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Effects of four different restoration treatments on the natural abundance of ^{15}N stable isotopes in plants

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$\delta^{15}N$ signals in plant and soil material integrate over a number of biogeochemical processes related to nitrogen (N) and therefore provide information on *net* effects of multiple processes on N dynamics. In general little is known in many grassland restoration projects on soil–plant N dynamics in relation to the restoration treatments. In particular, $\delta^{15}N$ signals may be a useful tool to assess whether abiotic restoration treatments have produced the desired result. In this study we used the range of abiotic and biotic conditions provided by a restoration experiment to assess to whether the restoration treatments and/or plant functional identity and legume neighborhood affected plant $\delta^{15}N$ signals. The restoration treatments consisted of hay transfer and topsoil removal, thus representing increasing restoration effort, from no restoration measures, through biotic manipulation to major abiotic manipulation. We measured $\delta^{15}N$ and %N in six different plant species (two non-legumes and four legumes) across the restoration treatments. We found that restoration treatments were clearly reflected in $\delta^{15}N$ of the non-legume species, with very depleted $\delta^{15}N$ associated with low soil N, and our results suggest this may be linked to uptake of ammonium (rather than nitrate). The two non-legume species differed considerably in their $\delta^{15}N$ signals, which may be related to the two species forming different kinds of mycorrhizal symbioses. Plant $\delta^{15}N$ signals could clearly separate legumes from non-legumes, but our results did not allow for an assessment of legume neighborhood effects on non-legume $\delta^{15}N$ signals. We discuss our results in the light of what the $\delta^{15}N$ signals may be telling us about plant–soil N dynamics and their potential value as an indicator for N dynamics in restoration.

Keywords: stable isotopes, restoration, topsoil removal, functional type, legumes, plant–soil interactions

INTRODUCTION

Species-rich grasslands on nutrient-poor soils form important cultural landscapes of high conservation value in Europe but their area has declined due to the opposing drivers of land abandonment or agricultural intensification (Poschlod and Wallis De Vries, 2002; Isselstein et al., 2005). As a consequence considerable effort has gone into their restoration with various levels of success (Bakker and Berendse, 1999) and limitations often include seed dispersal and abiotic constraints (e.g., in ex-arable fields where restoration often takes place). Recent restoration experiments have shown that seed limitation can be overcome by measure of species introduction but long-term success of restoration projects also depends on the suitability of the abiotic conditions (Kiehl et al., 2010). Since the high diversity of such grasslands is linked to adaptation to their often extreme abiotic conditions, including N limitation and low water availability, knowledge of plant–soil N dynamics of the ecosystem should play a key role in ecosystem functioning and hence restoration success.

Stable isotope methods using the heavy isotope of nitrogen ^{15}N, either as natural abundance (NA) or added in enriched form, are traditionally used in terrestrial ecosystems to follow paths or sources of N in systems (tracer studies). There are advantages and disadvantages of using either NA or enriched ^{15}N methods. For nitrogen, the NA method measures the ratio of the naturally occurring rare and heavy ^{15}N and the naturally prevalent lighter ^{14}N isotopes and provides the $\delta^{15}N$ signal of a sample. The $\delta^{15}N$ in plants is *per se* a function of $\delta^{15}N$ of all N sources and thus an integrator of net N dynamics in a system (Robinson, 2001). A number of studies have used ecosystem $\delta^{15}N$ as a proxy of N availability, and higher availability of N or more active N transformation rates have been linked to enriched $\delta^{15}N$ values (Coetsee et al., 2011). As such ^{15}N NA studies have the potential to provide insights into a number of plant–soil processes of importance to restoration: e.g., issues of effectiveness of restoration actions, such as whether topsoil removal does significantly alter soil–plant N dynamics and hence diversity in systems with high nutrient loads as often found in Central Europe. In addition, restoration experiments and projects, especially those with drastic manipulation of abiotic conditions such as topsoil removal on ex-arable land (Marrs, 2002), can provide a broad range of different abiotic and

biotic conditions within which to test how variability of δ^{15}N in plants may relate to their functional identity (FI) or to the soil conditions they experience.

The complexity of the N cycle in soils (including lack of knowledge of δ^{15}N of various pools in the N cycle) as well as relatively small discrimination during natural processes (compared to the isotope ^{13}C) make the δ^{15}N NA method more appropriate for providing integrated information rather than a clear signal of particular process or source of N in nature (Handley and Raven, 1992). Exceptions are that δ^{15}N in plants enables species to be identified as N-fixers (δ^{15}N around zero) or non-fixers (δ^{15}N significantly different to zero, e.g., Virginia and Delwiche, 1982). The NA method can also be used for estimating the percent of N derived from atmosphere (%Ndfa) in aboveground plant parts of N_2-fixing species (Shearer and Kohl, 1986) and to study facilitative legume neighbor interactions (Temperton et al., 2007).

In a broader context and depending of the study-scale, δ^{15}N in plants and soils can also provide information on climate (Amundson et al., 2003), nutrient retention in ecosystems (openness or closedness of the N cycle; Frank and Evans, 1997), and site history including N cycling and plant development (Chang and Handley, 2000; Coetsee et al., 2011). The study of Coetsee et al. (2011) in grazed pastures found that grazing can directly increase δ^{15}N in plants even if soil N remains the same, and linked this to altered rates of N transformation during grazing. Other examples of the integrative use of NA methods are studies on C and N dynamics in plant–mycorrhizal ecosystems but "questions remain about how different N forms, fungal symbionts, and N availabilities influence δ^{15}N signatures" (Hobbie et al., 2008).

Nevertheless, the limitations of such methods need to be kept in mind and we need to explicitly test what information these methods can and cannot provide, as well as their potential and relevance for addressing key issues in ecology and restoration. In order to do this, we first need additional information on the variability of δ^{15}N signatures in plants and soil and preliminary information on how these signatures may be affected by abiotic and biotic conditions at a site. Although NA methods using δ^{15}N signatures have been widely used as integrators of the N cycle within a whole range of ecosystems (Amundson et al., 2003, or southern *Nothofagus* forests in Peri et al., 2012), in grasslands most of the data on typical δ^{15}N signatures and their variability within and between plant species derives from relatively nutrient-rich grasslands (so-called mesic grasslands; Spehn et al., 2002; Kahmen et al., 2008; Gubsch et al., 2011). There are very few studies in nutrient-poor dry grasslands, such as primary succession grasslands on dunes (van der Heijden et al., 2006) or dry acidic grassland (Beyschlag et al., 2009). The few studies in dry grasslands have found, however, that δ^{15}N signatures of plants in these systems are more depleted in δ^{15}N than in mesic grasslands.

We therefore used a calcareous grassland restoration experiment established on ex-arable land that provided a range of abiotic and biotic conditions (ranging from relatively nutrient-rich soils to nutrient-poor soils) to screen the δ^{15}N signatures of plant species across the treatments. This allowed us to test whether similar patterns of more negative (depleted) δ^{15}N plant signals would be found in more nutrient-poor conditions compared to the more nutrient-rich conditions in mesic grassland habitats. In addition it allowed us to investigate the link between δ^{15}N plant signals with both soil characteristics and functional plant characteristics. The restoration experiment provided sites with and without topsoil removal and sites with and without hay transfer for the introduction of target species, thus representing a gradient in restoration effort from no action (no topsoil removal, no hay transfer) to maximum action (topsoil removal and hay transfer).

In particular we aimed to see if

(a) δ^{15}N signals in plant species at a calcareous grassland restoration site would clearly reflect the different restoration treatments, particularly whether the abiotic topsoil removal treatment could be clearly separated from the treatment without topsoil removal?
(b) Whether δ^{15}N signals could be used to clearly separate legume species (N_2-fixers) from non N_2-fixing species?
(c) Whether the vicinity of a legume species to a plant affected the δ^{15}N signature of the plant?

To test this we sampled a range of grassland species in the four different restoration treatments and measured N concentration and δ^{15}N in leaves of these species, as well as the soil nutrient availability and δ^{15}N in bulk soil. Species included either stress-tolerant target species (typical dry grassland species as indicators for restoration success) as well as non-target species more typically found in more nutrient-rich (mesic) grasslands.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

The study site consists of ex-arable fields in the vicinity of the nature reserve "Garchinger Heide" (48°18′N, 11°39′E, 469 masl, Germany), located in the Munich gravel plain on pararendzina soil (see **Figure 1**). The fields had been used as arable fields since the beginning of the twentieth century and were converted in 1993 in the course of a large-scale restoration project to reestablish nutrient-poor, species-rich calcareous grasslands (Pfadenhauer et al., 2000). The climate is temperate, mean annual temperature is 7.8°C, total annual precipitation is ∼865 mm (data for Oberschleißheim and Haimhausen-Ottershausen, DWD, 2009).

The restoration treatments were hay transfer (from the reference grassland nature reserve "Garchinger Heide") and topsoil removal, thus representing increasing restoration effort, from no restoration measures, through biotic manipulation to major abiotic manipulation. Restoration treatments were performed at field scale (approximately 200–300 m long, 70 m wide) in two different sets of fields providing two experimental blocks: blocks 1 and 2, with all four restoration treatments within each block (see **Figure 1**). The four restoration treatments were topsoil removal with hay transfer ($+r+h$) and without hay transfer ($+r-h$), no topsoil removal with hay transfer ($-r+h$) and no topsoil removal without hay transfer ($-r-h$). Each restoration treatment covered an area of around 70 m × 150 m depending on the exact field in question (see **Figure 1**). Treatment $-r-h$ represents the natural succession from old field to grassland and hence forms a restoration control. Topsoil removal consisted of mechanical removal of 40 cm agricultural topsoil down to the calcareous gravel and resulted in a strong reduction of total N and exchangeable P and

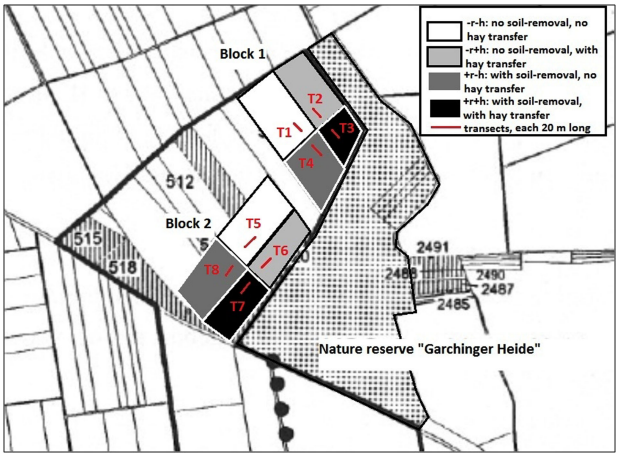

FIGURE 1 | The Garchinger Heide restoration site near Munich showing the experimental design with the four restoration treatments repeated over two blocks, and the eight transects used for sampling plant material. Transects (each 20 m long) were positioned within the treatment fields (size approximately 70 m × 150 m): T1–T4 are in Block 1, transects T5–T8 are in block two. The restoration treatments ranging from topsoil removal and hay transfer (+*r*+*h*) to no action (−*r*−*h*) are shown in different colors on the fields.

K in the substrate (**Table 1A**). Hay transfer aimed to overcome dispersal limitation of calcareous grassland species, and indeed number and cover of calcareous grassland species (including many legume species) was much higher on +*h* than on −*h* sites, even 13 years after start of the restoration (**Table 1B**; Kiehl, 2009).

Since 1995, the −*r* areas were either grazed by sheep or mown annually in July/August and the +*r* areas were mown only occasionally to remove woody species as mowing was usually not possible due to low biomass production (Pfadenhauer et al., 2000; Pfadenhauer and Kiehl, 2003). Sheep grazing occurred only in Block 2 (not Block 1) and then only for a period of 2 weeks. Different management types showed only minor effects on flora and fauna compared to the major treatments topsoil removal and hay transfer (see Kiehl and Wagner, 2006).

PLANT SAMPLING AND ANALYSIS

Plant and soil material were sampled along a 20-m transect in the middle of each treatment area. In August 2007, we sampled plant material (1–2 individuals) at eight equally distributed measuring points along each transect or within a distance of maximum 2 m perpendicular to the transect when species were not found close to the transect. This gave a total transect area sampled of 80 m^2 per treatment per block. An exception to this sampling method was made in the +*r*−*h* treatment, where plant cover was very low, such that we sampled in an area of approximately 50 m × 100 m. We used every sample per treatment per block as a replicate giving $n = 6$–17 per species and restoration treatment (**Table 2**; varying n depended on how many individuals per species were found along the transect).

To address the question of whether δ^{15}N signatures in plants could be used to clearly separate legume species (N_2-fixers) from non N_2-fixing species we sampled four legume species and two non-legume forb across the restoration treatments. To address the question of whether the vicinity of a legume species to a plant affected its δ^{15}N signature, we collected leaves of legume/non-legume pairs (<10 cm distance between each other) and control plants of the non-legume species (>30 cm distance to the next legume species). As far as possible we collected pairs (legume/non-legume) of stress-tolerant target species (typical for calcareous grasslands) as well as pairs of non-target species (typical for mesic grasslands) in all restoration treatments (but not all species were present in all treatments, as ecological sorting had occurred in response to the different abiotic conditions produced by the topsoil removal).

We sampled four legume and two non-legume species overall (see **Table 2**). The stress-tolerant target species were: *Anthyllis vulneraria* L. (hereafter *ant*), *Dorycnium germanicum* (Gremli) Rikli (hereafter *dor*), and *Helianthemum nummularium* (L.) Mill. (small shrub, hereafter *hel*; two legume and one non-legume forb respectively). Mesic, non-target species were: *Trifolium pratense* L. (hereafter *tri*), *Lotus corniculatus* (hereafter *lot*), and *Galium mollugo* (forb, hereafter *gal*; again two legume and one non-legume species respectively; Oberdorfer, 2001). Plant samples were dried (60 h/60°C), ground to fine powder and analyzed for δ^{15}N and N concentration (hereafter %N). We collected root samples in November 2008 and estimated whether they had been colonized by mycorrhizae and measured δ^{15}N and %N in the soil samples.

SOIL SAMPLING AND ANALYSIS

In November 2008, one mixed soil sample (0 to −15 cm) was obtained at four positions along each transect for analysis of δ^{15}N and other abiotic parameters in the bulk soil. Soil samples were sieved (<2 mm) to homogenize the substrate and exclude roots and stones. An aliquot of the sieved soil was dried (72 h/30°C), ground to fine powder and analyzed per restoration treatment for %N, δ^{15}N ($n = 8$) and P concentration ($n = 2$, hereafter %P). For analysis of mineralized soil N ($n = 8$, N_{min}: plant-available NH_4^+ and NO_3^-; [ppm]) 5 g of fresh soil were shaken with 50 ml 1 M KCl for 6 h and N_{min} was determined chromatographically in the soil solution using ion chromatography. P concentration in soil was determined with ICP-OES (Thermo Fisher Scientific, Waltham, USA).

δ^{15}N AND %N ANALYSES

For analyses of δ^{15}N NA signals (‰; hereafter δ^{15}N) and %N, ground plant or soil material was measured using an element analyzer coupled with an isotope ratio mass spectrometer (EA-IRMS; EA = EURO-EA 3000 by HEKAtech GmbH, Wegberg, Germany, IRMS = IsoPrime by Micromass UK Limited, Manchester, UK).

The δ^{15}N of a sample denotes the ratio of the heavier over the lighter stable isotope of nitrogen (^{15}N over ^{14}N) in a sample in relation to a standard (atmospheric N_2; see Mariotti, 1983): $\delta^{15}N = [(R_{sample}/R_{standard}) - 1] * 1000$ [‰], R_{sample} or $R_{standard}$ is the ratio of ^{15}N over ^{14}N for sample or standard, respectively.

STATISTICAL ANALYSIS

The restoration experiment was conducted at landscape scale on restoration fields of several hectares and thus, according to

Table 1 | Compilation of abiotic and biotic characteristics in different restoration treatments from previous evaluations at the field site: (A) abiotic soil properties in 2000 (0–10 cm, summarized from Pfadenhauer and Kiehl, 2003; Kiehl, 2005), note that nutrient contents and ratios relate to the *weight of the fine soil fraction* only not the total soil volume, and (B) species cover and species richness in 2006 (summarized from Hummitzsch, 2007).

(A)	With topsoil removal	Without topsoil removal
Skeletal content (fraction >2 mm) [% dry weight]	84.5 ± 5.8	69.9 ± 0.3
P_2O_5 [mg/100g]	4.1 ± 1.8	43.8 ± 5.7
K_2O [mg/100g]	9.1 ± 4.2	58.1 ± 7.6
N_{total} [%]	0.09 ± 0.07	0.43 ± 0.04
C_{org} [%]	0.9 ± 0.6	4.6 ± 0.5
C:N	10.1	10.6
pH	7.2 ± 0.1	6.9 ± 0.1

(B)	With topsoil removal		Without topsoil removal	
	With hay (+*r*+*h*)	Without hay (+*r*−*h*)	With hay (−*r*+*h*)	Without hay (−*r*−*h*)
Vegetation height [cm]	11.4 ± 5.0	14.1 ± 5.2	52.5 ± 7.3	58.3 ± 9.0
Cover [%]				
Litter	2.8 ± 1.3	2.4 ± 1.2	43.8 ± 24.1	30.0 ± 9.7
Bare soil	24.2 ± 13.6	74.8 ± 11.0	1.1 ± 2.3	1.5 ± 1.3
Vascular plants	48.4 ± 12.0	20.0 ± 14.2	85.7 ± 9.2	84.8 ± 5.8
Target GL species	50.0 ± 14.0	22.2 ± 12.9	95.9 ± 15.1	35.5 ± 23.6
Mesic GL species	0.5 ± 0.4	3.3 ± 4.2	34.3 ± 11.1	77.4 ± 26.5
Ruderal species	0.3 ± 0.5	1.3 ± 1.6	1.3 ± 1.3	5.6 ± 3.7
Legume species	18.5 ± 6.8	5.9 ± 8.1	19.4 ± 6.5	12.4 ± 10.1
Forb species	30.7 ± 9.6	17.9 ± 4.6	46.0 ± 12.8	40.7 ± 10.6
Grass species	1.6 ± 1.6	2.3 ± 1.9	66.2 ± 10.5	65.3 ± 11.4
SPECIES RICHNESS				
Total	23.1 ± 4.2	20.3 ± 6.9	27.9 ± 5.1	24.0 ± 3.6
Target GL species	21.1 ± 3.8	15.2 ± 4.0	19.1 ± 5.1	9.5 ± 3.3
Mesic GL species	1.5 ± 1.6	2.7 ± 2.6	7.5 ± 1.4	11.6 ± 1.8
Ruderal species	0.6 ± 0.6	2.4 ± 2.6	1.2 ± 0.6	2.9 ± 1.0
SPECIES RICHNESS PER RESTORATION TREATMENT				
Total	69.5 ± 4.9	85.0 ± 12.1	71.3 ± 1.8	78.7 ± 2.8
Target GL species	52.5 ± 2.7	51.0 ± 2.2	43.7 ± 1.8	34.3 ± 3.9
Mesic GL species	6.5 ± 0.6	9.5 ± 3.8	17.7 ± 1.8	22.0 ± 1.5
Ruderal species	10.5 ± 2.7	23.5 ± 4.9	10.0 ± 2.6	22.7 ± 2.7

Values are means with standard deviations, n = 10–20 from permanent plots (4 m^2) for all parameters except "species richness per restoration treatment" where n = 2 from the two blocks.

Restoration treatments: with (+) or without (−) topsoil removal (r) and hay transfer (h).

Target species = calcareous grassland species, GL = grassland.

Oksanen (2001), replications per site can be considered as independent samples in statistical analyses and not as pseudoreplicates *sensu* Hurlbert (1984). Due to the size of the sites, we were able to avoid edge effects and undesired between-treatment dispersal which often confound results on small plots. We tested effects of restoration treatments in a two factor (topsoil removal, hay transfer) factorial design on N parameters in plants and soil. The block effect was negligible for most subsets of data.

Since not all species combinations were present in all treatments, we used all data together only for a few analyses (for all species over all restoration treatments) testing for effects of species identity (SI), and functional identity (FI; whether a species was a legume or not) on leaf $\delta^{15}N$ and %N in a one-way ANOVa (and data were $\log_{10}$ transformed when variances found to be inhomogeneous).

All other analyses were performed using subsets of data separated either by FI, SI, neighborhood (NH), or restoration treatment. Here we performed an overall two-way ANOVA (Type III Sum of Squares) with topsoil removal (r), hay transfer (h), and an interaction between both ($r \times h$) as fixed factors. In general for soil parameters, $n = 8$ (four replicates per transect per treatment in each block) except for P where only two samples were taken (one pooled sample per transect; since P was not the main focus of the study). For plant leaf data the exact number of replicates

Table 2 | Overview of the number of plant pairs (non-legume species and their legume neighbor), control plants (non-legume species without legume neighbor) and legume species without non-legume neighbor sampled along transects in different restoration treatments, depending on availability of each species along transects.

Block	Transect	Treatment	Mesic grassland species				Target species			
			tri + gal	lot + gal	gal	lot	ant + hel	dor + hel	hel	ant
Block 1	T1	$-r-h$	8	8	6					6
	T2	$-r+h$		9			9			
	T3	$+r+h$					8	8	5	
	T4	$+r-h$				8	8	2	8	
Block 2	T5	$-r-h$	8	8	6					
	T6	$-r+h$		8	8					
	T7	$+r+h$					8	8	6	
	T8	$+r-h$		8	8		8		8	

Gal and hel are non-legume species.

Restoration treatments: with (+) or without (−) topsoil removal (r) and hay transfer (h).

Target/calcareous grasslands species: ant = Anthyllis vulneraria (legume species), dor = Dorycnium germanicum (legume species), hel = Helianthemum nummularium (non-legume species) and non-target/mesic species: lot = Lotus corniculatus (legume species), tri = Trifolium pratense (legume species), gal = Galium mollugo (non-legume species).

depended on the number of individuals per species found along the transects (see below for exact replicate values per species). For plant %N (untransformed) and δ^{15}N (log transformed) we performed one ANOVA on the whole dataset (all species in all restoration treatments together, n of all samples = 293, **Table 3A**).

In addition, we tested effects of restoration treatments on legume ($n = 126$) and non-legume species ($n = 167$), separately using two-way ANOVAs (**Table 3B**). For effects on single species, the dataset was split into different species (*ant*: $n = 46$, *dor*: $n = 18$, *hel*: $n = 85$, *lot*: $n = 47$, *tri*: $n = 15$, *gal*: $n = 82$; **Table 3C**). We used PASW Statistics 18 (2009, SPSS, USA). All data were tested for homogeneity of variance (Levene's test) and normality, and $\log_{10}$ transformed if assumptions were not met.

RESULTS

RESTORATION TREATMENT EFFECTS ON SOIL

The restoration treatments represented increasing restoration effort, from no restoration measures (control, $-r-h$), through biotic manipulation (with hay transfer, $+h$) to major abiotic manipulation (with topsoil removal, $+r$). Topsoil removal was very effective in persistently reducing plant-available nutrients, whereas hay transfer had no effect on soil nutrient parameters (except for $^{NH_4^+ : NO_3^-}$-ratios: $p = 0.014$; **Table 1A**; **Figure 2**). Sites without topsoil removal had twice as much %N in the substrate than those with topsoil removal, and this difference remained constant over time (in 2003: **Table 1A** and in 2008: **Figure 2**). In this study we link 2007 plant data mainly to 2008 soil data (rather than earlier soil values). In 2008 (**Figure 2**) topsoil removal reduced plant-available mineral N $^{(N_{min} : NH_4^+ + NO_3^-)}$, %N and %P strongly by 60–70%, respectively ($p \leq 0.020$). In contrast, the ratio of N_{min} to %N (N_{min}/N_{total} [%]) was stable across the restoration treatments ($p = 0.162$; **Figure 2**), but the ratio of ammonium to nitrate $^{(NH_4^+ : NO_3^-)}$ was significantly higher in topsoil removal sites than in non-removal sites ($p < 0.001$) and this corresponded to extremely low nitrate concentrations after topsoil removal (**Figure 2**). Topsoil removal reduced $\delta^{15}N_{soil}$ in bulk soil on average by around 1.2‰ compared to non-removal sites ($+r$: 3.23‰ $<-r$: 4.51‰, $p = 0.005$).

RESTORATION TREATMENT EFFECTS ON LEAVES AND ROOTS

Restoration treatments affected %N as well as δ^{15}N significantly in leaf tissue (**Figures 3** and **4**) when tested over all six plant species (**Table 3A**). A significant interaction effect between topsoil removal and hay transfer ($r \times h$) was found for %N but not for δ^{15}N (**Table 3A**). Splitting data into subsets based on FI (legume versus non-legume species) produced the same overall result, except that the interaction effect ($r \times h$) on %N did not occur for legumes (**Table 3B**). Restoration treatment effects on single species had significant effects on %N in leaf tissue of three of the species *ant*, *hel*, and *gal* (**Table 3C**; **Figure 4**). Restoration treatment effects on leaf δ^{15}N were found for both non-legume species, *hel* and *gal* (**Table 3C**; **Figure 4**). Here both foliar %N and δ^{15}N decreased (δ^{15}N became more negative) with increasing restoration effort (**Figure 3** and reading **Figure 4** from right to left across restoration treatments). Changes in δ^{15}N of non-legumes were even more pronounced than changes in %N.

All unspecific root samples contained some nodulated legume roots and all root samples were heavily infected with mycorrhizal fungi (50–95% of root tissues). Other kinds of fungal material were also visible and some of the significantly thicker, brownish stained material may have been ectomycorrhizial fungi components which often occur in symbiosis with *H. nummularium* (*hel*; Harley and Harley, 1987). Root samples did not vary in their %N between restoration treatments ($p = 0.368$; **Figure 4**) but $\delta^{15}N_{root}$ values were significantly lower ($p = 0.016$) in topsoil removal sites than in non-removal sites.

DIFFERENCES IN δ^{15}N BETWEEN NON-LEGUME AND LEGUME SPECIES

We found a low variability of δ^{15}N signal in the legume species across restoration treatments (signatures were generally around

Table 3 | ANOVA results (Type III Sum of Squares) for effects of topsoil removal (*r*), hay transfer (*h*), and their interaction effect ($r \times h$) on N concentration and δ^{15}N in plant leaves.

	Factor	d.f.	%N		δ^{15}N	
			F	*p*	*F*	*p*
(A) ALL SPECIES						
	r	1	65.025	<**0.001**	194.035	<**0.001**
	h	1	61.378	<**0.001**	17.477	<**0.001**
	$r \times h$	1	15.664	<**0.001**	0.018	0.892
(B) FUNCTIONAL IDENTITY						
non-	*r*	1	34.737	<**0.001**	783.733	<**0.001**
legumes	*h*	1	57.936	<**0.001**	77.090	<**0.001**
	$r \times h$	1	25.901	<**0.001**	0.110	0.740
legumes	*r*	1	57.228	<**0.001**	12.082	**0.001**
	h	1	29.693	<**0.001**	4.028	**0.047**
	$r \times h$	1	0.631	0.428	0.113	0.737
(C) SPECIES IDENTITY						
ant	*r*	1	16.379	<**0.001**	0.001	0.976
	h	1	8.646	**0.005**	3.320	0.076
	$r \times h$	1	0.434	0.514	2.746	0.105
dor	*r*	0				
	h	1	0.391	0.541	0.383	0.544
	$r \times h$	0				
hel	*r*	1	4.501	**0.037**	74.492	<**0.001**
	h	1	15.994	<**0.001**	32.447	<**0.001**
	$r \times h$	0				
lot	*r*	1	2.192	0.146	1.944	0.170
	h	1	0.800	0.376	2.974	0.092
	$r \times h$	0				
tri	only in $-r-h$	na				
gal	*r*	1	40.278	<**0.001**	203.595	<**0.001**
	h	1	49.685	<**0.001**	11.124	**0.001**
	$r \times h$	0				
roots	*r*	1	0.679	0.418	42.869	<**0.001**
	h	1	1.860	0.185	1.366	0.254
	$r \times h$	1	1.457	0.239	0.476	0.497

Results show (a) all data analyzed together, (b) data split into plant functional identity (legumes versus non-legumes) and then tested for restoration treatment effects and (c) data analyzed per species and tested for effects of restoration treatments. Significant effects are shown in bold. Please note that this is not a split plot design, but that we use the table to present separate analyses at different hierarchical levels.

Target species: ant = Anthyllis vulneraria, dor = Dorycnium germanicum, hel = Helianthemum nummularium and mesic species: lot = Lotus corniculatus, tri = Trifolium pratense (not tested because it only occurred in one treatment), gal = Galium mollugo, roots = unspecific combined root samples.

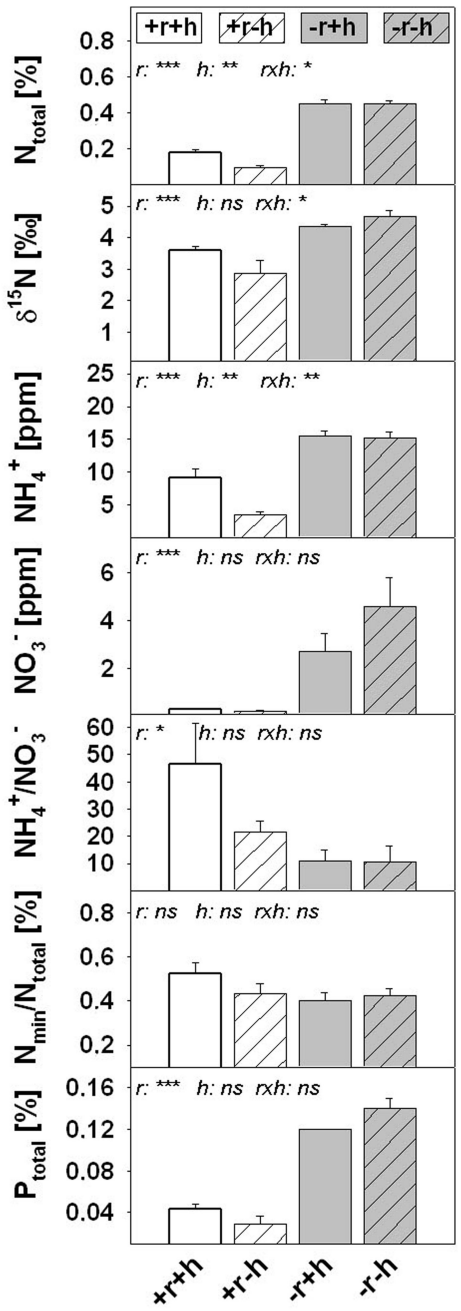

FIGURE 2 | Soil properties of the four restoration treatments in 2008 (see also Table 1A for comparison with data from earlier evaluations); parameters include N_{total} and P_{total} (concentrations [%] measured in bulk soil; fraction <2 mm) and mineral N forms [ammonium (NH_4^+), nitrate (NO_3^-) measured in soil solution). Values are means ± 1 SEM ($n = 8$ for *N* parameters, $n = 2$ for P_{total}). Restoration treatments: with topsoil removal and hay transfer ($+r+h$, white bars), topsoil removal and without hay transfer ($+r-h$, white striped bars), no topsoil removal and with hay transfer ($-r+h$, gray bars), no topsoil removal and without hay transfer ($-r-h$, gray striped bars). Significant effects of restoration treatments (r = topsoil removal, h = hay transfer, $r \times h$ = interaction between both factors) on soil properties are shown as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant; na, not available; *p*-values from ANOVA (Type III Sum of Squares).

−2‰, **Figures 3** and **4**). Values for the two non-legume species however, ranged overall from around −6 to +1‰ for *gal* and between around −4 and −10‰ for *hel*, the ectomycorrhizal species. In order to address our second aim of seeing whether δ^{15}N signals could be used to clearly separate legumes from non-legumes, we tested the whole dataset for effects of FI (legume or not) or effects of SI on leaf δ^{15}N and %N. We found that FI had

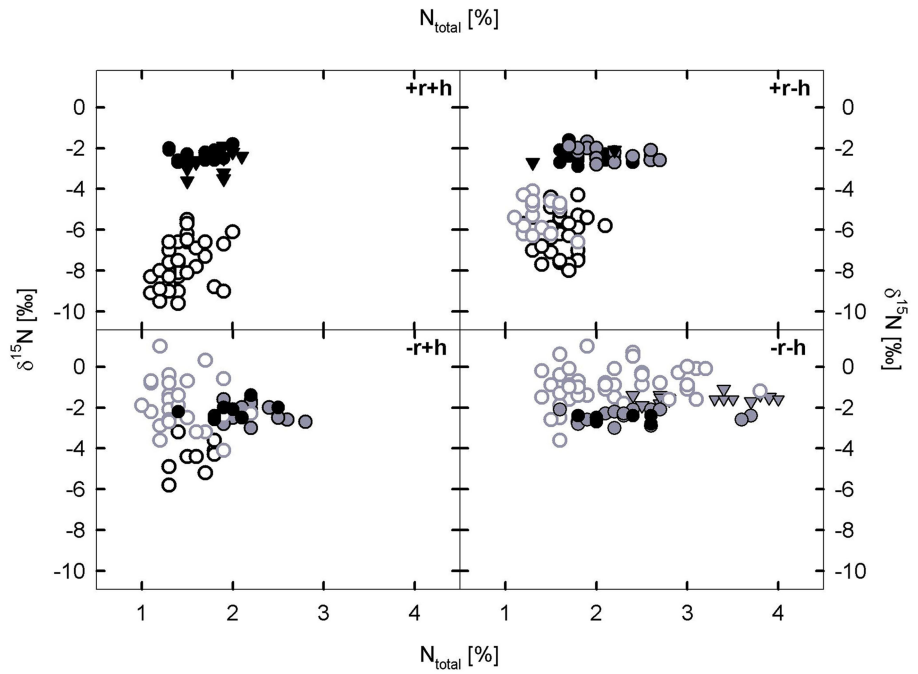

FIGURE 3 | The relationship between δ^{15}N values and N concentration [%] in leaves of six plant species across four restoration treatment. Restoration treatments are: with topsoil removal and hay transfer (+*r*+*h*), with topsoil removal and without hay transfer (+*r*−*h*), without topsoil removal and with hay transfer (−*r*+*h*), without topsoil removal and without hay transfer (−*r*−*h*). Closed symbols indicate legume species, open symbols non-legume species; black symbols indicate target species: *Anthyllis vulneraria* (●) and *Dorycnium germanicum* (▼), *Helianthemum nummularium* (black-open: ○) and gray symbols indicate mesic, non-target species: *Lotus corniculatus* (◉), *Trifolium pratense* (▼) and *Galium mollugo* (gray-open: ○). Every symbol in this figure represents one replicate per species and restoration treatment, mean values are given in **Figure 3**, neighborhood effects of legumes on non-legume species are shown in **Figure 4**.

a very significant effect on both leaf δ^{15}N ($p < 0.004$) and %N ($p < 0.0001$ for $\log_{10}$ transformed data). Effects of SI mirrored the results of FI in that significant differences were found between leaf δ^{15}N in legume species versus non-legume species ($p <= 0.05$).

When data were tested *within* each restoration treatment (data split into four subsets; see **Table 4**) for effects of FI or SI, both factors had a significant effect on foliar %N in topsoil removal sites, whereas there were more differentiated effects of the different factors in non-removal sites. FI almost always had a significant effect on foliar δ^{15}N (except in the +*r*+*h* treatment; $p = 0.267$; **Table 4**), whereas SI only sometimes affected δ^{15}N.

DETECTION OF EFFECT OF LEGUME IN NEIGHBORHOOD ON δ^{15}N OF NON-LEGUME SPECIES

δ^{15}N in legume species was generally around −2‰ (**Figures 2** and **3**) suggesting high levels of N_2 fixation (with little N derived from soil) such that legume species studied would in theory be able to provide a source of atmospherically fixed N_2 for non-legume neighbors and thus affect the δ^{15}N signal of neighbors (*sensu* Spehn et al., 2002; Temperton et al., 2007).

In the field, the abundance of legume species was much higher than expected across all treatments, however, such that collection of control plants of *hel* and *gal* unaffected by legume vicinity was difficult and was generally only possible at a distance of <30 cm to a legume. The non-legume control plants (not in vicinity of legume) seldom differed from plants growing as direct neighbor of a legume (see neighborhood, NH, effects in **Table 4** and **Figure 5**). Generally we found no legume neighbor identity effect except for *hel* growing in +*r*−*h*, where δ^{15}N was differently affected by growth next to *lot* versus *tri* ($p < 0.05$). Overall, however, the lack of clear separation of species neighborhoods did not allow us to adequately assess our third aim of testing whether the vicinity of a legume altered leaf δ^{15}N and %N.

DISCUSSION

DETECTION OF RESTORATION TREATMENT IN THE PLANT δ^{15}N SIGNAL

In our study, foliar δ^{15}N in both non-legumes *gal* and *hel* growing in topsoil removal sites were very δ^{15}N depleted and much more negative than δ^{15}N of legumes (**Figures 2** and **3**; compared with generally positive δ^{15}N signals in non-legumes in mesic grasslands; Spehn et al., 2002). In addition, we found clear differences in δ^{15}N signals in plants across the gradient of restoration effort (**Figures 3** and **4**), such that in combination with clear effects of topsoil removal on soil nutrients, this suggests that δ^{15}N signals in plants may be revealing interesting relationships between soil N dynamics and plant N use. A number of studies suggest that enriched δ^{15}N signals in plant may be linked to higher soil N availability or higher N transformation rates (Coetsee et al., 2011). Confirming this, Kahmen et al. (2008) combined the δ^{15}N NA method with enriched tracers in grasslands and found that low foliar δ^{15}N generally corresponded to low N availability in soil. For this reason we present **Figure 6** as an indication of how mean

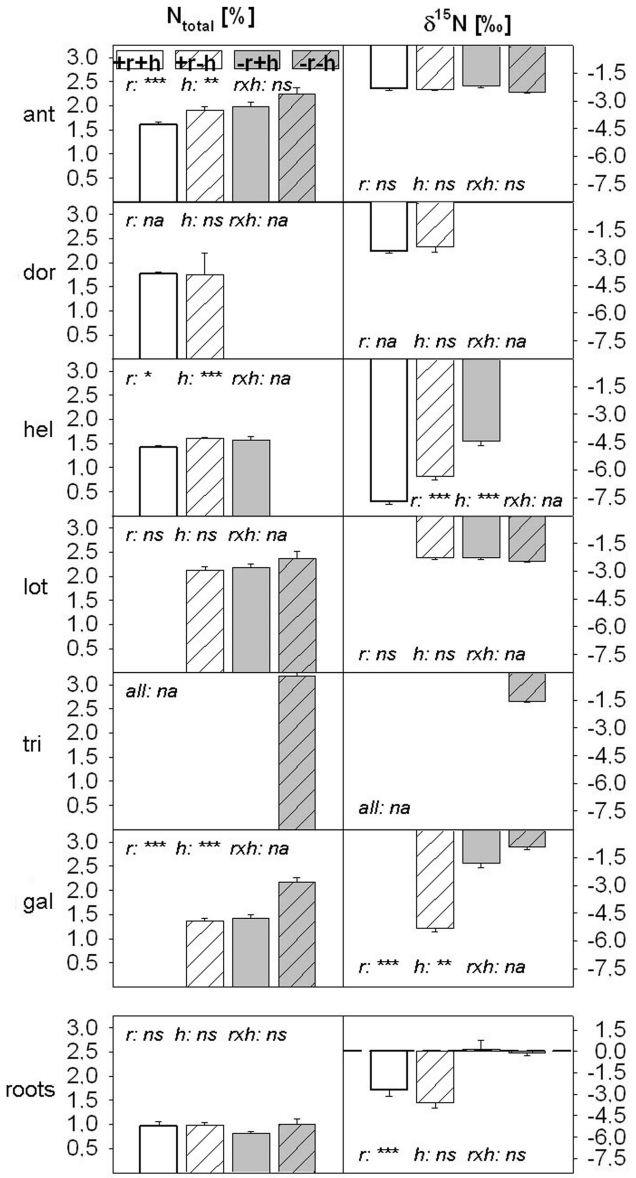

FIGURE 4 | N concentration [%] and δ^{15}N in plant leaves of all six species (three target calcareous grassland and three mesic species) and in root samples (species not identified) in the four restoration treatments. Values are means ± 1 SEM (plant: $n = 6 - 42$, except for *D. germanicum* in $+r-h$ treatment: $n = 2$; root: $n = 8$). Data for some species in some treatments are missing, as the species was not growing in that treatment (see **Table 2**). Restoration treatments: with topsoil removal and hay transfer ($+r+h$, white bars), with topsoil removal and without hay transfer ($+r-h$, white striped bars), without topsoil removal and with hay transfer ($-r+h$, gray bars), without topsoil removal and without hay transfer ($-r-h$, gray striped bars). Target species: ant = *Anthyllis vulneraria*, dor = *Dorycnium germanicum*, hel = *Helianthemum nummularium* (two legume and one non-legume species, respectively) and mesic species: lot = *Lotus corniculatus*, tri = *Trifolium pratense* and gal = *Galium mollugo* (two legume and one non-legume species, respectively). Significant effects of restoration treatments (r = topsoil removal, h = hay transfer, $r \times h$ = interaction between both factors) on plant *N* parameters are shown as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; ns, not significant; na, not available; *p*-values from ANOVA (Type III Sum of Squares, compare to **Table 3C**).

Table 4 | ANOVA (sequential Type I Sum of Squares) results for effects of functional identity (FI), species identity (SI), and effect of neighborhood (NH) on N concentration and δ^{15}N in plant leaves *in all species,* analyzed separately by restoration treatment [with (+) or without (−) topsoil removal (*r*) and hay transfer (*h*)].

	Factor	d.f.	%N		δ^{15}N	
			F	***p***	***F***	***p***
$-r-h$	FI	1	14.944	<**0.001**	52.241	<**0.001**
	SI	2	8.169	**0.001**	7.556	**0.001**
	NH	2	0.200	0.819	1.416	0.249
$-r+h$	FI	1	80.073	<**0.001**	1.257	0.267
	SI	2	2.542	0.088	27.872	<**0.001**
	NH	1	5.755	**0.020**	0.484	0.490
$+r-h$	FI	1	79.097	<**0.001**	465.050	<**0.001**
	SI	3	8.431	<**0.001**	6.559	**0.001**
	NH	4	4.745	**0.002**	2.334	0.064
$+r+h$	FI	1	37.154	<**0.001**	687.014	<**0.001**
	SI	1	5.650	**0.020**	0.954	0.332
	NH	2	8.225	**0.001**	2.497	0.090

*For detailed information on the effect of neighborhood on the two non-legume species, see **Figure 4**.*

δ^{15}N in leaves of the two non-legumes *hel* and *gal* ($\delta^{15}N_{non\text{-}legume}$) related to abiotic (see also **Figure 2**) aspects of the restoration sites. Due to low replication we could not perform robust correlation analysis and hence these relationships are provided as supporting data and pointers for future research needs. One can tentatively say that the higher the ratio of $NH_4^+ : NO_3^-$ was, the more negative was the $\delta^{15}N_{non\text{-}legume}$ (**Figure 6** last panel).

SEPARATING LEGUMES FROM NON-LEGUMES AND EFFECTS OF HAVING LEGUME NEIGHBORS ON PLANT δ^{15}N

Perhaps not surprisingly, we found that δ^{15}N in leaves of non-legumes differed significantly from that in legumes. Even if there were still sometimes significant differences in δ^{15}N *between* legume species, these differences were always largest between the legumes and the non-legumes (**Figures 3** and **4**). Although this may seem somewhat trivial, it does indicate that the legume species in the study were in all likelihood fixing atmospheric N_2 (which is used as a standard during isotope ratio mass spectrometry, hence producing values close to zero for N-fixing legumes when a sample is divided by standard ^{15}N). In addition, given the assumption that mycorrhizae may discriminate strongly against the heavier ^{15}N during nitrate uptake, we do not find clear evidence for this occurring in the legumes species studied here. Unfortunately, although the roots in general (not separable into species) were found to by mycorrhizal, we could not assess to what extent the legumes formed symbioses with mycorrhizae versus the non-legume species.

In our study, we could not come to any conclusions about legume neighborhood effects on the δ^{15}N of the two non-legume species studied, since the non-legume control plants (not in vicinity of legume) seldom differed in δ^{15}N from plants growing as direct neighbor of a legume. In addition, the relatively small

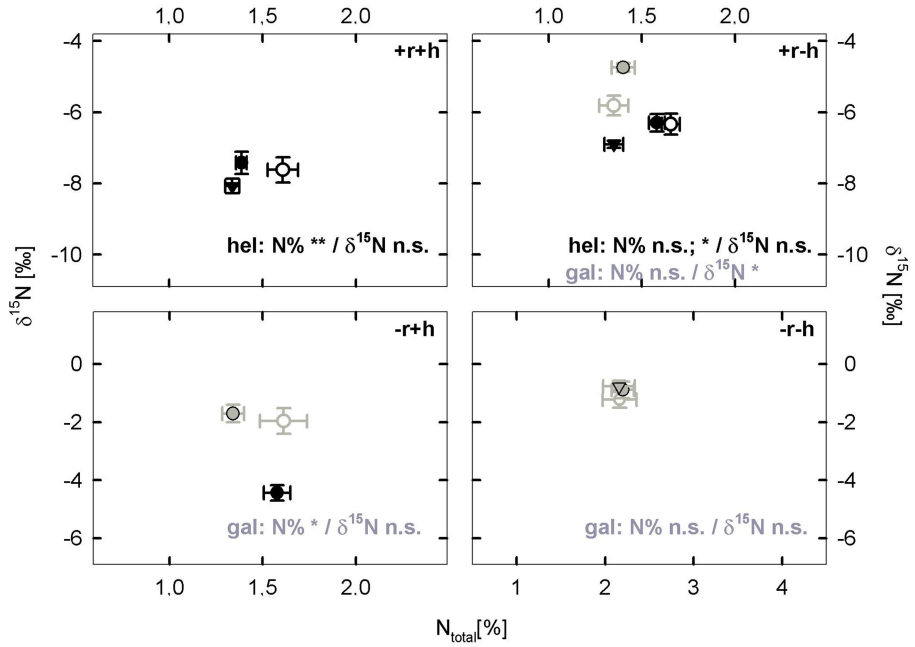

FIGURE 5 | Neighborhood legume effects on the two non-legume species (*hel*: *Helianthemum nummularium* and *gal*: *Galium mollugo*), showing the relationship between δ^{15}N and N concentration [%]. *Hel* (black type and symbols) was collected as a neighbor of the legumes *Anthyllis vulneraria* (●) or *Dorycnium germanicum* (▼) and as a control without legume neighbors (○). Similarly, *gal* (gray type and symbols) was collected as a neighbor of the legumes *Lotus corniculatus* (◉) or *Trifolium pratense* (▽) and as control without legume neighbors (○). Values are means ± 1 SEM. The number of samples per species (see **Table 2**) varied with species and treatment but in general $n = 6$–16 (only *hel* as neighbor of *D. germanicum* in $+r-h$ had $n = 2$). Significant effects of legume neighborhood on δ^{15}N and N concentration *within* each restoration treatment are shown as $^*p < 0.05$, $^{**}p < 0.01$; ns, not significant, *p*-values from LSD-tests following ANOVA (Type I Sum of Squares). Generally, there was no legume neighbor *identity* effect on δ^{15}N and %N, except for *hel* growing in $+r-h$, where %N was differentially affected by ID of the legume neighbor (hence two significance levels provided in graph).

differences in δ^{15}N between legumes and non-legumes *within* a restoration treatment in this study did not allow us to unequivocally show a facilitative effect of legume species on their neighbors in terms of improved N status. As such we cannot really answer our third question of to what extent the proximity of a legume or the identity of a legume neighbor affected leaf δ^{15}N. Interestingly, in other studies that were able to show a facilitative effect of legume neighborhood, foliar δ^{15}N of non-legume species was more positive than those of their legume neighbors (Spehn et al., 2002; Temperton et al., 2007; Gubsch et al., 2011), whereas in studies where δ^{15}N of non-legume species was lower (more depleted, negative) than those of their legume neighbor, no clear facilitation was evident (van der Heijden et al., 2006; Beyschlag et al., 2009 and this study). Thus, while it is possible, that the δ^{15}N of plant-available soil N in our study was close to that of legume δ^{15}N such that facilitation may have taken place, but was not visible in the data, our data on soil and plant N and δ^{15}N do point more toward a possible change in $\delta^{15}N_{\text{foliar non-legume}}$ in relation to changing plant N source (ammonium, nitrate) across the restoration gradient.

OTHER POSSIBLE INFLUENCES ON THE δ^{15}N SIGNAL IN PLANTS: SOIL N DYNAMICS AND PLANT N UPTAKE, GRAZING, AND MYCORRHIZAL DISCRIMINATION AGAINST HEAVY 15N

A number of studies using the δ^{15}N NA method (alone or combined with enriched tracers), provide some key pointers which may help to interpret our results and how they may relate to soil nutrient status and plant N source. Decreases in foliar δ^{15}N have been related to decreasing N availability in soils, lower nitrification, and mineralization rates and thus an overall more closed N cycle. Kahmen et al. (2008) investigated grassland plant's $\Delta\delta^{15}$N (i.e., foliar δ^{15}N standardized by background $\delta^{15}N_{soil}$) in relation to N uptake preferences from the soil, and found decreasing $\Delta\delta^{15}$N values with increasing proportion of NH_4^+ uptake (i.e., higher NH_4^+ : NO_3^--ratios). Our results seem to confirm this trend: more negative foliar $\delta^{15}N_{\text{non-legume}}$ was linked to increasing NH_4^+ : NO_3^--ratios in the soil at the restoration sites. In our study, NH_4^+ was present across all treatments, but NO_3^- was present only in sites without topsoil removal (**Figure 2**).

Curtis et al. (2011) synthesized stable isotope study results on N saturation and N leaching in terrestrial and aquatic systems and concluded that N has maximum retention in carbon-rich ecosystems, versus maximum leaching of nitrate N from carbon-poor systems, exposed to elevated atmospheric N inputs. Our restoration system may have been affected by elevated atmospheric N inputs, and it certainly is a carbon-poor system with low nitrate concentrations in the soil (**Figure 2**). Martinelli et al. (1999) also found within the tropics that more depleted δ^{15}N was correlated with lower N availability. Austin et al. (2006) inhibited nitrification and found significantly lower δ^{15}N in dominant vegetation with the switch in N form from NO_3^- to NH_4^+ taken up by plants. Schulze et al. (1994) showed that differences

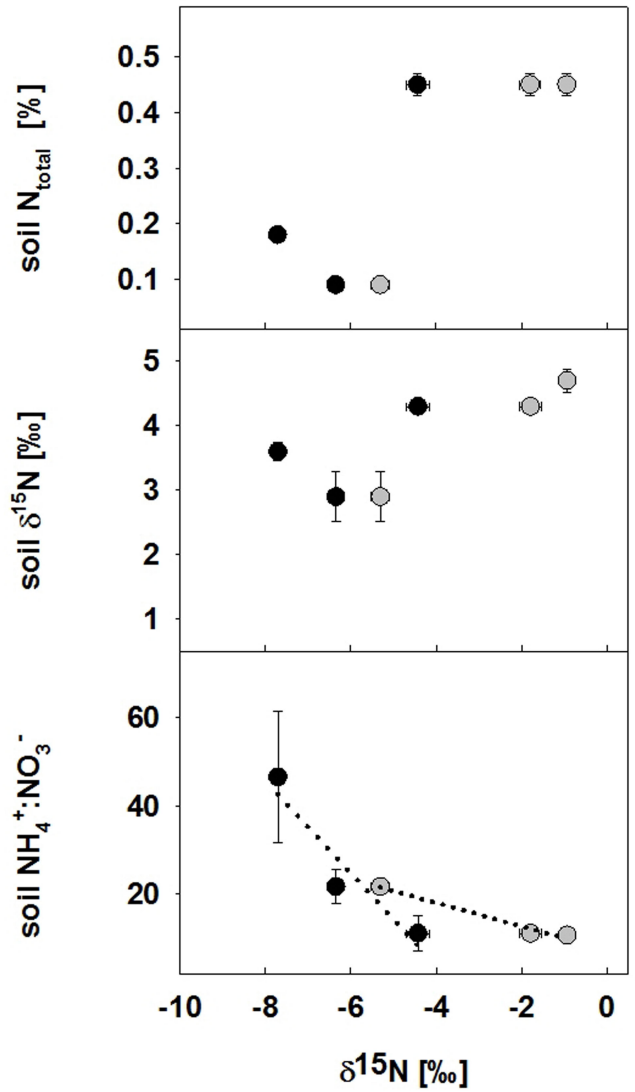

FIGURE 6 | Relationship between foliar δ^{15}N of the two non-legume species (hel and gal) and abiotic aspects of the four restoration treatments. Parameters include N concentration, $\delta^{15}N_{soil}$ values and NH_4^+ : NO_3^- ratios for bulk soil in all four restoration treatments in 2008. Tentative correlations are shown as dotted lines. Values are means ± 1 SEM and each symbol represents the δ^{15}N value of a species (without separation into different legume neighborhoods) in one of the four restoration treatments (note that hel was not present in the control treatment, and gal was not present in the topsoil and hay addition treatment); black symbols (•) represent hel (*Helianthemum nummularium),* gray symbols (•) gal (*Galium mollugo).*

in foliar δ^{15}N between different plant life forms disappeared with increasing N availability in the substrate. We also found a gradual convergence of δ^{15}N in non-legume and legume species when moving from the most restoration intensive sites (topsoil removal and hay transfer, $+r+h$) to control sites ($-r-h$; **Figures 3** and **4**). In addition to soil N dynamics, lower δ^{15}N in plants has also been linked to increasing environmental severity (by increasing elevation in the Alps Jacot et al., 2005; Huber et al., 2007) and to increasing species richness within plant communities (Gubsch et al., 2011). Jacot et al. (2005) showed an increase in the difference between δ^{15}N of legume species and non-legume species with increasing altitude which may reflect N cycling changes or other effects such as water availability and symbioses.

Grazing has been shown to affect the N cycle and N availability, with grazing found to both increase (Coetsee et al., 2011) as well as deplete (Golluscio et al., 2009) plant δ^{15}N. In our study, grazing played a very minor role compared to mowing, with sheep only grazing on one of the experimental blocks for a short time period. Any potential driving effects on plant δ^{15}N should have been picked up testing for block effects on δ^{15}N which were not significant.

A number of publications have addressed the potential for linking δ^{15}N signals to the level of openness of the N cycle (its "leakiness") in relation to the availability of N, with more N-rich systems predicted to have more open cycles than N-poor systems (Martinelli et al., 1999). Wetter and colder ecosystems seem to more efficient at retaining mineral N. Martinelli et al. (1999) predict that N losses in N-rich systems are more likely to discriminate against heavier ^{15}N, "because losses by fractionating pathways leave the remaining N within the system enriched." In our nutrient-poor, dry soils after topsoil removal N inputs and outputs were probably small compared to N cycling, and plants seemed to compete very effectively via help from mycorrhizae for the few available nutrients. It appears that the site of perhaps the largest discrimination against ^{15}N in the plant–fungi–soil system is by mycorrhizal fungi during plant N uptake (Hobbie and Hobbie, 2008). We hypothesize that the differences in δ^{15}N found between our two non-legume species may be attributable to their different mycorrhizal symbioses and life-histories. *Helianthemum* (*hel*), a woody shrub, forms a symbiosis with ectomycorrhizal fungi (ECM) and thus should have low δ^{15}N based on knowledge that δ^{15}N of non-fixing plants declines with longevity and woodiness of the species (Virginia and Delwiche, 1982), and that ECM normally discriminates against δ^{15}N more strongly than arbuscular mycorrhizal fungi (Michelsen et al., 1998).

CONCLUSION

We found that δ^{15}N values in plant species across four different restoration treatments in a calcareous grassland on ex-arable land were affected by the restoration treatments, particularly differences between topsoil removal and non-removal. Both foliar %N and δ^{15}N decreased (δ^{15}N became more negative) with increasing restoration effort (i.e., were most depleted in topsoil removal and hay addition) and this corresponded to a number of other NA ^{15}N studies that suggest more depleted plant δ^{15}N is associated with lower soil N availability and N transformations.

Legume species could be clearly separated from non-legume species using δ^{15}N signals indicating strong N_2 fixation, but we were not able to assess effects of legume neighborhood on δ^{15}N signals in non-legumes.

Very depleted (negative) plant δ^{15}N values were associated with low soil N but were also quite different between the two non-legume species, and this may have been a result of the different

mycorrhizal symbioses (VA versus ECM) these plant species enter into with fungi.

Our study provides evidence of how δ^{15}N in plants can inform us about plant-soil N dynamics, including successful soil N reduction in restoration settings but also the type of mycorrhizal symbioses plants enter into. Further research would endeavor to separate the link between soil N and water availability and δ^{15}N in plants from fractionation effects of mycorrhizal symbioses.

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