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Below and aboveground responses to lupines and litter mulch in a California grassland restored with native bunchgrasses

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ABSTRACT

Goals of ecosystem restoration in California grasslands include the reestablishment of plant communities with a high proportion of native species, and simultaneously improve soil nutrient cycling. Addition of annual lupines and a litter mulch layer were hypothesized to be factors that would promote the growth of the native perennial bunchgrass, *Nassella pulchra*, in a restored California grassland. To test this hypothesis, field mesocosms were installed, each encircling a *Nassella* plant, at a perennial grassland restoration site in Carmel Valley, California. Two sets of treatments were imposed: (1) seeding of the annual N-fixing legume, *Lupinus bicolor*; and (2) exchanging the grassland litter for a thicker mulch layer of C_4 grass litter (C/N = 99). Stable isotope analysis allowed the tracking of fates of N fixed by the legume and the C_4-litter derived C. Treatments continued for 28 months, from December 2002 to April 2005, when most of the destructive measurements were taken. In 2005, neither treatment had significantly increased the biomass of the annuals or the perennial bunchgrass, and there was little effect on total soil C and N. *Lupinus* decreased the $\delta^{15}N$ content, but did not affect the biomass, N and P content of the litter, which was largely composed of annual plants from the previous year. *Lupinus* resulted in higher soil microbial biomass carbon (SMB-C), and distinct effects on soil microbial communities, especially soil fungi, as measured by phospholipid fatty acid analysis (PLFA) and ergosterol. The high C/N litter mulch tended to increase biomass of *Nassella*, despite its lower P concentration, and it reduced SMB-C, presumably due to lower decomposition rates compared to the ambient litter. Using a high C/N litter mulch thus is ambiguous for grassland restoration. Repeated increases of legumes over a longer time frame may potentially increase soil fertility and soil C pools in California grasslands, but this study suggests that native perennial grasses may be slow to benefit.
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1. Introduction

Native perennial bunchgrasses dominated California grasslands prior to European settlement, but non-native annual grasses were able to outcompete the native perennial bunchgrasses after overgrazing and drought (Burcham, 1957; Jackson, 1985). Lowland grasslands in California are now annual grasslands, which are composed of non-native annual grasses from the Mediterranean Basin interspersed with some co-occurring native herbaceous species, and no or very few native perennials (Huenneke, 1989). For large parts of grasslands in California it is assumed that long-lived perennial grasses like *Nassella pulchra* are important keystones for successful reestablishment of native communities. Bunchgrasses provide native habitat conditions for a broad spectrum of native grassland organisms (Stromberg et al., 2007; Anderson and Anderson, 1996). The soil microbial community of the annual grasslands is different from that of relict perennial grasslands, or old-field grasslands that were tilled in the past. Based on a survey of sites across a land use gradient, using phospholipid fatty acid (PLFA) analysis, Steenwerth et al. (2003, 2006) found annual grassland to be more similar to each other and to be high in specific PLFA not abundant in perennial grasslands.

Restoration of ecosystems requires that both plant and microbial communities are considered (Potthoff et al., 2005a; Kardol et al., 2007), because the linkages between above- and belowground processes affect ecological functions (Wardle et al., 2004). For example, N-fixing legumes can increase the N status of neighboring plants and thus species composition in grasslands (Jacot et al., 2005; Temperton et al., 2007), as well as improve the N status of plants in subsequent years through decomposition and mineralization of N in plant material (Jensen, 1996). Soil biota mediate the plant-soil transfers that supply and retain nutrients in

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soil, and the composition of soil microbial communities is increasingly recognized as important for the stabilization of C from plant residues in soil (Guggenberger et al., 1999; Bailey et al., 2002).

Two approaches were taken to supply C and other nutrients to a restored perennial bunchgrass grassland ecosystem: (1) seeding of *Lupinus bicolor*, a native annual legume that, like other legumes, periodically has high abundance years (hereafter referred to as *Lupinus*); and (2) addition of a litter mulch of *Bouteloua gracilis*, a non-native perennial C_4 grass, used because it has a similar C:N ratio as the dominant native bunchgrass, *Nassella pulchra* (hereafter referred to as *Nassella*). Both approaches were hypothesized to facilitate nutrient cycling processes that would simultaneously increase the productivity of the native bunchgrasses and increase soil C storage (Potthoff et al., 2005a).

The litter mulch was expected to reduce the germination and establishment of annual grasses (Bilalis et al., 2003), and thus increase nutrient availability to the bunchgrass, as well as provide a C supply to the soil microbial biomass (SMB) that would gradually increase total soil C (Dornbush, 2007). Some litter types have been found to suspend annual grass seeds, limiting their contact with the mineral soil, and exposing germinated seedlings to periods of drying and death (Young et al., 1971). Seeding of *Lupinus* was expected to increase N availability due to N-fixation, and thereby facilitate plant productivity (Temperton et al., 2007) via N release from its decomposing litter (Yelenik et al., 2007), especially of *Nassella* in the C_4 litter treatment, as well as provide N to the soil microbial biomass that would lead to increased soil C assimilation (Mary et al., 1996). Since other species of *Lupinus* increase P availability to subsequent plant populations by rhizosphere secretion of organic anions into the rhizosphere (Kamh et al., 1999; Weisskopf et al., 2006), and also increase soil fungi (Appuhn and Joergensen, 2006), *Lupinus bicolor* also may affect soil processes that increase P availability to plants, again especially of *Nassella* in the C_4 litter treatment due to hypothesized mulch suppression of competing annual plants. Hence, from the perspective of practical restoration it is to ask, if the combination of lupine seeding and mulch addition brings specific benefit to the native bunchgrass.

The apparent transfer of the C from the C_4 litter mulch to the SMB can be tracked using the natural abundance of the stable isotope ^{13}C (Potthoff et al., 2003). The $^{12}C/^{13}C$ ratio is always lower in C_4 plants than in C_3 plants; all of these grassland species are C_3 plants. In C_3 plants, a substantial fractionation of C occurs during photosynthesis which discriminates against ^{13}C more strongly than the enzyme system of C_4 plants. Likewise, the transfer of N from *Lupinus* to other plant species can be followed with the natural abundance of $\delta^{15}N$ (Högberg, 1997). Due to fixation of N_2 by legumes, their isotopic signature is closer to that of atmospheric N_2 (zero ‰), than that taken up by the soil (more positive $\delta^{15}N$ values). Plants in the grassland community that utilize N that is provided by *Lupinus*, either from root exudates or from decomposition of its litter, will contain a more negative $\delta^{15}N$ signature.

By combining indicators of nutrient cycling with description of soil microbial communities, it is possible to infer the role of different microbial groups in processes that affect restoration. PLFA analysis uses cell membrane lipids as biomarkers to generate a profile or fingerprint of the microbial community (Bossio and Scow, 1995; Steenwerth et al., 2003; Joergensen and Potthoff, 2005; Potthoff et al., 2006). PLFA are quickly degraded in the soil environment and thus represent the living soil microbial community.

The main objective of this study was to determine how a one-time addition of litter mulch and/or seeding a legume affected: (1) the aboveground plant community composition; (2) the quantity and composition of the belowground microbial community; and (3) the fate and the availability of nutrients (N from legume fixation and C from added litter) of restored *Nassella* grassland, with an emphasis on the delayed responses that occurred 28 months after the treatments were imposed. The focus is on plant-soil interactions and litter-mediated processes.

2. Materials and methods

2.1. Site and treatments

The experiment took place at the UC Hastings Natural History Reservation in the foothills of the Santa Lucia Mountains in Upper Carmel Valley (121°33′45″E, 36°22′44″N). The study site was on a Sheridan coarse sandy loam soil (Coarse-loamy, mixed, thermic Pachic Haploxerolls; Cook, 1978) located on a level area that had been farmed between 1865 and 1937. From 1937 to 1996 the site supported annual grassland. The climate of this region is Mediterranean, with small annual amplitudes in daily mean temperatures (15.5 °C in summer and 13.0 °C in winter; Cook, 1978). Mean annual precipitation ranges between approximately 350 and 500 mm and occurs from September to May.

In November 1995, the restoration process started by tilling a 50 m × 20 m plot within the annual grassland (Potthoff et al., 2005a), which has been monitored for plant species composition since 1971 (Stromberg and Griffin, 1996). First, soil was intensively disked to 45 cm depth. Later, each time that annual seedlings colonized the field, it was rototilled to remove the annual plants before they could set seed. The harrow was adjusted to a depth of 25 cm. Tillage thus occurred four times per year during the wet season. In addition, glyphosate (Roundup Grass/Weed Killer, Ortho, Columbus, OH, USA) was applied once a year. The herbicide was usually sprayed in April to remove all sprouting vegetation when soil moisture did not allow vehicles to enter the plot. These procedures were effective in reducing the annual seed bank in soil. By the second year, the cover of annuals between tillage events was less than 10%. In December 1997, native perennial bunchgrasses were sown in a 600 m^2 area after the plot was harrowed. *Nassella pulchra* (*Agrostideae* tribe), *Elymus glaucus* (*Hordeae* tribe), and *Hordeum brachyantherum* ssp. *californicum* (*Hordeae* tribe) were seeded at 50, 75, and 38 kg/ha, respectively.

In December 2002, 20 *Nassella* plants were randomly selected in the plot and encircled with a PVC-tube (30 cm diameter and 30 cm in height) that was pounded down to a depth of 25 cm. From ten of the cylinders, C_3 surface litter was removed (40% C, C/N ratio = 51, 0.084% P) and exchanged by litter of the C_4-grass, *Bouteloua gracilis* (41% C, C/N ratio = 99, 0.042% P) that was grown nearby on the same soil type. To each of the 10 cylinders, 50 g/m^2 of litter were applied. This amount is within the typical range of litter amounts occurring in nearby grasslands (Potthoff et al., 2005a). For these cylinders, however, it turned out to be more than the existing litter, and it was utilized as a technique to suppress the germination and establishment of annual plants in the cylinders. *Bouteloua* has a higher natural abundance of ^{13}C in its litter tissue ($\delta^{13}C = -13.96$) than *Nassella pulchra*, a C_3 grass ($\delta^{13}C = -27.85$), and of the annual C_3 grasses ($\delta^{13}C = -28.84$), and therefore, final $\delta^{13}C$ values of recovered litter, SMB, and soil at the end of the experiment should indicate differences in the turnover and soil incorporation of surface inputs of perennial grass litter. About 50 pre-germinated *Lupinus bicolor* seeds were seeded per cylinder by hand to ten cylinders in total: five cylinders with non-exchanged C_3 litter (C3Lit) and five with exchanged C_4 litter (C4Lit). The investigation had a completely randomized design representing four treatments with five replicates: the encircled *Nassella* plant with: (1) existing C_3 litter and no seeded *Lupinus* (C3Lit, No LupAdded); (2)

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exchanged C_4 litter and no seeded *Lupinus* (C4Lit, No LupAdded); (3) existing C_3 litter and seeded *Lupinus* (C3Lit, LupAdded); and (4) exchanged C_4 litter and seeded *Lupinus* (C4Lit, LupAdded).

2.2. Plant sampling and analysis

Plant sampling was done in April/May 2003, 2004, and 2005. In 2004 and 2005, cover was recorded for all species in each cylinder. The diameter of the *Nassella* plant was measured in the first year.

In 2003 and 2004, the aboveground biomass of the annual species in the cylinders was estimated visually based on neighboring areas that were clipped and weighed. Separate estimates were also made for *Lupinus* spp., but it was impossible to accurately estimate biomass of *L. bicolor* and *L. nanus*, both of which occurred at the site before the experiment began. In 2005, aboveground biomass of annuals and *Nassella* was clipped, litter was removed, and plants and litter were weighed after drying. Nutrients (Ca, P, Al, B, Cu, Fe, K, Mg, Mn, Na, S, and Zn) were analyzed by microwave digestion with nitric acid (Sah and Miller, 1992) and analyzed by ICP-AES. Isotope signatures and total concentrations of C and N were determined on a Europa Hydra 20/20 IRMS (Crewe, UK) at the University of California at Davis Stable Isotope Facility in: (1) tissue from the *Nassella* plant in each cylinder including the dead leaves from the previous year that remain attached to the crown of the plant; (2) the standing annual plant biomass in each cylinder; and (3) litter residues left on the soil surface in each cylinder. For the latter, $\delta^{13}C$ values were used to calculate the proportion of *Bouteloua* C_4 litter vs. litter of annual plants grown in 2003 and 2004 by the following equation:

$$\delta^{13}C_{sa} = y\delta^{13}C_{C_4} + (1-y)\delta^{13}C_{C_3} \text{ or}$$

$$y = (\delta^{13}C_{sa} - \delta^{13}C_{C_3})/(\delta^{13}C_{C_4} - \delta^{13}C_{C_3});$$

where y is the biomass portion of C_4 litter C contributing to the mixed sample, sa indicates the mixed sample, C4 the C_4 litter, and C3 the C_3 litter.

2.3. Soil sampling and analysis

Soil samples were taken by subsampling from the intact cylinders in April 2003. Three subsamples, at two depths (0–7.5 and 7.5–15 cm), were taken from each cylinder using a 1.2 cm diameter steel tube. The subsamples of one cylinder were composited for each depth. After subsample removal, the core was filled with a PVC tube (1.1 cm in diameter) to prevent the soil from collapsing and to avoid preferential flow of rainwater into the holes. In 2005, the cylinders were removed from the site and sliced into three layers (0–7.5, 7.5–15, and 15–30 cm). Only the upper two layers were considered for further analysis. In December 2002, at the start of the experiment, a set of 10 randomly taken soil samples of corresponding depth and layers were analyzed as a time zero reference for isotopic signatures. Total soil C and N were determined on a Europa Hydra 20/20 IRMS (Crewe, UK) at the University of California at Davis Stable Isotope Facility along with isotope signatures for C and N.

Soil microbial biomass C was measured by fumigation extraction (Brookes et al., 1985; Vance et al., 1987). Field-moist soil was split into two portions (25 g for the fumigated and 25 g for the nonfumigated treatment), extracted by oscillating shaking at 250 rev m^{-1} with 100 mL 0.5 M K_2SO_4, and filtered through washed folded paper filter (Whatman No. 3, Springfield Mill, Maidstone, Kent, UK). Organic C in the K_2SO_4 extracts was measured using diluted extracts (1:10) and a Phoenix 8000 automatic analyzer (Dohrmann [Tekmar-Dohrmann], Manson, OH, USA) according to the method of Wu et al. (1990), and the calculation was: C in SMB = EC/k_{EC} where EC = [total C extracted from fumigated soil] − [total C extracted from nonfumigated soil] and k_{EC} = 0.45 (Wu et al., 1990; Joergensen, 1996).

Stable isotope ratios of C and N in the K_2SO_4-extracts were measured by continuous flow isotope ratio mass spectrometry. After the extracts were digested with persulfate, the CO_2 passed from an "OI Analytical" TOC analyzer (College Station, TX) in line with a PDZ Europa 20-20 isotope ratio mass spectrometer (Crewe, UK) at the University of California at Davis Stable Isotope Facility. Litter-derived C in total soil C and in the SMB was calculated following Potthoff et al. (2003).

Ergosterol was measured in 2 g of moist soil extracted with 100 ml ethanol for 30 min by oscillating shaking at 250 rev min^{-1} (Djajakirana et al., 1996). Quantitative determination of ergosterol was performed by reversed phase HPLC analysis at 26 °C using a column of 125 mm × 4 mm Sphereclone 5 μ ODS II with a Phenomenex guard column (4 mm × 3 mm). The chromatography was performed isocratically with 100% methanol and a resolution of detection of 282 nm.

The diverse set of PLFA from each soil sample, or PLFA profile, was analyzed by multivariate statistics, a semi-quantitative approach that generates fingerprints of the microbial community (ter Braak, 1995). Different microbial groups, e.g. types of bacteria or fungi, are characterized by different PLFA markers. The total concentration of PLFA is a measure of viable SMB (Zelles, 1997). The protocol for PLFA analysis followed Bossio and Scow (1995). Total lipids were extracted from moist soil samples (8 g dry soil) using a chloroform–methanol extraction (Bligh and Dyer, 1959) modified to incorporate a 0.05 M phosphate buffer. The PLFA were purified from the lipid extracts, quantified, and identified using a Hewlett Packard 6890 gas chromatograph fitted with a 25 m Ultra 2 (5% phenyl)-methylpolysiloxane column (J&W Scientific, Folsom, CA). Bacterial standards and identification software from the microbial identification system (Microbial ID, Inc., Newark, DE) were attributed to specific PLFA peaks. The Microbial ID protocol was modified to include an internal standard (19:0) of known concentration. The fatty acids were quantified by comparing the peak areas with those of the standard peak. Fatty acid terminology utilizes 'A:BuC', where 'A' indicates the total number of carbon atoms, 'B' the number of unsaturations, and 'u' precedes 'C', the number of C atoms between the closest unsaturation and the aliphatic end of the molecule. The suffixes 'c' and 't' indicate cis and trans geometric isomers. The prefixes 'i' and 'a' refer to iso and anti-iso methyl branching. Hydroxy groups are indicated by 'OH'. Cyclopropyl groups are denoted by 'cy', '10Me' refers to a methyl group on the tenth carbon from the carboxylic end of the fatty acid.

PLFA were grouped into bacterial (actinomycetes, Gram positive ($Gram^+$), Gram negative ($Gram^-$), fungal and unspecific origins following Kroppenstedt (1985), Federle (1986), O'Leary and Wilkinson (1988), Vestal and White (1989), Olsson et al. (1995), Zelles (1997), and Bossio and Scow (1998) (see below; Table 4). Actinomycetes were classified separately from $Gram^+$ bacteria.

2.4. Statistics

All results are given in arithmetic means. In the case of nutrient contents of soil or plant tissue results are expressed on an oven-dry basis (about 24 h at 105 °C). The significance of treatment effects was tested by two-way analysis of variance using the Tukey/Kramer HSD-test (honestly significant difference) with 'litter exchange' and 'lupin addition' as independent factors, or one-way analysis of variance for the factor 'lupin addition' in the case of $\delta^{13}C$ analysis (Statistica/w; Multrus and Lucyga, 1996).

Comparisons among PLFA profiles (taken as fingerprints of the microbial community) were analyzed by correspondence analysis using absolute contents (CA, Canoco 4.0; Microcomputer Power, Ithaca, NY) for visualization of sample similarities. In graphical

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outputs, the position of samples along the axes is determined by the loading scores, which describe the relative importance of a variable along the ordination axis. The CA algorithm constructs a theoretical variable that best explains the data for each ordination axis, and uses reciprocal averaging to assign values to the treatments to maximize the dispersion of the scores for the samples. Further ordination axes that are not correlated to the previous ones are constructed to explain the remaining variation. In addition to CA the PLFA contents were analyzed in a two-way-Anova procedure (see above) and a RDA (redandancy analysis) was applied in CANOCO to enable permutation tests (ter Braak, 1995). PLFAs that were detected in less than 10% of the samples were excluded from further analysis.

3. Results

3.1. Plant community composition and biomass

Total cover of *Lupinus* spp. ranged from ~25% to ~65% depending on the treatment in the first year (2003) (Fig. 1). In 2003, as expected, seeded plots had greater *Lupinus* cover than non-seeded plots. In 2003, the exchanged *Bouteloua* C_4 litter tended to reduce *Lupinus* spp. cover ($p = 0.06$; Fig. 1). This pattern was not apparent, however, in subsequent years. *Lupinus* spp. cover did not reach the performance of 2003; its cover was 6% or lower in 2004 and 2005 (Fig. 1; Table 1). No effects of the litter exchange on *Lupinus* spp. cover were observed in 2004, but in 2005, contrary to what was observed in 2003, the C_4 litter increased *Lupinus* spp. cover, but only slightly.

Aboveground biomass of *Nassella* bunchgrasses was not affected by *Lupinus* addition at the end of the experiment in 2005 (Table 2). Yet there was a strong trend ($p = 0.06$) toward higher biomass of *Nassella* plants in the cylinders with exchanged C_4 litter. With no added *Lupinus*, *Nassella* had two-fold higher biomass in the exchanged C_4 than C_3 litter treatment, and with

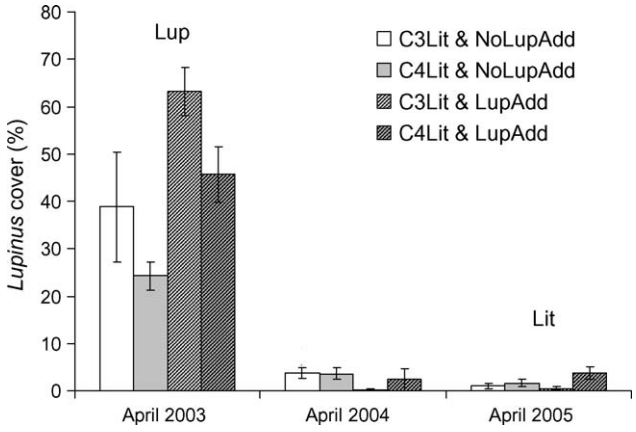

Fig. 1. Lupine cover (%) in each sampling year. For treatment abbreviations please refer to Table 1. Significant treatment effects ($p < 0.05$) calculated by two-way ANOVA using Tukey HSD are given on top of the columns compared ('Lup' for *Lupinus* addition, 'Lit' for litter exchange, and 'Lup&Lit' for significant interactions, $n = 5$. Error bars indicate standard error.

added *Lupinus*, 1.5-fold higher with C_4 litter compared to C_3 litter. Cover of *Nassella* in 2004 and 2005 was highest in the treatment with C_4 litter (27.6 and 21.4%, respectively) compared to 11–14% cover in the C_3 litter treatments (Table 1). Some caution is needed in interpreting these results, since no initial biomass measurements were possible. Similar diameters of *Nassella* bunches in all treatments in 2003 suggest that the plants in each treatment were initially similar in size (data not shown).

Standing biomass of annual plants (which included *Lupinus* spp.) was significantly affected by *Lupinus* seeding and added *Bouteloua* C_4 litter in the first year (2003) only (Fig. 2). Specifically, cylinders with the original C_3 litter left in place had about twice as much estimated biomass of annual plants as did the cylinders with the *Bouteloua* litter exchange. *Lupinus* addition increased the estimated annual

Table 1
Plant species and mean percent cover for each treatment in April, 2004 and 2005. (C3Lit = *N. pulchra* plant encircled by cylinder with original site C3 plant litter left in place, C4Lit = original litter in encircled areas exchanged with *B. gracilis* litter (50 g), LupAdd = Addition of *Lupinus* plants by seeding 1400 *L. bicolor* seed m^{-2}, NoLupAdd = No addition of *Lupinus* seed). Within each plant category, species are listed in order of descending percent cover observed in 2005.

	C3Lit & NoLupAdd		C4Lit & NoLupAdd		C3Lit & LupAdd		C4Lit & LupAdd	
	2004	2005	2004	2005	2004	2005	2004	2005
Native perennials								
Elymus glaucus	1.0	2.4	8.6	4.5	5.0	6.0	3.8	3.2
Hordeum brachyantherum	0.0	0.0	0.6	0.0	0.0	0.0	0	0.0
Nassella pulchra	11.4	14.0	27.6	21.4	12.6	12.4	14.6	11.9
Quercus douglasii	0.4	0.4	0.0	0.0	0.4	0.4	0	0.0
Native annuals								
Amsinckia menziesii	0.0	0.0	0.0	0.1	0.1	1.0	0	0.2
Clarkia purpurea	1.8	6.6	0.3	3.8	4.2	6.6	2.8	4.2
Escholtzia californica	0.42	10.2	10.0	0.0	0.0	0.3	0	0.6
Galium nuttallii	0.0	0.6	0.3	0.5	0.2	1.2	0	0.1
Lupinus bicolor[a]	5.8	1.2	3.8	0.4	0.0	0.1	2.6	0.7
Lupinus nanus	1.4	0.4	3.0	1.2	0.0	0.4	0.4	2.6
Phlox gracilis	0.0	4.2	0.1	1.7	0.0	1.2	1	4.0
Plagiobotrys nothofulvus	1.62	4.2	0.8	5.4	0.8	4.0	0.8	1.2
Platystemon californica	0.0	3.0	0.0	0.0	0.0	0.8	0	0.4
Silene galica	0.0	0.4	0.0	0.3	1.2	3.9	0	0.6
Thysanocarpus curvipes	0.0	0.0	0.0	0.0	0.1	0.8	0	0.0
Non-native annuals								
Annual grasses[b]	9.8	13.0	9.0	10.0	10.0	15.4	14.4	16.8
Anagalis arvensis	0.0	0.2	0.0	0.0	0.0	0.0	0	0.0
Erodium botrys	0.0	0.2	0.1	2.4	0.0	0.0	0	0.0
Erodium cicutarium	1.6	4.2	0.12	2.4	0.8	3.4	0.6	3.2
Hypochaeris glabra	12.3	15.2	4.4	15.4	6.3	12.6	5	11.6
Medicago spp.	0.0	0.4	0.0	0.2	0.0	0.6	0	0.7

[a] *Lupinus bicolor* was sown in the LupAdd-treatments in December 2002.
[b] Annual grasses included: *Aira caryophyllea*, *Avena barbata*, *Bromus diandrus*, *Bromus hordeaceus*, Vulpia spp.

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Table 2
Plant tissue harvested in 2005 (mean ± SE). For treatment abbreviations please refer to Table 1 *Nassella* tissue include both live biomass and dead leaves from previous seasons, which remain attached to the plant crown. Significant levels (ns = $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) are calculated by two-way ANOVA using Tukey HSD testing the effect of litter exchange (Lit) and Lupine addition (Lup); $n = 5$).

Plant tissue	Response variable	Lit	Lup	Lit*Lup	C3Lit & NoLupAdd	C4Lit & NoLupAdd	C3Lit & LupAdd	C4Lit & LupAdd
Recovered litter	Biomass (g m^{-2})	***	ns	ns	79 ± 21	303 ± 22	156 ± 31	291 ± 23
	μg P g^{-1} dry wt.	*	ns	*	813 ± 50	785 ± 26	836 ± 57	979 ± 32
	mg C g^{-1} dry wt.	*	ns	ns	401 ± 22	359 ± 19	428 ± 4	377 ± 27
	mg N g^{-1} dry wt.	ns	ns	ns	7.8 ± 0.6	8.5 ± 0.9	8.1 ± 0.8	9.7 ± 0.6
	$\delta^{13}C$§	n/a	*	n/a	−28.8 ± 0.2	−22.1 ± 0.8	−29.4 ± 0.1	−24.9 ± 0.4
	$\delta^{15}N$	**	**	ns	0.2 ± 0.3	1.4 ± 0.4	−0.8 ± 0.2	0.2 ± 0.2
Nassella	Biomass (g m^{-2})	ns	ns	ns	105 ± 31	216 ± 42	166 ± 85	235 ± 59
	μg P g^{-1} dry wt.	*	ns	ns	1984 ± 96	1605 ± 62	1779 ± 95	1653 ± 156
	mg C g^{-1} dry wt.	ns	ns	*	417 ± 4	400 ± 12	396 ± 16	458 ± 27
	mg N g^{-1} dry wt.	ns	ns	ns	11.5 ± 1.6	10.0 ± 0.8	10.2 ± 1.1	11.7 ± 2.2
	$\delta^{13}C$	ns	*	ns	−27.3 ± 0.4	−27.5 ± 0.2	−27.9 ± 0.2	−28.1 ± 0.2
	$\delta^{15}N$	ns	ns	ns	1.2 ± 0.2	0.4 ± 0.5	0.6 ± 0.4	1.1 ± 0.6
Standing biomass of annual plants	Biomass (g m^{-2})	ns	ns	ns	156 ± 38	114 ± 10	156 ± 16	139 ± 25
	μg P g^{-1} dry wt.	ns	ns	ns	3540 ± 180	3989 ± 190	3627 ± 205	3630 ± 260
	mg C g^{-1} dry wt.	ns	ns	ns	416 ± 6	398 ± 3	409 ± 4	402 ± 14
	mg N g^{-1} dry wt.	ns	ns	ns	9.6 ± 1.2	10.6 ± 0.4	9.5 ± 0.6	11.0 ± 0.7
	$\delta^{13}C$§	ns	ns	ns	−29.6 ± 0.3	−29.6 ± 0.1	−30.0 ± 0.1	−29.5 ± 0.2
	$\delta^{15}N$	ns	ns	ns	1.1 ± 0.5	2.0 ± 0.2	1.5 ± 0.2	1.6 ± 0.3

n/a, not applicable.
§ N.B. Analysis was split up by litter treatment to meet the assumptions of normality in ANOVA.

plant biomass in both litter treatments, which was mainly due to the higher *Lupinus* cover in these cylinders. There were no treatment effects in 2004 for the estimated standing biomass of annual plants, or in 2005 for the actual aboveground biomass.

In total, 4 native perennial, 11 native annual, and 10 non-native annual species occurred in the cylinders during Spring, 2004 and 2005 (Table 1). (Species richness was not measured in 2003). There were no marked differences in species composition among treatments in either year, nor were there clearly dominant annual species in any year; i.e., the highest cover of a native species was *Lupinus bicolor* (6% in 2004), and of a non-native species was *Hypochaeris glabra* (15% in 2005). The exchanged *Bouteloua* C_4 litter tended to reduce the cover of native annual species in 2005 (13% vs. 25% cover), whereas it had no effect on non-native annuals (Table 1). There were no comparable effects of adding *Lupinus* on either native or non-native annual species in either 2004 or 2005.

3.2. Nutrients in plants and litter

The total N and C content of all plant tissues and recovered litter were generally unaffected by the treatments at the end of the experiment in 2005, except that there were higher C concentrations in the recovered litter from the C_3 litter treatment than from the exchanged *Bouteloua* C_4 litter treatment (on average about 414 μg C/g vs. 367 μg C/g tissue; Table 2). The biomass of the recovered litter was also higher in the C_4 litter treatments, indicating that much of the added high C/N litter remained, since biomass of annual plants was either estimated or measured to be lower or not different than in the C_3 litter treatments in 2003, 2004, and 2005 (Fig. 2; Table 2).

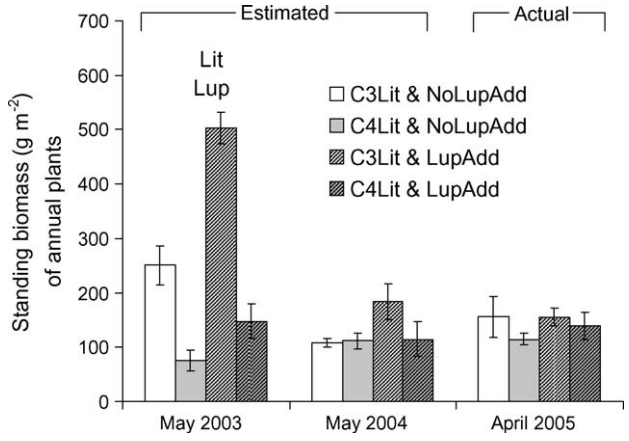

Fig. 2. Standing biomass (g m^{-2}) of annual plants in each sampling year. For treatment abbreviations please refer to Table 1. Significant treatment effects ($p < 0.05$) calculated by two-way ANOVA using Tukey HSD are given on top of the columns compared ('Lup' for *Lupinus* addition, 'Lit' for litter exchange, and 'Lup&Lit' for significant interactions, $n = 5$. Error bars indicate standard error.

Higher P concentrations in *Nassella* tissue occurred in treatments with existing C_3 litter compared to *Bouteloua* C_4 litter treatments (1881 μg P g^{-1} vs. 1629 μg P g^{-1} tissue) (Table 2). No significant treatment effects were found for micronutrients in any of the plant tissues or recovered litter (data not shown).

As expected, $\delta^{13}C$ values were less negative in recovered litter from the *Bouteloua* C_4 litter treatments than from the treatments without litter exchange (Table 2).

$\delta^{15}N$ values decreased in recovered litter due to *Lupinus* addition, on average from 0.78 to −0.31 (Table 2). Thus, the 2004 plants apparently had incorporated substantial *Lupinus*-derived N, or alternatively, there was immobilization from soil N by microbes on the litter in the fall/winter of 2004–2005. Adding *Bouteloua* litter significantly increased the $\delta^{15}N$ in the recovered litter.

Nassella plants with added *Lupinus* had slightly more negative $\delta^{13}C$ values than without added *Lupinus* (−28.0 vs. −27.4; Table 2). This indicates lower water use efficiency, i.e., lower C assimilation per unit water transpired in the *Lupinus* treatments (Tambussi et al., 2007) although it was not associated with higher N content. The annual plant litter, derived mainly from annual plants growing in 2004, showed the same pattern, i.e., *Lupinus* addition resulted in more negative $\delta^{13}C$ values.

3.3. Soil nutrient content

The total soil C content at both depths (0–7.5 and 7.5–15 cm) in the unamended treatment (C_3 litter and no added *Lupinus*) did not change throughout the 28-month period (Table 3). It ranged between 0.76 and 0.94% C in the upper layer, and 0.76 and 0.83% in the lower layer. Neither the increase of *Lupinus* nor the litter exchange affected the total C content of soil at 0–7.5 depth. The similarity between the C and N content of the two layers reflects the recent tillage to establish the restored perennial grassland.

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Table 3
ANOVA significance levels and treatment comparisons of total soil C, N and Bray P in the soil at each depth (2002, 2003 and 2005). For treatment abbreviations please refer to Table 1 Significant levels (ns = $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) are calculated by two-way ANOVA using Tukey HSD testing the effect of litter exchange (Lit) and Lupine addition (Lup); $n = 5$).

	Soil C mg g^{-1} soil (dw)		Soil N mg g^{-1} soil (dw)		Soil Bray-P μg g^{-1} soil (dw)	
	0–7.5 cm	7.5–15 cm	0–7.5 cm	7.5–15 cm	0–7.5 cm	7.5–15 cm
Treatment means						
December 2002						
t_0 (random $n = 8$)	8.2 ± 0.4	7.6 ± 0.3	0.65 ± 0.00	0.56 ± 0.00	25.2 ± 1.3	24.6 ± 0.5
April 2003						
C3Lit & NoLupAdd	7.8 ± 0.1	nd	0.57 ± 0.00	nd	nd	nd
C4Lit & NoLupAdd	8.0 ± 0.3	nd	0.59 ± 0.02	nd	nd	nd
C3Lit & LupAdd	7.8 ± 0.3	nd	0.62 ± 0.03	nd	nd	nd
C4Lit & LupAdd	7.6 ± 0.2	nd	0.54 ± 0.04	nd	nd	nd
April 2005						
C3Lit & NoLupAdd	8.5 ± 0.1	7.5 ± 0.4	0.49 ± 0.00	0.42 ± 0.03	27.7 ± 0.9	28.0 ± 1.2
C4Lit & NoLupAdd	9.0 ± 0.3	8.3 ± 0.4	0.58 ± 0.03	0.49 ± 0.04	32.0 ± 1.0	31.2 ± 1.6
C3Lit & LupAdd	9.4 ± 0.3	7.0 ± 0.1	0.55 ± 0.05	0.46 ± 0.01	30.2 ± 1.1	36.5 ± 1.2
C4Lit & LupAdd	8.9 ± 0.2	7.2 ± 0.3	0.54 ± 0.02	0.45 ± 0.02	31.0 ± 0.9	34.2 ± 1.2
ANOVA significance levels						
April 2003						
Lit	ns	nd	ns	nd	nd	nd
Lup	ns	nd	ns	nd	nd	nd
Lit × Lup	ns	nd	ns	nd	nd	nd
April 2005						
Lit	ns	ns	ns	ns	*	ns
Lup	ns	*			ns	***
Lit × Lup	ns	ns			ns	*

Adding *Lupinus* in 2002 resulted in slightly lower soil C at 7.5–15 cm depth in 2005, but had no effect on total soil N.

The *Bouteloua* C_4 litter increased P slightly in the surface soil (Table 3). In the lower layer, *Lupinus* increased the soil available P in the treatment with the existing C_3 litter, but not with the high C/N C_4 litter.

Table 4
ANOVA significance levels and treatment comparisons of soil microbial biomass C (SMB-C) and ergosterol contents at each depth (2002, 2003 and 2005). For treatment abbreviations please refer to Table 1 Significant levels (ns = $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) are calculated by two-way ANOVA using Tukey HSD testing the effect of litter exchange (Lit) and Lupine addition (Lup); $n = 5$).

	SMB-C μg g^{-1} soil (dw)		Ergosterol μg g^{-1} soil (dw)	
	0–7.5 cm	7.5–15 cm	0–7.5 cm	7.5–15 cm
Treatment means				
December 2002				
t_0 (random $n = 8$)	131 ± 17	73 ± 8	nd	nd
April 2003				
C3Lit & NoLupAdd	201 ± 59	nd	nd	nd
C4Lit & NoLupAdd	161 ± 35	nd	nd	nd
C3Lit & LupAdd	168 ± 48	nd	nd	nd
C4Lit & LupAdd	188 ± 49	nd	nd	nd
April 2005				
C3Lit & NoLupAdd	197 ± 33	93 ± 14	0.86 ± 0.13	0.31 ± 0.07
C4Lit & NoLupAdd	150 ± 9	93 ± 14	0.73 ± 0.10	0.29 ± 0.06
C3Lit & LupAdd	232 ± 56	107 ± 30	1.31 ± 0.11	0.35 ± 0.09
C4Lit & LupAdd	213 ± 20	77 ± 19	1.69 ± 0.57	0.32 ± 0.03
ANOVA significance levels				
April 2003				
Lit	ns	nd	ns	nd
Lup	ns	nd	ns	nd
Lit × Lup	ns	nd	ns	nd
April 2005				
Lit	*	ns	ns	ns
Lup	**	ns	*	ns
Lit × Lup	ns	*	ns	ns

3.4. Soil microbial biomass and communities

The SMB-C was ~130 μg g^{-1} dry soil in December 2002, right before installation of cylinders and treatments (Table 4). In the following Spring (April 2003), it was slightly higher (150–200 μg g^{-1} dry soil), with no differences among treatments. This range was generally seen again in April 2005. In spring 2005, soil in the C_3 litter treatments had higher SMB-C than the *Bouteloua* C_4 litter-exchange. *Lupinus* addition also increased the SMB-C. For the 7.5–15 cm layer, there were no differences among treatments on either date, and means ranged from 70 to 100 μg g^{-1} dry soil (data not shown). In 2005, a significant correlation was observed between the average treatment values of SMB-C and total PLFA of both depths ($p < 0.01$; $r = 0.96$; $n = 8$).

Using analysis of ^{13}C natural abundance (O'Leary, 1988; Farquhar et al., 1989), the contribution of the added *Bouteloua* litter to total soil C and the SMB-C was tracked. After 5 months of decomposition during the wet season, *Bouteloua*-derived C contributed 2–4% to total soil C (Table 5). By April 2005, this increased to 6–9%. There were no significant differences between the *Lupinus* and non-*Lupinus* addition treatments, but higher values tended to be seen with *Lupinus* addition. In the 7.5–15 cm layer, *Bouteloua* litter-derived C accounted for about 2% of the total soil C, and no significant treatment differences occurred.

Compared to total soil C, *Bouteloua* litter-derived C was much higher in the living part of the soil C, reaching 20–30% of the SMB-C in April 2003 (Table 5), however, these high contributions declined to 10–1% in April 2005. The lower layer showed comparable enrichments at that time. With *Lupinus* addition, a significantly higher amount of *Bouteloua* litter-derived C was present in the SMB-C of the upper layer in 2005 (Table 5).

Soil microbial community composition was strongly affected by *Lupinus* addition (Fig. 3). In an ordination of the PLFA profiles from the upper layer (0–7.5 cm) of the cylinders, Correspondence Analysis showed that 57% of the variance was explained by axis 1. *Lupinus* addition strongly influenced this axis, with much less effect from the type of litter treatment. The PLFA most highly

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Table 5
ANOVA significance levels and treatment comparisons of the amounts of litter derived C in soil microbial biomass C and the amounts of litter derived C in soil C at each depth (2003 and 2005) for the treatments with C4-litter (litter exchange). For treatment abbreviations please refer to Table 1 Significant levels (ns = $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) are calculated by one-way ANOVA using Tukey HSD testing the effect of lupine addition (Lup); $n = 5$).

	Litter derived C in SMB-C %		Litter derived C in soil C %	
	0–7.5 cm	7.5–15 cm	0–7.5 cm	7.5–15 cm
Treatment means				
April 2003				
C4Lit & NoLupAdd	29.1 ± 13.3	nd	2.2 ± 1.0	nd
C4Lit & LupAdd	19.9 ± 12.8	nd	3.2 ± 1.8	nd
April 2005				
C4Lit & NoLupAdd	8.3 ± 2.2	9.2 ± 0.6	6.5 ± 0.8	1.4 ± 0.9
C4Lit & LupAdd	13.5 ± 0.6 *Lup	8.2 ± 2.2	8.0 ± 1.7	1.0 ± 0.6

associated with axis 1 were of unknown or uncertain origin (negative scores: 14:1ω5t and 14:1ω5c; positive scores: unknown PLFA and 15:0 2OH). In addition to the CA biplot, an RDA checked for the significance of factors using Monte Carlo permutations in CANOCO (ordination not shown). RDA confirmed the CA. There was a strong *Lupinus* effect on PLFA profiles. The RDA was run with 'litter exchange' as a covariable for the factor '*Lupinus* addition' and, vice versa, with '*Lupinus* addition' as a covariable when testing for the effect of the litter exchange. In this RDA, 22% of variance was significantly explained by '*Lupinus* addition' ($F = 4.9$; $p = 0.03$) and only 1% was explained by the litter exchange ($F = 0.3$; $p = 0.63$).

Based on the relative proportions of individual PLFA, the relative abundance of bacteria was reduced with *Lupinus* addition in the upper layer (Table 6). This holds for Gram^{+} and Gram^{-} bacteria. Actinomycetes, however, when analyzed separately from other Gram^{+} bacteria, showed no differences among treatments. In contrast to bacterial PLFA, fungal PLFA increased in relative abundance in the *Lupinus* treatments. Nearly 35–40% of the PLFA were unspecific and not clearly related to any of the functional groups listed. At the lower depth, there was a trend toward higher relative abundance of bacterial PLFA and lower fungal PLFA. No

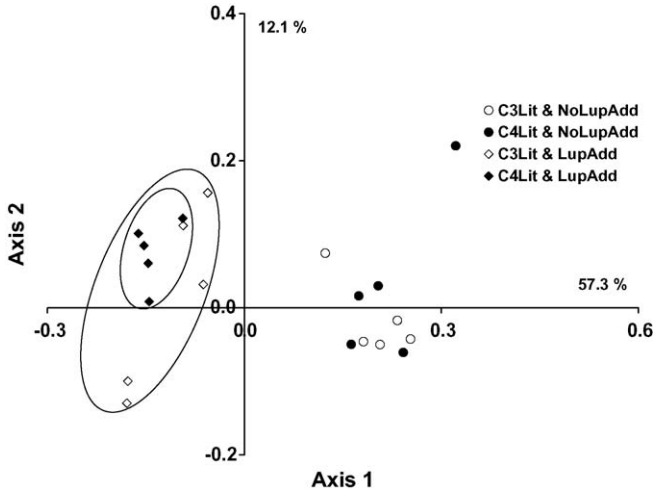

Fig. 3. Two-dimensional sample ordination using Correspondence Analysis of PLFA profiles in the 0–7.5 cm soil layer of the cylinders from April 2005. The two circles are hand-drawn clusters showing all the samples with seeded *Lupinus* (outer) and those with high C/N litter and seeded *Lupinus* (inner).

treatment effect was detected for the lower layer (7.5–15 cm), except for an increase in the relative abundance of actinomycetes markers with addition of *Lupinus*.

Soil ergosterol content, which serves as a fungal biomarker, confirmed the pattern observed from the PLFA profiles (Table 4). Ergosterol content of the soil samples from the upper 0–7.5 cm cm ranged between 0.7 and 1.3 μg g^{-1} dry soil. *Lupinus* addition caused higher amounts of ergosterol regardless of litter type. For the 7.5–15 cm layer, ergosterol was found to be ~0.3 μg g^{-1} dry soil.

4. Discussion

A one-time addition of high C/N litter or *Lupinus bicolor* seeds had longer-lasting effects on soil in this field mesocosm experi-

Table 6
Total PLFA and PLFA purportedly associated with bacteria, fungi, or from unspecific origin (refer to Table 1 for abbreviations). Bacterial PLFA are separated as actinomycetes, Gram^{+}, and Gram^{-}. Actinomycetes are listed apart from other Gram^{+} bacteria. PLFA with frequencies less than 10% in all samples are not listed. The relative proportions of PLFA from specific groups (rel.) are given in % of total PLFA. Significant levels (ns = $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) are calculated by two-way ANOVA using Tukey HSD testing the effect of litter exchange (Lit) and Lupine addition (Lup); $n = 5$).

Depth (cm)	Treatment	Total PLFA *abs*	All bacteria[a] *rel. %*	Actinomycetes[b] *rel.%*	Gram^{+}[c] *rel.%*	Gram^{-}[d] *rel.%*	Fungi[e] *rel.%*	Unspecific[f] *rel.%*
Treatment means								
0–7.5	C3Lit & NoLupAdd	30.98	44.35	5.15	21.65	15.79	20.03	35.63
	C4Lit & NoLupAdd	28.23	43.72	5.34	21.79	15.16	19.77	36.50
	C3Lit & LupAdd	44.44	39.58	5.26	18.71	13.96	23.58	36.84
	C4Lit & LupAdd	41.44	38.09	5.09	17.49	14.15	22.95	38.97
7.5–15	C3Lit & NoLupAdd	13.78	43.23	6.35	23.29	13.09	18.89	37.88
	C4Lit & NoLupAdd	13.04	43.12	6.49	22.67	13.08	17.57	39.31
	C3Lit & LupAdd	13.83	42.23	7.17	21.21	13.02	17.21	40.56
	C4Lit & LupAdd	9.20	44.46	7.34	23.64	12.65	17.13	38.41
ANOVA significance levels								
0–7.5	Lit	ns	ns	ns	ns	ns	ns	***
	Lup	ns	***	ns	***	***	**	***
	Lit × Lup	ns	ns	ns	ns	ns	ns	*
7.5–15	Lit	ns	ns	ns	ns	ns	ns	ns
	Lup	ns	ns	**	ns	ns	ns	ns
	Lit × Lup	ns	ns	ns	ns	ns	ns	*

[a] Sum of PLFA listed as actinomycetes, Gram^{+} and Gram^{-} + i15:1 (Federle, 1986; Zelles, 1997; Bossio and Scow, 1998).
[b] Sum of i17:1, 10Me16:0, 10Me17:0, and 10Me18:0 (Kroppenstedt, 1985; O'Leary and Wilkinson, 1988; Vestal and White, 1989).
[c] Gram^{+} bacteria excluding acinomycetes; sum of 12:1, i14:0, a16:0, i15:0, a15:0, i16:0, i17:0, a17:0 (Federle, 1986; Zelles, 1997).
[d] Sum of 16:1ω7t, 16:1ω7c, 17:1ω9c, cy17:0, and cy19:0 (Federle, 1986; O'Leary and Wilkinson, 1988; Zelles, 1997).
[e] Sum of 16:1ω5c, 18:3ω6, 9,12c, 18:2ω6,9c, 18:1ω9c (Federle, 1986; O'Leary and Wilkinson, 1988; Vestal and White, 1989; Olsson et al., 1995; Zelles, 1997).
[f] Sum of unspecific PLFAs 12:0, 13:0 3OH, 14:0, 14:0 3OH, 15:0, 15:0 3OH, 16:0, 17:0, i17:1ω5c, 16: 2OH, 16:1ω11c, 18:0, cy19:0ω10c, 20:2ω6,9c, 20:0.

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ment, than on plant community composition and biomass. *Lupinus* increased SMB-C and soil fungi, and had distinct effects on PLFA profiles 28 months after seeding (2005). Although some *Lupinus* N was recovered in litter derived from the 2004 plants, the biomass, N and P content of the litter was not significantly affected. Neither *Nassella* nor the 2005 annual plants were affected by *Lupinus* that was seeded 28 months before. The mulch of high C/N *Bouteloua* litter reduced SMB-C in the soil, possibly due to its capacity to immobilize nutrients. For *Nassella*, there was a trend toward higher aboveground biomass with high C/N litter, despite lower P content. The addition of high C/N litter is thus ambiguous in value as a restoration practice (cf. Eschen et al., 2007; cf. Kardol et al., 2008). Repeated occurrences of high *Lupinus* densities over multiple years may potentially improve soil fertility and soil C pools, but probably would only slowly affect the bunchgrass performance.

4.1. Plant community composition

High densities of *Lupinus* spp. occurred in the spring of 2003, the first spring after setting up the mesocosms in December 2002, as is typical of years with warm, wet weather during autumn and winter in California grasslands (Pitt and Heady, 1978). In the surrounding area and the cylinders without *Lupinus* addition, the cover of annual lupines was up to 40–50% groundcover. In subsequent years, this performance was not repeated.

Several studies suggest that it is common for leguminous species to dominate in years with low annual grass production, and for high grass years to occur during low legume years (Pitt and Heady, 1978; Jones and Winans, 1967). This might be due to specific climatic patterns occurring which favor grasses vs. legumes, but evidence also suggests that annual grasses have a high growth response to high inputs of N, whereas legumes decline with inputs of N (Jones and Winans, 1967). In other grassland systems, *Lupinus* spp. increases net N mineralization rates (Dijkstra et al., 2006). The high density of *Lupinus* in 2003 in this study, however, apparently did not have a significant fertilizer effect on annual plants in the subsequent year since neither estimates of annual plant cover (2004), nor litter from these plants (2005) increased significantly. The $\delta^{15}N$ ratio of the annual litter decreased with *Lupinus* addition indicating the use of N derived from legume N-fixation (Shearer and Kohl, 1986), however this was not true for *Nassella*, suggesting greater N availability to the annuals. The compact form of the bunchgrass, its coarse roots, and its root system with less root length in the surface layer (Potthoff et al., 2005a) may have limited its ability to compete with the annual grasses and microbes for *Lupinus*-derived N in the surrounding soil or to benefit from legume-induced nitrate sparing (Herridge et al., 1995).

Exchanging the existing litter with high C/N litter initially decreased *Lupinus* cover during the first year (2003), but this did not result in higher annual plant growth in the second year (2004), or either estimated cover or litter in the following year. The trend toward higher biomass of *Nassella* may have been due to mulch-induced changes in microenvironment, such as higher moisture content or lower temperature, given the lack of suppression of annual plants. The cover of native annual forbs tended to decrease with the mulch of high C/N litter, suggesting that seedling germination or establishment of these species was more affected than the exotic annual grasses. In a comparison of 42 paired grazed and ungrazed sites in California, grazing increased species richness and cover of native annual forbs, and this was related to lower vegetation height and litter depth (Hayes and Holl, 2003). Not all studies in California, however, show consistent effects due to grazing, because soil type, moisture regime, and grazing intensity are confounding factors (Jackson and Bartolome, 2007). Even so, the reduction in native annual forbs may be more related to litter amount and its effect on microenvironment, e.g., shading and temperature, than to litter quality.

4.2. Soil responses

Nitrogen is a limiting factor in the granite-derived soils in this region. The mean total N concentrations in these grassland surface soils range from 1.0 to 3.0 mg N g^{-1} dry soil depending on land use history (Steenwerth et al., 2003). With 0.5–0.6 mg N g^{-1} dry soil, this site is at the lower end of the range. High *Lupinus* biomass would be thus expected to make a significant contribution to available N pools in soil, mainly during the following year when most of the plant material decomposed. It is likely that the SMB may have competed favorably with annual grasses for this available N (Jackson et al., 1989). We did not measure SMB-N, but in 2005, *Lupinus* addition still had a positive effect on SMB-C. The SMB appears to act as an intermediate reservoir for available C and N in soil (Jenkinson and Ladd, 1981), in which the soil community could be immobilizing and retaining the *Lupinus*-derived N for long periods after it is fixed. Studies in other ecosystems have shown that N-fixing plants can rapidly increase soil C and N concentrations and microbial activity near the soil surface (Halvorson and Smith, 1995), and ultimately affect total C storage by minimizing N limitation and increasing plant productivity and belowground C inputs (Conant et al., 2001). Instead, positive feedback to plants was minor in this situation. One explanation is that the significant change in microbial composition caused by *Lupinus bicolor* may be associated with increased immobilization capacity, remaining high for a two-year period.

Other species of *Lupinus*, e.g., *Lupinus alba*, mobilize low concentrations of soluble soil P through secretion of organic anions, and this can increase P absorption of less P-efficient crops grown in association (Kamh et al., 1999). Carboxylate exudation releases phosphate from metal cations and displaces it from the soil matrix, increasing the availability of both inorganic P and organic P (Lambers et al., 2006). At the lower depth (7.5–15 cm), *Lupinus bicolor* increased the availability of soil Bray-P, mainly in the lower C/N litter treatment, and the high C/N litter contained more P. Thus, this litter appeared to reduce soil P availability, and thus may partially explain the lack of positive P feedback to plants, as was found for N.

Both rhizophere and litter-mediated effects could have contributed to the subsequent increase in fungal biomass following a year with abundant *Lupinus*. Earlier studies on the same soil type also showed that soil fungal biomass is reduced with increasing depth (Potthoff et al., 2006). In this study, this could be due to either decreased root length of *Lupinus*, which is known to increase soil fungi (see below) or to less contact with litter, which typically supports a high fungi:bacteria ratio (Ahl et al., 1998; Frey et al., 1999; Potthoff et al., 2005b; Scheller and Joergensen, 2008). Neither ergosterol content nor percentage of PLFA as fungal markers showed significant interactions between *Lupinus* addition and litter type. Although higher *Lupinus* cover occurred with existing litter in 2003, compared to high C/N exchanged litter, there was not a long-lasting effect on soil fungi. Appuhn and Joergensen (2006) found that in comparison to 14 other plants, *Lupinus luteus* was most effective in supporting fungal biomass in the rhizophere soil. Its rhizosphere soil was characterized by high SMB-C, a high C/N ratio, and high ergosterol contents. Moreover, fungal C was calculated to be about 10 times higher than bacterial C in the root material of *Lupinus luteus*. In the rhizophere soil, the ratio was 2.5. The ergosterol to SMB-C ratio was 0.18%, whereas it was as high as 0.6% in this study, indicating a very high fungal proportion in the bulk soil two years after most *Lupinus* rhizosphere activity had ceased. Analysing the nematode communities of different sites of an organic farm in Yolo county,

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California, Sánchez-Moreno et al. (2008) found the highest abundances of fungal feeding nematodes in a site dominated by lupines. This, again, supports indirectly the strong relation of lupines and fungal growth.

Lupinus not only influenced soil fungi two years later, but several other PLFA of unknown origin also were more abundant in the surface layer. Others have found that plant functional groups, including legumes, have clear effects on PLFA profiles and bacterial to fungal PLFA ratios (Wardle et al., 2003). Stands of different native and non-native plant species have different PLFA profiles in the rhizosphere (Batten et al., 2006). Not only is it difficult to determine whether litter or lingering rhizosphere effects were more important for determining soil microbial composition in this study, but an explanation for the adaptive significance of these long-term impacts for *Lupinus bicolor* also remains elusive.

4.3. Litter responses

Despite greater litter amounts in the high C/N litter treatment, SMB-C decreased with time. Apparently, litter quality decreased the available C compared to the existing lower C/N litter. Litter P was significantly increased by the high C/N litter mulch in combination with *Lupinus* addition, suggesting that microbial activity within this litter immobilized available P. Without *Lupinus* addition, the litter exchange had no effect on litter-P contents. The possibility of microbial P immobilization may explain why *Nassella*'s P content was generally reduced due to mulch addition. However, this reduction was independent from *Lupinus* addition and the available P in the upper soil layer slightly increased in the mulch treatments at the same time. It is possible that soil under the *Nassella* bunchgrass may have undergone gradual depletion in P availability with time since the restoration process began. Its high C/N litter remains attached to the plant, and may have also immobilized P in a similar fashion as the *Bouteloua* litter. More *Lupinus*-derived N tended to be found in the high C/N litter mulch than in the existing C_3 litter, as well.

For annual grasses, approximately one-third of the litter decomposes within a year after plant death based on repeated field sampling (Jackson et al., 1988). But for perennial bunchgrasses, some litter is still standing upright for several years, attached to the plant. A litter bag approach indicated annual decomposition rates of 40% and 50% for both *Nassella* litter in restored perennial grassland and annual litter in a nearby annual grassland, respectively (Jackson et al., 2007). Based on proportional calculations, the $\delta^{13}C$ values of the litter collected from cylinders of the litter-exchange treatments show that in total 127 g C_4 litter m^{-2} still remained in 2005, assuming no changes in the delta values of the litter. Given the initial addition of 715 g C_4 litter in 2003, the decomposition was 82% for the 2.5-year period. Since decomposition usually follows a second order dynamic with time, this is roughly consistent with the litterbag approach (Jackson et al., 2007). Phyllosphere organisms living in the leaf mass are apparently important for the decomposition of the upright *Nassella* litter *in situ* (Osono et al., 2003; Potthoff et al., 2005b; Minoshima et al., 2007), which may slow down the return of nutrients to soil and ultimately reduce nutrient availability to the roots of the bunchgrass.

The microbial uptake of litter C from the high C/N litter mulch was slightly increased for the *Lupinus* treatment in 2005 for the upper layer. This indicates a higher efficiency of microbial substrate use, possibly due to higher litter quality due to the contribution of *Lupinus* litter, or because of the increased amount of fungi or both. Typically, fungi are expected to be more effective in substrate use than bacteria (Sakamoto and Oba, 1994; Bailey et al., 2002).

4.4. Implications for restoration

Compared to soil microbial organisms, only weak responses by plants and total C and N pools were observed 28 months after seeding *Lupinus* and/or exchanging existing litter with high C/N litter. The target bunchgrass of restoration efforts, *Nassella pulchra*, only slightly profited from the treatments. Both the *Lupinus* addition and the litter mulch addition were expected to be more effective in promoting bunchgrass growth than was observed. Temperton et al. (2007) report positive effects of legumes to neighboring plants in a multiple plant experimental design; N fixation and transfer identified by lower $\delta^{15}N$ values (Shearer and Kohl, 1986), as well as reduced competition for nitrate were identified as pathways of promoting the growth of neighboring plants. The compact and dense bunchgrass form of *Nassella*, however, may restrict the access to nutrients released by *Lupinus* through rhizosphere interactions or decomposition, since *Lupinus* grows beside, rather than intermixed with the dense bunchgrass tillers and roots. Moreover, *Lupinus* N and P appear to be preferentially immobilized by microbes in soil and litter, which may be better competitors than plant roots. The high C/N litter attached to the *Nassella* plant itself may immobilize nutrients, as shown by the *Bouteloua* litter, further reducing nutrient availability to its roots. These processes may act to retain C and other nutrients in the ecosystem over the long-term, but are not conducive to rapid growth responses of the native perennial bunchgrass, and thus had little effect for increasing its dominance in the restored perennial grassland.

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