Chapter 3

Diversity of Cyanobacteria on Limestone Caves

Czerwik-Marcinkowska Joanna and
Massalski Andrzej

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Abstract

The caves are the biodiversity centers for different types of microorganisms, especially for cyanobacteria. They are also present in almost all extreme environments, and their importance in terrestrial ecosystems is greater because of the decreased competition from vascular plants. Cyanobacteria occurring on rocks are epilithic (colonizing the substrate surface), hypolithic (growing under pebbles and small stones), and endolithic (present in an upper layer of rock). There are three limiting factors for cyanobacteria growing in caves: light or its lack, high humidity, and constant temperature. In caves, one can find not only the cosmopolitan cavernicolous species but also rare taxa. Light, transmission, and scanning electron microscopy (SEM), laboratory cultures, as well as molecular phylogenetic studies are important tools in the study of cave cyanobacteria.

Keywords: cyanobacteria, cave, diversity, ecology

1. Introduction

Caves are highly specific environments scattered all over the world, and karst caves are considered a specific case of extreme environment [1, 2]. Caves are also biocenters for biological studies, and they are characterized by low natural light, uniform temperatures throughout the year, and high humidity [3]. A typical cave is described as having three major habitat zones based on light penetration and intensity: the entrance, transition, and dim light zone. Lamprinou et al. [2, 4] and Roldán and Hernández-Marinė [5] suggested that caves are the most typical and well known of the different karstic formations geologically created in limestone substrates and even caves with dim natural light were found to host phototrophic microorganisms. Lee et al. [6] and Falasco et al. [7] stated that biology of caves is not only a matter of exploring unique and extreme ecosystems but also understanding of the ecological balances on Earth. Unlike other nutrient-deprived environments where one might study oligotrophic conditions, most caves are readily accessible. The absence of photosynthetic organisms leads cave cavernicolous species to depend on allochthonous resources or, in rare cases, on chemoautotrophic bacteria and roots [8]. Allochthonous resources are carried by water, wind, gravity, or animals that are translocating between caves and external environments; thus cave communities are highly dependent on the adjacent epigean environments [3, 9]. Cave ecosystems are characterized by stable conditions, although cave habitats are extremely oligotrophic, receiving limited supplies of organic matter. Poulson and Lavoie [10] determined that organic matter in caves derives from plants and guano, whose bioavailability is largely dependent on its chemical properties and on environmental physical factors, such as temperature and light. Falasco et al. [7] described cave biological microorganisms as characterized by presence of resident and nonresident organisms (accidentals), which enter caves occasionally via water, sediments, wind, or air, as spores, or even carried in by animals. On the other hand, caves generally can be described as having relatively low species richness, biomass, and density, and where cavernicolous species are dominated by cyanobacteria, which are the first colonizers [11]. Such cave conditions attract cosmopolitan species, leading to the gradual elimination of particularly sensitive native species [12]. However, most caves, at least in Europe, are damp and the walls at the entrance are covered with a blue-green cyanobacterial gelatinous mass [11]. Entering a cave, one goes through a series of zones: entrance (strongly influenced by surface conditions), deeper, and the dim light zone. Deep in a cave, there is an absence of light, temperature at or near the MAST (mean annual surface temperature) for the region, and high humidity. At the entrance of limestone caves and on surfaces around an artificial lighting (caves made accessible to general public), cyanobacteria compete for light with algae, mosses, and sometimes also prothalli of ferns and flowering plants, but in the deepest recesses of caves, where the light is reduced to a minimum, there are only phototrophs [13, 14]. Hoffmann [15] divided species occurring in caves into three categories based on their ecological evolutionary: troglobiotic (obligatory cavernicolous that cannot survive outside the cave), troglophilic (growing and reproducing in caves), and trogloxenic (accidentally appearing in the cave environment). Lamprinou et al. [4] suggested that very few cyanobacteria are considered as obligatory cavernicolous such as *Geitleria calcarea, G. floridana, Herpyzonema pulverulentum*, and *Symphyonema cavernicolum.* However, Hoch and Ferreira [16] and Rabelo et al. [9] stated that the cave environments favor scenarios of high endemism, where species can be restricted even to a single cave. This high endemism leads several species to be threatened, particularly in tropical regions where the demand for mineral resources, food production, and energy generation has grown due to rapid development and population growth [17].

Cyanobacteria, the oldest cellular organisms on Earth, are very resistant to severe conditions in cave, including even darkness. Moreover, the cyanobacteria are able to survive in dark caves, as heterotrophic organisms, and are consumed by cave palpigrades [18]. It is interesting that cyanobacterial species, as suggested by Barton and Jurado [19], adapt to cave environment by interacting with minerals there and some of processes reshaping the mineral structure of the cave walls, floors, and ceilings—for instance, by forming speleothems such as stalactites and stalagmites. One of the most interesting problems, although not precisely known, is the origin of the global (cosmopolitan) distribution of cavernicolous cyanobacteria.

1.1. Study area

Europe is an exceptional continent for its abundance and variety of subterranean karstic forms comprising the natural geological heritage, and therefore many caves are protected and European countries have programs for the protection of caves and geodiversity. Some caves can be visited and are either ecologically or culturally a tourist attraction, and are important centers of mass touristic exploitation. While numerous caves can be visited all year round, others are difficult to get to and are explored only by speleologists and wild animals.

2. Material and methods

Cyanobacteria samples were collected from walls and ceiling of limestone caves where evidence of biological colonization was present. Algal crusts were scraped from the stone substrata (**Figure 1**) using nondestructive adhesive tape method also by removing the biofilm with sterilized scalpel, stored in labeled sterile plastic bags and used directly for observation under a light microscope (LM) or as inoculate for cultures on agar plates. Cyanobacterial material was cultured on agar plates or liquid medium in lab, and in consequence, filamentous and coccoid species were obtained. The samples were studied using a light microscope at 100× objective with oil immersion. Photomicrograph was taken using photomicroscope equipped with a digital camera.

For transmission electron microscopy (TEM), samples fixed in glutaraldehyde were postfixed in an osmium tetroxide solution, dehydrated in a graded ethanol series, and embedded in epoxy resin. Thin sections were collected on copper grids and stained with uranyl acetate and lead citrate. For scanning electron microscopy (SEM), cleaned sample was dried onto a coverslip, attached to an aluminum stub, and then gold coated, whereas environmental variables (temperature, light, and humidity) were measured using digital thermohygrometer and photoactinometer. For each parameter, the mean value with standard error was calculated.

Figure 1. Dark-green—colored cyanobacteria patches on walls in Sąspowska cave (Ojców National Park, Poland).

3. Results and discussion

Cavernicolous cyanobacteria belong to the orders Chroococcales, Nostocales, Oscillatoriales, and Stigonematales. Chroococcales are the most common order represented by genera: *Aphanocapsa, Aphanothece, Chondrocystis, Chroococcidiopsis, Chroococcus, Gloeocapsa, Gloeocapsopsis,* and *Gloeothece.* Whereas among Nostocales were present only three genera: *Hassalina, Nostoc,* and *Scytonema.* Coccoid and filamentous cyanobacteria species were dominant in the entrance zone, while in the dim light zone they were encountered only sporadically. Czerwik-Marcinkowska and Mrozińska [20] and Martínez and Asencio [21] stated that coccoid forms are more abundant in dark areas, whereas filamentous forms tend to be more diverse in illuminated part of cave, unlike the findings of Vinogradova et al. [22]. Epilithic cyanobacteria are the first colonizers and play an important role in the genesis of biofilms, being able to produce exopolymeric substances (EPS) that allow the adhesion to rocks and the consequent establishment of a microbial community [7]. Besides the colonization of various stone substrata and the production of pigments that are responsible for colored effects on rocky cave walls and erosion of the stone substrata, they can also serve as a food source for animals. Almost all cavernicolous cyanobacteria have gelatinous extracellular sheath layers of various thickness composed of polysaccharides. Keshari and Adhikary [23] observed that the gelatinous extracellular sheath of cyanobacteria plays a crucial role in adhesion to the substratum and also acts as a water reservoir, thus enabling the cyanobacteria to survive drought periods. Pattanaik et al. [24] suggested that water stress proteins, glycan, and UVA/B absorbing pigments are the main components of the EPS of cyanobacteria. The genus *Gloeocapsa* has the most various colorations due to the presence of a pigment called gloeocapsin. Another well-studied pigment, scytonemin, causes the dark coloration of cyanobacterial crusts [24]. Genera that usually dominate dark-colored crusts are *Scytonema, Nostoc,* and *Tolypothrix.* Some cyanobacteria from genus *Scytonema* have calcified trichomes [25]. In **Table 1**, the most frequent and abundant cyanobacteria species found in different European caves based on literature results since 2010 are reported.

3.1. Ecology of cyanobacteria in caves

Whitton [25] stated that cyanobacteria have existed for 3.5 billion years and they are the most important photosynthetic organisms on the planet for cycling carbon and nitrogen. Cyanobacteria living in limestone caves present a unique group of microorganisms, which developed adaptations against the more or less extreme conditions of their habitats. They play an important role in several aspects of the environment such as colonizers, nitrogen fixers, prey for micrograzers, or deterioration agents. Cyanobacteria are morphologically diverse group of prokaryotes successfully colonizing and inhabiting almost all kind of terrestrial and aquatic habitats including extreme microhabitats such as caves, rocks, external walls of monuments, and buildings [26, 27]. Wild caves and caves made accessible to general public are characterized by extreme conditions, and they also offer a unique habitat for cyanobacteria [20]. Cyanobacteria are prone to environmental stress such as desiccation, temperature, and UV radiation, and they adopted survival strategies by producing

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Cyanobacteria	Origin	References
Aphanocapsa fusco lutea	Božana Cave (Serbia)	Popović et al. [28]
Aphanocapsa muscicola	Božana Cave (Serbia)	Popovć et al. [28]
Aphanothece saxicola	Gelda Cave (Spain), Božana Cave (Serbia)	Martínez & Asencio [21], Popović et al. [28]
Asterocapsa divina	Gelda Cave (Spain)	Martínez & Asencio [21]
Calothrix fusca	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Chondrocystis dermochroa	Božana Cave (Serbia)	Popović et al. [28]
Chroococcidiopsis kashayi	Božana Cave (Serbia)	Popović et al. [28]
Chroococcus ercegovicii	Božana Cave (Serbia), caves from Ojców National Park (Poland)	Popović et al. [28], Czerwik-Marcinkowska et al. [26]
Chroococcus pallidus	Božana Cave (Serbia)	Popović et al. [28]
Chroococcus spelaeus	Gelda Cave (Spain)	Martínez & Asencio [21]
Chroococcus turgidus	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Chroococcus westii	Gelda Cave (Spain)	Martínez & Asencio [21]
Cyanobacterium cedrorum	Gelda Cave (Spain)	Martínez & Asencio [21]
Cyanosaccus aegeus	Gelda Cave (Spain)	Martínez & Asencio [21]
Cyanosaccus atticus	Gelda Cave (Spain)	Martínez & Asencio [21]
Cyanostylon microcystoides	Gelda Cave (Spain)	Martínez & Asencio [21]
Gloeocapsa atrata	Božana Cave (Serbia)	Popović et al. [28]
Gloeocapsa biformis	Gelda Cave (Spain), Božana Cave (Serbia), caves from Ojców National Park (Poland)	Martínez & Asencio [21], Popović et al. [28], Czerwik-Marcinkowska et al. [26]
Gloeocapsa compacta	Božana Cave (Serbia)	Popović et al. [28]
Gloeocapsa lignicola	Božana Cave (Serbia)	Popović et al. [28]
Gloeocapsa nigrescens	Gelda Cave (Spain)	Martínez & Asencio [21]
Gloeocapsa novacekii	Gelda Cave (Spain)	Martínez & Asencio [21]
Gloeocapsa punctata	Božana Cave (Serbia), caves from Ojców National Park (Poland)	Popović et al. [28], Czerwik-Marcinkowska et al. [26]
Gloeocapsa rupicola	Gelda Cave (Spain), Božnana Cave (Serbia), caves from Ojców National Park (Poland)	Martínez & Asencio (2010), Popović et al. [28], Czerwik-Marcinkowska et al. [26]
Gloeothece cyanochroa	Božana Cave (Serbia)	Popović et al. [28]
Gloeothece palea	Božana Cave (Serbia), caves from Ojców National Park (Poland)	Popović et al. [28], Czerwik-Marcinkowska et al. [26]
Gloeocapsopsis dvorakii	Božana Cave (Serbia)	Popović et al. [28]

Cyanobacteria	Origin	References
Hassalia byssoidea	Božana Cave (Serbia)	Popović et al. [28]
Leptolyngbya carnea	Gelda Cave (Spain)	Martínez & Asencio [21]
Leptolyngbya gracillima	Kastria Cave (Greece)	Lamprinou et al. [2]
Leptolyngbya leptotrichiformis	Gelda Cave (Spain)	Martínez & Asencio [21]
Lyngbya palikiana	Kastria Cave (Greece)	Lamprinou et al. [2]
Nodularia harveyana	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Nodularia sanguinea	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Nostoc commune	Selinits Cave (Greece), Božana Cave (Serbia), caves from Ojców National Park (Poland)	Lamprinou et al. [2], Popović et al. [28], Czerwik-Marcinkowska et al. [26]
Nostoc punctiforme	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Phormidium breve	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Phormidium formosum	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Phormidium vulgare	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Pleurocapsa minor	Gelda Cave (Spain)	Martínez & Asencio [21]
Pseudocapsa dubia	Gelda Cave (Spain), caves from Ojców National Park (Poland)	Martínez & Asencio [21], Czerwik-Marcinkowska et al. [26]
Pseudophormidium spelaeoides	Kastria Cave (Greece)	Lamprinou et al. [2]
Scytonema drilosiphon	Božana Cave (Serbia)	Popović et al. [28]
Scytonema julianum	Gelda Cave (Spain), Kastria Cave (Greece), caves from Ojców National Park (Poland),	Martínez & Asencio [21], Lamprinou et al. [2], Czerwik-Marcinkowska et al. [26]
Scytonema mirabile	Božana Cave (Serbia)	Popović et al. [28]
Symphyonema cavernicolum	Gelda Cave (Spain)	Martínez & Asencio [21]
Tolypothrix epilithica	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]

Table 1. Cyanobacteria species reported in caves based on literature results since 2010.

photoprotective pigments and bioactive compounds. Rock inhabiting cyanobacteria can be divided into “epilithic,” colonizing the substrate surface directly; “hypolithic,” living under pebbles and small stones lying on the rock; and “endolithic,” living in an upper layer of rock [29]. The cavernicolous cyanobacteria species reported from rock and stone wall surfaces, mostly coccoid and heterocytous types with (often colored) mucilaginous envelopes and sheaths, tend to be dominant in terms of biomass. Cyanobacteria play a main role in the species biodiversity of caves. However, characterizing the biodiversity of caves is challenging because cyanobacterial communities often have high richness and contain numerous species that have neither been isolated nor described using traditional culturing techniques. The culture-independent methods can be applied to study cave communities and are especially powerful if combined with culture-based information. Many studies [20, 30] have described cyanobacteria occurring in both terrestrial sediments and aquatic cave environments around the world. This widespread distribution reflects the tolerance of cyanobacteria toward environmental stress due to a broad spectrum of specific properties in physiology [31]. Jones and Motyka [32] noted that a single microorganism can change from an autotroph (utilizing light for food) to a mixotroph (autotrophic microorganism that grows more rapidly in the presence of certain organic substrate) to a heterotroph (growth with no light). Most of these cavernicolous species are nonresidents transported into caves by water, air, sediment, and animals. Moreover, these enrichment-based and cultural studies have focused on typical heterotrophic microorganisms, which grow in an extreme environment [33]. Culture-independent, molecular phylogenetic techniques have since shown that many previously unknown species can be found in caves [34]. Impact of cave tourism (artificial light) is altering the natural light gradient in cave ecosystems, which may have important repercussions on the composition of cyanobacteria communities inside the caves, and that is why, lampenflora can be regarded as invasive [35]. Tourists entering limestone caves are responsible for transferring cyanobacteria spores [36], leading to unintentional biological pollution and favoring, at the same time, the colonization of other cave microorganisms [37]. As a consequence, the alteration of the natural environmental conditions in caves may also modify the cyanobacteria communities. Hobbs et al. [38] demonstrated that lampenflora does not grow at close distance from incandescent lights due to high temperature. However, the artificial illumination also influences the water content of the substrate and air. Tourist presence leads to the increase of both temperature and CO_2 concentration inside cave [15], intensifying wall erosion [39]. Cyanobacteria communities in caves are mainly composed of cavernicolous species, generally characterized by small size, resistance to desiccation, specific preferences for pH, and tolerating low nutrient levels and high conductivity. Saiz-Jimenez [40] showed that cave environment is in a constant battle to remove cyanobacteria and lint left by visitors without damaging underlying formations. Bright artificial light installed in caves for the benefits of visitors may adversely effect on drying out surfaces and decreasing relative humidity, which may be lethal to cave adapted microorganisms. Moist, humid conditions favor the growth of species on soils and rocks, for instance, *Nostoc commune* colonies which typically grow on calcareous soils or depressions on limestone surfaces. In limestone caves, cyanobacteria can be found in water bodies [41] and in subaerophytic karst habitats [42]. Cavernicolous cyanobacteria can be observed in the cave entrance illuminated by direct or indirect sunlight and caves equipped with artificial illumination, as a part of a lampenflora community around lamps [12].

Limestone caves are under immense pressure from anthropogenic activities, especially in recent years [9], and are probably one of the centers of biodiversity for certain types of cyanobacteria [2], especially for species from families Hapalosiphonaceae and Symphyonemataceae [43].

This high diversity may partly be caused by the lack of photosynthetically active radiation which is almost the only stressor in caves, whereas subaerophytic habitats are significantly affected by many stress factors such as excessive irradiance, UV, desiccation, rapid temperature change, and their combinations.

It is well known that cyanobacteria are considered the pioneering inhabitants in the caves colonization. Cyanobacteria prevail in the cave entrances compared to the other microalgae [39]; however, they colonize the various parts of the cave entrances, where biodiversity of organisms is the lowest [22]. Among many factors influencing cavernicolous species, water relations in caves are important for cyanobacteria to growth and their colonization [44]. Lamprinou et al. [4] stated that cavernicolous species are dominated by cyanobacteria, which represent the first photosynthetic colonizers on the calcareous surfaces usually thriving both as epiliths and as endoliths. Epilithic communities form extensive dark-green coverings created by *Phormidium breve* as dominant species, or pale blue-green to whitish coverings consisting *Tolypothrix epilithica*. Lamprinou et al. [30] observed predominance of Oscillatoriales group over Chroococcales, in the dim light zone, and also in the entrance, especially in speleothems exposed to light, but their presence is attributed to the chasmoendolithic mode of life.

Round [45] differentiated the distribution of microorganisms depending on the access of either natural or artificial light. Growing of cyanobacteria visible in the form of different color patches on cave walls is undoubtedly connected with the availability of light and specific limestone cave microclimate. This microclimate in caves is influenced by air circulation, hydrological conditions, and isolation of cave from the outside thermal influences [21]. Microscopic observations [21] revealed that cyanobacteria are arranged in particular communities named patinas, which are blue, brown, green, or gray. These communities contain coccoid forms that are frequently accompanied by filamentous forms that are irregularly distributed and do not present stratification. Generally, there are two different areas of the caves. One area is the entrance, where the microclimate is influenced by the light, temperature, and relative humidity fluctuating throughout the year. Patina is greenish-bluish formed by coccoid species only, and there are also grayish patina constituted by coccoid and filamentous species. The second area is the inside with a stable temperature and relative humidity and very low light. The patina found there are greenish-bluish formed by only coccoid species, brownish-gray patina constituted by coccoid forms and filamentous forms, and bluish-grayish patina formed by coccoid forms and filamentous forms. On the other hand, the cave tourism is an important factor causing increase of temperature and environmental changes. Poulíčková and Hašler [11] observed the majority caves in Europe are characterized by an average humidity (circ. 70%), and their entrance walls usually are covered by cyanobacteria. The development of cave tourism requires alteration of natural corridors, installation of lighting, pathways, platforms, and associated infrastructure [46]. On the other hand, caves impacted by severe disturbances, including tourism and artificial illumination, have never been completely restored to their former ecological state [47]. Under such conditions, the oligotrophic nature of cave environments is expected to change through organic inputs that alter both the food web and the abundance and distribution of cave organisms [40].

Pentecost and Zhang [48] and Uher and Kovacik [49] noted that type of substratum is an important factor determining the species composition, distribution, and structure of cavernicolous species. They observed that growth of cyanobacteria such as *Anabaena oscillarioides, Gloeocapsa biformis,* and *Nostoc punctiforme* was dependent on the temperature, light, and humidity. These cyanobacteria prefer the humid places during their development, but they also display a considerable resistance to drying as well as to a low air temperature during winter. The adaptation mechanism of cyanobacteria living in a low temperature is not yet precisely known [39], but *Scytonema julianum* is reported as an atmophytic cyanobacterium grown in limestone cave walls in small clusters in shaded vadose settings throughout the world and is prone to rapid calcification [50].

The cyanobacteria species distribution in relation to cave morphology, lithic substrate, and microclimatic conditions still remain a challenge for further studies.

4. Conclusions

An investigation of the diversity and ecology of cyanobacteria in limestone caves has been conducted for many years. Cyanobacteria were the dominant group of phototrophs colonizing cave walls. Chroococcales was the most common cyanobacterial order (with *Gloeocapsa* as the most frequently encountered cyanobacterial genus), followed by Nostocales. The most widespread and abundant species were *Aphanocapsa muscicola, Gloeocapsa biformis,* and *Nostoc commune.* Caves impacted by severe disturbances, including tourism and artificial illumination, were never been completely restored to their former ecological state [47]. Principally, every visitor entering a cave, from the professional speleologist to tourists, has the potential to exert a negative impact on the cave ecosystem.

Author details

Czerwik-Marcinkowska Joanna* and Massalski Andrzej

*Address all correspondence to: marcinko@kielce.com.pl

Department of Botany, Institute of Biology, Jan Kochanowski University, Kielce, Poland

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