

# Changes to soil organic N dynamics with leguminous woody plant encroachment into grasslands

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**Abstract** Encroachment of nitrogen-fixing trees and shrubs into grasslands and savannas is a well-documented land cover change that occurs worldwide. In the Rio Grande Plains region of southern Texas, previous studies have shown woody encroachment by leguminous *Prosopis glandulosa* (mesquite) trees increases soil C and N, decreases microbial biomass N relative to soil N, and accelerates N mineralization and nitrification. We examined responses of the dominant organic N components in soil (amino acids and amino sugars) and two soil-bound protein-N acquiring enzymes (arylamidase and  $\beta$ -N-acetylglucosaminidase) along a grassland-to-woodland successional chronosequence to determine

changes to soil N chemistry and extractability. The proportion of total N held within amino compounds was significantly lower in the woodlands (47 %) relative to the grassland soils (62 %). This increase in non-hydrolysable N was accompanied by increases in plant cell wall derived amino acids (e.g. hydroxyproline, serine) and losses of microbial amino sugars, indicating the woodland organic N pool was altered in composition and potentially in quality, either because it was more structurally protected or difficult to degrade due to polymerization/condensation reactions. Soil carbon-normalized activities of both soil-bound N-acquiring enzymes were significantly higher in woodland soils, consistent with changes in the biochemical composition of organic N. Although soil total N increases following woody encroachment, this additional organic N appears to be less extractable by chemical hydrolysis and thus potentially in more refractory forms, which may limit microbial N accessibility, slow the cycling of soil organic carbon, and contribute to observed soil C and N accrual in these systems.

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## Introduction

Nitrogen cycling in semi-arid and arid grassland ecosystems is dramatically altered following the

encroachment of woody leguminous trees and shrubs, as nutrients become rapidly concentrated under the developing canopy and amplify a number of biogeochemical feedbacks within the system that can act to accumulate or reduce soil organic matter (SOM) stocks (Liu et al. 2010; Schlesinger et al. 1996; Schlesinger and Pilmanis 1998). This ecosystem shift has been documented in grasslands and savannas throughout the world (Archer 1995) and the response of SOM cycling to this land-cover change is potentially influenced by a number of factors, including an altered nitrogen cycle. Within such global change scenarios soil nitrogen dynamics have an under-investigated yet pivotal role in the stabilization or destabilization of SOM (Gårdenäs et al. 2011; Knicker 2011; Sollins et al. 2007).

In the Rio Grande Plains region of southern Texas, woody encroachment is initiated with the establishment of the leguminous *Prosopis glandulosa* (mesquite) tree, and progresses with the colonization by other tree and shrub species beneath its canopy (Archer et al. 1988). Compared to the grasslands they replace, these wooded landscapes have higher rates of above- and belowground net primary productivity (Archer et al. 2001; Hibbard et al. 2001) and significantly greater C and N stocks in the litter layer and the soil (Boutton et al. 2009; Boutton and Liao 2010). Additionally, soil microbial biomass-C and -N and the rates of soil N transformations (mineralization and nitrification) are higher in wooded soils (Hibbard et al. 2001; Liao and Boutton 2008; McCulley et al. 2004), although microbial respiration efficiencies are lower (Liao and Boutton 2008). An increased proportion of more difficult-to-degrade litter and SOM may explain in part the C and N accrual patterns observed in this system (Filley et al. 2008; Liao and Boutton 2008; McCulley et al. 2004), but additional water or nutrient limitations have also been suggested to play a dominant role (Creamer et al. 2011).

Amino acids and amino sugars are of particular significance to N cycling in soils because they store 35–80 % of total soil N (Amelung et al. 2001; Knicker et al. 1993; Martens and Loeffelmann 2003), and their form and type can indicate their source (e.g. microbial vs. plant residues). Amino acids are derived from both plant and microbial biomass (Kögel-Knabner 2006) while amino sugars are derived almost exclusively from microorganisms, and therefore are used a source

proxy for microbial residues (Amelung 2003; Liang et al. 2007). Additionally, changes in the proportion of certain amino acids, such as those associated with cell wall proteins, tend to increase with decomposition and may provide clues as to the extent of microbial recycling of organic N (Dauwe and Middelburg 1998; Keil et al. 2001).

Although free soil amino acids are rapidly consumed (McFarland et al. 2010), soil organic N, in general, is stabilized by adsorption onto or within clays (Kleber et al. 2007; Sollins et al. 2006), encapsulation within hydrophobic and recalcitrant biopolymers (Knicker and Hatcher 1997; Zang et al. 2001), intrinsic stability (e.g. microbial glycoproteins) (Rillig et al. 2007), and condensation reactions with phenolic compounds (Kraus et al. 2003; Olk et al. 2009; Schmidt-Rohr et al. 2004). Changes to the quantity and composition of plant inputs, such that occurs with woody encroachment, could change the importance of these various organic N stabilization mechanisms and impact soil organic N availability and subsequently SOM stability (e.g. de Graaff et al. 2006). This is particularly true in sandy loam soils, such as in south Texas, that have a decreased capacity for clay–protein interactions.

In soil and litter, N availability (as reflected by microbial N demand) can be indirectly measured through the activity of N-acquiring microbial and plant derived extracellular enzymes, as they regulate the breakdown of soil organic N (Sinsabaugh et al. 1993; Sinsabaugh 1994). The production of these enzymes is energetically costly, so there is a balance between microbial nutrient demand and the availability of C and N (Allison and Vitousek 2005; Sinsabaugh et al. 2008), where the activity of N acquiring enzymes should be stimulated in N-limiting systems and suppressed when labile or inorganic N is present (Olander and Vitousek 2000; Sinsabaugh et al. 2002; Sinsabaugh et al. 1993; Sinsabaugh and Moorhead 1994). The form (e.g. inorganic vs. organic) and availability of N, not just the quantity of N, regulates soil enzyme activity (Saiya-Cork et al. 2002; Sinsabaugh et al. 2008; Zeglin et al. 2007), so that changes in soil enzyme activity in response to woody encroachment should reflect changes in N availability and form that are experienced by the microbial community.

By combining measurements of soil enzyme activity with the amount and composition of amino acids

and amino sugars, we attempt to gain a more complete picture of N accessibility and dynamics, and to assess possible N-related controls on SOM accumulation in this system (e.g. Carreiro et al. 2000; Hu et al. 2001; Luo et al. 2004). Specifically, we examined the responses of the dominant organic N compounds (amino acids and amino sugars) and N acquiring enzymes ( $\beta$ -N-acetylglucosaminidase [NAG] and arylamidase) along a chronosequence of woody encroachment in a well-characterized semi-arid savanna ecosystem in southern Texas. Because of the low capacity for mineral associated amino compound stabilization in this region due to the low clay content in these soils ( $\sim 10\%$ ), and because wood-derived SOM is selectively accruing in non-mineral bound soil fractions (Filley et al. 2008), we hypothesized that the amino compound pool would reflect woody plant amino inputs. As previous studies have shown increases in N mineralization and nitrification with woody stand age (Hibbard et al. 2001; McCulley et al. 2004), we also hypothesized that amino content, as a proportion of total N, must progressively decrease to elicit the higher N cycling rates. Such a decrease in amino-N could shift microbial resource allocation strategies towards N-acquisition, resulting in higher N-acquiring enzyme activities, and allowing for SOM accrual.

## Materials and methods

### Site description

The Texas AgriLife La Copita Research Area is located in the Rio Grande plains region of southern Texas (27°40'N; 98°12'W). Topography is relatively flat, with 1–3 % slopes over elevations of 75–90 m. The mean annual temperature is 22.4 °C and the mean annual precipitation is 716 mm. Although this area was once open grassland, fire suppression and livestock grazing have caused a progressive encroachment of subtropical thorn woodland into the native grasslands during the past 150 years (Archer 1990; Boutton et al. 1998; McLendon 1993). The remnant grasslands are dominated by the grasses *Chloris cucullata*, *Bouteloua rigidisetata*, *Aristida purpurea*, *Panicum hallii*, *Eragrostis secundiflora*, and *Tridens muticus*. Dominant forb species include *Wedelia hispida* and *Ambrosia psilostachya* (Boutton et al. 1998). On

upland portions of the landscape, woody encroachment is initiated by the establishment of the nitrogen-fixing tree legume, *P. glandulosa* (honey mesquite), which appears to facilitate the establishment of other shrubs (such as *Condalia hookeri*, *Zanthoxylum fagara*, *Berberis trifoliata*, *Diospyros texana*, and *Celtis pallida*) beneath its canopy, resulting in the formation of discrete woody clusters within the grassland matrix (Archer et al. 1988). Soils under both the remnant grasslands and woody clusters are fine-loamy mixed hyperthermic Typic Argiustolls of the Runge series (Minzemayer 1979). More detailed site descriptions are given elsewhere (Archer et al. 1995; Boutton et al. 1998; Scifres and Koerth 1987).

### Plant sampling

Leaves, stems, and root tissues of *P. glandulosa* (the dominant plant species in each woody cluster) and *Z. fagara* (a dominant woody understory species) were sampled along with leaves/stems and roots of three dominant grass species within the grasslands (*B. rigidisetata*, *C. cucullata*, *T. muticus*). All soil was carefully removed from plant roots and care was taken to separate mesquite leaves from the stem. The bark of the woody plants was not removed from the stems. All plant tissues were air-dried at room temperature to constant weight and then ground to a fine powder using a steel ball mill (Retsch, Haan, Germany).

### Soil sampling

A space-for-time chronosequence approach was used to quantify soil total N, amino acids, enzyme activities, and their rates of change following woody plant proliferation into areas that were previously grassland. As *P. glandulosa* initiates woody cluster formation, the age of the mesquite tree within each woody cluster is equivalent to the age of the cluster (Archer et al. 1988). The age of the mesquite tree in each cluster was determined from its basal diameter using a site-specific regression equation developed by Stoker (1997).

In October 2006, soils were sampled from fifteen discrete woody clusters, ranging in age from 14 to 86 years, to establish the chronosequence of woody encroachment. Within each woody cluster, one soil core (5 cm diameter  $\times$  30 cm depth) was collected in each of the four cardinal directions around the base of

the central mesquite tree. Each core was within 50 cm of the mesquite tree. The litter layer was gently removed prior to taking each soil core. Soil cores were taken in a similar fashion around a randomly selected C<sub>4</sub> grass in the grassland adjacent to each woody cluster; these samples represent time 0 in the chronosequence. After sampling, cores were placed on ice until arrival at the laboratory, where they were divided into 0–5, 5–10, 10–15, and 15–30 cm depth increments. For each depth increment the four cores sampled from each site were combined, mixed, and passed through an 8-mm sieve. A portion of the 0–5 cm depth of this field-moist soil was frozen and later used in enzyme assays. Another portion of the 0–5 cm depth was air-dried and then ground with a ball mill grinder (Retsch, Haan, Germany). Amino acids and amino sugars were extracted from the air-dried ground soils (as described below). Soil C and N concentrations were measured using an elemental analyzer (Sercon, Crewe, UK) interfaced to a 20/22 isotope ratio mass spectrometer (Sercon, Crewe, UK).

#### Amino acid and amino sugar extraction

Amino acids and amino sugars were extracted and measured in triplicate using air-dried and ground soil, litter, and plant tissues, as described in Martens and Loeffelmann (2003) and modified by Olk et al. (2008). Briefly, 2 mL of 4 M methanesulfonic acid containing 0.2 % (wt) tryptamine [3-(2-aminoethyl)indole] was added to 250 mg of soil or 20 mg plant material in glass culture tubes and autoclaved for 90 min at 136 °C and 248 kPa pressure. After autoclaving, the solutions were neutralized, diluted, and centrifuged. An aliquot of the supernatant was further diluted and injected into a Dionex (Sunnyvale, CA) DX-500 anion chromatograph equipped with a Dionex AminoPac PA-10 column. Amino acids were detected with triple-pulsed amperometry (Johnson et al. 1993; Olk 2008). Using this method, asparagine and glutamine are converted to aspartate and glutamate, respectively, and tryptophan is not recovered. Yields of aspartate and glutamate are typically lower compared to the conventional procedure for soil amino acid analysis (HCl extraction, separation by cation exchange chromatography, ninhydrin derivatization and detection by light absorption) while yields of lysine, arginine, and histidine are typically higher (Olk et al. 2008). All of the other 20 standard amino acids, in addition to two

non-standard amino acids (ornithine, hydroxyproline) and two amino sugars (glucosamine, galactosamine) were identified.

#### Soil enzyme assays

Enzyme assays for  $\beta$ -*N*-acetylglucosaminidase (NAG, EC 3.2.1.52) and arylamidase ( $\alpha$ -aminoacyl-peptide hydrolase, EC 3.4.11.2) were performed on frozen, field most soils from all 30 woodland and grassland samples. The soil was brought to room temperature just before the addition of the appropriate substrate.

Soil NAG activity was measured following the procedure of Parham and Deng (2000) Briefly, 1 g soil was incubated at 37 °C for 1 h with *p*-nitrophenyl-*N*-acetyl- $\beta$ -glucosaminide as the substrate in acetate buffer (pH 5.5). The reaction was stopped with CaCl<sub>2</sub> (0.5 M) and NaOH (0.5 M) and the product, *p*-nitrophenol, was measured colorimetrically at 405 nm.

Enzyme assays for arylamidase were conducted following the method of Acosta-Martínez and Tabatabai (2000). Briefly, 1 g soil was incubated at 37 °C for 1 h with the substrate, L-leucine  $\beta$ -naphthylamide, in THAM (tris[hydroxymethyl]aminomethane) buffer (0.1 M, pH 8.0). The reaction was stopped with ethanol and the product,  $\beta$ -naphthylamide, was measured colorimetrically at 540 nm after its reaction with *p*-dimethylaminocinnamaldehyde. Enzyme activities were corrected using the appropriate blanks and controls.

#### Calculations and statistical analyses

Student's two-tailed, unpaired *t* tests were used to test for differences between grassland and woodland soils (enzyme activities and the amounts and concentrations of extracted amino compounds). Proportional amounts of amino compounds (relative to total aminos extracted) were arcsine transformed to meet assumptions of normality prior to statistical analysis. One-way analysis of variance (ANOVA) was used to determine differences between C content, N content, C/N ratios, and amino content between the soils and plant tissues. Linear regressions were performed using the REG procedure in SAS (version 9.2, SAS Institute, Cary, North Carolina, USA). In linear regressions with woody stand age, the mean of the grassland samples was treated as time 0 in the chronosequence. Significance was set at  $\alpha = 0.05$  unless otherwise reported.

## Results

### Amino compounds in plant inputs

In general, C and N contents and C/N ratios varied among plant tissues, but also between woody and grassland plants (Table 1). The C contents of the leaves, roots, and stems were generally consistent in the woody plant species while the N content decreased from leaves to roots to stems, thereby increasing the C/N ratios. Similarly, the N content in the grass species decreased from leaves and stems to roots while the C/N ratio increased. Although the C content was similar among *B. rigidiset*a tissues the other two grass species had less C in the roots than in the leaves and stems. The C/N ratios of woody plants tissues were generally lower than the C/N ratios of the corresponding grassland tissues, due to an increase in N in the woody tissues. In grassland plants about 1–7 % of the C and 18–87 % of the N was in amino compounds, while in woody plants amino compounds comprised

0.7–13 % of C and 6–91 % of N. Using measurements of root biomass in the grasslands (186–320 g C m<sup>-2</sup>) and woodlands (~1,300 g C m<sup>-2</sup>) from Hibbard et al. (2003) and the amino-C concentrations of woody plant and grassland roots from Table 1 as proxies for end member input, amino-C in belowground root biomass can be estimated to increase from 5 to 15 g C m<sup>-2</sup> in grassland soils to 40–80 g C m<sup>-2</sup> in woodland soils.

Generally, the distributions of amino compounds were similar between the plants and among the plant parts examined in this study, with some exceptions (Table 2). For example, *Z. fagara* leaves had substantially more methionine (6.5 %) than all other plants and plant tissues (0–0.9 %). Hydroxyproline in *P. glandulosa* roots and stems was 10× higher (9.5–10.3 %) than in the grassland tissues (~1 %) and higher than in *Z. fagara* tissues (0–3 %) and *P. glandulosa* leaves (1 %). In addition, arginine was present in nearly twice the concentration in *Z. fagara* roots (47 %) compared to tissues of the other plants

**Table 1** Carbon, nitrogen, and total amino compound contents of the plant tissues and soils examined in this study

Sample	C content (g kg <sup>-1</sup> )	N content (g kg <sup>-1</sup> )	C/N	Amino content	
				mg amino- C g <sup>-1</sup> C	mg amino- N g <sup>-1</sup> N
C <sub>4</sub> plants					
<i>B. rigidiset</i> a					
Leaf/Stem	405	8.3	49	52	831
Root	409	5.7	72	26	645
<i>C. cucullata</i>					
Leaf/Stem	397	12.7	31	11	182
Root	242	5.2	47	48	874
<i>T. muticins</i>					
Leaf/stem	400	11	35	68	784
Root	282	4.6	60	34	872
C <sub>3</sub> plants					
<i>P. glandulosa</i>					
Leaf	466	28.8	16	130	705
Root	456	17.8	26	65	596
Stem	472	11.9	40	62	919
<i>Z. fagara</i>					
Leaf	462	20.3	23	7	62
Root	468	15.7	30	31	527
Stem	477	9.1	53	36	704
Soils					
Grassland	6.1 ± 2 a	0.5 ± 0.1 a	12.3 ± 1.1 a	144 ± 25 a	620 ± 124 b
Woodland	25 ± 11 b	2.1 ± 0.9 b	12.5 ± 0.8 a	112 ± 23 bc	467 ± 95 a

Standard deviations are given for the soils. Analytical precision is not given for the plants. Within each column, different letters indicate significantly different means between soils

**Table 2** Amino compounds, expressed as a percentage of total amino compounds extracted (mg amino  $\text{mg}^{-1}$  total amino compounds), for plant tissues examined in this study

Amino compound	Percentage (%) of total amino compounds extracted								
	Grasses		<i>P. glandulosa</i>			<i>Z. fagara</i>			
	Leaf	Root	Leaf	Root	Stem	Leaf	Root	Stem	
Hydroxyproline	1 ± 1	0.9 ± 0.1	1.0	10.3	9.5	0	0.3	3.4	
Amino Sugars	6 ± 4	8 ± 2	0.34	1.4	1.3	7.9	8.4	5.9	
Galactosamine	2 ± 1	4 ± 1	0.04	0.2	0.3	1.3	4.0	3.9	
Glucosamine	4 ± 2	4 ± 1	0.3	1.2	1.0	6.6	4.4	2.0	
Acidic Aminos	18 ± 5	17 ± 5	10.1	11.8	7.6	34.1	18.9	17.9	
Glutamate	7 ± 3	7 ± 1	5.6	4.1	4.0	4.5	6.4	7.2	
Aspartate	12 ± 5	11 ± 4	4.5	7.7	3.6	29.6	12.5	10.7	
Basic Aminos	35 ± 15	29 ± 6	25.0	26.7	30.0	32.3	55.4	32.0	
Lysine	5 ± 1	4 ± 1	6.2	5.9	6.6	0	6.9	10.6	
Arginine	28 ± 15	23 ± 7	15.3	15.5	19.5	30.7	46.7	20.5	
Histidine	2 ± 1	1 ± 0.7	3.4	5.2	3.6	1.6	1.6	0.9	
Polar Aminos	9 ± 4	10 ± 1	13.4	15.2	15.5	7.2	7.3	12.1	
Threonine	3 ± 2	4 ± 1	5.0	3.7	4.2	0	1.5	4.0	
Serine	3 ± 2	3 ± 0.3	3.7	5.7	6.2	0.8	2.6	4.6	
Tyrosine	3 ± 0.2	3 ± 0.4	4.7	5.8	5.0	6.4	3.2	3.5	
Nonpolar Aminos	30 ± 18	34 ± 9	49.3	33.7	34.9	18.4	9.6	28.7	
Phenylalanine	5 ± 5	7 ± 7	11.2	2.7	4.3	2.9	0.6	1.6	
Proline	5 ± 3	5 ± 0.4	5.6	8.9	6.7	1.6	0.5	6.5	
Alanine	4 ± 3	5 ± 1	8.7	5.1	5.7	0	0.5	3.4	
Glycine	3 ± 2	5 ± 1	5.3	4.4	4.9	0	1.1	3.3	
Valine	4 ± 2	5 ± 0.5	5.6	4.8	5.1	0	0.3	4.1	
Isoleucine	3 ± 2	2 ± 0.3	3.8	2.2	2.4	3.0	0.8	3.4	
Leucine	5 ± 3	5 ± 0.6	7.7	4.2	4.7	3.2	0.8	5.7	
Methionine	0.4 ± 0.3	0.2 ± 0.3	0.9	0.6	0.3	6.5	0	0	
Cystine	0.4 ± 0.2	0.5 ± 0.3	0.5	0.7	0.8	1.2	5.0	0.7	

Analytical precision of replicates for woody plant tissues is not given. Standard deviation among the grasses (*B. rigidiseta*, *C. cucullata*, *T. muticus*) is given

(15–30 %). Amino sugars and acidic amino acids were lower in *P. glandulosa* relative to *Z. fagara* and the grasses, but *P. glandulosa* generally had higher nonpolar and polar amino acids. Even with these differences, the plants and plant tissues were generally compositionally similar and dominated by arginine, which constituted between 15 and 47 % of the total amino compounds extracted.

#### Amino compounds in soils: grasslands vs. woody clusters

The C and N concentrations were significantly higher in woody cluster soils relative to surrounding remnant grassland soils, while the C:N ratio was unchanged (Table 1). The total amount of amino compounds extracted was significantly higher in woody cluster soils than in grassland soils ( $6 \pm 2.4$  and

$2.0 \pm 0.7$  mg total amino compounds  $\text{g soil}^{-1}$ , respectively). This pattern was maintained for every individual amino compound extracted (data not shown). However, this relationship between ecosystem elements (grasslands vs. clusters) was altered when the mass of N contained within amino compounds was normalized to total N (i.e. mg amino N  $\text{g soil N}^{-1}$ , Table 3). During the conversion from grasslands to woody clusters, the total amino compound content exhibited significant ( $P < 0.0001$ ) decreases in C- and N-normalized concentrations, where 14 % of OC and 62 % of N held in amino compounds in grassland soils decreased to 11 % of OC and 47 % of N in woody cluster soils. Although significant decreases were observed for every group of amino acids extracted, the largest decreases were seen in acidic and basic amino acids, which decreased 38 and 31 %, respectively, with woody encroachment.

**Table 3** Amino acid N extracted from woodland and grassland soil and reported on the basis of soil N

Amino compound	Nitrogen (mg amino N g soil N <sup>-1</sup> )	
	Grassland	Woodland
Hydroxyproline	3.3 ± 1	9.0 ± 3***
Ornithine	4.6 ± 1***	3.1 ± 0.9
Amino sugars	58 ± 9***	44 ± 10
Galactosamine	16 ± 3***	12 ± 3
Glucosamine	43 ± 6***	32 ± 7
Acidic aminos	77 ± 25***	48 ± 13
Glutamate	32 ± 9***	21 ± 5
Aspartate	45 ± 17***	26 ± 8
Basic aminos	229 ± 55***	158 ± 40
Lysine	36 ± 8***	26 ± 6
Arginine	171 ± 44***	111 ± 31
Histidine	23 ± 6	21 ± 4
Polar aminos	57 ± 11**	50 ± 9
Threonine	25 ± 5***	20 ± 4
Serine	24 ± 5	22 ± 4
Tyrosine	8.6 ± 2*	7.7 ± 2
Nonpolar aminos	190 ± 33***	155 ± 28
Phenylalanine	3.1 ± 2	3.4 ± 2
Proline	20 ± 4	18 ± 3
Alanine	42 ± 7***	33 ± 7
Glycine	70 ± 12***	48 ± 11
Valine	20 ± 5*	18 ± 3
Isoleucine	9.4 ± 2	9.8 ± 2
Leucine	19 ± 4	19 ± 3
Methionine	1.7 ± 0.7	2.6 ± 0.7***
Cystine	5.4 ± 2**	4.0 ± 1
Total	620 ± 124***	467 ± 95

Standard deviations are given

Asterisks indicate significant differences of extracted amino-N between woodland and grassland sites

\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.0001$

Amino sugars also decreased substantially, with the two dominant amino sugars, glucosamine and galactosamine, experiencing a decrease of ~25 % in C- and N-normalized concentrations following woody encroachment.

Of the 21 individual amino compounds extracted, two amino acids (hydroxyproline, methionine) made significantly greater contributions to woody cluster soil N than to grassland soil N, while six amino acids (histidine, serine, phenylalanine, proline, isoleucine, leucine) made equal contributions (Table 3). The

remaining 13 compounds comprised greater proportions of soil N in grassland soils relative to cluster soils.

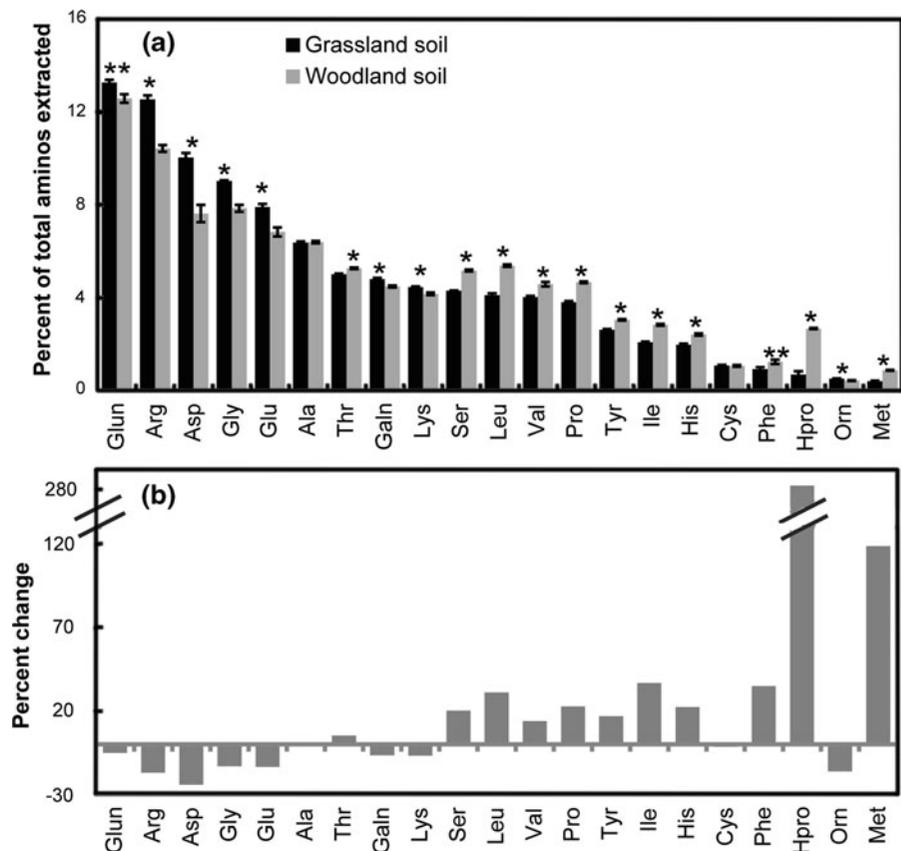
To determine whether woody encroachment changed the composition of the soil amino compound pool, the amount of each amino compound (mg amino g soil<sup>-1</sup>) was divided by the total amount of amino compounds extracted (Fig. 1a). Woody encroachment significantly increased the contributions from polar and nonpolar amino acids and significantly decreased basic and acidic amino acids and amino sugars in the soil. The amino pool also differed significantly between cluster and grassland soils ( $P < 0.01$ ) for every individual amino compound extracted except glucosamine ( $P = 0.02$ ), alanine, cysteine and phenylalanine ( $P = 0.03$ ) (Fig. 1a). Hydroxyproline and methionine displayed the greatest percent increases with woody encroachment (Fig. 1b), although they represented a small proportion of total amino compounds (Fig. 1a) so the actual magnitude of the change was small. Despite these differences, the sum of the five most abundant amino acids and amino sugars (glucosamine, arginine, aspartate, glycine, and glutamate) accounted for ~45 % of the total amino compounds extracted in woody cluster soils and ~53 % of the total amino compounds in grassland soils, making the overall profiles of the amino acid distributions in these two soils similar.

#### Amino compounds in soils: changes with woody stand age

Although the amount of every amino compound (mg amino g soil<sup>-1</sup>) was significantly ( $P < 0.0001$ ) correlated with woody stand age (e.g. Fig. 2a) ( $R^2 = 0.77$ ), the N-normalized concentrations of only two compounds, methionine ( $R^2 = 0.28$ ,  $P = 0.04$ ) and hydroxyproline (Fig. 2b) ( $R^2 = 0.79$ ,  $P < 0.0001$ ), displayed significant correlations with woody stand age. This relationship was also maintained with C-normalized amino-C from hydroxyproline ( $R^2 = 0.76$ ,  $P < 0.0001$ ), methionine ( $R^2 = 0.36$ ,  $P = 0.01$ ), serine ( $R^2 = 0.27$ ,  $P = 0.04$ ), and leucine ( $R^2 = 0.26$ ,  $P = 0.04$ ). No other linear correlations of normalized amino-C or amino-N with woody stand age were significant.

As C and N increase with woody encroachment, a lack of a relationship between woody stand age and amino-C or amino-N means that amino increases are

**Fig. 1** **a** The percent of total aminos extracted held within individual amino compounds and **b** the percent change in the amino pool on transition from grassland to woodland. Error bars indicate  $\pm$  one standard error. Asterisks indicate significant differences between grassland and woodland soils ( $*P < 0.01$ ,  $**P < 0.05$ ). *Gly* glycine, *Glun* glucosamine, *Arg* arginine, *Asp* aspartic acid + asparagine, *Glu* glutamic acid + glutamine, *Ser* serine, *Ala* alanine, *Leu* leucine, *Thr* threonine, *Val* valine, *Galn* galactosamine, *Pro* proline, *Lys* lysine, *Tyr* tyrosine, *Ile* isoleucine, *Hpro* hydroxyproline, *His* histidine, *Phe* phenylalanine, *Cys* cystine, *Met* methionine, *Orn* ornithine



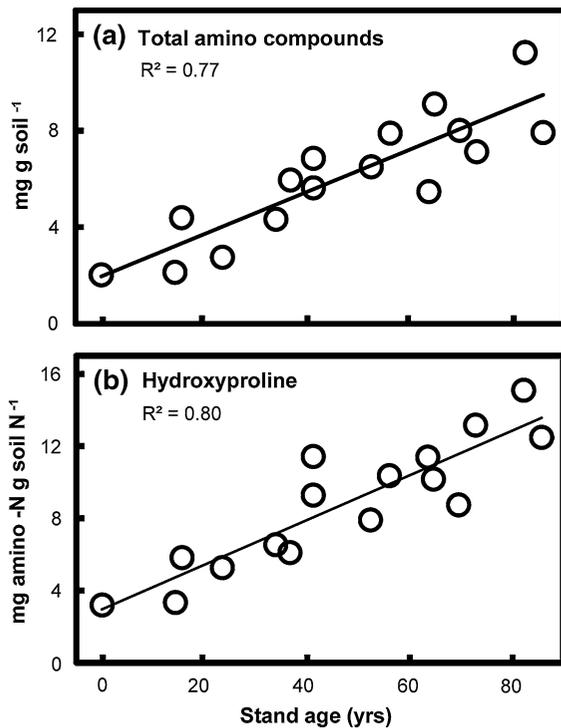
proportional to simultaneous increases in soil total N and SOC, and provides little information about the changing composition of soil amino compounds. Therefore, we examined whether the contribution of any individual amino acids to the total extractable amino pool changed in response to woody encroachment. Both positive and negative linear correlations relative to woody stand age were observed for 13 of the 21 extracted amino compounds (Table 4). The amino compounds with the largest decreases following the grassland to woodland transition (aspartate, glycine, glutamate) were also some of the most dominant amino compounds extracted from both soils (Fig. 1). In contrast, the compounds accruing at the most rapid rate in the woody cluster soils (hydroxyproline, leucine, serine) were not the dominant amino compounds extracted.

#### N-acquiring soil enzymes

The activity of NAG was significantly higher in the woody cluster soils relative to the grassland soils

( $76 \pm 42$  vs.  $20 \pm 12$   $\mu\text{g } p\text{-nitrophenol g soil}^{-1} \text{ h}^{-1}$ , respectively;  $P < 0.0001$ ) and also increased linearly with woody stand age (Fig. 3a) ( $R^2 = 0.52$ ;  $P = 0.002$ ), soil N concentration (Fig. 3b) ( $R^2 = 0.79$ ,  $P < 0.0001$ ), and soil C concentration (slope = 3.3;  $R^2 = 0.78$ ,  $P < 0.0001$ ). Normalized to soil C content, NAG activity was still higher in woodland soils ( $2.3 \pm 2.1$  versus  $0.1 \pm 0.05$   $\mu\text{g } p\text{-nitrophenol g SOC}^{-1} \text{ h}^{-1}$ ;  $P = 0.0004$ ), and was significantly correlated to woody stand age (Fig. 4a) ( $R^2 = 0.44$ ,  $P = 0.005$ ), despite three older woody clusters having surprisingly low C-normalized NAG activity.

Similarly, woodland soils had significantly higher arylamidase activity than grassland soils when expressed per g soil ( $11 \pm 6.3$  vs.  $5 \pm 1.5$   $\mu\text{g } \beta\text{-naphthylamide g soil}^{-1} \text{ h}^{-1}$ ;  $P = 0.007$ ). Unlike NAG activity, there were no significant correlations between arylamidase activity (per g soil) and woody stand age (Fig. 3c), or soil C and N concentrations (Fig. 3d). Arylamidase activity normalized to soil C content was also significantly higher in cluster versus grassland soils ( $P < 0.0001$ ), and although there was a



**Fig. 2** Milligrams of **a** total amino compounds extracted per gram of soil and **b** hydroxyproline-N per gram of soil N relative to woody stand age. Time 0 represents the average of all grassland samples. The average coefficient of variation is 3.7 % for amino compounds and 4.8 % for hydroxyproline

slight correlation with woody stand age, it was not significant due to the high activity observed in the 16-year old woody cluster (Fig. 4b) ( $R^2 = 0.21$ ,  $P = 0.08$ ).

## Discussion

### Accrual of non-hydrolysable amino-N

The proportion of soil N extracted as amino-N from woody cluster and grassland soils (47 and 62 %, respectively) was slightly higher but within range of the amount extracted from agricultural soils using the same method (41–52 %) (Martens et al. 2006; Martens and Loeffelmann 2003; Olk et al. 2008). Using less intensive extraction methods, Amelung et al. (1999, 2006) found that 24–32 % of total soil N was attributed to amino acids and up to 10 % of total N to amino sugars across a survey of grassland soils.

**Table 4** Results from regression analyses of the percentage of total amino compounds extracted versus woody stand age

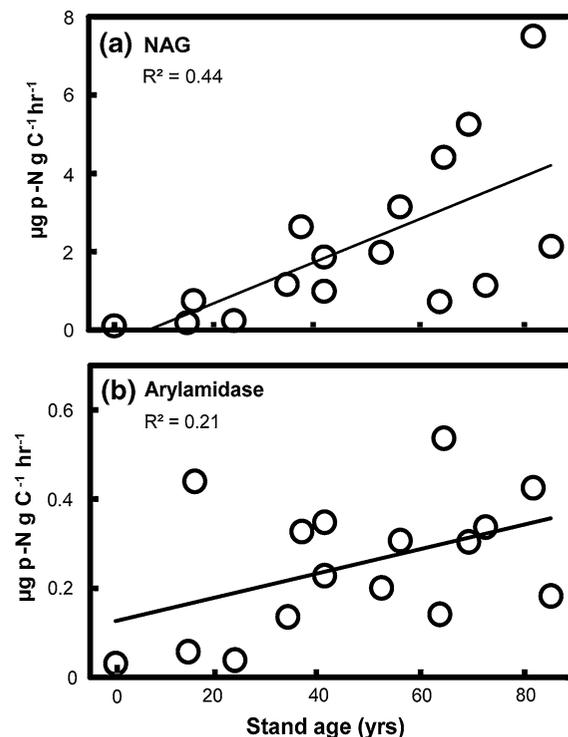
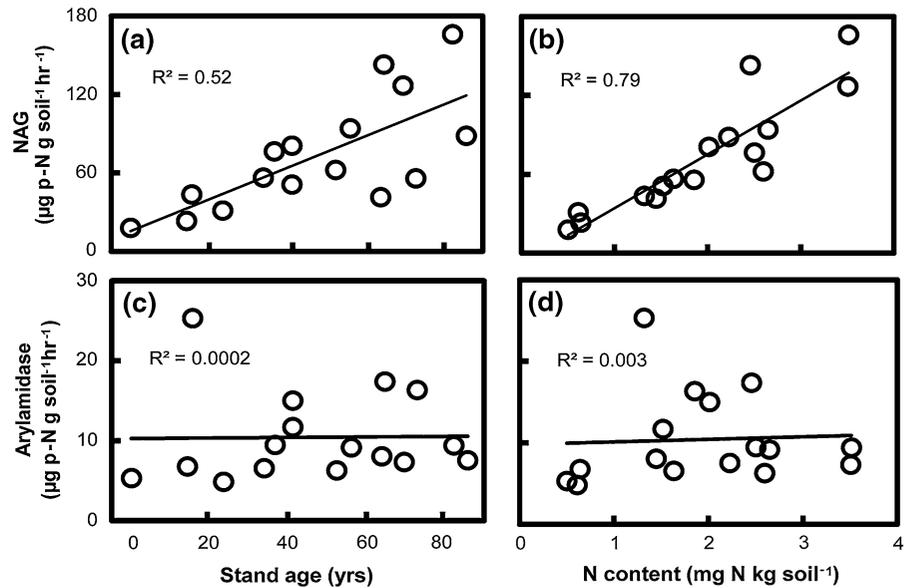
Compound	Regression parameters		
	Slope	$R^2$	$P$
Aspartate	−0.031	0.43	0.008
Glycine	−0.021	0.79	<0.0001
Glutamate	−0.017	0.34	0.02
Galactosamine	−0.010	0.47	0.004
Alanine	−0.007	0.36	0.02
Hydroxyproline	0.036	0.82	<0.0001
Leucine	0.019	0.72	<0.0001
Serine	0.018	0.77	<0.0001
Isoleucine	0.010	0.75	<0.0001
Phenylalanine	0.010	0.33	0.03
Proline	0.009	0.34	0.02
Valine	0.008	0.46	0.004
Methionine	0.005	0.42	0.007
Nonpolar amino acids	0.036	0.38	0.02
Polar amino acids	0.029	0.69	0.0003
Acidic amino acids	−0.040	0.39	0.009
Amino sugars	−0.021	0.35	0.02

Negative slopes indicate a decrease from the grassland to woodland soils with increasing woody stand age. Omitted compounds had  $P$  values >0.05

The higher proportion of soil N and C extracted as amino compounds in grassland soils (62 % of soil N, 14 % of soil C) relative to cluster soils (47 % of soil N, 11 % of soil C) (Table 3) indicates the larger proportion of N and C in woody cluster soils is non-hydrolysable. Both hydrolysable and non-hydrolysable N consists predominately of proteins, but the non-hydrolysable pool may also contain larger proportions of heterocyclic nitrogen compounds (Knicker and Hatcher 1997; Leinweber and Schulten 1998, 2000), particularly in regions prone to fire (Knicker 2004). Additionally, soil protein N can be rendered non-hydrolysable due to association with reactive soil surfaces such as aluminum and iron oxides (Leinweber and Schulten 2000) as well as encapsulation by hydrophobic compounds (Knicker and Hatcher 1997; Zang et al. 2001).

At La Copita, contributions of heterocyclic N to non-hydrolysable organic N should be minimal and consistent among landscape elements (grassland, cluster), since fire suppression was used as a land

**Fig. 3** Correlations between NAG activity (per g soil) and **a** woody stand age and **b** N content (mg N kg soil<sup>-1</sup>) and between arylamidase activity (per g soil) and **c** woody stand age and **d** N content (mg N kg soil<sup>-1</sup>). Time 0 represents the average of all grassland samples. Average coefficient of variation is 2.4 % for NAG and 5.7 % for arylamidase



**Fig. 4** Correlations between woody stand age and carbon-normalized enzyme activity of **a** NAG and **b** arylamidase. Time zero represents the average of all grassland samples. Average coefficient of variation is 2.4 % for NAG and 5.7 % for arylamidase

management process starting ~150 years ago (Archer 1990; Madany and West 1983; McLendon 1993). Changes in plant chemistry known to occur with mesquite encroachment, however, could potentially impact the extractability of soil N at this site. Phenols and polyphenols can stabilize organic nitrogen through hydrophobic interactions and hydrogen bonding as well as through covalent binding (Rillig et al. 2007; Schmidt-Rohr et al. 2004), leading to the formation of chemically stable N (Olk et al. 2006, 2009; Palm and Sanchez 1990). The high polyphenolic content of mesquite inputs (Holecheck et al. 1990; Lyon et al. 1988) combined with increases in lignin in response to woody encroachment in this region (Boutton et al. 2009; Filley et al. 2008) could potentially lead to decreased organic N availability and extractability in the woody clusters relative to the grasslands.

An accrual of non-hydrolysable N in the form of heterocyclic N (Leinweber and Schulten 1998), protein N bound to reactive soil surfaces (Kleber et al. 2007), encapsulated within hydrophobic domains (Knicker and Hatcher 1997; Zang et al. 2001), or chemically transformed amino N through covalent condensation and coupling reactions (Olk et al. 2009) could essentially remove this N from a microbially available reservoir making it harder to degrade, but not

necessarily inert (Nannipieri and Paul 2009). Decreases in organic N availability may result in a N-limitation of the microbial community, subsequently decreasing SOM decomposition (e.g. Hu et al. 2001). In the sandy loam soils examined in this study (~10 % clay), the potential for organic N stabilization on reactive clay surfaces (Kleber et al. 2007; Laird et al. 2001) is minimal and consistent between sites, and is likely not the mechanism for decreased extraction efficiency in older woody stands.

#### Amino response to changing plant inputs

Overall, the composition of soil amino compounds did not clearly reflect the composition of the dominant plant inputs (Fig. 1a, b; Table 3). For the majority of amino compounds, there were no definite trends in soil amino composition through the chronosequence that reflected changes in plant amino input composition. This is likely due to the fact that a good deal of variability existed in the amino composition of the woody plant tissues (Table 2), and so clear trends would be difficult to discern. There were, however, a few soil amino compounds that potentially responded to changing amino input from plants. For example *P. glandulosa* and woody cluster soils had higher amounts of serine, hydroxyproline, and polar amino acids (Table 2; Fig. 1) and *Z. fagara* leaves and woody cluster soils had higher amounts of methionine. However, arginine, which was in very high concentrations *Z. fagara* roots (Table 2), was found in higher concentrations in grassland soils (Fig. 1) although *Z. fagara* is not present in the grasslands.

Instead, the main differences in soil amino compounds between grassland and woody cluster soils seemed to be related to the proportion of soil N that was easily accessible to hydrolysis and thus possibly to microbial decomposition. This inaccessible pool of N can be in many forms and be reflective of both biotic and abiotic processes that control its stabilization. For example, the accumulation of microbial residues and plant and microbe cell wall-bound proteins in soils and sediments indicates these compounds may be more structurally protected from decay (Dauwe and Middelburg 1998; Knicker 2011; Laursen et al. 1996; Rillig et al. 2007). Hydroxyproline, and to a lesser extent, serine, are major amino acid components of plant cell wall structural proteins

(Cassab 1998; Showalter 1993), and their accumulation in soil may reflect the stabilization of cell wall associated proteins. They are particularly concentrated in extensins, which in dicotyledon plants are ubiquitous cell wall proteins but in monocot grasses are less abundant and hydroxyproline-poor (Carpita 1996). Although low in abundance, these amino acids, along with other cell wall associated amino acids such as valine, threonine, and proline (Carpita 1996; Cassab 1998; Showalter 1993), are more prevalent in woody cluster soils at La Copita (Fig. 1b), and increase with the extent of woody encroachment (Table 4). This supports the idea that some of the accumulating amino-N in woodland soils may be associated with plant cell walls. Other amino acids associated with extensins and ubiquitous plant cell wall proteins (such as tyrosine and histidine) are more prevalent in woody cluster soils as well (Fig. 1a), but do not increase with woody stand age (Table 4). Conversely, glycine, which can be associated with glycine-rich cell wall proteins of both monocot and dicot plants (Showalter 1993), and also with microbial biomass (Tremblay and Benner 2006), is in higher concentrations in grassland soils. Lysine, which is higher in grassland soils, is present in dicot extensins, although in monocot extensins it is in higher concentrations as it is often substituted for hydroxyproline (Carpita 1996). The increase in these plant cell wall associated amino acids in woody cluster soils suggests the pool of amino compounds that can be extracted is less altered by the microbial community.

Additionally, the higher proportions of amino sugars (glucosamine, galactosamine) in grassland soils (Fig. 1a; Table 4) indicate microbial contributions to the amino pool decrease with woody stand development, as these amino sugars have been used to estimate microbial contributions to SOM (Glaser et al. 2004; Kandeler et al. 2000). Therefore, increases in structurally-protected (e.g. plant cell-wall) amino acids and decreases in amino sugars diagnostic of microbial activity (e.g. glucosamine, galactosamine) suggest that the pool of extractable amino-N is less available to microorganisms. Combined with increases in non-hydrolysable amino-N in woody cluster soils, these data reveal a system with lower organic N availability and slower organic N turnover than the remnant grasslands.

## Increased N-acquiring enzyme activities reflect non-hydrolysable N accrual

The activities of both arylamidase and NAG were higher in woody cluster soils whether activity was normalized to soil mass (Fig. 3) or soil C (Fig. 4), indicating that the activities of NAG and arylamidase outpaced increases in soil C (and therefore also soil N). This is consistent with a scenario in which the microbial community is shifting enzyme activity towards N acquisition and potentially away from C consumption, due to organic N limitation caused by increases amino-N that are less available to microorganisms.

The activities of N acquiring enzymes have been shown to increase in the presence of complex N sources and decrease in the presence of labile N sources (Allison and Vitousek 2005). A greater amount of complex N in soils of woody clusters is evident through the higher proportion of non-hydrolysable N (Table 3) and buildup of dicotyledon cell wall amino acids (Table 4) and is reflected in the increasing C-normalized enzyme activities with woody stand age (Fig. 4). Therefore, we suggest that although N inputs are higher in woodland soils, the accumulating N is less accessible to the microbial community, requiring the observed higher enzyme production to maintain the appropriate N concentration in microbial biomass (Sinsabaugh et al. 2008). A microbial shift from C acquisition to N acquisition would be confirmed if the activity of C-acquiring enzymes, such as  $\beta$ -glucosidase, decreased with woody encroachment.

It is also possible that differences observed with N enzyme activities are due to changes in physical protection and microbial community structure with woody encroachment. The amount of particulate organic matter and the free light fraction (density  $< 1.0 \text{ g cm}^{-3}$ ) increase with woody encroachment (Creamer et al. 2011; Liao et al. 2006) and as enzyme activity is traditionally higher in these coarser fractions (Allison and Jastrow 2006; Muruganandam et al. 2009) and more related to SOM chemistry (Grandy et al. 2008) this could potentially contribute to the higher observed enzyme activities. In addition, changes in microbial community structure alters N enzyme activity (Grandy et al. 2009; Muruganandam et al. 2009) and the higher fungal biomass observed in woodland soils in this same ecosystem (Boutton et al. 2008) also may affect N enzyme activities.

## Conclusion

As grasslands undergo succession to woodlands in this subtropical landscape, microbial access to organic N decreases with the buildup of more chemically and structurally protected soil N (non-hydrolysable organic N and plant cell wall associated amino-N). The impact of this organic N limitation on the microbial community is confirmed by a decrease in microbial contributions to the extractable pool of amino-N, as well as a ramping up of N-acquiring enzyme activity well past the levels of soil N accrual. This apparent microbial N limitation, confirmed by multiple lines of evidence, is likely a mechanism facilitating SOM accrual in response to woody encroachment in this region. As we consider the responses of ecosystems to future climate and land cover changes, this research highlights the importance in relating the nutrient demands of the microbial community to the processes of decomposition and SOM accrual.

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