SOIL MICROBIAL COMMUNITIES AND SOIL ORGANIC MATTER: COMPOSITION AND ECOLOGICAL FUNCTIONS IN THE LUQUILLO CRITICAL ZONE

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Dedicated to Fred Scatena

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To get through the hardest journey we need only take one step at a time but we must keep stepping –Chinese proverb.

This quote was left above a desk that I inherited some years back from a former grad student. I kept several of her decorations in place in the hopes of channeling a bit of her work ethic over the past four and a half years. But for nearly all of the steps I've taken, I've relied on the guidance, advice and support of a smorgasbord of amazing people. Below I'd like to acknowledge and convey my heartfelt gratitude toward the people who helped make this dissertation possible.

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ABSTRACT

SOIL MICROBIAL COMMUNITIES AND SOIL ORGANIC MATTER: COMPOSITION AND ECOLOGICAL FUNCTIONS IN THE LUQUILLO CRITICAL ZONE

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Alain F. Plante

Tropical forest soils contain large pools of carbon, most of which is stored as soil organic matter. In spite of its significant role in the global carbon cycle, the dynamics of tropical soil organic matter, including the soil microbial communities that produce, maintain and decompose it, are poorly understood. This dissertation investigates controls on the structure and biogeochemical functions of soil microbial communities and soil organic matter, using a combination of laboratory experiments and natural gradients present at the Luquillo Critical Zone Observatory. First, we investigate linkages between soil enzyme activities, nutrient availability and plant roots in surface mineral soils through a greenhouse pot experiment. Three subsequent studies "dig deeper", by investigating microbial community structures and functions (carbon, nitrogen and phosphorus cycling capacity) along the upper 1.4 meters of soil profiles. Finally, we use ^{13}C NMR spectroscopy to profile the chemical composition of soil organic matter across various depths, soil and forest types, coupled with additional thermal and chemical analyses to evaluate acid-treatment effects on soil organic matter composition. We find that dynamic microbial communities exist along the upper 1.4 meters of tropical soil profiles and that, on a per biomass basis, subsoil microbial communities have similar capacity to participate in carbon and nutrient mineralization as their surface counterparts. While microbial activity is strongly correlated to soil carbon concentrations and hence energy availability, soil organic matter chemistry appears to be driven by landscape scale factors as well as pit-scale factors. Because even small amounts of active soil carbon below the surface few centimeters of the soil profile could produce significant carbon fluxes over large spatial and temporal scales, models that aim to predict the future changes to the global carbon cycle should begin to consider the capacity for carbon cycling to occur throughout the deep critical zone.

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CHAPTER 1 : Introduction

Soils are a dynamic component of the critical zone, representing an interface in which biological and geological processes drive Earth's cycles of carbon and nutrients. Soils are the largest reservoir of carbon on Earth's terrestrial surface (*Jobbagy and Jackson*, 2000), storing at least three times as much carbon as found in either the atmosphere or living plants (*Fischlin et al.*, 2007). Estimates of the amount of carbon stored in the top meter of soils worldwide range from 1,220 Petagrams (1 Pg = 1015 g, *Sombroek et al.* (1993)) to approximately 1,550 Pg (*Batjes*, 1996; *Jobbagy and Jackson*, 2000). Determining the mechanisms that govern the flow of carbon in and out of this massive reservoir is crucial not only for developing our conceptual understanding of ecosystems, but for predicting changes in ecosystem functions due to human activities, including land conversion and climate change.

Tropical forests, covering only 7-12% of Earth's land mass (*FAO*, 2001), represent a key player in terrestrial carbon biogeochemistry, containing between one third and half of the global soil carbon pool (*IPCC*, 2007; *Jobbagy and Jackson*, 2000). High temperatures and precipitation, coupled with a lack of interruption to their development during recent glacial cycles, place tropical forest soils among the deepest and most highly weathered on Earth. Unsurprisingly then, tropical forests are also large contributors to subsoil carbon stocks. Often present in minute concentrations, deep soil carbon is thought to have high potential for long-term stabilization and sequestration (*Rumpel and Kogel-Knabner*, 2011), as evidenced by radiocarbon ages on the order of thousands of years (*Rumpel et al.*, 2004; *Torn et al.*, 1997; *Trumbore*, 2000). However, tropical subsoils represent a knowledge gap in our understanding of the terrestrial carbon cycle. Compared with temperate and boreal ecosystems, a scant number of studies have investigated carbon cycling in tropical forest soils (*Balser et al.*, 2010), and most have been restricted to the upper 20-30 cm of the soil profile.

The majority of the carbon stored in soils is present as soil organic matter; a heterogeneous mixture of organic biomolecules derived from plant, microbial and animal residues. Soil organic matter influences soil structure and water-holding capacity, provides an energy source for soil biota, and

maintains a long-term nutrient pool for both soil biota and plants (*Brady and Weill*, 1996). Soil organic matter ranges in size and complexity from simple monomers or organic acids to mixtures of complex biopolymers aggregated together (*Baldock and Nelson*, 1999). In addition, the chemical structure of each component biomolecule can vary along a continuum of decomposition, ranging from unaltered structures identical to those found in precursor tissues, to highly decomposed residues bearing little resemblance to those from which they are derived (*Kelleher and Simpson*, 2006; *Piccolo*, 2001). The variable chemical nature of soil organic matter, coupled with numerous soil environmental factors that vary along a continuum (mineralogy, temperature, moisture, pH, microorganisms, etc.) makes it challenging to determine what governs the persistence of soil organic matter versus its decomposition (*Schmidt et al.*, 2011).

The complexity of soil organic matter is matched by the complexity of the microbial communities that produce, maintain and decompose it. The composition and diversity of soil microbial communities is controlled by their habitat, which consists of a matrix with pores and soil aggregates of different sizes (*Balser et al.*, 2010; *Sylvia et al.*, 2005). It is this environmental diversity that causes soils to be the most biologically diverse habitat on Earth, with current estimates suggesting there can be over ten thousand species of microorganisms in a single gram (*Fierer and Lennon*, 2011). The metabolic strategies these microorganisms employ to acquire energy and nutrients represent the biological drivers of biogeochemical processes (*Plante et al.*, In Press). Soil microorganisms produce a suite of extracellular enzymes that decompose organic matter and mineralize nutrients (*Wallenstein and Burns*, 2011). They break organic matter down further within their cells using numerous oxidizing agents, including oxygen, nitrate, sulfate and carbon dioxide (*Plante et al.*, In Press), and in doing so catalyze key soil redox transformations. A growing appreciation of the essential roles soil microorganisms play in biogeochemical cycles has led to rapid expansion of the field of soil microbial ecology, as well as the development of many new research methodologies aimed at characterizing the structures and functions of these communities (*Nannipieri et al.*, 2003; *Torsvik and Ovreas*, 2002).

An emerging paradigm suggests the persistence of organic matter in soils is governed by complex

interactions between organic matter and its environment (*Schmidt et al.*, 2011). Soil organic matter cycling can best be understood as the result of a confluence of numerous factors including climate, microorganisms, mineralogy, water availability, soil acidity, redox state and compound chemistry. In other words, the persistence of soil organic matter is an emergent ecosystem property governed by a complex suite of variables operating across different spatial and temporal scales.

It is from this broad context that this dissertation was inspired to characterize soil microbial communities and soil organic matter in the El Yunque National Forest, located in the Luquillo Mountains of northeastern Puerto Rico. This region is a mosaic of wet tropical forests and rainforests growing on steep slopes across gradients in elevation, temperature and rainfall (*McDowell et al.*, 2012; *Scatena*, 1989). It has been the site of extensive ecological and environmental research since the 1930's, with a large amount of infrastructure and accumulated knowledge that can facilitate the investigation of soil microbial ecology and biogeochemistry from an interdisciplinary perspective (*Brown et al.*, 1983). Recently, the El Yunque National Forest has been included in the Critical Zone Observatory Network. Critical Zone Observatories are the subject of studies addressing the interactions of biological and geological processes at the Earth's surface (*Banwart et al.*, 2013). The varied geology, climate, and vegetation across this landscape offer a unique opportunity to investigate state factor controls on what are often considered to be micro-scale phenomena associated with microorganisms and soil organic matter. While the primary goal of this dissertation was to provide new insights into tropical carbon cycling, recognition of the close linkage between soil carbon, nitrogen and phosphorus cycling through soil organic matter decomposition, as well as the stoichiometric constraints of soil microorganisms (*Cleveland and Liptzin*, 2007), resulted in the study of all three. Another major goal of this dissertation was to add to the small but growing body of knowledge on tropical subsoil organic matter and the microorganisms that inhabit this carbon and nutrient-poor environment. A recent survey of soil carbon stocks estimates an average carbon content of 22 kg m^2 to a depth of one meter in these forest soils, and an additional 4 kg m^2 of carbon stored from 100-140 cm (*Johnson et al.*, Under Review). These are substantial carbon stocks compared with the IPCC global estimate of 12.3 kg m^2 C stored in the upper 1 meter of tropical forest soils (*IPCC*, 2007), and the stability of this carbon therefore warrants study. Greater characterization of the microbial ecology and carbon

chemistry of tropical subsoils and will allow scientists to better predict the vulnerability of subsoil carbon pools to de-stabilization. From this interest in subsoil processes came the recognition that many of the standard methods for studying soil microbial ecology and biogeochemistry were developed for carbon and nutrient-rich surface soils. The variety of techniques presented in this dissertation underscore an additional goal of understanding how best to measure the biogeochemical processes occurring in carbon-poor, clay-rich tropical subsoils.

Chapter 1 investigates linkages between soil enzyme activities, nutrient availability and plant roots in surface Oxisol soils through a greenhouse pot experiment. The hypothesis that root carbon could increase microbial investment in nutrient acquisition was experimentally tested. While microbial responses to nutrients support the notion that enzyme production is regulated by soil resource availability, we did not observe any additional effect of roots on extracellular enzyme activities.

Chapters 2-5 investigate microbial ecology and carbon chemistry across the natural gradients present at the Luquillo Critical Zone Observatory, which is composed of two dominant parent materials (sedimentary volcaniclastic and igneous quartz-diorite) and several distinct forest types that occupy different elevations and climate zones.

Chapters 2 studies changes in major carbon, nitrogen and phosphorus cycling enzymes, microbial biomass and microbial community structure along the upper 1.4 meters of soil profiles, with the goal of elucidating whether the carbon and nutrient cycling capacity of microbial communities changes with soil depth, and whether such changes are purely functional, or if they can be related to shifts in community structure. Although enzyme activities and microbial biomass decline exponentially with soil depth, high specific enzyme activities suggest subsoil microorganisms retain the metabolic capacity to participate in biogeochemical processes.

Chapter 3's primary aim was to evaluate microbial contributions to the soil nitrogen cycle with depth across the two dominant parent materials and two major forest types present in the Luquillo Critical Zone. This chapter used DNA-based molecular techniques to fingerprint community structures and examine the microbial community's capacity to conduct specific nitrogen cycle transfor-

mations throughout soil profiles. We found greater abundances and a stronger vertical stratification of nitrogen cycle functional genes in soils derived from the quartz-diorite parent material, suggesting a more dynamic N cycle in these comparably young, sandy soils.

Chapter 4 was inspired by the finding that acid-phosphatase, the primary organic phosphorus-degrading enzyme in acid soils, exhibited high activity throughout soil profiles. This chapter examined in more detail the kinetics of acid-phosphatase in soils and the scaling relationship between enzyme kinetic parameters and soil resource availability.

Chapter 5 examined soil organic matter chemistry along depth profiles and across soil and forest types at the Luquillo Critical Zone Observatory, with a focus on assessing whether ^{13}C nuclear magnetic resonance spectroscopy (NMR) can be applied to low-carbon tropical subsoils to obtain an accurate representation of the chemical composition of soil organic matter. Broad differences in organic matter chemistry were observed between higher-elevation Colorado forest soils and lower-elevation Tabonuco forest soils, and patterns in organic matter chemistry with depth were evident at the level of individual soil profiles, underscoring the heterogeneity of the soil organic matter pool throughout this varied landscape.

CHAPTER 2 : Plant and nutrient controls on microbial functional characteristics in a tropical Oxisol

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Abstract:

Extracellular enzymes mediate the decomposition of organic matter and the release of plant-available nutrients. Current theory predicts that enzyme production by soil microbes is regulated by the stoichiometric demands of microbial biomass and the complexity of environmental resources, but most experiments ignore the potential effect of alleviated carbon limitation in the rhizosphere. Our objective was to investigate linkages between enzyme activities, soil nutrient availability and plant roots in a tropical Oxisol. We conducted a greenhouse experiment using soils from the Luquillo Experimental Forest and seedlings of *Tabebuia heterophylla*. Planted and unplanted pots were fertilized with different combinations of phosphorus and either mineral nitrogen (ammonia chloride) or a nitrogen-rich organic compound (casein). We measured changes in plant and soil nutrients and five extracellular enzyme activities. Phosphatase activity declined by 28% in the P and 40% in the complex nitrogen treatment, while *N*-acetyl glucosaminidase increased 162% in the complex nitrogen treatment. Beta-glucosidase, beta-xylosidase, cellobiohydrolase and *N*-acetyl glucosaminidase all increased significantly over time in the simple nitrogen treatment ($P<0.05$). Enzymatic responses support microbial resource allocation theory, that is, the concept that soil microbes regulate enzyme production based on scarcity of resources. However, we did not observe any additional effect of roots on extracellular enzyme activities. Enzymatic C:N, C:P and N:P ratios further support the notion that shifts in microbial stoichiometric demand drive responses to nutrients.

2.1. Introduction

Soil microbial communities produce extracellular enzymes that mediate the decomposition of organic matter and the release of assimilable nutrients such as carbon (C), nitrogen (N) and phosphorus (P, *Wallenstein and Burns* (2011)). Resource allocation theory, which predicts that enzyme synthesis increases in response to limiting nutrients and declines when nutrients are abundant (*Sinsabaugh and Moorhead*, 1994; *Allison et al.*, 2011) can be invoked to describe enzyme production. However, enzyme production itself can be constrained by nutrient availability, particularly C and N (*Schimel and Weintraub*, 2003). In accordance with resource allocation theory, *Allison and Vitousek* (2005) demonstrated that microbes increase production of N-acquiring enzymes in response to complex, organic N additions when labile C is also present. In contrast, simple mineral N additions led to declines in the activity of N-acquiring enzymes (*Allison and Vitousek*, 2005).

In surface soils, the resource allocation framework of enzyme production is complicated by plant roots exuding labile C compounds into their rhizospheres, thus potentially alleviating microbial C limitation (*Kuzyakov*, 2002). *Averill and Finzi* (2011) found that in a temperate forest, complex N additions enhance the production of N-degrading enzymatic activity only in the presence of roots. They proposed that C limitation is alleviated due to root exudates, thus allowing microbes to increase resource investment in N-acquiring enzymes. However, most studies of extracellular enzyme responses to nutrients have been conducted under laboratory conditions excluding roots, and the generality of these findings for ecosystems other than temperate forests remains to be tested. In low-nutrient status soils such as highly weathered, tropical Oxisols, nutrients cycle rapidly between plants and soils, suggesting that roots may exert a particularly strong influence on nutrient retention in such systems (*Anderson and Swift*, 1983; *Vitousek and Sanford*, 1986; *Templer et al.*, 2008). To date, few studies have examined the influence of roots on extracellular enzyme synthesis and microbial nutrient responses in tropical soils.

The first objective of this study was to test the resource allocation theory of microbial enzyme production in highly weathered, tropical Oxisol soils from the Luquillo Experimental Forest. We chose N as the focal nutrient for this study because of its relevance in a global change context. N

deposition is increasing rapidly in tropical regions (*Galloway et al.*, 2004), and its impact on soil microbial communities remains uncertain. We investigated how specific extracellular hydrolytic enzymes that target key reservoirs of C, N and P respond to simple and complex forms of N, with the hypothesis that simple N would repress N-acquiring enzyme activity and exacerbate microbial C-limitation, while complex N would lead to increased microbial production of N-acquiring enzymes (*Allison and Vitousek*, 2005). We predicted that we would observe microbial enzymatic responses to N in spite of the widespread notion that tropical forests are N rich or N saturated (*Brookshire et al.*, 2012) because previous long-term N addition studies in the Luquillo Forest have shown that microbial communities at this site are sensitive to added N (*Cusack et al.*, 2011).

The second objective was to evaluate whether plant roots mediate microbial enzymatic responses to simple or complex nutrients, with the hypothesis that plant root-derived labile C could alleviate microbial C limitation and, therefore, enhance enzymatic synthesis. Since plant C allocation is itself sensitive to nutrient availability (*Giardina et al.*, 2003), we predicted that simple N additions would decrease plant belowground production. By contrast, we predicted that plants would invest more C belowground (either in root biomass, exudates or both) in response to complex N that requires microbial degradation to become plant-available.

2.2. Methods

2.2.1. Site description and sample collection

This study was conducted using soils collected from the Bisley I and II watersheds in the Luquillo Experimental Forest of Puerto Rico (18°18' N, 65°50' W). The Bisley watershed forests are characterized as in the subtropical wet forest life zone, receiving approximately 3500 mm of mean annual precipitation. Elevations within the watersheds range between 260 and 400 m above sea level, and the landscape is characterized by steep, highly dissected terrain covered by mature Tabonuco (*Dacroydes excesla*) forest (*Scatena and Lugo*, 1995). Soils in the Bisley watersheds are classified as fine textured, kaolinitic, Aquic and Inceptic Hapludoxes (Soil Survey staff, 2013). Eight 10 × 10 m plots were constructed at randomly selected locations across both watersheds to capture as

much edaphic variation as possible. A-horizon soil was collected from each plot to a depth of 10 cm using a 5-cm diameter soil corer. Forty soil cores from each plot were combined and sieved to 5 mm to homogenize. These homogeneous plots were considered replicate blocks in the subsequent greenhouse experiment. The composited soil from each block was mixed with sterile (autoclaved) sand in 2:1 (dry mass soil : dry sand) ratio to improve drainage in greenhouse pots.

2.2.2. Seedling selection and germination

Tabebuia heterophylla, a native Puerto Rican early-successional tree, was chosen for this experiment for several reasons: its fast growth rate, early development of a complex root system, and ability to grow on nutrient-poor or degraded soils (USDA Forest Service, 2012). Rapid growth rates permitted us to conduct a nutrient manipulation experiment in a timely manner, while the early development of a complex root system was desirable as we were aiming to detect effects of root carbon on microbial activity. Since nutrient-rich O-horizon soils were not sampled, we desired a species that could grow effectively on relatively nutrient-poor soils. Seeds were sterilized by soaking in 70% ethanol for ~10 minutes, then germinated in sterilized sand and watered regularly with distilled water. Seedlings were transplanted into pots after 2-3 weeks and left for an additional four weeks prior to the initiation of nutrient treatments to ensure that the root system had recuperated from transplanting.

2.2.3. Experimental design

Soil from each replicate block (i.e., soil taken from the same field plot) was partitioned into 24 polypropylene pots, which were placed in a greenhouse where temperature and humidity conditions were kept constant. Nutrient and planting treatments were randomized among pots within each soil block to minimize effects of spatial variation in temperature, light or moisture availability. Soil was watered regularly with distilled water to maintain field moisture conditions. For each soil block, half of the pots were planted with *T. heterophylla* and half were left unplanted. Within each of these groups, pots were split into four different nutrient treatments: control, P, simple N+P and complex N+P. Sodium phosphate (Na_2PO_4) was used as a source of P, and ammonia chloride (NH_4Cl) was

used as a source of simple N. Casein protein, the source of complex N, requires specific enzymes for its degradation and has a low C:N ratio, thus minimizing the effects of added C on microbial communities. Nutrients were applied in 30 ml solutions every other week. Control pots received 30 ml of deionized water. N and P were added at rates of 1.6 mg N g^{-1} and 320 μg P g^{-1} dry soil (Table 2.1). The amount of N added was intended to increase soil N concentrations substantially (background N concentrations of 5-8 mg g^{-1} dry soil determined prior to experiment), while the amount of P added was intended to match the stoichiometric demands of microbial biomass and prevent microbial P-limitation (*Allison and Vitousek*, 2005).

Pots were harvested at 3, 6 and 9 weeks following the start of nutrient applications to measure effects of nutrients and plants over time. Seedlings were carefully excavated and separated into roots and shoots. To obtain rhizosphere soil, roots were shaken gently to remove any non-adhering soil; any that remained attached to roots was considered rhizosphere soil. Rhizosphere soil and bulk soil were collected from each planted pot, and bulk soil was collected from each unplanted pot.

Aliquots of these soil samples were frozen at -20 °C for subsequent enzyme assays. Separate aliquots were air-dried for chemical analyses. Due to the limited amount of rhizosphere soil collected, only microbial enzyme assays were performed on rhizosphere soils, while chemical analyses were performed on bulk soil taken from the same planted pots. Enzyme assays and chemical analyses were also performed on bulk soil from unplanted pots. Roots and shoots (consisting of stems and leaves) were washed carefully, weighed, dried and ground for C & N analysis.

2.2.4. Soil characteristics

To determine how nutrient applications influenced soil C and N availability, extractable C and N were measured by shaking 5 g of field-moist soil with 40 ml of 0.5 M K_2SO_4 buffer for 4 hours. The soil slurries were then filtered through Whatman #1 filter paper and extracts were kept frozen at -20 °C until total C and N levels were measured on a Shimadzu TOC-Vcsh TOC analyzer. Ammonia (NH_4^+) and nitrate (NO_3^-) levels were also measured on a subset of the extracts using the Hach Ammonia and Nitrate low-range testing kits and a Hach spectrophotometer. Organic nitrogen was

determined as the difference between total extractable N and ammonia + nitrate.

2.2.5. Enzyme assays

Total potential activities of three C-degrading enzymes (beta-glucosidase, beta-xylosidase, cellobiohydrolase), one organic N-degrading enzyme (*N*-acetyl glucosaminidase) and one organic P-degrading enzyme (acid phosphatase) were measured according to the fluorimetric protocol of *Saiya-Cork et al.* (2002) with modifications by *German et al.* (2011). Briefly, 1 gram of field-moist soil was homogenized in 125 ml of pH 5 sodium acetate buffer using a hand blender. Then, 200 μl of this homogenate was added to 8 replicate wells in a 96 well microplate. This was repeated 5 times per microplate for each of the enzyme assays performed. Next, 50 μl of fluorescent substrate proxies specific to each enzyme were added to the assay wells in optimal concentrations for measuring total potential activity (optimal concentrations determined prior to experiment). Assays were run with two standard columns containing soil homogenate and methylumbelliferone (MUB). Each assay microplate also contained substrate blank columns receiving 50 μl of substrate and 200 μl of sodium acetate buffer. Soil homogenate blanks were also measured simultaneously. Plates were incubated at 25 °C for 4 hours (optimal duration of assay determined prior to experiment). After incubation, 10 μl of 1 M NaOH was added to each well to terminate enzyme activity. Following reaction termination, fluorescence was measured using a fluorometer set at 365 nm excitation and 450 nm emission. From fluorescence values, enzyme activity was calculated as the rate of substrate converted in nmol g^{-1} dry soil h^{-1} (*German et al.*, 2011). We also calculated ratios of C:N, C:P and N:P acquiring enzyme activity to evaluate relative changes in microbial allocation to C, N and P resources *sensu Sinsabaugh et al.* (2008). We operationally defined total C-acquiring enzyme activity as the sum of beta glucosidase, beta xylosidase and cellobiohydrolase activities. We used *N*-acetyl glucosaminidase and acid phosphatase to represent N and P acquiring enzyme activity, respectively. For statistical analyses, C:N and C:P ratios were square-root transformed and the N:P ratio was log-transformed to achieve a normal distribution.

2.2.6. Statistical Approaches

Statistical analyses were performed using JMP v.10 (SAS Institute, 2012). Three separate ANOVA models were used to analyze the soil enzyme data, soil nutrient data, and plant data. The effects of collection block, nutrient treatment, plant presence (referred to as "planting"), time of harvest, and their interactions on soil enzyme activities and enzyme activity ratios were examined using a mixed-effects ANOVA and employing the restricted maximum likelihood (REML) approach. In this analysis, samples were either from rhizosphere soil or bulk soil from an unplanted pot. Nutrient treatment, time and planting were treated as fixed effects, and block was treated as a random effect. A separate ANOVA of the same structure was performed on extractable C and N data, where the planting treatment referred to whether bulk soil was from a planted or an unplanted pot rather than bulk unplanted soil versus rhizosphere soil. Finally, an ANOVA was performed to examine the effects of site, nutrient treatment and time on biomass and C and N content of leaf and root tissue. Tukey's HSD post-hoc test was used to evaluate how variables responded to the different treatments.

2.3. Results

2.3.1. Plant C, N and biomass

By 9 weeks, most plants in the simple N+P treatment had died, likely as a result of stress induced by high ionic strength in the soil matrix from the repeated addition of mineral fertilizer. All pots with plants in this combination of nutrient treatment were, therefore, excluded from statistical analyses. Aboveground and belowground plant biomass otherwise increased significantly over time ($P < 0.001$). P-treated plants had significantly greater root biomass than controls (Figure 2.1b), particularly at 9 weeks ($P < 0.02$ for time $\times$ nutrient interaction), while simple N+P treated plants exhibited the lowest root biomass of any treatment, though this treatment was not statistically different from controls. Complex N+P additions significantly increased aboveground plant biomass (Figure 2.1a), particularly at 9 weeks, where mean shoot biomass in the complex N+P treatment was more than $4 \times$ greater than mean shoot biomass for either control or P-fertilized plants ($P < 0.001$ for time $\times$ treatment effect). Shoot and root C:N ratios declined significantly in both N-fertilized treatments

and in P-treated plants relative to controls (Figure 2.2a-b, $P < 0.001$).

2.3.2. Soil Chemical Properties

Total extractable soil C increased significantly over time (Table 2.2) and was significantly greater in unplanted compared to planted soils ($P < 0.01$ for both time and planting effects). Complex N+P additions roughly doubled extractable C compared with all other treatments. This effect was most pronounced in unplanted soils receiving complex N+P.

Total extractable soil N increased significantly over time, driven by the accumulation of extractable N in the simple N+P and complex N+P treatments (Table 2.2, $P < 0.001$ for time $\times$ treatment effect). Tukey's HSD test revealed that soils given simple N+P exhibited the largest levels of extractable N, while soils given complex N+P exhibited significantly greater levels of extractable N than controls or soils given P only ($P < 0.001$). Ammonium concentrations were significantly greater in N addition treatments relative to controls ($P < 0.005$) while nitrate concentrations increased in the complex N+P treatment, though the effect was not statistically significant ($P = 0.09$). Soil organic N increased significantly over time, driven primarily by a statistically significant increase in organic N in the simple N+P treatment relative to controls, particularly at 6 and 9 weeks ($P < 0.05$ for time and treatment effects).

2.3.3. Extracellular Enzyme Activities

There were no significant overall effects of planting (i.e., no significant differences between bulk and rhizosphere soils) for any enzyme activities. However, phosphatase activity declined in planted soils relative to unplanted soils at 3 weeks, though this effect was not apparent at 6 or 9 weeks (Tukey's HSD, $P < 0.001$). Cellobiohydrolase activity was significantly greater in planted soils at 6 weeks (Tukey's HSD, $P < 0.001$).

For all enzymes except beta-glucosidase, there was a significant main effect of nutrient addition on enzyme activity ($P < 0.05$ for all significant nutrient effects), mostly reflecting a reduction in enzyme activity with nutrient addition. Phosphatase activity was reduced by 28% in the P and 40% in the

complex N+P treatments compared to control soils (Figure 2.3a, Tukey's HSD, $P < 0.001$). Beta-xylosidase activity was reduced in the complex N+P treatment compared with all other treatments (Figure 2.3c, Tukey's HSD, $P < 0.001$), and cellobiohydrolase activity was significantly reduced in the complex N+P treatment compared with the simple N+P treatment (Figure 2.3d, Tukey's HSD, $P = 0.02$). In contrast to the C-degrading enzymes, *N*-acetyl glucosaminidase activity increased 162% in in the complex N+P treatment (Figure 2.3e, $P < 0.001$).

Several enzyme activities displayed significant time × nutrient interaction effects. Specifically, Tukey's HSD test revealed that for all three C-degrading enzymes and *N*-acetyl glucosaminidase, activity increased over time in simple N+P treatment relative to controls. At 9 weeks, simple N+P treated soils exhibited 52% greater beta glucosidase activity, 50% greater beta xylosidase activity, 82% greater cellobiohydrolase activity, and 86% greater *N*-acetyl glucosaminidase activity compared with control soils (Figure 2.4 a-d).

Enzyme C:N, C:P and N:P ratios were all significantly affected by nutrient treatment. The C:N enzyme ratio was significantly lower in the complex N+P treatment compared with all other soils (Tukey's HSD, $P < 0.001$) and highest in the P treatment, which differed significantly from both N treatments (Figure 2.5, Tukey's HSD, $P = 0.03$). By contrast, both the C:P and N:P enzyme activity ratios were highest in the complex N+P treatment, followed by the P and simple N+P treatments (Figure 2.5, Tukey's HSD, $P < 0.01$).

2.4. Discussion

For all enzymes measured, we found support for resource allocation theory as applied to soil microbes. The decline in phosphatase activity with P additions is consistent with previous studies that P additions suppressed phosphatase activity (*Clarholm*, 1993; *Olander and Vitousek*, 2000). In our experiment, the likely cause is an increase in P availability relative to N availability, which increased microbial N demand relative to P. The shift toward higher N:P enzyme ratios with added P speaks to a shift in microbial resource allocation. In the complex N+P treatment, one might expect that increased N availability would maintain high microbial P demand and phosphatase activity. However,

complex forms of N require enzymatic degradation, while the supplemented P was in a labile form. It is, therefore, likely that P availability increased faster than N availability in this treatment, thus increasing microbial N demand more than P. This increased N demand was potentially alleviated over time as N availability increased. In the simple N+P treatment, extractable N accumulated rapidly in the soil, and phosphatase activity remained high, indicating that additions of labile N maintained microbial P demand. That the simple N treatment also contained excess P may explain why phosphatase activity did not, in fact, increase relative to control soils; by providing soil microbes with these two nutrients in immediately assimilable forms, we may have avoided inducing P-limitation.

The three C-degrading enzymes measured in this study (beta glucosidase, beta xylosidase and cellobiohydrolase) all behaved similarly in response to nutrients, increasing over time in response to simple N+P. High N availability, as indicated by high extractable N in this treatment, may have led to greater incorporation of N into microbial biomass, thus leading to the observed increase in organic N. Greater microbial biomass production can exacerbate microbial C-limitation, leading to the production of more C-acquiring enzymes, as has been observed in deciduous forests (*Waldrop et al.*, 2004; *Sinsabaugh et al.*, 2005) and grasslands (*Henry et al.*, 2005). In the complex N treatment, reduced beta xylosidase and cellobiohydrolase activity coupled with decreased C:N enzyme ratios suggests microbial allocation towards increased N acquisition and decreased C acquisition. However, given that our complex N treatment also contained added C, it is possible that declines in beta xylosidase and cellobiohydrolase activity in this treatment represent a shift towards acquisition of the added protein substrate that is independent of changes in microbial stoichiometric demand.

The one N-acquiring enzyme measured in this study (*N*-acetyl glucosaminidase) also responded to nutrient additions in a manner consistent with microbial resource allocation theory. The large increase in *N*-acetyl glucosaminidase activity in the complex N+P treatment suggests this enzyme's production was strongly induced in response to complex N. The decline in C:N and increase in N:P enzyme ratios support the notion of a shift towards N-acquisition in the complex N treatment. The specific function of this enzyme is the hydrolysis of β-*N*-acetylglucosamine residues from oligosaccharides (*German et al.*, 2011). While *N*-acetyl glucosaminidase is considered functionally

important in soils as a chitin or peptidoglycan-degrading enzyme, it is also involved in the deglycosylation of some proteins (*Stals et al.*, 2010). Our finding that this common soil enzyme appears inducible by intermediate degradation products that signal substrate availability is consistent with our understanding of microbial physiology (*Priest*, 1977) and with previous nutrient manipulation experiments in soils (*Allison and Vitousek*, 2005; *Averill and Finzi*, 2011). It should also be noted that the increase in *N*-acetyl glucosaminidase activity over time in the simple N+P treatment may speak to the dual role of this enzyme as a C and N-acquiring enzyme. All sources of organic N also contain C, and chitin, as the second most abundant biopolymer on earth (*Jolles and Muzzarelli*, 1999), is considered both an important C and N source for microorganisms.

The second objective of this study was to evaluate the role of roots in mediating enzymatic responses to nutrients. We hypothesized that in the complex N treatment, root-derived C in the form of increased root biomass or exudates would alleviate microbial C limitation and lead to greater enzyme activity, while additions of simple N would have the reverse effect. We found support for the first part of this hypothesis, in that mean root biomass in the complex N+P treatment was greater than controls and mean root biomass in the simple N+P treatment was less than controls. The fact that the largest increase in root biomass occurred in the P-only treatment may speak to the fact that P is a more limiting nutrient than N in these soils, as has been widely observed across tropical forests (*Vitousek et al.*, 2010). Additionally, the large increase in shoot biomass in the complex N relative to all other treatments suggests that seedling growth was substantially enhanced by microbial degradation of organic N. However, these shifting allocation patterns in plants did not appear to influence microbial enzyme activity very strongly in most cases, and in no instances did we find the anticipated planting $\times$ nutrient effect in response to complex N. There are several potential explanations for the lack of a strong effect of roots on enzyme activities. Firstly, our hypothesis that enzyme activity would be enhanced in the presence of roots is premised on the assumption that microbial biomass is C-limited, however, microbial biomass is often found to be P limited (*Cleveland and Liptzin*, 2007), particularly in tropical soils (*Cleveland and Townsend*, 2006). If microbial biomass were N or P limited, microbial responses to root-derived C additions would be constrained by N or P availability, as demonstrated in recent modeling studies (*Drake et al.*, 2013) and in studies testing

nutrient co-limitation of microbial activity (e.g., *Vance and Chapin* (2001); *Brown et al.* (2009); *Krashevska et al.* (2010)). In our simple N treatment, increases in C-acquiring enzyme activity over time indicate a shift towards microbial C-limitation, however, we were unable to determine whether roots had a positive effect on enzyme activities following repeated mineral N additions due to the death of most seedlings by the end of this treatment. Another possibility suggested by seedling death in the simple N treatment is that our N addition rate was high compared to *T. heterophylla* N requirements and that root priming effects were consequently reduced in N treatments. This is also consistent with the fact that we did not observe significant differences in extractable N between planted and unplanted soils, despite the increased incorporation of N into plant biomass as evidenced by the dramatically lower root and shoot C:N ratios in the N treatments compared with controls. Additionally, our sterilization of *T. heterophylla* seeds and subsequent germination under sterile conditions could have decreased mycorrhizal fungal colonization, which can substantially affect plant allocation patterns (*Rygiewicz and Andersen*, 1994). Finally, in the complex N treatment microbes may not have had to rely heavily on root exudates as a source of labile C due to the fact that casein also contains C that can be released through enzymatic degradation.

We recognize that a lack of microbial biomass measurements limits our interpretations with respect to microbial resource stoichiometry. Changes in enzyme activities can represent both shifts in total activity and shifts in activity per unit microbial biomass (specific activity), and differences in total enzyme activity can therefore be confounded with differences in the size of the microbial population. For instance, additions of mineral N fertilizer are often found to reduce microbial biomass (*Compton et al.*, 2004; *Treseder*, 2008; *Wallenstein et al.*, 2006), and reductions in enzyme activity in N-amended soils may therefore represent a decline in the size of the microbial biomass rather than a shift in resource allocation. Examining ratios of C:N, C:P and N:P acquiring enzyme activity provided us with an alterative method for evaluating nutrient demand in the absence of biomass data. However, enzymatic C:N:P ratios are generally found to converge on 1:1:1, suggesting that the plasticity of these ratios is relatively constrained (*Sinsabaugh et al.*, 2008, 2009). We also acknowledge limitations associated with extrapolating from laboratory enzyme assays, which can provide an index of potential enzyme activity, to activity *in situ*, which can be hindered by diffusion

limitation, enzyme-mineral interactions, variable substrate concentrations and other physical and chemical properties of the soil matrix (*Wallenstein and Weintraub*, 2008; *German et al.*, 2011).

Although the changes in enzyme activities we observed are consistent with resource allocation theory, there are alternative interpretations to our results that speak to the complexity of the interactions both within soil microbial communities and with their environment. Microbial community structure often shifts in response to nutrients (e.g., *Frey et al.* (2004); *Allison et al.* (2007); *Allison and Martiny* (2008); *Nemergut et al.* (2008)). Changes in community structure can occur due to changes in the relative fitness of various microorganisms under altered nutrient regimes (*Fierer and Jackson*, 2006; *Allison et al.*, 2007; *Fierer et al.*, 2007), leading to selection for organisms that can better exploit available resources. Such community shifts could alter the types of and abundances of enzyme producers. Shifts in bacterial:fungal ratios, which are often observed in N-addition studies, can alter the stoichiometry of the microbial biomass, which in turn can affect resource demand as well as the types of enzymes produced. N additions can also directly inhibit the production of some enzymes by soil fungi (*Fog*, 1988; *Carreiro et al.*, 2000; *Saiya-Cork et al.*, 2002).

2.5. Conclusions

In this study, we found that specific nutrient acquiring enzyme activities can be induced by the presence of a complex substrate, repressed in response to a labile degradation product, or induced in response to a shift in the availability of co-limiting nutrients. Although we observed shifts in plant C allocation (as measured by root biomass) and increased N-uptake by plants in both N addition treatments, we did not observe an effect of belowground C investment on soil enzyme activities in the complex N+P treatment. Increased aboveground plant biomass in the complex N+P treatment, decreased extractable soil C in planted soils, and the lack of a statistically significant increase in soil organic N in the complex N+P treatment suggest that in our experiment, plants may have been effective competitors for N released through microbial enzymatic degradation. Future studies using new methods of measuring soluble root exudates, microbial RNA transcripts and proteomics will shed more light on plant-soil interactions and the link between microbial community structure and function.

Table 2.1: Nutrients added, chemical forms and rates of nutrient addition. All nutrients were added bi-monthly for 9 weeks as 30 ml solutions.

Treatment	Chemical form	Rate of Addition (g^{-1} dry soil)
Control	none	none
P	Na_2PO_4	320 μg P
Simple N +P	NH_4Cl + Na_2PO_4	1.6 mg N + 320 μg P
Complex N +P	Casein + Na_2PO_4	1.6 mg N + 320 μg P

Table 2.2: Total extractable C and N by nutrient and planting treatments over time. Values are expressed in mg C or N g^{-1} dry soil. Values in bold are statistically different from unplanted control soils within their given time interval. n = 8 for all time × nutrient × planting combinations, except for 9 week unplanted controls (n = 4) and 9 week unplanted + P (n = 5).

Week	Treatment	C-extract. Planted	C-extract. Unplanted	N-extract. Planted	N-extract. Unplanted
3	Control	0.81±0.10	0.87 ± 0.13	0.64 ± 0.33	0.34 ± 0.04
3	P	0.88 ± 0.14	0.93 ± 0.15	0.67 ± 0.40	0.42 ± 0.13
3	Simple N+P	1.05 ± 0.27	0.94 ± 0.12	**3.52 ± 0.34**	**3.33 ± 0.57**
3	Complex N+P	**1.93 ± 0.37**	**2.90 ± 0.50**	**2.19 ± 0.34**	**2.73 ± 0.57**
6	Control	0.77 ± 0.06	0.85 ± 0.08	0.46 ± 0.03	0.45 ± 0.06
6	P	0.93 ± 0.08	0.91 ± 0.08	0.46 ± 0.02	0.80 ± 0.30
6	Simple N+P	0.93 ± 0.07	0.72 ± 0.05	**7.39 ± 0.67**	**5.95 ± 0.52**
6	Complex N+P	**1.22 ± 0.14**	**1.36 ± 0.14**	**2.92 ± 0.29**	**3.60 ± 0.43**
9	Control	1.39 ± 0.25	1.95 ± 0.24	0.53 ± 0.06	0.69 ± 0.09
9	P	1.62 ± 0.26	1.97 ± 0.25	1.47 ± 0.77	0.92 ± 0.21
9	Simple N+P	1.70 ± 0.24	1.53 ± 0.21	**6.42 ± 0.28**	**6.29 ± 0.24**
9	Complex N+P	1.86 ± 0.20	**3.42 ± 0.43**	**4.00 ± 0.30**	**4.43 ± 0.32**

Table 2.3: Ammonia, nitrate and organic N concentrations by nutrient and planting treatment over time. Values are expressed in mg N g^{-1} dry soil. Values in bold are statistically different from unplanted control soils within their given time interval. n = 3 for all time × nutrient × planting combinations

Week	Treatment	Ammonia	Ammonia	Nitrate	Nitrate	Organic N	Organic N
		Planted	Unplanted	Planted	Unplanted	Planted	Unplanted
3	Control	0.73 ± 0.63	0.10 ± 0.00	0.04 ± 0.02	0.07 ± 0.03	0.30 ± 0.12	0.20 ± 0.06
3	P	0.80 ± 0.70	0.1 ± 0.00	0.03 ± 0.02	0.03 ± 0.01	0.37 ± 0.22	0.17 ± 0.07
3	Simple N+P	2.43 ± 0.55	1.77 ± 0.85	0.03 ± 0.00	0.04 ± 0.00	1.30 ± 0.15	0.93 ± 0.43
3	Complex N+P	1.60 ± 0.38	1.77 ± 0.84	0.02 ± 0.01	0.02 ± 0.01	0.93 ± 0.29	1.20 ± 0.55
6	Control	0.10 ± 0.00	0.07 ± 0.03	0.01 ± 0.00	0.01 ± 0.01	0.33 ± 0.03	0.20 ± 0.06
6	P	0.01 ± 0.00	0.10 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.33 ± 0.03	0.37 ± 0.07
6	Simple N+P	1.87 ± 0.09	2.10 ± 0.26	0.02 ± 0.01	0.01 ± 0.00	4.40 ± 0.70	4.80 ± 0.87
6	Complex N+P	0.83 ± 0.22	1.07 ± 0.13	0.11 ± 0.08	0.01 ± 0.01	1.83 ± 0.43	2.77 ± 0.33
9	Control	0.07 ± 0.03	0.07 ± 0.03	0.03 ± 0.01	0.01 ± 0.00	1.87 ± 1.38	2.00 ± 1.31
9	P	0.73 ± 0.63	0.07 ± 0.03	0.02 ± 0.01	0.01 ± 0.00	1.57 ± 1.12	1.60 ± 0.87
9	Simple N+P	1.43 ± 0.07	1.57 ± 0.03	0.02 ± 0.02	0.02 ± 0.01	5.10 ± 0.71	3.77 ± 1.73
9	Complex N+P	1.40 ± 0.00	1.30 ± 0.20	0.21 ± 0.12	0.02 ± 0.01	3.00 ± 0.30	3.33 ± 1.04

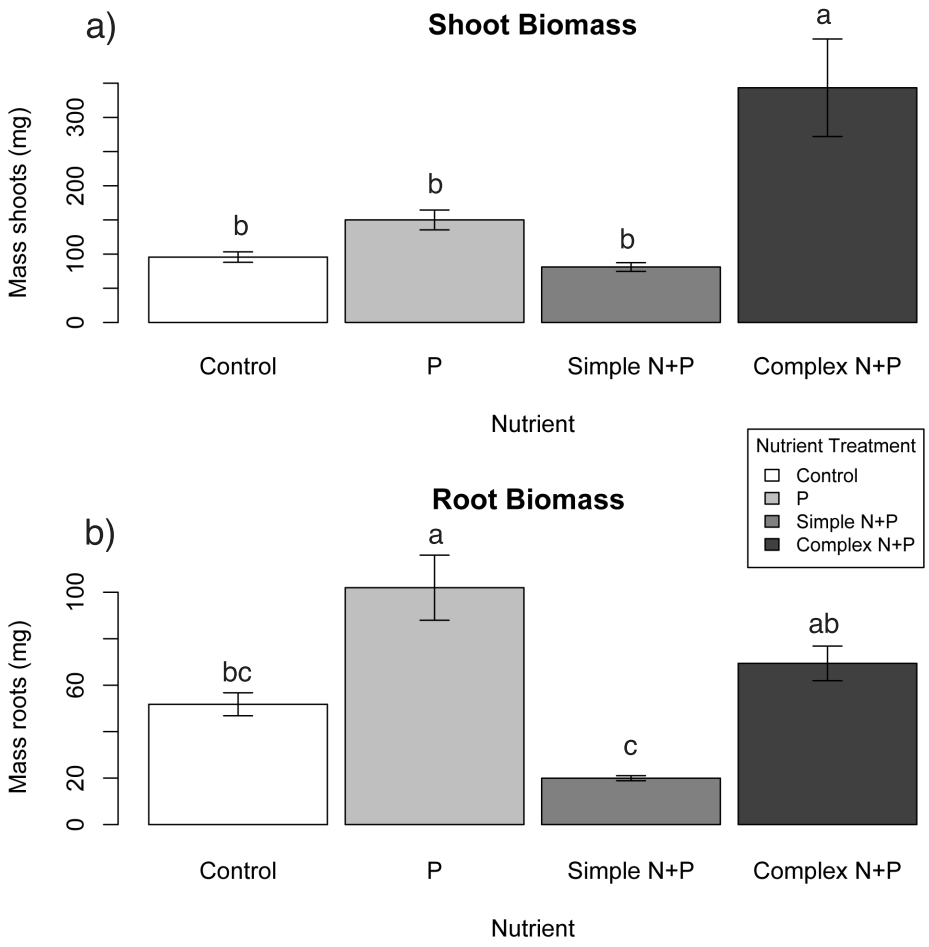

Figure 2.1: Mean a) shoot and b) root biomass across nutrient treatments. These values are averaged for all time intervals to highlight treatment effects. Bars that do not share a letter are statistically different (Tukey's HSD, $P < 0.05$).

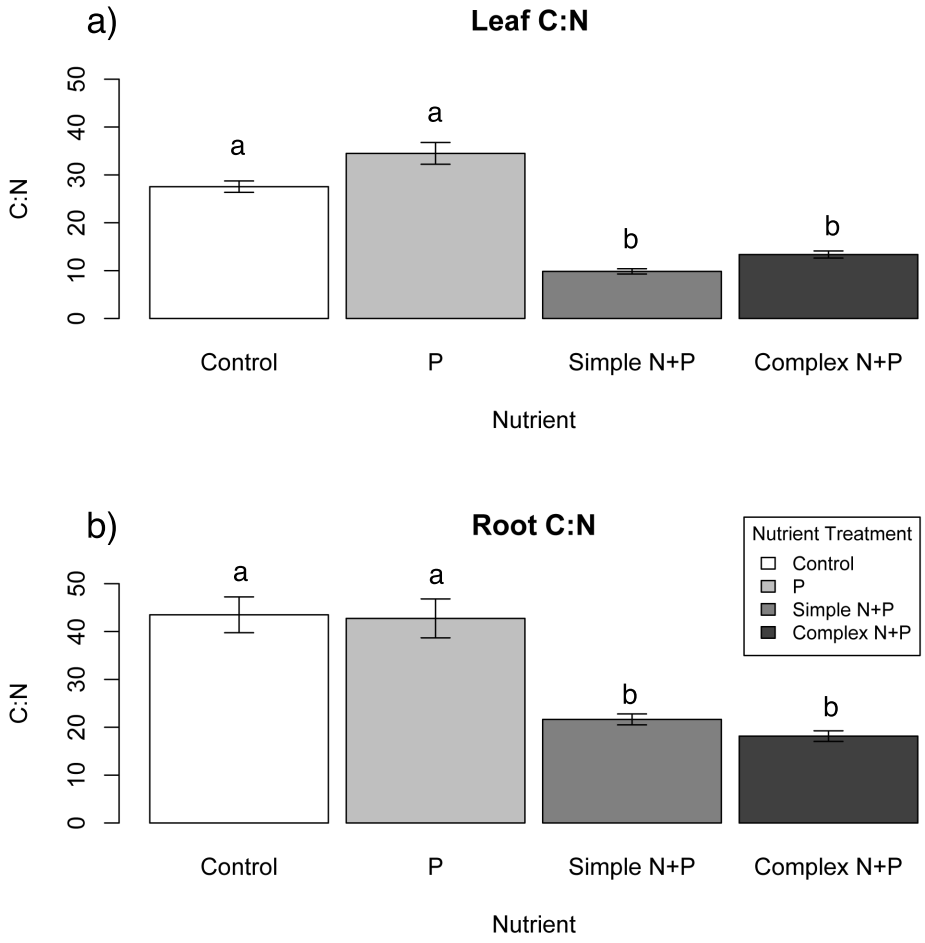

Figure 2.2: Mean a) leaf and b) root C:N ratios across nutrient treatments. These values are averaged for all time intervals to highlight treatment effects. Bars that do not share a letter are statistically different (Tukey's HSD, $P < 0.05$).

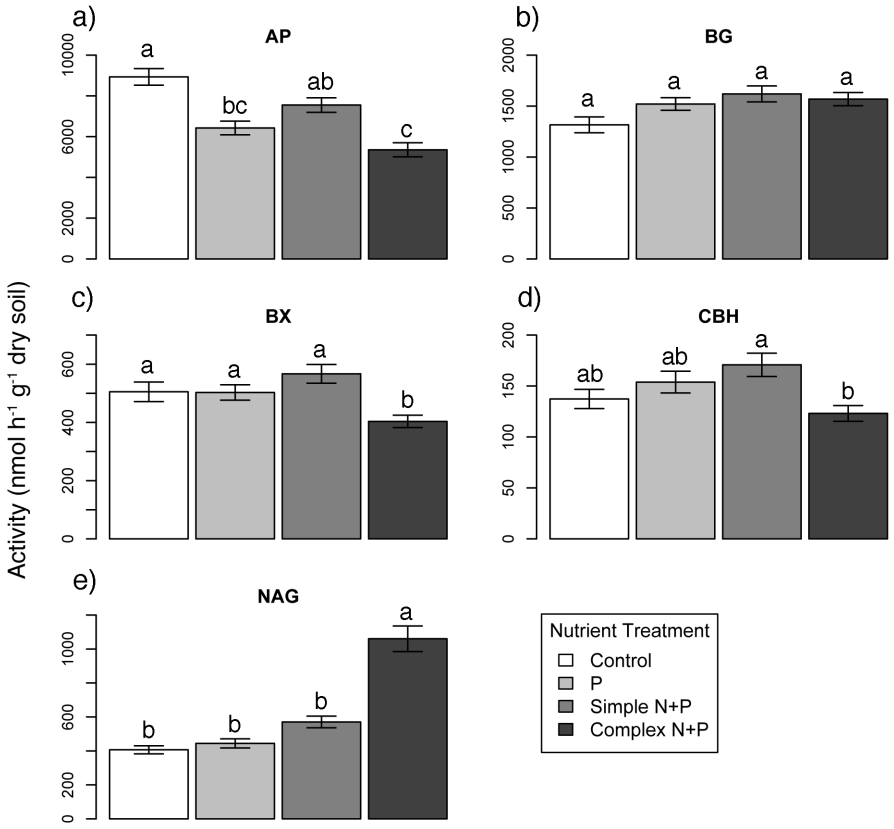

Figure 2.3: Enzyme activities across nutrient treatments for five hydrolytic enzymes studied: a) acid phosphatase (AP), b) beta-glucosidase (BG), c) beta-xylosidase (BX), d) cellobiohydrolase (CBH), e) *N*-acetyl glucosaminidase (NAG). Activity values are expressed in nanomoles h^{-1} g^{-1} dry soil. Values with different letters are statistically different (Tukey's HSD, $P < 0.05$). N values per treatment are as follows: control = 43, P = 44, simple N+P = 47, complex N+P = 47.

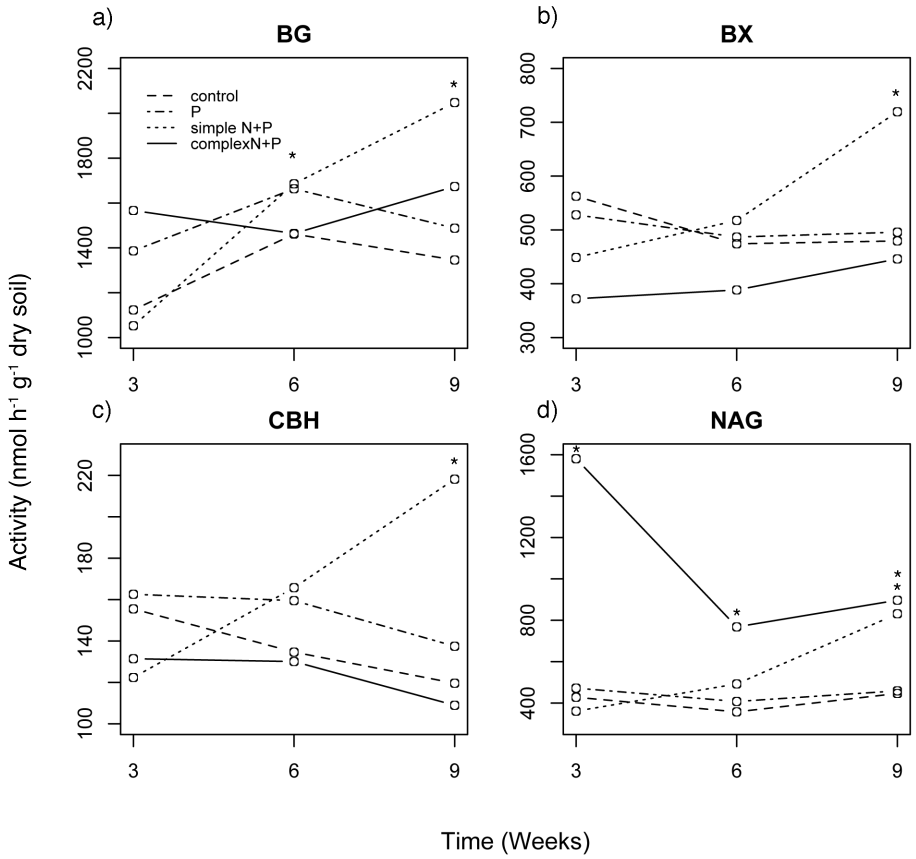

Figure 2.4: C and N-degrading enzyme activities by nutrient treatment over time. Stars indicate treatments that are significantly different from control soils at the given time interval (Tukey's HSD, $P < 0.05$).

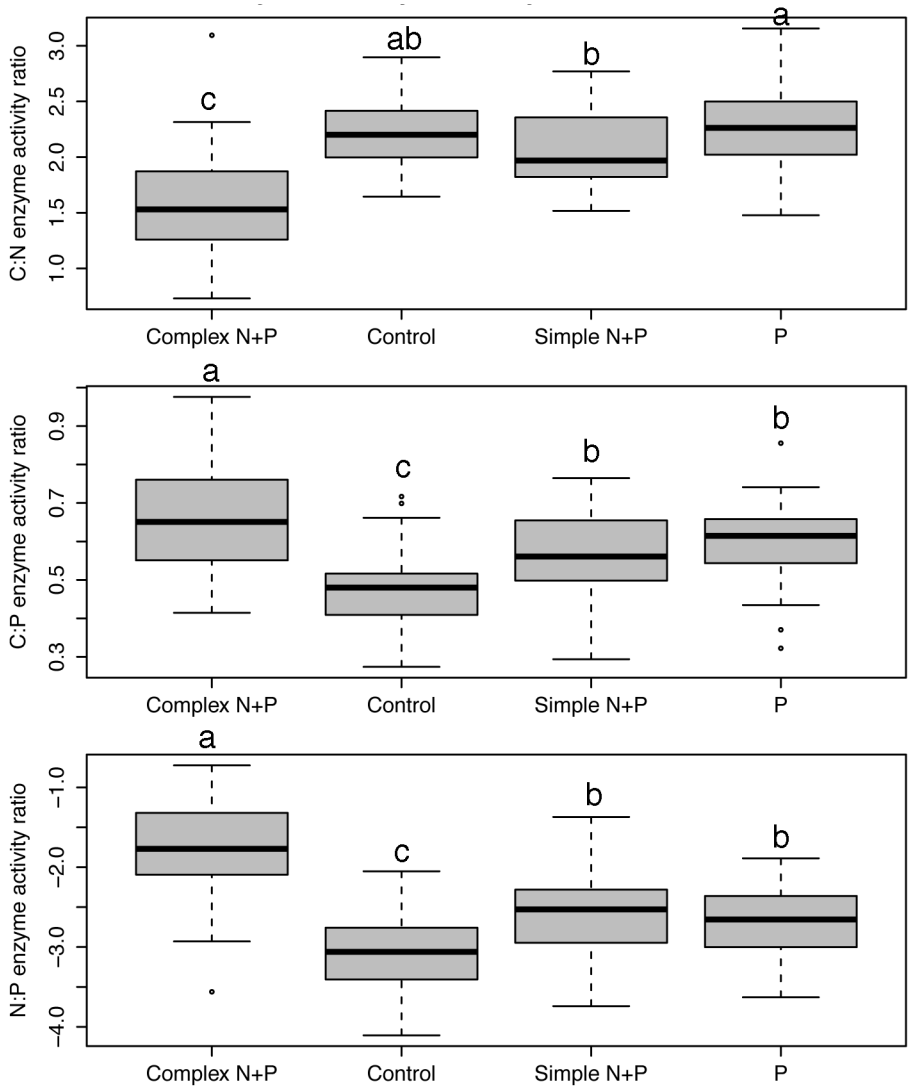

Figure 2.5: Enzyme activity C:N, C:P and N:P ratios by nutrient treatment. Box ranges represent the upper and lower quartile bounds of the data, while whiskers extend to the most extreme data point that is no more than 1.5 times the interquartile range for the box . Groups that are not connected by the same letter are significantly different (Tukey's HSD, $P < 0.05$).

CHAPTER 3 : Changes in extracellular enzyme activity and microbial community structure with soil depth at the Luquillo Critical Zone Observatory

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Abstract:

Extracellular enzymes in soils mediate the decomposition of organic matter and catalyze key transformations in carbon, nitrogen and phosphorus cycling. However, most studies of extracellular enzyme activity have focused exclusively on relatively carbon and nutrient-rich surface soils. In tropical forests, several centimeters of nutrient-rich surface soil can overlay meters of resource-poor subsoil, of which the microbial ecology is poorly characterized. The goal of this study was to determine how extracellular enzyme activity changes as a function of depth across two soil orders (Oxisols and Inceptisols) and two forest types that occur at different elevations (Tabonuco, lower elevation; Colorado, higher elevation) at the Luquillo Critical Zone Observatory in northeast Puerto Rico. We excavated three soil pits to 140 cm at four different sites representing the four soil × forest combinations, and measured potential activities of four carbon-acquiring enzymes (α-glucosidase, β-glucosidase, β-xylosidase, cellobiohydrolase), one nitrogen-acquiring enzyme (*N*-acetyl glucosaminidase) and one organic phosphorus-acquiring enzyme (acid phosphatase) at six discrete depth intervals. We used phospholipid fatty acid (PLFA) analysis to assess viable microbial biomass and community structure. Overall, microbial biomass, specific enzyme activities and community structure were similar across the two soil and forest types, in spite of higher carbon concentrations and C:N ratios in the Colorado forest soil. Soil nutrients, microbial biomass and potential enzyme activities all declined exponentially with depth. However, when normalized to

microbial biomass, specific enzyme activities either did not change with depth (β-glucosidase, β-xylosidase, cellobiohydrolase and *N*-acetyl glucosaminidase) or increased significantly with depth (α-glucosidase and acid phosphatase, $P < 0.05$). Principal components analysis of PLFA biomarkers revealed shifts in community structure with depth ($P < 0.01$), driven largely by a decline in fungal:bacterial ratios, an increase in gram positive and actinobacteria biomarkers, and a decrease in gram negative biomarkers. Shifts in community structure, upregulation of enzyme production in response to resource scarcity and decreased enzyme turnover rates may all contribute to high specific enzyme activities in subsoils. Our study indicates that low-carbon tropical subsoils contain small but metabolically active microbial communities, and that specific enzyme activities can be used to examine changes in microbial physiology across orders of magnitude gradients in soil carbon concentrations.

3.1. Introduction

Microbial communities in soils produce extracellular enzymes to acquire energy and resources from complex biomolecules in the soil environment (*Burns*, 1982). These enzymes are of interest on an ecosystem scale because they catalyze important transformations in the carbon (C), nitrogen (N) and phosphorus (P) cycles (*Wallenstein and Burns*, 2011). However, most studies of microbial communities and their associated enzymes have been restricted to the upper 15 cm of the soil, despite the fact that soil microbes influence biogeochemical processes throughout soil profiles (*Blume et al.*, 2002; *Buss et al.*, 2005; *Fierer et al.*, 2003a). Tropical forest soils are often many meters deep and tropical subsoils store large amounts of C, contributing approximately 50% of the C stored at depths >1 meter (*Jobbagy and Jackson*, 2000). It is thus reasonable to predict microbial communities may contribute to nutrient cycling in deeper parts of tropical soil profiles than have historically been measured.

Numerous soil properties can influence microbial communities, including carbon availability and composition (*Bending et al.*, 2002; *Griffiths et al.*, 1999), pH (*Rousk et al.*, 2010), temperature (*Zogg et al.*, 1997), redox status (*DeAngelis et al.*, 2010), texture (*Sessitsch et al.*, 2001), and mineralogy (*Heckman et al.*, 2009). All of these properties can change with depth, some by orders of magnitude. The environmental gradient represented by depth profiles influences the abundance, composition and functions of soil microbial communities. For example, microbial biomass and substrate pools generally decline with soil depth (*Blume et al.*, 2002; *Fang and Moncrieff*, 2005; *Fierer et al.*, 2003a; *Kramer et al.*, 2013). Furthermore, microbial community structure often changes with depth (*Eilers et al.*, 2012; *Ekelund et al.*, 2001; *Hansel et al.*, 2008; *Hartmann et al.*, 2009), possibly reflecting an increasing dominance of organisms that can maintain basal metabolism under conditions of low energy availability (*Hoehler and Jorgensen*, 2013). Several studies have found that potential enzyme activities decrease with depth (*Gelsomino and Azzellino*, 2011; *Snajdr et al.*, 2008; *Steinweg et al.*, 2013; *Taylor et al.*, 2002; *Venkatesan and Senthurpandian*, 2006). However, specific enzyme activities (activity normalized to biomass or substrate availability) have not been measured in most cases, and it is therefore difficult to separate physiologically adaptive changes in

enzyme activities from changes due solely to differences in biomass and substrate quantities (*Trasar-Cepeda et al.*, 2008). The few studies that have normalized microbial assimilation or mineralization activities to the size of the microbial biomass show either similar values throughout the soil profile or an increase in specific activity with depth (*Blume et al.*, 2002; *Gelsomino and Azzellino*, 2011; *Kramer et al.*, 2013; *Tate*, 1979). Additionally, increased mineral association in subsoils can lead to greater stabilization of organic materials (*Eusterhues et al.*, 2003; *Rasse et al.*, 2005). Changes in the fraction of enzymes that are sorbed to clay minerals or organo-mineral complexes with depth can influence both potential enzyme activities and enzyme turnover rates (*Allison*, 2006; *Taylor et al.*, 2002).

The primary objective of this study was to assess changes in the potential and specific activity of hydrolytic enzymes involved in C, N and P acquisition with increasing soil depth across the Luquillo Critical Zone Observatory (LCZO), a montane tropical forest in northeastern Puerto Rico. We hypothesized that energy (C) availability would be the major driver of the vertical distribution of enzyme activities. Specifically, we hypothesized that total enzyme activity would decline with depth; tracking declines in C availability and microbial biomass. We further hypothesized that enzyme activity per unit microbial biomass (i.e., specific activity) would increase with depth, reflecting greater microbial allocation to enzyme production in response to decreased resource (C and nutrient) availability (*Allison et al.*, 2011; *Sinsabaugh and Moorhead*, 1994). Our second objective was to investigate changes in microbial community structure with depth at the LCZO. By doing so, we hoped to evaluate whether changes in enzyme activities were functional, or attributable to changes in community structure. Lastly, our experimental design allowed us to examine how landscape-scale gradients in geologic parent material and vegetation at the LCZO mediate changes in soil enzyme activities with depth. We predicted that in surface soils, enzyme activity would be principally driven by forest type, due to differences in leaf litter chemistry (and therefore substrate chemistry) among forest types (*Cusack et al.*, 2011; *Fonte and Schowalter*, 2004). We predicted the influence of vegetation would decline with depth and that in subsoils, enzyme activity patterns would be influenced primarily by parent material.

3.2. Methods

3.2.1. Study site and sample collection

This study was conducted using soils from the Luquillo Critical Zone Observatory (LCZO) in north-eastern Puerto Rico (18°18' N, 65°50' W). The LCZO offers a natural experiment for studying changes in microbial community characteristics with depth in the context of landscape-scale gradients in geology, vegetation and climate. The LCZO is composed of two dominant parent materials of differing age and mineralogy: lower-Cretaceous volcaniclastic (VC) sediments of andesitic composition and an early-Tertiary age quartz-diorite (QD) pluton known as the Rio Blanco stock (*Seiders*, 1971a,b). The mountainous region is characterized by steep terrain and is highly dissected by slopes > 30°. The mean annual temperature decreases from approximately 24 °C at 300 masl to 21 °C at 800 masl and precipitation increases from 3000 mm y^{-1} to 4000 mm y^{-1} across the same elevation gradient (*Brown et al.*, 1983). Most of the vegetation falls into four climate-designated life zones *sensu Holdridge* (1967): subtropical wet forest, subtropical rainforest, lower montane wet forest, and lower montane rainforest. The LCZO is covered primarily by mature Tabonuco (*Dacroydes excesla* Vahl) forest at low elevations (<600 masl) and Palo Colorado (*Cyrilla racemiflora* L.) forest at intermediate elevations (600-800 masl). Sierra palm forest is found across all elevations and is dominant at the highest elevations (800-1000 masl, *Brown et al.* (1983); *Weaver* (1991)).

Soils in the LCZO are 0.5–1.5 m thick, underlain by saprolite that ranges in thickness from ~2 m on steep hillslopes to up to 23 m on ridgetops (*Buss et al.*, 2010; *Simon et al.*, 1990). The VC parent material weathers to produce Oxisols, which are classified as Humaquoxes on flat ridges, and Aquic and Inceptic Hapludoxes on slopes and in valleys (Table 3.1). The QD parent material weathers to produce Inceptisols, which are classified as Histic Humaquepts on ridges, and Aquic or Aquic Humic Dystrudepts on slopes and in valleys (Table 3.1, (Soil Survey staff, 2013)). All soils are moderately to strongly acidic and contain mostly kaolinitic minerals, with iron and aluminum oxyhydroxides in the clay fraction. The Oxisols are strongly weathered, fine-textured soils that are high in iron and aluminum and contain < 10% primary minerals, while the Inceptisols are coarser-textured and contain up to 40% primary minerals in surface soils (*Johnson and Hao*, 2013; *Scatena*,

1989; *Silver*, 1994).

To capture as much edaphic variability as possible in our study, we collected soils from four sub-watersheds (5–20 ha in size) throughout the LCZO that spanned 360–780 masl in elevation and represented Tabonuco and Colorado forest on VC and QD parent material (Table 3.1). Previous work has shown there are significant differences in soil properties associated with topographic position at the sub-watershed scale (*Johnson et al.*, 2011; *Scatena and Lugo*, 1995; *Silver*, 1994); we therefore deliberately incorporated topographic variation into our sampling design. Within each sub-watershed we sought pronounced local ridges (slope < 10°) that do not receive significant deposition from above. From each ridge, we sampled along catenas that descended into local ephemeral streams. Five soil pits were excavated along each catena; three on slope terrain, one on a ridgetop and one in a valley. Soil profiles were excavated to 140 cm or bedrock using a bucket augur. Samples were collected from the wall of the pit at discrete 10 cm intervals beginning at the surface of the mineral soil. Samples were immediately refrigerated at 4 °C following collection, and shipped on ice to the University of Pennsylvania where they were field-fresh sieved to 5 mm to homogenize. Samples from one slope pit, the ridge pit and the valley pit were used for enzyme and PLFA analysis. Soil chemical analyses (see below) were performed on samples from all five pits. One subsample was frozen at -20 °C until enzyme analysis, a second subsample was freeze-dried for phospholipid fatty acid analysis (PLFA), and a third subsample was air-dried and subsequently sieved to 2 mm for chemical analyses. We acknowledge that freezing may have a negative effect on enzyme activities as has been observed in other studies (*DeForest*, 2009; *German et al.*, 2011; *Turner and Romero*, 2010) but as it was not possible to assay this number of samples under field conditions in a timely manner, we focused on treating all samples identically so that sample storage did not bias our experimental outcome. Repeated measurements of the same samples indicated that activity within frozen samples varied < 10% over the duration of the experiment.

3.2.2. Soil chemical analyses

Prior to C and N analyses, a subsample of air-dried soil was ground (< 500 μm) with a mortar and pestle. Total C and total N concentrations of soils were determined by dry combustion analysis

using a Carlo-Erba NA 1500 elemental analyzer (Fisons Instruments, Beverly MA). In cases where carbon concentrations were < 0.1% as measured by combustion analysis, samples were re-run on a stable isotope mass-spectrometer (Delta Plus, Thermo-Finnigan MAT, Bremen, Germany) to improve the precision of our measurements. Given the low pH of the soils, total C concentrations can be equated to organic C concentrations. Available P was determined using a partial Hedley sequential fractionation based on the protocol of *Tiessen and Moir* (1993) with several modifications. First, 1–5 grams of sample were extracted by shaking soil in 30 ml of 0.25 M $NaHCO_3$ solution for 16 hours. Extracts were centrifuged and then filtered using a 0.45 μm nitrocellulose filter. The soil was then extracted again with 0.1 M NaOH following the same procedure. Total P concentrations in the $NaHCO_3$ and NaOH extracts were determined using ICP-OES spectrometry (Genesis ICP-OES, Spectro Analytical Instruments Gmbh, Kleve, Germany). Total extractable P is reported as the sum of $NaHCO_3$- and NaOH- extractable P. Soil pH was determined in a 1: 10 soil : distilled water slurry.

3.2.3. Phospholipid fatty acid analysis

We investigated soil PLFA composition both to evaluate changes in microbial community structure with depth, soil type and forest type, and as an index of viable microbial biomass (*Federle et al.*, 1986). Total lipids were extracted using 10 ml of methanol, 5 ml of chloroform and 4 ml of phosphate buffer from 5 g of freeze-dried soil (*DeForest et al.*, 2012; *White et al.*, 1979). Analytical recovery for the procedure was determined by adding phospholipid 19:0 as an internal standard (1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine, Avanit Polar Lipids, Inc.). Three blanks consisting of 5 g of combusted sterile sand were extracted along with the samples to identify potential contamination and validate low biomass samples. Polar lipids were separated from other lipids (e.g. neutral and glycolipids) using silicic acid solid-phase chromatography columns (Thermo Scientific). Separated polar lipids were then converted to fatty acid methyl esters (FAME) through methanolysis (*Guckert et al.*, 1985). FAMEs were separated using a HP GC-FID (HP6890 series, Agilent Technologies, Inc.) gas chromatograph. External FAME standards (K101 FAME mix, Grace) were used to determine concentrations.

A total of 87 biomarkers were identified across all samples using the Sherlock System (v. 6.1, MIDI, Inc.). Standard PLFA nomenclature was used where the first number refers to the number of carbon atoms, the second number refers to the number of carbon double bonds, and the third number (after the ϖ) is the location of the double bond on the fatty acid chain relative to the aliphatic end. We removed from analysis any PLFA biomarkers if they had low biomass (< 1% mol fraction) and only occurred in a single sample, reducing the number of biomarkers to 84. For several biomarkers (16:3ϖ6c, 16:0, *i*17:1ϖ9c, 17:0,18:0 and 21:0), a small amount of contamination was present in the blanks. We calculated the average blank value for each of these biomarkers and subtracted it from the measured value for each sample. Groups of microorganisms were assigned based on indicator PLFA, such as iso (i) and ante-iso (a) branched fatty acids (gram positive bacteria), methyl (Me) branched fatty acids (actinobacteria), monosaturated and cyclopropyl (cy) lipids (gram negative bacteria) and dimethyl acetyls (DMAs) (anaerobic bacteria, *Zelles* (1999)). Biomarker group abundance (% mol fraction) was calculated by dividing the sum of biomarker groups by total PLFA biomass. Fungal : bacterial ratios were calculated by dividing the sum of 16:1ϖ5c and 18:2ϖ6c by the sum of all biomarkers associated with bacteria (e.g., *i*15:0, *i*16:0, 17:0, cy17:0). Phospholipid fatty acid abundance is reported as μg C g^{-1} dry soil.

3.2.4. Enzyme Assays

The activities of six hydrolytic enzymes that are involved in C (α-glucosidase, (AG); β-glucosidase, (BG); cellobiohydrolase, (CBH); β-xylosidase (BX)), nitrogen (*N*-acetyl glucosaminidase, (NAG)) and P (acid phosphatase, (AP)) acquisition were measured according to the protocol of *Saiya-Cork et al.* (2002) with modifications by *German et al.* (2011). The specific functions of the enzymes assayed are listed in Table 3.2. Briefly, 0.5 g of field-moist, frozen soil was homogenized in 62.5 ml of sodium acetate buffer using a hand blender. The pH of the sodium acetate buffer was experimentally varied to approximate the pH of the soil samples being assayed, with pH values ranging from 3.7-5.0 (Table 3.3). 50 μl of fluorescent substrate proxies specific to each enzyme were added to eight replicate assay wells in optimal concentrations for measuring total potential activity (optimal concentrations determined prior to experiment, Table 3.1). Assays were run with two standard

columns containing soil homogenate and methylumbelliferone (MUB). Each assay microplate also contained substrate blank columns receiving 50 µl of substrate and 200 µl of sodium acetate buffer. Soil homogenate blanks were also measured simultaneously. Plates were incubated at 25 °C for 4 hours (optimal duration of assay determined prior to experiment). After incubation, 10 µl of 1M NaOH was added to each well to terminate enzyme activity. Within one minute of NaOH addition (*DeForest*, 2009), fluorescence was measured using a fluorometer set at 365 nm excitation and 450 nm emission. From fluorescence values, total enzyme activity (EA_{tot}) was calculated as the rate of substrate converted in nmol h^{-1} g^{-1} soil (*German et al.*, 2011), and total C-acquiring enzyme activity (C_{enz}) was operationally defined as the sum of AG, BG, BX and CBH activities.

Total enzyme activity was normalized to both soil C and microbial C concentrations to obtain two different specific enzyme activity indices. Enzyme activity per unit soil C (EA_{soc}) was calculated by dividing total potential activity (EA_{tot}) by the soil C concentration:

EA_{soc} (nmol activity mg^{-1} C h^{-1}) = EA_{tot} (nmol activity h^{-1} g^{-1} soil) / soil C (mg C g^{-1} soil)

Enzyme activity normalized per unit microbial C (EA_{mbc}) was calculated by dividing EA_{tot} by microbial biomass C:

EA_{mbc} (nmol activity μg^{-1} C_{mic} h^{-1}) = EA_{tot} (nmol h^{-1} g^{-1} soil) / C_{mic} (µg C g^{-1} soil)

Ratios of C, N and P acquiring enzyme activities were also calculated *sensu Sinsabaugh et al.* (2008). Enzyme ratios provide an index that is considered to be at the intersection of the metabolic theory of microbial ecology (based on energetic constrains and thermodynamics) and ecological stoichiometric theory (elemental resource availability, *Sinsabaugh et al.* (2009)). Thus, enzymatic ratios are a tool for examining relative allocation to energy versus nutrient acquisition. We refer to these ratios as the NAG : C_{enz}, NAG : AP and C_{enz} : AP ratio, respectively.

3.2.5. Statistical analyses

All statistics were performed using R (R Core Team, 2012). Soil chemical, microbial biomass and enzyme activity data were log-transformed, and ecoenzymatic ratios were log-log transformed

prior to statistical analyses to meet assumptions of normality. We used type II standard major axis (SMA) regression to evaluate relationships among soil C concentration, microbial biomass and enzyme activity using the "lmodel2" package (*Legendre*, 2013). Type II regression is useful when the two variables of interest are random and have comparable standard errors because in this case least squares (Type I) regression will underestimate the slope of the linear relationship between the variables (*Legendre*, 2013). A linear mixed effects model was fit to the enzyme data using the lme4 package in R (*Bates et al.*, 2012), to determine the effects of depth, soil type, forest type and their interactions on soil nutrient concentrations, microbial biomass, fungal : bacterial ratios, enzyme activities and ecoenzymatic ratios. Depth, soil and forest type were treated as fixed effects and soil pit (representing the field replication) was treated as a random effect nested within soil × forest type. Catena position (ridge, slope, or valley) was also included in the model as a random effect. For two soil pits used in this analysis, we did not have the deepest depth interval (140 cm) represented, and the design was therefore unbalanced. To overcome this, we determined Box's epsilon correction factors (GG values) for the model and residual degrees of freedom in order to calculate correct F ratios and P values for model terms including depth (*Greenhouse and Geisser*, 1959). Where significant effects were found, we ran post-hoc t-tests or Tukey's HSD test using the 'testInteractions' function in the "phia" package (*Rosario-Martinez*, 2012). Statistical significance was determined at the $\alpha = 0.05$ level. We corrected the P-value for significance associated with an α value of 0.05 using a Bonferroni correction.

Principal components analysis (PCA) was used to assess patterns in microbial community structure (i.e., PLFA biomarkers). Because we expected biomarker abundance to be strongly correlated with total biomass, biomass-normalized biomarker abundances (i.e., % mol fractions) were used for PCA. Biomarkers that were not indicative of a specific group of microorganism (e.g., gram positive, gram negative, etc.) were excluded from PCA to better investigate relationships among distinct microbial groups (*Zelles*, 1999). We performed PCA on the entire dataset and subsequently on a reduced version of the dataset that used average values for each soil × forest × depth class. Because the results of these two analyses match closely, we report the results of PCA on the reduced dataset, which provide a simpler visual representation. The first two principal components were extracted

for further analyses. A linear mixed-effects ANOVA model identical in structure to the model fit to enzyme data was used to explicitly test for effects of depth, soil type and forest type on community structure for the first two principal components. Lastly, we used type II regression to test for relationships between EA_{mbc} and community structure represented by principal components (discussed below).

3.3. Results

3.3.1. Effects of soil and forest types on soil C, N and P concentrations, microbial biomass and enzyme activities

Averaged across all depth intervals, soil C concentrations were 16% greater and soil N concentrations were 28% greater in the Oxisols compared with the Inceptisols (Table 3.3, $P < 0.05$). C concentrations were 96% greater in the Colorado forest compared with the Tabonuco forest across all soil depths (Table 3.3, $P < 0.01$). C : N ratios were also significantly greater in the Colorado forest (mean = 19.9) compared with the Tabonuco forest (mean = 11.4, $P < 0.01$) and particularly in Colorado forest Inceptisols (mean = 22.5, Table 3.3, $P < 0.05$ for soil × forest interaction). Extractable P did not differ among the four sites. Soil pH was less acidic in Inceptisols (mean = 5.1) compared with Oxisols (mean = 4.3) and in the Tabonuco forest (mean = 5.0) compared with the Colorado forest (mean = 4.4, Table 3.3, $P < 0.05$).

Soil type did not have a statistically significant effect on microbial biomass or any total or specific enzyme activities. Microbial biomass was also similar across the two forests. Several potential enzyme activities differed across the two forests (data not shown). AG_{tot} was 140% greater and CBH_{tot} was 44% greater in the Colorado forest than the Tabonuco forest ($P < 0.05$), driven by greater activity in Colorado forest Oxisols compared with all other sites. However, when normalized by microbial biomass, enzyme activities did not vary significantly with soil or forest type.

3.3.2. Depth trends in soil C, N, P concentrations, microbial biomass and enzyme activities

Soil C, microbial biomass C, and EA_{tot} were all highly correlated with each other across all soil and forest types and depth increments. The relationship between each of these variables was very strong (i.e., $r > 0.8$). In each case, approximately 80% of the variance in a given dependent variable (biomass or EA_{tot}) could be explained by its corresponding independent variable (biomass or soil C). Soil C, N and extractable P concentrations declined exponentially with depth (Figure 3.1, $P < 0.01$). The soil C : P ratio decreased by 47% and the soil N : P ratio decreased by 27% with depth ($P < 0.\ 01$ for each effect), while C : N ratios and pH were invariant with depth. Microbial biomass declined 98% from the surface to 140 cm (Figure 3.2a, $P < 0.01$), but only declined 20% with depth when normalized by soil C (Figure 3.2b, $P < 0.01$). EA_{tot} declined exponentially with depth (Figure 3.3a, $P < 0.01$). When normalized by soil C, EA_{soc} did not change significantly with depth (Figure 3.3b, $P = 0.12$) and when normalized by microbial biomass C, EA_{mbc} increased 19-fold with depth (Figure 3.3c, $P < 0.01$).

All potential enzyme activities declined exponentially with depth (Figure 3.4, top). However, trends were enzyme-specific when normalized by soil C (Table 3.5) or microbial biomass C (Figure 3.4, bottom). For each enzyme, the decline in activity with depth was diminished when normalized by soil C and specific activity either did not change or increased with depth when normalized by microbial biomass (Figure 3.4, Table 3.5). BG_{soc} and NAG_{soc} declined significantly with depth ($P < 0.01$) while BG_{mbc} and NAG_{mbc} did not change significantly with depth. BX_{soc}, CBH_{soc} and AP_{soc} did not change with depth, while BX_{mbc} and CBH_{mbc} both trended positively with depth ($P = 0.08$, 0.07 respectively) and AP_{mbc} increased 20-fold with depth ($P = 0.01$). AG_{soc} trended positively with depth ($P = 0.09$), while AG_{mbc} increased 48-fold with depth ($P = 0.03$). The NAG : C_{enz} ratio declined from 0.4 to 0.28 and the C_{enz} : AP ratio declined from 0.33 to 0.2 from the surface to 140 cm ($P = 0.01$, Table 3.5). The NAG : AP ratio declined approximately 5-fold with depth from 0.14 at the surface to 0.03 at 140 cm ($P < 0.01$, Table 3.5).

3.3.3. Microbial community structure

Community structure was assessed broadly on the basis of fungal : bacterial ratios and in more detail using PCA of all PLFA biomarker groups. Fungal : bacterial ratios declined 6-fold from the surface to 140 cm (Figure 3.2c, $P < 0.01$) and were 33% greater in Inceptisols (mean = 0.037) compared with Oxisols (mean = 0.028, $P = 0.03$). The first two principal components captured 67% of the variability in microbial community structure (Figure 3.5). PC1, which accounted for 49% of the variation in community structure, was significantly related to depth ($P < 0.01$), with deep soils shifted in positive x-space. PC1 was also negatively correlated with C, N and P concentrations, with correlation coefficients ranging from -0.43 to -0.62. Higher scores on PC1 were associated with greater relative abundance of gram positive bacteria and actinobacteria and smaller relative abundance of gram negative bacteria and fungi. PC2, which accounted for 18% of the variation in the data, represented a contrast between gram positive bacteria and fungi versus anaerobic bacteria and non-fungal eukaryotes. PC2 was not correlated with any soil variables. Soil and forest type did not have a significant effect on either principal component. No enzyme activities were significantly correlated with PC1. All EA_{mbc} values were weakly positively correlated with PC2, with AP being the most strongly correlated ($R^2 = 0.27$, $P < 0.01$). Correlation coefficients for C and N acquiring enzymes ranged from 0.08–0.15 ($P < 0.01$).

3.4. Discussion

We originally anticipated that forest type would be a primary driver of enzymes activities in surface soils due to differences in leaf litter chemistry (and therefore substrate availability or quality), while parent material would exert a stronger influence on enzyme activities in subsoils because of mineralogical differences that could influence physio-chemical processes such as sorption. However, in most cases we did not observe substantial differences in individual enzyme activities among soil or forest types. This is in spite of observed differences in bulk soil C concentrations and C : N ratios, as well as previously reported differences in the literature (*Harris et al.*, 2012). Moreover, the few differences we did observe were dwarfed by the dramatic change in microbial abundance and activity with soil depth that was consistent across all sites.

To our knowledge, this is the first study to examine both specific enzyme activities and ecoenzyme ratios as a function of depth in tropical soils. Our finding that specific enzyme activity increases with depth carries the implication that microbes may be expending more to acquire less in resource-poor subsoils. However, in order to make inferences about shifting physiological strategies along soil depth profiles, we first had to account for the exponential decline in total microbial abundance and activity. Other studies have also found exponential declines in soil C and microbial activity with depth in both temperate forest and agricultural soils (*Eilers et al.*, 2012; *Fierer et al.*, 2003a,b; *Taylor et al.*, 2002). Similar to these authors, we found that declining energy availability (soil C) is likely the principal driver of declines in microbial abundance and activity with depth. This is evidenced by the strong relationship between soil C, microbial biomass and enzyme activities. While correlations between biomass and soil C have been noted by many researchers, a meta-analysis showed the degree of correlation between these two variables is typically half (i.e., $r^2 = 0.39$) of what we observed in our soils (*Cleveland and Liptzin*, 2007). Compared with other tropical soils, the degree of correlation we observed between enzyme activities and biomass may be unique. A recent meta-analysis of tropical soil BG, NAG and AP activities found no significant correlations with microbial biomass C (*Waring et al.*, 2013).

We found that normalizing by soil C or microbial biomass causes a dramatic shift in enzyme activity trends with depth. Specific activity measurements have been used by others to compare biochemical activity in soils with very different organic C contents (*Barriuso et al.*, 1988; *Trasar-Cepeda et al.*, 2008) and have been shown to provide a more robust indicator of shifting microbial allocation patterns in tropical forests (*Weintraub et al.*, 2013). Our findings affirm this observation. In general, we observed a larger positive change in specific activity with depth when reported per microbial biomass instead of per unit soil C. The fraction of soil C that represents viable microbial biomass (i.e., PLFA C) declines with depth, therefore, declines in enzyme activity per unit soil C may be driven by changes in biomass. Alternatively, shifts in community structure with depth may result in an increase in the relative abundance of enzyme producers, resulting in increased specific activity when reported per unit biomass instead of per unit soil C. Additionally, the higher sensitivity of the PLFA technique to low C concentrations compared with combustion analysis could improve our

detection of shifting specific enzyme activities in the case of EA_{mbc} compared with EA_{soc}. We focus the rest of our discussion of depth trends in enzyme activities on EA_{mbc} values, which we find to be a stronger indicator of changes in microbial allocation strategies than EA_{tot} or EA_{soc}.

3.4.1. Depth trends in enzyme activities

Our finding that four C-acquiring enzymes measured in our study either did not change with depth (BG, BX and CBH) or increased with depth (AG) when normalized by microbial biomass (Figure 3.4, bottom) was unexpected. Typically, concentrations of plant-derived cellulosic compounds decline with depth in relation to microbial compounds, due to a combination of microbial processing, selective transport to subsoils in dissolved form, and preferential stabilization of microbial C in association with the soil mineral phase (*Kramer and Gleixner*, 2008; *Rumpel and Kogel-Knabner*, 2011; *Spielvogel et al.*, 2008). Moreover at our sites, root biomass is mostly concentrated in the upper mineral soil (*Lawrence*, 1996); we therefore expected contributions of root C to subsoil C pools to be small. Given C-acquiring enzyme activities are often stimulated in the presence of degradable substrates (*Allison and Vitousek*, 2005; *Hernandez and Hobbie*, 2010; *Weintraub et al.*, 2013), we expected that a lack of plant-derived C in subsoils would lead to a reduction in cellulolytic enzyme activity on a per-biomass basis.

An obvious explanation for our results is our assumption that the relative contribution of plant-derived C declines with depth is incorrect. In our sampling, we removed the organic rich O-horizon, which contains the least microbially processed C. It is possible that even in the surface A-horizon, organic matter is highly decomposed and compositionally more similar to subsoil organic matter than leaf litter. This explanation fits with our finding that the soil C : N ratio did not change with depth: we would expect to see higher C : N ratios in surface soils if they contained more plant-derived C compared with subsoils. C : N ratios throughout our soil profiles (mean = 15.6) were much closer to expected C : N ratios for microbial biomass (8-12, *Cleveland and Liptzin* (2007)) than for leaf litter (66, *McGroddy et al.* (2004)). However, the dramatic increase in specific AG activity, particularly when compared with other C-acquiring enzymes, may signal a subtle shift in C substrate availability. The relative contribution of AG_{mbc} to total specific C-acquiring enzyme

activity increased an order of magnitude with depth, from 3% in surface soils to 30% at 140 cm. This may reflect an increase in the relative abundance of starch, a labile polysaccharide that is both plant and microbially-derived (*Kogel-Knabner*, 2002), compared with cellulosic compounds. Additionally, many extracellular enzymes are produced constitutively at low levels unless environmental signals cause upregulation or repression to occur (*Allison et al.*, 2011; *Sinsabaugh and Shah*, 2012). It is possible that high specific activity of plant C-acquiring enzymes in subsoils reflects constitutive enzyme production. Finally, plant C-acquiring enzyme activity could persist in deep soils due to enhanced mineral stabilization of enzymes and reduced enzyme turnover rates. While turnover rates are not commonly measured in soils (*Wallenstein and Burns*, 2011), a large fraction of soil enzymes are immobilized as a result of adsorption or covalent bonding interactions with soil minerals or other colloids (*Burns*, 1982; *Gianfreda and Rao*, 2011; *Taylor et al.*, 2002). Immobilized enzymes often exhibit increased stability compared with free enzymes due to reduced accessibility to proteolytic degradation (*Allison*, 2006; *Ladd*, 1978; *Nannipieri and Gianfreda*, 1998).

Unlike the C-acquiring enzymes, we did not have an a priori expectation of how the N-acquiring enzyme NAG would trend with depth. NAG is considered primarily a chitin-degrading enzyme, but it is also involved in the degradation of peptidoglycan, an abundant component of bacterial cell walls. Constitutive NAG production and decreased turnover rates are potential explanations for our finding that specific NAG activity remains constant with depth. However, it is also possible that in deep soils bacterially-derived NAG enzymes play a role in recycling organic N from microbial biomass. Conversely, the NAG : C_{enz} and NAG : AP ratios declined with depth, indicating a downregulation of NAG production in relation to C and P-acquiring enzymes. It is unclear whether shifting enzyme ratios reflect a decline in microbial demand for organic N, a shift in fungal : bacterial ratios (fungal biomass being a major source of chitin in soils) or differences in turnover rates among enzymes. It is possible that other N-acquiring enzymes, such as proteases and ureases, may provide a better indicator of microbial N demand depending on substrate availability, particularly given the dual function of NAG as a C and N degrading enzyme. Clearly, more information on the size and composition of organic-N substrate pools is needed to fully assess the importance NAG throughout tropical soil profiles.

We found high AP activity and low NAG : AP and C_{enz} : AP ratios throughout the soil profiles, suggesting high microbial investment in P-acquiring enzymes. This finding is consistent with recent meta-analyses which have found ratios of BG : AP and NAG : AP are substantially lower in tropical soils compared with global averages (*Sinsabaugh et al.*, 2008; *Waring et al.*, 2013). Microbial investment in P-acquisition also appears to increase with depth, as evidenced by increased specific AP activity and decreases in NAG : AP and C_{enz} : AP ratios. Other studies have found increases in specific phosphatase activity with depth (*Taylor et al.*, 2002). However, to our knowledge our study is the first to examine changes in NAG : AP and C_{enz} : AP ratios with depth in tropical soils. A remarkable finding is that while in surface soils, our mean NAG : AP ratio (0.14) is similar to NAG : AP ratios found in other tropical systems (0.126 ± 0.045, *Waring et al.* (2013); *Weintraub et al.* (2013)), the NAG : AP ratio declines to an average of 0.03 with depth, far lower than literature NAG : AP ratios for even the most highly-weathered tropical soils.

From a resource allocation perspective, high microbial investment in P-acquiring enzymes is suggestive of microbial P-limitation, as has been proposed for other tropical soils (*Cleveland and Liptzin*, 2007; *Waring et al.*, 2013). However, in our soils extractable P concentrations did not decline as rapidly with depth as total C or N, and the ratio of extractable P to biomass increased substantially with depth (data not shown). Although the two pools of extractable P that we measured cannot be definitively related to P bioavailability, these fractions are considered to represent labile to moderately labile P available to microorganisms over the short to intermediate term (*Johnson et al.*, 2003). If soil P availability does increase with depth relative to C and N, the question arises of why microbes would invest resources in a P-acquiring enzyme. We propose an alternative explanation for high AP activity exists in our deep soils. Studies from other low-C environments such as deep ocean strata and marine sediments have demonstrated that microbes use phosphorylated organic compounds primarily as a C source (*Hoppe and Ullrich*, 1999; *Nausch and Nausch*, 2007; *Steenbergh et al.*, 2011). A recent study from temperate forest soils in Germany also demonstrated that soil microbes use the organic moiety of phosphorylated compounds as a C source, while mineralizing P without uptaking it (*Spohn and Kuzyakov*, 2013). It is possible that in our soils, increased AP_{mbc} with depth may be driven by microbial C demand. This explanation fits with our

finding that AP activity was slightly better correlated with soil C concentrations (R^2 = 0.71) than with either $NaHCO_3$- (R^2 = 0.67) or NaOH- extractable P (R^2 = 0.45). More generally, in environments characterized by extreme energy and resource limitation, enzymes that are typically classified as C, N or P acquiring may be used to target multiple nutrient reservoirs. However, we caution that a better understanding of what constitutes "microbially available P" is needed to determine whether AP activity results from C or P limitation in subsoils. Additionally, differences in turnover rates among enzymes need to be investigated in order to link shifting ecoenzyme ratios to changes in microbial nutrient demand with confidence.

In addition to shifting energy and resource demands, changes in specific enzyme activities may reflect compositional changes in the microbial community, a possibility which we explored using PLFA biomarkers. Changes in community structure can occur due to changes in the relative fitness of various microorganisms under altered C or nutrient regimes (*Allison et al.*, 2007; *DeForest et al.*, 2012; *Fierer and Jackson*, 2006; *Rinkes et al.*, 2011), leading to selection for organisms that can better exploit available resources. Such community shifts could alter the types and abundances of enzyme producers. Additionally, greater spatial separation between microorganisms in deep soil may result in lower numbers of microbial "cheaters" that do not produce enzymes but use enzyme products (*Allison*, 2005). Given that we observed changes in community structure with depth (discussed below) and some small correlations between community structure and enzyme activities, we believe it possible that shifts in community structure contribute to shifts in specific enzyme activity with depth.

3.4.2. Patterns in microbial community structure

Similar to our enzyme findings, depth, rather than soil or forest type, appears to be the major driver of microbial community structure in our study system. Other studies have found that subsurface microbial communities are distinct from surface communities (*Blume et al.*, 2002; *Eilers et al.*, 2012; *Hansel et al.*, 2008; *Hartmann et al.*, 2009). Interestingly, the depth trends we observed parallel findings from other studies conducted across a range of ecosystems. For example, many other researchers have observed decreased fungal : bacterial ratios with depth (*Ekelund et al.*, 2001;

Fierer et al., 2003b; *Taylor et al.*, 2002). Plant roots, which fungi are often associated with, are concentrated in the upper 30 cm of the soil in the LCZO and are nearly absent below 70 cm depth (*Lawrence*, 1996). Given that we excluded the shallow O-horizon soils, where we would expect the majority of fungal biomass to be concentrated, it is not surprising that we found low biomass of fungal biomarkers throughout soil profiles. The increase in actinobacterial abundance with depth across the upper ~80 cm of the soil also parallels results from other studies (*Federle et al.*, 1986; *Fierer et al.*, 2003b; *Fritze et al.*, 2000; *Hartmann et al.*, 2009). Often adapted to resource-limited conditions, actinobacteria are a metabolically versatile group of organisms that degrade high molecular weight components of SOM including lignin and cellulose (*McCarthy and Williams*, 1992). Shifts from large populations of gram negative bacteria in surface soils to gram positive bacteria in subsoils have also been observed by other researchers (*Blume et al.*, 2002; *Fierer et al.*, 2003b). Gram negative bacteria are most often associated with plant rhizospheres and high-organic matter environments (*Griffiths et al.*, 1999), possibly due to their preference for plant-derived C over gram positive bacteria, which typically utilize older, SOM-derived C (*Kramer and Gleixner*, 2008). Gram positive bacteria are also known for their ability to sporulate and resist harsh environmental conditions, including long-term desiccation (*Setlow*, 2007). In the Oxisol sites, dense clays impede subsurface infiltration and dry conditions typically prevail below 60 cm depth (*McDowell et al.*, 1992).

Similarly to its role in governing enzyme activities, it is likely that C quantity and chemical composition are dominant drivers of the changes in community composition that we observe with depth. Other environmental variables that are often linked to shifts in microbial community structure, such as pH (Table 3.3) and moisture content (data not shown), are relatively invariant in our profiles beneath the surface ~20 cm. The extremely low biomass of anaerobic bacteria throughout our soil profiles suggests that oxygen availability is not a major control on community structure, though redox status is an important microbial driver in surface soils, particularly in topographically low and frequently inundated valleys of the Luquillo forest (*Hall and Silver*, 2013).

We were surprised that we did not observe stronger correlations between community structure and enzyme activities, particularly given that both aspects of the microbial community changed with

depth. Several possible explanations exist. For one, PLFA may lack the resolution necessary for observing structure-function relationships, especially if biodiversity or biomass is very low and microbial communities exhibit fine-scale phylogenetic variation, or microdiversity, with respect to enzyme production. In such cases, examining community structure at a broad level can mask correlations between community structure and functions (*Zimmerman et al.*, 2013; *Frossard et al.*, 2012) due to a "convergence of function" among taxonomically diverse microorganisms. It is also possible that, while the microbial community as a whole shifted, the relative abundance of enzyme producers did not. In this case the primary driver of depth trends in enzyme activities may be physiological shifts among enzyme producers. Finally, the lack of relationship between enzyme activities and community structure may indicate other environmental mechanisms, such as enzyme immobilization and reduced turnover rates discussed above, are more important than community structure in accounting for the depth distribution of enzymes.

In spite of the community structure changes we observed with depth, it is important to note that a large fraction of the variance in community structure had no relationship with depth. Recent metagenomic studies have revealed enormous microbial diversity on the microscale (*Gans et al.*, 2005; *Torsvik and Ovreas*, 2002), which is often linked to the spatial heterogeneity of the soil habitat (*Ranjard and Richaume*, 2001). It is possible that in our soils, microscale heterogeneity in soil resources, moisture and redox status accounts for a large fraction of the variability in community structure independent of broad gradients in C availability. Additionally, our ability to resolve differences in community structure among samples is limited by the PLFA technique, which provides only a coarse view of community structure (*Frostegard et al.*, 2011). Assignment of PLFA biomarkers to classes such as gram positive or gram negative relies on assumptions about the broad applicability of relationships that have been developed based on a small subset of cultivable organisms (*Bossio and Scow*, 1998; *Zelles*, 1999). Another challenge with interpreting PLFA information is that changes in fatty acid biomarkers can indicate changes in community composition or physiological changes within the same community (*Bossio and Scow*, 1998). Nevertheless, we find that the high sensitivity of PLFA in detecting biomarkers in low biomass environments makes it a valuable technique for our study.

3.5. Conclusions

We observed strong and interrelated gradients in soil C, microbial biomass and enzyme activities with depth across two tropical forests underlain by two distinct geologic parent materials. Our study demonstrates that low-C tropical subsoils contain metabolically active microbial communities that contribute to biogeochemical cycles of C, N and P at rates similar to surface communities on a per-biomass basis. High phosphatase activity throughout soil profiles is consistent with other studies from the tropics, though in deep, low-C soils we suggest the possibility that phosphatase activity may be driven by microbial C demand, considering the relatively high P-availability. Shifts in community structure may also contribute to high specific enzyme activity in deep soils, although weak correlations between community structure and enzyme activities suggest community changes are secondary to physiological adaptations or changes in enzyme turnover rates. From an ecosystem perspective, high specific hydrolytic enzyme activity suggests tropical subsoil organic matter is composed of recognizable biomolecules, similar to those in surface soils. Future studies directly characterizing the stability and chemical composition of organic matter in tropical subsoils will provide further insight into controls on C and nutrient cycling throughout tropical soil profiles.

Table 3.1: General site and soil characteristics for the four soil × forest types. MAT = mean annual temperature, MAP = mean annual precipitation, ANPP = annual net primary productivity.

	Oxisol	Oxisol	Inceptisol	Inceptisol
	Tabonuco	Colorado	Tabonuco	Colorado
Location	18°18'50.0"N	18°16'39.1"N	18°15'46.3"N	18°17'28.7"N
	65°44'25.0"W	65°50'54.0"W	65°47'35.5"W	65°47'44.2"W
Elevation (m)	360	790	360	780
MAT (°C)[1]	24	21	24	21
MAP (mm yr^{-1})[1]	3500	4200	3500	4200
Aboveground biomass (t ha^{-1})[2]	190	130	190	130
ANPP(t ha^{-1} yr^{-1})[2]	10.5	7.6	10.8	4.05
Texture Class	Silty clay loam	Silty clay	Sandy loam	Sandy loam
Soil Taxonomy[3]	Humaquox, Aquic & Inceptic Hapludox	Humaquox, Aquic & Inceptic Hapludox	Histic Humaquepts, Aquic & Histic Dystrudepts	Histic Humaquepts, Aquic & Histic Dystrudepts

1. *McDowell et al.* (2012)

2. *Weaver and Murphy* (1990)

3. Classification of soils according to US Taxonomy.

Table 3.2: Summary of enzymes studied, their broad ecological functions, and substrate proxies used for laboratory assays. All substrate proxies used are methylumbelliferone (MUB)-linked. The appropriate concentration of substrate was determined in a preliminary experiment (*German et al.*, 2011).

Enzyme	Enzyme Function	Common substrates	Substrate Proxy	Substrate Concentration
Acid Phosphatase (AP)	Releases phosphate groups	Phospholipids, phosphosaccharides	4-MUB Phosphate	400 μM
α-glucosidase (AG)	Releases glucose from starch	Starch	4-MUB α-D-glucopyranoside	200 μM
β-glucosidase (BG)	Releases glucose from cellulose	Cellulose	4-MUB β-D-glucopyranoside	200 μM
β-xylosidase (BX)	Releases xylose from hemicellulose	Hemicellulose	4-MUB β-D-xylopyranoside	200 μM
Cellobiohydrolase (CBH)	Releases disaccharides from cellulose	Cellulose	4-MUB-β-D-cellobioside	200 μM
N-acetyl glucosaminidase (NAG)	Releases *N*-acetyl glucosamine from oligosaccharides	Chitin & peptidoglycan	4-MUB *N*-acetyl-β-D- glucosaminide	200 μM

Total C and N, and extractable P concentrations and soil pH of samples assayed for acid phosphatase activity and kinetic parameters. C and N concentrations are reported in mg g^{-1} soil, while P concentrations are reported in mg kg^{-1} soil. Values are reported as means of each soil × forest × depth combination ± 1 standard error.

Soil Order	Forest Type	Depth (cm)	n	Total C	Total N	C:N	$NaHCO_3$- P	NaOH- P	pH
Inceptisol	Colorado	0	5	68.72 ± 24.07	2.64 ± 0.72	24.59 ± 1.96	19.19 ± 3	68.14 ± 6.96	4.4 ± 0.18
		20	5	36.46 ± 8.87	1.7 ± 0.34	20.78 ± 1.07	6.25 ± 1.59	29.3 ± 8.98	4.38 ± 0.24
		50	5	27.54 ± 7.48	1.22 ± 0.31	27.49 ± 5.78	2.58 ± 0.67	16.97 ± 3.97	4.56 ± 0.3
		80	5	19.02 ± 8.53	0.84 ± 0.29	21.05 ± 3.69	2.25 ± 0.71	14.3 ± 3.97	4.68 ± 0.4
		110	4	9.73 ± 6.44	0.51 ± 0.3	18.23 ± 2.89	1.48 ± 0.84	14.73 ± 8.84	4.73 ± 0.45
		140	3	10.7 ± 9.15	0.52 ± 0.44	21.73 ± 3.42	2.82 ± 2	13.68 ± 8.53	4.25 ± 0.69
Inceptisol	Tabonuco	0	5	57.52 ± 13.64	4.44 ± 1.01	12.91 ± 0.36	21.67 ± 5.76	131.32 ± 44.39	5.76 ± 0.27
		20	5	17.82 ± 2.84	1.58 ± 0.31	11.61 ± 0.61	8.75 ± 2.19	73.82 ± 19.46	5.46 ± 0.45
		50	5	11.08 ± 0.55	1.02 ± 0.05	10.87 ± 0.14	2.77 ± 0.28	42.8 ± 5.89	5.62 ± 0.43
		80	5	4.42 ± 1.25	0.44 ± 0.13	10.39 ± 0.42	1.42 ± 0.24	41.25 ± 15.87	5.54 ± 0.53
		110	5	1.05 ± 0.3	0.18 ± 0.08	10.15 ± 4.1	1.87 ± 0.9	48.06 ± 38.08	5.5 ± 0.64
		140	5	0.58 ± 0.12	0.14 ± 0.08	8.31 ± 2.37	1.46 ± 0.78	25.2 ± 22.16	5.79 ± 0.47
Oxisol	Colorado	0	5	101.82 ± 15.9	5.62 ± 0.49	17.86 ± 1.42	28.97 ± 1.44	117.77 ± 11.44	4.2 ± 0.21
		20	5	50.44 ± 6.47	3.38 ± 0.37	14.87 ± 0.69	9.03 ± 3.23	40.32 ± 12.15	4.04 ± 0.1

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Table3.3 –*Continued from previous page*

Soil Order	Forest Type	Depth (cm)	n	Total C	Total N	C:N	$NaHCO_3$- P	NaOH- P	pH
		50	5	24.7 ± 2.56	1.64 ± 0.19	15.22 ± 0.61	2.29 ± 0.48	15.12 ± 3.73	4.29 ± 0.31
		80	5	13.26 ± 1.54	0.78 ± 0.15	18.01 ± 1.38	1.32 ± 0.39	10.85 ± 0.94	4.19 ± 0.31
		110	5	7.62 ± 0.86	0.44 ± 0.08	18.22 ± 1.85	1.26 ± 0.19	10.05 ± 1.56	4.37 ± 0.3
		140	4	5.78 ± 0.96	0.3 ± 0.08	20.81 ± 2.49	1.52 ± 0.26	10.03 ± 2.32	4.42 ± 0.48
Oxisol	Tabonuco	0	5	55.43 ± 3.92	4.38 ± 0.14	12.71 ± 0.97	18.74 ± 1.83	149.47 ± 30.92	4.23 ± 0.19
		20	5	28.78 ± 2.05	2.36 ± 0.1	12.16 ± 0.56	6.13 ± 0.32	56.5 ± 14.44	4.39 ± 0.2
		50	5	10.62 ± 1.59	0.8 ± 0.11	13.31 ± 0.95	1.74 ± 0.26	24.5 ± 6.57	4.28 ± 0.26
		80	4	4.35 ± 1.17	0.4 ± 0.1	10.79 ± 0.65	0.68 ± 0.17	20.4 ± 8.95	4.08 ± 0.25
		110	4	2.88 ± 0.79	0.28 ± 0.06	10.91 ± 1.42	0.87 ± 0.28	22.83 ± 11.08	4.34 ± 0.18
		140	4	2.8 ± 0.94	0.23 ± 0.07	12.06 ± 0.98	0.67 ± 0.13	11.22 ± 2.38	4.3 ± 0.15

Table 3.4: Total and specific enzyme activities and microbial biomass by depth. Values are reported as means of each depth combination ± 1 standard error. Units for total, biomass normalized and carbon normalized enzyme activities are: nmol hr^{-1} g^{-1} soil, nmol hr^{-1} μg^{-1} C_{mic} and nmol hr^{-1} mg^{-1} soil C, respectively. Units for total and carbon normalized biomasses are μg g^{-1} soil and μg mg^{-1} soil C, respectively.

	0	20	50	80	110	140
AG	39.5 ± 8.1	24.5 ± 6.5	15.5 ± 4.0	16 ± 5.3	8.4 ± 3.3	6.3 ± 2.3
AG_{mbc}	0.4 ± 0.1	0.5 ± 0.1	1.6 ± 0.5	4.6 ± 1.9	14.7 ± 10.7	95.9 ± 49.9
AG_{soc}	5.7 ± 1.4	7.5 ± 2.2	9.1 ± 3.0	19.1 ± 9	53.9 ± 30.6	28.5 ± 18.3
BG	873 ± 169.3	281.4 ± 82.0	87.2 ± 15.3	41.8 ± 10.4	10.6 ± 2.7	12.3 ± 3.4
BG_{mbc}	8.1 ± 1.9	5.3 ± 1.8	5.8 ± 1.7	7.6 ± 2.7	13.8 ± 9.2	75.2 ± 34.4
BG_{soc}	129.2 ± 31.4	92.1 ± 29.7	53.2 ± 14.6	47.2 ± 19.7	64.1 ± 30.7	44.0 ± 26.1
BX	256 ± 35.6	133.3 ± 39.0	50.7 ± 13.0	23.1 ± 6.1	7.9 ± 2.4	8.2 ± 2.5
BX_{mbc}	2.2 ± 0.4	2.5 ± 0.8	3.5 ± 1.2	5.4 ± 2	12.2 ± 8.3	80.2 ± 38.3
BX_{soc}	39.3 ± 7.9	43.8 ± 14.2	28.9 ± 9.6	28.6 ± 12.2	55.7 ± 28.2	30.8 ± 18.8
CBH	187 ± 48.3	46.5 ± 14.9	17.7 ± 4.0	10.6 ± 3.2	5.5 ± 2.1	4.6 ± 1.4
CBH_{mbc}	1.6 ± 0.5	0.8 ± 0.3	2 ± 0.6	3.3 ± 1.3	8.9 ± 6.5	59.1 ± 28.5
CBH_{soc}	26.2 ± 7.8	14.7 ± 5.1	10.1 ± 3.1	12.8 ± 5.8	32.5 ± 18.2	19.9 ± 12.2
NAG	583.1 ± 180.2	136.6 ± 27.5	32.0 ± 7.4	22.1 ± 5	7.7 ± 2.3	5.4 ± 2.8
NAG_{mbc}	4.8 ± 1.6	2.9 ± 0.8	2.6 ± 0.8	5.1 ± 1.8	14.3 ± 9.7	27.1 ± 17.1

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Table3.4 –*Continued from previous page*

	0	20	50	80	110	140
NAG_{soc}	82.8 ± 28.3	47.1 ± 11.5	20.2 ± 6.3	25.6 ± 10.4	59.0 ± 29.6	16.9 ± 12.4
AP	5013 ± 930.1	2340 ± 596	805.3 ± 186.0	437 ± 78.8	240.8 ± 31.5	155.4 ± 25.8
AP_{mbc}	50.4 ± 11.4	52.5 ± 15.7	73.0 ± 23.5	162.1 ± 51.5	441.9 ± 277.3	1062.8 ± 426.6
$APň_{soc}$	769.7 ± 181.7	799.1 ± 227.3	479.2 ± 150.3	660.8 ± 252.1	1626.1 ± 695.7	886 ± 488.1
NAG : AP	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.04 ± 0.01	0.03 ± 0.02
NAG : C_{enz}	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.2
C_{enz} : AP	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Biomass	106.4 ± 13.9	46.8 ± 7.8	16.8 ± 3.8	7.1 ± 1.9	3.0 ± 1.9	1.9 ± 0.7
Biomass : soil C	15.5 ± 3.0	15.4 ± 3.3	9.6 ± 3.0	7.4 ± 3.2	6.4 ± 4.7	12.5 ± 8
Bact biomass	74.8 ± 9.9	35.0 ± 6.0	12.3 ± 2.8	4.9 ± 1.3	2.2 ± 1.4	1.3 ± 0.5
Bact biomass : soil C	11.0 ± 2.2	11.5 ± 2.5	7.1 ± 2.2	5.3 ± 2.3	4.5 ± 3.4	8.7 ± 5.5
Fung biomass	4.8 ± 0.9	1.3 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.02 ± 0.01
Fung biomass : soil C	0.7 ± 0.2	0.4 ± 0.1	0.2 ± 0.09	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1

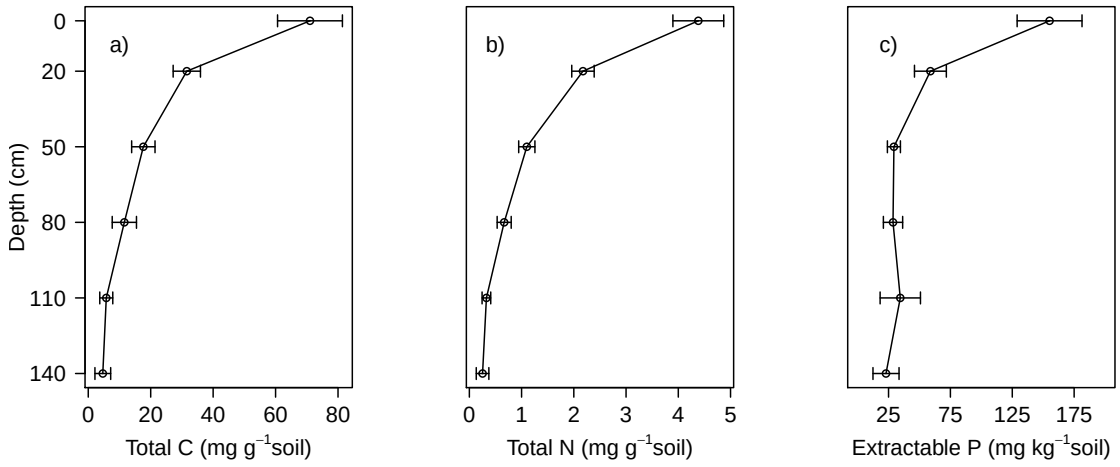

Figure 3.1: Declines in soil C, N and extractable P as a function of soil depth. Data points represent means across all soil and forest type combinations at each depth interval (n = 12, except n = 10 at 140 cm). Error bars represent standard errors of the mean.

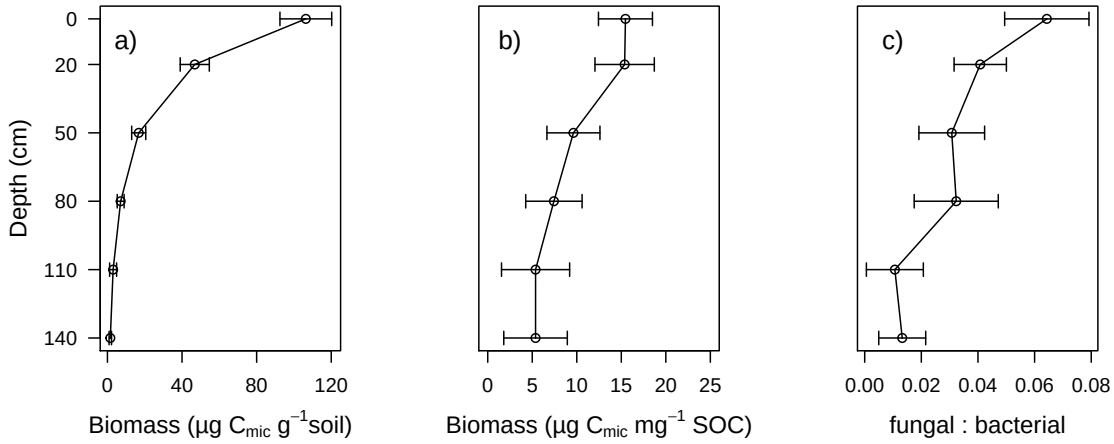

Figure 3.2: Declines in a) total microbial biomass, b) soil C normalized microbial biomass C and c) fungal : bacterial ratios as a function of soil depth. Data points represent means across all soil and forest type combinations at each depth interval (n = 12, except n = 10 at 140 cm). Error bars represent standard errors of the mean.

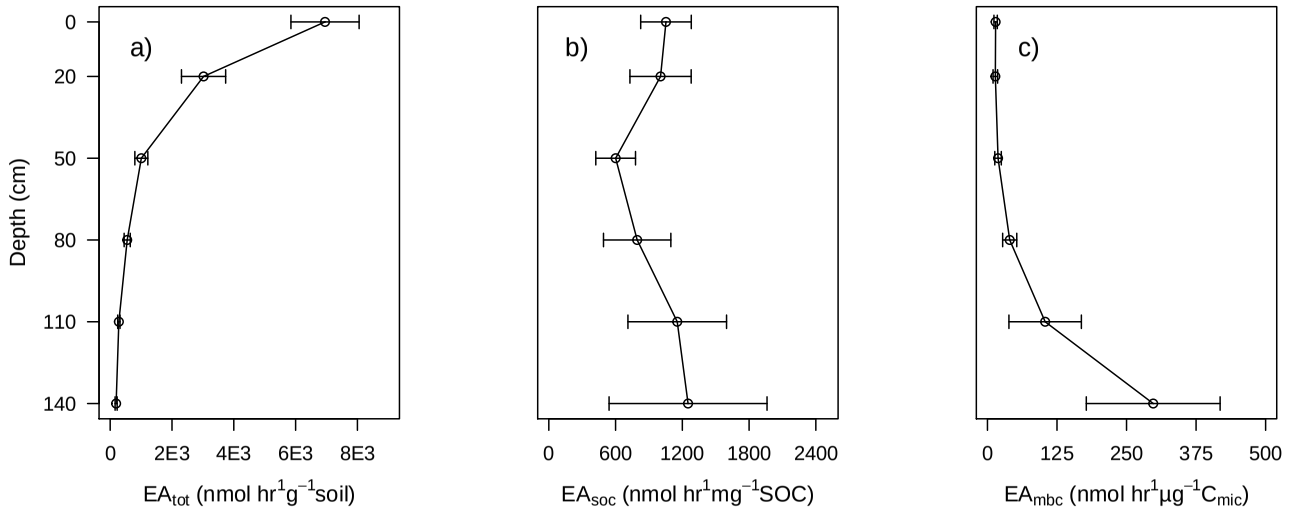

Figure 3.3: Declines in a) total, b) soil C normalized, and c) microbial biomass C normalized enzyme activity as a function of soil depth. Data points represent means across all soil and forest type combinations at each depth interval (n = 12, except n = 10 at 140 cm). Error bars represent standard errors of the mean.

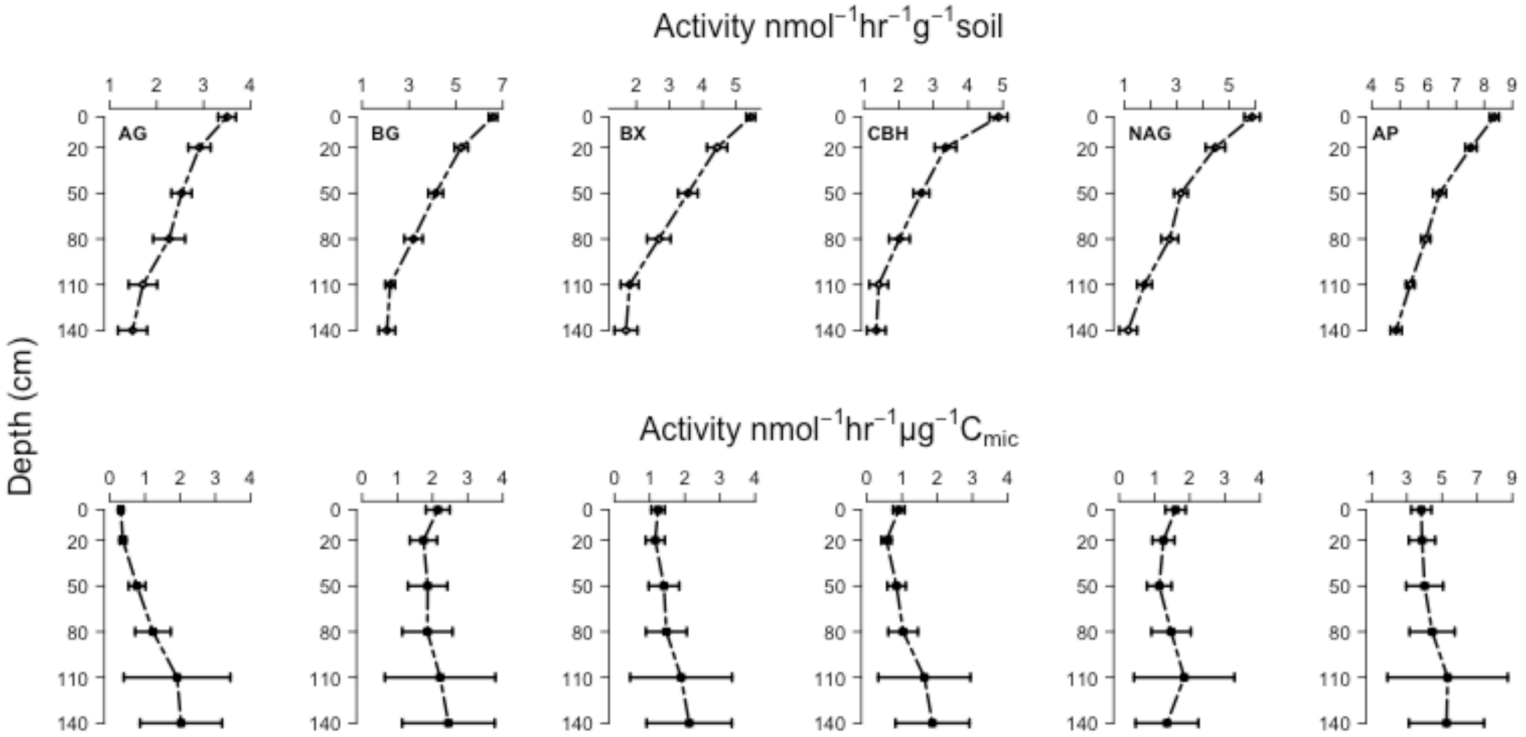

Figure 3.4: Changes in EA_{tot} (top) and EA_{mbc} (bottom) with depth for six enzymes. Left to right: α-glucosidase, β-glucosidase, β-xylosidase, cellobiohydrolase, *N*-acetyl glucosaminidase, acid phosphatase. Data points represent log-transformed means across all soil and forest type combinations at each depth interval (n = 12, except n = 10 at 140 cm). Error bars represent standard errors of the mean. Units are nmol substrate converted h^{-1} g $soil^{-1}$ for EA_{tot} (top) and nmol substrate converted h^{-1} μg^{-1} microbial C for EA_{mbc} (bottom).

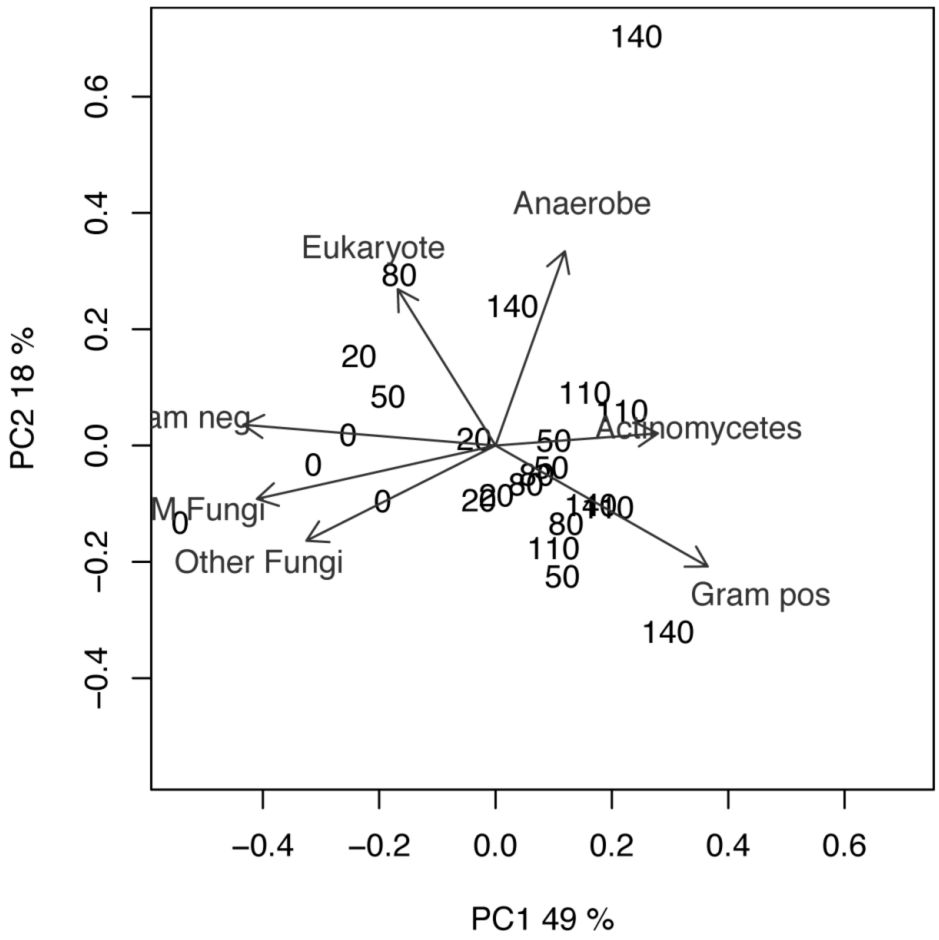

Figure 3.5: Principal components analysis of PLFA biomarkers. Seven biomarker classes were included in the PCA: gram positive bacteria, gram negative bacteria, actinobacteria, anaerobic bacteria, arbuscular mycorrhizal fungi, general fungi and non-fungal eukaryotes. All biomarker biomass data was normalized to total biomass for each sample and PCA was performed using the mol fraction of the various biomarkers as independent variables. Numbers on the plot refer to depth intervals and each represents an average of the three soil × forest replicates for a given depth. Percent variance explained by the first two principal components is written on the axes.

CHAPTER 4 : Parent material and vegetation influence bacterial community structure and nitrogen functional genes along deep tropical soil profiles at the Luquillo Critical Zone Observatory

Modified from paper accepted for publication as:

Stone M. M., J. Kan, and A.F. Plante (2014), Parent material and vegetation influence bacterial community structure and nitrogen functional genes along deep tropical soil profiles at the Luquillo Critical Zone Observatory. *Soil Biology and Biochemistry*.

Abstract:

Microbial communities mediate every step of the soil nitrogen cycle, yet the structure and associated nitrogen cycle functions of soil microbial communities remain poorly studied in tropical forests. Moreover, tropical forest soils are often many meters deep, but most studies of microbial nitrogen cycling have focused exclusively on surface soils. The objective of our study was to evaluate changes in bacterial community structure and nitrogen functional genes with depth in soils developed on two contrasting geological parent materials and two forest types that occur at different elevations at the Luquillo Critical Zone Observatory in northeast Puerto Rico. We excavated three soil pits to 140 cm at four different sites representing the four soil × forest combinations (n = 12), and collected samples at ten-centimeter increments from the surface to 140 cm. We used bacterial 16S rRNA gene-DGGE (denaturant gradient gel electrophoresis) to fingerprint microbial community structures, and quantitative PCR to measure the abundance of five functional genes involved in various soil nitrogen transformations: *nif*H (nitrogen fixation), *chi*A (organic nitrogen decomposition), *amo*A (ammonia oxidation), *nir*S (nitrite reduction) and *nos*Z (nitrous oxide reduction). Multivariate analyses of DGGE fingerprinting patterns revealed differences in bacterial community structure across the four soil × forest types that were strongly correlated with soil pH ($r^2 = 0.69$, $P < 0.01$) and nutrient stoichiometry ($r^2 \geq 0.36$, $P < 0.05$). Across all soil and forest types, nitrogen functional genes declined significantly with soil depth ($P < 0.001$). Denitrification

genes (*nir*S and *nos*Z) accounted for the largest proportion of measured nitrogen functional genes. Measured nitrogen functional genes were positively correlated with soil carbon, nitrogen and phosphorus concentrations ($P < 0.001$) and all genes except *amo*A were significantly more abundant in the Inceptisol soil type compared with the Oxisol soil type ($P < 0.03$). Greater abundances and a stronger vertical zonation of nitrogen functional genes in Inceptisols suggest more dynamic nitrogen transformation processes in this soil type. As the first study to examine bacterial nitrogen functional gene abundances below the surface 20 cm in tropical forest soils, our work provides insight into how pedogenically-driven vertical gradients control the nitrogen-cycling capacity of soil microbial communities. While previous studies have shown evidence for redox-driven hotspots in tropical nitrogen cycling on a watershed scale, our study corroborates this finding on a molecular scale.

4.1. Introduction

Microbial communities influence biogeochemical cycles throughout soil profiles, yet information on how community structure and functional characteristics change with soil depth is scarce (*Fierer et al.*, 2003b). Tropical forest soils are often many meters deep and subsoils store substantial quantities of soil organic carbon (C) in low concentrations, contributing approximately 50% of the estimated C pool below 1 meter (*Jobbagy and Jackson*, 2000). A large proportion of Bacteria and Archaea are able to live beneath the upper few meters of the Earth's surface in the so-called "deep biosphere" (*Hoehler and Jorgensen*, 2013; *Whitman et al.*, 1998), in environments that provide only marginal energy for cell growth and division. It is thus reasonable to predict that metabolically active microbial communities exist throughout the first several meters of tropical soil profiles. A more detailed characterization of these communities and their relationship to environmental variations is warranted to understand their roles in mediating biogeochemical cycles.

In surface soils, litter inputs principally define the energy resources and nutrients available to microbial communities (*Hobbie*, 1992; *Waldrop et al.*, 2006), and as such the amount and chemical quality of litter can have large impacts on microbial community structure and functions (*Chapman and Newman*, 2010; *Nemergut et al.*, 2010). Abiotic environmental properties such as redox potential (*DeAngelis et al.*, 2010), nutrient concentrations (*Cusack et al.*, 2011), soil texture (*Rousk et al.*, 2010; *Sessitsch et al.*, 2001), pH (*Rousk et al.*, 2010) and mineralogy (*Heckman et al.*, 2009) can also affect community structure. All of these properties can change dramatically with soil depth (*Hansel et al.*, 2008; *Holden and Fierer*, 2005). Microbial biomass generally declines exponentially with soil depth, following declines in soil C availability (*Blume et al.*, 2002; *Fierer et al.*, 2003b; *Stone et al.*, 2014). Molecular studies using both fingerprinting and metagenomic techniques have indicated that community composition also changes with depth (*Agnelli et al.*, 2004; *Eilers et al.*, 2012; *Hansel et al.*, 2008). Changes in both microbial community composition and the physiochemical environment with soil depth suggests that subsoil microbial communities are specialized for their environmental niches such that their metabolic functions cannot be easily inferred from their surface counterparts (*Ghiorse and Wilson*, 1988; *Zvyagintsev*, 1994).

A key ecological function of microbial communities is their participation in all steps of the soil nitrogen (N) cycle. For instance, nitrogen fixation, organic N decomposition, nitrification and denitrification are mediated largely by soil microbes (*Robertson and Groffman*, 2007). Tropical forests are often characterized by a dynamic microbial N cycle, with relatively high levels of N loss via denitrification (*Livingston et al.*, 1988; *Silver et al.*, 2000; *Vitousek and Sanford*, 1986) balanced by high levels of N fixation (*Cusack et al.*, 2009; *Reed et al.*, 2007). While tropical forests are often considered N-rich relative to temperate forests, N deposition is increasing rapidly in the tropics (*Galloway et al.*, 2004) and the response of soil microbial communities remains uncertain. Future changes to the N cycle of tropical forests will depend on the response of microbial N transformations to global change drivers. The environmental gradients represented by soil depth profiles may improve our understanding of the major environmental drivers of microbial N cycling. Several studies have found that abundances of N-fixing, nitrifying and denitrifying bacteria decline with depth in temperate forest soils (*Mergel et al.*, 2001), temperate grassland soils (*Marhan et al.*, 2011; *Regan et al.*, 2011), agricultural soils (*Forbes et al.*, 2009) and artificial Technosols (*Hafeez et al.*, 2012), but the depth distribution of N-cycling bacteria has not, to our knowledge, been investigated in tropical soils. Measurements of nitrogen functional genes (NFGs) using quantitative PCR (qPCR) provide a powerful tool for evaluating microbial contributions to different stages of the N cycle. The abundance of NFGs provides insight into the biological capacity for N-cycle processes to occur, including process rates, substrate availability and population density of functional groups of microorganisms (*Wallenstein and Vilgalys*, 2005).

The goal of this study was to evaluate how bacterial community structure and NFGs change with soil depth in the context of landscape-scale gradients. Our study site, the Luquillo Critical Zone Observatory (LCZO) in northeast Puerto Rico, offers a unique natural experiment setting for examining the influence of landscape-scale drivers (contrasting geologic parent materials and climactically-driven forest types) on microbial communities along deep soil profiles. Previous studies have found rapid rates of N cycling in this ecosystem (*Cusack et al.*, 2009; *Silver et al.*, 2005; *Templer et al.*, 2008) but research has been restricted to surface soils and above-ground vegetation. Here we coupled a molecular fingerprinting approach (DGGE) with measurements of NFGs using qPCR to

simultaneously assess changes in bacterial community structure and functions. The five NFGs we measured encode enzymes responsible for major N transformations in soils, including N fixation (*nif*H), nitrite reduction (*nir*S), nitrous oxide reduction (*nos*Z), ammonia oxidation (*amo*A) and organic N decomposition (*chi*A, *Lindsay et al.* (2010); *Wallenstein and Vilgalys* (2005)). We hypothesized that forest type would be an important control on community structure and NFGs in surface soils, as different vegetation assemblages lead to organic matter inputs of varying chemical quality. In subsoils, we predicted that community structure and NFG abundances would be relatively decoupled from forest type but that soil parent material will be more important. Finally, we predicted that the distribution of NFGs would change with depth to reflect variations in the dominant microbial processes along environmental gradients. We predicted that organic N decomposition and N fixation would be more important processes in surface soils, where organic matter is more abundant and N may be more limiting due to high plant demand. In subsoils, we predicted the importance of anaerobic metabolism and non-C energy sources would increase, which would be reflected by increased microbial denitrification activity.

4.2. Methods

4.2.1. Sample set

The samples used in this study comprise part of a larger sample set collected in January 2012 from four sites at the Luquillo Critical Zone Observatory (LCZO) in northeastern Puerto Rico (18°18' N, 65°50' W). A detailed descriptions of the study site and characterizations of these soils samples can be found in *Stone et al.* (2014) . Briefly, the sample sites were stratified across two dominant forest types, the lower-elevation (< 600 m) Tabonuco (*Dacroydes excesla* Vahl) forest, and the intermediate to high elevation (600-800 m) Palo Colorado (*Cyrilla racemiflora* L.) forest. Within each forest, samples were collected at sites representing the two dominant geologic parent materials in the region: lower-Cretaceous volcaniclastic (VC) sediments of andesitic composition that weather to produce Oxisols and an early-Tertiary age quartz-diorite (QD) pluton known as the Rio Blanco stock (*Seiders*, 1971a,b), that weathers to produce Inceptisols. Within each soil × forest combination, pits were excavated at each of three topographic positions (one ridge top, three slope and one

valley pit), and samples were collected at discrete depth intervals from the surface A-horizon (considered 0 cm depth) to 140 cm depth or bedrock. For the present study, subsamples from the three slope pits were immediately refrigerated at 4 °C following collection, and stored at -20 °C prior to DNA extraction.

4.2.2. DNA extraction and PCR-DGGE

Genomic DNA was extracted from 0.3-gram aliquots of each soil sample using the Power Soil DNA Extraction kit (MoBio Laboratories, Carlsbad, CA, USA). DNA was quantified by spectrophotometric absorption at 260 nm, and the purity was assessed from absorbance ratios at 260/280 and 260/230 nm using an ND-2000 Nanodrop spectrometer (Thermal Scientific, Wilmington, DE).

For our community fingerprinting analysis, DNA was pooled in equimolar from the three replicates at each soil × forest × depth combination to reduce the number of samples and reduce variation across replicates. Due to the low-DNA amount obtained from subsoils, a nested PCR strategy was used to improve PCR amplifications. We acknowledge that nested PCR can lead to amplification biases that distort the relative abundance of taxa in a sample. This can occur due to additional cycles introduced during nested PCR, and because different primer sets can result in different levels of amplification (*Tedersoo et al.*, 2010). For this reason, nested PCR was performed on all samples to avoid introducing additional bias to subsoil samples relative to surface samples, template DNA concentrations were normalized, and total number of amplification cycles was limited to 30 (*Kanagawa*, 2003). In subsequent discussion of DGGE patterns, we avoid quantitative statements about the relative abundance of different taxa and focus instead on the overall structure of the community fingerprint. For PCR amplification, we chose universal primers that gave us the highest rates of amplification success in preliminary trials. The Eubacteria-specific primer set 27F/1492r (*Lane*, 1991) was used for 20 cycles of the primary amplification. For the secondary PCR, an additional ten rounds of amplification were performed using 1 μl of PCR product from the first round and the 1070f (ATGGCTGTCGTCAGCT) and GC-clamped 1392r (ACGGGCGGTGTGTAC) primers (*Kan et al.*, 2006). The PCR reactions were performed using an automated Eppendorf Mastercycler Thermal Cycler (Perkin-Elmer, Norwalk, CT).

16S rRNA gene-denaturing gradient gel electrophoresis (DGGE) was performed using the Dcode system (Universal Mutation Detection System, Biorad) as previously described (*Kan et al.*, 2006; *Muyzer et al.*, 1993). First, we examined changes in community structure along a single depth profile, using samples at 10 cm increments from 0–140 cm to construct a detailed picture of how major bacterial populations change with depth. Based on our findings from this analysis, we selected five depth increments (0, 20, 50, 80 and 110 cm) as representatives that span the soil profiles and ran additional DGGE gels with samples from each soil × forest type combination at these five depths. This second analysis allows us to evaluate differences in community structure across soil and forest types, while capturing the variation in community structure that exists within individual depth profiles.

Equal amounts of PCR products were loaded onto an 8% vertical polyacrylamide gel containing a 50-70% denaturing gradient made of urea and formamide. Gels were electrophoresed at 60 °C and 70 V for 16 h and visualized with SYBR Gold staining (Life Technologies, NY). Digital photographs of gel images were analyzed using GelComparII v.5.10 (Applied Maths, Austin, TX, USA). A binary data matrix (presence or absence of bands) was used for all subsequent analysis to prevent patterns from being biased by variable band intensities.

4.2.3. Band excision and sequencing of DNA fragments

Dominant and unique bands were excised from DGGE gels, re-amplified using PCR, and sequenced with no-GC clamp primers 1070f using Bigdye-terminator chemistry by ABI PRISM3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were compared to the GenBank database using BLAST, and then deposited to the database under accession numbers KF937825-KF937872, KF951497-KF951502 and KJ142756-KJ142765. Because these bands represent individual taxonomic groups that we were able to isolate and do not reflect the relative abundances of different taxa in our samples, the results of this analysis are not discussed in the paper. A table containing sequenced, identifiable bands can be accessed here: Supplementary Table.

4.2.4. Quantitative PCR

Quantitative PCR (qPCR) was performed on samples from 0, 20, 50, 80, 110 and 140 cm depth to evaluate changes in N-cycling functional genes with depth and across the four soil × forest type combinations. Unlike our fingerprinting analysis, we did not pool samples across soil pits for qPCR, but measured gene copy numbers in each replicate depth interval separately. The five genes chosen for qPCR (*nif*H, *nir*S, *nos*Z, *chi*A and *amo*A) represent major transformations in the soil N cycle (*Lindsay et al.*, 2010; *Wallenstein and Vilgalys*, 2005). We followed the protocol of *Wallenstein and Vilgalys* (2005), with several modifications. Standard curves were constructed for each assay using DNA extracted from an organism containing the gene of interest (Table 4.1). DNA from each standard organism was amplified using the corresponding gene-specific primers, then serially diluted to obtain at least 5 concentrations that encompass the range observed in the samples (Table 4.1). For *chi*A, the primer concentration was experimentally optimized to 0.4 μM for both forward and reverse primers (Table 4.1). Amplification was performed using an ABI 7500 Sequence Detection System (Applied Biosystems) and in 10 μl reactions containing 5 μl of SYBR green (Applied Biosystems), primers, 1× BSA, 1 μl template DNA and PCR-grade water. A melting curve analysis was performed following each assay to ensure specificity of amplification. Copy numbers of genes present in a sample are reported per ng of total soil DNA present in the original DNA extract, yielding units of copy # ng^{-1} soil DNA.

4.2.5. Statistical analyses

All statistical analyses were performed using R v. 3.0.2 (R Core Team, 2012). The relationship between soil variables and DGGE banding patterns was analyzed using nonmetric multidimensional scaling (NMDS). NMDS allows the graphical representation of relationships between complex microbial community data and environmental variables. NMDS analysis was performed with the "metaMDS" function in the "vegan" package. The jaccard dissimilarity index was used because it produced the lowest stress compared with other indices and is considered most appropriate for binary datasets (*Oksanen*, 2013). The "envfit" function was used to overlay significant environmental vectors on a 2-D ordination of the samples. This tool is designed to fit environmental vectors onto

an NMDS ordination, with the magnitude of the vectors proportional to the correlation between ordination and environmental variable and their direction indicating the direction of the environmental gradient (*Oksanen*, 2013). Envfit also performs statistical significance tests on R^2 values by permutation.

All NFG measurements and environmental data (i.e., C, N, etc.) were log-transformed prior to regression analysis or analysis of variance to improve normality. Type II linear regression was used to evaluate relationships among absolute NFG abundances and environmental variables using the "lmodel2" package (*Legendre*, 2013). To better evaluate bacterial contributions to the N-cycle transformations investigated in this study, we examined the relative abundances of our NFGs by normalizing each NFG to the total NFG abundance in a given sample. We acknowledge that this approach provides an incomplete understanding of microbial contributions to the N-cycle since we did not measure all possible NFGs. Nevertheless, we believe it gives us valuable information about the relative biological capacity for specific N-cycle processes to occur.

We used analysis of variance (ANOVA) of linear mixed-effects models with the "lme4" package in R (*Bates et al.*, 2012) to determine the effects of soil type, forest type, depth and their interactions on NFGs. Soil and forest type were treated as fixed effects and soil pit (representing field replication) was treated as a random effect nested within soil × forest type. Where significant effects were found, we ran post-hoc t-tests or Tukey's HSD test using the 'testInteractions' function in the "phia" package (*Rosario-Martinez*, 2012). Statistical significance was determined at the $\alpha = 0.05$ level. We corrected the P value for significance associated with an α value of 0.05 using a Bonferroni correction.

4.3. Results

4.3.1. Community structures with depth

Dynamic changes in bacterial population structures with soil depth were observed as demonstrated by distinct DGGE band patterns (Figure 4.1, only Colorado Oxisol shown). Unique and dominant band numbers increased from 30 cm below the surface and decrease after 110 cm, indicating distinct

bacterial populations occurred at different soil layers. NMDS provided statistical validation for these visual observations (Figure 4.2): deeper samples were typically shifted to the right along axis 1, although there was a large amount of scatter among the samples. Bacterial community structure was highly correlated (i.e., $r^2 > 0.5$) with gradients in C and N concentrations, as well as with C:N ratios and pH.

4.3.2. Community structures across soil and forest types

Following our examination of a single depth profile, we selected five depth intervals (0, 20, 50, 80 and 110 cm) that provided good coverage of the soil profile and ran a gel containing samples from each soil × forest type at each of these five depth intervals (Figure 4.3). For this comparison, we found bacterial communities were moderately separated into four groups based on forest type (Colorado vs. Tabonuco) and soil type (Inceptisol vs. Oxisol, Figure 4.4). Colorado forest microbial communities formed tight clusters compared to Tabonuco forest communities, indicating forest type plays a significant role in shaping the major bacterial community structure. Within the Inceptisol soil type, bacterial communities formed particularly distinct groups based on forest types. Bacterial communities from different soil types (Inceptisol vs. Oxisol) were separated by axis 1, but overall the two soil types did not form distinct clusters. Further, no trends in community structure with depth were evident across the soil and forest types. Statistical analyses indicated that the NMDS ordination pattern was significantly correlated with soil pH, soil C:N, C:P and N:P ratios (Table 4.2), but not correlated with total C, N or P concentrations, or any of the NFGs.

4.3.3. Quantitative PCR

For all soil × forest combinations and depths combined, the abundance of each NFG declined significantly with depth. The relative abundance of all measured NFGs also changed with depth (Figure 4.5). Averaged across all soil × forest types, the denitrification genes accounted for the largest fractions of measured NFGs (average contributions of 49% for *nir*S and 31% for *nos*Z), and the relative abundance of these two genes also changed the most dramatically with depth. The relative abundance of *nir*S declined from 74% at 0 cm to 15% at 140 cm, while the relative abundance of

*nos*Z increased from 14% in surface soils to 54% at 140 cm (Figure 4.5). The relative abundance of the organic N decomposition gene *chi*A gene increased from 3% in surface soils to 17% at 140 cm, while the relative abundance of the nitrogen fixation gene *nif*H ranged from 9-20% from 0 to 80 cm but declined to 1% at 140 cm. The bacterial ammonia oxidation gene *amo*A was the least abundant NFG throughout our soil profiles, accounting for < 1% of total measured NFGs at each depth interval.

Across all soil depth intervals, forest type did not have an effect on the distribution of any NFGs. However, all NFGs except *amo*A were significantly more abundant in Inceptisols compared with Oxisols. Inceptisol subsoils contained markedly higher copy numbers of the denitrification genes *nir*S and *nos*Z compared with Oxisol subsoils (Figure 4.6, $P < 0.01$ for soil $\times$ depth interaction). At 110 cm, *nir*S copy numbers were 12.5 $\times$ higher and nosZ copy numbers 36 $\times$ higher in Inceptisols compared with Oxisols. This difference was even more exaggerated at 140 cm, where *nos*Z copy numbers were 200 $\times$ higher in Inceptisols compared with Oxisols, and *nir*S was entirely absent from Oxisols.

All NFGs were positively correlated with soil C, N and P concentrations. We were surprised to find that in each case, the relationship with soil P content was the strongest, with correlation coefficients ranging from 0.32-0.50 (Table 4.2). Correlation coefficients of NFGs with C and N ranged from 0.28-0.45 (Table 4.2). There were no significant correlations between NFGs and pH. Most NFGs were not correlated with soil nutrient ratios, with the exception of chiA, which was weakly but significantly correlated with soil N:P and C:P ratios (Table 4.2).

4.4. Discussion

4.4.1. Community structure

We were surprised that across the four sites we investigated, we did not observe trends in major bacterial community structure with soil depth. We expected the dramatic declines in soil C with depth to be a key driver of community structure. Rather, the variation in community structure associated with differences in resource stoichiometry and soil physio-chemical properties across the two soil

and forest types (discussed below) overwhelmed any depth-driven trends. However, within an individual Colorado Oxisol depth profile we did observe distinct changes in community structure with depth that related to gradients in C and N availability. It is likely that shifting resource availability and environmental conditions (e.g., moisture and oxygen availability) throughout this soil profile selects for different dominant taxa that fill different ecological niches. For example, other studies have found the abundance of gram-positive bacteria increases with depth (*Stone et al.*, 2014; *Blume et al.*, 2002; *Fierer et al.*, 2003b). Gram-positive organisms, including members of the phyla Firmicutes and Actinobacteria, are known for their ability to form endospores and survive harsh environmental conditions including desiccation (*Onyenwoke et al.*, 2004), that may prevail in subsoils. It is likely the depth-dependent changes in community structure we observed in the Colorado Oxisols are not unique to this site. Rather, we would expect dramatically shifting resource availability to result in community shifts at any of the sites in our study. Indeed, in several other preliminary DGGE gels that focused on single soil profiles, we also observed shifts in community structure with soil depth (data not shown). However, community shifts with depth are also likely to be site-dependent: each soil profile in our study represents a different combination of available resources and environmental parameters that relate to its unique combination of geology and vegetation. Therefore, that we did not observe one overarching depth trend across our four sites may simply indicate that the bacterial communities at each site are shifting with depth in different ways.

Alternatively, it is possible that, were we to include additional replicates at each depth interval for each soil $\times$ forest type, depth trends in community structure might have emerged across the sites. However, given the limited number of samples that can be included in a single gel and hence analyzed together, we chose to pool depth replicates for each soil and forest type rather than analyze their community structures separately. Other limitations of the DGGE technique may mask depth-dependent changes in community structure across our four sites. All PCR-based molecular approaches focus on the major components of microbial communities, possibly due to several biases introduced during amplifications, such as G+C content (*Dutton et al.*, 1993), copy number of the 16S rRNA gene (*Farrelly et al.*, 1995), primer selection and cycle numbers (*Suzuki and Giovannoni*, 1996; *Tedersoo et al.*, 2010), and chimera formation (*Kopczynski et al.*, 1994). "Universal" primers

are supposed to be able to amplify most of the phylogroups of bacteria from natural environments, however, none of these primers are able to cover all bacterial groups (*Hong et al.*, 2009). Minor groups may be beyond the detection limit and thus the richness of bacterial communities is likely underestimated (*Kisand and Wikner*, 2003). Indeed, we have only just begun to understand the diversity and distribution of bacterial communities in tropical soils and subsoils.

In addition to our single depth profile analysis, we observed clear differences in major bacterial community structure across the two soil types and two forest types investigated in this study. These effects are not necessarily separate. Rather, communities from each soil × forest combination could be visually separated by NMDS analysis. Differences in community structure across the four sites are proximally driven by differences in soil pH and resource stoichiometry. Overall, the Inceptisols were less acidic (pH = 5.6 ± 0.15) than the Oxisols (pH = 4.32 ± 0.1), and in particular, the Tabonuco Inceptisols (pH = 6.1 ± 0.21) were significantly less acidic than the Colorado Inceptisols (pH = 4.97 ± 0.1) or Oxisols. By correlating our NMDS analysis with environmental variables, we were able to verify that bacterial community structure within the Tabonuco Inceptisols was unique, and that this uniqueness was strongly related to the higher pH of these soils. pH differences across the two soil types are likely related to differences in pedogenic history across the two parent materials. The longer weathering history of the VC parent material has led to the development of Oxisol soils depleted in base cations that decrease acidity and rich in Fe and Al secondary minerals (*Scatena*, 1989). By contrast, the Inceptisols are primary-mineral rich soils, representing the product of rapid chemical weathering on the geologically younger QD parent material (*Boccheciamp et al.*, 1977; *Buss et al.*, 2005; *White et al.*, 1998) . Numerous other studies have found pH to be a key driver of bacterial community structure (*Baath and Anderson*, 2003; *Rousk et al.*, 2010) or diversity (*Fierer and Jackson*, 2006). One reason for this may be pH-dependent differences in organo-mineral interactions. With increasing acidity below pH 6-7, Fe and Al- oxyhydroxide solubility increases and complexation of Fe^{3+} or Al^{3+} with organic matter increases (*Lindsay*, 1979; *Wagai and Mayer*, 2007). This can reduce the bioavailability of soil C, resulting in indirect effects on microbial community structure (*Heckman et al.*, 2009). Additionally, Fe^{3+} and Al^{3+} bind and occlude inorganic P (*Chacon et al.*, 2006), leading to pH-dependent differences in P-availability, which can also drive

changes in community structure (*DeForest and Scott*, 2010; *DeForest et al.*, 2012). Although we did not observe a direct effect of P concentration on community structure, we found that total community structure was influenced by nutrient stoichiometry, including soil N:P and C:P ratios, both of which were higher in Oxisols. Overall, our results suggest differences in mineralogy and pedogenic history across the two soil types have resulted in different physio-chemical environments, including differences in acidity and P availability, with feedbacks on bacterial community structure (*Gelsomino and Azzellino*, 2011; *Heckman et al.*, 2009).

In addition to pH, separation of bacterial communities across the Tabonuco and Colorado forest types was related to differences in nutrient stoichiometry, with higher C:N, C:P and N:P ratios in the Colorado forest compared with the Tabonuco forest. The higher-elevation Colorado forest is characterized by a cooler, wetter climate than the Tabonuco forest and exhibits lower soil nutrient concentrations and higher foliar and soil C: nutrient ratios (*Cusack et al.*, 2011; *McGroddy and Silver*, 2000). A previous study using ^{13}C NMR found Colorado forest litter to be more highly processed and of poorer quality, as indicated by greater alkyl : O-alkyl C ratios and greater aromatic C content. Moreover, these authors found C quality differences can be related to differences in microbial community structure in surface soils (*Cusack et al.*, 2011). Our study is thus in agreement with previous work showing an effect of soil resource quality on community structure. We were surprised to find that both surface and subsoil bacterial communities could be differentiated according forest type. In subsoils, organic carbon is expected to be highly microbially processed and to compositionally reflect microbial biomass rather than fresh plant material (*Kramer and Gleixner*, 2008; *Rumpel and Kogel-Knabner*, 2011), therefore we did not expect differences in litter quality or nutrient stoichiometry across the two forests to influence subsoil communities. However, recent research has shown root C inputs exert a dramatic effect on subsoil C cycling through stimulation of microbial activity (*Fontaine et al.*, 2007). Differences in rooting depth and belowground C allocation patterns among the forests could have a profound impact on the structure of subsoil bacterial communities.

4.4.2. Nitrogen functional genes

The decline in all functional genes with depth indicates that N transformations are occurring primarily in the surface layers of the soil profile. While the vertical distribution of N-cycle processes is rarely reported in the literature, this result is not surprising given the orders of magnitude greater microbial biomass (*Stone et al.*, 2014) and soil C and N concentrations in surface soils. Nevertheless, our finding that NFG abundance declines sharply with depth may have important physiological implications. Our absolute NFG measurements, which are reported per ng of soil DNA, are analogous to specific metabolic activity measurements (i.e., activity per unit microbial biomass) that are commonly used when reporting CO_2 respiration or enzyme activities (*Barriuso et al.*, 1988; *Trasar-Cepeda et al.*, 2008). The decline in the "specific" abundance of NFGs with depth may speak to a general decrease in metabolic activity as energy becomes increasingly scarce (*Fierer et al.*, 2003a,b; *Hoehler and Jorgensen*, 2013). The metabolic processes reflected in the functional genes we measured included anaerobic respiration (*nos*Z and *nir*S), resource acquisition (*nif*H and *chi*A) and energy acquisition via substrate oxidation (*amo*A). If investment in any of these processes were too resource-costly in a low-C subsoil environment, we would expect microbes to decrease their energetic investment. For some genes, it is also possible that we reached the detection limit of qPCR due to the low DNA concentrations present in the reaction; a consequence of low concentrations of extractable DNA in deep soils. For instance, in surface soils the *amo*A gene only represented 0.07% of total measured NFGs. Bacterial ammonia oxidation genes may still be present in subsoils, but the concentrations in our deep soil DNA samples may have been too low to overcome the detection limit of qPCR. We also acknowledge that our exclusive focus on bacterial NFGs does not reflect the total biological potential for N cycling. Chitin degradation is commonly associated with fungi as well as bacteria (*Hodge et al.*, 1995; *Talbot and Treseder*, 2010) and in addition to ammonia oxidizing Bacteria (AOB), ammonia oxidizing Archaea (AOA) can contribute significantly to nitrification (*Chen et al.*, 2008; *Leininger et al.*, 2006) in many soils. Recent studies have shown that AOA have a broader range of habitats and may be the predominant ammonia-oxidizing populations in most natural environments (*Stahl and de la Torre*, 2012). In addition, there may be N transformation pathways we did not investigate that are important for tropical soils. For example,

dissimilatory nitrate (NO_3^-) or nitrite (NO_2^-) reduction to ammonium (NH_4^+), or DNRA, can be an important process for retaining N in humid tropical forest soils (*Silver et al.*, 2001; *Templer et al.*, 2008), as fluctuating redox conditions can lead to high rates of DNRA during periods of low oxygen availability (*Silver et al.*, 2001, 2005).

By examining the relative abundance of NFGs we were able to investigate trends in bacterial community investment in major N cycle processes that would otherwise be masked by the large declines in all NFGs with depth. The low relative abundance of *nif*H and its declining relative abundance with depth is consistent with previous studies which have reported N_2-fixing bacteria occur primarily in the upper soil layer and constitute 5% of the total bacterial population (*Mergel et al.*, 2001). N_2 fixation is likely to be more prevalent in surface soils due to the abundance of heterotrophic decomposers whose N requirements are high relative to the plant tissues being decomposed (*Roper and Ladha*, 1995; *Torres et al.*, 2005). The trend toward increased *chi*A with depth may speak to an increasing importance of organic N sources in deep soils. This pattern fits with recent work on a related set of soils by *Stone et al.* (2014). These authors found that *N*-acetyl glucosaminidase, an enzyme involved in chitin degradation, exhibited high specific activity in subsoils. Given that soil P concentrations increased with depth relative to C and N, increased chitinase production in subsoils may be the result of alleviated P limitation, as has been demonstrated in other studies (*Olander and Vitousek*, 2000; *Stone et al.*, 2013). The most dramatic shift was the decline in *nir*S relative abundance from 74% in surface soils to 15% in subsoils and concurrent increase in *nos*Z relative abundance, from 15% in surface soils to 54% in subsoils. This shift may speak to a vertical stratification of the denitrification process along an "oxidation gradient" within soil profiles. Each step in the denitrification pathway is inducible in response to O_2 partial pressure, C and substrate availability. Because enzyme production is sequential, there is usually a lag of hours to days between the production of an intermediate substrate and its consumption by the next enzyme in the pathway (*Robertson and Groffman*, 2007). Higher relative abundances of *nir*S (representing reduction of nitrite to nitric oxide) in shallower soils and increasing abundance of *nos*Z (representing the final reduction of nitrous oxide to N_2) with depth may reflect the increased reduction of mineral N substrates as they are mobilized down the soil profile.

Greater abundance of all NFGs in Inceptisols compared with Oxisols suggests a more dynamic soil N cycle in Inceptisols, with potentially greater N losses in subsoils balanced by greater rates of N fixation. Nitrogen fixation is an energetically expensive process, requiring large C inputs per mole of N fixed. The 150% greater abundance of the *nif*H gene in Inceptisols compared with Oxisols is strong evidence of greater microbial N demand on the younger soils. We propose two potential explanations for this pattern. Firstly, high N fixation rates could balance greater N losses from denitrification and export. The coarse texture and high conductivity of Inceptisol soils results in greater subsurface water penetration, fluctuating subsurface redox conditions and frequent inputs of dissolved N to subsoils (*McDowell et al.*, 1992; *McSwiney et al.*, 2001). By contrast, dense clays in the Oxisols impede subsurface infiltration and relatively dry, oxic conditions prevail beneath the rooting zone (*McDowell et al.*, 1992). With distinct redox zones (*McSwiney et al.*, 2001), Inceptisol subsoils may provide a more favorable environment for denitrification compared with their Oxisol counterparts. Previous research has shown greater stream NO_3^- export in the QD watershed (*McDowell et al.*, 1992; *McSwiney et al.*, 2001), which has been attributed to high nitrification activity in the stream banks. Higher N loss rates due to leaching and stream export in Inceptisol subsoils could produce a feedback on surface N-cycle processes, increasing microbial N demand and ultimately stimulating N-fixation. Secondly, given that we found strong correlations between each NFG and extractable P concentrations, high N demand in Inceptisols could be reflective of higher P availability resulting from differences in mineralogy, texture and weathering rates between the two soils. Although we did not detect differences in total extractable P between the two soil types, it is difficult to draw direct linkages between chemically-extractable and microbially-available P (*Johnson et al.*, 2003). However, we did observe lower soil C:P and N:P ratios, indicating relatively higher P availability in Inceptisols. Other studies in Amazonian forest soils have found increasing total P and strong-acid extractable P with increasing clay content, but larger labile P pools in sandier soils (*Silver et al.*, 2000; *Tiessen et al.*, 1994). These two explanations for greater bacterial N-cycling potential in Inceptisols are not mutually exclusive; rather, the same physiochemical features of Inceptisols that result in increased N export may also ameliorate P limitation, setting the stage for a more active N-cycling microbial community.

4.5. Conclusions

We found differences in dominant bacterial community structure across two soil and two forest types within the upper 1.4 meters of tropical soil profiles. Contrary to our initial hypothesis that forest type would be the primary driver of community structure in surface soils and soil type would be a more important driver in subsoils, bacterial communities could be differentiated according to soil and forest type across all depth intervals. These differences in community structure were related to differences in soil pH and resource stoichiometry across the four sites. We also found evidence for shifts in community structure with depth that relate to gradients in bulk soil resource availability (i.e., C & N). While concentrations of all NFGs declined with depth, corresponding with declines in C and nutrient concentrations, shifting relative abundances indicate that N-fixation occurs largely in surface soils, while denitrification and organic N decomposition are more important processes in subsoils. The greater overall abundance of NFGs in Inceptisols and their vertical distribution suggest that greater N inputs are balanced by greater N losses in the sandier soil that sits on a younger, more rapidly weathering parent material. Overall, both bacterial community structure and functional attributes are influenced by soil type and depth, underscoring the importance of pedogenically-driven vertical gradients in controlling microbial processes. Our study shows that in tropical forests, bacterial communities exhibit the capacity to perform N-cycle transformations in deeper parts of the soil profile than have previously been measured, and variable subsurface redox conditions may mediate these transformations.

Table 4.1: Nitrogen cycle genes investigated in this study, their functions, cultures used for positive controls, forward and reverse primers, amplicon lengths, annealing temperatures and standard curve ranges.

Gene	nifH	amoA	nirS	nosZ	chiA
Function	nitrogen fixation	ammonia oxidation	nitrite reduction	nitrous oxide reduction	organic N decomposition
Organism (ATCC (1)	Bradyrhizobium	Nitrosomonas	Paracoccus	Paracoccus	Serratia
or NRLL (2) number)	japonicum (B-4361)	europea (19,718*)	denitrificans (B-3785)	denitrificans (B-3785)	marcescens (B-2544)
Forward primer (μM)	AAAGGYGGWATCG	GGGGTTTCTACTG	CCTAYTGGCCGCC	CGYTGTTCMTCGA	CGTCGACATCGAC
	GYAARTCCACCAC (0.5)	GTGGT (0.4)	RCART (0.5)	CAGCCAG (0.5)	TGGGARTDBCC (0.4)
Reverse primer (μM)	TTGTTSGCSGCRT	CCCCTCKGSAAAG	CGTTGAACTTRCC	CATGTGCAGNGCR	ACGCCGGTCCAGC
	ACATSGCCATCAT (0.5)	CCTTCTTC (0.4)	GGT (0.5)	TGGCAGAA (0.5)	CNCKNCCRTA (0.4)
Amplicon length (bp)	432	592	1127	706	400
Annealing temperature (°C)	53	54	51.5	52	57
Standard curve range (copy no.)	10^6-10^0	10^5-10^0	10^7-10^1	10^5-10^0	10^5-10^0
Citation	Wallenstein and Vilgalys (2005)	Wallenstein and Vilgalys (2005)	Wallenstein and Vilgalys (2005)	Wallenstein and Vilgalys (2005)	Lindsay *et al.* (2010)

Table 4.2: R^2 values and P values for permutation analysis (NMDS ordination) or type II regressions between NFGs and environmental variables. Environmental correlations with the NMDS ordination including both soil types and forest types across five depth intervals (Figure 3) are displayed here. Values in bold represent statistically significant correlations ($P < 0.05$).

Variable	NMDS	chiA	nirS	nosZ	amoA	nifH
Carbon	0.21, 0.13	**0.42, < 0.001**	**0.35, < 0.001**	**0.36, < 0.001**	**0.28, < 0.001**	**0.29, < 0.001**
Nitrogen	0.13, 0.29	**0.45, < 0.001**	**0.33, < 0.001**	**0.39, < 0.001**	**0.30, < 0.001**	**0.32, < 0.001**
Phosphorus	0.01, 0.89	**0.47, < 0.001**	**0.50, < 0.001**	**0.48, < 0.001**	**0.32, < 0.001**	**0.39, < 0.001**
pH	0.69, 0.001	0.01, 0.47	0.03, 0.17	0.04, 0.12	0.0, 0.58	0.08, 0.09
C:N	0.36, 0.02	0.02, 0.26	0.004, 0.61	0.002, 0.70	0.006, 0.53	**<0.001, 0.97**
N:P	0.46, 0.004	0.06, 0.05	0.04, 0.11	0.03, 0.18	0.04, 0.11	0.03, 0.18
C:P	0.59, 0.001	0.07, 0.03	0.02, 0.22	0.03, 0.17	0.05, 0.06	0.02, 0.24

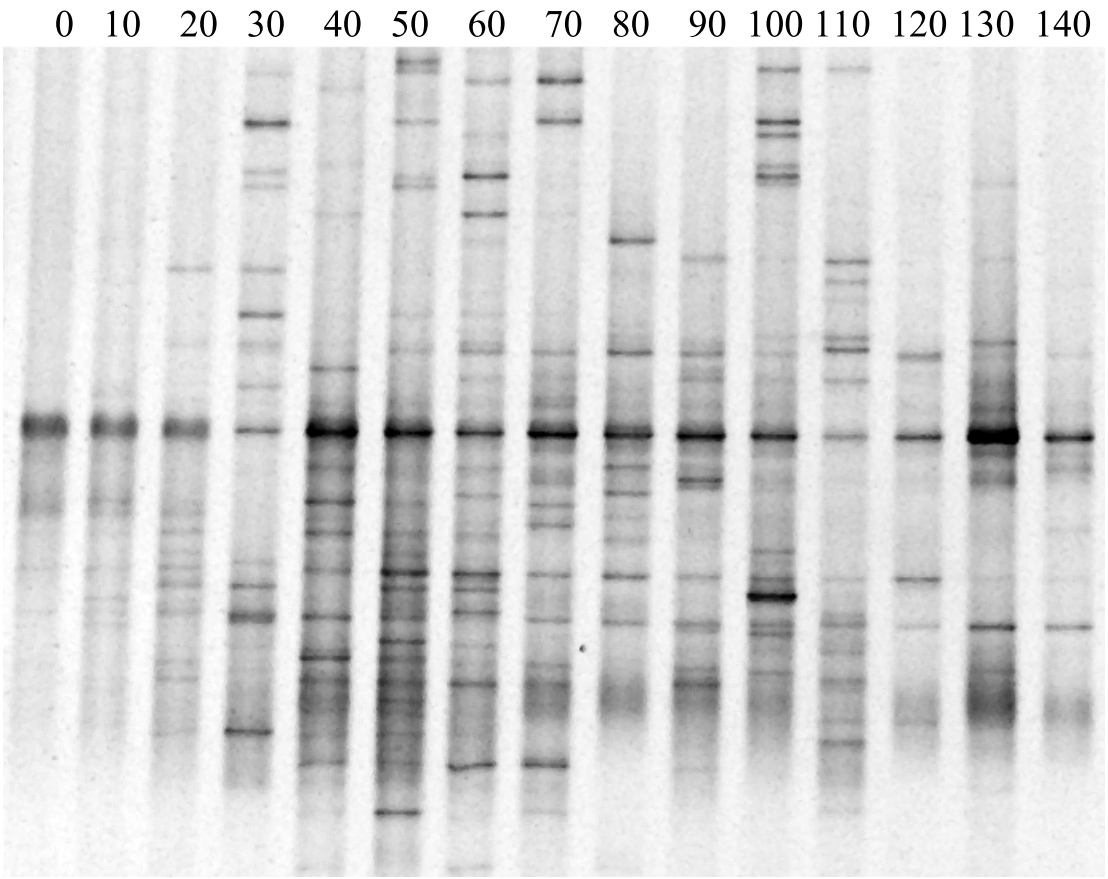

Figure 4.1: DGGE fingerprinting patterns for a Colorado Oxisol depth profile. Soil depth is written above each sample lane. Each sample lane contains PCR product from DNA pooled from three site replicates.

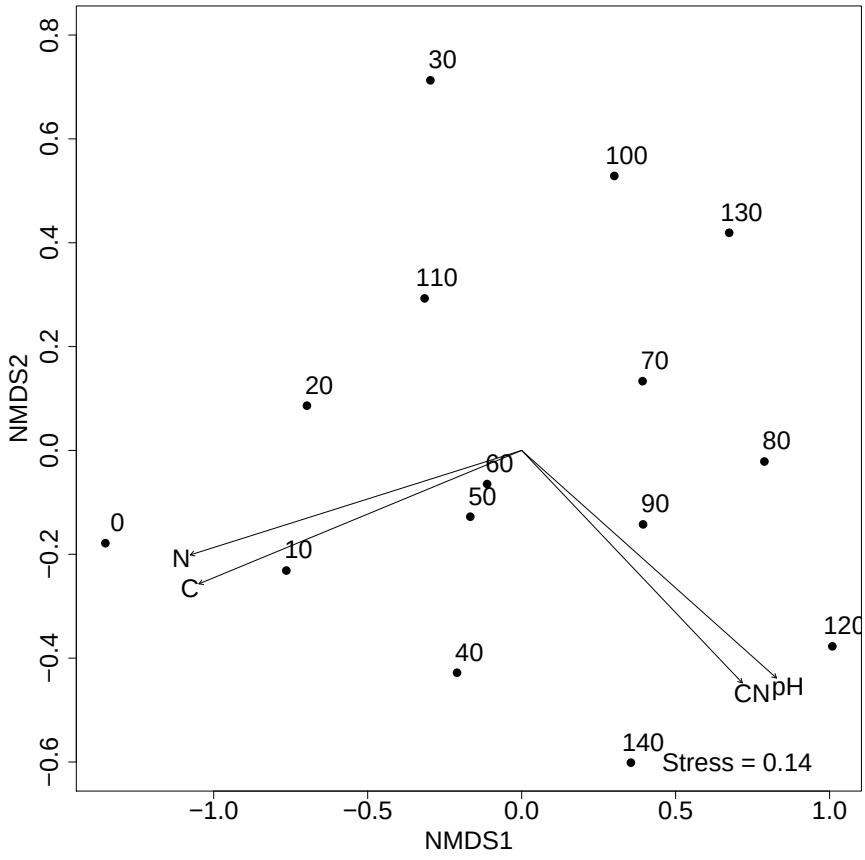

Figure 4.2: Nonmetric multidimensional scaling ordination plot of the DGGE fingerprinting patterns represented in 4.1. Circles represent individual community fingerprints, which are labeled on the plot by their corresponding depth interval. The length and position of the arrows on the plots indicates correlation strengths and directions of significant environmental variables ($P < 0.05$) with the microbial community. Solution NMDS stress = 0.14.

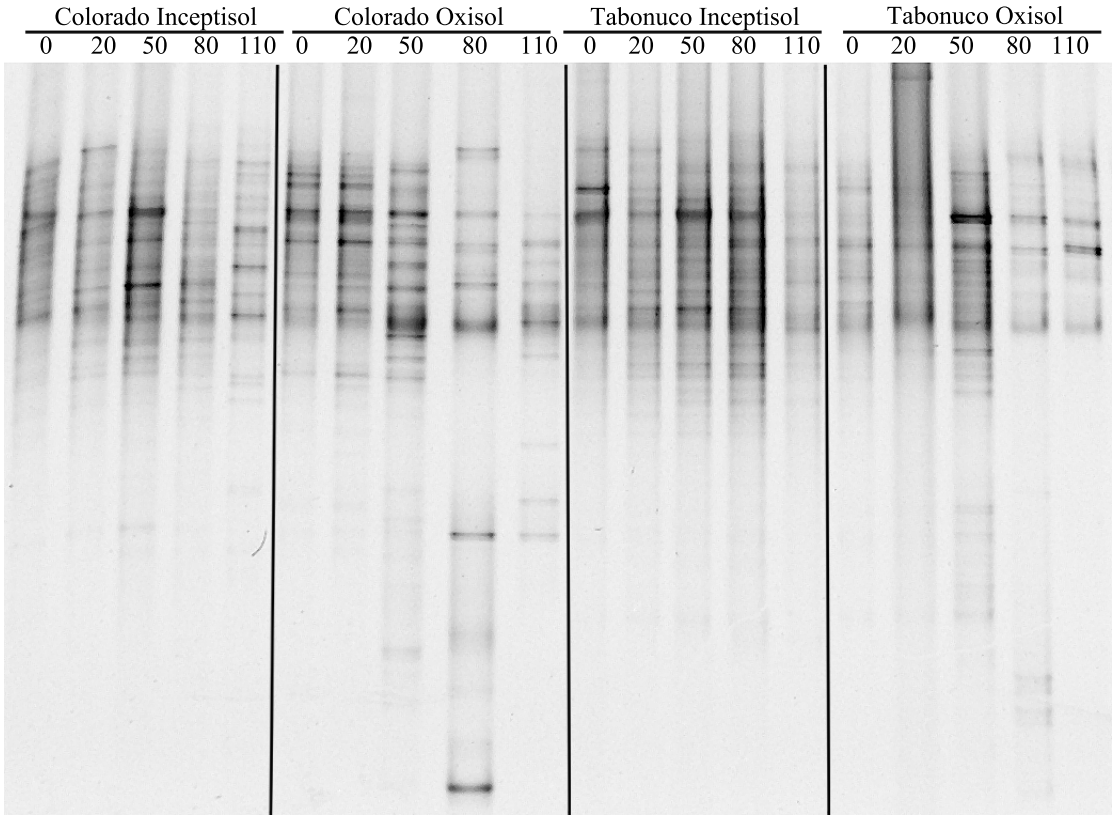

Figure 4.3: DGGE fingerprinting patterns for a Colorado Oxisol depth profile. Soil depth is written above each sample lane. Each sample lane contains PCR product from DNA pooled from three site replicates.

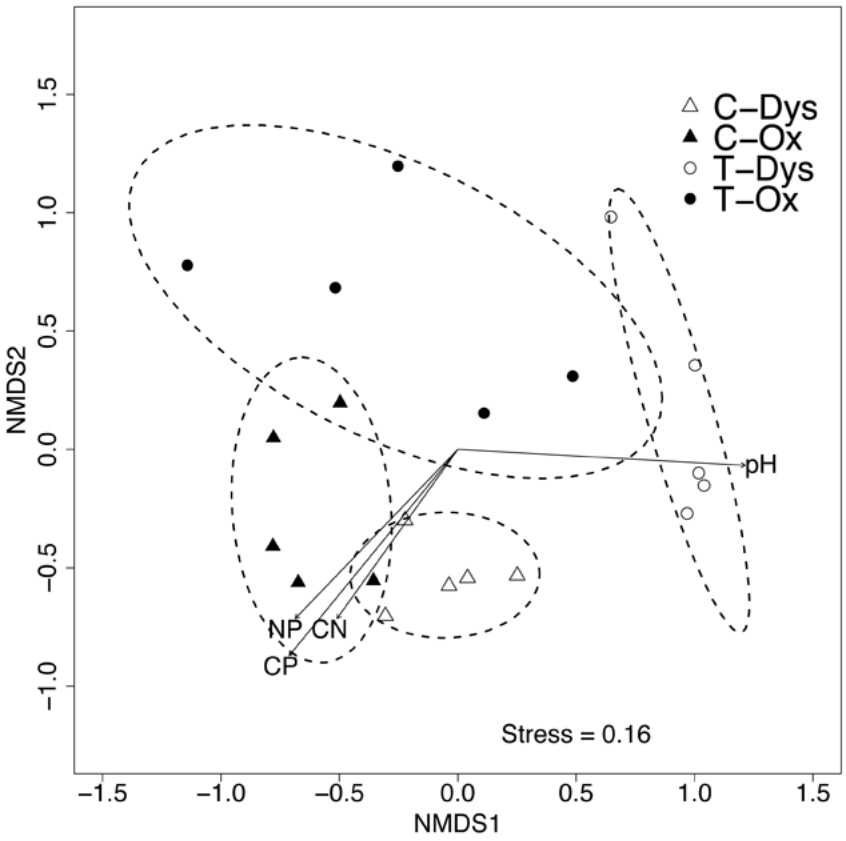

Figure 4.4: Nonmetric multidimensional scaling ordination plot of the DGGE fingerprinting pattern represented in 4.3, across two soil and two forest types for five depth increments (0, 20, 50, 80 and 110 cm). Symbols represent individual community fingerprints. C-Dys = Colorado Inceptisol, C-Ox = Colorado Oxisol, T-Dys = Tabonuco Inceptisol, T-Ox = Tabonuco Oxisol. 75% confidence ellipses are drawn around the four soil × forest types using the "dataEllipse" function in the calibrate package in R (*Graffelman*, 2013). The length and position of the arrows on the plot indicates correlation strength and direction of significant environmental variables ($P < 0.05$) with the microbial community. Solution NMDS stress = 0.16.

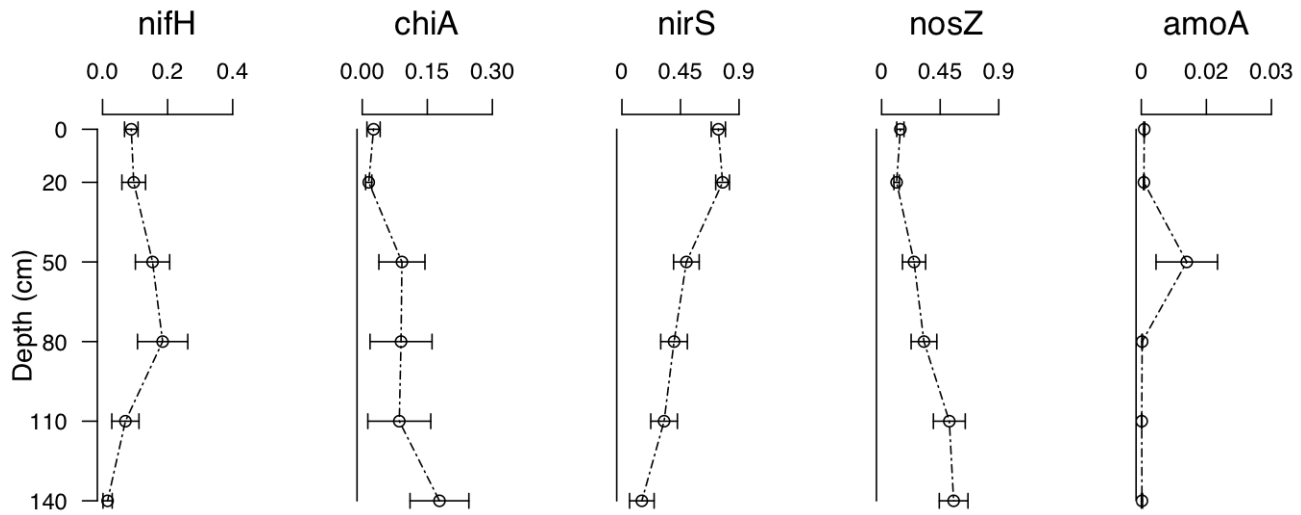

Figure 4.5: Changes in relative abundance of the five measured NFGs as a function of soil depth. NFG name is written above the x-axis and soil depth is written on the y-axis. Data points represent means across both soil types and both forest types at each depth interval (n = 12) and error bars represent standard errors of the mean.

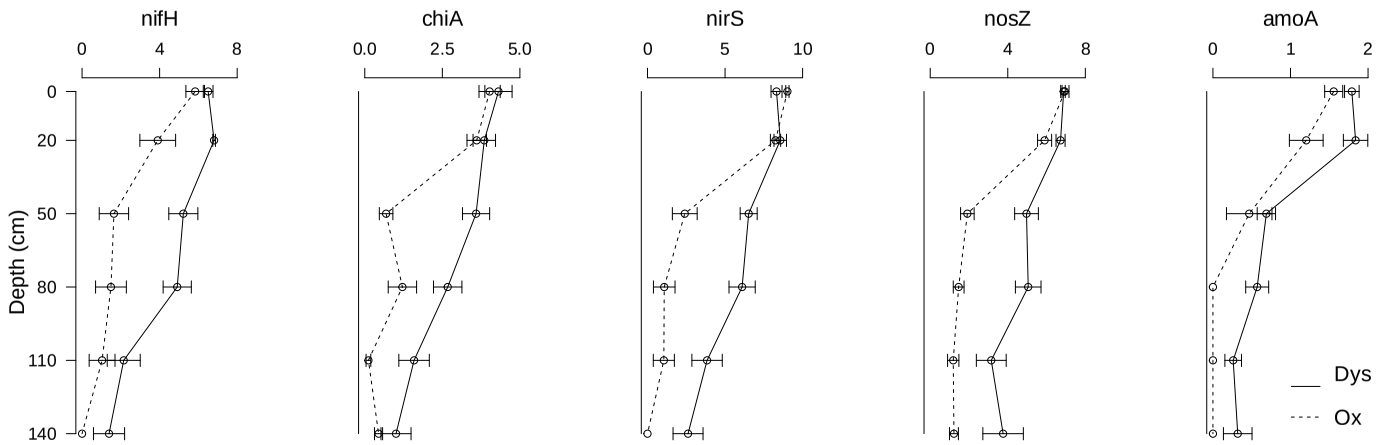

Figure 4.6: Changes in absolute abundance of NFGs as a function of soil depth, separated according to soil type. NFG name is written above the x-axis and soil depth is written on the y-axis. Solid lines indicate Inceptisols and dashed lines indicate Oxisols. Data points represent log-transformed means across both forest types at each depth interval (n = 6) and error bars represent standard errors of the mean. Units are copy # gene ng^{-1} soil DNA.

CHAPTER 5 : Changes in phosphatase kinetics with soil depth across a variable tropical landscape

Modified from paper previously published as:

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Abstract:

Phosphatase enzymes play a key role cycling phosphorus from organic to plant-available pools, particularly in tropical soils where inorganic phosphorus is often limited. However, most studies of phosphatase activity have focused only on surface soils, despite the large quantities of carbon and nutrients stored in tropical subsoils. The goal of this study was to determine how acid phosphatase kinetic parameters change with depth across two parent materials (represented by Oxisols and an Inceptisols) and two distinct forests (lower and upper montane) at the Luquillo Critical Zone Observatory in northeast Puerto Rico. We collected samples from five soil pits at each of four soil × forest types, and measured apparent phosphatase kinetic parameters ($^{App}V_{max}$ and $^{App}K_m$) and soil nutrients at 0, 20, 50, 80, 110 and 140 cm depths. Across all sites, $^{App}V_{max}$ declined 97% and $^{App}K_m$ declined 85% from the surface to 140 cm depth. The ratio of $^{App}V_{max}$ to $^{App}K_m$ (i.e., K_a) did not change through the first meter of soil profiles but was significantly reduced by 50% at 140 cm. Total carbon, nitrogen and extractable phosphorus all declined exponentially with depth. carbon concentrations and $^{App}V_{max}$ were both significantly greater in Oxisols compared with Inceptisols, and in the higher elevation montane forest compared to the lower elevation forest. The scaling relationship we observe between $^{App}V_{max}$ and $^{App}K_m$ is common for environmental systems, although the degree of correlation in our study (R^2 = 0.48) is unusually high, suggesting these parameters are both driven by changes in energy and nutrient availability along depth profiles. However, the consistency of K_a with depth indicates that overall catalytic capacity of phosphatase is

maintained across a range of substrate concentrations. The larger variability in ${}^{App}V_{max}$ compared with ${}^{App}K_m$ suggests microorganisms exert more control over phosphatase production than substrate availability. Our findings indicate that subsoil microbial communities are not metabolically dormant, but rather contribute to P-cycling at rates comparable to their surface counterparts. Further research on ecology of microorganisms in resource-limited tropical subsoils is warranted to better understand microbial contributions to biogeochemical cycles throughout tropical soil profiles.

5.1. Introduction

Phosphorus (P) availability can limit net primary production (NPP) in tropical forests where rock-derived inorganic phosphate is depleted or geochemically bound (*Cleveland et al.*, 2011; *Davidson et al.*, 2007; *Vitousek et al.*, 2010). In such ecosystems, plant-available P may be largely controlled by microbial mineralization of organic P (*Harrison*, 1987; *Johnson et al.*, 2003; *Turner and Engelbrecht*, 2011). Recent research indicates that organic P in tropical soils occurs mainly as phosphate monoesters and phosphate diesters (*Turner and Engelbrecht*, 2011), which can be mineralized by extracellular phosphatase enzymes (*Acosta-Martinez and Tabatabai*, 2011). The activity of these phosphatases therefore plays a key role in regulating plant and microbial P availability in tropical soils.

Numerous researchers have measured soil phosphatase activity (*Hui et al.*, 2013), and an inverse relationship with inorganic P availability is well established (*Clarholm*, 1993; *Olander and Vitousek*, 2000; *Sinsabaugh et al.*, 2008; *Treseder and Vitousek*, 2001). However, most studies have focused exclusively on surface soils where organic P, carbon (C) and other nutrients are relatively abundant. A wide range of biotic and abiotic factors can influence the activity and kinetic behavior of extracellular enzymes, including soil resource (C and nutrient) availability (*Allison and Vitousek*, 2005; *DeForest et al.*, 2012; *Sinsabaugh et al.*, 2008), microbial biomass (*Trasar-Cepeda et al.*, 2008), soil texture (*Allison and Jastrow*, 2006; *Marx et al.*, 2005), mineral association (*Allison*, 2006), moisture (*Baldrian et al.*, 2010; *Steinweg et al.*, 2012), pH (*Rao et al.*, 2000) and O_2 availability (*Hall and Silver*, 2013). All of these soil properties can change with soil depth (*Fierer et al.*, 2003b; *Rumpel and Kogel-Knabner*, 2011; *Spielvogel et al.*, 2008). Thus, microbial contributions to P cycling may also vary widely with depth in tropical soil profiles.

Kinetic parameters provide a more complete description of an enzyme's behavior than potential activities, which are typically measured at a single saturating substrate concentration (*German et al.*, 2011). These kinetic parameters can be used to improve uncertainty analysis and integrate microbial processes into biogeochemical models (*Allison et al.*, 2010; *Wieder et al.*, 2013). The kinetics of simple enzymes consisting of a single active site and substrate are described as a hyperbolic function

by the Michaelis-Menten equation:

$V = [V_{max} \times S] / [K_m + S]$

where V is the reaction rate, S is the substrate concentration, V_{max} is the maximum rate of substrate conversion, and K_m , the half-saturation constant, is defined as the substrate concentration at which the rate of substrate conversion is 1/2 V_{max} (*Michaelis and Menten*, 1913; *Tabatabai and Bremner*, 1971). K_m is also an indicator of enzyme-substrate binding affinity. In soils, these parameters are estimated for a pool of enzymes produced by many different organisms (*Glazer and Nikaido*, 1995; *Zimmerman et al.*, 2013), which may vary considerably in their individual kinetic properties (*Marx et al.*, 2005). Parameters estimated in soils are thus considered apparent V_{max} ($^{App}V_{max}$) and apparent K_m ($^{App}K_m$), with $^{App}V_{max}$ being a measure of enzyme abundance and $^{App}K_m$ being a measure of environmental substrate concentration (*Chrost*, 1991; *Sinsabaugh and Shah*, 2010). Ratios of $^{App}V_{max}$ to $^{App}K_m$ combine enzyme pool size and substrate concentration into a single metric. This metric has been defined as either the specificity constant K_a (*Esti et al.*, 2011; *Moscatelli et al.*, 2012) or the substrate turnover rate S_t (*Crottereau and Delmas*, 1998; *Sinsabaugh and Shah*, 2010). Larger values of K_a or S_t indicate superior catalytic performance.

Tropical subsoils store substantial quantities of C and nutrients (*Jobbagy and Jackson*, 2000). Extracellular phosphatases in tropical subsoils may therefore make an important contribution to P cycling, but we lack a basic understanding of their activity and kinetic behavior. The objective of this study was to evaluate how the kinetic parameters ($^{App}V_{max}$ and $^{App}K_m$) of acid phosphatase change with depth in the context of landscape-scale gradients in parent material and forest type present at the Luquillo Critical Zone Observatory. We predicted that $^{App}V_{max}$ and $^{App}K_m$ would decline with depth in accordance with declines in resource (C and nutrients) availability. Finally, we sought to determine whether the specificity constant (K_a) would shift (indicating an increase or decrease in overall catalytic performance) or remain constant with depth.

5.2. Methods

5.2.1. Sample set

The samples used in this study comprise part of a larger sample set collected in January 2012 from four sites at the Luquillo Critical Zone Observatory (LCZO) in northeastern Puerto Rico (18°18' N, 65°50' W). A detailed descriptions of the study site and characterizations of these soils samples can be found in *Stone et al.* (2014) . Briefly, the sample sites were stratified across two dominant forest types, the lower-elevation (< 600 m) Tabonuco (*Dacroydes excesla* Vahl) forest, and the intermediate to high elevation (600-800 m) Palo Colorado (*Cyrilla racemiflora* L.) forest. Within each forest, samples were collected at sites representing the two dominant geologic parent materials in the region: lower-Cretaceous volcaniclastic (VC) sediments of andesitic composition that weather to produce Oxisols and an early-Tertiary age quartz-diorite (QD) pluton known as the Rio Blanco stock (*Seiders*, 1971a,b), that weathers to produce Inceptisols. Within each soil × forest combination, pits were excavated at each of three topographic positions (one ridge top, three slope and one valley pit), and samples were collected at discrete depth intervals from the surface A-horizon (considered 0 cm depth) to 140 cm depth or bedrock. For the present study, these five pits were considered field replicates for each soil × forest type. We chose six depth intervals for enzyme assays: 0, 20, 50, 80, 110 and 140 cm, resulting in a total of 30 samples per soil × forest combination. Samples were field-fresh sieved to 5 mm to homogenize, and kept frozen at -20 °C until enzyme analysis.

5.2.2. Enzyme assays

Acid phosphatase activity was measured according to the protocol of (*Saiya-Cork et al.*, 2002) with modifications by *German et al.* (2011). Briefly, 0.5 grams of field-moist soil was homogenized in 62.5 ml of sodium acetate buffer using a hand blender. The pH of the sodium acetate buffer was experimentally varied to approximate the pH of the soil samples being assayed, with pH values ranging from 3.7 to 5.0. For each sample, we measured enzyme activity in a 96-well microplate. In each microplate, we performed six replicate assays at each of eight substrate concentrations to construct

Michaelis-Menten curves. The substrate concentration gradient was varied by depth because acid phosphatase activity declined exponentially with depth and preliminary tests indicated that activity was inhibited in many deep soils if assayed using the same concentration gradients as surface soils. Typically, we used a concentration gradient of 4 - 400 μM for surface soils (0 - 20 cm), 1-100 μM for shallow subsoils (50 - 80 cm) and 0.5 - 50 μM for deep subsoils (110 - 140 cm). Each assay well contained 200 μl of soil homogenate and 50 μl of substrate solution (diluted with deionized water to achieve the appropriate concentration). Each microplate also contained substrate blank columns receiving 50 μl of substrate diluted to the same eight concentrations and 200 μl of sodium acetate buffer. Soil homogenate blanks were also measured simultaneously. Plates were incubated at 25 °C for 4 hours (optimal duration of assay determined prior to experiment). After incubation, we added 10 μl of 1M NaOH to each well to terminate enzyme activity and immediately measured fluorescence values at 365 nm excitation and 450 nm emission using a fluorescence microplate reader (Synergy 2 Multi-Mode Microplate Reader, BioTek, Winooski, VT). From fluorescence values, we calculated enzyme activity as the rate of substrate converted in nmol g^{-1} soil hr^{-1} (*German et al.*, 2011). We estimated ${}^{App}V_{max}$ and ${}^{App}K_m$ values for each sample by fitting the Michaelis-Menten equation to the activity values using non-linear regression in R (R Core Team, 2012). We also calculated the specificity constant (K_a) as the ratio of ${}^{App}V_{max}$ and ${}^{App}K_m$.

5.2.3. Statistical analyses

Statistical analyses were performed using R (R Core Team, 2012). Phosphatase kinetic parameters and soil variables were log-transformed prior to statistical analyses to meet the assumptions of normality. Type II standard major axis (SMA) regression was used to evaluate relationships among log-transformed kinetic parameters and soil properties using the "lmodel2" package (*Legendre*, 2013). Type II regression is necessary when the two variables of interest are random because in this case least squares (Type I) regression will underestimate the slope of the linear relationship between the variables. To determine the effects of soil type, forest type, depth and their interactions on phosphatase kinetics parameters, we used analysis of variance (ANOVA) of linear mixed effects models using the lme4 package in R (*Bates et al.*, 2012). Soil and forest type were treated as fixed

effects, soil pit (representing field replication) was treated as a random effect nested within soil × forest type, and depth was treated as a repeated measure within each pit. For several soil pits used in this analysis, we did not have the deepest depth interval (140 cm) represented; the design was therefore unbalanced. We determined Greenhouse-Geisser epsilon correction factors for the model and residual degrees of freedom to correct F ratios and P values for model terms including depth (*Greenhouse and Geisser*, 1959). Where significant effects were found, we ran post-hoc t-tests or Tukey's HSD test using the 'testInteractions' function in the "phia" package (*Rosario-Martinez*, 2012). An alpha value of 0.05 was used as the cutoff for significant effects. We corrected the P value for significance associated with an alpha level of 0.05 using a Bonferroni correction.

5.3. Results

5.3.1. Phosphatase kinetics

Both ${}^{App}V_{max}$ and ${}^{App}K_m$ declined exponentially with depth (Figure 5.1, P < 0.001). ${}^{App}V_{max}$ declined 97% from the surface to 140 cm. There were significant main effects of soil and forest type on ${}^{App}V_{max}$ (Table 5.1). Averaged across all depths, ${}^{App}V_{max}$ was 65% higher in Oxisols (mean = 2155 nmol g^{-1} h^{-1}) compared with Inceptisols (mean = 1308 nmol g^{-1} h^{-1}), and 34% higher in the Colorado forest (mean = 1982 nmol g^{-1} h^{-1}) compared with the Tabonuco forest (mean = 1478 nmol h^{-1} g^{-1}). ${}^{App}K_m$ declined 85% from the surface to 140 cm (P <0.001), but there were no significant effects of soil type, forest type or their interactions (Table 5.1).

There was a statistically significant reduction in K_a with depth (Table 5.1, P = 0.01), but post-hoc pairwise comparisons indicated that differences were only significant between the surface and the deepest samples (140 cm). Further investigation with linear regression suggested that K_a did not decline substantially with depth across the entire dataset (slope = -0.006 , P = 0.002 , R^2 = 0.07). When examined separately for each soil × forest type, K_a declined with depth in Tabonuco Oxisols (slope = -0.02, P < 0.001, R^2 = 0.38), but not in Colorado Oxisols (slope = -0.004, P = 0.19, R^2 = 0.03) or Inceptisols (slope = -0.001, P = 0.56, R^2 = 0). K_a differed significantly between soil types, but not forest types. Averaged across all depths, K_a was twice as high in Oxisols (205 h^{-1})

compared with Inceptisols (106 h^{-1}).

$^{App}V_{max}$ and $^{App}K_m$ were significantly correlated with each other (Figure 5.2, R^2 = 0.48, P < 0.001) and with soil nutrients (i.e., C, N and P, Table 5.2). In general, $^{App}V_{max}$ was more strongly correlated with soil C, N and P concentrations than $^{App}K_m$. Both $^{App}V_{max}$ and $^{App}K_m$ exhibited a stronger correlation with the $NaHCO_3$- extractable P pool than either NaOH-extractable P or total extractable P. K_a was weakly but significantly correlated with C and N concentrations and $NaHCO_3$-extractable P, but not with total extractable P or NaOH-extractable P (Table 5.2).

5.4. Discussion

One of the most striking results of this study is the strong positive relationship between $^{App}V_{max}$, $^{App}K_m$, and soil nutrient concentrations. The scaling of $^{App}V_{max}$ and $^{App}K_m$ has been noted by other researchers (*Chrost*, 1991; *Sinsabaugh and Shah*, 2012; *Williams*, 1973). Although the original Michaelis-Menten model as applied to simple enzymes assumes V_{max} and K_m are independent, there are both ecological and methodological reasons why this assumption may not apply to $^{App}V_{max}$ and $^{App}K_m$ measured in soils. In ecological systems, apparent kinetic parameters represent a pool of enzymes produced by many different organisms that vary in their individual kinetic behavior (*Williams*, 1973). As such, $^{App}K_m$ is found to best describe environmental substrate availability rather than enzyme-substrate binding affinity (*Sinsabaugh and Shah*, 2010). Higher concentrations of substrate in the environment will stimulate microorganisms to produce more enzymes, thus increasing $^{App}V_{max}$ (*Sinsabaugh and Shah*, 2010, 2012). That $^{App}V_{max}$ and $^{App}K_m$ are both related to substrate concentration is evidenced by the strong correlations we observed between both parameters and soil C, N and $NaHCO_3$-extractable P (a measure of labile, microbially available P, (*Johnson et al.*, 2003)) concentrations. Additionally, $^{App}V_{max}$ and $^{App}K_m$ may scale due to shifts in community structure along the environmental gradient represented by our depth profiles. Different communities of microorganisms can produce isoenzymes that vary in their kinetic properties (*Farrell et al.*, 1994; *Khalili et al.*, 2011; *Knight and Dick*, 2004; *Tabatabai et al.*, 2002). In subsoils, where energy and nutrients are expected to strongly constrain microbial growth, natural selection may lead to a more efficient microbial community that produces enzymes with higher

substrate affinities (lower K_m values) so that fewer enzymes need to be produced overall (lowering V_{max}). Finally, as substrate concentrations in soil decrease, less competitive interactions occur between natural and artificial substrates used in assays, resulting in less artificial substrate required to achieve 1/2 V_{max} (*Chrost*, 1991). Nevertheless, a recent review of phosphatase kinetic parameters found only a weak correlation between V_{max} and K_m (*Hui et al.*, 2013). It is likely that we observed a strong correlation between ${}^{App}V_{max}$ and ${}^{App}K_m$ because our depth profiles represent a gradient of substrate concentrations that spans several orders of magnitude.

A question raised by this study is why ${}^{App}V_{max}$ declines more rapidly with depth than ${}^{App}K_m$, resulting in apparent declines in K_a with depth. Similar to the relationship between ${}^{App}V_{max}$ and ${}^{App}K_m$ discussed above, there are both ecological and methodological reasons for a slower decline in ${}^{App}K_m$ with decreasing substrate concentrations. Using a mathematical model, *Williams* (1973) demonstrated in a multispecies system where V_{max} and K_m were able to vary across species, the level of variability in ${}^{App}K_m$ values increased as ambient substrate concentration decreased. Therefore, it is possible that ${}^{App}K_m$ overestimates true K_m values in subsoils due to increasing measurement uncertainty. Additionally, the fraction of mineral associated organic materials, including enzymes, may increase with soil depth (*Eusterhues et al.*, 2003; *Taylor et al.*, 2002). Researchers have reported both positive and negative effects of mineral association on ${}^{App}K_m$ (*Gianfreda et al.*, 1992; *Kelleher et al.*, 2004; *Marx et al.*, 2005; *Rao et al.*, 2000; *Sarkar et al.*, 1989), probably because of the numerous ways in which mineral interactions can alter enzyme behavior. Mineral association can distort an enzyme's polymeric configuration, partially mask the enzyme's active site, or alter electrostatic interactions, all leading to increased or decreased substrate affinity (*Gianfreda et al.*, 1991; *Kelleher et al.*, 2004; *Quiquampoix*, 2000). Lastly, declines in ${}^{App}V_{max}$ relative to ${}^{App}K_m$ may reflect downregulation of enzyme production in response to changing stoichiometric requirements (*Sinsabaugh and Shah*, 2012). In our sites, the soil C:P ratio declines 49% with depth. Relatively greater available P in subsoils could cause microbes to shift resources away from P acquisition. Overall, the ecological ramifications of ${}^{App}V_{max}$ declining more rapidly than ${}^{App}K_m$ may be better expressed by the K_a parameter. We observed evidence for an apparent decrease of phosphatase catalytic performance in some subsoils (i.e., a decline in K_a,). Declines in K_a with depth

suggest that subsoil microbes receive less benefit from investment in phosphatase production than their surface counterparts. However, in three of the four soil × forest types, K_a was invariant with depth and in the one treatment where K_a declined (Tabonuco Oxisols) the decline was driven by several of the deepest soil samples. We conclude that catalytic performance remains constant across a wide range of substrate concentrations represented in our depth profiles but may be reduced at extremely low substrate concentrations.

Although we focused this study largely on depth trends, our sampling design was intended to capture as much edaphic variation as possible. We did so by sampling across two soil types underlain by distinct parent materials and two forest types that occur at different elevations corresponding with climactic differences. Larger ${}^{App}V_{max}$ values in the Colorado forest and in Oxisols compared with Inceptisols are consistent with greater C concentrations in these soils. Furthermore, the correspondingly higher K_a values in Oxisols compared with Inceptisols suggest increased catalytic capacity. In Oxisols, larger enzyme pools reflected in ${}^{App}V_{max}$ and higher substrate turnover rates reflected in K_a indicate increased microbial cycling of organic P when compared with Inceptisols. Although our P-extraction procedure did not separate organic and inorganic P pools, it is likely that mineral P is unavailable to microorganisms in Oxisols due to high concentrations of Fe-oxides that bind and occlude P under acid conditions (*Chacon et al.*, 2006). The Inceptisols occur on a geologically younger parent material experiencing rapid chemical weathering (*Buss et al.*, 2010; *White et al.*, 1998) and generally lack the secondary clay minerals responsible for adsorbing inorganic P. The various mechanisms and environmental conditions leading to P-occlusion underscore the challenge associated with defining microbially-available P pools and making inferences about ecosystem level nutrient limitation (*Johnson et al.*, 2003).

5.5. Conclusions

Phosphatase activity plays a key role in delivering P to both plants and microorganisms in tropical soils, but most studies have only characterized phosphatase kinetic properties in relatively nutrient-rich surface soils. To our knowledge, this is the first study that systematically investigates changes in phosphatase kinetic parameters as a function of depth in a tropical forest. We found a decline in

both ${}^{App}V_{max}$ and ${}^{App}K_m$ with depth that is consistent with vertical gradients in substrate availability. The correlation we observed between ${}^{App}V_{max}$ and ${}^{App}K_m$ is unusually strong but fits with the current understanding of the meaning of these ecological parameters. The larger variation in ${}^{App}V_{max}$ across soil, forest and depth gradients suggests greater plasticity in this parameter compared with ${}^{App}K_m$. Finally, although subsoil enzymes may perform less efficiently than surface enzymes at very low substrate concentrations, phosphatase catalytic capacity as measured by K_a is generally consistent across orders of magnitude changes in energy and resource availability. While on a per-gram soil basis subsoil microbial communities cycle small quantities of P compared with surface communities, P cycling on a per-unit microorganism basis may be equally dynamic.

Table 5.1: Acid phosphatase kinetic parameters ${}^{App}V_{max}$, ${}^{App}K_m$ and K_a . Parameters were estimated using non-linear regression and are reported as mean ± 1 standard error for each soil × forest × depth.

Soil Order	Forest Type	Depth (cm)	n	${}^{App}V_{max}$ (nmol h^{-1} g^{-1})	${}^{App}K_m$ (µmol L^{-1})	Ka (hr^{-1})
Inceptisol	Colorado	0	5	5730 ±2200	38.7 ±6.6	131 ±35.2
		20	5	1220 ± 230	19.4 ±2.3	72.6 ±20.4
		50	5	1140 ±467	12.1 ±3.8	91.6 ±19.8
		80	5	654 ±276	6.6 ±3.3	160 ±54.7
		110	4	354 ±70	5.3 ±2.2	133 ±66.6
		140	3	167 ±73	4.1 ±1.8	40.2 ±10.3
Inceptisol	Tabonuco	0	5	3430 ±381	41.7 ±5.0	85.4 ±10.8
		20	5	1210 ±316	14.9 ±2.4	89.3 ±26.0
		50	5	456 ±58	7.6 ±2.1	95.7 ±45.9
		80	5	350 ±84	6.3 ±3.8	137 ±45.2
		110	5	188 ±36	1.8 ±0.5	128 ±32.3
		140	5	150 ±24	3.3 ±1.5	82.1 ±23.1
Oxisol	Colorado	0	5	6570 ±1770	24.2 ±6.9	286 ±41.5
		20	5	3750 ±1260	26.0 ±9.0	210. ±72.2
		50	5	1460 ±264	9.2 ±2.6	185 ±27.7
		80	5	713 ±196	3.9 ±1.0	208 ±60.1
		110	5	336 ±73	3.2 ±1.5	222 ±65.6
		140	4	302 ±108	5.6 ±3.1	213 ±119
Oxisol	Tabonuco	0	5	6240 ±1830	21.1 ±2.7	290. ±73.7
		20	5	3280 ±1010	12.1 ±1.2	313 ±121.
		50	5	995 ±241	8.7 ±3.7	247 ±105
		80	4	276 ±28	4.7 ±1.8	98.2 ±42.8
		110	4	225 ±47	6.4 ±1.8	52.6 ±21.0
		140	4	193 ±68	5.8 ±2.1	56.8 ±36.0

Table 5.2: Summary of type II standard major axis regression of phosphatase kinetic parameters and soil nutrients. All variables were log-transformed prior to regression. Pearson correlation coefficients R^2 and P values are listed on the first line and 95% confidence intervals for slopes are listed on the second line. P values that are significant at the $\alpha = 0.05$ level and their corresponding R^2 values are in bold.

	Carbon	Nitrogen	P-total	$NaHCO_3$-P	NaOH-P
$^{App}V_{max}$	**0.69, < 0.001**	**0.71, < 0.001**	**0.44, < 0.001**	**0.62, < 0.001**	**0.41, < 0.001**
	0.83–1.04	0.93–1.14	1.07–1.41	1.25–1.58	1.07–1.4
$^{App}K_m$	**0.48, < 0.001**	**0.47, < 0.001**	**0.42, < 0.001**	**0.49, < 0.001**	**0.34, < 0.001**
	0.71–0.94	0.78–1.03	0.94–1.26	1.08–1.41	0.93–1.26
K_a	**0.09, 0.001**	**0.11, < 0.001**	0.01, 0.2	**0.05, 0.01**	0.03, 0.07
	0.58–0.83	0.64–0.92	0.74–1.08	0.87–1.45	0.76–1.10

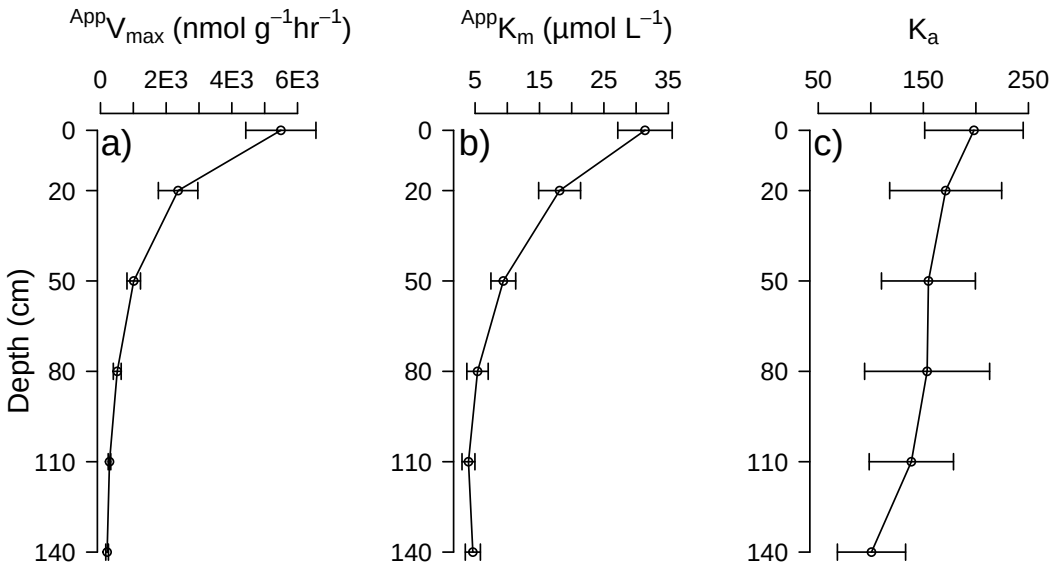

Figure 5.1: Changes in apparent kinetic parameters (${}^{App}V_{max}$ and ${}^{App}K_m$) and the specificity constant K_a (${}^{App}V_{max}$ / ${}^{App}K_m$) with depth. ${}^{App}V_{max}$ is a measure of enzyme abundance and ${}^{App}K_m$ is a measure of environmental substrate concentration. K_a is a measure of overall catalytic capacity. a) ${}^{App}V_{max}$, b) ${}^{App}K_m$ and c) K_a with depth. Values of kinetic parameters are reported as means across all soil × forest types at each depth interval with error bars representing ± 1 standard error.

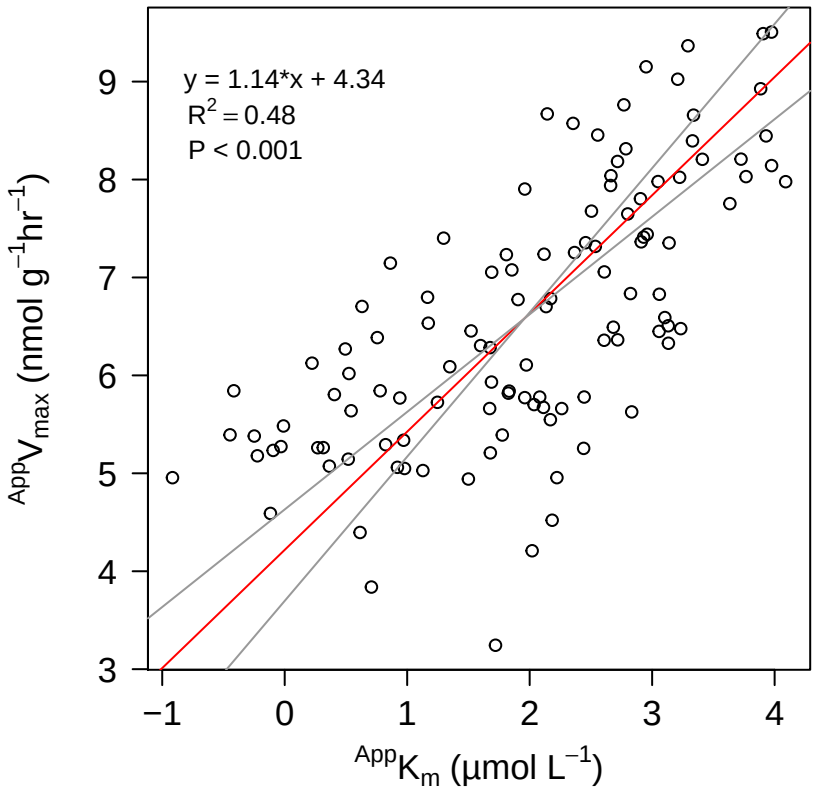

Figure 5.2: Relationship between apparent V_{max} ($^{App}V_{max}$) and apparent K_m ($^{App}K_m$) using Type II standard major axis regression. Regression line is plotted in red and 95% confidence interval is plotted in grey. Regression equation, R^2 value and P value are written on the plot.

CHAPTER 6 : Using ^{13}C NMR to evaluate the chemical nature of organic matter throughout tropical soil profiles

Chapter to be submitted for publication as:

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Abstract:

Tropical forest soils store large quantities of carbon (C) as soil organic matter (SOM), but the composition of tropical SOM remains poorly characterized, in part due to the analytical challenges associated high iron and low C concentrations. In this study, we used ^{13}C NMR to explore patterns in SOM chemistry across two soil and forest types and with depth at the Luquillo Critical Zone Observatory (LCZO) in northeast Puerto Rico. We used soils from pits excavated to 140 cm depth that were stratified across the two major soil types (Oxisols and Inceptisols) and two forest types (Tabonuco and Colorado) at LCZO. Thirty-four samples with an initial C concentration < 1% were chosen from discrete depth intervals (0, 30, 60, 90 & 140 cm) for ^{13}C NMR analysis. We compared soil ^{13}C NMR spectra to the ^{13}C NMR spectra of leaf litter, roots and fungal biomass from the same sites. Prior to ^{13}C NMR, soils were demineralized with hydrofluoric acid (HF) to remove paramagnetic compounds and concentrate organic matter. Given the scant information on tropical subsoil OM, a secondary goal of our study was to evaluate the effect of HF acid treatments on tropical subsoils. We measured C and nitrogen (N) concentrations, as well as ^{13}C and ^{15}N stable isotope abundances, before and after HF treatment to quantify C losses and assess whether C was fractionated during treatment. Finally, we used differential scanning calorimetry (DSC) to evaluate changes to the organomineral matrix before and after HF treatment.

HF treatments effectively enriched sample C and removed paramagnetic compounds, allowing us to obtain high-quality NMR spectra for low-C subsoils. C:N ratios before and after HF treatment

were nearly identical (mean = 16.6 ± 0.8), suggesting that the SOM pool was not substantially fractionated. However, large changes in the abundance of the ^{15}N stable isotope in several samples suggest acid treatment may lead to loss of some microbially-derived, N-rich compounds. Thermal analysis confirmed loss of a substantial fraction of the soil mineral matrix, however, retention of several endothermic regions in post-HF Inceptisol soils indicated that not all minerals were completely solubilized in this soil type. ^{13}C NMR spectra for soils were typically dominated by *O*-alkyl and alkyl C, representing labile polysaccharides (cellulose, aminosugars) and aliphatic compounds (fatty acids, cutin, suberin). SOM contained more alkyl C (mean = 32.2 ± 2.2%) than plant or microbial tissues (mean = 16.1 ± 1.4%), indicating preferential retention or stabilization of aliphatic C during microbial decomposition. The alkyl : *O*-alkyl C ratio was three times greater in soils from the higher-elevation Colorado forest (2.1 ± 0.3) compared with the lower-elevation Tabonuco forest (0.7 ± 0.1) despite similar alkyl : *O*-alkyl ratios in plant tissues from the two forests. Differences in SOM chemistry across the two forest types may relate differences in the soil decomposer community and its and substrate preferences, with microorganisms in the cooler, wetter Colorado forest soils preferentially decomposing labile polysaccharides (i.e., *O*-alkyl C). Changes in SOM chemistry with depth varied from pit to pit, and no major patterns were observed across all samples. This finding suggests the depth distribution of organic biomolecules is site-specific, with localized differences in C inputs, topography and transport acting as dominant controls. Overall, our findings suggest that aliphatic hydrocarbons may be particularly important for C storage in tropical soils, and that patterns in SOM composition with depth are likely mediated by a multitude of factors from the landscape scale to the pit scale.

6.1. Introduction

While our understanding of the chemical composition of soil organic matter (SOM) has advanced considerably in recent decades, the large carbon (C) reservoirs contained in tropical forest soils represent a knowledge gap. Moreover, only a handful of studies have examined subsoil OM chemistry in tropical forests, in part due to a historical lack of interest in quantifying and characterizing this pool (*Rumpel and Kogel-Knabner*, 2011; *Schmidt et al.*, 2011), but also because of the analytical challenges posed by Fe and Al-rich, C-poor soils (*Goncalves et al.*, 2003). However, recent forest carbon inventories show that substantial amounts of C can be stored to depths of 1 meter and beyond in tropical subsoils (*Johnson et al.*, Under Review). To better predict the vulnerability of tropical soil C to decomposition, the chemical composition of SOM, and particularly subsoil OM with high mean residence times (*Trumbore*, 2009), requires further study. While the emerging paradigm of soil C cycling suggests SOM chemistry is a secondary control on C stability (*Schmidt et al.*, 2011), chemistry can nonetheless influence vulnerability to microbial degradation (*Fontaine et al.*, 2007) and organomineral interactions, and allow us to make inferences about dominant transport processes and stabilization mechanisms (*Kaiser and Kalbitz*, 2012; *Wattel-Koekkoek et al.*, 2001).

There is a growing body of knowledge that describes how SOM chemistry changes with depth (*Rumpel and Kogel-Knabner*, 2011). In general, the proportion of plant-derived biomolecules (lignin, cellulose, hemicellulose) declines with depth in relation to microbial metabolites (carbohydrates and nitrogen-rich compounds, (*Kaiser et al.*, 2004; *Kleber et al.*, 2011; *Rumpel et al.*, 2004; *Spielvogel et al.*, 2008; *Torn et al.*, 2002)). This observation is attributed to a combination of the following: 1) Strong retention of plant-derived compounds in surface compartments, 2) Continual microbial processing of organic matter as it cycles downwards, and 3) Preferential sorption of microbial metabolites, particularly N-rich proteins, on mineral surfaces (*Kaiser and Kalbitz*, 2012; *Kleber et al.*, 2007; *Mertz et al.*, 2005). However, it should be noted that changes in SOM chemistry with depth are modulated by soil type and climate, which influence dominant transport processes, stabilization mechanisms and overall rates of decomposition (*Baldock and Skjemstad*, 2000; *Kaiser and Kalbitz*, 2012; *Wattel-Koekkoek et al.*, 2001). Indeed, the few studies that have characterized

tropical SOM along depth profiles highlight the importance of transport processes and preservation mechanisms, which vary with soil type and environmental conditions, in determining SOM chemistry. *Marin-Spiotta et al.* (2011) used ^{13}C NMR to characterize the chemical composition of SOM to 125 cm depth in volcanic Andisols on the Hawaiin archipelago. In this soil type, subsurface transport is dominated by macropores that bring fresh C to depth, where it is stabilized by allophane minerals, resulting in a mixture of modern and much older SOM in subsoils. By contrast, C transport along dense, clay-rich tropical Oxisols may be dominated by the slow percolation of dissolved organic matter (DOM), with changes in SOM chemistry throughout the soil profile dictated by a combination of selective sorption and microbial processing (*Kaiser and Kalbitz*, 2012). Tropical Oxisols are often dominated by kaolinite and Fe-oxides (hematite and goethite), which represent excellent sorbants for organic materials (*Kaiser and Guggenberger*, 2000; *Kaiser and Zech*, 2000; *Oades*, 1989), and may selectively preserve specific compounds (*Eusterhues et al.*, 2003; *Kleber et al.*, 2005).

In recent studies by *Stone et al.* (2014) and *Stone and Plante* (2014), changes in soil microbial biomass, community structure and extracellular enzyme activities with depth were investigated at the Luquillo Critical Zone Observatory, a montane, wet tropical forest in northeastern Puerto Rico. While these authors found exponential declines in microbial abundance and activity with depth, specific metabolic activities, normalized to microbial biomass or soil C, remain high in subsoils. In addition, shifts in the composition of the microbial community, the ratios of C : nutrient acquiring enzymes (*Stone et al.*, 2014) and the energy density of SOM as measured by thermal analysis (*Stone*, unpublished data) all suggest the presence of different organic substrates in subsoils compared with surface soils. If SOM stability is defined as resistance to microbial decomposition (*Plante et al.*, 2011), the discovery of metabolically active, and perhaps uniquely adapted microorganisms in low-C tropical subsoils suggests that some fraction of the SOM pool at depth is actively cycling. Understanding how SOM chemistry changes with depth and across this variable landscape can inform our understanding of the ecology of soil microorganisms and the inherent stability of the OM they consume.

In this study we used ^{13}C nuclear magnetic resonance (NMR) spectroscopy, a technique that provides information on the relative abundance of organic C functional groups (*Baldock et al.*, 1992; *Kogel-Knabner*, 1997), and has gained popularity in the characterization of a diverse range of organic materials found in natural environments (*Preston*, 1996; *Kogel-Knabner*, 2000), to assess how carbon chemistry changes with depth, and across landscape-scale gradients in geology and vegetation at the Luquillo Critical Zone Observatory. We predicted that we would observe distinct shifts in SOM chemistry with depth, from more plant derived chemical signatures in surface soils to microbial chemical signatures in subsoils (*Rumpel and Kogel-Knabner*, 2011). In surface soils we expected SOM chemistry to be strongly influenced by forest type, reflecting different compositions of the litter inputs (*Quideau et al.*, 2001). This hypothesis fits with prior research showing differences in bulk soil nutrient ratios and C chemistry across the two dominant forest types, particularly in surface soils (*Cusack et al.*, 2011; *Stone and Plante*, 2014). We predicted the influence of forest type on SOM chemistry would decline rapidly with depth. In subsoils, we predicted SOM chemistry would be more strongly influenced by parent material, reflecting differences in pedological processes (transport, mineral stabilization, etc.) across the two soil types (*Kaiser and Kalbitz*, 2012; *Rumpel and Kogel-Knabner*, 2011; *Wattel-Koekkoek et al.*, 2001; *Kaiser et al.*, 2002).

Obtaining high-quality NMR spectra often requires samples to be pre-treated with hydrofluoric acid (HF) to remove paramagnetic compounds and concentrate organic matter (*Schmidt et al.*, 1997). Given the scant information on tropical subsoil OM, a secondary goal of our study was to elucidate the impacts of soil pre-treatment with HF on the composition of OM determined by ^{13}C NMR. Acid treatments may be particularly important for tropical subsoils, which often contain high concentrations of Fe- associated organic matter (*Zech et al.*, 1997). However, there is concern over HF fractionating or otherwise altering the chemical composition of SOM and thus biasing NMR results (*Rumpel et al.*, 2006). Impacts of HF treatment on SOM composition vary in the literature, with some authors finding preferential losses of the stable organic matter pool (*Eusterhues et al.*, 2003), others observing a loss of labile polysaccharides (*Dai and Johnson*, 1999), and still others finding no substantial fractionation (*Goncalves et al.*, 2003; *Rumpel et al.*, 2002; *Schmidt et al.*, 1997). Previous researchers have evaluated SOM fractionation following acid treatment by examining C:N

ratios and spectral intensity distributions before and after HF (*Goncalves et al.*, 2003; *Schmidt et al.*, 1997) and in some cases abundances of ^{13}C and ^{15}N stable isotopes (*Schmidt and Gleixner*, 2005). We used C and N concentrations and ^{13}C and ^{15}N abundances to explore the possibility of SOM fractionation following HF. We also used thermal analysis to more closely examine alterations to the organomineral matrix following acid treatment. Thermal analysis integrates chemical composition and molecular structure of SOM as well as stabilization of SOM through interactions with soil minerals by measuring the energy inputs and outputs that occur as a bulk soil sample is heated (*Peltre et al.*, 2013). For our purposes, thermal analysis provides a rapid and inexpensive way to evaluate changes in both the organic and mineral fractions of a soil sample before and after demineralization. Performing these analysis using two soil types of contrasting mineralogy allows us to broaden our conclusions about the effects of pre-treatment on SOM composition.

6.2. Methods

6.2.1. Sample set

The samples used in this study comprise part of a larger sample set collected in January 2012 from four sites at the Luquillo Critical Zone Observatory (LCZO) in northeastern Puerto Rico (18°18' -N, 65°50' -W). Detailed descriptions of the study site and characterizations of these soils samples can be found in *Stone et al.* (2014). Briefly, the sample sites were stratified across two dominant forest types, the lower-elevation (< 600 m) Tabonuco (*Dacroydes excesla* Vahl) forest, and the intermediate to high elevation (600-800 m) Palo Colorado (*Cyrilla racemiflora* L.) forest. Within each forest, samples were collected at sites representing the two dominant geologic parent materials in the region: lower-Cretaceous volcaniclastic (VC) sediments of andesitic composition that weather to produce Oxisols and an early-Tertiary age quartz-diorite (QD) pluton known as the Rio Blanco stock (*Seiders*, 1971a,b), that weathers to produce Inceptisols. Within each soil × forest combination, pits were excavated at each of three topographic positions (one ridge top, three slope and one valley pit), and samples were collected at discrete depth intervals from the surface A-horizon (considered 0 cm depth) to 140 cm depth or bedrock. Samples were air-dried and sieved to 2 mm to homogenize prior to storage.

For the present study, samples from each of three soil pits (one ridge, slope and valley) at each of the four soil × forest types (12 pits total) were chosen from discrete depth intervals (0, 30, 60, 90 & 140 cm) for ^{13}C NMR analysis. We performed ^{13}C NMR at the 0 and 30 cm depth interval for each soil pit, with additional soil samples taken from deeper layers for pits where the initial soil C concentration as determined by combustion analysis was > 1%. Of the twelve pits, six were only sampled to 30 cm depth. Three were sampled to 60 cm depth, two pits were sampled to 80-90 cm depth, and one to 140 cm depth, for a total of 34 soil samples.

We also obtained samples of leaf litter, roots and fungal biomass from the study region, to compare the composition of SOM with the major source materials that contribute to its formation (*Kogel-Knabner*, 2002). Mixed leaf litter and fine root samples from each soil × forest type were obtained

from a related sample set collected in July 2010 (*Johnson et al.*, Under Review). Three different fungal specimens were obtained from Dr. Jean Lodge of the Sabana Research Station located near the Bisley Experimental Forests in Luquillo, Puerto Rico. The fungal specimens were: *Glomus* sp. (arbuscular mycorrhizae), *Psilocybe guilartensis* (soil basidiomycete) and *Psathyrella candolleana* (humus-decomposer).

6.2.2. Sample pre-treatments

Air-dried leaf litter, roots and fungal samples were ground with a mortar and pestle in liquid N prior to ^{13}C NMR analysis. Each soil sample was first pulverized to a fine powder using a ball mill, then 5 grams of sample was weighed into a 50 ml centrifuge tube. We acidified soil samples using 10 ml of 10% HCl acid to volatilize inorganic carbon (i.e., carbonates) and waited until the effervescence ceased. HCl-treated soil suspensions were centrifuged at 8000 rpm for 10 min followed by aspiration of the supernatant. We further treated the soil samples a mixture of 10% HF (w/w) and 10% HCl (w/w), a procedure which is intended to dissolve silicates and paramagnetic elements (Fe and Mn) while leaving acid-insoluble organic matter intact (*Schmidt et al.*, 1997; *Yao et al.*, 2014). We added 20 ml of the 10% HF/HCl solution to each centrifuge tube and placed tubes horizontally on an oscillating table shaker for 12 h. Samples were then centrifuged at 8000 rpm for 10 minutes, following which the acid supernatants were aspirated. We repeated the HF/HCl acid treatment at least twice for all samples, with additional treatments applied when the presence of iron-containing minerals could still be visually detected as a reddish color (Table 6.5). Following these acid treatments, the pellet was rinsed three times with deionized water and dried in a gravity convection oven at 50 °C for 48 h. The dried pellet was weighed and ground by mortar and pestle prior to NMR analysis.

The Inceptisol samples required additional pre-treatment prior to ^{13}C NMR. This soil type contained a substantial sand fraction consisting mostly of primary mineral quartz (Table 6.5). This fraction was removed by additional sieving (< 500μm) so that the sample could be packed evenly into zirconia rotors. In addition, preliminary NMR runs spectra from this soil type were very noisy, indicating the presence of paramagnetic compounds that were not dissolved during the acid treatment. Running

a magnet over these soil samples to remove visible, iron-bearing mineral particles substantially improved the NMR spectra; this procedure was therefore applied to all Inceptisol samples.

6.2.3. Soil chemical analyses

Total C and total N concentrations, ^{13}C and ^{15}N stable isotope abundances of soil samples both pre and post-acid treatment were determined using a stable isotope mass spectrometer (Delta Plus, Thermo-Finnigan MAT, Bremen, Germany). The enrichment in C and in N content after HF treatment (C_E and N_E, respectively) was calculated by dividing the element content in the treated sample by its content in the untreated sample. The recovery of C and of N after HF treatment (C_R and N_R, respectively) was calculated as:

$$C_R = (\theta \times C_{HF})/C_i$$

Where θ is the percent soil mass remaining following HF treatment, C_{HF} is the percent organic C weight in the HF treated soil and C_i is the organic C weight percent in the untreated soil (*Dai and Johnson*, 1999). In order to elucidate possible selective losses in organic matter, the ratio (R) between the C/N ratio before and after the treatment was calculated (Dick et al., 2005). From the ^{13}C and ^{15}N stable isotope abundance data we calculated the difference before and after HF treatment, Δ:

$\Delta = \delta$ after treatment - δ before treatment

6.2.4. NMR spectroscopy

Solid state ^{13}C NMR spectra of HF-treated soil samples were obtained using magic angle spinning techniques on a Bruker Avance III 300 MHz spectrometer operating at a ^{13}C resonance frequency of 75 MHz and using a Bruker double resonance probe. Approximately 250-450 mg of soil was packed into a 7-mm diameter cylindrical zirconia rotor, sealed with a Kel-F end-cap and spun at 5 k HZ. The CP NMR spectra were acquired following a 90°1H excitation pulse, a 1 ms 1H–^{13}C contact pulse, and with a 5 s delay between acquisitions for 3000 scans. The chemical shift frequency was referenced to glycine as an external standard. Line broadenings of 30 Hz were applied prior to

Fourier transformation. Each spectra was phase and baseline corrected prior to integration. The spin count observability (C_{obs}) was assessed as described by (*Yao et al.*, 2014).

Peak areas were integrated under the following seven chemical shift regions: 0-45 ppm (alkyl), 45-60 ppm (*N*-alkyl), 60-95ppm (*O*-alkyl), 95-110 ppm (di-*O*-alkyl), 110-145 ppm (aromatic), 145-165 (phenolic) and 165-215 ppm (carbonyl/amide C, *Knicker and Ludemann* (1995)). The area of each spectral region was divided by the sum of all spectral areas to obtain a relative proportion. The relative proportions of C attributed to the various spectral areas were used as variables for multivariate analysis. The ratio of alkyl : *O*-alkyl C was also calculated. This ratio is considered an index of decomposition, with higher ratios indicating greater levels of microbial processing and a loss of more labile C (*Baldock et al.*, 1992; *Baldock and Skjemstad*, 2000).

Samples with < 2 % C following acid treatment or a spectral signal : noise ratio < 10 were excluded from subsequent analysis. This resulted in the exclusion of 6 soil samples, reducing the total number of soil samples analyzed to 28.

6.2.5. Thermal analysis

To evaluate changing organomineral interactions and explore shifts in SOM energy content following acid treatment, we performed coupled thermal analyses (differential scanning calorimetry, DSC) and evolved gas analysis (CO_2-EGA) on untreated and acid-treated soils. Thermal analyses were performed as described by (*Peltre et al.*, 2013), using a Netzsch simultaneous TG and heat flux DSC thermal analyzer (STA 409PC Luxx) equipped with a type-S (Pt/PtRh) TG-DSC sample carrier supporting a PtRh10-Pt thermocouple (Netzsch-Gertebau GmbH). Briefly, samples were weighed to obtain approximately 1 mg C, with a maximum of 50 mg soil to avoid excess thermal disequilibrium. The heating program was as follows: a first step from ambient (~25 °C) to 105 °C at 10 °C min^{-1}, a second step at 105 °C for 15 min to drive off sample moisture, and a third heating step to 850 °C at 10 °C min^{-1}. The furnace atmosphere consisted of CO_2-free air flowing at 30 ml min^{-1} and N_2 protective gas flowing at 10 ml min^{-1}. The CO_2–EGA was performed by coupling a LI- 840 CO_2 /H2 O infrared gas analyzer (IRGA, LI-COR Biosciences) to the outlet of the STA

instrument. DSC thermograms were baseline corrected for the region between 120 and 850 °C using the non-parametric baseline fitting function of Peakfit (Systat Software). Qualitative differences in the thermograms of soils before and after acid treatment were used to infer changes to the soil mineral phase. In addition, several thermal indices were calculated, including the total energy released during combustion (mJ mg^{-1} soil), the energy density of SOM (energy release normalized to sample C, mJ mg^{-1} C), the temperature of peak energy release (DSC_{max}), the temperature of peak CO_2 evolution (CO_{2max}), and the proportion of total sample C evolved as CO_2 (% yield). Figures displaying thermal analysis results can be accessed here: Supplementary Figures.

6.2.6. Statistical analyses

Statistical analyses were performed using R v. 3.0.2 (R Core Team, 2012). We evaluated differences in thermal indices (i.e., CO_{2max}, DSC_{max}, etc.) and stable isotope abundances between untreated and acid-treated soils with 2-tailed, paired T-tests using the base stats package in R. In addition, we used principal components analysis (PCA) to visually separate samples based on the overall similarity of their CO_2 evolution curves over the temperature range 150-650 °C. For the NMR dataset, we applied PCA to the relative proportions of C in the various spectral regions *sensu* (*Smejkalova et al.*, 2008), to search for patterns in soil C chemistry across soil type, forest type, depth and soil pit. PCA was performed separately for thermal data and NMR data using the "calibrate" package in R (*Graffelman*, 2013).

6.3. Results

6.3.1. Mass loss, C, N, ^{13}C and ^{15}N pre and post-HF

The amount of sample mass lost due to demineralization by HF varied widely, ranging from 36–99%. Samples that received additional HF treatments invariably lost a greater amount of mass (Table 6.5). Typically, HF treatment concentrated soil C and N, with the exception of five Colorado Inceptisol samples that were relatively enriched in C and N (i.e., > 2%) prior to treatment, and another sample that was later excluded. Of the samples that were enriched, C and N enrichment factors (C_E and N_E) ranged from 1.05 to 18.3 (Table 6.5). As expected, deeper soil samples that received additional HF treatments typically became more enriched in HF. Carbon and nitrogen recovery values (C_R and N_R) varied widely, ranging from 14.5 to nearly 100%, however, the proportion of C and N recovered was typically similar within samples (Table 6.5). Proportionally more C and N were generally recovered from samples with higher initial C and N concentrations that received fewer HF treatments.

Across all samples, the C:N ratio of pre-treated soils (16.7 ± 1.2) and HF treated soils (16.5 ± 1.0) were statistically indistinguishable. The R value ranged from 0.76–1.50, with a mean of 1.0 (indicating no change in C:N ratios pre and post treatment). Typically, an R value of 1.0 ± 0.2 is accepted as an indication of no major alteration to the composition of organic matter following HF treatment (*Schmidt et al.*, 1997). Six samples had an R value outside this range (2 samples < 0.8 and 4 samples > 1.2), two of which were among those excluded from subsequent interpretation, resulting in four samples whose C:N ratios differed substantially following HF treatment.

The samples were also analyzed for δ ^{13}C and δ ^{15}N isotope abundances before and after HF (Table 6.5). The C isotope content for all samples before treatment varied between -26.5‰ and -29.4‰, which are typical values from organic C found in soils (*Schmidt and Gleixner*, 2005). In all but two surface soil samples, ^{13}C abundances were slightly less negative before HF (-27‰ ± 0.15) than following HF (-28.4‰ ± 0.13). Changes in the ^{15}N abundances following HF were more variable. Before HF treatment, soil ^{15}N abundances varied an order of magnitude (0.8 - to 8.0‰).

Variations in ^{15}N abundance following HF were also large (mean Δ = -0.29‰, min = -3.22‰, max = 4.03‰). All Inceptisol samples were more depleted in ^{15}N following HF, with an average reduction of 0.75‰, while most Oxisol samples were either enriched in ^{15}N or their ^{15}N content was not substantially altered (Table 6.5). Within individual pits, ^{15}N values tended to increase with depth, both before and after HF, although there was a large degree of variability among depth profiles.

6.3.2. ^{13}C NMR

Across all soil samples, the largest resonances occurred in the alkyl C region between 0 and 45 ppm, representing long-chain aliphatic compounds (fatty acids, waxes or resins Figure 6.1a, (*Baldock et al.*, 1992; *Kogel-Knabner*, 2002)), and in the *O*-alkyl C region between 60 and 95 ppm, representing ring carbon carbohydrates (72 ppm resonance) and secondary alcohols (~62 ppm resonance, (*Skjemstad et al.*, 1998)). Soil NMR spectra often exhibited a shoulder feature at 55 ppm, which is generally ascribed to carbon-nitrogen bonds in lignin or proteins (*Kogel-Knabner*, 2002). A small but prominent peak was often observed at 105 ppm, representing di-oxygenated polysaccharides, and a higher, distinct peak at 172 ppm, representing carboxyl groups in organic acids or amide groups in peptides (*Quideau et al.*, 2001; *Skjemstad et al.*, 1998). Other common resonances included at 130, 145 and 155 ppm (Figure 6.1a).

O-alkyl C peaks were the dominant feature in roots and leaf litter, indicating an abundance of polysaccharides in these plant tissues. Prominent peaks also occurred at 105, 155 and 170 ppm (Figure 6.1 b-c). Fungal tissue was more variable than roots or litter. All three fungal samples had large *O*-alkyl peaks between 70 and 90 ppm and another prominent peak in the carbonyl/amide C region near 172 ppm. Two of the fungal tissue samples, *Psilocybe* and *Psathoysella*, also displayed a prominent peak at 105 ppm. *Psathoysella* contained a substantial amount of alkyl C whereas the other two fungi contained very little alkyl C (data not shown).

We observed broad differences in the chemistry of SOM compared with litter, roots or fungal biomass (Table 6.4, Figure 6.2). The most prominent difference was the enrichment of alkyl C in soils compared with plant or microbial tissues, resulting in higher alkyl : *O*-alkyl ratios. Soils

contained nearly twice as much alkyl C as fungi and litter and 2.5 × more alkyl C than roots. Litter contained more aromatic and phenolic C than any other material, while roots were high in *O*-alkyl C and contained the smallest amounts of alkyl C. Fungi were high in *O*-alkyl and *N*-alkyl C, low in aromatic and phenolic C, and had the highest levels of amide/carboxylic C of any material. These differences in the chemical composition of different materials were validated by principal components analysis, with roots, litter and fungi each forming distinct and separate clusters (Figure 6.2). Root samples formed a tight cluster in negative space along PC1, which had large and negative loadings for *N*-alkyl (45-60 ppm), *O*-alkyl (60-95 ppm) and di-*O*-alkyl (95-110 ppm) C. Litter similarly formed a tight cluster in positive space along PC2, which had large and positive loadings for phenolic and aromatic C. The three fungal samples were more spatially separated along PC1, but still formed a distinct group in the lower left quadrant of the biplot, with large loadings for *N*-alkyl and carboxyl/amide C.

Across the two soil types, C chemistry could not be distinguished by PCA, although alkyl : *O*-alkyl C ratios were slightly greater, on average, in Inceptisols (mean = 1.89 ± 0.37) compared with Oxisols (mean = 1.33 ± 0.2). In addition, surface Inceptisol samples typically exhibited larger resonances in the aromatic C region than surface Oxisols (data not shown). By contrast, C chemistry was distinct across the two forest types (Figure 2). Due to larger amounts of alkyl C, Colorado forest soils had higher alkyl : *O*-alkyl ratios (mean = 2.04 ± 0.26) compared with the Tabonuco forest soils (mean = 0.70 ± 0.06).

We observed modest differences in the chemical composition of surface soil (0 cm) and subsoils (30 cm or deeper) organic matter. Averaged across all soil samples, the aromatic C chemical shift region was 26% smaller in subsoils (11.88 ± 0.46) compared with surface soils (16.13 ± 1.22) and the phenolic C region was 40% smaller in subsoils (5.0 ± 0.29%) compared with surface soils (8.3 ± 0.7%). PC2, which accounted for 32% of the variation in the dataset, indicated some separation of surface and subsoils. More notable than this overall depth effect was the fact that samples from the same pit tended to exhibit similar chemistries and cluster together in the biplot (trend not shown).

Because sample C chemistry appeared to be mediated by pit of origin, we examined our four most

complete depth profiles in more detail, to see if depth trends in C chemistry within profiles could be observed. These four profiles include two Oxisol (on slope and valley terrain) and two Inceptisol (on ridge and valley terrain) profiles, all located within the Colorado forest (Figure **??**–6.5). ^{13}C NMR spectra within the same profile were typically very similar. In each case, however, several distinct changes were observed with depth. All four profiles contained a large alkyl C peak, comprising anywhere from 27-55% of the total spectra. While this peak persisted with depth in all cases, for the Inceptisol profiles, the peak maxima shifted right, from ~30 ppm to ~40-45 ppm (Figure **??**). The Inceptisol valley profile was highly alkyl and homogenous with depth (Figure 6.3). In the Oxisol slope profile, the relative contribution of *O*-alkyl C increased 57% from the surface to subsoils (Figure 6.4), while in the Oxisol valley profile, the contribution of *O*-alkyl C decreased slightly from the surface (22.5%) to 30 cm (17.4%), but thereafter began increasing with depth to 29% at 90 cm (Figure 6.5). The two Oxisol profiles also featured an increasingly prominent amide peak at depth, centered at ~170 ppm.

6.3.3. Thermal analysis

In most cases, acid treatment dramatically changed the shape of a sample thermogram (e.g., Figure S1). With the exception of several high-C surface soils, most untreated samples showed a distinct endothermic region between 400 and 600 °C, which can be ascribed to the dehydroxylation of kaolinite (*Fernandez et al.*, 2012). Following acid treatment, this endotherm was significantly reduced or absent entirely, indicating that HF acid successfully removed a large fraction of the mineral matrix. One endothermic region remained a prominent feature of Inceptisol thermograms following acid treatment: a sharp peak at 573 °C, indicative of quartz minerals undergoing an α-β transition (*Fernandez et al.*, 2012). Several of the Tabonuco Inceptisol samples also retained endothermic regions between 700-800 °C following acid treatment (Figure S3). The loss of most endothermic regions, coupled with increased C concentrations, resulted in nearly three times as much energy release per gram acid-treated soil compared with untreated soils (Table 6.3, $P < 0.01$). This is in spite of the fact that CO_2 yields decreased from 80 ± 3% for untreated soils to 69 ± 2% for acid-treated soils (Table 6.3, $P < 0.01$) When normalized to soil C concentration, the energy density of SOM was

40% greater in acid-treated soils compared with untreated soils (Table 6.3).

The temperature region over which energy was released differed substantially between untreated and acid-treated soils. For untreated soil samples, the exothermic region was typically right-skewed, spanning a temperature range of 150-400 °C and with DSC_{max} occurring at 255 ± 4 °C. By contrast acid treated samples often exhibited narrower, left-skewed exothermic regions (e.g Figure S1), with DSC_{max} occurring at 324 ± 12 °C (Table 6.3, $P < 0.01$). Similar patterns were observed in the CO_2 evolution curves (e.g. Figure S2), although peak temperatures were shifted approximately 100 °C higher (Table 6.3, $P < 0.01$). In addition, rather than being unimodal, acid treated samples sometimes exhibited several distinct exothermic peaks (Figure S1). Principal components analysis of CO_2 evolution curves validated these observations (Figure S3), with acid-treated samples shifted in positive space along PC1, indicating greater amounts of CO_2 evolution at higher temperatures compared with untreated samples (Figure S5, $P < 0.01$).

6.4. Discussion

6.4.1. Acid Treatment Effects on Soil Organic Matter

HF acid effectively concentrated organic matter and allowed us to obtain high-quality spectra for most soil samples. Acid treatment was also necessary for removing paramagnetic compounds such as iron oxides. Paramagnetic compounds contain unpaired electrons, which reduce the efficiency of cross polarization such that ^{13}C nuclei in proximity to paramagnetics can be rendered "invisible" (*Baldock et al.*, 1992; *Schmidt et al.*, 1997). Moreover, because of selective interactions between paramagnetics and specific organic functional groups, the presence of paramagnetics can result in ^{13}C NMR spectra that do not accurately reflect the distribution of different chemical structures within a sample (*Baldock et al.*, 1992). However, HF acid was not effective in removing all iron-bearing minerals from the Inceptisols. While both soil types contain Fe-oxide secondary minerals (*McDowell et al.*, 2012), the Inceptisols also contain iron-bearing primary minerals, including Fe-silicates and hematite, particularly in subsoils and saprolite (*Buss et al.*, 2010). It is possible that our inability to remove all paramagnetic species in Inceptisols was due to differences in the dissolution rates of various iron-bearing minerals, which can vary widely (*Sidhu et al.*, 1981). For the Inceptsiols, we were able to physically separate some iron-bearing minerals using a magnet, resulting in substantially improved NMR spectra. This finding underscores the influence of mineralogy on the efficacy of acid treatments, as has been noted by other authors (*Goncalves et al.*, 2003).

While acid treatment allowed us to generate high quality NMR spectra for low-C, iron-rich soils, a caveat to the use of HF is the potential fractionation of the SOM pool (*Rumpel et al.*, 2006). We were particularly concerned about SOM fractionation in subsoils, where our carbon recovery values were typically low, increasing the potential for selective C loss. Other authors have also noted decreasing C recoveries with depth following HF (*Dick et al.*, 2005; *Goncalves et al.*, 2003; *Rumpel et al.*, 2006), with carbon losses greater than 90% observed for some subsoils (*Rumpel et al.*, 2002). Greater C losses from subsoils can be attributed largely to the additional acid treatments required to concentrate organic matter. In addition, larger proportions of SOM are often associated with Fe-oxides and clay minerals in subsoils (*Eusterhues et al.*, 2003; *Goncalves et al.*, 2003). We

explored the possibility that HF treatment altered the composition of SOM by examining C and N concentrations, stable isotope abundances, and the energy content of soil C before and after HF.

Our finding that R (representing the change in bulk soil C:N ratio following acid treatment) was typically close to 1 suggests that, overall, HF acid treatment did not substantially alter SOM quality (*Schmidt et al.*, 1997). We measured ^{13}C and ^{15}N stable isotope abundances to gain additional insight into possible SOM fractionation. Overall, ^{13}C abundances were similar before and after acid treatment. However, the small, consistent decline in ^{13}C may indicate some selective losses. Typically, higher soil ^{13}C contents are taken as an indication of greater microbial processing of organic matter, due to preferential decomposition of ^{13}C-depleted compounds, such as lipids and lignin, during decomposition (*Rumpel and Kogel-Knabner*, 2011; *Sollins et al.*, 2009). Hence, a slight decrease in ^{13}C abundances following acid treatment may indicate loss of microbially- derived compounds, such as proteins and amino acids. This may also help explain the large changes we observed in ^{15}N abundances following acid treatment. *Schmidt and Gleixner* (2005) also found larger and more variable changes in soil ^{15}N abundances following HF acid treatment compared with ^{13}C abundances. They attributed reductions in ^{15}N to selective loss of proteins, amino acids, and microbially-altered polysaccharides. Overall, while bulk SOM composition does not appear substantially altered following acid treatment, selective loss of certain components of the SOM pool may have occurred on a sample-by-sample basis. More generally, our findings demonstrate that C:N ratios alone are not enough to fully evaluate C fractionation following HF. However, given that there were no consistent trends in R values, $\Delta^{13}C$ or $\Delta^{15}N$ with soil type, forest type, or depth, we find it unlikely that HF procedures consistently altered the SOM pool in a manner that would bias the interpretation of our results.

6.4.2. Patterns in SOM chemistry across the Luquillo Critical Zone

SOM chemistry was distinct from plant and microbial tissues, indicating its mixed origin and suggesting the selective enrichment of various components. The most striking difference between the chemical composition of SOM compared with plant and microbial tissues is the greater contribution of alkyl C and correspondingly higher alkyl: *O*-alkyl ratios of SOM. The alkyl : *O*-alkyl ratio is

widely considered an index of SOM decomposition (*Baldock and Skjemstad*, 2000). The chemical composition of plant tissues in this study are similar to those found in other tropical forests, with relatively low levels of aromatic carbon and high levels of *O*-alkyl C in both litter and roots (*Cusack et al.*, 2011; *Marin-Spiotta et al.*, 2011; *Zech et al.*, 1997), indicating that polysaccharides (cellulose and hemicellulose) dominate these tissues. Likewise, the dominant chemical signatures in our fungal samples, *O*-alkyl and carboxyl/amide C, support the findings of previous researchers that fungal biomass is rich in carbohydrates and protein (*Baldock et al.*, 1990; *Kogel-Knabner*, 2002; *Martinez et al.*, 1991). The strong *O*-alkyl signature in most soils is likely the result of high levels of *O*-alkyl C in plant and microbial inputs. Roots and microbial biomass are concentrated in upper soils layers (*Lawrence*, 1996; *Stone et al.*, 2014), which may explain the particularly high *O*-alkyl C signatures observed in our surface soils.

Overall, SOM was dominated by alkyl and *O*-alkyl C, suggesting that lipids and carbohydrates comprise the majority of biomolecules present in these soils. Other researchers have noted the dominance of alkyl and *O*-alkyl C in tropical Oxisols (*Baldock et al.*, 1992; *Dick et al.*, 2005; *Krull and Skjemstad*, 2003). High levels of *O*-alkyl C in Oxisols have been attributed to preferential stabilization of polysaccharides on the Fe oxides found abundantly in this soil type (*Dick et al.*, 2005; *Neufeldt et al.*, 2002; *Wattel-Koekkoek et al.*, 2001). In addition, *Baldock et al.* (1992) suggests that rapid decomposition in the tropics leads to an accumulation of poorer-quality alkyl C in SOM. Aliphatic hydrocarbons, such as cutin, suberin and microbial lipids are considered relatively resistant to decomposition and tend to persist in soils (*Gleixner et al.*, 2001), accumulating in soil organic matter and at depth (*Kogel-Knabner et al.*, 1992a,b; *Zech et al.*, 1989). Across a forest successional gradient in SE Puerto Rico, *Ostertag et al.* (2008) found bulk soil C concentrations to be strongly correlated with hydrophobic leaf compounds. These authors concluded that aliphatic compounds might be particularly important for C storage in wet tropical forests. Hence, the SOM chemical signatures reported here are in accordance with previously published literature on tropical SOM chemistry and may be ascribed to preferential stabilization (*O*-alkyl C) and decomposition resistance (alkyl C).

Contrary to our expectation, we observed no major differences in SOM chemistry across the two soil types. While some studies have found soil type and mineral composition to be major drivers of SOM chemistry (*Baldock et al.*, 1992; *Wattel-Koekkoek et al.*, 2001), others have found little effect of mineralogy on SOM composition (*Dick et al.*, 2005). We predicted that soil type would be an important driver of SOM chemistry in subsoils, due to differences in DOM transport (*McDowell et al.*, 1992; *McSwiney et al.*, 2001), and organomineral interactions (*Baldock and Skjemstad*, 2000; *Kaiser and Kalbitz*, 2012). A previous study examining microbial biomass, community composition and enzyme activities found few differences across the two soil types (*Stone et al.*, 2014). This study concluded that soil resource availability, a major driver of microbial abundance and activity (*Allison et al.*, 2011; *Balser et al.*, 2010) is similar across the two mineralogically-distinct soil types, a conclusion which our ^{13}C NMR findings would seem to support. It is possible that similarities in climate, which co-varies with forest type but not soil type at the study site, drives similar decomposition patterns across the two soils, resulting in convergent SOM chemistries despite contrasting mineralogies.

In contrast to the similarity in SOM chemistry across the two soil types, we observed clear differences in SOM chemistry across the Tabonuco and Colorado forest types, with greater quantities of alkyl C in Colorado forest soils, supporting previous studies of C chemistry across the two forests (*Cusack et al.*, 2011). These two forest types occupy different elevation zones, with the higher elevation Colorado forest experiencing a slightly cooler and wetter climate than the lower-elevation Tabonuco forest (MAT = 24 °C, MAP = 3500 mm yr^{-1}, *McDowell et al.* (2012)). One explanation for differences in C chemistry across the two forests could be differences in the chemical composition of plant tissues between the two forest types. It is well-established that that the composition of plant litter influences the composition SOM (*Kogel-Knabner*, 2002; *Swift et al.*, 1979) and other studies have found direct links between forest vegetation composition and soil organic matter composition using ^{13}C NMR (*Quideau et al.*, 2001). However, our ^{13}C NMR results show litter and root tissues from the two forest types to be chemically similar. The alkyl : *O*-alkyl C ratio of Colorado forest litter was slightly greater (0.62) than Tabonuco forest litter (0.54), corresponding almost identically with previously reported alkyl : *O*-alkyl C ratios for these two forest litters (*Cusack et al.*,

2011), but we observed no difference in alkyl and *O*-alkyl C contents for roots. The slightly higher alkyl C content of Colorado forest litter cannot not fully account for the nearly three fold higher alkyl : *O*-alkyl C ratio in Colorado forest soils compared with Tabonuco forest soils. Moreover, this difference in alkyl: *O*-alkyl C ratios persists in subsoils, suggesting differences in pedogenic processes are altering the chemical nature of SOM across the two forest types.

Rather than initial plant inputs *per se* driving differences in SOM chemistry across the two forests, it seems likely the combination of differences in vegetation and climate drive broad differences in decomposition between the two forests. Previous researchers have demonstrated the substantial influence of climate factors on SOM chemistry (*Preston*, 1996). In particular, the accumulation of aliphatic C in soils with cooler and wetter climates has been observed (*Zech et al.*, 1989). Such changes might be ascribed to the effects of climate on soil decomposer community in terms of structure, activity and substrate preferences. Indeed, previous molecular fingerprinting work by *Stone et al.* (In Press) suggests differences in dominant bacterial community structure across the two forest types. In addition, *Stone et al.* (2014) found 140% greater potential activity of two labile C degrading enzymes in the Colorado forest compared with the Tabonuco forest : the starch-degrading enzyme alpha glucosidase and the cellulose degrading enzyme cellobiohydrolase, and particularly high specific activities for these enzymes in several Colorado forest subsoils. Different substrate utilization patterns can be driven by differences in community structure (*Fierer et al.*, 2007). Higher levels of starch and cellulose degrading enzyme activity suggest preferential use of labile polysaccharides as substrates in Colorado forest soils, which may account for diminished *O*-alkyl C contributions and relative enrichment in alkyl C. While enrichment in aliphatic compounds may be a result of differences in microbial substrate preferences across the two forests, loss of these labile substrates may produce a further negative feedback on decomposition, leading to the greater amounts of C stored in Colorado forest soils (*Johnson et al.*, Under Review).

6.4.3. Patterns in SOM chemistry with depth

We found weak evidence for an overall distinction between surface soil and subsoil C chemistry (slightly greater aromatic + phenolic signature in surface soils). Greater amounts of aromatic C in

surface soils suggest greater amounts of lignin arising from plant-residues in early stages of decomposition. However, we were surprised to find no consistent pattern in the dominant C compounds, alkyl C and *O*-alkyl C, or the alkyl : *O*-alkyl ratio with depth. This ratio increased in some pits and decreased in others, and no correlation with the soil characteristics could be established. *Dick et al.* (2005) examined the ^{13}C NMR spectra of Brazilian Ferrasols under native grassland and forest vegetation, and likewise found no consistent pattern in the alkyl: *O*-alkyl C ratio with depth. It is possible that in tropical forests, climactic conditions favoring rapid decomposition and transport of OM to depth result in may result in a high degree of OM decomposition throughout soil profiles. This hypothesis would fit with prior characterization of the soils in this study, which found C: N ratios to be low and invariant with depth (*Stone and Plante*, 2014). The notion that deeper soil C is substantially "more decomposed" than surface C has arisen largely from studies of temperate and boreal soils and may be less useful in describing the depth distribution of C in the tropics.

Patterns in SOM chemistry with depth could be observed at the level of individual soil pits. Samples from the same pit tended to have highly similar C chemistry, and different patterns in SOM chemistry with depth emerged for different pits. These observations suggest that local heterogeneity of soil conditions must be taken into account to understand changes in SOM chemistry along soil profiles. For instance, topographic position is known to play an important role in dictating stocks of C and nutrients across the LCZO (*Johnson et al.*, 2011; *Silver et al.*, 1994). Likewise, topography may play an important role in dictating the chemical composition of SOM, through its effects on transport processes, moisture levels and redox conditions. Overall, different mechanisms of transport and selective preservation may prevail in individual pits, due to each pit's unique combination of topographic position, soil type, vegetation, and other environmental factors. We focus the rest of the discussion of depth trends on patterns that emerged within soil profiles and how they may relate to environmental drivers.

Our two most complete Oxisol profiles contained high levels of alkyl C and increasing amounts of *O*-alkyl C with depth. This was initially surprising, because it seems contradictory to the notion of *O*-alkyl C as a labile, rapidly-degraded polysaccharide. A likely explanation is that the *O*-alkyl

C in deep soils represents newly synthesized microbial saccharides rather than the plant-derived polysaccharides that are expected to prevail in surface soils. Several studies have suggested that Fe-oxides and kaolinite in tropical Oxisols may selectively preserve non-cellulosic polysaccharides of microbial origin (*Neufeldt et al.*, 2002; *Wattel-Koekkoek et al.*, 2001). Microbial polysaccharides consist of both intracellular polysaccharides and a larger pool of extracellular polysaccharides (ECP), which have been shown to interact with primary clay minerals by gluing them together to form stable microaggregates (*Dorioz et al.*, 1993; *Kogel-Knabner*, 2002). Examining changes in SOM composition with depth along an Australian Oxisol profile, *Krull and Skjemstad* (2003) found increased ^{13}C, ^{15}N and *O*-alkyl concentrations, which the authors attributed to complexion of labile, microbial sugars with Fe oxide minerals. *Dick et al.* (2005) examined changes in organic matter with depth in Brazilian Ferrasols, finding relatively large amounts of *O*-alkyl and alkyl C in subsoils. The authors attributed this to stabilization of decomposed organic materials through interactions with Fe oxides and kaolinite. Both Oxisol profiles also showed a prominent amide peak at depth, possibly indicating greater amounts of protein-enriched microbial C. These findings are thus in accordance with previous literature and fit with our hypothesis that subsoil carbon is composed of microbial biomolecules (*Rumpel and Kogel-Knabner*, 2011). It is also interesting to note a topographic difference: while the valley profile showed a large *O*-alkyl C peak in surface soils, a decline in shallow subsoils, and a subsequent increase in deeper subsoils, the slope profile showed a continual increase in *O*-alkyl C from the surface to 80 cm depth. This difference may be due to a loss of plant-derived OM on slopes, either through downslope transport of surface organic matter, more rapid decomposition in well-oxygenated slope soils, or a combination of factors.

A very different picture of SOM chemistry emerged in our most extensively sampled profile, a valley profile from the Colorado Inceptisol site (Figure 6.3b). This profile was unique in that it was a highly saturated, topographic low with unusually high C concentrations all the way to bedrock, which occurred near 1.5 meters depth. SOM was strikingly alkyl and N-alkyl in composition throughout the profile, with the smaller *O*-alkyl peak at the surface entirely lost by 30 cm depth. In this particular soil profile, poor drainage due to shallow bedrock, combined with the soil profile's topographic position, might help explain this unusual chemical composition. This particular part of the landscape

may act as a trap for organic matter transported from upslope and deposited *in situ*. It is possible that fluctuating redox conditions in valley soils lead to the liberation and selective decomposition of more labile biomolecules, while aliphatic hydrocarbons and proteins may be selectively retained by the soil mineral phase (*Kleber et al.*, 2007; *Lichtfouse et al.*, 1998).

6.4.4. Thermal analysis of soils before and after HF

A final goal of our study was to explore whether thermal analysis could provide any additional information regarding the effect of acid treatment on organomineral associations and SOM composition. Thermograms of acid treated soils lend strong evidence to the near-complete loss of the soil mineral phase, as indicated by the disappearance of most energy-absorbing (endothermic) regions. However, thermal analysis also validated our observation that acid treatment did not entirely remove the soil mineral phase in the Inceptisols. All acid-treated Inceptisol samples retained a sharp endothermthic peak at 573 °C associated with a quartz α-β phase transition (*Fernandez et al.*, 2012), supporting the notion that quartz does not solubilize rapidly in acid (*Dick et al.*, 2005; *Goncalves et al.*, 2003). While quartz does not interfere with the acquisition of ^{13}C NMR spectra, large quantities of the mineral could dilute our SOM signal. The presence of additional endothermic regions at higher temperatures (700-800 °C) in several Inceptisol soils could indicate other minerals that were not completely solubilized, such as Mg-smectite (*Karathanasis and Harris*, 1994).

We explored the possibility that thermal analysis might provide additional insight into changes in SOM composition, by examining both the energy released and temperatures required to combust SOM before and after acid treatment. It is not surprising that greater amounts of energy were released following acid treatment, given that acid-treated soils had both higher C concentrations and a diminished soil mineral phase, which can mask exothermic reactions associated with SOM combustion (*Fernandez et al.*, 2012). However, we also observed a substantial increase in the energy density of soil C (energy content per unit C) following acid treatment, which may indicate alterations to the SOM pool itself. Greater energy densities are often associated with greater aromaticity, as greater heat inputs are required to combust aromatic structures compared with carbohydrates (*Leifeld*, 2007; *Peltre et al.*, 2013). However, other changes to the thermal behavior of soils can occur due to loss

of the soil mineral phase and concentration of OM (*Peltre et al.*, 2013; *Plante et al.*, 2011). In untreated soils, lower combustion temperatures may be the result of kaolinite catalyzing the thermal oxidation of OM (*Yariv et al.*, 2011). In addition, lower energy densities can result from energy adsorption by the mineral matrix and energy consumption during the desorption of SOM from the mineral phase (*Plante et al.*, 2011). Conversely, in acid treated soils with high C concentrations, oxygen availability may be insufficient for complete burning at lower temperatures (230-380 °C). In such cases, carbon and nitrogen atoms may form intercalated black residues (charcoals) that burn at higher temperatures (300-560 °C, *Yariv et al.* (2011)). Higher C soils may also experience more incomplete combustion as carbon monoxide, a phenomenon which would help explain the lower CO_2 yields of acid-treated soils. The presence of several distinct peaks in some acid-treated soils may likewise be the result of partial combustion followed by secondary condensation reactions (*Duguy and Rovira*, 2010). The question of whether SOM composition can be deconvoluted from other differences in the thermal behavior of soils with different organomineral associations and C concentrations remains to be explored as thermal analysis methods are further refined for SOM studies (*Fernandez et al.*, 2012; *Plante et al.*, 2009).

6.5. Conclusions

Our study is among the first to examine patterns in C chemistry in surface and subsoils in from tropical forests. Similarly to researchers working in Amazonian soils, SOM in these tropical forests appears dominated by carbohydrates and lipids. Differences in SOM composition across two soil types with contrasting mineralogies were small but differences across climactically driven forest types were pronounced. These findings suggest organomineral associations may play a smaller role than climate and vegetation in dictating the chemistry of SOM, possibly because climate can have such a dramatic effect on the soil decomposer community. The three fold higher alkyl: *O*-alkyl C ratio in soils from the Colorado forest suggests a greater degree of OM decomposition, preferential loss of labile polysaccharides and accumulation of poorer-quality aliphatic C, which may be an important component of the stable C pool in these soils. Trends in C chemistry with depth vary at the level of individual soil pits, suggesting topography, drainage and local vegetation are important controls. Similarly to its role influencing bulk soil C and nutrient stocks, topography may represent a major control on C chemistry and mediate the stability of C with depth across the Luquillo Critical Zone.

Fractionation of SOM by HF treatments is an important consideration and should continue to be investigated, particularly in low-C tropical subsoils where many rounds of acid are required to concentrate C. We did not find evidence for consistent patterns in SOM losses among our samples, although variations in ^{15}N abundances before and after acid treatment may point to selective loss or enrichment of N-rich components such as proteins. Thermal analysis confirmed substantial loss of the soil mineral matrix following acid treatment, but at the present thermal analysis alone cannot conclusively demonstrate changes in SOM composition following acid treatment because disruption of organomineral associations may lead to other changes in the thermal behavior of SOM.

Table 6.1: Number of HF acid treatments per sample, sample mass losses, C and N enrichment factors (C_E and N_E), percent C and N recovered (C_R and N_R), and R values indicating the degree of change in C:N ratio following HF

Soil Order	Forest Type	Topo	Depth	No. Acid treatments	Mass Loss %	C_E	N_E	C_R %	N_R %	R
Inceptisol	Colorado	Ridge	0	3	44.26	0.88	0.79	48.87	44.00	0.90
		Ridge	30	3	83.00	1.62	1.36	27.60	23.07	0.84
		Ridge	60	3	47.27	0.89	0.94	46.70	49.43	1.06
		Slope	0	3	36.21	1.77	1.87	>100	>100	1.05
		Slope	30 †	3	78.38	0.34	BDL	7.36	0.00	*
		Valley	0	3	40.93	0.68	0.64	39.99	37.97	0.95
		Valley	30	2	39.43	0.77	0.86	46.72	51.91	1.11
		Valley	60	3	24.47	0.65	0.78	48.81	59.11	1.21
		Valley	90	2	38.01	1.11	1.42	68.68	87.82	1.28
		Valley	140	3	34.12	1.76	2.31	>100	>100	1.32
Inceptisol	Tabonuco	Ridge	0	2	45.39	1.08	1.05	59.09	57.29	0.97
		Ridge	30†	3	85.55	1.30	1.29	18.86	18.58	0.99
		Slope	0	3	46.88	1.50	1.35	79.50	71.50	0.90
		Slope	30	3	80.27	1.47	1.36	29.08	26.78	0.92
		Valley	0	3	56.08	1.32	1.35	58.03	59.35	1.02

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Table6.1 –*Continued from previous page*

Soil Order	Forest Type	Topo	Depth	No. Acid treatments	Mass Loss %	C_E	N_E	C_R %	N_R %	R
		Valley	30	3	75.84	1.75	1.33	42.17	32.21	0.76
		Valley	60†	4	83.14	1.43	1.09	24.08	18.39	0.76
Oxisol	Colorado	Ridge	0	2	80.38	3.29	3.36	64.51	65.95	1.02
		Ridge	30	5	97.99	11.55	12.40	23.25	24.95	1.07
		Ridge	60†	5	89.97	2.22	2.77	22.26	27.82	1.25
		Slope	0	3	57.01	1.72	1.73	73.77	74.25	1.01
		Slope	30	4	90.76	3.43	3.35	31.71	30.94	0.98
		Slope	60	6	98.93	15.05	17.00	16.06	18.14	1.13
		Slope	80	5	96.52	10.55	15.86	36.67	55.14	1.50
		Valley	0	3	*	1.87	1.87	*	*	1.00
		Valley	30	3	94.87	7.32	6.69	37.53	34.30	0.91
		Valley	60	5	*	18.27	16.46	*	*	0.90
		Valley	90	6	99.08	15.54	15.86	14.28	14.58	1.02
Oxsiol	Tabonuco	Ridge	0†	3	54.06	2.28	1.91	>100	87.65	0.84
		Ridge	30	3	91.51	2.41	2.53	20.45	21.47	1.05
		Slope	0	4	63.98	2.14	2.13	76.99	76.66	1.00
		Slope	30	4	96.14	6.13	6.67	23.68	25.77	1.09

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Table6.1 –*Continued from previous page*

Soil Order	Forest Type	Topo	Depth	No. Acid treatments	Mass Loss %	C_E	N_E	C_R %	N_R %	R
		Valley	0	3	60.85	1.70	1.78	66.67	69.65	1.04
		Valley	30	5	98.54	16.76	14.69	24.50	21.47	0.88

† = sample excluded from subsequent NMR analysis

BDL = below detection limit

Table 6.2: ^{13}C and ^{15}N stable isotope abundances before and after HF treatment. Values are expressed in parts per thousand (‰)

Soil Order	Forest Type	Topo	Depth	δ ^{13}C Initial	δ ^{13}C Final	Δ	δ ^{15}N Initial	δ ^{15}N Final	Δ
Inceptisol	Colorado	Ridge	0	-28.22	-28.92	-0.71	4.24	2.90	-1.34
		Ridge	30	-26.76	-27.60	-0.84	6.01	6.05	0.04
		Ridge	60	-27.07	-27.62	-0.56	4.38	3.47	-0.91
		Slope	0	-28.85	-28.60	0.25	0.80	0.53	-0.28
		Slope	30†	-28.31	-29.55	-1.24	2.72	0.53	-2.19
		Valley	0	-29.04	-29.37	-0.33	1.88	0.24	-1.64
		Valley	30	-29.00	-29.34	-0.35	1.76	0.95	-0.81
		Valley	60	-29.25	-29.54	-0.28	1.36	0.28	-1.08
		Valley	90	-29.43	-29.70	-0.27	2.06	0.69	-1.37
		Valley	140	-28.76	-29.11	-0.35	1.75	0.50	-1.24
Inceptisol	Tabonuco	Ridge	0	-28.36	-28.77	-0.41	2.90	2.63	-0.27
		Ridge	30†	-26.76	-27.56	-0.80	6.44	6.21	-0.23
		Slope	0	-28.19	-28.42	-0.24	4.29	3.42	-0.87
		Slope	30	-27.14	-28.57	-1.43	6.34	3.12	-3.22
		Valley	0	-27.57	-29.14	-1.57	1.27	1.11	-0.16
		Valley	30	-28.15	-28.89	-0.74	3.91	4.62	0.71

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Table6.2 –*Continued from previous page*

Soil Order	Forest Type	Topo	Depth	δ ^{13}C Initial	δ ^{13}C Final	Δ	δ ^{15}N Initial	δ ^{15}N Final	Δ
		Valley	60†	-27.59	-28.89	-1.30	5.03	4.62	-0.41
Oxisol	Colorado	Ridge	0	-28.41	-27.75	0.66	4.75	8.77	4.03
		Ridge	30	-27.35	-27.83	-0.48	6.42	7.07	0.65
		Ridge	60†	-26.95	-27.75	-0.80	8.00	8.77	0.77
		Slope	0	-27.51	-27.83	-0.33	4.60	4.50	-0.10
		Slope	30	-26.80	-27.70	-0.90	6.81	6.62	-0.19
		Slope	60	-27.03	-27.32	-0.29	5.44	6.01	0.57
		Slope	80	-26.48	-27.49	-1.01	5.83	6.31	0.48
		Valley	0	-28.17	-28.58	-0.41	3.58	4.39	0.81
		Valley	30	-27.68	-28.51	-0.83	4.90	4.50	-0.40
		Valley	60	-27.41	-28.66	-1.25	4.97	4.11	-0.86
		Valley	90	-26.55	-28.00	-1.45	6.12	5.73	-0.39
Oxisol	Tabonuco	Ridge	0†	-28.02	-28.93	-0.90	4.63	3.13	-1.50
		Ridge	30	-27.34	-28.06	-0.72	7.51	8.21	0.70
		Slope	0	-28.07	-28.34	-0.27	4.58	4.52	-0.06
		Slope	30	-27.25	-27.36	-0.11	6.97	7.26	0.29
		Valley	0	-28.14	-28.28	-0.15	5.97	6.02	0.05

Continued on next page

Table6.2 –*Continued from previous page*

Soil Order	Forest Type	Topo	Depth	δ ^{13}C Initial	δ ^{13}C Final	Δ	δ ^{15}N Initial	δ ^{15}N Final	Δ
		Valley	30	-26.74	-27.43	-0.69	7.69	8.19	0.50

† = sample excluded from subsequent NMR analysis

BDL = below detection limit

Table 6.3: Average exothermic energy content and energy density of SOM as determined by DSC, maximum temperatures for energy flux and CO_2 evolution, and CO_2 yield as a % of total sample C. Values represent means ± one standard error.

	Untreated	HF Treated	T statistic, P value
n	34	34	
Exothermic energy (mJ mg^{-1} soil)	992 ± 178	2793 ± 440	4.86, < 0.001
Energy density (mJ mg^{-1} C)	15937 ± 1673	22367 ± 1126	3.4, < 0.01
DSC max (°C)	255 ± 4	324 ± 12	5.9, < 0.001
CO_2 max (°C)	346 ± 4	431 ± 9	9.6, < 0.001
CO_2 yield (%)	80 ± 3	68 ± 2	-3.4, < 0.01

Table 6.4: Percent size of the seven chemical shift regions integrated from ^{13}C NMR spectra. Values represent means ± one standard error.

	Soil (n=28)	Fungi (n=3)	Litter (n=4)	Root (n=4)
Alkyl	32.2 ± 2.2	16.9 ± 3.9	18.8 ± 1.4	12.9 ± 1.1
N-alkyl	8.4 ± 0.3	9.8 ± 1.2	7.2 ± 0.3	9.6 ± 0.1
O-alkyl	23.9 ± 1.2	45.8 ± 6.1	32.1 ± 1.1	46 ± 1.1
Di-*O*-alkyl	6.9 ± 0.3	11.7 ± 2	8.7 ± 0.1	8.8 ± 0.2
Aromatic	13.4 ± 0.6	4.1 ± 0.9	16.8 ± 0.5	11.5 ± 0.3
Phenolic	6.1 ± 0.4	1.1 ± 0.2	9.6 ± 0.6	5.7 ± 0.2
Amide	9.5 ± 0.3	10.6 ± 2	6.7 ± 1.4	5.5 ± 0.2
Alkyl: *O*-alkyl	1.6 ± 0.2	0.4 ± 0.2	0.6 ± 0	0.3 ± 0

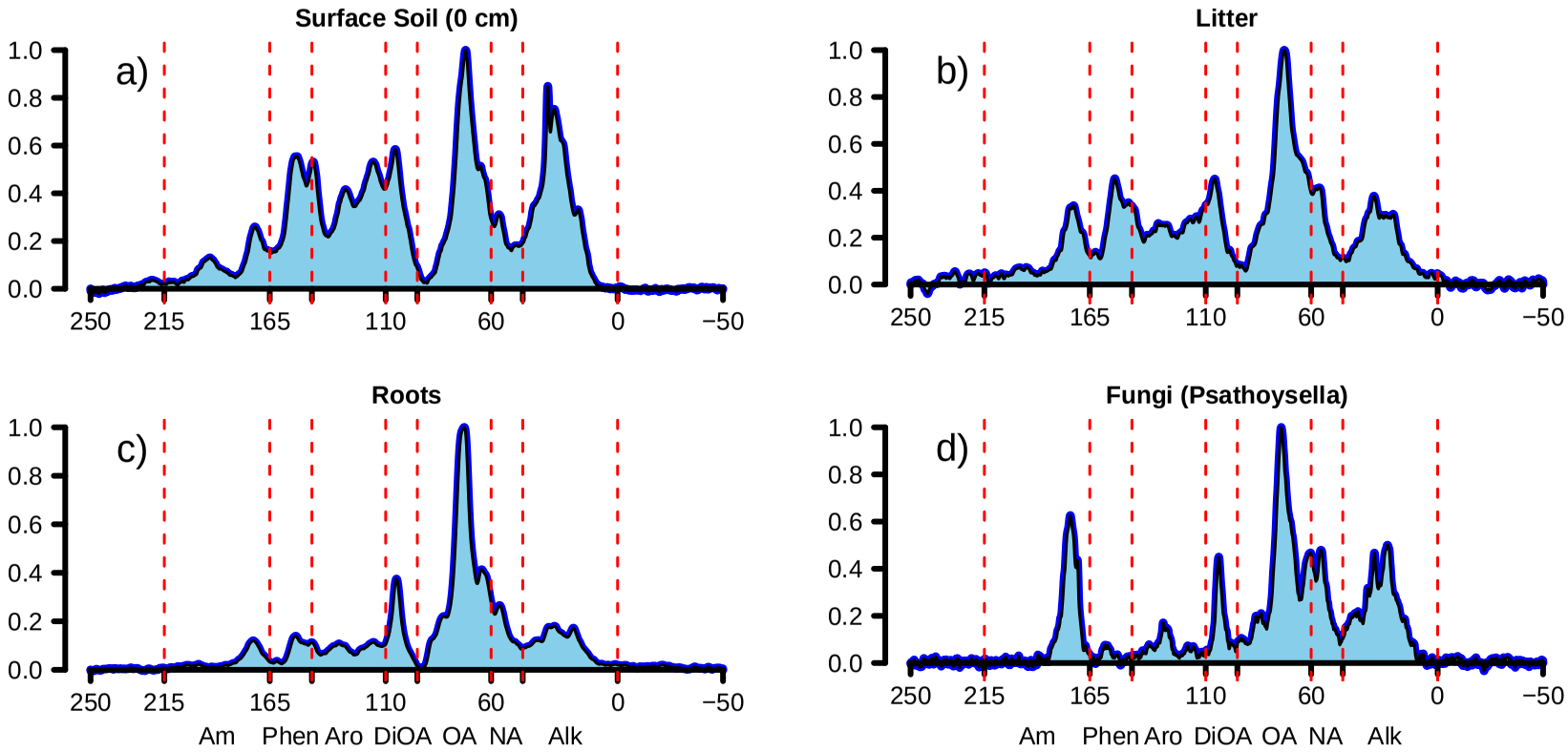

Figure 6.1: Representative ^{13}C NMR spectra from a) a surface soil, b) leaf litter, c) roots and d) fungal biomass. Dashed red lines denote boundaries of integrated chemical shift regions. Integrated chemical shift regions are as follows: Alk = alkyl (0-45 ppm), NA = N-alkyl (45-60 ppm), OA= *O*-alkyl (60-95 ppm), DiOA = Di-*O*-alkyl (95-110ppm), Aro= aromatic (110-145 ppm), Pheno = phenolic (145-165 ppm), Am = amide (165-215 ppm).

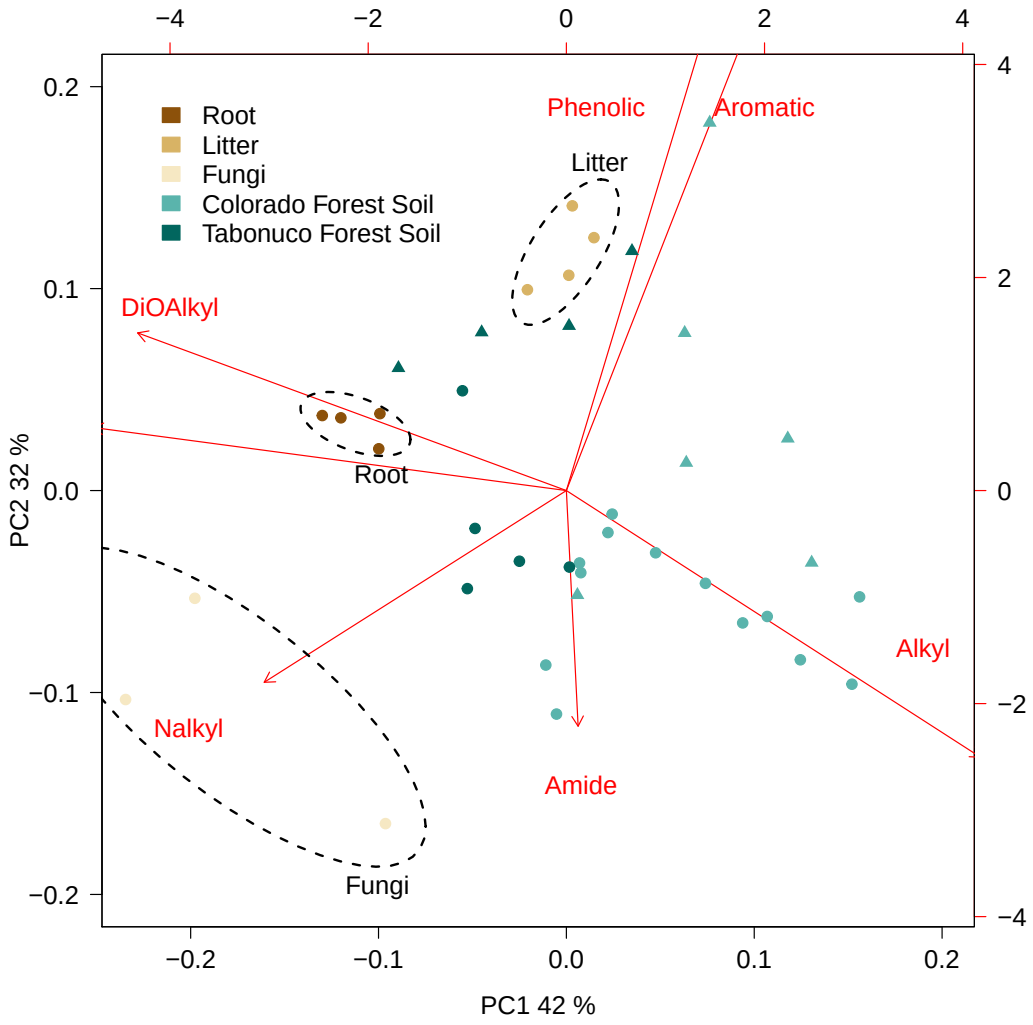

Figure 6.2: Principal components analysis of cleaned ^{13}C NMR dataset. Points represent individual samples and arrows represent correlation strengths and directions of major chemical shift regions. 70% confidence ellipses were drawn around distinct groups, including litter, roots and fungi. Triangles indicate surface soils (0 cm), while circles indicate subsoils (30–140 cm). Samples with < 2% C or a spectral signal : noise ratio < 10 were excluded, resulting in the exclusion of 6 soil samples and reducing the total number of soil samples analyzed to 28.

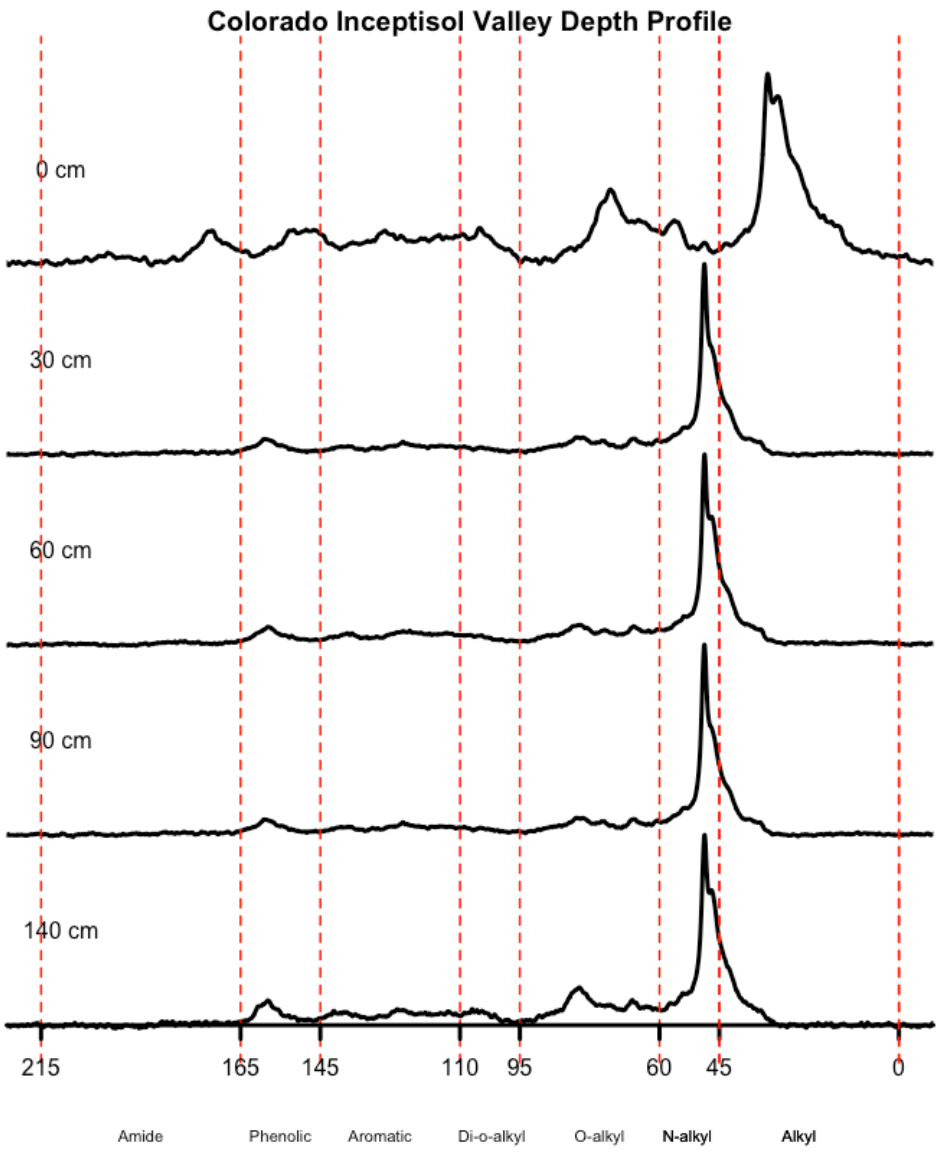

Figure 6.3: ^{13}C NMR depth profile sequence for a Colorado Inceptisol valley. Dashed red lines indicate boundaries of different integrated chemical shift regions. Numbers represent the chemical shift value (in ppm) of peak maxima.

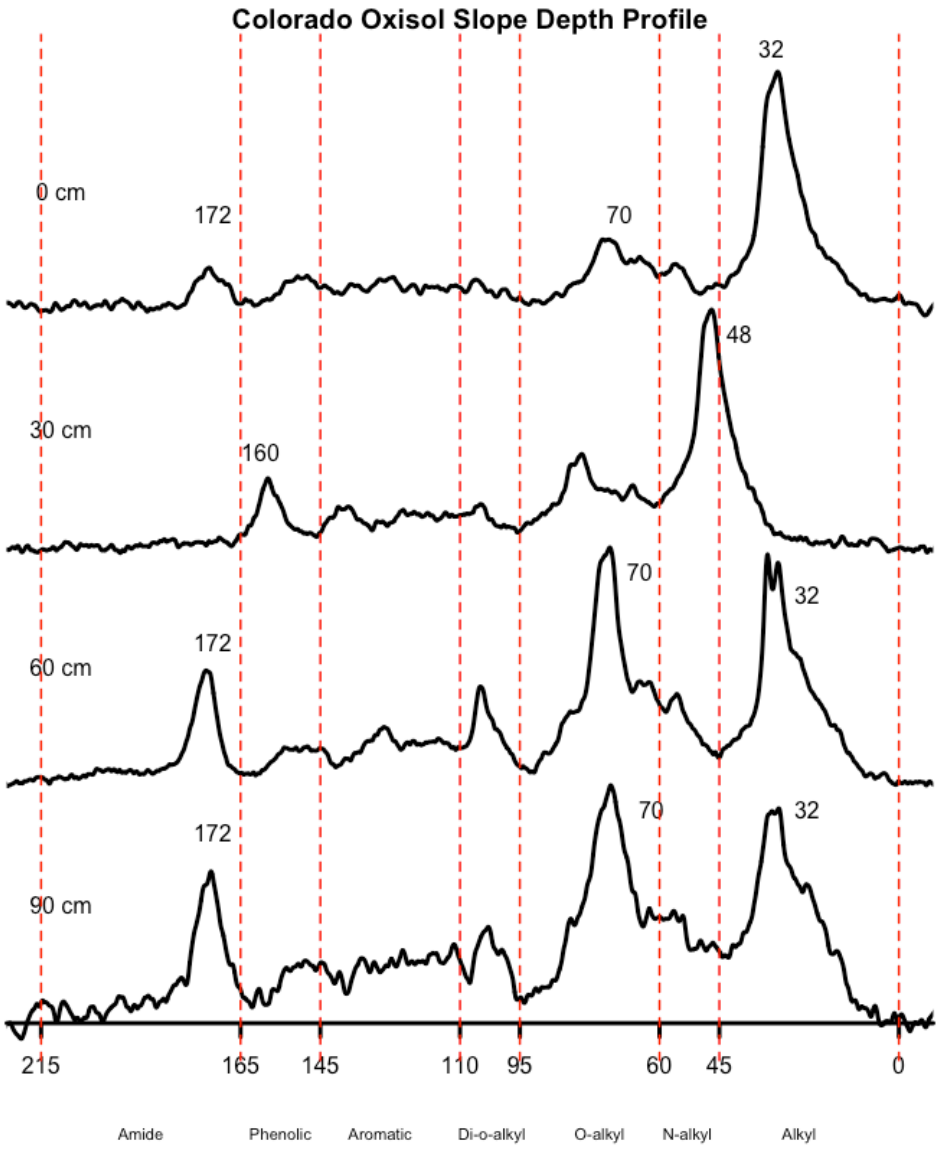

Figure 6.4: ^{13}C NMR depth profile sequence for a Colorado Oxisol slope. Dashed red lines indicate boundaries of different integrated chemical shift regions. Numbers represent the chemical shift value (in ppm) of peak maxima.

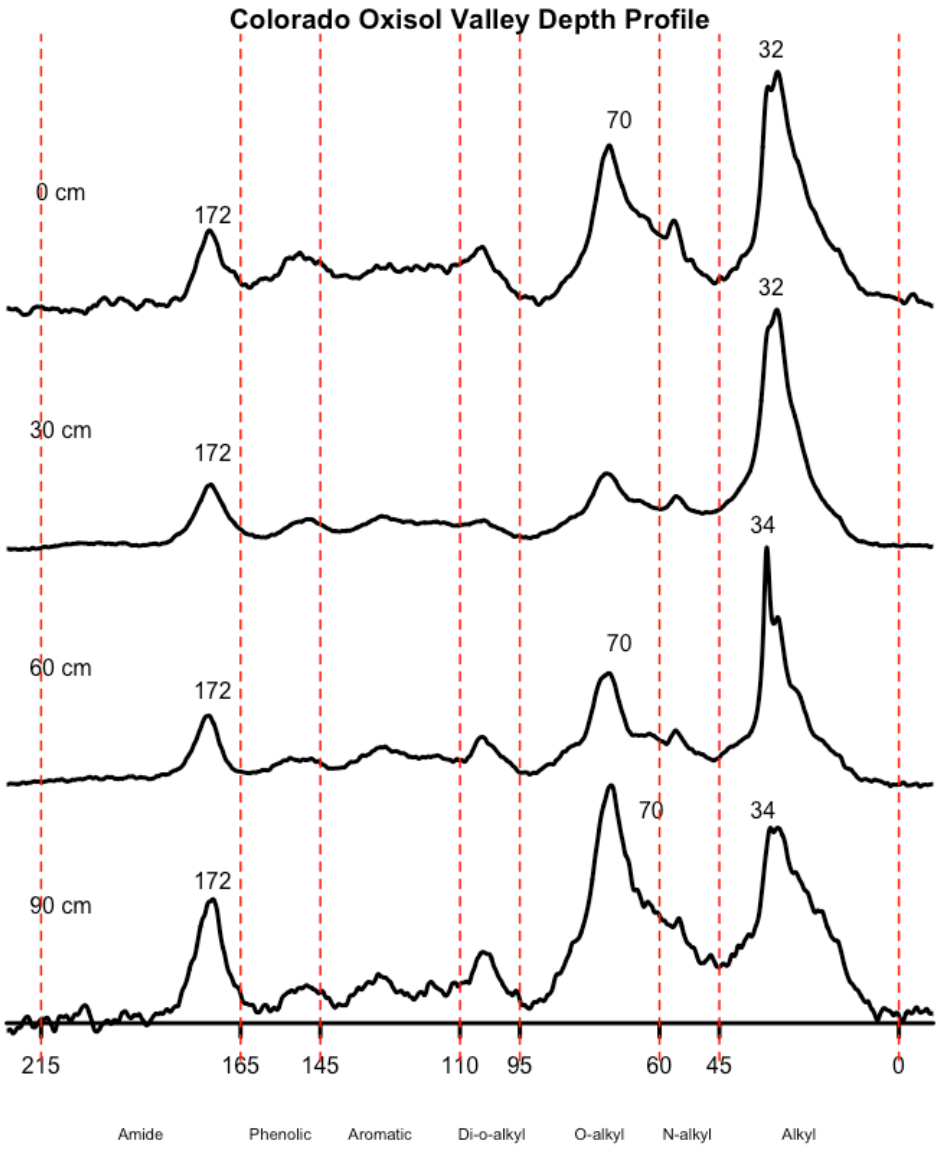

Figure 6.5: ^{13}C NMR depth profile sequence for a Colorado Oxisol valley. Dashed red lines indicate boundaries of different integrated chemical shift regions. Numbers represent the chemical shift value (in ppm) of peak maxima.

CHAPTER 7 : Conclusions

This dissertation characterizes the microbial ecology and soil organic matter composition found in soils throughout the Luquillo Critical Zone. Chapter 2 experimentally tests the resource allocation theory of microbial ecology to elucidate controls on microbial activity in surface soils. Subsequent chapters of this dissertation take advantage of natural gradients represented by depth profiles, contrasting parent materials and forest types to investigate environmental controls on soil microbial ecology and soil carbon. The major findings of the dissertation, their limitations, implications and potential avenues for future research are detailed below.

7.1. Effects of plant roots and nutrients on microbial functional traits in surface soils

In chapter 2, we found that microbial extracellular enzyme activities are regulated in response to carbon and nutrient availabilities, consistent with microbial resource allocation theory. Although we observed shifts in plant carbon allocation and tissue nutrient concentrations due to nutrient manipulations, plant roots did not appear to influence microbial responses to nutrients. Experimental artifacts, including relatively high nutrient addition rates, may be at play here, or microbial communities in these surface soils may not be carbon-limited. Longer-term field experiments can help unravel microbial functional responses to shifting resource availability in rhizospheres and bulk soils. Overall, our results suggest the potential for enhanced microbial carbon and nutrient cycling activity due to nitrogen deposition in tropical forest soils.

7.2. Patterns in soil microbial ecology and soil organic matter along depth profiles

A consistent finding throughout chapters 3-6 of this dissertation is the orders of magnitude decline in microbial abundance and activity with depth, which parallels declining energy availability in the form of organic carbon. While the relationship between biological activity and energy may seem self-evident, this association has important and often overlooked implications for the stability of deep SOM. The age of deep SOM is often taken as an indicator of its high stability (*Rumpel and Kogel-Knabner*, 2011; *Trumbore*, 2009), but the degree to which SOM is stabilized by physical

protection mechanisms, biochemical recalcitrance or diminished microbial activity has bearing for predicting its long-term persistence. Indeed, the high specific metabolic activities we observe in subsoils suggest that, on a per-biomass basis, the capacity of microorganisms to participate in carbon and nutrient mineralization remains constant or even increases with soil depth. Moreover, we found little difference in microbial metabolic activity across the two soil types, which differ markedly in their texture and mineralogy. This finding suggests that organo-mineral associations may not be a primary control on the stability of carbon with depth in these forest soils. Lastly, in chapter 6 we found the chemical composition of soil organic matter to be similar between surface and subsoils from the same pit. That subsoil carbon is not drastically different in its composition from surface carbon suggests that, despite being older and presumably more microbially processed, the deep soil carbon pool is still composed of recognizable biomolecules rather than "humified", refractory compounds.

Taken together, the results of this dissertation point to low energy availability, and hence suppressed microbial activity, rather than carbon chemistry or organo-mineral associations, as the primary control on deep soil carbon stability. Low energy availability can be a result of greater competition for limited substrates and increased spatial separation between microorganisms, substrates and exoenzymes (*Salome et al.*, 2010). While low energy availability may suppress microbial metabolic activity in situ, the potential for substrate metabolism can (and does) still exist, as indicated by laboratory assays of potential activity or measurements of functional metabolic genes. These findings fit into a broader picture emerging from microbial ecology studies of the "deep biosphere"–low energy subsurface environments including the continental crust and deep ocean sediments–which suggest that, while a substantial fraction of cells in these environments may be dormant, microbial communities are capable of re-starting their metabolism given small amounts of energy and nutrients (*Hoehler and Jorgensen*, 2013).

The possibility of an active carbon cycle to depth of 1 meter and beyond in tropical forests has broad implications for the terrestrial carbon cycle. Integrating even small concentrations of active carbon and biomass over the large volumes of soil below 20 cm depth means that the fluxes associated

with these pools can be significant, particularly on decadal and longer timescales (*Trumbore et al.*, 1995). Moreover, if the stability of subsoil carbon is essentially maintained by microbial starvation, additional inputs of carbon to subsoils may accelerate losses. It has been suggested that one effect of elevated atmospheric CO_2 will be an increase in root exudation, and therefore an increase in the supply of fresh organic matter to depth (*Philips et al.*, 2006). Increased belowground investment of plants could stimulate microbial metabolism and lead to the mineralization of older carbon–the so-called "priming" effect (*Fontaine et al.*, 2007). Land use change can lead to accumulation or loss of carbon in subsoils, with carbon stock increases of up to 48 % found in B-horizons after conversion from forest to grassland (*Don et al.*, 2009; *Mueller and Kogel-Knabner*, 2009). In addition, natural disturbances such as hurricanes and landslides occur frequently in the Luquillo Mountains and other montane tropical forests (*Scatena and Lugo*, 1995). Such disturbances can lead the exposure, and potential de-stabilization, of subsoil carbon. While landslide scars accumulate soil organic matter rapidly following disturbance, recovery of soil C pools to pre-disturbance levels can still take decades (*Zarin and Johnson*, 1995). Chronosequences of landslide scars may offer an opportunity to study the potential for loss of exposed subsoil carbon following disturbance. Finally, very few studies have addressed the potential effects of climate warming on tropical soil carbon cycling, despite projections of mean temperature increases of 1.7–5 °C over the 21st century (*Christensen et al.*, 2007), and the fact that even a slight change in the uptake and storage of C in these ecosystems could have substantial consequences for the global C cycle and present large feedbacks to future climate (*Lewis et al.*, 2009; *Wood et al.*, 2012). The construction of the very first *in situ* tropical soil warming experiment is currently underway in the El Yunque National Forest, with plans to warm soils to a depth of 1 meter (*Gonzalez et al.*). This experiment will offer a host of new opportunities for studies of tropical soil carbon dynamics and microbial responses to climate change. Despite the current lack of experimental evidence from field manipulations, the biogeochemical capacity of subsoil microorganisms demonstrated in this dissertation underscores the need for models that track carbon to depths greater than 20 cm in order to accurately simulate responses to global change (*Rumpel and Kogel-Knabner*, 2011; *Schmidt et al.*, 2011; *Trumbore*, 2009).

In addition to the implications of our depth profile studies for soil carbon cycling, the study of mi-

crobial community structure and functions along depth profiles advances our basic understanding of microbial ecology and the other biogeochemical processes soil biota perform. To this day it remains the case that the vast majority of soil microbial ecology studies focus on the upper 10 centimeters of the soil profile. However, if soil microbial populations shift with depth, the ecological strategies and metabolic capacities of these communities may change as well. In chapters 3-4 we observe changes in microbial community structure using phospholipid fatty acid analysis, and changes in community functions (as indicated by shifting ecoenzyme ratios and functional gene abundances) with soil depth. The vertical stratification of bacterial N-cycle genes found in chapter 4 suggests N-fixation to be an important process in surface soils, where plant demands for N are greatest, while denitrification may be relatively more important in subsoils due to fluctuating subsurface redox conditions. In addition, high specific phosphatase activity in subsoils may speak to the flexible use of exoenzymes to acquire various resources under energy-limited conditions, as discussed in chapter 4. By considering phosphatase activity strictly as an indicator of microbial phosphorus demand, an idea substantiated by microbial ecological studies in relatively high-carbon environments, we may be overlooking an important aspect of the microbial ecology of tropical subsoils. Taken together, the evidence presented in chapters 3 and 4 suggest that subsoil microbial communities are structurally and functionally distinct from surface communities. Soil profiles may be viewed as an environmental gradient that drives natural selection of communities best able to compete for resources, survive and reproduce at any given depth. Other researchers have found there can be equal or greater changes in microbial communities with soil depth than across surface soils taken from diverse and geographically disparate ecosystems (*Eilers et al.*, 2012). Such findings fit with the cosmopolitan view of the microbial world, which suggests spatial patterns in microbial diversity are driven by environmental heterogeneity more than geographic distance (*Green and Bohannan*, 2006).

7.3. Patterns in soil microbial ecology and soil organic matter across state factors

In most cases, the dramatic changes we observed in microbial activity with soil depth dwarfed differences across the two parent materials and forest types studied in this dissertation. Nonetheless, this

dissertation still finds evidence that state factors have important bearing for carbon biogeochemistry in the critical zone. Our ^{13}C NMR results suggest a greater degree of soil organic matter processing and a retention of aliphatic carbon in the cooler, wetter Colorado forest, which supports the findings of previous researchers in this study site (*Cusack et al.*, 2011; *Ostertag et al.*, 2008). The enrichment of alkyl carbon during SOM decomposition suggests aliphatic compounds may represent an important component of the stable carbon pool in these soils (*Mikutta et al.*, 2009; *Ostertag et al.*, 2008). Difference in soil organic matter chemistry across the two forests may relate to broad differences in the substrate use preferences of soil decomposers, as discussed further in chapter 6. In addition, our results suggest topography may play a key role in determining the composition of organic matter present in a soil profile. These findings fit with a recent forest carbon inventory that finds topographic position and forest type are the primary controls on soil carbon stocks at the Luquillo Critical Zone Observatory (*Johnson et al.*, Under Review). While this dissertation principally focused on the influence of geology and vegetation as state factors that may influence carbon cycling, the study of differences in microbial activity along topographic gradients represents a promising future direction that may help to better identify biogeochemical "hotspots".

While carbon chemistry and microbial carbon cycling activity appear similar across the two parent materials, chapters 4 and 5 present evidence for differences in the nitrogen and phosphorus cycling capacity of the microbial communities, possibly indicative of differences in nutrient availability across two soil types that differ in their texture and mineralogy. Consistently higher levels of nitrogen functional genes in the quartz-diorite parent material may speak to a direct linkage between pedogenesis, soil transport processes, and biogeochemical cycling, with younger, sandier tropical soils exhibiting a more dynamic nitrogen cycle due to more rapid transport of dissolved nitrogen species to depth and potentially greater fluctuations in subsurface redox conditions (*McDowell et al.*, 1992; *McSwiney et al.*, 2001). Greater nitrogen cycling capacity in Inceptisols could also be an indicator of greater phosphorus availability and hence alleviated phosphorus-limitation. Consistent with this second explanation are the lower soil C:P and N:P ratios in Inceptisols compared with the Oxisols, and the higher overall catalytic capacity (K_a) for acid phosphatase in Oxisol soils. A larger pool of organic phosphorus-acquiring enzymes in the Oxisols may be the result of greater

organic phosphorus demand in an environment where inorganic phosphorus is strongly bound to soil minerals (*Chacon et al.*, 2006).

7.4. Methodologies

A secondary goal of this dissertation was to explore the use of various methodologies for the study of microbial ecology and carbon biogeochemistry throughout tropical soil profiles. As discussed in chapter 6, tropical soils pose significant methodological challenges to soil organic matter research due to their high iron content. For microbial ecologists, subsoils pose the additional challenge of low biomass concentrations, making microbial biomass and community structure determiniations challenging. Many of the methods used in this dissertation had to be carefully tested and optimized, and often, our samples found us near the detection limit of our instruments. For instance, routine measurements of bulk soil carbon and nitrogen concentrations via combustion analysis become challenging when carbon concentrations approach 0.1 % or lower, as was the case for many of our samples. Samples with carbon concentrations this low were re-run on a stable isotope mass spectrometer to improve measurement precision. The molecular techniques used in chapter 4 required a great deal of optimization. While DNA extraction from soils is now a routine procedure in microbial ecology, standard DNA extraction protocols needed to be modified to concentrate DNA in low-biomass soils. A major challenge in performing DNA-based studies in clay-rich soils is evaluating whether the extraction procedure is able to capture a representative sample of the microbial community. DNA extraction biases, compounded with the PCR amplification biases discussed in chapter 4, may lead to significant distortion of microbial community composition (*Kopczynski et al.*, 1994; *Suzuki and Giovannoni*, 1996; *Tedersoo et al.*, 2010). Techniques such as quantitative PCR, which measure the concentration of genes present in a DNA extract, may be subject to less bias than traditional PCR-based approaches, however, quantitative PCR only provides information about the abundance of pre-selected genes, making it an appropriate tool for characterizing select functional attributes but not community structure. The gold standard for assessing microbial community composition is via next-generation sequencing methods such as 454 or Illumina sequencing, which can provide deep taxonomic information on millions of different organisms present in a sam-

ple (*Bartram et al.*, 2011). However, extraction and amplification biases are of major concern for soil metagenomic approaches as well (*Kakirde et al.*, 2010; *Lombard et al.*, 2011). Future studies using any molecular tools to characterize the microbial ecology of clay-rich tropical soils will have to take these limitations into consideration.

Phospholipid fatty acid analysis offered us an alternative approach for profiling community structure. An older and less taxonomically precise method than DNA-based approaches, we nevertheless found phospholipid analysis to be a highly valuable technique in this dissertation, due to 1) its relatively high detection limit, 2) the ability of PLFA to provide broad structural information and 3) the use of PLFA as an indicator of viable microbial biomass (*Frostegard et al.*, 2011). Phospholipid analyses are to this day widely used in studies of low-carbon sediments (*Zhu et al.*, 2013; *Zink et al.*, 2003).

We used solid-state ^{13}C NMR to assess carbon chemistry chapter 6. The methodological challenges associated with ^{13}C NMR as discussed in detail in chapter 6. Overall, we find this technique provides valuable information on the composition of soil organic matter, though for tropical soils and subsoils, the multiple acid treatments required to obtain high-quality spectra raise concerns about soil organic matter fractionation. While we did not find strong evidence of organic matter fractionation, we suggest that researchers continue to explore this possibility when applying ^{13}C NMR to soils, given that each soil type will respond to acid treatment somewhat differently. Although beyond the scope of the present dissertation, validation of ^{13}C NMR results with an additional chemical profiling technique, such as infrared spectroscopy, may aid researchers in determining whether ^{13}C NMR gives us a reliable characterization of soil carbon chemistry.

Tropical soils and subsoils contain unique biology, the ecology and composition of which are just beginning to be explored. The microorganisms inhabiting soils of the Luquillo Critical Zone dynamically contribute to carbon and nutrient cycling throughout at least the upper 1.4 meters of soil profiles and probably deeper. Despite facing resource scarcity in subsoils, resilience exists in the capacity of these microbial communities to mineralize carbon and nutrients. The high potential for subsoil microbial activity, and relatively small differences in carbon chemistry between surface and

subsoils suggest that researchers should question the inherent stability of deep soil carbon and the mechanisms that govern it. Models that aim to predict the future changes to the global carbon cycle should begin to consider the capacity for carbon cycling to occur throughout the deep critical zone.

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