

Rain-fed granite rock pools in a national park: extreme niches for protists

Blanca Pérez-Uz¹, Ismael Velasco-González¹, Antonio Murciano², Abel Sanchez-Jimenez², Manuel García-Rodríguez³, Juan D. Centeno⁴, Esperanza Montero⁴, Benito Muñoz², Cristina Olmedo², Pablo Quintela-Alonso¹, Pablo Refoyo², Richard A. J. Williams⁵ and Mercedes Martín-Cereceda^{1,*}

¹ Departamento de Genética, Fisiología y Microbiología, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid, Spain.

² Departamento de Biodiversidad, Ecología y Evolución, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid, Spain.

³ Departamento de Ciencias Analíticas, Facultad de Ciencias, Universidad de Educación a Distancia, Madrid, Spain.

⁴ Departamento de Geodinámica, Facultad de Ciencias Geológicas, Universidad Complutense de Madrid, Spain.

⁵ Human Science Research Centre, College of Life and Natural Sciences, University of Derby, UK.

* Corresponding author: cerecema@bio.ucm.es

Received: 10/03/19

Accepted: 21/10/19

ABSTRACT

Rain-fed granite rock pools in a National Park: extreme niches for protists

Rain-fed granite rock pools are geological landforms with a worldwide distribution. However, their role as habitats for microorganisms has been barely explored. We carried out a detailed morphological inventory of the ciliated protists in the sediments of three granite rain-fed rock pools from a Spanish National Park. The ciliate seed bank in the rock pools comprised 54 morphospecies. The species number inferred for each pool by rarefaction analysis was similar to that observed by microscopy. The most representative species were small bacterivorous ciliates, although the distribution of ciliate groups was significantly different in each rock pool. Testate amoebae were also found to be rich in species. This study demonstrates the existence of a diverse ciliate community adapted to persist in these ephemeral and extreme rock pool habitats through species resistance structures, many of which have not been described previously. The presence of competitive species in dormant stages prevents local extinctions through a sequential excystation over time. Our results provide a benchmark for studying protist and microbial biodiversity within rain-fed granite rock pools, a unique habitat that merits bio- and geo- conservation.

Key words: biodiversity, ciliates, dormancy, granite rock pools, conservation, testate amoebae

RESUMEN

Pilas graníticas ombrotóricas de un parque nacional: nichos extremos para protistas

Las pilas graníticas ombrotóricas son formas geológicas con distribución mundial. Sin embargo, apenas se ha explorado su papel como hábitats para los microorganismos. Hemos realizado un inventario morfológico detallado de los protistas ciliados en los sedimentos de tres pilas de granito en un Parque Nacional en España. La reserva de ciliados en las pilas graníticas fue de 54 morfoespecies. El número de especies inferidas para cada grupo por análisis de rarefacción fue similar al observado por microscopía. Las especies más representativas fueron los pequeños ciliados bacterívoros, aunque la distribución de los grupos de ciliados fue significativamente diferente en cada pila. Asimismo, las amebas testáceas presentaron una riqueza de especies elevada. Este estudio demuestra por primera vez la existencia de una diversa comunidad de ciliados adaptada a persistir en estos hábitats efímeros y extremos mediante estructuras de resistencia, muchas de las cuales no se han descrito anteriormente. La presencia de especies competitivas en estadios latentes evita las extinciones locales a través de procesos de exquistamiento

secuencial en el tiempo. Nuestros resultados proporcionan un referente para el estudio de la biodiversidad protista y microbiana en las pilas graníticas, un hábitat único que merece bio y geo conservación.

Palabras clave: *biodiversidad, ciliados, dormancia, pilas graníticas, conservación, amebas testáceas*

INTRODUCTION

The development of naturally formed depressions in solid bedrock of various types (granite, sandstone or limestone) is frequent around the world (Jocque *et al.*, 2010; Brendonck *et al.*, 2016). These depressions are typical on granite as minor landforms varying in diameter from centimetres to meters, and generally of circular or elliptical shape (Campbell, 1997). Different names, such as potholes (Chan *et al.*, 2005), weathering pits (Hall & Phillips, 2006), gnammas (Bayly, 1997), rock basins (Campbell, 1997) and rock pools (Meier & Soininen, 2014) are commonly used in the scientific literature. The current manuscript will adopt the term “rock pool”, which although non-geologically specific, complies with the terminology used in the most recent biological overviews on the topic (Brendonck *et al.*, 2010; Jocque *et al.*, 2010; Gabi & Matias-Peralta, 2015).

The genesis of the granite rock pool occurs at the geological scale on the surface of granitic outcrops or inselbergs. The process occurs due to the differential chemical dissolution of the minerals present in the rocks and mechanical and biological weathering, enhanced by the capacity to retain water once the pool is formed (Campbell, 1997; Chan *et al.*, 2005). Rain-fed granite rock pools are singular in that precipitation (rainwater, snow or ice) is the only source of water (Jocque *et al.*, 2010). Evaporation processes expose sediment-saprolites (*in situ* weathering products from granitoid rocks) into the cavity bed. The detritus left may contain a highly varied size of granite particles, organic crust of variable thickness and, in many cases, well-developed soil, growing bryophytes and vascular plants, that shape authentic ecological islands.

The alternation of desiccation and re-hydration cycles confers a transient nature to the aquatic biological communities inhabiting these granitic landforms. These communities are subjected, and have adapted, to extreme diurnal and season-

al changes in temperature and humidity (Jocque *et al.*, 2010). Several microhabitats are created in the sediments, which may be substantially different from those present in other natural formations (De Meester *et al.*, 2005; Brendonck *et al.*, 2010; Jocque *et al.*, 2010). Complex biotic-abiotic interactions develop at a fine-scale due to the heterogeneity of physical and chemical properties, built upon the highly unpredictable availability of rainwater, inundation and periods of drought (Chan *et al.*, 2005; De Meester *et al.*, 2005). Furthermore, rock pools may act as a starting point for the dispersal of organisms through the soil and watercourse, when excess water in the pool overflows, and some organisms may enter natural waterways, streams and rivers.

Previous studies on biological communities from rain-fed granite rock pools have explored the diversity patterns of vegetation (Baonza Díaz, 2009; Tuckett *et al.*, 2010) and invertebrates (Jocque *et al.*, 2010; Timms, 2014, 2016; Brendonck *et al.*, 2016). The literature on microbial communities from granite rock pool habitats is very scarce. Some research has been done on phytoplankton (Anusa *et al.*, 2012; Meier & Soininen, 2014), and on micro-parasites of invertebrates from coastal pools (Bengtsson & Ebert, 1998). However, taxonomic lists of free-living microbial heterotrophs from inland rock pools are patchy (Pinder *et al.*, 2000; Foissner *et al.*, 2002; Rylander, 2011). To date, no studies have compared the diversity, spatial/temporal distributions, or trophic dynamics of heterotrophic protists in the aquatic or sediment phases of these geological features.

Protists are eukaryotic microorganisms actively involved in diverse ecological activities (primary production, nutrient recycling, decomposition, bacterial predation) in close relation to animals and plants (Caron, 2009). Flagellates, ciliates and testate amoebae are the most important consumers of bacteria, both in aquatic and terrestrial environments (Jürgens & Matz, 2002; Corliss, 2004). In terrestrial ecosystems, as rock pools sediments

are, protists may play an essential link between the bacterial compartment and higher trophic level organisms such as nematodes, mites and earthworms (Ekelund & Rønn, 1994). Protists may also enhance terrestrial primary production as they stimulate plant nitrogen uptake and growth (Borkowski, 2004). A “soil microbial loop” hypothesis has been proposed to explain the increase of nitrogen uptake by plants in presence of protists: Bacterivorous protists facilitate the release into the soil inorganic nitrogen pool of at least part of the nitrogen accumulated in bacteria, thus increasing the nitrogen pool available for plant consumption (Clarholm, 1985). Ciliates include a large list of bacterivorous species, and several of their taxonomic groups show different oral structures that cause selective bacterial grazing, therefore influencing the composition of the bacterial community (Rønn *et al.*, 2002). The relevance that the type of feeding habits ciliates may have, has conducted to categorise the ciliates into either taxonomic and/or functional groups that allow the study of competitive traits among these microorganisms (Pratt & Cairns, 1985; Foissner & Berger, 1996). Moreover, many protists (ciliates and amoebae) are able to withdraw from the environmental stress of complete desiccation and/or high increase in temperature, because they go into dormant stages (cysts). These protists can provide large active populations immediately after the return of favourable conditions (rehydration). Some soil metazoan, such as nematodes and invertebrates, also undergo dormancy, but ciliates reproduce more rapidly, and together with bacteria are the first pioneers after a period of desiccation (Bamforth, 1995).

Rock pools (particularly rain-fed pools) have been recently proposed as “model habitats” due to their structural simplicity, global distribution, and stability in geological time scale (Brendonck *et al.*, 2010; Jocque *et al.*, 2010). A set of closely spaced rock pools like the ones studied here offer a model system to test ecological principles that remain virtually unexplored for rock pool microorganisms (De Meester *et al.*, 2005). These include spatial connectivity of meta-communities among nearby pools and r/K species selection and species competition. As they are transient habitats, rock pool may be good habitats to test r/K species traits. R-type

reproduction strategy is characterized by pioneer species with high multiplication rates, short generation times, rapid encystment/excystment cycles, and less sensitivity to abiotic stress. These species are capable of exploiting adverse conditions such as habitats where desiccation periods are unpredictable and intermittent more efficiently (Bamforth, 1995, 2001). Few data are available to date concerning r- and k-selection among ciliates (Lüftenegger *et al.*, 1985).

The aim of this work is to provide a novel detailed description of the ciliate community present in the dry sediments of rain-fed granite rock pools at a national park in Central Spain. The objectives are: (i) to monitor the spatial-temporal dynamics of the species after rehydration of the sediments in order to reveal the viable ciliate seed bank of the rock pools; (ii) to explore the differences between the ciliate meta-populations of closely located pools. Due to the transient and unpredictable environmental distinctiveness of the rock pools we hypothesise that small and highly efficient bacterial grazers would be the dominant ciliate species of these habitats.

MATERIALS AND METHODS

Study area

The rock-pools studied are located in La Pedriza, a huge granite batholith that formed around 300 Ma in the Spanish Central System mountain range (Fig. 1). La Pedriza is composed of equigranular granitoids rich in Carboniferous-Permian biotite (IGME, 2017). The fracture-controlled weathering of granites and erosion of the regolith created an etched landscape with boulders, kopjes and similar relief. The study rock-pools are on top of a granitic inselberg (40° 45' 5,20384" N, 3° 52' 54,46114" W, 1250 m altitude) covered by rock pools of various sizes (Fig. 1B).

The area is a singular and scenic landscape, was one of the first places to be protected in Spain as early as 1930, when it was listed as “Natural Site of National Interest”. In 1985, it became a Natural Park and, since 2013, it is part of the National Park “Sierra de Guadarrama”. (<https://www.parquenacionalsierraguadarrama.es/en/park/info-pnsg>).

Characterization of the rock pools studied

Three pools were selected for this study (hereafter P1, P2 and P3; Fig. 1C). Pool geometry (maximum axis, minimum axis, maximum depth) was determined for each pool. Area (m²) and volume (L) were calculated assuming ellipsoid geometric shapes. The sediment of each pool (approximately 10 g) was manually homogenized and gently scraped with a laboratory spatula in September 2013. The sediments were collected in sterile flasks protected from direct sunlight. In the laboratory, sediments were spread on sterile Petri dishes protected from light until completely dried at room temperature (20 °C ± 0.2) and then dried sediments were stored in sterile polyurethane flasks at room temperature for later analysis. Total organic carbon (TOC) was determined for each sediment by combustion analysis and

non-dispersive infrared detection (Schumacher, 2002) and expressed as milligram per gram of dry weight (mg TOC g DW⁻¹)” Temperature (T, °C) and relative humidity (RH) of the sediments were monitored for a complete year at one of the rock pools studied using i-button sensors (DS1923 Hydrochron), which record temperatures between - 20 °C and + 85 °C and relative humidity from 0 to 100 % (accuracy of ± 0.5 for temperature and 5 % relative humidity). Measurements were recorded at 10-minute intervals (see García-Rodríguez *et al.*, 2017) for a year. The i-button sensors were placed on the sediments within a waterproof capsule as indicated by manufacturer.

Analysis of ciliate assemblages

Two samples of varying weight (5 g in P1 and P2; 1 g in P3) of 1 year-dried sediment from each of

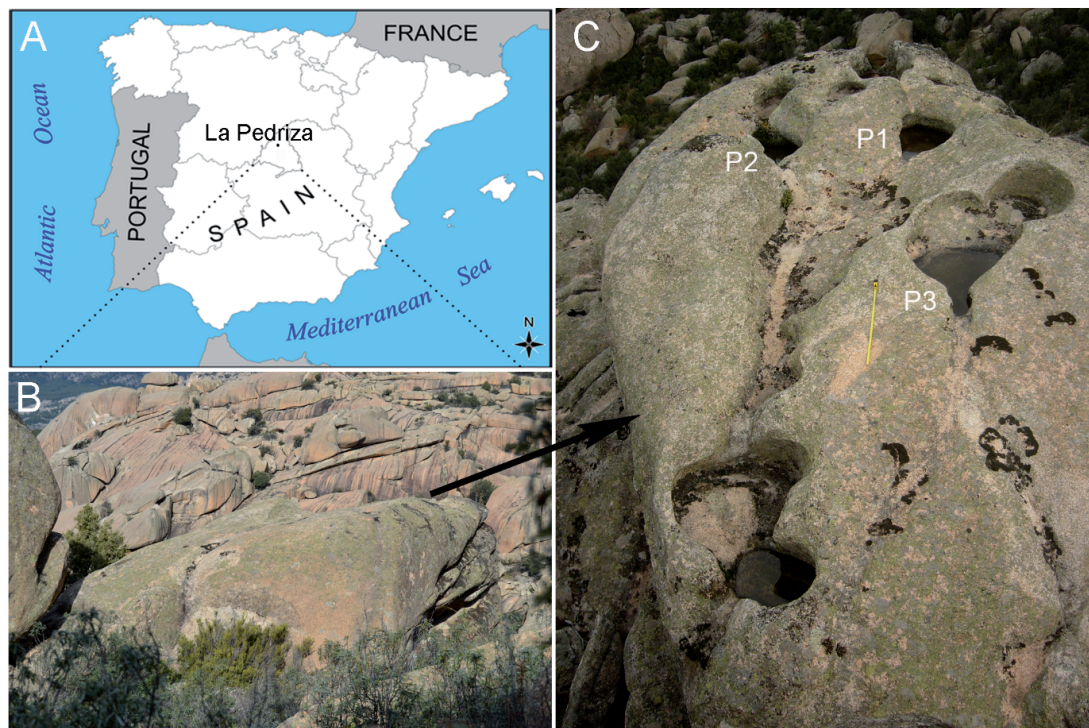


Figure 1. A) Location of La Pedriza (Parque Nacional Sierra de Guadarrama) in the Iberian Peninsula; B) Granite inselberg in La Pedriza. The inselberg is topped by rock pools; C) Location of the rock pools from this study (P1, P2 and P3) at the top of the inselberg featured in B). A) Localización de La Pedriza (Parque Nacional Sierra de Guadarrama) en la Península Ibérica; B) Inselberg de granito en La Pedriza. El inselberg presenta pilas graníticas en su cima; C) Localización de las pilas graníticas de este estudio (P1, P2 y P3) sobre el inselberg mostrado en B).

the three rock pools were rehydrated in the laboratory following the non-flooded Petri dish method (Foissner, 1987). Briefly, this method consists of adding a volume of distilled, autoclaved and filtered (0.22 μm pore) water into Petri dishes (5 cm diameter) containing the sample, until the sediment is slightly over-saturated but not flooded. These rehydrated samples were kept in the dark at 18 °C for 14 days and ciliates were counted on days 1, 2, 3, 7, 10 and 14 after rehydration, using bright field microscopy. 25 μl aliquot replicates from each sample were counted until no new species were found after whole slide screening under the microscope. This procedure involved a minimum of 5 and a maximum of 10 replicates depending on the sample. Ciliate abundance is expressed as number of ciliates per g of DW of sediment. Identification was carried out on obser-

vations of live ciliates under bright phase contrast or differential interference contrast microscopy and after using different silver impregnation techniques following Foissner (2014).

Testate amoebae were also identified, although these were not quantified. Identification was based on shell morphology (Ogden & Hedley, 1980; Mazei & Warren, 2012, 2014; Singer *et al.*, 2015) following the detailed online species identification literature within Microworld on amoeboid microorganisms (<https://www.arcella.nl>).

Statistical analyses

Chi-square test for homogeneity of proportions (STATA v.15) was used to determine whether the distribution of abundances of the main ciliate groups present in the sediments (Colpodids,

Table 1. Annual measurements of Temperature (T, °C) and Relative Humidity (RH, %) at the sediments of a model granite rock pool (P1) in La Pedriza (Parque Nacional Sierra de Guadarrama). *Medidas anuales de Temperatura (T, °C) y Humedad Relativa (RH, %) en los sedimentos de una pila granítica modelo (P1) en La Pedriza (Parque Nacional Sierra de Guadarrama).*

	(1) T Max.	(2) T Min.	(3) T Range	(4) RH Max.	(5) RH Min.	(6) RH Range	(7) days RH 100%
January	12 ^a	0 ^b	6 ^c	100	100	0	31
February	Ice (<0)*	Ice (<0)*	-	100	100	0	28
March	34	0	28	100	15	32	16
April	38	6	30	100	25	75	23
May	52	8	36	100	16	75	10
June	55	19	34	100	12	60	21
July	59	18	34	100	10	75	4
August	57	12	37	100	9	90	4
September	38	10	26	100	25	55	26
October	29	12	17	100	29	61	26
November	24 ^a	2 ^b	8 ^c	100	100	0	30
December	21	3	18	100	37	62	17

(1) Monthly maximum temperature measured in dry sediment.

(2) Monthly minimum temperature measured in dry sediment.

(3) Monthly average of the daily temperature variation in dry sediment.

(4) Monthly maximum humidity. Includes irregular precipitation.

(5) Monthly minimum humidity.

(6) Monthly average daily humidity variation.

(7) Numbers of days when 100 % saturation is reached. Includes the occasional rain and the saturation of the sensor by inundation.

^a Maximum water temperature.

^b Minimum water temperature

^c Monthly average water temperature variation.

* In February it was impossible to get access to the sensor to obtain the readings, due to the presence of a thick layer of ice covering the top of the pool.

Scuticociliates, Stichotrichs, Carnivores/Omnivores) was dependent on the rock pool type. The ciliate species richness capacity of the rock pools was estimated by rarefaction curves in function of the number of replicate counts (incidence data) for each of the sampling days. The expected number of species in the samples (interpolation) and in augmented replicates (extrapolation) was calculated according to Colwell *et al.* (2012). The asymptotic species richness was estimated by the Chao2 estimator (Chao, 1987). Analysis were performed using custom software written in Matlab[®] script.

Species accumulation curves were fitted to Beta-P and Weibull models (Thompson *et al.*, 2003) by the Levenberg-Marquardt nonlinear least squares algorithm. Both tolerance on residual sum of squares and tolerance on estimated coefficients were set to 10^{-12} . The maximum number of iterations was set to 10^5 but models were always re-run with new initial values to avoid convergence at local minimums. Goodness of fit to compare between fits was set by mean square error (MSE) as all models contained the same number of parameters. The Weibull model showed better fit in all cases than the Beta-P model, so the latter was used to calculate the asymptote in each case, taking the value of the function at 10 000 days.

RESULTS

Characterization of granite rock pool sediments

Sediment structure was different for the three rock pools despite their proximity. P1 sediments were mainly composed of coarse siliceous sand and soil, P2 sediments of well-established soil, and P3 sediments were mostly formed by dark mature soil containing some root and herb remnants. Nutrients in these pools come from autochthonous terrestrial vegetation, decaying invertebrates, and vertebrate faeces, mainly from wild goats (*Capra pyrenaica*, Schinz, 1838), which are abundant in the area in spring and autumn, and from visiting birds. TOC of the sediment varied greatly from pool to pool. It was much higher in P3 (200 mg TOC g DW⁻¹) than in P1 (11 mg TOC g DW⁻¹) and P2 (40 mg TOC g DW⁻¹).

Annual temperature and relative humidity values registered during a year in P1 sediments are shown in Table 1. The values of these parameters in the other two pools were not statistically different from P1 (data not shown). *La Pedriza* rock pools are subject to a characteristic continental climate, so pools are typically dry for a great part of the summer, and periodically filled, with ice or snow in winter, and rainwater in autumn and spring. Moreover, the surface of the granite rock pools is exposed to the incidence of direct sun. Therefore, high temperatures in periods of prolonged sunstroke can be frequently recorded in these environments.

Our results show that sediment temperature ranged from 59 °C in the hottest summer month in Central Spain (July) to below 0 °C (ice) in the peak of the winter months (February). Daily differences in both parameters were pronounced (see ranges in Table 1).

Ciliate species richness in granite rock pool sediments

A total of 54 ciliate species (morphospecies) were identified in the sediments of the granite rock pools (Table 2), of which 19 species were from P1, 24 species from P2 and 25 species from P3. Only four species were common to all three pools: *Cyclidium glaucoma*, *Halteria grandinella*, *Vorticella aquadulcis*, and *Uronema nigricans*. For two thirds of the species found in these three pools, the cyst, the structure required for the species to become viable again from the dry sediment, has not been described or reported at all.

Temporal occurrence of ciliate species after sediment rehydration in laboratory microcosms was different at each pool (Table 3). While the number of total species found per day in P1 was rather stable during the fortnightly period, P2 and P3 showed contrasting species succession. In P2 sediments, the lowest species number was found on day 1 and the maximum between days 7 and 10 after rehydration of the sediment. By contrast, P3 had the highest species richness on days 2 and 3 after rehydration, with a steady decrease in number thereafter.

The cumulative species number observed by microscope in the three pools is shown in figure

Table 2. Ciliate species identified in ombrotrophic granite rock pools (P1-P3) of La Pedriza (Parque Nacional Sierra de Guadarrama). *Especies de ciliados identificadas en pilas graníticas ombrotóricas (P1-P3) de La Pedriza (Parque Nacional Sierra de Guadarrama).*

Species	P 1	P 2	P 3	Reported cyst in literature ¹
<i>Bryometopus pseudochilodon</i>		+		Not described
<i>Colpoda cucullus</i>	+			Yes
<i>Colpoda steinii</i>			+	Yes
<i>Ctedoctema</i> sp.	+			Not described
<i>Cyclidium glaucoma</i>	+	+	+	Not described
<i>Cyclidium</i> sp1.		+		-----
<i>Cyclidium</i> sp2.			+	-----
<i>Cyrtolophosis minor</i>	+		+	Not described
<i>Disematostoma</i> sp.	+			Not described
<i>Drepanomonas sphagni</i>		+		Not described
<i>Enchelys</i> sp.		+		Yes
<i>Euploes</i> sp.			+	Yes
<i>Gastrostyla steinii</i>			+	Yes
<i>Gonostomum affine</i>		+		Yes
<i>Halteria grandinella</i>	+	+	+	Yes
<i>Homalogastra setosa</i>	+	+		Not described
Hypotrich 5	+			-----
<i>Metacystis</i> sp.	+			Not described
<i>Metopus hasei</i>			+	Yes/Not described
<i>Metopus palaeiformis</i>			+	Yes
<i>Microdiaphanosoma arcuatum</i>		+	+	Not described
<i>Nivaliella plana</i>		+	+	Not described
<i>Oxytricha curta</i>	+			Not described
<i>Oxytricha granulifera</i>	+			Yes
<i>Oxytricha longa</i>			+	Yes
<i>Oxytricha setigera</i>	+	+		Yes
<i>Oxytricha</i> sp.			+	-----
<i>Plagiocampa</i> sp.		+		Not described
<i>Platiophrya binucleata</i>			+	Not described
<i>Platiophrya macrostoma</i>		+		Not described
<i>Platiophrya</i> sp.		+		-----
<i>Prorodon</i> sp.			+	Yes
Prostomid 2	+			-----
<i>Pseudocyrtolophosis alpestris</i>			+	Not described
<i>Pseudoplatyophrya nana</i>		+		Not described
<i>Sathrophilus</i> sp.			+	Not described
Scutico (unidentified)		+		-----
<i>Spathidium</i> sp.			+	Yes
<i>Strobilidium</i> sp.	+			Not described
<i>Strombidium</i> sp.			+	Yes
<i>Tachysoma pellionellum</i>		+		Not described
<i>Trachelophyllum apiculatum</i>			+	Not described
<i>Trachelophyllum pusillum</i>		+		Not described
<i>Trachelophyllum</i> sp.		+		-----
Unidentified ciliate species		+		-----
<i>Uronema nigricans</i>	+	+	+	Not described
<i>Urosoma</i> sp.		+		Yes
<i>Urosomoida</i> sp.	+		+	Yes
<i>Urotricha farcta</i>	+			Not described
<i>Urotricha globosa</i>		+		Not described
<i>Urotricha</i> sp.			+	Not described
<i>Vorticella aquadulcis</i>	+	+	+	Yes
<i>Vorticella convallaria</i>	+			Not described
<i>Vorticella microstoma</i>			+	Yes

¹ See Supporting Information for further details and references for Table 2 (available at <http://www.limnetica.net/en/limnetica>)

Table 3. Number of observed ciliate species, estimated species richness and replicated incidence rarefaction for each granite rock pool (P1-P3). *Número de especies observadas, estimación de la riqueza de especies y rarefacción de incidencia de réplicas para cada pila granítica (P1-P3).*

Observed ciliate species	Day 1	Day 2	Day 3	Day 7	Day 10	Day 14
P1	9	7	9	7	9	9
P2	2	6	6	12	11	10
P3	7	11	11	9	7	5
Estimated* ciliate species	Day 1	Day 2	Day 3	Day 7	Day 10	Day 14
P1	13	7	10	11	9	10
P2	2	6	6	12	12	10
P3	8	19	14	10	7	6
N	Day 1	Day 2	Day 3	Day 7	Day 10	Day 14
P1	30	4	11	24	5	11
P2	2	3	7	5	8	7
P3	16	29	19	10	4	14

*Estimated species richness was calculated by rarefaction from replicated incidence data.

N: Number of replicate counts to get 95 % of the ciliate species at each pool

2. The asymptote of the curve was adjusted to 34 and 30 species respectively for P2 and P3. The number of cumulative species observed in these two pools were 26 at the end of the experimental period (Fig. 2), therefore we retrieved during the rehydration period 77 % of the species supposedly present in P2, and 87 % of the species supposedly present in P3. The results for P1 are clearly different because the accumulated species curve is very far from reaching the asymptote (Fig. 2): in this pool the accumulated number of species did not tend to stabilize after the two-week study (Fig. 2), as it did in the other two pools. The duration of the experiment was extended for 14 days for the following reasons: i) in two of the three pools, two weeks provided a reasonable time interval for retrieving most of the viable ciliate species; ii) a longer sampling period would have affected the experiment negatively, as quantitative determinations by microscope precise the removal of a given sample volume, and when done repeatedly, the initial experimental conditions may deteriorate and cause sample desiccation; iii) we considered that if the excystation of a ciliate species is not observed after two weeks of microscope observations it is very unlikely the

species is representative of the ecosystem.

Rarefaction analyses inferred the number of encysted ciliate species that each pool sediment is expected to harbour based on the sample size (number of replicate samples taken for microscopic observation) (Table 3). The estimated number of species in the three pools (visualized as the asymptote of the curve in Fig. 3) is similar to the number of species observed by the microscope, excepting for day 1 and 7 at P1 and days 2 and 3 at P3, where the estimated number of species was higher than that observed by microscopy. The estimated number of microscopic replicate counts required to reveal 95 % of the ciliate species in the sediments was also inferred at each day for each pool (Table 3). P2 needed the lowest number of replicates to disclose the species richness potential of the sediment, which is consistent with the rarefaction results showing that the observed versus estimated species ratio is close to one in this pool.

Ciliate community dynamics in granite rock pool sediments

To explore competitive traits for the ciliates, the species were classified into within four groups

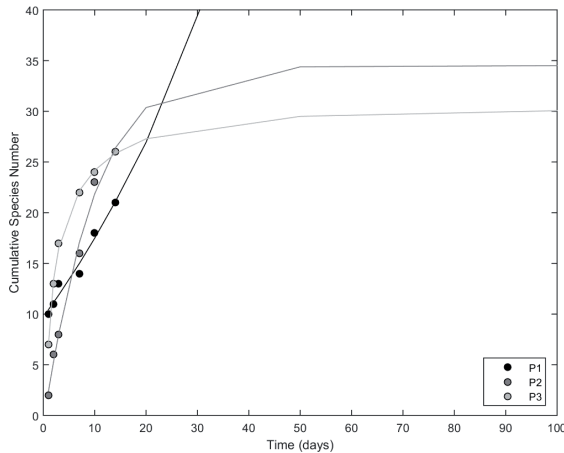


Figure 2. Cumulative number of ciliate species excysted at each rock pool (P1-P3) in the study period (14 days). *Número acumulativo de especies de ciliados exquistadas en cada pila granítica (P1-P3) durante el periodo de estudio (14 días).*

according to their main food and feeding strategy (based on Foissner & Berger, 1996): Scuticociliates (planktonic bacterivores), Colpodids (planktonic bacterivores), Spirotrichs (benthonic bacterivores) and Carnivores/Omnivores (feeding on a variety of algae, bacteria and other ciliates). The occurrence and abundance of these four groups were monitored over time. Results show that there was not a homogeneous distribution of these ciliate groups in the rock pools, as the frequencies of maximum abundances of each of the groups were significantly different depending on the pool analyzed ($X^2 471.6$; DF 6; $p < 10^{-4}$). Figure 4 illustrates that the most abundant groups, Colpodids and Scuticociliates, had diametrically opposed dynamics in pools P1 and P3. While P1 had a low ratio of Colpodids/Scuticociliates, P3 had a high ratio of Colpodids/Scuticociliates.

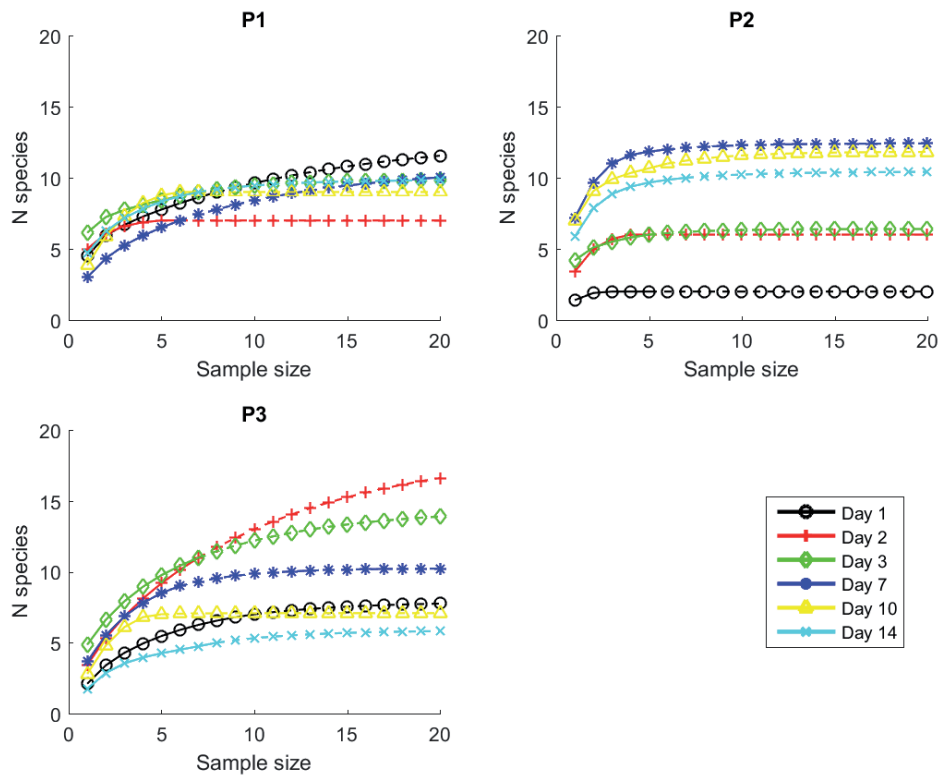


Figure 3. Rarefaction curves for each ciliate assemblage (site-day) with respect to the number of samples (replicated incidence data). Interpolation (continuous lines) and extrapolation (dashed lines) data are shown for the three pools. *Curvas de rarefacción para los ciliados (sitio-día) en relación al número de muestras (datos de incidencia de replicas). Se indican los datos de interpolación (líneas continuas) y extrapolación (líneas discontinuas) para las tres pilas.*

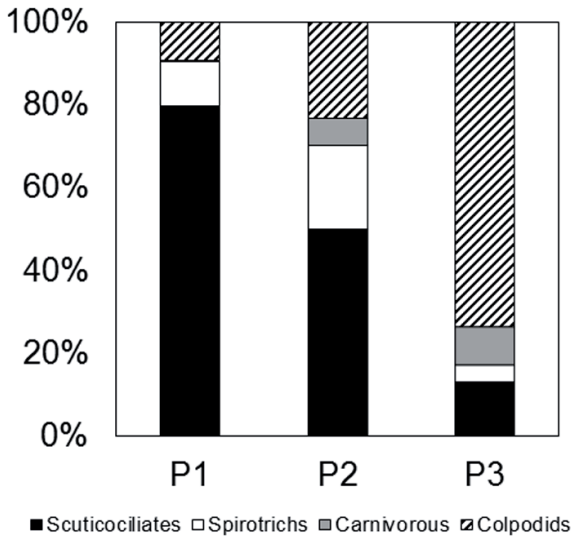


Figure 4. Relative abundance (%) of ciliate groups at each rock pool (P1-P3). *Abundancia relativa (%) de los grupos de ciliados en cada pila granítica (P1-P3).*

Temporal succession of the ciliate groups after sediment rehydration is detailed in figure 5. Colpodids always reached their highest abundances early in the population succession (day 3), although the species involved were not the same for the three pools. The dominant species were *Colpoda cucullus* in P1, *Platyophrya macrostoma* and *Platyophrya* sp. in P2, and a bloom of *Colpoda steinii* was found on day 3 in P3, although the species *Pseudocyrtolophosis alpestris* also appeared in P3. Only one species of Colpodids, *Microdiaphanosoma arcuatum*, appeared late in the succession in P2 and P3. Scuticociliates showed a wider exploitation range (from 3 and 10 days post-rehydration, depending on the pool) but in general tended to thrive later than the Colpodids. In P1, the dominant species were *Homalogastra setosa* (day 3) and *Cyclidium glaucoma* (day 7); in P2, *Homalogastra setosa* (day 7), and *Cyclidium glaucoma* and *Uronema*

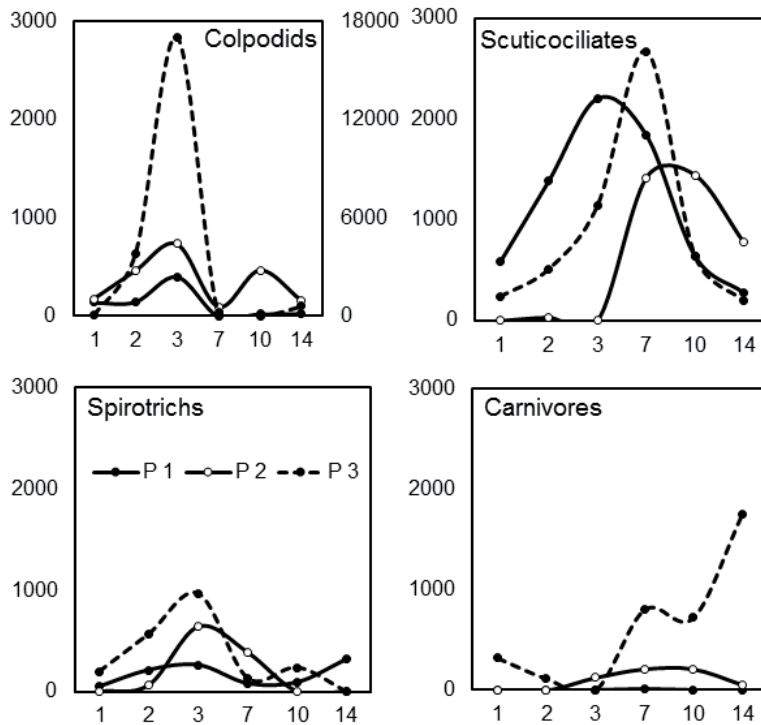


Figure 5. Temporal succession of ciliate groups after rehydration of the sediments at the three rock pools. X-axis represents time in days and Y-axis the abundance of each group (ind. g DW⁻¹); secondary Y-axis in Colpodids graph corresponds to the pool P3. *Sucesión temporal de los grupos de ciliados después de la rehidratación de los sedimentos en las tres pilas graníticas. El eje X representa el tiempo en días y el eje Y la abundancia de cada grupo (ind. g DW⁻¹); el eje Y secundario en la gráfica de Colpodidos corresponde con la pila P3.*

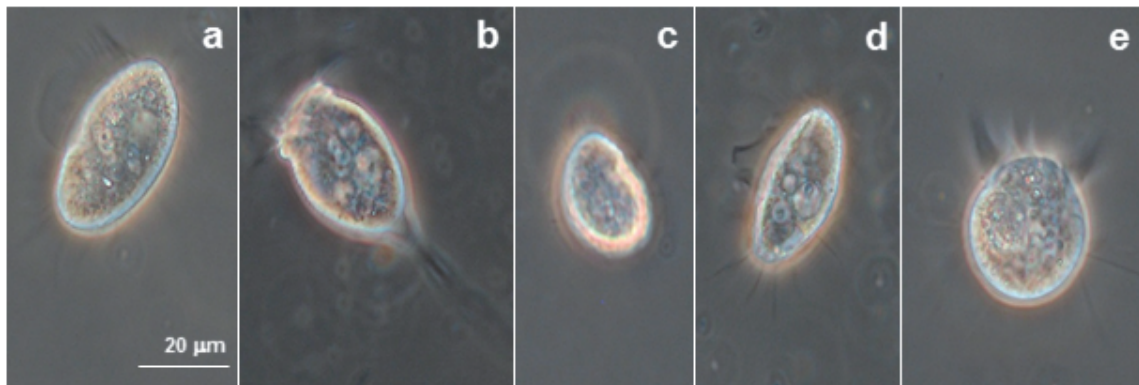


Figure 6. Micrographs of some representative ciliates in the granitic rock pools. a) *Homalogastra setosa*, b) *Vorticella aquadulcis*, c) *Colpoda steinii*, d) *Cyclidium glaucoma*, e) *Halteria grandinella*. Micrografías de algunos de los ciliados representativos de las pilas graníticas.

nigricans (day 10); in P3, *Cyclidium glaucoma* (day 7). Spirotrichs and Carnivores/Omnivores never reached density values as high as the other groups, and Spirotrichs tended to show up before the Carnivores (Fig. 5). Spirotrichs remained in P1 throughout the study period, reaching the highest abundance 14 days after sediment hydration. However, they disappeared entirely from P2 from day 10, and were only found in very low abundances in P3. The presence of Carnivores/Omnivores was only noteworthy in P3, where they were the most abundant group on the last day of sampling, when the species *Gastrostyla steinii* was actively preying *Metopus* sp. that was concurrently present in this pool. Figure 6 shows micrographs of some of the most abundant ciliate species in the pools.

Testate amoeba community in granite rock pool sediments

During this study, other protists were observed. Especially interesting were the testate amoebae found. Their abundance and temporal dynamics were not analysed as they appeared later in the experimental period and their study was not the focus of this work, but they were identified and their presence at each pool was recorded. Amoebae species richness varied greatly in the three rock pools: P2 showed much higher species

richness (22 morphospecies) than the other pools (13 morphospecies in P1, and only 4 morphospecies in P3). The main genera identified belonged to *Nebela*, *Trinema*, *Euglypha* and *Diffugia*. The genus *Nebela* was the most species rich and also showed the greatest intraspecific variability, mainly due to variable size and shape, with differential surface scale shapes (bacillar, ovoid, rounded, irregular, etc.). This variability has been recorded previously considering some species as complexes (i.e. *N. collaris*) with many pseudo cryptic characteristics among testate amoebae (Singer *et al.*, 2015; Luketa, 2017). The other genera with more morphospecies observed were in this order *Euglypha*, *Trinema* and *Diffugia*. Finally, other morphospecies belonging to different genera were recorded as well, although only single species were observed, like those from the genera *Argygnia*, *Padaungiella* and *Centropyxis*. Figure 7 shows the richness in species of each genus of testate amoeba found per pool and illustrates some of the most representative species.

DISCUSSION

Temperature and humidity represent two of the main factors controlling the presence and growth of biological communities in these landforms (Jocque *et al.*, 2010). In the present study, values recorded throughout the year show that

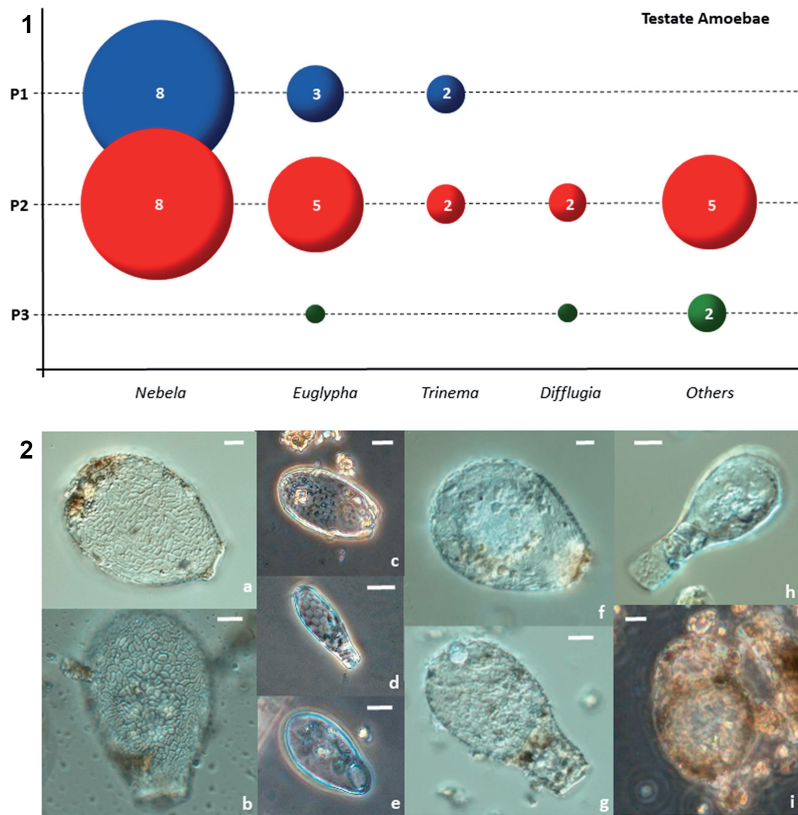


Figure 7. (1) Main genera of testate amoebae found in the three granite rock-pools (P1, P2 and P3) studied. Bubble size indicates number of morphospecies found (number inside bubbles). (2) Some testate amoebae species recorded. Micrographs under phase contrast (c, d, e, i) and interference contrast (a, b, f, h, g) microscopy. a) *Nebela tincta*, b) *Nebela* sp., c) *Euglypha rotunda*, d) *Euglypha cristata*, e) *Trinema enchelys*, f) *Argynnia vitraea*, g) *Padaungiella lageniformis*, h) *Padaungiella tubulata*, i) *Centropyxis platystoma*. Scale bars: 10 micrometres. Principales géneros de amebas testáceas de las tres pilas graníticas estudiadas (P1, P2 y P3). Tamaño de burbuja indica el número de morfoespecies (número en la burbuja). (2) Algunas especies de amebas testáceas registradas. Micrografías bajo microscopía de contraste de fases (c, d, e, i) y contraste de interferencia (a, b, f, h, g). Las mismas especies que las mencionadas arriba.

rock pool sediment environments studied are effectively governed by drastic changes in both parameters (Table 1).

Our study on granite rock pools at a National Park in Central Spain reveals that a considerable number of ciliate species colonized the pool sediments. The first record published of a ciliate found in rain-fed granitic rock-pool dates back to Reed & Klugh (1924), who found in the aquatic phase of a rock-pool in Ontario the species *Colpidium striatum* (Table 4), which has not been reported in these environments since. Oertel *et al.* (2008) and Foissner & Oertel (2009) described two new species of ciliates, respec-

tively in mosses and mud from the same roadside “laja” (local term for granitic rock-pool) in Venezuela. Rylander (2011) found 12 genera of ciliates in his comparative study of winters 1965 and 2010 in rain-fed granitic pools on top of an inselberg in Texas, four of the genera are also reported in the present study (*Vorticella*, *Urone-ma*, *Oxytricha*, *Halteria*). To date, the most detailed and similar to our study work was that by Foissner *et al.* (2002), who listed 63 different species of ciliates in the sediments of two granitic rock-pools (55 in one pool and 30 in the other pool) in Central Namibia dessert. Of those, eight species were described as new taxa and 17

species (27 % of the total species number recorded) were shared between both pools. In our study, only four species (7.4 % of the total) were found in all three rock pools analysed. Therefore, our results show a largely different ciliate community structure at each pool. This suggests little ciliate meta-community connectivity across the pools. Ciliates are rock pool inhabitants which can only arrive in a pool by passive dispersal and only survive the dry periods via resistant resting stages. If a ciliate species arrives in a pool, but the physical-chemical conditions are hostile, it will encyst in response to the non-favourable environment. As such, the species is likely to reappear in the next wet period unless the physical-chemical conditions or competition with autochthonous species is so extreme as to eliminate the species before encystation. However, this is very improbable as ciliates are able to encyst very rapidly in response to adverse condi-

tions. Therefore, despite the close proximity of these pools, limited ciliate dispersal seems to have occurred between them, probably via wind, transportation from mouth/legs of animals (phoresy) or water overflow.

Only 10 out of the 63 species found in Namibia desert (and seven additional genera) have been also observed in the present study: *Colpoda cucullus*, *Colpoda steinii*, *Gonostomum affine*, *Halteria grandinella*, *Homalogastra setosa*, *Metopus hasei*, *Nivaliella plana*, *Platyophra macrostoma*, *Pseudoplatyophra nana* and *Trachelophyllum pusillum*. 40 of the 54 species found by microscopy (Table 2) are recorded for the first time in granite rock pools habitats, due probably to the scarce number of previous detailed studies on ciliate communities of these habitats. In the present study, euryoecious, mainly bacterial grazers species, constitute most of the ciliate community of granitic rock-pools

Table 4. Literature review on ciliate and testate amoebae reported in granite rock pool sediments worldwide. *Revisión bibliográfica de ciliados y amebas testáceas hallados en sedimentos de pilas graníticas a nivel mundial.*

Reference	Sampling location	Sampling date	Ciliate (morphospecies number)	Testate amoebae (morphospecies number)	Abundance
Reed & Klugh (1924)	Kingston, Ontario (Canada)	October 1922	<i>Colpidium striatum</i> (1)	<i>Arcella vulgaris</i> , <i>Diffflugia pyriformis</i> , <i>Centropyxis aculeata</i> , (7)	Ciliate (rare) Amoebae (frequent)
Pinder <i>et al.</i> (2000)	9 granite pools in Southwestern Australia	Not indicated	-	(7)	Not indicated
Foissner <i>et al.</i> (2002)	2 granite rock pools in Central Namibia desert (21°45'S 15°08'E)	24.02.1994 (analysed 16.02.2000 and 17.03.2000 respectively)	¹ (63)	-	Not indicated
Foissner & Oertel (2009)	Mud from a roadside "Laja" (granite rock-pool) 150 km NE of Puerto Ayacucho, (Venezuela) (7°N, 66°56'W)	Not indicated	<i>Kamburophrys gibba</i> , <i>Enchelys lajacola</i> nov. spec. (2)	-	Not indicated
Oertel <i>et al.</i> (2008)	Mosses from a roadside "Laja" (granite rock-pool) 150 km NE of Puerto Ayacucho, (Venezuela) 7°N, 66°56'W)	Not indicated	<i>Apocoriplites lajacola</i> nov. spec. (1)	-	Not indicated
Rylander (2011)	3 granite rock pools in Texas, USA	1965 and 2010	12 genera	<i>Arcella</i> , <i>Centropyxis</i> , <i>Diffflugia</i> (3)	² Estimated in categories of abundance
Present study	3 granite rock pools in La Pedriza (³ PNSG), Madrid, Spain	16.09.2013 (analysed for 14 days, 19.11.2014 to 2.12.2014)	⁴ (54)	<i>Nebela</i> , <i>Trinema</i> , <i>Euglypha</i> and <i>Diffflugia</i> , (22)	Ranging from 8135 to 32 961 ind g ⁻¹ DW per basin for ciliates. Not quantified for amoebae

¹ 56 species in a deep pool covered with mud and grasses and 30 species in a flat rock pool covered with mud without grass; 8 of the species found were new to science; ² Abundance estimates based on the number of organisms counted in 15 drops of water in each of 26 samples: abundant, 4 or more specimens/coverslip; common, 2-3 specimens/coverslip; uncommon, 5-15 specimens/15 coverslips; rare, fewer than 5 specimens/15 coverslips. Small ciliates were not counted or identified. ³ PNSG: Parque Nacional Sierra de Guadarrama. ⁴ See Results section for more details on spatial and temporal distribution of the species in the pools.

sediments as shown for soil ciliates (Luftenegger *et al.*, 1985; Foissner, 1987; Aesch & Foissner, 1993; Bamforth, 1995; Bamforth, 2001).

Our results also highlight that the cyst stage has not been reported or characterized for most of the species found in this study (Table 2). Investigating the dormant (cyst) stages of ciliates is essential for species identification and adequate diversity approaches, as many species morphologically similar in their active stage can be separated by their distinctive cyst morphology. According to some authors (Foissner *et al.*, 2008), the characterization of “cyst species” might increase the number of free-living species of ciliates by as much as 50 %, and this may have important implications in biogeography and biodiversity.

The characterization of the most abundant ciliate groups show that bacterial-grazing species constitute most of the ciliate community in the sediments of all three granite rock pools. The most common species belong to small filter-feeding Colpodids and Scuticociliates, which have oral membranelles that can efficiently collect large quantities of planktonic bacteria. Rock pool sediments are patchy environments prone to contain different microhabitats or micro-niches. Niche compartmentalization, either in space or in time, allows the coexistence of species that use the same resources (Walker, 1987). Temporal niche compartmentalization was observed in this study. Colpodids and Scuticociliates despite using similar bacteria for their nutrition, can co-exist in the same pool because they excyst at different times—Colpodids earlier than Scuticociliates (Fig. 5). However, a single rock pool cannot sustain high populations of both bacterivorous groups at the same time (Fig. 5). This suggests that pool size and nutrient resources in exclusively rain-fed pools limit bacterial proliferation.

The results from our study provide one of the first quantitative and time-monitored evidence of r and k strategists among ciliates. Colpodids have a broad tolerance to abiotic factors and multiply rapidly (often within the cyst). They are typically the first ciliate colonizers of a habitat (Yeates *et al.*, 1991). In our study, we corroborate that Colpodid species are effectively pioneers of rock pool sediments after their re-hydration and can be regarded as rock pool r-strategists, as they are in

soil environments (Bamforth, 2001). Scuticociliates excysted from the sediments of the three pools later in time, so they can be considered intermediate species in the r/K continuum, which is consistent with previous results in other unpredictable environments (Bamforth, 2001). Carnivores/Omnivores appeared latest in the species succession, after the common bacterivorous ciliates, so they can be seen as K-strategists. The fact that the maximum abundances of Colpodids and Scuticociliates were observed in P3, which was also the only pool in which the succession matured enough for the appearance of Carnivores, is explained by the much higher organic content of this pool; this arguably allowed, first bacteria and consequently bacterial grazers to thrive, in turn supporting Carnivores.

There is a clear lack of studies concerning testate amoebae in granite rock pools (Table 4). We found the highest diversity of testate amoebae that has been recorded so far in these habitats (Table 4). Testate amoebae seem to have a role in humification because their presence in habitats with a high organic matter content TOC is common (Wilkinson & Mitchell, 2010). However, they can also be found in lower numbers in arid habitats with low levels of organic matter (Wilkinson & Smith, 2006; Bamforth, 2008). Our results do not corroborate an association of testate amoebae occurrence with high organic carbon content, because the rock pool with by far the highest content of TOC in their sediments (P3) was the pool with the lowest species richness of testate amoebae. Abundance values for amoebae were not determined in the present study, therefore a TOC relationship in terms of amoeba biomass is yet to be explored in granite rock pools. However, quantification of testate amoeba could also be relevant because some of the species identified in this study (*Trinema*, *Euglypha*) are known to have silica rich shells, made of small scales that can easily dissolve in soils and increase silica mineralization (Aoki *et al.*, 2007). Recently, it has been suggested that testate amoebae might play a role in creating silica hotspots in soils (Crevey *et al.*, 2016). As granite is a silica-rich rock, the potential contribution of testate amoebae to the silica cycle in granite landforms deserves further geo-microbiological exploration.

CONCLUSIONS

The present study on the characterisation and quantification of protists in three ombrotrophic granite rock pools demonstrates:

(i) The existence of a diverse ciliate community of primarily bacterivorous species adapted to persist in ephemeral rock pool habitats through resistance structures (cysts), many of which have not yet been described.

(ii) The presence of competitive ciliate species in dormant stages may constitute a microbial seed bank by preventing local extinctions through the different species excystation over time. Therefore, rain-fed granite rock pools are arguably potential reservoirs of durable and cryptic ciliate biodiversity.

FUTURE PERSPECTIVES

Rain-fed rock pools have been largely overlooked in nature conservation (Jocque *et al.*, 2010; Brendonck *et al.*, 2010; Gabi & Matias-Peralta, 2015). Our results show that rock pool environments offer a substantial opportunity to describe hitherto unknown ciliates' resting cysts, and to comprehensively investigate the life cycle of rare, endemic and newly identified ciliate species. Moreover, the role of bacterivorous protists in controlling bacteria involved in biological granite weathering (Song *et al.*, 2010; Olsson-Francis *et al.*, 2016) deserves further investigation for their relevance to bio- and geo-heritage. Including rock pools in conservation programs may be especially relevant considering their clear dependence on climate and their open exposure to climate change. This study provides a baseline for the conservation of these granitic landforms as unique habitats for microorganisms. Many factors may have affected the different spatial patterns observed in rock pool ciliates in this study. They may be the result of complex competition trade-offs, different nature and composition of sediment in different pools, and small habitat-size sampled. Further studies combining massive molecular sequencing with functional approaches using multi-trophic mesocosm experiments would help to elucidate the relative importance of these factors, and provide a detailed

appreciation of the microbial seed bank's role in shaping rock pool habitats.

ACKNOWLEDGEMENTS

This study was supported by Ministerio de Economía y Competitividad (MINECO- Spain), Project MICROEPICS (Ref: CGL2013-40851-P/BOS 2014-2018; PI. M. Martín-Cereceda). Permits to collect samples (References number: 140.13; 10/035066.9/17; Auto-Cient.52.2017) and facilities provided by El Parque Nacional Sierra de Guadarrama are gratefully acknowledged.

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