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Direct and indirect impacts of climate change on microbial and biocrust communities alter the resistance of the N cycle in a semiarid grassland

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Summary

1. Climate change will raise temperatures and modify precipitation patterns in drylands worldwide, affecting their structure and functioning. Despite the recognized importance of soil communities dominated by mosses, lichens and cyanobacteria (biocrusts) as a driver of nutrient cycling in drylands, little is known on how biocrusts will modulate the resistance (i.e., the amount of change caused by a disturbance) of the N cycle in response to climate change.

2. Here, we evaluate how warming (ambient vs. ~2.5 °C increase), rainfall exclusion (ambient vs. ~30% reduction in total annual rainfall) and biocrust cover (incipient vs. well-developed biocrusts) affect multiple variables linked to soil N availability (inorganic and organic N and potential net N mineralization rate) and its resistance to climate change during 4 years in a field experiment. We also evaluate how climate change-induced modifications in biocrust and microbial communities indirectly affect such resistance.

3. Biocrusts promoted the resistance of soil N availability regardless of the climatic conditions considered. However, the dynamics of N availability diverged progressively from their original conditions with warming and/or rainfall exclusion, as both treatments enhanced N availability and promoted the dominance of inorganic over organic N. In addition, the increase in fungal:bacterial ratio and the decrease in biocrust cover observed under warming had a negative indirect effect on the resistance of N cycle variables.

4. *Synthesis.* Our results indicate that climate change will have negative direct and indirect (i.e. through changes in biocrust and microbial communities) impacts on the resistance of the N cycle in dryland soils. While biocrusts can play an important role slowing down the impacts of climate change on the N cycle due to their positive and continued effects on the resistance of multiple variables from the N cycle, such change will progressively alter N cycling in biocrust-dominated ecosystems, enhancing both N availability and inorganic N dominance.

Key-words: *amoA* genes, fungal:bacterial ratio, lichens, mineralization, nitrogen cycling, plant–soil (below-ground) interactions, rainfall reduction, warming

Introduction

Ongoing climate change is characterized by increases in temperature and changes in precipitation patterns globally, albeit these alterations will vary across regions (IPCC 2013). Drylands (arid, semi-arid and dry-subhumid ecosystems) are the largest biome on the planet – they cover 41% of Earth's land surface and support over 38% of the total global population (Reynolds *et al.* 2007) – and are particularly sensitive to climate change (Maestre, Salguero-Gómez & Quero 2012a). Climate models forecast average (median) warming values ranging from 3.2 to 3.7 °C, and significant alterations in rainfall amounts and patterns, for drylands worldwide by the late

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21st century (Solomon *et al.* 2007). These changes are predicted to expand the area occupied by drylands globally by 10% at the end of this century (Feng & Fu 2013). Given the large proportion of the global population depending on ecosystem services provided by drylands that are tightly linked to soil fertility and plant productivity (e.g. grazing, grass fibre/wood collection, food production and game hunting; Safirel & Adeel 2005), understanding climate change effects on nutrient cycling is particularly important to establish effective management and mitigation actions in these areas (Reynolds *et al.* 2007; OECD/FAO 2011). Despite this, the literature on climate change effects on nutrient cycling is dominated by work conducted in other ecosystems, particularly the humid tropics and polar regions (Schimel 2010).

In drylands, nitrogen (N) is, after water, the most important factor limiting net primary production and organic matter decomposition (Robertson & Groffman 2007; Schlesinger & Bernhardt 2013). The influence of drylands on the global N cycle is well known. As an example, Bowden (1986) estimated that 30% of the total gaseous N-emissions on Earth come from these ecosystems. When studying the N cycle in drylands, surface soil communities dominated by mosses, lichens and cyanobacteria (biocrusts) are of particular interest since they occupy open spaces between plant canopies and play important roles in modulating key ecosystem processes worldwide (Eldridge & Greene 1994; Belnap & Lange 2003; Maestre *et al.* 2011). Water availability and temperature are key drivers of N mineralization, denitrification and microbial activity in dryland soils (Gallardo & Schlesinger 1993; Gallardo & Merino 1998), and hence, climate change will exert significant impacts on these processes through their effects on soil temperature and water availability (Robertson & Groffman 2007; Schlesinger & Bernhardt 2013). However, biocrusts can also play an important role as modulators of N cycle responses to climate change in drylands. These communities are known to affect the rate of processes such as N fixation (Belnap 2002), depolymerization (production of dissolved organic N; Delgado-Baquerizo *et al.* 2013a), nitrification (Reed *et al.* 2012) and gaseous N losses (e.g. N_2O; Barger *et al.* 2005) in dryland soils. Recent laboratory studies have also showed that the soil under biocrusts enhanced the resistance (the amount of change caused by a disturbance, Pimm 1984) of different aspects of the N cycle (e.g. depolymerization and mineralization rates) to changes in temperature and soil water availability under controlled laboratory conditions (Delgado-Baquerizo, Maestre & Gallardo 2013b). These results suggest that biocrusts are critical to maintain N availability in dryland soils under future climatic conditions. Therefore, an important question that remains unanswered is how biocrusts modulate the resistance of N cycle in response to simultaneous changes in temperature and water availability such as those predicted by climate change models.

Biocrusts also affect microbial communities linked to the N cycle. For example, biocrusts can positively affect the abundance of fungi (Bates *et al.* 2010), N-fixing cyanobacteria (Yeager *et al.* 2004) and ammonia-oxidizing archaea (AOA) and bacteria (AOB; Marusenko *et al.* 2013), as well as the functional diversity of microbial communities (Delgado-Baquerizo, Maestre & Gallardo 2013a). Recent studies have showed that changes in rainfall and temperature expected with ongoing climate change can dramatically alter the performance, composition and dominance of visible constituents in both lichen- and moss-dominated biocrusts in drylands (Maphangwa *et al.* 2012, Escolar *et al.* 2012; Reed *et al.* 2012) and that these changes are paralleled by alterations in C and N cycling (Johnson *et al.* 2012; Reed *et al.* 2012; Zelikova *et al.* 2012; Maestre *et al.* 2013). However, our knowledge of how the macroscopic component of biocrusts (e.g. lichens) modulate the response to climate change of key microbial communities associated with the N cycle such as AOB and AOA (that participate in the first step of nitrification; Verhamme, Prosser & Nicol 2011) is scarce. Several authors have found that climate change can enhance soil N mineralization (Rustad *et al.* 2001; Evans & Burke 2013), potential denitrification (Bai *et al.* 2013) and fungal abundance (Maestre *et al.* 2013) in drylands. However, the indirect impacts of climate change on N cycling, mediated by joint changes in particular groups of microorganisms (e.g. ammonia-oxidizing bacteria and archaea) and biocrust constituents, have not been evaluated yet.

As part of an ongoing field experiment aiming to evaluate the impacts of climate change on biocrusts and associated ecosystem processes (Escolar *et al.* 2012; Maestre *et al.* 2013; Ladrón de Guevara *et al.* 2014), we assessed the effects of changes in temperature (ambient vs. ~2.5 °C increase), rainfall (ambient vs. ~30% reduction in total annual rainfall) and biocrust cover on multiple variables from the N cycle (sum of dissolved organic and inorganic N, inorganic N and potential net N mineralization rates) and its resistance to climate change during the first 4 years of the experiment. We also evaluated the indirect impacts of climate change on the resistance of the studied N cycle variables mediated by changes in attributes of biocrust (cover) and microbial (abundance of AOA, AOB, bacteria and fungi and fungal:bacterial ratio) communities. We tested the following hypotheses: (i) biocrusts improve the resistance of the N cycle regardless of climate change impacts (Delgado-Baquerizo, Maestre & Gallardo 2013b); (ii) warming is more important than rainfall exclusion in determining the resistance to climatic change of N availability, enhancing both inorganic N and available N with increasing warming (Bai *et al.* 2013; Delgado-Baquerizo, Maestre & Gallardo 2013b) and (iii) climate-change-induced alterations in biocrust and microbial communities will modulate the resistance of the N cycle to modifications in temperature and rainfall amounts (Reed *et al.* 2012).

Materials and methods

STUDY SITE AND EXPERIMENTAL DESIGN

This study was conducted in the Aranjuez experimental station, located in the centre of the Iberian Peninsula (40°02′N – 3°32′W; 590 m.a.s.l.). The climate is Mediterranean semiarid, with a mean annual temperature and rainfall of 15 °C and 349 mm, respectively.

The soil is classified as Gypsiric Leptosol (IUSS Working Group WRB 2006). Perennial plant cover is lower than 40% and is dominated by the perennial grass *Stipa tenacissima* L (Castillo-Monroy *et al.* 2010). Open areas between plant patches contain a well-developed biocrust community dominated by lichens such as *Diploschistes diacapsis*, *Squamarina lentigera* and *Psora decipiens* (see Maestre *et al.* 2013 for a full species checklist).

We established a fully factorial experimental design with three factors, each with two levels: biocrust cover (cover < 10% vs. cover > 70%), warming ([WA]; ambient vs. ~2.5 °C increase) and rainfall exclusion ([RE]; ambient vs. ~30% reduction in total annual rainfall). Ten replicates per combination of treatments were established, resulting in a total of 80 experimental plots (1.2 × 1.2 m in size). The initial biocrust cover conditions were chosen from available sites. The situation in our study site is that there are biocrust patches of different sites dispersed among a 'matrix' of bare ground areas (Fig. S1 in Supporting Information). Half of these plots were randomly placed on bare ground areas with poorly developed biocrust communities (<10% cover) while the other half were placed in areas with well-developed biocrust communities (cover of visible biocrust constituents >70%) microsites. A minimum separation distance between plots of 1 m was ensured to minimize the risk of sampling non-independent areas. This experiment has been previously used to determine the effects of climate change on the composition, diversity and physiological performance of biocrusts (Escolar *et al.* 2012; Ladrón de Guevara *et al.* 2014) and on different variables of the C cycle (Maestre *et al.* 2013). The warming treatment was designed to simulate the average predictions of six atmosphere-ocean general circulation models for the second half of the 21st century (2040–2070) in central Spain (De Castro, Martín-Vide & Alonso 2005). To achieve a temperature increase within the 2–3 °C of average annual increment predicted by these models, we built open top chambers (OTCs) of hexagonal design with sloping sides of 40 × 50 × 32 cm (Escolar *et al.* 2012). We used methacrylate to build our OTCs because this material does not substantially alter the characteristics of the light spectrum (see Maestre *et al.* 2013 for details). Most climate models foresee significant reductions in the total amount of rainfall received during spring and fall in the study area (between 10% and 50%; De Castro, Martín-Vide & Alonso 2005). To generate these conditions, we set up passive rainfall shelters, which effectively reduced the total amount of rainfall reaching the soil surface by 33% (Escolar *et al.* 2012). Our rainfall shelters did have a negligible impact on soil or air temperatures (Figs S4 and S5 from Maestre *et al.* 2013). The OTCs and rainfall shelters were set up in July and November 2008, respectively, because of logistic reasons.

Our warming treatment promoted an average increase in air and surface soil (0–2 cm) temperature of 2.7 and 3.0 °C, respectively (Maestre *et al.* 2013). Warming effects were highest during the summer (June-September), when soil temperatures increased by warming up to 7 °C in some days (Fig. S5 from Maestre *et al.* 2013). Rainfall shelters caused an average reduction in surface soil moisture of 4% (0–5 cm depth), which was particularly noticeable after main rainfall events (Fig. S6 from Maestre *et al.* 2013). See Escolar *et al.* (2012) and Maestre *et al.* (2013) for further details on the effects of OTCs and rainfall shelters on these environmental variables.

SOIL INORGANIC N IONIC EXCHANGE MEMBRANES

The availability of ammonium and nitrate was measured *in situ* in all the experimental plots using ion-exchange membranes (IEMs; Subler, Parmelee & Allen 1995). We selected this technique because it generates minimal disturbances to the soil surface communities and because it provides good estimates of soil inorganic N production (Durán *et al.* 2012). Seasonal samplings (approximately every 3 months) were carried out between February 2009 and December 2012. During each sampling, two cationic and anionic IEMs (2.5 × 2.5 cm) were inserted into the soil at 0.5–3 cm depth in each of the plots and were incubated in the field for 23–25 days. After removal, IEMs were taken to the laboratory and dried at ambient temperature. They were carefully brushed to remove soil particles and placed into 125-mL flasks for extraction with 25 mL of 2 M KCL by orbital spinning (1 h at 200 rpm). The ammonium and nitrate were then colorimetrically analysed as described in Durán *et al.* (2012).

SOIL SAMPLING AND LABORATORY ANALYSES

Soil samples (top 0–1 cm depth located immediately under biocrust layer) were collected at the beginning of the experiment (July 2008), and 16, 34 and 46 months thereafter from 5 plots per combination of treatments randomly selected. Three soil samples per plot were sampled with a 5 cm diameter core, which were then bulked to obtain a unique sample per plot. Soil was sieved (2 mm mesh) and separated into two fractions. One fraction was immediately frozen at −80 °C in order to quantify the amount of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in our samples (see below). The other fraction was air-dried for 1 month in order to analyse dissolved inorganic (DIN; sum of ammonium and nitrate) and organic (DON) N, and potential net N mineralization rates. Previous studies have found that soil biochemical properties are hardly affected by air-drying in semi-arid Mediterranean soils (Zornoza *et al.* 2009), which otherwise are under dry conditions most of the year (see Maestre *et al.* 2013 for soil moisture data for our experiment). This storage approach is also commonly used when analysing soil variables, such as those evaluated here, in arid and semi-arid environments worldwide (e.g. Hbirkou *et al.* 2011; Maestre *et al.* 2012b).

The abundance of fungi, bacteria, AOA and AOB was measured using quantitative PCR. Soil DNA was extracted using the Powersoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the instructions provided by the manufacturer. However, instead of using 0.25 g of defrosted soil samples as suggested by the manufacturer, we used 0.5 g to improve yield. We conducted these analyses for each of our soil samples in the different climate (control, WA, RE and WA + RE) and biocrust cover (high and low) treatments and for the different soil samplings (0, 16, 34 and 46 months after the beginning of the experiment). We performed quantitative PCRs in triplicate using 96-well plates on an ABI 7300 Real-Time PCR (Applied Biosystems, Foster City, CA, USA). Bacterial 16S and fungal 18s rRNA genes were amplified with the *Eub 338 – Eub 518* and *ITS1 – 5.8S* primer sets, respectively, as described in Maestre *et al.* (2013). The *amoA* genes of AOB and AOA were amplified using the primer sets *amoA1F – amoA2R* and *Arch-amoAF – Arch amoAR*, respectively, as described in Delgado-Baquerizo *et al.* (2013c). Efficiencies for all quantification reactions were higher than 90%, with R^2 values ranging from 0.90 to 0.99. The abundance of fungi, bacteria, AOA and AOB was expressed as number of DNA copies gr^{-1} dry soil. To achieve these units, we calculated first the number of DNA copies per ng of DNA in our PCR. Then, we obtained the number of DNA copies in our whole DNA extraction (100 μL). Finally, we get the number of DNA copies per gram of dry soil.

Dissolved organic N, ammonium and nitrate were measured from K_2SO_4 0.5 M soil extracts in a ratio 1:5 (2.5 g of soil) following

Delgado-Baquerizo *et al.* (2013a). Soil extracts were shaken in an orbital shaker at 200 rpm for 1 h at 20 °C and filtered to pass a 0.45-μm millipore filter. The filtered extract was kept at 2 °C until colorimetric analyses were conducted, which was done within the 24 h following the extraction. Available N was calculated as the sum of ammonium, nitrate and DON. For measuring the potential net N mineralization rate, air-dried soil samples were rewetted to reach 80% of field water holding capacity and incubated in the laboratory for 14 days at 30 °C (Allen, Grimshaw & Rowland 1986). This rate was estimated as the difference between initial and final DIN concentrations, respectively (Delgado-Baquerizo *et al.* 2013a).

STATISTICAL ANALYSES

We first evaluated changes in the concentration of ammonium and nitrate in IEMs (IEM-measured ammonium and nitrate) through time by using a four-way (biocrust cover, WA, RE and time) ANOVA, with repeated measures of one of the factors (time). As the assumption of multisample sphericity was not met, the Huynh-Feldt adjusted degrees of freedom were used for within-subjects test (Quinn & Keough 2002). Data on the concentration of DIN, DON, potential net N mineralization rates and available N data did not meet ANOVA assumptions (normality and homogeneity of variances). Thus, we evaluated the effects of time (16, 34 and 46 months after the beginning of the experiment), initial biocrust cover, rainfall exclusion and warming by conducting a four-way semi-parametric PERMANOVA (Anderson 2001), with biocrust cover, WA and RE as fixed factors and time as random factor.

We then calculated the resistance of the N variables evaluated (DIN, DON, potential net N mineralization rate, available N and IEM-measured ammonium and nitrate) using the Orwin & Wardle (2004) resistance index (RS), according to the following equation:

$$\mathrm{RS} = 1 - \frac{(2 \cdot (D_0))}{((C_0) + (D_0))}$$

where D_0 is the difference between the control (C_0; value of each N variable in the absence of climate change treatments) and the disturbed (P_0, WA, RE and WA + RE treatments) soil at each sampling date. This index has the advantage of being standardized by the control, being bounded between −1 (less resistance) and +1 (maximal resistance); it remains bounded even when extreme values are encountered (Orwin & Wardle 2004). The RS values obtained for IEM-measured ammonium and nitrate were analysed using a three-way (biocrust cover, climate change treatments [WA, RE and WA + RE] and time) ANOVA, with repeated measures of one of the factors (time). Again, as the assumption of multisample sphericity was not met, the Huynh-Feldt adjusted degrees of freedom were used for within-subject tests (Quinn & Keough 2002). Climate change treatments (WA, RE, WA + RE) and initial biocrust cover (low and high) were included as fixed factors in these analyses. As the RS indexes of available N, DIN and potential net N mineralization rate did not meet ANOVA assumptions (normality and homogeneity of variances), we analysed them using a three-way semi-parametric permutational ANOVA (PERMANOVA). Time (16, 34 and 46 months after the beginning of the experiment) was considered as a random factor, while initial biocrust cover and climate change treatments (WA, RE, WA + RE) were considered as fixed factors in these analyses. Warming and rainfall exclusion treatments are collapsed when analysing resistance data because the RS indices are calculated in relation to control; hence, a proper control level is lost. In addition, we performed ANOVA and PERMANOVA *post hoc* analyses of our data to check for differences between climate change treatments (WA, RE, WA + RE) when analysing the RS index of IEM-measured ammonium and nitrate and soil (available N, DIN and potential net N mineralization rate) variables, respectively.

Temporal changes in the dynamics of the N cycling were evaluated using Spearman correlations between sampling date (0, 16, 34 and 46 months after the beginning of the experiment) and RS values obtained for available N, DIN and potential net N mineralization rate. These analyses were separately conducted for each climatic (WA, RE, WA + RE) and biocrust cover (low and high) treatment level.

Our microbial data did not meet ANOVA assumptions (normality and homogeneity of variances). Thus, we analysed the effects of the treatments on the abundance of bacteria, fungi, AOA and AOB and fungal:bacterial ratio variables by using a four-way PERMANOVA. We included time (16, 34 and 46 months after the beginning of the experiment) as a random factor and initial biocrust cover, RE and WA as fixed factors in these analyses.

Finally, we conducted structural equation (SE) modelling (Grace 2006) to evaluate how direct and total effects of climate change (WA and RE), biocrust cover and microbial communities determine the resistance of the N cycle variables evaluated (DIN, DON, potential net N mineralization rate and available N). In addition, because of the low DNA concentration present in some of our soil samples, we were not able to successfully analyse either fungi, bacteria, AOA and AOB in ca. 9% of our samples. Thus, we completed these missing data with the average of each variable for each treatment, sampling data and biocrust cover, prior to conducting SE models. We did not conduct any model for IEM-measured ammonium and nitrate because of the lack of temporal match between the IEMs and soil surveys. Additionally, we included in our models the percentage of biocrust cover measured in the different experimental plots at this time, available from Maestre *et al.* (2013). The total cover of the biocrust community was estimated in each plot using high resolution photographs (Maestre *et al.* 2013). Before conducting our SE models, fungi and biocrust cover were log-transformed to improve linearity. In these models, the WA and RE treatments are categorical exogenous variables with two levels: 0 and 1 (Grace 2006). As mentioned above, a proper control level is lost when obtaining RS values, so we included the controls for the RS indexes of available N, DIN and potential net N mineralization rates in the SE models as RS = 1 (maximum resistance, Orwin & Wardle 2004). Categorical exogenous variables are compatible with SE models because distributional assumptions do not apply to them (Grace 2006). After attaining a satisfactory model fit, we introduced the microbial community as a composite variable into our model. We included in this composite variable the abundance of fungi and the fungal:bacterial ratio, which were the only microbial variables affected by either WA, RE or WA + RE (Table S1). The use of composite variables does not alter the underlying assumptions of SE models, but collapses the effects of multiple conceptually-related variables into a single composite effect, aiding interpretation of model results (Shipley 2001). When data manipulations were complete, we parameterized our *a priori* model (Fig. S2) using our data set and tested its overall goodness-of-fit. There is no single universally accepted test of overall goodness-of-fit for SE models. Thus, we used the chi-square test (χ^2; the model has a good fit when $0 \leq \chi^2 \leq 2$ and $0.05 < P \leq 1.00$) and the root mean square error of approximation (RMSEA; the model has a good fit when *RMSEA* $0 \leq RMSEA \leq 0.05$ and $0.10 < P \leq 1.00$; Schermelleh-Engel, Moosbrugger & Müller 2003). Additionally, and because some variables

were not normally distributed, we confirmed the fit of the model using the Bollen-Stine bootstrap test (the model had a good fit when $0.10 <$ bootstrap $P \leq 1.00$; Schermelleh-Engel, Moosbrugger & Müller 2003). To aid with the final interpretation of our SE models, we also calculated the standardized total effects of the microbial components, biocrust cover and climate change treatments on the RS values of N variables. The net influence that a given variable has upon another is calculated by summing all direct and indirect pathways between these two variables. If the model fits the data well, the total effect should approximate the bivariate correlation coefficient for that pair of variables (Shipley 2001; Grace 2006).

PERMANOVA analyses were carried out using 9999 permutations (permutation of raw data) and the Euclidean distance with the PERMANOVA+ for PRIMER statistical package (PRIMER-E Ltd., Plymouth Marine Laboratory, UK). Structural equation modelling analyses were performed with AMOS 18.0 (SPSS Inc., Chicago, IL, USA). Other analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

Results

CLIMATE CHANGE AND BIOCRUST EFFECTS ON N AVAILABILITY AND THE RESISTANCE OF THE N CYCLE

The concentration of available N, DIN and IEM-measured nitrate increased with warming regardless of the initial biocrust cover ($P < 0.01$; Figs 1, 2 and S3; Tables S1 and S2). A similar trend was observed for the concentration of DON ($P = 0.085$; Fig. 1; Table S1). The magnitude of this warming effect, however, changed through time for IEM-measured nitrate, as indicated by a significant warming × time interaction ($P = 0.031$; Fig. 2; Table S2). Similarly, the potential net N mineralization rate increased with warming (WA), but only in the low biocrust cover plots (WA x biocrust cover interaction; $P = 0.006$; Fig. 1; Table S1). Warming effects also changed through time as indicated by the significant ($P = 0.027$) WA × time interaction found (Fig. 1; Table S1). Rainfall exclusion (RE) had no significant effects on the concentration of any of the N variables studied (Figs 1, 2 and S3; Tables S1 and S2).

The values of the RS index for DIN, potential net N mineralization rate and IEM-measured nitrate were higher in the high biocrust cover plots (Figs 3, 4 and S4; $P < 0.05$; Tables S3 and S4), albeit the magnitude of this biocrust effect changed through time for the later two variables (cover × time interaction, $P < 0.05$; Figs 3 and 4; Tables S3 and S4). High biocrust cover plots also tend to increase the resistance of the DON and available N (Figs 3 and S4; $P = 0.06$; Table S3). Differences between biocrust covers were not observed for the RS index of IEM-measured ammonium ($P = 0.855$; Fig. 4; Table S4). Overall, the RE and WA treatments showed the highest and lowest resistance, respectively, for the available N, DIN and IEM-measured ammonium and nitrate ($P < 0.01$; Figs 3, 4 and S4; Tables S3 and S4). The resistance of available N, DIN and IEM-measured nitrate was the highest under the RE treatment, as supported by *post hoc* analyses ($P < 0.05$). Similarly, the WA treatment showed the lowest resistance for DON and potential net mineralization rate (Fig. 3; $P < 0.08$; Table S3). However, differences between the WA and WA × RE treatments were not observed in any of the resistance variables evaluated ($P > 0.275$). Similarly, we did not find any differences between treatments when evaluating IEM-measured ammonium ($P > 0.084$).

The values of the RS index for IEM-measured ammonium and nitrate decreased with time in response to RE, WA and/or their combination, regardless of the initial biocrust cover (Table 1). Similarly, the RS indexes of available N and DIN decreased through time in the WA and/or WA + RE treatments for both low and high biocrust cover plots (Table 1). In addition, the values of the RS index for potential net N mineralization rate decreased with time in the WA, RE and WA + RE treatments, but only at the low biocrust cover plots (Table 1). Finally, the values of the RS index for DON decreased with time in the WA and WA + RE treatments, but only in the low biocrust cover plots (Table 1).

CLIMATE CHANGE IMPACTS ON MICROBIAL COMMUNITIES

The abundance of fungi tends to increase and decrease with WA ($P = 0.094$; Table S1) in the low and high biocrust cover plots, albeit this effect change with time (as indicated by the time × cover × WA interaction found; $P = 0.081$; Fig. 5, Table S1). The fungal:bacterial ratio tended to increase when with WA regardless of initial biocrust cover, as indicated by a marginally significant effect of this treatment ($P = 0.068$; Fig. 5, Table S1). In addition, WA did not affect the abundance of bacteria, AOA and AOB (Figs 5 and S5; Table S1). Similarly, RE did not have any effect on their abundance (Figs 5 and S5; Table S1).

INDIRECT IMPACTS OF CLIMATE CHANGE ON N CYCLE RESISTANCE

Warming and RE had negative direct effects on the RS indexes for DIN, DON, potential net N mineralization rates and available N (Figs 6 and S6; $P < 0.01$). Biocrust cover had a positive direct effect on the RS of DIN, potential net N mineralization rates and available N (Figs 6 and S6; $P < 0.05$). We also found that WA had a negative direct effect on biocrust cover (Fig. 6; $P = 0.08$), hence this treatment had a negative indirect impact on the resistance of the N cycle (Fig. 6). Rainfall exclusion did not have any effect on biocrust cover (Fig. 6; $P = 0.654$). Similarly, microbial variables had a negative direct effect on the RS of DIN and available N (Figs 6 and S6; $P < 0.05$). Both RE and WA had a positive direct effect on the fungal:bacterial ratio, thus having a negative indirect effect on the resistance of available N and DIN (Figs 6 and S6). However, we found a positive direct effect of biocrust cover on the fungal and bacterial abundances and on the fungal:bacterial ratio (Fig. 6; $P < 0.05$).

Overall, biocrust cover had a total positive effect on the resistance indexes for DIN, DON, potential net N mineralization rates and available N (Tables 2 and S6). Nonetheless,

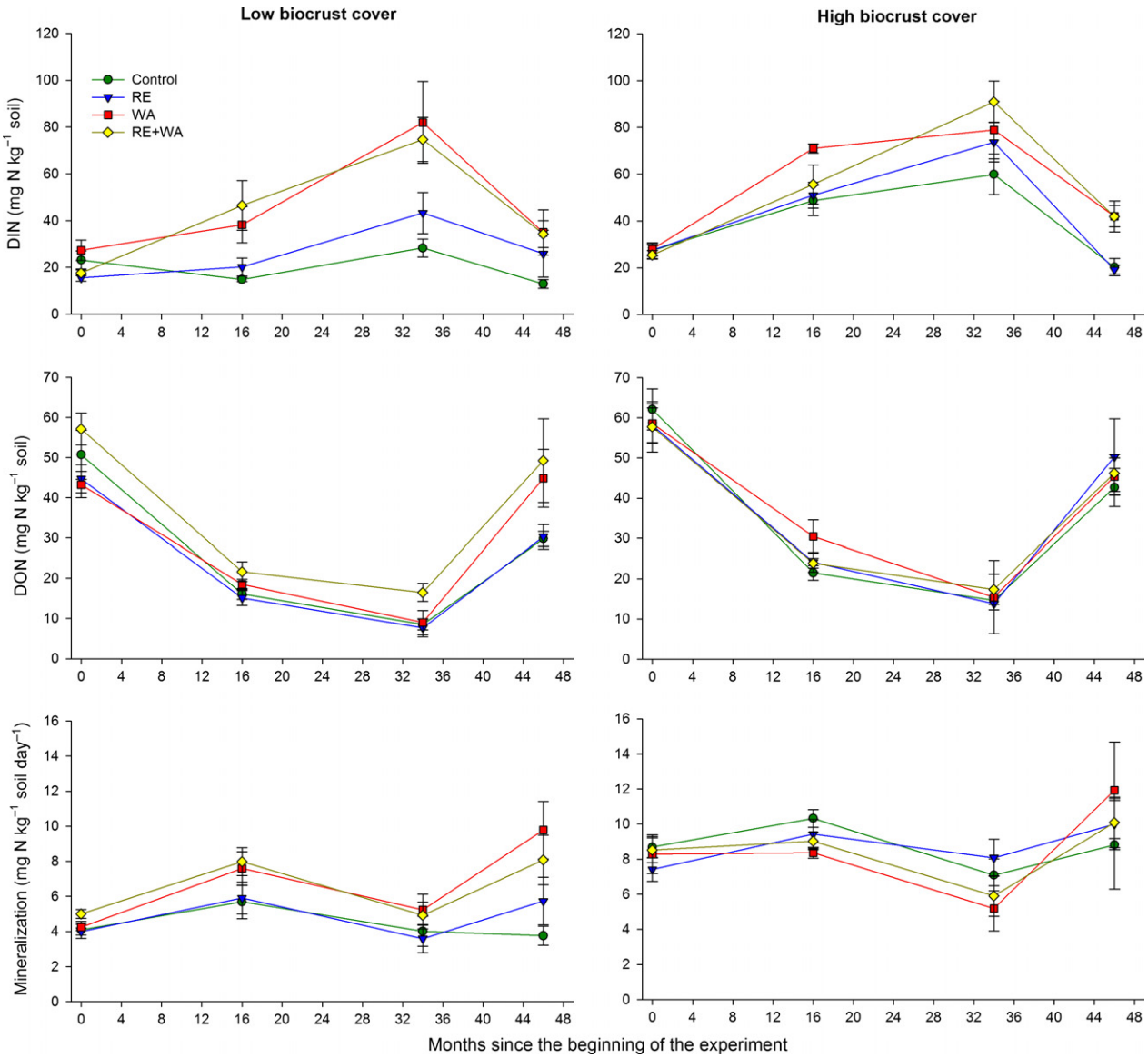

Fig. 1. Changes in the concentration of DIN, DON and the potential net N mineralization rate in response to increasing warming and rainfall reduction in both low and high biocrusts cover during the course of the experiment. Data are means ± SE (n = 5).

climate change treatments and soil microbial community had negative total effects on the resistance of the DIN and available N (Table 2; Table S5).

Discussion

Well-developed biocrusts enhanced the resistance of available N, DIN, DON, potential net N mineralization rates and IEM-measured nitrate in the soil surface, which is a hotspot of nutrients and biological activity in drylands (Belnap, Hawkes & Firestone 2003). However, and despite of the highest resistance of the N cycle found in high biocrust cover areas, our results show that the dynamics of multiple variables of this cycle (available N, DIN, DON, potential N mineralization rates, ammonium and nitrate) will progressively diverge from their original conditions with warming and/or rainfall exclusion. An air–soil surface warming of 2–3 °C increased the amount of available N, inorganic N and tended to enhance the concentration of DON, the abundance of fungi and the fungal:bacterial ratio. The impacts of warming on the resistance of the N cycle variables evaluated were more negative than those of rainfall exclusion. In addition, the impacts of warming on the abundance of microbes (i.e. fungi) were independent of those of rainfall exclusion, which overall had little effects on the different variables measured (except for the fungal:bacteria ratio). These results suggest that microbial communities are highly resistant to drought in drylands (Yuste *et al.* 2014). Indirect effects derived from climate change on microbial communities and biocrust cover may result in a lower N cycle resistance and a higher inorganic N dominance in soils. For example, warming tended to enhance the abundance of fungi and the fungal:bacterial ratio, increasing N availability and inorganic N contents in our study area.

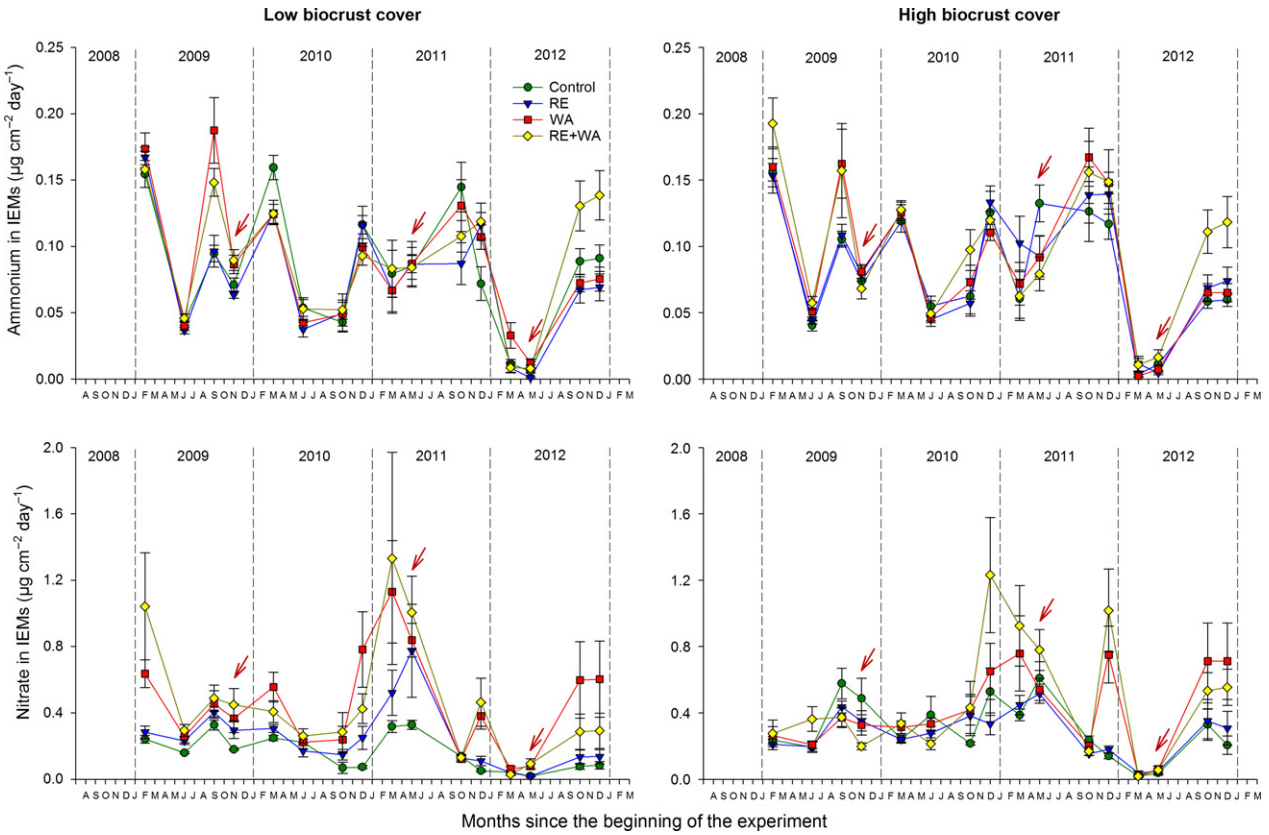

Fig. 2. Temporal changes in the amount of IEM-measured nitrate and ammonium in response to increasing warming and rainfall reduction in both low and high biocrusts cover throughout the study period. IEMs = ion-exchange membranes, RE = rainfall exclusion and WA = warming. Arrows indicate the closest date to the soil samplings in this study. Data are means ± SE (n = 10).

CLIMATE CHANGE IMPACTS ON THE RESISTANCE OF N CYCLE THROUGH TIME

Warming led to a 68% and 50% increase in the availability of N (relating to the control) and to a 157% and 46% increase in inorganic N, in the low and high biocrust cover plots, respectively, only 16 months after the beginning of the experiment. The greater resistance found under well-developed biocrusts may be the consequence of a higher soil stability and multifunctionality compared to bare ground areas (Bowker *et al.* 2011; Castillo-Monroy *et al.* 2011; Maestre *et al.* 2012b). Previous studies have showed how biocrusts enhance the resistance of some aspects of the N cycle (i.e. depolymerization and mineralization; Delgado-Baquerizo, Maestre & Gallardo 2013b) to warming under controlled laboratory conditions and how climate-change-induced modifications in the composition of biocrusts dramatically alters N cycling (i.e. increasing inorganic N dominance, Reed *et al.* 2012). Our study adds to this recent literature that the effect of biocrusts on the resistance of N availability (i.e. available N, inorganic and organic N and potential net N mineralization rates) in response to climate change is maintained during the first 4 years after the onset of the climatic manipulation.

Despite the observed positive effect of biocrusts on the resistance of multiple N cycle variables, our analyses indicate that the dynamics of available N, DIN, DON, potential N mineralization rates, ammonium and nitrate will progressively diverge from their original conditions with warming and rainfall exclusion. These impacts may derive into irreversible changes in the N cycle of dryland soils. For example, an increase in the available N and inorganic N dominance with warming may negatively impact native plant species richness and facilitate exotic plant invasions (Allen *et al.* 2009; Rao & Allen 2010; Castro-Díez *et al.* 2013; Porter *et al.* 2013) . It is interesting to note that we found an increase in the activity of the N-rich enzymes phosphatase and β-glucosidase with warming, which was linked to the augment of N availability found in this treatment (Table S6). These results suggest that the observed increase of N with warming could be already generating the shortage of other essential elements, such as phosphorus and carbon (Schlesinger *et al.* 1990; Bai *et al.* 2013). In addition, we did not find any changes in the N content of the microbial biomass with warming (Fig. S7), suggesting that the microbial community is not immobilizing the extra N promoted by this treatment. These results resemble those reported by previous experimental warming studies (see Bai *et al.* 2013 for thorough revision of the current literature). The accumulation of inorganic N forms (ammonium and nitrate) with warming may promote potential N losses through processes such as denitrification, leaching and run-off, reducing water and air

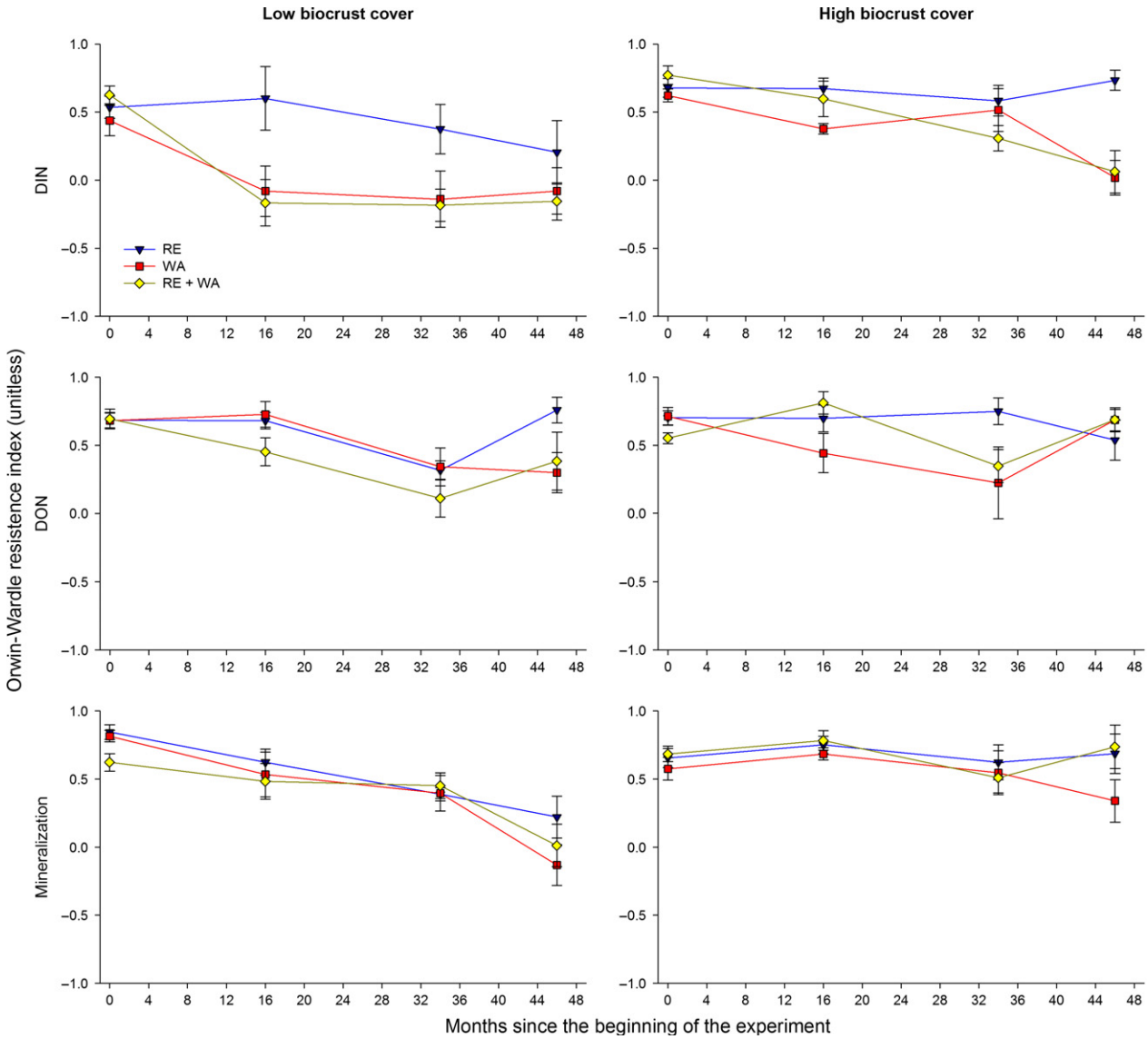

Fig. 3. Changes in the resistance indexes of DIN, DON and potential net N mineralization rate throughout the experiment for the different climate change treatments and biocrust covers evaluated. RE = rainfall exclusion and WA = warming. Data are means ± SE (n = 5).

quality (Schlesinger & Harley 1992; Robertson & Groffman 2007; Schlesinger *et al.* 2009).

Overall, the variables from the N cycle evaluated in this study were more resistant to rainfall exclusion than to warming. Small changes in temperature can quickly reduce the resistance of N availability in soil by favouring processes such as N mineralization, which are highly sensitive to soil temperature (Dalias *et al.* 2002; Wang *et al.* 2006; Bregliani *et al.* 2010). These results agree with previous laboratory experiments showing that changes in temperature have a higher impact on the N cycle than modifications in water availability (Delgado-Baquerizo, Maestre & Gallardo 2013b). However, we cannot discard that the lower impact of rainfall exclusion on the N cycle variables studied may be a consequence of a small reduction in soil moisture promoted by this treatment (average of 4%, Maestre *et al.* 2013), which may not have been significant enough to promote changes in N availability at our study site. A previous study showed that biocrusts can promote the accumulation of available N even in response to very low, dew-like, water pulses (Delgado-Baquerizo *et al.* 2013d). Similarly, while warming reduced the cover of biocrusts, rainfall exclusion did not have this effect 46 months after the beginning of the experiment. These results suggest that higher rainfall exclusion would be necessary to achieve similar impacts in N cycle resistance than those found for warming. For example, Evans and Burke (2012) found that reductions of 50–75% of incoming precipitation can promote an increase in the concentration of IEM-measured nitrate and ammonium in soil. Our results cannot be extrapolated to ecosystems dominated by other types of biocrusts given the important role that the composition of biocrust communities has on their effects on nutrient cycling (e.g. Bowker *et al.* 2011). For example, Reed *et al.* (2012) showed that the death of a dominant moss community after a

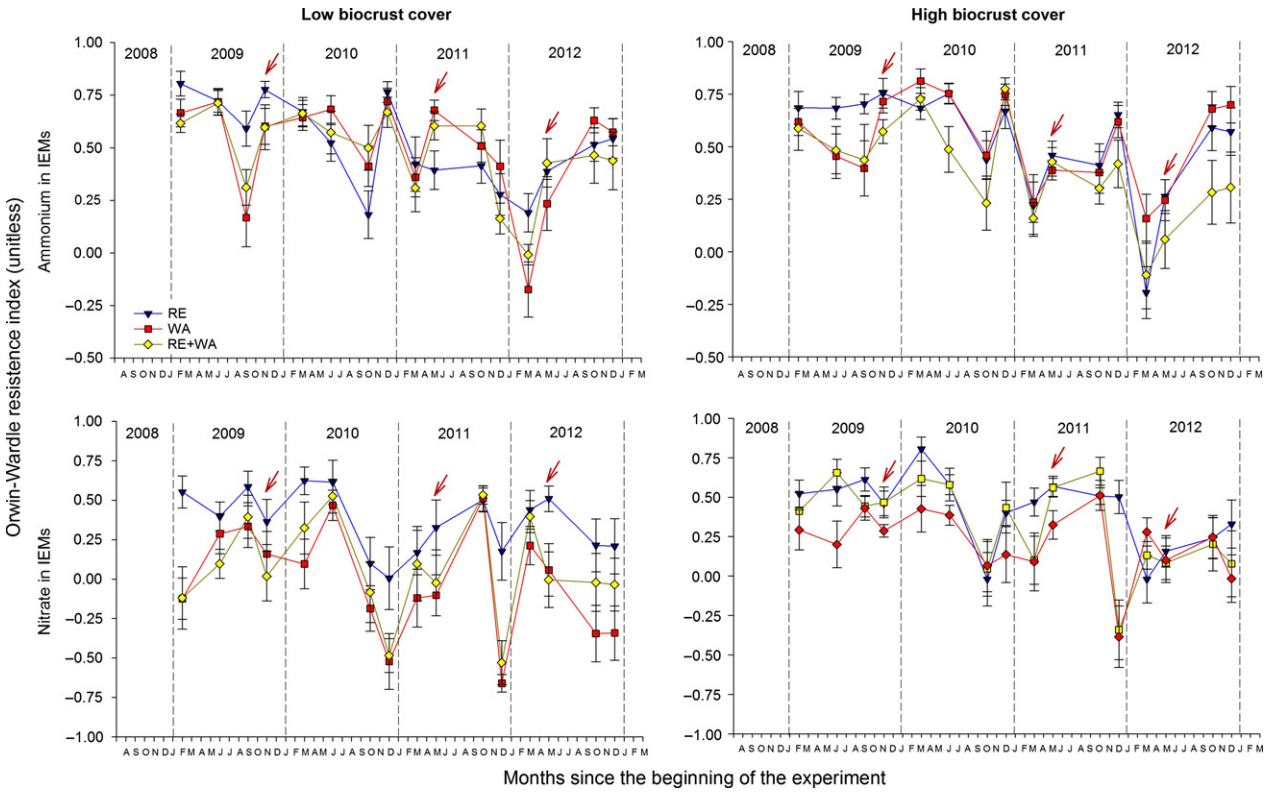

Fig. 4. Changes in the resistance indexes of IEM-measured nitrate and ammonium throughout the experiment in the different climate change and biocrust cover treatments evaluated. IEMs = ion-exchange membranes, RE = rainfall exclusion and WA = warming. Arrows indicate the closest date to the soil samplings in this study. Data are means ± SE (n = 10).

Table 1. Correlation coefficients (Spearman's ρ) between the resistance indexes of the studied N variables and the number of months after the beginning of the experiment (0, 16, 34 and 46 months) for each climate change treatment (WA, RE and WA + RE) and biocrust cover (low and high; n = 20). Data for ammonium and nitrate in IEMs include 16 sampling dates after the beginning of the experiment (n = 160). P values below 0.05 are in bold

	Low cover			High cover		
	RE	WA	RE + WA	RE	WA	RE + WA
Ammonium	−0.40 (**<0.001**)	−0.20 (**0.013**)	−0.25 (**0.001**)	−0.35 (**<0.001**)	−0.14 (0.084)	−0.32 (**<0.001**)
Nitrate	−0.14 (0.078)	−0.22 (**0.006**)	−0.07 (0.409)	−0.30 (**<0.001**)	−0.30 (<**0.001**)	−0.16 (**0.044**)
DIN	−0.22 (0.297)	−0.51 (**0.009**)	−0.65 (**<0.001**)	0.02 (0.939)	−0.62 (**0.001**)	−0.77 (**<0.001**)
DON	−0.11 (0.617)	−0.51 (**0.016**)	−0.49 (**0.012**)	−0.10 (0.625)	−0.15 (0.502)	0.13 (0.528)
Mineralization	−0.80 (**<0.001**)	−0.72 (**<0.001**)	−0.52 (**0.008**)	0.04 (0.852)	−0.20 (0.349)	0.06 (0.757)
Available N	−0.19 (0.383)	−0.57 (**0.003**)	−0.70 (**<0.001**)	−0.32 (0.117)	−0.33 (0.103)	−0.49 (**0.011**)

single summer of altered precipitation regime shifted the regular dynamics of N cycling, enhancing inorganic N and nitrification rates.

The observed seasonal differences in the resistance index of IEM-measured nitrate and ammonium in response to warming increasing also deserve a mention. Our results suggest that warming increasing had much more impacts on the N cycle during the wet season than during the dry season, despite this treatment having increased temperatures up to 7 °C during the latter (Maestre *et al.* 2013). Because of the harsh environmental conditions (very low water availability and high temperatures) typically found during the dry season in drylands, the highest plant and microbial activites (i.e. N mineralization) are typically found during the wet season (Austin *et al.* 2004; Schwinning & Sala 2004). These results indicate that the effects of climate change on N cycling may be more noticeable during particular seasons of the years (i.e. wet seasons); hence, future research must pay particular attention to these periods when studying the impacts of climate change on the N cycle in drylands.

We would like to highlight that the results derived from artificial warming studies like those presented here must be considered with caution because a sudden increase in temperature may overload compensatory processes such as biological

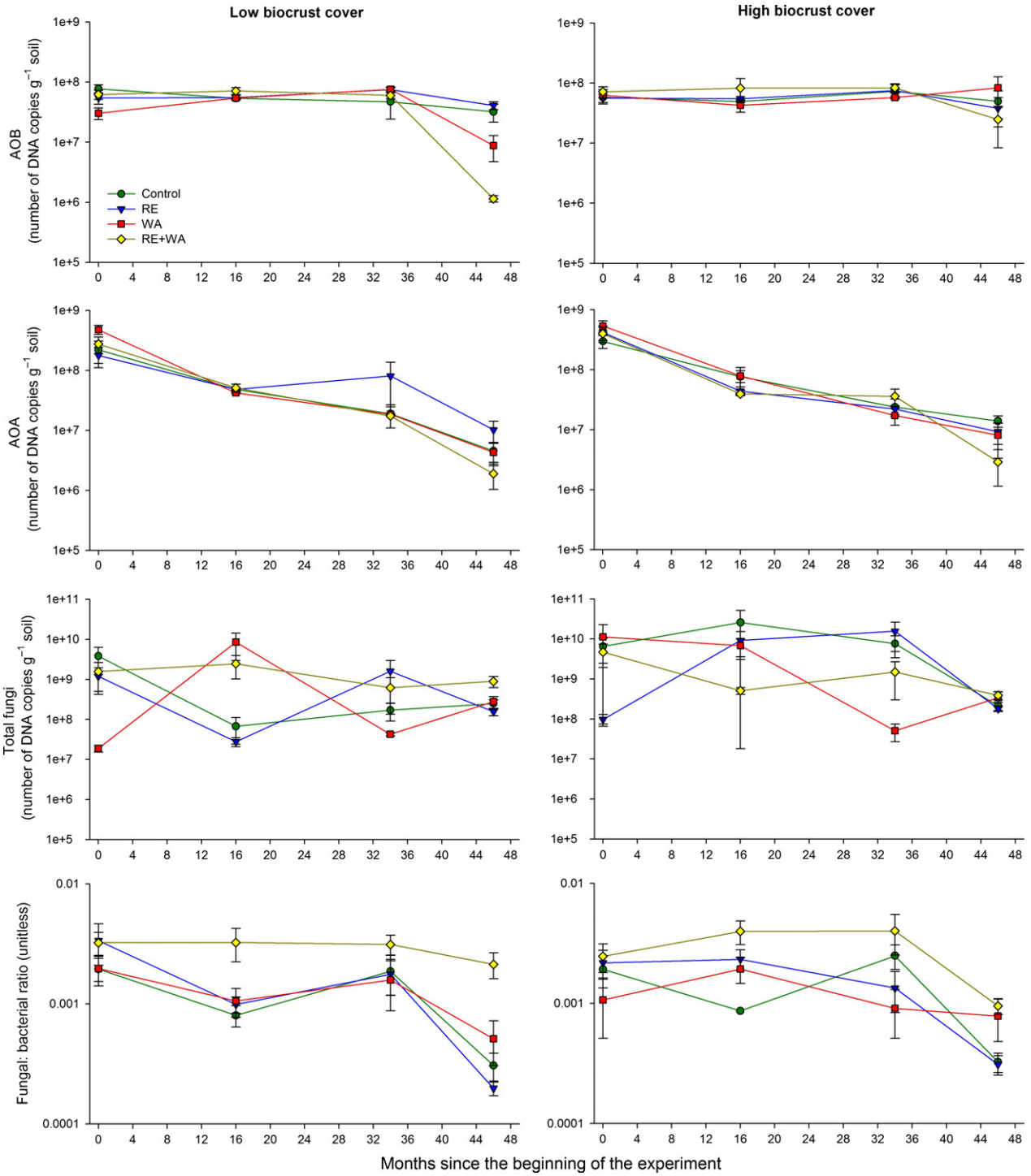

Fig. 5. Changes in the abundance of ammonia-oxidizing archaea (AOA) and bacteria (AOB), fungi and in the fungal:bacterial ratio throughout the experiment for the different climate change treatments and biocrust covers evaluated. RE = rainfall exclusion and WA = warming. Data are means ± SE (n = 5).

adaptation through genetic change, migration and adjustment of community structure (Doak & Morris 2010). Despite these limitations, our results mimic those of a meta-analysis of previous warming studies (Bai *et al.* 2013). These authors found increasing inorganic N and potential net mineralization, but small changes in microbial biomass N, in response to warming in multiple ecosystems, including grasslands, forests and shrublands. Our approach to simulate the effects of future changes in rainfall patterns on ecosystem processes also has limitations (Vicca *et al.* 2014). However, it can provide important insights on how phenomena such as sudden year-on-year weather variations and drought (a major component of the climatic change affecting drylands worldwide, Feng & Fu 2013) can affect the N cycle in drylands.

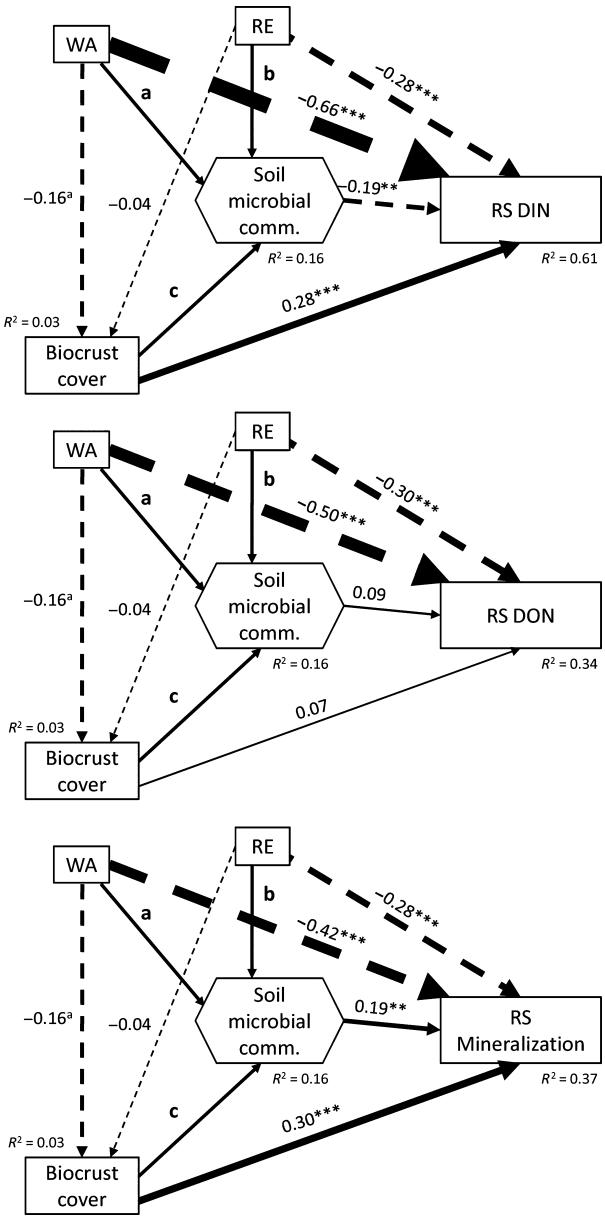

Fig. 6. Effects of warming increase (WA), rainfall exclusion (RE), biocrust cover and microbial community (soil microbial comm. is composed by abundance of fungi and the fungal:bacterial ratio) on the resistance (RS) index for DIN, DON and potential net N mineralization rate (including the data 16, 34 and 46 months after the beginning of the experiment). Numbers adjacent to arrows are path coefficients, analogous to regression weights and indicative of the effect size of the relationship. Continuous and dashed arrows indicate positive and negative relationships, respectively. Width of arrows is proportional to the strength of path coefficients. As in other linear models, R^2 denotes the proportion of variance explained and appears above every response variable in the model. For graphical simplicity, factors influencing microbial communities are as follows: a. WA → fungi = 0.09, WA → fungal:bacterial ratio = 0.29**; b. RE → fungi = 0.04, RE → fungal:bacterial ratio = 0.27**; c. biocrust cover → fungi = 0.25**, biocrust cover → fungal:bacterial ratio = 0.16*. The hypothetical model created was satisfactorily fitted to our data, as suggested by non-significant χ^2 values ($\chi^2 = 0.284$; $P = 0.594$; *d.o.f* = 1 in all cases), nonparametric bootstrap $P = 0.610$ and by values of RMSEA = 0.000 with a $P = 0.648$. Significance levels are as follows: [a]$P = 0.07$, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Table 2. Standardized total effects (direct plus indirect effects) derived from the structural equation modelling of warming increase (WA), rainfall reduction (RE), biocrust cover and microbial community (fungal abundances and fungal:bacterial ratio) on the resistance index (RS) of DIN, DON and potential net N mineralization rate

	RE	WA	Biocrust cover	Fungi	Fungal: bacterial ratio
RS DIN	−0.25	−0.67	0.25	−0.19	0.14
RS DON	−0.29	−0.49	0.09	0.05	0.05
RS mineralization	−0.25	−0.44	0.30	−0.15	0.18

INDIRECT EFFECTS OF CLIMATE CHANGE ON THE RESISTANCE OF THE N CYCLE

Our results provide evidence that indirect impacts of warming and rainfall exclusion on biocrusts and associated microbial communities will negatively impact the resistance of N cycle in drylands. For example, as warming continues the biocrust cover will be further reduced (Maestre *et al.* 2013), negatively affecting the resistance of the N cycle and favouring the inorganic N dominance in dryland soils. Thus, losses of lichen-dominated biocrusts with warming will limit the positive impacts that these communities exert on the resistance of the N cycle. Our results resemble those of Reed *et al.* (2012), who reported how the loss of the dominant biocrust component (mosses in their case) due to changes in the rainfall regime dramatically altered N cycling in the Colorado Plateau (USA), which shifted from NH_4^+ to NO_3^- dominance.

Similarly, any climate-change-induced alterations in microbial communities may cause an indirect impact on the resistance of N cycle variables. For example, we observed that rainfall exclusion had a positive impact on the fungal:bacterial ratio, which results in a negative indirect impact on the resistance of available N and DIN. Fungi are well known to be more tolerant to desiccation than bacteria (Austin *et al.* 2004), and this could explain the increase in the fungal:bacterial ratio observed with rainfall exclusion 36 months after the beginning of the experiment. However, the lack of effect of the rainfall exclusion on N availability, DIN, DON and potential net N mineralization rates suggest that the indirect effect of rainfall exclusion on N cycle through the fungal:bacterial ratio may only promote small changes in the N cycle between control and rainfall exclusion treatments. Similarly, warming had an indirect negative impact on this resistance by increasing this ratio. Additionally, we found that warming increased the abundance of fungi, but not of bacteria, 46 months after the beginning of the study. This augment may promote, at least in part, the observed increase in the availability of N observed with warming (Table S7). Fungal-dominated microbial communities use N more efficiently (i.e. have lower N requirements) than bacterial-dominated ones and thus accelerate N depolymerization and mineralization (Austin *et al.*

2004; Cookson *et al.* 2006), enhancing N availability (Paul & Clark 1996; Austin *et al.* 2004) and therefore reducing the resistance of the N cycle.

Concluding remarks

Biocrusts can play an important role slowing down the impacts of climate change on the N cycle, albeit the impacts of such change on biocrusts and associated microbial communities will negatively affect the resistance of the N cycle in dryland soils. As a consequence, the dynamics of N availability in dryland soils will progressively diverge from their original conditions in response to warming. Overall, our results provide solid evidence that considering biocrusts and microbial communities is of paramount importance when assessing the direct and indirect impacts of climate change on the N cycle in drylands and highlight the importance of biocrusts as modulators of these impacts.

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Data accessibility

Data available from the Dryad Digital Repository http://dx.doi:10.5061/dryad.vt84k (Delgado-Baquerizo *et al.* 2014).

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Supporting Information

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

Figure S1. Overall view of the vegetation in our study area.

Figure S2. *A priori* generic structural equation model used in this study.

Figure S3. Changes in the concentration of available N in response to increasing warming and rainfall reduction in both low and high biocrusts cover during the course of the experiment.

Figure S4. Changes in the resistance index of available N throughout the experiment in the different climate change and biocrust cover treatments evaluated.

Figure S5. Changes in the abundance of bacteria in response to increasing warming and rainfall reduction in both low and high biocrusts cover during the course of the experiment.

Figure S6. Effects of warming increase, rainfall exclusion, biocrust cover and microbial community from our structural equation modelling on the resistance index for available N.

Figure S7. Changes in the concentration of microbial biomass nitrogen in response to increasing warming and rainfall reduction in both low and high biocrusts cover at the beginning of the experiment and 46 months later.

Table S1. PERMANOVA analyses carried out with the concentration of the different N availability variables and microbial abundances in this study.

Table S2. PERMANOVA analyses carried out with the concentration of ammonium and nitrate in IEMs.

Table S3. PERMANOVA analyses carried out with the resistance index of the different N availability variables in this study.

Table S4. PERMANOVA analyses carried out with the resistance index of the different N availability variables in this study.

Table S5. Standardized total effects derived from the structural equation modelling of warming increase, rainfall reduction, biocrust cover and microbial community on the resistance index of available N.

Table S6. Correlation coefficients between the available N and the concentrations of proteins, amino acids and the enzyme activities of β-glucosidase and phosphatase in both the low and high biocrusts cover.

Table S7. Correlation coefficients between the abundance of total fungi, bacteria, ammonia-oxidizing bacteria (AOB) and archaea (AOA) and the fungal:bacterial ratio with the studied soil nitrogen variables in both low and high biocrusts cover.