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ECOSPHERE

Fate of N additions in a multiple resource-limited Mediterranean oak savanna

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Citation: Morris, K. A., R. K. F. Nair, G. Moreno, M. Schrumpf, and M. Migliavacca. 2019. Fate of N additions in a multiple resource-limited Mediterranean oak savanna. Ecosphere 10(11):e02921. 10.1002/ecs2.2921

Abstract. Mediterranean oak savannas, such as Spanish *dehesas*, are multiple resource-limited ecosystems found in semiarid regions which are key contributors to interannual variability of the global carbon (C) budget. Interactions between nitrogen (N) and phosphorus (P) cycles are expected to play a major role in overall ecosystem function as anthropogenic N deposition shifts ecosystems from N to P limitation, leaving unknown how increased N availability might influence C uptake. Therefore, the fate of N additions in dehesas is important for understanding global C cycling. Using a ^{15}N tracer experiment within fertilized (N or N + P) plots of a Holm oak dehesa, we tested the effects of ecosystem spatial heterogeneity (habitat), P addition, and time on the fate of added N. We expected that open pasture areas would retain more of the added N in biological components due to greater N limitation, that the addition of P would enhance N retention in biological components relative to N alone, and that added N would shift from being within the microbial biomass immediately after addition to being predominantly within plants at the beginning of the following growing season. We found that open pasture plots with N only had the greatest label recovery seven months after the start of the experiment, supporting the idea that open pasture was more N-limited than under-canopy areas. However, soil was the largest sink for added N, regardless of habitat, treatment, or time. Our results suggest that abiotic fixation of N may play an important role in modifying the effects of N deposition in dehesas.

Key words: ^{15}N; dehesa; microbial N; phosphorous; stoichiometry; tree–grass.

Received 16 August 2019; accepted 29 August 2019; final version received 1 October 2019. Corresponding Editor: Jason B. West.

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INTRODUCTION

Atmospheric nitrogen (N) deposition causes a variety of downstream effects on ecosystems, ranging from increased productivity to groundwater contamination (LeBauer and Treseder 2008, Schlesinger 2009, Schulte-Uebbing and de Vries 2018). One major consequence of N deposition is a potential shift in the biological availability of N relative to phosphorus (P) because of differential deposition rates from anthropogenic activity, driving ecosystems from N into P limitation (Peñuelas et al. 2012). The consequences of N:P imbalances are largely unknown (Sardans et al. 2012), especially in the context of how such imbalances might affect ecosystem N allocation.

In their 2011 review, Pardo et al. found that ecosystems dominated by low-biomass components (e.g., grasslands, desserts) are more sensitive to N deposition than ecosystems dominated by high-biomass components (e.g., forests) due to differing generation times and buffering abilities. Shorter generation times lead to faster responses at the community level, which, along with lower biomass, means small changes in ecosystem N availability can result in large

changes in individual biomass components (Pardo et al. 2011). This contrast in sensitivity makes predicting the response of mixed tree–grass ecosystems to shifts in N and P availability difficult (Sardans et al. 2012). Tree–grass ecosystems are distributed globally, making up roughly one-third of the terrestrial land cover (Di Castri 1991, Mistry and Beradi 2000, Hanan and Lehmann 2010). Many of these mixed cover ecosystems are located in semiarid regions which have recently received heightened attention as key players in the interannual variability of the global C budget (Ahlström et al. 2015). Increased N availability has the potential to increase C uptake of such systems, but it is not yet clear if P availability might limit this potential. Therefore, shedding light on the linkages between C, N, and P cycles is of growing importance.

Dehesas are a type of tree–grass ecosystem comprised of open oak woodland with an herbaceous layer consisting mostly of annuals (typically) or in some cases crops. They are analogous to the Portuguese *montado*, other Mediterranean wood pastures (den Herder et al. 2017), and the Californian oak woodlands of the United States (Mistry and Beradi 2000). Dehesas are multiple resource-limited ecosystems, being limited by nutrient availability early in the growing season and water later in the growing season (García and Mata 2000, Moreno 2008). This is due to the relatively nutrient-poor parent material of the soil and the extremely dry summer conditions typical of the Mediterranean climate (Olea and San Miguel-Ayanz 2006, Moreno 2008, Vitousek et al. 2010). Multiple resource limitation is characterized by sensitivity of the ecosystem to addition of any limiting resource (Wang et al. 2014, He and Dijkstra 2015, Wurzburger and Wright 2015). Previous work has highlighted the vulnerability of dehesas to N deposition due to differing responses of individual vegetative components (Ochoa-Hueso et al. 2013, Perez-Priego et al. 2015) and the possibility of exacerbated fire-cycles due to increased biomass, such as that seen in the equivalent Californian ecosystem (Ochoa-Hueso et al. 2011, Rivest et al. 2011). However, effects of N and P additions on N allocation and retention have not yet been studied in dehesas.

The response of dehesa systems to N deposition is further complicated by the extreme heterogeneity of the herbaceous layer and uneven distribution of soil properties (Delgado-Baquerizo et al. 2013, Moreno et al. 2013, El-Madany et al. 2018). Soils developing underneath oak canopies are noticeably enriched in nutrients and soil organic matter (SOM) compared to soils in the open pasture areas (Gallardo 2003, Howlett et al. 2011, Moreno et al. 2013), often with as much as three times greater soil organic carbon concentrations. Under tree canopies, soils tend to stay moist longer after rainfall, due to protection from solar radiation by the tree. However, when dry conditions persist, soil under the canopy is often drier than that in the open areas because of the additional water demands of the perennial oaks (Cubera and Moreno 2007, Breman and Kessler 2012, Dubbert et al. 2014). Therefore, these two spatial locations represent two distinct habitats, and the fate of N is expected to differ between them.

Much work has been done to determine how ecosystems allocate N, especially within the context of N deposition (Hart et al. 1993, Fenn et al. 2003, Templer et al. 2012). One of the most common methods is the use of a stable isotope tracer (^{15}N). But in the most recent review, ^{15}N tracer studies in mixed tree–grass ecosystems, such as dehesas, were still lacking (Templer et al. 2012). Additionally, many studies only look for short-term fates of tracers (days to weeks), which can differ from long-term N sinks (months–years). These longer timescales are especially important for tree–grass systems where the herbaceous layer is able to respond to nutrition additions much more quickly than the trees (Rivest et al. 2011). Therefore, we set out to explicitly test the effects of ecosystem (1) spatial heterogeneity (hereafter referred to as "habitat"), (2) P addition, and (3) time on the fate of added N in a dehesa.

1. Because nutrients are not evenly distributed throughout dehesa soil, biotic uptake may be faster or of greater magnitude outside of the tree canopy where the soil is of poorer quality. We expect that pasture areas will retain more of the added N in both plant tissues and microbial biomass due to their presumed greater N limitation and a more closed N cycle than underneath tree canopies.
2. Given the general trend observed of greater biomass production when both N and P are

added (Vitousek et al. 2010), we expect greater N uptake and retention in biotic components of the system (both plants and soil microbes) when P is added.

3. Immediately after application, we expect added N would be taken up mostly by microbes, because microbes are thought to be better competitors for mineral nutrients than plant roots in the short term (Kuzyakov and Xu 2013). In the dry season, we expected most of the label to accumulate in the soil, as inorganic N accumulation is often seen in arid soils during the dry period (Austin et al. 2004). In the following spring growing season, we expected that plants would obtain the majority of the remaining label, because it is the peak of plant N demand, as seedlings are actively investing in nutrient uptake (Otieno et al. 2011, Jongen et al. 2013). Overall, label recovery is expected to decrease with time due to gaseous losses and label transfer below the measured soil zone.

To test these hypotheses, we fertilized plots within a Holm oak (*Quercus ilex* L.) dehesa with either N or N + P followed by a ^{15}N label to trace the recovery of ^{15}N within the herbaceous layer vegetation and surface soil for one year. Because previous work in Mediterranean oak savannas found up to 90% of ecosystem N (not associated with tree biomass) was located in the top four cm of soil (Jackson et al. 1988), we focused on this active section of the ecosystem as we were interested in short-term competition between plants and soil microbes, rather than long-term competition strategies that might be utilized by the slow-responding oak trees (Rivest et al. 2011).

Methods

Site description

Our study site was located in a publicly accessible dehesa at Majadas de Tiétar (39°56′25″ N 5°46′29″ W) in Extremadura, Spain, 258 m above sea level. The tree density is roughly 20 trees/ha, and the site is grazed from early December to late June by cattle at an intensity of <0.3 livestock units·ha^{-1}·yr^{-1} (El-Madany et al. 2018). The herbaceous layer is a native biodiverse pasture, dominated by annual species (e.g., annual vernalgrass, *Anthoxanthum aristatum* (Boiss.) and soft brome, *Bromus hordeaceus* (L.)) and nonleguminous forbs (e.g., European umbrella milkwort, *Tolpis barbata* (L.) Gaertn.) grow in an Abruptic Luvisol and sustained by ~650 mm average annual precipitation, which falls mostly between winter and early spring. Since 2003, the site has been the location of an eddy covariance tower belonging to the FLUXNET network with the site identifier ES-LMa (Pacheco-Labrador et al. 2017, El-Madany et al. 2018, Luo et al. 2018). It is worth mentioning that while this study took place at the same site as several others, the sampling plots were well outside of the tower footprints and the experiment itself was separate from nutrient manipulation experiments reported elsewhere.

^{15}N tracer experiment

An area was selected at the site with a sufficient number of mature oak trees for our sampling scheme (about 1.3 ha) and then divided in half, one half for N addition and the other for the N + P addition treatment. Within each half, 18 sampling plots of 4 × 4 m were established (Fig. 1). On 20 March 2017, to prevent targeted grazing pressure on the sampling plots, the surrounding area (excluding the treatment plots) was fertilized using pelleted ammonium nitrate (NH_4NO_3) and monopotassium phosphate (KH_2PO_4) at a rate of 50 kg N/ha, with an additional 25 kg P/ha within the N + P treatment. Mixtures were hand-thrown, specifically avoiding the sampling plots. Sampling plots were then fertilized using pelleted fertilizer pre-weighed to create exactly the fertilization loads approximated by the hand application. N addition plots received 129 g potassium nitrate (KNO_3) and 183 g NH_4NO_3 (equivalent to 50 kg N/ha), while N + P plots received 232 g NH_4NO_3 and 176 g KH_2PO_4 (equivalent to 50 kg N/ha + 25 kg P/ha). These doses are approximately five times the current N deposition rate, with sufficient P addition in the N + P plots to maintain the ecosystem's original herbaceous layer N:P stoichiometry (Migliavacca et al. 2017). Within each treatment area, nine plots were under tree canopies and nine were in open pasture areas. For each set of nine plots, three remained unlabeled while the other six received 1.1 g of 99.9% ^{15}N ammonium nitrate (Berry & Associates, Dexter,

Michigan, USA). On 21 March, pre-weighed aliquots of labeled salt were dissolved in 2.5 L of distilled water and sprayed onto plots in 0.5-m strips using guidelines and a hand-sprayer. After the 2.5 L of labeled salt solution was distributed, the hand-sprayer reservoir was rinsed with an additional 0.5 L of water, which was also sprayed onto each plot.

While our design is technically pseudoreplicated, there are a several arguments that support its validity. First of all, spatial heterogeneity is extremely high at this site (Nair et al. 2019) and multiple assessments of ecosystem parameters (ranging from root mass, chlorophyll content of vegetation, and carbon and water fluxes) have found that variability on the scale of 1–2 m (such as our sampling plots) is equal to variability on the scale of hundreds of meters (El-Madany et al. 2018, Nair et al. 2019). Additionally, because the site is grazed during the vegetative period of the herbaceous layer, it was necessary to fertilize additional surface area to prevent preferential grazing pressure on fertilized sampling plots. However, scattering N-only plots within a N and P fertilized area runs the risk that P from the surrounding fertilization could influence perceived N-only treatment effects if added P is relocated by bioturbation or surface water flow. Finally, pre-fertilization data on bulk soil and plant (above and below) N pools and $\delta^{15}N$ signatures were collected (Table 1) and showed no significant difference between plots before nutrient addition treatments when tested for differences using a fixed-effects model with component type (foliar, root, or soil), habitat (i.e., under tree canopy and in open pasture), and future plot designation to explain variation in N content (mg/g). We therefore feel confident that with this experimental design, the sampling plots represent independent measurements of the desired ecosystem components, despite their nontraditional spatial layout.

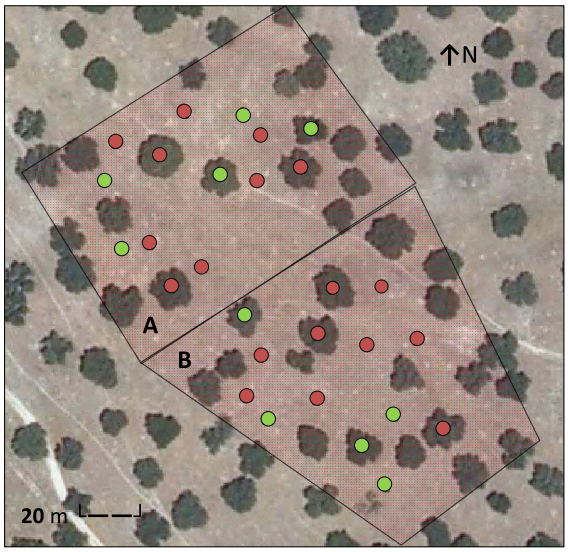

Fig. 1. Aerial view of the experimental setup in the dehesa near Majadas de Tiétar, area A received the N addition treatment, and area B received the N + P addition treatment. Green points are sampling plots that did not receive any ^{15}N label, and red points are labeled plots. Points over trees represent two distinct 4 × 4 m sampling plots, one on either side of the trunk oriented east–west. Points over open pasture represent only one distinct 4 × 4 m sampling plot. N addition was 50 kg N/ha, with an additional 25 kg P/ha in the N + P treatment.

Sampling and sample processing

Plots were sampled five times: one day, seven weeks, seven months, and one year after label was applied, as well as one pre-label sampling which took place one day before fertilization. Sampling took place on 20 March 2017, 22 March 2017, 10–12 May 2017, 19 October 2017, and 21 March 2018, respectively. Samples in May were collected over 3 d with control plot samples being collected the first day, half of the treatment plots sampled the next (evenly distributed across treatments and habitats), and the second half being collected on the final day. This was done due to difficulty in collecting cores with the

Table 1. Summary of pretreatment (19 March 2017) N concentrations by habitat of plant material and surface soil (0–5 cm).

Habitat	Future treatment	Foliar	Root	Soil
Open pasture	N plots	7.68 ± 2.0	9.82 ± 1.5	0.95 ± 0.08
	N + P plots	6.08 ± 1.0	9.80 ± 1.0	1.03 ± 0.05
Under canopy	N plots	18.1 ± 1.8	13.2 ± 1.2	2.62 ± 0.4
	N + P plots	19.5 ± 1.8	13.2 ± 0.9	2.92 ± 0.5

Notes: Values are mg N/g, mean ± standard error, $n = 6$. There were no significant differences between plots prior to nutrient additions.

equipment available and to accommodate laboratory space for processing multiple experimental sets at one time. For each sampling, one 0–5 cm soil core with a 5.5 cm diameter was collected from a random location within each plot, avoiding sampling directly next to edges and previously sampled locations as needed.

The intact cores were placed in a cooler and taken directly to the laboratory (University of Extremadura, Plasencia, ~40 min transfer time) where the aboveground plant biomass was separated, washed, and placed in a drying oven at 45°C for 48 h. The soil portion was sieved through a 2-mm sieve, and the coarse fragment (<1%) removed. All root material was removed, washed, and placed in a drying oven at 45°C for 48 h. Sieved soil was stored field-moist at 4°C overnight. The following day soils were subsampled as follows to determine extractable and chloroform-labile extractable N content (hereafter referred to as microbial biomass N as a more ecologically intuitive term, discussed further below). One 40 g subsample went into 200 mL of 0.5 mol/L K_2SO_4 which was shaken for one h, and then, the supernatant was filtered through Whatman #1 filter paper and frozen. A second 40 g subsample was placed in a glass beaker and fumigated with chloroform in the dark for 72 h and then extracted in 0.5 mol/L K_2SO_4 as above. A final 10 g subsample was taken to determine gravimetric water content and for later stable isotope analysis. All exact weights were recorded for later mass calculations. Frozen extracts and dry soil and plant materials were weighed and then shipped to the Max Planck Institute for Biogeochemistry in Jena, Germany, for further processing. There, dry plant samples were picked through to remove litter and small stones. All dry samples were ground to a fine powder using a ball mill. Subsamples of plant and soil powder were weighed out for ^{15}N, N, and C content analysis.

Chemical analysis of C, N, and ^{15}N

Dry soil and plant samples were run on a Delta-Plus isotope ratio mass spectrometer (Thermo Fisher, Bremen, Germany) coupled via a ConFlow III open split to an elemental analyzer (Carlo Erba 1100 CE analyzer; Thermo Fisher Scientific, Rodano, Italy) to measure their total N and ^{15}N content. Standard deviation of the measured standards was 0.2‰ or better. Soil and plant tissue C concentrations were determined on a Vario EL II (Elementar Analysensysteme GmbH, Hanau, Germany). Extracts from fumigated and unfumigated soils were run on a TN-100 (A1 Envirotech, Düsseldorf, Germany) to determine total extractable N content. Microbial biomass N was calculated as the difference in total extractable N content in fumigated and unfumigated samples (Brookes et al. 1985). These values were not adjusted using any *k* factor because all comparisons using these values are internally referenced. The remaining volume of extracts was frozen again and shipped to the Stable Isotope Laboratory at Utah State University in Logan, UT, USA, for measurement of ^{15}N content. There, samples were persulfate-digested (Cabrera and Beare 1993) and the digest solution was diffused using the diffusion technique of Stark and Hart (1996). The filter paper products of the diffusion were run on a Europa 20-20 IRMS (Sercon Limited, Crewe, UK) to measure their ^{15}N content with relative deviation of the standards equal to 0.05‰ or better. Due to shipping the extracts multiple times, 12 of the 96 samples for microbial biomass ^{15}N recovery were not available for final ^{15}N analysis (Appendix S1: Table S1).

Label recovery calculations

For each ecosystem component investigated, ^{15}N excess (atom percent excess) was calculated by taking the absolute content of ^{15}N in the sample (atom percent enrichment) minus the natural abundance of ^{15}N found in the same component from unlabeled plots in the same sampling campaign. Recovery for this pool was then calculated as the total amount of excess ^{15}N calculated at the plot level (atom percent excess multiplied by N pool size) divided by the known quantity of ^{15}N that was added to the plot. The exceptions to this were the soil total extractable N and microbial N pools, which were calculated relative to the total ^{15}N excess recovered in the soil of the same plot. Ambient $\delta^{15}N$ of pretreatment (fertilization and labeling) plants and soils were calculated as follows:

$$\delta^{15}N = \left(\left(R_{\text{sample}} - R_{\text{standard}}\right)/R_{\text{standard}}\right) \times 1000$$

where R is the ratio of ^{15}N to ^{14}N in the sample (R_{sample}), and the standard is the ratio in atmospheric air (R_{standard} = 0. 3677% ^{15}N).

Statistical analysis

Data were analyzed in R Studio using the R version 1.3.2 (R Core Team 2019), using a split-plot design with nutrient addition treatment as the whole-plot factor and habitat nested within whole plots. We used a mixed-effects model with treatment (N only and N + P), habitat, time, and their interactions as fixed effects and plot as a random effect. As we sampled a relatively short time series (four campaigns post-labeling) over a pronounced seasonal cycle and collected new soil cores, avoiding previously sampled locations within each plot, we treated campaign as a fixed factor without a time series autocorrelation term in the models. Data were tested for normality using visual inspection of histograms and q–q plots, and then transformed when needed by applying Tukey's ladder of powers to transform the original non-normal distribution into one that maximizes the Shapiro–Wilk W statistic. This was accomplished using the function transformTukey() from the package rcompanion (Mangiafico 2019). If the function was unable to produce an optimum transformation, the log or square-root transformation was used as appropriate. Analysis of variance (ANOVA) was then computed using the function lmer() from the package lmerTest (Kuznetsova et al. 2017), and Tukey's honest significant difference post hoc comparisons on estimated marginal means were carried out using the function emmeans() from a package by the same name (Lenth 2016, 2019). Degrees of freedom were calculated using Satterthwaite's method for ANOVAs and the Kenward-Roger method for Tukey tests. Effects and comparisons were considered statistically significant at $P \leq 0.05$. Our original data and example code can be found in the supplementary material (Data S1).

Results

Ecosystem N

The N concentration (mg N/g) of foliar material varied with time and habitat ($P < 0.05$, campaign × habitat, Table 2). The herbaceous layer underneath tree canopies had higher N concentrations in all sampling campaigns, but the magnitude was greatest in March 2017. Foliage had significantly more N in March 2018 than in March 2017 ($P < 0.001$, campaign, Table 2). There was also a decrease in foliar N concentrations in the fall, which statistically differed from the two early-spring campaigns for samples underneath canopies. Nutrient additions resulted in different foliar N concentrations when averaged over sampling campaigns, although the effect was only near significance ($P = 0.051$, treatment). Foliage from plots where only N was added had 1.5–3 mg N/g more N on average than in N + P plots throughout the four sampling campaigns.

Root N concentration followed roughly the same pattern as the aboveground plant pool, but the values were generally less variable. Notably, root N was not higher in March 2018 than in March 2017, as was seen in plant foliage. However, root N concentration did vary significantly across time ($P < 0.001$, campaign, Table 2) with the highest root N concentrations measured in March 2017 and the lowest in October 2017. Root N was significantly higher underneath tree canopies than in open pasture ($P < 0.001$, habitat, Table 2). There was significantly more N in roots when N alone was added, with on average 1–2 mg N/g more than roots in N + P plots throughout the four sampling campaigns ($P < 0.05$, treatment).

Plant tissue C:N did not differ by habitat or nutrient addition treatment, but did differ significantly between plant roots and foliage, and both the magnitude and direction of the difference varied over time ($P < 0.001$, type × time; Appendix S1: Fig. S1). The highest foliar C:N was 36 ± 3 in October 2017, which corresponded to the lowest root C:N of 23 ± 2 (averaged over nutrient addition treatment and habitat). Soil C:N was unaffected by nutrient addition treatments or time, but did vary by habitat ($P < 0.001$, habitat; Appendix S1: Fig. S2). Under-canopy soil had higher C:N (13 ± 0.1) than open pasture soil (11 ± 0.1), mostly due to higher absolute C concentration under canopies (44 ± 4 vs. 12 ± 1 mg C/g soil for under canopy and open pasture, respectively).

Soil N concentration was much higher underneath canopies than in open pasture ($P < 0.001$, habitat, Table 2). There was a significant interaction between treatment and time ($P < 0.01$), in spring 2017 (for the first two sampling campaigns), there was more N in soil from the N-only plots, while in March 2018 there was

Table 2. Nitrogen content of measured ecosystem components, values represent mean ± standard error with combined nutrient treatments, $n = 12$, except for foliar samples for 19.10.2017 where only 7 and 11 samples had sufficient plant material for processing in open grassland and under-canopy plots, respectively.

Habitat	Campaign	Foliar (mg N/g)	Root (mg N/g)	Soil (mg N/g)	Total E (μg N/g) soil	Microbial (μg N/g) soil
Open pasture	March 2017	9.46 ± 0.8 a	10.32 ± 0.7 a	1.26 ± 0.1 a	43.9 ± 10.2 a	52.10 ± 5.0 ab
	May 2017*	10.68 ± 1.4 a	8.77 ± 0.6 ab	1.10 ± 0.1 ab	14.83 ± 1.5 b	42.19 ± 2.6 ab
	October 2017	8.08 ± 1.1 a	6.19 ± 0.8 a	1.33 ± 0.2 a	33.61 ± 3.8 a	54.97 ± 4.0 a
	March 2018	22.15 ± 1.8 b	7.31 ± 0.9 b	0.87 ± 0.1 b	14.13 ± 1.3 b	39.13 ± 1.8 b
Under canopy	March 2017	19.22 ± 1.8 a	12.64 ± 0.8 a	3.41 ± 0.2 a	128.75 ± 28.6 a	117.19 ± 14.4 a
	May 2017*	15.20 ± 0.6 ab	9.77 ± 0.8 b	3.60 ± 0.5 ab	55.34 ± 10.9 ab	112.59 ± 16.7 a
	October 2017	11.75 ± 0.7 b	9.06 ± 0.7 b	3.03 ± 0.3 ab	37.62 ± 5.0 b	110.19 ± 10.5 a
	March 2018	28.12 ± 1.9 c	10.95 ± 0.6 ab	2.55 ± 0.2 b	24.22 ± 2.6 b	107.49 ± 9.6 a

Notes: Root and soil samples are from 0 to 5 cm depth. Letters represent Tukey post hoc groupings within a single spatial location and ecosystem component.

*Half of the samples (distributed evenly across treatments) were collected on 21 May 2017, the second half on 22 May 2017.

significantly more N in the N + P plots (2.0 ± 0.3 vs. 1.4 ± 0.2 mg N/g soil in March 2018 for N + P and N only, respectively). Overall, the lowest soil N pools were measured in March 2018.

Total extractable soil N was generally higher under canopies ($P < 0.001$, habitat, Table 2). Time affected the total extractable N of the two habitats differently. This pool consistently decreased across the sampling campaigns underneath canopies but oscillated in the open pasture soil ($P < 0.01$, habitat × campaign, Table 2). Total extractable N was not affected by the nutrient addition treatments.

Microbial biomass N (chloroform-labile N) was most strongly affected by habitat, with much larger microbial N pools under tree canopies ($P < 0.001$, habitat, Table 2). There was a significant interaction between the nutrient addition treatments and sampling campaign. This was driven by the final sampling campaign in March 2018 where the N + P plots averaged 86 ± 9 μg microbial N/g soil and the N-only plots averaged 61 ± 14 μg microbial N/g soil ($P < 0.05$, averaged over habitat). Degrees of freedom, F statistics, and *P*-values for all ecosystem N pools are available in Appendix S1: Table S2.

^{15}N label recovery

Label recovery in both foliar and root pools was greatest in March 2017, the first sampling campaign after label application ($P < 0.05$, time, Table 3). In foliage, there was consistently greater recovery in open pasture plots than under-canopy plots ($P < 0.001$, habitat, Table 3). Label recovery in roots had a slightly different trend, with consistently higher recovery in open pasture plots in the first three sampling campaigns but the opposite in March 2018 ($P < 0.001$, habitat × time, Table 3). There was no significant effect of the nutrient addition treatments on label recovery in foliage or roots.

Label recovered in the bulk soil was nearly significantly affected ($P = 0.057$) by the three-way interaction of habitat, treatment, and time (Fig. 2). This is because time significantly influenced recovery in open pasture samples from the N addition plots, but not open pasture samples from N + P plots nor any samples from under-canopy soils. This carried over into a significant habitat by time interaction ($P < 0.01$) and a significant effect of time on its own ($P < 0.01$). Soil label recovery was greater in October than in May 2017 or March 2018 (Table 3).

Percent of label recovered in soil pools was calculated relative to recovery in soil from which that sample was extracted, because any label found in soil N pools would by definition also be part of the label recovered in the bulk soil. There was a significant interaction between habitat and time for label recovered in the total extractable N pool. Overall, there was more label recovered in the total extractable N pool underneath canopies (22% ± 4% on average across treatments and time, Fig. 3) compared to open pasture (14% ± 2%, $P < 0.05$). For open pasture samples, the most label was recovered in this pool in

Table 3. Percent of added ^{15}N recovered in main ecosystem components, values represent median ± standard error with combined nutrient treatments, n = 12, except for foliar samples for 19 October 2017 where only 7 and 11 samples had sufficient plant material for processing in open grassland and under-canopy plots, respectively.

Habitat	Campaign	Time since label application	Foliar	Root	Soil	Sum
Open pasture	March 2017	24 h	13.2 ± 2.5	7.2 ± 1.5	23.0 ± 5.1	41.3 ± 6.7 a
	May 2017†	9 weeks	4.3 ± 2.5	5.4 ± 1.3	18.9 ± 4.0	27.4 ± 6.5 ab
	October 2017	7 months	3.5 ± 1.0	2.9 ± 1.0	47.6 ± 5.2	**62.8 ± 6.2 a**
	March 2018	1 yr	3.1 ± 0.6	1.2 ± 0.6	20.8 ± 3.2	25.0 ± 3.5 b
Under canopy	March 2017	24 h	9.7 ± 1.7	3.8 ± 0.8	29.1 ± 8.5	40.9 ± 9.8 a
	May 2017†	9 weeks	3.0 ± 0.4	2.2 ± 0.5	20.8 ± 2.2	27.8 ± 2.6 b
	October 2017	7 months	1.8 ± 0.3	1.4 ± 0.4	22.9 ± 5.1	**24.7 ± 5.6 b**
	March 2018	1 yr	2.7 ± 0.4	2.5 ± 0.7	20.1 ± 3.4	27.7 ± 3.7 ab

Notes: Root and soil samples are from 0 to 5 cm depth. Final column is the sum of the above ground, root, and soil percent label recovery and represents the total amount of added label recovered in the herbaceous layer down to five cm soil depth in all measured pools. Letters represent Tukey post hoc groupings within a single spatial location. The bold values are a significant vegetative cover difference in label recovery 7 months after application, P = 0.002.

† Half of the samples (distributed evenly across treatments) were collected on 21 and the second half on 22 May 2017.

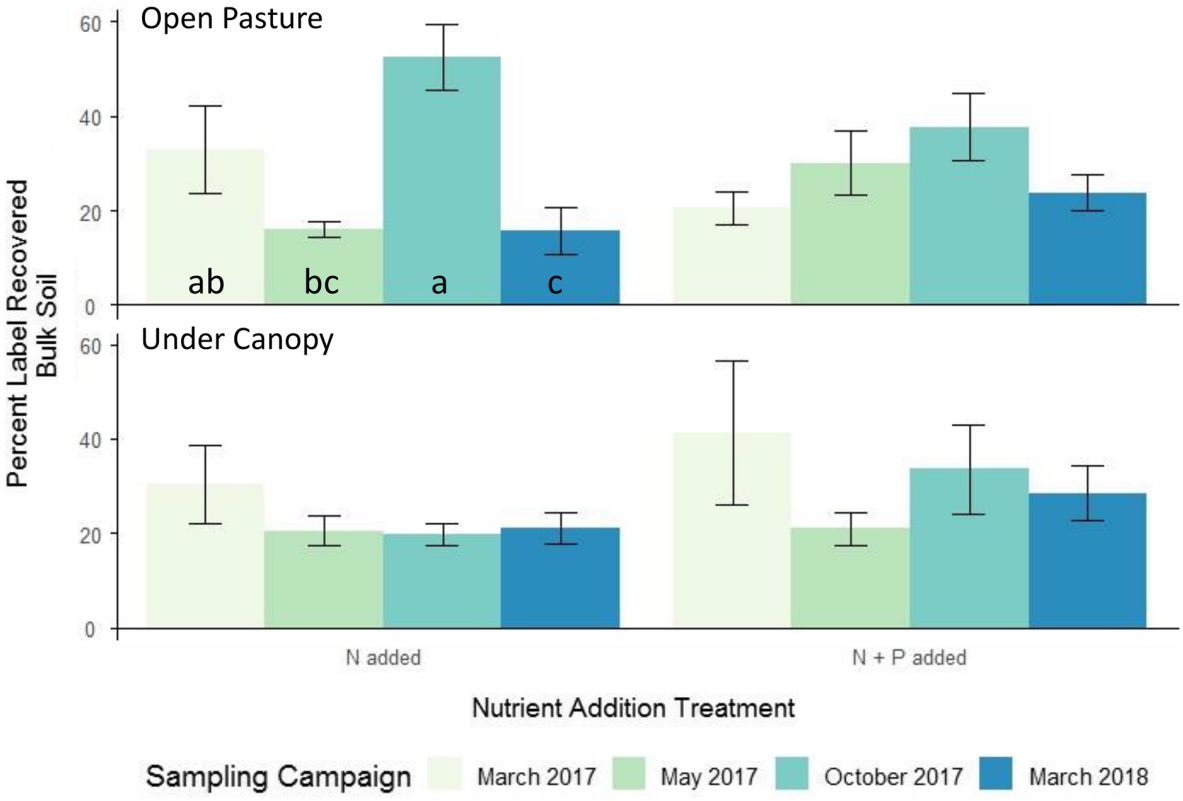

Fig. 2. Label recovery in bulk soil over four sampling campaigns for open pasture (above) and under-canopy (below) plots with two different nutrient additions. Values are mean ± standard error, n = 6. Samples are from 0 to 5 cm depth. Letters represent statistically different Tukey's post hoc groupings within the open pasture N addition treatment.

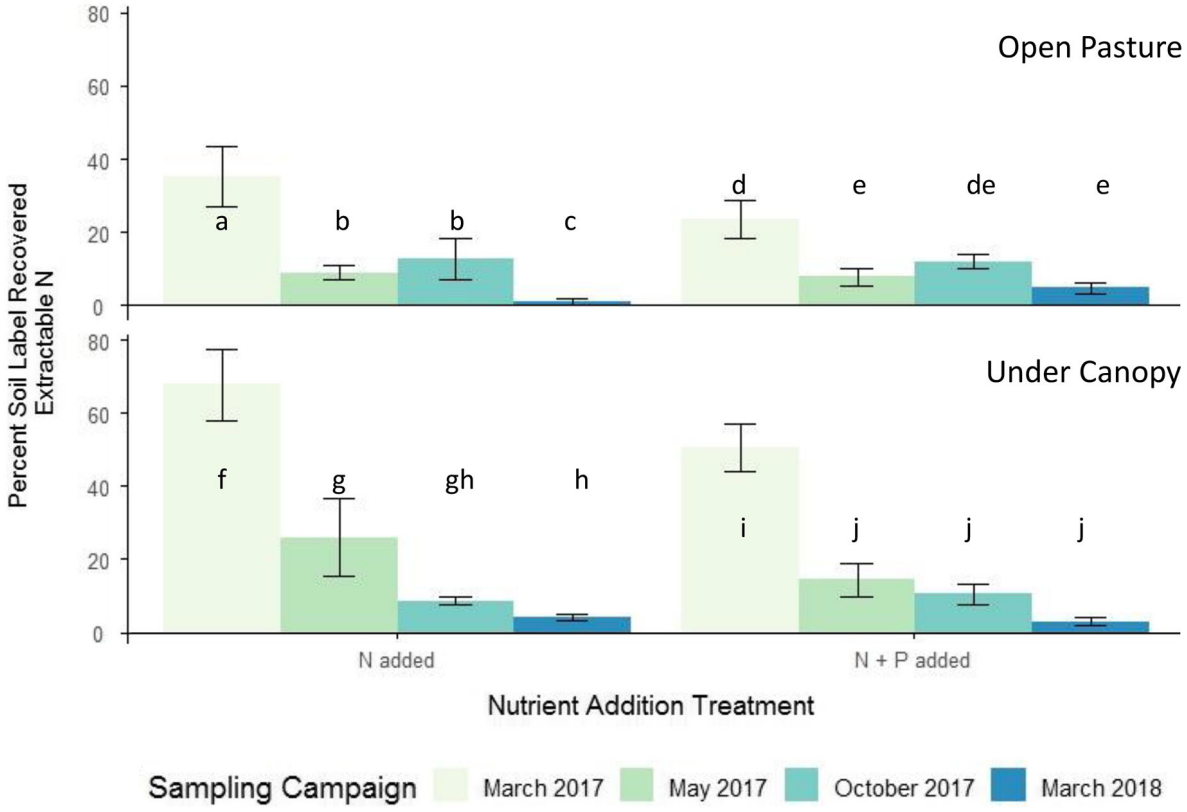

Fig. 3. Label recovery in extractable N over four sampling campaigns for open pasture (above) and under-canopy (below) plots with two different nutrient additions. Values are mean ± standard error, see Appendix S1: Table S1 for sample sizes. Samples are from 0 to 5 cm depth. Letters represent Tukey post hoc groupings within one habitat × nutrient addition combination.

March 2017, statistically more than recovery in any subsequent sampling campaign, decreasing to near the detection limits of the method by March 2018 ($P < 0.05$, habitat × time, Fig. 3). For under-canopy samples, the greatest recovery in total extractable N was also in the first sampling, but because this pool was overall larger, amounts were easily detectable even in March 2018.

Microbial biomass ^{15}N recovery (recovery from the chloroform-labile pool) was significantly influenced by the three-way interaction between habitat, time, and treatment, although marginally so ($P = 0.048$). However, due to the sensitivity of this measurement to sample loss (both the unfumigated and fumigated samples have to be carried successfully through to the end of the diffusion procedure), some habitats by nutrient addition time steps were only represented by a few samples (Appendix S1: Table S1). Therefore, we focus on results of the lower order ANOVA effects (single factors and two-factor interactions). Unlike other soil pools, label recovery in microbial biomass N was significantly affected by habitat and treatment ($P < 0.05$, for the interaction, Fig. 4). This is because microbial biomass recovery was greater in open pasture when N alone was added, but there was no treatment effect under trees. In general, the label recovered in microbial biomass decreased with time, from 24% ± 9% in March 2017 to 10.8% ± 2% in March 2018 (averaged across nutrient addition treatments and habitat).

Total ^{15}N label recovery

Total label recovery was calculated as the sum of label recovered in foliage, roots, and soil. Overall recovery was affected by the interaction

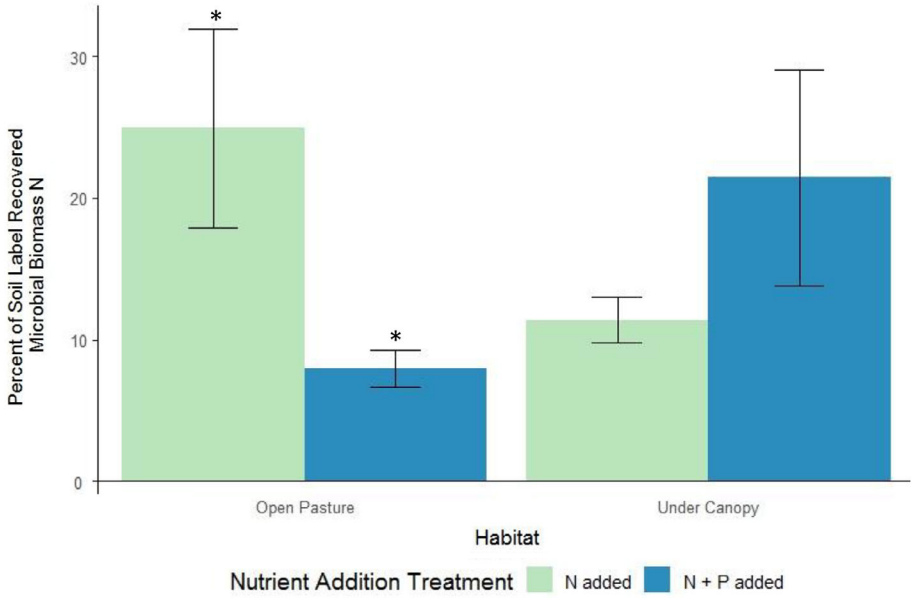

Fig. 4. Label recovery in microbial biomass for two habitats under two different nutrient addition treatments. Values are mean ± standard error over all sampling campaigns, n = 12–18 (see Appendix S1: Table S1 for details).

between habitat and time ($P < 0.01$, Table 3). Within one habitat type, open pasture plots had the greatest label recovery in October 2017, but below-canopy plots had the greatest recovery immediately after label application in March 2017. Comparing between habitats, open pasture had significantly greater label recovery than under canopy, and this effect was strongest in October 2017 (Table 3). The overall mean of open pasture recovery was 40.5%, compared to 34.9% for under canopy (averaging across sampling campaigns). Maximum recovery in plots where N alone was added was 54% ± 7% in March 2017, and the minimum recovery was 24 ± 4% in March 2018. In comparison, maximum recovery in N + P plots was 46 ± 9% in March 2017 with a minimum of 33% ± 4% in March 2018. Degrees of freedom, F statistics, and P-values for all ^{15}N label recovery variables are available in Appendix S1: Table S3.

Discussion

Total ^{15}N recovery in this study

We set out to determine the effects of habitat, P availability, and time on the fate of N additions in a dehesa using a ^{15}N tracer. Compared to other ^{15}N tracer experiments, our total label recovery was relatively low, around 40% one day after onset, while typical grassland tracer experiments recover 52% in the short term (one week to one month, Templer et al. 2012). There are four potential reasons for this: initial sample processing, grazing, leaching, and unmeasured pools. In March 2017, immediately after label application, foliar samples were rinsed with distilled water before further processing. This step, while critical to accurately measuring the true ^{15}N content of the plant material, likely removed residual label on the surface of the foliage. We minimized this effect by adding an additional 0.5 L of water to the plots after label application in the field, but this volume may have been insufficient. This label loss is only relevant to the first sampling campaign, because rain showers at the end of March 2017 (Appendix S1: Fig. S3) would have washed residual label off the foliage surface.

Between March and May 2017, we believe the primary loss of label was through grazing. Estimated biomass removal from grazing, based on exclusion cages within the same dehesa, is 50 kg

dry matter·ha^{-1}·month^{-1} during the spring. Assuming 1% N content of the vegetation and an atom percent excess of 0.14 (grand average for foliage in this study; Appendix S1: Table S4), 28 mg $^{15}N/m^2$ would have been removed by cattle between March and May 2017, 54% of our added label. This estimate is conservative as atom percent excess of foliage in March 2017 was 0.37 for open pasture and 0.20 for under canopy and measures of material removed by grazing in 2017 for other fertilized sites in the area were 120 kg dry matter·ha^{-1}·mo^{-1} (G. Moreno, *personal observation*, but see also ancillary information reported for sites ES-LM1 and ES-LM2 in the European Fluxes Database Cluster). However, it must be noted that some label was likely returned to plots as urine and dung, and we do not have grazing estimates for the experimental area itself, only sites nearby.

Leaching also likely accounts for some label loss, especially given our shallow (0–5 cm) sampling depth. Due to seasonal rainfall, leaching most likely occurred from March to May 2017 and October 2017 to March 2018 (Appendix S1: Fig. S3). If soil rewetting happens before plants and microbes are physiologically active in the fall, tracer present in the form of nitrate (NO_3^-) would have readily transferred below five cm. Indeed, in Mediterranean systems, rainfall with dry prior conditions leads to increased NO_3^- in local watersheds due to accumulated soil NO_3^- flushed from pore spaces (Bernal et al. 2005, Llorens et al. 2011).

Finally, one potential unmeasured pool is litter (standing and flattened dead material from the herbaceous layer, including tree litter under canopies). When sampling vegetation, browned and browning components were intentionally discarded. However, given our data we believe the role of litter as an N sink in this system is not insignificant (discussed in *Fate of added ^{15}N*). This oversight was due to the low ground cover of litter at the site (3% cover in May 2017, data not shown). In other studies, litter accounted for 25.5% of grassland tracer recovery (Templer et al. 2012); however, this is likely higher than our site, given the low litter cover in dehesas (Casals et al. 2010). A second unmeasured pool is label taken up by oak trees. However, Holm oak trees do not respond readily to fertilization even after two years (Rivest et al. 2011); therefore, this ecosystem component is likely a small sink for ^{15}N.

Despite the factors contributing to low recovery, this phenomenon is not unique to this experiment. Hart et al. (1993) had 44% total recovery one year after application to forest soil. Nadelhoffer et al. (1999) was similar with 45.5% total recovery in an oak forest, despite sampling to 20 cm soil depth. Mauritz et al. (2014) found just 10–12% recovery in annual plants of a semiarid chaparral system one year after label application. These studies cite various reasoning for their low label recovery such as dry conditions at onset of label application (Hart et al. 1993), horizontal mixing with natural abundance material diluting a relatively low-level tracer (Nadelhoffer et al. 1999), and sample processing error (Mauritz et al. 2014), the first and the last of which are similar to contributing factors in our study. All of these examples are systems ungrazed by livestock. It is also known that there is a negative correlation between the abundance of fine roots and tracer recovery (Templer et al. 2012) and fine roots are especially abundant in our site within the surface soil (Moreno et al. 2005, Rolo and Moreno 2012, Nair et al. 2019).

Ecosystem N pools

There was a seasonal pattern in N concentrations of bulk surface soil and plants, which declined in each sampling campaign throughout the year following the first growing period. At the peak of the second growing period, in March 2018, N concentration in plants increased (dramatically in foliage, Table 2). At the same time, there was a slight decrease in soil N and a very noticeable decrease in extractable soil N pools which indicate that plant N uptake was great enough to reduce other surface N pools. Likely, the increase in plant N is a result of interannual variability rather than a delayed fertilization effect as the growing period of 2017 was characterized by low productivity due to low precipitation during the spring and summer periods (Luo et al. 2018) with rainfall only 60% of the annual average (data not shown, but see Appendix S1: Fig. S3). The timing and magnitude of the dehesa springtime productivity peak are extremely sensitive to rainfall and temperature conditions of that year (Luo et al. 2018). Because soil was already relatively dry during fertilization in

2017, it is likely that plants were not able to fully utilize added nutrients until the following growing season. However, this is not supported by our low tracer recovery in plant pools in March 2018 (foliar N content is high, Table 2, when foliar ^{15}N recovery is low, Table 3), possibly because much of the biologically available tracer had been removed from the system due to grazing of the initially highly labeled material.

Ecosystem N concentrations were strongly affected by habitat with all pools having on average higher N concentrations underneath tree canopies. Surface soil N and microbial biomass N were between two and three times greater under tree canopies. This is as expected as dehesa trees create islands of fertility with enhanced nutrient content and higher microbial biomass (Dahlgren et al. 1997, Lopez-Sangil et al. 2011), despite a slightly higher C:N ratio of the under-canopy soil, likely due to higher C:N ratio of tree-derived litter (Cardinael et al. 2018). As our site has relatively low tree density (<20% tree cover), in general effects seen in the open pasture areas would be more representative of overall ecosystem behavior.

For plant tissues, addition of N alone resulted in slightly higher N concentrations than N + P addition. This was not the case for microbial biomass, which did not differ between nutrient addition treatments except for the last sampling campaign where the N + P treatment had higher biomass N. The treatment effect in plant tissues is possibly a result of the form of fertilizer applied, which was dominated to a greater extent by NO_3^- in the N-only plots. NO_3^- is more soluble than NH_4^+ and may have been more readily taken up by plants during the relatively dry 2017 growing season. Alternatively, it is possible that the many different species that make up the dehesa herbaceous layer on average prefer NO_3^- (Britto and Kronzucker 2013). Previous work has found that dehesa pasture vegetation is nutrient-limited, but responses are easily confounded by other limiting factors such as light or water limitation (Moreno 2008). Generally, plant biomass is the metric used to gauge nutrient limitation in response to fertilization, but this is not possible for this study because of active grazing. It is known that the herbaceous layer of the Majadas dehesa is more productive with N addition and shows an additional increase with N + P fertilization (Perez-Priego et al. 2015, Migliavacca et al. 2017).

The greater microbial biomass N found in N + P plots in March 2018 indicates that either microbe was able to take up more N in the presence of P or there was greater microbial biomass when N and P were added, but that the effect was not immediate. However, because the label recovery in microbial biomass did not follow the same pattern, we cannot be sure where this increased biomass N came from. Because plant root tissue also increased in N concentration in March 2018, the source may be higher N rhizodeposition, but one would have expected to see this pattern in both treatments. Because we only see it in microbes in the N + P treatment, perhaps it is a result of a subtle change in plant rhizosphere chemistry (Dijkstra et al. 2011) in the presence of added P. Alternatively, it could be a shift in plant community composition and corresponding rhizosphere chemistry due to N + P addition vs. N-only addition over the year (Ochoa-Hueso et al. 2013).

Fate of added ^{15}N

We hypothesized that open pasture areas would retain more added N due to their lower SOM and presumed greater N limitation compared to under-canopy areas (H1). The label recovery in plants was significantly higher in the open pasture plots for most sampling campaigns (Table 3). This supports the idea that more N was retained in this lower nutrient availability environment, but the magnitude of the difference is small compared to that seen in the soil data. While the overall mean (combining nutrient addition treatments) for total label recovery was greater in open pasture plots, this effect was driven by soil label recovery in October 2017 (Table 3).

The peak in soil ^{15}N recovery seen in October 2017 may relate to a peak in decomposition and degradation of the herbaceous layer litter throughout the intensely irradiative summer. The litter layer would have been composed almost entirely of recently senesced, labeled foliage from the previous growing season. Physical fragmentation and photodegradation control litter degradation rates in semiarid systems (Coûteaux et al. 1995, Austin and Vivanco 2006), and label stored in this pool would have reached

its maximum transfer to the soil pool by October 2017. Additionally, direct spraying is not the only way label can get into the litter pool. Initial decomposition involves an increase in litter N content (Parton et al. 2007) from investment of exoenzymes (which have high N content). This was likely greater for N-only plots because of increased microbial demand for P, which could also be obtained from litter. Therefore, we believe that low soil label recovery of N-only plots compared to N + P plots in May 2017 (Fig. 2) is due to greater litter N immobilization in the absence of increased P availability. Somewhat surprisingly, in Parton et al. (2007), arid grassland litter was the only litter type which did not show a pattern of initial N investment. However, they only tested one litter sample from one arid grassland. Our results suggest that the pattern may hold at least for some semiarid grassland litters. In total, these data imply that there is more complete biomass turnover throughout the year in open pasture areas of dehesas, which might be enhanced in the presence of greater N availability.

We also hypothesized that increased P availability would prevent P limitation and enhance N retention in plant and microbial pools relative to addition of N alone (H2). We found little evidence for co-limitation, given that there was no consistently greater recovery in N + P plots compared to N-only plots. We did find that there was greater label recovery in open pasture microbial biomass of N-only plots (Fig. 4). We interpret this as slower growth and lower turnover of the microbial biomass pool in open pasture plots when N and P are imbalanced. This is supported by statistically lower microbial biomass N concentrations in the N-only plots compared to N + P plots in March 2018, indicating that potentially microbes in N-only plots were growing slower. Reduced growth is a fairly common response of soil microbes to N addition (Treseder 2008, Riggs and Hobbie 2016). A broad-scale assessment of grassland microbes found that the addition of N alone affected the microbial community differentially than N + P addition (Leff et al. 2015), which could lead to differential turnover. Different SOM concentrations and chemical compositions underneath tree canopies also support a distinct microbial community (Ho et al. 2017), which did not show this differential response to stoichiometric imbalance. However, because the nutrient addition treatments may have affected communities in both habitats, it is not possible to say what role this played. Overall, low label recovery in microbial biomass when N and P were added was surprising given that previous research done at the site indicated that soil microbes were co-limited by N and P (Weiner et al. 2018).

With regard to the effect of time (H3), we expected to find that added N would first be taken up in microbial biomass (Kuzyakov and Xu 2013), then move into the extractable soil N pool as a result of inorganic N accumulation (Austin et al. 2004), and end mostly in the plant pool one year later (Kuzyakov and Xu 2013). Contrary to our expectation, the highest plant recovery was in March 2017, just 24 h after label was applied. High recovery immediately after application suggests that much of the label was taken up through leaf tissue (Sparks 2009, Nair et al. 2016), meaning that plants were never in competition with microbes for this N. The slight increase of label recovery in plant tissues under tree canopies from fall 2017 to spring 2018 could indicate the use of N leached to soil below five cm.

Regardless of season, the largest individual sink for added N was the soil. This is consistent with previous tracer studies in grasslands for weeks to months after addition (Templer et al. 2012). However, none of the studies in the 2012 review deal directly with seasonal effects, which played an important role in our study, because we found the highest soil label recovery in the fall. A study similar to ours, which addresses temporal effects on the fate of N additions in Mediterranean shrublands, also found a peak in N recovery within the soil inorganic N pool in the fall (Dias et al. 2012) and attribute it to litter decomposition. This fall peak was only found in plots with added N, not in unfertilized plots, with a rate of N application very similar to ours (their study had 40 or 80 kg N/ha, ours 50 kg N/ha). We did not measure inorganic N pools, but it is likely that the peak in total extractable N (inorganic N plus organic N) concentrations seen in October 2017 is due to increased inorganic N, which generally increases in arid and semiarid soils throughout the dry season (Gallardo et al. 2000, Austin et al. 2004). Given this pattern, if the onset of autumn rain is acute, N would be susceptible to loss from the system via leaching

(Bernal et al. 2005, Llorens et al. 2011, Dijkstra et al. 2012). However, our label recovery in total extractable N and microbial N does not follow the same pattern (see Table 3, Fig. 3) so we cannot rule out the possibility that high soil label recovery in the fall is purely from physical breakup of labeled litter, with mineralization playing little to no role. Additional experiments would be needed to make this distinction.

In a Californian Mediterranean grassland, Hart et al. (1993) had relatively high label retention (33%) in non-extractable soil N, which they attributed to abiotic fixation by vermiculite and SOM. Vermiculite is not present at our site, but smectite is (Pérez Arias 1992, Muñoz et al. 1995), and likely contributed to soil ^{15}N retention in addition to SOM. This is evident by the fact that only 2.5–7.5% of total soil N was extractable (including chloroform-labile N). In other words, as much as 97.5% of soil N was not extractable by 0.5 mol/L K_2SO_4, indicative of high abiotic N fixation. Additionally, the highest recovery of ^{15}N in total extractable and microbial N pools was immediately after label application, indicating little reverse flow of label after abiotic fixation. Previous work comparing biotic vs. abiotic fixation rates found that on average 76% of added NH_4^+-N was abiotically fixed in an N-poor, sandstone-derived soil (Johnson et al. 2000), similar to that at our field site. If the liquid application of labeling solution led to faster or more efficient fixation of ^{15}N, relative to the pelleted fertilizer N, this could explain the low ^{15}N recovery in plant tissues during March 2018 when tissue N concentration was highest. This is because the abiotically fixed ^{15}N, if initially quickly sorbed, would have been less available for biological uptake throughout the remainder of the experiment.

Conclusion

We found no strong effect of increased P availability on the fate of N additions; rather, we found generally equal N allocation between the two nutrient addition treatments. However, one should bear in mind that 2017 was a dry and low productivity year. More work should be done to determine how water availability might influence N allocation and potential N:P imbalances in dehesas and similar ecosystems. We show that habitat plays a fundamental role in how N is cycled in this system. However, although soil N content was up to three times higher beneath canopies, this barely affected the fate of N during the spring growing season when most grass biomass is produced. Our major finding is that soil retained most of the added N, regardless of time since application, P availability, or habitat. If excess N is mostly abiotically fixed in soil, as our results imply for a dry year, moderate rates of N deposition may not have much impact on ecosystem function because the majority of added N would not be biologically available. The strong seasonal effect we found in open pasture plots (especially with only N added) implies that the timing of rainfall onset relative to the onset of plant growth will be critical to ecosystem N retention in the future. Additional work is needed to determine whether these findings are generalizable to wetter years and therefore how this phenomenon could feedback into interannual ecosystem C dynamics.

Acknowledgments

The authors thank the Alexander von Humboldt Stiftung for financial support of the MaNiP project through the Max Planck Research Prize to Markus Reichstein. Thanks also go to Kirsten Küsel, Markus Reichstein, and Susan Trumbore for stimulating discussion of the data. Finally, the authors thank Yonatan Cáceres Escudero, Heike Geilmann, Jinhong Guan, Tiana W. Hammer, Iris Kuhlman, Mónica Lorente, Adrian Mateos, and Rolf Rödger for tireless support in the field and in the laboratory. The authors declare no conflict of interest.

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