Chlorophyll α fluorescence and Raman spectroscopy can monitor activation/deactivation of photosynthesis and carotenoids in Antarctic lichens

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Lichens survive harsh weather of Antarctica as well as of other hostile environments worldwide. Therefore, this investigation is important to understand the evolution of life on Earth in relation to their stress tolerance strategy. We have used chlorophyll α fluorescence (ChlF) and Raman spectroscopy, respectively, to monitor the activation/deactivation of photosynthesis and carotenoids in three diverse Antarctic lichens, Dermatocarpon polyphyllizum (DP), Umbilicaria antarctica (UA), and Leptogium puberulum (LP). These lichens, post 4 h or 24 h of hydration, showed differences in their ChlF transients and values of major ChlF parameters, e.g., in the maximum quantum efficiency of PSII photochemistry (ΦPSII), yields of fluorescence and heat dissipation (ΦNPQ), of effective quantum efficiency of PSII photochemistry (ΦPSII,e) and of non-photochemical quenching (ΦNPQ), which may be due to quantitative and/or qualitative differences in the composition of their photobionts. For understanding the kinetics of hydration-induced activation of photosynthesis, we screened ΦNPQ of these lichens and reported its non-linear stimulation on a minute time scale; half of the activation time (t1/2) was fastest ~4.05 ± 0.29 min for DP, which was followed by 5.46 ± 0.18 min for UA, and 13.95 ± 1.24 min for LP. Upon drying of fully activated lichen thallus, there was a slow decay, in hours, of relative water content (RWC) as well as of Fv/Fm. Raman spectral signatures were different for lichens having algal (in DP and UA) and cyanobacteria (in LP) photobionts, and there was a significant shift in ν1 C=C (Raman band of carotenoids post 24 h hydration as compared to their value at a dry state or post 4 h of hydration; this shift was decreased, when drying, in DP and LP but not in UA. We conclude that hydration nonlinearly activated photosynthetic apparatus/reactions of these lichens in minute time range but there was a de-novo synthesis of chlorophylls as well as of carotenoids post 24 h. Their dehydration-induced deactivation, however, was comparatively slow, in hours range, and there seemed a degradation of synthesized chlorophylls and carotenoids post dryness. We conclude that in extremophilic lichens, their photosynthetic partners, in particular, possess a complex survival and photoprotective strategy to be successful in the extreme terrestrial environments in Antarctica.

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1. Introduction

Photosynthesis is the base of life on this planet, Earth; it is highly diverse and powered by sunlight, which includes the assimilation of CO2 in the presence of H2O and light; light is harvested by antenna pigments and an array of protein and chlorophyll molecules embedded in the thylakoids to produce chemical energy, in the form of NADPH and ATP, and then saccharides and other biomolecules. Regulation of photosynthesis, comprises of a chain of naturally selective photochemical and biochemical reactions, is highly complex and far from being fully understood. For understanding the evolution of life on Earth or for investigating possibilities of extraterrestrial life, it is a prerequisite for understanding how photosynthesis works in organisms facing harsh environments. Organisms from Antarctica might be a good choice since photosynthesis takes place in extremely harsh conditions which are associated with rapid changes in dryness, temperature, and wind [1]. Lichens are symbiotic organisms; they create a life form with the mutual partnership of fungi and photobionts (algae and/or cyanobacteria) in extreme conditions where it is impossible to survive individually. The majority of Antarctic lichens are considered extremophiles [2] since they may revive their metabolic functions in a suitable environment; these distinct features allow lichens to survive under extreme environmental conditions, where vascular plants struggle and ultimately die [3]. Lichens are genetically diverse; they have different thallus morphotypes and produce a
wide variety of secondary chemical substances. They can easily colonize the surfaces of soil, wood, rocks, high mountain, polar regions, or even in a desert, and are well adapted to their prevailing environmental situations [4–8]. Lichens were probably contributed to the formation of atmosphere and climate during early evolutionary phases of life on Earth [9,10], and the photobionts are, still, predominantly contributing to the carbon cycle of the terrestrial ecosystem of Antarctica [11].

In contrast to the vascular plants, poikilohydric autotrophic organisms, e.g., bryophytes and lichens, do not have water regulation systems (e.g., stomata and vascular bundles). These autotrophic organisms can gain and lose water frequently, but their survival depends upon a complex strategy and their cells’ capacity of activating and de-activating the metabolism of constitutive molecules in prevailing environmental situations. Molecular mechanisms that cause physiological changes, e.g., activation and de-activation of photosynthesis and protective processes, are highly interesting but are poorly understood in Antarctic lichens [12]. Recently, in Mishra et al. [6], we have used three non-invasive optical methods, e.g., reflectance, chlorophyll a fluorescence (ChlF), and Raman spectroscopy, to monitor hydration-induced changes in photosynthesis and biophysical characteristics in an Antarctic lichen Dermatocarpon polyphyllizum (DA). We compared different parameters of these optical methods at different stages after hydration (e.g., 4 h, 24 h, and 48 h), and reported that functions of DA photobionts were already activated post 4 h of hydration and there was a de-novo synthesis of carotenoids post 24 h or 48 h of hydration. In this study, we have investigated the kinetics of activation/deactivation of photosynthesis in three diverse Antarctic lichens, Dermatocarpon polyphyllizum (DA), Umbilicaria antarctica (UA), and Leptogium puberulum (LP). To do so, we have used ChlF signals, specifically, to monitor the dynamics of activation of photosynthetic activity. ChlF is a prominent non-invasive tool for measuring the photosynthetic activity of photosynthetic organisms including lichens or analogous species under different situations [13–17]. Further, we have used Raman spectroscopy, another powerful tool, frequently being used in lichen research [18–20], to monitor hydration-induced changes in the carotenoid’s composition. We have also measured the effects of dehydro- dration on the dynamics of activated photosynthesis. We expected that hydration-induced progression of photochemical activity and quantitative/qualitative changes in photosynthetic apparatus and carotenoid compositions would be reflected in respective signals of ChlF and Raman spectra. We intended to provide experimental evidence on the kinetics of activation and/or de-novo synthesis of photosynthetic apparatus and carotenoid post hydration and their reversibility after drying.

2. Material and methods

2.1. Lichen specification

Dermatocarpon polyphyllizum (DA), a foliose, multi-lobed chlorolichen, grows not only at high altitudes, on rock and stony surface, but also at low altitudes or even close to a coastal line in polar and/or in sub-polar regions [21]. In Antarctica, D. polyphyllizum is found in the South Shetlands [22] and several places along the western coast of the Antarctic Peninsula (see Australian Antarctic Data Center, Global Biodiversity Information Facility, https://data.aad.gov.au/[23,24]). Thallus of D. polyphyllizum is dark brown or even black in the “wet state”, and its color changes to grey or even brown in the “dry state”; this chlorolichen includes unicellular green alga Diplosphaera sp. [25] as a photosynthesizing partner, in contrast to most others that have Trebouxia sp. as the presence of Diplosphaera sp. in D. polyphyllizum thallus provides an opportunity to study the physiological properties of this lichen since only a limited number of studies have, thus far, focused on Diplosphaera sp. (e.g. [26] or Diplosphaera-possessing lichens (see e.g. [27]). Umbilicaria antarctica (UA) is a macrolichen with a foliose morphotype of thallus and its diameter may reach up to 15 cm. In Antarctica, it grows on inclined rockfaces, typically close to cracks and depressions. In such a habitat, UA benefits from water originated from melted snow and ice accumulation formed during Austral winter. Color of the upper side of the thallus is typically whitish-grey in the dry state which turns into greyish-green when wet; the lower surface is black with dense coverage of rhizines. Thallus thickness varies between 90 and 350 μm according to the spot of measurement within a thallus with a mean value of 202 μm (our unpublished data). UA is a chlorolichen that possesses symbiotic alga Trebouxia sp. as photosynthesizing photobiont [8]. Leptogium puberulum (LP) is a foliose macro-lichen, often lobate. The thallus can be of a lead-grey, dark-olive, brownish color and it can change to dark brown or even black post hydration. The lower surface of the thallus has the same color as the upper one but usually slightly lighter and its photobiont is cyanobacteria Nostoc commune. LP is reported from Antarctica, South Georgia, and several sub-Antarctic islands [28]. In the James Ross Island, LP is quite abundant on the slopes and plateaus of table mountains. It grows typically of wet or moist stony ground in rocks crevices. Studies on physiological responses of LP to environmental perturbations are rather few; however, dehydration-induced changes observed in nuclear magnetic resonance (NMR) signal [29] and spectral reflectance [30] were already addressed.

2.2. Collection and handling of the lichen

The lichens, Dermatocarpon polyphyllizum (DA) and Leptogium puberulum (LP) were brought from the James Ross Island, Antarctica. DA was collected from a long-term research plot (LTRP) which is close to the Mendel station. The LTRP is positioned at the northern coast of the island, at coordinate, 63 48 03 S, 57 52 50 W, and at an altitude of 7 m asl (above sea level). LP was collected from the stony slope (facing NS) located below the Berry Hill mesa (63 48 50 S, 57 51 15 W, 125 m asl). Umbilicaria antarctica (UA) was collected from rock walls at the Cape Tuxen, Antarctica (65 16 S, 64 8 W). The collection site is rocky cape forming the south side of the entrance to the Waddington Bay on the Kiev Peninsula, the west coast of Graham Land. These lichens were collected and dried, in Antarctica, under natural conditions, and they were further stored at 5 °C. For investigating activation of photosynthetic activity, we measured chlorophyll a fluorescence (ChlF) of these lichens on a black paper, beneath which filter papers filled with distilled water inside a Petri dish. We further sprayed distilled water over the lichen thallus and then kept beneath the fluorescence imaging camera, at room temperature ~22 °C and under low irradiance (~50 μmol (photons) m⁻² s⁻¹).

2.3. Chlorophyll a fluorescence for measuring activation and deactivation of photosynthesis

A transect fluorescence imaging system (Photons Systems Instruments, Brno, CZ; for detailed on instrumentation see Nedbal et al. [31] and Mishra et al. [32]) was used to screen ChlF transients of these lichens. We measured slow ChlF transients of these samples, after 10 min of dark adaptation, by an imaging camera (with protocol modified from Mishra et al. [32]) at different stages of hydration (i.e., 4 h and 24 h; for more details of used protocol see, Mishra et al. [6]). Recorded ChlF transients of lichens were used to evaluate important parameters, e.g., in the maximum quantum efficiency of PSII photochemistry (Fv/Fm), and yields of fluorescence and heat dissipation (ΦPSII), of effective quantum efficiency of PSII photochemistry (ΦPSII) and of non-photochemical quenching (qN) as given in Hendrickson et al. [33] (also see [34]). For investigating activation of photochemical activity in these lichens, we measured basic ChlF parameters: 1) terminal-state steady fluorescence, F0, by using short flashes of measuring flashes (30 μs, ~3 μmol (photons) m⁻² s⁻¹) and 2) maximum fluorescence, Fm, by using a strong saturating pulses (~2000 μmol (photons) m⁻² s⁻¹), in the presence of actinic irradiance of ~10 μmol (photons) m⁻² s⁻¹ post
hydration. Effective quantum efficiency of PSII photochemistry ($\Phi_{PSII} = (F'_{m} - F_{T})/F'_{m}$) of these lichens was calculated to present the activation of photochemical efficiency post hydration.

2.4. Raman spectroscopy for measuring structural changes in carotenoids

We have used an InVia Raman spectrometer (Renishaw, Wotton-under-Edge, UK) equipped with a Leica confocal microscope to measure Raman spectra (single-point measurement) of these lichens. The instrument was calibrated to a silicon Raman band at 520.5 cm$^{-1}$, and a 514.5 nm laser line was used with power ~1.25 mW at the source. We have used an objective with the magnification of 50×, the exposure time was 3 s, and at each point, spectra were accumulated 10 times. The Raman spectra of dry lichens were measured at 20 °C after they were taken from the freezer at 5 °C. Then, we measured the Raman spectra of lichens surface areas post 4 h, 24 h, 48 h, and 144 h of hydration, and that after subsequent dehydration of 24 h. The measurement was accomplished in three replicates in each studied condition (for a more detailed description see [6]).

2.5. Relative water content (RWC) in lichens

The relative water content (RWC) of lichen was calculated as ($FW - DW$) / ($TW - DW$) x 100; where $FW$ = fresh weight, $TW$ = turgid weight (~24 h in water in darkness), and $DW$ = dry weight (24 h drying at 60 °C). Three samples of each lichen were taken at dry state, and at a deferent time interval of hydration and drying, for RWC measurements in triplicates.

2.6. Data processing

We have used a FluorCam 7 software (Photons Systems Instruments, Brno, CZ) to process the ChlF transients to calculate averaged ChlF parameters over the selected surface area of the lichens. Raman spectra were processed using a Wire 3.4 software (Renishaw, Wotton-Under-Edge, UK) and Grams AI 9.1 software (Thermo Fisher Scientific, Wal-tham, USA). Further, Graphpad Prism Software, version – 5 (GraphPad Software-La Jolla, CA, USA), was used for calculating the statistical tests to evaluate the statistical significance of differences between the parameters and to fit the different stimulation and decay curves by using non-linear regression models.

3. Results and discussion

3.1. Lichens possess a diverse range of structural and functional characteristics and stay alive in harsh conditions of Antarctica

Poikilohydric lichens do not possess functional and structural ability to regulate intrathalline water content, but their survival in extreme situations depends on their ability to activate and deactivate their metabolism and photosynthetic processes while being in equilibrium with a relative humidity of ambient air. In terrestrial ecosystems along the eastern and western side of the Antarctic peninsula, the sources of water are mainly ice and snow fields which melt during austral summer. Therefore, water for hydration of poikilohydric exists only for a short period, typically of about two months. The period of water availability might be even shorter, ranging from weeks to several days, depending on different sites topography and prevailing weather, surface temperature, and wind speed. During austral winter, thus, lichens are typically frozen,

Fig. 1. Diversity in dry upper (A, D, G), dry lower (B, E, H), and 24 h hydrated upper (C, F, J) surfaces of three Antarctic lichens that can be visualized in the photographs: (1) Dermatocarpon polyphyllizum (A, B, C), (2) Umbilicaria antarctica (D, E, F), and (3) Leptogium puberulum (G, H, J).
dried, and inactive under snow for a long time. Lichens can equilibrate their metabolic or photosynthetic activity by air humidity alone in the absence of ice [14]; however, even below the snow cover, they are well protected and may activate their photosynthesis up to the depth of 15 cm and even at the subzero temperature of around −10 °C [16,35,36]. They often exhibit full physiological recovery when rehydrated [37]. We have presented RGB (red-green-blue) image of the dry upper-surface (dark black) and dry lower-surface (light black or brown) of three lichens, *D. polyphyllizum* (DP, see Fig. 1A, B), *U. antarctica* (UA, see Fig. 1D, E) and *L. puberulum* (LP, see Fig. 1G, H), to show the variations in their structure and associated protective pigments; the color of upper surface might be associated with tightly compacted hyphae forming the upper cortex and secondary metabolites located in it. RGB images of these lichens post 24 h hydration showed patches of dark green areas on the rough upper surfaces of DP (Fig. 1C) and LP (Fig. 1J), but the upper surface of UA (Fig. 1F) has patches of green photobionts on its thin layer. Similarly to Mishra et al. [6], we observed that hydration changes optical characteristics and activated photosynthetic apparatus on thallus of these lichens; dynamics of ChlF transient, i.e., variation of ChlF transients (slow Kautsky kinetics) with peak *F*~p~ and beyond, in the presence of actinic irradiance (−160 μmol (photons) m$^{-2}$ s$^{-1}$), was observed in DP (blue line of Fig. 2A) and UA (purple line of Fig. 2A) but not in LP (red line of Fig. 2A). The appearance of a secondary peak, M, after primary peak *P* (*F*~p~), in UA (purple line of Fig. 2A), is highly prominent. The effective quantum efficiency of PSII photochemistry (ΦPSII) post 4 h hydration was 0.464 ± 0.03, 0.544 ± 0.007 and 0.195 ± 0.022, respectively, for DP, UA, and LP. The dynamic behavior of ChlF transients, post 24 h of hydration, is fully visible in all three lichens (Fig. 2B, C, D). For investigating photochemical and non-photochemical behavior of these lichens, we have measured ChlF transients of these lichens post 24 h hydration under three different actinic irradiances (*L*1 = 40 μmol (photons) m$^{-2}$ s$^{-1}$ (Fig. 2B), *L*2 = 80 μmol (photons) m$^{-2}$ s$^{-1}$ (Fig. 2C), and *L*3 = 160 μmol (photons) m$^{-2}$ s$^{-1}$ (Fig. 2D)). This enabled us to compare the contribution of quantum yields of effective efficiency of PSII photochemistry (ΦPSII), of regulatory non-photochemical quenching (Φnpq) and of non-regulatory basal constitutive thermal dissipation and fluorescence (Φf,d) of these lichens according to the energy

3.2. Hydration-induced activation of photosynthesis and their photochemical/non-photochemical characteristics are different for diverse Antarctic lichens

We have already reported in Mishra et al. [6] that hydration activated the functions photosynthetic apparatus in *D. polyphyllizum* after 4 h, and there was essentially no change in the values of ΦPSII post 4 h, 24 h or 48 h of hydration; however, *F*~m~/*F*~o~ significantly changed between 4 h and 24 h hydration, but it had almost similar values post 24 h or 48 h hydration. Therefore, for comparing the activation status of their photochemical activities in the three lichens (e.g., DP, UA, LP), we measured slow ChlF transients post 4 h and 24 h of hydration (Fig. 2). Presence of variable fluorescence, in all three lichens post 4 h of hydration, indicates active photosystems on thallus of these lichens; dynamics of ChlF transient, i.e., variation of ChlF transients (slow Kautsky kinetics) with peak *F*~p~ and beyond, in the presence of actinic irradiance (−160 μmol (photons) m$^{-2}$ s$^{-1}$), was observed in DP (blue line of Fig. 2A) and UA (purple line of Fig. 2A) but not in LP (red line of Fig. 2A). The appearance of a secondary peak, M, after primary peak *P* (*F*~p~), in UA (purple line of Fig. 2A), is highly prominent. The effective quantum efficiency of PSII photochemistry (ΦPSII) post 4 h hydration was 0.464 ± 0.03, 0.544 ± 0.007 and 0.195 ± 0.022, respectively, for DP, UA, and LP. The dynamic behavior of ChlF transients, post 24 h of hydration, is fully visible in all three lichens (Fig. 2B, C, D). For investigating photochemical and non-photochemical behavior of these lichens, we have measured ChlF transients of these lichens post 24 h hydration under three different actinic irradiances (*L*1 = 40 μmol (photons) m$^{-2}$ s$^{-1}$ (Fig. 2B), *L*2 = 80 μmol (photons) m$^{-2}$ s$^{-1}$ (Fig. 2C), and *L*3 = 160 μmol (photons) m$^{-2}$ s$^{-1}$ (Fig. 2D)). This enabled us to compare the contribution of quantum yields of effective efficiency of PSII photochemistry (ΦPSII), of regulatory non-photochemical quenching (Φnpq) and of non-regulatory basal constitutive thermal dissipation and fluorescence (Φf,d) of these lichens according to the energy

![Fig. 2.](https://example.com/fig2.png) Chlorophyll a fluorescence transient of 4 h (A) and 24 h (B, C, D) hydrated *Dermatocarpon polyphyllizum* (DP, blue lines), *Umbilicaria antarctica* (UA, purple lines), and *Leptogium puberulum* (LP, red lines). Twenty-four hours hydrated lichens were exposed at three different irradiances, 40 μmol (photons) m$^{-2}$ s$^{-1}$ (B), 80 μmol (photons) m$^{-2}$ s$^{-1}$ (C), and 160 μmol (photons) m$^{-2}$ s$^{-1}$ (D). All measurements were done on ~20 min dark adapted lichens, and curves are average of three independent replicas where each replicate were comprised of 3-5 photosynthetic active green areas on hydrated lichen surface.
partitioning approach of Hendrickson et al. [33] (see Fig. 3). We report that $\Phi_{f,d}$ differed significantly between DP versus LP and UA versus LP but not between DP versus UA (after 4 h of hydration, $\Phi_{f,d} = 0.425 \pm 0.038$ for DP, $0.398 \pm 0.014$ for UA, and $0.669 \pm 0.010$ for LP). The $\Phi_{f,d}$ differed between 4 h and 24 h post hydration only in DP; however, it was almost constant under different irradiance in all lichens (Fig. 3). For cyanolichen, LP, the $\Phi_{psii}$ was lowest and it did not change significantly between 4 h and 24 h post hydration (Fig. 3C). However, we report −36% increase and −14.5% decrease in the $\Phi_{psii}$ of DP and UA post 24 h of hydration as compared to their respective value post 4 h hydration. Further, as expected high actinic irradiance of 160 $\mu$mol (photons) m$^{-2}$ s$^{-1}$ decreased the $\Phi_{psii}$ of both DP (Fig. 3A) and UA (Fig. 3B), but not of LP (Fig. 3C). As expected, the yield of regulatory non-photochemical quenching, $\Phi_{npq}$, increased with increasing actinic irradiance in both DP and UA (Fig. 3A, B); however, in LP, $\Phi_{npq}$ was almost constant for L$_1$ versus L$_2$ and its value declined for high irradiance by 12% as compared to low actinic irradiance (Fig. 3C). It is to note that photobionts in LP are cyanobacteria having an abundance of phycobilisome antenna systems, and it may hamper actual values of important parameters during interactions with light [38] that might be the reasons for the lower value of $\Phi_{npq}$ with increasing irradiance, in LP. Moreover, a continuous rise of terminal steady-state fluorescence, $F_{v}$, following semi-steady state (S) is visible (see red lines in Fig. 2B, C, D beyond $F_{v}$) in the ChlF transients of lichen LP, which is generally a characteristic of cyanobacteria.

### 3.3. Kinetics of activation and deactivation of photosynthesis in Antarctic lichens

Activation and deactivation of metabolism in photosynthetic organisms, which is specifically related to the switching on and off photosynthesis, respectively, in the favorable and unfavorable environments, is the basic strategy for their survival in extreme environmental situations of Antarctica. Lichens do not possess water regulatory mechanisms within its thallus; therefore, it is more cumbersome to understand their survival mechanisms. However, the absence of water regulatory systems in lichens provided us an opportunity to investigate their adaptive photosynthetic mechanisms. For understanding metabolisms of hydration-induced activation and deactivation of photosynthesis, we initially measured terminal-steady state fluorescence, $F_{m}$, and maximum fluorescence, $F_{m}$, under low ambient irradiance (−10 $\mu$mol (photons) m$^{-2}$ s$^{-1}$) at every 5 min interval post hydration. We observed that the $\Phi_{psii}$ changes during the first few minutes after hydration and further it stayed almost constant (data not shown). Thereafter, we measured $\Phi_{psii}$ on minute time range starting from the dry state of lichens (t = 0) to 100 min post hydration (see Fig. 4). Similarly to our earlier reports presented in Mishra et al. [6], in the dry state, the value of minimum fluorescence, as well as maximum fluorescence, is within the limits of measurement errors and there was no variable fluorescence. Non-linear changes in $\Phi_{psii}$ post hydration are obvious in all three lichens (Fig. 4). Half of the time required to achieve maximum $\Phi_{psii}$ post hydration (see bar diagram inside Fig. 4) confirms that activation of photosynthesis is fastest in DP ($t_{1/2}$ = 4.05 ± 0.29 min) which is followed by UA ($t_{1/2}$ = 5.46 ± 0.42 min), and it is slowest in LP ($t_{1/2}$ = 13.95 ± 1.24 min). This result confers that photosynthesis rapidly activated in these lichens with the occurrence of favorable situations.

We further measured deactivation of photosynthesis of 48 h fully wet samples of the three lichens (Fig. 5): we found that there was a slow decay in the ChlF parameter, $F_{v}/F_{m}$, as well as in RWC during drying of fully activated lichen thallus. We already knew that lichens quickly suck water and increase RWC, and there is not much change in its water content after full hydration (see [6]) Here, we observed that RWC (Fig. 5B), post 16 h of drying, was reduced to 43%, 25%, and 17% respectively for UA, LP, and DP, whereas their photosynthesis measured by the ratio of variable fluorescence to maximum fluorescence, $F_{v}/F_{m}$ (Fig. 5A), was fully deactivated following 5 h in UA and following 12 h in DP and LP. Half of the time ($t_{1/2}$) required to inhibit $F_{v}/F_{m}$ evaluated from one phase exponential decay equation, was lowest in LP ($t_{1/2}$ = 0.48 h) which is followed by DP ($t_{1/2}$ = 0.77 h) and UA ($t_{1/2}$ = 1.45 h) (see insert in Fig. 5A where data were presented with 95% of confidence interval); however, $t_{1/2}$ of RWC decay is fastest in UA ($t_{1/2}$ = 1.81 h) and it possesses almost similar value for DP (2.45 h) and LP (2.49 h) (see insert in Fig. 5B where data were presented with a 95% of confidence interval). Typically, it is difficult to measure fluorescence signals and $F_{v}/F_{m}$ after RWC drop below 20% [39]. Lichen possesses a diverse range of structural and morphological features to be successful in the extreme and peculiar conditions of Antarctica. It is to note that lower surface of UA is fully covered by rhizines, and its upper cover, where green patches were visible post hydration, is quickly dried to inactivate its photosynthetic activity; therefore, although, UA possesses high RWC it was not

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*Fig. 3.* Estimated yields of effective quantum efficiency of PSII photochemistry ($\Phi_{psii}$), of regulatory non-photochemical quenching ($\Phi_{npq}$), and of non-regulatory basal constitutive thermal dissipation and fluorescence ($\Phi_{c}$) for (A) Dermatocarpon polyphyllizum, (B) Umbilicaria Antarctica and (C) Leptogium Puberulum, measured at different state of their hydration. Data are average of three independent replicas where each replicate were comprised of 3–5 photosynthetic active green areas on hydrated lichen surface.
compared the wavenumber position of the cyanobacteria as an extracellular screening compound [42]. We have a protective pigment that is produced exclusively by some bands are as per corroborative bands of scytonemin [41] which is a UV different positions of the structures via oxidation or enzymatic degradation [43] which may cause differences in the carotenoid polyene chain. For the three lichens, in the dry state (non-hydrated), the \( \nu(C=C) \) Raman band was located at different wavenumber positions, i.e., \( 1517 \text{ cm}^{-1} \) for DP, \( 1516 \text{ cm}^{-1} \) for LP, and a significantly lower wavenumber position, \( 1509 \text{ cm}^{-1} \), was registered for UA (see Fig. 6B; Table 1). Long stay in the inactive state under snow cover in Antarctica may disintegrate the carotenoid structures via oxidation or enzymatic degradation [43] which may cause different positions of \( \nu(C=C) \) Raman band in the dry state. Further, similarly to what was obtained in Mishra et al. [6] in lichen DP, we observed the shift of this carotenoid \( \nu(C=C) \) band with hydration in these lichens (Fig. 6E, F; Table 1). Fig. 6E showed significant changes in the position of \( \nu(C=C) \) Raman band in DP post 24 h or 48 h hydration; however, here, in addition, a reversible change in the position of this Raman band after 24 h dehydration was obtained. In cyanolichen LP (Fig. 6G), there was a slight shift in \( \nu(C=C) \) band position towards higher wavenumber post 24 h hydration; however, the position of this band decreased after 48 h and longer hydration (Fig. 6G). Further, 24 h dehydration resulted in a slightly lower \( \nu(C=C) \) band position in LP as compared to that post 24 h hydration. Compared to DP, where the \( \nu(C=C) \) position varies within \( -5 \text{ cm}^{-1} \) between dry state and post 24 h hydration, in LP, the band shifts only \( -1.5 \text{ cm}^{-1} \) (see Table 1). In lichen UA, a huge shift in \( \nu(C=C) \) band position towards higher values (\( -13 \text{ cm}^{-1} \)) was registered post 24 h hydration and this shift stay almost similar values post 48 h or 144 h hydration (Fig. 6F; Table 1). Therefore, in contrast to DP and LP, dehydration did not reverse the shift of \( \nu(C=C) \) band in UA (cf. Fig. 6E, F, G; Table 1). It is possible to measure fluorescence signal post 5 h onwards. Furthermore, ChlF signal in cyanolichen LP was lowest even when they are fully hydrated, and thus, deactivation of is photosynthesis with slightly higher RWC as compared with DP might be reasonable.

4. Raman spectroscopy revealed changes in carotenoids during hydration and subsequent dehydration

Typical Raman spectra obtained within the three-lichen species, i.e., in DP, LP, and UA are shown in Fig. 6A. Raman spectra of lichens having algal photobionts, i.e., in DP and UA, are specifically represented by two strong Raman bands, in the spectral regions of 1509–1522 cm\(^{-1}\) and 1155–1157 cm\(^{-1}\), respectively, due to in-phase \( \nu(C-C) \) and \( \nu(C-C) \) stretching vibrations of the polyene chain in carotenoids; besides, there is a medium intensity band at around 1005 cm\(^{-1}\) which corresponds to the in-plane rocking modes of the CH\(_2\) groups attached to the polyene chain [40]. Raman spectra of LP, with cyanobacteria as a photobiont, show, in addition to the carotenoid bands, another band around 1600 cm\(^{-1}\), 1555 cm\(^{-1}\), and a shoulder at 1174 cm\(^{-1}\). These bands are as per corroborative bands of scytonemin [41] which is a UV protective pigment that is produced exclusively by some cyanobacteria as an extracellular screening compound [42]. We have compared the wavenumber position of the \( \nu(C-C) \) Raman band of these lichens in the dry state with hydrated (post 4 h, 24 h, 48 h and 144 h) and dehydrated (post 24 h) states of the lichens (see Table 1). Variations of the wavenumber position generally reflect the structural changes in the carotenoid polyene chain. For the three lichens, in the dry state (non-hydrated), the \( \nu(C-C) \) Raman band was located at different wavenumber positions, i.e., 1517 cm\(^{-1}\) for DP, 1516 cm\(^{-1}\) for LP, and a significantly lower wavenumber position, 1509 cm\(^{-1}\), was registered for UA (see Fig. 6B; Table 1). Long stay in the inactive state under snow cover in Antarctica may disintegrate the carotenoid structures via oxidation or enzymatic degradation [43] which may cause different positions of \( \nu(C=C) \) Raman band in the dry state. Further, similarly to what was obtained in Mishra et al. [6] in lichen DP, we observed the shift of this carotenoid \( \nu(C=C) \) band with hydration in
Table 1

Wavenumber positions of Raman carotenoid ν1(C=C) band (cm⁻¹). Data are average (n = 3 of three measurements from distinct zones) ± SD. Superscripts denote significant difference of hydrated/dehydrated samples with respect to their respective data of dry state: (for a = p < 0.05, b (p < 0.01), and c (p < 0.001).  

<table>
<thead>
<tr>
<th>Lichen</th>
<th>Hydration status</th>
<th>Dry state</th>
<th>4 h</th>
<th>24 h</th>
<th>48 h</th>
<th>144 h</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP</td>
<td></td>
<td>1517.0 ± 0.5</td>
<td>1518.5 ± 0.2</td>
<td>1521.1 ± 0.5a</td>
<td>1521.8 ± 1.5b</td>
<td>1522.1 ± 0.1b</td>
<td>1518.0 ± 1.4</td>
</tr>
<tr>
<td>UA</td>
<td></td>
<td>1509.4 ± 0.2</td>
<td>1509.3 ± 0.4</td>
<td>1520.4 ± 4.3</td>
<td>1521.5 ± 1.0c</td>
<td>1522.2 ± 1.3c</td>
<td></td>
</tr>
<tr>
<td>LP</td>
<td></td>
<td>1516.3 ± 0.6</td>
<td>1516.7 ± 0.3</td>
<td>1517.8 ± 1.8</td>
<td>1516.8 ± 0.7</td>
<td>1515.6 ± 1.8</td>
<td>1514.9 ± 0.9</td>
</tr>
</tbody>
</table>

Fig. 6. Raman spectra (averaged from three different spots) of the three lichens are compared in (A). “S” points to the bands assigned to scytonemin. Magnified region of carotenoid ν1(C=C) vibration band of three lichens is presented for the dry state (B), post 24 h hydration (C), and post 24 h dehydration of fully wet samples (D). Variation of carotenoid ν1(C=C) band position with hydration status in Antarctic lichens: (E) Dermatocarpon polyphyllizum, (F) Umbilicaria antarctica, and (G) Leptogium puberulum. Data in column represent mean (n = 3 of three measurements from distinct zones) ± SE. Asterisks denote statistical significance of hydrated/dehydrated samples with respect to their respective data of dry state or 4 h hydrated one (* p < 0.05, ** p < 0.01, *** p < 0.001; one-way Anova with Tukey’s multiple comparisons test).
note that there is no substantial change in the position of ν1(C=C) band for dry state versus 4 h hydration in all three lichens.

We have already reported interpretation of hydration-induced activation of carotenoid ν1(C=C) band shift in *D. polyphyllum* in Mishra et al. [6]. In short, the Raman spectrum reflects signals from a mixture of carotenoids, and, thus, relative changes in the content of carotenoids or its composition may cause a shift in ν1(C=C) band position and/or affect the Raman spectral intensity. The shift in ν1(C=C) band wavenumber position of carotenoids is dependent on several factors, especially the number of conjugated double bonds, bonding with other cellular components, then isomerization or molecular termination (for more details see de Oliveira et al. [44]). Among these factors, the number of conjugated double bonds in the polyene chain plays the most important role in the shift of the wavenumber position. Typically, longer polyene chain, with the high number of conjugated double bonds, shifts towards lower positions and vice versa (see Withnall et al. [45]). Common xanthophyll C-40 carotenoids with 11 conjugated double bonds exhibit the position of ν1(C=C) band slightly above 1520 cm⁻¹, whereas β-carotene features may shift to lower positions; a broad range of wavenumber position of specific carotenoids, i.e., between ~1515–1525 cm⁻¹, is reported elsewhere (see [40,46,47]. Ruban et al. [48] observed that the ν1(C=C) region was at the lowest position for zeaxanthin and shifted towards lower wavenumber with lutein, violaxanthin, and neoxanthin, and the position of the band depends on the resonance excitation wavelength. Therefore, we interpret the shift of carotenoid ν1(C=C) towards higher wavenumber positions as a possible consequence of xanthophyll cycle activation in the case of DP and UA, i.e. lichens with algal photobionts. On the other hand, the subtle variability of this parameter in the case of LP and even decrease of ν1(C=C) position after 24 h can be assigned to completely different protective mechanisms in cyanobacterial photobiont. It is possible that water supersaturation [49] in cyanolichen, LP, may cause disintegration of carotenoids and that is considered as one of the possible reasons for the reversible shift of ν1(C=C) Raman band position even in the fully hydrated state. In another investigation, obtained on rock-inhabiting (endolithic) cyanobacterial colony from the polyextreme environment of the Atacama Desert, Vitek et al. [50] reported a relation between ν1(C=C) band position with depth, i.e., a vertical profile of the cyanobacterial colony with lower positions of ν1(C=C) wavenumber prevailing at the upper portions of the colony. The authors interpreted this shift as an increased need for photoprotection within the upper position, where higher doses of solar radiation occur. This may be provided by carotenoids with more conjugated double bonds that act as more effective antioxidants. The slight shift towards the lower position may be hypothetically related to the activation of the cyanobacterial photoprotective mechanisms [50]. It is completely different compared to the xanthophyll cycle in algae and vascular plants and is based on orange carotenoid protein conversion to the red form [51]. This conversion is accompanied by the change in polyene chain conjugation with one more double bond in the red form of the carotenoid protein [52]. The completely different protective mechanism in LP compared to the two experimental chlorolichens (UA, DP) is a likely reason for a different pattern of carotenoid ν1(C=C) band shift in LP.

5. Conclusions

Likewise, in our previous study on *D. polyphyllum* [6], in the dry state, photosynthetic apparatus in the thallus of lichens LP and UA also remained fully inactivated since there was no variable fluorescence or dynamics of ChlF transients. In the dry state or inactivated state, the position of ν1(C=C) Raman band for DP and LP is almost similar, located at 1516–1517 cm⁻¹, but it is significantly different, ~1509 cm⁻¹, for UA; this points to differences in carotenoid compositions with different structures or bindings among lichens. Hydration, in the presence of light, does activate primary photosynthetic processes, in a minute time scale, in all investigated lichens; however, their activation time measured by half time needed for stimulation of effective quantum efficiency of PSII photochemistry (ΦPSII) is species-specific. This fast activation of photochemical activity suggests that the presence of favorable situations (i.e., early phases of thallus rehydration), in Antarctic lichens, stimulates inactive photosynthetic apparatus rather than *de-novo* synthesis of components of chloroplastic apparatus. This experiment further supports our earlier hypothesis that carotenoids may not be synthesized *de-novo* during the early activation phase of photosynthesis; however, there is a *de-novo* synthesis of structurally different carotenoids, e.g., post 24 h of hydration, that may be responsible for the enhancement in the position of ν1(C=C) Raman band. Furthermore, this process is species-specific and restoration of different metabolic, biochemical, and physiological processes might depend on the duration of their past stay in the inactivated states. In both DP and LP there is a decline in ν1(C=C) Raman band position post 24 h of dehydration, but not in UA, although we observed a huge shift (~12 cm⁻¹) of ν1(C=C) band towards upper positions post 24 h hydration. Moreover, in LP, Raman spectral features of scytonemin, a UV protective cyanobacterial sheath pigment, was detected. This different Raman spectroscopic record can be assigned to the distinct photoprotective mechanisms in algal and cyanobacterial photobionts. The different activation and deactivation kinetics of these lichens reflected in chlorophyll a fluorescence or Raman signals reflect their differential survival strategies, activation, and co-action of different mechanisms during hydration or dehydration.

**CRediT authorship contribution statement**

Kumud Bandhu Mishra:Conceptualization, Investigation, Formal analysis, Writing - original draft.
Petr Vítek:Conceptualization, Investigation, Formal analysis, Writing - original draft.
Anamika Mishra:Investigation, Formal analysis, Writing - original draft.
Josef Hájek:Conceptualization.
Milosl Barták:Conceptualization, Writing - original draft.

**Declaration of competing interest**

The authors declare that they have no competing interests.

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