

## ORIGINAL PAPER

# Protist Diversity Responses to Experimental N Deposition in Biological Crusts of a Semiarid Mediterranean Ecosystem



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**Biological soil crusts (BSC) are associations of different macro and microorganisms and aggregated soil particles located on the surface of soils in many different habitats. BSC harbour a diverse and complex community of ciliates and testate amoebae. These phagotrophic protists play an important role in C and N recycling in soil ecosystems but have not been frequently studied in BSC. In this context, the effects of three increasing N inputs on ciliates and testate amoebae in crusts from a semi-arid Mediterranean ecosystem were evaluated. A field experiment with artificial N-deposition was designed to mimic the effects caused by anthropogenic N depositions. The results have shown that the protist populations of these semi-arid Mediterranean environments have lower species richness than other soil environments. The increase in N produces a net loss of diversity in the populations studied and shifts in the community structure. It has also been shown that some ciliates and testate amoebae, due to their population responses to increased N concentrations, could potentially be used as bio-indicators of N contamination in these BSCs.**

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**Key words:** Ciliate; testate amoebae; diversity; N-deposition; semi-arid ecosystems.

## Introduction

Biological soil crusts (biocrusts or BSC) have been defined as "... an intimate association between soil

particles and differing proportions of photoautotrophic (e.g. cyanobacteria, algae, lichens, bryophytes) and heterotrophic (e.g. bacteria, fungi, archaea) organisms, which live within, or immedi-

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ately on top of the uppermost millimetres of soil. Soil particles are aggregated through the presence and activity of these often extreme-tolerant biotas that desiccate regularly, and the resultant living crust covers the surface of the ground as a coherent layer. . . .” (Belnap et al. 2003 in Weber et al. 2022). These micro-ecosystems occur in very different dry environments, from deserts and polar regions to temperate zones, and now it is widely recognized their significance in maintaining the structure and stability of soil (Belnap et al. 2016; Chamizo et al. 2016; Maestre et al. 2011; Rogers 2020; Veste et al. 2021) where vascular plants are not found (Barger et al. 2013; Jeong et al. 2018). In addition to the macroscopic components of the biocrusts (mosses and lichens), the main microbial communities involved in these consortia (cyanobacteria, phototrophic protists) are as well highly engaged in the processes of C and N fixation (Elbert et al. 2012). Heterotrophic protists, also play a key role in remineralization processes that take place through their predatory activities on prokaryotes or other eukaryotes, especially in the recycling of N and P because of their trophic interactions (Geisen et al. 2017). These mechanisms become additional N inputs besides biological fixation and atmospheric deposition (Hawkes 2003), particularly important in these N-limited semi-arid ecosystems (Castillo-Monroy et al. 2010; Holst et al. 2009).

The preservation of these habitats is nowadays considered crucial as BSCs fulfil a wide range of ecological functions and ecosystem services such as maintaining soil fertility and a protective role against erosion and evaporation, especially in arid or semi-arid regions (Bethany et al. 2019; Szyja et al. 2019; Veste et al. 2021; Xiao et al. 2022). Numerous studies have used BSCs as a model ecological system and have been devoted to elucidating different aspects of their ecological and functional processes, such as their composition ecophysiology, biotic interactions, ecosystem functions (nutrient cycling, water fluxes and erosion) (Bowker et al. 2014; Castillo-Monroy and Maestre 2011; Chamizo et al. 2016; Lafuente et al. 2020; Maestre et al. 2011; Maestre et al. 2016; Ochoa-Hueso et al. 2016; Rosentreter and Root 2019). These studies have led to major experimental research focused on the restoration and conservation of ecosystem function in different dryland landscapes as these sites are very sensitive to physical disturbance (Belnap et al. 2016; Chock et al. 2019; Ferrenberg et al. 2015; Maestre et al. 2011; Slate et al. 2020).

Different studies have focused on the type of BSC, the effects of the presence or absence and distribution of the organisms participating in its structure or enzymatic activities and its relationship with abiotic factors (Lafuente et al. 2020; Ochoa-Hueso et al. 2011b; Yang et al. 2019). Some functional aspects of BSC depend directly on the type of communities involved in semi-arid ecosystems. BSCs have been classified according to their ecological succession, from the simplest, such as those with only a cyanobacteria crust, which are considered early successional biocrusts and tend to have lower microbial diversity and abundance, and BSCs with cyanobacteria, lichens and bryophytes considered within the late-successional biocrusts that harbour more abundant and diverse microbiota (Belnap and Lange 2003; Darby and Neher 2016; Lan et al. 2013), with a higher abundance of bacteria, but lower abundance of fungi. Between these two types, a number of intermediate stages have been described depending on the addition of other cyanobacteria, lichens, or a further step of bryophytes to the BSC association (Bamforth 2008). This functional diversity is associated with other characteristics, such as variable porosity and small-scale hydrological properties (Tighe et al. 2012), which are determinants for the development of microbiota and would be very important for protist populations.

The microbial community composition of the biocrust has been recorded and identified, questioning the individual functional contributions of different organisms (Büdel 2005; Darby et al. 2006; Darby and Neher 2016; Darby et al. 2010; Dumack et al. 2016; García-Pichel et al. 2003; Housman et al. 2007; Khanipour Roshan et al. 2020; Lan et al. 2013; Nagy et al. 2005). The role of bacteria, other than cyanobacteria, and other microorganisms has not often been considered in detail until recently (Miralles et al. 2012; Ochoa-Hueso et al. 2016). Protists, especially heterotrophic species, are important members of this soil community and some works have investigated their diversity in different types of BSCs from a morphological point of view (Bamforth 2004, 2008; Darby et al. 2006; Dumack et al. 2016; Robinson et al. 2002). Lately, studies of metagenomic diversity have been performed in bacteria, fungi, and certain protists, such as flagellates, Amoebozoa and Cercozoa (Cano-Díaz et al. 2018; Couradeau et al. 2019; Khanipour Roshan et al. 2021; Maier et al. 2018; Moreira-Grez et al. 2019; Rippin et al. 2018). While these high-

throughput methods have certainly provided new insights into the diversity and ecology of microorganisms, however, they have not yet achieved enough taxonomic resolution due to the paucity of properly curated sequences in the databases and this is an important point that needs to be resolved.

The effects of N-deposition on the different components of BSC, and other soil communities, have given rise to numerous studies in these N-limited environments. This research has ranged from ecosystem properties to microbial abundance and diversity, especially in semi-arid ecosystems (Castillo-Monroy et al. 2010; Lo Cascio et al. 2021; Ochoa-Hueso et al. 2011a, b; Wang et al. 2015), in this framework of recovery from disturbances that are important for the ecosystem services provided by the BSC.

The study of protist diversity (mainly  $\alpha$ -diversity) in biocrusts has been explored previously (Bamforth 2004; 2008; Darby et al. 2006; Darby and Neher 2016; Darby et al. 2010; Robinson et al. 2002) although the functional aspects of these microorganisms related to BSC have been overlooked in comparison to those of the main organisms involved in these habitats (Darby et al. 2010; Darby and Neher 2016; Jia et al. 2021; Khanipour Roshan et al. 2020, 2021). Here, we have studied the effects of N deposition on the diversity of ciliates and testate amoebae, within a larger experimental set-up conceived to investigate the possible effects that N pollution would cause on the biological soil crust of a semi-arid Mediterranean ecosystem. Since most arid and semi-arid ecosystems are N-limited, much effort has been devoted to studying the effects of N on BSC. Previous works have developed long term field experiments manipulating N content to assess the effects on the diversity and composition of microbial communities in terrestrial ecosystems. These works have been focused mainly on bacterial and fungal communities (Lo Cascio et al. 2021; Ochoa-Hueso et al. 2011b; 2016; Wang et al. 2015; Zhou et al. 2017). There have been inconsistent microbial responses to N inputs reported in other studies, producing either increased or decreased populations (Demoling et al. 2008; Fierer et al. 2012), suggesting that N fertilization on soil microbial diversity is likely site dependent as well (Andretta et al. 2022; Lo Cascio et al. 2021). The research about microbial eukaryotic communities has shown that fungal biomass was reduced as N fertilization increased (Lan et al. 2013; Lo Cascio et al. 2021). However,

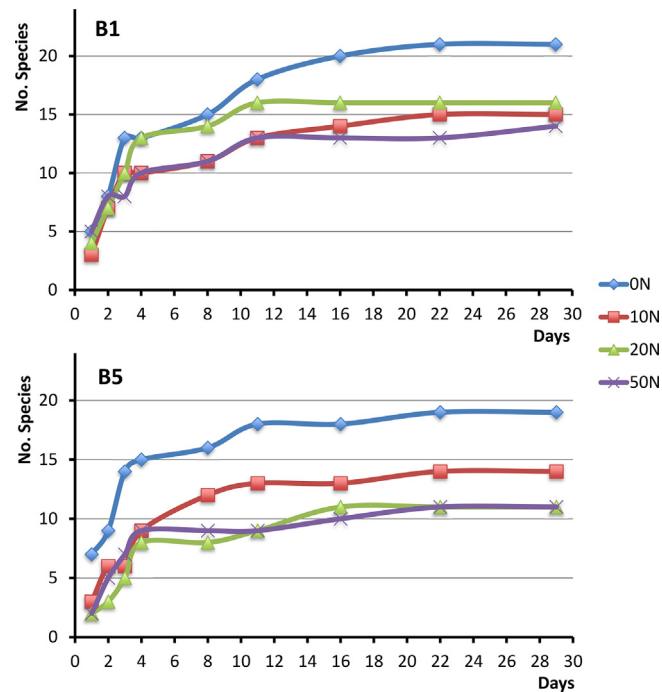
few similar studies have been conducted to investigate the effects of N deposition in BSC on protist populations (Wang et al. 2015). The main objective of our research was to assess the effects that this type of deposition would cause, at three different concentrations, over ciliate and testate amoebae communities. To meet this aim, the species richness, abundance, and diversity of these groups were studied. The identification of N deposition effects has also made it possible to identify potential bioindication protistan species or populations associated with N content.

## Results

### Accumulation Curves

The accumulation curves showed an exponential increase in the occurrence of new species until the fourth day of sampling, and thereafter a slow decline in the presence of new species was observed (Fig. 1). These curves showed that species richness was lower with N deposition treatment in both blocks studied (B1 and B5), i.e. the control treatments (0N) were the plots with the highest number of species (23 in B1 control and 20 in B5 control). The plots with the highest N-deposition treatments (50N) were, in both blocks, those with the lowest number of species (Table 1; Fig. 1). During the first 4 sampling dates the number of species increased rapidly for each experimental N-deposition, and then stabilised reaching asymptotically a characteristic maximum depending on the N deposition treatment as K-selected or r-selected species appeared.

Differences in total ciliate abundance as a function of treatment and sampling days were tested through simple regression. Models obtained were statistically significant in sampling dates 1, 3 and 29 (Day 1:  $F = 6.79$ ;  $p < 0.04$ ; day 3:  $F = 6.88$ ;  $p < 0.04$ ; day 29:  $F = 45.91$ ;  $p < 0.0005$ ). These results indicated that the variability found in the abundance of ciliates was significantly explained in those days by N-deposition treatments ( $R^2$ : 53% of the variability explained for 1st day;  $R^2$ : 53.4% of the variability explained for 3rd day and finally 88.44% explained for 29th day). The abundance of ciliates was negatively correlated to N treatment (correlation coefficients:  $-0.73$  for days 1 and 3) and positively correlated ( $0.94$ ) to N for day 29. These results seem to indicate that in 50N deposition treatment, ciliate species were growing well at these N concentrations.



**Figure 1.** Cumulative number of species obtained in blocks studied (B1, B5) with different N-deposition regimes (0N, 10N, 20N, 50N).

In the case of amoebae, no accumulation curves were used, as the study of diversity required a long-term procedure, and the number of species was only assessed on the last day of the experimentation.

#### Ciliate Diversity

A total of 45 species of ciliates distributed in 28 genera were recorded throughout this study (Table 1). The most frequent group was the Class Colpodea, followed by the Subclass Scuticociliatia and the third was the Class Litostomatea with 10 species recorded. Litostomatea with 6 species recorded showed a higher frequency than hypotrichs, however, both had similar abundance (Fig. 2A, B). Other ciliates found in the minority were the Classes Prostomatea, Heterotrichea and Phyllopharyngea with abundances of less than 6% of the total ciliate population (Fig. 2B). These classes also occurred at a lower frequency (<11%) and were represented by a low number of species: 3, 2 and 2 species respectively (Fig. 2A).

The highest species richness of ciliates (30 species) was found in the control plots (0N), not receiving any N treatment. The control plot in B1 had a higher diversity with a higher number of species compared to B5. Both control plots (0N) had 13 species in common and differed in 10 species for B1

and 7 species for B5. Certain species did not appear in the control plots and showed up only in N-treated plots (Table 1).

Considering the distribution in the N-deposition treatments, certain species were found in all samples, such as *Cyclidium muscicola* and *Trachelophyllum* sp.1 (Table 1), while others were less common and were only recorded in one sample and in a particular N concentration (Fig. 3), such as:

- Treatment 0N: *Colpoda inflata*, *Cyrtolophosis alpestris*, *Blepharisma* sp., *Tachysoma granuliferum*, *Enchelydium* sp., *Spathidium procerum*, *S. spathula* y *Paragas-tronauta clatratus*.
- Treatment 10N: *Bryometopus pseudochilodon*, *Cinetochilum margaritaceum*, *Cristigera* sp., *Enchelydium fusidens*, *Trachelophyllum* sp.2 y *Urotricha furcata* in treatment 10N.
- Treatment 20N: *Trihymena terricola*, *Hypotrich* sp., *Fuscheria terricola*.
- Treatment 50N: *Colpoda aspera*, *Holophrya* sp.1.

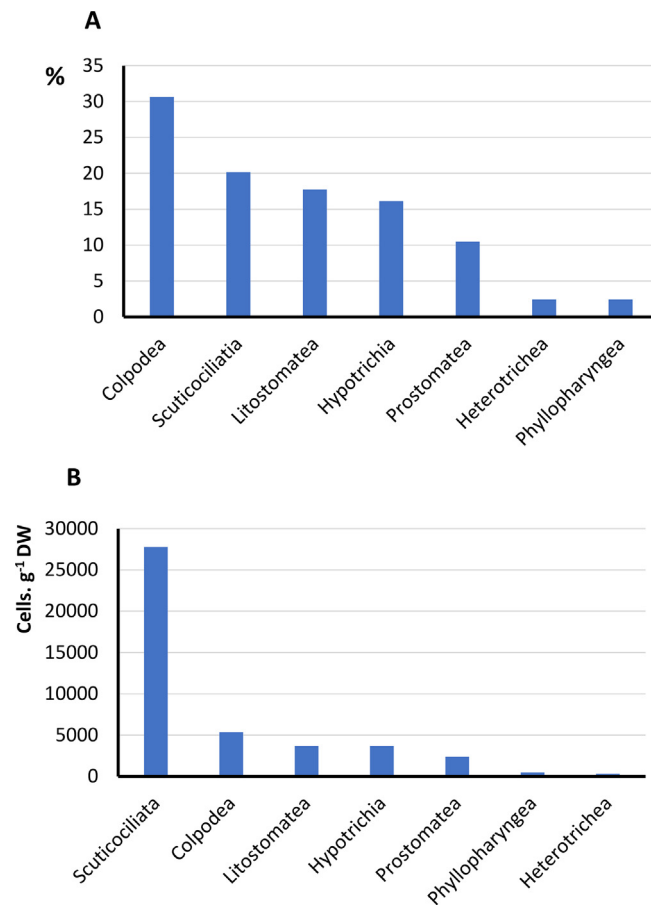
There were also species records appearing in just a single treatment (in both B1 and B5), for instance, *Avestina* sp. was only recorded in samples from plots receiving an intermediate N treatment (20N) (Fig. 3A). *Colpoda colpidiopsis* and *Apertospathula similis* were only observed in samples from plots treated with the highest N deposition treatments

**Table 1.** Ciliate species occurrence in experimental blocks B1 and B5 and N-deposition treatment plots (0N, 10N, 20N and 50N).

Taxon	Species	Block 1				Block 5			
		0N	10N	20N	50N	0N	10N	20N	50N
Class Colpodea	<i>Avestina</i> sp.			+				+	
	<i>Bryometopus pseudochilodon</i>						+		
	<i>Bryometopus</i> sp.	+	+		+	+			
	<i>Trihymena terricola</i>			+					
	<i>Colpoda aspera</i>				+				
	<i>Colpoda cucullus</i>	+		+	+	+	+	+	+
	<i>Colpoda colpidiopsis</i>				+				+
	<i>Colpoda ecaudata</i>	+					+		
	<i>Colpoda inflata</i>	+							
	<i>Colpoda steinii</i>	+	+	+		+			
	<i>Cyrtolophosis alpestris</i>					+			
	<i>Cyrtolophosis mucicola</i>	+	+		+	+		+	+
	<i>Maryna atra</i>				+	+			
	<i>Platyophrya paeletti</i>	+	+		+	+			
Subclass Scuticociliatia	<i>Cinetochilum margaritaceum</i>						+		
	<i>Cristigera</i> sp.						+		
	<i>Cyclidium citrullus</i>	+	+	+	+			+	
	<i>Cyclidium heptatrichum</i>	+		+		+			
	<i>Cyclidium muscicola</i>	+	+	+	+	+	+	+	+
	<i>Homalogastra setosa</i>	+		+	+	+			
	Unidentified Scuticociliate sp.			+	+	+			
Class Heterotrichea	<i>Blepharisma</i> sp.	+							
	<i>Metopus es</i>						+	+	
Subclass Hypotrichia	<i>Gonostomum affine</i>	+	+	+		+	+	+	
	<i>Tachysoma granuliferum</i>	+							
	<i>Urosoma similis</i>	+	+						+
	<i>Urosomoida agilis</i>	+				+	+	+	+
	<i>Urosomoida minima</i>	+		+			+		+
	Hypotrich sp.			+					
Class Litostomatea	<i>Apertospathula similis</i>				+				+
	<i>Arcuospathidium vermiforme</i>	+	+			+			
	<i>Enchelydium fusidens</i>		+						
	<i>Enchelydium</i> sp.	+							
	<i>Enchelys pupa</i>			+		+	+		
	<i>Fuscheria terricola</i>			+					
	<i>Spathidium procerum</i>					+			
	<i>Spathidium spathula</i>					+			
	<i>Trachelophyllum</i> sp.1	+	+	+	+	+	+	+	+
<i>Trachelophyllum</i> sp.2		+							
Class Prostomatea	<i>Holophrya</i> sp.				+				
	<i>Plagiocampa mutabilis</i>	+	+	+			+	+	+
	<i>Urotricha furcata</i>		+						
	<i>Urotricha ondina</i>	+	+			+	+	+	
Class Phyllopharyngea	<i>Leptopharynx</i> sp.	+							+
	<i>Paragastronauta clatratus</i>					+			

(50N). Finally, four species were never observed in the highest N concentration (50N) treatment: *Colpoda steinii*, *Gonostomum affine*, *Arcuospathidium*

*vermiforme* and *Urotricha Ondina* (Fig. 3B). These observations could be used as a first approximation of their bioindication possibilities.



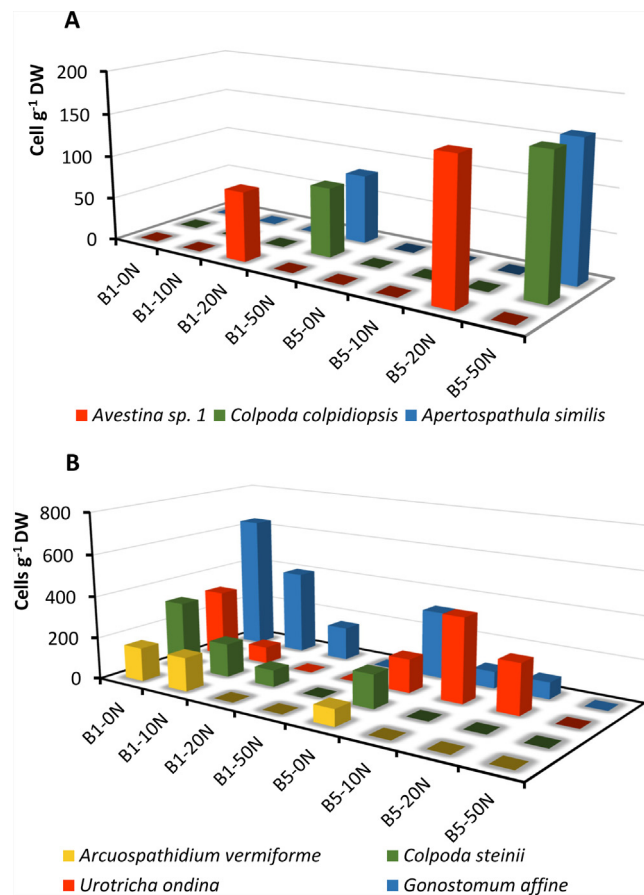
**Figure 2.** (A) Frequency of species appearance (%); (B) total abundance (Cells  $g^{-1}$  DW) of ciliate groups recorded in this study.

On the other hand, regarding species richness and  $\alpha$ -diversity related to N deposition treatments (Fig. 4A, B), there was a difference between B1 and B5, as the former always had a higher number of species than the latter in all plots. However, the total ciliate abundance (cells.  $g^{-1}$  DW) was higher in B5 than in B1, approximately 31.4% (1.31 times) higher (Fig. 4C).

At the same time, within blocks, the highest number of ciliate species was always found in the control plots (0N) in both blocks. This suggests that N-deposition treatments (10N, 20N, 50N) might have a reduction effect on some of the species richness as the N concentration increased in the plots (Fig. 1, Fig. 4). The diversity measurements (Shannon-Weaver index -H), also showed the same trend, decreasing diversity with increasing N-deposition, except for diversity in sample 50N from B5 (Fig. 4B). However, significant differences were

only found between the ciliate abundance in the different N deposition treatments (Wilcoxon-Mann-Whitney test) for were found for the control treatment and 20N and 50N for block B1.

The information on  $\beta$ -diversity (Table 2) provided an idea of the degree of dissimilarity of the plots studied with respect to the controls (0N) and between them. It allowed assessing the heterogeneity of the communities found in the blocks and treatments studied. A high  $\beta$ -diversity index indicated a low level of similarity, while a low  $\beta$ -diversity index showed a high level of similarity. Differences between control plots (0N) and the treatments increased with the N treatment, indicating that communities after N-treatment diverged from the original control community (0N). These results were very clear in the case of B1, however, in the case of B5, the treatment 10N was not so different from the 20N, so between those treatments there was a

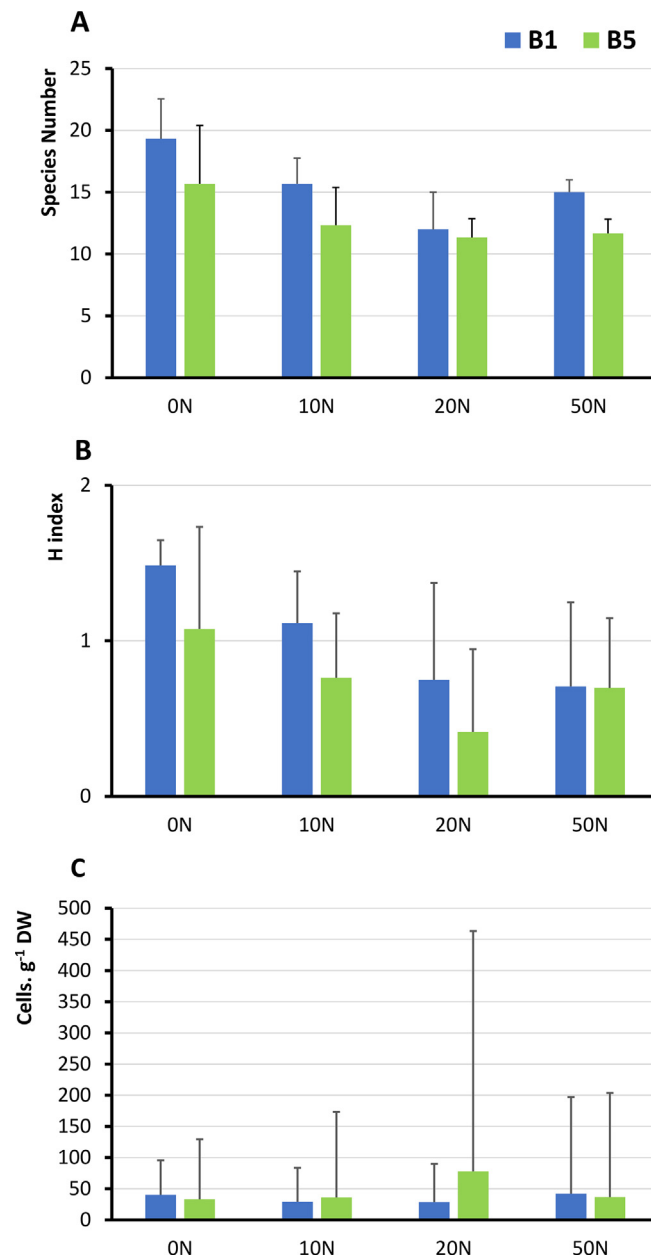


**Figure 3.** (A) Abundance (cells g<sup>-1</sup> DW) of ciliate species appearing in single treatments (20N and 50N); (B) ciliate species always excluded from 50N treatment.

low b-diversity index, while 10N was very different from the 0N treatment and very different from the same treatment in B1.

The N deposition experiments affected greatly scuticociliates increasing their numbers as experimental N deposition increased. In B5, Scuticociliatia had an increasing and greater predominance in treatments 0N, 10N and 20N than Block 1 (B1), and then a reduction in 50N was observed (Fig. 5). The genus *Cyclidium* was the most abundant and frequent of the five genera found (Table 1; Supplementary Material Fig. S1), and two of the species in this genus became dominant in the 20N and 50N deposition treatments, with populations reaching between 65–90% of the total ciliate abundance specially in B5. This effect was due to two species: *Cyclidium muscicola* and *C. citrulus*. The most abundant and frequent species of scuticociliate in B5 was *C. muscicola* (Table 1; Supplementary Material Fig. S1), where it reached 63% of the total population of ciliates. The plots treated with N then showed higher abundance of this ciliate than con-

trols (0N). It is interesting to mention that in this block, this species coincided with the presence of a heliozoan population of the genus *Acanthocystis*. In the case of B1, the most abundant scuticociliates were *C. citrullus*, and *C. muscicola* which contributed 81,5% of the total ciliate population (Supplementary Material Fig. S1). The population of these species also increased in abundance as N-deposition loads increased. Multivariate correspondence analysis (MCA) (Supplementary Material Fig. S5) for B1 showed that the first two axes (dimensions) explained 96.96% of the variability of the ciliate groups found within the N treatments. The first dimension showed the gradient from low N concentration (10N or control 0N) to high N concentration (20N and 50N). Scuticociliates appeared close to the high N concentrations, while Colpodea and Litostomatea were located between 0N and 10N, Prostomatea closer to 10N and Hypotrichia closer to 0N. Heterotrichea and Phylopharyngea were in the low end of N concentration while they were also displaced in one extreme of dimension



**Figure 4.** (A) Mean ciliate species richness and standard deviation; (B) Mean diversity index (Shannon-Wiener H index) and standard deviation; (C) Mean Ciliate abundance and standard deviation (Cells. g<sup>-1</sup> of dry weight) in both blocks (B1 and B5) for different N-treatments (0N, 10N, 20N, 50N).

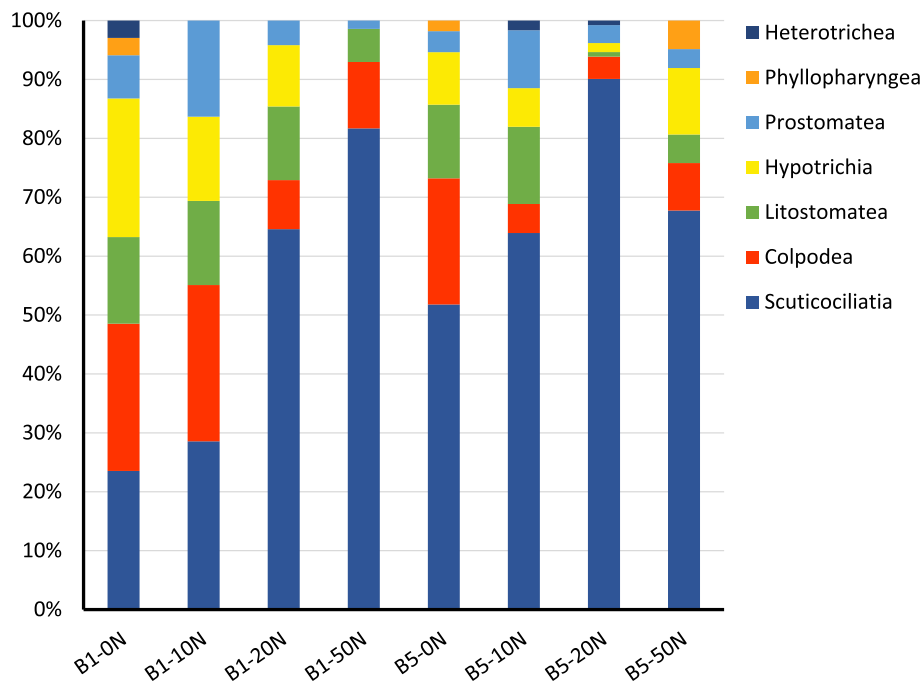
2, which seems to indicate that the appearance of these groups was not related to N deposition. In B5 results were similar (not shown), although the differences in species compositions modified the position of the groups in both dimensions.

The Colpodea group with 13 species (Table 1, Supplementary Material Fig. S2), although showed higher diversity than the Scuticociliatia group, had a lower abundance. This group seemed to be affected by increasing nitrogen concentrations in

the plots, in an opposite way to what happened with scuticociliates, as higher N loads reduced their populations, this was also observed in the multivariate correspondence analysis (Supplementary Material Fig. S5). *Colpoda* was the most abundant and frequent colpodean genus of the 13 species observed in this group (Table 1; Supplementary Material Fig. S2). The most abundant and frequent species in B5 was *Colpoda cucullus*, reaching a percentage of 2.3% of the total population of ciliates (Supple-

**Table 2.** b-diversity (Whittaker index:  $b_w$ ) values between blocks (B1 and B5) and treatment plots (0N, 10N, 20N and 50N).

		B1				B5			
		0N	10N	20N	50N	0N	10N	20N	50N
<b>B1</b>	<b>0N</b>	0.00							
	<b>10N</b>	0.37	0.00						
	<b>20N</b>	0.49	0.61	0.00					
	<b>50N</b>	0.57	0.59	0.60	0.00				
<b>B5</b>	<b>0N</b>	0.40	0.49	0.50	0.47	0.00			
	<b>10N</b>	0.51	0.66	0.53	0.79	0.59	0.00		
	<b>20N</b>	0.47	0.46	0.48	0.60	0.55	0.36	0.00	
	<b>50N</b>	0.47	0.62	0.63	0.52	0.68	0.52	0.45	0.00

**Figure 5.** Relative abundance (%) of ciliate groups for both blocks (B1 and B5) in N-deposition treatments (0N, 10N, 20N, 50N).

mentary Material Fig. S1). While in B1 the most abundant and frequent species was a very characteristic morphotype of *Bryometopus* sp. with a 4.3% (Supplementary Material Fig. S1) which only appeared in the control treatment (0N) in B5.

Litostomatea and Hypotrichia showed similar total abundance during the study, however, the latter was less diverse than the former (Fig. 2A, B). Litostomatea species did not seem to show a clear trend associated with N deposition (Supplementary Material Fig. S3). The most abundant species was *Trachelophylum* sp1, which occurred in both blocks

(B1 and B5) in all experimental plots and represented 5.5% of the total ciliate population in B1, and 3.2% in B5. The abundance of this species tended to be lower in the N-treatments than in the control treatment (0N) in B1, but there was a high increase in 10N compared to 0N in B5, and then a high decrease towards the higher N concentrations (20N and 50N). The most abundant and frequent species of Hypotrichia was *Gonostomum affine*, which reached a percentage of 6.4% of the total population of ciliates in B1 and 1.9% in B5. This species showed a clear and consistent tendency in B1

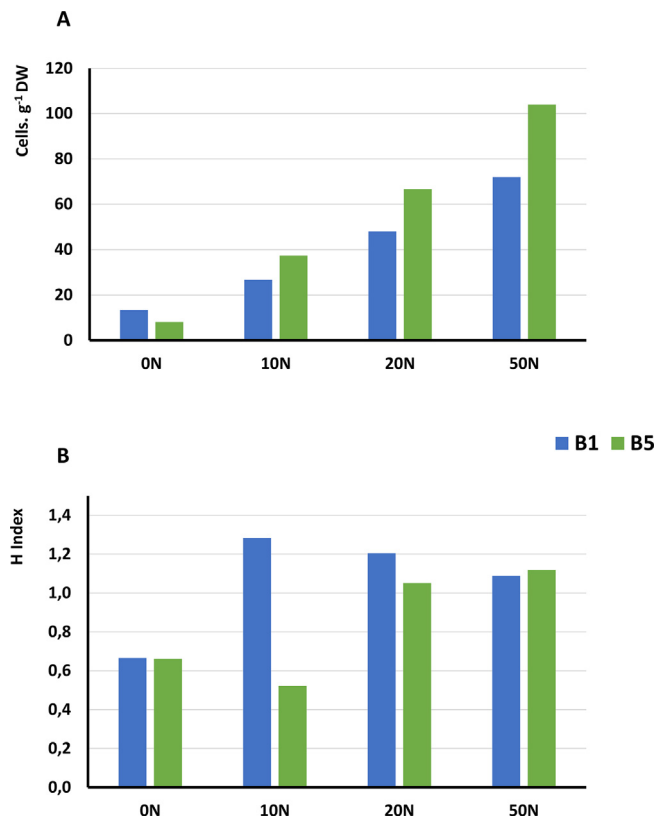
and B5 to reduce their abundance as N concentration increased in the N-deposition treatments, disappearing in 50N (Supplementary Material Fig. S4).

The remaining ciliate groups observed, Prostomatea, Phyllopharyngea and Heterotrichea had lower abundances (Fig. 2A) and did not follow a distinctive pattern in their distribution. Multivariate correspondence analysis showed the same result, as it has been mentioned above (Supplementary Material Fig. S5). The species generally occurred in the first half of the sampling days, as observed for Litostomatea. The most abundant and frequent species in Prostomatea was *Urotricha ondina* with a percentage of 2.1% of the total population of ciliates in B1, and 3.2% in B5, followed by *Plagiocampa mutabilis*. Phyllopharyngea and Heterotrichea included only two low-abundance species which were found in three separate counts.

Four statistically significant models were obtained by stepwise multiple regression with a backward elimination approach. This procedure allowed us to prospect significant predictors of the relationships between ciliates. The abundance of the main ciliate groups was selected as explanatory variables in the multiple regression model for the presence/abundance of a single group. The backward elimination procedure initially included all possible explanatory ciliate populations (variables) in a model and discarded, one by one, those that were less statistically significant as explanatory variables. The procedure ended when the variables remaining in the model were all statistically significant (Table 3). Four significant models were obtained that explained a high percentage of the variability ( $R^2$ ) found for the abundance of Scuticociliatia, Heterotrichia, Hypotrichia and Litostomatia. The first variables eliminated in all models were Prostomatea, Phyllopharyngea and Colpodea, and no significant models were found to explain the presence/abundance of these groups. This indicated that these populations were not significant explanatory variables for the abundance of any of the aforementioned groups and that none of those groups appears to be related to their presence or abundance. Finally, Litostomatia and Hypotrichia were excluded from the model for Hypotrichia and Litostomatia respectively. The highest percentage of explanation was found for Scuticociliatia and Heterotrichia. These models provide information that can be used to interpret the functional activity of some of the ciliate groups found in the BSC samples (Scuticociliatia, Heterotrichia, Litostomatia and Hypotrichia) (Table 3).

**Table 3.** Stepwise multiple regression models, variability explained by the model ( $R^2$ ) for every ciliate group, F-value of the ANOVA and the corresponding p-value.

Model	$R^2$ (%)	F stat / p value
Scuticociliatia = 7861.09 + 29.5613*Heterotrichia - 8.77783*Litostomatia - 3.33856*Hypotrichia	97.27	F = 47.50; p = 0.001
Heterotrichia = -243.347 + 0.271296*Litostomatia + 0.111384*Hypotrichia + 0.0309262*Scuticociliatia	94.45	F = 22.68; p = 0.006
Hypotrichia = 616.492 - 0.0924562*Scuticociliatia + 4.10827*Heterotrichia	76.62	F = 8.19; p = 0.026
Litostomatia = 650.693 - 0.0756239*Scuticociliatia + 1.79304*Heterotrichia	89.29	F = 20.84; p = 0.004



**Figure 6.** (A) Testate amoebae total abundance; (B) diversity (Shannon-Wiener H index) for the different plots of the N-deposition field experiment (0N, 10N, 20N, 50N) of both blocks, B1 and B5.

### Testate Amoebae Diversity

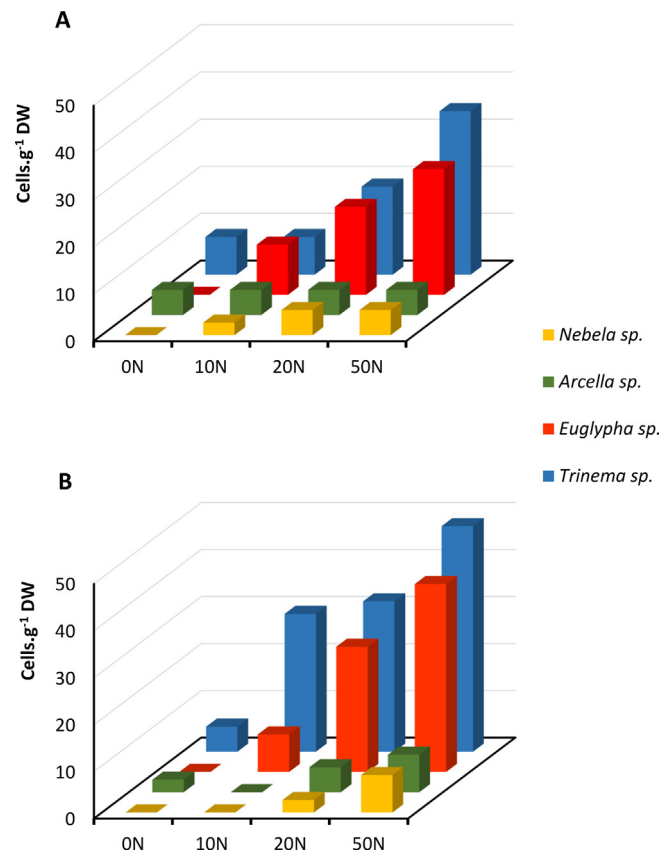
The diversity and species richness of testate amoebae were very low compared to that of ciliates (Figs 6, 7). Only 4 species were found: *Nebela* sp., *Arcella* sp. (Amoebozoa) and *Euglypha* sp. and *Trinema* sp. (Rhizaria). Control plot (0N) diversities in both blocks were low. The N-deposition gave place to a high increase in diversity in B1 due to the appearance of two new species: *Euglypha* sp. and *Nebela* sp. and then decreased steadily towards the 50N plot due probably to the increase in abundance and dominance of two of the species, *Trinema* sp. and *Euglypha* sp., although it remained higher than in the 0N treatment since the four species remained. In Block B5, however, the effect was a progressive increase in those N-treated plots towards 50N treatment where also *Euglypha* sp. and *Trinema* sp. dominated the testate amoebae populations. The 10N treatment in this case showed a light diversity reduction compared to 0N probably due to the absence of *Arcella* sp.

The abundance of testate amoebae was in this case 35% higher in B5 than in B1 (Fig. 7). There was also an increase in the abundance of amoebae with high N deposition in the treatments, with a greater increase in the B5 than in B1 (Fig. 7A, B). *Trinema* sp. and *Euglypha* sp., both with self-secreted siliceous scales (idiosomes), were, in that order, the most abundant testate amoebae species in all nitrogen deposition treatments studied, clearly increasing in abundance as N deposition increased from 10N, 20N to 50N (Fig. 7A, B). The other species, *Nebela* sp. and *Arcella* sp., remained almost constant or increased very slightly as N deposition increased, but with much lower abundances.

### Discussion

#### Abundance and Diversity of Ciliates and Testate Amoebae

The protists recovered in these ecosystems were 45 ciliate species (in 216 sampling units), and 4 species of testate amoebae (in 24 sampling units). These



**Figure 7.** Testate amoebae species abundance for the different plots of the N-deposition field experiment (0N, 10N, 20N, 50N) of both blocks, (A) B1 and (B) B5.

numbers are similar to previously studied BSC in arid environments from the United States and Australia (Bamforth 2004, 2008; Robinson et al. 2002) to South Africa (Dumack et al. 2016), although this last work is not comparable to ours since no ciliates or testate amoeba were surveyed. These results seem to indicate that the BSC from the semi-arid Mediterranean areas have a lower number of species and lower diversity than those observed in other terrestrial ecosystems (Esteban et al. 2006; Finlay et al. 2001; Finlay and Fenchel 2001; Foissner 2000; Ledeganck et al. 2003).

Ciliate species richness observed in this work was the same found by Bamforth (2008), who obtained 45 species as well, and it was 10% lower than the species richness found by Bamforth (2004) and 20% lower than that retrieved by Robinson et al. (2002), who obtained 50 and 56 species respectively. The relative frequency of rare species, which we have considered here as those below 1%, and those that were only observed once, were the lowest in the work by Robinson et al. 2002 that accounted for only 2% of the observed species,

while the frequency of rare species found here was higher (15%). This difference is probably related to the number of sites that were explored by Robinson et al. (2002). These authors explored 17 areas, most of them under different types of vegetation, while here eight sites were explored but six had different N-treatments.

Robinson et al. (2002) worked on Australian arid areas, in different sites and soil types, with an alkaline pH. However, it is important to consider that most of the sites they included were BSC under shrubs canopies which always show a higher abundance and diversity of protists, especially in the case of testate amoebae since higher concentrations of organic matter are found in these areas. There were only three interspace soils (bare soils) and they showed extremely low richness of both ciliates (4–12 species) and testate amoebae species (6–8) so, in these cases, results were closer to those obtained here. They mentioned that they also studied early-stage cyanobacterial crusts in which they found four species of ciliates, but the number of testate amoebae species present was not identified. As

for the abundance of testate amoebae, they were two orders of magnitude higher than those of ciliates, which is completely different from what we found. Therefore, for comparison purposes, the samples most similar to the ones we worked with, were those labelled as interspace soils, not affected by litter or interactions with plants' canopy and these had even lower species richness than ours. The cyanobacterial crust had the lowest ciliates species richness, which is consistent with an early-stage BSC, different from our observations on late-stage BSC.

Bamforth (2004, 2008) working in different types of BSC from arid ecosystems (Arizona and Utah respectively) observed similar pH values to ours as well (Ochoa-Hueso et al. 2011b, 2013b; 2017). They found the highest ciliate species richness in cyanobacteria-lichen crusts. When lichens were present, their results on testate species richness were much lower than those for ciliates, as occurred in Bamforth (2004). However, these results were higher in his second paper, where he used a different methodology to count testate amoebae (Bamforth 2008). Therefore, the results from Bamforth (2004) are somehow very similar to ours, not only in terms of total species richness but also in the r-K strategist appearance and species present, although they found in general a much higher diversity of testate amoebae as there were surveyed a larger number of sites.

Khanipour Roshan et al. (2020) working on the biological crusts of the Baltic Sea coastal dunes, reported 34 morphotypes of heterotrophic protists, mainly amoeba, amoeba-flagellates, and flagellates (Cercozoa and Stramenopiles). These results were obtained in a very different environment to that shown here and found a comparatively very low ciliate diversity that was only recorded as a tiny percentage in their samples, although they did not include detailed information in this regard. Comparison in this case was not possible because ciliate groups were not identified.

As mentioned above, other authors working in edaphic ecosystems have detected higher species richness, up to 102 species of ciliates and 105 species of testate amoebae in samples from Scotland (Finlay et al. 2001), 270 species of ciliates in Germany (Foissner 2000), 128 species of ciliates in Namibia (Foissner et al. 2002) or 114 species of ciliates in China (Li et al. 2010). The former works, represent different environmental conditions from those of the BSC studied here, however, the work by

Foissner et al. (2002) included areas with semi-arid characteristics, as in this study. For comparison purposes, the results obtained on ciliates in our research and other works in BSC represent less than 50% of the diversity found in other soil ecosystems. In the case of testate amoebae, this work shows approximately 26 times fewer testate amoebae than those found in other soil environments. Bamforth (1973) pointed out that species diversity depends on the stability of the terrestrial ecosystem, which in turn depends on the continuity of moisture, meaning that wetter regions would have more species of protists. In this sense, a semi-arid or arid ecosystem would be expected to have lower protist diversity. This lower diversity has been common to all BSCs as recorded in the few works exploring protists (Bamforth 2004, 2008; Dumack et al. 2016; Robinson et al. 2002), but has also been found in N deposition treatments (Wang et al. 2018).

The comparison of the community structure of the untreated (ON) plots studied here with those previous works on BSC showed several differences. The abundance of ciliates was low and in the range with other works, however, the abundance of testate amoebae was low in our case, since other works show 18 times more abundant testate amoebae than ciliates (Bamforth 2004; Robinson et al. 2002). Considering the ciliate groups reported in other works on BSC, the most frequently occurring groups were colpodids (40–58%), followed by hypotrichs (20–30%) and litostomatids (9–13%) (Bamforth 2004, 2008; Robinson et al. 2002). However, in our BSC, colpodids had a lower frequency (33%), with a similar number of species, followed by scuticociliates and litostomatids, both with a 19% and finally hypotrichs with a 16%. The scuticociliates, although were also observed in other works too, had always less than half the frequency with the same number of species or less. Therefore, these populations do appear to be important in our BSCs. This is interesting, because scuticociliates are, like colpodids, r-strategies bacterivores, and in our case, seemed to appear in the successions with high populations after colpodids decreased. The most abundant scuticociliate found, *Cyclidium muscicola*, was also present in the other works mentioned.

The colpodid/polyhymenophoran (C/P) ratio has been used to assess the predominance of ciliate groups in the populations (Li et al. 2010). This ratio represents the relationship between r-selected Colpodida with a wide tolerance to adverse conditions, as they can encyst, and the Polyhy-

menophorea, K-selected ciliates which are very sensitive to fluctuations and more abundant under stable conditions. Therefore, this ratio also indicates the adverse/disturbed or favourable/stable conditions of the environment for soil protozoa. The total C/P ratio here was always below 1 indicative of undisturbed conditions, as it happened in all the previous studies (Bamforth 2004, 2008) except in the case of Robinson et al. (2002) where C/P was over 1.

Regarding testate amoebae, it was common to find in the control treatments (0N) only two species of the genera *Arcella* and *Trinema*, one K-selected (lobose) and one r-selected (filose), respectively. Although the literature records that *Arcella* species (i.e. *A. vulgaris*) are indicators of acidic pH in lacustrine environments (Marcisz et al. 2020), the opposite has been found in other lakes (Neville et al. 2010; Sigala Regalado et al. 2018). Results from BSC studies show that certain species can thrive in desert BSC with alkaline pH of 7.5–8 (Bamforth 2008). The species *A. arenaria* was found in BSC on soil pH between 7.2–8.4 or 7–10 (Bamforth 2004; Robinson et al. 2002 respectively). *Trinema* species also appear in all BSC studies showing taxonomic details. The most frequently encountered species has been *T. enchelys*, however, *T. lineare*, *T. plenum*, and *T. complanatum* have also been cited in Australian soils (Bamforth 2004, 2008; Robinson et al. 2002). Therefore, it can be suggested that these species may probably occur in alkaline conditions and carbonated soils. Other studies characterizing environmental conditions for a range of alkaline-mineral rich and higher pH have found *T. lineare* and *Euglypha rotunda* under similar conditions to those found here (Marcisz et al. 2016; Šimová et al. 2022). These studies developed models to estimate biotic-abiotic associations and these species were typical of higher pH and drier, calcareous conditions, as is also the case in our study.

The low abundance of testate amoebae and species richness found seems to be a characteristic of the semi-arid Mediterranean ecosystem studied, although the differences with previous works might be a consequence of a low number of sites investigated in this study or the use of different methodologies for testate amoebae abundance assessment. These results should be investigated further.

#### Effects of N Deposition on Ciliates

The N treatments affected the ciliate community structure. An overall decrease in the number of spe-

cies and diversity was observed as N deposition was increased when compared to the control treatment (0N). The diversity (Shannon-Wiener index) values were similar to that found in other studies in soil litters (Jia et al. 2021), although other works have observed a reduction in microbial diversity (Fiore-Donno et al. 2022; Wang et al. 2015, 2018).

The experimental N fertilization conditions most similar to our N-deposition in the field were those of Gilbert et al. (1998a) although they worked in a very different habitat. These authors applied an enrichment gradient of 5N, 15N and 50N to a short-term deposition experiment in peatland microbial communities. Their results showed an increase in protozoa biomass when N supply increased. We did not estimate biomass but our ciliate abundance results, indicate that deposition of N did not produce a clear effect on this group of protists.

Environmental pollution due to N-deposition has been reported to acidify soils. This could be a priori a possible reason for the observed ciliate diversity reduction. However, the studied site did not show a significant pH reduction due to experimental N-deposition, probably due to the soil type present in the area (calcareous with slightly basic pH between 7.79 and 8.01) which could have buffered the acidification (Lo Cascio et al. 2021).

The changes in the community structure can be explored considering the responses of the different ciliate groups to the increase in N-deposition. Other studies on litter have found that protist richness and most dominant groups were positively correlated with TN content (Jia et al. 2021). Similar results about ciliate abundances were reported by Li et al. (2010) with respect to total N. We found that some scuticociliate species, the most abundant group, increased in abundance with increasing N deposition. However, other groups, Colpodea, Hypotrichia or Litostomatea showed the opposite trend to scuticociliates. And some groups only appeared sporadically at the highest N concentrations, such as certain Phyllopharyngea.

Interestingly, some studies have argued that although total N (as well as organic matter, phosphorus, or potassium) has been found to influence soil protists, these effects, especially in heterotrophic protists, are indirect effects, as changes in resource availability would be more important, i.e., the abundance and richness of prey would be determinant (Li et al. 2010; Zhou et al. 2017). In biocrusts microbial species richness and diversity, mainly of prokaryotes, are lower than the underlying soil,

suggesting a strong selection within crusts (Moreira-Grez et al. 2019; Nagy et al. 2005). The most abundant ciliate species mentioned (scuticociliates) are exclusively bacterivorous. Therefore, an increase in their abundance must be related to the presence of a suitable prey (Ingham et al. 1986).

Scuticociliates did not reach high numbers until colpodids reduced their abundance in the N-treatments. However, they survived longer. The inverse relationship between *Cyclidium muscicola* and *Colpoda* spp. found in this work, has also been described in other field experiments of soil fertilization with manures (Aesch and Foissner 1993) but in the opposite direction. The authors concluded that the dynamics of both ciliates were related to changes in the number and quality of the food, although the soil conditions were very different from those in this experimental set-up.

On the other hand, during the study to evaluate diversity in the samples of plot 20N (B5), it was observed that the population of *Cyclidium muscicola* decreased rapidly from the 7th to the last day of sampling. Although no other protist populations were counted, a large population of an *Acanthocystis* (Heliozoa) species coincided with the bloom of *C. muscicola*. The main prey of the heliozoan, as a diffusion feeder, was probably this scuticociliate. The type of feeding mechanism of Centrohelids requires an abundance of prey (in this case ciliates) for efficient feeding. This effect could have led to the disappearance of this scuticociliate, as it was observed during the last two counting dates. This indicated that *Acanthocystis* was able to survive and thrive at 20N concentrations and functionally be able to capture prey. Bamforth (2004) also recorded the presence of Heliozoa in BSC and although there are no records of this feeding activity in soils, feeding experiments of another heliozoan (*Actinophrys sol*) on scuticociliates have demonstrated a high feeding capacity on *Cyclidium* at high concentrations of prey (Pierce and Coats 1999) as it was in this case. Other heliozoan predation experiments have demonstrated the ability of these microorganisms and especially *A. sol* to predate on *Urosomoida* sp. (Weithoff and Bell 2022). Although in our observations these *Urosomoida* species coincided as well with *Cyclidium* and *Actinophrys*, they did not show high populations, but could have been predated as well.

Colpodids which are usually the most abundant group in soils, were in our study the second in abundance and decreased as N deposition increased;

these are r-selected bacterivores, filter feeders with an upstream mechanism, with smaller feeding structures and a different oral organization that provides them with lower bacterial clearance rates, so they would need a high concentration of longer rod-shaped bacteria to capture them and be able to grow on them (Fenchel 1987; Foissner 1987). The type of bacteria that *Cyclidium* species can feed on, due to their downstream filter-feeding strategies and oral structures, are in the smallest range size, between 0.25 to 0.35  $\mu\text{m}$  (Fenchel 1987). However, they require high bacterial concentrations to grow. Accordingly, their preys and survival strategy are slightly different, and they would encyst straight away once the bacterial concentration is below the optimal level to feed effectively. An indirect effect of an increased N deposition may have reduced their potential bacterial prey, then their abundance would equally decrease with increasing N concentration. Although we have not measured bacteria, other works have shown a bacterial succession occurring during N-deposition experimentation, so this could have been an important factor to consider. Other works on soils have found that the presence of *Colpoda* species was correlated with bacterial abundance (Xiong et al. 2019).

The most abundant colpodid species was *Colpoda cucullus*. These species are generally the first to appear since excystation is quite fast in *Colpoda* species. Early work on *C. cucullus* indicated that after addition of appropriate media and temperature, excystation proceeded in less than 2 hours (Hall 1913). These species are r-strategists with high reproductive output, fast generation times and short life spans. Although speculative, their reduction (B1) with N treatment or maintenance in low abundance (B5) probably had something to do with the reduction of their optimal bacterial food, as mentioned above, especially if there was a shift in the bacterial population structure due to the N-fertilization.

The other groups that appeared were Litostomatea and Hypotrichia. All litostomes found were haptorids, typical of soils with a slender body shape that allow them to move easily in edaphic environments. These ciliates are predators or raptorial feeders, of large preys, like other ciliates or other phototrophic protists. Most of the litostomes appeared at the control plot (0N) or in the low N-deposition plots, so it appears that increased N-deposition affected the development of these populations. The most abundant litostome was *Trachelophyllum* sp.1 which appeared exceptionally in both plots in all treat-

ments, so it did not seem to be greatly affected by increased N-deposition.

Hypotrichia were the fourth most representative group in the BSC, equally as abundant but less species-rich than Litostomatea. Hypotrichia is a predatory group, suspension feeders or filter feeder with upstream mechanism (Fenchel 1987). Because this mechanism does not allow them to retain very small preys, they are specialised in larger preys, although they may feed on larger bacteria, flagellates, photrophic protists or other small ciliates, and are generally considered omnivores. They are also considered K-selected, so they usually appear in successions later than an r-selected protist (Lüftenegger et al. 1985). This may allow them to feed on r-selected protists that were growing earlier. However, the most abundant hypotrich found in the BSCs studied was *Gonostomum affine*, which due to its oral configuration, is considered a bacterivorous species (Kamra et al. 2008).

The stepwise multiple regression model for Scuticociliatia (Table 3) showed that an increase in Hypotrichia or Litostomatia had a reducing effect on Scuticociliatia. This suggests that both groups preyed on Scuticociliatia, and that Litostomatia was a more effective predator than Hypotrichia. However, an increase in Heterotrichia meant an increase in Scuticociliatia, probably because both are bacterivores, so both were related to bacteria and behaved similarly. No significant results were obtained for any relationship of Colpodida with any of the other groups present. This effect can be interpreted to mean that they have no effective predators within ciliates.

#### Effects of N Deposition on Testate Amoebae

Our results show that N addition over 20N load resulted in a higher diversity of testate amoebae and that N increase resulted in a steady increase of the biomass of testate amoebae when compared with the control. To our knowledge, there have been no similar works evaluating testate amoebae in semi-arid environments, in BSC or even in soils with N deposition.

In the BSC of a semi-arid Mediterranean environment, one would be expected to find testate amoeba species adapted to dry conditions, higher pH and calcareous soils. The morphotypes found in the BSC -*Trinema*, *Euglypha*, *Arcella* and *Nebela* - all include species adapted to these conditions. Although no identification at species level was

made, other studies show that most *Trinema* species are adapted to dry conditions (e.g., *T. lineare*, *T. enchelys*), while only some *Euglypha* species would be adapted to these dry environments. *Arcella vulgaris* or *A. hemisphaerica* are also adapted to dry conditions (Šimová et al. 2022).

In the plots treated with nitrogen deposition, in addition to *Arcella* sp. and *Trinema* sp., two other species were found: *Euglypha* sp. and *Nebela* sp. *Euglypha* sp. has also been found in other crusts, however, it is the first time *Nebela* sp. is cited in this type of habitat. The most common species of *Euglypha* sp. found in other BSC have been *Euglypha rotunda* and *E. laevis*. Although eight other species of *Euglypha* have been cited in these habitats. The highest diversity of *Euglypha* species (9 species) was found by Robinson et al. (2002) in Australia, who also noted that the genera *Euglypha* and *Trinema* (among two other species) were the most prevalent and provided more than half of the individuals in the sites studied.

The small increase of *Nebela* sp., parallel to a large increase of *Euglypha* sp. and *Trinema* sp., could be related through a predatory relationship which has been found in other works. Although we cannot confirm this association with our data, Gilbert et al. (2003) found that both *Trinema* and *Euglypha* were preyed upon by *Nebela tincta*. This activity was found within the marginal groups on which this species preyed (included predation on small testate amoebae; 4%, including *Trinema* spp. and *Euglypha* spp.). These previous data could be an indication why *Nebela* did not have a notable increase in our observations.

Mitchell and Gilbert (2004) working on testate amoeba in *Sphagnum*, and with a very different community structure of testate amoebae to that described here, found no changes in species richness with the addition of N. They used two concentrations within the range of those used in this study and one higher concentration. No significant trends of individual species associated with the N fertilization experiment were found, although there was an increase in *E. rotunda* and *T. lineare* in some of the increased N concentration treatments shown. However, Gilbert et al (1998b) found a decrease in the relative proportion of testate amoeba in fertilized peatland plots.

Song et al. (2015) also developed their work in peatlands to assess the effect of N fertilization on testate amoebae. This work used much higher N concentrations than the ones used here. They found

that N addition decreased the biomass of *Euglypha* and especially *E. rotunda*. We obtained opposite results with *Euglypha* sp., so results are not easy to compare as these differences could be a consequence of the higher N concentrations used or the environment in which they worked on.

#### Potential Bioindicators: Protist Species Associated to a N Deposition Gradient

Although the identification of indicator species was not the main objective of this work, we have found consistent trends in the appearance or disappearance of species that could be associated to N concentrations and could be useful for future bioindication studies or to provide further autecological data of protists in these environments.

Some works assessing relationships with soil physical–chemical conditions have found that ciliate abundance correlates positively with total N or Ammonia-N (Jia et al. 2021; Li et al. 2010). However, these results are not consistent for all groups of ciliates, since not all groups or species respond in the same way, as we have shown in this work. Some species only appeared in the highest N depositions used (50N), such as *Aperthospathula similis* or *Colpoda colpidiopsis*, however, we have not found detailed data about environmental conditions where these species have been previously found. We could hypothesize that these species could be related to high N concentration in BSC, as we have observed here; these results should be verified in future research.

Certain species never appeared in the plots of the highest N deposition plots (50N). These species were *Arcuospathidium vermiforme*, *Colpoda steinii*, *Urotricha ondina* and *Gonostomum affine*. *A. vermiforme* only occurred at low N concentrations. However, there are no N data recorded in the literature from where this species has been found previously, that could contribute to establishing a bioindicator value for this species.

*C. steinii* has been widely used as a model ciliate for ecotoxicological studies. It is a ciliate also considered ubiquitous or generalist and has been cited with high abundances in different mangrove belts designed for municipal sewage treatment, with high N content (both of Ammonium-N and Nitrate-N) (Chen et al. 2008). Therefore, it is strange that our study only showed higher abundance in control plots, with low N content, or decreased as N deposition increased.

The prostomid ciliate *U. ondina* was excluded as well from the highest N concentration plot. No data have been found in the literature about autoecological information on this species in soils.

*G. affine* consistently reduced its abundance with increased N deposition (10N-20N) and did not occur at the highest N concentration (50N). There are no previous data indicating any relationship of this species with environmental N concentration. Only one paper has found *Gonostomum* sp. at sites with high ciliate diversity where soils had extremely high total N concentrations in India (Abraham et al. 2019). Other works in different environments have found *Gonostomum* in acid mine drainage at very low pH (Mesa et al. 2017) and were considered a novel environmental feature for this genus. However, the *G. affine* populations found here were mainly found in soil with basic pH.

Testate amoebae have been shown to have strong relationships between morphological characteristics and environmental variables related to soil moisture (Fournier et al. 2012). This group has been extensively studied in peatlands to assess the relationship between the abundance and diversity of populations and environmental variables (Carballeira and Pontevedra-Pombal 2021).

Only *Euglypha* sp. and *Trinema* sp. showed a possible relationship with increased N-deposition since both showed increased abundances in BSC with increasing N-deposition, reaching the highest abundance in the highest deposition treatment (50N). Although identification to species level was not achieved, there is much information that seems to support our observations regarding these species and their relationship with N deposition, although most data have not been obtained from soils but from other environments, mainly peatlands (Šímová et al. 2022), but also in wastewater treatment plants (de Góes Cohn Freitas et al. 2022).

## Conclusions

The diversity, abundance, and species richness of ciliates and testate amoebae in biological soil crusts are lower than those observed in other soil ecosystems. This might be associated with the specific characteristics of this habitat and the effects of other organisms involved in these ecosystems.

The increase in nitrogen concentration caused by the deposition experiment decreased the number of species, abundance, and diversity of ciliate populations in BSC. The only exceptions to this

observation were the Scuticociliatia whose abundance increased at elevated nitrogen concentrations, and Prostomatida, which only increased at low N concentrations.

The number of species, abundance and diversity of testate amoebae were very low in the BSC studied. The increase in N concentration brought by the deposition experiment increased the species richness, the abundance, and the diversity of the testate amoeba populations.

## Methods

**Site description and experimental treatments:** Biological soil crust samples were collected in May 2010 from the Nature Reserve 'El Regajal-Mar de Ontigola' (Aranjuez, Spain; 40° 00' N, 3° 36' W), an area in the south of the Madrid region. This is a Mediterranean semi-arid location, around 580 m above sea level with an average annual precipitation of 425 mm. The soil of the area is alkaline (limestone and gypsum) and the dominating vascular plants are evergreen shrubs such as *Quercus coccifera* L. or *Rosmarinus officinalis* L. Soil was covered by a well-developed early- and late-successional cyanobacteria-lichen-bryophyte biological crust composed of cyanobacteria, lichens and mosses characteristic of calcareous soil and with a calcicole BSC distribution pattern, i.e. the majority of lichens and bryophytes were typical of basic soils (Ochoa-Hueso et al. 2011b; 2013b; Ochoa-Hueso et al. 2017 for further information).

Long-term field experiments were carried out at this site to emulate N deposition for 8 years since 2007. The main objective was to assess the possible effects of increased N deposition from atmospheric pollution on plant communities and the function of these ecosystems in general. The experimental design is described in detail elsewhere (Ochoa-Hueso et al. 2011b, 2013a, Ochoa-Hueso et al. 2017). Briefly, a randomized block design was used, in which blocks included grouped experimental units (plots). There were six blocks with 4 plots each (2.5 × 2.5 m) separated with buffer areas of approximately 1 m. Each plot was randomly subjected to N additions of 10, 20, and 50 kgN ha<sup>-1</sup> yr<sup>-1</sup> respectively, keeping one unfertilized control per block to which no N was added. Background deposition was estimated at 6.4 kg N ha<sup>-1</sup> yr<sup>-1</sup> (Ochoa-Hueso et al. 2013a).

N-deposition treatments were carried out once a month with 2 L of 0-, 19-, 37- and 93- mM ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>) solution. The procedure was intended to reproduce the N enrichment due to dry deposition that causes sudden peaks in N concentration with rainfall. In this experimental context, two of the blocks (B1 and B5) were chosen for their similar characteristics and evaluated for this study. The N deposition treatment in each plot is indicated throughout this work as 0N, 10N, 20N and 50N according to the N concentration used.

Biological crusts were collected for each plot from the top 3 cm of soil and stored in sealed plastic bags. They were kept in the dark at 4 °C until processing. The biocrusts were then air-dried and sieved with a 4 mm mesh.

**Enumeration and identification of ciliates and amoebae:** Ciliate diversity within plots was assessed by enumeration and identification of ciliates using the non-flooded Petri dish method described by Foissner (1987, 1992) slightly modified and following the succession of populations during a previously determined time. Briefly, three sampling units (evaluation units) per plot of 5 g sieved dry sample were set in three different sterile plates and rehydrated,

saturation but not flooding it, with 10 ml of filtered (0.2 μm) deionized water to emulate the rehydration of a biological crust with rainwater in the natural environment (Finlay et al. 2000). Rehydrated samples were kept at 18 °C during the experimental period.

A preliminary study using this protocol was developed to evaluate the sampling effort. Samples were incubated for 30 days at the same temperature to estimate the potential abundance of protists in the trophic state. This protocol allowed us to obtain a species accumulation curve and estimate a minimum sampling period of 15 days and a maximum sampling period of 29 days for ciliates. The final protocol to assess ciliate diversity in BSC samples included rehydration and sampling for 9 days, distributed within the established sampling period.

Abundance and diversity of testate amoeba were carried out in a similar way, however, enumeration was instead carried out at the end of a 6-week incubation period, as described by Finlay et al. (2000).

Enumeration of ciliates in the rehydrated samples using the non-flooded protocol mentioned above was performed in three 25 ml aliquots, one per sampling unit (evaluation unit) and per day in each plot, and it was carried out, after evaluating the accumulation curves, on day 1, 2, 3, 4, 8, 10, 16, 22 and 29 after rehydration.

The identification of ciliates was made on the same rehydrated original samples or after the culture of aliquots of those samples in SES medium (CCAP) or in wheat grain medium (Kirby 1950). This procedure required in vivo observation (bright field, phase contrast and interference contrast) and the use of silver impregnation techniques such as quantitative protargol impregnation (Skibbe 1994), silver carbonate method (Fernandez-Galiano 1994), and dry silver nitrate (Klein 1958) when necessary.

Testate amoebae counts were assessed in three 1 ml aliquots (1 per plate) in a Sedgewick-Rafter chamber (Finlay et al. 2000) after incubating rehydrated samples for 6 weeks and staining with lactophenol aniline blue before the enumeration procedure. Identification was based on the shell or test morphology, shape, and size (Kosakyan et al. 2016).

Observations were performed on an Olympus microscope BX50 with bright-field and phase-contrast microscopy and on a Nikon Eclipse 80i equipped for bright-field and Nomarski differential interference contrast microscopy. Live measurements were made with the Carl Zeiss Axiovision Rel. 4.7 software or on photographs with Adobe Photoshop. The nomenclature used is based on Lynn (2008) and Adl et al. (2012).

**Statistical analysis:** Descriptive statistical analysis of abundance and diversity was performed with StatGraphics Centurion v16.2.04. Simple regression analyses were used to assess differences in the total abundance of ciliates, species numbers and diversity due to sampling dates and treatment. Kruskal-Wallis and Wilcoxon-Mann-Whitney tests were used to evaluate differences in the abundance distributions between N-deposition treatments. Stepwise multiple regression was used to search, through models, for significant predictors of ciliate relationships. Multivariate Correspondence Analysis was used to visualize the relationship between categories of ciliate group abundances and N deposition treatments. Diversity indices were calculated using the data analyser software PAST v2.17c: α-diversity was estimated using the Shannon-Wiener H index. Whittaker's index ( $b_{w}$ ) was used to estimate β-diversity, especially useful in the study of environmental gradients and spatial turnover implying the replacement of species between different treatments within the same habitat (Baselga 2010; Koleff et al. 2003).

## Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships

that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**Blanca Pérez-Uz:** Conceptualization, Methodology, Supervision, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Virginia C. Galfione:** Investigation, Data curation. **Raul Ochoa-Hueso:** Methodology, Resources. **Mercedes Martín-Cereceda:** Conceptualization, Methodology, Writing – review & editing.

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## Appendix A. Supplementary Data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.protis.2022.125929>.

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