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A new aspect of grassland vegetation dynamics: cyanobacterium colonies affect establishment success of plants

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Abstract

Aims: Cyanobacteria may have considerable effects on community functioning, mostly because they produce various metabolites that adversely affect other organisms. Here we synthesized existing knowledge about the effects of toxic cyanobacteria on the germination and growth of terrestrial plants. We also aimed to test the chemical effects of a *Nostoc* (Cyanobacteria) extract on the germination and growth of species of alkali habitats to investigate whether cyanobacteria can alter community structure and diversity via affecting the establishment success of plants.

Location: Cyanobacterium colonies from the Hortobágy National Park, east Hungary; indoor experiments at the University of Debrecen, Hungary.

Methods: To review the effects of toxic cyanobacteria on terrestrial plants, we conducted a literature search. To test these effects on native plants, field-collected *Nostoc* colonies were used to prepare a cell-free water extract, and treatments (watering with *Nostoc* extract and watering with tap water) were tested on 3 × 100 seeds of nine alkali grassland species. After 5 wk, seedling number, seedling length and fresh and dry weights were measured.

Results: We collected data on the effects of cyanobacteria on 27 species, but they were mostly focused on crops irrigated with cyanobacteria-containing water, not on floras native to natural ecosystems. In the germination experiment species identity and treatment had a significant effect on almost all variables, but their interaction only affected germination rate and fresh weight. Fresh weight decreased significantly only in the invasive *Hordeum jubatum*, but germination rate decreased significantly in five species.

Conclusions: Based on our findings, terrestrial cyanobacterium colonies can affect the establishment success of grassland plants, through which they may be important in determining which species can be incorporated into the community. Thus, cyanobacteria might play an important role in shaping diversity, species composition and the structure of natural plant communities.

Introduction

Community assembly and biotic interactions such as facilitation and competition are among the most important topics of plant ecology (Onipchenko et al. 2009; Spasojevic & Suding 2012; le Roux et al. 2013; Martorell & Freckleton 2014). However, when studying factors that affect terrestrial plant communities, mainly plant–plant or plant–animal interactions are considered; interactions with photoautotrophic organisms other than vascular plants (e.g. cyanobacteria) are mostly overlooked (van der Heijden et al. 2008). The effects of cyanobacteria on other organisms have been mostly studied in aquatic ecosystems (Casanova et al. 1999; Mitrovic et al. 2005; Máthé et al. 2007). However, cyanobacteria are important primary producers and N-fixers in numerous terrestrial habitats (Coxson & Kershaw 1983; Dodds & Gudder 1995; Hrouzek et al. 2011).

Terrestrial cyanobacteria occur in almost every alkali habitat type, both on solonetz and solonchak soils (Török

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et al. 2011), where mostly *Nostoc* species are typical on the soil surface (Komáromy 1984). These habitats can be found under continental climates in areas with high groundwater levels and with moderate to high soil salt content (Eliáš et al. 2013; Deák et al. 2014a; Valkó et al. 2014). In the European Union, these habitat types cover approximately 209 152 ha, and more than 98% of their stands are located in Hungary (Šefferová-Stanová et al. 2008). These habitats are characterized by a fluctuating moisture regime (they are covered by surface water in spring, but dry out until midsummer) and by bare soil surfaces enabling *Nostoc* species to form colonies of considerable size (Appendix S1). *Nostoc* colonies usually appear in early spring when the soil surface is covered by water. Colonies swell and form mucous mats on the surface, then, during the summer they dry out and break into pieces. They are most numerous in alkali meadows (*Agrostio stoloniferae–Alopecuretum pratensis*), open alkali swards (*Puccinellietum limosae*, *Plantagini tenuiflorae–Pholiuretum pannonici* and *Camphorosmetum annuae*) and alkali marshes (*Bolboschoenetum maritimi*); however, they can also be widespread in other alkali habitat types (Deák et al. 2014a, b,c). Colonies can be found in every year, but rainfall fluctuations between years can considerably affect their cover (Lukács et al. 2015).

Cyanobacteria produce a wide variety of cyanotoxins and several alkaloid and peptide components with strong bioactivity (Shunmugam et al. 2014; Sanz et al. 2015). The main function of cyanotoxins is usually thought to be defence against planktivores (Codd 1995), but it is also conceivable that they function as allelochemicals against aquatic plants (i.e. produce biochemicals that negatively influence the germination, growth, survival and reproduction of other organisms; Mitrovic et al. 2004; Willis 2007). Adverse effects of these biochemicals on aquatic macrophytes have already been demonstrated (Pflugmacher et al. 1999, 2001; Mitrovic et al. 2004). In addition, the reaction of terrestrial crop plants to cyanotoxins and cyanobacteria has received some interest among researchers, as the water bodies used for irrigation can contain a significant amount of cyanobacteria (Wiegand & Pflugmacher 2005). Consequently, there is growing evidence of growth inhibition induced by cyanotoxins in a number of terrestrial crop plants (Saqrane & Oudra 2009). However, besides being potentially important in irrigated croplands, cyanobacteria can have considerable impact on a number of natural terrestrial habitats. In habitats with a high abundance of cyanobacterium colonies plants can experience the advantages of N accumulation due to the N fixation of the cyanobacteria, and it may also facilitate the establishment of plant species by ameliorating soil conditions (Dodds & Gudder 1995). In some aspects, cyanobacterium cover may have effects similar to those of litter as it (1) forms a physical barrier; (2) decreases fluctuations in temperature and soil humidity; and (3) increases the nutrient content of the soil (Xiong & Nilsson 1999). Along with these possible positive effects, the presence of toxic cyanobacteria presumably has negative effects on terrestrial species of natural ecosystems, but this has not yet been studied.

Based on the above considerations, terrestrial cyanobacterium colonies may have various effects on the establishment success of vascular plants. On the one hand, in stressed environments, such as alkali grasslands they can facilitate vascular plants in a way somewhat similar to that of a moderate amount of litter (Bertness & Hacker 1994; le Roux & McGeoch 2008; Butterfield 2009; Kelemen et al. 2013). On the other hand, via the production of a variety of allelopathic compounds, they can also have negative effects on the species of natural terrestrial communities, which are presumably similar to the previously detected adverse effects of cyanobacteria on a number of terrestrial crops (Codd 1995; Saqrane & Oudra 2009).

Our aim was to (1) summarize previous studies on the effects of cyanotoxins on the germination and growth of terrestrial plants; and (2) to test the chemical effects (i.e. by excluding potential physical effects) of a *Nostoc* extract on the germination and seedling growth of selected species occurring in alkali habitats in an indoor experiment. We conducted the experiments in order to investigate whether the presence of cyanobacterium colonies can alter community structure and diversity via decreasing the establishment success of vascular plants.

Methods

Literature search

We conducted a systematic literature search of studies dealing with the effects of toxic cyanobacterial extracts and cyanotoxins, using the search terms 'cyanobacteria' AND 'toxin' AND ('plant growth' OR 'germination') in Google Scholar, which yielded 5720 hits (last accessed: 8th Jun 2016). We scanned the first 500 papers by title and abstract, the next 500 papers were scanned by title. Inclusion criteria were the following: (1) treatment with cyanotoxin or toxic cyanobacterial extract; (2) dealing with terrestrial vascular plant(s); (3) measurement of the effects on seedling growth and/or germination; and (4) comparisons with untreated control. We excluded studies in which non-toxic strains of cyanobacteria were used to study the fertilizing effect of cyanobacterial extracts.

Identification, isolation and culturing of *Nostoc*

Nitrogen-fixing cyanobacterium species forming terrestrial colonies in alkali grasslands were collected in Nyírőlapos,

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Hortobágy (East Hungary), and identified to genus level as *Nostoc* sp. according to Komárek (2013). The filaments were isolated and purified by streaking on agar plates containing BG-11 medium (a medium optimized for cyanobacteria; Stanier et al. 1971). The plates were placed at 25 ± 2 °C and under an irradiance of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After 1 wk, the filaments were cut at their ends with sterilized forceps, streaked again on the agar plates containing BG-11 medium and grown under the same conditions. To obtain a large quantity of the species, filaments were transferred from agar plates to a 250-ml flask containing 100 ml BG-11 medium and incubated for 1 wk. The 100-ml culture was scaled up by transferring this culture to a flask containing 1 L BG-11 medium. The cultures were then aerated with sterilized air and grown under the same conditions as isolation. The filaments were harvested after 3 wk by centrifugation (Beckman Avanti; 8000 *g*) and for calculating the dry weight an aliquot was lyophilized.

Cyanobacterial crude extract preparation

Throughout the experiment a cell-free extract of *Nostoc* BGSD-2012 was used. The harvested sample was frozen and stored at −25 °C. After thawing, the sample was ultrasonicated on ice (3 kHz × 10 min) until the cells lysed, which was checked with light-microscopy. A 20 $g \cdot L^{-1}$ water extract of *Nostoc* BGSD-2012 was used for the experiments.

Characterization of the *Nostoc* extract

The carbohydrate composition and content of the crude extract were determined using the method of Gyémánt & Nánási (2003). The concentration and composition of carotenoids were measured with the method of Deli et al. (2014). A detailed description of these methods can be found in Appendix S2. The detection of plant inhibitory metabolites and the calculation of IC_{50} values (i.e. the concentration which causes 50% of maximum growth inhibition) were based on the *Sinapis* test (Vasas et al. 2002). The *Sinapis* test uses white mustard (*Sinapis alba*) seeds exposed to different concentrations of a certain substance to determine the inhibitory effect of the substance based on the growth of the seedlings. Exacted mass of the purified toxin was measured using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, US) with the method of Stranska-Zachariasova et al. (2016).

Germination experiment

We selected seven characteristic species of alkali grasslands (*Agrostis stolonifera, Aster tripolium* ssp. *pannonicus, Beckmannia eruciformis, Hordeum hystrix, Lepidium ruderale, Plantago schwarzenbergiana* and *Puccinellia limosa*) to represent the life forms typical of alkali grasslands and a relatively broad spectrum of seed size (Thousand-seed weights from 0.025 to 3.512 g). In addition, we also selected an invasive grass (*Hordeum jubatum*) recently invading alkali grasslands in the study region and an easily germinated perennial forb (*Trifolium repens*). Seeds were collected in alkali grasslands in east Hungary, cleaned and dry-stored until the germination tests.

We aimed to test specifically for the chemical effects of *Nostoc.* Accordingly, the following treatments were used: (1) watering with tap water containing *Nostoc* extract; and (2) watering with only tap water (control treatment). These two treatments were tested on seed sets of 100 seeds in three replicates for each of the nine species, altogether 54 pots. Pots were filled with steam-sterilized potting soil and 100 seeds were sown in each of them (altogether 5400 seeds). We also used ten additional pots of the same size (filled with steam-sterilized soil only) to detect airborne seed contamination. All pots were randomly placed on germination shelves under natural light. The pots were watered 5 days a week, twice a day. Depending on the treatment, pots were irrigated with 27 ml tap water containing *Nostoc* extract or 27 ml tap water (control). The germination experiment lasted 5 wk from 29 Apr 2013. At the end of the experiment all germinated seedlings were counted and removed. We measured shoot length (20 randomly chosen seedlings) and fresh and dry weights (all seedlings separately) with an accuracy of 0.001 g. We also calculated dry matter content (dry weight/fresh weight × 100) of the seedlings.

Statistical analysis

We tested the effect of treatments and species identity on germination and seedling growth with linear models, where 'species identity' ($df = 8$), 'treatment' ($df = 1$) and the interaction between treatment and species identity ($df = 8$) were used as fixed factors, and germination rate (%), fresh and dry weight per seedling (mg), dry matter content per seedling (%) and seedling length (mm) were dependent variables. To indicate significant differences ($P < 0.05$) in dependent variables between treatments within a species, *t*-tests were used (Zar 1999). All calculations were performed using SPSS 17.0 (SPSS, Chicago, IL, US).

Results

Results of the literature search

We found 45 studies that met the inclusion criteria. These studies dealt with 27 species from eight plant families (Table 1). Out of these 45 studies, 29 reported only

Table 1. Terrestrial plant species for which the effects of toxic cyanobacteria on germination and/or growth were previously studied.

Species	Family	Number of Studies
Allium cepa	Alliaceae	1
Brassica napus	Brassicaceae	3
Brassica narinosa	Brassicaceae	1
Brassica oleracea	Brassicaceae	2
Brassica rapa-chinensis	Brassicaceae	1
Eruca sativa	Brassicaceae	1
Festuca rubra	Poaceae	1
Lactuca sativa	Asteraceae	8
Lens esculenta	Fabaceae	2
Lepidium sativum	Brassicaceae	1
Lolium perenne	Poaceae	3
Lycoperiscon esculentum	Solanaceae	4
Malus pumila	Rosaceae	1
Medicago sativa	Fabaceae	3
Nasturtium officinale	Brassicaceae	1
Oryza sativa	Poaceae	9
Phaseolus vulgaris	Fabaceae	3
Pisum sativum	Fabaceae	3
Sinapis alba	Brassicaceae	7
Solanum tuberosum	Solanaceae	1
Spinacia oleracea	Chenopodiaceae	2
Trifolium repens	Fabaceae	1
Triticum aestivum	Poaceae	2
Triticum durum	Poaceae	2
Vicia faba	Fabaceae	3
Vigna radiata	Fabaceae	1
Zea mays	Poaceae	4

For the results regarding species of natural floras see Table 2, for the results of crops see Appendix S3.

Table 2. Results of studies dealing with the effects of cyanotoxins and toxic cyanobacterial extracts on the germination and growth of species also occurring in natural grasslands.

Species	Effects of Cyanobacteria/ Cyanotoxin	Reference
Festuca rubra	No effect on germination and root growth	Pereira et al. (2009)
Lolium perenne	Root dry weight increased, shoot dry weight decreased	Crush et al. (2008)
Lolium perenne	No effect on germination and root growth	Pereira et al. (2009)
Lolium perenne	Fresh weight decreased in field experiment, root and shoot length decreased in laboratory experiments	Bácsi et al. (2011)
Trifolium repens	No effect on root and shoot dry weight	Crush et al. (2008)

negative effects of cyanotoxins and extracts on the germination and/or growth of the studied plants, and 13 studies found positive or neutral effects along with negative ones. Five studies reported no effect for at least one species (Järvenpää et al. 2007; Crush et al. 2008; Pereira et al. 2009; Lefebvre et al. 2013; Azavedo et al. 2014), while root fresh and dry weights were sometimes positively affected (*Lactuca sativa* and *Lolium perenne*, Crush et al. 2008; *Oryza sativa*, Prieto et al. 2011; *L. sativa*, Freitas et al. 2015). We summarized the findings of these studies for natural floras in Table 2 and for crop species in Appendix S3.

Characterization of the *Nostoc* extract

Altogether there were 387 $mg \cdot g^{-1}$ total carbohydrates in the strain. We determined a polysaccharide composition of xylose, arabinose, mannose and galactose in the gelatinous mucilage (in ratio: erythrose 16, fucose 2, arabinose 36, xylose 97, mannose 21, galactose 15, GX [unidentified] 12, glucose 6). In the strain total carotenoid content was 0.324 $mg \cdot g^{-1}$. Similar to previous findings, our *Nostoc* isolate contained echinenone (35%) and β-carotene (36%) as major compounds. The minor carotenoids were canthaxanthin, β-carotene 5,6-epoxide, β-carotene 5,8-epoxide and (9Z)- and (13Z)-β-carotenes. Two metabolites were detected from the cyanobacterial extract with plant inhibitory effects. The metabolites with exact masses 852.330209 [M + H] and 772.370529 [M + H] were purified and tested with the *Sinapis* test (Kós et al. 1995). The IC_{50} value of the extract was 15 $mg \cdot ml^{-1}$.

Indoor experiment

Out of the total 54 pots, seedlings emerged in 52 pots; seedlings emerged in only two of the three *Nostoc*-treated pots of *P. limosa* and *T. repens.* Species identity had a significant effect on each measured variable except dry matter content, and *Nostoc* treatment on each variable except dry weight (Table 2). Their interaction had a significant effect only on germination rate and the fresh weight of seedlings (Table 2), i.e. not all species reacted in the same way to the different treatments. Germination rate of five species was significantly lower in the *Nostoc* extract treatment compared to the control (Fig. 1). Regarding fresh weight, we detected a significant difference between treated and control plants only in *H. jubatum* (Fig. 2). For mean + SE values of dry weight, dry matter content and seedling length for all studied species see Appendix S4.

Discussion

According to the literature search, until now a total of 45 papers have dealt with the effects of toxic cyanobacteria on the germination and growth of terrestrial plants. However,

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Table 3. Results of the germination experiment: the effect of species identity, treatment (control or treated) and their interaction on germination rate (%), fresh and dry weight per seedling (mg), dry matter content per seedling (%) and seedling length (mm); tested with GLM.

	Species			Treatment			Species × Treatment		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Germination Rate	41.966	8.45	**<0.001**	39.853	1.52	**<0.001**	4.694	8.36	**0.001**
Fresh Weight	34.094	8.43	**<0.001**	21.417	1.50	**<0.001**	2.317	8.34	**0.042**
Dry Weight	35.583	8.43	**<0.001**	3.398	1.50	*0.074*	1.671	8.34	0.142
Dry Matter Content	1.756	8.43	0.121	10.947	1.50	**0.002**	2.192	8.34	*0.053*
Seedling Length	135.020	8.43	**<0.001**	22.650	1.50	**<0.001**	1.290	8.34	0.281

Significant effects are marked with bold, marginally significant effects are marked with italics.

all these studies dealt with crop plants or with a few other economically important grassland species (*F. rubra*, *L. perenne* and *T. repens*). They focused mostly on sites and species contaminated by cyanotoxins via spray irrigation with cyanobacteria-containing water (Wiegand & Pflugmacher 2005). Even though we found 45 papers examining the effects of toxic cyanobacteria on the germination and growth of terrestrial plants, their effects on natural flora and plant communities remained unclear.

Our results suggest that the presence of *Nostoc* colonies might affect the establishment of grassland species. We found that the effect of the *Nostoc* extract was species-specific, which suggests that *Nostoc* species may be an indirect driver of the interspecific competition between grassland plants. Our results also suggest that *Nostoc* species can have an important role in biotic filtering and in determining which species of the local species pool can be assembled into the community (Keddy 1992; Díaz et al. 1998).

The Species × Treatment interaction was significant in the case of germination rate and fresh weight, thus, regarding these two variables, *Nostoc* treatment affected species differently. For example, while the germination rate of four species decreased due to the treatment, *B. eruciformis* germination tolerated the potential allelopathic effects of *Nostoc*, and it even seems to have benefited from the presence of a toxic but also N-fixing cyanobacterium. In contrast, we observed the most severe adverse effects in the germination and establishment of the adventive, invasive grass *H. jubatum*. This finding indicates that this species is probably not adapted to the potential allelopathic effects of the studied *Nostoc* species, which may constrain its colonization in natural grasslands with a high amount of terrestrial *Nostoc* colonies. *Nostoc* treatment had a significant and generally positive effect on the dry matter content (dry weight/fresh weight × 100) of the seedlings, i.e. mostly indicating a reduction of water content in seedlings. This result might be attributed to the fact that cyanotoxins often reduce growth and cause morphological alterations in the roots of vascular plants that severely affects water uptake (found, e.g. in *S. alba* by M-Hamvas et al. 2003 and *Phragmites australis* by Máthé et al. 2007). Our results

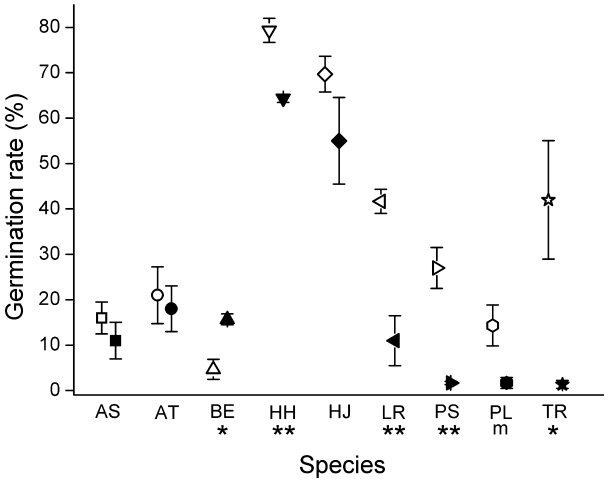

Fig. 1. Germination rate (%) of the studied species. Empty symbols represent germination of control seeds, filled symbols represent germination of *Nostoc*-treated seeds. Significant differences are marked with asterisks: m – $P < 0.1$, *$P < 0.05$, **$P < 0.01$. AS, *Agrostis stolonifera*; AT, *Aster tripolium* ssp. *pannonicus*; BE, *Beckmannia eruciformis*; HH, *Hordeum hystrix*; HJ, *Hordeum jubatum*; LR, *Lepidium ruderale*; PS, *Plantago schwarzenbergiana*; PL, *Puccinellia limosa*; TR, *Trifolium repens*.

regarding *T. repens* were also in accordance with the results of Crush et al. (2008) who found that cyanobacteria treatment did not have a significant effect on the root and shoot dry weight of this species.

A number of studies have assessed the potential impact of cyanobacteria on natural macrophyte communities in aquatic ecosystems (Mitrovic et al. 2005; Kinnear et al. 2008; Jámbrik et al. 2010; Ha & Pflugmacher 2013). Some of these findings can be used as a comparison with terrestrial plants. These studies found that cyanotoxins and extracts have concentration-dependent negative effects on a number of macrophytes, e.g. *Chara* spp., *Nitella* spp. and *Myriophyllum variifolium* (Casanova et al. 1999); *Phragmites australis* (Máthé et al. 2007); *Spirodela oligorrhiza* (Kinnear et al. 2007); *Chara* spp. and *Nitella hyalina* (Rojo et al. 2013). However, a few papers suggested that cyanobacteria might have stronger negative effects on aquatic

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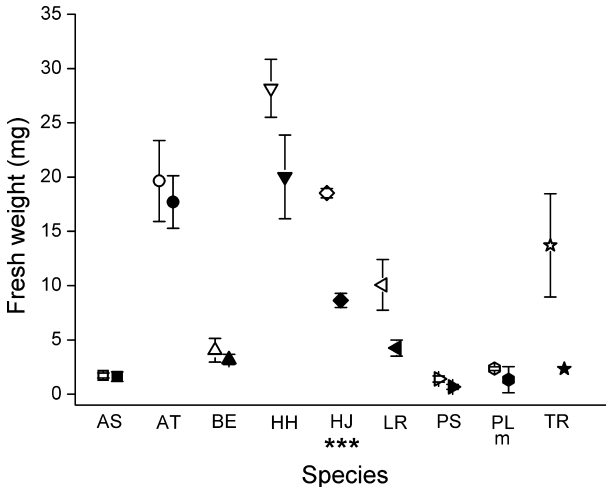

Fig. 2. Seedling fresh weight of the studied species. Empty symbols represent fresh weight of control plants, filled symbols represent fresh weight of *Nostoc*-treated plants. Significant differences are marked with asterisks: m – $P < 0.1$, ***$P < 0.001$. AS, *Agrostis stolonifera*; AT, *Aster tripolium* ssp. *pannonicus*; BE, *Beckmannia eruciformis*; HH, *Hordeum hystrix*; HJ, *Hordeum jubatum*; LR, *Lepidium ruderale*; PS, *Plantago schwarzenbergiana*; PL, *Puccinellia limosa*; TR, *Trifolium repens*.

macrophytes by covering than by allelopathic compounds (Casanova et al. 1999; Roijackers et al. 2004). Nevertheless, papers studying the effects of cyanobacteria on macrophytes mostly concluded that cyanobacteria might have substantial impact on the composition and structure of aquatic macrophyte communities (Mitrovic et al. 2004; Kinnear et al. 2007; Máthé et al. 2007; Rojo et al. 2013).

In grasslands *Nostoc* colonies may have substantial negative effects when they are present at high density due to the above-mentioned chemical effects and through the formation of a physical barrier similar to litter (Facelli & Pickett 1991). When only smaller or scattered colonies are present they may have neutral or positive effects, similar to the density-dependent effects of plant–plant interactions (Callaway & Walker 1997). Their potential positive effects can be expected to be stronger in environments where plants face severe abiotic stress (le Roux & McGeoch 2008; Butterfield 2009; Kelemen et al. 2015), thus the amelioration of soil properties and microclimate by *Nostoc* colonies can be quite important for the germination and establishment of plants (Hawkes 2004; Serpe et al. 2006). Moreover, Belnap (2002) found that N fixation rates of cyanobacteria are higher in habitats with heavily fluctuating soil moisture (such as alkali grasslands). This may imply that those plants of the previously described habitats that tolerate the allelopathic effects can benefit from the increased N fixation.

In conclusion, the potential effects of cyanobacterium colonies on grassland vegetation and their role in vegetation dynamics have been largely overlooked so far. Although many other factors must be kept in mind when assessing the effects of cyanobacteria, our results draw attention to the fact that cyanobacteria should be considered not just in aquatic ecosystems, but also in terrestrial ecosystems where they occur. Our results suggest that by altering crucial measures of plant performance *Nostoc* colonies might play an important role in shaping the diversity, species composition and structure of natural plant communities.

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Supporting Information

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

Appendix S1. Photograph of an alkali grassland with *Nostoc* colonies.

Appendix S2. Detailed description of the methods used for determining the carbohydrate and carotenoid content and composition of the *Nostoc* extract.

Appendix S3. Results of previous papers studying the effects of different cyanobacteria on terrestrial vascular plants.

Appendix S4. Results of the germination tests: means and standard errors based on the data of seedlings (mean ± SE).