Response of the estuarine cyanobacterium *Lyngbya aestuarii* to UV-B radiation

Jnanendra Rath · Siba P. Adhikary

Abstract Growth response and changes in the spectral properties of methanolic extract of an estuarine cyanobacterium, *Lyngbya aestuarii Agardh*, to UV-B radiation were studied. Increase in growth accompanied by increase in chlorophyll *a*, protein and carbohydrate content was observed up to 12 h of UV-B irradiation followed by a decline with further increase in the duration of UV exposure. Carotenoid content progressively increased with the UV-B dose. The organism synthesized, to a significant extent, mycosporine amino acid-like substances (MAAs) upon UV-B exposure. The cells in the trichome became coiled followed by formation of small bundles as a response to UV-B radiation. SDS protein profile of the UV irradiated cells showed repression of 20 and 22 kDa proteins. However, irradiation with UV-B for 6–24 h led to overproduction of 84, 73, 60, 46, 40, 37 KDa proteins, possibly conferring protection to the organism from UV-B. UV irradiated cells cultured in florescent light for up to 7 days showed revival from UV damage of the pigments and macromolecular contents, suggesting existence of a repair mechanism in the organism.

Keywords Absorption spectra · Chlorophyll *a* · Cyanobacteria · *Lyngbya aestuarii* · Protein profile · UV-B

Introduction

Enhanced solar ultraviolet radiation (UVR) due to stratospheric ozone depletion is a major stress factor for phototrophic organisms in aquatic and terrestrial ecosystems

(Franklin and Forster 1997). Ultraviolet-B (UV-B) is a biologically effective component of solar radiation which, because of its absorption by biomolecules such as nucleic acids, proteins, and lipids, causes deleterious effects on biological systems (Karentz et al. 1991; Williamson 1995; Sinha et al. 2001). An increase in the level of UV-B radiation (280–320 nm) reaching the earth's surface has thus caused a risk of exposure of living organisms to this harmful radiation (Kerr and McElroy 1993; Vincent and Roy 1993; Bothwell et al. 1994).

Cyanobacteria are oxygen producing photosynthetic prokaryotes dominating the microbial communities of almost all the ecosystems on earth. They are present in habitats of great diversity, such as hot springs, Antarctic ice shelves, deserts, and also in marine and freshwater environments, and are important contributors to global photosynthetic biomass production (Fogg and Stewart 1968; Potts and Friedmann 1981). Due to increase of incidence of UV-B on the earth, these organisms are often exposed to high solar radiation including UV-B in their natural habitats (Ehling-Schulz and Scherer 1999). There are reports that growth, cell differentiation, motility and photo-orientation of cyanobacteria are affected by UV irradiation (Tevini 1994; Franklin and Forster 1997). However, it is difficult to generalize the precise influence of UV radiation on their growth since these organisms show large differences in UV sensitivity, their physiological responses to UV, and also photo-repair mechanisms.

It is presumed that cyanobacteria faced more intense solar UVR because of their presence prior to the development of the stratospheric ozone layer. Therefore, it has been postulated that cyanobacteria as a group may possess efficient mechanisms to prevent or counteract the harmful effects of solar UVR. There are reports that cyanobacteria synthesize UV-absorbing mycosporine amino acid-like

J. Rath · S. P. Adhikary (✉)
Department of Biotechnology, Utkal University,
Bhubaneswar 751004, India
e-mail: adhikary2k@hotmail.com

substances (MAAs) which protect them from harmful UV-B radiation (Scherer et al. 1988; Karentz et al. 1991; Ehling-Schulz et al. 1997). *Lyngbya aestuarii* is the dominant cyanobacterium in Chilika Lagoon, India, floating on the water surface throughout the year and so exposed to elevated levels of solar radiation and also UV. Thus, the effect of UV-B radiation on the growth, photosynthetic pigments, cellular carbohydrate and protein of the organism was studied. The reversal of the UV-B effect, if any, after incubating the irradiated cells under control culture conditions was also analyzed.

Materials and methods

L. aestuarii floating on the surface of lake water and also attached to fishing nets was isolated from Chilika Lagoon, Orissa, India, (19°58′58.7″N, 86°19′25.7″E), the largest brackish water lagoon in Asia, and obtained in axenic culture. The organism was routinely grown in ASN-III medium with inorganic nitrogen (Rippka et al. 1979) at $25\pm1°C$ under 7.5 W m^{-2} light intensity provided from daylight fluorescent tubes and maintained in the laboratory. For UV exposure experiments, 50 mL homogenized suspension of the organism from its exponential growth phase (10 mg fresh weight) was used and irradiated with UV-B (UV-B lamp, NIS, G15T8; maximum output at 315 nm; 5 W m^{-2} at the surface of the petri dish). The irradiance of UV-B was measured using a double monochromator spectroradiometer (Type 752; Optronic Laboratories, Fla., USA). The culture suspension was placed in glass petri dishes of 75 mm diameter (triplicate for each treatment) over a magnetic stirrer and irradiated with UV-B for 3, 6, 12, 24 and 48 h continuously in a locally fabricated UV radiation chamber with a provision for maintenance of the temperature at $25\pm1°C$. The volume of the culture suspension was measured and the loss, if any, due to evaporation during treatment was equilibrated by ASN-III medium. Cultures maintained at $25\pm1°C$ under 7.5 W m^{-2} light intensity provided from fluorescent tubes were always treated as control. Pigments of the control and UV-B irradiated cells were extracted in 90% methanol (v/v) for measurement of absorption spectra in a Hitachi U2000 UV-visible double beam spectrophotometer in the wavelength range of 750 to 250 nm using quartz cuvettes. Quantity of chlorophyll-*a* was estimated using the extinction co-efficient of Mackinney (1941), and the amount of total carotenoids was determined following Davis (1976). The quantity of MAAs was expressed as absorption maxima of the extract in the UV region of the spectrum at 360 nm per 3 ml extract from 10 mg fresh weight of cells. Carbohydrate content was estimated using anthrone reagent as described by Herbert et al. (1969). Cell protein was estimated following Lowry et al. (1951). Cells soon after irradiation

for different hours were inoculated into fresh culture media containing 10 ml ASN-III media and cultured for 7 days at $25\pm1°C$ under 7.5 W m^{-2} light intensity.

Changes in the protein profile of the cyanobacterium soon after UV-B irradiation and after culturing the irradiated cells in light was studied using SDS-PAGE. The cells were lysed by boiling for 5 min in 200 μL of cracking buffer [1:1 v/v approx, containing 50 μL of 0.5 M Tris-HCl (pH 6.8), 20 μL of 5% (m/v) ß-marcaptoethanol, 40 μL of 10 % (m/v) sodium dodecyl sulphate (SDS), 40 μL of 10% glycerol, 0.26 mg of 20 mM sodium azide, 40 μL of 1 mM phynyl methyl sulphonyl fluoride (PMSF), 1.52 mg of 20 mM ethylene glycol tetra-acetic-acid (EGTA) and 4 μL of 0.1% (w/v) bromophenol blue]. The supernatant was extracted by centrifugation and the quantity of protein was determined. Proteins were electrophoresed on 30% poly-acrylamide SDS linear slab gels in vertical system (Biotech) overlaid with a gel prepared by 12% resolving gel [9.9 mL H_2O, with 7.5 mL Tris/HCl (pH 8.8), 12 mL 30% acrylamide, 300 μL 10% SDS, 300 μL of 10% APS (ammonium persulphate) and 12 μL TEMED (N′,N′,N′, tetramethyl ethylenediamine) and 5% stacking gel [6.8 ml of H_2O with 1.25 mL Tris/HCl (pH 6.8), 1.7 mL 30% acrylamide, 100 μL 10% SDS, 160 μL 10% APS and 8 μL TEMED]. The Electrophoresis buffer contained 250 mL 25 mM tris-glycine (pH 8.3) and 5 mL 20% SDS. Equal amounts of proteins were loaded into each well. Electrophoresis was carried out using a Biotech power pack (Yercaud, India) with a power supply of 100 mV/20 miliampere for 1 h, followed by 30 miliampere for 3 h. The gel was stained with 0.1% comassie brilliant blue overnight (1 g coomassie brilliant blue R 250 in the solution of 400 mL methanol, 100 mL acetic acid and 500 mL distilled water), and was destained with 7% acetic acid, 20% methanol and 2% glycerol solution. Molecular weight of the proteins was determined by comparison of their mobilities with those of marker proteins of known molecular weight (Merck protein standard mixture IV, 1.15791.0001, batch K 90925591 containing cytochrome C-12.3, myoglobin- 16.9, carbon anhydrase -30.0, ovalbumin-42.7, albumin-66.2, ovotransferin - 78.0 kDa). Photograph of the gel was taken in a Bio-Rad Geldoc system and analyzed using 1D analysis software. All the experiments were carried out in triplicates and the values ± S.D are presented in the text.

Results

Chlorophyll-*a*, carotenoid, carbohydrate and cell protein content of the UV-B irradiated cells for different durations and that of UV-B irradiated cells cultured in florescent light for 7 days is shown in Fig. 1. Chlorophyll-*a* in comparison

to the control cells was increased on irradiation with UV-B. With 6 h of continuous UV-B exposure, a 32% increase in chlorophyll-a was observed, although this decreased thereafter. Upon exposure of 48 h, chlorophyll-a declined by 35% in comparison to the control. Carotenoid content progressively increased with the duration of irradiation. After 48 h of UV-B irradiation, a 125% increase in carotenoid content with respect to control observed. In contrast, cell protein and carbohydrate content increased gradually up to 12 h of UV-B exposure and decreased thereafter. However, even after 48 h of continuous irradiation with UV-B the values were still higher than those of the control (Fig. 1). The pigment and macro-molecular content of the respective pre-UV-B irradiated cells cultured in ASN-III medium under florescent light for 7 days were almost equal to the UV-B unexposed cultures.

The absorption spectra of the methanolic extract showed prominent absorption at 680 nm due to chlorophyll *a*, at 480 nm due to carotenoid and also at 360 nm due to MAAs (Fig. 2a). Spectroscopic data showed that, upon UV-B irradiation, the ratio of absorption of MAAs (360 nm): chlorophyll-*a* (680 nm) increased up to 12 h followed by a decline with increased duration of UV exposure. Absorption spectra of methanolic extract of respective pre-UV-B irradiated cells after culturing for 7 days showed additional absorption peaks at 526 nm and 327 nm (Fig. 2b) due to carotenoids and MAAs, respectively.

Changes in morphological features of the organism soon after irradiation with UV-B for different durations are shown in Fig. 3a–f. Upon continuous UV-B exposure up to 6 h there was almost no change in the morphology of the cells/trichome. However, coiling of the cells within the trichome was observed after 12 h of irradiation (Fig. 3b). With further increase in the duration of UV-B irradiation, the sheath layer became diffused after 24 h (Fig. 3c) and, after 48 h of irradiation, the trichome was fragmented into small cell groups and packed inside the sheath (Fig. 3f). The sheath of the control cells was almost hyaline, but the sheath layer became yellowish with an increase in the duration of UV-B exposure (Fig. 3d, e). Migration of the trichome from the sheath was also observed after 48 h of
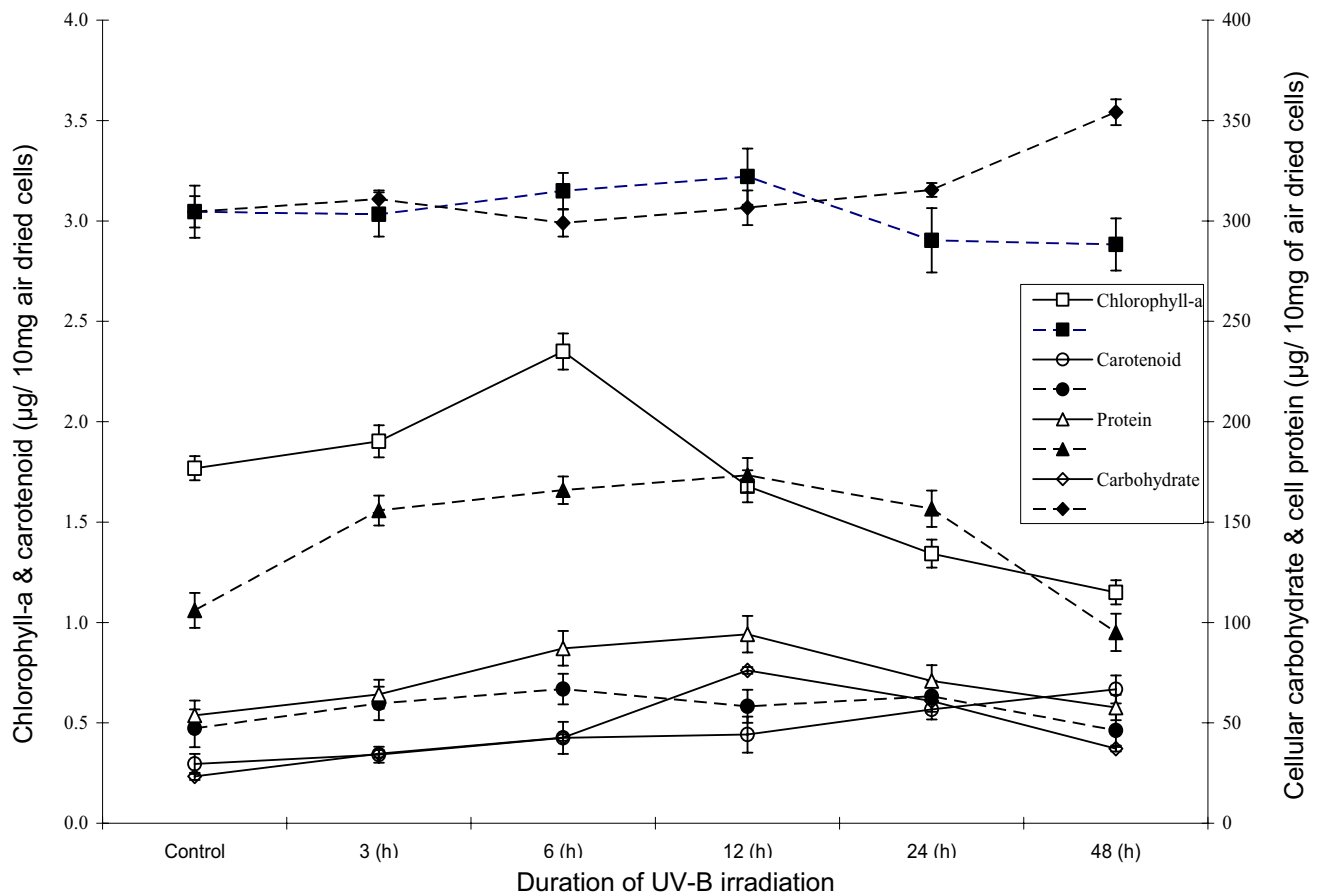

Fig. 1 Chlorophyll-*a*, carotenoid, cellular carbohydrate and cell protein content of *Lyngbya aestuarii* after exposure to UV-B for different durations (-, open) and after culturing the UV-B irradiated cells in ASN- III medium for up to 7 days under fluorescent light (− − − − ,closed). —□—, ┄■┄ chlorophyll- *a*; —○—, ┄●┄ carotenoid; ┄◇┄, —◆— carbohydrate; —△—, ┄▲┄ cell protein

Fig. 2 Absorption spectra of methanolic extract (90% v/v, 45°C, 1 min) of equal amount of *Lyngbya aestuarii* **a** after irradiation with UV-B for different times and, **b** after culturing the UV-B irradiated cells in ASN-III medium for up to 7 days under fluorescent light. *1* control, *2* 3 h, *3* 6 h, *4* 12 h, *5* 24 h, *6* 48 h, *(a)* chlorophyll-*a*, *(b)* carotenoid, *(c)* MAAs
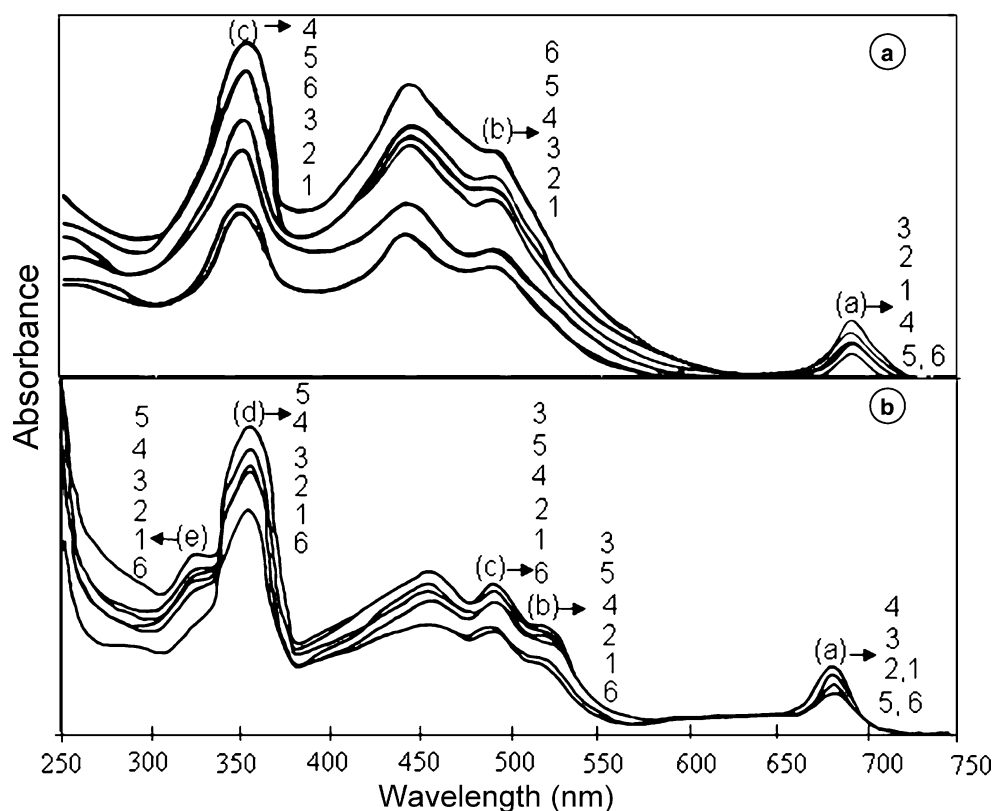

irradiation. Morphological features of 48-h pre-UV-B irradiated cells after culturing in fluorescent light (Fig. 3g–l) showed repair of damaged cells in a definite sequence. Soon after transferring the UV-B irradiated cells into control culture, small healthy trichomes without sheath developed first from the fragments (Fig. 3g, h). These trichomes then join in diverse patterns (Fig. 3i–k) forming a full grown filament. Upon culturing the 48-h pre–UV-B irradiated cells for 7 days under routine culturing conditions (control), the filaments showed almost normal structure, although the sheath layer was much wider (Fig. 3l) than the control (Fig. 3a).

Changes in SDS protein profile of *L. aestuarii* after different periods of UV-B irradiation were analyzed and the results are shown in Fig. 4. It was observed that the 20 kDa and 22 kDa proteins were repressed on UV exposure even for 3 h, and hence were the UV sensitive proteins. With exposure of the organism to 6 and 12 h of UV-B, several proteins, e.g., 84, 73, 60, 46, 40, 37 kDa, were over-produced, which possibly protected the cells from UV-B for the extended durations. After culturing the respective UV-B irradiated cells for different periods for 7 days under routine culturing conditions, the SDS protein profiles were almost identical, irrespective of the pre- UV-irradiation dosage, indicating repair of UV-injury in their subsequent culture in fluorescent light.

Discussion

Since cyanobacteria originated early, during the Precambrian era, i.e. before the existence of the present ozone shield, it is presumed that these organisms faced more intense solar UVR as compared to other phylogenetically much younger phototrophs. Therefore, these organisms must possess efficient mechanisms to prevent or counteract the harmful effects of solar UVR. *Lyngbya aestuarii* is a dominant organism in Chilika Lagoon, occuring in a free floating condition and as an epiphyte throughout the year and simultaneously exposed to the high solar incidence of a tropical country like India. Upon exposure to UV-B for different durations, the quantity of chlorophyll-*a* declined and the carotenoid content progressively increased. Carotenoids are well known for their antioxidant activity. In cyanobacteria, carotenoids occur in

Fig. 3 Photographs of *Lyngbya aestuarii* showing morphological features after irradiation with UV-B for different durations and after culturing the UV-B irradiated cells in ASN- III medium for up to 7 days under fluorescent light. *a* control, *b* 12 h, *c* 24 h, *d–f* 48 h; *arrow* indicates diffused sheath layer around the trichome and bundled cells packed inside the sheath. Scale bars a, b, e, f=10 μm, c, d=30 μm. *g* UV-B irradiated cells up to 48 h, *h* release of trichome after culturing the UV-B irradiated cells in ASN-III medium, *i–k* different types of joining of small fragments and full grown filaments after culturing the UV-irradiated cells up to 7 days. *Scale bars* g, h=30 μm, i–l=10 μm
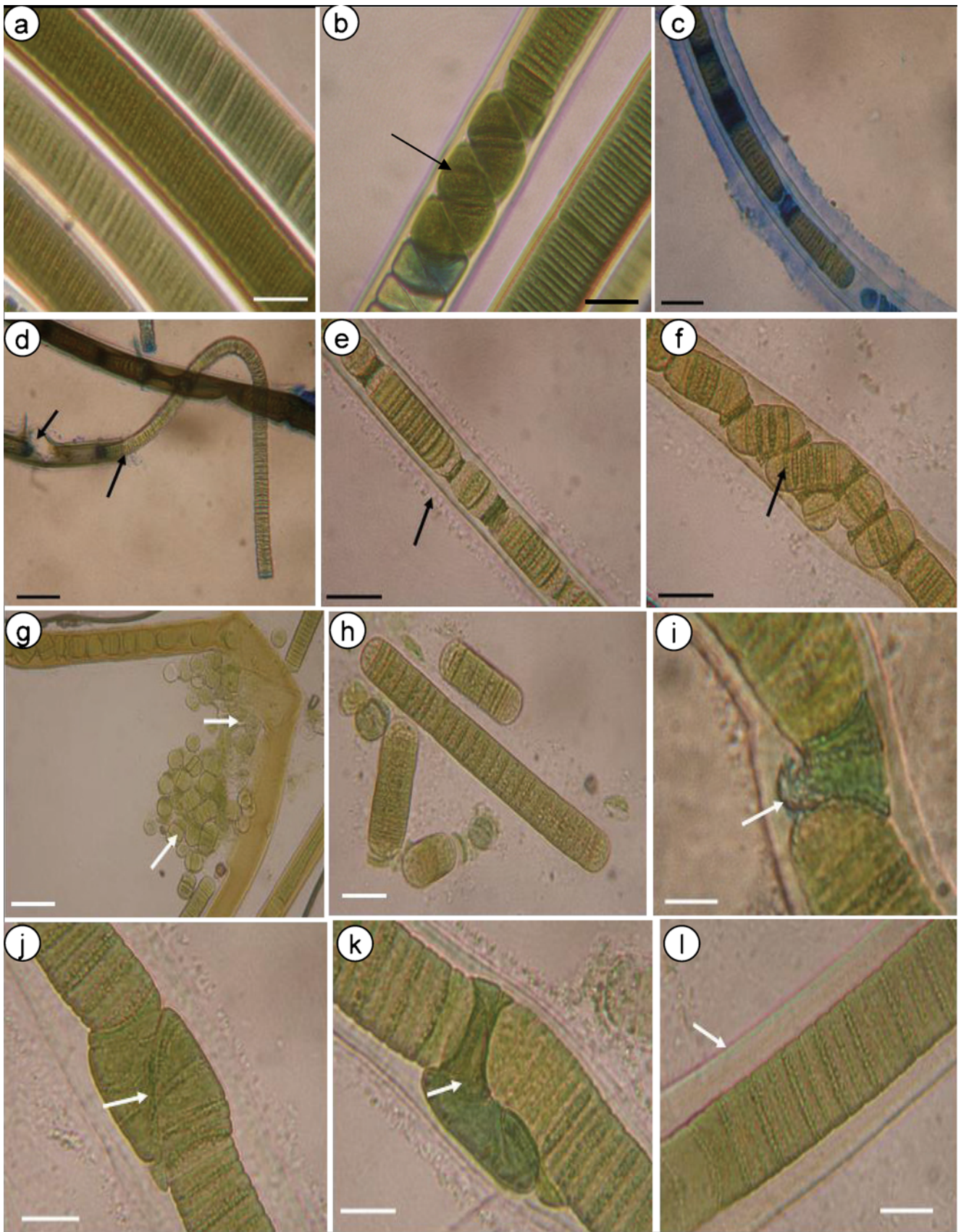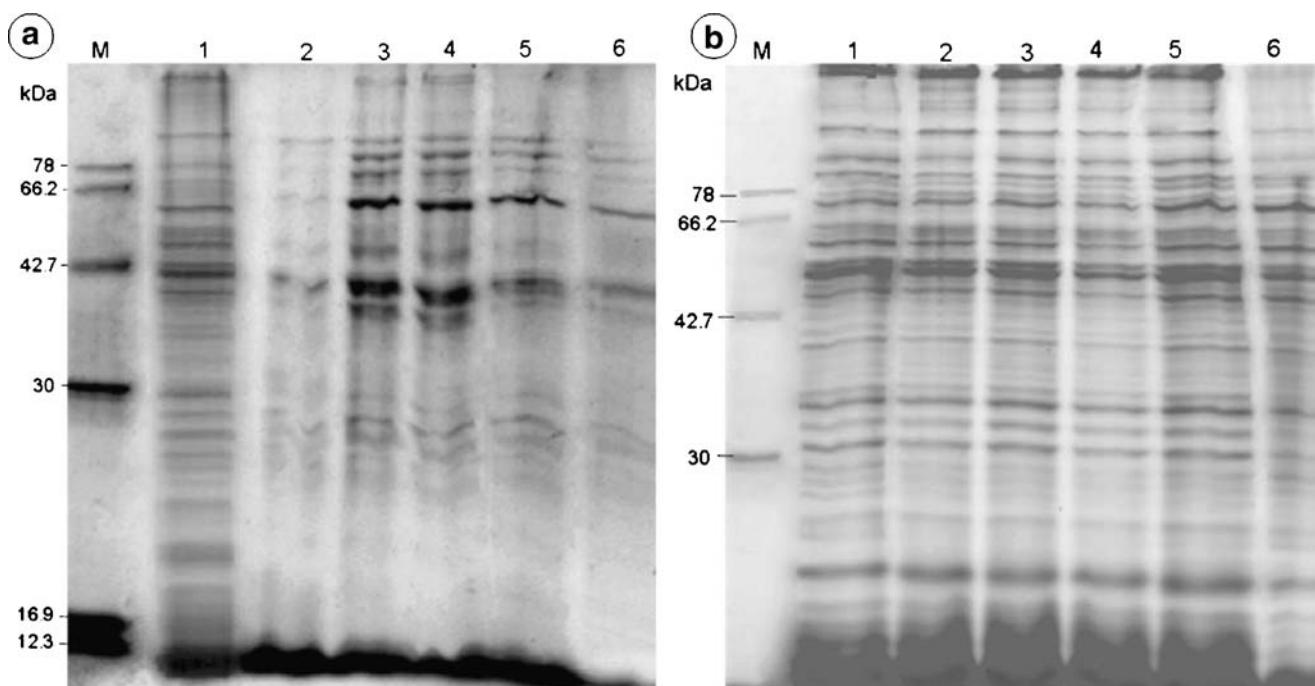
Fig. 4 Protein profile of *Lyngbya aestuarii* **a** after irradiation with UV-B for different durations and **b** after culturing the UV-B irradiated cells in ASN-III medium for up to 7 days under fluorescent light.

Lanes: *1* control, *2* 3 h, *3* 6 h, *4* 12 h, *5* 24 h, *6* 48 h. *M* Molecular masses of standard proteins in Kilodaltons

the outer cellular membrane as well as in thylakoids and, during long-term exposure to high natural or artificial irradiance, higher carotenoid to chlorophyll *a* ratios have been reported in other species of cyanobacteria (Paerl 1984; Ehling-Schulz et al. 1997; Quesada and Vincent 1997; Garcia-Pichel and Castenholz 1991; Ehling-Schulz and Scherer 1999). Synthesis of carotenoids is possibly an adaptive strategy of the organism to counteract the effect of Reactive Oxygen Species that might have been generated due to exposure to UV-B and solar irradiance (Xue et al. 2005). Though UV-B is a small component of the solar spectrum (<1% energy) it is highly reactive and therefore might have induced chlorophyll *a* synthesis as well as an increase in cellular macromolecules with immediate exposure (up to 6 h). However, with a further increase in the duration of UV-B irradiation the contents were reduced. In contrast, the absorption ratio of MAAs (360 nm): chlorophyll *a* (680 nm) increased after a longer period of UV-exposure. Several studies have shown that MAAs act as a potential UV-sunscreen by absorbing harmful radiation providing protection to the cells from UV injury (Scherer et al. 1988; Ehling-Schulz et al. 1997; Ehling-Schulz and Scherer 1999). Cells with high concentrations of MAAs were reported to be approximately 25% more resistant to UV-B than those with no or low concentrations of MAAs (Garcia-Pichel and Castenholz 1993). Hence, synthesis of MAAs in *L. aestuarii* upon UV-B exposure may be advantageous for this organism by giving protection to high solar irradiation.

Upon UV exposure for different periods, certain proteins, e.g., 20 and 22 KDa, were selectively repressed and a few others were overproduced, conferring protection to the cells from UV injury. The cyanobacterium *Synechocystis* sp. showed a capability for *de novo* formation of D1 and D2 proteins, the key proteins in photosystem II (PSII) in response to UV stress (Vass et al. 2000). The terrestrial cyanobacterium, *Tolypothrix byssoidea*, also induced 43, 49 and 58 kDa proteins upon UV exposure, conferring protection to the organism to survive prolonged UV exposure (Adhikary 2003). Several other reports have shown that induction and overproduction of specific proteins are closely correlated with the development of stress tolerance (Close and Lammers 1993; Hill et al. 1994), and are important in the maintenance of vital cellular functions of the organisms when subjected to environmental stress conditions existing in the natural habitats. Despite these reports on the widespread existence of stress proteins, very little is known about their precise physiological function. One of the major functions of the stress proteins is to enable the organism to cope with the stressor. However, with the existing information, it is difficult to demonstrate unambiguously that a particular observed change in protein expression contributes to the survival process.

Changes in morphological features with response to UV-B show interesting results. *Lyngbya aestuarii* tolerated a wide duration of UV-B exposure. With UV-B exposure for 6 h

there was almost no change in morphological features. However, with further increase in the radiation dose, coiling of the cells within the trichome was observed. Changes in the morphological features in another cyanobacterum, *Arthrospira platensis*, from alteration of helical orientation to development of straight forms with decreasing helix pitch to a more compact structure was observed after UV-B exposure (Wu et al. 2005). This was suggested as an effective protective mechanism from photo-inhibition resulting from self-shading of the cells. However, the results of the present investigation, with formation of cell coiling in *L. aestuarii* and fragmented trichomes with small groups of cells packed inside the sheath with increased duration of UV exposure, seems like a hibernation process, which has not been reported in cyanobacteria. Further, diffusion of the sheath layer, breakage of sheath and finally the escape of trichome from the sheath (Fig. 3c, d) after 48 h of UV-B irradiation might also be an efficient strategy of this organism to avoid long-term exposure to UV-B.

Exposure of living cells to UVR has been shown to cause several types of DNA lesions. However, these were reported to be repaired by photoreactivation and excision repair mechanisms (Rozema et al. 2002). Such a process was also observed in *L. aestuarii* from the fact that the pigment and macromolecular content, and the morphological alterations, were considerably revived after culturing of UV-B irradiated cells under florescent light. These results suggest that *L. aestuarii* occurring in Chilika Lagoon possesses efficient survival strategies to cope with UV-B stress. In addition to the increase in carotenoids and MAAs in the cells upon increased duration of UV-B exposure, the trichomes became coiled with formation of several separating discs, finally broke up into small fragments embedded with in a sheath, and hibernated waiting for the onset of favorable conditions. Upon culture of the UV irradiated cells, the sheath broke, releasing the fragmented trichomes that finally developed into full grown filaments. Such a hibernation mechanism to survive UV-B stress as observed in *L. aestuarii* is uncommon in cyanobacteria.

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