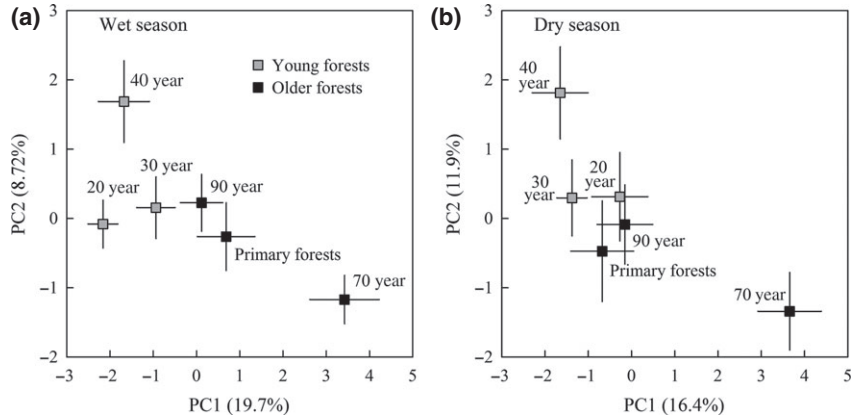

Fig. 5 Principal component analysis of forest litter layer microbial community structure using all PLFA biomakers; (a) Points represent mean community structure for the wet seasons (across 3 years) averaged by site means, with three replicate sites per forest age, except in the 40 year secondary forests where there are only two sites; (b) Points represent mean community structure for the dry seasons (across 2 years) averaged by site means, with three sites per land cover type, except in the 40-year secondary forests where there are only two sites. Error bars represent one standard error from the mean.

communities shift, differences in the chemistry and quantity of leaf and root litter biomass and exudates have been shown to influence belowground microbial composition and activity (Carney & Matson, 2006; De Graaff *et al*., 2010; Potthast *et al*., 2010). As these variables may all change during forest succession, it can be difficult to determine which is most important.

Successional changes in tree species composition along our chronosequence were not reflected in the C chemistry of aboveground litter inputs (Ostertag *et al*., 2008). In fact, the greatest differences in plant chemistry occurred during the transition from pastures to forest cover (Marín-Spiotta *et al*., 2008), with no consistent chemical differences in leaf litterfall with forest age. Litter-associated microbes in our current study did not show the same trends with reforestation as soil communities. Differences in litter microbial communities may be more strongly affected by decomposition stage of the standing stock of litter on the forest floor (DeAngelis *et al*., 2013). An earlier litter-transplant field decomposition experiment at our reforestation chronosequence showed that site differences were more important in explaining rates of mass loss than initial litter chemistry (Ostertag *et al*., 2008). This previous study and our current findings suggest that changes in soil microbial communities are not influenced by successional shifts in litterfall chemistry during reforestation. On the other hand, individual plant species' effects may be masked by interactions among mixed species litter, which can influence microbial processes (Hickman *et al*., 2013).

Differences in microbial community composition or activity during land-use and land-cover change also have been attributed to differences in soil properties rather than to changes in plant communities (Bossio *et al*., 2005; Acosta-Martínez *et al*., 2007; Tischer *et al*., 2014b). A study in the Loess Plateau, China, reported a rapid increase in microbial biomass with gains in soil organic C and total N during the first two decades of forest succession (Jia *et al*., 2005). Our data did not reveal successional trends in total soil C or N, C:N, pH, moisture, or texture or significant relationships between soil properties and microbial parameters. The distribution of SOM fractions and their radiocarbon-based turnover time did vary across the chronosequence (Marín-Spiotta *et al*., 2008, 2009). The amount of particulate organic matter was smallest in the pastures and youngest forests (then 10-years old) than in all other forested sites, reflecting the chemical composition of pasture plant tissue and faster cycling of physically unprotected SOM. In our study, Gm+ bacteria were relatively more abundant in the late successional and primary forests than in the pasture sites. Gm+ bacteria have been linked to more complex or diverse sources of C (Griffiths *et al*., 1999; Fierer *et al*., 2003b; Kramer & Gleixner, 2006), consistent with heterogeneous plant inputs in the forested sites (Marín-Spiotta *et al*., 2008; Ostertag *et al*., 2008). These results may help explain the observed microbial differences between the pastures and forested sites, but not among forest of different ages.

Microbial communities shift on seasonal and yearly time scales

Successional differences in microbial community composition persisted through time, with different PLFA biomarkers accounting for seasonal and yearly varia-

tions in community structure. The interannual differences in microbial communities in our study could be due to year to year differences in litterfall production (Ostertag *et al*., 2008) and hence inputs to the litter layer and soil, in response to changes in precipitation. Differences in soil moisture and the specific microbial groups responding on seasonal time scales suggest soil microsite conditions were an important driver of microbial community composition and activity in these forests.

The PLFA biomarker for anaerobic, Gm− bacteria was the strongest indicator of microbial community differences during wet seasons, suggesting temporal variations in anaerobic soil environments. Shifts in precipitation and soil moisture have been shown to regulate microbial community composition and function (Fierer *et al*., 2003a; Evans & Wallenstein, 2011; Bouskill *et al*., 2012). In other forest sites in Puerto Rico on similar soils receiving greater rainfall (3500 mm MAP), soil oxygen concentrations showed large spatial and temporal fluctuations, driving changes in bacterial populations (Pett-Ridge & Firestone, 2005; Bouskill *et al*., 2012). Our soils, while not as wet, showed visible evidence of redoximorphic features at the microsite scale, likely facilitated by the high clay and iron (Fe) content and well-developed aggregate structure. Gm+ bacteria, which were the strongest indicator of community differences among our sites during the dry seasons, have been shown to be most resistant to moisture stress (Kaur *et al*., 2005; Schimel *et al*., 2007; Bouskill *et al*., 2012).

Our study also revealed seasonal differences in soil extracellular enzyme activities. Contrary to our expectations that extracellular enzyme activity would increase during the wet season, the activity of all soil enzymes measured was greatest in the dry season. Reduced moisture during the dry season may inhibit microbial access to soil resources and trigger extracellular enzyme production (Alster *et al*., 2013). Alternatively, enzyme turnover may decrease in dry soil conditions, resulting in a larger pool of active enzymes (Geisseler *et al*., 2012). Variations in the amount of extracellular enzymes may alter decomposition rates and nutrient cycling across seasons, with implications for soil C turnover.

Shifts in microbial composition are not reflected in enzyme activity

Changes in microbial community composition along the reforestation chronosequence did not result in differences in enzyme activities. Enzyme activity was also not correlated with measured soil and microbiological properties. Despite many studies reporting a positive, linear relationship between enzyme activity and microbial biomass (Bossio *et al*., 2005; Acosta-Martínez *et al*., 2007), at our sites total PLFA biomass, a proxy for active microbial biomass (Vestal & White, 1989; Frostegård & Bååth, 1996), was decoupled from activity measurements. The lack of land-cover effects on enzyme activities and of statistical relationships between our measures of microbial composition and function could be explained by proposed functional redundancy in the microbial community (Allison & Martiny, 2008; Chaer *et al*., 2009) or high environmental heterogeneity in these tropical soils. Data on soil microbial functional gene diversity suggest a certain amount of redundancy for the majority of genes involved in C, N, and P cycling, although some specific genes did show differences between early successional and late successional forests (Smith, 2013).

High diversity in soil microsite conditions may help explain the large variability in extracellular enzyme activity. Highly-weathered clay soils under diverse tropical forest vegetation may show large micro-scale variability in soil C, nutrient concentrations and redox potential (Pett-Ridge & Firestone, 2005; Townsend *et al*., 2008; DeAngelis *et al*., 2010). Microbial composition and functional activities can also very spatially in the soil matrix (Ettema & Wardle, 2002). At our sites, we previously reported that microbial community composition differed more among soil physical fractions than among forest ages, suggesting microbes are responding to heterogeneity in resources at a fine scale (Smith *et al*., 2014). This environmental heterogeneity can confound treatment effects as enzyme assays are often performed on a small quantity (~1 g) of soil (Sinsabaugh *et al*., 1999). The ability to effectively homogenize a sample for enzyme analysis can be limited in highly heterogeneous and clay-rich soils.

Methodological constraints in the measurements of extracellular enzyme activity may also conceal significant relationships with soil microbiological variables. Extracellular enzyme assays are a measure of potential activity vs. realized or *in situ* activity (Sinsabaugh *et al*., 1999; Burns & Dick, 2002), and thus reflect sample and assay conditions, which are often set at optimal enzyme reaction conditions (Deforest, 2009; German *et al*., 2011). Enzyme assays measure the pool of all available enzymes in a sample, which includes those that were actively being produced in response to an added substrate and residual enzymes that may have become stabilized on soil mineral surfaces (Quiquampoix *et al*., 2002; Tate, 2002; Allison, 2006). The activation of enzymes on mineral surfaces has been reported in soils dominated by variably charged clay minerals and high

concentrations of Fe and aluminum (Al) oxides and hydroxides (Quiquampoix *et al.*, 2002; Zimmerman & Ahn, 2011), such as the Oxisols at our sites. This fact can often confound any significant relationship between microbial PLFAs or potential enzyme pools (Waring *et al.*, 2013).

The high activity of phosphatase relative to other enzymes measured in all land uses is consistent with studies in both temperate (Saviozzi *et al.*, 2001; Trasar-Cepeda *et al.*, 2008) and tropical ecosystems (Acosta-Martínez *et al.*, 2007; Stone *et al.*, 2014; Tischer *et al.*, 2014a). Expectations of phosphatase activity with changes in land cover are less predictable, with some studies reporting higher values in forests (Sicardi *et al.*, 2004; Trasar-Cepeda *et al.*, 2008), pastures (Chen *et al.*, 2003) or no difference between forest and pastures (Acosta-Martínez *et al.*, 2007). These patterns have been attributed to differences in soil C, N, and P, rather than to changes in land use or land cover (Waring *et al.*, 2013; Tischer *et al.*, 2014a). Preliminary data from our sites show no successional trend in the distribution of different P pools (E. Marín-Spiotta, unpublished data), suggesting that phosphatase activities most likely reflect a combination of soil and microbial stoichiometry, moisture-driven turnover rates and clay-mineral interactions.

Few studies of land-use change effects on microorganisms incorporate repeated sampling, despite recent recognition that shifts in microbial composition over time can play an important role in ecosystem response to global change (Allison & Martiny, 2008; Treseder *et al.*, 2011; Gutknecht *et al.*, 2012). Our high frequency sampling revealed strong seasonal and interannual differences in microbial community composition during pasture reforestation in highly weathered soils in Puerto Rico. Successional patterns in the soil microbial community composition persisted through changes in the makeup of the community between wet and dry seasons and from year to year. Patterns in soil microbial properties were not explained by pH or total soil C and N, which did not vary over 90 years of forest regeneration, but responded to differences in soil moisture. Microbial community composition from the forest litter differed greatly from those in the soil and did not show the same successional trends.

In this study, PLFA proved an effective tool for identifying ecologically relevant trends in microbial community composition. We showed that the microbial groups responsible for explaining differences among land cover types changed over time. Our findings indicate that conclusions made from studies with samples collected at only one time point may provide only part of the story. The role of different biomarkers in driving temporal variability of community composition suggests individual microbial groups respond differentially to changes in environmental conditions. Understanding heterogeneity in microbial response to environmental drivers can improve predictions of adaptability or resilience of ecosystems to environmental change.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Mean and standard error of (a) soil and (b) forest litter properties across a tropical reforestation chronosequence.

Table S2. Mean and standard error of the relative percent value of select soil PLFA biomarkers averaged by site (n = 3, except for 40-year forests where n = 2).

Table S3. Mean and standard error of forest litter PLFA guilds (relative percent) by year, season, and land cover type averaged by site (n = 3, except for 40-year forests where n = 2).

Table S4. Mean and standard error of forest litter extracellular enzyme activities ($\mu mol\ g^{-1}\ h^{-1}$) by year, season, and land cover type.