Rain-fed Granite Rock Basins Accumulate a High Diversity of Dormant Microbial Eukaryotes

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Abstract

Rain fed granite rock basins are ancient geological landforms of worldwide distribution and structural simplicity. They support habitats that can switch quickly from terrestrial to aquatic along the year. Diversity of animals and plants, and the connexion between communities in different basins have been widely explored in these habitats, but hardly any research has been carried out on microorganisms. The aim of this study is to provide the first insights on the diversity of eukaryotic microbial communities from these environments. Due to the ephemeral nature of these aquatic environments, we predict that the granitic basins should host a high proportion of dormant microeukaryotes. Based on an environmental DNA diversity survey, we reveal diverse communities with representatives of all major eukaryotic taxonomic supergroups, mainly composed of a diverse pool of low abundance OTUs. Basin communities were very distinctive, with alpha and beta diversity patterns non-related to basin size or spatial distance respectively. Dissimilarity between basins was mainly characterized by turnover of OTUs. The strong microbial eukaryotic heterogeneity observed among the basins may be explained by a complex combination of deterministic factors (diverging environment in the basins), spatial constraints, and randomness including founder effects. Most interestingly, communities contain organisms that cannot coexist at the same time because of incompatible metabolic requirements, thus suggesting the existence of a pool of dormant organisms whose activity varies along with the changing environment. These organisms accumulate in the pools, which turns granitic rock into high biodiversity microbial islands whose conservation and study deserve further attention.

Keywords Granite rock basins · Microbial reservoirs · Protists · Fungi · Dormancy · Conservation

Introduction

Biodiversity is a crucial component in the functioning of ecosystems. The more diverse biological communities are, the more stable, productive and resilient to perturbations the ecosystems are thought to become [1]. Microbial eukaryotic communities are an essential component of all ecosystems. They are actively involved in biogeochemical cycles because of their wide taxonomic and metabolic versatility as autotrophs, heterotrophs (predators, decomposers and parasites) and mixotrophs [2,3]. Protists are actively involved in nutrient turnover in ecosystems and are therefore essential to the microbial loop. The photosynthetic representatives (*i.e.* eukaryotic microalgae) are, together with cyanobacteria, thought to account for around 40% of the primary productivity in oceans [4]. Non-photosynthetic protists are the main consumers of bacteria in aquatic and terrestrial environments, transferring carbon to higher levels of the food chain [5]. Microscopic fungi drive the soil carbon cycling and plant nutrition [6]. Both microbial groups include numerous taxa that produce resistance structures through which they stay in the habitats under metabolic dormancy surviving this way the environmental stress [7]. Evaluating microbial eukaryote diversity is of major importance for understanding key ecological processes occurring in all environments on Earth.

The development of molecular tools has provided new approaches for the study of microbial diversity and distribution [8,9]. High-throughput sequencing (HTS) has become a powerful tool to undertake large-scale environmental inventories of microorganisms [10]. Taking advantage of the volume of data provided, metabarcoding approaches have become very effective in the analysis of microbial biodiversity [11]. Several genetic markers exist for eukaryotic microorganisms, the 18S rDNA V4 and V9 variable regions being among the most commonly used [12, 13]. Molecular surveys have estimated that the microbial eukaryotic diversity could be by several orders of magnitude greater than the retrieved by exclusively morphological approaches [14]. However, some researchers are recently suggesting that modern genetic methods may be overestimating diversity, at least for protistan groups, and claim for investigating intraspecies sequence variability [15]. The refinement and implementation of metabarcoding approaches have allowed the identification of cryptic, invasive or toxic species, and the discovery of hitherto unknown lineages and unexpectedly diverse microbial communities [16, 17].

Microbial biodiversity debates are presently focused on the description of biogeographical gradients at global scale [8, 18], as well as to reveal the local and regional diversity and spatial distribution patterns of the microorganisms [19-22]. Understanding the drivers of microbial diversity and distribution is still a challenging aspect to understand the functioning of ecosystems [23]. Several factors have been studied as responsible of the spatial heterogeneity in ecosystems: local adaptation by changing environment (biotic and abiotic) [24], dispersal limitation by spatial constraints [25], and random placement of species [26], including founder effects [27]. Recently, geogenic factors (e.g. landform and underlying lithology) have been also revealed as important drivers of microbial (bacterial) diversity [28]. The drivers of microbial eukaryotic diversity remain however largely under-studied in many environments. The factual diversity, spatial distribution and ecological meaning of many lineages and taxa, has not been yet deciphered [10].

Ephemeral, shallow freshwater systems are among the still under-sampled environments for microorganisms [29, 30]. The organisms inhabiting these systems must face relatively long periods of unsuitable conditions (such as drought, freezing, lack of light in winter, or varying levels of oxygen), and active communities vary strongly along the year [31]. Still, the communities encountered in these systems are surprisingly diverse and heterogeneous, including clades that were previously thought to occur only in the ocean [29].

It has been argued that the coexistence of these organisms is made possible by their capacity of entering dormancy and activating when conditions are suited [30].

Rain-fed (ombrotrophic) granite rock basins represent one of the most variable habitats in terms of environmental parameters. Rock basins are hollows eroded on horizontal surfaces by weathering and dissolution of the granite [32,33]. The leftover sandy sediment often harbours light development of soil, organic crust, bryophytes and vascular plants. In ombrotrophic basins, rainfall is the only source of water, creating temporary pools on the rocks [34]. Basins undergo several cycles of inundation-desiccation throughout the year. Temperature, humidity, pH and availability of nutrients, beside of changing seasonally –sometimes erratically– may have drastic fluctuations throughout the day due to the variation in the intensity of diurnal insolation on the basins [34]. The high variability of these factors may determine the nature of the biological communities occupying these habitats. Research on eukaryotic microorganisms colonizing ombrotrophic rock basins has been very scarce and focused on phytoplankton [35]. Barely anything is known on the presence of heterotrophic protists and fungi in these habitats [36-38]. To our knowledge, there are not previous molecular studies addressing the study of microbial communities in rock basins.

The aim of this work is to describe the occurrence, diversity, and distribution patterns of microbial eukaryotic OTUs retrieved from the sediments of rain-fed rock basins by HTS. Based on the few previous studies on ephemeral aquatic environments [29-31] we predict that our granitic basins should host many dormant organisms. Separating the dormant from the metabolically active fraction of the population is not possible based on environmental DNA sequencing, nor with RNA [39]. However, there are several hints that can indicate the existence of an important dormant community: (1) the presence of microorganisms that cannot be active at the same time (for instance because of different oxygen optima) can be an indirect proof for the existence of a dormant population (2) rare (very low abundant) OTUs should constitute the greatest part of the diversity and (3) because of the strong influence of stochastic processes in building communities, inherited from ancient individual history of each basin, communities should not share a high number of OTUs, and that beta diversity is mostly characterised by a turnover of communities.

Materials and Methods

Study Area

Sampling sites are located in *La Pedriza del Manzanares* (UTM: 30N 425279 4511417. DATUM: ETRS89), a preserved area within the National Park *Sierra de Guadarrama*. *La Pedriza* has an average annual rainfall of 850 mm with an average annual temperature of 12 °C between 800 and 1200 m. At high altitudes (1200 to 1800 m) rainfall and average annual temperature is 1250 mm and 9 °C respectively. The climate of the zone is Mediterranean temperate-cold, humid [40]. Our scale of study is the granitic basin, the emblematic minor geoform of *La Pedriza*. The basins are developed on medium to coarse grain size leucogranites, formed by quartz crystals, orthoses, microcline, plagioclase and biotite [41]. Granite texture is equi-granular without phenocrysts.

Selection and Morphometric Analysis of Granitic Rock Basins

We conducted a random sampling of 21 basins, of which 20 were active and 1 was a non-active basin (i.e. where water does not accumulate because of its morphology).Photographs

of all studied basins were taken before sampling (Supplementary Fig. 1). The identification criteria for an active basin were: (i) not to present any fractures that prevent water to be retained; (ii) not to be completely covered by lichens, mosses or vascular plants [42]. All the chosen basins had standard forms, and those with complex morphology were not considered for sampling. The non-active basin was chosen as control, as it has a depth equals 0 and therefore it cannot retain water. In order to facilitate the study of metapopulation connectivity among the basins, the sampling area (0.2 km^2) was limited to a maximum distance between basins of 1.02 km and minimum distance of 1 m. Altitude variation in the area ranged from 1080 m to 1250 m above sea level. The major and minor basin axes (length and width of the basins), the maximum (h) and the minimum (u) depth were measured. The area (A) and the volume (V) were estimated using the most similar geometric figure: ellipsoid (in 18 basins) and spheroid (in 3 basins). Basin volume capacity ranged from 623.1 cm^3 to 18899.8 cm^3. Basins were grouped into two rather homogeneous groups according to their Area/Volume ratio: those with A/V ≥1 (12 basins) and those with A/V <1 (8 basins). The higher this ratio is, the faster the basin should dry since more surface is exposed to water loss by evaporation and insolation. (Supplementary Table 1).

Collection of Samples

Samples of the sediments were taken after the summer period (September-October) when the basins were dry and before the first autumn rains. The sediment of each basin (aprox. 4 g) was manually homogenized with a spatula and one sample per basin was collected in sterile polypropylene containers. In the laboratory sediments were spread on sterile Petri dishes protected from light and left to completely dry at room temperature (20 ^{0} C ± 0.2) until further analysis.

Chemical Analysis

Elemental composition of sediments was determined for total organic carbon (TOC) (by combustion method and nondispersive infrared analysis), Kjeldahl nitrogen (N) (by Kjeldahl method) and total phosphorous (P) (by inductively coupled plasma-optic emission spectrometry – ICP-OES).

Extraction of DNA, Amplification and Sequencing of 18rDNA

Aliquots of approximately 1 g of dry sediment from each basin were mixed with 500 ml of LifeGuard soil Preservation SolutionTM (MoBIO, Carlsbad, CA, USA)) to preserve nucleic acids. DNA was extracted within a month using the PowerSoil ® DNA Isolation Kit (MoBIO, Carlsbad, CA, USA) according to the manufacturer´s instructions. The V4 variable region of the 18S rRNA genes was amplified using the primers V4F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC) and V4R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA) [13]. PCR amplifications were conducted in a total reaction volume of 20 µl, using Promega's GoTaq® PCR kit (Promega, Wood's Hollow, WI, USA). Amplification was carried out for 30 cycles. Amplicons from independent PCR amplifications for each sample were pooled together. Each pool was then purified using Wizard® SV Gel and PCR Clean-Up System purification Kit (Promega, Wood's Hollow, WI, USA) according to the manufacturer´s instructions. ~ A DNA library was prepared from the pools using the TruSeq Nano

PCR-free Library Preparation kit and the paired-end 2x300 bp sequencing was done on an Illumina® MiSeq at the University of Geneva (Molecular Systematics & Environmental Genomics Laboratory) [43].

Bioinformatics

The reads obtained were analysed following the pipeline by de Vargas et al [16], to delete sequencing errors, sequences without rDNA reads, detect chimeras and other pollutant sequences. Singletons were also eliminated [44]. The remaining sequences were allocated to each sample and taxonomically classified by global sequence alignment using the GGSearch algorithm [45] *versus* the sequences of the curated ribosomal eukaryotic database PR^2 [46]. Sequences were also aligned against the SILVA bacterial and archaeal database to remove possible prokaryotic OTUs. Dereplicated sequence readings (metabarcodes) were grouped into operating taxonomic (OTUs) or phylotypes by the Swarm algorithm v2 [47]. OTUs were numbered from the least to the most abundant, and a database was generated with a sequence similarity threshold (OTUs vs PR^2) $\geq$ 90% for microbial eukaryotes (M_{Ek}), that is all sequences but Metazoa and Embryophyta. Sequences will be made available upon acceptance of the manuscript.

Statistical Analysis

Statistical analysis was conducted using the R software (R Development Core Team, 2013) (vs 3.5.0). To determine whether the sampling performed was sufficient to get a reasonable estimate of OTU richness, the non-parametric estimator Chao 1 [48] was calculated (R package iNEXT 2.0.14). Rarefaction curves were plotted to make comparable the OTUs richness of each basin with that of the others, by referring to the lowest OTU abundance value found among the basins [49] (R package iNEXT 2.0.14). Kruskal-Wallis (K-W) test was used to compare the differences among phylotype abundances in the basins, and Pearson *Chi*-square test to test whether the relative OTUs richness were homogeneous or not among basins. To correlate OTUs richness to the number of times they appear in the basins, multiple regression analysis were used adjusting the phylotype occurrence to an inverse exponential model ($y = e^{a+bx^{-1}}$).
Diversity estimates were computed based on raw reads and OTUs using the R packages Vegan 2.5-2 for alpha diversity and Beta part 1.5.0 for beta diversity. Alpha diversity indexes used were Gini-Simpson ($D = 1 - \Sigma\ p_i^2$) [50], Shannon- Weaver ($H = -\Sigma p_i \times \ln(p_i)$) [51] and Evenness ($E=H/\ln S$) [52]. To assess the correlation between OTUs richness, alpha diversity indexes and geomorphological variables, Spearman rank correlations were calculated. Mann Whitney-Wilcoxon test (U) was used to compare the alpha diversity indexes and richness in the two Area/Volume ratio groups of basins (A/V $\geq$1 or A/V <1). Non-metric MultiDimensional Scaling (NMDS, package Vegan 2.5-2) was applied to visualise comparatively the OTUs abundances in function of the two A/V basin groups. Ellipses were drawn on NMDS plots using the R package (envfit function in vegan package vers. 2.3-1 [53]. Beta diversity indexes were calculated using the Sorensen index (β_{sor}) for the occurrence of OTUs and the Bray-Curtis index (β_{BC}) for the abundance-based dissimilarity. In both cases the diversity was separated into the two independent components: species turnover (β_{sim}) and nestedness (β_{sne} ($\beta_{sor} - \beta_{sim}$)) [54], and balanced variation in abundance ($\beta_{BC.BAL}$) and abundance gradient ($\beta_{BC.GRA}$ ($\beta_{BC} - \beta_{BC.BAL}$)) [55]. To evaluate if the differences in any of the components of the beta diversity between the basins were dependent on the geographic distances, we used the function 'decay.model' of the package Beta part 1.5.0. The function adjusted a GLM (exponential decay model) with dissimilarity as response variable, spatial distance as predictor, log link and Gaussian error [56].

Results

Overall Taxonomic Affiliation of OTUs

A total of 2,090,236 quality-filtered reads of the V4 regions of the 18S rRNA gene were obtained, 51% (1,059,658) belonging to microorganisms. The reads grouped into 4,153 OTUs of which 66% (2,761) were microbial OTUs (Supplementary Data 1). The most representative non-microbial OTUs (Metazoa and Embryophyta) in the basins belonged to the eutardigrade *Ramazzottius oberhauseri* species complex, to several species of rotifers including both bdelloids and monogononts (impossible to differentiate based on 18S rRNA sequences [57]), to the oribatid mite *Scutovertex sculptus*, and to the bryophytes *Grimmia* sp. and *Ptychomitrium gardneri*. The phylogenetic spectrum of microbial OTUs covered all current eukaryotic supergroups [58]: Amoebozoa, Archaeoplastida, Excavata, Opisthokonta, and SAR (Stramenopiles–Alveolata–Rhizaria) (Fig. 1a). In terms of number of OTUs the richest supergroup was the Rhizaria, represented by Cercozoa (37.2% of all OTUs), followed by the Opisthokonta (22.1%), of which the fungi Chytridiomycota (9.7%) and Ascomycota (7.3%) were the most diverse. Alveolata represented 19.9% of all OTUs, most of them belonging to Ciliophora (12.5%) and Dinophyta (6.1%). Archaeoplastida constituted 11.1% of all OTUs, of which Chlorophyta were the most rich in OTUs group (9.4%). Some OTUs belonging to less represented taxa were the Cryptophyta and Centroheliozoa (0.80% of all OTUs each), Katablepharophyta (0.33%) and Apusomonads (0.25%). A few OTUs (less than 0.9% of the total) representing deep-branching lineages (Rozellida, Colpodellida) in the eukaryotic tree were also found. Also, 13 OTUs belonging to the Marine Stramenopiles (MAST-12 lineage) were also present in our dataset

This order of importance changed noticeably when considering the abundances of the reads (Fig. 1b). The most abundant groups were the Archaeoplastida (46.2% of all reads, of which 38.8% were Chlorophyta), Alveolata (27.2%, of which Ciliophora represented 25.2%), and Opisthokonta (9.9%, of which Chytridiomycota reached 8.5%). The species rich Cercozoa only represented 2.9% of the total 18S rDNA reads. When analyzed per individual basin, the protistan groups Chlorophyta (in 10 basins) and Ciliophora (in 9 basins) were also the most abundant microbial eukaryotes (Fig. 2).

Distribution of the Abundance and Richness of OTUs

A large proportion of the microbial OTUs (95%) had an abundance distribution in the rock basins between 1 and 512 reads (Supplementary Fig. 2). Moreover, 55% of the OTUs occurred only in two of the 21 basins studied (Supplementary Fig. 3). Phylotype occurrences adjusted significantly to an inverse exponential distribution curve when relating the OTUs to the number of times they appeared in the basins.

The abundance and richness of OTUs were not distributed homogeneously among the basins (Kruskal-Wallis OTUs abundances, K-W = 1934.6; df = 20; p-value < 10^{-4}; Chi-square OTUs richness, χ^2 = 1526.3; df = 20; p-value < 10^{-4}). The lowest number of rDNA reads (1229) and OTUs (267) was found in the the non-active basin 9 (Supplementary Fig. 4), used as control for our study. Rarefaction curves in combination with Chao 1 estimator showed that the OTUs richness was not retrieved for any of the samples. No rarefaction curves of any of the basins reached saturation (Fig. 3). Also, the sampling depth and sequencing coverage was different for

each basin. The retrieved microbial OTUs represented a percentage of Chao1 estimator that ranged from a minimum of 42.7% to a maximum of 71.8% (Supplementary Fig. 5).

The OTUs were classified according to their relative abundance in the basins [18]: Rare (R): OTUs with abundance values ≤ 0.01%; Abundant (A): OTUs with abundance values ≥ 1%; Non Rare Non Abundant (NRA): OTUs with abundance values > 0.01% and < 1%. Figure 4 represents comparatively the distribution among basins of reads and richness of OTUs within each of the three abundance categories. Within each category, both the DNA reads (Kruskall-Wallis test; p-value < 0.001) and the richness of OTUs (*Chi*-Square test; p-value < 0.05) were neither evenly distributed among the basins.

A clear partitioning of the microbial abundance was observed in the rock basins (Table 1). There were not exclusively Abundant (A) OTUs (OTUs that appeared only as Abundant in the basins), while the exclusively Rare (R) OTUs represented 52.3% of all OTUs. The OTUs appearing exclusively in the intermediate category (NRA) represented 1.9% of the total. No OTUs found to be Rare was ever detected as Abundant or viceversa. However, 41.9% of the OTUs were found with abundances in the basins ranging between R and NRA categories. In terms of occurrence (Table 1), there were no OTUs exclusively A, R or NRA that occurred in all the rock basins. The basins only shared 2.1 % of the total microbial eukaryotes. (Supplementary Table 2).These common OTUs were present in a different abundance category depending on the basin.

Only 105 OTUs were at least once an Abundant OTU in a basin. These OTUs covered more than 70 % of the total reads of each basin (representing more than 90% of the reads in four of the basins), excepting for the control basin where the Abundant OTUs represented only half (50.3%) of the total number of sequences. By contrast, a high number of OTUs (2,680) were at least once a Rare OTU in a basin. However, for most of the basins the Rare OTUs meant less than 2% of the abundance. The control basin did not have Rare OTUs. The maximum representation of the Rare OTUs was 4.0% of the basin total reads and only occurred in one basin (basin 17) (Supplementary Table 3).

Analysis of Alpha and Beta Diversity

Clear differences in the values of the alpha diversity indexes (Shannon (H), Simpson-Gini (D) and Evenness-Pielou (E)) indexes were found among the basins. For all indexes the basin 9 (control) had the highest values of alpha diversity (Supplementary Table 4). Correlation analysis revealed strong association among the three diversity indexes (Spearman Rho > 0.90). The correlation between the OTUs richness and the alpha diversity indexes was lower (Spearman Rho < 0.70) although statistically significant in all cases (Supplementary Table 5).

No statistically significant correlations were found between alpha diversity indexes and any of the morphometric descriptors of the basins (Fig. 5). Alpha diversity indexes were also compared in basins grouped in function of their A/V ratios as an indirect measure of the likelihood of basin desiccation. Significant differences were not found (p-value > 0.05 in Mann-Whitney (Wilcoxon) W-test; results not shown). Spearman tests were used to test for significant correlations between microbial diversity metrics and OTUs richness to the TOC, N and P profile in the basins. Positive correlations existed between Shannon and Evenness indexes and both nutrients (N and P) (Supplementary Table 6).

Multiple-sites dissimilarity measures of beta diversity revealed high differences in the OTU composition between basins. The variation in OTU composition was mainly characterized by a turnover of OTUs (β_{sim}) rather than by OTU nestedness (β_{sne}) (Table 2). Regarding abundance-based dissimilarity (β_{BC}), most of the dissimilarity was due to balanced variation in abundance ($\beta_{BC.BAL}$), that is, individuals of some OTUs in one basin are substituted by the same number of individuals of different OTUs in another basin.

Abundance gradient contribution ($\beta_{BC.GRA}$), that implies the loss of some individuals of a given OTU from one basin to another, was very small (Table 2).

Pairwise comparison of beta diversity (Fig. 6) showed no significant response to distance (p-value > 0.1) for any of the GLM distance-decay similarity models of beta diversity components. Additionally, the abundance-based values of beta diversity in the basins (Bray-Curtis distances) were ordinated by Non-Metric MultiDimensional Scaling (NMDS). Analysis were performed for the total OTUs abundance, and for their partitioning into the three different abundance categories (Abundant, Rare and NRA OTUs). In all cases, basins were neither discriminated (stress values < 0.1) in function of their A/V ratio, as indicated by the overlapped Ordihull convex ellipses for both groups of basins (Fig. 7 and Supplementary Fig. 6).

Discussion

Surveys on microbial communities from small and transient inland ecosystems are scarce when compared to those on large freshwater or marine environments [29,30,59]. The structural simplicity, isolated nature, global distribution, and permanence in geological time of rain-fed granite rock basins posit them as archetypes for testing a wide variety of ecological, biogeographic and evolutionary hypotheses on the metacommunities inhabiting these habitats [60]. There are very few studies that describe the presence of microorganisms in rain-fed granitic basins, and they have only addressed the morphological characterization of some genera of free-living protists [35,37,38,61] and obligate parasitic fungi (Microsporidia) [62]. The present work on ombrotrophic granite basins located within a National Park is the first comprehensive 18S rDNA gene study of eukaryotic microbial communities in these habitats.

Description of the Communities

Our results revealed rich and diverse microbial communities in the sediments of the granitic basins. These communities represented more than half of the abundance and number of the eukaryotic OTUs retrieved. The large OTUs richness found is potentially encompassed by a wide range of ecological functions, which supports the diverging conditions generated even in a single basin in function of biotic and abiotic factors and temporal availability of water. Cercozoa, by a lot the richest group in number of OTUs in these habitats, are a broad Phylum of physiologically and morphologically diverse protists [63,64]. Cercozoa are found abundantly in terrestrial, marine and freshwater habitats [65]. Encountered OTUs belong in majority to Glissomonadida, a species-rich assemblage of small gliding flagellates living in soils and formerly largely grouped into the morphospecies *Heteromita globosa* [66]. These organisms have typically high growth rates and very efficient encystment capacities [67]. They appeared as rare OTUs in most basins, which corroborates the hypothesis that most remain dormant waiting for appropriate conditions to develop. We found also abundant reads from a specialized parasitoid genus, *Viridiraptor* (OTU 154), whose abundance pattern seemed to be similar with the chlorophyte *Desmodesmus* (OTU 14) [68]. Likewise, several small testate amoebae more or less specialized on algae (genera *Rhogostoma* and *Trinema*) were represented in most of the basins [69]. In spite of being the richest protist group in the study, Cercozoa reads were typically present in low abundances unlike what it was found in other ephemeral shallow freshwater ecosystems [29,30].

By contrast, Chlorophyta were the most abundant microorganisms but were represented by four times less OTUs than Cercozoa. A plausible explanation to these results is related to the diurnal insolation cycle on the basins. During some periods of the day it was common to

observe blooms of Chlorophyta in the aquatic phases, whose resting stages accumulated in the sediments and were therefore retrieved by the 18S rDNA sequencing. This is the case of the planktonic species *Stephanosphaera pluvialis*, a relatively little known protist and the only species described for the genus which we isolated from the aquatic phase of some of the basins of this study. *S. pluvialis* has been observed in garden birdbaths [70] and rain pools [71]. For this reason, it is thought the species might be transferred from place to place on bird plumage (phoresis). This species is very likely to be represented in the sediment for the OTU 2, the most abundant microbial in our study that had a 100% homology in PR^2 with the Chlorophyta *Stephanosphaera* sp. In freshwater systems, strict phototrophs like the Chlorophyta are often found in nutrient-rich environments (eutrophic), while less productive water bodies are rather dominated by mixotrophs such as Chrysophyta [72], which were scarce in the present study. Our sediments had variation coefficients around 50% or higher for TOC and nutrients (N and P) values (Supplementary Table 7, showing the heterogeneity of the basins regarding the CNP content). P is considered the main responsible element of eutrophication in freshwaters [73]. The sediments were generally characterised by over-enrichment of nutrients in particular by P, which had values equivalent to those considered within hypereutrophic levels in water samples [74]. The abundant presence of animal droppings (birds, plus a thriving population of *Capra pyrenaica*) may explain the high P and N values measured in many of the sediments, which were otherwise only fed by rainfall.

Also, high levels of primary productivity in the basins, provoked by the blooms of Chlorophyta, may ultimately lead to hypoxia (or anoxia). This would also explain the presence in our survey of several protistan taxa associated to low oxygen conditions. OTUs were associated with sequences belonging to MAST-12 lineage, a group originally thought to be exclusively marine but now being increasingly detected in a variety of ecosystems, including ephemeral small freshwater systems [29], and often characterized by low oxygen amounts [75-77]. Other typical anaerobic/microaerophilic organisms were also encountered, such as sequences related to the flagellate *Trimastix* [78], and the ciliates *Brachonella galeata* and *Metopus violaceus* within the Armophorida [79]. These ciliates are known to enter dormancy under high oxygen pressure, awaiting anoxia [80]. Other ciliate taxa, such as *Halteria grandinella* (OTU 8) and Oxytrichidae (OTUs 4,16 and 27, among many others) are well-known aerobes that may respond to adverse anaerobic conditions by encysting. These organisms probably activate when respiration increases and depletes locally oxygen, for instance when nutrient pulses occur (for instance with animal droppings). However, given the size and depth of the granitic pools, and the fact that they dry out regularly, the existence of permanent anoxic microniches can be practically ruled out. This suggests the existence of two communities present in a single environment that can be active at different times and the existence of a dormant pool of encysted organisms.

The most likely explanation for the resilience of microbial communities in these rather extreme and drought-prone habitats, is therefore their capacity to go into metabolic dormancy [59]. Dormancy reflects a selected reservoir of metabolically-quiescent organisms, which can be revived under different environmental conditions [7]. This microbial encysted pool may help in explaining ecological events such as microbial bloom dynamics, biogeographical patterns, and microbial resilience under cyclical and drastic environmental perturbations [7], as occurred in the rock basins habitats here studied. The fact that the basins act as natural receptacles, together with the existence of dormant microorganisms that may activate at different moments, would explain the wide variety of microbial sequences encountered in the granitic pools sediments, as microorganisms can be progressively added to the basins without any effect from competition. In agreement with this hypothesis, inactive basin 9 (effectively a soil sample where a "wash-away" of populations by water happens) hosted significantly lower richness of OTUs, and no rare sequences appeared.

These results suggest that the lack of a washing-away effect occurring in the granitic basins resulted in high levels of microbial richness.

Patterns of Abundance and Diversity of OTUs

Our results show that basin populations were mainly constituted by a high richness of OTUs which generally were present at low abundances. The pool of exclusively rare OTUs was never detected as abundant OTUs. Moreover, about 50% of the rare OTUs were found only in two basins, but hardly ever in the same two. Our study was based in a single sample taken per basin, and although it was collected as a composite (homogenised) sample of all the sediment occupying the bottom of each basin, the rarefaction curves did not reach saturation in any of the basins. Therefore, β diversity may have been overestimated, especially in its turnover component. In addition, it is plausible and cautious to think that some of the *a priori* absent rare OTUs may have been overlooked in our sampling because organisms were too rare to be detected by our approach. Taking this in mind, our results are globally in line with the recent molecular studies carried out in other microbial habitats [44,81], which show that communities are dominated by a very infrequent and low abundant microbial community that has been termed "rare biosphere" [82-84]. Some hypotheses have been proposed on the nature and function of this "rare biosphere": an active microbial reservoir, a dormant seed bank, including to an unknown extent extracellular DNA and dead organisms [18,85,86]. It has been acknowledged that certain protist lineages can present different copies of the 18S rRNA in a single genome. This is most likely to be observed in organisms where ribosomal genes include many insertions in comparison with those of canonical eukaryotes, like foraminifera [87] and amoebozoa [88], but it has also been detected in other groups such as dinoflagellates [89]. Intra-genomic polymorphism within genomes should nevertheless be infrequent, as concerted evolution of the different copies is quickly eliminated in evolutionary times [90]. As a consequence, rare sequence should correspond, most of the times, to organisms present in low numbers in samples. However, the ecological significance, if any, of this pool of rare OTUs is still to be understood. Unravelling the real microbial diversity and the ecological role of rare microorganisms remains a current challenge in microbial ecology [18].Whether, as we hypothesized, at least some of the rare OTUs found in the dried sediments in this study have the potential to become dominant OTUs in response to returning water availability or if they prevail as chronically rare after rewetting, are questions still to be explored that will add valuable information to the nature and function of the rare microbial pool.

A relevant characteristic showed by the eukaryotic microorganisms of the rain-fed granitic basins studied is their stochastic and heterogeneous population dynamics, which is reflected by the high levels of beta diversity dissimilarity between basins. Most of the dissimilarity we found among the populations was due to species spatial turnover. The contribution of nestedness was minimal, which shows that there was a high degree of OTUs replacement, and the basins having less number of OTUs were not just subsets of the most diverse basins (βsne and $\beta_{BC.GRA}$ very low; [54-56]. A striking large among-basin variation in OTUs identity was found; only 59 OTUs (2.1%) were shared by the basins. That is, most OTUs were replaced while very few OTUs co-occurred regardless of the spatial closeness between the basins, highlighting the small level of connectivity among the populations of the metacommunity.

The high species turnover observed can be explained by the joint effects of fine-scale local adaptation to the diverging environment characterising the basins, and also random processes, may these last include resilient founder effects [27, 91, 92]. Local adaptation in response to changing of environmental variables is an important mechanism for population differentiation [24]. As environmental variables shape habitats at a very fine scale, adaptation allows closely related microbial populations to coexist providing high levels of biodiversity, as we found here. Moreover, the observed microbial heterogeneity among the basins may also be caused

by resilience of founder events [27]. Ombrotrophic rock basins are discrete (spatially isolated) habitats, and the colonisation by passive dispersers –as microorganisms are – must be constrained to chance (transportation by air, or via vectors such as invertebrates, birds or mammals). After initial colonisation, microbial eukaryotes may establish rapidly a large population by cell division. Random genetic drift owing to founder effects in spatial isolated habitats, between which gene flow is restricted, may create bottlenecks that lead to high genetic divergence among populations [93] resulting in a very high number of different OTUs.

The partitioning of beta diversity into its two components, nestedness and spatial turnover, may also be very informative for habitat conservation strategies [54]. A pattern of OTUs subsets (nestedness) would theoretically allow selecting a small number of the richest basins for preserving most of the biodiversity of the habitats, while a pattern of OTUs substitutions dominated by turnover, as the one observed in our study, involves conservation efforts devoted to a larger assemble of basins, not only the species richest ones.

The influence of the basin morphometry on the biodiversity has been often defined following the known principle of species- area relationship [94]. Research on invertebrate communities in rock basins has shown that a larger basin size determines a greater diversity of these communities [35,95]. In our study, depth, volume or area are not useful predictors of the diversity at the microbial eukaryote level. These results concur with those found for phytoplankton in rock pools [60] and by Soininen and Luoto [96] in lakes. Our findings may look surprising because larger habitats are intuitively related to higher heterogeneity and, therefore, to the possibility of new resources to be exploited by a more diverse number of microorganisms. Results may be explained for the permanent reservoir of dormant OTUs which, even in the smaller basins, may allow the coexistence of multitude of similar physiological types through a different temporal excystation or return to active state. Some authors have also proposed that a higher likelihood of basin desiccation determines less diversity for the biological communities [97]. Our study is the first that explores this hypothesis in microorganisms, using the A/V as a proxy of basin desiccation (the higher A/V ratio, the greater the likelihood of desiccation or evaporation rate). Contrary to expected, the results show no significant differences for diversity indexes or abundance of OTUs on the basis of the A/V ratio. It should be pointed out that we measured desiccation rate indirectly. The extent to which our results mean that the rate of desiccation does not influence the distribution of the microbial populations in the sediments should be confirmed in future research by *in situ* hydroregime measurements on a temporal scale.

Distances Between Basins

The negative relationship between biological similarity and spatial distance is a common macroecological pattern [98]. To further investigate the dissimilarity of the microbial communities in relation to the distance separating the basins, we produced distance-similarity decay models [56]. Our results show that spatial distance between the basins did not explain the differences observed in the beta diversity values for any of the components. These results agree with those found by Simon et al [29] also in ephemeral habitats, but differ from Lepère et al [20], who did found distance–decay patterns for rare and dominant taxa of small protists in lacustrine ecosystems. Results obtained are in agreement with the hypothesis that each basin has an independent history of colonization, localized events that provide individuality to the basins. Our similarity decay-models results support the idiosyncrasy of these habitats. Rain-fed granite basins are already so microbially unique that distance between them does not provide more variability to the innate microbial dissimilarity they hold.

Altogether, our results suggest that ombrotrophic granite rock basins may be hotspots of regional/local microbial diversity in the ecosystems. Because of drastically (and independently) changing environmental conditions, each ombrotrophic basin can host distinctive and unique communities, which may co-exist under different metabolic states. Each basin can be considered as a repository of the accumulated diversity of protists that were once active with the potential of blooming again, provided that conditions become suitable. This emphasises the biological value of these habitats and the interest to delve into their study and conservation.

Conflict of Interest The authors declare no conflict of interest.

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Figure captions

Fig. 1 Distribution of the number of microbial eukaryotic OTUs (a) and reads (b) among the taxonomic groups present in the basins. Red line represents the cumulative percentage (%) of OTUs (a) and reads (b). Only groups with abundances ≥ 0.1 % of the total were represented in b

Fig. 2 Distribution of the abundance of main taxonomic groups of Protists and Fungi at each basin. Only groups with abundances ≥ 0.1 % of the total were represented

Fig. 3 Rarefaction curves of the number of microbial eukaryotic OTUs for each granite rock basin with extrapolation (dashed line) to the asymptotic Chao1 value

Fig. 4 Variation of the reads (median and standard error) and total OTUs number (black squares) in the basins for each OTUs abundance category. Abundant (a); Rare (b); Non Rare non Abundant (c). See Results section for a detailed description of the categories

Fig. 5 Spearman correlations between the diversity indexes, OTUs richness and geomorphology indicators. Circles with asterisks represent significant correlations; * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001. A: Basin Area; V: Basin Volume; H: Shannon diversity index; D: Simpson diversity index; E: Pielou evenness index; R: Richness of OTUs; Ek: Eukaryotes; MEk: Microbial Eukaryotes; L: Length of the basins; W: Width of the basins; h: Maximum basin depth (h); u: Minimum basin depth

Fig. 6 Relationship (exponential decay model [56]) between Beta diversity components and the spatial distance among basins. Adjusted regression lines are represented in red; a: Beta Simpson (β_{sim}); b: Beta balanced Bray-Curtis($\beta_{BC.BAL}$); c: Beta nestedness (β_{sne}); d: Beta Gradient Bray-Curtis ($\beta_{BC.GRA}$). See Materials and Methods section for a detailed description of Beta diversity components

Fig. 7 Bray-Curtis based non-metric multidimensional scaling (NMDS) plot for the total OTUs (a) and the three abundance categories (b, c, d). Blue (1) represents basins with A/V ratio ≥ 1 and red (0) represents basins with A/V ratio <1