ISSN: 2278-5213

RESEARCH ARTICLE

Distribution pattern of Cyanobacteria in hot water springs of Tattapani, Himachal Pradesh, India

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Abstract

The water samples from hot water springs of Tattapani, HP, India were analysed for distribution pattern of cyanobacteria. Results revealed that the hot water springs which are a good medium containing all essential inorganic ions supports considerable growth of both nitrogen and non-nitrogen fixing cyanobacteria. However, the water of spring used in lab for growth of cyanobacteria does not support their growth. Along the temperature gradient from 65°C to 35°C, the water showed a gradual decrease in salt residue per unit volume. This indicates that the decrease in water temperature leads to salt precipitation. These changes in the quality of water and variation of temperature along the spring affected the distribution and occurrence of cyanbacterial population.

Keywords: Hot water springs, Tattapani, cyanobacteria, temperature gradient, salt precipitation.

Introduction

Cyanobacteria (blue-green algae) are one of the most interesting groups among the algae from structural, functional, adaptation, distribution and economic point of view. Some of their physiological characteristics are similar to higher plants and green algae in respect of chlorophyll, biosynthesis, oxygenic photosynthesis and essential amino acids (Fogg *et al*., 1973). They are only nitrogen fixing organisms that have oxygen generating photosynthetic system having capacity to fix the atmospheric nitrogen in the form of ammonia at the expense of photosynthesis (Fogg *et al*., 1973; Whitton and Roger, 1989) which offer a unique biological material for an understanding of physiological processes operating in extreme environments (Edwards *et al*., 1968). They share similarities in light reactions mediated by photosystem I and II, protein pigment complex (Phycobilisomes), chlorophyll and carotenoids. Their ancestors are possibly the oldest primary producer organisms common in the distant past and they perhaps used thermal springs as refugia (Gold, 1992; 1999; Plescia *et al*., 2001; Adhikary 2006; Izagiurre *et al*., 2006; Hindak, 2008).

Among aquatic habitats, streams of hot water springs are one of the best habitats for blue-green algae (Stewart, 1970). Hot water springs occur worldwide except Antarctica (Waring, 1965) and most of them occur in Yellow-stone Plateau of North America (Keefer, 1971), Iceland (Barth, 1950), New-Zealand and Japan (Uzamasa, 1965). In our country too, hot water springs of varied temperature regimes are concentrated in the state of Himachal Pradesh and rest of states, Haryana, Maharashtra, Gujarat, Bihar, West Bengal and Orrisa (Vasishta, 1968).

Although the systematic of various Indian hot springs have been studied (Prasad and Srivastava, 1965; Vasishta, 1968), their ecological study has been rather neglected. Among the predominant forms of cyanobacteria common to all hot water springs in India are *Chroococcus yellowstonesis, Synchococcus elongatus.* Var. *amphigranulatus, Oscillatoria jasorvensis, O. tenus, O. filiforms, Phormidium laminosus, Lyngbya nigra* and *Mastigocladus laminosus* (Vasishta, 1968). The form of *Synechococcus* (*S. elongatus* Var. *amphigranulatus* and *S. vuleanus*) are considered as strictly thermal forms as they have not been recorded from non-thermal situations. In hot water spring both heterocystous and non-heterocystous cyanobacteria are found. The non-heterocystous cyanobacteria are lacking N_2-fixation capacity. However, unicellular non-heterocystous algae such as Gloeocapsa (Wyatt and Silvey, 1969) and Aphanothece (Singh, 1973) are important because they fix nitrogen under aerobic conditions.

Cyanobacteria possess various biomedical applications. Thermophilic cyanobacteria, *Phormidium* sp. produced an anti-microbial material against gram negative and positive bacteria *Candida albicans* and *Cladosporidium resinae* (Fish and Codd, 2004). Another *Phormidium* sp., immobilized in calcium alginate was used for treatment of dye-rich wastewater (Ertugrul *et al*., 2008). Cancer drugs were produced from thermophilic cyanobacteria by Javor (1999). Some unusual Fe-proteins, siderophores were identified in *Mastigocladus laminosus* (Mohamed, 2008). Thermophilic *Synechococcus* sp. is a potential producer of poly-b-hydroxybutyrate which is the basis of biologically degradable plastics (Miyake *et al*., 1996).

Production of hydrogen by some cyanobacteria is a promising source of energy for the future (Mitsui, 1987). The exploitation of natural hot water springs with a large content of CO_2 is highly profitable for algal biotechnology, e.g. production of *Spirulina* (Fournadzhiev *et al*., 2002). Precipitation of travertine using cultures of thermal cyanobacteria is a promising method for capturing and sinking CO_2 of anthropogenic origin (Hayashi *et al*., 1994; Ono and Cuello, 2007). Several species of cyanobacteria and algae are known to produce novel compounds which are used in drug development for human and other uses (Aguilera, 2012). Keeping in view of currently reported potential of cyanobacteria in tissue engineering and regenerative medicines, this investigation was undertaken to assess the effect of physio-chemical parameters of hot water springs of "Tattapani" on the distribution of thermal cyanobacteria along the thermal gradient of spring. The investigation will be helpful for cultivation and characterization of themophilic cyanobacterial strains for possible biomedical engineering exploration.

Materials and methods

Study area and water sample collection: Water samples were collected from Tattapani, one of the important hot water sulphur spring located in district Mandi about 48 km from Shimla, Himachal Pradesh, India (Fig. 1).

Fig. 1. Map of Himachal Pradesh showing Tattapani.

Water sample analysis: The physico-chemical properties of the thermal spring water collected from the site No. 1 (65°C) were analyzed according to standard methods for the examination of water and waste water (Arnold *et al*., 1985).

Collection of algal samples: Algal samples were collected from the different sites of thermal springs of Tattapani in many replicates. Pre-sterilized screw cap glass vials of 15 mL capacity were used for collection purpose. Randomly selected 1 cm^2 blocks of algal patch were scrapped and collected in separate vials. Remaining volume of the vials was filled with natural spring water and brought to laboratory for isolation.

Isolation of algae: Streak plate technique was used for isolation of species of *Mastigocludus***,** *Chroococcus, Lyngbya, Phormidium, Microcystis, Oscillatoria* and *Synechococcus.* Algal population was fragmented with sterilized awl pins on the rotator for about 5 min. The algal suspension was then diluted and aliquots (a drop) of different dilutions were streaked on different petri-dishes containing Allen and Arnon nutrient agar medium (1.0% w/v) with the help of a sterilized inoculation needle. Both nitrate free as well as nitrate containing media was used for this purpose and petri-plates were incubated at 45°C under continuous illumination of 2500 lux and regularly observed for development of individual colonies.

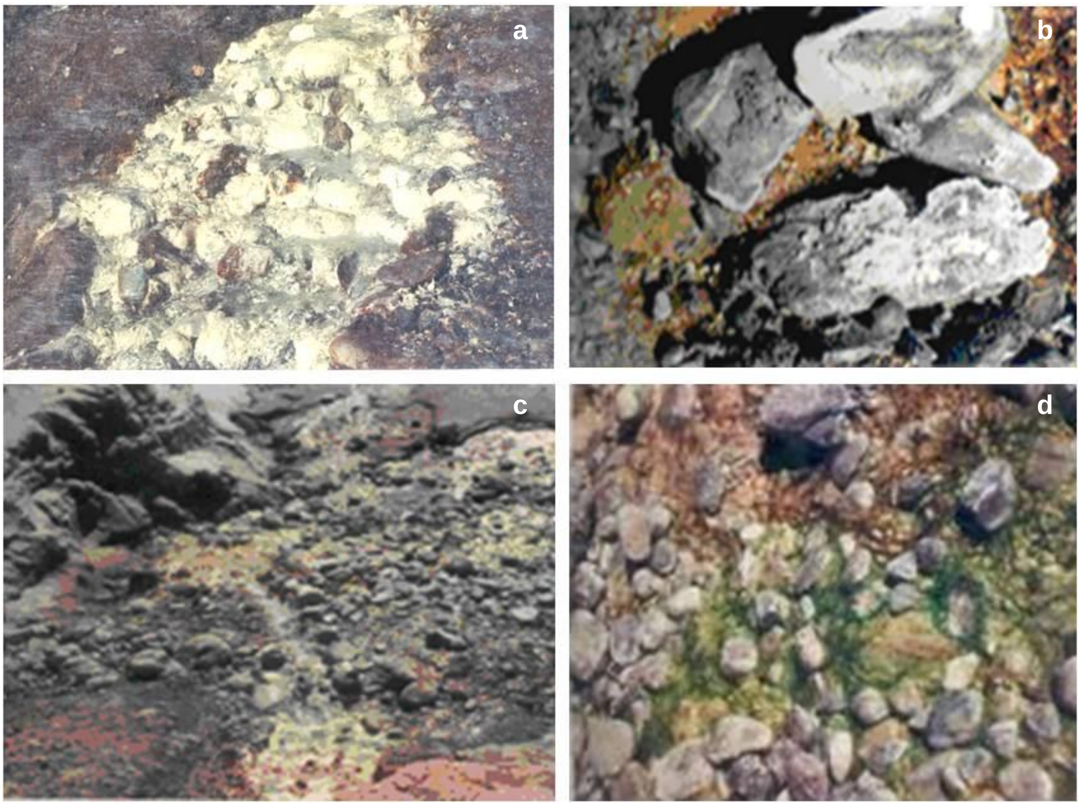
Discrete colonies seemingly free from contaminants were sucked up with the help of sterilized micropipettes and transferred separately pre-sterilized culture tubes, each containing 10 mL of nitrate free or nitrate containing medium (AA medium). These culture tubes were transferred unshaken to the incubator. After about a fortnight of incubation, many colonies were started growing in the respective liquid media. The same were observed under a microscope for purity. If contaminates were detected, the algal suspension was streaked again and the process was repeated until pure lines were established.

Identification of algae: Taxonomic identification of algal forms collected from different sites of Tattapani hot springs were done using standard reference works of Desikachary (1959). Some cyanobacterial forms were identified at national facility for marine cyanobacteria, Bharathidasan University, Tiruchirapalli-620024, TN, India.

Results and discussion

The temperature at the emergent site of the spring was as high as 65^{0}C which gradually declined to 33^{0}C at the junction where hot spring water mixed with the Sutlej river water (Fig. 2). The maximum and minimum (day/night) ambient air temperature during winter months in district Mandi, H.P was recorded to be within the range of 22/12^{0}C (maximum/minimum) temperature. The high water temperature recorded at the emergent site (65^{0}C) gradually equilibrates with air temperature resulting in the formation of a descending temperature gradient along the spring before it finally joins with the flow of river water. This descending temperature gradient, having a variation in the mineral composition forms small groups of different ecological niche along the stream that influence the distribution and type of cyanobacterial population.

Fig. 2. Sites of Tattapani hot water springs.



a. Site 1: 65°C, the emergent site of the thermal spring; b. Site 2: 55°C, represent minerals deposition and occasional cyanobacterial patches over rocks; c. Site 3: 45°C, represent numerous rocks with cyanobacterial colonies; d. Site 4: 35°C, represents a small pool with floating filamentous cyanobacterial mats adhered to rocks.

At high temperature at the emergent site of thermal spring, salts were dissolved due to high temperature (65^0C) which subsequently found to be deposited on the rocks during gradual cooling of water from 65^0C to 55^0C. As a consequence, numerous rocks with salts deposited over them were found at site 2 (Fig. 2).

Physico-chemical composition of water of Tattapani hot spring: Since most of the precipitation of dissolved salts took place at site 3 and 4, the water analysis was not conducted at these sites. Therefore in order to have the composition of water of the stream, samples were taken from site 1 "65°C" for analysis. The water of this site was clear with smell of sulphur (Table 1). The pH of water was 7.2 which showed that the spring is slightly alkaline in nature. Total dissolved solids were 10,120 mg/L which are higher than the total solid reported from other hot springs (Vasishta, 1968). The concentration of nitrate was also quite high (2 mg/L). In many hot water springs, studied by other researchers, nitrate was found to be very low or absent (Brock and Brock, 1969a; b). In India many hot water springs in various states studied by Vasishta (1968) don't contain any appreciable amount of nitrate. This may be due to the fact that nitrate estimation in this study was done at the first or emergent site of the hot spring which showed high amounts. On the other hand, it is possible that the content is very low where cyanobacterial population was abundant (site 3 and 4) simply because of the precipitation of the salts. Apart from temperature, some researchers (Ward and Castenholz, 2002; Sompong *et al.*, 2005; debnath, 2009) also focussed on the role of pH and combined nitrogen (especially ammonium) on the species distribution in cyanobacterial mat community below 60°C. Ammonia was found to be another nitrogen source in the spring. The concentration was 0.2 mg/L which is higher than the earlier reports which ranged from 0.001 to 0.080 mg/L (Bharti, 1991). In this spring Tattapani, average value was recorded to be 1.60 mg/L.

Ammonia was more abundant than nitrate at least at spring source in both alkaline and acid springs, but particularly in case of later. Diazotrophic cyanobacteria are able to colonize springs where nitrogen levels are low to support other taxa. Conversely they may be out-competed by non-diazotrophic cyanobacteria in spring with sufficient combined nitrogen (Ward and Castenholz, 2002). There are three sources for the supply of carbon in natural water such as free CO_2, HCO_3 and CO_3. The carbon status of water affects the growth and photosynthesis of the phytoplanktons of hot water spring.

Table 1. Physical and chemical characteristics of Tattapani hot water spring.

Physical examination	
Appearance	Clear
Colour (Hazen scale unit)	Normal
Taste and odour (qualitative)	Salty taste
Turbidity (Naphtha turbidity unit)	6
Conductivity (microelements in cm)	11,800
pH	7.2
Temperature(during collection)	65°C
Chemical examination (mg/L)	
Total solids dried at 105°C	10120
Free carbon dioxide as CO_2	55
Phenolphthalein alkalinity as $CaCO_3$	Nil
Methyl Orange alkalinity as $CaCO_3$	256
Total hardness as $CaCO_3$	430
Carbonate hardness as $CaCO_3$	256
Non-carbonate hardness as $CaCO_3$	174
Free and saline ammonia as N	0.2
Nitrate as N	2.0
Nitrite as N	0.005
Chloride as Cl	3500
Sulphate as SO_4	115
Sulphide as S_2	8.5
Sulphite as SO_3	7.8
Fluoride as Fl	3.0
Iron as Fe	0.2
Calcium as Ca	164
Magnesium as Mg	4.8
Phosphate as PO_4	0.5
Potassium as K	240
Sodium as Na	270
Silicate	119

Table 2. Variation in dissolved oxygen, free CO_2 and bicarbonate alkalinity along the temperature gradient of hot water spring Tattapani.

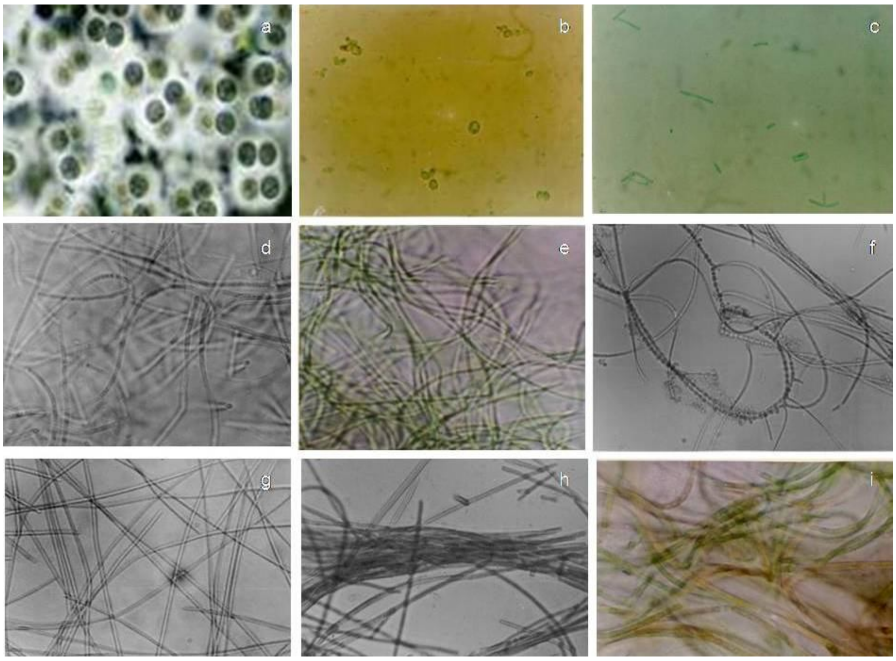
Water temperature (°C)	Oxygen (mg/L)	Dissolved free CO_2 (mg/L)	Bicarbonate alkalinity (mg/L)
65	2.3	55.0	21.6
55	3.5	25.6	21.0
45	4.2	12.7	20.0
34	4.9	2.6	19.8

Free CO_2 was 55 mg/L at the emergent site of the thermal spring (65°C) while there was a rapid fall along the thermal gradient (Table 2). In sites 3 and 4, where the free CO_2 was less than at the emergent site, the dissolved bicarbonate had greater significance as alternative carbon source for photosynthesis at higher temperature and alkaline pH, since; no phenolphthalein alkalinity was detected in this hot water spring. The alkalinity entirely was assumed to be due to methyl orange alkalinity. Bicarbonate hardness was 174 mg/L which showed that free CO_2 is not a limiting factor to control the growth of phytoplanktons in this spring. Inorganic phosphate was found in large quantity in the water of the thermal spring in comparison to the surface fresh water. This is similar to the report of Brock (1969b) and this spring is also characterized by high amount of silica. Faulty rocks and their dissociation may be the cause of high silicate values as also reported earlier for the Manikaran hot water spring (Singh and Sharma,1986) sulphide concentration of some hot springs of North Iceland and New Zealand (Castenholz, 1976).

Table 3. Occurrence of cyanobacterial species along temperature gradient of hot water spring Tattapani.

Site 3 (45°C)	Site 4 (35°C)
Aphanocapsa grevillei	*Calothrix brevissima*
A. thermalis	*Chroococcus ansuyensis*
Calothrix parietina	*C. minor*
Chroococcus ansuyensis	*C. minutus*
C. minor	*Coelosphaerium kuetzingiana*
C. yellowstonensis	*C. dubium*
Entophysalis granulose	*Lynqbya calotrichicola*
Leptochaete hansqirgi	*L. diqueti*
Lynqbya aerugino-coerulea	*L. holdenii*
L. diqueti	*L. kuetzingiana*
L. nigra	*Mastigocladus laminosus*
Mastigocladus laminosus	*Microcytis stagnalis*
Oscillatoria brevis	*Oscillatoria cortiana*
O. laete-virens	*O. filiformis*
O. onimatis	*O. lativana*
O. princeps var. *tenella*	*O. lamnosus*
O. proboscidea var. *westii*	*O. onimatis*
Phormidium africanum	*O. princeps* var. *tenella*
P. cebennese	*O. proboscidea*
P. tenue	*O. tenuis*
Plectonema notatum var. *africanum*	*Phormidium valderianum*
Scytonema leptobasis Var. *thermalis*	*Spirulina subsalsa*
Spirulina subsalsa	*Synechococcus elonatus* var. *Amphigranulatus*
Synchococcus elongates Var. *amphigranulatus*	

Fig. 3. Tattapani hot water spring microbial mats dominated by cyanobacterial population.



a. *Chroococcus minor;* b. *Microcystis* sp.; c. *Synechococcus elongates;* d. *Phormidium tenue* sp. 1; e. *Phormidium tenue* sp. 2; f. *Mastigoclasdus lamionsus;* g. *Oscillatotria laete–virens;* h. *Oscillatotria onimatis;* i. *cyanobacterium Lyngbyadigueti.*

In the present investigation, the total concentration of sulphide and sulphite were 8.5 and 7.8 mg/L respectively. The observed high concentration of sulphide and sulphite in this spring indicates the prevailing anaerobic and reducing conditions of the spring, which reflect a reminiscent of archaic environment of Pre-cambrian era (Castenholz, 1967). These conditions are well suited for the growth of blue-green algae (Brock, 1970), some of which might have been the direct descendant of Pre-cambrian thermophiles (Castenholz, 1967).

The physico-chemical analysis of water revealed the presence of nutrients such as nitrogen, phosphorus, sodium, potassium and others which though present in low concentration, were conducive for fair growth of the blue-green algae and thermophilic bacteria. These organisms formed compact benthetic mats in the effluent of hot springs. Such thermal environments are conducive for many photosynthetic prokaryotic algae which are known to grow at constant temperature as high as 75°C in neutral or alkaline water (Brock, 1967a; b) and up to 55°C in acidic water (Doemel and Brock, 1970). Abundant blue-green flora have been found in various hot springs e.g, Tiverias hot springs of the Israel (Dor,1967), hot springs in Greece (Anagnostidis, 1961) and the Yellowstone thermal springs (Wiegert and Mitchell, 1973). The predominant cyanobacteria found in site 3 and 4 are mentioned in Table 3. It was interesting to note that with exception to few common species found in both site 3 and 4, the cyanobacterial flora in site 3 was largely different from the flora of site 4. The differences might be due to the thermo-tolerance of species or due to the variations in mineral composition of the spring. In addition to this, the common species found in both the sites showed a better adaptability towards the flexible environment. It was observed that a low temperature (40-47°C), Synechococcus mat from Yellowstone National park was found to support Synechococcus, Phormidium, Pseudanabaena and Spirulina like cyanobacteria and this was more diverse than their higher temperature(60-80°C) counterparts (Norris *et al.,* 2002). This trend is also observed in this study (Table 3). Cyanobacterial communities have been divided in to 2 categories according to Yoneda (1952): i) mesothermophilous community (≤ 45°C, sites 4 of thermal spring and ii) euthermophilous community (45-65°C, site 3 of the thermal spring). Of the cyanobacterial species belonging to these two categories, *Aphanocapsa grevillei, A. thermalis, Calothrix parietina, Oscillatoria laete-virens, Lynqbya nigra, L. aerugino-coerulea, Leptochaete hansqirgi, Chroococcus yellowstonensis, Entophysalis granulose, Oscillatoria proboscidea* var. *westii, Phormidium africanum, P. cebennese, Plectonema notatum* var. *africanum, Scytonema leptobasis* Var. *thermalis,* Synchococcus elongates Var. amphigranulatus, *S. elongatus, S. lividus, G. gelatinosa, Fischerella thermalis* and *Calothrix thermalis* are the true thermal species. Facultative species of great tolerance are *Chroococcus ansuyensis, C. minor, Lynqbya diqueti, L. holdenii, Mastigocladus laminosus, Oscillatoria lamnosus, O. onimatis, O. princes* var. *tenella, O. proboscidea, O. tenuis, Spirulina subsalasa,* *Synechococcus elonatus* var. *amphigranulatus* which are also found in fresh water bodies. This indicates the temperature dependence of cyanobacterial species in geothermal spring. *Synchococcus elongatus, S. lividus, G. gelatinosa, Fischerella thermalis* and *Calothrix thermalis* are the true thermal species and facultative species of great tolerance are *O. amphibia, O. princes*, *P. fragile* and *P. laminosum* has been also reported by Debnath *et al.* (2009). Few filamentous and unicellular forms of cyanobacteria were isolated and cultured in axenic forms in Allen and Arnon media with or without nitrogen source. These were *Chroococcus minor*, *Microcystis*, *Synechococcus elongatus* as unicellular forms. *Phormidium tenue* and *Mastigocladus laminosus,* Oscillatoria laete-virens, *Oscillatoria ornimatis* and *Lyngbya digueti* as filamentous forms (Fig. 3). They were abundant and mostly colonized within the temperature range of 35-45°C. However, among the various cyanobacterial population studied, unicellular *Synechococcus elongatus* and filamentous *Mastigocladus* and *Phormidium* sp. were uniformly distributed at all the temperature ranges (35-65°C) indicating their higher thermo tolerance nature compared to other species. Except *Synechococcus elongatus*, filamentous *Phormidium* and *Mastigocladus laminosus* there was a seasonal variation in the occurrence and population density of the other species and occasionally some species were found to have disappeared completely for years. No new cyanobacterial sp. was found during intensive microscopic examination of the spring water and algal mats during this investigation. Most of them were already reported in other thermal springs in India and in rest of the world. These cyanobacterial forms were morphologically identical to their similar non-thermal species; therefore, they were designated as thermal strains of the respective non-thermal forms in the text. Among the filamentous forms, heterocystous *Mastigocladus laminosus* was abundantly found in all the cyanobacterial mats collected from randomly selected submerged rocks at different sites of the spring. This species was found to be nitrogen fixing, having branched filaments with heterocysts and was often found in dessicated form. Dessicated filaments revived within a few days in suitable nutrient media. By virtue of its dual physiological characteristics i.e. photosynthesis and nitrogen fixation often in the same or in different branches of the common filament, this strain of *M. laminosus* has a competitive advantage over other cyanobacteria with regards to sustaining its own growth and increasing primary productivity under limited nutrient level in the spring. *Phormidium* sp. and *Oscillatoria* have never been found in isolation and they always coexisted with *M. laminosus*. *Mastigocladus laminosus* has become a hot model organism studied in laboratories also with a potential for biotechnological applications e.g. gliding of its hormogonia through agar (Robinson *et al.*, 2007) and production of capsular polysaccharides and their anti-cancer activity (Gloaguen *et al.,* 1999; 2007). It is also interesting that its ferredoxin activity was optimal at 65°C (Fish *et al.,* 2005) but the alga was shown to grow as a branched form at <53°C, and unbranched form in 60°C (Castenholz, 1972). *Mastigocladus laminosus* was found to produce antibacterial component in methanolic extract. The methanolic extract was observed to inhibit *Escherichia coli, Pseudomonas aeruginosa, P. syringe, Enterobacter* sp. and *Enterococcus fecalis* while, *Bacillus subtilis* and *Agrobacterium tumefaciens* were found resistant (Bhardwaj *et al.,* 2010). *Mastigocladus laminosus* has been demonstrated as a pioneer species for a laboratory model of cyanobacterial mat (Bryanskaya *et al.,* 2008). The problem of *M. laminosus* obviously needs further observation in the field, but also followed with cultivation experiments and sequencing in the lab (data not shown). This strain is deposited in the culture collection Centre, NFMC Tiruchirappalli along with other isolated cyanobacteria: *Chroococcus minor, Microcystis, Synechococcus elongatus, Phormidium tenue, Oscillatoria laete-virens, Oscillatoria ornimatis and Lyngbya digueti.*

Conclusion

"Tattapani" is one of the important hot water sulphur spring located in district Mandi about 48 Km from Shimla, Himachal Pradesh. The spring is slightly alkaline in nature (pH 7.2) and temperature ranges between 35-65°C and always higher than the ambient air temperature. The salinity of the spring water is very high (10,120 mg dissolved solids/L) and contains all the essential salts e.g. nitrate, phosphate, carbonate, sulphate, chloride, silicate, fluoride, sulphite, sulphide and micronutrients such as Fe, Mn, Ca, Mg, K and Na conducive for fair growth of cyanobacteria.

A large number of cyanobacteria species either forming a thick mat adhered to submerged rocks or floating forms were found along the thermal gradient (35-65°C) in this stream. They were abundant and mostly colonized within the temperature range of 35-45°C, except *Synechococcus elongatus*, filamentous *Phormidium* and *Mastigocladus laminosus*, there was a seasonal variation in the occurrence and population density of the other species and occasionally some species were found to have disappeared completely for whole of years. No new cyanobacterial species was found during intensive microscopic examination of the spring water and algal mats during this investigation. Most of them were already reported in other thermal springs in India and in rest of the world.

These cyanobacterial forms were morphologically identical to their similar non-thermal species therefore; they were designated as thermal strains of the respective non-thermal forms in the text. No growth of any of the thermal strains of cyanobacteria was obtained under laboratory conditions using spring water as nutrient medium.

Acknowledgements

The author is grateful to Professor Dr. G. Subramanian of NFMC, Tiruchirapalli, Dr. P.S. Basu, Principal Scientist, ICAR and Dr. Hari Om Agrawal, Professor of HPU, Shimla for their guidance and suggestions and UGC and DST for financial grant and fellowship. This communication is a part of the Ph. D programme of the author.

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