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ASSESSMENT OF EPILITHIC CYANOBACTERIAL COLONIZATION OF ANCIENT TEMPLES OF GARHWAL REGION, UTTARAKHAND, INDIA

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ABSTRACT

Colonization and growth of cyanobacteria on exposed surfaces of stone-made temples and monuments result in their biodeterioration in form of both aesthetic and structural damage. This study investigates the epilithic cyanobacterial colonization of exteriors of 11 different ancient stone temples, showing varying degree of deterioration, located in Garhwal region of Uttarakhand. Cyanobacteria-dominated biofilms/ crusts occurring on the exteriors of the temples were sampled and analyzed. The intensity of cyanobacterial colonization of temples, as assessed by quantifying the chlorophyll *a* (Chl *a*) content of biomass of phototrophic biofilms/crusts developed on wall surfaces of temples, ranged from 6.8 µg Chl *a* cm^{-2} to 14.5 µg Chl *a* cm^{-2}. A total of 22 epilithic cyanobacterial species (17 genera) representing unicellular, colonial and filamentous (heterocystous and non-heterocystous) forms were isolated from epilithic biofilms/crusts and identified. The results showed that epilithic cyanobacteria dwelling on investigated temples have ample diversity.Among the recorded taxa, the commonly occurring and widely distributed taxa with percentage occurrence of 50% and above comprised *Asterocapsa* sp., *Gloeocapsaatrata*, *Gloeocapsa* sp., *Phormidium* sp. and *Scytonema ocellatum*.

***Keywords*:** Cyanobacteria, Colonization, Epilithic, Lithobionts, Biofilms, Biodeterioration

Introduction

Colonization, growth and activities of cyanobacteria and other microorganisms on exposed surfaces of lithic (stone-built) monuments, architectural buildings and artefacts of historical, religious and cultural importancelead to their biodeterioration, which includes both aesthetic and structural damage (Scheerer *et al.*, 2009; Warscheid and Braams, 2000; Crispim and Gaylarde, 2005; Lamenti *et al*., 2000; Crispim *et al*., 2006; Macedo *et al*., 2009). Biodeterioration of monuments and buildings is a serious problem all over the world as it results not only in reduction in their aesthetic value, due the undesirable discoloration of the wall surface and biofilm formation, and generation of structural deformities but also in economic loss due to high expenses involved in their conservation.

Cyanobacteria (Blue-green algae) constitute a major group of prokaryotes which are remarkably diverse in their morphology, physiology and metabolism (Stanier and Cohen-Bazire, 1977; Carr, and Whitton, 1982; Castenholz and Waterbury, 1989). They are ecologically versatile and widely distributed organisms which are abundantly found indiverse habitats under different climatic zones (Whitton and Potts, 2000; Gaysina *et al*., 2019). They successfully colonize and inhabit lithic habitats, such as natural rocks/stones and various man-made stone monuments, buildings and heritage structures as lithobionts (rock-dwelling organisms), which include both epiliths (those growing on the external surface of rocks) and endoliths (those growing inside rocks) (Büdel, 1999; Crispim *et al*., 2006; Macedo *et al*., 2009). Because of their peculiar features, they are usual primary colonizers or pioneer inhabitants of nutrient-poor, water-limited and light-exposed lithic substrates. Cyanobacteria growing on exposed rock surfaces and external walls of monuments and buildings frequently encountermultiple abiotic stresses, such as desiccation, high light intensities combined with increased levels of solar UV radiation, high and fluctuating temperature, oxidative stress and scarcity of nutrients (Büdel, 1999). As growth or survival strategies on/in lithic habitats, they possess protective mechanisms against desiccation (Potts, 1994, 1999), oxidative stress (Latifi *et al*., 2009; Richa and Sinha, 2011), high light intensities and UV-radiation (Groniger *et al*., 2000; Ehling-Schulz and Scherer, 1999), and high temperature (Adhikary, 2003, 2004).

Epilithic cyanobacteria are aeroterrestrial organisms which inhabit interface between rock and the atmosphere. They comprise majority of the rock-dwelling cyanobacteria. As photoautotrophs and characteristically primary colonizers of lithic substrates, they facilitate and promote the growth of heterotrophic micro-organisms, like bacteria and fungi, resulting in the formation phototrophic biofilms or sub-aerial biofilms (SAB) on monuments and buildings or on bare rocks held together by extra cellular polymeric substances (EPS). Cyanobacterial EPSplay role in adhesion, surface colonization, cell aggregation, and biofilms formation and stabilization on lithic surfaces (Rossi and De Philippis, 2015; Rossi *et al*., 2012). The assemblages of epilithic cyanobacteria on lithic surfaces are commonly known as crusts or patinas in cultural heritage studies.Terrestrial epilithic cyanobacteria have been reported to colonize and

grow on natural rock/stone surfaces (Marquardt and Palinska, 2007; Adhikary, 2000) as well as on walls of temples (Adhikary, 2000; Adhikary *et al*., 2015; Roy *et al*., 1997; Gaylarde *et al*., 2012; Lan *et al*., 2010), monuments (Samad and Adhikary, 2008; Pattanaik and Adhikary 2002; Ortega-Morales, 2006), historic (ancient) and modern buildings (Tripathi *et al*., 1990; Gaylarde *et al*., 2007; Crispim *et al*., 2003; Gaylarde and Gaylarde, 1999; Gaylarde and Gaylarde, 2005; Barberousse *et al*., 2006), cave walls (Albertano, 1993, 2012; Mulec *et al*., 2008; Martinez and Asencio, 2010), statues (Lamenti *et al*., 2000) church walls (Crispim *et al*., 2004; Schlichting, 1975) and cathedrals (Ortega-Calvo *et al*., 1993; Tomaselli *et al*., 2000).

Uttarakhand, a mountainous state of India, is often referred to as the "Devbhumi" (literally 'Land of the Gods') due to its religious significance and the presence of numerous temples and pilgrimage centres which attract tourists and pilgrims throughout year. The state is rich in ancient stone temples, monuments and heritage structures many of which exhibit unaesthetic surface discoloration and other symptoms of deterioration. Studies investigating the diversity of cyanobacteria colonizing stone temples located in the state is scarce. The aim of this study was to investigate epilithic cyanobacterial colonization as well as their occurrence and distribution on selected stone temples located in Garhwal region of Uttarakhand. The assessment of the degree or intensity of cyanobacterial colonization of stone temples or monuments is an important step for the understanding of their biodeterioration as well as for the implementation of suitable control measures and restorative treatment.

Materials and Methods

Study area and sampling sites

Uttarakhand is situated in the Western Himalaya between $28^0$43'N to $31^0$28'N latitude and $77^0$34'E to $81^0$03'E longitude, comprise significant part of the Indian Himalayan Region (IHR) which is characterized by varied topography and climatic conditions, and is rich in biological and cultural diversity. The altitude varies from 200 m to more than 8,000 m above mean sea level. The climate and vegetation vary greatly with elevation. The temperature ranges from sub-zero to 43^0C. The average annual rainfall is 1550 mm. The state with 13 administrative districts is divided in to two regions/ divisions-Garhwal and Kumaun. Out of 13 districts, 7 districts, namely Haridwar, Dehradun, Pauri Garhwal, Tehri Garhwal, Chamoli, Rudrapryag and Uttarkashi lies in Garhwal region.The sampling sites for the present study included various places with ancient stone temples distantly located at varying altitudes in Garhwal region of Uttarakhand. The investigated temples comprised Bharat Mandir, Rishikesh, Dehradun (30º06'25"N, 78º17'56" E; alt. 345 masl), Tapkeshwar Mahadev, Dehradun (30º21'26"N, 78º01'00"E; alt. 680 masl), Lakhamandal Shiva Temple, Lakhamandal, Chakrata, Dehradun (30º43'53"N, 78º04'04"E; alt.1140 masl), Raghunath ji temple, Devpryag, Tehri Garhwal (30º08'44"N, 78º33'59"E; alt.531masl), Gaura Devi temple, Devalgarh, Pauri Garhwal (30º13'22"N, 78º51'44"; alt.1337 masl), Narayankoti group of temples, Guptakashi, Rudrapryag (30º32'36"N, 79º04'26"; alt.1393masl), Tungnath Temple, Chopta, Rudrapryag (30º29'22"N, 79º12'55"; alt. 3471masl); Gopinath Temple, Gopeshwar, Chamoli (30º24'49"N, 79º18'57"; alt. 1447masl), Koteshwar Mahadev Temple, Rudrapryag, Rudrapryag (30º18'07"N, 79º00'19"; alt. 885masl), Maa Uma Devi Temple, Karnapryag, Chamoli (30º15'45"N, 79º13'02"E; alt. 784 masl) and Omkeshwar Temple, Ukhimath, Rudrapryag (30º31'06"N, 79º05'43"; alt. 1304 masl),

Collection and treatment of samples

Samples of phototrophic biofilms or crusts rich in epilithic cyanobacteria were taken from the visually discoloured external surfaces of temples using the adhesive tape method (Gaylarde and Gaylarde, 1998; Urzi and De Leo, 2001) as well as by scraping off with a sterile scalpel. Samples were taken from the different faces and height of temples above 20 cm above ground level, each point covering an area of 4 cm^2. Intending to isolate maximum number of possible taxa of epilithic cyanobacteria, representing great diversity, biofilms or crusts of different appearance/colour and texture comprising both dry and moist biofilms, if observed, were collected. The collected samples were kept in labelled clean and sterile wide-mouthed screw-cap tubes and polybags and transported to the laboratory for further analysis. They were thoroughly wiped with a wet cotton swab to remove adherent dust or particles. Each sample was divided in to sub samples for various analysis. After 12-24 hours of rehydration of biofilms/crusts in agarized (0.8 %) or liquid BG-11 medium (Rippka *et al*., 1979) in glass Petri plates incubated at 25 ^{0}C under continuous light in growth chamber, they were examined microscopically, using binocular and optical microscope, for the evaluation and identification of major biomass and cyanobacterial species. Subsamples of each sample were air-dried and stored in desiccator at room temperature.

Biofilm analyses and identification of cyanobacteria

The biofilms/crusts sampled from temples were analyzed to determine the species composition of epilithic cyanobacteria. Cyanobacteria in rehydrated phototrophic biofilms and in culture were identified microscopically to the genus/species level on the basis of morphological characteristics (nature, shape and dimensions of cells, colonies and filaments; presence/absence and position of heterocysts and akinetes; shape of intercalary and end cells; presence/absence and pattern of sheaths; polarity) with the help of standard literature (Desikachary, 1959; Rippka *et al*., 1979).

Isolation, purificationand growth of cyanobacteria

Epilithic cyanobacteria were isolated from rehydrated phototrophic biofilms/crusts and established as clonal (unicyanobacterial) and axenic cultures by micromanipulation, streaking and repeated sub-culturing on solidified and in liquid BG-11 medium with antibiotic treatment following standard microbiological methods as described by Rippka (1988). Solidified culture medium was prepared by supplementing liquid BG-11 medium with 1.2% (w/v) separately sterilized agar. The antibiotic was omitted during growth of cultures. The clonal and axenic cultures of cyanobacteria were grown photo autotrophically in sterilized BG-11 culture medium (Rippka *et al*., 1979) in cotton-stoppered Erlenmeyer flasks (100 ml, 250 ml) in a growth chamber at 26±2 °C, and under continuous illumination (PAR) with the light intensity of 1.5 Klux at the surface of culture flasks provided by cool white fluorescent tubes/lamps (Phillips, India). The pH of the medium was maintained at

7.6 with HEPES-NaOH buffer (20 mM). For the growth of nitrogen-fixing heterocystous cyanobacteria, the source of combined nitrogen ($NaNO_3$) was omitted from the medium. Cultures were shaken for 15 min twice a day on rotary shaker (80 rpm).

Assessment of cyanobacterial colonization

The intensity of cyanobacterial colonization of temples was assessed by quantifying the chlorophyll *a* (Chl*a*) content of biomass of cyanobacteria-dominated phototrophic biofilms/crusts developed on external wall surfaces of temples, and expressed as µg Chl*a* cm^{-2}. The primary photosynthetic pigment Chl*a* is regarded as the good estimator of biofilm biomass (Prieto *et al*., 2004). The phototrophic biofilms/crusts sampled from temples were thoroughly dusted and washed to remove dusts and grits/particles of the substrata. The biofilms were air dried, if required, weighed, and kept in vacuum desiccator until analysis. Chl*a* was exhaustively extracted with aqueous acetone (80%, v/v) by grinding, overnight incubation at 4^0C and centrifugation (5000×g; 10 min.) at room temperature in dark. Absorbance of the cell-free extract, containing Chl*a*, was measured using UV-Vis spectrophotometer (Elico SL159) at 663 nm. The amount (µg ml^{-1}) of Chl*a* was calculated using specific absorption coefficient (α) of 82.04 for chl*a* (Myers and Kratz, 1955; Allen, 1968). Values were expressed in µg/mg dry weight of biofilm sample, multiplying by the solvent volume divided by sample dry weight. Finally, results were expressed as µg Chl*a* cm^{-2} for the assessment of the colonization.

Results and Discussion

Assessment of cyanobacterial colonization of temples

The external and exposed wall surfaces of investigated temples showed visually conspicuous colonization by epilithic cyanobacteria in form phototrophic biofilms/crusts (**Fig. 1**). The temples exhibited aesthetic damage due undesirable discoloration at varying degree from low to high of their exposed surfaces due to the development of cyanobacteria-dominated phototrophic biofilms which varied in texture, consistency, thickness, color or appearance. Some of the temples showed structural damage due to the deterioration, which may be physical, chemical or biological. Biofilms were irregularly distributed in patches and streaks over the surface. Temples were observed to be in different stages of deterioration. In addition to discoloration (bluish-green, blackish-brown and black) of wall surfaces, the observed symptoms of deterioration or biodeterioration included texture changes, cracking, crumbling, pitting or biopitting, exfoliation and appearance of uneven surfaces. Based on the observed level or intensity of biocolonization, discoloration and deterioration, the status of deterioration of the investigated monuments were given on a 3-point scale (1-3) and marked as 1 (low), 2 (moderate) and 3 (high). Accordingly, the investigated monuments were classified as lowly deteriorated, moderately deteriorated and highly deteriorated. The intensity of cyanobacterial colonization, as assessed by quantifying the Chl*a* content of biomass of phototrophic biofilms/crusts developed on external wall surfaces, and the deterioration status of the temples are given in **Table 1**. Cyanobacteria-dominated phototrophic biomass varied from 6.8 µg Chl*a* cm^{-2} for Lakhamandal Shiva temple to 14.5 µg Chl*a* cm^{-2} for Narayankoti group of temples. This indicates that the cyanobacterial colonization and growth provides a significant input of organic matter to the lithic temples. A great variety of epilithic cyanobacterial genera/species were detected in the phototrophic biofilms or crusts sampled from the temples. The epilithic cyanobacteria showed variable pattern of colonization or growth on the external walls of temples. This is presumably due to the prevailing microclimatic conditions as well as structural features of temples or properties of constructional stones, such as roughness and porosity. As a quantitative biomarker, Chl*a* has been used by various workers to estimate the degree or intensity of cyanobacterial and algal colonization of stone surfaces and building facades (Prieto *et al*., 2004; Schumann *et al*., 2005; Fernandez-Silva *et al*., 2011).

Occurrence and distribution of epilithic cyanobacteria on temples

The investigation undertaken revealed the occurrence and distribution of a wide variety of cyanobacterial species growing epilithically in biofilms/crusts on temples (**Table 2**). A total of 22 epilithic cyanobacterial species belonging to 17 genera were recorded from the exteriors of 11 different investigated temples. Of the 22 species encountered, 9 were coccoid, 4 were non-heterocystous simple filamentous, 5 were heterocystous simple filamentous, 3 were heterocystous false branched filamentous and 1 was heterocystous true branched filamentous. The results showed that the epilithic cyanobacteria dwelling on investigated stone monuments and artefacts have ample diversity. **Fig. 2** shows the percentage occurrence of epilithic cyanobacteria on temples. Among the recorded epilithic cyanobacteria, the commonly occurring and widely distributed taxa with percentage occurrence of 50% and above comprised *Asterocapsa* sp., *Gloeocapsaatrata*, *Gloeocapsa* sp., *Phormidium* sp. and *Scytonema ocellatum*. The most widespread and recurring genus was Gloeocapsa, occurring with 2 species (*Gloeocapsa* sp. and *Gloeocapsaatrata*). *Gloeocapsa* sp., showed 72.72 % (maximum value) occurrence followed by *Gloeocapsaatrata*, *Asterocapsa* sp. and *Scytonema ocellatum* all of which showed 63.63% occurrence. *Gloeocapsa* sp., was able to colonize maximum 8 temples followed by *Gloeocapsaatrata*, *Asterocapsa* sp. and *Scytonema ocellatum*, all of which were observed to colonize 7 temples out 11 temples investigated. Among the temples, maximum number of taxa (13 species) were recorded at Koteshwar Mahadev temple (KM), followed by Tapkeshwar Mahadev (TM) and Gaura Devi temple (GD), each with 12 species. The lowest number of taxa (7 species) were encountered on Lakhamandal Shiva Temple (LS), Tungnath temple (TN) and Gopinath temple. The range of cyanobacterial taxa found in this study is in consonance with those reported in other studies on stone temples and monuments elsewhere (Adhikary, 2000; Adhikary *et al*., 2015; Roy *et al*., 1997; Gaylarde *et al*., 2012; Lan *et al*., 2010; Samad and Adhikary, 2008; Pattanaik and Adhikary, 2002; Ortega-Morales, 2006).

Conclusion

Deterioration of stone-built temples, monuments and heritage structures caused by the colonization, growth and activities of various micro-organisms, along with various physical and chemical agents/factors, constitutes a major problem world-wide. The results of the study revealed the colonization of a wide range of stone-built temples with variable intensities by epilithic cyanobacteria, showing notable biodiversity.Knowledge of the types of species

colonizing and inhabiting monuments and historical buildings, and their activity is important in evaluating the potential damage or biodeterioration and suitable treatment measures for their conservation and restoration.Among microbial communities, cyanobacteria constitute pioneer organisms which readily colonize exposed lithic surfaces leading to the development of biofilms or crusts. The tremendous colonizing ability of cyanobacteria can be attributed to their rapid growth rate, simple growth requirements, efficient nutrient uptake mechanisms, easy dispersal, production of EPS, remarkable ability to tolerate varying environmental conditions, enormous surviving capacity and the ability of many of them to perform both photosynthesis and nitrogen fixation. Studies focusing on cyanobacterial colonization of stone temples or monuments in a particular climatic or microclimatic condition is important to understand the process and pace of their biodeterioration.

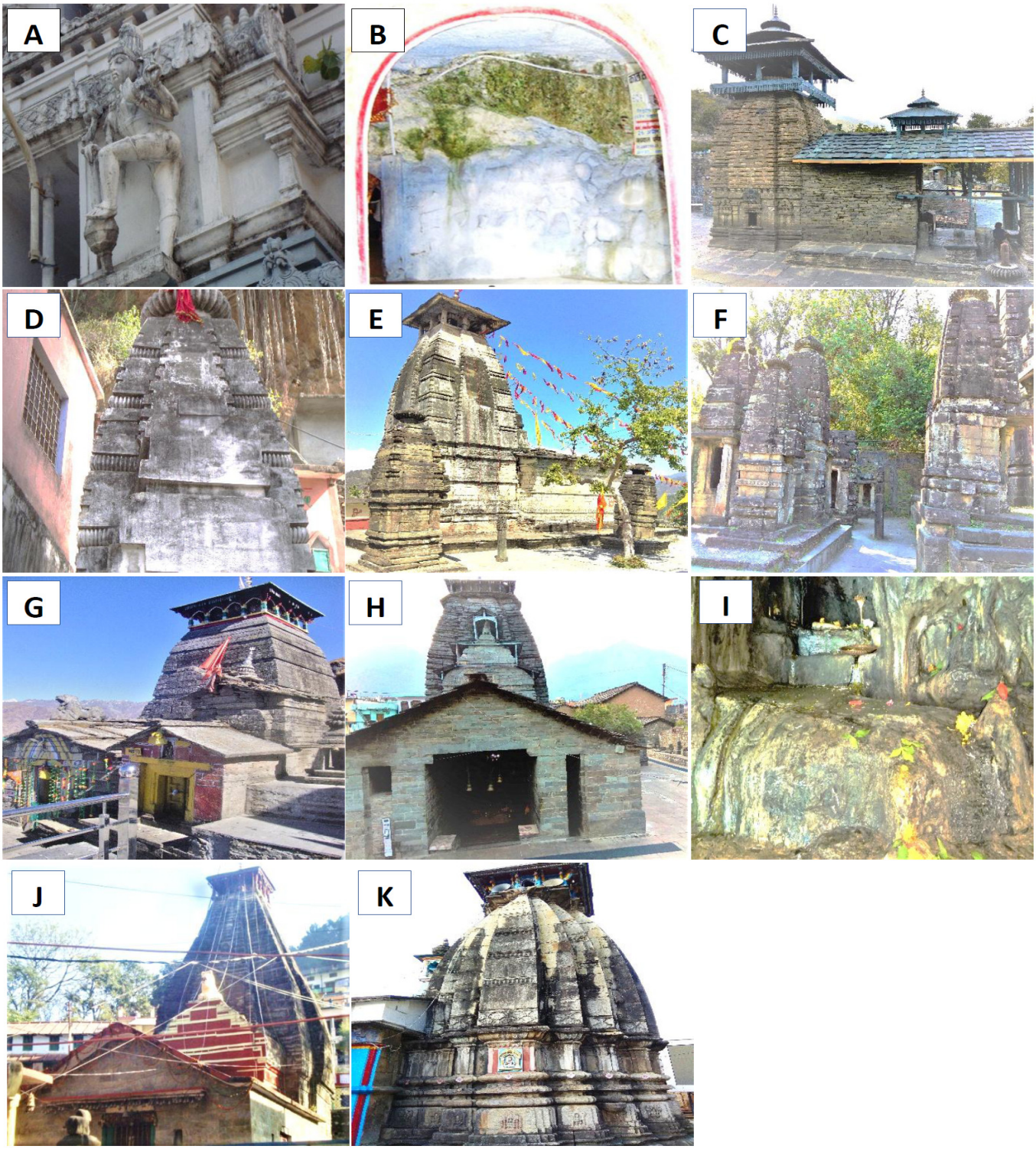

Fig. 1(A-K):Photographs of investigated temples (A) Bharat Mandir, Rishikesh, (B) Tapkeshwar Mahadev, Dehradun, (C) Lakhamandal Shiva temple, Lakhamandal, (D) Raghunath Ji temple, Devpryag, (E) Gaura Devi temple, Devalgarh, (F) Narayankoti group of temples, Guptakashi, (G) Tungnath temple, Chopta, (H) Gopinath temple, Gopeshwar, (I) Koteshwar Mahadev temple, Rudrapryag, (J) Maa Uma Devi temple, Karnapryag, (K) Omkareshwar temple, Ukhimath.

Table 1 : Intensity of cyanobacterial colonization and deterioration status of temples

S. No.	Temple	#Phototrophic biomass (µg Chl*a* cm^{-2})	*Deterioration status
1	Bharat Mandir	9.7±2.20	3
2	Tapkeshwar Mahadev	14±0.9	3
3	Lakhamandal Shiva temple	6.8±0.16	2
4	Raghunath Ji temple	13.4±0.9	3
5	Gaura Devi temple	12±0.66	3
6	Narayankoti group of temples	14.5±0.54	3
7	Tungnath temple	10.4±0.32	3
8	Gopinath temple	8±0.18	2
9	Koteshwar Mahadev temple	11±2.22	3
10	Maa Uma Devi temple	7.5±0.55	2
11	Omkareshwar temple	14±0.75	3

#Values are mean ±SD (n=3)
*1: low; 2: moderate; 3: high

Table 2 : Occurrence and distribution of epilithic cyanobacteria on temples
(+) indicates presence

S.No.	Cyanobacterial species	*Temples										
		BM	TM	LS	RJ	GD	NK	TN	GN	KM	MU	OK
1	*Chroococcus turgidus*	+	+		+	+				+		
2	*Asterocapsa* sp.			+			+	+	+	+	+	+
3	*Gloeocapsa atrata*	+		+		+	+		+	+	+	
4	*Gloeocapsa* sp.	+	+		+	+	+	+	+			+
5	*Synechococcus elongatus*	+	+			+	+			+		
6	*Synechocystis* sp.		+				+				+	
7	*Aphanothece stagnina*		+			+			+			
8	*Gloeothece* sp.	+		+						+		+
9	*Chroococcidiopsis* sp.			+				+				+
10	*Phormidium* sp.		+		+	+		+		+	+	
11	*Lyngbya* sp.	+				+	+			+		+
12	*Oscillatoria proboscidea*		+							+		+
13	*Oscillatoria irrigua*		+		+		+		+		+	
14	*Nostoc calcicola*		+							+		
15	*Nostoc commune*				+	+		+	+			
16	*Calothrix fusca*		+			+						+
17	*Calothrix marchica*			+	+					+	+	
18	*Cylindrospermum* sp.		+							+		
19	*Scytonema ocellatum*	+		+	+	+		+		+	+	
20	*Scytonema* sp.				+		+		+		+	+
21	*Tolypothrix* sp.	+			+	+		+			+	
22	*Hapalosiphon* sp.		+	+		+				+		

*BM: Bharat Mandir; TM: Tapkeshwar Mahadev; LS: Lakhamandal Shiva temple; RJ: Raghunath Ji temple; GD: Gaura Devi temple; NK: Narayankoti group of temples; TN: Tungnath temple; GN: Gopinath temple; KM: Koteshwar Mahadev temple; MU: Maa Uma Devi temple; OK: Omkareshwar temple

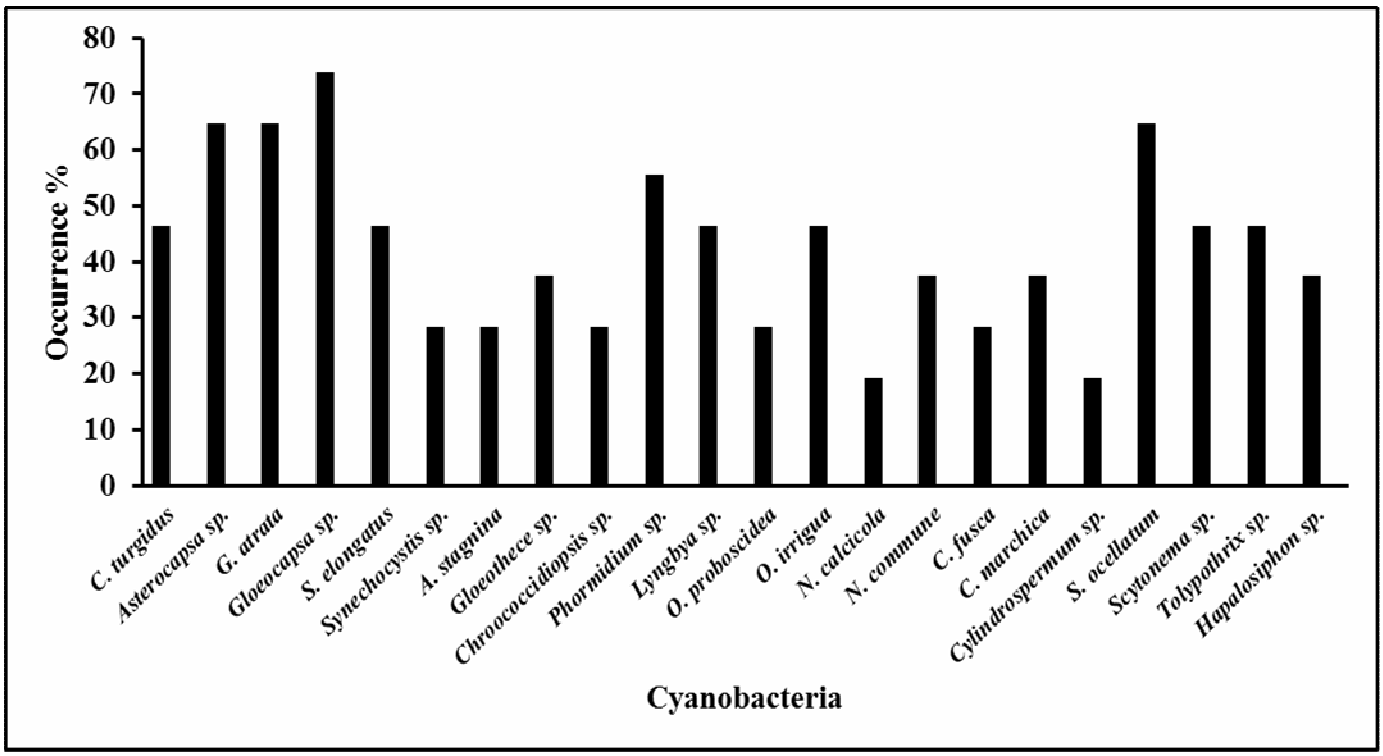

Fig. 2 : Percentage occurrence of epilithic cyanobacteria on temples

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