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Nitrogen deposition reduces the cover of biocrust-forming lichens and soil pigment content in a semiarid Mediterranean shrubland

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Abstract Biocrusts are key drivers of the structure and functioning of drylands and are very sensitive to disturbance, including atmospheric nitrogen (N) deposition. We studied the impacts of simulated N deposition on biocrust community composition and soil photosynthetic and photoprotective pigment content after five years of N application in a European semiarid Mediterranean shrubland. The experiment consisted in six experimental blocks with four plots, each receiving 0, 10, 20, or 50 kg NH_4NO_3-N ha^{-1} $year^{-1}$ + 6–7 kg N ha^{-1} $year^{-1}$ background. After 5 years of N application, total lichen cover decreased up to 50% compared to control conditions and these changes were only clearly evident when evaluated from a temporal perspective (i.e. as the percentage of change from the first survey in 2008 to the last survey in 2012). In contrast, moss cover did not change in response to N, suggesting that biocrust community alterations operate via species- and functional group-specific effects. Interestingly, between-year variations in biocrust cover tracked variations in autumnal precipitation, showing that these communities are more dynamic than previously thought. Biocrust species alterations in response to N were, however, often secondary when compared to the role of ecologically relevant drivers such as soil pH and shrub cover, which greatly determined the composition and inter-annual dynamics of the biocrust community. Similarly, cyanobacterial abundance and soil pigment concentration were greatly determined by biotic and abiotic interactions, soil pH for pigments, and organic matter content and shrub cover for cyanobacteria. Biocrusts, and particularly the lichen component, are highly sensitive to N deposition and their responses to pollutant N can be best understood when evaluated from a temporal and multivariate perspective, including impacts mediated by interactions with biotic and abiotic drivers.

Keywords Abiotic and biotic interactions · Biocrusts · Mediterranean ecosystems · Nitrogen deposition · Soil pigments · Temporal dynamics

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Introduction

The global nitrogen (N) cycle has been widely altered in both terrestrial and aquatic ecosystems due to human activities (Gruber and Galloway 2008; Fowler et al. 2013). Atmospheric N deposition is predicted to continue increasing globally in future scenarios due to the ongoing process of agricultural intensification, associated with the emissions of reduced N (NH_3), and to fossil fuel demand and use, which are directly related to the emissions of oxidised N (NO_x), particularly in countries like China and India (Gruber and Galloway 2008; Fowler et al. 2013). Nitrogen deposition causes ecosystem eutrophication, soil acidification, base cation depletion, and solubilisation of toxic metals such as aluminium (Horswill et al. 2008). These undesired consequences of N deposition are, taken together, currently contributing to the ongoing global biodiversity loss and the widespread ecosystem degradation (Sala et al. 2000; Bobbink et al. 2010).

Manipulation and observational studies have contributed to demonstrate the negative impacts that N deposition can exert on the structure and functioning of terrestrial ecosystems worldwide (Bobbink et al. 2010), including tropical forests (Lü et al. 2010), Mediterranean shrublands (Vourlitis et al. 2009; Ochoa-Hueso et al. 2017), European grasslands (Stevens et al. 2004), and temperate and boreal forests (Waldrop and Zak 2006; DeLuca et al. 2007). These effects are not only dependent on the N loads received but also on the dominant type of atmospheric deposition (dry vs. wet and oxidised vs. reduced) as well as on the different sensitivity of the species present in the ecosystem (Stevens et al. 2010; Bobbink et al. 2010). Moreover, it has also been shown that cumulative N deposition loads since the industrial revolution can explain lower-than-expected levels of plant diversity in acid grasslands of Europe (Duprè et al. 2010) and in boreal ecosystems (Gundale et al. 2011), whereas even low N deposition loads can be enough to negatively impact on the most sensitive elements of the ecosystems, such as mycorrhizal infection rates and diversity (Gordon et al. 2002) and lichen communities (Reed et al. 2016). For example, in a fertilisation study carried out in a low-N deposition boreal forest, the epiphytic lichen community was significantly altered in response to the addition of only 6 kg N ha^{-1} $year^{-1}$ and the community-level response was attributed to physiological responses of the individual species rather than to changes in competitive interactions (Johansson et al. 2012).

Biological soil crusts (hereafter, biocrusts) are an important constituent of plant communities from arid and semiarid ecosystems worldwide (Belnap et al. 2008), including semiarid Mediterranean shrublands of central Spain (Maestre et al. 2011). These usually species-rich communities, composed of several species of cyanobacteria, algae, protozoa, fungi, mosses, and lichens, are found in the uppermost millimetres of the soil surface and play an important ecological role by contributing to C and N fixation (Castillo-Monroy et al. 2010), protecting against erosion, and regulating topsoil water dynamics (Rodríguez-Caballero et al. 2012; Concostrina-Zubiri et al. 2013). The impacts of N deposition on biocrusts are also particularly relevant, given the ability of some of their constituent species to fix atmospheric N_2 through the nitrogenase enzymatic complex (Belnap 2002). In temperate zones, a multitude of studies have evaluated the impacts of increased N deposition on moss and lichen communities (Arróniz-Crespo et al. 2008). However, the amount of studies devoted to examining the effects of global change drivers on late-successional biocrust communities from arid and semiarid zones is very limited (Belnap et al. 2008; Escolar et al. 2012), particularly in relation to N deposition (Ochoa-Hueso and Manrique 2011, 2013; Reed et al. 2016; Ochoa-Hueso 2017; Ochoa-Hueso et al. 2017). In fact, to the best of our knowledge, no study has yet simultaneously evaluated the effects of increased N deposition on the composition and physiology of highly biodiverse biocrust communities under realistic field conditions.

In this study, we aimed at filling this knowledge gap by evaluating the impacts of five years of simulated N deposition on the composition, abundance (including mosses, lichens, and cyanobacteria), and soil pigment concentration of the biocrust community from a Mediterranean semiarid shrubland in central Spain. In the semiarid shrublands developed on calcareous soils of central Spain, biocrust cover and distribution is highly determined by soil properties and interactions with shrubs (Ochoa-Hueso et al. 2011). In particular, soil pH and the availability of micronutrients are positively related to lichen cover due to a lack of plant competition under high pH conditions and their characteristic nutritional demands, whereas shrub cover tends to favour mosses at the expense of lichens (Ochoa-Hueso et al. 2011). Given that N deposition can influence soil pH, micronutrient availability (Horswill et al. 2008) and shrub cover (Cabal et al. 2017), an increase in N deposition should have, at least, indirect impacts on biocrust communities via changes in soil chemistry and shrub cover. Increased N deposition could also cause direct negative impacts associated with the toxic effects produced by high levels of N, especially ammonium (Ochoa-Hueso et al. 2013a). Finally, greater nitrophilous plant biomass in response to increased N could also contribute to the extirpation of biocrust communities through competitive exclusion (Ochoa-Hueso and Manrique 2014). Therefore, we hypothesise that simulated N deposition would significantly reduce biocrust cover, particularly the lichen element, despite clear associations between biocrust cover and soil physicochemistry and shrub cover (H1). However, we also hypothesise that the initially highly heterogeneous distribution of biocrusts at the study site will delay the appearance of the effects of N addition, thus partially buffering the response to N (H2). This will result in effects becoming only clearly evident when evaluated from a temporal perspective. In addition, we predict that simulated N deposition would significantly reduce soil pigment content and cyanobacterial abundance (H3). However, given that these parameters were only evaluated once after five years and thus no temporal evaluation is possible, the effects of N addition will be less clear than in the case of moss-lichen communities (H4). Finally, based on the role of soil pH on soil pigment abundance (see the "Results" section) and the incipient acidification detected at the field site under high N addition loads (Ochoa-Hueso et al. 2013a), we hypothesise that pigment responses would be dependent upon within-site soil pH spatial variability (H5).

Materials and methods

Study area

This study was carried out in the Nature Reserve 'El Regajal-Mar de Ontígola' (Aranjuez, Spain; 40° 00′ N, 3° 36′ W). The

Reserve is located in a semirural area that is approximately 50 km away from the city of Madrid and 580 m above the sea level. The climate of the area is semiarid Mediterranean, characterised by a long, dry summer period (from May to September) and a rainier period during winter. The average annual precipitation is around 425 mm, and the average annual temperature is approximately 15 °C. Yearly temperature variations are large; air temperatures can reach over 40 °C in summer and below 0 °C in the coldest months. The soil is alkaline (seasonal variations in pH range between 7.79 and 8.01), and therefore, nitrate is the most dominant form of inorganic N in soil (Ochoa-Hueso et al. 2013a). The predominant vegetation at the top of the limestone hills is characterised by the presence of woody species such as *Quercus coccifera* L. (kermes oak) and *Rosmarinus officinalis* L. (rosemary; 29.4 ± 2.8% SE cover on average within the plots in 2008; Cabal et al. 2017) and includes the presence of well-developed early- and late-successional biocrusts composed of cyanobacteria, lichens (especially *Cladonia foliacea* (Huds.) Willd., *Squamarina lentigera* (Weber) Poelt, *Diploschistes diacapsis* (Ach.) Lumbsch, etc.), and mosses (especially *Tortella squarrosa* (Brid.) Limpr.; Ochoa-Hueso et al. 2011). All the species mentioned are very common in Mediterranean soils. Here, we use *Tortella squarrosa* (Brid.) Limpr. as the newly accepted name for *Pleurochaete squarrosa* (Brid.) Lindb. in Ros et al. (2013), the most recent moss European checklist (which considers the systematic conclusions of Werner et al. 2005 and Grundmann et al. 2006). In autumn 2008, the average late-successional biocrust cover at the site was 19.7% (6.1% lichens and 13.6% mosses).

Nitrogen addition experiment

In October 2007, we selected six experimental blocks corresponding to different open areas dominated by rosemary shrubs scattered between dense kermes oak scrubs. Each block was divided into four 2.5 × 2.5 m plots. Since then, each plot has been fertilised with N loads equivalent to 0, 10, 20, and 50 kg N ha^{-1} $year^{-1}$ in order to simulate N deposition scenarios equivalent to those predicted for 2050 in the Mediterranean basin or to N deposition rates measured in other Mediterranean regions (Fenn et al. 2003; Phoenix et al. 2006). The estimated background N deposition for the area is 6–7 kg N ha^{-1} $year^{-1}$ (reduced and oxidised inputs are almost equivalent: 56% NH_4^+-N and 44% NO_3^--N, based on modelling and on-site data; Ochoa-Hueso et al. 2013a). From October 2007 to September 2011, N was applied monthly as ammonium nitrate (NH_4NO_3), except in the summer period (July and August) during which no N was added. Each monthly treatment consisted in 2 l of water with N concentrations of 0, 0.019, 0.037, and 0.093 M. Ammonium nitrate was selected to mimic N deposition over other N-based fertilisers because its oxidised-to-reduced N ratio is comparable to that of the study site. In September, a three-month N load was applied to simulate the peak of N mobilisation that typically takes place with the autumn rains, when all accumulated dry N is solubilised and, therefore, made available. From September 2011 onwards, N was applied quarterly.

Biocrust cover

Biocrust cover estimates were carried out in autumn 2008, autumn 2009, and autumn 2012. Surveys were consistently carried out in autumn because this is the growing season for biocrust-forming organisms and they are easier to distinguish (they are usually hydrated). For surveying purposes, each plot was sub-divided into 12 squares (0.5 × 0.5 m). The cover (%) of all moss and lichen species present was visually estimated to the nearest 1% in six alternate squares as extensively described in Ochoa-Hueso et al. (2011). Shrub cover was also visually estimated for each square, but this variable was only used as a predictor variable in our statistical analyses. We did not carry out initial cover measurements before the commencement of the experiment in autumn 2007, which could, potentially, have limited our ability to detect and properly interpret biocrust responses to N; however, in autumn 2008, biocrust cover and physiology was still tightly linked to natural variations in the soil environment and shrub cover at the study site (Ochoa-Hueso et al. 2011) and we did not detect any significant effect of N additions (this study). Therefore, in this study, we consider autumn 2008 as our baseline survey.

Soil sampling

The soil sampling for cyanobacterial counts and pigment determinations was carried out in autumn 2012. Given that biocrust organisms inhabit the first millimetres of soil, all collected samples were superficial. We combined between four and eight 0–0.5-cm-depth and 5-cm-diameter-wide subsamples for each plot. Samples were transported to the laboratory into plastic bags where they were sieved through a 2-mm mesh. Samples were kept frozen at − 20 °C until further analyses were done.

Pigment analyses

To determine the amount of pigments in soil, 1 g from each sample was diluted in 0.5 ml distilled water, after which 4 ml of high-performance liquid chromatography (HPLC)-acetone was added. Samples were then transferred to test tubes, which were filled with acetone to complete 10 ml. The rest of the tube was filled with helium and covered with Parafilm™. After this, samples were refrigerated at 4 °C for 24 h. Samples were then filtered using GF/F filter paper and concentrated to 3 ml. Soil pigments were separated by HPLC

according to a modification of the methods in Val et al. (1994). We injected 25 μl of extract into a C18 column. The mobile phase velocity was 1.2 ml min^{-1}, and the elution time was 30 min. The peak identification and quantification was determined by commercial standards (VKI, Hørsholm, Denmark) of neoxanthin; violaxanthin; diadinoxanthin; myxoxanthophyll; antheraxanthin; lutein; zeaxanthin; cantha-xanthin; chlorophyll *a*, *b*, and c_2; echinenone; and β-carotene. As scytonemin is not available commercially, its peak was estimated from its peak area at 436 nm following Bowker et al. (2002).

Cyanobacterial counts

Soil cyanobacterial counts were done based on the protocol described in Bowker et al. (2002). Firstly, 3 ml of distilled water was added to 1.000 ± 0.005 g of each soil sample. The resultant slurry was thoroughly mixed, after which aliquots of soil were taken with a Pasteur pipette and deposited on a microscope slide. For each sample, 20 optical fields were observed using the × 40 objective and the genera of all different filamentous cyanobacteria present were recorded. The presence of diatoms was also registered.

Statistical analysis

Nitrogen addition effects on total and individual biocrust-forming species cover were analysed by means of linear mixed-effects models using the 'lme' function of the *nlme* package in R version 3.4.0 (R Core Team 2017), with time and N as fixed factors and block and plot as random factors. We also carried out general linear mixed models separately for each year and for the percentage of change in cover (%) between the last (2012) and first (2008) biocrust sampling campaigns, with N as a fixed factor and block as a random factor. Soil pigment and microscope count data were also analysed by means of linear mixed models, with N as the fixed factor and block as the random factor, respectively. We tested the potential role of soil pH as a mediator of soil pigment and cyanobacterial community response to N by means of covariance analyses with both *soil pH* and the *N addition* × *soil pH* interaction as covariates. We carried out this analysis only in the case of variables that showed statistically significant associations with pH in a multiple regression analyses (see "Results" below).

Additionally, we carried out two redundancy analyses (RDA) using the % of change in cover of the ten most dominant biocrust-forming species recorded (from 2008 to 2012), on the one hand, and the cyanobacterial counts, on the other hand. As environmental drivers, we included N deposition and previously identified ecologically meaningful variables for biocrust communities at the study site such as soil pH, soil organic matter (SOM) content, non-base cation availability (hereafter referred to as non-bases), and shrub cover (Ochoa-Hueso et al. 2011). Plot-level soil chemical data was obtained from Ochoa-Hueso et al. (2013a). The permutation-based significance of the predictor variables was evaluated using the 'envfit' function of the *vegan* package. We also carried out multiple linear regression analyses to relate biocrust species-level cover (2012) and % of change, soil pigment abundance, and cyanobacterial counts to the same environmental variables (i.e. soil pH, SOM, non-bases, and shrub cover). Models were retained based on their lowest Akaike information criterion (AIC). Multimodels were done using the 'stepAIC' function of the *MASS* package.

Results

Biocrust cover

Biocrust cover fluctuated over time in response to autumnal precipitation (Fig. 1). The experimental plots contained three moss species, of which *Tortella squarrosa* (Brid.) Limpr. was the most abundant (mean annual cover ranged between 5.5 and 13.6%). The other two species were *Didymodon vinealis* (Brid.) R.H. Zander (mean cover ranged between 0.06 and 0.34%) and *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr (mean cover ranged between 0.34 and 0.36%). We also found 12 species of lichens, of which *Cladonia foliacea* (Huds.) Willd. (mean cover ranged between 0.74 and 1.28%), *Cladonia subrangiformis* Sandst. (mean cover ranged between 0.28 and 0.64%), *Collema* sp. (mean cover ranged between 0.54 and 0.66%), *Endocarpon pusillum* Hedw. (mean cover ranged between 0.19 and 0.33%), *Diploschistes diacapsis* (Ach.) Lumbsch (mean cover ranged between 0.73 and 1.12%), *Fulgensia fulgens* (Sw.) Elenkin (mean cover ranged between 0.20 and 0.28%), *Psora decipiens* (Hedw.) Hoffm. (mean cover ranged between 0.21 and 0.35%), *Squamarina* sp. (mean cover ranged between 0.59 and 0.94%), and *Toninia sedifolia* (Scop.) Timdal (mean cover ranged between 0.20 and 0.29%) were dominant.

In agreement with our first hypothesis, five years of experimental N deposition significantly reduced the cover of lichens up to 50% compared to the reference conditions (i.e. calculated as the % of change from the first to the last survey), and this effect was significant for all the N treatments (Table 1, Figs. 1 and 2). In contrast, these impacts were not significant when evaluated as the percentage of soil surface covered by lichens, despite the similar trend found in the latest data collected (2012), as predicted by our second hypothesis (Fig. 1). In contrast to lichens, moss cover (mainly represented by *T. squarrosa*) was not significantly affected by N addition (Tables 1 and 2). Spatial variations in biocrust cover from autumn 2008 to autumn 2012 were mainly driven by shrub cover and soil properties, particularly soil pH (Table 3). Similar to % cover, changes over time in the community

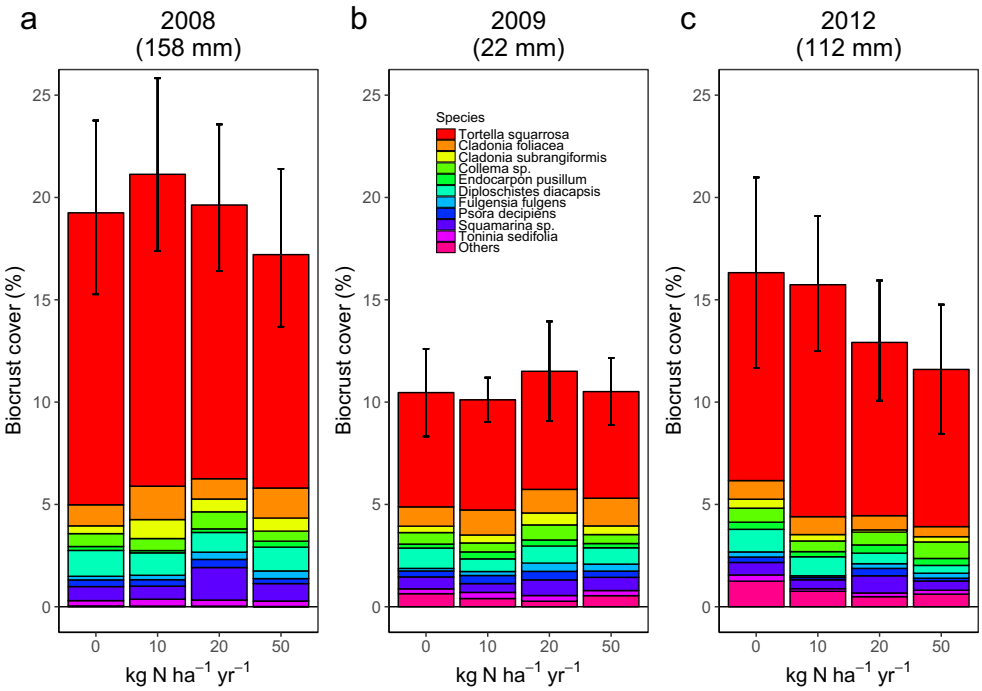

Fig. 1 **a**–**c** Nitrogen addition and time effects on the biocrust cover after five years of N addition. Precipitation values for each sampling year correspond to the summed precipitation of the September and October months, which are the months of the year where biocrust growth and physiology are at their peak. For the sake of clarity, standard error bars ($n = 6$) are only represented at the community level. *Tortella squarrosa* (red bars) is the only moss species; the rest are lichens

structure were primarily driven by soil properties, although N deposition played a much more significant role in this case (Table 4, Fig. 3). Impacts of N deposition were also species-specific and year-dependent (Tables 2 and 3). For example, *F. fulgens* showed a transient increase in cover after two years of fertilisation that had disappeared by year 5, while *C. foliacea* and *D. diacapsis* cover were negatively correlated with N fertilisation after five years. Additionally, *T. squarrosa* and most lichen species were very sensitive to temporal dynamics regardless of simulated N deposition (Table 2, Fig. 1).

Table 1 Nitrogen addition effects on the percentage (%) of change of biocrust cover between the years 2008 and 2012

% change	Nitrogen	
	df	*F*-value
Tortella squarrosa	3, 20	0.17
Cladonia foliacea	3, 20	1.74
Cladonia subrangiformis	3, 20	3.01† (↓)
Collema sp.	3, 20	1.74
Diploschistes diacapsis	3, 20	0.23
Endocarpon pusillum	3, 20	1.63
Fulgensia fulgens	3, 20	1.27
Psora decipiens	3, 20	1.04
Squamarina sp.	3, 20	0.40
Toninia sedifolia	3, 20	2.79† (↓)
Total lichen	3, 20	*4.56** (↓)
Total biocrust	3, 20	0.92

The arrow indicates the direction of the effect. Significant effects are in italics. *Tortella squarrosa* is the only moss species; the rest are lichens

df degrees of freedom

$^{\dagger}P < 0.1$; $^{*}P \leq 0.05$

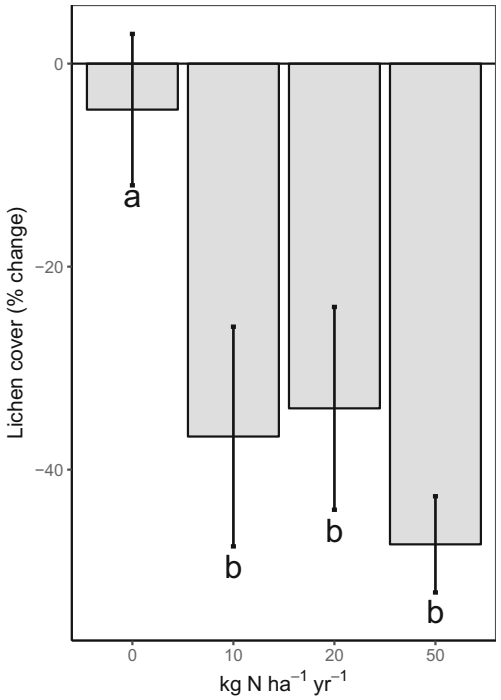

Fig. 2 Nitrogen addition effects on the percentage (%) of change of lichen cover between the years 2008 and 2012 (i.e. in 2012, lichen cover was reduced in approximately 50% under the highest N addition load in relation to the initial conditions). Different letters indicate statistically significant differences ($P \leq 0.05$). Error bars indicate 1 SE

Table 2 Nitrogen addition and time effects on the total biocrust and species-specific (ten most abundant species) cover after 5 years of N addition. Nitrogen effects are also separately shown for each sampling year

Species cover (%)	All years						2008		2009	2012
	Nitrogen		Time		N × T		Nitrogen		Nitrogen	Nitrogen
	df	*F*-value	*df*	*F*-value	*df*	*F*-value	*df*	*F*-value	*F*-value	*F*-value
Tortella squarrosa	3, 20	0.20	2, 40	*16.56***	6, 40	0.23	3, 20	0.21	0.02	0.27
Cladonia foliacea	3, 20	0.12	2, 40	*5.54***	6, 40	0.94	3, 20	0.29	0.15	0.53
Cladonia subrangiformis	3, 20	0.09	2, 40	*5.03**	6, 40	1.25	3, 20	0.34	0.35	0.64
Collema sp.	3, 20	0.80	2, 40	0.74	6, 40	0.74	3, 20	1.50	1.38	0.33
Diploschistes diacapsis	3, 20	0.56	2, 40	*5.53***	6, 40	1.24	3, 20	0.12	0.60	*3.10** (↓)
Endocarpon pusillum	3, 20	0.15	2, 40	*5.55***	6, 40	1.68	3, 20	0.74	0.70	0.48
Fulgensia fulgens	3, 20	1.19	2, 40	1.72	6, 40	1.19	3, 20	0.86	2.94† (↑)	0.60
Psora decipiens	3, 20	0.53	2, 40	*3.30**	6, 40	0.62	3, 20	0.20	0.69	1.02
Squamarina sp.	3, 20	0.38	2, 40	2.67†	6, 40	0.58	3, 20	0.46	0.34	0.32
Toninia sedifolia	3, 20	0.07	2, 40	*4.41**	6, 40	1.75	3, 20	0.52	0.22	1.15
Total lichen	3, 20	0.18	2, 40	*13.14***	6, 40	1.35	3, 20	0.26	1.77	0.77
Total biocrust	3, 20	0.16	2, 40	*19.89***	6, 40	0.51	3, 20	0.18	0.10	0.40

The arrow indicates the direction of the effect. Significant effects are in italics. *Tortella squarrosa* is the only moss species; the rest are lichens

T time, *df* degrees of freedom

† $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$

Cyanobacterial diversity

Nostoc Vaucher ex Bornet & Flahault was the most abundant cyanobacterial genus in soil (Fig. 4a). Other genera found, in decreasing order of abundance, were the following: *Microcoleus* J.B.H.J. Desmazières ex M. Gomont, *Leptolyngbya* C.A. Agardh ex Gomont, *Scytonema* Agardh ex Bornet & Flahault, and *Calothrix* Agardh ex Bornet & Flahault (Fig. 4a). In contradiction with our third hypothesis and despite the trend towards a decrease, we did not find significant effects of N addition on cyanobacterial community structure and abundance (Tables 4 and 5, Figs. b, 4a), although the multiple regression analyses showed that *Microcoleus* increased in response to N deposition while *Calothrix* decreased (Table 6). Overall, these findings support our fourth hypothesis of less clear effects of N addition on cyanobacterial communities than in the case of moss-lichen communities. Similar to the biocrust response and also in support of our hypothesis of the buffering role of spatial heterogeneity (H2), shrub cover and SOM were main drivers of soil cyanobacterial and diatom abundance (Tables 4 and 6).

Soil pigments

Scytonemin, a photoprotective pigment exclusively found in cyanobacteria, was the most abundant pigment in soil, with a concentration of approximately 28 times the concentrations of chlorophylls *a* and *b* (Fig. 4b). Together with scytonemin, other characteristic pigments of cyanobacteria (canthaxanthin and echinenone), diatoms (diadinoxanthin), and green algae (violaxanthin, chlorophyll *b*, and lutein) were present in the soil, although in much lower concentrations (pooled as others in Fig. 4b). Zeaxanthin (vascular plants and cyanobacteria) or chlorophyll c_2 (diatoms) were not detected in the analysis. Similar to biocrust cover, soil pH played an important role in determining the abundance of pigments in soil, as has already been described for lichens (Ochoa-Hueso et al. 2011). In this sense, scytonemin increased with soil pH, whereas lutein and chlorophylls decreased with increasing soil pH values. Soil pigment concentration was not significantly altered by N fertilisation in the mixed models, despite the trend towards a reduction (Fig. 4b). However, both the covariance and multiple regression analyses showed that scytonemin, chlorophyll, and lutein contents were negatively impacted by simulated N deposition, which is in agreement with our fifth hypothesis (Tables 5 and 6). In the case of scytonemin, we detected a decoupling between soil pH and pigment content in the highest N treatment, as indicated by a significant N × pH interaction (Fig. 5) that was highly driven by one plot fertilised with 50 kg N ha^{-1} $year^{-1}$ in which soil acidification was particularly marked (Ochoa-Hueso et al. 2013a). When this plot was removed from the analysis, the effects of N and pH were still marginally significant ($P = 0.06$) and significant ($P < 0.01$), respectively, but the N × pH interaction term was

Table 3 Backward multiple regression analyses between moss and lichen cover in 2012 and moss and lichen percentage (%) of change after five years of N addition and selected ecologically relevant environmental variables (shrub cover, pH, SOM, and non-bases) and N deposition

2012	Regression model with the lowest AIC	R^2	P
Tortella squarrosa	9.80–1.61 × SOM + 0.33 × shrubs	0.52	< 0.01
Cladonia foliacea	2.93–*0.01 × N deposition* + 0.09 × SOM − 0.42 × pH + 0.08 × non-bases + 0.01 × shrubs	0.51	< 0.01
Cladonia subrangiformis	− 0.78 + 0.13 × non-bases	0.54	< 0.01
Collema sp.	NS	–	–
Diploschistes diacapsis	1.02–*0.01 × N deposition* − 0.14 × SOM + 0.08 × non-bases	0.29	0.02
Endocarpon pusillum	NS	–	–
Fulgensia fulgens	− 1.20 − 0.04 × SOM + 0.21 × pH	0.29	< 0.01
Psora decipiens	0.52–0.06 × SOM	0.15	0.04
Squamarina sp.	− 3.07 − 0.14 × SOM + 0.56 × pH	0.23	0.03
Toninia sedifolia	NS	–	–
Total lichen	NS	–	–
Total biocrust	15.90–1.99 × SOM + 0.36 × shrubs	0.52	< 0.01
% change (2012–2008)			
Tortella squarrosa	− 2.89 + 0.32 × pH − 0.04 × non-bases + 0.02 × shrubs	0.41	< 0.01
Cladonia foliacea	4.57–*0.01 × N deposition* − 0.51 × pH − 0.08 × non-bases	0.36	< 0.01
Cladonia subrangiformis	− 5.13 + 0.62 × pH	0.30	< 0.01
Collema sp.	NS	–	–
Diploschistes diacapsis	− 6.44 + 0.49 × pH + 0.32 × non-bases	0.48	< 0.01
Endocarpon pusillum	NS	–	–
Fulgensia fulgens	NS	–	–
Psora decipiens	NS	–	–
Squamarina sp.	1.41–0.31 × pH + 0.10 × non-bases	0.28	0.01
Toninia sedifolia	NS	–	–
Total lichen	− 0.16 − *0.007 × N deposition*	0.23	< 0.01
Total biocrust	− 1.89 + 0.22 × pH − 0.04 × non-bases + 0.01 × shrubs	0.32	0.01

Nitrogen addition-related effects are in italics. *Tortella squarrosa* is the only moss species; the rest are lichens

NS no significant model found

Table 4 Permutation-based (n = 999) significance of the relationship between environmental drivers and canonical axes 1 and 2 in redundancy analyses (RDA) with biocrust (change in % cover from 2008 to 2012) and microscope count data (2012)

Biocrust	RDA1	RDA2	R^2	P value
Shrubs	− 0.16	− 0.99	0.15	0.17
Organic matter	− 0.83	0.56	0.33	*0.015**
Soil pH	0.96	− 0.28	0.24	0.054†
Non-base cations	− 0.89	0.46	0.53	*0.003***
N deposition (log + 1)	− 0.76	− 0.65	0.47	*0.006***
Microscope counts				
Shrubs	0.96	0.28	0.24	0.065†
Organic matter	− 0.81	0.59	0.41	*0.014**
Soil pH	− 0.99	0.02	0.05	0.54
Non-base cations	− 0.69	0.72	0.14	0.19
N deposition (log + 1)	0.58	− 0.81	0.01	0.92

† $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$

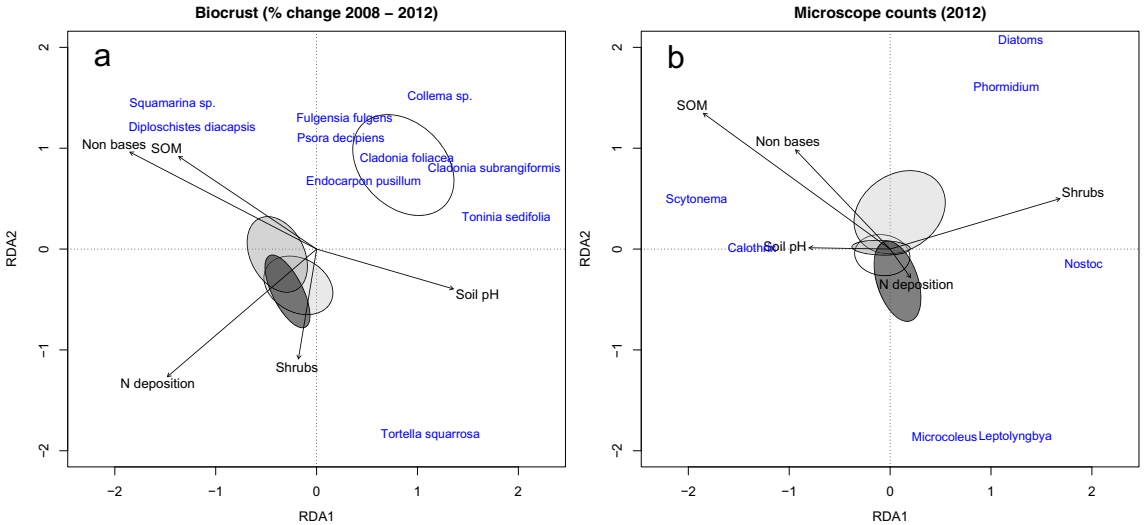

Fig. 3 Biplot showing results of redundancy analyses with **a** biocrust (change in % cover from 2008 to 2012) and **b** cyanobacterial community data (2012). Circles represent the SE of experimental treatments. The colour of the circles denotes the treatment: 0 N (white), 10 N (light grey), 20 N (grey), and 50 N (dark grey). The black arrows and their associated label indicate the magnitude of the relationships between canonical axes and the environmental variables evaluated as well as the direction of the relationship (see Table 4 for permutation-based R^2 and P values). Species loadings have been re-scaled (× 3.75) to ease visualisation

no longer significant ($P = 0.79$). The AIC was, however, lower in the case of the model containing all replicates (AIC = − 114.6) than in the case of the model excluding the outlier (AIC = − 110.2).

Discussion

Our data clearly demonstrate that, in contrast to the commonly accepted static view, biocrust communities from semiarid ecosystems are highly dynamic and that, at the local scale, they can track annual variations in autumnal precipitation (growing season for biocrusts). Intra-annual (i.e. seasonal) variations were, however, not investigated here, although they are also likely to be relevant (Belnap et al. 2006). At the spatial scale considered in this study (2.5 × 2.5 m plots), biocrust community abundance and composition were highly variable and their dynamics were mainly driven by soil properties, particularly soil pH, and shrub cover, both factors previously identified as determinants of the abundance and distribution of biocrusts at the study site (Ochoa-Hueso and Manrique 2011; Ochoa-Hueso et al. 2011). Nitrogen addition also contributed to reduce lichen cover and soil pigments over time (H1 and H3), indicating a great sensitivity of this type of communities to increased N deposition. Moreover, community alterations in response to N were more evident when evaluated from a temporal and multivariate perspective, suggesting cumulative effects and complex interactions between N deposition and the local environmental conditions (H2). This is due to the fact that all plots started off with different values of lichen cover and, although they all followed similar response trajectories, these changes are not easily detected unless the initial conditions (in our case, autumn 2008) are accounted for. This also suggests the role of spatiotemporal environmental variability to buffer against the impacts of N deposition in highly heterogeneous semiarid Mediterranean ecosystems, a phenomenon that has also been observed in English calcareous grasslands subjected to simulated climate change (Fridley et al. 2011).

In the present study, mosses and lichens responded differently to N addition, which conditioned responses at the biocrust community level. Lichens were more sensitive to N, decreasing their cover up to 50% as compared to their reference condition in 2008, whereas the moss cover did not significantly change after 5 years of N fertilisation. Although the lichen cover only decreased from 6%, a number that is highly comparable to other sites where a well-developed late-successional biocrust community is assumed to be relevant, to 3%, this reduction can have far-reaching implications in terms of ecosystem function, given the disproportionately important role that these communities can play in arid and semiarid ecosystems (Jimenez Aguilar et al. 2009). Lichen responses were also species-specific, with the two foliose, oligotrophic lichen species present at the site (*Cladonia foliacea* and *C. subrangiformis*) showing negative responses to N. Previous research at the study site had established a critical load for lichen physiology at approximately 26 kg N ha^{-1} $year^{-1}$ after 2 years of study (Ochoa-Hueso et al. 2013b). Based on the significant response of lichen cover to the lowest level of N addition (plus the background deposition), this critical load can now be reduced to a

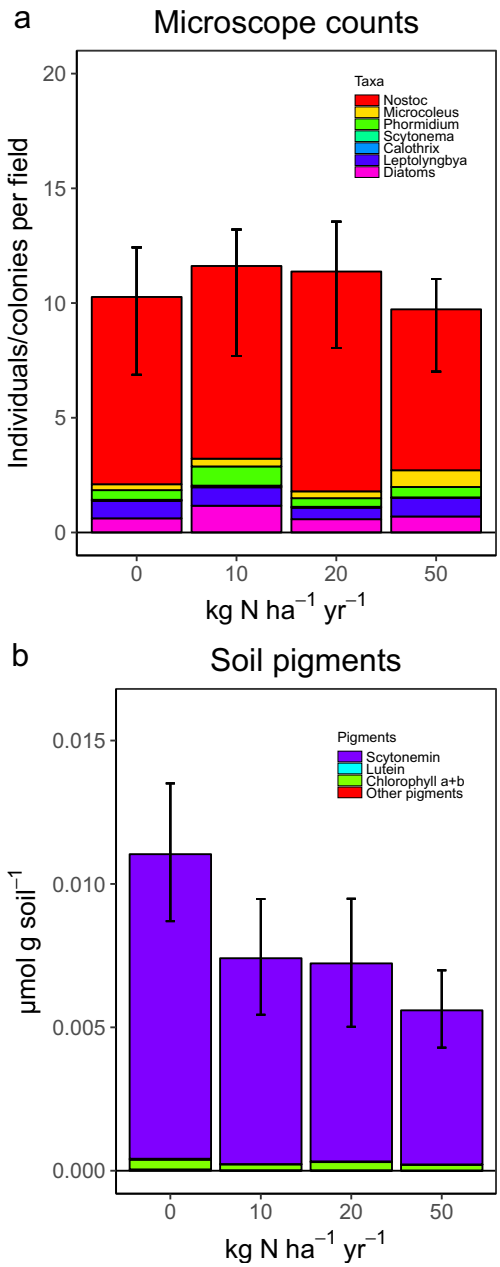

Fig. 4 Nitrogen addition effects on microscope counts (cyanobacteria and diatoms) (**a**) and soil pigments (**b**) after 5 years of N addition. For the sake of clarity, standard error bars are only represented at the group level ($n = 6$)

value between 6 and 16 kg N ha^{-1} $year^{-1}$. This number is still higher than the critical load established for epiphytic lichen communities from boreal forests (< 6 kg N ha^{-1} $year^{-1}$; Johansson et al. 2012) but at least 10 kg N ha^{-1} $year^{-1}$ lower than the critical load established for Mediterranean woodlands based on epiphytic lichen functional groups (26 kg N ha^{-1} $year^{-1}$; Pinho et al. 2011). Nevertheless, this value is still limited by methodological constraints of our experimental design (i.e. relatively high N loads for a sensitive group of organisms such as lichens and moderately high background N deposition at the study site) and so future observational and manipulation studies carried out along representative N deposition gradients will help refine this number.

The lack of clear effects on the moss cover is remarkable. Nitrogen is very rarely a limiting nutrient for bryophytes, as they are able to assimilate it efficiently, especially from atmospheric deposition (Turetsky 2003), and so an increase in N deposition has usually negative effects on the abundance of terrestrial mosses (Arróniz-Crespo et al. 2008). In addition, previous studies on *T. squarrosa* at the same study site (Ochoa-Hueso and Manrique 2013) and along realistic N deposition gradients (Ochoa-Hueso et al. 2014; Izquieta-Rojano et al. 2016) have shown that N deposition can significantly alter the physiology of this moss species. Therefore, significant alterations in moss abundance in the long term in response to N can be expected. In this sense, a trend towards a reduction in the abundance of *T. squarrosa* was evident after five years of fertilisation, despite the lack of statistically significant results. However, the absence of consistent results can be attributed to the high variability of the moss cover across the site and also to complex interactions with the local biotic (e.g. vascular plant cover) and abiotic environment. Supporting this argument, Ochoa-Hueso and Manrique (2013) demonstrated that, under controlled greenhouse conditions, responses of *T. squarrosa* to N fertilisation were dependent upon soil moisture as well as on the competition with herbaceous plants. For example, under high soil moisture conditions, the moss cover increased with N fertilisation. In contrast, its cover decreased when N addition was combined with low water availability. In semiarid Mediterranean areas, water scarcity and drought are the norm and dry N deposition frequently accumulates on biocrusts during rainless periods, becoming available as high-N concentration pulses (Fenn et al. 2003). This may cause toxicity effects and, therefore, reduce the moss cover in the coming years, which would be consistent with our lichen results. Finally, Ochoa-Hueso and Manrique (2013) also showed that high water and high N conditions can reduce the moss cover when N fertilisation coincides with the optimum N load for plant growth, suggesting that competitive exclusion effects can also play an important role in the response of terricolous mosses to N deposition. In any case, the current lack of statistical effects of N deposition on the moss cover in the 5-year period suggests that N can accumulate in *T. squarrosa* tissue without reducing its performance, at least transiently (Pearce et al. 2003; Ochoa-Hueso and Manrique 2013).

Although less clearly than in the case of moss-lichen communities, N deposition significantly contributed to reduce chlorophyll and lutein concentration in soil (H4), whereas a significant decrease in scytonemin content was also evident

Table 5 Nitrogen addition effects on soil pigments and microscope counts (cyanobacteria + diatoms) after 5 years of N addition. In the case of soil pigments, covariance analyses are reported for those cases in which soil pH was a significant explanatory variable in the multiple regression analysis (i.e. scytonemin, chlorophylls, and lutein; see Table 6)

Soil pigments	Nitrogen	pH	N × pH
Scytonemin	*3.13*† (↓)	*26.08***	*4.16**
Chlorophylls *a* + *b*	1.24	*5.20**	1.08
Lutein	1.74	1.74	0.47
β-Carotene	0.73	NE	NE
Microscope counts			
Nostoc sp.	0.20	NE	NE
Microcoleus sp.	1.42	NE	NE
Phormidium sp.	1.90	NE	NE
Scytonema sp.	0.26	NE	NE
Calothrix sp.	0.88	NE	NE
Leptolyngbya sp.	0.30	NE	NE
Diatoms	0.59	NE	NE

The arrow indicates the direction of the effect. Significant effects are in italics

NE not evaluated

† $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$

after the modulating effect of soil pH was accounted for (H5). Belnap et al. (2008) studied the effects of N addition on biocrust pigments presents in the Mojave Desert (California) and also found a decrease in scytonemin and echinenone concentration in soil with increasing N fertilisation loads. Similarly, Ochoa-Hueso et al. (2016) found a significant decrease in echinenone content in soils collected from semiarid ecosystems located along a N pollution gradient in central and eastern Spain. In this study, the impacts of N on scytonemin content were also associated with a decoupling between soil pH and pigment content in the highest N treatment. This decoupling can be easily visualised in Fig. 5, where scytonemin content and soil pH appear to be still closely related to one another in the control and the 10 and 20 kg N ha^{-1} $year^{-1}$ treatments, whereas this relationship did not longer exist under the highest N addition treatment. These results also suggest that, in agreement with our initial hypothesis, the impacts of N deposition on soil pigment abundance

Table 6 Backward multiple regression analyses between soil pigment abundance and microscope counts in 2012 and selected ecologically relevant environmental variables (shrub cover, pH, SOM, and non-bases) and N deposition

Soil pigments	Regression model with the lowest AIC	R^2	*P*
Scytonemin	− 0.05 + 0.008 × pH	0.45	< 0.01
Chlorophylls *a* + *b*	1.94*E*−03 − *3.08E−06* × *N deposition* − 1.94*E*−04 × pH − 4.45*E*−06 × shrubs	0.30	0.02
Lutein	2.44*E*−04 − *6.64E−07* × *N deposition* − 2.44*E*−04 × pH − 1.01*E*−06 × shrubs	0.46	< 0.01
Carotene	NS	–	–
Microscope counts			
Nostoc sp.	16.27–1.63 × SOM	0.30	< 0.01
Microcoleus sp.	5.92 + *0.008* × *N deposition* − 0.09 × SOM − 0.65 × pH − 0.008 × shrubs	0.47	< 0.01
Phormidium sp.	NS	–	–
Scytonema sp.	− 0.004 + 0.01 × SOM − 0.001 × shrubs	0.41	< 0.01
Calothrix sp.	0.04–*0.001* × *N deposition* + 0.02 × SOM − 0.01 × non-bases	0.21	0.05
Leptolyngbya sp.	1.09–0.16 × SOM + 0.02 × shrubs	0.35	< 0.01
Diatoms	0.04 + 0.03 × shrubs	0.23	0.01

Nitrogen addition-related effects are in italics

NS no significant model found

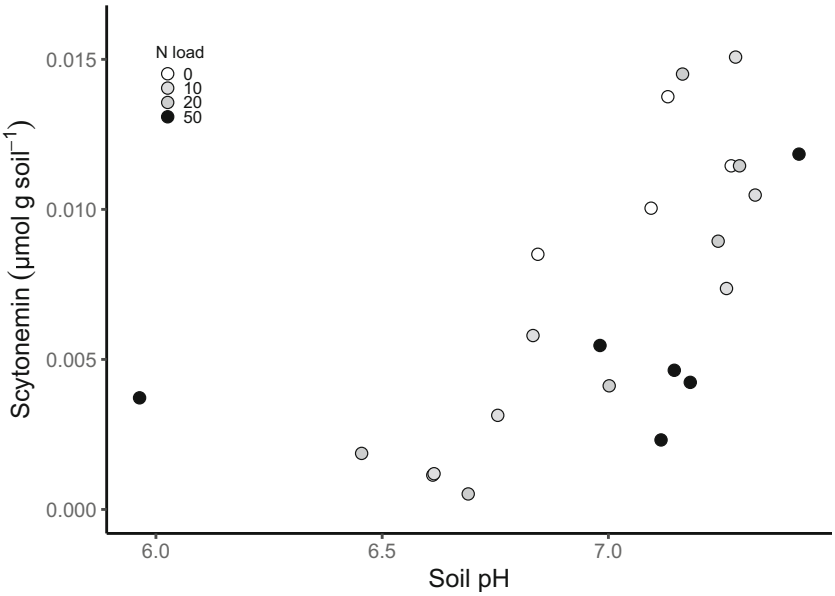

Fig. 5 Relationship between soil pH and scytonemin content depending on the N load applied

could operate via alterations in soil pH (Ochoa-Hueso et al. 2016). In this sense, an incipient soil acidification has already been described as a result of the N addition treatments at our experimental site (Ochoa-Hueso et al. 2013b). Similar to soil pigments and lichens, two cyanobacterial genera were affected by simulated N deposition, although microscope counts were still more closely related to soil fertility (SOM content) and shrub cover.

Conclusions

Five years of experimental N deposition negatively impacted on the biocrust community present in the study area, a semi-arid calcareous shrubland highly representative of other shrublands from the Mediterranean basin, including Spanish matorrales and garrigas, Portuguese matos, Italian macchias, and Greek phryganas. This suggests a previously undescribed high sensitivity of this widespread type of ecosystem to increased N deposition, which can have far-reaching important implications in the context of interactions with climate change and climate change mitigation strategies. For example, a reduction in biocrust cover (including cyanobacteria and lichens) could reduce the ability of dryland ecosystems to stabilise soils and sequester C, creating a positive feedback with climate change (Ochoa-Hueso et al. 2016). Similarly, a reduction in the ability of biocrusts to retain N could also mean higher N leaching rates, therefore hampering the buffering role of these communities against aquifer and watercourse pollution. We also found that community-level alterations in response to simulated N deposition were more evident when evaluated from a temporal and multivariate perspective, which suggests complex interactions between N deposition and the local biotic and abiotic environments, such as vascular plant cover and soil physicochemical properties.

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