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Distribution survey of Cyanobacteria in three Greek caves of Peloponnese

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Abstract:

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Caves and hypogean environments host various phototrophic microorganisms, with Cyanobacteria constituting the major group. The spatial and temporal distribution of Cyanobacteria (156 taxa in total) from three Greek caves, located in the limestone arc of Peloponnese and differing in morphology, was studied. The community patterns in different ecological niches were analyzed in relation to environmental parameters (Photosynthetically Active Radiation, Temperature, and Relative Humidity). Cyanobacterial communities were found to thrive in patchy biofilms and showed known protective strategies against desiccation and irradiation. The nMDS analysis of the cumulative seasonal samples per sampling site showed no general pattern of distribution, with a clear differentiation of cyanobacterial communities among the three caves. Only in the typical cave 'Kastria', cyanobacterial taxa showed growth habits in accordance with the gradient of light from entrance inwards.

Keywords: cyanobacterial communities; hypogean habitat; environmental parameters; ecological niches; Greek caves

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INTRODUCTION

Caves are classified according to the composition of the lithic substrate, the proximity to the ground-water table, the spelaeogenic history, and the overall passage morphology. Limestone caves are highly specific environments scattered all over the world (Hoffmann, 1989; Hernández-Mariné et al., 2001; Roldán et al., 2004), and karst caves are considered a specific case of extreme environment (Culver et al., 2004; Mulek & Kosi, 2008). The term 'extreme' is usually defined in a mathematical sense as belonging to the outer parts of a Gaussian distribution, i.e., as 'extraordinary'. However, among biologists it should be clear that this definition has to be handled carefully. As soon as there are organisms able to settle down, those habitats are no longer extreme in a biological sense (Seckbach et al., 2007).

Most caves represent stable environments characterized by uniform temperatures throughout the year, high humidity and low natural light. A typical cave is described as having three major habitat zones based on light penetration and intensity: the entrance-, transition-, and dim light zone. Even caves with dim natural light have been found to host phototrophic mi-

croorganisms where the low Photosynthetically Active Radiation (PAR) is the pressure and maybe one of the divergence forces for selection (Roldán & Hernández-Mariné, 2009). Moreover, cave characteristics such as dimensions, morphology, location, orientation and lithic substrate can play important role to the biocommunity structure.

The taxa recorded in caves have been categorized (e.g. Hoffmann, 2002) into three groups: a) 'troglobitic' species, obligatory cavernicoles that cannot survive outside the cave, b) 'troglophilic' species, growing and reproducing in caves, and c) 'trogloxenic' species, accidentally reaching the cave environment. Concerning cyanobacteria, some species such as *Loriella osteophila* Borzi, *Scytonema julianum* (Kützinger) Meneghini Richter can be considered as troglophiles (Hoffmann, 2002). Very few cyanobacteria are considered as obligatory cavernicolous such as *Geitleria calcarea* Friedmann, *G. floridana* Friedmann, *Herpyzonema pulverulentum* Hernández-Mariné & Canals, and *Symphyonema cavernicolum* Asencio, Aboal & Hoffmann. Hypogean environments including caves have been studied worldwide: Brasil (Sant'Anna et al., 1991), Israel (Friedmann, 1964; Vinogradova et al., 1998), Hungary (Claus, 1964; Hajdu, 1966), France (Bourelly & Dupuy, 1973; Leclerc et al., 1983), Germany (Dobat, 1977), Italy (Borzi, 1917; Skuja, 1970; Abdelahad, 1989), Romania (Șerbănescu & Decu, 1962), Slovenia (Mulek & Kosi, 2008), Spain

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(Ariño et al., 1997; Ascencio & Aboal, 1996, 2000a,b; Martínez & Asencio, 2010; Hernández-Marín et al., 2001; Roldán et al., 2004), and Greece (Anagnostidis et al., 1982; Lamprinou et al., 2009, 2011, 2012a,b,c). Despite all the aforementioned mainly floristic studies, it is only recently that cave microbiology has been established as a new field combining aspects of microbial life, geology and chemistry that influence the natural cave processes (Mulek & Kosi, 2008).

The aim of this study is to present the spatial and temporal distribution of cyanobacteria in three Greek caves differing in location and morphology, to analyze and correlate the observed community patterns with the environmental parameters, and to study the various growth habits in relation with the ecological niches.

MATERIAL AND METHODS

The caves 'Kastria', 'Selinitza' and 'Franchti' differ in morphology and in the proximity to the sea, and are all located in the limestone arc of Peloponnese, Greece. (Fig. 1). 'Kastria' (37°57'37.54"N, 22°08'26.91"E, altitude 827 m a.s.l.) is a typical cave in terms of light gradient from the entrance inwards, located in Achaia; it actually represents an old subterranean river with an explored length of 1980 m, 500 m of which are touristically exploited with a separate entrance. 'Selinitza' (36°48'10.44"N, 22°17'51.18"E, altitude 2 m a.s.l.), located in Messinia, is a typical cave in terms of light gradient but influenced by the sea spray. 'Franchti' (37°25'21.01"N and 23°07'51.81"E; altitude 12.5 m a.s.l) is an exposed, non-typical cave with partly collapsed roof, located in Argolida; according to the archaeologists, this cave represents a remarkable site on the coast of southeast Greece since its deposits cover the period from 20.000 BC down to 3.000 BC (far prehistoric).



Fig. 1. Map of Greece showing the location of caves: 'Franchti' (dot), 'Kastria' (circled dot) and 'Selinitza' (double circled dot).

Sampling was made at selected sites 1-7 in each cave starting from the physical cave entrance, hosting various growth habits of cyanobacteria. Sampling was conducted seasonally during a biannual survey (2009-2010). Air Temperature (T °C), Relative Humidity (RH%), and Photosynthetically Active Radiation (PAR $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) were measured by a LI-1400 data logger (LI-COR Biosciences, USA) during the survey (Table 3). Four subsamples were collected under sterile conditions from each sampling site. Two of them were incubated in situ inside transparent sterile vials, and the other two were partly fixed with formaldehyde solution at a final concentration of 2.5%. Enrichment cultures were obtained in flasks and petri-dishes with BG11 and BG 11₀ (Stanier et al., 1971). Cultures were maintained in incubator (SANYO, GALENKAMP) under stable conditions (23 °C, 80% RH, 7 $\mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ PAR) and under daylight (north facing window) at room temperature for 2 years. For Light Microscopy (LM) fresh and cultured material was observed on glass slides under a high-resolution light microscope (Axiolab, Zeiss, Germany). Species presence/absence data were analyzed by non-metric Multidimensional Scaling Ordination (nMDS) and Analyses of Similarities (SIMPER) based on Jaccard similarity index and the application of PRIMER software v.6. Species richness, as number of species, was used as a proxy of cyanobacterial diversity and comparisons were made through ANOVA (Statgraphics Centurion).

RESULTS

A photosynthetic microflora consisting of cyanobacteria, green algae, diatoms and bryophytes was found inhabiting sites with adequate light in all caves studied and near the cave entrances; these taxa were preserved in formaldehyde solution and are available for further taxonomic study. Cyanobacterial communities were found to prevail mainly on the cave walls, and on spelaeothems such as stalagmites and stalactites, especially in the low light zone.

After a seasonal survey in each cave and in each sampling site (1-7) the microscopic analysis (LM) revealed a total number of 156 cyanobacterial taxa (Table 1 available online at <http://dx.doi.org/10.5038/1827-806X.41.2.12>) belonging to the orders Chroococcales (69), Oscillatoriales (59), Nostocales (26), and true-branched Stigonematales (2). Despite the almost similar annual biodiversity in terms of number of species in the three caves ('Kastria' 76, 'Selinitza' 71, 'Franchti' 65 taxa) only 19 taxa were common, with 12 of them belonging to Oscillatoriales.

The nMDS analysis of the cumulative seasonal samples per sampling site showed a clear distinction of the cyanobacterial community in the three caves (Fig. 2), with average dissimilarity among pairs of caves ranging between 86-89% (for the distinctive species see also the results of the analysis of similarities in Table 2).

Analysis of the average species richness per season showed differentiation among the three caves (Fig. 3): In 'Franchti' and 'Selinitza' the average species richness per season was stable throughout the year; on the other hand, 'Kastria' showed significantly



Fig. 2. nMDS analysis of the cumulative seasonal samples per sampling sites 1-7. (K = 'Kastria', S = 'Selinitsa', F = 'Franchti').

higher values in autumn and winter and especially low values in spring with two sites (K1 & K2) lacking noticeable cyanobacterial growth. The summer average species richness was not significantly different from that of either autumn or spring. The nMDS analysis performed for each cave revealed a different seasonal pattern of cyanobacterial community. In particular:

In 'Kastria' the seven sampling sites were grouped in three clusters (Fig. 4): (i) the entrance zone consisting of sites 1, 2, 3, (ii) the transition zone consisting of sampling site 5, and (iii) the dim light zone consisting of sampling sites 6, 7. The samples of site 4 were more dispersed indicating seasonal variation, still the nMDS 3D configuration (not shown here) revealed the site's distinction from the rest. The entrance community was the richest (51 taxa) followed by the transition and the dim light zone (33 and 18 taxa respectively).

Table 2. Results of SIMPER analysis among pairs of caves based on the cumulative seasonal species composition, showing the percentage (%) average dissimilarity (AD) and the responsible distinctive species.

86.25%	86.35%	88.98%
Kastria <i>Scytonema julianum</i> (Kützting) Meneghini <i>Iphinoe spelaeobios</i> Lamprinou & Pantazidou <i>Pseudophormidium spelaeoides</i> (Čado) Anagnostidis <i>Lyngbya palikiana</i> Claus	Franchti <i>Pseudophormidium</i> sp. (Forti) Anagnostidis & Komárek <i>Cyanosarcina</i> (sp.3) Kováčik <i>Nostoc</i> (sp.3) Vaucher ex Bornet & Flahault <i>Asterocapsa aerophytica</i> Lederer <i>Chroococcus</i> cf. <i>lithophilus</i> Ercegović Selinitsa <i>Leptolyngbya tenuis</i> (Gomont) Anagnostidis & Komárek <i>Cyanosarcina</i> (sp.1) Kováčik <i>Nostoc macrosporum</i> Meneghini ex Bornet & Flahault	Kastria <i>Scytonema julianum</i> (Kützting) Meneghini <i>Iphinoe spelaeobios</i> Lamprinou & Pantazidou <i>Pseudophormidium spelaeoides</i> (Čado) Anagnostidis <i>Leptolyngbya gracillima</i> (Zopf ex Hansgirg) Anagnostidis & Komárek <i>Lyngbya palikiana</i> Claus
Franchti <i>Chroococcidiopsis kashaii</i> Friedmann <i>Pseudophormidium</i> sp. (Forti) Anagnostidis & Komárek <i>Leptolyngbya gracillima</i> (Zopf ex Hansgirg) Anagnostidis & Komárek		Selinitsa <i>Chroococcidiopsis kashaii</i> Friedmann <i>Nostoc commune</i> Vaucher ex Bornet & Flahault

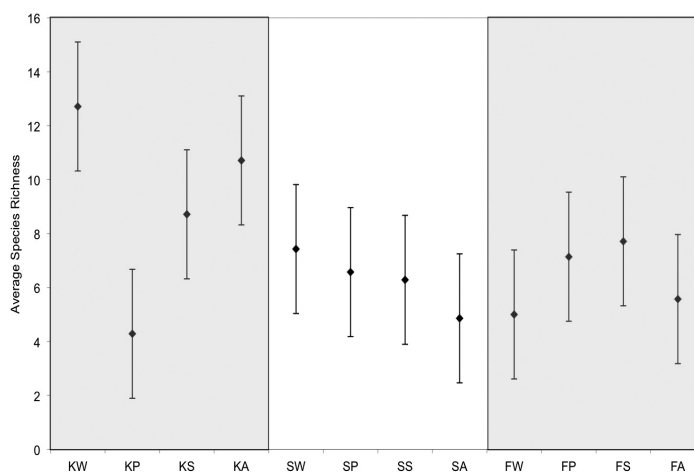


Fig. 3. ANOVA of the average species richness per season (error bars=95% LSD) showing the differentiation among caves (K = 'Kastria', S = 'Selinitsa', F = 'Franchti', W = Winter, P = Spring, S = Summer, A = Autumn).

In 'Franchti' the seven sampling sites were grouped in two major clusters (Fig. 5): (i) the first one consisting of sampling sites 1-5 with seasonal sub-grouping, and (ii) the second one with sampling sites 6 (stalagmite) and 7 (stalactite). Analysis of species richness revealed 52 and 32 taxa in the above clusters, respectively.

In 'Selinitsa' the seven sampling sites were grouped in three clusters (Fig. 6): (i) the entrance zone, mostly affected by the sea spray, consisting of sampling sites 1,3, (ii) the transition zone, in terms of PAR and influence of the salt spray, consisting of sampling site 2, and (iii) the dim light zone consisting of sampling sites 4,5,6,7. The species richness was higher at the entrance zone (48 taxa); the transition and the dim light zones followed with 11 and 35 taxa, respectively.

Table 3. Average seasonal values and standard deviation of Photosynthetically Active Radiation (PAR), Temperature (T) and Relative Humidity (RH) measured in the three caves (K = 'Kastria', S = 'Selinita', F = 'Franchti', W = Winter, P = Spring, S = Summer, A = Autumn).

	PAR $\mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$		T $^{\circ}\text{C}$		RH %	
	average	sd	average	sd	average	sd
FA	2.58	2.70	19.50	2.06	89.00	5.23
FP	0.71	0.56	12.53	0.53	53.66	2.46
FS	1.32	0.86	25.10	0.81	62.18	4.86
FW	7.75	12.83	15.92	1.15	59.97	4.96
KA	0.40	0.60	12.01	0.35	90.17	4.59
KP	4.24	7.85	12.37	1.37	92.04	5.59
KS	4.70	8.15	16.21	3.56	81.63	13.78
KW	6.20	15.07	6.60	0.69	92.35	4.42
SA	12.08	16.89	20.08	0.43	75.90	2.76
SP	4.46	7.28	16.73	0.40	68.11	10.95
SS	10.10	12.25	20.66	4.03	64.26	6.53
SW	2.77	4.15	16.05	0.48	86.31	2.20

DISCUSSION

Although a photosynthetic microflora consisting of various algal groups was found in sites of the three caves with adequate light, Cyanobacteria were found to prevail especially in the low light zone (see also Albertano, 1999; Hernández-Marín et al., 2001; Lamprinou et al., 2009). A general pattern of cyanobacterial distribution in relation to the environmental parameters examined (PAR, T, RH) cannot be determined in the caves investigated herein. The morphology of the cave, the water availability, the type and the coherence of the substratum apart from the abiotic parameters might thus explain the variations in species composition (see also Roldán et al., 2004).

However, in the typical cave 'Kastria' where light shows a clear gradient from the entrance inwards, the cyanobacterial community is organized into mosaics following the expected 'typical' (entrance, transition and dim light zone) pattern of cyanobacterial distribution. The entrance community zone shows biofilms with the highest number of species mostly of the order Chroococcales (see also Roldán & Hernández-Marín, 2009). Mucilaginous, 'temporary' biofilms protected against dessication and direct light were prevailing at the entrance zone, with the following characteristic taxa: *Aphanocapsa* spp., *Chroococcus* spp., *Eucapsis minor*, *Leptolyngbya gracillima*, *L. perelegans* and *Pseudophormidium spelaeoides*. On the contrary, the dim light community zone is characterized by a lower number of species with Oscillatoriales prevailing over Chroococcales (see also Vinogradova et al., 1998; Roldán & Hernández-Marín, 2009). Biofilms there become thinner and less mucilaginous, and taxa with calcified filaments able to survive at dim light, such as *Scytonema julianum* and *Iphinoe spelaeobios*, are prevailing. Presence of species with carbonate precipitates on the polysaccharide sheaths has been previously reported from dim light zones of caves (Hernández-Marín et al., 2001; Roldán et al., 2004). It is noted that *Scytonema julianum* together with the species *Leptolyngbya palikiana*, *L. gracillima*, *Pseudophormidium spelaeoides*, were almost always present from the entrance to the deeper part of cave

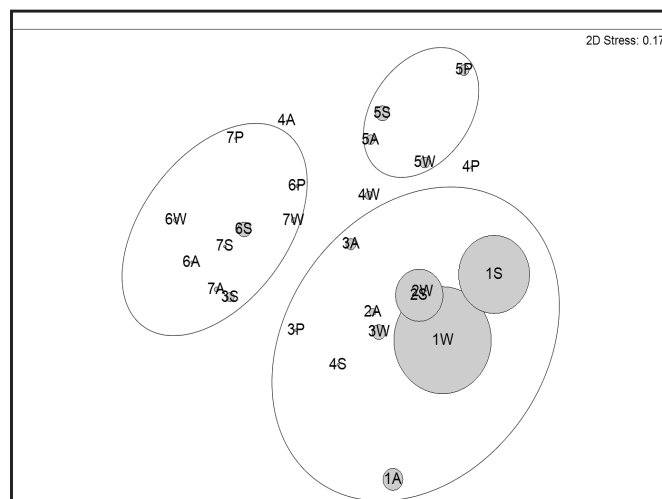


Fig. 4. In cave 'Kastria', nMDS analysis of the cumulative seasonal samples per sampling site with superimposed the average value of PAR. Grouping of sampling sites (1-7) in three clusters is shown. (W = Winter, P = Spring, S = Summer, A = Autumn).

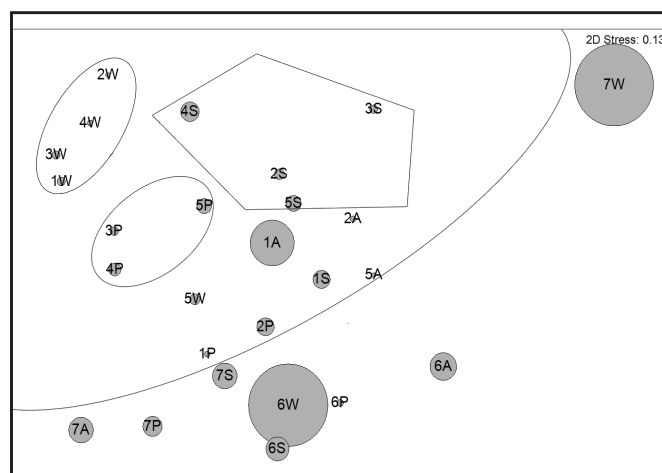


Fig. 5. In cave 'Franchti', nMDS analysis of the cumulative seasonal samples per sampling site with superimposed the average value of PAR. Grouping of sampling sites (1-7) in two clusters is shown. (W = Winter, P = Spring, S = Summer, A = Autumn).

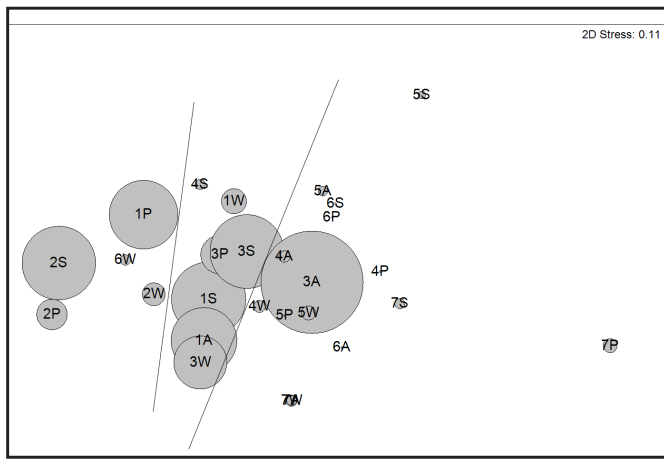


Fig. 6. In cave 'Selinitza', nMDS analysis of the cumulative seasonal samples per sampling site with superimposed the average value of PAR. Grouping of sampling sites (1-7) in three clusters is shown. (W = Winter, P = Spring, S = Summer, A = Autumn).

'Kastria'. According to Hoffmann (2002) *Scytonema julianum* can be characterized as a troglophile species, and according to previous reports from caves (Couté & Bury, 1988; Ariño et al., 1997; Roldán & Hernández-Marín, 2009) this species seems to bear strong environmental fluctuations.

The presence of filamentous heterocytous cyanobacterial species with ability to fix atmospheric N_2 and produce exopolymeric polysaccharide substances (EPSs), like those of the genus *Nostoc* observed in most sites of 'Selinitza' - a 'typical' cave but affected by the sea spray-, might be a clear advantage for in-cave environment. It is known that nitrogen is a nutrient limiting algal growth; as a result the capacity of the heterocytous cyanobacteria to fix nitrogen plays an important role especially in these poor environments (Asencio & Aboal, 2011). The same is true for *Chroococcidiopsis kashaii*, a species always present in this cave in biofilms with abundant EPS, since these substances are crucial for increasing dessication tolerance (see also Zammitt et al., 2011).

Predominance of Oscillatoriales over Chroococcales, like that observed in the dim light zone of 'Kastria', was also observed in the open air cave 'Franchi', especially in sites of speleothems (stalagmites, stalactites) exposed in light, but their presence there is attributed to the chasmoendolithic mode of life.

It is worth noticing that new genera and species have already been established from the three investigated Greek caves, i.e. *Iphinoe spelaeobios* Lamprinou & Pantazidou (Lamprinou et al., 2011), *Iphinoe* cf. *spelaeobios* Lamprinou & Pantazidou (Lamprinou et al., 2012b), *Toxopsis calypsus* Lamprinou & Pantazidou (Lamprinou et al., 2012a), *Phormidium melanochrouron* Lamprinou & Pantazidou (Lamprinou et al., 2012c), enhancing the view that hypogean environments in general may favor speciation or physiological adaptation (Lamprinou et al., 2009; Zammitt et al., 2011). Therefore, the species distribution in relation to cave morphology, lithic substrate, and microclimatic conditions still remain a challenge for further research.

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