

# Long-term outcome of nitrogen immobilization to restore endemic sand grassland in Hungary

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## Summary

1. Soil nitrogen immobilization by carbon amendment is a management technique used for conservation purposes to increase the competitive ability of late-seral plant species over early-seral species based on their different tolerance of low soil nitrogen content.

2. We immobilized nitrogen over six growing seasons on three ex-arable fields with poor sandy soils in the Hungarian lowland in order to restore endemic sandy grassland. Sucrose and sawdust were applied at rates based on previous laboratory experiments using local soils. We tested the efficacy of long-term carbon amendment for lowering soil nitrogen availability and favouring late-seral native species over early-seral weed species.

3. Carbon amendments resulted in significant increase in soil microbial biomass C and reduced soil nitrogen availability after 2 years.

4. Total vegetation cover was reduced by reducing soil nitrogen availability, but total species richness was not impacted. Cover of early-seral species decreased, and species richness and cover of late-seral species increased irrespective of nitrogen immobilization. However, after 4–6 years reducing soil nitrogen availability hampered the spread of moss under vascular vegetation.

5. *Synthesis and applications.* This study supports the efficacy of carbon amendment as a tool to immobilize available soil nitrogen in the upper soil layers. However, the desired impact on vegetation was not fully achieved despite application over several years. Nitrogen immobilization was most relevant to bryophytes, lacking deep root systems, which may explain the responsiveness of this group to N limitation. The different impact of N availability on the complex of early-seral, late-seral vascular species and that of the bryophyte layer provides opportunity for directing state transformations in arid grasslands. Bryophyte cover can be suppressed through carbon amendments in order to enhance the germination and establishment of grassland species. The advantage of the method is that it opens bryophyte cover gradually without disturbing the soil surface, possibly avoiding the establishment of invasive species. However, further studies are required for deeper insight.

**Key-words:** biological soil crust, carbon amendment, drought, early-seral, ion exchange resin, late-seral, mosses, sawdust, sucrose

## Introduction

Habitats world-wide are subject to biotic homogenization, resulting in the replacement of native by non-native and specialist by generalist biota (McKinney & Lockwood 1999; Olden & Poff 2003; Olden *et al.* 2004; Devictor

*et al.* 2008; Ross *et al.* 2012). Global trade, biotic invasion and human activities such as travel accelerate the process. This homogenization combined with human disturbances largely contributes to global biodiversity loss. Currently, managers lack sufficient methods for reducing non-native vegetation while favouring native species. Disturbance to soil (i.e. previous cultivation) often results in the increase in available nitrogen (Eschen *et al.* 2007; Perry *et al.* 2010), thus favouring invasion by nitrophilic plant species

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(Pyšek & Richardson 2007). Often, nitrophilic non-native species become invasive and generate conservation problems (Balogh, Botta-Dukát & Dancza 2003). In contrast, late-seral species better tolerate low soil nutrient content, especially in nutrient-poor water-stressed ecosystems (Paschke, McLendon & Redente 2000). Observations of different competitive ability of native late-seral species over non-native early-seral species (mostly invasive plants) based on their different tolerance of low soil nutrient content (Morphan & Seastedt 1999; Craine 2005; Doll *et al.* 2011; Eldridge, Redente & Paschke 2012; Cleland, Larios & Suding 2013) has led to nitrogen immobilization through carbon amendment as a possible management technique. Carbon amendments increase soil microbial biomass (Zink & Allen 1998; Eschen *et al.* 2007), especially fungal biomass (Kardol *et al.* 2008), resulting in immobilization of available soil nitrogen in microbial biomass (Michelsen *et al.* 1999; Eschen *et al.* 2007; Kirkpatrick & Lubetkin 2011).

Several experiments have demonstrated immobilization of soil nitrogen with carbon amendments, however, the desired vegetation response is often not achieved (Corbin & D'Antonio 2004; Huddleston & Young 2005; James *et al.* 2010; Doll *et al.* 2011; Kirkpatrick & Lubetkin 2011). Reviews have been conducted on optimal conditions and application (Morphan & Seastedt 1999; Miller & Seastedt 2009; James *et al.* 2010; Perry *et al.* 2010), yet it is difficult to predict the efficiency of nitrogen immobilization due to site differences and influence of uncontrolled effects in the field (Perry *et al.* 2010; Desserud & Naeth 2013). The outcome of nitrogen immobilization differs if tested for seedling establishment and survival (Averett *et al.* 2004; Bleier & Jackson 2007; Biederman & Whisenant 2009; Brunson, Pyke & Perakis 2010; Desserud & Naeth 2013) vs. effects on established vegetation (Morphan & Seastedt 1999; Prober *et al.* 2005; Spiegelberger *et al.* 2009; Larchevêque *et al.* 2010; Mitchell & Bakker 2011). Studies generally focus on vascular species response; however, biological soil crusts might have important roles in certain habitat types (Langhans, Storm & Schwabe 2009; Su *et al.* 2009; Serpe *et al.* 2013). Soil crusts create special microenvironment with altered light, microclimate and soil characteristics, thus affecting seed dispersal, germination and survival of vascular plants (Jeschke & Kiehl 2008; Su *et al.* 2009). A dense moss layer may inhibit regeneration processes such as germination (Jeschke & Kiehl 2008) and seedling establishment in calcareous grasslands (Otsus & Zobel 2004). Restricted contact of bryophytes with the upper soil surface may lead to differential response to nitrogen immobilization compared with vascular plants. In plant communities where bryophytes reach high cover, their response to nitrogen immobilization treatments becomes more important.

In our study, the target community for restoration is the Pannonian endemic open sand grassland, protected at European level. The disturbance history of this low

productive grassland includes cultivation, abandonment and invasion by exotic N<sub>2</sub>-fixing black locust *Robinia pseudoacacia* L. These disturbances contribute to significant changes in the nitrogen balance of soils, indicating that N immobilization may represent a novel restoration approach. After disturbance multiple pathways of vegetation development are possible in this sand grassland (Bartha, Molnár & Fekete 2008; Choi *et al.* 2008). Bryophytes, as native invaders, can form crusts and dominate patches (with a cover of 40–80%) stalling community change to grassland (Bartha, Molnár & Fekete 2008). Knowledge on the mechanism is scarce; however, the influence of droughts is assumed.

Carbon amendments were applied over six growing seasons on three ex-arable fields with poor sandy soils in the Hungarian lowland, distributed along an elevation gradient. Sucrose and sawdust application rates were based on previous laboratory experiments with local soils (Török *et al.* 2000). The purpose of this experiment was to test: 1) efficacy of long-term carbon amendment for lowering soil nitrogen availability on poor sandy soils and 2) effects of nitrogen immobilization on old-field vegetation development, including bryophytes, relative to an endemic European grassland community.

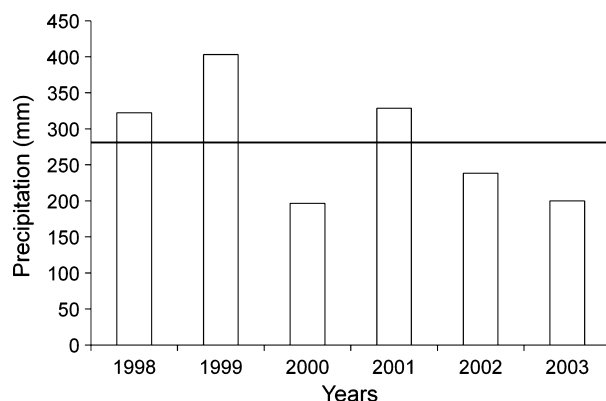
We hypothesized that soil N immobilization would alter competitive interactions between early-seral, late-seral vascular plants and bryophytes resulting in the regeneration of endemic open sand grassland.

## Materials and methods

### SITE DESCRIPTION

The study site was located in the Kiskunság inland sand dune region of Hungary, which has a diverse, mosaic of vegetative communities, largely dependent on water-table depth. Significant decline (92%) of endemic Pannonian sand grasslands occurred in the 20th century due to cultivation, afforestation and spontaneous invasion by exotic plants (Biró *et al.* 2013). In the 1990s, cultivation was ceased for economic reasons, leaving weedy grassland patches behind. Climate of the study area is temperate with sub-Mediterranean influence (Csécsécs *et al.* 2011). Mean annual temperature is 10.5 °C, and mean yearly precipitation is 500–550 mm. The weather conditions of the study period were diverse; in 1999, precipitation was high, while drought conditions prevailed in 2000 and 2003 (Pálfai 2007). Precipitation during the vegetation period (from March to August) is shown in Fig. 1. Calcareous sandy soils with humus content of less than 1% dominate the site (Kröel-Dulay & Kovács-Láng 2008).

Experimental fields were located at an abandoned homestead near Fülöpháza (N 46°52'; E 19°24'), within the Kiskunság National Park. The goal of ecological restoration in this area is an endemic open sandy grassland community (*Festucetum vaginatae* Rapaics ex Soó 1929) that occurred before cultivation. Three sites (Meadow, Depression and Hummock), c. 200 m apart, were selected for treatments, and a semi-natural grassland site located within 800 m of the treatment sites was selected as a reference. Cultivation ceased at the Meadow site in 1991 and at the



**Fig. 1.** Total growing season (March to August) precipitation during the study years measured close to the site (Kecskemét station). The 10-year average for this location is 281 mm, which is indicated by the horizontal line.

Depression and Hummock sites in 1995. Minor elevation differences occurred among sites Meadow, Depression, Hummock and reference grassland were at 105, 106, 107 and 108 m a.s.l., respectively. This elevation gradient corresponded to differences in humus content and productivity of soils, the lowest being the richest, and the highest the least productive (Török *et al.* 2000). Soil in the upper 0.2-m layer contains 0.14–0.36% organic C, 0.018–0.054% total N and pH of 7.9–8.1 on average in the study sites.

#### EXPERIMENTAL DESIGN AND TREATMENTS

Six treated and six control plots (10 × 10 m each) were placed randomly at each site within a 30 × 40 m area. Sucrose and sawdust were selected for nitrogen immobilization, as the most easily accessible and most effective carbon sources for restoration experiments. Sawdust was composed of oak sawdust and wood chips (with particle size of 0.01–3 cm), and sucrose was commercial beet sugar. Based on results of laboratory immobilization experiments (Szili-Kovács *et al.* 2000), 700 kg carbon ha<sup>-1</sup> yr<sup>-1</sup> was spread on the surface of plots by hand in the first year (1998). The lack of significant effect at two sites (Hummock and Meadow) was attributed to low application rate. The amount of added C was increased dependent on soil organic matter content in subsequent years. Hummock, Depression and Meadow received a total of 1408, 2011 and 2816 kg C ha<sup>-1</sup> yr<sup>-1</sup> on average, respectively. Rate of sawdust was 26.6% in the amendment. Sucrose was hand-broadcasted eight times, sawdust two times from April to October between 1999 and 2003.

#### SAMPLING

Soil cores were taken regularly at seven randomly selected points in each plot from the upper 20 cm during the growing seasons between September 1998 and May 2003. Soil moisture, temperature, available N (ammonium and nitrate), soil organic C, microbial biomass and total N were measured (for baseline data see Török *et al.* 2000). Concentrations of extractable NH<sub>4</sub>-N and NO<sub>3</sub>-N were measured using a Tecator autoanalyzer for analysis of 1 M KCl extractions of air-dried samples. Soil microbial biomass C was estimated by chloroform fumigation extraction according to Vance, Brookes & Jenkinson (1987). Soil N

availability was assessed by *in situ* ion exchanger resin (IER) bag method (Binkley & Matson 1983): 10 g mixed-bed IER (MB-3, Merck®, Darmstadt, Germany) was sewed into nylon mesh bags and buried into soil at a depth of 5–8 cm in the centre of each plot in two repetitions and changed before sucrose amendments every 6 weeks during the growing season (four times per year) between 2001 and 2003. Collected IER bags were dried and cleaned in the laboratory then extracted with 1 M KCl solution, and NH<sub>4</sub>-N and NO<sub>3</sub>-N were measured by steam distillation.

Vegetation sampling was carried out in three 2 × 2 m permanent quadrats in each plot, resulting in 18 control and 18 treatment samples per site. The reference grassland was sampled by 18 permanent 2 × 2 m quadrats placed randomly at the site. Cover of vascular plant species and moss was estimated by eye two times per year: in late May and early September between 1998 and 2003. Mosses were not identified to species level; the dominant species in the study sites was *Tortula ruralis* (Hedw.) Gaertn. *et al.* (Lhotsky *et al.* 2008).

#### DATA ANALYSES

Microbial biomass C, available NH<sub>4</sub>-N and NO<sub>3</sub>-N, and soil water content were analysed at each site separately using repeated-measures ANOVA with treatment as fixed factor for the period 1999–2003 (SPSS 9.0 provided by SPSS Inc., Chicago, IL, USA). May and September samples were analysed to see seasonal effect. Resin-derived-N data were summed for each year between 2001 and 2003, and these cumulative values were evaluated. Data for soil moisture content were natural logarithm-transformed to meet the criteria for ANOVA. All other variables did not require any transformation according to normality and homogeneity of variance tests. Statistical significance was assigned at  $\alpha = 0.05$ .

To visualize secondary succession in terms of vascular species composition, principal coordinate analysis (PCoA) was implemented with Bray–Curtis dissimilarity index and logarithm transformation of cover data (Podani 2001). Besides vascular plants, mosses were also included in the analysis as a single variable. We used data from all plots at the restoration and reference sites between 1998 and 2003. Summer and fall data were combined using maximum cover values of each species from the two sampling periods within each year. Successional trajectories show coordinates of samples averaged for each site and treatment per year. We assessed the temporal effects of carbon addition on species richness and cover of early-seral (i.e. weeds and non-native) and late-seral vascular species groups based on the classification of Simon (2000) and Csecserits *et al.* (2011) (Appendix S1, Supporting information).

Linear mixed effect (lme) models were used to investigate effects of treatment on vegetation data in the R statistical environment ver. 2.15.2 (R Development Core Team 2010). Treatment and year were treated as fixed factors, while 2 × 2 m quadrats were used as nested random effect within 10 × 10 m plots. Each site was analysed separately. Cover data were analysed with nlme::lme function (Pinheiro *et al.* 2013) with logit transformation to fulfil assumptions of normality and homoscedasticity (Warton & Hui 2010). Species richness data were analysed using lme4::lmer function with Poisson error distribution and identity link function (Bates, Maechler & Bolker 2012). Model residuals were checked for heterogeneity of variances and normality by visual assessment of diagnostic plots. In case of significant factor effects, Tukey's HSD tests were implemented for *post*

hoc pairwise comparisons using multcomp::glht (Hothorn, Bretz & Westfall 2008). Means and standard errors were calculated using non-transformed data. We conducted multiple significance tests; therefore, we controlled for false discovery rate by the

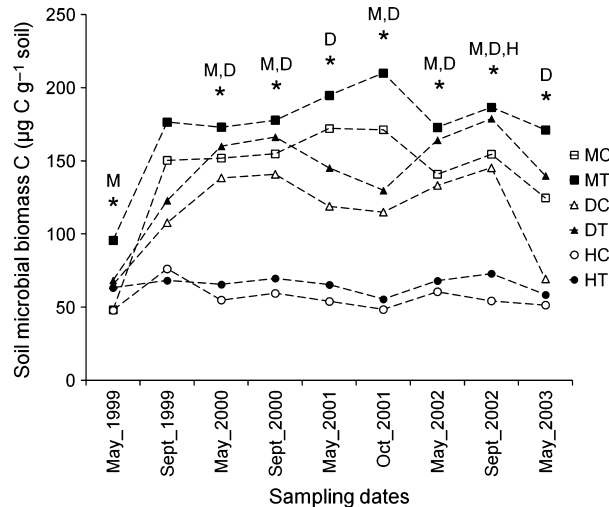
method of Benjamini and Hochberg (1995). Adjusted *P*-values are reported.

## Results

### SOIL PROPERTIES

Carbon treatment resulted in a significant increase in microbial biomass C at soil depth 0–20 cm during most sampling occasions between 1999 and 2003 for Depression and Meadow sites, and once for the Hummock site, where microbial biomass was the lowest (Fig. 2). Carbon treatment had a significant effect on soil microbial biomass C at all sites (Table 1). Soil mineral N (as the sum of ammonium-N and nitrate-N) was significantly decreased in C-treated plots at Depression and Meadow sites, mainly due to a reduction in nitrate-N. Soil moisture was significantly increased by treatment at sites Hummock and Meadow. Season effect (difference between May and September samples) was significant for all the variables except for microbial biomass C at Hummock site.

Ion exchange resin bags adsorbed significantly more soil nitrate at 5–8 cm depth in control plots than in C-treated plots at all sites over the years (Fig. 3). The greatest influence of treatment was detected at Meadow site, the lowest at Hummock site. There was little difference among sites



**Fig. 2.** Mean soil microbial biomass C at three study sites (Hummock, H, Depression, D and Meadow, M) in control (C) and carbon-treated plots (T) between 1999 and 2003. Significant differences ( $\alpha = 0.05$ ) between control and treatment plots within sites are shown by asterisks.

**Table 1.** Results of repeated-measures ANOVA of soil microbial biomass and soil parameters in control and carbon-treated plots based on soil samplings in May and September between 1999 and 2003

		Site H		Site D		Site M	
	d.f.	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Soil microbial biomass C							
Treatment	1	8.43	0.003	43.38	<0.001	39.09	<0.001
Year	4	0.99	0.415	40.20	<0.001	30.94	<0.001
Season	5	2.04	0.064	14.82	<0.001	22.12	<0.001
Treatment × Year	4	0.39	0.862	4.88	<0.001	4.10	0.001
Soil ammonium-N							
Treatment	1	0.19	0.751	2.51	0.099	3.49	0.057
Year	4	23.69	<0.001	38.97	<0.001	19.39	<0.001
Season	5	20.64	<0.001	41.20	<0.001	18.95	<0.001
Treatment × Year	4	1.26	0.275	1.45	0.195	1.74	0.129
Soil nitrate-N							
Treatment	1	1.11	0.333	7.85	<0.001	12.09	<0.001
Year	4	4.11	<0.001	5.70	<0.001	6.82	<0.001
Season	5	3.36	<0.001	2.91	0.001	8.27	<0.001
Treatment × Year	4	1.04	0.405	2.48	0.009	1.02	0.411
Soil mineral N							
Treatment	1	1.11	0.292	15.01	<0.001	16.32	<0.001
Year	4	9.89	<0.001	14.50	<0.001	12.57	<0.001
Season	5	15.45	<0.001	25.85	<0.001	25.76	<0.001
Treatment × Year	4	1.33	0.243	2.30	0.050	1.19	0.307
Soil moisture							
Treatment	1	8.04	0.003	3.06	0.074	5.68	0.013
Year	4	102.80	<0.001	263.30	<0.001	188.40	<0.001
Season	5	14.21	<0.001	29.81	<0.001	18.85	<0.001
Treatment × Year	4	2.51	0.025	0.78	0.566	2.27	0.050

Shown are degrees of freedom, *F*-ratios and *P*-values. Experimental sites Hummock, H, Depression, D and Meadow, M.



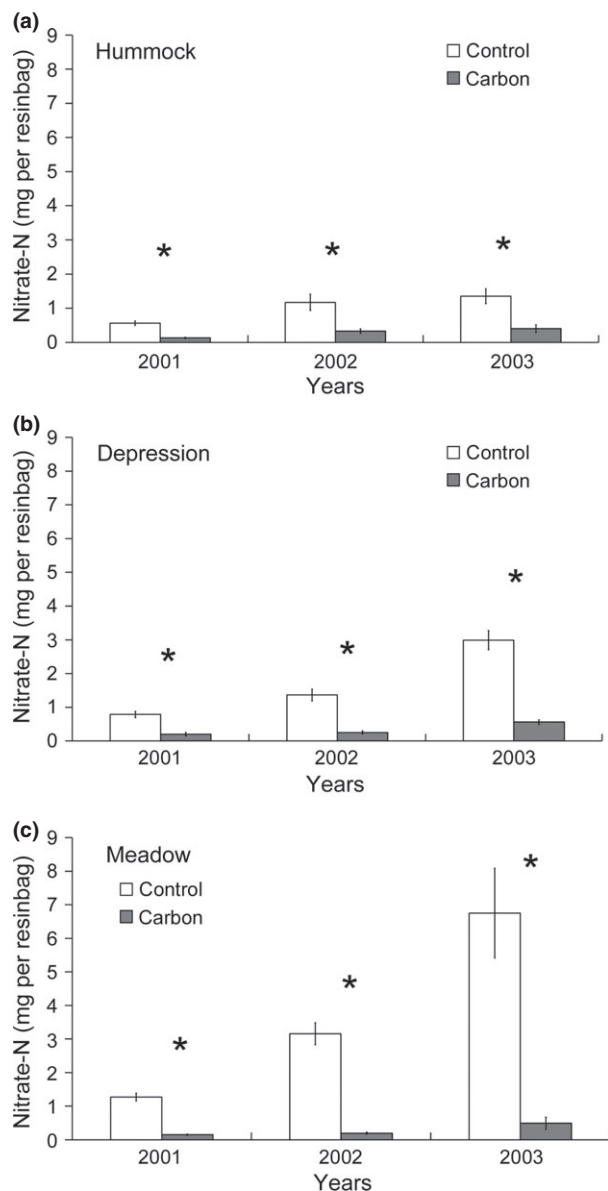
in cumulative nitrate-N in IER bags from carbon-amended plots. Rather, site differences were due to elevated and gradual increase in nitrate-N over the years in control plots.

#### VEGETATION

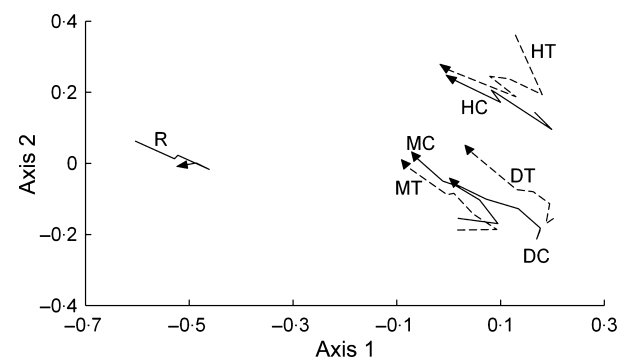
Ordination for plant species cover explained 16.6% of variability for the first axis and 9.7% for the second axis (Fig. 4). Reference grassland was clearly separated from old fields, and its trajectory demonstrated a relatively stable state during the 6 years. The direction of change

was similar for all three old-field sites: trajectories approached the reference site along the first axis. However, despite 8–12 years of secondary succession, including 6 years of carbon amendment, old fields were still very different from the reference grassland. Carbon amendment only had an effect at the Depression site, where trajectories gradually separated. Distance between the coordinate averages of control and treatment quadrats along the second axis increased to 0.091 by the last year at this site, while it reached only 0.014 and 0.032 at Hummock and Meadow sites, respectively. The least productive site (Hummock) differed from the other two along the second axis.

Carbon addition significantly decreased total cover at the Meadow site (Table 2). At the Hummock and Depression sites, significant treatment/year interaction was detected, total cover was significantly lower in carbon-amended plots only in 2001 ( $z = 3.846$ ,  $P < 0.01$  and  $z = 3.746$ ,  $P < 0.01$ , respectively). The cover of early-seral species decreased over time in treated plots in comparison with control, but the patchy structure of the vegetation contributed to high variability, so significant differences were only detected in 2001 at Depression site (Fig. 5a). At the Depression, early-seral cover changed significantly with year: after 2000, early-seral species became less abundant in treated plots. Cover of early-seral species was higher at both Depression and Meadow sites every year compared to Hummock and reference grassland, although it showed a gradual temporal decrease after 2000. Irrespective of carbon addition, we observed a temporal increase in average cover of late-seral species in each old field (Fig. 5b). At the reference grassland, cover of late-seral species decreased, the delayed die-off in response to the droughts of 2000 and 2003 being responsible. Carbon amendment had no significant effect on either total species richness or richness of late-seral and early-seral species at any sites (Table 2). Species richness of late-seral



**Fig. 3.** Average cumulative nitrate-N content ( $\pm$  SE) of ion exchanger resin (IER) bag samples. Four incubation periods within each year are summed. White bars represent control plots, and grey bars represent carbon-treated plots. Significant difference ( $\alpha = 0.05$ ) between control and carbon-treated samples is indicated by asterisk.



**Fig. 4.** Result of principal coordinate analysis based on plant cover data. Carbon-treated (T, dashed lines), untreated control (C, solid lines) and reference grassland (R) plot coordinates are averaged per year for each site (Hummock, H, Depression, D and Meadow, M); trajectories link these points in time between 1998 and 2003.

species increased significantly in time at each site, irrespective of treatment.

Significant decrease in moss cover was observed in relation to carbon amendment at each site. The effect of treatment developed gradually, it was significant from year 2001 at Hummock, from 2002 at Depression and in 2003 at Meadow (Fig. 6). By the fifth year of treatment, moss cover reached an average of 50% in control plots and was only between 2.8 and 26.4% in treated plots. This difference was quite apparent in the field

(Fig. 7). Moss cover increased over time at the reference grassland, except for the droughts in 2000 and 2003 (Fig. 6d).

## Discussion

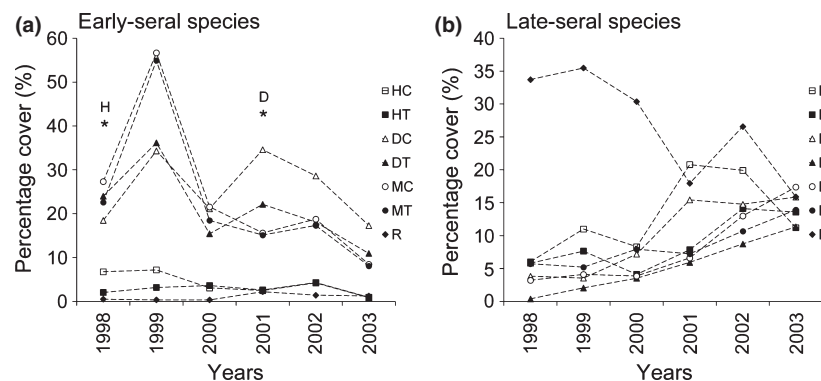
The present study supported the efficacy of carbon amendment as a tool to immobilize available soil nitrogen in upper soil layers; however, the desired impact on vegetation was not fully achieved within 6 years. Total

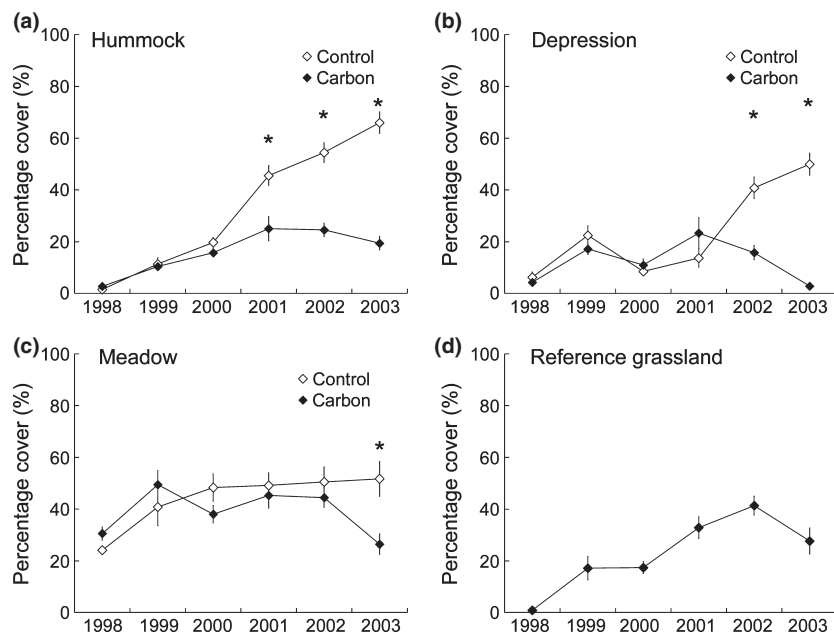
**Table 2.** Effect of carbon amendment on cover and species richness of different species groups based on linear mixed effect models between 1998 and 2003

	d.f.	Site H		Site D		Site M	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Total cover							
Treatment	1	2.60	0.241	8.7	0.032	13.66	0.010
Year	5	67.81	<0.001	49.6	<0.001	33.65	<0.001
Treatment × Year	5	5.46	<0.001	3.0	0.030	0.94	0.611
Cover of late-seral species							
Treatment	1	1.23	0.439	1.9	0.320	0.0	0.944
Year	5	10.57	<0.001	12.4	<0.001	15.7	<0.001
Treatment × Year	5	3.39	0.015	0.9	0.674	1.5	0.320
Cover of early-seral species							
Treatment	1	3	0.241	4.9	0.103	0.17	0.841
Year	5	8	<0.001	18.6	<0.001	72.32	<0.001
Treatment × Year	5	5	0.002	4.0	0.005	0.20	0.994
Cover of mosses							
Treatment	1	22.8	<0.001	10.1	0.023	1.14	0.454
Year	3	108.0	<0.001	19.3	<0.001	5.76	<0.001
Treatment × Year	3	31.9	<0.001	25.6	<0.001	3.80	0.007
Total species richness							
Treatment	1	0.03	0.933	2.20	0.241	2.51	0.210
Year	5	12.57	0.058	31.81	<0.001	9.36	0.182
Treatment × Year	5	3.12	0.834	2.37	0.896	0.70	0.999
Species richness of late-seral species							
Treatment	1	0.10	0.874	0.09	0.882	0.71	0.559
Year	5	37.99	<0.001	87.97	<0.001	20.28	0.003
Treatment × Year	5	2.72	0.874	3.78	0.732	0.07	0.999
Species richness of early-seral species							
Treatment	1	0.05	0.915	0.40	0.679	3.02	0.162
Year	5	29.92	<0.001	8.09	0.250	4.95	0.578
Treatment × Year	5	5.16	0.559	6.27	0.432	1.35	0.976

Shown are degrees of freedom, *F*-ratios and *P*-values. Experimental sites Hummock, H, Depression, D and Meadow, M.

**Fig. 5.** Average total cover ( $\pm$ SE) of early-seral (a) and late-seral species (b) in control (C) and carbon-treated (T) plots at experimental sites (Hummock, H, Depression, D, Meadow, M) and a reference grassland (R) between 1998 and 2003. Significant differences ( $\alpha = 0.05$ ) between control and treatment plots within sites are shown by asterisks.





**Fig. 6.** Average percentage cover ( $\pm$ SE) of moss at the three sites (a-c) in control and carbon-treated plots, and at the reference grassland (d) during 6 years of study. Significant differences ( $\alpha = 0.05$ ) between control and carbon-treated plots are shown by asterisks.



**Fig. 7.** Control (green) and carbon-treated plots at Hummock in April 2002.

vegetation cover decreased with treatment, but the expected increase in late-seral species and decrease in early-seral species was observed irrespective of treatment. Despite the promising changes, the composition of old-field vegetation remained different from the reference grassland. Nitrogen immobilization was most relevant to bryophytes, without deep root systems, which may explain the responsiveness of this group to N limitation.

Induced soil N immobilization by carbon amendment was significant in the upper 20 cm at two sites (Depression and Meadow), similar to other experiments (Eschen *et al.* 2007; Brunson, Pyke & Perakis 2010; Doll *et al.* 2011; Cleland, Larios & Suding 2013). The low level of soil nitrate-N and ammonium-N, close to detectable levels at site Hummock (soil mineral N around  $4 \mu\text{g N g}^{-1}$ , Szili-Kovács *et al.* 2011), may be the reason for lack of significant N immobilization in treated soils; however, the

increase in microbial biomass C was significant even at this site. Cumulative measures by IER bags provide better estimates of nitrogen availability (Miller & Seastedt 2009; Rowe, Brown & Paschke 2009). IER bag monitoring provided evidence of significant decrease in nitrate availability as a result of carbon amendment at all sites. As for the ammonium component of available N, treatment effect was less consistent (data not shown) because of ammonia volatilization in calcareous soils (Fenn & Kissel 1973). Total cumulative soil nitrate increased gradually from 2001 to 2003 in control plots as succession proceeded. This development was stalled by carbon amendment. Biological soil crusts contribute to the nutrient content of soil (Langhans, Storm & Schwabe 2009). In our case at two of the sites (Hummock and Depression), this could explain the parallel increase in moss cover and that of increasing soil nitrate content in control plots.

We found that nitrogen immobilization influenced vascular vegetation by lowering total cover, while total species richness was not influenced. We hypothesized that soil N immobilization would alter competitive interactions between early-seral and late-seral vascular plants; however, changes in abundance and species richness were irrespective of treatments. The difference between treated and control vegetation largely depended on year, which can be attributed to drought events. Despite the tolerance of native species to drought, massive local extinctions were observed in the region as a result of the unusually dry years of 2000 and 2003 (Rédei *et al.* 2008). This together with extremely low water-holding capacity of sandy soils may be a reason for weak treatment effect (Blumenthal 2009). In such systems, the effect of carbon amendment is dependent on the availability of species taking advantage of short nutrient pulses with precipitation events

(Larchevêque *et al.* 2010; Kirkpatrick & Lubetkin 2011). Influencing established vegetation through manipulation of soil nitrogen is difficult. The sequence of seed arrival and seedling emergence determines species priority, thus fundamentally influencing species composition (James *et al.* 2010). Because of the deep rooting system of established vegetation (Doll *et al.* 2011), impact of nitrogen immobilization is less pronounced on established vegetation and is often restricted to general biomass decrease and not species turnover (Morphan & Seastedt 1999; Gendron & Wilson 2007; Blumenthal 2009). The low response of early-seral species in our case might be attributed to the fact that this group included perennials with deeper root system (like common milkweed *Asclepias syriaca* L.) that might not react to N immobilization (Spiegelberger *et al.* 2009).

The impact of carbon amendment on moss was an unexpected outcome of our treatment. Carbon amendment had a negative effect on moss cover from the fourth year on even at the lowest amounts applied. Vascular species strive to develop longer roots against the lack of water in sandy soil, whereas bryophytes benefit from dew and minor precipitation events. Therefore, mosses in contact with the surface are likely to be more directly affected by nitrogen immobilization in upper soil layers. The decrease in mosses that we observed with carbon amendment may also be the result of direct osmotic shock caused by sucrose on moss leaves or increased osmotic potential of soils (Kirkpatrick & Lubetkin 2011). It is assumed that young mosses are even more sensitive to physiological stress than mature mosses, so establishment and growth of mosses may have been prevented in treated plots. Sawdust cover, like litter, can reduce light and induce dieback of mosses (Serpe *et al.* 2013). In our experiment, it is unlikely that light was a limiting factor because of the delayed effect of application for 3, 4 or 5 years. The discovery that C addition negatively impacts moss cover could have implications for vegetation manipulation.

Patches of mosses and lichens (called 'black spot' due to the dark colour of dry mosses) represent a state in grassland ecosystems (Choi *et al.* 2008) that can stall succession (Bartha, Molnár & Fekete 2008). Moss cover can enhance germination of certain species (Su *et al.* 2009), but biological soil crust has been shown to inhibit germination of sand grassland perennial species in ex situ experiments (Langhans, Storm & Schwabe 2009). Furthermore, Lhotsky, Kovács-Láng & Veres (2008) found higher seedling mortality of the dominant, late-seral grass *Festuca vaginata* within cryptogam crust compared to bare soil in open sand grassland community. Removal of cryptogam crust increases microsite availability for germination of vascular plants in sandy habitats (Jeschke & Kiehl 2008; Langhans, Storm & Schwabe 2010) and may provide a competitive advantage to vascular plants. We conclude that nitrogen immobilization can be a promising tool to guide secondary succession of open sand grassland based on the sensitivity of bryophytes. The advantage of

nitrogen immobilization is that it opens the moss layer gradually without disturbing the soil surface, possibly avoiding the establishment of invasive species. Conservation management implications include manipulations of the bryophyte layer in order to influence vascular species survival, especially when extreme drought events result mass dieback of dominant grasses. With climate change, drought events may become more abundant resulting in the relative dominance of mosses in sand grasslands (Rédei *et al.* 2008). Climate manipulation experiments combined with N immobilization could shed light on species-specific responses that could guide the conservation of endemic vascular species.

## Acknowledgements

We are grateful to several undergraduate students for their help with field work. This research was supported by the US-Hungarian Joint Fund JFNo. 639. The valuable comments of anonymous reviewers are acknowledged.

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Received 24 July 2013; accepted 6 January 2014

Handling Editor: Paul Kardol

## Supporting Information

Additional Supporting Information may be found in the online version of this article.

**Appendix S1.** Species assigned to late-seral and early-seral groups.