CHARACTERIZATION OF AEROPHYTIC OXYPHOTOTROPHS ISOLATED FROM GUA TEMPURUNG, MALAYSIA: POLYPHASIC AND BIOCHEMICAL APPROACHES

ZULAIHAT HAMZA ABDULLAHI

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ZULAIHAT HAMZA ABDULLAHI

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LIST OF ABBREVIATIONS AND SYMBOLS

EPS	Extracellular polymeric substances
CCA	Complementary chromatic adaptation
ML	Maximum likelihood
BI	Bayesian inference
Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
PBP	Phycobiliprotein
PC	Phycocyanin
APC	Allophycocyanin
PE	Phycoerythrin
BLAST	Basic local alignment search tool
PCR	Polymerase chain reaction
WGS	Whole genome sequence
QC	Quality control
CRISPR	Clustered regularly short palindromic repeats
TEM	Transmission Electron Microscopy
°C	Degree centigrade
psi	Pounds per square inch
h	hour
d	day
mL	Milliliter
H’	Shannon-Weiner diversity index
D	Margalef’s richness index
µmol	Micromole
%	Percentage
pH	Potential hydrogen
min	minute
µm	micrometer
µl	Microliter
mg/ml	Milligram per milliliter
rpm	Revolution per minute

s	second
g	gram
Kb	Kilobyte
bp	Base pair
cell mL^{-1}	Cell per milliliter
mg	milligram
nm	Nanometer

LIST OF APPENDICES

PENCIRIAN OKSIFOTOTROFIK AEROFIT YANG DIPENCILKAN DARI GUA TEMPURUNG, MALAYSIA: PENDEKATAN BERFASA DAN BIOKIMIA

ABSTRAK

Gua adalah antara persekitaran oligotropik yang ekstrem dengan nutrisi yang terhad dan keamatan cahaya yang rendah, tetapi sesetengah mikroalga aerofit mendapati persekitaran ini sesuai untuk pertumbuhan dan kemandirian mereka. Gua Tempurung adalah gua batu kapur terbesar di Malaysia, tetapi malangnya tiada sebarang kajian dijalankan ke atas kepelbagaian alga, maklumat taksonomi, penyesuaian, komposisi fisiologi dan biokimianya. Kajian ini dijalankan bertujuan untuk menilai ciri dan penyesuaian oksifototrof aerofitik yang diasingkan dari Gua Tempurung. Mikroalga aerofitik telah disampelkan dari pelbagai zon cahaya di dalam Gua Tempurung. Sampel ini dikultur di dalam media tertentu untuk menggalakkan pertumbuhan pelbagai jenis mikroalga. Parameter alam sekitar seperti kelembapan, keamatan cahaya dan suhu diukur secara in-situ. Tambahan pula, kajian ini menggunakan pendekatan polifasa untuk mencirikan secara menyeluruh strain alga yang dipilih. Sebanyak 25 morfospesis mikroalga aerofitik yang berbeza di Gua Tempurung, telah dikenalpasti melalui kajian ini. Sianobakteria adalah Kumpulan dominan, dengan 14 morfospesis berbeza, dan Stesen 1 menunjukkan kepelbagaian tertinggi dengan 19 morfospesis. Antara parameter alam sekitar yang dikaji, variasi cahaya memberi kesan kepada kepelbagaian mikroalga di empat stesen pensampelan. Strain alga terpilih, strain *Chroococcidiopsis thermalis* ZHB 1 dan *Stichococcus bacillaris* WMOUSM 1, telah diklasifikasi menggunakan analisis genetik penjujukan gen 16S rRNA dan 18S rRNA masing-masing. Strain *C. thermalis* ZHB 1 didapati secara genetik bersamaan dengan *Chroococcidiopsis* sp., manakala strain *S. bacillaris* WMOUSM 1 adalah bersamaan dengan *Tetratostichococcus* sp. Kajian ini juga mengkaji tindak balas biokimia dan fisiologi strain ini di bawah keadaan pertumbuhan yang berbeza, termasuk keamatan cahaya, tahap nitrogen, dan fotokala. Kedua-dua strain menunjukkan kadar pertumbuhan tertinggi mereka pada keamatan cahaya 25 µmol m^{-2} s^{-1}, peringkat pegun lewat, dengan kandungan nitrogen berbeza, di bawah fotokala 24L:00D. Strain *C. thermalis* ZHB 1 var non glomerorum mempunyai hasil kuantum terbaik pada keamatan cahaya 15 µmol m^{-2} s^{-1}, nitrogen sederhana, dan fotokala 8L:16D pada peringkat pegun awal. Strain *Tetratostichococcus* sp. WMOUSM 1 merekodkan hasil kuantum tertinggi pada 5 µmol m^{-2} s^{-1} keamatan cahaya, nitrogen sederhana, dan fotokala 12L:12D pada peringkat eksponen. Selain itu, strain *C. thermalis* ZHB 1 var non glomerorum mempunyai kandungan Chl *a* (1.04 mg L^{-1}) dan karotenoid (1.1 mg L^{-1}) tertinggi pada 5 µmol m^{-2} s^{-1} keamatan cahaya, nitrogen rendah, fotokala 8L:16D. Strain ini mempunyai lebih banyak fikobiliprotein dalam nitrogen tinggi, dan pada fotokala 24L:00D. Strain *S. bacillaris* mempunyai kandungan klorofil *a, b,* dan karotenoid tertinggi pada cahaya 15 µmol, nitrogen rendah, dan pada fotokala 12L:12D. Strain *C. thermalis* ZHB 1 var non glomerorum mempunyai lebih banyak kandungan karbohidrat, protein dan lipid pada keamatan cahaya 5 µmol m^{-2}s^{-1}. Walau bagaimanapun, strain *Tetratostichococcus* sp. WMOUSM 1 mempunyai paras tertinggi kandungan karbohidrat, protein dan lipid (8.66%, 9.22% dan 66.43%, masing-masing) pada fotokala 8L:16D. Analisis genom strain *C. thermalis* ZHB 1 var non glomerorum mendedahkan gen yang dikaitkan dengan toleransi pengeringan, CRISPR, dan penyesuaian tekanan, adalah penyumbang kepada kemandiriannya dalam persekitaran gua yang ekstrim. Kesimpulannya, sebanyak 25 alga aerofitik ditemui di empat stesen pensampelan. Strain *C. thermalis* ZHB 1 var non glomerorum dan strain *Tetratostichococcus* sp. WMOUSM 1 yang mana berjaya disusun dan dianalisis, menunjukkan tindak balas kepada kondisi parameter yang berbeza. Sebilangan gen kesesuaian dan gen tekanan juga dikenal pasti dalam genom *C. thermalis*. Penemuan kajian ini memberikan maklumat asas yang penting tentang kepelbagaian mikroalga aerofitik di Gua Tempurung, taburan, tindak balas, dan penyesuaiannya yang menyumbang kepada pemahaman penerokaan alga di dalam gua, pertumbuhan, dan toleransi terhadap habitat gua yang ekstrim. Kajian ini telah mendedahkan kehadiran 25 alga aerofitik di empat stesen pengambilan sampel dalam Gua Tempurung. Penjujukan yang berjaya dan analisis terhadap strain *Tetratostichococcus* sp. WMOUSM 1 dan strain *C. thermalis* ZHB 1 var non glomerorum telah Responsif menonjolkan kepekaan mereka terhadap pelbagai keadaan alam sekitar. Selain itu, pengenalpastian gen adaptif dan tekanan dalam genom strain *C. thermalis* ZHB 1 var non glomerorum menambah pandangan yang ketara. Penemuan ini berperanan sebagai maklumat asas untuk memahami kepelbagaian, penyebaran, tindak balas, dan penyesuaian alga mikro aerofitik di Gua Tempurung. Ia memberikan sumbangan yang besar kepada pengetahuan kita tentang bagaimana alga-alga ini menjalani proses penempatan, pertumbuhan, dan perkembangan dalam persekitaran gua yang ekstrim.

CHARACTERIZATION OF AEROPHYTIC OXYPHOTOTROPHS ISOLATED FROM GUA TEMPURUNG, MALAYSIA: POLYPHASIC AND BIOCHEMICAL APPROACHES

ABSTRACT

Caves are among the extreme oligotrophic environments with limited nutrients and low light intensity, but some aerophytic microalgae find this environment suitable for their growth and survival. Gua Tempurung is the largest limestone caves in Malaysia, but unfortunately no study was conducted on its algal diversity, taxonomic information, adaptation, physiological, and biochemical composition. This study aimed to assess the characteristics and adaptation of aerophytic oxyphototrophs isolated from Gua Tempurung. Aerophytic microalgae were collected from various light zones within Gua Tempurung. These samples were subjected to culture in specific media to promote the growth of different types of microalgae. Environmental parameters like humidity, light intensity, and temperature were measured *in-situ*. Furthermore, the study employed a polyphasic approach to thoroughly characterize the selected algal strains. This study identified 25 different morphospecies of aerophytic microalgae in Gua Tempurung. Cyanobacteria were the dominant group, with 14 different morphospecies, and Station 1 showed the highest diversity with 19 morphospecies. Among the environmental parameters studied, light variation impacts microalgal diversity at four sampling stations. These indices were found to be highest at the entrance of the cave. The selected algal strains, *Stichococcus bacillaris* WMOUSM 1 and *Chroococcidiopsis thermalis* ZHB 1, were characterized using genetic analysis of 16S rRNA and 18S rRNA genes sequencing, respectively. Strain *C. thermalis* ZHB 1 was found to be genetically similar to *Chroococcidiopsis* sp., while strain *S. bacillaris* WMOUSM 1 was similar to *Tetratostichococcus* sp. The study examined the biochemical and physiological responses of these strains under different growth conditions, including light intensity, nitrogen levels, and photoperiod. Both strains showed their highest growth rates at 25 µmol m^{-2} s^{-1} light intensity, late stationary stage, with different nitrogen, under 24L:00D photoperiod. Strain *C. thermalis* ZHB 1 var non glomerorum had its best quantum yield at 15 µmol m^{-2} s^{-1} light intensity, medium nitrogen, 8L:16D photoperiod at early stationary stage. Strain *Tetratostichococcus* sp. WMOUSM 1 recorded highest quantum yield at 5 µmol m^{-2} s^{-1} light intensity, medium nutrients, 12L:12D photoperiod at exponential stage. Additionally, strain *C. thermalis* ZHB 1 var non glomerorum had the highest Chl *a* (1.04 mg L^{-1}) and carotenoid (1.1 mg L^{-1}) at 5 µmol m^{-2} s^{-1} light intensity, low nitrogen, 8L:16D photoperiod. The strain had more phycobiliproteins in high nitrogen, at 24L:00D photoperiod. Strain *Tetratostichococcus* sp. had highest chlorophylls *a, b,* and carotenoid at 15 µmol light, low nitrogen, at 12L:12D photoperiod. Strain *C. thermalis* ZHB 1 var non glomerorum had more carbohydrates, proteins, and lipids at 5 µmol $m^{-2}s^{-1}$ light intensity. However, strain *Tetratostichococcus* sp. WMOUSM 1 had the highest levels of carbohydrates, proteins, and lipids (8.66%, 9.22%, and 66.43%, respectively) at 8L:16D photoperiod. Genomic analysis of strain *C. thermalis* ZHB 1 var non glomerorum revealed genes associated with desiccation tolerance, CRISPRs, and stress adaptation, which contributed to its survival in the cave's extreme environment. This study has revealed the presence of 25 aerophytic algae at the four sampling stations within Gua Tempurung. Successful sequencing and analysis of strain *Tetratostichococcus* sp. WMOUSM 1 and strain *C. thermalis* ZHB 1 var non glomerorum have highlighted their responsiveness to various environmental conditions. Additionally, the identification of adapted and stress genes in the genome of strain *C. thermalis* ZHB 1 var non glomerorum adds significant insights. These findings serve as fundamental information for understanding the diversity, distribution, responses, and adaptation of aerophytic microalgae in Gua Tempurung. They contribute significantly to our knowledge of how these algae colonize, grow, and thrive in the extreme cave environment.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Caves are the biodiversity centres for different types of microorganisms, with highly specific environments, scattered all over the world and considered specific cases of the extreme environment (Roldán & Hernández-Mariné, 2009). They are considered as an extreme environment for autotrophs that need light for photosynthesis. These extremophilic phototrophs comprise eukaryotes (various types of algae) and prokaryotes i.e. cyanobacteria (Seckbach & Oren, 2007a). Oxygenic phototrophic microorganisms are found abundantly in environmental extremes of pH, radiation, salt concentration, and temperature. Several extremophilic phototrophs are greatly resistant to radiation. They are dominant in structural diversified photosynthetic biofilms dwelling in a low-illuminated environment. Possession of oxyphototrophic type of photosynthesis in algae makes them to be one of the main primary colonizers, which are adapted to live in extreme natural conditions in terrestrial environments (Chen *et al.*, 2020). These phototrophs are not necessarily photosynthetic because some of them are heterotrophs. Most of them are found in caves, both under artificial and natural light illuminations, which are affected by biological, physical, and chemical conditions (Lamprinou *et al.*, 2009; Roldán & Hernández-Mariné, 2009). Thus, aerophytic algae and cyanobacteria occupying the lithophytic habitats of these localities offer a wealth of information, on their survival and adaptations, characterized by the fragile dynamic balance linked to environmental factors. Research on algae and cyanobacteria has been mostly directed to examine the isolated strains from the water system; however, recently, the importance has been moved to organisms that do not primarily depend on the aquatic environment for their reproduction and survival. Additionally, some terrestrial cyanobacteria extracts have been found to have greater biological activities (Patterson *et al.,* 1994; Reisser, 2000).

Algae and cyanobacteria play a significant role in the cave ecosystem such as nitrogen fixers, colonizers, deterioration agents, or prey for micro grazers. They are responsible for the erosion of the stone substrate and the production of pigments that cause the colour effects on rocky cave walls. They also serve as a source of food for animals (Grobbelaar, 2000; Joanna & Mrozińska, 2009). The production of the extracellular sheath by cyanobacteria plays a key role in adhesion to the substratum and acts as a water reservoir, thus enabling the cyanobacteria to survive the drought period (Keshari & Adhikary, 2013; Macedo *et al.,* 2009). This extracellular sheath known as extracellular polymeric substances (EPS) and scytonemin (UV absorbing component) allow some microalgae to protect themselves from desiccation and UV radiation and protect other taxa that may not exhibit those features (Pattanaik *et al.,* 2007). Aerophytic algae that inhabit calcareous rock can deposit crystals of calcium carbonate ($CaCO_3$) in their sheaths (Poulíčková & Hašler, 2007). These autotrophic algae are also important part of the cave ecosystem as they are essential for ecological succession in the most external cave areas. They secrete pigments, acids, and other secondary metabolites which cause the degradation and deterioration of the rock. They also provide oxygen to heterotrophic organisms while reducing carbon dioxide and other substances (Kosznik-kwaśnicka *et al.,* 2022).

The presence of seeping or running water in caves also accelerates the growth of aerophytic algae, because water is one of the essential factors that influence colonization and growth of aerophytic algae. They have developed a variety of strategies to deal with water scarcity and humidity. They have also possessed a colourful strategy known as complementary chromatic adaptation (CCA). During chromatic adaptation, cyanobacteria change their pigment composition and thereby their colour (Kehoe & Gutu, 2006). The sheaths of their colour appeared, because of the presence of pigments which act as a filter to diminish the amount of incident light (Krumbein *et al.,* 1978). This complementary adaptation optimizes light absorption and thus favours their photosynthesis and growth.

1.2 Statement of research problem

Natural caves are characterized by low nutrient and natural light input (Simon *et al.*, 2007). This condition change with the introduction of artificial light that directly and indirectly influence the cave ecosystem and its fauna and flora. There are a lot of data sets on algal flora in caves from many countries located in different continents, as they play a lot of roles in nitrogen fixation, colonization and causes erosion of the stone substrate. However, no information is available about algal communities in Gua Tempurung, Malaysia, and even less about its environmental conditions.

Provision of artificial light for cave tourism adversely change the natural condition of the cave, thereby decrease its relative humidity and at the same time increases the temperature, which may be dangerous to the cave-adapted microorganisms (Janez & Gorazd, 2009). Due to the harsh conditions of the cave, the diversity of aerophytic algae may be lower inside the cave in comparison to those growing naturally outside. Thus, this unique habitat of a cave with low nutrients and light intensity, uniform temperature, and high relative humidity that may harbour wealth of information on the occurrence and survival of aerophytic algae is worth exploring.

In addition to the distribution of aerophytic algae in Gua Tempurung, no study has been conducted to determine the taxonomic information base on DNA sequencing, pigment extraction, and morphological adaptation in the cave. Also, there is no study on the growth rate, physiological and biochemical compositions of aerophytic algae in Gua Tempurung. Therefore, increasing our knowledge on the phylogeny, ecology, the morphological and biochemical status of the aerophytic algae in the cave will help us determine how aerophytic algae respond and adapt to survive in Gua Tempurung.

1.3 Aim and objectives

This research aims to assess the characteristics and adaptations of aerophytic oxyphototrophs isolated from Gua Tempurung, Malaysia.

The objectives are as follows:

i. To determine the diversity and compositions of aerophytic algae in Gua Tempurung.
ii. To characterise *Chroococcidiopsis thermalis* ZHB 1 AND *Stichococcus bacillaris* WMOUSM 1 using a polyphasic approach.
iii. To investigate the response of strain *Chroococcidiopsis thermalis* ZHB 1 var non glomerorum and strain *Tetratostichococcus* sp. WMOUSM 1 towards various environmental conditions (light intensity, photoperiod and nitrogen concentration) based on their biochemical and physiological states.
iv. To identify the gene responsible for the survival of strain *Chroococcidiopsis thermalis* ZHB 1 var non glomerorum under extreme conditions of the cave.

1.4 Research question

i. How diverse are the species of aerophytic algae surviving in the cave?
ii. Is there any variation in the community structure of aerophytic algae across different zones of the cave?
iii. To what extent do the isolated algal strains tolerate the extreme environmental factors?
iv. Are there any gene of aerophytic algae influence their adaptation and survival in the cave environment?

1.5 Research hypothesis

i. There is a difference in terms of aerophytic algae community structure across different zones of the cave.
ii. Extreme environmental factors (light intensity, temperature, nutrient, and relative humidity) affect the adaptation of the algal strains in the cave.
iii. Genes of aerophytic algae influence their survival and adaptation in the cave.

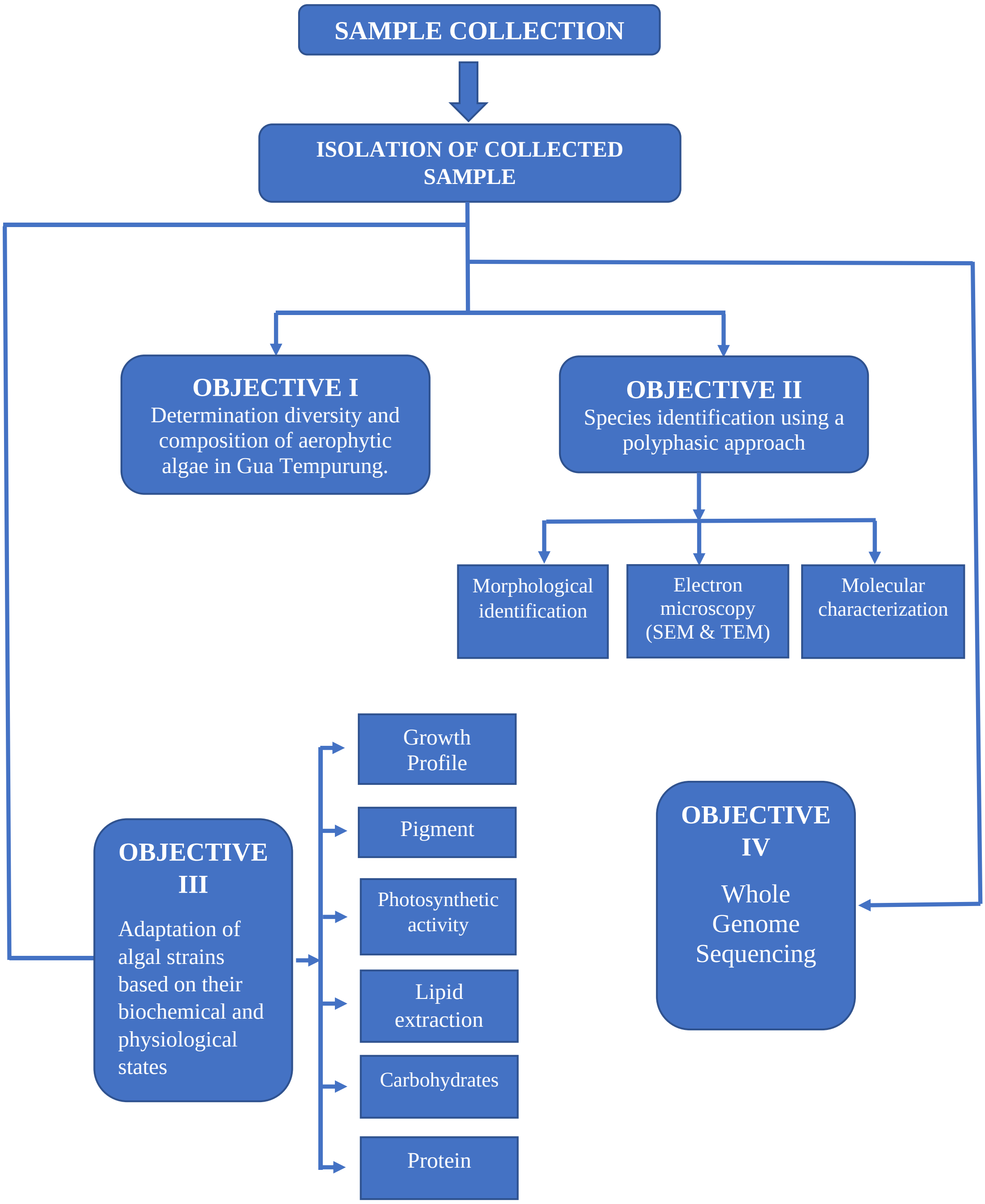

Figure 1.1 Flow of ideas aim for this research.

1.6 Thesis structure

‘Why aerophytic algae in an extreme cave habitat are important and what makes them survived in this extreme environment?’ serves as the primary focus of this thesis This study extensively analyzed the morphological, molecular, and physiological characteristics of strain *Chroococcidiopsis thermalis* ZHB 1 and strain *Stichococcus bacillaris* WMOUSM 1.

Chapter 2 provides a reviewed background literature for the described studies.

Chapter 3 Introduces and describes the locations where samples were collected, along with the procedures for sample collection, isolation, and maintenance.

Chapter 4 described the environmental parameters and species diversity in the four distinct zones of Gua Tempurung, utilizing electron microscopy and identification references.

Chapter 5 briefly outlines the process of identifying selected algal strains through a polyphasic approach, which integrates both morphological and molecular characterization, alongside an examination of ultrastructural features. The analyses confirm identities of the selected algal strains (strain *Chroococcidiopsis thermalis* ZHB 1 and strain *Stichococcus bacillaris* WMOUSM 1) at species level.

Chapter 6 examined the responses biochemical and physiological state of strain *Chroococcidiopsis thermalis* ZHB 1 and strain *Stichococcus bacillaris* WMOUSM 1 at different growth stages under various environmental conditions such as light intensity, nutrient concentration, and photoperiod. The results obtained answer the question “to what extent do the isolated algal strains tolerate the extreme environmental factors.

Chapter 7 identified and described the genes responsible for survival of strain *Chroococcidiopsis thermalis* ZHB 1 through whole genome sequencing. The findings answer the question “Are there any gene of aerophytic algae influence their adaptation and survival in the cave environment”.

Chapter 8 and 9 provide a comprehensive discussion and conclusions that integrate various components of this research, placing them within a broader context.

CHAPTER 2

LITERATURE REVIEW

2.1 Aerophytic algae

Aerophytic algae represent a major ecological group that includes the highly specialized lithobionts (hypoliths, epi-, and endo) as well as the soil crust and bark dweller (Hauer *et al.*, 2015). They are known to colonize different substrata such as rocks, buildings, bark, wood etc., and as epiphytes on living organisms on shrubs, leaves of trees (Hanus Ettl & Gärtner, 1995). These oxygenic phototrophic microorganisms, both eukaryotic and prokaryotic (cyanobacteria) can also be found in extreme environments. They occur both at extremely low and high temperatures. Some of the phototrophs have occupied strongly acidic, hypersaline waters or alkaline habitats, and some extremophilic phototrophs are highly resistant to radiations (Seckbach & Oren, 2007a).

Aerophytic algae, occur in caves, rocks, and limestone walls, at the entrance in which the growth conditions are best for their survival (Mulec *et al.*, 2008). They also play an important role in the tropic network processes and colonization of rocky habitats where they produce colourful patches on the cave walls (Mulec *et al.*, 2008). Algae inhabit terrestrial environment as microbiotic crust, subaerial algae, terrestrial algae, cryptogrammic crusts, and aerophytic algae (Lemes-Da-Silva *et al.*, 2010). The majority of aerophytic algae spend most of their life out of aquatic habitat, but their reproduction stages cannot be complete without water (Lewis & McCourt, 2004). Aerophytic algae in the cave are observed usually by indirect or direct illumination of light which is found around the artificial light available to tourists (Czerwik-Marcinkowska & Mrozińska, 2011; Mulec *et al.*, 2008).

In algal evolution, phycologists have advocated the use of phylogenetic species concepts, in which species are the smallest group of organisms that are separate and share a unique combination of state character (Nixon & Wheeler, 1990). Physiological and molecular studies, demonstrate several morphological characters of high plasticity which are used in the delimitation of species. The presence of pyrenoid has been used to distinguish green algal species and genera (Ettl, 1983). They also manufacture scytonemin, which refers to as a pigment that provides protection against solar ultraviolet (UV) radiation, short wavelength and increases their suitability in a comparatively exposed terrestrial environment (Rastogi *et al.,* 2015).

Generally, the presence of seeping and running water accelerates the growth of plants in caves (Janez & Gorazd, 2009). Another important ecological parameter that affects algal growth is the type of substratum and the presence of sediment (Chang & Chang-Schneider, 1991). Algae found around light (i.e., lampenflora) can reproduce, survive in low light intensities than the compensation point of photosynthesis (i.e., the amount of light intensity at which CO_2 is fixed in sugars during photosynthesis is equal to the amount of CO_2 that is released during respiration) (Janez & Gorazd, 2009).

2.2 Show cave

In ancient times, our ancestors have a special bond with caves. Caves were primarily used as a permanent home or a temporary refuge mainly for practical purposes (Gillieson, 2021). They also used it as a source of minerals, water and even as places of worship or burial ground (Germinario *et al.,* 2020). From the lower Pleistocene era to the present days, evidence of their early occurrence can be seen in various caves where human remains are found (Mazza *et al.,* 2022). These caves have an outstanding cultural and natural importance, such as the Mammoth Cave National

Park (Kentucky, USA) (Niemiller *et al.,* 2021), Las Manos Cave (Argentina), and the Škocjan Caves (Slovenia) (Estévez *et al.,* 2019). These caves are compiled by the United Nations Educational Scientific and Cultural Organization (UNESCO) on the list of World Heritage sites (Estévez *et al.,* 2019). Caves have become a successful tourist attraction, due to their cultural content, geological formations of their natural beauty, and their historical background. In some cases, they also constitute prominent scientific resources, as many contain valuable geological records and paleo environmental information, as in the case of Nerja Cave in Spain (Estévez *et al.,* 2019) and Tempurung cave in Malaysia (Muhammad, 2010).

Show cave can be defined as "any opening where payment is given to visit and gain access to it" it was earlier originated in the Vilenica Cave, Slovenia in 17th century, which is the first world show cave recognized in 1633 (Chiarini *et al.,* 2022; Mulec *et al.,* 2008). Presently, almost every country has at least one show cave. It was predicted that there are over 250 million visitors in a year, with almost 500 larger show caves distributed worldwide (Gessert *et al.,* 2018). Also, around 100 million people who get direct or indirect income from related activities around the show caves area such as accommodation, and transportation (Estévez *et al.,* 2019). It also provides income from health care, speleotourism, marketing and religious activities, as in the case of Batu cave in Malaysia (Paniandi *et al.,* 2018). In certain areas, increase in tourists represent the main source of income and become important economic resources for its inhabitants (Epuran *et al.,* 2021; Mulec *et al.,* 2008). For centuries, "underground tourism" was done in a very simple way using oil lamps as a source of light (Estévez *et al.,* 2019). Some caves where fashioned incorporated gradually with a piece of lighting equipment, an artificial entrance from the outside, and marked out a safer way on their inside, these actions improved the enjoyment and observation of the caves, which also represent a very significant change in the conditions in a natural environment (Estévez *et al.*, 2019). Provision of light to areas that are in total darkness for years as in the case of Las Maravillas Cave in Spain for example, decreases the relative humidity and increases the environmental temperature in the interior parts of the cave (Giordano *et al.*, 2005).

Conservation of Paleolithic paintings in caves is of great interest because they represent cultural heritage for all humankind (Dans & González, 2018). Cave paintings are a unique illustration of humans' close association with caves, of which some of the most remarkable are illustrative sites such as the Altamira Cave and Lascaux Cave (Spain) (Lawson, 2012). Niah cave in northern Sarawak, Malaysia, is one of the painted caves whose popularity is due to the ancient rock painting and natural attractions from stalactites and stalagmites (Gabriel & Northup, 2013). A large variety of organisms may be exploited in open site painting. They make use of a large variety of inorganic and organic components of the substrate, which are combined and accumulated on the painted surface with other environmental contaminants and dirt. Depending on the environmental conditions of the site, specific microbial communities developed on the capacity of the substratum, which provides different ecological niches (Ciferri, 1999).

Algae and Cyanobacteria growing on paintings are exposed to light such as facades on the frescoes of buildings that may cause considerable damage (Ciferri, 1999). Grobbelaar, (2000) identified *Chlorella* sp (Chlorophyta) in the carbonated formations in the Congo cave (South Africa). Autotrophic microflora, which is composed of algae and cyanobacteria, were mainly found in paintings higher portions, and is characterized by the presence of large porosity of cavities and fissures (Zucconi *et al.*, 2012).

Trentepohlia sp. and *Stichococcus* sp. are known to be among the most abundant genera of green algae in caves. It has been reported that *Trentepohlia* sp. is responsible for serious discoloration on surfaces and paintings (Nowicka-Krawczyk *et al.*, 2022). Algal microflora was mixed with cyanobacteria *Nostoc* sp. and *Chroococcus* sp. in humid cavities. These two genera were reported to be among the most widespread contributing to the decay of historic caves (Macedo *et al.*, 2009).

Plate 2.1 The stalactites and stalagmites ((a) and (b), respectively), and limestone rocks ((c) and (d)) in Gua Tempurung

2.3 Speleological installation and lighting system

The level of adaptation of autotrophic in a show cave varies depending on the type of tourism practiced and the actions undertaken (Giraud & Fleischman, 2004). The impacts tend to be greater in caves that are adapted for general tourism, than in caves adapted totally for the practice of speleotourism and scientific activities, with restricted groups of participants who only require a minimal portable lighting system and a speleological installation. "Speleology" is the study or exploration of caves. The caves which are firstly equipped with electric lighting systems (Borderie *et al.,* 2015) were the Luray Caverns cave in Virginia, the USA in 1881, followed by Kraushöhle cave in Austrian 1883 and then the Postojna Cave in Slovenia in 1884. Soon, As reported by Aley (2004), this phenomenon was firstly studied by Dalby an Austrian scientist in 1966 and Kermode a French scientist in 1975 and it was not until the 1960s when primary initiated the word "lampenflora" (which is initially a German term coined in English vocabulary) meaning "the flora of the lamps" (Aley, 2004). This word lampenflora is presently used internationally to recognize this phenomenon, however, in French, it is also known as "maladieverte" or "mal verde" in Spanish (Mulec *et al.,* 2008).

The problems of lampenflora in show caves are rarely overlooked, where economic interests occasionally take priority over management (Estévez *et al.,* 2019; Alam *et al.,* 2001). It was observed that the regular installation of sources of artificial lighting inside the caves promoted the proliferation of a complex community and development of phototrophic organisms near the lamps (Borderie *et al.,* 2015). In some caves for example as in the case in the karstic caves of the bay of Ha Long (Vietnam) or Natural Bridge Caverns (Texas, USA) (Smith & Olson, 2007). Moreover, lampenflora causes a serious problem, because it constitutes an opportunistic and invasive community in anthropized underground environments (Bontemps *et al.,* 2022). Except for the cave entrance, these organisms grow in places where they would not occur naturally, therefore, they use artificial light to develop (Janez & Gorazd, 2009). These, new dwellers of the caves that have grown in artificial lighting successfully compete to inhabit this ecological niche (Mulec, 2019). In many cases, the problem is aggravated because the places of the greatest attention to tourists (high-value geological formations or cave paintings) tend to be more illuminated than the rest parts of the cave (Estévez *et al.*, 2019). These areas seem to increase their attractiveness to the public, but they are more prone to be colonized by lampenflora. Numerous works have been published in recent decades on lampenflora communities, and on the methods that have been used to control and prevent their growth in caves.

Plate 2.2 Gua Tempurung (a) Installation of artificial light (b) growth of lampenflora around artificial lamp

2.4 Colonization and development of biofilms

Cyanobacteria and algae are the most common photosynthetic organisms identified in caves, with some elements may also form part of the lampenflora communities which include lichens, mosses, and sometimes even higher plants and ferns (Mulec, 2018; Nugari *et al.*, 2009). In the early stages of colonization, eukaryotic algae and cyanobacteria tend to play the most significant role in the processes of forming the biofilm and are the original species in the ecological succession (Estévez *et al.*, 2019; Gaylarde & Gaylarde, 2000; Mulec *et al.*, 2008).

From a metabolic point of view, the presence of cyanobacteria in lampenflora communities is very important. These are the photosynthetic microorganisms that grow in illuminated underground environments most successfully; and they require no organic matter, with the presence of accessory pigments that can absorb a wide spectrum of light radiation (Barresi & Palla, 2017). These qualities present a significant improvement in the colonization of new areas (Hebelka, 2014). However, this group of algae may survive independently in nature, but most of the microorganisms tend to form multicellular communities known as “biofilms”. Biofilms function in CO_2 and nitrogen fixation and their photosynthetic capacity (Miyauchi *et al.,* 2020). Biofilms stimulate the growth of ferns and mosses in the area around them, which also benefit from environment enriched in nutrients and organic matter, and in turn promote the proliferation of some heterotrophic microorganisms (Casamayor *et al.,* 2008; Garofano & Govoni, 2012). In the case of caves that are adapted for tourism other elements of organic nature, serve as a source of nutrients such detritus (dust from shoes, hairs, and dry skin) and fluffs which are also introduced by visitors (Aley, 2004; Constantin *et al.,* 2021). Simon *et al.* (2007) reported that between 95% - 99% of microorganisms live in the form of biofilms in the natural environment which signifies a privileged lifestyle for most microorganisms.

Biofilms may compose of complex biocoenoses, in which algae and cyanobacteria coexist with yeasts, bacteria, and fungi (Pfendler *et al.,* 2018). Danin & Caneva (1990) reported that biofilms with the highest diversity are more resistant to unfavourable external conditions. Chemically, they are mainly composed of water (approx. 70-90%), organisms with a hydrated matrix of extracellular polymeric substances (EPS) and a diverse metabolism that they separate and composed of enzymes, lipopolysaccharides, polysaccharides, glycoproteins, proteins, fatty acids, lipids, and glycolipids (Faimon *et al.,* 2003). This matrix confers a series of advantages, which help in increasing the resistance of its members to external agents, the survival of the community in hostile environments, or other organisms. These benefits are of greater protection, against sources of environmental stress (exposure to UV rays or desiccation processes, atmospheric contaminants, and heavy metals), enhanced circulation and concentration of nutrients, and greater water retention within the biofilm. It has been demonstrated that these single-cell microorganisms use a process of intracellular communication through chemical signals call "quorum sensing", which makes them act in a coordinated way as multicellular organisms (Costerton *et al.,* 1999; Hebelka, 2014).

2.5 Morphological phylogeny of aerophytes

The algal taxonomy is based on a polyphasic approach which includes morphological characters (e.g., thallus morphology, polarity, and cell division, and branching type), ecological parameters, molecular data, and ultrastructural arrangement, are combined to describe the groups. Complex assessment of molecular sequencing and morphological variability, of algal samples have related problems to their artificial cultivation (e.g., low competitive ability under artificial conditions before isolation, environmental exigencies not understood and slow growth). The studies of true branched cyanobacteria taxonomy are not abundant. The knowledge of these organisms is devoted to few papers which include some genera (e.g. *Fischerella*, *Capsosira*, *Westiellopsis*, *Nostochopsis*, *Chlorogloeopsis*, and *Stigonema*) with the number of species reduce, about the populations mainly from the temperate regions, and is considered insufficient to resolve the evolutionary history of the true branched cyanobacteria (Glime, 2007).

Over the last 20 years, taxonomic studies of cyanobacteria based on a polyphasic approach have revealed a much greater genetic diversity than previously estimated, and many recently described genera would have been mistakenly identified as members of traditional genera (e.g., *Phormidium, Lyngbya,* and *Leptolyngbya*), if only evaluated on their morpho-anatomical features. DNA sequences and their secondary structure, play a key role in identifying cyanobacterial taxa and resolving their phylogenetic relationships.

In the last decade, there has been considerable progress in understanding the different ecological interactions in various habitats. However, there are still only a few ecophysiological studies on subaerial algae and their adaptations. Environmental factors in aerophytic habitats are not always friendly to algae and do not always support the successful establishment of a phototrophic community (Glime, 2007). Recently, the intensified tourist visitation as experienced by many caves and to make the caves more attractive for visitors, artificial lighting was fixed, which changes the physicochemical conditions in the caves. Also, on the surfaces around electrical lights and at the entrance of limestone caves, algae and cyanobacteria compete for light with ferns, and bryophytes, but they are usually the only phototrophs at the deepest recesses of the caves (Round, 1981).

2.6 Identified aerophytic microalgae in caves

A cave is a place that is characterized by high humidity, low temperature, and low nutrients, which is more or less stable throughout the year (Popović *et al.*, 2020). These conditions favourable for the growth of aerophyte in suitable cave environments. The eco-physiological characteristics of lampenflora communities and their composition have been the focus of several studies (Estévez *et al.*, 2019). The most common microorganisms recognized in lampenflora communities are the golden (*Chrysophyta*) and green *(Chlorophyta*) algae together with cyanobacteria (Blatnik *et al.*, 2020; Hebelka, 2014), but their composition differ from one cave to another.

In American region, Aley (2004) compared the lampenflora composition of two different communities in the Oregon Caves National Monument and the Carlsbad Caverns National Park in New Mexico, both located in the United States of America. Oregon Caves National Monument recorded a total of 200 species, and it was estimated that 70% of species were cyanobacteria and 20% of the population were green algae. The percentage abundance was also similar in Carlsbad Caverns National Park in New Mexico, where a total of 100 species were identified, which include cyanobacteria (40%), green algae (35%) and very small percentage of golden algae.

Furthermore, in European region, several researchers conducted studies on cave aerophytic algae. In a study conducted by Mulec *et al.* (2008) the authors identified 60 algal species, with cyanobacteria being recorded from eight different underground environments in the karst region of Slovenia (i.e. two mines and six caves). The authors reported the dominance of cyanobacteria (47%), followed by green algae (30%) and 23% of golden algae. The representatives of cyanobacteria were mainly the species of *Synechocystis* sp, *Aphanocapsa muscicola*, and *Lyngbya* sp. The genera and species found in algae are mainly the green algae (*Trentepohlia aurea* and *Stichococcus bacillaris*), and golden algae (*Navicula mutica* and *Chlorocloster* sp). In a work conducted by Janez & Gorazd (2009), the study showed that around 50% of the total photosynthetic microorganisms in European caves were cyanobacteria. The most common cyanobacteria species was *Gloeocapsa sanguine*, green algae was *Stichococcus bacillaris*, and golden algae was *Aphanothececa stagnei*. Green algae like *Chlorella* have been reported in lampenflora communities in several caves around the world (Mulec, 2014; Nugari *et al.*, 2009).

In a report conducted by Pfendler *et al.* (2018) in 6 caves (1 Swiss cave and 5 caves in France) identified seven groups of eukaryote algae Trebouxiophyceae

(51.2%) which is the most abundant, followed by Eustigmatophyceae (24.6%) and Chlorophyceae (23.8%). Six orders of cyanobacteria were also identified, with Chroococcales (40.7%) being the most numerous, followed by Nostocales (38.8%), Pleurocapsales (15%), Oscillatoriales (1.6%), and Prochlorales (0.1%). The scientists also identified Phaeophyceae, Ulvophyceae, Xanthophyceae, and Klebsormidiophyceae, which accounted for only 0.4% of the total. Fistulifera (17.4%), Didymosphenia (12.8%), and Phaeodactylum (11.3%) were the most common diatoms discovered in the caves.

Garbacki *et al.,* (1999), reported in Belgian caves that phototrophic organisms consisted of *Cyanobacteria* (54%), *Chrysophyta* (30%), and *Chlorophyta* (16%). A study conducted in Slovenia cave by Mulec *et al.* (2008); Smith & Olson (2007) also reported that *Cyanobacteria* prevailed which contributed 69% of the species while *Chlorophyta* (19%) and *Crysophyta* (12%) represented the lowest part of the population. Czerwik-Marcinkowska & Mrozińska (2011) reported mass occurrence of aerophytic algae in 25 caves of the Polish Jura were *Chlorophytes* (32.9%) were dominant, followed by diatoms (11.8%), and *Xanthophyte*. Popović *et al.* (2015) reported that at Božana Cave, Serbia, the most predominant form of cyanobacteria at the sampling site with the lowest light intensity were the coccoid form, while the abundant species at the sampling site is with the highest light intensity recorded was the *Nostacles*. The phototrophs that colonized the cave walls were dominated by cyanobacteria with 29 taxa, in with *Chroococcales* (21 taxa) prevailed. The most common species of cyanobacteria documented were from the genera *Aphanocapsa, Gloeocapsa, Chroococcus,* and *Scytonema*, while the only recorded green algae on the cave walls were *Trentepohlia aurea* and *Desmococcus olivaceus.*

Also, in Asian region, similar study was conducted by Fatma *et al.* (2019) at Niah cave in Malaysia reported the growth of *Synechococcus* sp (18.78%), *Chroococcus* sp (4.94%), and *Gleocapsa* sp (12.20%) in which *Synechococcus* sp. prevailed. Another study conducted by Hajong *et al.* (2021) identified several aerophytic algae Meghalaya caves, India as shown in Table 2.1. Similar aerophytic algae were identified by a research conducted by Vuuren *et al.* (2019) in African region as also stated in Table 2.1.

Table 2.1 Some of the identified aerophytic algae in caves reported in several countries/locations.

Species	**Location**	**References**
Chlorococcum sp. *Coelastrum astroideum* *Scenedesmus abundans* *Anabaena subcylindrica* *Aphanocapsa annulata* *Aphanothece* sp. *Calothrix* sp. *Chroococcus* sp. *Leptolyngbya* sp.	Meghalaya caves, India	Hajong *et al.,* 2021
Anabaena sp. *Aphanocapsa Nägeli* *Lyngbya* sp. *Nostoc* sp. *Oscillatoria* sp. *Phormidium* sp. *Scytonema* sp. *Chlorella* sp. *Chlorococcum* sp. *Desmococcus* sp. *Stichococcus Nägeli* *Trentepohlia* sp. *Ulotrix Kützing* sp.	Skilpad and Bushmen cave, South Africa	Vuuren *et al.,* 2019
Chroococcus minor *Cyanothece aeruginosus* *Gloeocapsa aeruginosa* *Oscillatoria rupicola* *Scytonema julianum* *Chlorella miniata* *Cylindrocystis crassa*	Mammoth cave, Kentucky, USA	Smith & Olson, 2007

Desmococcus olivaceus *Trentepohlia aurea* *Aphanothece saxicola* *Chroococcus pallidus* *Gloeocapsa biformis* *Chroococcidiopsis* sp. *Nostoc commune* *Scytonema drilosiphon*	Bozana cave, Serbia	Popović *et al.*, 2015
Aphanocopsa muscicola *Chroococcidiopsis doonensis* *Chroococcus minor* *Chroococcus turgidus* *Hassalia byssoidea* *Leptolyngbya gracillima* *Phormidium* sp. *Schizothrix* sp.	Leontari cave, Greece	Lamprinou *et al.*, 2009
Desmococcus olivaceum *Leptosira terricola* *Klebsonmidium flaccidum* *Stichococcus bacillaris* *Trentepohlia aurea* *Heterococcus caespitosus* *Orthoseira roseana*	Krakowsko-Czestochowska cave, Southern Poland	Joanna & Mrozińska, 2009
Gleocapsa sp. *Chroococcus minutus* *Chroococcus turgidus* *Scytonema drilosiphon* *Oscillatoria limosa* *Phormidium* sp. *Chlorella vulgaris* *Stichococcus minor* *Navicula* sp.	Otap Head cave, Republic of Abkhazian	Popkova & Mazina, 2019

2.7 Factors affecting the growth of aerophyte in caves

Development of aerophytic vegetation is mostly influenced by environmental conditions such as temperature, light, seeping water, high relative humidity, and substratum characteristics (Chang & Chang-Schneider, 1991). Effects of these environmental conditions are briefly discussed.

2.7.1 Light

Light quantity and quality are among the most essential parameters for phototrophic organisms, as they are necessary for the regulation of several cellular processes and photosynthesis (Oldenhof *et al.,* 2006). Growth of algae under different light intensities exhibits differences in pigments, biochemical composition, ratio, and rate of biomass synthesis (Maltsev *et al.,* 2021). Algae and cyanobacteria are considered the first colonizers of various exposed environments (Falasco *et al.,* 2014; Righini *et al.,* 2022) they are phototrophic organisms commonly found in cave habitats (Mulec *et al.,* 2008; Sacco Perasso *et al.,* 2022). In show caves that are equipped with artificial illumination, aerophytic algae are simply observed around lamps as a part of a lampenflora community, they grow and colonise quickly the illuminated parts of a caves (Mulec *et al.,* 2008). The instalment of artificial light in caves for the benefit of visitors affects the drying out surface and decrease the relative humidity, which may be lethal to cave adapted microorganisms (Joanna & Andrzej, 2018). Light intensity influenced the zonation of aerophytic algae in caves (Burgoyne *et al.,* 2021) which depends on factors such as cave entrance covered with or without vegetation, cave entrance orientation and size, and exposure to sampling stations (PopoviĆ *et al.,* 2017). The intensity of light that penetrates the cave determines whether the aerophytic algae will be heterotrophic or autotrophic (Albertano, 2012). It influences the functional stability, growth, and reproduction of photoautotrophic plants and microalgae, as well as the metabolism of mixotrophic microalgae, which can combine heterotrophic, autotrophic, and photoheterotrophic nutrition (Abiusi *et al.,* 2020).

The frequency and duration of light have a significant impact on the metabolism and growth of photoautotrophic microalgae (Oostlander *et al.,* 2020). The effects of light duration on microalgal does not only changes its growth but also biochemical composition. The utilization of lighting systems with dark cycles promotes microalgae productivity by decreasing energy consumption and increasing light absorption efficiency, which is essential for increasing the profitability of microalgae production for biotechnology (Maltsev *et al.,* 2021). Continuous lighting promotes photoinhibition processes in algal development, whereas the use of dark periods, particularly short-term ones, can aid restore photosystem damage (Yustinadiar *et al.,* 2020). Whereas alterations with the intensity of light and duration of photoperiod make microalgae to change the production of carotenoids (Maltsev *et al.*, 2021).

2.7.2 Temperature

Temperature is also a physical factor that impacts the cell size, nutrients requirement, growth rate, and biochemical composition of microalgae. It strongly influences the nutrients uptake, cellular composition, and carbon dioxide fixation of algae (Juneja *et al.,* 2013; B. Zhang *et al.*, 2020). Temperature changes the microalgal process such as microbial yield, death, and rate of reaction (Huang *et al.,* 2015).

In caves, lampenflora does not develop near strong lights, due to a very high temperature that kills the organisms in the vicinity (Janez & Gorazd, 2009). Also, visitors increase the temperatures of the cave because of mass tourism in caves (Fernandez-Cortes *et al.,* 2006). Mais (2004) reported that the presence of visitors in ice caves can melt the ice in the cave due to temperature increase. In addition to the corrosion in caves of natural processes, the combined effect of temperature variation and increase in CO_2 concentration induced by visitors can directly intensify the development of the wall corrosion processes and light intensity (Columbu *et al.,* 2021). Temperature fluctuations caused by visitors promote the growth of lampenflora, which changes the stable cave microclimate and leading to the migration of other organisms by invading surface species (Constantin *et al.,* 2021). Temperature is also a factor that