BIOGEOCHEMISTRY OF WOODY PLANT INVASION:

PHOSPHORUS CYCLING AND MICROBIAL COMMUNITY COMPOSITION

A Dissertation

by

ILSA BETH KANTOLA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Rangeland Ecology and Management

Biogeochemistry of Woody Plant Invasion:
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Approved by:

Co-Chairs of Committee,	Thomas W. Boutton
	Terry J. Gentry
Committee Members,	C. Thomas Hallmark
	Frank M. Hons
Head of Department,	Steven G. Whisenant

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ABSTRACT

Biogeochemistry of Woody Plant Invasion:
Phosphorus Cycling and Microbial Community Composition. (May 2012)

Ilsa Beth Kantola, BS, California Polytechnic State University, San Luis Obispo

Co-Chairs of Advisory Committee: Dr. Thomas W. Boutton
Dr. Terry J. Gentry

Woody plant encroachment is a globally-prevalent vegetation change phenomenon that has shifted grass-dominated ecosystems to mixed grass and woody plant matrices over the last century. In the Rio Grande Plains of Texas, the introduction of N-fixing woody legumes has increased above- and belowground primary productivity and changed the litter chemistry of the system, accelerating rates of belowground biogeochemical processes. The purpose of this study was to assess the impact of grassland to woodland transition on i) P concentrations in soil physical fractions that differ in their organic matter turnover rates, ii) P availability within the soil over the course of woody encroachment and across the landscape, and iii) microbial community composition and diversity. Soil samples were collected in remnant grasslands and four woody landscape elements (clusters, groves, drainage woodlands, and playas) along a 135-yr chronosequence of woody plant encroachment. P was fractionated by the Hedley method and P concentrations were determined by alkaline oxidation and lithium fusion coupled with ascorbic acid colorimetry. Bacterial and fungal communities were characterized by molecular methods. Whole soil P concentrations were 2-5X greater in woody landscape elements than in grasslands, and nutrient concentrations increased linearly with time following woody plant invasion in all but the slowest-cycling physical fractions. Plant-available P and organic P increased dramatically with time following encroachment. Changes in P availability were more pronounced in drainages and playas

than in upland clusters and groves. Analysis of the bacterial and fungal communities demonstrated that microbial communities in grasslands differ at both phylum and genus level from the flora of the wooded landscape elements. This study demonstrates that woody encroachment strongly influences the distribution and availability of soil P and indicates that nutrient cycles in the soil are closely linked and similarly affected by increased woody plant abundance. Microbial communities under woody species differ in composition from those of the grasslands, and are likely contributing to the observed changes in nutrient availability. Since N and P are generally the most limiting nutrients in terrestrial ecosystems, increased stores of P are likely to alter rates of microbial processes, plant-microbe and plant-plant interactions, and successional dynamics in this ecosystem and similar landscapes around the world.

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CHAPTER I

INTRODUCTION

Woody plant encroachment is a global phenomenon that has replaced many grass-dominated ecosystems that historically supported pastoral farming and grazing communities around the world with savanna and woodland vegetation. The expansion of woody plants into grasslands is often described as 'encroachment', as in many of these ecosystems encroachment consists of native species that have altered distribution, rather than the introduction of a non-native species. In the last century, increased woody plant abundance has been documented in grasslands of the Americas, Africa, Europe, Oceana, and the Arctic (eg. Archer et al., 1988; Hudak and Wessman, 1998; Briggs et al., 2005; Allison et al., 2006; Chen et al., 2008; Maestre et al., 2009; Lorenzo et al., 2010; Barger et al., 2011). A number of factors contribute to this change in vegetation, including livestock grazing, fire suppression, rising atmospheric CO_2 levels that promote growth in C_3 woody species, and increased atmospheric N deposition (Archer 1994; Archer et al., 2001; Polley et al., 1994; Scholes and Archer, 1997; Köchy and Wilson 2000; Van Auken, 2000; Roques et al., 2001). The shift from grassland to woody plant-dominated vegetation has been shown to result in increased above- and belowground primary productivity, changes in soil structure and organic matter content, increased nutrient concentrations, altered nutrient cycling rates, and changes in microbial community structure and function (Padien and Lajtha, 1992; Hibbard et al., 2001; McCulley et al., 2004; Liao et al., 2006a,b; Lorenzo et al., 2010; Dickie et al., 2011; Eldridge et al., 2011). The presence of woody plants accumulates nutrients and organic material beneath the shrub canopy, producing a matrix of soil properties that reflects the aboveground vegetation in grassland/shrub ecosystems (Schlesinger et al., 1990; Padien and Lajtha, 1992; Hibbard et al., 2001).

This dissertation follows the style of Soil Biology and Biochemistry.

Tropical and temperate grasslands and shrublands account for 40-45% of the C fixation within the terrestrial biosphere (Beer et al., 2010) and 30% of the absorption of anthropogenic CO_2 emissions as soil organic matter and plant biomass (Canadell et al., 2007). Increases in above- and belowground biomass in these ecosystems due to woody encroachment have serious implications for the global carbon budget as well as coupled cycles of C, N, and P. The plant species responsible for encroaching in grasslands vary by ecosystem, and the response of each ecosystem is determined by the particular climate, soil, and vegetation properties of that ecosystem. However, several trends are common to woody plant encroachment world-wide: increasing shrub cover results in increases in aboveground biomass, soil C and N, and N mineralization (Liao et al., 2008; Eldridge et al., 2011). Examination of the effects of vegetation change on ecosystems around the world has shown that capacity of woody and N-fixing plants to thrive in low-nutrient and marginal environments increases the impact of invasion on the nutrient cycling of an ecosystem over than of herbaceous and non-N-fixing species (Liao et al., 2008; Houlton et al., 2008).

The Texas Agrilife La Copita Research Area in south Texas offers the opportunity to observe the effects of woody plant encroachment on soil biogeochemical cycling across a range of landscape types and a 135-year timeline of vegetation change. Livestock grazing, fire suppression, and climate and atmospheric chemistry changes have led to the replacement of C_4 grasslands by C_3 subtropical thorn woodlands on the site (Scholes and Archer, 1997; Archer et al., 1998, 2001; Boutton et al., 1998, 1999). Over the past century, the abundance of N-fixing *Prosopis glandulosa* (honey mesquite) has increased on the site, creating a matrix of woody plant elements and remnant grasslands. The progressive nature of woody encroachment has facilitated the use of a chronosequence approach to woody plant encroachment research, wherein mesquite tree age can be used as proxy for the period of woody plant influence on the soil. The chronosequence approach has demonstrated that a number of the effects of woody encroachment progress at steady rates. Woody encroachment has increased net primary productivity (NPP), increasing C storage in both plant biomass and soil organic matter and shifting the

organic matter chemistry toward more recalcitrant compounds that resist decomposition (Hibbard et al., 2001; McCulley et al., 2004; Liao et al., 2006a,b; Filley et al., 2008). Furthermore, the development of woody plant assemblages dominated by N-fixing tree legumes has increased the pool of N cycling in the system through the production of N-rich litter and roots. Woody plants support larger pools of microbial biomass C and N, higher soil respiration rates, and higher C and N mineralization rates than historic grassland vegetation (McCulley et al., 2004; Liao and Boutton, 2008). Inputs of organic matter have changed the distribution of C and N among the water-stable aggregate fractions in the soil, influencing the soil fertility and organic matter turnover on the site (Boutton et al., 1998, 2009; Archer et al., 2001; Liao et al., 2006b; Boutton and Liao, 2010).

The effect of woody encroachment on the P cycle is less understood than the C and N cycles, but the critical role P plays in plant growth is clear. Inputs to the P cycle in soils occur primarily at the rate of weathering of P-containing parent materials, much more slowly than C and N, as there is no atmospheric gaseous source of P (Walker and Syres, 1976; Parton et al., 1988; Chapin et al., 2002). Within the soil, P is bound in organic matter and forms of insoluble mineral complexes (Haussling and Marshner, 1989; Chapin et al., 2002). Plants liberate P from organic materials and some mineral compounds through the production of phosphatase enzymes excreted by plant roots (Houlton et al., 2008; Vitousek et al., 2010). The large rhizospheres of woody species increase the volume of soil that can be mined by plant roots for P, increasing the amount of P cycling biologically in woody plant biomass over that of grass species. In all plants, P is an essential component of adenosine triphosphate (ATP), the chemical energy plants use in metabolism. In non-N-fixing plants, limits on the production of ATP are the primary effect of P limitation on plant growth (Allison et al., 2006;, Finzi and Rodgers, 2009). In N-fixing plants like mesquite, feedbacks occur between the P and N cycles within the plants themselves: the energy consumption and nitrogenase production of N-fixation requires P, therefore plants allocate N to the production of P-liberating

phosphatase enzymes (Chapin et al., 2002; Allison et al., 2006; Elser et al., 2007; Wang, Y.-P. et al., 2007, Houlton et al., 2008; Vitousek et al., 2010).

Investigations into the effects of woody species on P cycles in the soil have shown that shrubs can concentrate nutrients, including inorganic and organic P, under their canopies, creating islands of fertility that are important for nutrient conservation (Cross and Schlesinger, 1995, 2001; Padien and Lajtha, 1992; Hibbard et al., 2001; Dossa et al., 2010). Low P soils force plants and the associated microbes to work to acquire P from the environment, investing N that would otherwise be used for biomass production into enzymes that decompose organic P compounds in soil organic matter (Chapin et al, 2002; Wang, Y.-P. et al., 2007; Houlton et al., 2008; Vitousek et al., 2010. Potential mechanisms of nutrient accrual in shallow soil include litter production, mining from deep soil by plant roots, atmospheric interception of P-containing dust and particulates, and lateral transfer from nearby soils (Vitousek et al., 2010). Studies of shrub encroachment in other ecosystems have shown increases in P under shrub canopies (Allison et al., 2006; Dossa et al., 2010; Lorenzo et al, 2010) and significant increases in phosphatase activities that indicate P cycling (Allison et al., 2006).

Total phosphorus measurements in soil are a poor indicator of plant-available P because many of the chemical forms of P present in soil are relatively insoluble and therefore unavailable for plant uptake (Haussling and Marshner, 1987; Cross and Schlesinger, 1995, 2001). In some ecosystems, P availability is constrained by organic compounds, from which P must be mineralized before becoming plant-available. Previous research has shown that organic matter produced by woody species following woody plant encroachment at La Copita is both chemically recalcitrant and increasingly physically protected in water stable aggregates as the temporal influence of woody vegetation increases (Liao et al, 2006a, Filley et al. 2008, Boutton et al. 2009, 2010). Therefore P may be both physically protected within aggregates and chemically occluded within the soil. Measurement of P content of soil physical fractions describes where soil P is found in fast and slow SOM turnover pools (Liao et al., 2006a). Through sequential extraction of different P fractions, a gradient of P pools ranging from plant-

available to relatively recalcitrant can be quantified, and soil P can be subdivided into organic and inorganic forms (Hedley et al., 1982; Tiessen and Moir, 1993; Lajtha et al., 1999; Dossa et al., 2010).

Vegetation change is a known driver of microbial community shifts in a wide range of ecosystems (Nusslein and Tiedje, 1999; Kourtev et al., 2002; Bossio et al., 2005; Lorenzo et al., 2010). Within a landscape, microbial communities respond to differing nutrient sources and soil conditions under contrasting vegetative cover (Wallenstein et al., 2007; Biederman and Boutton, 2009; Dickie et al., 2011). Alterations in chemistry and nutritional quality of organic matter, including the production of decomposition-resistant secondary aliphatic compounds during the grass-to-woodland vegetation, shift modify the microbial environment in the soil during woody plant encroachment (Hibbard et al., 2001; Obi et al., 2002; Steenkamp et al., 2007; Filley et al., 2008; Boutton et al., 2009). Change from grass to woody shrub vegetation modifies the physical environment by shading sub-canopy soil (altering temperature and moisture regimes) and by root distribution and activity in the belowground environment (potentially liberating and redistributing water and nutrients from deep soil) (Padien and Lajtha, 1992; Canadell et al., 1996; Cross and Schlesinger, 2001; Hibbard et al., 2001). These changes in organic matter quality and the physical environment due to plant succession have the potential to alter the diversity, abundance, and function of microbial communities in the soil environment, creating a community adapted for decomposing recalcitrant woody litter and N fixation (Filley et al., 2008; Boutton et al., 2009; Lorenzo et al., 2010; Dickie et al., 2011).

Shifts in makeup of belowground microbial communities at La Copita have already been observed in the nematode community, where a shift in the population of nematodes from root-feeding to bacterial-feeding species suggests that the woody plant roots are more resistant to feeding by soil biota, or that the microbial community has changed to support more bacterial-feeders. In addition, the increased pressure of bacterivores may accelerate nutrient turnover in the wooded systems, and may indicate a shift in the bacterial community toward populations more attractive to nematodes and other

predators (Biederman and Boutton, 2009). It stands to reason that these same factors will affect the structure of the bacterial and fungal communities, as they respond to changes in plant-supplied nutrients and grazing pressure from predatory soil fauna. It remains to be seen whether woody plant-adapted bacteria will monopolize the bacterial portion of the soil biota, creating a less-diverse, more specialized bacterial community, or whether increased litter and N inputs to the soil will increase the overall biomass and diversity of microbes in the community. Previous research in invasive *Acacia dealbata* (a related tree legume) predicts decreases in fungal species richness and diversity as mesquite establishes in the grasslands and the microbial community becomes more specialized (Allison et al., 2006; Lorenzo et al., 2010). It is not yet known how quickly the microbial community changes after the introduction of woody plants, and to what extent the community remains stable over the lifespan of a mesquite tree on the site.

While the effects of vegetation and land cover change on soil C and N have been widely examined within the scope of global climate change, research into the importance of P in the C and N cycles has lagged behind, likely due to challenges in measuring soil P and the perception of P as having a lesser role in OM turnover than C and N. Recent P research has indicated that in many systems, P is the nutrient responsible for limiting plant growth and microbial activity, prompting the need for further research into the forms of P and the relative availability of the nutrient in soils under different vegetation types (Elser et al., 2007; Wang, Y.-P. et al., 2007; Vitousek et al., 2010). In addition to the effects of woody plant encroachment on soil bacteria and fungi, encroachment by mesquite at La Copita offers the opportunity to examine the effects of a N-fixing legume on soil biota. Recent advancements in molecular methods for microbial characterization allow for identification of bacteria and fungi at the genus level, effectively identifying the microbial groups and allowing for comparison between landscape elements and other woody encroachment-affected ecosystems.

This study will evaluate the effects of woody plant encroachment into grasslands on the ecosystem biogeochemistry and soil microbial communities through soil physical fractionation, measurements of soil C, N, and P, P fractionation, and molecular methods

for microbial community analysis. The specific objectives of the study are to a) quantify the effects of woody plant influence on soil C, N, and P concentrations in whole soils and soil physical fractions; b) use P fractionation procedures to describe the changes in the chemical forms of phosphorus in mesquite-invaded soils i) with time and ii) across different landscape elements produced by woody encroachment; and c) characterize the diversity and composition of the soil microbial community using quantitative polymerase chain reaction (qPCR), automated ribosomal intergenic spacer analysis (ARISA), and 454 pyrosequencing and identify differences that may be associated with changes in soil properties and nutrient cycles under the influence of woody plant encroachment. The results of this study will enhance the understanding of the coupling of soil biogeochemical cycles by incorporating P into C and N research, predicting the roles of the three nutrients in grassland and wooded systems in the face of progressive vegetation change. The results of this study will illustrate the response of the plant/microbe continuum to the introduction of woody plants, increasing understanding of the short- and long-term effects of woody plant encroachment on storage and cycling of soil nutrients.

CHAPTER II

CARBON, NITROGEN, AND PHOSPHORUS INCREASE IN SOIL PHYSICAL FRACTIONS FOLLOWING VEGETATION CHANGE FROM GRASSLAND TO WOODLAND

1. Synopsis

Woody plant encroachment has been pervasive in grass-dominated ecosystems around the world during the past century due to livestock grazing, fire suppression, and/or changes in climate and atmospheric chemistry. In the Rio Grande Plains of Texas, subtropical thorn woodlands dominated by N-fixing tree legumes have largely replaced grasslands. This dramatic land cover change has increased above- and belowground primary productivity and accelerated rates of biogeochemical processes in the soil. The purpose of this study was to assess the impact of this grassland to woodland transition on C, N, and P concentrations in soil physical fractions that differ in their organic matter turnover rates. Soil samples (0-10 cm) were collected in remnant grasslands and near the centers of woody plant clusters ranging in age from 15 to 90 yrs in a subtropical savanna parkland in southern Texas. Soils were fractionated by wet sieving into five size and density classes: un-sieved whole soil, free light fraction (density <1 g/cm^3), macroaggregates (>250 μm), microaggregates (53-250 μm), and free silt and clay (<53 μm). C and N concentrations in each of the fractions were determined by combustion/gas chromatography and total P concentrations were determined by alkaline oxidation and sulfuric acid digestion coupled with ascorbic acid colorimetry. Carbon, N, and P concentrations in whole soil were 2-3X greater in woody clusters than in grasslands. In addition, C, N, and P concentrations increased linearly with time following woody plant invasion in all fractions except free silt and clay. Most of the newly accrued C, N, and P was found in the relatively more labile light fractions and macroaggregates. C:P and N:P ratios increased following woody encroachment, indicating C and N accumulated at a faster rate than P. Since N and P are generally the most limiting nutrients in terrestrial ecosystems, increased stores of these elements are

likely to alter rates of microbial processes, plant-microbe and plant-plant interactions, and successional dynamics in this ecosystem.

2. Introduction

Woody plant encroachment is a geographically widespread land cover change that has resulted in the conversion of many grass-dominated ecosystems to shrublands, savannas, and woodlands. In the last century, woody plant abundance has increased in grasslands on every continent except Antarctica (Archer et al., 1988; Hudak and Wessman, 1998; Briggs et al., 2005; Allison et al., 2006; Knapp et al., 2008; Lorenzo et al., 2010). Several factors contribute to this change in vegetation, including livestock grazing, fire suppression, and rising atmospheric CO_2 levels that promote growth in C_3 woody species (Polley et al., 1994; Scholes and Archer, 1997; Köchy and Wilson, 2000, 2001; Van Auken, 2000; Roques et al., 2001). Examination of the effects of vegetation change on ecosystems around the world has shown that the introduction of woody plants has more effect than herbaceous invaders, and N-fixing capacity in woody plants has an even greater impact on the NPP, plant N, and litter production of an ecosystem. (Liao et al., 2008).

Woody plant encroachment has been shown to increase both C and N storage and availability in soils (Padien and Lajtha, 1992; Hibbard et al., 2001; McCulley et al., 2004; Liao et al., 2006b; Wheeler et al., 2007; Knapp et al., 2008; Liao et al. 2008; Springsteen et al., 2009; Scharenbroch et al., 2010; Eldridge et al., 2011; Barger et al., 2011). Woody plants increase aboveground NPP, thereby increasing the capacity for site vegetation to sequester atmospheric C and N in plant tissues (Hibbard et al., 2001; Köchy and Wilson, 2000; Wheeler et al., 2007). The litter produced by woody plants has been shown to be higher in recalcitrant secondary chemical compounds which resist degradation and preserve organic matter in the soil (Hibbard et al., 2001; McCulley et al., 2004; Liao et al, 2006b; Springsteen et al., 2009). This results in alteration of the soil nutrient landscape as the vegetation landscape shifts. The presence of woody plants

serves to establish islands of fertility, where nutrients accumulate beneath the shrub canopy (Schlesinger et al., 1990; Padien and Lajtha, 1992; Hibbard et al., 2001).

Compared to the available literature on the effects of woody plant encroachment on C and N, relatively little is known about the response of soil P to vegetation change. Investigations into the effects of woody plant encroachment on P cycles in the soil have attributed nutrient increases under shrub canopies to litter production, mining from deep soil by plant roots, atmospheric interception, or inputs from birds or other animals (Padien and Lajtha, 1992; Hibbard et al., 2001; Dossa et al., 2010). Phosphorus plays a critical role in the energy-intensive processes of plant metabolism, namely photosynthesis and N-fixation, creating feedbacks between the N and P cycles (Robson et al., 1981; Chapin et al., 2002; Schulze, 2004; Turner et al., 2005; Allison et al., 2006; Elser et al., 2007; Wang, Y.-P. et al., 2007; Chen et al., 2008; Vitousek et al., 2010; Ceulemans et al., 2011). Due to the high P demands of N-fixation, woody encroachment by N-fixing species is of special interest. In systems where woody plant encroachment has led to a rapid change in the abundance and availability of C and N, P may become the limiting nutrient as biotic requirements exceed the rate of P weathering from minerals in the soil (Walker and Syers, 1976; Chapin et al., 2002; Vitousek et al., 2010).

In the Rio Grande Plains of southern Texas, subtropical thorn woodlands dominated by N-fixing tree legumes have largely replaced grasslands as a result of livestock grazing and fire suppression during the past 150 yrs (Archer et al., 1988, 2001, Boutton et al, 1998, 1999). In this region, woody encroachment has increased soil C and N storage (Liao et al., 2006a; Boutton and Liao, 2010), enlarged the size of the soil microbial biomass C and N pools (McCulley et al., 2004; Liao and Boutton, 2008), accelerated soil respiration (McCulley et al., 2004), and increased rates of soil N transformations (Hibbard et al., 2001; McCulley et al., 2004). Phosphorus has been shown to accumulate under the canopies of woody species (Schlesinger and Pilmanis, 1998; Dossa et al., 2010), but the effect of woody plant encroachment on the soil P at La Copita has not been investigated. The mechanism of woody encroachment at this site-- by a single, primary N-fixing woody species-- offers the opportunity to observe C, N, and P along a

chronosequence representing the invasion of woody species into grasslands over the last century. Though feedbacks between the C and N cycles are widely accepted, the importance of P as a control for N fixation rates (Schulze, 2004; Wang, Y.-P. et al., 2007; Vitousek et al., 2010), and the relationship between N availability and C mineralization (Henriksen and Breland, 1999), make it essential to incorporate measurements of P into the framework of the La Copita ecosystem.

The primary objectives of this study were to quantify the effects of woody plant encroachment on C, N, and P in whole soil and soil physical fractions following woody plant encroachment of grasslands and to investigate the association of soil nutrients with different aggregate sizes within the soil structure as the structure is altered by the presence of woody species. Through nutrient analyses of whole soil and soil physical fractions sampled along a chronosequence of woody plant encroachment, this study seeks to show i) increased abundance of mesquite alters the relative proportions of water-stable aggregates in the soil, ii) woody plants increase the size of nutrient pools and the rates of nutrient cycling in wooded soils, and iii) the association of nutrients with fast-turnover aggregate size classes indicates the availability of C, N, and P is increasing with woody encroachment.

3. Materials and methods

3.1. Study area

Field sampling was conducted in October 2006 at the Texas AgriLife La Copita Research Area (27º 40'N, 98º 12'W) in the Rio Grande Plains approximately 65 km west of Corpus Christi, TX. Climate is subtropical with mean annual temperature of 22.4 ºC and mean annual precipitation of 716 mm. The precipitation pattern is bimodal, with peaks in May-June and September. Topography consists of nearly level uplands that grade (1-3% slopes) into lower-lying drainage woodlands and playas. Elevation ranges from 75 to 90 m across the site. Sequential aerial photography, tree ring analyses, vegetation dynamic modeling, and isotopic analyses of soils indicate that during the last 150 years, vegetation on the site has shifted from open grasslands to mixed

grassland/thorn woodland, with woody plant dominance continuing to increase over time (Archer et al*,* 1988, 2001, 2004; Boutton et al*.,* 1998, 1999; Bai et al., 2009, 2012a).

Upland portions of the landscape have loamy sand soils (Typic and Pachic Argiustolls) with a nearly continuous subsurface argillic (Bt) horizon at 30-40 cm depth. These upland surfaces are covered by a relatively open grassland matrix that also includes small woody clusters (1-5 m diameter) and larger woody groves (10-100 m diameter). Grasslands are dominated by C_4 grasses including *Bouteloua, Chloris, Panicum, Tridens, Eragrostis,* and *Paspalum.* Discrete woody clusters (approx. 1-5 m in diameter) occur within the grassland matrix and are dominated by *Prosopis glandulosa* Torr. (honey mesquite), and include as many as 15 other tree/shrub species including *Condalia hookeri* M.C. Johnst*., Zanthoxylum fagara* (L.) Sarg*., Ziziphus obtusifolia* (T.&G.) Gray*,* and *Berberis trifoliolata.* On portions of the upland landscape where the argillic horizon is absent, woody clusters expand laterally and often fuse to form larger woody groves (Archer et al., 1988; Bai et al., 2009, 2012a). Lower-lying portions of the landscape consist of sandy loam and sandy clay loam soils (Pachic Argiustolls). Although once covered by C_4 grassland (Boutton et al., 1998; Bai et al., 2012b), these lowland areas are now closed-canopy woodlands similar in species composition to upland clusters and groves. This study focused on quantifying changes in soil C, N, and P storage during the development of woody clusters within the upland grassland matrix.

3.2. Chronosequence approach

To evaluate changes in soil C, N, and P concentrations following woody plant encroachment, a space-for-time chronosequence approach was utilized. On this site, the formation of woody clusters within the grassland matrix is initiated only after the establishment of mesquite (Archer et al., 1988). Thus, the age of a woody cluster corresponds to the age of the mesquite tree in that cluster. The ages of mesquite trees were determined by measuring their basal diameters and then substituting those values into a regression equation to predict tree ages (Stoker, 1997). Woody plant clusters were selected to encompass the full range of mesquite basal diameters, corresponding to tree

ages ranging from approximately 10 to 85 years. Samples from remnant grasslands were assumed to be representative of the grassland ecosystems that dominated the region prior to woody plant invasion (Time 0).

3.3. Sample collection

For C, N, and P analyses, soil samples were collected in upland portions of the landscape from fifteen woody clusters and fifteen neighboring remnant grasslands. Four soil cores (5 cm diameter x 10 cm deep) were collected from near the bole of each mesquite in each cluster, and around the base of a neighboring C4 grass located at least 5 m beyond the canopy edge of the cluster. Soil cores were stored in plastic bags, transported on ice and stored at 4 ºC. Litter was collected from a 0.25 m^2 grid square at each sample site and stored in paper bags at 4 ºC. For clusters, the grid square was located within 0.5 m of the bole of the central mesquite tree. For grasslands the grid square was located at least 5 m outside of the dripline of the cluster. Woody debris and foliar litter were collected together. Litter was dried at 50 ºC and pulverized in a centrifugal mill (Angstrom, Inc., Belleville, MI) and saved for elemental, isotopic, and chemical analysis.

3.4. Soil physical and chemical characterization

Bulk density was determined by the core volume method and corrected for field moisture content. An aliquot of each soil was passed through an 8-mm sieve to remove large organic matter fragments and roots. The sieved soil was dried at 65 ºC and used for chemical, elemental, and isotopic analyses. Two hundred grams of whole soil was set aside for whole soil analyses, while the remainder was physically fractionated. Roots and organic matter fragments > 8 mm were dried at 50 ºC and pulverized in a dental amalgamator (Dentsply Rinn, Elgin, IL) and reserved for elemental, isotopic, and chemical analysis.

3.5. Soil physical fractionation

Changes in distribution of soil physical fractions with time following woody plant encroachment were determined by wet sieving. In a method adapted from Elliott (1986), 30-g aliquots of each sample were spread on the surface of a 250-µm sieve and submerged in 5 cm of deionized water for five minutes (Figure 1). The free light fraction (particulate organic matter that floats on water, density < 1.0 g/cm^3) was separated by aspiration and retained on a nylon filter. The soil sieves were lifted out of the water bath and then resubmerged in the water 50 times (for approximately 2 minutes) to isolate macroaggregates. Aggregates remaining on the sieve were oven dried at 50 ºC. The process was repeated using a 53-µm sieve to isolate microaggregates from the water that passed through the 250 µm sieve, and the remaining free silt and clay material was centrifuged and oven dried. Wet sieving fractionated soil into three size categories: macroaggregates (>250 µm diameter), microaggregates (53-250 µm diameter), and free silt and clay (unincorporated mineral fraction <53 µm diameter), and isolated the organic matter (called "free light fraction") from mineral soil material. After drying, samples were pulverized in an Angstrom ring and puck mill and reserved for C, N, and P analyses.

3.6. Carbon and nitrogen concentrations and isotopic composition

Elemental and isotopic analyses of C and N in whole soils, soil physical fractions, litter samples, and root samples were conducted at the Stable Isotope Laboratory at Texas A&M University, College Station. Elemental and isotopic analyses were performed on a Carlo Erba EA-1108 elemental analyzer (CE Elantech, Lakewood, NJ, USA) interfaced with a Finnigan Delta Plus isotope ratio mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) operating in continuous flow mode. Soils, soil fractions, litter, and root samples collected from the field site were analyzed for % C, % N, $\delta^{13}C$, and $\delta^{15}N$ (Ehlringer and Rundel, 1989).

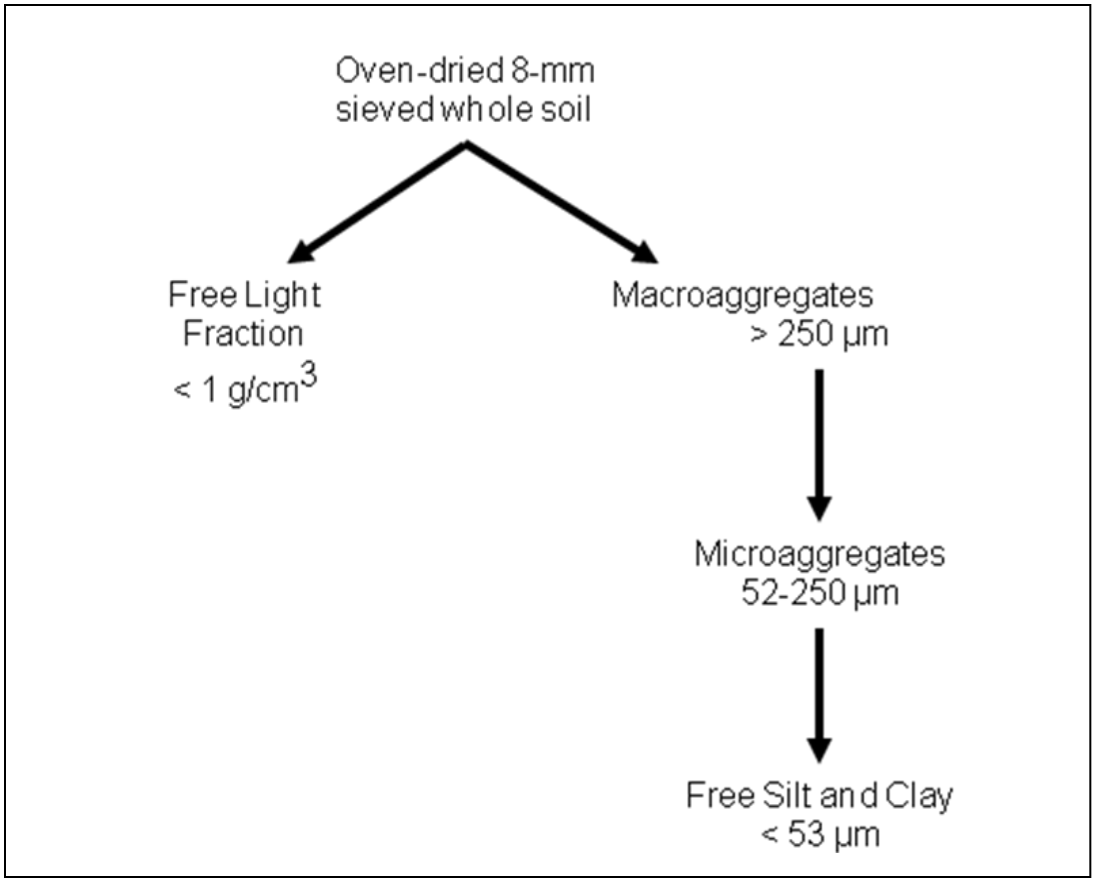

Figure 1. Schematic for physical fractionation of whole soil into size and density fractions by wet sieving (adapted from Elliot et al., 1986.)

3.7. Phosphorus analyses

Total P analyses of whole soils and macroaggregates, microaggregates, and free silt and clay were performed in the Texas AgriLife Research Soil Characterization Laboratory at Texas A&M University (College Station, TX, USA). Finely ground soil and soil fractions were alkaline oxidized and analyzed for total phosphorus concentration by colorimetry (Dick and Tabatabai, 1977). Ground, dried, 200-mg samples from physical fractionation were boiled to dryness with NaOBr-NaOH (3ml bromine in 100 ml 2M NaOH) in a sand bath at 260-280 ºC, then heated for an additional 30 minutes to digest P from the sample. The dried soil was resuspended in 4 ml deionized H_2O and 1 ml 88% formic acid to remove any remaining bromine, then combined with 25 ml 5N H_2SO_4 to re-suspend the boiled material. The flasks were stoppered and the contents mixed on a tabletop vortex, then centrifuged at 12,000 rpm for 5 minutes to remove soil particles. Phosphorus concentration was determined using a modified molybdenum blue method (Murphy and Riley, 1962; Dick and Tabatabai, 1977). Color was developed by combining 2 ml of the digested samples with ammonium molybdate ($(NH_4)_6Mo_7O_{24}$-$4H_2O$, 40 g in 1000 ml H_2O) and antimony potassium tartrate ($C_8H_4K_2O_{12}Sb_2$-$3H_2O$, 1.454 g in 500 ml H_2O) to produce a blue color, and the concentration of P was measured on a Spectronic 20 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 720 nm (Dick and Tabatabai, 1977) and referenced with a standard curve of potassium phosphate solution (KH_2PO_4, at 0, 2.5, 5.0, 7.5, 10.0, and 12.5 µg/ml). Phosphorus concentrations of free light fraction were determined by sulfuric acid digestion followed by inductively coupled plasma spectrometry, as insufficient free light material was available in the fractionated samples to perform alkaline oxidation.

Total P was measured in the litter and root samples by lithium fusion in the Stable Isotope Laboratory at Texas A&M University. Two hundred mg of pulverized litter or root material was combined with 750 mg of lithium metaborate in a graphite crucible heated to 1000 ºC in a muffle furnace. The white-hot flux was poured into 50 ml of 10% HNO_3 and stirred to dissolve for two hours (Lajtha et al., 1999). The cooled mixture was diluted to 100 ml with water and analyzed by phosphomolybdate colorimetry on a

Spectronic 20D+ (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 720 nm (Murphy and Riley, 1962; Dick and Tabatabai, 1977).

3.8. Statistical analyses

Linear regression was used to determine relationships between C, N, and P concentrations in soil fractions and woody plant stand age. ANOVA was used to test for differences between soil physical fraction distribution, soil nutrient ratios, and soil nutrient concentrations in grasslands vs. woody landscape elements using JMP software (SAS Institute Inc., Cary, NC, USA). Significance level was $p<0.05$.

4. Results

4.1. Soil physical and chemical characteristics

Soil pH was not significantly different between remnant grasslands and clusters in the upper 15 cm of the soil profile (Table 1). Soils beneath grasslands and clusters were loamy sands approximately 80% sand, 10% silt, and 10% clay. Bulk density of the 0-10 cm depth interval decreased significantly from 1.2 g cm^{-3} in grasslands to 0.9 g cm^{-3} in clusters.

4.2. Distribution of soil fractions by mass

The distribution of soil fractions from wet sieving increased linearly as a proportion of whole soil mass in the order free light fraction < free silt and clay < macroaggregates < microaggregates (Figure 2). The relative proportions of the free light fraction and macroaggregates increased with time following commencement of woody plant encroachment in the upper 10 cm of the soil profile. The free light fraction (density < 1.0 g cm^{-3}) increased from < 1% of whole soil weight to 4.5% of whole-soil weight in the oldest clusters (>80 yr). The macroaggregate fraction (>250 um) in the upper 10 cm increased from <10% of whole soil weight in grasslands to >30% of whole soil weight in the oldest clusters. The relative proportions of microaggregates (53-250 um) and free

Table 1
Soil physical and chemical characteristics of surface soil of grasslands and clusters at the La Copita Research Area.

Characteristic	Landscape Element	
	Grassland	Cluster
pH	6.5 (0.1)	6.4 (0.1)
Texture	Loamy sand	Loamy sand
Sand (mg kg^{-1} soil)	805 (4)	813 (5)
Silt (mg kg^{-1} soil)	105 (20)	96 (16)
Clay (mg kg^{-1} soil)	90 (20)	91 (16)
Bulk Density (g cm^{-3})	1.2 (0.01)	0.9 (0.02)

Standard errors of the mean are in parentheses. Data for pH and texture from Liao et al., 2006a, 0-15 cm. Bulk density measured for 0-10 cm.

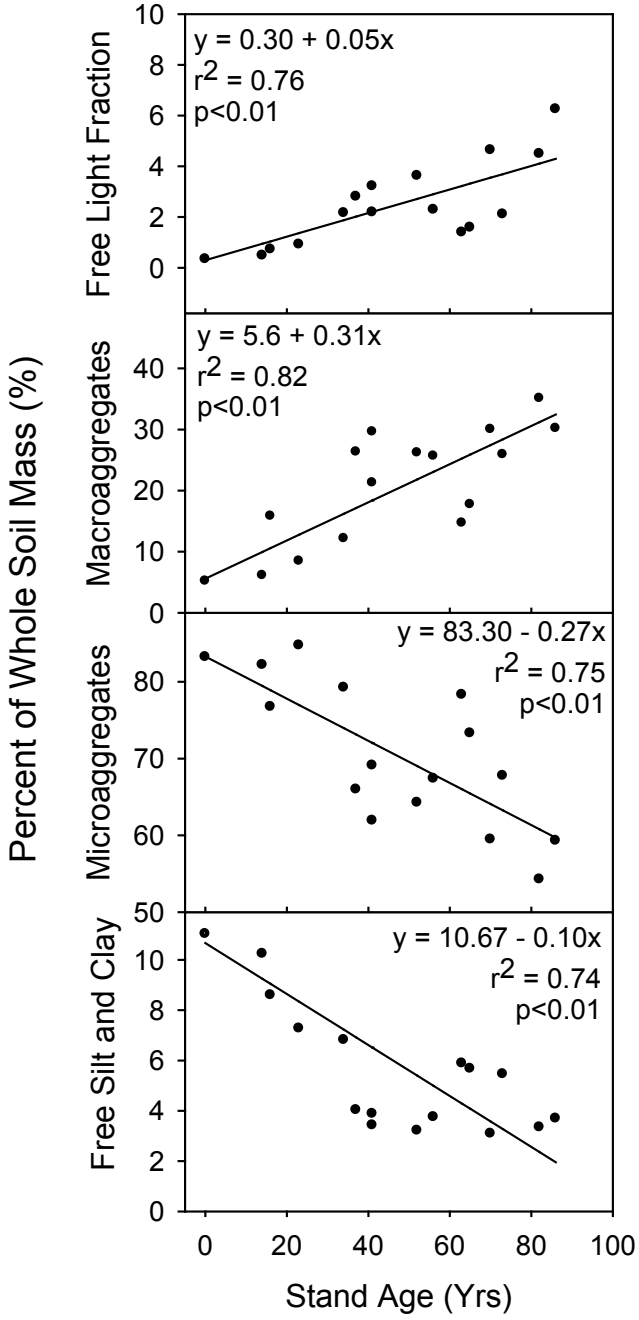

Figure 2. Changes in distribution of soil physical fractions with cluster age, as a percentage of whole soil mass (0-10 cm) at the La Copita Research Area. Time zero values are representative of the average remnant grassland value.

silt and clay (< 53 um) decreased linearly with time after woody plant encroachment. Microaggregates decreased from 80% to 60% of whole-soil weight from grasslands to >80-yr clusters. Free silt and clay proportions decreased from 11% of whole-soil weight in grasslands to 3% of whole-soil weight in the oldest clusters.

4.3. Carbon, nitrogen, and phosphorus concentrations in litter and roots

Concentrations of C, N, and P increased with time in plant litter samples. Carbon concentration in litter increased 1000%, from 36.8 g m^{-2} in grasslands to 375.0 g m^{-2} in the oldest clusters (>80 yr) (Figure 3). Nitrogen concentration in litter increased from 0.81 g m^{-2} in grasslands to 17.1 g m^{-2} in the oldest clusters. Phosphorus increased from 14.4 mg m^{-2} in remnant grasslands to 130.6 mg m^{-2} in clusters >80 yr.

Carbon concentration in roots collected from the upper 10 cm of the soil cores showed no significant difference between the average grassland C concentration and the concentration of the oldest clusters, but within the clusters, the concentration of C in roots increased from an average of 60.0 g m^{-2} in clusters < 25 years to 374.9 g m^{-2} in clusters > 80 years. Nitrogen concentrations in roots increased from 9.4 g m^{-2} in remnant grasslands to 38.6 g m^{-2} in the oldest clusters. Root P concentrations increased from 59.7 mg m^{-2} in grasslands to 236.0 mg m^{-2} in the oldest clusters.

4.4. Carbon, nitrogen, and phosphorus concentrations in whole soil and soil fractions

Soil organic C, soil total N, and total digested P (0-10 cm) increased approximately 3-, 3-, and 2-fold respectively along the chronosequence following the commencement of woody plant encroachment in whole soil. The distribution pattern of nutrients was the same for C, N, and P in the grasslands: the largest pool of each nutrient was in the microaggregates, followed by free silt and clay, macroaggregates, and free light fraction. In the clusters, the distribution was more variable. For C and N pools in the clusters, the macroaggregate and microaggregate pools were not significantly different in size, while the microaggregate P pool was significantly larger than the macroaggregate P. In addition, cluster C and N pools in the free light fraction were significantly larger than the

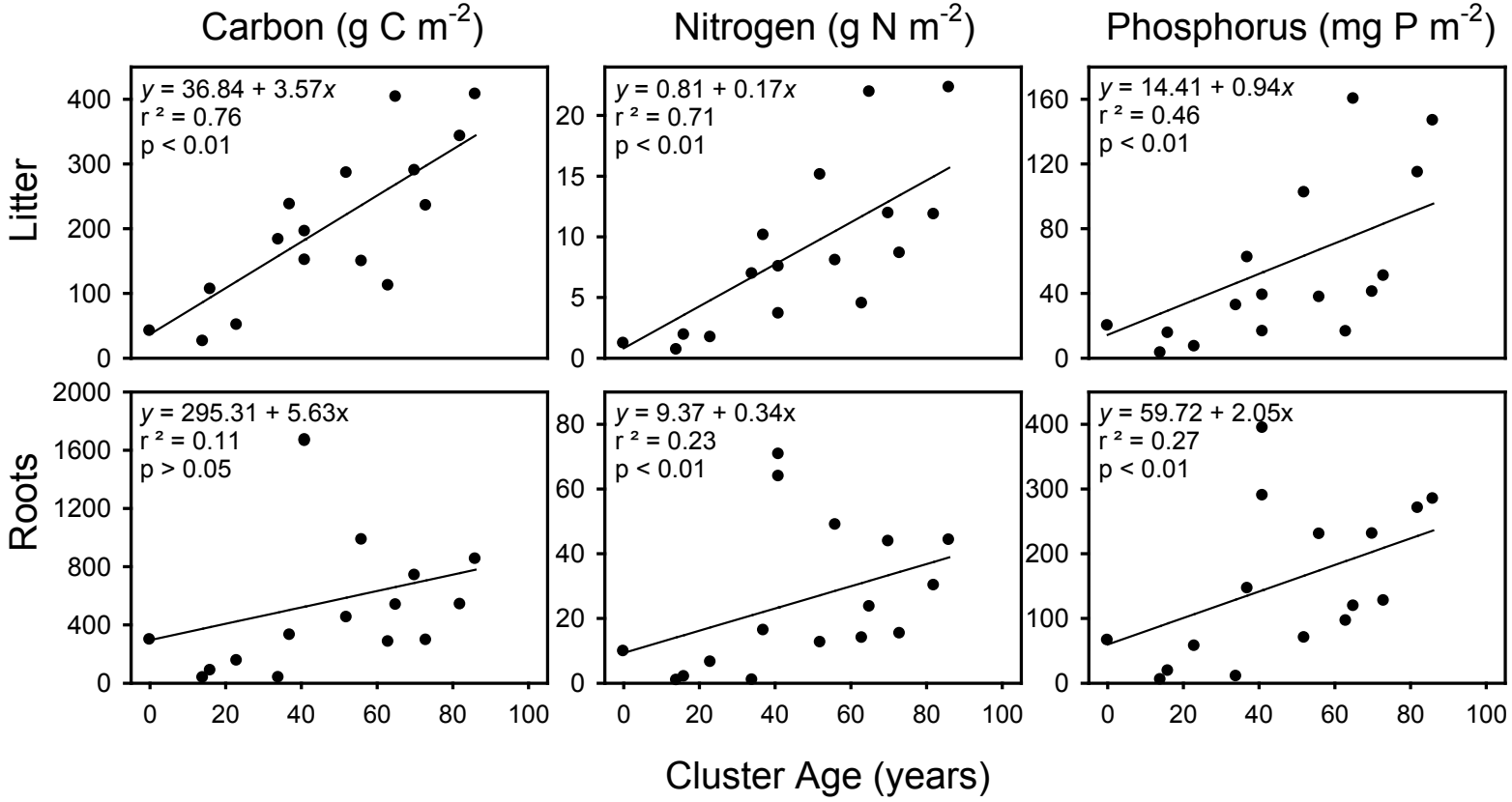

Figure 3. Carbon (g C m^{-2}), nitrogen (g N m^{-2}), and phosphorus (mg P m^{-2}) in roots (0-10 cm) and surface litter (0.25 m^2) relative to woody plant stand age at the La Copita Research Area in southern Texas. Time zero values are mean remnant grassland values.

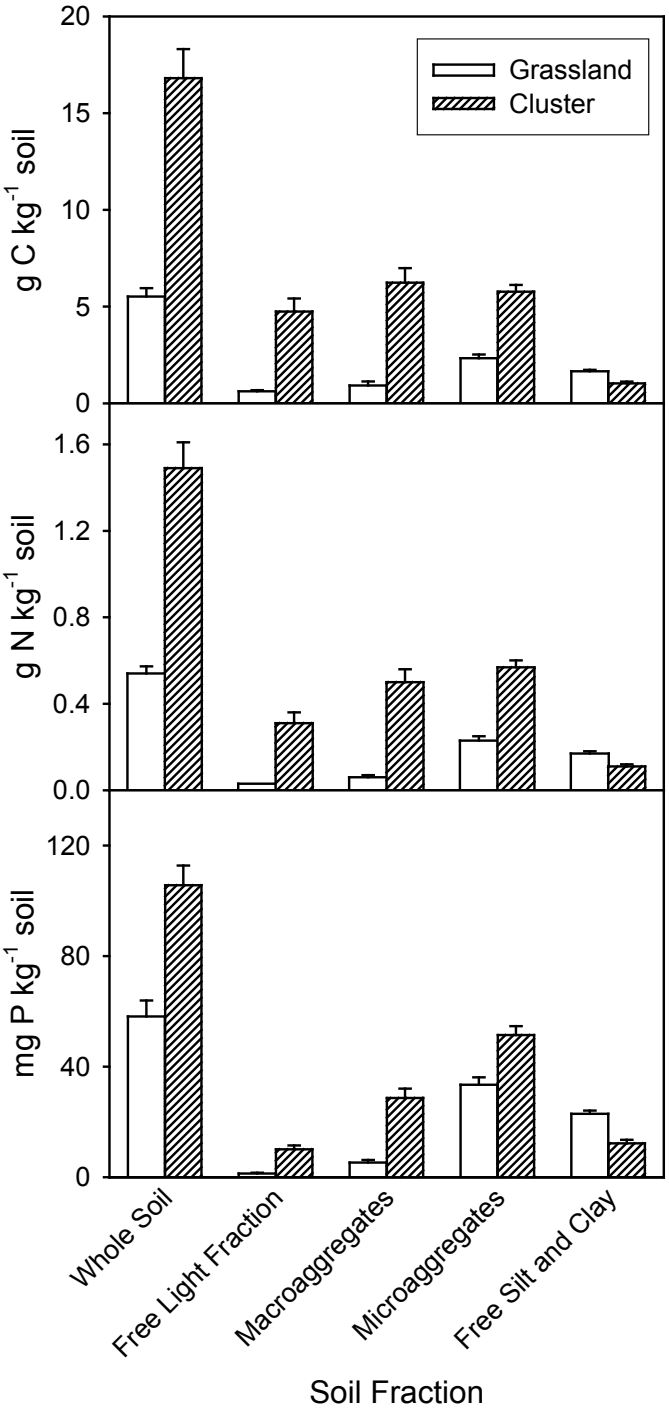

Figure 4. Average carbon (g C kg^{-1} soil), nitrogen (g N kg^{-1} soil) and phosphorus (mg P kg^{-1} soil) in whole soils and soil physical fractions for grasslands and clusters (0-10 cm). Error bars are standard errors of the mean.

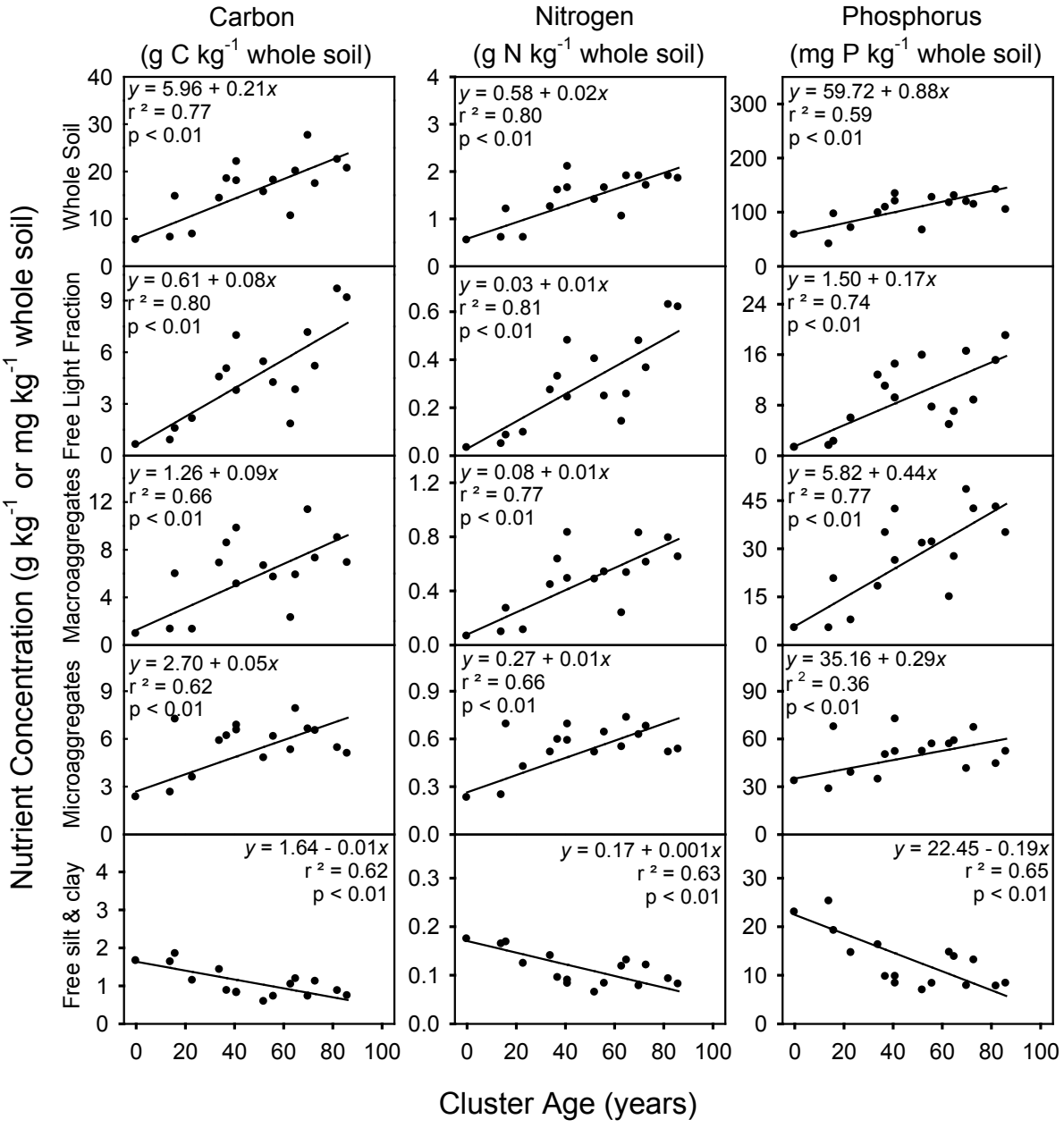

Figure 5. Changes in concentration of carbon (g C kg^{-1} soil), nitrogen (g N kg^{-1} soil), and phosphorus (mg P kg^{-1} soil) with cluster age in whole soil and soil physical fractions (0-10 cm). Time zero values are mean remnant grassland values.

free silt and clay pools, in contrast with P, where the two pools were not significantly different in size (Figure 4). Carbon concentrations of all fractions isolated from wet sieving, except free silt and clay, increased linearly with age of woody cluster (Figure 5). The greatest increases of C were in macroaggregates. Macroaggregate C increased from 1.3 g C kg^{-1} whole soil in grasslands to 9.0 g C kg^{-1} whole soil in the >80-yr clusters. Carbon associated with the free silt and clay fraction increased at a similar rate, from approximately 0.6 g C kg^{-1} whole soil in remnant grasslands to 7.5 g C kg^{-1} whole soil in the oldest clusters. Carbon concentrations in microaggregates increased from 2.7 g C kg^{-1} whole soil in grasslands to 7.0 g C kg^{-1} whole soil in clusters. Free silt and clay C concentrations decreased following commencement of woody plant encroachment, from 1.6 g C kg^{-1} whole soil in remnant grasslands to 0.8 g C kg^{-1} in the oldest clusters (Figure 5).

Changes in N paralleled those of C, with the greatest increase in N in the macroaggregates, which increased from >0.08 g N kg^{-1} whole soil in remnant grasslands to 0.8 g N kg^{-1} whole soil in the oldest clusters (Figure 5). Nitrogen concentrations in free light fraction increased from 0.03 g N kg^{-1} whole soil in grasslands to 0.5 g N kg^{-1} in >80-yr clusters, and concentrations in microaggregates increased from 0.3 g N kg^{-1} whole soil in grasslands to 0.7 g N kg^{-1} whole soil in clusters. In free silt and clay, N decreased from 0.2 g N kg^{-1} whole soil in remnant grasslands to 0.08 g N kg^{-1} whole soil in the oldest clusters.

Changes in P reflected the same pattern as C and N, with the greatest change observed in the macroaggregate fraction. Total digested P in macroaggregates increased from 5.8 mg P kg^{-1} whole soil in remnant grasslands to 44.0 mg P kg^{-1} whole soil in the oldest clusters. Free light fraction P concentrations increased from 1.5 mg P kg^{-1} whole soil to 16.0 mg P kg^{-1} whole soil with woody plant encroachment. In the microaggregates, P increased from 35.0 mg P kg^{-1} whole soil in grasslands to 60.0 mg P kg^{-1} whole soil in clusters > 80 years. Free silt and clay P decreased from 23.0 mg P kg^{-1} whole soil in grasslands to 6.0 mg P kg^{-1} whole soil in the oldest clusters.

4.5. Nutrient accumulation rates

Concentrations of C, N, and P (g kg^{-1} whole soil-basis) were converted to C, N, and P stocks (g m^{-2} basis) using soil bulk densities. Linear regressions fit to the data provided the accumulation rates reported in Table 2. Rates of whole-soil C, N, and P accumulation in the upper 10 cm of the profile averaged 16.0 g C m^{-2} yr^{-1}, 1.3 g N m^{-2} yr^{-1}, and 0.05 g P m^{-2} yr^{-1}, respectively (Table 2). The highest rates of accrual occurred in the macroaggregate fraction for all nutrients. Macroaggregates and free light fraction each contributed 40-60% of C and N accrual in whole soil, while macroaggregates alone were responsible for > 80% of P accrual. The microaggregate and free silt and clay fractions contributed little (<10%) to the overall accumulation rates for C and N, and just over 20% to the rate of P accumulation.

4.6. Unprotected vs. aggregate-protected nutrients

Absolute concentrations of both protected and unprotected C, N, and P were higher in wooded clusters than in remnant grasslands for both young (< 50 yr) and old (> 50 yr) clusters (Figure 6). Relative proportions of protected C (macroaggregate + microaggregate + free silt and clay-associated C) were not significantly different in grasslands than in young clusters at 88% and 87%, respectively. In clusters > 50 yr, relative proportions of protected C were significantly lower than those of grasslands (88%) at 73% due to increasing size of the unprotected fraction. Relative proportions of protected nitrogen were not significantly different in grasslands and young clusters (86 and 87%, respectively), but were significantly different between grasslands and old clusters (77%). Relative proportions of protected phosphorus were significantly different in both young and old clusters. Protected phosphorus accounted for 98% of total phosphorus in grasslands, 92% of young clusters and 89% of old clusters.

4.7. C:N:P ratios of whole soil and soil fractions

Nutrient ratios were compared using ANOVA. C:N ratios of whole soils and soil fractions were significantly lower in grasslands than in clusters for whole soil, and

Table 2
Soil organic carbon, total nitrogen, and total phosphorus accumulation rates from linear correlations between cluster ages and nutrient stocks of soil fractions in woody clusters at the La Copita Research Area.

	Nutrient accumulation rates (g m^{-2} yr^{-1})		
	Soil Organic Carbon	Total Nitrogen	Total Phosphorus
	0-10 cm	0-10 cm	0-10 cm
Whole soil	16.0 (2.55)	1.31 (0.18)	0.05 (0.003)
Free Light Fraction Density <1.0 g cm^{-2}	7.01 (0.78)	0.49 (0.06)	0.01 (0.003)
Macroaggregates	7.77 (1.90)	0.69 (0.12)	0.05 (0.01)
Microaggregates	3.82 (1.20)	-0.02 (0.06)	0.01 (0.01)
Free Silt and Clay	-1.72 (0.26)	-0.17 (0.03)	-0.03 (0.01)

Numbers in parentheses are the standard errors of the estimates.

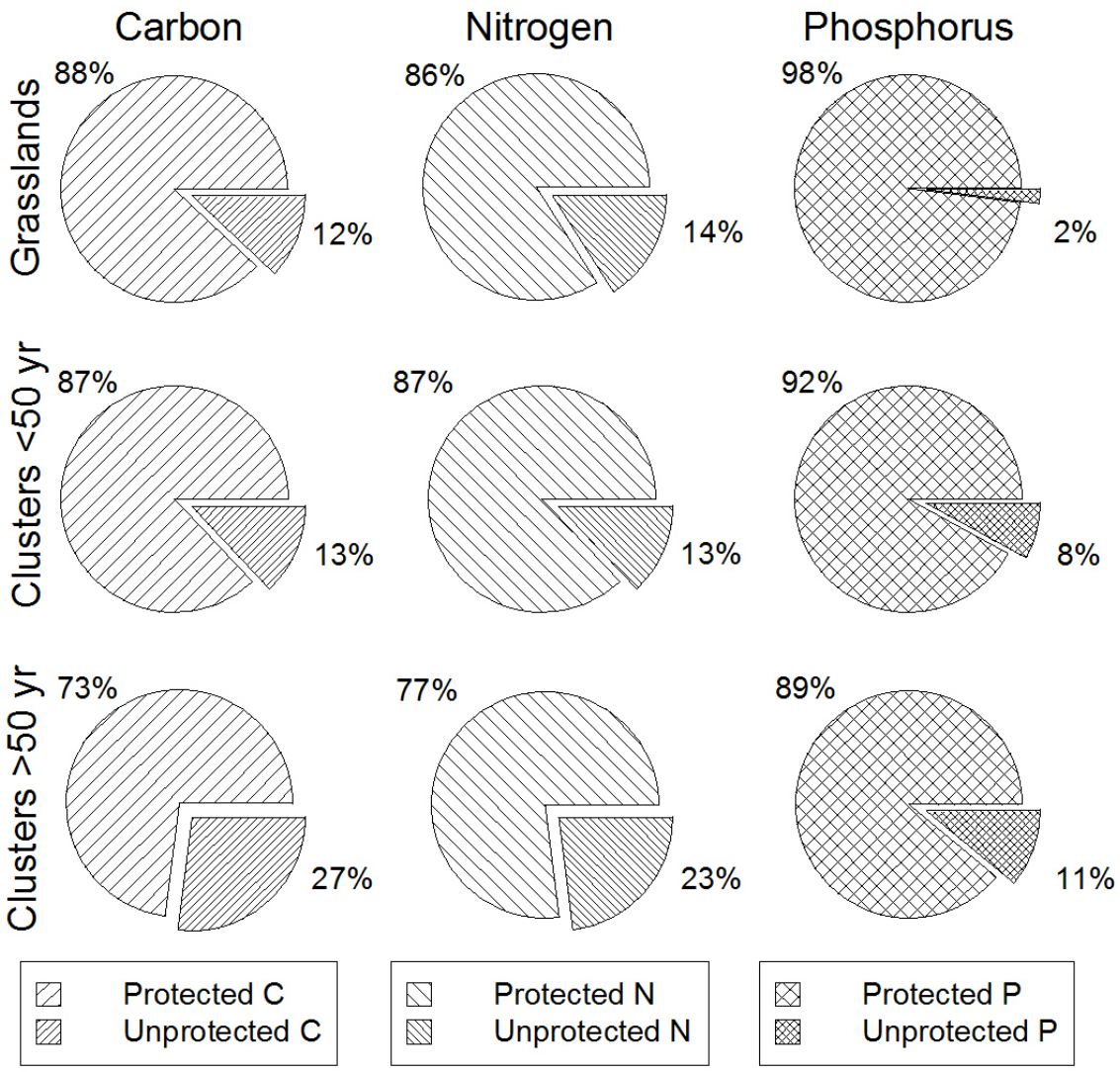

Figure 6. Comparison of proportions of unprotected (free light fraction) vs. physically protected (within macroaggregates, microaggregates, and free silt and clay) carbon, nitrogen, and phosphorus in the upper 10 cm of the soil profile in grasslands and young (< 50 yrs) and old (>50 yrs) clusters at the La Copita Research Area.

significantly higher in free light fraction (Figure 7). C:P ratios were significantly lower in grasslands than in whole soil, macroaggregates, microaggregates, and free silt and clay. N:P ratios were significantly lower in grasslands than in clusters for whole soil and all fractions except free light fraction.

5. Discussion

5.1 Soil organic matter following woody plant encroachment

Woody plant encroachment has increased over the last century and has increased C, N, and P concentrations by 180-305% in the soils of south Texas. These increases are observed in the roots and litter of the woody plants as well as in the soils beneath the invading species. The increases in nutrients are likely due to the higher rates of organic matter input from increased NPP, increased root biomass, and increased litter production in the woody clusters compared to remnant grasslands. Aboveground NPP for La Copita has been measured at 150-320% of the NPP of remnant grasslands (Archer et al., 2001), and though belowground productivity was not measured for this experiment, the comparatively large root biomass produced by woody plants on this site indicates increased belowground NPP as well (Boutton et al., 1999; Hibbard et al., 2001). Greater masses of roots were recovered for elemental analysis from clusters than from grasslands, indicating that in the upper 10 cm the root biomass produced by woody plants exceeds that of grassland plants. The average mass of litter collected beneath woody plants was 600% of the average grassland litter (data not shown), indicating that the increase in total C, N, and P in the upper 10 cm of the soil can be attributed to an increase in the organic matter in woody clusters.

5.2 Sources of soil C, N and P in the system

The accumulation of nutrients in wooded areas is owed in part to the quality of the organic matter produced: the woody plant litter tends to be more recalcitrant than grassland litter. Woody plant material contains more lignin, tannins, and secondary aliphatic compounds that resist decomposition than grassland litter (Köchy and Wilson,

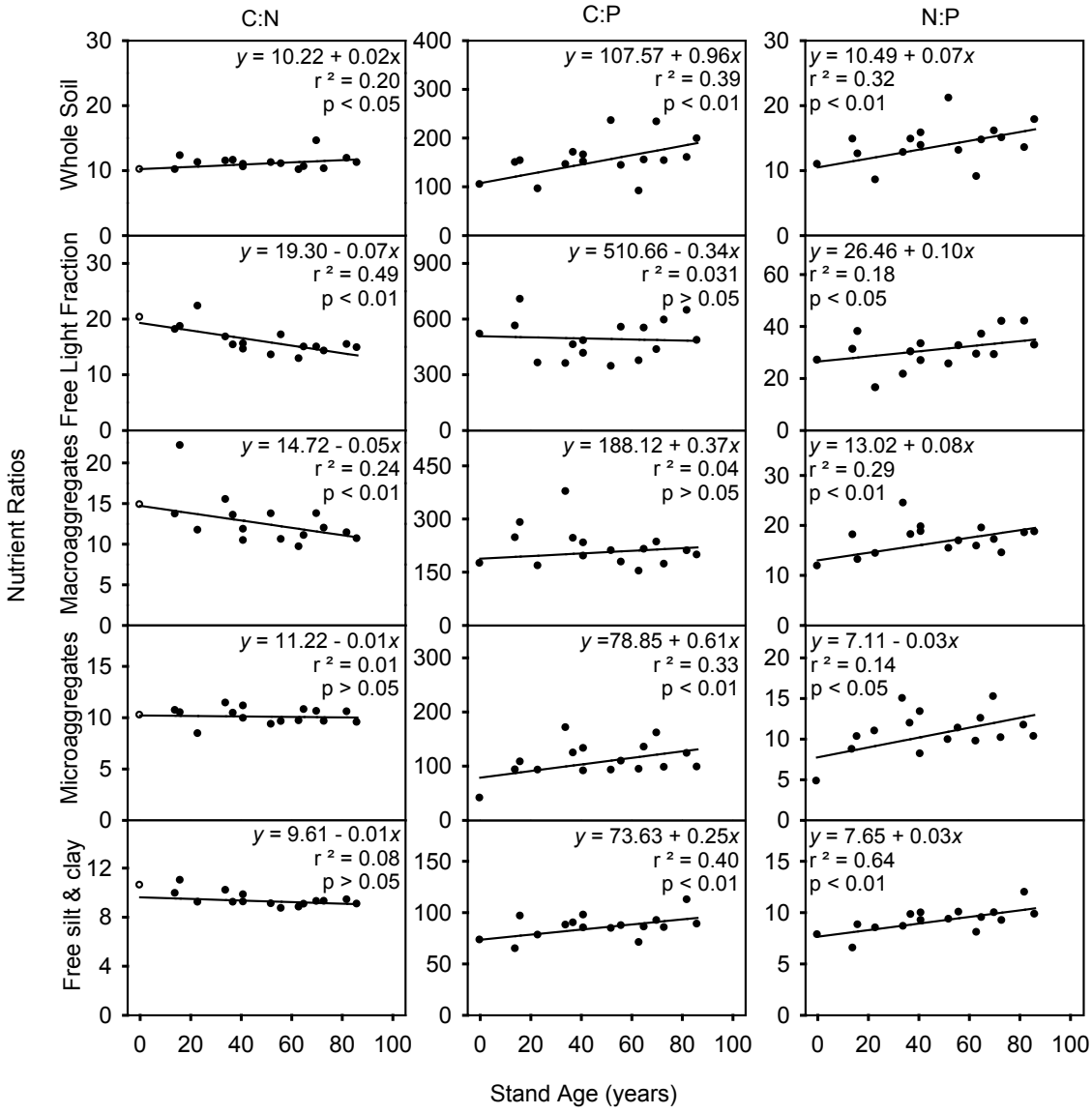

Figure 7. C:N, C:P, and N:P ratios with respect to cluster age for whole soil and soil physical fractions in clusters and grasslands at the La Copita Research Area.

1997; Filley et al., 2008). Soil respiration rates (McCulley et al., 2004) and long-term incubation studies (Boutton et al., 2002) have demonstrated that soil organic carbon turnover is slower in clusters than in grasslands. The pattern of nutrient increase in litter and roots indicate that C, N and P are being incorporated into the organic matter of the woody plants before entering the soil organic matter. The linear increases in root and litter nutrient (Figure 3) shows inputs to soil nutrient pools from organic matter inputs in the soil continue to increase with time following woody encroachment. Carbon in the soils is increasing at an average rate of 17.7 g m^{-2} yr^{-1}, and linear trends of C concentration over time indicate that the rate is essentially steady.

The presence of tree legumes among the encroaching woody plants is likely to be a significant factor in the accumulation of nutrients following woody plant encroachment. Previous research has shown that the presence of N-fixing woody species has more effect on the biogeochemical cycling of invaded ecosystems than woody plants alone (Liao et al., 2008). Plants capable of fixing N contribute to overall N concentrations in the soil, as well as avoiding N limitation that could retard the rates of accumulation of C and P in the plants and subsequently in the soils. Rates of N fixation in similar mesquite-invaded grasslands have ranged from 4-15 g N m^{-2} yr^{-1} (Rundel et al., 1982; Johnson and Mayeux, 1990). In addition to this input of atmospheric nitrogen to the system, previous studies have shown that net N-mineralization is increased in the woody clusters, indicating that the N cycle on the site is being altered by woody plant presence (Hibbard et al., 2001; McCulley et al., 2004; Liao and Boutton, 2008). Along the 86-year chronosequence, N is increasing in whole soils at a rate of 1.4 g m^{-2} yr^{-2}, similar to rates reported by Hibbard et al. (2001).

Phosphorus is an essential component of plant tissue and soil nutrition, and is likewise affected by woody plant encroachment. Phosphorus limitation has historically been associated with aquatic ecosystems while N was considered the limiting nutrient for terrestrial ecosystems (eg., Smith, 1984; Vitousek and Howarth, 1991; Vitousek et al. 2002). However, research has shown that P plays a critical role in the energy-intensive processes of plant metabolism, namely photosynthesis and N-fixation, creating

feedbacks between the N and P cycles (Robson et al., 1981; Chapin et al., 2002; Schulze, 2004; Turner et al., 2005; Allison et al., 2006; Elser et al., 2007; Wang, Y.-P. et al., 2007; Vitousek et al., 2010; Ceulemans et al., 2011). Due to the high P demands of N-fixation, woody encroachment by N-fixing species are of special interest: while P is necessary to meet the energy needs of N fixation, N is required for plants to produce phosphatase, the enzyme responsible for liberating P from the soil for plant uptake (Raghothama and Kartikeyan, 2005; Vitousek et al., 2010; Finzi et al., 2011). In systems where woody plant encroachment has led to a rapid change in the abundance and availability of C and N, P may become the limiting nutrient as biotic requirements exceed the rate of P weathering from minerals in the soil (Walker and Syers, 1976; Chapin et al., 2002; Vitousek et al., 2010).

While C and N are obtained from the atmosphere, P has no stable gaseous form, and plant P acquisition is therefore limited to terrestrial sources. The source of the increased P in woody-encroached soils is likely deep mining by mesquite roots, which can exceed the depth of the grasses they replace on the site (Jobbagy and Jackson, 2001; Porder and Chadwick, 2009). Phosphorus deposition as wind-borne dust and particulate matter is also a source of P, but deposition in Texas occurs at less than 2% of the rate of P increase observed in the La Copita soils (Mahowald et al., 2008). Animals may introduce P through waste, however the linear increases in P do not demonstrate the 10-15 year lag that is expected as the mesquite grows large enough to produce shade or to support bird roosts. The rhizosphere of woody shrubs is much larger than that of grass species, allowing plants to access P deeper in the soil profile (Canadell et al., 1996; Jackson et al., 1996, Boutton et al., 1998), as well as increasing nutrient concentrations in the deeper soil due to root turnover. Phosphatase enzymes produced by mesquite roots may liberate P from a larger volume of soil than the grasses were capable of accessing.

5.3 Physical protection of nutrients

Physical protection of organic matter in the soil contributes to the storage of nutrients. Macroaggregates are formed from microaggregates and free silt and clay which are bound together by organic matter, roots, fungal hyphae, or exudates from roots and microbes. Some of these binding materials may be more labile than the components which they adhere to, but the overall result of macroaggregate formation is the protection of organic matter from microbial degradation within the aggregate structure (Tisdall and Oades, 1982; Oades and Waters, 1991). During woody plant encroachment, the proportions of microaggregates and free silt and clay decrease in the La Copita soils while macroaggregates and free light fraction increase. While macroaggregates represent a protected, slow-turnover aggregate pool, the free light fraction is unprotected, and increases in these pools together contribute to an overall increase in soil P. The capacity of soils to incorporate organic matter is finite (Tisdall and Oades, 1982), but the linear increases and decreases in soil physical fraction percentages for clusters indicate that organic matter saturation of the landscape has not yet occurred, and the effects of woody encroachment on soil aggregation are still advancing.

The concentrations of C, N, and P in the soils at La Copita increased in all soil fractions except free silt and clay following woody plant encroachment (Figure 5). While nutrient concentrations increased in both protected and unprotected portions of the soil organic matter pool (Figure 5), the proportion of C, N, and P in the protected pool decreased with woody plant encroachment. This change was more dramatic when comparing older clusters to grasslands (Figure 6), due to the advancing nature of the effects of woody encroachment, but in both young (<50 years) and old (>50 years) clusters this represented a 160-225% increase in C and N, and a 550% increase in P compared to the grasslands.

In other shrub/grass systems, woody plant litter with high C:N ratios contributed to slower turnover of organic matter under shrubs than under grasses (Köchy and Wilson, 1997). However, no significant change occurred in the C:N ratios of microaggregates

and free silt and clay with the arrival of woody species at La Copita, and the C:N ratios of the free light fraction and macroaggregates both decreased with woody plant encroachment (Figure 7). As opposed to a decrease in C concentrations, this indicates an increase in the N concentration of the organic matter entering the soil. This result reflects the inputs from N-fixing legumes on the site, and points to secondary compounds rather than a high C:N ratio in the woody litter as the source of slow-turnover organic matter on the site. The N:P ratios increased in all soil fractions, affirming that nitrogen is accumulating in the soil fractions more quickly than phosphorus. As N and P are generally the limiting nutrients in terrestrial systems (Walker and Syres, 1976; Koerselman and Meuleman, 1996; Elser et al., 2007; Vitousek et al., 2010), the rate of nitrogen accumulation indicates that in these legume-invaded soils phosphorus is likely to replace nitrogen as the primary limiting nutrient. As both N and P continue to increase in a linear fashion in the soils, neither the phosphorus limitation on nitrogen fixation nor the nitrogen limitation on phosphatase enzyme production seems to be limiting nutrient acquisition at this point.

While the accumulation of C and N in the soils is fairly evenly divided between increases in the macroaggregates and in the free light fraction, the increase in soil P is found primarily in the macroaggregate fraction (Table 2). This P is being stored in slower turnover soil fractions, and the majority of P in the upper 10 cm of the soil is more stable than C and N, where approximately half of the nutrients are found in quick-turnover fractions. This may also indicate that the forms of P found in plant litter following the initial uptake of P from the deep soil are strongly divided between plant-available and recalcitrant forms. Unlike C and N, which can be lost to the atmosphere from the soil, P does not have a gaseous form that is produced during OM decomposition. Primary mechanism for P loss from the shallow soils is erosion of the surface soil by wind and water, and the added protection of inter-aggregate storage may help conserve P in the shallow soil after relocation from deeper in the soil profile.

6. Conclusions

Woody plant encroachment into grasslands of the Rio Grande Plains of Texas during the last century has led to increases of 180-305% in C, N, and P storage in the upper 10 cm of the soil profile. These changes in nutrient stores are likely due to: (i) higher rates of above- and belowground primary productivity in the wooded areas compared to grasslands, (ii) more recalcitrant organic matter produced by woody plants, (iii) the physical protection of organic matter within soil aggregates, and (iv) uplift of N and P by the more deeply rooted woody species. Like C and N, P is being stored in both fast-cycling and slow-cycling soil pools, as the pool of unincorporated organic matter, the free light fraction, is increasing along with macroaggregates. In the C and N pool, the free light fraction accounted for roughly half of the nutrient accumulation, while the P pool heavily favored the macroaggregate fraction.

Phosphorus accumulation under woody plant encroachment coincides with C and N accumulation, though the mechanism of acquisition differs. Unlike C and N which are freely available to plants from the atmosphere, P is mined by plant roots from deeper in the soil. While C and N will remain available in the atmosphere at concentrations in excess of what the plants require, the extent of the rhizosphere of the woody plants will determine the volume of soil the plants can mine for P. This source is therefore finite, and may lead to P limitation in the system as woody plant encroachment advances, but at this time there is no evidence that the rate of P increase in the shallow soil is slowing on this site. These woody plant-encroached soils are acting as a sink for atmospheric C, are storing N, and are redistributing soil P from deep soil to shallow soils where it can be used by vegetation. The combination of these biogeochemical shifts has the potential to be significant world-wide due to the global extent of woody plant encroachment on Earth's grasslands.

CHAPTER III

CHANGES IN SOIL PHOSPHORUS FRACTIONS FOLLOWING WOODY PLANT INVASION OF GRASSLAND

1. Synopsis

Many grass-dominated ecosystems around the world have experienced woody plant encroachment over the last century due to livestock grazing, fire suppression, and/or changes in climate and atmospheric chemistry. In the Rio Grande Plains of Texas, subtropical thorn woodlands dominated by N-fixing tree legumes have largely replaced grasslands and altered the biogeochemistry of this region. The purpose of this study was to assess the impact of this grassland-to-woodland transition on the size, distribution, and availability of soil P pools. A modified Hedley method was employed to fractionate soil P into pools based on organic and inorganic forms and relative plant-availability. Soil samples (0-10 cm) were collected in remnant grasslands and near the centers of woody plant clusters ranging in age from 14 to 86 yrs in a subtropical savanna parkland in southern Texas. Soil P was fractionated into resin-extractable inorganic P, bicarbonate-extractable organic and inorganic P, hydroxide-extractable organic and inorganic P, acid-extractable inorganic P, and residual inorganic P forms. Phosphorus concentrations in these fractions were determined by colorimetry, and soil total P was determined by lithium fusion. Organic P was calculated from the difference between total and inorganic P. Total P in whole soils increased dramatically from 98 mg P kg^{-1} soil in remnant grasslands to 168 mg P kg^{-1} soil in the oldest woody plant stands (70-85 yrs). Phosphorus concentrations of all pools increased linearly with increasing woody plant stand age except acid-extractable phosphorus. The most dramatic increases were observed in the resin-extractable fraction (plant-available P), which increased from 3 to 13 mg P kg^{-1} soil, and in hydroxide-extractable P (the majority of the organic P in the system), which increased from 15 mg P kg^{-1} soil in grasslands to 26 mg P kg^{-1} soil in the wooded clusters. Although the exact mechanisms by which soil P increases following woody invasion remain unknown, we suggest that the more deeply rooted woody plants

are acquiring P from deep in the soil profile and transferring it into the upper portion of the profile via litterfall and root turnover. Because P is a limiting nutrient in many terrestrial systems, increases in its availability could alter rates of biogeochemical processes, affect species interactions, and influence the future trajectory of woody invasion in this region.

2. Introduction

The invasion of grass-dominated ecosystems around the world by woody vegetation has been among the most striking and geographically widespread land cover changes during the past century (Van Auken, 2000, 2009; Knapp et al., 2008; Maestre et al., 2009; Barger et al., 2011; Eldridge et al., 2011). Woody plant cover has increased in grasslands of North America, South America, Africa, Europe, and Asia (Archer et al., 1988; Hudak and Wessman, 1998; Briggs et al., 2005; Allison et al., 2006; Knapp et al., 2008; Lorenzo et al., 2010), converting these landscapes to savannas and woodlands. It has been hypothesized that increased woody plant abundance in grasslands is driven by livestock grazing (Roques et al., 2001), reduced fire frequency (Scholes and Archer 1997), changes in atmospheric N-deposition (Köchy and Wilson, 2001), rising atmospheric CO_2 concentration (Polley et al., 1994; Bond et al., 2003), and climate change (Knapp et al., 2008).

Encroachment by woody plants increases aboveground NPP, thereby increasing the capacity for site vegetation to sequester atmospheric C and N in plant tissues (Hibbard et al., 2001; Köchy and Wilson, 2000; Wheeler et al., 2007). Woody plant litter has been shown to be higher in recalcitrant secondary chemical compounds which resist degradation and preserve organic matter in the soil (Köchy and Wilson, 1997; Hibbard et al., 2001; McCulley et al., 2004; Liao et al, 2006b; Springsteen et al., 2009). Increased organic inputs lead to increased soil aggregation, further protecting organic matter from microbial decomposition (Chapter II; Six et al., 2000; Liao et al., 2006a). This results in alteration of the soil nutrient landscape as the vegetation landscape shifts, increasing both C and N storage and availability in soils (Padien and Lajtha, 1992; Hibbard et al.,

2001; McCulley et al., 2004; Liao et al., 2006b; Wheeler et al., 2007; Knapp et al., 2008; Liao et al. 2008; Springsteen et al., 2009; Scharenbroch et al., 2010; Eldridge et al., 2011; Barger et al., 2011). The presence of woody plants serves to establish islands of fertility, where nutrients accumulate beneath the shrub canopy (Schlesinger et al., 1990; Padien and Lajtha, 1992; Hibbard et al., 2001).

Phosphorus is a critical component of plant tissue and soil biogeochemistry. While P has long been associated with nutrient limitation in aquatic environments, it is becoming clear that P has potential to limit a wide range of terrestrial environments as much or more than N (Vitousek and Howarth, 1991: Vitousek et al., 2002, 2010; Elser et al., 2007). P is essential for plant metabolic processes, particularly photosynthesis and N-fixation, which require large amounts of energy (McGroddy et al., 2004; Schulze, 2004; Wang, Y.-P. et al., 2007, Vitousek et al., 2010.) Unlike C and N, which are available to plants both in the soil and in the atmosphere, plant acquisition of P is restricted to soil P pools, of which up to 90% may be in forms plants cannot take up (Haussling and Marshner, 1989).

Woody encroachment in southern Texas has converted the landscape from subtropical grassland composed of C_4 species to thorn woodland. Increasing abundance of N-fixing *Prosopis glandulosa* (honey mesquite) and associated woody species has resulted in higher rates of above- and below-ground NPP and changed the distribution of C and N in the soil (Boutton et al., 1998, 2009: Archer et al., 2001; Liao et al., 2006a; Boutton and Liao, 2010). Woody clusters support larger pools of microbial biomass and higher soil respiration rates than grassland vegetation (McCulley et al., 2004; Liao and Boutton, 2008). Carbon and N have increased in the soil in both labile and recalcitrant pools due to the influx of N-rich organic matter (Chapter II, Liao et al., 2006a). The increased respiration rates in the soil do not deplete the carbon pool due to the quality of this litter: both foliar litter and root litter inputs from woody species are higher in secondary aliphatic compounds, making them more chemically recalcitrant than the grassland vegetation (Filley et al., 2008).

The N-fixing capacity of mesquite at La Copita is of particular interest when studying the effects of woody plant encroachment on P. When N-fixing vegetation is present, N accumulates in plant tissues through biological fixation and is incorporated into the soil N pool through inputs of N-rich root matter and foliar litter until equilibrium with amounts of P is reached (Vitousek et al., 2002). As N fixation continues, the weathering rate of P in the soil will eventually limit biomass production (Walker and Syers, 1976). Low P soils force plants and the associated microbes to work to acquire P from the environment, investing N that would otherwise be used for biomass production into phosphatase enzymes that decompose organic P compounds in soil organic matter (Chapin et al., 2002; Allison et al., 2006; Wang, Y.-P. et al., 2007; Vitousek et al., 2010; Ceulemans et al., 2011). Woody plants, with different architecture and nutrient requirements than grasses, change the way P is acquired and cycled in the system. Phosphatase liberates phosphorus from deeper in the soil profile due to deeper rooting shrubs (Canadell et al., 1996; Jackson et al., 1996; Boutton et al., 1998), while higher NPP contributes to a larger pool of organic matter turning over in the surface soil (Hibbard et al, 2001; Liao et al., 2006a). This behavior may eventually deplete the soil P in the vicinity of the plant roots, creating pressure on the microbial community to increase the decomposition of organic matter and the weathering of phosphate minerals to meet their own nutrient needs (Gahoonia et al., 1994; Schachtman et al., 1998).

Investigations into the effects of woody species on P cycles in the soil have shown that shrubs can concentrate nutrients, including inorganic and organic P, under their canopies (Padien and Lajtha, 1992; Schlesinger and Pilmanis, 1998; Hibbard et al., 2001; Dossa et al., 2010; Lorenzo et al., 2010). Potential mechanisms of P accrual include litter production and turnover, mining from deep soil, atmospheric interception, and inputs from excrement of birds or other animals that may roost in the mesquite or under the canopy. In studies of tropical N-fixing invasive shrubs in Hawaii, available P concentrations increased 2 to 28-fold under shrub canopies, along with significant increases in phosphatase activities that suggest accelerated P mineralization rates (Allison et al., 2006). Similarly, studies of invasive *Acacia* species showed that

exchangeable P content increased under the shrub canopies compared to the inter-canopy spaces (Lorenzo et al., 2010).

Total phosphorus measurements in soil are a poor indicator of plant-available P because many of the chemical forms of P present in soil are relatively insoluble and therefore unavailable for plant uptake. In some systems, upwards of 90% of soil P may be in organic forms, which are inaccessible to most plants (Haussling and Marshner, 1989). By using increasingly aggressive extraction solutions, estimates have been made of the relative availability of portions of the P pool in the soil (Hedley et al., 1982; Tiessen and Moir, 1993; Lajtha, et al., 1999; Dossa et al., 2010). Through sequential extraction of different P fractions, a gradient of P pools ranging from plant-available to relatively recalcitrant can be quantified. Resin-extractable P represents the plant-available phosphate in the soil solution, while bicarbonate extractable P is easily accessible to microbes (Hedley et al., 1982). This fraction is associated with phosphatase activity, making it an indicator of the ability of plants to cycle P (Dossa et al., 2010).

The relationship between P concentration and N fixation, and the potential for P limitation in woody plant-invaded systems make it important to incorporate measurements of P availability into the framework of the La Copita ecosystem, and to understand the dynamics of P in the soil in order to understand the overall effects of woody plant encroachment on soil. Using sequential fractionation of soil phosphorus, this study will i) demonstrate the different soil P pools associated with grassland and shrub-invaded soils and ii) show how the size and distribution of soil phosphorus fractions change along a chronosequence of woody plant invasion.

3. Materials and methods

3.1. Study area

Samples were collected October 2006 at the Texas AgriLife La Copita Research Area (27º 40'N, 98º 12'W) in the Rio Grande Plains of southern Texas, located 65 km west of Corpus Christi. Mean annual temperature is 22.4 ºC and mean annual

precipitation is 716 mm, with the majority of the precipitation falling in May-June and September. Elevation ranges from 75 to 90 m across the site, with nearly level uplands grading (1-3% slopes) into lower-lying drainage woodlands and playas. Sequential aerial photography, tree ring analyses, vegetation dynamic modeling, and isotopic analyses of soils indicate that during the last 150 years vegetation on the site has shifted from open grasslands to mixed grassland/thorn woodland, with woody plant dominance continuing to increase over time (Archer et al., 1988, 2001, 2004; Boutton et al., 1998, 1999; Bai et al, 2009, 2012a,b).

Upland soils are sandy loams (Typic and Pachic Argiustolls) with a subsurface argillic (Bt) horizon. The upland vegetation consists of a grassland matrix dominated by the genera *Bouteloua, Chloris, Panicum, Tridens.* The grasslands include discrete woody clusters dominated by the woody legume *Prosopis glandulosa* Torr. (honey mesquite) and including several other woody species beneath its canopy including *Condalia hookeri* M.C. Johnst., *Zanthoxylum fagara* (L.) Sarg., *Ziziphus obtusifolia* (T.&G.) Gray, and *Berberis trifoliolata.* Shrub clusters often expand and fuse to form larger woody groves in the uplands where the argillic horizon is absent. Grassland-to-woodland conversion has gone to completion on lowland portions of the landscape by similar mechanisms. Additional details on plant communities, soils, and successional dynamics have been presented elsewhere (Scifres and Koerth, 1987; Archer et al., 1988, Boutton et al., 1998).

3.2. Chronosequence approach

In order to evaluate changes in the storage and chemical composition of soil P in response to woody plant encroachment, a space-for-time chronosequence approach was utilized. On this site, the formation of wooded landscape elements is initiated by the establishment of mesquite in grassland (Archer et al., 1988). Thus, the age of a woody plant stand corresponds to the age of the largest mesquite tree in that stand. The ages of mesquite trees were determined by measuring their basal diameters and then substituting those values into regression equations to predict tree ages, using equations developed by

tree ring analysis (Stoker, 1997). Clusters were selected to encompass the full range of mesquite basal diameters, corresponding to tree ages ranging from approximately 15 to 85 yr. Samples from remnant grasslands were considered to be representative of the grassland ecosystems that dominated the region prior to woody plant invasion (Time 0).

3.3. Collection of soil samples

Soil samples were collected from fifteen mesquite clusters and fifteen neighboring remnant C_4 grasslands in the upland portion of the landscape. Four soil cores (5 cm diameter x 10 cm deep) were collected from near the bole of each mesquite tree, and four additional cores were collected around the base of a neighboring grass plant located at least 5 m beyond the dripline of the cluster. Soil cores were stored in plastic bags, transported on ice, and stored at 4 ºC prior to analyses.

3.4. Soil characterization

Wet weights were determined on all soil cores, after which an aliquot of each sample was dried at 105°C to determine moisture and bulk density. The remainder of the soil was passed through an 8-mm sieve to remove large organic matter fragments, dried at 65 ºC, pulverized in a centrifugal mill (Angstrom, Inc., Belleville, MI, USA), and used for subsequent chemical analyses. Soils were analyzed for C and N concentrations using a Carlo Erba EA-1108 elemental analyzer (CE Elantech, Lakewood, NJ, USA).

3.5. Phosphorus fractionation

The chemical forms of P in whole soil were analyzed using a modified Hedley method (Figure 8; Hedley et al., 1982; Tiessen and Moir, 1993; Lajtha et al., 1999; Dossa et al., 2008). A 1-gram sample was placed in a 50-ml centrifuge tube with two 2.5 cm^2 anion exchange membranes and 30 ml deoinized water (AR204-SZRA, Ionics, Watertown, MA) (Cross and Schlesinger, 2001). Samples were shaken for 16 hours at 21 ºC. The anion exchange resin strips were removed and the adsorbed P was eluted by shaking in a separate tube with 30ml of 0.5 M HCl for 4 hours to collect resin-

extractable P. The soil-water solution was centrifuged and the water removed. Bicarbonate-extractable P was extracted by shaking for 16 hours with 30 ml of 0.1 M $NaHCO_3$ in the 50 ml centrifuge tube, centrifugation and collection of the extraction solution. The process was repeated with 30 ml of 0.1 M NaOH and 30 ml of 1.0 M HCl. Following extraction with HCl, the remaining soil was dried and re-pulverized for residual phosphorus analysis. The $NaHCO_3$ and NaOH extracts were divided and a portion was digested with potassium persulfate ($K_2S_2O_8$) to yield total P. All extracts were analyzed for orthophosphate (inorganic P) by colorimetry (Murphy and Riley, 1962) on a Spectronic 20D+ spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 712 nm (Dossa et al. 2010). Organic P was determined by subtracting inorganic P from total P for the digested ($NaHCO_3$ and NaOH) fractions (Lajtha et al., 1999). Total P values calculated from the sum of the individual fractions (Tiessen and Moir, 1993) averaged 25% less than total P values determined by lithium fusion due to limitations of the colorimetry technique in measuring low concentrations of P and loss of material from the residual fraction during drying and re-grinding.

3.6. Total phosphorus

Total P analyses of whole soil and residual soil were performed using the lithium fusion method (Lajtha et al., 1999). Ground, dried, 250-mg samples were mixed with 750 mg of lithium metaborate in graphite crucibles and heated to 1000 ºC in a muffle furnace. The molten flux was poured into 50 ml 10% HNO_3 and stirred to dissolve for two hours. Phosphorus concentration of the HNO_3 solution was determined using a modified molybdenum blue method (Murphy and Riley, 1962; Dick and Tabatabai, 1977; Lajtha et al., 1999). Color was developed by combining the oxidized samples with ammonium molybdate ($(NH_4)_6Mo_7O_{24}$-$4H_2O$, 40g in 1000 ml H_2O) and antimony potassium tartrate ($C_8H_4K_2O_{12}Sb_2$-$3H_2O$, 1.454g in 500 ml H_2O) to produce a blue color, and the concentration of P was measured on a Spectronic 20D+ (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 712 nm (Dick and Tabatabai, 1977; Dossa et al.,

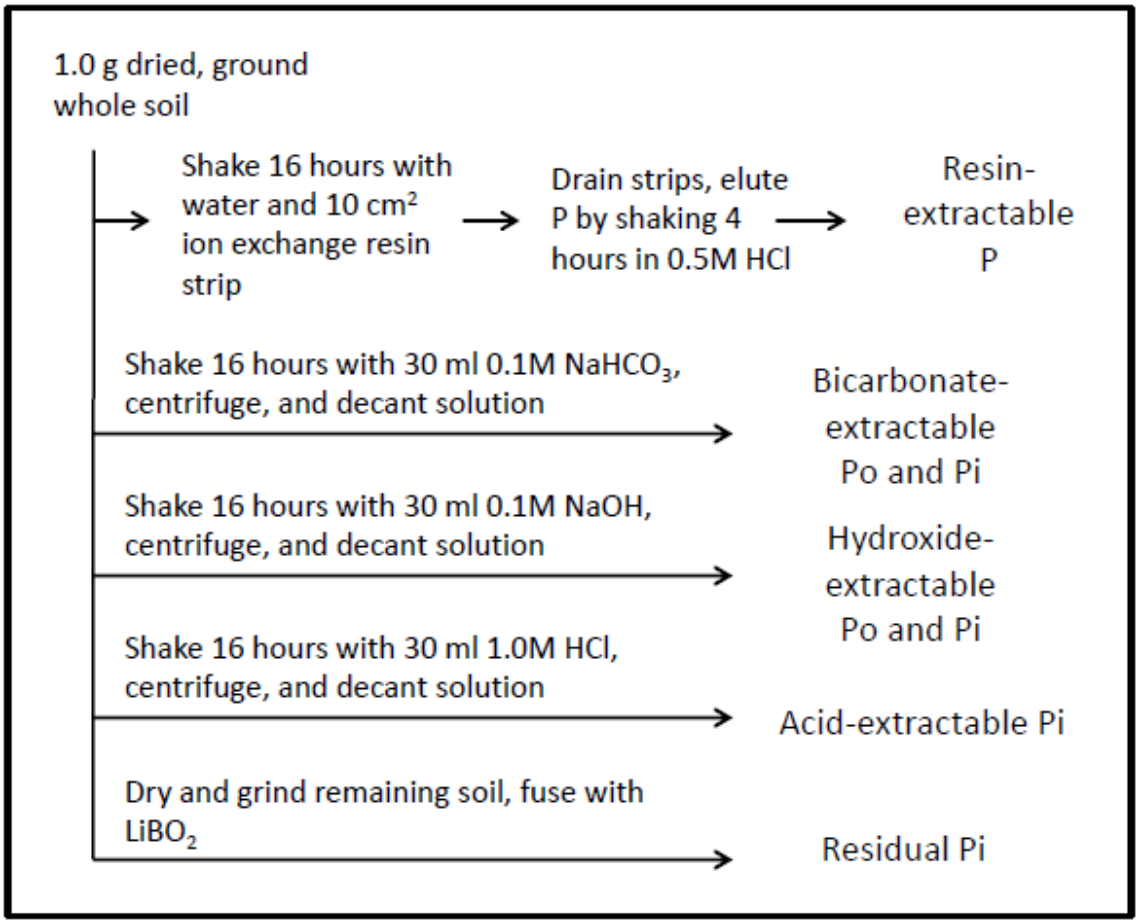

Figure 8. Modified Hedley method for fractionation of soil phosphorus (adapted from Lajtha et al., 1999).

2010) and referenced with a standard curve of potassium phosphate solution (KH_2PO_4, at 0, 2.5, 5.0, 7.5, 10.0, and 12.5 µg/ml).

3.7. *Statistical analyses*

Linear regression was used to describe the relationships between cluster age and P concentrations and P fraction concentrations in soil with increasing woody plant stand age. ANOVA was used to demonstrate differences between P concentrations and phosphorus fraction distributions in grasslands vs. woody clusters using JMP software (SAS, Institute Inc., Cary, NC, USA). Significance level was $p<0.05$.

4. Results

4.1. Soil characteristics

Soil pH was not significantly different between remnant grasslands and clusters in the upper 15 cm of the soil profile (Table 3). Soils were loamy sands with approximately 80% sand, 10% silt, and 10% clay, and did not differ between grasslands and woody clusters. Bulk density of the 0-10 cm depth interval decreased significantly from 1.2 g cm^{-3} in grasslands to 0.9 g cm^{-3} in clusters. Soil organic C in the upper 10 cm of the soil profile increased an average of 230%, from 643 g C m^{-2} to 1495 g C m^{-2} between grasslands and clusters. Soil total N increased 210%, from 63 g N m^{-2} in grassland soils to 133 g N m^{-2} in the associated clusters.

4.2. Phosphorus concentrations in whole soil and phosphorus fractions

The concentration of soil total P measured by lithium fusion of whole soil (0-10 cm) increased 150%, from 95.5 mg P kg^{-1} soil in the remnant grasslands to 143.6 mg P kg^{-1} in the oldest clusters (Figure 9). Pool sizes of soil total P increased from an average of 10.8 g P m^{-2} to 14.0 g P m^{-2} in the oldest clusters (> 80 yr), which represents an accumulation rate of 0.04 g P yr^{-1}.

Mean concentrations of P (0-10 cm) were significantly higher in cluster soils than in grasslands for all fractions except acid-extractable P, where the change was not

Table 3
Physical and chemical characteristics of surface soil of grasslands and clusters at the La Copita Research Area.

Characteristic	Landscape Element	
	Grassland	Cluster
pH	6.5 (0.1)	6.4 (0.1)
Texture	Loamy sand	Loamy sand
Sand (%)	80.5 (0.4)	81.3 (0.5)
Silt (%)	10.5 (2.0)	9.6 (1.6)
Clay (%)	9.0 (2.0)	9.1 (1.6)
Bulk Density (g cm^{-3})	1.2 (0.01)	0.9 (0.02)
SOC (g C m^{-2})	642.9(43.9)	1494.5(115.6)
Total N (g N m^{-2})	63.4(3.3)	132.7(9.1)

Standard errors of the mean are in parentheses. Data for pH and texture are from Liao et al., 2006a, 0-15 cm. All other characteristics represent the 0-10 cm depth increment.

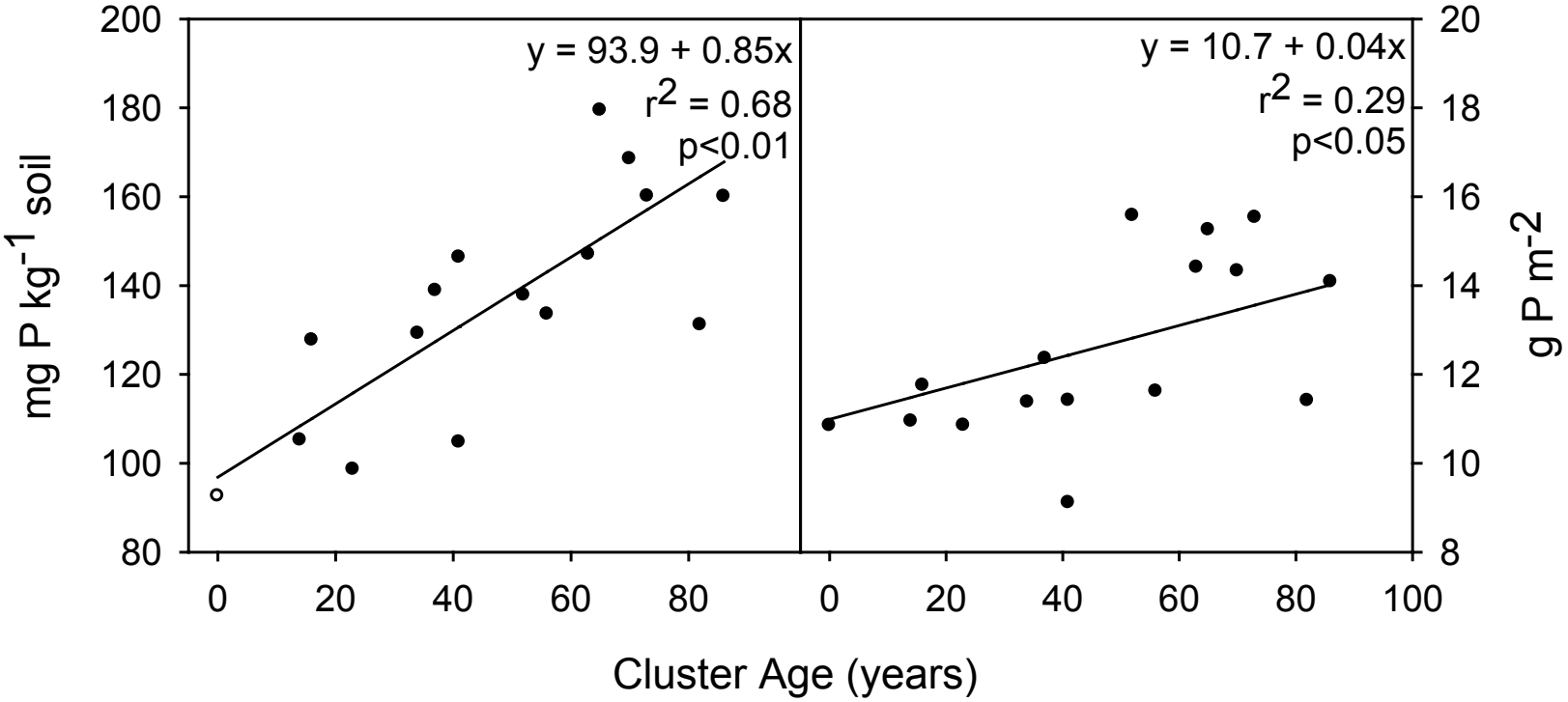

Figure 9. Soil total phosphorus concentrations and pool sizes with respect to cluster age in whole soil (0-10 cm) at the La Copita Research Area. Time 0 is the mean of all grassland samples.

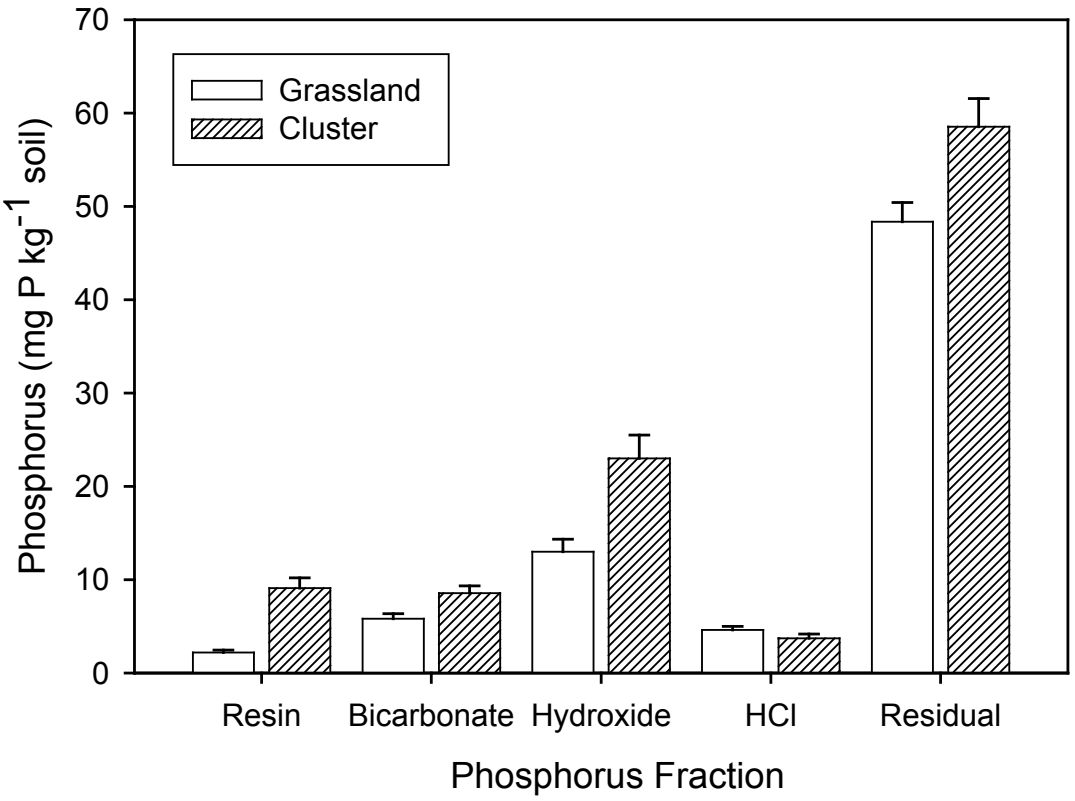

Figure 10. Average phosphorus concentration (mg P kg^{-1} soil) in phosphorus fractions for grasslands and clusters (0-10 cm). Error bars are standard errors of the means.

significant (Figure 10). The largest increases were observed in the resin-extractable and hydroxide-extractable fractions. The plant-available, labile inorganic P (resin-extractable Pi) increased from 2.0 mg P kg^{-1} soil in grasslands to 8.8 mg P kg^{-1} in clusters, an increase of 430%. Total bicarbonate-extractable P (labile P, Pi + Po) increased from 5.9 mg P kg^{-1} in grasslands to 8.3 mg P kg^{-1} in clusters. Total hydroxide-extractable P (Pi +Po) increased from 13.4 mg P kg^{-1} in grassland to 22.0 mg P kg^{-1} in clusters. Acid-extractable Pi showed no significant trend, and individual samples occasionally showed higher acid-extractable P concentrations in grasslands than in the associated clusters. Residual P increased from 47.4 mg P kg^{-1} in grasslands to 58.7 mg P kg^{-1} in clusters.

The proportion of extracted soil P in labile fractions (resin-extractable Pi and bicarbonate extractable Pi and Po) accounted for 10.8% of P found in all fractions (calculated from the sum of the individual P fractions) in remnant grasslands, and averaged 17% of P found in all fractions in clusters, with a maximum of over 20% in clusters >80 yrs (Table 4, Figure 11). The non-occluded fractions (hydroxide-extractable Pi and Po and HCl-extractable Pi) summed to 24.5% of the grassland P and 29.9% of the cluster P. HCl-extractable P was 6.3% in grasslands and 3.7% in clusters. The largest fraction in both vegetation types was the occluded, residual P which accounted for 64.7% of P in grasslands and 57.8 % of P in clusters (Table 4, Figure 12). The organic fractions summed to 19.8% of the grassland P and 23.2% of cluster P (Table 4). Pairwise t tests indicated significant differences between grasslands and clusters for resin Pi, bicarbonate Pi+Po, NaOH Pi+Po, and residual Pi (Table 4). All fractions except HCl-extractable Pi showed higher concentrations of P in clusters compared to grasslands. The change in HCl-extractable Pi was insignificant.

4.3. Organic phosphorus in extractable fractions

In the bicarbonate-extractable P fraction, the increase in P concentration was significant in only the inorganic fractions (Table 4, Figure 12). Inorganic P accounted for slightly more than half (56% in grasslands, 57% in clusters) of total bicarbonate-

Table 4
Mean soil P concentrations by fraction in grassland and cluster soil samples. Means were averaged from 14 soil samples in each landscape element. A one-way ANOVA was conducted with 26 degrees of freedom. C/G indicates the ratio of each average P concentration in the mesquite cluster to that of the grasslands. Fraction P is the sum of the extracted fractions. Whole soil P is total P measured by lithium fusion. All values are reported as mg P kg^{-1} soil.

P extract	Grassland	Cluster	C/G	F-statistic	p value	Fraction
Resin Pi	2.04	8.77	4.3	33.60	<0.01*	plant-available
Bicarb P	5.88	8.29	1.4	5.90	0.02*	labile
Bicarb Pi	3.23	4.75	1.5	3.36	0.08	labile
Bicarb Po	2.65	3.55	1.3	0.78	0.39	labile
NaOH P	13.36	21.99	1.6	8.15	<0.01*	non-occluded
NaOH Pi	1.86	2.09	1.1	0.25	0.62	non-occluded
NaOH Po	11.50	19.90	1.7	7.98	<0.01*	non-occluded
HCl	4.58	3.79	0.8	1.61	0.22	non-occluded
Residual P	47.37	58.69	1.2	10.24	<0.01*	occluded
Fraction P	73.23	101.55	1.4	26.72	<0.01*	
Whole soil P	95.45	142.59	1.5	26.52	<0.01*	
Total Pi	59.08	78.06	1.3	17.94	<0.01*	
Total Po	14.15	23.48	1.7	6.88	0.01*	

Asterisks indicated significance $p<0.05$.

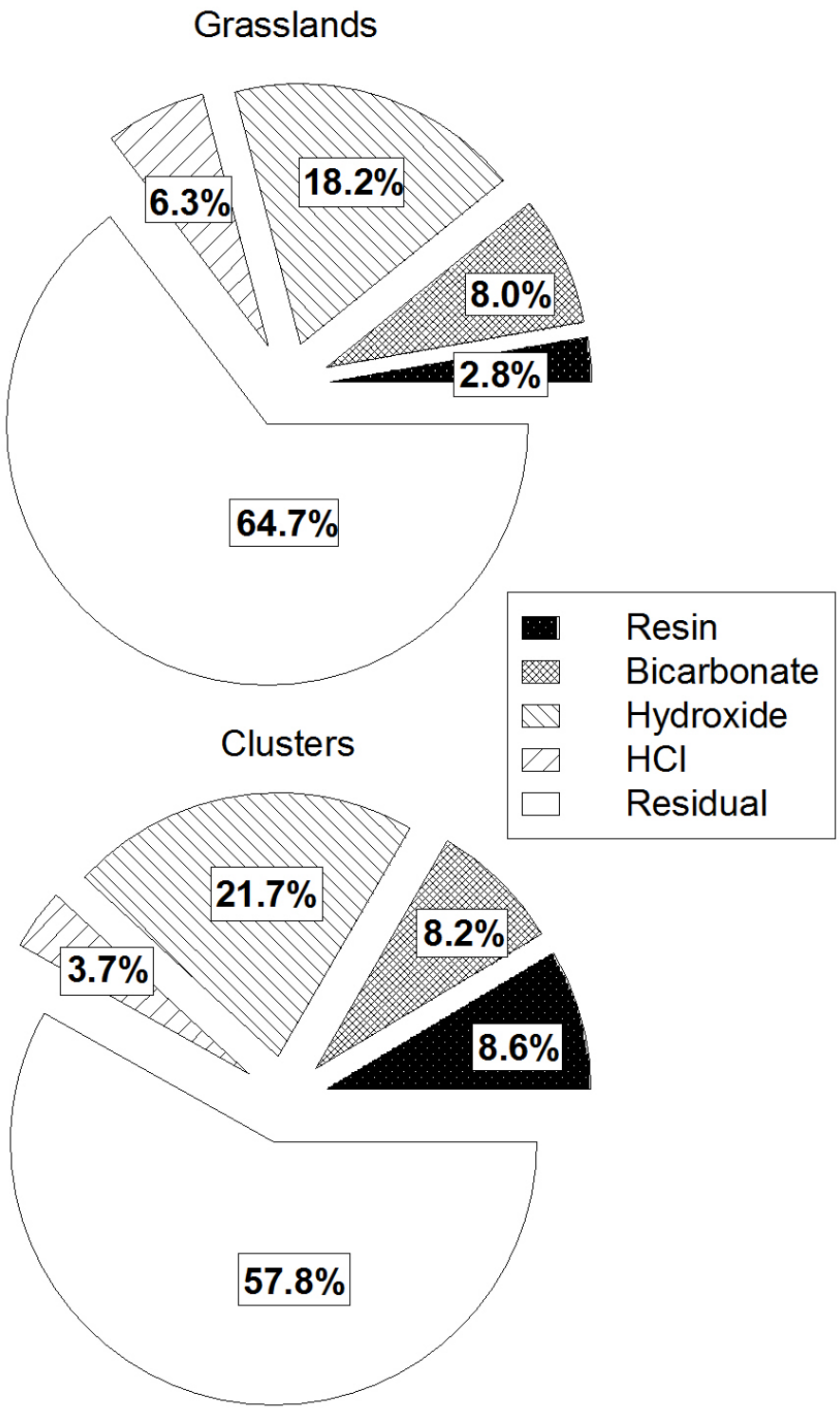

Figure 11. Phosphorus fractions as percentages of total soil P for grasslands and clusters (0-10 cm) at the La Copita Research Area.

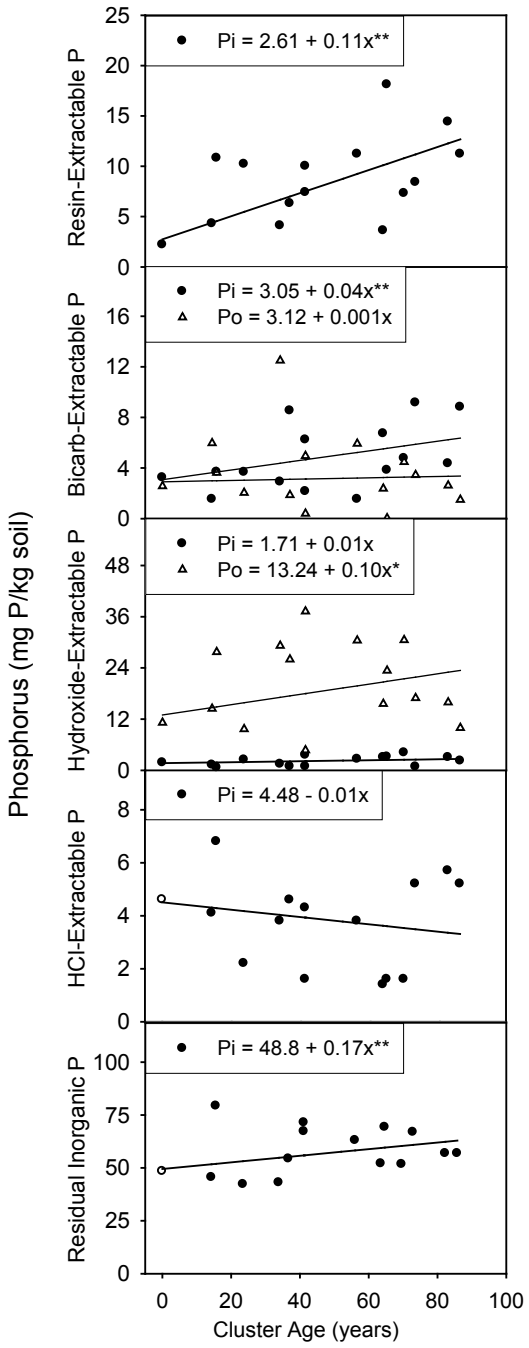

Figure 12. Changes in P_i (inorganic P) and P_o (organic P) concentrations within phosphorus fractions with respect to the age of woody clusters (0-10 cm). Time 0 is the mean of all grassland samples. One asterisk indicates significance at $p<0.05$, two asterisks indicate significance at $p<0.01$.

extractable P. Inorganic P concentration increased more quickly and to a larger extent than organic P in the bicarbonate-extractable fraction. While the overall increase in P in the hydroxide-extractable fraction was significant, when divided into organic and inorganic components, only the organic fraction showed a significant increase in nutrient concentration between the clusters and the grasslands. Organic P comprised 92% of the hydroxide-extractable P in grasslands and 91% in clusters. Hydroxide-extractable organic P represented 94% of total Po in the grassland soils and 85% of total Po in cluster soils.

4.4. Phosphorus accumulation rates

Rates of accumulation varied widely between P fractions (Figure 12). Phosphorus accrued most rapidly in the residual P fraction, with a rate of 0.17 mg P kg^{-1} soil yr^{-1}. NaOH-extractable organic P and resin-extractable Pi accumulated at similar rates, 0.13 and 0.12 mg P kg^{-1} y^{-1}, respectively. Slow accumulation (0.04 mg P kg^{-1} soil yr^{-1}) occurred in the bicarbonate-extractable Pi. Little accumulation occurred in the bicarbonate-extractable Po and hydroxide-extractable Pi, and data trends suggest loss may be occurring in the HCl-extractable Pi.

5. Discussion

Over the course of 85 years of woody plant encroachment into grasslands, significant changes in soil P have occurred, both as total P increases and the redistribution of soil P fractions beneath the mesquite canopies. Measurements of total P (0-10 cm) along the chronosequence indicate that nutrient accumulation in mesquite clusters begins with the earliest establishment of the mesquite trees, prior to the recruitment of non-N-fixing woody species. As early as 14 years after woody plant encroachment, the total P concentrations of soil samples are higher than the average grassland values (Figure 9). This agrees with measurements of C and N along the same chronosequence (Table 4, Chapter I, Liao et al., 2006a,b). The formation of these “islands of fertility” has been documented in a number of arid and semiarid systems (Schlesinger et al., 1990; Padien

and Lajtha, 1992; Hibbard et al., 2001), and is the result of a combination of biological and physical factors that conserve nutrients beneath woody plants. Increased soil moisture and temperature moderation through shading reduce erosion and losses to the atmosphere while canopy interception of nutrient containing particles represents a small addition to the soil nutrient pools. Additionally, woody plants take up soil nutrients and store them in plant tissue, shifting nutrients to organic forms in the soil as roots and litter turn over (Schlesinger et al., 1990). Potential initial soil nutrient loss in the shallow soil due to rapid uptake by woody plants during vegetation change reverses within the 14-year gap between the remnant grasslands and the youngest mesquite clusters. Though it is conceivable that mesquite preferentially grows in pre-existing areas of localized high nutrient content, extensive sampling of this site (Hibbard et al., 2001; Archer et al., 2001, 2004; McCulley et al., 2004; Liao et al., 2006a,b; Bai et al., 2009; Boutton et al., 2009; Boutton and Liao, 2010) and areas with similar vegetation patterns (Padien and Lajtha, 1992; Allison et al., 2006; Dossa et al., 2010) indicate that the mesquite is driving nutrient increases in these soils and the phenomena is also found in other landscapes affected by woody plant encroachment.

The response of plants to nutrient limitation differs by species, and plant-induced differences in nutrient distribution are most striking in contrasting vegetation patterns like the cluster development of woody plant encroached soils. Plant cycled nutrients have been shown to concentrate in the upper 20 cm of the soil profile as they are dependent on organic matter turnover for translocation (Jobbagy and Jackson, 2001). Phosphorus is particularly prone to redistribution by plants due to the absence of a gaseous atmospheric component of the P cycle. The importance of vegetation influence on the P cycle is evident at La Copita, where the increase in total P coincides with increases in NPP and organic matter inputs to the soil (McCulley et al., 2004; Liao et al., 2006a). Soil P acquired by woody plants is concentrated in the above- and belowground biomass of mesquite and understory woody species. Litter and root turnover incorporate the woody plant litter into SOM, shifting litter quality toward more nutrient rich, recalcitrant compounds (Archer et al., 2001; Hibbard et al., 2001; McCulley et al., 2004;

Liao et al., 2006b; Filley et al., 2008; Boutton et al., 2009). Though OM decomposition has been shown to affect soil pH, resulting in differences in phosphorus availability (Brady and Weil, 2007; Dossa et al., 2010), there was no significant effect of shrub invasion on soil pH in the clusters (Table 3).

The increase in organic matter in the soils is reflected in the increase in P_o following encroachment. P_o increased 170% between average grassland and cluster soils (Table 4). The proportion of soil total P allotted to the organic fraction increased significantly from 19% of total P in the grasslands to 23% in the woody clusters. The P_o fraction in grasslands was slightly higher than the average measured for Mollisols by Cross and Schlesinger (1995), which estimated 15% organic P for grassland soils. The average P_o and P_i concentrations of cluster soils at La Copita most closely resembled average values for Alfisols and Ultisols (Cross and Schlesinger, 1995). Measurements of total P and organic and inorganic P only begin to scratch the surface of the puzzle of P availability. Though composed of plant and microbial material, P_o is often bound to mineral soil, making it inaccessible to plants for uptake (Haussling and Marshner, 1989, Turner et al., 2005). Through fractionation of the soil P, the relative availability of P in the soil can be quantified (Hedley et al., 1982; Tiessen and Moir, 1993; Lajtha, et al., 1999; Cross and Schlesinger, 2001; Dossa et al., 2010, De Schrijver et al., 2011).

Soils around the bole of the mesquite trees had significantly higher concentrations of resin-extractable and bicarbonate-extractable P. These fractions represent the labile, plant-available pools of soil P, which can be readily assimilated by plants and microbes. The results in the La Copita soils were similar to those found in tropical shrublands in Senegal (Dossa et al., 2010), and contrasted with shrub-invaded desert soils (Cross and Schlesinger, 2001) and forest soils (De Schrijver et al., 2011), which showed a decrease in resin-extractable P following woody plant establishment. In forest ecosystems, labile P decreased significantly over 40 years of forest development at 0-15 cm soil depth, while in 0-10 cm depth of the desert soil, labile P decreased under shrub vegetation. However, the forest study showed that labile P concentrations remained high in the 0-5 cm depth, perhaps more shallow than the zone of P acquisition by tree roots. This may

account for the increases in resin-extractable P in the 0-10 cm depths at La Copita and in the Senegalese soil, where shading by woody plants prevents shallow-rooted herbaceous species from using shallow soil P in the mesquite understory.

The proportion of the Po that can be mineralized by biological processes [(bicarbonate-extractable Po) / ((bicarbonate-extractable Po + Pi) + resin-extractable Pi)] was similar to values reported by Cross and Schlesinger (1995) for Mollisols in the grasslands (33.5%), but decreased in the clusters (20.5%) (data not shown). In this case, the apparent decrease in biologically-available Po may be misleading—the higher concentration of resin-extractable Pi in clusters contributes to the calculated decrease in mineralizable Po, but the overall concentration of bicarbonate-extractable Po (3.6 mg P kg^{-1} soil in grasslands, 3.4 mg P kg^{-1} soil in clusters) is not significantly different between the two landscape elements.

Hydroxide-extractable P represented the largest non-residual pool in the La Copita soils. Though the increase in hydroxide-extractable Pi was not significant, hydroxide-extractable Po increased significantly in the clusters. Hydroxide-extractable P is primarily Fe- and Al-sorbed organic phosphate compounds (Cross and Schlesinger, 1995; Dossa et al., 2010). As there is no indication of differences in Fe or Al content between the grassland and cluster soils, the increase in hydroxide-extractable Po likely represents the influence of the OM introduced to the system by the productivity of the mesquite trees. The increased in hydroxide-extractable P reflects the increase in SOC between grasslands and clusters (Table 4).

The Hedley method for phosphorus fractionation is traditionally used to measure labile, plant-available forms of P (resin-extractable Pi and bicarbonate-extractable Pi and Po) for agricultural purposes. Cross and Schlesinger (1995) postulated that the method can be used to indicate the relative importance of biological and geochemical soil weathering processes in the availability of P in different soils. The Hedley method indicates the time scale at which P may become available based on the process that will be required to liberate the P from the soil matrix. Both grassland and cluster soils at La Copita rely primarily on geochemical processes to liberate P, as roughly 80% of the total

P is in recalcitrant forms. However, the increase in P fractions prone to biological weathering (resin-extractable Pi, bicarbonate-extractable Po and Pi) with woody plant encroachment indicates that biological nutrient cycling processes play a larger role in the clusters than in the grasslands.

There are four potential sources for the increasing P in shallow soils: atmospheric deposition, horizontal transfer from nearby soils, root uplift/redistribution, and animal activity (food storage and waste production) (Jobbagy and Jackson, 2001; Porder and Chadwick, 2009). Both atmospheric deposition and horizontal transfer are unlikely to significantly contribute to the P increases observed during woody encroachment at La Copita. As P lacks a stable gaseous atmospheric form (contrasted with C and N), deposition of atmospheric P consists of mineral dust and organic matter, with lesser contributions of particulate matter produced by combustion of fossil and biological fuels (Mahowald et al., 2008). The rate of P deposition for Texas and the southern United States ranges from 0.5 to 1.0 mg m^{-2} yr^{-1}, a minute fraction of the roughly 50 mg m^{-2} yr^{-1} increases observed at La Copita (Mahowald et al., 2008). The data do not support the horizontal transfer of P from the grassland soils to the wooded soils, as there is no observable decrease in P concentrations in grasslands with time across the chronosequence. While animals are present on the site, the preferential use of mesquite trees as animal habitat requires a tree large enough to provide shade or support roosting birds, therefore a lag would develop in the phosphorus increase data while the mesquite reached sufficient size to attract animals. No lag is evident in the La Copita data. Phosphorus increases at La Copita are likely due to a combination of root uplift and enzymatic weathering of P from deep in the soil profile. The enlarged rhizosphere (up to 10 m deep in mesquite compared to 1.5 m in grasslands) offers both the ability to access nutrients deeper in the soil and root volume with which to produce P-weathering enzymes (Boutton et al., 1998). Phosphorus taken up by plant roots is transported to the aboveground plant tissues and becomes part of the shallow soils during litter turnover.

This theory reflects the “islands of fertility” hypothesis (Schlesinger et al., 1990; Padien and Lajtha, 1992; Hibbard et al., 2001), wherein the larger aboveground biomass

of the mesquite trees contributes to the cycling of larger amounts of nutrients between the foliage and the soil. While the depth sampled at La Copita represents only the shallow soil, observations deeper in the soil profile (Jobbagy and Jackson, 2001) have shown that the increase in the surface layers is likely accompanied by a decrease in P concentration in the deeper soil. As the soil P stocks are essentially finite, over time the loss of deep soil P to biological cycling will limit P acquisition by plants to P cycling in the shallow soil (Gahoonia et al., 1994; Schachtman et al., 1998).

6. Conclusions

Woody encroachment is frequently viewed from the perspective of grassland loss, however this study shows that woody plant establishment in grasslands results in increased soil fertility through acquisition and redistribution of soil P. Over 86 years of mesquite encroachment, whole soil total P increased 174%, and labile and plant-available P increased 184%. The dramatic increase in P present in plant-available forms indicates that woody encroachment in this region strongly enhances the availability of this limiting nutrient. Phosphorus acquisition strategies developed by N-fixing woody plants to support the energy-intensive metabolic process of N-fixation likely contribute to the increases in plant-available P, liberating P from organic matter through the production of phosphatase enzymes. As these phosphatase enzymes are N-rich, the nutrient tradeoffs between N fixation and P acquisition represent a coupling of the biogeochemical cycles of the two nutrients. Phosphorus accumulation in these soils show no sign of slowing after 86 years of mesquite influence, indicating that the effects of mesquite continue to progress. This alteration in the soil nutrient environment is likely to favor the invading woody species and advance the extent of woody plant encroachment in this system. Due to the similarity of this woody plant encroachment scenario to the vegetation changes occurring in other parts of the world, understanding of the changes in the P cycle driven by grassland-to-woodland conversion is essential to understanding the future of the P cycle on global scales.

CHAPTER IV

CHANGES IN SOIL C, N, AND P STORAGE FOLLOWING WOODY PLANT INVASION IN A SUBTROPICAL SAVANNA PARKLAND LANDSCAPE

1. Synopsis

Many grass-dominated ecosystems around the world have experienced woody plant encroachment over the last century due to livestock grazing, fire suppression, and/or changes in climate and atmospheric chemistry. In the Rio Grande Plains of Texas, subtropical thorn woodlands dominated by N-fixing tree legumes have largely replaced grasslands and altered the biogeochemistry of this region. The purpose of this study was to assess the impact of this grassland-to-woodland transition on the size and distribution of soil C, N, and P pools across five landscape elements of a mesquite-invaded grassland. Soil samples (0-7.5 cm) were collected in remnant grasslands and near the centers of woody plant clusters, groves, drainage woodlands, and playas ranging in age from 14 to 134 years in a subtropical savanna parkland in southern Texas. Soil organic C and total N were measured by elemental analysis, and total P was measured by lithium fusion followed by phospho-molybdate colorimetry. Soil P was fractionated into resin-extractable inorganic P, bicarbonate-extractable organic and inorganic P, hydroxide-extractable organic and inorganic P, acid-extractable inorganic P, and residual inorganic P forms. Phosphorus concentrations in these fractions were determined by colorimetry, and soil total P was determined by lithium fusion. Organic P was calculated from the difference between total and inorganic P. Concentrations of soil C, N, and P increased linearly in all wooded landscape elements with time following woody encroachment. Mean soil organic C increased between 320 and 460% in wooded areas compared to remnant grasslands. Similarly, soil total N increased 285-535% and total P increased 160-340% in wooded areas. The most dramatic increases were observed in the lowland drainages and playas, while C, N, and P increased at slower rates in upland woody clusters and groves. P concentrations in all pools increased linearly with increasing

woody plant stand age except acid-extractable phosphorus. The most dramatic increases were observed in the resin-extractable fraction (plant-available P), which increased from 3 to 13 mg P/kg soil, and in hydroxide-extractable P (the majority of the organic P in the system), which increased from 15 mg P/kg soil in grasslands to 26 mg P/kg soil in the wooded clusters. Woody plant invasion increases C and N pools in the soil through increased NPP, litter chemistry changes, and symbiotic N-fixation by legume species, respectively. Although the exact mechanisms by which soil P increases following woody invasion remain unknown, we suggest that the more deeply rooted woody plants are acquiring P from deep in the soil profile and transferring it into the upper portion of the profile via litterfall and root turnover. Because P is generally the most limiting nutrient, increases in its availability could alter rates of biogeochemical processes, affect species interactions, and influence the future trajectory of woody invasion in this region.

2. Introduction

Woody plant encroachment has been observed in grassland ecosystems worldwide over the course of the last century (Archer et al., 1988; Hudak and Wessman, 1998; Briggs et al., 2005; Allison et al., 2006; Knapp et al., 2008; Lorenzo et al., 2010). This phenomenon is driven by number of factors, including livestock grazing, fire suppression, and rising atmospheric CO_2 levels that promote growth in C_3 woody species, outcompeting historic C_4 grassland vegetation (Archer et al., 1994, 2001; Scholes and Archer, 1997; Boutton et al., 1998, 1999; Van Auken, 2000; Roques et al., 2001). As grasslands represent 40% of the terrestrial land area and up to 30% of the planet's C storage, alterations of the biogeochemistry of grassland ecosystems has global implications for terrestrial nutrient cycles (Schlesinger et al., 1990, Canadell et al., 2007).

Woody plants differ from the grasslands they invade in structure as well as functional processes. The increased above- and belowground biomass of woody plants alters the cycle of organic matter and associated nutrients in the soil. Woody litter is more chemically recalcitrant than grass (Köchy and Wilson, 1997; Hibbard et al., 2001;

McCulley et al., 2004; Filley et al., 2008; Springsteen et al., 2009), and the influx of organic matter associated with litterfall and root turnover in woody plants promotes aggregation in the soil (Chapter II; Six et al., 2000, Liao et al., 2006a), leading to further retention of organic matter in the soil (Boutton et al., 1998, 2009; Archer et al., 2001; Liao et al., 2006b; Boutton and Liao, 2010). Woody plant encroachment has been shown to increase both C and N storage and availability in soils, as both slow- and fast-cycling C and N pools increase in size (Padien and Lajtha, 1992; Hibbard et al., 2001; McCulley et al., 2004; Liao et al., 2006b). Studies along a gradient of woody plant influence have shown that the magnitude of soil response to woody encroachment depends on environmental conditions, soil characteristics, and existing vegetation (Köchy and Wilson, 2001; Liao et al., 2008; Liu, Z. et al., 2010; Lorenzo et al., 2010; Scharenbroch et al., 2010).

Investigations into the effects of woody plant encroachment on P cycles in the soil have shown that shrubs can concentrate inorganic and organic P under their canopies through litter turnover, mining from deep soil, atmospheric interception, and inputs from birds or other animals (Schlesinger et al., 1990; Padien and Lajtha, 1992; Schlesinger and Pilmanis, 1998; Dossa et al., 2010). The large rhizospheres of woody species increase the volume of soil that can be mined by plant roots for P-weathering minerals (Canadell et al., 1996), increasing the amount of P cycling organically in woody plant biomass over that of grasses. The P and N cycles are strongly coupled in N-fixing plants, where the high P requirements of N-fixation lead to allocation of N for the production of phosphatase enzymes (Robson et al., 1981; Chapin et al., 2002: Allison et al., 2006; Wang, Y.-P. et al., 2007; Houlton et al., 2008; Vitousek et al, 2010). The strategies employed by plants to acquire phosphorus from soil minerals lead to alterations in the distribution and availability of soil P fractions (Dossa et al., 2010; Lorenzo et al., 2010).

At the La Copita Research Area in Texas, woody plant encroachment by N-fixing mesquite creates four distinct vegetation patterns: upland clusters and groves, and lowland drainages and playas. Above- and belowground biomass production is greater

following woody plant encroachment on both upland and lowland sites, and significantly lower bulk density than remnant grasslands indicates the incorporation of organic matter into the surface soil (Chapter II; Hibbard et al., 2001; McCulley et al., 2004; Liao et al., 2006a). The introduction of N-fixing legumes has been shown to contribute to increased overall N pool size through inputs of N-rich root matter and foliar litter (Hibbard et al., 2001; McCulley et al., 2004; Liao et al., 2006a). Woody plants at La Copita support larger pools of microbial biomass, higher soil respiration rates, and higher N mineralization rates than historic grassland vegetation (McCulley et al., 2004; Liao et al., 2006a). While soil P has not been quantified in the different landscape elements at La Copita, P measurements in clusters have shown that the P requirements of N-fixation by mesquite are altering the soil P cycle in the uplands. Differing vegetation patterns and differing rates of C and N storage and cycling between the landscape elements at La Copita indicate that the soils at each site respond differently to woody plant encroachment (Chapter II and III; Hibbard et al., 2001; Liao et al., 2006a, Liu, F. et al., 2010). This difference in nutrient response to vegetation change is likely to carry over to the P cycle.

The investigation of P concentration across a landscape offers several challenges. The P biogeochemical cycle differs from those of C and N, which plants can acquire from the atmosphere. Inorganic P availability is limited by the rate of weathering of P-containing minerals in the soil (Walker and Syres, 1976; Parton et al., 1988). Regardless of concentration of total P in soil, plant-available P may be limited, as a considerable amount of soil P is bound in insoluble Fe, Al, and Ca complexes. Additionally, upwards of 90% of soil P may be in organic forms, which are inaccessible to most plants (Walker and Syers, 1976; Haussling and Marshner, 1989). Therefore measurements of total P do not accurately reflect the availability of the nutrient in soils. Soil P fractionation allows examination of total P concentrations as well as different availability classes of P in the soils under different degrees of woody encroachment on remnant grasslands and each of four wooded landscape elements.

At the La Copita Research Area in southern Texas, the effects of woody plant encroachment on soil biogeochemistry can be observed across five landscape elements: remnant C_4 grasslands, mesquite clusters, mesquite groves, closed-canopy drainage woodlands, and playas. This ecosystem offers the opportunity to compare the effects of woody plant encroachment on a potentially limiting nutrient. The objectives of this study were to demonstrate the progressive effects of woody plant encroachment on soil nutrient distributions by measuring i) SOC, total N, and total P and ii) P fractions with respect to time and vegetation class along a chronosequence of mesquite encroachment on five landscapes in southern Texas.

3. Materials and methods

3.1. Study area

Texas AgriLife La Copita Research Area (27º 40'N, 98º 12'W) is located in the Rio Grande Plains of southern Texas, 65 km west of Corpus Christi. Climate is subtropical with mean annual temperature of 22.4 ºC and mean annual precipitation of 716 mm, with rainfall peaks in May-June and September. Elevation ranges from 75 m in the lowland playas and drainage woodlands to 90 m in the upland grasslands, clusters, and groves. The vegetation at La Copita has shifted from C_4 grassland to C_3 shrub woodlands over the last 150 years, primarily due to cattle grazing and fire suppression. Sequential aerial photography, tree ring analysis, vegetation dynamic modeling, and isotopic analysis of soils have confirmed increasing presence of woody vegetation on the site (Archer et al., 1988, 2001, 2004; Boutton et al., 1998, 1999.)

The landscape at La Copita is topographically divided into upland and lowland areas. Upland soils consist of sandy loams (Typic Argiustolls) with a subsurface argillic (Bt) horizon, with inclusions of Typic Ustochreps that lack the argillic horizon. The uplands consist of a grassland matrix dominated by C_4 grasses (such as *Bothriochloa, Bouteloua, Chloris, Eragrostis*), and include discrete clusters of C_3 woody plants. The woody clusters are dominated by *P. glandulosa* (honey mesquite) and may include as many as 15 other tree/shrub species *(*such as *Condalia hookeri, Zanthoxylum fagara, Zizyphus*

obtusifolia, Berberis trifoliolata). Where the argillic horizon is absent, clusters often expand laterally and fuse to form larger upland groves of woody vegetation (Archer et al., 1988; Bai et al., 2009, 2012a). Grassland-to-woodland succession has gone to completion in the lower-lying portions of the landscape where the soils are finer-textured sandy clay loams (Pachic Argiustolls), and these areas are now covered by closed-canopy drainage woodlands (Bai et al., 2012b). Playas are oval-shaped basins that occupy the lowest portion of the landscape and have no external drainage, and are covered primarily by grassland with scattered solitary mesquite trees (Farley, 2000; Parker et al., 2010). Additional details on plant communities, soils, and successional dynamics have been presented elsewhere (Scifres and Koerth, 1987; Archer et al., 1988; Boutton et al., 1998).

3.2. Chronosequence approach

The responses of soil C, N, and P to woody plant encroachment at La Copita were evaluated using a space-for-time chronosequence approach. The formation of wooded landscape elements at La Copita is initiated only after the establishment of mesquite in grassland (Archer et al., 1988). Therefore, the maximum age of woody plant assemblages can be estimated from the age of the mesquite trees. Basal diameters of mesquite trees were measured and substituted into regression equations to predict tree ages, using equations derived from tree ring analysis for each landscape element (Flinn et al., 1994; Stoker, 1997). Soil samples from remnant grasslands were used to represent the historic grassland soil conditions prior to woody plant encroachment (Time 0). Woody plant stands sampled in each of the landscape elements (clusters, groves, drainages, and playas) were selected to encompass the full range of mesquite basal diameters, corresponding to tree ages ranging from approximately 10 to 135 yr.

3.3. Collection of soil samples

Samples were collected in October 2006. For C, N, and P analyses, soil samples (0-7.5 cm) were collected from five landscape elements: remnant grasslands (n=11),

mesquite clusters (n=11), groves (n=10), drainages (n=11), and playas (n=6). In the wooded landscape elements, four soil cores (5 cm diameter x 7.5 cm deep) were collected from near the bole of each mesquite, and in grassland soils four cores were collected from around the base of a dominant perennial C_4 grass plant at least 5 m beyond the dripline of any neighboring woody vegetation. The four soil cores collected at each sampling point were combined and mixed in the field, stored in plastic bags, transported on dry ice and stored at -20 ºC.

3.4. Soil C and N analyses

An aliquot of soil was passed through an 8-mm sieve to remove large organic matter fragments, dried at 55 ºC, and pulverized in a centrifugal mill (Angstrom Inc, Belleville, MI, USA). Carbon and N concentrations in whole soils were determined by combustion-gas chromatography using a Carlo Erba EA-1108 elemental analyzer (CE Elantech, Lakewood, NJ, USA).

3.5. Phosphorus fractionation and analyses

Soil P was sequentially fractionated using a modified Hedley method (Hedley et al., 1982; Tiessen and Moir, 1993; Lajtha et al., 1999; Dossa et al., 2010) (Figure 13). A soil sample (1 g) was placed in a 50-ml centrifuge tube with two 2.5 cm^2 anion exchange resin membranes (AR204-SZRA, Ionics, Watertown, MA) and 30 ml deionized water. Samples were placed on a horizontal shaker for 16 hours at 21 ºC (Cross and Schlesinger, 2001). Anion exchange resin strips were removed from the solution and placed in a 50-ml centrifuge tube with 30 ml 0.5 M HCl and shaken for four hours to elute adsorbed P. The original soil-water solution was centrifuged for 5 minutes at 5,000 rpm and the water was removed by pipette. The soil sample was sequentially extracted with 0.1 M $NaHCO_3$, 0.1 M NaOH, and 0.1 M HCl to extract bicarbonate-extractable P, hydroxide-extractable P, and HCl-extractable P. Each extraction solution was added to the centrifuge tube, shaken for 16 hours, centrifuged, and the solution pipetted into a

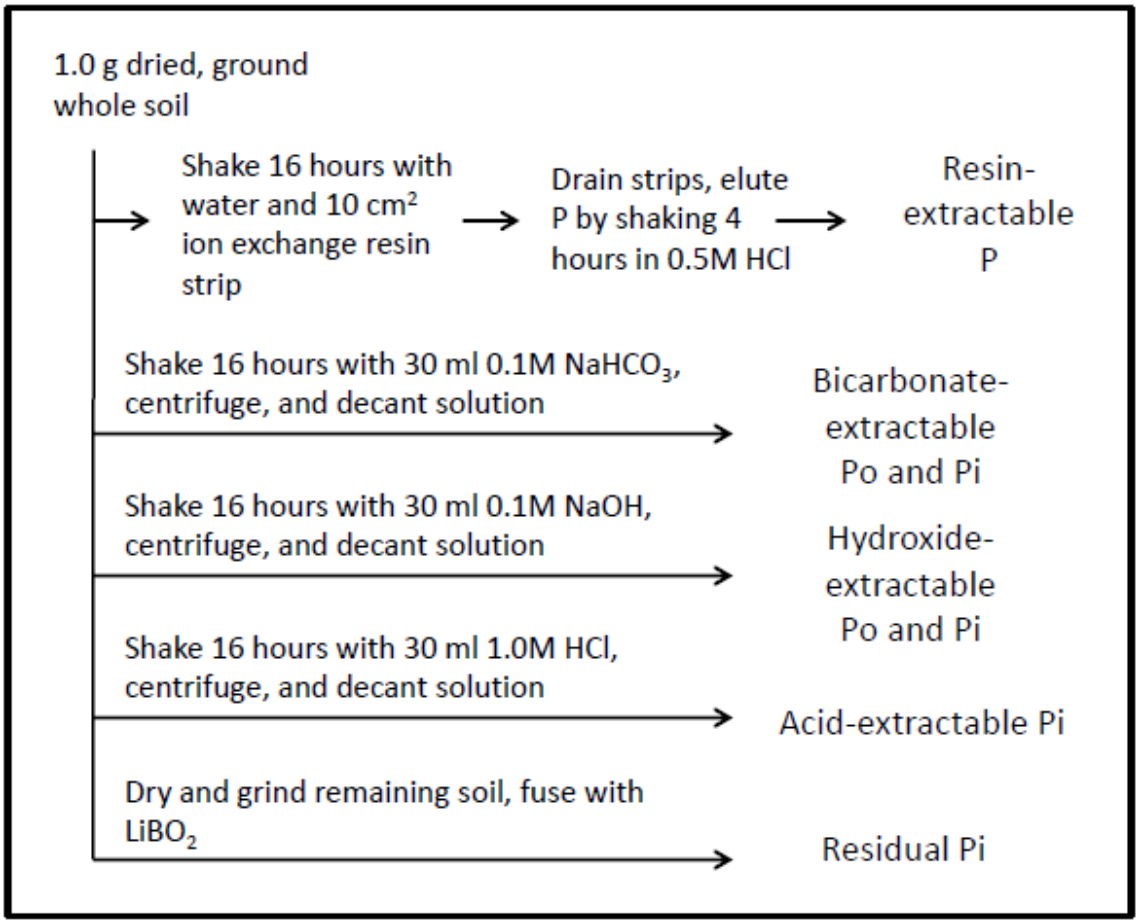

Figure 13. Flow chart for fractionation of soil phosphorus (adapted from Lajtha et al., 1999).

separate tube for analysis. Soil remaining after HCl extraction was dried, ground, and analyzed by lithium fusion for residual P. Aliquots of the bicarbonate and hydroxide extraction solutions were digested with potassium persulfate ($K_2S_2O_8$) to oxidize organic P and analyzed by phospho-molybdate colorimetry (Murphy and Riley, 1962) to determine total P (inorganic Pi + organic Po). Pi was measured by the same method for the other extractants. The values for Pi were subtracted from the total P values for digested samples to determine relative proportions of Po and Pi. Fractionated total P values (sum of the individual fractions, Tiessen and Moir, 1993) averaged 22-30% lower than P values determined by lithium fusion of whole soil. Whole soil and residual soil samples were analyzed for total P by lithium fusion (Lajtha et al., 1999). Approximately 200-250 mg of dried, ground soil was mixed with 750 mg of lithium metaborate in graphite crucibles and heated to 1000 ºC in a muffle furnace. The molten flux was dissolved in 10% HNO_3 by stirring for two hours (Lajtha et al., 1999). Phosphorus content of the flux/acid mixture was determined by phospho-molybdate colorimetry (Murphy and Riley, 1962; Dick and Tabatabai, 1977). Aliquots of extraction solutions were combined with ammonium molybdate, antimony potassium tartrate, and ascorbic acid, and the resulting blue color was compared to a standard curve of potassium phosphate solution on a Spectronic 20 D+ (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 720 nm (Dick and Tabatabai, 1977).

3.6. Statistical analyses

The relationships between soil nutrients and woody plant stand age were evaluated by linear regression. ANOVA was used to test for differences in nutrient concentrations between different landscape elements using JMP software (SAS Institute, Inc., Cary, NC, USA.)

Table 5
Characteristics of surface soils of five landscape elements at the La Copita Research Area.

	Grassland	Cluster	Grove	Drainage	Playa
pH	6.5(0.1)	6.4(0.06)	6.3(0.1)	6.2(0.1)	6.2(0.2)
Texture	Loamy Sand	Loamy Sand	Loamy Sand	Sandy Loam	Sandy Clay Loam
Sand (g kg^{-1}soil)	805(40)	813(50)	814(06)	660(30)	525(13)
Silt (g kg^{-1}soil)	105(20)	96(16)	120(13)	154(27)	190(50)
Clay (g kg^{-1} soil)	90(20)	91(16)	66(13)	186(19)	285(104)
Bulk Density (g cm^{-3})	1.2(0.02)	1.0(0.03)	1.0(0.03)	1.0(0.04)	1.2(0.03)

Standard errors of the mean are in parentheses. Data from Liao et al., 2006a, and Liu, F. et al., 2010, 0-15 cm.

4. Results

4.1. Soil characteristics

Bulk density of the soils decreased with woody plant encroachment from 1.2 g cm^{-3} to around 1.0 g cm^{-3} in the wooded landscape elements (Table 5). The bulk densities were similar within the wooded landscape element wooded elements. The lowland soils were differentiable from the upland soils by soil texture: upland soils were loamy sands while lowland soils were sandy loams. Grassland, cluster, and grove soils averaged 81% sand while drainages were 66% sand and playas only 52%. Soil pH (measured for the upper 15 cm of the soil profile) was not significantly different between sample sites.

4.2. Soil organic carbon and total nitrogen concentrations

Soil organic C in the upper 7.5 cm of the soil profile increased significantly between 3- and 7-fold following woody plant encroachment. Grasslands averaged 8.3 g kg^{-1} while the wooded landscape elements ranged from 36.6 g kg^{-1} in the oldest groves to 71.7 g kg^{-1} in the oldest drainages. Clusters and playas showed similar increases, with 43.3 and 46.2 g kg^{-1}, respectively (Figure 14, Table 6).

Soil total N increased between 4- and 9-fold in the wooded landscape elements. Total N averaged 0.7 g kg^{-1} in the grasslands, significantly lower than all wooded landscape elements. Like SOC, the greatest increase in N was found in the drainages, where N increased to 6.81 g kg^{-1} in the oldest drainages. Nitrogen concentrations in clusters, groves, and playas increased 430-470%, to 3.35, 3.49, and 4.29 g kg^{-1}.

4.3. Soil total phosphorus and distribution in phosphorus fractions

Average concentrations of P were significantly higher in wooded landscape elements than in grasslands for most extraction fractions. Notable exceptions were acid-extractable P, where the change was not significant for any wooded landscape element, and hydroxide-extractable P in the groves, which decreased compared to grasslands (Figure 15). The largest increases were observed in the resin-extractable, hydroxide-extractable, and residual fractions. In general, the phosphorus fraction concentrations

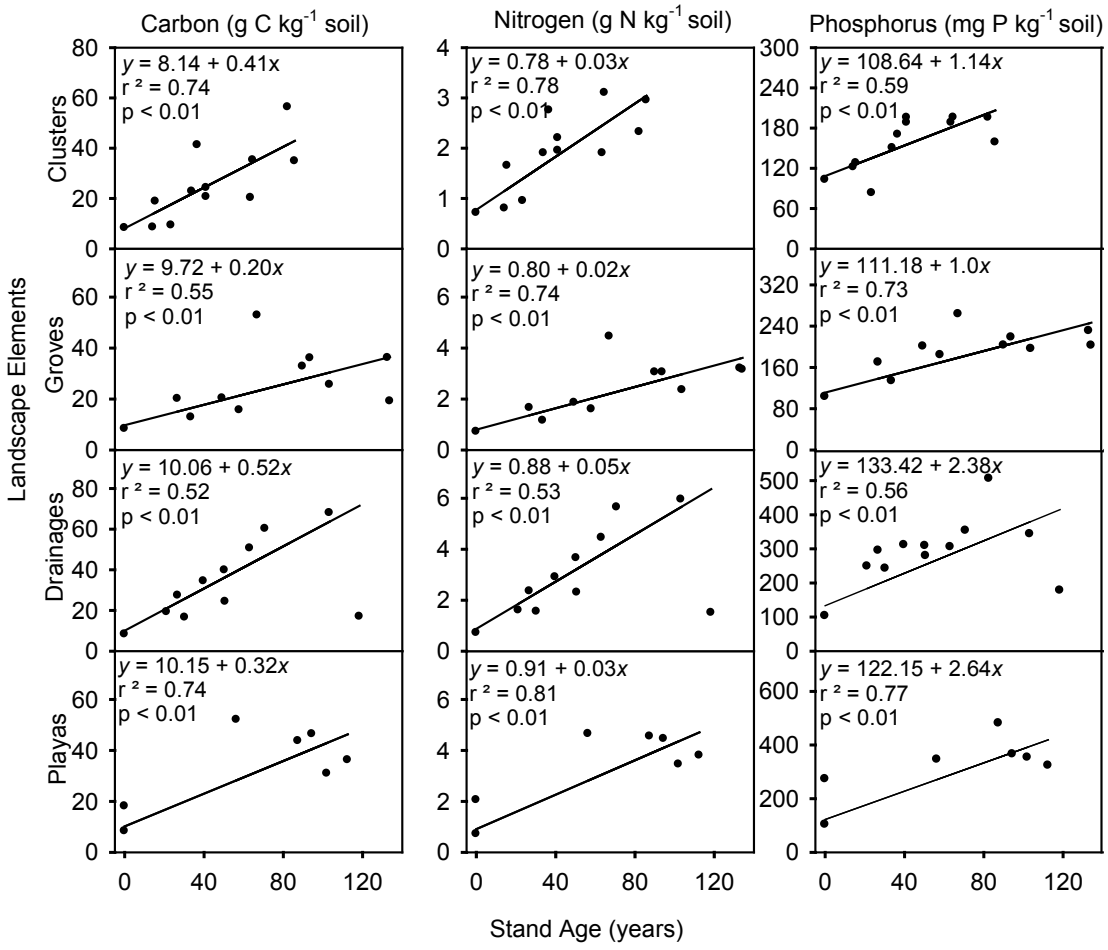

Figure 14. Changes in concentrations of soil organic C, total N, and total P in the 0-7.5 cm depth increment vs. woody plant stand age in four different landscape elements at the La Copita Research Area. Time zero is the mean value for remnant grasslands.

Table 6
Mean soil nutrient concentrations (0-7.5 cm) for five landscape elements at the La Copita Research Area. Percentages indicate the average increase in nutrient concentration between remnant grasslands and wooded landscape elements.

	Grassland	Cluster	Grove	Drainage	Playa
SOC (g C kg^{-1} soil)	8.3 (1.0)[a]	26.5 (4.3)[b]	27.1 (3.9)[b]	42.9 (9.0)[c]	37.9(5.0)[bc]
Increase		321%	328%	520%	456%
Total N (g N kg^{-1} soil)	0.7 (0.1)[a]	2.0 (0.2)[b]	2.6 (0.3)[bc]	3.81(0.78)[d]	3.8(0.4)[cd]
Increase		287%	359%	537%	539%
Total P (mg P kg^{-1} soil)	90.0 (5.5)[a]	118.7 (8.6)[ab]	143.2 (5.7)[b]	211.10(14.96)[c]	277.0(21.9)[d]
Increase		132%	159%	235%	308%

Standard errors of the mean are in parentheses. Significant differences in nutrient concentrations between landscape elements are indicated by different superscript letters.

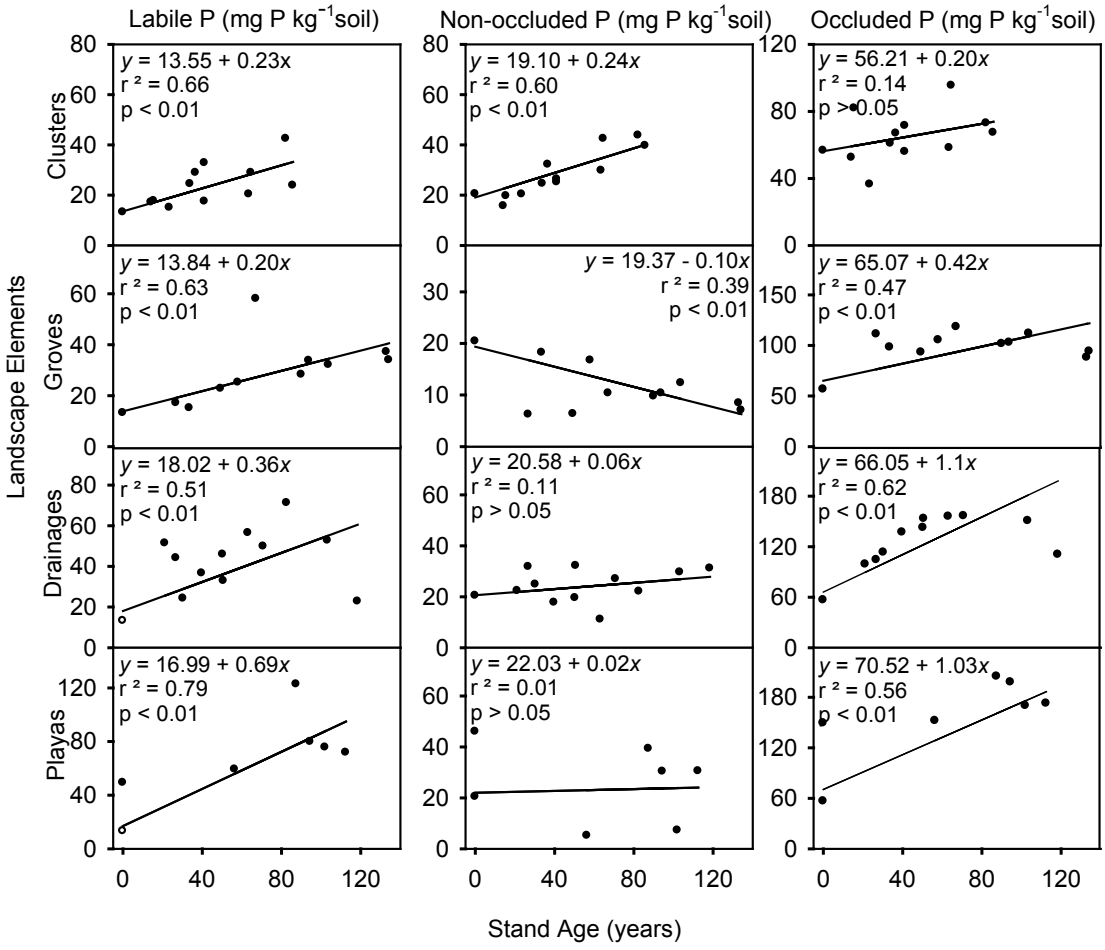

Figure 15. Changes in phosphorus concentration (mg P kg^{-1} soil) by availability category with respect to woody plant stand age at the La Copita Research Area. Labile P represents resin- and bicarbonate-extractable P, non-occluded P is hydroxide- and acid-extractable P. Soils samples were collected at 0-7.5 cm.

increased grassland<clusters<groves<drainages<playas. The average concentration of total soil P for whole soil (0-7.5 cm) increased between 132 and 308% with woody plant encroachment on the La Copita site, from 90.0 mg kg^{-1} soil in the remnant grasslands to 277.0 mg kg^{-1} in the playas (Table 6).

4.3.1 Phosphorus fractions in upland soils

Average plant-available, labile inorganic P (resin-extractable Pi) increased significantly from 3.4 mg kg^{-1} soil in grasslands to 9.0 mg kg^{-1} in clusters and 14.6 in groves, an increase of 262 and 409% (Table 7, Figure 16). Total bicarbonate-extractable P (labile, Pi + Po) increased significantly from 9.7 mg kg^{-1} in grasslands to 15.4 mg kg^{-1} in clusters and 15.7 mg kg^{-1} in groves. Total hydroxide-extractable P (Pi +Po) increased significantly from 20.4 mg kg^{-1} in grassland to 29.0 mg kg^{-1} in clusters, but decreased significantly to 10.6 mg kg^{-1} in groves. Acid-extractable Pi showed no significant trend, and individual samples occasionally showed higher acid-extractable P concentrations in grasslands than in clusters and groves. Residual P increased significantly in the groves, from 52.0 mg kg^{-1} in grasslands to 98.9 mg kg^{-1}, but the change in P was not significant between grasslands and clusters (61.3 mg kg^{-1}).

The proportion of extracted soil P in labile fractions (resin-extractable Pi and bicarbonate extractable Pi and Po) accounted for 14.6% of mean fractionated P (calculated from the sum of the individual P fractions) in remnant grasslands, and averaged 20.6% of total fractioned P in clusters and 21.2% in groves (derived from Table 7, Figure 17.) The non-occluded fractions (hydroxide-extractable Pi+Po and HCl-extractable Pi) summed to 27.6% of the grassland P and 27.8% of the cluster P, but only 9.8% of the grove P. The largest fraction in both upland wooded elements was the occluded, residual P which accounted for 58.0% of P in grasslands, 51.7 % of P in clusters, and 69.1% in groves. The organic fractions summed to 20.7% of the grassland P and 23.2% of cluster P, but only 8.2% of grove P (Figure 18). Pairwise t-tests indicated significant differences between grasslands and clusters for bicarbonate Pi+Po and hydroxide Pi+Po (Table 7). Grasslands and groves were significantly different for resin-

Table 7
Mean P concentrations (mg P kg^{-1} soil) by fraction in the 0-7.5 cm depth interval at the La Copita Research Area. Total P is the sum of the fractions. Parentheses indicate the standard error of the mean.

P fraction	Grassland	Cluster	Grove	Drainages	Playa
Labile P					
Resin Pi	3.4 (0.4)^{a}	9.0 (1.5)^{ab}	14.6 (2.6)^{bc}	22.2 (2.9)^{c}	45.5 (7.9)^{d}
Bicarb P	9.7 (0.3)^{a}	15.4 (1.6)^{b}	15.7 (1.4)^{b}	22.2 (1.7)^{c}	30.8 (2.9)^{d}
Bicarb Pi	8.3 (0.4)^{a}	11.1 (1.1)^{ab}	9.7 (1.4)^{a}	15.1 (1.8)^{b}	22.4 (2.9)^{c}
Bicarb Po	1.5 (0.3)^{a}	4.3 (0.9)^{ab}	6.0 (0.8)^{bc}	7.1 (1.3)^{bc}	8.4 (0.7)^{c}
Non-occluded P					
NaOH P	20.4 (2.1)^{a}	29.0 (2.9)^{b}	10.5 (1.3)^{c}	24.4 (2.0)^{ab}	26.4 (6.8)^{ab}
NaOH Pi	3.2 (0.4)^{a}	5.8 (1.0)^{b}	4.8 (0.2)^{ab}	4.5 (0.7)^{ab}	5.3 (0.9)^{ab}
NaOH Po	17.2 (2.0)^{a}	23.2 (2.5)^{a}	5.8 (1.3)^{b}	19.9 (1.8)^{a}	21.1 (7.1)^{a}
HCl	4.4 (0.5)^{a}	4.0 (0.8)^{a}	3.5 (0.5)^{a}	9.3 (4.7)^{a}	4.6 (1.2)^{a}
Occluded P					
Residual P	52.0 (4.3)^{a}	61.3 (4.3)^{a}	98.9 (2.9)^{b}	133.1 (8.3)^{c}	169.7 (28.3)^{d}
Total P	90.0 (5.5)^{a}	118.7 (8.6)^{ab}	143.2 (5.7)^{b}	211.1 (15.0)^{c}	277.0 (21.9)^{d}

Different letters indicate significant differences between the means for each row, $p<0.05$.

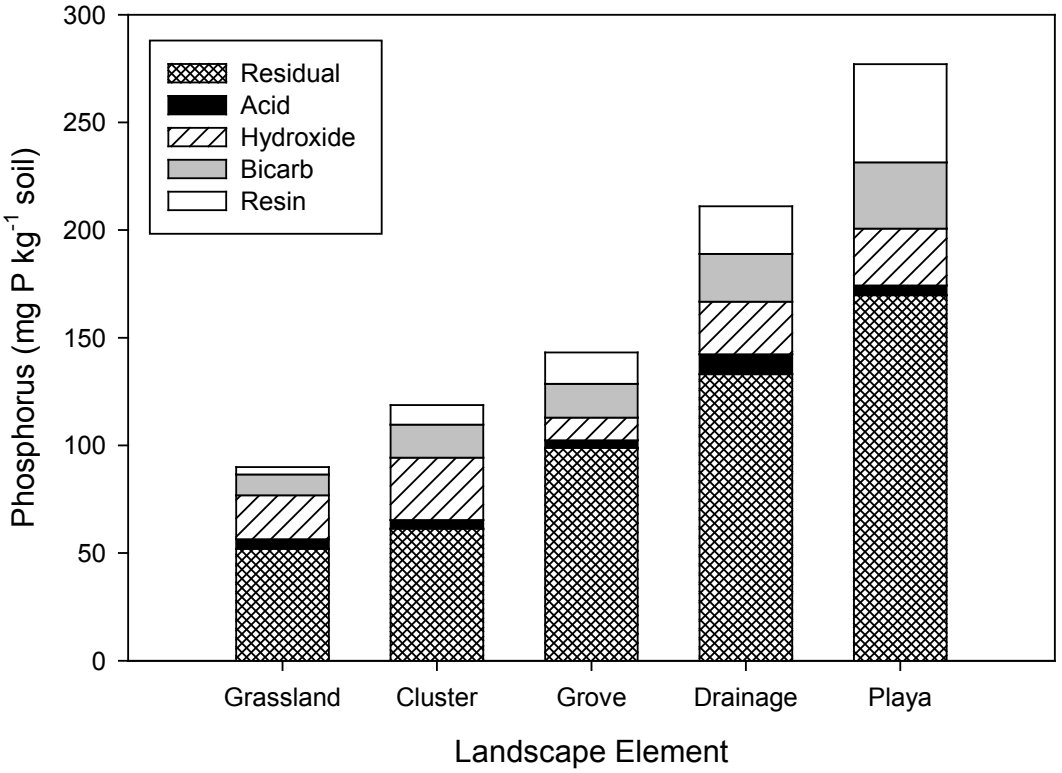

Figure 16. Soil total P and its distribution within P fractions (mg P kg^{-1} soil) for each landscape element (0-7.5 cm) at the La Copita Research Area.

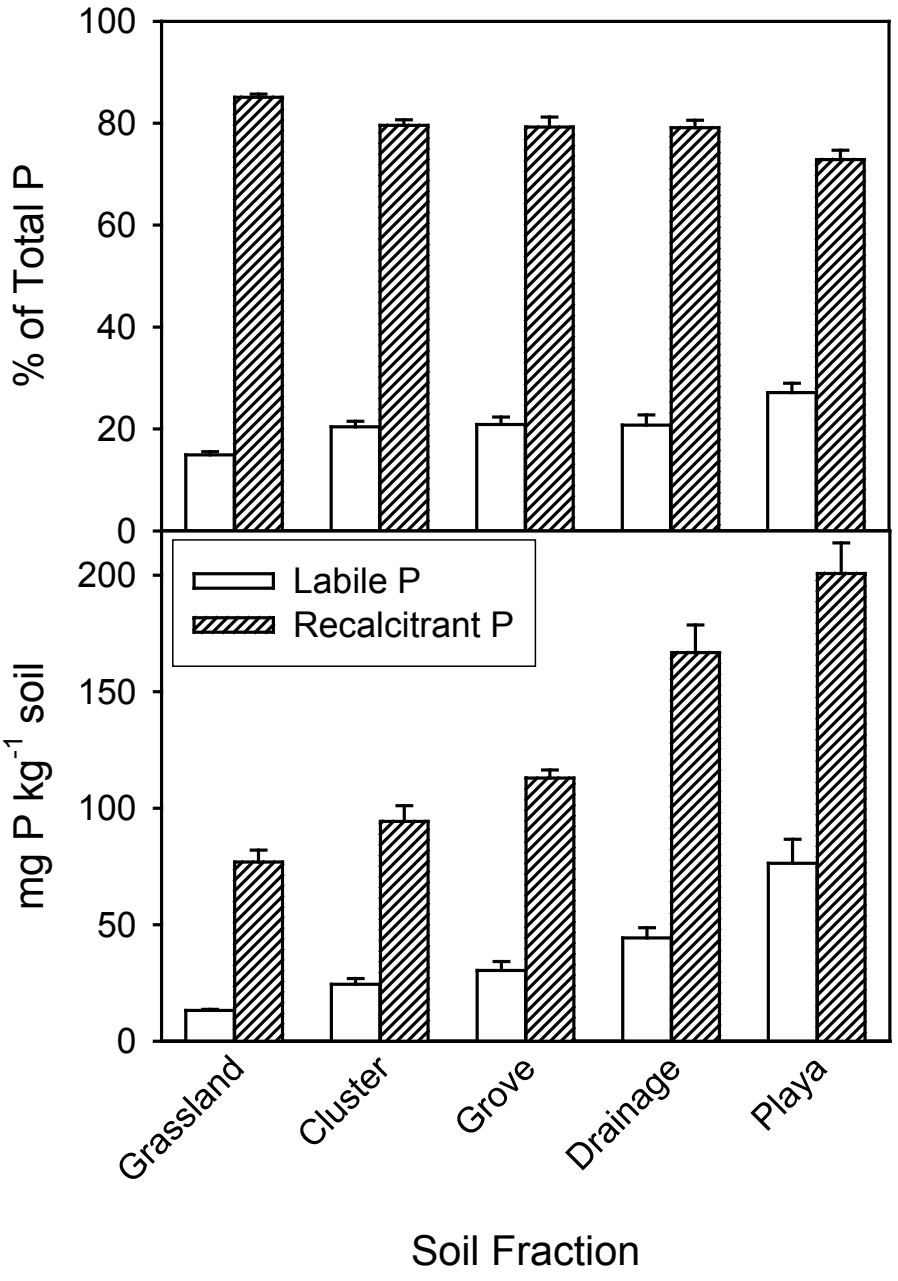

Figure 17. Comparison of proportion and concentration of labile (resin- and bicarbonate-extractable) and recalcitrant (hydroxide-extractable, acid-extractable, and residual) phosphorus (mg P kg^{-1} soil) found in 0-7.5 cm soil cores at the La Copita Research Area. Error bars are standard errors of the estimates.

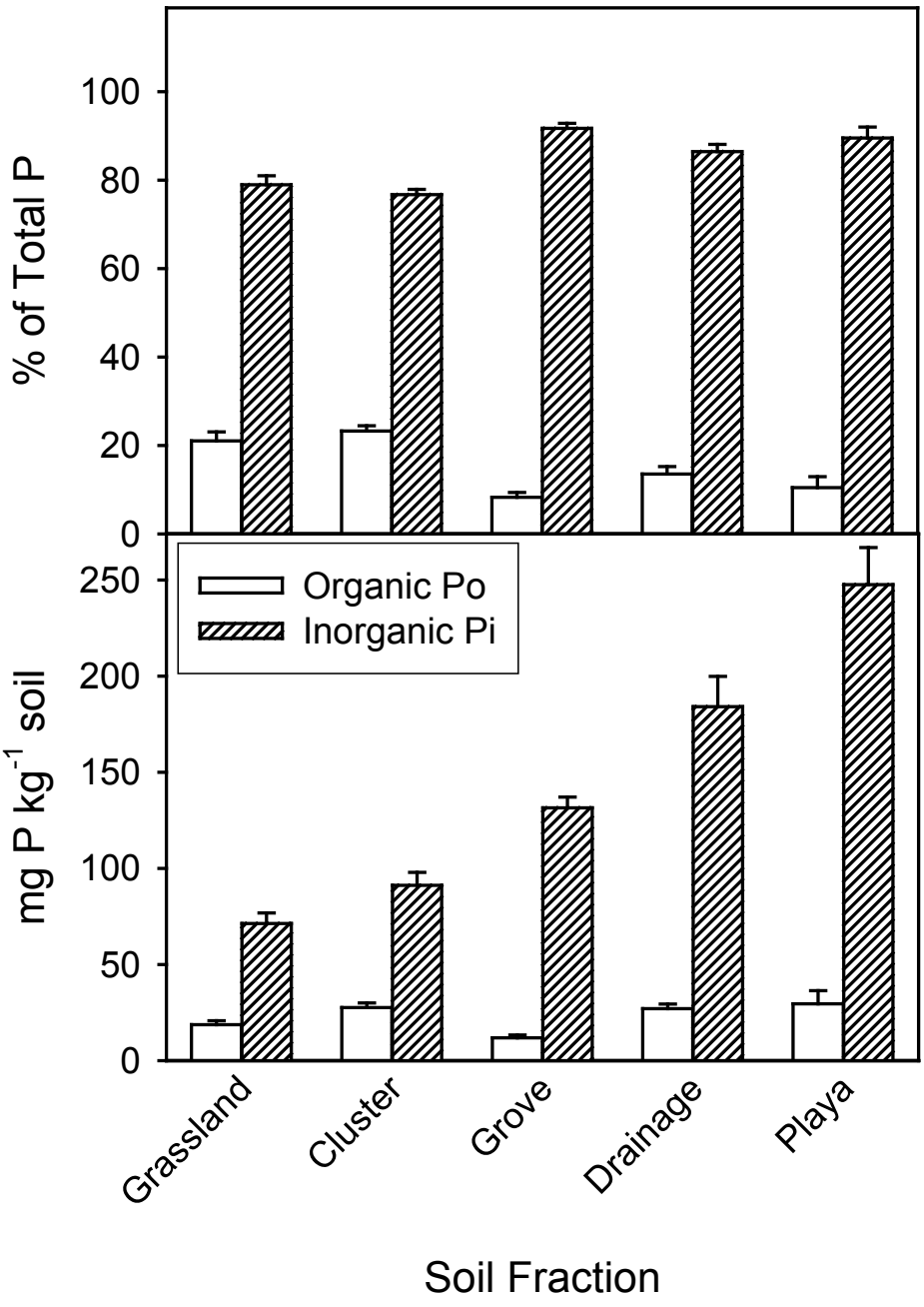

Figure 18. Comparison of proportion and concentration of organic (bicarbonate- and hydroxide-extractable P_o) and inorganic (P_i fractions) phosphorus (mg P kg^{-1} soil) found in 0-7.5 cm soil cores at the La Copita Research Area. Error bars are standard errors of the estimates.

extractable Pi, bicarbonate-extractable Pi+Po, hydroxide-extractable Pi+Po, and residual P. All fractions except HCl-extractable Pi showed increased phosphorus in clusters over the concentrations in grasslands. The change in HCl-extractable Pi was insignificant.

4.3.2. Phosphorus fractions in lowland soils

The average plant-available, labile inorganic P (resin-extractable Pi) increased significantly from 3.4 mg kg^{-1} soil in grasslands to 22.2 mg kg^{-1} in drainages and 45.5 mg kg^{-1} in groves, an increase of 644% in drainages and 1324% in playas, by far the largest single-fraction increase observed on the site (Table 16). Total bicarbonate-extractable P (labile, Pi + Po) increased significantly from 9.7 mg kg^{-1} in grasslands to 22.2 mg kg^{-1} in drainages and 30.8 mg kg^{-1} in playas. Total hydroxide-extractable P (Pi +Po) increases were not significant, from 20.4 mg kg^{-1} in grasslands to 24.4 mg kg^{-1} in drainages and 26.4 mg kg^{-1} in playas. Acid-extractable Pi showed no significant trend between the grassland and lowlands soils. Residual P increased significantly in the drainages and playas, from 52.0 mg kg^{-1} in grasslands to 133.1 mg kg^{-1} in the drainages and 169.7 mg kg^{-1} in the playas.

The proportion of extracted soil P in labile fractions accounted for 14.6% of total fractionated P in remnant grasslands, 21.0% of total fractioned P in drainages, and 27.5% in groves (derived from Table 7, Figure 17). The non-occluded fractions summed to 27.6% of the grassland P, and decreased to 16.0% of the drainage P and 11.2% in the playas. As in the upland soils, the largest individual fraction was the residual P, accounting for 63.0% of P in drainages and 61.3% in groves. The organic fractions summed to 12.8% of drainage P, and 10.6% of playa P, significantly lower than the 20.7% organic P measured in the grasslands (Figure 18). Pairwise t-tests indicated significant differences between grasslands and drainages for resin Pi, bicarbonate Pi+Po and residual P (Table 7). Grasslands and playas were significantly different for resin Pi, bicarbonate Pi+Po, and residual P. All fractions except HCl-extractable Pi showed increased phosphorus in clusters over the concentrations in grasslands. The change in HCl-extractable Pi was insignificant.

4.4. Organic phosphorus in extractable fractions

Organic P accounted for 15-28% of total bicarbonate-extractable P (Table 7, Figure 18). The organic P concentration increased more quickly and to a larger extent than inorganic P in the bicarbonate-extractable fraction. With woody encroachment, the ratio of organic to inorganic P in the bicarbonate-extractable fraction increased from $^1/_8$ to nearly $^1/_3$. The differences in total P in the hydroxide-extractable fraction were significant only for the upland landscape elements, an increase occurred in the cluster soils while the concentration of P in groves decreased. Organic P comprised 85% of the hydroxide-extractable P in grasslands and 90-82% clusters, drainages, and playas. In the groves, where the concentration of hydroxide-extractable P was lower than in the grasslands, organic P was 55% of total P.

5. Discussion

5.1 Effects of woody encroachment on the landscape

The effects of woody plant invasion vary with the characteristics of the invaded ecosystem and the invasive species themselves, but on a global scale a number of trends hold true: encroachment by woody species has resulted in increases in NPP, organic matter turnover, and acquisition and cycling of soil nutrients (Hibbard et al., 2001; Köchy and Wilson, 2000; Liao et al., 2006a; Liao et al., 2008; Wheeler et al., 2007). The functional differences between grasses and N-fixing woody plants enhance the effects of woody plant encroachment on soil nutrient cycles, particularly N (Liao et al., 2008). Growing insight into linkages between P and N cycles in terrestrial ecosystems (Chapin et al., 2002; Wang, Y.-P. et al., 2007; Vitousek et al., 2010) indicate that P is as essential as N in encroachment by N-fixing species.

Though research in other landscapes has indicated that increases in soil organic matter lead to decreased pH through organic decomposition, the soils at La Copita do not differ significantly in pH between grasslands and wooded landscape elements (Brady and Weil, 2007; Dossa et al., 2010; Table 5). Bulk densities of the surface soils at La

Copita decrease with increased organic matter concentrations, illustrating the effects of fresh and decomposing roots and litter on the surface soil (Archer et al., 2001; Boutton et al., 1999, 2009; Hibbard et al., 2001; McCulley et al., 2004; Liao et al., 2006a). The quality of organic matter differs between the grasslands and woody plants due to the concentrations of secondary compounds (lignins, cutins, and tannins), altering the rates at which organic matter is decomposed in the woody plant landscapes (Köchy and Wilson, 1997; Filley et al., 2008). The soils under woody plants also conserve organic matter through aggregation, as evidenced by water stable soil aggregate fractionation of grassland and cluster soils (Liao et al., 2006a; Chapter 2). This relationship has been observed elsewhere in soils that are stabilized by organic material (Oades and Waters, 1991).

Both soil and plant type may affect the rates of nutrient accumulation during woody plant encroachment (Köchy and Wilson, 2001; Dossa et al., 2010; Lorenzo et al., 2010; Scharenbroch et al., 2010). At La Copita, clusters, groves, and drainages support similar combinations of mesquite and woody species, while playas host mesquite alone. The presence of mesquite and N-fixing species is critical to C and N accumulation in the soil; however,the effect of individual species on P accumulation remains to be investigated. Unlike previous studies which examine differences between invading species (Dossa et al., 2010) or the effects of woody plant encroachment across a range of soil types (Cross and Schlesinger, 1995, 2001), this study compares the effects of four examples of woody plant encroachment within a single ecosystem under increasing degrees of encroachment.

5.2. Changes in whole soils carbon, nitrogen, and phosphorus

Woody plant invasion resulted in linear increases in concentrations of C, N, and P over time in whole soils across all landscapes at La Copita (Table 6, Figure 14). Increases in average C and N concentrations between grasslands and wooded landscape elements were significant for all elements. The increase in total P was significant for groves, drainages, and playas when compared with remnant grasslands, though not for

grasslands compared to clusters. For all nutrients, the largest increases in concentration were between the grasslands and the lowland landscape elements, the drainages and playas. Carbon, N, and P concentrations were not significantly different between clusters and groves, indicating that the upland soils respond similarly to the input of organic matter from woody plants. Higher concentrations of C, N, and P in lowland landscapes (drainages and playas) compared to uplands was likely due to the higher clay content of the soil (Table 5), which conserves soil water and slows decomposition of organic matter.

Carbon and N accumulation in woody-encroached ecosystems is attributed to increased ANPP; the large biomass of woody species incorporates more atmospheric C and N than grass tissues, creating a larger pool of organic matter cycling in the system through litterfall and root turnover (Hibbard et al., 2001; McCulley et al., 2004; Liao et al., 2006a; Liao et al., 2008). Measurements of soil microbial biomass C and N confirm increases in the organic C and N pools (McCulley et al., 2004). Carbon and N mineralization rates contrast with mean residence times, indicating that both slow and fast-turnover C and N pools are increasing in size under mesquite vegetation (McCulley et al., 2004; Liao et al., 2006b). Though the P cycle in woody encroachment is less well understood, linkages between C, N, and P cycles and the ability of plants to acquire P from the soil environment indicate that plant tissue is the key driver of P cycling (Jobbagy and Jackson, 2001).

5.3 Effects of woody plant encroachment on P fractions

In all vegetation classes, the largest P fraction increases on a percentage basis were observed in the labile P fractions (Figure 17). Resin-extractable P and bicarbonate-extractable P represent the plant-available P pools, which are rapidly cycled between plants and microbes in the soil (Cross and Schlesinger, 1995). The concentration of labile P at La Copita in grasslands is similar to Mollisol values reported by Cross and Schlesinger (2001), but in contrast with desert soils, labile P increased under woody vegetation at La Copita. As microbes are responsible for liberating P from organic

compounds (Parton et al., 1988), the larger labile P pool suggests a larger or more active microbial community in the La Copita soils than the desert soil, and an increasing effect of biological cycling as woody encroachment advances on the site.

The recalcitrant P pool consists of all fractions that are not labile and plant-available: the hydroxide-extractable, acid-extractable, and residual P. Between 70 and 85% of the phosphorus in the 0-7.5 cm soil depths at La Copita is in recalcitrant forms (Figure 17). Increases in P concentration were measured in the hydroxide-extractable organic fraction, the largest non-residual fraction, which represents the majority of the Fe- and Al-adsorbed organic phosphates (Cross and Schlesinger, 1995; Dossa et al., 2010). Organic P accounted for 9-21% of total P, and concentrations were significantly higher than grasslands in clusters, drainages, and playas. Increased organic phosphates in the lowland soils are likely related to the increase in clay in the playa and drainage soils, as there is no apparent difference in Fe or Al concentrations between the landscape elements at La Copita. Surprisingly, the hydroxide-extractable pool in the groves was smaller than the other woody elements. While no explanation can be reached for this phenomenon in the upper 7.5 cm of soil, groves differ from the other elements by virtue of the lack of argillic horizon at around 30-cm depth and lower clay content than grassland and cluster soils (Table 5). This difference in soil characteristics could indicate that soil nutrients are less confined to the shallow soils in groves than in other landscape elements.

In desert soils, the largest fraction is frequently the acid-extractable pool, which represents P bound to $CaCO_3$ in the soil. However, at La Copita, the absence of soil carbonates makes the acid-extractable pool the smallest of the fractions and the least-affected by woody encroachment. Recalcitrant P is liberated from the soil by geochemical processes; therefore the Hedley method of P fractionation indicates that 70-85% of the soil P at La Copita will become available to the system at rates of decades to centuries (Cross and Schlesinger, 1995). However, as the duration of mesquite presence on the site the increases, so does the size of the labile phosphorus fractions (resin-

extractable Pi, bicarbonate-extractable Po and Pi), and biological nutrient cycling plays a larger role in the cycling P in the soil (Figure 17).

5.4. Coupling of the nitrogen and phosphorus cycles

In all landscape elements, the percent increases in C and N were similar, while the increases in total P were significantly lower. The slower rate of P increase is likely due to limited stocks of P for cycling by woody plants. Both C and N are atmospherically available nutrients, while the overall supply of P in the soil is largely unaffected by small amounts of atmospheric deposition of P-containing particles (Mahowald et al., 2008). The P stocks of the soil system are essentially finite: new labile P becomes available in the soil at the rate of weathering of P-containing minerals in the soil, but there are few external sources of P (Walker and Syres, 1976). The distribution of P can be altered by plant roots taking up nutrients from deeper in the soil profile and redistributing them at the surface in plant litter. It has been proposed that mesquite, as an N-fixing species, is adapted for P acquisition. N-fixing plants require P at higher rates than grassland species in order to have the energy to fix atmospheric N, and in return they allot a portion of the fixed nitrogen for the production of P-liberating phosphatase enzymes (McGroddy et al., 2004; Wang, Y.-P. et al., 2007; Houlton et al., 2008; Vitousek et al., 2010). Phosphorus measurements at La Copita indicate that mesquite has a high capacity for P acquisition and translocation, most likely from the deeper soil below the rooting depth of grassland species. The linear trend in nutrient increases with time following woody encroachment indicates a steady transfer of P to the soil surface. No lag in nutrient increase can be observed after 14 years, and there is no sign of slowing of the rate of nutrient increase up to 134 years. The continued growth of soil P pools in the shallow soil indicates that the source of P has not yet been depleted.

6. Conclusions

Woody plant encroachment increases the size of soil pools of C, N, and P in the upper 7.5 cm of soil under four distinct vegetation regimes in Texas. Significant increases occurred in all landscape elements, though changes were most dramatic in the

lowland, finer-textured soils. This study indicates that there are subtle variations in the effect of woody plant encroachment on grassland soils within ecosystems. Under the same species of invading woody plants, the accumulation of soil nutrients increases with time following encroachment, and clay content. Additionally, the distribution of soil P fractions shifts toward more labile, plant-available forms of P as woody encroachment progresses. Regressions indicate that rates of accumulation of C, N, and P remain linear throughout the 135-year chronosequence in all landscape elements, and do not appear to be reaching equilibrium at the present time. While the global trends of increasing nutrient stores and nutrient availability with woody plant encroachment hold true, this study shows that the magnitude of responses to woody vegetation are variable within this ecosystem due to variation in soil characteristics and vegetation patterns. The individual characteristics of grasslands must be evaluated when predicting the ecosystem response to woody plant encroachment.

CHAPTER V

COMPOSITION AND DIVERSITY OF SOIL MICROBIAL COMMUNITIES FOLLOWING VEGETATION CHANGE FROM GRASSLAND TO WOODLAND: AN ASSESSMENT USING MOLECULAR METHODS

1. Synopsis

Woodlands dominated by N-fixing tree legumes have largely replaced grasslands in many regions of the world over the last century. This vegetation change has been linked to significant increases in above- and belowground primary productivity, soil C and N storage, and the size and activity of the soil microbial biomass pool following woody plant proliferation in areas that were once grassland. The relationships and interactions between plant communities and soil microbial communities are of particular importance to nutrient cycling, organic matter turnover, and carbon sequestration on these sites. Research was conducted on plots in a successional chronosequence of grassland to mesquite woodland representing the stages of woody plant encroachment. Remnant grasslands representing the historic vegetation of the site or original condition (time 0) and woody plant stands differing in age (approx. 10-100 years) were sampled for analysis of microbial characteristics. Ages of woody plant stands were determined by dendrochronology and aerial photography. Bacterial and fungal community DNA was extracted from whole soil, PCR amplified, and the microbial diversity analyzed with quantitative polymerase chain reaction (qPCR) and automated ribosomal intergenic spacer analysis (ARISA) for each of five vegetation-based landscape elements: remnant grasslands, mesquite clusters, mesquite groves, closed-canopy drainage woodlands, and playas. Pyrosequencing was performed on samples from grasslands, clusters, groves and drainages. Variation in nutrient pools under similar vegetation elements indicates non-vegetative components of the plant-soil continuum are altering below-ground dynamics. Non-metric multi-dimensional scaling of ARISA data confirmed a separation of grassland bacteria and fungi from the flora of the wooded landscape elements. Pyrosequencing of replicate samples from four of the landscape elements revealed

microbial identities and differences in species composition in both bacterial and fungal communities between the grassland and wooded landscape elements.

2. Introduction

Grassland ecosystems around the world are being chemically and physically altered by woody plant encroachment as the phenomenon spreads around the globe. Woody plant encroachment has expanded to encompass every continent except Antarctica over the last 150 years (Archer et al., 1988; Hudak and Wessman, 1998; Van Auken, 2000; Allison et al., 2006; Maestre et al., 2009; Lorenzo et al., 2010). In many ecosystems, livestock grazing and suppression of natural fire cycles are the primary drivers of grassland loss and woody plant expansion (Scholes and Archer, 1997; Van Auken, 2000; Roques et al., 2001). The success of woody plants in former grasslands is also associated with atmospheric chemical changes and global climate variation, which relate to rising concentration of atmospheric CO_2 and increased N deposition (Archer, 1994, 2001; Polley et al., 1994; Köchy and Wilson 2000, 2001; Van Auken, 2000; Knapp et al., 2008). Studies in woodlands, tropical forest, arctic tundra, and savannas have demonstrated that within a site, differences in soil physical and chemical characteristics can be attributed to herbaceous and woody vegetation regimes (Padien and Lajtha, 1992; Nusselein and Tiedje, 1999; Wallenstein et al., 2007; Dossa et al., 2010).

Woody plant encroachment increases aboveground NPP in most invaded grassland systems, increasing pools of nutrients through sequestration in plant tissues. In turn, belowground nutrient and organic matter pools are increased through litter and root turnover (Padien and Lajtha, 1992; Hibbard et al., 2001; McCulley et al., 2004; Liao et al., 2006a; Wheeler et al., 2007; Knapp et al., 2008; Liao et al., 2008; Maestre et al., 2009). Islands of fertility form around woody plants as nutrients accumulate beneath the shrub canopy (Padien and Lajtha, 1992; Schlesinger and Pilmanis, 1998; Hibbard et al., 2001). These “islands” also produce heterogeneity in the soil environment through shading, temperature moderation, changes in soil pH, and redistribution of soil moisture (Schlesinger et al., 1990; Schlesinger and Pilmanis, 1998; Maestre et al., 2009). The

larger rhizophere of woody plants compared to grass species increases the zone of contact between roots and soil, fostering the exchange of nutrients and root exudates (Canadell et al., 1996; Jackson et al., 1996). Belowground microbial communities reflect differing nutrient inputs and soil conditions under contrasting vegetative cover (Nusselein and Tiedje, 1999; Fierer and Jackson, 2006; Wallenstein et al, 2007; Lauber et al., 2008; Biederman and Boutton, 2009, 2010; Lorenzo et al., 2010; Scharenbroch et al., 2010).

At the Texas AgriLife La Copita Research Area in southern Texas, woody plant encroachment takes the form of N-fixing mesquite (*Prosopis glandulosa*) woodlands replacing coastal prairie, converting the dominant vegetation from C_4 annual species to nitrogen-fixing C_3 perennials (Boutton 1998, 1999; Archer, 2001). Current vegetation patterns, sequential aerial photography, tree ring analyses, and stable isotope measurements of soil organic matter all indicate that open grasslands at La Copita have been largely replaced by woody vegetation on the site during the past century (Archer et al., 1988, 2001; Boutton et al., 1999). At La Copita, C, N, and P pools are larger in the soils beneath woody plant species compared to the remnant grasslands due to increases in aboveground and belowground biomass production. Wooded sites show nutrient increases of 100-500% and significantly lower bulk density than remnant grasslands (Chapters II-IV; Hibbard et al., 2001; McCulley et al., 2004; Liao et al., 2006a). Microbial biomass C and N pools increase in response to the introduction of woody species, as do C and N mineralization rates (Hibbard et al., 2001; McCulley et al., 2004; Liao et al., 2006b; Liao and Boutton, 2008). Measurements of soil respiration rates indicate that soil microbial communities respond to changed litter inputs: both foliar and root litter inputs from woody plants are more chemically recalcitrant than grassland vegetation (McCulley et al., 2004; Liao et al., 2006b; Filley et al., 2008).

In the case of woody encroachment in Texas, examining he changes in the microbial community under different of woody plant encroachment is essential to understanding the influence mesquite is having on the landscape. The objective of this study is to characterize the microbial community in the soils of contrasting landscape elements in a

mesquite-invaded south Texas grassland in order to observe differences in the communities based on soil type and vegetation pattern. The development of molecular methods for microbial characterization in the past few decades has allowed scientists to make culture-independent surveys of soil microbial communities (Janssen, 2006.) While still limited compared to the vast diversity of soil microbes, PCR-based community libraries present a larger picture of the belowground drivers of organic matter turnover and nutrient cycling than traditional culturing techniques alone. This study will compare the structure and diversity of the bacterial and fungal communities using automated ribosomal intergenic spacer analysis, quantitative PCR, and 454 pyrosequencing of the 16s rRNA gene and large ribosomal subunit gene. We hypothesized that changes in the vegetation communities would be reflected in the composition of the microbial communities of the soil, indicating changes in community function as the microbial community adapts to changing composition of substrates and quantity of nutrients. Increased understanding of the long-term ecosystem changes during the mesquite invasion will influence land management decisions and contribute to model forecasting of the effects of global climate change on the region.

3. Materials and Methods

3.1. Study site

Soil samples were collected at the La Copita Research Area near Alice, Texas (27'40'N, 98'12'W), in the Rio Grande Plains of southern Texas in the spring of 2007. The climate at the site is subtropical with mean annual temperature of 22.4 ºC and mean annual precipitation of 716 mm. Elevation ranges from 90 m in the uplands (grasslands, clusters, and groves), descending by 1-3% slopes to about 75 m in the lowlands (drainages and playas.) Soils are Typic and Pachic Argiustolls of the Runge series with a relatively continuous argillic horizon at 30-40 cm in the upland zones and Pachic Argiustolls in the lowlands (Boutton et al., 1998). Typical grassland species include *Bouteloua, Chloris, Panicum, Tridens, Eragrostis,* and *Paspalum*, while the woody clusters are *Prosopis gladulosa,* (honey mesquite) with *Condalia hookeri* M.C. Johnst.,

Zizyphus obtusifolia (T.&G.) Gray*, Zanthoxylum fagara* (L.) Sarg.*,* and *Berberis trifoliolata* beneath the mesquite canopy (Archer et al., 1988).

The La Copita Research Area is comprised largely of five distinct landscape elements distinguished by vegetation structure, topographic position, and soil characteristics: (1) remnant C_4 grasslands which represent the historic vegetation of the area; (2) small woody clusters consisting of a single mesquite tree and associated non-legume woody C_3 species; (3) larger groves of multiple mesquite and associated C_3 woody species; (4) closed-canopy drainage woodlands; and (5) playas, where mesquite trees grow in moist soils without the accompaniment of other woody plants. Grasslands, clusters, and groves occur in upland topographic positions on sandy loam soils, while drainage woodlands and playas occupy the lowest portions of the landscape on sandy clay loam and clay loam soils (Table 8). Grassland soils have significantly higher bulk density than soils beneath woody plant stands. Upland soils are significantly higher in sand and lower in clay than the lowland soils. The lowland soils (drainages and playas) were significantly lower pH than grasslands, but were not significantly different than cluster and grove soils (Table 8; Liao et al., 2006a, Liu, F. et al., 2010).

3.2. Sample Collection

In order to characterize changes in the soil microbial community composition in response to woody plant encroachment over time, a space-for-time chronosequence approach was utilized. On this site, the formation of mixed-species wooded landscape elements was initiated only after the establishment of mesquite in grassland (Archer et al., 1988). Thus, the age of a woody plant stand corresponds to the age of the largest mesquite tree in that stand. The ages of mesquite trees were determined by measuring their basal diameters and then substituting those values into regression equations to predict tree ages, using equations specific to each landscape element (Stoker, 1997). Woody plant stands sampled in each of the landscape elements (clusters, groves, drainages, and playas) were selected to encompass the full range of mesquite basal

Table 8
Representative soil physical and chemical characteristics of surface soils (0-15 cm) of five landscape elements at the La Copita Research Area. Data from Liao et al., 2006a, Liu, F. et al., 2010.

	Grassland	Cluster	Grove	Drainage	Playa
pH	6.5(0.1)	6.4(0.06)	6.3(0.1)	6.2(0.1)	6.2(0.1)
Bulk Density (g cm^{-3})	1.2(0.02)	1.0(0.03)	1.0(0.03)	1.0(0.04)	1.2(0.03)
Texture	Loamy Sand	Loamy Sand	Loamy Sand	Sandy Loam	Sandy Clay Loam
Sand (%)	80.5(0.4)	81.3(0.5)	81.4(0.6)	66.0(3.0)	52.5(1.3)
Silt (%)	10.5(2.0)	9.6(1.6)	12.0(1.3)	15.4(2.7)	19.0(0.5)
Clay (%)	9.0(2.0)	9.1(1.6)	6.6(1.3)	18.6(1.9)	28.5(1.0)

Standard errors of the mean are in parentheses.

diameters, corresponding to tree ages ranging from approximately 14 to 130 yr. Samples from remnant grasslands represent the grassland ecosystems that dominated the region prior to woody plant invasion (Time 0).

Soil cores (5 cm diameter x 7.5 cm deep) were collected from five landscape elements: grasslands (n = 10), clusters (n = 10), groves (n = 11), drainage woodlands (n = 11), and playas (n = 6). In the wooded landscape elements, four cores were collected from around the bole of the oldest mesquite tree in each stand; in grasslands, four cores were collected around the base of a dominant perennial grass plant. The four cores collected at each sampling location were combined and mixed in storage bags immediately in the field. Samples were then frozen and transported to Texas A&M University on dry ice to minimize community shifts and/or DNA degradation during transit. The soils were stored frozen at -20 ºC.

3.3. Elemental analyses of soil carbon, nitrogen, and phosphorus

Aliquots of whole soil were oven dried at 50° C and finely ground in a centrifugal mill prior to analysis. Elemental analyses of carbon and nitrogen in whole soils were performed on a Carlo Erba EA-1108 elemental analyzer (CE Elantech, Lakewood, NJ, USA) interfaced with a Finnigan Delta Plus isotope ratio mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) operating in continuous flow mode (Ehlringer and Rundel, 1989).

Total P analyses of whole soils were performed by lithium fusion as described in Chapter 3. Briefly, finely ground soil was mixed with lithium metaborate ($LiBO_4$), fused in a 1000 ºC muffle furnace, dissolved in 5N HNO_3, and the solution analyzed for total phosphorus concentration by a modified molybdenum blue method (Murphy and Riley, 1962; Dick and Tabatabai, 1977; Lajtha et al., 1999; Dossa et al., 2010) measured against a potassium phosphate reference curve on a Spectronic 20D+ (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

3.4. Soil DNA Extraction

DNA was extracted from the 7.5-cm soil cores collected in grasslands, clusters, groves, drainage woodlands, and playas. Community DNA was extracted from each soil sample with a MoBio PowerMax Soil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA). Aliquots (5 g) of each soil were prepared according to the manufacturer's protocol. The DNA concentrations were quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and diluted to a standard concentration. Samples were frozen for subsequent analysis by ARISA, pyrosequencing, and qPCR.

3.5. Community q-PCR analyses of soil DNA

Relative bacterial and fungal abundances in soil DNA were quantified by community q-PCR assays on soil DNA extracted from soils in woody plant stands along the vegetation chronosequence described above. qPCR was performed according to Fierer et al (2005) with primer set EUB338/518 for bacteria and ITS1f/5.8s for fungi (Boyle et al., 2008). Each assay was performed in triplicate in an Eppendorf Mastercycler Realplex thermocycler (Eppendorf, Westbury, NY, USA). Each 10 µl reaction contained 4.5 µl 2.5x RealMasterMix with 20x SYBR Green solution (5Prime, Inc., Gaithersburg, MD, USA), 0.5 µl of each primer (10 µM), 1.0 µl BSA (10 mg ml^{-1}), 2.5 µl molecular grade water, and 1.0 µl template DNA (2.5 ng μl^{-1}). The thermocycling conditions consisted of 15 minutes of initial denaturation at 95 °C, followed by 40 cycles of 95 °C for 1 min and 53 °C for 30 s, and 72 °C for 1 min (Fierer et al., 2005). Plasmid standards for bacteria were generated from *Escherichia coli* genomic DNA (obtained from Carlos Gonzales, Texas A&M University). Fungal standards were generated from *Neurospora crassa* (obtained from Heather Wilkinson, Texas A&M University). Standards were prepared according to Fierer et al. (2005). Concentrations of DNA were converted to copy numbers of genes as described by Smits et al (2004).

3.6. ARISA analyses of soil DNA

Soil rRNA genes were amplified for ARISA using the general bacterial primers 27F and 1492R (Cardinale et al., 2004) and fungal primers 2234C and 3126T (Ranjard et al., 2001). The forward primers were labeled with HEX (6-carboxy-1,4-dichloro-2',4',5',7'-tetra-chlorofluorescein). PCR reactions were optimized using a FailSafe PCR Enzyme kit (Buffer B; Epicentre, Illumina, Madison, WI) and performed in an Eppendorf MasterCycler after a hot start at 94 ºC for 3 min, followed by 30 cycles of denaturation at 94 ºC for 1 min, 55 ºC for 30 s, and 72 ºC for 1 min, and extension of incomplete products for 5 mins at 72 ºC (Ranjard et al., 2001). Amplifications were performed in triplicate, and products were combined for downstream use.

Frozen standardized DNA samples were shipped to the Research Technology Support Facility at Michigan State University (MSU) for automated ribosomal intergenic spacer analysis (ARISA) of the 16S and internal transcribed spacer (ITS) regions of the bacterial and fungal rRNA genes respectively (Fisher and Triplett, 1999; Ranjard et al., 2001; Cardinale et al., 2004; Kennedy et al., 2005). Reactions contained 1-3 µl of the PCR product and 0.5 µl of internal size standard (GeneScan 1000-pb ROX; Applied Biosystems, Foster City, CA, USA) were denatured in deionized formamide for 5 min and size-separated by capillary electrophoresis. The electrophoregrams created from the fluorescence of the sample represented the fragment lengths and the proportion of total PCR product of each length. The ARISA fragments were analyzed by the GeneScan 3.1 software program at MSU (Perkins-Elmer, Waltham, MA, USA).

3.7. Pyrosequencing of soil DNA

Pyrosequencing was performed by the Research and Testing Laboratory (Lubbock, TX) on twelve soil samples from grasslands, clusters, groves, and drainage woodlands according to Roche 454 Titanium chemistry protocols (Acosta-Martinez et al., 2008). Samples were amplified with primers 27F and 519R for bacteria (Somenahally et al., 2011) and primers ITS1F and ITS4 for fungi (Amend et al., 2010). Sequences were aligned using BioEdit (Ibis Biosciences, Carlsbad, CA, USA) and the RDP Pipeline

(Ribosomal Database Project, Cole et al., 2007) and analyzed in MOTHUR (mothur.org, Schloss et al., 2009). Identities were assigned to the sequences by the RDP 16S Bacterial Classifier and the LSU Fungal Classifier (Wang, Q. et al., 2007) (accessed 18 October, 2011).

Alignment of 16S and LSU sequences was performed using the MOTHUR and RDP pipeline (mothur.org, Schloss et al., 2009; Ribosomal Database Project, Cole et al., 2007). Bacterial and fungal diversity statistics were calculated using operations in MOTHUR. Pairwise community comparisons, principal components analysis, and non-metric multidimensional scaling were performed in PAST and JMP. The Classifier function of the RDP was used to characterize the bacterial and fungal sequence libraries. Distance matrices were calculated in MOTHUR using the dist.seqs function. Bacterial and fungal sequences showing at least 97% sequence identity to existing sequence libraries were defined as operational taxonomic units (OTUs) using MOTHUR (Rousk et al., 2010; Lekberg et al., 2012).

3.8. Data analyses

Linear regression was used to determine relationships between C, N, and P concentrations in soil fractions and increasing woody plant stand age. ANOVA was used to demonstrate differences between soil physical fraction distribution, C:N:P ratios, and nutrient concentrations of grasslands and woody landscape elements using JMP software (SAS, Institute Inc., Cary, NC, USA). Non-metric multidimensional scaling of the ARISA and 454 pyrosequencing data was used to compare the composition of the bacterial and fungal communities in each of the landscape elements.

4. Results

4.1. Soil characteristics

Soil organic C, total N, and total P were all significantly higher in the wooded landscape elements than in the grasslands, and concentrations increased with mesquite age (Figure 19). The soil C concentration of clusters was not significantly different from that of

groves, though both were significantly lower than drainages and playas. All landscape elements showed significantly different concentrations of N and P. The C, N, and P increased with mesquite age when compared to grassland soils (Chapter 1).

4.2. Community qPCR results

Bacteria were numerically dominant, as compared to fungi, on the basis of gene copies detected by community qPCR in the La Copita soils. Bacteria accounted for >96% of rRNA gene copies in all landscape elements. Bacterial 16S copy numbers varied less than two-fold across the landscapes, with the lowest numbers being in the grasslands and the highest numbers in the clusters (Table 9). Fungal ITS copy numbers ranged two-fold, with the lowest numbers being in the drainages and the highest numbers in the clusters. The qPCR analysis of fungal to bacterial ratios showed that clusters had the highest fungal:bacterial ratio at 0.034 (Table 9, Figure 20). This ratio was not significantly different from that of the grasslands and groves at 0.026 and 0.028. The fungal:bacterial ratio of drainages was significantly lower than all other landscape elements at 0.017.

4.3. Bacterial and fungal community composition - ARISA results

A total of 1453 bacterial fragments were produced with ARISA, with 13 to 56 unique fragments per sample site, and 1230 total fungal fragments, with 19 to 37 unique fragments per sample site. Diversity statistics and richness estimations performed on the results reflect differences in library size between the grasslands and the mesquite clusters, and could not be used to determine differences in the microbial communities. The number of unique fragments isolated from grasslands and woody clusters did not vary significantly by landscape element.

NMDS analysis was performed to determine the relatedness of the bacterial and fungal communities in the different landscape elements (Fig. 21). While the bacterial samples were not clearly divided by any one landscape element, the fungi results

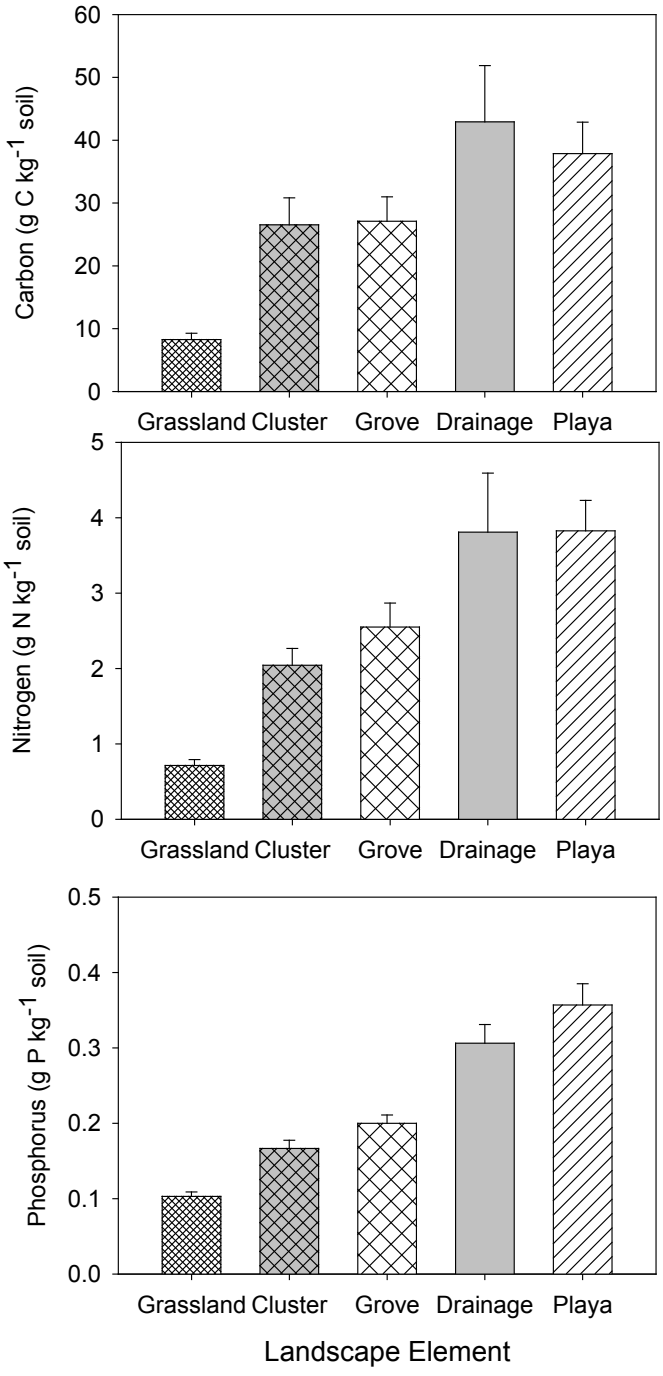

Figure 19. Average carbon (g C kg^{-1} soil), nitrogen (g N kg^{-1} soil) and phosphorus (g P kg^{-1} soil) concentrations in whole soils for grasslands, clusters, groves, drainages, and playas at the La Copita Research Area. Error bars represent the standard error of the mean.

Table 9
Results from qPCR sampling of four landscape elements at the La Copita Research Area. The abundances of bacteria and fungi are represented as average gene copies per nanogram DNA plus or minus the standard error of the mean.

	Grassland	Cluster	Grove	Drainage
Bacteria	$1.09\pm0.09 \times 10^6$	$2.12\pm0.25 \times 10^6$	$1.17\pm0.04 \times 10^6$	$1.35\pm0.07 \times 10^6$
Fungi	$2.80\pm0.26 \times 10^4$	$7.11\pm1.24 \times 10^4$	$3.22\pm0.63 \times 10^4$	$2.33\pm0.29 \times 10^4$
Fungal:bacterial ratio	0.026±0.001	0.034±0.006	0.028±0.006	0.017±0.001

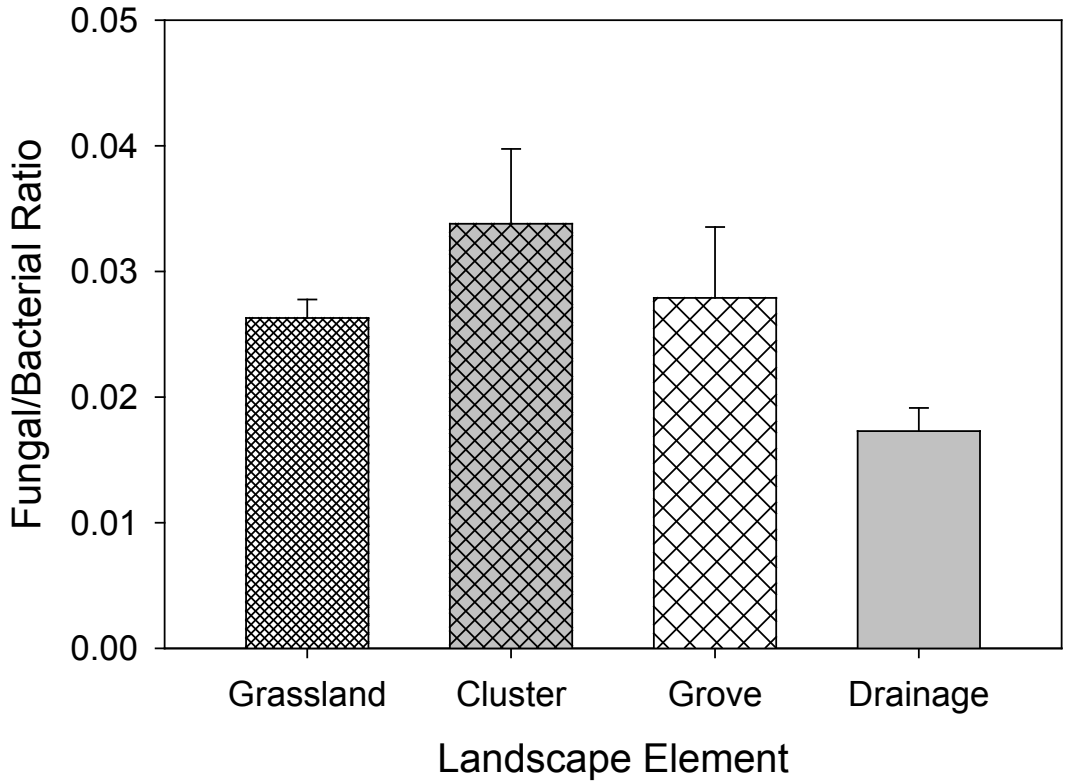

Figure 20. Comparison of fungal/bacterial ratios across four landscape elements at the La Copita Research Area (0-7.5 cm). Values are based on community qPCR assays, error bars represent the standard error of the mean.

revealed two general groups: grassland soils, and soils with a woody vegetation component.

4.4. Pyrosequencing results

Pyrosequencing resulted in a total of 32850 16S rRNA sequences; individual sequence libraries ranged from 1983 to 3227 sequences, and contained between 933 and 1724 OTUs. Sequence libraries were standardized to 1983 bacterial and 1575 fungal sequences for data analysis using the sub.sample command in MOTHUR. A total of 13191 unique bacterial OTUs and 11427 fungal OTUs were identified among the twelve sequence libraries (Table 10). The number of OTUs sequenced did not vary significantly by landscape element or by concentration of C, N, or P. The NMDS analysis of the OTUs identified for both bacteria and fungi showed definite clustering of the grassland communities (Figure 22).

4.4.1. Bacterial sequences

Phylum-level classification of the 16S sequences showed that five phyla (*Acidobacteria, Actinobacteria, Proteobacteria, Bacteroidetes,* and *Gemmatiomonadetes*) represented 85-91% of classifiable sequences in all landscape elements (Figure 23). The relative abundances of *Acidobacteria* were significantly higher ($p<0.05$) in grassland samples (29.5%) than in all wooded landscape elements(17.1-25.6%), and the relative abundance of *Actinobacteria* was lower (8.8%) than wooded elements (16.1-19.7%). A total of 12.7 to 25.7% of bacterial sequences could not be classified in RDP.

As an overview of the bacteria represented in each sample, the ten most common OTUs from each were identified and compared (Table 11). Collating the ten most common genera from each of the bacterial samples yielded 23 individual genera in ten phyla. Four genera (Gp 4, Gp 6, *Gemmatimonas*, and *Opitutus*) were represented in all twelve samples. These four genera accounted for 13.5-35.2% of the bacterial sequences in the samples. The vast majority of genera (by relative abundance) were in the phylum

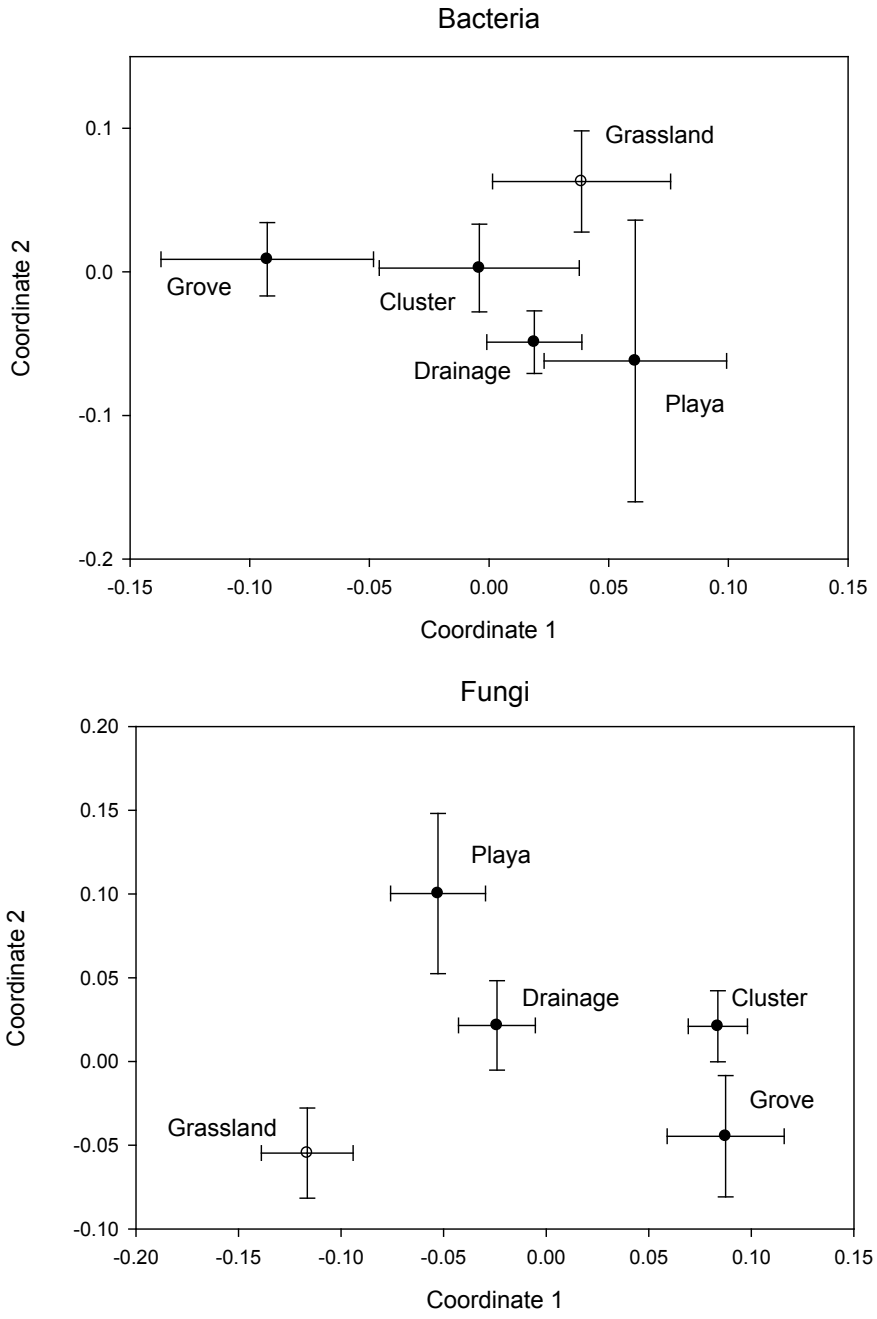

Figure 21. Non-metric multidimensional scaling (NMDS, Bray-Curtis) of the bacterial and fungal communities of the La Copita Research Area based on fragments identified by ARISA showing separation of grassland communities from communities associated with wooded landscape elements. Error bars are standard errors of the mean.

Table 10
Summary of normalized sequence library sizes, operational taxonomic units (OTUs) identified, and diversity and richness estimates for four landscape elements at the La Copita Research Area. The OTUs were defined as sequences sharing >97% sequence identity and served as the basis for the Shannon, Simpson, and Chao I calculations. Numbers in parentheses are standard errors of the mean.

Landscape element	Library size	OTUs identified	Shannon (H')	Simpson (1/D)	Chao I est. richness	Shared genera		
						Grass	Cluster	Grove
(A) Bacteria								
Grassland	1983	1060	6.41(0.04)	243.7(29)	2853(219)			
Cluster	1983	1032	6.44(0.16)	346.9(97)	2763(472)	116		
Grove	1983	1143	6.58(0.10)	379.1(112)	3564(332)	118	132	
Drainage	1983	1162	6.65(0.07)	459.8(110)	3698(108)	115	122	123
(B) Fungi								
Grassland	1575	993	6.34(0.16)	244.2(86)	6206(665)			
Cluster	1575	903	6.02(0.08)	100.4(13)	5558(218)	78		
Grove	1575	983	6.31(0.03)	195.9(22)	6282(816)	58	78	
Drainage	1575	930	6.11(0.10)	122.7(35)	5091(259)	56	69	73

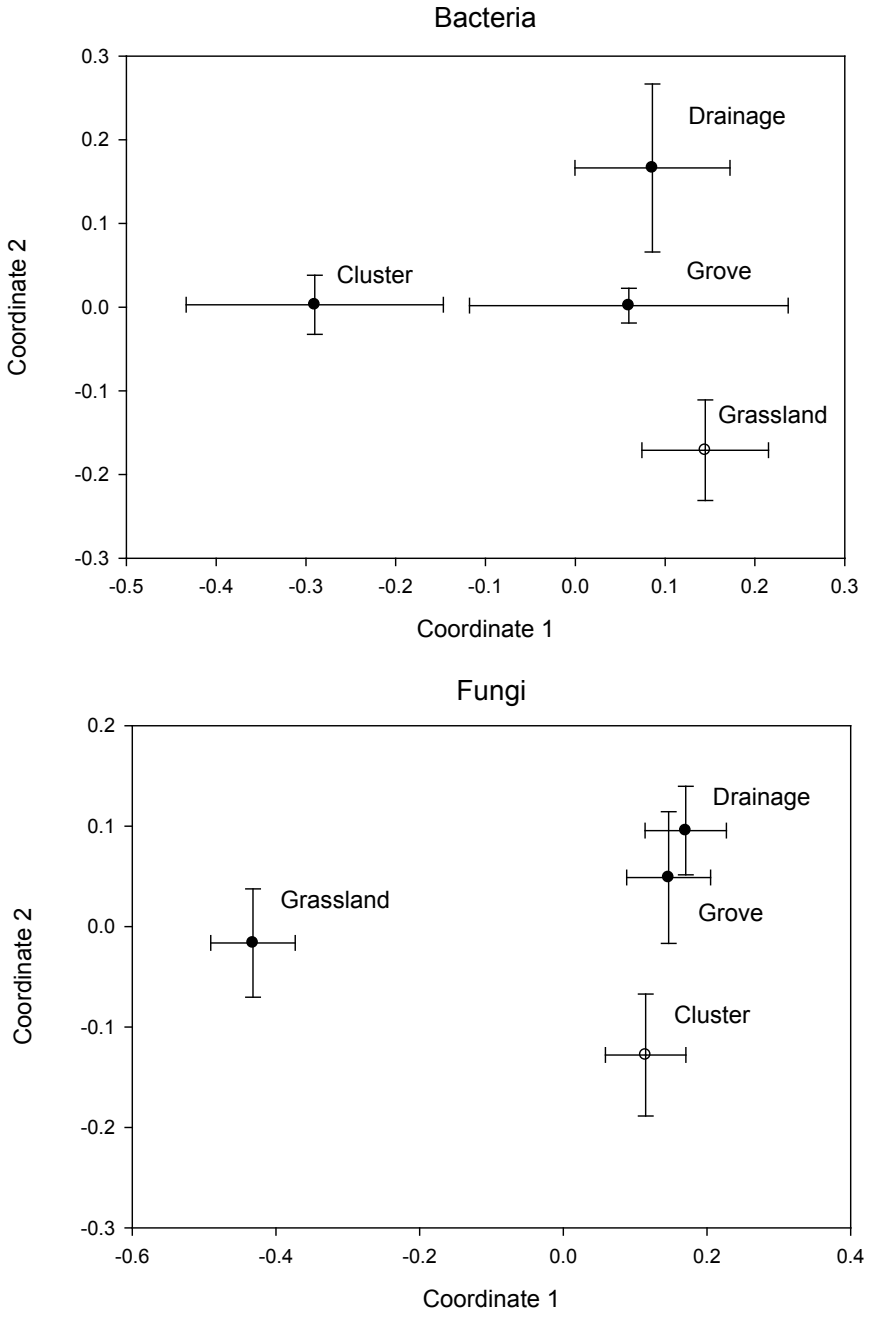

Figure 22. Non-metric multidimensional (NMDS, Bray-Curtis) scaling of bacterial and fungal communities based abundance of OTUs identified by pyrosequencing at the La Copita Research Area. Error bars are standard errors of the mean.

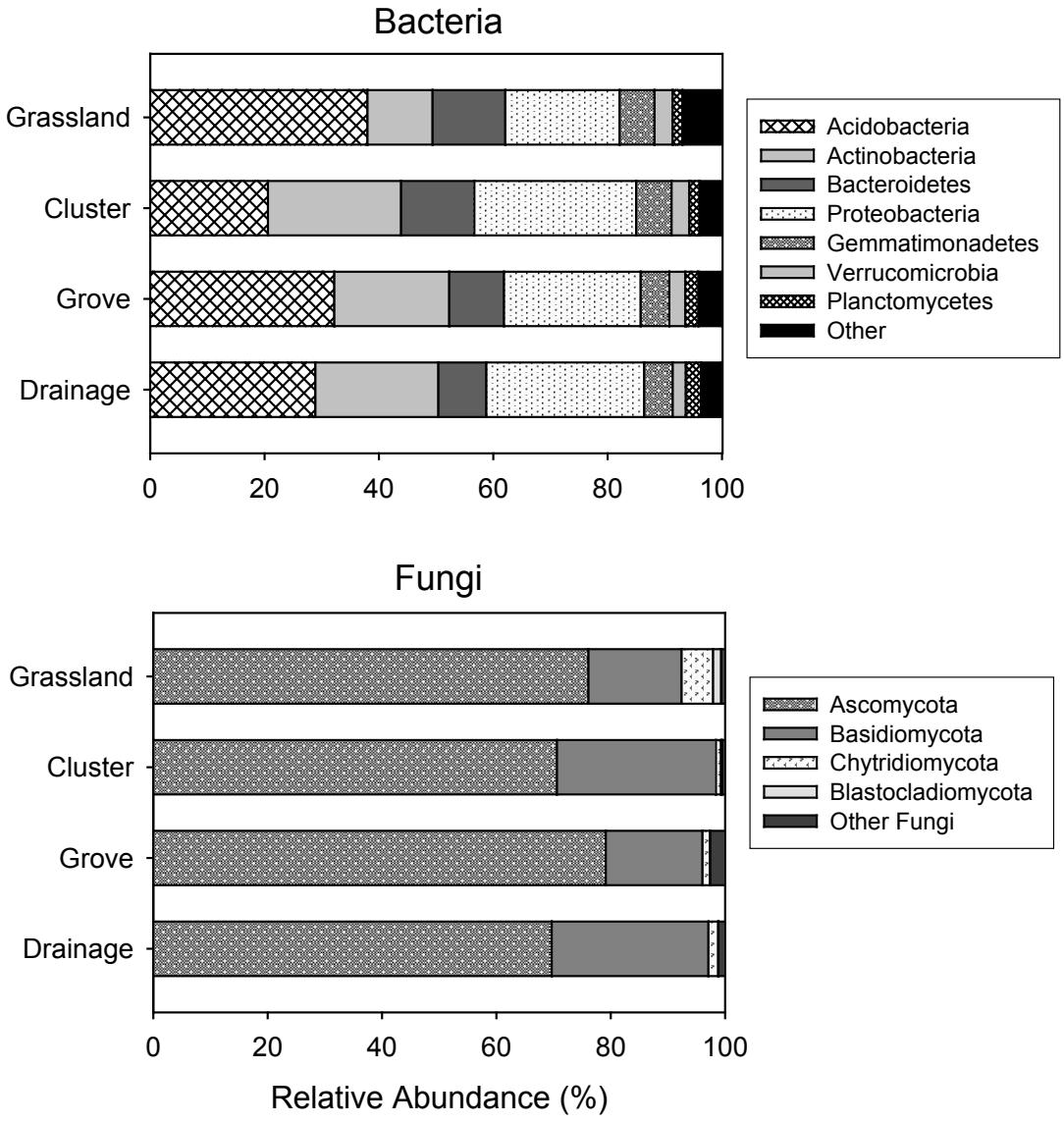

Figure 23. Distribution and relative abundance of bacterial and fungal phyla at the La Copita Research Area based on analyses of rRNA pyrosequencing libraries.

Table 11. The ten most abundant bacterial OTUs found in each soil sample from four landscape elements. A representative sequence for each OTU was assigned a putative identity by the Ribosomal Database Project Classifier (accessed October 18, 2011).Values represent the proportion (%) of sequences in each library that were attributed to the abundant OTUs. Phylum and genus designations were assigned by the RDP classifier. OTU # refers to each OTU's identifier within the larger data set.

	Grasslands			Clusters			Groves			Drainages				
OTU#	G2	G11	G20	C21	C20	C14	GV21	GV14	GV51	DR3	DR1	DR11	Phylum	Genus
B 214	3.8	3.3		1.8	7.6	6.3	4.3	--	--	--	--	--	*Acidobacteria*	Gp1
B 211	21.6	13.0	16.0	8.0	2.9	3.8	6.3	21.2	17.3	7.0	10.4	15.7	*Acidobacteria*	Gp4
B 216	2.6	3.2	8.2	7.7	2.2	2.8	2.7	8.7	6.8	7.7	6.2	8.3	*Acidobacteria*	Gp6
B 215	2.1	6.1	2.3	1.3	--	--	--	1.4	1.7	0.8	1.6	1.8	*Acidobacteria*	Gp7
B 220	--	0.6	--	0.8	--	--	--	0.9	0.6	1.3	1.1	1.1	*Acidobacteria*	Gp16
B 044	--	--	--	--	1.9	--	1.2	--	--	--	--	--	*Actinobacteria*	*Actinoallomurus*
B 046	--	--	--	--		--		--	--	0.8	0.9		*Actinobacteria*	*Kribbella*
B 056	--	--	--	1.0	2.4	1.7	2.0	--	--	0.9	--	0.7	*Actinobacteria*	*Mycobacterium*
B 017	--	--	--	--	1.3	--	--	--	--	--	0.6	--	*Actinobacteria*	*Pseudonocardia*
B 005	1.0	1.0	3.2	--	--	--	--	0.9	--	--	--	4.0	*Actinobacteria*	*Rubrobacter*
B 008	0.7	--	--	--	--	--	--	--	0.6	--	--	--	*Actinobacteria*	*Solirubrobacter*
B 036	--	--	--	--	2.7	1.2	1.2	--	--	--	--	--	*Actinobacteria*	*Streptomyces*
B 089	2.6	2.6	2.2	--	--	1.5		1.0	--	--	--	--	*Bacteroidetes*	*Flavisolibacter*
B 243	--	0.8	--	--	--	--	--	--	--	--	--	--	*Cyanobacteria*	*Bacillariophyta*
B 209	5.1	4.7	4.3	6.1	5.2	4.3	6.2	2.8	3.1	3.5	5.9	2.4	*Gemmatimonadetes*	*Gemmatimonas*
B 246	--	--	0.8	--	--	--	--	--	--	--	--	--	OP10	OP10 *genera incertae sedis*
B 239	--	--	0.8	--	--	--	--	--	--	--	--	--	*Planctomycetes*	*Pirellula*
B 146	--	--	--	--	--	--	--	--	0.5	1.2	--	--	*Proteobacteria*	*Balneimonas*

Table 11. (Continued)

	Grasslands			Clusters			Groves			Drainages				
OTU#	G2	G11	G20	C21	C20	C14	GV21	GV14	GV51	DR3	DR1	DR11	Phylum	Genus
B 147	--	--	--	--	--	1.4	--	--	--	--	--	--	*Proteobacteria*	*Bradyrhizobium*
B 122	1.0	--	1.2	1.4	--	--	--	0.7	0.6	--	0.6	0.9	*Proteobacteria*	*Nitrospira*
B 199	--	--	--	1.2	--	--	0.8	1.1	1.5	3.1	1.9	0.8	*Proteobacteria*	*Seroidobacter*
B 082	--	--	--	--	1.2	1.2	1.0	--	--	--	--	--	*TM7*	*TM7_genera_incertae_sedis*
B 229	1.5	2.5	1.4	1.7	2.5	2.6	1.2	2.5	1.5	1.3	2.1	1.2	*Verrucomicrobia*	*Opitutus*
% of OTUs	36.7	32.0	39.0	27.5	19.8	17.9	21.4	38.7	32.7	26.3	29.2	35.7		

Acidobacteria, represented in the same samples by five primary genera. Seven genera of *Actinobacteria* comprised 0.7-8.3% of the bacterial sequences.

4.4.2. Fungal sequences

Phylum-level classification of the fungal sequences showed that four phyla accounted for 61.8 to 86.3% of identified sequences. *Ascomycota* was the most common phylum in all samples (55.8-62.1%), with significant contributions from *Basidiomycota*, *Chytridiomycota*, and *Blastocladiomycota.* Unclassified fungi accounted for 13.2-27.1% of sequences (Figure 23).

In an assessment of the ten most common sequences present in the fungal samples, the fungi showed more variety than the bacteria (Table 12). Isolating the ten most common genera from the twelve samples resulted in forty-two distinct genera, compared to twenty-three genera found in the bacterial samples. Only one OTU was ubiquitous among the ten most abundant genera for all twelve samples, *Gibberella* in the phylum *Ascomycota*. Thirty-one sequences (70.5%) of the common genera were in the phylum *Ascomycota*, with the remaining genera representing the phyla *Basidiomycota* (nine sequences), *Blastocladiomycota* (one sequence), and fungi incertae sedis (one sequence). Seventeen genera were unique to a single sample site within the ten most common sequences.

5. Discussion

In a variety of ecosystems, it has been shown that both individual plant species and assemblages of plants can alter the structure and function of the microbial community (Fierer and Jackson, 2006; Wallenstein et al., 2007; Boyle et al., 2008; Hollister et al., 2010). The response to vegetation change in any given ecosystem will depend on the unique microbial community supported by that system (Strickland et al., 2009; Hollister et al., 2010). Bacteria and fungi are drivers of organic matter turnover and biogeochemical cycling, and taken in conjunction with the increased ANPP, SOC, total

Table 12. The ten most abundant OTUs for fungi at each landscape sampled at the La Copita Research Area. A representative sequence for each OTU was assigned a putative identity by the RDP Classifier (accessed October 18, 2011). Values represent the proportion (%) of total sequences attributed to the abundant OTU. OTUs were defined at a similarity level of 97% and were calculated using MOTHUR. OTU # refers to the OTU identifying number within the larger data set.

	Grasslands			Clusters			Groves			Drainages				
OTU#	G2	G11	G20	C21	C20	C14	GV21	GV14	GV51	DR3	DR1	DR11	Phylum	Genus
F 134	--	0.6	--	--	--	--	--	--	--	--	--	--	*Ascomycota*	*Anthostomella*
F 154	--	--	--	--	1.3	--	--	--	--	--	--	--	*Ascomycota*	*Arachnomyces*
F 076	--	--	--	--	--	--	--	--	0.8	--	--	--	*Ascomycota*	*Ascobolus*
F 077	--	--	--	--	--	--	1.4	--	--	--	0.5	--	*Ascomycota*	*Ascodesmis*
F 150	--	--	--	0.7	1.3	0.4	0.5	--	--	--	0.9	0.5	*Ascomycota*	*Aspergillus*
F 122	--	--	--	--	--	--	--	1.2	0.9	--	--	--	*Ascomycota*	*Bionectira*
F 113	0.5	--	--	0.7	--	--	--	--	--	--	--	--	*Ascomycota*	*Calonectria*
F 100	0.7	1.2	1.3	--	--	--	--	--	--	--	--	--	*Ascomycota*	*Cercophora*
F 096	--	--	--	--	--	--	--	--	1.3	--	5.8	--	*Ascomycota*	*Chaetomidium*
F 145	--	--	--	--	--	--	0.5	--	--	--	--	--	*Ascomycota*	*Cladophialophora*
F 167	0.7	--	0.5	--	--	0.5	--	1.2	0.8	--	0.6	0.5	*Ascomycota*	*Cladosporium*
F 200	--	0.9	--	--	--	--	--	--	--	--	--	--	*Ascomycota*	*Edenia*
F 140	--	0.9	0.6	2.1	1.4	2.7	2.9	2.2	1.2	2.3	3.9	2.1	*Ascomycota*	*Exophiala*
F 122	7.2	3.7	4.7	3.6	3.0	3.7	2.2	3.0	1.6	2.9	1.8	3.0	*Ascomycota*	*Gibberella*
F 186	--	--	--	--	0.9	--	--	--	--	--	--	--	*Ascomycota*	*Heleiosa*
F 107	--	--	0.4	--	1.3	--	--	0.7	--	1.2	0.9	1.2	*Ascomycota*	*Hypocrea*
F 090	--	--	--	--	--	--	0.5	--	--	--	0.8	--	*Ascomycota*	*Melanopsammella*
F 177	--	--	1.0	--	--	--	--	--	--	--	--	--	*Ascomycota*	*Montagnula*
F 125	--	--	--	0.7	1.8	0.6	1.9	0.8	--	--	--	--	*Ascomycota*	*Nectriopsis*
F 120	--	--	--	--	--	--	--	0.8	--	0.7	--	--	*Ascomycota*	*Paecilomyces*

Table 12. (Continued)

	Grasslands			Clusters			Groves			Drainages				
OTU#	G2	G11	G20	C21	C20	C14	GV21	GV14	GV51	DR3	DR1	DR11	Phylum	Genus
F 078	0.6	7.5	0.9	--	--	--	--	--	--	--	--	--	*Ascomycota*	*Peziza*
F 181	1.4	1.0	1.3	--	--	--	--	--	--	--	--	--	*Ascomycota*	*Phaedothis*
F 190	--	--	0.4	--	--	--	--	--	--	--	--	--	*Ascomycota*	*Preussia*
F 194	--	--	--	0.8	--	0.8	--	--	0.9	0.7	0.9	0.9	*Ascomycota*	*Pyrenochaeta*
F 058	--	--	--	1.4	--	--	--	--	--	--	--	--	*Ascomycota*	*Satchmopsis*
F 103	1.2	--	--	--	--	--	--	--	--	--	--	--	*Ascomycota*	*Schizothecium*
F 059	0.4	--	--	--	--	--	--	--	--	--	--	--	*Ascomycota*	*Sclerotinia*
F 153	--	--	--	--	1.1	--	0.5	--	--	--	--	--	*Ascomycota*	*Spiromastix*
F 098	--	0.7	--	--	--	--	--	--	--	--	--	--	*Ascomycota*	*Triangularia*
F 189	0.6	0.9	--	--	--	--	--	--	--	--	--	--	*Ascomycota*	*Westerdykella*
F 135	--	--	--	--	3.0	--	--	--	--	--	--	--	*Ascomycota*	*Xylaria*
F 007	--	--	--	0.9	--	0.3	--	--	--	--	--	--	*Basidiomycota*	*Atractiella*
F 031	--	--	--	--	--	--	--	--	--	0.8	--	1.5	*Basidiomycota*	*Clitocybe*
F 041	--	--	--	--	--	--	--	1.0	--	--	--	--	*Basidiomycota*	*Clitopilus*
F 018	--	-	--	4.1	--	3.6	0.5	--	3.1	0.6	--	0.7	*Basidiomycota*	*Geastrum*
F 032	--	--	--	--	--	--	--	--	--	--	--	0.5	*Basidiomycota*	*Melanoleuca*
F 026	--	--	--	--	--	--	--	--	1.1	--	--	--	*Basidiomycota*	*Panaeolus*
F 051	--	--	--	--	--	--	--	--	--	0.9	--	1.6	*Basidiomycota*	*Pterula*
F 046	--	--	--	--	1.7	1.5	0.9	--	--	0.6	--	--	*Basidiomycota*	*Stephanospora*
F 043	--	5.1	0.6	0.7	--	--	--	1.9	--	--	--	--	*Basidiomycota*	*Thanatephorus*
F 055	1.1	--	--	--	--	--	--	--	--	--	--	--	*Blastocladiomycota*	*Catenomyces*
F 053	--	--	--	--	--	0.8	--	4.2	1.1	0.7	1.8	--	F. incertae sedis	leaf
% of OTUs	14.4	21.9	11.7	15.7	16.8	14.9	11.8	17.0	12.8	11.4	17.9	10.9		

N, and total P associated with mesquite encroachment, investigation of the microbial landscape offers an insight into the extent of the ecosystem alteration that occurs with woody plant invasion.

Previous studies have shown that approximately 50% of the increase in soil C and N occurs in the unprotected free light fraction of the soil organic matter. This increase contributes to increased cycling dynamics, as well as a 40% attributed to the increase in the abundance of macroaggregates which physically protect organic matter from microbial decomposition (Chapter II, Liao et al, 2006a). Fractionation of phosphorus by availability categories shows that both plant- and microbially-available P and recalcitrant P increase under woody encroachment (Chapters III and IV). The increase in recalcitrant nutrients may be attributed to litter chemistry: woody plant litter at La Copita contains higher concentrations of decomposition-resistant suberin-derived fatty acids than litter and roots from grasslands (Hibbard et al., 2001; Filley et al., 2008; Liao and Boutton, 2008; Boutton et al., 2009) and the suite of secondary woody species contains members of the genus *Zanthoxylum,* which is known to produce compounds with antimicrobial properties (Obi et al., 2002; Steenkamp et al., 2007). In addition to chemical recalcitrance, the organic matter produced by woody species at La Copita is increasingly physically protected in water stable aggregates (Chapter II; Liao et al, 2006a; Filley et al. 2008; Boutton et al., 2009, 2010). Measurements of soil respiration rates have shown that physical protection of carbon may in fact exert more control over rates of carbon turnover than the biochemistry of the woody litter (Creamer et al., 2011).

Fierer and Jackson (2006) showed that a higher C:N ratio and available P both contributed to changes in fungal communities, while pH and soil texture contributed to changes in bacterial community composition (Lauber et al., 2008, 2009; Jones et al., 2009; Rousk et al., 2010). At La Copita, the variation in pH among the soils is very limited (6.2-6.5), though both texture and nutrient availability are altered by the presence of mesquite and woody understory plants. The differing substrate composition (C, N, and P) beneath the mesquite is likely to result in a microbial community is adapted for feeding on different qualities and quantities of organic matter than the community in the

grassland soils (Lauber et al., 2008; Rousk et al., 2010). Composition of substrate beneath the wooded landscape elements (clusters, groves, and drainages) should be more similar to other wooded landscape elements than to grassland substrate.

Measurement of fungal and bacterial gene copies ratios by qPCR does not reliably estimate microbial biomass ratios, as gene copies may vary between microbial taxa (Lauber et al., 2008), but studies in these soils have demonstrated higher annual soil respiration and higher soil microbial biomass C in wooded landscape elements than in remnant grasslands at La Copita (McCulley et al., 2004; Liao et al., 2006b; Liao and Boutton, 2008). Measurements have shown that drainages differ from the upland soils in terms of texture, which may contribute to variation in nutrient availability (as described in Chapter IV) and the observed difference in fungal/bacterial ratio. Though the total number of gene copies was lower in La Copita soils than similar sites for both bacteria and fungi, qPCR fungal/bacterial ratios for both grassland and woodland sites were similar to those found by the same technique in other ecosystems, including nitrogen-fixing red alder (Boyle et al., 2008), cultivated and pasture soils (Lauber et al. 2008), and in desert shrubland (Fierer et al., 2005). Boyle's (2008) experiment also indicated that higher productivity sites in the Oregon forests showed lower fungal/bacterial ratios. At La Copita, only the fungal/bacterial ratios of the drainages are significantly lower than those of the grasslands. Ratios were lower (within one order of magnitude) than those found at La Copita by Brewer (2010) using phospholipid ester-linked fatty acid analysis (PLFA), likely due to measurement technique. Boyle et al. (2008) also showed lower fungal/bacterial ratios for replicate soil samples when using qPCR than when using PLFA techniques.

ARISA provided a rough estimate of community diversity through the measurements of length and abundance of PCR-amplified DNA fragments. As described in Kaplan and Kitts (2003), the length of sequences is not a reliable method for identifying individual species within a sample, as the difference of a single base pair can be indicative of two separate species, or of two identical species and a sequencing error. However, taken as an analysis of community composition, ARISA allows comparison of the bacterial and

fungal communities found in each landscape at La Copita. Previous PLFA work by Brewer (2010) showed distinct microbial communities between grassland and bole soil samples for a depth of 0-15 cm, analogous to grassland and cluster soils in this study. NMDS of the ARISA sequence libraries showed weak clustering of grassland samples in both the bacterial and fungal samples (Figure 21), but indicated that further investigation into community composition would be required to quantify differences in the communities.

Pyrosequencing provided a much clearer picture of the microbial communities. Non-metric multidimensional scaling of the OTUs identified by pyrosequencing confirmed that the bacterial and fungal communities of the grassland soils are distinct from those of the woody plants (Figure 23). The data produced though pyrosequencing was used to quantify community richness and evenness scores and to compare communities between the landscape elements. Pyrosequencing differs from traditional culturing techniques as molecular methods are not limited by the need to provide living organisms for study. This is essential for studying *Acidobacteria,* a notoriously difficult bacterial phylum to culture, which makes up a significant portion of the soil microbial community at La Copita (Jones et al., 2009; Figure 22).

Studies of invasive legumes in Spain causing similar changes in soil nutrient chemistry have shown an increase in bacterial community richness, accompanied by a decrease in fungal community richness and diversity as woody species invaded grasslands (Lorenzo et al., 2010). Shannon bacterial diversity was higher in the grove and drainages than the grasslands, though clusters and grasslands were similar. As soil nutrient content increases grasslands < clusters < groves < drainages, the increase in bacterial diversity roughly correlates with increasing C, N, and P. Fungal diversity showed a weaker but opposite trend: diversity was higher in grasslands and groves than in clusters and drainages. Chao I estimate of richness showed the bacterial community in grasslands to be similar to the other upland landscape elements (clusters and grove), while drainages were similar only to groves. In the fungal community, Chao I showed the drainages to be less rich than grasslands and groves. Though bacterial evenness and

diversity were not significantly different between landscape elements, measurements of bacterial community richness indicated that clusters and groves were significantly different (Table 10).

Soil bacterial communities in the wooded landscape elements showed smaller relative abundances of *Acidobacteria* than those in grasslands, and larger abundances of *Proteobacteria* and *Actinobacteria*. *Proteobacteria* contains the genera associated with a wide range of nutrient cycling processes, including nitrogen fixation, and while not absent in the grasslands, the phylum is larger and more diverse in the wooded areas. Mycorrhizal fungi liberate nutrients from decomposing organic matter and often make those nutrients available to plants and other microbes in the soil. Ectomycorrhizal fungi are primarily *Basidiomycota, Ascomycota,* and *Zygomycota.* At La Copita the fungi are primarily *Basidiomycota* and *Ascomycota,* which are associated with the breakdown of lignified plant detritus. Arbuscular mycorrhizal fungi are solely *Glomeromycota* (Schussler et al., 2004), which make up 0.2-0.7% of the grassland and 0.1-0.2% of the wooded landscape element sequences (data not shown). The largest change in fungal community composition at the phylum level is observed in the *Chytridiomycota*, which decline from 4.0% in the grasslands to 0.8-1.4% in the wooded landscape elements. *Chytridiomycota* are primarily plant parasites in terrestrial soils, though they may also feed on pollen and cellulose (James et al., 2006). The reduced relative abundance of *Chytridiomycota* may indicate that the woody species are more resistant to these parasites than the grassland vegetation.

Investigations of the soil fauna at La Copita reveal that the nematode community changes with the presence of woody species. Biederman and Boutton (2009) showed that in clusters >30 years, bacterivore nematodes increased from 30% to 70-80% of the total nematode community, and root-parasitic nematodes decreased from 40% to less than 10%, suggesting litter quality may limit root feeders. Though quantification of the diversity and richness of the bacterial community does not change significantly between the grasslands and the woody landscape elements, analysis of the composition of the

community indicates that there is a species shift which likely reflects the altered litter composition and which may contribute to the change in nematode food source.

6. Conclusions

The use of molecular methods for the characterization of microbial communities is shedding light on the unseen world of soil microbiology. Though the amplification and identification of genetic sequences, we are able to demonstrate that the composition of microbial communities changes with the introduction of woody vegetation to historically grassland ecosystems even when community diversity is unaffected. As these bacteria and fungi play key roles in the decomposition of organic matter and the cycling of C, N, and P in the soils, any change in the soil biota indicates a change in the rate or manner of nutrient cycling below the soil surface. While many studies have examined the effects of different soils on soil microbial community, this study offers a look at soils of similar edaphic properties undergoing an invasion of woody vegetation. The effects of vegetation change on soil nutrients and bacterial and fungal populations they support appear widespread. Whether the changes in soil nutrient availability are the drivers of changes in microbial community composition or a result of the efforts of the microbial community remains to be determined.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Woody plant encroachment has dramatic effects on the biogeochemistry of grassland soils. It has been shown that the presence of woody species in former grasslands contribute to the formation of islands of fertility, in which nutrients, organic matter, and even moisture become concentrated beneath the canopies of woody species. The effects of woody encroachment have been seen in grasslands around the world, and due to anthropogenic influences and the trajectory of climate behavior, these changes are likely to persist and intensify. The objective of this study was to demonstrate the effects of woody plant encroachment on P and on the microbial communities of south Texas, to show that P pools are altered by woody plants in a similar manner as C and N despite differences in soil P chemistry and cycling mechanisms. The results of this investigation confirmed that the introduction of woody species to the ecosystem has resulted in increased C, N, and P accumulation in plant litter, plant roots, and the upper 7.5-10 cm of the soil profile in both fast and slow turnover pools. Organic matter inputs to the soil from woody plants increase soil aggregation, further protecting soil nutrients within the physical structure of the soil. Over the course of woody encroachment, the distribution of soil P shifts, yielding a larger pool of labile, plant-available P in the surface soil under woody plants than in grasslands. Finally, significant shifts occurred in the microbial communities with the establishment of woody plants, likely a result of the changes in soil biogeochemistry driven by woody encroachment.

The presence of mesquite is associated with an increase in three essential soil nutrients, C, N, and P, in woody clusters, and the increases are significant and linear with time following woody encroachment. Rates of nutrient accumulation show no signs of tapering off across a chronosequence of woody encroachment spanning more than 85 years. The linear progression of C, N, and P indicates that the chronosequence approach is useful in determining the degree to which an ecosystem has been affected, and to predict the trajectory of effects of woody encroachment. Fractionation of soils

under mesquite trees along the chronosequence indicates that the effects of organic input are progressive: macroaggregates, which are formed from mineral soil particles glued together by decomposed organic material, increase linearly with time during encroachment, as does the concentration of free light fraction, the less decomposed organic matter. Within the fractions, average C, N, and P increased in all fractions except free silt and clay, the fraction containing the smallest proportion of the nutrient pools. Increases in the free light fraction, the fastest turnover fraction, and the microaggregates, which turn over slower than all fractions except silt and clay (Liao et al., 2006b), confirms that nutrients are increasing in both slow and fast-cycling pools under mesquite. Records of P deposition in the area and measurements of the soil P around mesquite clusters at La Copita support the hypothesis that woody plants are responsible for mining P from deep soils through root uptake and incorporation in plant tissues. The P is then transferred to the surface soil through litter turnover.

Fractionation of soil P in the grasslands and clusters indicates that as woody encroachment advances, increases occur in all P fractions except acid-extractable P. While mean P increase in the clusters was 160% of the grassland P concentration, within individual fractions the changes varied from no change in acid-extractable fraction to 430% increase in the resin-extractable P. The magnitude of the increase is greatest in the resin-extractable P, which indicates that woody clusters are shifting P from recalcitrant pools to biologically-cycled P: in addition to increasing total P in soil, P is increasing in the portions of the soil that cycle the fastest (Liao et al., 2006b). Unlike C and N which are liberated to the atmosphere, phosphorus liberated from the fast-cycling organic matter in the wooded clusters becomes available to plants and microbes in the shallow soil, enhancing the shallow soil P pool. Fractionation results in these grassland soils contrast the findings of a similar method in desert and agricultural soils, demonstrating the importance of soil chemistry on P availability (Cross and Schlesinger, 2001; Dossa et al., 2010). Fractionation of soils along the chronosequence of woody encroachment is the first experiment of its kind to demonstrate the steady progression of soil nutrient alteration.

Analyses of total C, N, P, and P fractions across the five landscape elements at La Copita show that within an ecosystem, subtle variations in vegetation cover and soil characteristics can alter the response to woody encroachment. Mesquite is prevalent in all of the wooded landscape elements, but the distribution of secondary woody plants is different for each. Additionally, the soils at La Copita are divided between upland and lowland topographic features. Increases in C, N, and P were linear and significant for all landscape elements. The largest changes in nutrient concentration were between the grasslands and the lowland landscape elements, the drainages and playas. Mean soil organic C increased 320-520%, N increased 287-539%, and total P increased 132-308%. P increased in the labile and occluded P fractions in all landscape elements, but non-occluded P (hydroxide- and acid-extractable P) either decreased significantly (in groves) or showed no significant trend (drainages and clusters) in soils with finer texture. The proportion of P in the organic pool (primarily hydroxide-extractable P) reflects this changed in non-occluded P.

Woody encroachment shifts a larger portion of the soil nutrients in the shallow soil at La Copita to the fast-cycling soil physical fractions and into plant- and microbially-available forms under mesquite landscape elements, providing a new nutrient environment for microbes. In addition, the presence of woody litter provides an additional slow-turnover pool. With this bifurcated nutrient pool arrangement, it stands to reason that the microbial community of the site would change to optimize use of the new food sources. Soil microbially communities are notoriously difficult to culture in laboratory settings, but molecular approaches to community characterization provide identities and abundances of soil microbes and their proxy data. Analysis of fungal:bacterial gene copy ratios by qPCR showed the grove soils to have a smaller ratio than clusters and drainages. Fragment analysis by ARISA indicated that at the community level, the fungi in grassland soils were different than those under wooded landscape elements. Pyrosequencing identified the bacteria and fungi of the soil communities to the genus level, demonstrating conclusively that the wooded landscape

elements support fungal and bacterial communities that are significantly different from the bacteria and fungi in the grassland soils.

The data presented in this study points to mesquite roots as vital players in the cycling of P in woody-invaded ecosystems. Unfortunately, this research was restricted to surface soil and any roots contained therein. Further studies should examine the roots and rhizosphere of grass and mesquite at La Copita to better understand the mechanisms of P relocation. As P is being translocated from deep soil mineral pools to the soil surface through plant litter, the pool of P at the soil surface increases, as should the P incorporated into woody tissue. Soil P is essentially a closed cycle, therefore the deeper soil pool should be depleted in response. The shallow sampling of this study cannot demonstrate conclusively that mesquite is removing P from deeper soil layers; additional, deeper sampling is required to quantify soil P losses elsewhere in the profile. The development of molecular techniques for microbial characterization has given scientists the power to produce and sequence hundreds of thousands of sequences for a given soil, however the data is limited by the reference libraries that are still in development. A sizeable portion of the sequence library created for a given soil will identify as "unknown" when queried through sequence identity databases. However, this is changing rapidly with the boom in sequence analysis and identification, and the accuracy and robustness of the databases will increase with time.

The results of this study indicate that woody plants affect the P cycle as strongly as they affect the C and N cycles, increasing the concentrations of all three nutrients over time. P fractions in the soil respond to differences in soil characteristics beneath woody landscape elements of the same species. As woody encroachment is a global phenomenon that is expected to progress with environmental conditions and anthropogenic facilitation of grazing and fire cycle interruption, these nutrient changes could potentially be observed in landscapes around the globe. Climate change scenarios also predict increased woody plant abundance in higher latitudes and elevations around the globe, increasing the potential for these changes in C, N, and P to become important in ecosystems that are not yet woody-encroached. Molecular investigation of the effects

of woody encroachment shows that the belowground communities reflect aboveground vegetation changes. As bacteria and fungi play a role in much of the biogeochemical cycling in soils, it is imperative that microbial influences on nutrient cycling be considered when predicting the trajectory of woody plant encroachment in ecosystems.

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VITA

Ilsa Beth Kantola

Education:

2006 – 2012 Texas A&M University, College Station, TX
Ph.D. in Rangeland Ecology and Management, May 2012

2001 – 2006 California Polytechnic State University, San Luis Obispo, CA
B.Sc. in Environmental Engineering, March 2006

Mailing Address: Department of Ecosystem Science and Management
Texas A&M University, 2138 TAMU
College Station, TX 77843

Email address: ikantola@tamu.edu

Honors and Awards:

1st place Poster, Ecology/Cons. Div., Student Research Week, Texas A&M (2009)
2nd place Poster, Soil Biology/Biochem. Division, Soil Science Soc. of America (2008)
Tom Slick Graduate Research Fellowship (2010-2011)
Department of Ecosystem Science and Management Wasko Fellowship (2009-2010)
Texas A&M University Graduate Diversity Fellowship (2006-2009)

Selected presentations and abstracts:

Kantola, IB, Boutton, TW, Filley, TR, Hallmark, CT. 2011. Changes in soil C, N, and P storage following woody plant invasion of grassland. *Abstracts, Ecological Society of America Meeting* PS 66-130.

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