

ORIGINAL ARTICLE

A niche for cyanobacteria producing chlorophyll *f* within a microbial mat

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Acquisition of additional photosynthetic pigments enables photosynthetic organisms to survive in particular niches. To reveal the ecological significance of chlorophyll (Chl) *f*, we investigated the distribution of Chl and cyanobacteria within two microbial mats. In a 7-mm-thick microbial mat beneath the running water of the Nakabusa hot spring, Japan, Chl *f* was only distributed 4.0–6.5 mm below the surface, where the intensity of far-red light (FR) was higher than that of photosynthetically active radiation (PAR). In the same mat, two ecotypes of *Synechococcus* and two ecotypes of Chl *f*-producing *Leptolyngbya* were detected in the upper and deeper layers, respectively. Only the *Leptolyngbya* strains could grow when FR was the sole light source. These results suggest that the deeper layer of the microbial mat was a habitat for Chl *f*-producing cyanobacteria, and Chl *f* enabled them to survive in a habitat with little PAR.

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Introduction

Light quality is one of the most important drivers in niche differentiation of phototrophs, and the spectrum of available light determines the distribution and community composition of photosynthetic organisms in natural environments. These niche differentiations are generally caused by variations in pigment composition and/or photosynthetic apparatus, which have diverged to adapt to various colors of light. Close relationships between the absorption spectra of photosynthetic organisms and the surrounding light environments and niche differentiation have been reported in many previous studies, such as those observed in *Synechococcus* with different types of phycobilisomes (Wood *et al.*, 1998; Stomp *et al.*, 2007), planktonic bacteria with green and blue types of proteorhodopsins in ocean water columns (Béjà *et al.*, 2001; Sabehi *et al.*, 2007), and cyanobacteria containing chlorophyll (Chl) *a* and *b* (*Prochloron*) and Chl *d* (*Acaryochloris*) coexisting with colonial ascidians (Kühl *et al.*, 2005).

Chl *f* is a newly discovered red-shifted Chl with an absorption peak in the far-red light (FR) region (Chen *et al.*, 2010). The first reported Chl *f*-containing organism was a filamentous cyanobacterium, *Halomicronema hongdechloris*, isolated

from stromatolites in Australia (Chen *et al.*, 2012). To date, this strain has been used to study the extinction coefficient (Li *et al.*, 2012), biochemical structure (Willows *et al.*, 2013) and biophysical characteristics (Li *et al.*, 2013; Tomo *et al.*, 2014; Akimoto *et al.*, 2015) of Chl *f*. Several other cyanobacteria producing Chl *f* have also been found from various environments. A unicellular cyanobacterium strain, KC1, was isolated from Lake Biwa in Japan (Akutsu *et al.*, 2011; Miyashita *et al.*, 2014), and a phylogenetically related species was found in biofilms at Jenolan Caves in Australia (Behrendt *et al.*, 2015). The *Leptolyngbya* sp. strain JSC-1 from a hot spring in Yellowstone National Park in the United States (Gan *et al.*, 2014) and *Chlorogloeopsis fritschii* from a rice paddy in India (Airs *et al.*, 2014) were reported to contain both Chl *d* and Chl *f*.

Some previous studies have reported that Chl *f* in various cyanobacterial species was reversibly induced and decreased in laboratory experiments (Chen *et al.*, 2012; Airs *et al.*, 2014; Gan *et al.*, 2014; Miyashita *et al.*, 2014). In all newly discovered cyanobacterial strains, Chl *f* was produced when their cells were grown under FR as the sole light source and decreased to an undetectable level under light conditions of white or another color. Five previously identified cyanobacterial strains were also reported to accumulate Chl *f*, as well as to synthesize a small amount of Chl *d* and FR shifted phycobiliproteins when they were cultured under FR (Gan *et al.*, 2015). The mechanism of this reversible switch in pigmentation was referred to as FaRLiP (far red light photoacclimation). Some

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key genes of this FaRLiP response and the Chl *f* synthesis enzyme have previously been identified (Zhao *et al.*, 2015; Ho *et al.*, 2016). The available data suggest that the distribution of Chl *f* in natural environments must be restricted to habitats in which FR dominates, such as soils, under the plant canopy, microbial mats and aquatic blooms (Gan and Bryant, 2015). Indeed, the presence of Chl *f* in nature has recently been confirmed in cyanobacterial biofilms located in a cave environment and beachrock in Australia (Behrendt *et al.*, 2015; Trampe and Kühl, 2016).

The light spectra in natural and artificial microbial mats have been measured (Pierson *et al.*, 1990; Boomer *et al.*, 2000; Kühl and Fenchel, 2000; Ferris *et al.*, 2003) and found that photosynthetically active radiation (PAR) decreased rapidly within a few millimeters through its absorption by cyanobacteria and/or anoxygenic photosynthetic bacteria living in the mats. Light with wavelengths longer than 700 nm remained, even in deeper layers of the mats. Based on these observations, we expected Chl *f*-producing cyanobacteria to be distributed in microbial mats and Chl *f* to be produced in deeper layers of the mat, where organisms use FR for photosynthesis. The Nakabusa hot spring in Japan is famous for its thick, colorful microbial mats that contain cyanobacteria (Nakagawa and Fukui, 2002; Kubo *et al.*, 2011). The present study was conducted to reveal the niche of Chl *f*-producing cyanobacteria and the ecological role of Chl *f* in microbial mats from this hot spring. We investigated the spectra and strength of penetrated light, the vertical distribution of Chl, and the community structure of cyanobacteria at each depth of the mat. After isolating the cyanobacteria from the mat, we also evaluated the presence of Chl *f* and growth characteristics of those isolates under white light (WL) and FR.

Materials and methods

Sampling

We collected microbial mat samples from the Nakabusa hot spring, Nagano, Japan (36.394367 N, 137.747183 E), on 14 October 2012. At this site, dense colorful microbial mats are formed on the wall of the sand control dam, on which hot spring water falls continuously. Two pieces of green-colored mats Nb3 and Nb4 (ca 5 × 10 cm) were cut from the wall with a knife and put in different plastic containers. The distance between these two mat samples was ~10 cm, and their *in situ* temperatures were 49.9 °C and 46.8 °C, respectively. The samples were transported to the laboratory under dark, ambient temperature conditions and stored in a refrigerator at 4 °C for 2 days until the culturing experiment was carried out, after which they were frozen at -20 °C for further experiments.

Measurement of light spectra in microbial mats

The downward spectral transmittances as a function of depth in the microbial mats were measured using a USB4000 spectrophotometer (Ocean Optics, Inc., Dunedin, FL, USA) with a fiber optic tip (Pierson *et al.*, 1990). Solar spectral irradiance was measured at the sampling site with USB4000 at 10:00, 17 June 2012. The downward spectral irradiances in the microbial mats were calculated from these data. The light environments in the microbial mats at different times of day and under different weather conditions were also estimated. For those calculations, we used the solar radiation spectra data collected at Gifu (~140 km away from Nakabusa) by the New Energy and Industrial Technology Development Organization (NEDO) for 1 week before sampling the mats.

Pigment composition analyses

The frozen microbial mats were cut into a rectangular parallelepiped, whose base was 1 cm square, and embedded in 2% agarose gel. The gel blocks were then bored 19 mm in diameter, frozen again at -20 °C and sliced horizontally into sections 0.5 mm thick from the surface down to 6.5 mm (Nb3) and 4.0 mm (Nb4) using a simple microtome (Artec Co. Ltd, Osaka, Japan). Pigments were extracted twice from each freeze-dried section with 100 µl of methanol for 15 min by sonication in the dark. Next, a total of 200 µl of extract was centrifuged for 5 min at 16 000 g, after which its supernatant was analyzed by reverse phase high performance liquid chromatography using a Spherisorb 5 µm ODS2 column (4.6 × 250 mm, Waters, Milford, MA, USA) at 40 °C and a methanol–water–acetone gradient program (2.5 ml min⁻¹, 0–10 min linear gradient 90–100% methanol, 10–20 min 100% methanol, 20–22 min linear gradient methanol to acetone, 22–23 min 100% acetone). The absorption spectra of eluted pigments (350–800 nm) were obtained by using a Waters 2996 PDA detector (Waters, Milford, MA, USA). Chls *a* and *f* and BChl *a* were eluted at retention times of 9.8, 8.3 and 7.3 min, respectively. The specific absorption coefficients of 78.36 (665.5 nm), 78.36 (707 nm) and 60 (771 nm) L g⁻¹ cm⁻¹ in methanol were used to calculate the relative amounts of Chl *a*, Chl *f* and BChl *a*, respectively (Namsaraev, 2009; Li *et al.*, 2012).

Microscopy

Cross-sectioned images of the microbial mat Nb3 were acquired using a BX51 microscope equipped with a U-MWU2 mirror unit and a DP72 digital camera (OLYMPUS, Tokyo, Japan). The range of excitation wavelengths was 330–385 nm and fluorescence at >420 nm could be observed. Cells of the newly isolated cyanobacteria were observed using an ECLIPSE 80i microscope and a DS-5Mc digital camera (Nikon, Tokyo, Japan).

PCR-DGGE

DNAs were extracted from each horizontally sliced and pigment-extracted section of mats using the method described by Ohkubo and Miyashita (2012). PCR and denaturant gradient gel electrophoresis (DGGE) were performed with the Cyanobacteria-specific primer set CYA353F-CYA781R as described in Ohkubo *et al.* (2006), except for the concentrations of polyacrylamide (9%) and denaturant (30–70%) in gel, and the electrophoresis conditions (60 °C, 100 V, 13 h). The gel was stained with SYBR Gold Nucleic Acid Gel Stain (Life Technologies Japan, Tokyo, Japan) and photographed on a Visi-Blue transilluminator (LLC., Upland, CA, USA) with a CCD camera AE-9000 N E-Graph (ATTO CORPORATION, Tokyo, Japan).

Isolation of cyanobacteria

A piece of mat Nb3 was homogenized in a microtube containing BG11 medium using sterile pestles. Aliquots of homogenates were inoculated to liquid BG11 medium in two 24-well plastic culture plates that were placed in 22 °C or 35 °C under FR LEDs as a sole light source. After 3 months, cyanobacteria that had increased under these conditions were isolated under microscopy were transferred to new media with sterile pipette tips and cultured under FR or WL.

Sequencing and phylogenetic analyses

Sequencing of DGGE bands was performed as described in Ohkubo and Miyashita (2012). DNA extraction from cyanobacterial strains was conducted as described above. Two primer sets, CYA359F-1510R and 9F-CYA781R (Nübel *et al.*, 1997; Ahmed *et al.*, 2007), were used for PCR amplification of 16S rDNA fragments from strains Nb3F1 and Nb3U1. In the case of strain Nb3F2, a Bacteria-universal primer set, 341 F-907 R (Nakagawa and Fukui, 2002) was used. Direct sequencing was performed with the primers 9 F, 341 F, CYA359F, 515 F, 536 R, CYA781R, 907 F, 907 R, 936 R and 1510 R (Nübel *et al.*, 1997; Ahmed *et al.*, 2007; Nakagawa and Fukui, 2002). The sequences obtained were compared with GenBank sequences by NCBI BLAST searches (Altschul *et al.*, 1990) and deposited in the DDBJ database under accession numbers LC192821–LC192830, LC270317. These sequences and 68 known cyanobacterial sequences were aligned using PyNAST (Caporaso *et al.*, 2010), and the phylogenetic relationship was analyzed using the maximum likelihood method in the RAxML program (Stamatakis, 2014) with the GTRGAMMA model and a rapid bootstrap procedure (500 replicates). The constructed tree was visualized using FigTree ver. 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Growth experiment

Cyanobacterial strains Nb3U1, Nb3F1 and Nb3F2 were pre-cultured in BG11 medium (pH 8.0) with aeration under continuous irradiation (110 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$) provided by a WL LED (Color Changing LED Light Bulb, Lighting EVER, Las Vegas, NV, USA). After 20–30 days, pre-cultured cells were harvested and homogenized using plastic pestles. The same volumes of homogenates were then inoculated into glass tubes containing 50 ml of BG11 and cultivated for 30–35 days with aeration. All three strains were incubated under 110 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ provided by WL LED and FR LED (MR60L-5LED, Aster, Toyohashi, Japan), and Nb3F1 and Nb3F2 were incubated in the dark, too. Each experiment was triplicated. The emission spectra of LEDs are shown in Supplementary Figure S1. Strains Nb3U1 and Nb3F1 were cultured at 45 °C, whereas Nb3F2 was cultured at 25 °C. Cell growth was monitored by measuring the OD₇₈₀ with a V-650 spectrophotometer (JASCO, Tokyo, Japan).

Results

Penetrating light spectra and distribution of Chls within the microbial mats

The spectral compositions and the photon flux density of the downward irradiance changed with depth within the microbial mats (Figure 1). The on-site solar radiation was 1.4–3.3 $\mu\text{mol photon m}^{-2} \text{s}^{-1} \text{nm}^{-1}$ over the wavelength range of 400–900 nm. There were some attenuation peaks in the spectra within the microbial mats. Those peaks could be assigned to light absorption by photosynthetic pigments such as Chl *a* (440 and 678 nm), phycobiliproteins (588 and 615 nm), BChl *a* (802 and 880 nm) and BChl *c* (742 nm) based on previous reports (Boomer *et al.*, 2000; Ferris *et al.*, 2003). The results showed that the PAR (400–700 nm) was depleted within both microbial mats, and the depletion was faster than that of FR (700–750 nm), indicating that FR of certain strengths could penetrate deeper layers (Figure 1). Although PAR (813.4 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$) was 5.4 times higher than that of FR (149.3 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$) at the surface, the photon flux density of FR exceeded that of PAR at a depth of 3.5 mm in Nb3 (PAR; 0.185, FR; 0.223 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$) and 2.5 mm in Nb4 (PAR; 2.808, FR; 1.767 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$). Although the solar radiation spectra changed depending on the time and the weather for one week before sampling of mats, the calculated light spectra within the mats did not differ significantly (Supplementary Figure S2).

Chl compositions in each horizontal serial section (0.5 mm thickness) of both microbial mats are shown in Figure 2. The amounts of Chl *a*, BChl *a* and Chl *f* in each section were expressed as relative values with respect to the amount of Chl *a* in the surface (0–0.5 mm) section of each mat. In mat Nb3, the Chl *a*

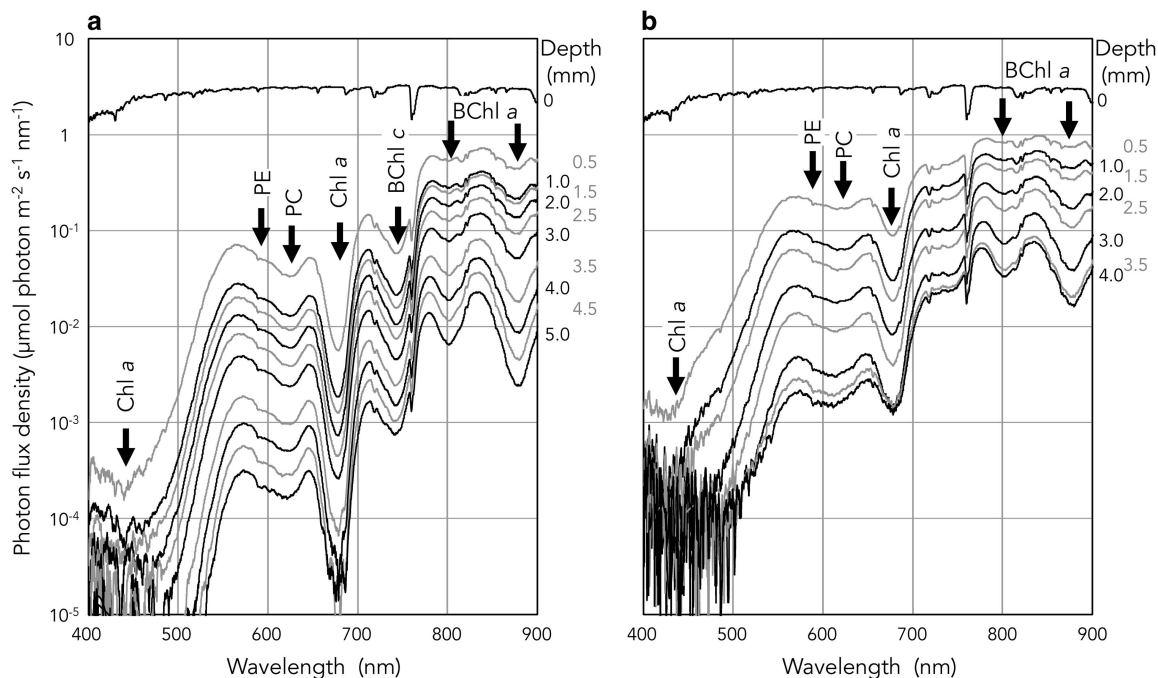


Figure 1 Downward irradiance spectra with depth in microbial mats Nb3 (a) and Nb4 (b) from Nakabusa hot spring. Arrows indicate major attenuation peaks from absorption of photosynthetic pigments (Chl a, 440 and 678 nm; phycoerythrin (PE), 588 nm; phycocyanin (PC), 615 nm; BChl c, 742 nm; BChl a, 802 and 880 nm).

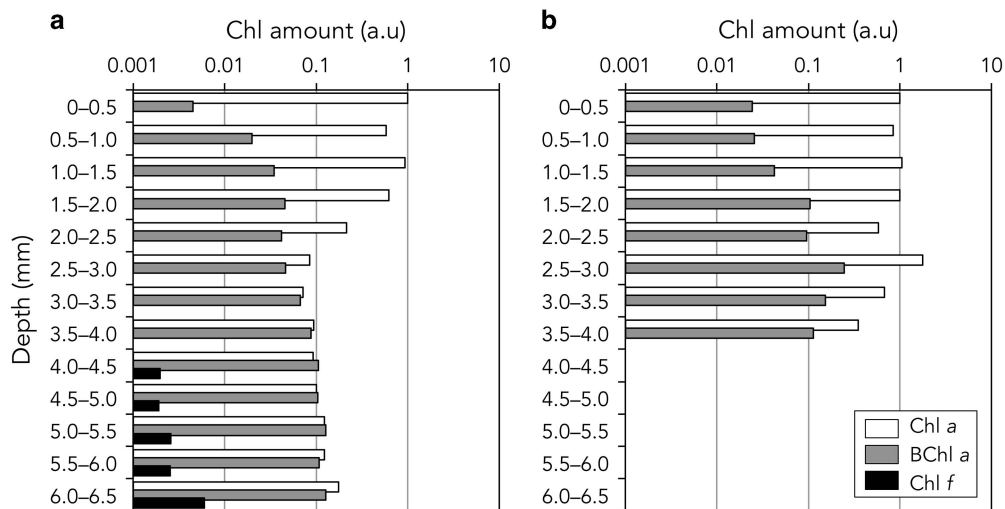


Figure 2 Relative amounts of chlorophyll in each 0.5 mm thick horizontal section of microbial mats Nb3 (a) and Nb4 (b). This graph shows relative amounts of chlorophyll a (white), bacteriochlorophyll a (gray) and chlorophyll f (black).

content was similar from the surface to 2 mm in depth. The content in sections deeper than 2.5 mm were less than one-tenth that of the surface. BChl a increased in proportion to depth, with the relative amount of BChl a in the 2.5–3.0 mm section being 10 times higher than that in the 0–0.5 mm section. Chl f was only detected from five sections deeper than 4.0 mm. The amounts of Chl f detected were 1.9–3.4% of those of Chl a detected at each depth. In the mat Nb4, Chl a content varied in the range of 35.2–176% from the surface to bottom. BChl a gradually increased from the surface to deep layers.

Chl f were below the detection limits in all sections of the mat.

Vertical distribution of cyanobacteria within the microbial mats

The vertical distribution of cyanobacteria in microbial mat Nb3 was observed by both microscopy and PCR-DGGE analysis. The mat appeared to consist of four layers, a bright-green surface layer, a glossy white or gelatinous brown layer, a black-green layer and a white or brown layer that was similar to the

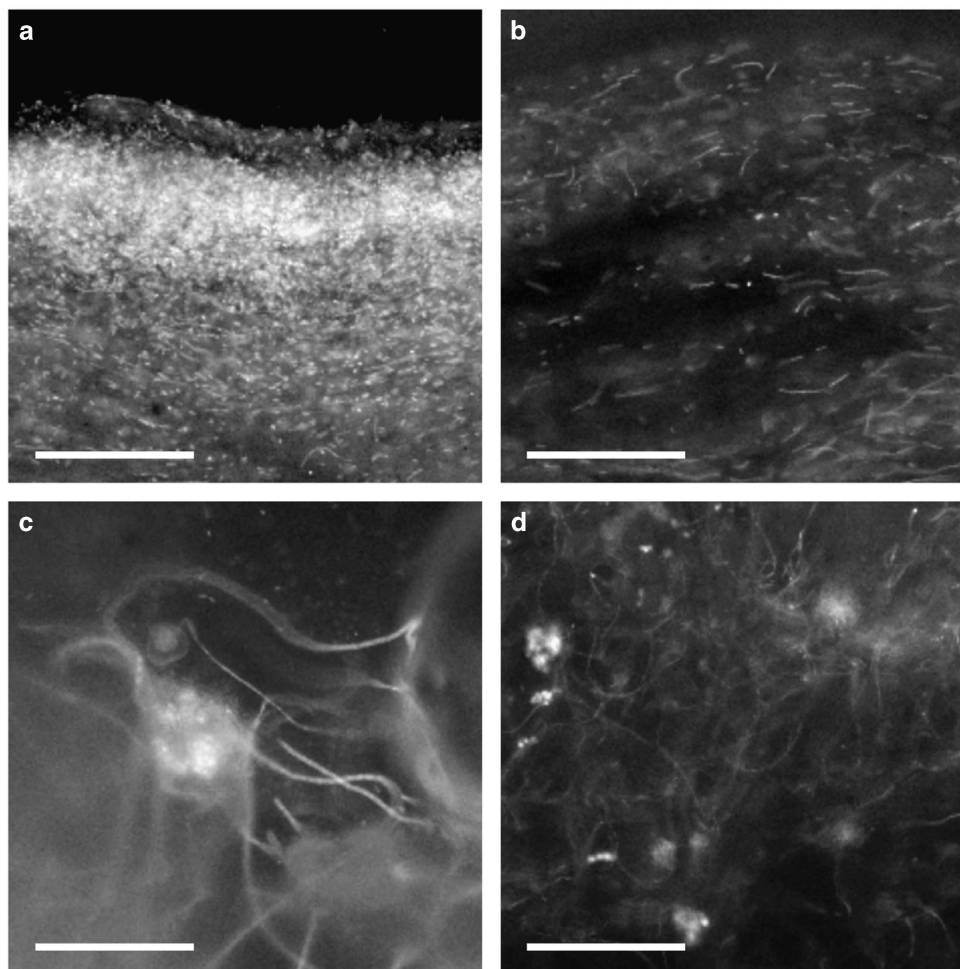


Figure 3 Images from vertical sections of microbial mat Nb3 shown under epifluorescence microscopy at depths of 0–0.2 mm (a), 0.4–0.7 mm (b), 4.8–5.0 mm (c) and 6.4–6.7 mm (d). Scale bars indicate 100 μm .

second layer. Upon microscopic observation, strong red autofluorescence was only observed at the surface of the mat (Supplementary Figure S3). Cells observed in this layer were unicellular (0.8–3 μm in diameter) and rod shaped or elongated (0.8–1 μm in diameter and 5–30 μm in length) (Figures 3a and b). The cells were scattered within the surface layer of the mat. Below the greenish surface layer, the green color and the red autofluorescence weakened with increasing depth (Supplementary Figure S3). Although the clearly visible green color was not observed in the mat at depths of > 1.0 mm upon light microscopy, weak red fluorescence was observed at depths of 4.0–7.0 mm under fluorescence microscopy. Cells in these layers were long, thin filaments 1–3 μm in diameter and > 100 μm in length that were loosely tangled together (Figures 3c and d).

The PCR-DGGE profile showed that mat Nb3 contained four dominant cyanobacteria (Figure 4a). Two cyanobacteria (band3 and band4) were predominant at depths of 0–4.0 mm, whereas two others (band1 and band2) were detected in deeper layers of the mat. The total intensity of band1 and band2 accounted for 17% of all bands in the 4.0–

4.5 mm section, then increased to 70% with increasing depth. Four cyanobacteria detected by PCR-DGGE were phylogenetically distinct from one another. Cyanobacteria from band1 and band2 were independently clustered with filamentous cyanobacteria in the phylogenetic tree (Figure 5). Band1 was closely related to *Leptolyngbya* sp. 0413, whereas band2 was clustered with *Leptolyngbya* sp. 0412 and *Pseudanabaena* sp. 0411. They were isolated from Russian hot springs (Sorokovikova *et al.*, 2008). Band3 was clustered within the *Synechococcus* T1 clade containing strain CR_L35 and the uncultured clone YCB100, which were originated from hot springs in Costa Rica and Tibet, respectively (Lau *et al.*, 2009; Ward *et al.*, 2012). Band4 was also closely related to thermophilic *Synechococcus* spp. strains SH-94-5 and C9, which were both isolated from hot springs in North America (Ferris *et al.*, 1996; Miller and Castenholz, 2000). These results show that at least two species of unicellular *Synechococcus* (band3 and band4) were predominant cyanobacteria in the surface to middle layers of mat Nb3 (0–4.0 mm), and that two species of filamentous *Leptolyngbya*

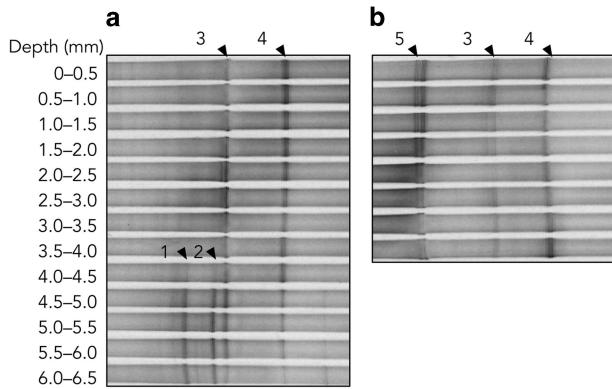


Figure 4 Vertical distribution of cyanobacterial phylotypes detected by PCR-DGGE within microbial mats Nb3 (a) and Nb4 (b). DNA was extracted from each 0.5 mm thick horizontal section of the microbial mat shown in Figure 2, after pigment extraction.

(band1 and band2) were present in the deep layer of the same mat (> 4.0 mm).

Three DGGE bands were detected from mat Nb4 (Figure 4b). Two bands were identical to band3 and band4 in mat Nb3, whereas band5 was detected from all depths. Although two bands were present at the position of band5, the DNA sequences of both bands were identical. Band5 showed an identical sequence to some unicellular cyanobacterial strains containing the thermophilic cyanobacteria, *Thermosynechococcus elongatus* BP-1 and *Thermosynechococcus* sp. NK55 (Figure 5), which were isolated from Beppu and Nakabusa hot springs in Japan, respectively (Nakamura *et al.*, 2002; Stolyar *et al.*, 2014). Conversely, neither of the *Leptolyngbya* spp. (band1 and band2) were detected from the mat. Therefore, the dominant cyanobacteria in mat Nb4 were three unicellular thermophilic cyanobacteria, which were all distributed from the surface to the bottom of the mat.

Characteristics of isolated cyanobacteria

To reveal which cyanobacteria detected by PCR-DGGE produced Chl *f* in mat Nb3, we cultured and isolated cyanobacteria from the mat. Analysis of the cultured organisms revealed three strains, Nb3U1, Nb3F1 and Nb3F2. Strain Nb3U1 was a blue-green (under both WL and FR LEDs) ellipsoidal or rod-shaped unicellular cyanobacterium 1.5–1.9 μm in diameter and 3.0–5.7 μm in length (Figure 6a). Strain Nb3F1 was a blue-green (under WL) or yellow green (under FR) thin filamentous cyanobacterium 1.7–2.0 μm in width (Figure 6b). Strain Nb3F2 was filamentous, 1.6–1.8 μm in width and blackish green and green under WL and FR, respectively (Figure 6c). Trichomes of both filamentous strains were not constricted at the cross walls nor coiled into aggregates or biofilms in flasks. Phylogenetic analysis showed that the 16S rDNA sequence of strain Nb3U1 was closely related to that of DGGE band3 (99.2%, 381/384). The 16S rDNA sequence of strains Nb3F1 and Nb3F2 revealed identical or similar

sequences to that of DGGE band1 (100%, 386/386) and band2 (97.2%, 344/354), respectively. Based on these observations, these three strains were identified at the genus level as *Synechococcus* sp. Nb3U1, *Leptolyngbya* sp. Nb3F1, and *Leptolyngbya* sp. Nb3F2.

When the three isolated strains were cultured under FR as the sole light source, Chl *f* accumulated in the cells of *Leptolyngbya* spp. strains Nb3F1 and Nb3F2, but not in *Synechococcus* sp. Nb3U1. The absorption spectra of strain Nb3U1 cells grown under WL and FR were almost identical (Figure 6d). However, there was a distinct shoulder at ~715–750 nm in the absorption spectra of strains Nb3F1 and Nb3F2 cells grown under the FR (Figures 6e and f). Absorptions at 450–500 nm were also enhanced, but they decreased under FR at 550–650 nm, and the peak at 568.5 nm in Nb3F2 disappeared completely. High performance liquid chromatography analyses also showed that strain Nb3F1 accumulated Chl *f* when the cells were grown under FR, whereas no peak in Chl *f* was detected from strain Nb3U1 (Supplementary Figure S4).

The growth characteristics of these cyanobacteria were compared under WL and FR. *Synechococcus* sp. strain Nb3U1 grew under WL, but showed almost no growth under FR (Figure 6g). The maximum specific growth rate (μ_{max}) was 0.40 ± 0.01 per day, and cell growth reached a plateau in ~1 month under WL. Conversely, *Leptolyngbya* spp. grew under both colors of light, and did not grow in the dark (Figures 6h and i). The lag phase of cell growth under FR was 3–5 days longer when compared with that of cells grown under WL. The growth rate under FR was slower than that under WL. The μ_{max} of *Leptolyngbya* sp. strain Nb3F1 under WL and FR was 0.30 ± 0.05 and 0.26 ± 0.02 per day, respectively. Cell densities plateaued in ~20 days under both light conditions, and the OD_{780} in the stationary phase under FR was ~80% of that under WL. In the case of *Leptolyngbya* sp. strain Nb3F2, the μ_{max} under FR (0.21 ± 0.02 per day) was ~50% of that under WL (0.42 ± 0.05 per day). However, the density of cells grown under FR rose to approximately the same level as that of cells grown under WL about 10 days later (Figure 6i).

Discussion

We investigated the distribution of Chl *f* within microbial mats in a stream flowing from the hot spring at Nakabusa, Japan. In a 7-mm-thick microbial mat (Nb3), we only detected Chl *f* from 4.0–6.5 mm below the surface (Figure 2), where the photon flux density of FR was higher than that of PAR (Figure 1). Chl *f* may be produced to use the FR that can reach to deeper layers of the mat. Our findings confirm an exception that Chl *f* is distributed in microbial mats as described in a previous study (Gan and Bryant, 2015).

The vertical distribution of cyanobacterial species and Chl relative to light quality at each depth showed that the habitat of Chl *f*-producing cyanobacteria is an environment dominated by FR.

Microbial mat Nb3 contained four dominant species of cyanobacteria, two *Leptolyngbya* species (DGGE band1 and band2) and two *Synechococcus* species (band3 and band4). Of these, *Synechococcus* was

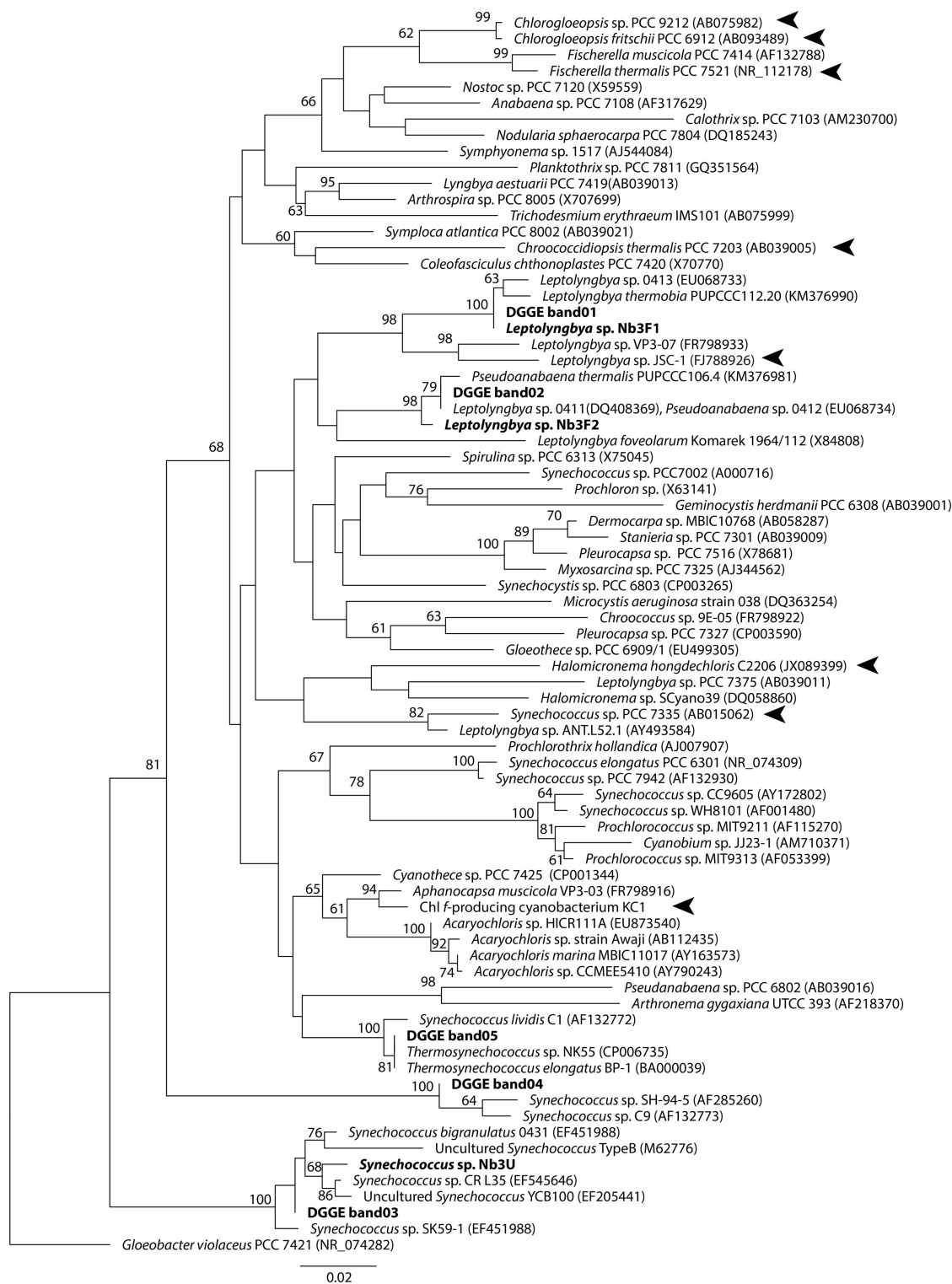


Figure 5 Phylogenetic relationship of sequences obtained from PCR-DGGE bands in Figure 4 and strains newly isolated in this study. Bold text indicates the bands and strains isolated from the microbial mat, and arrowheads indicate the sequences of reported Chl *f*-producing cyanobacteria. Numbers at each node show the bootstrap values represented as a percentage. Nodes without any values received <60% support.

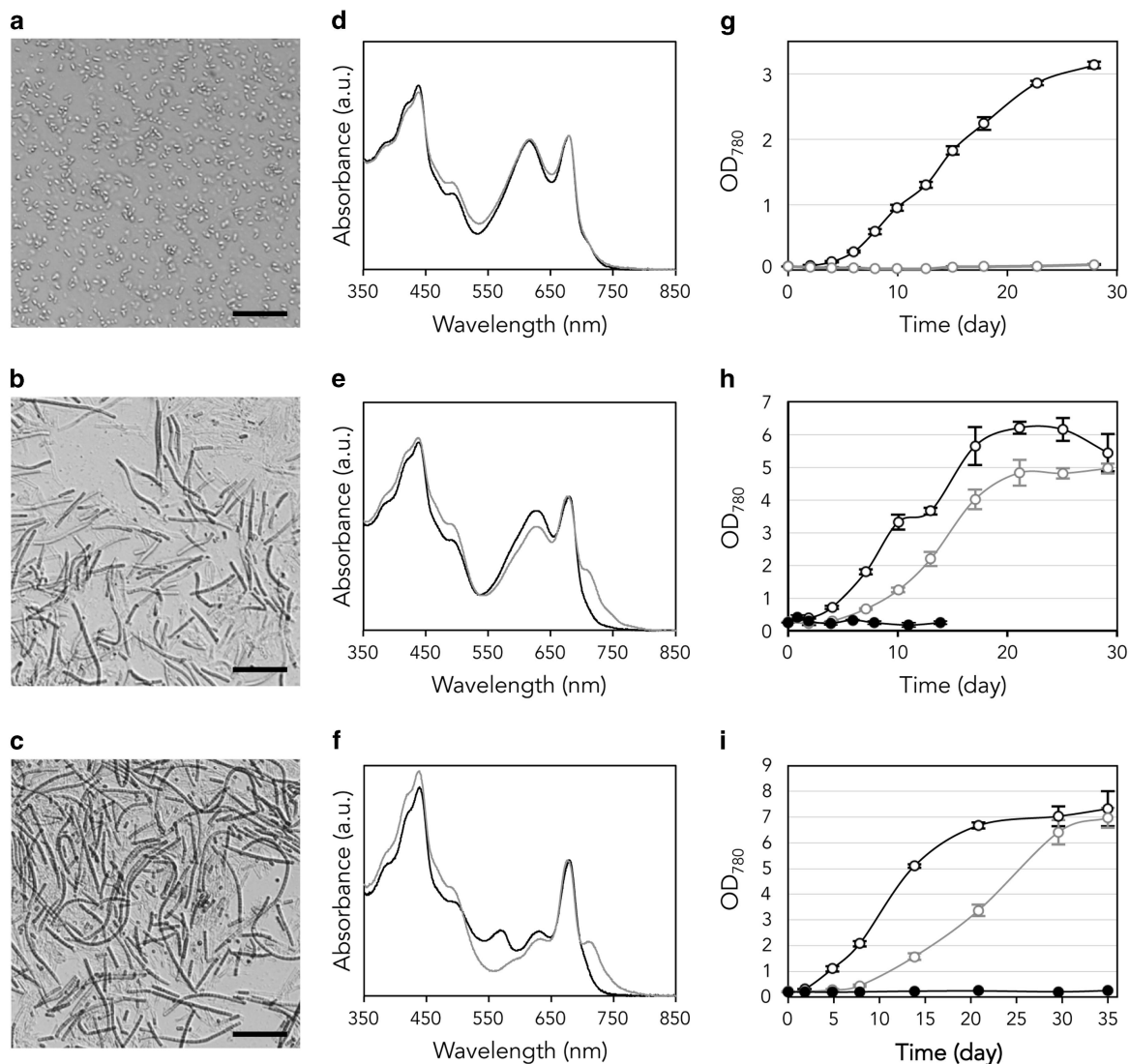


Figure 6 (a–c) Microscopic observation of *Synechococcus* sp. strain Nb3U1 (a), *Leptolyngbya* spp. strain Nb3F1 (b), and Nb3F2 (c). Scale bars indicate 30 μm. (d–f) Cell absorption spectra of strains Nb3U1 (d), Nb3F1 (e) and Nb3F2 (f) grown under WL (black lines) and FR (gray lines) LEDs. (g–i) Growth curves of strains Nb3U1 (g), Nb3F1 (h) and Nb3F2 (i) under conditions of WL (black open circles), FR (gray open circles) and dark (black filled circles).

mainly distributed in the surface and middle layers (0–4.0 mm below the surface), whereas *Leptolyngbya* was in the deep layers (4.0–6.5 mm), corresponding to the presence of Chl *f* (Figure 4). These distribution patterns indicate that Chl *f* in the mat originated from both *Leptolyngbya* species, which supports our analysis of the pigment composition in the isolated cyanobacterial strains. Two strains of *Leptolyngbya* spp. (Nb3F1 and Nb3F2) accumulated Chl *f* under FR, whereas *Synechococcus* sp. Nb3U1 did not (Figures 6d–f). Not all strains were identical to the cyanobacteria detected by PCR-DGGE based on their 16S rDNA sequences. However, strains Nb3F1, Nb3F2 and Nb3U1 are thought to be equivalent to band1, band2 and band3, respectively, based on the species level. Therefore, the physiological characteristics are thought to be similar between each isolated

strain and cyanobacterium within the mat. These results indicate that the spatial segregation between unicellular and filamentous cyanobacteria within the mat was caused by differences in the light wavelength they could use for photosynthesis. In fact, both strains of *Leptolyngbya* grew under FR, whereas strain Nb3U1 could not grow under the same conditions (Figures 6g–i). Previous studies have suggested that two *Synechococcus* strains have the capacity to grow at deep layers in microbial mats of Mushroom Spring in Yellowstone National Park (Nowack *et al.*, 2015; Olsen *et al.*, 2015). These *Synechococcus* strains have genes involved in a special form of allophycocyanin and IsiA-like protein, which can probably absorb FR. It is unclear whether the cyanobacteria living in mat Nb3 have these genes, but strain Nb3U1 has no capacity to

grow under FR-dominated environments. The growth rate of strain Nb3U1 was similar to or greater than that of filamentous strains under WL, indicating that *Synechococcus* cells might be able to grow much faster than *Leptolyngbya* spp. by using PAR. In contrast, in deep layers in which FR is dominant, *Synechococcus* could be out-competed by *Leptolyngbya*. It is possible that *Synechococcus* were detected from the deep layer by PCR-DGGE because their small unicellular cells could be easily carried by water flow within the mat. We concluded that Chl *f* caused niche differentiation in two types of cyanobacteria within microbial mat Nb3, and that the deep layer was a niche for Chl *f*-producing cyanobacteria.

Even though the distance between the two mats analyzed was only about 10 cm, Chl *f* was not detected from mat Nb4 (Figure 2). DGGE bands of *Leptolyngbya* (band3 and band4) were also absent from mat Nb4 (Figure 4). These results correspond to the above suggestion that Chl *f* in mat Nb3 originated from these *Leptolyngbya* species. However, it is still unknown whether *Thermosynechococcus* sp. in mat Nb4 (band5) has the capacity to synthesize Chl *f*; therefore, this should be investigated in future studies. The *in situ* light environments in mat Nb4 might also cause a lack of Chl *f*. Although FR was enriched in both mats, the light intensity (400–900 nm) in mat Nb4 was always higher than that at a depth of 4.0 mm in Nb3 (Figure 1). If the intensity of PAR in Nb4 is too high for a FaRLiP response, Chl *f* should not be synthesized, even if Chl *f*-producing cyanobacteria are present. Therefore, the microbial mats in hot springs do not always contain Chl *f*, and both the presence of Chl *f*-producing organisms and environmental factors in the mats would be important.

Previous studies have confirmed that anoxygenic photosynthetic bacteria are major components in the microbial mats at Nakabusa hot spring (Nakagawa and Fukui, 2002; Kubo *et al.*, 2011; Everroad *et al.*, 2012). In this study, we observed attenuation peaks from bacteriochlorophylls (BChls *a* and *c*) in downward irradiance spectra within mats (Figure 1). A bacteria-targeted PCR-DGGE profile also indicated the presence of anoxygenic photosynthetic bacteria (Supplementary Figure S5). Supplementary Table S1 shows that the sequence of bandS1 was closely related to the 16S rRNA gene sequences of *Chloracidobacterium thermophilum* strains A, E and S (96.6%, 515/533), which were recently discovered in microbial mats at hot springs in Yellowstone National Park (Bryant *et al.*, 2007). It was also related to *C. thermophilum* strain B (96.1%, 512/533), the type strain of this species (Tank and Bryant, 2015a). BandS2 showed an identical sequence (534/534) to *Roseiflexus castenholzii* DSM 13941 (Supplementary Table S2), which was isolated from a microbial mat at Nakabusa (Hanada *et al.*, 2002). *C. thermophilum* is a unicellular aerobic anoxygenic phototrophic bacterium containing BChl *a* and *c*, and a

peak at 745 nm was observed in the cell absorption spectrum (Tank and Bryant, 2015b). In contrast, *R. castenholzii* is a filamentous anoxygenic facultative phototrophic bacterium containing BChl *a*, but it lacks BChl *c* and chromosomes, whereas it produces *in vivo* absorption peaks at 801 and 878 nm (Hanada *et al.*, 2002). This bacterium is photoheterotrophic under anaerobic light conditions. Judging from the DGGE band densities and the downward irradiance spectra, *Chloracidobacterium* sp. was distributed in the upper layers (0–4.0 mm) and *R. castenholzii* was dominant in deeper layers (3.0–6.5 mm) of the mat corresponding to their oxygen requirements. Accordingly, there were four types of photosynthetic bacteria within mat Nb3: *Synechococcus* (Chl *a*), *Leptolyngbya* (Chl *a+f*), *Chloracidobacterium* (BChl *c+a*) and *Roseiflexus* (BChl *a*), which could coexist within same mat by avoiding competition for light.

We thought that cyanobacteria acquired the capacity to synthesize Chl *f* as a result of evolutionary adaptation to environments within microbial mats. Both strains of *Leptolyngbya* isolated in this study could grow photoautotrophically under FR, which is the dominant light in the deeper layers of microbial mats (Figure 6). This characteristic would have allowed Chl *f*-producing cyanobacteria to expand their distribution range vertically within microbial mats. The acquisition of Chl *f* by cyanobacteria would have occurred just once during the early stage of their evolutionary process, as at least eight species of Chl *f*-producing cyanobacteria were widespread in the phylogenetic tree of the Cyanobacteria phylum (Figure 5). Moreover, the deep layer of microbial mats might have been a safe habitat for cyanobacteria in the early Earth, where UV radiation was probably much greater than it is today (Garcia-Pichel, 1998; Nisbet and Sleep, 2001).

This study provides a new example of niche differentiation along the light spectrum. Within a microbial mat, Chl *f* was distributed in the deeper layer, where FR was dominant. Two species of Chl *f*-producing filamentous cyanobacteria containing Chl *a* and *f* were also distributed there and able to grow under FR. Two other species of unicellular cyanobacteria containing only Chl *a* were distributed in the upper layer reached by PAR, whereas two species of anoxygenic phototrophic bacteria containing only BChl *a* or BChl *c+a* also existed within the same mat. The results suggest that Chl *f* contributes to cyanobacterial survival in habitats with little PAR, as well as to coexistence with other photosynthetic organisms in microbial mats. However, our results only showed a temporary state of the microbial mat, as well as the physiological characteristics of cyanobacteria isolated and cultured in the laboratory. Further studies are needed to reveal how Chl *f*-producing cyanobacteria increase and decrease in the life cycle of microbial mats and the expression patterns of genes of FaRLiP and Chl *f*-synthesis (Gan *et al.*, 2014, 2015; Ho *et al.*, 2016).

Conflict of Interest

The authors declare no conflict of interest.

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