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N immobilization treatment revisited: a retarded and temporary effect unfolded in old field restoration

Running head: Long-term effect of N immobilization

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Abstract

Aim:

There is increasing recognition that plant-soil feedbacks drive species coexistence, therefore the belowground compartment should be better considered in ecological restoration. Addition of carbon is a restoration measure that relies on indirect plant-soil relationships by soil nitrogen immobilization in microbial biomass to manipulate competitive hierarchies between plant species. The aim of the present work was to evaluate the impact of six years of carbon amendment in old-field restoration in the long term, twenty years after the first application.

Location:

Old-fields and reference sand steppe on dry calcareous sandy soil in Fülöpháza, Kiskun LTER site (46°53′ N, 19°24′ E), Hungary, Pannonian biogeographic region, Europe.

Methods:

We applied carbon amendment in the form of sucrose and sawdust on three abandoned agricultural fields between 1998 and 2003. Vegetation was surveyed in 1998-2006 and re-sampled in 2008, 2010 and 2018 by permanent 2 m x 2 m coenological relevés for carbon amended, control and reference plots (n=144). We used PRC to describe vegetation development trajectories and linear mixed effects models to test changes in cover of vascular plants and cryptogams, nitrogen requirement groups and restoration species groups with time and treatment.

Results:

Carbon amendment resulted in lower soil nitrogen availability, lower cover of vascular plants and cryptogams compared to control, but these differences became visible only four-five years after the first application and disappeared three years after the cessation of treatments. Minor treatment effects on the cover of oligotrophic, mesotrophic and target species were found.

Conclusions:

Carbon amendment did not speed up the recovery of sand grasslands, however, the reduction of cover (vegetation and cryptogam) can be a window of opportunity for other species to colonize that can be used as a complement to other treatments. Long-term monitoring is especially important in evaluating restoration interventions that focus on indirect above ground-below ground linkages.

Keywords: aboveground-belowground linkages, carbon amendment, grassland restoration, invasive species, long-term, nitrogen requirement, old-field restoration, sand grassland, soil available nitrogen

Introduction

There is increasing recognition that plant-soil feedbacks drive species coexistence, invasiveness and ecosystem processes, and therefore the belowground compartment should be better considered in the context of ecological restoration (Aronson, 2020). Soil organisms that are directly connected to plants (i.e. pathogens, mutualists and herbivores) are easier to manipulate through restoration interventions (Kardol and Wardle, 2010). When considering indirect interactions, nitrogen cycling is the most promising for restoration interventions, since this cycling is almost exclusively regulated by biological processes and is mainly controlled by the soil microbial community (Stevens *et al.*, 2011).

Disturbed lands (either for natural or anthropogenic reasons) tend to have an elevated level of nitrogen in the soil, which alters mineralization and immobilization (Stevens *et al.*, 2011). Disturbed and nitrogen-rich systems tend to facilitate the growth of exotic species (Davis *et al.*, 2000). Management approaches such as removing invasive species chemically or mechanically are unlikely to limit reinvasion in many ecosystems, while soil legacy effects remain (Nsikani *et al.*, 2018). The remaining high nitrogen availability may favour plant re-invasion, while the reestablishment of native communities require a shift from the fast growing system to a slow, steady-release cycle (Davis *et al.*, 2000). This might be accomplished by lowering soil nitrogen availability that increases the competitive abilities of native perennials (Morghan and Seastedt, 1999; Lowe *et al.*, 2003; Corbin and D’Antonio, 2004; Uddin *et al.*, 2020). There are several management approaches to promote nitrogen immobilization, e.g. burning, grazing, topsoil removal or biomass removal, and carbon addition is one of the most reliable methods (Perry *et al.*, 2010).

Carbon amendment can stimulate carbon-limited microbes to reproduce and grow and in parallel accumulate soil available nitrogen in microbial biomass (Zink and Allen, 1998;

Török *et al.*, 2000). This process limits inorganic nitrogen for plant uptake (Török *et al.*, 2014). The induced nitrogen limitation would slow the growth of nitrophilous plant species, particularly annuals or early successional species (Paschke *et al.*, 2000). Since fast growing exotic species often dominate nitrogen rich habitats and typically benefit from increased nitrogen levels when compared to native perennials, carbon amendment may be particularly likely to control their spread (Eschen *et al.*, 2007; Perry *et al.*, 2010).

The impact of carbon amendment on ecological restoration and particularly invasive species is contradictory. That carbon amendment reduces invasive species and favours target species was confirmed by several studies, however, different results are also known from the literature (Perry *et al.*, 2010). The results depend partly on the type and rate of carbon sources used, and partly on the site properties, like developmental phase, species composition or climate and soil properties. For instance, quickly and slowly decomposing carbon sources can have a different impact (e.g. Eschen *et al.*, 2007), and the response of vegetation in the establishment phase is different from that of already established vegetation (Török *et al.*, 2014). The results also depend on the length of application and the length of monitoring, since some time is required until the indirect impact of microbial nitrogen immobilization is expressed, and microbial immobilization is known to be only temporary, as nitrogen is released via microbial respiration or the decay of microbial biomass (Perry *et al.*, 2010).

The present work aimed to evaluate the impact of six years of carbon amendment in old-field restoration in the long term, twenty years after the first application. The target of restoration was the low productive Pannonic sand steppe (Natura 2000 priority habitat 6260). The basic concept of the research was that the temporarily increased competitive advantage of native grassland species over nitrophilous and invasive species induced by carbon amendment can lead to long-term restoration success if desired species can establish during the application phase (cf. Perry *et al.*, 2010). Based on this, we have tested the following hypotheses: 1. Carbon amendment decreases soil available nitrogen during the application period. 2. Carbon amendment decreases the cover of vascular plants (as a result of lower productivity) and cryptogam species (direct impact of sucrose) on the long-term (twenty years after the first application). 3. As a result of induced temporary competitive advantage, carbon amendment increases the cover of target species and decreases the cover of nitrophilous and invasive species in the long term. 4. Carbon amendment accelerates vegetation development towards reference grassland.

Materials and methods

Site description

The experiment was carried out in Fülöpháza, Kiskun LTER site (46°53′ N, 19°24′ E), Hungary, Pannonian biogeographic region, Europe (Fig. 1.). The climate is warm temperate with a mean annual temperature of 10.5 °C and yearly precipitation ranging from 500 to 550 mm, which reaches maximum levels in May and November. Wind-formed sand dunes provide the base of the typical low humus (less than 1%) and calcareous sandy soils (Kovács-Láng *et al.*, 2008). The vegetation composition of the region is strongly dependent on soil water availability. The potential vegetation is forest-steppe with a mosaic of open oak forests, juniper–poplar woodlands and dry sand grasslands (Erdős *et al.*, 2018). The actual vegetation is a mosaic of agricultural fields, forest plantations, old-fields, and remnants of primary sand grasslands and wetlands (Biró *et al.*, 2013).

Three abandoned agricultural sites were chosen on sandy substrate for the carbon amendment experiment in 1998. The sites belonged to the same farm that was close to the strictly protected Fülöpháza sand dune area and was surrounded by black locust stands and abandoned arable fields. The three sites were approximately 200 m apart, arranged along an elevation and productivity gradient: Meadow site being at the lowest altitude (105 m a.s.l.) and relatively the most productive (0.44 soil organic C%, 6.1 mg kg^{-1} available nitrogen, NH_4+NO_3-N), Depression intermediate (106 m a.s.l., 0.26 soil organic C%, 6.0 NH_4+NO_3-N mg kg^{-1}) and Hummock at the highest elevation (107 m a.s.l.) and being the least productive (0.12 soil organic C%, 4.6 NH_4+NO_3-N mg kg^{-1}). The sites were abandoned between 1991 and 1995. The exact crops that had been planted are unknown, but typical crops are rye and maize in the region. The estimated cover of vascular species at the beginning of the experiment was 39, 42, and 8% for Meadow, Depression and Hummock, respectively.

Two reference areas were also selected for comparison in the nearby remnants of primary sand grasslands. These were located at the highest elevation (107 m a.s.l.). Most of the studied abiotic features were within the range of that of the studied old-fields, except for plant available nitrogen (1.5-1.7 NH_4+NO_3-N mg kg^{-1}). Further details are presented in Török *et al.* (2000; 2014).

Experimental design

Restoration trials started in 1998. A 30 m x 40 m experimental block was established at each site that was divided into 12 experimental plots of 10 m x10 m. Of these six were randomly assigned for carbon amendment (further referred to as C-amended) and six remained untreated control. We used a combination of an easy available (commercial beet sugar) and a slowly degrading carbon source (a mixture of sawdust and wood chips of 0.01-3.00 cm particle size obtained from oak (*Quercus* spp.) to induce microbial nitrogen immobilization. The initial application rate (700 kg carbon ha^{-1} year^{-1}) was based on previous laboratory studies using the soil of the sites (Török *et al.*, 2000). Sucrose was applied four times during the growing season with a total amount of 1300 kg ha^{-1} year^{-1}, and sawdust (300 kg ha^{-1} year^{-1}) was added to the soil only once in 1998. In later years (1999-2003) the application rate was increased according to site productivity to 2816, 2011 and 1408 C kg ha^{-1} year^{-1} for Meadow, Depression and Hummock, respectively (Török *et al.*, 2014). Sucrose was applied eight times, sawdust twice from April to October 1999-2003. The rate of sawdust was 26.6% of total carbon amendment. All carbon sources were hand-broadcasted on the surface without disturbing the soil or vegetation.

Monitoring

To estimate soil nitrogen availability, soil cores were taken from the corners and the centre of each 10 m x 10 m experimental plot from the upper 10 cm of the soil in the May or June vegetation peak between 1998-2004, at the end of April in 2005, and in June 2019. Extractable NH_4-N and NO_3-N concentrations of air-dried samples were measured using 1 M KCl extractions by a Tecator autoanalyzer. We used total available nitrogen, the sum of NH_4-N and NO_3-N for further analysis.

Three 2 m x 2 m permanent quadrats were selected for vegetation monitoring in each plot, resulting in 18 C-amended and 18 control samples per site. The two reference areas were also monitored by 18 randomly placed permanent quadrats of 2 m x 2 m each. Percent cover was visually estimated for each plant species together with the total cover of vascular plants and cryptogams in late May and early September between 1998-2006 and in 2008, 2010 and 2018. Cover data were pooled based on the yearly maximum of the two samplings per species per plot. Nomenclature follows Király (2009).

Data analyses

To evaluate vegetation development over time, the principal response curve method (PRC , van den Brink and ter Braak 1999) was implemented per each site separately using the package ‘vegan’ (Oksanen *et al.*, 2018). The untreated control was used as zeroline, and the “treatments” were the C-amended plots and the reference grassland to be reached as target. The time factor included the twelve monitoring years. We used the cover data of all 126 species identified during the experiment without standardization or data transformation as response variable. The first axis of each PRC was inspected with randomization tests using the reduced model and 999 permutations. We selected the first axis of PRC (PRC1) to be displayed with all species with a total sum of cover higher than 85 % for all plots and monitoring years per site. For more information on species contribution see Supplementary Information Appendix S3.

We have selected four indicators to describe vegetation development: 1. total cover of vascular plants, 2. total cover of cryptogams, 3. nitrogen requirement of species, and 4. role of species in restoration. We used Borhidi’s (1995) nitrogen supply categories summarized in Horváth *et al.* (1995) to detect vegetation response to nitrogen manipulation by carbon amendment. The original nine categories were merged into three nitrogen requirement groups: species of oligotrophic habitats (NB 1-3, further referred to as oligotrophic species); species of mesotrophic habitats (NB 4-6, further referred to as mesotrophic species); and species of hypertrophic habitats (NB 7-9, further referred to as hypertrophic species). Species were categorized into three groups according to their role in restoration (restoration species groups). Target species were selected based on species fidelity to open or closed sand grasslands in the Kiskunság (Csecserits *et al.*, 2011). Unwanted neophyte species were defined by Balogh *et al.* (2004). The rest of the species were considered indigenous, but non-target. For the complete list of species and their categorization see Appendix S1.

We used separate linear mixed effects models (lme) to investigate the differences in soil available nitrogen, cover of vascular plants, cover of cryptogams, and relative cover of N requirement categories (oligotrophic, mesotrophic, hypertrophic) and restoration species groups (target, non-target, neophyte) among the treatments using the nlme package. All statistical analyses were performed using R v 3.5.1 (R Core Team, 2018). Means and SE reported in figures and in the text are based on untransformed data.

Treatment and year were treated as fixed categorical explanatory variables, while plots within sites were included as nested random factor in the models. In case of total available nitrogen, treatment included two levels (C-amended and control) and year nine levels (1998-2005, 2019). For all vegetation indicators treatment included three levels (C-amended, control and reference), apart from neophytes that included only two levels (C-amended and control) given that this group had mostly zero cover in reference plots, which therefore were excluded from the statistical analyses. For the vegetation indicators year included twelve levels according to the monitoring years (1998-2003, 2004, 2006, 2008, 2010, and 2018). Total available nitrogen was log-transformed, cover of vascular plants and the relative cover of mesotrophic, hypertrophic, and non-target species were square-root transformed, the relative cover of target, oligotrophic, and neophyte species were arcsine transformed to meet assumptions of normality and homoscedasticity. We used varIdent variance structure for all indicators given the high variance of residuals within analysed groups. Finally, in the case of significant interactions between fixed factors, the ‘contrast’ package based on Wald test (Kuhn *et al.*, 2016) was used as post hoc pairwise test to detect significant differences across within year treatments. If no interactions were found, fixed factors were analyzed separately by Tukey HSD test, using the “multcomp” package (Hothorn *et al.*, 2008).

Results

Changes in soil nitrogen availability

There was a significant impact of year, treatment, and treatment and year interaction (all $p<0.01$) when analysing total available nitrogen (Table 1). Mean total available nitrogen values were below 7 mg kg^{-1} soil, except for the last measurement in 2019 when it was above 15 mg kg^{-1} soil. Carbon amendment resulted in significantly lower total available nitrogen compared to control in 1998, 2000, 2001 and 2003 (Fig. 2.).

Vegetation development over time

The first Principal Response Curve (Meadow: PRC 1 = 15 %; F = 169.19, p = 0.001; Depression: PRC 1 = 13 %; F = 149.8, p = 0.001 Hummock: PRC 1 = 20 %; F = 239.8, p = 0.001) indicated similar community composition for the control and the C-amended plots that both remained different from the reference grassland during the investigated period, although the difference decreased at Depression and Hummock site over time (Fig. 3.). The reference grassland had high abundance of *Euphorbia segueriana*, *Festuca vaginata* and *Fumana procumbens* that negatively correlated with the canonical coefficients, plus *Secale sylvestre* and *Stipa borysthenica* that have positively correlated with PRC1 in some sites (Appendix S2). Species that positively correlated with the canonical coefficients and represented the vegetation of the experimental sites included generalist grasses (*Bromus tectorum*, *Cynodon dactylon*, *Poa angustifolia*, *Secale sylvestre*) and forbs (*Ambrosia artemisiifolia*, *Artemisia campestris*, *Crepis rhoeadifolia, Gypsophila paniculata, Medicago minima*). Besides the basic similarities, there were important differences between sites. Perennial grasses, like *Elymus repens, Poa bulbosa* and *Stipa borysthenica* differentiated for Meadow and an invasive alien species, *Asclepias syriaca* for Depression.

Changes in the cover of vascular plants and cryptogams

There was a significant impact of year, treatment and treatment and year interaction (all $p<0.0001$) when analysing the cover of vascular plants and cryptogams (Table 1). The cover of vascular plants fluctuated with time. The reference grassland had significantly less overall cover of vascular plants (32%) than C-amended (52%) and control (58%) on average. C-amended plots had significantly lower cover of vascular plants than controls during 2001-2005, similar to reference in 2001-2003, but significantly higher than that of the reference in most years after 2004 (Fig 4.a).

The cover of cryptogams was increasing with time in control, C-amended and reference plots. Significantly higher cover of cryptogams was found in the reference (50%) than in the C-amended (35%) or control (48%) plots on average. When comparing treatments within years, C-amended plots had significantly lower cover of cryptogams than control between 2002-2006 and than reference between 2002-2005 (Fig 4.b).

Nitrogen requirement groups

A significant impact of year, treatment and treatment and year interaction (all $p<0.0001$) was found for all nitrogen requirement groups, except for treatment in case of hypertrophic species (Table 1). The cover of oligotrophic species increased, the cover of mesotrophic species decreased with time, while the cover of hypertrophic species showed only fluctuations with years in both C-amended and control plots (Fig. 5). Reference had a significantly higher cover of oligotrophic (92%) and lower cover of mesotrophic (4%) and hypertrophic (4%) species compared to control (56, 32 and 12%) and C-amended (55, 34 and 11%) plots on average and also in some separate years. Differences were significant between control and C-amended plots in some years only for oligotrophic (1998 and 2005) and mesotrophic species (1998-99, 2005, 2018) and no difference was shown for hypertrophic species (Fig 5).

Restoration species groups

A significant impact of year, treatment and treatment and year interaction (all $p<0.05$) was found also for all restoration groups, except in case of neophytes (Table 1). The latter did not show a significant difference with treatment or interaction (Table 1). The cover of target species increased from ca. 40% to over 75% and in turn the cover of non-target species decreased from ca. 40% to 10 % on average with time in treated and control plots. Reference plots (99%) had significantly higher cover of target species than control (61%) and C-amended (62%) on average and also in all years (Fig. 6.a). At the same time, reference plots (0.65%) had significantly lower cover of non-target species than control (26%) and C-amended (26%) on average, and also in each year and contained no neophyte cover. Significantly higher cover of target species was found in C-amended than in control plots only in 2005 (Fig. 6.a). The cover of neophyte species basically showed no difference in treatments and the cover of non-target species showed significant difference between C-amended and control plots in 2005 (Fig. 6.b,c).

Discussion

Long-term monitoring revealed a delayed impact of carbon amendment on the vegetation, and the fading of treatment effects both in the soil and in the vegetation after the cessation of applications. Previous results showed that carbon amendment successfully induced soil microbial activity and lowered nitrogen availability that resulted in a decreased cover of vascular plants and cryptogams (Török *et al.*, 2000; 2014), but did not influence nitrogen requirement groups (Szabó *et al.*, 2008), and basically the composition of old-field vegetation remained different from the reference grassland in the short term (Török *et al.*, 2014). Based on the long-term evaluation, differences in nitrogen availability disappeared with the cessation of carbon applications, but the lowered cover of vascular plants and cryptogams remained for two and three years after treatment, respectively. The initial vegetation reflected higher soil nitrogen availability in the old-fields compared to the reference. Dominant species of the three old-fields were mesotrophic or hypertrophic, except for the Depression, compared to the oligotrophic grassland (Török *et al.*, 2000). Oligotrophic species increased and mesotrophic species decreased in cover with succession both in C-amended and control plots. Similar trends were visible for target and non-target species. These changes together with the trajectory analysis show that the secondary succession continues on the studied sites, and the compositional differences between old-fields and reference grassland were still visible 22 years after the start of the experiment.

Several factors are assumed to be responsible for the lack of a more articulated response to carbon amendment in our study. The first factor is soil nitrogen availability. Carbon amendment must lower soil N levels favouring the initial competitive dynamics (Blumenthal *et al.*, 2003). Agricultural practices generally enrich the soil by adding fertilizers that result in an excess of nutrients compared to low productivity grasslands (Walker *et al.*, 2003). In our study, the fields probably did not receive a high fertilizer input characteristic of intensive agriculture in the past, because the usual practice in the area included mostly animal manuring (Csecserits *et al.*, 2007). Some measurements in arable fields, under rye or alfalfa show a range of total available nitrogen between 2-10 mg/kg soil (Szili-Kovács pers. comm.). Also the mobile nitrogen forms can be easily leached out from the loose sandy soil (Tilman and Isbell, 2015) after cessation of cultivation that could result in lower initial differences in soil nitrogen content between old-fields (4.6-6.1 mg/kg) and reference grasslands (1.5-1.7 mg/kg) in our case. This low initial difference could result in a lack of changes in competitive dynamics due to carbon amendment;while treatments caused significantly reduced soil nitrogen, they did not result in nitrogen values within the range of the reference plots (Török *et al.*, 2000; 2014). It is also possible that carbon addition did not work as expected, because the main limiting resource for plant growth can be phosphorus or the N:P ratio (Suding *et al.*, 2004).

The second factor is the vegetation. For successful application of carbon amendment, non-native species must be more competitive in environments with high nitrogen availability, whereas native species should be adapted to low N availability (Blumenthal *et al.*, 2003). The dominant neophyte species (*Conyza canadensis*, *Asclepias syriaca*) in our study are considered as mesotrophic, so that the lowered nitrogen availability could not induce strong competitive advantage of oligotrophic target species as expected. Also, carbon amendment is reported to influence mainly annual invasive species (Davis *et al.*, 2000). This is confirmed also by our results for annual neophytes (data not published), however, there were also deep rooting perennial species present among the neophytes in our case (e.g. *Asclepias syriaca*).

Furthermore, the old-fields in our study were abandoned three to seven years prior carbon addition, so that there was already established vegetation with both annual and perennial species present (Török *et al.*, 2000). The low response of vegetation to nitrogen immobilization might be attributed to the presence of perennials (including neophytes) with deep root system which cannot benefit from immobilization in upper soil layers (Török *et al.*, 2014). This is in line with the initial floristic composition concept (Egler, 1954) and the priority hypothesis (Chase, 2003) that state that the species that arrive first in a habitat significantly affect the establishment, growth, or reproduction of species arriving later and this way the already established vegetation determines further vegetation development.

On the other hand, the similarity of control and carbon amended plots can be attributed also to spontaneous recovery processes. Surveys on the regeneration of sandy old-fields in the Pannonian biogeographic region reported that at a decadal time scale the vegetation of old-fields became similar to sand grasslands in terms of generalist species (Halassy, 2001; Csecserits *et al.*, 2011; Albert *et al.*, 2014; Valkó *et al.*, 2016). However, the cover of neophytes might not decrease significantly during secondary succession (Csecserits *et al.*, 2011) and some specialist species might not be able to establish (Molnár and Botta-Dukát, 1998). This implies that generalist species can establish without carbon amendment, but the specialist species cannot arrive at the old-fields without assisted dispersal (Halassy *et al.*, 2019), therefore the impact of carbon addition might not be detectable because of the lack of propagules of specialist species.

Finally, the weak response to carbon amendment can also be due to the complex nature of aboveground-belowground linkages (Wardle *et al.*, 2004). Nitrogen immobilization through the activation of soil microorganisms by carbon amendment is known to be temporary, and plant available nitrogen is released when the microbial biomass decomposes (Perry *et al.*, 2010). The delayed impact can be attributed to the time required until an ecologically significant difference in soil available nitrogen was expressed in the vegetation. The carbon amendment, soil microbial immobilization, lowered plant available nitrogen, vegetation response loop can be influenced by several unknown factors, such as the impact of changing drought and water availability of the soil on microbial status or links to other elements of the soil biota.

Conclusion

Our results highlight the importance of long-term monitoring in restoration ecology. Several years are required before the impacts can be visible in the restored system (Herrick *et al.*, 2006), and continuous monitoring can show if the development trajectories remain in the desired direction, or adaptive management is needed to correct the restoration trajectory (Viani *et al.*, 2017). This is especially true when restoration aims at indirect above ground-below ground linkages, such as in the case of carbon amendment.

We conclude that several aspects should be assessed before taking into consideration carbon amendment as a method for restoration of abandoned fields in low productive areas. Firstly, carbon addition is more effective when soil nitrogen availability is much higher before restoration due to artificial fertilizers used in intensive agriculture compared to the target community and in more compacted soils. Another point before considering carbon amendment is the temporary and delayed effect on nitrogen immobilization. This implies that carbon sources should be added repeatedly over the years, which is not economically feasible and appropriate for restoration at large scale. Considering the vegetation, carbon amendment is more effective on open ground before the establishment of perennial plants, including neophytes and if no propagule limitation exists for target species. Finally, carbon amendment is primarily suggested as a complement to other treatments, seed introduction on the first place, since the lowering of the cover of vascular plants and cryptogams can be a window of opportunity for other species to colonize.

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Author contributions

KT, MH conceived and designed the study; all authors did collections and other field work; KSZ advised on statistical analysis; ZS, BPR performed statistical analyses; ZS, MH prepared the first version of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data accessibility

The data that support the findings of this study are openly available in ZENODO at http://doi.org/10.5281/zenodo.3895274

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Table 1. Results of linear mixed effects models (LME) according to the different indicators. Significant differences are indicated in bold.

	Fixed effects		
	year	**treatment**	**year * treatment**
Total available nitrogen	**F=114.208, df=8, p<0.0001**	**F=34.113, df=1, p<0.0001**	**F=2.516, df =8, p=0.0118**
Cover of vascular plants	**F=170.039, df=11, p<0.0001**	**F=11.525, df=2, p<0.0001**	**F=30.980, df =22, p<0.0001**
Cover of cryptogams	**F=109.18321, df=11, p<0.0001**	**F=13.95900, df=2, p<0.0001**	**F=14.85277, df = 22, p<0.0001**
Species of oligotrophic habitats	**F=47.7644, df=11, p< 0.0001**	**F=50.0337, df=2, p<0.0001**	**F=26.9087, df=22, p<0.0001**
Species of mesotrophic habitats	**F=35.940, df=11, p< 0.0001**	**F=266.064, df=2, p<0.0001**	**F=20.677, df=22, p<0.0001**
Species of hypertrophic habitats	**F=40.78124, df=11, p< 0.0001**	F=1.43818, df=2, p=0.2409	**F=18.61759, df=22, p<0.0001**
Target species	**F=17.556, df=11, p< 0.0001**	**F=219.108, df=2, p<0.0001**	**F=25.556, df=22, p<0.0001**
Non-target species	**F=30.6575, df=11, p<0.0001**	**F=454.1904, df=2, p<0.0001**	**F=31.7263, df=22, p<0.0001**
Neophyte species	**F=27.89494, df = 11, p<0.0001**	F=0.19088, df=1, p=0.6631	F=1.54383, df=1, p=0.1101

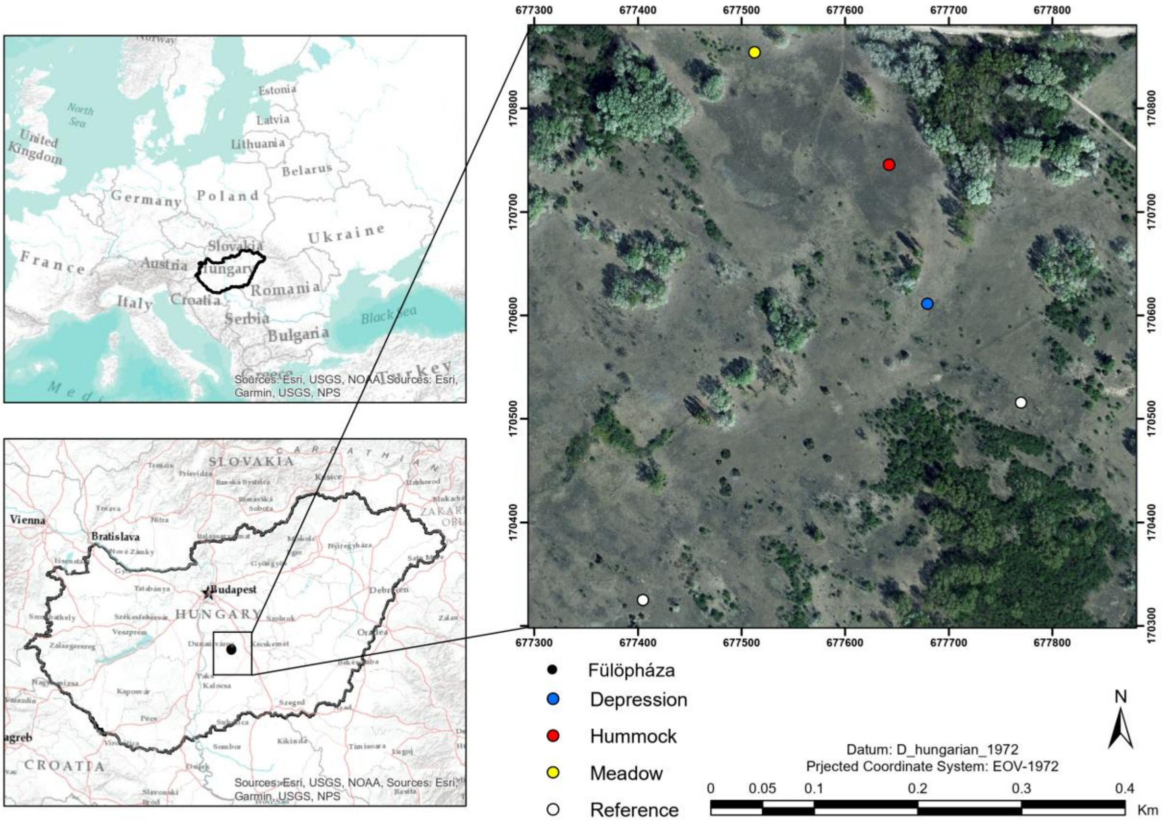


Figure 1. Location of the experimental sites in Fülöpháza, Hungary.

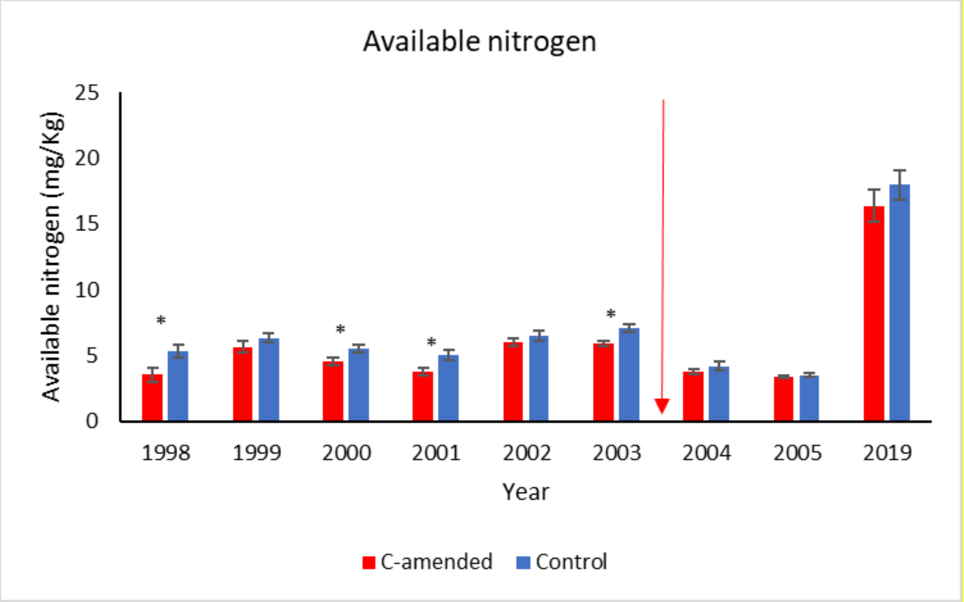
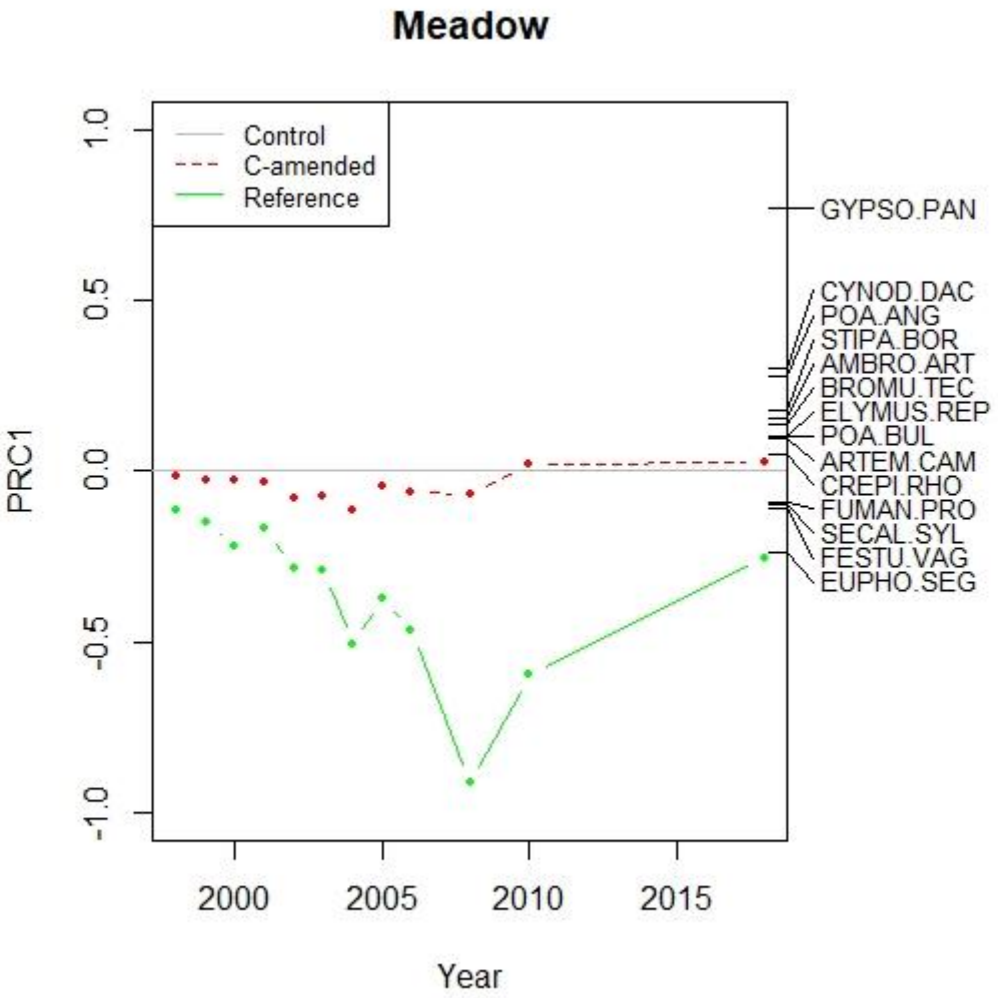
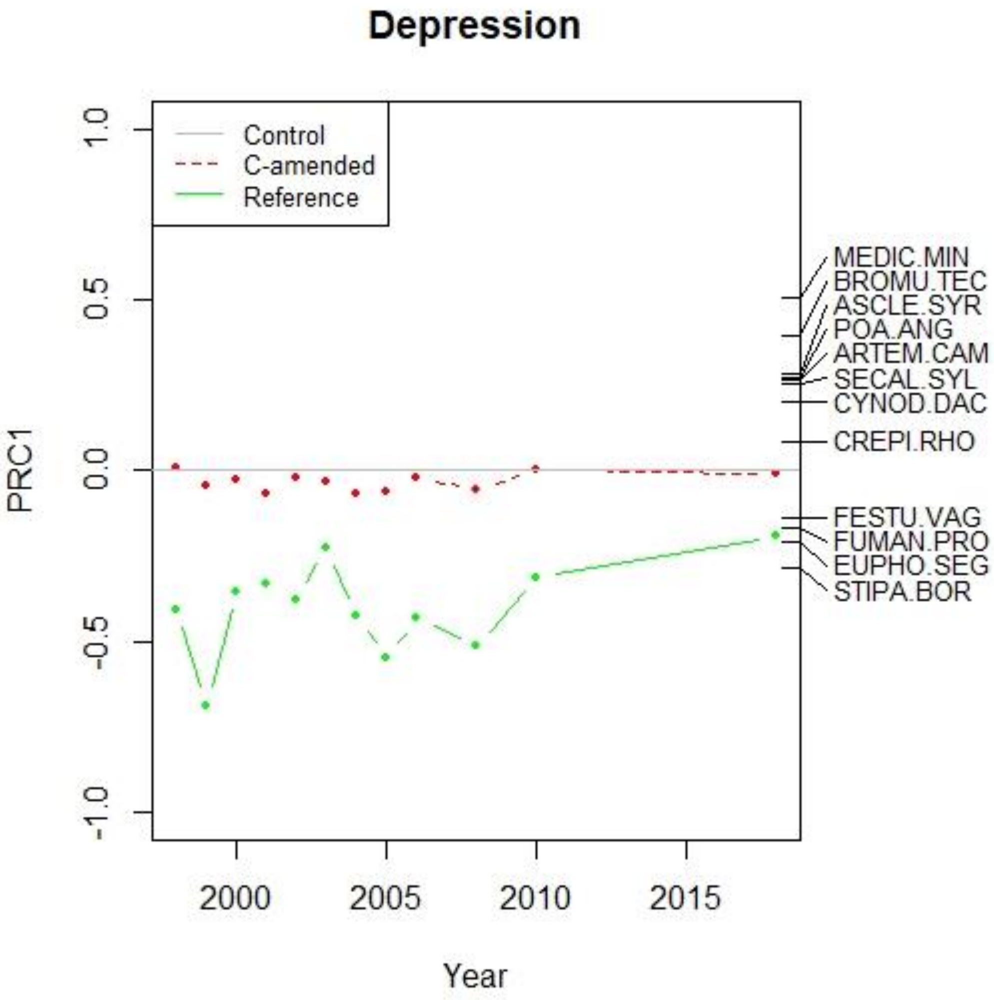


Figure 2. Changes of total soil available nitrogen (NH_4-N+NO_3-N) with year and treatment between 1998 and 2019. Years with significant ($p < 0.05$) differences between C-amended and control plots are marked with asterisks. The red arrow shows the cessation of the treatment.

a


Meadow
Control
C-amended
Reference
PRC1
1.0
0.5
0.0
-0.5
-1.0
2000
2005
2010
2015
Year
GYPSO.PAN
CYNOD.DAC
POA.ANG
STIPA.BOR
AMBRO.ART
BROMU.TEC
ELYMUS.REP
POA.BUL
ARTEM.CAM
CREPI.RHO
FUMAN.PRO
SECAL.SYL
FESTU.VAG
EUPHO.SEG

b


Depression
Control
C-amended
Reference
PRC1
1.0
0.5
0.0
-0.5
-1.0
2000
2005
2010
2015
Year
MEDIC.MIN
BROMU.TEC
ASCLE.SYR
POA.ANG
ARTEM.CAM
SECAL.SYL
CYNOD.DAC
CREPI.RHO
FESTU.VAG
FUMAN.PRO
EUPHO.SEG
STIPA.BOR

c

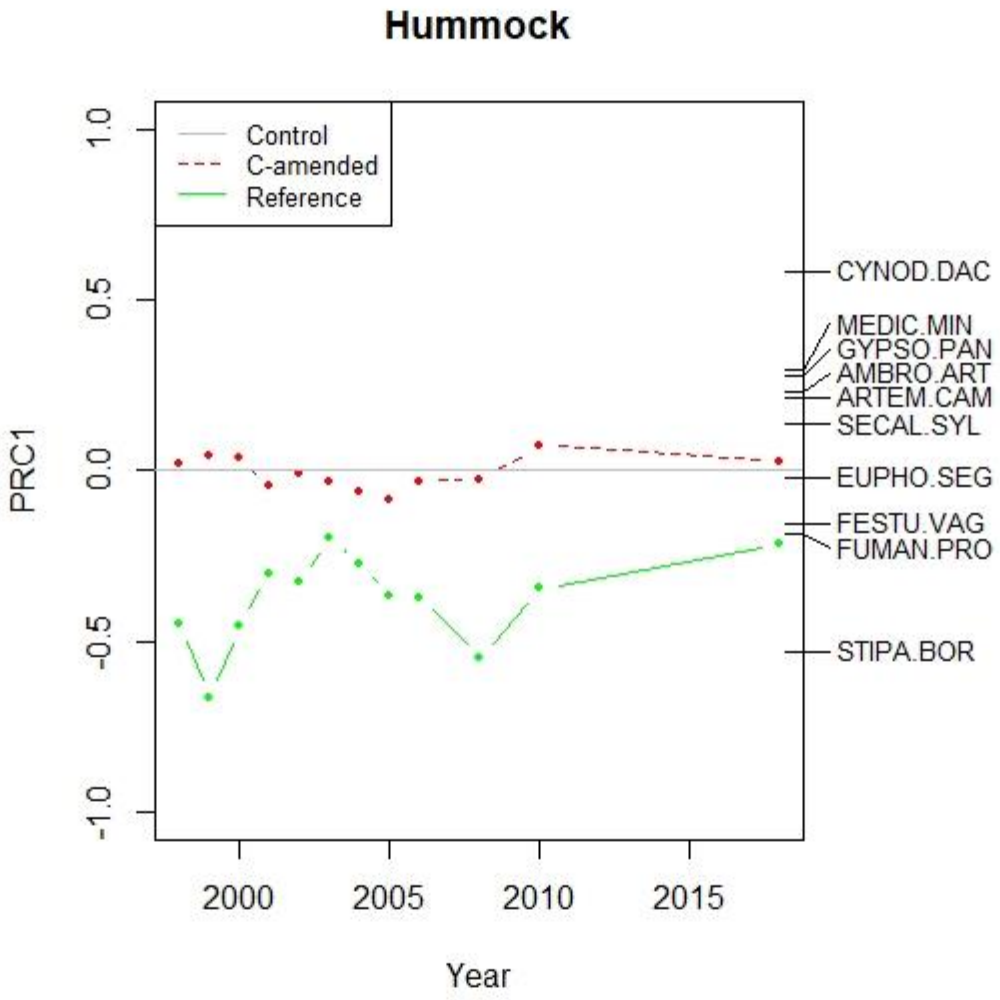


Figure 3. Principal Response Curves showing the effect of carbon amendment over time on vegetation development. (a) Meadow site, (b) Depression site and (c) Hummock site. Zero-lines show control, the other lines show the course of C-amended and reference grassland plots over time. The vertical axes show the canonical coefficient for PCR1 and species weight. For species names and species weights refer to Appendix S2.

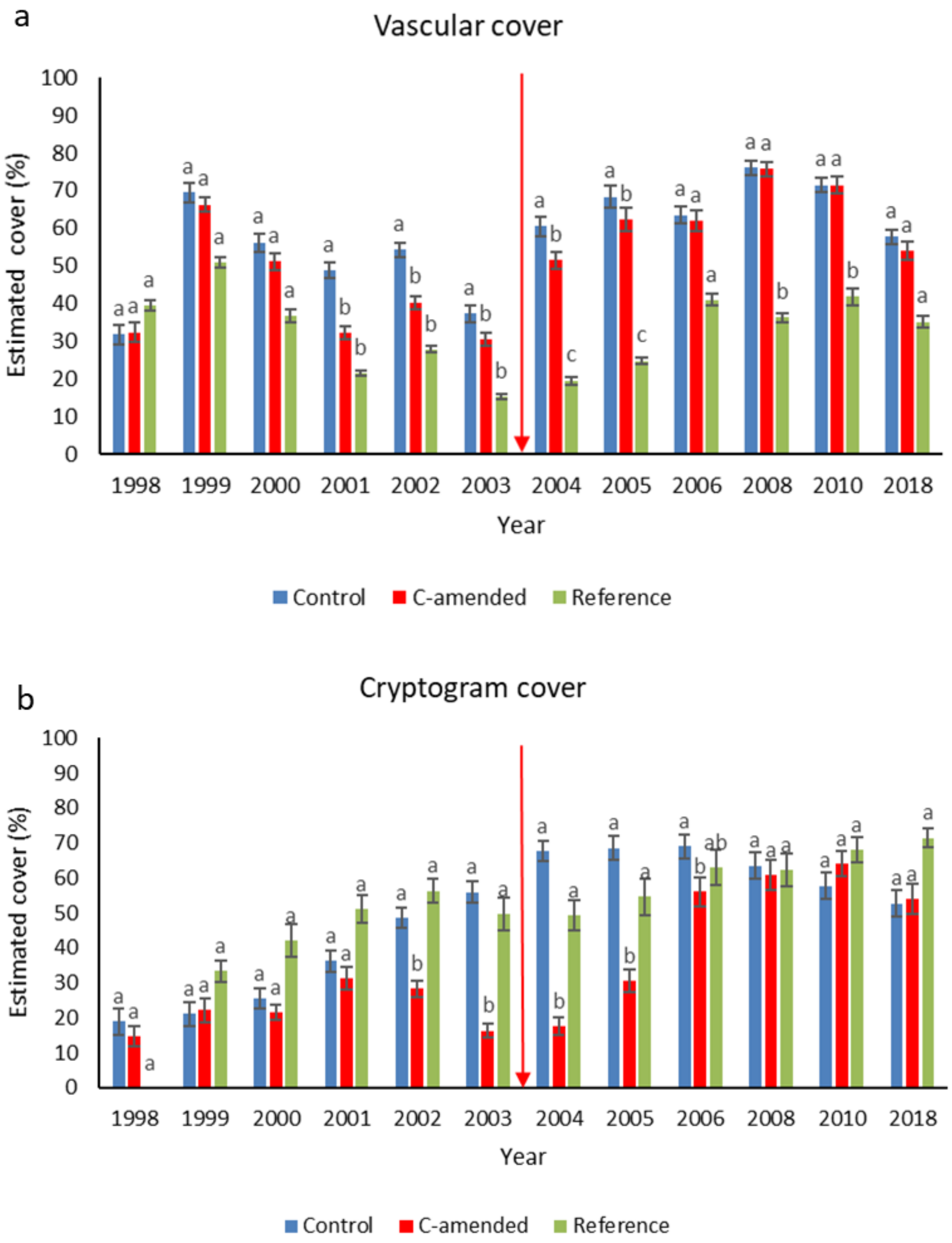
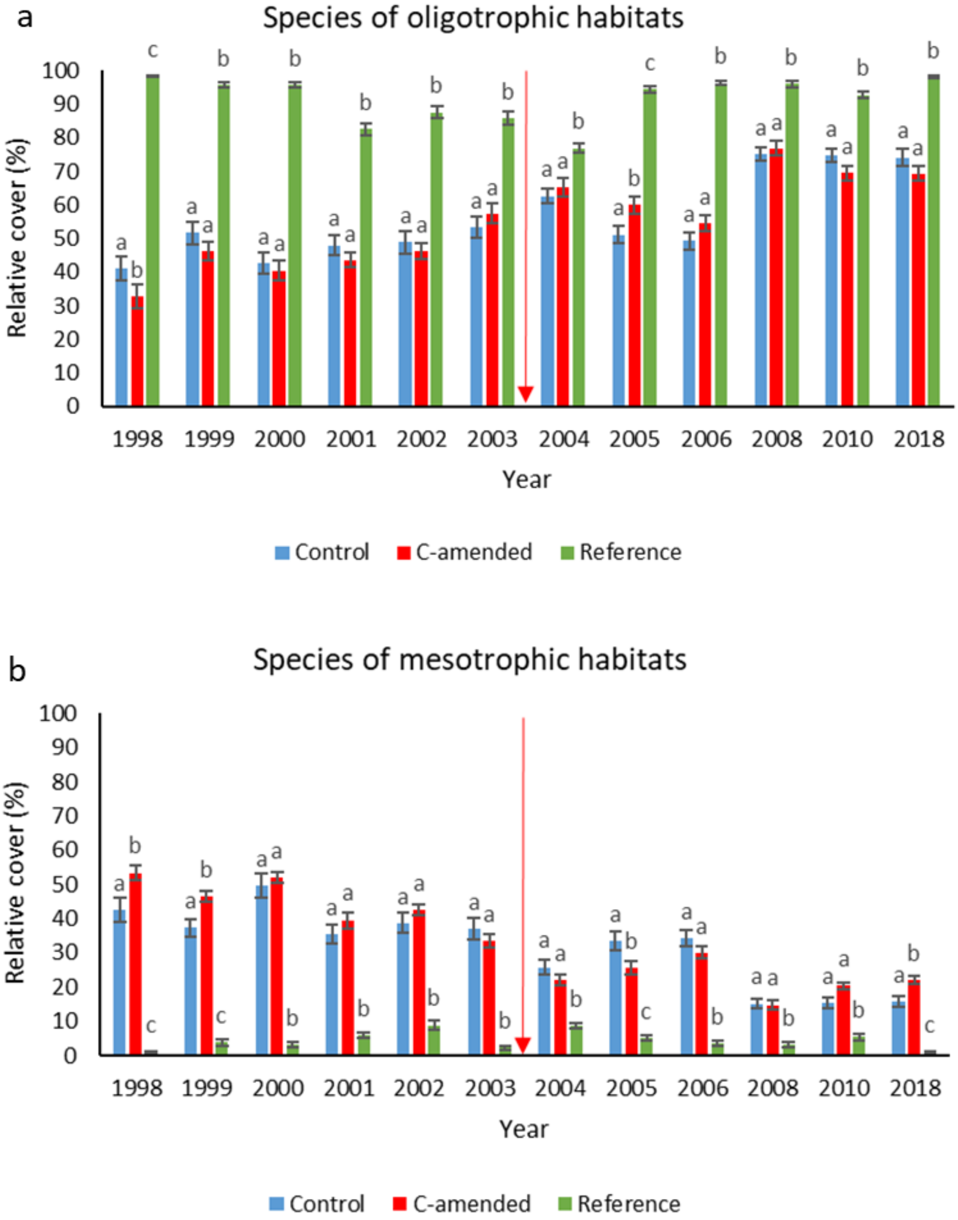


Figure 4. Changes of a) total cover of vascular plants and b) total cover of cryptogams with year and treatment between 1998 and 2018. Within year significant difference ($p < 0.05$) between C-amended, control and reference plots based on Wald test is indicated by lowercase letters. The red arrow shows the cessation of the treatment.


a
Species of oligotrophic habitats
Relative cover (%)
0
10
20
30
40
50
60
70
80
90
100
1998
1999
2000
2001
2002
2003
2004
2005
2006
2008
2010
2018
Year
Control
C-amended
Reference
b
Species of mesotrophic habitats
Relative cover (%)
0
10
20
30
40
50
60
70
80
90
100
1998
1999
2000
2001
2002
2003
2004
2005
2006
2008
2010
2018
Year
Control
C-amended
Reference

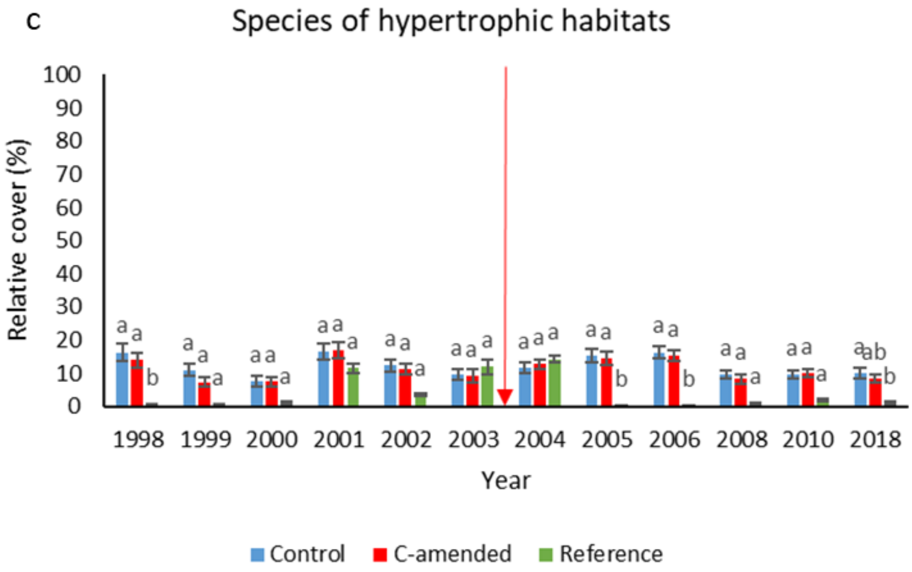
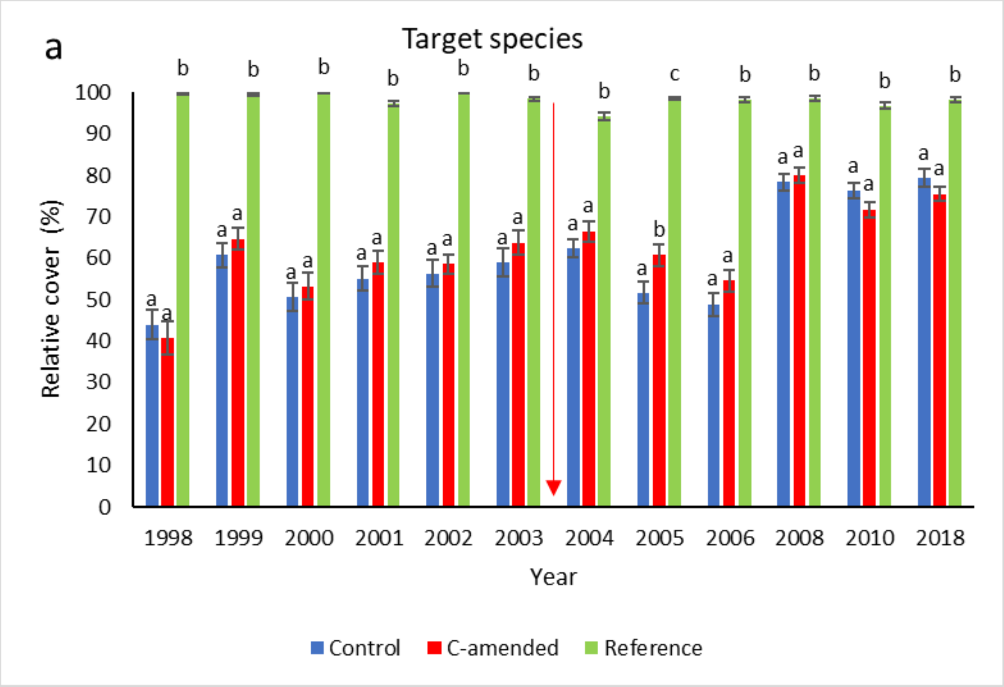
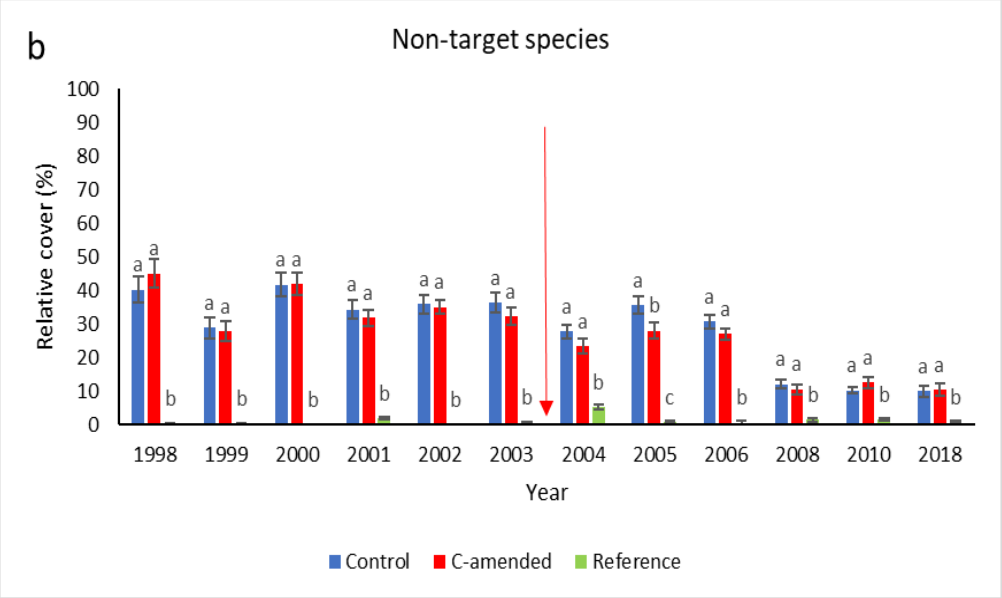


Figure 5. Changes in the relative cover of nitrogen requirement groups with year and treatment between 1998 and 2018. a) Species of oligotrophic; b) mesotrophic; and c) hypertrophic habitats. Within year significant difference ($p < 0.05$) between C-amended, control and reference plots based on Wald test is indicated by lowercase letters. The red arrow shows the cessation of the treatment.


a
Target species
Relative cover (%)
0
10
20
30
40
50
60
70
80
90
100
1998
1999
2000
2001
2002
2003
2004
2005
2006
2008
2010
2018
Year
Control
C-amended
Reference


b
Non-target species
Relative cover (%)
0
10
20
30
40
50
60
70
80
90
100
1998
1999
2000
2001
2002
2003
2004
2005
2006
2008
2010
2018
Year
Control
C-amended
Reference

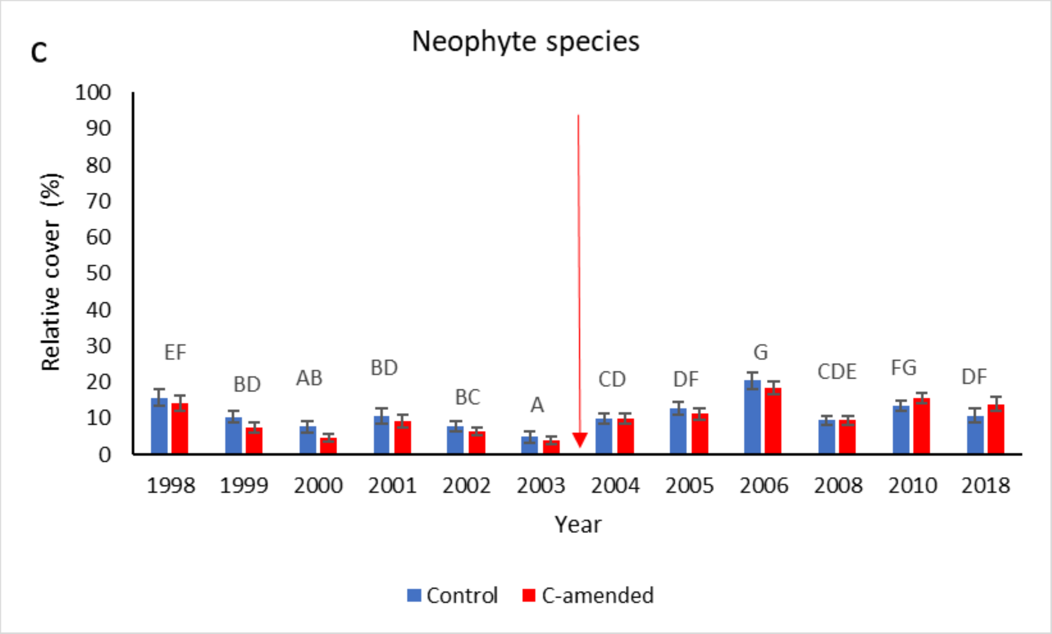


Figure 6. Changes in the relative cover of restoration species groups with year and treatment between 1998 and 2018. a) Target; b) indigenous; and c) neophyte species. Within year significant difference ($p < 0.05$) between C-amended, control and reference plots based on Wald test is indicated by lowercase letters for target and non-target species. Between-year significant difference ($p < 0.05$) is indicated by capital letters based on Tukey HSD for neophyte species. Red arrow shows the cessation of the treatment.

Supplementary Information

Additional supporting information may be found in the online version of this article:

Appendix S1. List of species and their categorization according to nitrogen requirement and role in restoration.

Appendix S2. The species weights for PRC1.

Appendix S3. PCA biplot for the vegetation development of the three sites